Micropollutant removal from municipal wastewater - From conventional treatments to advanced biological processes

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Micropollutant removal from municipal wastewater – From conventional treatments to advanced biological processes

[Elimination des micropolluants des eaux usées municipales – Des traitements classiques aux procédés biologiques avancés]

PhD thesis

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à Núria, ma plus belle découverte

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Thesis abstract

Many micropollutants present in municipal wastewater, such as pharmaceuticals and pesticides, are poorly removed in conventional wastewater treatment plants (WWTPs), and may generate adverse effects on aquatic life and contaminate drinking water resources. To reduce the release of these substances into the environment, advanced treatments are necessary. The objective of this thesis was to study and develop various options to improve micropollutant removal from municipal wastewaters, with a special focus on biological oxidation. A special emphasis was devoted to the development of a process affordable for small WWTPs that requires low chemical inputs, maintenance and energy, and ease of operation. Various technologies were investigated, from conventional biological treatments to advanced physico-chemical and biological processes such as ozonation, activated carbon adsorption, enzymatic bio-oxidation with laccase and biodegradation with white-rot fungi.

The potential of two physico-chemical treatments, (i) oxidation by ozone and (ii) powdered activated carbon (PAC) adsorption followed by either ultrafiltration or sand filtration (SF), was assessed in a study made in collaboration with several other institutions with two large-scale pilot systems operated in parallel over more than one year at the municipal WWTP of Lausanne. The micropollutants studied were removed on average over 80% compared to raw wastewater, with an average ozone dose of 5.7 mg O_3 l^{-1} or a PAC dose between 10 and 20 mg l^{-1}. Both advanced treatments led to a clear reduction in toxicity of the effluents. Implementation of ozonation and PAC-SF in municipal WWTPs appears to be feasible in terms of operation and costs. However, due to their relative technical complexity and possibly too high relative costs for smaller installations, these two processes may not be suitable for small WWTPs with non-permanent staff.

The role of nitrification on micropollutant removal in WWTPs was investigated with two identical aerobic granular sludge sequential batch reactors (AGS-SBRs), operated with or without nitrification (inhibition of the ammonia monooxygenase (AMO)). Out of the 36 micropollutants studied, five were significantly better removed in the reactor with nitrification, probably due to co-oxidation catalysed by AMOs. However, for the removal of all the other pollutants, ammonia-oxidizing bacteria did not play a significant role. The higher removal efficiencies of many pollutants observed in nitrifying WWTPs, highlighted during the pilot study performed at Lausanne WWTP, are therefore probably related to the presence of a more diversified aerobic heterotrophic microbial community, which is favoured in the conditions required for the growth of nitrifying bacteria.

The potential of laccase, an oxidative enzyme produced by many white-rot fungi and bacteria, was assessed for the removal of a wide range of micropollutants. Out of the 39 substances tested, nine could be oxidized by laccase alone, and three others were degraded in the case of addition of a mediator, a compound acting as an electron shuttle between the pollutant and the enzyme. Despite the limited range of potentially degraded pollutants, mainly phenols and anilines, laccase (with or without mediators) is able to oxidize several substances of concern (high toxicity or poor removal in WWTPs), such as natural and synthetic estrogens, diclofenac (DFC), mefenamic acid (MFA), triclosan (TCN), bisphenol A (BPA), isoproturon (IPN) and sulfamethoxazole (SMX).

The influence of pH, temperature, laccase concentration and reaction time on the oxidation kinetics of DFC, MFA, TCN and BPA was investigated. All four factors have a significant effect on the micropollutant oxidation with the greatest influence shown by pH. Results for single compounds were different from those obtained for mixtures of micropollutants. Optimal conditions for micropollutant oxidation are compound-dependent, ranging between pH 4.5 to 6.5 and between 25°C to more than 40°C. A laccase concentration of 730 U l^{-1} allowed obtaining high removal rates (> 90%) of the four

compounds in a short time (40 min to 5 h), showing the potential of laccases to remove, in acidified wastewater, several persistent or toxic compounds.

The influence of pH, mediator, enzyme and pollutant concentrations on SMX and IPN oxidation kinetics with laccase-mediator systems (LMS) was investigated with three mediators: 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), syringaldehyde (SA) and acetosyringone (AS). Both pollutants were completely transformed within a few hours in the presence of laccase and ABTS, as well as, for SMX, in the presence of AS or SA. The three mediators were consumed during the reactions (no catalytic cycles observed). Faster oxidation kinetics were observed at lower pH values, but also higher mediator/pollutant ratios were required. Transformation product mixtures were always less toxic to algae than untreated pollutants. Based on these findings, LMS appears to be a promising option to treat concentrated and potentially toxic industrial effluents, but does not seem adapted for the treatment of low micropollutant concentrations in municipal wastewaters.

To select a laccase-producing organism that could be used in an advanced biological treatment, four strains of the bacterial genus *Streptomyces* and the white-rot fungus *Trametes versicolor* were studied. Due to its higher laccase production, its laccase activity on a wider pH range and at lower temperatures, higher laccase stability and better efficiency for micropollutant oxidation, the fungus *T. versicolor* appeared to be the better candidate to be inoculated in a biological post-treatment.

Finally, a laboratory-scale sequential batch fungal filter (SBFF), composed of woodchips as substrate and support for the mycelium, was designed and tested in continuous operation with two white-rot fungi, *Trametes versicolor* and *Pleurotus ostreatus*. The SBFFs with *P. ostreatus* could be operated during several months (up to 140 d) with unsterile wastewaters, without addition of any external substrate, acidification or re-inoculation. A wide range of micropollutants was removed well by a combination of fungal and microbial degradation and adsorption. The SBFFs were able to compete with ozonation and PAC adsorption regarding the average removal efficiency (up to 82%) of 27 micropollutants in municipal wastewaters. However, the relatively long hydraulic retention times required (24 to > 48 h, versus < 40 min for PAC or ozone) and the relatively short life-span of the fungus resulted in relatively high energy consumption (> 0.4 kWh m^{-3}) and high woodchips requirement (> 1 g l^{-1}). Therefore, despite good efficiency to treat micropollutants, simple technical equipment, ease of operation, only woodchips and electricity as sole inputs, and low maintenance, future research has to focus on the optimization of the fungal filters in order to implement them in WWTPs.

This thesis opened new perspectives regarding biological treatment of micropollutants in wastewater, highlighting also the challenges of applying fungal and oxidative enzyme treatments in WWTPs.

Keywords: micropollutants, pharmaceuticals, pesticides, wastewater, ozonation, activated carbon, adsorption, oxidation, biodegradation, laccase, white-rot fungi, fungal treatment, WWTP.

Résumé de la thèse

De nombreux micropolluants présents dans les eaux usées municipales, tels que médicaments et pesticides, ne sont que partiellement éliminés dans les stations d'épuration (STEP), et peuvent engendrer des effets néfastes sur la faune aquatique et contaminer les ressources en eau potable. Pour réduire leurs apports dans l'environnement, des traitements avancés sont nécessaires. L'objectif de cette thèse était d'étudier et de développer différentes options pour améliorer l'élimination des micropolluants dans les eaux usées municipales, notamment par oxydation biologique. Une attention particulière a été consacrée au développement d'un procédé abordable pour les petites STEP, qui ne demande que peu d'apports en produits chimiques, de maintenance et d'énergie, et facile d'exploitation. Différentes technologies ont été étudiées, des traitements biologiques conventionnels aux procédés physico-chimiques ou biologiques avancés tels que l'ozonation, l'adsorption sur charbon actif, la bio-oxydation enzymatique par des laccases et la biodégradation par des champignons lignivores.

Le potentiel de deux traitements physico-chimiques, (i) l'oxydation par l'ozone et (ii) l'adsorption sur charbon actif en poudre (CAP) suivi soit d'ultrafiltration ou de filtration sur sable (FS), a été évalué dans une étude menée en collaboration avec plusieurs autres institutions avec deux installations pilotes à échelle industrielle exploitées en parallèle durant plus d'une année à la STEP de Lausanne. Les micropolluants étudiés ont été abattus en moyenne à plus de 80% par rapport aux eaux usées brutes, avec une dose moyenne de 5.7 mg O_3 l^{-1} ou de 10 à 20 mg l^{-1} de CAP. Ces deux procédés ont permis une réduction significative de la toxicité des effluents. L'implémentation de l'ozonation et du CAP-FS dans les STEP municipales apparait être réalisable en termes d'exploitation et de coûts. Cependant, à cause de leur complexité technique et de leurs possibles coûts relativement élevés pour de petites installations, ces deux procédés pourraient ne pas être adaptés pour de petites STEP sans personnel permanent.

Le rôle de la nitrification dans l'élimination des micropolluants dans les STEP a été étudié avec deux réacteurs séquentiels discontinus à boues granulaires aérobiques (AGS-SBR), exploités avec ou sans nitrification (inhibition de l'ammonia monooxygenase (AMO)). Parmi les 36 micropolluants analysés, cinq ont été significativement mieux éliminés dans le réacteur avec nitrification, probablement par co-oxydation par l'AMO. Cependant, pour l'élimination des autres polluants, les bactéries nitrifiantes n'ont pas joué un rôle significatif. Le meilleur abattement de nombreux micropolluants observé dans les STEP avec nitrification, mis en évidence durant les essais pilotes à la STEP de Lausanne, est donc probablement lié à la présence d'une communauté plus diversifiée d'hétérotrophes aérobiques, favorisée par les conditions nécessaires pour la croissance de bactéries nitrifiantes.

Le potentiel des laccases, enzymes oxydatives produites par de nombreux champignons lignivores et bactéries, a été évalué pour l'élimination d'une large gamme de micropolluants. Parmi les 39 substances testées, neuf ont pu être oxydées par la laccase seule, et trois autres ont été dégradées en cas d'addition d'un médiateur, un composé agissant comme intermédiaire dans le transfert des électrons entre le polluant et l'enzyme. Malgré la gamme restreinte de polluants potentiellement dégradés, principalement des phénols et des anilines, la laccase (avec ou sans médiateur) est capable d'oxyder plusieurs substances préoccupantes (haute toxicité ou faible élimination dans les STEP), telles que les œstrogènes naturelles et synthétiques, le diclofénac (DFC), l'acide méfénamique (MFA), le triclosan (TCN), le bisphénol A (BPA), l'isoproturon (IPN) et le sulfamethoxazole (SMX).

L'influence du pH, de la température, de la concentration de laccase et du temps de réaction sur les cinétiques d'oxydation du DFC, MFA, TCN et BPA a été étudiée. Ces quatre facteurs ont un effet significatif sur l'oxydation des micropollutants, le plus marqué étant pour le pH. Les résultats pour les

composés seuls étaient différents de ceux obtenus avec des mélanges de polluants. Les conditions optimales pour l'oxydation des micropolluants dépendent des polluants, se situant à un pH de 4.5 à 6.5 et une température de 25°C à plus de 40°C. Une concentration de laccase de 730 U l^{-1} a permis d'atteindre des taux d'élimination élevés (> 90%) des quatre composés dans un temps restreint (40 min à 5 h), montrant le potentiel des laccases pour l'élimination, dans des eaux usées acidifiées, de plusieurs composés toxiques ou persistants.

L'influence du pH et des concentrations de médiateurs, d'enzyme et de polluants sur les cinétiques d'oxydation du SMX et de l'IPN avec des systèmes laccase-médiateur (SLM) a été étudiée avec trois médiateurs : le 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), le syringaldehyde (SA) et l'acetosyringone (AS). Les deux polluants ont été complètement transformés en quelques heures en présence de laccase et d'ABTS, ainsi que, pour le SMX, en présence de SA et d'AS. Les trois médiateurs ont été consommés durant les réactions (pas de cycles catalytiques observés). Des cinétiques d'oxydation plus rapides ont été observées à des pH plus acides, mais des ratios médiateur/polluant plus élevés étaient également nécessaires. Les mélanges de produits de transformation étaient toujours moins toxiques pour les algues que les polluants non traités. Basé sur ces résultats, le SLM semble être une option prometteuse pour traiter des effluents industriels concentrés et potentiellement toxiques, mais ne semble pas être adapté pour le traitement de faibles concentrations de micropolluants dans des eaux usées municipales.

Afin de sélectionner un organisme produisant de la laccase qui pourrait être utilisé dans un traitement biologique avancé, quatre souches du genre bactérien *Streptomyces* et le champignon lignivore *Trametes versicolor* ont été étudiés. De part sa plus grande production de laccase, l'activité de sa laccase sur une gamme de pH plus large et à plus basses températures, une laccase plus stable et une meilleure efficacité pour l'oxydation des micropolluants, le champignon *T. versicolor* est apparu comme étant le meilleur candidat pour être inoculé dans un post-traitement biologique.

Finalement, un filtre fongique séquentiel discontinu (FFSD), composé de copeaux de bois servant de substrat et de support pour le mycélium, a été conçu et testé à échelle de laboratoire avec deux champignons lignivores, *Trametes versicolor* et *Pleurotus ostreatus*. Le FFSD avec *P. ostreatus* a pu être exploité en continu durant plusieurs mois (jusqu'à 140 j) avec des eaux usées non-stériles, sans addition d'aucun substrat externe, d'acidification ou de réinoculation. Une large gamme de micropollutants a pu être bien éliminée par une combinaison de dégradations fongiques et microbiennes et d'adsorption. Les FFSD étaient en mesure de rivaliser avec l'ozonation ou l'adsorption sur CAP concernant l'élimination moyenne (jusqu'à 82%) de 27 micropolluants dans des eaux usées municipales. Cependant, les relativement long temps de séjour hydraulique nécessaires (de 24 à > 48 h, comparé à < 40 min pour l'ozone ou le CAP) et la relative courte durée de vie du champignon ont engendré une consommation d'énergie relativement élevée (> 0.4 kWh m^{-3}) et des besoins en copeaux de bois importants (> 1 g l^{-1}). Par conséquent, malgré sa bonne efficacité pour le traitement des micropolluants, des équipements techniques simples, la simplicité d'exploitation, peu de maintenance et seulement des copeaux de bois et de l'électricité comme consommable, le filtre fongique doit encore être significativement optimisé afin de pouvoir être implémenté dans les STEP.

Cette thèse a ouvert de nouvelles perspectives concernant le traitement biologique des micropolluants dans les eaux usées, soulignant également les défis liés à l'application de traitements fongiques ou enzymatiques dans les STEP.

Mots-clés : micropolluants, médicaments, pesticides, eaux usées, ozonation, charbon actif, adsorption, oxydation, biodégradation, laccase, champignons lignivores, traitement fongique, STEP.

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Chapter 1 Introduction

The increasing worldwide consumption of chemical products has led to increasing chemical pollution of surface and groundwaters, with still largely unknown effects on human health and aquatic life. Contamination of natural water by thousands of chemical compounds despite, for most of them, very low concentrations (pg - µg l^{-1}), raises considerable ecological issues and is a major public concern almost all around the world (Schwarzenbach et al., 2006). Once in the environment, these compounds are referred to as "trace contaminants" or "micropollutants". Micropollutants are usually defined as "chemical compounds present at low concentrations (e.g., nano- to micrograms per litre) in the environment, and which, despite their low concentrations, can generate adverse effects for living organisms" (Chèvre and Erkman, 2011). This includes several hydrophobic pollutants such as heavy metals, dioxins, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), flame retardants, etc., but also more polar (hydrophilic) compounds designed to be biologically active such as pesticides and pharmaceuticals. Many of these micropollutants, such as detergents, pharmaceuticals, biocides or personal care products, are mainly rejected into municipal sewer systems and, because of only partial removal in conventional wastewater treatment plants (WWTPs) (Verlicchi et al., 2012), reach the aquatic ecosystems. WWTP effluents are thus considered as the main vector of these compounds into the environment (Kasprzyk-Hordern et al., 2009).

1.1 Impact of micropollutants

1.1.1 Toxicity to aquatic life

If the global issue of persistent, bioaccumulative and toxic (PBT) compounds is already partially handled through international legislations (Stockholm Convention POP, Rotterdam Convention PIC, European regulation REACH, etc.), impacts on wildlife related to less persistent but continuously emitted substances, such as pharmaceuticals, personal care products, biocides and endocrine disrupters were recently reported worldwide. For instance, feminization of fish and mussels, as well as intersex and reproductive disruption in fish, were observed in several rivers downstream of WWTP outfalls, probably related to the release of estrogenic endocrine disrupters, such as the active ingredient of the contraceptive pill (ethinylestradiol), natural estrogens, nonylphenol or bisphenol A (Alan et al., 2008; Gagné et al., 2011b; Jobling et al., 1998; Jobling et al., 2006; Tetreault et al., 2012; Tetreault et al., 2011; Tyler and Jobling, 2008; Vethaak et al., 2005). Several other adverse effects were observed downstream of WWTP outfalls, especially in the case of low dilution of effluents, such as neuroendrocinal alterations and oxidative stress in freshwater mussels (Gagné et al., 2011a; Gillis et al., 2014), histopathological effects in fish (Galus et al., 2013; Tetreault et al., 2012), alteration of macroinvertebrate communities and gammarid health (fecundity, sex ratio, stress) (Englert et al., 2013; Peschke et al., 2014), or reduction in leaf litter breakdown by gammarid crustaceans, which may impact the whole aquatic food web downstream of WWTPs (Bundschuh et al., 2011b).

Although it is very difficult to link these effects with specific micropollutants, there is evidence that these impacts were mainly due to micropollutant toxicity and not due to other macropollutants (organic matter, nutrients, etc.) found in treated wastewaters. Indeed, it was reported that some of these adverse effects were clearly reduced after degradation of most micropollutants by ozonation (which did not affect the concentration of macropollutants) (Bundschuh et al., 2011b; Bundschuh and Schulz, 2011). Moreover, several studies showed that micropollutants can have toxic effects already at the concentrations found in WWTP effluents (see below).

For instance, the fish population of a small lake was strongly affected (male feminization, decrease in the reproductive success) and collapsed after exposure to the synthetic estrogen ethinylestradiol at 5-6 ng l^{-1} (Kidd et al., 2007). Ethinylestradiol was reported to decrease egg fertilization and to change the sex ratio in fish even at 0.3 ng l^{-1} (Parrott and Blunt, 2005).

The anti-inflammatory drug diclofenac, the antiepileptic carbamazepine and the beta-blocker metoprolol were reported to generate cytological alterations in fish liver, kidney and/or gills already at 0.5-1 µg l^{-1}, concentration found in municipal WWTP effluents (Hoeger et al., 2005; Triebskorn et al., 2004; Triebskorn et al., 2007). Diclofenac is indeed known for its potential toxicity to wildlife and was associated to the collapse of the vulture population in Pakistan, caused to renal failures linked to the consumption of diclofenac-treated livestock (Oaks et al., 2004). The antiepileptic carbamazepine may also possibly alter freshwater community structure and ecosystem dynamics at a concentration of 0.2-2 µg l^{-1} (Jarvis et al., 2014).

Several antibiotics (e.g., azithromycin, clarithromycin, erythromycin and ciprofloxacin), in addition to potentially favouring the development of antibiotic resistant pathogens (if present at concentrations above the minimum selective concentration (MSC)) (Sandegren, 2014), were also reported to significantly inhibit algae and cyanobacteria growth at low concentrations (1-5 µg l^{-1}), close to the concentrations found in WWTP effluents (Ebert et al., 2011; González-Pleiter et al., 2013; Harada et al., 2008; Isidori et al., 2005).

The biocide triclosan affects river biofilms and algae community structure at concentrations potentially lower than 0.5 µg l^{-1} (Franz et al., 2008; Ricart et al., 2010). The biocide irgarol was also reported to significantly affect algae at concentrations as low as 8-25 ng l^{-1} (Nyström et al., 2002).

Most of these impacts are chronic sub-lethal effects that are difficult to extrapolate to real impacts on aquatic ecosystem functions. Nevertheless, this demonstrates that constant exposure to very low levels of micropollutants found in municipal WWTP effluents is not harmless, especially in the case of low dilution of effluents. Therefore, release of micropollutants should be avoided to protect sensitive aquatic ecosystems.

1.1.2 Drinking water contamination

Contamination of surface waters by micropollutants released from municipal WWTPs raised the question of drinking water contamination, as surface waters are one of the main sources of drinking water all around the world. Several micropollutants of wastewater origin, such as pharmaceuticals, biocides, personal care products, plastic additives or sweeteners have been detected, usually at low

concentrations (<1 – 100 ng l^{-1}), in finished drinking waters in many countries (Benner et al., 2013). In order to estimate the potential human health risks, the lifelong human exposure to these micropollutants via drinking water was assessed in a few studies (Houtman et al., 2014; Sanderson, 2011; Webb et al., 2003). The dose of micropollutants ingested during a whole life (70 years) by drinking 2 l of water per day varies between < 5 µg up to 4 mg, corresponding, for most of the 58 pharmaceuticals studied, to less than 10% of one defined daily dose (DDD), the dose a patient is administered on one day. The exposure to pharmaceuticals via drinking water is thus considered to be very low in comparison with therapeutic doses or other exposure routes. Due to the very low concentrations of micropollutants in drinking waters, all the studies concluded that appreciable adverse impacts on human health are unlikely at current levels of exposure, even if the potential effect of low level chronic exposure to chemical mixtures is still mainly unknown. Concerns over drinking water contaminated by micropollutants of municipal wastewater origin should therefore not divert water suppliers and regulators from real water quality issues regarding human health: contamination by pathogens, toxic disinfection by-products, algal toxins or high concentrations of regulated industrial/agricultural pollutants (Richardson, 2003; Sanderson, 2011). Nevertheless, even if no human health impacts are expected, drinking water resources are valuable and have to be protected and preserved to provide water of high quality in the future. Moreover, a trend to a decrease in public acceptance of the presence of micropollutants in drinking waters appeared, linked to increased awareness of these issues. Public awareness of the contamination of drinking water is likely to be a stronger driver for political actions than potential adverse effects for aquatic organisms, even though the latter are more consequential (Eggen et al., 2014).

1.2 Strategies to reduce the release of micropollutant in surface waters

In order to protect aquatic ecosystems and to preserve drinking water resources, reduction of the inputs of micropollutants in surface waters is necessary. For micropollutants of mainly urban origins, such as pharmaceuticals, personal care products, household products and biocides, several levels of action are proposed: (i) prevention and source control, (ii) source separation and decentralized treatments, or (iii) centralized end-of-pipe treatments (Eggen et al., 2014; Larsen et al., 2004).

1.2.1 Prevention and source control

Source control aims to avoid the use and the dispersion of chemicals of concerns. This includes (i) regulations, at national or international levels, to ban or restrict the use of harmful compounds; (ii) collaboration with industries to develop chemicals harmless for the environment (biodegradable, not toxic, etc.) (“green chemistry”) or technologies requiring less chemicals (e.g., washing machines requiring few detergents, thermal or mechanical weeders); (iii) substitution of harmful chemicals by more environmentally friendly existing substances; and (iv) users awareness and promotion of best management practices (via information campaigns or incentive taxes) to change consumer behaviours, such as proper disposal, moderate use, optimal usage, choice of chemicals with lower environmental impacts, etc.

Obviously, source control should be the first strategy to apply to limit the dispersion of critical substances. However, success can only be expected in the long-term, due to the time required to adopt

new regulations, to develop cleaner products or to change consumer habits. Moreover, compound-specific regulations, even coupled to changes in consumer behaviours, are unlikely to be sufficient to reduce the loading of hundreds of micropollutants that come from various usages and applications. In addition, restriction of pharmaceutical consumption for environmental reasons seems difficult to justify. For this class of compounds, substitution of critical drugs with others with the same therapeutic properties but more biodegradable and moderate usage are probably more realistic. In any case, source control must be complemented with other measures (Eggen et al., 2014).

1.2.2 Source separation and decentralized treatments

This strategy consists to apply target treatment of the most polluted and concentrated wastewaters, such as hospital effluents, patient urine, industrial effluents, etc., before their dilution with high volumes of less polluted wastewaters. These point-source measures allow treating much smaller volumes and with better efficiency than in diluted wastewater. Decentralized treatments are thus interesting options in the case of high participation of these punctual sources to the global load of pollutants.

Urine, which represents less than 1% of the wastewater volume (Larsen et al., 2004), contains around 50% of the non-metabolized pharmaceuticals excreted after human consumption (Lienert et al., 2007). Source separation and specific treatment of urine were thus proposed (Larsen et al., 2004). However, although this could reduce half of the load of pharmaceutical in wastewater and allow recovering valuable nutrients, complementary treatments will be necessary to treat the part not contained in urine, and urine collection will require a major change of the sanitation system, which might be feasible only in the long-term.

Decentralized treatment of hospital effluents was also suggested to avoid the spread of specific ecotoxic pharmaceuticals, pathogens and multiantibiotic resistant bacteria (Lienert et al., 2011). However, the contribution of hospital effluents to the total load of pharmaceuticals at WWTPs is usually less than 10-15% (Langford and Thomas, 2009; Ort et al., 2010). This is not surprising as pharmaceuticals are widely used throughout the population. Even if some drugs are only administered in hospitals, they are mainly excreted at home in case of ambulatory care. Treatment of hospital effluents would thus not be sufficient to reduce the load of pharmaceuticals released in the environment.

Therefore, for micropollutants of mainly households or diffuse origins, such as pharmaceuticals, personal care products, biocides, detergents, etc., centralized treatments, in combination with source control, appear to be the best solution.

1.2.3 Centralized end-of-pipe treatments

As in most developed countries, municipal wastewater is collected and routed via the sewer networks towards centralized WWTPs, the easiest and most efficient strategy is to upgrade WWTPs with advanced technologies able to treat micropollutants (see below). This strategy was adopted in March 2014 by the Swiss government, which decided to implement, over the next 20 years, technical measures for micropollutant reduction in (i) large WWTPs (> 80,000 population equivalent (PE)) for

which larger load reductions can be achieved, (ii) all WWTPs > 8000 PE that are characterized by low dilution (< 10 times by dry weather) in the receiving waters (ecotoxicity reduction), and (iii) WWTPs > 24,000 PE discharging into sensitive waters or important water reservoirs (drinking resources protection). The goal of these measures is to reduce by 80% on average (based on indicator substances) the load of micropollutants present in raw wastewater before its discharge in the environment (Eggen et al., 2014).

To be relevant for micropollutant removal in municipal WWTPs, advanced technologies have to meet several criteria (Abegglen and Siegrist, 2012):

- Efficiency on a wide range of micropollutants, covering most of the substances concerned
- Absence of formation of undesirable or toxic by-products and wastes
- Feasible in WWTPs without strong modification of existing installations
- The local staff should be able to operate it with minimal training and it should not impact the efficiency of the WWTP
- The costs and the energy consumption of the advanced treatment should be reasonable and proportional to the benefits of the treatment.

The efficiency of centralized solutions depends also on the efficiency of the wastewater collection systems. Indeed, a significant load of micropollutant can potentially reach the environment via sewer leakages or combined sewer overflows (CSOs). In combined sewer systems, stormwater runoff is collected in the same pipe as domestic wastewater. During rain events, once the maximal capacity of the WWTP is reached, the additional flow is frequently directly diverted, via CSOs and without treatment, into surface waters. The quantity of non-treated municipal wastewater (excluding stormwater) that reaches the environment depends on the sewer network configuration and the climate, but ranges usually around 2-4% of the total wastewater collected (in dry weather equivalent). Thus, for some micropollutants well removed in WWTPs, CSOs may appear as the main source into the environment (Buerge et al., 2006; Weyrauch et al., 2010). Urban stormwater contains also significant concentrations of micropollutants, such as heavy metals, PAHs or pesticides (Gasperi et al., 2014). Therefore, optimization of the sewer network and the stormwater collection and treatment has also to be considered to reduce the release of micropollutants.

1.2.4 Advanced physico-chemical treatments

To face the water-quality problem caused by wastewater micropollutants, efficient but cost effective centralized treatment technologies have to be developed. Mature solutions already exist and are ready to be used in WWTPs, such as ozonation (oxidation of the pollutant with ozone) or adsorption onto activated carbon. Other advanced physico-chemical technologies, such as filtration on tight membranes (reverse osmosis and nanofiltration) or advanced oxidation processes (AOPs) showed good potential for micropollutant removal, but are still at the research level or are too expensive for implementation in municipal WWTPs. These technologies are presented in Chapter 2.

1.2.4.1 Limitations of advanced physico-chemical treatments for small WWTPs

Advanced physico-chemical treatments, despite their efficiency, are often considered to be relatively expensive, especially for small installations, and require technical skills for their operation (Eggen et al., 2014). These technologies are thus not well adapted for small WWTPs without permanent staff. Moreover, they consume significant energy (increasing up to 30% the energy consumption in WWTPs) (Eggen et al., 2014) and potentially non renewable resources (e.g., coal for activated carbon production), which goes against efforts made for the reduction of climate change.

Alarming concentrations of micropollutants are usually found in small watercourses receiving large amounts of WWTP effluents (low dilution). In the canton of Vaud (Switzerland), 47 WWTPs present a dilution factor smaller than 10 during dry weather. Among them, 30 have less than 2000 PE (Jaquerod et al., 2010). Even if these small WWTPs are not important in terms of load of micropollutants, they can impact the quality of many small streams. Therefore, in order to avoid negative impacts in the receiving waters, research has to be invested in the development of a treatment affordable for small WWTPs, with low equipment needs, maintenance, skills, and energy requirements.

1.2.5 Advanced biological oxidation processes

Some bacteria and fungi have developed powerful oxidative enzymes such as oxygenases, laccases and peroxidases that have broad substrate spectra and are usually involved in the degradation of complex and highly resistant natural molecules such as lignin and lignin-derived aromatic compounds (Baldrian, 2006; Conesa et al., 2002; Dwivedi et al., 2011). Many studies showed that these enzymes are also able to oxidize several micropollutants recalcitrant to bacterial degradation, including pharmaceuticals and biocides (Rodarte-Morales et al., 2011; Yang et al., 2013b). Therefore, oxidative enzymes could possibly be employed in wastewater as unspecific oxidation catalysts able to transform many different organic micropollutants, converting them to less toxic compounds or more prone to further biodegradation. Oxidative enzymes have, however, lower redox potentials (between 0.2 and 1.4 V, see below) than chemical oxidants like hydroxyl radicals (E^0 = 2.33 V) or ozone (E^0 = 2.07 V) (Gogate and Pandit, 2004). Therefore, slower oxidation rates and a narrower substrate range can be expected with oxidative enzymes.

As for most chemical oxidants, oxidative enzymes do not mineralize the substrate, but create radicals which can break down to smaller transformation products or couple with other molecules through non-enzymatic processes (oxidative coupling reactions) to form higher molecular weight compounds (Auriol et al., 2008; Garcia et al., 2011; Huang and Weber, 2005). In most cases, the transformation products were reported to be less toxic or more biodegradable than the parent compounds, showing the potential of biological oxidative treatment to improve wastewater quality (Auriol et al., 2008; Cabana et al., 2007a; Gaitan et al., 2011; Kim and Nicell, 2006a; Kim and Nicell, 2006c; Lloret et al., 2013; Murugesan et al., 2010; Palvannan et al., 2014; Saito et al., 2004; Suda et al., 2012; Tsutsumi et al., 2001).

The development of advanced biological oxidation processes (with oxidative enzymes), potentially less demanding in energy and resources than physico-chemical treatments, but requiring probably

longer reaction times and thus more space, may thus possibly be an option for the treatment of micropollutants in small WWTPs, where size is not a limiting factor.

1.2.5.1 Type of oxidative enzymes

Several oxidative enzymes from bacteria, plant and fungi have been described and could be potentially used to remove pollutants from the environment. The main families are oxygenases, peroxidases and polyphenol oxidases (Burton, 2003; Duran and Esposito, 2000; Torres et al., 2003). The characteristics of these enzymes are presented below.

1.2.5.1.1 Oxygenases

Oxygenases can be divided into monooxygenases and dioxygenases. Monooxygenases use molecular oxygen to insert one oxygen atom into a substrate while the second oxygen is reduced by the cofactor NAD(P)H (nicotinamide adenine dinucleotide (phosphate)) to water. Dioxygenases catalyze the regio-selective insertion of two oxygen atoms from molecular oxygen into a substrate in the presence or not of cofactors (Burton, 2003; Li et al., 2002). Oxygenases include a large number of different enzymes families, which are able to oxidize a broad spectrum of aromatic compounds. Examples of monooxygenase are the well-known *cytochrome P450* superfamily which is involved in many reactions, like bioconversion of xenobiotics, metabolism, biosynthesis of biological compounds, etc. (Bernhardt, 2006); *ammonia and methane monooxygenases* that were used to degrade several micropollutants (chlorinated solvents, biocides, plastic additives, pharmaceuticals) by cometabolism (Erwin et al., 2005; McFarland et al., 1992; Roh et al., 2009; Tran et al., 2009; Yi and Harper, 2007); or *toluene monooxygenases* that can hydroxylate a broad variety of aromatic compounds (Gullotto et al., 2008; Li et al., 2002). Examples of dioxygenases are *catechol dioxygenases* which catalyze the oxidative cleavage of the aromatic ring of hydroxylated aromatic compounds (Guzik et al., 2011); *toluene and naphthalene dioxygenases* that have broad substrate spectrum transforming more than 70 compounds in cometabolic activities (http://eawag-bbd.ethz.ch/index.html, last accessed 15.10.2014) ; or many other aromatic *hydrocarbon dioxygenases*, like biphenyl, benzoate, or phthalate dioxygenases families that can oxidize many aromatic hydrocarbons (Gibson and Parales, 2000).

These enzymes are mainly intracellular (Arras et al., 1998) and require usually other cellular components as cofactors (e.g., NAD(P)H), which can only be efficiently regenerated inside cells or in the presence of intact cellular membranes (Torres et al., 2003). The use of pure oxygenase enzymes is thus not interesting and only processes using whole living microorganism cells can be applied for large scale applications (Gullotto et al., 2008).

1.2.5.1.2 Peroxidases

Peroxidases are oxidoreductases (hemoproteins) produced by many organisms like mammals, plants, fungi and bacteria. Theses enzymes utilize hydrogen peroxide (H_2O_2) to catalyze the oxidation of various organic and inorganic compounds (Conesa et al., 2002). Hydrogen peroxide first oxidizes (activates) the enzyme, which in turn oxidizes the substrate (AH_2) into radicals ($AH\cdot$) (Karam and Nicell, 1997). The reaction involves a two-electron transfer reaction to reduce H_2O_2 to two molecules of water (Battistuzzi et al., 2010; Conesa et al., 2002; Veitch, 2004).

Peroxidases are readily inactivated by excess of H_2O_2, which is a serious constraint for the industrial or environmental application (Baciocchi et al., 2002). A strict regulation of the H_2O_2 concentration is necessary, with a "feed on demand" system or by in situ generation (Conesa et al., 2002). For the latter, several fungal or bacterial enzymes like aryl alcohols oxidases, glyoxal oxidases, galactose oxidases or glucose oxidases can generate H_2O_2 by reducing oxygen and oxidizing different substrates (Sinsabaugh, 2010). H_2O_2 can thus be produced in situ, for instance by addition of glucose and glucose oxidase (Inoue et al., 2010). Inactivation of peroxidases can be also due to destruction of the heme prophyrin ring by the oxidant (suicide inhibition) (Burton, 2003).

A potential strong limitation for the application of peroxidases in wastewater is the fast consumption of H_2O_2 by the wastewater matrix (reaction with the organic matter) (Ksibi, 2006), which may strongly decrease the enzymatic activity or require the addition of high concentrations of H_2O_2.

Four main types of extracellular peroxidases have been widely studied so far: horseradish peroxidase, lignin peroxidase, manganese peroxidase and versatile peroxidase.

Horseradish peroxidase (HRP), found in the root of the horseradish plant (Veitch, 2004), can catalyze the oxidation of a wide range of toxic aromatic compounds like phenols, biphenols, anilines, benzidines and related heteroaromatic compounds (Karam and Nicell, 1997). The reaction products can be polymerized through a non-enzymatic process that leads to the formation of water-insoluble precipitates (Karam and Nicell, 1997). HRP is suitable for wastewater treatment because it retains its activity over a broad pH and temperature range (Karam and Nicell, 1997). HRP has many physiological roles including lignification, cross-linking of cell wall polymers, resistance to infection and metabolism (Veitch, 2004).

Lignin peroxidase (LiP) is part of the extracellular enzymes system of wood-degrading fungi and is involved in lignin degradation (Karam and Nicell, 1997). LiP was shown to oxidize a broad range of polycyclic aromatic and phenolic compounds. LiP is the only known extracellular peroxidases capable of oxidizing non-phenolic aromatic substrates with high redox potential (1.45 to 1.49 V (Burton, 2003)) (Oyadomari et al., 2003). The presence of a mediator like veratryl alcohol (a secondary fungal metabolite) can increase the oxidation of organic substrates that are recalcitrant towards the oxidation by LiP alone. The mediator is oxidized by LiP to a radical cation which in turn can react with the aromatic rings of recalcitrant compounds.

Manganese peroxidase (MnP) is another enzyme involved in lignin degradation by ligninolytic (wood-degrading and soil litter-decomposing) fungi. MnP has more specific substrates and preferentially oxidizes manganese(II) ions (Mn^{2+}), always present in wood and soils, into highly reactive Mn^{3+}, which is stabilized by fungal chelators such as oxalic acid. Chelated Mn^{3+} in turn acts as low-molecular weight, diffusible redox mediator that can oxidize phenolic compounds and transform them into instable free radicals that tend to degrade spontaneously (Hofrichter, 2002). MnP can catalyse the oxidation of several phenolic and amino-aromatic compounds, as well as a few nonphenolic aromatic substances with low redox potential (Hofrichter, 2002). However, the enzyme requirement for high concentrations of manganese ions, hydrogen peroxide and chelators makes its feasibility for wastewater treatment application doubtful (Karam and Nicell, 1997).

Versatile peroxidase (VP), also isolated from several white-rot fungi and involved in lignin degradation, is a structural and functional hybrid of LiP and MnP, showing both Mn^{2+}-dependant and Mn^{2+}-independent activities. VP is able to oxidize both LiP and MnP substrates. Moreover, it can directly oxidizes hydroquinones, substituted phenols and even some high redox-potential dyes that are not efficiently oxidized by LiP or MnP in the absence of veratryl alcohol or Mn^{2+} respectively (Martínez, 2002).

1.2.5.1.3 Polyphenol oxidases

Polyphenol oxidases belong to another family of oxidoreductases that can catalyze the oxidation of phenolic compounds in the presence of molecular oxygen without the need of cofactors (Karam and Nicell, 1997). They are divided into two subclasses: laccases and tyrosinases.

Laccases

Laccases (EC 1.10.3.2) are multicopper enzymes that catalyze the oxidation of various aromatic compounds, particularly those with electron-donating groups such as phenols (-OH) and anilines (-NH_2), with the concomitant reduction of oxygen to water (Gianfreda et al., 1999). Laccases usually contain four copper ions distributed in three active sites, which are involved in the electron transfer from the substrate (T1 active site) toward oxygen (T2/T3 active sites) (Baldrian, 2006). Laccase withdraws one electron from the substrate (RH) and converts it into a free radical (R·), which can break down to a smaller molecule or be polymerized. After receiving four electrons, the enzyme transfers them to molecular oxygen to form water (Eq. 1.1) (Dwivedi et al., 2011):

$$4RH + O_2 \rightarrow 4R\cdot + 2H_2O \qquad (1.1)$$

Laccase has a low substrate specificity and can oxidize a wide range of compounds, with a preference for first *ortho*, then *para* and at the end *meta* substituted phenols (Baldrian, 2006).

Laccase enzymes are widespread among (wood-rotting) fungi, bacteria and plants, and have various biological functions (Claus, 2004; Dwivedi et al., 2011):

1. *Degradation of polymers*: laccase is involved in the degradation of complex natural polymers such as lignin or humic acids to gain carbon and other nutrients. The reactive radicals generated by the enzymatic oxidation lead to the cleavage of covalent bonds and to the release of monomers.
2. *Cross-linking of monomers*: radicals generated by the enzymatic oxidation of phenolic compounds and anilines can react with each other to form dimers, oligomers or polymers covalently coupled by C-C, C-O or C-N bonds. This process is involved in lignin polymerization and humic substance formation (Gianfreda et al., 1999), or polymerization of melanin and spore coat resistance (Strong and Claus, 2011), and can be use in soils to bound xenobiotics to the organic humic matrix and thus detoxify contaminated soils.
3. *Ring cleavage of aromatic compounds*: laccase can catalyze the ring cleavage of some aromatic compounds, which is useful for the degradation of aromatic xenobiotics.

The relatively low redox potential of laccase (E^0 of 0.45 to 0.8 V) compared to those of peroxidases (> 1 V) allows only the direct degradation of compounds with a low-redox potential (Baldrian, 2006). The substrate range of laccase can be, however, widened to non-phenolic compounds or pollutants with higher redox potentials by the addition of mediators (Husain and Husain, 2008). Mediators are low molecular weight compounds (such as ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)), syringaldazine or hydroxybenzotriazole (HBT)) that are easily oxidized by laccase, producing free reactive radicals that can oxidize in turn more complex substrates (Torres et al., 2003). In this laccase-mediator process, binding between the enzyme and the substrate is not necessary enabling therefore the oxidation of more recalcitrant substrates. If synthetic mediators such as ABTS or HBT are very efficient, natural compounds like phenolic molecules generated during fungal degradation of complex substrates like lignin may also act as natural mediators. Thus addition of mediator might not be required if the culture broth contains some lignocellulosic substrates (Majeau et al., 2010).

The structural properties of fungal laccases have been well described with median values of acidic isoelectric point around 3.9, a molecular weight around 66 kDa and, for laccase activity, a temperature optimum around 55°C (range from 50°C to 70°C) and pH optima usually in the acidic pH range, between 3 and 7 depending on the substrate and the source of enzymes (Baldrian, 2006; Dwivedi et al., 2011; Majeau et al., 2010). The enzymatic activity at higher pH is decreased by the binding of a hydroxide anion on the active copper centre of laccase that interrupts the internal electron transfer (Baldrian, 2006). Laccases are considered as relatively stable enzymes, with half-lives of several days in treated municipal wastewater at 20°C (Majeau et al., 2010; Zimmermann et al., 2011b).

Various reagents can inhibit laccases, such as small anions as halides, azide, cyanide and hydroxide, which can bind to the active copper and interrupt the electron transfer. Other inhibitors like divalent metal ions, fatty acids, EDTA, cationic quaternary ammonium detergents, glutathione, thiourea, humic acid, etc. have also been reported (Baldrian, 2006; Dwivedi et al., 2011).

Tyrosinases

Tyrosinases are copper-containing enzymes that are ubiquitously distributed in nature. The best documented function of this enzyme is the formation of melanin pigments, which protect the cell against UV radiation and oxidants, and bind toxic heavy metals (Claus and Decker, 2006). Tyrosinase catalyzes two different reactions using molecular oxygen: (1) the hydroxylation of monophenols to form o-diphenol and (2) the oxidation of o-diphenols to o-quinones by dehydrogenation (Claus and Decker, 2006). The reactive quinones undergo then a non-enzymatic polymerization to form soluble oligomers and colored compounds like melanin (Karam and Nicell, 1997). These transformation products are less toxic than the parent phenols (Torres et al., 2003). Thus tyrosinase can be used in environmental technology for the detoxification of phenol-containing wastewater or contaminated soils (Claus and Decker, 2006). The redox potential E^0 of tyrosinase is estimated to be at 0.26 V, much lower than the one reported for laccase (Baldrian, 2006). This enzyme seems therefore to be less interesting for industrial applications.

Laccases have the advantage compared to other oxidative enzymes to be readily available (extracellular enzymes produced by many fungi and bacteria). They are highly stable compared to

fungal peroxidases, have a broad substrate range and do not require cofactors such as hydrogen peroxide (but mediators can increase their efficiency) (Burton, 2003). Thus, despite slightly lower redox potential than peroxidases, laccases are interesting catalysts for many industrial and environmental applications, including wastewater treatment (Dwivedi et al., 2011).

1.2.5.2 Pure enzymes versus whole living microorganisms

The most important issue precluding the practical use of pure oxidative enzymes for wastewater treatment is the high cost of enzyme production, as large quantities of enzyme would be required (Majeau et al., 2010). Solutions to reduce these costs are for instance the production of these enzymes with cheap substrates like agricultural or forestry wastes, and the immobilization of the enzymes in order to reuse them several times (Majeau et al., 2010). Another potentially more interesting option would be to use the whole organisms producing these enzymes for the treatment of wastewater. Indeed, combined effects of the actions of laccases, peroxidases and other extracellular or intracellular enzymes produced by these organisms could be advantageous in terms of broader substrate range and further mineralization of toxic compounds (Majeau et al., 2010). Several organisms producing oxidative enzymes could be used for this purpose, mainly fungi and bacteria.

Fungal laccases and peroxidases are widely distributed in ascomycetes, deuteromycetes and basidiomycetes, especially in wood degrading fungi such as many members of the white-rot fungi genus *Trametes* (particularly *Trametes versicolor*) (Baldrian, 2006). These basidiomycetes produce constitutively small amounts of extracellular laccase that can be augmented by an inducer such as aromatic or phenolic compounds related to lignin (Majeau et al., 2010). Laccases are also reported in saprophytic ascomycetes of composts (e.g., *Aspergillus*) and in soil hyphomycete (Baldrian, 2006; Dwivedi et al., 2011). The main role of fungal oxidative enzymes (laccase and peroxidase) is to depolymerize the complex cell-wall constituents of wood such as lignin in order to gain carbon and nutrients. This degradation process involves also other enzymes, such as glucose oxidase and glyoxal oxidase for H_2O_2 production and cellobiose-quinone oxidoreductase for quinone reduction (Dwivedi et al., 2011). Due to the properties of their substrate, ligninolytic enzymes are almost exclusively extracellular, but intracellular laccases have also been observed in several fungi (Baldrian, 2006). The white-rot fungus *Trametes versicolor* was reported to be one of the most promising fungi for the degradation of persistent compounds (Marco-Urrea et al., 2009).

Laccase and peroxidase activities have also been identified in many soil bacteria, particularly in *Streptomyces* sp., *Bacillus* sp. and *Pseudommonas* sp., but also in several other bacteria. Many forms of bacterial laccases have been described with intracellular, periplasmic, on spore coats or extracellular enzymes (Sharma et al., 2007). Bacterial laccases have usually low level of expression, more restricted substrate range (Majeau et al., 2010) and lower redox potential than fungal laccases (Bugg et al., 2011; Dwivedi et al., 2011). However, novel bacterial laccases have been reported to successfully oxidize a wide range of substrates. In contrast to fungal laccases, some bacterial laccases can be highly active and much more stable at high temperature, at high pH as well as at high concentration of chloride (Bugg et al., 2011; Dwivedi et al., 2011; Reiss et al., 2011; Sharma et al., 2007).

The possibility to use laccase producing organisms, such as white-rot fungi or *Streptomyces* bacteria, for municipal wastewater treatment, faces, however, many challenges due to treatment conditions that are far from ideal for these organisms (dead wood, soil), such as competition with native microorganisms, predation, or long-term survival in stressful conditions (Libra et al., 2003).

1.3 Objectives and structure of the thesis

The global objective of this thesis was to study and develop various options to **improve micropollutant removal from municipal wastewaters**, with a special focus on oxidative biotransformation. The main idea was to improve biodegradation of recalcitrant micropollutants after the final WWTP stage using microorganisms that produce oxidative enzymes. The goal was to develop a treatment process that requires low energy, low or no chemical inputs, low maintenance and ease of operation, in order to provide an environmentally friendly solution affordable for small WWTPs to improve the quality of natural waters.

To reach this goal, many open questions had to be investigated:

1. What is the fate of various classes of micropollutants in conventional WWTPs, what are their removal efficiencies and which classes of pollutants are of most concern?
2. What is the efficiency of existing but still rarely applied advanced treatments such as ozonation and activated carbon adsorption? Can they be implemented in municipal WWTPs and what are their limitations?
3. Are oxidative enzymes such as ammonia monooxygenases and laccases able to transform target micropollutants usually not easily biodegradable? If yes, in which conditions?
4. Are bacteria or fungi able to produce a sufficient quantity of these enzymes to transform target micropollutants? Can we grow and maintain these microorganisms in systems treating municipal wastewater?
5. Is it possible to maintain and promote these organisms for long-term operations in real wastewater in a post-treatment system? What are the optimal conditions? Is this microbial system still efficient to treat micropollutants?
6. Is this process relevant for small municipal WWTPs? What are the main limitations and in which conditions could it work?

The investigations of all these questions are described in the following eight chapters of this thesis.

In *Chapter 2*, a literature review evaluating the fate of more than 160 micropollutants of various classes (surfactants, pharmaceuticals, personal care products, pesticides, household products, heavy metals, etc.) in conventional WWTPs is described in order to better understand the removal mechanisms, the possibilities to improve them and the classes of pollutants of most concern.

In *Chapter 3*, the efficiency for micropollutant and toxicity removal and the technical feasibility in WWTPs of two advanced treatments, ozonation and adsorption onto powdered activated carbon, were assessed, based on the results of a pilot-scale study performed during more than one year at the WWTP of Lausanne, Switzerland, in collaboration with several other institutions.

In *Chapter 4*, the role of nitrification and ammonia monooxygenase oxidation in micropollutant removal in WWTPs was studied with a laboratory-scale aerobic granular sludge sequencing batch reactor.

In *Chapter 5*, the range of pollutants oxidized by laccase and laccase-mediator systems was assessed, and the influence of the treatment conditions on the removal of these micropollutants by laccase (*Chapter 6*) and laccase-mediator systems (*Chapter 7*) was determined.

In *Chapter 8*, several laccase-producing microorganisms, including *Streptomyces* bacteria and the white-rot fungus *Trametes versicolor*, were tested for their ability to produce laccase in wastewater. The activity and the efficiency of their respective laccases were assessed in various conditions to determine which, of bacteria or fungi, have the highest potential for wastewater treatment applications.

Finally, in *Chapter 9*, a sequencing batch fungal filter was designed and tested in continuous operation with two white-rot fungi, *Trametes versicolor* and *Pleurotus ostreatus*, to determine long-term removal efficiencies of a wide range of micropollutants in synthetic and real wastewater.

The last chapter, *Chapter 10* with *General conclusions and perspectives*, summarises the main conclusions and discussions drawn from all the work presented.

Each chapter is structured as a scientific publication, with its own materials and methods section and its own supporting information section. However, all the references are combined and presented at the end of the document. The final appendixes contain mainly protocols used during this thesis.

Chapter 2 Fate of micropollutants in municipal wastewater treatment plants

*This chapter is a literature review. An adapted version was submitted for publication in **WIREs Water**, with the name "Fate of micropollutants in municipal wastewater treatment plants", by Jonas Margot, Luca Rossi, D. Andrew Barry and Christof Holliger.*

2.1 Introduction

A wide range of chemicals used daily in homes, workplaces or in the urban environment ends up in sewers. This is obviously the case for "down the drain" products, such as detergents and their additives, or personal care products, but also for pharmaceuticals and their metabolites that are excreted in urine and faeces, and several household chemicals such as food or plastic additives, or flame retardants contained in textiles. Municipal wastewaters are also contaminated by non-domestic pollutants such as heavy metals, pesticides or hydrocarbons, leached during rain runoff from roads, buildings, and urban parks and gardens. The fate of these pollutants during wastewater treatment depends mainly on their physico-chemical characteristics (hydrophobicity, biodegradability, volatility) and the type of treatment. In order to reduce the release of micropollutants into the aquatic environment, understanding what happens for different classes of micropollutants in conventional wastewater treatment plants (WWTPs) is necessary. This chapter aims to explain the main removal mechanisms in conventional treatment systems and the fate of certain classes of micropollutants during treatment. Finally, processes to improve micropollutant removal, by optimizing biological treatments or with advanced physico-chemical treatments, are discussed.

2.2 Removal mechanisms in conventional WWTPs

Every day in Switzerland, each inhabitant produces on average (including infiltration/runoffwater inflow inputs) around 300 to 350 litres of wastewater that need to be treated (DGE, 2013; VSA-FES/ORED, 2006). This last century, different treatment technologies were developed as a function of the observed environmental issues related to wastewater contamination (Table 2.1).

Table 2.1 Chronology of environmental impacts generated by the pollution emitted from municipal wastewater in industrialized countries, and examples of technical measures taken in Switzerland. Adapted from Weissbrodt (2012).

Detection period	Phenomenon	Undesired pollutants	Technical measure	Application year
1920	Mud accumulation in rivers	Total suspended solids (TSS)	Mechanical treatment	1920
1950	Rivers anoxia	Organic matter as biological oxygen demand (BOD)	Biological carbon removal	1950
1965	Eutrophication of lakes	Total phosphorus	Chemical dephosphatation	1965
1975	Fish toxicity	Ammonium	Biological nitrification	1975
1980	Pollutants in agriculture	Heavy metals	Interdiction of sewage sludge farming	2000
1990	Eutrophication of the North Sea	Nitrates	Biological denitrification	1995
1900-2010	Waterborne diseases, antibiotic resistances	Pathogens, resistant bacteria	Disinfection	(1970)-foreseen 2025?
2000	Aquatic toxicity and hormonal disturbances	Micropollutants	Advanced treatments (oxidation, adsorption)	Foreseen 2016
2010	Accumulation in biological systems	Nanoparticles	Membrane filtration	Foreseen 2025?

Currently, the classical configuration for domestic wastewater treatment (Fig. 2.1) is composed of (i) pre-treatments to remove coarse wastes (bar screen), sand (decantation channel) as well as fat and grease (tank where skimmers collect the floating fat), (ii) primary treatment composed of a primary clarifier (or sedimentation tank) to remove most of the suspended solids by sedimentation (primary sludge), (iii) secondary treatment designed to remove the dissolved or residual solid easily biodegradable contaminants by biodegradation, and (iv) tertiary treatments to remove nutrients such as ammonium (by biological nitrification), nitrate (by biological denitrification) and phosphate (mostly by chemical precipitation), or in some cases to eliminate residual suspended solids (by sand filtration) or pathogens (by disinfection).

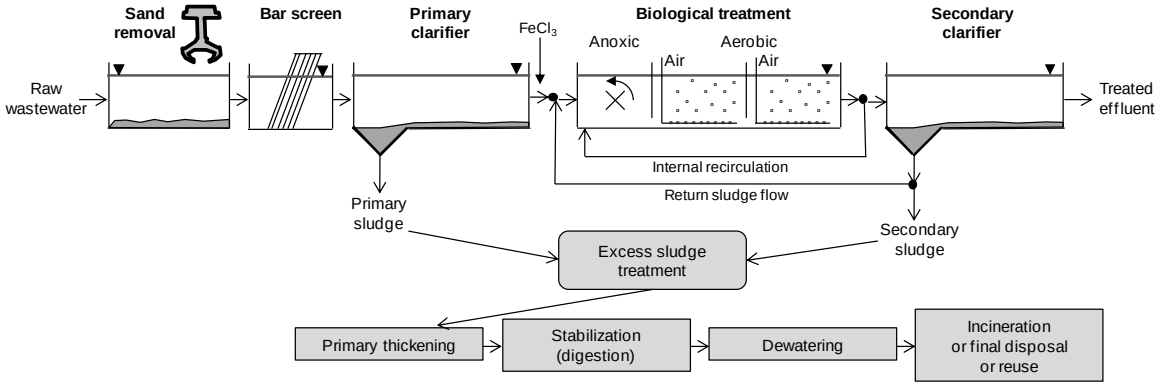

Fig. 2.1 Scheme of a conventional WWTP with activated sludge for the removal of biodegradable organic matter, nitrification, denitrification and chemical phosphorus removal (precipitation with $FeCl_3$).

Biological treatments consist of managing indigenous water-borne microorganisms to promote fast degradation of organic matter, either in suspend-growth systems (e.g., activated sludge), or in fixed-film systems (e.g., trickling filters or biofilters). Both systems are generally aerated, in order to

increase the degradation rates of the dissolved organic matter. The excess microbial biomass produced (secondary sludge) is then separated from the water by a secondary clarifier and all the sludge produced are (eventually) stabilized (anaerobic digestion), de-watered (by filter presses or centrifugation), and then either reused as a fertilizer (landfarming), disposed in landfills or incinerated. Conventional WWTPs are thus designed to remove the solid wastes, suspended solids, easily biodegradable dissolved organic matter and nutrients (phosphorus and nitrogen) from wastewater. Despite the fact that they were not designed to treat other kind of pollutants, many micropollutants are affected by the physical, chemical and biological processes occurring during conventional wastewater treatment.

The main mechanisms for micropollutant removal in conventional wastewater treatment are (Fig. 2.2): (i) sorption onto particulate matter, (ii) biological transformation, (iii) volatilization and (iv) abiotic degradation. Sorption and volatilization consist of a transfer of the micropollutant from one compartment (water) to another (solid or gas) whereas degradation leads to the transformation of the micropollutant. Complete mineralization produces water, CO_2 and minerals.

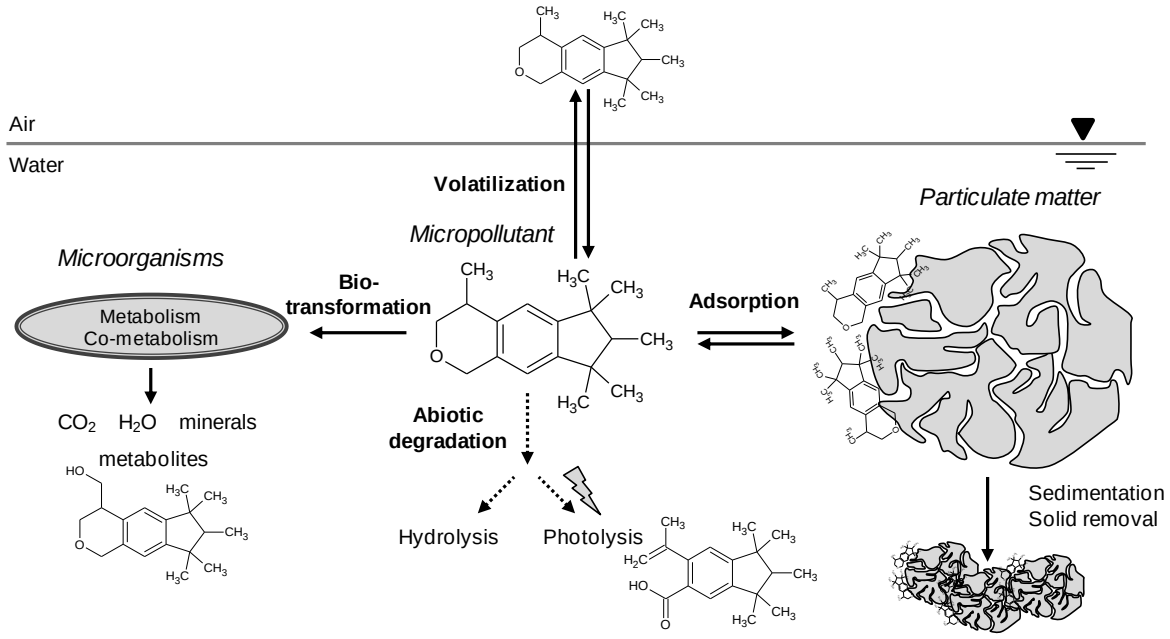

Fig. 2.2 Main removal mechanisms of micropollutants in conventional WWTPs (example of the polycyclic musk galaxolide). This compound is mainly eliminated by sorption on particulate matter and removed with the excess sludge. Biological degradation and volatilization processes may play also a role (10-15%) in the elimination of this compound (see Table 2.2).

2.2.1 Sorption

Sorption onto sludge or particulate matter can be an important removal mechanism for hydrophobic or positively charged micropollutants, especially if they are poorly biodegradable. Adsorption onto biological sludge can be differentiated into two main processes (Joss et al., 2006a):

- *Hydrophobic interaction*s between pollutants and suspended solids or sludge components, such as extracellular polymeric substances (EPS) or the lipophilic cell membrane of microorganisms.

- *Electrostatic interactions* between positively charged groups of the pollutant and the mainly negatively charged surfaces of microorganisms or effluent organic matter (EfOM).

Other phenomena such as active/passive cells uptake (absorption by microorganisms), cationic exchanges, cationic bridges, surface complexation and hydrogen bridges may also play an important role in sorption mechanisms (Pomiès et al., 2013). Adsorption is therefore a complex process dependent on the physico-chemical properties of the pollutant (charge, hydrophobicity) and the properties of the sludge (surface charge, specific surface area, EPS content, oxidation degree of the organic matter, mineral content). Different adsorption capacities are thus observed among different sludge (primary or secondary, flocs or biofilms) (Barret et al., 2010a; Mailler et al., 2013). Electrostatic interactions are influenced by the pH of the wastewater as slight variation in the pH can lead to either protonation (positively charged or neutral) or deprotonation (neutral or negatively charged) of compounds containing functional moieties with a pKa around 6-9.

Micropollutants not only sorb to particulate matter, but also onto colloidal particles (1 nm to 1 µm), which are considered as part of the "dissolved" phase (Pomiès et al., 2013). Sorption onto dissolved or colloidal matter (DCM) increases the solubility of hydrophobic substances, such as persistent organic pollutants (POPs), polycyclic aromatic hydrocarbons (PAHs) or heavy metals (Barret et al., 2010a; Barret et al., 2010b; Katsoyiannis and Samara, 2007). This means that the presence of DCM or dissolved organic carbon (DOC) in wastewater can significantly affect the partitioning of these pollutants between the "dissolved" and the "particulate" phases, limiting their removal by adsorption onto the sludge and therefore facilitating their discharge into the environment together with the treated effluent. A diagram of the adsorption process in wastewater is presented in Fig. 2.3.

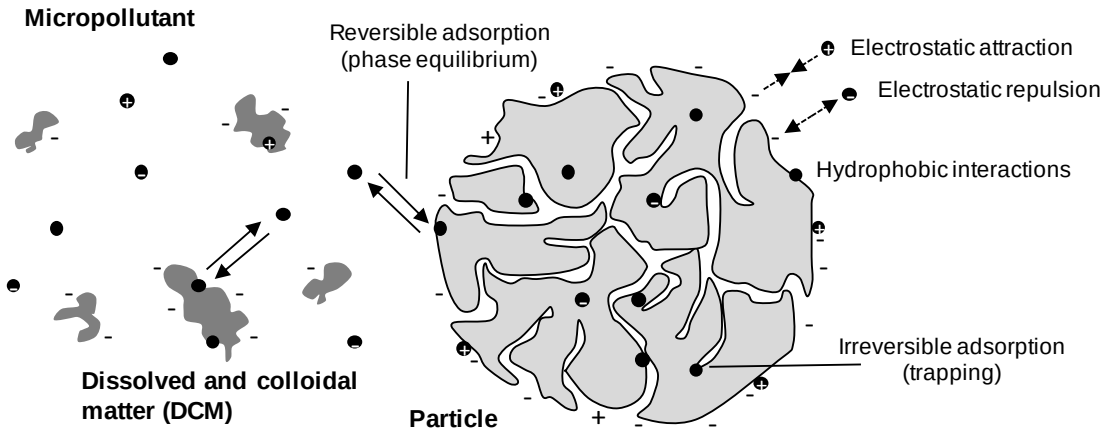

Fig. 2.3 Micropollutant adsorption onto sludge and onto dissolved and colloidal matter.

Sorption is usually a reversible process composed of two reactions which occur simultaneously: adsorption and desorption. Sorption equilibrium is reached when the rate of both reactions is equal (Joss et al., 2006a). The sorption kinetics of various pollutants onto secondary sludge, including hydrophobic PAHs and hydrophilic substances such as polar pharmaceuticals and pesticides, are reported to be fast, with sorption equilibrium reached in less than 0.5 to 2 h (Barret et al., 2010b; Ternes et al., 2004; Wick et al., 2011). The sorption equilibrium on colloids is reached even faster (<

5 min) (Maskaoui et al., 2007). Due to longer hydraulic retention time (HRT) in biological treatments, equilibrium can be assumed for solid-liquid partitioning in WWTPs.

Sorption equilibrium onto sludge can be described by empirical sorption isotherms, such as the Langmuir model, the Freundlich model or the linear model. At low pollutant concentrations (e.g., < 1µg l^{-1}) relatively to the amount of sludge (> 100 mg l^{-1}), the linear model is often considered (saturation of the sludge neglected). This model is, however, not appropriated in all cases, especially for higher pollutant concentrations. In this case, the Freundlich model was shown to be more accurate, as it considers saturation of the adsorption sites (Wick et al., 2011). In the linear model, under equilibrium conditions, the concentration sorbed onto the suspended solid (SS) (C_s in [µg kg^{-1} SS]) is assumed to be proportional to the concentration in solution (C_w in [µg l^{-1}]) (Pomiès et al., 2013):

$$C_S = K_d \, C_W \tag{2.1}$$

where K_d is the sorption (or distribution) coefficient (in [l kg^{-1} SS]), which has to be determined experimentally for each specific sludge.

The total micropollutant concentration C_T (in [µg l^{-1}]) is defined by:

$$C_T = C_W + C_S \, SS \tag{2.2}$$

where SS is the suspended solids concentration (dry weight) (in [kg SS l^{-1}]). At equilibrium, the fraction of pollutant removed by sorbtion, γ_s [-], is thus defined by:

$$\gamma_S = \frac{C_S \, SS}{C_T} = \frac{K_d \, SS}{1 + K_d \, SS} \tag{2.3}$$

The fraction of pollutant removed by sorption (if not degraded or stripped) is therefore mainly dependent on (i) the sorption coefficient K_d of the pollutant on the specific sludge, which depends on the pollutant and sludge characteristics and the colloids/DOC content of the water, and (ii) the suspended solids concentration *SS*. The main factors influencing micropollutant adsorption onto sludge are synthesised in Fig. 2.4.

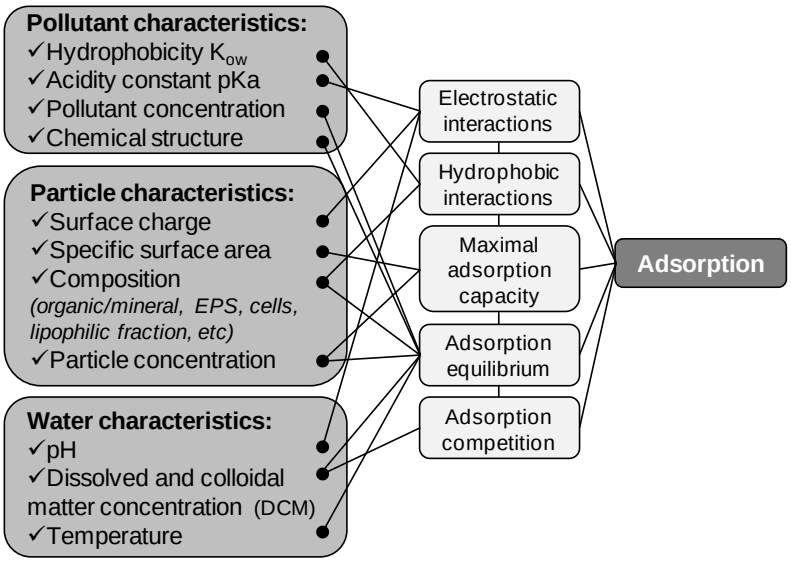

Fig. 2.4 Main factors influencing pollutant adsorption onto particles or sewage sludge

For WWTPs, the specific sludge production *SP*, which is the excess sludge withdrawn in primary and/or secondary treatment per litre of wastewater treated [kg SS l^{-1}], can be used in Eq. 2.3 instead of *SS* to estimate the removal by sorption on the excess sludge (Joss et al., 2006a). The specific sludge production *SP* in the secondary treatment depends on the influent composition (TSS content and the growth substrate concentration (BOD)), the sludge age, the temperature, and the amount of coagulant added for phosphorus removal. *SP* can be estimated by empirical formula, such as the one proposed by ATV-DVWK (2000). Typically, values between 100 and 400 mg SS l^{-1} can be expected for municipal wastewater (Joss et al., 2005). For a *SP* around 200-300 mg SS l^{-1} (average value in canton of Vaud (DGE, 2013)), micropollutant removal with excess sludge can be estimated with Eq. 2.3, according to their sludge affinity K_d:

- K_d < 400 [l kg^{-1} SS]: negligible removal by sorption (< 10%) (e.g., polar pharmaceuticals such as diclofenac, metoprolol, carbamazepine, sotalol) (Hörsing et al., 2011; Joss et al., 2006a).
- 400 < K_d < 4000 [l kg^{-1} SS]: low to moderate removal (10-50%) (e.g., heavy metals such as Ni, or pharmaceuticals such as azithromycin, oxazepam) (Hörsing et al., 2011; Joss et al., 2006a; Katsoyiannis and Samara, 2007).
- 4000 < K_d < 40,000 [l kg^{-1} SS]: moderate to high removal (50-90%) (e.g., heavy metals such as Fe, Pb, fragrances AHTN and HHCB, or pharmaceuticals such as ciprofloxacin, norfloxacin, fluoxetine) (Hörsing et al., 2011; Joss et al., 2006a; Katsoyiannis and Samara, 2007).
- K_d > 40,000 [l kg^{-1} SS]: more than 90% removal by adsorption can be expected (e.g., persistent organic pollutants such as heptachlor, hexachlorobenzene or several PCB congeners) (Katsoyiannis and Samara, 2005; Katsoyiannis and Samara, 2007).

For non-charged pollutants (in the pH range 6-8 found in wastewater), K_d, and therefore the removal by sorption, can be reasonably estimated by the octanol-water partition coefficient K_{OW} of the substance (indicator of hydrophobicity). Nonionic compounds are predicted to be not significantly

removed (< 10%) by sorption for log K_{OW} values < 3.5, partially removed (10-95%) for log K_{OW} of 3.5-6, and almost completely removed (> 90%) for log K_{OW} > 6 (Wick et al., 2011). Positively charged substances tend to have stronger affinity for the sludge than expected based on their log K_{OW}, due to the electrostatic attraction with the mainly negatively charged surface of the sludge. And negatively charged substances tend to have, at the opposite, weaker affinity with the sludge due to charge repulsion (Wick et al., 2011).

A fraction of pollutants chemically bound in the sludge is possibly not in equilibrium with the liquid concentration. Indeed, a fraction of the pollutants can be strongly sequestered in the solid (irreversible sorption) and thus the part of pollutant removed with the solids can be higher than what expected based on the sorption coefficient K_d (Plósz et al., 2012). On the other hand, polar micropollutants (especially pharmaceuticals) trapped in faeces particles can be release in the water phase during biodegradation of these particles, leading to an increase of the soluble concentration during the treatment (Göbel et al., 2007).

Coagulants and flocculants are often added during primary or secondary treatments to precipitate phosphate or to improve sedimentation of fine particles by charge neutralization of particles and colloids. Coagulation/flocculation have very low impact (< 15%) on the removal of polar micropollutants such as many pharmaceuticals, as these compounds are mostly in the soluble fraction (Luo et al., 2014; Ternes et al., 2002). Addition of coagulant can however improve the removal (up to > 90%) of more hydrophobic (and potentially sorbed) substances (such as musk fragrance, heavy metals) during the primary decantation, due to a better removal of particulate (and possibly colloidal) matter (El Samrani et al., 2008; Fu and Wang, 2011; Luo et al., 2014). It is expected that this enhanced removal in the primary treatment would also happen to a large extent in the secondary treatment (due to the high removal of particulate matter) without any coagulant addition (Joss et al., 2006a).

Adsorption is a transfer of pollutant from the liquid to the solid phase. Therefore, the fate of sorbed pollutants will depend on the fate of the solids. In Switzerland, since 2006, all the sludge produced in WWTPs is incinerated (ORRChim, 2005), leading to a high degree of mineralization of organic pollutants and sequestration of heavy metals in ash (stored in controlled landfills). In many countries, sludge is, after stabilization, used in agriculture as fertilizer. This option reuses the valuable nutrients (phosphorus, nitrogen) contained in the sludge but carries the risk of releasing contaminants in soil, food, or in the aquatic environment if pollutants are not degraded or immobilized in the soil compartment (Passuello et al., 2010; Wilson et al., 1996).

2.2.2 Biological transformation

For many hydrophilic organic micropollutants, biological transformation is the main removal mechanism during wastewater treatment. Micropollutant concentrations in wastewater are usually too low (ng l^{-1} to µg l^{-1}) to support the growth of microorganisms or to induce the corresponding enzymes and/or cofactors for their biodegradation (probably no acclimatization / adaptation occurs at these concentrations). Therefore, biological transformation of micropollutants generally requires the presence of other growth substrates (carbon and energy sources) (Tran et al., 2013b).

Biotransformation of trace contaminants can be separated in two main processes: (i) metabolic reactions on mixed substrate or (ii) co-metabolic reactions (Fig. 2.5).

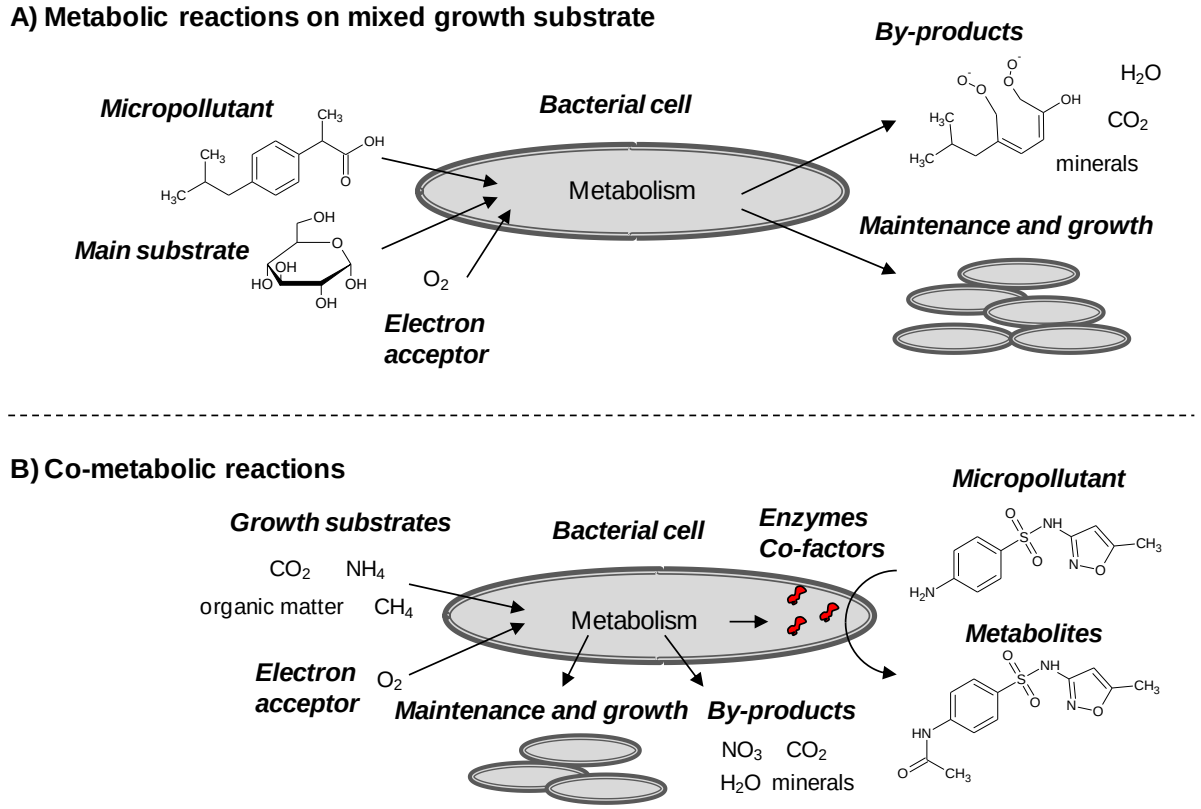

Fig. 2.5 Micropollutant biotransformation by (A) metabolic (e.g., ibuprofen) or (B) co-metabolic processes (e.g., sulfamethoxazole).

During metabolic reactions on mixed substrates, microorganisms use organic micropollutants as a growth substrate, together with other organic compounds. These substrates are used as energy (catabolism) and/or carbon source (anabolism) for their cell development (maintenance, growth and reproduction). Catabolic reactions lead to transformation of the pollutant to smaller molecules, ultimately until their complete bio-mineralization, i.e., their conversion to water, carbon dioxide and other minerals (Benner et al., 2013). Many bacterial strains able to utilize and mineralize specific pollutants as the sole energy source have been isolated, meaning that metabolic pathways exist for these substances. The degradation of these pollutants at very low concentrations requires the presence of other substrates that, together, will sustain the growth of cells. High concentrations of easily biodegradable substrates in wastewater can, however, repress the expression of these specific catabolic pathways. This preferential substrate selection may thus reduce micropollutant degradation until all the readily degradable substrates are consumed (Benner et al., 2013).

During co-metabolic reactions, micropollutants are not used as a growth substrate but are biologically transformed, by side reactions catalysed by unspecific enzymes (such as mono- or di-oxygenases, N-acetyltransferases, hydrolases) or cofactors produced during the microbial conversion of the growth substrate. Co-metabolism can thus be defined as "the transformation of a non-growth substrate in the obligate presence of a growth substrate or another transformable compound" (Fischer and Majewsky,

2014). Co-metabolism often leads to the formation of transformation products (TPs) (and not to mineralization), but these TPs may possibly be used as growth substrates for other microorganisms (Benner et al., 2013; Tran et al., 2013b). Although co-metabolic transformations require the presence of a growth substrate, if present at high concentrations the substrate can reduce the transformation of some micropollutants by competitive inhibition, i.e., competition between the growth and the co-metabolic substrate (the pollutant) to the nonspecific enzyme active site (Plósz et al., 2012; Plósz et al., 2010).

Clear separation between metabolism and co-metabolism is hardly feasible in complex systems such as activated sludge as both reactions probably occur simultaneously due to the diversity of microorganisms present (Fischer and Majewsky, 2014). Biodegradation and biotransformation are thus considered as a whole and usually quantified by the biological removal of the parent molecule (primary biodegradation).

Micropollutant removal in wastewater by biodegradation in batch conditions can usually be modelled by pseudo first order kinetics, meaning that the concentration decreases exponentially over the time (Joss et al., 2006b):

$$\frac{dC_T}{dt} = -k_{bio} SS\, C_W \tag{2.4}$$

Where C_T is the total micropollutant concentration [µg l^{-1}], C_w the micropollutant concentration in solution [µg l^{-1}], SS the suspended solids concentration (dry weight) [g l^{-1}], t the reaction time [d] and k_{bio} the reaction rate constant [l g^{-1} SS d^{-1}] (including both metabolic and co-metabolic reactions).

The transformation rate is directly proportional to the amount of microorganisms present (i.e. indirectly the sludge concentration SS) and to the soluble pollutant concentration (C_w). As the sludge concentration can be assumed to be constant for short-term batch experiments (pseudo first order kinetic) (Joss et al., 2006b), the residual pollutant concentration C_t after a defined reaction time t can be estimated by Eq. 2.5, with C_0 the initial pollutant concentration. The fraction of pollutant removed by biodegradation γ_{bio} [-], assuming no sorption or volatilization, can be estimated by Eq. 2.6.

$$C_t = C_0 \exp\left(-k_{bio} SS\, t\right) \tag{2.5}$$

$$\gamma_{bio} = 1 - \frac{C_t}{C_0} = 1 - \exp\left(-k_{bio}\, SS\, t\right) \tag{2.6}$$

The reaction rate constant k_{bio} is influenced by the sludge type and has to be determined experimentally for each specific biological treatment. The following sludge characteristics are thought to influence k_{bio} (Joss et al., 2006a; Joss et al., 2006b):

- *Microbial diversity and composition of the sludge*. Longer sludge retention times (SRTs), i.e., longer mean residence times of the microorganisms in the system, are correlated with better removal of many micropollutants. Longer SRTs are associated with increasing microbial

diversity, including slow-growing organisms (such as autotrophic nitrifying bacteria), leading to more diverse enzymatic activity and metabolic pathways for the degradation of complex molecules. The lower food-to-microorganism ratios obtained at longer SRTs (due to usually higher sludge concentrations) lead to oligotrophic conditions (shortage of biodegradable compounds) and therefore may induce microorganisms to metabolise also poorly degradable micropollutants (Maeng et al., 2013).

- *Fraction of active biomass within the sludge*, which depends mainly on the sludge age. For instance, we can expect 50% of active heterotrophic biomass in sludge of 10 d but only 20% in sludge of 80 d (80% of inert matter).
- *Floc size of the sludge*, due to diffusive mass transfer limitation in big flocs for rapidly degrading compounds.

k_{bio} can also be influenced by temperature (higher degradation rate at 20°C compared to 10°C), pH (influences enzymatic activity and cell uptake, with usually higher uptake of the neutral (non-charged) species), redox conditions (usually higher under aerobic conditions), and the availability of a co-substrate (Cirja et al., 2008; Joss et al., 2006a).

k_{bio} is an global empirical parameter which does not allow to differentiate the biodegradation mechanisms (metabolism or co-metabolism) and which is very dependent on the sludge characteristics. But due to its simple determination, it can be use to estimate the biological removal of a pollutant in specific secondary treatments. For example, for a typical nutrient-eliminating plant with a sludge age of 10-15 d, SS of 3.5 g l^{-1}, an HRT of 12 h and a sludge recycle ratio of 2, Joss et al. (2006b) divided the micropollutants according to their degradability:

- k_{bio} < 0.1 [l g^{-1} SS d^{-1}]: no substantial removal by biodegradation (< 20%) (e.g., the pharmaceuticals diclofenac or carbamazepine, or the fragrances AHTN and HHCB) (Joss et al., 2006a).
- 0.1 < k_{bio} < 10 [l g^{-1} SS d^{-1}]: partial removal (20-90%) (e.g., the pharmaceuticals bezafibrate, gemfibrozil, 17α-ethinyl estradiol) (Joss et al., 2006a).
- k_{bio} > 10 [l g^{-1} SS d^{-1}]: more than 90% removal by bio-transformation (e.g., the pharmaceuticals paracetamol and ibuprofen) (Joss et al., 2006a).

Bio-transformation of micropollutants in WWTPs will thus depend on the sludge concentration (*SS*), their biodegradability (k_{bio}) in this sludge, and the hydraulic retention time within the reactor (which depends on the reactor configuration, the flow and the sludge recycled ratio).

Bioavailability of the pollutant is a prerequisite for bio-transformation. The soluble fraction is considered as being available but the bioavailability of the sorbed fraction is assumed to be much lower (Pomiès et al., 2013). As the sorbed fraction is in equilibrium with the dissolved one, desorption (which is relatively fast) will occur during the degradation of the soluble fraction. Thus, part of the sorbed fraction can also be degraded (if not sequestered in the sludge).

A synthesis of the main factors affecting biodegradation/transformation of micropollutants in WWTPs (activated sludge treatment type) is presented in Fig. 2.6.

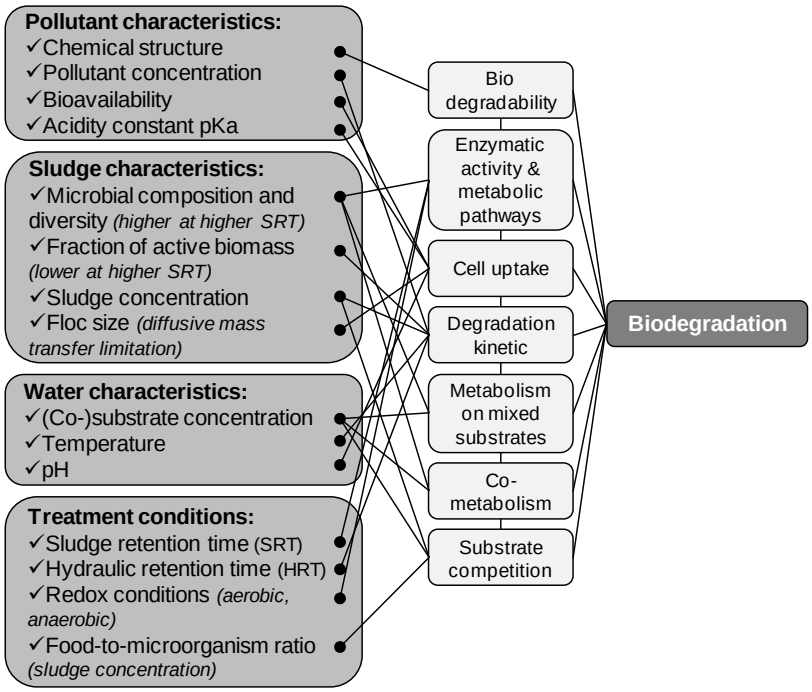

Fig. 2.6 Main factors influencing micropollutant biodegradation in WWTPs (activated sludge system).

2.2.3 Volatilization

Volatilization of micropollutants can occur during wastewater treatment, occurring as surface volatilization but more significantly by stripping during aeration. The transfer of the pollutant from water to air depends essentially on the volatility of the compound (Henry's law constant) and the operation conditions of the process (aeration, agitation, temperature and atmospheric pressure) (Pomiès et al., 2013).

The air-water partition coefficient K_{AW} of a substance [-], a dimensionless version of the Henry's law coefficient K_H [l Pa mol^{-1}], is defined by:

$$K_{AW} = \frac{C_g}{C_w} = \frac{K_H}{RT} = \frac{M\ p_p}{C_w\ RT} \tag{2.7}$$

Where C_g is the pollutant concentration in air [µg l_{air}^{-1}], C_w the soluble pollutant concentration [µg l_{water}^{-1}], M the molar weight of the substance [µg mol^{-1}], p_p the partial pressure of the pollutant in the gas phase [Pa], R the universal gas constant: 8.314 ×10^3 [l Pa mol^{-1} K^{-1}], and T the temperature [K].

The fraction $\gamma_{stripped}$ of a compound stripped from the water during aeration, assuming no degradation or sorption and equilibrium between the gas concentration in the rising bubbles and the dissolved concentration, can be evaluated by Eq. 2.8 (Joss et al., 2006a):

$$\gamma_{stripped} = 1 - \exp(-K_{AW}\ q_{air}) \tag{2.8}$$

where q_{air} [m^3_{air} $m^{-3}_{wastewater}$] is the air required for the aeration per m^3 of wastewater treated. q_{air} varies from 5 to 15 [m^3_{air} $m^{-3}_{wastewater}$] in conventional activated sludge, up to 25 [m^3_{air} $m^{-3}_{wastewater}$] for membrane bioreactors (Joss et al., 2006a).

Stripping of micropollutants during aeration in activated sludge systems (max around 15 m^3_{air} $m^{-3}_{wastewater}$), assuming no sorption or biodegradation, can be thus estimated based on the K_{AW} or K_H of the substances (at 20°C):

- $K_{AW} < 3 \cdot 10^{-3}$ [-] ($K_H < 8$ [m^3 Pa mol^{-1}]): negligible stripping (< 5%), as observed for hydrophilic substances such as pharmaceuticals ($K_{AW} < 10^{-5}$) (Hörsing et al., 2011).
- $3 \cdot 10^{-3} < K_{AW} < 5 \cdot 10^{-2}$ [-] ($8 < K_H < 120$ [m^3 Pa mol^{-1}]): low to moderate stripping (5-50%), as predicted for pollutants such as methyl TERT-butyl ether (MTBE) (K_{AW} of 0.02) (Fischer et al., 2004), several aromatic hydrocarbons (Altschuh et al., 1999), volatile polyfluorinated compounds (PFCs) (Lei et al., 2004) or musk fragrances AHTN and HHCB (K_{AW} $5 \cdot 10^{-3}$ to $1.5 \cdot 10^{-2}$ [-])(Artola-Garicano et al., 2003; Upadhyay et al., 2011; Weinberg et al., 2011).
- $K_{AW} > 5 \cdot 10^{-2}$ [-] ($K_H > 120$ [m^3 Pa mol^{-1}]): stripping higher than 50% can be expected, especially for hydrophobic volatile organic compounds (VOCs) such as benzene, toluene, ethylbenzene and xylene (BTEX) (K_{AW} around 0.2 [-]) (Sieg et al., 2009) or some chlorinated solvents (K_{AW} up to 1.1 [-]) (Chen et al., 2012).

Stripping should not be considered as an option for water treatment if the gas flow is not treated afterwards, otherwise the WWTP could cause atmospheric pollution.

2.2.4 Abiotic degradation

Organic micropollutants can potentially be degraded during wastewater treatment by abiotic reactions, such as photolysis, hydrolysis or reaction with other chemicals.

Direct *photolysis* occurs when a photon is absorbed by a compound, leading to bond cleavage to form a new compound. Pollutants can be also degraded by indirect photolysis, due to the production, during sun irradiation of dissolved organic matter, NO_2^-/NO_3^- or HCO_3^-/CO_3^{2-}, of transient excited species (reactive oxygen, radicals) which can react with the pollutants (Wang and Lin, 2014). In conventional treatments, photolysis by natural sunlight is very restricted due to the low surface-to-volume ratio available for sunlight irradiation (only the surface of the clarifiers or the biological tanks, in case of open tanks) and the high turbidity of the wastewater, which strongly limits the penetration of light into the water. Phototransformation is thus not expected to be a significant degradation mechanism in conventional systems. Photolysis can play a significant role in wastewater treatment with open water lagoons for compounds having aromatic rings, heteroatoms, and other functional chromophore groups that can either absorb solar radiation or react with photogenerated transient species (Verlicchi and Zambello, 2014).

Hydrolysis is the result of the cleavage of chemical bonds by substitution of an atom or group of atoms in an organic compound by a water molecule (or hydroxide ion) (Schwarzenbach et al., 2003). Hydrolysis can be a significant degradation pathway in aquatic environments for some organic

compounds, especially esters and amides, such as several sulfonamide, tetracycline, macrolide and β-lactam antibiotics (Ying et al., 2013). But not all micropollutants can be hydrolyzed. Rates of hydrolysis in water are strongly dependent on the pH and the temperature (Mabey and Mill, 1978). Rates usually increase rapidly with the temperature, and hydrolysis at high pH (base-catalyzed) is often faster than acid-catalyzed or neutral hydrolysis for many compounds (Mitchell et al., 2014). Hydrolysis half-lives ($t_{1/2}$) of micropollutants at neutral pH and 25°C vary from few seconds (e.g., *tert*-butyl chloride) to thousands of years (e.g., trichloromethane) (Schwarzenbach et al., 2003). Pollutants with very fast hydrolysis rates are expected to be completely transformed in sewers before reaching the WWTP. On the other hand, compounds with $t_{1/2}$ > 7 days will not be significantly hydrolyzed (< 10%) during wastewater treatment (HRT < 24h). In domestic wastewater (pH 6.5-8 and 10-25°C), hydrolysis rates are relatively slow for most micropollutants ($t_{1/2}$ > 7 d) compared to biodegradation or sorption (Schwarzenbach et al., 2003). Thus, except for a few compounds such as some β-lactam, macrolide and tetracycline antibiotics ($t_{1/2}$ 2-5 d) (Ying et al., 2013), hydrolysis can be considered as a negligible removal mechanism in WWTPs.

Other chemical transformation may also happen during wastewater treatment, such as abiotic nitration where aniline or phenolic compounds react with nitric oxide (NO) or nitrite (NO_2^-) (formed during the nitrification/denitrification process) to produce nitrophenols or unstable diazonium cations (Jewell et al., 2014; Nödler et al., 2012). This reaction is however not expected to be a major removal mechanism.

2.3 Fate of selected classes of micropollutants in conventional WWTPs

Micropollutants found in municipal wastewater are mainly (but not exclusively) surfactants, pharmaceuticals, personal care products, household chemicals, biocides and pesticides, heavy metals, polycyclic aromatic hydrocarbons, as well as other persistent organic pollutants. The fate of these compounds during conventional wastewater treatments (equivalent to activated sludge with partial nitrification), their average removal efficiency and their average concentrations in influent and effluent are discussed in this chapter and synthesized in Table 2.2 (p. 38). Average values come, if available, from national studies on many WWTPs in Europe or North America. Different removal efficiencies or concentrations may be, however, observed in specific WWTPs due to different operation conditions or sources of pollutants. Table 2.2 summarises also other key information on the different classes of micropollutants in order to estimate their risk for the environment. For each substance, chronic Environmental Quality Standards for inland water (EQSs) are presented, if available. EQSs are limits of concentrations in surface water, validated by national and international experts, below which no adverse effect of the substance on sensitive aquatic organisms is expected. As EQSs are not yet proposed for all the compounds studied, Predicted No-Effect Concentrations (PNEC) were also determined. PNECs have the same meaning as EQSs, except that they were not validated by experts and thus have a lower reliability. PNECs were calculated automatically based on a wide range of ecotoxicity studies using AiiDA (http://aiida.tools4env.com), one of the largest ecotoxicity databases available. Finally, with this information we made a rough prioritization of the compounds based on their persistence in WWTPs, their potential toxicity to aquatic organisms in the

case of low effluent dilution, and their load released into the environment (Table 2.2). The different classes of pollutants are described below.

2.3.1 Surfactants

Surfactants are widely used, mostly in household applications for detergents and cleaners but also for industrial and institutional cleaning, personal care, textiles, paint additives, lacquers and plastics (Berna et al., 2007). Surfactants are classified into four main classes: anionic (61% of the consumption in Western Europe in 2005, including 24% soaps), non-ionic (30%), cationic (7%) and amphoteric (2%) (Berna et al., 2007). According to Berna et al. (2007), the most consumed surfactants in 2005 were soaps (23.5%), linear alkylbenzene sulfonates (LAS, 16.6%), alcohol ethoxylates (AE, 17.9%) and alcohol ether sulfates (AES, 13.5%). The remaining surfactants used were mostly secondary alkane sulfonates (SAS, 2.2%), alcohol sulfates (AS, 2%), alkyl phenol ethoxylate (APEO, 1%), cationic (6.8%) and amphoteric (2.5%) surfactants. Once used, most of these chemicals are directly discharged into sewers ("down the drain" pathway). Therefore, due to their high consumption (> 7.5 g d^{-1} $capita^{-1}$), concentrations of surfactants in raw wastewater are relatively high (> 40 mg l^{-1}) (Matthijs et al., 1999), which may represent 20-30% of the dissolved organic carbon (DOC) of the wastewater (assumption of 100 mg DOC l^{-1}). Fortunately, most household surfactants are easily biodegradable and well removed (> 95%) in WWTPs. Due to their low volatility (high surface-active properties and polarity), they are mainly removed is by biodegradation or adsorption (Knepper and Berna, 2003). The fate of these surfactants in municipal WWTPs is presented below and synthesized in Table 2.2.

2.3.1.1 Soaps

The most consumed anionic surfactants, soaps, are salts of fatty acid with various number of carbon atoms (C_{10}-C_{22}), made with different source of fats or oil (e.g., tallow, coconut oil, palm oil, laurel oil, olive oil). Soaps are usually made of a mixture of lauric, myristic, palmitic, stearic, oleic, linoleic, or linolenic acids, with sodium or potassium counterions (Cantarero et al., 2010). Due to their wide consumption, they can be found in municipal wastewater at relatively high concentrations, in average around 28 mg l^{-1} (González Casado et al., 1998; Matthijs et al., 1999). Soaps and their fatty acids are readily biodegradable and almost completely mineralized in both aerobic and anaerobic conditions, and, therefore, highly removed (> 99%) in WWTPs (Berna et al., 2007; Scott and Jones, 2000). Due to their high influent concentrations, WWTP effluent soap concentrations are usually in the range 100 – 200 µg l^{-1} (Matthijs et al., 1999), which is higher than their reported PNEC (22-44 µg l^{-1}, Table 2.2). Thus, despite their degradability, the constant release (pseudo-persistence) of such compounds means that effects on sensitive aquatic organisms in the proximity of the discharge point cannot be excluded in the case of low effluent dilution.

Although biodegradation is reported to be the main removal pathway, soaps made of sodium or potassium salts are likely to precipitate in wastewater containing calcium and magnesium ions, due to the formation of relatively insoluble calcium and magnesium fatty acid salts (González Casado et al., 1998). Precipitated soap is not anymore available for biodegradation and therefore can be found at relatively high concentrations in the sewage sludge (sum of the fatty acid salts on average around 10 g

kg^{-1} dry matter (dm)) (Cantarero et al., 2010). Assuming a sludge production of around 200 g SS $m^{-3}_{wastewater}$ and a wastewater soap concentration of 10 mg l^{-1}, this means about 20% of soap removed with the excess sludge.

2.3.1.2 Linear alkylbenzene sulfonate (LAS) and secondary alkane sulfonate (SAS)

LAS are the most widely consumed synthetic anionic surfactant. They replaced the anionic branched alkylbenzene sulfonates (ABSs) which had poor biodegradability and were the cause of strong foam formation in treated waters and rivers (Knepper and Berna, 2003). LAS are complex mixtures of homologues with different alkyl chain lengths (C_{10} to C_{14}) that give them different chemical and physical properties. Their concentrations in municipal wastewater have been reported to range from 3 to 21 mg l^{-1}, with an average around 5 mg l^{-1} (Mungray and Kumar, 2009). LAS are easily biotransformed under aerobic conditions (but not under strict anaerobic conditions) in shorter-chain homologues. The oxidation of the alkyl chain generates sulfophenyl carboxylates (SPCs), intermediates much less toxic and without interfacial activity (Oya and Hisano, 2009), which can be then completely mineralized to CO_2 and H_2O (Hampel and Blasco, 2002; Mungray and Kumar, 2009). LAS removal in WWTPs (activated sludge, trickling filters) are reported to range between 95 to 99.9%, with 20 to 40% of the influent concentration retained in the sludge (average around 5-10 g kg^{-1} dm) (Feijtel et al., 1996; Mungray and Kumar, 2009; Schowanek et al., 2007). Indeed, a significant fraction of LAS (> 20%) is reported to be either associated with suspended solids (SS), or, as for soaps, in a precipitated form of insoluble Mg/Ca-salts (Mungray and Kumar, 2009). Despite their good removal, LAS concentrations in WWTP effluents were reported to be in the range of 10 to 400 µg l^{-1}, with an average around 50 µg l^{-1} (Feijtel et al., 1996; Mungray and Kumar, 2009). Although the degradation intermediates SPC are easily degradable, they were also found at high concentrations (median at 57 µg l^{-1}) in European WWTP effluents (Reemtsma et al., 2006).

The toxicity of LAS for aquatic organisms depends on the number of carbon in the alkyl chain, $C_{12\text{-}14}$ LAS being more toxic than $C_{10\text{-}11}$ (Hampel and Blasco, 2002). A PNEC of 21-27 µg l^{-1} was proposed (Mungray and Kumar, 2008) (Table 2.2), which is in the range of concentrations found in WWTP effluents. Thus, in the case of low effluent dilution in the receiving water, LAS may generate risk for the sensitive aquatic organisms, especially in the case of only anaerobic treatment of the wastewater. But LAS will undergo further (fast) degradation in natural waters (half-lives of few hours to few days (Perales et al., 1999; Takada et al., 1994)), limiting their potential impact only in the proximity of the WWTP outfall (Scott and Jones, 2000).

Secondary alkane sulfonates (SAS) are other sulfonated anionic surfactants, consumed in lower quantities than LAS. Their average concentrations in municipal WWTPs were reported to be around 840 µg l^{-1} in influents and around 3 µg l^{-1} in effluents (which is lower than the PNEC, Table 2.2), with more than 99% removal. 84% of the removal was attributed to biodegradation and 16% was exported in the sludge (around 500 mg kg^{-1} dm) (Field et al., 1995).

2.3.1.3 Alcohol ethoxylate (AE)

AE are the most consumed non-ionic surfactants and are widely present in household detergents. Commercial AE are composed of a mixture of several homologues with alkyl chain length from 12 to

18 carbons and various degree of ethoxylation (from 0 to 18) (Belanger et al., 2006). Their total concentrations in municipal wastewater (in the USA) range from 0.6 to 3.7 mg l^{-1}, with an average around 2.5 mg l^{-1} (McAvoy et al., 1998; McAvoy et al., 2006). AE are easily biodegradable, under both aerobic and anaerobic conditions (Berna et al., 2007; Federle and Itrich, 2006). The half-lives of the parent compounds are reported to be very short (a few minutes) in activated sludge, AE being first degraded to polar metabolites such as polyethylene glycols (PEGs), which are then further mineralized (complete biodegradation) (Battersby et al., 2001; Federle and Itrich, 2006). Therefore, AE are usually well removed (> 99%) in activated sludge treatments (McAvoy et al., 1998; McAvoy et al., 2006). Although a fraction of AE, specially the one with long alkyl chains which is more hydrophobic, can sorb to the suspended matter (van Compernolle et al., 2006), their concentrations in European sewage sludge are relatively low (average at 190 mg kg^{-1} dm for digested sludge and at 1160 mg kg^{-1} dm for undigested sludge) (Matthijs et al., 2004), suggesting that the main removal pathway (90 – 99%) is biodegradation. The average reported AE concentration in WWTP effluents in Europe and North America is around 3.5–6.8 µg l^{-1} (Belanger et al., 2006), much higher concentrations (100 – 500 µg l^{-1}) being measured in the effluents of trickling filters (McAvoy et al., 1998). Aquatic toxicity of AE depends mainly on their alkyl chain length (the longer, the more toxic) and their degree of ethoxylation (the more, the less toxic), with a PNEC value varying from 1.6 µg l^{-1} to 2.9 mg l^{-1} (Belanger et al., 2006) (Table 2.2). The global risk for aquatic organisms of the mixture of AE in undiluted effluents is considered to be low, except after fixed biofilm processes (such as trickling filters), but this risk disappears after 2-4 times effluent dilution (Belanger et al., 2006) and is spatially limited as AE are rapidly degraded in the receiving waters (Larson and Games, 1981).

2.3.1.4 Alkyl ethoxy sulfate (AES) and alkyl sulfate (AS)

Alkyl ethoxy sulfates (or alcohol ether sulfates, AES), and alkyl sulfates (or alcohol sulfates, AS) are, together, the second most consumed synthetic anionic surfactants after LAS (Berna et al., 2007). They are composed of a mixture of homologues with diverse alkyl chain lengths ($C_{12\text{-}18}$ for AS and $C_{12\text{-}16}$ with 3 or 4 ethylene oxide units for AES) (Fernández-Ramos et al., 2013). Their average concentrations in Dutch municipal wastewater were at 3.2 mg l^{-1} for AES and 0.6 mg l^{-1} for AS (Matthijs et al., 1999). Due to the good (complete) biodegradability of both AES and AS under aerobic and anaerobic conditions (Scott and Jones, 2000), these two surfactants are almost completely (> 99%) removed in WWTPs. Their average effluent concentrations in Dutch WWTPs were at 6.5 µg l^{-1} for AES and 5.7 µg l^{-1} for AS (Matthijs et al., 1999), but higher concentrations (30-60 µg l^{-1} AES) were reported at the outlet of trickling filters (McAvoy et al., 1998). These concentrations should however not have harmful effect on aquatic organisms, as they are lower than their respective PNECs (Table 2.2) (van de Plassche et al., 1999). The total concentrations of AES and AS in sewage sludge are reported between 50 to 100 mg kg^{-1} dm (Bruno et al., 2002), suggesting that most of these surfactants are degraded (> 97%) and only very little adsorbed.

2.3.1.5 Alkylphenol ethoxylate (APE)

APEs are non-ionic surfactants that have been widely used in domestic detergents and in industrial production (pulp and paper, textile), as well as in paints, pesticides, emulsifiers, wetting and dispersing agents (Bergé et al., 2012).

Two types of APEs are commonly used: nonylphenol ethoxylates (NPE, representing 80% of the APEs) and octylphenol ethoxylates (OPE, 20% of the APEs). NPEs and OPEs are mixtures of homologues with chain lengths varying from 5 to 40 ethoxylate units, depending on the targeted application (Bergé et al., 2012). Due to the environmental and health concerns of NPEs and their transformation product nonylphenol (NP), use of NPEs and NPs was restricted (but not ceased) in Europe since 2003, by fixing a maximum concentration of 0.1 % (w/w) of NPEs in products used for industrial and house cleaning, textile processing or biocides (EC, 2003). Despite a clear decrease in the load released into the environment, NPEs are still found in municipal wastewater throughout Europe (Bergé et al., 2012). One of the main sources of NPEs in wastewater might come from washing processes of textiles and leathers in households. Indeed, the European textile market is dominated by imports from countries where EU restriction are not applicable and thus high concentrations of NPEs coming from the manufacturing process are still found in new home textiles, towels and clothes (average 230 mg kg^{-1}). This source could contribute to more than 60% of the load that arrives in municipal wastewater (Månsson et al., 2008). Average concentrations of the sum of APE in raw municipal wastewater were found at 680 µg l^{-1} for $NP_{0-16}E$ and at 6.4 µg l^{-1} for $OP_{0-5}E$ (Loyo-Rosales et al., 2007).

APEs are subject to rapid primary biodegradation under aerobic conditions. A shortening of the ethoxylate chain is first observed, leading to APEs with 1 to 4 ethoxylate units ($AP_{1-4}E$). Complete de-ethoxylation leading to the formation of alkylphenols (APs) such as nonylphenol (NP) or octylphenol (OP) might then happen, especially under anaerobic conditions. The main APE degradation intermediates reported were (i) alkylphenols (NP and OP), (ii) short-chain alkylphenol ethoxylates (1-4 ethoxylate units), and (iii) different carboxylate derivatives including alkylphenoxyethoxy carboxylates (APECs) and carboxylated alkylphenoxyethoxy carboxylates (CAPECs) (Petrovic and Barceló, 2010). These intermediates can be then further degraded under aerobic conditions, while under anaerobic conditions, AP are reported to be the ultimate degradation products (Ying, 2006). APE metabolites are usually more persistent than the parent compound. Moreover, AP and the short-chain $AP_{1-2}E$ exhibit much higher estrogenic activity and toxicity than the parent compounds, leading to the classification of NP and OP as priority hazardous substance in Europe (Bergé et al., 2012; Ying et al., 2002).

Although average $NP_{0-16}E$ removal in activated sludge is reported to be high (80-99%) (Loyo-Rosales et al., 2007; Ying et al., 2002), the overall removal including the transformation products NPEC is much lower (60-80%), with on average 40 to 60% of all nonylphenolic compounds degraded and 20% being removed with the excess sludge (Loyo-Rosales et al., 2007). OPE, which are found in much lower (around 30 times) concentrations in raw wastewater, are less removed than NPE in WWTPs (removal of $OP_{0-5}E$: 72-82%), probably due to their lower affinity for solids (Loyo-Rosales et al., 2007). The average APE concentrations in American sewage sludge was determined at 534 mg kg^{-1} dm for NP, 62 mg kg^{-1} dm for NP_1E, 60 mg kg^{-1} dm for NP_2E and < 1.1 µg kg^{-1} dm for OP (Venkatesan and Halden, 2013). The higher NP sludge concentration could be caused by degradation of NPE to NP during anaerobic digestion (Bergé et al., 2012).

All APEs and their transformation products APs and APECs are biodegradable, but due to their formation during the treatment, these intermediates may not have time to be fully degraded and thus can be found in the effluent. Their median concentrations in WWTPs effluent in developed countries were at 1.28 µg l^{-1} for NP and 4.5 µg l^{-1} for NP_1E, with concentrations slightly higher for NPEC (Bergé et al., 2012). Both short-chain NPE and NPEC can be further degraded in the river to form the toxic NP, less easily degradable. Thus, NP concentration in the receiving water may spatially increase, and despite the dilution of the effluent, largely exceed the Swiss Environmental quality standard (EQS) for surface water (13 ng l^{-1}) (Table 2.2) (Petrie et al., 2013).

2.3.1.6 Cationic and amphoteric surfactants

Quaternary ammonium compounds (QACs) are a wide group of cationic surfactants used for instance in detergents, disinfectants, fabric softeners, or hair conditioners (Kreuzinger et al., 2007). The main QACs are alkyldimethylbenzyl (BACs), dialkyldimethyl (DDAC) and alkyltrimethyl (ATAC) ammonium chlorides, with varying alkyl chain lengths (Boethling, 1984; Clara et al., 2012). The average concentration of QACs in Austrian municipal wastewater were reported at 106 µg l^{-1} for the sum of BAC-C_{12-18}, 91 µg l^{-1} for DDAC-C_{10-18} and 21 µg l^{-1} for ATAC-C_{12-16} (Clara et al., 2012). Hospital and laundry wastewater are specially an important source of QACs in municipal wastewater (Kreuzinger et al., 2007). Due to their positive charge, QACs have a strong affinity for the sewage sludge or suspended solids (predominantly negatively charged). Elimination of QACs by sorption in the excess sludge can thus be important and is reported to increase with the alkyl chain length. QACs are moreover aerobically biodegradable (but poorly anaerobically) (Ying, 2006). Thus, QACs removals higher than 95% are usually observed in WWTPs (Boethling, 1984; Clara et al., 2012; Kreuzinger et al., 2007). For DDAC, the main pathway is by the elimination (60-90%) with the excess sludge, but for BAC and ATAC, 80 to 99% are reported to be biodegraded (Clara et al., 2012). Their average Austrian WWTP effluent concentrations were around 0.5 µg l^{-1} for BAC-C_{12-18}, 1.2 µg l^{-1} for DDAC-C_{10-18} and 0.24 µg l^{-1} for ATAC-C_{12-16} (Clara et al., 2012; Kreuzinger et al., 2007). QACs were found in the range of 20-100 mg kg^{-1} dm in sewage sludge (Martínez-Carballo et al., 2007). Despite their lower effluent concentrations compared to other surfactants, QACs are of concern due to their higher toxicity. For instance, ditallow dimethyl ammonium chloride (DTDMAC), a widely used ingredient in fabric softeners, was voluntarily phased out by industry due to its toxicity to aquatic organisms and its low biodegradability (Clarke and Smith, 2011). PNECs for BACs, ATACs and DDACs are between 100 and 1000 ng l^{-1} (Table 2.2), thus toxic effect on sensitive aquatic species cannot be excluded in the case of low dilution of the effluents. New generations of QACs, with faster biodegradation kinetics, have thus been developed to lower the environmental exposure levels: the esterquats. Esterquats are QACs with two weak ester linkages that improve their biodegradability. They are thus more environmentally friendly and replace slowly the older QACs (Mishra and Tyagi, 2007).

Amphoteric surfactants, with their properties to be either cationic, neutral or anionic depending on the pH, are dermatologically mild surfactants and thus mainly used in cosmetics and hand dishwashing liquids, but also for wool care detergents. The main amphoteric surfactants are the alkyl and alkylamido betaines and the alkyl imidazoline derivatives. They are reported to be readily mineralized under aerobic and, apart alkyl betaines, also under anaerobic conditions. They are thus expected to be

well removed (> 80%) in WWTPs. They have the same range of (low to moderate) toxicity than anionic surfactant (Garcia et al., 2008).

2.3.1.7 Other relevant surfactants

2,4,7,9-tetramethyl-5-decyne-4,7-diol (TMDD) is a non-ionic surfactant not used in households but used as defoamer or wetting agent in many industrial processes, such as, among others, the formulation of printing ink. Recycled toilet papers contain significant concentrations of TMDD. TMDD is thus introduced to domestic wastewater through toilet paper, paper tissues and paper towels (Guedez and Püttmann, 2013). TMDD average concentrations in German raw municipal wastewater were reported in the range of 0.5 to 1.9 µg l^{-1}, but, due to its low biodegradability, high solubility and low tendency to sorb onto sludge, it was only partially removed during the treatment (33-68%). TMDD concentrations were reported to be in the range 0.3-0.95 µg l^{-1} in domestic WWTP effluents and up to 20-140 µg l^{-1} in printing ink and paper-recycling factory effluents, indicating that WWTPs were a dominating source of TMDD in the aquatic environment (Guedez and Püttmann, 2011; Guedez and Püttmann, 2013). High TMDD concentrations were found for instance in Germany in the rivers Rhine (200-1000 ng l^{-1}) or Rhur (up to 16 µg l^{-1}) (Guedez et al., 2010; Guedez and Püttmann, 2013), occasionally exceeding its PNEC (10 µg l^{-1}, Table 2.2)

2.3.2 Pharmaceuticals

Municipal WWTPs are reported to be the main source of pharmaceuticals into surface waters (Kasprzyk-Hordern et al., 2008), although local releases from pharmaceutical industries or veterinary drugs residues coming from farming area can also contribute significantly (but to lesser extent) to the load of certain pharmaceuticals, especially antibiotics, anti-parasitic drugs, anti-fungal and hormones (Iglesias et al., 2014). About 3000 pharmaceutical compounds are commercially available in Europe (Ternes and Joss, 2006). In Western Europe, over 300 mg of active ingredients are, on average, consumed every day per inhabitant, of which 99% of the mass is dominated by around 60 compounds (Besse et al., 2008; Ortiz de García et al., 2013). Once ingested, these pharmaceuticals find their way into urine and faeces, partially as the original molecule (the part not metabolized in the body) and partially as metabolites, which are mainly hydroxylated, hydrolysed or conjugated forms of the parent compound (Ikehata et al., 2006). The estimated total load of pharmaceuticals (parent compounds) into sewers is around 70 mg d^{-1} $capita^{-1}$, which corresponds to about 200-250 µg l^{-1} (Oosterhuis et al., 2013; Ortiz de García et al., 2013). Depending on the quantity of drugs consumed and their excretion rates (0 to 100%), concentrations of individual pharmaceuticals in raw wastewater can vary from less than 1 ng l^{-1} to over 100 µg l^{-1}. The most abundant pharmaceuticals in wastewater (found at 0.1 to more than10 µg l^{-1}) are, not surprisingly, those that are most consumed. This includes *analgesic and anti-inflammatory drugs* (such as paracetamol, ibuprofen, tramadol, (acetyl)salicylic acid, naproxen, codeine, diclofenac, mefenamic acid) with average concentrations between 1-100 µg l^{-1}, *antibiotics* (such as ciprofloxacin, clarithromycin, erythromycin, ofloxacin, azithromycin, sulfamethoxazole, trimethoprim, with 0.5-5 µg l^{-1}), *iodinated contrast media* (for X-ray radiography, such as iohexol, iomeprol, iopamidol, iopromide, with 2-20 µg l^{-1}), *antidiabetics* (such as metformin with 60-100 µg l^{-1}), *antihypertensives / diuretic* (such as irbesartan, eprosartan, losartan, valsartan, hydrochlorothiazide, furosemide, with 1-5 µg l^{-1}), *beta-blockers* (for heart problems, such as atenolol, metoprolol, sotalol,

with 0.5-1.5 µg l^{-1}), *lipid regulators* (anti-cholesterol, such as bezafibrate, gemfibrozil, simvastatin, with 0.5-1 µg l^{-1}), *psychiatric drugs* (such as carbamazepine, gabapentin, levetiracetam, with 0.5-4 µg l^{-1}), and *antihistamines* (e.g., gastric anti-acid, such as cimetidine, ranitidine, with 2-4 µg l^{-1}) (Margot et al., 2013b; Verlicchi et al., 2012). Pharmaceuticals used for less common diseases (e.g., anti-cancer) or consumed at lower doses (e.g., contraceptive pills) are usually detected at lower concentrations (< 1-100 ng l^{-1}).

The fate of pharmaceuticals in WWTPs is very dependent on their characteristics, such as their sorption affinity and their biodegradability. The removal rates and mechanisms of the most abundant pharmaceuticals usually reported in the effluents are presented in Table 2.2 (48 active ingredients representing around 70% of the mass of drugs consumed (Besse et al., 2008)). Removal efficiencies from 0 to 100% can be observed, depending on the compound. Pharmaceuticals have low volatility ($K_{AW} < 10^{-5}$) (Hörsing et al., 2011) and thus are not expected to be stripped during the WWTP treatment. Very hydrophobic pharmaceuticals such as the anti-cancer drug tamoxifen (log $K_{OW} = 6.3$) enter WWTPs associated to the particulate phase and are mainly removed with the removal of suspended solids (Tauxe-Wuersch et al., 2006). Removal by sorption can be significant (10-80%) for a few other hydrophobic drugs such as mefenamic acid (log $K_{OW} = 5.1$), gemfibrozil (log $K_{OW} = 4.8$) or fenofibrate (log $K_{OW} = 5.2$), or for positively charged pharmaceuticals such as several quinolone and macrolide antibiotics (ofloxacin, norfloxacin, ciprofloxacin, azithromycin, clarithromycin), and few other drugs such as tetracycline, fluoxetine, simvastatin, atorvastatin, or diazepam (Jelic et al., 2011; Lara-Martín et al., 2014; Lubliner et al., 2010; Yan et al., 2014). Most other pharmaceuticals have high solubility, low hydrophobicity and often negative charge at neutral pH (acidic compounds), which means low sorption affinity on biological sludge (negatively charged). They are thus mostly found in the "dissolved" phase and their removal by sorption is often negligible (< 5%) (Verlicchi et al., 2012). Their removal in the primary treatment is usually very poor. Biodegradation or biotransformation is therefore the main removal mechanism for most pharmaceuticals. Pharmaceuticals can potentially be used directly by microorganisms as carbon or energy sources and therefore be partially mineralized, or be transformed co-metabolically to different metabolites. It was reported that most of these intermediate metabolites were then further degraded, leading in many case to complete mineralization (Quintana et al., 2005). Stable metabolites are, however, also formed during the biological treatment and release into the environment together with WWTPs effluents (NEPTUNE, 2010).

Biotransformation processes are strongly dependent on the properties of the compound (biodegradability). A few pharmaceuticals (e.g., some analgesic/anti-inflammatory drugs and natural hormones) are well removed during the biological treatment, but most are only partially or not removed at all (Table 2.2). The majority of the drugs studied are on average removed less than 50%. Removal efficiencies are not related to the therapeutic classes, but are linked to drug chemical structure (persistency increases with the complexity of the molecule, presence of halogen, nitro and azo groups, etc.) (Verlicchi et al., 2012).

Highly variable removal efficiencies are observed among different WWTPs for the same compound. This is attributed to different operation conditions such as biomass concentration (varying usually

from 2 to 5 g TSS l^{-1}, and thus changing the food-to-microorganism ratio), SRT (from 2 to 20 d), HRT (from 2 to 24 h), pH (from 6 to 8), temperature (from 10 to 25°C), configuration and type of WWTP (Verlicchi et al., 2012). Removal rates presented in Table 2.2 are averages of several studies. Different removal efficiencies can be however observed in individual plants due to different operation conditions.

Many authors have reported that better degradation of several drugs (such as hormones, ibuprofen, ketoprofen, naproxen, bezafibrate, gemfibrozil, atenolol and some antibiotics) occurs in WWTPs with higher SRT (> 10 d compared to 2 d) (Verlicchi et al., 2012). This was, as already discussed, possibly due to the enrichment, at higher SRT, of certain microbial communities able to break down pharmaceuticals. WWTPs incorporating nitrification, with thus longer SRTs, also showed better removal efficiencies for these compounds (cf. Chapter 3).

The influence of HRT on the removal of pharmaceuticals was reported for compounds with low sludge affinity (low K_d) and medium to good biodegradability (half-lives in the range of the HRT). For these substances, higher removal efficiencies were observed at higher HRT. Substances that are highly biodegradable (half-lives much shorter than the HRT, and thus always removed) or hardly degradable (half-lives much longer than HRT), as well as compounds mainly removed by sorption, are unlikely to be influenced by HRT (Verlicchi et al., 2012).

Temperature stimulates microbial activity and thus higher removal efficiencies of biodegradable pharmaceuticals are usually observed in summer compared to winter. The pH of the wastewater can also impact the removal (by sorption and by biodegradation, due to the expected higher cell uptake of uncharged species) of some ionisable substances if the change in pH can affect their charges (switch to another form: cationic, neutral, anionic, or zwitterionic), which concerns mostly substances with a pKa value close to the pH of the wastewater (such as quinolone antibiotics) (Verlicchi et al., 2012).

The configuration and the type of biological treatments can also affect the removal of pharmaceuticals. Membrane bioreactors (MBRs) were often reported to have slightly better removal efficiencies than conventional activated sludge reactors (CAS), probably due to the longer SRT usually used in MBRs. Indeed, similar efficiencies were reported for CAS and MBR working at the same SRT (Clara et al., 2005a). Trickling filters, conversely, were reported, in some but not all studies, to be less efficient than CAS for pharmaceutical removal (Kasprzyk-Hordern et al., 2009), probably because of the much lower HRT in trickling filters (3-20 min) (Séguret et al., 2000). Similar micropollutant removal efficiencies were, however, reported between CAS and biofilters, despite lower HRT in the latter (Mailler et al., 2013). It is difficult to conclude which configuration is the best as the same removal efficiencies can be reached in all cases. The presence of a diverse microbial community in the system, with sufficient biomass (low food-to-microorganism ratio) and long enough HRT seem to be the key parameters affecting pharmaceutical removal, more than the treatment configuration. Anoxic zones (e.g., for denitrification) do not seem to affect the removal of micropollutants significantly as most pharmaceuticals are reported to be better removed (or at least as well removed as) under oxic conditions (Falås et al., 2013).

Low apparent removal of certain compounds may be due to the reformation of these substances during the treatment, by the biological cleavage of the conjugated molecules. Indeed, many human drugs metabolites are the hydroxylated (-OH), amine (-NH_2), carboxylated (-COOH) or conjugated (with glucoronic or sulphuric acids) forms of the parent molecules. Deconjugation of the metabolite during the treatment can thus reform the parent drugs. This is for instance reported for N^4-acetyl sulfamethoxazole, reconverted to the antibiotic sulfamethoxazole (Göbel et al., 2007), oxazepam-glucoronide to the anxiolytic oxazepam (Bijlsma et al., 2012), as well as for several other pharmaceutical metabolites (ibuprofen, diclofenac, carbamazepine or oestrogens) (Verlicchi et al., 2012). Some pollutants can also be converted to other pollutants. For instance, the biological oxidation of the hormone estradiol leads to the formation of the hormone estrone (Shi et al., 2013). Low apparent removal of the dissolved fraction can also be due to the release in the dissolved phase during the treatment of pharmaceuticals trapped into faeces particles. This is mostly suspected for drugs mainly excreted with bile and faeces, such as macrolide and fluoroquinone antibiotics (erythromycin, azithromycin, ciprofloxacin, norfloxacin) (Göbel et al., 2007; Verlicchi et al., 2012).

The average concentrations of some of the most abundant pharmaceuticals measured in WWTP effluents (average concentrations between 100 ng l^{-1} to a few µg l^{-1}) in various countries are presented in Table 2.2. Pharmaceuticals found at concentrations higher than 1 µg l^{-1} (on average) are the anti-diabetic metformin, the anti-hypertensives valsartan and irbersartan, the anticonvulsant gabapentin, the diuretic hydrochlorothiazide and the contrast media iomeprol, iopamidol, and iopromide. Most of the other pharmaceuticals are found, on average, at lower concentrations (< 1µg l^{-1}). Concentrations can, however, vary strongly depending on the country (consumption habits) and the type of treatment. Some compounds well removed during the treatment are still present at relatively high concentrations in the effluent due to their high presence in the influent (e.g., for ibuprofen).

The risk for aquatic organisms generated by this mixture of pharmaceuticals at low concentrations discharged permanently in WWTP effluents is difficult to assess, as the safety thresholds for many substances are not really known and the cocktail effect is difficult to evaluate. By comparison with PNEC values and the environmental quality standards (EQS) for surface waters proposed for some pharmaceuticals in Switzerland (Table 2.2), a significant risk for the sensitive aquatic organisms in the case of low dilution of the effluent (< 2-10 times) may be induced by several antibiotics (azithromycin, ciprofloxacin, clarithromycin, erythromycin, sulfamethoxazole), anti-inflammatory drugs (ibuprofen and especially diclofenac), carbamazepine and propranolol.

The dominant pharmaceuticals found in sewage sludge are usually the one with strong sorption affinity and high concentrations in wastewater. A wide survey, monitoring the concentration of 72 pharmaceuticals in sewage sludge of 94 WWTPs in the USA, showed that antibiotics were the drugs found at higher concentrations (0.8-6.8 mg kg^{-1}dm), with, in order of decreasing concentration, ciprofloxacin, ofloxacin, 4-epitetracycline, tetracycline, minocycline, doxycycline and azithromycin (McClellan and Halden, 2010). Other pharmaceuticals are usually found in sludge at concentrations between < 1 to 200 µg kg^{-1} dm (Gardner et al., 2013; Guerra et al., 2014; Jelic et al., 2011; McClellan and Halden, 2010). In the case of soil amendment with sewage sludge, some drugs, especially

antibiotics (azithromycin, ciprofloxacin, norfloxacin, ofloxacin, tetracycline) may persist several years in the soil due to their low degradation kinetics (half-lives from 500 to 2300 d) (Walters et al., 2010).

Human pharmaceutical metabolites, which are mainly hydroxylated, hydrolyzed or conjugated forms of the parent compounds, are frequently found in raw wastewater in the same range of concentrations as the active pharmaceuticals (from < 10 ng l^{-1} up to 3-4 µg l^{-1}). Human drug metabolites are usually more polar and hydrophilic than the parent compounds due to their transformation in the liver or kidney in order to be readily excreted in the urine or bile (Ikehata et al., 2006). They are thus not expected to be significantly removed by sorption. Some of these metabolites are well degraded during the biological treatment (e.g., > 90% for N-acetyl sulfamethoxazole), but many others are not. Human drug metabolites can thus be an issue in WWTPs effluents (concentrations up to 1-4 µg l^{-1}) (Evgenidou et al., 2015; Gracia-Lor et al., 2014; Margot et al., 2013b). Several human pharmaceutical metabolites have (negligible or) lower pharmacological activities than the parent compounds. Their risk for aquatic organisms is therefore considered to be lower. Others, like norfluoxetine, fenofibric acid or salicylic acid, which are metabolites of, respectively, fluoxetine, fenofibrate and aspirin, have similar or even higher activities and thus could be of environmental concern (Besse et al., 2008).

Natural and synthetic hormones are found in domestic wastewater due to their excretion in urine and faeces. The most studied hormones are estrogens (mostly female hormones) such as estrone (E1), 17β-estradiol (E2), and estriol (E3), and the synthetic 17α-ethinylestradiol (EE2) (active substance in contraceptive pills). These estrogens are found on average at relatively low concentrations in raw wastewater (around 80, 20, 300 and 1-15 ng l^{-1} for E1, E2, E3 and EE2, respectively, 10-20% associated to particles). Natural estrogens are well removed in WWTPs, usually over 80%, mostly by biodegradation but also partially (10-40%) by sorption onto secondary sludge (2-30 µg kg^{-1} dm), resulting in low concentrations in effluents (1-35 ng l^{-1}). EE2 is usually not so well removed (on average at 50-60%) and is found on average at 0.5 to 3 ng l^{-1} in effluents (Gabet-Giraud et al., 2010; Gardner et al., 2013; Lubliner et al., 2010). Despite these very low concentrations, estrogens may still impact aquatic organisms (e.g., fish and mussel feminization or vitellogenin production in males) (Kidd et al., 2007; Tyler and Jobling, 2008) as they are still at levels more than 10 times above their respective EQS for surface waters (Table 2.2). Several other natural hormones are present in municipal wastewaters, such as testosterone (3 µg l^{-1}), androsterone (1.5 µg l^{-1}) or progesterone (280 ng l^{-1}). Typical removals in excess of 97% in WWTPs have been reported and thus their concentrations in effluents are below 15 ng l^{-1} (Lubliner et al., 2010), which is much lower than their PNEC values (Table 2.2).

Illicit drugs such as amphetamine, cocaine and its main metabolite benzoylecgonine, MDMA (ecstasy) or THC-COOH (cannabis metabolite) are present on average in the range 100-2000 ng l^{-1} in raw wastewater, with the highest values usually observed in large cities and during weekends. These illicit compounds are on average well removed in conventional WWTPs (from 79% for cocaine to > 98% for amphetamine and THC-COOH, probably by biodegradation/transformation), except for MDMA (0-26%). Concentrations of illicit drugs in the effluents are thus relatively low (on average between < 2 ng l^{-1} up to 100 ng l^{-1}, Table 2.2) (Been et al., 2014; Bijlsma et al., 2012; Kasprzyk-Hordern et al., 2009).

Table 2.2 Fate of 168 micropollutants (main surfactants, most abundant pharmaceuticals (effluent concentration usually > 100 ng l^{-1}), several personal care products and household chemicals, main pesticides/biocides used in urban environment, heavy metals, and other pollutants) in conventional WWTPs.

Substance	Family	Typical WWTP removal[a]	Ref.	Removal mechanisms[b]			Typical WWTP effluent concentration[c]	Ref.	EQS[d]	Ref.	PNEC[e]	AF[f]	CAS[g]	Prioritization[h]		
		[%]		B	S	V	[ng/l]		[ng/l]		[ng/l]			L	T	P
Surfactants																
Soap	Anionic surfactant	99	[1,2]	80	20	0	**150,000**	[3]	-		22,000 / 44,000	10 / 10	57-10-3 (palmitic acid) / 143-07-7 (lauric acid)	X	X	
Linear alkylbenzene sulfonate (LAS)	"	97	[4-6]	70	30	0	**50,000**	[4,5]	-		21,000 (C_{10-13})	10	68411-30-3	X	X	
Secondary alkane sulfonate (SAS)	"	99	[7]	84	16	0	3,000	[7]	-		7,200 (C_{14-17})	50	97489-15-1	X		
Alkyl ethoxy sulfate (AES)	"	99	[2,3]	97	3	0	6,500	[3]	-		18,000	10	68081-91-4	X		
Alkyl sulfate (AS)	"	99	[2,3]	97	3	0	5,700	[3]	-		84,000	50	68955-20-4	X		
Alcohol ethoxylate (AE)	Non-ionic surfactant	99	[8,9]	91	9	0	**5,000**	[10]	-		1,560 (C_{12-18}) / 71,100 (C_{9-11})	50 / 10	68213-23-0 / 68439-46-3	X	X	
Nonylphenol ethoxylates (NPEs)	"	90	[11,12]	80	20	0	**6,000**	[13]	13 (NP)	CH	500 (NPEs) / 30 (NP)	10 / 10	26027-38-3 / 25154-52-3	X	X	
Octylphenol ethoxylates (OPEs)	"	80	[11]	90	10	0	**1,000**	[11]	100 (OP)	EU	21,000 (OPEs) / 0.21 (OP)	10 / 10	9036-19-5 / 140-66-9	X	X	
2,4,7,9-tetramethyl-5-decyne-4,7-diol (TMDD)	"	50	[16]	95	5	0	600	[16]	-		10,000	100	126-86-3			
Alkyldimethylbenzyl ammonium chlorides (BACs)	Cationic surfactant	95	[14,15]	90	10	0	**500**	[14,15]	-		120 (C_{12-14})	10	85409-22-9		X	
Alkyltrimethyl ammonium chlorides (ATAC)	"	95	[14,15]	90	10	0	**240**	[14,15]			136 (C_{16-18})	50	68002-61-9		X	
Dialkyldimethyl ammonium chlorides (DDAC)	"	95	[14,15]	25	75	0	**1,200**	[14,15]			910 (C_{16-18})	10	92129-33-4	X	X	
Pharmaceuticals																
Removal usually >70%																
Caffeine	Stimulant	95	[19,24]	100	0	0	**820** / 191 / 184	[19/21/23]	-		500	10	58-08-2		X	
Ibuprofen	Anti-inflammatory	80	[17, 24, 25]	97	3	0	**394** / **460** / **81** / **330** / **120**	[18/20/21/22/23]	300	CH	1	10	15687-27-1		X	
Paracetamol	Analgesic	100	[19,24,25]	100	0	0	< 8 / 79 / 178	[19/20/23]	-		500	10	103-90-2			
Salicylic acid	Analgesic	99	[24,25]	100	0	0	78	[24,25]	-		3,200	10	69-72-7			
Simvastatin	Anti-cholesterol	77	[19]	70	30	0	98 / < 41	[19/20]	-		62,500	100	79902-63-9			
Removal usually between 30-70%																
Atenolol	Beta blocker	41	[17,19,25]	98	2	0	843 / 940	[18/20]	150,000	CH	6,400,000	50	29122-68-7			X
Azithromycin	Antibiotic	39	[17,19,25]	80	20	0	**220** / **408**	[17/23]	90	CH	-		83905-01-5		X	X
Bezafibrate	Anti-cholesterol	41	[17,19]	95	5	0	**320** / 25	[17/21]	460	CH	*(0.03)*	1,000	41859-67-0		X	X
Ciprofloxacin	Antibiotic	69	[17,19,25]	20	80	0	**170** / **67** / **96** / **179**	[17/20/21/23]	89	CH	1.2	10	86393-32-0		X	
Clarithromycin	Antibiotic	33	[17,19,25]	70	30	0	**276** / **130**	[18/23]	60	CH	20	100	81103-11-9		X	X
Diltiazem	Anti-hypertensive	67	[25]	100	0.3	0	85 / 155	[20/23]	-		*(1,640)*	5,000	42399-41-7			
Eprosartan	Anti-hypertensive	37	[19]	100	0	0	880 / 227	[19/21]	-		-		133040-01-4		?	X
Erythromycin	Antibiotic	45	[24,25]	98	2.3	0	**42** / **830** / **213**	[18/22/23]	40	CH	200	10	114-07-8		X	X
Furosemide	Diuretic	51	[25]			0	280	[20]	-		*(0.6)*	1,000	54-31-9		?	
Gemfibrozil	Anti-cholesterol	39	[17,19,25]	87	13	0	180 / 420 / 138 / 1,433	[17/20/21/23]	-		312,500	10	25812-30-0			X
Iohexol	Contrast medium	31	[19]	99	1	0	15,191	[19]			32,000,000	100	66108-95-0	X		X
Iomeprol	Contrast medium	34	[17,19]	99	1	0	10,534 / 376	[19/21]	-		-		78649-41-9	X	?	X
Iopromide	Contrast medium	41	[17,19]	99	1	0	2,460 / 2,700	[17/21]	-		-		73334-07-3	X	?	X
Ketoprofen	Anti-inflammatory	40	[17,19,25]	100	0	0	190 / 86	[17/21]	-		-		22071-15-4		?	X
Lisinopril	Anti-hypertensive	50	[27]			0	180	[20]	-		-		76547-98-3		?	
Losartan	Anti-hypertensive	(50)	[19,25,27,28]			0	510	[19]	-		2,860,000	50	114798-26-4			
Mefenamic acid	Anti-inflammatory	42	[17,19,25]	80	20	0	870	[18]	4,000	CH	*(790)*	5,000	61-68-7			X
Metformin	Anti-diabetic	57	[17]	100	0	0	10,347 / 27,800	[18/23]	1,000,000	CH	2,200,000	50	657-24-9	X		
Metronidazole	Antibiotic	45	[19,25]	100	0	0	680	[17]	-		250,000	50	443-48-1			X
Naproxen	Anti-inflammatory	40	[17,19]	100	0	0	**462** / 27 / 193	[18/21/23]	1,700	CH	320	100	22204-53-1		X	X

Table 2.2 (Continuation)

Substance	Family	Typical WWTP removal[a] [%]	Ref.	Removal mechanisms[b] B	S	V	Typical WWTP effluent concentration[c] [ng/l]	Ref.	EQS[d] [ng/l]	Ref.	PNEC[e] [ng/l]	AF[f]	CAS[g]	Prioritization[h] L	T	P
Pharmaceuticals (continuation)																
Removal usually between 30-70%																
Norfloxacin	Antibiotic	69	[17,19,25]	20	80	0	70 / 39	[17/23]	-		160	10	70458-96-7			
Ofloxacin	Antibiotic	58	[17,19,25]	20	80	0	70 / 160 / 10 / 251	[17/20/22/23]	-		2,100	10	82419-36-1			
Pravastatin	Anti-cholesterol	37	[17]	100	0	0	420	[17]	-		-		81093-37-0		?	X
Ranitidine	Aastric antacid	52	[25]	100	0	0	120 / 7 / **842**	[20/21/23]	-		*(0.25)*	1,000	66357-35-5		X	
Sulfamethoxazole	Antibiotic	44	[17,19,25]	100	0	0	**238** / **330** / **280** / **1,190**	[18/20/21/23]	600	CH	13	50	723-46-6		X	X
Tramadol	Analgesic	33	[24]	100	0	0	256	[21]	-		-		36282-47-0		?	X
Trimethoprim	Antibiotic	35	[17,19,25]	87	13	0	150 / 170 / 229 / 482	[17/20/21/23]	60,000	CH	100,000	10	738-70-5			X
Valsartan	Anti-hypertensive	(50)	[19,25,27,28]			0	2'100 / 1'600	[19/20]	560,000	CH	-		137862-53-4	X		
Venlafaxine	Anti-depressant	40	[19]	100	0	0	150 / 119	[19/21]	-		*(5.7)*	5,000	93413-69-5		?	X
Removal usually <30%																
Carbamazepine	Anticonvulsant	16	[17,19,25]	0	100	0	**482** / **140** / **832** / **731**	[18/20/21/23]	500	CH	89	10	298-46-4		X	X
Clindamycin	Antibiotic	10	[17,19,25]			0	115 / 50 / 70	[19/20/21]	-		*(0.22)*	10,000	18323-44-9		?	X
Diatrizoic acid	Contrast medium	29	[17,19]	99	1	0	598 / 619	[18/21]	-		-		117-96-4		?	X
Diclofenac	Anti-inflammatory	20	[17,19,25]	80	20	0	**647** / **260**	[18/22]	50	CH	500	10	15307-86-5		X	X
Fluconazole	Antifungal	15	[19]	100	0	0	110 / 108	[19/21]	-		*(6)*	5,000	86386-73-4		?	X
Gabapentin	Anticonvulsant	15	[17,19]			0	1,910	[17]	-		*(10,000)*	10,000	60142-95-2	X		X
Hydrochlorothiazide	Diuretic	30	[25,28]	0	100	0	1,100	[20]	-		*(3)*	10,000	58-93-5	X	?	X
Iopamidol	Contrast medium	28	[17,19]	99	1	0	1,610 / 144	[17/21]	-		-		60166-93-0	X	?	X
Irbersartan	Anti-hypertensive	10	[27,28]	0	100	0	1,700 / 480	[19/21]	704,000	CH	-		138402-11-6	X		X
Metoprolol	Beta blocker	25	[17,19,25]	100	0	0	240 / 410	[17/20]	64,000	CH	146,000	50	51384-51-1			X
Oxazepam	Anxiolytic	13	[19,28]	0	100	0	350 / 162	[19/21]	-		100,000,000	100	604-75-1			X
Primidone	Anticonvulsant	16	[19,28]			0	200	[17]	-		15,000,000	100	125-33-7			X
Propranolol	Beta blocker	28	[17,19,25]	50	50	0	**120** / 33 / **140**	[17/20/22]	160	CH	100	10	525-66-6		X	X
Sotalol	Beta blocker	22	[17,19,25]	85	15	0	435	[18]	-		-		3930-20-9		?	X
Hormones																
Estrone (E1)	Natural hormone	76	[23]	87	13	0	**15** / **12** / **217**	[18/22/23]	3.6	CH	3.6	10	53-16-7		X	
17β-estradiol (E2)	Natural hormone	90	[19,23]	89	11	0	**3** / **1.3** / **4**	[18/22/23]	0.4	CH	0.042	10	50-28-2		X	
Estriol (E3)	Natural hormone	99	[23]	89	11	0	1	[23]	-		67	100	50-27-1			
17α-ethinyl estradiol (EE2)	Contraceptive	60	[19,26]	83	17	0	**2** / **0.5** / **2**	[18/22/23]	0.037	CH	0.004	10	57-63-6		X	
Androsterone	Natural hormone	99.9	[23]	100	0.3	0	0.1	[23]	-		-		53-41-8			
Progesterone	Natural hormone	97	[23]	52	48	0	8	[23]	-		1,000	100	57-83-0			
Testosterone	Natural hormone	99	[23]	99	1	0	12	[23]	-		200	50	58-22-0			
Illicit drugs																
Amphetamine	Nervous system stimulant	98	[30,31]	100	0	0	2	[30,31]	-		*(4,901)*	1,000	60-13-9			
Cocaine	Nervous system stimulant	79	[30,31]	100	0	0	30	[30,31]	-		-		50-36-2		?	
Ectasy (MDMA)	Psychedelic drug	15	[30,31]			0	100	[30,31]	-		-		64057-70-1		?	X
THC-COOH (cannabis metabolite)	Psychoactive drug	98	[30,31]	100	0	0	13	[30,31]	-		-		64280-14-4		?	
Personal care products																
Galaxolide (HHCB)	Fragrances	85	[32,33]	10	76	14	850	[32,34,36]	-		6,800	10	1222-05-5			
Tonalide (AHTN)	"	85	[32,33]	10	76	14	250	[32,34,36]	-		3,920	50	21145-77-7			
Iso E Super (OTNE)	"	65	[34,35]	50	40	10	**1,400**	[32,34,36]	-		560	50	54464-57-2 (EC: 915-730-3)	X	X	
Musk ketone	"	46	[34,35]	0	100		17	[32,34,36]	-		*(0.33)*	10,000	81-14-1		?	
Musk xylene	"	54	[34,35]	0	100		17	[32,34,36]			*(1.0)*	10,000	81-15-2		?	

Table 2.2 (Continuation)

Substance	Family	Typical WWTP removal[a]	Ref.	Removal mechanisms[b]			Typical WWTP effluent concentration[c]	Ref.	EQS[d]	Ref.	PNEC[e]	AF[f]	CAS[g]	Prioritization[h]		
		[%]		B	S	V	[ng/l]		[ng/l]		[ng/l]			L	T	P
Personal care products (continuation)																
Methyl-paraben	Preservatives	95	[37]	95	5	0	19	[37]	-		4,000	50	99-76-3			
Ethyl-paraben	"	95	[37]	95	5	0	2.5	[37]	-		21,000	100	120-47-8			
Triclocarban	Biocides	90	[26,38]	10	90	0	**70**	[21,22,38]	-		1.1	50	101-20-2		X	
Triclosan	"	90	[26,38]	35	65	0	**200**	[21,22,38]	20	CH	1.5	10	3380-34-5		X	
Chloroxylenol	"	95	[31]				**300**	[31]	-		*(26)*	5,000	88-04-0		X	
N,N-diethyl-m-toluamide (DEET)	"	62	[17]	100	0	0	700	[17,21]	41,000	CH	*(800)*	5,000	134-62-3			
4-methylbenydlidene camphor (4-MBC)	UV filters	72	[33,39]	70	30	0	800	[33,39]	-		-		38102-62-4		?	
Benzophenone-3 (BP-3)	"	90	[33,39]	95	5	0	270	[33,39]	-		1,600	50	131-57-7			
Ethylhexyl methoxycinnamate (EHMC or OMC)	"	98	[33,39]	90	10	0	36	[33,39]	-		*(27)*	1,000	5466-77-3		?	
Octocrylene (OC)	"	96	[33,39]	50	50	0	52	[33,39]	-		1,000,000	100	6197-30-4			
Octyl-tirazone (OT or ethylhexyl triazone)	"	96	[33]	5	95	0	< 34	[33]	-		800,000	100	88122-99-0			
Household chemicals																
Acesulfame	Sweeteners	5	[40,41]	0	0.1	0	30,000	[40,41]	-		2,000,000	50	55589-62-3	X		X
Saccharin	"	99	[40,41]	100	0	0	500	[40,41]	-		9,983,000	50	81-07-2			
Sucralose	"	5	[40,41]	0	0.1	0	10,000	[40,41]	-		-		56038-13-2	X	?	X
Cyclamate	"	95	[40,41]	100	0	0	500	[40,41]	-		*(1.0)*	10,000	139-05-9		?	
Di-(2-ethylhexyl) phthalate (DEHP)	Plastic additives	94	[42]	60	40	0	**2,400**	[42]	1,300	EU	10	10	117-81-7	X	X	
Dimethyl phthalate (DMP)	"	62	[42]	95	5	0	340	[42]	800,000	UK	960,000	10	131-11-3			
Diethyl phthalate (DEP)	"	92	[42]	99	1	0	800	[42]	200,000	UK	102,000	10	84-66-2			
Di-n-butyl phthalate (DnBP)	"	69	[42]	88	12	0	**570**	[42]	8,000	UK	340	10	84-74-2		X	
Butyl benzyl phthalate (BBP)	"	86	[42]	96	4	0	180	[42]	20,000	UK	1,810	10	85-68-7			
Bisphenol A (BPA)	"	80	[19,26]	95	5	0	**200**	[18,22]	1,500	CH	175	10	80-05-7		X	
Benzotriazole	Corrosion inhibitors	26	[17,19]	100	0	0	6,600	[21]	30,000	CH	40,000	10	95-14-7	X		X
Methylbenzotriazole	"	30	[19]	100	0	0	2,900	[21]	75,000	CH	40,000	10	64665-57-2	X		X
Benzothiazole	"	80	[19,43]	100	0	0	300	[43]	238,000	CH	30,000	50	95-16-9			
Benzothiazole-2-sulfonic acid (BTSA)	"	15	[43]	100	0	0	1,600	[43]	-		-		941-57-1	X	?	X
2-hydroxybenzothiazole (OHBT)	"	60	[43]	99	1	0	100	[43]	-		-		934-34-9			
2-methylthiobenzothiazole (MTBT)	"	0	[43]	99	1	0	100	[43]	-		-		615-22-5			X
Ethylenediaminetetraacetatic acid (EDTA)	Chelating agents	5	[14,43-46]	0	0	0	**90,000**	[14,43-46]	2,200,000	CH	39,000	10	60-00-4	X	X	X
Nitrilotriacetatic acid (NTA)	"	95	[14,43-46]	100	0	0	10,000	[14,43-46]	190,000	CH	156,000	10	139-13-9	X		
Decabromodiphenyl ether (BDE-209)	Brominated flame retardants	90	[49]	0	100	0	2	[48]	-		40	50	1163-19-5			
2,2',4,4'-Tetrabromodiphenyl ether (BDE-47)	"	91	[26]	0	100	0	**0.7 / 8**	[22/47]	0.5	EU	500	10	5436-43-1		X	
Pentabromodiphenyl ethers (BDE-99)	"	93	[26]	0	100	0	**0.6 / 9**	[22/47]	0.5	EU	*(10,000)*	10,000	32534-81-9		X	
Tetrabromobisphenol A (TBBA)	"	90	[50]	20	80	0	2	[50]	-		1,700	10	79-94-7			
Tris(2-chloro,1-methylethyl) phosphate (TCPP)	Phosphorus flame retardants	1	[51]	0	100	0	1,500	[21,51]	-		120,000	50	13674-84-5	X		X
Tris(2-chloroethyl) phosphate (TCEP)	"	1	[51]	0	100	0	350	[21,51]	-		1,440,000	50	115-96-8			X
Tris(1,3-dichloro-2-propyl) phosphate (TDCP)	"	1	[51]	0	100	0	150	[21,51]	-		10,000	50	13674-87-8			X
Tri-iso-butyl phosphate (TiBP)	"	86	[51]	99	1	0	160	[21,51]	-		341,000	100	126-71-6			
Tris(butoxyethyl)-phosphate (TBEP)	"	88	[51]	98	2	0	440	[21,51]	-		76,000	100	78-51-3			
Tri-n-butyl phosphate (TnBP)	"	60	[51]	99	1	0	300	[21,51]	50,000	UK	37,000	10	126-73-8			
Triphenyl phosphate (TPP)	"	65	[51]	85	15	0	50	[21,51]	-		140	10	115-86-6			
Short chain chlorinated paraffins (SCCPs, C_{10-13})	Flame retardants	99	[52]	20	80	0	60	[52]	-		500	10	85535-84-8			
Perfluorooctanoic acid (PFOA)	Perfluorinated compounds	1	[53]	0	100	0	13	[21,53]	-		650	10	335-67-1			X
Perfluorooctane sulfonic acid (PFOS)	"	1	[53]	0	100	0	**12**	[21,53]	0.65	EU	10	10	1763-23-1		X	X

Table 2.2 (Continuation)

Substance	Family	Typical WWTP removal[a]	Ref.	Removal mechanisms[b]			Typical WWTP effluent concentration[c]	Ref.	EQS[d]	Ref.	PNEC[e]	AF[f]	CAS[g]	Prioritization[h]		
		[%]		B	S	V	[ng/l]		[ng/l]		[ng/l]			L	T	P
Pesticides																
Atrazine	Herbicide	23	[19,29]	95	5	0	**10**	[19,21]	600	EU	0.011	10	1912-24-9		X	X
(Aminomethyl)phosphonic acid (AMPA)	Degradation product	0	[26]			0	4,000	[22,26]	1,500,000	CH	11,000	100	1066-51-9	X		X
Carbendazim	Fungicide	30	[17,19,29]	95	5	0	**100**	[17,19,29]	340	CH	0.114	10	10605-21-7		X	X
Diazinon	Insecticide	40	[17,29]	94	6	0	**40**	[17,21,29]	15	CH	0.005	10	333-41-5		X	X
Diuron	Herbicide, algicide	33	[17,19,29]	90	10	0	**70**	[17,19,21,29]	20	CH	0.026	10	330-54-1		X	X
Glyphosate	Herbicide	30	[26]			0	**850**	[22,26]	108,000	CH	200	10	1071-83-6		X	X
Irgarol 1051	Algicide	48	[17,19,29]			0	**7**	[17,19,29]	2.3	CH	0.0045	10	28159-98-0		X	X
Isoproturon	Herbicide, algicide	42	[17,19,29]	80	20	0	25	[17,19,21,29]	320	CH	174	10	34123-59-6			X
(4-chloro-2-methylphenoxy)acetic acid (2,4-MCPA)	Herbicide	28	[17]			0	300	[17,21]	1,340	CH	1,400	10	94-74-6			X
Mecoprop	Herbicide	25	[17,19,29]	80	20	0	500	[17,19,21,29]	3,600	CH	514,700	10	93-65-2			X
Terbuthylazine	Herbicide	24	[29]	70	30	0	50	[21,29]	220	CH	60	10	5915-41-3			X
Terbutryn	Herbicide, algicide	60	[19,29]	80	20	0	20	[19,29]	60	CH	200	10	886-50-0			
Persistent organic pollutants (POPs)																
∑209 PCBs	Dielectric and coolant fluids	75	[54,55]	0	100	0	**20**	[47,56]	-		1	10	11097-69-1		X	
Aldrin	Insecticide	86	[55]	0	100	0	1	[55]			36	10	309-00-2			
Dieldrin	Insecticide	77	[55]	0	100	0	**8.9**	[55]	∑=10	EU	1	10	60-57-1		X	
Endrin	Insecticide	81	[55]	0	100	0	2.8	[55]			3.4	10	72-20-8			
Dichlorodiphenyltrichloroethane (DDT)	Insecticide	83	[55]	0	100	0	1	[55]	25	EU	1	10	50-29-3			
Endosulfan	Insecticide	84	[55]	0	100	0	**2.7**	[55]	5	EU	1	10	115-29-7		X	
Heptachlor	Insecticide	91	[55]	0	100	0	**6.4**	[55]	0.0002	EU	46	10	76-44-8		X	
Hexachlorobenzene	Fungicide	90	[55]	0	100	0	1.7	[55]	10	EU	4	10	118-74-1			
Hexachlorocyclohexanes (HCHs)	Insecticide	79	[55]	0	100	0	6.2	[55]	20	EU	60	10	58-89-9			
Pentachloronitrobenzene (PCNB)	Fungicide	75	[55]	0	100	0	14	[55]	-		1,300	10	82-68-8			
Polycyclic aromatic hydrocarbons (PAHs)																
Acenaphthene		85	[57]	4	95	1	23	[47,57]	-		1,000	10	83-32-9			
Anthracene		90	[57]	1	98	1	2	[22,57]	100	EU	200	10	120-12-7			
Fluoranthene		80	[57]	1	99	0	**10**	[22,47,57]	6.3	EU	60	10	206-44-0		X	
Fluorene		90	[57]	10	90	0	13	[47,57]	-		1,000	10	86-73-7			
Naphthalene		60	[57]	10	80	10	49	[57]	2,000	EU	37,000	10	91-20-3			
Phenanthrene		80	[57]	5	95	0	**12**	[57]	-		0.02	10	85-01-8		X	
Pyrene		70	[57]	1	99	0	15	[47,57]	-		120	10	129-00-0			
Benzo[b]fluoranthene		80	[57]	0	100	0	3	[22,57]	∑=30	EU	*(54)*	10,000	205-99-2			
Benzo[k]fluoranthene		90	[57]	0	100	0	2	[22,57]			*(0.2)*	5,000	207-08-9			
Benzo[a]pyrene		70	[57]	0	100	0	**1.1**	[22,57]	0.17	EU	2	10	50-32-8		X	
Indeno[1,2,3-cd]pyrene		80	[26,57]	0	100	0	**1.4**	[22,57]	∑=2	EU	-		193-39-5		X	
Benzo[ghi]perylene		80	[26,57]	0	100	0	**1.1**	[22,57]			*(0.01)*	10,000	191-24-2		X	
Volatile organic compounds (VOCs)																
Ethylbenzene		98	[57]	35	5	60	63	[57]	-		20,000	50	100-41-4			
Styrene		96	[57]	35	5	60	100	[57]	-		6,300	10	100-42-5			
Toluene		75	[57]	35	5	60	750	[57]	50,000	UK	100,000	10	108-88-3			
m-Xylene + p-Xylene		97	[57]	35	5	60	5	[57]	-		26,000	50	108-38-3			
o-Xylene		70	[57]	35	5	60	170	[57]	-		26,000	50	95-47-6			
1,2,4-Trimethylbenzene		82	[57]	35	5	60	340	[57]	-		23,560	100	95-63-6			

Table 2.2 (Continuation)

Substance	Family	Typical WWTP removal[a] [%]	Ref.	Removal mechanisms[b] B	S	V	Typical WWTP effluent concentration[c] [ng/l]	Ref.	EQS[d] [ng/l]	Ref.	PNEC[e] [ng/l]	AF[f]	CAS[g]	Prioritization[h] L	T	P
Heavy metals		Total / dissolved					Total / dissolved									
Aluminium (Al)		93 / 44	[26]	0	100	0	68,000 / 20,000	[22]	-		400	10	7429-90-5	X		
Cadmium (Cd)		85 / 89	[26]	0	100	0	50 / **24**	[22]	< 80-250[i]	EU	13	10	7440-43-9		X	
Chromium (Cr)		73 / -	[58]	0	100	0	700 / 600	[22]	3400	UK	2,400	10	7440-47-3			
Copper (Cu)		86 / 63	[26]	0	100	0	8,300 / **5,600**	[22]	1,000-28,000[i]	UK	50	10	7440-50-8	X	X	
Iron (Fe)		78 / 61	[26]	0	100	0	170,000 / 59,000	[22]	1,000,000	UK	56,000	10	7439-89-6	X		
Lead (Pb)		89 / 57	[26]	0	100	0	800 / **400**	[22]	1,200	EU	21	10	7439-92-1		X	
Mercury (Hg)		79 / 32	[26]	0	94	6	16 / 11	[22]	50	EU	25	10	7439-97-6			X
Nickel (Ni)		31 / 6	[26]	0	100	0	4,900 / **4,300**	[22]	4,000	EU	110	10	7440-02-0	X	X	X
Zinc (Zn)		75 / 16	[26]	0	100	0	30,900 / **24,000**	[22]	8,000-125,000[i]	UK	8.5	10	7440-66-6	X	X	X

[a] Average removal in conventional WWTPs (mainly activated sludge) observed, if available, in national studies on a wide range of WWTPs in Europe or in the USA. In bracket: very variable values reported (median)

[b] Main removal mechanisms in WWTPs. **B**: biodegradation, **S**: sorption, **V**: volatilization. Scale: White: less then 10%, Light grey: 10-40%, Dark grey: 40-70%, Black: >70% of the total removal (100%) (values are estimation of the removal percentage attributed to each mechanism) ([/]: no data). The fraction removed by sorption was estimated based on the concentration of the pollutant in sewage sludge, assuming an average sludge production of 240 mg/l. References for pharmaceutical and personal care products in sludge: [23,25,26,59,61-63]; for pesticides in sludge: [23,60]; for PAHs and heavy metals: [23]. For others compounds, see [64]

[c] Average effluent concentrations observed, if available, in national studies on a wide range of WWTPs in Europe or in the USA. Ref. [17-19]: 10-28 WWTPs in Switzerland, Ref. [20]: 50 WWTPs in the USA, Ref. [21]: 90 WWTPs in Europe, Ref. [22]: 162 WWTPs in UK, Ref. [23]: 5 WWTPs in the USA. **In bold:** effluent concentrations higher than their respective EQS or PNEC (risk for sensitive aquatic organsisms in case of low dilution of the effluent)

[d] Chronic environmental quality standards (EQS) for inland surface waters (annual average value). Proposition for Switzerland (CH), United Kingdom (UK) and Europe (EU). For metals: standards for the dissolved or bioavailable concentrations. Reference: **CH**: http://www.oekotoxzentrum.ch/expertenservice/qualitaetskriterien/vorschlaege/index_EN (last accessed June 2014), **UK**: https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/298245/geho0810bsxl-e-e.pdf (last accessed June 2014), **EU**: Directives 2013/39/EU and 2008/105/EC. (-): no value proposed

[e] Predicted No-Effect Concentration (PNEC): concentration below which exposure to a substance is not expected to cause adverse effects. Automatically calculated by the ecotoxicity database AiiDA (http://aiida.tools4env.com) based on the most sensitive species (values not validated by external experts). In bracket: PNEC for which an AF ≥ 1000 is used (less reliable). (-): no value proposed

[f] Assessment or security factor (AF), used to calculate the PNEC (PNEC=[Concentration affecting the most sensitive species] / AF), which depends on the number and the quality of the available ecotoxicity tests. For AF ≥ 1000, only acute ecotoxicity data are available, and thus the PNEC is less reliable

[g] Chemical Abstracts Service number (CAS) of the substance for which the PNEC was determined in AiiDA (http://aiida.tools4env.com)

[h] Prioritization of the micropollutants based on their **load** (**L**) (for substances with effluent concentration > 1000 ng/l), their **toxicity** (**T**) (for substances with effluent concentration > PNEC) and their **persistance** (**P**) in WWTPs (for substances removed less than 50% in conventional treatments)

[i] Hardness related values

References: [1](Berna et al., 2007), [2](Scott and Jones, 2000), [3](Matthijs et al., 1999), [4](Feijtel et al., 1996), [5](Mungray and Kumar, 2009), [6](Schowanek et al., 2007), [7](Field et al., 1995), [8](McAvoy et al., 1998), [9](McAvoy et al., 2006), [10](Belanger et al., 2006), [11](Loyo-Rosales et al., 2007), [12](Ying et al., 2002), [13](Bergé et al., 2012), [14](Clara et al., 2012), [15](Kreuzinger et al., 2007), [16](Guedez and Püttmann, 2011), [17](DGE, 2013), [18](Kase et al., 2011), [19](Margot et al., 2013b), [20](Kostich et al., 2014), [21](Loos et al., 2013), [22](Gardner et al., 2012), [23](Lubliner et al., 2010), [24](Oulton et al., 2010), [25](Verlicchi et al., 2012), [26](Gardner et al., 2013), [27](Huerta-Fontela et al., 2010), [28](Oosterhuis et al., 2013), [29](Singer et al., 2010), [30](Bijlsma et al., 2012), [31](Kasprzyk-Hordern et al., 2009), [32](Clara et al., 2011), [33](Kupper et al., 2006), [34](Bester et al., 2008), [35](Yang and Metcalfe, 2006), [36](Fatta-Kassinos et al., 2010), [37](González-Mariño et al., 2011), [38](Lozano et al., 2013), [39](Balmer et al., 2005), [40](Kokotou and Thomaidis, 2013), [41](Lange et al., 2012), [42](Bergé et al., 2013), [43](Reemtsma et al., 2006), [44](Alder et al., 1990), [45](Kari and Giger, 1996), [46](Knepper, 2003), [47](Hope et al., 2012), [48](North, 2004), [49](Nyholm et al., 2013), [50](Potvin et al., 2012), [51](Meyer and Bester, 2004), [52](Zeng et al., 2013), [53](Guo et al., 2010), [54](Blanchard et al., 2004), [55](Katsoyiannis and Samara, 2004), [56](Balasubramani et al., 2014), [57](Fatone et al., 2011), [58](Ziolko et al., 2011), [59](Jelic et al., 2011), [60](Campo et al., 2013), [61](McClellan and Halden, 2010), [62](Yan et al., 2014), [63](Hörsing et al., 2011), [64](Margot, 2015)

2.3.3 Personal care products

Personal care products (PCPs) include ingredients found in shampoos, washing lotions, skin care products, dental care products, sunscreen agents, cosmetics, perfumes, hair styling products, etc. The most studied PCPs are fragrances (such as polycyclic and nitro musks), UV filters, antimicrobial/disinfectants, preservatives and insect repellents. Due to their wide consumption and their type of usage (often skin application), they enter municipal wastewater mainly via wash-off during showering or bathing (Ternes and Joss, 2006). The fate of these PCPs is described below.

2.3.3.1 Fragrances

Fragrances such as synthetic musks are widely used in cosmetics, perfumes, body lotions, shampoos, detergents and fabric softeners. The main synthetic musks detected in the environment are the polycyclic musks galaxolide (HHCB) and tonalide (AHTN), produced in high quantities (300-1400 tons/y in Europe in 2002); the nitro musks ketone and xylene, though their use has been significantly reduced during the past decades due to their potential toxic health effects; and the bicyclic hydrocarbon fragrance compound OTNE (Kubwabo et al., 2012; Kupper et al., 2006). Musks are relatively lipophilic as they are made to sorb on surface (skin, fabric, etc). Due to their uses, they usually arrive in wastewater through wash-off during laundry or shower. Concentrations of musks in raw wastewaters are usually around 1-13 µg l^{-1} for HHCB (average of 1.6 mg d^{-1} $capita^{-1}$), 0.2-1.8 µg l^{-1} for AHTN (average of 0.36 mg d^{-1} $capita^{-1}$), 2.5-13 µg l^{-1} for OTNE (average of 3.4 mg d^{-1} $capita^{-1}$), and around 5-60 ng l^{-1} for musks ketone and xylene (Bester et al., 2008; Clara et al., 2011; Clara et al., 2005b; Fatta-Kassinos et al., 2010; Kupper et al., 2006). Due to their hydrophobicity (log K_{OW} > 5.7) and their relatively high sludge distribution coefficient (K_d = 5000 – 20,000 l kg^{-1}SS), polycyclic musks are usually well removed in WWTPs, from 70 to 99%, mainly by sorption (Clara et al., 2011; Horii et al., 2007; Kupper et al., 2006). A significant portion (up to 50-70%) is removed during the primary treatment, together with suspended solids, and the remaining with the secondary sludge (Kupper et al., 2006). Polycyclic musks are also possibly slightly biodegraded during the secondary treatment (5-10%), or during the sludge treatment (up to 50%), but are not considered to be easily biodegradable (Clara et al., 2011; Kupper et al., 2006). Due to their relative volatility (K_{AW} around $5 \cdot 10^{-3}$ to $1.5 \cdot 10^{-2}$ [-]), a fraction of AHTN and HHCB (up to 14%) volatilizes from the aeration basins, leading to the presence of relatively high concentrations of musks in the indoor (up to 300 and 3 µg m^{-3} air for HHCB and AHTN respectively) and outdoor atmosphere of WWTPs. WWTPs can therefore be regarded as significant sources of these compound to the atmosphere (Upadhyay et al., 2011; Weinberg et al., 2011). Removal of nitro musks and the fragrance OTNE in WWTPs is reported to be slightly lower than for polycyclic musks (around 50% for musks xylene and ketone, and between 50% to 80% for OTNE) (Bester et al., 2008; Yang and Metcalfe, 2006), probably, for nitro musks, due to their lower hydrophobicity and volatility (Upadhyay et al., 2011). Concentrations of fragrances in WWTP effluents are usually reported in the range 300-1400 ng l^{-1} for HHCB, 50-500 ng l^{-1} for AHTN, 800-1800 ng l^{-1} for OTNE, and 0.5-35 ng l^{-1} for nitro musks (Bester et al., 2008; Clara et al., 2011; Fatta-Kassinos et al., 2010). Risks for sensitive aquatic organisms cannot be excluded for OTNE in the case of low dilution of the effluent as its concentration is higher than its PNEC (560 ng l^{-1}, Table 2.2). Due to their lipophilicity, polycyclic musks can then bioconcentrate in fish. The average

concentrations found in fish filets in the United States were 1100 ng g^{-1} tissue for HHCB and 110 ng g^{-1} for AHTN (Ramirez et al., 2009).

Sewage sludge contains relatively high concentrations of synthetic musks and fragrances, especially for HHCB (3-20 mg kg^{-1} dm), AHTN (0.4-3 mg kg^{-1} dm) and OTNE (2.9-4.5 mg kg^{-1} dm) (and around 40-80 µg kg^{-1} dm for nitro musks) (Bester et al., 2008; Clara et al., 2011; Kupper et al., 2006). In the case of soil amendment, these compounds are, however, reported not to persist in soils (removal in a few weeks) (Yang and Metcalfe, 2006).

Many PCPs and other products (e.g., air fresheners) that contain fragrances contain also fragrance dispersants such as dimethyl phthalate (DMP) and diethyl phthalate (DEP) (Bergé et al., 2013). The concentration and the fate of phthalates in wastewater are discussed in the sub-chapter 2.3.4.2.

2.3.3.2 Preservatives, antimicrobials and insect repellents

Parabens are widely employed as antimicrobial *preservatives* in PCPs such as body lotions, shampoos, tooth pastes, deodorants, etc. The most commonly used parabens include methyl- (MeP), ethyl- (EtP), propyl- (PrP), butyl- (BuP) and benzyl-parabens (BzP). Due to their light estrogenic effect and their ubiquitous presence in human tissues, they are possibly substances of concern for human health (Kirchhof and de Gannes, 2013). Median concentrations of parabens in raw municipal wastewater in Spain were at 2500 ng l^{-1} for MeP, 1400 ng l^{-1} for PrP, 760 ng l^{-1} for EtP and 200 ng l^{-1} for BuP. BzP was found at very low concentrations (< 2 ng l^{-1}) (González-Mariño et al., 2011). Parabens are well (> 95%) removed in WWTPs. A small fraction may be removed during the primary treatment by sorption to particles (Sun et al., 2014), but the main removal mechanism reported (> 95%) is biodegradation. Parabens are indeed readily biodegradable (González-Mariño et al., 2011). Parabens were found in sewage sludge, but at relatively low concentrations (74 µg kg^{-1} dm for MeP, 3.4 µg kg^{-1} dm for PrP and less than 1 µg kg^{-1} dm for the others) (Liao et al., 2013). Concentrations of parabens in WWTP effluents are usually lower than 100 ng l^{-1} (much more below their PNEC, Table 2.2), with median concentrations in Spain at 19 ng l^{-1} for MeP and 2.5 ng l^{-1} for EtP, the only two parabens detected in the effluents (González-Mariño et al., 2011).

Antimicrobial agents are widely used in PCPs, mainly in soaps (liquids and bars), toothpastes, deodorants and shave gels. Triclocarban and especially triclosan are among the most common antimicrobials used. Their concentrations in raw wastewater are relatively high, on average between 1 and 10 µg l^{-1} for triclosan and slightly lower for triclocarban (0.1 to 6 µg l^{-1}) (Gardner et al., 2013; Guerra et al., 2014; Lozano et al., 2013). Due to their hydrophobicity (log K_{OW} around 4.9), 70 to 80% of their influent concentration is associated to particles. A significant fraction (up to 60-80%) of triclosan and triclocarban is thus removed during the primary treatment, together with suspended solids. The residual fraction is further removed in the biological treatment, either by sorption to the secondary sludge or by biodegradation (around 10 to 35% of the total removal) (Lozano et al., 2013). The overall removal in WWTPs is usually over 80%, and often over 95% (Gardner et al., 2013). Average concentrations in WWTP effluents (mostly on the dissolved fraction) in Europe, Australia and the USA are reported to be around 100-250 ng l^{-1} for triclosan and 10-120 ng l^{-1} for triclocarban (Gardner et al., 2012; Loos et al., 2013; Lozano et al., 2013; Ying and Kookana, 2007). Triclosan concentrations in the receiving water may thus exceed the proposed Swiss EQS of 20 ng l^{-1}, and both

compounds are expected to exceed their PNEC (1-2 ng l^{-1}, Table 2.2) in the case of low dilution of the effluents (Ecotox Center, 2014).

Despite a fraction biodegraded, most of these antimicrobial agents accumulate in the sewage sludge. The median concentrations found in sludge coming from 94 US WWTPs were 36 mg kg^{-1} dm and 12.6 mg kg^{-1} dm for triclocarban and triclosan, respectively (McClellan and Halden, 2010). Triclosan was found on average around 5 mg kg^{-1} dm in sludge from Australia and United Kingdom (Gardner et al., 2013; Ying and Kookana, 2007). When the sludge is used as fertilizer, triclocarban is reported to persist for long time in soil (no apparent degradation in three years), while triclosan can be slowly degraded (half-live of 190 d) (Walters et al., 2010).

Chloroxylenol is another antibacterial agent found at high concentrations in raw wastewater (10-30 µg l^{-1}). Despite good removal (> 95%) in WWTPs, its concentration in effluents was reported to be around 300 ng l^{-1} (Kasprzyk-Hordern et al., 2009).

N,N-diethyl-m-toluamide (DEET) is the active ingredient of most commercial *insect repellents*. Showering and bathing after application and laundering of clothes are considered to be a major source of DEET in wastewater. Concentrations in raw wastewater are in the range 0.1 to 10 µg l^{-1}, with the highest values observed usually in summer (Aronson et al., 2012). Removal efficiency of DEET in WWTPs is highly variable, ranging from 10 to 99% depending on the plant or season. Due to its low sorption affinity (K_d = 30-100 l kg^{-1} SS) and low volatility ($K_{AW} = 8 \cdot 10^{-7}$) (Aronson et al., 2012; Stevens-Garmon et al., 2011), the main removal mechanism is expected to be biodegradation (DEET is moderately biodegradable). Higher removal rates were observed in plants with longer SRTs, probably due to higher microbial diversity, but the reasons for these high removal efficiency variations are not well understood (Aronson et al., 2012). Average concentrations of DEET in WWTP effluents are between 100 to 900 ng l^{-1} in Europe (Aronson et al., 2012; DGE, 2013; Loos et al., 2013), which is far below the proposed EQS value for Switzerland (41 µg l^{-1}).

2.3.3.3 UV filters

Organic ultraviolet (UV) filters are widely used in sunscreen agents and cosmetics to protect against sunburn or as a preservative to prevent UV degradation of other cosmetics ingredients. They mainly enter aquatic environments either directly during recreational activity (bathing in lakes and rivers) or indirectly through municipal wastewater (wash-off from the skin during showering). Many (about 30) different UV filters are frequently used. Some of the most common are benzophenone-1, -3 (also called oxybenzone) and -4 (BP-1, BP-3, BP-4), 4-methylbenydlidene camphor (4-MBC), ethylhexyl methoxy cinnamate (EHMC, also called octyl-methoxycinnamate (OMC)), octocrylene (OC), octyl-triazone (OT) and butyl methoxydibenzoylmethane (BMDM) (Balmer et al., 2005; Kupper et al., 2006; Tsui et al., 2014). Concentrations of UV filters in Swiss raw wastewater vary seasonally, from < 100 ng l^{-1} up to 20 µg l^{-1}, with usually higher concentrations after sunny summer days (1-10 µg l^{-1} for 4-MBC, BP-3, EHMC, OT and OC) (Balmer et al., 2005; Kupper et al., 2006). Average concentrations (summer and winter) measured in some Chinese raw wastewaters were, for instance, at 250 ng l^{-1} for BMDM, 438 ng l^{-1} for EHMC, 284 ng l^{-1} for BP-3 and 643 ng l^{-1} for BP-4 (Tsui et al., 2014).

Most UV filters (e.g., BP-3, EHMC, OC, OT) are usually over 90% removed in conventional WWTPs, although 4-MBC has lower average removal rates (around 70%) (Balmer et al., 2005; Kupper et al., 2006). Some UV filters (such as OC or OT) are lipophilic as they are often used as additives in cosmetics (usually composed of lipids or oils). They tend then to sorb onto particles and are usually partially (50-90%) removed during primary treatments. UV filters are thus found in stabilized sewage sludge at relatively high concentrations, around 0.6-6 mg kg^{-1} dm for 4-MBC, EHMC, OC and OT (Kupper et al., 2006). This is however not the case for benzophenones (BP-1, 3, 4) which are less hydrophobic (Tsui et al., 2014). Due to their biodegradability, most UV filters are further removed by degradation during the secondary treatment (Kupper et al., 2006). Concentrations in WWTPs effluents are thus reduced and usually in the range 10 ng l^{-1} to 1 µg l^{-1} (Balmer et al., 2005), which is lower than their respective PNECs (Table 2.2). Due to their lipophilicity, UV filters can bioconcentrate in aquatic organisms. 4-MBC and BP-3 were for instance found in fish in Swiss lakes at concentrations around 100 ng g^{-1} lipid (Balmer et al., 2005).

2.3.4 Household and industrial chemicals

Many other chemicals are used daily in homes or workplaces (sweeteners, anticorrosives or chelating agents) or are present in household equipment (plasticizers, flame retardants, perfluorinated compounds). They often find their way into sewers.

2.3.4.1 Food and beverage additives

Artificial sweeteners such as acesulfame, aspartame, cyclamate, neotame, neohesperidine dihydrochalcone (NHDC), saccharin and sucralose, are widely used (increasing over time) in food, beverages and toothpaste, where they act as sugar substitutes. Artificial sweeteners are designed not to be metabolized in the human body (their goal is to provide a negligible energy source). Thus, except for aspartame, neotame and NHDC, which are mostly excreted in metabolite forms, 90 to 100% of all other sweeteners consumed are then released in urine and faeces. The estimated total load of sweeteners in sewers is around 10 to 60 mg d^{-1} $capita^{-1}$ (Lange et al., 2012), which is in the same range as the total load of pharmaceuticals. Concentrations of acesulfame, cyclamate, saccharin and sucralose in raw municipal wastewaters are relatively high, varying usually between 1 to 200 µg l^{-1}, with average concentrations around 20-30 µg l^{-1} (Kokotou and Thomaidis, 2013; Lange et al., 2012). Cyclamate and saccharin are easily biodegradable and are removed from 90 to more than 99% in WWTPs, resulting in effluent concentrations typically below 1 µg l^{-1}. Acesulfame and sucralose are, on the contrary, very persistent, and due to their high hydrophilicity (log K_{OW} around -1), not significantly removed by sorption to the sludge (< 0.1%, with sludge concentration around 50 to 150 µg kg^{-1} dm) (Ordoñez et al., 2013). These two sweeteners are thus not significantly removed during treatment, and their concentrations in effluents are therefore relatively high (10-50 µg l^{-1} for acesulfame and 0.4 to 20 µg l^{-1} for sucralose) (Kokotou and Thomaidis, 2013; Lange et al., 2012). Sucralose and especially acesulfame are detected in surface, ground and drinking waters in relatively high concentrations (up to a few µg l^{-1}). Their concentrations in some drinking waters are among the highest concentrations of anthropogenic trace pollutants found, but are, however, around 1000 times lower than their organoleptic (sweetness) threshold values (Lange et al., 2012). Sweeteners are not expected to be toxic to aquatic organisms at these concentrations (Sang et al., 2014).

2.3.4.2 Plasticizers and plastic additives

Plasticizers are added in plastics to improve their flexibility. *Phthalates* (phthalic acid esters) are common plasticizers, although phthalates are also used as fragrance dispersants in cosmetics, or as additive in epoxy resins, food packaging, building materials, etc. Phthalates are pollutants of concern due to their disruption of endocrine activity and their association with many human health problems (alteration of reproduction, development and neurodevelopment) (Huang et al., 2012). The most studied phthalates are di-(2-ethylhexyl) phthalate (DEHP) (widely used as PVC plasticizer, for instance in PVC shower curtains), dimethyl phthalate (DMP) and diethyl phthalate (DEP) (used as fragrance dispersants), and di-n-butyl phthalate (DnBP) and butyl benzyl phthalate (BBP) (used as an additive in many products). Due to their widespread use, median concentrations of phthalate in raw municipal wastewater are around 40 µg l^{-1} for DEHP, 10 µg l^{-1} for DEP and 1-2 µg l^{-1} for DMP, DnBP and BBP. Phthalates are partially to well removed (60 to 95%) in WWTPs (Table 2.2). For the hydrophobic DEHP (log K_{OW} = 7.5), sorption on particles and sludge is considered to be the main removal mechanism (> 40% removal). For more hydrophilic DEP and DMP (log K_{OW} < 2.5), biodegradation seems to play an important role (less than 3% removed by sorption). For BBP and DnBP (log K_{OW} = 4.7-4.8), both sorption (5-10%) and biodegradation can participate to the observed removal. Median concentrations in WWTP effluents were reported to be around 2.4 µg l^{-1} for DEHP and between 200 to 800 ng l^{-1} for DMP, DEP, DnBP and BBP. In the case of low dilution of the effluent, DEHP may exceed the European EQS of 1.3 µg l^{-1} in inland waters (EC, 2013) and DnBP may exceed its PNEC (Table 2.2). DEHP accumulates in sewage sludge, with a median concentration around 60 mg kg^{-1} dm. The other phthalates are also detected in sludge but at much lower median concentrations (0.2 to 0.5 mg kg^{-1} dm) (Bergé et al., 2013).

Bisphenol A (BPA) is produced in large quantities. It is mainly (about 95%) used in the production of synthetic polymers such as polycarbonates (transparent hard plastic) and epoxy resins. These polymers are widely used in households, for instance for inner water-pipe coating, food containers, bottles, inner coatings for tins (canned food) and beverage cans, toys, etc. BPA is also used as a stabilizer in PVC (e.g., in shower curtains) and as a colour developer in thermal papers (e.g., shop receipts, faxes) (Michałowicz, 2014). Recycling of thermal paper was reported to contaminate recycled papers with BPA. Up to 46 µg g^{-1} (average 19 µg g^{-1}) of BPA was found in recycled toilet paper (Liao and Kannan, 2011), which may contribute significantly to the load of BPA in wastewater. BPA was found on average at 0.8-1 µg l^{-1} in raw wastewater in the United Kingdom (UK) and in Switzerland (Gardner et al., 2013; Margot et al., 2013b). BPA is moderately to easily biodegradable with removals of over 80% observed in WWTPs, especially in those that have a nitrification step (Gardner et al., 2013; Margot et al., 2013b). BPA has a moderate sorption capacity (K_d 300-500 l kg^{-1}) (Banihashemi and Droste, 2014), suggesting that the removal is mostly by biodegradation (Zhao et al., 2008). Concentrations in sewage sludge are indeed relatively low (around 70 µg kg^{-1} dm) (Gardner et al., 2013). BPA concentrations in WWTPs effluents were reported on average at around 100-300 ng l^{-1} (Gardner et al., 2012; Kase et al., 2011). BPA is an endocrine disrupter and can affect fish (impact on gonad morphology) at very low concentrations (1 µg l^{-1}) (de Kermoysan et al., 2013), with a PNEC of 175 ng l^{-1} (Table 2.2). A risk for aquatic organisms can thus not be excluded in the case of low effluent dilution.

2.3.4.3 Anticorrosives

Benzotriazoles are high-production-volume polar chemicals mostly used as corrosion inhibitors in de-icing fluids for aircrafts, automotive antifreeze formulation, brake fluids, industrial cooling systems, but also in households for silver protection and as a polishing agent in dishwashing detergent. The main benzotriazoles reported are benzotriazole (BTr) itself, and 4- and 5- methylbenzotriazoles (MBTr). Due to their wide usage in dishwashing products (on average 12.5 mg per tablet), it was estimated that around 3 mg d^{-1} $capita^{-1}$ of benzotriazoles are released in the sewers (Vetter and Lorenz, 2013). Concentrations of benzotriazoles in raw wastewater are thus relatively high, on average around 10 µg l^{-1} (usually between 5-15 µg l^{-1}) for BTr and around 5 µg l^{-1} for MBTr. Removal of benzotriazoles in WWTPs is usually low, on average between 20 and 30% (DGE, 2013; Margot et al., 2013b; Reemtsma et al., 2006; Weiss and Reemtsma, 2005). They are not considered to be rapidly biodegraded and due to their low hydrophobicity (log K_{OW} < 2), they are not significantly removed by sorption (K_d < 200 l kg^{-1}, 15-30 µg kg^{-1} dm) (Stasinakis et al., 2013). Concentrations of benzotriazoles in WWTP effluents are therefore relatively high, and were found on average in 90 European WWTPs at 6.6 µg l^{-1} for BTr and 2.9 µg l^{-1} for MBTr (Loos et al., 2013). These levels are, however, lower than the proposed Swiss EQS for surface waters (30 and 75 µg l^{-1} for BTr and MBTr, respectively) (Ecotox Center, 2014).

Benzothiazoles are also high-production-volume chemicals with various applications, the main one being vulcanization accelerators in rubber, but they are used also as corrosion inhibitors in antifreeze and cooling liquids, in wood preservation or in industrial processes. The main benzothiazoles reported in municipal wastewater, coming from urban runoff (tire abrasion on roads) and unknown sources in households, were benzothiazole-2-sulfonic acid (BTSA), benzothiazole (BT), 2-hydroxybenzothiazole (OHBT) and 2-methylthiobenzothiazole (MTBT). Their individual concentrations in raw wastewater and in WWTP effluents are in the range of 0.2-2 µg l^{-1}, which are much lower than the proposed Swiss EQS value for surface waters (238 µg l^{-1}) (Ecotox Center, 2014). Removal efficiencies reported for benzothiazoles in WWTPs are very variable, from 0 to 80%, mostly due to biotransformation/degradation. Very low adsorption on sludge are indeed reported for these polar pollutants (K_d = 3-200 l kg^{-1}, 20-60 µg kg^{-1} dm) (Asimakopoulos et al., 2013; Kloepfer et al., 2005; Reemtsma et al., 2006; Stasinakis et al., 2013).

2.3.4.4 Synthetic chelating agents

Ethylenediamine tetraacetatic acid (EDTA) and *nitrilotriacetatic acid (NTA)* are synthetic chelating agents designed to "sequester" metal ions such as Fe^{3+}, Ca^{2+} or Mg^{2+}. EDTA and NTA are widely used in laundry and household detergents as builders to reduce water hardness (Ca^{2+}, Mg^{2+}), or as stabilizers in personal care products and detergents. Both compounds are very hydrophilic and with a low volatility (log K_{OW} around -3.8 and K_{AW} < 5 × 10^{-9})[1]. EDTA and NTA were found at very high concentrations in raw municipal wastewater, with average concentrations between 70 and 950 µg l^{-1} (Alder et al., 1990; Clara et al., 2012; Gardner et al., 2013). NTA is biodegradable with good removal efficiencies (90 to 97%) reported for WWTPs. EDTA is, on the contrary, not biodegradable, and less than 10% removal is usually reported. Concentrations of chelating agents in WWTP effluents are on

[1] Source: SRC PhysProp Database: http://esc.syrres.com/fatepointer/search.asp, last accessed 17.03.2014

average around 3-50 µg l^{-1} for NTA and around 50-130 µg l^{-1} for EDTA (Alder et al., 1990; Clara et al., 2012; Kari and Giger, 1996; Knepper, 2003; Reemtsma et al., 2006). EDTA has therefore one of the highest (together with some surfactants) average concentrations reported for synthetic chemicals in municipal WWTP effluents. EDTA and NTA were measured in surface waters at concentration up to 19 µg l^{-1} and around 1 µg l^{-1}, respectively. Median EDTA concentrations of 3.7 µg l^{-1} and 0.9 µg l^{-1} were reported in some European surface waters and in Lake Geneva, respectively (CIPEL, 2005; Knepper, 2003; Reemtsma et al., 2006). These concentrations are however lower than the proposed Swiss EQS for surface waters (2200 µg l^{-1} for EDTA and 190 µg l^{-1} for NTA) (Ecotox Center, 2014). EDTA concentrations in effluents surpass the relevant PNEC (39 µg l^{-1}, Table 2.2) and thus potential impacts of this compound cannot be neglected.

Phosphonates, other chelating agents widely used in laundry detergents but still few studied, were also measured on average at high (individual) concentrations (50-150 µg l^{-1}) in raw municipal wastewater. Phosphonates were usually not detected in WWTPs effluents (< 15-29 µg l^{-1}), with a removal efficiency over 80%, mostly by sorption on activated sludge (Nowack, 1998).

2.3.4.5 Flame retardants

Flame retardants are chemicals incorporated in various household equipments, such as building materials (e.g., insulation), electrical/electronic devices, upholstered furniture (e.g., sofas), textiles, plastics or polyurethane foams, to inhibit fires. The main families of organic flame retardants are based on brominated, organophosphorus and chlorinated paraffin compounds (van der Veen and de Boer, 2012). Flame retardants can reach the sewers during cleaning of textiles or household surfaces (flame retardants can accumulate in dust).

Brominated flame retardants have been used for several decades but, due to their environmental persistence, bioaccumulative potential and toxicity, some congeners of polybrominated biphenyls (PBBs) (hexa-BB) and polybrominated diphenyl ethers (PBDEs) (tetra-, penta-, hexa- and hepta-BDEs) were classified in 2009 as persistent organic pollutants (POPs) in the Stockholm Convention (van der Veen and de Boer, 2012). Their use is now restricted in many countries (including Switzerland), but due to their presence in high quantities in existing furniture and electric devices, they are expected to continue to contaminate the environment for many years.

PBDEs are a family of 209 congeners with a structure similar to the toxic polychlorobiphenyls (PCBs). The main congeners in commercial mixtures were tetra BDE-47, penta BDE-99 and -100, hepta BDE-153 and -175/183, octa BDE-197, nona BDE-207 and deca BDE-209 (La Guardia et al., 2006). Therefore these congeners, and especially BDE-47,-99, -100, -153 and -209, are the most frequently detected in wastewater (North, 2004). Median concentrations of BDE-47 and -99 in raw wastewater were around 10 ng l^{-1} in the UK (16 WWTPs) (Gardner et al., 2013) and between 10-140 ng l^{-1} in Norway (3 WWTPs), where BDE-209 was also found on average at 40-110 ng l^{-1} (Nyholm et al., 2013). PBDEs are usually well removed (median removal around 90%) in conventional WWTPs, mostly during secondary treatments (Gardner et al., 2013). As they are poorly biodegradable and relatively hydrophobic (log K_{OW} 4-10), the main removal mechanism is sorption onto sludge. Due to its high hydrophobicity (log K_{OW} around 9), the large congener BDE-209 has a very strong sludge affinity. BDE-209 was reported to be the dominant PBDE in sewage sludge, with average

concentrations of 300-500 µg kg^{-1} dm in Switzerland and Spain (33 WWTPs) and 5300 µg kg^{-1} dm in the US (94 WWTPs). BDE-47 and -99 were the second most abundant PBDEs in sludge, with average concentration between 800-1000 µg kg^{-1} dm in the US and around 10-50 µg kg^{-1} dm in UK, Spain and Switzerland (Gardner et al., 2013; Gorga et al., 2013; Kupper et al., 2008; Venkatesan and Halden, 2014). In the case of sludge application on land, PBDEs will persist in soils for years (< 1% attenuation in 3 y) (Venkatesan and Halden, 2014). Concentrations of PBDEs in WWTP effluents are dominated by congeners BDE-47 and -99, found at a median concentrations around 9 ng l^{-1} in the US (52 WWTPs) and 0.7 ng l^{-1} in the UK (162 WWTPs) (Gardner et al., 2012; Hope et al., 2012). Despite these very low concentrations (sum of PBDEs usually lower than 30 ng l^{-1}) (North, 2004), PBDEs may bioaccumulate in aquatic organisms at levels much higher (e.g., average 30 to 200 µg kg^{-1} wet weight in fish from the St. Lawrence River) (Houde et al., 2014) than the European EQS for biota (8.5 ng kg^{-1} wet weight) (EC, 2013).

Other brominated flame retardants (BFRs) such as tetrabromobisphenol A (TBBA) and hexabromocyclododecanes (HBCDs) are still widely used. TBBA was found on average at around 20 ng l^{-1} in wastewater and is reported to be well removed (> 90%) in WWTPs, probably by a combination of sorption and biotransformation, leading to effluent concentrations < 2 ng l^{-1} (Potvin et al., 2012). TBBA and HBCDs were found on average around 20-100 µg kg^{-1} dm in Spanish and Swiss sewage sludge (Gorga et al., 2013; Kupper et al., 2008). Many new BFRs are now also emerging due to the ban of several PBDE congeners (Covaci et al., 2011). Concentrations and fate of these new BFRs in WWTPs are still little studied, but it seems that concentrations of most of them are still below 10 ng l^{-1} in raw wastewater (Nyholm et al., 2013).

Organophosphorus flame retardants (OFRs) are, after BFRs, the second most consumed organic flame retardants[2]. They were proposed as an alternative to BFRs, and their consumption is expected to increase. They are also commonly used as plasticizers, lubricants, hydraulic fluids, floor polish or concrete additives (0.002% w/w as antifoam) (Andresen et al., 2004; Holmgren, 2013). The most frequently detected organophosphorus flame retardants and plasticizers are the *non-chlorinated* trimethyl phosphate (TMP), tri-n-butyl phosphate (TnBP), tri-iso-butyl phosphate (TiBP), tris(butoxyethyl)-phosphate (TBEP), triphenyl phosphate (TPP), and 2-ethylhexyl diphenyl phosphate (EHDPP), and the *chlorinated* tris(1,3-dichloro-2-propyl) phosphate (TDCP), tris(2-chloro,1-methylethyl) phosphate (TCPP), and tris(2-chloroethyl) phosphate (TCEP). All these compounds were present in raw municipal wastewater at average concentrations from 100 ng l^{-1} up to 19 µg l^{-1}, which is much higher than reported for brominated flame retardants. The highest average concentrations were observed for TnBP and TiBP (1-19 µg l^{-1}), plasticizers used also as antifoam in concrete from where they may leach in the sewers (Holmgren, 2013); TBEP (4-13 µg l^{-1}), a plasticizer and floor polish; and TCPP (1-4 µg l^{-1}), a flame retardant mostly used in polyurethane foam and building insulation (Marklund et al., 2005; Meyer and Bester, 2004). The chlorinated OFRs TCPP, TCEP and TDCP are not significantly (< 5 %) removed in WWTPs. TiBP and TBEP were usually well removed (> 80%), probably by a combination of sorption onto secondary sludge and biotransformation, whereas TnBP and TPP had removal efficiencies between 50 and 75% (Meyer and Bester, 2004). Much lower

[2] Data for 2011 (last accessed 19.03.2014):
http://www.flameretardants-online.com/web/en/106/7ae3d32234954e28e661e506e284da7f.htm

removal efficiencies for these last four compounds were, however, observed in other studies (Marklund et al., 2005; Rodil et al., 2012). Concentrations of OFRs in WWTP effluents are thus still relatively high, with average values observed between 50 ng l^{-1} for TPP, up to 0.5-10 µg l^{-1} for TCPP and TBEP (Table 2.2) (Loos et al., 2013; Marklund et al., 2005; Meyer and Bester, 2004). ORFs were among the most relevant substances detected in the effluents of 90 European WWTPs (Loos et al., 2013). OFRs were also found in sewage sludge at average concentrations between 40 µg kg^{-1} dm for TCEP, up to 1400 µg kg^{-1} dm for EHDPP (Marklund et al., 2005). It is not expected that the levels of OFRs found in effluents generate impacts on aquatic organisms as they are much lower than their PNECs (Table 2.2). But several OFRs (TCEP, TCPP, TDCP and TBEP) are carcinogens or possible carcinogens, and some are neurotoxic and/or can accumulate in liver and kidneys (van der Veen and de Boer, 2012). It is therefore not desirable to release them into the environment.

Chlorinated paraffins (CPs) are the third most consumed family of organic flame retardants[2]. CPs are also used as plasticizers or as additives in paints or sealants. Short chain CPs (SCCPs, C_{10-13}) have received growing global attention in recent years for their long-range transport, persistence in the environment, bioaccumulation and potential toxicity to aquatic organisms. Their presence in raw municipal wastewater is little studied but average concentrations (sum of C_{10} to C_{13} chain lengths) around 6 µg l^{-1} were reported in Beijing, China, 97% of them being associated with suspended particles (around 200 ng l^{-1} in the dissolved phase). SCCPs seem to be well removed (total removal > 99%) in WWTPs, 70-80% by sorption and elimination with the sludge and 20-30% probably by biotransformation/degradation. The dissolved concentration was also removed at around 80% during the treatment. Final effluent concentrations of SCCPs (sum of C_{10} to C_{13}) were around 60 ng l^{-1}, 60% in the dissolved phase (Zeng et al., 2013), which is lower the PNEC (500 ng l^{-1}, Table 2.2). In Europe, lower CPs concentrations were found in raw wastewater, on average (15 WWTPs) 140 ng l^{-1} for the sum of C_{10-13}, 841 ng l^{-1} for C_{14-16} and 650 ng l^{-1} for C_{17-28}, almost completely associated with suspended solids. CPs were not detected in the effluent (Coelhan, 2010).

2.3.4.6 Perfluorinated compounds

Perfluorinated compounds (PFCs) are a large family of synthetic chemicals used in many types of household products that utilise their properties of creating *water-repellent, grease-repellent and dirt-repellent surfaces*. They are for instance used in non-stick cookware (polytetrafluoroethylene (PTFE) known as Teflon®), water-proofing sprays, Gore-Tex® clothing, stain- or water-resistant textiles (clothes, carpets, tablecloths, upholstered furniture, etc), some cosmetics (nail polish, eye make-up), floor polish and waxes, window cleaners, degreasers, or paper packages for oily foodstuffs (pizza and pop-corn boxes) (KemI, 2006). PFCs are a complex group of organic compounds characterised by a carbon chain in which all hydrogen atoms have been replaced by fluorine atoms. This characteristic makes PFCs very persistent in the environment and non-degradable. PFCs can be classified into three families: perfluoroalkyl sulphonates (PFAS), perfluorocarboxylic acids (PFCA) and fluorotelomers. The PFAS perfluorooctane sulfonic acid (PFOS) was classified as a persistent organic pollutant in the Stockholm convention and as a priority hazardous substance in the EU due to its very high persistence in the environment, its bioaccumulation potential and its toxicity. Its use is now restricted in many countries and its production has decreased drastically in recent years. The PFCA perfluorooctanoic acid (PFOA) has also recently received more attention due to its toxic and eco-toxic properties and its

high persistence (Post et al., 2012). PFOA and PFOS are among the most abundant PFCs observed in raw municipal wastewaters, with average concentrations around 5-50 ng l^{-1}. The sum of the concentrations of the most common PFCs is usually reported in the range 30-150 ng l^{-1}. PFCs are usually not significantly removed (< 5%) in WWTPs (despite variable removal efficiencies). Concentrations in WWTP effluents are thus relatively similar those in the influents (Ahrens et al., 2009; Arvaniti et al., 2012; Bossi et al., 2008; Guo et al., 2010). Median PFOA and PFOS concentrations in effluents from 90 European WWTPs were for instance at 12-13 ng l^{-1}, and slightly lower for other PFCs (Loos et al., 2013). Despite these very low effluent concentrations, PFOS is still present at a level 20 times higher than its European EQS for surface waters (0.65 ng l^{-1}) (EC, 2013) and may persist for a very long time in the environment. PFCs are found at low concentrations in sewage sludge (total 10 to 50 µg kg^{-1} dm) (Arvaniti et al., 2012; Guo et al., 2010).

2.3.4.7 Others synthetic chemicals

Several other families of synthetic chemicals are found in municipal wastewater, but they are still few studied and probably less specific to domestic wastewater, as their main sources in the environment are expected to come from industries, contaminated sites or diffuses sources (traffic, atmospheric depositions, etc). We can however mention methyl-tert-butyl ether (MTBE), a *gasoline additive* known to contaminate groundwater, found at 100-400 ng l^{-1} in raw domestic wastewater and not well removed (30-35%, possibly by volatilization) in WWTPs (Achten et al., 2002); or naphthalene sulfonates, a family of *industrial dispersants*, found on average in raw municipal wastewaters at 200-1100 ng l^{-1}, with good WWTP removal efficiencies (> 90%) for the mono-sulfonates (NSAs, < 100 ng l^{-1} in effluents), but low removal (0-35%) for the di-sulfonates (NDSAs, 200-400 ng l^{-1} in effluents) (Reemtsma et al., 2006; Weiss and Reemtsma, 2008).

2.3.5 Biocides, pesticides and persistent organic pollutants (POPs)

Biocides and pesticides are designed to destroy or control the growth of targeted organisms, such as plants (herbicides), algae (algaecides), insects (insecticides) or fungi (fungicides). More than 500 biocide- and pesticide-active ingredients are approved for use in Europe[3]. 95% of the total use of biocide in Switzerland is however dominated by only 30 active compounds (Bürgi et al., 2009), and over 60% of the total European pesticide (fungicide, herbicide and insecticide) consumption (by weight) by only 20 active substances (Eurostat, 2007). The term pesticide is commonly used for chemicals applied to protect plants (mainly for agricultural use), whereas the term biocide is usually applied for all other purposes (mainly urban use). Biocides are for instance applied in bituminous roof sealing membranes to avoid root penetration, in external facade paintings to avoid algae and moss development, in grass or plant-management (golf, parks, cemeteries), or weed control (roadways, railroads). During rain events, biocides and pesticides can leach from buildings, parks and gardens, and are partly drained to the combined sewer network (Burkhardt et al., 2012; Coutu et al., 2012a). Pesticides in surface waters were often considered to be of agricultural origin, but new studies showed that urban contributions to the river pesticide loads can be in the same range as from the agriculture in

[3] Pesticides (active substances) approved in Europe (last accessed 14.03.2014):
http://ec.europa.eu/sanco_pesticides/public/?event=activesubstance.selection
Biocides (active substances) approved in Europe (last accessed 14.03.2014):
http://ec.europa.eu/environment/chemicals/biocides/active-substances/approved-substances_en.htm

mixed land use watersheds (urban and agricultural use such as the Swiss Plateau) (Wittmer et al., 2011).

Concentrations of pesticides/biocides in municipal wastewater are highly variable as their inputs are influenced by rain events (higher load during rain) and the season (application periods). Constant inputs of several compounds are also observed during dry weather, suggesting household uses (Wittmer et al., 2011). Apart from concentration peaks reaching several µg l^{-1} during special events (e.g., disposal activities), average pesticide/biocide concentrations in raw wastewater are usually lower than 1 µg l^{-1}, and for most compounds lower than 100 ng l^{-1} (Campo et al., 2013; Köck-Schulmeyer et al., 2013; Singer et al., 2010). Removal of pesticides/biocides in WWTPs is highly variable, but on average poor efficiencies (< 50%) are reported (Campo et al., 2013; Köck-Schulmeyer et al., 2013). Table 2.2 presents the average removal efficiencies of some frequently detected pesticides/biocides in municipal wastewaters (compounds mainly used for material protection, parks and gardens, but also in agriculture). The fate of these compounds during the treatment is not clear, some being possibly partially degraded and some partially sorbed to the sludge. Due to their low to moderate hydrophobicity (log K_{OW} from -3.2 to 3.6) (Wittmer et al., 2011), they are on average found at low concentrations in the sludge (< 1 to 44 µg kg^{-1} dm) (Campo et al., 2013). Removal by sorption is therefore expected to be lower than 15%. Their concentrations in WWTP effluents are relatively similar to those in influents, ranging on average for most compounds between 5 and 300 ng l^{-1} (Table 2.2). A few exceptions were observed for glyphosate, a widely used herbicide (active substance of the Roundup®), its degradation product AMPA, and the herbicide mecoprop (average concentrations often observed above 500 ng l^{-1}). Despite their low concentrations, some pesticides such as diazinon, diuron and irgarol are still at concentrations higher than their proposed EQS values for surface waters in Switzerland (Table 2.2), leading to potential risks for sensitive aquatic organisms in the case of low dilution of the effluents.

In mixed land use watersheds, contribution of WWTP effluents to the total load of pesticides in surface water can be significant, varying from 8-30% for terbuthylazine, isoproturon and atrazine (mainly used in agriculture), up to 40-70% for mecoprop, diazinon, diuron, carbendazim and terbutryn (mainly used in urban environment) (Gerecke et al., 2002; Wittmer et al., 2011).

Several pesticides from the "old" generation (more hydrophobic, with log K_{OW} from 3.6 to 6.2) were classified as *persistent organic pollutants (POPs)* in the Stockholm convention due to their persistence in the environment, their accumulation in living organisms and their toxicity to human and wildlife. This includes aldrin, chlordane, chlordecone, dieldrin, endrin, heptachlor, hexachlorobenzene (HCB), alpha and beta hexachlorocyclohexane (HCH), lindane, mirex, pentachlorobenzene, endosulfan, toxaphene, and dichlorodiphenyltrichloroethane (DDT)[4]. The use of these pesticides is now banned or strongly limited in many countries since the convention entered into force on May 2004. Concentrations of these pesticides in raw municipal wastewater in 2001-2003 in Greece were on average between 10 to 50 ng l^{-1}, mostly (50-80%) associated with particles (Katsoyiannis and Samara, 2005). All these compounds were well removed (50 to 80%) with the suspended solids during the

[4] Listing of POPs in the Stockholm convention (last accessed 14.03.2014): http://chm.pops.int/TheConvention/ThePOPs/ListingofPOPs/tabid/2509/Default.aspx

primary treatment and further removed (probably by sorption) during the biological step. The overall removal varied from 75 to 91%, resulting in concentrations between 1 and 14 ng l^{-1} in the effluent (Katsoyiannis and Samara, 2004), which is lower than the European EQS for inland waters for these substances (5-25 ng l^{-1}), except for the sum of aldrin, dieldrin and endrin, which is close to their EQS, and for heptachlor which exceeds more than 50,000 times its EQS (0.0002 ng l^{-1}) (EC, 2013). Concentrations of these persistent pesticides in sludge were found at 5 to 40 µg kg^{-1} dm (Katsoyiannis and Samara, 2005).

Other non-pesticide POPs, such as the toxic *polychlorinated biphenyls (PCBs)*, used over many years as heat exchange fluids in electric transformers or as additives in paint and oil, are still found in municipal wastewaters despite their ban in many countries since 1970-1990 (Balasubramani et al., 2014). A total of 209 PCB congeners exist, where 1 to 10 chlorine atoms are attached in different configurations to the two benzene rings. Concentrations of PCBs in raw municipal wastewaters are reported in the low ng l^{-1} range, with the sum of the 209 congeners estimated on average around 50 to 100 ng l^{-1} (Balasubramani et al., 2014; Blanchard et al., 2004). An average removal of 75% of the sum of PCBs in conventional WWTPs was typically reported (Blanchard et al., 2004; Katsoyiannis and Samara, 2004). Due to their hydrophobicity and low biodegradability, PCBs are mostly removed by sorption, at similar level during primary and secondary treatments. They tend then to accumulate in the sludge where they are found (sum of PCBs) on average around 500 µg kg^{-1} dm (Blanchard et al., 2004; Katsoyiannis and Samara, 2004). PCBs in WWTP effluents are usually found at concentrations lower than 1 ng l^{-1} for individual congeners, with the sum of the 209 congeners at around 20 ng l^{-1}, which is higher than their PNEC (1 ng l^{-1}, Table 2.2) (Balasubramani et al., 2014; Hope et al., 2012). PCBs released in surface waters can accumulate in fish, rendering them unfit for human consumption (Bodin et al., 2014). Sources of PCBs to the aquatic environment are however diverse and the highest inputs are often coming from contaminated sites, stormwaters and combined sewer overflows (CSOs) (due to atmospheric deposition) (Rossi et al., 2004). Inputs from WWTP effluents are usually low but may contaminate sediments in the vicinity of the plant (Poté et al., 2008). WWTP outfalls were for instance contributing to 5% of the total load of PCBs to the Hudson River estuary (New York), whereas 41% were coming from the Hudson River (contaminated by former industrial activities) and 30% from stormwater and CSOs (Rodenburg et al., 2011).

2.3.6 Heavy metals

Heavy metals are elements that are not biodegradable, tend to accumulate in living organisms and are known to be toxic if present in excessive levels. Some of these elements, such as zinc or copper, are essential for life in trace concentrations but accumulation in the organism can lead to serious diseases (Fu and Wang, 2011). The term "heavy metal" refers generally to (post-)transition metals with a density greater than 5 kg l^{-1} (Fu and Wang, 2011), but some other metals or metalloids are also sometime included in this category, such as aluminium or arsenic. Toxic heavy metals of particular concern in wastewater include zinc (Zn), copper (Cu), nickel (Ni), mercury (Hg), cadmium (Cd), lead (Pb) and chromium (Cr) (Fu and Wang, 2011).

Historically, heavy metals in wastewater have been strongly associated with industrial emissions. In recent years, probably due to more stringent regulations and displacement of industrial activities out of cities, industries are no longer considered to be the main source of heavy metals in municipal wastewater (Ziolko et al., 2011). Household sewage is reported to be an important source of heavy metals such as Cu (corrosion of pipes and taps, food), Zn (leaching from galvanized material, food) and Hg (amalgam), and contribute also to the load of Pb (leaching from old lead plumbing), Cr and Ni (stainless steel products) or Cd (artist paint pigments). Stormwater runoff can also be a significant source of heavy metals, especially from building materials, such as Zn (from galvanized metal) or Cu (roofs, catenaries of trains/trolleybuses), but also from traffic (Zn from tires, Cu and Pb from brake linings or asphalt) or agricultural runoff (Cu used as a fungicide). Business and industry sewage can contribute significantly to the total load of heavy metals. For instance, dentists can be a source of Hg; car washes a source of Cu, Zn, Pb, Cr or Cd; and art school a source of Cd (paint pigments in colours yellow-red) (Chèvre et al., 2011; Sörme and Lagerkvist, 2002).

Concentrations of heavy metals in municipal wastewater are highly variable as metals come from very diverse sources. In raw wastewater, metal concentrations are in the range of µg l^{-1} to mg l^{-1}, except for Cd and Hg (ng l^{-1} to µg l^{-1}), with abundances usually observed as follows (median total/dissolved concentrations in µg l^{-1} in 16 WWTPs in the UK) (Gardner et al., 2013; Rule et al., 2006): Al (1470 / 40) > Fe (1097 / 215) > Zn (160 / 37) > Cu (65 / 17) > Cr (12 / -) = Pb (12 / 2) = Ni (11 / 6.3) > Cd (0.45 / 0.1) > Hg (0.053 / 0.014). High concentrations of Al and Fe may be due to addition of these chemicals as coagulant to treat water or as constituents of several natural clay minerals. Heavy metals are mostly associated with suspended solids (> 75% of the total concentration), except for Ni (> 50% in the dissolved phase). Therefore, removal of heavy metals in WWTPs is strongly associated with the removal of suspended solids (TSS), as illustrated in Fig. 2.7. As presented in Table 2.2, high metal removal efficiency (> 75%) can be achieved in most WWTPs due to important removal of TSS (> 90%, around 10 mg l^{-1} in effluent), except for Ni, with median removal around 30%. Poor nickel removal (or even enrichment in the process) may also be caused, to some extent, by impurities (about 40 ppm of Ni, Cu and Cr) in the chemicals (e.g., iron chloride) added during the treatment for phosphate removal (Buzier et al., 2006). Despite good elimination of metals associated with particles, conventional treatments have, however, little effect (usually less than 60%) on the dissolved concentration. Thus, in WWTP effluents, most of the metals (except for Al and Fe) are found predominantly (50-90%) in the dissolved phase. Concentrations in effluents are usually in the low µg l^{-1} range, Zn being the most abundant toxic heavy metal, followed by Cu and Ni (Table 2.2). In some effluents, these concentrations can slightly exceed the proposed environmental quality standards (EQS) for surface waters, especially for Zn, Cu and Ni (Table 2.2), leading to a risk for aquatic organisms in the case of low dilution in the receiving waters. High sediment contaminations with heavy metals in the proximity of WWTP outfalls in lakes were reported, representing a significant source of toxicity for benthic organisms (Poté et al., 2008).

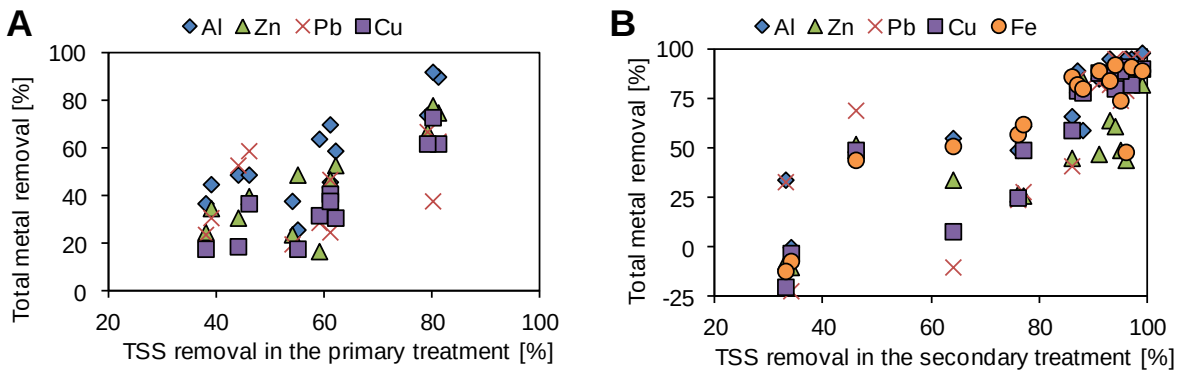

Fig. 2.7 Removal of heavy metals (total concentration) as a function of the removal of suspended solids in (A) primary treatments and (B) secondary treatments. Results of an extensive study made on 16 WWTPs in the UK during 2010/2011. Data adapted from Gardner et al. (2013).

The "dissolved" fraction of heavy metals is composed of "free" ions but also of metals bound to dissolved organic or inorganic ligands or to colloids (Buzier et al., 2006; Worms et al., 2010). Due to the relatively high concentrations of dissolved organic matter (DOC) in wastewater, and thus its large complexation capacity, free metal ions are expected to be rare and dissolved metals are more likely to occur as organometallic complexes (Ziolko et al., 2011). Partition of metals between particles and the dissolved phases is thus influenced by the presence of DOC: complexation of metals by dissolved ligands can lower their affinity for particle surfaces and increase their solubility, leading to a decrease of the coefficient of partition K_d and therefore their removal with the sludge (Katsoyiannis and Samara, 2007). Synthetic chelating agents such as EDTA or NTA, present in significant concentrations in WWTP effluents (30-500 µg l^{-1}) (Gardner et al., 2012), may also increase the solubility of metals, possibly remobilizing the fraction sorbed onto the sludge, and thus increasing their rejection into the environment (Alder et al., 1990).

As metals are not biodegradable, removal of dissolved heavy metals in secondary treatments depends on their affinity for the settleable organic matter, such as bacterial cell walls and extracellular polymers, or on the active cellular uptake by microorganisms (Ziolko et al., 2011). Copper has, for instance, a strong affinity for organic matter, leading to a significant removal of its dissolved concentration in the biological treatment, which is not the case for dissolved zinc (Gardner et al., 2013).

Volatilization during the biological treatment is not expected to be a significant removal mechanism for most metals (Ziolko et al., 2011). For mercury, which is a volatile metal (K_{AW} around 0.3 [-] for elemental Hg), volatilization is reported to be only a minor (but not negligible) component of the total flux (2-10%), due to its high sorption affinity for the sludge (Gbondo-Tugbawa et al., 2010; Yoshida et al., 2013).

Most of the metals entering WWTPs will end up in the excess sludge. Concentrations in primary and secondary sludge are more or less similar (Gianico et al., 2013), despite slightly higher metal loads are removed with primary sludge (Yoshida et al., 2013). Zinc is reported to be the predominant toxic heavy metal in fresh sludge (median concentration around 400 mg kg^{-1} dm), followed by Cu (160 mg kg^{-1} dm), Pb, Ni and Cr (20-40 mg kg^{-1} dm), and finally Cd and Hg (0.6 mg kg^{-1} dm) (Gardner et al.,

2013; Gianico et al., 2013). Concentrations in digested sludge are reported to be higher due to weight loss of fresh sludge during anaerobic digestion (degradation of organic matter) (Karvelas et al., 2003). In the case of sludge incineration with flue gas treatment, heavy metals will finally end in the ash (less than 0.5% in the flue gas) (Yoshida et al., 2013) and then in control landfills. In the case of agricultural soil amendment, heavy metals will mostly accumulate in the soil, slowly mobilized by rainfalls to surface or ground waters (Kwon et al., 2014).

2.3.7 Polycyclic aromatic hydrocarbons (PAHs)

Polycyclic aromatic hydrocarbons are a family of over one hundred organic compounds comprised of two or more fused benzene rings without any heteroatoms. They come primarily from incomplete combustion or pyrolysis of organic material such as oil, petroleum, coal and wood, both from natural and anthropogenic sources, the latter being the most dominant (Rubio-Clemente et al., 2014). Most PAHs do not have commercial uses, apart for naphthalene used in products such as lubricants, bathroom products, deodorant discs, wood preservatives, fungicides, or concrete plasticizers (Fatone et al., 2011). They are usually released into the atmosphere via gaseous emissions and are then subject to wet and dry deposition. They are widely spread throughout the environment, causing water, soil, and air pollution. Some of them are highly carcinogenic, mutagenic and teratogenic, and relatively persistent. Eight PAHs have been identified as priority pollutants in water in Europe (EC, 2013). Due to their hydrophobicity, low water solubility, and variable volatility (which decreases with the number of condensed benzene rings), they have tendency to bind to particles and accumulate in organisms. PAHs are not specific domestic wastewater pollutants but, due to their wide presence in urban environment, are especially adsorbed onto particles on roads. They can thus reach municipal sewers during rain events (urban runoff). PAHs concentrations in raw wastewater are however relatively low, with median concentrations in UK and Italian wastewater around 20 ng l^{-1} for heavy PAHs such as benzo(a)pyrene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(g,h,i)perylene and indeno(1,2,3.cd)pyrene, and slightly higher for lighter PAHs such as anthracene (50 ng l^{-1}), fluoranthene (110 ng l^{-1}) and naphthalene (1100 ng l^{-1}) (Fatone et al., 2011; Gardner et al., 2013). The sum of the concentrations of the 16 PAHs (recommended by the US-EPA) in Italian wastewater was between 0.2 and 1.5 µg l^{-1} (Fatone et al., 2011).

PAHs are typically associated with particle matter present in the raw wastewater, with, for most of them (with log K_{OW} of 4.5 to 7), less than 20% in the dissolved phase. Only naphthalene, and to a smaller extent acenaphthene and fluorene are found in higher percentage in the dissolved phase (25-100%), due to their lower hydrophobicity (log K_{OW} < 4.2) (Fatone et al., 2011).

PAH removal during the primary treatment is thus significant (30-60%) and associated with the removal of suspended solids (Fatone et al., 2011). In the biological treatment, the remaining PAHs are mainly removed by adsorption onto the sludge due to their strong affinity for activated sludge particles. Biodegradation and volatilization seem not to be important removal mechanisms (< 2%), except for naphthalene which is expected to be partially removed by stripping during the aeration (up to 5-20%) due to its relative volatility (K_{AW} of $2 \cdot 10^{-2}$ [-] compared to < $2 \cdot 10^{-3}$ [-] for heavier PAHs) (Fatone et al., 2011; Liu et al., 2011a; Manoli and Samara, 2008; Sander, 1999). Overall PAH removal in different WWTPs is highly variable, but the median removal varies between 60% to more than

90%, depending on the compound (Table 2.2). High molecular weight PAHs, which are also the most toxic, are usually over 80% removed (Fatone et al., 2011; Gardner et al., 2013). The sum of the 16 US-EPA PAHs is reported to be reduced over 70% in most WWTPs (Fatone et al., 2011; Liu et al., 2011a).

Effluent concentrations of UK and Italian WWTPs were around 1-3 ng l^{-1} for heavy PAHs (benzo(a)pyrene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(g,h,i)perylene and indeno(1,2,3.cd)pyrene) and anthracene, and between 10 to 50 ng l^{-1} for lighter compounds (naphthalene, acenaphthene, fluorene, phenanthrene, fluoranthene, pyrene). The sum of the 16 PAHs concentrations is reported to be lower than 200 ng l^{-1} (Fatone et al., 2011; Gardner et al., 2012). In the final effluent, PAHs are mostly (> 50%) found in the dissolved phase (Fatone et al., 2011).

Despite their very low concentrations in the effluents, many PAHs are still above their European EQS (annual average) for surface waters, which range from 0.17 ng l^{-1} for benzo(a)pyrene to 6.3 ng l^{-1} for fluoranthene (Table 2.2) (EC, 2013). Thus, PAHs may pose a risk to the environment in the case of low dilution (< 10 times) of the effluent. Sediment contaminations with PAHs in the proximity of WWTP outfalls in lakes were also reported (Poté et al., 2008).

As adsorption is the main removal mechanisms, PAHs tends to accumulate in the sludge. Concentrations of individual PAHs in fresh sludge are found in the range 0.1 to 0.5 mg kg^{-1} dm (median UK concentration) (Gardner et al., 2013), with the sum of the 16 PAHs around 1.2-1.8 mg kg^{-1} dm (Fatone et al., 2011). Stabilization of the sludge by anaerobic digestion and composting was reported to be ineffective to reduce the PAHs sludge concentrations (Villar et al., 2006). Application of sludge as fertilizer at doses > 150 Mg ha^{-1} can lead to accumulation of PAHs in soil, due to the low degradation rate of PAHs in soil (50% removal in 4 years) (Oleszczuk, 2006). PAHs are degraded during incineration of the sludge and they are found only in very low concentrations (sum 16 PAHs < 0.2 mg kg^{-1} dm) in ashes. However, light PAHs can be formed during the incomplete combustion of the sludge, leading to significant emission of PAHs in the flue gas (0.02-50% of the mass of PAHs contained in the sludge), despite the presence of air control devices (flue gas treatment) (Park et al., 2009).

2.3.8 Volatile organic compounds (VOCs)

Volatile organic compounds have a high vapour pressure at ambient temperature, resulting to a low boiling point (usually between less than 50°C to 250°C at 1 atm). They are extensively used in fuels, paints, aerosols, cosmetics, solvents, disinfectants and pesticides, and are often present in significant concentrations in municipal wastewater. Aromatic VOCs, such as benzene, toluene, ethylbenzene, xylene and styrene (BTEXS group), are especially of concern due to their toxicity. In a survey made in Italy (Fatone et al., 2011), toluene was the most abundant aromatic VOC in raw municipal wastewater, with 3 to 5 µg l^{-1}. Xylene, styrene and ethylbenzene were found at slightly lower concentrations (100-300 ng l^{-1}) and benzene usually below 60 ng l^{-1}. Other aromatic VOCs were present in raw wastewater, such as 4-chlorotoluene, a drain pipe solvent (up to 300 ng l^{-1}), or 1,2,4-trimethylbenzene and 1,4-dichlorobenzene (up to 1000 ng l^{-1}). These aromatic VOCs were mainly present in the dissolved fraction due to their low affinity for suspended solids. The removal of these aromatic VOCs was high

(usually over 70%) during conventional treatment, with around 50% of the removal observed during pre-treatments and primary sedimentation, and 50% during the biological treatment. Only toluene was still found in significant concentrations (0.5 – 2.7 µg l^{-1}) in the effluent. O-xylene was the most persistent aromatic VOC (0-70% removal) due to its lower volatility (Fatone et al., 2011). Volatilization (surface volatilization in primary treatment and stripping during aeration) is likely to be the major removal mechanism for aromatic VOCs (Byrns, 2001), but biodegradation can also play an important role (Mozo et al., 2012; Yang et al., 2014). Volatilization is influence by the wastewater temperature and is reported to be higher in summer (Oskouie et al., 2008). Chlorinated VOCs were also found in municipal WWTP effluents at concentrations in the ng l^{-1} - µg l^{-1} range: chloroform and dichloroacetonitrile (200-800 ng l^{-1}), bromodichloromethane, dibromochloromethane, dichloromethane (DCM), trichloromethane (TCM), tetrachloroethylene (PCE) and trichloroethylene (TCE) (20-350 ng l^{-1}) (Antoniou et al., 2006; Martin Ruel et al., 2011). Chlorinated VOCs (except chloroform, PCE and TCE which are very volatile) are reported to be less volatile than aromatic VOCs and thus less removed from the water phase during the treatment (Yang et al., 2014). Since the off-gas is usually not treated in municipal WWTPs, transfer of VOCs to the atmosphere can contribute to air pollution around WWTPs (Yang et al., 2014). The concentrations found in the effluent are not expected to cause aquatic toxicity (values for DCM, TCM, PCE, TCE and benzene lower than the European EQS of 2.5 to 20 µg l^{-1}) (EC, 2013).

2.3.9 Synthesis

The average removal efficiencies in conventional WWTPs and the average effluent concentrations of 168 micropollutants presented in this chapter are synthesised in Table 2.2 and in Fig. 2.8. The sum of the concentrations of these 168 micropollutants in the effluents is on average around 0.75 mg l^{-1}, with 0.46 mg l^{-1} only for organic pollutants. Half of the load of organic pollutants is dominated by surfactants, one third by a few household chemicals (2 sweeteners, 2 corrosion inhibitors and 2 chelating agents), and 13% by pharmaceuticals. The highest effluent concentrations (> 10 µg l^{-1}) were observed for several heavy metals (Al, Fe, Zn), surfactants (soap, LAS), some household chemicals (chelating agents EDTA and NTA, sweeteners acesulfame and sucralose), and some pharmaceuticals (iomeprol, iohexol, metformin).

Hydrophobic pollutants (heavy metals, PAHs, POPs, several household chemicals like brominated flame retardants, several personal care products), and easily biodegradable pollutants (surfactants, plastic additives, hormones, several PCPs, some pharmaceuticals and household chemicals) are in general largely removed (> 70%) during treatment. Their effluent concentrations can, however, still be higher than their respective EQS or PNEC values for surface waters. Despite their possible degradation in the environment, risks for sensitive aquatic organisms cannot be excluded in the vicinity of WWTP outfalls due to the constant input of these chemicals (pseudo-persistence).

More hydrophilic (polar) and hardly biodegradable pollutants, e.g., most pharmaceuticals and pesticides/biocides as well as several household chemicals (sweeteners, EDTA, corrosion inhibitors, some phosphorus flame retardants and PFCs), are only poorly removed (< 50%) during treatment. These compounds thus present a greater risk of contamination of receiving waters and persistence within them.

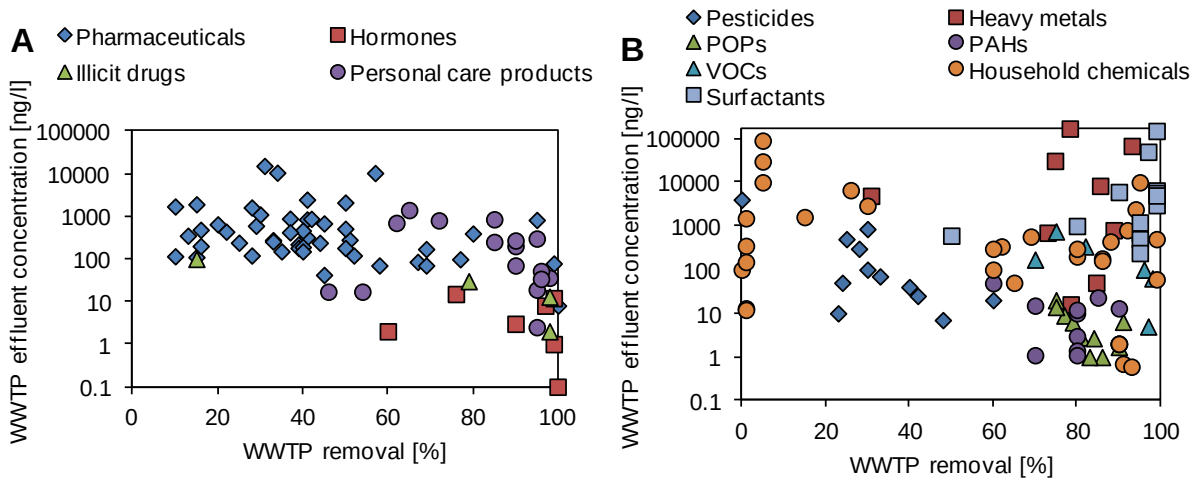

Fig. 2.8 Synthesis of average WWTP removal efficiencies and effluent concentrations of (A) 48 pharmaceuticals (Swiss data), 16 personal care products (5 fragrances, 2 preservatives, 3 antimicrobial agents, 1 insect repellent, 5 UV filters), 7 hormones and 4 illicit drugs; and (B) 12 pesticides/biocides, 9 heavy metals, 10 persistent organic pollutants (POPs, mainly hydrophobic pesticides and PCBs), 12 polycyclic aromatic hydrocarbons (PAHs), 6 volatile aromatic organic compounds (VOCs), 32 household chemicals (4 sweeteners, 6 plastic additives, 6 corrosion inhibitors, 2 chelating agents, 12 flame retardants and 2 perfluorinated compounds) and 12 surfactants. Average values from European and American WWTPs, with primary and secondary treatments (equivalent to activated sludge with partial nitrification). Sources of the data are given in Table 2.2.

The impact of these micropollutants on aquatic organisms in receiving waters is difficult to evaluate due to the diversity of pollutant and modes of action (mixture effect). The risk of a specific compound (without considering the mixture effect) depends on (i) its aquatic toxicity, usually assessed by quality criteria such as the PNECs or EQSs, and (ii) its concentration in the surface water, which depends on the effluent concentration, the dilution factor and the stability (persistence) of the compound in the environment.

Concentrations of several micropollutants (55 out of 168) in WWTP effluents are higher than their respective proposed EQS or PNEC for surface waters (Table 2.2). This is for instance the case for most surfactants (8), several pharmaceuticals (13) and hormones (3), some PCPs (4), PBDEs (2), PFOS, EDTA, plastic additives (3), some pesticides/biocides (6), POPs (4), several heavy metals (5) and PAHs (5). Individual risks can thus not be excluded for these compounds in the case of low dilution of the effluents in the receiving waters.

In streams, dilution factors are estimated from the ratio of the flow rate of the receiving water to that of the effluent, and can vary from less than 2 (meaning that more than 50% of the total flow in the stream comes from the effluent) to more than 10^6. The annual median dilution factor is usually between 10 to 100 in Europe (except northern countries where it is over 500), but it can vary by more than two orders of magnitude in the same area depending on the situation of the WWTP or the season (Keller et al., 2013). Higher surface water concentrations are expected in area with high population density and/or low river flows. For WWTPs discharging their effluent into lakes or the sea, a gradient of concentration is expected, with high concentrations close to the outfall, and then decreasing with distance until a residual concentration corresponding to the total dilution of the effluent in the whole water volume (Bonvin et al., 2013b).

The stability of micropollutants in the environment is expected to be higher for substances poorly removed in WWTPs, as removal mechanisms in the natural environment (biodegradation and trapping in sediment) are relatively similar to those (at different scales and different rates) in conventional WWTPs (expect for photolysis, which is higher in natural waters). A compound poorly removed in WWTPs is to be considered more problematic than the same effluent concentrations of a compound degraded extensively in the plant (from a much higher influent concentration) (Reemtsma et al., 2006). Persistence in WWTPs is therefore also a key factor to determine the risk of micropollutants.

Prioritization of micropollutants released from WWTPs should therefore be assessed based on their load in the receiving media (contamination of drinking resources), their potential ecotoxicological impacts (ratio concentration/PNEC) and their persistence in the environment. Based on these three parameters, a basic prioritization is proposed in Table 2.2. All classes of micropollutants studied (except VOCs) present an issue regarding their load, their ecotoxicological impacts or their potential persistency in the environment.

2.4 Enhanced treatment of micropollutants in WWTPs

Two main options exist to enhance the removal of micropollutants from wastewater: (i) improvement and optimization of the existing technologies and (ii) addition of complementary advanced treatments.

2.4.1 Optimization of conventional treatments

As described in chapter 2.3, several micropollutants are at best only partially removed in conventional WWTPs. The presence of pollutants in WWTP effluents can be due to high persistence of the molecule (hydrophilic and not biodegradable compounds), but also to too short contact times (HRTs) with microorganisms to lead to complete biodegradation, not enough diverse bacterial communities to metabolise/cometabolise the substance, or poor separation of suspended solids (sorbed fraction). Optimization of conventional treatments to achieve good removal of total suspended solids (TSS), biochemical oxygen demand (BOD), chemical oxygen demand (COD), dissolved and total organic carbon (DOC, TOC) and ammonium (NH_4^+) may thus improve the removal of less persistent micropollutants that are poorly removed due to the reasons mentioned above.

2.4.1.1 Improvement of hydrophobic pollutant removal

Removal of several micropollutants such as heavy metals, PAHs, PCBs, hydrophobic pesticides, flame retardants (PBDEs), phthalates (DEHP), UV filters, triclosan, or polycyclic musks, is strongly linked to the removal of suspended solids. Therefore, achieving low concentrations of TSS in the effluent, for instance by sand filtration or advanced decantation, will significantly reduce concentrations of micropollutants associated with solids. The removal of the dissolved fraction is challenging but, as a large fraction of dissolved metals and hydrophobic pollutants is associated with dissolved organic matter, it is expected that improving the removal of DOC (and thus the complexation capacity of the water phase), for instance with an effective biological treatment, will also improve the removal of dissolved hydrophobic pollutants (by decreasing their solubility) (Katsoyiannis and Samara, 2007; Ziolko et al., 2011). It was found, for instance, that effluent concentrations of PAHs and PBDEs were

correlated with effluent DOC concentrations, suggesting that these substances might be associated with DOC rather than with suspended solids (Gardner et al., 2012).

2.4.1.2 Improvement of biodegradable pollutant removal

Removal efficiencies of (moderately) biodegradable micropollutants are reported to vary from one WWTP to another. This suggests that their removal may be improved. Indeed, correlations between the removal of BOD, COD and ammonia, and the removal of several pollutants were reported. Higher BOD degradation was linked to better removal of the relatively easily-biodegradable compounds ibuprofen, estrogens (E2), salicylic acid and triclosan (Fig. 2.9 A). Good removal of ammonium (complete nitrification) was linked to better removal of bisphenol A and estrogens (E1) (Fig. 2.9 B), but also of many other compounds (e.g., atenolol, bezafibrate, norfloxacin, ofloxacin, metronidazole, methylbenzotriazole, simvastatin, gemfibrozil, naproxen, ketoprofen, mefenamic acid, or iomeprol) (Gardner et al., 2013; Margot et al., 2013b). Most of these compounds were removed < 40% in WWTPs without nitrification and > 70-80% in WWTPs with complete nitrification (cf. Chapter 3). It is not clear (suggestion proposed in Chapter 4) if these better removal efficiencies observed in plants with nitrification are due to longer HRT (more time for biodegradation), longer SRT (more microbial diversity in the sludge, including slow growing organisms), cometabolism by the enzyme ammonia monooxygenase (responsible for the nitrification), or the combination of the three. Each of these factors can improve the removal efficiencies of several (moderately biodegradable) micropollutants in conventional treatments. Thus, WWTPs that can achieve high removal of TSS, BOD, DOC and ammonium will certainly perform better in removing several (adsorbable or moderately biodegradable) micropollutants.

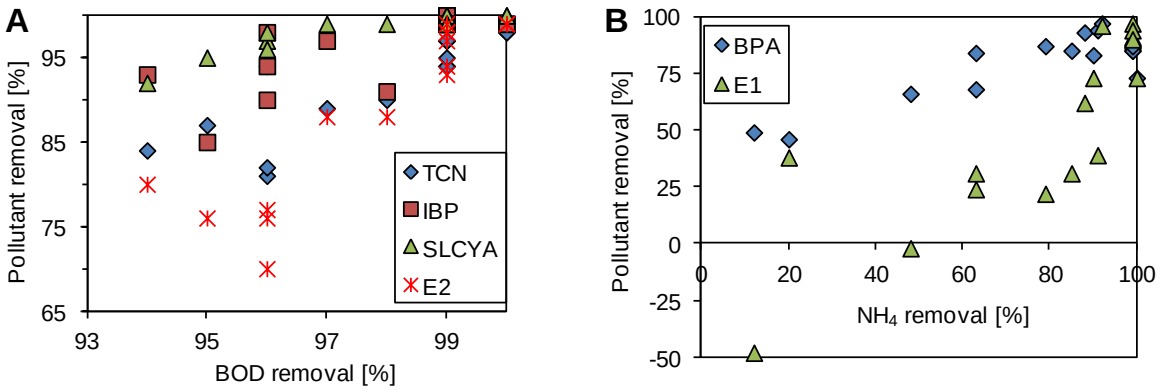

Fig. 2.9 Removal of (A) triclosan (TCN), ibuprofen (IBP), salicylic acid (SLCYA) and 17β-estradiol (E2) as a function of BOD removal in conventional WWTPs, and removal of (B) bisphenol A (BPA) and estrone (E1) as a function of ammonium removal in secondary biological treatments. Results of an extensive study made on 16 WWTPs in UK during one year in 2010/2011. Data adapted from Gardner et al. (2013).

2.4.2 Advanced treatments

Significant removal improvement of hardly biodegradable and hydrophilic (low sorption affinity) micropollutants such as pharmaceuticals, pesticides, phosphorus flame retardants, sweeteners or corrosion inhibitors, seems not to be feasible by optimization of existing conventional treatments. It appears that, for the removal of these substances, complementary treatments, called advanced treatments, are necessary. Up to now, mainly physico-chemical advanced processes are available,

removing pollutants from water either physically by adsorption or tight membranes filtration, or chemically (mainly by oxidation). Currently, two main technologies with a potential for large-scale application in terms of efficiency, cost and energy requirements have been identified: (i) oxidation of micropollutants with ozone and (ii) adsorption onto activated carbon (Abegglen and Siegrist, 2012).

2.4.2.1 Ozonation

Ozone (O_3) is a powerful and selective oxidant that attacks mainly electron-rich moieties of organic compounds such as phenols, amines or alkenes, or inorganic ions such as Fe^{2+}, Mn^{2+}, HS^-, NO_2^- or Br^-. Ozone is unstable in wastewater and decomposes rapidly (half life of a few minutes) due to its reaction with effluent organic matter (EfOM), leading to the formation of very reactive hydroxyl (OH) radicals. OH radicals are powerful and unspecific oxidants able to degrade almost any kind of organic compound. This indirect oxidation by OH radicals increases the global efficiency of ozonation, making this technology applicable to the removal of a wide range of micropollutants from WWTP effluents, with doses from 3 to 8 mg O_3 l^{-1} (Buffle et al., 2006; Hollender et al., 2009). Some pollutants with low ozone and OH radical reactivity are, however, not significantly removed at these doses (Table 2.3).

Table 2.3 Second-order rate constants with ozone and OH radical at pH 7 for several micropollutants (Huber et al., 2003; Real et al., 2009; Rosal et al., 2010; Zimmermann et al., 2011a). Estimation of their removal by ozonation, calculated for an example of a reactor with a dose of 4.9 mg O_3 l^{-1}, a R_{ct} of 10^{-7} [-] (typical value) and an ozone exposure of 1.52 [mg min l^{-1}] (or 1.9 mM s). f_{O3} and f_{OH} [-] are the fractions removed due to the reaction with ozone and OH radical respectively.

Substance	**k_{O3}** **[M^{-1} s^{-1}]**	**k_{OH}** **[M^{-1} s^{-1}]**	**Removal** **[%]**	***f_{O3}*** **[%]**	***f_{HO}*** **[%]**
Carbamazepine	3×10^5	8.8×10^9	**100%**	100%	0%
Diclofenac	6.8×10^5	7.5×10^9	**100%**	100%	0%
Naproxen	2×10^5	9.6×10^9	**100%**	100%	0%
Paracetamol	4.1×10^6	2.2×10^9	**100%**	100%	0%
Sulfamethoxazole	5.5×10^5	5.5×10^9	**100%**	100%	0%
Trimethoprim	2.7×10^5	6.9×10^9	**100%**	100%	0%
Metoprolol	2000	7.3×10^9	**99%**	73%	27%
Atenolol	1700	8×10^9	**99%**	68%	32%
Bezafibrate	590	7.4×10^9	**92%**	44%	56%
Benzotriazole	230	7.9×10^9	**86%**	23%	77%
5-Methyl-benzotriazole	400	5×10^9	**82%**	44%	56%
Ketoprofen	0.4	8.4×10^9	**80%**	0%	100%
Ibuprofen	9.6	7.4×10^9	**76%**	1%	99%
Primidone	1	6.7×10^9	**72%**	0%	100%
Fenofibric acid	3.43	6.55×10^9	**71%**	1%	99%
Metronidazole	350	1.98×10^9	**65%**	64%	36%
Diuron	16.5	4.6×10^9	**60%**	3%	97%
Iopromide	0.8	3.3×10^9	**47%**	0%	100%
Atrazine	6	2.8×10^9	**42%**	2%	98%
Diatrizoate	0.05	5.4×10^8	**10%**	0%	100%

The removal R [-] of a pollutant by ozonation depends on its reactivity with ozone and the hydroxyl radical, and the ozone exposure. The ozone exposure depends mainly on the ozone dose, the exposure time and the wastewater composition (its oxidative demand). Indeed, EfOM and nitrite react rapidly with ozone, reducing the quantity of ozone available for micropollutant oxidation. In a plug flow reactor with a known *HRT* [s], R can be calculated by Eq. 2.9, knowing the second order rates constants k_{O3} and k_{OH} [M^{-1} s^{-1}] of the pollutant, with ozone and the hydroxyl radical, respectively (Zimmermann et al., 2011a). k_{O3} and k_{OH} are determined in pure water and can often be found in literature.

$$R = 1 - \exp\left(-\left(k_{O3} + R_{ct}\, k_{OH}\right) \int_0^{HRT} [\mathrm{O_3}]\,\mathrm{d}t\right) \tag{2.9}$$

with $R_{ct} = \dfrac{\int_0^{HRT} [\mathrm{OH}]\,\mathrm{d}t}{\int_0^{HRT} [\mathrm{O_3}]\,\mathrm{d}t}$ [-],

the ratio of OH radical and ozone exposures, assumed constant for a specific wastewater. R_{ct} and the ozone exposure $\int_0^{HRT} [\mathrm{O_3}]\,\mathrm{d}t$ have to be determined experimentally (batch test or measured) in the water to be treated, as they are strongly influenced by the matrix composition. The main factors influencing micropollutant oxidation by ozone in wastewater are presented in Fig. 2.10.

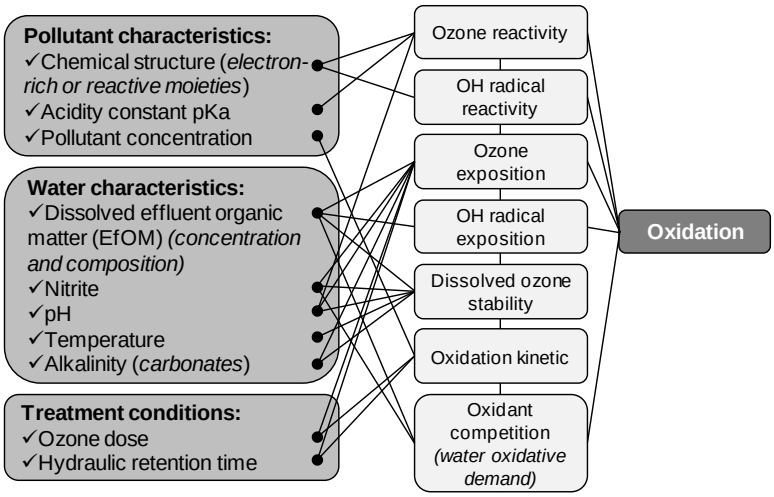

Fig. 2.10 Main factors influencing micropollutant oxidation by ozone in wastewater.

The fractions of the compound removed by direct reaction with ozone or by reaction with OH radical, f_{O3} and f_{OH} [-], respectively, can be calculated with Eqs. 2.10 (Huber, 2004):

$$f_{\mathrm{O3}} = \frac{k_{\mathrm{O3}}}{k_{\mathrm{O3}} + R_{ct} k_{\mathrm{OH}}}, \quad \text{and} \quad f_{OH} = \frac{R_{ct}\, k_{\mathrm{OH}}}{k_{\mathrm{O3}} + R_{ct} k_{\mathrm{OH}}} \tag{2.10}$$

Second-order rate constants with ozone and OH radicals are presented in Table 2.3 for several micropollutants. An example of their calculated removal in a specific reactor is also shown. Reactive substances such as carbamazepine, diclofenac or sulfamethoxazole should be in theory completely

removed due to direct reaction with ozone, whereas compounds not very reactive with ozone, such as ibuprofen, ketoprofen or iopromide, are predicted to be mostly removed by reaction with the OH radical.

Oxidation of organic pollutants could lead to their complete mineralization into CO_2, H_2O and other minerals but, with ozone doses typically used for wastewater treatment (3-8 mg O_3 l^{-1}), only partial pollutant oxidation occurs, producing many (mostly unknown) oxidation by-products. Some of these by-products are undesirable due to their toxicity (and carcinogenicity), such as nitrosamine N-nitrosodimethylamine (NDMA), formaldehyde or bromate (Hollender et al., 2009; Wert et al., 2007). Higher aquatic toxicity of ozonated wastewaters compared to non-ozonated effluents was reported in several studies, this toxicity decreasing in the case of filtration of the ozonated water on sand filters (degradation of the reactive and biodegradable by-products) (Petala et al., 2006; Petala et al., 2008; Stalter et al., 2010a; Stalter et al., 2010b). A final filtration step is thus recommended after ozonation. With proper design, ozonation decreases the toxicity of WWTP effluents, making this technology suitable for municipal wastewater application (cf. Chapter 3).

2.4.2.2 Activated carbon adsorption

Activated carbon is a charcoal produced by pyrolysis of organic materials (wood, coal, etc.) which is then activated chemically or thermally to develop its porosity (removal of the tar from the pores). Due to its very high specific surface area (500-1500 m^2/g), this material has a very strong sorption affinity and can therefore remove a wide range of water pollutants by adsorption. Activated carbon can be applied either in a granular form (GAC) in a compact filter, or added as a powder (PAC) to the water and then removed by either filtration or flocculation/decantation. Spent PAC or GAC are then incinerated (or regenerated for GAC) to destroy the adsorbed pollutants. A PAC dose between 10 to 20 mg l^{-1} proved to be sufficient to remove over 80% a wide range of micropollutants, although a few compounds with low PAC affinity (very hydrophilic and/or negatively charged) are not well removed at these doses (Boehler et al., 2012). Due to the physical removal (and not transformation) of many micropollutants, a clear decrease in aquatic toxicity of the effluents was observed after PAC treatment (cf. Chapter 3).

Removal of a pollutant by sorption onto PAC depends mainly on its affinity (hydrophobicity, charge) for the activated carbon, its concentration in the dissolved phase and the PAC concentration. As for sorption onto sludge, adsorption onto PAC is considered as a reversible process that reaches equilibrium. For many pollutants, equilibrium is almost (80-95%) reached in 1-2 days, meaning that the PAC retention time in the system should be as least as long to optimize the use of the PAC. Adsorption equilibrium follows in many case a Freundlich isotherm (Eq. 2.11) where the pollutant concentration sorbed onto PAC, C_s [µg g^{-1} PAC], depends on the dissolved concentration C_w [µg l^{-1}] at equilibrium and the Freundlich coefficients K_f [μg^{1-n} l^n g^{-1}] and n [-] (NEPTUNE, 2010).

$$C_S = K_f \, C_w^n \tag{2.11}$$

K_f and n have to be determined experimentally for each pollutant, for the specific PAC tested and in the wastewater that have to be treated. Indeed, the wastewater composition has a strong influence on the adsorption capacity, as the EfOM can compete with micropollutants for the adsoption sites, or can

block the pore accessibility. The main factors affecting micropollutant adsorption onto PAC in wastewater are presented in Fig. 2.11.

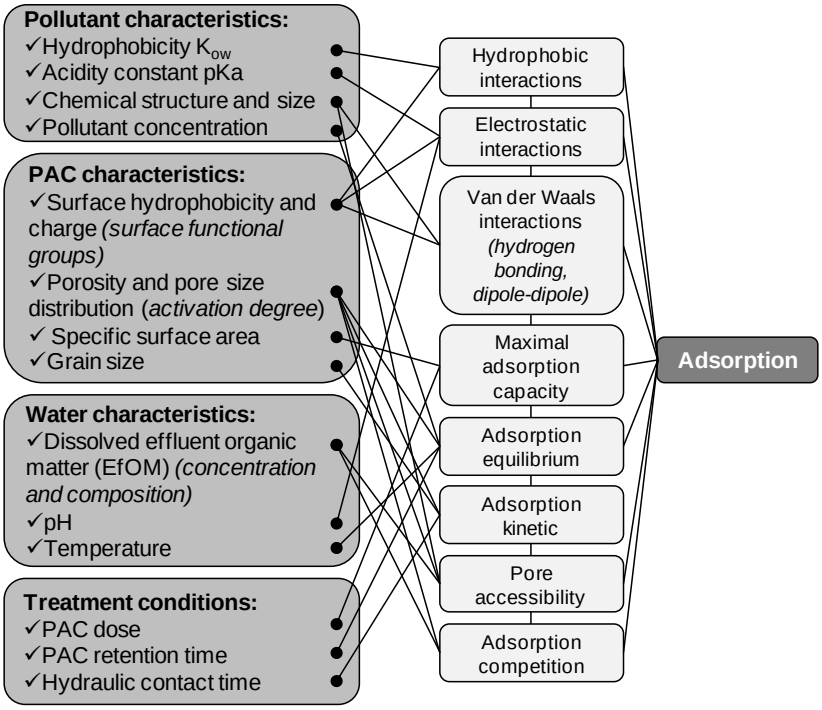

Fig. 2.11 Main factors influencing micropollutant adsorption on powdered activated carbon.

Knowing K_f and n, the PAC dose [PAC] (in [g l^{-1}]) needed to reach a target removal efficiency R [-] of a specific compound by sorption (assuming equilibrium) can be determined with Eq. 2.12, with C_0 [µg l^{-1}] the initial dissolved pollutant concentration.

$$[\mathrm{PAC}] = \frac{R C_0}{K_f ((1-R) C_0)^n} \tag{2.12}$$

Table 2.4 Freundlich sorption isotherms for six pharmaceuticals calculated from measurements (batch experiments) with biologically treated wastewater (DOC of 11 mg l^{-1}, pH 6.8) for the PAC "Norit SAE Super" (NEPTUNE, 2010). Estimation of the PAC dose needed to remove 80% of each substance, assuming an initial concentration of 1 µg l^{-1} and equilibrium conditions.

Substance	K_f [$\mu g^{1-n} l^n g^{-1}$]	n [-]	PAC dose for 80% removal [mg l^{-1}]
Clarithromycin	160	0.22	7.1
Sulfamethoxazole	40	0.21	28.0
Benzotriazole	450	0.39	3.3
Primidone	76	0.4	20.0
Carbamazepine	380	0.34	3.6
Oxazepam	280	0.36	5.1

An example of Freundlich coefficients and PAC doses needed to remove 80% of a few micropollutants in a biologically treated wastewater is presented in Table 2.4. For this particular water and with initial pollutant concentrations at 1 µg l^{-1}, a PAC dose below 30 mg l^{-1} should, in theory, assure > 80% removal of these 6 compounds. The PAC dose necessary is, however, strongly influence by the DOC content of the wastewater, which enters in competition with the pollutants for the sorption sites, and, according to the isotherm, strongly dependent on the initial micropollutant concentration (higher doses needed for higher concentrations).

2.4.2.3 Others technologies

Other technologies have proven to be also efficient in micropollutant removal, but they are not yet adapted for municipal WWTPs, either due to high costs or to efficiencies limited to only some classes of compounds.

Filtration on tight membranes (reverse osmosis and nanofiltration) is one of the most efficient technologies, allowing removals above 95% of almost all organic and inorganic pollutants (Martin Ruel et al., 2011; Urtiaga et al., 2013). This technology has, however, a high cost (high energy consumption) and produces a large amount of concentrate (15-25% of the flow with high micropollutant concentrations) that need to be treated separately.

Advanced oxidation processes (AOPs) are technologies able to remove a wide range of organic micropollutants by oxidation with the very reactive and unspecific OH radical. The principle of AOPs is to produce OH radicals using usually hydrogen peroxide (H_2O_2) combined with other oxidants such as ozone (O_3), ultraviolet light (UV) or ferrous iron (Fenton). OH radicals react very rapidly with micropollutants, but also with EfOM, limiting the treatment performance. Despite the good efficiency of AOPs to oxidise micropollutants, their performance in wastewater is quite similar to that of ozonation alone (without H_2O_2 addition). Indeed, in wastewater, ozone reacts with the EfOM, generating a high proportion of OH radicals. Ozonation of treated wastewater behaves thus like an AOP, without the cost of adding H_2O_2. AOPs are therefore not an improvement in treating wastewater compared to ozonation alone (Lee and von Gunten, 2010).

Other oxidation processes, for instance by ferrate, chlorine, chlorine dioxide or photolysis have also been shown to oxidise several micropollutants. These technologies act either on a narrower range of micropollutants than ozone or activated carbon, or are still only at the research level (Kim et al., 2009; Lee and von Gunten, 2010).

2.5 Conclusions

The fate of micropollutants in conventional WWTPs depends on their physico-chemical characteristics, in particular their hydrophobicity and sludge sorption affinity, their biodegradability, and their volatility.

Relatively hydrophobic pollutants such as heavy metals, PAHs, POPs, several household chemicals like brominated flame retardants and several personal care products, are usually well removed (> 70%), mostly by sorption onto sewage sludge. Easily biodegradable pollutants such as surfactants, plastic additives, hormones, several PCPs, some pharmaceuticals and household chemicals, are also well removed during the treatment by biodegradation/transformation. Some VOCs seem to be significantly removed from the water by volatilization. Despite good removal of these substances, effluent concentrations of some of them (surfactant, heavy metals) can still be relatively high due to their high concentrations in raw wastewater. Good removal efficiencies do, however, not mean that the effluent concentrations will not potentially affect aquatic life, as some of these compounds are toxic at very low concentrations (hormones, POPs, PAHs).

More hydrophilic and poorly-to-moderately biodegradable pollutants are not well removed during conventional treatments. The removal efficiency of some compounds can be improved with modern biological treatments, which are able to achieve high removal of BOD and ammonium (nitrification). Many of these polar and hardly biodegradable substances, e.g., most pharmaceuticals, pesticides, and several household chemicals (corrosion inhibitors, sweeteners, EDTA, phosphorus flame retardants, PFCs), are, however, not significantly removed even in modern biological treatments. To decrease their discharge into surface waters, advanced treatments such as ozonation and adsorption onto activated carbon are necessary.

Nevertheless this does not solve all the problems as some substances will still be not well eliminated by these advanced treatments and others may still be at concentrations above their no-effect toxicity threshold, despite good removal (> 80%) in the WWTP. Particular attention will also be needed for new products. Moreover, estimation of the potential effect of substances usually focused on individual substances, without considering the synergetic or antagonist effects of the cocktail of micropollutants present in wastewater. Closer collaboration between disciplines like WWTP engineering, ecotoxicology, chemistry, and biology is therefore needed to limit the discharge of micropollutants and to indentify problematic substances.

2.6 Table of abbreviations

4-MBC	4-methylbenydlidene camphor	EQS	Environmental quality standard
AE	Alcohol ethoxylates	EtP	Ethyl-paraben
AES	Alcohol ether sulfates	GAC	Granular activated carbon
AHTN	7-Acetyl-1,1,3,4,4,6-hexamethyltetralin (tonalide)	HHCB	Hexahydrohexamethyl cyclopentabenzopyran (galaxolide)
AMPA	Aminomethylphosphonic acid	HRT	Hydraulic retention time
AOP	Advanced oxidation process	LAS	Linear alkylbenzene sulfonates
APEO	Alkyl phenol ethoxylate	MBTr	4- and 5- methylbenzotriazoles
AS	Alcohol sulfates	MDMA	3,4-methylenedioxy-N-methylamphetamine
BBP	Butyl benzyl phthalate	MeP	Methyl-paraben
BDE	Brominated diphenyl ether	MTBT	2-methylthiobenzothiazole
BFR	Brominated flame retardant	NDMA	N-nitrosodimethylamine
BMDM	Butyl methoxydibenzoylmethane	NHDC	Neohesperidine dihydrochalcone
BOD	Biochemical oxygen demand	NTA	Nitrilotriacetatic acid
BP-1, -3, -4	Benzophenone-1, -3, -4	OC	Octocrylene
BPA	Bisphenol A	OFR	Organophosphorus flame retardant
BT	Benzothiazole	OHBT	2-hydroxybenzothiazole
BTEXS	Benzene, toluene, ethylbenzene, xylene and styrene	OMC	Octyl-methoxycinnamate
BTr	Benzotriazole	OT	Octyl-triazone
BTSA	Benzothiazole-2-sulfonic acid	OTNE	1-(1,2,3,4,5,6,7,8 Octahydro-2,3,8,8-tetramethyl-2-naphthalenyl)ethanone
BuP	Butyl-paraben	PAC	Powdered activated carbon
BzP	Benzyl-paraben	PAH	Polycyclic aromatic hydrocarbon
COD	Chemical oxygen demand	PBB	Polybrominated biphenyl
CP	Chlorinated paraffin	PBDE	Polybrominated diphenyl ether
CSO	Combined sewer overflow	PCB	Polychlorobiphenyl
DCM	Dissolved or colloidal matter	PCP	Personal care product
DDT	Dichlorodiphenyltrichloroethane	PFAS	Perfluoroalkyl sulphonate
DEET	N,N-diethyl-m-toluamide	PFC	Perfluorinated compound
DEHP	Di-(2-ethylhexyl) phthalate	PFCA	Perfluorocarboxylic acid
DEP	Diethyl phthalate	PFOA	Perfluorooctanoic acid
DMP	Dimethyl phthalate	PFOS	Perfluorooctane sulfonic acid
DnBP	Di-n-butyl phthalate	PNEC	Predicted no-effect concentration
DOC	Dissolved organic carbon	POP	Persistent organic pollutant
E1	Estrone	PrP	Propyl-paraben
E2	17β-estradiol	PTFE	Polytetrafluoroethylene
E3	Estriol	PVC	Polyvinyl chloride
EDTA	Ethylenediaminetetraacetatic acid	SAS	Secondary alkane sulfonates
EE2	17α-ethinylestradiol	SCCP	Sort chain chlorinated paraffin
EfOM	Effluent organic matter	SP	Sludge production
EHDPP	2-ethylhexyl diphenyl phosphate	SRT	Sludge retention time
EHMC	Ethylhexyl methoxy cinnamate	TBBA	Tetrabromobisphenol A
EPA	Environmental protection agency	TBEP	Tris(butoxyethyl)-phosphate
EPS	Extracellular polymeric substance	TCEP	Tris(2-chloroethyl) phosphate

TCPP	Tris(2-chloro,1-methylethyl) phosphate	TP	Transformation product
TDCP	Tris(1,3-dichloro-2-propyl) phosphate	TSS	Total suspended solids
THC	Tetrahydrocannabinol	UK	United Kingdom
TiBP	Tri-iso-butyl phosphate	US	United States
TMP	Trimethyl phosphate	UV	Ultraviolet
TnBP	Tri-n-butyl phosphate	VOC	Volatile organic compound
TOC	Total organic carbon	WWTP	Wastewater treatment plant

Chapter 3 Advanced treatment of micropollutants in municipal wastewater: Ozone or powdered activated carbon?

This study was coordinated by the City of Lausanne and the experimental part was performed in collaboration with several other institutions. This thesis contributed mainly to the analysis, interpretation, and synthesis of all the results gathered during this study.

An adapted version of this chapter was published in ***Science of the Total Environment (2013) 461–462: 480-498****, with the name "Treatment of micropollutants in municipal wastewater: Ozone or powdered activated carbon?", by Jonas Margot, Cornelia Kienle, Anoys Magnet, Mirco Weil, Luca Rossi, Luiz Felippe de Alencastro, Christian Abegglen, Denis Thonney, Nathalie Chèvre, Michael Schärer and D. Andrew Barry.*

Results of this study were also published in French in a technical report (Margot et al., 2011) and in an engineering journal (Margot and Magnet, 2011).

3.1 Introduction

About 3000 pharmaceutical compounds and more than 300 pesticides and biocides are commercially available in Switzerland (OPBio, 2005; OPPh, 2010; Swissmedic, 2012). They can enter urban sewer systems via human excretion in urine and faeces, by improper disposal, or through leaching of pesticides and biocides from urban areas during rain events. As described in Chapter 2, many of these hydrophilic organic compounds are poorly removed in conventional wastewater treatment plants (WWTPs), and are thus characterized by a relatively constant input at low concentrations (ng l^{-1} to µg l^{-1}) into the aquatic environment. As most of these substances are designed to be biologically active, they can affect sensitive aquatic organisms even at very low concentrations (cf. Chapter 1). Furthermore, as lakes and rivers are used in many places for drinking water supply, pharmaceuticals and pesticides can therefore be found in tap water at very low concentrations, even after drinking water treatment (Huerta-Fontela et al., 2011; Mompelat et al., 2009; Stackelberg et al., 2007). Therefore, the release of these compounds into the environment should be avoided.

Effluents of WWTPs are the main source of pharmaceuticals in the aquatic environment (Bartelt-Hunt et al., 2009; da Silva et al., 2011). Since it is unrealistic to limit the consumption of pharmaceuticals, additional steps during wastewater treatment are one of the best options to reduce the release of these compounds into surface waters. Currently, as presented in Chapter 2, two main technologies with a potential for large-scale application in terms of efficiency, costs and energy requirements have been identified (Abegglen and Siegrist, 2012; Joss et al., 2008): oxidation of micropollutants with ozone or adsorption onto activated carbon.

Through the strong oxidative properties of ozone and of the hydroxyl radicals produced spontaneously in its decomposition, ozonation was found to degrade efficiently most micropollutants present in treated wastewater with a dose of 3-8 mg O_3 l^{-1} (Hollender et al., 2009; Lee et al., 2012; Nakada et al., 2007; Reungoat et al., 2012; Reungoat et al., 2010; Rosal et al., 2010). A potential disadvantage of this process is the formation of unknown reactive by-products due to partial oxidation of the compounds and reaction with matrix components (von Gunten, 2003a). For example, undesirable toxic oxidation by-products such as nitrosamines N-Nitrosodimethylamine (NDMA), bromate or formaldehyde can be formed (Hollender et al., 2009; Richardson, 2003; Wert et al., 2007), potentially increasing the toxicity compared to non-ozonated wastewater (Petala et al., 2006; Petala et al., 2008; Stalter et al., 2010a; Stalter et al., 2010b). These oxidation products are usually more easily biodegradable and can be partially removed during biological post-filtration (Hollender et al., 2009; Richardson et al., 1999; Stalter et al., 2010a; Stalter et al., 2010b).

Activated carbon allows removal of a broad spectrum of micropollutants via adsorption to its high specific surface area and is thus widely used in drinking water treatment (Snyder et al., 2007; Westerhoff et al., 2005). As organic matter present in wastewater effluent can compete for adsorption sites, larger amounts of activated carbon are required. The efficiency of granular activated carbon (GAC) filtration to remove micropollutants has been studied in some WWTPs, showing a mitigated efficiency depending on the compound and the frequency of GAC regeneration/replacement (Grover et al., 2011; Nguyen et al., 2012; Reungoat et al., 2012; Reungoat et al., 2010; Snyder et al., 2007). Powdered activated carbon (PAC) adsorption, with a dosage of 10-20 mg l^{-1}, has been proposed as a more efficient alternative compared to GAC treatment (Boehler et al., 2012; Metzger et al., 2005; Nowotny et al., 2007; Serrano et al., 2011). However, to date, very few large scale studies evaluating the efficiency of micropollutants removal via PAC treatment in municipal wastewater have been reported.

In order to find a feasible and efficient solution for the removal of pharmaceuticals and pesticides in wastewater, a global pilot study was conducted at the municipal WWTP of Lausanne, Switzerland. The goals were to evaluate and compare the efficiency of ozonation and PAC adsorption (i) to remove a broad range of micropollutants in WWTP effluents, and (ii) to reduce ecological impacts of the effluent. Finally, we aimed to determine the feasibility of these advanced treatments at the WWTP scale in terms of operation, energy consumption and costs.

3.2 Materials and methods

3.2.1.1 Lausanne wastewater treatment plant

The municipal WWTP of Lausanne, Switzerland, is the largest in the Lake Geneva watershed and treats on average 95,000 m^3 d^{-1} of wastewater representing a population equivalent (PE) of 220,000 individuals. The sewer system is only partially separated, collecting a significant amount of urban runoff during rain events. The watershed includes a major hospital and several clinics, which are a potential source of specific pharmaceuticals. The wastewater treatment consists of pre-treatments (grit removal and screening at 1 cm), primary clarifiers, biological activated sludge treatment (AS, sludge age of 2 d) without nitrification, or, for 5% of the flow, a moving bed bioreactor (MBBR) with partial

to complete nitrification (< 1 mg $N\text{-}NH_4$ l^{-1}). In both treatments, phosphorus is removed by precipitation with iron chloride. Treated wastewater (WWTP effluent) is then discharged in Lake Geneva, which is the main drinking water reservoir for more than 600,000 inhabitants (www.cipel.org, last accessed 7 May 2013).

Table 3.1 Characteristics of the effluent of the biological treatments (feed water for the advanced treatments). Average and standard deviation of 33 24-h composite samples taken after the biological treatment with low to complete nitrification depending on the campaigns.

Conventional parameters		
Total suspended solids (TSS)	[mg l^{-1}]	14.8 (± 5.3)
Dissolved organic carbon (DOC)	[mg l^{-1}]	7.3 (± 1.9)
Chemical oxygen demand (COD)	[mg l^{-1}]	24.4 (± 12)
Biochemical oxygen demand (BOD_5)	[mg l^{-1}]	11.2 (± 10.2)
$N\text{-}NH_4$	[mg l^{-1}]	7.7 (± 7.7)
$N\text{-}NO_3$	[mg l^{-1}]	9.9 (± 5.6)
$N\text{-}NO_2$	[mg l^{-1}]	0.4 (± 0.3)
P_{total}	[mg l^{-1}]	0.7 (± 0.6)
$P_{soluble}$	[mg l^{-1}]	0.09 (± 0.08)
pH	[-]	7.2 (± 0.4)
Temperature	[°C]	17.1 (± 3.5)
Conductivity	[µS cm^{-1}]	914 (± 96)

3.2.1.1.1 Ozonation pilot plant

The pilot plant for ozonation was designed to treat a maximum flow rate of 100 l s^{-1} (13,000 PE) and consisted of a plug flow reactor (volume of 129 m^3) separated into four chambers (nine compartments) in series (Fig. 3.1 A) to assure optimal hydraulic conditions and a minimal reaction time of 20 min. Characteristics of the feed water (effluent of the conventional WWTP) are presented in Table 3.1. Ozone-containing gas (2-14% w/w) was continuously produced by an ozone generator (Effizon SMO 600 from ITT Wedeco, Wallisellen, Switzerland) fed with pure oxygen. 60% of the gas was injected counter currently into the 1st or 2nd chamber depending on the water flow rate and 40% in the 3rd chamber. The reaction time in the reactor ranged between 20 and 60 min. The ozone dosage was automatically adjusted to the water quality (oxidative demand) by varying the gas flow to maintain a constant residual concentration of dissolved ozone (around 0.1 mg O_3 l^{-1}), measured with an online sensor (AMI codes II, from Swan, Hinwill, Switzerland), and confirmed with a second probe (AquaTector from Mesin, Winterthur, Switzerland) at the outlet of the 3rd chamber. Corresponding initial ozone doses varied between 2 and 13 mg O_3 l^{-1}, with on average 5.7 mg O_3 l^{-1}. Ozone concentrations in the feed and off gas were continuously measured with BMT 964 probes (Berlin, Germany). The transfer efficiency of ozone into the dissolved phase was between 70 to over 90% depending on the gas flow. In this paper, the ozone dose refers to the amount of gaseous ozone injected and not to the ozone dissolved into the water. The remaining gaseous ozone was catalytically converted to oxygen before its release into the atmosphere. The effluent of the ozone reactor was then filtered through a rapid sand filter (flux of 8 m h^{-1}, characteristics described in the next section) with biological activity to remove reactive oxidation products.

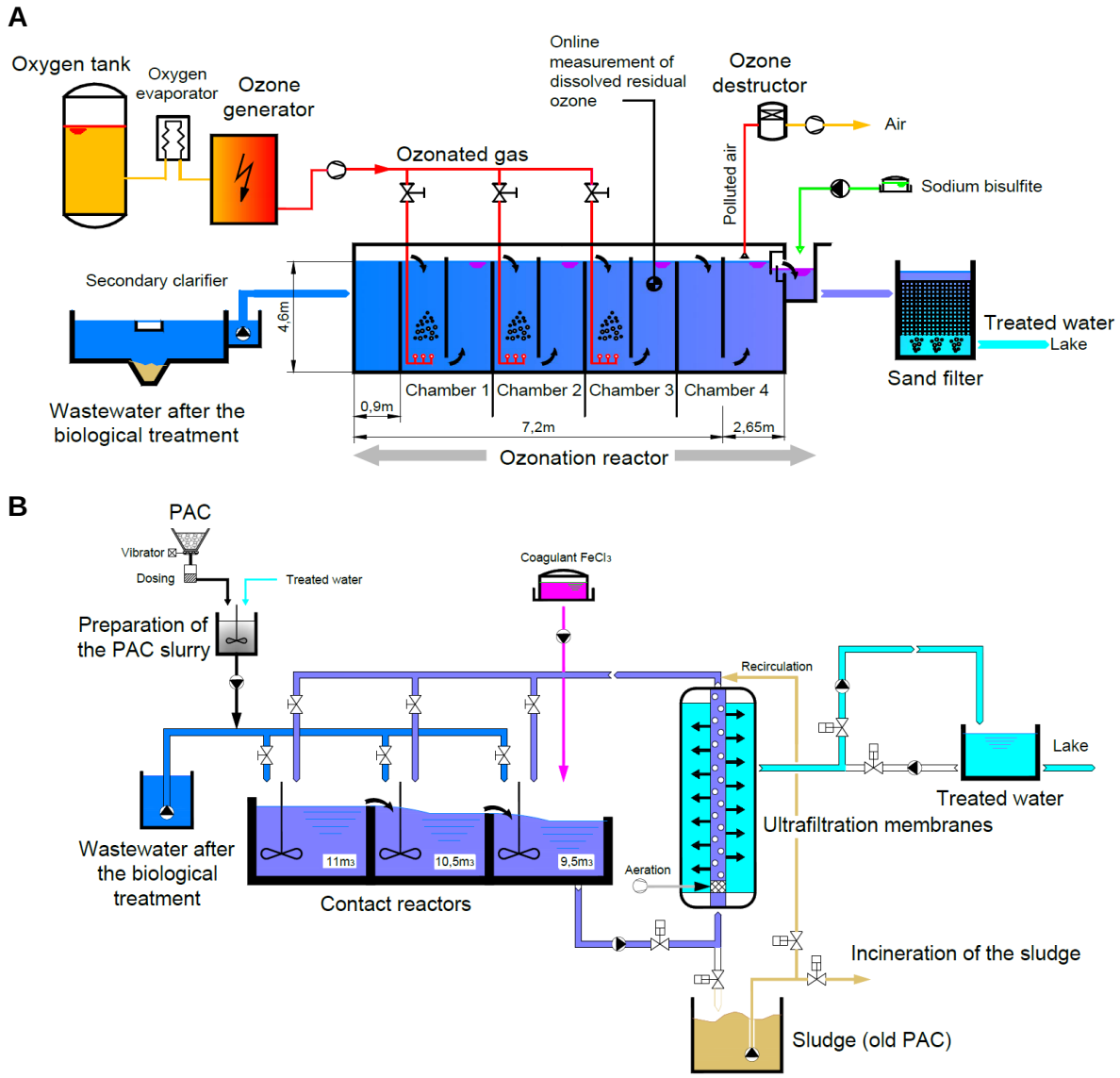

Fig. 3.1 (A) Ozonation installation (B) Powdered activated carbon (PAC) installation with ultrafiltration separation.

3.2.1.1.2 Powdered activated carbon treatment pilot plant

The pilot plant for PAC treatment was designed to treat WWTP effluent, in parallel to the ozonation, at a maximum flow of 10-15 l s^{-1} (ca. 1700 PE). Based on bench-scale batch adsorption tests on five different PACs (Omlin and Chesaux, 2010), two PACs were selected for the pilot study to assess if the treatment efficiency was influenced by the type of PAC: Norit SAE SUPER™ (Norit Activated Carbon, The Netherlands), with grain size d_{50} of 15 µm, specific surface area of 1150 m^2 g^{-1}, pH of point of zero charge pH_{PZC} > 7.3, and ash content of 12%; and SORBOPOR™ MV-125 (Enviro Link SA, Switzerland) with grain size d_{80} < 45 µm, specific surface area of 1100 m^2 g^{-1}, pH_{PZC} of 9-11, and ash content < 6%. Norit SAE SUPER and SORBOPOR MV-125 were used during the first and the second half of the study respectively with ultrafiltration separation. The installation was composed of a well-mixed contact reactor of 30 m^3 where PAC slurry (3-5 g l^{-1}) was added continuously in proportion to the wastewater flow to reach a final dosage of 10 to 20 mg PAC l^{-1}. A coagulant ($FeCl_3$ at 4-15 mg l^{-1}) was added to improve the subsequent separation of the PAC. Treated water was then filtrated in low transmembrane-pressure (0.1-0.3 bar) cross-flow hollow fibres ultrafiltration (UF)

membranes (Norit AirLift^{TM}, in PVDF, molecular weight cut-off of 100-300 kDa, total filtration surface of 660 m^2) to remove the PAC (Fig. 3.1 B). The tangential filtration process allowed increasing the concentration (up to 1-2 g PAC l^{-1}) and the residence time of the PAC in the system. Every four hours, the system was partially drained (volume removed proportional to the PAC dose) to maintain a constant PAC concentration in the reactor and to remove excess old PAC, which was then incinerated with the sewage sludge from the conventional treatment. The hydraulic residence time in the contact reactor varied between 40 and 170 min, depending on the flow rate. The corresponding solid (PAC) residence time was between 2 and 17 d in order to reach adsorption equilibrium. UF membranes were backwashed every 10 min for 10 s, and chemical cleaning with citric acid and sodium hypochlorite was performed every month to avoid fast clogging of the membranes. The PAC separation was also studied over a 5-month period with a pilot sand filter (SF) without concentration and recirculation of the PAC (with the PAC SORBOPOR MV-125). The filter, also used after ozonation, consisted of 1.2 m of expanded shale (grain size 1.6-2.4 mm), and 60 cm of quartz sand (grain size 0.7-1.2 mm), with a filtration flux of 8-16 m h^{-1} and one backwash per day. Supplementary information concerning the operation of the ozonation and PAC-UF pilot plants can be found in Margot et al. (2011).

3.2.1.2 Sampling campaigns

The pilot systems were operated continuously for more than one year. To monitor long term efficiency and to optimize the treatments, 25 sampling campaigns of one day (2-3 per month) and four seasonal campaigns of one week were performed between June 2009 and October 2010. During the campaigns, 24-h to 72-h composite samples (taken time proportional every 15 min) were collected with refrigerated automatic samplers (ISCO 6712 FR, Teledyne, USA, and WS 316, Watersam, Germany) at 5 locations: 1. Influent of the WWTP after grit removal and screening (Influent), 2. Effluent of the biological activated sludge treatment (only the first seasonal campaign) or effluent of the biological MBBR with nitrification (BIO), 3. Effluent of the ozone reactor (OZ), 4. Effluent of the sand filter following the ozonation (SF), and 5. Effluent of the PAC with ultrafiltration (PAC-UF) or with sand filter (PAC-SF) treatment (the last seven one-day campaigns). Composite samples were stored at 4°C and transferred in less than 12 h (or 24 h for the bioassays) to the laboratories performing analyses.

3.2.1.3 Chemicals and reagents

High purity micropollutants, deuterated standards and reagents used for micropollutant analysis have been listed previously (Morasch et al., 2010).

3.2.1.4 Analyses of micropollutants

Upon arrival in the laboratory, samples were immediately acidified to pH 2.5 with 5 N HCl and filtered at 0.7 µm through glass fibre filters (type GF/F, Whatman). Analysis of 58 hydrophilic micropollutants (36 pharmaceuticals, 13 biocides and pesticides, 2 corrosion inhibitors and 7 endocrine compounds, Table S 3.1, Supporting information (SI)), identified in Switzerland as priority micropollutants (Morasch et al., 2010; Perazzolo et al., 2010), were conducted on the filtrate by solid phase extraction (SPE) followed by ultra-performance liquid chromatography coupled to tandem quadrupole mass spectrometer (UPLC-MS/MS). The analytical method, described in Morasch et al.

(2010), was developed and validated for wastewater matrix. Briefly, the target compounds were extracted less than 1 h after acidification by an automated solid phase extraction (SPE) system (GX-274 ASPEC, Gilson, USA) on hand-assembled two-layered cartridges (Oasis HLB and mixture of Strata X-CW, Strata X-AW and Isolute ENV+ phases). The eluent was then analysed by ultra-performance liquid chromatography (UPLC) (Acquity UPLC system, with HSS T3 or BEH C18 column depending on the compounds, from Waters, USA) coupled to a tandem quadrupole mass spectrometer (MS/MS) (Acquity TQ Detector, Waters). To account for losses during SPE and the matrix effect, samples were spiked with deuterated surrogates, as described by Morasch et al. (2010). UPLC-MS/MS conditions, extraction efficiency of the associated deuterated standards and repeatability of the method are detailed by Morasch et al. (2010). Uncertainties of the micropollutant analyses, including recovery and repeatability uncertainties, were compound- and concentration-dependent with a decreased reproducibility close to the limit of detection (LOD). For the large majority of the compounds, the relative standard deviation was < 30% (Bonvin et al., 2011). Compounds detected with this method are presented in Table 3.3 (analytical method A) with their respective LODs.

Chemical properties of these 58 micropollutants are reported in Table S 3.1, SI. Hydrophobicity was expressed by the log D_{ow} at pH 7, a corrected form of the octanol-water partition coefficient (log K_{ow}) determined for non-ionic substances, to account for the molecule dissociation or protonation at pH 7 (de Ridder et al., 2010). The log D_{ow} values were calculated from the corresponding pK_a values following Schwarzenbach et al. (2003). For neutral molecules, log D_{ow} = log K_{ow}, for ionic compounds, log D_{ow} < log K_{ow}.

During two seasonal campaigns, a broader range of 120 micropollutants, including human pharmaceutical metabolites, were analysed on filtered 7-d composite samples (glass fibre filter APFD09050, Millipore) following Hollender et al. (2009) and Kern et al. (2009). The method consists of SPE, with the same cartridges as for method A, followed by LC-MS/MS with an XBridge C-18 column (Waters) and Linear Trap Quadrupole orbitrap mass spectrometer with electrospray ionization (Thermo Fisher Scientific Corporation, USA). Compounds detected with this method are presented in Table 3.3 (analytical method B) with their respective LODs.

Analyses of the endocrine disrupting compounds 17β-estradiol (E2) and 17α-ethinylestradiol (EE2) (analytical method C in Table 3.3) were done on filtered 7-d composite samples during two seasonal campaigns by solid phase extraction (LiChrolut® EN-RP18 cartridge, Merck, Germany) followed by LC-MS/MS detection (API 4000 LC-MS/MS, Applied Biosystems, USA). The method used is described in Table S 3.2 and Table S 3.3, SI.

In the case when the effluent concentration was below the LOD of the compound, the removal rate was calculated as a minimum value using the LOD as effluent concentration. These minimum removal rates were not integrated into the global removal average unless they were above 80%, in order not to bias the results.

3.2.1.5 Bioassays

In this pilot study, a broad range of bioassays was performed, showing that most acute toxicity bioassays were not sensitive enough to detect the effects of low micropollutant concentrations in wastewater. An overview on these bioassays can be found in Kienle et al. (2011). Two kinds of assays were therefore selected based on their sensitivity: i) bioassays on enriched samples and ii) chronic toxicity tests in the whole effluent. These two approaches can be seen as complementary for evaluating the effects of the effluents: the first mentioned assays are very sensitive and focus on the effects produced by specific pollutants, while the second assays evaluate the long-term toxicity of the effluent, including the effect of very polar compounds not well extracted during the enrichment process, such as ozonation by-products (Stalter et al., 2011). For the first approach, two bioassays were performed on enriched samples: the *Yeast Estrogen Screen* (YES) to evaluate the estrogenicity (Routledge and Sumpter, 1996) and the *Combined Algae Assay* to evaluate the global toxicity and the presence of photosynthesis inhibitors (Escher et al., 2008b). For the second approach, a fish early life stage test (FELST, (OECD, 1992b)) with rainbow trout was performed by exposing the fish for 67 d to the effluent from the different treatments under flow-through conditions.

3.2.1.5.1 Sample enrichment (YES and combined algae assay)

The sample enrichment was done by solid phase extraction (SPE), which allows for increased pollutant concentrations in the extracts and thereby enables a better detection in the bioassays. It also limits the impact of the matrix components and metals, which are partially separated during the extraction (Macova et al., 2010). 7-d composite samples were enriched using SPE as described in Escher et al. (2008b) and as presented in Table S 3.2, SI. Briefly, 200 ml (influent samples) or 500 ml (all others) were enriched 200 and 500 times respectively using LiChrolut® EN-RP18 cartridges (Merck, Germany), and then stored in 1 ml of a solvent mixture (~50% ethanol, ~50% acetone and methanol) at -20°C until analysis.

3.2.1.5.2 Yeast Estrogen Screen (YES)

The yeast estrogen screen with the recombinant yeast *Saccharomyces cerevisiae* was performed according to Routledge and Sumpter (1996) in 96-well microtitre plates using yeast cells provided by J. Sumpter (Brunel University, Uxbridge, UK). In brief, yeast cells were cultured in minimal medium on an orbital shaker at 30°C for 24 h before the onset of the test. At the beginning of the test, 1:2 dilution series of the reference substance, the enriched wastewater samples and the solvent control were pipetted onto the plates. The solvent was evaporated completely on a sterile bench. In the meantime the cell density of the yeast cells was determined, and an assay medium prepared (seeded with 4×10^7 yeast cells). Subsequently, the yeast-cell suspension was pipetted on the test plate (200 µl/well). The plate was incubated at 30°C. After 72 h, cell density ($OD_{620\ nm}$) and colour change ($OD_{540\ nm}$) were measured using a plate reader (Synergy 4, Biotek, Winooski, USA). The estrogenic activity in the wastewater samples was assessed relative to a reference substance (17β-estradiol, a potent estrogen) and expressed as 17β-estradiol equivalent concentrations (EEQ). Both, the reference substance and the wastewater samples, were tested in triplicates in a 1:2 dilution series. The highest tested concentration of 17β-estradiol was 1.25×10^{-9} M (340 ng l^{-1}, in ethanol) and the maximum enrichment factors of the wastewater samples were 5 for the WWTP influent and 50 for all additional treatment steps. Ethanol served as solvent control (50 µl/well, 8 wells/plate).

3.2.1.5.3 Combined Algae Assay

The combined algae assay on the green algae *Pseudokirchneriella subcapitata* was conducted as described by Escher et al. (2008a). The herbicide diuron served as the reference substance and ethanol as the solvent control (50 µl/well, 8 wells/plate). Both, the reference substance and the wastewater samples, were tested in triplicates in a 1:2 dilution series, with the highest concentration of diuron being 3×10^{-7} M (69.9 µg l^{-1}, in ethanol) and maximum enrichment factors of the wastewater samples of 20 (WWTP influent) and 83.3 (all additional treatment steps). After a complete ablation of the solvent, the samples were re-suspended in 100-µl algae medium. Finally, 100 µl of algae suspension with an OD_{685} of 0.1 were added to each well. Photosynthesis inhibition was measured by means of effective quantum yield after 2 h of exposure using a Maxi-Imaging PAM (pulse amplitude modulation, IPAM) device (Walz, Effeltrich, Germany) as described by Schreiber et al. (2007). Algae growth was measured by means of absorbance at 685 nm in a microtitre plate photometer (Synergy 4, Biotek, Winooski, USA) at the test start and end (after 24 h of exposure) as well as on two occasions in between. The toxicity of the wastewater samples was expressed as diuron-equivalent concentrations (DEQs) for the endpoint "inhibition of Photosystem II" and toxic equivalent concentrations (TEQs, virtual baseline toxicant) for growth inhibition (Escher et al., 2008a).

Comparison of the measured photosynthesis inhibition with the concentration of photosynthesis inhibitors was based on the concept of concentration addition for substances with similar mode of action according to Chèvre et al. (2006). The concentrations of the four most abundant photosynthesis inhibitors included in the analytical list, the herbicides atrazine, diuron and isoproturon, and the algicide terbutryn were converted to diuron-equivalents based on their relative potency (HC_{50}-EC_{50}: hazardous concentration affecting 50% of the species with 50% effect, (Chèvre et al., 2006)) and then summed up. One µg l^{-1} of atrazine, diuron, isoproturon and terbutryn corresponds to 0.084, 1, 0.559 and 0.881 µg l^{-1} DEQs respectively.

3.2.1.5.4 Fish early life stage test with rainbow trout (Oncorhynchus mykiss)

This test was performed according to OECD guideline 210 (OECD, 1992b). Details of the methodology are described by Stalter et al. (2010b). In brief, freshly fertilized eggs (< 1 h, 40 eggs per replicate) of rainbow trout (*Oncorhynchus mykiss*) were exposed to the test waters in 8-l stainless steel vessels in a flow-through system. Reconstituted water (OECD guideline 203, (OECD, 1992a)) served as the control medium. At the start of the test, 70 eggs/replicate were randomly distributed to the test vessels and gradually reduced to 40 eggs the next day. The fish embryos were exposed at 10 ± 2°C and in darkness. Flow of test media into each test vessel was adjusted to 11 ml min^{-1}, corresponding to two test vessel volume exchanges per day. For the post hatch period the temperature was raised to 12 ± 2°C and a 12/12 h photoperiod was set. Flow-through rates in the test vessels were adjusted weekly depending on the fish developmental stage to reach 44 ml min^{-1} seven days before the test end, achieving a eight-fold medium exchange in the test vessels per day (OECD, 1992b). From the beginning of swim-up onwards, the fish were fed four times per day (trout starter, 4% body weight per day). In total, four controls and three replicate treatments for each wastewater were assessed. During the test period several endpoints were determined daily, namely: hatching, mortality, swim up, malformations and abnormal behaviour. After the end of the test fish were humanely killed with an overdose of MS222 (tricaine methanesulfonate, Sigma–Aldrich, St. Louis, USA). Afterwards,

individual fish were blotted dry and fresh weight and length were measured. Plasma vitellogenin concentration was determined in whole body homogenates of 20 fish per control and wastewater as described by Holbech et al. (2006) using a vitellogenin ELISA test kit for rainbow trout (Biosense, Bergen, Norway) in a 1:20 dilution.

The significance of the difference in the response between the treatments was assessed with the Tukey's test for single-step multiple comparison. Significant differences are reported for p values < 0.05. All calculations were performed using R (R Foundation for Statistical Computing, Vienna, Austria).

3.2.1.6 Laboratory-scale batch adsorption experiment

The influence of wastewater dissolved organic carbon (DOC) concentration on micropollutant removal efficiency with PAC was assessed in laboratory-scale batch adsorption experiments. Adsorption tests were conducted in triplicates on 24-h composite wastewater samples collected at the Lausanne WWTP after either simple coagulation-precipitation treatment (DOC of 17 mg l^{-1}), activated sludge treatment without nitrification (DOC of 11 mg l^{-1}), or moving-bed bioreactor treatment with full nitrification (three composite samples with DOC of 5, 7 and 8 mg l^{-1}). PAC (10 mg of SORBOPOR^{TM} MV-125, Enviro Link SA, Switzerland) was added to one litre of the different types of wastewater and agitated at 140 rpm during 24 h in the dark at 20°C. Analyses of initial and final sample concentrations, after filtration at 0.45 µm, of carbamazepine, diclofenac, benzotriazole, mecoprop and iopamidol were done by SPE followed by UPLC-MS/MS as described above. DOC was analysed by catalytic combustion oxidation method (Shimadzu TNM1 device).

3.2.1.7 Other analyses

Standard wastewater quality parameters (TSS, DOC, COD, BOD_5, NH_4, NO_3, NO_2, P_{total}, and $P_{soluble}$) were regularly analysed on 24 h-composite samples by standard methods recommended in Switzerland (DFI, 1983). Temperature, pH and conductivity were continuously analysed on-line with E+H measurement systems (Endress+Hauser, Switzerland). Indicator bacteria (*Escherichia coli*, intestinal enterococci and total viable bacteria) and coliphages (F-specific (RNA) and somatic phages) were analysed by standard plate count methods. Bromide and bromate were analysed by High Performance Ion Chromatography (HPIC) with a post column-reaction, with UV-detection for bromate and suppressed conductivity detection for bromide.

3.3 Results and discussion

3.3.1 Micropollutant concentrations in WWTP influent and effluent

3.3.1.1 Raw wastewater

Most of the micropollutants analysed were detected in the raw wastewater, with 70 compounds quantified in at least one sample (Table 3.3). The highest average concentrations of pharmaceuticals were observed for the analgesics paracetamol (51 µg l^{-1}) and ibuprofen (4.1 µg l^{-1}), the iodinated contrast media family (3.3 to 21 µg l^{-1}), the antidiabetic metformin (> 10 µg l^{-1}) and the antihypertensive irbesartan (4.7 µg l^{-1}). High concentrations (> 5 µg l^{-1}) were also detected in raw

wastewater for food components (aspartame and caffeine), corrosion inhibitors (benzotriazole and methylbenzotriazole) and an industrial additive (benzothiazole). On average, 25 compounds reached an influent concentration > 1 µg l^{-1}. A similar range of concentrations was observed in other Swiss municipal wastewater (Hollender et al., 2009), with the exception of the contrast media. In the present study, these showed higher concentrations probably due to the presence of many hospitals and clinics in the watershed.

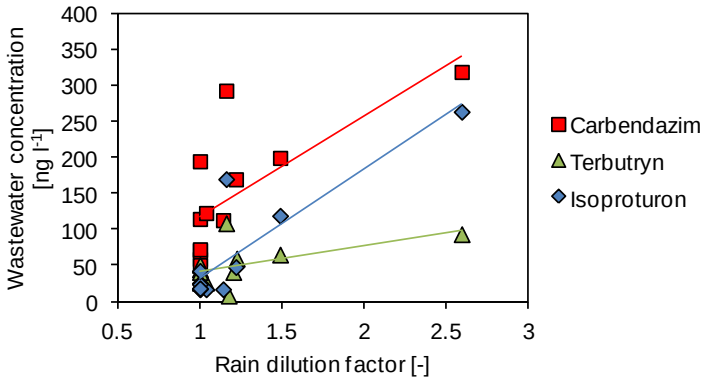

Fig. 3.2 Concentration of selected pesticides in raw wastewater as a function of wastewater dilution by runoff water. Correlations with the dilution factor (wet weather flow/dry weather flow): Isoproturon ($r = 0.875$, $p < 0.001$), carbendazim ($r = 0.712$, $p < 0.01$), terbutryn ($r = 0.612$, $p < 0.05$).

High variations of the influent daily average concentration of the same compound were observed between the different campaigns. A factor > 4 in the 10-90 percentile range of the concentrations was observed for half of the compounds due, inter alia, to variations of the consumption of these compounds (Coutu et al., 2013). These variations highlight the importance of long term sampling campaigns, lasting at least one year, to cover the different consumption habits of the respective substances. During rain events, no noticeable different variations of the influent concentration could be detected compared to the background variability despite dilution by runoff water. Only the pesticides isoproturon, carbendazim and terbutryn showed a significant concentration increase during wet weather (p-value < 0.05 for the correlation with the dilution factor, Fig. 3.2), presumably due to the leaching of facades and runoff of pesticides used in gardens in the urban area (Burkhardt et al., 2007; Coutu et al., 2012b).

3.3.1.2 Biological treatment

As presented in Fig. 3.3 A and Table 3.3, most of the micropollutants were not well removed in the conventional biological wastewater treatment. Average removals of less than 50 % were found for 50 (i.e., 71 %) of the 70 compounds detected, with 16 having an average concentration in the effluent above 1 µg l^{-1}, and 52 a concentration above 100 ng l^{-1}. Only the analgesic paracetamol was completely eliminated in all the campaigns. The most persistent micropollutants (less than 10% removal on average) were the pharmaceuticals carbamazepine, clindamycin, diclofenac, gabapentin and metoprolol, the pesticides carbendazim and diuron, and most of the pharmaceutical metabolites. All these compounds have been reported as persistent in many studies (Kupper et al., 2006; Oulton et al., 2010; Singer et al., 2010; Verlicchi et al., 2012). Some compounds such as the antibiotic clindamycin, the beta blocker metoprolol and most of the pharmaceutical metabolites were found at higher concentrations (in the dissolved phase) in the effluent of the biological treatment than in the

influent. Similar observations in other studies were attributed to (i) release during the treatment of compounds trapped in faeces particles (Göbel et al., 2007), (ii) biological cleavage in the treatment of pharmaceutical conjugates (human metabolites) producing again the parent compound (Onesios et al., 2009), (iii) formation of bacterial metabolites during the biological treatment or (iv) analytical uncertainties.

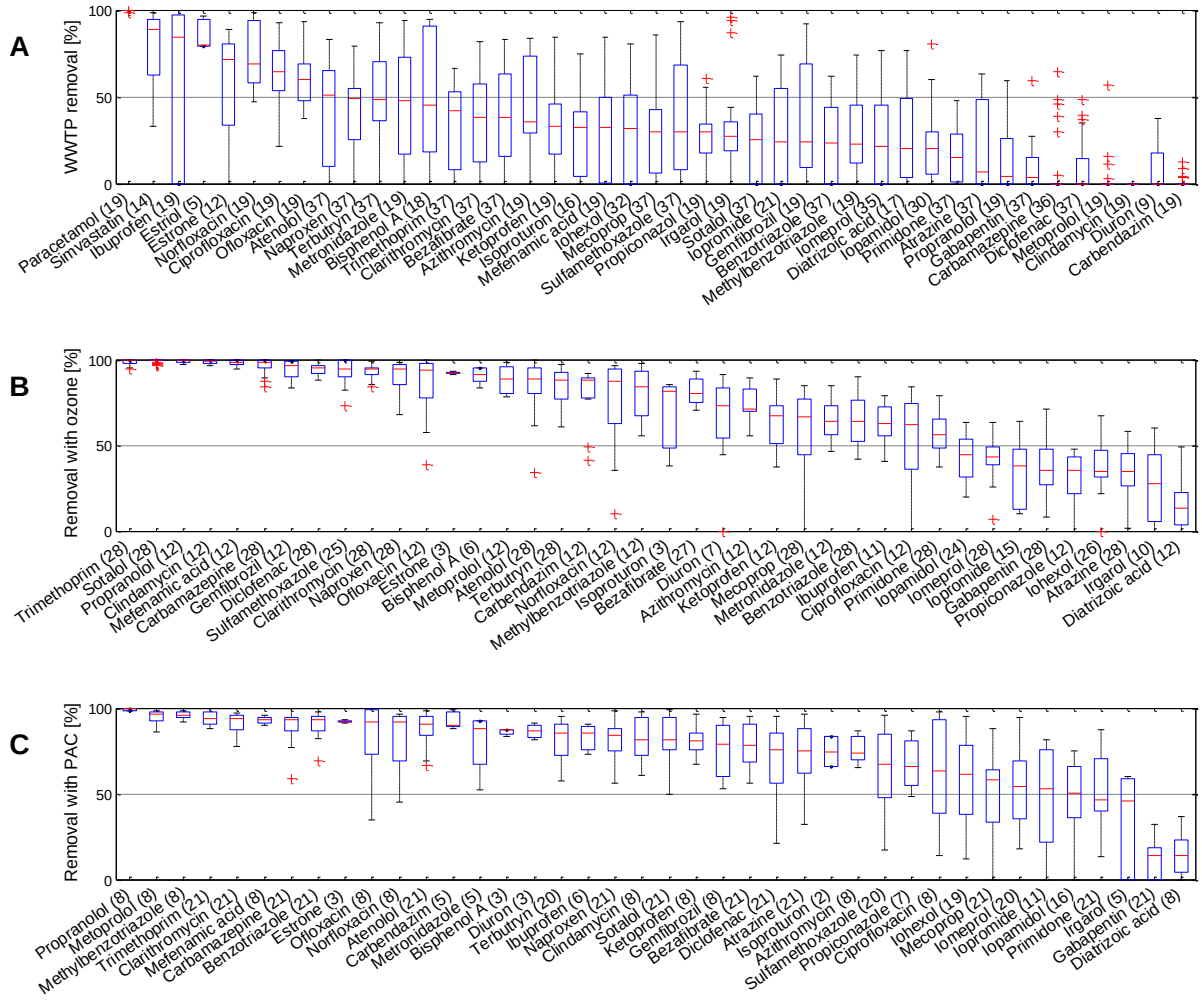

Fig. 3.3 Removal efficiency of 40 to 43 micropollutants during **(A)** the conventional biological wastewater treatment with either activated sludge without nitrification or moving bed bioreactor with partial to complete nitrification (average removal of 35%), **(B)** the ozonation (ozone dose between 2.3 to 9.1 mg O_3 l^{-1}, median 5.9 mg O_3 l^{-1} or 0.83 g O_3 g^{-1} DOC, average removal of 71%) and **(C)** the PAC-UF treatment (PAC dose between 10 to 20 mg PAC l^{-1}, median 12 mg l^{-1}, average removal of 73%). Results of (n) analyses (24 h to 72 h composite samples) conducted between June 2009 and October 2010. Representation of the median removal, the quartiles 25-75 %, the minimum and maximum values and the outliers.

Large variations of the removal rate in the biological treatment were observed among the different campaigns (Fig. 3.3 A). For 24 of the 42 regularly studied micropollutants, these removal efficiency variations could be explained in part by the different levels of nitrification reached in the biological treatment. Indeed, significant positive correlations were observed between the removal of those compounds and the degree of nitrification of ammonium (Table 3.2), with especially strong correlations ($r > 0.8$) for 11 substances and medium correlations ($0.6 < r < 0.8$) for 7 others. The influence of the ammonium removal on the abatement of 20 pollutants (with $r > 0.5$) is presented in Fig. 3.5. Less than 30% removal in a non-nitrifying sludge compared with more than 60% elimination

in a treatment with complete nitrification was observed for instance for atenolol, bezafibrate, bisphenol A, gemfibrozil, methylbenzotriazole or metronidazole. Similar observations were reported for some of these substances by Clara et al. (2005a). The higher micropollutant removal observed at high nitrification levels is presumably due to (i) the longer hydraulic residence time in the reactor (Fig. 3.4), leading to a longer time available for biodegradation processes, as well as to (ii) the presence of a more diverse microbial population with different metabolisms and a higher activity of nitrifying bacteria. These bacteria have the ability to degrade many micropollutants, probably by cometabolic oxidation by the ammonium monooxygenase enzyme (Fernandez-Fontaina et al., 2012). But, even for the most efficient biological treatment with complete nitrification (< 1 mg N-NH_4 l^{-1}), less than 50% removal was observed for 21 out of 43 compounds, with an average removal of only 50%. Among them, 18 compounds were not significantly influenced by the nitrifying efficiency of the biological treatment, including the very common pollutants carbamazepine, diclofenac, gabapentin, sulfamethoxazole, benzotriazole and mecoprop (Table 3.2). These results confirm the need for advanced treatments.

Table 3.2 Correlation coefficients between the removal of 42 micropollutants and the level of nitrification (% of ammonium removal) in the biological treatment. Pearson correlation on 19 to 36 analyses. Correlations were considered significant for p values < 0.05.

Substance	Correlation	Substance	Correlation
Bisphenol A	0.97 ***	Irgarol	0.48 *
Norfloxacin	0.95 ***	Clarithromycin	0.43 **
Atenolol	0.93 ***	Terbutryn	0.36 *
Ofloxacin	0.90 ***	Paracetamol	0.29 ns
Bezafibrate	0.88 ***	Isoproturon	0.27 ns
Methylbenzotriazole	0.87 ***	Benzotriazole	0.26 ns
Metronidazole	0.87 ***	Carbendazim	0.24 ns
Trimethoprim	0.86 ***	Estrone	0.20 ns
Simvastatin	0.86 ***	Propiconazol	0.20 ns
Gemfibrozil	0.83 ***	Mecoprop	0.19 ns
Ketoprofen	0.83 ***	Iopamidol	0.16 ns
Ibuprofen	0.76 ***	Diclofenac	0.14 ns
Iohexol	0.75 ***	Carbamazepine	0.12 ns
Mefenamic acid	0.71 ***	Ciprofloxacin	0.12 ns
Naproxen	0.69 ***	Gabapentin	0.05 ns
Azithromycin	0.67 **	Clindamycin	0.00 ns
Sotalol	0.66 ***	Sulfamethoxazole	-0.08 ns
Iomeprol	0.65 ***	Diatrizoic + iothalamic acid	-0.13 ns
Propranolol	0.57 *	Metoprolol	-0.22 ns
Primidone	0.53 ***	Atrazine	-0.41 *
Iopromide	0.50 *	Diuron	-0.42 ns

ns: no significant, * p < 0.05, ** p < 0.01, *** p < 0.001

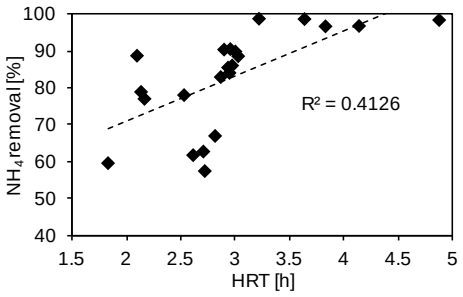

Fig. 3.4 Ammonium removal in the moving bed bioreactor as a function of the hydraulic retention time (HRT) in the reactor. Daily averages of 22 campaigns. Although a significant correlation is observed, nitrification depends on the presence of nitrifying microorganisms, which depends also on the conditions (HRT, aeration) a few days before the measurement campaigns (time for development).

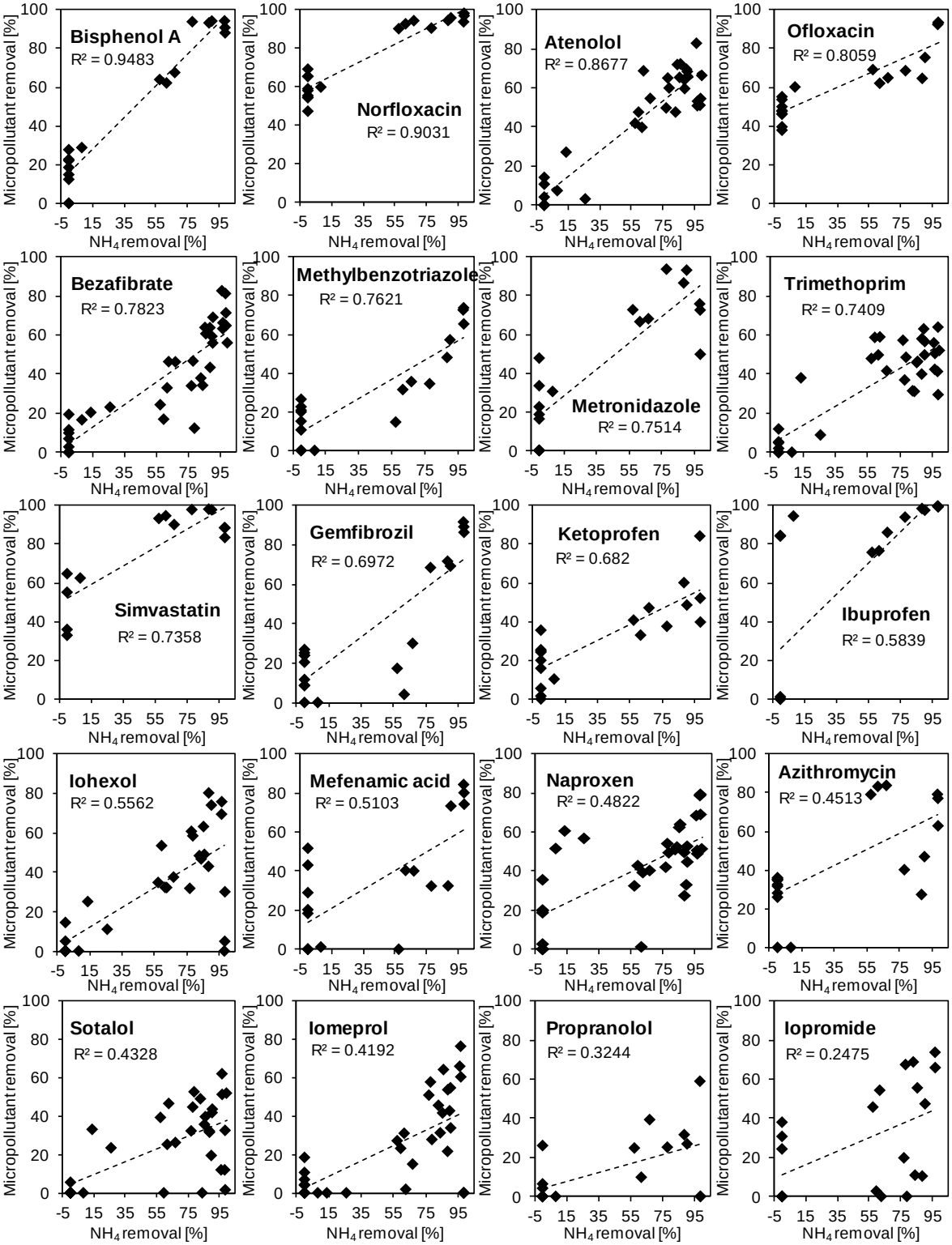

Fig. 3.5 Removal of 20 micropollutants in the biological treatment as a function of the level of nitrification (ammonium removal). Results of 19 to 36 campaigns on 24 to 72-h composite samples at the entrance of the WWTP and at the outlet of the biological treatment. Diverse levels of nitrification were obtained by varying the hydraulic residence time and/or the aeration either in an activated sludge tank with a sludge age of 2 d (0 to 26% of nitrification, 9 to 21 mg $N\text{-}NH_4$ l^{-1} in the effluent) or in a moving bed bioreactor (57 to 99% of nitrification, 0.1 to 10 mg $N\text{-}NH_4$ l^{-1} in the effluent).

Table 3.3 Concentrations of 70 micropollutants in raw wastewater and after biological treatment (WWTP effluent), and removal rate obtained with the conventional (with low to complete nitrification) or the advanced treatments (in reference to the concentration in the effluent of the biological treatment) (ozone doses between 2.3 and 9 mg l^{-1} (median 5.9 mg l^{-1}) and PAC doses between 10 and 20 mg l^{-1} (median 12 mg l^{-1})). Average with standard deviation of n analyses (24-h composite samples) conducted between June 2009 and October 2010. Compounds with analytical method A were regularly analysed, while compounds with analytical methods B and C correspond to one or two analyses of a 7-d composite sample taken for a larger screening campaign (with partial nitrification, 6 mg O_3 l^{-1}, or 12 mg PAC l^{-1}). Comparison with removal rates obtained in other studies in similar conditions is presented for the two advanced treatments (Abegglen and Siegrist, 2012; Bundschuh et al., 2011a; Hollender et al., 2009; Huber et al., 2005; Huerta-Fontela et al., 2011; Ormad et al., 2008; Reungoat et al., 2012; Reungoat et al., 2010; Rosal et al., 2010; Senta et al., 2011; Sudhakaran et al., 2012; Ternes et al., 2003; Wert et al., 2009; Yang et al., 2011; Zwickenpflug et al., 2010).

Compound	Compound class	LOD (ng l^{-1})	Analytical method	Number of analysis (n)	Influent concentration (ng l^{-1})	(n)	Effluent concentration (ng l^{-1})	(n)	WWTP removal (%)	(n)	Ozone removal (%)	(n)	PAC-UF removal (%)
Pharmaceuticals													
Atenolol	Beta blocker	1.2	A	37	1274 (±436)	37	682 (±267)	37	42 (±27)	28	85 (±14)[a]	21	88 (±9)[e]
Azithromycin	Antibiotic	75.6	A	19	2272 (±1472)	19	935 (±333)	19	44 (±26)	12	74 (±10)[d]	8	76 (±8)[c]
Bezafibrate	Lipid regulator	1.5	A	37	953 (±262)	37	595 (±314)	37	38 (±26)	27	81 (±8)[a]	21	79 (±12)[e]
Carbamazepine	Anticonvulsant	0.1	A	37	482 (±586)	37	461 (±292)	37	7.6 (±18)	28	97 (±4)[a]	21	90 (±9)[e]
Ciprofloxacin	Antibiotic	36.5	A	19	2291 (±600)	19	779 (±372)	19	63 (±18)	12	53 (±29)[b8]	8	63 (±32)[f]
Clarithromycin	Antibiotic	0.4	A	37	709 (±418)	37	440 (±302)	37	37 (±26)	28	93 (±4)[a]	21	92 (±5)[e]
Clindamycin	Antibiotic	0.2	A	19	65 (±33)	19	115 (±69)	19	0 (±0)	12	99 (±1)[a]	8	82 (±13)[c]
Diatrizoic and iothalamic acid	Iodinated contrast medium	32.8	A	17	597 (±628)	19	370 (±366)	17	28 (±25)	12	16 (±16)[b2]	8	15 (±13)[e]
Diclofenac	Analgesic / Anti-inflammatory	1.2	A	37	1197 (±497)	37	1187 (±389)	37	9 (±14)	28	94 (±3)[a]	21	69 (±19)[e]
Eprosartan	Antihypertensive	20	B	2	1055 (±488)	1	880	1	37	1	98[c]	1	65[c]
Fluconazole	Antifungal	20	B	2	120 (±14)	1	110	1	15	1	27[d]	1	> 64[c]
Gabapentin	Anticonvulsant	1.8	A	37	3867 (±1339)	37	3692 (±1456)	37	9.2 (±12)	28	38 (±16)[b5]	21	11.8 (±11)[f]
Gemfibrozil	Lipid regulator	2.9	A	19	411 (±128)	19	265 (±159)	19	36 (±32)	12	94 (±5)[b11]	8	76 (±16)[d]
Ibuprofen	Analgesic / Anti-inflammatory	13.4	A	19	4101 (±2465)	19	952 (±759)	19	57 (±46)	11	63 (±12)[b11]	6	83 (±7)[e]
Iohexol	Iodinated contrast medium	2177.3	A	35	21275 (±6975)	34	15191 (±7294)	32	31 (±27)	26	38 (±16)[a]	19	57 (±25)[e]
Iomeprol	Iodinated contrast medium	306.9	A	35	14467 (±9657)	35	10534 (±6338)	35	25 (±24)	28	43 (±12)[b2]	20	54 (±21)[c]
Iopamidol	Iodinated contrast medium	145.4	A	30	3360 (±2574)	30	2535 (±1587)	30	21 (±20)	24	42 (±13)[a]	16	49 (±21)[e]
Iopromide	Iodinated contrast medium	2044.6	A	22	6408 (±2663)	23	4141 (±2086)	21	29 (±27)	15	34 (±19)[a]	11	47 (±30)[c]
Irbesartan	Antihypertensive	20	B	2	4700 (±4808)	1	1700	1	79	1	51[b7]	1	98[c]
Ketoprofen	Analgesic / Anti-inflammatory	6.0	A	19	1119 (±1328)	19	669 (±757)	19	32 (±21)	12	63 (±16)[a]	8	81 (±9)[c]
Levetiracetam	Anticonvulsant	10	B	2	2100 (±566)	1	330	1	87	1	18[a]	1	> 97[c]
Losartan	Antihypertensive	20	B	2	2405 (±2256)	1	510	1	87	1	> 96[b7]	1	80[c]
Mefenamic acid	Analgesic / Anti-inflammatory	2.6	A	19	946 (±455)	19	581 (±299)	19	33 (±29)	12	98 (±2)[a]	8	93 (±2)[e]
Metformin	Antidiabetic	< 1000	B	2	> 10000	1	> 4000	1	-	0	-	1	> 55[c]
Metoprolol	Beta blocker	4.4	A	19	561 (±299)	19	653 (±400)	19	4.6 (±13)	12	88 (±8)[a]	8	95 (±4)[f]
Metronidazole	Antibiotic	21.0	A	19	1168 (±866)	19	567 (±497)	19	45 (±34)	12	64 (±12)[b6]	5	79 (±17)[c]
Morphine	Analgesic / Anti-inflammatory	20	B	1	270	1	190	1	30	1	> 90[c]	1	> 90[c]
Naproxen	Analgesic / Anti-inflammatory	9.4	A	37	697 (±249)	37	380 (±110)	37	41 (±23)	28	90 (±8)[a]	21	81 (±12)[e]
Norfloxacin	Antibiotic	1.9	A	19	334 (±167)	19	59 (±35)	19	76 (±19)	12	75 (±29)[b9]	8	82 (±21)[c]
Ofloxacin	Antibiotic	0.4	A	19	234 (±60)	19	84 (±36)	19	61 (±17)	12	85 (±20)[c]	8	83 (±24)[c]
Oxazepam	Anxiolytic	20	B	2	305 (±134)	1	350	1	13	1	9[d]	1	69[c]
Paracetamol	Analgesic / Anti-inflammatory	7.9	A	18	51438 (±31884)	18	< 7.9	19	100 (±0)	1	> 85[b11]	0	-
Primidone	Anticonvulsant	0.7	A	37	114 (±39)	37	97 (±21)	37	16 (±15)	28	57 (±11)[a]	21	51 (±19)[f]
Propranolol	Beta blocker	0.3	A	19	127 (±37)	19	114 (±17)	19	13 (±17)	12	99 (±1)[a]	8	99 (±1)[c]
Ritonavir	Antiretroviral	20	B	2	110 (±14)	1	90	1	25	1	> 78[c]	1	> 56[c]

Table 3.3 (Continuation)

Compound	Compound class	LOD (ng l^{-1})	Analytical method	Number of analysis (n)	Influent concentration (ng l^{-1})	(n)	Effluent concentration (ng l^{-1})	(n)	WWTP removal (%)	(n)	Ozone removal (%)	(n)	PAC-UF removal (%)
Pharmaceuticals													
Simvastatin	Lipid regulator	29.7	A	14	736 (±503)	14	98 (±96)	14	77 (±23)	8	> 70[c]	4	> 65[c]
Sotalol	Beta blocker	0.5	A	37	337 (±175)	37	247 (±63)	37	23 (±20)	28	99 (±1)[a]	21	81 (±15)[c]
Sulfamethoxazole	Antibiotic	0.2	A	37	340 (±261)	37	171 (±127)	37	38 (±30)	25	93 (±7)[a]	20	64 (±25)[e]
Trimethoprim	Antibiotic	0.2	A	37	235 (±52)	37	158 (±73)	37	35 (±23)	28	99 (±2)[a]	21	94 (±4)[f]
Valsartan	Antihypertensive	5	B	2	2250 (±354)	1	2100	1	16	1	61[b7]	1	65[c]
Venlafaxine	Antidepressant	10	B	2	235 (±21)	1	150	1	40	1	75[d]	1	46[d]
Pharmaceutical metabolites													
10,11-dihydro-10,11-dihydroxy carbamazepine	Drug metabolite	10	B	2	975 (±106)	1	1000	1	0	1	47[b10]	1	52[c]
Atenolol acid	Drug metabolite	10	B	2	1550 (±212)	1	1700	1	0	1	72[d]	1	> 99[c]
Fenofibric acid	Drug metabolite	20	B	2	390 (±57)	1	490	1	0	1	57[b1]	1	78[c]
Formyl-4-aminoantipyrine	Drug metabolite	10	B	2	445 (±92)	1	700	1	0	1	> 99[b6]	1	59[c]
N,N-didesvenlafaxine	Drug metabolite	10	B	1	250	1	330	1	0	1	> 97[c]	1	61[c]
N-acetyl sulfamethoxazole	Drug metabolite	20	B	2	570 (±156)	1	50	1	93	1	50[b2]	1	> 20[c]
N-acetyl-4-aminoantipyrine	Drug metabolite	20	B	2	920 (±28)	1	1200	1	0	1	> 98[b6]	1	34[c]
Valsartan acid	Drug metabolite	10	B	2	125 (±21)	1	150	1	0	1	39[c]	1	43[c]
Endocrine disrupting compounds													
17α-Ethinylestradiol	Hormonal contraceptive	1.9	C	2	5.3 (±4.3)	1	< 1.9	1	> 18	0	-	1	-
17β-Estradiol	Hormone	0.5	C	2	14 (±1)	1	1.3	1	91	1	> 61[b2]	1	> 61[c]
Bisphenol A	Plastic component	48.9	A	18	834 (±460)	18	338 (±311)	18	50 (±36)	3	> 95[b4]	3	> 83[c]
Estriol	Hormone	97.5	A	12	306 (±140)	12	< 97.5	11	> 75 (±12)	0	-	0	-
Estrone	Hormone	15.6	A	12	134 (±87)	12	71 (±83)	12	58 (±31)	3	> 90[b2]	3	> 92[c]
Biocides - pesticides													
Atrazine	Herbicide	0.2	A	37	21 (±16)	37	14 (±8)	37	20 (±24)	28	34 (±13)[a]	21	74 (±17)[c]
Carbendazim	Fungicide	16.1	A	19	106 (±92)	19	132 (±79)	19	1.5 (±3.5)	12	79 (±17)[c]	5	> 93[e]
Diuron	Herbicide	13.7	A	9	69 (±49)	9	70 (±41)	9	10 (±16)	7	73 (±16)[a]	3	> 82[f]
Irgarol	Algicide	1.0	A	19	16 (±14)	19	7.5 (±6.2)	19	34 (±29)	10	32 (±21)[d]	5	0 to > 60[c]
Isoproturon	Herbicide	16.9	A	16	62 (±67)	16	39 (±32)	16	27 (±22)	3	68 (±26)[b3]	2	75 (±12)[e]
Mecoprop	Herbicide	9.6	A	37	386 (±408)	37	245 (±239)	37	29 (±25)	28	60 (±22)[a]	21	48 (±27)[e]
Propiconazole	Fungicide	6.9	A	19	59 (±28)	19	40 (±17)	19	28 (±16)	12	32 (±14)[c]	7	66 (±15)[c]
Terbutryn	Algicide	0.1	A	37	38 (±21)	37	19 (±16)	37	49 (±25)	28	85 (±10)[a]	20	80 (±13)[c]
Other common chemicals													
Aspartame	Sweetener	< 100	B	2	> 10000	1	> 4000	1	-	0	-	1	-
Benzothiazole	Industrial additive	400	B	2	6500 (±566)	1	1400	1	80	1	7[d]	1	> 71[c]
Benzotriazole	Corrosion inhibitor	4.1	A	37	9224 (±3112)	37	6948 (±1846)	37	24 (±22)	28	64 (±14)[a]	21	90 (±7)[e]
Caffeine	Food component	< 50	B	2	> 10000	1	820	1	> 92	1	> 92[b11]	1	65[f]
Galaxolidone	Fragrance (HHCB) metabolite	40	B	2	335 (±177)	1	220	1	52	1	0[d]	1	77[c]
Methylbenzotriazole	Corrosion inhibitor	48.5	A	19	5720 (±2810)	19	4201 (±2488)	19	29 (±24)	12	80 (±15)[a]	8	96 (±2)[e]
N,N-diethyl-3-methylbenzamide (DEET)	Insect repellent	< 50	B	2	805 (±445)	1	290	1	74	1	48[b8]	1	66[f]
Oxybenzone	UV filter	20	B	2	425 (±290)	1	60	1	90	1	> 67[a]	1	50[c]

[a] Similar removal (<10% difference) obtained with about 0.6 g O_3 g^{-1}DOC by Hollender et al. (2009). [b] Similar range of removal obtained in other studies ([1]Ternes et al. 2003, [2]Huber et al. 2005, [3]Ormad et al. 2008, [4]Wert et al. 2009, [5]Reungoat et al. 2010, [6]Rosal et al. 2010, [7]Huerta-Fontela et al. 2011, [8]Yang et al. 2011, [9]Senta et al. 2011, [10]Bundschuh et al. 2011, [11]Sudhakaran et al. 2012). [c] Not reported in other studies. [d] Contradictory to other studies (>10% lower removal) (Hollender et al. 2009, Reungoat et al. 2010 and 2012). [e] Similar removal (<10% difference) obtained with 10 to 20 mg PAC l^{-1} by Zwickenpflug et al. 2010 (in Abegglen et al. 2012). [f] Similar range of removal obtained with granular activated carbon (GAC) filters (Reungoat et al. 2010 and 2012, Yang et al. 2011)

3.3.2 Removal of micropollutants with advanced treatments

Both advanced treatments were able to reduce the micropollutant concentrations in the effluent significantly (Fig. 3.3 B and C, Table 3.3). The number of micropollutants with an average concentration above 1 µg l^{-1} in the effluent of the advanced treatments was reduced from 16 in the biologically treated wastewater to nine after ozonation and to seven after PAC-UF. Substances with concentrations > 1 µg l^{-1} after both treatments were the contrast media iohexol, iomeprol, iopamidol and iopromide, the pharmaceuticals gabapentin and metformin and the sweetener aspartame, and after ozonation additionally the chemicals benzotriazole and benzothiazole. The number of micropollutants with an average concentration above 100 ng l^{-1} was reduced from 52 (out of 70) in the biologically treated wastewater to 30 after both advanced treatments.

3.3.2.1 Ozonation

The removal percentages during the ozonation of the 40 micropollutants routinely analysed are presented in Table 3.3 (method A) and Fig. 3.3 B.

Substances with high ozone reactivity

Twelve substances were eliminated to over 90% even with the lowest ozone dose (2.3 mg O_3 l^{-1}, eq. 0.3 g O_3 g^{-1} DOC), including 4 antibiotics (trimethoprim, clindamycin, sulfamethoxazole and clarithromycin), 2 beta-blockers (sotalol and propranolol), 2 anti-inflammatory drugs (mefenamic acid and diclofenac), carbamazepine, gemfibrozil, estrone and bisphenol A. All these compounds contain electron-rich moieties such as phenols, anilines, olefins or amines (except gemfibrozil with a benzene derivate), which are known to have high ozone reactivity (second-order rate constant $k_{O3} > 10^4$ M^{-1} s^{-1}) (Lee and von Gunten, 2012). The removal of substances with lower reactivity was more dependent on the operational conditions, such as the ozone dose (from 2.3 to 9.1 mg O_3 l^{-1}) and the wastewater quality (presence of ozone and hydroxyl radical scavengers or competitors, pH, etc.), leading to higher variations in the transformation rate between the different sampling campaigns.

The macrolide azithromycin showed lower removals (average of 74%) than expected based on its reported high ozone reactivity (k_{O3} 6×10^6 M^{-1} s^{-1}) (Lee and von Gunten, 2012). One potential explanation is the possible sorption (up to 15% in WWTP effluent) of this substance to colloid particles (1 nm to 1 µm, considered as being part of the aqueous phase) (Maskaoui et al., 2007; Worms et al., 2010), which could protect it against ozone attack (Zimmermann et al., 2011a). Potential short-circuiting of a small water fraction through the reactor, which could reduce the exposure to ozone, may also explain the incomplete removal of very reactive substances such as azithromycin, diclofenac or carbamazepine.

Substances with low ozone but high OH radical reactivity

Ibuprofen, ketoprofen, metronidazole, primidone, mecoprop and benzotriazole, which have low reactivity with ozone ($k_{O3} < 350$ M^{-1} s^{-1} (Beltrán et al., 1994; Real et al., 2009; Rosal et al., 2010; Zimmermann et al., 2011a)) showed moderate average removals (around 60%), mainly due to reaction with the strong and unselective oxidant OH hydroxyl radical originating from reaction of ozone with

the organic wastewater matrix (Huber et al., 2003; Rosal et al., 2010). As the OH radical formation in wastewater is mainly due to the reaction of ozone with specific moieties of the effluent organic matter (EfOM), variation in the composition of EfOM, for instance by addition of coagulant, can lead to different amounts of OH radical formed per unit of ozone (Gonzales et al., 2012; Wert et al., 2011). Moreover, OH radical exposure varies with the concentration of $HO^{\cdot}$ scavengers (such as carbonate) and pH (Buffle et al., 2006). Reactions of micropollutants with OH radicals are thus more affected by the quality of the wastewater than direct ozone oxidation (Wert et al., 2011), which could explain the high removal variation observed for compounds with low ozone reactivity.

Substances with low ozone and low OH radical reactivity

Under the applied ozone doses (average 5.7 mg O_3 l^{-1} or 0.8 g O_3 g^{-1} DOC), only low removals (average of 34 to 43%) of the iodinated contrast media iohexol, iopromide, iomeprol, and iopamidol were obtained, with particularly low elimination (16%) of diatrizoic and iothalamic acids. Diatrizoate, the anionic form of diatrizoic acid, is one of the most ozone-resistant pharmaceuticals, having, as other contrast media, very low ozone reactivity, but also a low OH radical reactivity (Huber et al., 2005; Real et al., 2009). Low removals of atrazine (34%), gabapentin (38%), irgarol (32%) and propiconazole (32%) were also observed. Atrazine is reported to have low reactivity with ozone and OH radicals (Acero et al., 2000). Poor oxidation of gabapentin was also obtained in other studies (Reungoat et al., 2010). Low removals of the pesticides irgarol and propiconazole during ozonation were also observed by Bundschuh et al. (2011a), but for irgarol, higher removals were reported by Hollender et al. (2009). Irgarol is expected to have low ozone reactivity due to its triazine ring, which is very resistant to oxidation (Chen et al., 2008). The very low concentration of this substance in WWTP effluent (2 to 17 ng l^{-1}), close to the limit of quantification, leads however to high analytical uncertainties and could be the cause of the divergences observed. Resistance to ozonation is particularly of concern for the contrast media and gabapentin due to their high concentrations in wastewater (above 1 µg l^{-1}) and their persistence even for efficient biological treatment.

Removal efficiency with higher ozone doses

A higher ozone dose, 17.6 mg O_3 l^{-1}, equivalent to 2.6 g O_3 g^{-1} DOC, was tested during one campaign (data not illustrated). At this dosage, much better removal of the recalcitrant micropollutants was found, with 88% gabapentin elimination, 66% atrazine, and 84, 82 and 81%, respectively, of iopamidol, iohexol and iomeprol. Higher doses lead however to higher costs and a higher risk of forming bromate, a toxic by-product (see below "*Formation of toxic oxidation by-products*"), and therefore were not further tested.

Removal of other micropollutants and human drugs metabolites measured in a screening campaign

Table 3.3 presents also the removal of 23 other micropollutants (analytical method B), which were analysed only once on a 7-d composite sample (with 6 mg O_3 l^{-1}). Half of them were removed at a rate of over 70%. One can notice however the lower efficiency (< 51% removal) of ozone for the antihypertensive irbesartan, the anticonvulsant levetiracetam, the anxiolytic oxazepam and the insect repellent DEET. These substances contain amide functions that exhibit low reactivity with ozone (Lee and von Gunten, 2012). The human pharmaceutical metabolites, which are mainly hydroxylated, hydrolysed or conjugated forms of the parent compound (Ikehata et al., 2006), were mostly not as well removed as the parent compound. This is especially the case for the 10,11-dihydro-10,11-dihydroxy carbamazepine and N-acetyl sulfamethoxazole, with an elimination of only 50% compared to > 90% for carbamazepine and sulfamethoxazole. The lower ozone reactivity of the metabolites can be explained by the protective effect of the hydroxyl or acetyl group on the reactive moiety, which changes the electron density and thus slows down the reaction (Huber et al., 2005).

Influence of the pH on the oxidation process

Reactivity of a substrate with ozone is strongly influenced (up to 4 orders of magnitude) by the protonation of the reactive amine or phenol (Lee and von Gunten, 2012). Dissociated moieties have a higher electron density and thus are more reactive towards ozone (Lee and von Gunten, 2012). Due to their two pKa values close to the pH of wastewater (Table S 3.1, SI), the reactivity of fluoroquinolone antibiotics is particularly susceptible to pH variations. The variations of pH measured in the wastewater, from 6.3 to 8, can thus increase the reactivity of ciprofloxacin, norfloxacin and ofloxacin by 1 or 2 orders of magnitude (Dodd et al., 2006), explaining partially the high variation in the removal rates of these compounds during the different campaigns. This assumption is supported by the significant positive correlations observed between the pH and the removal rate of these three compounds (Fig. 3.6).

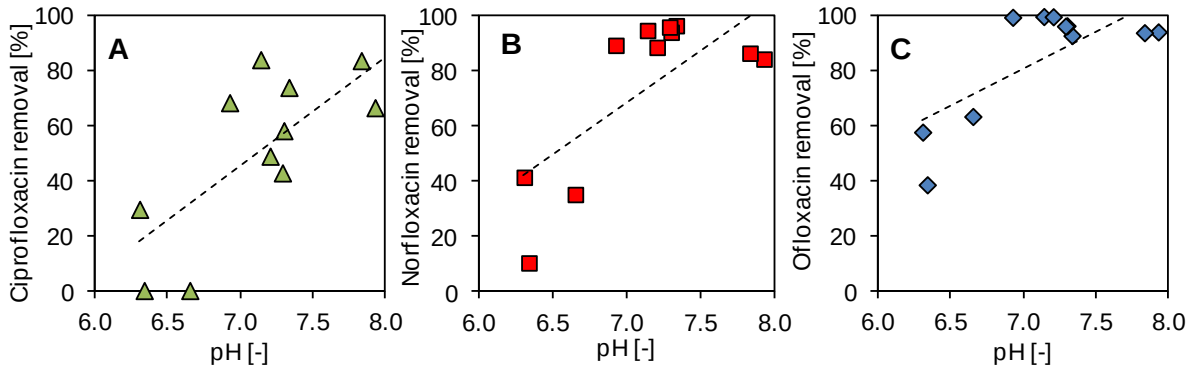

Fig. 3.6 Removal of fluoroquinolone antibiotics by ozonation (in the pilot plant) as a function of the feed water pH. (A) Ciprofloxacin. (B) Norfloxacin. (C) Ofloxacin. Ozone doses varied between 3 and 7 mg O_3 l^{-1} to maintain the same residual dissolved ozone concentration in the third chamber of the reactor. As the pH influences the ozone decomposition, higher ozone doses were usually required at higher pH to maintain a similar residual ozone concentration. These potentially higher ozone exposures at higher pH may thus also partially explain the correlation observed. However, no clear link between the ozone dose and the removal of these three compounds was evident, suggesting that the pH was the most influential factor. Correlations of the removal rate with the pH: ciprofloxacin ($r = 0.76$ $p = 0.004$), norfloxacin ($r = 0.73$, $p = 0.007$), ofloxacin ($r = 0.74$, $p = 0.006$).

Relation between ozone dose and micropollutant removal efficiency

Effluent organic matter containing electron-rich organic moieties and nitrite react rapidly with ozone, contributing to the ozone demand with 0.2-0.6 mg O_3 mg^{-1} C and 3.4 mg O_3 mg^{-1} $N\text{-}NO_2$ respectively (Wert et al., 2011; Wert et al., 2009). Thus, in order to have enough residual ozone for the oxidation of micropollutants and to assure a sufficient and relatively constant ozone exposure, the ozone dosage was regulated to maintain a constant ozone residual concentration near the end of the reactor. During the campaigns, the dosage varied from 2.3 to 9.1 mg O_3 l^{-1} depending mainly on the DOC (0.38 g O_3 g^{-1} C) and NO_2 (3.4 g O_3 g^{-1} N) concentrations (Fig. 3.7), but also due to the residence time of the water in the reactor and the choice of the chamber in which ozone was injected. No clear relation between ozone dose (in mg O_3 l^{-1}) and micropollutant transformation rate was evident. However, when the ozone dose was normalized by the concentration of scavenger equivalent, a weighted sum of DOC and NO_2 concentrations (3.4 [$N\text{-}NO_2$] + 0.38 [DOC]), higher doses (in g O_3 g^{-1} scavenger equivalent) tended to lead to higher removal rates for most micropollutants (Fig. 3.8). An average ozone dose of 5.7 mg O_3 l^{-1}, corresponding to 1.6 g O_3 g^{-1} scavenger equivalent or around 0.85 g O_3 g^{-1} DOC in the case of 0.3 mg $N\text{-}NO_2$ l^{-1} (or 0.61 g O_3 g^{-1} DOC in absence of NO_2), was sufficient (minimum dose) to achieve an average reduction of 80% of the 65 studied micropollutants in the WWTP (compared with raw wastewater).

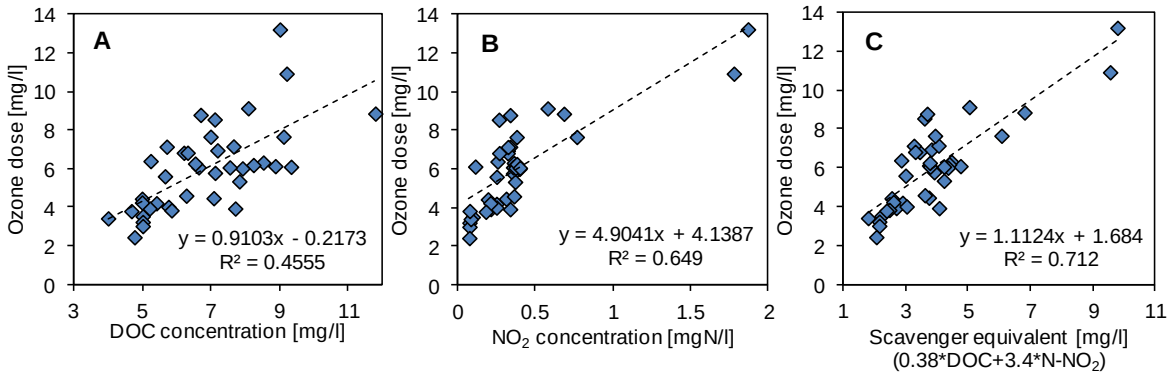

Fig. 3.7 Daily average ozone dosage in the reactor as a function of daily average concentrations of (A) dissolved organic carbon (DOC), (B) nitrite, and (C) scavenger equivalent, calculated by the optimal (maximizing R^2) weighted sum of DOC and NO_2 concentrations (in mg l^{-1}): 0.38 DOC + 3.4 $N\text{-}NO_2$. The ozone dose was regulated to maintain the same residual dissolved ozone concentration (~0.1 mg l^{-1}) in the third chamber of the reactor and thus varied depending on the oxidative demand of the water, mainly due to DOC and nitrite concentration.

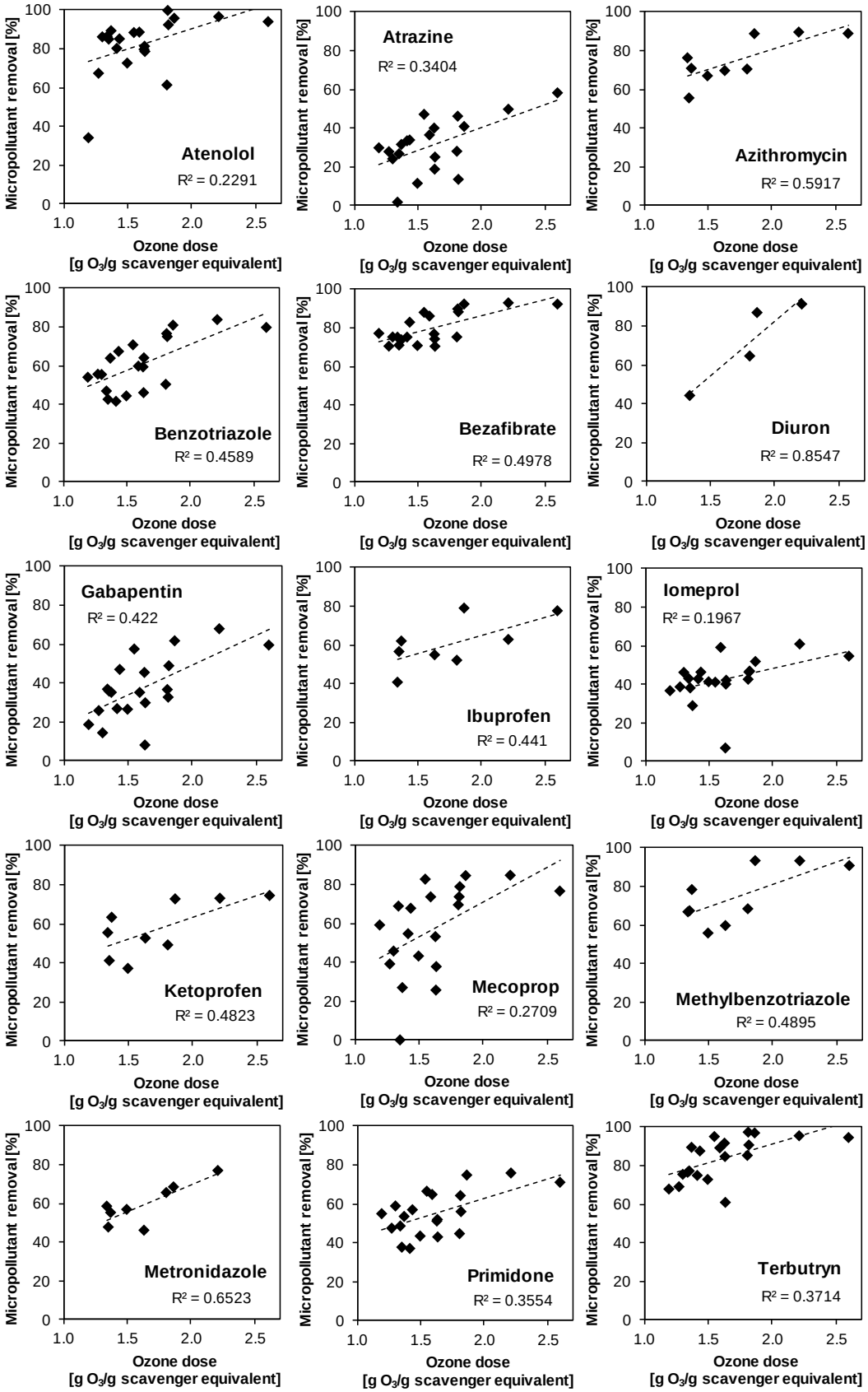

Fig. 3.8 Influence of the daily average ozone dose on the removal of 15 micropollutants by ozonation. Results of 20 campaigns conducted on the effluent of a moving bed bioreactor with partial nitrification. The ozone dose is normalized by the scavenger equivalent concentration, calculated by the weighted sum of DOC and NO_2 concentrations (in mg l^{-1}): 0.38 DOC + 3.4 N-NO_2.

Effect of the sand filter on micropollutant removal

The sand filter following the ozonation had only a limited effect on micropollutant removal, with a slight improvement in the average removal of 36 compounds from 73.2% for ozone alone to 75.8% for ozone combined with the sand filter. Higher removals (> 10%) were observed mainly for compounds that were well eliminated in an efficient biological treatment, such as ibuprofen, metronidazole and ciprofloxacin, and for two pesticides carbendazim and propiconazole, possibly due to sorption on the biofilm (Fig. 3.9).

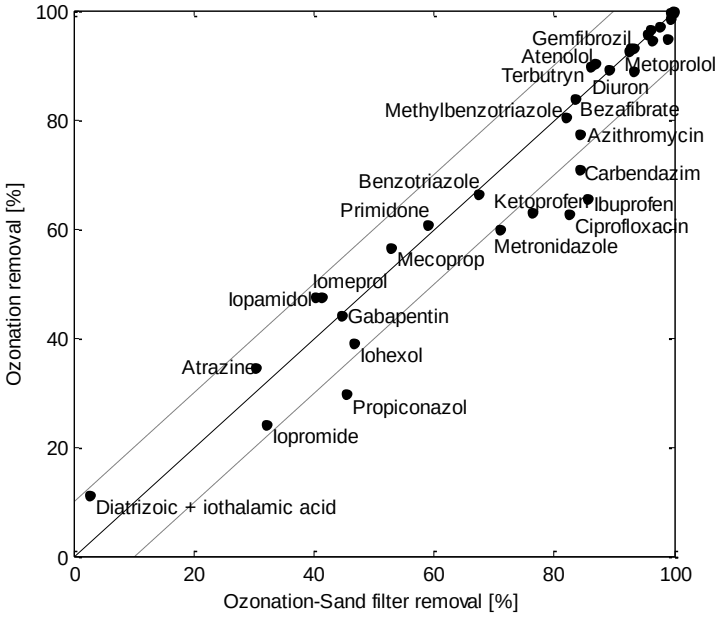

Fig. 3.9 Comparison of the removal of 36 micropollutants with ozone alone or with ozone followed by a sand filter (SF). Black line: similar removal by ozone alone or by ozone + SF. Dashed line: 10% difference between the removal by ozone alone or by ozone + SF. Average of 8 sampling campaigns (24 to 72-h composite samples). Average removal of the 36 compounds was 73.2% for ozone and 75.8% for ozone + SF.

Formation of toxic oxidation by-products

Formation of toxic oxidation by-products can occur during ozonation of wastewater, such as carcinogenic bromate, nitrosamines or formaldehyde (Wert et al., 2007; Zimmermann et al., 2011a). High concentrations of bromide (350 µg l^{-1}) measured in a 7-d composite sample in the wastewater suggested that excessive bromate formation could occur during ozonation (von Gunten, 2003b). The concentration of bromate was below the detection limit (1 µg l^{-1}) in the effluents of the biological treatment and PAC-UF. After ozonation (6 mg O_3 l^{-1}, equal to 0.8 g O_3 g^{-1} DOC) and sand filtration, the bromate concentration increased to 3.7 and 5.1 µg l^{-1} respectively. These concentrations remained however below the Swiss drinking water standard of 10 µg l^{-1} (OSEC, 1995) and far below the proposed ecotoxicologically relevant concentration of 3 mg l^{-1} (Hutchinson et al., 1997). The formation of bromate was dependent on the ozone dose applied, exceeding the drinking water standard for an ozone dose above 1.8 mg O_3 mg^{-1} scavenger equivalent (7 mg O_3 l^{-1}, with 70 µg l^{-1} bromide), as shown in a laboratory scale experiment (Fig. 3.10). Unlike nitrosamines that can be partially removed in a sand filter (Hollender et al., 2009), the bromate concentration was not reduced during the sand

filtration, and therefore a high ozone dose should be avoided to ensure low bromate concentrations in the effluent.

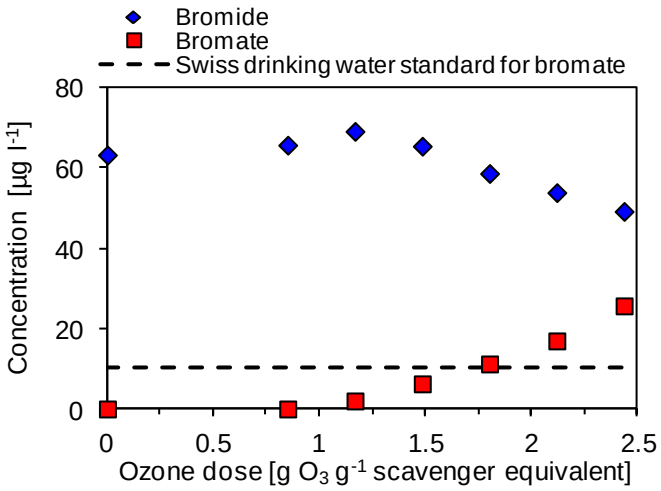

Fig. 3.10 Influence of the ozone dose on bromate formation. The ozone dose is normalized by the scavenger equivalent concentration, calculated by the weighted sum of DOC and NO_2 concentrations (in mg l^{-1}): 0.38 DOC + 3.4 N-NO_2. Laboratory-scale oxidation experiments were conducted on 24-h composite wastewater samples collected at the Lausanne WWTP after biological treatment with full nitrification (5 mg DOC l^{-1}, 0.6 mg N-NO_2 l^{-1}). Different amounts of a stock solution of dissolved ozone (in water) were added to the samples to reach the desired ozone concentration (from 0 to 9.6 mg O_3 l^{-1}). At low doses (< 1.2 g O_3 g^{-1} scavenger equivalent), only negligible oxidation of bromide to bromate occurred due to fast ozone consumption by nitrite and reactive DOC. Above 1.2 g O_3 g^{-1} scavenger equivalent, a linear relation between the ozone dose and bromate formation was observed. At 1.8 g O_3 g^{-1} scavenger equivalent (7 mg O_3 l^{-1}, or 1.4 g O_3 g^{-1} DOC), the Swiss drinking water standard for bromate (10 µg l^{-1}) was exceeded.

3.3.2.2 Powdered activated carbon treatment

The removal percentage during the PAC-UF treatment of the 40 micropollutants routinely analysed is presented in Table 3.3 (method A) and Fig. 3.3 C. High variations in the removal rate, especially for compounds with lower PAC affinity, were observed among the different campaigns. Indeed, to optimize the treatment the PAC dose was increased from 10 to 20 mg l^{-1} during the study. Moreover, the DOC concentration in the feed water was not constant, leading to variable competition for the adsorption sites between EfOM and micropollutants. As the type of PAC (Norit SAE SUPER and SORBOPOR MV-125) did not significantly influence the removal rate compared to other variables, results are presented for both PAC types together.

Substances with high PAC affinity

Seven substances were removed at a rate of more than 90% in almost all the campaigns, including the beta-blockers propranolol and metoprolol, as well as methylbenzotriazole, trimethoprim, mefenamic acid, estrone and carbendazim. In 50% of the campaigns, over 90% of the following compounds were removed as well: clarithromycin, carbamazepine, benzotriazole, ofloxacin, norfloxacin and atenolol. These 13 micropollutants have a very good affinity for PAC, with high elimination rates even with 10 mg PAC l^{-1}. Apart from the hydrophobic mefenamic acid, all those compounds were either positively charged (five substances) or neutral (seven substances) at the pH of the wastewater, covering a broad range of hydrophobicity (log D_{ow} from -1.3 to 3.7).

Substances with medium PAC affinity

A second group of 15 substances (from metronidazole to azithromycin on Fig. 3.3 C) had, on average, between 70 and 90% removal, including six neutral and six negatively charged compounds. The medium PAC affinity for diclofenac and gemfibrozil was reported elsewhere (Snyder et al., 2007; Westerhoff et al., 2005), but better removal of ibuprofen was observed in our case, either due to different PAC characteristics or to biodegradation phenomena in the reactor.

Substances with variable or low PAC affinity

The 12 remaining substances (from sulfamethoxazole to diatrizoic acid on Fig. 3.3 C), composed of neutral or negatively charged compounds (including all the hydrophilic contrast media), showed poor or very variable affinity for PAC with an average removal between 11 and 66%. The high removal variation observed for sulfamethoxazole, ciprofloxacin, mecoprop, primidone and the contrast media were partly due to the different PAC doses applied, with increasing removal when the dose increased from 10 to 20 mg l^{-1}. High variations (< 20% to > 60% removal) occurred also within the same PAC dose (mainly at 10 mg l^{-1}), which could not be explained by the different parameters monitored (water quality and operational parameters such as residence time, PAC concentration, PAC type, etc.). These high variations may be due to different EfOM content and composition, as discussed below.

The anionic contrast media diatrizoic and iothalamic acids and the anticonvulsant gabapentin showed less than 20% removal by PAC-UF. The low PAC affinity of these hydrophilic (log D_{ow} of -1.2 to -0.4) and charged substances were reported by Reungoat et al. (2010) and Boehler et al. (2012). Low adsorption of gabapentin could be caused by the absence of an aromatic ring (de Ridder et al., 2010). The variable elimination of irgarol (0% to > 60%), despite its hydrophobicity (log D_{ow} of 4), is probably due to its very low concentration in the feed water, leading to high uncertainties in estimates of the removal rate.

Removal efficiency with higher PAC dose

A higher PAC dose of 60 mg l^{-1} was tested during one campaign, leading to more than 90% removal of substances with a low PAC affinity (e.g., sulfamethoxazole, mecoprop, primidone and the contrast media iohexol, iomeprol and iopromide). Even this high dose was unable to remove gabapentin satisfactorily (56% removal, data not illustrated). Higher doses of PAC lead however to higher costs and larger amounts of sludge produced.

Removal of other micropollutants and human drug metabolites measured in a screening campaign

Table 3.3 shows the removal of 24 other micropollutants (analytical method B), which were analysed once on a 7-d composite sample (12 mg PAC l^{-1}). About half of them were removed at a rate of over 70%. We observe, however, a lower efficiency (< 60% removal) for most of the human pharmaceutical metabolites. Indeed, pharmaceutical compounds are usually transformed in the liver or kidney to more polar and hydrophilic metabolites in order to be readily excreted in the urine or bile (Ikehata et al., 2006). For instance, the metabolite 10,11-dihydro-10,11-dihydroxycarbamazepine has a log K_{ow} of 0.13 compared to 2.45 for the parent compound carbamazepine (Miao et al., 2005).

Therefore, due to the low hydrophobicity of human metabolites, a lower PAC affinity is expected. The low removal of the UV filter oxybenzone and the antidepressant venlafaxine is not explained, however, given the good PAC affinity for those substances reported in the literature (Reungoat et al., 2012; Snyder et al., 2007).

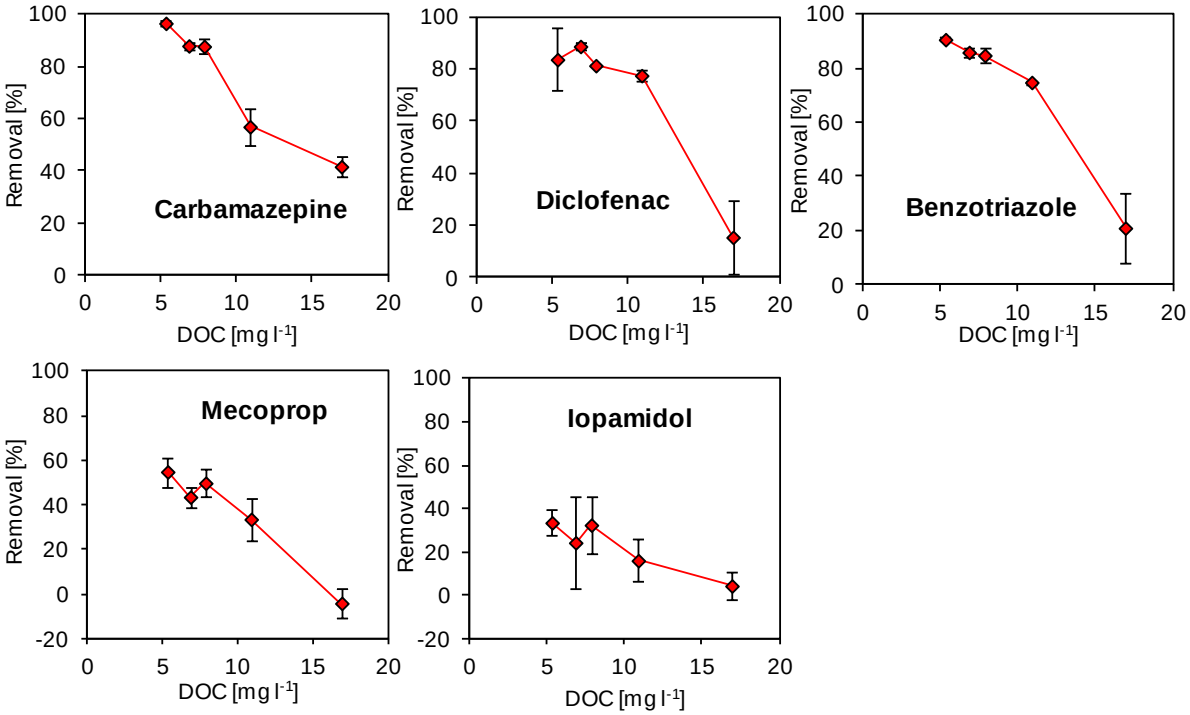

Fig. 3.11 Influence of dissolved organic carbon (DOC) wastewater concentration on powdered activated carbon (PAC) removal efficiency of five micropollutants in wastewater. Average (diamonds) and standard deviation (error bars) of triplicates. Laboratory-scale batch adsorption experiments were conducted on 24-h composite wastewater samples collected during the same period at the Lausanne WWTP after either simple coagulation-precipitation treatment (DOC of 17 mg l^{-1}), activated sludge treatment without nitrification (DOC of 11 mg l^{-1}), or moving-bed bioreactor treatment with full nitrification (DOC of 5, 7 and 8 mg l^{-1}). (10 mg l^{-1} PAC, SORBOPORTM MV-125).

Possible influence of effluent organic matter on removal efficiency

The adsorption process in complex matrix is not yet fully understood and can be influenced by many parameters, the main one being the competitive effect of the EfOM, either by direct competition for the adsorption sites or by pore blockage/constriction (Delgado et al., 2012). EfOM characteristics, mainly the concentration of low molecular weight and hydrophobic molecules, determine the competitiveness of the organic matter (de Ridder et al., 2011; Newcombe et al., 1997). Variation in the concentration and composition of the EfOM, due to different treatments of the wastewater (biodegradation, chemical coagulation, etc.) can thus lead to different micropollutant removal rates at the same PAC dose. This issue was investigated with laboratory batch adsorption experiments. Five micropollutants in Lausanne wastewater treated to different levels (coagulation/precipitation, biological treatment without nitrification or with full nitrification) were examined. A strong influence of the feed water DOC (from 5 to 17 mg l^{-1}) on the substance removal with PAC was observed for all the compounds (Fig. 3.11), confirming the high competitive effect of EfOM for the adsorption sites. The highest PAC efficiency was observed in the effluent of the biological treatment with full nitrification (DOC of 5 mg l^{-1}), significantly higher than in wastewater coming from a treatment

without nitrification (DOC of 11 mg l^{-1}). Wastewater treated only with coagulation/precipitation (DOC of 17 mg l^{-1}) led to a strong reduction of the PAC adsorption capacity, probably due to the presence of smaller biodegradable competitive molecules. Thus, different degrees of secondary treatment can lead to variable adsorption rates. Consequently, the PAC dose necessary to achieve an average overall micropollutant removal above 80% (whole treatment) in wastewater with a DOC of 5 to 10 mg l^{-1}, was variable: 10 mg l^{-1} was sometimes sufficient but in most cases 20 mg l^{-1} was required. These minimum doses were noted in other studies as well (Boehler et al., 2012; Nowotny et al., 2007).

Role of electrostatic and hydrophobic interaction in the adsorption process

Electrostatic and hydrophobic interactions seem to play an important role in the adsorption process. As presented in Fig. 3.12, on average more than 80% (most more than 90%) of all the positively charged molecules were removed, independently of their hydrophobicity. Only the large molecule azithromycin, diprotonated at pH 7, was eliminated to a lower extent despite its higher hydrophobicity, possibly by size exclusion in the micropores of the PAC (Ji et al., 2010). The removal of the negatively charged and neutral substances was more dependent on their hydrophobicity, the most hydrophilic compounds being eliminated to a lesser extent. For the same log D_{ow}, neutral and especially negatively charged compounds were on average less adsorbed than those that were positively charged.

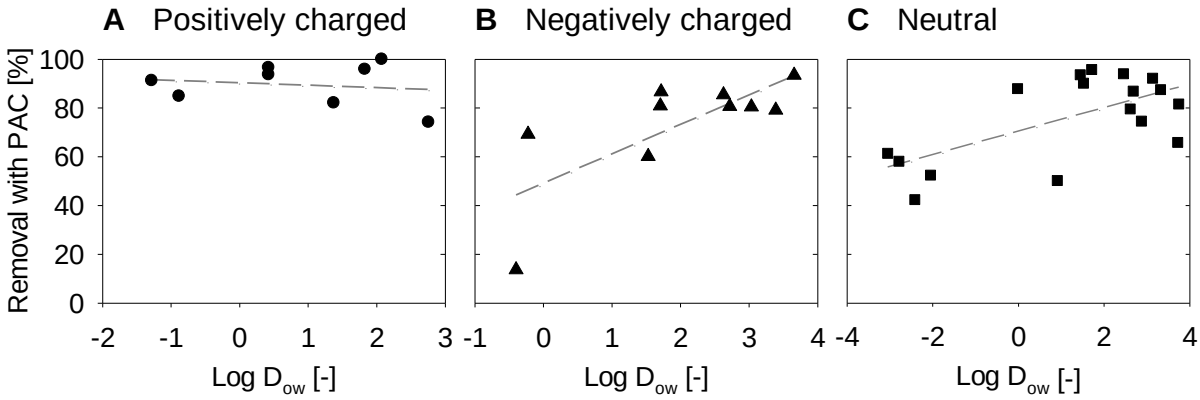

Fig. 3.12 Removal of 35 micropollutants with PAC-UF treatment as a function of micropollutant hydrophobicity (log D_{ow}) and charge at pH 7. (A) positively charged, (B) negatively charged, and (C) neutral. Median removal of eight 48-72 h composite samples. Correlation r between PAC removal and log D_{ow} not significant (p-value > 0.05) for positively charged compounds and zwitterions, and significant for negatively charged (r = 0.743, p = 0.014) and neutral compounds (r = 0.648, p = 0.005).

The two PACs studied have a point of zero charge pH_{PZC} > 7.3, thus the fresh PAC is expected to be neutral or slightly positively charged at the pH tested. However, in wastewater, the adsorption of EfOM, negatively charged at neutral pH, leads to a decrease in the PAC pH_{PZC} due to the EfOM coverage, resulting from a net negative surface charge on the loaded PAC (Newcombe, 1994; Yu et al., 2012). As both EfOM and micropollutant adsorption occurred simultaneously, electrostatic attraction between the cationic compounds and the negatively charged surface of the loaded PAC are expected, even for hydrophilic substances. Conversely, charge repulsion should occur for the anionic substances. These electrostatic repulsions can be offset by hydrophobic partitioning (expulsion in the solute-water system) at high log D_{ow}. Thus, in wastewater, hydrophobic interaction is expected to be

more significant for negatively charged and neutral compounds than for positively charged substances, as observed in our results. This assumption was tested in batch tests by de Ridder et al. (2011) where very similar behaviour was observed, confirming that both log D_{ow} and charge interaction have a significant influence on micropollutant adsorption in wastewater. But, for neutral or negatively charged substances, log D_{ow} was not by itself sufficient to explain the observed removals. Although hydrophobic partitioning has been reported as the dominant mechanism leading to PAC adsorption for compounds with log D_{ow} > 3.7, other adsorption mechanisms such as hydrogen bond formation and pi-pi interaction between micropollutants and the PAC surface have been reported to be more prominent as log D_{ow} decreases (de Ridder et al., 2010). Thus, for hydrophilic compounds with the same log D_{ow}, very different PAC affinities can be expected depending on the characteristics of the molecules.

Separation of PAC with ultra- or sand filtration – Influence on micropollutant removal

As observed in other studies (Snyder et al., 2007; Yoon et al., 2007), the influence of ultrafiltration on the removal of hydrophilic micropollutants (log K_{ow} < 2.8) is expected to be negligible due to the relatively high molecular weight cut-off of the membrane (100-300 kDa) compared to the molecular mass of micropollutants (< 1 kDa). For more hydrophobic compounds, significant adsorption on the membranes can occur (Yoon et al., 2007), but at a much lower level than on PAC. Therefore, PAC adsorption is considered to be by far the main removal process in the PAC-UF system. To check this assumption and to evaluate another (cheaper) separation system, a sand filter was used instead of the UF membrane during seven campaigns. Good PAC retention (> 90%) was observed with less than 1-3 mg TSS l^{-1} in the effluent. Similar micropollutant removal rates were measured with both separation systems (UF and sand filter), on average around 80%, indicating that the PAC, and not the ultrafiltration, was responsible for micropollutant removal.

3.3.3 Ecotoxicological evaluation

In addition to chemical analysis, the results of bioassays provided information on potential effects of the mixture of compounds. Both advanced treatments were able to reduce significantly the toxicity of the biological treatment effluent, both in bioassays with algae on enriched samples and in a chronic test on fish with continuous exposure to the raw effluent.

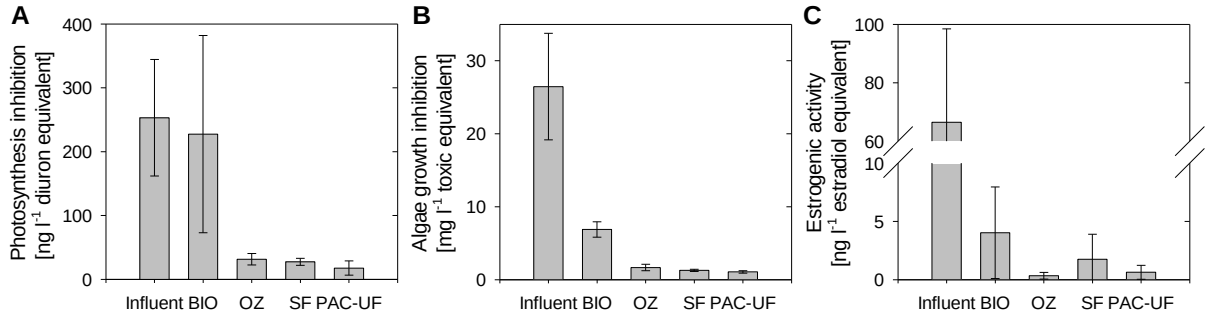

Fig. 3.13 (A) Inhibition of photosynthetic activity (diuron-equivalent concentration) and (B) inhibition of growth (toxic-equivalent concentration) of the green algae *Pseudokirchneriella subcapitata*. (C) Estrogenic activity (YES, estradiol-equivalent concentration). Average results (±standard deviation) of three campaigns of one week in the raw wastewater (influent) and in the effluents of the biological treatment (BIO), the ozonation (OZ), the sand filter following the ozonation (SF) and the PAC-UF treatment. Ozone doses of 3.5, 6.0 and 6.7 mg O_3 l^{-1} (eq. 0.76, 0.91, 0.92 g O_3 g^{-1} DOC), and PAC doses of 10, 12 and 20 mg l^{-1} for, respectively, campaigns 1, 2 and 3.

3.3.3.1 Combined algae assay on enriched samples

3.3.3.1.1 Photosynthesis inhibition

As presented in Fig. 3.13 A, raw wastewater induced photosynthesis inhibition equivalent to 253 ± 92 ng l^{-1} of diuron. This specific effect of substances acting on the photosystem II (Escher et al., 2008a) was not strongly reduced during the biological treatment (14 ± 37%, 228 ± 155 ng DEQ l^{-1}), suggesting low biodegradability of these compounds. However, both advanced treatments led to a clear decrease in this effect with 82 ± 8% removal (32 ± 9 ng DEQ l^{-1}) during ozonation and 87 ± 11% removal (18 ± 11 ng DEQ l^{-1}) during PAC-UF treatment. The residual toxicity was significantly lower ($p < 0.05$) after PAC-UF compared to OZ in campaigns 1 and 3 (no significant difference in campaign 2). Photosynthesis inhibition was not significantly reduced after the sand filter following ozonation (27 ± 5 ng DEQ l^{-1}), presumably due to the low biodegradability of those compounds. The overall removal in the WWTP was 87 ± 4% with ozonation followed by sand filtration and 92 ± 9% with PAC-UF treatment, showing the ability of these two treatments to improve the quality of the WWTP effluent.

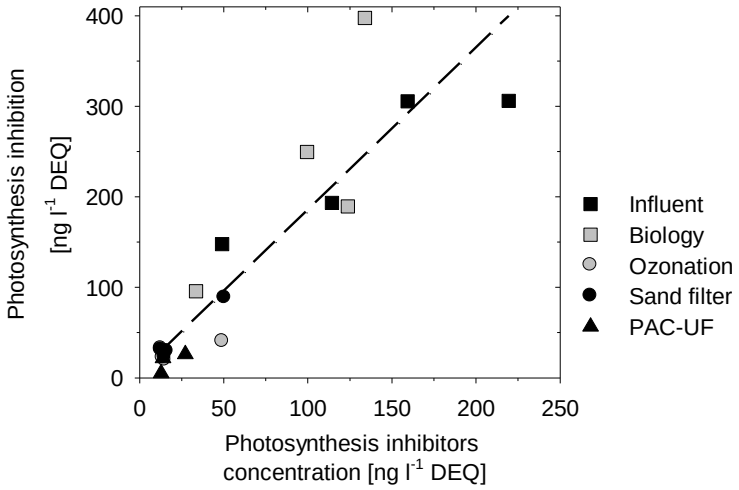

Fig. 3.14 Comparison of the green algae photosynthesis inhibition (in diuron-concentration equivalent DEQ) with the sum of the wastewater concentrations of the four most abundant photosynthesis inhibitors included in the analytical list (atrazine, diuron, isoproturon, and terbutryn), converted to DEQ based on their relative potency. Results of 19 analyses on 7-d composite samples taken after the different treatments. Dashed line: linear regression.

The herbicides atrazine, diuron and isoproturon, and the algicide terbutryn act as photosystem II inhibitors in plants and algae and can have a cumulative effect when present in a mixture (Brust et al., 2001; Knauert et al., 2010; Nyström et al., 2002). A clear relation (correlation $r = 0.909$, $p < 0.001$) between inhibition of the photosystem II and the concentration of relevant pesticides measured in the samples was observed (Fig. 3.14). The sum of the relative potency of these four compounds, expressed as diuron equivalents, could explain, on average, 56% of the total inhibition observed. The other (unmeasured) compounds participating in the remaining photosynthesis inhibition are expected to be eliminated to the same extent as these four inhibitors. Indeed, a reduction of the concentrations of these inhibitors in advanced treatments led to a similar reduction of the photosynthesis inhibition. Similar effects were observed for ozonation in a previous study at the Regensdorf WWTP, Switzerland (Escher et al., 2009).

3.3.3.1.2 Algae growth inhibition

A relatively high algae growth inhibition was observed in the raw wastewater (Fig. 3.13 B), with a non-specific toxicity of 26 ± 7.3 mg l^{-1} (baseline toxic equivalent concentration (Escher et al., 2008a)). This was clearly reduced (73 ± 6%, 6.9 ± 1 mg l^{-1}) during the biological treatment. This non-specific toxicity, contrary to the photosynthesis inhibition, can thus be partially attributed to biodegradable or adsorbable compounds that were removed in this treatment. The advanced treatments were able to reduce the residual toxicity (attributed to non-readily biodegradable micropollutants) by 75 ± 7% during ozonation (1.67 ± 0.45 mg l^{-1}) and 84 ± 5% during PAC-UF treatment (1.07 ± 0.17 mg l^{-1}). This toxicity was significantly lower after PAC-UF compared to OZ in campaigns 2 and 3 (no significant difference in campaign 1). The sand filtration following ozonation was also able to reduce the growth inhibition from 10 to 46% (mean: 1.28 ± 0.16 mg l^{-1}), the highest improvement being observed when the biological treatment was not effective, meaning that biodegradable toxic compounds remained in the ozone effluent. This resulted in a mean overall elimination (compared to WWTP influent) of 96 ± 1% with ozonation followed by sand filtration and 97 ± 0.1% with PAC-UF treatment. In a comparable study, Escher et al. (2009) detected a higher maximum reduction of non-specific toxicity during biological treatment (70 - 99.5%) at the Regensdorf WWTP and a subsequently lower removal efficiency during ozonation.

3.3.3.2 Estrogenic activity on enriched samples

High estrogenic activity was detected with the YES in raw wastewater (37-100 ng l^{-1} estradiol equivalents, EEQ), which was then strongly reduced (88 ± 10%) during the biological treatment (Fig. 3.13 C). The removal of estrogenic activity was dependent on the level of nitrification, from 75% without nitrification to 99% with full nitrification (< 1 mg $N\text{-}NH_4$ l^{-1}) (Fig. 3.15). The low estrogenicity level measured in the effluent of the biological treatment (0.7-8.3 ng l^{-1} EEQ) could, however, be sufficient to affect the fertility of sensitive fish species (Lahnsteiner et al., 2006), as shown also with the fish test (cf. §3.3.3.3). Estrogenic activity was further significantly diminished by 89 ± 4% during ozonation and 77 ± 17% with PAC-UF, which is similar to results obtained by Stalter et al. (2011) and Escher et al. (2009). This resulted in a mean overall elimination (compared to WWTP influent) of 99 ± 1% with both advanced treatments. The residual estrogenicity observed in the effluents, significantly lower after OZ (0.1-0.65 ng l^{-1} EEQ) than after PAC-UF (0.29-1.32 ng l^{-1} EEQ) in campaigns 1 and 2 (no significant difference in campaign 3), was in most cases below the environmental quality standard of 0.4 ng l^{-1} proposed for 17-β-estradiol (Kase et al., 2011). Therefore, advanced treatments or biological treatment with full nitrification are efficient means to reduce the release of endocrine compounds, and thus to reduce the risk of feminization of fish and mussel populations. As the estrogenic activity was already very low after the ozonation, there was no improvement due to the sand filter. During one campaign an increase in estrogenicity was observed, presumably due to contamination of the new sand by estrogenic compounds. Indeed, an unexplained increase in bisphenol A concentration was measured after the sand filter for this case.

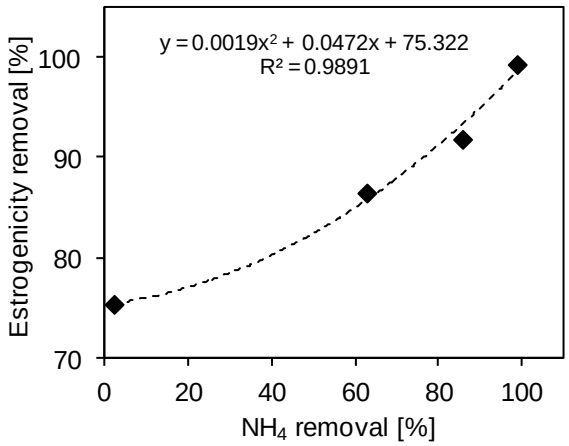

Fig. 3.15 Estrogenic activity removal in the biological treatment (activated sludge or moving bed bioreactor) as a function of the level of nitrification (NH_4 removal). Estrogenic activity was measured with the YES on four 7-d composite samples in the influent and effluent of the biological treatment with various levels of nitrification. Dashed line: fitted quadratic trend line.

3.3.3.3 Fish early life stage toxicity

Both advanced treatments significantly decreased the toxicity of the WWTP effluent on the development of rainbow trout embryos for all endpoints measured: the overall survival of the fish, the hatching success, the swim up, the individual development (weight and size) and the induction of estrogenic effects.

Overall survival

The overall survival of the rainbow trout after 69 d of continuous exposure in the effluent of the biological treatment (BIO) was relatively low, with only 58 ± 6.6% survival (Fig. 3.16 A). The survival was significantly improved after the ozonation (OZ) (85 ± 6.6% survival) and the PAC-UF treatment (93 ± 3.8%) reaching a level statistically similar to the control (95 ± 2% survival). The subsequent sand filtration (SF) step did not improve the survival of the fish compared to the ozonation alone.

Hatching success

The hatching success of the fish reached 80 ± 5% in BIO effluent, which was significantly lower than in the control (100% success). Both advanced treatments improved the hatching success to a level statistically similar to the control, with 97 ± 3.8% for OZ, 98 ± 2.9% for SF and 100 ± 0% for PAC-UF. However, the hatching progress was on average delayed for 2 d in OZ and SF effluents compared to PAC-UF or the control, and delayed for one week in the BIO effluent (Fig. 3.17 A). Delay in hatching after ozonation was also observed by Stalter et al. (2010b), and not notable in the sand filter effluent, as discussed below.

Swim-up

The swim-up, which is the developmental transition from larval stage to juvenile fish stage, appeared after 60 d in BIO effluent, delayed by 8 d compared to the control (Fig. 3.17 B). Both advanced treatments reduced the delay in the swim-up. The beginning of the swim-up appeared simultaneously

in PAC-UF effluent and in the control, but was delayed by 3 d compared to the control in OZ and SF effluents. A notable delay in the swim-up was also observed after ozonation by Stalter et al. (2010b), possibly due, in their case, to the presence of toxic oxidation by-products. 28% of the fish died during the larvae stage in BIO effluent, with only 45 ± 9% of the larvae reaching the juvenile stage at the end of the test. This was much improved after the advanced treatments, with 93.3 ± 3.8% of the larvae in PAC-UF effluent, 88.2 ± 10% in OZ, and 85.5 ± 10.4% in SF that swam up, showing no significant difference with the control (93.1 ± 3.1%).

Weight and length of the fish

Weight and length of the fish at the end of the test was relatively low in BIO effluent and increased significantly after the advanced treatments. Those parameters were however still significantly lower in OZ and SF effluents compared to the control, while no difference was observed in PAC-UF effluent (Fig. 3.16 B and C). The fish were on average 6.7% longer and 22% heavier in PAC-UF effluent than in OZ or SF effluents, and 32% longer and twice as heavy as in BIO effluent. The sand filter did not improve growth of the fish compared to the ozonation alone.

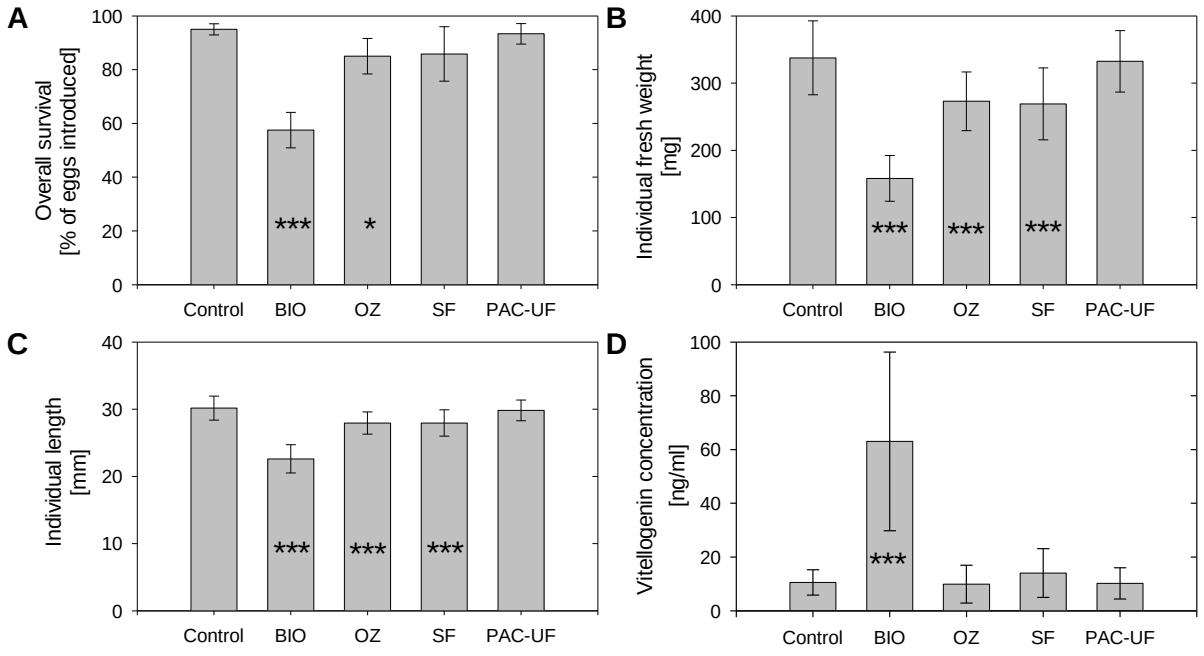

Fig. 3.16 Results of the Fish Early Life Stage Test (FELST) with (A) the overall survival (average of three replicates per treatment), (B) the individual fresh weight (average of 69 to 152 fish per treatment), (C) the individual length (average of 69 to 152 fish per treatment) and (D) the vitellogenin concentration (average of 20 fish per treatment) of the fish larvae at the end of the test (after 69 d). Significant differences with the controls are represented by * (p value < 0.05), ** (p < 0.01), *** (p < 0.001). All the endpoints for the control, OZ, SF and PAC-UF were significantly different from the endpoints of BIO. Ozone dose: 4.7 ± 1.5 mg O_3 l^{-1}. PAC dose: 13.1 ± 2.6 mg l^{-1}.

Vitellogenin concentration

The vitellogenin (VTG) concentration in the juvenile fish was significantly higher in the BIO effluent (63.1 ± 33.2 ng ml^{-1}) compared to the fish in the control (10.6 ± 4.7 ng ml^{-1}) (Fig. 3.16 D). Similar VTG concentrations (67.3 ± 26.9 ng ml^{-1}) were found by Stalter et al. (2010b) in juvenile rainbow trout exposed to secondary effluent. VTG, an egg yolk precursor normally produced by mature female

fish, can be used as a biomarker for exposure to exogenous estrogens for juvenile and male fish (Jobling et al., 2006; Thorpe et al., 2000). The increase of VTG content in juvenile fish in BIO effluent indicates the presence of environmentally- relevant concentrations of estrogenic compounds. This effect was not observed after both advanced treatments, the VTG content in the fish being on par with the control in PAC-UF (10.2 ± 5.8 ng ml^{-1}), OZ (9.9 ± 7.1 ng ml^{-1}) and SF effluent (14.1 ± 9.1 ng ml^{-1}). These results confirm the ability of ozonation and PAC-UF to eliminate the estrogenicity in wastewater, as presented in Fig. 3.13 C. The minor increase of VTG in the fish exposed to SF effluent compared to OZ effluent, also observed in the YES, is probably due to contamination of the new sand by endocrine active compounds.

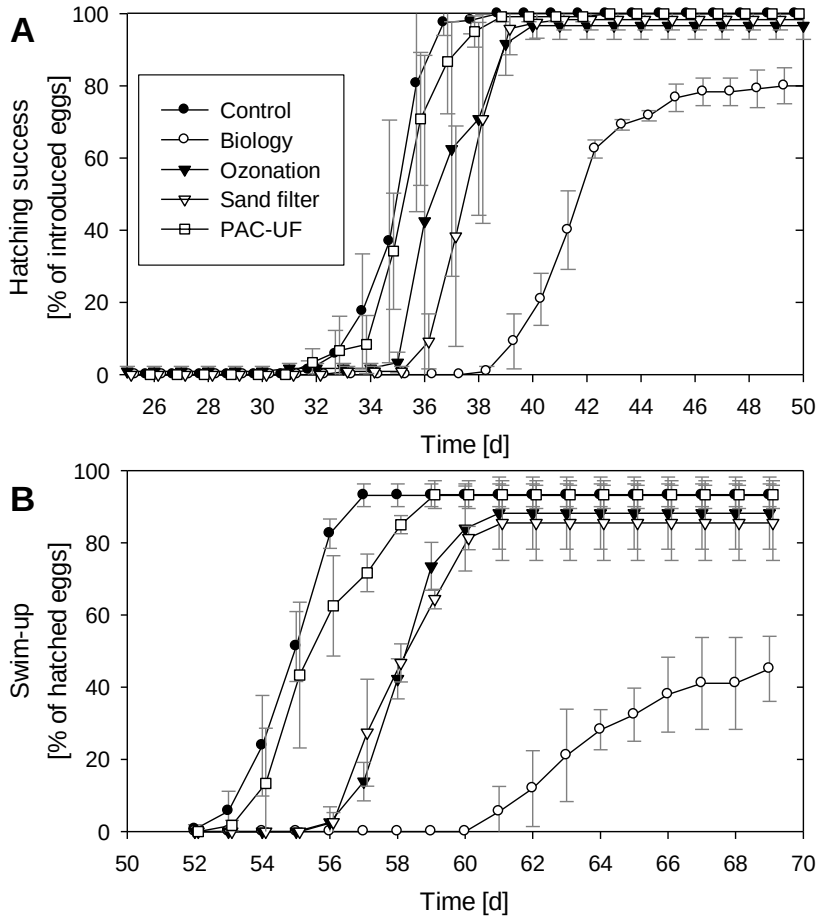

Fig. 3.17 Hatching success (A) and swim-up (B) of the eggs and larvae of the rainbow trout in the effluent of the different treatments. Average and standard deviation of 3 replicates. Ozone dose: 4.7 ± 1.5 mg O_3 l^{-1}. PAC dose: 13.1 ± 2.6 mg l^{-1}.

Toxicity of the biologically treated effluent and possible influence of nitrite and ammonia

As presented above, the effluent of the biological treatment impaired the survival and the development of rainbow trout, delaying their swim-up and their growth as expressed by lower biomass and body length. Besides the mortality observed (43%), a delay in the development can, for instance, increase the risk for predation in natural systems since larvae are unable to escape before the swim-up (Stalter et al., 2010b). Moreover, changes in VTG concentrations in fish can be an indicator for an effect on their reproduction system (Miller et al., 2007; Thorpe et al., 2007). Therefore, effluents from conventional WWTPs can have a significant impact on salmonid fish in natural environments in the

case of low dilution of the effluent. Besides the estrogenic substances and other micropollutants present in the effluent, macropollutants such as nitrite and ammonia or bacterial contamination could also affect the fish.

Rainbow trout are sensitive to nitrite (NO_2^-), with lower growth rates observed at 0.3 mg $N\text{-}NO_2^-$ l^{-1} and 65% mortality at 0.91 mg $N\text{-}NO_2^-$ l^{-1} (with 10 mg Cl^- l^{-1}) (Kroupova et al., 2008). The toxicity can, however, be strongly inhibited by chloride ions (Lewis and Morris, 1986). The relatively high concentration of chloride in the investigated wastewater (80-170 mg l^{-1}) could have therefore drastically reduced (up to a factor of 10) the toxic effect of nitrite. In the present study, NO_2^- concentrations varied between 0.04 and 0.55 mg $N\text{-}NO_2^-$ l^{-1} in BIO and OZ effluents and around 0.22 mg $N\text{-}NO_2^-$ l^{-1} in PAC-UF effluent. Those concentrations are very unlikely to have induced significant lethal and sub-lethal effects on the fish.

Embryos and larvae of rainbow trout are additionally very sensitive to ammonia (NH_3), the unionized form of ammonium NH_4^+. Sub-lethal effects such as a decrease in the larvae weight were observed after 20 d of exposure at 0.006 to 0.18 mg $N\text{-}NH_3$ l^{-1} (Vosylienė and Kazlauskienė, 2004) and a delay in development to the swim-up stage appeared at concentrations above 0.01 mg $N\text{-}NH_3$ l^{-1} (Brinkman et al., 2009). Lethal effects were reported for concentrations above 0.022 to 0.13 mg $N\text{-}NH_3$ l^{-1} (Brinkman et al., 2009; Solbé and Shurben, 1989). In the present study, the concentrations of unionized ammonia, calculated according to Armstrong et al. (2012), were relatively high in the BIO, OZ and SF effluents, varying between 0.02 and 0.06 mg $N\text{-}NH_3$ l^{-1} during the first 10 d, decreasing then below 0.01 mg $N\text{-}NH_3$ l^{-1} in all effluents until the end of the test. The NH_3 concentration in the PAC-UF effluent was always < 0.01 mg $N\text{-}NH_3$ l^{-1} due to further nitrification in the reactor. Ammonia concentrations in BIO, OZ and SF effluents at the beginning of the test were therefore high enough to induce sub-lethal effects and even mortality. Ammonia could be thus partly responsible for the lower weight and length of the fish exposed to OZ and SF effluents, as well as for their delay in reaching the swim-up stage. The clear impact on fish development and the high mortality observed in the BIO effluent is, however, not attributable to ammonia toxicity alone as much smaller impacts and mortality rates were observed with the same ammonia concentration in OZ effluent. Therefore, the toxicity observed in the BIO effluent can presumably be related to compounds oxidized during ozonation, such as pharmaceuticals and pesticides. This demonstrates that several compounds influencing rainbow trout development and survival in the BIO effluent were removed in the advanced treatment. Ozonation and activated carbon are therefore efficient techniques to reduce effects of micropollutants on fish.

Ozonation influence on fish toxicity

Stalter et al. (2010b) reported that fish toxicity increased during the ozonation process, probably due to the formation of labile oxidative by-products such as toxic aldehydes or metabolites. These adverse effects were reduced after the sand filtration, probably due to biodegradation or spontaneous degradation of the reactive products. Unlike Stalter et al. (2010b), in our case ozonation clearly reduced the fish toxicity compared to the BIO effluent to a level close to the control. Moreover, the sand filter did not affect the residual toxicity of the OZ effluent. These contradictory results are likely due to different ozone reactor configurations and/or different water compositions. Indeed, the reactor

used in Stalter et al. (2010b) contained 3 chambers with an HRT of only 3 to 15 min (Zimmermann et al., 2011a), risking release of toxic reactive products or even residual ozone in the effluent. In our case, the fourth large contact chamber (Fig. 3.1 A) ensured complete reaction of ozone and reactive products within the reactor, with an overall hydraulic residence time (HRT) of 20 to 60 min depending on the flow.

3.3.4 Costs and energy needs

The costs of the construction and the operation of the pilot plants are presented in Table 3.4 for an average micropollutant removal of 80% compared to raw wastewater. An average ozone dose around 5.7 mg O_3 l^{-1} and a PAC dose around 15 mg l^{-1} (between 10 and 20 mg l^{-1}) was needed to reach this level, remembering that the doses required varied according to the feed water quality. Although some substances were poorly eliminated with those doses, an average removal of 80%, as recommended by Swiss authorities, is a good compromise to reduce the load of micropollutants significantly while keeping the cost of the treatment in an acceptable range. Ozone-SF and PAC-SF had a similar cost (0.16-0.18 € m^{-3}) with a similar average removal rate. Compared to the average price and energy consumption of wastewater treatment in Switzerland (0.54 € m^{-3}, 0.33 kWh m^{-3}) (Abegglen and Siegrist, 2012), these two advanced treatments increased the costs and the electricity consumption by about 30%, which represents an annual increase of about 20 € per inhabitant. The PAC separation by ultrafiltration was not optimized, generating high electricity consumption and high costs because of the rapid clogging of the membrane. Additional tests on other more efficient ultrafiltration systems (12 months of operation) showed, however, that these prices could be reduced by a factor 4 to 5, reaching 0.16-0.25 € m^{-3}, with an electricity consumption of 0.1-0.2 kWh m^{-3} (Magnet et al., 2014). If these costs can be maintained for long term operation, UF separation will become a very competitive alternative, enabling high effluent quality. The costs of these advanced treatments (ozone-SF or PAC-SF) for larger WWTPs could be reduced to less than 0.12 € m^{-3} due to the scale effect (Abegglen and Siegrist, 2012).

Table 3.4 Costs and energy needs for construction and operation of the pilot plants. Costs are given excluding VAT, based on local (Swiss) prices in 2010 (0.17 € kWh^{-1} of electricity, 0.25 € Nm^{-3} O_2, 2 € kg^{-1} PAC, 66 € h^{-1} staff costs) for an average removal of 80% of the 65 studied micropollutants (compared to raw wastewater). Investment costs are calculated with an interest rate of 4.5% y^{-1}, with amortization periods of 10, 20 and 30 y for, respectively, electromechanical, mechanical and structural equipment.

		Ozonation with sand filter	**PAC with sand filter**	**PAC with ultrafiltration**
Dosage		5.7 mg O_3 l^{-1}	15 mg PAC l^{-1}	15 mg PAC l^{-1}
Capacity (average flow)	[l s^{-1}]	60	15	5
Electricity consumption	[kWh m^{-3}]	0.117	0.08	0.9
Operating costs	[€ m^{-3}]	0.043	0.054	0.404
Investment costs	[€ m^{-3}]	0.133	0.107	0.399
Total costs (excluding VAT)	**[€ m^{-3}]**	**0.176**	**0.161**	**0.803**

3.3.5 Comparison of the advanced technologies

3.3.5.1 Micropollutant removal

As presented in Table 3.3, ozone and activated carbon were both able to reduce of 80% or more the concentration of the majority of the micropollutants monitored. The average removal of the 40 substances routinely studied was very similar between ozone (71% with an average dose of 5.65 mg O_3 l^{-1}) and PAC-UF treatment (73% with an average dose of 13 mg PAC l^{-1}). However, for some compounds, different removal rates can be observed (Fig. 3.18). For instance, PAC-UF gave on average better removal of compounds without specific reactive moieties such as atrazine, propiconazole, ibuprofen or benzotriazole. On the other hand, ozone gave better removal of hydrophilic or negatively charged compounds such as gabapentin, sulfamethoxazole or diclofenac.

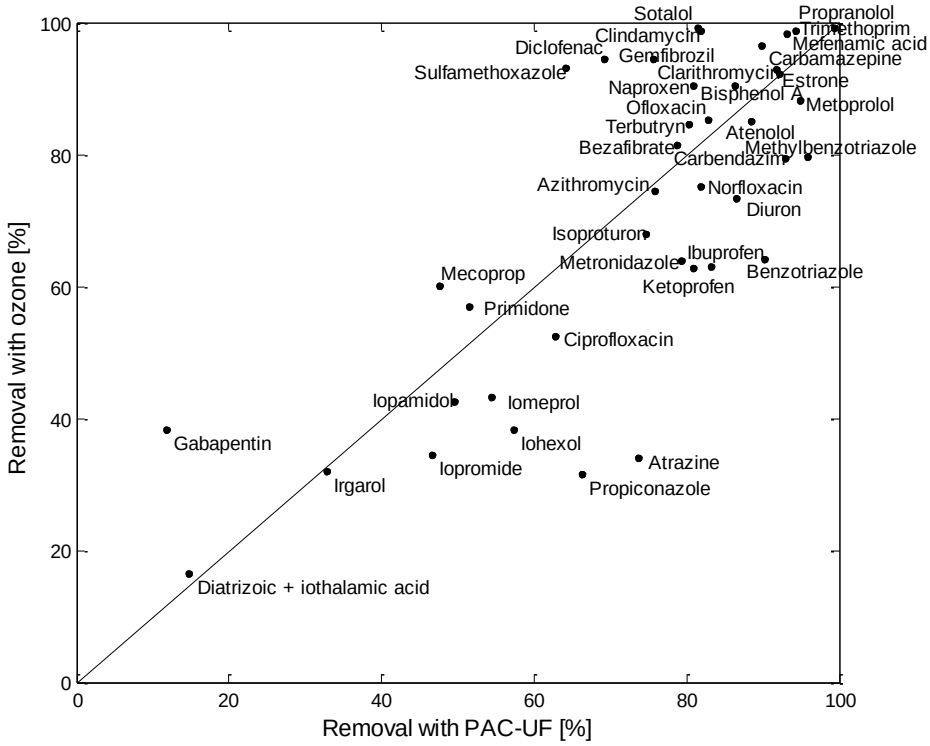

Fig. 3.18 Comparison of the average removal of 40 micropollutants with PAC-UF treatment (dose of 10-20 mg PAC l^{-1}, median 12 mg l^{-1}), or ozonation (dose of 2.3-9.1 mg O_3 l^{-1}, median 5.9 mg O_3 l^{-1} or 0.83 g O_3 g^{-1} DOC) during one year of operation (3 to 28 analyses depending on the substance).

Some micropollutants were resistant to both treatments, although they could be mostly removed with higher ozone and PAC doses. A more economically feasible alternative would be to avoid their release into the sewer system. For instance, collection of patient urine in separate containers within the 24 h after X-ray examinations, and treatment of this urine in a separate system (such as incineration) could avoid the release of persistent iodinated contrast media in wastewater (Heinzmann et al., 2008).

For the tested operation conditions and the micropollutants studied, ozone appeared to be more compound-specific than PAC. Many reactive compounds could be eliminated by more than 95% with the ozone dose applied but substances with low ozone reactivity were only partially removed. With PAC, fewer compounds were removed above 95% but also fewer substances were removed below

80%. Thus, for a same average removal of the 40 substances, PAC removed a broader range of compounds but to a lower degree than ozone. PAC efficiency was in general less predictable than for ozone, especially for compounds with low PAC affinity where high variations in the removal rate were observed, probably due to variation of the wastewater composition (competition for the adsorption sites).

Removal mechanisms are different in ozone and PAC. At the ozone dose applied, no mineralization to CO_2 seemed to take place (cf. §3.3.5.3), meaning that micropollutants were presumably transformed to (unknown) oxidation products. The transformation products are expected to lose their biological activity (Dodd et al., 2009; Larcher et al., 2012), but higher toxicity of the metabolites has also been reported in some cases (Larcher et al., 2012; Luster-Teasley et al., 2002; Rosal et al., 2009). Unlike ozone, PAC physically removes the micropollutant from the water, which avoids the release of unknown transformation products.

3.3.5.2 Toxicity removal

Ozone, with an average of 5.38 mg O_3 l^{-1} (eq. 0.86 g O_3 g^{-1} DOC), and activated carbon, with an average of 14 mg l^{-1}, were both able to reduce the toxicity of WWTP effluent significantly and with a relatively similar efficacy. PAC-UF was slightly more effective than ozone in reducing toxicity to algae (PAC: 84 % [79-88%], OZ: 75% [67-81%]), photosynthesis inhibition (PAC: 87% [77-99%], OZ: 82% [77-92%]) and fish development impact (PAC: similar to control, OZ: delay in the development). On the other hand, ozone was slightly better in reducing estrogenic activity (PAC: 77% [58-90%], OZ: 89% [85-92%]).

In other studies, increases in toxicity after ozonation compared to the feed water were observed, leading to mortality and delays in development of juvenile rainbow trout (Stalter et al., 2010b), reproduction inhibition of lumbriculus worms (Stalter et al., 2010a), mortality of zebra mussels (Stalter et al., 2010a) and growth inhibition of duckweed (Magdeburg et al., 2012). Increases of genotoxic and mutagenic potential after ozonation were also reported (Petala et al., 2008; Stalter et al., 2010a). These effects were attributed to the formation of toxic oxidation by-products during ozonation, such as aldehydes, which could then be removed after sand filtration. Our study gave different results, with a clear decrease of the toxicity after ozonation in all bioassays. No genotoxicity or mutagenicity (Micronucleus, UmuC and Ames test) was detected in OZ effluents (Kienle et al., 2011) despite the formation of bromate. This could be attributed to the longer reaction time in our OZ reactor, promoting the degradation of labile intermediate products (Petala et al., 2006). Reduction of toxicity during ozonation was also observed by Misík et al. (2011), Reungoat et al. (2010) and Takanashi et al.(2002), confirming that ozonation, if carefully designed, is comparable to PAC-UF treatment to improve the effluent quality.

3.3.5.3 General improvement of water quality

Advanced treatments had a positive impact on macropollutants and bacterial contamination, as presented in Fig. 3.19 and Fig. 3.20. The PAC-UF treatment, working as a bioreactor with addition of coagulant, enabled a significant reduction of the residual DOC (54 ± 10%), phosphorus (> 90%), NH_4 (85 ± 20%) and BOD_5 (72 ± 18%), and complete removal of TSS, intestinal bacteria and coliphages (<

5 UFP ml^{-1}, indicator of human viruses). The PAC-SF treatment had similar removal efficiencies for COD, TOC, DOC and NH_4, but was less effective in removing TSS and phosphorus, and afforded only very limited disinfection with no elimination of total viable bacteria, only 11% removal of *E. coli* and 79% removal of enterococci (data not illustrated). Effluent colour intensity was greatly reduced after PAC-SF and disappeared after PAC-UF. PAC alone had an influence only on DOC (20-35% removal) and colour removal. The biologically active filtration steps (UF or sand filter) were the main cause for improvement of general water quality, UF being more efficient than the sand filter.

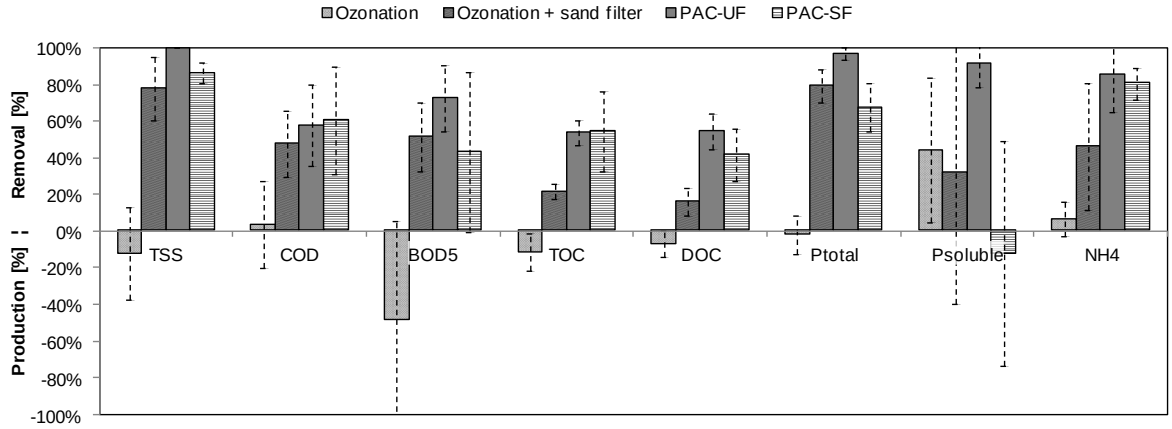

Fig. 3.19 Removal of macropollutants with ozone, ozone/sand filter, PAC-UF and PAC-SF. Average and standard deviation of 14 (9 for PAC-SF) 24-h composite samples. Ozone dose of 3.8-7.0 mg O_3 l^{-1}, PAC dose of 10-20 mg l^{-1}, coagulant (for PAC-UF only): 5-15 mg $FeCl_3$ l^{-1}. TSS: total suspended solid, COD: chemical oxygen demand, BOD_5: 5-d biochemical oxygen demand, TOC: total organic carbon, DOC: dissolved organic carbon, P_{total}: total phosphorus, $P_{soluble}$: dissolved phosphorus, NH_4: ammonium.

Ozonation was able to disinfect the effluent partially, with removal of coliphage virus below the detection limit (5 UFP ml^{-1}) (> 95% removal) and a reduction over 97% in the concentration of fecal bacteria; this level being below the European standard for good bathing water quality (European Commission, 2006) (Fig. 3.20). Ozonation alone was able to reduce the colour of the effluent but not to reduce the concentration of macropollutants (Fig. 3.19), with little effect only on soluble phosphorus probably due to residual precipitation with $FeCl_3$. The absence of DOC removal and the significant increase in BOD_5 (49 ± 54%) suggest that organic pollutants were not mineralized but transformed to more biodegradable compounds, which were then partially removed in the sand filter. The sand filter was responsible for most of the macropollutant removals, with 80 ± 13% of TSS, 79 ± 10% of P_{total}, 59 ± 21% of BOD_5, 44 ± 34% NH_4 and 20 ± 8% of DOC.

Due to its nonspecific removal mechanism, PAC is able to eliminate other kinds of micropollutants not analysed here, such as dissolved heavy metals (Cr, Fe, Zn or Pb), which is not the case for ozone even with a sand filter (Martin Ruel et al., 2011; Renman et al., 2009).

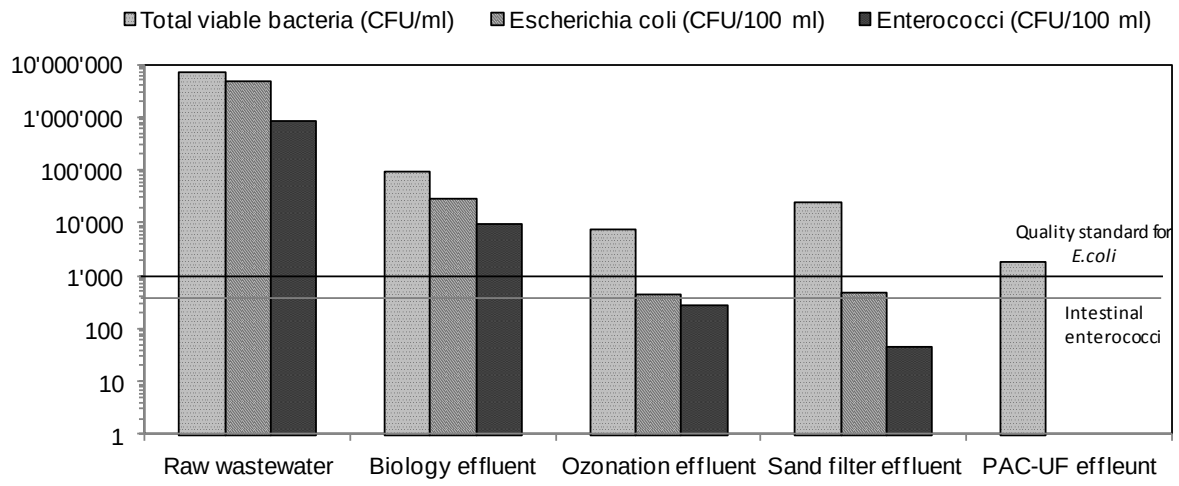

Fig. 3.20 Influence of the treatments on the concentration of indicator bacteria in the effluent. Average of two campaigns (grab samples) with 6.9 mg O_3 l^{-1} or 20 mg PAC l^{-1}. European standards for good bathing water quality (Directive 2006/7/EC) are given for E. coli (1000 CFU/100 ml) and intestinal enterococci (400 CFU/100 ml) as comparative values.

3.3.5.4 Feasibility and implications for WWTP

Both advanced treatments proved to be technically feasible at large scale in the municipal WWTP, with reasonable and relatively similar costs (0.16-0.18 € m^{-3}) in the case of PAC separation by sand filtration.

PAC with ultrafiltration separation was not economically competitive although this could change for this rapidly improving technology, especially considering the other beneficial effects of membranes on water quality (disinfection, total PAC and suspended solid retention). PAC separation by sand filtration showed a good retention of the suspended solids, but release of low amounts of loaded PAC into the effluent cannot be excluded, thus membrane systems represent a safer alternative.

The spent PAC has to be eliminated. Incineration with the sewage sludge is a good solution assuring complete mineralization of organic pollutants. Recirculation of the spent PAC to the biological treatment before its elimination can additionally improve the global micropollutant removal efficiency without impacting the quality of the biological treatment (Boehler et al., 2012; Zwickenpflug et al., 2010), improving by the way sludge dewaterability (Satyawali and Balakrishnan, 2009). Addition of 10 to 20 mg l^{-1} of PAC increased the WWTP sewage sludge production (dry matter) by 5 to 10% respectively. For plants that dispose sewage sludge on agricultural land (stopped in Switzerland in 2006), separate treatment of the PAC is necessary, increasing the costs.

Unlike ozonation where the dose was regulated by the oxidative demand of the water, PAC addition was only regulated by the flow to maintain a constant dose. Short pollution variations (< 1 d) are expected to be buffered by the long residence time and the high concentration of PAC in the system. But, in the case of longer pollution peaks, the treatment efficiency would likely be reduced. Regulation of the PAC dose by the amount of DOC in the feed water should be studied as DOC was shown to influence PAC efficiency.

Operation of the ozone reactor required staff training as well as specific safety measures due to the toxicity of ozone gas. As such, ozonation is not suitable for small WWTPs with non-permanent staff.

Optimization of these treatments in terms of energy and resource consumption remains. Although they were able to reduce aquatic toxicity, their energy and resource consumption is still significant and should for example be balanced by energy efficiency measures on the WWTP and in the sewer system. In all cases, the application of the treatment should be proportional to its benefit. Additional studies on the environmental impact of these advanced treatments taking into account their life cycle are thus necessary, with a special focus on the PAC due to its energy-intensive production (Larsen et al., 2010).

Given that the performance of these advanced treatments is relatively similar, selection of an optimal solution is nuanced. For a given WWTP the choice thus depends mainly on local conditions, involving consideration of multiple factors in a cost-benefit analysis.

3.4 Conclusions

- Of the 70 dissolved organic micropollutants detected in untreated wastewater, 50 were removed on average less than 50% in conventional treatment. Addition of a nitrification step significantly improved the removal of 24 substances.
- Both advanced treatments, ozonation and PAC-UF, reduced the concentration of the remaining compounds on average by more than 70%, with an average ozone dose of 5.65 mg O_3 l^{-1} or an average PAC dose of 13 mg l^{-1}.
- For the studied operation conditions, ozone appeared to be more compound-specific than PAC. Ozone was more effective in removing almost completely certain compounds and PAC acted better on a broad spectrum of micropollutants. Removal rates of micropollutants with low ozone reactivity or PAC affinity were depending more directly on variations in the feed water quality.
- Ozone efficiency was strongly dependent on the presence of micropollutants with electron-rich moieties. PAC efficiency was improved for hydrophobic or positively charged compounds.
- Both advanced treatments significantly reduced the toxicity of WWTP effluent, with PAC-UF performing slightly better overall.
- Both treatments proved to be feasible at large scale and for long term operation in real WWTP conditions, with similar costs if sand filters were used for the PAC retention.
- For sensitive receiving waters, such as recreational waters or drinking water resources, the PAC-UF treatment seemed to be the most suitable technology, despite its current higher costs and energy consumption. Indeed, PAC-UF treatment led to a good removal of most micropollutants and macropollutants without forming problematic by-products, the strongest decrease in toxicity and a total disinfection of the effluent.

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3.5 Supporting information

Supporting information associated with this chapter is presented below.

Table S 3.1 Physico-chemical properties of the 58 micropollutants routinely analysed. References: (Escher et al., 2011; Morasch et al., 2010; Reungoat et al., 2012; Schwarzenbach et al., 2003)

Compound	CAS-No	M [g/mol][a]	Log K_{OW}[a]	pK_a[a]	Charge at pH 7[b]	Log D_{ow} (pH 7)[c]	Type[d]
Pharmaceuticals							
Acipimox	[51037-30-0]	154.1	-0.52	3.3	-1	-2.1	A
Atenolol	[29122-68-7]	266.3	0.16	9.6	1	-1.3	B
Azithromycin	[83905-01-5]	749	4.02	8.7; 9.5	2	2.8	B
Bezafibrate	[41859-67-0]	361.8	4.25	3.7; 13.6	-1	2.7	A
Carbamazepine	[298-46-4]	236.3	2.45	13.9	0	2.5	N
Ciprofloxacin	[85721-33-1]	331.4	0.28	6.1; 8.8	1; Z; 0; -1	0.3	Z
Clarithromycin	[81103-11-9]	748	3.16	9.0	1	1.8	B
Clindamycin	[18323-44-9]	425	2.16	7.5	1; 0	1.4	B
Clofibric acid	[882-09-7]	214.7	2.57	3.5	-1	1.0	A
Diatrizoic acid	[117-96-4]	613.9	1.37	1.2; 7.9; 11.7	-1	-0.4	A
Diclofenac	[15307-86-5]	296.2	4.51	4.1	-1	3.0	A
Fenofibrate	[49562-28-9]	360.8	5.19	NA	0	5.2	N
Gabapentin	[60142-96-3]	171.2	-1.1	3.7; 10.0	Z	-1.1	Z
Gemfibrozil	[25812-30-0]	250.3	4.77	4.7	-1	3.4	A
Ibuprofen	[15687-27-1]	206.3	3.97	4.9	-1	2.6	A
Iohexol	[66108-95-0]	821.1	-3.05	NA	0	-3.1	N
Iomeprol	[78649-41-9]	777.1	-2.79	11.7; 12.6; 13.6	0	-2.8	N
Iopamidol	[60166-93-0]	777.1	-2.42	11.1; 12.9	0	-2.4	N
Iopromide	[73334-07-3]	791.1	-2.05	11.4	0	-2.1	N
Iothalamic acid	[2276-90-6]	613.9	0.5	2.1; 11.2;12.6	-1	-1.2	A
Ketoprofen	[22071-15-4]	254.3	3.12	4.5	-1	1.7	A
Mefenamic acid	[61-68-7]	241.3	5.12	4.2	-1	3.7	A
Metoprolol	[37350-58-6]	267.4	1.88	9.7	1	0.4	B
Metronidazole	[443-48-1]	171.2	-0.02	2.5	0	0.0	N
Nadolol	[42200-33-9]	309.4	0.81	9.7	1	-0.6	B
Naproxen	[22204-53-1]	230.3	3.18	4.2	-1	1.7	A
Norfloxacin	[70458-96-7]	319.3	-1.03	6.4; 8.7	Z; 0; -1	-1.0	Z
Ofloxacin	[82419-36-1]	361.4	-0.39	5.7; 7.1	Z; 0; -1	-0.4	Z
Paracetamol	[103-90-2]	151.2	0.46	9.4	0	0.5	N
Pravastatin	[81093-37-0]	424.5	3.1	4.5	-1	1.7	A
Primidone	[125-33-7]	218.3	0.91	NA	0	0.9	N
Propranolol	[525-66-6]	259.3	3.48	9.4	1	2.1	B
Simvastatin	[79902-63-9]	418.6	4.68	13.5	0	4.7	N
Sotalol	[3930-20-9]	272.4	0.24	8.2; 9.1	1	-0.9	B
Sulfadimethoxine	[122-11-2]	310.3	1.63	2.0; 6.7	-1	1.0	A
Sulfamethoxazole	[723-46-6]	253.3	0.89	1.8; 5.8	-1	-0.2	A
Trimethoprim	[738-70-5]	290.3	0.91	1.3; 7.2	1; 0	0.4	B
Endocrine disrupting compounds							
17α-Ethinylestradiol	[57-63-6]	296.4	3.67	10.4	0	3.7	N
Bisphenol A	[80-05-7]	228.3	3.32	10.1	0	3.3	N
Estriol	[50-27-1]	288.4	2.45	10.4	0	2.5	N
Estrone	[53-16-7]	270.4	3.13	10.3	0	3.1	N
Nonylphenol	[84852-15-3]	220.4	5.92	11.1	0	5.9	N
β-Estradiol	[50-28-2]	272.4	4.01	10.5	0	4.0	N
Pesticides and other common chemicals							
Atrazine	[1912-24-9]	215.7	2.61	1.7	0	2.6	N
Benzotriazole	[95-14-7]	119.1	1.44	8.4	0	1.4	N
Carbendazim	[10605-21-7]	191.2	1.52	4.2	0	1.5	N
Chloridazon	[1698-60-8]	221.6	1.14	3.4	0	1.1	N
Diazinon	[333-41-5]	304.4	3.81	2.4	0	3.8	N
Diuron	[330-54-1]	233.1	2.68	13.6	0	2.7	N
IPBC	[55406-53-6]	281.1	2.54	NA	0	2.5	N
Irgarol	[28159-98-0]	253.4	4.07	NA	0	4.1	N
Isoproturon	[34123-59-6]	206.3	2.87	NA	0	2.9	N
Mecoprop	[93-65-2]	214.7	3.13	3.1	-1	1.5	A
Methylbenzotriazole	[29385-43-1]	133.2	1.71	8.8	0	1.7	N
Propiconazole	[60207-90-1]	342.2	3.72	1.1	0	3.7	N
Tebufenozide	[112410-23-8]	352.5	4.25	NA	0	4.3	N
Terbutryn	[886-50-0]	241.4	3.74	4.3	0	3.7	N
Triclosan	[3380-34-5]	289.5	4.76	7.8	0; -1	4.8	N

[a] Source: Morasch et al. (2010), completed with Escher et al. (2011) and Reungoat et al. (2012). [b] Source: www.chemicalize.org (last accessed 25.10.2012) [c] $\log D_{ow} = \log K_{ow} - \log(1+10^{(pH-pKa)})$ for acids and $\log D_{ow} = \log K_{ow} - \log(1+10^{(pKa-pH)})$ for bases (Schwarzenbach et al. 2003). [d] A: acidic, B: basic, N: neutral, Z: zwitterion

Table S 3.2 Sample preparation for estrogens analyses and enrichment for the bioassays (YES, algae assay).

	Solid phase extraction for estrogens	Solid phase extraction for bioassays
General Information		
Sample type	Water samples	
Sample volumes	250 ml wastewater influent 500 ml wastewater effluent	200 ml wastewater influent 500 ml wastewater effluent
Blank	500 ml ultrapure water	
Sample preparation		
Filtration	Yes, with glass fibre filter type APFD 09050 (1 µm) (Millipore)	
Acidification	Yes, with HCl to pH 3	
Addition of isotope-labelled internal mixed standard solution (IS)	30 ng EE2-D4, E2-13C2, E1-D4, BPA-D16 and NP-13C6 to each sample	No
Sample enrichment	Solid phase extraction (SPE)	
SPE cartridges	LiChrolut EN RP-18 (bottom: 100 mg LiChrolut EN, top: 200 mg LiChrolut RP 18)	
Conditioning	6 ml Hexane 2 ml Acetone 6 ml Methanol 10 ml Water (pH 3.0)	2 ml Hexane 2 ml Acetone 6 ml Methanol 6 ml Water (pH 3.0)
Washing	8 ml Methanol/Water (70:30, v/v) 6 ml Acetonitrile/Water (30:70, v/v)	No, only filling of the cartridge with water (pH 3.0)
Elution	4 ml Acetone	4 ml Acetone 1 ml Methanol
Evaporation	With N_2 to ca. 100 µl	With N_2 to ca. 500 µl, then completing to 1000 µl with ethanol
Enrichment factor	1250 × wastewater influent 2500 × wastewater effluent	200 × wastewater influent 500 × wastewater effluent
Purification and storage of sample extract		
Sorbent	Mini silica gel columns (1.00 ± 0.01 g)	No
Application of sample	100 µl sample + 2 × 0.2 ml Hexane/Acetone (60:40, v/v)	
Elution	7.1 ml Hexane/Acetone (60:40, v/v)	
Evaporation	To dryness, fill-up with 200 µl Ethanol	
Storage	In the dark, at -20°C	

Table S 3.3 Specification for LC-MS/MS analytics of estrogenic active substances.

LC-MS/MS analysis	
LC-MS/MS instrument	API 4000 LC-MS/MS (Applied Biosystems, Warrington, UK)
HPLC separation	Gradient elution Eluent A = water/acetonitrile (90:10, v/v) Eluent B = acetonitrile/water (90:10, v/v)
HPLC column	MS C18 HPLC column (2.1 mm x 100 mm, particle size 3.5 µm)
Ionisation	Negative electrospray ionisation (ESI^-)
Calibration	0 - 200 ng/ml E1, E2 and EE2 mixed standards 0 - 2500 ng/ml NP+BPA standards
Replicates	2
Limit of quantification	E1 0.6 ng/l; E2 1.1 ng/l; EE2 3.0 ng/l; BPA 4.9 ng/l; NP 22.9 ng/l

Chapter 4 Role of nitrification in micropollutant removal - Aerobic granular sludge as an example

4.1 Introduction

While conventional wastewater treatment plants (WWTPs) are not specifically designed to treat polar and hardly biodegradable compounds, several studies highlight attenuation across biological treatment processes (Verlicchi et al., 2012). As shown in Chapter 3, high variability in micropollutant removal was observed in Lausanne WWTP, Switzerland, possibly partially attributed to the level of nitrification reached during the biological treatment. Good ammonium removal (complete nitrification) was linked to better removal of many micropollutants (e.g., bisphenol A, atenolol, bezafibrate, norfloxacin, ofloxacin, metronidazole, methylbenzotriazole, simvastatin, gemfibrozil, naproxen, ketoprofen, mefenamic acid, iohexol or iomeprol). Most of these compounds were removed less than 40% in the WWTP without nitrification and above 70-80% in the WWTP with complete nitrification. Similar results were observed in several other WWTPs. For instance, a wide study conducted on 28 WWTPs in canton Vaud, Switzerland, showed that 10 out of the 27 micropollutants studied (mefenamic acid, atenolol, bezafibrate, gemfibrozil, iomeprol, ketoprofen, metformin, metoprolol, naproxen and pravastatin) were significantly better removed in WWTPs with nitrification than without (DGE, 2013). Clara et al. (2005a) reported that the removal of several pollutants (bisphenol A, estrone, estradiol, estriol, ibuprofen and bezafibrate) in activated sludge were strongly correlated with the sludge retention time (SRT), with low removal (< 50%) at SRTs < 2 d, and high removal (> 80%) at SRTs > 10 d (SRT of 10 d corresponding to the minimum sludge age recommended for nitrogen removal in WWTPs (at 10°C)). An extensive study made on 16 WWTPs in the United Kingdom during one year showed a strong positive correlation between bisphenol A and estrone removal efficiencies and the level of ammonium removed via nitrification (Gardner et al., 2013). Similar correlations were found for ciprofloxacin, ibuprofen, triclocarban and 2-hydroxy-ibuprofen in six WWTPs in Canada (Guerra et al., 2014), and for iopromide and trimethoprim in a WWTP in USA (Batt et al., 2006). Better micropollutant removal in nitrifying WWTPs was also reported in several other studies (Drewes et al., 2002; Kreuzinger et al., 2004).

It is still not clear if these better removal efficiencies are due to (i) longer hydraulic retention times (HRTs) in nitrifying WWTPs (more time for biodegradation), (ii) longer SRTs (higher microbial diversity in the sludge, including slow growing organisms, increasing the number of possible metabolic pathways), (iii) lower food-to-microorganisms ratios (stimulation to metabolize less biodegradable compounds), (iv) co-oxidation by the enzyme ammonia monooxygenase (AMO) (responsible for ammonia oxidation), or (v) a combination of all these processes.

AMOs are intracellular oxidative enzymes produced by autotrophic ammonia-oxidizing bacteria (AOBs) or archaea (AOAs) to catalyse the oxidation of ammonium to nitrite, which is further oxidized to nitrate by nitrite-oxidizing bacteria (NOBs). AMOs are relatively unspecific enzymes and have been

reported to co-oxidize also aromatic compounds, probably via hydroxylation reactions (Khunjar et al., 2011; Yi and Harper, 2007). The role of AOBs in the removal of micropollutants in synthetic or spiked wastewater was investigated in several studies by selectively inhibiting the enzyme AMO with allylthiourea (ATU) in AOB-enriched activated sludge. A wide range of micropollutants was reported to be better removed with active (usually >70% removal) than inhibited (< 40% removal) AMOs, including naproxen, ketoprofen, gemfibrozil, diclofenac, indomethacin and fenoprofen (Tran et al., 2009), bisphenol A and nonylphenol (Kim et al., 2007), bezafibrate and synthetic estrogens (Maeng et al., 2013), triclosan (Roh et al., 2009), trimethoprim (Batt et al., 2006), atenolol (Sathyamoorthy et al., 2013) and artificial sweeteners (Tran et al., 2014), suggesting that this enzyme was involved in the degradation of these compounds. Several studies showed also that micropollutant degradation rates were positively linearly correlated with ammonia oxidation rates (Fernandez-Fontaina et al., 2012; Helbling et al., 2012; Tran et al., 2014; Yi and Harper, 2007) or with the initial ammonium concentration (Tran et al., 2009), indicating a link between nitrification and micropollutant removal. Most of these compounds were, however, also degraded (at a slower rate) with inhibited AMOs, showing that other metabolic or co-metabolic reactions occurred, probably linked to the heterotrophic microbial activity. Moreover, with real (not enriched) municipal activated sludge, despite better removal of several micropollutants were observed in sludge with higher nitrification capacities, inhibition of AMO did not significantly affect micropollutant (bisphenol A, triclosan, ibuprofen, ketoprofen, naproxen, atenolol, ranitidine, venlaflaxine and valsartan) removal rates (except for isoproturon), suggesting that the better removal observed with nitrifying sludge was not due to AMO oxidation but rather to differences in the heterotrophic bacterial community or to other enzymatic reactions in autotrophic organisms (Falås et al., 2012a; Helbling et al., 2012; Roh et al., 2009).

Although it has been proven that AMOs play an important role in micropollutant removal in strongly AOB-enriched sludge, it is still not clear what the role of AOBs (or AOAs) is in municipal activated sludge, where they make up less than 2-5% of the total biomass (Limpiyakorn et al., 2005; Limpiyakorn et al., 2011; Sathyamoorthy et al., 2013).

Aerobic granular sludge sequencing batch reactors (AGS-SBRs) are regarded as a promising technologies for municipal wastewater treatment due to their high compactness, simultaneous biological N- and P- removal ability (in only one tank), reduced energy and chemical consumption, reduced costs and simplicity/flexibility of operation compared to conventional activated sludge processes (Giesen et al., 2013; van der Roest et al., 2011). Aerobic granules are formed by self-aggregation of microorganisms which perform different and specific functions, such as chemical oxygen demand (COD) abatement, nitrification, denitrification and phosphate accumulation. All the processes can occur simultaneously within the same granules due to the variety of redox conditions present: aerobic in the outside layer and anoxic/anaerobic in the inside layers, due to limitation of oxygen penetration depth related to heterotrophic and nitrifying activity (Lochmatter et al., 2013). As the COD concentration in the influent is often the limiting parameter to allow high P- and N-removal, AGS-SBR are mainly operated in a way to avoid aerobic heterotrophic activity, in order to save COD for anoxic heterotrophic denitrifying and phosphate accumulating organisms (Lochmatter et al., 2013). The good nitrogen removal ability of granular sludge and their relatively low content of strict aerobic heterotrophic organisms make this system a good model to study the direct effect of nitrification on

micropollutant abatement. Indeed, AGS-SBRs allow treating a wastewater with high COD content (close to real municipal wastewater), while limiting potential micropollutant biodegradation by aerobic heterotrophic organisms. Moreover, the potential of AGS-SBRs for micropollutant removal in municipal-like wastewater is still largely unknown, as only few studies have been performed on this topic (Amorim et al., 2014).

The aims of this study were thus (i) to assess the potential of aerobic granular sludge for micropollutant removal in wastewater, and (ii) to study to direct effect of nitrification on the removal of micropollutant, in order to clarify the reasons leading to better micropollutant removal in nitrifying WWTPs: co-oxidation by AMO, longer reaction time or more diverse microbial community. To answer to these questions, two similar laboratory-scale AGS-SBRs were operated in parallel, one with complete nitrification and the other with inhibition of ammonia oxidation, treating a synthetic wastewater spiked with 36 micropollutants. Their micropollutant removal efficiencies were then compared with the ones of a full-scale WWTP with various level of nitrification, to corroborate the results.

4.2 Materials and methods

4.2.1.1 Synthetic wastewater composition

A synthetic wastewater, with general characteristics (COD, P and N concentrations) similar to municipal wastewaters, was used to exclude any strong fluctuation of the matrix composition and to allow good development of the granular sludge (controlled conditions).The influent wastewater consisted of a mixture of two synthetic media (one containing the carbon source: acetate and propionate, one with the nutrients N and P) diluted nine times in lake water (from Lake Geneva, filtered at 1 mm), as described by Lochmatter et al. (2013). The final wastewater contained 286 mg l^{-1} sodium acetate, 194 mg l^{-1} sodium propionate, 48 mg l^{-1} $MgSO_4$, 40 mg l^{-1} KCl, 201 mg l^{-1} NH_4Cl, 78 mg l^{-1} K_2HPO_4, 31 mg l^{-1} KH_2PO_4, and trace elements (in addition to the elements already present in lake water: 53 mg l^{-1} EDTA, 1.3 mg l^{-1} $ZnSO_4$, 6.5 mg l^{-1} $CaCl_2$, 3.4 mg l^{-1} $MnCl_2$, 2.9 mg l^{-1} $FeSO_4$, 1.6 mg l^{-1} $(NH_4)_6Mo_7O_{24}$, 1.1 mg l^{-1} $CuSO_4$, and 0.9 mg l^{-1} $CoCl_2$). This composition resulted in concentrations of about 450 mg l^{-1} of COD (half due to acetate, half due to propionate), 50 mg $N\text{-}NH_4$ l^{-1} and 20 mg $P\text{-}PO_4$ l^{-1}, thus comparable to concentrated municipal wastewater (Metcalf and Eddy, 2003). The initial pH of the synthetic wastewater was at 7.2.

For the micropollutant degradation experiment, the synthetic medium containing the carbon sources was spiked with a mixture of 36 micropollutants (mainly pharmaceuticals and pesticides), resulting in a final concentration in the synthetic wastewater of around 1 µ l^{-1} for each pollutant (cf. Table 4.1). These micropollutants were selected due to their ubiquity in municipal wastewaters (cf. Chapter 3). As the stock solution of micropollutants was prepared in methanol, this resulted to add 44 mg l^{-1} of methanol in the synthetic wastewater, increasing the theoretical COD up to 515 mg l^{-1}.

Allylthiourea (ATU, $C_4H_8N_2S$), a selective inhibitor of ammonia oxidation by AOBs, was added in the synthetic wastewater of one reactor at a final concentration of 11.1 mg l^{-1} (95 µM) to inhibit nitrification. This concentration was reported to instantaneously and selectively inhibit ammonia

oxidation in activated sludge by chelation of the copper from the active site of the ammonia monooxygenase (AMO) (Temizer Oguz, 2005). ATU is reported to have no effect on the second step of nitrification, that is oxidation of nitrite to nitrate by nitrite oxidizers (NOBs) (Temizer Oguz, 2005).

Table 4.1 Micropollutants studied, with their initial concentration in the synthetic wastewater, and their analytical limit of quantification and recovery rates.

Compound	Compound class	Analytical method[a]	LOQ[b] [ng l^{-1}]	Recovery rate[c] [%]		Initial concentration [ng l^{-1}]			
				Influent	Effluent	Theoretical[d]	Measured[e]	CV[f] [%]	Losses[g] [%]
Atenolol	Beta blocker	A	1	100	99	1197	1247 (± 16)	1	-4
Atrazine	Herbicide	A	1	108	108	1205	1202 (± 74)	6	0
Azithromycin	Antibiotic	A	1	95	86	1108	775 (± 88)	11	30
Benzotriazole	Corrosion inhibitor	A	1	119	106	1179	1318 (± 168)	13	-12
Bezafibrate	Lipid regulator	N	5	111	107	1118	1122 (± 23)	2	0
Bisphenol A	Plastic component	N	6	217	54	1233	843 (± 193)	23	32
Carbamazepine	Anticonvulsant	A	1	105	100	1353	1372 (± 66)	5	-1
Clarithromycin	Antibiotic	A	1	112	101	1190	1092 (± 65)	6	8
Diclofenac	Analgesic / Anti-inflammatory	A	1	95	94	1149	1160 (± 75)	7	-1
Diuron	Herbicide	A	1	78	72	1084	863 (± 20)	2	20
Estriol	Hormone	N	100	116	42	1105	713 (± 81)	11	35
Estrone	Hormone	N	70	44	102	1188	567 (± 168)	30	52
Gabapentin	Anticonvulsant	A	1	93	99	1074	1050 (± 57)	5	2
Gemfibrozil	Lipid regulator	N	1	98	95	1036	1018 (± 22)	2	2
Ibuprofen	Analgesic / Anti-inflammatory	N	20	113	96	1248	1160 (± 48)	4	7
Iohexol	Iodinated contrast medium	N	20	187	103	4662	5484 (± 1384)	25	-18
Iomeprol	Iodinated contrast medium	N	5	97	116	5500	5421 (± 311)	6	1
Iopamidol	Iodinated contrast medium	N	4	63	94	4454	2413 (± 61)	3	46
Iopromide	Iodinated contrast medium	A	30	100	114	6860	7344 (± 437)	6	-7
Irgarol	Algicide	A	1	102	86	1064	604 (± 44)	7	43
Isoproturon	Herbicide	A	1	84	78	1197	1000 (± 22)	2	16
Ketoprofen	Analgesic / Anti-inflammatory	N	1	104	98	1134	1145 (± 59)	5	-1
Mecoprop	Herbicide	N	1	94	100	1178	1184 (± 34)	3	-1
Mefenamic acid	Analgesic / Anti-inflammatory	N	1	118	135	1145	1156 (± 26)	2	-1
Metformin	Antidiabetic	A	1	77	78	1216	1393 (± 50)	4	-15
Methylbenzotriazole	Corrosion inhibitor	A	1	104	106	1145	1206 (± 35)	3	-5
Metoprolol	Beta blocker	A	1	84	85	1219	1160 (± 18)	2	5
Metronidazole	Antibiotic	N	1	83	84	1311	1040 (± 34)	3	21
Naproxen	Analgesic / Anti-inflammatory	N	1	80	110	1207	1200 (± 33)	3	1
Paracetamol	Analgesic / Anti-inflammatory	A	1	113	117	1195	1234 (± 55)	4	-3
Primidone	Anticonvulsant	A	1	106	99	1217	1327 (± 64)	5	-9
Sotalol	Beta blocker	A	1	84	79	1147	1107 (± 130)	12	4
Sulfamethoxazole	Antibiotic	A	1	89	99	1062	1041 (± 19)	2	2
Terbutryn	Algicide	A	1	133	112	1283	857 (± 68)	8	33
Triclosan	Biocide	N	30	100	116	1115	362 (± 47)	13	68
Trimethoprim	Antibiotic	A	1	97	97	1229	1254 (± 376)	30	-2

[a] A: acid UPLC mobile phase, column HSS T3. N: neutral UPLC mobile phase, column BEH C18

[b] Limit of quantification (LOQ), based on the lowest standard that could be clearly quantified

[c] Recovery rate (after correction with deuterated standards) of the samples (influent and effluent of the reactor) spiked with a known amount of pollutant (ratio measured/theoretical concentrations)

[d] Initial theoretical concentration (based on the quantitiy of micropollutant spiked)

[e] Initial real concentration, measured 5 d after the preparation of the synthetic wastewater (storage at ambient temperature, non-sterile conditions). Average and standard deviation of four analyses

[f] Coefficient of variation (CV) (reproducibility) of four analyses of the influent concentrations

[g] Differences between the theoretical and measured initial concentrations. Due to losses during the storage time or analytical uncertainties

4.2.1.2 Reactor setup and sequencing batch operation

The reactor setup was similar to the one used by Lochmatter et al. (2013). Two identical double-walled bubble column reactors (glass columns, 5.2 cm internal diameter, 142 cm height) with a working volume of 2.6 l each were used. The reactors were fed with the synthetic wastewater and continuously operated in sequential batch (SBR) mode. The SBR cycle, of a duration of 4 h 12 min, was composed of four steps (Fig. 4.1): (i) anaerobic/anoxic feeding with synthetic wastewater during 60 min (21.7 ml min^{-1}), followed by 2 min of anaerobic mixing (with nitrogen gas); (ii) intermittent

aeration during 180 min, with alternation of 25 min aerated (3.6 l_{air} min^{-1}) and 5 min without aeration; (iii) 5 min settling; and (iv) 5 min for withdrawal. The effluent was withdrawn at the half of the height of the column, resulting to a water exchange ratio of 50% per cycle. The temperature was regulated in both reactors at 20 ±1°C, and the pH was controlled between 7.0 and 7.5 (by injection of NaOH or HCl 1 M). An intermittent aeration strategy (Fig. 4.2) was chosen to stimulate the denitrification in the reactor, according to Lochmatter et al. (2013). Each reactor was treating 7.44 l d^{-1}, resulting in an average hydraulic retention time (HRT) of about 8 h.

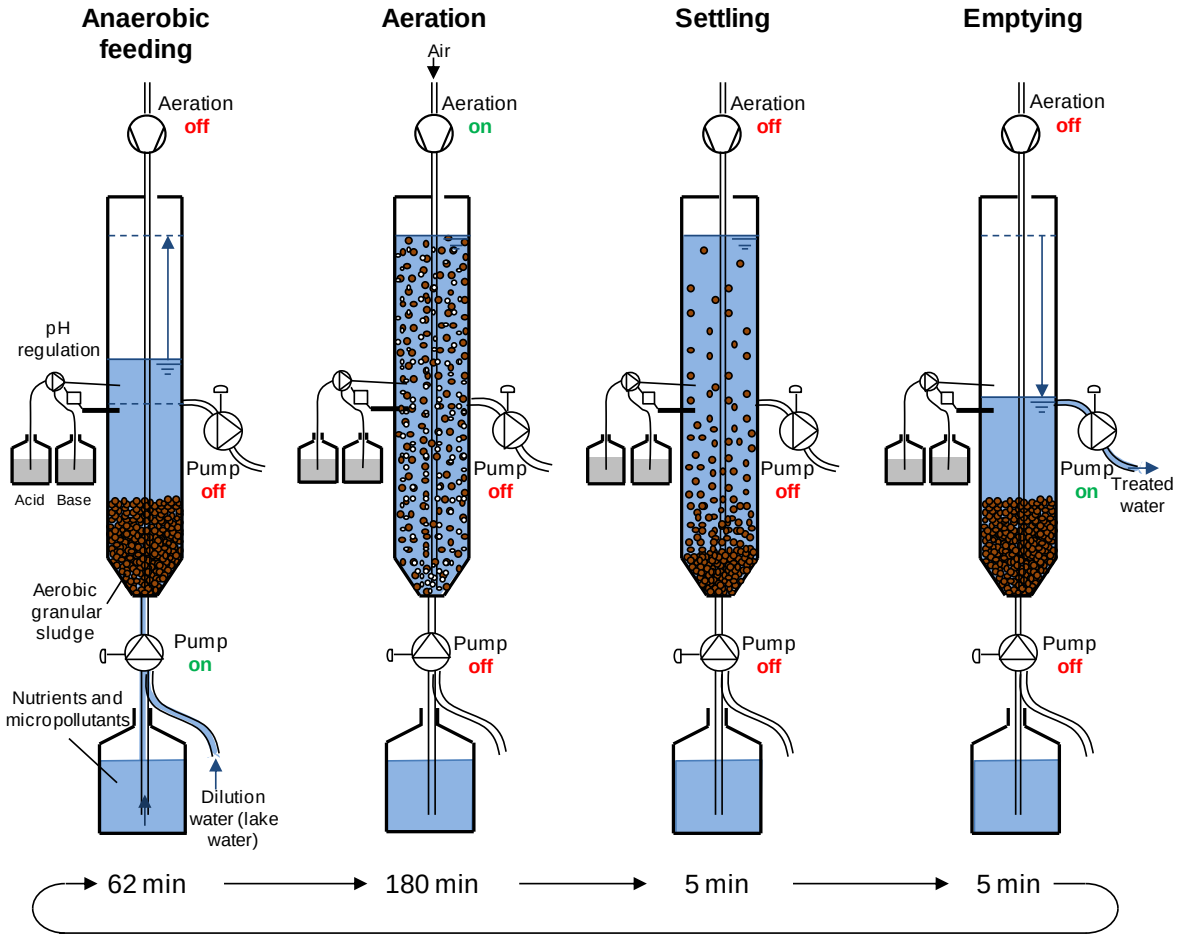


Fig. 4.1 Configuration and operation of the aerobic granular sludge sequencing batch reactor (AGS-SBR)

The reactors were inoculated with flocculent sludge from the municipal WWTP of Thunersee (Switzerland), which treats N and P biologically (in activated sludge systems). Granular sludge (1-2 mm diameter) was obtained in the reactors after around 30 d by progressive washout of bacteria not able to granulate, following the strategy developed by Lochmatter and Holliger (2014). The reactors were continuously operated during 55 d with synthetic wastewater (without micropollutant) to achieve stable and high nutrient removal efficiencies. During this period, the excess granular sludge produced was not purged to increase the biomass concentration. After 55 d, the biomass from both reactors was mixed and re-partitioned equally between the two reactors to assure identical sludge composition and concentration. At the same time, micropollutants (at around 1 µg l^{-1}), and, for one reactor, ATU (at 11.1 mg l^{-1} to inhibit nitrification) were added in the synthetic wastewater. The two reactors were then

operated during 5 d more (15 HRTs) to assure stable conditions prior the sampling campaign for micropollutant analysis (at day 60).

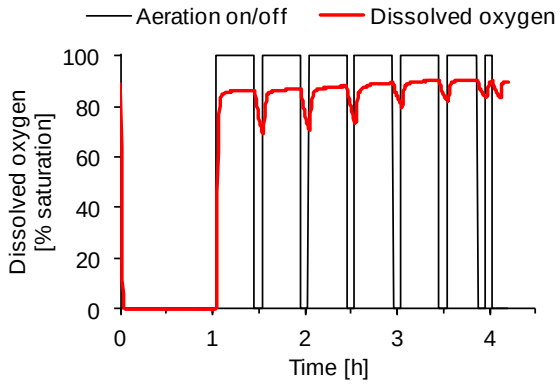


Fig. 4.2 Aeration sequence (0: off, 100: on) during the batch cycle and equivalent dissolved oxygen concentration in the reactor with complete nitrification.

4.2.1.3 Sampling campaign for micropollutant analysis

Five days after the addition of micropollutants and the inhibition of nitrification in one reactor, a sampling campaign was performed to evaluate the micropollutant removal efficiencies with and without nitrification. Two successive batch cycles were monitored in both reactors. Four samples were collected per cycle and per reactor: (i) in the raw wastewater (influent), (ii) after 1 h at the end of the anaerobic feeding, (iii) after 2.5 h at the half of the aeration phase, and (iv) after 4.2 h at the end of the batch cycle (effluent). Two extra samples were collected in the influent and effluent of the reactor not inhibited for quality control of the analytical method (recovery rate measurement). For the two sampling points taken during the batch cycles (after 1 and 2.5 h), the samples were withdrawn at the middle of the reactor during a mixing phase to assure homogeneous micropollutant concentrations and to estimate the biomass concentration. During each sampling, 300 ml were collected, directly centrifuged 20 min at 15,900 × *g* (at 4°C) and filtered at 0.7 µm (glass microfibers filters, Whatman). The recovered biomass (centrifugation pellets and filters) was used to determine the total suspended solids (TSS) concentrations. The filtered water was directly used for the analyses of micropollutants (280 ml), inorganic anions/cations (2 ml filtered at 0. 22 µm) and organic acids (acetate and propionate) (1 ml filtered at 0. 22 µm). At the end of the sampling campaign (after the two cycles), the residual biomass of each reactor was used to determine the sludge volume index after 10 min ($SVI_{10\ min}$) and a fraction of the biomass was frozen (at -18°C) for microbial community characterization.

4.2.1.4 Analytical methods

4.2.1.4.1 Micropollutant analysis

Analysis of micropollutants was performed with a screening method for 44 compounds (Table IX.3, Appendix IX) with off-line solid phase extraction (SPE) followed by ultra-performance liquid chromatography (UPLC) (Acquity UPLC system, with HSS T3 or BEH C18 column depending on the methods, from Waters, USA) coupled to a tandem quadrupole mass spectrometer (MS/MS) (Xevo TQ MS, Waters).

The off-line SPE method, with hand-assembled two layers cartridges (Oasis HLB and mixture of Strata X-CW, Strata X-AW and Isolute ENV+ phases), was similar to the one used in Chapter 3 and described by Morasch et al. (2010), at the difference that only 230 ml of sample were extracted, and that the samples were not acidified but adjust to neutral pH prior the extraction (to avoid pollutant degradation in acidic conditions). The cartridges were therefore also conditioned with non-acidified water and methanol. The extraction was performed within 24 h after the sampling campaign. After the extraction, cartridges were dried 30 min under air stream and frozen (-18°C during 11 d) until the elution. Elution of the cartridges was performed just before the analysis as described by Morasch et al. (2010). Eluate fractions were concentrated at 40°C under a gentle N_2 stream to a volume of 500 µl and then diluted 2.5 times with the aqueous UPLC eluent, prior to the injection (10 µl) in the UPLC column. Two different UPLC methods, either with acidic or neutral eluents, were used depending on the compounds. The conditions of the UPLC gradient and the compounds analysed by each methods are presented in Appendix IX.

Target compounds were identified and quantified using tandem mass spectrometry (MS/MS) (Xevo TQ MS, Waters) in positive and negative electrospray ionization modes (ESI), and detected in multiple reaction monitoring mode (MRM), according to Morasch et al. (2010). Losses during extraction and matrix effects were corrected by adding internal standard (deuterated pollutants at 500-1000 ng l^{-1}) before processing the samples. MS/MS conditions for each pollutant and for their associated deuterated standards are presented in Appendix IX. A set of six standards (corresponding to 1 – 2000 ng l^{-1}) was used to determine the calibration curves. The standards (spiked with the deuterated surrogates) were prepared in the aqueous UPLC eluent and directly injected into the UPLC-MS without passing by an SPE step.

The limit of quantification (LOQ) was between 1-5 ng l^{-1} for most compounds (cf. Table 4.1), except for ibuprofen (LOQ: 20 ng l^{-1}), iohexol (20 ng l^{-1}), iopromide (30 ng l^{-1}), triclosan (30 ng l^{-1}), estrone (70 ng l^{-1}) and estriol (100 ng l^{-1}).

Quality control

Prior processing the samples, two samples (one raw and one treated wastewater) were split in two, and one half was spiked with known concentrations (~500 ng l^{-1}) of micropollutants to calculate the final recovery rates (after correction with the internal deuterated standards, calculated as the concentration difference between the spiked and unspiked samples). The final recovery rate in both samples varied between 80 to 120% for 31 out of the 36 compounds quantified (cf. Table 4.1), showing the relatively good accuracy (< ±20%) of the analytical method. Only for bisphenol A (recovery rates between 54-200%), estriol and estrone (42-116%), iohexol (103-187%) and iopamidol (63-94%), the recovery rates were very variable. Therefore, for these five compounds, results have to be considered with precaution. The reproducibility of the analytical method, assessed by analysing four times the synthetic wastewater, was relatively good, with a coefficient of variation (CV) lower than 13% for all compounds except bisphenol A (23%), estrone (30%), iohexol (25%) and trimethoprim (30%) (cf. Table 4.1).

4.2.1.4.2 Organic acid analysis

Acetate and propionate concentrations were determined by high performance liquid chromatography (HPLC) (Co-2060 Plus, Jasco, Tokyo, Japan) equipped with an ORH-801 column (Transgenomic, Glasgow, United Kingdom) and a refractive index (RI) detector (RI-2021plus, Jasco, Tokyo, Japan). 20 µl of sample were injected and separation of the compounds was conducted under isocratic condition at 0.5 ml/min with a mobile phase composed of 5 mM H_2SO_4 in pure water, during 18 min at 35°C. Limits of detection were at 0.1-0.2 mM for both acetate and propionate.

4.2.1.4.3 Standard parameters analysis

TSS were determined by centrifugation (20 min at 15,900 *g*) of a known volume of sample, followed by filtration of the supernatant at 0.7 µm (GMF Whatman filters). The dry weights of the centrifugation pellets, as well as the one of the biomass retained on the filters, were used to determine the dry suspended solids. Dry matter and water content were determined by overnight drying the sample at 105 °C. The mineral content was determined after 2 h combustion of the samples at 550 °C.

Major inorganic anions and cations, such as ammonium (NH_4^+), nitrite (NO_2^-), nitrate (NO_3^-), and orthophosphate (PO_4^{3-}), were determined by ion chromatography (anions: ICS-90, IonPacAS14A column; cations: ICS-3000A, IonPacCS16 column) with conductivity detector (Dionex DX 500, Olten, Switzerland).

Dissolved oxygen (DO) and pH were continually monitored with online electrodes (from Mettler Toledo).

4.2.1.4.4 Bacterial community composition

The relative composition of the bacterial communities in the granular sludge was characterized by terminal-restriction fragment length polymorphism (T-RFLP) analysis, targeting the hypervariable region of the Eubacteria 16S rRNA gene pool (FAM-labelled 8-F forward primer and 518-R reversed primer), with the method described by Weissbrodt et al. (2012). The bacterial community structures were expressed as relative contribution of all operational taxonomic units (OTUs) contributing to the total measured fluorescence. T-RF with size lower than 32 pair bases (pb) were not considered (back-ground noise)

4.3 Results and discussion

4.3.1 Biomass concentration and nutrient removal

At the time of the sampling campaign, both reactors contained relatively similar biomass concentrations, with, in the reactor with nitrification, 3.6 and 2.4 g l^{-1} TSS for the first and second batch cycle, respectively; and in the reactor with inhibited nitrification, 4.7 and 2.5 g l^{-1} TSS for the first and second cycle, respectively. The biomass decreased between the first and second cycles due to the sampling of high volume of water (total 0.6 l out of 2.6 l) for micropollutant analysis. The mineral content of the TSS was at 35-37% in both reactors. Despite homogenization of the biomass five days before, the granular sludge of both reactors was visually different, with more loose and filamentous granules in reactor without inhibition and more compact granules in the inhibited reactor. This resulted

in lower settleability in the first reactor (not inhibited), with a sludge volume index $SVI_{10\ min}$ of 95 ml g^{-1} compared to 38 ml g^{-1} in the inhibited reactor.

Prior to homogenizing the biomass of the two reactors, five days before the sampling campaign, both reactors showed good nutrient removal efficiencies, with complete (> 97%) phosphate removal and high nitrification-denitrification rates (> 90%). During the biomass homogenization, a thick biofilm was removed from the wall of the reactors, resulting in a strong loss of the nitrification capacity (< 65% NH_4 removal). As the goal was to reach complete nitrification in the reactor not inhibited, the intermittent aeration phases were extended. The addition of ATU in one reactor five days before the sampling campaign resulted in direct inhibition of the nitrification. At the time of the sampling campaign, the nutrient removal efficiencies were stable in both reactors.

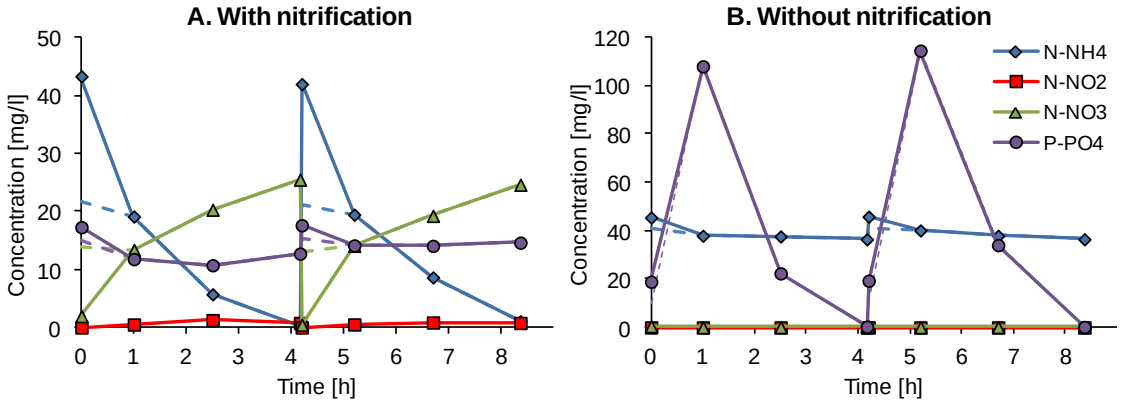


Fig. 4.3 Nutrient removal during two subsequent sequential batch cycles (starting at time 0 and 4 h 12 min). (A) In the reactor with nitrification and (B) in the reactor with inhibited nitrification. Dash-lines: equivalent initial concentrations in the reactor after dilution (1:1) with the previous batch cycle.

Fig. 4.3 presents the evolution of N- and P- concentrations during two successive sequential batch cycles during the sampling campaign. In the reactor without inhibition (Fig. 4.3 A), complete nitrification was observed (> 99% NH_4 removal, with less than 1 mg N-NH_4 l^{-1} in effluent) during the two cycles, as well as partial denitrification of the nitrate produced (40% removal of total dissolved nitrogen). In the inhibited reactor (Fig. 4.3 B), as expected, no nitrification was observed, with only 20% of NH_4 and total dissolved nitrogen removal, probably by biomass uptake (production of new sludge). For the phosphate, opposite behaviours were observed, with only 22% of PO_4^{3-} removed in the reactor with nitrification and 99% in the inhibited reactor. In the latter, classical behaviour for biological phosphorus removal was observed (Furumai et al., 1999), with release of the phosphates accumulated in the cells (in form of polyphosphates) during the anaerobic phase (first hour) and ortho-phosphate uptake by polyphosphate-accumulating organisms (PAOs) during the aerobic phase.

The low P-removal reached in the reactor without inhibition was probably linked to the intensive aeration applied to reach complete nitrification, which limited the denitrification rate during the aerobic phase. This resulted in the presence of relatively high nitrate concentrations in the reactor during the first anaerobic/anoxic feeding, deteriorating the P-removal efficiency (more efficient with complete anaerobic conditions) (Peng et al., 2010). Very different behaviours for nutrient removal were thus observed between the two reactors.

Acetate and propionate, the two main carbon substrates, were completely (> 95%) consumed in both reactors during the anaerobic/anoxic feeding, confirming the unfavourable growth conditions for strict aerobic heterotrophic organisms in this system.

4.3.2 Micropollutant removal

The removal of the 36 micropollutants studied in the AGS-SBRs (average of two batch cycles) is presented in Fig. 4.4. In the reactor with complete nitrification, nine micropollutants were removed above 80%, while 17 were removed less than 20%. The average removal of the 36 pollutants was at only 42%, showing the restricted potential of AGS-SBRs for the treatment of micropollutants in wastewater. Apart for five compounds, very similar removal efficiencies were measured with or without inhibition of the nitrification, showing the limited role of AMO oxidation for the removal of a wide range of pollutants. Very good removal reproducibility was observed between the two batch cycles, despite lower biomass concentration in the second cycle. The kinetics of micropollutant removal, grouped as a function of the removal efficiencies, are presented below.

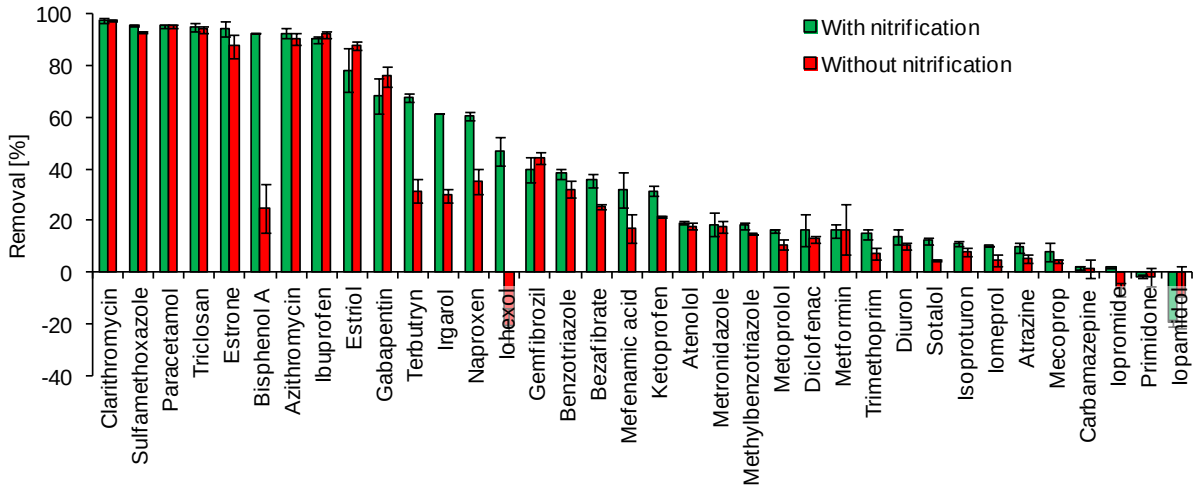


Fig. 4.4 Micropollutant removal in the aerobic granular SBR (average and values of two batch cycles) in the reactor with or without (inhibited) nitrification. Average removal of the 36 pollutants: 42% with nitrification and 33% without nitrification.

4.3.2.1 Micropollutants well removed (>70%) in all conditions

Nine pollutants were well removed (> 70%) in both reactors, with or without nitrification (Fig. 4.5). The two macrolide antibiotics azithromycin and clarithromycin, as well as the biocide triclosan were rapidly removed already during the anaerobic/anoxic feeding. These three compounds are relatively hydrophobic (log K_{ow} of 3.2-4.8) (cf. Table S 3.1, Chapter 3), which suggests that they were at least partially removed by adsorption. Indeed, although these pollutants can be biologically degraded, they are reported to have a good (azithromycin and clarithromycin) to strong (triclosan) adsorption affinity for activated sludge (Banihashemi and Droste, 2014; Jelic et al., 2011; Yan et al., 2014; Ying and Kookana, 2007). Their fast removal in the anoxic phase is coherent with the rapid adsorption kinetics observed for micropollutants onto activated sludge, with adsorption equilibrium usually reached in less than 30 min (Ternes et al., 2004) (longer time to reach equilibrium are, however, expected in granular sludge due to diffusion limitation). The positive charge of the two marcolides may favour electrostatic attraction with the granule surface, mainly negatively charged (Liu et al., 2014). It was

reported that both nitrifying bacteria (via AMO oxidation) and other non-ammonia oxidizing microorganisms play a role in triclosan degradation in nitrifying activated sludge (Roh et al., 2009). Thus, although this could not be seen in our results, it is likely that the three compounds were further degraded in the sludge after their initial fast adsorption.

The two natural estrogens estrone (E1) and estriol (E3) were also rapidly removed (close to their limit of quantification) during the anaerobic/anoxic feeding. Due to their moderate hydrophobicity (log K_{ow} of 2.5-3.1), neutral charge at pH 7 and good biodegradability, it is expected that they were initially rapidly adsorbed onto granular sludge and then progressively degraded during the aerobic phase. Indeed, it is reported that estrogens tend to sorb rapidly to activated sludge and then are further degraded in the sludge phase, especially under aerobic conditions (low degradation occurred under anoxic conditions) (Hashimoto and Murakami, 2009). The absence of effect of AMO inhibition on E1 and E3 removal suggests that heterotrophic bacteria may have played a significant role in their degradation, as also reported by Maeng et al. (2013).

Although ibuprofen is a relatively hydrophobic compound (log K_{ow} of 4.0), it seemed to be mainly removed by biodegradation, as very low removal in the initial anaerobic/anoxic phase was observed (by considering the dilution effect with the previous batch). Its low adsorption affinity could be explained by its negative charge (at pH 7), which may lead to electrostatic repulsion with the negatively charged surface of the granules. Indeed, several other pollutants, negatively charged at pH 7, such as diclofenac, mefenamic acid, gemfibrozil or bezafibrate, were only poorly removed in the AGS-SBRs, confirming that adsorption was not significant for these anionic compounds, despite their relatively high hydrophobicity (log K_{ow} > 4.0). The good removal of ibuprofen in both reactors (without or with ATU) is consistent with the results of other studies, where presence of ATU did not significantly affect its removal (Falås et al., 2012a; Maeng et al., 2013; Tran et al., 2009). Moreover, pure culture of nitrifying bacteria (*Nitrosomonas europaea*) were unable to degrade ibuprofen (Roh et al., 2009), confirming that ibuprofen degradation was due to the activity of heterotrophic bacteria.

The removal of paracetamol, sulfamethoxazole and gabapentin is expected to be due to biodegradation as these compounds have low hydrophobicity (log K_{ow} < 0.9) and low sludge affinity (Yan et al., 2014). Paracetamol is known to be easily degraded in WWTPs with or without nitrification (DGE, 2013; Maeng et al., 2013; Margot et al., 2013b) and seemed to be well degraded also during the anoxic/anaerobic feeding. Sulfamethoxazole (SMX) was removed at a slightly higher rate in the nitrifying reactor but this was probably not due to AMO oxidation as complete removal was observed also in the inhibited reactor. SMX was reported to be only poorly removed (< 30%) in AOB-enriched nitrifying sludge (Suarez et al., 2010), confirming that the removal observed with the granular sludge was mainly due to heterotrophic bacteria. SMX removal in WWTPs is highly variable, ranging from 0 to 70%, with average removal usually lower than 50% (DGE, 2013; Margot et al., 2013b). This lower efficiency in WWTPs may be related to the biological cleavage of the human metabolite N^4-acetyl sulfamethoxazole, reconverted to the antibiotic sulfamethoxazole during the biological treatment in real wastewater (Göbel et al., 2007), while this could not occur in the synthetic wastewater in the AGS-SBRs. Gabapentin, well removed in the AGS-SBRs (with or without nitrification), was reported to be poorly removed (< 30%) in several WWTPs (DGE, 2013; Margot et al., 2013b; Reungoat et al.,

2011), while it was well removed (> 80%) in few others (Kasprzyk-Hordern et al., 2009; Yu et al., 2006). This variable removal rate in WWTPs cannot be explain by variable rates of nitrification, or by reformation of gabapentin by biological cleavage of a conjugated molecule, as gabapentin is not metabolized in the human body (no conjugate formed) (Kasprzyk-Hordern et al., 2009). Therefore, better gabapentin removal in AGS-SBRs or in some WWTPs may be due to the presence of specific heterotrophic microorganisms able to degrade this compound.

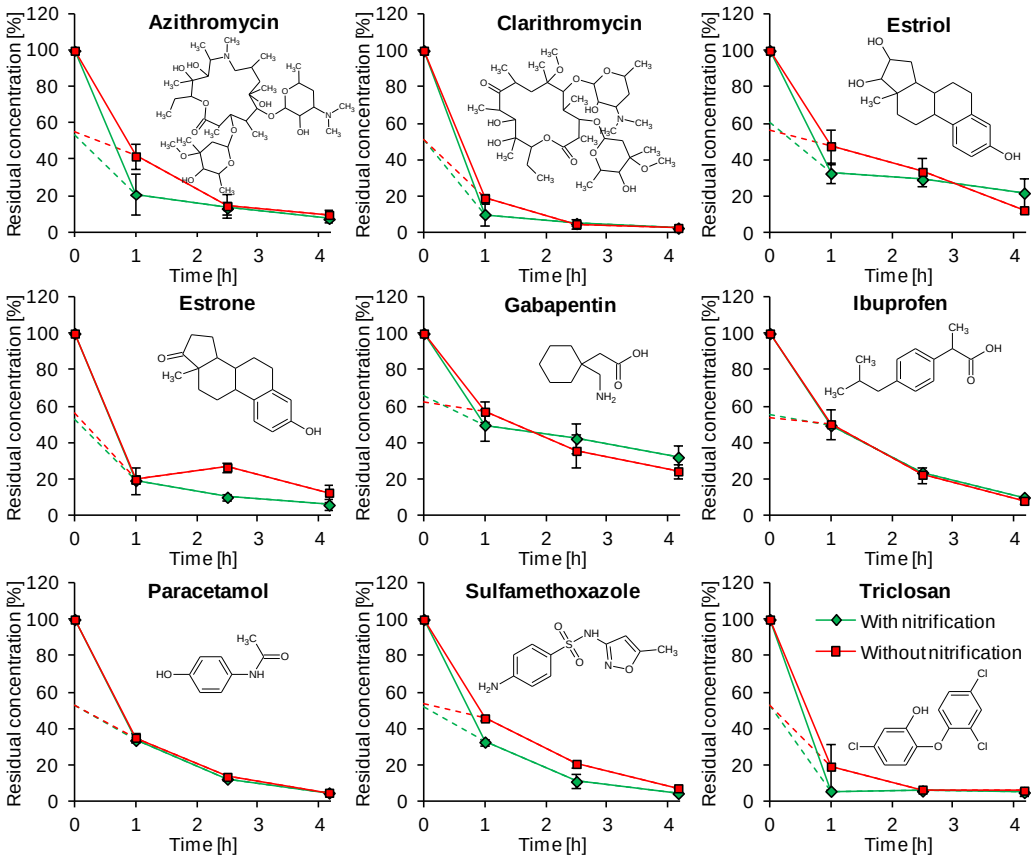


Fig. 4.5 Kinetics of micropollutant removal with or without nitrification. Compounds well removed (> 70%). Average and values (error bars) of duplicates. Dash-lines: equivalent initial concentrations in the reactor after dilution (1:1) with the previous batch cycle.

4.3.2.2 Micropollutants better removed with than without nitrification

Five compounds were significantly better removed in the reactor with complete nitrification than in the one without (Fig. 4.6). This difference was particularly important for bisphenol A (BPA), even taking into account the moderate analytical reproducibility (± 23%) for this compound. The role of AMO in BPA oxidation was confirmed in other studies (Kim et al., 2007; Roh et al., 2009), where pure cultures of nitrifying bacteria (*N. europaea*) were able to degrade BPA only in absence of ATU. The good removal of BPA observed only in nitrifying WWTPs (Gardner et al., 2013; Margot et al., 2013b) may thus be due to oxidation by AMO. However, in nitrifying sludge, BPA was reported to be also slowly degraded by heterotrophic organisms (Kim et al., 2007; Roh et al., 2009). It is thus likely that both ammonia-oxidizing organisms and other heterotrophic organisms, which might be favoured under condition favourable for nitrification, are responsible for BPA removal in WWTPs.

Even considering the moderate analytical reproducibility (± 25%) for iohexol, this compound was still significantly better removed without than with AMO inhibition. Oxidation of iohexol by AMO was not previously reported but biodegradation of this compound with nitrifying sludge was already observed (Hapeshi et al., 2013). Higher iohexol removal efficiencies were also observed in WWTPs with higher nitrification levels (cf. Chapter 3). AOB might thus play a role in iohexol degradation. Batt et al. (2006) and Pérez et al. (2006) observed that iopromide, another iodinated contrast medium with very similar structure than iohexol, was transformed by nitrifying sludge to a dehydroxylated metabolite, this metabolite being not observed when nitrification was inhibited. As iohexol contains two more hydroxyl groups than iopromide, it is possibly a better substrate for organisms present in nitrifying sludge, which may explain why it was better removed than the other iodinated contrast media.

Naproxen (NPX) was slowly removed in the inhibited reactor and degraded significantly faster in the reactor with nitrification, suggesting that it could be partially oxidized by AMO. Similar results were observed in other studies with enriched nitrifying sludge (Tran et al., 2009). It was suggested that AMO might oxidize NPX through O-dealkylation mechanism, as observed for other aromatic ethers (Alvarino et al., 2014). In full-scale WWTPs, NPX removal was also reported to be positively correlated with ammonium removal (DGE, 2013; Fernandez-Fontaina et al., 2012; Margot et al., 2013b), supporting the fact that nitrification might play a role in its removal. However, this might not only be because of the activity of AOBs but also to heterotrophic degradation, as no effect of nitrification inhibition was observed on NPX removal with activated sludge from full-scale nitrifying WWTPs (Falås et al., 2012a).

The two triazine herbicides irgarol and terbutryn, with relatively similar chemical structures, were also clearly better removed in the reactor not inhibited, suggesting their potential oxidation by AMO. Very few studies have been conducted on the degradation of these two herbicides in WWTPs. It has recently been reported that both compounds were transformed in nitrifying activated sludge to a sulfoxide metabolite (oxidation of the sulfur group) (Luft et al., 2014), which may explain why atrazine, another structurally similar triazine herbicide but without the sulfur group, was not oxidize in the AGS-SBRs. Moreover, a slight but significant positive correlation between their removal and ammonium removal in WWTPs has previously been observed (cf. Chapter 3), supporting the possible link between irgarol and terbutryn removal and AMO oxidation.

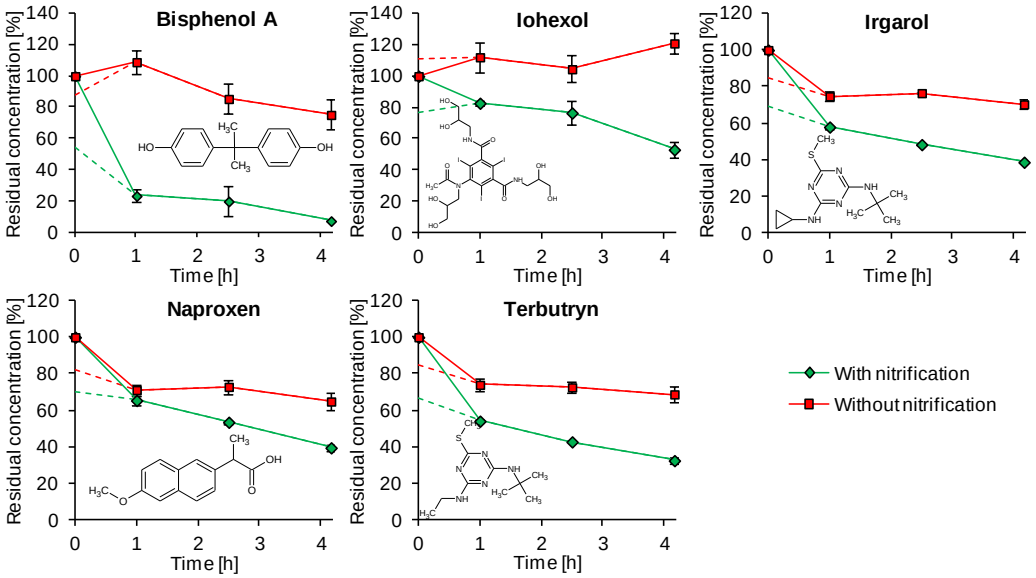


Fig. 4.6 Kinetics of micropollutant removal with or without nitrification. Compounds better removed with nitrification (47-93%) than without (< 35%). Average and values (error bars) of duplicates. Dash-lines: equivalent initial concentrations in the reactor after dilution (1:1) with the previous batch cycle.

4.3.2.3 Micropollutants only slightly removed (20-40%)

Five micropollutants were slowly removed in both reactors, with no clear difference between the reactors (inhibited or not), despite a slightly better removal in the one with complete nitrification for bezafibrate, ketoprofen and mefenamic acid (Fig. 4.7). It has been reported that the removal of gemfibrozil, bezafibrate and ketoprofen in nitrifying sludge was reduced in presence of ATU, although these compounds were also partially removed with inhibited nitrification (Maeng et al., 2013; Tran et al., 2009). However, in activated sludge from full-scale WWTPs, ketoprofen removal was not impacted by ATU addition (Falås et al., 2012a). Therefore, both AOBs and other heterotrophic organisms may play a role in their degradation. The slow removal kinetics observed indicate that better removal efficiencies would be reached with longer HRTs. The higher removal efficiencies for these compounds (except for benzotriazole) observed in WWTPs with higher ammonium removal efficiencies (DGE, 2013; Margot et al., 2013b) (cf. Chapter 3) may thus be partially related to longer HRT in these WWTPs. HRTs are, however, also related to the microbial community composition, as shorter HRTs in a WWTP mean higher nutrient loads, which may affect the microbial composition. Thus both HRTs and the microbial composition may play a role for the removal of these compounds in WWTPs.

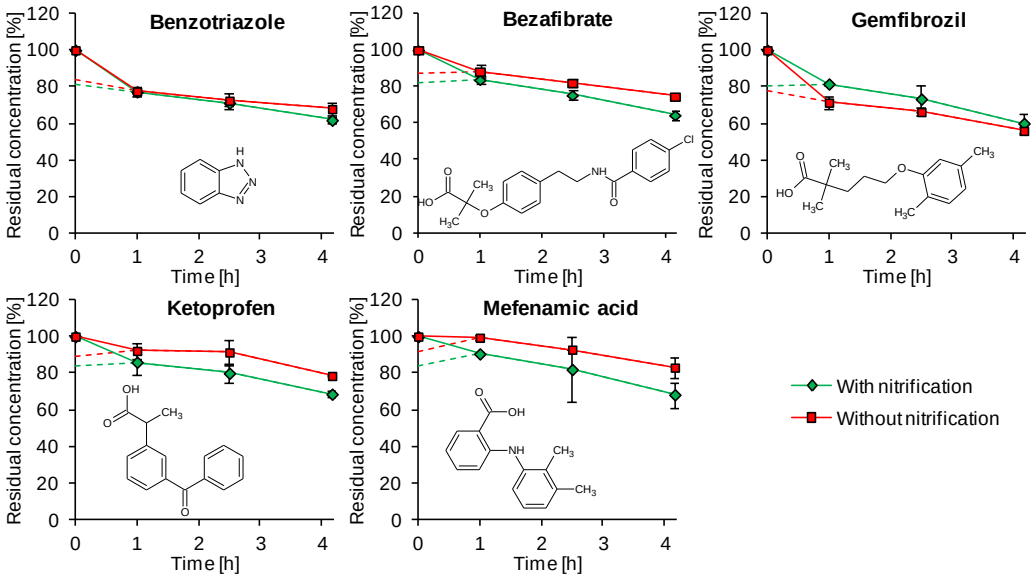


Fig. 4.7 Kinetics of micropollutant removal with or without nitrification. Compounds slightly removed (20-40%). Average and values (error bars) of duplicates. Dash-lines: equivalent initial concentrations in the reactor after dilution (1:1) with the previous batch cycle.

4.3.2.4 Micropollutants not or poorly removed (<20%)

Almost half of the micropollutants studied (17 out of 36) were not or only poorly removed in both AGS-SBRs (Fig. 4.8). Some of them were reported to be degraded in nitrifying activated sludge in batch experiments (biomass concentration 1-5 g TSS l^{-1}), such as atenolol and metoprolol (~80% removal in 25 h) (Falås et al., 2013; Sathyamoorthy et al., 2013), iopromide and trimethoprim (~70% in 24 h and 100 h, respectively) (Batt et al., 2006), diclofenac (75% in 72 h) (Tran et al., 2009) and metronidazole (90% in 24 h) (Phan et al., 2014). Lower removals observed in this study may be due to shorter HRTs in the AGS-SBRs (~8 h), and to different bacterial communities in the granular sludge, with possibly a lower fraction of AOBs than in enriched nitrifying sludge and less diverse aerobic heterotrophic microbial community. The microbial community composition has, indeed, a strong influence on the degradation of some micropollutants in municipal WWTPs. For instance, diclofenac, which is hardly degraded in most activated sludge systems, was reported to be well degraded (> 90% in less than 24 h) with the biomass of some moving bed bioreactors (MBBRs) (Falås et al., 2012b; Falås et al., 2013).

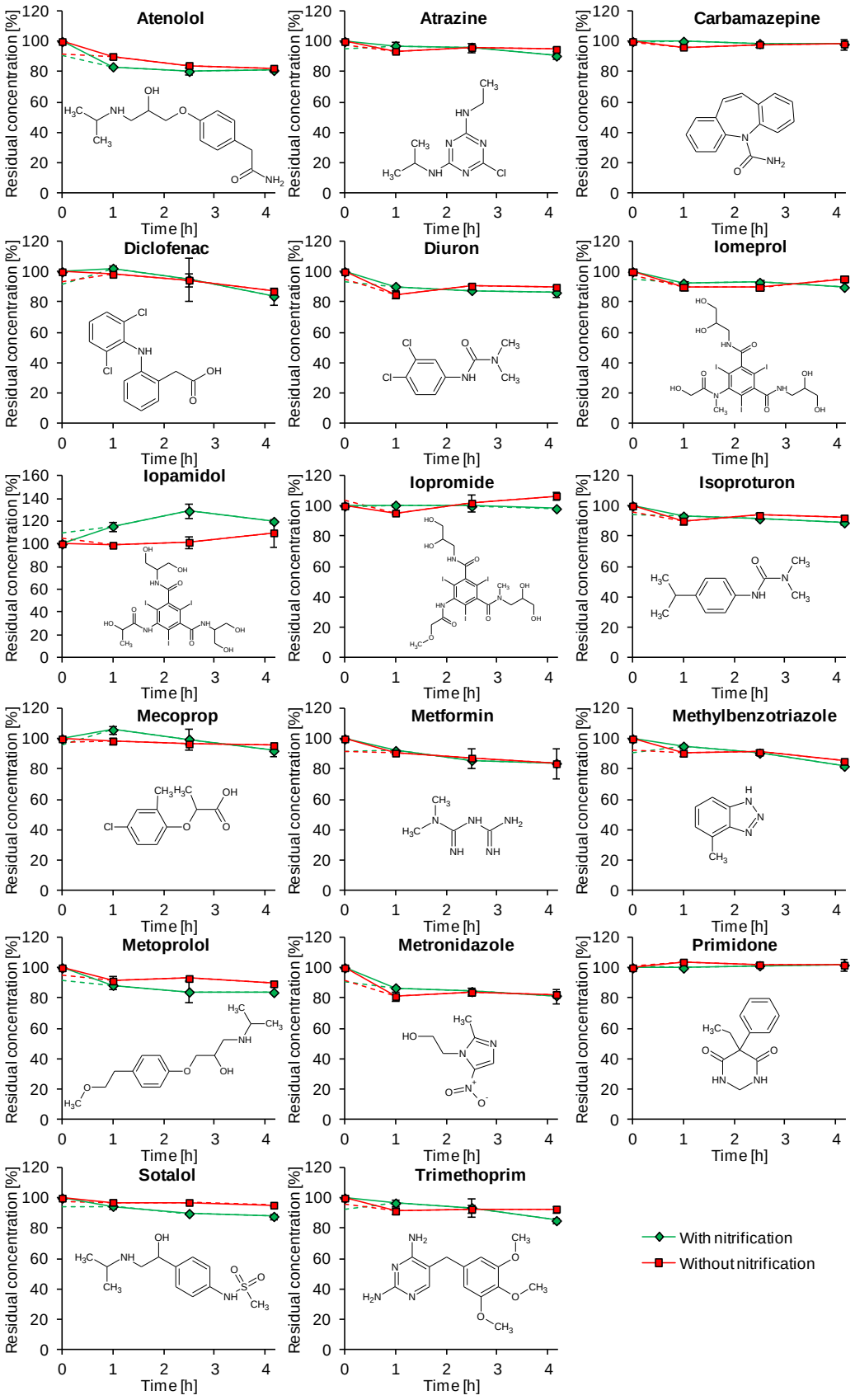


Fig. 4.8 Kinetics of micropollutant removal with or without nitrification. Compounds poorly removed (< 20%). Average and values (error bars) of duplicates. Dash-lines: equivalent initial concentrations in the reactor after dilution (1:1) with the previous batch cycle.

4.3.2.5 Removal under aerobic versus anoxic/anaerobic conditions

Except for the compounds rapidly removed by adsorption, faster (or at least similar) degradation rates were observed during the aerobic phase than during the anoxic/anaerobic step (considering the dilution effect with the previous batch). This was particularly the case for ibuprofen, iohexol, naproxen, bezafibrate, gemfibrozil, ketoprofen and mefenamic acid. Most of these compounds (apart iohexol), as well as several others (e.g., natural and synthetic estrogens, metoprolol, benzotriazole, methylbenzotriazole, valsartan, salicylic acid, erythromycin, roxithromycin) were indeed reported to be much better degraded under aerobic than anoxic/anaerobic conditions (Alvarino et al., 2014; Falås et al., 2013; Phan et al., 2014; Suarez et al., 2010). Only a few micropollutants were reported to be sometimes better degraded under anaerobic conditions: trimethoprim, sulfamethoxazole and possibly naproxen and clarithromycin (Alvarino et al., 2014; Falås et al., 2013). Aerobic conditions should thus be favoured to increase micropollutant removal in WWTPs.

4.3.3 Comparison with full scale WWTPs

The micropollutants removal potential of the AGS-SBRs was compared to the one of a full-scale municipal WWTP, with complete (> 97% NH_4 removal) or without (< 25% NH_4 removal) nitrification. Data for the WWTP came from the study presented in Chapter 3 at Lausanne WWTP, Switzerland, which has two biological treatments in parallel, a high load activated sludge without nitrification (SRT of 2 d) and a MBBR with partial to complete nitrification (could be adjusted by changing the flow treated). Even if not the same wastewater was treated with the AGS-SBRs and the WWTP (synthetic versus real municipal wastewater, respectively), both processes were operated in continuous with similar treatment objectives: good removal of COD and complete nitrification. The MBBR (with complete nitrification) had a lower HRT than the AGS-SBR (3.9 h versus 8 h in the biological part) but a higher biomass concentration (not measured but usually between 5-13 g TSS l^{-1} in MBBRs (Falås et al., 2012b) versus ~ 3 g TSS l^{-1} in the AGS-SBR).

As presented in Fig. 4.9 A, the MBBR was on average more efficient than the AGS-SBR regarding the removal of most micropollutants (average removal of the 34 compounds of 50% and 41%, respectively). Only three compounds were better removed with the AGS-SBR: clarithromycin, sulfamethoxazole and gabapentin. As clarithromycin was suspected to be mainly removed by adsorption onto the granular sludge, its higher removal may be related to uncompleted saturation of the adsorption sites of the granules, which were in contact with the pollutants only for a few days compared to several weeks in the MBBR (where adsorption is expected only on the new fresh biomass produced). Clarithromycin may also enter the WWTP trapped into faeces particles and then released in the water phase during the biological treatment, resulting in a low apparent removal of the dissolved fraction (Göbel et al., 2007). For sulfamethoxazole, the low removal rate in real municipal wastewater may be related, as discussed before, to the production of this compound during the treatment by biological cleavage the conjugated metabolite. Thus, for these two antibiotics, lower removal efficiencies in the AGS-SBRs would be expected after long-term operation with real wastewater. The good removal of gabapentin is promising as this compound is only poorly removed in conventional WWTPs.

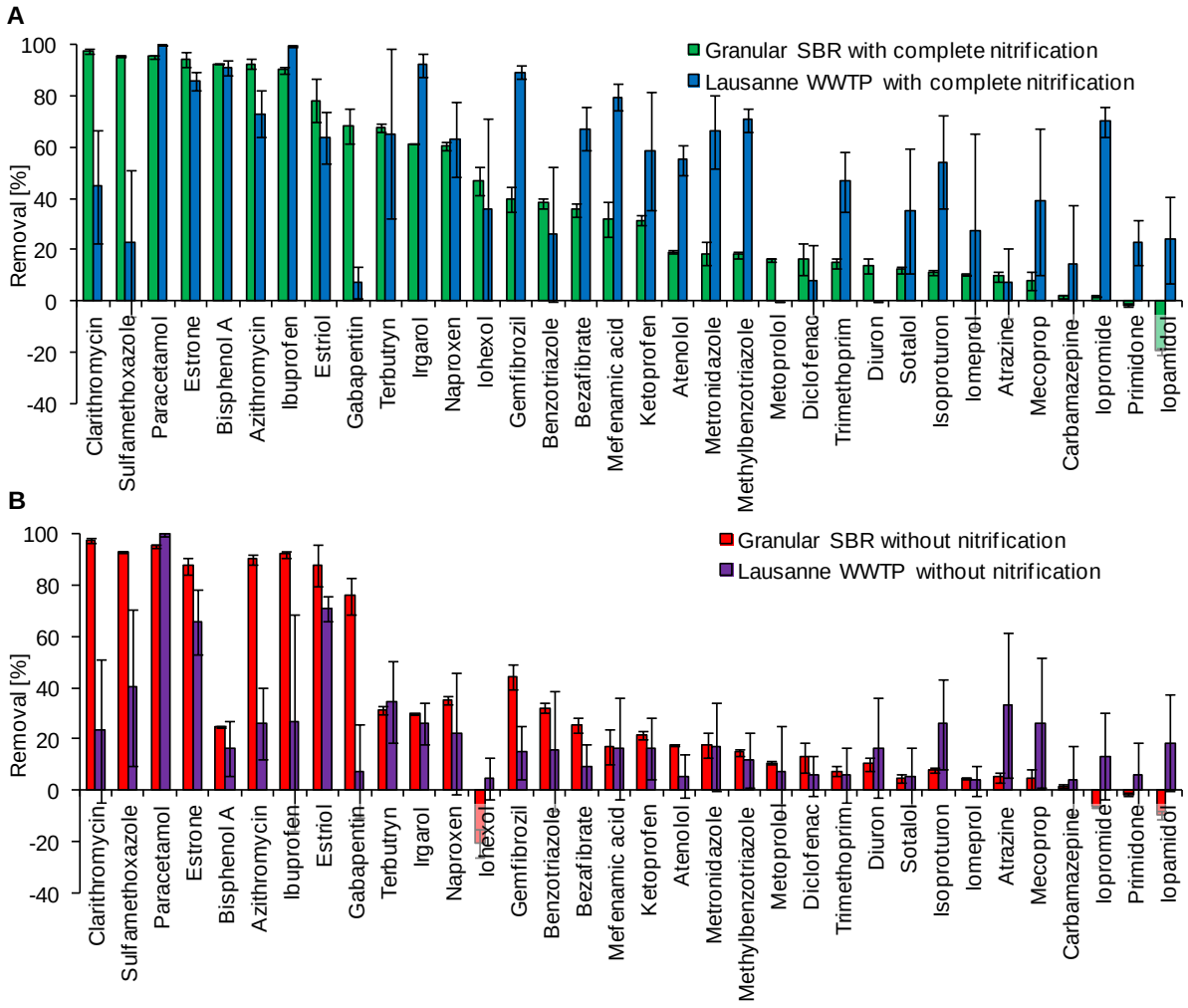


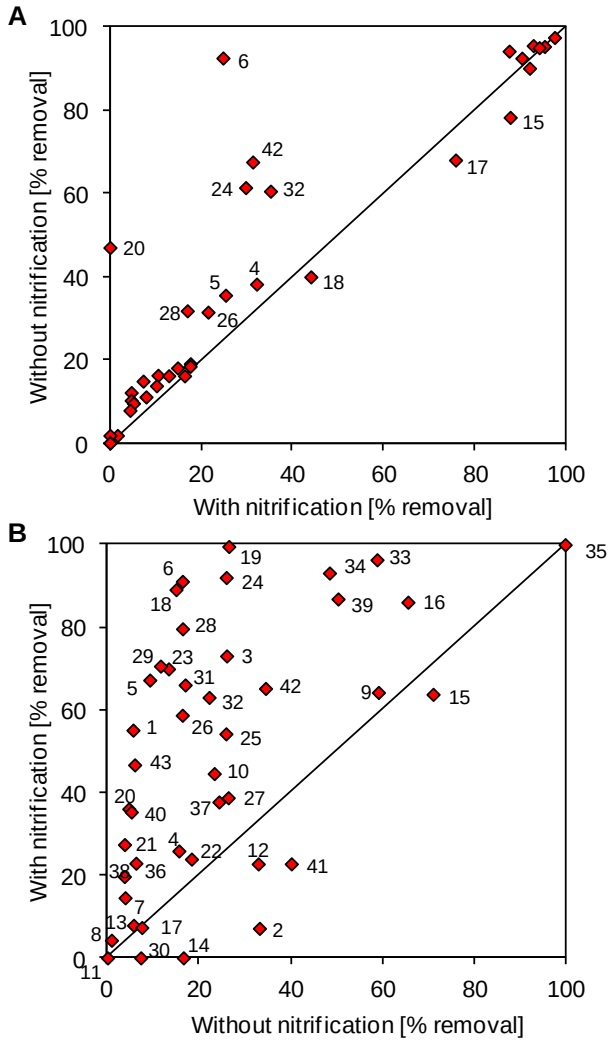
Fig. 4.9 Comparison of micropollutant removal efficiencies between the granular SBR (synthetic wastewater) and Lausanne WWTP (real municipal wastewater), (A) with complete nitrification (> 97% NH_4 removal, < 1 mg N-NH_4 l^{-1} in effluents), (B) without nitrification (< 25% NH_4 removal). For Lausanne WWTP, average and standard deviation of 6 campaigns (24-72 h-composite samples) for complete nitrification (moving bed bioreactor), and 12 campaigns (24-72 h-composite samples) without nitrification (activated sludge) (data from Chapter 3).

A group of micropollutant was significantly better removed with the MMBR than the AGS-SBR (Fig. 4.9 A): gemfibrozil, bezafibrate, mefenamic acid, ketoprofen, atenolol, metronidazole, methylbenzotriazole, trimethoprim, isoproturon and iopromide. Most of these compounds were reported to be better removed in WWTPs with high nitrification capacities (DGE, 2013; Margot et al., 2013b). As the AGS-SBR had similar ammonium removal efficiency than the MBBR (complete nitrification on both cases), this gives a clue that the better removal observed in nitrifying WWTPs is very likely not due to AOB activity, but probably to the presence of a more diverse microbial community. As discussed before, aerobic conditions are favouring micropollutant degradation, and most pollutants are better (or at least as well) removed by heterotrophic organisms than by AOBs. Thus, the presence of a diversified aerobic heterotrophic microbial community seems to be the main reason for better micropollutant removal in nitrifying WWTPs, as also suggested by Falås et al. (2012b). The growth of these organisms is unfavoured in AGS-SBRs, which may explain the lower removal efficiencies reached. The presence of only simple carbon sources in the synthetic wastewater

does also not favour the development a diversified heterotrophic microbial community in the granular sludge.

Fig. 4.9 B shows a comparison of the removal efficiencies between the AGS-SBR inhibited with ATU and the activated sludge reactor without nitrification. In this case, the AGS-SBR was more efficient for the removal of most micropollutants (average removal of 32% vs 22%). This demonstrates that, even if the granular sludge had lower degradation potential than conventional nitrifying sludge, it had higher potential than non-nitrifying sludge, confirming that conditions favourable for the growth of nitrifying bacteria might also be favourable for other microorganisms able to degrade micropollutants.

As synthesized in Fig. 4.10, nitrification inhibition in the AGS-SBRs only affected the removal of a few pollutants (Fig. 4.10 A), while conditions allowing complete nitrification in WWTPs favoured the degradation of most micropollutants compared to WWTPs without nitrification (Fig. 4.10 B).



ID	Compound	ID	Compound
1	Atenolol	23	Iopromide
2	Atrazine	24	Irgarol
3	Azithromycin	25	Isoproturon
4	Benzotriazole	26	Ketoprofen
5	Bezafibrate	27	Mecoprop
6	Bisphenol A	28	Mefenamic acid
7	Carbamazepine	29	Methylbenzotriazole
8	Carbendazim	30	Metoprolol
9	Ciprofloxacin	31	Metronidazole
10	Clarithromycin	32	Naproxen
11	Clindamycin	33	Norfloxacin
12	Diatrizoic acid	34	Ofloxacin
13	Diclofenac	35	Paracetamol
14	Diuron	36	Primidone
15	Estriol	37	Propiconazole
16	Estrone	38	Propranolol
17	Gabapentin	39	Simvastatin
18	Gemfibrozil	40	Sotalol
19	Ibuprofen	41	Sulfamethoxazole
20	Iohexol	42	Terbutryn
21	Iomeprol	43	Trimethoprim
22	Iopamidol		

Fig. 4.10 Comparison of micropollutant removal efficiencies between biological treatments without nitrification (< 25% NH_4 removal) or with complete nitrification (> 97 % NH_4 removal, < 1 mg N-NH_4 l^{-1} in effluents). (A) In the granular SBR with or without nitrification inhibition. **(B)** At Lausanne WWTP, with either activated sludge (without nitrification) or moving bed bioreactor (with complete nitrification) (average removal of 6-12 campaigns, data from Chapter 3).

4.3.4 Microbial community

The relative compositions of the microbial community in the granular sludge and in the sludge from Lausanne WWTP are presented in Fig. 4.11. The composition is presented in terms of operational taxonomic unit (OTU) based on the results of the T-RFLP analysis. One OTU can correspond to several bacterial species, but also one species can be present in several OTUs. Affiliation of OTUs to specific bacterial species is thus not possible without other information (e.g., by pyrosequencing). Nevertheless, these results allow comparing the bacterial community composition between different sludge.

As presented in Fig. 4.11, nitrification inhibition did not strongly affect the microbial compositions in the granular sludge. The same main OTUs were present in both sludge, but at different proportions, especially for OTU 302, much more present in the sludge not inhibited. Nitrifying population (AOBs and NOBs) can usually not be assessed by T-RFLP due to their relatively low abundance (< 1%) in granular sludge (Weissbrodt et al., 2012). Therefore, the changes in bacterial composition observed between the granular sludge inhibited or not were not due to a change of the proportion of AOBs. Filamentous granules were observed in the reactor not inhibited, suggesting that the dominant OTU 302 was linked to filamentous bacteria. While a relatively diversified bacterial community was observed in the granular sludge (90% of the total abundance dominated by 26 and 45 OTUs, without or with inhibition, respectively), much lower diversity was measured in the non-nitrifying activated sludge, with only 11 OTUs dominating 90% of the total abundance. In the MBBR biomass (taken from the first tanks with low nitrification), higher diversity was observed, with 34 OTUs dominating 90% of the total abundance. Although it is not possible to make a link between specific OTUs and micropollutant degradation, it can be observed that the sludge from the two AGS-SBRs had relatively similar composition and showed relatively similar removal efficiencies, while the two sludge from the WWTP had relatively different microbial communities and showed very different micropollutant removal efficiencies, with higher removal with the more diversified sludge.

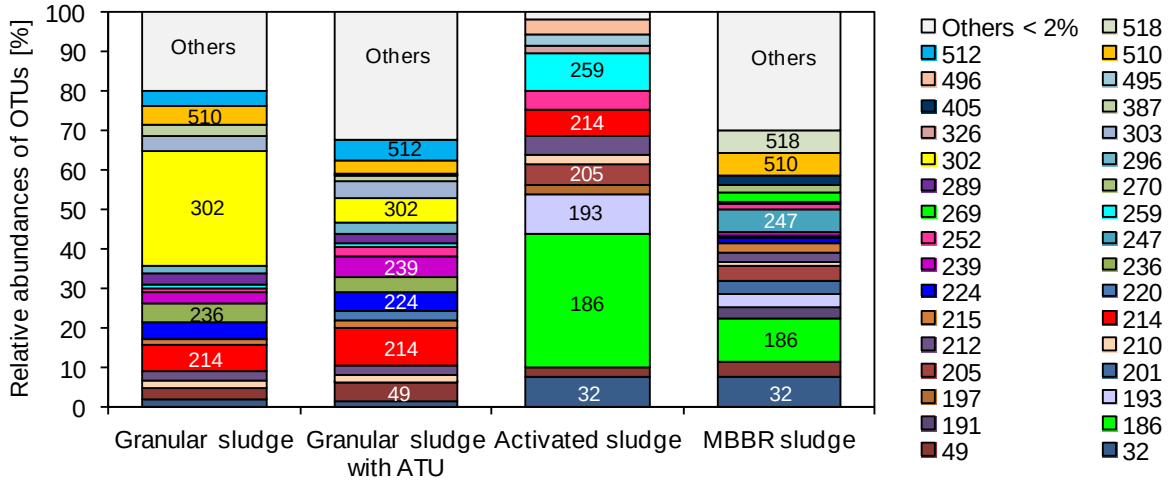


Fig. 4.11 Fingerprint (T-RFLP profiles) of the microbial community in the aerobic granular sludge (with or without nitrification inhibition with ATU) and in the sludge from Lausanne WWTP (activated sludge without nitrification, or sludge from the moving bed bioreactor (first reactor with low nitrification)). Numbers: size (in base pairs (bp)) of the TRFs for each operational taxonomic unit (OTU). Only OTUs with relative abundance >2% in at least one sample are listed.

4.4 Conclusions

The main goals of this study were to assess the potential of aerobic granular sludge for micropollutant removal from wastewater and to clarify the reasons leading to better micropollutant removal in nitrifying WWTPs. Based on the results of this study, it can be concluded that:

- AGS-SBRs treating synthetic wastewater showed a moderate potential for micropollutant removal, with only nine pollutants removed over 80% and 17 less than 20% (out of 36).
- Autotrophic nitrifying organisms (especially AOBs) did not play a significant role in micropollutant removal, apart for a few compounds: bisphenol A, naproxen, iohexol, irgarol and terbutryn.
- Several micropollutants were degraded faster during the aerobic phase than during the anoxic/anaerobic step, confirming the higher potential of aerobic conditions for micropollutant removal.
- The operating conditions of AGS-SBRs, chosen to be unfavourable for aerobic heterotrophic organism growth in order to save COD for denitrification and biological phosphorus removal, may play a role in the low-to-moderate efficiency of this process for micropollutant removal.
- The better removal efficiencies observed for several micropollutants in nitrifying WWTPs compared to nitrifying AGS-SBRs, despite similar nitrification efficiency, suggests that aerobic heterotrophic organisms may play an important role in micropollutant removal in nitrifying WWTPs, significantly more than the one of nitrifying organisms (present in much lower abundance).
- In WWTPs, conditions favourable for the growth of nitrifying bacteria, such as long SRTs, low food-to-microorganisms ratio and good aeration, seem to be also favourable for the development of a diversified heterotrophic microbial community able to degrade micropollutants. Moreover, the longer HRT required in nitrifying WWTPs is expected to increase the removal efficiencies of pollutants with low to moderate degradation kinetics.
- Nitrifying WWTPs have, therefore, a much higher potential than non-nitrifying WWTPs regarding micropollutant removal from municipal wastewater. However, even up-to-date nitrifying WWTPs are not able to remove all the micropollutants, with still almost half of those studied removed less than 50%. If better removal efficiencies are required, complementary treatments will be necessary.

Acknowledgments

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Chapter 5 Screening of the range of pollutants oxidized by laccase

5.1 Introduction

As shown in Chapter 3, ozonation and adsorption onto powdered activated carbon proved to be effective technologies to treat micropollutants and are feasible in terms of implementation and operation on a large scale in WWTPs. But, these advanced treatments appeared not to be adapted for small WWTPs due to the cost of the treatment and the skills required for their operation. Moreover, they consume significant energy, which goes against the efforts made for the reduction of climate change. Thus, effort has still to be invested in research for the development of a treatment affordable for small WWTPs, with low equipment needs, skills and energy requirements. One potential means to reduce the impacts of micropollutants released by small WWTPs is to improve their biodegradation in a post-treatment step using microorganisms that produce oxidative enzymes such as laccases (Blánquez et al., 2008; Zhang and Geißen, 2012).

The ability of fungal laccases to catalyse (alone or with the help of mediators) the oxidation of pharmaceuticals and biocides was demonstrated for several substances, such as endocrine compounds (Auriol et al., 2008; Cabana et al., 2007a), analgesic and anti-inflammatory drugs (Hata et al., 2010; Lu et al., 2009; Marco-Urrea et al., 2010a; Marco-Urrea et al., 2010b), antibiotics (Schwarz et al., 2010; Suda et al., 2012), UV filter (Garcia et al., 2011), biocides (Kim and Nicell, 2006c) and various halogenated pesticides (Torres-Duarte et al., 2009). Due to their wide range of substrates and the sole requirement of oxygen as the co-substrate, laccases appear to be a promising biocatalyst to enhance the biodegradation of micropollutants in wastewater in a complementary treatment step.

The goal of this preliminary study was thus to assess the potential of laccases and laccase-mediator systems for the removal of a wide range of micropollutants commonly found in municipal wastewater. More specifically, the goals were: (i) to screen which pollutants can be oxidized by laccase or laccase-mediator systems, at high (20 mg l^{-1}) and at environmentally relevant (1 µg l^{-1}) concentrations in synthetic acidic wastewater, and (ii) to assess the potential of laccase and laccase-mediator systems for micropollutant removal in real treated wastewater at near neutral pH.

5.2 Materials and methods

5.2.1.1 Chemicals and enzyme

Micropollutants, laccase powder from *Trametes versicolor* (ref. 38429, Sigma), 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), and all other chemicals were purchased from Sigma-Aldrich Chemie GmbH (Buchs, Switzerland).

5.2.1.2 Laccase activity test

Laccase activity was determined using a colorimetric assay by measuring the oxidation of 0.5 mM ABTS in oxygen-saturated acetate buffer (0.1 M) at pH 4.5 and 25°C. Laccase preparation was added to the solution after which the initial linear reaction rate, calculated from the formation of ABTS cation radicals, was measured as the increase of absorbance at 420 nm in a temperature-controlled spectrophotometer (U-3010, Hitachi, Tokyo, Japan). One unit of activity (U) was defined by the oxidation of one µmol of ABTS per min, using the extinction coefficient ε_{420nm} of 36,000 M^{-1} cm^{-1} (Childs and Bardsley, 1975).

5.2.1.3 Micropollutant analysis at high concentrations (mg l^{-1} range)

Determination of micropollutant concentrations at the mg l^{-1} range was carried out by reverse phase liquid chromatography with a diode-array detector (HPLC-DAD) (LC-2000plus, Jasco, Tokyo, Japan, equipped with Bondapack-C18 column, 15-20 µm, 3.9 mm × 300 mm, Waters™, Milford, USA). An aliquot of 50 µl of sample was injected. Separation of the compounds was conducted with a 1-h gradient, at 1 ml min^{-1}, of pure H_2O containing 0.1% acetic acid and increasing concentration of methanol (with 0.1% acetic acid) from 5 to 65% (v/v). Detection of the compounds was done by DAD at 200 nm. The limit of detection (LOD) was, for most compounds, around 0.3 mg l^{-1} (~1 µM).

5.2.1.4 Micropollutant analysis at low concentrations

Low micropollutant concentrations (ng l^{-1} to µg l^{-1}) were analysed by online solid phase extraction (SPE), followed by ultraperformance liquid chromatography coupled with tandem mass spectrometry (UPLC-MS/MS). Five ml of sample were loaded via a 5 ml loop at a flow rate of 2 ml min^{-1} into the SPE cartridge (2.1 × 20 mm, with Oasis HLB 25 µm phase, Waters), which was previously washed 2 min at 2 ml min^{-1} with pure methanol and conditioned 1 min at 2 ml min^{-1} with H_2O with 1% (v/v) formic acid. The cartridge was then progressively eluted in back-flush mode with the gradient of solvents used for the UPLC at 0.4 ml min^{-1} during 12 min. The SPE effluent was directly injected into the UPLC column and served as UPLC mobile phase. Compounds were separated on the UPLC column (Acquity UPLC system, with BEH C18 column, 2.1 × 50 mm, 1.7 µm, Waters) at 30°C, eluted with an aqueous-organic mobile phase (SPE effluent) composed of (v/v) 94.8% H_2O, 5% acetonitrile and 0.2% NH_4OH (solvent A) and 5% H_2O, 94.8% acetonitrile and 0.2% NH_4OH (solvent B) in gradient mode, from 5 to 95% solvent B (v/v) in 12 min at 0.4 ml min^{-1}. The column was previously equilibrated during 5 min at 0.4 ml min^{-1} with 95% solvent A and 5% solvent B. Target compounds were identified and quantified using tandem mass spectrometry (MS/MS) (Xevo TQ MS, Waters) as described in Appendix IX. Losses during extraction and matrix effects were corrected by adding internal standard (deuterated pollutants) before processing the samples. The detection limit was between 6 and 60 ng l^{-1} depending on the substances (cf. Table 5.1).

5.2.1.5 Screening of the range of pollutants oxidized by laccase or laccase-mediator

For this screening, a selection of 28 micropollutants commonly found in municipal wastewater (Table 5.2, compounds with superscript a), including 16 pharmaceuticals, 10 pesticides/biocides, and two industrial chemicals, was tested. These compounds were selected due to the possibility to be analyzed easily by a cheap HPLC-DAD method. The reaction solution was prepared by diluting a stock solution

(1 g l^{-1} in methanol) of a mixture of micropollutants to a final concentration of 20 mg l^{-1} each in a 20 mM citrate-phosphate buffer at pH 5.2. The 28 pollutants were separated in four mixtures of seven compounds each with different polarity in order to be able to separate them correctly with the HPLC method. Relatively high concentrations were tested to avoid a pre-concentration step before the analysis. Batch reactions were conducted in 2-ml glass vials containing 1 ml of oxygen-saturated reaction solution. Reactions were initiated by adding commercial laccase to reach a final laccase activity of 600 U l^{-1}. A parallel experiment in the same conditions but with addition of a mediator, ABTS, at 100 µM (about 1 mole ABTS per mole of micropollutants) was also performed. The flasks were incubated for 7 to 24 h in the dark at 25°C under static conditions. After defined reaction times, aliquots (50 µl) were withdrawn from each vial and directly injected into the HPLC column to analyse micropollutant concentrations.

5.2.1.6 Micropollutant oxidation assay with laccase in synthetic wastewater at low concentrations

The reaction solution was prepared by diluting a stock solution (100 µg l^{-1} in pure water) of a mixture of micropollutants to a final concentration of 1 µg l^{-1} each in a 10 mM acetate buffer at pH 4.5. The mixture of compounds was composed of 31 micropollutants commonly found in municipal wastewater (Table 5.2, compounds with superscript b), including 17 pharmaceuticals, 8 pesticides/biocides, 5 endocrine disruptors and one corrosion inhibitor. Batch reactions were conducted in 250 ml Erlenmeyer flasks containing 50 ml of oxygen-saturated reaction solution. Reactions were initiated by adding 5 ml of commercial laccase stock solution (1 g l^{-1} in pure water) to reach a final laccase activity of 500 U l^{-1}. A parallel experiment in the same conditions but with addition of a mediator, ABTS, at 1.4 µM (about 10 moles ABTS per sum of the moles of micropollutants) was also performed. The flasks were incubated for 20 h in the dark at 30°C on a rotating shaker at 150 rpm. Samples (8 ml per replicate) were then filtered at 0.22 µm (glass micro fiber filter, BGB Analytik) and immediately processed by online solid phase extraction (SPE) and analyzed by UPLC-MS/MS. Experiments were done in duplicate with four controls: i) mixture of pollutants without laccase, ii) mixture of pollutants with heat-inactivated laccase, iii) mixture of pollutants with ABTS and without laccase, and vi) mixture of pollutants with ABTS and heat-inactivated laccase. The first control was to assess the physico-chemical loss during the experiment, and the others to assess the influence of the presence of inactivated laccase or mediator alone on the removal rates. Removal rates by laccase were calculated relative to the second control, and by laccase-mediator relative to fourth one.

5.2.1.7 Micropollutant oxidation assay with laccase in real wastewater

Treated wastewater (TWW) (24-h composite sample, time proportional sampling every 15 min) from the effluent of the municipal WWTP of Lausanne (activated sludge without nitrification) was filtrated at 0.45 µm (glass microfiber filters) and then used for the tests. Two sets of experiments were performed: (i) with TWW slightly acidified (with HCl 1 M) to a pH of 6.5 (increasing to pH 7 at the end of the test), and (ii) with TWW not acidified, with an initial pH of 7.6 (reaching 7.8 at the end of the test). As for the test with synthetic wastewater, batch reactions were conducted in 250 ml Erlenmeyer flasks containing 50 ml of TWW. Reactions were initiated by adding commercial laccase to reach a high final laccase activity of 1000 to 1700 U l^{-1}. A parallel experiment in the same

conditions (both pH values) but with addition of a mediator, ABTS, at 100 µM was also performed. The flasks were incubated for 12 h in the dark at 23°C on a rotating shaker at 150 rpm. Samples (10 ml per replicate) were then filtered at 0.22 µm (glass micro fiber filter, BGB Analytik) and the reaction was stopped by addition of 5 mM sodium azide (laccase inhibitor). Samples were then processed by online SPE and analyzed by UPLC-MS/MS for the determination of 41 micropollutants. Experiments were done in duplicate with two controls: i) TWW (pH 6.5) with heat-inactivated laccase, and ii) TWW (pH 6.5) with ABTS and heat-inactivated laccase.

5.3 Results and discussion

5.3.1 Micropollutants degraded by laccase and laccase-mediator systems

The screening performed in buffer solution (pH 5.2) with relatively high concentrations of pollutants (20 mg l^{-1}) and laccase (600 U l^{-1}), with or without mediator (100 µM ABTS), showed that five pollutants (out of the 28) were completely oxidized by laccase alone (Fig. 5.1 A and B), and three by laccase and ABTS (Fig. 5.1 C and D). The 20 other pollutants tested were not significantly removed (< 10%) during laccase or laccase-mediator treatments (Table 5.2, compounds with superscript a).

All the pollutants oxidized by laccase alone were already reported to react and to be detoxified with this enzyme: the anti-inflammatory drugs diclofenac (DFC) (Lloret et al., 2013; Marco-Urrea et al., 2010b) and mefenamic acid (MFA) (Hata et al., 2010), the plastic component bisphenol A (BPA) (Cabana et al., 2007a; Saito et al., 2004), the biocide triclosan (TCN) (Inoue et al., 2010; Kim and Nicell, 2006c), and the analgesic paracetamol (PCL) (Lu et al., 2009). The ability of laccase to oxidize DFC, a pollutant usually not significantly removed in conventional WWTPs and already toxic at low concentrations; MFA, a pollutant poorly removed in WWTPs and found in relatively high concentrations in effluents; or BPA and TCN, two substances with potential toxicity already at low concentrations (c.f. Table 2.2, Chapter 2), is promising and shows the potential of laccases for WWTP effluent quality improvement. The optimal conditions for the oxidation of these four substances were thus determined and are presented in Chapter 5. PCL, despite its very high concentrations in raw wastewater, is easily biodegradable and already almost completely removed in conventional WWTPs (c.f. Chapter 3), thus PCL treatment by laccase does not make really sense in municipal wastewater.

To confirm that PCL was removed by laccase oxidation and not by other chemical or biological processes, controls with laccase inhibited with sodium azide (Fig. 5.1 C) or without laccase (data not shown) were performed. No PCL removal was observed in the controls, confirming the role of laccase in the removal process.

Both PCL and BPA were oxidized very rapidly (> 90% removal in 30 min) by laccase at pH 5. TCN was also removed in a short time (> 90% removal in 2 h), while DFC and MFA were degraded at a slower rate. As shown in Fig. 5.4, PCL, BPA and TCN contain a phenol group which is known to be a good substrate for laccase (Gianfreda et al., 1999). This may explain their fast reaction. DFC and MFA contain both an (electron donating) aniline group, which is probably the group oxidized by laccase. This suggests that anilines react slower than phenols. PCL, with both a phenol and an aniline group, appeared to be a very good substrate for laccases.

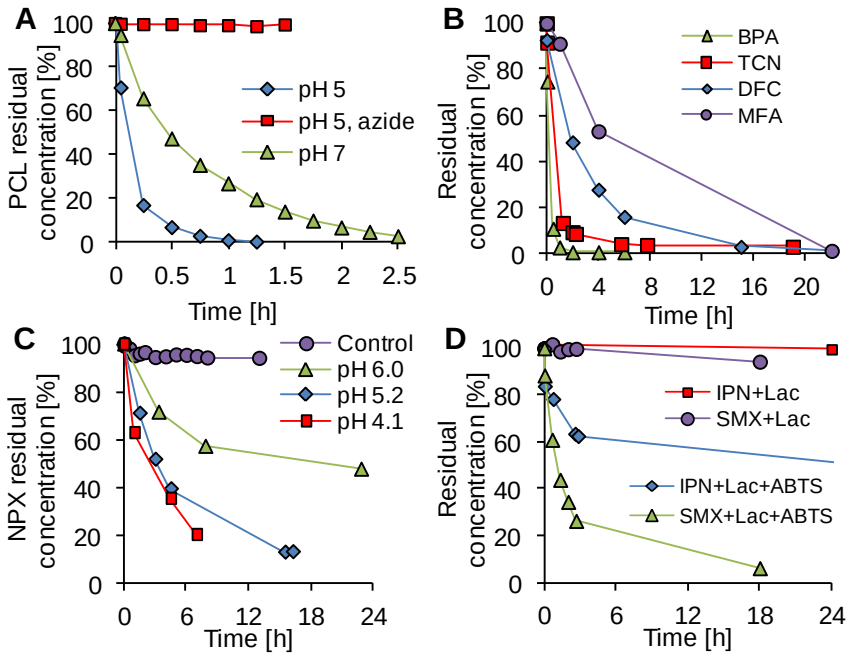


Fig. 5.1 (A and B): Micropollutants removed by laccase catalyzed oxidation (600-700 U/l). (C and D): Micropollutants removed by laccase (600 U/l) – mediator (ABTS 100 µM) reactions. (A) Paracetamol (PCL) with laccase at pH 5 and pH 7 and at pH 5 with inhibition of laccase activity with sodium azide. **(B)** Bisphenol A (BPA), triclosan (TCN), diclofenac (DFC) and mefenamic acid (MFA) with laccase at pH 5. **(C)** Naproxen (NPX) with laccase and ABTS at pH 4.1, 5.2 and 6.0, and at pH 5.2 with laccase but without mediator (Control). **(D)** Isoproturon (IPN) and sulfamethoxazole (SMX) at pH 5, with laccase (Lac), with or without ABTS.

Among the three pollutants oxidized by laccase-mediator systems (LMS) (Fig. 5.1 C and D), only the anti-inflammatory drug naproxen (NPX) was previously reported to be degraded by LMS (at the time of the study) (Lloret et al., 2010). The ability of LMS to degrade NPX, usually found in relatively high concentrations in WWTP effluents, as well as the oxidation of the antibiotic sulfamethoxazole and the herbicide isoproturon, two pollutants potentially toxic at low concentrations (PNEC < 200 ng l^{-1}) (c.f. Table 2.2, Chapter 2), suggests that laccase-mediated reactions may be of interest to improve effluent quality. This question was therefore addressed in Chapter 7.

The effect of the pH on micropollutant oxidation by laccase or laccase-mediator was evaluated for NPX and PCL. For the other pollutant, this effect was studied more in details in the next chapters. As presented in Fig. 5.1 A and C, both compounds were oxidized faster at lower pH (pH 4-5). The lower oxidation rates under neutral-alkaline conditions may thus impact the removal efficiencies in municipal wastewaters (pH 6.5-8).

During a screening with laccase and two different mediators, syringaldehyde (SA) and 1-hydroxybenzotriazole (HBT), Nguyen et al. (2014a) recently observed that the antiepileptic primidone (with HBT) and especially the herbicide atrazine (with both SA and HBT) were removed during laccase-mediator treatment. As this was not observed in our screening with ABTS, a complementary experiment with atrazine and primidone with the mediators SA and acetosyringone (AS), as well as with various concentrations of ABTS were performed. As presented in Fig. 5.2 A, contrary to the observation of Nguyen et al. (2014a) with SA, atrazine was not degraded by any of the mediators tested, confirming our first screening. Primidone was not oxidized by laccase-mediator reactions with

SA and AS, but a low removal (around 20%) was observed with ABTS (Fig. 5.2 B). To confirm this, a test with different concentration of ABTS was performed, showing that no more than 20% primidone removal could be reached, even at high mediator concentrations (1 mM) and long reaction times (up to 7 d) (Fig. 5.2 C). It cannot be excluded that the mediator HBT would perform better, but in any case, it seems that primidone is not easily degraded by laccase-mediator reactions.

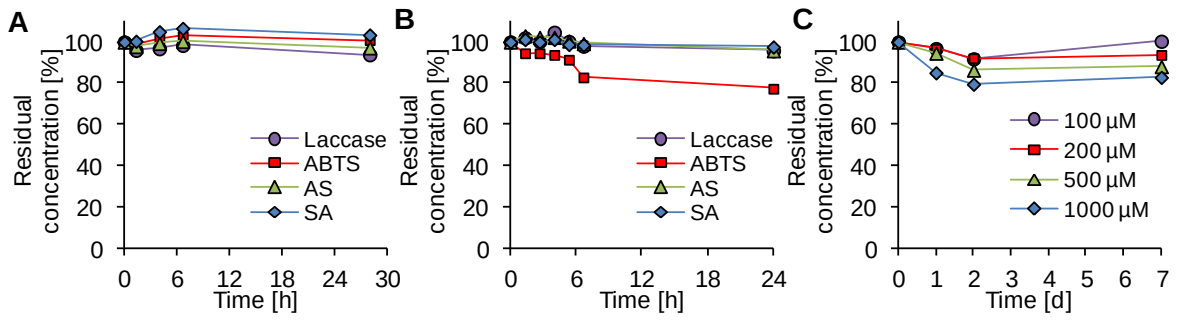


Fig. 5.2 Residual concentrations of (A) atrazine and (B) primidone during the treatment with laccase alone (●, 550 U/l), laccase with ABTS (■, 500 µM), laccase with AS (▲, 500 µM) and laccase with SA (◊, 500 µM) (at pH 6, 20 mg/l initial pollutant concentration). (C) Evolution of primidone concentrations during the treatment with laccase (550 U/l) and different concentrations of ABTS (from 100 to 1000 µM) (at pH 5).

5.3.2 Transformation of micropollutants at environmentally relevant concentrations

Out of the 31 micropollutants tested at low concentrations (1 µg l^{-1}) in acidic synthetic wastewater, seven were removed during the laccase treatment (Table 5.1): bisphenol A (BPA), diclofenac (DFC), mefenamic acid (MFA), and four estrogenic compounds, including the natural and synthetic hormones estrone (E1), β-estradiol (E2), estriol (E3) and 17α-ethynil estradiol (EE2). Losses of micropollutants were observed in both controls, without enzyme or with inactivated laccase. The losses were especially high for MFA and relatively high for DFC, two compounds that are not stable under acidic conditions (cf. Chapter 8). Thus, due to the long incubation at pH 4.5, acidic degradation or hydrolysis could occur, and seemed to be significantly more important at these low concentrations compared to what was observed at 20 mg l^{-1}. Losses of the other compounds, stable at acidic pH, are not explained but could be partially due to sorption to the vessels or the filters, or to chemical degradation. The removals observed in the samples treated with laccase were significantly higher than the one observed in the controls, showing the ability of laccase to oxidize these substances even at very low concentrations (ng l^{-1} to µg l^{-1}). The seven compounds were not anymore detected (concentrations below the LOD) in the samples treated with laccase, corresponding to a removal rate of over 90% compared to the samples treated under the same conditions with inactivated laccase.

Table 5.1 Concentrations (average ± SD of duplicate) of micropollutants before (Initial) and after 20 h reaction time at 30°C, pH 4.5, with 500 U l^{-1} laccase (Laccase treatment). Control 1: same conditions than the laccase treatment but without laccase. Control 2: same conditions than the laccase treatment but with inactivated (boiled) laccase. Removal rates were calculated based on the concentration of Control 2 to take account only of the transformation by laccase. LOD: analytical limit of detection. BPA: bisphenol A, DFC: diclofenac, MFA: mefenamic acid, E1: estrone, E2: β-estradiol, E3: estriol and EE2: 17α-ethynil estradiol.

Concentration		**BPA**	**DFC**	**MFA**	**E1**	**E2**	**E3**	**EE2**
LOD	[ng l^{-1}]	20	20	6	40	48	60	60
Initial	[ng l^{-1}]	1084 (±24)	1078 (±25)	1026 (±13)	1145 (±20)	1065 (±81)	971 (±20)	1196 (±80)
Control 1 (without laccase)	[ng l^{-1}]	678 (±14)	283 (±5)	78 (±14)	553 (±58)	545 (±29)	927 (±15)	497 (±134)
Control 2 (boiled laccase)	[ng l^{-1}]	765 (±20)	449 (±13)	118 (±16)	656 (±74)	680 (±34)	836 (±16)	365 (±4)
Laccase treatment	[ng l^{-1}]	< 20	< 20	< 6	< 40	< 48	< 60	< 60
Removal	**[%]**	**> 97 %**	**> 96 %**	**> 95 %**	**> 94 %**	**> 93 %**	**> 93 %**	**> 84 %**

The 24 other micropollutants were not significantly removed (< 20%) by laccase or laccase-mediator treatments compared to the controls (Table 5.2, compounds with superscript b). On the contrary to the results at higher concentrations (20 mg l^{-1}), isoproturon, naproxen and sulfamethoxazole were not significantly removed even by the laccase-mediator treatment. This may be due to the very low concentration of mediator (ABTS) used (1.4 µM), which may be not sufficient to oxidize these pollutants (c.f. Chapter 7).

5.3.3 Micropollutant removal in real wastewater

Due to analytical problems with the UPLC column, only five micropollutants could be unambiguously quantified in TWW: DFC, MFA, gemfibrozil, NPX and bezafibrate. These compounds were not removed in the controls with heat-inactivated laccase, or heat-inactivated laccase and ABTS. As presented in Fig. 5.3, none of them was neither clearly removed (less the 30% at pH 6.5 and not at all at pH 7.6) by laccase treatment at both pH values tested during the 12 h of reaction. The addition of the mediator ABTS allowed almost complete removal of both DFC and MFA at pH 6.5, enhancing strongly the reaction. ABTS did not, however, improve the removal of the other pollutants. At pH 7.6, the mediator did not enhance the reaction, even for DFC and MFA, as no significant removal of any pollutant was observed.

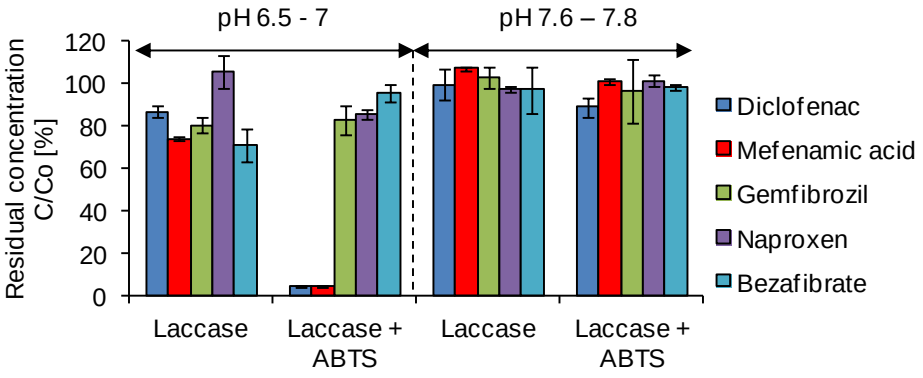


Fig. 5.3 Diclofenac (initial concentration 1540 ng/l), mefenamic acid (810 ng/l), gemfibrozil (130 ng/l), naproxen (87 ng/l) and bezafibrate (302 ng/l) residual concentrations in real treated wastewater after 12 h reaction with laccase (1000 to 1700 U/l), or laccase (1000 U/l) and ABTS (100 µM), at 23°C, at either pH 6.5-7 (slightly acidified) or pH 7.6-7.8 (not acidified). Average and values of duplicates.

The low removal efficiencies of DFC and MFA in TWW (pH 6.5 – 7.6) by laccase compared to the good removals observed at the same concentrations in acidic (pH 4.5) synthetic wastewater, as well as the low effect of laccase-mediated reaction in TWW on NPX removal compared to the good NPX removal observed under acidic conditions with LMS, confirm the strong effect of pH on laccase and laccase-mediator oxidation capacity. Treating micropollutants by laccase, alone or together with a mediator, in municipal wastewater (pH 7 – 7.5) will thus require either to acidify the water, or to find a laccase from another organism, more active at neutral to alkaline pH.

5.4 Synthesis of the removal of micropollutants with laccase or laccase-mediator systems

Out of the 39 micropollutants tested in this study, nine could be oxidized by laccase, and three with laccase-mediator reactions (Table 5.2). In addition to these twelve compounds, seven other micropollutants frequently found in municipal wastewater have been reported in the literature to be oxidized by laccase or laccase-mediator reactions (Table 5.2). All the compounds oxidized by laccase contained either a phenol or an aniline (electron donating) group (Fig. 5.4).

Table 5.2 Micropollutants degraded or not with laccase or laccase-mediator. Synthesis of the pollutants investigated in this study and of other micropollutants frequently found in wastewater (literature data)

Degraded with:				**Not degraded**		
Laccase	Ref.	**Laccase - mediator**	Ref.			Ref.
17α-ethinylestradiol[b]		Isoproturon[ab]		Atenolol[a]	Iopromide[b]	
17β-estradiol[b]		Naproxen[ab]		Atrazine[ac]	Irgarol[ab]	
Bisphenol A[ab]		Sulfamethoxazole[ab]		Benzotriazole[ab]	Ketoprofen[ab]	
Diclofenac[ab]		*Ciprofloxacin*	[4]	Bezafibrate[ab]	Mecoprop[ab]	
Estriol[b]		N,N-*diethyl*-m-*toluamide (DEET)*	[2]	Carbamazepine[ab]	Metformin[a]	
Estrone[b]		*Norfloxacin*	[4]	Carbendazim[ab]	Metoprolol[a]	
Mefenamic acid[ab]		*Oxybenzone*	[3]	Clindamycin[b]	Nadolol[a]	
Paracetamol[a]				Clofibric acid[b]	Oxazepam[b]	
Triclosan[a]				Diazinon[ab]	Propranolol[b]	
Nonylphenol	[1]			Diuron[ab]	Propiconazole[ab]	
Octylphenol	[6]			Gemfibrozil[a]	Sotalol[ab]	
Tertacycline	[5]			Ibuprofen[b]	Terbutryn[ab]	
				Iomeprol[ab]	Trimethoprim[ab]	
				Iopamidol[ab]	*Metronidazole*	[6]

In italic: pollutants not investigated in this study. Results from the literature

References: [1] (Cabana et al., 2007a), [2] (Tran et al., 2013a), [3] (Garcia et al., 2011), [4] (Prieto et al., 2011), [5] (De Cazes et al., 2014), [6] (Nguyen et al., 2014a)

[a] Conditions of the test: pH 5.2 (citrate-phosphate buffer, 20 mM), 25°C, 600 U/l laccase activity, 20 mg/l pollutant, with or without 100 µM ABTS (mediator). Reaction time: 7-24 h.

[b] Conditions of the test: pH 4.5 (acetate buffer, 10 mM), 30°C, 500 U/l laccase activity, 1 µg/l pollutant, with or without 1.4 µM ABTS (mediator). Reaction time: 20 h.

[c] Atrazine was reported to be oxidized with the mediator HBT [6], not tested here

Despite the wide range of chemicals oxidised by laccase, and the potential of this enzyme to degrade pollutants even at environmentally relevant concentrations, treatment of non-phenolic or non-aniline compounds seems to be very limited, even with the addition of mediators. Indeed, the majority of the pollutants tested could not be removed by laccase or laccase-mediator treatment. Moreover, the strong pH effect on laccase reactions may strongly limit the feasibility of such treatment in real not-acidified wastewater.

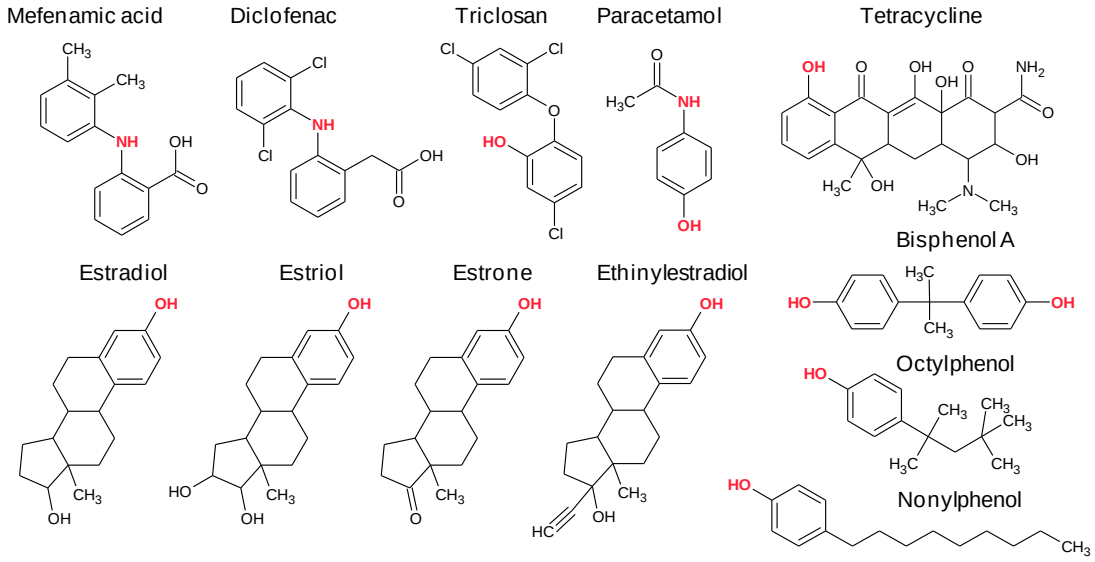


Fig. 5.4 Structures of the pollutants degraded by laccase. All are phenol or aniline compounds (on red).

Although a pure enzymatic treatment with laccase to decrease the load of micropollutants in municipal WWTP effluents does not seem to be an interesting option (too narrow range of pollutant affected), using laccase in combination with other removal processes (e.g., biodegradation or adsorption) may significantly improve the quality of the effluent. Indeed, among the micropollutants degraded by laccase, six were proposed as priority pollutants in the European water framework directive (EC, 2011): nonylphenol, octylphenol, isoproturon, diclofenac, 17β-estradiol and 17α-ethinylestradiol. Moreover, laccase treatment may be a very interesting option to remove diclofenac and mefenamic acid from wastewater, as these compounds are only poorly removed in conventional treatment and found in relatively high concentrations in WWTP effluents (c.f. Chapter 3). The ability of laccase to degrade oestrogenic compounds (hormones, bisphenol A, nonylphenol, octylphenol, triclosan) is also of great interest as these substances are linked to strong impacts on aquatic populations (e.g. fish and mussels feminization) downstream of WWTP outfalls (c.f. Chapter 1). Therefore, using laccase to treat micropollutants in wastewater may be a potential alternative to other advanced treatments, if the goal is to remove targeted toxic compounds and not to decrease the load of a wide range of pollutants.

The assessment of the feasibility of laccase or laccase-mediator systems for the treatment of specific micropollutants in wastewater required first (i) to determine the operational conditions (pH, temperature, laccase activity, reaction time, mediator concentration) for which the treatment is efficient, and to compare them with the conditions found in municipal wastewater, and (ii) to investigate how to produce or maintain the enzymes inside the reactor and by which organisms. These questions were addressed in the three following chapters.

Chapter 6 Influence of treatment conditions on the oxidation of micropollutants by laccase

*An adapted version of this chapter was published in **New Biotechnology (2013) 30: 803-813**, with the name "Influence of treatment conditions on the oxidation of micropollutants by Trametes versicolor laccase", by Jonas Margot, Julien Maillard, Luca Rossi, D. Andrew Barry and Christof Holliger.*

6.1 Introduction

In order to develop an efficient biooxidative treatment, the influence of operational conditions on micropollutant oxidation by laccases has to be investigated. This study is a step towards this goal. Specifically, we aimed to understand better the combined effect of pH, temperature, reaction time (kinetics) and enzyme concentration on the removal of diclofenac (DFC), mefenamic acid (MFA), bisphenol A (BPA) and triclosan (TCN), catalysed by the laccase from *Trametes versicolor*. Special attention was paid to pH due to its potentially high influence on transformation rates (Kim and Nicell, 2006b; Kim and Nicell, 2006c). The experimental design involved the response surface methodology (RSM) so as to cover a wide range of different experimental conditions with maximum information gain (Bezerra et al., 2008). A Doehlert design was chosen due to its flexibility with the number of factors studied, the possibility to move the range of values easily if the optimal conditions were not included in the first set-up, and the low number of experiments needed compared with other designs (Bezerra et al., 2008; Ferreira et al., 2007; Ferreira et al., 2004). As pollutant mixtures are common, the second objective of this study was to evaluate the effect of the presence of other pollutants in the reactive solutions compared to single-compound solutions.

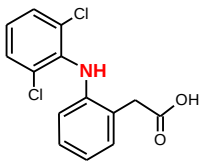
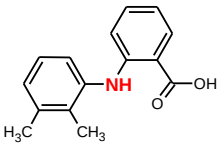
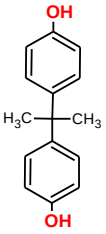
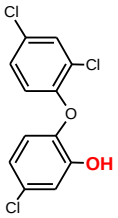
6.2 Materials and methods

6.2.1.1 Selection of the micropollutants

Four common wastewater micropollutants were selected considering their tendency to be oxidised by laccase (Table 6.1). DFC, a common anti-inflammatory drug, has been identified as one of the most important active pharmaceuticals present in the environment (Letzel et al., 2009). DFC is poorly removed in conventional WWTPs (Tauxe-Wuersch et al., 2005) and can affect fish at concentrations usually found in WWTP effluents (Letzel et al., 2009; Stülten et al., 2008; Triebskorn et al., 2004). MFA, another common anti-inflammatory drug, is found at relatively high concentrations in WWTP effluents (0.8 to 2.4 µg l^{-1}) (Kase et al., 2011; Tauxe-Wuersch et al., 2005). TCN, an antimicrobial agent widely used as preservative in many personal care products, is ubiquitous in municipal wastewater (Singer et al., 2002). Despite its high removal rate (72-94%) in WWTPs (Singer et al., 2002; Ying and Kookana, 2007), the concentration found in effluents can still be toxic for sensitive organisms (Ricart et al., 2010). BPA, a compound mainly used in plastic and thermal paper products, is detected in most WWTP effluents despite its relatively high removal rate (61-98%) during treatment

(Melcer and Klečka, 2011; Weltin et al., 2002). Due to its endocrine-disrupting ability at low concentrations (Crain et al., 2007) and its potential risk for human health, BPA is of special concern and has been banned in many products in Europe and North America (Canadian Governor General in Council, 2010; European Commission, 2011).

Table 6.1 Main properties of the four micropollutants studied, which include two aniline (DFC and MFA) and two phenolic (BPA and TCN) compounds. In the molecular structure, groups in bold (in red) are considered to be oxidized by laccase.

Compound	Diclofenac	Mefenamic acid	Bisphenol A	Triclosan
CAS	15307-86-5	61-68-7	80-05-7	3380-34-5
Molecular structure				
Molecular weight [g mol^{-1}][a]	296.16	241.29	228.29	289.55
Water solubility [mg l^{-1}][a]	2.4	20	120	10
Log K_{ow} [-][a]	4.51	5.12	3.32	4.76
pKa [-][a]	4.15	4.2	10.3 [d]	7.9 [b]
Average Swiss WWTP effluents concentration [ng l^{-1}][c]	647	870	331	116
Environmental quality standards (EQS) [ng l^{-1}][c]	50	4000	1500	100 [b]

[a] SRC PhysProp Database : http://www.srcinc.com/what-we-do/databaseforms.aspx?id=386 (last accessed 09.07.2012)
[b] Water Framework Directive - United Kingdom: http://www.wfduk.org/sites/default/files/Media/Triclosan%20-%20UKTAG.pdf (last accessed 09.07.2012)
[c] (Kase et al., 2011), [d] (Clara et al., 2004)

6.2.1.2 Chemicals and enzyme

Purified laccase from *T. versicolor* (ref. 38429, Sigma), BPA, DFC sodium salt, MFA and TCN (purity > 97%) were purchased from Sigma-Aldrich Chemie GmbH (Buchs, Switzerland), as well as acetate sodium salt, acetic acid, citric acid, sodium phosphate dibasic, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), methanol (HPLC gradient grade), acetone and tris(hydroxymethyl)aminomethane (Tris).

6.2.1.3 Laccase activity test

Laccase activity was determined as described in Chapter 5. One unit of activity (U) was defined by the oxidation of one µmol of ABTS per min, at pH 4.5 and 25°C. Enzymatic assays were performed in triplicate with a coefficient of variation always smaller than 5%. The specific activity of the commercial laccase was measured as 7.3 U mg^{-1}.

6.2.1.4 Micropollutant analysis

Determination of BPA, DFC, MFA and TCN concentrations was carried out by reverse-phase liquid chromatography with a diode-array detector (HPLC-DAD), as described in Chapter 5. Separation of

the compounds was conducted at 25°C with a 20-min gradient of pure H_2O containing 0.1% acetic acid (pH 3.3) and increasing concentration of methanol from 40 to 65% (v/v) at a flow rate of 1 ml min^{-1}. The quantification was done with DAD by summing the area of the peaks at 200, 224 and 278 nm. The limit of detection (LOD) was around 0.3 mg l^{-1} (1 µM). Since TCN and MFA had the same retention times with this method, experiments with mixtures of micropollutants were only conducted with BPA, DFC and MFA.

Low micropollutant concentrations (LOD of 6 to 60 ng l^{-1}) were analysed by online solid phase extraction (SPE), followed by ultraperformance liquid chromatography coupled with tandem mass spectrometry (UPLC-MS/MS), as described in Chapter 5.

6.2.1.5 Micropollutant oxidation assay with laccase

The reaction mixture was prepared by diluting stock solutions (1 g l^{-1}) of pollutants to a final concentration of 20 mg l^{-1} (67 to 87 µM, depending on the compound) in different buffers made of citric acid (1 to 16 mM) and dibasic sodium phosphate (7 to 37 mM) for pH 3 to 7.5, and Tris-HCl (40 mM) for pH 8 to 9. Relatively high concentrations of micropollutants were tested in order to use a fast and simple analytical method (HPLC-DAD). As the studied micropollutants have limited water solubility, especially at low pH for MFA and DFC, the stock solutions were prepared in methanol (except for BPA, which was prepared in acetone). The final concentrations of methanol in the reaction mixture were between 6 and 12% (v/v) and 2% acetone, respectively, which prevented precipitation of the compounds. The effect of the organic solvents on laccase was tested and no difference in activity or stability between the solvent mixture and the aqueous control was observed after 24 h. Laccase stability was moreover not significantly influenced by the addition of micropollutants. Batch reactions were conducted in 8-ml glass vials containing 2 ml of oxygen-saturated reaction mixture. Diffusion of oxygen from the air was sufficient to maintain a high level of dissolved oxygen during the reaction (> 69% saturation). Reactions were initiated by adding a desired amount of laccase stock solution (1 g l^{-1} in pure water), the vials were well mixed (vortex mixer) and then incubated in darkness at different temperatures in thermostatic baths (10°C) or in thermostatic rooms (20 to 40°C) under static conditions. After defined reaction times, the oxidation was stopped by direct injection into the HPLC column. This allowed for rapid halting (less than 1 min) of the reaction without the need to add inhibitor compounds that could interfere with the analysis. Unlike other studies (Garcia et al., 2011; Kim and Nicell, 2006c; Lloret et al., 2010), samples were not acidified to stop the reaction so as to prevent the degradation or precipitation of MFA and DFC observed below pH 3 and to avoid increasing laccase activity at low pH prior to acidic inactivation of the enzyme.

6.2.1.6 Experimental design

In order to determine the optimal conditions and the combined effect of pH, temperature, reaction time and initial enzyme activity on the degradation of the four pollutants, a Doehlert experimental design (Ferreira et al., 2007; Ferreira et al., 2004) with Response Surface Methodology (RSM) was applied to minimize the number of experiments. Doehlert design involves conducting experiments in a hyperspherical experimental space filled uniformly. The number of experiments, N, that were conducted was calculated using $N = k^2 + k + C_0$, where k is the number (here four) of factors and C_0 the number of experiments at the centre point (Ferreira et al., 2004). Reaction time was studied at five

different levels between 1 and 7 h. pH and enzyme concentrations were each studied at 7 different levels (pH 3 to 9 and enzyme concentrations 73 to 1380 U l^{-1}). Temperature effects were tested at three levels (10, 25 and 40°C). Three experiments were conducted at the centre point to calculate the variance of the experimental response. A total of 23 experiments in different conditions were conducted (cf. Table 6.4 and Table 6.5).

Two sets of experiments were conducted according to this design (23 experiments each, all performed in less than 36 h), one with a mixture of BPA, DFC and MFA and one with TCN alone. For the latter set of experiments, 28 additional experiments under the same conditions but with longer reaction times were also conducted to confirm the reaction kinetics (cf. Table 6.5).

The same 23 experiments were also performed with a mixture of four other compounds, benzotriazole, carbamazepine, metoprolol and naproxen. As no significant removal (<10%) was observed in any of the tested conditions, these results are not presented here.

To study in more detail the pH effect as a function of the reaction time and to evaluate the effect of mixtures, a new set of experiments was conducted with the four compounds separately, at 25°C and 730 U l^{-1}, and by varying only two parameters, i.e. pH from 3 to 9 and the reaction time from 0 to 26 h.

In addition, the effect on the degradation of a mixture of pollutants compared to the compound alone was assessed at pH 7.2, 26°C, 730 U l^{-1}, and different reaction times from 0 to 20 h with BPA, DFC or MFA alone, in mixtures with the three possible pairs of compounds, and the three compounds together. Experiments were conducted in duplicate. Removal values of the duplicates did not differ from each other by more than 3%.

To evaluate the efficiency of laccase treatment at very low micropollutant concentrations (ng to µg l^{-1}), an experiment was conducted at pH 4.5 and 30°C with a mixture of BPA, DFC and MFA, and four estrogenic compounds, estrone (E1), β-estradiol (E2), estriol (E3) and 17α-ethynilestradiol (EE2), each at a concentration of 1 µg l^{-1}. Micropollutants were analysed after reacting for 20 h with 500 U l^{-1} laccase. This experiment is detailed in section 5.2.1.6, Chapter 5.

Controls without laccase and with boiled-inactivated laccase showed no removal of the compounds even after 40 h, except at low pH where partial removal (degradation or precipitation) of DFC was observed at pH 3, and of MFA below pH 5. In these cases, the calculation of the removal rate was corrected according to the control with inactivated laccase.

6.2.1.7 Statistical analysis

The results of the Doehlert experimental matrix were analysed by fitting a quadratic model (Eq. 6.1):

$$Y = a_0 + \sum_{i=1}^{4} a_i X_i + \sum_{i=1}^{4} \sum_{j=i+1}^{4} a_{ij} X_i X_j + \sum_{i=1}^{4} a_{ii} X_i^2, \quad (6.1)$$

to the results (RSM) in order to estimate the effect of each factor X_i (reaction time, pH, initial enzyme activity and temperature) and the two-by-two interactions (X_iX_j) on the removal of the compound Y. This model contains 15 coefficients (a_0, a_1, a_2, ...), which were determined by least squares with

multiple regression. Each of them corresponds either to the linear effect of the coded factor X_i, to the quadratic effect X_i^2, or to the two-by-two interactions between the factors X_jX_i. The significance of these coefficients was evaluated using ANOVA with correction of the sum of the squares for non-perpendicular elements. Coefficients with a p-value higher than 0.05 (F-test) were considered as non-significant. The factors were coded between -1 (minimum of the range) and +1 (maximum of the range), 0 being the centre of the experimental domain, to facilitate comparison amongst them.

The relationship between coded values used in Eq. 6.1 and real values presented in Table 6.4 and Table 6.5 is given by Eq. 6.2:

$$X_i = \frac{U_i - U_i^0}{\Delta U_i}, \tag{6.2}$$

where X_i is the coded value, U_i the real value of the variable, U_i^0 the real value at the centre of the interval and ΔU_i half of the interval (cf. Table 6.2 and Table 6.3).

Table 6.2 Main factors influencing enzymatic degradation, with the range of the values studied, and U_0 and ΔU used to code the factors for the Doehlert design with a mixture of DFC, MFA and BPA.

Factors	Variable	Unit	Minimum	Maximum	Centre value U_0	Range ΔU around the centre
Reaction time	X_1	[h]	1.1	7.0	4.1	3.00
pH	X_2	[-]	3.5	9.0	6.4	3.20
Laccase activity	X_3	[U l^{-1}]	73	1378	725	799.1
Temperature	X_4	[°C]	10	40	25	18.97

Table 6.3 Main factors influencing enzymatic degradation, with the range of the values studied, and U_0 and ΔU used to code the factors for the Doehlert design for TCN alone.

Factors	Variable	Unit	Minimum	Maximum	Centre value U_0	Range ΔU around the centre
Reaction time	X_1	[h]	0.5	5.7	3.1	2.61
pH	X_2	[-]	3.1	9.0	6.0	3.48
Laccase activity	X_3	[U l^{-1}]	62	1184	623	686.7
Temperature	X_4	[°C]	10	40	25	18.97

6.2.1.8 Modelling the transformation rate

Model including the effects of pH, temperature, enzyme concentration and reaction time

To approximate the oxidation process and to allow predictions for a wide range of conditions, which is not feasible with the quadratic statistical model, a semi-empirical model was developed based on the behaviour observed in the experimental data. The influence of pH, temperature, enzyme concentration

and reaction time on the biooxidation of micropollutants by laccase was modelled by a global semi-empirical model that was built by combining different sub-models, as presented below.

For the reaction time, the order of the reaction kinetics appeared to be pH-dependent. At neutral pH, the data were well fitted by first-order kinetic models, but as the pH decreased, the order of reaction seemed to increase (Fig. S 6.1, Supporting information (SI)), probably due to the involvement of protons and hydroxyl anions in the reaction. Thus, to best fit the data, a kinetic model with a pH-dependent reaction order was developed.

By integrating the standard model of a chemical reaction rate (Eq. 6.3), describing the transformation of a reactant *A* as a function of the time *t*, *k* being the reaction rate constant and *x* the order of the reaction, we obtain the residual concentration of the compound [*A*] after time *t*, as a function of the order of the reaction (Eq. 6.4):

$$v = \frac{\mathrm{d}[A]}{\mathrm{d}t} = k[A]^x \tag{6.3}$$

$$[A] = \left(\frac{d}{k\,t + \frac{d}{[A]_0^{1/d}}} \right)^d \quad \text{with } d = \frac{1}{x-1},\ \forall x \neq 1 \tag{6.4}$$

The removal rate *Y* of the compound *A* can thus be described by Eq. 6.5:

$$Y = 1 - \frac{[A]}{[A]_0} = 1 - \left(\frac{d}{k\,t[A]_0^{1/d} + d} \right)^d \quad \text{with } d = \frac{1}{x-1},\ \forall x \neq 1 \tag{6.5}$$

By assuming that the initial concentration of the reactant $[A]_0 = 1$ (arbitrary concentration units as the initial concentration was always constant), the influence of the reaction order on the removal could be assessed by a simple model (Eq. 6.6). When *x* is close to 1, this model is very close to the exponential model (first-order reaction).

$$Y = 1 - \left(\frac{d}{kt + d} \right)^d \text{ with } d = \frac{1}{x-1},\ \forall x \neq 1. \tag{6.6}$$

By fitting Eq. 6.6 to the experimental data (e.g., Fig. S 6.2, SI), the optimised reaction order for each pH was determined. The results of the fitting show that the apparent order of the reaction was influenced by the pH (Fig. 6.1). The apparent order of the reaction was close to 1 at around pH 7, but it increased to above 2 at pH 3. According to these results, the relationship between the apparent reaction order *x* and pH is roughly linear (Eq. 6.7), where *b* is the order of reaction at pH 0 (usually between 1 and 3) and *a* the slope of the decrease of the order of reaction with the increasing pH (usually between -0.03 to -0.3):

$$x = a\ pH + b \tag{6.7}$$

The global kinetic model with the pH-dependent reaction order could thus be described by using Eq. 6.7 in Eq. 6.6.

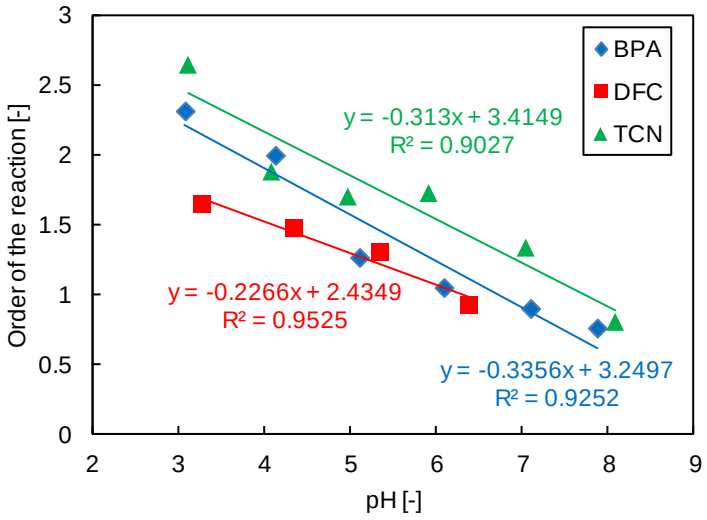


Fig. 6.1 Influence of the pH on the order of the kinetic reaction (determined with the best fitting of the experimental data) and approximation with linear regressions (lines).

As observed in the experimental part, the pH and the temperature influence the reaction rate constant, *k*. The bell-shape effect of the pH on laccase activity was well approximated by a standard acid-base speciation model for weak polyacids with two *pKa* values (Eq. 6.8), which possibly correspond to the ionization of the amino acids involved in the catalytic process (Joshi et al., 2000). The values of pK_1 and pK_2 gave the optimal pH range, the optimum being at the centre. C_1 was used as an adjustment coefficient.

$$k_1 = C_1\left(\frac{1}{10^{pK_1-pH}+1}-\frac{1}{10^{pK_2-pH}+1}\right). \tag{6.8}$$

The enzyme concentration *E*, which, obviously, influences the reaction rates, should in theory be included in Eq. 6.3 as another reactant. As the enzyme is a catalyst, its concentration is not expected to decrease with time (deactivation neglected). Therefore, the effect of *E* can be integrated in the apparent rate constant *k*. The influence of *E*, which showed a saturation behaviour at high concentrations, was well approximated by a simple Monod-like model with two coefficients *K* and C_2 (Eq. 6.9), with C_2 being the constant rate at enzyme saturation (k_{max}) and *K* the half-velocity constant. For the initial value, $K >> E$ could be used to simulate a linear effect of the enzyme concentration.

$$k_2 = C_2\frac{E}{K+E}. \tag{6.9}$$

A simple Arrhenius model could be used to predict the effect of temperature on the constant rate, giving increasing activity with increasing temperature. However, for enzymatic reactions, denaturation of the protein occurs when temperature exceeds a certain level. Thus, a more realistic model was used. A generalized Arrhenius function, developed by Alexandrov et al. (2007) for enzymatic reactions was chosen amongst others (Santos et al., 2007) due to its straightforward estimation of the initial values of

the coefficients. In addition to the standard coefficients of the Arrhenius model, including the pre-exponential factor C_3 and the activation energy *Ea* (usually between 30 and 100 kJ mol^{-1} (Huber et al., 2003; Kobelnik et al., 2010; Tita et al., 2009)), the generalized function (Eq. 6.10) needed two more coefficients, the optimal temperature *To* [°K] (usually in the range 30-60°C) and a parameter η determining the shape of the curve (usually between 1 and 10). *R* is the universal gas constant (8.314 J mol^{-1} K^{-1}).

$$k_3 = C_3\left(\frac{\eta f}{\eta - 1 + f^{\eta}}\right) \text{ where } f = \exp\left[\frac{Ea}{R}\left(\frac{1}{To} - \frac{1}{T}\right)\right]. \quad (6.10)$$

The **global model**, which was able to describe the effect of the four operational parameters over a wide range of values, was built by combining the abovementioned sub-models in Eq. 6.11, with nine coefficients available for fitting.

$$Y = 1 - \left(\frac{d}{kt + d}\right)^{d} \text{ with } d = \frac{1}{(a\,pH + b) - 1}, \quad (6.11)$$

with the rate constant, *k*, combining the effect of the various factors, described by Eq. 6.12:

$$k = C\left(\frac{\eta f}{\eta - 1 + f^{\eta}}\right)\left(\frac{E}{K + E}\right)\left(\frac{1}{10^{pK_1 - pH} + 1} - \frac{1}{10^{pK_2 - pH} + 1}\right), \text{ where } f = \exp\left[\frac{Ea}{R}\left(\frac{1}{To} - \frac{1}{T}\right)\right] \quad (6.12)$$

Here, *Y* is the removal fraction [-] of the compound, for the reaction time *t* [h], the pH [-], the initial enzyme activity *E* [U l^{-1}], and the temperature *T* [°K]. The nine coefficients to fit are *a* and *b* for the effect of the pH on the order of the reaction, *C* a global adjustment coefficient, η, *Ea* and *To*, the coefficients of the generalized Arrhenius function, *K* the Monod empirical coefficient for the enzyme concentration, and pK_1 and pK_2, the empirical coefficients of the acid-base speciation model.

The fitting of this model to the data was done by non-linear least squares regression (Levenberg-Marquardt algorithm). Initial coefficient values were estimated based on the results of the quadratic model. All the calculations were performed using Matlab (MathWorks, Natick, USA).

Model including only the effects of pH and reaction time

The results of the additional experiments performed at fixed temperature (25°C) and enzyme concentration (700 U l^{-1}) and variable pH and reaction times were fitted with a simple version of the semi-empirical model, by using Eq. 6.11 with the rate constant *k* defined in Eq. 6.13, as a function of the *pH* [-] only; *C*, pK_1, pK_2, *a* and *b* being the five coefficients that were fitted.

$$k = C\left(\frac{1}{10^{pK_1 - pH} + 1} - \frac{1}{10^{pK_2 - pH} + 1}\right) \quad (6.13)$$

6.3 Results and discussion

6.3.1 Micropollutant removal by laccase under various conditions

The percentages of removal of the micropollutants by laccase obtained under the different conditions are reported in Table 6.4 for DFC, MFA and BPA, and in Table 6.5 for TCN. A wide distribution of the removal between 0 and 100% was observed for the four compounds, demonstrating the ability of laccase to oxidise these compounds as well as the influence of the experimental conditions on degradation rates. The standard deviation of the three centre points was less than 3% for all the compounds.

Table 6.4 Observed and predicted (with the semi-empirical model) removal rates of DFC, MFA and BPA present in a mixture and oxidized by laccase under different experimental conditions (Doehlert design). Lines 1-3 present the three centre points. The validation experiments were not included in the fitting process.

Doehlert	Factors				DFC removal		MFA removal		BPA removal	
experiments	pH	Reaction time	Laccase activity	Temperature	Observed	Predicted	Observed	Predicted	Observed	Predicted
#	[-]	[h]	[U l^{-1}]	[°C]	[%]	[%]	[%]	[%]	[%]	[%]
1	6.4	4.25	725	25	**80**	83	**94**	96	**90**	93
2	6.4	4.08	725	25	**79**	83	**94**	96	**92**	93
3	6.4	4.08	725	25	**82**	82	**97**	96	**94**	93
4	6.5	1.12	725	25	**49**	46	**65**	56	**82**	74
5	6.4	7.03	725	25	**89**	92	**100**	100	**92**	96
6	3.6	2.67	725	25	**84**	81	**13**	30	**67**	59
7	9.1	5.60	725	25	**6**	2	**0**	1	**22**	19
8	8.9	2.45	725	25	**0**	1	**0**	1	**16**	12
9	3.5	5.58	725	25	**88**	91	**46**	43	**63**	71
10	5.5	2.48	73	25	**31**	21	**80**	78	**37**	36
11	7.3	5.52	1378	25	**72**	73	**52**	52	**95**	96
12	7.3	2.52	1378	25	**50**	49	**37**	28	**89**	90
13	4.7	4.10	1378	25	**92**	95	**100**	100	**96**	95
14	5.4	5.47	73	25	**34**	39	**91**	96	**47**	56
15	8.1	4.08	73	25	**5**	1	**0**	2	**9**	11
16	5.7	2.53	562	10	**42**	43	**95**	98	**62**	63
17	7.2	5.55	888	40	**66**	72	**39**	45	**95**	98
18	7.3	2.53	888	40	**36**	43	**18**	20	**88**	93
19	4.4	4.05	888	40	**100**	93	**100**	89	**99**	95
20	6.3	4.13	236	40	**71**	61	**83**	83	**93**	90
21	5.7	5.53	562	10	**57**	66	**98**	100	**81**	80
22	8.6	4.13	562	10	**2**	1	**8**	2	**5**	12
23	6.6	4.10	1214	10	**64**	63	**93**	87	**93**	87
Validation experiments										
1	7.3	4.00	1450	25	**69**	67	**50**	44	**93**	95
2	7.4	8.15	1450	10	**50**	50	**45**	51	**86**	91
3	7.4	25.15	1450	10	**92**	82	**88**	89	**93**	98
4	5.5	4.07	1015	29	**88**	94	**59**	100	**95**	96

Table 6.5 Observed and predicted removal rates with the semi-empirical model of TCN alone and oxidized by laccase under different experimental conditions (Doehlert design). Lines 1-3 present the three centre points. Second section: conditions of the additional experiments with longer reaction time. Bottom section: validation experiments, not included in the fitting process.

Doehlert experiments	Factors				TCN removal	
	pH	Reaction time	Laccase activity	Temperature	Observed	Predicted
#	[-]	[h]	[U l^{-1}]	[°C]	[%]	[%]
1	5.9	3.05	623	25	**84**	83
2	6.0	3.10	623	25	**86**	83
3	6.0	3.12	623	25	**89**	83
4	6.0	0.53	623	25	**37**	36
5	6.0	5.70	623	25	**91**	92
6	3.2	1.80	623	25	**33**	29
7	9.0	4.43	623	25	**1**	2
8	8.9	1.80	623	25	**3**	1
9	3.1	4.40	623	25	**47**	51
10	5.0	1.82	62	25	**27**	18
11	7.0	4.47	1184	25	**78**	83
12	7.0	1.83	1184	25	**54**	59
13	4.1	3.10	1184	25	**91**	88
14	5.0	4.48	62	25	**27**	36
15	8.0	3.08	62	25	**8**	1
16	5.2	1.83	483	10	**45**	37
17	6.8	4.45	763	40	**93**	94
18	6.9	1.83	763	40	**81**	79
19	3.9	3.10	763	40	**96**	93
20	5.8	3.13	202	40	**90**	82
21	5.2	4.47	483	10	**61**	62
22	8.6	3.15	483	10	**5**	1
23	6.2	3.18	1044	10	**66**	65
Additional experiments with longer reaction time						
24	6.0	7.25	623	25	**96**	94
25	3.2	8.50	623	25	**72**	70
26	8.9	8.50	623	25	**2**	4
27	5.9	9.73	623	25	**95**	96
28	5.0	8.50	62	25	**53**	54
29	7.0	8.50	1184	25	**94**	92
30	4.1	9.25	1184	25	**96**	97
31	8.0	9.22	62	25	**13**	3
32	6.0	8.97	623	25	**96**	96
33	9.0	9.50	623	25	**1**	4
34	3.1	9.47	623	25	**72**	72
35	7.0	9.52	1184	25	**94**	94
36	5.0	9.52	62	25	**50**	57
37	6.0	10.22	623	25	**96**	97
38	6.0	24.67	623	25	**96**	99
39	3.2	25.92	623	25	**88**	91
40	8.9	25.92	623	25	**9**	13
41	5.9	27.15	623	25	**98**	99
42	5.0	25.92	62	25	**79**	82
43	7.0	25.92	1184	25	**97**	99
44	4.1	26.67	1184	25	**98**	99
45	8.0	26.63	62	25	**19**	9
46	6.0	26.38	623	25	**96**	99
47	9.0	26.92	623	25	**10**	10
48	3.1	26.88	623	25	**92**	90
49	7.0	26.93	1184	25	**96**	99
50	5.0	26.93	62	25	**77**	83
51	6.0	27.63	623	25	**98**	99
Validation experiments						
1	7.1	8.12	1457	10	**69**	70
2	7.1	26.00	1457	10	**85**	92
3	5.1	4.07	1020	29	**97**	96

6.3.2 Significance of the experimental conditions on pollutant oxidation

The fitting of the quadratic models (Table 6.6) showed that all factors had a significant influence on DFC, BPA and TCN oxidation by laccase. The pH had the largest effect, followed by enzyme concentration and temperature. The reaction time had a smaller influence, due to the high degradation rate observed after reacting for only an hour (lower limit of the domain). All quadratic terms were significant except for reaction time, clearly showing a non-linear response. A bell shape effect with an optimal value was observed for the removal of the compounds as a function of pH, and a plateau response was observed for the removal as a function of the initial enzyme activity (Fig. 6.2 for BPA and Fig. S 6.3, Fig. S 6.4 and Fig. S 6.5, SI, for the other compounds). MFA oxidation by laccase was, in the range of the conditions tested, only significantly influenced by the pH. Indeed, a temperature variation between 10 and 40°C did not show any significant effect, and high degradation rates were already observed at the lower limit of the tested domain for the reaction time and the amount of enzyme. These quadratic models gave valuable information on the general behaviour of the oxidation, but results could not be extrapolated outside of the tested range and the predictive capacity of these models appeared to be very poor. Polynomial models are moreover not appropriate to fit responses with asymptotic behaviour for low or high variable values or when the response should be non-negative (Rawlings et al., 1998), which is the case for micropollutant degradation. The semi-empirical model developed here has greater predictive utility, and has some potential to help understand the degradation process.

Table 6.6 Best set of coefficients for the quadratic models with their significance (p-value) based on ANOVA, their 95% confidence intervals (according to (Rawlings et al., 1998) [1]) and their coefficient of determination R^2.

Coefficients		Diclofenac		Bisphenol A		Mefenamic acid		Triclosan	
Constant	a_0	80.20 [69 91]	***	92.30 [84 100]	***	96.49 [67 126]	***	86.00 [75 97]	***
Time	a_1	16.12 [8 25]	**	6.89 [1 13]	*	15.88 [-7 39]	ns	17.79 [9 26]	**
pH	a_2	-49.10 [-58 -41]	***	-32.07 [-38 -26]	***	-51.57 [-75 -28]	**	-23.30 [-32 -15]	***
Enzyme	a_3	27.78 [19 36]	***	36.21 [30 42]	***	3.39 [-20 26]	ns	28.76 [20 37]	***
Temperature	a_4	13.97 [6 22]	**	18.61 [13 25]	***	-11.62 [-35 11]	ns	25.47 [17 34]	***
Time^2	a_{11}	-11.11 [-29 7]	ns	-4.80 [-18 8]	ns	-12.21 [-60 36]	ns	-22.92 [-41 -5]	ns
pH^2	a_{22}	-47.84 [-65 -30]	**	-69.17 [-82 -57]	***	-116.50 [-164 -69]	***	-84.24 [-103 -66]	***
Enzyme^2	a_{33}	-35.14 [-52 -19]	**	-28.02 [-40 -16]	***	-25.22 [-70 19]	ns	-31.69 [-48 -15]	**
Temperature^2	a_{44}	-20.39 [-36 -5]	*	-1.25 [-12 10]	ns	-15.78 [-58 26]	ns	-0.39 [-16 15]	ns
Time-pH	a_{12}	1.83 [-20 24]	ns	6.48 [-9 22]	ns	-17.21 [-77 42]	ns	-6.67 [-29 16]	ns
Time-Enzyme	a_{13}	9.31 [-15 34]	ns	-6.02 [-24 12]	ns	10.14 [-56 76]	ns	16.01 [-8 40]	ns
Time-Temperature	a_{14}	5.36 [-20 30]	ns	-8.50 [-26 9]	ns	11.71 [-56 79]	ns	-5.92 [-31 19]	ns
pH-Enzyme	a_{23}	-12.59 [-38 13]	ns	2.79 [-15 21]	ns	-5.88 [-74 63]	ns	-26.97 [-52 -2]	*
pH-Temperature	a_{24}	-18.32 [-44 7]	ns	6.27 [-12 24]	ns	-43.30 [-112 25]	ns	-7.30 [-33 18]	ns
Enzyme-Temperature	a_{34}	-27.97 [-53 -3]	*	-25.81 [-44 -8]	*	-19.69 [-89 49]	ns	-9.17 [-35 17]	ns
	# of coef.	**15**		**15**		**15**		**15**	
	R^2	**0.975**		**0.987**		**0.884**		**0.977**	
	adj. R^2	**0.918**		**0.957**		**0.613**		**0.924**	

Significance: *** = p-value < 0.001, ** = p-value < 0.01, * = p-value < 0.05, ns = non significant

In square barckets, 95% univariate confidence intervals [lower-limit upper-limit] according to [1]

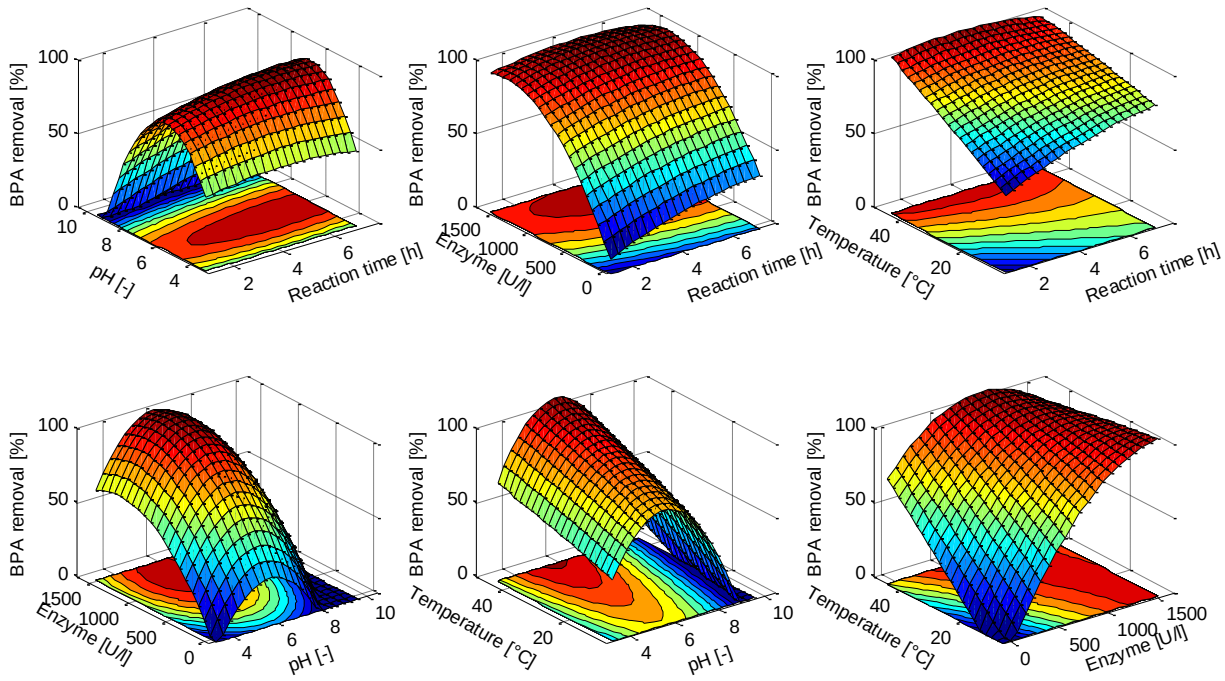


Fig. 6.2 Influence of the pH, reaction time, temperature and enzyme concentration on the removal of BPA according to the quadratic model. For each plot, the two parameters that did not vary are fixed at the centre of their domain (pH 6, 4-h reaction time, 725-U l^{-1} laccase and 25°C).

6.3.3 Validation of the semi-empirical models

Model including the effects of pH, temperature, enzyme concentration and reaction time

The results of the fitting to the experimental results of the Doehlert design using the semi-empirical model are presented in Fig. 6.3, Table 6.4 and Table 6.5. The best-fit coefficients for each compound are shown in Table 6.7. For the four compounds, the semi-empirical model fitted the data well, with coefficients of determination, R^2, above 0.97, meaning that more than 97% of the variability of the results could be explained by the model. In most cases, deviations between observed and predicted values were less than 10%.

Table 6.7 Set of best-fit coefficients for the semi-empirical model. These coefficients were used to predict the removal of each of the compounds present together in the solution (DFC, MFA and BPA) or alone (TCN).

Compound	pK_1	pK_2	C	K	Ea [kJmol^{-1}]	To [°K]	η	a	b
DFC	1.84	6.56	7044	3785900	108	317.6	1.02	-0.064	1.885
MFA	5.40	5.42	425	352	55	295.3	1.62	-0.279	2.223
BPA	4.09	7.29	5037400	726850000	49	312.0	9.57	-0.099	2.370
TCN	3.84	6.54	50722	5275400	53	322.5	20.36	-0.030	1.750

To validate the model, new experiments were conducted in a set of conditions not explored previously (Table 6.4 and Table 6.5). The predictions of the model for these new experiments were very close to the experimental data (Fig. 6.3), apart from one experiment with MFA at pH 5.5, where the removal was much lower than predicted. MFA is in fact not stable at low pH. It showed inconsistent behaviour, which lowered the reproducibility of the results. Apart from MFA at low pH, the model was adequate

for all the other experimental results and thus can be used to estimate the effect of the treatment conditions on the oxidation of these compounds by laccase.

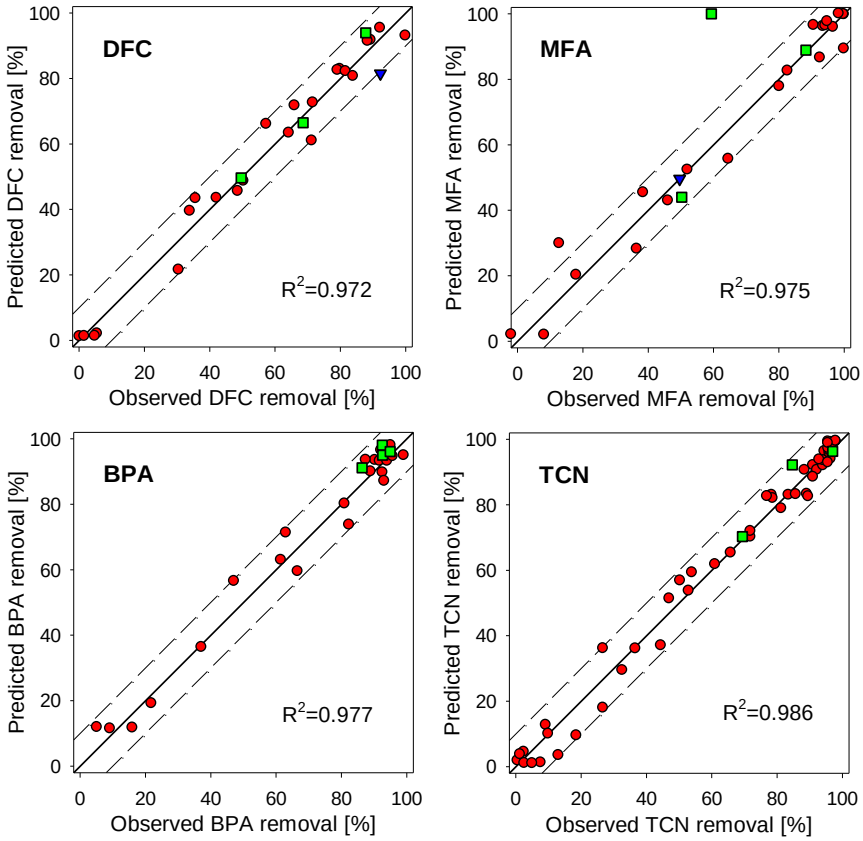


Fig. 6.3 Observed versus predicted removal rates with the semi-empirical model for diclofenac (DFC), mefenamic acid (MFA), bisphenol A (BPA) and triclosan (TCN). Circles ●: data used for the fitting. Squares ■: data used for the validation. Triangles ▼: addition of new data (done during the validation) into the fitting to improve the model. Dashed line: 10% deviation around the predicted values. Coefficients of determination R^2 are given for the data used for the fitting.

Model including only the effects of pH and reaction time

A simplification of the global model was developed for the compounds present alone in the solution, as a function of pH and reaction time only. The best set of coefficients for the prediction of the removal of each compound with this model is presented in Table 6.8. A very good model fit to the experimental data (R^2 > 0.99 for DFC and BPA) was obtained, apart for the prediction of some experiments with MFA (Fig. 6.4).

Table 6.8 Set of best-fit coefficients for the time-pH semi-empirical model. These coefficients were used to predict the removal of each of the four compounds present alone in the solution. R2: coefficient of determination of the fitting.

Compound	pK_1	pK_2	C	a	b	R^2
DFC	3.41	5.47	0.827	-0.191	2.208	0.994
MFA	6.19	6.36	14.855	-2.641	17.067	0.960
BPA	3.62	6.68	6.601	-0.332	3.151	0.994
TCN	3.97	6.60	2.480	-0.215	2.736	0.986

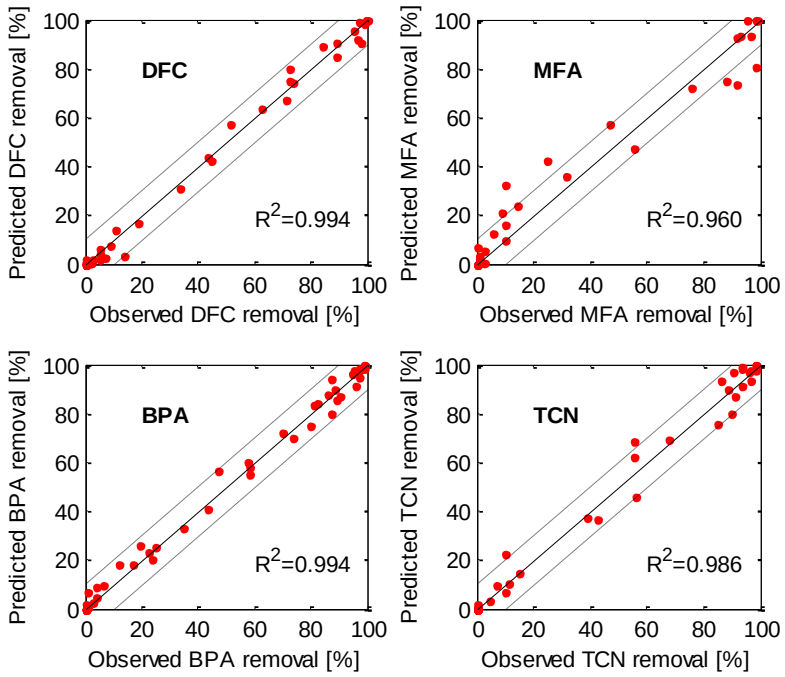


Fig. 6.4 Observed versus predicted removal with the time-pH semi-empirical model for DFC, MFA, BPA and TCN alone in the solution. Dash line: 10% deviation between observed and predicted values.

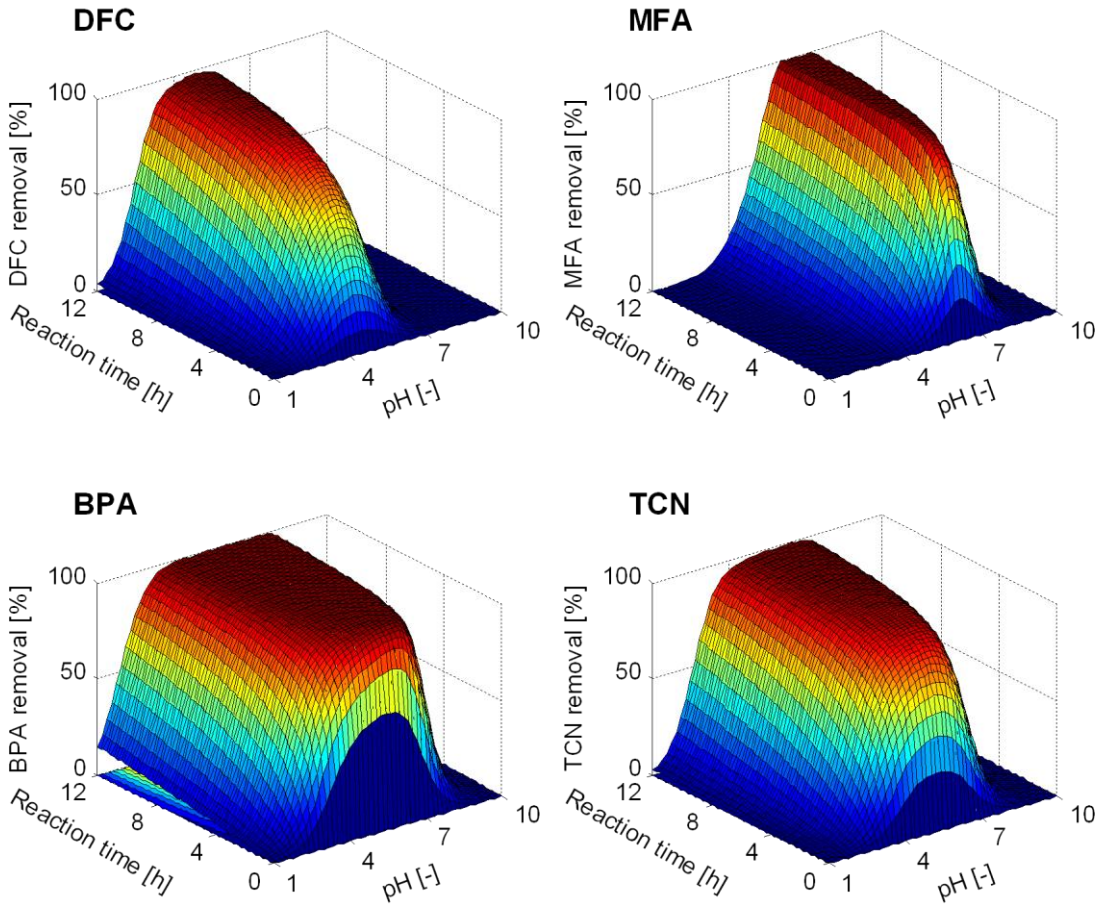


Fig. 6.5 Influence of the pH and the reaction time (at 25°C and 700 U l^{-1} laccase) on the removal of DFC, MFA, BPA and TCN in pure solution according to the time-pH semi-empirical model.

The combined effects of the pH and the reaction time on the removal of the four compounds are presented in Fig. 6.5 according to the time-pH semi-empirical model. Comparisons between the experimental results and the prediction of the semi-empirical model are presented in Fig. 6.6.

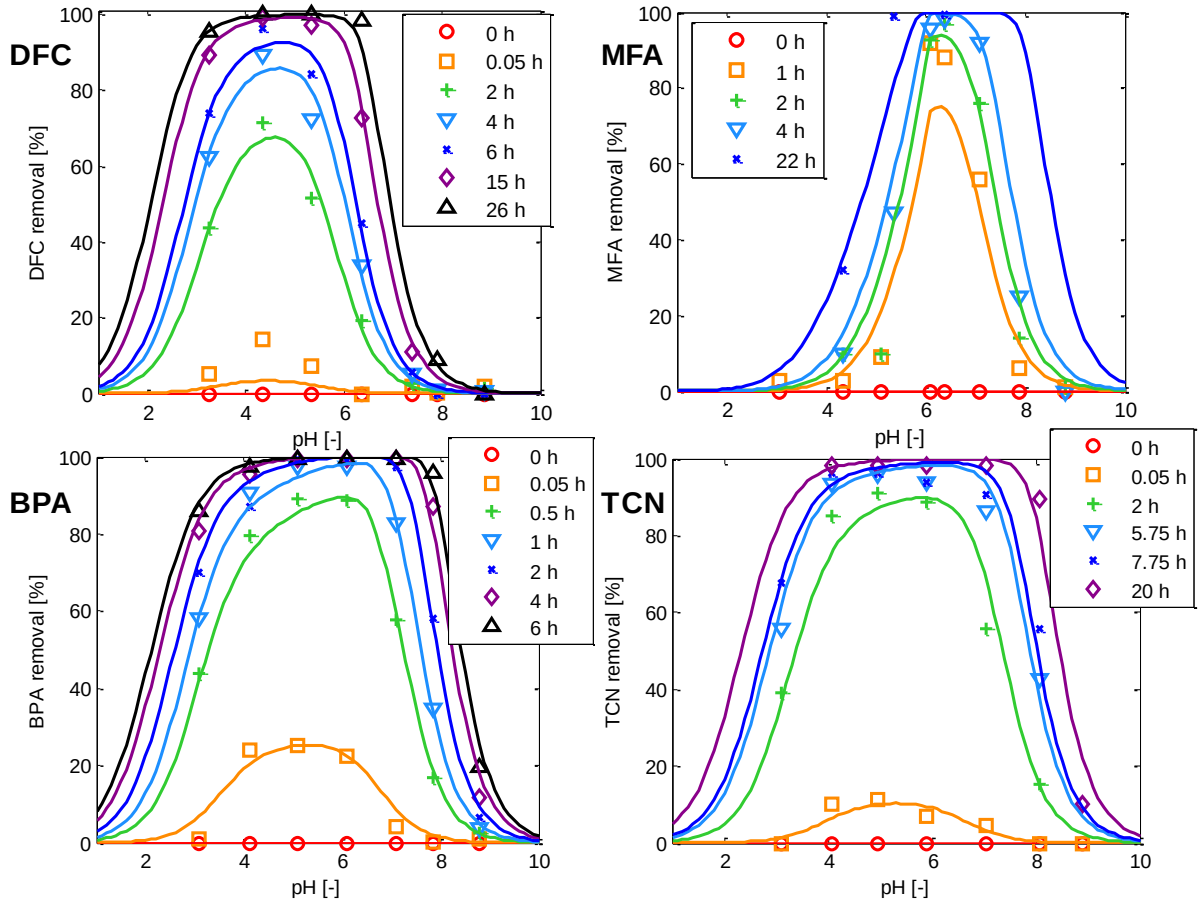


Fig. 6.6 Comparison between the experimental results (symbols) and the prediction of the time-pH semi-empirical model (line) for the removal of DFC, MFA, BPA and TCN in pure solution as a function of pH and reaction time, at 25°C and 700 U l^{-1} laccase for DFC and MFA, 730 U l^{-1} for BPA and 630 U l^{-1} for TCN.

6.3.4 Effect of treatment conditions on the transformation of micropollutants

Fig. 6.7 (and Fig. S 6.6, Fig. S 6.7, Fig. S 6.8 and Fig. S 6.9, SI) show the combined effects of the treatment conditions on the oxidation of the four compounds (DFC, MFA and BPA in a mixture, TCN alone), according to the semi-empirical model.

The influence of the enzyme concentration on the transformation rate was not proportional. The rapid increase of the removal rate observed with increasing amounts of enzyme at low concentrations faded out above 600 to 700 U l^{-1}, reaching a plateau (Fig. 6.7). Similar behaviour was observed by Kim and Nicell (2006c) for TCN oxidation. This saturation effect can appear when the substrate concentration becomes the limiting factor.

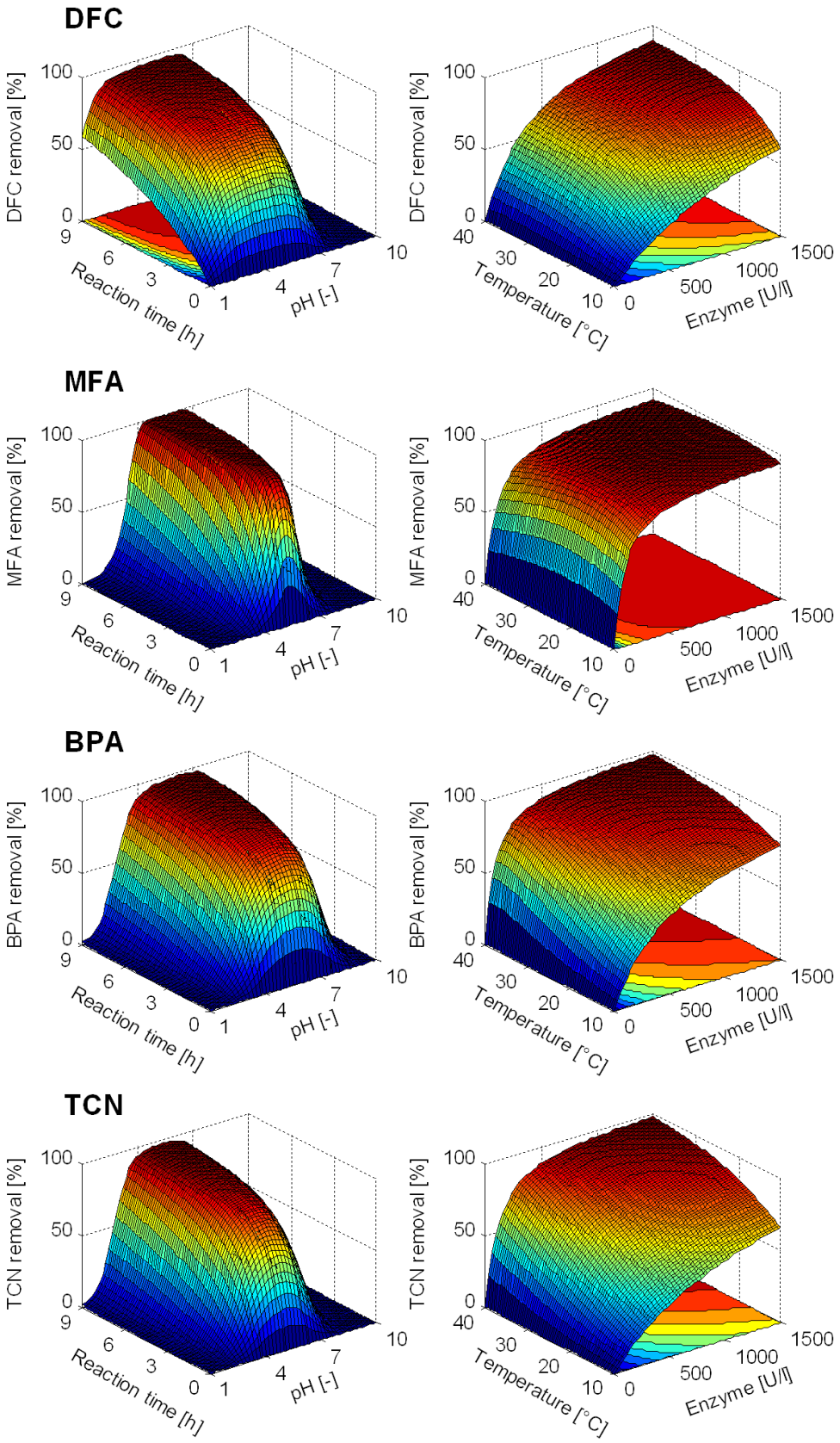


Fig. 6.7 Influence of pH and reaction time (left, at 25°C and 725 U l^{-1} laccase) or laccase concentration and temperature (right, at pH 6 and reaction time of 2 h) on the removal of diclofenac (DFC) (in a mixture with MFA and BPA), mefenamic acid (MFA) (in a mixture with DFC and BPA), bisphenol A (BPA) (in a mixture with MFA and DFC) and triclosan (TCN) (alone) according to the semi-empirical model.

The influence of temperature on the removal was studied for the range 10-40°C. No extrapolation outside this range was possible due to the limited amount of data used to calibrate the model. For the phenolic compounds (TCN and BPA), the temperature had a significant and almost linear effect (Fig. 6.7). The removal of TCN and BPA increased for instance from 20% or 30%, respectively, at 10°C to more than 70% at 40°C when 700 U l^{-1} of laccase reacted for 0.5 h at the optimal pH. These results are consistent with other studies for TCN and BPA (Kim and Nicell, 2006b; Kim and Nicell, 2006c), where very similar behaviour was observed between 25°C and 40°C. For the aniline compounds (DFC and MFA), the temperature effect was non-linear in the range studied (Fig. 6.7). DFC removal increased in a similar way to TCN from 10 to 25°C, but then a plateau was reached, and no additional removal occurred at higher temperatures. For MFA, the effect of temperature was smaller, with a slight increase in the removal from 10 to 20°C, followed by a slight decrease above 25°C. Our results suggest that the optimal temperature is mainly dependent on the substrate properties since denaturation of the laccase probably did not occur in the temperature range tested (Dodor et al., 2004).

The pH had a very strong influence on laccase activity, showing a clear bell-shaped response, with very low removal at very acidic (< 2) or alkaline pH (> 8) conditions, and complete removal around pH 5-6 (Fig. 6.6 and Fig. 6.8). The strongest pH effect was observed for MFA, where only a narrow pH range (between 6 and 7, optimum at pH 6.3) led to good removal of this compound in a single-compound solution (Fig. 6.8). The optimal pH range for DFC removal in a single-compound solution was in a more acidic range, as also observed by Lloret et al. (2010), from pH 3.5 to 5.5 with an optimum at pH 4.5. For the phenolic compounds (BPA and TCN), the optimal pH range was from pH 4 to 7, with an optimum around pH 5.5, similar to results reported in other studies (Cabana et al., 2007a; Kim and Nicell, 2006b; Kim and Nicell, 2006c). Outside these ranges, compound removal decreased drastically. According to Xu (1997), the bell-shape pH profile can be explained by the balancing of two opposing effects: i) the pH influence on the redox potential of the enzymatic reaction, and ii) the enzyme inhibition by hydroxide anions at high pH. In fact, the redox potentials of the aniline compounds DFC and MFA and the phenolic compounds BPA and TCN are pH-dependent and decrease when the pH increases, due to the transfer of a proton during the reaction (Daneshgar et al., 2009; Kuramitz et al., 2001; Liu and Song, 2006). As the redox potential of the active copper T1 of the laccase is less influenced by pH (Xu, 1997), the redox potential difference between the aniline or phenolic compounds and the T1 copper of laccase increases with pH, which increases the substrate oxidation rate. However, at higher pH the hydroxide anion is more prone to bind to the T2/T3 active coppers of laccase, stopping the electron transfer and inhibiting the activity (Xu, 1997). The pH can also alter the ionization of the amino acids and the charge of the compound, changing the shape and the electrostatic interactions of the enzyme and the ability of the substrate to bind to the active site (Joshi et al., 2000). The optimal pH is thus dependent not only on the laccase properties but also on the properties of the compound, as presented in Fig. 6.8. Even if laccase from *T. versicolor* seemed to be more active under acidic conditions (pH 5 to 6), its stability decreased at more acidic pH (Fig. S 6.10 and Fig. S 6.11, SI). For slow kinetics, a compromise should be made between the activity and the stability to find the pH that allows the highest removal rate.

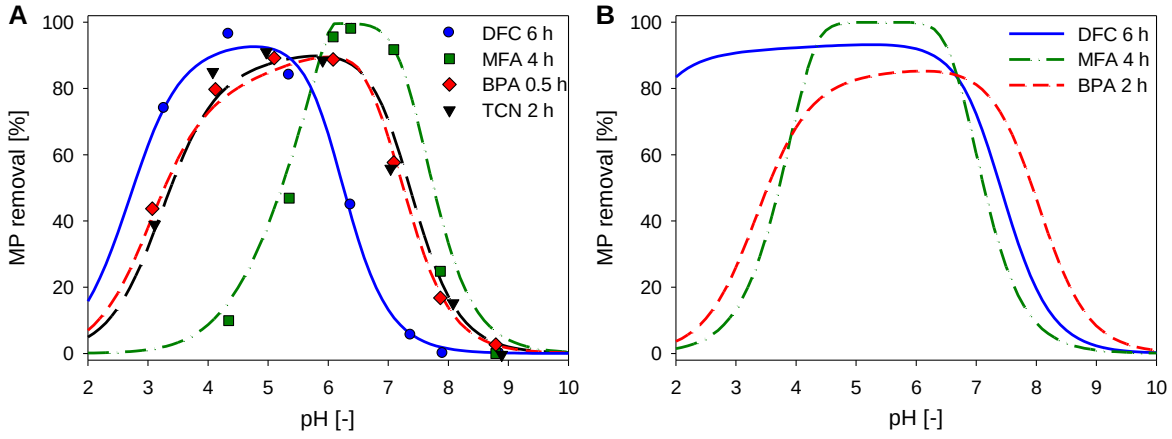


Fig. 6.8 Effect of pH on the removal of DFC, MFA, BPA and TCN in (A) single-compound solutions or (B) in a mixture of DFC, MFA and BPA at 25°C and 730 U l^{-1} laccase at different reaction times. Symbols: experimental data (not possible to represent in a 2D plot for the mixture due to the variation of the other factors). Lines: predictions of the semi-empirical model. DFC: diclofenac, MFA: mefenamic acid, BPA: bisphenol A, TCN: triclosan.

At the optimal pH, fast removal was observed during the first hours of the reaction for the four compounds (Fig. 6.7, detailed in Fig. 6.9 for DFC and BPA), especially for MFA and BPA. The removal rate, which followed first- to second-order kinetics depending on the pH, then decreased for increasing reaction times before reaching a plateau. At the optimal pH, 25°C, and for 730 U l^{-1} laccase, 90% removal of single-compound solutions was reached within 40 min for BPA, 1 h 40 min for MFA, 2 h 20 min for TCN and less than 5 h for DFC, all of which are in the range of hydraulic residence times used in biological wastewater treatments (Choubert et al., 2011). Under alkaline conditions, however, the transformation kinetics were very slow compared to acidic conditions (Fig. 6.9)

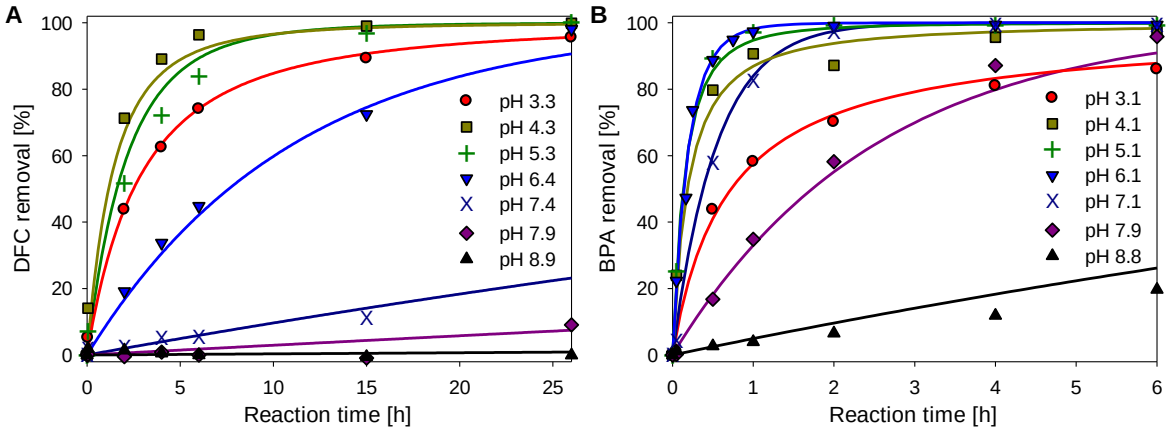


Fig. 6.9 Kinetics of (A) DFC and (B) BPA transformation when present in single-compound solutions at 25°C, 730 U l^{-1} laccase and different pH. Symbols: experimental data. Lines: predictions with the semi-empirical model. DFC: diclofenac, BPA: bisphenol A.

6.3.5 Effect of micropollutant mixtures

Transformation rates were different if the compounds were present in single-compound solutions or in a mixture (DFC, MFA and BPA). The presence of other compounds caused a shift of the optimal pH for MFA degradation towards more acidic conditions (between pH 4.5 to 6.5, optimum at pH 5.4) (Fig. 6.8), probably due to competitive effects observed under alkaline conditions when DFC was present (see below). The presence of other compounds particularly affected the transformation of DFC, where a much wider optimal pH range was observed in a mixture (from pH 2.5 to 6.5, optimum at pH 4.5) compared to a single-compound solution. The optimal pH range for BPA was only slightly influenced by the presence of other compounds, but the rate was slower - a mixture needed 2 h to attain the same removal rate at that observed for a single-compound solution after 0.5 h (Fig. 6.8).

The presence of other compounds can thus have a strong influence on the kinetics by either increasing (for DFC) or decreasing (for MFA and BPA) the reaction rate. For instance, at pH 7.2, 25°C, 730 U l^{-1} laccase, and 20-h reaction time (Fig. 6.10), only 25% of DFC was removed in a single-compound solution, but 60% in the presence of BPA, and 95% in the presence of MFA. For BPA and MFA, by contrast, the presence of other compounds seemed to create a competitive effect, with reduced removal compared with single-compound solutions (Fig. 6.10).

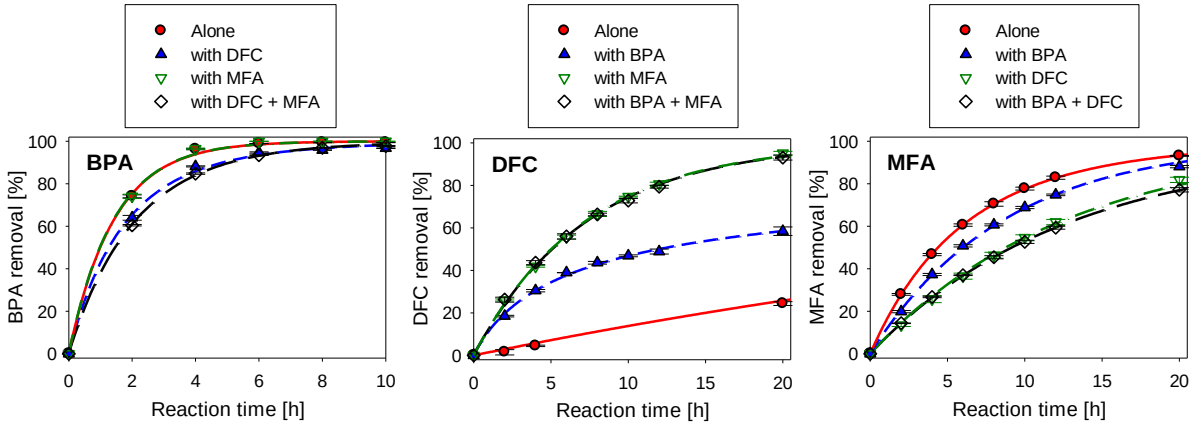


Fig. 6.10 Influence of micropollutant mixtures on the removal of BPA, DFC and MFA at pH 7.2, 26°C with 730 U l^{-1} of laccase. Symbols: experimental data from duplicates with standard deviation. Lines: adjustment of kinetic models with variable order of reaction. DFC: diclofenac, MFA: mefenamic acid, BPA: bisphenol A.

Additional experiments (not presented in the published version) were performed with other mixtures of pollutants, including TCN and paracetamol (PCL). As presented in Fig. 6.11 A, at pH 6.6, the presence of PCL did not influence the (slow) oxidation rate of DFC, despite PCL was completely oxidized in 1 h. But a strong increase in DFC oxidation rate was again observed when MFA was present. On the contrary, PCL affected the oxidation of MFA, which started only once all PCL was removed (after 1 h) (Fig. 6.11 C). As PCL is very good substrate for laccase (c.f. Chapter 5), it is very likely that there was a competition between PCL and MFA for the enzyme. Similar to the results at pH 7.2 or 6.6, at pH 5.2, DFC removal was also enhanced in presence of other pollutants, especially with TCN (Fig. 6.11 B). TCN was, however, strongly affected by the presence of other pollutants, with 90% removal in 5 h in single-compound solution but only 10% removal in mixture with BPA and DFC (Fig. 6.11 D).

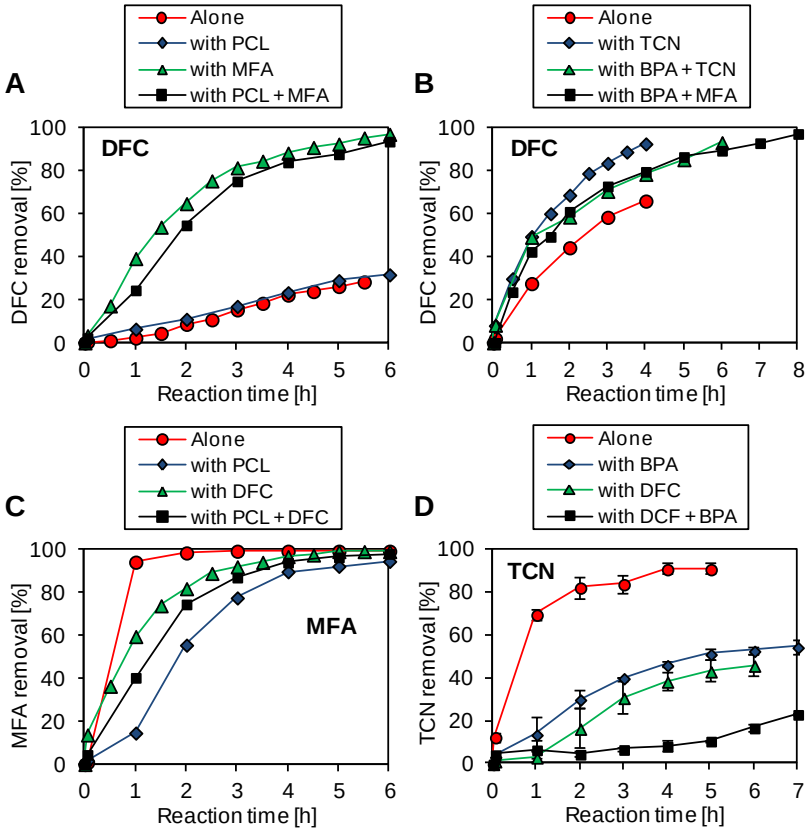


Fig. 6.11 Influence of micropollutant mixtures on the removal of DFC, MFA and TCN at 25°C, at different pH values and in different mixtures. (A) DFC removal alone or in mixture with PCL and/or MFA at pH 6.6 and 700 U l^{-1} of laccase. **(B)** DFC removal alone or in mixture with TCN, BPA and/or MFA at pH 5.2 and 900 U l^{-1}. **(C)** MFA removal alone or in mixture with PCL and DFC at pH 6.6 and 700 U l^{-1}. **(D)** TCN removal alone or in mixture with BPA and DFC at pH 5.2 and 750 U l^{-1} (average and values of duplicates). DFC: diclofenac, PCL: paracetamol, MFA: mefenamic acid, TCN: triclosan, BPA: bisphenol A

Although most studies on laccase-mediated oxidation considered single-compound solutions (Cabana et al., 2007a; Huang and Weber, 2005; Inoue et al., 2010; Kim and Nicell, 2006b; Kim and Nicell, 2006c; Lloret et al., 2010; Tsutsumi et al., 2001), our results show that the transformation rate can be noticeably different in mixtures. The application of laccase for wastewater treatment requires efficient removal in complex matrices containing a mixture of pollutants and other organic and inorganic compounds. Thus, the effect of mixtures should be better understood. Laccase produces reactive radicals that can break down to smaller molecules, polymerise or react with other molecules (Claus, 2004; Dwivedi et al., 2011). As MFA and BPA are oxidised relatively rapidly by laccase, their unstable radicals could react with DFC, either as a mediator before returning to their initial stage, or by cross-linking to form a bigger molecule that is more prone to precipitate. This last assumption is more likely as more precipitates were visible in the mixtures. Moreover, the visually observed yellow colour of DFC transformation products was much lighter in the mixture, indicating formation of other transformation products. As MFA was a strong enhancer of DFC removal and possibly acted as a mediator, experiments were performed (at pH 5) with MFA mixed with naproxen, sulfamethoxazole or isoproturon, three compounds which can be degraded by laccase-mediator systems (c.f. Chapter 5). However, none of these three compounds was degraded in 5 h of reaction, suggesting that MFA was not acting as a strong mediator. The mechanistic description of the mixture phenomenon requires further investigations and may be very different at much lower pollutant concentrations (lower chance

to have cross reaction between two pollutants), but these preliminary results highlight the important role of mixture effects in the fate of micropollutants in a complex matrix such as wastewater.

6.3.6 Transformation at low micropollutant concentrations

The rate of enzyme-catalysed reactions is dependent on the substrate concentration, following in most cases a Michaelis-Menten model (Johnson and Goody, 2011). Thus, at lower micropollutant concentrations, even if similar pH and temperature effects are expected, slower transformation kinetics are expected. The experiment conducted at a concentration of 1 µg l^{-1} with a mixture of BPA, DFC, MFA, and four estrogenic compounds, estrone (E1), β-estradiol (E2), estriol (E3) and 17α-ethynilestradiol (EE2) (c.f. Chapter 5), showed that over 90% removal of these seven compounds could be obtained in less than 20 h of reaction time with 500 U l^{-1} laccase, at pH 4.5 and 30°C. This demonstrates the potential of laccase to treat micropollutants, including natural (E1, E2 and E3) and synthetic (EE2) hormones, at very low concentrations (ng to µg l^{-1}). The effect of the substrate concentration on the removal rate was therefore much lower than the effect of other parameters such as pH. For instance, 96% DFC removal was reached in less than 20 h at a concentration of 1 µg l^{-1} whereas, under these conditions and according to the prediction of the semi-empirical model, this same removal level would be attained after 12 h for an initial DFC concentration that was 20,000 times higher. However, this experiment does not lead to conclusions regarding reaction kinetics at low substrate concentrations. Further experiments would be necessary to clarify this point.

6.4 Conclusions

Laccase from *Trametes versicolor* was able to transform completely four problematic micropollutants usually found in wastewater: DFC, MFA, BPA and TCN. Operational conditions such as pH, enzyme concentration, temperature and reaction time were found to have a strong influence on the removal rate. The optimal pH for degradation was compound-dependent but always in the acidic range (4.5 to 6.5), with slow removal kinetics for alkaline conditions. The influence of temperature was less significant but higher for phenolic (BPA and TCN) than for aniline compounds (DFC and MFA), the latter showing an optimum between 25 and 30°C. The semi-empirical model described well the experimental data and satisfactorily predicted the removal efficiency of additional experiments. Removal rates were quite different in micropollutant mixtures compared with single-compound solutions. Mixtures led to either increases or decreases in degradation rates, depending on the compound. Although conditions in municipal wastewater (pH 7-8, 10-25°C) are outside the optimal range, the results show that removal above 85% is possible for the four compounds tested under pH and temperature conditions found in wastewater, with sufficient enzyme concentration and reaction time. The ability of laccase to transform recalcitrant micropollutants such as DFC and MFA, even at low concentration, is very promising and could be a first step for further mineralization and improvement of the quality of WWTPs effluents.

Acknowledgements

A special thank to the co-authors of this study, for their help in the experimental part, their advice and comments on the manuscript: Julien Maillard, Luca Rossi, D. Andrew Barry and Christof Holliger.

6.5 Supporting information

The supporting information of this chapter is presented below.

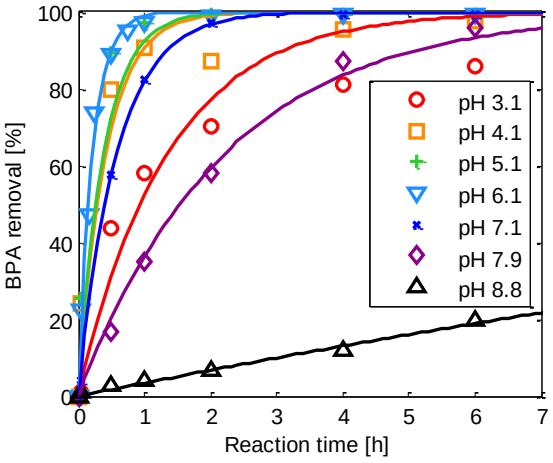


Fig. S 6.1 Fitting of a first-order model (lines) to the removal of BPA (symbols) measured at different pH values (at 25°C with 730-U l^{-1} laccase).

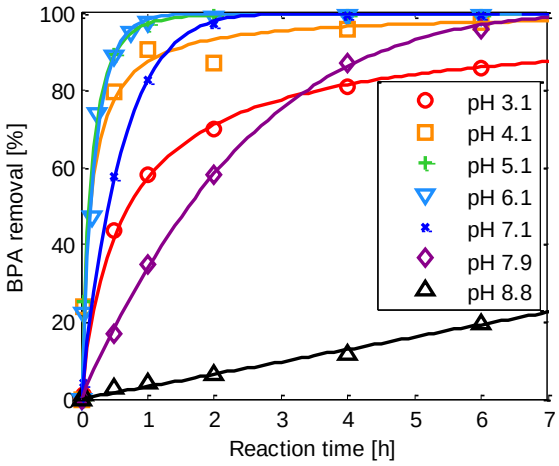


Fig. S 6.2 Fitting of the best-order model (lines) to the removal of BPA (symbols) measured at different pH values (at 25°C with 730-U l^{-1} laccase).

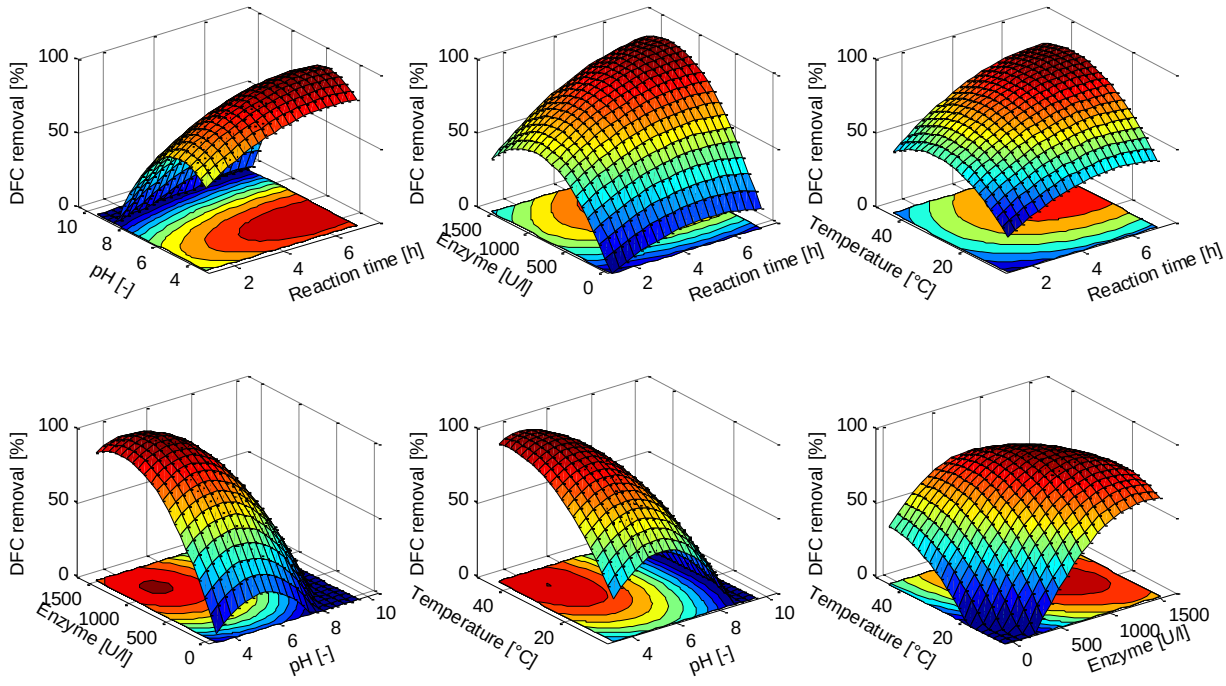


Fig. S 6.3 Influence of the pH, reaction time, temperature and enzyme concentration on the removal of DFC according to the quadratic model. For each plot, the two parameters that did not vary are fixed at the centre of their domain (pH 6, 4-h reaction time, 725-U l^{-1} laccase and 25°C).

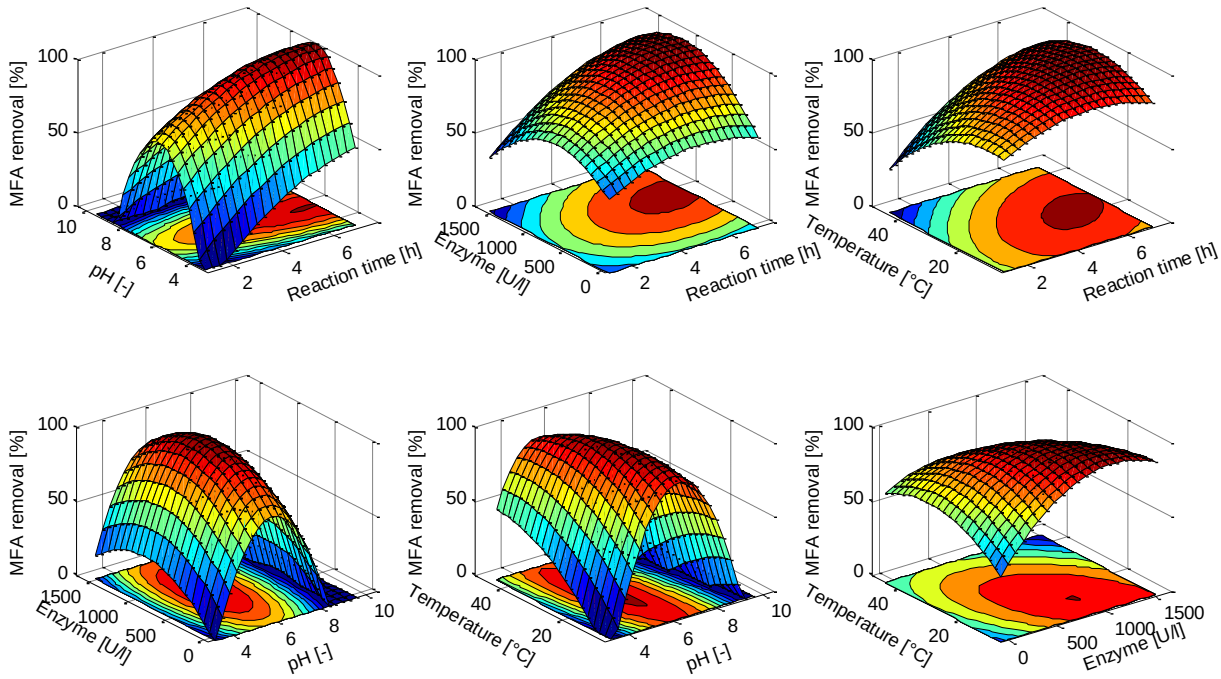


Fig. S 6.4 Influence of the pH, reaction time, temperature and enzyme concentration on the removal of MFA according to the quadratic model. For each plot, the two parameters that did not vary are fixed at the centre of their domain (pH 6, 4-h reaction time, 725-U l^{-1} laccase and 25°C).

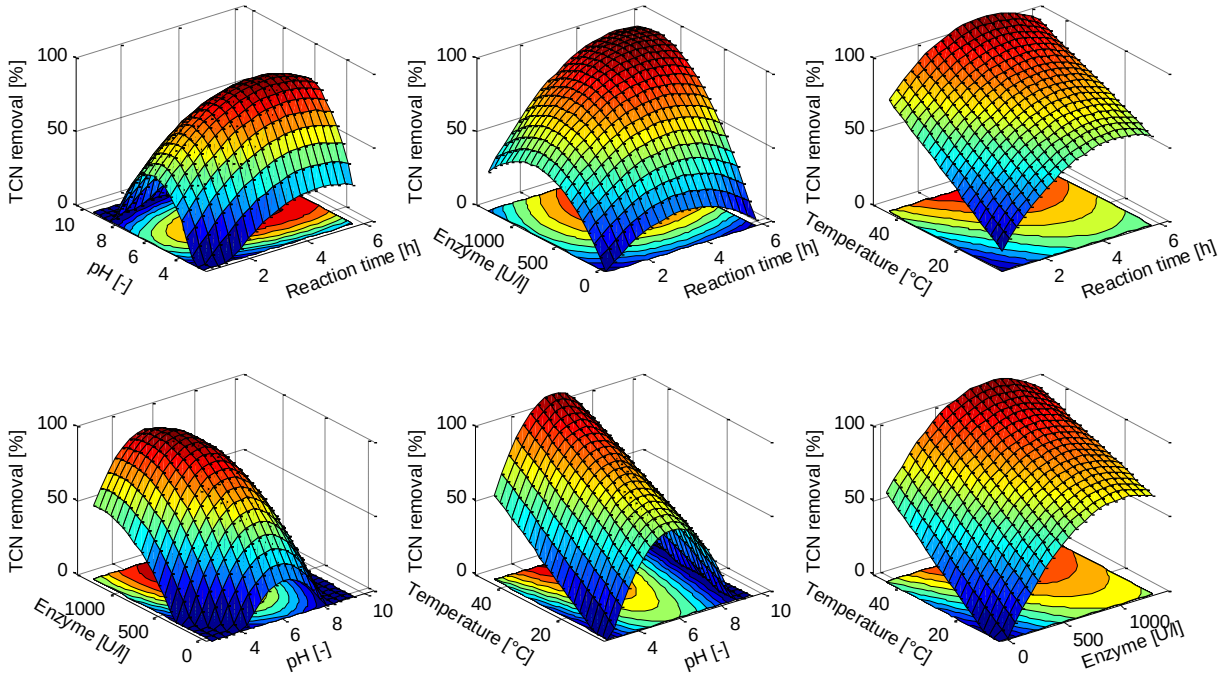


Fig. S 6.5 Influence of the pH, reaction time, temperature and enzyme concentration on the removal of TCN according to the quadratic model. For each plot, the two parameters that did not vary are fixed at the centre of their domain (pH 6, 3-h reaction time, 623-U l^{-1} laccase and 25°C).

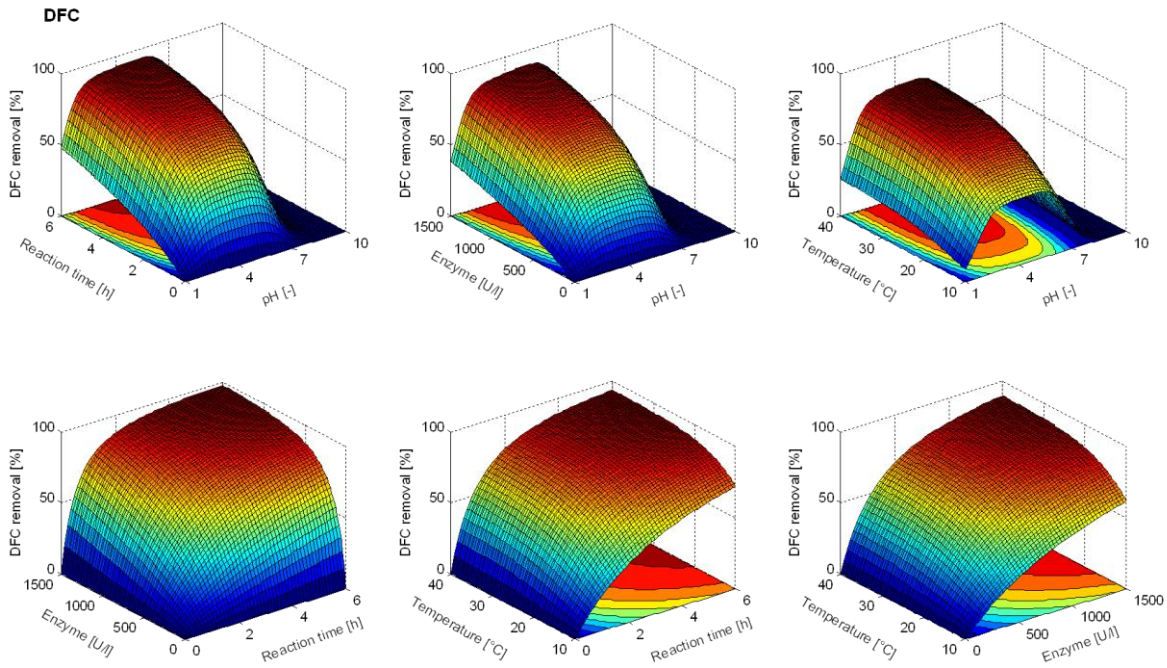


Fig. S 6.6 Influence of the pH, reaction time, temperature and enzyme concentration on the removal of DFC according to the semi-empirical. For each plot, the two parameters that did not vary are fixed at the centre of their domain (pH 6, 4 h reaction time, 725 U l^{-1} laccase and 25°C).

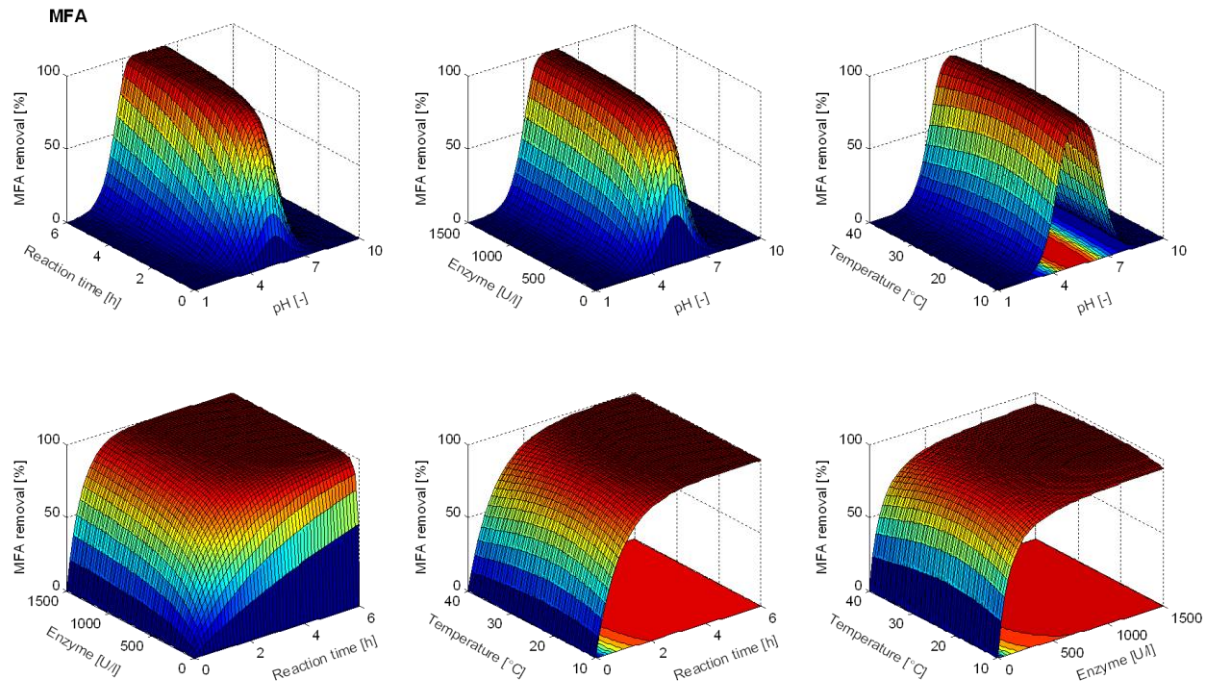


Fig. S 6.7 Influence of the pH, reaction time, temperature and enzyme concentration on the removal of MFA according to the semi-empirical. For each plot, the two parameters that did not vary are fixed at the centre of their domain (pH 6, 4 h reaction time, 725 U l^{-1} laccase and 25°C).

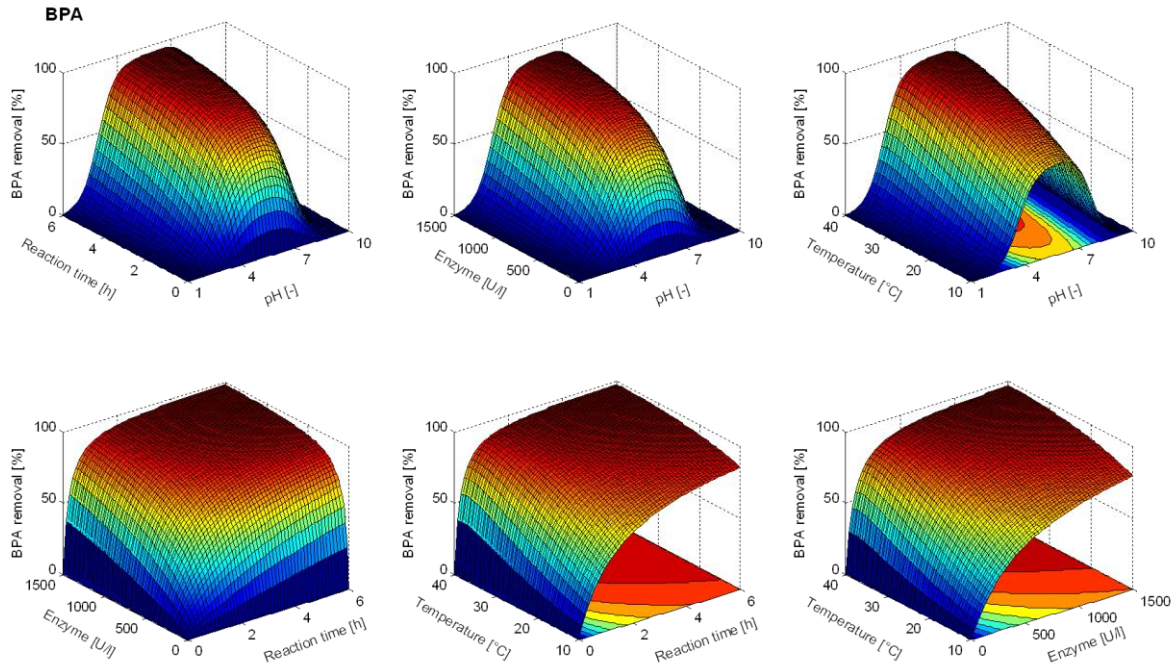


Fig. S 6.8 Influence of the pH, reaction time, temperature and enzyme concentration on the removal of BPA according to the semi-empirical. For each plot, the two parameters that did not vary are fixed at the centre of their domain (pH 6, 4 h reaction time, 725 U l^{-1} laccase and 25°C).

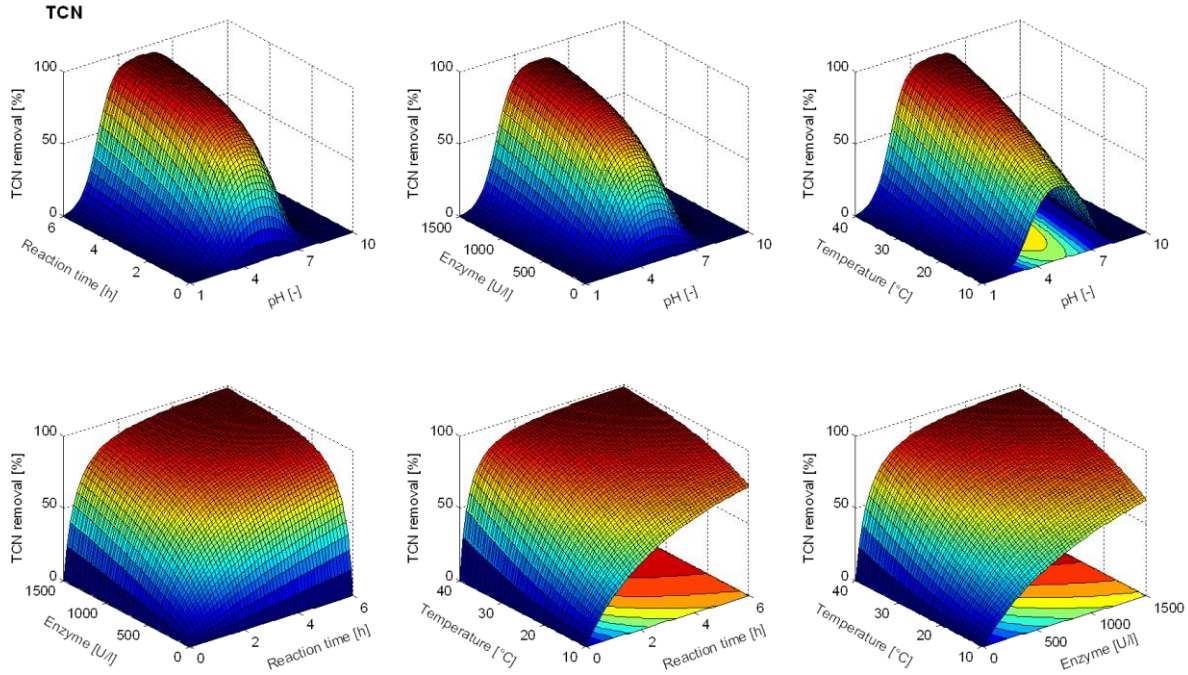


Fig. S 6.9 Influence of the pH, reaction time, temperature and enzyme concentration on the removal of TCN according to the semi-empirical. For each plot, the two parameters that did not vary are fixed at the centre of their domain (pH 6, 4 h reaction time, 725 U l^{-1} laccase and 25°C).

Influence of the treatment conditions on laccase activity

Fig. S 6.10 presents the stability of laccase in buffer solution at different pH and different incubation times while Fig. S 6.11 shows the residual laccase activity in different operational conditions after the reaction with the micropollutants.

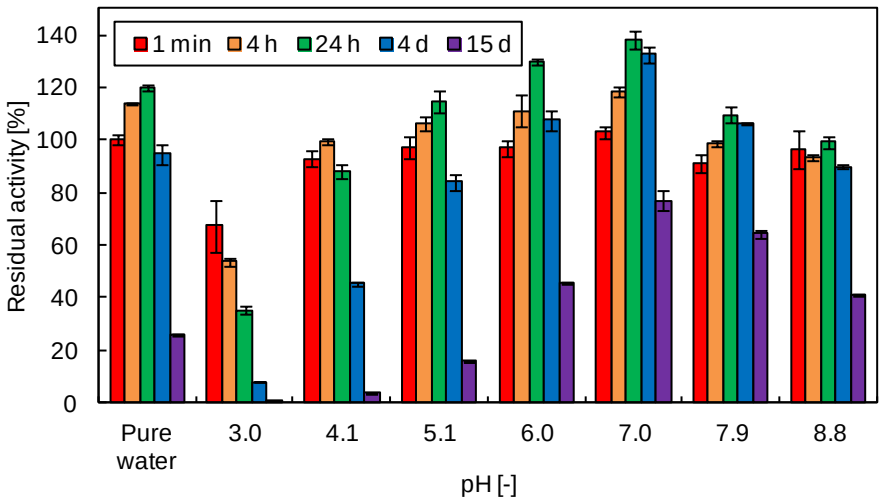


Fig. S 6.10 Stability of laccase from *T. versicolor* (560 U l^{-1} initial activity) at different pH (buffer citrate-phosphate 20-40 mM pH 3-7, Tris-HCl 40 mM pH 8-9) as a function of incubation time at 25°C. The residual activity is relative to the initial activity in pure water. High stability is observed at neutral and alkaline pH values, but inactivation is observed for acidic pH values, with complete loss of activity in less than 15 d at pH 3. Error bars: standard deviation of duplicate.

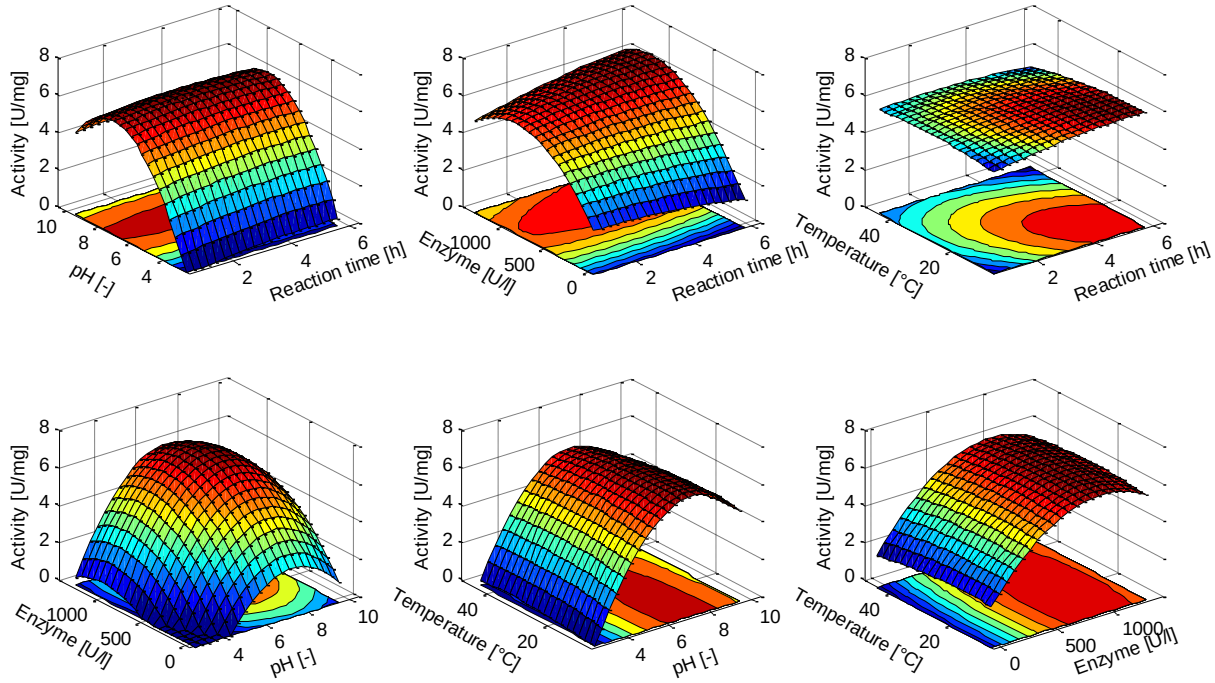


Fig. S 6.11 Simulation of the residual laccase activity [U mg^{-1} of laccase] at the end of the experiments of the Doehlert experimental design based on the results of the 23 experiments conducted with triclosan. For each plot, the two parameters that did not vary are fixed at the centre of their domain (pH 6, 3-h reaction time, 623-U l^{-1} laccase and 25°C).

The quadratic model can reproduce correctly the observed data, with a good fit (R^2 = 0.988), as presented in Fig. S 6.12. Laccase stability seems to be mainly influenced by the pH, with the highest stability at neutral pH. In the ranges studied, the temperature and the reaction time did not have a significant influence on the stability. Laccase seems to be less stable when present at low concentration in the reaction mixture.

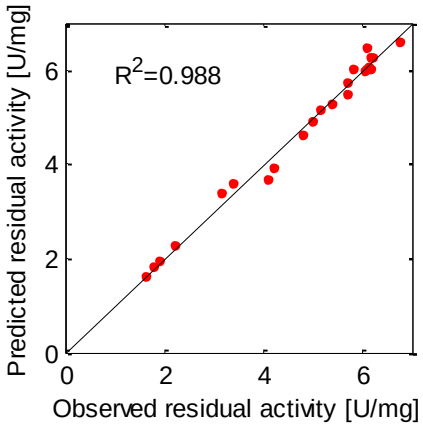


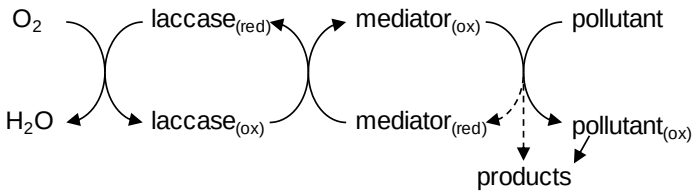
Fig. S 6.12 Observed versus predicted residual laccase activities with the quadratic model at the end of the experiments of the Doehlert experimental design conducted with triclosan.

Chapter 7 Sulfamethoxazole and isoproturon degradation and detoxification by a laccase-mediator system

An adapted version of this chapter was submitted for publication in ***Biochemical Engineering Journal****, with the name "Sulfamethoxazole and isoproturon degradation and detoxification by a laccase-mediator system: Influence of treatment conditions and mechanistic aspects", by Jonas Margot, Pierre-Jean Copin, Urs von Gunten, D. Andrew Barry and Christof Holliger.*

7.1 Introduction

Bio-oxidation of micropollutants, catalyzed by oxidative enzymes such as laccase, attracted recently a growing interest from the scientific community (Nyanhongo et al., 2007). Laccases can oxidize a wide range of pollutants containing phenol and aniline moieties, including several pharmaceuticals and pesticides, requiring only oxygen as a co-substrate (cf. Chapter 5). Despite the promising potential of this class of enzymes, many pollutants are recalcitrant to laccase oxidation. One way to increase their range of action is to use redox mediators (Husain and Husain, 2008). Mediators are compounds that can be oxidized by laccase to free radicals, which in turn can oxidize pollutants less specifically, increasing the spectrum of compounds potentially degraded by these enzymes. Mediators are often described as "electron shuttles", that, once oxidized to radicals by laccase, may be reduced back to their parent compound during the oxidation of a pollutant (Fabbrini et al., 2002).



Scheme 1 Ideal laccase-mediator reaction model

This ideal catalytic cycle (Scheme 1), where only oxygen is consumed during pollutant oxidation, is a means to increase the range of action of laccase. The mediator recycling does, however, not always happen and consumption of mediators during the reaction is possible. In this case, the term "laccase enhancer" is a better descriptor (González Arzola et al., 2009). Nevertheless, mediators or enhancers notably widen the substrate range of laccases. Despite several studies on laccase-mediator systems (LMS) for micropollutant removal in wastewater (Auriol et al., 2007; Garcia et al., 2011; Murugesan et al., 2010; Nguyen et al., 2014a; Tran et al., 2013a), the mechanistic aspects of the kinetics of pollutant oxidation, the fate of the mediator during the reaction, and the conditions required for an optimal pollutant oxidation are not completely understood.

A variety of organic compounds may act as mediators, as long as they can be oxidized by laccase and the free radical formed is stable enough to diffuse away from the enzymatic pocket. Furthermore, the reduction potential has to be high enough to oxidize a target compound (Medina et al., 2013). One of the most commonly used synthetic mediators is 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) (González Arzola et al., 2009). This compound is oxidized by laccases to a stable radical cation $ABTS^{\bullet+}$ (Fig. 7.1 B). Natural mediators have also been identified, mostly lignin-derived phenolic compounds, some of the most effective of which are syringaldehyde (SA) and acetosyringone (AS) (Medina et al., 2013). SA and AS have similar structures and are oxidized by laccase to an unstable phenoxy radical (Fig. 7.1).

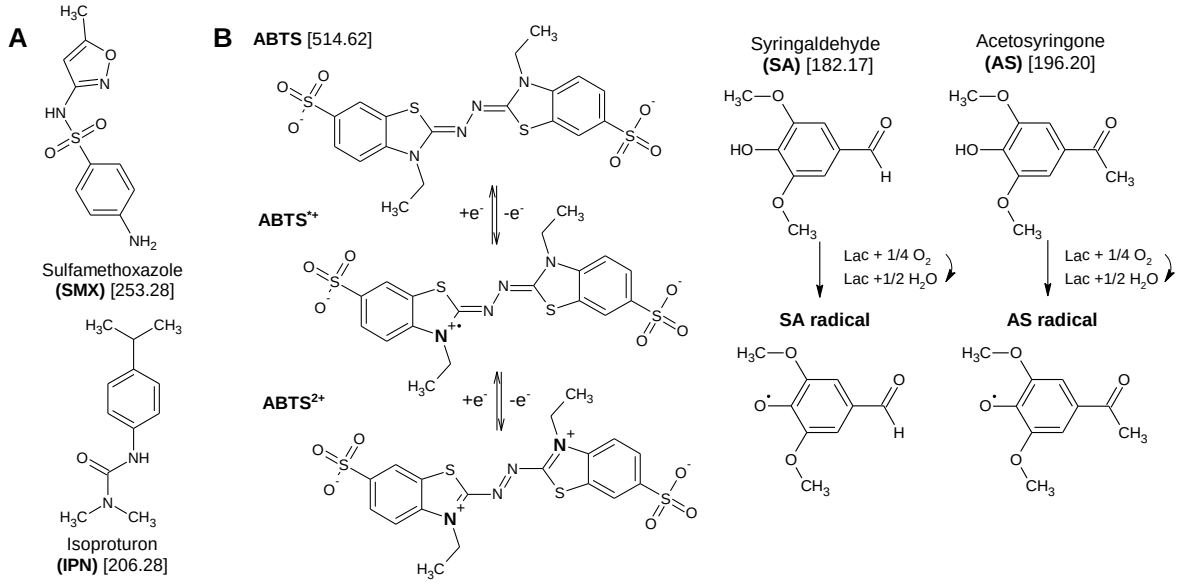


Fig. 7.1 Selected micropollutants (A) and mediators (B). In (B), ABTS oxidation by laccase to its stable radical cation $ABTS^{\bullet+}$ and eventually to the di-cation $ABTS^{2+}$ (according to Fabbrini et al. (2002)); and oxidation of the mediators syringaldehyde and acetosyringone to their respective unstable reactive phenoxy radicals (according to Martorana et al. (2013)). In [], exact molar mass of the compounds (in g mol^{-1}).

To assess the potential of LMS for micropollutant removal in wastewater, two pollutants of environmental concern are investigated in this study: the sulfonamide antibiotic sulfamethoxazole (SMX) and the herbicide isoproturon (IPN) (Fig. 7.1 A). Due to its wide consumption and only poor removal in WWTPs, SMX is ubiquitous in municipal wastewater effluents (in average around 200 ng l^{-1} in Switzerland) (Kase et al., 2011) and may generate risks for sensitive aquatic organisms in the receiving waters (Bonvin et al., 2013b). IPN is a herbicide commonly used in urban areas (parks, gardens, cemeteries) and therefore also frequently detected in municipal WWTP effluents (Kase et al., 2011). Due to its toxicity at very low concentrations, it was selected as a priority substance by the European Union (EC, 2013).

The aims of this study were (i) to assess the potential of LMS for SMX and IPN degradation and detoxification with three mediators: ABTS, AS and SA, and (ii) to determine the influence of the operational parameters (pH, laccase, mediator and pollutant concentrations) on LMS-based oxidation kinetics. Based on these experiments (a) optimal conditions for pollutant degradation were identified,

(b) mechanistic aspects of LMS-based systems were elucidated, and (c) the potential of LMS for the treatment of micropollutants in wastewater was assessed.

7.2 Materials and methods

7.2.1.1 Chemicals and enzyme

SMX and IPN (purity > 99%), laccase powder from *Trametes versicolor* (ref. 38429, Sigma), ABTS diammonium salt, SA, AS, and all other chemicals were purchased from Sigma-Aldrich Chemie GmbH (Buchs, Switzerland). Stock solutions of SMX (3.95 mM, 1 g l^{-1}), IPN (4.85 mM, 1 g l^{-1}), AS (10 mM, 1.96 g l^{-1}) and SA (10 mM, 1.82 g l^{-1}), were prepared in pure methanol and stored at -18 °C. Stock solutions of ABTS (10 mM, 5.14 g l^{-1}) and laccase (2 g l^{-1}) were prepared in pure water, stored at 4°C and replaced for any new set of experiments. A stock solution of aqueous chlorine (OCl^-, 8.16 mM) was prepared by diluting a sodium hypochlorite solution (~ 5%, Sigma) 100 times. The final OCl^- concentration was measured at pH 10.9 by spectrophotometry at 292 nm (extinction coefficient ε_{292nm} of 362 M^{-1} cm^{-1}) (Furman and Margerum, 1998).

A stock solution of the ABTS radical cation (at 5 mM) was produced by chemical oxidation of ABTS (6.9 mM in pure water) with aqueous chlorine (2.5 mM), under acidic conditions (pH < 5), at a slightly under-stoichiometric ratio to avoid potential residual chlorine in the solution (Pinkernell et al., 2000). Another solution of ABTS radical cations was produced by ultrafiltration (Vivaspin 20 centrifugation devices, PES, MWCO: 3 kDa, from Startorius AG, Göttingen, Germany) of a solution of ABTS (500 µM) oxidized by laccase, in order to remove (most of) the enzyme (> 60 kDa). The exact $ABTS^{\bullet+}$ concentration was determined by spectrophotometry at 420 nm (ε_{420nm} of 36,000 M^{-1} cm^{-1}) (Childs and Bardsley, 1975).

7.2.1.2 Laccase activity test

Laccase activity was determined as described in Chapter 5. One unit of activity (U) was defined by the oxidation of one µmol of ABTS per min, at pH 4.5 and 25°C.

7.2.1.3 Micropollutant, mediator and transformation product analyses

Determinations of SMX, IPN, AS, and SA concentrations were carried out by reverse phase liquid chromatography with a diode-array detector (HPLC-DAD) (LC-2000plus, Jasco, Tokyo, Japan, equipped with Bondapack-C18 column, 15–20 µm, 3.9 mm × 300 mm, Waters™, Milford, USA). Aliquots of 50 µl were injected. Separation of the compounds and the transformation products was conducted with a 30-min gradient, at 1 ml min^{-1}, of pure H_2O containing 0.1% acetic acid (pH 3.23) and increasing concentration of methanol (with 0.1% acetic acid) from 5 to 52% (v/v) or 15 to 60%, for SMX, AS and SA, or IPN determination, respectively. The compounds were detected at 268, 305, 305 and 242 nm for SMX, AS, SA, and IPN, respectively. The limit of quantification (LOQ) was around 0.1 mg l^{-1} (~ 0.5 µM), and the accuracy of the measurements (coefficient of variation of 10 injections) was around 1-2%.

Characterization of the transformation products formed during the laccase-mediated reaction was carried out by HPLC coupled to a mass spectrometer (UPLC-MS). Aliquots of 10 µl of each sample

were injected in the column (Acquity UPLC system, with a HSS T3 (C_{18}) column, 2.1 × 100 mm, 1.8 µm, Waters), which was eluted at 30°C in 20 min at 0.4 ml min^{-1} with a mobile phase composed of pure water and methanol, in a gradient mode, from 2 to 95% methanol. Transformation products were characterized (m/z ratio and retention time) and quantified (signal intensity) by MS (Xevo TQ MS, Waters) in scan mode (40 – 2000 m/z, scan time 0.4 s) and a positive electrospray ionization mode (ES+, cone voltage 30 V). Based on the retention times, some of the transformation products characterized by MS could be related to transformation products observed by HPLC-DAD, which gave further information about their UV/visible absorption spectrum.

7.2.1.4 Micropollutant oxidation assay in laccase-mediator systems under various conditions

Micropollutant oxidation assays were performed at different pH values (3 to 9) in citrate or phosphate buffers (30 – 40 mM) containing the pollutant at around 100 µM (20 – 25 mg l^{-1}) and variable concentrations of mediator (10 – 1000 µM). Batch reactions were conducted in 2-ml glass vials containing 1 ml of an oxygen-saturated reaction mixture. Reactions were initiated by adding laccase to obtain an initial activity between 100 and 650 U l^{-1}. Vials were incubated in the dark at 25°C under static conditions for several hours (usually around 20 h). After defined reaction times (every 40 to 160 min), aliquots (50 µl) were withdrawn from each vial and directly injected into the HPLC column to analyze micropollutant and mediator concentrations. Controls without laccase or without mediators were performed to assess micropollutant degradation by mediators or by laccase alone, respectively. Experiments were typically carried out in duplicate.

Several experiments were performed under various conditions to better understand laccase-mediated reactions: (i) three mediators were tested (namely ABTS, AS and SA) with either SMX or IPN; (ii) for each mediator, degradation kinetics were studied at various pH values (from 3 to 9), diverse mediator concentrations (from 10 to 1000 µM), various laccase activities (100 to 650 U l^{-1}), and various pollutants concentrations (50 to 150 µM).

For the transformation product analyses by UPLC-MS, lower micropollutants concentrations were used: (i) IPN at 20 µM with 500 µM ABTS and 560 U l^{-1} laccase at pH 5, and (ii) SMX at 10 µM with 50 µM mediator (ABTS, AS or SA) and 560 U l^{-1} laccase at pH 6. After defined reaction times (about every hour), 10 µl were withdrawn from each vial and directly injected into the UPLC-MS to follow the kinetics of transformation product formation. Controls with laccase and mediators without pollutants were also performed.

Dissolved oxygen consumption experiments were conducted in a closed (airtight) cell containing an oxygen probe and 3 ml of reactive solution of pollutant, laccase and mediator. The cell was closed just after addition of laccase in an air-oxygen saturated solution, without any headspace.

7.2.1.5 Evaluation of the role of the ABTS radical cation

The role of the ABTS radical cation ($ABTS^{\bullet+}$) in the oxidation reaction was assessed by comparing degradation kinetics in solutions containing micropollutants (100 µM) and (i) only ABTS (550 µM) chemically oxidized by HOCl, (ii) chemically oxidized ABTS (550 µM) and laccase (280 U l^{-1}), (iii) ABTS oxidized by laccase (160-200 µM, ultrafiltered) with very low laccase activity (< 10 U l^{-1}), and

(iv) ABTS oxidized by laccase (160-200 µM, ultrafiltered) with further addition of laccase (200-250 U l^{-1}). These experiments were conducted in duplicate, at pH 5 with IPN and pH 5 or 6 with SMX.

7.2.1.6 Ecotoxicity test

A growth inhibition assay on the green alga *Pseudokirchneriella subcapitata* was selected to evaluate the toxicity of the micropollutants before and after treatment with the laccase-mediator system, as green algae are among the most sensitive organisms to the herbicide IPN but also to the antibiotic SMX (Ecotoxicity database AiiDA: www.aiida.tools4env.com).

For the toxicity test, mediators (AS and SA) and SMX stock solutions were prepared in pure water, as methanol is toxic to the green algae at the level present in the solutions (50% growth inhibition at 1 g l^{-1} (0.125% v/v), data not shown).

The samples tested consisted of IPN (100 µM) or SMX (150 µM) in a citrate-phosphate (20-40 mM) buffer at pH 5 or 6, respectively, with or without reaction during 40 h (with IPN) or 88 h (with SMX) at 25°C with a mediator concentration of 500 µM (either ABTS, AS or SA with SMX and only ABTS with IPN) and laccase (540 U l^{-1}). Controls were performed with each mediator and laccase incubated without pollutant, at the same concentrations. Long reaction times were used to assure complete reaction of the pollutants and stabilisation of the transformation products. These solutions were then diluted 200 times (with IPN) or 20 times (with SMX) in the algae growth medium prior to the toxicity tests to achieve concentrations of pollutants (0.5 and 7.5 µM for IPN and SMX, respectively) in the algae medium that still allow them to grow (Pavlić et al., 2006; Yang et al., 2008).

The algae growth inhibition test was performed according to the OECD guideline 201 (OECD, 2011) with the green algae *Pseudokirchneriella subcapitata* (Chlorophyceae ; strain SAG 61.81, from the Culture Collection of Algae, Göttingen, Germany), maintained as described by Valloton et al. (2009). Briefly, exponentially growing algae (initially about 50,000 cells ml^{-1} in sterile mineral AAP growth medium, pH 7.5) were exposed to the diluted samples over a period of 72 h, in an incubation shaker (Infors HT, Bottmingen, Switzerland) at 25°C and 90 rpm, with continuous illumination (70 µmol m^{-2} s^{-1}) by cool-white fluorescent lamps. Algae growth was determined as described by Daouk et al. (2013) by measuring the optical density at a wavelength of 690 nm (linearly correlated to the cell density) with a microplate reader (ELx800 ™, BioTek® Instruments, Winooski, Vermont) at the beginning and at the end of the test. The growth rate was calculated as the natural logarithmic increase in the optical density over time, and the growth inhibition was then determined by relative comparison of the growth rate of algae exposed to the sample to the one of algae growing in pure mineral media (controls), according to the OECD 201 guideline. Tests were carried out in triplicate, with a coefficient of variation for the growth rates below 16% and 2.5% for the samples and the controls, respectively, meeting the validity criteria of the OECD guideline. SMX and IPN were analysed before and after 72 h of incubation in the samples not treated by laccase-mediator. Less than 3-6% loss was observed for both compounds, confirming a constant exposure during the test.

7.2.1.7 Reaction modelling of laccase-mediator systems

Based on the results of the experiments, a kinetic model of laccase-mediator reactions was established, considering Michaelis-Menten type kinetics for laccase/mediator reactions and second-order rate

kinetics for mediator/pollutant reactions (see Section 7.3.9). The various differential equations were solved numerically with the ode45 solver (variable step Runge-Kutta method) within MATLAB (MathWorks, USA).

7.3 Results and discussion

7.3.1 Oxidation of IPN and SMX with various mediators

IPN or SMX did not react with the enzyme or the mediators alone during the time of incubation (up to 72 h) (Fig. 7.2). Recalcitrance of SMX to laccase oxidation was recently reported (Shi et al., 2014), although this compound was oxidized by crude *Phanerochaete chrysosporium* laccase extract in another study (Guo et al., 2014), possibly due to the presence of other oxidative enzymes or mediators in the extracted solution.

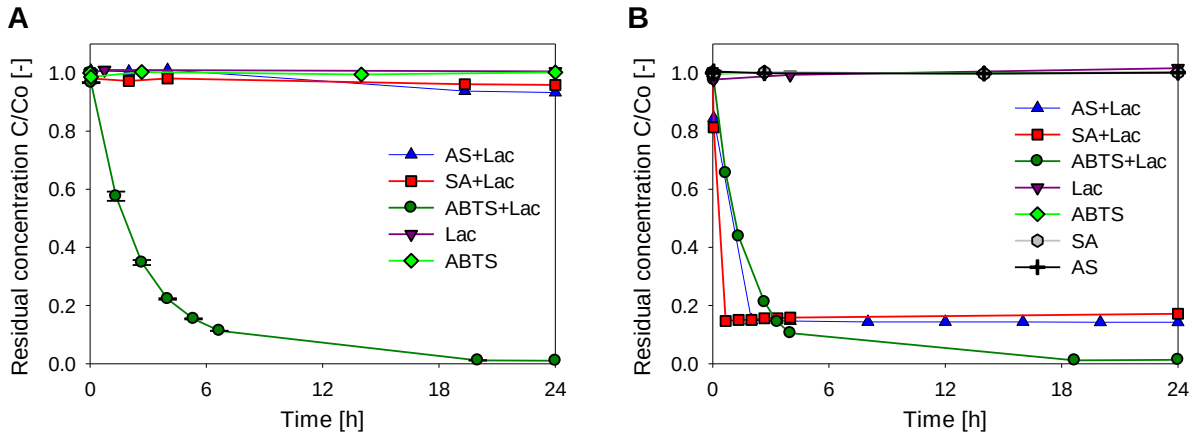


Fig. 7.2 Relative residual concentrations of (A) isoproturon (100 µM) at pH 5 in presence of the 3 mediators at 500 µM and 630 U l^{-1} laccase activity, and **(B) sulfamethoxazole** (80-100 µM) at pH 6 in presence of the 3 mediators at 100 µM and 320 U l^{-1} laccase activity. Controls with only mediators (at 500 µM) and micropollutants (without laccase), as well as with micropollutants and laccase (without mediators) are also presented. Lac: laccase, AS: acetosyringone, SA: syringaldehyde, ABTS: 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid).After 24 h, stable concentrations were observed up to 3-4 d.

With LMS, IPN was completely oxidized in the presence of ABTS in less than 20 h at pH 4-6 (Fig. 7.3 A). The two other natural mediators (AS and SA) were on the contrary not able to mediate the oxidation of IPN, even at high concentrations (500 µM, pH 5) and for long reaction times (up to 96 h) (Fig. 7.2 A).

SMX appeared to be much more reactive to LMS oxidation, with reasonable oxidation rates in presence of ABTS and very fast oxidation (almost complete removal in less than 1h) in the presence of both AS or SA at pH 6 (Fig. 7.2 B and Fig. 7.3 B-D). Very few studies have been published on SMX oxidation by LMS. Recently, a few studies showed that SMX could be oxidized by laccase in presence of several mediators (1-hydroxybenzotriazole (HTB), syringic acid, AS and SA) (Nguyen et al., 2014b; Shi et al., 2014; Yang et al., 2013c). Oxidation by LMS of other sulfonamides (sulfadimethoxine and sulfamonomethoxine) was also reported (Weng et al., 2012). The mechanisms and the conditions for the oxidation of these pollutants by LMS are, however, still largely unknown.

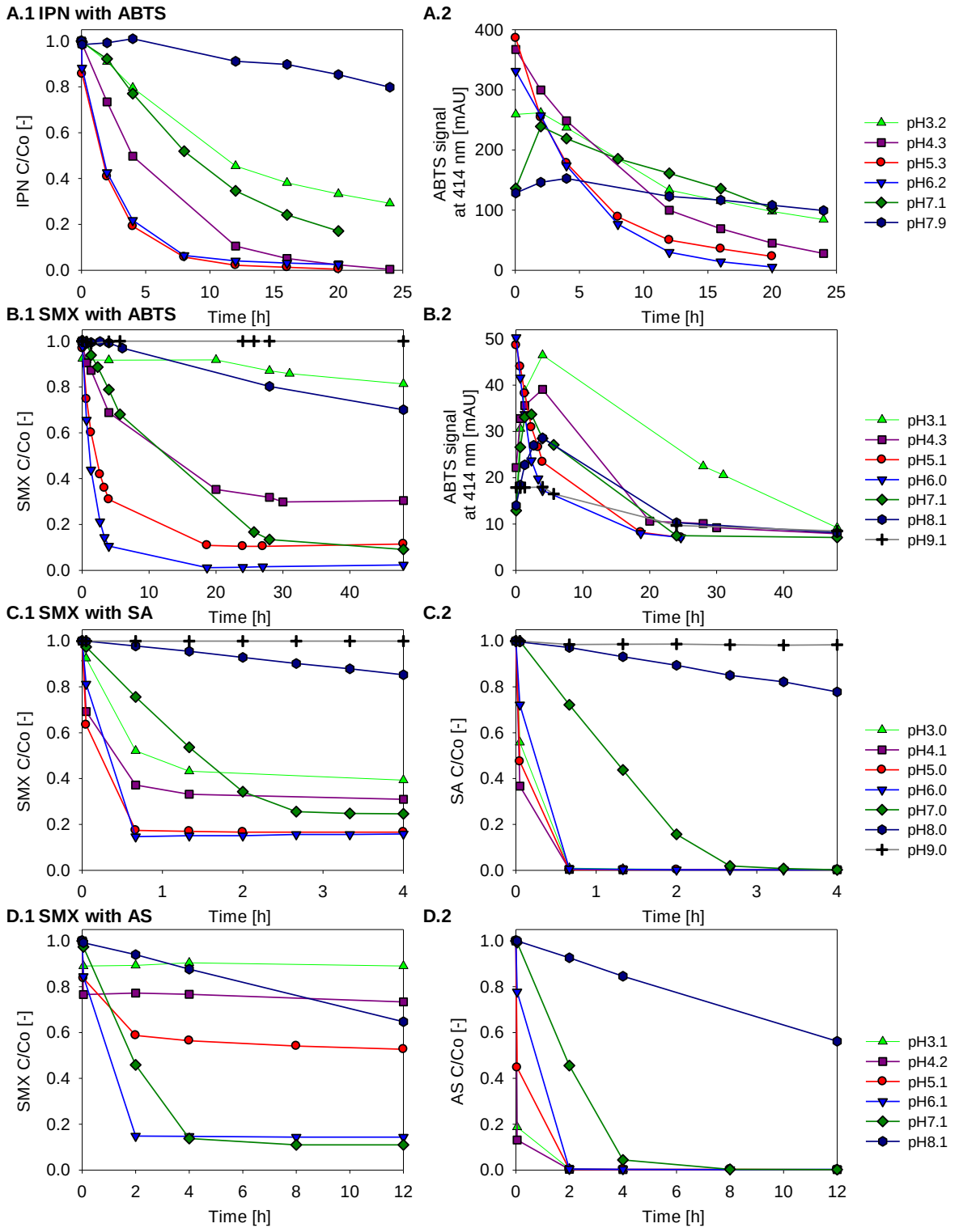


Fig. 7.3 Pollutant and mediator residual concentrations during laccase-mediated reactions at various pH. (A) IPN (A.1) in presence of ABTS (A.2: oxidized ABTS HPLC signal). **(B)** SMX (B.1) in presence of ABTS (B.2). **(C)** SMX (C.1) in presence of SA (C.2). **(D)** SMX (D.1) in presence of AS (D.2). *Experimental conditions:* (A) 100 µM IPN, 500 µM ABTS, 560 U l^{-1} laccase (lac). (B) 73 µM SMX, 100 µM ABTS, 315 U l^{-1} lac. (C) 75 µM SMX, 110 µM SA, 320 U l^{-1} lac. (D) 93 µM SMX, 201 µM AS, 560 U l^{-1} lac. SMX: sulfamethoxazole, IPN: isoproturon, AS: acetosyringone, SA: syringaldehyde.

7.3.2 pH influence on the oxidation kinetics

As shown in previous studies (Margot et al., 2013a; Margot et al., 2013c) (cf. Chapters 6 and 8), pH has a strong influence on the laccase activity, with higher activities (for *T. versicolor* laccase) under acidic conditions (pH 4-6) and almost no activity in alkaline solutions (pH > 7.5-8). It was therefore expected that LMS oxidation would also be strongly influenced by pH, especially during the first stage of the mediator oxidation by laccase. To further elucidate the effect of the pH, experiments were performed in the pH range 3 to 9. As presented in Fig. 7.3, kinetics of SMX and IPN abatement varied significantly as a function of pH. Fast oxidation of both compounds was observed at pH 5-6 for all three mediators, with decreasing rates at lower (3-4) or higher (7-8) pH-values. No significant oxidation was observed at pH 9. The optimal pH range for IPN and SMX oxidation was around 5-6 (Fig. 7.4 and Fig. 7.6 C), significantly higher than the optimal pH for mediator oxidation (pH < 3-4) (Fig. 7.6 B). This difference could be related to a higher self-reaction of mediator radicals at lower pH (produced locally at high concentration), leading to a decreasing pollutant exposure with reactive species.

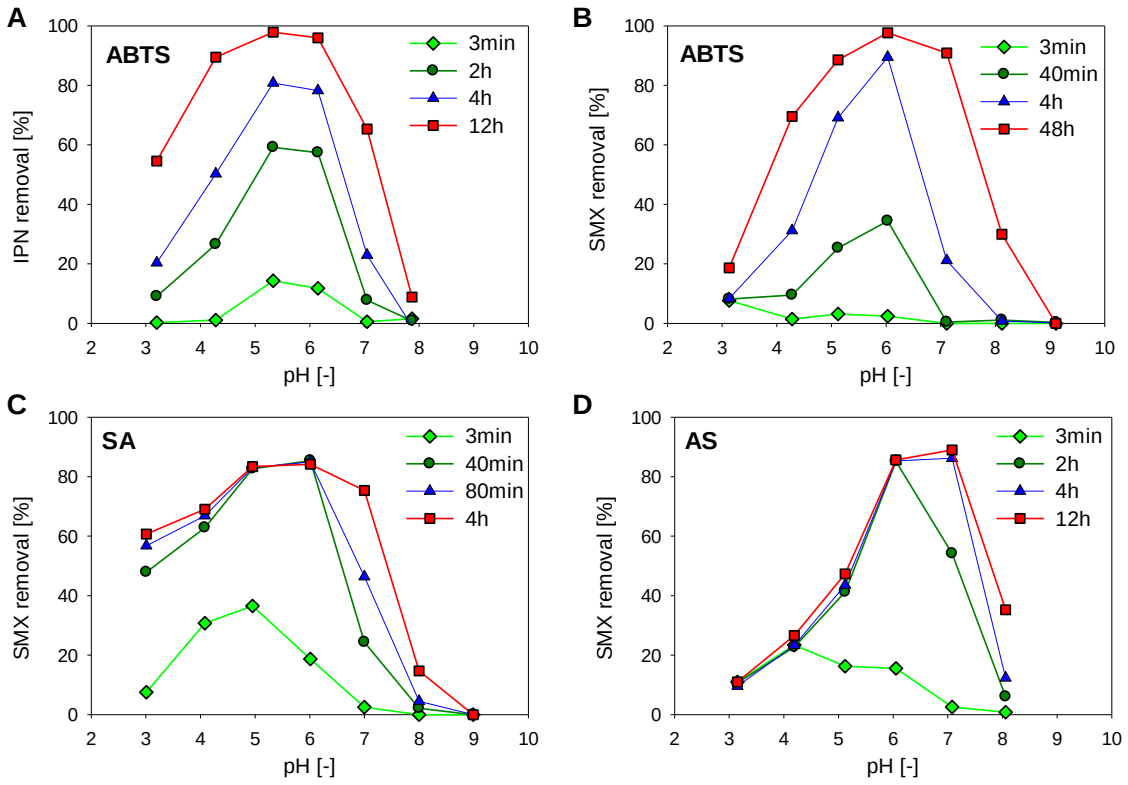


Fig. 7.4 Influence of pH on the abatement of **(A)** isoproturon (IPN, 100 µM) with ABTS (500 µM, 560 U l^{-1} laccase), **(B)** sulfamethoxazole (SMX, 80 µM) with ABTS (100 µM, 315 U l^{-1} laccase), **(C)** SMX (80 µM) with syringaldehyde (100 µM, 320 U l^{-1} laccase), and **(D)** SMX (100 µM) with acetosyringone (200 µM, 560 U l^{-1} laccase). Results for different reaction times.

7.3.3 Mediator consumption and effect of pH on the ratio mediator/pollutant

Mediators are often described as electron-shuttles between laccase and the substrate, with catalytic action of the mediator (Scheme 1) (Fabbrini et al., 2002). However, it appears from our results that neither ABTS, SA or AS acted as catalysts. These three mediators were consumed during the reaction, with a mediator/pollutant molar ratio in excess of unity. For AS and SA, a clear decrease in their concentration was observed as the reaction progressed, up to their complete disappearance (Fig. 7.3 C.2 and D.2). This consumption was independent on the pollutant concentration (Fig. 7.9 A.2, B.2). Although ABTS has been described as a catalytic mediator, with a constant recycling of its radical cation $ABTS^{\bullet+}$ during the oxidation of various substrates (Solís-Oba et al., 2005), no catalytic reactions were observed in this study.

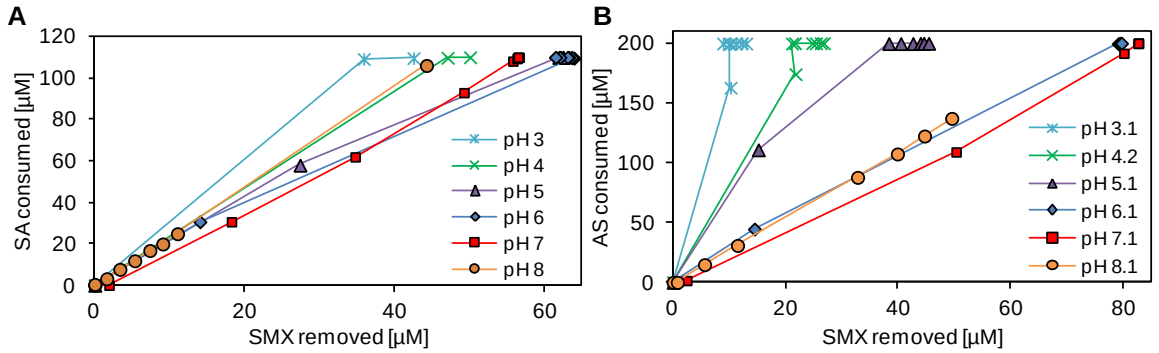


Fig. 7.5 Correlation between sulfamethoxazole (SMX) removal and the mediator consumption at various pH values. (A) With syringaldehyde (SA) (initial concentrations of 75 and 110 µM for SMX and SA, respectively). **(B)** With acetosyringone (AS) (initial concentrations of 93 and 201 µM for SMX and AS, respectively).

The disappearances of the mediators AS and SA (Fig. 7.3 C.2 and D.2) were proportional to the disappearance of SMX (in most cases linear correlation r > 0.99) (Fig. 7.5). This allowed determining the stoichiometry of the mediator-SMX reaction (molar ratio of the SMX oxidized relative to the mediator consumed). These ratios were pH-dependent, varying from 1.7 (pH 5-6) to 2.4-2.5 (pH 3 and 8), and from 2.4 (pH 6-7) to 16 (pH 3), for SA and AS, respectively (Fig. 7.6 A).

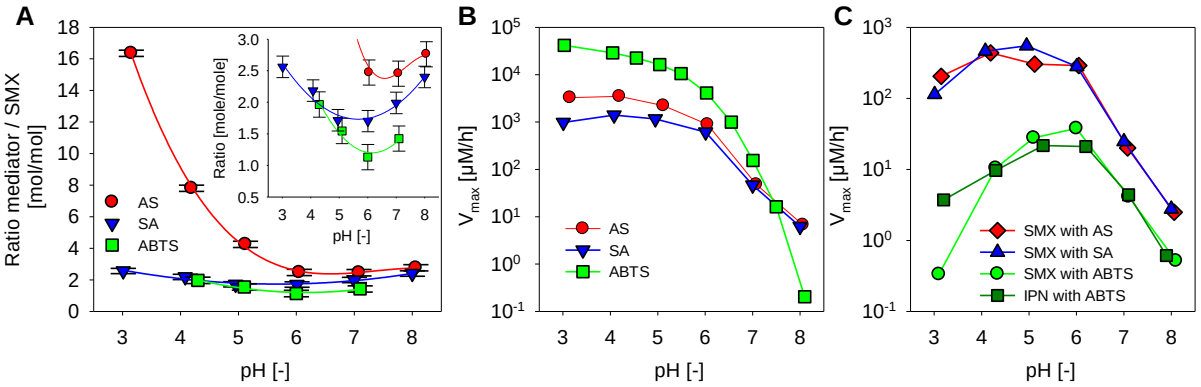


Fig. 7.6 pH influence on the ratio mediator/pollutant and the rate of laccase-mediated reactions. (A) Stoichiometric ratio of mediator consumed per mole of SMX oxidized in presence of AS, SA and ABTS. Insert: zoom into the low ratio range. Error bars: standard deviation. **(B)** Initial maximum oxidation rates (V_{max}, in µM h^{-1}) of the mediators AS, SA and ABTS as a function of the pH. **(C)** Initial maximum oxidation rates (V_{max}, in µM h^{-1}) of SMX in presence of AS, SA and ABTS, and initial maximum oxidation rates of IPN in presence of ABTS as a function of the pH. *Experimental conditions:* (B) 201 µM AS with 560 U l^{-1} lac, 110 µM SA with 320 U l^{-1} lac, and 500 µM ABTS with 370 U l^{-1} lac. (C) 73-93 µM SMX with 201 µM AS (560 U l^{-1} lac), 110 µM SA (320 U l^{-1} lac) and 100 µM ABTS (315 U l^{-1} lac), and 100 µM IPN with 500 µM ABTS (560 U l^{-1} lac). SMX: sulfamethoxazole, IPN: isoproturon, AS: acetosyringone, SA: syringaldehyde.

ABTS was not quantified during the reaction, but the radical cation $ABTS^{\bullet+}$ could be determined semi-quantitatively (HPLC-DAD signal intensity at 414 nm). The disappearance of the $ABTS^{\bullet+}$ signal (Fig. 7.3 A.2 and B.2) was closely linked to the removal of SMX and IPN (Fig. 7.3 A.1 and B.1), suggesting that the radical cation was involved and consumed during the reaction. The possibility that $ABTS^{\bullet+}$ was reduced back to ABTS was ruled out as laccase activity stayed relatively constant until the end of the experiments, and thus ABTS was oxidized back to $ABTS^{\bullet+}$ at a much higher rate (~ 300 µM per min) than its observed consumption (less than 1 µM per min). The molar ratio of ABTS consumed per SMX oxidized was also pH-dependent, with 1.1 at pH 6 and up to 2 mol/mol at pH 4 (Fig. 7.6 A), which is lower than for SA and AS. With IPN, this ratio could not be determined as a function of pH, but at pH 5, a molar ratio ABTS/IPN of 2.28 (±0.3) was found (based on Fig. 7.8 B). IPN required thus 1.5 times more mediator than SMX for a similar extent of oxidation.

The increase in the mediator/pollutant ratio at low pH, especially important for AS (from 2.4 to 16), is thought to be related to the very fast oxidation of the mediators at low pH, leading to fast production of reactive species that are more likely to react with each other than with the pollutants (presence in lower abundance).

7.3.4 Effect of the concentrations of enzyme, mediators and pollutants on the pollutant oxidation rate

To better understand the reactions involved in laccase-mediator systems, several experiments were performed in which either the mediator or the enzyme concentration was varied.

7.3.4.1 Effect of the enzyme concentration

It was observed that, at pH 5 (Fig. 7.7 A), the oxidation of IPN with ABTS was not significantly influenced by a variation of the laccase activity by a factor of five (from 120 to 600 U l^{-1}), while a strong influence on the rates was observed when reducing the mediator concentration by a factor of five (from 500 to100 µM). Therefore, it can be assumed that at pH 5 the rate-limiting step was not the oxidation of the mediator by laccase but the reaction of the oxidized mediator with the pollutant. However, at higher pH, when the mediator oxidation by laccase becomes limiting, higher laccase activity is expected to increase the pollutant oxidation rate.

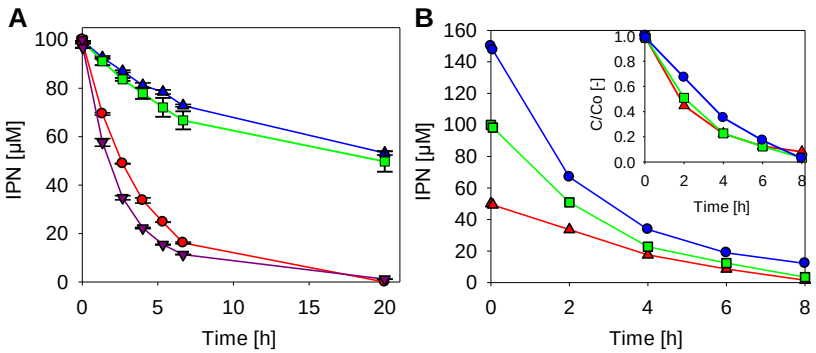


Fig. 7.7 Influence of enzyme and mediator concentrations on isoproturon (IPN) oxidation at pH 5. (A) 100 µM ABTS and 120 U l^{-1} laccase (▲), 100 µM ABTS and 600 U l^{-1} laccase (■), 500 µM ABTS and 120 U l^{-1} laccase (●), and 500 µM ABTS and 600 U l^{-1} laccase (▼). **(B)** 560 U l^{-1} laccase and different concentrations of ABTS and IPN, but with a ratio of five (ABTS:IPN): 250:50 µM (▲), 500:100 µM (■) and 750:150 µM (●). Insert: relative concentration. Error bars: range of values of duplicates.

7.3.4.2 Effect of the mediator and pollutant concentration

A strong influence of the mediator concentration on the pollutant oxidation rates under acidic conditions was observed for both SMX and IPN (Fig. 7.8 A and B). As shown in Fig. 7.8 C, the oxidation rates increased proportionally with increasing mediator concentrations, reaching a plateau at high mediator levels. The assumption behind this saturation effect (plateau) was that, at high mediator concentrations (and sufficient laccase activity), high levels of reactive radicals are quickly produced, with a tendency to react with each other (possibly already in the enzymatic pocket) rather than with the pollutants. However, this saturation effect was not observed when, instead of keeping the pollutant concentration constant, the pollutant level was varied in proportion to the mediator concentration so as to keep the mediator/pollutant ratio constant (Fig. 7.8 C and Fig. 7.7 B). In this case, a linear increase in the oxidation rate was observed as a function of the mediator (and pollutant) concentration, which was expected since the radical/pollutant ratio stayed constant.

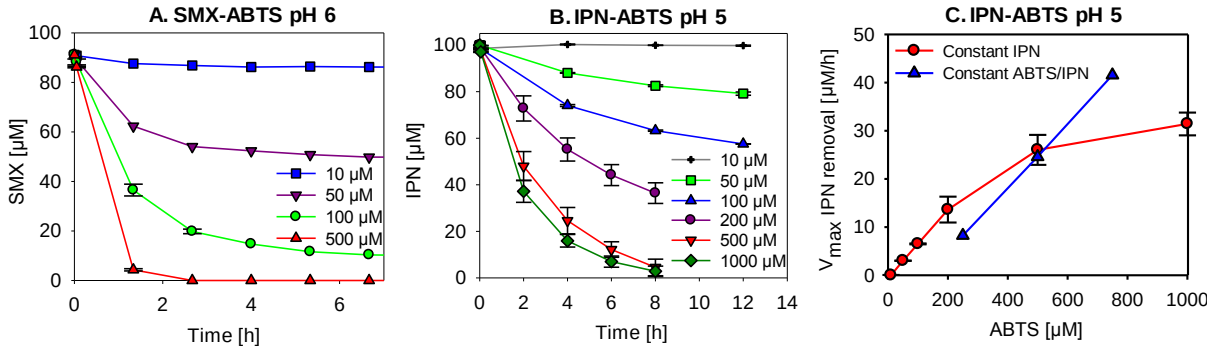


Fig. 7.8 Influence of mediator concentrations on sulfamethoxazole (SMX) and isoproturon (IPN) oxidation in presence of laccase at acidic pH. (A) Residual SMX concentrations (in µM) at pH 6 with various concentrations of ABTS (450 U l^{-1} laccase). **(B)** Residual IPN concentrations (in µM) at pH 5 with various concentrations of ABTS (560 U l^{-1} laccase). **(C)** Initial maximum IPN oxidation rates (Vmax, in µM h^{-1}) as a function of the ABTS concentration with constant initial IPN concentration (100 µM, ●), and variable IPN concentrations (50, 100 and 150 µM, ▲) to maintain a ratio ABTS/IPN of five (pH 5, 560 U l^{-1} laccase). Error bars: range of values of duplicates.

In contrast to the results under acidic conditions, at pH 7, no effect of mediator concentrations on the rate of SMX oxidation by the three mediators was observed (Fig. 7.9). At the three mediator concentrations tested (100, 200 and 500 µM), SMX was oxidised at the same constant rate (zero order reaction) until all the mediator was consumed. As laccase is several orders of magnitude less active under neutral-alkaline than under acidic conditions (Fig. 7.6 B), this observation points towards rate limitation of the oxidation of the mediator by laccase. Enzymatic reactions follow Michaelis-Menten kinetics. Therefore, the rate of the reaction does not vary with the mediator concentration when it is present at sufficiently high levels to saturate all the reactive sites of the enzymes. The radicals constantly produced are thus expected to react directly with SMX, leading to zero-order kinetics as long as sufficient mediator is present.

At a constant mediator/pollutant ratio (at pH 5) and with a sufficiently high mediator concentration, the relative pollutant removal rate appeared to be independent on the initial pollutant concentration (similar to first order kinetics) (Fig. 7.7 B). Although this was not tested with low mediator/pollutant concentrations, it is expected that this independency would not be valid once the mediator

concentration becomes lower than a certain threshold, related to the affinity constant of the enzyme for the mediator (Michaelis constant K_m).

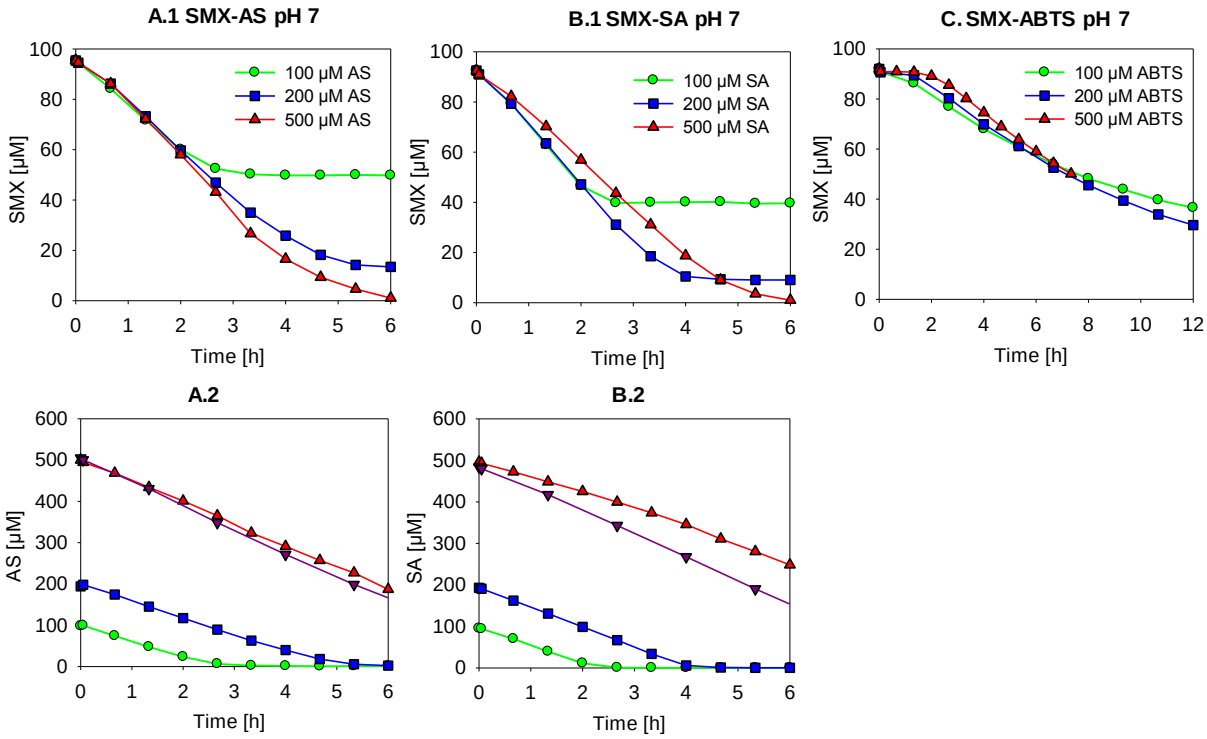


Fig. 7.9 Influence of mediator concentrations on sulfamethoxazole (SMX) oxidation in presence of laccase at neutral pH. Residual SMX concentrations (in µM) at pH 7 with various concentrations of **(A.1)** acetosyringone (AS) (520 U l^{-1} laccase), **(B.1)** syringaldehyde (SA) (455 U l^{-1} laccase), and **(C)** ABTS (450 U l^{-1} laccase). Residual concentrations of **(A.2)** AS and **(B.2)** SA at initial concentrations of 100 (●), 200 (■) or 500 (▲) µM with SMX (91-95 µM), or 500 µM without SMX (▼) (same experiments as in A.1 and B.1, respectively).

7.3.5 Oxygen consumption during laccase-mediated reactions

Some experiments, presented in Fig. 7.10, were performed in closed (airtight) cells to assess oxygen consumption during the laccase-mediated reactions. With AS and SA (at 500 µM, pH 7) (Fig. 7.10 A), a decrease in dissolved oxygen concentration was observed, resulting to about 40% consumption of the oxygen available to oxidize 100 µM of SMX (completely removed in 6 h, data not shown). This shows that no oxygen limitations are expected for SMX oxidation. Approximately 0.8 and 1 mole of oxygen were consumed per mole of SMX oxidized, with SA and AS, respectively. Oxygen consumption stopped once SA was oxidized, while, with AS, almost complete oxygen depletion was observed, possibly due to further oxidation of the transformation products. With ABTS (Fig. 7.10 B), fast oxygen consumption was observed with SMX, which was completely removed in about 3 h (still 60% oxygen saturation), and slightly slower oxygen consumption was observed with IPN, which was also completely removed in about 18 h (20% oxygen saturation). Therefore, in both cases, oxygen should not limit the reaction. In presence of ABTS and laccase without pollutant, significantly slower oxygen consumption was observed. This suggests that oxygen was not only consumed for the oxidation of ABTS but also for further pollutant oxidation. About 1 and 2 moles of oxygen were consumed per mole of SMX and IPN oxidized, respectively. Complete oxygen depletion was observed during the reaction with ABTS, this occuring a long time after complete oxidation of ABTS to its

radical cation. This suggests that the ABTS radical cation, as well as possibly the other transformation products formed, were further slowly oxidized to unknown products.

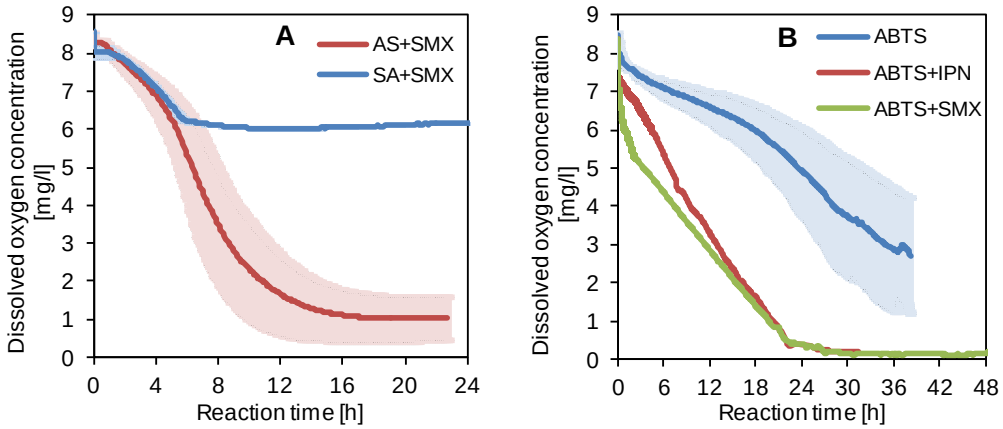


Fig. 7.10 Dissolved oxygen consumption during laccase-mediated oxidation of sulfamethoxazole (SMX) and isoproturon (IPN). (A) Oxidation of SMX (100 µM) in presence of acetosyringone (AS) or syringaldehyde (SA) (500 µM) and 300 U l^{-1} laccase, at pH 7. **(B)** Oxidation of IPN or SMX (100 µM) in presence of ABTS (450 µM) and 300 U l^{-1} laccase, at pH 5, or under the same conditions but without pollutants (ABTS alone). Average and values (interval) of duplicates.

Although the setup was not designed to calculate precisely the stoichiometry of the reaction (oxygen diffusion from the air was possible before closing the cell), it was observed that about 0.25 mole of oxygen was consumed per mole of mediator (SA, AS and ABTS) oxidized during the initial phase of the reaction (when the mediator was still present at high concentration), suggesting a one electron transfer from each mediator molecule.

7.3.6 Role of the ABTS radical cation for the oxidation of SMX or IPN

For AS and SA, it is expected that the unstable and reactive phenoxy radicals generated during the oxidation by laccase (Fig. 7.1) are the reactive species causing the pollutant oxidation. For ABTS, it is not clear which reactive species are involved in pollutant oxidation. It is reported that $ABTS^{\bullet+}$ can oxidize several compounds, mainly polyphenols, phenols or anilines (Osman et al., 2006a; Solís-Oba et al., 2005). To elucidate the fate of the selected target compounds, we investigated if $ABTS^{\bullet+}$ alone (oxidized chemically) could also oxidize SMX and IPN. As presented in Fig. 7.11 C and Fig. 7.12 C, no SMX or IPN removal was observed with $ABTS^{\bullet+}$ alone, while addition of laccase (280 U l^{-1}) in the same solution led to complete removal of both compounds in a few hours. Low laccase activity (7-9 U l^{-1}) enabled pollutant oxidation but when laccase was inhibited no degradation was observed with $ABTS^{\bullet+}$ (Fig. 7.11 and Fig. 7.12 A). This demonstrates that $ABTS^{\bullet+}$ is not directly responsible for SMX or IPN oxidation and that laccase is necessary to catalyze this reaction.

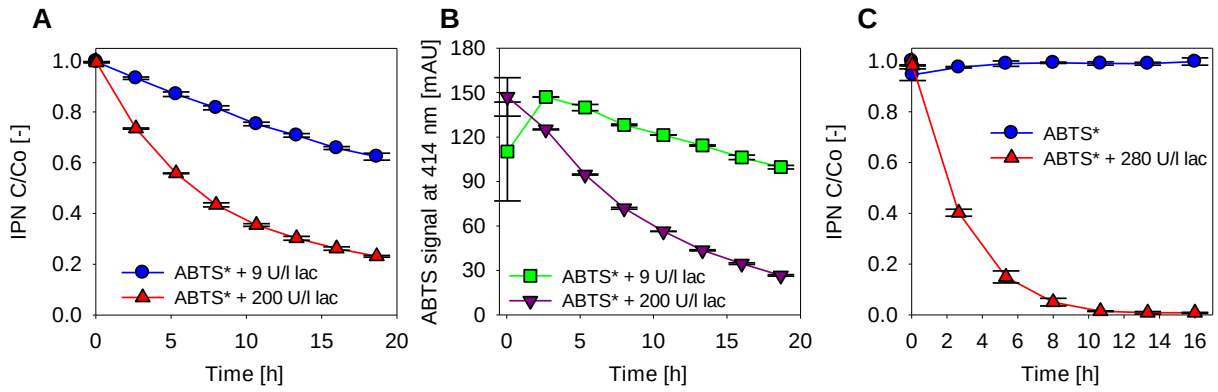


Fig. 7.11 Oxidation of isoproturon (IPN, 100 µM) with oxidized ABTS (radical cation $ABTS^{\bullet+}$) at pH 5, with or without laccase. (A) Residual IPN concentrations in presence of 205 µM $ABTS^{\bullet+}$ (500 µM ABTS oxidized by laccase and ultrafiltered to remove the enzyme): (●) ultrafiltered solution (9 U l^{-1} residual laccase activity), (▲) ultrafiltered solution with addition of 200 U l^{-1} laccase. **(B)** $ABTS^{\bullet+}$ concentration (UV-Vis signal at 414 nm) for the experiment described in A; (■) with ultrafiltered solution, (▼) with ultrafiltered solution and 200 U l^{-1} laccase. **(C)** Residual IPN concentrations with (●) 540 µM $ABTS^{\bullet+}$ (oxidized chemically with HOCl), or (▲) 540 µM $ABTS^{\bullet+}$ and 280 U l^{-1} laccase. Error bars: range of values of duplicates.

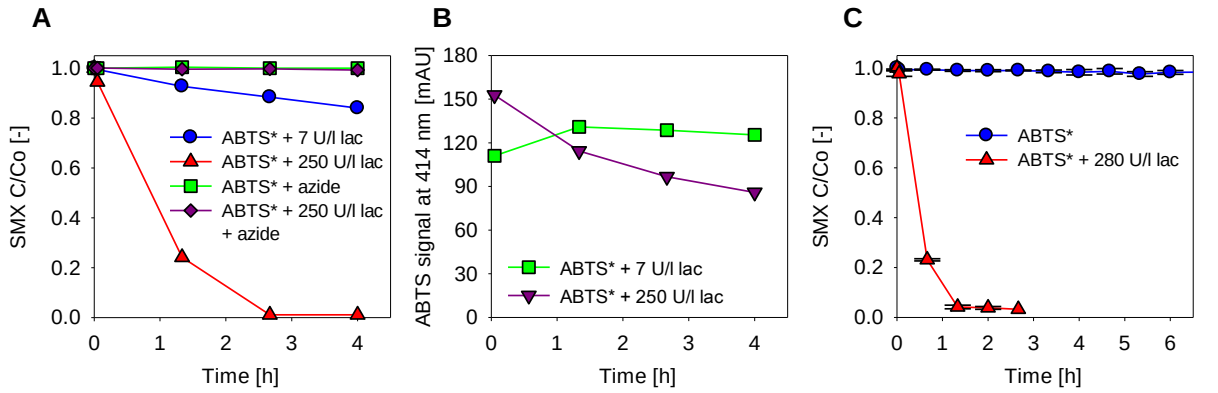


Fig. 7.12 Oxidation of sulfamethoxazole (SMX, 100 µM) with oxidized ABTS (radical cation $ABTS^{\bullet+}$), with or without laccase. (A) Residual SMX concentrations at pH 6 in presence of 160 µM $ABTS^{\bullet+}$ (500 µM ABTS oxidized by laccase and ultrafiltered to remove the enzyme): (●) ultrafiltered solution (7 U l^{-1} residual laccase activity), (▲) ultrafiltered solution with addition of 250 U l^{-1} laccase, (■) controls with $ABTS^{\bullet+}$ and sodium azide (10 mM), (◊) control with $ABTS^{\bullet+}$, laccase (250 U l^{-1}) and sodium azide (10 mM). **(B)** $ABTS^{\bullet+}$ concentration (UV-Vis signal at 414 nm) for the experiment described in A; (■) with ultrafiltered solution, (▼) with ultrafiltered solution and 250 U l^{-1} laccase. **(C)** Residual SMX concentrations at pH 5 with (●) 540 µM $ABTS^{\bullet+}$ (oxidized chemically with HOCl), or (▲) 540 µM $ABTS^{\bullet+}$ and 280 U l^{-1} laccase. Error bars in C: range of values of duplicates.

To investigate if the reactive product responsible for pollutant degradation was formed during ABTS or $ABTS^{\bullet+}$ oxidation by laccase, ABTS was chemically oxidized by chlorine to form a solution containing only $ABTS^{\bullet+}$, which was then used to treat the pollutants by addition of laccase. A comparison between the removal efficiencies of IPN and SMX by laccase with oxidized $ABTS^{\bullet+}$ or laccase with ABTS showed almost identical results (Fig. 7.13), suggesting that the reactive species were formed from the reaction of $ABTS^{\bullet+}$ with laccase. Moreover, $ABTS^{\bullet+}$, which is quite stable in pure solution (half-life of 47 h at 20-23°C) (Pinkernell et al., 2000), was degraded in presence of the pollutants and laccase, at a rate strongly correlated with the removal rates of the micropollutants (Fig. 7.3 A.2 and B.2 and Fig. 7.11 B). This suggests that laccase reacts with $ABTS^{\bullet+}$ producing reactive species, which in turn react with the pollutants.

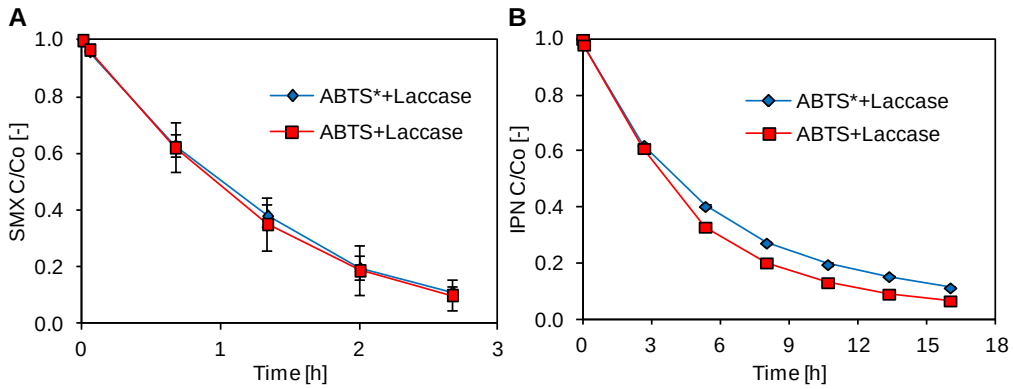


Fig. 7.13 Oxidation of (A) sulfamethoxazole (SMX, 100 µM), and (B) isoproturon (IPN, 100 µM) with laccase (260 U l^{-1}) and either ABTS (■, 450 µM), or ABTS radical cation (◊, $ABTS^{\bullet+}$, 450 µM), at pH 5. $ABTS^{\bullet+}$ was produced before the reaction by chemical oxidation (at a stoichiometric ratio) of 450 µM ABTS with HOCl. Average and values (error bars) of duplicates.

A transformation product with a UV/Vis spectrum similar to a degradation product of the ABTS di-cation ($ABTS^{2+}$) was detected in samples incubated with ABTS and laccase, suggesting an $ABTS^{2+}$ formation (see section 7.3.7). Although the direct oxidation of $ABTS^{\bullet+}$ (reduction potential E^0 = 0.6 V) to the stronger oxidant $ABTS^{2+}$ (E^0 = 1.1 V) by laccase (E^0 ~0.8 V) is thermodynamically unfavourable, it was suggested that this reaction could slowly happen inside of the enzymatic pocket (the electrostatic interaction in the binding site may lower the reduction potential of the ABTS di-cation) (Branchi et al., 2005). $ABTS^{2+}$ is reported to oxidize several compounds such as aromatic alcohols that cannot be oxidized by $ABTS^{\bullet+}$ (Bourbonnais et al., 1998; Majcherczyk et al., 1999). $ABTS^{2+}$ (low solubility and very low stability in water) or one of its degradation products could therefore be the reactive species responsible for pollutant oxidation in the combined laccase/ABTS system (Branchi et al., 2005; Majcherczyk et al., 1999). A slow production of $ABTS^{2+}$ may explain why the pollutant oxidation in the laccase/ABTS system takes several hours while the enzyme oxidizes ABTS completely to $ABTS^{\bullet+}$ within a few minutes. These results indicate that in the present case, the real mediator is not ABTS but its radical cation $ABTS^{\bullet+}$, which is oxidized by laccase to a reactive species with higher reduction potential (possibly $ABTS^{2+}$) which, in turn, reacts with IPN or SMX.

7.3.7 Characterization of the transformation products

Oxidation by LMS does not lead to complete pollutant mineralization because laccase and the oxidized mediators react mainly with some specific (electron donating) moieties of organic compounds. As shown in the chromatograms in Fig. 7.14, several transformation products were detected by UPLC-MS for the reaction of SMX in presence of the three mediators and for the reaction of IPN in presence of ABTS.

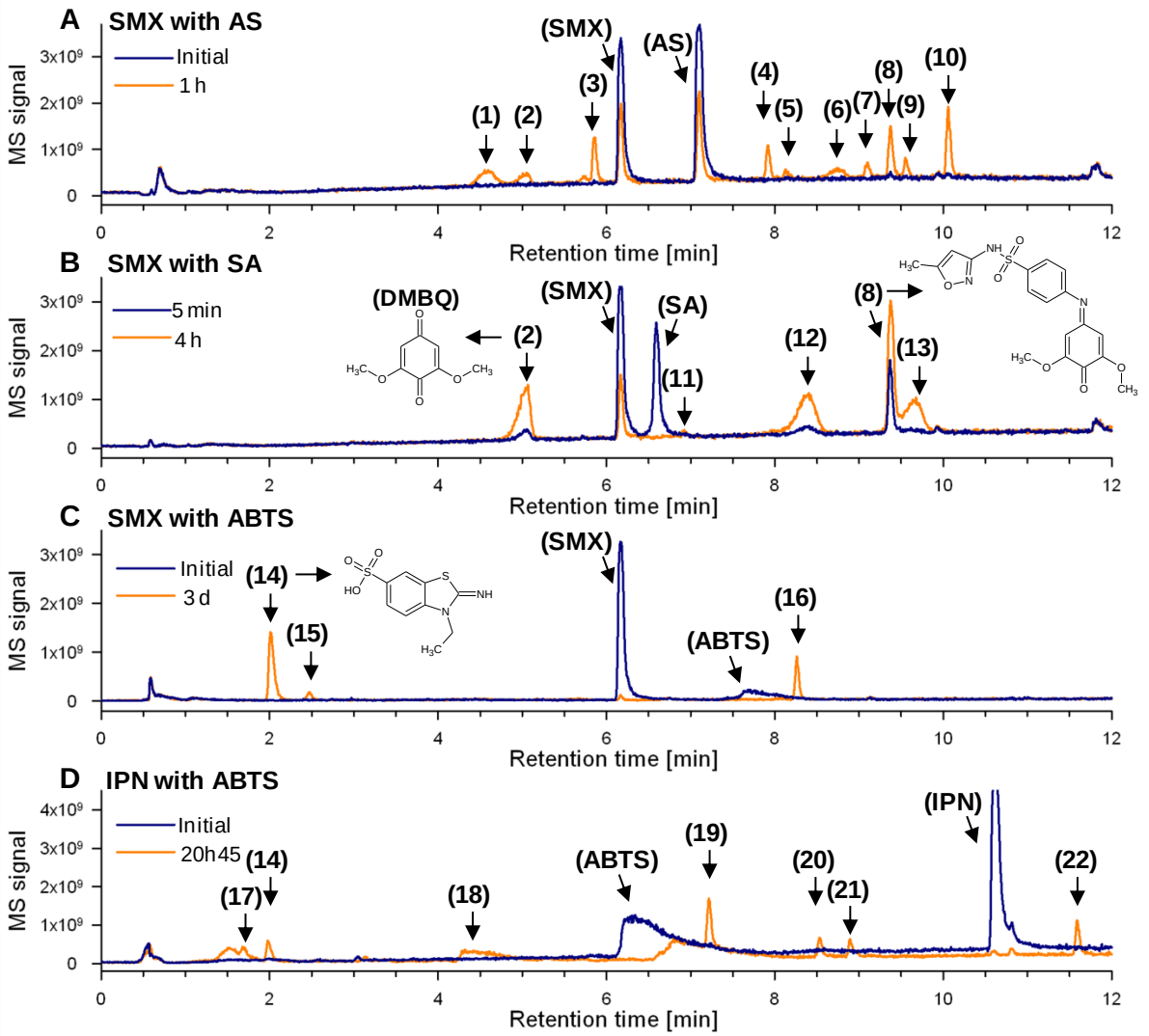


Fig. 7.14 UPLC-MS chromatograms before and during laccase-mediated reactions. (A) sulfamethoxazole (SMX) in presence of acetosyringone (AS), **(B)** SMX in presence of syringaldehyde (SA), **(C)** SMX in presence of ABTS, and **(D)** isoproturon (IPN) in presence of ABTS. Numbers in bracket: ID of the main transformation products (TPs). The structures of TPs 2, 8 and 14 (confirmed by other studies) is presented as well. Suggested structures for some of the other transformation products are shown in Fig. 7.16.

7.3.7.1 Transformation products formed in the laccase-AS-SMX system

During SMX oxidation by laccase in presence of AS, 10 main transformation products were detected (ID number 1 to10, Fig. 7.14 A). Six of them had a molar mass higher than SMX or AS suggesting that they were oxidative coupling products (Table 7.1). Several products (1, 2, 3 and 6) were also generated during the oxidation of AS by laccase. Product 2, with a mass of 168 g mol^{-1} and a maximum UV/Vis absorbance at 290 nm (Table S 7.1, Supporting information (SI)), was identified as 2,6-dimethoxy-1,4-benzoquinone (DMBQ) (Fig. 7.14 B), as observed and confirmed in other studies (Ibrahim et al., 2013; Weng et al., 2012). Product 6, with a mass of 332 g mol^{-1}, is likely (but exact structure not confirmed) a dimeric product of AS, as proposed in Fig. 7.16. Similar dimeric products were observed by Ibrahim et al. (2013). Products 4-5 and 7-10 were observed only when SMX was present. Product 8, with a mass of 403 g mol^{-1} and UV-Vis adsorption spectrum maxima at 200, 314 and 405 nm, was identified based on similar studies (Shi et al., 2014; Weng et al., 2012) as a coupling

product between DMBQ and SMX (Fig. 7.14 B). The masses of products 5, 7, 9 and 10 (Table 7.1) suggest that they were also coupling products between AS radicals and SMX (structure suggestion in Fig. 7.16), as also proposed by Shi et al. (2014). All these transformation products appeared rapidly during the first hour of reaction, linked to the degradation of SMX and AS (Fig. 7.15 A and B). Concentrations of products 2, 4, 6, 7 and 8 were stable for more than 72 h. In contrast, products 1, 3, 5, 9 and 10 vanished within this time (Fig. 7.15 B) suggesting that these products were either not stable or further oxidized by laccase, which is consistent with the additional oxygen consumption observed (cf. section 7.3.5). After 72 h, the two main (in signal intensity) products still present in solution were 2 and 8 (Fig. 7.15 A).

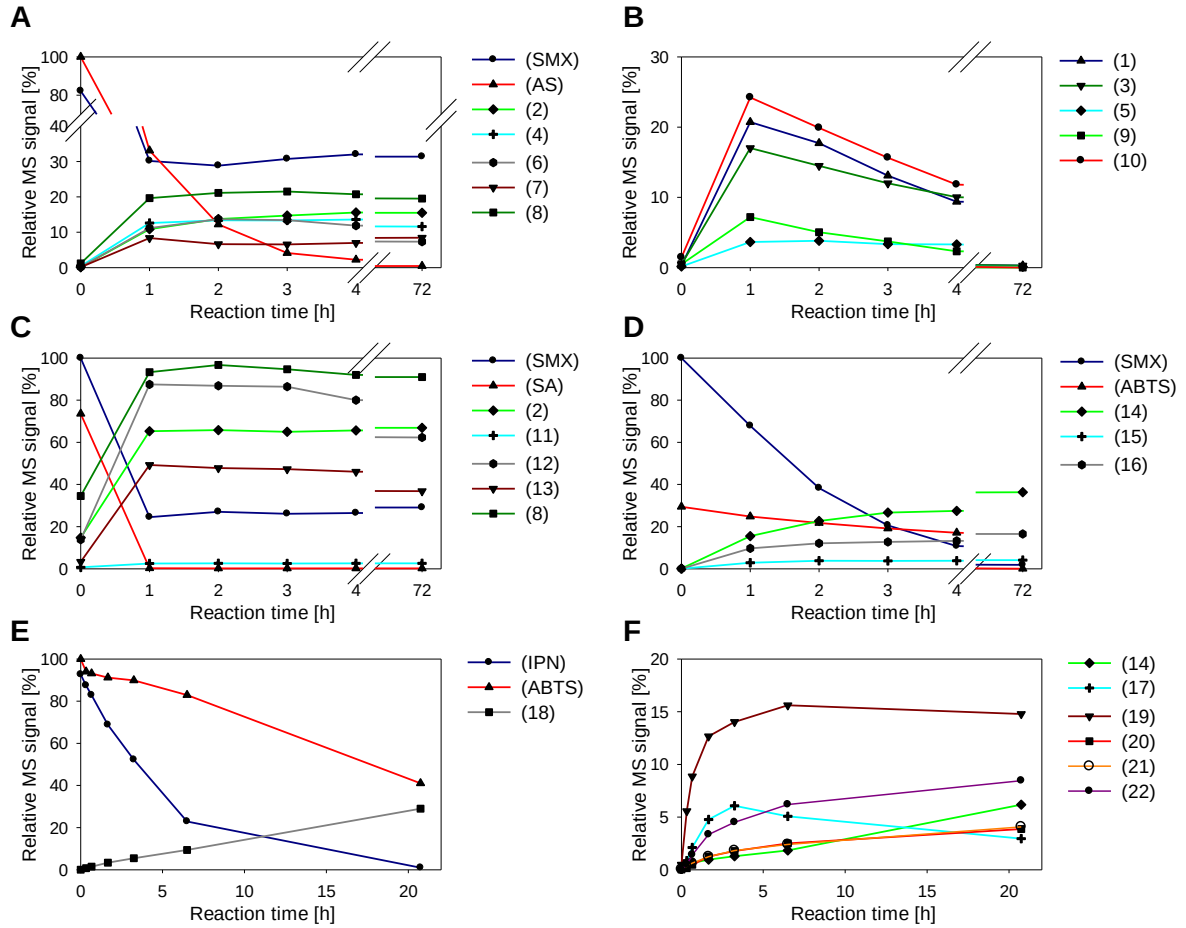


Fig. 7.15 Kinetics of sulfamethoxazole (SMX) and isoproturon (IPN) oxidation and transformation products formation by laccase-mediator systems. (A and B) SMX in presence of acetosyringone (AS), **(C)** SMX in presence of syringaldehyde (SA), **(D)** SMX in presence of ABTS and **(E and F)** IPN in presence of ABTS. Numbers in brackets: ID of the transformation products, corresponding to Fig. 7.14.

7.3.7.2 Transformation products formed in the laccase-SA-SMX system

During SMX oxidation by laccase in presence of SA, only five main transformation products were detected (Table 7.1). Two of them were the same as found with AS, namely products 2 and 8, which is not surprising because product 2 (DMBQ) is a typical product of SA oxidation (Ibrahim et al., 2013). These two dominant products were much more abundant with SA than with AS, with a 3.5 to 3.8 times higher signal intensity (Fig. 7.14 A and B). The third most abundant transformation product was 12, which is very likely a dimeric product of SA, with a structure similar to the dimeric AS (product 6)

(Fig. 7.16). Similar to AS, the five transformation products appeared during the first hour of reaction together with SMX and SA removal, and were then stable for more than 72 h (Fig. 7.15).

Table 7.1 Retention time, molar mass and m/z of the parent compounds and the transformation products (TPs) detected by UPLC-MS during the laccase-mediated transformation of sulfamethoxazole (SMX) in presence of either acetosyringone (AS), syringaldehyde (SA) or ABTS, or isoproturon (IPN) in presence of ABTS. The numbers of the transformation products correspond to Fig. 7.14. Structures of compounds with similar masses are suggested for some transformation products and presented in Fig. 7.16.

ID	Retention time	Molar mass[a]	m/z of adducts[b]	Type of products[c]	Structures proposition[d]
	[min]	[g/mol]	[g/mol]		
Parent compounds					
(AS)	7.11	196	197+219	Acetosyringone	(AS)
(SA)	6.6	182	183+205	Syringaldehyde	(SA)
(ABTS)	6.44 to 7.69	514	514+515+536+558	ABTS	(ABTS)
(SMX)	6.19	253	254+276	Sulfamethoxazole	(SMX)
(IPN)	10.62	206	207+229+435	Isoproturon	(IPN)
Transformation products AS+SMX					
(1)	4.58	180	181+203	AS TP	-
(2)	5.05	168	169+191	2,6-Dimethoxy-1,4-benzoquinone (DMBQ)	(2)
(3)	5.85	182	183+205	AS TP	(V)
(4)	7.91	248	249+271+519	-	-
(5)	8.11	447	448+470	*Coupling AS-SMX*	(III)
(6)	8.77	332	333+355+687	Dimeric AS TP	(I)
(7)	9.1	415	416+438+454	*Coupling AS-SMX*	-
(8)	9.37	403	404+426	Coupling SMX-DMBQ	(8)
(9)	9.56	417	418+440	*Coupling AS-SMX*	(IV)
(10)	10.06	445	446+468	*Coupling AS-SMX*	-
Transformation products SA+SMX					
(2)	5.05	168	169+191	2,6-Dimethoxy-1,4-benzoquinone (DMBQ)	(2)
(11)	6.93	281	282+304	-	-
(12)	8.38	318	319+341+659+351+373	Dimeric SA TP	(II)
(8)	9.37	403	404+426+829	Coupling SMX-DMBQ	(8)
(13)	9.66	348	349+371+367+381+719+403	-	-
Transformation products ABTS+SMX					
(14)	2.01	258	259+281	ABTS TP	(14)
(15)	2.48	98	99	*SMX fragment*	(VI)
(16)	8.26	238	239+261	*SMX fragment*	(VII)
Transformation products ABTS+IPN					
(17)	1.69	273	274+296	ABTS TP	(VIII)
(14)	1.99	258	259+281	ABTS TP	(14)
(18)	4.72	546	547+569	ABTS TP	-
(19)	7.23	222	223+245	*Hydroxy-isoproturon*	(IX)
(20)	8.56	445	446+468	*Coupling IPN+fragment ABTS*	-
(21)	8.9	447	448+470	*Coupling IPN+fragment ABTS*	-
(22)	11.61	232	233+255	-	-

[a] Molar mass M deduced from the m/z of the adducts

[b] m/z of ESI MS adducts with positive ion mode: M+H: [M+1], M+Na: [M+23], 2M+Na: [2M+23], M+CH_3OH: [M+32], M+NH_4: [M+18]

[c] Transformation products may come from the mediator degradation by laccase (also observed without pollutant), or by reaction with the pollutant. *In italics*: suggestion based on the mass of the by-product. (-): no suggestion

[d] Refers to the structures proposed in Fig. 7.16, based on the mass and the relation log Kow/retention time of the proposed molecule. (-): no suggestion

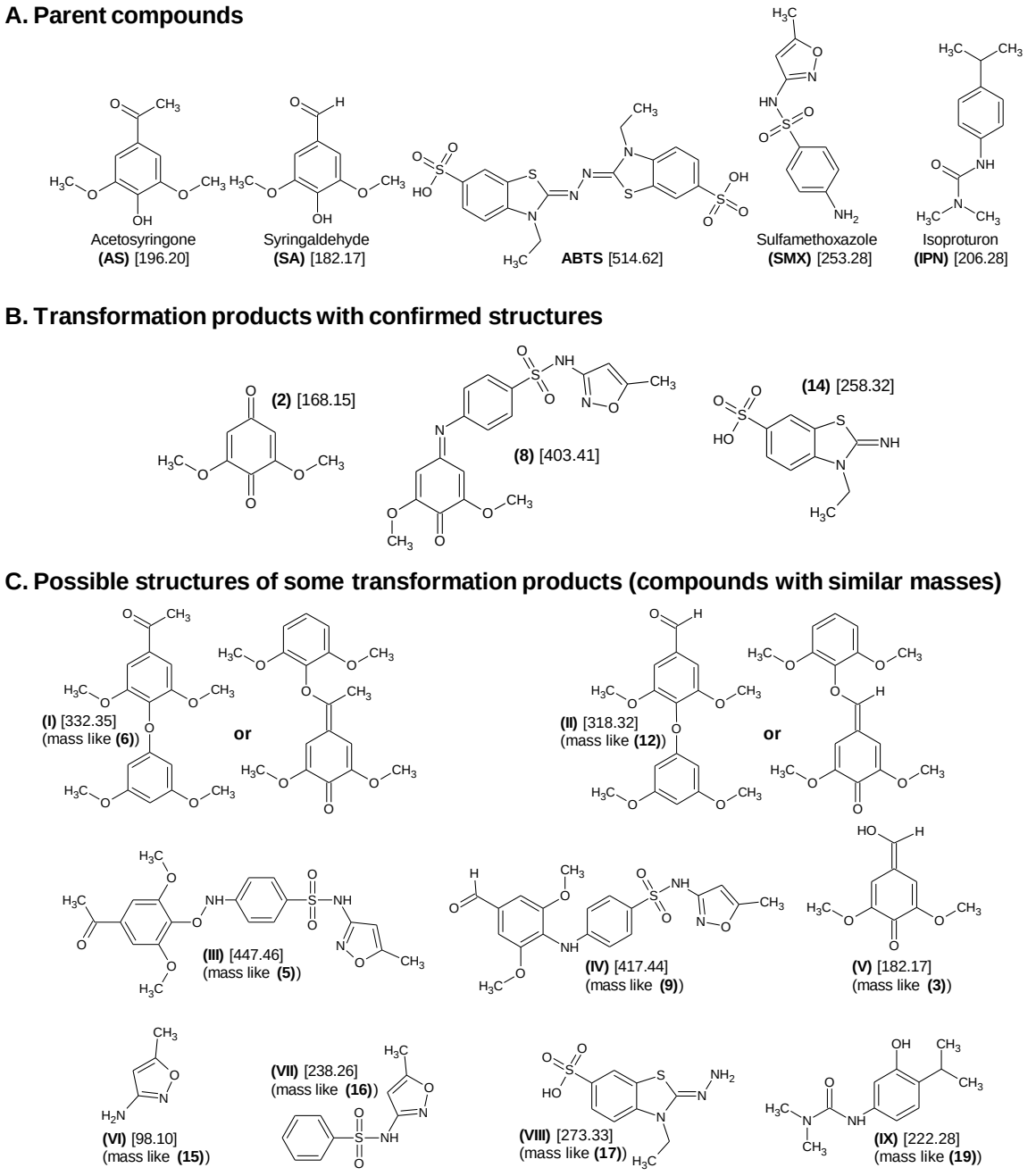


Fig. 7.16 Structures and molar mass of (A) the parent compounds, (B) the transformation products with confirmed structures (by other related studies) and (C) compounds (numbered with roman numbers) with similar masses and properties (polarity) as some transformation products (Arabic numbers refer to the ID of the transformation products, cf. Fig. 7.14) (hypothetical structures).

7.3.7.3 Transformation products formed in the laccase-ABTS-SMX system

During SMX oxidation by laccase in presence of ABTS, only three transformation products were clearly visible (Fig. 7.14 C, Table 7.1). Product 14 was also observed during ABTS oxidation by laccase without any pollutant and was identified as 3-ethyl-6-sulfonate benzothiazolinone imine, an ABTS fragment. Its chemical structure (Fig. 7.14 C) was elucidated in other studies (Marjasvaara et al., 2008; Osman et al., 2006b). Products 15 and 16 were possibly, based on their mass and retention time (relative to their log K_{ow}), degradation products of SMX (Fig. 7.16). No coupling products with a

mass higher than the parent compounds were detected. The three products appeared gradually during 72 h in parallel to the disappearance of SMX and ABTS (sum of ABTS and $ABTS^{\bullet+}$) (Fig. 7.15 D). The highest signal intensity was observed for the ABTS fragment 14, followed by SMX fragment 16.

7.3.7.4 Transformation products formed in the laccase-ABTS-IPN system

During IPN oxidation by laccase in presence of ABTS, seven transformation products were detected (Fig. 7.14 D, Table 7.1). Three of them (14, 17 and 18) were ABTS degradation products, also observed during ABTS oxidation without pollutant, and (at very low concentrations for 17 and 18) with SMX. Product 14 was the same ABTS fragment as detected in the ABTS-SMX system and product 17 was probably also an ABTS fragment (Fig. 7.16). Product 18 had a higher mass (546 g mol^{-1}) than ABTS (514 g mol^{-1}), but could not be identified. Products 14 and 17 had UV/Vis absorption spectra with maxima at 200, 258, 286, 294 and 218, 258, 284, 292 nm respectively (Table S 7.1, SI). Similar adsorption spectra (220, 254, 284, 292 nm) were found for a decomposition product of $ABTS^{\bullet+}$ in neutral-alkaline solutions ($ABTS^{\bullet+}$ is unstable under alkaline conditions) (Majcherczyk et al., 1999), suggesting that products 14 and 17 were related to $ABTS^{\bullet+}$ degradation. Product 18 had an absorption spectrum with maxima at 222, 264, 292, 300 nm, which corresponds to the adsorption spectrum of a (not clearly identified) decomposition product of $ABTS^{2+}$ observed in other studies (Majcherczyk et al., 1999). As proposed by Majcherczyk et al. (1999) and as discussed before, this result may suggest that $ABTS^{2+}$ was involved in the laccase-mediated reaction. Apart from product 17, which appeared rapidly (within 3 h) and then disappeared slowly, both products 14 and 18 appeared gradually at a rate proportional to ABTS (and $ABTS^{\bullet+}$) degradation (Fig. 7.15).

The four other detected transformation products (19-22) had all a mass higher than IPN, suggesting the potential formation of coupling products. None of them could be identified but, according to its mass and retention time, product 19 could possibly be a hydroxylated IPN (Fig. 7.16).

7.3.7.5 Influence of pH on the type of transformation products

Several transformation products detected by UPLC-MS were related (by retention time comparisons) to transformation products observed by HPLC-UV/vis (chromatograms presented in Fig. S 7.1 to Fig. S 7.3, SI). Their relative abundance after complete reaction of the mediators with SMX could thus be determined at various pH values (Fig. 7.17).

For SMX in the laccase-SA system (Fig. 7.17 A), the abundance of the dimeric SA (product 12) observed at low pH (pH 3) was a factor two higher compared to pH 4-7, and almost no dimeric SA was detected at pH 8. In contrast, the coupling product SMX-DMBQ (product 8) was much more abundant (10 times) at pH 6-8 than at pH 3. The production of DMBQ (product 2) during SA oxidation was the highest at pH 6-7.

Similar observations were made in the laccase-AS system in presence of SMX (Fig. 7.17 B) where the coupling product SMX-DMBQ (8) was observed in high abundance at pH 7-8 but almost not detected at pH 3-4. The dimeric AS (product 6) was also almost absent at pH 8 but present in high abundance at pH 3-6. The production of DMBQ (product 2) during AS oxidation was highest at pH 6-7.

These results support the assumption that, at low pH, the reactive mediator radicals are rapidly formed, favouring their coupling to form dimeric SA and AS rather than reacting with SMX. Under neutral to alkaline conditions, most of the reactive SA or AS radicals (slowly produced) react directly with SMX to form the coupling product SMX-DMBQ (product 8) or decompose to DMBQ (product 2).

The pH influenced also the relative product distribution in the laccase-ABTS system in the presence of SMX (Fig. 7.17 C). Product 17, an ABTS fragment, was more abundant at low pH while the ABTS fragment 14 was present at higher levels at a higher pH. This suggests that ABTS decomposes into different products depending on the pH. The abundance of the SMX fragment 16 was correlated with the percentage SMX removal observed at the different pH values.

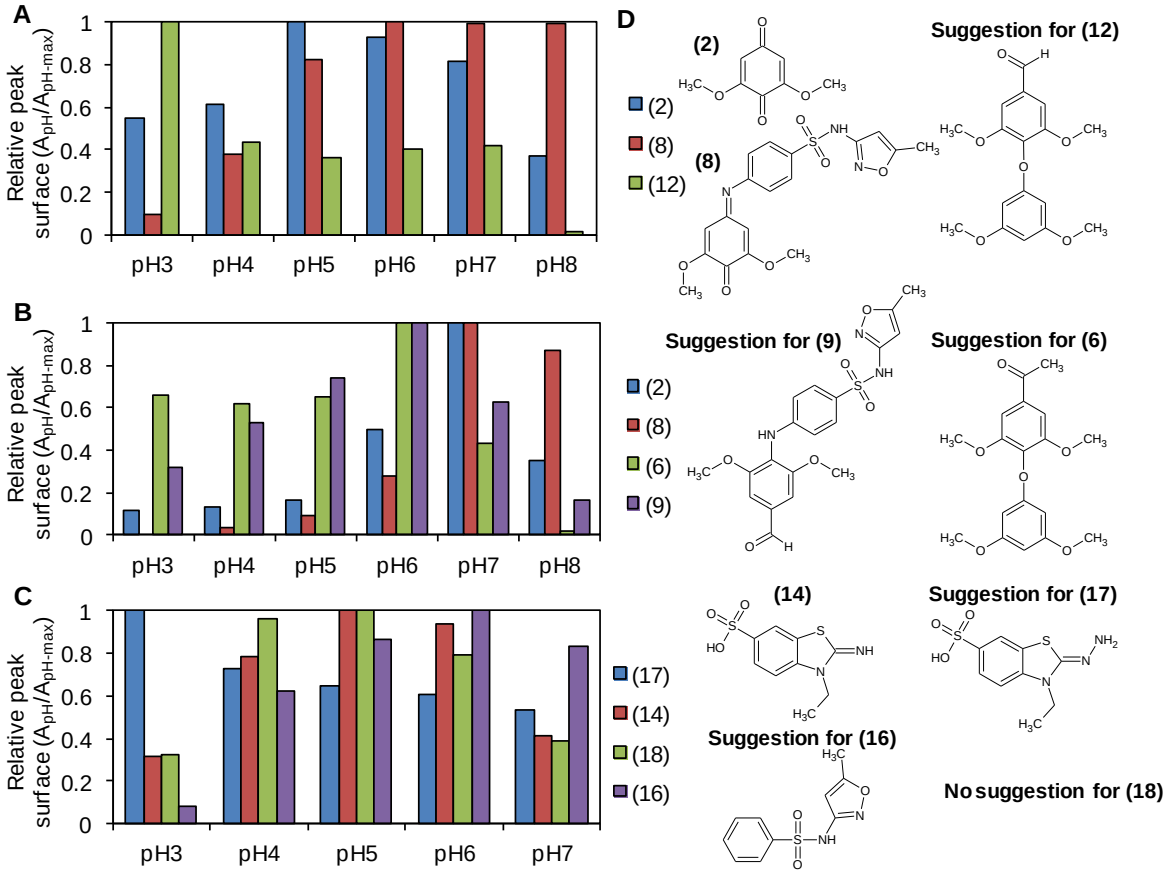


Fig. 7.17 Relative abundance of the main transformation products detected by HPLC-DAD after the complete reaction of sulfamethoxazole (SMX) in presence of laccase and mediators at various pH (peak surface of the compound relative to the maximum surface observed for the same compound at different pH). **(A)** In presence of syringaldehyde. **(B)** In presence of acetosyringone. **(C)** In presence of ABTS. **(D)** Structural propositions for the various products. Numbers in brackets refer to the ID of the transformation products detected by UPLC-MS (Fig. 7.14). For product 9, maximum peak area observed during the reaction (not stable, decrease with the time).

7.3.8 Toxicity of transformation products

The evolution of the toxicity of the transformation products formed from IPN and SMX in laccase-mediated systems was assessed by ecotoxicity tests with green algae. After the treatment, both pollutants were not detected in the solutions (> 99% abatement). The toxicity of the solutions containing mixtures of transformation products (section 7.3.7) was compared to solutions containing the parent compounds, or the mediator oxidized by laccase in the absence of the pollutant (Fig. 7.18).

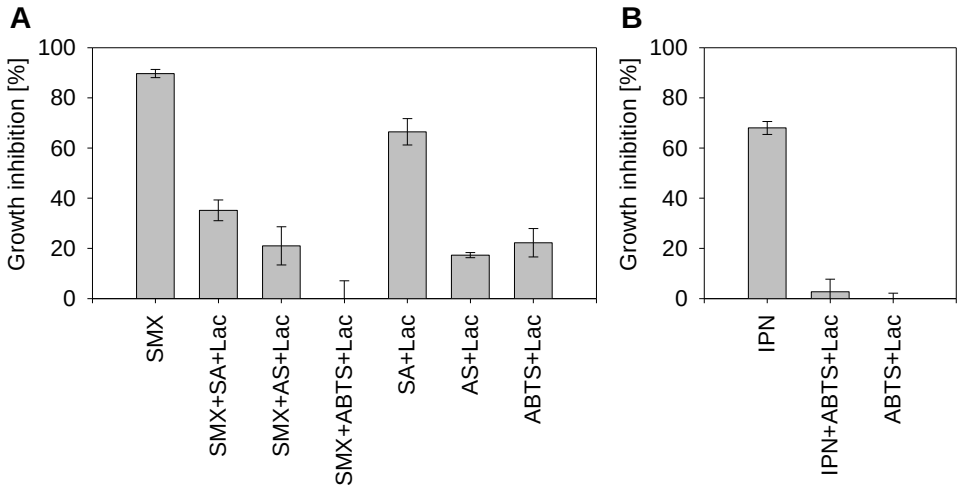


Fig. 7.18 Growth inhibition of the green algae *Pseudokirchneriella subcapitata*. After 72 h exposure to **(A)** sulfamethoxazole (SMX) (7.5 µM); SMX treated with a laccase (Lac)-mediator (SA, AS or ABTS) system (mixture of transformation products, TPs); or laccase with mediators in absence of SMX (mediators at 25 µM. SA: syringaldehyde, AS: acetosyringone); and **(B)** isoproturon (IPN) (0.5 µM); IPN treated with a laccase-mediator (ABTS) system (mixture of TPs); or laccase with ABTS (at 2.5 µM) in absence of IPN. Average and standard deviation of triplicates.

SMX at 7.5 µM (1.9 mg l^{-1}) inhibited 90% of algae growth compared to the control. Similar SMX toxicity to *Pseudokirchneriella subcapitata* were observed in other studies, with EC_{50} (concentration inhibiting 50% of the growth) reported in the range of 0.15 – 0.5 mg l^{-1} (García-Galán et al., 2009) or at 1.9 mg l^{-1} (Yang et al., 2008). Laccase-mediated treatments reduced this toxicity by 61% in presence of SA, 77% in presence of AS and 100% in presence of ABTS (Fig. 7.18 A), demonstrating the much lower algal toxicity of the mixture of transformation products compare to the non-treated SMX.

To evaluate if the residual toxicity observed in presence of SA and AS was due to SMX transformation products or to mediator transformation products, the same bioassays were conducted in absence of SMX. High algae growth inhibition (66%) was observed in the solution with SA (25 µM), and lower but significant inhibition was observed with laccase-induced oxidation of AS and ABTS (17 and 22%, respectively). Especially with SA, the residual toxicity was higher in absence than in presence of SMX transformation products. The product 2 (DMBQ) was produced in lower quantities in presence of SMX (Fig. S 7.2, SI) due to the reaction of the SA phenoxy radical with the pollutant, and also present in lower concentrations (2-8 times) in the oxidized AS solution (which was less toxic). This suggests that DMBQ might be responsible for a part of the toxicity observed with SA and AS. The toxicity of the oxidized SA was also reported in other studies (20% bacterial inhibition at 0.25 µM) (Nguyen et al., 2014b). Mediator transformation products were thus probably the reason for

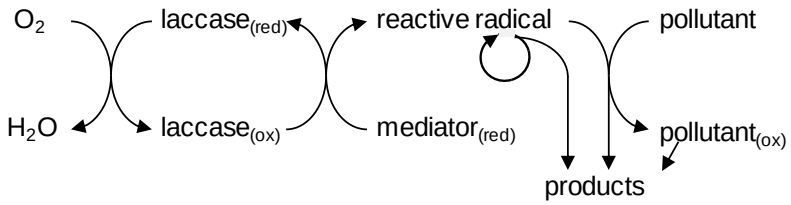
the residual toxicity observed after complete SMX oxidation. As the pH influences the relative abundance of each product (section 7.3.7.5), different toxicity may thus also be observed at various pH values, with possibly lower toxicity at alkaline pH (lower concentrations of DMBQ).

IPN at 0.5 µM (103 µg l^{-1}) inhibited 68% (± 3%) of algae growth (Fig. 7.18 B), which is similar to what was reported by Pavlić et al. (2006) (70% inhibition at 100 µg l^{-1}). After the treatment in the laccase-ABTS system, more than 95% of this toxicity disappeared, showing the very low toxicity of the transformation product mixture compared to IPN.

These results show that laccase-mediated reactions can significantly reduce toxicity of SMX and IPN to algae (among the most sensitive organisms for these pollutants) despite the formation of several transformation products. The synthetic mediator ABTS was most efficient with almost complete IPN and SMX toxicity removal, while residual toxicity was still observed with the natural mediators AS and SA. Laccase-mediated systems appear thus to be an interesting way to decontaminate effluents which are toxic to sensitive aquatic organisms. However, precautions must be taken when treating effluents with low toxicity because oxidized mediators (especially SA) may generate significant residual toxicity at low concentrations (< 25 µM).

7.3.9 Mechanistic aspects of laccase-mediated reactions

The ideal scheme of laccase-mediated reactions where the mediator is continuously recycled during the redox process (Scheme 1) does not correspond to the observations in this study. Based on our results, an alternative laccase-mediated oxidation model is proposed (Scheme 2).



Scheme 2 Proposition of a laccase-mediator reaction model

As illustrated in Scheme 2 and described in Eqs. 7.1-7.4, our results suggest that the mediator (*med*) is oxidized by laccase (*lac*) to reactive radicals ($R^{\bullet}$) (Eq. 7.1) that will either react by a radical-radical coupling reaction, producing products P_1 (such as dimeric AS and SA) (Eq. 7.2), further react to more stable products (P_2) (e.g. 2,6 DMBQ for AS and SA) (Eq. 7.3), or react with other compounds present in the solution, such as the pollutants (*poll*), at a stoichiometric ratio *a* (number of moles of radical needed to oxidize one mole of pollutant) to produce products P_3 (e.g., oxidation products of SMX or coupling products SMX-DMBQ) (Eq. 7.4). k_1 to k_4 are the rate constants for reactions 7.1-7.4, respectively. This mechanistic description is coherent with the nature of the transformation products detected (section 7.3.7), as illustrated in Fig. 7.19 for SA and SMX.

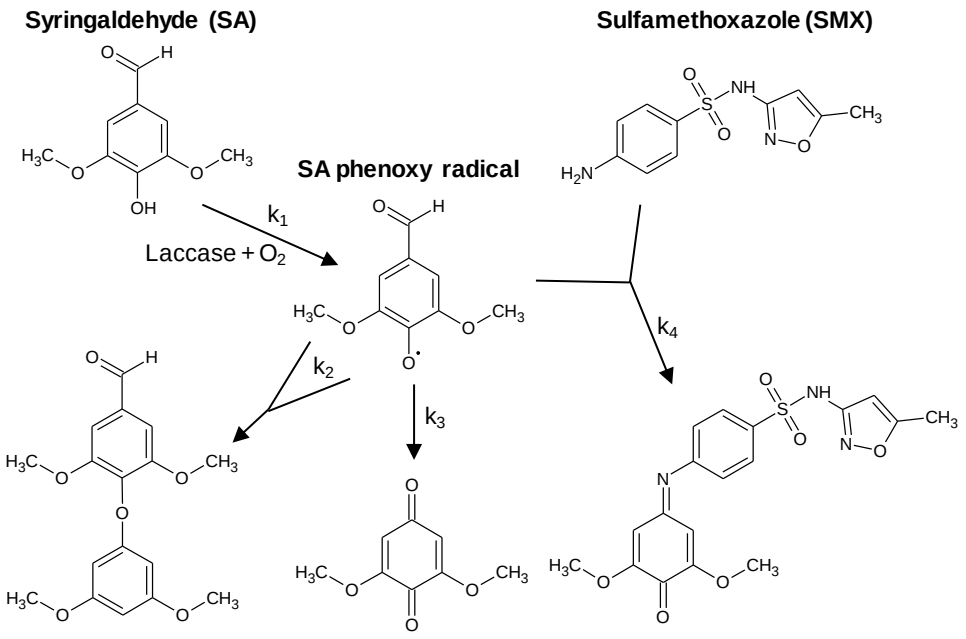


Fig. 7.19 Laccase-mediated reactions in solution containing syringaldehyde and sulfamethoxazole, based on the main transformation products observed by UPLC-MS. The structure of the dimeric SA is only hypothesized (not confirmed). k_1 to k_4: rate constants used in the kinetic model.

$$lac + med + \tfrac{1}{4}\mathrm{O_2} \xrightarrow{k_1} lac + R^{\cdot} + \tfrac{1}{2}\mathrm{H_2O} \qquad k_1 \qquad (7.1)$$

$$R^{\cdot} + R^{\cdot} \xrightarrow{k_2} P_1 \qquad k_2 \qquad (7.2)$$

$$R^{\cdot} \xrightarrow{k_3} P_2 \qquad k_3 \qquad (7.3)$$

$$R^{\cdot} + \tfrac{1}{a}\, poll \xrightarrow{k_4} P_3 \qquad k_4 \qquad (7.4)$$

Based on this reaction model, a kinetic model was established (Eqs. 7.5-7.9), assuming a constant laccase recycling (catalytic cycle with no loss of activity during the reaction) (Eq. 7.5). As the oxidation rate of the mediator by laccase is influenced by the mediator concentration (rate increasing with the concentration until reaching a saturation with a plateau) (Fig. S 7.4, SI), a Michaelis-Menten type kinetics was used to model the mediator removal rate, with K_m, the specific half-saturation constant of the laccase for a mediator (Eq. 7.6).

$$\frac{\mathrm{d}[lac]}{\mathrm{d}t} = 0 \qquad (7.5)$$

$$\frac{\mathrm{d}[med]}{\mathrm{d}t} = -k_1[lac][\mathrm{O_2}]\frac{[med]}{K_m + [med]} \qquad (7.6)$$

$$\frac{\mathrm{d}[\mathrm{O_2}]}{\mathrm{d}t} = -\tfrac{1}{4}k_1[lac][\mathrm{O_2}]\frac{[med]}{K_m + [med]} \qquad (7.7)$$

$$\frac{\mathrm{d}[R^{\cdot}]}{\mathrm{d}t} = k_1[lac][\mathrm{O_2}]\frac{[med]}{K_m + [med]} - k_2[R^{\cdot}]^2 - k_3[R^{\cdot}] - k_4[R^{\cdot}][poll] \qquad (7.8)$$

$$\frac{\mathrm{d}[poll]}{\mathrm{d}t} = -\tfrac{1}{a}\,k_4[R^{\cdot}][poll]. \qquad (7.9)$$

This model was used to simulate the behaviour of the laccase-mediated reactions under various conditions by assigning arbitrary values (based on rough fitting of the data) to the six undetermined variables k_1, k_2, k_3, k_4, K_m and a. The five differential equations were solved numerically with the ode45 solver (variable step Runge-Kutta method) within MATLAB. The values of the parameters used for the simulations shown in Fig. 7.20 and Fig. 7.21 are presented in Table 7.2. The results of selected simulations are presented in Fig. 7.20.

Table 7.2 Modelling parameters (arbitrary values) used to simulate the reactions presented in Fig. 7.20 and Fig. 7.21.

Parameters		**Fig. 7.20 A**	**Fig. 7.20 B**	**Fig. 7.20 C**	**Fig. 7.20 D**	**Fig. 7.21**
k_1	[μM^{-1} h^{-1}]	0.1 or 0.001	0.1 or 0.005	0 to 1	0.1	0.1
k_2	[μM^{-1} h^{-1}]	0.005	0.0005	0.005	0.0005	0.005
k_3	[h^{-1}]	0.005	0.0005	0.005	0.0005	0.005
k_4	[μM^{-1} h^{-1}]	0.05	0.005	0.001	0.0015	0.05
K_m	[µM]	10	10	10	10	10
a	[-]	1.7	2.2	1	2.2	1.7
Initial conditions						
O_2	[µM]	250	250	250	250	250
Laccase	[µM]	200	40-200	200	200	200
Pollutant	[µM]	100	100	110	2-200	0.1-150
Mediator	[µM]	10-500	100-500	100	10-1000	0.5-750

The significant effect of the mediator concentrations on the laccase-induced oxidation rates of SMX under acidic conditions and the absence of such an effect under neutral conditions (Fig. 7.8 and Fig. 7.9) were correctly reproduced by the model by varying the rate constant k_1 (Eq. 7.1) (Fig. 7.20 A).

As discussed before, under acidic conditions, increasing the mediator concentration increased the pollutant oxidation rate, reaching progressively a plateau (a maximum) at high mediator concentrations (Fig. 7.8 C). This saturation effect was attributed to significant cross-reactions between the radicals produced in high quantities (reactions of Eq. 7.2 favoured over Eq. 7.4), and were correctly reproduced by the model (Fig. 7.20 D). This saturation effect was not observed with constant mediator/pollutant ratios (Fig. 7.8 C), a phenomenon confirmed with the model (Fig. 7.20 D). Indeed, reaction rates of Eq. 7.2 and Eq. 7.4 were enhanced in the same way with the parallel increase in both mediator and pollutant concentrations.

As discussed before, under acidic conditions a strong increase in laccase activity (up to a factor 5) did not significantly increase the pollutant oxidation rates, while an increase in the mediator concentration strongly enhanced the reaction (Fig. 7.7), suggesting that the oxidation of the mediator by laccase was not the limiting step. This phenomenon was well reproduced with the model by choosing a relatively high (non-limiting) reaction rate constant k_1 (Fig. 7.20 B). At higher pH (lower k_1), when the mediator oxidation by laccase becomes rate-limiting, the model shows that a higher laccase activity is necessary to increase the pollutant oxidation rate (Fig. 7.20 B).

The increase in the required mediator/pollutant ratio observed at low pH (Fig. 7.6 A) was reproduced with the model by increasing the reaction rate constant k_1 (as observed at low pH) (Fig. 7.20 C). Indeed, at low k_1 values ($-\log(k_1) > 5$), the reaction is limited by the oxidation of the mediator by

laccase (similar to the observations at neutral-alkaline pH) and the ratio mediator/pollutant is close to the minimum set for this simulation (ratio of 1). As k_1 increases (-log(k_1) < 5), corresponding to a decrease in pH, the reaction becomes more and more limited by the oxidation of the pollutant by the radical (k_4). The radicals, rapidly produced, tend to accumulate in the solution and to react with each other or be further transformed (k_2 and k_3) rather than reacting with the pollutant. This leads to an increase in the mediator/pollutant ratio.

The proposed model was able to qualitatively reproduce all the different scenarios observed in the experiments, confirming that the mechanistic description proposed is adequate to describe laccase-mediated reactions.

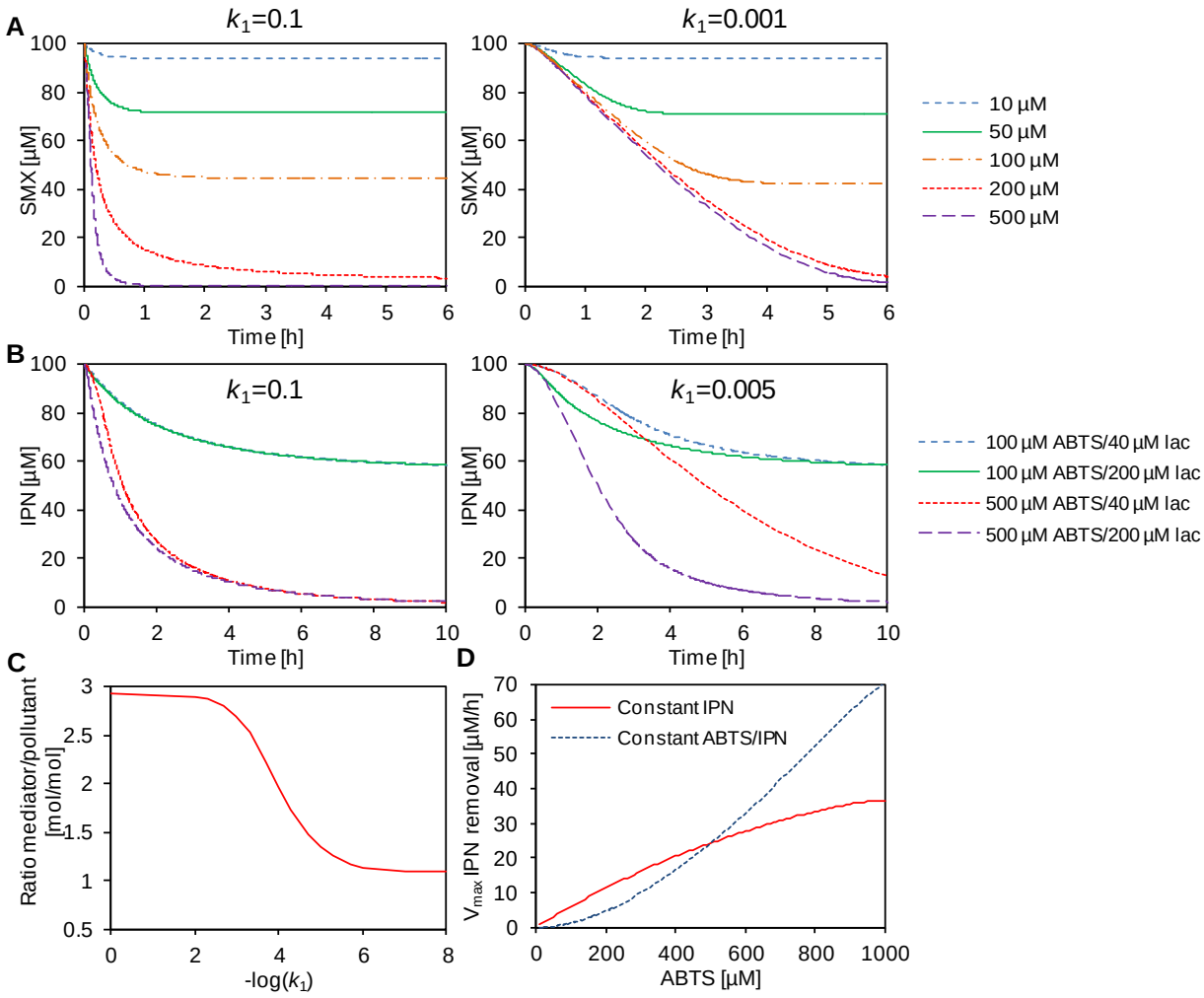


Fig. 7.20 Results of the kinetic model. (A) Influence of the mediator concentration (from 10 to 500 µM) on the pollutant (SMX) oxidation kinetics, with either the pollutant oxidation as the limiting step (k_1 = 0.1 μM^{-1} h^{-1}, similar to what is observed at pH 5-6, Fig. A, left) or mediator oxidation limiting step (k_1 = 0.001 μM^{-1} h^{-1}, similar to what is observed at pH 7, Fig. A, right). **(B)** Influence of ABTS and laccase (lac) concentrations on the pollutant (IPN) oxidation kinetics, with either the pollutant oxidation as the rate limiting step (k_1 = 0.1 μM^{-1} h^{-1}, similar to what is observed at pH 5-6, Fig. B, left) or mediator oxidation limiting step (k_1 = 0.005 μM^{-1} h^{-1}, similar to what is observed at pH 7, Fig. B, right). **(C)** Stoichiometric ratio between the mediator consumed per mole of pollutant oxidized, as a function of the reaction rate constant k_1 (in μM^{-1} h^{-1}). **(D)** Initial maximal pollutant (IPN) oxidation rates (V_{max} in µM h^{-1}) as a function of the mediator concentration (ABTS), with either constant IPN concentration (100 µM) or constant ABTS/IPN ratio (5 mol mol^{-1}). The modelling parameters are presented in Table 7.2.

7.3.10 Practical implications

This study highlights several points regarding the potential application of laccase-mediated systems for the treatment of micropollutants in contaminated waters. In particular, it is possible to assess the feasibility of treating very low pollutant concentrations in wastewater and how to enhance oxidation rates.

7.3.10.1 Treatment of very low pollutant concentrations

The model developed allowed assessing the oxidation kinetics for very low concentrations of pollutants. The time required to remove 90% of a (fictive) pollutant was modelled as a function of the pollutant concentration using two scenarios: (i) constant ratio mediator/pollutant (ratio of five), and (ii) constant mediator concentration (at 500 µM) (Fig. 7.21).

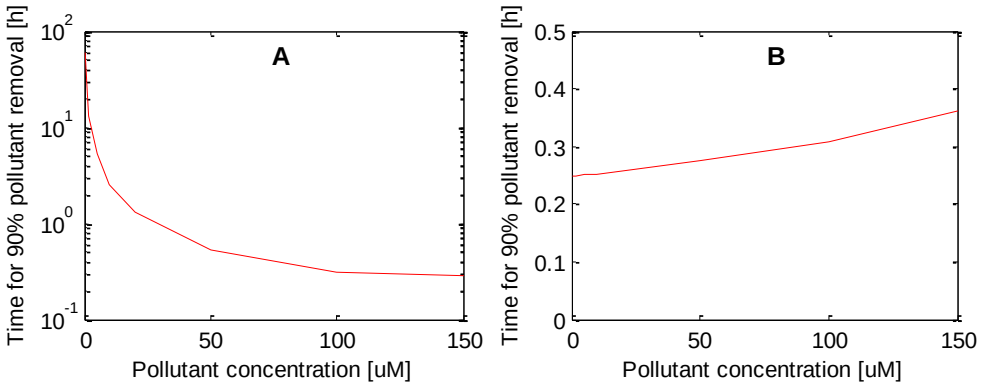


Fig. 7.21 Modelling of the time needed to remove 90% of the pollutant as a function of the pollutant concentration. (A) With a constant mediator/pollutant ratio of five (log scale for the y-axis). **(B)** With a constant mediator concentration of 500 µM. The modelling parameters are presented in Table 7.2.

In the first scenario, when the pollutant, and therefore the mediator, were present at high concentrations (> 100 and 500 µM, respectively), the removal time was independent of the pollutant concentration (similar to the experimental results, Fig. 7.7 B). But, as shown in Fig. 7.21 A, this was valid only for pollutant concentrations > 100 µM, corresponding to mediator concentrations > 50 times the affinity constant of the enzyme for the mediator (Michaelis constant K_m, chosen at 10 µM). At lower pollutant (and therefore mediator) concentrations, the time required to remove 90% of the pollutant was predicted to increase by a factor around 10 every time the mediator concentration was divided by 10 (18 min at 500 µM up to 60 h at 2.5 µM). The relatively low affinity of the enzyme for the mediator implies that adding mediator at concentrations lower than the K_m of the enzyme (which is for instance around 20 µM for ABTS with this laccase, Fig. S 7.4, SI) will require excessively long reaction times (> 50 times the minimum).

When the mediator concentration was kept constant (at a value far above the K_m, scenario 2), only a limited effect of the pollutant concentration on the removal time was predicted (Fig. 7.21 B), showing the possibility, with high mediator doses, to treat pollutants rapidly even at very low pollutant concentrations.

Treatment of low pollutant concentrations (e.g. 1 µg l^{-1} or 0.005 µM) will thus require very high mediator/pollutant ratios to avoid too long reaction times (e.g., a ratio above 4000 to keep a mediator

concentration above 20 µM), which may lead to increase the toxicity of the water due to the release of high quantities of mediator transformation products.

7.3.10.2 Strategy to improve oxidation rates

The results presented above show that, depending on the pH, different strategies have to be applied to optimize the rate of pollutant degradation in laccase-mediated processes. If the reaction is limited by the oxidation of a pollutant by the radical (pH < 6), increasing the laccase activity has almost no effect and the best strategy is to increase the mediator concentration. However, this increase should stay below a certain threshold (mediator/pollutant ratio < 10) because at higher mediator concentrations reaction rates will reach a plateau due to high losses of the radicals formed from the laccase-mediator reaction (self-reactions). If the reaction is limited by the mediator oxidation by laccase (pH > 7), increasing the mediator concentration will not affect the oxidation rate of the target compound, as long as the mediator concentration is 50 times the K_m value of the enzyme. The strategy in this case is thus to increase the laccase activity. When both reactions are limiting (pH 6-7), an increase in both laccase and mediator concentrations should be considered.

7.3.10.3 Limitations of laccase-mediator systems for municipal wastewater

Despite fast oxidation of SMX and IPN in laccase-mediated systems and their related toxicity removal, addition of laccase and mediators in real treatment systems to increase micropollutant removal faces many limitations in terms of its feasibility: (i) The high concentration of mediator required (> 10 µM) due to mediator consumption during the reaction, the relatively low affinity of laccase for the mediator and the possible loss of radicals by reaction with other matrix components. (ii) The potential formation of toxic transformation products due to the oxidation of the mediator. (iii) The formation of several mediator transformation products at concentrations possibly much higher than the target pollutant. Therefore, an application of LMS to treat municipal wastewater with very low micropollutant concentrations appears to be unrealistic. However, LMS may be an option for treatment of industrial wastewater that contains concentrated and toxic pollutants, such as effluents of pharmaceutical or pesticide industries. Indeed, in contrast to the biological water treatment, LMS are not subject to intoxication and may be used to reduce the toxicity of highly polluted effluents, prior to further biological treatment of the transformation products.

7.4 Conclusions

The use of laccase-mediator systems effectively transformed IPN (with ABTS) and SMX (with ABTS, AS ad SA) to less toxic transformation products, consisting mostly of coupling products. The pH had a strong influence on the oxidation kinetics (faster at low pH) and on the required mediator/pollutant ratio (higher at low pH). Indeed, the three mediators tested did not act as catalysts and were therefore consumed in the process. Our results suggest that laccase oxidizes mediators to reactive radicals, which either spontaneously degrade into more stable products, react with each other (coupling reactions between radicals) or with the pollutants. Despite the requirement of high amount of mediators, LMS appears to be a potentially promising technology to treat concentrated and toxic effluents.

Acknowledgements

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7.5 Supporting information

HPLC-DAD chromatograms

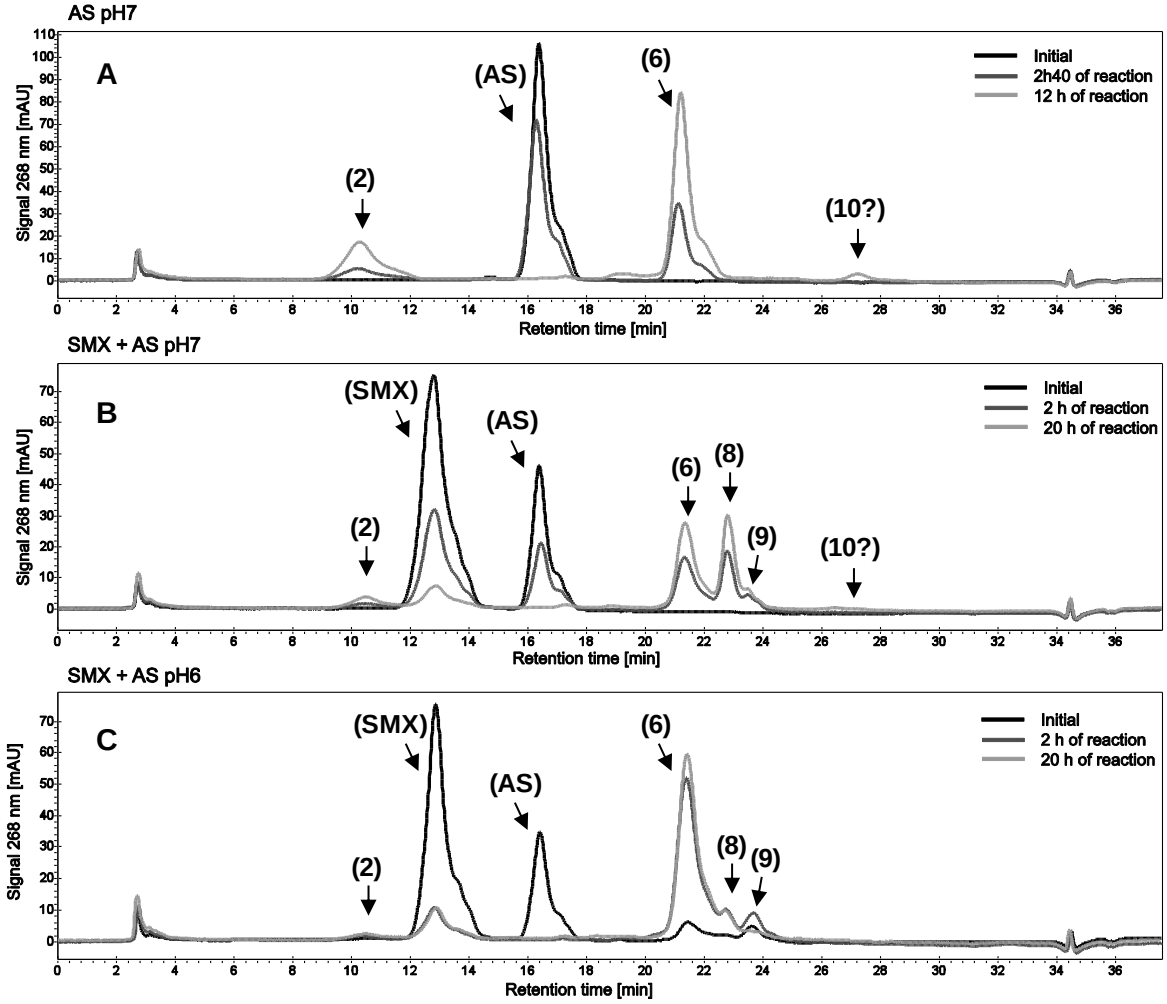

Fig. S 7.1 HPLC-DAD (268 nm) chromatograms during the oxidation of acetosyringone (AS) by laccase with or without sulfamethoxazole (SMX). (A) Oxidation of AS (500 µM) by laccase (450 U l^{-1}) at pH 7, without SMX. **(B)** Oxidation of AS (200 µM) by laccase (560 U l^{-1}) at pH 7, in presence of SMX (100 µM). **(C)** Similar conditions as in (B) but at pH 6. Numbers in brackets refer to the ID of the transformation products detected by UPLC-MS (Fig. 7.14).

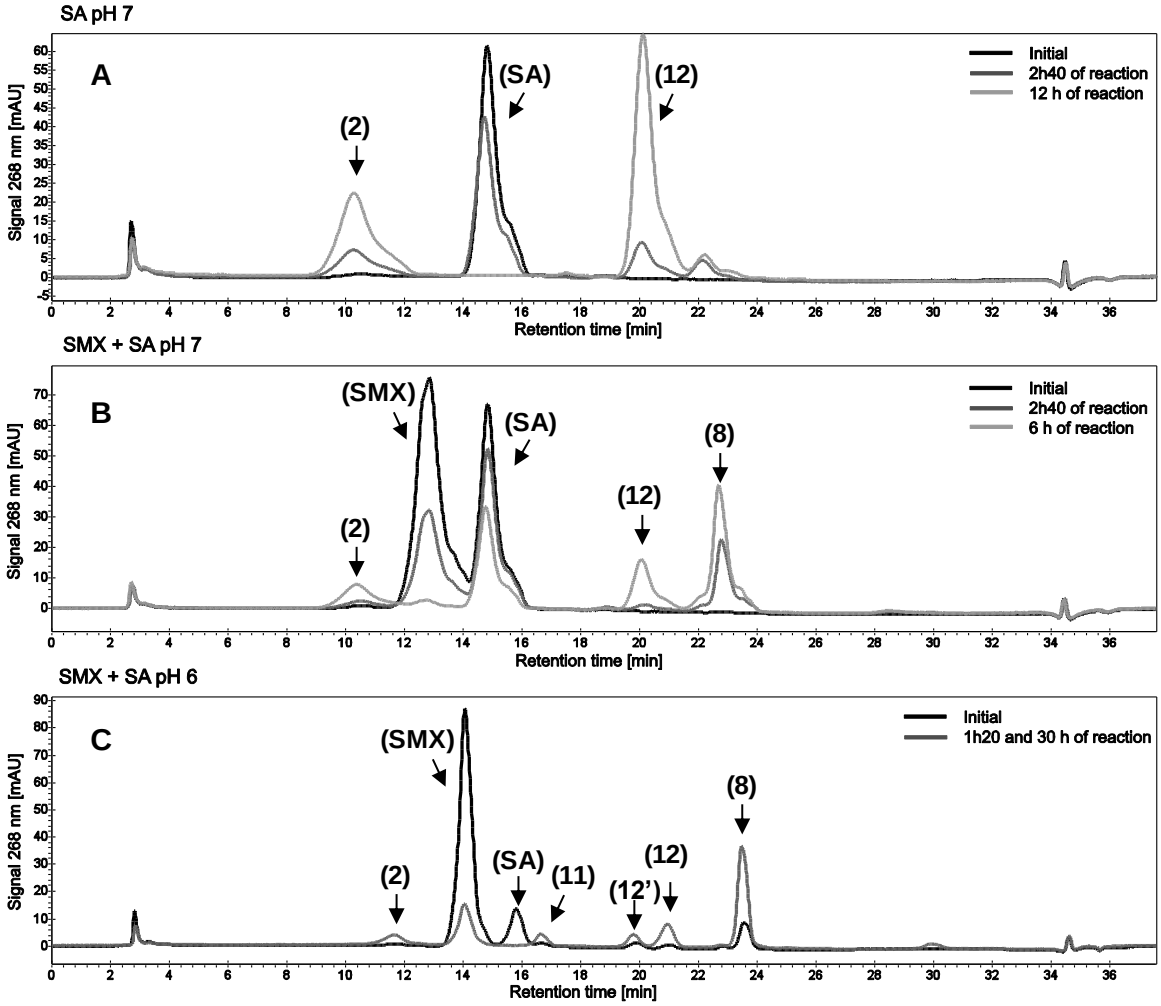

Fig. S 7.2 HPLC-DAD (268 nm) chromatograms during the oxidation of syringaldehyde (SA) by laccase in presence or absence of sulfamethoxazole (SMX). (A) Oxidation of SA (500 µM) by laccase (450 U l^{-1}) at pH 7, without SMX. **(B)** Oxidation of SA (500 µM) by laccase (455 U l^{-1}) at pH 7, in presence of SMX (100 µM). **(C)** Oxidation of SA (100 µM) by laccase (320 U l^{-1}) at pH 6, in presence of SMX (80 µM). Numbers in brackets refer to the ID of the transformation products detected by UPLC-MS (Fig. 7.14).

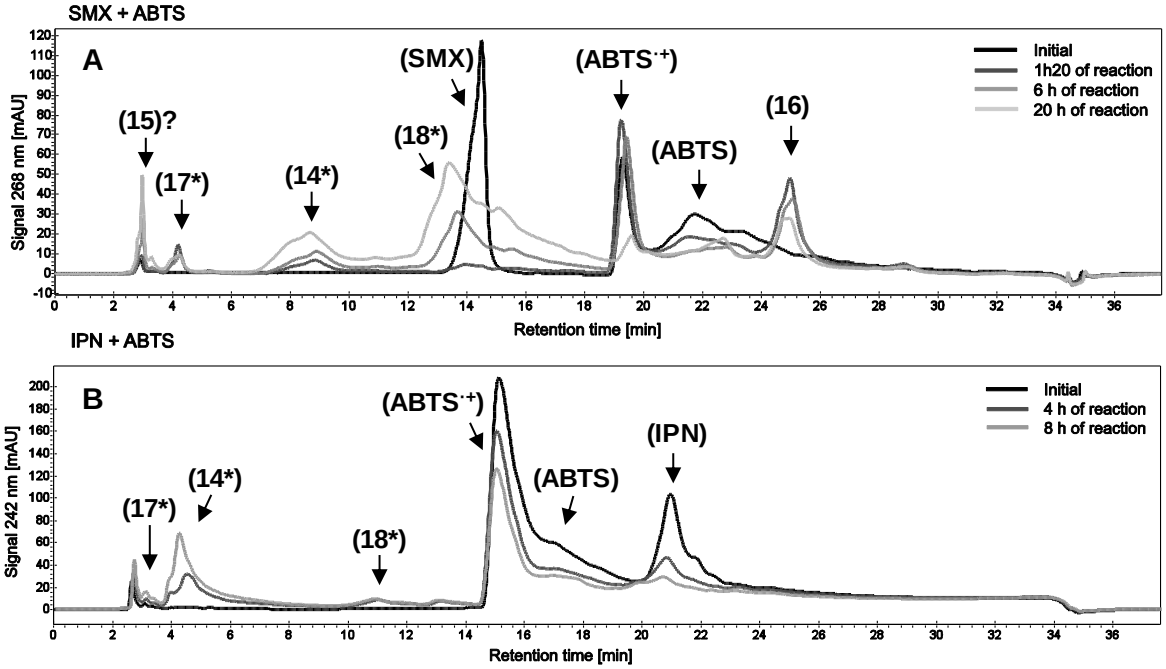

Fig. S 7.3 HPLC-DAD chromatograms (242-268 nm) during the oxidation of ABTS (500 µM) by laccase in presence of **(A)** sulfamethoxazole (SMX, 100 µM, pH 6, 450 U l^{-1} laccase, signal at 268 nm), and in presence of **(B)** isoproturon (IPN, 100 µM, pH 5, 503 U l^{-1} laccase, signal at 242 nm). Numbers in brackets refer to the ID of the transformation products detected by UPLC-MS (Fig. 7.14). Different HPLC methods were used in A and B (peaks not at the same retention time). Transformation products with (*): correspondence between the compounds appearing on the chromatograms by UPLC-MS and HPLC-DAD not completely confirmed.

Characteristics of parent compounds and transformation products

Table S 7.1 Characteristics of the parent compounds and the detected transformation products (TPs)

ID	Retention time [min]	Molar mass [g/mol]	Type of products[a]	Structures proposition[b]	Log K_{ow} of proposed structures[c] [-]	Maxima UV/Vis absorbance[d] [nm]
Parent compounds						
(AS)	7.11	196	Acetosyringone	(AS)	1.23	**218, 302**
(SA)	6.6	182	Syringaldehyde	(SA)	0.86	**218, 308**
(ABTS)	6.44 to 7.69	514	ABTS	(ABTS)	1.99	**224, 344**, oxidized: **414, 648**
(SMX)	6.19	253	Sulfamethoxazole	(SMX)	0.43	**200, 268**
(IPN)	10.62	206	Isoproturon	(IPN)	2.32	**200, 242**
Transformation products AS+SMX						
(1)	4.58	180	AS TP	-		**208, 390-400**
(2)	5.05	168	2,6-Dimethoxy-1,4-benzoquinone (DMBQ)	(2)	0.28	200, **290**
(3)	5.85	182	AS TP	(V)	-0.36	
(4)	7.91	248	-	-		
(5)	8.11	447	*Coupling AS-SMX*	(III)	3.23	
(6)	8.77	332	Dimeric AS TP	(I)	0.04/1.97	**200, 260-270**, 340-350, 500
(7)	9.1	415	*Coupling AS-SMX*	-		
(8)	9.37	403	Coupling SMX-DMBQ	(8)	2.28	**200**, **314**, 405
(9)	9.56	417	*Coupling AS-SMX*	(IV)	3.14	**200, 306-310**
(10*)	10.06	445	*Coupling AS-SMX*	-		200, 218
Transformation products SA+SMX						
(2)	5.05	168	2,6-Dimethoxy-1,4-benzoquinone (DMBQ)	(2)	0.28	200, **290**
(11)	6.93	281	-	-		**200, 262**, 502
(12)	8.38	318	Dimeric SA TP	(II)	-0.37/1.42	**200, 280**, 360, 560
(8)	9.37	403	Coupling SMX-DMBQ	(8)	2.28	**200**, **314**, 405
(13)	9.66	348	-	-		
Transformation products ABTS+SMX						
(14*)	2.01	258	ABTS TP	(14)	0.08	**200, 258**, 286, 294
(15*)	2.48	98	*SMX fragment*	(VI)	-0.74	**200, 265**
(16)	8.26	238	*SMX fragment*	(VII)	1.91	**200**, 220, 304, **410-420**
Transformation products ABTS+IPN						
(17*)	1.69	273	ABTS TP	(VIII)	0.4	**218, 258**, 284, 292
(14*)	1.99	258	ABTS TP	(14)	0.08	**200, 258**, 286, 294
(18*)	4.72	546	ABTS TP	-		200, **222, 264**, **292**, 300
(19)	7.23	222	*Hydroxy-isoproturon*	(IX)	1.85	
(20)	8.56	445	*Coupling IPN+fragment ABTS*	-		
(21)	8.9	447	*Coupling IPN+fragment ABTS*	-		
(22)	11.61	232	-	-		

[a] Transformation products may come from the mediator degradation by laccase (also observed without pollutant), or by reaction with the pollutant. *In italics*: suggestion based on the mass of the by-product. (-): no suggestion

[b] Refers to the structures proposed in Fig. 7.16, based on the mass and the relation log Kow/retention time of the proposed molecule. (-): no suggestion

[c] Calculated for unionisated species with the software ACD/ChemSketch

[d] Determined by HPLC-DAD. In bold: main maxima. Transformation products with (*): correspondence between the compounds detected by UPLC-MS and HPLC-DAD not certain

Determination of the Michaelis-Menten constant K_m

The half-saturation constant K_m for the oxidation of ABTS by commercial laccase from *Trametes versicolor* was determined at pH 4.5 at various ABTS concentrations (1-1000 µM) using a Lineweaver-Burk plot. As shown in Fig. S 7.4, ABTS oxidation followed Michaelis-Menten kinetics, with a K_m value of 19 µM. Similar values (13-25 µM) were reported in other studies for other *Trametes versicolor* laccases (Han et al., 2005).

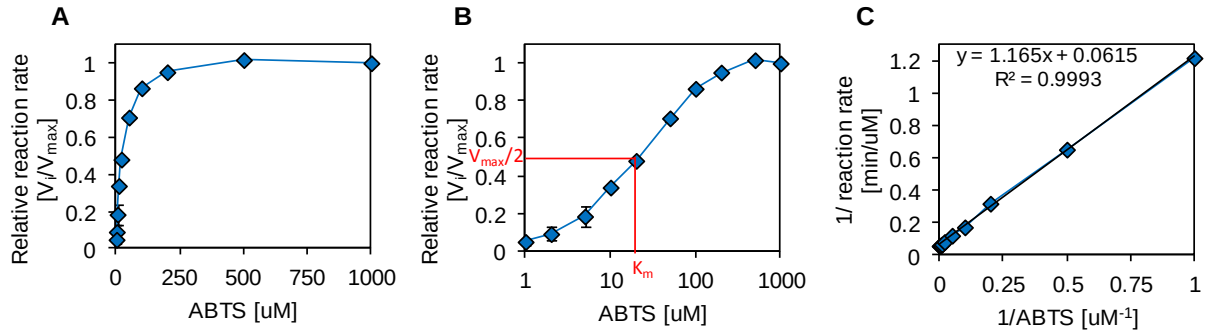

Fig. S 7.4 Influence of the initial ABTS concentration on the initial ABTS oxidation rate (V_i) by laccase. (A) Linear x-axis scale. **(B)** Log x-axis scale. **(C)** Lineweaver-Burk plot of $1/V_i$ as a function of 1/ABTS (linearization of the Michaelis-Menten equation: $(1/V_i) = (K_m/ V_{max})(1/[ABTS]) + (1 / V_{max})$). V_{max}: maximum rate achieved at saturating substrate concentrations. K_m: substrate concentration at which the reaction rate is half of V_{max}. Conditions: pH 4.5, 25°C, 10 mg l^{-1} comercial laccase from *Trametes versicolor*. The K_m value for ABTS with the selected laccase was found to be 19 µM.

Chapter 8 Bacterial *versus* fungal laccase: Potential for micropollutant degradation

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8.1 Introduction

In order to overcome the cost associated with the large amount of free laccase required in real applications (due to losses during the treatment), two strategies have been envisaged, i.e., (i) immobilization of the enzymes on solid supports in order to reuse them several times (Fernández-Fernández et al., 2012) or (ii) production of the enzyme during wastewater treatment using laccase-producing microorganisms and cheap substrates (e.g., agriculture or forestry waste) (Libra et al., 2003). The latter option avoids expensive immobilization processes while it could further improve the degradation of micropollutants along with other oxidative enzymes produced by these organisms, such as peroxidases or oxygenases. It would, however, require growing and maintaining the laccase-producing organisms in the wastewater treatment plants (WWTPs), a process that is still little studied (Blánquez et al., 2006; Libra et al., 2003; Zhang and Geißen, 2012). While the extensively studied white-rot wood-degrading fungi such as *Trametes versicolor* are attractive candidates with their high production rates of extracellular lignolytic enzymes (Nyanhongo et al., 2007), very little is known about the potential of bacterial laccases for bioremediation applications. Wastewater treatment involving bacteria is, however, considered to be more stable, as bacteria generally tolerate a broader range of habitats and grow faster than fungi (Harms et al., 2011). Moreover, in contrast to fungal laccases, some bacterial laccases can be highly active and much more stable at high temperatures, at high pH as well as at high chloride concentrations (Bugg et al., 2011; Dwivedi et al., 2011; Reiss et al., 2011; Sharma et al., 2007).

Most bacterial laccases studied so far are located intracellularly, which is a disadvantage for micropollutant degradation (Sharma et al., 2007). However, some strains of *Streptomyces* spp. produce extracellular laccases, such as *S. psammoticus* MTCC 7334 (Niladevi et al., 2008a), *S. cyaneus* CECT 3335 (Arias et al., 2003), *S. ipomoea* CECT 3341 (Molina-Guijarro et al., 2009) or *S. griseus* NBRC 13350 (Endo et al., 2002). Moreover, laccases from *S. psammoticus* and *S. ipomoea* showed unusually high activity at the slightly alkaline pH values (7-8) found in wastewater, as well as tolerance to high NaCl (> 1 M) concentrations (Molina-Guijarro et al., 2009; Niladevi et al., 2008a). High laccase activity was also observed in the culture supernatant of *S. psammoticus* and *S. cyaneus* (Arias et al., 2003; Niladevi et al., 2009), suggesting suitability of these strains for bioremediation applications.

The goal of this study was thus to assess the potential of four laccase-producing strains of *Streptomyces* bacteria, namely *S. cyaneus* CECT 3335, *S. psammoticus* MTCC 7334, *S. ipomoea* CECT 3341, and *S. griseus* NBRC 13350, together with the white-rot fungus *T. versicolor*, to select the best candidate for future use in municipal wastewater post-treatment, e.g., in a biotrickling or sand filter. More specifically, the goals were to study: (i) their ability to produce laccase in biologically treated wastewater on cheap substrates, such as agricultural, forestry or food industry wastes, in a sufficient quantity to oxidize the pollutants in a reasonable time (< 1 d), (ii) their laccase activity at different pH and temperature in order to determine optimal conditions for wastewater treatment, (iii) the inhibition of laccase activity by compounds present in wastewater such as salts, (iv) laccase stability in the pH range potentially found in the treatment, and finally (v) the laccase substrate range and their ability to oxidize different phenolic and aniline micropollutants in the pH range found in wastewater.

8.2 Materials and methods

8.2.1.1 Chemicals, choice of micropollutants, and commercial laccase enzyme

Three micropollutants were selected as model compounds for this study because of their regular presence in municipal WWTP effluent at relatively high concentrations (average between 300-1000 ng l^{-1}) (Kase et al., 2011), their potential toxicity (Crain et al., 2007; Triebskorn et al., 2004) and because they are prone to oxidation by the laccase of *T. versicolor* (cf. Chapter 6): the anti-inflammatory drugs mefenamic acid (MFA) and diclofenac (DCF), both aniline compounds, and the plastic additive bisphenol A (BPA), a phenolic substance.

BPA, DFC sodium salt, and MFA (purity > 97%), laccase preparation from *T. versicolor* (ref. 38429, Sigma), 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), 2,6-dimethoxyphenol (DMP), syringaldazine and guaiacol were purchased from Sigma-Aldrich Chemie GmbH (Buchs, Switzerland). All other chemicals used were purchased from either Sigma-Aldrich or Fisher Scientific AG (Wohlen, Switzerland). Soy flour, spelt flour and oat bran, all from organic production, and spruce wood chips were purchased at a local supermarket (Coop, Lausanne, Switzerland). Wheat straw flour was purchased from Provimi Kilba (Cossonay, Switzerland). Dry rushes (*Juncus* genus, stem diameter: 0.2-0.4 mm), dry ash branches (*Fraxinus* genus, with bark, diameter of the branches: 0.3-0.7 mm) and dry beech sawdust (*Fagus* genus) were collected in a wetland and in the forest next to L'Isle (Switzerland). Oat bran and spruce wood chips were ground to obtain fine particles (< 1 mm). Ash branches and rushes were cut into sections of 0.5-1.0 cm, washed with tap water and oven-dried for 24 h at 60°C.

8.2.1.2 Microorganisms and inoculum preparation

Pure strains of *S. cyaneus* CECT 3335 and *S. ipomoea* CECT 3341 (from Spanish Type Culture Collection, Valencia, Spain), *S. griseus* NBRC 13350 (from NITE Biological Resource Center, Chiba, Japan) and *S. psammoticus* MTCC 7334 (from Microbial Type Culture Collection, Chandigarh, India) were cultivated in GYM *streptomyces* medium (DSMZ, medium 65 (in g l^{-1}): glucose – 4, yeast extract – 4, malt extract – 10, pH 7.2) at 30°C, 140 rpm during 4 d. Cell pellets were collected by centrifugation, washed 3 times with phosphate-buffered saline (PBS (in g l^{-1}): NaCl – 8, KCl – 0.2,

Na_2HPO_4 – 1.44, KH_2PO_4 – 0.24, pH 7.4) and then stored as cells suspension (with typical cell density of ~7 × 10^3 CFU ml^{-1}) in PBS with 5% glycerol at -80°C to be used as inoculum. The strain *T. versicolor* ATCC 42530 (from American Type Culture Collection, Manassas, Virginia, USA) was maintained by sub-culturing it every 30 d on 20 g l^{-1} malt extract agar (15 g l^{-1}) slants (pH 4.5) at 25 °C. A mycelial suspension of *T. versicolor* was prepared by homogenizing 5-7 d grown mycelium in malt extract medium (20 g l^{-1}, pH 4.5) as described by Blánquez et al. (2004), and then stored in saline solution (NaCl – 8 g l^{-1}) at 4°C until use as inoculums (8.5 g l^{-1} dry volatile solid mycelium).

8.2.1.3 Laccase production

Production of laccase by the four *Streptomyces* strains was done in ISP9 mineral medium (Shirling and Gottlieb, 1966) composed of (in g l^{-1}): $(NH_4)_2SO_4$ – 2.64, KH_2PO_4 anhydrous – 2.38, $K_2HPO_4 \cdot 3H_2O$ – 5.65, $MgSO_4 \cdot 7H_2O$ – 0.1, with the following trace elements (in mg l^{-1}): $FeSO_4 \cdot 7H_2O$ – 1.1, $ZnSO_4 \cdot 7H_2O$ – 1.5, $CuSO_4 \cdot 5H_2O$ – 6.4 and $MnCl_2 \cdot 4H_2O$ – 7.9, pH 6.6 – 6.9. In this mineral medium, five different carbon sources were tested at 10 g l^{-1}: soy flour, oat bran, glucose, wheat straw flour and spruce sawdust. Production of laccase activity by *S. cyaneus* was also tested in a modified and optimized ISP9 mineral medium, with 6.4 times less copper (1 mg l^{-1} $CuSO_4 \cdot 5H_2O$), and with the same five different carbon sources (at 10 g l^{-1}) except glucose, which was replaced by spelt flour.

Finally, to test the ability of *S. cyaneus* and *T. versicolor* to produce laccase activity in wastewater, secondary treated wastewater was collected (grab sample) at the Lausanne (Switzerland) municipal WWTP in the effluent of a moving bed bioreactor with full nitrification. The ionic wastewater composition, measured by ion chromatography–conductivity detector (Dionex DX 500), was (in mg l^{-1}): $P\text{-}PO_4^{2-}$ – 1.0, SO_4^{2-} – 229, Cl^- – 837, $N\text{-}NO_3^-$ – 93, $N\text{-}NH_4^+$ – 0.09, Mg^{2+} – 11.5, Ca^{2+} – 83, Na^+ – 74, K^+ – 15.4. In this wastewater, five different sources of carbon were tested: soy flour (10 g l^{-1}, initial pH after substrate addition: 6.8), spelt flour (10 g l^{-1}, pH 7.1), rushes (20 g l^{-1}, pH 5.5), ash branches (100 g l^{-1}, pH 4.8) and beech sawdust (20 g l^{-1}, pH 5.9).

The liquid media and the wastewater, together with their carbon sources, were autoclaved 30 min at 121°C and then inoculated with 0.33% or 0.67% (v/v) of, respectively, *Streptomyces* and *T. versicolor* inocula. Cultures were incubated at 30°C for 23 d and shaken at 140 rpm to ensure aerobic conditions. Every 1-3 d, 1.5 ml was withdrawn in aseptic conditions from each culture flask and centrifuged at 10,000 *g* for 20 min. Cell-free culture supernatants were then used directly to determine laccase activity and pH. At the end of the incubation period, cell-free supernatants from the remaining cultures showing the highest activity were collected with the same procedure, filtered at 0.45 µm (Filtropur, Sarstedt), and stored at -20°C until they were used as extracellular crude enzyme preparations.

8.2.1.4 Concentrated *S. cyaneus* laccase preparation

For micropollutant degradation and laccase stability assays, as laccase activity in the extracellular crude enzyme preparation was not always high enough, 100 ml of cell-free culture supernatant of *S. cyaneus* (in modified ISP9 medium with soy flour), filtered at 0.45 µm, were concentrated 33 times by ultrafiltration (Vivaspin 20 centrifugation devices, PES membranes, MWCO: 30 kDa, from Startorius AG, Göttingen, Germany) to obtain 3 ml of laccase concentrated at ~2000 U l^{-1}.

8.2.1.5 Laccase activity test

Laccase activity was determined as described in Chapter 5, by measuring the oxidation of 0.5 mM ABTS in oxygen-saturated acetate buffer (0.1 M) at pH 4.5 and 25°C. Crude laccase preparation was added to the solution and the increase of absorbance at 420 nm was monitored with a temperature-controlled spectrophotometer (U-3010, Hitachi, Tokyo, Japan). One unit of activity (U) was defined by the oxidation of one µmol of ABTS per min, using the extinction coefficient ε_{420nm} of 36,000 M^{-1} cm^{-1} (Childs and Bardsley, 1975).

The laccase ability to oxidise other substrates was determined by the same procedure, monitoring the oxidation at 468 nm (ε_{468nm}: 27,500 M^{-1} cm^{-1}) (Muñoz et al., 1997), 470 nm (ε_{470nm}: 26,600 M^{-1} cm^{-1}) (Koduri and Tien, 1995) and 526 nm (ε_{526nm}: 65,000 M^{-1} cm^{-1}) (Palmieri et al., 1997) for, respectively, 2,6-dimethoxyphenol (DMP, at 0.5 mM), guaiacol (at 0.5 mM) and syringaldazine (at 0.01 mM, stock solution of 0.216 mM in methanol).

8.2.1.6 Influence of the pH on laccase activity

Laccase activity was measured at different pH values, from 2.6 to 8, in citric acid (2 – 40 mM) - dibasic sodium phosphate (8 – 130 mM) buffers, with four different substrates: ABTS (0.5 mM), DMP (0.5 mM), syringaldazine (0.01 mM) and guaiacol (0.5 mM). Aliquots of 200 µl of *S. cyaneus* crude laccase preparation (L_{Sc}), or 30 to 200 µl of *T. versicolor* commercial laccase solution (L_{Tv}, 0.1 g l^{-1}) were added to a total of 1200 µl of reaction mixture, the activity of which was measured at 25°C as described above. The pH was measured in the solution after addition of the laccase preparation. Measurements were conducted in duplicate.

8.2.1.7 Temperature influence on the activity

Laccase activity was measured at different temperatures, from 10 to 80°C, in acetate buffer (0.1 M, pH 4.5 at 25°C), with 0.5 mM ABTS. Aliquots of 30 to 200 µl of *S. cyaneus* crude laccase preparation, or 30 µl of *T. versicolor* commercial laccase solution (0.1 g l^{-1}) were added to 1200 µl of reaction mixture after which the activity was measured as described above. The temperature and pH were checked in the spectrophotometer cuvettes before and after the reaction. Measurements were conducted on 2 to 3 replicates. As the pH of the acetate buffer is influenced by the temperature, the measured activities were corrected to an equivalent activity at pH 4.5, as described below.

8.2.1.7.1 Correction of the activity to pH 4.5

During the test, the pH of the acetate buffer in the cuvettes decreased when the temperature increased, from pH 4.62 at 10°C to pH 4.05 at 70°C (Fig. 8.1 A), following a linear relation (valid between T = 2°C and 70°C, R^2: 0.993): pH = -0.0099 T (°C) + 4.715.

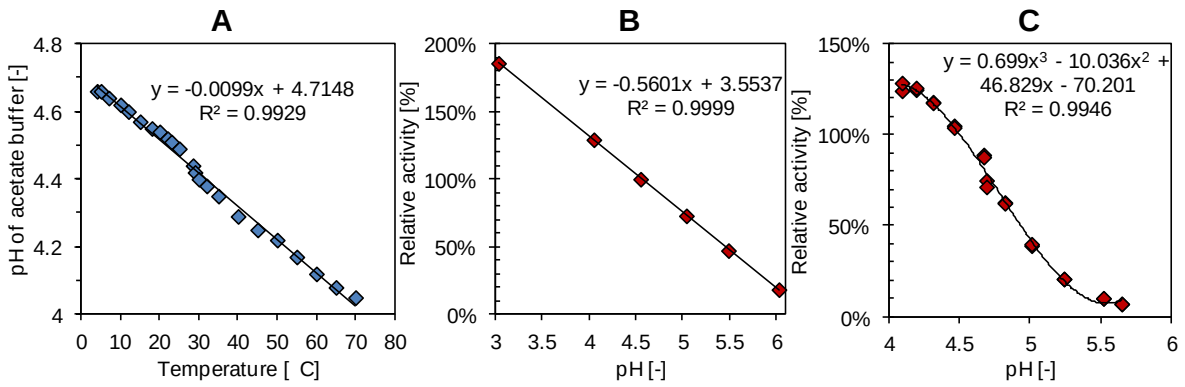

Fig. 8.1 (A) Influence of temperature on the pH of 100-mM acetate buffer (pH 4.5 at 25°C). (B) Influence of the pH on the ABTS activity of *T. versicolor* laccase. (C) Influence of the pH on the ABTS activity of *S. cyaneus laccase*. Activities are given relative to that at pH 4.5 (set at 100%).

The laccase activity with ABTS increases when the pH decreased from 5 to 4 (Fig. 8.1 B and C). Therefore, to assess the temperature effect alone without the pH effect, the measured activity values (A_{pH}) were corrected to an equivalent activity at pH 4.5 ($A_{4.5}$) with the following relation: $A_{4.5} = f_{4.5} A_{pH}$. The correction factors $f_{4.5}$, determined by regression, were, for *T. versicolor* laccase (valid from pH 3 to 6, R^2: 0.999): $f_{4.5} = -0.5601\ pH + 3.5537$, and for *S. cyaneus* laccase (valid from pH 4.1 to 5.6, R^2: 0.995): $f_{4.5} = 0.699\ pH^3 - 10.036\ pH^2 + 46.829\ pH - 70.201$.

8.2.1.8 Stability at various pH

Laccase stability was assessed in pure water, as well as in buffer solutions at various pH. Citric acid (5 – 20 mM) - dibasic sodium phosphate (10 – 40 mM) buffers were used for pH 3 to 7, and Tris – HCl buffers (50 mM) for pH 8 and 9. Concentrated *S. cyaneus* crude laccase preparation or commercial *T. versicolor* laccase were added to the buffers to reach an initial laccase activity of 130 U l^{-1} and then incubated in the dark at 25°C for 55 d. The laccase activity and the pH in the solutions were monitored regularly. Experiments were conducted in duplicate.

8.2.1.9 Inhibition by sodium chloride

The inhibitory effect of sodium chloride was assessed by measuring the laccase activity with ABTS in acetate buffer (0.1 M, pH 4.5) containing from 0 to 600 mM (0-35 g l^{-1}) of NaCl. Crude *S. cyaneus* laccase preparation or commercial *T. versicolor* laccase were added to the solution (initial laccase activity without inhibitors of 10 U l^{-1}), incubated for 30 s, before measuring the activity with the addition of ABTS (0.5 mM).

8.2.1.10 Micropollutant analysis

Determination of BPA, DFC and MFA concentrations was carried out by HPLC-DAD as described in Chapter 6.

8.2.1.11 Micropollutant oxidation assay with laccase at different pH

Micropollutant oxidation assays were performed as previously described in Chapter 6, in citrate phosphate buffer (30-40 mM) at three different pH values (5, 6 and 7) with a mixture of the three compounds at 20 mg l^{-1} each: DFC, MFA and BPA. Relatively high concentrations were tested to use a fast and simple analytical method (HPLC-DAD). Batch reactions were conducted in 2-ml glass vials containing 1 ml of oxygen-saturated reaction mixture. Reactions were initiated by adding laccase preparation to obtain an initial activity of 210-220 U l^{-1}. For *T. versicolor* laccase, a stock solution of commercial enzyme (1 g l^{-1} in pure water) was used. For *S. cyaneus* laccase, concentrated crude enzyme preparation (laccase activity of 2000 U l^{-1}) was added. The vials were incubated in the dark at 25°C under static conditions for 12 d. As shown in Chapter 6, diffusion of oxygen from the air space was sufficient to maintain a high level of dissolved oxygen during the reaction. After defined reaction times, aliquots (50 µl) were withdrawn from each vial and directly injected into the HPLC column to analyse micropollutant concentrations. Controls without laccase were performed at the three pH values to assess chemical degradation. Duplicate experiments were conducted. Laccase activity and pH were analysed at the beginning and at the end of the incubation period in each vial. The pH stayed stable during the experiments in all the vials.

8.2.1.12 Comparison of "commercial" versus "in house-produced" *Trametes versicolor* laccases

Commercial *T. versicolor* laccases (ref. 38429, from Sigma) and laccases produced on wood substrate were compared for their micropollutant oxidation potential.

Laccase production on wood substrate

T. versicolor was grown in a glass column (used as a trickling filter) on oak wood by addition of mycelium inoculum on moistened autoclaved wood chips. Once the wood was completely colonized by the mycelium, a synthetic wastewater containing micro and macro nutrients (Borràs et al., 2008), 4 g l^{-1} of glucose and 10 mM MOPS buffer (pH 7), was filtered through the colonized wood chips as in a trickling filter. The water was continuously recirculated and laccase activity was regularly monitored. After 3 d of recirculation, when the activity reached 2000 U l^{-1}, the solution was filtrated at 0.22 µm and used as "produced on-site" laccase preparation.

Micropollutants oxidation assay

Oxidation of a mixture of three micropollutants, BPA, DFC and MFA, at 20 mg l^{-1}, was conducted as described above, in 20 mM citrate-phosphate buffer at two different pH values: 5.8 and 6.8. "Produced" or "commercial" laccase preparations were added to the reaction mixture at the same initial activity of 570 to 580 U l^{-1}. To have similar reaction mixture compositions between both experiments, the same amount of "produced" laccase preparation was also added, after heat inactivation, to the solution containing commercial laccase. Indeed, the "produced" preparation contained some organic substances leached from the wood substrate that may have an effect on the oxidation kinetics. Micropollutant concentrations were then monitored during 10 h. Duplicate experiments were conducted at 25°C.

8.3 Results

8.3.1 Production of laccase activity by *Streptomyces* strains

Among the four strains of *Streptomyces* tested in ISP9 medium, laccase activity was only detected in the culture supernatant of *S. cyaneus* (with soy flour: 35 U l^{-1}, oat bran: 2.75 U l^{-1} and glucose: 3.75 U l^{-1}) and *S. ipomoea* (with soy flour: 0.75 U l^{-1} and oat bran: 0.5 U l^{-1}), despite notable growth of all four strains in the media containing soy flour and oat bran. No laccase activity was detected in the cultures of *S. psammoticus* and *S. griseus*, neither in ISP9 medium with the five different carbon sources, nor in another specific medium with wheat straw and yeast extract, as described by Niladevi and Prema (2008b). The absence of activity with *S. psammoticus* strain MTCC 7334 contrasts with studies of Niladevi et al. (2008a; 2008b; 2009). Although *S. griseus* was reported to produce extracellular laccase, this enzyme is assumed to be mainly localized in the cell wall (Endo et al., 2002), which could explain the absence of activity detected in the culture supernatant. No activity was detected in any culture when wheat straw flour and spruce sawdust were used as the sole carbon source. Depending on the substrate, *S. cyaneus* produced from 5.5- to 46-times more laccase activity than *S. ipomoea*, making this strain the best candidate among the tested *Streptomyces* strains for laccase production during wastewater treatment. Thus, only *S. cyaneus* was selected for further characterization.

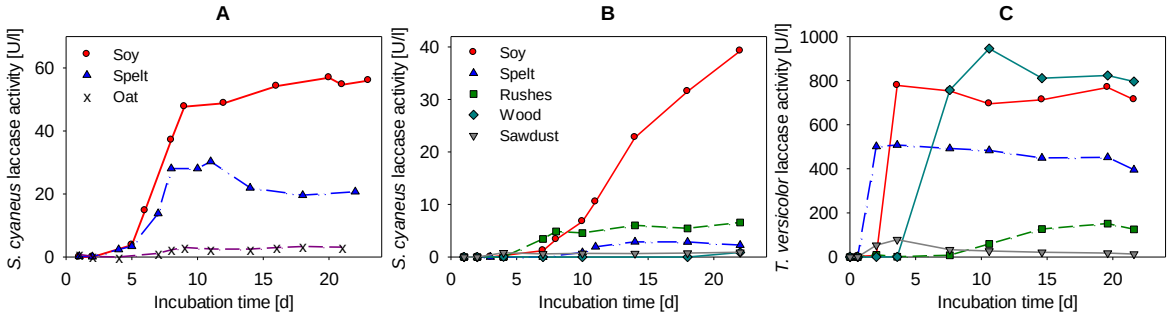

Fig. 8.2 Laccase activity in the culture supernatant of (A) *S. cyaneus* cultivated in modified ISP9 medium with soy flour, spelt flour and oat bran as carbon sources, **(B)** *S. cyaneus* cultivated in secondary treated sterile municipal wastewater with soy flour, spelt flour, rushes pieces, ash branches pieces (wood) and beech sawdust, and **(C)** *T. versicolor* cultivated in treated wastewater with the same carbon sources.

Laccase activity in the supernatant of *S. cyaneus* cultures was enhanced in modified ISP9 medium (containing 6.4 times less copper) (Fig. 8.2 A), reaching on average 57 U l^{-1} with soy flour and 30 U l^{-1} with spelt flour. Similar activities (200 U l^{-1} at 50°C, equivalent to about 50 U l^{-1} at 25°C) were measured with the same strain after 14 d of growth with soy flour by Moya et al. (2010). The activity increased rapidly after 4-5 d of incubation, once the strain had reached the stationary phase (Fig. S 8.1, Supporting information (SI)). Similar observations were made by Arias et al. (2003), who suggested that this increase in activity was related to cell death and lysis releasing intracellular laccase. After 8-9 d of incubation, laccase production decreased and the activity reached a plateau, staying at a similar level until the end of the incubation (23 d).

8.3.2 Laccase production in treated wastewater

Both *S. cyaneus* and *T. versicolor* were able to grow in sterile secondary treated wastewater containing different carbon sources. In *S. cyaneus* culture supernatant (Fig. 8.2 B), laccase activity was observed with soy flour (with a similar level to that in ISP9 medium but delayed by 2 weeks) and spelt flour (10 times lower than in ISP9), but also with rushes (6.6 U l^{-1}), suggesting that lignocellulose-containing waste could serve as substrate for laccase production. However, no or only very low activity levels (< 1 U l^{-1}) were observed with wood branches or sawdust, possibly due to the low pH (4.7) present in the wood medium and probable lack of essential nutrients (nitrogen and phosphorus) with sawdust as the sole substrate.

Laccase activity of *T. versicolor* cultures in wastewater increased very rapidly after only 2-3 d of incubation (Fig. 8.2 C), reaching a maximum of 508, 778 and 945 U l^{-1} for spelt flour, soy flour and wood branches, respectively. Lower activity was observed with rushes (151 U l^{-1}) and sawdust (79 U l^{-1}) but, unlike *S. cyaneus*, all lignocellulose substrates led to the presence of laccase activity in culture supernatant. High activity (e.g., 550 U l^{-1} with wood branches and soy flour) was still measured after 45 d of incubation (data not shown), showing the ability of this fungus to survive in the long term on these lignocellulosic substrates. Laccase activity was 20-times higher in *T. versicolor* culture supernatants with soy flour and rushes than for *S. cyaneus*, and 175-times higher with spelt flour. Wood branches were the best substrate for *T. versicolor* laccase production.

8.3.3 Comparison of "commercial" versus "in house-produced" *T. versicolor* laccases

T. versicolor produces two main laccase isoenzymes, the proportions of which differ depending on the growth substrate or the presence of inductors (Bourbonnais et al., 1995; Moldes et al., 2004; Nakatani et al., 2010). As the kinetic properties of these two main isoenzymes differ slightly (Bourbonnais et al., 1995; Moldes and Sanromán, 2006), different proportions of isoenzymes in the mixture can lead to slightly different oxidation behaviour. Therefore, the commercially available laccase preparation from *Trametes versicolor* obtained from Sigma (Ref. 38429) may not be fully representative of the laccase produced in a biofilter system with wood chips as the substrate/support. To assess if there was significant difference on micropollutant oxidation kinetics by both laccase preparations, we compared the oxidation kinetics of three micropollutants, bisphenol A (BPA), diclofenac (DFC) and mefenamic acid (MFA), by either the commercial laccase (from Sigma) or laccase produced on wood substrate.

As presented in Fig. 8.3, for both pH values tested, both laccase preparations had very similar oxidation kinetics for BPA and MFA, with no significant difference in the degradation rates. For DFC, the commercial laccase preparation was slightly less efficient at both pH values than the "produced" one, but with less than 10% difference in the removal rates. These very similar oxidation kinetics observed at two different pH values on three different micropollutants show that the commercial laccase preparation, which is a mixture of different proteins with at least two distinct enzymes displaying laccase activity (Fig. S 8.3, SI), is representative, for micropollutant oxidation, of the laccase produced on wood substrate in a trickling filter. Thus, to allow comparison with literature data

and to have a reproducible and constant proportion of the different laccase isoenzymes, commercial *T. versicolor* laccase preparation was used for the following experiments instead of culture supernatants.

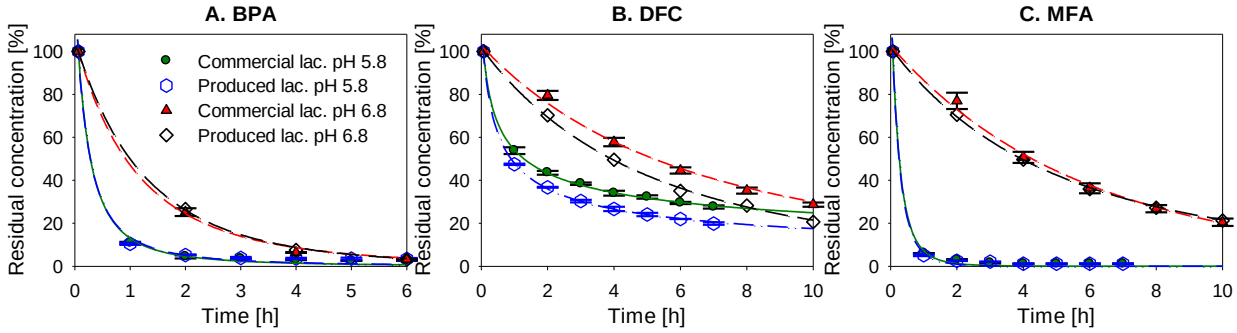

Fig. 8.3 Residual concentrations of (A) bisphenol A (BPA), (B) diclofenac (DFC) and (C) mefenamic acid (MFA), as a function of the reaction time with commercial (from Sigma) (●,▲) and produced (on wood substrate) (○,◊) laccase preparations from *T. versicolor*, at pH 5.8 and 6.8, 570-580 U l^{-1}, 25°C. Average and values (error bars) of duplicate. Lines: variable order reaction model fitted to the data.

8.3.4 Influence of pH on laccase activity with different substrates

As shown in Fig. 8.4, laccase preparations of *S. cyaneus* (L_{Sc}, from the culture supernatants) and commercial laccase of *T. versicolor* (L_{Tv}) were both able to oxidize the four substrates tested, as also observed in other studies (Arias et al., 2003; Eichlerová et al., 2012). Compared to its activity with ABTS at pH 4.5 (close to the optimum), L_{Sc} was 4-, 10- and 46-times less active with DMP, syringaldazine and guaiacol, respectively. L_{Tv} was only 1.3-, 2- and 12-times less active with these three substrates compared to ABTS, showing a broader substrate specificity than L_{Sc}.

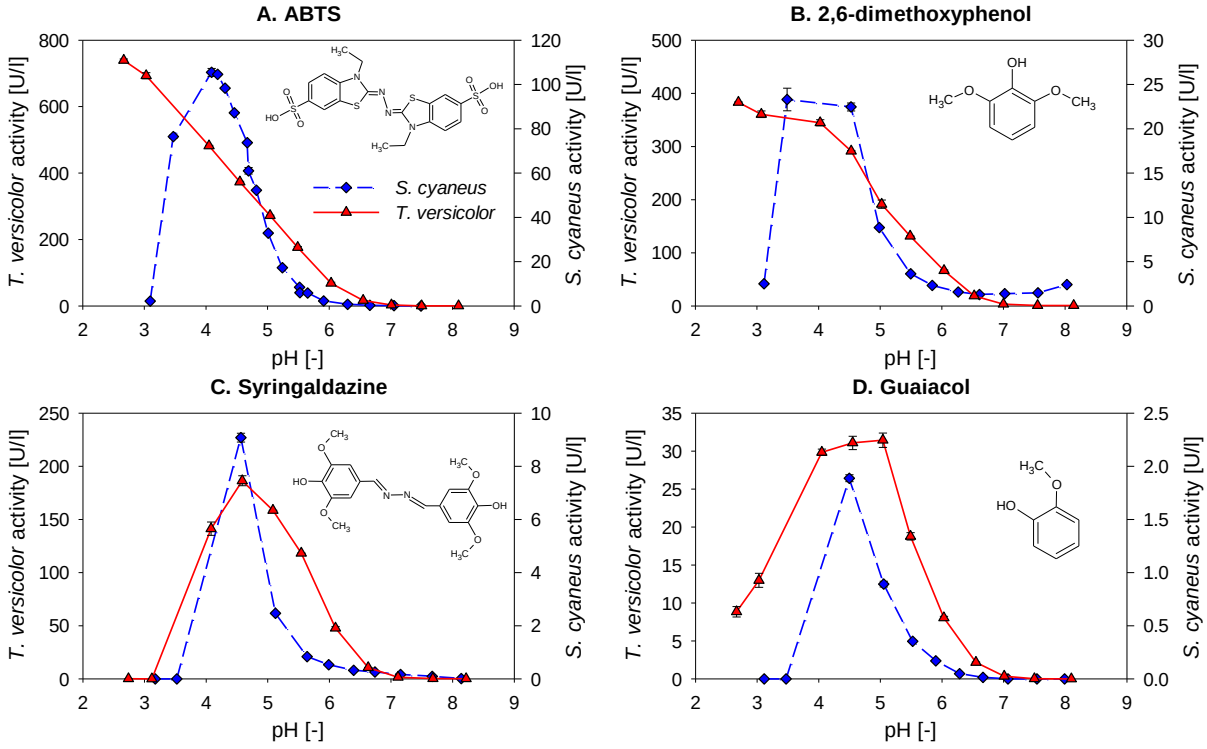

Fig. 8.4 Influence of pH on *S. cyaneus* (♦) and *T. versicolor* (▲) laccase activity with different substrates: (A) ABTS, (B) 2,6-dimethoxyphenol, (C) syringaldazine and (D) guaiacol. Average and values of duplicates, at 25°C.

For the four laccase substrates, the pH had a very strong influence on the activity of both laccase preparations, with very low activity in slightly alkaline conditions (pH > 7) and maximum activity between pH 4 and 4.5 for L_{Sc}, and from less than 2.7 to 5 for L_{Tv} (Fig. 8.4). L_{Sc} activity was strongly dependent on the pH, with, for instance, an order of magnitude increase between pH 5.5 and 4.5 with ABTS (from 8.4 to 87 U l^{-1}) and with syringaldazine (from 0.8 to 9 U l^{-1}), and a rapid decrease of activity below pH 3.5 with all substrates.

8.3.5 Influence of temperature on laccase activity

Maximum activities (with ABTS, pH 4.5) were observed at 60°C and 50°C for L_{Sc} and L_{Tv}, respectively (Fig. 8.5 A), which is 10°C lower than optimal temperatures reported in other studies (70°C and 60°C respectively) (Arias et al., 2003; Rancaño et al., 2003). A rapid decrease in activity was observed above 70°C for both preparations, probably due to heat denaturation of the enzymes. For L_{Sc}, a rapid decrease in activity was also observed when the temperature decreased below 50°C, with only 25% of its maximum activity remaining at 25°C, compared to 73% for L_{Tv}. Both laccase preparations were still active at 10°C, showing 13 and 44% of their maximum activity for L_{Sc} and L_{Tv}, respectively.

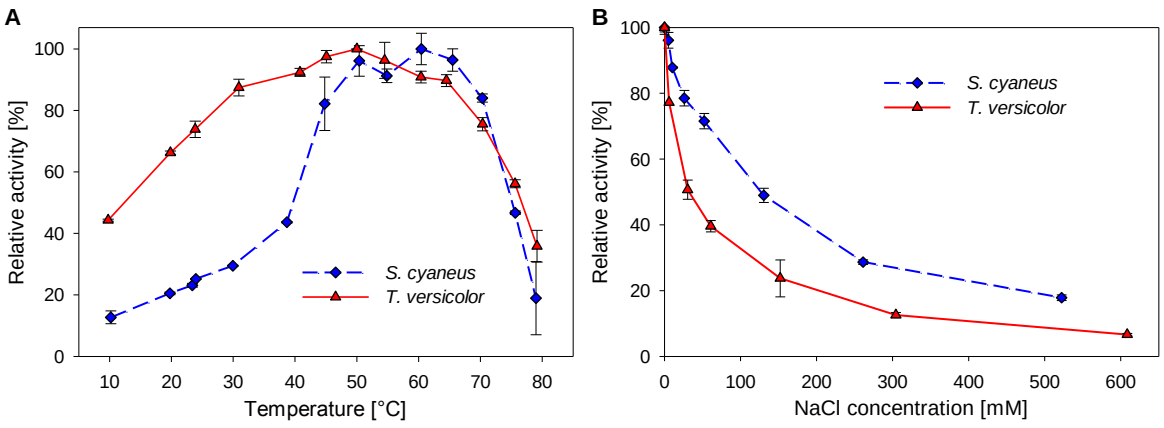

Fig. 8.5 (A) Influence of temperature on *S. cyaneus* (♦) and *T. versicolor* (▲) laccase activity. Average ± standard deviation of 2 to 3 replicates, at pH 4.5. **(B) Influence of sodium chloride concentration on *S. cyaneus* and *T. versicolor* laccase activity.** Average and values of duplicates, at pH 4.5, 25°C. Initial laccase activity (without NaCl) of 10 U l^{-1}.

8.3.6 Inhibition of the laccase activity by NaCl

Both laccase preparations were sensitive to sodium chloride (Fig. 8.5 B), with, for L_{Sc} and L_{Tv} respectively, 4 and 20% of activity inhibition at 5 mM, a typical concentration for municipal wastewater, and more than 80 and 90% at 550 mM, a concentration found in various industrial wastewaters and in seawater (Lefebvre and Moletta, 2006; Leutz, 1974). The IC_{50} (inhibition concentration for which the activity was reduced by 50%) was observed at 130 mM for L_{Sc} and at 30 mM for L_{Tv}, showing the higher sensitivity of the latter towards chloride ions. Similar IC_{50} (around 20 mM Cl^-) were observed for L_{Tv} by Enaud et al. (2011), but no information on L_{Sc} chloride inhibition was reported previously.

8.3.7 Stability of laccase at various pH

The stability of laccases incubated at various pH and 25°C is presented in Fig. 8.6. The data were fitted with a bi-exponential equation to model various mechanisms of enzyme inactivation (Eq. 8.1) (Aymard and Belarbi, 2000) by non-linear least squares regression using MATLAB (MathWorks, USA), with A_0 and A_t the activity at time 0 and at incubation time t respectively, a and b the pre-exponential factors, and k_1 and k_2 the apparent first order rate constants:

$$\frac{A_t}{A_0} = a\,\exp(-k_1\,t) + b\,\exp(-k_2\,t) \tag{8.1}$$

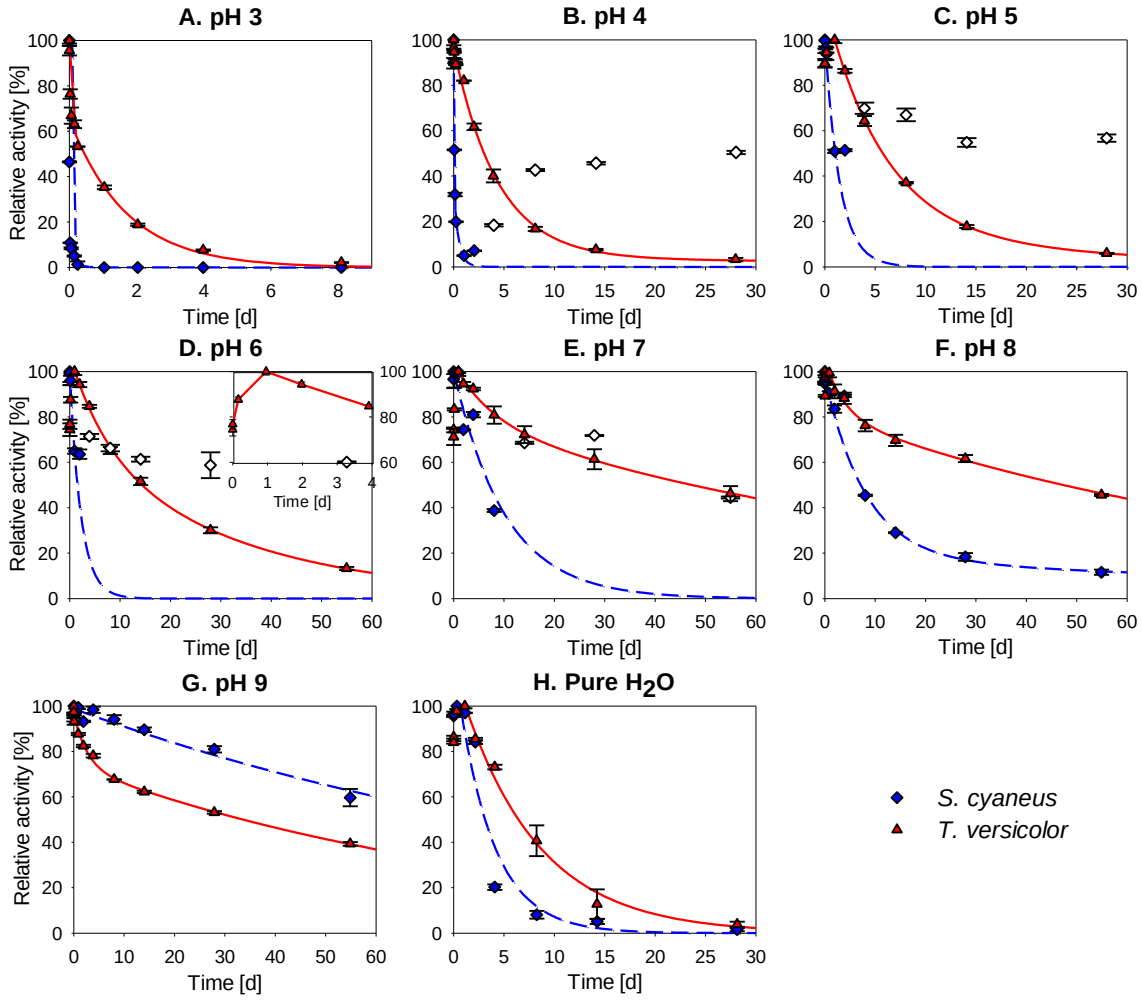

Fig. 8.6 *S. cyaneus* (♦) and *T. versicolor* (▲) laccase residual activity as a function of incubation time at different pH in buffer solution at 25°C. Plots show the average and values of duplicates. Relative activity refers to the maximum activity measured during the test. The initial laccase activity was 130 U l^{-1} in all the tests. Lines: bi-exponential inactivation model fitted to the data. The white symbols (◊) represent *S. cyaneus* laccase residual activity in the tests where the pH increased gradually to 7.3-7.7 due to microbial growth. Due to change in pH, these data were not used to fit the model. The insert at pH 6 shows the increase of *T. versicolor* laccase activity during the first days of incubation, also observed at pH 5, 7 and 8.

The results of the fitting and the estimated half-life of laccase at different pH are presented in Table 8.1. In pure water (both enzymes) and at pH 9 for L_{Sc}, the inactivation followed a simple exponential decay, k_1 and k_2 being equal (Table 8.1). Except for pH 5, 6 and 7 for L_{Sc} where the time series were too short to have confidence in the fitted model, a bi-exponential model was necessary to reproduce

the behaviour observed. In these cases, a fast initial inactivation rate, represented by a high apparent first-order rate constant (k_2), followed by slower decay kinetics (k_1) were observed.

Table 8.1 Best-fit set of coefficients of the bi-exponential model (Eq. 8.1) fitted to the laccase stability results and calculated half-life at different pH values.

S. cyaneus	pH 3	pH 4	pH 5	pH 6	pH 7	pH 8	pH 9	Pure H_2O
R^2	1.000	0.993	0.985	0.990	0.883	0.997	0.955	0.939
a	0.132	0.351	0.509	0.503	0.502	0.175	0.426	0.428
k_1 [d^{-1}]	7.581	2.035	0.674	0.444	0.098	0.007	0.008	0.284
b	1.641	0.614	0.509	0.504	0.506	0.806	0.561	0.792
k_2 [d^{-1}]	915.486	18.518	0.674	0.444	0.098	0.123	0.008	0.284
$t_{1/2}$ [d]	**0.0016**	**0.063**	**1.1**	**1.6**	**7.1**	**7.2**	**81.9**	**3.1**
T. versicolor	pH 3	pH 4	pH 5	pH 6	pH 7	pH 8	pH 9	Pure H_2O
R^2	0.998	0.997	1.000	0.999	0.997	0.994	0.992	0.994
a	0.653	0.038	0.149	0.682	0.791	0.807	0.736	0.531
k_1 [d^{-1}]	0.591	0.012	0.038	0.030	0.010	0.010	0.012	0.132
b	0.343	0.930	1.013	0.388	0.241	0.244	0.233	0.637
k_2 [d^{-1}]	36.717	0.230	0.170	0.134	0.133	0.256	0.382	0.132
$t_{1/2}$ [d]	**0.45**	**3.0**	**5.8**	**14.4**	**47.3**	**47.3**	**33.6**	**6.4**

R^2: coefficient of determination of the fitting, *a* and *b*: pre-exponential factors, k_1 and k_2: apparent first-order rate constants [d^{-1}], and $t_{1/2}$: calculated half-life of the laccase [d]

L_{Tv} was more stable than L_{Sc} at acidic and neutral pH. Very fast inactivation of L_{Sc} was observed at pH 3, with a half-life shorter than 3 min compared to 11 h for L_{Tv}. At this pH, instant precipitation appeared when L_{Sc} was added. At pH 4, no precipitate was visible, but L_{Sc} was still rapidly inactivated, with a half-life of 90 min. L_{Sc} and L_{Tv} stability increased as the pH increased, reaching the highest stability at pH 9 for L_{Sc}, with an estimated half-life of 82 d, and at pH 7-8 for L_{Tv}, with a half-life of 47 d. When laccase was incubated in pure water (pH 6.5-7.5), the stability was significantly reduced compared to storage in a buffer at neutral pH; with half-lives of only 3 and 6 d for L_{Sc} and L_{Tv}, respectively.

From pH 5 to 8, an increase in L_{Tv} activity was observed during the first 24 h of incubation, as illustrated for pH 6 in Fig. 8.6 D. At pH 6 and 7, this increase in activity was as high as 34%. A similar increase in activity (24 ± 1%) was also observed after sonication (15 pulses of 3 s at 100 W) of fresh laccase solutions, suggesting that this increase was due to a better dispersion of the enzymes that were initially partly aggregated (presence of particles with strong laccase activity). This phenomenon was also noticed in other studies (Margot et al., 2013c; Silvério et al., 2013). Changes of the storage conditions (pH and temperature) could also gradually influence laccase activity, possibly due to slow reorganisation of laccase structure or conformation (Kurniawati and Nicell, 2008).

As the tests were not conducted under sterile conditions, bacterial growth (turbidity, confirmed by microscopy) was observed after 2 d in the incubation tubes containing L_{Sc} at pH 4 to 7, resulting in an increase in the pH to 7.3-7.7 in all these tubes (Fig. S 8.2, SI). An increase in activity following the increase in pH was observed (Fig. 8.6 B-E), suggesting a partially reversible pH inactivation of the enzyme. To verify this hypothesis, L_{Sc} and L_{Tv} were again incubated at pH 3.5 and 3, respectively, and

their residual activity was followed over time. At a certain time, the pH of the solution of two of the four replicates was increased to 7.5 by addition of concentrated NaOH. The effect of this artificial increase in pH on laccase stability is presented in Fig. 8.7. For L_{Sc}, as expected, a very fast inactivation was observed in the four replicates, with more than 90% of inactivation within 1 h (Fig. 8.7 A). However, when the pH was increased to 7.5 after 3 h of incubation at pH 3.5, L_{Sc} activity increased again, reaching 86% of the initial activity after 4 d. At the same time, the precipitate observed at pH 3.5 was again solubilised at pH 7.5. In the replicates maintained at pH 3.5, the activity reduced to an undetectable level after 1 d. After 2 d at pH 3.5, pH was increased to 7.5 in one replicate, leading to a slow L_{Sc} activity increase from the no-detect level to 10% of the initial activity. These results indicate that L_{Sc} is affected by two inactivation types, one that is fast but reversible and another that is slower but irreversible. The former type is exactly what was observed in the stability experiment (depicted in Fig. 8.6 B-E) when the pH increased due to bacterial activity. The irreversible L_{Sc} inactivation seemed to be relatively similar to L_{Tv} inactivation (Fig. 8.6 E), which seemed to be only affected by pH in an irreversible manner (Fig. 8.7 B).

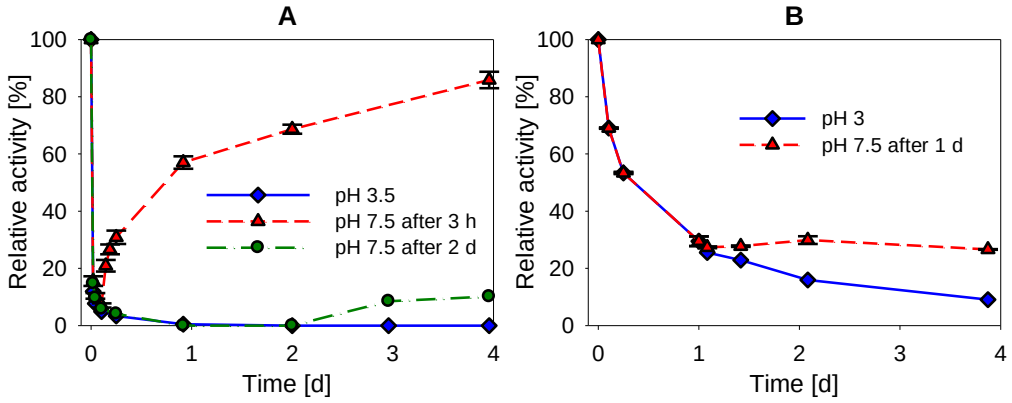

Fig. 8.7 (A) *S. cyaneus* laccase residual activity as a function of incubation time (at 25°C in buffer solution) at pH 3.5 (♦), and reversible inactivation due to alkalinisation (NaOH addition) at pH 7.5 after 3 h (▲) or after 2 d (●). (B) *T. versicolor* laccase residual activity as a function of incubation time at pH 3 (♦), and laccase stabilisation due to alkalinization at pH 7.5 after 1 d (▲). Average and values (error bars) of duplicates.

8.3.8 Micropollutant oxidation by laccase preparations

The kinetics of laccase-mediated degradation of the plastic additive BPA and the two anti-inflammatory drugs DFC and MFA at different pH are presented in Fig. 8.8. The residual concentrations were fitted with a variable order reaction model (two coefficients, Eq. 8.2), as proposed in Chapter 6, taking an initial concentration C_0 of 1 (arbitrary units as the initial concentration was always constant). C_t is the residual concentration after a reaction time t, x the order of the reaction, and k the apparent rate constant of variable order.

$$\frac{C_t}{C_0} = \left(\frac{d}{k\, t\, C_0^{1/d} + d} \right)^d \quad \text{with } d = \frac{1}{x-1}, \forall\, x \neq 1 \tag{8.2}$$

The results of the fitting and the estimated half-life of the pollutants at different pH are presented in Table 8.2. The order of reaction varied mainly between 1 and 3, as observed also in Chapter 6.

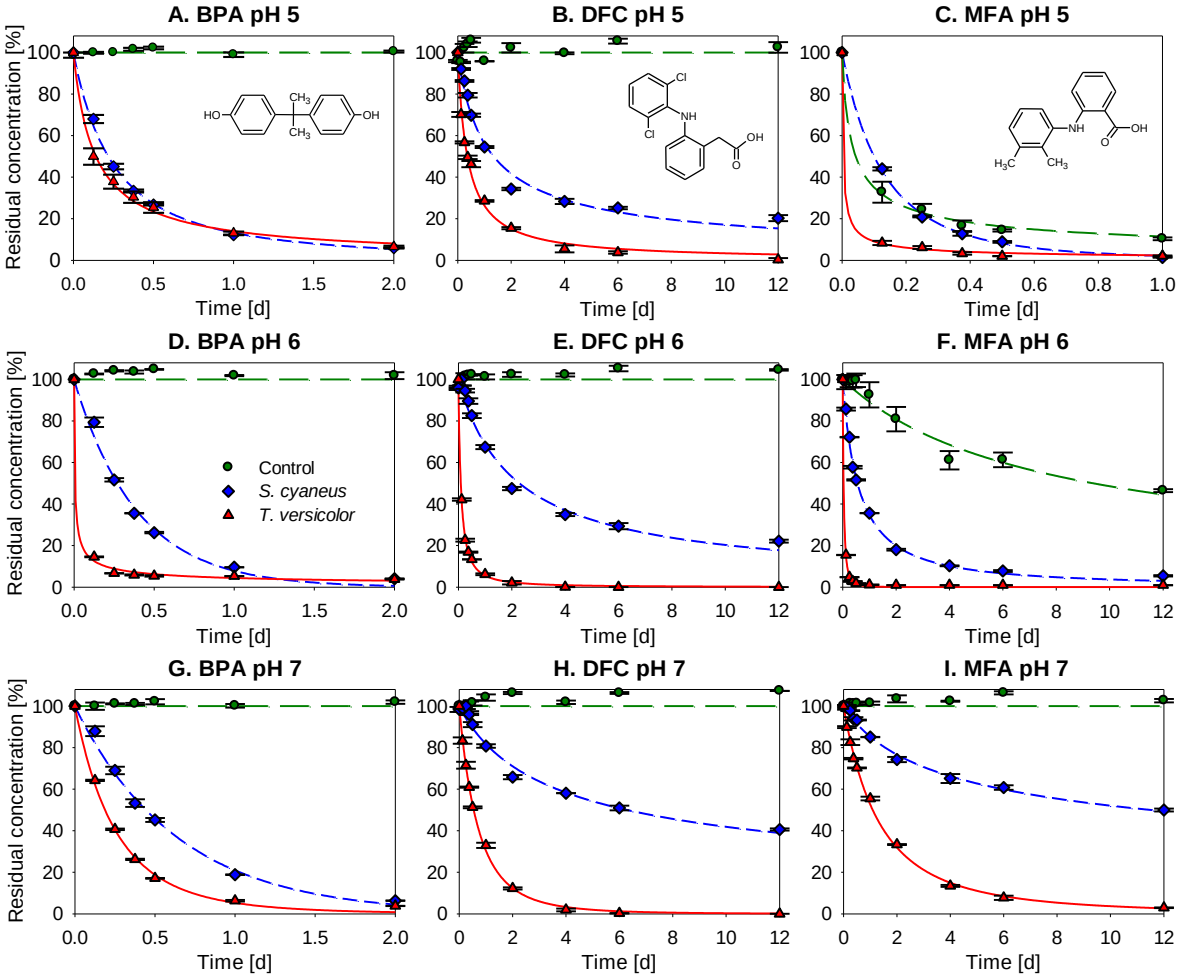

Fig. 8.8 Residual concentrations of three micropollutants as a function of the reaction time with laccase preparations from *S. cyaneus* (♦) and *T. versicolor* (▲), and in a control without laccase (●). Degradation of bisphenol A (BPA), diclofenac (DFC) and mefenamic acid (MFA) at three different pH values (5, 6 and 7), at 25°C. Average and values of duplicates. The initial micropollutant concentration, present in mixture, was at 20 mg l^{-1} for the three compounds. The initial laccase activity was 210 and 220 U l^{-1} for *T. versicolor* and *S. cyaneus* laccases, respectively. Lines: variable order reaction model fitted to the data.

Both laccase preparations were able to oxidize the three pollutants at all pH values studied. Except for BPA at pH 5, where both laccases had a very similar efficiency, L_{Tv} provided more efficient micropollutant oxidation, especially at pH 6 and 7. As observed previously (cf. Chapter 6), MFA was degraded in the control without laccase under acidic conditions, with a half-life of 1 h at pH 5 and 9 d at pH 6. Thus, at pH 5, it was difficult to distinguish between laccase oxidation and abiotic degradation. However, at pH 6 and 7, MFA was significantly oxidized by both laccases. The highest oxidation rates of BPA and DFC were observed at pH 6 for L_{Tv}, with half-lives of 9 min and 2.2 h, respectively, and at pH 5 for L_{Sc} with half-lives of 5.2 h and 32 h, respectively.

Table 8.2 Best-fit coefficients of the variable order reaction model for the degradation of bisphenol A, diclofenac and mefenamic acid and their respective half-lives at different pH values.

	Bisphenol A		**Diclofenac**		**Mefenamic acid**		
	S. cyaneus	*T. versicolor*	*S. cyaneus*	*T. versicolor*	*S. cyaneus*	*T. versicolor*	Control
	pH 5		**pH 5**			**pH 5**	
R^2	0.997	0.997	0.985	0.993	0.992	0.999	0.996
x	1.596	2.167	2.547	1.986	1.321	2.696	2.936
k	3.936	7.623	0.925	2.763	7.733	339.154	34.015
$t_{1/2}$ **[h]**	**5.2**	**3.4**	**32.2**	**8.6**	**2.4**	**0.09**	**1.0**
	pH 6		**pH 6**			**pH 6**	
R^2	0.993	0.998	0.982	0.999	0.997	1.000	0.969
x	0.998	2.964	2.175	1.729	1.814	1.437	2.382
k	2.553	233.949	0.469	9.868	1.667	23.333	0.124
$t_{1/2}$ **[h]**	**6.5**	**0.15**	**54.8**	**2.2**	**13.4**	**0.83**	**225**
	pH 7		**pH 7**			**pH 7**	
R^2	0.995	0.994	0.980	0.999	0.993	0.998	
x	0.984	1.190	3.120	1.270	4.014	1.438	
k	1.566	3.939	0.251	1.402	0.204	0.741	
$t_{1/2}$ **[h]**	**10.6**	**4.5**	**151**	**13.0**	**276**	**26.2**	

R^2: coefficient of determination of the fitting, *x* : order of the reaction, *k* : apparent variable-order rate constant, and $t_{1/2}$: calculated half-life of the micropollutants [h]

The residual laccase activities in the reaction mixtures at the end of the test, after 12 d of incubation, are presented in Table 8.3. Loss of 97% of the initial activity was observed at pH 5 for L_{Tv}. Additional laccase inactivation (from 0 to 17%) seemed to occur over the course of the reaction, especially at pH 5, compared to incubation in solutions without micropollutants (stability test). Unlike what was observed for oxidation of phenols (Kurniawati and Nicell, 2008), the laccase inactivation due to the catalytic reaction was, however, much lower than the inactivation due the test conditions (pH and temperature). At the end of the test, L_{Tv} activity was 17-times higher in the mixture at pH 6 than at pH 5. The residual activity of L_{Sc} was slightly higher than that for L_{Tv}. This contrasts with the results of the stability test, where L_{Sc} was rapidly, but reversibly, inactivated at these pH values.

Table 8.3 Residual laccase activity in the reaction vials after 12 d of reaction with the mixture of micropollutants at 25°C and at different pH

	Residual activity [a]		**Residual predicted activity** [b]
	T. versicolor [%]	*S. cyaneus* [%]	*T. versicolor* [%]
pH 5	3.3 (± 0.2)	10.4 (± 0.2)	20
pH 6	53.7 (± 0.9)	52.0 (± 2.1)	53
pH 7	68.4 (± 1.0)	79.1 (± 0.1)	74

[a] Average and standard deviation of duplicates, relative to the initial activity

[b] Residual activity after 12 d predicted with the bi-exponential model based on the stability test (incubation at 25°C at the same pH but without micropollutants)

8.4 Discussion

For selection of microorganisms able to produce laccase on-site for treating micropollutants in wastewater, the potential of four strains of *Streptomyces* bacteria together with the white-rot fungus *T. versicolor* was assessed for (i) their ability to produce laccase in treated wastewater on cheap substrates, (ii) their laccase activity at different pH and temperature, (iii) laccase inhibition by chloride salt, (iv) laccase stability, and (v) the laccase substrate range and their ability to oxidize different micropollutants.

8.4.1 Laccase production on different substrates

Among the four *Streptomyces* strains assessed, only *S. cyaneus* produced laccase to a level potentially sufficient for the targeted application, for instance in a system with a long hydraulic residence time (of a few days to a few weeks such as wetlands). Despite the attractive possibility of producing (low amounts of) laccase in treated wastewater on lignocellulosic substrates (rushes) with *S. cyaneus* without the addition of other nutrients, the activity levels reached were not comparable with those obtained in *T. versicolor* cultures, which were more than 20-times higher. *T. versicolor* was found to produce high amounts of laccase (up to 945 U l^{-1}) in treated wastewater with ash branches (including the bark) as the sole substrate, which is promising for the development of a fungal trickling filter for wastewater post-treatment. Indeed, this forestry waste is cheap and widely available in Switzerland, for example, and the activity reached in the supernatant is high enough – according to the study presented in Chapter 6 – to allow high removal (> 90%) of various micropollutants (BPA, DFC, MFA and triclosan) in an appropriate time range (less than 10 h) at pH 7 and 25°C, conditions that are found in municipal wastewaters. Maintaining *T. versicolor* in unsterile biologically treated wastewater is, however, still a challenge due to competition/predation by other microorganisms. This competition can probably be limited in the case of use of lignocellulosic materials as the sole substrate, as only few organisms can use them as carbon source.

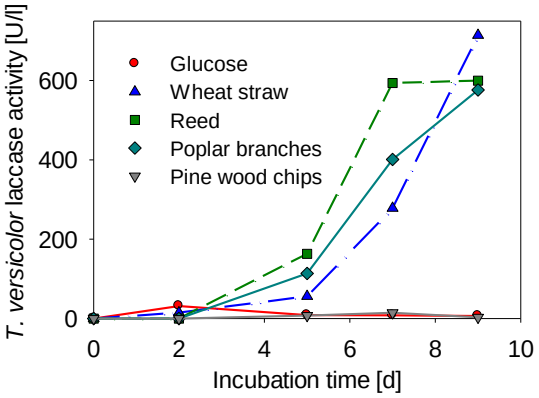

Fig. 8.9 Evolution of laccase activity in the supernatant of *T. versicolor* cultures (25°C, 140 rpm, pH 5-6.8) in sterile treated (activated sludge without nitrification) municipal wastewater with diverse substrates: glucose (10 g l^{-1}), wheat straw pieces (47 g l^{-1}), reed pieces (*Phragmites australis*, 153 g l^{-1}), poplar (*Populus* spp., 124 g l^{-1}) branches with the bark and pine wood chips (without bark, 123 g l^{-1}).

The composition of the growth substrate had a strong influence on laccase production by *T. versicolor*, as also observed in other studies on lignocellulosic materials (Özşölen et al., 2010). As only complex

substrates were used here (wood with bark, integral soy flour, etc.), it was not possible to identify which components induced laccase production. It seemed that the role of wood bark was significant as much lower activity (79 U l^{-1}) was observed in *T. versicolor* cultures with only beech sawdust compared to that with ash branches (with the bark). These results were also confirmed by an additional experiment in treated wastewater (Fig. 8.9), where high *T. versicolor* laccase activity (> 550 U l^{-1}) was observed in culture supernatant after 9 d of cultivation on poplar (*Populus* spp.) branches with the bark, on reed pieces (*Phragmites australis*) and on wheat straw, and low activity (< 30 U l^{-1}) on pine wood chips (without bark) and glucose. Bark contains in general more lignin and polyphenols than wood (Harkin and Rowe, 1971), and aromatic or phenolic compounds related to lignin or lignin derivatives such as ferulic acid or vanillin, are known to induce laccase production by white-rot fungi (De Souza et al., 2004; Parenti et al., 2013). For *S. cyaneus*, induction of laccase production on lignocellulosic substrate was not observed. The role of laccase in *Streptomyces* spp. is, however, not clear and might be related more to morphogenesis than to lignin degradation (Endo et al., 2002). This is also supported by the means by which *S. cyaneus* likely produces extracellular laccase (cell lysis rather than active secretion). Indeed, the *S. cyaneus* laccase sequence deposited by Moya and co-workers (GenBank HQ857207) does not harbour any secretion signal peptide (J. Maillard, unpublished data).

8.4.2 Laccase activity at different pH values and temperatures

Both laccases had optimal activity under acidic conditions (pH < 5) for all substrates. Slight variations of the optimal pH (< 3 to 5) were observed for the phenolic substrates, which are assumed to be related to the protonation/deprotonation state of the compound (Rosado et al., 2012). The pH range in which significant activity was measured was wider for L_{Tv} than for L_{Sc}, with L_{Tv} showing higher activity in the pH range 5.5 to 7. Both laccases showed, however, rather low activity under slightly alkaline conditions, impairing their use in non-acidified municipal wastewater (pH 7-8).

The strong pH influence observed on the activity of both laccases on all the substrates could be related, as discussed in Chapter 6, to a balance between two opposing phenomena (Xu, 1997): (i) the increase in redox potential difference (and thus oxidation rate) between laccase type 1 copper site (T1, where the substrate oxidation takes place) and the phenolic or aniline substrates when the pH increases, and (ii) the increase in hydroxyl inhibition of laccase (binding of the hydroxide anion to the T2/T3 Cu, where the reduction of oxygen to water take place) at higher pH, possibly leading to a bell-shape activity profile. Despite their relatively low amino acid sequence homology (Fig. S 8.5, SI), similar mechanisms are expected for both laccases as the three-dimensional structure of the active site of *S. cyaneus* laccase is presumably very similar to the one of *T. versicolor* laccase (see SI for details). This bell-shape profile was observed for both laccases on the phenolic substrates syringaldazine and guaiacol, but not on ABTS with L_{Tv}, which is consistent with the fact that the redox potential of ABTS is not dependent on the pH in the range tested (no protons involved in the oxidation) (Xu, 1997). Due to its phenolic structure, a bell-shape profile was expected for DMP, but not observed with L_{Tv}. The decrease in activity with the pH was, however, possibly out of the pH range studied (appearing at lower pH). The low L_{Sc} activity below pH 3.5 with all substrates was probably rather due to the fast inactivation of the enzyme at these pH values (50% inactivation in 2 min at pH 3) than to thermodynamic and kinetic considerations (variation of redox potential).

Both laccases had optimal activity on ABTS at 50 to 60°C. These optimal temperatures can, however, differ depending on the substrate (Margot et al., 2013c; Yang et al., 2013d). L_{Tv} showed significant activity in a wider temperature range than L_{Sc}, especially at lower temperatures (from 10 to 40°C). It retained 44% of its maximum activity at 10°C, making this enzyme more attractive for municipal wastewater treatment (10-25°C).

8.4.3 Laccase inhibition by chloride

Municipal and especially industrial wastewaters can contain relatively high chloride concentrations. Chloride (Cl^-), similar to other halide anions (F^-, Br^-) or to the hydroxide anion (OH^-), has been reported to either bind to the T2 Cu of laccase and to interrupt the internal electron transfer between T1 and T2/T3 active site (Xu, 1996), and/or to bind near the T1 active site, blocking the access of the substrate to T1 Cu or inhibiting the electron transfer (Enaud et al., 2011). Both laccases considered here were inhibited by sodium chloride, with L_{Sc} being slightly more tolerant. The chloride concentration in municipal wastewater which is around 2.5 to 5 mM (unpublished data, Lausanne WWTP), is not expected to affect laccase activity significantly (< 20%). However, chloride inhibition can be an issue for the treatment of industrial effluents from, for example, the pharmaceutical industry (around 90 mM Cl^- (Rajkumar and Palanivelu, 2004)), especially for L_{Tv} (> 60% inhibition).

8.4.4 Laccase stability

For all biotechnological applications, good stability of the enzyme under the treatment conditions is required. Enzyme inactivation is influenced by many different factors, the pH being an important one due to its effect on the structures of proteins (influence on the balance of electrostatic and hydrogen bonds between the amino acids) (Sadana, 1988). L_{Sc} incubated at 25°C in buffer solutions was relatively rapidly inactivated ($t_{1/2}$: 0-2 d) at acidic pH (< 7) compared to L_{Tv}. However, this fast inactivation was reversible and L_{Sc} could recover most of its activity when the pH was switched again to alkaline conditions. The mechanism of this reversible pH inactivation is unknown, but could be due to refolding of the tertiary structure of the enzyme when the pH increases (Kurniawati and Nicell, 2008), or possibly resolubilization of precipitated laccase. The irreversible L_{Sc} pH inactivation seemed to be in the same range as that observed for L_{Tv}. Thus, if the reversible pH inactivation can be avoided, both laccases would have relatively similar stability. This was observed during the micropollutant degradation test. Indeed, the 12 d stability of L_{Sc} at pH 5, 6 and 7 during this test was similar or even higher than that of L_{Tv}, and much higher than what was observed for L_{Sc} during the stability test. This suggests that the different incubation conditions, such as the presence of micropollutants (laccase substrate) and solvents (4% methanol and 2% acetone), prevented the reversible inactivation, possibly by limiting L_{Sc} precipitation/aggregation or by increasing the stability due to pollutant binding to the active centre of the enzyme (Mai et al., 2000). Laccase stability is thus not only dependent on the pH but also on the composition of the solution. This was also confirmed by the much higher laccase stability in buffer solution at pH 7 than in pure water at the same pH.

Similar stability results for L_{Tv} were previously reported (Kurniawati and Nicell, 2008; Mai et al., 2000). In most cases, a fast initial inactivation rate followed by slower decay kinetics was observed. Some authors explained this behaviour by the possible presence of two isoenzymes of laccase, one

being unstable and rapidly inactivated and the other being more stable (Kurniawati and Nicell, 2008). However, other mechanisms may also explain this biphasic behaviour (Aymard and Belarbi, 2000), such as a fast reversible inactivation followed by a slower irreversible one. *T. versicolor* is known to produce at least four different isoenzymes, which can differ significantly in their stability (Koschorreck et al., 2008b). The commercially available *T. versicolor* laccase preparation used in this study contains, as shown in Fig. S 8.3 (SI), at least two distinct enzymes with laccase activity, possibly explaining the observed biphasic behaviour. As the proportion of the different isoenzymes is reported to be influenced by the culture conditions (Moldes et al., 2004), stability may differ for other sources of *T. versicolor* laccase. The higher and relatively good laccase stability observed at neutral to alkaline pH values for both laccase preparations, L_{Tv} and L_{Sc}, is advantageous for the targeted applications in municipal wastewater (pH 7-8). Although laccases are known to be relatively stable at ambient temperatures and near-neutral pH, this is, however, the first time that long-term stability (45-60% remaining activity after incubation 55 d at 25°C) was reported for these two particular laccase preparations.

8.4.5 Laccase substrate range and oxidation of micropollutants

The broader the laccase substrate range is, the greater the potential for the enzyme to be used to remove micropollutants. Both laccases were able to oxidise the four aromatic model substrates tested, showing higher activity against the non-phenolic ABTS, followed by the phenolic compounds DMP, syringladazine and finally guaiacol. L_{Sc} was much less active on the phenolic substrates than on ABTS compared to L_{Tv}. These differences in reactivity are reported to be related to differences in shape and chemical composition of the substrate binding site of the enzymes (Rosado et al., 2012; Xu et al., 1996). Differences in the phenol substitution seemed also to influence the activity. The electron-donating property of the methoxy group is reported to reduce the redox potential of phenolic compounds, guaiacol (1 methoxy group) having a higher redox potential E^0 than the two other substrates (2 methoxy groups) (Xu, 1996). For small *o*-substituted phenols, the redox potential difference (ΔE^0) between laccase type 1 copper site (T1) and the substrate seems to be the main driving force for the oxidation (Xu, 1996). Therefore, the lower the E^0 value of the phenolic substrate, the faster will be the reaction rate, which is consistent with the results obtained here. For larger *o*-substituents, other significant mechanisms such as steric hindrance may be observed (Xu, 1996).

BPA, DFC and MFA are three common micropollutants found at relatively high concentrations in municipal WWTP effluent (average between 300-1000 ng l^{-1}) (Kase et al., 2011). DFC is of special concern because it is not removed in conventional biological treatments (cf. Chapter 3) and can affect fish at typical WWTP effluent concentrations (1 µg l^{-1}) (Triebskorn et al., 2004). Despite their very low activity at pH 7 on the model substrates, both laccases were able to reduce the concentration of these micropollutants significantly at neutral pH, which is for the first time reported for bacterial laccases. The oxidation rates were much higher at pH 7 with L_{Tv} compared to L_{Sc}, especially for the two aniline pollutants (DFC and MFA), confirming the wider pH range of this enzyme. L_{Sc} was less reactive with aniline (DFC and MFA) than with phenol compounds (BPA) compared to L_{Tv}. Both laccases rapidly oxidized BPA, with a similar rate at pH 5, while L_{Tv} was more effective for the oxidation of the two aniline compounds at all pH values. This difference in oxidation rate is thought to be either related to different affinity for the aniline substrates or to a lower redox potential of the T1

copper site of L_{Sc}, as observed for many other bacterial laccases (E^0 < 0.5 V vs. SHE, compared to 0.785 V for L_{Tv}) (Hong et al., 2011; Telke et al., 2009).

The higher DFC degradation with L_{Tv} at pH 6 than at pH 5 contrasts with the study presented in Chapter 6, where the highest removal was observed below pH 5. This shift in the optimal pH is likely due to the different initial enzyme concentrations used, 3.5 times higher in the previous study. At low enzyme concentrations, the oxidation rate was slower and the time to reach a defined level of micropollutant removal longer. The longer the reaction time, the higher was the loss of laccase by inactivation, especially under acidic conditions. Thus, in the case of low enzyme concentrations, the gain associated with higher laccase activity at lower pH was offset by the loss of activity at these pH values due to the long reaction time. Higher degradation levels were thus observed at higher pH, where laccase was more stable. The loss of activity was limited in the case of high enzyme concentrations (fast reaction) and thus higher degradation levels were obtained at a lower pH value (close to the optimal pH for laccase activity). Therefore, depending on the laccase concentration, a compromise between laccase stability (higher at high pH values) and laccase activity (higher at low pH values) has to be found to determine the optimal pH for the treatment.

8.5 Conclusion

The evaluation of five laccase-producing organisms to improve micropollutant degradation in wastewater showed that *T. versicolor* was the most promising strain. This fungus produced more than 20-times more laccase activity than *S. cyaneus*, the best candidate of the *Streptomyces* strains evaluated, and this especially in treated wastewater with forestry waste as the sole substrate, a cheap and widely available product. Laccase from *T. versicolor* (L_{Tv}) was moreover more active than that from *S. cyaneus* (L_{Sc}) near neutral pH and between 10 to 25°C, conditions usually found in municipal wastewater. Despite an optimal activity under acidic conditions (pH < 6), which limits their use in non-acidified wastewater, both laccases had the ability to degrade common wastewater micropollutants, BPA, DFC and MFA even at neutral pH, which is for the first time reported for a bacterial laccase. Micropollutant oxidation was faster with L_{Tv}, especially for aniline pollutants, showing the greater potential of this enzyme for the target application. Both laccases were relatively stable at slightly alkaline pH values, conditions found in municipal wastewater. Thus, altogether, despite a slightly lower resistance of its laccase to chloride, *T. versicolor* appeared to be the best candidate to be used in a post-treatment, such as a fungal trickling filter composed of wood support, for micropollutant degradation in wastewater.

Acknowledgments

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8.6 Supporting information

Evolution of extracellular laccase activity and biomass in *S. cyaneus* cultures

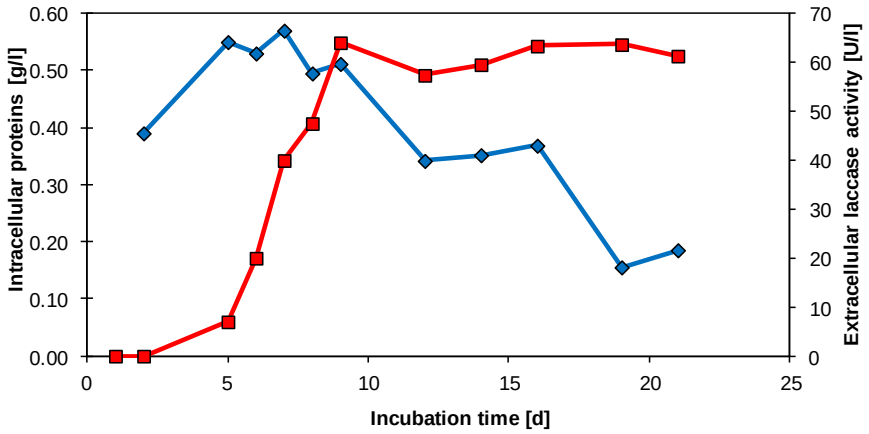


Fig. S 8.1 Evolution of the extracellular laccase activity (■, right axis) and intracellular protein content (♦, left axis, indicator of the biomass) of *S. cyaneus* culture (in modified ISP9 medium with soy flour 10 g l^{-1}). Intracellular proteins were measured with the Bio-Rad DC protein assay kit in the supernatant of pre-washed cells, lyzed by sonication (15 pulses of 3 s at 100 W).

Evolution of pH during the laccase stability test - Example for the incubation at initial pH of 4

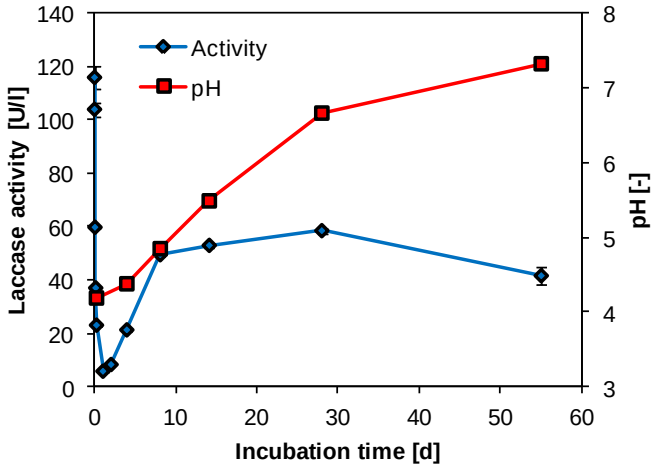


Fig. S 8.2 Evolution of *S. cyaneus* laccase activity incubated at 25°C in citrate-phosphate buffer at an initial pH value of 4 and evolution of the pH due to bacterial growth. Average and values of duplicates (difference in the duplicate pH values lower than 0.06 unit).

Characterization of the commercial laccase preparation from *Trametes versicolor* from Sigma

The commercially available laccase preparation from *Trametes versicolor* obtained from Sigma (Ref. 38429) was analyzed by separating the proteins of 5 µl of concentrated laccase solutions (40 and 5 g l^{-1}) by sodium dodecylsulfate polyacrylamide (12%) gel electrophoresis (SDS-PAGE), following Sambrook et al. (1989). The SDS-PAGE was done with and without 10 min boiling of the proteins. Prior to staining the proteins with Coomassie brilliant blue, one of the duplicate gels was incubated in acetate buffer 100 mM, pH 4.5, with 0.5 mM ABTS to detect the laccase activity.

As presented in Fig. S 8.3, the commercially available laccase preparation contains a mixture of different proteins, from 17 to ~80 kDa, with a major band around 66 kDa, which corresponds approximately to the reported mass of the best-characterized *T. versicolor* laccase isoenzymes (Bourbonnais et al., 1995; Moldes et al., 2004). Similar protein bands from this laccase preparation were also observed by Wang et al. (2012). Despite the denaturing properties of the SDS gel, laccase activity was observed in at least two distinct bands in the gel with unboiled samples, around 40 kDa and 66-70 kDa, suggesting the presence of at least two enzymes with laccase activity in the preparation. The 40 kDa protein showed lower intensity with Coomassie staining but had high laccase activity, suggesting that this protein is thus either very active or more resistant to denaturation than the 66-70 kDa protein. These data show clearly that the commercially available laccase preparation contains a mixture of different proteins, several of which displaying laccase activity.

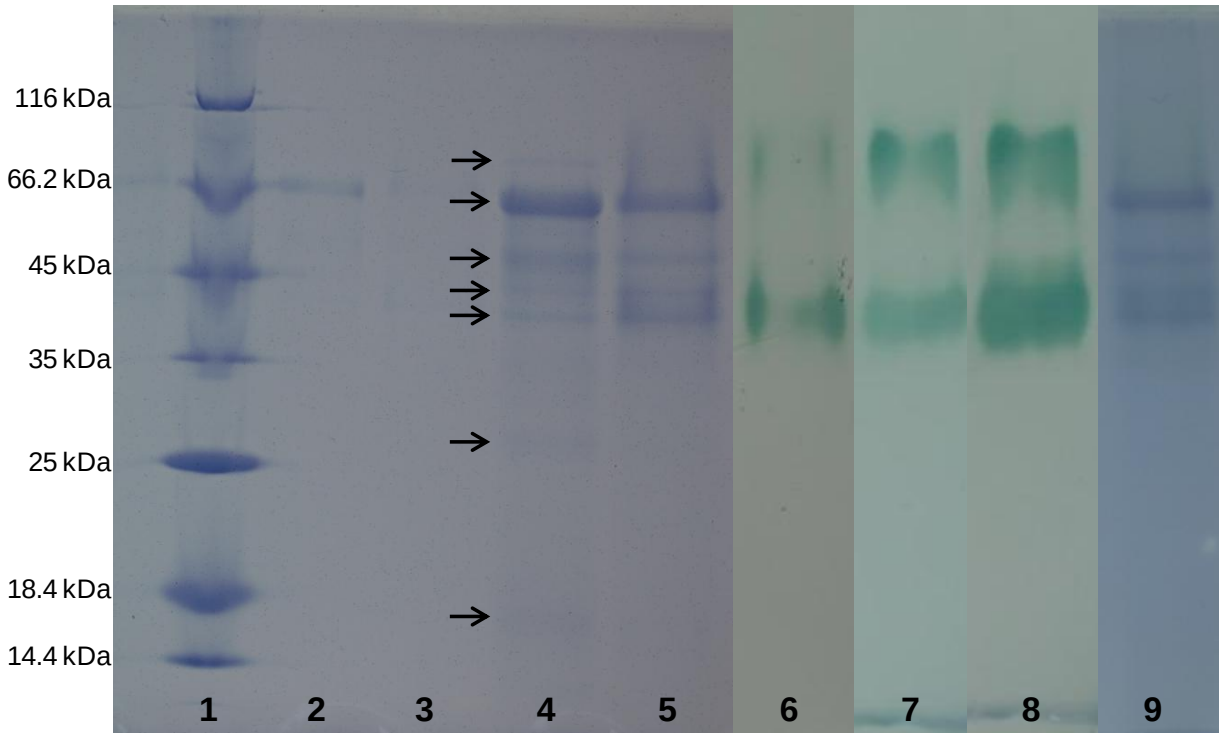


Fig. S 8.3 SDS-PAGE of commercially available laccase preparation from *T. versicolor* from Sigma (Ref. 38429). Lanes 1-5 and lane 9: Coomassie staining; Lanes 6-8: laccase activity with ABTS (0.5 mM in acetate buffer 100 mM pH 4.5). Lane 1: protein ladder (Fermentas); Lanes 2 and 3: 125 µg boiled and unboiled laccase, respectively; Lanes 4 and 5: 1 mg of boiled and unboiled laccase, respectively; Lanes 6-8: 1 mg of unboiled laccase recorded after increasing incubation time in ABTS solution (in green); Lane 9: same lane as 6-8 after additional Coomassie staining. Arrows: bands of proteins present in the commercially available laccase preparation.

Identification of one laccase candidate from *S. cyaneus* culture supernatant

Extracellular crude enzyme preparation of *S. cyaneus* culture supernatant was concentrated 80 times by ultrafiltration as described in the manuscript, and then separated by sodium dodecylsulfate polyacrylamide (12%) gel electrophoresis (SDS-PAGE) following Sambrook et al. (1989). A protein band around 75 kDa corresponding to the predicted *S. cyaneus* laccase molecular mass (Arias et al., 2003) was analysed by mass spectrometry (MS) after trypsin digestion and compared to profiles of peptides generated from available *Streptomyces* genomes and from the deposited *S. cyaneus* laccase sequence (GenBank HQ857207). This analysis was performed by the PCF laboratory (EPFL, Switzerland).

MS analysis of the excised 75 kDa protein band obtained after concentrating *S. cyaneus* culture supernatant showed a profile matching with nine unique peptides (20% coverage) of the deposited laccase sequence (GenBank HQ857207). This latter protein sequence shows 84% amino acid sequence identity with the phenoxazinone synthase (PHS) of *S. antibioticus* (Hsieh and Jones, 1995) (Fig. S 8.4). This laccase, along with several other *Streptomyces* proteins, form a distinct multi-copper oxidase family either classified as laccase (EC 1.10.3.2) or phenoxazinone synthase (EC 1.10.3.4). Functional differentiation between these two classes is unclear (Le Roes-Hill et al., 2009). The reported *S. cyaneus* laccase shows 33% sequence identity with the well-characterized CotA laccase of *Bacillus subtilis* (GenBank AAB62305) (Martins et al., 2002), and only very limited sequence identity with the EpoA laccase of *S. griseus* (GenBank BAB64332) (Endo et al., 2003) or the laccase of *T. versicolor* (GenBank CAA77015) (Fig. S 8.5). Despite its relatively low sequence homology with other well-characterized laccases, the structure and active site configuration of *S. antibioticus* PHS, a close parent of *S. cyaneus* laccase, is reported to be very similar to other laccases, with three conserved cupredoxin-like domains, T1 (type 1 Cu centre) where the substrate oxidation takes place, and a trinuclear Cu cluster T2 and T3 where the electrons are transferred and where the reduction of oxygen to water take place (Enguita et al., 2003; Smith et al., 2006). Thus, similar catalytic mechanisms for these enzymes are expected. It is, however, important to mention that the *S. cyaneus* laccase activity was measured in the culture supernatant, which might also contain several other laccases not yet identified or reported in databases.

Comparison of sequences of *S. cyaneus* laccase and *S. antibioticus* phenoxazinone synthase

The amino acids sequences of *S. cyaneus* laccase and *S. antibioticus* phenoxazinone synthase are presented below. A high sequence identity (84%, in black) exists between both enzymes. A fifth copper centre, not present in other laccases, was identified. This copper is thought to participate in the stability of the structure but not in the oxidation mechanisms (Smith et al., 2006).

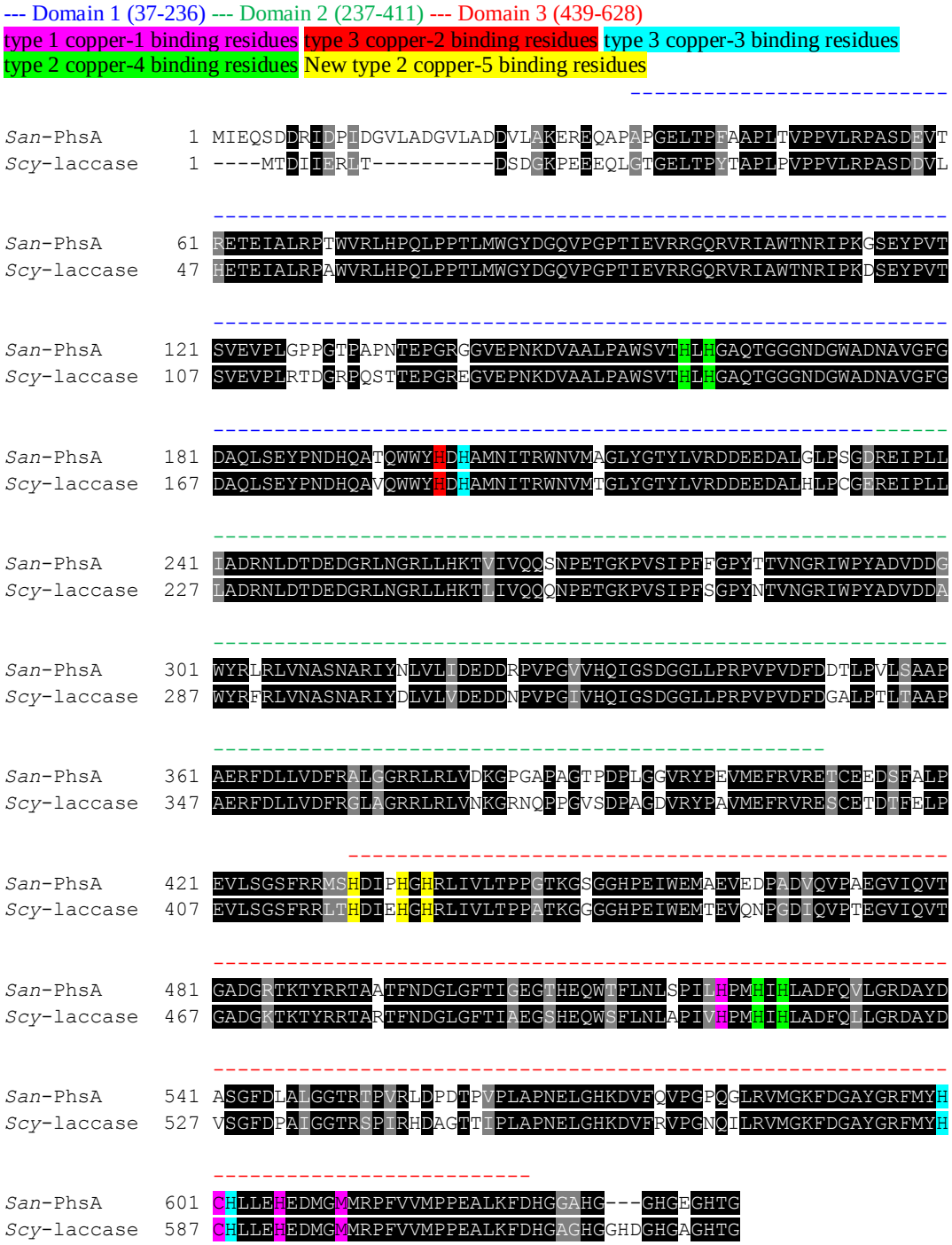


Fig. S 8.4 Comparison of the amino acids sequences of *S. cyaneus* laccase (*Scy*-laccase, GenBank HQ857207) and *S. antibioticus* phenoxazinone synthase (*San*-PhsA, GenBank AAA86668). The residues that bind the different copper atoms (active sites T1 and T2, mononuclear, and T3, binuclear) are presented in colour, following Smith et al.(2006). The proteins are separated into three main domains, presented with the dashed lines with different colours.

Phylogenetic tree between different multicopper oxidases

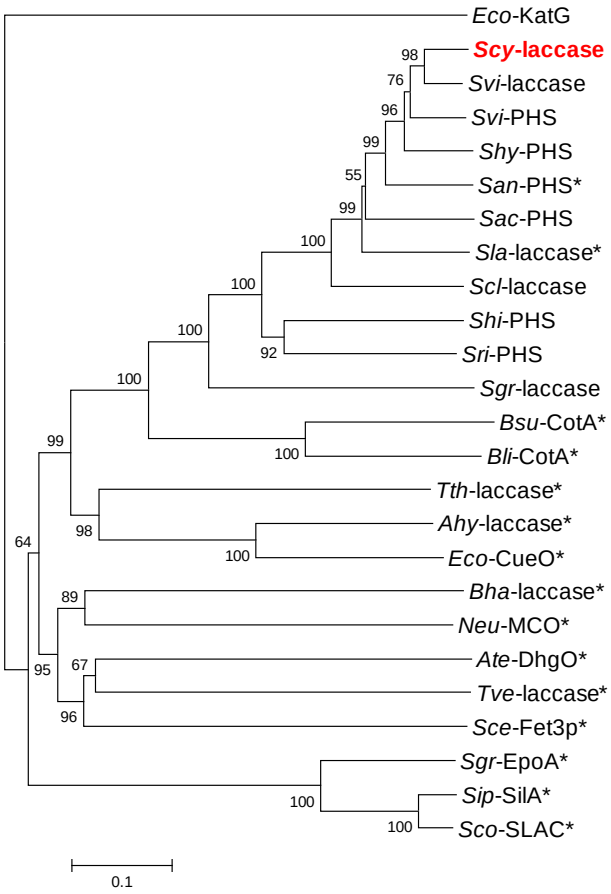


Fig. S 8.5 Sequence likelihood analysis of laccases. The identified laccase of *Streptomyces cyaneus* (*Scy*-laccase, in red, GenBank HQ857207) was compared (using ClustalX and MEGA4) to characterized multicopper oxidases (indicated by *), and to protein sequences found in databases which show a minimal sequence identity of 50% (with >90% sequence length coverage)

Legend for microbial species and sequence references: ***Eco*-KatG**: *Escherichia coli* (GenBank: YP_491509), used here to root the tree; ***Svi*-PHS**: *Streptomyces viridochromogenes* phenoxazinone synthase (WP_003993803); ***Shy*-PHS**: *Streptomyces hygroscopicus* (YP_006248289); ***San*-PHS**: *Streptomyces antibioticus* (AAA86668) (Smith et al., 2006); ***Sac*-PHS**: *Streptomyces acidiscabies* (WP_010360990); ***Sla*-laccase**: *Streptomyces lavendulae* (BAC16804) (Suzuki et al., 2003); ***Scl*-laccase**: *Streptomyces clavuligerus* (WP_003957540); ***Shi*-PHS**: *Streptomyces himastatinicus* (WP_009715166); ***Sri*-PHS**: *Streptomyces rimosus* (WP_004571981); ***Sgr*-laccase**: *Streptomyces griseus* (YP_001821963); ***Bsu*-CotA**: *Bacillus subtilis* (AAB62305) (Martins et al., 2002); ***Bli*-CotA**: *Bacillus licheniformis* (YP_077905) (Koschorreck et al., 2008a); ***Tth*-laccase**: *Thermus thermophilus* (YP_005339) (Miyazaki, 2005); ***Ahy*-laccase**: *Aeromonas hydrophila* (ACX47357) (Wu et al., 2010); ***Eco*-CueO**: *Escherichia coli* (YP_488426) (Grass and Rensing, 2001); ***Bha*-laccase**: *Bacillus halodurans* (AAP57087) (Ruijssenaars and Hartmans, 2004); ***Neu*-MCO**: *Nitrosomonas Europaea* multicopper oxidase (PDB 3G5W) (Lawton et al., 2009); ***Ate*-DhgO**: *Aspergillus terreus* dihydrogeodin oxidase (BAA08486) (Huang et al., 1995); ***Tve*-laccase**: *Trametes versicolor* (CAA77015) (Jönsson et al., 1995); ***Sce*-Fet3p**: *Saccharomyces cerevisiae* (CAA89768) (Stoj et al., 2007); ***Sgr*-EpoA**: *Streptomyces griseus* (YP_001822531) (Endo et al., 2002); ***Sip*-SilA**: *Streptomyces ipomoeae* (ABH10611) (Molina-Guijarro et al., 2009); ***Sco*-SLAC**: *Streptomyces coelicolor* small laccase (CAB45586) (Machczynski et al., 2004).

Chapter 9 Development of a fungal filter to improve micropollutant removal in wastewater

9.1 Introduction

As shown in the previous chapters, the potential of laccase and laccase-mediated reactions for treating micropollutants in municipal wastewater is limited to specific compound classes (mainly phenols and anilines) and is strongly influenced by the pH, with very slow reaction rates in alkaline conditions. Thus, despites their high potential for specific bioremediation purposes (e.g., decontamination of effluents containing targeted toxic pollutants) (Majeau et al., 2010), pure enzymatic treatments with laccase or laccase-mediator systems appear not to be adapted for removing a broad range of pollutants present at very low concentrations in municipal wastewater (near neutral pH) (cf. Chapter 5).

One option to face these limitations while keeping the high potential of laccase would be to treat the water directly with laccase-producing organisms, such as with the white-rot fungi *Trametes versicolor* or *Pleurotus ostreatus*. Indeed, white-rot fungi showed to have a much broader substrate range than laccase alone, due to the combined action of their various non-specific extracellular (laccase, manganese and lignin peroxidases) and intracellular (cytochrome P450 monooxygenases) oxidative enzymes (Yang et al., 2013b). Using the whole fungal cells instead of only some of their specific extracellular enzymes, may thus have many advantages to degrade pollutants (Harms et al., 2011): (i) the fungal cells can produce (via several extracellular oxidases) the hydrogen peroxide (H_2O_2) required for pollutant oxidation with peroxidases (Baldrian, 2008); (ii) compounds oxidized by laccases or peroxidases can be potentially further metabolized inside the cells (e.g., with quinone reductases) and possibly completely mineralized, avoiding accumulation of transformation products such as quinones; (iii) intracellular degradation may directly happen, initiated by the attack of cytochrome P450 or other intracellular enzymes (nitroreductases, phenol-monooxygenases, etc.), leading to mineralization or conjugate formation; and (iv) small fungal metabolites or by-products resulting from lignocellulosic material degradation (e.g., lignin-related phenols) may act as natural mediators, expanding the oxidative potential of laccases (Li et al., 2014).

Due to their powerful and unique oxidative enzymatic system, white-rot fungi have the ability to degrade several micropollutants found in municipal wastewater (Cruz-Morató et al., 2014), including some hardly degradable for bacteria, such as carbamazepine (Jelic et al., 2012), diclofenac (Badia-Fabregat et al., 2014), iopromide (Gros et al., 2014), mefenamic acid (Hata et al., 2010), clofibric acid (Cruz-Morató et al., 2013b), ketoprofen (Marco-Urrea et al., 2010c) or naproxen (Marco-Urrea et al., 2010a).

Using white-rot fungi to treat municipal wastewater is, however, challenging due to the operation conditions very far from their natural habitat (dry dead wood). So far, only few studies have been performed with fungal reactors treating real municipal wastewater under non-sterile conditions. Zhang

and Geissen (2012) were able to maintain the fungus *Phanerochaete chrysosporium* active up to 45 d in a plate reactor treating pre-treated municipal wastewater, but only with the addition of external carbon (glucose) and nitrogen (ammonium) sources. Cruz-Morató et al. (2013a) showed that *Trametes versicolor* could degrade several micropollutants in real municipal wastewater in an air-pulsed fluidized-bed bioreactor, provided that the water was acidified (pH 4.5) and nutrients (glucose, ammonium) supplied. Long-term operation in non-sterile municipal wastewater with *Trametes versicolor* was not reported but experiments with textile wastewater showed that a periodic renewal of the fungal biomass (every 21 d) was necessary for continuous operation under non-sterile conditions (Blánquez et al., 2008). Competition with and predation by other microorganisms appeared to be detrimental for the survival of the fungus. Several strategies have been proposed to deal with non-sterile conditions (Libra et al., 2003): (i) acidification of the medium to pH 3-4.5 to limit bacterial growth (fungi are tolerant to low pH), (ii) limiting nitrogen in the medium to reduce the bacterial development (white-rot fungi can grow on substrates with very low amount of nitrogen (C:N > 350:1) (Boddy and Jones, 2008)), or (iii) growing the fungi on lignocellulosic substrates not easily degraded by bacteria (selective advantage).

Several types of fungal bioreactors have been tested for continuous operation with municipal or industrial wastewaters, including air-pulsed fluidized-bed bioreactors (Blánquez et al., 2008; Cruz-Morató et al., 2014), polyether foam plate bioreactors (Zhang and Geißen, 2012), fungal membrane bioreactors (Yang et al., 2013a), bubble column reactor (Spina et al., 2014), fixed-bed and stirred tank reactors (Rodarte-Morales et al., 2012) or rotating biological contactors (Pakshirajan et al., 2011; Šíma et al., 2014).

Designing a fungal reactor to treat micropollutants in municipal wastewater is challenging due to the low competitiveness of white-rot fungi for simple substrates compared to native microorganisms and their short survival in unsterile wastewaters. In order to be competitive with other advanced treatments, the fungal treatment should, moreover, be affordable for small WWTPs, with low equipment needs, skills and energy requirements.

The main goal of this study was therefore to design a fungal reactor which did not require glucose or expensive substrates addition, without pH adjustment and artificial aeration, and working in the long term under non-sterile conditions.

The survival of the fungus in raw wastewater, with its high content of easily degradable compounds, is expected to be more challenging (strong competition with bacteria) than in treated wastewater (WWTP effluent), where most of the easy substrates for the bacteria are already consumed. Therefore, the fungal treatment should be placed at the outlet of the secondary treatment of the WWTP. An additional substrate for the fungus will thus be required. To avoid too much competition with other organisms and to give a competitive advantage to white-rot fungi, a lignocellulosic substrate could be used. The concept was therefore to develop a filter composed of woodchips, which will serve as support for the mycelium (fixed biomass) and substrate for the fungus. Indeed, white-rot fungi have the unique ability to degrade lignin in wood, a very complex natural polymer, in order to get access to their main substrates: cellulose and hemicelluloses (Baldrian, 2008). As only few organisms (mostly fungi) are

able to grow on wood, this strategy should limit the competition for the substrate and allow longer survival of the fungus in the system.

In order to develop an efficient fungal reactor, the potential of the fungus to degrade micropollutants had first to be evaluated, in comparison with laccase and laccase-mediated reactions. The detailed goals of this study were therefore:

1. To test the ability of *Trametes versicolor* to degraded pollutants degraded by laccase-mediator systems (in batch tests)
2. To assess the range of micropollutants degraded by *Trametes versicolor* in real wastewater with lignocellulosic substrates (in batch tests)
3. To design and test a fungal filter for long-term operation with synthetic and real wastewater

9.2 Materials and methods

9.2.1.1 Choice of micropollutants, chemicals and wastewater

Eleven micropollutants which could be analysed by HPLC-DAD were selected as model compounds to assess and monitor the efficiency of the fungal systems for the treatment of micropollutant in wastewater. Three compounds were selected due to their possible oxidation by laccase-mediator systems but not by laccase alone (cf. Chapter 5): the anti-inflammatory drug naproxen (NPX), the antibiotic sulfamethoxazole (SMX) and the herbicide isoproturon (IPN). NPX and SMX are found in relatively high concentrations in WWTP effluents (cf. Chapter 3) and are thus interesting compounds to monitor. Moreover, degradation of NPX was proposed as an indicator of white-rot fungal activity as this drug is easily degrade by the fungi but not that well by bacteria (Rodríguez-Rodríguez et al., 2010). Three other pollutants were selected for their property to be oxidized by extracellular laccase: diclofenac (DFC), mefenamic acid (MFA) and bisphenol A (BPA). DFC and MFA are poorly removed in conventional treatment and found in relatively high concentrations in WWTP effluents (cf. Chapter 3). DFC may generate, moreover, impacts on sensitive organisms at very low concentrations (cf. Chapter 1). Finally, five other pollutants poorly removed in WWTPs and among the most concentrated in effluents (up to several µg l^{-1}) (cf. Chapter 3) were chosen: the anti-epileptic carbamazepine (CBZ), the corrosion inhibitor benzotriazole (BTZ), the antidiabetic metformin, the beta-blocker metoprolol and the antibiotic trimethoprim.

Most of the chemicals used were purchased from either Sigma-Aldrich (Buchs, Switzerland) or Fisher Scientific AG (Wohlen, Switzerland). Deuterated micropollutant standards were purchased from Sigma-Aldrich, Dr. Ehrenstorfer, TRC (Toronto Research Chemicals), Sequoia Research Products, Acros Organics, and TCI, or were obtained from Ciba-Geigy and Altana Pharma.

Real treated wastewater (TWW) was collected at the outlet of Lausanne municipal WWTP (220,000 population equivalent). 24-h composite samples (time proportional sampling every 15 min) were collected after the biological treatment, with either activated sludge without nitrification or moving bed bioreactor with partial nitrification. Depending on the experiment, the sampled wastewater, if not

used directly, was either frozen (-18°C, for experiment with real micropollutant concentration) or stored at 4°C (for experiments with spiked wastewater).

9.2.1.2 Laccase activity test

Laccase activity was determined as described in Chapter 5. One unit of activity (U) was defined by the oxidation of one µmol of ABTS per min, at pH 4.5 and 25°C.

9.2.1.3 Analyses of micropollutant, glucose methanol and standard parameters

9.2.1.3.1 Micropollutant analysis at high concentrations

Determination of micropollutant concentrations at the mg l^{-1} range was carried out by reverse phase liquid chromatography with a diode-array detector (HPLC-DAD) as described in Chapter 5. To analyse mixtures of pollutants (metformin, trimethoprim, metoprolol, BPA, DFC and MFA), a 1-h HPLC gradient from 98:2 to 35:65 of H_2O:methanol (% v/v, with 0.1% acetic acid) was used. To analyse individual compounds, shorter isocratic methods (around 20 min) were developed, with a ratio H_2O:methanol (% v/v, with 0.1% acetic acid) of 53:47 for NPX, 86:14 for SMX, 58:42 for IPN, 63:37 for CBZ, and 92:8 for BTZ. Detection of the compounds was done at 232 nm for NPX, 268 nm for SMX, 242 nm for IPN, 214 nm for CBZ, 202 nm for BTZ, 234 nm for metformin, 208 nm for trimethoprim, 276 nm for DFC, 354 nm for MFA, and 200 nm for BPA and metoprolol. The limit of detection (LOD) was, for all compounds, between 0.1 and 0.3 mg l^{-1} (~1 µM).

9.2.1.3.2 Micropollutant analysis at low concentrations

Analysis of micropollutants at the low ng l^{-1} to µg l^{-1} range in real municipal wastewater was performed with a screening method for 44 compounds (Table IX.3, Appendix IX) by two different methods, either with (i) off-line solid phase extraction (SPE) or (ii) on-line SPE, both followed by ultra-performance liquid chromatography (UPLC) (Acquity UPLC system, with HSS T3 or BEH C18 column depending on the methods, from Waters, USA) coupled to a tandem quadrupole mass spectrometer (MS/MS) (Xevo TQ MS, Waters).

The off-line SPE method, with hand-assembled two layers cartridges (Oasis HLB and mixture of Strata X-CW, Strata X-AW and Isolute ENV+ phases), was similar to the one used in Chapter 3 and described by Morasch et al. (2010), at the difference that only 50 ml of sample were extracted, and that the samples were not acidified but adjusted to neutral pH prior the extraction (to avoid pollutant degradation in acidic conditions). The cartridges were therefore also conditioned with non-acidified water and methanol. After the extraction, cartridges were dried 30 min under air stream and frozen (-18°C) (up to one month) until the elution. Elution of the cartridges was performed just before the analysis as described by Morasch et al. (2010). Eluate fractions were concentrated at 40°C under a gentle N_2 stream to a volume of 500 µl and then diluted 2.5 times with the aqueous UPLC eluent, prior to the injection (10 µl) in the UPLC column. Two different UPLC methods, either with acidic or neutral eluents, were used depending on the compounds. The conditions of the UPLC gradient and the compounds analysed by each methods are presented in Appendix IX.

For the on-line SPE (with cartridge 2.1 × 20 mm, Oasis HLB 25 µm phase, Waters) and the following UPLC separation, the same method than the one presented in Chapter 5 was used, with 5 ml of sample

extracted and injected. Two different UPLC methods, either with basic or neutral eluents, were applied depending on the compounds. The conditions of the UPLC gradient and the compounds analysed by each methods are presented in Appendix IX.

Target compounds were identified and quantified using tandem mass spectrometry (MS/MS) (Xevo TQ MS, Waters) in positive and negative electrospray ionization modes (ESI), and detected in multiple reaction monitoring mode (MRM), according to Morasch et al. (2010). Losses during extraction and matrix effects were corrected by adding internal standard (deuterated pollutants at 250 ng l^{-1}) before processing the samples. MS/MS conditions for each pollutant and for their associated deuterated standards are presented in Appendix IX. Extraction efficiencies and repeatability of the method in municipal wastewater are detailed by Morasch et al. (2010). A set of seven standards covering a wide range of concentrations (factor 200 to 450 between the lowest and highest concentrations tested) was used to determine the calibration curves. With the off-line SPE method, the standards (spiked with the deuterated surrogates) were prepared in the aqueous UPLC eluent and directly injected into the UPLC-MS/MS without passing by an SPE step. With the on-line SPE method, the standards (spiked with the surrogates) were prepared in Evian bottle water (due to its composition close to surface waters) and then processed as the other samples by SPE prior to be injected into the UPLC column.

The advantage of the off-line method was the better purification of the samples (less matrix effect than with the on-line SPE) and the possibility to adapt the SPE cartridge for the analysis of very polar pollutants (such as metformin). This method required, however, relatively big volumes of samples (min 80 ml) and was time consuming, thus not very adapted for long-term monitoring of the fungal filters. The second method, with the on-line SPE, reduced the volume of sample required to only 12 ml and simplified substantially the cleaning and concentration procedures. This method, despite the stronger matrix effect, was thus selected for the fungal filter monitoring. Both methods had relatively similar sensitivity, with LOD, in samples with strong matrix effect, between 1 and 30 ng l^{-1} for most compounds (cf. Table 9.4).

9.2.1.3.3 Methanol and glucose analysis

Methanol and glucose concentrations were determined by HPLC equipped with an ORH-801 column (from Transgenomic) and with a refractive index (RI) detector (RI-2021plus, Jasco, Tokyo, Japan). A total of 20 µl of sample were injected and separation of the compounds was conducted under isocratic condition at 0.5 ml min^{-1} with a mobile phase composed of 5 mM H_2SO_4 in pure water, during 18 min at 35°C. Limits of detection were at 0.1 g l^{-1} (3 mM) and 0.01 g l^{-1} (0.05 mM) for methanol and glucose, respectively.

9.2.1.3.4 Standard parameters analysis

Total suspended solids (TSS) were determined by filtration of a known volume of sample at 0.45 µm (mixed cellulose ester Whatman filters). The dry weight of the filter (beforehand washed and dry at 105°C) was measured before and after the filtration to determine the dry suspended solids.

Dry matter and water content were determined by overnight drying the sample at 105 °C. The mineral content was determined after 2 h combustion of the samples at 550 °C.

Total viable bacteria were determined by counting the number of colonies (colony forming unit, CFU) at different dilutions of the sample (1 to 1000 times in NaCl – 8 g l^{-1}) after incubation 1-2 d at 30°C on Plate count agar medium (from Merck: peptone from casein – 5 g l^{-1}, yeast extract – 2.5 g l^{-1}, D(+)glucose – 1 g l^{-1}, agar-agar – 14 g l^{-1}, pH 7) (plate count method).

Major anions and cations were determined by ion chromatography with conductivity detector (Dionex DX 500), and dissolved organic carbon (DOC) and total dissolved nitrogen were analysed by catalytic combustion (Shimadzu TNM1).

9.2.1.4 Correction of the micropollutant losses by filtration

Prior to the analysis by HPLC-DAD, all samples had to be filtered at 0.22 µm to remove particles and microorganisms. The effect of the filtration on micropollutant concentrations was tested with several different syringe filters: filters in polyethersulfone (PES) (Filtropur S, 0.2 µm, diameter 25 mm, sterile, from Sarstedt), in nylon (Simplepure Nylon 66, 0.22 µm, 13 mm, from BGB), or in polypropylene (PP) with glass microfibers (GMF) (Simplepure PP-GMF, 0.22 µm, 25 mm or 13 mm, from BGB). The pollutants of interest were spiked at 10 mg l^{-1} in citrate-phosphate buffer solution (20-40 mM) at different pH and the solutions were analysed with or without filtration (1 ml filtered). A first test with different filters with NPX showed (Fig. 9.1 A) that important losses occurred, especially with PES (Filtropur) and nylon (BGB) filters in acidic pH, with more than 90% losses below pH 4. Fewer losses were observed with GMF filters and only below pH 5.5. GMF – 13 mm filters were thus selected for the experiments. As NPX losses were pH dependent, a sigmoid curve modelling the losses with GMF – 13 mm filters as a function of the pH was fitted to the data (Fig. 9.1 B). This model allowed correcting the NPX chromatogram surfaces for the losses due to the filtration (Eq. 9.1). The UV/Vis absorbance of NPX was also affected by pH, with less absorbance at lower pH, due to the protonation of NPX (pKa around 4.2). This loss of signal was also modelled with a sigmoid curve (Eq. 9.2) (Fig. 9.1 C). These two models allowed correcting the NPX chromatogram surface for the losses due to filtration and the losses of signal due to pH (compared to pH 7) (Eq. 9.3), and therefore avoiding overestimating NPX removal due to analytical and processing artefacts. This correction was applied for all NPX analyses by HPLC-DAD. An example of the unfiltered, filtered and corrected HPLC-DAD signal for NPX at different pH is presented in (Fig. 9.1 D).

Equations used to correct the NPX signal as a function of pH

$$S = \frac{0.528}{1 + 48603\ \exp(-2.286\ pH)} + 0.476 \tag{9.1}$$

$$F = 1 - \left(0.7478 \left(1 - \frac{0.9895}{1 + 20000\ \exp(-2.1699\ pH)} \right) \right) \tag{9.2}$$

$$Corrected\ signal_{pH7} = \frac{Signal}{S\ F} \tag{9.3}$$

With *S*, the correcting factor for the losses of UV-Vis signal as a function of pH of the solution (*pH*) compared to pH 7; *F*, the correcting factor for the losses of NPX by filtration with GMF-13mm filters; and *Signal*, the chromatogram NPX surface measured at the pH of the solution.

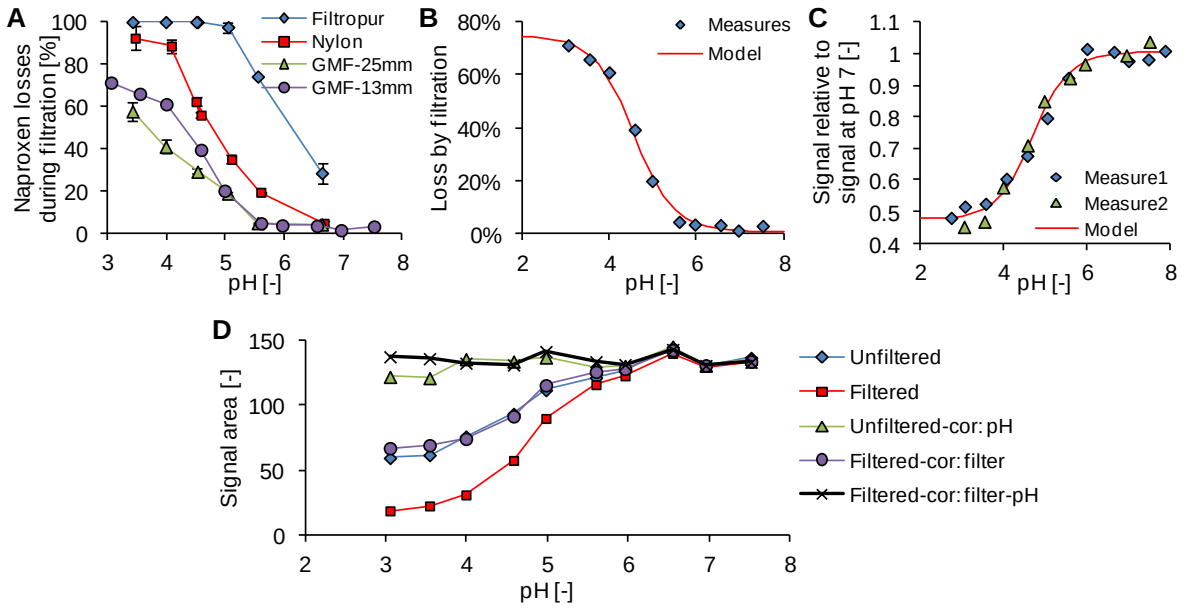


Fig. 9.1 (A) Naproxen losses during filtration with different filters as a function of the pH. Filtration of 1 ml with filters Filtropur S (PES, 0.2 µm, 25 mm, sterile), filters BGB Nylon (0.22 µm, 13 mm) and filters BGB PP-GMF (0.22 µm, 25 or 13 mm). **(B)** Adjustment of a sigmoid curve (model) to the losses by filtration with the GMF-13mm filters. **(C)** Adjustment of a sigmoid curve to the losses of UV-Vis signal due to change of pH (compared to pH 7). **(D)** Example of corrections of naproxen losses during filtration (cor: filter) and UV-Vis signal change (cor: pH) as a function of the pH of the solution.

For most of the other pollutants tested (SMX, IPN, BTZ, CBZ, metformin, trimethoprim, metoprolol, BPA and DFC), no strong effect (< 15% losses) of the filtration (with GMF filters 13 mm) or losses of UV/Vis signal (< 20%) with change of pH were observed from pH 5 to 8. However, for MFA, high losses (up to 90%) were observed during filtration at pH 5, but only 16% losses were measured at pH 5.8. As degradation of this compound was tested mostly at pH higher than 5.8, no correction of the filtration was performed for MFA.

9.2.1.5 Fungal strain and inoculum preparation

Pure strains of two white-rot fungi, *Trametes versicolor* (ATCC 42530 from the American Type Culture Collection) and *Pleurotus ostreatus* (wild strain isolated by Daniel Job in the 80s and conserved at the university of Neuchâtel, kindly provided by the same person), were maintained by sub-culturing them every 1-2 months on 20 g l^{-1} malt extract agar (15 g l^{-1}) slants (pH 4.5) at 25°C. A mycelial suspension of each fungus was prepared by homogenizing 5-11 d grown mycelium in malt extract medium (20 g l^{-1}, pH 4.5) as described by Blánquez et al. (2004) (cf. Appendix V), and then stored up to a few months in saline solution (NaCl – 8 g l^{-1}) at 4°C until use as inoculums (8.5 g l^{-1} dry volatile solid mycelium).

9.2.1.6 Fungal pellet preparation

Fungal pellets (1-3 mm) were prepared by growing the fungal mycelium in malt extract medium (20 g l^{-1}, pH 4.5) at 25°C with agitation at 130 rpm during 5-7 d, until pellets were well developed. Pellets were then collected, rinsed with sterile water and used directly for the experiments (solid concentration of the pellets (dry weight): 17.5 to 22 g l^{-1}, with 14% mineral). (Protocol detailed in Appendix VI).

9.2.1.7 Woodchips selection and preparation and wood inoculation

Several wood species were tested as substrate for *T. versicolor* and *P. ostreatus* mycelium development: oak (*Quercus* genus), beech (*Fagus* genus), ash (*Fraxinus* genus), birch (*Betula* genus) and spruce (*Picea* genus). Dry branches of 10 to 40 mm of diameter were collected in the forests of the Swiss Plateau and shredded with the bark in woodchips of 5 – 15 mm length with a garden shredder (BioQuick 2500, Atika). Before use, woodchips were washed several times with tap water to remove the dust and small particles and soaked 30 min in water to saturate them. The water surplus was drained and the wet woodchips were autoclaved 30 min at 121°C. Sterile woodchips were then inoculated with 4% (v/v) of mycelial preparation and well mixed under sterile conditions. The wood was then incubated at 25°C during 5 – 7 d for *Trametes versicolor* or 2 – 3 weeks for *Pleurotus ostreatus*. When the woodchips were completely colonized by the mycelium (all white), they were well mixed to homogenize the mycelium and transferred to non-sterile glass columns. In each column, 65 g of inoculated wood (about 20 -21 g dry weight) was added. The columns were then slightly shaken (but not compacted) to distribute the woodchips evenly and avoid large voids, and again incubated at 25°C for 2 – 4 d for *Trametes versicolor* or 1 – 2 weeks for *Pleurotus ostreatus*. Once the mycelium has covered again completely the wood substrate, the columns were ready to be use to treat wastewater. The inoculated wood contained 65 – 70 % water (w/w) and 2% (dry weight) of inorganics.

9.2.1.8 *Trametes versicolor* growth inhibition assay

In order to use fast and cheap analytical HPLC-DAD methods for micropollutant analysis, relatively high initial micropollutant concentrations (up to 10 mg l^{-1}) were necessary. The effect of such concentrations on *Trametes versicolor* growth was tested for 9 pollutants prior the degradation tests: NPX, SMX, IPN, DFC, MFA, BPA, CBZ, metoprolol and triclosan. Fungal growth inhibition was performed in 96 well plates with an adapted version of the method developed by Stephen Mackay (EPFL-LBE, personal communication) to determine filamentous fungi growth kinetics (method described in Appendix X). Briefly, a sterile growth medium consisting of malt extract at 2 g l^{-1} at pH 5, spiked with individual micropollutants at 10 mg l^{-1} (with 1% v/v methanol), was inoculated with 3×10^5 cells (or spores) ml^{-1} of *Trametes versicolor* in the wells of the plate. The plate was then incubated at 28°C and the optical density (OD) was monitored at 405 nm during 96 h. Eight replicates per pollutants and per control were performed, with one control without pollutant and one control without pollutant but with 1% (v/v) methanol. At the end of the test, the growth curves were compared with the ones of the controls to determine the growth inhibition.

9.2.1.9 Micropollutant degradation assay in submerged cultures in UAB medium

To test the ability of *Trametes versicolor* to degrade pollutants which can be oxidized by laccase-mediator systems, pure fungal culture in sterile synthetic wastewater was used. The synthetic wastewater was composed of a mineral medium (UAB medium) with micro and macronutrients, as presented in Appendix III (adapted with minor changes from Blánquez et al. (2004) and Borràs et al. (2008)), glucose – 8 g l^{-1} as carbon source, $(NH_4)_2SO_4$ – 2.3 g l^{-1} as nitrogen source, (2-(*N*-morpholino)ethanesulfonic acid) (MES) or (3-(N-morpholino)propanesulfonic acid) (MOPS) buffers at 10 mM for pH range from 5.5 to 6.7 and 6.7 to 7.9, respectively, and micropollutants spiked at 10

mg l^{-1} (with 1% (v/v) methanol). The pH was adjusted to the desired value with HCl or NaOH (1 M), and the synthetic wastewater was sterilized by filtration (Filtropur S, 0.2 µm, sterile) and inoculated with 20% (v/v) of *Trametes versicolor* pellets (final: 3.5 g l^{-1}, dry weight). Batch experiments were performed in 100-ml Erlenmeyer flasks containing 50 ml of inoculated synthetic wastewater. Cultures were incubated at 25°C for 15 d and shaken at 130 rpm to ensure aerobic conditions. After defined time periods (every 1-2 h during the first 7 h, then every day), aliquots of 2 ml were withdrawn in aseptic conditions, filtered at 0.22 µm (BGB PP-GMF filters), and used to determine micropollutant (by HPLC-DAD) and glucose concentrations, laccase activity, and pH. Three micropollutants were tested individually, all at 10 mg l^{-1}: NPX (at initial pH 5.5, 6.5 and 7.5), SMX (pH 5.5) and IPN (pH 5.5). For each pollutant, one control in the same conditions but with fungus inhibited by 10 mM sodium azide was performed, and for NPX, a second control with not inoculated synthetic wastewater was also done. Experiments were performed in duplicates.

9.2.1.10 Micropollutant degradation assay in submerged batch cultures in real wastewater

Degradation of micropollutants in real wastewater by *Trametes versicolor* was tested in batch experiments with treated wastewater (TWW) from the municipal WWTP of Lausanne. TWW was collected (10 l of 24-h composite sample) at the outlet of the activated sludge tank with partial nitrification (12 mg N-NH_4 l^{-1}) the 7th of December 2012. TWW was centrifuged (15 min at 15,900 × g and 15°C), filtered (not in aseptic conditions) at 0.22 µm (mixed cellulose ester membranes, from Millipore) to remove most of the microorganisms, and stored for 3 d at 4°C before the experiment. TWW was used without any pH adjustment (initial pH 7.89) or micropollutant spiking to be close to real conditions.

As shown in Chapter 8, *Trametes versicolor* was able to survive in sterile TWW with lignocellulosic material as sole substrate. Using lignocellulosic substrates is thus a strategy to avoid strong competition for the substrate with other microorganisms present in wastewater. Based on previous experiments (Fig. 8.9, Chapter 8), wheat straw was selected as lignocellulosic substrate. The straw (*M classic for rabbit litter* from Migros) was chopped in section of 0.5 to 2 mm, washed with deionised water to remove the dust, dried overnight at 105°C, and autoclaved 20 min at 121°C.

In 5-l sterile Erlenmeyers, 1.5 l of filtered TWW containing 15 g l^{-1} of autoclaved wheat straw was inoculated with 150 ml of fresh pellets from *Trametes versicolor* to reach a final biomass concentration of 2.0 g l^{-1} (dry weight). These batch cultures were then incubated in the dark at 25°C during 14 d on a rotary shaker at 150 rpm. After defined time intervals (2 h, 1, 2, 4, 7, 10, and 14 d), aliquots of 80 to 160 ml were withdrawn in aseptic conditions from each culture, centrifuged 10 min at 15,000 *g*, and filtered at 0.22 µm on mixed cellulose ester membranes the first two days and then at 0.45 µm with PES Filtropur S or GMF 0.45 Whatman syringe filters. Samples (50 ml) were then processed by off-line SPE and concentrations of 44 micropollutants were determined by UPLC-MS/MS. At the same time, laccase activity and pH were analysed. Two controls were performed: (i) with TWW and wheat straw without inoculation with *Trametes* pellets, to evaluate the losses of pollutants by adsorption onto the straw or by microbial degradation, and (ii) with TWW, wheat straw and inactivated (autoclaved) pellets, to evaluate the loss by adsorption onto the pellets. The degradation test and the controls were performed in duplicates.

9.2.1.11 Long term survival experiment

To evaluate how long *Trametes versicolor* could survive in TWW with only lignocellulosic substrates as carbon source, the fungus (pure culture) was cultivated in batch submerged culture in sterile TWW with either wheat straw or wood chips as sole substrate. Erlenmeyers (1 l) containing 400 ml of autoclaved TWW (collected at the outlet of the moving bed bioreactor with nitrification (pH 6.84) in Lausanne WWTP), and either 20 g l^{-1} of chopped wheat straw or 100 g l^{-1} of dry beech woodchips, were inoculated with 4% (v/v, 0.34 g l^{-1} dry volatile solid) of *Trametes* mycelial preparation. Erlenmeyers were then incubated at 25°C and shaken at 130 rpm during one year. Samples of 1 ml were regularly withdrawn in aseptic conditions to monitor pH and laccase activity.

9.2.1.12 Design of a fungal filter

Several configurations of fungal filters were tested to find a system that allows the fungus to survive while treating efficiently the wastewater. The first configuration tested was a trickling filter, where the water, spread at the surface with a shower system, trickled through the unsaturated woodchips/mycelium support (detailed results presented by Vargas (2013)). Although this solution allowed good development and survival of the mycelium on the wood, probably due to the constant unsaturated conditions (closer to their natural habitat), this system was ineffective or not reliable to treat water. Indeed, in trickling filters, water follows preferential pathways (unsaturated medium). If this is not a big issue in conventional trickling filters, as the biofilm develops where the water (which contains the substrate) flows, it was the opposite in our system: the mycelium disappeared where the water was flowing (too stressful conditions) and developed well in the more dry wood parts. Addition of a soluble substrate, glucose, allowed the growth of *Trametes* mycelium in the whole filters, especially where the water flowed. This led, however, to the clogging of the filter by the mycelium and to a short survival (less than 10 d) of *Trametes* in the system, probably due to the strong competition for the glucose. The low and unpredictable contact between the mycelium and the water in trickling filters made this system not reliable and not adapted for our purpose.

As completely saturated conditions, which allow a good contact water-mycelium, were too stressful for the fungi (short survival), the idea was to alternate saturated (for good contact water-mycelium) and unsaturated conditions (closer to the natural habitat of the fungi). A sequential batch filter was thus developed (Fig. 9.2). This filter was composed of a glass column (35.9 mm internal diameter, 250 mm height) filled at 60% (21 g dry weight) with beech woodchips colonized by the fungal mycelium. An electro-valve allowed automatically closing or opening the bottom of the filter. A pump, also controlled automatically, allowed filling the column and recirculating the water. This filter worked with sequential operations repeated every 5 min (Fig. 9.2): (i) the water to treat, 150 ml per batch cycle stored in a 500-ml bottle, was pumped in the filter at a flow of 66.7 ml min^{-1} during 1 min with the bottom valve of the filter closed (Filling of the filter). (ii) Water was then left in contact with the mycelium during 1 min to allow micropollutant degradation (Reaction time). (iii) The bottom valve was then open to drain the filter in the storage tank (Emptying), and (iv), the filter was maintained 3 min in unsaturated conditions to allow for fungus recovery (Resting time). These sequential operations were repeated during 24 h up to several days until micropollutants were degraded. The treated water was then discarded manually and replaced by 150 ml of fresh wastewater to start a new batch cycle.

The filters were operated during several months with the same wood/mycelium substrate. The duration of each operation (1 min reaction time, 3 min resting time) was arbitrary defined based on previous experiments. Longer resting times than reaction times were chosen to give more chance to the fungus to survive in the system. The phase durations were programmed in an automat which was regulating the pump and the valve. This sequential mode of operation was applied for all the experiments described below. The set-up was composed of eight columns in parallel (Fig. 9.4 E), working in synchronisation, which allowed testing several conditions at the same time. Columns and bottles were open to the ambient air (not in a closed sterile environment) to be closer to real conditions in WWTPs and the system was operated at ambient temperature (22-24°C).

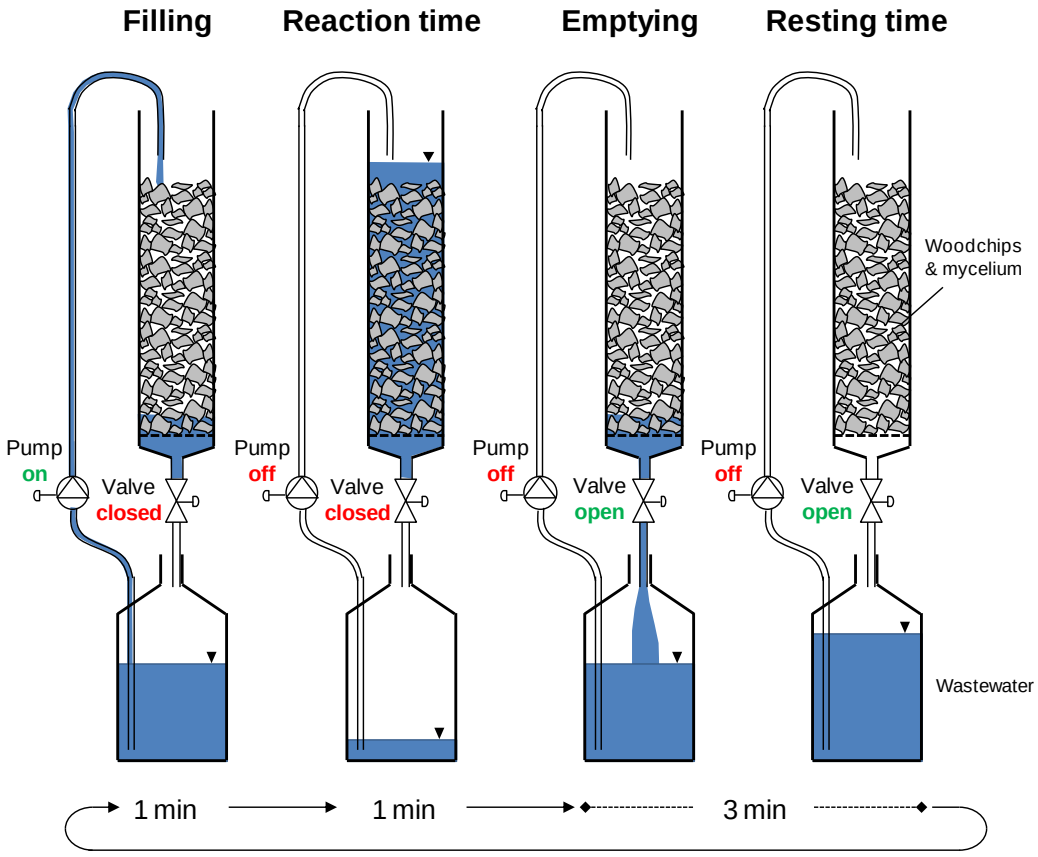


Fig. 9.2 Configuration and sequential operation of the fungal wood filter. Water was treated in batch mode with periodic recirculation (every 5 min) in the filter. Every 1-3 days, the treated water was replaced by raw wastewater and a new batch cycle was started. Illustration of the same filter during different times of the process.

9.2.1.13 Selection of the fungal strain – competition and survival in the filter

Although *Trametes versicolor* is known for its ability to degrade many micropollutants, this fungus is not necessarily the best suited organism for a fungal filter treating municipal wastewater. Indeed, in addition to good degradation capacities, it should also be able to survive long enough in the filter. As, in preliminary experiments, we did not succeed to make survive *Trametes versicolor* in our systems more than 1 – 2 weeks (Vargas, 2013), another white-rot fungus was also tested. Based on the advices of Dr. Daniel Job (University of Neuchâtel), an expert in applied mycology for industrial processes, the fungus *Pleurotus ostreatus* was selected. *Pleurotus ostreatus* is known to be more resistant and more competitive than *Trametes versicolor* (D. Job, personal communication and Stamets (2005)) and

therefore could possibly resist longer in the fungal filter. These two fungi (Fig. 9.3) are common species in temperate climates in Europe (Borgarino and Hurtado, 2011; Stamets, 2005).

Fig. 9.3 *Trametes versicolor* (left) and *Pleurotus ostreatus* (right) in their natural environment. Sources: *Trametes*: mycorance.free.fr/valchamp/champi61.htm; *Pleurotus*: www.mykoweb.com/CAF/species/Pleurotus_ostreatus.html.

Comparisons between these two white-rot fungi were made for (i) their ability to colonize and to compete for woodchips in unsaturated conditions (Competition experiment) and (ii) their performance for NPX degradation in synthetic wastewater in the sequential batch fungal filter (Fungal filter experiment). The first experiment is described below, the second in the next section (9.2.1.14).

9.2.1.13.1 Competition experiment

Sterile wet beech woodchips were inoculated by mycelium of either *Trametes versicolor* or *Pleurotus ostreatus* (in pure culture). Once completely colonized, the woodchips were disposed in each side (one for each fungus) of a horizontal glass column which contained in the middle fresh autoclaved wet woodchips (not inoculated). The column was then incubated in dry conditions at 25°C for 120 d. The colonization of the fresh wood by the two fungi or by other organisms present in the column (green mould) and any change in the column were regularly visually monitored. This experiment was conducted in duplicate.

9.2.1.14 Micropollutant removal in the fungal filters – continuous operation in synthetic wastewater

The micropollutant removal efficiency of the fungal filters inoculated with *P. ostreatus* mycelium was tested in several long-term experiments (continuous operation) with 10 different micropollutants in synthetic wastewater. Synthetic wastewater consisted of (unsterile) tap water in which different micropollutants (depending on the experiment) were dissolved at concentration between 5 to 20 mg l^{-1}. Micropollutants were not spiked from a concentrated stock solution to avoid adding methanol in the water. The initial pH of the synthetic wastewater was around 7.8. Tap water, coming mainly from Geneva Lake, was selected as a simple model for TWW, as tap water is the main component of wastewater and has therefore relatively similar ionic composition. Of course, TWW has a more complex matrix, with, in particular, much higher microorganism, DOC and TSS concentrations (cf. Table 9.2). Therefore, experiments in real TWW were also conducted in a second stage.

The fungal filters were operated in sequential reaction/resting phases (5 min cycle) as explained before, with water renewed (batch cycles) every 24 h to 7 days. The filters were operated continuously

during 40 to 140 d (7 to 31 batch cycles depending on the experiments). Micropollutant residual concentrations as well at laccase activity and pH were regularly monitored by withdrawing 2 ml of treated water during each sampling. 1 ml was filtered (PP-GMF filters, 0.22 µm) for micropollutant analysis (in the few hours following the sampling) and the other was directly used for laccase and pH determination.

Naproxen in synthetic wastewater: Fungi comparison and long term experiment

Three wood filters inoculated with *P. ostreatus* mycelium and three other inoculated with *T. versicolor*, as well as two wood filters not inoculated (controls) were tested in parallel for their efficiency to treat NPX at 10 mg l^{-1} in synthetic wastewater. After 16 d, only two filters with *P. ostreatus* were maintained and their long term efficiency was monitored during 120 d (31 batch cycles).

Sulfamethoxazole in synthetic wastewater

Two wood filters inoculated with *P. ostreatus* mycelium and one wood filter not inoculated (control) were tested in parallel for their efficiency to treat SMX at 10 or 5 mg l^{-1} in synthetic wastewater during a period of 80 d (21 batch cycles).

Carbamazepine in synthetic wastewater

The three filters (two inoculated with *P. ostreatus* and one control) used for the 80-d experiment with SMX were then used to treat CBZ at 10 mg l^{-1} for 60 d more (7 cycles).

Benzotriazole in synthetic wastewater

One wood filter inoculated with *P. ostreatus* mycelium and one wood filter not inoculated (control) were tested in parallel for their efficiency to treat BTZ at 10 mg l^{-1} during 64 d (7 batch cycles).

Mixture of mefenamic acid, diclofenac, bisphenol A, metoprolol, trimethoprim and metformin

Two wood filters inoculated with *P. ostreatus* mycelium and one wood filter not inoculated where the microbial activity was inhibited with 10 mM sodium azide (inhibited control), were tested in parallel for their efficiency to treat a mixture of MFA (20 mg l^{-1}), DFC (5 mg l^{-1}), BPA (20 mg l^{-1}), metoprolol (5 mg l^{-1}), trimethoprim (5 mg l^{-1}) and metformin (10 mg l^{-1}) in synthetic wastewater during a period of 42 d (14 batch cycles). As MFA, DFC and BPA are oxidized by extracellular laccase, 10 mM of sodium azide were added in the samples (1% v/v) for micropollutant analysis just after the sampling to stop the reaction (inhibition of laccase activity).

Near the end of some of these experiments (with NPX, CBZ and BTZ), methanol at 1% (v/v) was added in the synthetic wastewater to assess the effect of an external carbon source on micropollutant degradation. Methanol consumption was also monitored.

9.2.1.15 Identification of the removal mechanisms

Several removal mechanisms may happen in the filters: adsorption, fungal degradation (by the whole cells or by extracellular enzymes) or microbial degradation by native microorganisms. To identify the

main removal mechanisms, several strategies were applied: (i) control experiments with wood filters not inoculated with fungal mycelium, to study the adsorption on the wood and the microbial degradation, (ii) biological activity inhibition in the filters by addition of 10 mM of sodium azide (azide anions inhibit several metabolic functions, such as the activity of cytochrome oxidases (respiratory inhibition), catalases, peroxidases, laccase, ATPases, etc. (Rachutin Zalogin and Pick, 2014)) to evaluate the effect of adsorption alone (on the wood or on the wood colonized by the mycelium), (iii) desorption experiments (batch cycle with synthetic wastewater without pollutant) to measure the desorption of the pollutants from the wood/mycelium, and thus evaluate the importance of the adsorption phenomenon, (iv) modelling of adsorption kinetics/equilibrium and biodegradation kinetics to confirm the main removal mechanisms, and (v) degradation experiments with cell-free supernatants from the filters to evaluate the role of extracellular enzymes, or with whole supernatants to evaluate the role of the free compared to the fixed biomass. These different strategies were applied for some micropollutants, mainly NPX, during the experiments described in section 9.2.1.14.

9.2.1.16 Micropollutant removal in the filter –continuous operation with real municipal wastewater

As the synthetic wastewater contains much less microorganisms, TSS and DOC than real treated wastewater, two experiments in real TWW were performed to evaluate their impact on the efficiency of the fungal filters: (i) with TWW spiked with NPX at 10 mg l^{-1}, and (ii) with TWW at the real micropollutant concentrations (not spiked).

NPX in treated wastewater

TWW was collected (20 l of 24-h composite sample) on 16 January 2014 in the effluent of the moving bed bioreactor (with partial nitrification, 6.6 mg $N\text{-}NH_4$ l^{-1}) at the WWTP of Lausanne. As there was a problem with the secondary clarifier, the sample contained high concentrations of TSS and was therefore centrifuged 20 min at 6200 *g* to reduce the TSS content. The whole TWW was then stored at 4°C and progressively used as raw water for each new batch cycles of the experiment.

Two wood filters inoculated with *P. ostreatus* mycelium and one wood filter not inoculated (control) were tested in parallel for their efficiency to treat NPX at 10 mg l^{-1}, dissolved in real TWW, during a period of 140 d (29 batch cycles). NPX, laccase activity and pH were regularly monitored.

Treated wastewater in real conditions

TWW was collected (20 l of 24-h composite sample) on 12 June 2014 in the effluent of the moving bed bioreactor (with partial nitrification, 9.2 mg $N\text{-}NH_4$ l^{-1}) at the WWTP of Lausanne. The composite sample was well mixed (homogenized) and then separated in 20 individual bottles of 1 l each, directly frozen at -18°C. One day prior starting a new batch cycle with the fungal filters, one bottle of TWW was thawed at 4°C and then used for the batch cycle (150 ml per filters). This procedure allowed having very similar raw TWW for every batch cycles.

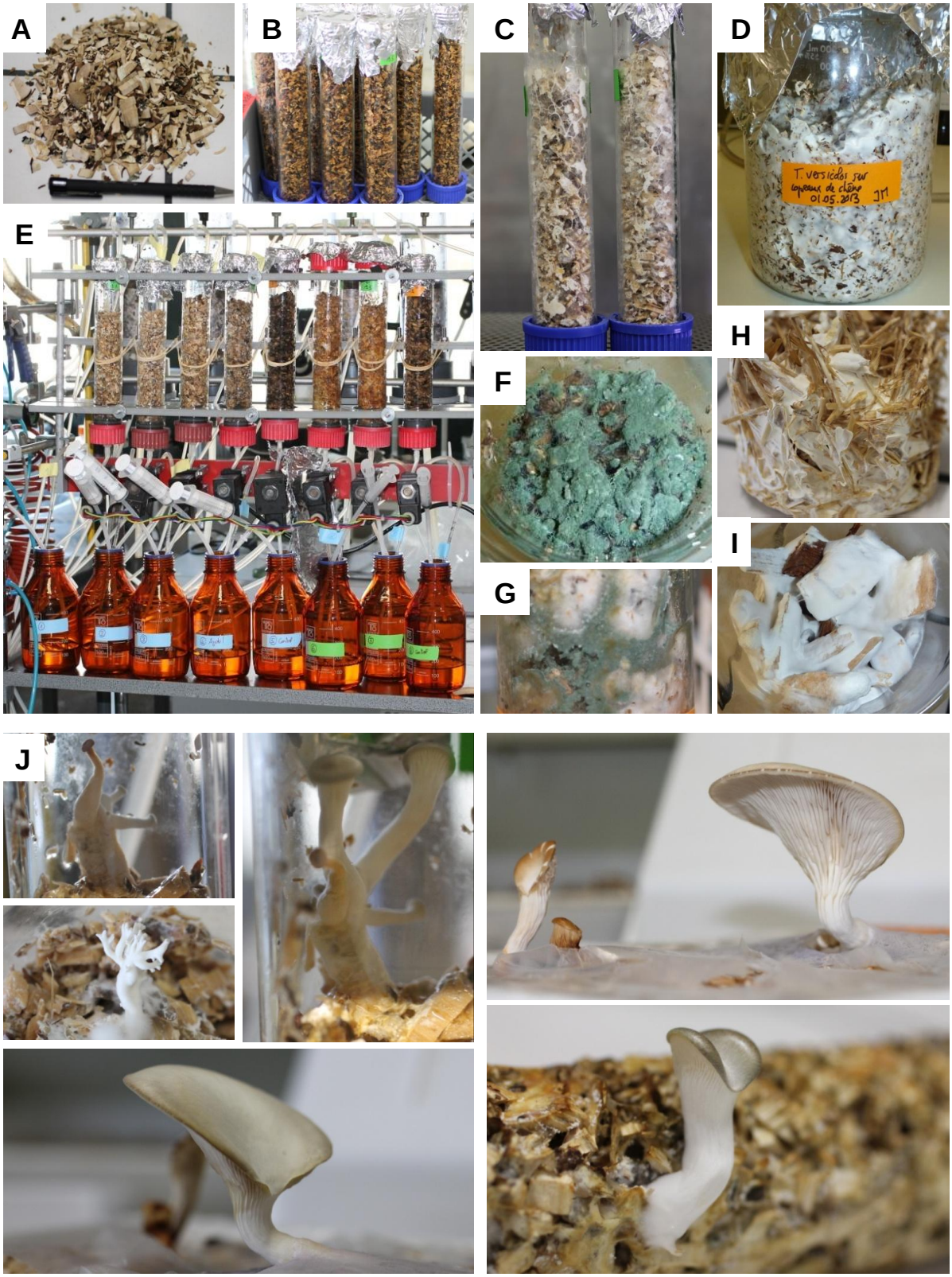


Fig. 9.4 Pictures of (A) beech woodchips, (B) columns used for the wood filters, (C) columns with woodchips colonized by *P. ostreatus* mycelium, (D) woodchips colonized by *T. versicolor* mycelium, (E) setup of eight filters in parallel with the electrovalves and the bottles, (F) woodchips colonized by a green mould, (G) development of a green mould in a fungal filter, (H) wheat straw colonized by *T. versicolor* mycelium, (I) large woodchips colonized by *T. versicolor* mycelium, (J) occasional appearance of *P. ostreatus* mushrooms out of the woodchips colonized by the mycelium.

Three wood filters inoculated with *P. ostreatus* mycelium, one wood filter inoculated with the fungus but inhibited with 10 mM sodium azide (inhibited fungus control) and one not-inoculated wood filter (wood control) were tested in parallel for their efficiency to treat micropollutants at real concentrations in TWW, during a period of 64 d (14 batch cycles). Micropollutant concentrations, laccase activity and pH were regularly monitored. During each sampling, 16 ml (out of 150 ml initially) were withdrawn from each filter and filtered at 0.22 µm (PP-GMF filters). 2 ml were directly used to determine laccase activity and pH, and the other 14 ml were spiked with 10 mM sodium azide to inhibit laccase activity and then frozen at -18°C until micropollutant analysis. A total of 44 micropollutants were analysed by on-line SPE followed by UPLC-MS/MS. At the end of each cycle, 15 ml, also filtered at 0.22 µm, were sampled for DOC analysis. Eight short batch cycles of 48 h each were performed at the beginning of the experiment to progressively reach the adsorption equilibrium in the wood, and then three long cycles of 5 and 18 d were carried out to assess slow degradation kinetics. Finally, three last batch cycles were performed after 55 d of operation. For these three cycles, the microbial activity in the wood control was inhibited with 10 mM sodium azide. Micropollutant analyses were done at the beginning (raw TWW) and at the end (after 48 h) of each cycle (apart for the 12th and 13th cycles, not analysed). During the 9th and 14th cycles, kinetics of micropollutant removal were monitored during 5 d.

9.3 Results and discussion

9.3.1 Growth inhibition assay for *Trametes versicolor*

As presented in Fig. 9.5, no growth inhibition of *Trametes versicolor* was observed with eight out of the nine micropollutants tested at 10 mg l^{-1}. These micropollutants did not have any toxic effect at this concentration. Any significant difference in growth rate was neither observed between the control and the medium with 1% methanol. Methanol appeared to be not toxic at this concentration. However, with the antibiotic sulfamethoxazole (SMX), the growth was completely inhibited in the eight replicates tested. SMX was thus already toxic at 10 mg l^{-1}, meaning that lower concentrations should be used in degradation experiments.

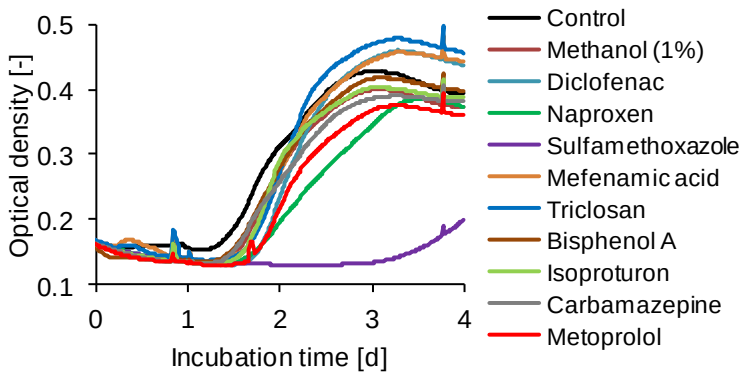


Fig. 9.5 Growth curve (optical density) of *Trametes versicolor* in malt extract medium with 10 mg l^{-1} of micropollutant (with 1% methanol). Control: inoculum in malt extract medium without any pollutant. Average of 4 to 8 replicates.

9.3.2 White-rot fungi as a replacement of laccase-mediator systems

Oxidation of micropollutants by laccase with the help of mediators showed interesting potential for the treatment of toxic and highly concentrated wastewaters (cf. Chapter 7). However, as also shown in Chapter 7, high concentrations of mediators are required and the mediator itself can generate toxic effects. An alternative to adding mediators would be to use directly the organisms that produce laccase. Indeed, it is known that white-rot fungi such as *Trametes versicolor*, in addition to produce high amount of laccase, have the ability to degrade pollutants by several other mechanisms, such as with other oxidative enzyme (cytochrome P450, peroxidases) (Yang et al., 2013b), or by producing natural mediators during degradation of complex substrates (lignin) which could enhance the action of laccase (Li et al., 2014). The potential of *Trametes versicolor* to degrade three pollutants oxidized by laccase-mediator systems, NPX, IPN and SMX, was therefore tested, as a possible alternative treatment to laccase-mediated systems.

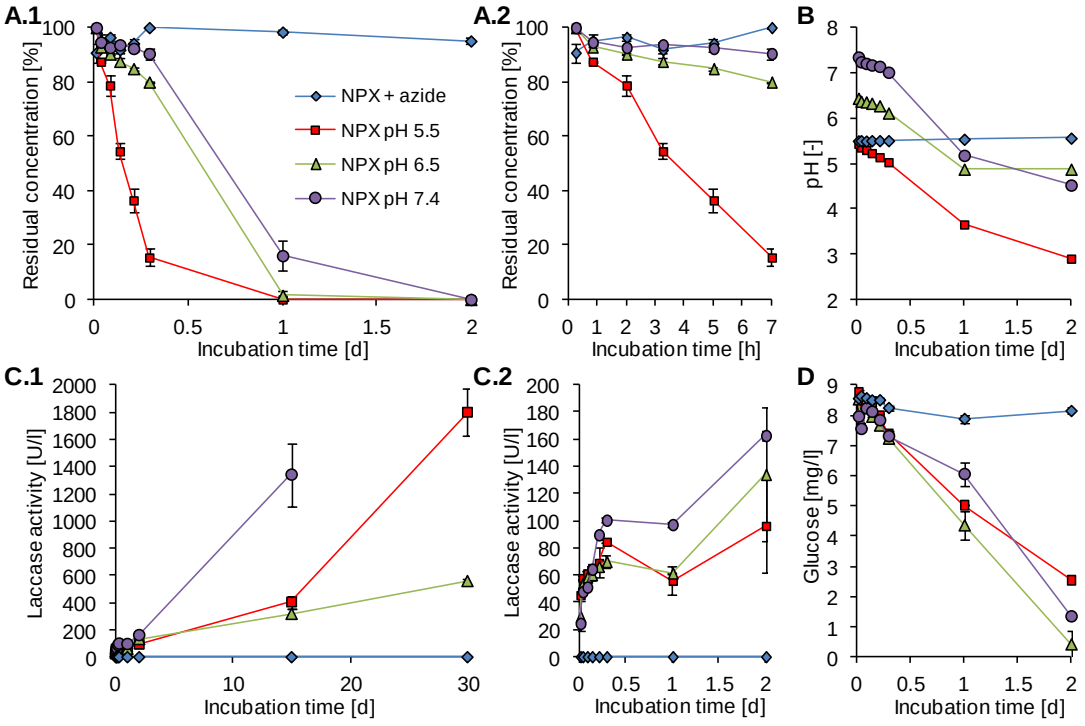


Fig. 9.6 (A) Naproxen (NPX, at 10 mg l^{-1}) degradation by *Trametes versicolor* at different initial pH values in UAB medium. (A.2) Zoom on NPX degradation during the first hours of incubation. (B) pH evolution during NPX degradation tests. (C.1) Laccase activity evolution during the test. (C.2) Zoom on laccase activity during the first 2 d of the test. (D) Evolution a glucose concentrations during the experiment. Average and values (error bars) of duplicate. Azide: control with inhibited fungus with 10 mM sodium azide.

As presented in Fig. 9.6 A.1, NPX was completely removed in less than 12 h at pH 5.5 in a defined growth medium containing glucose, and not significantly removed in the control with inhibited fungal pellets. NPX was therefore rapidly degraded by *Trametes versicolor*, as also reported in other studies (Marco-Urrea et al., 2010a). At pH 6.5 and 7.4, the degradation kinetics were much slower during the first hours of incubation (Fig. 9.6 A.2), but accelerated subsequently, together with a drop of pH (Fig. 9.6 B), to reach 98% and 84% removal after 24 h, at pH 6.5 and 7.4, respectively (Fig. 9.6 A.1). The degradation kinetics were therefore pH dependant, becoming faster in acidic pH, similar to what was observed with laccase or laccase-mediated reactions (cf. Chapter 6 and Chapter 7). This indicates that

enzymatic reactions were probably participating in the degradation. Laccase may be involved (in a mediated reaction), but it is more likely due to other oxidative enzymes such as cytochrome P450 (Marco-Urrea et al., 2010a). No significant difference in glucose consumption rates (Fig. 9.6 D) and extracellular laccase production (Fig. 9.6 C.2) were, however, observed between the cultures at three different initial pH values. Although pH affected NPX degradation kinetics, it did not seem to impact the fungal metabolism. Laccase production continued several days after complete glucose consumption, reaching very high activity, up to 2000 U l^{-1}, after 30 d of incubation (Fig. 9.6 C.1).

As presented in Fig. 9.7 A, both IPN and SMX were completely degraded in less than 4 d by *Trametes versicolor*. Despite high initial SMX concentrations were used (10 mg l^{-1}), no fungal activity inhibition was observed, whereas the same concentrations inhibited completely fungal growth (cf. section 9.3.1). SMX and IPN are known to be degraded by several white-rot fungi (Del Pilar Castillo et al., 2001; Guo et al., 2014; Rodarte-Morales et al., 2011), but this is the first time that *Trametes versicolor* shows IPN degradation ability. The removal could be attributed to degradation/transformation and not to adsorption onto the mycelium as no (for IPN) or only limited removal (for SMX) were observed in the control with inactivated biomass. The removal rate correlated well with the glucose consumption (Fig. 9.7 D) but not with the laccase activity (Fig. 9.7 C), which increased only once all glucose was consumed. This suggests that laccase was not directly involved in the degradation.

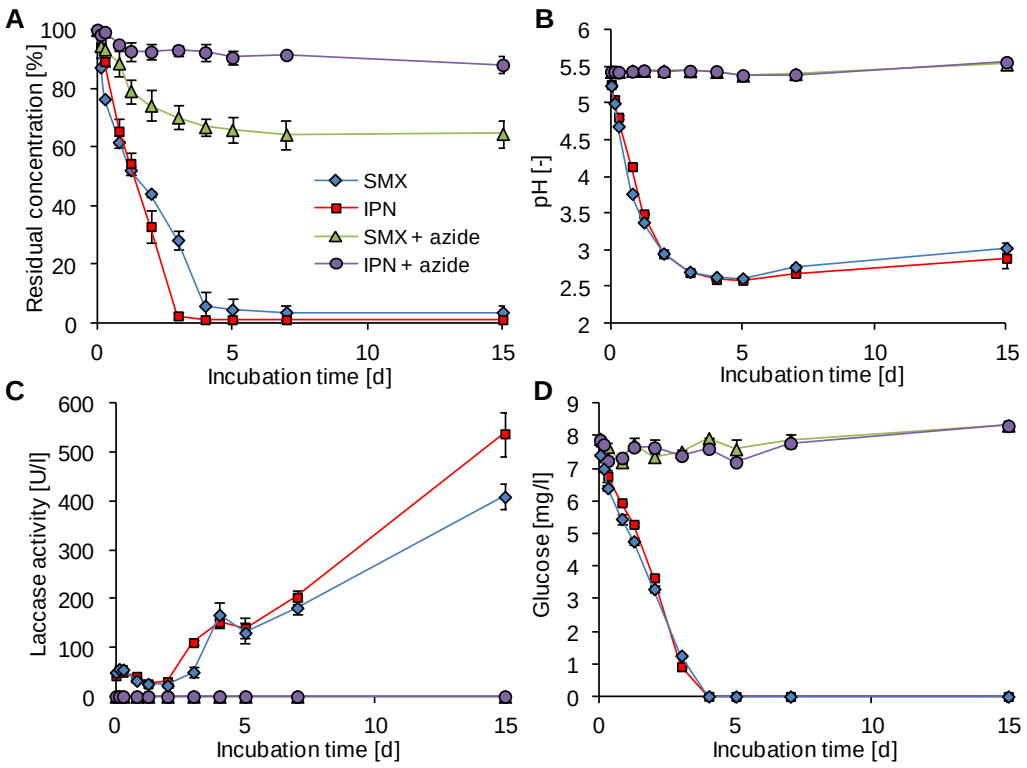


Fig. 9.7 (A) Sulfamethoxazole (SMX, 10 mg l^{-1}) and isoproturon (IPN, 10 mg l^{-1}) degradation by *Trametes versicolor* in UAB medium. (B) pH, (C) laccase activity and (D) glucose concentration evolutions during the experiment. Average and values (error bars) of duplicate. Azide: controls with inhibited fungus with 10 mM sodium azide.

The drop of pH observed in all the culture media (at the three initial pH values, with the three pollutants), reaching at the end pHs between 2.5 and 5, was related to glucose metabolism, as the decrease in pH stopped once all glucose was consumed. Indeed, it was observed in several previous

experiments (Contijoch, 2014; Vargas, 2013) that glucose consumption by with-rot fungi was related to pH drop in the medium. This pH drop was not due to the production of CO_2, which could acidify the water by dissolution (carbonic acid formation), as no increase in pH was observed after stripping the CO_2 with nitrogen gas. White-rot fungi are known to produce extracellular organic acids (such as oxalic acid) during their metabolism (Mäkelä et al., 2002). Oxalic acid is thought to be produced by wood-degrading fungi to weaken the wood structure and thus increasing the pore size to permit penetration by lignocellulolytic enzymes (Hastrup et al., 2012) and possibly to inhibit bacterial growth (de Boer and van der Wal, 2008). Other studies showed that the pH decrease in the growth media of a mixed culture of fungi and bacteria was related to the excretion of gluconic acid, which results from the conversion of glucose by glucose dehydrogenase. No pH decrease was reported with other sugars (mannose, galactose) (Romano and Kolter, 2005). Although organic acid excretion was not analyzed, this was the most probable explanation for the pH drop observed.

The ability of *Trametes versicolor* to degrade these three pollutants suggests that white-rot fungi may have higher potentials for micropollutant degradation in (not highly toxic) wastewater compared to laccase-mediator systems.

9.3.3 Micropollutant degradation in real wastewater by *Trametes versicolor* in submerged batch cultures

The potential of *Trametes versicolor* for micropollutant removal in real treated wastewater was tested in a batch experiment of 14 d in submerged cultures with wheat straw as sole external substrate addition. Although TWW was filtered to limit microbial competition and the wheat straw autoclaved, the water was not sterile. This resulted in important microbial growth, especially in the control with heat-killed fungal pellets (lots of substrate available). As it became very difficult to filter the samples (due to the microbial development), not all samples from each duplicate of the controls were analysed. Moreover, some samples at the beginning of the experiment (with the two controls) were lost due to a problem during the SPE. Despite these analytical problems, the removal kinetics could be determined for 23 micropollutants detected in the TWW (Fig. 9.8, Fig. 9.9 and Fig. 9.10).

Three micropollutants, DFC, MFA and NPX were completely removed in less than 2 d with the active fungus, while no clear removal was observed in the controls (Fig. 9.8 A). These three pollutants were present in relatively high concentrations in TWW (400-800 ng l^{-1}). Two other pollutants, IPN and gemfibrozil, were also clearly better removed with the active fungus (Fig. 9.8 A). For gemfibrozil, complete disappearance was observed after 2 d, followed by an increase in the concentration after 4 d. This increase, apart from possible analytical issues, may be due to up-take of gemfibrozil inside the fungal cells when the fungus was active and then release in the medium when the fungus died (cell lysis). In any case, gemfibrozil was partially removed from the water by *T. versicolor*, probably by degradation as reported by Nguyen et al. (2013). DFC and MFA are known to be degraded by laccase and high laccase activity was measured after one day of experiment (280 U l^{-1} and pH 6.3) (Fig. 9.8 B), suggesting that laccase was, at least partially, involved in their removal. Degradation of NPX and IPN by *T. versicolor* was already demonstrated at high concentrations (cf. section 9.3.2).The ability of the fungus to degrade all these pollutants even at very low concentrations (ng l^{-1} to µg l^{-1}) is therefore very promising.

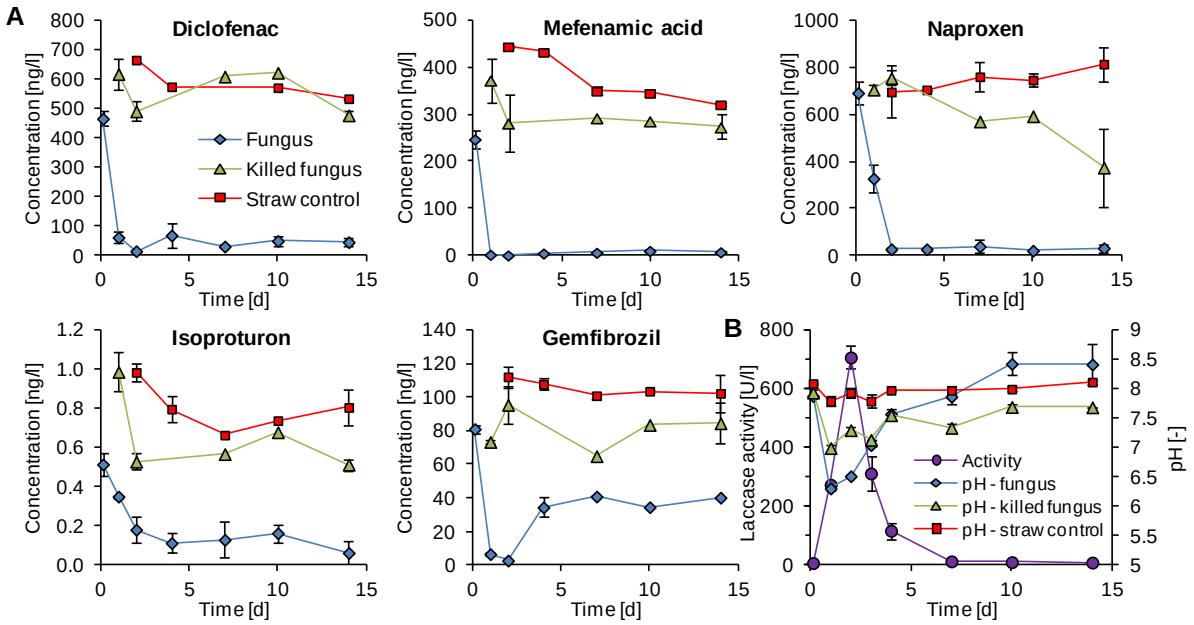


Fig. 9.8 (A) Micropollutants well removed only with the active fungus (fungal degradation). Average and standard deviation of the residual micropollutant concentrations in wastewater of duplicates with (i) *Trametes versicolor* pellets and wheat straw (◊, Fungus), (ii) killed (autoclaved) *Trametes versicolor* pellets and wheat straw (▲, Killed fungus), and (iii) only wheat straw (■, Straw control). **(B)** Evolution of laccase activity (with the active fungus) and pH in the medium during the experiment.

Six other micropollutants were partially removed from the TWW treated with the active fungus but also in the two controls: SMX, BTZ, gabapentin, atenolol, methylbenzotriazole and metronidazole (Fig. 9.9). Their removal can therefore not be attributed only to fungal degradation. As shown in section 9.3.2, SMX, at high concentrations, can be degraded by *T. versicolor*, and was therefore probably also degraded by the fungus in TWW. For atenolol, the removal was probably due to microbial degradation as very different removal kinetics were observed between the two replicates with the killed fungus: the native microbial community needed probably more time to developed in one replicate compared to the other. For BTZ and methylbenzotriazole, two pollutants found in high concentrations in TWW (4-8 µg l^{-1}), as well as for metronidazole, it is not clear if the removal was due to slow adsorption onto the wheat straw, slow microbial degradation or a combination of both. These three compounds had good adsorption affinities for powdered activated carbon (PAC) (cf. Chapter 3), and, therefore, adsorption onto wheat straw may be significant. Gabapentin, on the contrary, had very low affinity for PAC and was thus not expected to be significantly adsorbed. The lower removal in the control with wheat straw, which presented less microbial growth than the two other media, suggests also that microbial degradation was probably the main removal mechanism for gabapentin.

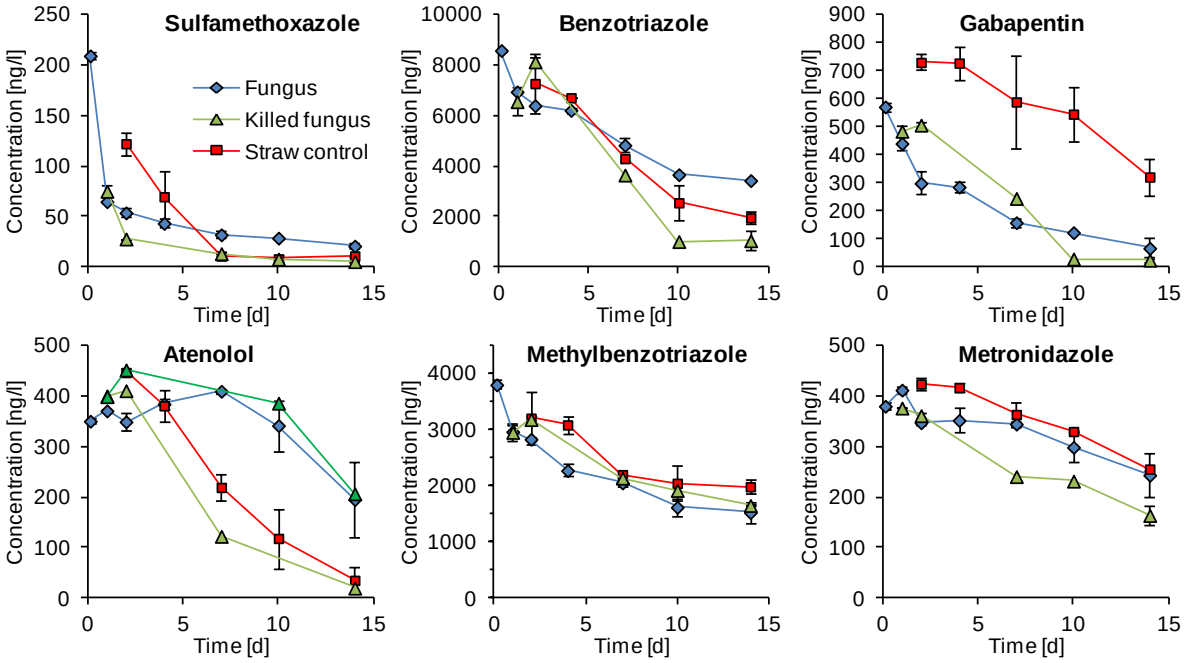


Fig. 9.9 Micropollutants removed in all conditions (by adsorption or biodegradation). Average and standard deviation of the residual micropollutant concentrations in wastewater of duplicates with (i) *Trametes versicolor* pellets and wheat straw (◊, Fungus), (ii) killed (autoclaved) *Trametes versicolor* pellets and wheat straw (▲, Killed fungus), and (iii) only wheat straw (■, Straw control). For Atenolol, the two replicates with the killed fungus are presented, as they were very different.

Twelve other micropollutants were not or only poorly (< 50%) removed during the 14 d of experiment (Fig. 9.10). This included the X-ray contrast media iopamidol, iohexol and iomeprol, as well as the antidiabetic metformin, found in very high concentration in TWW (4 – 60 µg l^{-1}), several other pharmaceuticals (carbamazepine, bezafibrate, metoprolol, sotalol and primidone) and three pesticides (mecoprop, atrazine and terbutryn). The persistence of carbamazepine and the X-ray contrast media, compounds which were reported to be degraded by *T. versicolor* (Gros et al., 2014; Jelic et al., 2012), suggests that either (i) these compounds could not be degraded by the fungus in the conditions tested (near neutral pH, real TWW), (ii) other external energy sources or addition of nutrients (nitrogen) were necessary to degrade these compounds (Zhang and Geißen, 2012), or (iii) the fungus did not survive long enough to degrade significantly these pollutants. Indeed, as shown in Fig. 9.8 B, the fungus was very active the first two days of the experiment (strong increase in laccase activity and decrease in pH), but, after two days, laccase activity dropped rapidly and was negligible after 7 d. At the same time (after 2 d), a strong microbial development was observed (high turbidity) in the water containing the fungal pellets, together with an increase in pH (Fig. 9.8 B). This suggests that the fungus died after 2-3 d and that the fungal pellets and enzymes were used as substrate for the development of other microorganisms.

This experiment showed that, although several micropollutants were not affected, several others, including persistent compounds such as DFC, MFA and NPX, could be rapidly removed from real TWW by means of a fungal treatment with *T. versicolor*. However, *T. versicolor* was not able to survive more than one week in submerged cultures in real unsterile treated wastewater. The long-term survival of the fungus in the process was therefore the main challenge in order to potentially develop a fungal treatment able to remove micropollutant from municipal wastewater.

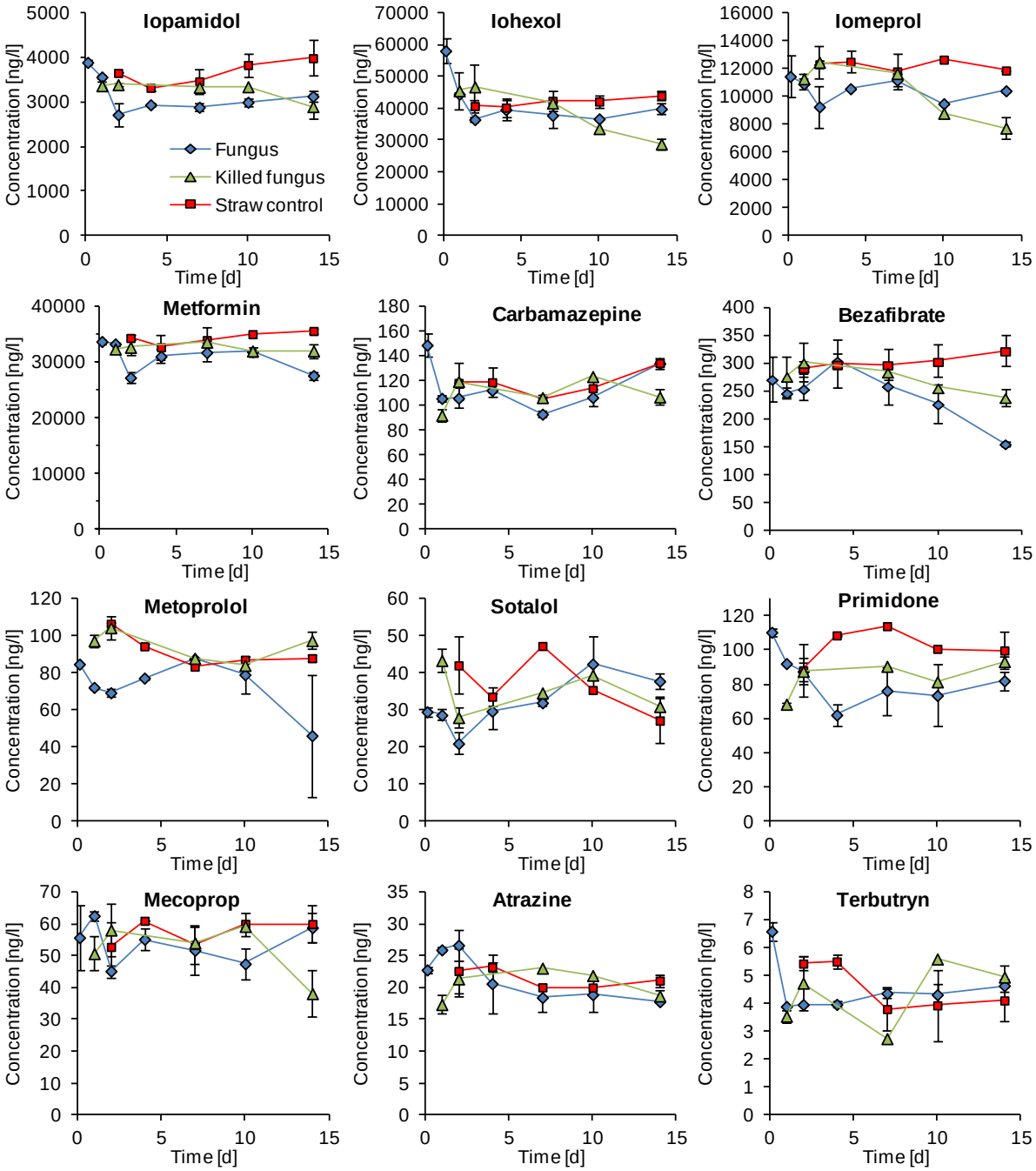


Fig. 9.10 Micropollutants not or poorly removed (less than 50%) in all conditions during the 14 d of experiment. Average and standard deviation of the residual micropollutant concentrations in wastewater of duplicates with (i) *Trametes versicolor* pellets and wheat straw (◊, Fungus), (ii) killed (autoclaved) *Trametes versicolor* pellets and wheat straw (▲, Killed fungus), and (iii) only wheat straw (■, Straw control).

9.3.4 Long term survival of *T. versicolor* in submerged cultures

The short survival time of *T. versicolor* in real TWW with lignocellulosic substrate as sole carbon source, observed in the previous section, could be due to two main causes: (i) the substrate (wheat straw) was not adapted to maintain the fungus alive (or active) more than one week, or (ii), the fungus was not able to survive in the presence of other microorganisms (competition for the substrate, predation, etc). The first assumption was tested by growing *T. versicolor* mycelium in sterile TWW (submerged culture) with either wheat straw or beech woodchips as sole carbon source and by

monitoring its long term activity. These conditions were close to the ones of the previous test (section 9.3.3), with the difference that a pure culture of *T. versicolor* was used (no competition or predation possible). As presented in Fig. 9.11, laccase activity stayed at a relatively stable level during one year of incubation, with both wheat straw and woodchips. As laccase is not stable (half-life of 3 d) at the pH found in the culture media (pH 4.2 with woodchips) (cf. Chapter 8), a continuous laccase production was necessary to maintain this level of activity. Therefore, *T. versicolor* could survive and was still active after 363 d of incubation in TWW with wheat straw or woodchips as sole carbon sources. This suggests that the low survival of *T. versicolor* in unsterile TWW was due to competition or predation by other microorganisms, together with the stress of prolonged immersion in water.

By comparison, *T. versicolor* was reported to survive 2 – 5 y in wood logs in natural environment (Boddy and Heilmann-Clausen, 2008). In natural conditions, high water contents in the wood impose, however, poor aeration, restricting aerobic processes and resulting in mycelia death if prolonged (Boddy and Heilmann-Clausen, 2008).

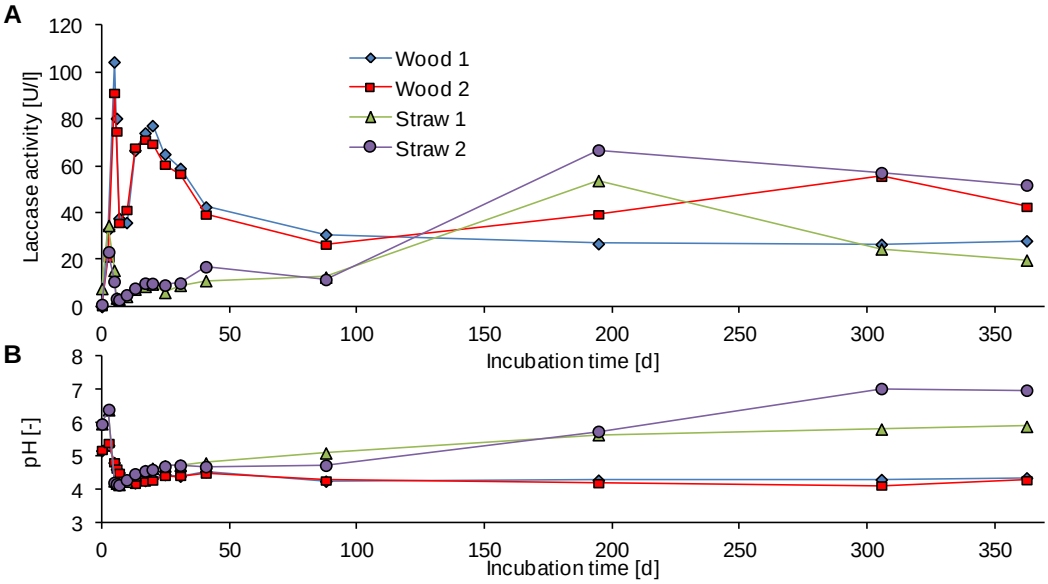


Fig. 9.11 Evolution of (A) laccase activity and (B) pH in the submerged pure cultures of *Trametes versicolor* inoculated in sterile treated wastewater with either wood chips (Wood) or chopped wheat straw (Straw) as sole substrate. Experiment in duplicates (1 and 2). Incubation at 130 rpm and 25°C.

9.3.5 Strategy to increase the survival of white-rot fungi in unsterile systems

The survival of white-rot fungi in submerged environment in unsterile systems is a real challenge. Indeed, aqueous media are very far from the natural habitat of these fungi, which live on dry dead wood. Despite the strategy to use lignocellulosic substrate in submerged culture to avoid strong competition for the substrate with other organisms, submerged conditions in TWW were too harsh for the fungi, not allowing them to resist against predation or competition with other microorganisms. The strategy chosen was therefore to develop a process where the fungi would be closer to their natural habitat. Hence the idea to develop a fungal filter made of woodchips colonized by the fungal mycelium, with an alternation of saturated (to treat the pollutants) and unsaturated phases (to allow for fungus recovery). This sequential batch fungal filter is presented in details in section 9.2.1.12.

9.3.6 Selection of the wood species as support/substrate in the fungal filters

Prior to testing the fungal filter, it was necessary to choose a wood species adapted for the growth of the mycelium. Observations made in the forests of the Swiss Plateau revealed that *Trametes versicolor* was growing mainly on oak and beech logs or tree stumps. These two wood species, as well as a few other species (ash, birch and spruce) were tested for the mycelium development of *Trametes versicolor* and *Pleurotus ostreatus*. Both fungi were able to colonize all the wood species, but the rate of colonization and the biomass produced (only visually quantified) were higher on beech and oak wood for both fungi. Beech was indeed reported to be the most common host tree species for wood-degrading fungi in Switzerland (Küffer et al., 2008). Spruce woodchips was the substrate with least mycelial development, which is coherent with the natural habitat of these two fungi, mainly on dead deciduous and only very rarely on dead conifers (Borgarino and Hurtado, 2011). As beech is one of the most common tree species in the Swiss plateau, and thus easily available, it was selected as support/substrate for the fungal filters.

Although the mycelium of both fungi developed well on the wet and sterile woodchips without addition of any other substrates or nutrients (Fig. 9.4 C, D, H, I), *T. versicolor* produced much more biomass and grew much faster than *P. ostreatus*, covering completely the substrate in 5 days and clogging completely the woodchips in 2-3 weeks. *P. ostreatu*s grew more in a filamentous way (mycelium propagation), colonizing slowly the wood substrate (complete colonization in three weeks) without producing a lot of biomass (no clogging observed). More detailed results of this experiment are presented elsewhere (Contijoch, 2014; Vargas, 2013).

9.3.7 Selection of a fungal strain - Colonization and competition for fresh woodchips

As described in section 9.2.1.13, *T. versicolor*, despite its ability to degrade many micropollutants, was not necessarily the most adapted strain for the fungal filter, as it was very challenging to make it survive more than 10 d in an unsterile environment. Another more resistant and more competitive white-rot fungus, *P. ostreatus*, was therefore also tested.

The ability of the two fungi to colonize and compete for fresh wet woodchips (incubated in dry conditions in a glass column, with the two fungal mycelium inoculated in each side) was compared. As presented in Fig. 9.12, after 7 d of incubation, the fresh wood started to be colonized by a green mould, naturally present in the ambient environment. After 11 d, this green mould started to colonize also the area already covered by *T. versicolor* mycelium (parasitism), but not the one with *P. ostreatus*. After about 21 d, *P. ostreatus* started to colonize the fresh wood, despite the presence of the green mould and finally dominated all the woodchips in the column.

This experiment showed that *P. ostreatus* had the ability to resist and to compete with the green mould, and to colonize slowly all the fresh wood, whereas *T. versicolor* mycelium was rapidly colonized by the green mould, without showing any mycelial invasion in the fresh wood area. These two fungi had therefore different strategies of wood colonization, with, for *T. versicolor*, fast growth and low competitiveness, and for *P. ostreatus*, longer lifespan, slower growth and higher competitiveness.

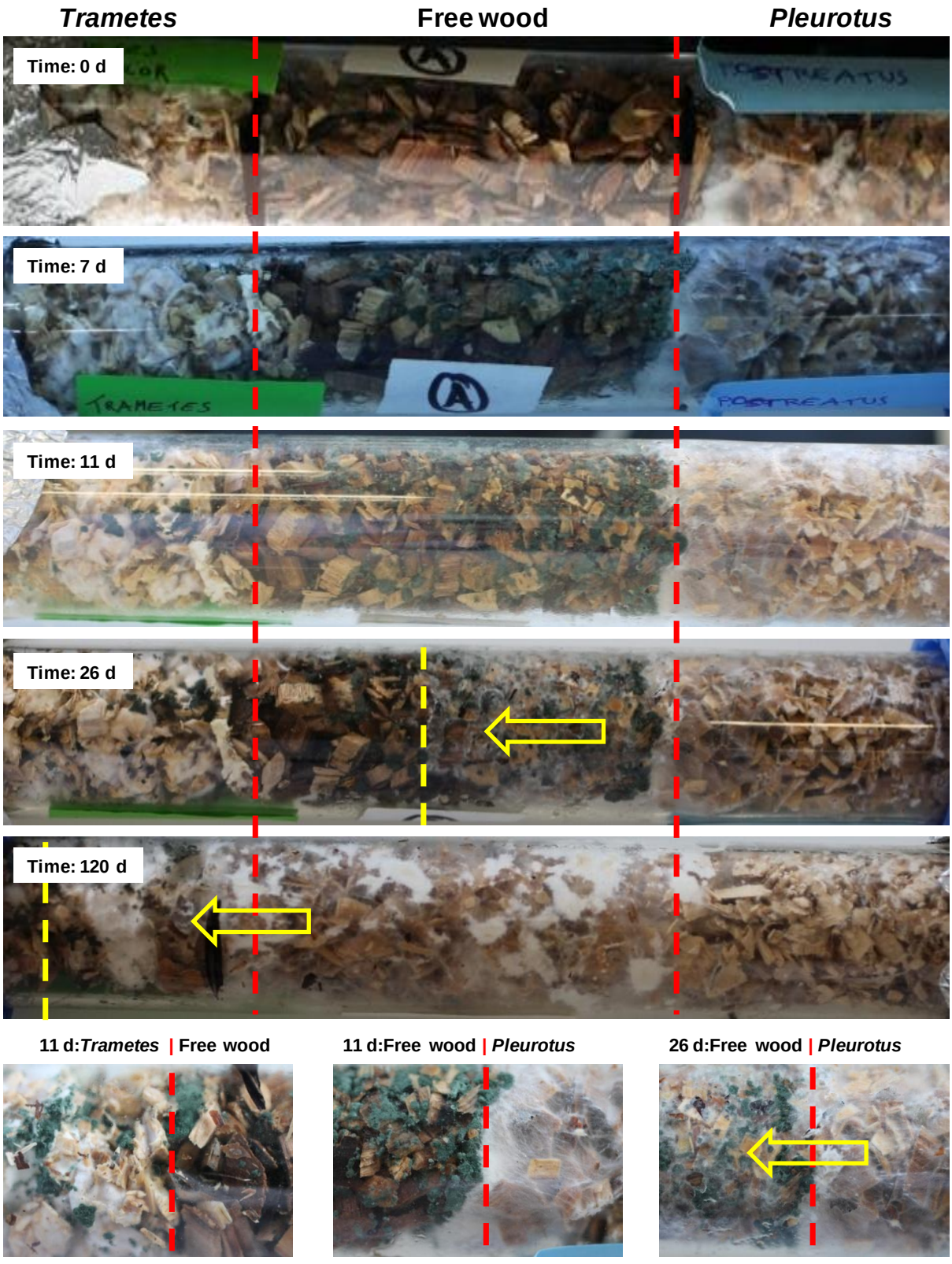


Fig. 9.12 Evolution of the wood colonization by *Trametes versicolor* (inoculated on the left of the first red line), *Pleurotus ostreatus* (inoculated on the right of the second red line) and the green mould (not inoculated, naturally present in the wood). Yellow line and arrow: evolution of the front line of the *Pleurotus* colonization. Same results were observed in the duplicate experiment.

The type of green mould was not identified but it is known that several fungi of the genus *Cladosporium*, *Aspergillus*, *Penicillium* or *Trichoderma* can produce green spores forming the mould. *Trichoderma* spp. are especially known for their ability to attack other fungi to access to nutrients present in their cells, causing their death (Hatvani et al., 2012) . Moreover, *Pleurotus* is known to be more resistant than *Trametes* to the infection by *Trichoderma* spp. (Daniel Job, UNINE, personal communication), which is consistent with our observations.

To better understand the effect of the presence of other microorganisms on the development of white-rot fungi in natural environment, the interactions between fungi and between white-rot fungi and bacteria are discussed below.

Interaction between fungi

In wood, interactions between fungi are mainly (i) parasitism, where the parasitic fungus penetrates or lyses the cells of the host fungus to access to nutrients (Woodward and Boddy, 2008), or (ii) competition for space, as occupancy of territory (wood) is the only other way for the fungus to access the nutrients (Boddy and Heilmann-Clausen, 2008). Wood-inhabiting fungi have thus developed several different strategies to colonize dead wood, classified in three main categories: (i) competitive (C-selected), (ii) stress-tolerant (S-selected) and (iii) ruderal (R-selected), depending on the speed of the mycelium invasion, the tolerance to nutrient stress, the rate of growth, etc. One fungus can apply several of these strategies during its life-cycle and can thus have some characteristics of the three categories. *R-selected* fungi are favoured in the relative absence of stress in uncrowded environments resulting of a disturbance. They are characterized by their ability for rapid reproduction, effective dispersal, utilisation of simple easily available organic substrates (narrow enzymatic ability), and rapid growth. *C-selected* fungi are combative fungi favoured in relatively non-stressed, undisturbed conditions. They increasingly extend their territory, leading to competition with other fungi. They are characterized by long-life expectancy and wide enzymatic ability. *S-selected* fungi have the ability to cope with a particular abiotic stress or set of stresses, slow or intermittent reproduction and often slow growth (Boddy and Heilmann-Clausen, 2008). As the wood properties changes during its decay, different fungal species may succeed each other when the conditions become more adapted for another species. When fresh dead wood is available in non-stressful conditions, fungi with a preponderance of R-selected characteristics will first colonize the wood (early stage of decomposition). As colonization proceeds leading to no more uncolonized territory available, fungi with more C-selected characteristics begin to dominate (middle stage of decomposition). Once the wood resource is used up (which can take 3 to 9 years in small or large logs) and the main proportion of nutrient is contained within living mycelium or bacteria, C-selected fungi begin to decline and are possibly replaced by other fungi with more R-selected properties (Boddy and Heilmann-Clausen, 2008).

Trametes spp. are classified as secondary combative invaders, with a preponderance of both R and C-selected characteristics (Boddy and Heilmann-Clausen, 2008). This is coherent with our observation, with a rapid growth and reproduction (R-selected), and wide enzymatic ability (C-selected). *Pleurotus ostreatus* seems to have mostly C-selected characteristics (very competitive, long-life expectancy and wide enzymatic ability).The green mould can be clearly classified as a R-selected fungus, with rapid growth on uncolonized wood, probably narrow substrate range (fungal parasite) and effective

dispersal. After the early stage of colonization, the green mould was replaced by *Pleurotus ostreatus* (secondary invader).

The ecological characteristics of the two white-rot fungi suggest that *P. ostreatus* is more adapted for a long term survival in the fungal filter. Contrary to *T. versicolor*, it has the ability to compete with the green mould, to colonize fresh wood, and, due to its slow growth, it will not clog rapidly the filter with its mycelium.

Interaction between white-rot fungi and bacteria

During wood degradation by white-rot fungi, the environmental conditions become very selective for bacteria because of rapid and strong acidification (production of organic acid by the fungi), production of reactive oxygen species and secretion of fungal secondary metabolites with antimicrobial activity. Bacteria that resist to these conditions may profit from the degradation activity of the fungi. Indeed, white-rot fungi exude many different extracellular enzymes and secondary metabolites, involved in the degradation of wood polymers (lignin, cellulose, hemicelluloses, etc.). The oligomers released are the main growth substrate for the fungi. These compounds, as well as the secondary metabolites and the extracellular enzymes released, are also appropriate substrate for bacteria. Thus, despite most bacteria cannot degrade wood, they can compete with the fungi for the lower molecular weight compounds released, depriving them for their main growth substrate (de Boer and van der Wal, 2008). Moreover, predation of fungal mycelium by some bacteria was also reported (bacterial mycophagy). However, predation of bacteria (cell lysis) by white-rot fungi seems more frequent. Fungi may use bacteria as a valuable source of nitrogen (de Boer and van der Wal, 2008). In short, bacteria may compete for the growth substrate of the fungi. To deal with that, white-rot fungi developed different strategy to inhibit bacterial growth (predation, acidification, or antimicrobial compounds production). These strategies are expected, however, to be less efficient in submerged environment, due to rapid dilution of the organic acids and antimicrobial agents produced (loss of the inhibitory properties).

Most bacteria cannot degrade complex and difficult to access wood polymers. Fresh dead wood is, however, rapidly colonized by bacteria, which grow on easily degradable substrates like sugars, organic acids, pectin and easily accessible cellulose. The bacterial community that develops on the fresh dead wood may therefore inhibit or delay colonization of the wood by white-rot fungi (de Boer and van der Wal, 2008). Addition of dead wood in wastewater may also support the growth of bacteria for a certain period.

9.3.8 Selection of a fungal strain - Comparison of the efficiency of the two fungi to remove naproxen in the fungal filter

Although *P. ostreatus* seemed, according to its ecological characteristics, more adapted for long term survival in the filter, it was necessary to test its efficiency for micropollutant removal and its real survival in the filter, in comparison to the well known *T. versicolor*. NPX was selected as a model compound, due to its good degradability by *T. versicolor* and its low degradability by bacteria. The filters were operated in sequential batch mode during 16 d (8 batch cycles) with synthetic wastewater, with three filters with *P. ostreatus* and three with *T. versicolor*.

As presented in Fig. 9.13 (A.1 for *Trametes* and B.1 for *Pleurotus*), NPX was completely removed by both fungi in less than 24-48 h during the three first batch cycles. During the first cycle, important and fast removal was also observed in the wood controls (filters not inoculated), due to adsorption onto the wood chips. In the next cycles, the removal in the controls strongly decreased, as the adsorption equilibrium with the initial concentration was progressively reached (no more removal after three cycles). This effect was not observed in the filters with the active fungi, demonstrating that, in these filters, the removal was mainly by fungal biodegradation.

Both fungi were well active (laccase production) during the three first cycles (7 d) (Fig. 9.13 A.2 and B.2), but from the 4th cycle on, the activity of *T. versicolor* decreased drastically in all the replicates. At the same time, NPX removal efficiency decreased rapidly in all the filters with *Trametes*. From the 4th cycle on, NPX was probably not any more degraded but only removed by adsorption onto the wood/mycelium. This was confirmed by inhibiting *Trametes* in one replicate (Fungus 1) at the 5th cycle: no differences in NPX removal between the active and inhibited filters were observed, meaning that adsorption was the main removal mechanism. After 6 cycles (11 d), NPX was not anymore removed in the filters with *T. versicolor*.

With *P. ostreatus*, on the contrary, no decrease in fungal activity or in NPX removal efficiency was observed during the 16 d of operation. Even in the last cycles, NPX was still almost completely removed in less than 24 h. To confirm that the removal was due to fungal degradation and not adsorption onto *Pleurotus* mycelium, at the 5th cycle, the fungus was inhibited in one replicate (Fungus 1). A rapid decrease in NPX removal efficiency was observed in the inhibited filter (no more removal after three cycles), while the two other active filters performed as well as before, confirming the fungal degradation.

As also observed in pure submerged cultures (section 9.3.2), the pH seemed to influence NPX degradation kinetics in the filters. Indeed, despite an initial pH in the synthetic wastewater of 7.8, fast drop of pH to 4.5 – 5 was observed in all the filters during the first cycle (Fig. 9.13 A.3 and B.3). This initial pH drop, also observed in the wood controls, was probably related to the release of soluble organic acids from the wood (Risholm-Sundman et al., 1998) or to the natural acidity of wood (Landi and Staccioli, 1992). Acidification was stronger, especially after the first cycle, in the filters containing the fungi compared to the wood controls. This was probably due to the fungal production of low molecular weight acids (such as oxalate) (Plassard and Fransson, 2009), as discussed in section 9.3.2. NPX removal in the fungal filters was faster during the two first cycles, when the pH was below 6, which was probably related to stronger adsorption and faster degradation at low pH. NPX was, however, still relatively rapidly degraded by *Pleurotus* in the next cycles, although the pH stayed between 6.5 and 8. The ability of *P. ostreatus* to degrade NPX even in neutral-alkaline conditions is therefore a great advantage for municipal wastewater treatment compared to laccase-mediator systems (efficient only in acidic pH).

Very similar results were observed between the three replicates for both fungi, showing the very good reproducibility of the treatment in the sequential batch fungal filters, and this even with open system (not sterile) with complex removal mechanisms (adsorption and biodegradation). This filters

configuration, with alternation of submersion/drainage, was thus much more reliable than the trickling filters (cf. section 9.2.1.12).

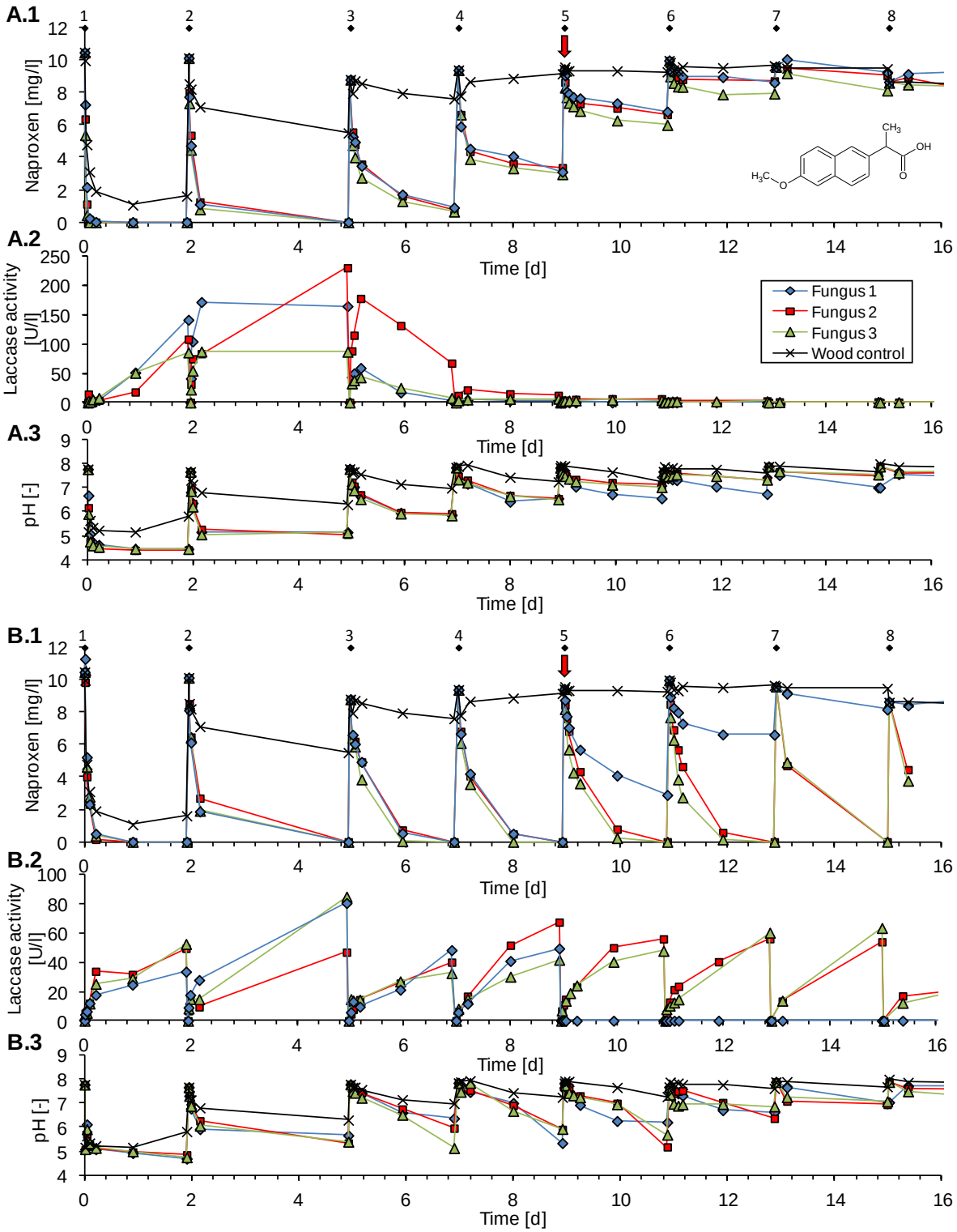


Fig. 9.13 Kinetics of naproxen removal (A.1, B.1) during the batch cycles (◊ numbered) in three filters (Fungus 1, 2 and 3) inoculated with (A) *Trametes versicolor* and (B) *Pleurotus ostreatus*, and in a wood filter not inoculated (Wood control). Evolution of laccase activity (A.2, B.2) and pH (A.3, B.3) during the batch cycles. The synthetic wastewater was renewed at each batch cycle. Arrow (batch cycle 5): inhibition of the biological activity (azide addition) in the fungal filter 1.

Despite, at the beginning, laccase activity in the filters with *Trametes* was almost three times higher than with *Pleurotus*, leading to slightly faster NPX degradation kinetics, *T. versicolor* was not active anymore in the filters after 7 d of operation. The sequential batch fungal filter was therefore unsuccessful to keep *T. versicolor* active in the system. On the contrary, and confirming the results of the colonization/competition test, *P. ostreatus* efficiently removed NPX and kept the same activity during the 16 d of operation. *P. ostreatus* was therefore clearly more adapted for this type of filter and these conditions. This fungus was thus selected for all the following experiments with the fungal filter.

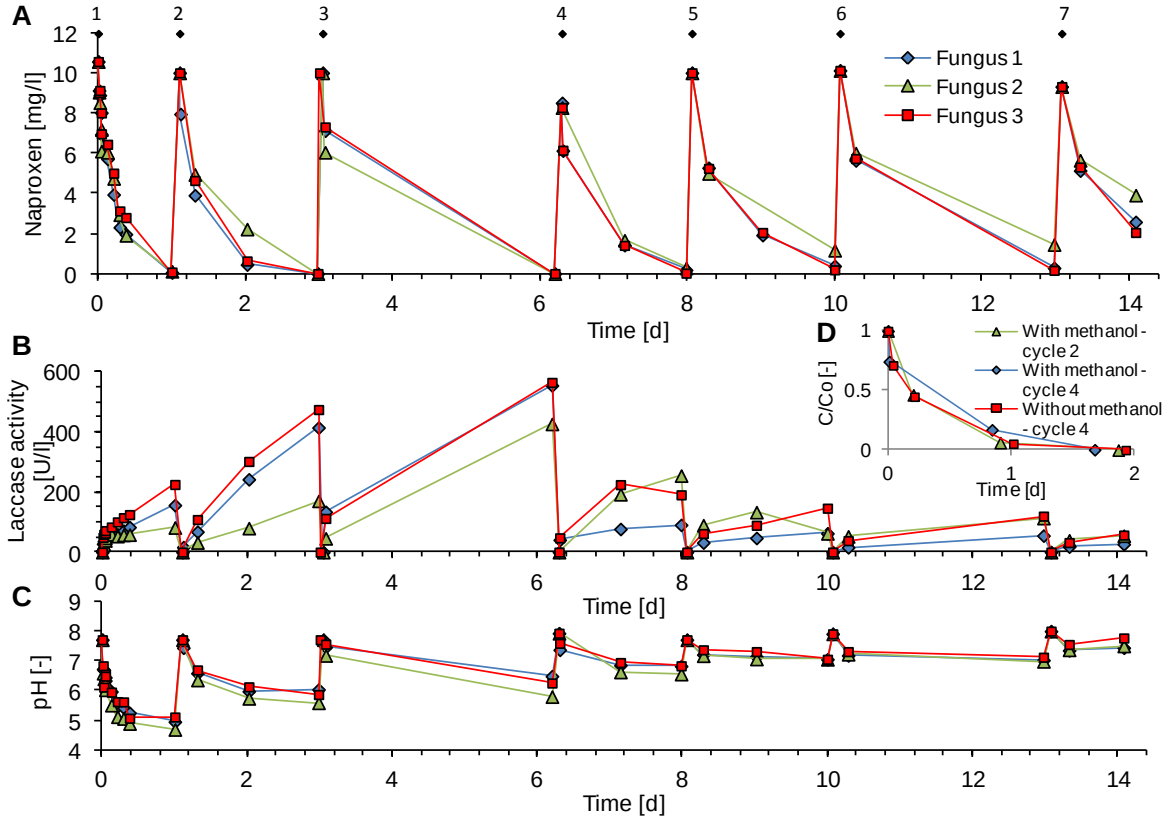


Fig. 9.14 Kinetics of naproxen removal (A) during the batch cycles (◊ numbered) in three filters (Fungus 1, 2 and 3) inoculated with *Pleurotus ostreatus*, in synthetic wastewater containing 1% methanol. Evolution of laccase activity (B) and pH (C) during the batch cycles. (D) Comparison of removal kinetics with or without methanol.

9.3.8.1 Effect of methanol addition

Methanol, due to its low cost, is often used as an external substrate to stimulate processes such as denitrification in WWTPs. Addition of another easy substrate in the fungal filters, such as methanol, could also maybe stimulate the fungal activity. Three fungal filters with *P. ostreatus* were thus operated in parallel to treat NPX in synthetic wastewater containing 1% of methanol. As shown in Fig. 9.14, NPX was well removed in the three filters during the seven batch cycles performed, similar to the results of the filters without methanol (Fig. 9.13 B.1). In the three first cycles, laccase activity increased rapidly up to 565 U l^{-1}, which was seven times higher than the maximum observed in the same filters without methanol. Despite this much higher laccase activity, the kinetic of NPX removal was, however, not different than the one in the filters without methanol (Fig. 9.14 D). Moreover, in the three filters, a green mould appeared on the wood and mycelium after 6 days, and developed with time. This coincided with a rapid decrease in laccase activity in the next cycles. Methanol was thus

probably stimulating laccase activity, but did not increase NPX removal kinetics, and favoured the development of the green mould (parasite of the fungus). Similar observations (laccase activity stimulation but green mould development) were made in a previous study with *T. versicolor* when glucose was added in the columns (Vargas, 2013). Addition of an easy substrate should thus be avoid to allow long-term operation (and activity) of the fungal filter.

9.3.9 Efficiency of the fungal filter to remove micropollutants from synthetic wastewater

As the sequential batch fungal filter with *P. ostreatus* proved to be efficient to treat NPX in synthetic wastewater during 16 d of operation, longer-term operation with several other micropollutants (at high concentrations in synthetic wastewater) were tested to evaluate the potential of this system.

9.3.9.1 Naproxen

As the two fungal filters with *P. ostreatus* of the previous experiment (section 9.3.8) were still active after 16 d (8 cycles), their operation was continued during almost 120 d to study their long term behaviour and efficiency to treat NPX (Fig. 9.15). From day 16 to 28 (13 d), the filters were not controlled and the synthetic wastewater was not renewed. During this period, the valve of one filter (Fungus 2) got blocked and the mycelium stayed in complete submersion during several days. When the filters were again operated normally (from the 9th cycle onward), filter 2 was less efficient to treat NPX, and its efficiency decreased with time until no more NPX was removed (23rd cycle, Fig. 9.15 A). Complete submersion of *P. ostreatus* during a prolonged time was thus detrimental for the fungus and led to its premature death. The other filter (Fungus 1) kept a very good efficiency during 80 d (24 cycles), with more than 90% NPX removal in less than 48 h (Fig. 9.15 A). Afterward, the efficiency started to decrease rapidly, and the laccase activity became negligible. NPX was still degraded until day 96, but at a much lower rate. To try to stimulate the activity of the fungus, 1% methanol was added as substrate in the synthetic wastewater of both filters from the 29th cycle onward. No stimulation of laccase production was observed and a slight decrease in NPX kinetic removal was notice for filter 1 (Fungus 1). In filter 2, NPX started to be again slightly removed, probably due to stimulation of the microbial activity. Indeed, as shown in Fig. 9.15 D, methanol was completely consumed, and at a same rate in both filters, very likely by the microbial community. Addition of an external carbon source was therefore more favouring the indigenous microbial community than the white-rot fungus, leading probably to its faster death.

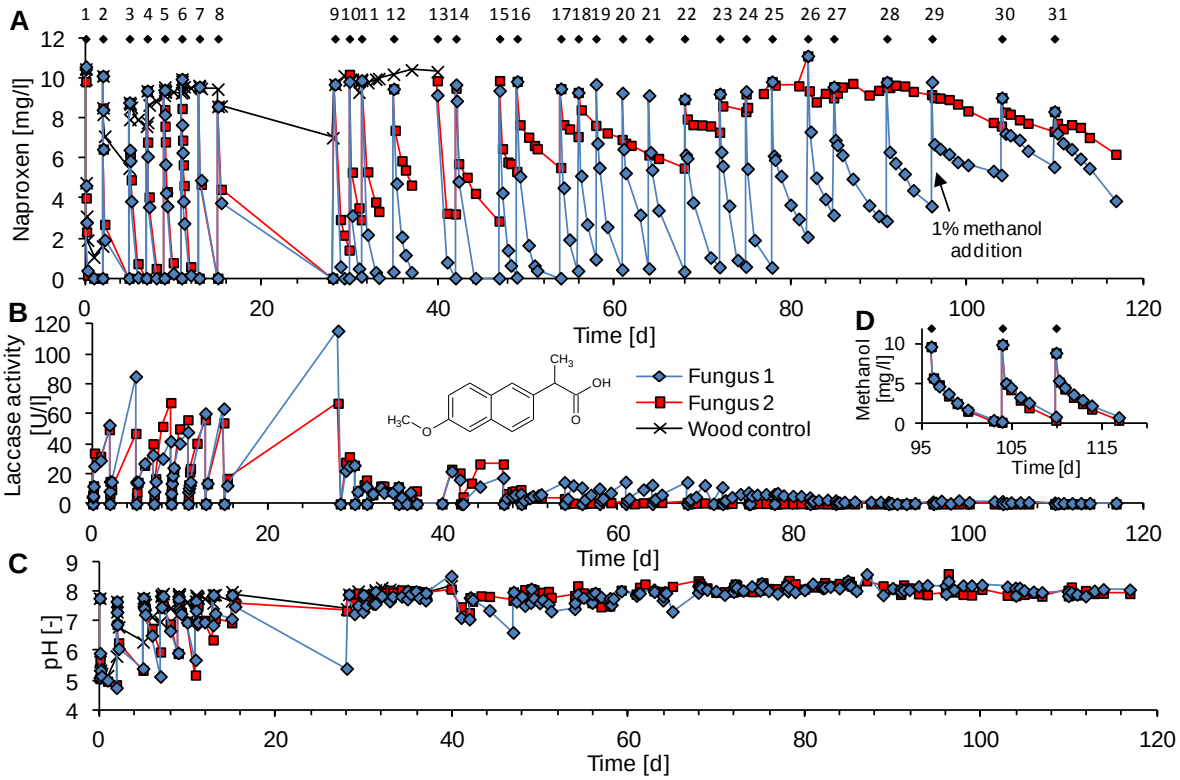


Fig. 9.15 (A) Kinetics of naproxen removal during the batch cycles (◊ numbered) in two filters (Fungus 1 and 2) inoculated with *Pleurotus ostreatus*, and in a wood filter not inoculated (Wood control). Evolution of laccase activity (B) and pH (C) during the batch cycles. The synthetic wastewater was renewed at each batch cycle. From the 29th cycle, an external carbon source (methanol at 1% v/v) was added in both fungal filters. (D) Evolution of methanol concentration in the fungal filters.

9.3.9.2 Sulfamethoxazole

The removal of the antibiotic SMX, which can be degraded by *T. versicolor* (cf. Section 9.3.2), was also tested in the fungal filter with *P. ostreatus*. As presented in Fig. 9.16, SMX was continuously degraded (> 90% in 48 h) by the fungus in the filters during 80 d (21 cycles). During the first 8 cycles, SMX was added at initial concentrations around 9 mg l^{-1}. Rapidly, after 3-4 cycles, one of the replicate (filter 1) started to be less efficient than the other. This was possibly due to a toxic effect of SMX at these concentrations, as observed for *T. versicolor* (cf. section 9.3.1). As, from the beginning, filter 1 was slightly less active than the second replicate, SMX was not always completely removed at the end of the cycle, and thus, the fungus in filter 1 was more and more exposed to SMX. This higher exposure could possibly impact the fungal activity and progressively decrease its efficiency to remove SMX, as observed during cycles 5 to 8 (with filter 1). It was therefore decided to decrease the initial concentration by half, to 4.5 mg l^{-1} (from cycle 12). This strategy was effective, as both replicates behaved then in a similar way, with constant SMX degradation until the end of the experiment.

Similar to NPX, SMX was strongly removed by adsorption in the wood control during the first cycle, and then progressively less and less adsorbed in the next cycles. However, constant (slow) removal was observed in the control even once the adsorption equilibrium with the initial concentration should be reached, meaning that SMX was degraded. To check if it was degradation by photolysis (SMX is sensitive to photodegradation (Bonvin et al., 2013a)) or biodegradation by the native microbial community, the microbial activity was inhibited with azide from the 19th cycle onward. Directly, the

removal in the control stopped. SMX could therefore also be degraded by the native microbial community, but at a slower rate than with the fungus.

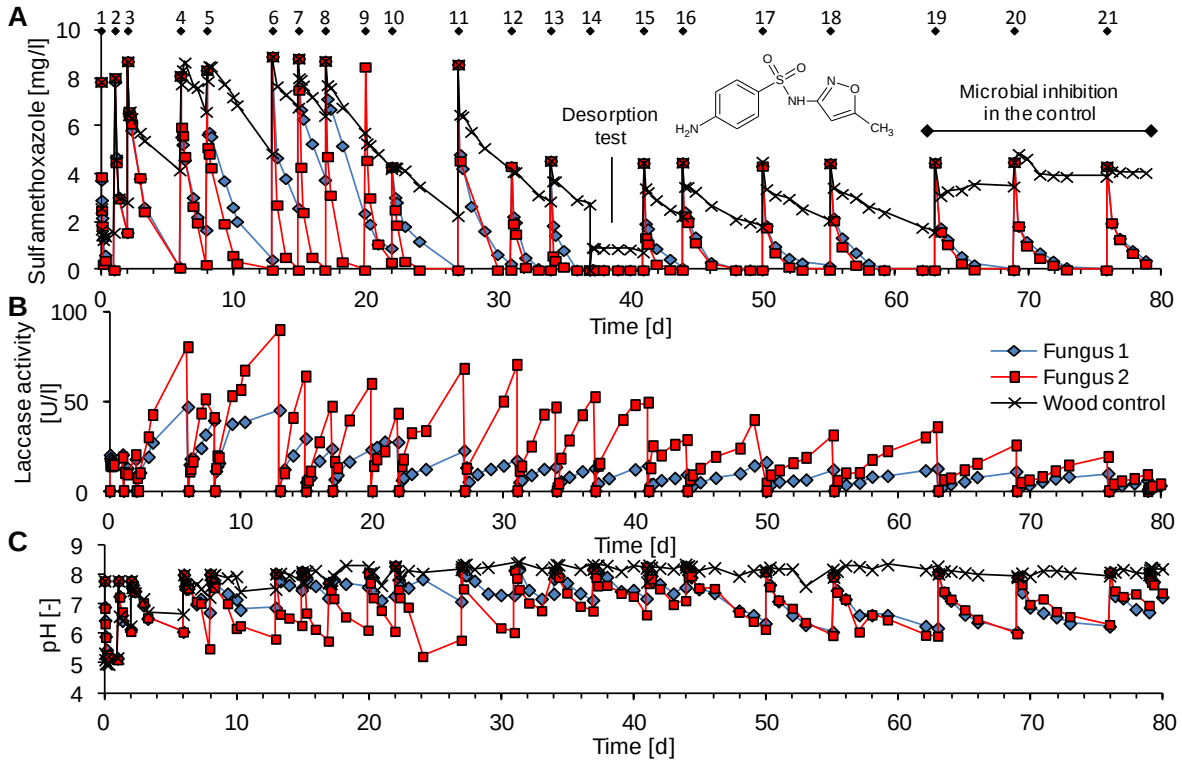


Fig. 9.16 (A) Kinetics of sulfamethoxazole removal during the batch cycles (◊ numbered) in two filters inoculated with *P. ostreatus* (Fungus 1 and 2) and in a wood filter not inoculated (Wood control). Evolution of laccase activity (B) and pH (C) during the batch cycles. The synthetic wastewater was renewed at each batch cycle, except for Fungus 1 and the wood control at the 9th batch cycle. The microbial activity in the wood control was inhibited (azide 10 mM) during the cycles 19, 20 to 21.

To evaluate the fraction removed by adsorption on the wood/mycelium, a desorption test was performed during the 14th cycle: the synthetic wastewater was renewed without addition of SMX (initial concentration at 0 mg l^{-1}). In the wood control, a rapid increase in the concentration (up to 0.9 mg l^{-1}) was observed (Fig. 9.16 A), whereas SMX was not detected in the two fungal filters. This showed that SMX was completely degraded during the previous cycle in the fungal filters (no desorption), whereas, in the wood control, desorption occurred, showing that adsorption was in important removal mechanism.

9.3.9.3 Carbamazepine

At the end of the experiment with SMX (Fig. 9.16), after 80 d of operation, the two fungal filters were still well active. They were therefore used to evaluate their potential to treat CBZ, a very persistent antiepileptic. As shown on Fig. 9.17, CBZ was very rapidly removed in both fungal filters, but at a similar rate as in the wood control (90 % removal in the first cycle). The three filters behaved indeed in a very similar way during the 60 d of operation (7 cycles). As, in each cycle, the removal stopped before complete CBZ elimination (reaching a plateau corresponding to the adsorption equilibrium), it could be attributed to adsorption and not to degradation. At each new cycle, CBZ was slightly less removed, due to a progressive saturation of the support. At the end of the last (7th) cycle, still 50%

CBZ removal was observed. The good absorption affinity of CBZ for the wood supports was also observed with PAC (cf. Chapter 3), confirming that this compound can be well remove by adsorption.

During the long 5th cycle, which lasted 7 d, a constant, but very slow, decrease in CBZ concentration was observed in the two fungal filters, whereas a plateau was reached in the wood control (constant concentration). The same observation could be done for the long 7th cycle, with a constant slow decrease in the fungal filter 2, the one that was still active, and no degradation in the wood control and the fungal filter 1 (which did not have anymore laccase activity). This suggested that CBZ was very slowly degraded by the fungus. Indeed, CBZ was reported to be degraded by *P. ostreatus*, probably by a combined action of the extracellular manganese peroxidase and intracellular cytochrome P450 (Golan-Rozen et al., 2011).

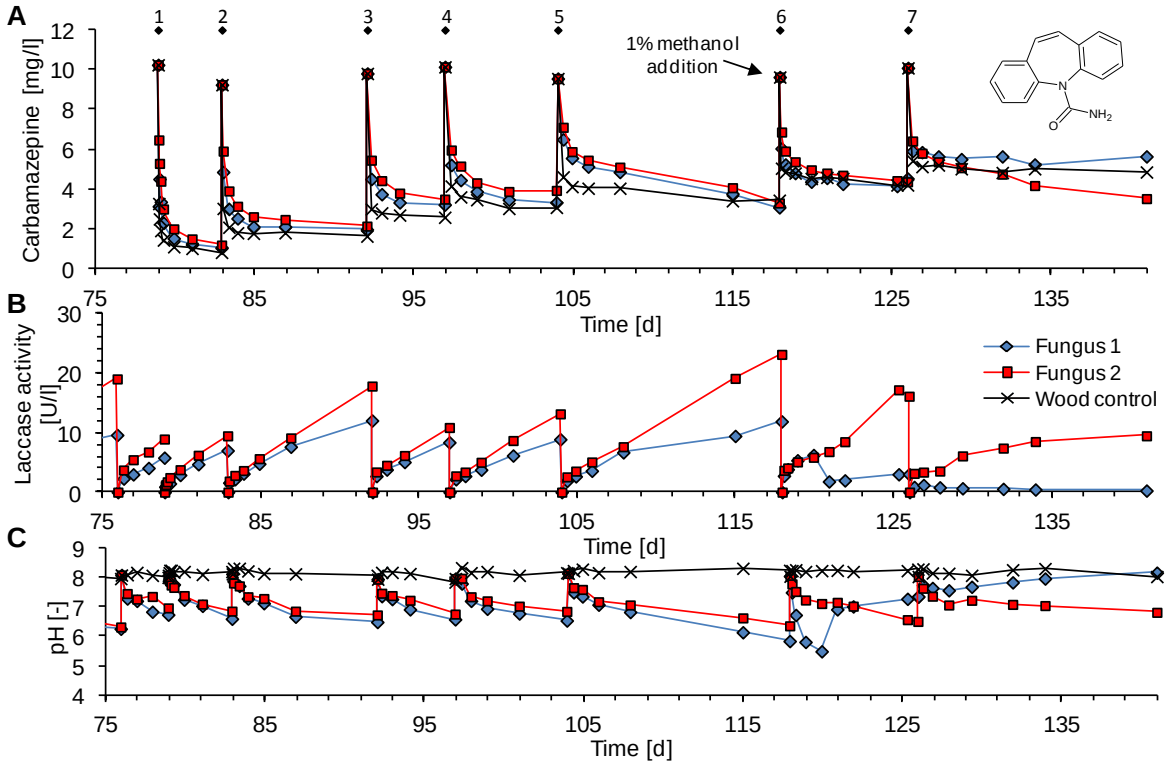


Fig. 9.17 (A) Kinetics of carbamazepine removal during the batch cycles (◊ numbered) in a filter inoculated with *P. ostreatus* (Fungus) and in a wood filter not inoculated (Wood control). Evolution of laccase activity (B) and pH (C) during the batch cycles. The synthetic wastewater was renewed at each batch cycle. From the 6th cycle on, an external carbon source (methanol at 1% v/v) was added in the fungal filter 1 (Fungus 1) and the wood control. The experiment with carbamazepine was started on day 79 with the filters tested previously with sulfamethoxazole.

In order to possibly stimulate the fungal activity, methanol (at 1% v/v) was added as external substrate in the synthetic wastewater during the 6th and 7th cycles, in the fungal filter 1 and in the wood control. This resulted, in the fungal filter 1 (6th cycle), of, first, a stimulation of laccase production (increase in activity) together with a drop of pH, followed, 2 d later, by a drop of activity together with an increase in pH. During the 7th cycle, the fungus was not anymore active in the fungal filter 1 (with methanol). All these effects were not observed in the fungal filter 2 (without methanol), which kept the same activity until the end of the experiment. Addition of methanol did, therefore, not increase CBZ removal and led rapidly to the death of the fungus, probably by predation/competition by other

microorganisms. Indeed, at the end of the 6th cycle, a green mould (probably a fungal parasite) appeared in the fungal filter with methanol, but not in the one without methanol or in the wood control with methanol. This confirmed again that the addition of an external easy carbon source was not in the favour of the white-rot fungus. The fungal filter without methanol addition was still active after 140 days of operation in unsterile systems, without re-inoculation or addition of fresh woodchips. Several fruiting bodies (sporocarps) even appeared after 150 d out of the woodchips removed from the filter 2 (Fig. 9.4 J).These results show the potential long durability of this system. As long as the fungus was active, the water was slowly acidified (from pH 8.0 to 6.5) during each batch cycle, which was not the case in the wood control or in the filter with inactive fungi.

Following methanol addition during the 6th cycle, methanol was consumed at a relatively similar rate in the wood control and the fungal filter (Fig. 9.18 A), suggesting that methanol was not specially used as substrate by the fungus. This was confirmed by an additional experiment in pure culture, were methanol was not significantly used as growth substrate by *P. ostreatus* and *T. versicolor*, but it stimulated laccase production by *P. ostreatus* (Contijoch, 2014). Together with methanol consumption, the appearance of one by-product was observed in all the samples coming from the fungal filters with methanol, but not in the other samples (wood filter with methanol or fungal filter without methanol). This by-product was detected by HPLC-RI with an ORH-801 column designed for organic acids, alcohols and carbohydrates analysis. The by-product appeared rapidly at the beginning of the batch cycle, coinciding with a fast drop of pH, and then disappeared completely after 3 d (probably by degradation), together with, in this case, a sharp increase in pH (Fig. 9.18). This suggested that the by-product formed following methanol addition was probably (but not confirmed) an organic acid produced by the fungus and degraded later by other microorganisms. Similar phenomena were observed following the addition of methanol in a filter treating BTZ (next section).

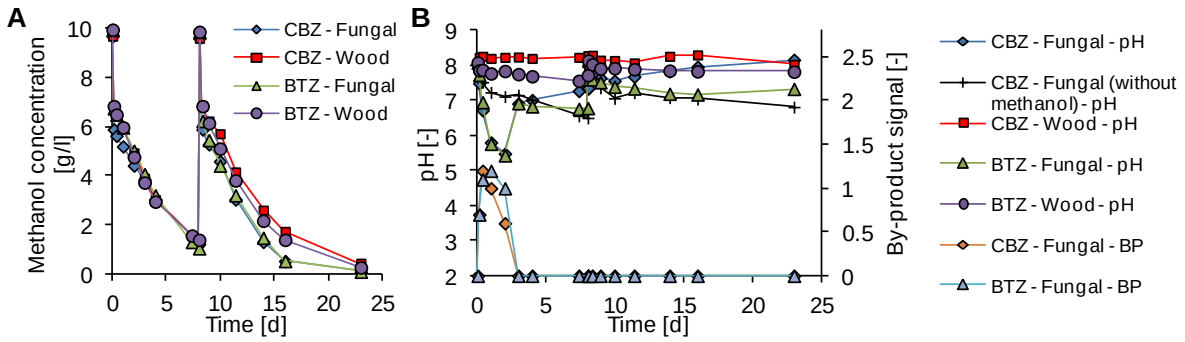


Fig. 9.18 (A) Methanol consumption and (B) evolution of the pH and by-product (BP, probably organic acid) formation in the fungal and wood filters treating carbamazepine (CBZ) or benzotriazole (BTZ) in synthetic wastewater, after 10 g/l methanol addition. Results of the two last batch cycles of the experiment with CBZ (Fig. 9.17) and with BTZ (Fig. 9.19).

9.3.9.4 Benzotriazole

The removal of the corrosion inhibitor BTZ in the fungal filter was also tested. As shown in Fig. 9.19 A, BTZ was rapidly removed (> 70%) during the first cycle in both the fungal filter and the wood control, probably by adsorption. Unlike for the other micropollutants, a progressive saturation of the adsorption on the wood control was not observed. Indeed, in each cycle, after a very fast removal by adsorption at the beginning of the cycle, BTZ was then still slightly continuously removed, without

reaching a plateau. This was a sign that BTZ was degraded, in both the wood and the fungal filters. Despite the degradation was slightly higher in the fungal filter, it was not possible to say if it was due to degradation by the fungus or by the native microbial community. During the 7th cycle, whereas the fungus was almost dead, this slow degradation was still clearly visible, suggesting it was done by the microbial community.

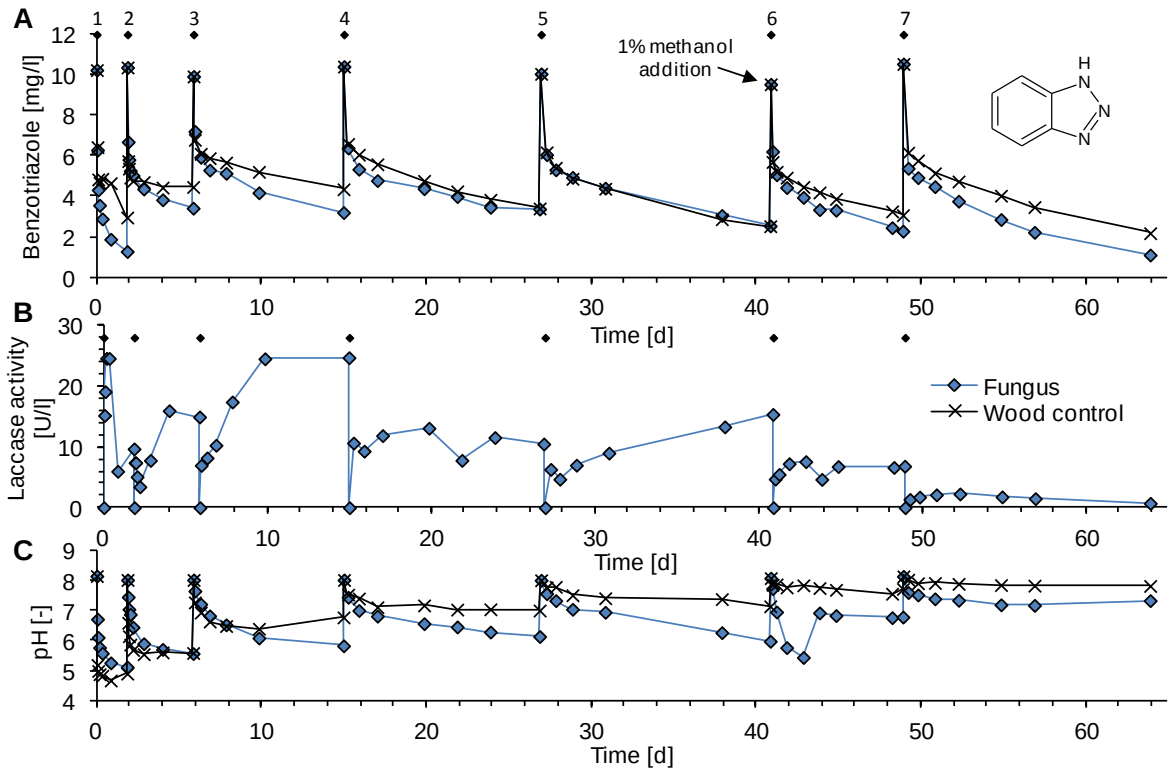


Fig. 9.19 (A) Kinetics of benzotriazole removal during the batch cycles (◊ numbered) in a filter inoculated with *P. ostreatus* (Fungus) and in a wood filter not inoculated (Wood control). Evolution of laccase activity (B) and pH (C) during the batch cycles. The synthetic wastewater was renewed at each batch cycle. From the 6th cycle on, an external carbon source (methanol at 1% v/v) was added in the fungal filter and the wood control.

From the 6th cycle onward, methanol was added as an external carbon source in both, the fungal and the wood control. Exactly as it happened with CBZ in the previous experiment, the methanol addition led first to a short stimulation of the fungus (with production of organic acid), but this was rapidly followed by the premature death of the fungus, as well as the appearance of a green mould on the mycelium. Unlike the effect with CBZ, methanol slightly stimulated the removal of BTZ in the fungal filter, probably due to the development of the microbial community. Despite a fraction of BTZ (around 40%) rapidly removed by adsorption during all the cycles, further removal by biodegradation (although possible) was very slow, with 90% removal observed only after 15 d (7th cycle).

9.3.9.5 Mixture of six micropollutants

The last test of the fungal filters with synthetic wastewater was performed with a mixture of six micropollutants: two anti-inflammatory drugs – DFC and MFA, one plastic component – BPA, one beta-blocker – metoprolol, one antibiotic – trimethoprim, and one anti-diabetic – metformin. The removal of these compounds during the 42 d of operation of the fungal filters is presented in Fig. 9.20 for MFA, DFC and BPA, and in Fig. 9.21 for metoprolol, trimethoprim and metformin.

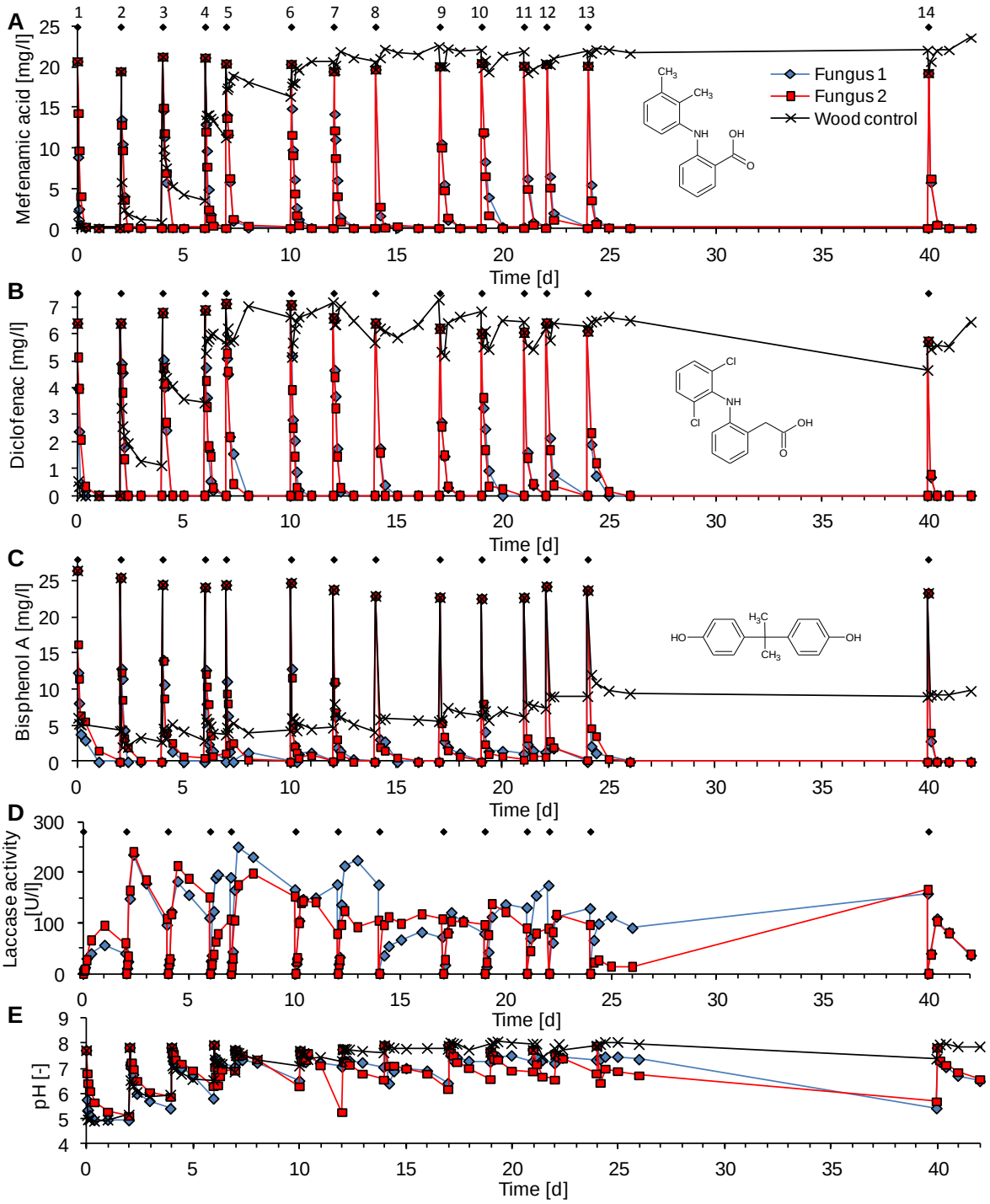


Fig. 9.20 Kinetics of micropollutant removal during the batch cycles (◊ numbered) in the two filters inoculated with *P. ostreatus* (Fungus 1 and 2) and in the wood filter not inoculated (Wood control). The microbial activity was inhibited (10 mM azide) in the wood control. (A) For mefenamic acid, (B) for diclofenac, and (C) for bisphenol A. Evolution of (D) laccase activity and (E) pH. The synthetic wastewater containing the mixture of pollutants was renewed at each batch cycle.

MFA, DFC and BPA were almost completely removed at each cycle in less than 12 h in the fungal filters during the 42 d of operation (Fig. 9.20 A, B, C). In the wood control, in which the microbial activity was this time inhibited from the beginning with azide to assess the effect of adsorption only, MFA and DFC were also completely removed by adsorption during the first cycle. At each next cycle, a progressive saturation of the filter was observed and after 4-5 cycles, no more removal of MFA and

DFC was observed in the wood control (the adsorption equilibrium with the initial concentration was reached). Adsorption of BPA in the wood control behaved differently, with less adsorption during the first cycles, but with only very slow saturation of the filter. Adsorption played thus an important role in BPA removal in the wood control during the 42 d of operation, with still 60% removal even after 14 cycles. For MFA and DFC, but also for BPA, adsorption could not explain the complete removal observed in the fungal filter, indicating that degradation by the fungus was the main removal mechanism. Part of the removal by degradation could be possibly attributed to laccase oxidation, as laccase activity was relatively high (50-250 U l^{-1}) (Fig. 9.20 D) and these three compounds are known to be oxidize by laccase (cf. Chapter 6). However, as also confirmed in section 9.3.10.6, extracellular laccase oxidation was not the main removal mechanism, as fast removal was observed also when laccase activity was still low (for instance during the 13th cycle with the fungal filter 2). It is indeed reported that the degradation of these three compounds by white-rot fungi is mainly due to other enzymes than extracellular laccase (Cabana et al., 2007b; Hata et al., 2010).

Long term removal of DFC and BPA in unsterile synthetic wastewater by white-rot fungi (*T. versicolor*) was also reached by Yang et al. (2013a) in a fungal membrane bioreactor operated continuously (hydraulic retention time of 2 d) during 3 months, but with a much lower efficiency (80-90% for BPA and around 55% for DFC). The fungal filter developed here seems thus more adapted for long term operations.

The beta-blocker metoprolol was only slightly removed (< 40%) in the fungal filters, and with the same efficiency than in the wood control (inhibited with azide), indicating that adsorption was the main removal mechanism (Fig. 9.21 A). Adsorption in the wood control reached very slowly equilibrium with the initial concentration (no more removal after 10 cycles), whereas, in the fungal filters, slow removal by degradation appeared after 9-10 cycles, probably due to the development of the native microbial community. The fungal filters were thus ineffective to remove metoprolol, neither by adsorption or fungal degradation. The highest removal achieved, 60% after 16 d (fungal filter 2, 13th cycle), was probably mainly due to the microbial community present in the filter.

The antibiotic trimethoprim was relatively well removed in the fungal filter during the 40 d of operation, with, on average, 77% removal (Fig. 9.21 B). During the first cycles, similar removal efficiencies were observed in the wood control, suggesting that the removal was, at the beginning, mainly due to adsorption. However, the wood control became progressively saturated, with less than 25% removal observed during the 13th cycle. This saturation did not happen in the fungal filters, indicating that trimethoprim was degraded, either by the fungus or by the native microbial community. The absence of complete removal after 16 d (cycle 13) in the fungal filters, which should not happen in the case of biodegradation, was possibly due to interferences during the quantification (strong noise on the chromatograms due to high release of wood components).

The anti-diabetic metformin, a pollutant found in very high concentrations in WWTP effluents (> 10 µg l^{-1}, cf. Chapter 3), was only very poorly removed (< 40%) in the fungal filters during the 40 d of operation (Fig. 9.21 C). A slight adsorption was observed during the first cycle, but mainly in the wood control (60% removal). A progressive saturation of the filters was then observed, with no more

removal after 8 cycles. The fungal filter was thus ineffective to remove metformin, neither by adsorption or biodegradation by the fungus or the native microbial community.

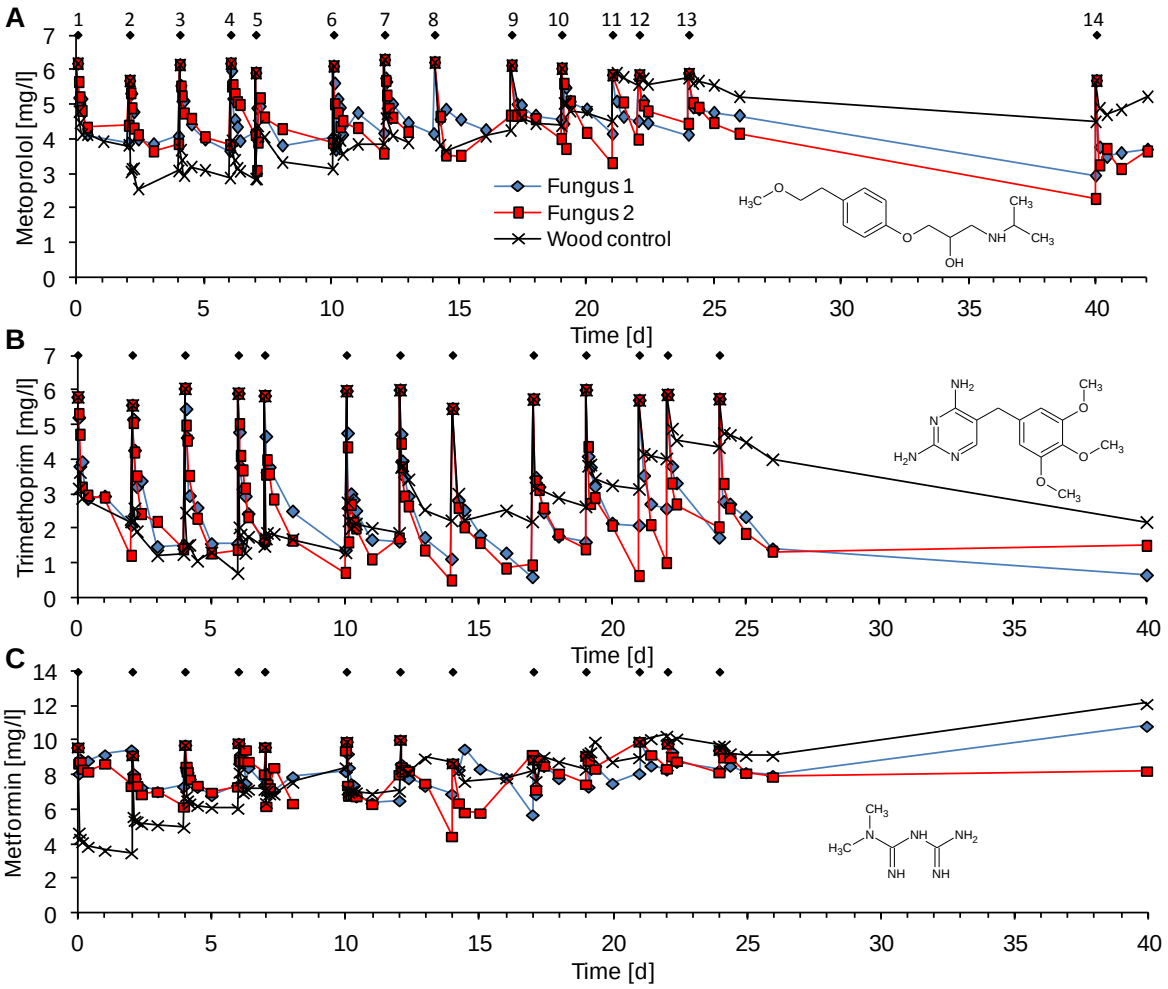


Fig. 9.21 Kinetics of micropollutant removal during the batch cycles (◊ numbered) in the two filters inoculated with *P. ostreatus* (Fungus 1 and 2) and in the wood filter not inoculated (Wood control). The microbial activity was inhibited (10 mM azide) in the wood control. (A) For metoprolol, (B) for trimethoprim, and (C) for metformin. The synthetic wastewater containing the mixture of pollutants was renewed at each batch cycle.

9.3.9.6 Synthesis of the removal efficiency in synthetic wastewater

As shown in the previous sections, the fungal filters were very effective to completely remove NPX, SMX, MFA, DFC and BPA in continuous operation during 40 to 80 d, with more than 90% removal in 12 to 48 h (depending on the compound) in synthetic non-sterile wastewater. Fungal degradation was clearly the main removal mechanism for these five pollutants. Trimethoprim was also well removed in the fungal filters, with on average around 80% removal in 48 h, mainly by biodegradation (by the fungus or the native microorganisms). BTZ and CMZ were partially removed in the filters (between 50 to 90% in 5 to 15 d), but mainly by adsorption on the wood/mycelium supports, despite a very slow degradation (possibly by the fungus) could be observed. Finally, metoprolol and metformin were only poorly removed (on average less than 40%), due to their low sorption affinity and low biodegradability. These experiments in synthetic wastewater showed that *P. ostreatus* could be maintained active in the fungal filters during more than 140 d under non-sterile conditions, in

continuous operation without addition of new substrate or re-inoculation of the fungus, while removing completely half of the pollutants tested in less than 48 h.

9.3.10 Removal mechanisms

As already discussed in the previous sections, micropollutants are removed by two main mechanisms in the fungal filters: adsorption onto the wood/mycelium and biodegradation by the fungus or the microbial community. The relative contribution of these mechanisms changed at each batch cycle. In order to understand better these two mechanisms and their evolution during the long-term operation of the filters, a modelling approach was used.

9.3.10.1 Adsorption

During the first batch cycles with almost all the pollutants, adsorption onto the wood/mycelium was the main removal mechanism, as relatively similar removal efficiencies were observed between the fungal filters and the wood controls. As described in Chapter 1, sorption processes are composed of two simultaneous reversible reactions, adsorption and desorption. Adsorption equilibrium is reached when the rate of both reactions becomes equal, meaning that the concentration on the solid phase, q, is in equilibrium with the concentration in the liquid phase, C. The rate of adsorption/desorption, as well as the adsorption equilibrium can be approximated by simple models.

9.3.10.2 Adsorption kinetic modelling

Two approaches were compared to model the adsorption kinetics of NPX in the wood control, one derived from a Langmuir equilibrium model (with a maximum adsorption capacity), and the other from a Freundlich equilibrium model.

For the "Langmuir type" kinetic model, based on the equations proposed by Oh et al. (2012), rates of adsorption and desorption are assumed to follow second-order and first-order kinetics, respectively. The variation of the concentration in the liquid, C [mg l^{-1}], at a time t [d], is described by Eq. 9.4, while the change in the concentration on the solid, q [mg g^{-1}], is described by Eq. 9.5. m [g l^{-1}] is the concentration of adsorbent (dry wood), k_a [l mg^{-1} d^{-1}] and k_d [d^{-1}] are the second order adsorption and first order desorption rate constants, respectively, and q_m [mg g^{-1}] is the maximum adsorption capacity of the adsorbent.

$$\frac{\mathrm{d}C}{\mathrm{d}t} = m\,k_d\;q - m\,k_a\;C\,(q_m - q) \tag{9.4}$$

$$\frac{\mathrm{d}q}{\mathrm{d}t} = k_a\;C\,(q_m - q) - k_d\;q \tag{9.5}$$

At the equilibrium the derivatives are equal to zero, and q can be expressed as a function of C, resulting to a Langmuir isotherm (Eq. 9.10, see below) with $K = k_d/k_a$, the Langmuir coefficient.

For the "Freundlich type" kinetic model, according to Skopp (2009), the variation in the time of C and q are described by Eqs. 9.6 and 9.7, respectively, with k_a [l^{n1-1} mg^{1-n1} d^{-1}] and k_d [l^{n2-1} mg^{1-n2} d^{-1}] the adsorption and desorption rate constants, respectively, and n_1 and n_2 [-], two constants of the model.

$$\frac{dC}{dt} = -k_a\, C^{n_1} + k_d\, (m\, q)^{n_2} \tag{9.6}$$

$$\frac{dq}{dt} = \frac{k_a}{m} C^{n_1} - \frac{k_d}{m} (m\, q)^{n_2} \tag{9.7}$$

At the equilibrium the derivatives are equal to zero, and q can be expressed as a function of C, resulting to a Freundlich isotherm (Eq. 9.8, see below), with K_f = (1/m) $(k_a/k_d)^{1/n2}$ and $n=n_1/n_2$, the Freundlich coefficients.

These two models were successfully applied to reproduce the adsorption kinetic of NPX in the two wood controls (Fig. 9.22). Indeed, the adsorption kinetics and the progressive saturation effect were both very well reproduced by the models, even in the wood control 2, where, at the 5th cycle, a desorption test was performed. This demonstrated that the removal of NPX in the wood controls was only due to reversible adsorption phenomena onto the wood support. The sorption kinetics were relatively fast, reaching equilibrium in 8-9 h. Although the model was not fitted to other micropollutant concentrations, similar adsorption kinetics were observed for most of them in the wood controls. Both models appeared thus to be a good tool to predict the progressive saturation of the filters until they reach the adsorption equilibrium with the initial concentration.

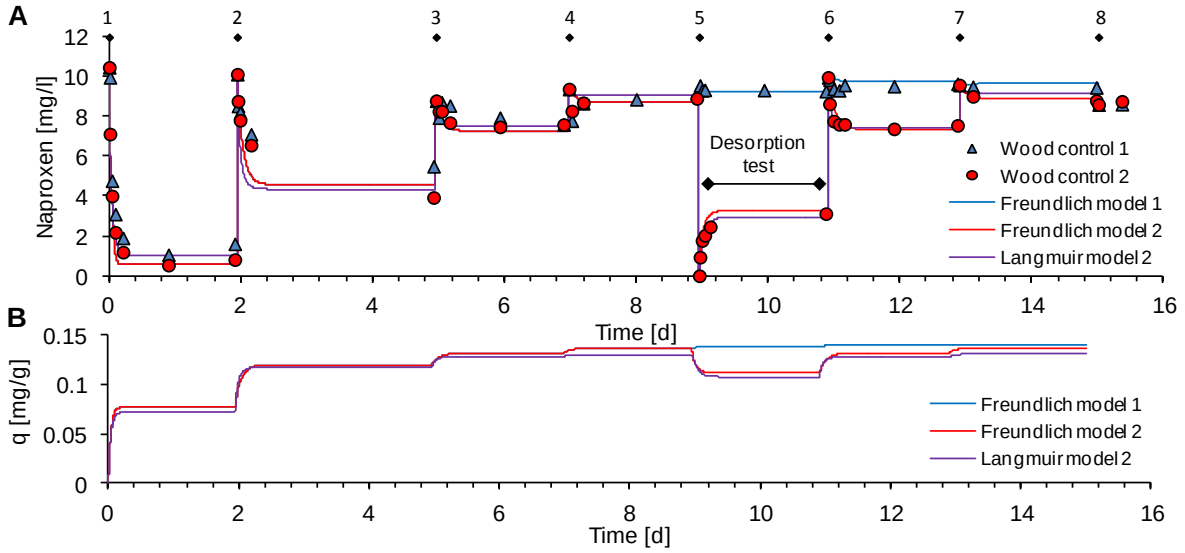


Fig. 9.22 Modelling of naproxen adsorption kinetics in the two wood filters (Wood control 1 and 2). Symbols: measured data. Lines: modelled values. Coefficients for the Freundlich kinetic model: k_a – 837 [l^{n1-1} mg^{1-n1} d^{-1}], k_d – 618 [l^{n2-1} mg^{1-n2} d^{-1}], n_1 – 0.026 [-], n_2 – 0.1252 [-] , m – 130 [g l^{-1}] (Equivalent to K_f – 0.0872 [l^n mg^{1-n} g^{-1}], n – 0.2069 [-]). Coefficients for the Langmuir kinetic model: k_a – 1.6681 l [mg^{-1} d^{-1}], k_d – 1.6957 [d^{-1}], q_{max} – 0.145 [mg g^{-1}] , m – 130 [g l^{-1}].

9.3.10.3 Adsorption equilibrium modelling

Once the equality between the adsorption and desorption rates was reached, the concentrations onto the solid q and in the liquid C stayed constant. If C increased or decreased, then q (at equilibrium) also increased or decreased (cf. Fig. 9.22), following a defined relation (at a constant temperature and pH). This relation can be described by an adsorption isotherm, i.e., a function describing the concentration on the solid according to the one in the liquid, once the equilibrium is reached. The most commonly used adsorption isotherms are the two parameters models from Freundlich and Langmuir (Foo and Hameed, 2010).

The adsorption equilibrium of the micropollutants in the wood control was thus modelled using a Freundlich isotherm (Eq. 9.8):

$$q_i = K_f \; C_i^n \tag{9.8}$$

with K_f [l^n mg^{1-n} g^{-1}] and n [-], the Freundlich coefficients, C_i [mg l^{-1}], the concentration in the liquid phase at the equilibrium at the end of the cycle i, and q_i [mg g^{-1}], the concentration sorbed onto the solid at the equilibrium at the end of the cycle i. The concentration onto the solid q_i was calculated for each cycle with Eq. 9.9, with C_o and q_o, the initial concentrations in the liquid and the solid, respectively, and m [g l^{-1}], the concentration of adsorbent (solid medium).

$$q_i = q_{i-1} + \frac{C_0 - C_i}{m} \quad \text{with } q_0 = 0 \tag{9.9}$$

The coefficients K_f and n were then determined by linear regression of $\log(C_i)$ versus $\log(q_i)$. The slope of the regression line gave n and the y-intercept gave $\log(K_f)$.

By equalling Eq. 9.8 and 9.9, the theoretical concentration in the liquid at the equilibrium C_i could then be determined (by numerical solving of this implicit equation) for each cycle i as a function of the initial concentration C_o.

Freundlich isotherms are often able to reproduce well adsorption phenomena in wastewater, as they account for multisite adsorption onto heterogeneous surfaces (various adsorption sites). The theory behind this isotherm is that the stronger binding sites are occupied first, followed by the sites with lower adsorption energy, exponentially decreasing the adsorption capacity of the adsorbent. Complete saturation of the support is thus never reached (Foo and Hameed, 2010). But it is an empirical model which is valid only for the range of concentration used to calibrate it. Indeed, as the shape of the isotherm changes very rapidly, using it outside its calibration range may give completely wrong values. This was indeed observed when the coefficient n was far from 1 (data not shown). Several other models with more physical meaning may also reproduce well the adsorption phenomena observed. A two parameters Langmuir model (Eq. 9.10) (Foo and Hameed, 2010) was therefore also tested.

$$q_i = q_m \frac{C_i}{K + C_i} \tag{9.10}$$

with q_m [mg g^{-1}], the maximum concentration onto the solid (maximal adsorption capacity), and K [mg l^{-1}], the Langmuir coefficient. The Langmuir model assumes monolayer adsorption, which occurs only on a limited number of sites. Once all these sites are occupied (saturation), no more adsorption can occur (plateau of the isotherm) (Foo and Hameed, 2010).

These two models were compared for NPX adsorption on the wood controls. Both models fitted well the measures (Fig. 9.23). As the Freundlich model showed slightly better fitting, it was used to determine the isotherms for each pollutant (Fig. 9.24). The Langmuir model may, however, enable better extrapolations at lower concentrations (slopes not so steep and isotherm more linear).

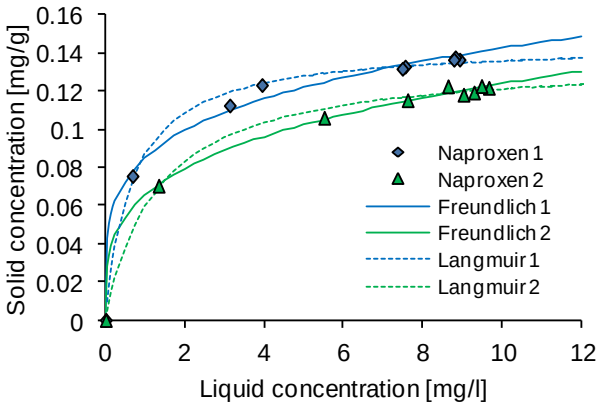


Fig. 9.23 Comparison of Freundlich and Langmuir isotherms to model the adsorption equilibrium of naproxen on two wood filters (1 and 2) in synthetic wastewater.

As presented in Fig. 9.24 and in Table 9.1 (for the values of the coefficients), very different adsorption isotherms were found for each micropollutant. Some, like NPX, DFC and MFA, presented a very good adsorption affinity at low concentrations, but reached very fast the saturation (coefficient $n < 0.3$). NPX and DFC had very similar adsorption affinity, while MFA was adsorbed almost 3.8 times more. BPA, CBZ and metformin, on the contrary, showed lower adsorption affinity at low concentrations, but had (almost) linear isotherms, with no saturation effect observed at high liquid concentrations. BPA showed a very high adsorption affinity, with, for instance, a concentration adsorbed onto the wood 15 times higher than the one of NPX (for a concentration in the liquid around 10 mg l^{-1}). CBZ had a lower adsorption affinity than BPA, but still much higher than metformin, poorly removed by adsorption. The adsorption of trimethoprim was comparable to the one of CBZ. Trimethoprim behaved, however, very strangely during the two first batch cycles (Fig. 9.21 B), with less adsorption than in the third cycle. This may be related to the pH, which was low in the two first cycles (pH 5-6) and then increased to neutral values (pH 6.5-7.8). As trimethoprim has a pKa at 7.2 (cf. Table 9.1), its charge changes with change in pH (trimethoprim is positively charged under acidic conditions and not charged in alkaline media) (Fig. 9.25), which may affect its adsorption affinity (possibly higher for the neutral form).

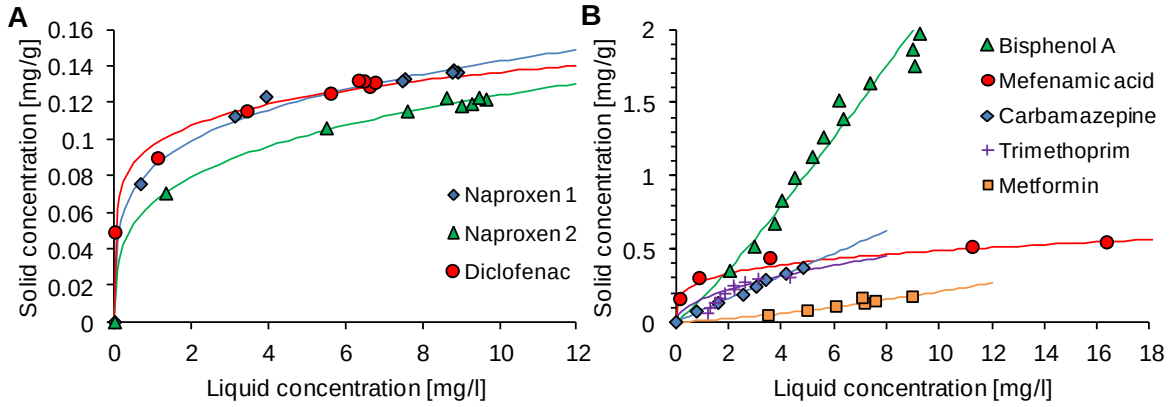


Fig. 9.24 Freundlich isotherms for micropollutant adsorption in the wood filter in synthetic wastewater.

Table 9.1 Freundlich coefficients for the isotherms of micropollutant adsorption in the wood filter in synthetic wastewater and high pollutant concentrations (5-20 mg/l), and physico-chemical properties of the pollutants.

	Naproxen 1	Naproxen 2	Diclofenac	Mefenamic acid	Bisphenol A	Carbamazepine	Metformin	Trimethoprim
K_f [l^n mg^{1-n} g^{-1}]	0.0848	0.0653	0.0967	0.2794	0.1616	0.0811	0.0083	0.1557
n [-]	0.225	0.278	0.150	0.241	1.144	0.978	1.400	0.508
Log K_{ow} [-][a]	3.18	3.18	4.51	5.12	3.32	2.45	-2.64	0.91
pKa [-][a]	4.2	4.2	4.1	4.2	10.1	13.9	12.4	7.2
Charge at pH 7[b]	-1	-1	-1	-1	0	0	+2	+1 and 0

[a] Source: compilation by Margot et al. (2013)

[b] Source: www.chemicalize.org (last accessed 27.08.2014)

These results show that the adsorption is strongly influenced by the characteristics of the pollutant. All the acidic compounds (NPX, DFC, MFA), mainly negatively charged at pH above 4.2 (cf. Table 9.1), had strong adsorption affinity at low concentrations but reached fast saturation ($n << 1$), while neutral or positively charged pollutants showed mostly linear adsorption isotherms ($n \approx 1$). This suggests that the adsorption on wood is mainly driven by electrostatic interactions. As wood is mainly acid, it is expected to be mainly negatively charged at neutral pH, which should limit the adsorption of negatively charged pollutants (electrostatic repulsion), hence, possibly, the fast saturation effect observed for them.

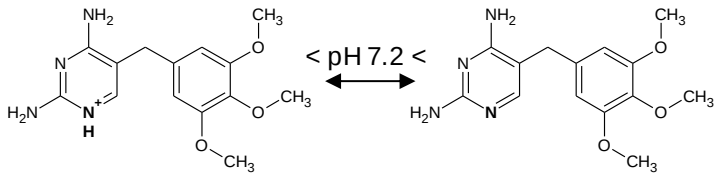


Fig. 9.25 Protonated and neutral forms of trimethoprim (depending on the pH, pKa of 7.2) (according to the database on www.chemicalize.org)

The Freundlich isotherms allowed calculating how many batch cycles were necessary to reach the adsorption equilibrium with the initial concentration in the wood control (no more removal by adsorption). This information can be very useful to determine how many cycles have to be taken into account to evaluate the effect of biodegradation alone. As presented in Fig. 9.26 A, if no more removal by adsorption was predicted after 5-9 cycles for NPX, DFC and MFA; 20, 50 and 150 batch cycles were necessary to reach the ultimate adsorption equilibrium for metformin, CBZ and BPA, respectively.

The effect of the initial pollutant concentration (10 mg l^{-1} versus 1 µl^{-1}) on the removal efficiency by adsorption was (theoretically) assessed for two pollutants with very different isotherms: CBZ and NPX (Fig. 9.26 B). For CBZ, which had a linear isotherm, same removal efficiencies were predicted independently of the initial concentration. However, for NPX, complete removal by adsorption during 1000 cycles was predicted at 1 µl^{-1} compared to no more removal after 5 cycles at 10 mg l^{-1}. This huge difference was due to the very strong adsorption affinity of NPX at very low concentrations (according to the Freundlich isotherm), and the fast saturation at higher levels. However, as mentioned before, it is very risky to extrapolate the Freundlich isotherm outside of the range from where it was calculated, as the slope of the isotherms changes very rapidly, especially when n is far from 1. Therefore, results with NPX at 1 µl^{-1} give only an indication that the removal efficiency can vary a lot depending on the

initial concentration, but very different values will probably be predicted for an isotherm determined at these low concentrations.

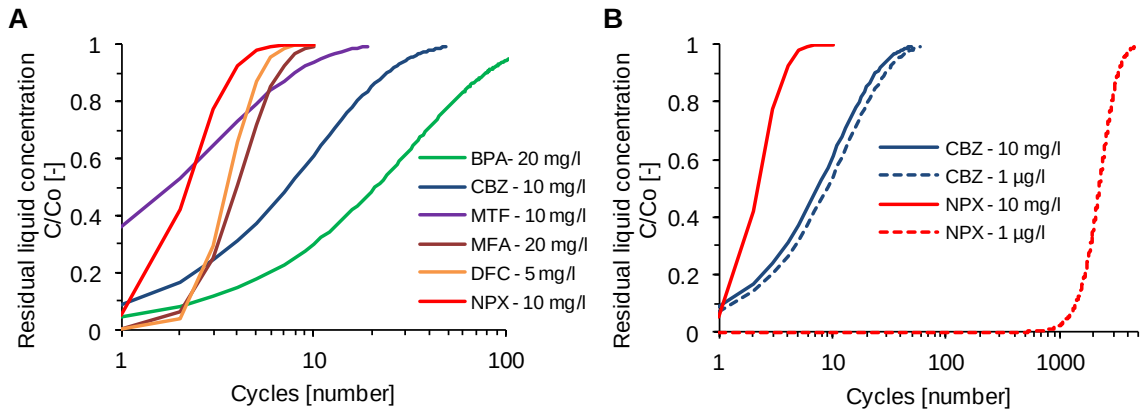


Fig. 9.26 Modelling of the residual micropollutant concentration in the liquid phase at the end (equilibrium) of the batch cycle with the wood filter (only adsorption) as a function of the number of cycles performed (based on the Freundlich equations). Conditions: 130 g l^{-1} dry wood, reused in each cycle. **(A)** With the real concentrations tested in synthetic wastewater. **(B)** Test of the Freundlich model with real and 10,000 time lower (1 µg l^{-1}) concentrations. BPA: bisphenol A, CBZ: carbamazepine, MTF: metformin, MFA: mefenamic acid, DFC: diclofenac, NPX: naproxen.

The isotherms were determined for adsorption onto the wood alone. The adsorption capacity on the wood covered by the mycelium may be different. According to the results with NPX (with inhibited fungus) (Fig. 9.13 B1), CBZ (Fig. 9.17), metoprolol and metformin (Fig. 9.21), slightly lower adsorption capacity was observed on the wood covered by the mycelium than on the not inoculated wood, probably due to the reduced access to the wood adsorption sites, covered by the mycelium.

9.3.10.4 Biodegradation

For pollutants such as NPX, SMX, DFC, MFA, BPA and trimethoprim, apart from the first cycles where adsorption dominated, biodegradation (by the fungus or by the native microbial organisms) was the main removal mechanism in the fungal filters. The degradation pathways and the possible metabolites produced were not studied (out of the goals of this study), but the evolution of the biodegradation rate with time was determined. For that, biodegradation (combined with the adsorption) of NPX in the fungal filters was modelled by pseudo first order kinetics (Eq. 9.11), assuming that fungal active biomass X [g l^{-1}] was constant during the batch cycle (no growth or decay).

$$\frac{\mathrm{d}C}{\mathrm{d}t} = k_s \; X \; C = k \; C \qquad (9.11)$$

With C [mg l^{-1}], the residual concentration in the liquid, t [d], the reaction time, k_s [l g^{-1} d^{-1}], the second order rate constant, and $k = k_s X$ [d^{-1}], the pseudo first-order rate constant.

Solving Eq. 9.11 gave the residual pollutant concentration as a function of the reaction time and the initial pollutant concentration C_o [mg l^{-1}] (Eq. 9.12):

$$C = C_0 \; \exp(-k \; t) \qquad (9.12)$$

This pseudo first order model was fitted to the data by minimizing the sum of the square of the difference between the measures and the model and the rate constant *k* was calculated for each batch cycle.

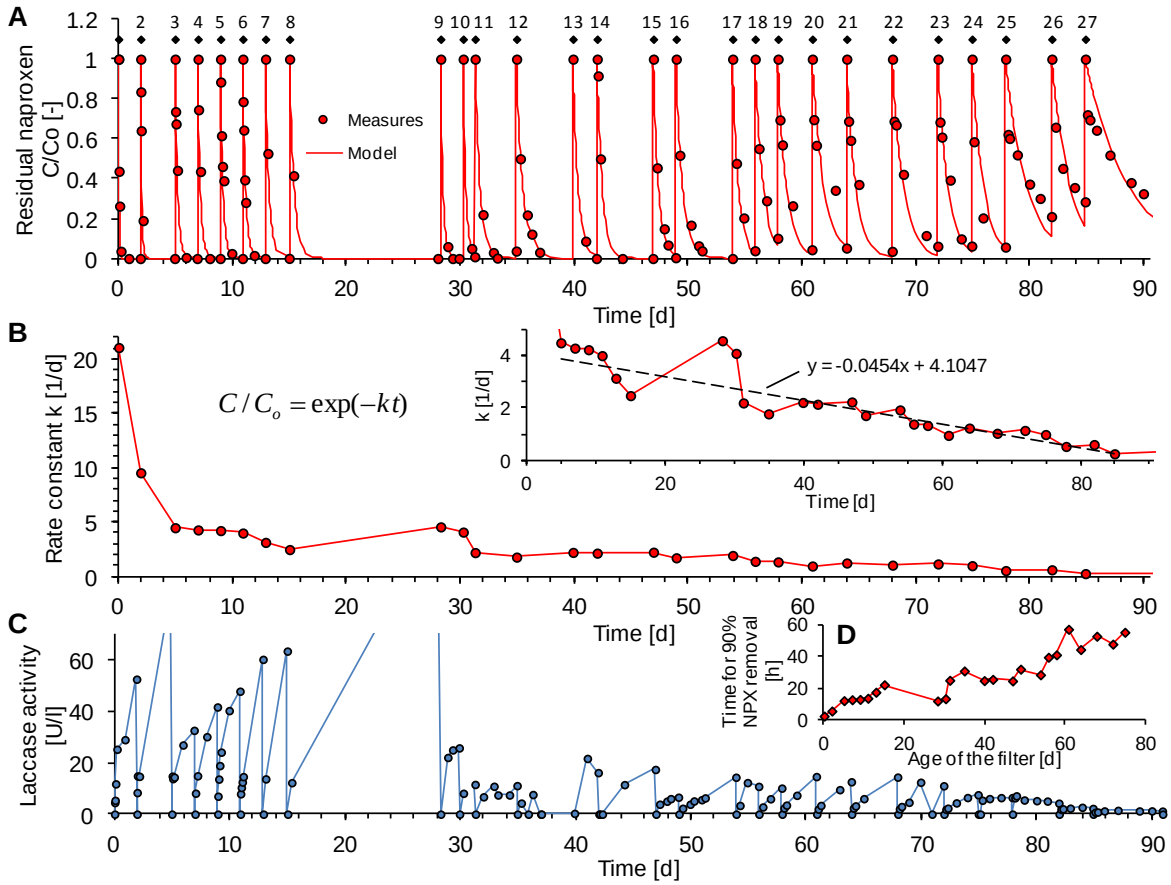


Fig. 9.27 Modelling of the kinetic of naproxen removal (degradation and adsorption) with a first order model. (A) Comparison between the model and the measures. (B) Evolution of the first order rate constant *k* as a function of the operation time of the filter (Insert: zoom on the lower k values). (C) Evolution of laccase activity in the filter. (D)Time for 90%naproxen removal as a function of the age of the filter. Results of the long term experiment with naproxen in synthetic wastewater (Fig. 9.15).

As presented in Fig. 9.27 A, NPX removal followed first order kinetics very well, up to 80 d of operation, when the fungal activity started to decline seriously. The removal rate constant was very high during the two first cycles, probably by the combined action of both strong adsorption and low pH (which should increase the fungal activity) (Fig. 9.27 B). From the third cycle onward, the pH stayed close to neutral conditions and the removal rate constant was more stable, decreasing slowly with time from 4.5 to 0.27 d^{-1} after 85 d. The increase of the removal rate after 28 d was not due to faster degradation but to higher adsorption, resulting probably to the complete liberation of the adsorption sites during the previous long cycle. Apart from this phenomenon, a linear decrease in removal efficiency was observed, linked to the slow decay of the fungus, less and less active (laccase production) in the filter (Fig. 9.27 C). Modelling the removal rate allowed therefore quantifying the decay of the fungus and the long term efficiency of the filter. As the decay was progressive, starting from the beginning of the operation, it indicates that the conditions in the filter were not optimal for the fungus, allowing it surviving but too stressful to develop. Indeed, no mycelium development was

observed once the filter was immerged (which is an advantage to avoid clogging the filter), whereas it was growing very well in dry conditions. The decrease in the degradation rate with the age of the filter has for direct consequence to increase the time required to remove the pollutants. As shown in Fig. 9.27 D, 90% NPX removal was reached in less than 12 h during the first cycles while it required up to 55 h when the filter was 75 d old. As very long degradation times require important volumes of filters, the filters should be re-inoculated with fresh mycelium once a defined maximum reaction time is exceeded (possibly around 48 h).

A trial was made to model at the same time adsorption and biodegradation kinetics, by combining Eqs. 9.4-9.5 and Eq. 9.11 to evaluate the effect of each mechanism in the fungal filter. It was assumed that only the fraction in the liquid was degraded (Eq. 9.13), and that the fraction adsorbed was only behaving as a source of pollutant for the fungus, released in the liquid phase by desorption as the dissolved pollutants were consumed (Eq. 9.14). It was, however, not possible to fit correctly this model to the measurements (data not shown). This suggested that the adsorbed fraction of the pollutants was also degraded by the fungus. A more complex model with many parameters to fit (data not shown) did also not result in a better fitting than model with a simple first order kinetic. It appeared to be very complex to separate adsorption and biodegradation mechanisms.

$$\frac{\mathrm{d}C}{\mathrm{d}t} = m\,k_d\;q - m\,k_a\;C\,(q_m - q) - k_{bio}\;X\;C \tag{9.13}$$

$$\frac{\mathrm{d}q}{\mathrm{d}t} = k_a\;C\,(q_m - q) - k_d\;q \tag{9.14}$$

9.3.10.5 Influence of the duration of the resting time on NPX removal

To possibly increase the removal rate of NPX in the fungal filters, it was tried, during one batch cycle, to shorten the resting time used in the sequential operation. The resting time was initially fixed arbitrary to 3 min, with 1 min feeding and 1 min reaction (cycle of 5 min). The resting was decreased to 1 min (cycle of 3 min), thus increasing the reaction time percentage to 1/3 of the cycle instead of 1/5. On the contrary to what was expected, this change did not lead to a faster removal rate of NPX (Fig. 9.28), probably due to complex interactions between adsorption and degradation, as the adsorbed fraction could be possibly degraded even during the resting time. As shorter resting time may lead to faster decline of the fungus (more stressful conditions), the initial resting time of 3 min was kept for all the experiments.

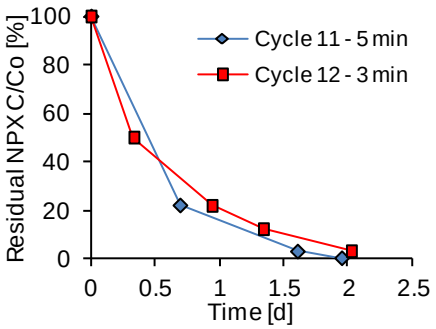


Fig. 9.28 Influence of the length of the resting time on naproxen (NPX) removal kinetics. Comparison between the 11th cycle (sequences of 5 min: 1 min feeding, 1 min reacting and 3 min resting) and the 12th cycle (sequences of 3 min: 1 min feeding, 1 min reacting and 1 min resting). Results of the long term experiment with naproxen in synthetic wastewater (Fig. 9.15).

9.3.10.6 Role of laccase in micropollutant removal

Some micropollutants, namely BPA, DFC and MFA, are subject to laccase oxidation. To evaluate the role of extracellular laccase on their removal in the filter, supernatants of the two fungal filters treating the mixture of six micropollutants (cf. section 9.3.9.5, Fig. 9.20) were collected during the 10th cycle and filtered at 0.22 µm to remove all microbial cells but not extracellular laccase. They were then spiked with a mixture of BPA, DFC and MFA, and the removal rate due to laccase oxidation was then compared to the one monitored in the filters during the 10th cycle. As shown in Fig. 9.29 A, BPA was rapidly oxidized by laccase in both supernatants, at a rate close to the removal observed in the filters. DFC and MFA were also oxidized by laccase in the supernatants, but at a rate much slower than the one observed in the filters (Fig. 9.29 B and C). Although extracellular laccase was probably involved in the removal of BPA in the filters, these results confirm that laccase oxidation was not the main removal mechanisms for DFC and MFA, probably degraded by the fungus via the action of other intracellular enzymes, such as cytochrome P450 (Hata et al., 2010). Laccase activity is very sensitive to the pH, with usually very slow oxidation rates under alkaline conditions (cf. Chapter 6). For MFA, the effect of the pH of the supernatants was clearly visible, with lower removal at pH 7.5 (supernatant 1) than pH 6.9 (supernatant 2). In the fungal filters, however, the pH was not strongly influencing the reactions, with fast removal observed for the three pollutants even in alkaline conditions. The lower sensitivity of the fungal filter to the pH is a clear advantage, compared to an enzymatic process with laccase, for municipal wastewater treatment.

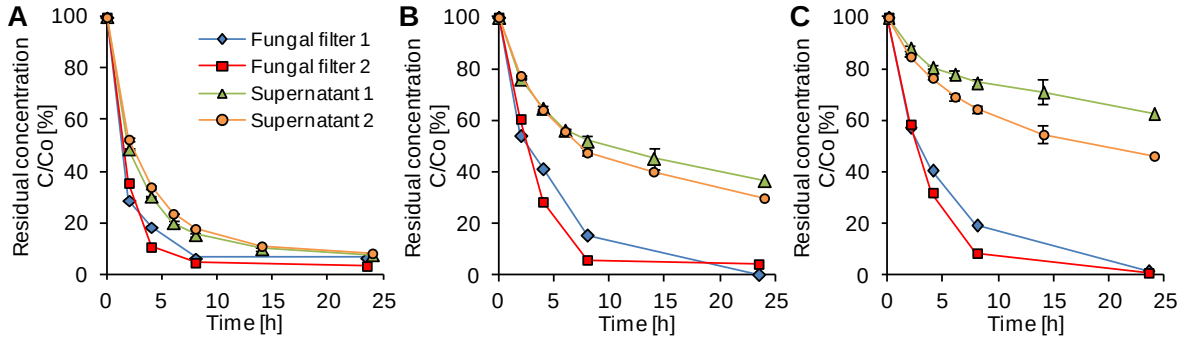


Fig. 9.29 Comparison of (A) bisphenol A (BPA), (B) diclofenac (DFC) and (C) mefenamic acid (MFA) removal in the filters 1 and 2 inoculated with *P. ostreatus* (fungal filter) and in their respective filtered (cell free) supernatants. For the supernatants, average and values of duplicates. Laccase activity and pH: 136 U l^{-1} and pH 7.5, and 122 U l^{-1} and pH 6.9, in filter 1 /supernatant 1 and filter 2 /supernatant 2, respectively. Initial concentrations: BPA – 20 mg l^{-1}, DFC – 10 mg l^{-1}, MFA – 20 mg l^{-1}.

To evaluate the potential of extracellular enzymes produced in the filters for the degradation of other micropollutants, the supernatant of a fungal filter with *T. versicolor* with high activity (943 U l^{-1}) and low pH (pH 5) was collected, filtered to remove the cells and spiked with eight micropollutants. Six of them, paracetamol, BPA, DFC, MFA and triclosan were rapidly oxidized by the extracellular enzymes (probably laccase), while IPN, NPX and SMX were not significantly removed (Fig. 9.30). This suggests that several pollutants, all phenols or anilines, may be potentially removed in the fungal filter by laccase oxidation. Several other extracellular enzymes may be also involved, such as manganese-peroxidase or lignin-peroxidase, but all require the presence of H_2O_2, which was probably not present in the supernatant (fast reaction with organic matter). The participation of these extracellular

peroxidases to the degradation of micropollutant in the fungal filters was not studied but could be significant (Golan-Rozen et al., 2011).

Despite the possible production of redox mediators by the fungus during lignocellulosic substrate degradation (Li et al., 2014), none of the compounds potentially degraded by laccase-mediator systems (IPN, SMX and NPX) were significantly removed in the supernatant (Fig. 9.30). Extracellular degradation by natural laccase mediator reactions in the filters is thus not expected to be a main removal mechanism.

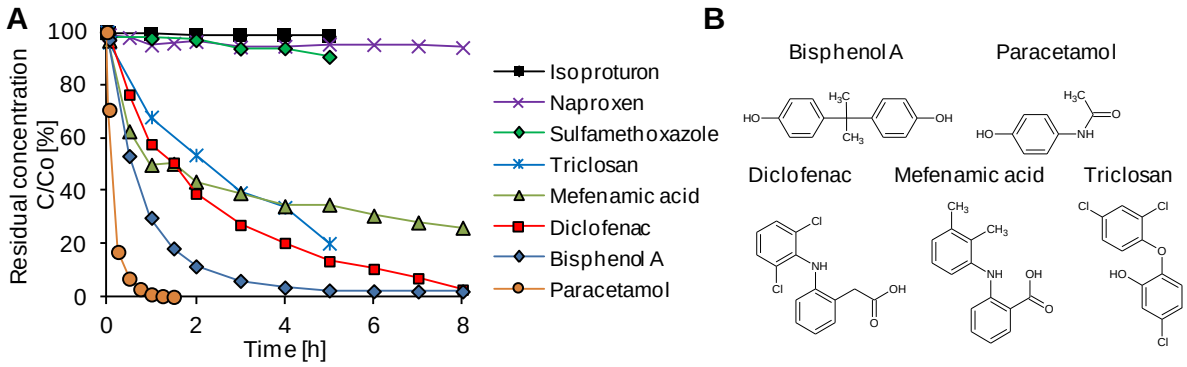


Fig. 9.30 (A) Micropollutant degradation in the cell free (filtered at 0.2 µm) supernatant from a wood filter inoculated with *Trametes versicolor*, with 943 U l^{-1} laccase activity, pH 5, spiked with 10 mg l^{-1} of pollutants. (B) Structures of the pollutants degraded.

9.3.11 Micropollutant removal in spiked real wastewater

The ability of *P. ostreatus* to degrade several micropollutants in the fungal filters treating synthetic wastewater in continuous operation during more than 140 d in non-sterile conditions showed the potential of this system. However, the synthetic wastewater was composed of tap water spiked with the pollutants, which, despite a relatively similar mineral composition (Table 9.2), is not fully representative of real treated wastewater (TWW). The main differences is found in the content of dissolved organic carbon (DOC) and dissolved nitrogen, present at 10 to 20 times higher concentrations in TWW, and total bacteria, more than 1000 times more concentrated in TWW (Table 9.2). The presence of these nutrients (DOC and nitrogen), although at relatively low levels, and the high amount of bacteria may thus impact the efficiency of the fungal filter, by increasing the development and the competition/predation of other microorganisms. Therefore, the fungal filters were tested with real treated municipal wastewater (composition in Table 9.2), spiked with NPX at 10 mg l^{-1}.

Table 9.2 Composition of the treated wastewater (TWW) and the tap water used in this study. For the tap water, average published values for 2013 for Lausanne, Switzerland (eauservice, 2014).

Conventional parameters		TWW[a]	Tap water[b]
Dissolved organic carbon (DOC)	[mg l^{-1}]	10.8	< 1
Total dissolved nitrogen (TN)	[mg l^{-1}]	15.2	< 1
N-NH_4	[mg l^{-1}]	6.6	< 0.025
N-NO_3	[mg l^{-1}]	8.0	0.9
N-NO_2	[mg l^{-1}]	0.5	0.001
Phosphate (PO_4)	[mg l^{-1}]	< 0.2	0.023
Sodium	[mg l^{-1}]	64	7
Magnesium	[mg l^{-1}]	9.9	8
Potassium	[mg l^{-1}]	12.3	1.5
Calcium	[mg l^{-1}]	77	59
Fluoride	[mg l^{-1}]	0.16	n.a.
Chloride	[mg l^{-1}]	112	12
Sulfate	[mg l^{-1}]	53	33
pH	[-]	7.7	7.8
Conductivity	[µS cm^{-1}]	914	337
Total viable bacteria	[CFU ml^{-1}]	10^5	80^c

[a] Measured in the treated wastewater (TWW), effluent of the Lausanne WWTP (24-h composite sample)

[b] Average quality of the tap water in Lausanne in 2013

[c] Measured in the tap water used during the experiment

n.a.: not analysed

As presented in Fig. 9.31, NPX in TWW was completely degraded in each batch cycle in less than 3 d in the two fungal filters, and this during the 140 d of continuous operation (21 batch cycles). In the wood control, NPX was completely removed by adsorption during the first cycle, but then, fast saturation of the filter occurred, with no more NPX removal observed in the wood filter from the third cycle onward. During the 50 first days (16 cycles), the filters behaved as expected, with similar degradation rates for both fungal filters and no removal in the wood control. Laccase activity, however, rapidly decreased, reaching a very low level (< 10 U l^{-1}) after 20 to 30 d (depending on the filter). During these first 50 d of operation with real TWW, the filters behaved in a similar way as with synthetic wastewater. Therefore, apart from the faster decline of laccase activity in TWW, probably partially due to the degradation of the enzyme by the microbial community, operation of the filters with TWW did not impact the global efficiency of the system.

After 50 d, starting from the 17th cycle onward, a change of behaviour occurred in the three filters: (i) very fast degradation of NPX was observed in the fungal filter 2 (complete removal in less than 24 h), and, after a few cycles, also in the filter 1, and (ii) appearance of NPX degradation in the wood control. The fast removal in filter 2 was correlated with a significant increase in laccase activity and a strong acidification (down to pH 5) of the TWW at each cycle, suggesting that something stimulated the fungus, which became again very active. The removal in the wood control was probably related to the emergence of a microorganism able to degrade NPX. Attempts were made to isolate and identify

this organism, by plating the treated water in agar-medium containing NPX as sole carbon source, without success.

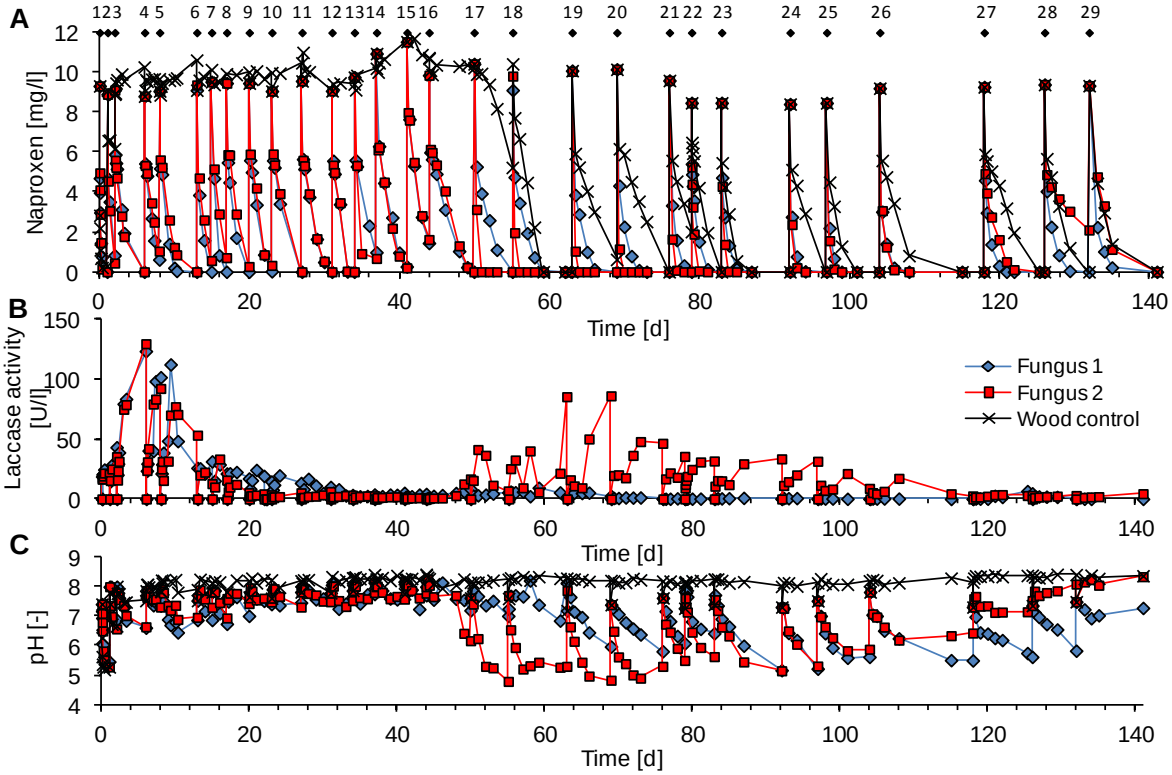


Fig. 9.31 (A) Kinetics of naproxen removal during the batch cycles (◊ numbered) in two filters inoculated with *P.ostreatus* (Fungus 1 and 2) and in a wood filter not inoculated (Wood control). Evolution of laccase activity (B) and pH (C) during the batch cycles. The real treated wastewater, spiked with naproxen, was renewed at each batch cycle.

The coincidence of the simultaneous change of behaviour in the wood control and the fungal filter 2 at the 17th cycle suggests that is was possibly related to a change in the (microbial) composition of the raw TWW, although this was not expected as the same TWW (stored at 4°C) was used during the whole experiment. To know if the organisms responsible for NPX degradation were either (i) attached to the wood supports (fixed biomass), meaning that they were slowly selected in the system (biofilm development to avoid the washout at each new cycle), or (ii) in suspension in the liquid (free cells), meaning that they came mainly from the raw wastewater (renewed at each cycle), a new experiment was performed. The supernatant of the fungal filter 2 and of the wood control were collected at the end of the 22nd cycle, once NPX was completely degraded. One part of the supernatant was filtered at 0.22 µm to remove the microorganisms and the other part was used as is. The two fractions were then spiked with NPX at 10 mg l^{-1} (without methanol addition), and incubated 52 d at 25°C and 130 rpm to ensure aeration of the media. NPX concentration, laccase activity and pH were monitored with time. As presented in Fig. 9.32 A, NPX was not removed in any of the supernatants neither from the fungal nor the wood filters, filtered or not, and even an increase in concentration was observed along the 50 d, related to the evaporation of water in the cultures. This showed that the cells in suspension, as well as the extracellular enzymes, were not able to degrade significantly NPX. The fixed biomass was therefore responsible for the removal observed in the column, suggesting that, in the wood control, microorganisms were slowly selected for their ability to degrade NPX.

This experiment showed also that laccase was inactivated at a faster rate in the raw than filtered supernatants (Fig. 9.32 B). In both cases, 50% activity was lost during the three first days, showing the low stability of *P. ostreatus* laccase at pH 5.8-6. Then, the decline slowed down in the cell free supernatant, with still 21% of the initial activity after 24 d compared to only 2% in the raw supernatant. The higher inactivation in the raw supernatant was probably due to laccase degradation by the microbial community.

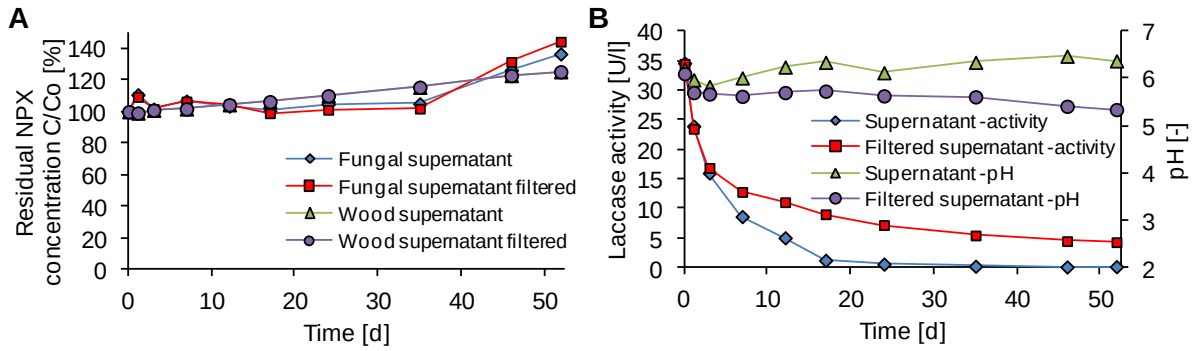


Fig. 9.32 Test of naproxen (NPX) degradation spiked in the supernatants collected from the fungal filter 2 and the wood filter at the end of the 22nd batch cycle, filtered or not at 0.2 µm (cell free). (A) Residual NPX concentration. **(B)** Laccase activity and pH in the supernatant of the fungal filters. pH in the wood supernatant was stable at pH 8.4.

Although no clear explanation could be deduced for this sudden change of behaviour in the filters, these results demonstrated that NPX can be very well degraded also by some native microorganisms present in TWW. It was also demonstrated that *Pleurotus* can survive four months in the filter treating real wastewater. After 120 d, the fungal activity in filter 2 became, however, very low, coinciding with a decrease in the acidification of the water and a decrease in NPX removal rate. Nevertheless, NPX was still completely removed in less than 5 d in the filters after 140 d of operation with real TWW.

9.3.12 Micropollutant removal in real conditions

The previous section showed that the fungal filters can be continuously operated during several months to treat micropollutants spiked at high concentrations in real wastewater. The last experiment consisted thus to test their efficiency for the treatment of a wider range of pollutants, at real (very low) concentrations in real wastewater.

9.3.12.1 Wastewater composition

The treated wastewater used came from a composite sample collected in the effluent of the municipal WWTP of Lausanne, at the outlet of a biological treatment with partial nitrification (moving bed bioreactor). Its mineral and physico-chemical composition is presented in Table 9.3. The composition was relatively similar to the TWW used in the previous section (cf. Table 9.2), with also a high concentration of bacteria. We can notice the relatively high concentration of TSS (25 mg l^{-1}), due to a poor efficiency of the secondary clarifier.

Table 9.3 Composition of the treated wastewater (TWW) used for this experiment (24-h composite sample collected at the outlet of the municipal WWTP of Lausanne) and composition of the effluents of the fungal and the wood filters, after 48 h of treatment (cycles 8 or 9).

Conventional parameters		Raw TWW[a]	Effluent[b] of the Fungal filter	Wood filter
Total suspended solids (TSS)	[mg l^{-1}]	25.2 (±2.3)	11.7 (±5.2)	< 1
Dissolved organic carbon (DOC)	[mg l^{-1}]	9.4 (± 0.55)	45	28
Total dissolved nitrogen (TN)	[mg l^{-1}]	21.2	30.4	7.4
N-NH_4	[mg l^{-1}]	9.2 (±0.04)	1.5 (±1.23)	< 0.1
N-NO_3	[mg l^{-1}]	10.2 (±0.1)	11.4 (±0.4)	0.36
N-NO_2	[mg l^{-1}]	< 0.06	< 0.06	< 0.06
P-phosphate	[mg l^{-1}]	< 0.06	< 0.06	< 0.06
Sodium	[mg l^{-1}]	66.3 (±0.3)	72.4 (±0.3)	71.9
Magnesium	[mg l^{-1}]	8.8 (±0.2)	8.0 (±0.6)	7.7
Potassium	[mg l^{-1}]	14.1 (±0.06)	15.9 (±2.7)	11.7
Calcium	[mg l^{-1}]	35.9 (±0.4)	31.8 (±3.0)	47.9
Fluoride	[mg l^{-1}]	0.2 (±0.02)	0.6 (±0.17)	0.43
Chloride	[mg l^{-1}]	102 (±0.2)	109 (±3.6)	111.3
Sulfate	[mg l^{-1}]	60.8 (±0.5)	62.7 (±1.6)	61.0
Bromide	[mg l^{-1}]	< 0.1	< 0.1	< 0.1
pH	[-]	7.8	6.9 - 7.3	7.8 - 8.0
Total viable bacteria	[CFU ml^{-1}]	3×10^4	$1\text{-}4 \times 10^5$	5×10^3

[a] Average of 6 analyses for DOC, 3 for the anions/cations, 2 for TSS and 1 for TN

[b] After 48h of treatment, in cycles 8 or 9 (expected for TN, cycle 2). Average of 3 analyses for the fungal filter (1 for DOC and TN), one for the wood filter

The concentrations of micropollutants in the raw TWW are presented in Table 9.4. A total of 35 micropollutants out of 44 could be quantified. Their concentrations varied between 1 ng l^{-1} (some pesticides) up to 11 µg l^{-1} (X-ray contrast media). Similar concentrations (less than 2.5 times variation) were found during a large monitoring study in the effluent of the same WWTP (cf. Chapter 3), apart for a few compounds present in concentration 4 to 10 times lower in the present study (azithromycin, clarithromycin, ibuprofen, metronidazole, simvastatin, atrazine, irgarol and isoproturon). The TWW used was thus well representative of the average quality of WWTP effluents.

The TWW was analysed 12 times (at each new batch cycle), with, on average on the 35 micropollutants, a coefficient of variation between the concentrations in each sample of only 10%. This demonstrated the low influence of the storage time (frozen samples) and the good reproducibility of the analytical method.

Table 9.4 Concentrations of 35 micropollutants in the raw treated wastewater and their removal efficiencies by the fungal or wood filters.

Compound	Compound class	LOD ($ng\ l^{-1}$)[a]	Analytical method[b]	TWW concentration ($ng\ l^{-1}$)[c]	Removal (%) in 48 h[d]		Removal (%) in 5 d[d]	
					Fungal filter	Wood filter	Fungal filter	Wood filter
Pharmaceuticals								
Atenolol	Beta blocker	10	B	312 (± 41)	78 (± 22)	99	85 (± 10)	99
Azithromycin	Antibiotic	5	B	88 (± 22)	40 (± 20)	77	51 (± 21)	83
Bezafibrate	Lipid regulator	1	B	179 (± 21)	82 (± 28)	100	95 (± 8)	99
Carbamazepine	Anticonvulsant	10	B	248 (± 27)	38 (± 9)	31	34 (± 4)	n.a.
Ciprofloxacin	Antibiotic	30	B	403 (± 25)	88 (± 2)	92	89 (± 3)	91
Clarithromycin	Antibiotic	5	B	77 (± 12)	n.a.	n.a.	n.a.	n.a.
Diatrizoic acid	Iodinated contrast medium	100	N	174 (± 16)	n.a.	n.a.	n.a.	n.a.
Diclofenac	Analgesic / Anti-inflammatory	30	B	1308 (± 207)	**98 (± 1)**	**13**	**97 (± 4)**	**15**
Gabapentin	Anticonvulsant	80	N	1699 (± 28)	92 (± 10)	98	98 (± 1)	99
Gemfibrozil	Lipid regulator	5	B	79 (± 7)	96 (± 2)	81	98 (± 0)	97
Ibuprofen	Analgesic / Anti-inflammatory	20	B	199 (± 28)	> 96	> 96	> 96	> 96
Iohexol	Iodinated contrast medium	3000	N	11621 (± 350)	48 (± 10)	> 80	71 (± 5)	> 80
Iomeprol	Iodinated contrast medium	2000	N	11239 (± 50)	64 (± 24)	82	76 (± 19)	78
Iopamidol	Iodinated contrast medium	500	N	3062 (± 99)	43 (± 13)	83	61 (± 11)	77
Iopromide	Iodinated contrast medium	500	N	2497 (± 49)	48 (± 37)	97	77 (± 19)	96
Ketoprofen	Analgesic / Anti-inflammatory	200	N	267 (± 34)	n.a.	n.a.	n.a.	n.a.
Mefenamic acid	Analgesic / Anti-inflammatory	5	B	520 (± 63)	98 (± 1)	94	99 (± 1)	95
Metoprolol	Beta blocker	10	B	425 (± 18)	70 (± 12)	86	75 (± 13)	90
Metronidazole	Antibiotic	10	N	120 (± 2.6)	46 (± 6)	91	83 (± 11)	97
Naproxen	Analgesic / Anti-inflammatory	15	B	778 (± 50)	**75 (± 4)**	**30**	**98 (± 2)**	**26**
Ofloxacin	Antibiotic	30	B	166 (± 22)	81 (± 10)	91	89 (± 13)	96
Paracetamol	Analgesic / Anti-inflammatory	20	N	22 (± 8)	n.a.	n.a.	n.a.	n.a.
Primidone	Anticonvulsant	20	N	57 (± 6)	n.a.	n.a.	n.a.	n.a.
Simvastatin	Lipid regulator	20	N	25	n.a.	n.a.	n.a.	n.a.
Sotalol	Beta blocker	10	B	147 (± 13)	64 (± 15)	82	74 (± 6)	85
Sulfamethoxazole	Antibiotic	20	N	132 (± 20)	64 (± 8)	65	79 (± 6)	62
Trimethoprim	Antibiotic	1	B	55 (± 5.5)	93 (± 3)	100	96 (± 5)	100
Biocides - pesticides - chemicals								
Atrazine	Herbicide	2	N	2.4 (± 2.4)	n.a.	n.a.	n.a.	n.a.
Benzotriazole	Corrosion inhibitor	50	N	5983 (± 400)	**63 (± 3)**	**-15**	**72 (± 3)**	**-17**
Diuron	Herbicide	10	N	68 (± 0.6)	80 (± 2)	85	86 (± 6)	85
Irgarol	Algicide	0.5	B	1.5 (± 0.7)	n.a.	n.a.	n.a.	n.a.
Isoproturon	Herbicide	0.5	B	6.1 (± 0.7)	80 (± 3)	71	86 (± 6)	76
Mecoprop	Herbicide	20	B	319 (± 46)	35 (± 4)	36	79 (± 5)	58
Methylbenzotriazole	Corrosion inhibitor	50	N	3111 (± 227)	72 (± 2)	80	79 (± 1)	82
Terbutryn	Algicide	0.3	B	14 (± 1)	99 (± 2)	100	99 (± 2)	100

[a] LOD: limit of detection in the samples with the strongest matrix effect (highest LOD)

[b] B: basic HPLC mobile phase. N: netural HPLC mobile phase

[c] Average and standard deviation of 12 and 2 analyses, for compounds with method B and N, respectively, of the raw treated wastewater (TWW)

[d] Average (and standard deviation) values of three fungal filters inoculated with *P. ostreatus* and one wood filter not inoculated, measured durging the 9th batch cycle and, for filter 2, also during the 14th cycle. In bold, much higher removal with the fungus than without

n.a.: not analysed in the effluent of the filters, due to strong matrix effect or concentrations in the raw wastewater too close to the LOD

9.3.12.2 Impact of the filters on the global quality of the water

The evolution of the conventional water quality parameters during the fungal treatment are presented in Table 9.3. After 48 h of treatment in the fungal or the wood filters, the TSS content decreased significantly, especially in the wood filter, probably by trapping and degradation of the particles. The low amount of TSS after the fungal treatment was coherent with the absence of growth of fungal mycelium observed in submerged condition (no sludge production). A strong increase in dissolved organic carbon (DOC) was observed in the effluent of both filters, but especially in the one inoculated with the fungus. This phenomenon is discussed later in section 9.3.12.10. Total dissolved nitrogen increased in the fungal filters, probably due to the release of extracellular enzymes, while it was consumed in the wood filter. Ammonium was almost totally consumed in the fungal and in the wood filters, while it stayed at stable concentrations in the inhibited filter (11.1 mg $N\text{-}NH_4$ l^{-1}, data not shown). Nitrate was also consumed in the wood filter, but stayed stable in the fungal filters. The major

minerals were not strongly influenced by the treatment and stayed at relatively stable concentrations. The wood filter significantly reduced (> 80%) the amount of total viable bacteria, while their concentration increased (4-10 times) in the effluent of the fungal filters, probably due to the release of dissolved substrates by the fungus (wood oligomers, extracellular enzymes, secondary metabolites) (de Boer and van der Wal, 2008). On the whole, the fungal filters released DOC and total dissolved nitrogen, which probably promoted bacterial growth, but decreased the concentrations of TSS and NH_4.

9.3.12.3 Fungal activity in the filters

The evolution of laccase activity and pH during the 63 d of continuous operation (14 cycles) are presented in Fig. 9.33. Similar to what happened in the experiment with NPX spiked in TWW, laccase activity rapidly declined in the fungal filters, reaching very low level (< 5 U l^{-1}) already after 17 d for filters 1 and 3, and after 22 d for filter 2.This fast decrease in activity was probably related, as proposed before, to microbial degradation of the extracellular laccase produced, together with the decline of the fungus in the filters. After 50 d, the fungus was, however, not completely dead as several fruiting bodies (sporocarps) appeared out of the woodchips of filter 1 (Fig. 9.4 J). During each batch cycle, the water was acidified, at the beginning also in the wood control and in the filter with the inhibited fungus (linked to the intrinsic acidity of the wood), and then only in the active fungal filters. This constant acidification was probably related to continuous production of organic acids by the fungus, suggesting that the fungus was still active at the end of the experiment, despite very low laccase activity.

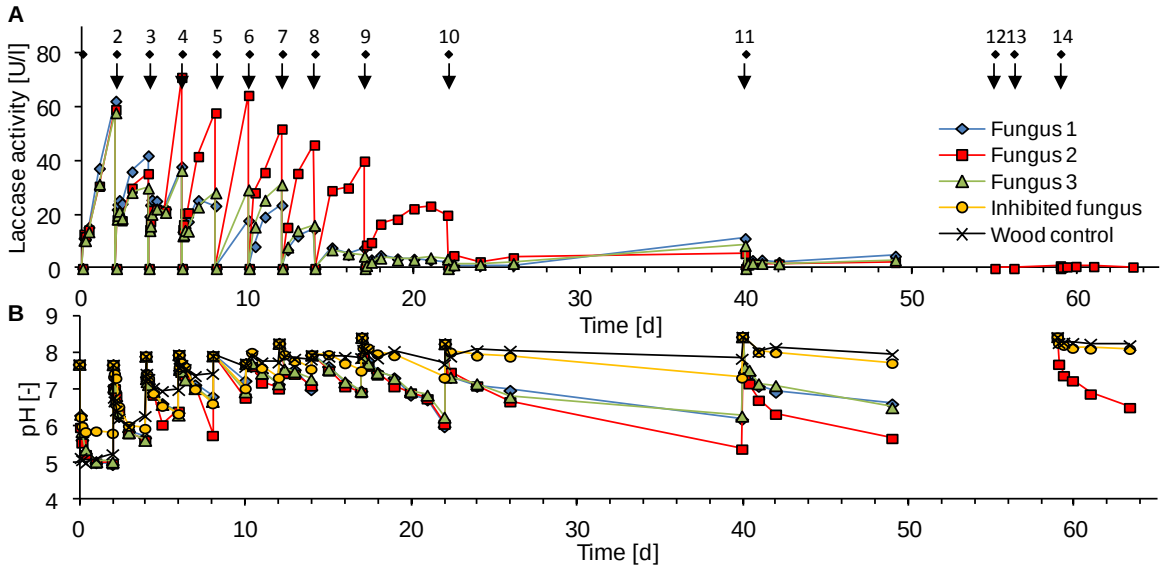


Fig. 9.33 Laccase activity (A) and pH (B) in the treated waters from the three filters inoculated with *P. ostreatus* (Fungus 1, 2 and 3) and from the controls with the inhibited fungus or the wood not inoculated. Arrows: beginning of a new batch cycle (water changed), with the number of the cycle.

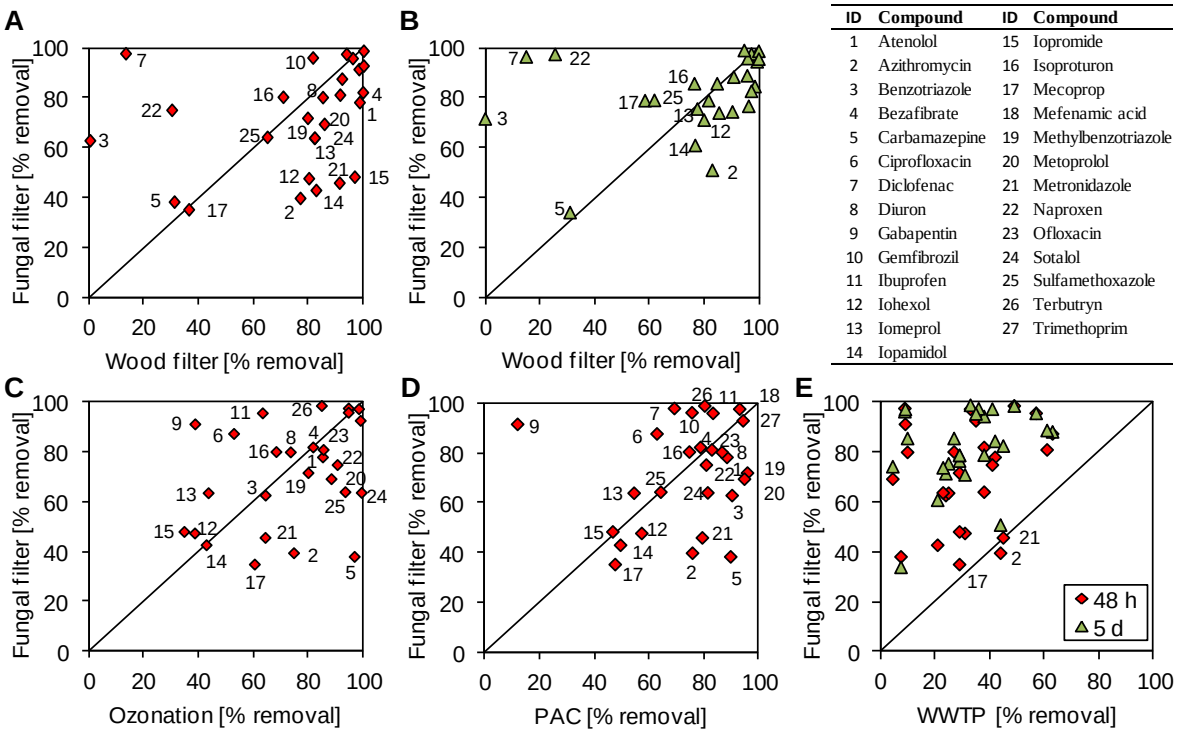
9.3.12.4 Micropollutant removed in the filters in comparison with other technologies

Out of the 35 pollutants detected in raw wastewater, 26 to 27 (with LOD low enough) could be quantified in the effluents of the filters and were studied in details. Their average removals after 48 h and 5 d during the 9th cycle (once adsorption was less important), in the filters with or without inoculation with *P. ostreatus*, are presented in Table 9.4. Both types of filters (inoculated or not) were

very efficient to remove most of the micropollutants, with an average removal of the 27 pollutants of 72% and 75% after 48 h, and of 82% and 77% after 5 d, in the fungal and wood filters, respectively. After 48 h, the wood filter was thus even more efficient than the fungal filters, with 19 micropollutants removed at more than 80%, compared to 12 in the fungal filters. The comparison of the removal efficiencies (Fig. 9.34 A and B) showed that the fungal filter was much more efficient to remove DFC, NPX and BTZ, and slightly more efficient to remove IPN, SMX, gemfibrozil and mecoprop, while the wood filter removed better most of the other pollutants, especially (after 48 h) the iodinated contrast media, azithromycin and metronidazole. After 5 d, most of these pollutants were also degraded in the fungal filter, which became on average more efficient: only CBZ was still removed less than 50%, while it was the case for CBZ, DFC, BTZ and NPX in the wood filter.

After 48 h of treatment, the wood and fungal filters reached already similar average removal efficiencies as other advanced treatments such as ozonation and activated carbon adsorption. Indeed, the average removal of the same 27 pollutants during the pilot assays at Lausanne WWTP (cf. Chapter 3) was of 74% with both ozonation (5.9 mg O_3 l^{-1}) and powdered activated carbon adsorption (12 mg PAC l^{-1}), compared to only 32% in the conventional treatment (biology with partial nitrification). Despite relatively similar average removal efficiencies, not the same pollutants were well removed with the fungal filter or with the advanced treatments. Compared to ozonation (Fig. 9.34 C), gabapentin, ciprofloxacin, ibuprofen, the iodinated contrast media, and several herbicides were better removed in 48 h with the fungal filter, while most of the other compounds were better removed by ozonation, especially CBZ, azithromycin, sotalol, SMX, metoprolol, mecoprop and metronidazole. Compared to PAC (Fig. 9.34 D), the fungal filter (after 48 h) performed better especially for gabapentin, but also, inter alia, for DFC, gemfibrozil, ciprofloxacin or terbutryn, while PAC treatment removed better CBZ, BTZ, azithromycin, metronidazole, atenolol, NPX and, to a lesser extent, several other pollutants. After 5 d of treatment, the fungal filter performed better then PAC or ozone for most pollutants, apart mainly for CBZ and azithromycin, still poorly removed in the filters. As shown in Fig. 9.34 E, all the micropollutants were clearly better removed in the fungal filters than in conventional WWTPs.

Despite the long reaction time required (over 48 h), the sequential batch filters appeared to be a very promising technology, able to reach similar removal efficiencies as the one reached (in 20 to 40 min) with ozonation (at 5.9 mg O_3 l^{-1}) and PAC (at 12 mg l^{-1}). Similar reaction times (hydraulic retention times from 24 to 96 h) were applied in other fungal bioreactors operated in continuous mode (Blánquez et al., 2008; Cruz-Morató et al., 2013b; Jelic et al., 2012; Rodarte-Morales et al., 2012; Yang et al., 2013a; Zhang and Geißen, 2012), confirming that relatively long reaction times are necessary when using fungal processes. Higher removal efficiencies could be reached by increasing the reaction time in the filters (by increasing the size of the plant), or by increasing the dose of ozone and PAC in the advance treatments (more feasible solution in terms of operation). The fact that the wood filters performed, on average for the 27 pollutants, as well as the fungal filters will be discussed later.



ID	Compound	ID	Compound
1	Atenolol	15	Iopromide
2	Azithromycin	16	Isoproturon
3	Benzotriazole	17	Mecoprop
4	Bezafibrate	18	Mefenamic acid
5	Carbamazepine	19	Methylbenzotriazole
6	Ciprofloxacin	20	Metoprolol
7	Diclofenac	21	Metronidazole
8	Diuron	22	Naproxen
9	Gabapentin	23	Ofloxacin
10	Gemfibrozil	24	Sotalol
11	Ibuprofen	25	Sulfamethoxazole
12	Iohexol	26	Terbutryn
13	Iomeprol	27	Trimethoprim
14	Iopamidol		

Fig. 9.34 Comparison of the removal efficiencies of 27 micropollutants between the fungal filters and (A) the wood filters after 48 h, (B) the wood filters after 5 d, (C) ozonation with 5.9 mg O_3 l^{-1}, (D) powdered activated carbon (PAC) at 12 mg l^{-1}, (E) conventional WWTP (with partial nitrification). Data for ozonation, PAC and the WWTP are average values from one year of operation of a pilot installation in Lausanne WWTP (Margot et al., 2013b). Average removal efficiency of the 27 pollutants: 72% and 82% after 48 h and 5 d, respectively, in the fungal filter; 75% and 77% after 48 h and 5 d, respectively, in the wood filter; 74% with both ozonation and PAC.

9.3.12.5 Kinetics of micropollutant removal

The kinetics of micropollutant removal in the filters were studied during the 9th and 14th batch cycles for 26 micropollutants. To identify the main removal mechanism, the kinetics were compared between the fungal filters, the wood filters, as well as with the fungal and wood filters where the microbial activity was inhibited with azide. This allowed classifying the pollutants according to the main removal mechanisms: adsorption, fungal degradation, microbial degradation, or a combination of all these mechanisms. As the main mechanism changes with time (saturation of the adsorption sites and development of the native microbial community), this analysis represents what happens after 20 (9th cycle) to 60 (14th cycle) days of operation

Micropollutants mainly removed by adsorption

Five pollutants, ciprofloxacin, ofloxacin, terbutryn, diuron and isoproturon (Fig. 9.35), were rapidly removed (> 80% in less than 24 h) in the wood and the fungal filters, and this, at a similar rate in the active and inhibited filters, suggesting that adsorption onto the woodchips was the main removal mechanism. High removal by adsorption in activated sludge was also reported for ciprofloxacin and ofloxacin (cf. Chapter 2), and good adsorption affinity of the three pesticides onto PAC (>75% removal) was observed in Chapter 3, corroborating the assumption of the removal by adsorption in the filters. Abiotic degradation (photolysis) could not be completely excluded but was probably not as significant as adsorption, as discussed later.

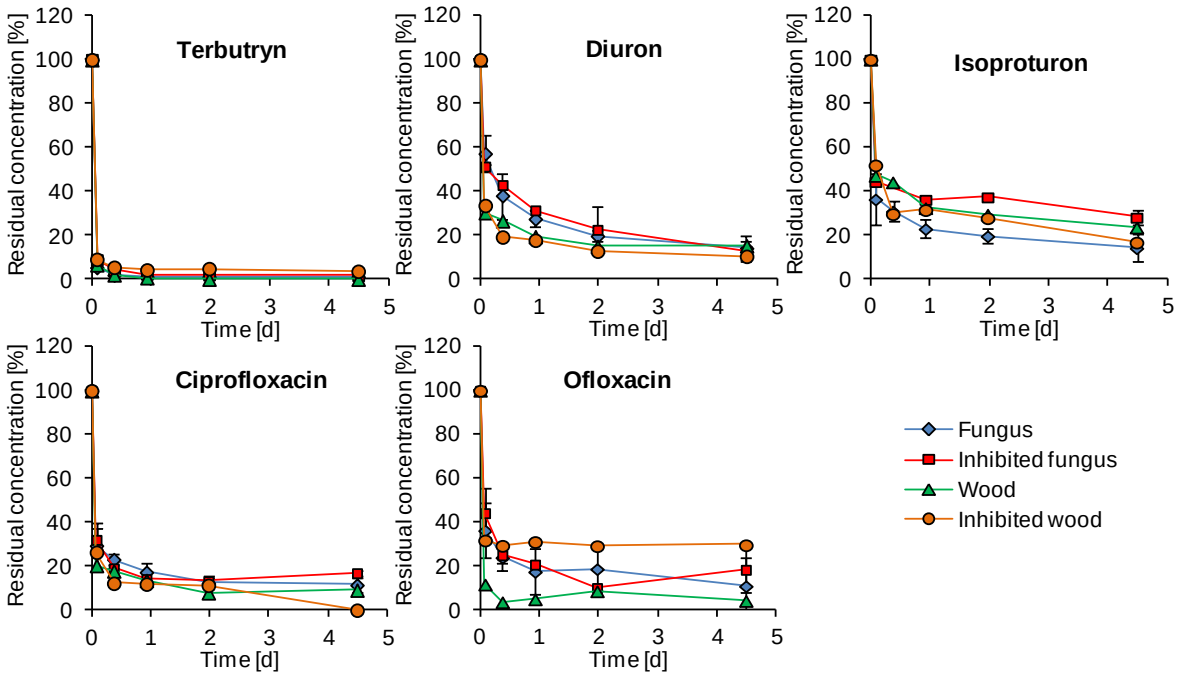


Fig. 9.35 Micropollutants well removed in all conditions, probably by physical processes (adsorption). Average and standard deviation of the residual concentrations of (i) 4 replicates for the filters inoculated with *P. ostreatus* (◊, Fungus), (ii) duplicates for the filter with *P. ostreatus* inhibited with sodium azide (■), and (iii) only one filter with wood (without inoculation, ▲) or with inhibited wood (●). Results of the 9th and 14th batch cycles.

Micropollutants mainly removed by fungal degradation

Three micropollutants were well removed in the active fungal filters but not (< 25%) in the wood or inhibited filters: DFC, NPX and BTZ (Fig. 9.36), suggesting that biodegradation by *P. ostreatus* was the main removal mechanism. The ability of the fungus to degrade DFC and NPX in the filters was shown (at higher concentration) in section 9.3.9, but this was not clearly observed for BTZ. Indeed, BTZ was certainly slowly removed in the filters with synthetic wastewater, but probably more by native microbial than fungal degradation. This assumption is supported by the results of the experiment with *T. versicolor*, where degradation of BTZ by native microorganisms was also observed (Fig. 9.9). In any case, biodegradation was the main removal mechanism, as no removal was observed in the inhibited filters.

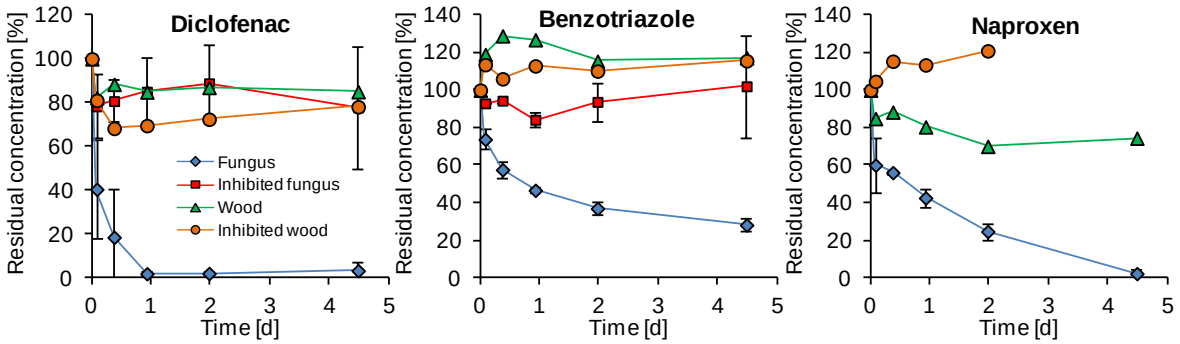


Fig. 9.36 Micropollutants well removed only in the fungal filters (fungal degradation). Average and standard deviation of the residual concentrations of (i) 4 replicates for the filters inoculated with *P. ostreatus* (◊, Fungus), (ii) duplicates for the filter with *P. ostreatus* inhibited with sodium azide (■), and (iii) only one filter with wood (without inoculation, ▲) or with inhibited wood (●). Results of the 9th and 14th batch cycles.

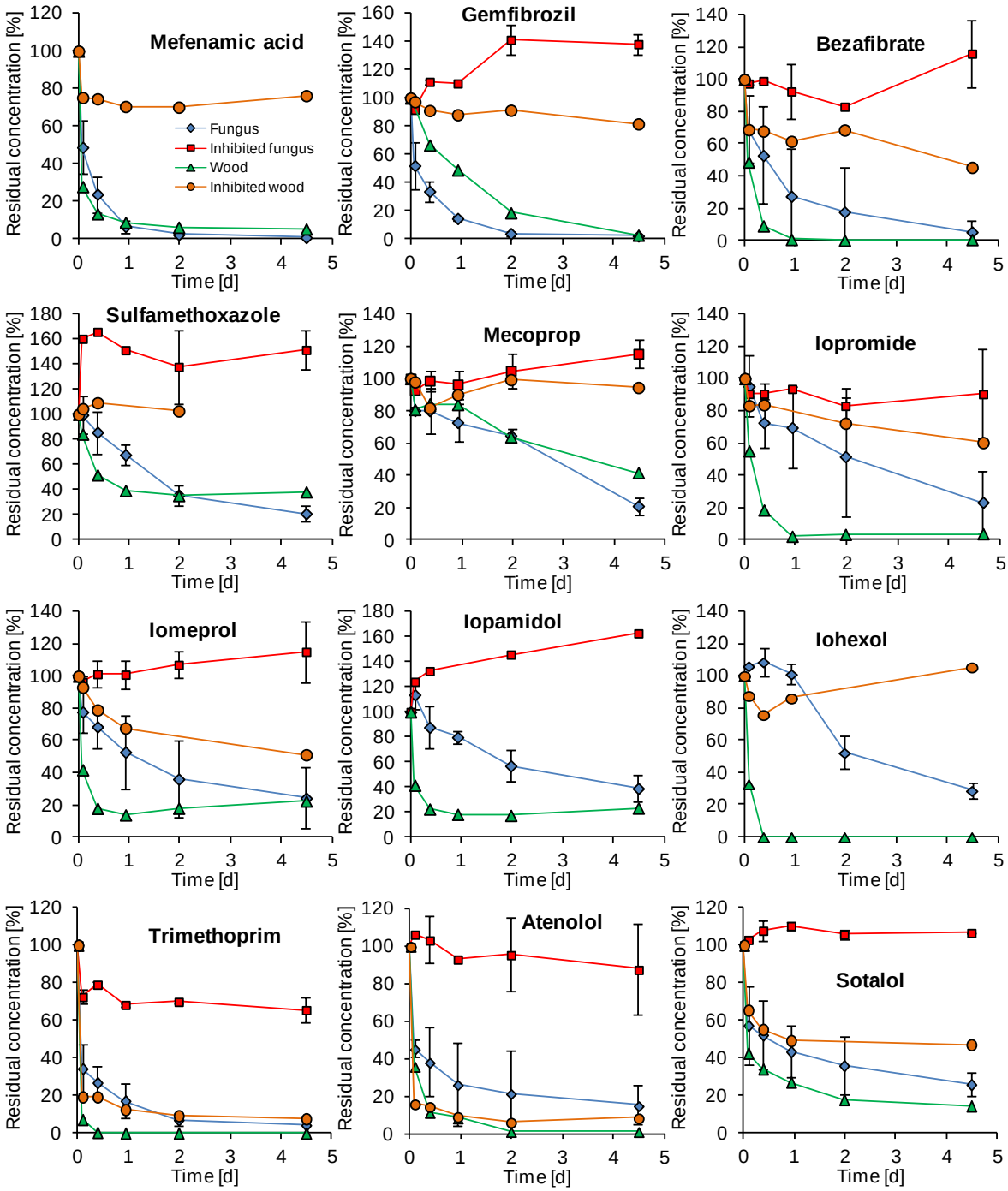


Fig. 9.37 Micropollutants well removed only in filters not inhibited (biological degradation). Average and standard deviation of the residual concentrations of (i) 4 replicates for the filters inoculated with *P. ostreatus* (◊, Fungus), (ii) duplicates for the filter with *P. ostreatus* inhibited with sodium azide (■), and (iii) only one filter with wood (without inoculation, ▲) or with inhibited wood (●). Results of the 9th and 14th batch cycles.

Micropollutants mainly removed by biodegradation

Twelve micropollutants were removed in the active fungal and wood filters, but not (or only poorly) in the inhibited filters (Fig. 9.37), suggesting that they were mainly removed by biodegradation. As degradation occurred in both the fungal and the wood filters, it was not possible to determine who was involved: *P. ostreatus*, the native microbial community or both. MFA and SMX, two compounds potentially degraded by the fungus, were removed at the same rate in the wood and the fungal filters,

showing that not only *P. ostreatus*, but also the native microbial community had the ability to degrade these pollutants. Gemfibrozil was removed faster in the fungal filter, suggesting that it was also degraded by the fungus (also observed in the experiment with *T. versicolor*, Fig. 9.8), while bezafibrate and the iodinated contrast media were clearly removed faster in the wood filters, suggesting that they were probably only degraded by the native microorganisms. For trimethoprim, atenolol and sotalol, although no removal was observed in the inhibited fungal filter (inhibited since the beginning of the experiment), high removal was observed in the inhibited wood filter (inhibited only at the end of the experiment, two cycles before the measurements). This removal was probably partially due to adsorption, as the wood was possibly not yet (in only two cycles) at equilibrium with the initial concentration, but this has to be confirmed.

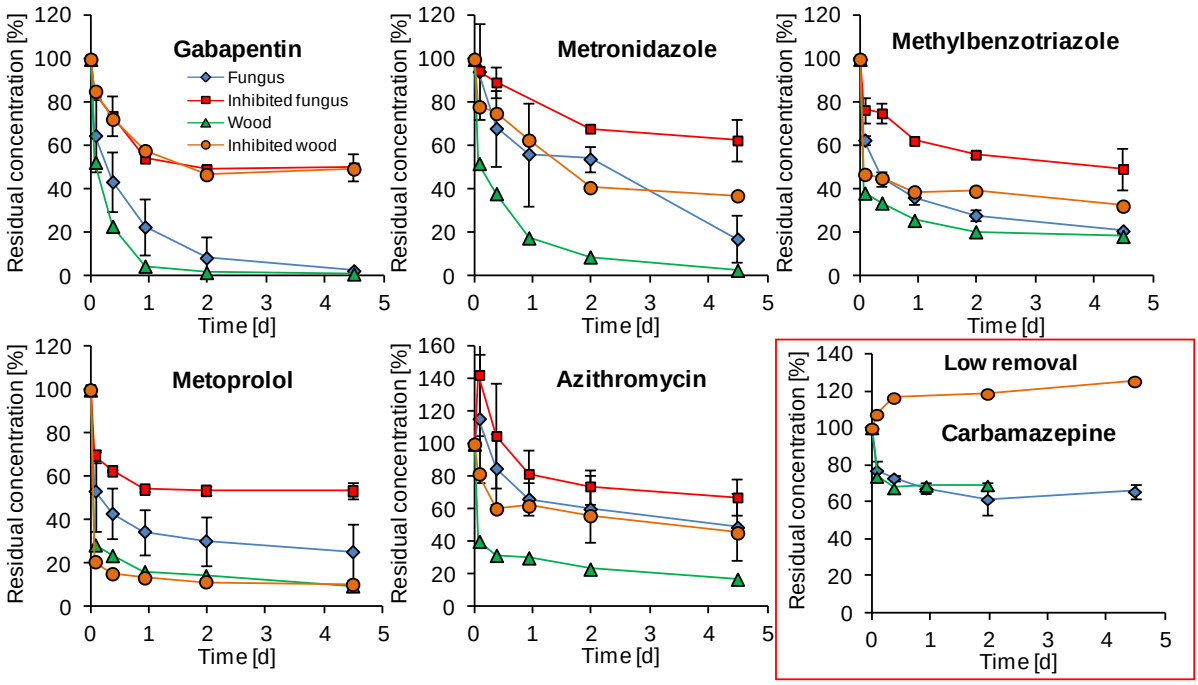


Fig. 9.38 Micropollutants removed by combinations of biodegradation and physical processes (adsorption, etc.). Average and standard deviation of the residual concentrations of (i) 4 replicates for the filters inoculated with *P. ostreatus* (◊, Fungus), (ii) duplicates for the filter with *P. ostreatus* inhibited with sodium azide (■), and (iii) only one filter with wood (without inoculation, ▲) or with inhibited wood (●). In red: compound with only low removal in all filters. Results of the 9th and 14th batch cycles.

Micropollutants removed by a combination of microbial degradation and adsorption

For the five micropollutants gabapentin, methylbenzotriazole, metronidazole, metoprolol, and azithromycin (Fig. 9.38), a combination of biodegradation (most probably by the native microorganisms) and adsorption was probably the cause of the removal observed in the filters. Indeed, although faster removals were observed in the active filters, significant removal was also observed in the inactivated filters, but reaching a plateau before complete removal (sign of adsorption and not degradation). Thus, the filters were not yet at equilibrium with the initial concentration, still leaving some potential for more adsorption. The almost complete removal of gabapentin in 48 h in the active filters (while less than 50% removal was observed in the inactivated one) demonstrates that this compound can be degraded by the native microbial community (also observed in the experiment with

T. versicolor, Fig. 9.9). This result is of great interest as gabapentin is usually only poorly removed in conventional WWTPs, as well as by ozonation or PAC adsorption (cf. Chapter 3).

Finally, CBZ was not or only poorly removed (probably by adsorption) in all the filters, active or inactive, confirming the high persistence of this pollutant to biodegradation.

One analysis performed after 18 d of reaction in the inhibited fungal filters at the end of the 10th cycle, showed that for metoprolol, atenolol, sotalol, trimethoprim, azithromycin, mecoprop, MFA, gemfibrozil, DFC and bezafibrate, no further removal in the inhibited fungal filter occurred during these 18 d (stable concentrations, apart from a low removal observed for sotalol and trimethoprim, data not shown). This confirmed that no significant biotic or abiotic degradation mechanisms occurred in the inhibited filters for these compounds

9.3.12.6 Evolution of the removal efficiencies as indication of the removal mechanisms

The removal rates of 15 micropollutants (compounds with analytical method B, Table 9.4) at the end of each batch cycle (after 48 h) were monitored during 12 cycles in all the filters to assess the evolution of the removal efficiency with time. Due to analytical interferences (strong matrix effect), this was not performed for the other compounds (with analytical method N, Table 9.4). Different evolution patterns of the removal efficiency between the different filters (wood, active or inhibited fungus) provide an indication of the removal mechanisms. For instance, the pattern observed for the inhibited fungal filter gave indication on the evolution of the removal by adsorption. The pattern of the fungal filter and the wood filter gave information on the removal by biodegradation by the fungus and the native microorganisms, respectively.

As presented in Fig. 9.39 and Fig. 9.40, for most of the micropollutants studied, a strong removal by adsorption was observed in all the filters during the first batch cycles, decreasing then at each cycle in the inhibited fungal filter due to progressive saturation of the adsorption sites of the woodchips/mycelium. In some cases (e.g., gemfibrozil), even desorption from the wood occurred after a few cycles (negative removal), probably due to competition for the adsorption sites with wastewater components with stronger adsorption affinity. The behaviour of the inhibited fungal filter gave thus a good indication of the evolution of the adsorption mechanism.

For some pollutants, such as gemfibrozil, MFA, NPX and DFC, the removal efficiency in the fungal filter was high (60-100%) and stayed more or less constant during the 12 cycles, while it (temporally) decreased in the wood or inhibited filters (Fig. 9.39 and Fig. 9.40 A). This suggests that these pollutants were mainly removed by fungal degradation. For gemfibrozil and MFA, and less clearly for NPX, a very interesting pattern was observed in the wood filter: the removal efficiencies, high during the first cycle, decreased then rapidly in the next cycles in the same way than in the inhibited filter, showing that the removal observed was only due to adsorption. After 5-6 cycles, however, the removal efficiencies started to increase again in the wood control, reaching high removal rates after 8-9 cycles. This phenomenon could be attributed to microbial degradation, as no significant removal was observed when the microbial activity in the wood control was inhibited (14th cycle). This demonstrated that a microbial community, able to degrade MFA, gemfibrozil and NPX, slowly developed in the filters (biofilm formation on the wood substrate, which became darker). These organisms were very

likely coming from the TWW. The removal observed in the wood control was thus clearly due to two different and separated mechanisms: adsorption during the first cycles and degradation by the native microorganisms that slowly developed in the last cycles. An illustration of these different phenomena is presented in Fig. 9.39 A, with gemfibrozil as an example.

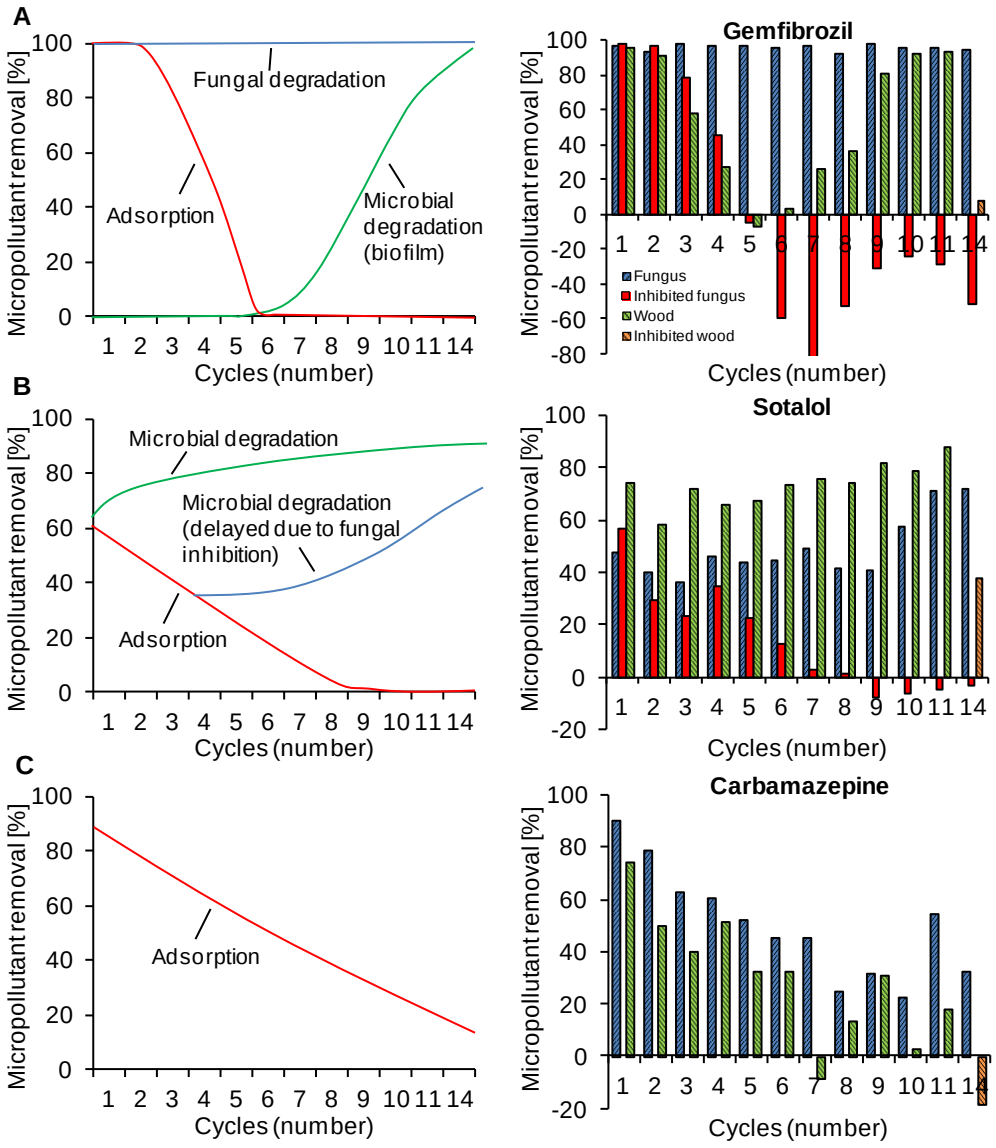


Fig. 9.39 Evolution of the removal mechanisms (adsorption, microbial or fungal degradation) as a function of the number of batch cycles (48 h) performed. Example for **(A)** a pollutant (gemfibrozil) degraded by the fungus and by the biofilm (native wastewater microorganisms) that slowly develops, **(B)** a pollutant (sotalol) not degraded by the fungus but degraded by the native microorganisms, and **(C)** a pollutant (carbamazepine) neither degraded by the fungus or the native organisms. For the fungal filter: results for the filter 2.

Several other micropollutants (Fig. 9.39 B and Fig. 9.40 B), such as sotalol, atenolol, metoprolol, bezafibrate, and, to a lesser extent, azithromycin and mecoprop, were very likely removed mainly by degradation by the native microbial community. Indeed, the removal efficiency in the fungal filter decreased (temporarily) during the first cycles to a similar way as in the inhibited filter, suggesting it was due to adsorption, while it increased rapidly in the wood filter, probably due to the development of the microbial community. The removal efficiency increased again after 9 cycles in the fungal filter

(coinciding with a strong decrease in laccase activity), very likely due to the (late) development of the native microbial community (microbial degradation). The removal observed in the fungal filter was thus not due to the fungus, but to, first, adsorption, and then, microbial degradation. The delay observed between the appearance of important microbial degradation in the fungal and the wood filters, faster in the latter, was probably linked to the fungal inhibition of the microbial growth when the fungus was still active (until the 9th cycle), as discussed later in section 9.3.12.8. An illustration of these different phenomena is presented in Fig. 9.39 B, with sotalol as an example.

Trimethoprim (Fig. 9.40 B) was almost completely removed during every cycle in the fungal and in the wood filters, while it was less removed in the inhibited filter, with decreasing efficiency at each cycle (saturation effect). Trimethoprim was therefore biologically degraded in the active filters. It was, however, not possible to determine if it was only due to native microbial degradation or also to fungal degradation. Similar to the observations made at higher concentrations (Fig. 9.21 B, section 9.3.9.5), trimethoprim behaved very strangely in the inhibited filter during the 2 first batch cycles, with less adsorption than in the third cycle. This was, as already discussed, probably related to a pH effect (change of the charge of the molecule).

Terbutryn, ciprofloxacin and ofloxacin were almost completely removed in all the filters (inhibited or not) during each batch cycle (Fig. 9.40 C), suggesting that adsorption was the main removal mechanism, although abiotic degradation cannot be completely excluded, as ciprofloxacin and ofloxacin are very sensitive and terbutryn is relatively sensitive to direct photodegradation (Lányi and Dinya, 2005; Wang and Lin, 2014). However, these compounds are not more photosensitive than DFC, SMX or NPX (Andreozzi et al., 2003; Wang and Lin, 2014), not significantly removed in the inhibited filters. Moreover, rapid adsorption of ofloxacin and ciprofloxacin may protect them from fast photodegradation (Belden et al., 2007). It is therefore expected that these compounds were mainly removed by adsorption, and possibly partially by photodegradation.

CBZ was relatively well removed (70-90%) in the wood and the fungal filters during the first cycle, the removal efficiency decreasing then slowly at each cycle, at a similar rate in both filters (Fig. 9.39 C). The progressive saturation of both filters was an indication that the removal was mainly (if not only) due to adsorption. This slow saturation of the adsorption sites was confirmed by the similar results obtained at higher concentrations (Fig. 9.17).

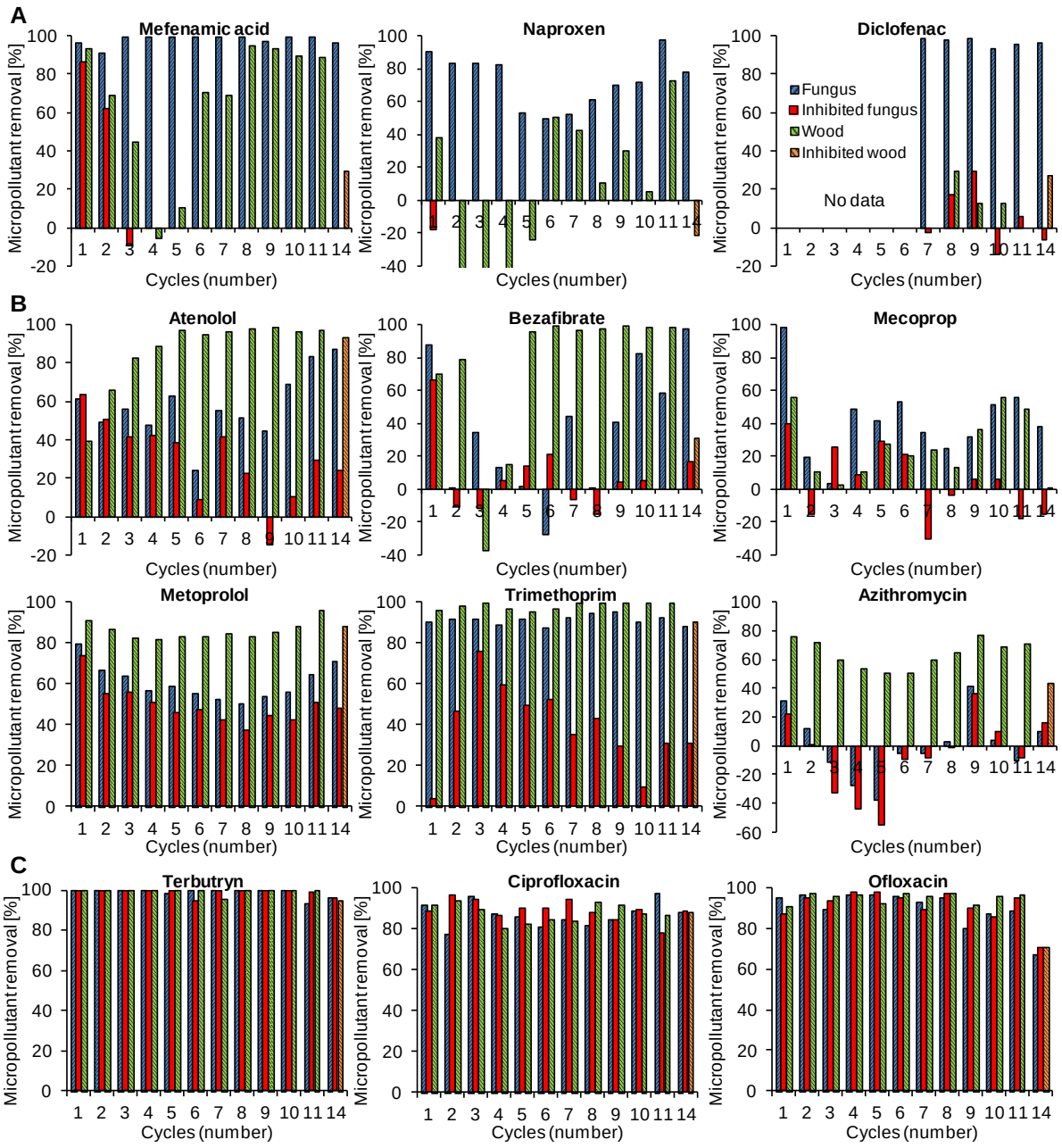


Fig. 9.40 Evolution of the removal mechanisms (adsorption, microbial or fungal degradation) as a function of the number of batch cycles (48 h) performed. (A) Micropollutants degraded by the fungus. **(B)** Micropollutants not degraded by the fungus but degraded by the native microorganisms. **(C)** Micropollutants removed by adsorption. For the fungal filter: results for the filter 2.

9.3.12.7 Freundlich adsorption isotherms at low concentrations

The progressive saturation of the adsorption sites in the inhibited filters allowed determining (cf. section 9.3.10.3) the Freundlich adsorption isotherms at the low concentration ranges for a few micropollutants (Fig. 9.41 and Table 9.5). For most of them (CBZ, metoprolol, atenolol, trimethoprim and sotalol), the isotherms were relatively linear at low concentrations (ng - µg l^{-1}). This was expected as, in synthetic wastewater (cf. section 9.3.10.3), a saturation effect was observed for trimethoprim only at concentrations above 2 mg l^{-1} (40,000 times higher) and no saturation effect was reached for CBZ even at 5 mg l^{-1}. Different results were, however, observed for MFA and gemfibrozil. These two

pollutants reached a saturation of the adsorption capacity already at low concentrations (100-400 ng l^{-1}) in real wastewater, while they were (at least for MFA), still adsorbed at concentrations 10,000 times higher (> 4 mg l^{-1}) in synthetic wastewater. For MFA, the maximum adsorption capacity on the woodchips reached around 10 ng g^{-1} in real wastewater, while it was 50,000 higher in synthetic wastewater (around 500 µg g^{-1}). These strong differences of maximum adsorption capacities may be possibly due to competitive adsorption with the dissolved organic matter present in real wastewater, but also to a pH effect or to other more complex adsorption phenomena (which may differ at different concentrations) not covered by the simple Freundlich model. In any case, these results showed that, to be reliable, adsorption isotherms have to be determined in a water matrix similar to the real water and at concentration ranges that cover the real concentrations. Any extrapolation outside these concentration ranges may lead to completely wrong estimations, especially for non-linear isotherms ($n << 1$).

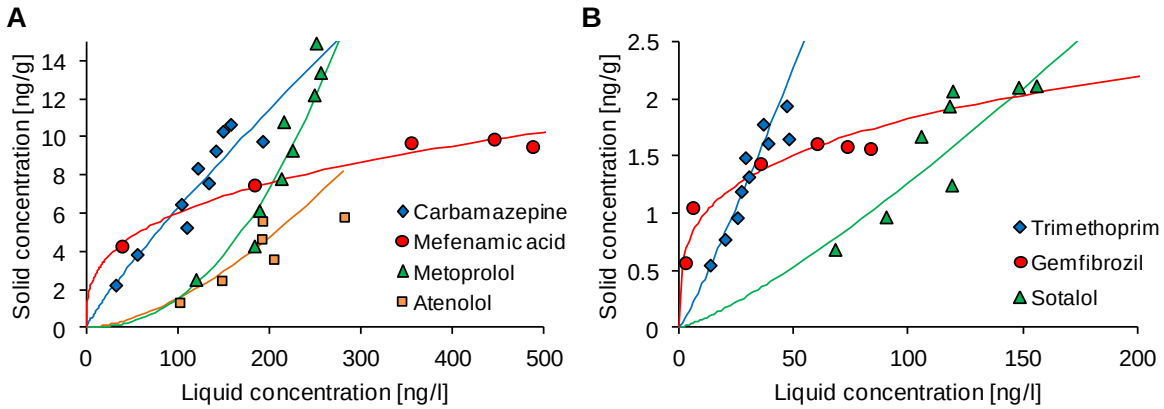


Fig. 9.41 Freundlich isotherms for micropollutant adsorption in the wood filter (for carbamazepine, mefenamic acid and gemfibrozil) or in the inhibited fungal filter (for metoprolol, atenolol, sotalol and trimethoprim) in real wastewater, at real concentrations.

Table 9.5 Freundlich coefficients for the isotherms of micropollutant adsorption in the wood filter (for carbamazepine, mefenamic acid and gemfibrozil) or in the inhibited fungal filter (for metoprolol, atenolol, sotalol and trimethoprim) in real wastewater at real concentration (0.1-1 µg/l), and physico-chemical properties of the pollutants.

	Carbamazepine	Mefenamic acid	Gemfibrozil	Trimethoprim	Metoprolol	Atenolol	Sotalol
K_f [l^n mg^{1-n} g^{-1}]	0.0191	1.284×10^{-4}	2.235×10^{-5}	0.1309	1641	5.297	0.1222
n [-]	0.871	0.332	0.272	1.107	2.258	1.635	1.247
Log K_{ow} [-][a]	2.45	5.12	4.77	0.91	1.88	0.16	0.24
pKa [-][a]	13.9	4.2	4.7	7.2	9.7	9.6	8.2 ; 9.1
Charge at pH 7[b]	0	-1	-1	+1 and 0	+1	+1	+1

[a] Source: compilation by Margot et al. (2013)

[b] Source: www.chemicalize.org (last accessed 27.08.2014)

9.3.12.8 Fungal inhibition of the native microbial biofilm development

As presented in section 9.3.12.6, micropollutants that were mainly removed by degradation by the native microbial community, were clearly better removed only after the 9th cycle in the fungal filter 2, while they were already well removed after 3-4 cycles in the wood filter. This resulted probably from a delay in the development of the native microbial community in the fungal compared to the wood filter. The increase in removal efficiency for these compounds after the 9th cycle in the fungal filter coincided also with a strong decrease in laccase activity (filter 2, Fig. 9.33). These results suggested that the fungus was preventing the growth of other microorganisms, which developed mainly once the fungus was no more active.

This assumption was confirmed by the comparison of the efficiency of the three fungal filter replicates during the 9th and 14th cycles. Filters 1 and 3 (9th cycle) and filter 2 (14th cycle) had very low fungal activity (0.8-4 U l^{-1} laccase activity), while in filter 2 (9th cycle), the fungus was still well active (23 U l^{-1}). As presented in Fig. 9.42, the removal of sotalol, atenolol, metoprolol (not shown), bezafibrate, metronidazole, gabapentin, iopromide, and iohexol was much faster in filters 1 and 3 (with low fungal activity) than in filter 2 (well active). Moreover, when the fungus in filter 2 became inactive (14th cycle), the removal rates increased to a level similar or better than in filters 1 and 3. These results showed that the active fungus was not responsible for the degradation of these compounds, and confirmed that the active fungus probably prevented or inhibited the development of the microbial community, which was responsible for the removal of these micropollutants. Indeed, *P. ostreatus*, as well as several other basidiomycetes, are known for their ability to inhibit the development of bacteria in their surrounding (e.g., by predation, acidification of the medium or production of antimicrobial metabolite) (de Boer and van der Wal, 2008).

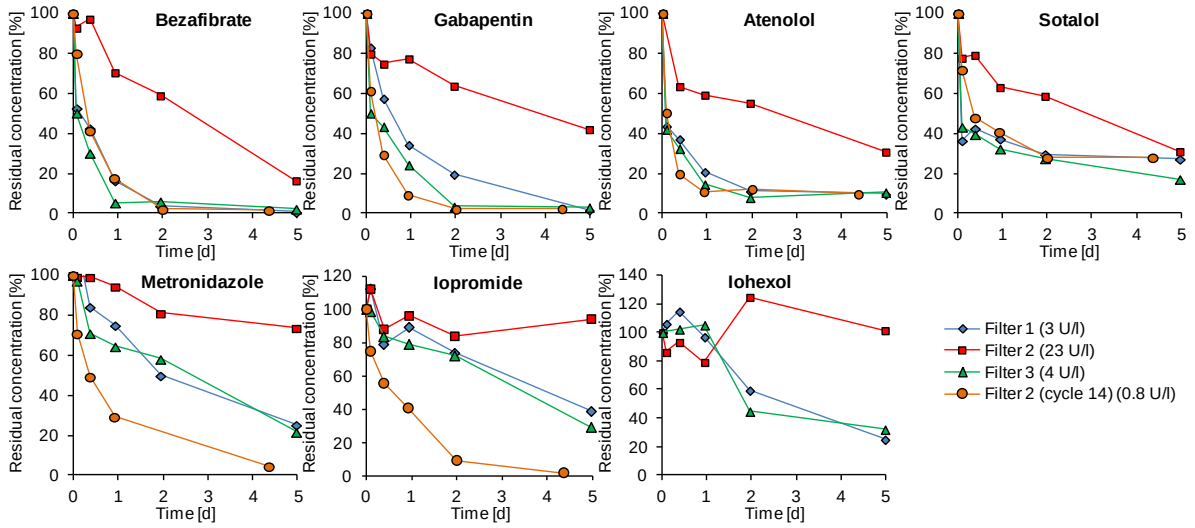


Fig. 9.42 Difference in micropollutant removal kinetics between the three replicates (filters 1, 2 and 3) inoculated with *P. ostreatus* during the 9th batch cycle, and comparison with the kinetic observed in filter 2 during the 14th batch cycle. Filters 1 and 3 (9th cycle) and filter 2 (14th cycle) had very low fungal activity (0.8-4 U l^{-1} laccase activity), while in filter 2 (9th cycle), the fungus was still active (23 U l^{-1}).

It seemed that only the development of the attached microbial biofilm was impaired by the presence of the fungus (direct competition for space with the mycelium), but not the growth of free bacteria in the liquid phase, on the contrary stimulated by the fungus. Indeed, much higher (20 to 80 times) concentrations of free bacteria in the liquid phase were found in the effluents of the fungal filters than in those of the wood filter (cf. Table 9.3). Moreover, during the 8th cycle, 2.6 to 3 times higher concentrations of total viable bacteria were measured in the effluent of the fungal filter 2 (4×10^5 CFU ml^{-1}) than in the effluents of the fungal filters 1 and 3 ($1.3\text{-}1.5 \times 10^5$ CFU ml^{-1}). This higher bacteria concentration in the effluent of the filter 2 may be due to the much higher fungal activity in this filter (40 U l^{-1} laccase activity) compared to the two others (< 8 U l^{-1}), and therefore to the release of higher amount of dissolved organic substrates for the bacteria (extracellular enzyme, metabolites, dead fungal cells, etc.).

For the removal of pollutants mainly degraded by the native microorganisms, a wood filter with a well established microbial community will thus perform much better than an active fungal filter. On the other hand, the fungal filter will allow the removal of compounds difficult to degrade by the microbial community, such as DFC, BTZ or NPX. Therefore, combining fungal and native microbial degradation in two separated serial processes is the key to reach good micropollutant biodegradation in municipal wastewater.

9.3.12.9 Synthesis of the main removal mechanisms in the fungal filters

Based on all the previous results, the 27 micropollutants analyzed in this study could be classified according to their main removal mechanism in the fungal filter (Table 9.6). Five to seven pollutants were clearly degraded by the fungus, while at least 14 others were mainly removed by microbial degradation and six mainly removed by adsorption. Despite the good efficiency of the fungal filter to remove a wide range of micropollutants, the contribution of the fungus itself was only limited to a few compounds. Most of the efficiency of the filters was due to the native microbial community.

Table 9.6 Synthesis of the main removal mechanisms in the fungal filters, determined for 27 micropollutants.

Fungal degradation	Native microbial degradation	Adsorption
diclofenac, gemfibrozil, mefenamic acid, naproxen, sulfamethoxazole, (benzotriazole, trimethoprim)	atenolol, azithromycin, bezafibrate, gabapentin, ibuprofen, iohexol, iomeprol, iopamidol, iopromide, mecoprop, methylbenzotriazole, metoprolol, metronidazole, sotalol, (benzotriazole, trimethoprim)	carbamazepine, ciprofloxacin, diuron, isoproturon, ofloxacin, terbutryn

Compounds in bracket: clear distinction between fungal and native microbial degradation could not be done

9.3.12.10 Release of organic compounds

One of the main drawbacks of the fungal filters, as designed here, is the release of high amount of dissolved organic matter in the treated water. Indeed, soluble wood components and organic matter (fungal metabolites accumulated during the incubation period) are leached in the water during the operation of the filter. As presented in Fig. 9.43 A, during the second batch cycle (not measured for the first cycle), the DOC concentration increased by 63 times (up to 600 mg l^{-1}) and 27 times (255 mg l^{-1}) in the effluents (after 48 h) of the fungal and wood filters, respectively. The release of DOC decreased then rapidly at each new cycle, due to progressive washing of the wood/mycelium. After 8 cycles, the release of DOC was, however, still relatively high, with 45 and 28 mg l^{-1} in the effluent of the fungal and wood filters, respectively (compared to 9.4 mg l^{-1} in the raw TWW). The amount of DOC released was also dependent on the length of the cycle (Fig. 9.43 B). In a batch cycle of 5 d, 2.5 times more DOC was released in the fungal filter than in a cycle of 2 d.

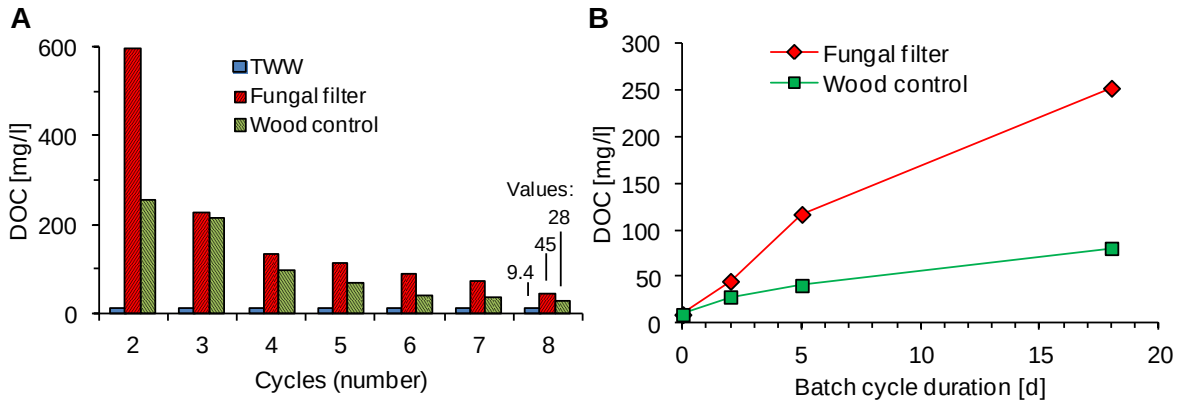


Fig. 9.43 Release of dissolved organic carbon (DOC) during the batch cycle by the wood filters, inoculated (fungal filter) or not (wood control) with *P. ostreatus*. (A) Evolution of the DOC released as a function of the number of batch cycles (48 h) performed. **(B)** Evolution of the DOC released as a function of the duration of the batch cycle (compilation for cycles 8-10). TWW: raw treated wastewater.

The soluble wood compounds released coloured also the treated water in a dark brown-yellow during the first cycles, the colour intensity decreasing then at each cycle to very light yellow after 10 cycles (Fig. 9.44).

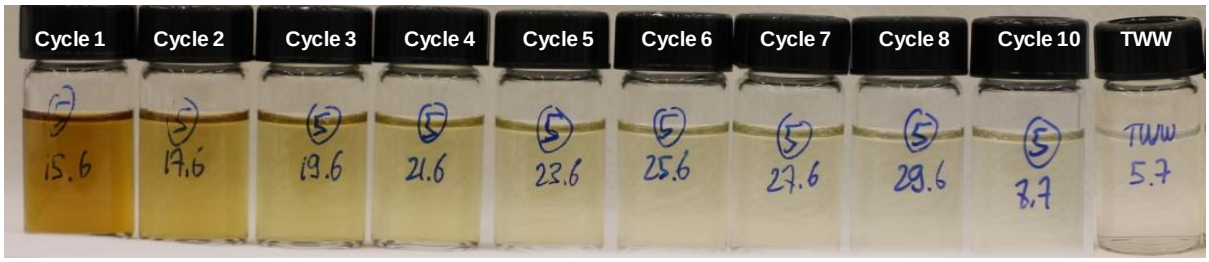


Fig. 9.44 Evolution of the colour of the water treated by the wood filter (not inoculated) as a function of the number of batch cycles (of 48 h) performed. TWW: raw initial treated wastewater.

Despite the release of dissolved organic matter, bacteria did not develop in the water treated by the wood filter (decrease in total viable bacteria, Table 9.3), indicating that the soluble wood components were not easily biodegradable (possibly due to lack of essential nutrients). The release of these

compounds should therefore not lead to fast oxygen consumption in the receiving waters. Nevertheless, their toxicity for aquatic organisms should be assessed before further development of this system. Soluble wood component (hydrophilic wood extractives) are expected to be mainly lignin-derivative compounds (Liu et al., 2011b), condensed tannins, flavonoids, proanthocyanidins and several other polyphenols, as well as low molecular weight organic acids such as acetic acid (Piškur et al., 2009; Rowe, 1989; Vek et al., 2013).

In addition to the release of wood extractives, the fungal filters released probably also dissolved fungal metabolites, extracellular enzyme and wood oligomers, due to the fungal metabolism (de Boer and van der Wal, 2008). These compounds, despite their probable participation to the DOC released, seemed relatively easily biodegradable (bacteria developed well in the filters with high fungal activity), and therefore, potentially easily removed in an additional step by microbial degradation.

The impact that the DOC released could have on the receiving waters is not clear. Similar compounds (such as refractory tannins, humic or lignin-derivative substances) are also present at high concentrations (from 10 up to 100 mg l^{-1} DOC) in many natural water bodies without generating ecological problems, known as dystrophic waters or blackwaters (due to the brown colour of the tannins, humic substances or organic acids lixiviated from forest soils or swamps) (Kerr et al., 2013; Liu et al., 2011b). However, high input of organic matter in an aquatic ecosystem not used to have such high content of DOC (oligotrophic waters) may generate several impacts, even if the DOC is of low bioavailability (Andreasson et al., 2009). High input may still possibly generate hypoxia (low dissolved oxygen level due to microbial activity) in poorly aerated rivers, which can be harmful or lethal to a wide range of organisms. Moreover, the DOC may contain compounds that can be toxic, at high concentrations, to sensitive organisms. Finally, high concentrations of DOC reduce light penetration through the water column, which may inhibit the growth and survival of submerged plants and algae (Kerr et al., 2013). It will be therefore important to determine, in additional studies, if the DOC released from the fungal filters will possibly generate such impacts or will be harmless for the aquatic organisms.

Strategy to limit the release of organic compounds

To face the problem of DOC released in the water, different strategies could be applied, such as (i) washing well and soaking the woodchips in water during several days (or weeks) before their inoculation, to extract most of the soluble organics prior their use in the fungal filter, (ii) avoiding too long reaction time (cycle duration < 2-3 d) in the fungal filter, and (iii) removing the bark of the dead wood branches before preparing the woodchips. Indeed, hardwood (such as beech) contains around 4% of extractives while the bark contains around 20% (Helm, 2000). As bark can comprise between 14 to 37% of the tree mass (Pérez Cordero and Kanninen, 2003), using woodchips without bark could possibly decrease the release of soluble organics by more than half. Applying these three strategies should reduce drastically the DOC of low bioavailability released by the woodchips and the coloration of the water. For the dissolved compounds produced by the fungus (metabolites, wood oligomers and extracellular enzymes), their production cannot be avoided, but most of these compounds can probably be degraded by the native microbial community in an additional step. These strategies still have to be tested to evaluate their efficiency.

9.3.13 Energy consumption of the fungal filters and practical implications

The global aim of this study was to develop a process for micropollutant removal in municipal wastewater affordable for small WWTPs, with low equipment needs, skills and energy requirements. The sequential batch fungal filters go in this direction, with simple technical equipments (only a tank, pumps, valves and a simple control system), no continuous input of reagents, no need of specific skill for the operation and low maintenance (only for the pumps and valves, and every few months to renew the wood supports). The energy consumption for the operation of the filters was therefore also assessed to evaluate the full potential of this system.

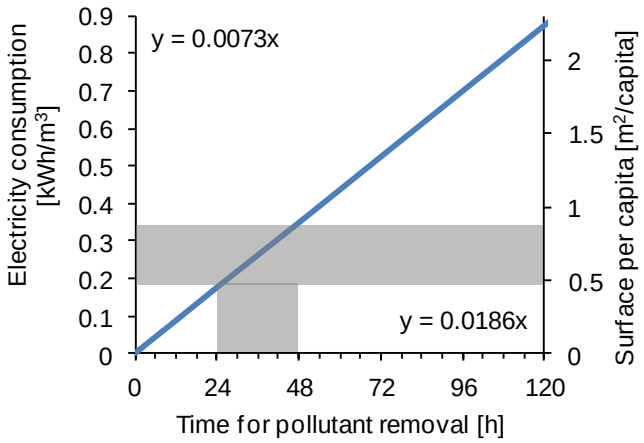


Fig. 9.45 Electricity consumption (for water recirculation) and space requirement by the fungal filter as a function of the duration of the batch cycle. Filter characteristics (scale up of the laboratory columns, but in the same operational conditions): Height: 125 cm, divided in 5 layers (25 cm) saturated sequentially (1 min immersion, 4 min drainage). Pump yield (energy): 0.5 [kWh/kWh]. Ratio volume of water treated per bacth cycle / volume of reactor (filter): 0.59 [m^3/m^3]. Percentage of volume of water recirculated every 5 min per volume treated per batch cycle: 9%. Equations: x : duration of the batch cycle (h), y: electricity consumption (kWh per m^3 treated) (left) or space requirement (m^2 / capita, with 330 litres consumed per capita per day) (right). Grey zone: reaction time required for good micropollutant removal.

Energy consumption for the operation

During the fungal filter operation, the only energy consumption came from the pump used for the water recirculation. As about 45% of the water treated in a batch cycle was recirculated every 5 min on a height of 25 cm, this was equivalent to pump all the water of the batch cycle on about 135 cm every hour. The energy (electricity) required to pump the water, including the pump yield (50%) and the head losses, was of 7.27 Wh m^{-3} of water treated, per hour of treatment. The total electricity consumed per volume of water treated was thus dependant on the duration of the batch cycle, ranging from 0.17 kWh m^{-3} for a cycle of 24 h, up to 0.87 kWh m^{-3} for a cycle of 5 d (Fig. 9.45). As a cycle of 48 h was sufficient to remove well (> 80%) many micropollutants, in synthetic but also in real wastewater, this would result in an electricity consumption of 0.35 kWh m^{-3}, which is similar to the total electricity consumed actually in conventional WWTPs (Abegglen and Siegrist, 2012). By comparison, for similar average removal efficiencies, PAC adsorption and ozonation (both with sand filtration) consume for their operation around 0.08 and 0.12 kWh m^{-3}, respectively (cf. Chapter 3), and PAC adsorption followed by ultrafiltration potentially less than 0.2 kWh m^{-3} (Magnet et al., 2014). In terms of energy consumption (for the operation), the fungal filters, as designed here (not optimized), are thus not competitive with other advanced treatments (3 times more electricity consumption) for a batch cycle

of 48 h, but start to be competitive when the batch cycle is shorter than 24 h (which allowed already a good removal of most pollutants).

To be more competitive, the energy requirement of the process has thus to be reduced. Instead of recirculating every 5 min the water, which consumes a lot of energy, it would be much more efficient to keep the water static and to sequentially immerge and emerge the woodchips/mycelium. Similar systems, such as rotating biological contactors (RBC), are already used for treating wastewater and were recently proposed for fungal reactors (Šíma et al., 2014). RBC are composed of rotating disks, only partially immerged in wastewater, on which biomass grows as a static biofilm. Rotation leads to natural aeration of the biofilm, fluid mixing, convection through the media biofilm pores, and compound diffusion to the biofilm. RBCs are known for their low energy and maintenance requirement, with less than half the energy consumption of an activated sludge plant (Hassard et al., 2014). Developing a rotating fungal contactor (RFC), where the woodchips/mycelium support is packed with a mesh in a rotating drum, partially immerged (one third) in the water, may drastically reduce the energy consumption of the system. Indeed, with a slow rotation speed (one rotation every 3 min, to have 1 min immersion and 2 min resting), the resistance to rotation (friction) inside the water will be low and water will have time to drain before being too much dragged along with the drum in the emerged part. Rotation of the drum should therefore not require strong power. Such systems, still to be tested, should be able to compete easily for energy consumption with other advanced treatments, despite a reaction time of 24 to 48 h.

Energy consumption for the woodchips/mycelium preparation

Apart for the operation, energy is also required to prepare the supports (woodchips/mycelium). Energy consumption to shred the dry wood to produce woodchips is not expected to be important compared to other processes. The main energy cost for the support preparation was the sterilisation of the wood before its inoculation. Although the mycelium could sometime develop well in non-sterile wood, it was often not able to compete with a green mould, which develops much faster (Fig. 9.4 F). Sterilisation was thus a guarantee of good mycelium development. Based on the data of Kerry (2010), the wood sterilisation (laboratory-scale autoclave) required around 0.25 kWh kg^{-1} dry wood. Assuming a life expectancy of the filters up to 140 d, with a batch cycle duration of 48 h (70 cycles performed), 2 g l^{-1} of dry wood per treated water were necessary, corresponding to an energy consumption of 0.5 kWh m^{-3} just for the wood sterilisation. This wood was then inoculated with 1-4% v/v of mycelium, which would correspond to adding between 0.14 to 0.57 ml of mycelium per litre of water treated. The additional energy consumption related to the mycelium preparation (medium sterilization) would correspond to 0.03 kWh m^{-3} treated (for 1% v/v mycelium in the wood). Although, at industrial scale, sterilisation is probably more efficient, and that significant optimization of the woodchips preparation is still possible, this shows that preparation of the inoculated wood support may consume more electricity than the operation of the filter itself. Same conclusions were drawn for a fungal bioreactor, where preparation of the inoculums was the most critical point (Gabarrell et al., 2012). Production of PAC and ozone consumes also a significant amount of energy. A detailed life-cycle assessment of the three processes would allow comparing the ecological impacts of these three treatments.

Footprint

The space (footprint) required by the fungal filters was also estimated for a scale-up version of the laboratory columns used (but with the same operational conditions), of 125 cm high composed of five layers of 25 cm sequentially saturated. As presented in Fig. 9.45, a surface of 0.45 m^2 and 0.89 m^2 per capita for a batch cycle of 24 h and 48 h, respectively, would be necessary. This is lower than for constructed wetlands (2-4 m^2 $capita^{-1}$) (Matamoros et al., 2007), but more than 100 times higher than for ozonation or PAC adsorption (around 0.005 m^2 $capita^{-1}$) (Margot et al., 2011). Therefore, the high space requirement of this advanced biological system limits its potential application in WWTPs were space is not a limiting factor (mostly small WWTPs).

Life expectancy of the filters and woodchips management

The life expectancy of the fungal filters (fungal activity) varied between 50 to more than 140 days, depending on the test conditions. As the fungus was still well active after 140 d in some filters, this could be considered as an operation duration possible to reach in an optimized system. Even once the fungus was not anymore active, the filters were still efficient to remove many pollutants (degradation by the native microbial community). Thus longer operation times could be reached depending on the targeted pollutants.

For a life-span of the fungal filters of 140 d and with batch cycles of 48 h, relatively high quantity of woodchips would be necessary (per volume of water treated): 2 g l^{-1} dry weight. Beech wood is widely available in Switzerland and forestry waste can be used to produce the woodchips, so their cost should be low. Once the filter loses its efficiency, the woodchips/mycelium will have to be renewed. This means that big quantities of waste will be produced, multiplying by ten the sludge production of WWTPs (around 0.2 g l^{-1} dry weight) (DGE, 2013). Woodchips, once slightly dried, are expected to be a good combustible and could improve the incineration of the sludge (wet woodchips contain around 30% dry matter, so similar than dewatered sludge (Metcalf and Eddy, 2003)).

To be more competitive with other advanced treatments, the fungal filters still have to be optimized to (i) reduce the time of treatment (to reach a batch cycle < 24 h) while maintaining the same efficiency (possibly by using another white-rot fungi), (ii) increase the life-span of the fungus (more than 140 d) and the long-term stability of the process, and (iii) significantly reduce the energy consumption. For that, other filter configurations should be tested, such as the rotating fungal contactor. Therefore, despite the promising possibilities to treat biologically many refractory micropollutants during several months, effort has still to be invested in research to design a fungal filter able to compete, in terms of feasibility and full-scale operation, with other advanced treatments such as ozonation and activated carbon adsorption.

9.4 Conclusions

The main results of this study can be synthesised in the following points.

Trametes versicolor was able to degrade several micropollutants (DFC, MFA, NPX, IPN, SMX and gemfibrozil) even at low concentrations in real treated wastewater, but it was not able to survive more than one week in unsterile water, due to competition/predation by other microorganisms.

The other white-rot fungus, *Pleurotus ostreatus*, was much more resistant and competitive in an unsterile environment than *Trametes versicolor*. Despite its much slower growth and lower laccase activity, it was also able to degrade many micropollutants. It was therefore selected for the fungal filter development.

A sequential batch fungal filter, composed of beech woodchips as support/substrate for the fungal mycelium, and working in alternation of saturated/unsaturated conditions, was designed. This system was very reliable, allowing good reproducibility of the treatment efficiencies.

These fungal filters could be operated in continuous mode during several months (up to 140 d) to treat micropollutants in unsterile wastewaters (synthetic and real municipal treated wastewater), while keeping the fungus active without addition of any external substrate (including fresh woodchips), acidification, or re-inoculation.

A wide range of micropollutants could be removed well in the fungal filters, including hardly degradable compounds such as DFC, NPX, SMX, gabapentin and X-ray contrast media, by a combination of fungal and microbial degradation and adsorption. The average removal efficiency of 27 micropollutants (in real wastewater) reached 72 and 82% after 48 h and 5 d of treatment (batch cycle duration), respectively. After 5 d, only CMZ was removed less than 50%. This process was thus able to compete with other advanced treatments such as ozonation (at 5.9 mg O_3 l^{-1}) and PAC adsorption (at 12 mg l^{-1}) (both with 74% average removal efficiency).

Despite the good efficiency of the fungal filter to remove a wide range of micropollutants, the contribution of the fungus itself was only limited to a few compounds (DFC, NPX, MFA, SMX, gemfibrozil). Most of the efficiency of the filters was due to the native microbial community. Moreover, the fungus prevented or slowed down the growth of the microbial biofilm, reducing the removal of some pollutants compared to a wood filter not inoculated with the fungus. Therefore, combining fungal and native microbial degradation in two separated serial processes is the key to reach good micropollutant biodegradation in municipal wastewater.

The main drawbacks of the fungal filters were the release of significant amount of DOC, leached from the woodchips, as well as important energy consumption (water recirculation) due to the long reaction times (24-48 h) needed for the removal of the pollutants. The long reaction time and the moderate life-span of the filters (up to 140 d) resulted also in relatively high consumption of woodchips per litre of water treated (2 g l^{-1}).

Despite good efficiency to treat micropollutants, simple technical equipments, ease of operation, only woodchips and electricity as sole inputs, and low maintenance, the fungal filters still have to be significantly optimized in order to eventually compete one day with ozone or PAC regarding the long-term operational feasibility and stability at full-scale for the treatment of micropollutants in municipal wastewater.

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We specially thank Andreu Contijoch and Micaela Vargas (EPFL) for their help with the monitoring of the filters, as well as Jean-Pierre Kradolfer (EPFL) for the construction of the filter set-up. We acknowledge Paqui Blánquez and Teresa Vicent (UAB) for their advices regarding the design of fungal reactors and their kind gift of the *Trametes versicolor* strain. And last but not least, a special acknowledgement to Daniel Job (UNINE) for his recommendation to use the white-rot fungus *Pleurotus ostreatus*, key of the success of this study, for the kind gift of the fungal strain and for helpful discussions.

Chapter 10 General conclusions and perspectives

This last chapter gives an overview of the main achievements of this thesis concerning micropollutant removal from municipal wastewater, and highlights the needs for further research regarding different issues raised during this work.

10.1 Main achievements of this thesis

10.1.1 Micropollutant removal in conventional WWTPs

This work confirmed that the removal of hydrophilic (low sorption affinity) and hardly biodegradable micropollutants, such as pharmaceuticals, pesticides and several household products, is particularly challenging with conventional biological treatments.

The pilot study conducted at Lausanne WWTP demonstrated that undiluted WWTP effluents can impact the development and the survival of juvenile fish (rainbow trout). These effects were very likely linked to micropollutants toxicity as they could be strongly reduced after treatment of micropollutants with ozone or activated carbon. This confirms that advanced treatments are necessary to improve quality of surface waters.

The role of nitrification in micropollutant removal was investigated in laboratory-scale reactors and in full-scale WWTPs. It was shown that the addition of a nitrification step improves significantly the removal of many micropollutants (cf. Table 10.1), and this, probably rather because of a higher diversity of aerobic heterotrophic microorganisms and longer HRTs in nitrifying WWTPs than to the action of nitrifying organisms. For WWTPs that do not plan to have advanced treatments, up-grading with a nitrification step is therefore recommended. However, even in WWTPs with complete ammonium removal, around half of the pollutants studied were still removed less than 50% (cf. Table 10.1). Thus, nitrification is not sufficient for the protection of the receiving waters.

10.1.2 Micropollutant removal with advanced physico-chemical processes

Two advanced treatments were assessed in a comprehensive study based on large-scale pilot systems at Lausanne WWTPs: ozonation and powdered activated carbon (PAC) adsorption. Both technologies were efficient to remove most of the pollutants studied (cf. Table 10.1), with similar average removal efficiencies. They both significantly decreased the toxicity of the effluents towards a wide range of organisms. Both treatments proved to be feasible at large scale and for long-term operation in real WWTP conditions, with similar and reasonable costs if sand filters were used for the PAC retention.

Although these two treatments are relatively mature, a few issues should be clarified to optimize these processes:

- PAC and ozone efficiencies appeared to be strongly affected by the dissolved organic carbon (DOC) content of wastewater. Regulation of the dose of reactant as a function of the DOC concentration, which was performed for ozone, has still to be tested for PAC.
- Efficient and reliable separation of the spent PAC at reasonable costs is challenging and was not achieved in this study. Improvement in ultrafiltration technologies may be the key-point to solve this issue.
- Despite ozonation decreased significantly the toxicity of the wastewater, contradictory results were observed in other studies, suggesting that the wastewater matrix composition could affect the toxicity of the by-products. This should be further assessed.

10.1.3 Micropollutant removal with oxidative enzymes

The potential of the oxidative enzyme laccase for micropollutant removal in wastewater and the optimal conditions for an efficient enzymatic treatment were determined. Despite the ability of laccase to degrade in a few hours several critical pollutants such as oestrogenic compounds, triclosan, mefenamic acid and diclofenac, and this even at environmentally relevant concentrations, the majority of the pollutants tested could not be removed. Moreover, the strong pH effect on laccase activity may strongly limit the feasibility of such treatment in real not-acidified wastewater. Indeed, the two fungal (from *Trametes versicolor*) and bacterial (from *Streptomyces cyaneus*) laccases tested were mainly active under acidic conditions. Under neutral-alkaline conditions, high enzyme concentrations and long reaction times were necessary, which restrains the application of this process in WWTPs. Therefore, enzymatic treatment with solely laccase to decrease the load of a wide range of micropollutants in municipal WWTP effluents does not appear as an interesting option.

However, laccase treatment may be of interest if the goal is to remove only a few target pollutants, such as the endocrine disrupters responsible for fish feminization in many rivers. In this case, further research is needed especially (i) to increase the stability of the enzymes (relatively rapidly degraded) in municipal wastewater, (ii) to immobilized the enzymes on a support that stays in the system (to avoid the lost of free enzymes in effluents), (iii) to select laccases highly active and stable under neutral-alkaline conditions, (iv) to produce high quantity of laccase by a cheap process, and (v) to design a system feasible for long-term operation in WWTPs, without too high costs, energy consumption or maintenance needs, for instance by filtration through a fixed-bed containing high concentration of immobilized laccases. The semi-empirical model proposed in this thesis could become a useful tool to optimize the design and predict the efficiency of such systems as a function of the operation conditions (pH, temperature, HRT, enzymatic activity).

The strong effect (enhancement or inhibition depending on the compounds) of mixtures of pollutants on the degradation kinetics with laccase, highlighted in this work, should also be further investigated to assess the impact of the wastewater matrix as well as the presence of other pollutants on laccase oxidation efficiency.

A comprehensive study on laccase-mediator systems (LMS) was performed, allowing a better understanding of the mechanistic aspects of LMS reactions. Addition of mediators was shown to widen the range of pollutants oxidized by laccase, allowing complete degradation and partial to complete detoxification (towards algae) of the herbicide isoproturon and the antibiotic sulfamethoxazole. Depending on the pH, different strategies were proposed to increase the oxidation rates. It was demonstrated that the synthetic mediator ABTS, in addition to be more efficient, allowed better removal of the toxicity of the mixture of transformation products than the natural mediators syringaldehyde and acetosyringone. Instead of acting as catalysts, as initially thought, the mediators were consumed during the reaction. Therefore, treatment of low micropollutant concentrations in municipal wastewater (especially at neutral-alkaline pH) would require relatively high doses of mediator, which might increase the toxicity of the effluent (due to the toxicity of the mediator itself) and generate significant costs. Thus, despite their interesting potential for concentrated industrial effluents, LMS appear not to be suitable for municipal wastewater treatment.

10.1.4 Micropollutant removal with advanced biological processes

Treatment of micropollutants with the laccase-producing white-rot fungus *Pleurotus ostreatus* appears to be a much more promising option. Indeed, the sequential batch fungal filters developed, with woodchips as substrate and support for the fungal mycelium, could be operated in continuous mode during several months (up to 140 d) to treat micropollutants in unsterile wastewaters, while keeping the fungus active without addition of any external substrate, acidification or re-inoculation. A wide range of micropollutants could be well removed by a combination of fungal and microbial degradation and adsorption. This process was able to compete with other advanced treatments such as ozonation and PAC adsorption regarding the average removal efficiency of 27 micropollutants in municipal wastewaters (cf. Table 10.1). This demonstrated that biological processes can be used to remove a wide range of micropollutants from wastewater. However, biological processes are rather slow and long reaction times (24 to > 48 h, versus < 40 min for PAC or ozone) were required for the removal of most pollutants. These long reaction times and the moderate life-span of the filters resulted in relatively high energy consumption (> 0.18 kWh m^{-3} for water recirculation and > 0.25 kWh m^{-3} for woodchips inoculation) and high woodchips requirement (> 1 g l^{-1}).

Therefore, despite good efficiency to treat micropollutants, simple technical equipments, ease of operation, only woodchips and electricity as sole inputs and low maintenance, the fungal filters have to be optimized in order to compete with ozone or PAC regarding their application in municipal WWTPs. More research is especially needed (i) to reduce the reaction times to less than 24 h, possibly by stimulating the growth (nutrient addition) of the active biomass, (ii) to decrease the energy consumption, for instance by changing the design of the filters (e.g., using a rotating fungal contactor), (iii) to increase the life-span of the fungus in the filters (for instance by testing other more resistant white-rot fungi), or, indirectly, to increase the quantity of water treated per filter (by a factor 10 minimum), and (iv) to reduce the release of wood components in the treated water, for instance by a prior washing of the woodchips, and by removing the bark of the wood. In the case these objectives would be reached, which is not without challenges, fungal treatment of micropollutants in municipal wastewater might become a reality.

Table 10.1 Synthesis of the removal efficiencies of 43 micropollutants in municipal wastewater achieved by various biological and physico-chemical processes during this thesis. WWTP: wastewater treatment plant, PAC: powdered activated carbon.

Removal efficiency [%]	WWTP without nitrification[a]	WWTP with complete nitrification[b]	Ozonation[c]	PAC treatment[d]	Fungal filter[e]
Paracetamol	100	100	n.a.	n.a.	n.a.
Estriol	71	64	n.a.	n.a.	n.a.
Estrone	66	86	92	92	n.a.
Ciprofloxacin	59	64	53	63	89
Norfloxacin	59	96	75	82	n.a.
Simvastatin	50	87	n.a.	n.a.	n.a.
Ofloxacin	48	93	85	83	89
Sulfamethoxazole	40	23	93	64	79
Terbutryn	34	65	85	80	99
Atrazine	33	7	34	74	n.a.
Diatrizoic acid	33	23	16	15	n.a.
Ibuprofen	26	100	63	83	96
Mecoprop	26	39	60	48	79
Azithromycin	26	73	74	76	51
Irgarol	26	92	32	33	n.a.
Isoproturon	26	54	68	75	86
Propiconazole	24	38	32	66	n.a.
Clarithromycin	23	45	93	92	n.a.
Naproxen	22	63	90	81	98
Iopamidol	18	24	42	49	61
Metronidazole	17	66	64	79	83
Diuron	17	0	73	87	86
Mefenamic acid	16	80	98	93	99
Bisphenol A	16	91	91	86	n.a.
Ketoprofen	16	59	63	81	n.a.
Benzotriazole	16	26	64	90	72
Gemfibrozil	15	89	94	76	98
Iopromide	13	70	34	47	77
Methylbenzotriazole	12	71	80	96	79
Bezafibrate	9	67	81	79	95
Gabapentin	7	7	38	12	98
Metoprolol	7	0	88	95	75
Primidone	6	23	57	51	n.a.
Trimethoprim	6	47	99	94	96
Diclofenac	6	8	94	69	97
Atenolol	6	55	85	88	85
Sotalol	5	35	99	81	74
Iohexol	5	36	38	57	71
Carbamazepine	4	15	97	90	34
Iomeprol	4	27	43	54	76
Propranolol	4	20	99	99	n.a.
Carbendazim	1	4	79	93	n.a.
Clindamycin	0	0	99	82	n.a.
Average removal (27 pollutants)[f]	18	48	74	74	82

[a] Average removal of 12 campaings (24-72 h composite samples) in WWTP with less than 25% NH_4 removal

[b] Average removal of 6 campaings (24-72 h composite samples) in WWTP with > 97% NH_4 removal (< 1 mg N-NH_4 l^{-1} in effluents)

[c] Average removal of 12-28 campaings (24-72 h composite samples) during ozonation with an average dose of 5.7 mg O_3 l^{-1} (eq. 0.8 g O_3 g^{-1} DOC)

[d] Average removal of 8-24 campaings (24-72 h composite samples) during PAC treatment with an average dose of 12 mg l^{-1}

[e] Average removal of 4 batch cycles after 5 d of treatement in the fungal filter with *P. ostreatus*

[f] Average removal of the 27 micropollutants analysed in the fungal filter

Grey scale: white: < 25%, light grey: 25-50%, dark grey: 50-75%, black: >75% removal. n.a.: not analysed

10.2 General conclusions

This thesis opened new perspectives regarding biological treatment of micropollutants in wastewater, highlighting also the challenges of applying fungal and oxidative enzyme treatments in WWTPs. This work contributed to answer, at least partially, to several fundamental questions regarding micropollutant removal in WWTPs, as discussed below.

10.2.1 Is biological treatment of micropollutants a feasible option?

As demonstrated with the fungal filters, significant biological degradation/transformation is possible for most of the micropollutants studied. White-rot fungi can potentially degrade pollutants hardly degradable by bacteria, such as diclofenac, benzotriazole or naproxen, while the native wastewater microbial community can potentially degrade most of the other micropollutants. The ability of the native microbial community to degrade several pollutants poorly removed in WWTPs, such as X-ray contrast media, gabapentin, beta-blockers or pesticides, is very promising for the further development of biological filters used as post-treatment in WWTPs. Combining fungal and microbial processes opens therefore new perspectives for biological degradation of a wide range of pollutants in municipal wastewater.

Although biodegradation of most micropollutants appears to be feasible, the question remains if these advanced biological treatments are a realistic option for WWTPs. Based on the results of this work, the answer is probably "*not yet*". Indeed, several constraints were raised concerning the feasibility of their implementation in real WWTPs.

For instance, keeping white-rot fungi active in real wastewater is highly challenging due to the conditions very far from their natural habitat. Expensive solutions for this issue were proposed in other studies, such as acidification or addition of nutrients (glucose, ammonium), which do not appear to be realistic in municipal WWTPs. The idea proposed in this thesis, using cheap and widely available woodchips as substrate for the mycelium, was relatively successful and allowed operating the filters in continuous mode during several months. However, once in contact with wastewater, the mycelium was not able to grow anymore and decayed slowly in a few months. The self-sustainability of white-rot fungi in wastewater seems to be very difficult to achieve, meaning that a periodical re-inoculation of the system would be necessary. Therefore, a strong improvement of the life-span of the fungus in the system is a prerequisite to limit the relative high costs of fungal mycelium preparation and inoculation.

Another constrain of biological micropollutant treatment is the relative long hydraulic retention times (HRTs) required, up to several days, which may considerably increase (by a factor of 2 to > 5) the size of WWTPs. The potential application of such treatments is thus limited to WWTPs having sufficient space available. Long HRTs are also synonym of higher financial and operation costs (low volume of water treated per equipment and energy inputs). Therefore, optimization of advanced biological processes in terms of energy and resource consumption, as well as decreasing the treatment time, for instance by increasing the active biomass in the system, are necessary before considering real applications. Increasing the active biomass without frequent re-inoculations is, however, limited by the quantity of nutrients available (or added) in WWTP effluents. Therefore, achievement of short HRTs

in biological processes treating very low concentrations of micropollutants seems difficult without addition of (potentially costly) external substrates, which may drastically change the microbial community composition and its efficiency for micropollutant degradation.

Finally, the characteristics of the transformation products formed during biodegradation processes are still relatively unknown. Further research is needed to determine the degree of micropollutant mineralization and the relative toxicity of these products, as well as the products released by the wood filters.

10.2.2 Which technology to apply for micropollutant removal in WWTPs?

None of the technologies assessed in this thesis were able to remove efficiently all the micropollutants studied. The choice of one technology will thus depend on the target pollutants and on the benefits / drawbacks of each system. For the removal of target phenolic and anilines compounds, such as several endocrine disrupters, an enzymatic treatment with laccase might become interesting and should be further developed. However, if the objective is to remove (on average at 80%) a wide range of pollutants, pure enzymatic treatments are not adapted, but other solutions can be proposed, depending on the size of the WWTP.

For WWTPs larger than about 2000-10,000 population equivalents (PE), both ozonation and PAC adsorption are feasible options, with possibly similar costs. The choice will thus depend on local constraints and secondary objectives, such as the need for disinfection, safety considerations, building constraints, adaptability of existing installations, availability of PAC or pure oxygen, incineration capacities for the spent PAC, type of treatment wanted (transformation versus removal), risk of releasing toxic by-products, waste production, etc. Advanced biological treatments, if further developed, will probably not be adapted for large WWTPs due to their high requirements in space.

For small WWTPs (< 2000 PE) with non-permanent staff, ozonation is not an interesting option for safety reasons (ozone is a harmful gas that has to be handled with caution). PAC processes are also expected to be not adapted due to the potential technical issues related to handling of fine powders (for dosage and separation from water). Advanced biological treatments with white-rot fungi might become an interesting option but still have to be significantly optimized. Therefore, none of the options tested are, according to the current state-of-the-art, well adapted for small WWTPs and further research is needed.

Combining biological degradation and adsorption processes appeared as a successful strategy in the fungal filters. Another technology, especially based on this combination of processes and probably more realistic than fungal treatments, should deserve more attention: biological activated carbon filtration (BAC). Indeed, using granular activated carbon (GAC) as a support in a slow gravity filter for the development of a self-sustaining native wastewater microbial community appears to be a potentially very interesting option for small WWTPs. Combining the high adsorption capacity of GAC and the interesting degradation potential of an adapted wastewater microbial community in a slow gravity filter, without frequent backflush (to avoid disturbing the biofilm) and with long enough HRTs (to allow degradation), may allow the removal of a wide range of pollutants with a relatively simple technology, and with low maintenance and energy consumption. Of course, GAC, like PAC, has to be

periodically renewed once completely saturated, but the possibility to regenerate GAC may reduce its costs and its environmental impacts.

10.2.3 Objective of 80% micropollutant removal – Is it sufficient to protect aquatic organisms?

Reducing by five, on average, the concentration of micropollutants in raw wastewater is the objective targeted by the new Swiss regulation. Is this reduction sufficient to protect surface waters? The pilot study performed at Lausanne WWTP, as well as other studies (Bundschuh et al., 2011b), demonstrated that an average removal of 80% of a wide range of micropollutants by ozonation or PAC adsorption improves substantially the quality of the effluent, reducing significantly potential adverse effects on aquatic organisms. Therefore, this objective seems to be appropriate, being a good compromise between environmental benefits and costs of the treatments.

However, for limiting the risk for aquatic organisms, objectives in terms of concentrations in the receiving waters and not only in terms of removal efficiencies should fixed. Indeed, even if removed over 80% during the treatments, some micropollutants, either present at high concentrations in raw wastewater or toxic at very low concentrations, may still generate adverse effects at the concentrations found in effluents. Moreover, an average removal efficiency of 80% means that some compounds will be better removed but also some not well eliminated. Regarding only ecological impacts and not the reduction of the load, removing only a few compounds of high ecological risk may already be sufficient to reduce significantly potential adverse effects on sensitive aquatic organisms. Therefore, establishment of environmental quality standards (EQS) to protect aquatic life in surface waters, already existing for several micropollutants, has to be generalised for all relevant compounds in order to identify critical substances and to determine if the treatment objectives are adapted for the protection of surface waters.

Estimation of the potential effect of micropollutants usually focused on individual substances, without considering the synergetic or antagonist effects of the cocktail of micropollutants present in wastewater. Closer collaboration between disciplines like WWTP engineering, ecotoxicology, chemistry, and biology is therefore needed to identify problematic substances and to limit the discharge of critical micropollutants.

Reducing by 80% the concentration of micropollutant in wastewater will also lead to decrease, by maximum a factor of five, the contamination of drinking water resources. However, despite this significant reduction, this should not change the perception of the public concerning presence of micropollutant in their drinking water, as these substances will still be detected in tap water (especially with the new generation of analytical devices with lower limits of detection). Therefore, public information should emphasize the potential benefits of advanced treatments for the aquatic life rather than the very unlikely potential impacts on human health.

The efficiency of advanced processes was mainly assessed for polar organic micropollutants such as pharmaceuticals and pesticides. Their efficiency for the treatment of other classes of micropollutants, such as heavy metals, PAHs, PCBs, surfactants, flame retardants, etc. should also be investigated as these compounds might also generate adverse effects on aquatic organisms.

10.2.4 Avoided versus induced impacts of advanced treatments

Finally, a fundamental question without currently any clear answers has to be addressed: what are the potential environmental benefits of treating micropollutants in wastewater compared to the potential impacts induced?

The benefits of advanced treatments for the aquatic environments downstream of WWTPs are certainly significant and are not questioned. But what about the pollution induced during the production of activated carbon (which requires high energy inputs and possibly non-renewable resources) or ozone, or for the operation of the processes and the elimination of the wastes produced? Are these impacts significant compared to the benefits of the advanced treatments?

Although it is difficult to compare different environmental impacts, such as aquatic ecotoxicity with air pollution or climate changes, life-cycle assessment (LCA) methodology allows giving some rough comparisons. Based on LCA results of several studies, advanced treatments such as ozonation and activated carbon adsorption may generate significant additional environmental impacts (related to energy and chemical consumption), which might be higher or lower than the relative benefits of the treatment, depending on the methodology used (Høibye et al., 2008; Igos et al., 2013; Igos et al., 2012; Larsen et al., 2010; Muñoz et al., 2009; Papa et al., 2013; Wenzel et al., 2008). In any case, all the studies concluded that the environmental impacts of advanced treatments are not negligible. Therefore, significant optimization of these processes in terms of energy and chemical/resource consumption has to be performed. This could be achieved, for instance, by regulating the dosage of reactant as a function of the water quality, avoiding technical configurations that require more energy, recycling the wastes produced (off-gas rich in oxygen for ozonation, or spent PAC) in the biological treatment to increase the global efficiency of the system, choosing PAC made from renewable resources (forestry or agriculture wastes), regenerating and reusing the PAC, and limiting the dosage to the minimum necessary to achieve the protection objectives.

High removal rates (> 90%) of most micropollutants can be reached with high doses of PAC or ozone, or with long HRTs for advanced biological treatments. These higher dosages or longer HRTs will, however, induce higher environmental impacts, which might become more important than the relative benefits of increasing removal efficiencies (Larsen et al., 2010). Therefore, an environmental trade-off between the reduction of ecotoxicity in the receiving waters and an increase in resources and energy consumptions has to be determined.

In parallel to the uncontested necessity to remove toxic micropollutant from WWTP effluents (but with reasonable means), source control has to be implemented. Indeed, the best solution to avoid environmental impacts is always to limit the use and the dispersion of critical substances. Even for pharmaceuticals, whose benefits are obvious for human health, source control could be applied. For the same therapeutic effects, usually various drugs exist, some being easily biodegradable and others being persistent in WWTPs. Substitution of some of the most critical drugs (or pesticides, household products, etc.) by others more environmentally friendly (e.g., substituting the anti-inflammatory drug diclofenac by the other anti-inflammatory drug ibuprofen), as promoted in Sweden[5], might already

[5] Reference: www.janusinfo.se/In-English/, last accessed 24.10.2014

significantly decrease the risk for aquatic organisms without the need for advanced treatments. Therefore, political actions and public awareness are necessary in addition to technical measures at the WWTPs.

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Appendix

The main protocols used for the preparation of the reagents and the culture of white-rot fungi , as well as the parameters used for micropollutants analysis by UPLC-MS/MS are described below.

I. Preparation of buffer solutions

The pKa values (and thus the pH values) of the buffers change as a function of the temperature. The recipes below are for a pH at 25°C. The pH should always be measured at the same temperature than the one used in the experiment.

Stock solutions

Prepare first the stock solutions of the following reactants before preparing the corresponding buffers. Use always pure water (demineralised or miliQ).

Stock solutions (50 ml)		
Citric acid 0.1 M		
M_mol	Citric acid	H_2O
[g/mol]	mg	ml
192.12	960.6	50
Sodium phosphate dibasic Na_2HPO_4 0.2 M		
M_mol	Na_2HPO_4	H_2O
[g/mol]	mg	ml
141.96	1419.6	50
Sodium phosphate monobasic dihydrate NaH_2PO_4*2H_2O 0.2 M		
M_mol	NaH_2PO_4*2H_2O	H_2O
[g/mol]	mg	ml
156.01	1560.1	50
Tris 0.2 M (tris(hydroxymethyl)aminomethane)		
M_mol	Tris	H_2O
[g/mol]	mg	ml
121.14	1211.4	50
Sodium acetate anhydrous CH_3COONa 0.1 M		
M_mol	CH_3COONa	H_2O
[g/mol]	mg	ml
82.03	410.15	50
Acetic acid glacial CH_3COOH 0.1 M (density: 1.049 g/ml)		
M_mol	CH_3COOH	H_2O
[g/mol]	µl	ml
60.05	286.2	49.714

HCl 1 M (from HCl 37%, 440.3 g/l, 12 M, density 1.19 g/ml)		
M_mol [g/mol]	HCl 37% ml	H_2O ml
36.46	4.140	50
NaOH 1 M (from anhydrous NaOH pellets)		
M_mol [g/mol]	NaOH g	H_2O ml
40.00	2	50

Citrate-phosphate buffers

To prepare citrate-phosphate buffer, use the citric acid and sodium phosphate stock solutions prepared before. By varying the volume of these two solutions, buffer at **pH from 2.6 to 8** can be prepared. Adjust the pH to the desired value with NaOH or citric acid.

Citrate phosphate buffer, pH 2.6 to 8, 25-50 mM, 40 ml, at 25°C
Check the pH and adjust at 25°C

pH	**Citric acid 0.1 M ml**	**Na_2HPO_4 0.2 M ml**	**H_2O ml**
2.6	8.92	1.08	30
3	8.234	1.766	30
3.5	7.25	2.75	30
4	6.266	3.734	30
4.5	5.708	4.292	30
5	5.15	4.85	30
5.5	4.971	5.344	30
6	4.164	5.836	30
6.5	3.45	6.76	30
7	2.316	7.684	30
7.5	1.5	8.5	30
8	0.608	9.392	30

Tris-HCl buffers

Tris-HCl buffers can be used for **pH 7.0 to 9.0**. The pKa of Tris (tris(hydroxymethyl)aminomethane) is at 8.07 at 25°C.

Tris-HCl buffer, pH 8-9, 40 mM, 40 ml, at 25°C

pH	**Tris 0.2M ml**	**HCl 0.2M ml**	**H_2O ml**
8	8	5.23	26.77
9	8	1.33	30.67

Acetate buffers

Acetate buffers can be used for **pH 3.6 to 5.6**. The pKa of acetate is at 4.76 at 25°C. The molecular weights are 60.05 g/mol for acetic acid glacial (1.049 g/ml), and 82.03 g/mol for anhydrous sodium acetate.

To prepare large volume of acetate buffer pH 4.5, do as presented below.

Acetate buffer, pH 4.5, 100 mM, 400 ml, at 25°C			
pH	**Acetic acid glacial (60.05g/mol)**	**Sodium acetate CH_3COONa pur**	**H_2O**
	µl	**mg**	**ml**
4.50	1441.1	1443.8	400

For the preparation of a 100 mM buffer, mix different proportions of sodium acetate 0.1 M with acetic acid 0.1 M until you reach the desired pH.

Acetate buffer 100 mM, pH 3.6 to 5.6, 50 ml, at 25°C		
	Acetic acid 0.1 M	**Sodium acetate 0.1 M**
	ml	**ml**
3.6	46.3	3.7
3.8	44.0	6.0
4.0	41.0	9.0
4.2	36.8	13.2
4.4	30.5	19.5
4.6	25.5	24.5
5.0	14.8	35.2
5.2	10.5	39.5
5.4	8.8	41.2
5.6	4.8	45.2

MOPS buffers

MOPS (3-(N-morpholino)propanesulfonic acid) is a biological buffer useful in the **pH range from 6.5 to 7.9** (pKa 25°C: 7.2).

Molecular weight of anhydrous MOPS: **209.26 g/mol**.

To prepare 100 mM MOPS solution, dissolve 20.93 g MOPS in one litre of demineralised water. Adjust then the pH with NaOH or HCl 1 M (0.1 M) to the desired value.

MES buffers

MES (2-(*N*-morpholino)ethanesulfonic acid) is a biological buffer useful in the **pH range from 5.5 to 6.7** (pKa 25°C: 6.1).

Molecular weight of anhydrous MES: **195.24 g/mol**.

To prepare 100 mM MES solution, dissolve 19.52 g MES in one litre of demineralised water. Adjust then the pH with NaOH or HCl 1 M (or 0.1 M) to the desired value.

Phosphate buffers

Phosphate buffers are useful for the **pH range from 5.8 to 8**.

Phosphate buffer 100 mM, pH 5.8 to 8, 100 ml, at 25°C

pH	Na_2HPO_4 0.2 M ml	NaH_2PO_4 0.2 M ml	H_2O ml
5.8	4	46	50
6	6.15	43.85	50
6.2	9.25	40.75	50
6.4	13.25	36.75	50
6.6	18.75	31.25	50
6.8	24.5	25.5	50
7	30.5	19.5	50
7.2	36	14	50
7.4	40.5	9.5	50
7.6	43.5	6.5	50
7.8	45.75	4.25	50
8	47.35	2.65	50

II. Protocol to determine laccase activity with ABTS

In presence of oxygen, laccase catalyze the oxidation of the substrate ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid), colourless, to its radical cation $ABTS^{\cdot+}$, dark green. Laccase activity can thus be determined by monitoring the change of absorbance in the green domain. In this study, one unit of enzyme activity [U] is defined by the oxidation of 1 µmol of ABTS per minute at 25°C and pH 4.5.

Equipment, reactants and material

Thermo-regulated (25°C) spectrophotometer (420 nm, for kinetic studies). Acetate buffer 100 mM pH 4.5 (see previous section), ABTS solution 10 mM (see below), 1 ml cuvettes for spectrophotometer.

Protocol

In a spectrophotometer cuvette of 1.5 ml, maintained at 25°C, add, in this order, the following reactants to obtain a final volume of 1000 µl:

- 850 to 925 µl (depending on the amount of sample tested) of acetate buffer 100 mM, pH 4.5, well aerated
- 25 to 100 µl of sample (culture supernatant), eventually centrifuged 5 min at 6000 *g*, or filtered at 0.2 µm
- 50 µl of ABTS 10 mM (final concentration: 0.5 mM). Mix rapidly and start the measure.

With a spectrophotometer, measure the absorbance *A* at 420 nm, 25°C, during 1 to 10 minutes. Calculate the initial slope of the reaction (Δ*A*/min) in the linear part. If the slope is too steep (> 1 Δ*A*/min), reduce the amount of sample (100, 50 or 25 µl).

The slope of the curve is then used to calculate the enzymatic activity in [U/l] (µmol of ABTS oxidized par minute and per litre of sample), by the following equation:

$$Activity\,[\mu\text{mol min}^{-1}\,\text{l}^{-1}] = \frac{slope\,[\Delta\text{A min}^{-1}]\,10^6\,[\mu\text{mol mol}^{-1}]}{\varepsilon\,[\text{mol}^{-1}\,\text{l cm}^{-1}]\,\ell\,[\text{cm}]}\,\frac{V_{tot}\,[\mu\text{l}]}{V\,[\mu\text{l}]}$$

With :

- ε the molar absorptivity (also called molar extinction coefficient), expressed in $M^{-1}\cdot cm^{-1}$, who changes for each compound and wave-length, and is equal to **36,000 $M^{-1}cm^{-1}$** for ABTS at 420 nm
- ℓ the optical pathlength, which is **1 cm** with these cuvettes
- V the volume of sample added (in µl)
- V_{tot} the final volume in the cuvette (usually 1000 µl)

Preparation of ABTS solution 10 mM in pure H_2O

Solution 10 mM, 5 ml	Molar mass [g/mol]	Pure H_2O [ml]	ABTS [mg]
ABTS diammonium salt	548.68	5	27.434

III. Preparation of fungal culture media

Preparation of UAB defined medium

Prepare first a solution of micronutrient and macronutrient, as follow (do not autoclave) (adapted with minor changes from Blánquez et al. (2004), Borràs et al. (2008), and personal communication from the staff of the Universitat Autonoma de Barcelona (UAB)):

Micronutrient	Concentration [g/l]	Concentration [mM]	Molar mass [g/mol]
Nitrilotriacetic acid (NTA, chelating agent)($C_6H_9NO_6$)	1.5	7.85	191.14
$MnSO_4 \cdot H_2O$	0.5	2.96	169.02
NaCl	1	17.11	58.44
$FeSO_4 \cdot 7H_2O$	0.1	0.36	278.02
$CoSO_4 \cdot 7H_2O$	0.181	0.65	281.1
$ZnSO_4 \cdot 7H_2O$	0.1	0.35	287.55
$CuSO_4 \cdot 5H_2O$	0.01	0.04	249.69
$AlK(SO_4)_2 \cdot 12H_2O$	0.01	0.02	474.39
H_3BO_3	0.01	0.16	61.83
$NaMoO_4 \cdot 2H_2O$	0.012	0.05	218.98

Macronutrient	Concentration [g/l]	Concentration [mM]	Molar mass [g/mol]
KH_2PO_4	20	146.96	136.09
$MgSO_4 \cdot 7H_2O$	5.3	21.50	246.48
$CaCl_2 \cdot 2H_2O$	1.335	9.08	147.02

Then, in one litre of final solution, add the following ingredients:

Defined medium	Concentration [g/l]	Concentration [mM]	Molar mass [g/mol]
Glucose ($C_6H_{12}O_6 \cdot H_2O$)	8.80	44.40	198.17
$(NH_4)_2SO_4$	2.368	17.92	132.14
MES buffer ($C_6H_{13}NO_4S$)	1.952	10.00	195.23
Macronutrients	100 ml l^{-1}	Dilution	10
Micronutrients	10 ml l^{-1}		100
For pellets formation, add also: Thiamine (vitamin B1)	10 mg l^{-1}		300

The final UAB medium concentrations of selected compounds are presented below:

Final major elements concentration	Concentration [mg/l]	Concentration [mM]	Molar mass [g/mol]
Glucose	8000	44.40	180.16
N	502.1	35.84	14.01
P	455.1	14.70	30.97
Cl^-	70.4	1.99	35.45
SO_4^{2-}	1932.2	20.11	96.06

For the preparation of UAB medium for **micropollutant degradation experiments** with *T. versicolor*, proceed as follow:

Preparation of the defined medium (per litre of final medium)	
In a beaker, add:	**Volume [ml/l]**
- Macronutrients	100
- Micronutrients	10
- Glucose stock solution (444 mM, 88 g/l)	100
- Ammonium sulfate stock solution (179.2 mM, 23.68 g/l)	100
- MES (or MOPS buffer) 100 mM stock solution (19.52 g/l)	100
- Micropollutant stock solution (1 g/l in methanol) (final 10 mg/l)	10
- Demineralised water	380
Adjust the pH to the desired value with NaOH or HCl 1M	
Sterilize by filtration (0.2 µm) in sterile erlenmeyers	
Add, under sterile conditions:	
- *Trametes versicolor* pellets (not diluted): final goal 3-4 g dry weight /l	200

Preparation of malt extract medium (20 g/l)

For the preparation of white-rot fungi mycelium and pellets, a malt extract medium at 20 g/l is used, and prepared as presented below.

In 2 litres beaker, add:

- 1 l of demineralised water
- 20 g of malt extract
- Mix well (magnetic stirrer) and adjust the pH to 4.5 with HCl 1 M
- Split the medium in the erlenmeyers and autoclave

To prepare the **malt extract (20 g/l) – agar (15 g/l) Petri plates**, the same medium is used, as described below:

In 1 litre beaker, add:

- 0.5 l of demineralised water
- 10 g of malt extract
- 7.5 g of agar
- Mix well (magnetic stirrer) and adjust the pH to 4.5 with HCl 1 M
- Transfer the medium in a glass bottle and autoclave
- When the medium is still warm, transfer it to the petri dishes under sterile conditions (in the laminar flow). Let it cool down (solidify) and store it at 4°C.

IV. Subculture of white-rot fungi in malt extract-agar plate

White-rot fungi are maintained on malt extract – agar Petri plate, and subculture every 1-2 months. To inoculate a new Petri plate, proceed as follow:

- Prepare new malt extract (20 g/l) – agar (15 g/l), pH 4.5, Petri plates as described above.
- Cut a square (0.5 x 0.5 cm, with a sterile knife) in an old Petri plate containing mycelium of the white-rot fungus.
- Transfer the square of agar to the centre of a new malt extract – agar Petri plate, with the old mycelium facing the new agar medium.
- Incubate at 25°C during 7 days, until the mycelium covers the whole plate.
- Store the plate at 4°C during 1-2 months until the next sub-culture.

V. White-rot fungi mycelium preparation

Equipments and reactants

Scale, magnetic stirrer, autoclave, laminar flow, incubator at 25°C, rotating stirrer at 130 rpm, homogenizer (mixer), malt extract, NaCl, HCl (1 M), 7 days old white-rot fungi mycelium on agar plate.

Material to autoclave

- 5 erlenmeyers of 0.5 l with 200 ml of malt-extract medium (20 g/l, pH 4.5)
- A bottle with 0.5 l of malt-extract medium
- A knife or scalpel to cut the agar plate
- A tablespoon
- A big metallic sieve
- An empty beaker of 3 l
- A bottle with 1 l of demineralised water
- A bottle with 0.5 l of NaCl solution at 8 g/l
- An empty bottle of 0.5 l
- A mixer

Protocol

Prepare 1.5 l of malt-extract medium (pH 4.5) as described above. Transfer 0.5 l in a bottle and split the remaining litre in 5 erlenmeyers (of 0.5 l) (200 ml/erlenmeyer). Autoclave with the other material.

Once the medium has cooled down, in the laminar flow (under sterile conditions), inoculate each erlenmeyer with 6 squares (0.5 x 0.5 cm) of agar plate containing 7 days old (or older) mycelium of the white-rot fungus. Cut the squares of agar with the sterile knife.

Incubate the erlenmeyers at 25°C, 130 rpm, during 5 to 7 days.

If the white-rot fungus grows in very small pellets, add again after 5 to 7 days 100 ml of sterile malt-extract (20-40 g/l, pH 4.5) in each erlenmeyer and incubate them again for 2 to 4 more days until the size of the pellets increases (easier to collect with the sieve).

After 5 to 11 days, bring all the equipment in the laminar flow (sterile conditions), and:

- Put the metallic sieve on the sterile beaker
- Empty the erlenmeyers (2 to 3 in one time) in the sieve to collect the mycelium (pellets), and let it drain
- Rinse the mycelium with sterile water (use 1 l of water for 1 l of growth medium) and let it drain
- Collect the mycelium with the spoon and transfer it to a sterile 0.5 l bottle
- Add to the mycelium (around 250 ml) the same volume of NaCl solution (8 g/l)
- Homogenize the mycelium preparation with a sterile mixer (10’000 rpm) to break the pellets
- Store the mycelium preparation at 4°C until use as inoculums (storage time: a few months).

VI. White-rot fungi pellets preparation

Equipment

Scale, magnetic stirrer, autoclave, laminar flow, incubator at 25°C, rotating stirrer at 130 rpm

Reactants

Malt extract, NaCl, HCl (1 M), white-rot fungi mycelium preparation

Material to autoclave

- 5 erlenmeyers of 2 l with 1 l of malt-extract medium (20 g/l, pH 4.5)
- A tablespoon
- A big metallic sieve
- An empty beaker of 3 l
- 5 bottles with 1 l of demineralized water
- A bottle with 1 l of NaCl solution at 8 g/l
- An empty bottle of 1 l
- 10 ml tips cut at their end

Protocol

Prepare 5 l of malt-extract medium (pH 4.5) as described above. Split the 5 litres in 5 erlenmeyers of 2 l (1 l/erlenmeyer). Autoclave with the other material.

Once the medium has cooled down, in the laminar flow (under sterile conditions), inoculate (with sterile 10 ml tips cut at their end) each erlenmeyer with 6 ml of white-rot fungus mycelium preparation.

Incubate the erlenmeyers at 25°C, 130 rpm, during 5 to 7 days until pellets are well developed. If pellets are too small, add sterile concentrated malt-extract (40 g/l) and incubated again until the pellets are bigger.

After 5 to 7 days, bring all the equipment in the laminar flow (sterile conditions), and:

- Put the metallic sieve on the sterile beaker
- Empty the erlenmeyers (one at the time) in the sieve to collect the pellets, and let it drain
- Rinse the pellets with sterile water (use 1 l of water for 1 l of growth medium) and let it drain
- Collect the pellets with the spoon and transfer it to a sterile 1 l bottle
- Eventually, in the case of long storage, add to the mycelium the same volume of NaCl solution (8 g/l). But as chloride inhibit laccase activity, avoid this step if you can use the pellets directly for the experiment
- Store the pellets at 4°C until use in an experiment (storage time: a few weeks).

VII. Woodchips inoculation with white-rot fungi

Equipment

Scale, autoclave, incubator at 25°C

Reactants

Dry woodchips, white-rot fungi mycelium preparation

Material

2 l beaker, a big sieve, a spoon, 10 ml tips cut at their end, columns (biofilters)

Protocol

- In a 2 l beaker, add and weight 1 l (not compacted) of dry wood chips
- Clean well the wood chips with tap water to remove the dust: fill the beaker with water, mix well the wood in the water, remove the water by using a sieve to retain the wood, and repeat this as many times as necessary to have relatively clear water at the end
- Let the wood soak in the water during 30 min to 1 h to saturated it, and then remove the water with the help of a sieve
- Autoclave the wet wood in the beaker and at the same time autoclave a spoon and 10 ml tips cut at their end
- When the sterile wood is at ambient temperature, add 4% (v/v) (40 ml per litre of wood) of white-rot fungus mycelium preparation under sterile conditions (with a sterile 10 ml tip cut at the end to avoid the clogging of the tip by the mycelium)
- Mix the mycelium with the wood with the sterile spoon in the beaker
- Incubate the inoculated wood at 25°C during 5 to 7 days. Mix eventually after 3-4 days to aerate the support
- When the wood is completely colonized by the mycelium (all white), transfer the wood to (non-sterile) columns. Weight the amount of wood added and add the same amount in all the columns. Shake slightly the column to distribute well the wood supports and to avoid large voids. Avoid to compact too much the supports (fast clogging of the column and heterogeneous fungal growth at the surface only of the supports)
- Let the mycelium develop again for 2-4 days
- When the wood is completely covered by a white mycelium, but not too clogged, the column can be used to treat water.

VIII. Ergosterol extraction and analysis

Ergosterol extraction

Equipment

Sonicator with water bath at 45°C, water bath at 70°C, centrifuge, vortex, N_2 gas evaporator, coffee grinder

Material

Note: All solvents must be HPLC-grade.

- cyclohexane
- potassium hydroxide 10% (w/v) in methanol (23.5 g KOH pellets 85% in 200 ml of MeOH)
- methanol
- deionized water
- glass pipettes
- 10-ml glass centrifuge tubes w/screw caps
- 10-ml glass tubes for the N_2 gas evaporator
- autoanalyzer vials

Methods

Sample preparation

1. Take minimum 10 g of wet wood chips-mycelium sample. Homogenize well and eventually grind it with a coffee mil. Homogenize again and take 5 g to measure the fresh and dry weight (to allow estimation of the dry weight of the sample analyzed).
2. In a 10 ml centrifugation glass tube, add 0.5 to 1 g of ground sample and note the fresh weight.
3. In the case of storage, flush with nitrogen gas, close the tube and freeze directly at -18°C to store the sample (ergosterol is not stable in contact with oxygen and light).

Extraction

Note: Always work with cyclohexane in the fume hood. Retain all cyclohexane waste (including spent sample) for appropriate disposal.

1. Preheat water bath to 70°C and sonicator bath to 45°C.
2. In the centrifuge tubes containing the sample, add 1-ml cyclohexane and 3-ml 10% (w/v) potassium hydroxide (KOH, in methanol), and seal tubes with screw caps.
3. Vortex to dissolve.
4. Sonicate for 15-20 min at 45°C.
5. Place tubes in water bath at 70°C for 90-min. Do not close completely the lids to avoid too high pressure in the tubes. Add regularly 1 ml cyclohexane to compensate the evaporation (check every 5-10 min).
6. Remove from bath and add 1-ml deionized water and 2-ml cyclohexane to each tube.
7. Vortex for 30-sec. (Important to be thorough.)
8. Equilibrate the weight of the tubes (with water or cyclohexane) and centrifuge 5-min at 3500 rpm (2170 *g*) (ambient temperature).
9. Collect the top transparent (cyclohexane) phase with a glass pipette and transfer it to a clean glass tube (for evaporation). Do not collect the water phase!
10. Add again 2-ml cyclohexane to sample solution.
11. Vortex for 30-sec.
12. Equilibrate the weight of the tubes and centrifuge 5-min at 3500 rpm.
13. Collect the top phase and combine cyclohexane fractions from each sample in the tube for evaporation.
14. Repeat a third time the point 10 to 13.
15. Evaporate the cyclohexane phase collected completely under N_2 gas. Evaporator plate should be set at 40°C.
16. Dissolve the extract in 1-ml methanol. Seal the evaporator tube with Parafilm (to avoid too much evaporation) and vortex.
17. Heat in 40°C water bath for 15-min to help dissolve the ergosterol.
18. Vortex for 30-sec. Weigh first an ependorf tubes (1.5 ml) and then transfer all the content in it. Weigh the amount of sample added. This weight corresponds to the final volume of the extraction (methanol density at 25°C: 0.791 g/ml). Centrifuge 3 min at 6000 rpm to remove the suspended solids.
19. Transfer the supernatant (in the case of presence of solids, take care not to pipette the solid phase) in an amber HPLC vial. Eventually, in the case of still visible suspended solids in the supernatant, filter through a 0.2-µm PTFE filter (attached to a syringe) in the amber HPLC vial.
20. Store at 4°C in a dark box (ergosterol is light- and air-sensitive) before the analysis.

Ergosterol standards preparation

Ergosterol standards should be prepared in methanol (HPLC grade). A stock solution at 400 mg/l is initially prepared and is then used for preparing all the range of concentrations (dilution in pure methanol), from 0.4 mg/l to 400 mg/l.

Preparation of a stock solution at 400 mg/l in methanol:

1. Preheat water bath to 40°C.
2. Weigh 20 mg ergosterol standard into a 50-ml glass bottle (always close the bottle).
3. Add 50-ml methanol (check the weight, with methanol density of 0.791 at 25°C). Vortex.
4. Place in water bath at 40°C for 15-min. Vortex.
5. Place in water bath at 40°C for another 15-min.
6. Vortex until ergosterol dissolves completely. Repeat if necessary point 5.
7. Store at 4°C protected from the light.

HPLC separation and analysis

Ergosterol is analyzed by HPLC-DAD, at 282 nm, with a reverse phase C18 column, with isocratic 1 ml/min 88% methanol – 12% pure H_2O. Injection volume: 50 µl. Retention time: 10 min. Length of the analysis: 20 min.

IX. UPLC-MS/MS parameters

Table IX.1 UPLC conditions for the acidic and neutral methods used with the off-line SPE.

	Method acidic (pH 2.2)	**Method neutral (pH 7)**
Eluent A	94% H_2O, 2.5% MeOH, 1% formic acid, 2.5% (5mM) NH_4-formate 200 mM	95% H_2O, 5% acetonitrile
Eluent B	91.5% MeOH, 5% H_2O, 1% formic acid, 2.5% (5mM) NH_4-formate 200 mM	95% acetonitrile, 5% H_2O
Gradient	92% A isocratic for 0.5 min, linear gradient to 100% B in 19 min	86% A isocratic for 1.5 min, linear gradient to 99% B in 20.5 min
Column	HSS T3, 2.1 x 100 mm, 1.8 µm (Waters)	BEH C18 column, 2.1 x 100 mm, 1.7 µm (Waters)
Column Temperature	30 °C	30 °C
Flow rate	0.3 ml/min	0.3 ml/min

Table IX.2 UPLC conditions for the basic and neutral methods used with the on-line SPE.

	Method basic (pH 12)	**Method neutral (pH 7)**
Eluent A	94.8% H_2O, 5% acetonitrile, 0.2% NH_4OH	95% H_2O, 5% acetonitrile
Eluent B	94.8% acetonitrile, 5% H_2O, 0.2% NH_4OH	95% acetonitrile, 5% H_2O
Gradient	95% A, linear gradient to 95% B in 12 min	95% A, linear gradient to 95% B in 12 min
Column	BEH C18 column, 2.1 x 50 mm, 1.7 µm (Waters)	BEH C18 column, 2.1 x 50 mm, 1.7 µm (Waters)
Column Temperature	30 °C	30 °C
Flow rate	0.4 ml/min	0.4 ml/min

Table IX.3 Tandem mass spectrometry (MS/MS) parameters for the screening of 44 micropollutants

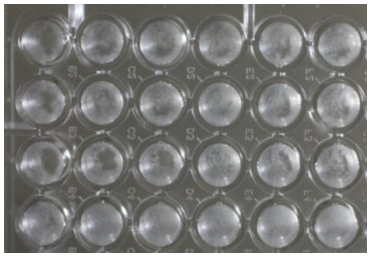
Substance	Parent ion	Daughter ion	Cone voltage	Collision energy	Surrogate to calculate recovery	Parent ion	Daughter ion	Cone voltage	Collision energy	UPLC-MS/MS method[a]	
	[m/z]	[m/z]	[V]	[eV]		[m/z]	[m/z]	[V]	[eV]	SPE off-line	SPE on-line
17α-Estradiol	271.2	145.1 / 143.1	-55	40 / 45	17b-Estradiol-d3	274.24	145.09	-64	40	N	B
17α-Ethinylestradiol	295	145.0 / 159.1	-60	35 / 25	Ethynylestradiol-d4	299.27	147.04	-55	44	N	B
17β-Estradiol	271.2	145.1 / 143.1	-55	40 / 45	17b-Estradiol-d3	274.24	145.09	-64	40	N	B
Atenolol	267.3	145 / 190	31	28 / 17	Atenolol-d7	274.42	145.18	36	36	A	B
Atrazine	216.1	96.1 /174	29	23 / 18	Atrazine-d5	221.2	179.1	34	18	A	N
Azithromycin	749.88	83.08 / 116.1	53	56 / 46	Azithromycin-d3	753.84	116.16	52	50	A	B
Benzotriazole	120.15	92.08/ 65.02	39	15 / 16	Benzotriazole-d4	124.16	68.76	40	20	A	N
Bezafibrate	360.1	154 / 274	-20	30 / 15	Bezafibrate-d4	364.1	278	-26	18	N	B
Bisphenol A	227.1	133 / 212	-31	25 / 17	Bisphenol A-d6	233.21	215.1	-29	18	N	B
Carbamazepine	237.3	165.15 / 179.07	33	36 / 35	Carbamazepine-d10	248.33	204.35	36	24	A	B
Ciprofloxacin	332.1	231.1 / 288.1	30	40 / 18	Ciprofloxacin-d8	340.42	235.19	40	36	A	B
Clarithromycin	748.5	158.1 / 590.3	32	33 / 20	Clarithromycin-d3	751.5	161	28	26	A	B
Diatrizoic acid	615.07	233.17 / 361.09	35	36 / 14	Diatrizoic acid-d6	620.74	239	32	38	A	N
Diclofenac	296	214.9 / 250	24	18 / 15	Diclofenac-d4	300.06	218.9	25	20	A	B
Diuron	233	46.3 / 72.1	30	14 / 30	Diuron-d6	239.2	78.04	25	20	A	N
Estriol	287.2	145 / 171	-53	39 / 37	Estriol-d3	290.19	147.2	-75	67	N	B
Estrone	269.2	143 / 145	-47	48 / 36	Estrone-d4	273.21	147.05	-56	38	N	B
Gabapentin	172.27	119.11 / 137.13	25	22 / 15	Gabapentin-d4	176.15	158.11	24	12	A	N
Gemfibrozil	249	121 / 127	-20	15 / 10	Gemfibrozil-d6	255.1	121 /133	-22	18 / 12	N	B
Ibuprofen	205	161	-20	10	Ibuprofen-d3	208.14	164.09	-16	8	N	B
Iohexol	822	804.1 / 822	33	22 / 5	Iohexol-d5	827	809	35	20	N	N
Iomeprol	778	405 / 778	35	40 / 5	Iomeprol-d3	781	408.1	36	45	N	N
Iopamidol	778	559 / 778	35	25 / 5	Iopamidol-d3	780.9	562	36	26	N	N
Iopromide	792	573 / 792	38	25 / 5	Iopromide-d3	795	795	38	5	A	N
Irgarol	254.34	83.08 / 198.14	32	29 / 18	Irgarol-d9	263.18	199.09	30	20	A	B
Isoproturon	207	47 / 72	25	16 / 22	Isoproturon-d6	213.35	78.08	29	18	A	B
Ketoprofen	255.07	104.89	22	22	Ketoprofen-d3	258.29	105.06 / 212.22	30	24 / 15	N	N
Mecoprop	213.05	70.9 / 140.9	-16	12 / 18	Mecoprop-d3	216.07	144	-22	12	N	B
Mefenamic acid	240.15	192 / 196	-32	25 / 18	Mefenamic acid-d3	243.15	195 / 199	-32	25 / 18	N	B
Metformin	130.1	60 / 71	22	12 / 16	Metformin-d6	136.17	60	22	12	A	-
Methylbenzotriazole	134.18	79.06 / 106.12	35	17 / 12	Methylbenzotriazole -d6	140.05	81.06	36	22	A	N
Metoprolol	268.2	116 / 133	30	18 / 24	Metoprolol-d7	275.26	123.06	34	18	A	B
Metronidazole	172	82 / 128	22	21 / 15	Metronidazole-d4	176.05	127.95	24	14	N	N
Naproxen	229	170 / 185	-15	20 / 5	Naproxen-d3	231.9	173	-14	16	N	B
Norfloxacin	320.1	233 / 276.1	30	25 / 18	Norfloxacin-d5	325.1	281.1	27	18	A	B
Ofloxacin	362.1	261.1 / 318.1	30	26 / 20	Ofloxacin-d8	370.41	265.31	36	28	A	B
Paracetamol	152	93 / 110	20	25 / 15	Paracetamol-d3	155.16	93.03	28	22	A	N
Primidone	219.28	91.08 / 162.16	20	20 / 11	Primidone-d5	224.29	167.6	21	12	A	N
Simvastatin	419.54	199.25 / 285.31	24	17 / 12	Simvastatin-d6	425.24	199.09	22	12	A	N
Sotalol	273.33	133.2 / 213.16	20	26 / 18	Sotalol-d6	279.1	261.1	20	12	A	B
Sulfamethoxazole	254	92 / 156	27	26 / 16	Sulfamethoxazole-d4	258.2	96.09	26	28	A	N
Terbutryn	242.1	91 / 186.1	28	28 / 19	Terbutryn-d5	247.1	191.1	30	18	A	B
Triclosan	287	34.8	-23	12	Triclosan-d3	292	34.8	22	11	N	B
Trimethoprim	291	123 / 230	39	27 / 24	Trimethoprim-d3	294.23	230.15	40	24	A	B

[a] Method - A: acidic, N: neutral, B: basic

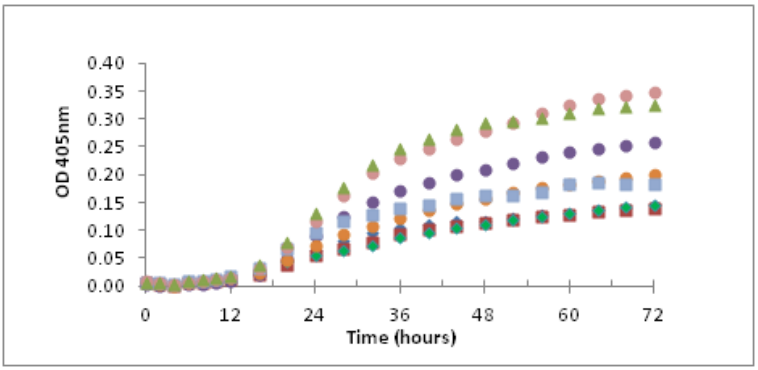
X. Protocol for filamentous fungi growth kinetics using a high-through-put 96 well assay

Description

The method, developed by Stephen Mackay (LBE, EPFL, 01.07.2013), determines kinetic growth, rapidly and cost effectively running 8-12 variables simultaneously and comparatively. Carbon sources, pollutants, inhibitors, pH and defined media conditions can be rapidly assessed. The experiment uses a quantified spore inoculum added to a growth medium. Cultures are grown in 96 well plates over several hours to obtain a sigmoidal growth curve read at 405 nm. The filamentous fungi grow as a mat over the base of the plate which can be measured at OD 450. Data can be analysed directly as OD vs. time or indirectly as relative growth. OD Max can be compared directly, or alternatively, the gradient of the curve can be calculated. OD can be measured up to 1.0 but will level off before that depending on carbon concentrations. 96 well plates are sealed using a clear airtight sealing film with low background (OD 405nm = 0.05). The temperatures can be set, however due to internal temperatures; 28°C is the lowest constant temperature. Condensation negatively affects readings, therefore holes need to be pierced in the film (6-8 for each well) to allow aerobic growth and reduce condensation. This however leads to evaporation; therefore experiments need to be run over short periods (48-96 hours for minimal evaporation). Antibiotics are important as the microtiter plates, sealing film and spectrophotometer are not sterile.



OD =0.35



Materials

- Spore inoculum
- 0.1% Tween 80 in ddH_2O, autoclaved aliquots in 10ml test tubes
- growth media with antibiotics filtered (Camp, Kan and Amp).
- Biotek filter spectrophotometer
- Neubauer cell counter + 0.1% Nile blue (filtered and aliquoted)
- 96 well microtiter plate (sterile or non-sterile)
- Clear transparent adhesive qPCR sealing film for the 96 well plates

Method

Preparation of the media

1. Autoclave 0.1% tween 80 in ddH_2O in >10ml test tubes
2. Prepare aliquots of growth media sterilized by autoclaving or filtration. Add, if necessary, respective antibiotics (chloramphenicol 34µg/ml 1:100 (prepared in EtOH); Kanamycin 50µg/ml 1:100 (ddH_2O); Ampicillin 100µg/ml (ddH_2O). Prepare 25-50 ml media and filter aliquots into sterile 2ml tubes. Media should not be too rich. Preferably, use defined minimal media when comparing substrates (i.e. 2mM final concentration of carbon source). Buffers can be included such as 50-100 mM MOPS or MES to the maintain pH. Minimal media allows for a stationary phase to plateau. For rich medium, compare the gradients.

Preparation of the spore culture and plate

a) Inoculate starter cultures for spore inoculum.
b) Grow pellets in rich culture medium. After 3-5 days, remove spores suspended in the liquid medium (~20ml). Centrifuge spores at high speed (9000 *g*) for 15 min. Carefully remove the liquid supernatant. There should be no visible pellet (or very small). If there is, it is due to smaller pellet mass (if homogenized well, it does not affect the assay).
c) Resuspend the spores in 0.1% Tween 80 (10ul/10ml). Count cells using the haemocytometer. Use between 2 x 10^4 (2 spores per 25 squares) and 2 x 10^5 (20 spores per 25 squares). Stain spores for 5 min with 1:10 of 0.1% Nile blue stain (non-toxic) at room temperature (stain 500µl-1ml for consistent counts; count 6-10 times to determine average). Add 10 ul on the edge of the plate (diffuse per capillarity). Visualize on the microscope at 400 X. Spores are small but distinguishable from media precipitates. Dilute accordingly.
d) Add 100 µl of growth media (at the concentration or with the compounds to test) per well in the 96 well plate. Preferably run 7 replicates per variable and use a spore-free control per medium variant (0.1% Tween 80) to monitor bacterial contamination.
e) Add 100 µl of spore inoculum to each well (inoculate across the different media or at random to reduce uneven spore concentrations due to settling). Vortex intermittently.
f) Seal the plate. Condensation will appear on the film. Pierce each well with a sterile needle 6-8 times around the edges of each individual well. Rub the surface of the sealing film, or use the underside of a pre-warmed heating block to remove condensation. Condensation is difficult to remove, requiring ~30 min for the temperature of the medium to adjust. Mark the level of the medium in the side of the wells to monitor evaporation.

Setup and reading the spectrophotometer

a) Open the Gen 5 program, create a new protocol. Set the incubation temperature minimum of 28°C for a constant temperature. Set the OD at 405nm. Read every 20 min for 48-96 hours. (NB. Do not set shaking). Validate the protocol.
b) 30 min after inoculation (to standardize the time it takes to remove condensation and setup the spec), place the micro-titre plate in the pre-warmed spectrophotometer and start reading.
c) The machine will read-every 20 min for several days. Monitor the curves real time on the program. Do not open the machine as this could result in condensation and changes in environmental oxygen/carbon dioxide which cause spikes.

Note: It is best to use 7 or more replicates, due to the nature of non-uniform growth of the filamentous fungi as outliers do occur regularly. (Use at least 6 of the 7 readings).

Curriculum Vitae

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Education

06.2010 – 01.2015 **PhD in Environmental Science**. *Ecole Polytechnique Fédérale de Lausanne (EPFL), Switzerland. Academic visit (2 months) at the Universitat Autònoma de Barcelona (UAB), Spain*

Dissertation: "Micropollutant removal from municipal wastewater – From conventional treatments to advanced biological processes"

10.2003 – 08.2008 **Master in Environmental Science and Engineering**. Specialization in environmental chemistry and bioprocess. *EPFL, Switzerland*

Master thesis: "Impacts of urban drainage on receiving waters"

08.1999 – 07.2002 **Baccalaureate in "physics and maths".** *Gymnase de Burier, Vaud, Switzerland*

Work experience

06.2010 – 01.2015 **Researcher and teaching assistant**. *EPFL, Ecological Engineering Laboratory (ECOL) and Laboratory for Environmental Biotechnology (LBE), Switzerland*

- Developing solutions for the treatment of micropollutants in municipal wastewater (biological systems, ozonation and activated carbon adsorption)
- Participation to several international scientific meetings
- Writing reports and scientific publications
- Teaching assistant for "Wastewater treatment", "Urban hydrology" and "Soil sciences" courses at EPFL

01.2010 – 05.2010 **Collaborator (trainee) at the Natural regional park Gruyère Pays-d'Enhaut**. *Château-d'Oex, Switzerland*

- Geographic information system (GIS) manager. Elaboration of maps of the park, participation to several ecological projects.

09.2008 – 05.2009 **Scientific collaborator (civil service) at EAWAG (Swiss institute for aquatic research).** *Dübendorf, Switzerland*

- Research project about "Decentralized membrane based drinking water systems for developing countries"

09.2007 – 02.2008 (employed at 20%) **Consultant at SETEMIP-Environnement** (now Cycleco). *Ambérieu-en-Bugey, France*

- Training companies in "life cycle assessment"

2006 – 2007 **Teaching assistant**. *EPFL, Switzerland*

(employed at 10%)

- Courses : "Environmental process design and operation" and "Life cycle assessment"

Extracurricular activities

09.2004 – 07.2009 Active member of the student association Unipoly for sustainable development. *EPFL, Lausanne, Switzerland*

- Development of projects to raise awareness to environmental issues. Member of the executive committee (2005-2007)

Languages

French	Native tongue
English	Good oral and writing skills
German	Basic oral and writing levels

Computing

Detailed knowledge	Word, Excel, Power Point, Matlab, Mathcad, SimaPro, REBEKAII
Basic knowledge	MicroStation, Aquasim, Adobe Illustrator, Adobe Photoshop, Idrisis, Manifold, MySQL, Routing System, Mapinfo

Laboratory skills

Solid phase extraction (SPE), high performance liquid chromatography (HPLC and UPLC), tandem mass spectrometry (MS/MS), UV/Visible (DAD) and refractive index (RI) HPLC detection, spectrophotometry, ion chromatography (IC), filed sampling of wastewater, standard physico-chemical analyses of wastewater, basic ecotox methods (algae, daphnia and microtox tests), basic microbiological methods (solid and liquid cultures, microscopy, protein and ergosterol quantification), bio-reactor design and operation.

Diverse

07.2009 – 12.2009 **Bicycle trip** from Switzerland to Ethiopia (13,200km) during 6 months with fundraising for a development project (water and sanitation) in Ethiopia (www.cycling-together.org)

07.2002-12.2002 **Bicycle trip** from Alaska to Mexico (12,000 km) during 5 months

Interests

- Hiking, cycling, skiing, outdoor activity, travelling, nature and environment

List of scientific publications

- **Margot J**, Bennati-Granier C, Maillard J, Blánquez P, Barry DA, Holliger C. *Bacterial versus fungal laccase : Potential for micropollutant degradation*. AMB Express 2013; 3: 1-14.
- **Margot J**, Maillard J, Rossi L, Barry DA, Holliger C. *Influence of treatment conditions on the oxidation of micropollutants by Trametes versicolor laccase*. New Biotechnology 2013; 30: 803-813.
- **Margot J**, Kienle C, Magnet A, Weil M, Rossi L, de Alencastro LF, et al. *Treatment of micropollutants in municipal wastewater: Ozone or powdered activated carbon?* Science of the Total Environment 2013; 461–462: 480-498.
- Rossi L, Queloz P, Brovelli A, **Margot J**, Barry DA. *Enhancement of Micropollutant Degradation at the Outlet of Small Wastewater Treatment Plants.* PLoS ONE 2013; 8.
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- Chèvre N, Coutu S, **Margot J**, Wynn HK, Bader H-P, Scheidegger R, et al. *Substance flow analysis as a tool for mitigating the impact of pharmaceuticals on the aquatic system*. Water Research 2013; 47: 2995-3005.
- Peter-Varbanets M, **Margot J**, Traber J, Pronk W. *Mechanisms of membrane fouling during ultra-low pressure ultrafiltration.* Journal of Membrane Science 2011; 377: 42-53.

Reports and engineering publications

- **Margot J**, Magnet A, Thonney D, Chèvre N, de Alencastro F, Rossi L. *Traitement des micropolluants dans les eaux usées - Rapport final sur les essais pilotes à la STEP de Vidy (Lausanne).* Ville de Lausanne, 2011.
- **Margot J**, Magnet A. *Elimination des micropolluants dans les eaux usées - Essais pilotes à la station d'épuration de Lausanne*. gwa 2011; 7: 487-493.
- Magnet A, **Margot J**, Corbaz M, Bonvin E. *Traitement des micropolluants à la STEP - Séparation du charbon actif en poudre par ultrafiltration en mode frontal*. Aqua & Gas 2014; 1: 24-29.

Conferences – Scientific presentations

- **24th SETAC European Meeting.** Basel, Switzerland. May 11-15, 2014. *Treatment of micropollutants in municipal wastewater using white-rot fungi*. Margot J, Vargas M, Contijoch A, Barry DA, Holliger C. Oral presentation.
- **Global Young Scientists Summit (GYSS@one-north) 2014.** Singapore. January 19-24, 2014. Theme: *Advancing Science, Creating Technologies for a Better World*. Selected with four other scientists to represent EPFL. Workshop participation.
- **International Conference on Chemistry in the Environment (ICCE)**, Barcelona, Spain. June 25-28, 2013. *Laccases: Potential treatment for removal of micropollutants in municipal wastewater?* Margot J, Bennati-Granier C, Maillard J, Barry DA, Holliger C. Oral presentation.
- **Environmental Microbiology and Biotechnology (EMB)**, Bologna, Italy. April 10-12, 2012. *Determination of optimal conditions for the degradation of micropollutants by laccase from Trametes versicolor.* Margot J, Maillard J, Rossi L, Barry DA, Holliger C. Oral presentation.

- **21st SETAC European Meeting.** Milan, Italy, May 15-19, 2011. *Elimination of micropollutants in wastewater treatment plants: Ozonation or activated carbon? Conclusions of a one-year pilot project.* Margot J, Magnet A, Thonney D, Chèvre N, de Alencastro F, Kienle C, Abegglen C, Barry DA, Rossi L. Poster presentation.
- **21st SETAC European Meeting.** Milan, Italy, May 15-19, 2011. *Evaluation of bioassays and wastewater quality: In vitro and in vivo bioassays for the performance review in the Project "Strategy MicroPoll".* Kienle C, Kase R, Abegglen C, Margot J, Magnet A, Thonney D, Werner I, Schärer M. Oral presentation.
- **21st SETAC European Meeting.** Milan, Italy, May 15-19, 2011. *Seasonal variations of antibiotics measured in the environment from sales data analysis.* Coutu S, Margot J, Chèvre N, Rossi L. Poster presentation.
- **7th Swiss Geoscience Meeting**. Neuchâtel, Switzerland, November 20-21, 2009. *Water temperature increase in receiving waters due to the increase of imperviousness: a multidisciplinary assessment approach.* Rossi L, Margot J, Hari ER. Oral presentation.

Other oral presentations as invited lecturer

- **Technical day of the Groupement romand des exploitants de stations d'épuration des eaux (GRESE)**. Yverdon, June 6, 2013. Lecture : *Micropolluants et impact de la nitrification*
- **Summer school HES SO Valais**. Sion, August 26, 2013. Lecture : *Micropolluants: évaluation du risque et traitement dans les eaux usées*
- **Course Master level, University of Lausanne**. October 30, 2013. Lecture : *Traitement des micropolluants dans les eaux usées*
- **5 à 7, Holinger AG**. Lausanne, March 27, 2014. Lecture: *Traitement des micropolluants dans les eaux usées : point de vue scientifique*

Awards

- Selected with four other scientists to represent EPFL at the Global Young Scientists Summit (GYSS@one-north) 2014. Singapore, January 2014
- Co-winner of the EDCE PhD Mobility Award to "encourage good PhD students to go for an academic visit to an external research institution", December 2011
- Prize "CSD pour l'environnement" for "excellent master project whose quality stands out for its solutions that increase quality of life and environment", October 2008
- Prize "Société suisse de mensurations et améliorations foncières" for "the best SIE average mark for the Master cycle including the Master Project", October 2008

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