Sulfamethoxazole and isoproturon degradation and detoxification by a laccase-mediator system: Influence of treatment conditions and mechanistic aspects

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ABSTRACT

The potential of laccase-mediator systems (LMS) for the removal and detoxification of two wastewater micropollutants, the antibiotic sulfamethoxazole (SMX) and the herbicide isoproturon (IPN), was assessed. The influence of various parameters on micropollutant oxidation rates, such as pH, mediator, enzyme and pollutant concentrations, 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 presence of laccase and ABTS, as well as, for SMX, in presence of AS or SA. The three mediators were consumed during the reaction (no catalytic reactions observed), at a ratio mediator/pollutant between 1.1 and 16 mol/mol. Faster oxidation kinetics were observed at lower pH values, but also higher mediator/pollutant ratios were required. Several transformation products were formed, including cross-coupled products. Product mixtures were always less toxic to algae than untreated pollutants. Finally, a kinetic model that could explain the experimental observations was established. Based on the findings in this study LMS appears to be a promising option to treat concentrated and potentially toxic industrial effluents.

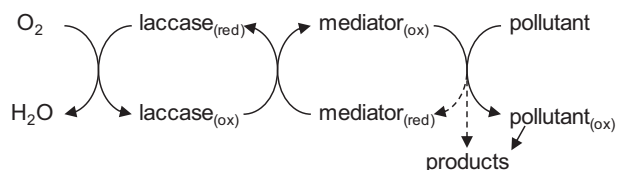© 2015 Elsevier B.V. All rights reserved.

1. Introduction

The presence of polar organic micropollutants such as pharmaceuticals and pesticides in wastewater, their poor removal in municipal wastewater treatment plants (WWTPs), and their potential impact on aquatic organisms motivate development of new treatment processes able to deal with these substances [1–4]. Bio-oxidation of these pollutants, catalyzed by oxidative enzymes such as laccase, is a potentially attractive treatment option [5]. Laccases, multi-copper oxidases produced by many fungi and bacteria, can oxidize a wide range of pollutants containing phenol and aniline moieties, including several pharmaceuticals and pesticides, requiring only oxygen as a co-substrate [6].

Many pollutants are recalcitrant to laccase oxidation. One way to increase their range of action is to use redox mediators [7]. Mediators are organic compounds that can be oxidized by laccase to free radicals. As they are less specific, these radicals can oxidize other pollutants and thus increase 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 [8]. 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 [9]. Nevertheless, mediators or enhancers notably widen the substrate range of laccases. Despite several studies on laccase-mediator systems (LMS) for micropollutant removal in wastewater [10–14], 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.

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Scheme 1. Ideal laccase-mediator reaction model.

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 [15]. One of the most commonly used synthetic mediators is 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) [9]. This compound is oxidized by laccases to a stable radical cation ABTS$^{\bullet+}$ (Fig. S1, Supporting information (SI)) [8]. Natural mediators have also been identified, mostly lignin-derived phenolic compounds, the most effective of which are syringaldehyde (SA) and acetosyringone (AS) [15]. SA and AS have similar structures and are oxidized by laccase to an unstable phenoxy radical (Figs. S1, SI) [16].

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) (Figs. S1, SI). 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) [17] and may generate risks for sensitive aquatic organisms in the receiving waters [18]. IPN is a herbicide commonly used in urban areas (parks, gardens, cemeteries) and therefore also frequently detected in municipal WWTP effluents [17]. Due to its toxicity at very low concentrations, it was selected as a priority substance by the European Union [19].

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.

2. Materials and methods

2.1. Chemicals and enzyme

SMX and IPN (purity > 99%), laccase powder from Trametes versicolor (ref. 38.429, 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 (around 5%, Sigma) 100 times. The final OCl$^-$ concentration was measured at pH 10.9 by spectrophotometry at 292 nm (extinction coefficient $\epsilon_{292\,nm}$ of 362 M^{-1} cm^{-1}) [20].

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), in acidic conditions (pH < 5), at a slightly under-stoichiometric ratio to avoid potential residual chlorine in the solution [21]. 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 ($\epsilon_{420\,nm}$ of 36,000 M^{-1} cm^{-1}) [22].

2.2. Laccase activity test

The laccase activity was determined using a colorimetric assay by measuring the oxidation of 0.5 mM ABTS in an oxygen-saturated acetate buffer (0.1 M) at pH 4.5 and 25 °C as described by Margot et al. [23]. A sample aliquot containing laccase 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.

2.3. 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$_2$O 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} (around 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.

2.4. Micropollutant oxidation assay in laccase-mediator systems under various conditions

Micropollutant oxidation assays were performed at different pH values (3–9) 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 40–160 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 (from 100 to 650 U l^{-1}), and various pollutants concentrations (from 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 (around 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. The cell was closed just after addition of laccase in an air–oxygen saturated solution, without any headspace.

2.5. 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.

2.6. 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 [24,25].

The algae growth inhibition test was performed according to the OECD guideline 201 [26] 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. [27]. Briefly, exponentially growing algae (initially around 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. [28] by measuring the optical density at a wavelength of 690 nm (linearly correlated to the cell density) with a microplate reader (ELx800^{TM}, 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.

2.7. 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 13, SI). The various differential equations were solved numerically with the ode45 solver (variable step Runge-Kutta method) within MATLAB (MathWorks, USA).

3. Results and discussion

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. S2, SI). Recalcitrance of SMX to laccase oxidation was recently reported [29], although this compound was oxidized by crude *Phanerochaete chrysosporium* laccase extract in another study [30], possibly due to the presence of other oxidative enzymes or mediators in the extracted solution.

With LMS, IPN was completely oxidized in the presence of ABTS in less than 20 h at pH 4–6 (Fig. 1 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. S2 A, SI).

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 1 h) in the presence of both AS or SA at pH 6 (Fig. 1B–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) [29,31,32]. Oxidation by LMS of other sulfonamides (sulfadimethoxine and sulfamonomethoxine) was also reported [33]. The mechanisms and the conditions for the oxidation of these pollutants by LMS are, however, still largely unknown.

3.2. pH influence on the oxidation kinetics

As shown in previous studies [23,34], pH has a strong influence on the laccase activity, with higher activities (for *T. versicolor*
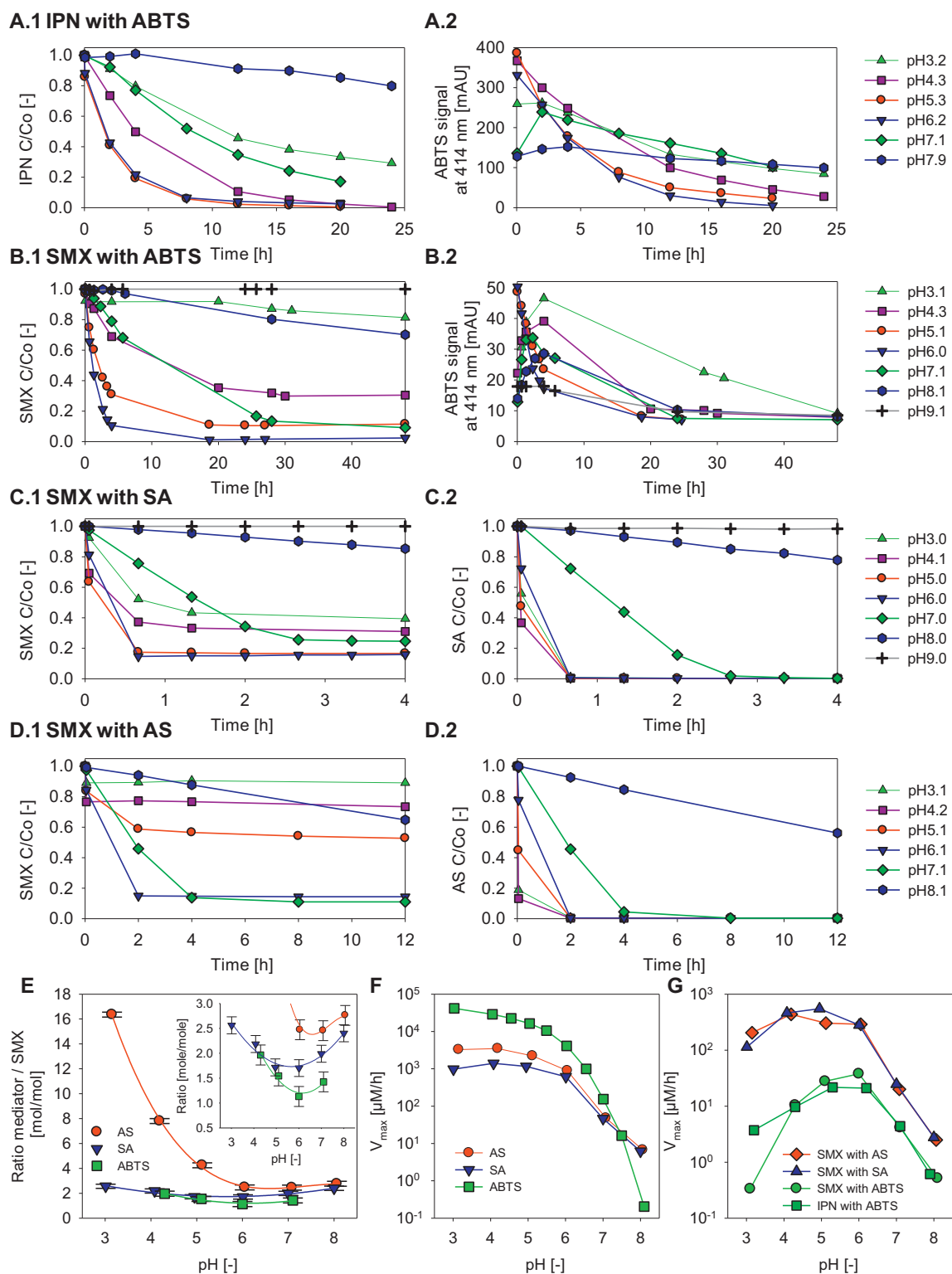
Fig. 1. pH effect on laccase-mediated reactions. (A) Isoproturon (IPN, A.1) in presence of ABTS (A.2: oxidized ABTS HPLC signal). (B) Sulfamethoxazole (SMX, B.1) in presence of ABTS (B.2). (C) SMX (C.1) in presence of syringaldehyde (SA, C.2). (D) SMX (D.1) in presence of acetosyringone (AS, D.2). (E) 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. (F) Initial maximum oxidation rates (V_{max}, in μM/h) of the mediators AS (●), SA (▼) and ABTS (■) as a function of the pH. (G) Initial maximum oxidation rates 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: (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. (F) 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. (G) 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).

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 from 3 to 9. As presented in Fig. 1, 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. 1G or Fig. S3, SI), significantly higher than the optimal pH for mediator oxidation (pH < 3–4) (Fig. 1F). This difference could be related to a higher self-reaction of mediator radicals at lower pH (produced locally at high concentrations), leading to a decreasing pollutant exposure with reactive species (cf. Section 3.7.5).

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) [8]. However, it appears from our results that neither ABTS, SA nor 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 (Figs. 1 C.2 and D.2). This consumption was independent of the pollutant concentration (similar decay rates were observed with or without the presence of SMX, Fig. S5, SI), suggesting that the pollutant was not involved in the disappearance of the mediators. The removal of SA and AS was thus probably caused by an irreversible oxidation by laccase. 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 [35], no catalytic reactions were observed in this study.

The disappearances of the mediators AS and SA (Figs. 1 C.2 and D.2) were proportional to the disappearance of SMX (linear correlation $r > 0.99$) (Fig. S4, SI). 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. 1E).

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 (Figs. 1 A.2 and B.2) was closely linked to the removal of SMX and IPN (Figs. 1 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 (around 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. 1E), 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. 2B). IPN required thus 1.5 times more mediator than SMX for a similar extent of oxidation.

The increase in the mediator/pollutant ratio observed at low pH, most pronounced for AS, was probably related to the very fast oxidation of the mediators at low pH, leading to the rapid production of a high level of reactive mediator species. These reactive species reacted then probably more easily with each other than with the pollutants (present in lower concentrations), increasing therefore the mediator/pollutant ratio required at low pH (see Section 3.9 for further discussion).

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.

3.4.1. Effect of the enzyme concentration

It was observed that, at pH 5 (Fig. S6 A, SI), 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 to 100 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.

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 IPN and SMX (Figs. 2 A and B). As shown in Fig. 2C, 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. 2C and Fig. S6 B, SI). 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.

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 (Figs. 2 D–F) was observed. 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 (Fig. S 5, SI). As laccase is several orders of magnitude less active in neutral-alkaline than in acidic conditions (Fig. 1F), this observation points towards a 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 of the initial pollutant concentration (similar to first-order kinetics) (Fig. S 6 B, SI). Although this was not tested with low mediator/pollutant concentrations, it is expected that this independence 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).
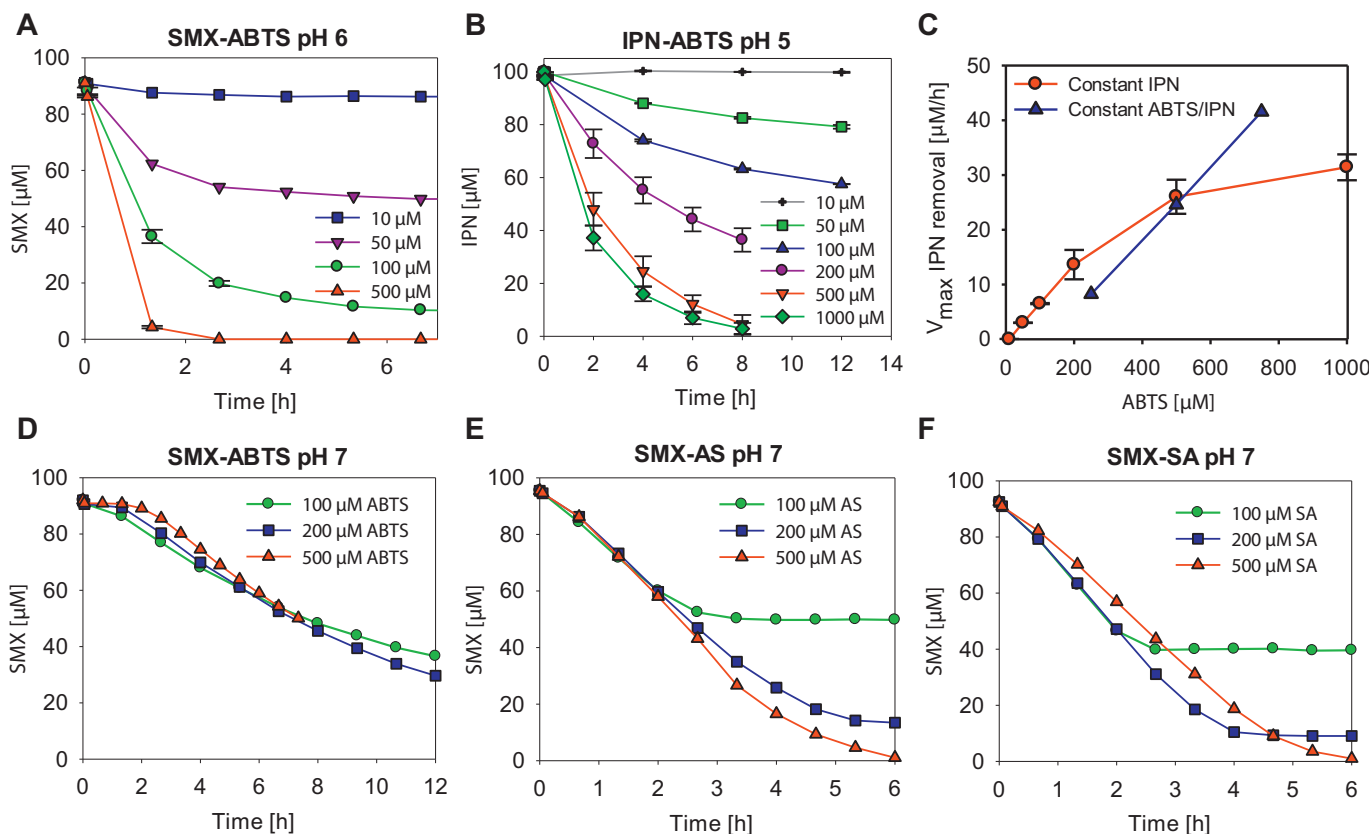
Fig. 2. Influence of mediator concentrations on sulfamethoxazole (SMX) and isoproturon (IPN) oxidation in presence of laccase. (A) Residual SMX concentrations at pH 6 with various concentrations of ABTS (450 U l^{-1} laccase). (B) Residual IPN concentrations at pH 5 with various concentrations of ABTS (560 U l^{-1} laccase). (C) Initial maximum IPN oxidation rates (V_{max}, 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). (D-F) Residual SMX concentrations at pH 7 with various concentrations of (D) ABTS (450 U l^{-1} laccase), (E) acetosyringone (AS) (520 U l^{-1} laccase), and (F) syringaldehyde (SA) (455 U l^{-1} laccase). Error bars: range of values of duplicates.

3.5. Oxygen consumption during laccase-mediated reactions

In the laccase-mediated reaction systems, around 0.25 mole of oxygen (one mole of electrons transferred) was consumed per mole of mediator (ABTS, AS and SA) oxidized during the initial phase of the reaction (when the mediator was still present at high concentration). Oxygen consumption stopped once SA was oxidized while, in presence of AS and ABTS, almost complete oxygen depletion was observed (Fig. S7, SI). This further oxygen consumption suggests that additional oxidation of the transformation products of AS and ABTS occurred slowly. In all cases, complete oxygen depletion was observed only after complete pollutant oxidation, suggesting no oxygen limitation in the reaction.

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 their oxidation by laccase (Fig. S1, SI) 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$^{•+}$ can oxidize several compounds, mainly polyphenols, phenols or anilines [35,36]. To elucidate the fate of the selected target compounds, we investigated if ABTS$^{•+}$ alone (oxidized chemically) could also oxidize SMX and IPN. As presented in Figs. S8 C and S9 C (SI), no SMX or IPN removal was observed with ABTS$^{•+}$ 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$^{•+}$ (Figs. S8 A and S9 A, SI). This demonstrates that ABTS$^{•+}$ is not directly responsible for SMX or IPN oxidation and that laccase is necessary to catalyze this reaction.

To investigate if the reactive product responsible for pollutant degradation was formed during ABTS or ABTS$^{•+}$ oxidation by laccase, ABTS was chemically oxidized by chlorine to form a solution containing only ABTS$^{•+}$, 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$^{•+}$ or laccase with ABTS showed almost identical results (Fig. S10, SI), suggesting that the reactive species were formed from the reaction of ABTS$^{•+}$ with laccase. Moreover, ABTS$^{•+}$, which is quite stable in pure solution (half-life of 47 h at 20–23 °C) [21], was degraded in presence of the pollutants and laccase, at a rate strongly correlated with the removal rates of the micropollutants (Figs. 1 A.2 and B.2 and Fig. S8 B, SI). This suggests that laccase reacts with ABTS$^{•+}$ producing reactive species, which in turn react with the pollutants.

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 3.7). Although the direct oxidation of ABTS$^{•+}$ (reduction potential $E^0 = 0.6$ V) to the stronger oxidant ABTS^{2+} ($E^0 = 1.1$ V) by laccase (E^0 around 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) [37]. ABTS^{2+} is reported to oxidize several compounds such
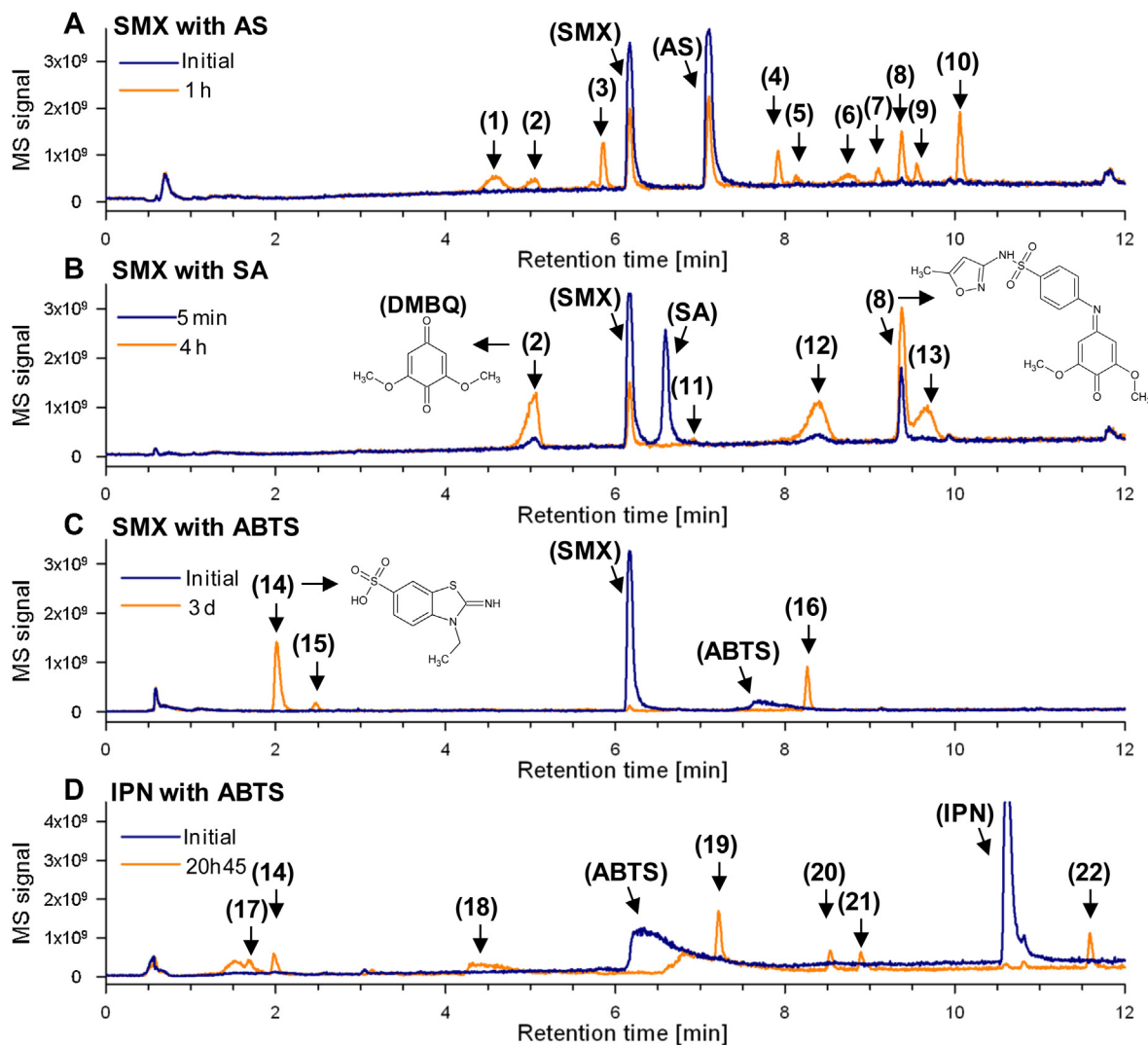
Fig. 3. UPLC-MS chromatograms before and during laccase-mediated reactions. (A) sulfamethoxazole (SMX) in presence of acetosyringone (AS), (B) SMX in presence of syringaldehyde (SA), (C) SMX in presence of ABTS, and (D) isoproturon (IPN) in presence of ABTS. Numbers in bracket: ID of the main transformation 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. S11 C, SI.

as aromatic alcohols that cannot be oxidized by ABTS[•+] [38,39]. 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 [37,39]. 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[•+] within a few minutes. These results indicate that in the present case, the real mediator is not ABTS but its radical cation ABTS[•+], which is oxidized by laccase to a reactive species with higher reduction potential (possibly ABTS[2+]) which, in turn, reacts with IPN or SMX.

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. 3, 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.

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 1–10, Fig. 3A). Six of them had a molar mass higher than SMX or AS suggesting that they were coupling products (Table 1). Several products (1–3 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 S1, SI), was identified as 2,6-dimethoxy-1,4-benzoquinone (DMBQ) (Fig. 3B), as observed in other studies [33,40]. 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. S11, SI. Similar dimeric products were observed by Ibrahim et al. [40]. 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 absorption spectrum maxima at 200, 314 and 405 nm, was identified based on similar studies [29,33] as a coupling product between DMBQ and SMX (Fig. 3B). The masses of products 5, 7, 9 and 10 (Table 1) suggest that they were also coupling products between AS radicals and SMX (structure suggestion in Fig. S11, SI), as also proposed by Shi et al. [29]. All these transformation products appeared rapidly during the first hour of reaction, linked

Table 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. 3. Structures of compounds with similar masses are suggested for some transformation products and presented in Fig. S11, SI.

ID	Retention time [min]	Molar mass [g/mol][a]	m/z of adducts[b]	Types of product[c]	Structure propositon[d]
Parents compounds					
(AS)	7.11	196	197 + 219	Acetosyringone	(AS)
(SA)	6.6	182	183 + 205	Syringaldehyde	(SA)
(ABTS)	6.44–7.69	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 ABT + 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)
(18)	1.99	258	259 + 281	ABTS TP	(14)
(19)	4.72	546	547 + 569	ABTS TP	–
(20)	7.23	222	223 + 245	*Hydroxy-isoproturon*	(IX)
(21)	8.56	445	446 + 468	*Coupling IPN + fragments ABTS*	–
(22)	8.9	447	448 + 470	*Coupling IPN + fragments ABTS*	–
(23)	11.61	232	233 + 255	–	–

[a] Molar mass M deduced from the m/z of the adducts.

[b] m/z of ESI MS products with positive ion mode: M + H: [M + 1]; M + Na: [M + 23]; 2M + Na: [2M + 23]; M + CH$_3$OH: [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 Supporting information, Fig. S 11, based on the mass and the relation log Kow/retention time of the proposed molecule. (-): no suggestion.

to the degradation of SMX and AS (Fig. S12, SI). 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. S12, SI) suggesting that these products were either not stable or further oxidized by laccase, which is consistent with the additional oxygen consumption observed (cf. Section 3.5). After 72 h, the two main (in signal intensity) products still present in solution were 2 and 8 (Fig. S12, SI).

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 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 [40]. These two dominant products were much more abundant with SA than with AS, with a 3.5–3.8 times higher signal intensity (Figs. 3A 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. S11, SI). 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. S12, SI).

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. 3C, Table 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. 3C) was elucidated in other studies [41,42]. Products 15 and 16 were possibly, based on their mass and retention time (relative to their log K_{ow}), degradation products of SMX (Fig. S11, SI). 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$^{\bullet+}$). The highest signal intensity was observed for the ABTS fragment 14, followed by SMX fragment 16.

3.7.4. Transformation products formed in the laccase-ABTS-IPN system

During IPN oxidation by laccase in presence of ABTS, 7 transformation products were detected (Fig. 3D, Table 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
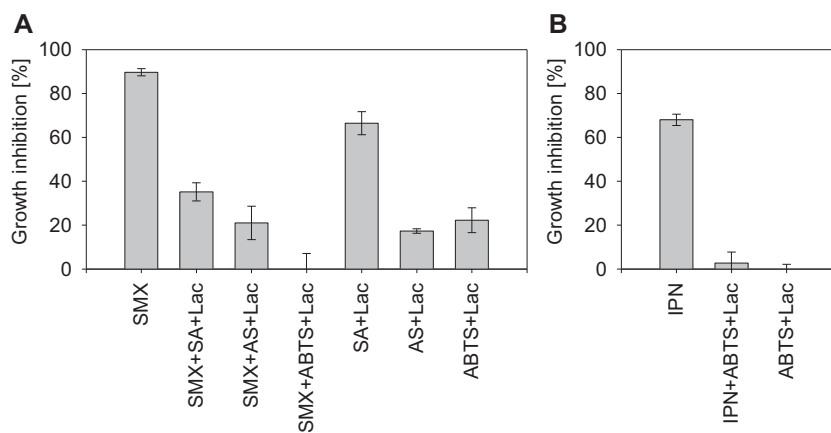
Fig. 4. Growth inhibition of the green algae *Pseudokirchneriella subcapitata*. After 72 h exposition to (A) sulfamethoxazole (SMX) (7.5 μM); SMX treated with a laccase (Lac)-mediator (SA, AS or ABTS) system (mixture of transformation products, TPs); or laccase with mediators in absence of SMX (mediators 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.

ABTS fragment as detected in the ABTS-SMX system and product 17 was probably also an ABTS fragment (Fig. S11, SI). 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 S1, SI). Similar absorption 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) [39], 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 absorption spectrum of a (not clearly identified) decomposition product of ABTS^{2+} observed in other studies [39]. As proposed by Majcherczyk et al. [39] 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. S12, SI).

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. S11, SI).

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 Figs. S13–15, SI). Their relative abundance after complete reaction of the mediators with SMX could thus be determined at various pH values (Fig. S16, SI).

For SMX in the laccase-SA system (Fig. S16 A, SI), 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. S16 B, SI) 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. S16 C, SI). 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.

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 3.7) was compared to solutions containing the parent compounds, or the mediator oxidized by laccase in the absence of the pollutant (Fig. 4).

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} [43] or at 1.9 mg l^{-1} [24]. Laccase-mediated treatments reduced this toxicity by 61% in presence of SA, 77% in presence of AS and 100% in presence of ABTS (Fig. 4A), demonstrating the much lower algal toxicity of the mixture of transformation products compared 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
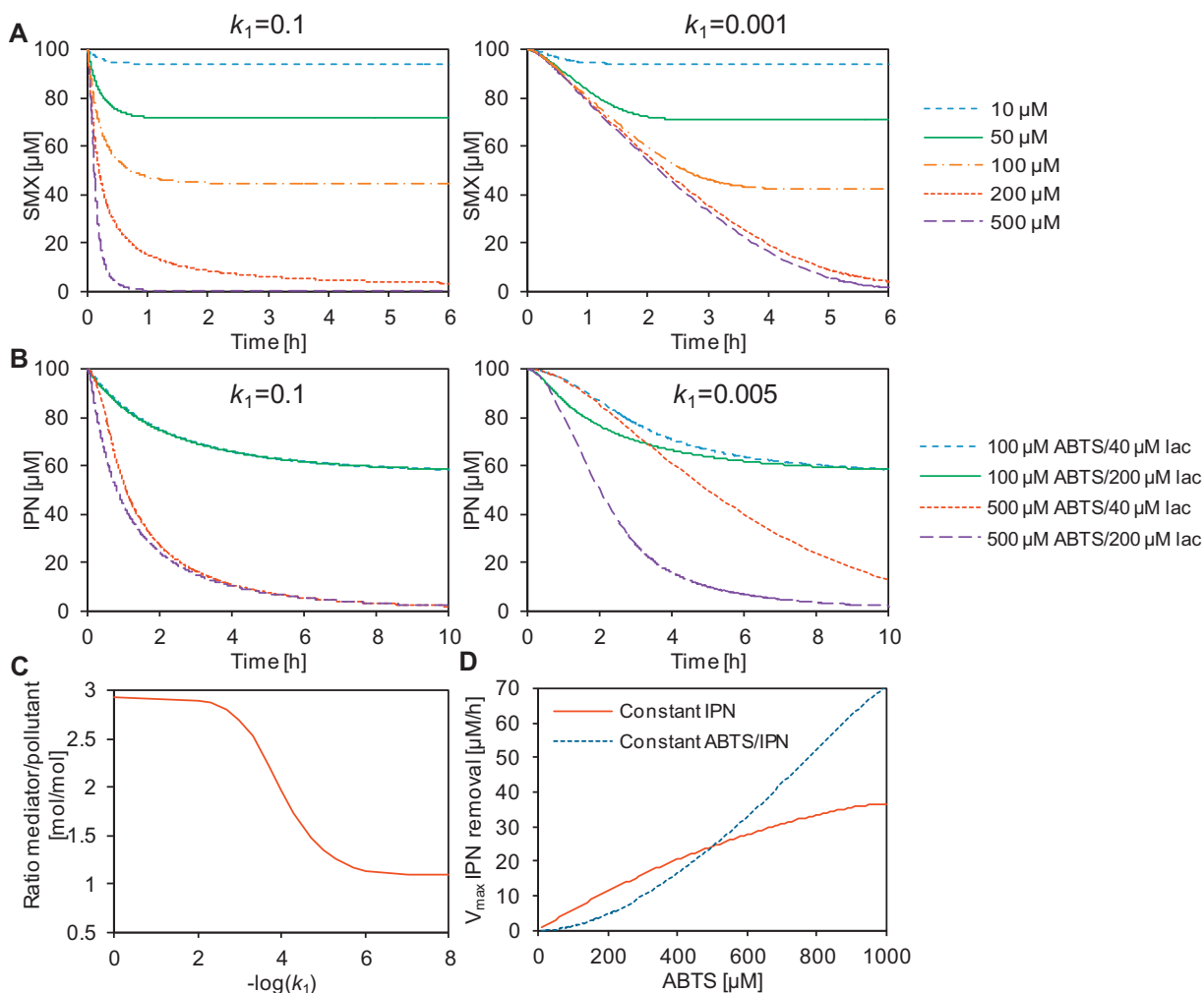
Fig. 5. Results of the kinetic model. (A) Influence of the mediator concentration (from 10 to 500 μM) on the pollutant (SMX) oxidation kinetics, with either the pollutant oxidation as the limiting step ($k_1 = 0.1$ μM 1 h^{-1}, similar to what is observed at pH 5–6, Fig 5A, left) or mediator oxidation limiting step ($k_1 = 0.001$ μM^{-1} h^{-1}, similar to what is observed at pH 7, Fig 5A, 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. 5B, left) or mediator oxidation limiting step ($k_1 = 0.005$ μM^{-1} h^{-1}, similar to what is observed at pH 7, Fig. 5B, 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 modeling parameters are presented in Table S2, SI.

products. The product 2 (DMBQ) was produced in lower quantities in presence of SMX (Fig. S14, 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) [32]. 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 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. 4B), which is similar to what was reported by Pavlić et al. [25] (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.
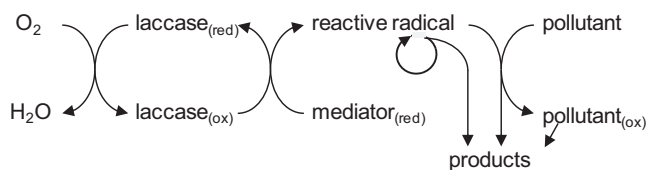
These results show that laccase-mediated reactions can significantly reduce toxicity of SMX and IPN to algae (among

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).

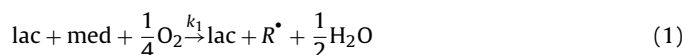### 3.9. Mechanistic aspects of laccase-mediated reactions

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).

As illustrated in Scheme 2 and described in Eqs. (1)–(4), our results suggest that the mediator (med) is oxidized by laccase (lac) to reactive radicals ($R^{\bullet}$) (Eq. (1)) that will either react by a

Scheme 2. Proposition of a laccase-mediator reaction model.

radical–radical coupling reaction, producing products P_1 (such as dimeric AS and SA) (Eq. (2)), further react to more stable products (P_2) (e.g., 2,6 DMBQ for AS and SA) (Eq. (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. (4)). $k_1 - k_4$ are the rate constants for Reactions (1)–(4), respectively. This mechanistic description is coherent with the nature of the transformation products detected (Section 3.7) (illustration for SA and SMX in Fig. S17, SI).

$$lac + med + \frac{1}{4}O_2 \overset{k_1}{\rightarrow} lac + R^\bullet + \frac{1}{2}H_2O \tag{1}$$

$$R^\bullet + R^\bullet \overset{k_2}{\rightarrow} P_1 \tag{2}$$

$$R^\bullet \overset{k_3}{\rightarrow} P_2 \tag{3}$$

$$R^\bullet + \frac{1}{a}poll \overset{k_4}{\rightarrow} P_3 \tag{4}$$

Based on this reaction model, a kinetic model was established (Section 13, SI) and used to simulate the behavior of the laccase-mediated reactions under various conditions. The results of selected simulations are presented in Fig. 5.

Both the effect of the mediator concentrations on the oxidation rates of SMX under acidic conditions and the absence of such an effect under neutral conditions (Fig. 2) could be correctly reproduced by the model by varying the rate constant k_1 (Eq. (1)) (Fig. 5A).

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. 2C). This saturation effect was attributed to significant cross-reactions between the radicals produced in high quantities (reactions of Eq. (2) favoured over Eq. (4)), and were correctly reproduced by the model (Fig. 5D). This saturation effect was not observed with constant mediator/pollutant ratios (Fig. 2C), a phenomenon confirmed with the model (Fig. 5D). Indeed, reaction

rates of Eq. (2) and Eq. (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. S6 A, SI), suggesting that the oxidation of the mediator by laccase was not the limiting step. This phenomenon was reproduced with the model by choosing a relatively high (non-limiting) reaction rate k_1 (Fig. 5B). 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. 5B).

The increase in the required mediator/pollutant ratio observed at low pH (Fig. 1E) was reproduced with the model by increasing the reaction rate constant k_1 (as observed at low pH) (Fig. 5C). 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.

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.

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. 6).

In the first scenario, when the pollutant, and therefore the mediator, were present at high concentrations (>100 and 500 μM,
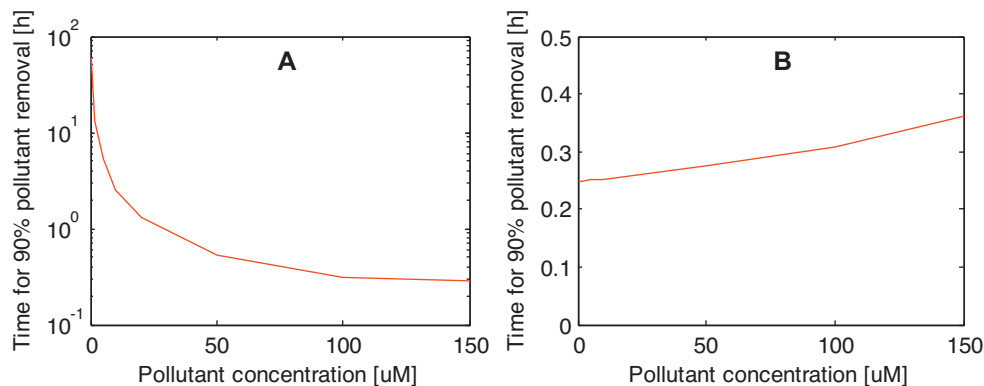

Fig. 6. Modeling of the time needed to remove 90% of the pollutant as a function of the pollutant concentration. (A) With a constant mediator/pollutant ratio of five (log scale for the *y*-axis). (B) With a constant mediator concentration of 500 μM. The modeling parameters are presented in Table S2, SI.

respectively), the removal time was independent of the pollutant concentration (similar to the experimental results, Fig. S6 B). But, as shown in Fig. 6A, 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. S18, 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. 6B), 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.

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.

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 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.

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.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bej.2015.06.008

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