A STRATEGY FOR XENOBIOTIC REMOVAL USING PHOTOCATALYTIC TREATMENT, MICROBIAL DEGRADATION OR INTEGRATED PHOTOCATALYTIC-BIOLOGICAL PROCESS

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Summary

According to the limited natural resources and due to the risks of anthropogenic pollution, it appears necessary to react efficiently in order to remove existing contaminations and avoid the creation of new ones. Therefore, the purpose of this thesis is to propose a sustainable strategy for treating problematic pollutants with the most adequate process.

First, an overview of the different treatment processes has been given. In particular, biological, photocatalytic and integrated biological-photocatalytic treatments have been described as efficient methods to remove xenobiotics. Biological treatment of contaminated environments and industrial effluents has been presented as the most attractive method on a cost-benefit extent. But for biorecalcitrant substances, the Advanced Oxidation Processes (AOP) have remained the most efficient methods although expensive. Therefore, the coupling of biological-photocatalytic has emerged as the best compromise for removal of recalcitrant xenobiotics.

The first step of the treatment strategy has consisted of evaluating the biotraitability of 19 chemicals: pesticides, pharmaceuticals and volatile organic compounds (VOCs) have been assessed according to three biodegradability methods (Zahn-Wellens, Manometric Respirometry, Closed-Bottle tests), whose official protocols had to be adapted to the compound specificities (volatility, hydrophobicity). Structure-Activity Relationship (SAR) estimation models have also been used to compare and validate the experimental results.

For the compounds which have been assessed as biotraitable (VOCs), further biodegradation studies have been led in order to improve the treatment (batch with pulses of substrate): automated monitoring of the biodegradation process, increased degradation yields and biomass productivity have been successfully completed for Toluene, Ethylbenzene, Xylenes, Chlorobenzenze and Dichlorobenzene removal.

Conversely, the compounds assessed as biorecalcitrant have been oriented towards the TiO_2 and photo-Fenton photocatalytic treatments. Thus, the degradation of four *p*-halophenols by TiO_2 photocatalysis has been investigated and has demonstrated the halogen effect on removal rates, aromatic intermediates and toxicity variations. Finally, the treatment of five

bioreacalcitrant pesticides (Alachlor, Atrazine, Chlorfenvinphos, Diuron, Pentachlorophenol) has been studied in order to develop a coupled photocatalytic-biological system: first, enhanced biotreatability has been evaluated using the Zahn-Wellens procedure and then fixed-bed bioreactors have been operated and permitted to find out the best moment for coupling.

Key words: biodegradability, xenobiotics, photocatalysis, pollution removal, wastewater, fed-batch, pesticides, VOCs.

Résumé

En raison de la limitation des ressources naturelles et des risques de pollutions anthropogéniques de l'environnement, il apparaît nécessaire d'agir efficacement en vu d'évincer les contaminations existantes et d'éviter la dissémination de nouvelles sources polluantes. Le but de cette thèse est alors de proposer une stratégie permettant de déterminer le processus le plus adéquat pour traiter des polluants problématiques.

Un survol des différents procédés de traitement a d'abord été présenté. L'efficacité des méthodes biologiques, photocatalytiques et de couplage biologique-photocatalytique a alors été établie pour l'élimination des composés xénobiotiques. Le traitement biologique des sites contaminés et des effluents industriels s'est imposé comme la méthode la plus attractive en terme de coûts-bénéfices. Néanmoins, les procédés d'oxydation avancée (POA) s'avèrent encore être les plus efficaces pour traiter les substances biorécalcitrantes, bien qu'ils occasionnent des coûts élevés. De ce fait, le couplage biologique-photocatalytique semble être le meilleur compromis pour l'élimination des composés xénobiotiques récalcitrants.

La première étape de la stratégie de traitement établie dans le cadre de cette thèse a consisté a évaluer à la biotraitabilité de 19 produits chimiques : pesticides, composés pharmaceutiques et composés organiques volatils (COVs) ont été évalués sur la base de trois méthodes (Zahn-Wellens, Respirometrie Manometrique, Tests des bouteilles fermées), dont les protocoles officiels ont dû être adaptés aux spécificités des composés étudiés (volatilité,

hydrophobicité). Des modèles de relation « Structure-Activité » ont également été employés, pour comparaison et validation des résultats expérimentaux.

Pour les composés évalués biotraitables (COVs), des études de biodégradation ont été menées dans l'optique d'améliorer le procédé de traitement (système batch avec pulses de substrat) : le monitoring automatisé du procédé de dégradation, l'augmentation des rendements de dégradation et de la productivité de biomasse ont été réalisés avec succès pour l'élimination du toluène, de l'ethylbenzène, des xylenes, du chlorobenzène et du dichlorobenzène.

Au contraire, les composés évalués biorécalcitrants ont été orientés vers un traitement photocatalytique par TiO_2 ou photo-Fenton. La photodégradation de quatre *p*-halophénols par TiO_2 a été investiguée et a permis de démontrer l'effet de l'halogène sur les taux d'élimination du polluant, la nature des intermédiaires aromatiques produits et les variations de toxicité de la solution traitée. Enfin, un système de couplage biologique-photocatalytique a été développé et étudié pour le traitement de cinq pesticides biorécalcitrants (Alachlor, Atrazine, Chlorfenvinphos, Diuron, Pentachlorophénol) : tout d'abord, une biotraitabilité améliorée des pesticides a été réalisée et évaluée selon le protocole du test de Zahn-Wellens, puis leur traitement en bioréacteurs sur lits fluidisés a été accompli et a permis de déterminer le moment le plus approprié au couplage.

Mots-clés: biodegradabilité, xénobiotiques, photocatalyse, dépollution, eaux résiduaires, fed-batch, pesticides, COV.

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CHAPTER 1.

Introduction and background of the thesis

1.1. Context

The total annual world production of synthetic organic chemicals is over 300 million tons (Fewson, 1988) with more than 1000 new compounds fabricated every year, so that the actual diversity of chemicals reaches approximately 20 millions of substances. For instance, in Switzerland, 250 000 chemicals are commercialized.

If not treated, all these compounds sooner or later appear in the environment. In 1995, Swoboda-Golberg (1995) surveyed the vast multitude of compound classes with enormous number of functional groups and their possible combination and arrangements. In this review, the major sources of synthetic organic chemicals have been summarized, including industry, household and agriculture, with a special regard to compound chemical stability or biodegradability.

In fact, during the past three decades, research on the impact of chemical pollution has focused almost exclusively on the conventional 'priority' pollutants (i.e. persistent organic pollutants, POPs) and this has been extensively reviewed recently (Birkett and Lester, 2002). Today, these compounds are less relevant for many first world countries because emissions have been substantially reduced through the adoption of appropriate legal measures and the elimination of many of the dominant pollution sources. The focus has consequently switched to compounds present in lower concentrations but which nevertheless might have the ability to cause harm (Larsen et al., 2004). One of the interesting characteristics of many of the chemicals that might cause this type of pollution is that they do not need to be persistent in the environment to cause negative effects (Ayscough et al., 2000). This is because their high transformation and removal rates can be offset by their continuous introduction into the environment, often through sewage treatment works (Suter and Giger, 2001).

In particular, volatile organic compounds (VOCs) of fuels and industrial solvents are commonly found contaminants in the subsurface, pesticides and phenols in groundwater. Persistent organic pollutants are long-lived organic compounds that become concentrated as they move through the food chain. They are also known as persistent toxins that bioaccumulate or as pseudo-oestrogenic chemicals. They have toxic effects on animal reproduction, development, and immunological function. In a recent review, a special attention has been focused on priority organic environmental contaminants and experimental approaches for determination and studies of specific toxicity mechanisms (Janošek et al., 2006).

In fact, although the risks associated with exposure to chemicals are probably most significant with regard to the natural environment, the public's concern is understandably more focused on human exposure. However, the issue of xenobiotics (and their metabolites) in the environment, notably the aquatic compartment, has been a growth area in environmental chemistry for several years (Fent et al., 2006; Jones et al., 2001). To date, most of the published literature has addressed the occurrence of organic pollutants in sewage effluent and receiving waters. This is especially important for human health in areas that practice indirect water reuse, where sewage effluent is released to streams and rivers that are in turn used as a source of raw water for the production of potable supplies for communities living downstream (Klöppfer, 1996a; Van Dijk-Looijaard and Van Genderen, 2000). Unfortunately, there are extremely few data available on the occurrence of pollutants in point-of-use drinking waters (that is, tap water at the sink).

Of the Earth's 1386 million cubic kilometers of water, only 2.5% of that quantity is freshwater and nearly one third of this smaller amount is available for human use (Postel et al., 1996). Against this backdrop of a finite water supply, total water withdrawn for human uses has almost tripled in the last 50 years from 1382 $km^3\ yr^{-1}$ in 1950 to 3973 $km^3\ yr^{-1}$. Projections anticipate that the worldwide human water consumption will further increase to 5235 $km^3\ yr^{-1}$ by 2025 (Clarke and King, 2004): an amount greater than nine times the annual flow of the Mississipi River (Goudie, 2000). Over 50% of the available freshwater supplies is already used for human activities and this proportion is increasing in response to growing agricultural, industrial, and residential demands (Postel et al.,

1996; Vorosmarty and Sahagian, 2000). By 2025 an estimate of 5 people out of 8 will be living in conditions of water stress or scarcity (Arnell, 1999). Limiting water availability will certainly alter ecosystems, human health, agricultural and industrial output, and unfortunately increase the potential for conflict (Postel et al., 1996).

In the light of such alarming reports, two strategies are promising:

- developing and applying curative treatments is necessary to reduce the existing pollution,
- adopting a prevention policy is a sustainable approach to preserve the overall future life on Earth.

From the recent environmental awareness and legislative restrictions on uncontrolled discharges of wastes, the best strategy for treatment of high toxic hazardous and xenobiotic wastes may be their treatment at source with a special stress put on the industry (Grüttner, 1997). A large group of aromatic compounds generally not produced by natural processes has to be completely degraded and mineralized because of their high persistence. This may be achieved through the development of particular solutions for each ecological problem.

1.2. Conventional treatments for pollution removal

1.2.1. Biological treatments

1.2.1.1. Characteristics of biodegradation

It is clear that microbial-based treatment of contaminated environments and industrial effluents are economically attractive in comparison with other conventional treatment methods such as adsorption on granular activated carbon, air stripping or reverse osmosis, combustion, and strong oxidative processes (Gogate and Pandit, 2004). In natural waters and soils, the mineralization or complete biodegradation of an organic molecule is almost always a consequence of microbial activity (Alexander, 1981). The most rapid and complete degradation of the majority of pollutants is brought about under aerobic conditions (Riser-Roberts, 1998).

The essential characteristics of aerobic microorganisms degrading organic pollutants are illustrated in Figure 1.1. They consist of:

1. Metabolic processes for optimizing the contact between the microbial cells and the organic pollutants. The chemicals must be accessible to the organisms having biodegrading activities. For example, hydrocarbons are water-insoluble and their degradation requires biosurfactants.

2. The initial intracellular attack of organic pollutants is an oxidative process, the activation and incorporation of oxygen is the enzymatic key reaction catalyzed by oxygenases and peroxidases.

3. Peripheral degradation pathways convert organic pollutants step by step into intermediates of the central intermediary metabolism, e.g., the tricarboxylic acid cycle.

4. Biosynthesis of cell biomass from the central precursor metabolites, e.g., acetyl-CoA, succinate, pyruvate. Sugars required for various biosyntheses and growth must be synthetized by gluconeogenesis.

A huge number of bacteria and fungia generally possess the capability to degrade organic pollutants. Biodegradation is defined as the biologically catalyzed reduction in complexity of chemical compounds (Alexander, 1994). It is based on two processes: growth and cometabolism.

In the case of growth, organic pollutants are used as sole source of carbon and energy. This process results in complete degradation (mineralization) of organic pollutants.

Cometabolism is defined as the metabolism of an organic compound in the presence of a growth substrate which is used as the primary carbon and energy source.

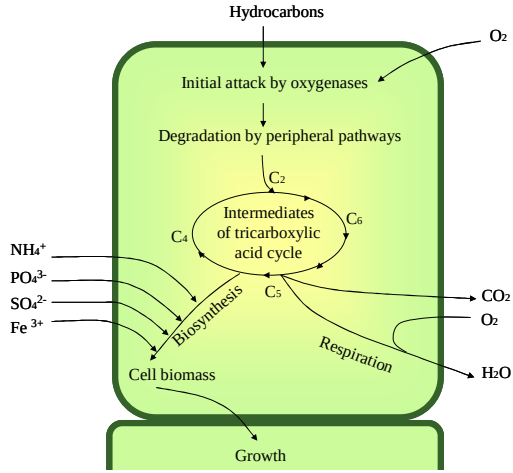


Figure1.1. Main principle of aerobic biodegradation of hydrocarbons: growth associated processes.

Enymatic key reactions of aerobic biodegradation are oxidations catalyzed by oxygenases and peroxidases. Thus in the presence of molecular oxygen, compounds such as benzoates, phenols, anilines and aromatic hydrocarbons are initially oxidized by mono- or dioxygenases and converted to catechols or protocatechuates with subsequent mineralization. The different pathways which are implicated to degrade xenobiotics have been largely elucidated (Dagley, 1985). A special interest is now devoted to gene expression for the regulation of biodegradative processes.

Fewson (1988) in his review presented the biodegradation process as environmentally compatible; Singleton (1994) referred to “The Green Option”. In fact, biodegradation may imply advantages as well as disadvantages for human beings and nature (Table 1.1).

Table 1.1. Beneficial and deleterious effects of biodegradation

Beneficial effects	Deleterious effects
Detoxification, decontamination and bioremediation of hazardous or unpleasant materials	Biodegradation of useful materials
Conversion to non-toxic products of compounds that might give rise to harmful substances	Production of toxic compounds from innocuous precursors and conversion to more recalcitrant forms
Formation of useful products	Enhanced biodegradation of pesticides after repeated applications

The two first beneficial effects are quite well known, the third one only gained its importance in recent years. It has been shown that microbial biotransformation could be used to synthesize commercially useful products such as carboxylate diols and fluorinated compounds (Ribbons et al., 1990). For example, Kiener (1995) reported a process of microbial biotransformation of xylene and toluene via selective oxidation of alkyl groups on aromatic N-heterocycles with following ring hydroxylation to intermediates used for antipolytic and antidiabetic drug production.

1.2.1.2. Factors influencing microbial degradation of xenobiotics

Numerous factors can affect the biodegradation processes. They depend on the nature of chemical molecules to be degraded (e.g. molecule size, charge, number and position of functional groups, solubility and toxicity) as well as the environmental conditions. For instance, some molecular features can increase recalcitrance (Fewson, 1988); environmental factors influence the growth of organisms, the availability of xenobiotics

and more subtly, they can affect the gene expression (Van der Meer et al., 1998; Daubaras and Chakrabarty, 1992).

Therefore, basic information is required to achieve a successful biotreatment:

- nature of the xenobiotic chemicals,
- concentration of the xenobiotic chemicals,
- presence and activity of the xenobiotic degrading microorganisms,
- appropriate conditions of cultivation which are required for the growth of the target microorganisms.

Whenever they are identified and controllable, chemical and environmental factors should be taken into account during the engineering development process and all along the xenobiotic degradation (Liu et al., 2000).

Usual factors which play a significant role during biological treatments are:

- compound bioavailability,
- pH and temperature of the medium,
- nutrients,
- oxygen availability,
- residence time (dilution rate).

Laboratory experiments, which aim to study the xenobiotic biodegradation, are preferably carried out at pH and temperature which are optimal for the cultivated microorganisms in order to maximize their degradation activity. Many bacteria have their optimum pH near neutrality and are mesophilic, whereas fungi prefer more acidic environments (Singleton, 1994). However, under suboptimal environmental conditions, an increased resistance of microorganisms can be achieved by immobilization of the bacterial cells (Hörtemann and Hempet, 1991). This is interesting for wastewater treatment.

Improving the monitoring of these parameters will turn out as an improved ability to perform environmental risk assessments, to design environmental strategies and to optimize wastewater treatment.

1.2.1.3. Biodegradability of xenobiotic compounds

Basically, biodegradability for a chemical compound refers to its potential for biological degradation.

Biorecalcitrance for a chemical can (1) be due to the lack of the microbial enzymatic pool necessary to degrade the molecule or (2) be attributed to its toxical properties against microorganisms.

(1) In case of strains deficient in the adequate enzyme activities, biotreatment can finally be achieved after involved acclimation and induction with the chemical (Haigler et al., 1992; Spain and Gibson, 1988). This technique has been widely used for degradation of halogenated aromatics (Reineke and Knackmuss, 1984; Spain and Nishino, 1987; Seignez et al., 2001; Moos et al., 1983; Kle ka and Maier, 1985).

(2) In many situations, toxicity determination provides reliable information about biodegradability of chemicals. This characteristic has been considered as a convenient tool to predict the behavior of many substances in both environmental and treatment plant situations (Mantis et al., 2005; Pessala et al., 2004; Chen et al., 1997; Boluda et al, 2002).

Several tests have been developed in order to assess the toxicity of chemical compounds at different levels of ecotoxicity. For this purpose, many living organisms have been selected for their relevance, accuracy and applicability. In particular, luminescent bacteria bioassays (for eg., Microtox^{TM} using *Vibio fischeri*) have emerged as a simple, fast and comparatively inexpensive alternative to *in-vivo* bioassays with higher organisms (Parvez et al., 2006; Steinberg et al., 1995; Manusadžianas et al., 2003; Kaiser and Palabrica, 1991).

Simultaneously, structure-activity relationships (QSAR) have been investigated in order to assess toxicity and biodegradability, and therefore predict the potential ecological effect of pollutants on various trophic levels (Schultz et al., 1988; Sixt et al., 1995; Sarakinos et al., 2000; Chen et al., 1999). Various estimation programs even provide qualitative and/or quantitative information concerning the biodegradability characteristics of chemicals (Howard, 2000).

Considering the enormous quantity of scientific investigations related with microbial degradation for diverse recalcitrant chemicals, it is presumable that every chemical are biodegradable under specific conditions (presence or absence of oxygen; temperature; pH; nutrient medium; liquid, gaseous or solid phases; microorganisms strains; etc.).

However in a context of bioremediation and/or pollution control (contaminated air, soils, wastewaters, surface and groundwaters), biodegradability should mean biotreatability. Indeed many methods which are aimed to assess biodegradability in fact prospect for their possible removal in realistic conditions of treatment processes.

In chapter 2, the different methods for biodegradability assessment are proposed and discussed.

In practice, it is frequent that no specific treatment methods are known so far for many pollutants. In the worst cases their biological treatment is simply impossible to achieve. In particular hardly-biodegradable anthropogenic substances accumulate and saturate the auto-depurative conditions of the water cycle which becomes perturbed and overloaded. The situation worsens because adequate water treatment systems are still missing or are unable to lower the concentration of the toxic substances that represent a chronic chemical risk (Klöpffer, 1996b, Andreadakis et al., 1997). In the meantime, numerous water sources are deteriorated and compromise the overall supply and quality of drinking water.

1.2.2. Conventional wastewater treatments

Water treatments have to focus on :

- contaminated drinking, ground and surface waters,
- wastewaters which contain toxic or biorecalcitrant compounds.

For this purpose, the actual and available technologies are very diverse, but conventional processes are often classified as preliminary, primary, secondary and tertiary treatments (Horan, 1990):

- During the preliminary step, debris and sandy materials are removed from the wastewater.

- During primary treatment, wastewater is cleared of suspended solids (settlement) and greases (floatation).

- Secondary treatment is a biological process: microorganisms absorb the dissolved organic matter from wastewater and use them as food supply. Two strategies are possible for aerobic wastewater treatment:

  - the fixed biomass systems grow microorganisms on solid supports such as rocks, sand or plastic. Wastewater is spread over the supports and by the way provides organic matter and nutrients to the biomass: the biofilm grows by thickening. Trickling filters, rotating biological contactors and sand filters are examples of fixed biomass systems,

  - suspended biomass systems maintain the biomass stirred during all the treatment: microorganisms grow in size and number. After the treatment, the biomass is settled out as sludge. Some of it is pumped back into the incoming wastewater to provide seed microorganisms. The rest is sent to a further treatment process. Activated sludge, extended aeration, oxidation ditch and sequential batch reactor systems are all examples of suspended biomass systems.

- Tertiary treatment focuses on the removal of the disease-causing organisms from wastewater. Treated wastewater can be disinfected by chlorine adjunction or ultraviolet lightening. High levels of chlorine may be harmful to the aquatic life present in receiving streams. Therefore treatment systems often add a neutralizing chemical to the treated wastewater before stream discharge.

The incapability of conventional biological wastewater treatment to remove effectively many industrial biorecalcitrant and/or toxic pollutants evidences that new efficient treatment systems are needed. For the last 30 years, the water purification research has been extensively growing. Rigorous pollution control and legislation in many countries (for instance EEC, 1992; Conseil Fédéral Suisse, 1998) have resulted in an intensive search for new and more efficient water treatment technologies.

1.2.3. Advanced Oxidation Processes (AOP)

In the last 15 years, a lot of research projects have been addressed to a special class of oxidation techniques defined as Advanced Oxidation Process (AOP), pointing out its potential prominent role in the wastewater purification (Ollis and Ekabi, 1993). It was shown that AOP could successfully solve the problem of biorecalcitrant water pollutants working at or near ambient temperature and pressure (Bahnemann et al., 1994; Bolton and Cater, 1994).

All AOP are characterized by the same chemical feature: the production of hydroxyl radicals $\bullet OH$. These radicals are extremely reactive species and attack mainly every organic molecules with rate constants usually in the order of 10^6-10^9 mol l^{-1} s^{-1} (Hoigne, 1997). $\bullet OH$ radicals are also characterized by a low selectivity of attack which is a useful attribute for an oxidant used in wastewater treatment and for solving pollution problems. The versatility of AOP is also enhanced by the fact that they offer different possible ways for $\bullet OH$ radicals production, thus allowing a better compliance with the specific treatment requirements.

Table 1.2 shows that $\bullet OH$ has the highest thermodynamic oxidation potential, which is perhaps why $\bullet OH$-based oxidation processes have gained the attention of many advanced oxidation technology developers. In addition, most environmental contaminants react 1 million to 1 billion times faster with $\bullet OH$ than with O_3, a conventional oxidant (US EPA, 1998).

Table 1.2 Oxidation potential of several oxidants in water (CRC Handbook, 1985)

Oxidant	Oxidation potential (eV)
$\bullet OH$	2.80
$O(^1D)$	2.42
O_3	2.07
H_2O_2	1.77
Permanganate ion	1.67
Chlorine	1.36
O_2	1.23

The oxidation reactions involving hydroxyl radical and organic substrates (RH or PhX) in aqueous solutions may be classified with respect to their character (Boossmann et al., 1998):

- abstraction of hydrogen: $\bullet OH + RH \rightarrow R\bullet + H_2O$ (Eq 1.1)
- addition reactions: $\bullet OH + PhX \rightarrow HOPhX\bullet$ (Eq 1.2)
- electron transfer: $\bullet OH + RH \rightarrow [R-H]^{+\bullet} + HO^-$ (Eq 1.3)

Application of AOP depends on the polluting load of wastes, as illustrated in Figure 1.2.

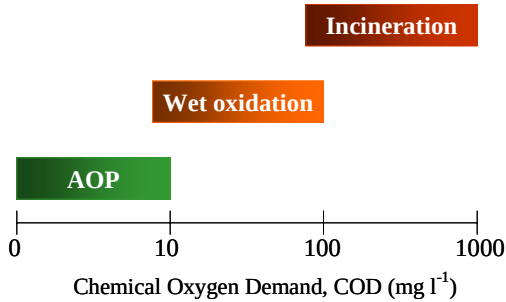


Figure 1.2. Suitability of water treatment technologies according to the Chemical Oxygen Demand, COD (Andreozzi et al., 1999)

Only wastewater with relatively small Chemical Oxygen Demand (COD) contents (5 g l^{-1}) can be suitably treated by these techniques (Andreozzi et al., 1999) in order to avoid an excessive consumption of expensive reactants. Wastes with massive pollutant contents are more conveniently treated by wet oxidation or incineration (Mantzavinos et al, 1997; Luck, 1999)

AOP can be classified according to the reaction phase (homogeneous or heterogeneous) or to the methods used to generate the $\bullet OH$ radicals (chemical, electrochemical, sonochemical or photochemical). The main used AOP are illustrated in Figure 1.3.

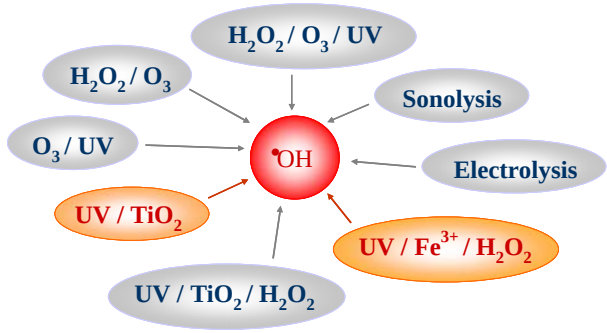


Figure 1.3. Main advanced oxidation processes (AOP).

An interesting class of AOP consists of the so-called Advanced Photochemical Oxidation (APO) processes (US EPA, 1998). APO processes are characterized by a free radical mechanism initiated by the interactions of photons of a proper energy level with the molecules of chemical species present in the solution or with a catalyst.

The most used APO technologies can be broadly divided into the following groups: (1) heterogeneous photocatalysis, (2) homogeneous photocatalysis, and (3) the photo-Fenton process. These APO technologies are briefly described below.

1.2.3.1. Heterogeneous photocatalysis

Since 1972, when Fujishima and Honda (1972) reported the photocatalytic decomposition of water on TiO_2 electrodes, photocatalysis has been used with great success in the degradation of a wide variety of contaminants, including alkanes, alcohols, carboxylic acids, alkenes, phenols, dyes, PCBs, aromatic hydrocarbons, halogenated alkanes and alkenes, surfactants, and pesticides (Ollis and Ekabi, 1993; Blake, 2001).

Heterogeneous photocatalysis is a technology based on the irradiation of a catalyst, usually a semiconductor, which may be photoexcited to form electron-donor sites (reducing sites) and electron-acceptor sites (oxidizing sites), providing great scope as

redox reagents. The process is heterogeneous because there are two active phases, solid and liquid.

Among several semiconductors, titanium dioxide has proven to be the most suitable for widespread environmental applications. TiO_2 is stable to photo- and chemical corrosion, and inexpensive. TiO_2 has an appropriate energetic separation between its valence and conduction bands (VB and CB, respectively), which can be surpassed by the energy of a solar photon. The VB and CB energies of the TiO_2 are estimated to be +3.1 and -0.1 volts, respectively, which means that its band gap energy is 3.2 eV and absorbs in the UV light (wavelength λ<387 nm). Hydroxyl radical ($\bullet OH$) is the main oxidizing species responsible for photo-oxidation of the organic compounds (Legrini et al., 1993; Valavanidis et al., 2006). The first event, after absorption of near ultraviolet radiations at λ<387 nm, is the generation of electron/hole pairs (Eq 1.4) separated between the CB and VB.

$$TiO_2 + h\nu \rightarrow TiO_2\left(e^- + h^+\right) \qquad \text{(Eq 1.4)}$$

Some of the numerous events which take place after the UV light absorption by TiO_2 particles are presented in figure 1.4. The subsequent generation and separation of electrons (e^-_{CB}) and holes (h^+_{VB}) are also mentioned. In particular, three oxidation reactions have been experimentally observed: electron transfer from R, H_2O and ^-OH adsorbed on the catalyst surface.

It has been shown that the photocatalytic degradation of a water contaminant can be enhanced by addition of H_2O_2, a better electron acceptor than O_2 (Ollis et al., 1991; Pichat et al., 1995; Diller et al., 1996). This effect has been explained by the formation of hydroxyl radicals during the reaction with CB electrons by Eq 1.5:

$$H_2O_2 + e^-_{CB} \rightarrow \bullet OH + OH^- \qquad \text{(Eq 1.5)}$$

It limits the electron-hole recombination rate and increases the hydroxyl radical concentration at the TiO_2 surface.

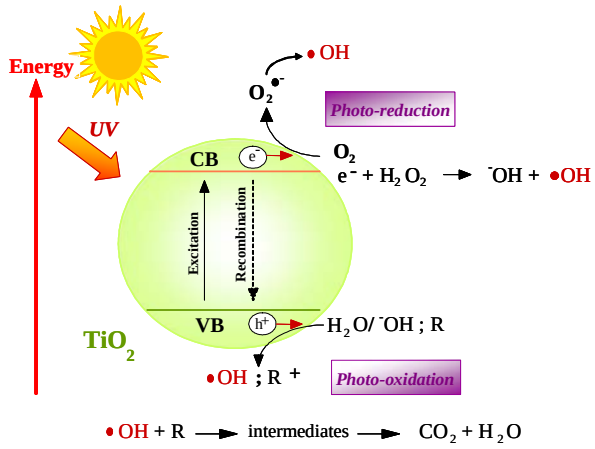


Figure 1.4. Schematic of an irradiated TiO_2 semi-conductor particle with some possible photo-chemical and photo-physical processes.

1.2.3.2. Homogeneous photodegradation

The former applications of homogeneous photodegradation (single-phase system) to treat contaminated waters concerned the use of UV/H_2O_2 and UV/O_3. The use of UV light for photodegradation of pollutants can be classified into two principal areas:

- direct photodegradation, which proceeds from direct excitation of the pollutant by UV light, and
- photo-oxidation, where light leads oxidative processes principally initiated by hydroxyl radicals. This process involves the use of an oxidant to generate radicals, which attack the organic pollutants to initiate oxidation. The three major oxidants used are: hydrogen peroxide (H_2O_2), ozone, and photo-Fenton system (Fe^{3+}/H_2O_2).

1.2.3.3. Fenton and photo-Fenton assisted reactions

The Fenton reaction was discovered by H.J.H. Fenton in 1894. In 1934, Haber and Waiser postulated that the effective oxidative agent in the Fenton reaction was the hydroxyl radical ($\bullet OH$). Since then, some groups have tried to explain the whole mechanism (Walling, 1975; Prousek, 1995; Sychev and Isak, 1995).

The Fenton reaction can be outlined as follows:

$$M^{n+} + H_2O_2 \rightarrow M^{(n+1)+} + OH^- + \bullet OH \qquad \text{(Eq 1.6)}$$

where M is a transition metal such as Fe or Cu.

In absence of light and complexing ligands other than water, the most accepted mechanism of H_2O_2 decomposition which occurs in acid homogeneous aqueous solution, involves the formation of hydroxyperoxyl ($HO_2\bullet / O_2^-$) and hydroxyl radicals ($\bullet OH$). (DeLaat and Gallard, 1999; Gallard and De Laat, 2000)

The $\bullet OH$ radical, once in solution, attacks almost every organic compound. The metal regeneration can follow different pathways. For Fe^{2+}, the most accepted scheme is described in Eq 1.7 to Eq 1.14 (Sychev and Isak, 1995).

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \bullet OH$ K= 63 l mol^{-1} s^{-1} (Eq 1.7)

$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + HO_2\bullet + H^+$ K= 3.1 x 10^{-3} l mol^{-1} s^{-1} (Eq 1.8)

$Fe^{2+} + \bullet OH \rightarrow Fe^{3+} + OH^-$ K= 3 x 10^{8} l mol^{-1} s^{-1} (Eq 1.9)

$\bullet OH + H_2O_2 \rightarrow HO_2\bullet + H_2O$ K= 3.3 x 10^{7} l mol^{-1} s^{-1} (Eq 1.10)

$Fe^{3+} + HO_2\bullet \rightarrow Fe^{2+} + H^+ + O_2$ K= 2 x 10^{3} l mol^{-1} s^{-1} (Eq 1.11)

$Fe^{3+} + O_2\bullet^- \rightarrow Fe^{2+} + O_2$ K= 5 x 10^{7} l mol^{-1} s^{-1} (Eq 1.13)

$Fe^{2+} + HO_2\bullet \rightarrow Fe^{3+} + HO_2^-$ K= 1.2 x 10^{6} l mol^{-1} s^{-1} (Eq 1.14)

Fenton reaction rates are strongly increased by irradiation with UV/visible light (Rupert et al., 1993; Sun and Pignatello, 1993; Bandara et al., 1996). This type of photoassisted reaction is referred to as the “photo-Fenton reaction” (Zepp et al., 1992).

$$Fe^{3+} + H_2O + h\nu \rightarrow Fe^{2+} + \bullet OH + H^+ \qquad \text{(Eq 1.15)}$$

The positive effect of irradiation on the degradation rate is due to the photochemical regeneration of ferrous ions (Fe^{2+}) by photoreduction of ferric ions (Fe^{3+}) (Faust and Hoigne, 1990). The new generated ferrous ion reacts with H_2O_2 producing a second $\bullet OH$ radical and ferric ion (Eq 1.7), and the cycle continues. In these conditions, iron can be considered as a real catalyst.

The main advantage of the photo-Fenton process is the light sensitivity up to a wavelength of 600 nm (35% of the solar radiation) (Safarzadehamiri et al., 1997). The depth of light penetration is high and the contact between pollutant and oxidizing agent is close, since homogeneous solution is used (Bauer et al., 1999; Fallmann et al., 1999).

Fenton and photo-Fenton processes have been applied with great success to treat a wide variety of contaminants, including phenols (Kiwi et al, 1994; Gernjak et al., 2003), dyes (Bandara et al., 1996), halogenated alkanes and alkenes (Buyuksonmez et al., 1999), and pesticides (Fallmann et al., 1999; Parra et al., 2000).

Several parameters governing or influencing the kinetics of the photo-Fenton system have been studied: pH (Scott et al., 1995), iron concentration (Krutzler and Bauer, 1999), iron species (Cuzzola et al., 2002), H_2O_2 concentration (Safarzade-Hamiri et al., 1997), initial pollutant concentration (Rupert et al., 1993), temperature (Sagawe et al., 2001; Rodriguez et al., 2002), irradiation source (Rodriguez et al., 2002), presence of radical scavengers (Chen and Pignatello, 1997; Kiwi et al., 2000).

1.3. Aim of the thesis

The aim of this thesis is to propose a strategy to treat xenobiotic compounds. As introduced in part 1.2., the Advanced Oxidation Processes (AOP) appear as interesting tools, in comparison with the well-established techniques like activated carbon, air stripping, reverse osmosis, combustion, etc. Indeed, many of these techniques only transfer the pollutants from one phase to another without destroying them. Other processes are selective but rather slow in degradation rates, or rapid but not selective, thus increasing the engineering, maintenance and energy costs. Other restrictive factors are economic reasons, oxidative potential, effluent characteristics or tendency to form harmful disinfection by-products as, for example, the case of formation of trihalomethanes (THMs) when a chlorination procedure is used for drinking water treatment.

Biological treatment is limited to wastewaters which contain biodegradable substances and which are not inhibitive nor toxic to the bioculture.

Although AOP are cheaper than combustion or wet oxidation technologies, the most serious drawback of AOP is their relatively high operational costs, compared to those of biological treatments.

For these reasons, their use as a pre-treatment step for the enhancement of the biodegradability of wastewater containing recalcitrant or inhibitory compounds can be justified when the intermediates resulting from the AOP are readily degradable by microorganisms. Thus considering AOP as a preliminary treatment before inexpensive biological process seems very promising from an economical point of view (Robertson, 1996; Scott and Ollis, 1995; Scott and Ollis, 1997; Sarria et al., 2002).

Therefore, the approach proposed in this thesis consists of:

1. Determining the potential of a target xenobiotic to be degraded through a biological process,
2. Depending on the previous response, applying the appropriate treatment among the three most promising processes described before: biodegradation, photocatalysis or integrated biological-photocatalytic treatment.

Figure 1.5 presents the schematic of this strategy.

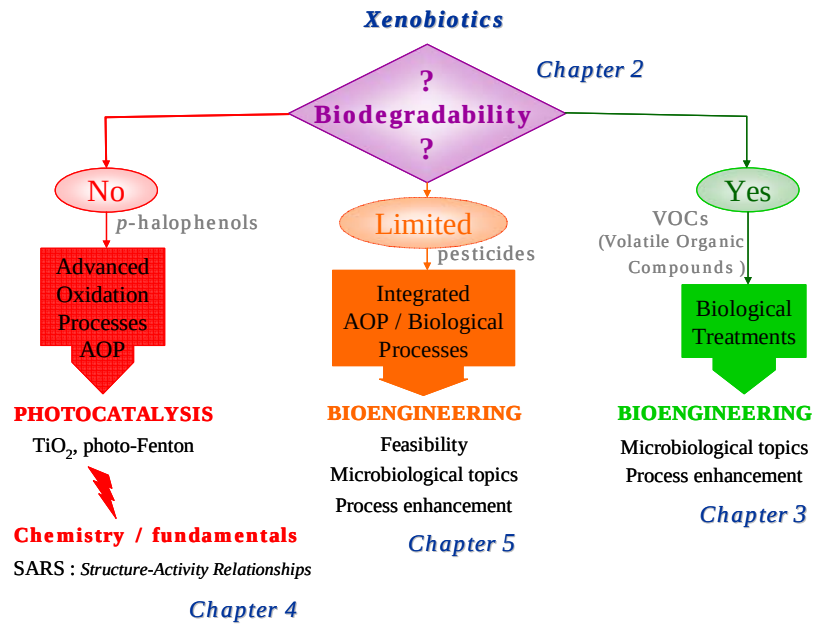


Figure 1.5. Overview of the strategy proposed to treat xenobiotics

Each situation will be illustrated in this thesis. For this purpose, the manuscript is organized in six chapters. In the first chapter, background and fundamentals of the research are presented. The second chapter clarifies the assessment methods used to determine the biodegradability of chemicals. Chapters 3, 4 and 5 are devoted to biodegradation, photocatalytic process and the integrated biological-photocatalytic treatment, respectively. These methods are applied to specific xenobiotic substances.

Each definite topic is subject to publication. By the way, chapters 2 to 5 provide the information through separated scientific articles. The following paragraph 1.4 details the several target xenobiotics which have been studied in this work. It is also aimed to introduce the different aspects related to both the substances and their appropriate degradation methods.

1.4. The target xenobiotics compounds

1.4.1. Volatile Organic Compounds

It is worth mentioning that there is no agreement about the definition of Volatile Organic Compounds (VOCs). In spoken language, VOCs are often used as synonyms for organic solvents. An effect-oriented definition, mainly used in the USA, states that VOCs are all organic compounds contributing to photochemical ozone creation. More general definitions are based on physical and chemical properties of the compounds, such as chemical structure, boiling point, air/water partitioning, and vapor pressure.

Definitions based on vapor pressure are frequently used. In the USA, VOCs are defined as organic compounds that have a vapor pressure more than 13.3 Pa at 25°C, according to ASTN test method D3960–90. In the European Union, a common definition is that VOCs are organic compounds with a vapor pressure above 10 Pa at 20°C (European VOC Solvents Directive 1999/13/EC). The Australian National Pollutant Inventory defines a VOCs as chemical compounds based on carbon chains or rings (and also containing hydrogen) with a vapor pressure greater than 2 mm of mercury (0.27kPa) at 25°C, excluding methane.

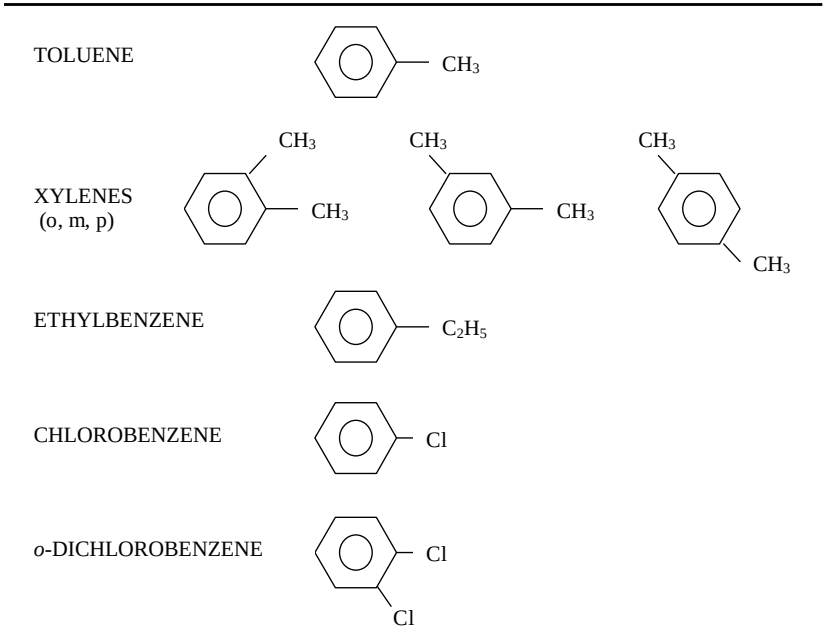
VOCs are a topic of interest in many disciplines, such as food, flavor, fragrance and medical sciences. The main area dealing with VOCs may be the environmental chemistry, because VOCs contribute to stratospheric ozone depletion, tropospheric ozone formation, toxic and carcinogenic human health effects, etc.

Chlorobenzenes and TEX compounds (toluene, ethylbenzene and xylenes) are among the most important contaminants present in surface and groundwater which usually originate from the leakage of tanks containing petroleum-derived products and industrial wastewaters (Akunar-Askar et al., 2003). Because they are both toxic and relatively water soluble compared with other petroleum constituents, their entry into surface and drinking water is of major concern.

Many technologies were developed and optimized for removing either chlorinated benzenes or TEX compounds from industrial effluents and groundwater in order to limit the contamination of downstream drinking water resources. Among all remediation

technologies for treating TEX-contaminated groundwater, bioremediation appears to be an economical, energy-efficient and environmentally sound approach (Devinny et al., 1999; Shim et al., 2002; Nishino et al., 1992). Microorganisms are able to degrade chlorinated benzenes and TEX under aerobic, microaerobic or hypoxic, as well as anaerobic conditions. However these substances are highly toxic and easily provoke inhibitions to the microorganisms which degrade them (Sardessai and Bhosle, 2002). Therefore developing and upgrading biotechniques for simultaneous and efficient removal of all TEX remains challenging (Rula and Cohen, 1999; Shim and Yang, 1999; León et al., 1999).

In this thesis, the target VOCs consisted of chlorobenzene, *o*-dichlorobenzene, toluene, ethylbenzene and *o*-, *m*-, *p*-xylenes (TEX). Their molecular structure is illustrated in Scheme 1.1. Two other chlorinated solvents (dichloroethane, dichloromethane) are occasionally mentioned since they are studied for specific topics.



Scheme 1.1. Structures of the VOCs studied in Chapters 2 and 3.

In chapter 2, these substances will be shown biodegradable. As biological treatment prevails over the other mentioned-processes, it is hence proposed to study and enhance their biodegradation. The basis of the work dealing with VOCs is three-fold:

1. Develop and test a reliable engineering process with the aim to produce a bacterial consortium able to degrade chlorobenzenes and/or TEX,

2. Characterize and optimize the degradation process, focusing on microbial, kinetic and biochemical aspects,

3. Automate the cultivation procedure of the efficient chlorobenzenes/TEX-degrading bacterial consortium, using LabVIEW© software.

This topic is presented in chapter 3.

1.4.2. Phenols

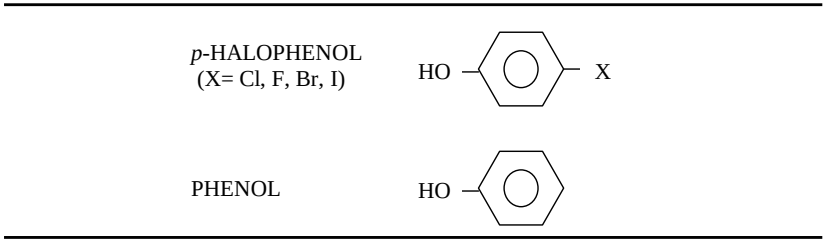
Phenols, another group of organic compounds, are considered as priority pollutants since they are harmful to organisms at low concentrations and many of them have been classified as hazardous pollutants because of their potential to harm human health. It should be noted that the contamination of drinking water by phenolics, at even a concentration of 0.005 mg l^{-1} could bring about significant taste and odor problems making it unfit for use. Human consumption of phenol-contaminated water can cause severe pains leading to damage of capillaries ultimately causing death. Phenol containing water, when chlorinated during disinfection of water also results in the formation of chlorophenols. The most important pollution sources containing phenols and phenolic compounds such as nitrophenols, chlorophenols and other halophenols, are the wastewaters from the iron-steel, coke, petroleum, pesticide, paint, solvent, pharmaceutics, wood preserving chemicals, and paper and pulp industries (Kahru et al., 1998a).

Current methods for removing phenolics from wastewater include microbial degradation, adsorption on activated carbon, chemical oxidation (using agents such as ozone, hydrogen peroxide or chlorine dioxine), deep-well injection, incineration solvent extraction and irradiation. Solvent extraction methods are expensive and deep-well injection may lead to contamination of groundwater. Adsorption and oxidation treatments

become exceedingly expensive when low effluent concentrations must be achieved. Wastewaters containing phenol in the range of 5-500 mg l^{-1} are considered suitable for treatment by biological processes. A wide variety of pure and mixed cultures of microorganisms are even capable of degrading phenols and phenolic compounds under both aerobic and anaerobic conditions. Surprisingly, although biological treatment has shown great promises, microbial degradation of these compounds is seen as a cost effective method. Indeed biological treatment of phenolics is limited by the intrinsic properties of these compounds owing to their toxicity; they are slow to biodegrade and the degrading microorganisms must be exposed to only low concentrations of the substrates (Kahru et al., 1998b; Grau and Darin, 1997).

This is the reason why alternative technologies have to be explored (Bandara et al., 2001; Espuglas et al., 2002; Ghaly et al., 2001; Tang and Huang, 1996). In this context, and according to the strategy proposed in this thesis, AOP was selected for treatment of phenolic substances.

In chapter 4, TiO_2 photocatalysis is considered for the removal of *p*-halophenols (see Scheme 1.2) in contaminated water, with the aim to better understand the chemical mechanisms involved during their photocatalytic degradation. For that purpose, structure-activity relationships and molecular descriptors are employed to characterize photodegradability of *p*-halophenols. Toxicological properties are also investigated. Phenol is used as reference compound in order to assess the implication of halogen in the photodegradation of *p*-halophenols.



Scheme 1.2. Structures of the phenolic substances studied in Chapters 2 and 4.

1.4.3. Pesticides

Advancing increase of production and application of pesticides for agriculture as well as for plant protection and animal health has caused the pollution of soil, ground and surface water which involves a serious risk to the environment and also to the human health due to direct exposure or through residues in food and drinking water (Ma et al., 2005; Ma et al., 2002).

In the world, alarming levels of pesticides have been reported to be persistent, toxic, mutagenic, carcinogenic, and tumorogenic (Costa, 2005). Pesticide contamination of water systems has been of major concern in recent years. Pesticide residues reach the aquatic environment through manufacturing plants, direct surface run-off, leaching, careless disposal of empty containers, equipment washings, etc. (Canna-Michaelidou and Nicolaou, 1996).

Pesticides are divided into 3 families depending on the living organisms that they target. They can also be classified according to their molecular structure (organochlorine, organophosphorus, phenyl urea, etc.). Due to the wide range of pesticides it is extremely difficult to apply one single method for pesticide removal.

Chemical treatments can be inefficient for the removal of synthetic organochlorine pesticides from waters and may also lead to hazardous final products (Drzewicz et al., 2004; Chiron et al., 2000). On the other hand, advanced water treatment processes, and mainly the adsorption onto activated carbon, have proved to be efficient and reliable methods for removal of aqueous-dissolved organic pesticides. The main drawbacks of these techniques are their cost and the fact that substances remain untouched.

In recent years, the ability of microorganisms to metabolize some pesticides has also received much attention due to the environmental persistence and toxicity of these chemicals. Although in some cases, microbial metabolism of contaminants may produce toxic metabolites, a variety of microorganisms (mainly aerobic bacteria and fungi) are known to utilize organic pesticides as the sole carbon or energy source, but in specific conditions (Perrin-Ganier et al., 1996). Practically conventional activated sludge systems often fail to achieve high efficiency in removing pesticides from wastewater. It is mainly

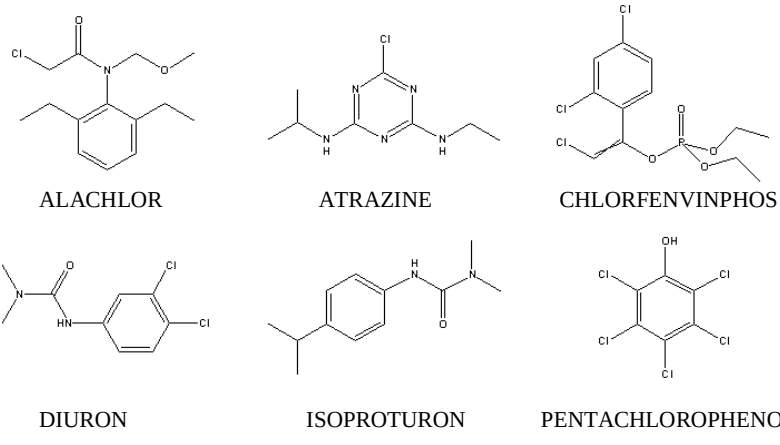
due to the low biodegradability of organic pesticides, their toxicity or their tendency to inhibit microorganisms (etkauskaité et al., 1998).

Besides the Advanced Oxidation Processes, and particularly the photocatalytic processes, were shown particularly efficient for degradation of the organic pollutants which are recalcitrant to conventional treatment because of their high chemical stability and/or low biodegradability (Gogate et al., 2004a; Konstantinou et al., 2003; Pera-Titus et al., 2004; Rodriguez et al., 2002; Sarria et al., 2002).

Due to their relatively high operational costs compared to biotreatments, their use as a pre-treatment step has been considered as a solution to enhance biodegradability of problematic wastewater. Such a technology is justified if the intermediates resulting from the degradation of the toxic or recalcitrant compounds are readily degraded by microorganisms. Therefore, combinations of AOP as preliminary treatments with inexpensive biological processes become very promising from an economical and environmental point of view (Pulgarin et al., 1999; Parra et al., 2002; Pulgarin and Kiwi, 1996).

Several water-soluble pesticides were selected: Alachlor, Atrazine, Chlorfenvinphos, Diuron, Isoproturon and Pentachlorophenol (of special interest because of their extremely easy transport in the environment, seriously threatening all surface and groundwater).
These substances are included among the Priority Substances (PS) listed by the European Commission (OJEC, 2001).
Their molecular structure is illustrated in Scheme 1.3.

In chapter 2, the above-mentioned pesticides are tested for their biodegradability.
As their biotreatability is shown difficult to achieve, the third case proposed by the strategy (Figure 1.5), i.e. the integrated photocatalytic-biological treatment, is applied to the pesticides.
Chapter 5 is devoted to present these experiments.



Scheme 1.3. Structures of the pesticides studied in Chapters 2 and 5.

The basis of the work dealing with pesticides is three-fold:

1. Assess the feasibility of the integrated photocatalytic-biological technology for the removal of the single or mixed pesticides using several analytical tools and testing methods,

2. Improve the modelling relation between chemical, biological and toxicological parameters,

3. Optimize the coupling strategy, that is to say select the best moment for coupling.

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CHAPTER 2.

Biodegradability Assessment of Several Priority Hazardous Substances: Choice, Application and Relevance Regarding Toxicity and Bacterial Activity

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2.1. Abstract

Nineteen compounds listed in the category of Priority Substances (PS) were selected for a biodegradation study using standardized tests. The compounds consist of pesticides, chlorinated solvents and Volatile Organic Compounds (VOCs). In this chapter, the choice of the most suitable method is discussed in relation to the physico-chemical properties of each substance. Zahn-Wellens, Manometric Respirometry and Closed-Bottle tests are alternatively used. Experimental results are presented and interpreted. Toxicity (Microtox^{TM}) and bacterial viability (Bac-light^{TM}) are also used as tools to investigate the influence of each substance on the microbial population (activated sludge). In addition, experimental values are compared with predictive data calculated according to Quantitative Structure Activity Relationships (QSARs) models. *Biodeg Models* were permitted to correctly estimate 17 substances; *Survey Models* and screening tests also revealed the same behavior for 16 target compounds.

2.2. Introduction

Chemical release into environment is one of the major patterns of the anthropogenic pollution. Xenobiotics tend to persist in the natural cycles unless they are degraded. Therefore developing efficient treatment systems enables to reduce pollution, since these systems possibly lead to innocuous substances. Considering both natural and laboratory-controlled scales, bacteria are the most significant tool involved in ultimate

biodegradation (Howard et al., 1975; Alexander, 1994). For that topic, mixed microbial culture prevail over pure cultures. Indeed even if pure cultures are useful in identifying possible pathways of degradation, organisms isolated by enrichment may not necessarily be active in the environment.

Biodegradability prospect of a target substance has to meet three main ecological points (Pitter and Chudoba, 1990): Is bacterial adaptation necessary? What are the rate and the degree of biodegradation under given conditions? Is it possible to reach the ultimate mineralization or are toxic compounds produced? To answer these questions, many testing strategies have been evolved. In 1990, Pitter and Chudoba proposed a classification in accordance with the Organization of Economic Cooperation and Development (OECD, Paris).

Three groups of tests were defined:

- ready biodegradability (or screening),
- inherent biodegradability, and
- simulation.

Screening tests indicate if a compound is degradable under natural conditions without any problem. Six methods permit the screening of chemicals for ready biodegradability in an aerobic aqueous medium: Dissolved Organic Carbon (DOC) Die-Away, Modified OECD Screening -DOC Die-Away, Carbon dioxide (CO_2) Evolution (Modified Sturm Test), Manometric Respirometry, Closed Bottle and MITI (Ministry of International Trade and Industry -Japan). These informative tests basically discriminate readily biodegradable compounds from others, but they often underestimate the potentiality of degradation in environmental systems.

Therefore when the result is negative, inherent (potential) biodegradability tests are required. In this category of tests, the concentrations of both the target compound and inoculum (usually activated sludge) are higher than with the screening tests, and the ratio is shifted in favor of microorganisms which are adaptable for long periods.

Inherent biodegradability tests provide information concerning the biotraitability of a substance. For example, the Zahn-Wellens procedure is an interesting test to prospect the

"behavior" of a chemical in an activated sludge treatment plant, since the experimental conditions are similar to this process.

Finally, simulation test methods confirm the positive or negative results of the previous tests.

Reproducibility and reliability of all the tests were improved consequently with the use of standardized protocols. Indeed, some test guidelines were developed by various national and international organizations such as OECD, U.S. Environmental Protection Agency (EPA) Office of Pollution Prevention and Toxics (OPPT), and the European Commission. They list experimental conditions, analytical methods, and criteria for whether a chemical is considered to be biodegradable (pass) and non-biodegradable (fail) (Howard et al., 1987).

Nevertheless sometimes it is difficult to obtain precise informative data concerning the biodegradability of pesticides or volatile organic compounds (VOCs) (Cowan et al., 1996). Literature still provides heterogeneous results, mainly due to various initial concentrations of target chemicals or to the many studies devoted to natural or industrial matters. However a methodology for translating OECD tests to degradation rates into realistic environmental situations has been presented, but only applied to 20 chemicals (Struijs and Van den Berg, 1995) or a limited number of substances (Federle et al., 1997).

Since the 1980s, many studies have prevailed to build up estimation programs which able to provide qualitative and/or quantitative information concerning the biodegradability characteristics of chemicals (Howard, 2000). They aimed to bypass experimental limitations and to better understand the implemented mechanisms. However, probably due to their novelty, only a few external validations have been completed until now (Boethling et al., 2003).

In this chapter, first, a procedure for selecting the most suitable test of biodegradability regarding the physico-chemical properties of 19 xenobiotics and considering a processing in an industrial wastewater treatment plant (WWTP) is discussed. Then the adequate methods for the testing of the biotraitability of the target compounds are presented. Finally the results of the experimental biodegradability testing are commented and

compared with some estimates which are calculated with the most relevant predicting methods according to the review completed by Howard (2000).

The 19 xenobiotics consist of seven medium/high soluble pesticides (Alachlor, Atrazine, Chlorfenvinphos, Diuron, Isoproturon, Lindane and Pentachlorophenol) listed as Priority Substances (PS) according to the European Commission (OJEC, 2001). The others are chlorinated solvents (Dichloroethane, Dichloromethane), Volatile Organic Compounds, VOCs (Chlorobenzene, *o*-Dichlorobenzene, Toluene, Ethylbenzene and *o*-, *m*-, *p*-Xylenes). Finally three other pharmaceutical substances were also tested ("T824", $C_{11}H_{11}Cl_2N_2O$, (±)-alpha-(2,4-dichlorophenyl)-1H-imidazole-1-ethanol; "R113675"; $C_{17}H_{21}NO_3$; (-)-[4aS-(4a alpha, 6 beta, 8aR*)]- 4a, 5, 9, 10, 11, 12- hexahydro- 3- methoxy- 11- methyl- 6H-benzofuro [3a,3,2-elf] [2] benzazepin- 6-ol hydrobromide; "FEMAM", $C_9H_{11}NO_2$, alpha-methyl-alpha-amino-phenyl-acetinamide).

2.3. Material and methods

2.3.1. Chemicals

The pesticides Alachlor (purity: 95%), Chlorfenvinphos (93.2%) and Diuron (98.5%) were supplied by Aragonesas Agro (Spain); Atrazine (95%) and Isoproturon (98%) by Ciba (Switzerland); Lindane (90%) by Exagama 90 Rhône-Poulenc (France) and Pentachlorophenol (98%) by Aldrich (Switzerland). Dichloromethane (99.8%), Dichloroethane (99.5%), Chlorobenzene (98%) and *o*-Dichlorobenzene (>98%) were from Merck (Switzerland). Toluene (99.5%) and Ethylbenzene (99.8%) were provided by Acros Organics (Belgium) and *o*-, *m*- and *p*- Xylenes (>98%) were from Fluka (Switzerland). Other chemicals $C_{11}H_{10}Cl_2N_2O$ "T824", $C_{17}H_{21}NO_3$ "R113675" and α-methylphenylglycynamide "Femam" were provided by pharmaceutical industries. Their physico-chemical properties are presented in Table 2.1. Glucose (97%) was from Sigma (Switzerland). Diethylene glycol $C_4H_{10}O_3$ (>98%) used as reference compound in the Zahn-Wellens test came from Fluka (Switzerland).

Table 2.1. Physico-chemical properties of the target compounds (Tomlin, 1997; Boublick and Cervenkova, 1984; SRC, Hansch and Leo, 1995). P_{atm} = 1 atm = 760 mmHg

Compound	Molecular formula	Molecular weight, g/mol	Solubility in water at 25°C, mg/l	Vapor pressure, mm Hg at 25 °C	Octanol-water partition coefficient, log K_{ow}
Alachlor	$C_{14}H_{20}ClNO_2$	269.77	239	$2200\ 10^{-8}$	3.52
Atrazine	$C_8H_{14}ClN_5$	215.69	34	$28.9\ 10^{-8}$	2.61
Chlorfenvinphos	$C_{12}H_{14}Cl_3O_4P$	359.58	125	$750\ 10^{-8}$	3.81
Chlorobenzene	C_6H_5Cl	112.56	490	12.00	2.84
o-Dichlorobenzene	$C_6H_4Cl_2$	147.00	150	1.50	3.38
Dichloroethane	$C_4H_8Cl_4$	197.92	5101	-	1.83
Dichloromethane	CH_2Cl_2	84.93	13001	435.00	1.25
Diuron	$C_9H_{10}Cl_2N_2O$	233.10	41	$6.9\ 10^{-8}$	2.68
Ethylbenzene	C_8H_{10}	106.17	160	9.75	3.15
FEMAM	$C_9H_{12}N_2O$	164.00	94300		
Isoproturon	$C_{12}H_{18}N_2O$	203.29	65	$2.47\ 10^{-8}$	2.87
Lindane	$C_6H_6Cl_6$	290.83	8	$4200\ 10^{-8}$	3.72
Pentachlorophenol	C_6HCl_5O	266.34	15	$11000\ 10^{-8}$	5.12
R113675	$C_{17}H_{21}NO_3$	368.27	31000		1.09
Toluene	C_7H_8	92.14	530	28.50	2.73
T824	$C_{11}H_{10}Cl_2N_2O$	257.12	131		2.67
o-Xylene	C_8H_{10}	106.17	170	6.75	3.12
m-Xylene	C_8H_{10}	106.17	160	8.25	3.20
p-Xylene	C_8H_{10}	106.17	180	9.00	3.15

2.3.2. Biodegradability methods

2.3.2.1. Zahn-Wellens test

The Zahn-Wellens test was carried out according to the EC protocol (Directive 88/302/EEC). The inoculum –activated sludge- came from a secondary effluent of the treatment plant of Morges (Switzerland). The fresh sample was centrifuged at 10 000 RPM during 7 minutes at 20 °C, and washed once with mineral medium.

The official protocol of the Zahn-Wellens test recommends that compound concentrations fit with the limits of the sensibility of the Dissolved Organic Carbon, DOC determination (50 mg_c/l) and also with the hydrosolubility thresholds of each substance (see Table 2.1).

According to the guidelines of the Zahn-Wellens test, the ratio between the carbon content of the tested substance and the dry-weight of the inoculum was ranged between 1 and 4 (experimental values averaged 3.3).

Aeration and homogenization were guaranteed.

Preliminary experiments were realized to check that neither volatilization nor adsorption occurred during the testing period.

2.3.2.2. Closed bottle and Manometric respirometry tests

The Closed bottle test (Directive 92/69/EEC), also called BOD_{28} – Biochemical Oxygen Demand, measured during 28 days – and the Manometric respirometry test (Directive 92/69/EEC) were executed using a Hg free WTW 2000 Oxitop© unit thermostated at 20 °C.

Experiments were carried out according to official protocols, except for the composition of the mineral medium (the same as for the Zahn-Wellens test) and the use of the Oxitop© material.

This equipment facilitated the manipulation and minimized the quantity of vessels required. Indeed when O_2 is consumed, the CO_2 produced is trapped by sodium hydroxide pellets and a depression in the bottle is automatically recorded.

The volume of the solutions poured in the bottles was determined according to the theoretical oxygen demand without nitrification ($ThOD_{NH3}$). The formula is:

$$ThOD_{NH_3} = \frac{16\left(2c + (h - cl - 3n)/2 + 3s + 5p/2 + na/2 - o\right)}{M},$$

with C_c H_h Cl_{cl} N_n Na_{na} O_o P_p S_s, the formula of the test compound, and M, its molecular weight.

The average concentration of biomass was 25 mg of suspended solids per liter of mineral nutrient medium.

2.3.3. Initial concentrations

All the target compound concentrations were fixed to 50 mg/l, except for the FEMAM (500 mg/l) or whenever the hydrosolubility threshold was lower (Lindane 2 mg/l, Atrazine 20 mg/l, Pentachlorophenol 15 mg/l). These concentrations corresponded to carbon contents higher than the minimal threshold prescribed by the official guidelines for the biodegradability testing.

2.3.4. Analytical methods

Pesticide concentration was analysed using reverse-phase liquid chromatography (flow 1 ml/min) with UV detector in a HPLC-UV (Varian 9012, 9100, 9065) with an ODS-2 column (Waters 4.6x250 mm, from Phenomenex) and a guard column (Waters 4.6x10 mm): Alachlor (H_2O/Acetonitrile 40/60, 224nm), Atrazine, Isoproturon, Chlorfenvinphos and Diuron (Acetic acid(1%)/Acetonitrile 80/20-40/60 (0-30 min) and 40/60 (30-35 min), 249 nm).

Concerning Pentachlorophenol, the HPLC system was constituted by a LC-10 AT VP pump (Shimadzu) and a UV–Visible diode array detector (Agilent 1100 Series). The stationary phase was a Hypersil ODS Teknokroma column (250x4.6 mm). A methanol/water mixture (80/20) was used as the mobile phase, which was degassed by sonication and filtered (0.45 μm) before using.

The VOCs concentrations were determined by using a gas chromatograph (Varian Star 3400 Cx, Palo Alto, USA), equipped with a flame ionization detector and a DB-624 capillary column (30m x0,53mm) (J&W Scientific, Folsom, USA).

The Dissolved Organic Carbon, DOC was measured using a Total Organic Carbon, TOC analyser (Shimadzu model 5050A, Japan) calibrated with standard solutions of potassium hydrogen phthalate, sodium carbonate and sodium hydrogen carbonate. Limit of detection of the apparatus was 0.2 mg_C/l. The mineralization degree (degradation percent) corresponded to the ratio of DOC disappearance between sample and blank.

The pH was determined using a combined electrode and pH Meter from Radiometer Copenhagen PHM 92 (Denmark).

The toxicity was assessed using the Microtox™ test system. This test was carried out using a Microtox model 500 analyzer (Canada); this laboratory-based temperature controlled photometer (15–27 °C) maintains luminescent bacteria reagent (*Vibrio fischeri*) and test samples at the appropriate test temperature. This self-calibrating instrument measures the light production from the luminescent bacteria reagent. The sample toxicity is determined by measuring the effective concentration from which 50% of the light is lost due to compound toxicity (EC_{50}).
The Toxic Unit (TU) is used to express the data, as proposed by U.S. EPA (Lankford and Eckenfelder, 1990): $TU = 100/EC_{50}$.

The LIVE/DEAD® BacLight™ Bacterial Viability Kit L-7007 (Molecular Probes Corporation, Canada) provided a quantitative index of bacterial viability. In fact it uses a mixture of SYTO® 9 green fluorescent nucleic acid stain which labels bacteria with both intact and damaged membranes. Besides, the red fluorescent nucleic acid stain Propidium Iodide penetrates only bacteria with damaged membranes. The bacterial samples are centrifuged and filtered through 0.2 µm PC Memb 13 mm (Nucleopore CORP., Canada). The Epi-FluoroMicroscopic observation is carried out under UV light with emission filters BP 450-490 nm, reflector FT 510 and stop-filter LP 520 (Eclipse 800, Nikon, Switzerland).

In all cases the 28 day-tests were duplicated and gave similar results.

For each experimental sample, the chemical analyses were replicated thrice. Standard deviation was less than 5%.

In this study, reported results correspond to the average value of the measurements.

2.4. Results and discussion

2.4.1. Selecting the appropriate test of biodegradability

In this study biodegradability of 19 chemicals is prospected to assess their biotraitability in an industrial WWTP. Considering this objective, the Zahn-Wellens test is the most adequate procedure, because the experimental conditions are close to a WWTP process.

Unfortunately this method is inappropriate for many compounds due to their physico-chemical properties. Indeed this method is only applicable to water-soluble and non-volatile substances.

Concerning hydrosolubility, threshold is prescribed by the minimal carbon content of the tested solution. Since 50 mg of carbon per liter are requisite, Atrazine, Diuron, Lindane and Pentachlorophenol are out of range (Table 2.1).

Besides VOCs and many target pesticides tend to volatilize. More precisely without any value recommended in the Zahn-Wellens guidelines, compounds which present a vapor pressure included between the atmospheric pressure (P_{atm} = 760 mm Hg) and 7.5 10^{-2} mm Hg (threshold for VOC) can be considered as moderately volatile (Alachlor, Atrazine, Chlorfenvinphos, Lindane, Pentachlorophenol).

Therefore the use of the Zahn-Wellens protocol turns out to be inconsistent for these substances, as well as for VOCs.

Consequently two adapted Ready-Biodegradability methods were chosen to test them.
Actually target compounds are supposed biorecalcitrant or at least difficult to treat (they are defined as hazardous substances (OJEC, 2001)). So Ready-Tests experiments are likely to fail. However as previously stated, negative results obtained with Ready-Tests do not prove unbiotraitability, whereas positive results guarantee the feasibility of a biotreatment in an industrial WWTP.

Table 2.2. Test methods and results for the biodegradability assessment of the target compounds. Degradation percent is related with chromatographic results (1), DOC measurements (2) or theoretical oxygen uptake (3).

Test Methods	Compounds	Degradation (%)	Time (d)	Initial Concentration (mg/l)	Conclusion
Zahn-Wellens	Chlorfenvinphos	60 (1+2) 30 (1+2)	22 28	10-50 125	Inhibitive factor
	Diuron	<0 (2)	28	50	Cellular lyses
	FEMAM	16 (1+2)	28	500	
	Isoproturon	0 (1+2)	28	50	
	R113675	94 (2)	6	50	Biodegradation
	T824	<0 (2)	56	50	Cellular lyses, enzymatic lacks, no bacterial adaptation
Manometric Respirometry	Alachlor	0 (1+3)	28	50	Inhibitive factor
	Chlorfenvinphos	0 (1+3)	28	50	Cellular lyses, chlorine toxicity
	Dichloroethane	25 (1+3)	26	50	Microbial adaptation
	Dichloromethane	0 (1+3)	28	50	Cellular lyses
BOD_{28}	Atrazine	100 (1+3)	21	20	Biodegradation
	Chlorobenzene	73 (1+3)	20	50	Biodegradation
	o-Dichlorobenzene	<0 (1+3)	28	50	No cellular damage
	Ethylbenzene	81 (1+3)	14	50	Biodegradation
	Lindane	100 (1+3)	9	2	Biodegradation
	Pentachlorophenol	0 (1+3)	28	15	
	Toluene	93 (1+3)	14	50	Biodegradation
	m-, *p*-Xylene	85 (1+3)	14	50	Biodegradation
	o-Xylene	80 (1+3)	19	50	Biodegradation

Therefore a compromise is aimed between the more selective experimental conditions of the Ready-Tests and the unsuitable conditions of the Zahn-Wellens test.

To approximate to the Zahn-Wellens test conditions, two modifications are considered:

- a slight concentration of the test compound (due to its low hydrosolubility threshold)
- a closed system (in order to avoid volatilization).

In fact the slightly adapted screening tests applied are the Closed-Bottle Test (BOD_{28}) and the Manometric Respirometry. They are favored because both are operated in closed vessels.

In particular, the Closed-Bottle test lengthened up to 28 days is favorably disposed towards low hydrosolubility values.

Table 2.2 summarizes the different methods applied for each compound.

2.4.2. Zahn-Wellens test

The Zahn-Wellens method was used to test the biodegradability of Diuron, Isoproturon, and the three pharmaceutical compounds (R113675, T824 and FEMAM).

During 28 days, the contents of DOC were measured in both samples and blanks (only sludge in mineral medium). As indicated in the protocol, the mineralization degree, expressed as the “degradation percent”, was calculated regularly. It corresponds to the ratio of DOC disappearance between sample and blank, taking into account the initial carbon content in both sample and blank. In fact samples were performed every two or three days.

Values indicated in Figure 2.1 correspond to average values calculated from at least six experimental data. Standard deviation and experimental error were less than 0.05 mg_c/l. They are not pictured in order to clarify the experimental results.

Diethylene Glycol (DEG) was used as a reference substance to check the correct functioning of experimental system. As shown in Figure 1, DEG was degraded up to 70% in ten days, which validates the initial activity of the activated sludge used in the Zahn-Wellens test. One of the pharmaceutical compounds, R113675 was also degraded up to 94% after six days, with a constant pH. Therefore this substance is considered as

potentially biodegradable. On the contrary, no DOC was removed in 28 days for Diuron, Isoproturon, FEMAM and T824.

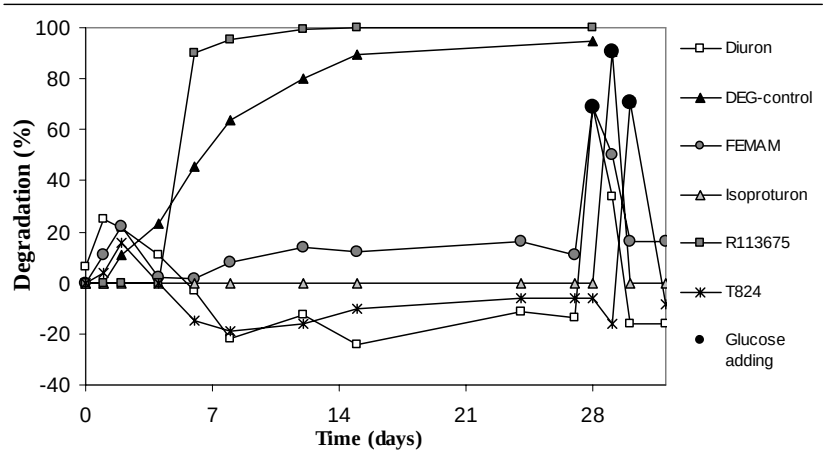

Figure 2.1. Zahn-Wellens biodegradability test. Evolution of the degradation percent as a function of time for R113675, T824, FEMAM, diuron, isoproturon and diethylene glycol DEG (control). Glucose adding is symbolized with the dark circles. Data are related with DOC analyses and correspond to the average value of at least six measurements.

First of all, Isoproturon biorecalcitrance confirms the conclusions obtained previously with the same test (Parra et al., 2002).

Regarding Diuron, this pesticide is considered as non-ready biodegradable, according to MITI database (www.cerij.or.jp/ceri_en/otoiawase/otoiawase_menu.html). Nevertheless many studies have been completed to improve biodegradation carried out by specific bacteria or fungi, such as *Aeromonas sp.*, *Arthrobacter sp.* and other commercially available strains (Tixier et al., 2000; Tixier et al., 2002).

Precise pathways have even been described (Turnbull et al., 2001) and many material supports have been tested: soil, natural waters, etc. (Sumpono et al., 2003). It means that microbial degradation can be performed with specific and acclimated strains and reinforces the need of biodegradability studies in order to assess the potential risks of

substances in the environment (Liu et al., 2000). In fact cellular lysis is suspected for bacteria unacclimated to Diuron and T824, because basification and carbon release were recorded during the experiment.

Furthermore, microscopic observations revealed that almost 80% of the bacterial population showed damaged membranes on day 28 with the BacLight™ assay. This statement needs to be distinguished from an apparent cellular damage due to starvation. Indeed it is likely that the bacteria have long ago used up any available nutrients, and have in fact been starving for a long time. But blanks (bacteria with mineral medium) revealed that only 20% of the total population was affected by this factor. So 60% of the damaged cells remained assigned to the presence of hazardous substances.

As presented in Figure 2.1, the consumption of glucose added after 28 days of experiment reveals that many cells able to degrade this easily biodegradable substrate were still viable.

Biorecalcitrance of T824 and Diuron in the tested conditions should more result from their structural stability towards microbiological attack rather than from the toxicity of the solution.

This statement was concomitant with the evolution of the overall toxicity of the tested solutions. For example, the Microtox™ assay carried out with T824-samples which were harvested on days 0 and 28 revealed that toxicity ($1/EC_{50}$) decreased from 14.5 to 0.4 TU in the course of the biodegradability testing (results not shown).

2.4.3. "Ready-biodegradability" tests

Ready-biodegradability testing was used to assess the biotraitability of 14 xenobiotics using two modified screening methods: BOD_{28} and Manometric Respirometry (Table 2.2).

The 14 target compounds evaluated with the tests fall into four classes. Degradation could be inexistent, complete or partial; the last case was shown to be favoured by bacterial adaptation or not.

Figure 2a indicates the evolution of the degradation percent for Dichloroethane and Dichloromethane (Manometric Respirometry) and for VOCs (BOD_{28}) during 28 days. Figure 2b illustrates the evolution of the degradation percent during the 28 days of

experiment, i.e. Manometric Respirometry for Alachlor and Chlorfenvinphos, and BOD_{28} for Pentachlorophenol, Atrazine and Lindane.

In both tests the degradation percent was evaluated according to the compound concentration (chromatographic analyses) and to the oxygen consumption due to the biodegradation of the target compound. Indeed oxygen consumption was measured in the closed vessels containing either compound with sludge, compound without sludge or sludge without compound. As for the Zahn-Wellens test (Figure 2.1), Figures 2.2a and 2.2b indicate average values in order to simplify them.

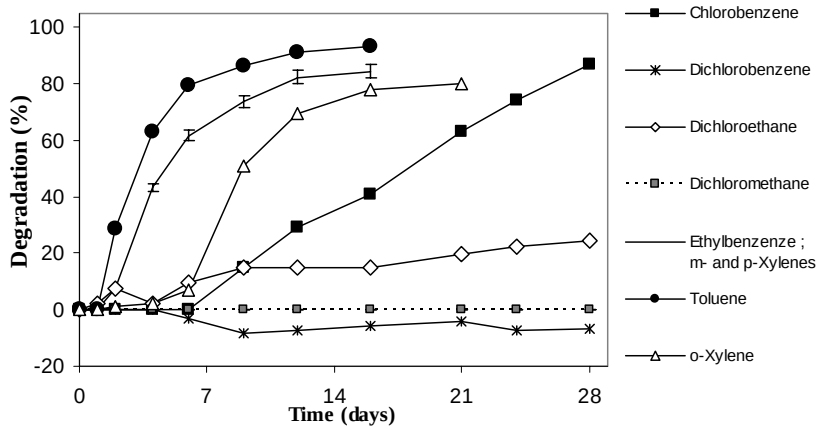

Figure 2.2a. Evolution of the VOC degradation percent during 28 days for the Ready-Biodegradability testing (BOD_{28}). Data are related with chromatographic results and correspond to the average value of at least six measurements.

First of all, VOCs illustrated in Figure 2.2a (Toluene, Ethylbenzene, *o*-, *m*- and *p*-Xylenes) passed over 70% of degradation in approximately one week. So they can be considered as biodegradable. Chlorobenzene was also degraded within 3 weeks. Both stripping and adsorption were avoided.

As shown in Figure 2a, Dichloroethane was degraded up to 25% in 26 days (GC analyses). This partial elimination performed in drastic conditions for biodegradation

testing indicates a possible biodegradation in a WWTP. A faster process would certainly be achieved with a pre-adapted biomass. This finding is in consistency with a reported bio-oxidative activity of wastewater microbiota under a specific incubation method, which led to the same range of degradation (Tabak et al., 1981).

On the contrary, no degradation of Dichloromethane was observed after 28 days of experiment (chromatographic analyses and oxygen depletion measurements). During the testing of Dichlorobenzene, carbon release has been observed, due to cellular lyses.

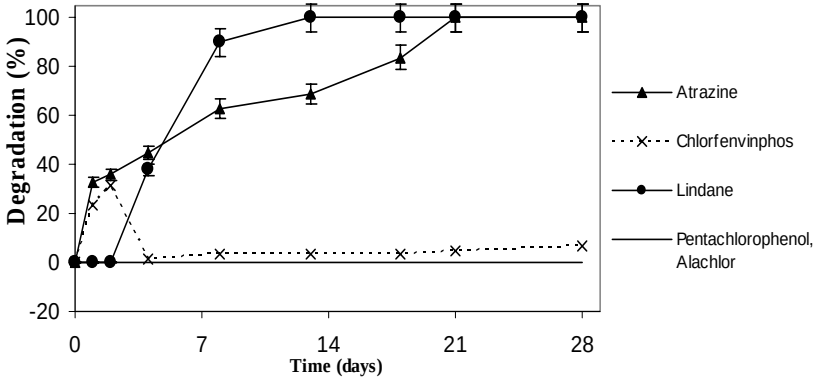

Figure 2.2b. Evolution of the degradation percent during 28 days for the Ready-Biodegradability testing of Alachlor, Chlorfenvinphos (Manometric Respirometry), Atrazine, Lindane and Pentachlorophenol (BOD_{28}). Data are related with chromatographic results and correspond to the average value of at least six measurements.

Concerning Pentachlorophenol and Alachlor (Figure 2.2b), neither oxygen consumption nor disappearance of the initial compound (HPLC analysis) were observed during 28 days. Thus, no degradation occurred and bacteria appeared to be inhibited, as glucose remained untouched when added after the test.

Alachlor was previously shown to inhibit the biodegradation ability of mixed bacterial cultures up to 18% at a 50 mg/l concentration (Zargoc-Koncan, 1996); and Pentachlorophenol is considered relatively resistant to biodegradation due to its high

chlorine content. Incidentally its biodegradation can be performed with pre-adapted bacteria (*Arthrobacter sp*., for eg.), but remains limited by the substrate inhibition (Klecka and Maier, 1985; Liu et al., 2000).

As illustrated in Figure 2.2b, Atrazine was degraded up to 100% within 21 days. This tendency is supported by previous results (Zagorc-Koncan, 1996). Particularly they showed that despite its harmful effect on algae, Atrazine (0-70 mg/l) caused no effect on biodegradation processes occurring with mixed bacterial populations.

The low hydrosolubility of Lindane (see Table 2.1 and Figure 2.2b) imposes a maximal concentration of 2 mg/l, which corresponds to an oxygen demand of 1,3 mg/l. Despite this minimal value, a depletion of oxygen was recorded and maintained after 7 days of experiment, taking into account blanks containing either biomass without Lindane or compound without inoculation.
The aerobic inactivity observed in the controls suggests a bacterial involvement. Furthermore, the recovered sludge was able to consume glucose, proving its state of health.
Kipopoulou et al. (2004) confirmed the occurrence of the combined sorption and biodegradation processes on sludge, which is different to photolysis, hydrolysis and air stripping (Mills, 1985; Dorussen and Wassenberg, 1997). They also strengthened that biosorption was the main removal process involved in the environment and that it was linked with the bioconcentration of Lindane.

Concerning Chlorfenvinphos, no degradation was observed after 28 days of experiment (Figure 2b). Despite the measured-20% losses due to volatilization, biodegradation has occurred up to 60% within 22 days for 10 to 50 mg/l of Chlorfenvinphos, and only up to 30% for 125 mg/l (results not shown). Each bacterial culture exhibited a first step of cellular lyses detected by microscopic observations (damaged cells), basification of the cultivation medium and carbon release. The lower the ratio compound/bacteria, the further the biodegradation occurred. So it seems that Chlorfenvinphos implies irreversible cellular damages, which can be overwhelmed if the bacterial population is concentrated enough.

Experimental conclusions are summarized in Table 2.2.

2.4.4. Predictive estimation models

Some predictive models can provide screening-level estimates of biodegradability. Their benefits consist in their large applicability, their relevance and their obvious technical advantages. Their development also results from many studies devoted to link the chemical structure of a compound with its biodegradability (Mani et al., 1991).

Howard (2000) reviewed the most reliable estimation methods. In this study, five of them were applied to each of the 19 target compounds and the conclusions were matched to the experimental data obtained with the Zahn-Wellens and Ready-Biodegradability tests stated above.

2.4.4.1. Qualitative Substructure Model

First, the Qualitative Substructure Model, QSM (Niemi et al., 1987) focuses on the presence of some specific structural sub-units which are associated with predicting half-lives ($T_{1/2}$).

This method was built on the basis of BOD data converted into $T_{1/2}$. For eg., a chemical with a theoretical BOD of 50% or greater in five days was assumed to have a $T_{1/2}$ of five days and a 25% theoretical BOD in ten days corresponded to a $T_{1/2}$ of 20 days.

Using these data, specific structural units exhibited by persistent chemicals were identified and classified.

In this study, each target compound was divided into its molecular sub-units. Each of them was associated with the corresponding $T_{1/2}$ as indicated in the QSM. This analysis revealed that every target compound had at least one persistent molecular segment.

As indicated in Table 2.3, QSM overestimated $T_{1/2}$ for only one compound, R113675.

In fact, the estimated $T_{1/2}$ greater than 100 days was due to the highly branched molecular structure of R113675, but experimentally, the Zahn-Wellens test showed that the compound was biodegradable in approximately six days (Table 2.2).

Conversely the presence of a “benzene ring with at least two substitutions (non-hydroxyl) and a log K_{ow} >2.18” (Niemi et al., 1987) appeared to be a crucial molecular characteristic for many of the studied compounds. This characteristic corresponds to a

half-life longer than 100 days in the QSM and indeed each involved molecule was experimented biorecalcitrant with our screening-tests (Alachlor, Chlorfenvinphos, Diuron, *o*-DCB, Isoproturon, T824 and Pentachlorophenol, see Table 2.2).

So it can be concluded that experimental data agreed with QSM predictions for 18 xenobiotics.

2.4.4.2. Biodegradability Probability Program

The second type of predictive methods concerns the Biodegradability Probability Program (Howard et al., 1992; Boethling et al., 1994), which requires more calculation.
In these four models, some coefficients are attributed to specific structural fragments. Then they are introduced into mathematical formulas.

Biodeg Models, also named BioWIN 1 and 2 (Boethling et al., 2003) provide coefficients which were developed by linear (BW1) and non-linear (BW2) regressions. They forecast a "fast" or a "not fast biodegradation".

Concerning the Survey Models, also named BioWIN 3 and 4, they distinguish primary biodegradation (BW3) from ultimate biodegradation (BW4).

In this study, fragment coefficients were calculated separately for each target compound according to the proposed mathematical equations and taking into account both types and quantities of specific molecular fragments. Then a probability of biodegradation was calculated, depending on molecular weight, empirical equation constants and fragment coefficients.

All detailed information concerning the specific fragments and their regression-derived coefficients are provided in the corresponding model descriptions (Boethling et al., 2003; Howard, 2000).

Table 2.3 summarizes the results obtained for the biodegradability assessment of the 19 xenobiotics using the four BioWIN models (noted BW 1-4), the Qualitative Structural Model (noted QSM) and the Zahn-Wellens and screening tests (noted Exp).

Table 2.3. Comparison of experimental results and estimated data for the biodegradability assessment of the 19 xenobiotics (Exp:Experimental, NB:NonBiodegradable, B:Biodegradable, QSM:Qualitative Structural Model ($T_{1/2}$ indicated in days), BW:BioWIN, F:Fast, NF:Not Fast, D:Days, W:Weeks, M:Months)

Compounds	Biodegradability Assessment Methods						Conclusion
	Exp	QSM ($T_{1/2}$)	BW 1	BW 2	BW 3	BW 4	
Alachlor	NB	>100 d	NF	NF	W	M	+
Atrazine	**B**	>100 d	NF	NF	W	M	-
Chlorfenvinphos	NB	>100 d	NF	**F**	W	M	+/-
Chlorobenzene	B	>5 d	F	**NF**	D	W	+/-
o-Dichlorobenzene	NB	>100 d	NF	NF	W	M	+
Dichloroethane	NB	>15 d	NF	NF	W	M	+
Dichloromethane	NB	>15 d	NF	NF	W	M	+
Diuron	NB	>100 d	NF	NF	W	M	+
Ethylbenzene	B	2-15 d	F	F	DW	W	+
FEMAM	NB	**>15 d**	**F**	**F**	W	**W**	-
Isoproturon	NB	>100 d	**F**	**F**	W	M	+/-
Lindane	**B**	>40 d	NF	NF	W	M	-
Pentachlorophenol	NB	>100 d	NF	NF	W	M	+
R113675	B	**>100 d**	F	F	W	**M**	+/-
Toluene	B	15 d	F	F	D	W	+
T824	NB	>100 d	NF	NF	W	M	+
m-, *p*-Xylene	B	2-15 d	F	F	D	W	+
o-Xylene	B	2-15 d	F	F	D	W	+

First, if each compound is considered separately, the comparison between the five estimates shows that the five predictive methods give coherent results for every target compounds, except for Chlorfenvinphos, Chlorobenzenze and R113675.

In fact, Chlorfenvinphos was expected to biodegrade fast according to the sole BW2, because this model attributed an excessive contribution to the phosphate ester group. In this case, both experiments and other models invalidated this prediction.

Conversely, BW2 attributed a slow biodegradability to Chlorobenzene because of the presence of the aromatic chloride.

Concerning R113675, it was due to the highly branched molecular structure which had already been noticed by the QSM. However, as for Chlorfenvinphos, the other results obtained for Chlorobenzene and R113675 were opposite.

Thus the five predictive models provided coherent results for 16 xenobiotics.

Secondly, the comparison between experiments and estimates shows that experiments agreed with predictions for 14 xenobiotics.

In fact, Atrazine and Lindane were surprisingly experimented biodegradable whereas they were predicted resistant. Their ability to be degraded by a bacterial community was discussed previously. However since the predictions were pessimistic, no environmental problem would have resulted from this inaccuracy.

On the contrary, FEMAM and Isoproturon were determined degradable according to models but were experimented biorecalcitrant with the Zahn-Wellens test.

For these two substances, BW1 and BW2 performed poorly.

Concerning Isoproturon, BW4 and QSM predicted that mineralization would be achieved in three months at least, and so the compound was presented rather resistant, which was not the case for Femam. The remark concerning BW1 and BW2 was already noticed by Rorije et al. (1999). The authors also explained that both consistency and overall accuracy could be improved by optimizing the classification criterion for these two models using the MITI data (Tunkel et al., 2000).

Finally the overall results suggest that the QSM and Survey Models (BW3 and 4) are generally suitable for use in biodegradability assessment, regarding a consecutive industrial WWTP process.

2.5. Conclusion

In this chapter, the most suitable procedures for testing biodegradability of 19 xenobiotics were selected regarding the physico-chemical properties of the compounds and considering a consecutive processing in an industrial wastewater treatment plant (WWTP). The adequate methods have been successfully performed in order to test the substance biotraitability.

In this study experimental results agreed with predictions for 16 xenobiotics, whatever their biodegradability may be.

The usefulness of estimation models was shown to be considerably increased whenever several methods are performed comparatively.

This conclusion reinforces the strategy suggested by Boethling et al. (2003) who proposed to allow prediction only when several models give unanimous results.

By this approach, screening level experiments may be avoided with limited environmental risks.

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CHAPTER 3.

Biodegradation of Volatile Organic Compounds

PART A.

ENHANCING PRODUCTION OF ADAPTED BACTERIA TO DEGRADE CHLORINATED AROMATICS

Accepted for publication in *Industrial & Engineering Chemistry Research*

3.A.1. Abstract

The study was aimed to enhance the production of adapted bacteria for degrading chlorobenzene (CB) and *o*-dichlorobenzene (DCB). Batch cultures fed by pulses of substrate (SPB, Substrate Pulse Batch process) were successfully performed in a 12 liter-bioreactor: 240 mg l^{-1} h^{-1} of CB/DCB mixture was eliminated and 50 mg l^{-1} h^{-1} of dry weight produced. Studying the substrate removal dynamics, concentration of metabolic intermediates, chlorides and cellular integrity allowed the correct proceeding of bacterial cultivation. The substrate loading rate was optimized to obtain the highest biomass productivity with the lowest toxicity and inhibiting effects. The time interval between each pulse was decreased gradually so that a continuous substrate feeding was finally attained. In the continuous feeding technique, both cell productivity and CB/DCB elimination capacity were twice the values obtained with the SPB. Results come up to industrial prospects and present the continuous feeding technique as an attractive adaptation of SPB to degrade chlorinated solvents.

3.A.2. Introduction

At present, the purification of industrial waste air containing volatile organic compounds (VOCs) increasingly refers to microbiological methods, due to the rapid development of industrial biotechnology. The use of biofilters is based on the microbial degradation of organic substrates (xenobiotics) to carbon dioxide and water. Contrary to chemical methods (catalytic or high-temperature burning), adsorption or filtration through selective membranes which only transfer the pollution, the microbial mineralization allows a sustainable pollution control of the environment. Biological techniques for waste gases purification are operated onto a great variety of organic compounds, including chlorinated substances. Indeed chlorinated benzenes are widely used as organic solvents, heat transfer agents and insecticides, and their production as intermediates in the synthesis of chemicals, such as pesticides and dyes also contributed to spread out these compounds into the environment (Pearson, 1982). In particular Chlorobenzene (CB) and 1, 2-dichlorobenzene (DCB) were identified as priority pollutants by the US Environmental Protection Agency (Oh and Bartha, 1994).

The biomass responsible for the degradation of the gaseous contaminant fed to the biofilter can be supplied by the support material itself (Barona et al., 2004). Nevertheless, the filter-bed inoculation with active microorganisms is a frequent and necessary prerequisite for successful operation (Kennes and Thalasso, 1998; Kennes and Veiga, 2001). Consequently, prior to the inoculation itself, the proper selection, acclimation and activation of the microorganisms is crucial to ensure the successful operation of the biofilters. Although pure strains of microorganisms can be purchased, mixed cultures can be obtained from the sludge originated in wastewater treatment plants or similar origins (Prado et al., 2003; Prado et al, 2005). Once the biomass is in the laboratory, the best process to grow specific strains was shown to be batch or microcosms cultures (León et al, 1999).

This study was aimed to adopt Substrate Pulse Batch (SPB) technique for the efficient biomass production for the biodegradation of chlorobenzene (CB) and *o*-dichlorobenzenze (DCB). SPB technique is a batch cultivation process in which substrates are supplied in pulses. This technique can be considered as a special case of a

conventional, semi-continuous extended culture operation: total volume remains virtually constant because substrate is fed in a gaseous, solid or very concentrated liquid form, according to an intermittent profile.

In this chapter, the substrate loading rate in SPB technique was optimized to obtain the highest biomass productivity with the lowest toxicity and inhibiting effects. For this purpose the time interval between each pulse was decreased gradually so that a continuous substrate feeding was finally attained. Finally, the results of SPB technique were compared with that of continuous process in terms of CB/DCB elimination capacity and biomass productivity.

3.A.3. Materials and methods

*3.A.*3.1. Inoculum Preparation

The bacterial consortium used to inoculate the bioreactor was selected in our laboratory in a previous work (Seignez et al, 2001). The bacteria were originally issued from the sludge of a wastewater treatment plant (Novartis and Rohner AG, Basel, Switzerland) and from the biotrickling filters treating chlorinated aromatic solvents and preserved at -80°C (Rohner AG, Basel, Switzerland).

A sample of this adapted mixed culture was cultivated in flasks containing nutrient medium (CM67, Oxoid LTD, Basingtoke, England) at 30°C for 1 day.

Then the obtained biomass was collected by centrifugation and transferred into the batch reactor.

The nutrient medium consisted of inorganic salts and vitamins necessary for the growth of microorganisms, the same composition as previously reported with ammonium as the nitrogen source (León et al, 1999). The medium was composed of: 3.3 g l^{-1} K_2HPO_4, 1.9 g l^{-1} $NaH_2PO_4.2H_2O$, 4.5 g l^{-1} $(NH_4)_2SO_4$, 0.2 g l^{-1} $MgSO_4.7H_2O$, 0.5 g l^{-1} $CaCl_2.2H_2O$, 10 ml l^{-1}trace-elements and 2.5 ml l^{-1} vitamins. The trace-element solution contained 5 g l^{-1} $CaCl_2.2H_2O$, 1 g l^{-1} EDTA.$2H_2O$, 1 g l^{-1} $FeSO_4.7H_2O$, 160 g l^{-1} $MnCl_2.4H_2O$, 40 g l^{-1} $ZnSO_4.7H_2O$, 30 g l^{-1} H_3BO_3, 40 g l^{-1} $CoCl_2.2H_2O$, 40 g l^{-1} $CuCl_2.2H_2O$, 4.6 g l^{-1} $NiCl_2.H_2O$ and 40 mg l^{-1} $NaMoO_4.2H_2O$. The vitamin solution consisted of 8 g l^{-1} biotin,

100 mg l^{-1} p-aminobenzoic acid, 0.2 g l^{-1} nicotinic acid, 0.2 g l^{-1} thiamin-Cl, 0.2 g l^{-1} Ca-pantothenate, 0.2 g l^{-1} pyridoxine-HCl, 20 mg l^{-1} cyanocobalamin, 80 mg l^{-1} riboflavine, 40 mg l^{-1} folic acid, 0.2 g l^{-1} choline-Cl and 0.8 g l^{-1} myo-inositol.

The sole carbon and energy source consisted in 80.8 % (m/m) chlorobenzene (CB) and 19.2 % *o*-dichlorobenzene (DCB). The volumetric ratio of 4:1 corresponds to industrial gas effluents emission of two CBs from Rohner SA chemical industry (Basel, Switzerland). Compounds were supplied by Fluka, Buchs, Switzerland. Experiments were made under non-sterile conditions. The volatile organic carbons (VOCs) i.e., CB and *o*-DCB were supplied by a precision feeding pump (SpectraPhysics, San José, Calif.).

3.A.3.2. Bioreactor and Automation Program

The experimental set-up used in this study is presented in Figure 3.A.1.

Bioreactor is made of stainless steel, with a total volume of 20 l. The working liquid volume was 12 l. Numerous ports along its height and at the bottom allow feeding, sampling and connecting the measuring probes.

The characteristics of the reactor and the operating conditions for the experiments are described in Table 3.A.1.

The overall set-up was monitored by a system of programmable relays (Bioline, Inceltech, F-Toulouse).

Table 3.A.1. Operational data for the bioreactor

Parameter	Value	Material
Temperature	30 °C	PT 100, *Digital thermostat*
pH	7 +/- 0.2	Mettler Toledo, CH-Greifensee
Dissolved Oxygen	0.8-98 %	Oximetric probe (L=16.4 cm)
(DO)	*100 %=7.14 mg/l*	Mettler Toledo, CH-Greifensee
Agitation	400 RPM	2 Rushton impalers
Aeration	5 l/min Recirculation 13 l/min K_{La}*=34* h^{-1}	Diaphragm pump, Haussman pumpen, CH-Schlieren

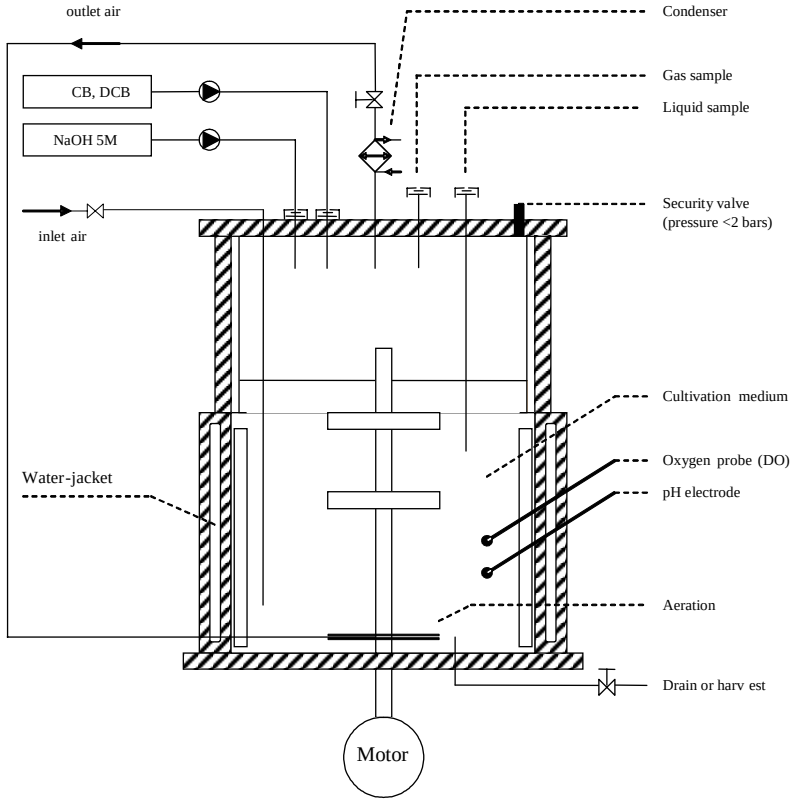


Figure 3.A.1. Schematic presentation of the laboratory bioreactor

*3.A.*3.3. Analytical methods

Concentrations of CB and DCB were determined by capillary gas chromatography (Varian Star 3400 Cx, Palo Alto, USA; Integrator C-R65A, Shimadzu, France), with a flame ionization detector fitted and a DB-624 capillary column (30 m length, 0.53 mm diameter) (J&W Scientific, Folsom, California). Samples were analyzed at a 5.8 ml/min nitrogen flow rate and at 30 ml/min split injection. Injection and detector temperatures were 220°C. Oven temperature was increased at a rate of 10°C/min from 80°C to a final

temperature of 145°C, which was maintained for 2.5 min. Retention times were 4.5 and 7.8 min for CB and DCB, respectively. The culture was sampled by means of a 1 ml Gas-tight syringe (Dynatech, Serie A2, Bâton-Rouge, USA).

Chloride and ammonium ions concentrations were determined photometrically with a flow injection analyzer (FIA, Tecator, Höganas, Sweden) at 463 and 590 nm, respectively. Conductivity was determined directly in the culture medium with a conductimeter (WTW, Weiheim, Germany). Concentrations of water-soluble metabolites were measured by high performance liquid chromatography (HPLC) (Varian 910, Palo Alto, USA), on a ORH801 column (Interaction chromatography Inc., San Jose, USA) with sulfuric acid (0.01 N) as the mobile phase at a flow rate of 1ml min^{-1}. Compounds were detected by the IR refractivity index detector (Varian 9065 Polychrom, Palo Alto, USA) and their retention times were compared with *trans–trans* muconic acid p.a. quality, supplied by Fluka, CH-Buchs.

The accumulation of UV-absorbing metabolites was monitored by UV-spectrophotometer (U-2000, Hitachi, Tokyo, Japan) at 255 nm after filtration (0.22 µm filter, Schleicher and Schuell, G-Dassel). The dissolved organic carbon (DOC) concentration was measured after filtration, HCl-acidification and CO2 elimination, using a TOC analyzer (Shimadzu, Tokyo, Japan). The oxygen uptake rate was determined by measuring the concentration of dissolved oxygen in the culture media using a polarographic probe (Ingold AG, Urdorf, Switzerland) after reducing the agitation rate.

BOD_5 – Biochemical Oxygen Demand – was measured during 5 days using an Hg free WTW 2000 Oxitop© unit thermostated at 20 °C. Experiments were carried out according to official protocol (Dir 92/69/EEC, O.J. L383A). The volume of the solutions poured in the bottles was determined according to the theoretical oxygen demand without nitrification ($ThOD_{NH3}$). It corresponded to 97 ml of medium.
The acute toxicity was assessed using a Microtox™ Model 500 Analyzer. This analyzer is based on the use of a luminescent bacterium, viz. the strain *Vibrio fischeri* NRRL B-11177. When properly grown, luminescent bacteria produce light as a by-product of their cellular respiration. Any inhibition of cellular activity results in a decreased rate of respiration and a corresponding decrease in the rate of luminescence. The toxicity is

determined by measuring, after 5 and 15 minutes of exposure, the concentration at which 50% of the light is lost because of the toxicity of the solution examined (EC_{50}). The Toxic Unit (TU) is used to express the data, as proposed by U.S. EPA (Lankford and Eckenfelder, 1990): $TU = 100/EC_{50}$.

The LIVE/DEAD® BacLight™ Bacterial Viability Kit L-7007 (Molecular Probes Corporation, Canada) provides a quantitative index of bacterial viability. It uses a mixture of SYTO® 9 green fluorescent nucleic acid stain which labels bacteria with both intact and damaged membranes. Besides, the red fluorescent nucleic acid stain Propidium Iodide penetrates only bacteria with damaged membranes. The bacterial samples were centrifuged and filtered through 0.2 µm PC Memb 13 mm (Nucleopore CORP., Canada). The Epi-Fluoro-Microscopic observation was carried out under UV light with emission filters BP 450-490nm, reflector FT 510 and stop-filter LP 520 (Eclipse 800, Nikon, Switzerland).

Acute toxicity immobilization tests were performed with *Daphnia magna* according to OECD Guideline 202 (2004). Temperature was maintained at 20 °C and replicate groups of 10 samples were carried out.

VOCs lost by stripping were continuously quantified by FID analyses during the process. VOCs lost by stripping were taken into account for all the calculations.

The biomass concentration was monitored by dry-weight measurement according to Dutch standard methods (NEN 32355.3) and by absorbance at 650 nm.

3.A.4. Results and discussion

*3.A.*4.1. Substrate Feeding Pattern

The process of substrate feeding by pulse (SPB) consists of injecting a small but precise amount of CB/DCB mixture so that the global volume of the liquid medium remains constant, while bacteria are allowed to mineralize the substrate.

SPB cultivation process is characterized by the successive sequences of pulses. The total period of cultivation is determined by the overall behavior of the microbial community.

Usually bacteria are progressively inhibited by the accumulation of chlorides which results from the degradation of the chlorinated substances or by the production of intermediate compounds which are not converted into carbon dioxide and water.

The cultivation time consist of the repetition of a sequence which is called “pulse”.

Basically, one typical cycle started by aeration, until the medium was saturated by oxygen (overall 5 minutes were needed to achieve the oxygen saturated conditions). Then one pulse of substrate was added, with a special care to avoid CB and DCB stripping. Then the substrate concentration in the medium sharply decreased until total substrate disappearance.

A typical response of the process during one pulse is illustrated in Figure 3.A.2.

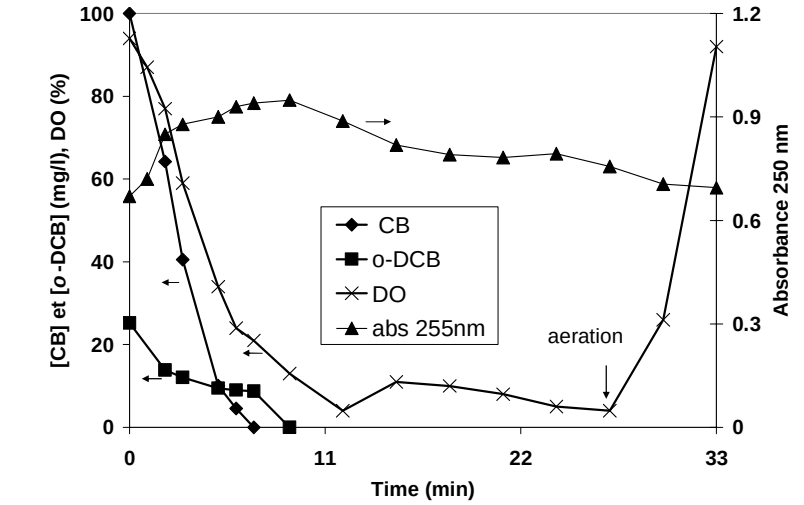


Figure 3.A.2. Disappearance of CB and DCB in the liquid phase, consumption of Dissolved Oxygen, and evolution of absorbance at 255 nm during one pulse.

As shown in Figure 3.A.2, by introducing the compounds to the reactor at t = 0, the oxygen concentration sharply decreased and remained constant even after disappearance of CB/DCB. The consecutive low oxygen concentration was stated to be due to the degradation of intermediate metabolites (León et al, 1999).

The maximum amount of substrate added by one pulse was determined by the available oxygen concentration in the liquid medium. The pulse duration was the time between two injections of substrate; it should be long enough to degrade the substrate and its metabolites in order to avoid their accumulation in the liquid phase and consequently their inhibitive effect.

During the first set of SPB, experiments were run with constant cycle time and loading rates of 35 min and 125 mg l^{-1} of CB/DCB mixture, respectively. Then the optimal biomass growth yield and productivity were achieved either by increasing the amounts of substrates added by each pulse, or by reducing the cycle time. It should be noticed that reducing cycle time results in a continuous approach.

In this study, both techniques (SPB and continuous feedings) were investigated and compared. The results will be discussed below in more details.

*3.A.*4.2. Parameters of Control

The correct proceeding of the bacterial cultivation requires regular analyses of concentration of metabolic intermediates, chlorides, ammonium, and cellular integrity in the system.

3.A.4.2.1. Metabolic intermediates

UV absorbance at 255 nm (A_{255}) measures the concentration of the aromatic intermediates produced consequently to the degradation of the chlorinated solvents. Seignez et al.[12] presented A_{255} as a reliable parameter to control and optimize the cultivation process. A_{255} can be indicative of the removal efficiency of the biosystems and was measured regularly. Intermediates such as muconic acid, mono and dichlorocatechols were analyzed by HPLC, using both columns, for aromatic and aliphatic detection. Scheme 3.A.1 illustrates the most relevant pathways used to explain CB and *o*-DCB degradation (Reineke and Knackmuss, 1984; Haigler et al, 1988). Proteins and dissolved organic carbon were also quantified during the experiment. Table 3.A.2 presents 3 typical metabolic behaviors observed during batch and continuous cultivation processes.

Table 3.A.2. Production of intermediate metabolites as the bacterial response to continuous or batch cultivation processes. Substrate loadings are indicated in parenthesis

Cultivation technique	**Intermediate productivity ($mg_c\ l^{-1}\ h^{-1}$)**	**Intermediate production rate ($mg_c\ g_{CB/dCB}^{-1}$)**	**Maximum intermediate concentration ($mg_c\ l^{-1}$)**	**Detected metabolites**	**Toxicity (DBO_5, *Daphnia)***
SPB (200 mg $l^{-1}\ h^{-1}$)	0.56 ± 0.05	10 ± 2	250	muconic acid	No
SPB (600 mg $l^{-1}\ h^{-1}$)	7.5 ± 0.08	370 ± 20	600	3-chlorocatechol dichlorocatechol	No
Continuous (200 mg $l^{-1}\ h^{-1}$)	1 ± 0.05	20 ± 10	200	muconic acid proteins (2 g l^{-1})	No

As shown in Table 3.A.2, intermediate metabolites appear to be produced and accumulate in all cases.

Even though stœchiometric chloride concentrations are attained, the complete mineralization seems to be achieved lately by the further degradation of intermediates.

However, both productivity and production rates of intermediate compounds depend on the substrate loading.

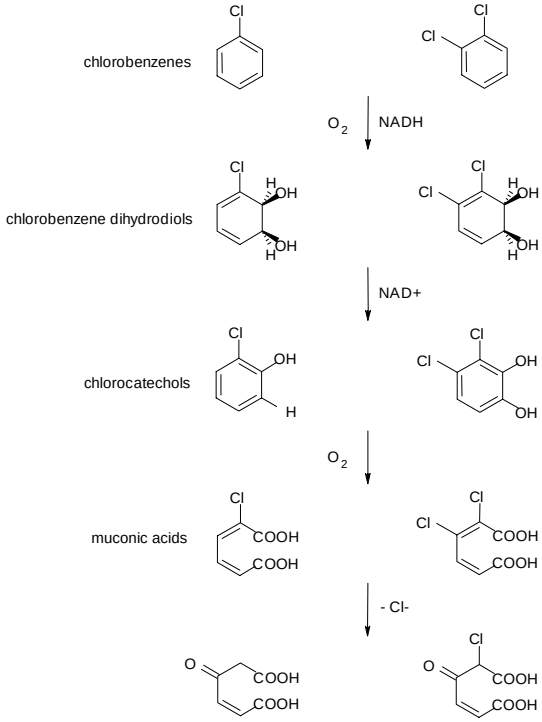
With the SPB process, increasing 3 times the amount of CB/DCB mixture injected by pulse gives more than 30 times the production rates of metabolites.

When SPB process with 600 mg l^{-1} of CB/DCB was applied, metabolites tended to accumulate in the system. After 8 hours (± 2) of total cycle run, 600 $mg_c\ l^{-1}$ of intermediates was measured. This maximum intermediate concentration was concomitant with the accumulation of CB/DCB in the system, the color of the cultivation medium was observed to turn black and the acid addition rate needed for the pH controller sharply increased. These observations were proved to characterize the typical inhibition of the bacterial community (León et al, 1999).

Muconic acid was only detected in the 200 mg l^{-1} SPB and in the continuous processes, but not with the 600 mg l^{-1} SPB technique.

It means that the metabolic pathway at 600 mg l^{-1} SPB process has already been stopped before production of muconic acid. Indeed mono and dichlorocatechols accumulated (see Scheme 3.A.1).

In this case, only 60% of stœchiometric chlorides were recovered.



Scheme 3.A.1. CB and *o*-DCB degradation routes achieved by *Pseudomonas* sp. (Haigler et al, 1988)

The Daphnia bioassay showed that the intermediate compounds were not toxic. The obtained cultivation media was even proved to be partially biodegradable, according to

the DBO_5 test. So industrial effluents could be pretreated with such a process and consequently be rejected into a classical sewage treatment plant with no risk of disruption.

Finally the two main factors which determine the viability of the overall cultivation process are:

- the amount of substrate injected into the cultivation medium and
- the time needed for the bacteria to degrade intermediates.

If the two points are deficient, intermediate compounds tend to accumulate, which lowers the removal of CB and DCB and provokes the inhibition of the CB/DCB conversion. Consequently the bacterial community loses its ability to eliminate CB and DCB, and cellular integrity is damaged.

Therefore the particular case of continuous feeding becomes relevant whenever the amount of substrate approaches the optimal substrate loading determined with the SPB process (i.e. 200 mg l^{-1} h^{-1}).

In this context, high concentrations of proteins were detected (2 g l^{-1}). No further investigation was realized in this study but Part B in Chapter 3 will report the production of Exo Polymeric Substances (EPS) by similar bacterial communities (*Pseudomonas* sp.) which are able to degrade a mixture of toluene, ethylbenzene and xylenes.

These metabolites could protect the bacterial cells against the omnipresence of CB and DCB. Indeed the two molecules as well as their metabolites of degradation are lipophilic and toxic; they are known to be responsible of cellular and wall damages (De Bont, 1998).

3.A.4.2.2. Chloride concentration

The chloride ion concentration was determined either by FIA or conductivity of the medium: both methods were satisfying.

Experimental measurements fitted well with the theoretical quantity of substrate added to the medium and stœchiometric amounts of chlorides were released into the medium during CB/DCB conversion.

This correct correlation confirmed the complete degradation of the parent molecules. Since the measured values were compared with the FID analyses of the off-gases, approximately 10 % of the injected substrate was estimated to be lost by stripping.

Oh and Bartha (1994) have reported that a chloride concentration higher than 7 g l^{-1} could inhibit the bacterial growth. The above-mentioned threshold was systematically overwhelmed in our experiments without disturbance. Indeed, during the SPB experimentation tests, the chloride production rate was approximately 4 mg l^{-1} h^{-1} and the maximal concentration was estimated to be 8.9 g l^{-1}. Since higher substrates loadings were supplied during the continuous cultivation process, the average production rate of chlorides was at least 125 mg l^{-1} h^{-1} and the above-mentioned toxicity threshold was passed after 95 h with a final concentration of chlorides of 11.8 g l^{-1}.

3.A.4.2.3. Nitrogen contents

Ammonium concentration in the liquid phase was measured every day to avoid nitrogen limitation for bacterial growth.

Nitrogen was supplied to the microorganisms by adding ammonium salts; nitrate concentration in the reactor was 4.5 g l^{-1} and was never allowed to reach below 3 g l^{-1}. The overall consumption of ammonium was 150 mg g_{DW}^{-1}, and no significant difference was observed between SPB and continuous techniques.

Nitrification was noticed once with the SPB process but not observed in the continuous experiments.

3.A.4.2.4. Cellular Integrity

The cellular viability was investigated with the Baclight™ microscopic assay in the course of cultivation process. This easy tool was particularly useful to optimize the cell productivity.

Indeed, a high active cell ratio is indication of a high removal capacity. In this case it is possible to increase the substrate loading rate, which permits to enhance the removal capacity. On the contrary, when low active cell ratio is observed, substrate loading rate should be decreased in order to avoid inhibition.

The overall evolution of the cellular viability was similar for both types of substrate feeding (SPB or continuous).

A ratio of 70 % of healthy bacteria indicated that the process had reached a convenient steady-state. In this context, even though the experiments were performed at constant temperature and pH, bacteria showed to be resistant to unexpected short periods changes of temperature (up to 40°C for about one day), or pH (4.5 during 10 hours).

*3.A.*4.3. CB and DCB Conversion

3.A.4.3.1. SPB Technique

Figure 3.A.2 is representative of a typical SPB degradation experiment during one pulse. In the example, 10 minutes were necessary for the bacteria to degrade 100 and 25 mg l^{-1} of CB and DCB, respectively. CB disappeared immediately, whereas two steps were observed before the complete removal of DCB.

In fact, different metabolic pathways are implicated to degrade CB and *o*-DCB (Haigler et al, 1988; see Scheme 3.A.1).

Investigations on *Pseudomonas sp*. showed that growth on CB involves conversion of the substrate to chlorocatechols via dioxygenase and diol dehydrogenase reactions. Concerning *o*-DCB, it is converted by a dioxygenase to a hydrodiol, which is converted to 3,4-dichlorocatechol. According to Sigel et al. (1992), a negative effect is exerted by the two chlorides onto the aromatic ring, which slows down the overall reaction compared to CB degradation.

During the first phase of substrate degradation, absorbance at 255 nm (A_{255}) increased from 0.6 to 0.9 due to the production of metabolic intermediates, and then decreased progressively to the initial value when substrate and metabolites were totally degraded.

This was the criteria to start a pulse because A_{255} evolution could be linked with the risk of inhibition due to toxic intermediate metabolites as well as initial molecules, and absorbance is also easy to measure.

The oxygen uptake rate was measured by using the biological oxygen monitor (BOM) to be 5.2 (± 0.5) g $l^{-1}h^{-1}$ at the start of the cycle during the VOCs uptake, due to the aerobic degradation of the substrate.

Specific degradation rates of CB and DCB were investigated during the overall SPB process.

The initial substrate uptake rate ($q_{s,i}$) is calculated from the slope of the time course of the CB/DCB removal from the gas phase (GC analyzes). Only the first 6 minutes after injection of substrates were considered for this calculation.

The gas-liquid mass transfer coefficient was obtained from physical absorption of N_2O as described by Ebrahimi et al. (2003).

At 400 RPM, K_La was calculated to be about 0.015 s^{-1} and mass transfer rate was calculated to be at least 4 times higher than the uptake rate of the compounds. So using the concentration of the gas phase for initial uptake rate calculation is not a problem, but by increasing biomass concentration and decreasing the compound concentration in the gas phase, it is risky and mass transfer coefficient should be taken into account.

Figure 3.A.3 plots some typical slopes used to calculate the initial substrate uptake rates for CB and *o*-DCB. Global substrate uptake rate ($q_{s,g}$) was calculated based on the total amount of CB/DCB removed during one pulse (by correcting for stripping). As $q_{s,g}$ also took into account the time needed for degradation of both CB/DCB and intermediate metabolites, it was lower than the initial rate ($q_{s,i}$).

Figure 3.A.3 shows the typical time course of the substrate uptake rates during the SPB cultivation process.

Initial substrate uptake rates were maximal at the beginning of the cultivation process and sloped down to a mean value of 0.07 and 0.02 g g_{DW}^{-1} h^{-1} for CB and DCB, respectively. The same behavior has already been reported (Bielefeldt and Stensel, 1999a; Bielefeldt and Stensel, 1999b).

In the case of chlorinated solvents, the substrate removal can be directly correlated with the chloride released in the cultivation medium during the reaction phase. Note that this should be distinguished from the full mineralization of the substrate.

In the present study, stœchiometric amounts of chlorides were always completed, except before an observed inhibition of the bacterial community, due to an excess of substrate loading. Thus, as the release of chlorides happens during the last steps of CB/DCB

degradation (see Scheme 3.A.1), it means that substrate was degraded and not just absorbed inside the bacterial cell.

Therefore decreasing q_s reflects a metabolic inhibition and not an increasing difficulty for the bacteria to incorporate the substrate.

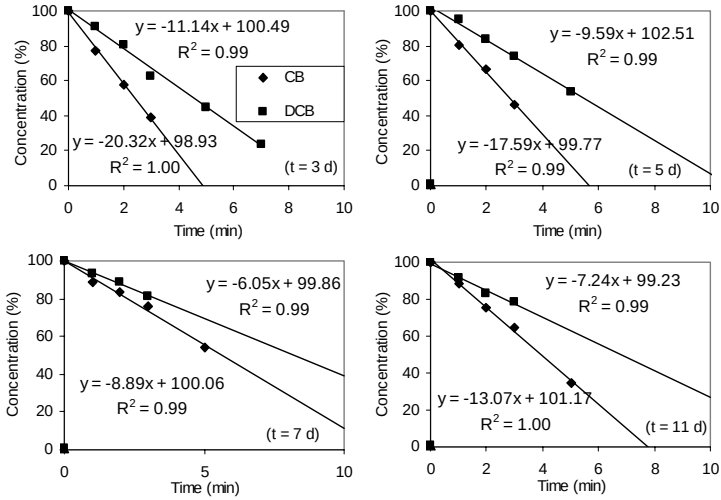


Figure 3.A.3. Calculation and evolution of the degradation rates q_s during the cultivation process

3.A.4.3.2. Continuous Feeding

System was operated in a fed-batch mode with a continuous substrate feeding.

Change in the total liquid volume was negligible because of dosing a concentrated substrate mixture.

During the continuous cultivation process, the CB/DCB loading was progressively increased along the time course and ranged between 200 and 520 mg l^{-1} h^{-1}.

Consequently to every load changes, at least one hour was necessary for the biological system to reach a steady-state. For instance, with 430 mg l^{-1} h^{-1} of CB/DCB injected

inside the bioreactor, the measured residual substrate concentration stabilized to 3.6 and 7.6 mg l^{-1} of CB and DCB, respectively.

Along with the microbial adaptation to the CB/DCB degradation, the residual concentration was systematically observed to decrease until zero.

However 520 mg l^{-1} h^{-1} of CB/DCB was found to be the threshold for the community to simultaneously degrade CB and DCB. Indeed, above this load (corresponding to 100 mg l^{-1} h^{-1} of DCB), 7.4 mg l^{-1} of DCB remained into the liquid phase; this residual concentration being stable. Higher loads provoked the inhibition of the both substrate degradation.

As well as for SPB process, substrate uptake rates decreased in the course of continuous cultivations. Obtained maximal global CB and DCB uptake rates reached respectively 53 and 13 mg g_{DW}^{-1} h^{-1} for continuous feeding, versus 61 and 14 mg g_{DW}^{-1} h^{-1} for SPB technique.

The above-mentioned data were the maximum substrate uptake rates which could be obtained, regardless of the cultivation period. Regarding these values, the two cultivation processes appeared to be comparable on kinetics aspects.

*3.A.*4.4. Biomass Yield and Productivity

Biomass yield and productivity were determined and compared for both SPB and continuous feeding techniques (Table 3.A.3). SPB was performed with either pure oxygen or air during the aeration step (See section 3.A.4.1).

Every parameter presented in Table 3.A.3 shows that the continuous feeding of substrate was the most productive approach for bacterial cultivation: the obtained biomass productivity (100 mg_{DW} l^{-1} h^{-1}) was twice the global productivity conducted with the SPB technique.

Both SPB processes with air and pure oxygen were found to produce similar biomass contents regarding time (global productivity averaged 50 mg_{DW} l^{-1} h^{-1}), but not regarding the substrate loading. Thus considering global yields, oxygenated SPB was comparable to the continuous process (i.e. 400 mg_{DW} $g_{CB/DCB}^{-1}$).

Table 3.A.3. Biomass yields and productivities achieved by cultivation processes operated within aerated SPB, oxygenated SPB or continuous feeding techniques.

	SPB (air)	**SPB (O_2)**	**Continuous**
Maximal productivity ($mg_{DW}\ l^{-1}\ h^{-1}$)	66 ± 6	56	208 ± 6
Global productivity ($mg_{DW}\ l^{-1}\ h^{-1}$)	48 ± 2	51	100 ± 3
Global yield ($mg_{DW}\ g_{CB/DCB}^{-1}$)	280 ± 8	400 ± 25	360 ± 20
Maximal substrate loading ($mg_{CB/DCB}\ l^{-1}\ h^{-1}$)	240	155	520
Optimal substrate removal rate ($mg_{CB/DCB}\ l^{-1}\ h^{-1}$)	235 ± 5	100 ± 20	470 ± 6
Maximal biomass concentration ($g_{DW}\ l^{-1}$)	13	8	20

However, the continuous feeding mode could perform at least twice the maximal substrate loading that SPB could carry out. Moreover the continuous system was more relevant compared to the others because its optimal substrate removal rate was twice the optimal rate obtained with the SPB process operated with air and five times the optimal substrate removal rate of SPB operated with oxygen.

Hence biomass production of the continuous reactor was higher than for sequential batch reactors.

This behavior was surprising, due to the toxicity of CB and *o*-DCB.

In particular, Microtox™ acute toxicity testing was performed on these substances. The obtained EC_{50} (5 min) were 16.94 (± 0.9) and 3.13 (± 0.2) mg l^{-1} for CB and *o*-DCB, respectively, which is in accordance with literature (Bazin, 1985; Blum and Speece, 1991; Warne et al., 1999).

Besides, these substances and some of their metabolic byproducts are known to damage the bacterial membranes and provoke inhibition (De Bont, 1998).
This is the main reason why SPB (Substrate Pulse Batch) processes were aimed for treating VOCs, such as BTEX and halo-aromatics (León et al, 1999; Shim and Yang, 1999).

Therefore it was risky to test the continuous feeding of CB and *o*-DCB.
First, it allowed residual amounts of CB and *o*-DCB, and their possible accumulation nearby and inside the cells.
Second, it favored the use of the parent molecules instead of the subsequent intermediate metabolites. In this case, it was shown that the metabolic degradation chain could be blocked (Klecka and Gibson, 1981).

In the present study, these phenomena were avoided. It was certainly due to the correct adaptation of the bacterial community able to degrade CB and *o*-DCB, and also because the substrate loadings were progressively increased in the course of the cultivation process.

3.A.5. Conclusion

Active bacterial community was produced to degrade chlorinated solvents. Four regular analyses were used as reliable parameters in order to control bacterial growth and CB/DCB degradation. They consist of UV absorbance at 255 nm (intermediate contents), chloride production, nitrogen consumption and cellular viability.

Biomass yield and productivity as well as the CB/DCB degradation rates were determined and compared for both SPB and continuous techniques. Between the two experimented processes, the continuous feeding of substrate was found to enhance both biomass productivity and CB/DCB removal capacity.

However, two different endpoints can be considered. First, a biotechnological approach tends to maximize both biomass productivity and global yields. In this case, the continuous substrate feeding process was the most relevant, as the obtained values were

more than twice those obtained for the SPB process (360 mg of dry biomass produced per g of CB/DCB consumed versus 280 mg_{DW} $g_{CB/DCB}^{-1}$). The second strategy concerns either environmental or industrial topics and focuses the maximal removal capacity of the biotreatment. Substrate degradation rates are to be optimized so that the process allows a sustainable treatment of polluted gaseous effluents (i.e. efficient and stable). In this case, the continuous feeding process was also the most relevant technique with 470 ± 6 mg $_{CB/DCB}$ l^{-1} h^{-1}. Finally, the continuous supply of substrate corresponds to an enticing mode of biomass production regarding industrial prospects and it is usually easier to operate compared to batch processes.

3.A.6. Nomenclature

A_{255} = abs 255 nm = absorbance at 255 nm
BOD = biochemical oxygen demand
DO = dissolved oxygen
DW = dry weight
K_{La} = mass transfer coefficient
$q_{s,g}$ = global substrate uptake rate
$q_{s,i}$ = initial substrate uptake rate
RPM = rotation per minute
SPB = substrate pulsed batch
TEX = toluene, ethylbenzene, *o*-, *m*-, *p*-xylene

3.A.7. References

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CHAPTER 3.

Biodegradation of Volatile Organic Compounds

PART B.

MASS PRODUCTION OF BACTERIAL COMMUNITIES ADAPTED TO THE DEGRADATION OF VOLATILE ORGANIC COMPOUNDS (TEX)

Submitted to *Biodegradation*

3.B.1. Abstract

This study focuses on the mass cultivation of bacteria adapted to the degradation of a mixture composed of toluene, ethylbenzene, *o*-, *m*- and *p*-xylenes (TEX). For the cultivation process Substrate Pulse Batch (SPB) technique was adapted under well automated conditions. The key parameters to be monitored were handled by LabVIEW software including, temperature, pH, dissolved oxygen and turbidity. Other parameters, such as biomass, ammonium or residual substrate concentrations needed offline measurements.

SPB technique has been successfully tested experimentally on TEX. The dynamic behavior of the mixed bacterial population was observed and discussed under different operational conditions. Carbon and nitrogen limitations were shown to affect the integrity of the bacterial cells as well as their production of exopolymeric substances (EPS). Average productivity and yield values reached 0.45 $kg_{DW}\ m^{-3}\ d^{-1}$ and 0.59 $g_{DW}\ g_C^{-1}$, respectively. Obtained data come up to the industrial specifications and indicate the feasibility of controlled SPB technique.

3.B.2. Introduction

Toluene, ethylbenzene, and xylene (TEX) are among the most important contaminants present in surface and groundwater which usually originate from the leakage of tanks containing petroleum-derived products and industrial wastewaters (Akunar-Askar et al., 2003).

Because they are both toxic and relatively water soluble compared with other petroleum constituents, their entry into surface and drinking water is of major concern. In spite of governmental intervention in many countries, their emission to the environment is still escalating.

Therefore, there is a need to develop and optimize technologies for removing TEX from groundwater, especially when downstream drinking water resources are a concern.

Among all remediation technologies for treating TEX-contaminated groundwater, bioremediation appears to be an economical, energy-efficient and environmentally sound approach.

Microorganisms are able to degrade TEX under aerobic, microaerobic or hypoxic, as well as anaerobic conditions (Villatoro-Monzon et al. 2003). Development and upgrading of the biotechniques for simultaneous and efficient removal of all TEX with a mixed culture are of major challenges.

The substrate pulse batch (SPB) technique is attractive for the production of a specialized biomass used to inoculate biosystems which treat volatile organic compounds (VOCs). SPB technique is a batch cultivation process in which substrates are supplied in pulses.

This technique can be considered as a special limit case of a conventional, semi-continuous extended culture operation: total volume remains virtually constant because substrate is fed in a gaseous, solid or very concentrated liquid form, according to an intermittent profile.

This technique aims to save both heterogeneity and kinetic dynamics of the mixed population thanks to the continual variation of substrate concentration in the medium. Short contacts time due to rapid degradation also avoid toxic damages which may result from the solvent effects onto bacterial cells (De Bont 1998). This process is

recommended for achieving a more stable and efficient biomass culture (León et al. 1999; Seignez et al. 2001). However, industrial implementation of SPB technique is limited because of its rules of thumb.

This study was aimed to improve the productivity and yield of a particular microbial culture for degrading a TEX mixture using SPB technique. This research was in context of an European Project, "Bioreactor For Innovative Mass Bacteria Culture, BIOMAC" (see: www.eureka.be, project E!2497). This study presents the kinetics of an adapted bacterial consortium to degrade TEX in an automated bioreactor using SBP technique.

3.B.3. Materials and methods

*3.B.*3.1. Inoculum Preparation

The bacterial consortium used to inoculate the bioreactor was selected in our laboratory in a previous work (León et al. 1999). The bacteria were originally issued from the sludge of a wastewater treatment plant (Novartis and Rohner AG, Basel, Switzerland) and from the biotrickling filters (Rohner AG, Basel, Switzerland), adapted to the TEX degradation and cultivated on TEX or toluene as sole source of carbon then preserved at -80°C.

A sample of this adapted mixed culture was cultivated in flasks containing nutrient medium (CM67, Oxoid LTD, Basingtoke, England) at 30°C for 1 day.
Then the obtained biomass was collected by centrifugation and transferred into the batch reactor.

The nutrient medium consisted of inorganic salts and vitamins necessary for the growth of microorganisms, the same composition as previously reported in Chapter 3, Part A, with ammonium as the nitrogen source.
In all the experiments, the sole carbon and energy source consisted of 90 % (v/v) toluene (Tol), 2.5 % ethylbenzene (EB), 6.5 % m- and p-xylenes (X), 1.3 % o-xylene. This mixture is called "TEX". Compounds were supplied by Fluka, Buchs, Switzerland. Experiments were made under non-sterile conditions. TEX were supplied by a precision feeding pump (SpectraPhysics, San José, USA).

3.B.3.2. Bioreactor and Automation Program

The experimental set-up used in this study is presented in Figure 3.B.1.

Bioreactor is made of stainless steel and Pyrex glass; with a total volume of 14.5 l. The working liquid volume was 10 l. Numerous ports along its height and at the bottom allow feeding, sampling and connecting the measuring probes.

The characteristics of the reactor and the operating conditions for the experiments are described in Table 3.B.1.

Table 3.B.1. Operational data for the bioreactor

Parameter	Value	Material
Temperature	35 °C	PT 100, *Digital thermostat*
pH	6.5-7	Electrode, Liquisys S, *Threshold contact* e
Dissolved Oxygen	0.8-98 %	Oximetric probe (L=31.7 cm)
(DO)	*100 %=7.14 mg/l*	Ingold Messtecknick
Agitation	170-800 RPM	Motor : Lust
Aeration	0.5-0.7 l/min K_{La}=3-9 h^{-1}	Brooks Instrument

The overall set-up was controlled by the so-called BioOPT program. This LabVIEW computer-based monitoring program allows data acquisition and was used to optimize the biodegradation process.

Modulable FieldPoints (National Instruments) were the connecting tools between computer and instrumental systems. The main electrical components are listed in Table 3.B.2.

Table 3.B.2. List of the National Instruments modules

FP-2000	Ethernet network module capable of running LabVIEW Real-Time embedded code and 3 Mb Flash Memory for user
FP-AI-110	8 analog inputs 0-20 /4-20 mA, 16 bits, 50/60 Hz filtering
FP-AO-200	8 analog outputs 0-20 /4-20 mA, 12 bits
FP-RLY-420	8 relays, SPST (form A), 3 A at 250 VAC or 35 VDC
FP-RTD-PT100	Dual Channel I/O: 4-wire RTD input modules (-50 to 350 °C)
FP-TB-10	Dual-Channel terminal base for 6 dual-channel I/O modules

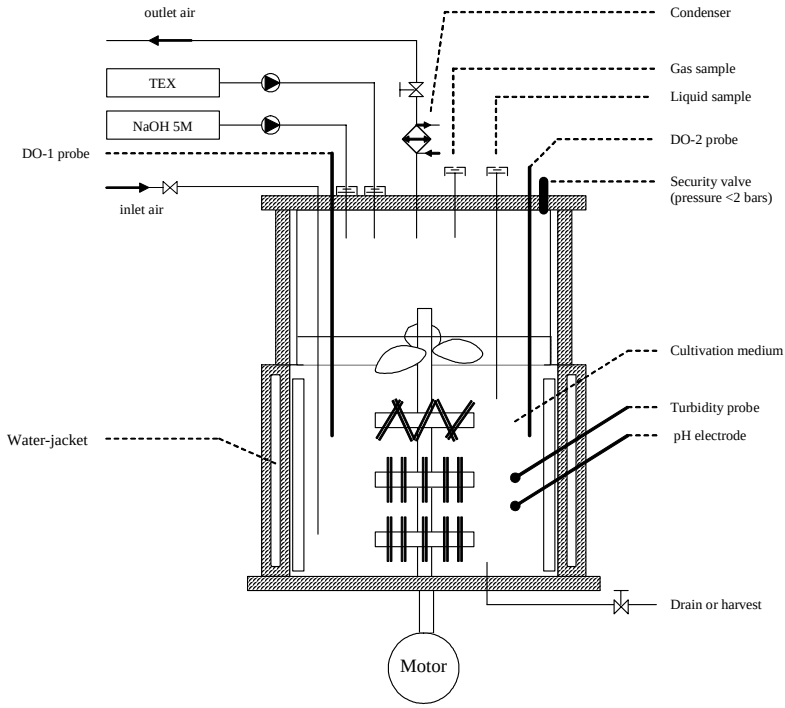

Figure 3.B.1. Schematic presentation of the laboratory bioreactor

3.B.3.3. Analytical methods

Concentrations of TEX mixture were determined by capillary gas chromatography (GC-14B, Integrator C-R65A, Shimadzu, France), with a flame ionization detector fitted and a DB-624 capillary column (30 m length, 0.53 mm diameter) (J&W Scientific, Folsom, California). Samples were analyzed at a 32 ml/min nitrogen flow rate and at 30 ml/min split injection. Injection and detector temperatures were 220 and 300°C, respectively. Oven temperature was increased at a rate of 4°C/min from 85°C to a final temperature of

110°C. The culture was sampled by means of a 250 µl Gas-tight syringe (Dynatech, Serie A2, Bâton-Rouge, USA).

Volatile Organic Compounds (VOCs) lost by stripping were continuously quantified by FID analyses during the process. The amounts were systematically taken into account for any calculation of the TEX conversion rate.

Dissolved organic carbon concentration was measured by an IR detector Shimadzu TOC-500 (Burkard Instrumente, Switzerland). The reactor sample was filtrated at 0.45 µm (Schleicher&Schuell, Germany), acidified by HCl 2 M and the CO_2 was purged before analysis.

Metabolites estimation was made by measuring the optical density of the filtered sample at 255 nm in an UV spectrophotometer (Hitachi U-2000, Tokyo, Japan). Ammonium and nitrate in the liquid phase of the batch reactor were determined by the enzymatic method (Boehringer, Manheim, Germany) to control the nitrogen measurement for TEX degradation.

The biomass concentration was monitored by dry-weight measurement according to Dutch standard methods (NEN 32355.3) and by absorbance at 650 nm.

The LIVE/DEAD® BacLight™ Bacterial Viability Kit L-7007 (Molecular Probes Corporation, Canada) provides a quantitative index of bacterial viability. It uses a mixture of SYTO® 9 green fluorescent nucleic acid stain which labels bacteria with both intact and damaged membranes. Besides, the red fluorescent nucleic acid stain Propidium Iodide penetrates only bacteria with damaged membranes. The bacterial samples were centrifuged and filtered through 0.2 µm PC Memb 13 mm (Nucleopore CORP., Canada). The Epi-FluoroMicroscopic observation was carried out under UV light with emission filters BP 450-490nm, reflector FT 510 and stop-filter LP 520 (Eclipse 800, Nikon, Switzerland).

Extracellular polymeric substances (EPS) were extracted as follows. Samples of the culture (10 ml) were centrifuged at 16.000 x g for 30 min at 4°C. The supernatant polysaccharides (dissolved EPS) were isolated by precipitation with 3 volumes of ethanol, and kept overnight at 4°C (Duenas et al. 2003). The pellets (bound EPS +

bacteria) were resuspended in nutrient medium containing 10 mM EDTA (Platt et al. 1985). After sedimentation of the bacterial cells by centrifugation, the supernatant was treated as for the dissolved EPS. After centrifugation of both bound- and dissolved-EPS tubes (5.000 x g, 20 min, 4°C), the pellets were dispersed in aqueous 80 % ethanol and centrifuged again (3 times). The final precipitates were dissolved in distilled water (50 ml). The carbohydrate content was determined by the Anthrone method modified from Standard Methods (Raunkjauer et al. 1994) with glucose as the standard.

The determinations were done in triplicate and the average values were calculated with less than 5 % standard deviation.

3.B.4. Results and discussion

*3.B.*4.1. Bioengineering

To automate the production of adapted bacteria able to degrade TEX, a LabVIEW computer-based monitoring program was developed for command and data acquisition. First, the key parameters implicated in the biodegradation process are presented. Then schedules are proposed to reinforce the interaction between command and control in order to provide a completely autonomous system.

3.B.4.1.1. SPB technique for biomass cultivation

SPB technique has already been explained and illustrated in Chapter 3, Part A for chlorobenzene and dichlorobenzene biodegradation. In Part B, the target VOCs are TEX.

SPB technique aims to save both overall heterogeneity and kinetic dynamics of the mixed population thanks to the continual variation of substrate concentration in the medium. Short contacts due to rapid degradation also avoid toxic damages which may result from the solvent effects onto bacterial cells (De Bont, 1998). Therefore such a process allows a better stability to the culture and improved biomass productivity.

However it is mainly built on empiricism. In this context, the operator plays a crucial role, which limits the industrial implementation. This is the reason why this study focuses on the automation of the process.

In this study, the cultivation of mixed bacteria was handled in a batch culture with the pulse feeding technique. The overall process can be categorized into two periods.

- First, microorganisms are progressively fed with increasing but still low amounts of TEX. This step allows the induction of the enzymatic pool and strongly influences the further TEX degradation (Grady et al., 1996).
- Second, the most efficient substrate loading is investigated in order to improve both productivity and degradation yields.

During the cultivation, four parameters are checked to have the best conditions for microbial growth:

- Concentrations in the gas phase of TEX compounds;
- Concentration in the liquid phase of the produced intermediate compounds;
- Oxygen concentration in the liquid phase. It should be at least 20 % of the saturation concentration. It is measured in two places in the bioreactor (Dissolved Oxygen probes DO-1 and DO-2);
- Nitrogen concentration (ammonia) in the batch medium. It shall be higher than 100 mg/l to avoid limitation of the bacterial growth.

In this study, volatile substrates (TEX) are injected by repeated pulses in the gaseous phase. 0.15 m above the liquid surface is the selected height to perform that crucial step: it favors and regulates the injection of the convenient amount of solvents (no overpressure) and limits any uncontrolled volatilization losses (stripping process).

The equilibrium between gas and liquid phases is reached in 2 minutes after the end of the TEX injection. The stripping process depends on both aeration and agitation.

Three steps are successively performed:

- Phase 1 – aeration, for about 5 minutes to saturate the liquid phase.

Air flow was set at 7 to 10 l/min. If TEX were injected at the beginning of this phase, 80% of the substrate would be stripped.

- Phase 2 – injection of TEX, within 15 seconds.

The substrate amounts provided to the culture can be modified according to the magnitude and/or the frequency of the injected pulses.

- Phase 3 – reaction.

During this phase, biodegradation of the parent molecules and their intermediate metabolites occur. The time period allowed for this phase depends on the microbial activity.

During phases 2 and 3, the air flow was set at 0.5 l/min to minimize the stripping (evaluated at respectively 2% and 7 to 23% of the initial TEX amount).

3.B.4.1.2. Automation parameters

Each of the three above-mentioned phases can be precisely defined at the beginning of the cultivation process using a basic LabVIEW computer-based monitoring program.

Experimental data are thus automatically recorded, so that the instrumental system is completely autonomous, except for the decision to increase the substrate loading.

Indeed, the increase in substrate loading depends on the biomass behavior. It is difficult to forecast the substrate needed, because it would mean that bacteria should consume the solvents as expected.

In fact, bacterial consumption means (1) incorporation of the substrate, and only after (2) degradation of the parent molecules and the intermediate metabolites. In particular, aerobic biodegradation of TEX is correlated with oxygen consumption.

Experimentally, a minimum concentration of dissolved oxygen (DO_{min}) is recorded when no residual concentration of TEX is measured in the gaseous phase (manual GC analyses).

Therefore it is possible to indirectly control the accurate degradation of TEX by the automatic and continuously recorded DO evolution.
Hence, two parameters can be used to characterize the effectiveness of the bacterial cultivation:

- time necessary to degrade TEX during phase 3,
- decrease of DO.

First, time delay of phase 3 can be forecasted according to the cellular yield of biomass production $Y_{X/S}$. Indeed, the global rate of substrate degradation q_s ($q_s = \mu/Y_{X/S}$) may be calculated. Then the frequency of pulses is determined so that the charge of TEX is inferior to the maximum degradation capacity of consortium ($= q_s.[X]$). Unfortunately, this method is limited by the evolution of the consortium characteristics during the cultivation. Moreover, concentration of biomass still needs to be determined (manual operation).

Second, as previously mentioned, concomitant consumption of oxygen and TEX permits to regulate the overall process according to the variations of DO values. Indeed when DO_{min} is reached, it means that no more substrate is available for bacteria. Hence, a new pulse of substrate can be injected.
However an extra-delay of time appears necessary after having reached DO_{min}. This period of time corresponds approximately to 1/3 of the delay necessary for the TEX to disappear from the aqueous phase; i.e. 1/3 of the time necessary to reach DO_{min}.
This delay is necessary for the degradation of the intermediate by-products inside the bacterial cells.
By this way, the succession of pulses can be automatically monitored and adapted to the real progression of the cultivation.

Monitoring of the cultivation process by the DO variations implies the reliability of DO data. However, DO measurements can be faltered in two circumstances.

The first case happens when biomass growth implies an increasing O_2 demand: O_2 transfer becomes limiting into the liquid phase if the agitation remains the same. Thus DO variation is attenuated and no DO_{min} is clearly noted (Figure 3.B.2a).

It outlines the need to link DO variation and agitation. For eg., ΔDO > 50 % can be specified in BioOPT program. Underneath this value, agitation would be automatically increased.

The second case happens when microorganisms become unhealthy: O_2 consumption decreases but shortening the cycles would favor the bacterial inhibition. On the contrary, phase 3 should be lengthened (Figure 3.B.2b).

In both cases, it is supposed that DO probes are cleaned regularly (no bacterial film).

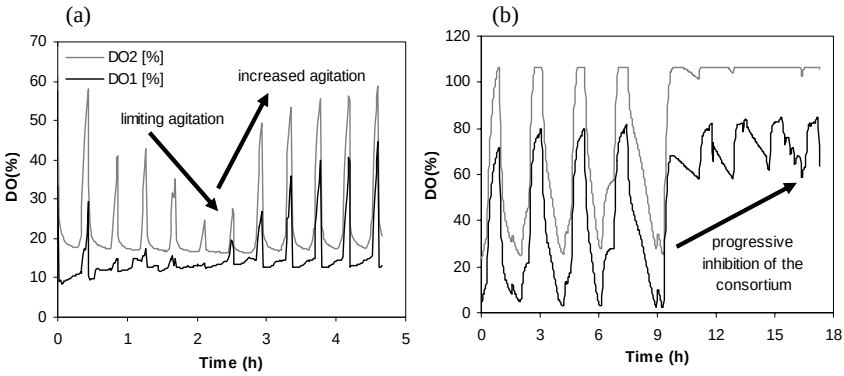

Figure 3.B.2. Evolution of DO during automatic runs of biomass cultivation

The obtained results indicate that using SPB method under automated conditions (computer control) gives at least similar results to those obtained with an operator's supervision (unpublished results).

*3.B.*4.2. Microbiology

To optimize the production of adapted bacteria able to degrade TEX according to industrial specifications, the cultivation of a bacterial community was carried out under different operational conditions and kinetics features were followed.

3.B.4.2.1. Microbial growth

Several cultivations were carried out in order to automate the process. The key point was to meet a convenient relation between (i) the amount of solvents which are the carbon source and (ii) their toxic effects, which inhibit the bacterial growth.

For cultivation process, SPB was adopted because this technique allows the bacteria to degrade a low amount of the initial molecule into less toxic metabolites. Each pulse of substrate favors the bacterial growth and the acclimation to the solvents.
Higher substrate amounts are progressively introduced into the biological system. It was shown that low amounts of TEX (326 mg l^{-1} every 2 h during 6 days) were necessary to acclimate bacteria to TEX. After this step, the shorter the lag phase, the more resistant the bacterial consortium to high substrate loading (696 mg l^{-1} every 5 minutes for instance).

Evolution of biomass concentration and substrate mass conversion rate during the overall production process were investigated. Figure 3.B.3 illustrates the time course of the increase on biomass concentration: 23 g/l of biomass was produced within 17 days.

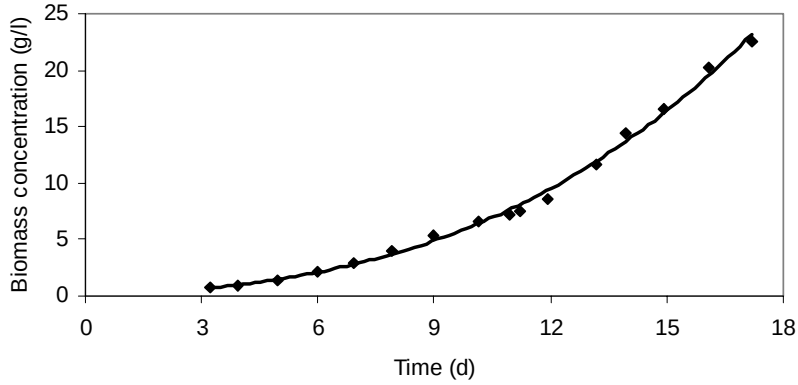

Figure 3.B.3. Evolution of the biomass concentration during the cultivation process

During this period approximately 0.8 l of TEX mixture was injected into the biological system. According to the carbon contents of the off-gases which were continuously

measured (FID analyses), 6.5 % of the loaded TEX was stripped. Total biomass yield and biomass productivity were estimated to be 0.3 $g_{DW}\ g_{TEX}^{-1}$ and 0.06 $g_{DW}\ l^{-1}\ h^{-1}$, respectively.

Specific growth rates (μ_{max}) were calculated to be about 0.007 h^{-1}. Overall, calculated growth rates ranged between 0.003 and 0.011 h^{-1} depending on carbon or nitrogen contents in the nutrient medium. These values are comparable with other reports. For instance Shim and Yang (1999) have reported that specific growth rates ranged between 0.001 and 0.002 h^{-1} with only toluene or *o*-xylene as substrate.

After the initial period of induction (6 days) and during 30 days of cultivation, interval of time between successive pulses was reduced from 1.5 h until 0.5 h.
Besides and alternatively to the higher frequency of pulses, the amounts of TEX were also increased from an initial concentration in the liquid phase of 24 mg_c/l until a final concentration of 67 mg_c/l (stripping losses already subtracted). Therefore average productivity and yield values reached 0.45 $kg_{DW}\ m^{-3}\ d^{-1}$ and 0.59 $g_{DW}\ g_c^{-1}$, respectively.
These data come up to the industrial specifications and confirm the benefits of such an improved technology.

3.B.4.2.2. Impact of C and N concentration on cell viability

The cellular viability was investigated during the cultivation process. After inoculation, the ratio of healthy cells was approximately 50 %. It reached a climax (90 %) after 24 hours and then progressively decreased to 60 % within 16 days. Besides, it was shown that limiting the amounts of carbon and nitrogen provoked cellular damages and was concomitant with a lower productivity. This effect was reversible, since higher N and C loadings allowed the cells to recover their integrity: this bacterial property is called resilience.

Exo-Polymeric Substances (EPS) were also studied at different cultivation conditions. Table 3.B.3 shows that limiting C or N contents of the nutrient medium change both the composition and the total amount of EPS.
For instance, when the C source was limiting, the total amount of EPS was twice the amount measured with the adequate supply of C and N.

Besides, limiting N provoked an increase of carbohydrates contents (92 % instead of 40 % with balanced C and N contents in the nutrient medium).

Since EPS are predominantly composed of carbohydrate and protein, the ratio between each constituent depends on the relative C and N amounts in the nutrient medium.

Their active secretion is also linked to the environmental conditions: carbon starvation provokes the production of dissolved EPS as a response to an energetic deficiency. On the contrary, a nitrogen limited culture tends to minimize the overall EPS production and certainly favors its consumption.
The similar link between nutrients, bacterial viability and growth has been demonstrated by Durnaz and Sanin (2001) and Liu and Fang (2003).

Table 3.B.3. Influence of C and N on the cellular metabolism of the mixed population.

Conditions	**Limiting C**	**Limiting N**	**Balanced C and N**
Viability	30 %	50 %	65 %
Dissolved EPS	2.5 g/g_{DW}	0.2 g/g_{DW}	1.0 g/g_{DW}
Carbohydrates contents	*40 %*	*92 %*	*40 %*
Bound EPS	0.8 g/g_{DW}	0.1 g/g_{DW}	0.5 g/g_{DW}
Carbohydrates contents	*25 %*	*67 %*	*40 %*

3.B.4.2.3. Substrate conversion

Typical kinetics of degradation of TEX during one pulse is presented in Figure3.B.4.

Three steps can be distinguished during elimination of TEX after injection into the liquid phase:

- fast removal of TEX, followed by an increase of oxygen consumption rate during the first 15 minutes,
- slower removal of TEX and stabilization of DO (lower bacterial consumption),
- no residual concentration of TEX, significant increase of both DO and A_{255} (absorbance at 255 nm). This step should correspond to the exportation of intermediate metabolites (León 1999).

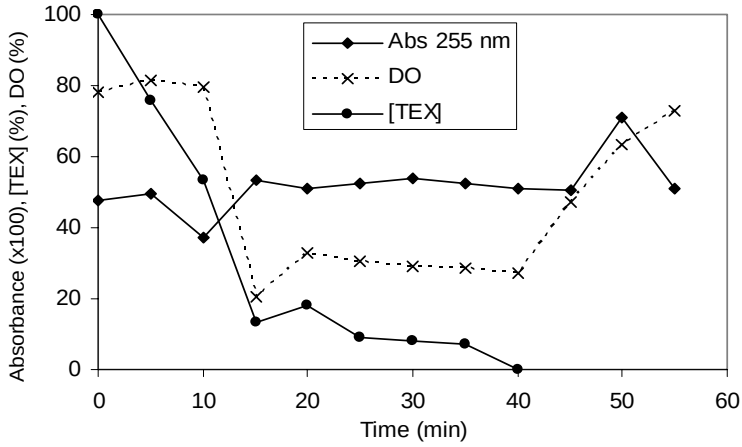

Figure 3.B.4. Disappearance ratio (%) of TEX, oxygen uptake and A_{255} evolution during a representative pulse, after 4 days of cultivation.

$[TEX]_{l0}$ = 25 mg_C/l, [X] = 0.85 g/l, Agitation = 200 RPM, Q_{air} = 0.5 l/min

Such studies were carried out during 30 days of process and focused on the removal of each compound separately (Tol, EB, *o*-, *m*-, *p*-X).
They revealed some modifications of the bacterial behavior in the course of cultivation.

As illustrated in Figure3.B.5, from day 1 until day 20, toluene exhibited the fastest elimination. It was followed by EB, *m*- and *p*-X, and finally *o*-X. (Figure 3.B.5 a and b).

Between days 22 and 25, this ranking was changed: EB reached first the lowest residual concentration and Tol was only removed after Xylenes (Figure 3.B.5 c).

Then from day 26 until the end, the ranking was the same as at the beginning (Figure 3.B.5 d).

For all that, global rates of TEX disappearance remained unchanged (see Figure 3.B.6 below). Similar evolutions were observed thrice.

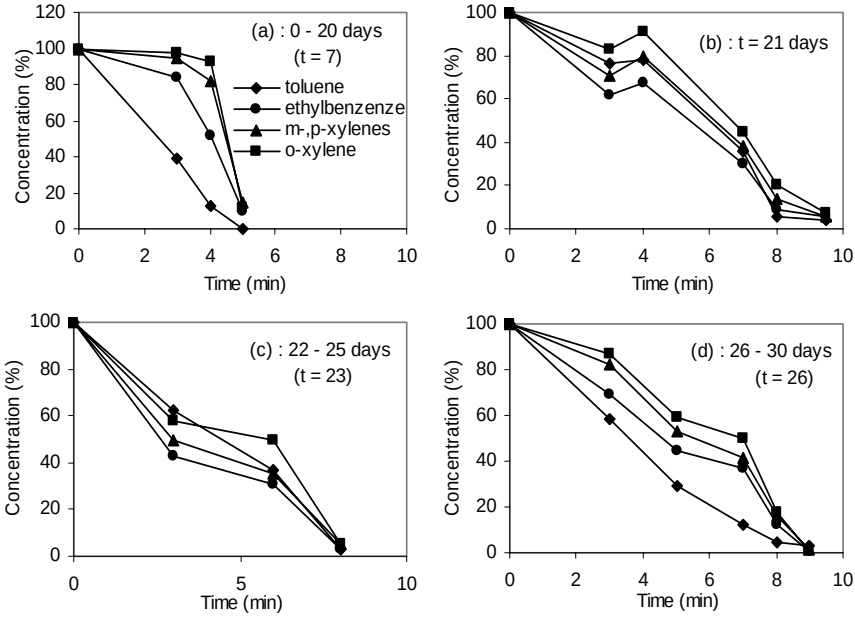

Figure 3.B.5. Disappearance of TEX checked along 30 days of cultivation

Three points are tentatively proposed to comment the differences in the TEX removal.

- First, as the substrate loading is continuously accommodated to the biomass growth, it is reasonable to consider the saturation and full expression of available enzymes (Grady et al. 1996). Since global degradation and growth parameters remain constant (see Figure 3.B.6 below), sporadic delays between supply and demand would only tend to orientate the metabolic activity towards the EPS production.

- Second, the greater the amounts of TEX injected in the medium, the higher its overall toxicity. Solvents are known to accumulate in and disrupt the bacterial cell membrane thus affecting the structural and functional integrity of the cell (Sikkema et al. 1995). Therefore the major metabolic pathway could be changed. Indeed two distinct

substrate interactions are to be considered: toluene and ethylbenzene are inhibitive competitors, whereas xylenes transformation is necessarily cometabolic and so secondary to toluene and/or ethylbenzene degradation (Yu et al., 2001).

- Three, the studied biodegradation is carried out by a mixed bacterial community. Many strains are involved, and their proportion can be modified as well as the metabolic dynamism during the cultivation process. Biomolecular techniques such as RT-PCR (Real Time Polymerase Chain Reaction) or T-RFLP (Terminal Restriction Fragment Length Polymorphims) would provide an idea of the bacterial composition as the process goes along.

3.B.4.2.4. Substrate uptake rate

TEX degradation rates were investigated during the overall process.

The initial substrate uptake rate ($q_{s,i}$) is calculated from the slope of the time course of the TEX removal from the gas phase (GC analyzes). Only the first 15 minutes after injection of TEX were considered for this calculus.
Global substrate uptake rate ($q_{s,g}$) was calculated based on the total amount of TEX removed during one pulse (by correcting for stripping).
As $q_{s,g}$ also took into account the degradation of both TEX and intermediate metabolites, it was lower than the initial rate ($q_{s,i}$).

Figure 3.B.6 shows the typical time course of the substrate uptake rates during the cultivation process.

Both substrate uptake rates were maximal at the beginning of the cultivation process and sloped down to a mean value of 0.015 $g_c\ g_{DW}^{-1}\ h^{-1}$.

It should be noticed that the substrate uptake rate presents the rate of removal of the TEX from the gas phase.

However it is difficult to argue if the substrate is either degraded or only absorbed inside the bacterial cell. Therefore decreasing q_s reflects a metabolic inhibition and/or an increasing difficulty for the bacteria to incorporate the substrate. The same behavior has

also been reported by Bielefeldt and Stensel (1998), the longer the cultivation time, the lower the degradation rates during the cultivation process.

A progressive deterioration of the bacterial walls is prospected to explain this evolution, which is due to the well-known toxicological characteristics of the lipophilic substrates.

In this case, to accelerate the adaptation phase, the most efficient conditions have to be selected. A more stable process can be achieved quickly which improves the overall biomass productivity.

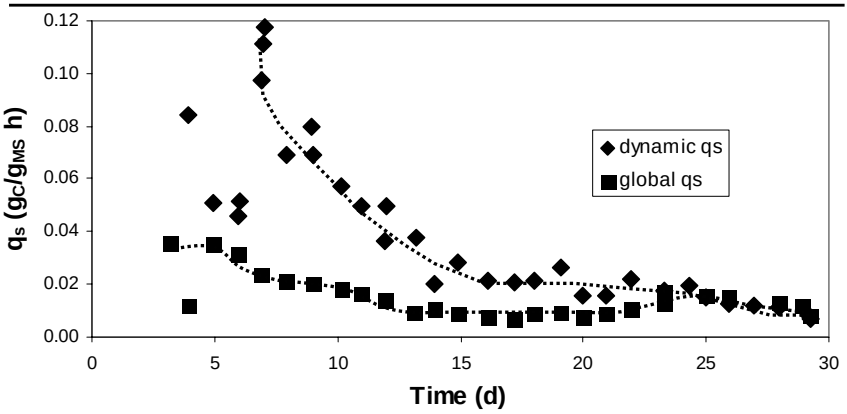

Figure 3.B.6. Evolution of the degradation rates q_s during the cultivation process

3.B.5. Conclusion

In this study, SPB technique has been adopted for the cultivation of bacteria for degradation of TEX. Obtained results indicated that relatively higher yields and productivity could be achieved in computer controlled SPB technique compared to the manually controlled one. Automated SPB cultivation technique benefits form an easier maintenance coupled with improved performances. The subsequent increase of reliability presents this process as a ready-way to produce large quantities of cell mass..

Microorganisms produced in this way can play a major role for the removal of hydrocarbons from industrial off-gases, degradation of substitute organic solvents, reduction in odors from wastewater plants and the food industry or even the removal of pollutant mixtures containing chlorinated solvents.

3.B.6. Nomenclature

A_{255} = abs 255 nm = absorbance at 255 nm
BOD = biochemical oxygen demand
DO = dissolved oxygen
DW = dry weight
K_{La} = mass transfer coefficient
$q_{s,g}$ = global substrate uptake rate
$q_{s,i}$ = initial substrate uptake rate
RPM = rotation per minute
SPB = substrate pulsed batch
TEX = toluene, ethylbenzene, *o*-, *m*-, *p*-xylene

3.B.7. References

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CHAPTER 4.

Photocatalytic Degradation of *p*-halophenols in TiO_2 Aqueous Suspensions: Halogen Effect on Removal Rate, Aromatic Intermediates and Toxicity Variations

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4.1. Abstract

The influence of the halogen upon the TiO_2 photocatalytic degradation of *p*-halophenols in water has been investigated in this chapter. Phenol was used as the reference compound.

Compared with its value for phenol, the apparent first-order rate constant of removal, k, was slightly but significantly higher for *p*-fluorophenol and *p*-chlorophenol, and slightly but significantly lower for *p*-bromophenol. For *p*-iodophenol, k was about half that of phenol. The relative values confirm that k is roughly correlated to the Hammett constant; this constant reflects the electron density on the aromatic ring and, accordingly, the reactivity towards electrophilic species generated by UV-irradiation of TiO_2.

All compounds were found to be poorly adsorbed on TiO_2. Accordingly, k was not related to the differences observed in the very low adsorbed amounts.

The detected aromatic intermediate products included hydroquinone (HQ), benzoquinone (BQ) and various halodihydroxybenzenes. HQ, BQ, 4-chloro-1,2 (and 1,3)-dihydroxybenzenes and 4-bromo-1,3-dihydroxybenzene were quantified. Mechanisms are tentatively suggested to interpret the differences in the degradation pathways of the *p*-halophenols. The organic intermediate products accounted for only a few percents of the total carbon during the degradation.

The toxicity ($1/EC_{50}$) measured by the Microtox™ test almost did not vary in the course of the degradation of phenol, *p*-chlorophenol and *p*-bromophenol until complete removal

of these compounds. By contrast, the value of $1/EC_{50}$ was multiplied by *ca.* 2.5 when *ca.* 45% of *p*-iodophenol had been removed; concentrations of BQ higher than with the other *p*-halophenols are tentatively suggested to be at the origin of this increase. Interpretation of a surprising substantial increase in the toxicity value when the removal of *p*-fluorophenol increased from 80 to 95% requires further investigation.

4.2. Introduction

The photocatalytic degradation of chloroaromatic pollutants in water has been widely studied (Blake, 1999; Pichat, 2003; Fujishima et al., 1999). In particular, it has been shown that benzenes substituted, inter alia, by chlorine can be dechlorinated.

However, investigations regarding benzene derivatives substituted by other halogens are rarer (Stafford et al., 1996), principally because fluorinated, brominated and iodinated compounds are less often employed. Nevertheless, studying their photocatalytic behaviour is also of interest to better assess the capabilities of this purification method and conceivably to provide insights into the degradation mechanisms.

In this chapter, the photocatalytic degradation of *p*-halophenols in TiO_2 aqueous suspensions is reported.

The study includes: the extent of adsorption of these compounds, the disappearance rates, the analyses of the primary aromatic intermediate products, and the variations in total organic carbon and toxicity (evaluated by the Microtox^{TM} test) during the degradation.

The purposes were to determine how the nature of the halogen affects these characteristics and whether the potential differences depend on the experimental conditions.

In particular, it was wished to further document the effects on the removal rates (i) of adsorption for these poorly adsorbed compounds, and (ii) of electron density on the aromatic ring, which varies with the halogen substituent.

It was also expected that changing the halogen might provide additional information on the basic photocatalytic degradation mechanisms, which are still controversial.

Another objective was to assess to what extent the variations in toxicity during the photocatalytic treatment differ for structurally closely related pollutants.

4.3. Materials and methods

*4.*3.1. Materials

All organic compounds were purchased from Aldrich, except 4-chlorophenol obtained from TCI. They were used without further purification (purity > 98%). The photocatalyst was TiO_2 Degussa P25 (about 80 % anatase, 20% rutile, 50 $m^2 g^{-1}$, non porous). To check the photocatalyst effect on the ranking of the *p*-halophenols degradation rates, Millennium PC 50 (100% anatase, 50 $m^2 g^{-1}$, average pore diameter: 20 nm) was used.

4.3.2. Adsorption isotherms in the dark

The batch adsorption equilibrium experiments were conducted at natural pH (ca. 6.5). Preliminary experiments, performed during 24 h under constant magnetic stirring, showed that the equilibrium was reached within 1 h. The equilibrium concentrations, C_{eq}, were determined by liquid chromatography after centrifugation and filtration (0.45 µm Millipore filters) of the suspension.

The number n_M of moles adsorbed per gram of TiO_2 was calculated as follows:

$$n_M = \frac{V \cdot \Delta C}{W}, \qquad \text{(Eq 1.1)}$$

where ΔC was the difference between the initial concentration (up to 30 mmol L^{-1}) and C_{eq}, V was the suspension volume (0.02 l), and W was the TiO_2 mass (0.2 g).

4.3.3. Photocatalytic degradation experiments

Unless otherwise indicated, a Philips HPK 125 W high-pressure mercury lamp was used. Its output was filtered by a 2.2 cm thick circulating-water cuvette to absorb the infrared wavelengths and a 340 nm cut-off filter (Corning 0.52) to avoid direct photolysis of the organic pollutants. The radiant flux thus entering the reactor was 47±4 mW cm^{-2} (as measured with a UDT 21 A powermeter). The magnetically stirred, aqueous suspensions were UV-irradiated in a cylindrical flask (total volume: ca. 90 ml), opened to air, with a bottom optical window whose surface was approximately 11 cm^2. The solutions were prepared with deionized, doubly-distilled water. The volume of aqueous phenol or *p*-

halophenol solution (0.155 mmol l^{-1}) introduced into the photoreactor was 20 ml. The minimal amount of TiO_2 necessary to have no UV radiant flux above the suspension was 0.5 g l^{-1}. That meant that the UV photons entering the reactor were absorbed, scattered or reflected mainly by the slurry and secondarily by the reactor. The degradation was carried out at room temperature and at natural pH (*ca*. 6.5). The aerated suspension was first stirred in the dark for 60 min to reach equilibrated adsorption.

To check the effects of the experimental conditions on the ranking of the degradation rates for the four *p*-halophenols and phenol, another type of reactor and a xenon lamp were also used. The total radiant flux, measured with a YSI Corporation powermeter, was 80 mW cm^{-2}. About 0.5% of the photons were emitted at wavelengths shorter than 300 nm and about 7% between 300 and 400 nm. This lamp was part of a Hanau Suntest Simulator. A 290 nm cut-off filter was used. The photocatalytic experiments were performed in open-to-air Pyrex flasks placed in the Suntest Simulator and containing 40 ml of suspension.

4.3.4. Analytical methods

The high performance liquid chromatography (HPLC) system comprised a LDC constametric 3000 isocratic pump and a LDC Spectro Monitor D UV detector adjusted at 225 nm. A reverse-phase column, 25 cm long, 4.6 mm i.d., packed with Spherisorb 5 ODS-2 was used. The mobile phase was composed of methanol (40 v/v %) and deionized doubly distilled water at pH 3 (adjusted with orthophosphoric acid).

Under these conditions, the retention times in min^{-1} were approximately: 10 (*p*-fluorophenol); 21 (*p*-chlorophenol); 26 (*p*-bromophenol); 43 (*p*-iodophenol); 3 (hydroquinone); 5 (benzoquinone); 3 (1,2,4-trihydroxybenzene); 7 (4-chloro-1,3-dihydroxybenzene); 11.5 (4-chloro-1,2-dihydroxybenzene); 8.5 (4-bromo-1,3-dihydroxybenzene).

Experience shows that retention times vary with trademarks, previous uses of the column and other settings of the HPLC apparatus.

Consequently, the aforementioned values must be regarded as indicative, and differences should not be disquieting for someone attempting to copy the method.

Identification of the intermediate organic products was performed both by comparing their UV spectrum to those of commercial compounds by use of both a Varian-9065 Polychrom diode-array HPLC detector and by GC-MS (HP 5890 and 5971 A).
For non-commercialised compounds, samples were analysed with liquid chromatography / mass spectrometry (LC-MS: HP Series 1100; Inersil ODS-2 column, 10 cm long, 3 mm i.d.). The mobile phase was a mixture of methanol and deionized, doubly distilled water, the v/v percentage of methanol varying from 10 to 90 over 30 min.

Dissolved Organic Carbon measurements were performed using a TOC analyzer (Shimadzu, model 5050A) equipped with an ASI automatic sample injector. A potassium phthalate solution was utilised for calibration. The precision and limit of detection of the apparatus were 1 and 0.2 ppm, respectively.

For analysing the halide anions, a Sarasep AN1 anion exchange column (Touzart, 10 cm long, 4.6 mm i.d.) with a solid phase chemical suppressor cartridge (Alltech) was employed. The mobile phase was a mixture of sodium hydrogenocarbonate (1.7 mmol l^{-1}) and sodium carbonate (1.8 mmol l^{-1}) in deionized, doubly distilled water. The detector was a Waters 431 apparatus. pH values were measured with a Iono Porcessor II (Tacussel).
The acute toxicity was assessed using a Microtox^{TM} Model 500 Analyzer. This analyzer is based on the use of a luminescent bacterium, viz. the strain *Vibrio fischeri* NRRL B-11177. When properly grown, luminescent bacteria produce light as a by-product of their cellular respiration. Any inhibition of cellular activity results in a decreased rate of respiration and a corresponding decrease in the rate of luminescence. The toxicity is determined by measuring, after 5 and 15 minutes of exposure, the concentration at which 50% of the light is lost because of the toxicity of the solution examined (EC_{50}). The Toxic Unit (TU) is used to express the data, as proposed by U.S. EPA (Lankford and Eckenfelder, 1990): $TU = 100/EC_{50}$.

In all cases, the analyses were at least duplicated. The standard deviation was less than 5%. The results reported in this study correspond to the average value of the measurements.

4.4. Results and discussion

4.4.1. Adsorption of phenol and p-halophenols on TiO_2

Figure 4.1 shows the results obtained for the extent of adsorption of *p*-halophenols on TiO_2 as a function of C_{eq}, the concentration in bulk solution at adsorption/desorption equilibrium in the dark.

The number n_M of organic molecules adsorbed per gram of TiO_2 can be derived from the following relationships (Cunningham and Srijaranai, 1994; Chen and Ray, 1999; Al-Sayyed et al., 1991):

$$\frac{C_{eq}}{n_M} = \frac{1}{K.n_{M\,max}} + \frac{C_{eq}}{n_{M\,max}} \qquad \text{Eq 4.2}$$

$$\frac{C_{eq}}{n_M} = \frac{N_A.\sigma^o}{A_{sp}.K} + \frac{N_A.\sigma^o}{A_{sp}}.C_{eq} \qquad \text{Eq 4.3}$$

where *K* is the adsorption equilibrium constant for the compound considered, n_{Mmax} is the maximum value of n_M, σ° is the average area occupied by one adsorbed organic molecule, A_{sp} is the TiO_2 surface area, and N_A is the Avogadro's number.

The plots of C_{eq}/n_M versus C_{eq} are shown for the four *p*-halophenols using the right-hand scale in Figure 4.1.

The values of n_{Mmax} and σ° are calculated from the slope and ordinate at the origin of the straight lines in Figure 4.1 according to Equations 2 and 3. These values are listed in Table 4.1.

The number of molecules adsorbed by surface area unit of catalyst, viz. $(1/\sigma^\circ)$ is in the order:

p-iodophenol > phenol > *p*-chlorophenol > *p*-bromophenol > *p*-fluorophenol

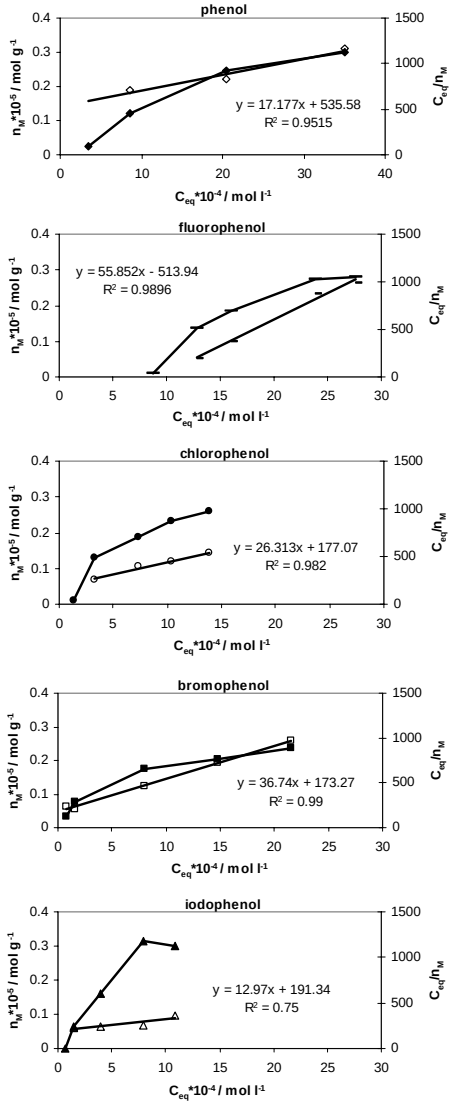


Figure 4.1. Room temperature isotherms of adsorption on TiO_2 for phenol and *p*-halophenols. For the meaning of n_M and C_{eq}, see equations (2-3). The thicker curves refer to the variations of n_M.

For *p*-chlorophenol, it agrees with previous reports (Al-Sayyed et al., 1991; D'Oliveira et al., 1990). The values are lower than those measured for *p*-chloro and *p*-fluoromethoxybenzenes (anisoles), viz. about 0.2 molecules per nm^2 (Amalric et al., 1996).

Considering that the geometric area of one molecule of *p*-halophenol is *ca.* 0.5 nm^2 (20 to 25% larger than benzene, i.e. 0.4 nm^2; Gregg and Sing, 1967), about two molecules would be needed to cover 1 nm^2 of catalyst. In other words, the surface coverage measured was comprised between 1.0 and 4.5%.

Low coverage is expected because pollutant molecules need to break the three-dimensional network of hydrogen-bound water molecules close to the TiO_2 surface and to reorganize the water molecules around them. Therefore an unfavorable change in entropy occurs.

Also, if it is assumed that *p*-halophenol molecules are hydrogen-bound to the surface hydroxyl groups and that the maximum concentration is on the order of 5 OH groups per nm^2 of TiO_2 (Boehm, 1966), at most 5 molecules of halophenol per nm^2 could be adsorbed. With respect to this maximum, the values measured would correspond to only 0.2 to 0.9 %.

Table 4.1. Measured extents of adsorption (Conditions: as for Figure 4.1), molecular characteristics (Handbook of Chemistry and Physics, 1992) and measured values of k for phenol and *p*-halophenols. (Conditions: as for Figure 4.2)

	n_{Mmax} (µmol g^{-1})	molecules adsorbed per nm^2 of TiO_2 $(1/\sigma^o)x\ 10^2$	C-H and C-F bond energy (kJ mol^{-1})	Hammet constant σ	k (h^{-1})
phenol	5.8	7.0	368 (C-H)	0	1.98
p-fluorophenol	1.8	2.2	523 (C-F)	0.15	2.17
p-chlorophenol	3.8	4.6	397 (C-Cl)	0.24	2.09
p-bromophenol	2.8	3.5	335 (C-Br)	0.26	1.76
p-iodophenol	7.7	9.3	272 (C-I)	0.28	0.88

4.4.2. Photocatalytic removal rates of phenol and *p*-halophenols

4.4.2.1. Results

The influence of the halogen substituent on the photocatalytic degradation of the *p*-halophenols was investigated by comparing their apparent rate constants of disappearance (*k*). In all cases, first-order kinetics was observed for up to 15 minutes, with linearization coefficients generally > 0.99 (see inset in Figure 4.2). The first 15 min have been used to focus on rates of elimination of the parent compound and to minimize competition with the removal of intermediate products.

The values of k (Table 4.1) are in decreasing order as follows:

p-fluorophenol ≈ *p*-chlorophenol ≈ phenol > *p*-bromophenol > *p*-iodophenol.

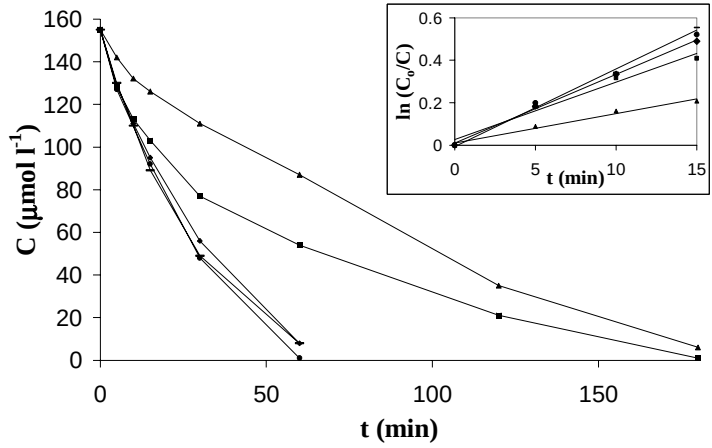


Figure 4.2. Kinetics of the photocatalytic disappearance of phenol (diamonds), *p*-fluorophenol (hyphens), *p*-chlorophenol (circles), *p*-bromophenol (squares), and *p*-iodophenol (triangles). The inset shows linear transforms. C is the concentration of the pollutant considered. Subscipt 0 refers to the initial conditions.
Conditions: 20 ml; 0.155 mmol l^{-1}; TiO_2 Degussa P25 (0.5 g l^{-1}); 47 mW cm^{-2}

This ranking is similar to that previously reported (O'Shea and Cardona, 1994; Parra et al., 2000).

This order did not change when TiO_2 Millennium PC 50 was used in place of TiO_2 Degussa P25 under otherwise identical conditions. Note that the two samples have the same surface area but PC 50 is porous, whereas P25 is not. The k values corresponding to P25 were about 1.6 times higher on the average.

When another reactor and lamp were employed (see Experimental section), the k ranking was still the same when increasing, under otherwise identical conditions, either the P25 concentration by a factor of 2 or the pollutant initial concentration by a factor of 2.5.

However, the ratio of k for each halophenol with respect to k for phenol depended to some extent on the conditions.

This shows that attempts to correlate results obtained under various conditions have limitations (Serpone et al., 1996).

4.4.2.2. Correlation between the photocatalytic degradability and the adsorbed amounts of p-halophenols

It is a priori reasonable to assume that the closer the pollutant to the photocatalyst surface, the higher the probability of reaction with holes or other oxidizing species formed on that surface.

In fact, *p*-iodophenol has the lowest k and the highest, though very low, number of molecules adsorbed per nm^2 of TiO_2 (Table 4.1).

And comparison of the other values of k and n_M for the other *p*-halophenols clearly shows that there is no relationship.

Indeed, all *p*-halophenols are poorly adsorbed as aforementioned.

4.4.2.3. Correlation between the photocatalytic degradability and the Hammett constant of p-halophenols

The Hammett constant σ represents the electron density over the aromatic ring. The higher σ the lower the electron density. In agreement with previous reports (O'Shea and

Cardona, 1994; Parra et al., 2000; D'Oliveira et al, 1993), a rough negative correlation was found between σ and k with a R^2 coefficient comprised between 0.57 (case of the k listed in Table 4.1) and 0.95 depending on the conditions (type of reactor, lamp and TiO_2, etc.).

This correlation suggests an attack by electrophilic species, i.e. hydroxyl radical or hole (Walling et al., 1978).

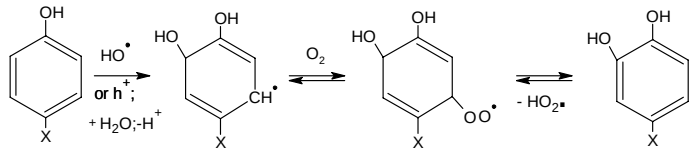
4.4.3. Aromatic intermediate products of degradation

The aromatic intermediate products were identified by LC-MS using commercially available standards, viz. hydroquinone (HQ), benzoquinone (BQ), 1,2,4-trihydroxybenzene, 4-chloro-1,2 (and 1,3)-dihydroxybenzenes, and 4-bromo-1,3-dihydroxybenzene.

Besides, the formation of another bromodihydroxybenzene, a fluorodihydroxybenzene and a dihydroxyiodobenzene was inferred from the mass spectra by comparison with the spectra of the commercialized halodihydroxybenzenes.

The additional brominated derivative was assumed to be 4-bromo-1,2-dihydroxybenzene as a result of the directive effect of the OH group of 4-bromophenol towards the ortho position for an electrophilic attack (Scheme 4.1).

As the positional selectivity of the F atom is the strongest and that of the I atom the weakest, 4-fluoro-1,3-dihydroxybenzene and 1,2-dihydroxy-4-iodo-benzene were likely to be the other halogenated derivatives detected.



Scheme 4.1. Suggested mechanism for the formation of 4-halo-1,2-dihydroxybenzene

Standards allowed us to quantify the amounts of the two halogenated aromatic intermediate products of 4-chlorophenol.

Figure 4.3 shows that 4-chloro-1,3-dihydroxybenzene reached a higher percentage than 4-chloro-1,2-dihydroxybenzene in the course of the degradation. In principle, the directive effect of the OH group should prevail over that of the Cl atom, therefore the percentages should have been the opposite of those observed. Lower amounts of 4-chloro-1,2-dihydroxybenzene may be explained by either a higher extent of adsorption of this compound on TiO_2 (K_{ads} = 0.13 l μmol^{-1}; Pichat, 2003), or a higher degradation rate. The first hypothesis seems more likely (Pichat, 2003).

The concentration of 1,2,4-trihydroxybenzene remained very low in accordance with the well-known photocatalytic instability of this compound.

The sum of the concentrations of HQ and BQ was higher than the concentrations of the aromatic halogenated intermediate products (Figures 4.3 and 4.4). Moreover, the concentration of BQ was higher than that of HQ in the case of *p*-iodophenol, whereas it was the opposite for the other *p*-halophenols and phenol (Figure 4.4). In particular, for *p*-fluorophenol the maximum concentration of BQ reached only 0.3 $\mu mol\ l^{-1}$, that is, approximately 60 times less than the maximum concentration of HQ.

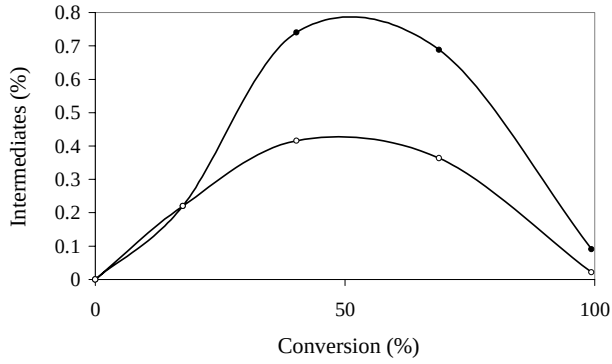


Figure 4.3. Percentages of 4-chloro-1,3-dihydroxybenzene (solid circles) and 4-chloro-1,2-dihydroxybenzene (open circles) formed with respect to the initial pollutant concentration versus the percentage of pollutant degraded. Conditions: as for Figure 4.2.

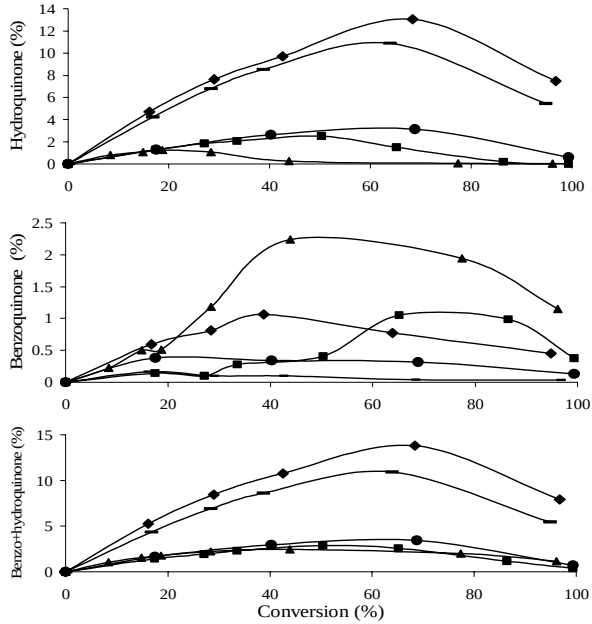


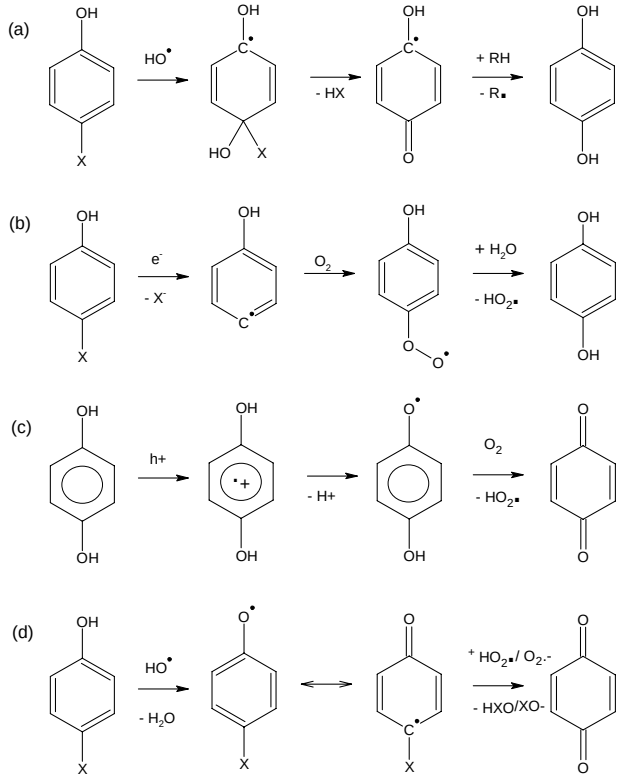
Figure 4.4. Percentages of BQ, HQ and BQ + HQ (with respect to the initial pollutant concentration) versus the removed percentage of phenol (diamonds), *p*-fluorophenol (hyphens), *p*-chlorophenol (circles), *p*-bromophenol (squares), and *p*-iodophenol (triangles). Conditions: as for Figure 4.2

Scheme 4.1 shows the mechanism usually proposed for hydroxylation of a benzene nucleus via the formation of a hydroxyhexadienyl radical. It accounts for the formation of the various 4-halodihydroxybenzenes.

In the case of phenol, formation of HQ can occur through a hydroxycyclohexadienyl radical if the attack occurs at position 4.

Replacing attack by an OH radical by attack by a hole, addition of H_2O and elimination of H^+ would yield to the same initial organic radical. In the case of the *p*-halophenols, the C atom in position 4 is not prone to be attacked by an electrophilic species because of the

electron-withdrawing effect of the halogen X. Consequently, formation of HQ via the sequence of reactions depicted in Scheme 4.2a is unlikely.



Scheme 4.2**.** Possible degradation pathways of *p*-halophenols, during TiO_2 photocatalysis, leading to either hydroquinone (HQ) (a and b) or benzoquinone (BQ) (d), and HQ oxidation into BQ (c). For discussion, see text.

To account for the formation of HQ from *p*-halophenols and for the differences induced by the nature of the halogen, we tentatively suggest a reductive attack at position 4 by a

photoproduced electron with the release of a X^- anion. As shown in Scheme 4.2b, the resulting aryl radical, after O_2 addition, would yield HQ, H_2O being used as a H source. The higher the electronegativity of X, the easier the reductive attack. That is in agreement with the concentration of HQ observed during the degradation, the highest concentration corresponding to *p*-fluorophenol and the lowest to *p*-iodophenol (Figure 4.4).

The mechanism suggested implies the cleavage of the C-X bond whose strength depends on the electronegativity of X. On the basis of this only argument, *p*-iodophenol should yield the highest amount of HQ. But the C-X bond strength intervenes in a second step, the primary step being the reductive attack which is favored by the partial positive charge on the C atom and, accordingly, by the electronegativity of X (inductive effect).

BQ can be formed by oxidation of HQ (Scheme 4.2c). If this oxidation was the only pathway, the ratio between BQ and HQ concentrations would be independent of the *p*-halophenol. As this is not the case, a mechanism implying position 4 on the aromatic ring must be involved.

The mechanism tentatively suggested (Scheme 4.2d) includes first the removal of the H atom of the phenolic OH group, second the use of $HO_2°/O_2^{°-}$ as a source of O with the release of HXO/XO^- (in the case of phenol: H_2O/OH^-), at least for X = I, Br, Cl. Note that this mechanism implies a reaction between two radicals, which diminishes its probability to occur; however, $HO_2°/O_2^{°-}$ are relatively abundant radicals during UV irradiation of TiO_2 aqueous suspensions in the presence of air. As the cleavage of the C-X bond occurs, the lower this bond strength (Table 4.1), the higher the reaction rate.

This is in agreement with the results (Figure 4.2 and Table 4.1). In addition, hypo-iodous, -bromous and -chlorous acids and the corresponding conjugate base anions are stable compounds, which might also explain a markedly higher rate of formation of BQ from *p*-iodo, bromo, and chlorophenol compared with *p*-fluorophenol (Figure 4.4).

The fluorous equivalents are highly unstable and their decomposition to HF/F^- and O_2 makes *a priori* the mechanism less likely to take place.

4.4.4. Toxicity

According to the Microtox™ tests, the toxicity ranking of the initial solutions from the less to the most toxic was:

phenol < *p*-fluorophenol < *p*-chlorophenol < *p*-iodophenol < *p*-bromophenol

(see Figure 4.5).

This tendency is in agreement with previous results (McCloskey et al., 1996; Cronin and Schultz, 1996), except for *p*-bromophenol, which was found less toxic than *p*-iodophenol.

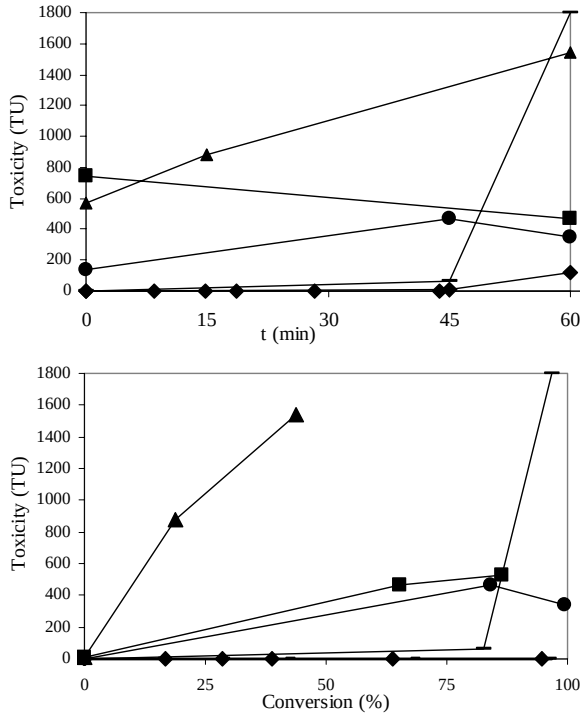


Figure 4.5. Evolution of the acute toxicity during the photocatalytic degradation of phenol (diamonds), p-fluorophenol (hyphens), p-chlorophenol (circles), p-bromophenol (squares), and p-iodophenol (triangles). Top: Kinetics. Bottom: Variation as a function of the percentage of pollutant removed. Conditions: as for Figure 4.2.

During the photocatalytic degradation of phenol and *p*-halophenols, distinct toxicity variations were observed depending on the initial compound. For phenol and *p*-chlorophenol, the toxicity increased slightly and for *p*-bromophenol it decreased slightly. A progressive rise was measured during the degradation of *p*-iodophenol, so that the Microtox^{TM} toxicity value was *ca.* 2.5 times higher when *ca.* 45% of *p*-iodophenol had been eliminated. A very pronounced increase in toxicity was observed when the removal of *p*-fluorophenol increased from 80 to 95%.

Though toxic (Corti and Snyder, 1998; De Caprio, 1999), HQ had not a significant effect on the overall toxicity. Indeed, HQ attained its highest, though very low concentrations in the course of the degradation of phenol and *p*-fluorophenol (Figure 4.4), while the measured toxicity remained the weakest of all the solutions examined (Figure 4.5).

The concentration of BQ, a compound more toxic than HQ (Den Besten et al., 1994; Colinas and Burkart, 1994), might explain the increase in the toxicity of the *p*-iodophenol-containing solution during degradation.

But this suggestion is only tentative as the percentage of BQ, with respect to the quantity of *p*-iodophenol eliminated, remained very low (Figure 4.4).

It has recently been reported that the cytotoxicity of iodoacetic acid is much higher than that of chloroacetic acid (Plewa et al., 2004). Accordingly, the observed increase in toxicity might be due to traces of I-containing organic intermediate products.

In the case of the *p*-fluorophenol degradation, the sudden increase in toxicity (Figure 4. 5) corresponded to a decrease in the concentration of F^- anions. Further work would be needed to determine whether these phenomena are coincidental or related, e.g. the formation of a SiF_6^{2-}complex might be assumed to occur as a result of an attack of the reactor glass by HF.

This discussion is meant to be only suggestive. Interpreting the toxicity variations would imply a complete knowledge of the nature and toxicity - on the bacteria employed - of the degradation products, which cannot be achieved. However, it is known that total mineralization can be reached by use of sufficiently prolonged UV-irradiation.

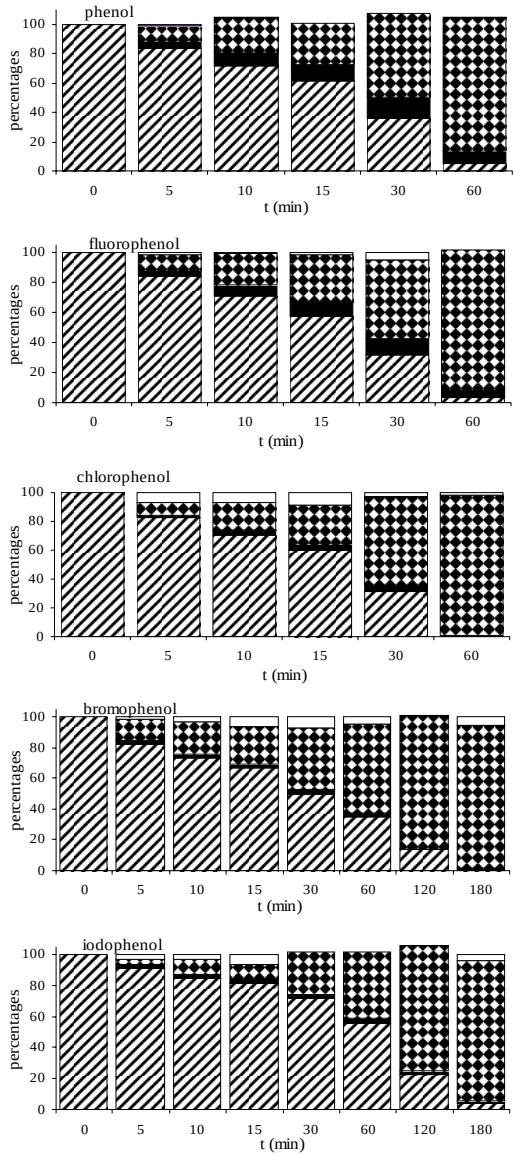


Figure 4.6. Percentages of the relative carbon forms in the TiO_2 suspensions during the photocatalytic degradation of *p*-halophenols (*p*-halophenols in gray, quantified intermediate products in black, CO_2 in striped and the difference in white). Conditions: as for Figure 4.2.

4.4.5. Carbon balance

Figure 4.6 shows the kinetic evolutions in the various forms of carbon. The time scale shown coincides with the total or almost total disappearance of the original compound. As pointed out in several papers dealing with the photocatalytic degradation of *p*-chlorophenol or its isomers (Chen and Ray, 1999; Al-Sayyed et al., 1991; D'Oliveira et al., 1990):

- the amount of aromatic intermediate products corresponded to only a few % of the initial amount of the original pollutant (less than 5% of TOC in the present case all along the degradation), and
- these aromatics disappeared at about the same time as the original compound.

The present study indicates that these features were similar for the other *p*-halophenols.

4.5. Conclusion

Beyond confirming the importance of the overall electron density on the ring for the photocatalytic removal rate of substituted benzenes in water, comparison of the primary aromatic products of *p*-halophenols has allowed us to suggest degradation mechanisms whose relative significance depends on the halogen.

Also, even though the total quantity of these aromatic intermediate products was found to be similar regardless of the halogen, at equal removal percentage of *p*-halophenol the variations in toxicity markedly differed for solutions that initially contained *p*-iodophenol.

This observation illustrates that halogen-induced differences in mechanisms and reactivity can be significant for the detoxification of waters polluted by these types of compounds.

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CHAPTER 5.

Integrated Photocatalytic-Biological Process for Treatment of Pesticides

PART A.

EVALUATING MICROTOX© AS A TOOL FOR BIODEGRADABILITY ASSESSMENT OF PARTIALLY TREATED SOLUTIONS OF PESTICIDES USING Fe^{3+} AND TiO_2 SOLAR PHOTO-ASSISTED PROCESSES

Submitted to *Ecotoxicological and Environmental Safety*

5.A.1. Abstract

To shorten photo-treatment time is of major concern for the cost and energy benefits of the xenobiotics degradation performed by photocatalytic processes. Using photo-Fenton and TiO_2 phototreatments, partially photodegraded solutions of 6 separate pesticides (alachlor, atrazine, chlorfenvinphos, diuron, isoproturon and pentachlorophenol) were tested for biocompatibility, which was evaluated according to the Zahn-Wellens procedure. This study investigated if Microtox© could be considered as a suitable global indicator capable of giving information on the evolution of biocompatibility of the water solution contaminated with organic pollutants during the phototreatment in order to promote biotreatment. The obtained results demonstrated that biodegradability increased significantly after short photo-Fenton treatment times for alachlor, diuron and pentachlorophenol. Uncertain results were obtained with atrazine and isoproturon. Microtox© acute toxicity testing was shown to correctly represent dynamics and efficiency of photo-treatment.

5.A.2. Introduction

For mixed household and industrial wastewater treatment plants, the contribution from industry must be managed properly in order to avoid toxic or persistent substances end up in the effluent or sludge. (Sarakinos et al, 2000; Chen et al., 1999; Grüttner, 1997; Grau and Da-Rin, 1997)

The magnitude of the potential risk of these toxicants on environment and human health is widely evaluated through biological monitoring. (Mantis et al., 2005; Pessala et al., 2004; Ma et al., 2005) In case of pesticides, the substances differ from most other industrial organic chemicals in that they are produced with the explicit intention of exerting toxic effects on one or more target organisms. Unfortunately, they can adversely affect other living beings that happen to be nearby, because these compounds are brought into the environment through various possibilities (Jones et al., 2004; Fent et al., 2006; Aksu, 2005; Welp and Brümmer, 1999).

Advanced Oxidation Processes (AOPs) have been proposed as an alternative for the treatment of biorecalcitrant wastewater. Many studies have concentrated on this goal (Legrini et al., 1993; Andreozzi et al., 1999; Espulgas et al., 2002; Pera-Titus et al., 2004). They pointed out that these processes, while making use of different reacting systems, are all characterized by the same chemical feature: production of strong oxidant species, such as hydroxyl radicals, which are able to oxidize almost all organic pollutants (Valavanidis et al., 2006). The photo-assisted catalysis involves the irradiation of a semi-conductor like TiO_2 with UV light at a wavelength below 387 nm, generating electron-hole pair on the catalyst surface and inducing the formation of radical reduced oxygen species. These radicals are produced in situ and are highly reactive and unselective oxidants. These OH radicals can also be produced by the Fenton reagent (addition of H_2O_2 to Fe^{2+} salts). Photo-Fenton (Safarzadeh-Amiri et al., 1996) combines Fenton and UV–VIS light. The photolysis of Fe^{3+} complexes enables Fe^{2+} regeneration. Under these conditions iron can be considered a real catalyst. Both iron and TiO_2 catalytic systems are of special interest because solar light can be used (Malato et al., 2002; Sarria et al., 2005). References and patents related to the photocatalytic removal of toxic compounds from

water published during the last decade can be counted in thousands (Blake, 2001): however, solar technologies have been most extensively studied and developed for heterogeneous TiO_2 photocatalysis and homogeneous photo-Fenton (Malato and Agüera, 2003). Particularly, investigation upon the photocatalytic oxidation of pesticides in aqueous media irradiated by near-UV light has been successful and many chemicals were reported to be degraded efficiently by this method (Malato et al., 1999; Hincapié et al., 2005; Pichat et al., 2004).

The major limitation of the AOPs is their operational cost. Indeed, for the total oxidation of hazardous organic compounds or non-biodegradable effluents, financial support remains relatively high compared to those of a biological treatment (Muñoz et al., 2005). Therefore it is potentially attractive to exploit these processes as a pre-treatment step to enhance the biodegradability of wastewater containing recalcitrant or inhibitory compounds (Scott and Ollis, 1997). Nevertheless this procedure is only justified if the resulting intermediates are easily degradable by microorganisms in a consecutive biological treatment because incomplete oxidation may result in more toxic intermediates than the parents (Amorós et al., 2000; Drzewicz et al., 2004). Therefore the toxicity assessment of the photodegradation products is the critical point in evaluating the possibility of photocatalysis to be a pre-treatment process.

Toxicity assessment of a chemical using a single species testing reflects the sensitivity of that test only: it may over or underestimate the potential toxicity for that particular substance. Accordingly, recent research has focused on the development of representative, cost-effective, and quantitative test batteries, which can detect different effects using a variety of endpoints (Manusadžianas et al., 2003; Davoren and Fogarty, 2004; Escher et al., 2005). However such complete battery of bioassays for mixed chemical compounds is not yet feasible, in particular for industrial effluents. Furthermore potential synergistic or antagonist effects may occur and can be problematic to study (Isnard, 1998; Kungolos et al., 1999; Hauser et al., 1997).

Microbial tests, due to several factors such as short exposure time, ease of handling and reproducibility of results between laboratories, have been widely used in toxicity screening procedures. Strains of luminescent bacteria and culture media were studied and

evaluated: in 1979, Anthony Bulich proposed a specific test for rapid assessment of the toxicity of aquatic samples using the light emitting bacterium *Vibrio fisch*eri (former *Photobacterium phosphoreum*). In 1982, the system was developed commercially under the trade mark Microtox™ (Berckman Instruments Inc.). Now this test is widely accepted as a standard for this bioassay type (Parvez et al., 2006; Radix et al., 2000) and has already been used for evaluation of industrial wastewaters (Lin et al., 1994; Hao et al., 1996) and study of toxicity of pesticides (Kahru et al., 1996; Jones and Huang, 2003).

Shortening time of phototreatment is of major concern for the cost and energy benefits of total treatment process (coupling of photocatalysis and biotreatment). The Zahn-Wellens procedure is the most appropriate method for biodegradation assessment and can be used for testing biocompatibility of partially photodegraded solutions of pesticides. But this analytical tool is quite time-consuming (28 days). The main objective of this study was to evaluate Microtox© as an alternative indicator for biodegradation assessment of partially phototreated waters contaminated with pesticides. In this study six pesticides (alachlor, atrazine, chlorfenvinphos, diuron, isoproturon and pentachlorophenol) were considered separately. Experiments on photocatalytic oxidation of these substances were performed over photo-Fenton and TiO_2, and results are presented. Dissolved organic carbon analysis was used as a measure of mineralization of pesticides to assess the efficiency of photocatalytic process. Toxicity assessment of the original pesticides and their photodegradation products were tested with acute Microtox© testing. Regarding the evolution of the toxicological level of the photodegraded solutions of pesticides in the course of the photo-treatment, different stages of photo-Fenton process were taken for testing their biodegradability with the Zahn-Wellens procedure.

5.A.3. Experimental

*5.A.*3.1. Chemicals

The chemical compounds used in this study consist of: Alachlor (95% technical grade $C_{14}H_{20}ClNO_2$, Aragonesas Agro S.A.), atrazine (95%, technical grade $C_8H_{14}ClN_5$, Ciba-Geigy), chlorfenvinphos (93.2%, technical grade $C_{12}H_{14}Cl_3O_4P$, Aragonesas Agro S.A.),

diuron (98.5%, technical grade $C_9H_{10}Cl_2N_2O$, Aragonesas Agro S.A.), isoproturon (98%, technical grade $C_{12}H_{18}N_2O$, Aragonesas Agro S.A.) and pentachlorophenol (98%, analytical grade C_6HCl_5O, Aldrich Chemical). Analytical standards of all pesticides (for chromatographic analyses) were purchased from Sigma-Aldrich. Water used in the pilot plant was obtained from the Plataforma Solar de Almería (PSA) distillation plant (conductivity<10μS/cm, Cl^- = 0.7-0.8 mg/L, organic carbon <0.5 mg/l). Ferrous iron sulfate ($FeSO_4.7H_2O$), hydrogen peroxide (30% w/v) and sulphuric acid for pH adjustment (around 2.7-2.8) were reagent grade. TiO_2 was Degussa P-25.

*5.A.*3.2. Analytical determinations

Mineralization was followed by measuring the dissolved organic carbon (DOC) by direct injection of filtered samples into a Shimazu-5050A TOC analyser provided with an NDIR detector and calibrated with standard solutions of potassium phthalate. Pesticide concentration was analysed using reverse-phase liquid chromatography (flow 0.5 ml/min) with UV detector in a HPLC-UV (Agilent Technologies, series 1100) with C-18 column (LUNA 5 μm, 3 mm x 150 mm, from Phenomenex). Ultra pure distilled-deionized water obtained from a Milli-Q (Millipore Co.) system and HPLC-grade organic solvents were used to prepare all the solutions. Cation concentrations were determined with a Dionex DX-120 ion chromatograph equipped with a Dionex Ionpac CS12A 4 mm x 250 mm column. Isocratic elution was done with H_2SO_4 (10 mM) at a flow rate of 1.2 ml min^{-1}. Anion concentrations were measured with a Dionex DX-600 ion chromatograph using a Dionex Ionpac AS11-HC 4 mm x 250 mm column. The gradient programme was pre-run for 5 min with 20 mM NaOH, injection of the sample, 8 min 20mM NaOH and 7 min with 35mM of NaOH at a flow rate of 1.5 ml min^{-1}. H_2O_2 concentration was determined by iodometric titration.

*5.A.*3.3. Toxicity Measurements

Microtox© acute toxicity testing was performed with *Vibrio fischeri* using a Model 500 Analyzer (Azur Environment, Workingham, England). Hydrogen peroxide present in the samples from photo-Fenton experiments was removed prior to toxicity analysis using

catalase (2500 U/mg bovine liver; 100 mg l^{-1}) acquired from Fluka Chemie AG (Buchs, Switzerland) after adjusting the sample pH to 7. Both samples from photo-Fenton and TiO_2 phototreatments were filtrated (0.22 μm filter, Schleicher and Schuell, G-Dassel) before toxicity testing. Measurement of toxicity was performed within 24 h after irradiation. Stored samples should be frozen before analysis. Toxicity is expressed as toxicity units, $TU=100/EC_{50}$, where EC_{50} is the concentration which causes 50% reduction of the bioluminescence (*Vibrio fischeri*). All chemicals for the bioassays were obtained from a commercial supplier (Tetra Technique, Veyrier, Switzerland).

5.A.3.4. Biodegradability assessment

The Zahn-Wellens test was carried out according to the EC protocol (Directive 88/302/EEC). The activated sludge obtained from a secondary effluent of the treatment plant of Almería (Spain) or Morges (Switzerland) was used as inoculum. The fresh activated sludge was centrifuged at 10 000 RPM during 7 minutes at 20 °C, and washed once with mineral medium. According to the guidelines of the Zahn-Wellens test, the ratio between the carbon content of the experimental sample and the dry-weight of the inoculum was ranged between 1 and 4 (average 3). Aeration and homogenization were guaranteed. Preliminary experiments were performed to check that neither volatilization nor adsorption occurred during the testing period of 28 days.

5.A.3.5. Photoreactor set-up

All the experiments were carried out under sunlight, in a pilot plant at the PSA (latitude 37ºN, longitude 2.4ºW). The pilot plant was operated in batch mode and had three compound parabolic collectors (CPCs), one tank and one recirculation pump (Malato-Rodrıguez et al., 2004). Schematic of the photoreactor is illustrated in Figure 5.A.1. Each collector (1.03 m^2 each) was mounted on a fixed platform tilted 37º (local latitude). The water flowed at 20 l min^{-1} directly from one module to another and finally into the tank, from which the pump recirculated the fluid back to the CPCs. The total volume (V_T) of 35 l was separated into two parts: 22 l (glass-transparent tubes in the CPC) of total irradiated volume (V_i) and the dead reactor volume (tank + high density polyethylene

tubes), which was not illuminated, as recently described in detail elsewhere (Kositzi et al., 2004). Solar ultraviolet radiation (UV) was measured by a global UV radiometer (KIPP&ZONEN, model CUV 3), mounted on a platform tilted 37º (the same as the CPCs), which provides data in terms of incident W_{UV} m^{-2}. In this way, the energy reaching any surface in the same position with regard to the sun could be measured. Based on Eq 5.A.1, the actual time for the irradiation was calculated for the experiment.

$$t_{30W,n} = t_{30W,n-1} + \quad t_n \frac{UV}{30} \frac{V_i}{V_T}; \qquad t_n = t_n - t_{n-1} \qquad \text{Eq 5.A.1}$$

where t_n is the experimental time for each sample, UV the average solar ultraviolet radiation measured during Δt_n, and t_{30W} the "normalized illumination time". In this case, time refers to a constant solar UV power of 30 W m^{-2} (typical solar UV power on a perfectly sunny day around noon). This calculation allows the comparison with other photocatalytic experiments. As the system was outdoors and was not thermally controlled, the temperature inside the reactor was continuously recorded by a PT-100.

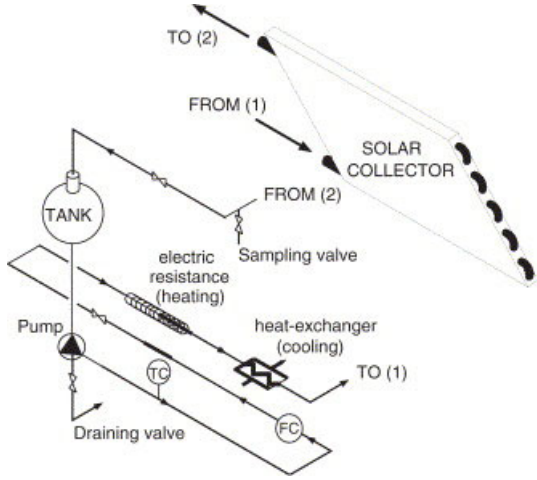


Figure 5.A.1. Flow diagram of photoreactor

The characteristics (mainly water solubility) of the six pesticides tested, made the use of "simulated wastewater" preparation procedures necessary.
Solutions of alachlor, chlorfenvinphos and isoproturon were tested at 50 mg l^{-1}. Since atrazine, diuron and pentachlorophenol are soluble in water at less than 50 mg l^{-1} at ambient temperature, these compounds were tested at the maximum of their hydrosolubility. These solutions consisted of the filtrated supernatant of a saturated solution of the target chemical. Exact concentration was measured by HPLC.

During the photocatalytic experiments, DOC analyses were used to monitor the degradation and mineralization of each pesticide tested in this work. In some cases, concentration of the inorganic species produced (mainly chloride, nitrate and ammonia) and direct pesticide concentrations (provided by HPLC) were used for mass balance calculation.

Concerning the photo-Fenton set of experiments, several prior tests (Hincapié et al., 2005) were performed with very different Fe concentrations (2 and 55 mg l^{-1}) to find out the best conditions for comparison with TiO_2 and for evaluating toxicity and biodegradability with both photo-treatments. It has been demonstrated that 2 mg l^{-1} of Fe were enough for mineralizing the pesticides under illumination.

5.A.3.6. Data analyses

Validation of toxicological bioassays as well as biodegradability assessments was ensured since all the quality control data were considered acceptable according to the official guidelines (OECD, U.S. Environmental Protection Agency (EPA) Office of Pollution Prevention and Toxics (OPPT), and the European Commission) and other established criteria (e.g., response to the negative controls, use of reference substances: phenol for Microtox©, diethylene glycol for Zahn-Wellens testing (Lapertot and Pulgarin, 2006)).

Photocatalytic and biodegradability experiments were at least duplicated and all samples were analyzed in triplicate.

Toxicity data were computed and EC_{50} values were calculated according to the gamma method, using linear regression analysis of transformed DOC concentrations as natural logarithm data versus percentage inhibition. All correlation coefficients were > 0.90.

Statistical analysis of all experimental results was carried out using analysis of variance (ANOVA). For all laboratory experiments, α was set at 0.05.

Concerning toxicity data, outliers were first detected using Student's *t* test. The 5-min and 15-min EC_{50} values were compared using one-way ANOVA with Dunett's multiple comparison procedure. Control and samples were also compared. Data were tested for normality and homogeneity of variances. In the case of a failed normality or equal variance test (e.g. between 5- and 15-min EC_{50} values or for chlorfenvinphos), data were rank transformed and tested again. If data were still not normal (it only concerned 5- and 15-min EC_{50} values), they were compared using a non-parametric Kruskal-Wallis test. Following the Kruskal-Wallis test, a non-parametric analog of the Dunnett's procedure for multiple comparisons was used (Zar, 1996). Finally, each endpoint corresponds to an averaged value of 4 to 12 validated data.

Such a statistical analysis was performed to strongly confirm the indicated endpoints, with a significant level of probability ($P< 0.05$).

5.A.4. Results

*5.A.*4.1. Photodegradation

The results obtained with the TiO_2 and photo-Fenton treatments for each pesticide are shown in Figures 5.A.2 and 5.A.3, respectively.

Concerning photo-Fenton (Figure 5.A.3), all the experiments were done in the same way.

Once the pesticide dissolved in water had been added to the pilot plant, it was homogenized in the system.

Then a sample was taken (initial concentration, t_{30W} = -0.75 h) and concentrated sulfuric acid was added for pH adjustment, so that volume changes could be ignored.

After 15 min a second sample was taken (t_{30W} = -0.5 h) to check if the pH was around 2.7.

After that, iron salt ($FeSO_4.7H_2O$) was added and mixed for 15 min (t_{30W} = -0.25 h).
Finally, the necessary amount of hydrogen peroxide was added, and after 15 min a sample was taken to evaluate the dark Fenton reaction (t_{30W} = 0).
At that moment collectors were uncovered and the photo-Fenton experiments began ("illumination", Figure 5.A.3a).
In the case of pentachlorophenol, acid was not added in order to avoid precipitation, but pH evolved very quickly to acidic pH (due to release of chloride) and photo-Fenton was effective (no Fe precipitation was detected).

In the case of TiO_2 tests the initial procedure was shorter.
All the experiments were started recirculating the compound(s) solution, with the collectors covered, for about 15 min until a constant concentration was achieved throughout the system.
After sampling, the catalyst, titanium dioxide, was added (final concentration = 200 mg/L) and the mixture recirculated for more than 15 min.
Just before uncovering the CPCs, to start the photocatalytic process, another sample was collected to characterise the initial condition of the solution.

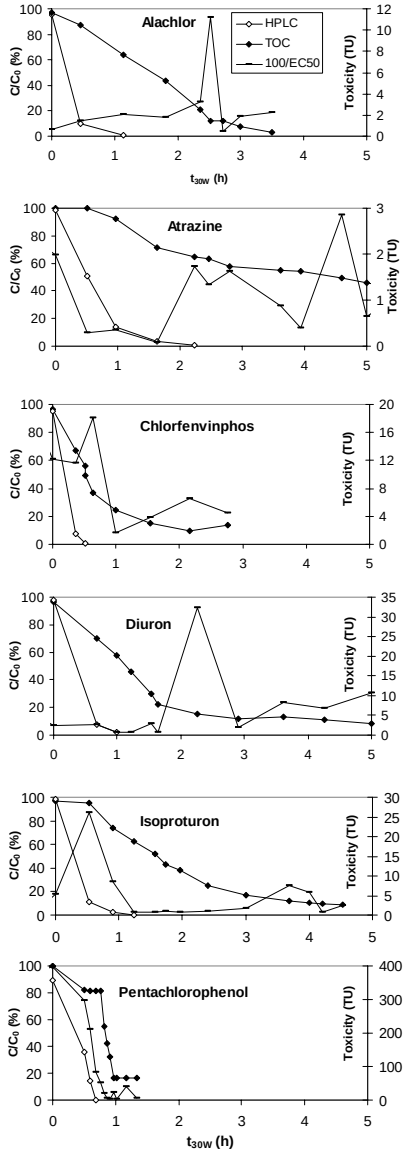
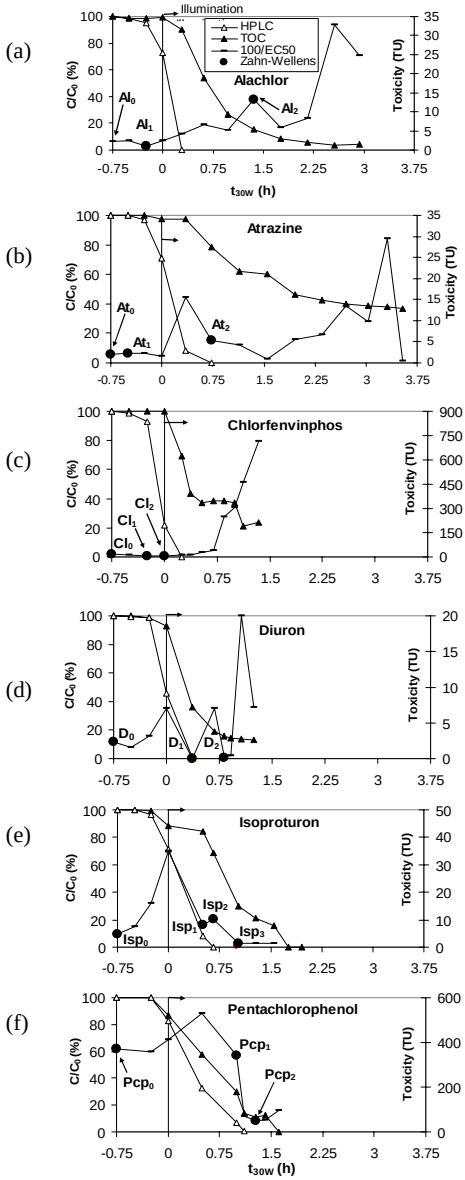


Figure 5.A.2. Evolution of mineralization (DOC), compound removal (HPLC) and toxicity ($100/EC_{50}$) during the TiO_2 photocatalytic process of single pesticide solutions tested for alachlor, atrazine, chlorfenvinphos, diuron, isoproturon and pentachlorophenol.



Negative illumination time (t_{30W}) corresponds to the dark period of experiment. Start of illumination is indicated by the vertical line (t_{30W}=0) and the arrow on top of each graph. Points Al_x, At_x, D_x, Cl_x, Isp_x, Pcp_x indicate samples which were taken for a further biodegradability assessment (See Table V.A.2).

Figure 5.A.3. Evolution of mineralization (DOC), compound removal (HPLC) and toxicity ($100/EC_{50}$) during the photo-Fenton process of single pesticide solutions tested for alachlor, atrazine, chlorfenvinphos, diuron, isoproturon and pentachlorophenol.

It is important to note that during the dark period of photocatalytic treatments neither mineralization by Fenton nor degradation by TiO_2 was observed for all the tested compounds.

Comparing the time course of photodegradation of pesticides between Figures 5.A.2 and 5.A.3 brings out that in all cases photo-Fenton was quicker than TiO_2.

During both photocatalytic treatments, some intermediate products are generated. The relative amounts of these by-products correspond to the difference between the curves of HPLC and TOC, on Figures 5.A.2 and 5.A.3. The molecular composition of the partially degraded solutions of pesticides was not determined but Figures 5.A.2 and 5.A.3 show that TiO_2 and photo-Fenton generate similar proportions of intermediate by-products in the course of the photodegradation process.

Complete mineralization could be achieved in all cases, except for atrazine.

Comments concerning atrazine and the overall photoreactivity of test pesticides have been previously detailed and discussed (Farré et al., 2005; Hincapié et al., 2005; Lapertot et al., 2006b).

*5.A.*4.2. Toxicity Measurements

The study aimed to assess if the Microtox© analysis could be a useful tool suitable to detect when a water pre-treated by a phototreatment could be biodegradable.

For that reason Microtox© bioassay was first tested for its reliability to evaluate the acute toxicity (to *Vibrio fischeri*) of each pesticide.

Table 5.A.1 gives the EC_{50} obtained for pure pesticide diluted in MilliQ water after 5-min exposure. Statistics and literature data for comparison are also listed in Table 5.A.1.

The cumulative results and variation coefficients < 10% indicate that Microtox© bioassay provides a suitable evaluation of acute toxicity.

Among all the target compounds, it was found that atrazine, alachlor and diuron are the less toxic substances, whereas pentachlorophenol is the most toxic. As indicated in Table 5.A.1 (see references), obtained data are concomitant with literature. In particular, atrazine was already designated to be poorly toxic at environmentally relevant concentrations (Lawton et al., 2005).

Table 5.A.1. Experimental and reference data for acute Microtox© EC_{50} testing for the target pesticides.

Test Compound	Experimental			Literature	
	EC_{50} (mg/l)	Repetition	Variation Coefficient (%)	Reference	EC_{50} (mg/l)
Alachlor	105 ± 8 [(1)]	6	9.44	Canna-Mickaelidou and Nicolaou, 1996	135 ± 14 [(2)]
Atrazine	89 ± 6 [(1)]	6	8.44	Boggaerts et al., 2001 Kross et al., 1992	150 13 [(3)]
Chlorfenvinphos	18 ± 1 [(1)]	6	4.46	Not Found	-
Diuron	86 ± 5 [(1)]	8	8.74	Boggaerts et al., 2001	58
Isoproturon	29 ± 2 [(1)]	6	7.09	Parra et al., 2002	25
Pentachloro-phenol	1 ± 0.1 [(1)]	4	5.59	Boggaerts et al., 2001 Kahru et al., 1996 De Zwart and Slooff, 1983	0.7 1.1 0.9

[(1)]: 95% Confidence Interval (mg/l), [(2)]: EC_{20}, 10 min, [(3)]:EC_{10}, 5 min

As illustrated in Figures 5.A.2 and 5.A.3, toxicity was evaluated regularly in the course of photocatalytic processes. Frequent samples were collected in order to observe the impact of the oxidative treatment on DOC content, residual pesticide concentration and toxicological level. Toxicity evolution was considered as an indicator of a progressive molecular transformation of the solution. It was already demonstrated that variations of toxicity were representative of the molecular transformations which result from photocatalytic reactions (Parra et al., 2002) or from other treatments (Drzewicz et al., 2004) and even biological processes (Lin et al., 1994). As illustrated in Figures 5.A.2 and 5.A.3, each pesticide exhibited a particular toxicological behavior, which also depends on the applied photocatalytic process (TiO_2, photo-Fenton). For instance, overall toxicity increased at the first steps of oxidation process. But this trend was alternative, depending on both the type of compound and the photocatalytic process. The toxicity could be leveled off or up as treatment was proceeding.

An increasing toxicity reveals the production of some intermediate metabolites which are more toxic than the initial molecule (Lapertot et al., 2006a). Thus, whenever the phototreated solution was considered sufficiently modified regarding its toxicological variations, the photodegradation was interrupted and a consecutive biodegradability assay was carried out.

*5.A.*4.2. Biodegradability testing

Taking into account the higher efficiency of the photo-Fenton with regard to TiO_2 process for degrading the target pesticides, it was decided to evaluate biodegradability for photo-Fenton only.

For each target pesticide, two or three steps were identified and selected for further biodegradability assessment with the Zahn-Wellens test (Figure 5.A.3, see dark circles indicated on toxicity curves).

Table 5.A.2 indicates the results obtained for the biodegradability testing with Zahn-Wellens. The test was considered positive whenever 70% of carbon elimination was obtained within 28 days maximum according to the EC protocol (Directive 88/302/EEC). The intermediate results noted (+/-) in Table 5.A.2 correspond to a carbon elimination of approximately 60% achieved within 28 days, the 70%-threshold being attained later on.

Concerning the biodegradability assessments performed on the initial solutions of single pesticides, several prior tests were carried out, using either Zahn-Wellens or ZW-slightly modified methods (Lapertot and Pulgarin, 2006). All pesticides were shown biorecalcitrant, except atrazine.

Despite the good biodegradability of atrazine, it has been preferred including it in the study because it has been demonstrated at both lab (Farré et al., 2005) and pilot plant scales (Hincapié et al., 2005) that when treated by photo-Fenton, the toxicity (measured by *Vibrio fischeri*) of the water containing atrazine (around 20 mg/l) increases during the process, then decreases again after a certain time.

Toxicity was not yet related to biodegradability, but one of the purposes of this paper was to explore the relationship between these two parameters. Moreover the EU aims to

reduce pollution from discharges of hazardous substances (European Commission, 2002), in which atrazine is concerned.

In this context, any type of AOP applied should demonstrate the complete removal of the target substance and the harmlessness of the treated effluent.

Table 5.A.2. Biodegradability assessment performed with the partially phototreated solutions of single pesticide.

Test Compound	Sample[(1)]	Characteristics phototreated solutions			Zahn-Wellens Result
		Presence of initial compound	t_{30W} (h)	DOC/DOC_o (%)	
Alachlor	Al_0	Yes	0	100	-
	Al_1	Yes	0	99	-
	Al_2	No	1.36	16	+
Atrazine	At_0	Yes	0	100	+
	At_1	Yes	0	100	-
	At_2	No	0.75	78	+/-
Chlorfenvinphos	Cl_0	Yes	0	100	-
	Cl_1	Yes	0	100	-
	Cl_2	Yes	0	100	+
Diuron	D_0	Yes	0	100	-
	D_1	No	0.37	36	+/-
	D_2	No	0.81	15	+
Isoproturon	Isp_0	Yes	0	100	-
	Isp_1	Yes	0.50	85	-
	Isp_2	No	0.66	69	+/-
	Isp_3	No	1.03	30	+
Pentachloro-phenol	Pcp_0	Yes	0	100	-
	Pcp_1	No	1.0	30	+
	Pcp_2	No	1.27	11	+

[(1)]: Samples are taken at the stages of the photo-Fenton treatment indicated in Figure 5.A.3

In this study, the solutions resulting from the final stages of both photo-treatments (photo-Fenton and TiO_2) were supposed biocompatible, because either mineralization was achieved or at least only minimum organic carbon contents were still remaining, except for atrazine.
For that reason and also because it is aimed to minimize the duration of the photo-treatment, no biodegradability assessment was performed at the final stages of the oxidative processes.

From Table 5.A.2 it can be concluded that the photo-treatment period can be significantly shortened because of the positive Zahn-Wellens results obtained with the partially degraded solutions of all pesticides.

5.A.5. Discussion

*5.A.*5.1. Toxicity assessment

To shorten phototreatment time is of major concern for the cost and energy benefits of total treatment process (coupling of photocatalysis and biotreatment).
Therefore the first aim of this study was to investigate if Microtox© could be considered as a suitable global indicator capable of giving information on the evolution of biocompatibility of the water solution contaminated with organic pollutants during the phototreatment in order to promote biotreatment.
But due to the complexity of the studied process and the specificity and sensibility of the Microtox© testing, this approach was considered and discussed with caution.
Besides, a more detailed study of the intermediate metabolites as well as other inorganic species produced such as chloride, ammonium or nitrate contents in addition with toxicity analyses of these compounds should be achieved in order to improve the knowledge of the implicated degradation pathways and molecular interactions.
Some studies have already been devoted to similar topics (Hermann et al., 1999; Amorós et al, 2000).
However tentative statements are proposed in order to explain some specific toxicity variations.

In fact, due to the chemical complexity of the solutions produced during photocatalytic processes, it seems more interesting to focus on the deviation trend which occurs between DOC and HPLC data in the course of the photo-degradation (expressed in % C-gram of the initial concentration) rather than observe the sole overall evolution of toxicity. Indeed, the difference between the two curves (HPLC and DOC) pictured in Figures 5.A.2 and 5.A.3 indicates the amount of the intermediate metabolites which are generated during the photocatalytic process.

Considering the removal of the initial molecule on one hand and the disappearance of DOC on the other hand, two main categories of behaviors can be outlined.
When the produced intermediates demineralize shortly, toxicity decreases regularly in the course of the photodegradation. This is the case of isoproturon and pentachlorophenol (photo-Fenton, Figure 5.A.3).
But when the intermediate metabolite degradation takes a long time (after disappearance of the original compound), level of toxicity is not predictable, but overall in the end, it tends to level out (for eg. in Figure 5.A.3: atrazine, alachlor, chlorfenvinphos, and diuron).

As it is illustrated in Figures 5.A.2 and 5.A.3, Microtox© acute toxicity testing demonstrate the overall dynamics and efficiency of photo-treatment.

*5.A.*5.2. Biodegradability Assessment

As indicated in Table 5.A.2, for each target compound, several steps of photo-Fenton treatment were evaluated for biocompatibility.

First of all, as expected, longer process advancement results in a higher potential for the consecutive biotreatment.

The obtained results show that biodegradability of some compounds can be achieved even in the presence or absence of the original molecule. For instance alachlor biodegradation was not observed until alachlor was completely removed by photo-Fenton. On the contrary, for atrazine and isoproturon, partial degradation results in biodegradable solution even in presence of original compound. Actually, as shown in Table 5.A.2, biodegradability was negative for atrazine and isoproturon in the samples

where the parent compound was still present. Except for the initial solution of atrazine as it is a biodegradable compound. Chlorfenvinphos could be also included here.

The obtained results show that chlorfenvinphos was biodegraded even without illumination. In fact, 80% of chlorfenvinphos was removed during dark-Fenton. It was transformed into intermediate by-products with no mineralization (DOC constant). Therefore biodegradability was achieved due to lower amounts of initial compound. It confirms the results presented in Chapter 2 in which it was stated that chlorfenvinphos implies irreversible cellular damages that could be overwhelmed whenever the pesticide concentration was decreased from 50 down to 10 mg l^{-1} (Lapertot and Pulgarin, 2006).

In case of alachlor, diuron and pentachlorophenol, the progressive decrease of DOC/DOC_0 during photo-Fenton could be related with an increase of biodegradability (see Table 5.A.2). Indeed biodegradability has been achieved for alachlor, diuron and pentachlorophenol when DOC/DOC_0=16%, 15% and 11% respectively, which means that the DOC may decrease more than in the case of isoproturon (DOC/DOC_0=30%).
For isoproturon, biotreatability of the contaminated water could be finally achieved after more than 0.7 h of illumination time ($t_{30W,}$ photo-Fenton, Table 5.A.2).

Concerning atrazine, as illustrated in Figure 5.A.3, the intermediate metabolites produced during photo-Fenton provoked an increasing toxic response to *Vibrio fischeri*. Besides after 0.75 h of irradiation, the obtained solution of atrazine was found to be biorecalcitrant. Surprisingly, the initial solution of pure atrazine has already been proved biodegradable (Lapertot and Pulgarin, 2006). More precisely, only 40% of carbon content was eliminated during the Zahn-Wellens test (results not shown). Attending to the results of DOC, HPLC, nitrogen and chloride analyses, the intermediates were completely dechlorinated and only the triazine ring was remaining.
As cyanuric acid is known to be biodegradable, other non-chlorinated atrazine intermediates should be biorecalcitrant (Kontchou and Gschwind, 1999; Horikoshi and Hidaka, 2003; Lawton et al., 2005). Among the photogenerated intermediates, ammelide and ammeline are two well-known metabolites produced during photo-treatment of atrazine (Konstantinou and Albanis, 2003; Chan and Chu, 2005). A specific analysis of Microtox© acute toxicity performed on these two pure substances showed that their EC_{50}

(5-min) is lower than pure atrazine, i.e. 0.12 ± 0.02 and 0.18 ± 0.01 mg l^{-1}, respectively. This result is concomitant with the observed increasing toxicity during the photo-treatment of atrazine (Figures 5.A.2 and 5.A.3). Cyanuric acid, which is the final product formed during oxidation process after long irradiation times (Pellizzetti et al., 1990) exhibited an EC_{50} (5-min) of 148 ± 12 mg l^{-1}. The lower toxicity (in comparison with atrazine and its degradation products) has also been reported by Hiskia et al. (2001).

5.A.6. Conclusions

In this study, it has been shown that photo-Fenton was more efficient than TiO_2 for pesticide degradation and TOC mineralization.

It has also been demonstrated that photo-Fenton treatment is a very consistent method for enhancing biodegradability of water contaminated with pesticides, and therefore reducing the costs of the treatment (hydrogen peroxide consumption, photoreactor size, operation time, etc.).

Indeed biodegradability increases significantly after short photo-Fenton treatment times for alachlor, diuron and pentachlorophenol.

Uncertain results were obtained with atrazine and isoproturon, which also indicates the need to perform previous tests before coupling photo-degradation and biotreatment.

The obtained results demonstrate the Zahn-Wellens test as a useful tool for determining the optimal DOC range in which wastewater becomes biodegradable.

Overall Microtox© acute toxicity testing was shown to represent dynamics and efficiency of photo-treatment. Hence it was observed that during phototreatment the relative increase of toxicity could be accompanied by a concomitant increase of biodegradability.

Thus even if Microtox© can not provide a reliable biodegradability assessment by itself, it can help for selecting samples to be tested by the Zahn-Wellens biodegradability assessment method.

5.B.7. References

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CHAPTER 5.

Integrated Photocatalytic-Biological Process to Treat Pesticides

PART B.

PHOTO-FENTON AND BIOLOGICAL INTEGRATED PROCESS FOR DEGRADATION OF A MIXTURE OF PESTICIDES

Submitted to *Journal of Photochemistry and Photobiology, A: Chemistry*

5.B.1. Abstract

A solution of mixed pesticides (alachlor, atrazine, chlorfenvinphos, diuron, isoproturon) was considered for degradation employing photo-Fenton as a preliminary step before biotreatment. Photo-Fenton degradation is an important sub-process in the integrated photobiological process for removal of biorecalcitrant chemicals. Shortening the photo-Fenton treatment time has a critical impact on the economical feasibility of the integrated process. In this study, photo-Fenton was proved to enhance biotraitability of a mixture of biorecalcitrant substances (pesticides). During the photocatalytic process, dissolved organic carbon (DOC) measurements, liquid and ionic chromatography analyses, acute toxicity evaluation using Microtox^{TM} and the Zahn-Wellens biodegradability testing were provided to characterize the photo-treated solutions. In order to find out the best moment for coupling of photocatalytic and biological processes, different times of photo-Fenton pretreatment were tested for biotreatment. The partially photo-treated solutions were fed to 8 parallel continuously operated packed-bed bioreactors during 28 days. Considering the coupled system, it was shown that the pre-treated solutions obtained with only 1 g l^{-1}

H_2O_2 (irradiation time, t_{30W}= 0.6 h) exhibited higher than 80% of DOC degradation. In this case, the packed-bed bioreactors contributed to more than 50% of the total carbon conversion of the pesticide solution.

5.B.2. Introduction

Agrochemicals such as insecticides, herbicides and fungicides are classified among the most dangerous toxicants. Due to their high toxicity, biological treatment of agro-industrial effluents is often perturbed and sometimes blocked. It is more important to test the mixture of these substances rather than the individual chemical species because of the possible presence of combine synergistic and/or antagonistic effects.

In this study, five pesticides were chosen including alachlor, atrazine, chlorfenvinphos, diuron and isoproturon. These compounds are considered Priority Substances by the EU (European Commission, 2001) not only from their intrinsic toxicity but also because of their extremely easy transport in the environment that represents a risk to both surface and groundwater.

Several oxidative degradation procedures (AOPs, Advanced Oxidation Processes) have been developed in the field of the chemical treatment of water for the elimination of pesticides: TiO_2/UV, Fenton reagent, O_3, O_3/UV and O_3/H_2O_2. Of these, photo-Fenton process was demonstrated particularly efficient for mineralization of pesticides (Farré et al., 2005 ; Hincapié et al., 2005 ; Malato et al., 1999 ; Pichat et al., 2004). The Fenton method requires H_2O_2, Fe^{2+} salts and acidic pH. Under these conditions highly reactive and unselective oxidants, OH radicals, are produced. With UV-VIS light, the formed Fe^{3+} complexes are photolysed, which enables regeneration of Fe^{2+} (catalyst): the process is called photo-Fenton.

Photo-Fenton and other AOPs for wastewater treatment have been extensively studied (Blake, 2001), but their use remains limited due to high operational costs, generation of UV radiations by lamps, H_2O_2 consumption in case of photo-Fenton (Pulgarín et al., 1999 ; Bressan et al., 2004 ; Da Hora Machado et al., 2005). Hence the combination of photo-Fenton as a preliminary treatment, followed by a biotreatment was considered to be a feasible process for pesticide removal. Previous papers related the feasibility of the

coupling, using the Zahn-Wellens procedure to assess the biocompatibility of the photo-treated solutions of individual pesticides (Lapertot et al., 2006 ; Sarria et al., 2003a ; Sarria et al., 2003b). In Part A of Chapter 5, the Microtox^{TM} acute toxicity measurement was considered as a predictive tool to facilitate the biocoupling. In this study, the biotraitability of the photodegraded solutions was more precisely investigated using packed-bed bioreactors operated during 28 days.

This paper reports:

(1) the results of the preliminary experiments that demonstrated the biological incompatibility of the solution of mixed pesticides without any photo-Fenton pretreatment;

(2) the photo-Fenton degradation of the mixed pesticides. The chemical and biological characteristics of the photo-treated solutions were evaluated in order to select different stages of the photocatalytic reaction to be tested for the subsequent biotreatment;

(3) the results of the biotreatment, which was carried out using several bioreactors operated in parallel and continuously fed by various pre-treated solutions of mixed pesticides,

(4) the overall carbon degradation efficiency of the tested photo-Fenton / bioreactor coupled systems.

5.B.3. Materials and methods

*5.B.*3.1. Chemicals

The chemical compounds used in this study consist of: alachlor (95% technical grade $C_{14}H_{20}ClNO_2$, Aragonesas Agro S.A.), atrazine (95%, technical grade $C_8H_{14}ClN_5$, Ciba-Geigy), chlorfenvinphos (93.2%, technical grade $C_{12}H_{14}Cl_3O_4P$, Aragonesas Agro S.A.), diuron (98.5%, technical grade $C_9H_{10}Cl_2N_2O$, Aragonesas Agro S.A.) and isoproturon (98%, technical grade $C_{12}H_{18}N_2O$, Aragonesas Agro S.A.). Analytical standards of all pesticides (for chromatographic analyses) were purchased from Sigma-Aldrich.

Demineralised water was used to prepare the mixture of pesticides. Ferrous iron sulfate ($FeSO_4.7H_2O$), hydrogen peroxide (30% w/v) and sulphuric acid used for pH adjustment (around 2.7-2.8) were reagent grade.

5.B.3.2. Analytical determinations

Mineralization was followed by measuring the dissolved organic carbon (DOC) by direct injection of filtered samples into a Shimazu-5050A TOC analyser provided with an NDIR detector and calibrated with standard solutions of potassium phthalate. Pesticide concentration was analysed using reverse-phase liquid chromatography (flow 1 ml/min) with UV detector in a HPLC-UV (Varian 9012, 9100, 9065) with an ODS-2 column (Waters 4.6x250 mm, from Phenomenex) and a guard column (Waters 4.6x10 mm): Alachlor (H_2O/Acetonitrile 40/60, 224nm), Atrazine, Isoproturon, Chlorfenvinphos and Diuron (Acetic acid(1%)/Acetonitrile 80/20-40/60 (0-30 min) and 40/60 (30-35 min), 249 nm). Ultra pure distilled-deionised water obtained from a Milli-Q (Millipore Co.) system and HPLC-grade organic solvents were used to prepare all the solutions. Cation concentrations were determined with a Dionex DX-120 ion chromatograph equipped with a Dionex Ionpac CS12A 4 mm x 250 mm column. Isocratic elution was done with H_2SO_4 (10 mM) at a flow rate of 1.2 ml min^{-1}. Anion concentrations were measured with a Dionex DX-600 ion chromatograph using a Dionex Ionpac AS11-HC 4 mm x 250 mm column. The gradient programme was pre-run 5 min with 20 mM NaOH, injection, 8 min 20mM NaOH and 7 min NaOH 35mM, flow rate 1.5 ml min^{-1}. H_2O_2 concentration was determined by iodometric titration.

5.B.3.3. Toxicity measurements

Microtox™ acute toxicity testing was performed with *Vibrio fischeri* using a Model 500 Analyzer (Azur Environment, Workingham, England). Hydrogen peroxide present in the samples from photo-Fenton experiments was removed prior to toxicity analysis using catalase (2500 U/mg bovine liver; 100 mg l^{-1}) acquired from Fluka Chemie AG (Buchs, Switzerland) after adjusting the sample pH to 7. Samples from photo-Fenton treatment were filtrated (0.22 μm filter, Schleicher and Schuell, G-Dassel) before toxicity testing. Measurement of toxicity was performed within 24 h after irradiation. Stored samples

should be frozen before analysis. Toxicity is expressed as toxicity units, $TU=100/EC_{50}$, where EC_{50} is the concentration which causes 50% reduction of the bioluminescence (*Vibrio fischeri*). All chemicals for the bioassays were obtained from a commercial supplier (Tetra Technique, Veyrier, Switzerland).

5.B.3.4. Biodegradability assessment

The Zahn-Wellens test was carried out according to the EC protocol (Directive 88/302/EEC). The activated sludge obtained from a secondary effluent of the treatment plant of Morges (Switzerland) was used as inoculum. The fresh activated sludge was centrifuged at 10 000 RPM during 7 minutes at 20 °C, and washed once with mineral medium. According to the guidelines of the Zahn-Wellens test, the ratio between the carbon content of the experimental sample and the dry-weight of the inoculum was ranged between 1 and 4 (average 3.2). Aeration and homogenization were guaranteed. Preliminary experiments were performed to check that neither volatilization nor adsorption occurred during the testing period of 28 days.

5.B.3.5. Experimental set-up

5.B.3.5.1. Photo-Fenton experiments

The photocatalytic experiments were performed using 0.5 l Pyrex flask with a cut-off at λ=290 nm placed into Hanau Suntest Simulator.

The radiation source employed was a xenon lamp and the total radiant flux (80 mW cm^{-2}) was measured with a YSI Corporation powermeter. The lamp had a λ distribution with about 0.5% between 300 and 400 nm. The profile of the photons emitted between 400 and 800 nm followed the solar spectrum. Based on Eq.5.B.1, the actual time for the irradiation was calculated for the experiment.

$$t_{30W,n} = t_{30W,n-1} + t_n \frac{UV}{30}; \qquad t_n = t_n - t_{n-1} \qquad \text{Eq 5.B.1}$$

where t_n is the experimental time for each sample, UV the average solar ultraviolet radiation emitted during Δt_n, and t_{30W} the "normalized illumination time". Thus time

refers to a constant solar UV power of 30 W m^{-2} (typical solar UV power on a perfectly sunny day around noon). This calculation allows the comparison with other photocatalytic experiments.

The aqueous suspensions were magnetically stirred throughout irradiation, opened to air. Extreme care was taken to ensure uniform experimental conditions during the partial degradations, since several experiments were necessary to be run in order to continuously feed the bioreactors.

5.B.3.5.2. Biotreatment experiments

As illustrated in Figure 5.B.1, 8 aerated packed-bed bioreactors were operated in parallel. Each bioreactor consisted of a 0.24 l-glass column containing ca. 0.15 l packing expanded clay colonized by activated sludge from the wastewater treatment plant of Morges (Switzerland). Liquid in the columns was circulated with peristaltic pumps.

The effluent of the photocatalytic stage was circulated through the bottom of the column, which operated as an up-flow reactor. In order to homogenize the bacterial population throughout each column and between all of them (in fact, 8 biofilters were run simultaneously), wastewater from the primary decantor was recirculated inside the biofilter from upside down during a few days.

The pH was controlled and adjusted at 7 using 1M H_2SO_4 or 2 M NaOH solutions.

The required nutrients for bacterial activity were provided after photo-treatment with a concentrated mineral medium, so that volume remained unchanged.

The aeration was about 0.03 l h^{-1} and the O_2 concentration was checked by means of a dissolved oxygen (DO) probe (Ingold AG, Urdorf, Switzerland) on top of the column. All the glass vessels were protected from illumination with aluminum sheets. Agitation was maintained before and after biotreatment using magnetic stirring. Temperature was 25° C.

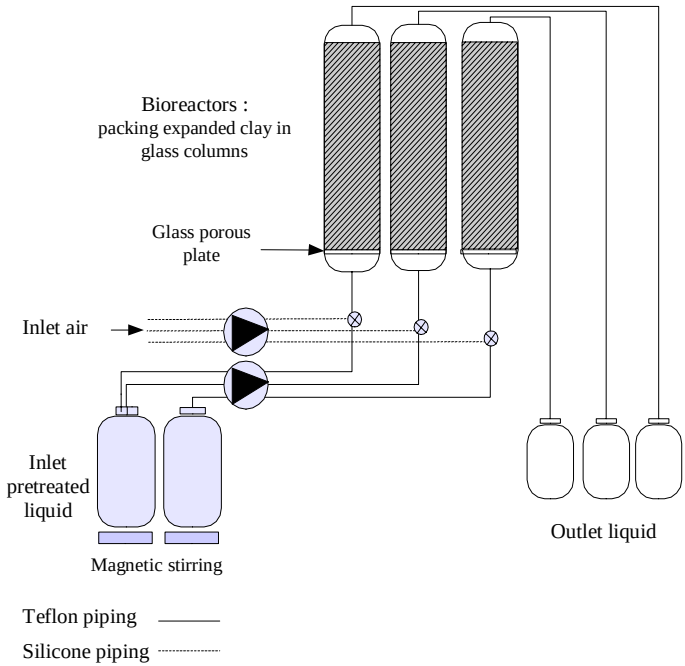

Figure 5.B.1. Experimental setup used for investigation of the photocatalytic-biological coupling treatment of the mix of pesticides.

5.B.3.6. Data analyses

Validation of toxicological bioassays as well as biodegradability assessments was ensured since all the quality control data were considered acceptable according to the official guidelines (OECD, U.S. Environmental Protection Agency (EPA) Office of Pollution Prevention and Toxics (OPPT), and the European Commission) and other established criteria (e.g., response to the negative controls, use of reference substances: phenol for Microtox^{TM}, diethylene glycol for Zahn-Wellens testing).

Photocatalytic and biodegradability experiments were at least duplicated and all samples were analyzed in triplicate. Toxicity data were computed and EC_{50} values were calculated

according to the gamma method, using linear regression analysis of transformed DOC concentrations as natural logarithm data versus percentage inhibition. All correlation coefficients were > 0.90.
Statistical analysis of all experimental results was carried out using analysis of variance (ANOVA). For all laboratory experiments, α was set at 0.05.

5.B.4. Results and discussion

*5.B.*4.1. Biocompatibility of the mixed pesticide solution

Before considering any photocatalytic treatment of the mixture of pesticides, a solution of the mixed compounds (alachlor, atrazine, chlorfenvinphos, diuron, isoproturon) was first tested for biodegradability using the Zahn-Wellens procedure (see Chapter 2). This test was carried out under similar conditions that of a wastewater treatment plant using activated sludge. A parallel control experiment using diethylene glycol (0.5 g/l) was carried out to test if the sludge was active. The diethylene glycol was in fact degraded as much as 96% in 6 days.
Under the tested conditions, the mixture of pesticides was proved biorecalcitrant. The concentrations of DOC and of each pesticide (HPLC) in the mix remained unchanged, even after 28 days. This observation also indicates that bacteria cannot adapt to degrade these chemicals. This result is concomitant with previous experiments performed with the single compounds and using the same biodegradability testing procedure (Chapter 2). In that study, no biodegradation has been observed for each compound tested alone, except for atrazine (see Table 5.B.1).

A supplementary test to measure the biodegradation of the mix of pesticides was carried out in batch mode in the packed-bed bioreactors shown in Figure 5.B.1.
Even under theoretically favorable conditions, such as the presence of co-substrates and adapted bacteria, as well as a strict control of pH, temperature and aeration, the test confirmed that the solution of mixed pesticides is non-biodegradable in the tested conditions.

Three techniques were used to follow this test:

(a) respirometric measurements with an O_2 probe in both the inlet and outlet of the biofilters,

(b) determination of single pesticide concentrations by HPLC and

(c) measurements of DOC as a function of time.

Table 5.B.1. Physico-chemical and biological properties of the pesticides.
(NB: Non-Biodegradable, B: Biodegradable)

Compound	Molecular formula	Solubility in water at 25°C (mg/l)	EC_{50} ± 95% Confidence Interval (mg/l)	Biodegradability (Zahn-Wellens test)	Initial concentration (mg/l)
Alachlor	$C_{14}H_{20}ClNO_2$	239	105 ± 8	NB	30
Atrazine	$C_8H_{14}ClN_5$	34	89 ± 6	B	30
Chlorfenvinphos	$C_{12}H_{14}Cl_3O_4P$	125	18 ± 1	NB	30
Diuron	$C_9H_{10}Cl_2N_2O$	41	86 ± 5	NB	30
Isoproturon	$C_{12}H_{18}N_2O$	65	29 ± 2	NB	30

*5.B.*4.2. Chemical and biological characteristics of the photo-treated pesticide solution

Photo-Fenton degradation is an important sub-process in the integrated photocatalytic-biological coupled process for removal of biorecalcitrant chemicals.

Shortening the photo-Fenton treatment time has a critical impact on the economical feasibility of the integrated process.

For this purpose, it is very important to gather information concerning both chemical and biological characteristics of the solution during the photocatalytic pre-treatment.

DOC analyses, concentrations of the initial compounds and of other inorganic species produced during the photo-Fenton process were monitored in the course of the photocatalytic experiments.

Acute toxicity was also measured using the Microtox^{TM} system and biodegradability was assessed according to the Zahn-Wellens method.

Decreasing DOC concentrations are representative of the oxidative reactions that occur in the experimental solution during the photocatalytic treatment.

As shown in Figure 5.A.2, evolution of global mineralization can be well illustrated by both H_2O_2 consumption and the illumination time. Therefore the progress of the photo-Fenton process could be equally defined by one of the two parameters. In fact, the concentration of H_2O_2 was precisely followed in order to avoid any residual amount of H_2O_2 in the photo-treated solution, because H_2O_2 may damage the bacterial cells and thus limit the consecutive biotreatment. No remaining H_2O_2 was also required for toxicity analyses.

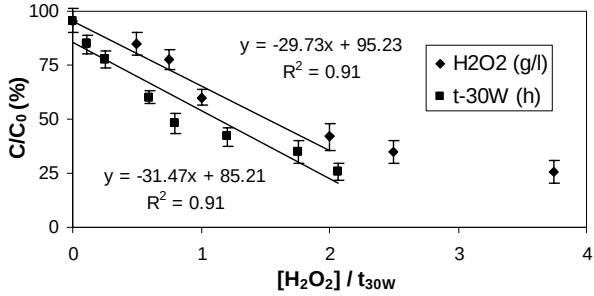

Figure 5.B.2. DOC evolution of the solution of mixed pesticides as a function of the irradiation time (t_{30W}) and the consumed amounts of H_2O_2 during photo-Fenton. Error bars represent 95% confidence intervals.

Figure 5.B.2 shows that the degradation ratio tends to stabilize at H_2O_2 concentrations above 2 g l^{-1}. Beyond this threshold, higher amounts of H_2O_2 as well as a higher irradiation time are necessary to extend degradation.

In fact, the mineralization of the mixed pesticide solution was difficult to achieve.

Long irradiation period and its consequent high time and energy consumption, as well as the high H_2O_2 amounts result in a rather expensive treatment. Electricity represents about

60% of the total operational cost when photoprocesses are driven by electrical generation of photons instead of direct solar irradiation.
This underlines the aim of the study to shorten the photocatalytic treatment time in order to promote the biodegradation.

In this study, the illumination time (t_{30W}, described in section 5.B.3.5.2.) was chosen to characterize the advancement of the photo-Fenton process, because it is a useful parameter in practical applications.

Figure 5.B.3 represents the chemical degradation of the mix of pesticides as a function of the illumination time course (t_{30W}). Evolution of the overall Microtox™ acute toxicity is also pictured, toxicity being expressed in TU (=100/EC_{50}, Lankford and Eckenfelder, 1990).

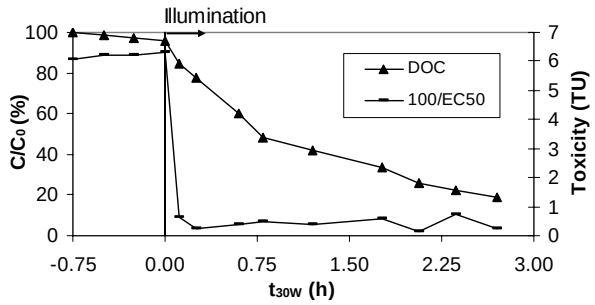

Figure 5.B.3. Evolution of toxicity (expressed as 100/EC_{50}) and DOC of the solution of the mixed pesticides as a function of the irradiation time (t_{30W}) during photo-Fenton. Data correspond to the average value of at least six measurements.

A sharp decrease in toxicity was observed at the beginning of the photo-Fenton process, followed by a stable toxic level during all the rest of the photo-treatment.
This indicates that the intermediates formed during the photo-assisted pre-treatment are less toxic than the initial compounds.
This is different from the behavior observed with the photo-Fenton degradation of the single pesticides (Chapter 5, Part A). Indeed, even if many fluctuations have been

mentioned, mainly depending on the intermediate by-products generated by each tested compound, it has been shown that toxicity of single pesticide solutions tends to increase during the first stages of the photo-treatment and then decreases.

So it seems that whenever the substances are mixed, toxicity presents a global behavior which tends to decrease as soon as the photo-Fenton begins. It has already been stated that toxicity is not an additive data (Kahru, et al., 1996).

Moreover, as illustrated in Table 5.B.2, comparison between the irradiation times necessary to degrade the single and the mixed compounds reveals that the photo-Fenton process is more efficient for treating the mixing solution, even if incomplete mineralization was achieved.

Table 5.B.2. Comparison of the illumination time (t_{30W}) necessary to remove 100% of the parent molecule of pesticide (HPLC data) and 80% of DOC during the photo-Fenton treatment of both single and mixed pesticides.

Compound	Illumination time,t_{30W} (in h)			
	100 % removal of initial compound		80 % DOC removal	
	Single	In mixture	Single	In mixture
Alachlor	0.28 ± 0.01	0.05 ± 0.04	1.20 ± 0.02	
Atrazine	0.94 ± 0.05	0.85 ± 0.03	>3.50	
Chlorfenvinphos	0.25 ± 0.01	0.05 ± 0.01	1.13 ± 0.03	2.50 ± 0.02
Diuron	0.37 ± 0.02	0.25 ± 0.01	0.68 ± 0.02	
Isoproturon	0.66 ± 0.03	0.50 ± 0.02	1.28 ± 0.30	

The evolution of the 5 initial compounds was followed during the pre-treatment process by HPLC measurements.

At 0.15 h of photo-treatment, when about 20% of DOC removal was observed, mainly every parent pesticide was eliminated (only 3% atrazine and 4% chlorfenvinphos were

left). Thus complete disappearance and total dechlorination (23 mg l^{-1} chloride was expected for the tested concentrations) of all pesticides were attained very easily.
The elimination of the initial bio-recalcitrant compound was required in order to test the biocompatibility of the photo-treated solution (Parra et al., 2002).

The slow decrease of DOC compared with the pesticide evolution indicated an accumulation of intermediate products.
The nitrogen balance was also checked during the photo-Fenton process, because the release of ammonia and nitrate was indicator of the by-product degradation.
The slow mineralization rate observed at the end of the photo-treatment was concomitant with the incomplete release of nitrogen as NH_4^+ or NO_3^-.
From previous experiments (Chapter 5, Part A), it has been concluded that the most resistant intermediates were due to the phenylurea pesticides (diuron and isoproturon) and atrazine. In particular, the stable triazine ring and the formation of urea could justify the remaining 10% DOC measured at the end of the photo-Fenton process.

In the case of the mixed pesticide solution, toxicity and biodegradability were found to be related.
Indeed, as illustrated in Figure 5.B.3, before irradiation (i.e. during the Fenton process), the solution of the mixed pesticides was toxic up to 6 TU and was also biorecalcitrant according to the test of Zahn-Wellens.
On the contrary, after illumination (i.e. during the photo-Fenton process) the partially treated solutions exhibited a lower toxicity, approximately 0.5 TU (see Figure 5.B.3), and were biodegradable according to the Zahn-Wellens test.
These results are in accordance with previous experiments which demonstrated that biocompatibility was achieved after complete disappearance of the initial biorecalcitrant substance (Lapertot et al., 2006).

Therefore it is interesting to test the biotreatability of the solutions partially degraded by the photo-Fenton process.

*5.B.*4.3. Photochemical-biological coupled flow treatment

The above-mentioned results presenting the concomitant decrease of toxicity and increase of biodegradability of the treated solution of mixed pesticides suggest the photo-Fenton as a promising pre-treatment process.
Therefore, a photochemical-biological coupled flow reactor can be considered for the complete mineralization of the solution of the mixed pesticides.

The aim of the study was also to determine the best moment for coupling, in order to limit the cost of the photocatalytic treatment.
For that purpose, successive steps of pre-treatment were selected during the photo-Fenton process and tested for biotreatment in several biological fixed bed reactors.

The bioreactors (see Figure 5.B.1) were operated in continuous mode, whereas the photo-chemical treatment was operated in batch mode.

Table 5.B.3 gives the characteristics of different pre-treated solutions which were tested for biocoupling.

Table 5.B.3. Photocatalytic characteristics and biodegradability assessment of the pre-treated solutions tested with the photo-Fenton / Bioreactor coupled system.
(NB: Non-Biodegradable, PB: Partially-Biodegradable, ND: Not-Determined)

Sample	t_{30W} (h)	H_2O_2 (g l^{-1})	C/C_0 (%)	Biodegradability (Zahn-Wellens)
A	0	0	96	NB
B	0.11	0.50	85	PB
C	0.25	0.75	76	PB
D	0.60	1.00	60	PB
E	0.80	1.23	48	ND
F	1.20	2.00	42	ND
G	1.76	2.50	35	ND
H	2.06	3.75	26	PB

Each partially-treated solution was tested in triplicate: 3 columns were operated in parallel and were compared to the control bioreactors.
Control bioreactors consisted of a bioreactor fed with only the mineral medium and a bioreactor operated with the initial solution of no photo-treated mixed pesticides.
A preliminary test using diethylene glycol was performed in each bioreactor in order to check both the activity of the bacteria and the correct operating mode of each column.

The pre-treatment of the solution was made in photocatalytic reactors using the photo-Fenton process.
After photo-treatment, the solutions were neutralized (pH around 6.5-7), which also provoked the precipitation of the iron. Solutions were centrifuged and filtrated before biotreatement.
Sequential batches of the photo-treatment process were carried out so that the input flow rate into the biological reactors was 0.12 l d^{-1}. This flow rate was maintained during at least 28 days. A relatively steady-state condition for DOC removal was observed in the columns after 48 hours.

Various experiments were performed in order to compare the efficiency of the coupled photo-catalytic and biological treatments of the mixture of pesticides based on the advancement of photodegradation.

Figure 5.B.4 illustrates the experimental results obtained with the coupled system. Data correspond to the average values obtained after 28 days of biotreatment in the columns.

Figure 5.B.4 shows the DOC removal efficiency which was attributed to both photo-Fenton and biotreatment, as a function of the photoreaction time presented in Table 5.B.3.

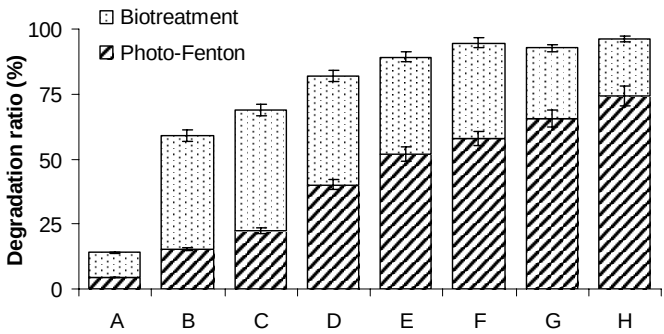

Figure 5.B.4. Relative contribution of photocatalysis and biotreatment for the degradation of the solution of the mixed pesticides. Characteristics of the samples A-H are listed in Table 5.B.2. Error bars represent 95% confidence intervals.

A maximum degradation efficiency of 90% was obtained with the coupling system. It was achieved with pre-treated solutions E, F, G and H.

Thus beyond an irradiation time of 0.80 h (1.25 g l^{-1} H_2O_2, sample E), intensifying the photo-Fenton reaction is useless to enhance the global degradation ratio. So it means that beyond this threshold, the more intensive the photo-treatment, the less important the biological contribution to achieve the global degradation.

In fact, more than 80% of DOC were degraded in the coupled system with pre-treated solutions using only 1 g l^{-1} H_2O_2 (t_{30W}=0.6 h, sample D). This global degradation degree is already an interesting result for an industrial application of the integrated process. In this case, the biological treatment contributes to more than 50% of the total carbon conversion of the pesticide solution.

It should be noticed that using a recirculation loop in biological system can have important effect on the total removal capacity and intensification of the integrated process. Since the toxicity of the pesticide solution becomes low after partial photo-Fenton process, the adaptation of bacteria is expected to enhance the biotreatment.

5.B.5. Conclusion

The photocatalytic treatment of the solution of the mixed pesticides was successfully achieved with the photo-Fenton process. In this study, photo-Fenton degradation was considered as a sub-process in the integrated photocatalytic-biological coupled system for removal of the biorecalcitrant pesticides. In particular, it was aimed to shorten the photo-treatment time in order to enhance the economical feasibility of the integrated process.

The Microtox^{TM} acute toxicity evaluation and the Zahn-Wellens tests for biodegradability assessment were shown to be two relevant indicators to determine the best moment for coupling. Biotreatability of the partially photo-degraded solutions was investigated more accurately using several biological fixed bed reactors which were continuously operated during 28 days.

The obtained concomitant decrease of toxicity and increase of biodegradability of the partially photo-treated solution of mixed pesticides confirmed that photo-Fenton is a promising pre-treatment process capable to enhance the biotreatability of waters contaminated with biorecalcitrant chemicals (pesticides).

Finally more than 80% of DOC was degraded in the coupled system for the pesticide solutions which were photo-treated during only 0.6 h (t_{30W}) and needed 1 g l^{-1} H_2O_2. This step of photo-Fenton treatment proved to be the most relevant moment for coupling because in this case, the biological treatment contributed to more than 50% of the total carbon conversion of the pesticide solution. Recirculation of the bioreactor effluents is now prospected in order to improve the global degradation degree of the contaminated waters.

5.B.6. References

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CHAPTER 6.

Conclusion and perspectives of the thesis

During the XXth Century, legislation has been developed to protect the quality of the limited environmental resources. However, obvious and repetitive ecological catastrophes reveal the urgency to react efficiently.
Overall more stringent pollution controls have resulted in promotion of research topics related with pollution removal. For this, up-to-date approaches for industrial processes as well as for pollution treatments now need to be ecologically sustainable.

In that sense, this thesis was aimed to propose a strategy for treating problematic pollutants with the most adequate process.

In Chapter 1, an overview of the different treatment processes has been given. Among the existing processes, biological treatment of contaminated environments and industrial effluents has been presented as the most attractive method on a cost-benefit extent. The Advanced Oxidation Processes (AOP), and particularly TiO_2 and photo-Fenton photocatalytic treatments, have also been described as they are really efficient for removal of recalcitrant substances. But the economical drawback of such processes has contributed to develop new hybrid technologies combining both AOP and biological processes for the removal of recalcitrant and/or toxic substances. Therefore in this thesis, the three above-mentioned processes - biological, photocatalytic and integrated biological-photocatalytic processes - have been tested for degradation of target xenobiotics. These chemical compounds were pesticides, phenolic substances and volatile organic compounds (VOCs). The schematic of the general strategy proposed in this thesis has been illustrated in this Chapter 1.

The first step of the proposed strategy has intended to evaluate biotraitability for 19 chemicals, which were pesticides, pharmaceuticals and volatile organic compounds (VOCs).

Therefore Chapter 2 was aimed to enhance the procedure of biodegradability assessment, so that both volatile and hydrophobic substances could be tested. It has been shown that the most suitable test of biodegradability could be selected and adapted in accordance with the physicochemical properties of each compound. In particular, three official protocols - Zahn-Wellens, Manometric Respirometry and Closed-Bottle tests - have been adapted and performed. They have permitted to evaluate the biotreatability of xenobiotics in industrial wastewater treatment plant conditions. Acute toxicity (Microtox^{TM}), bacterial viability (Bac-light^{TM}) and Structure Activity Relationships (SARs) models have also been used to investigate the influence of each substance on the microbial population (sludge).

The main contribution of this study has been to adapt biodegradability assessment methods for the testing of chemical compounds which were considered unsuitable regarding the existing protocols. The comparison between the screening level experiments and the several SARs predictive models has also been performed, so that experimental results have agreed with estimates for 16 xenobiotics. Finally, this study has permitted to demonstrate that comparison between several estimation models was beneficial in order to limit overall environmental risks.

The next step of the proposed strategy has depended on the results of the biodegradability test procedure presented in Chapter 2.

A positive result means that the target compound can be degraded by microorganisms.

Among the 19 xenobiotics which have been tested in Chapter 2, the VOCs have revealed biodegradable. Therefore their biodegradation has been studied in order to improve the treatment process.

In Chapter 3, the Substrate Pulse Batch (SPB) process has been presented as a suitable technique for cultivation of mixed bacteria capable to degrade chlorinated aromatics and TEX (toluene, ethylbenzene, xylenes) compounds. It has been shown that batch cultivation feeded by pulses of substrate (SPB technique) permitted to overwhelm both toxicity and inhibition of VOCs, so that biomass production could be enhanced. Indeed, in chapter 3 part A, 240 mg l^{-1} h^{-1} of CB/DCB mixture has been eliminated and 50 mg l^{-1} h^{-1} of dry weight have been produced using SPB process. In particular, studying substrate removal dynamics, concentration of metabolic intermediates, chlorides and cellular integrity has allowed the correct proceeding of bacterial cultivation. The time interval between each pulse of substrate has been decreased gradually during the CB/DCB biodegradation process, so that a continuous substrate feeding has finally been attained. In the continuous feeding technique, both cell productivity and CB/DCB elimination capacity have been twice the values obtained with the SPB. In chapter 3 part B, automation of the process has been successfully achieved for removal treatment of TEX compounds. A monitoring program based on LabVIEW software has been developed in order to automate the process and to limit the operator contribution. In Part B, the dynamic behavior of the mixed bacterial population has also been observed and discussed under different operational conditions. Carbon and nitrogen limitations have been shown to affect the integrity of the bacterial cells as well as their production of exopolymeric substances (EPS). Average productivity and yield values have reached 0.45 kg_{DW} m^{-3} d^{-1} and 0.59 g_{DW} g_C^{-1}, respectively. Finally, obtained data have come up to the industrial specifications and have indicated the feasibility of controlled SPB technique for TEX removal.

As for CB/DCB removal, it would be interesting to perform a continuous process for TEX biodegradation with the automated technology. More specifically, it could be useful to clarify the dynamics of the bacterial population, in order to avoid the observed decrease in degradation rates which have systematically occurred during the biological removal of VOCs. For instance, it is assumable that the composition of the bacterial consortium changes along the process. Indeed specific strains are certainly more capable to tolerate either the initial substrates or the intermediate metabolites. Genetic adaptations

might certainly be associated with the evolution of the substrate degradation kinetics. Tools of molecular biology could provide the expected information.

In case of negative results to biodegradability assessment, the biorecalcitrant compound needs to be treated by other treatment technologies.

TiO_2 and photo-Fenton photocatalytic treatments are two efficient and ecologically friendly advanced oxidation processes (AOPs). They have been considered in Chapters 4 and 5.

Chapter 4 has focused the degradation of halogenated phenolic substances by means of TiO_2 photocatalysis. The influence of the halogen upon the degradation of *p*-halophenols in water has been investigated. Phenol has been used as the reference compound. Compared with its value for phenol, the apparent first-order rate constant of removal, k, has been shown slightly but significantly higher for *p*-fluorophenol and *p*-chlorophenol, and slightly but significantly lower for *p*-bromophenol. For *p*-iodophenol, k has been about half that of phenol. The relative values have thus confirmed that k was roughly correlated to the Hammett constant. Conversely, since all compounds have been poorly adsorbed on TiO_2, their various degradation rates have not been related to the difference of halogen. The organic intermediate products have been detected; they included hydroquinone (HQ), benzoquinone (BQ) and various halodihydroxybenzenes. Mechanisms have been tentatively suggested to interpret the differences in the degradation pathways of the *p*-halophenols. The Microtox^{TM} acute toxicity has also been investigated during the TiO_2 photocatalytic removal of *p*-halophenols. Interpretations have been tentatively suggested to link the observed variations in toxicity and the intermediate metabolites produced according to the halogen. Finally, even though the total quantity of these aromatic intermediate products has been found to be similar regardless of the halogen, at equal removal percentage of *p*-halophenol the variations in toxicity has markedly differed for solutions that initially contained *p*-iodophenol.

This study has illustrated how the molecular structure of a chemical could influence its reactivity. However, since the molecular composition becomes more and more complex along the degradation process, further investigations are required in order to provide more relevant assessments about the overall variations in toxicity and the influence of the halogen upon this parameter.

In Chapter 5, the combination of photocatalytic and biological systems has been explored for treatment of water contaminated with bioreacalcitrant pesticides. For the development of this coupled process, several points have been considered:

- the biotraitability of the initial pollutant,
- the chemical and biological characteristics of the photo-treated solutions,
- the definition of the optimal pretreatment time,
- the efficiency of the coupled process.

Shortening phototreatment time has been of major concern for the cost and energy benefits of the xenobiotics degradation performed by photocatalytic processes. Using photo-Fenton and TiO_2 phototreatments, partially photodegraded solutions of 6 separate pesticides (alachlor, atrazine, chlorfenvinphos, diuron, isoproturon and pentachlorophenol) have been tested for biocompatibility, which have been evaluated according to the Zahn-Wellens procedure. This study has been the topic of Chapter 5 part A. In particular, it has been investigated if Microtox© could be considered as a suitable global indicator of the evolution of biocompatibility of the contaminated water during the phototreatment. The obtained results have demonstrated that biodegradability was significantly increased after short photo-Fenton treatment times and that Microtox© acute toxicity testing was useful to correctly represent overall dynamics and efficiency of photo-treatment.

Therefore, it has been aimed to find out the best moment for coupling the photocatalytic and biological processes. Thus, in Chapter 5 part B, the biotraitability of partially photodegraded solutions of mixed pesticides has been more precisely investigated using several packed-bed bioreactors, which have been successfully operated during 28 days.

The obtained concomitant decrease in toxicity and increase in biodegradability for the partially phototreated solutions have confirmed that photo-Fenton was a promising pre-treatment process, capable to enhance the biotreatability of waters contaminated with biorecalcitrant chemicals (pesticides). Finally, more than 80% of DOC has been degraded in the coupled system for pesticide solutions which had been photo-treated during only 0.6 h (t_{30W}) with 1 g l^{-1} H_2O_2. This step of photo-Fenton treatment has been proved to be the most relevant moment for coupling, because in this case, the biological treatment had contributed to more than 50% of the total carbon conversion of the pesticide solution. Recirculation of the bioreactor effluents has been presented as an outlook to improve the global degradation degree of the contaminated waters.

In order to prospect for an industrial application of the integrated photocatalytic-biological process, the development, installation and operation of an hybrid reactor has been achieved in the framework of the CADOX project (European Commission). At present, the testing of real industrial wastewaters is scheduled to provide interesting information about the limiting factors due to realistic experimental conditions.

Finally, in the light of these studies, three main points have been clearly demonstrated:

- Biodegradability assessment is crucial to select the appropriate process for removing problematic xenobiotics,
- Biological processes can be induced and enhanced for treating biodegradable xenobiotics,
- Photocatalysis using TiO_2 and photo-Fenton methods can be used as preliminary treatment before a consecutive biological process for removing biorecalcitrant xenobiotics.

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Curriculum Vitae

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