Environ Sci Pollut Res
DOI 10.1007/s11356-016-7602-4

4TH INTERNATIONAL SYMPOSIUM ON ENVIRONMENTAL BIOTECHNOLOGY AND ENGINEERING-2014

Degradation alternatives for a commercial fungicide in water: biological, photo-Fenton, and coupled biological photo-Fenton processes

Elsa López-Loveira[1] · Federico Ariganello[1] · María Sara Medina[1] · Daniela Centrón[2] · Roberto Candal[1] · Gustavo Curutchet[1]

Received: 6 April 2016 / Accepted: 5 September 2016

Abstract Imazalil (IMZ) is a widely used fungicide for the post-harvest treatment of citrus, classified as "likely to be carcinogenic in humans" for EPA, that can be only partially removed by conventional biological treatment. Consequently, specific or combined processes should be applied to prevent its release to the environment. Biological treatment with adapted microorganism consortium, photo-Fenton, and coupled biological photo-Fenton processes were tested as alternatives for the purification of water containing high concentration of the fungicide and the coadjutants present in the commercial formulation. IMZ-resistant consortium with the capacity to degrade IMZ in the presence of a C-rich co-substrate was isolated from sludge coming from a fruit packaging company wastewater treatment plant. This consortium was adapted to resist and degrade the organics present in photo-Fenton-oxidized IMZ water solution. Bacteria colonies from the consortia were isolated and identified. The effect of H_2O_2 initial concentration and dosage on IMZ degradation rate, average oxidation state (AOS), organic acid concentration, oxidation, and mineralization percentage after photo-Fenton process was determined. The application of biological treatment to the oxidized solutions notably decreased the total organic carbon (TOC) in solution. The effect of the oxidation degree, limited by H_2O_2 concentration and dosage, on the percentage of mineralization obtained after the biological treatment was determined and explained in terms of changes in AOS. The concentration of H_2O_2 necessary to eliminate IMZ by photo-Fenton and to reduce TOC and chemical oxygen demand (COD) by biological treatment, in order to allow the release of the effluents to rivers with different flows, was estimated.

Keywords Imazalil · Bioremediation · Photo-Fenton · Coupled treatment

Responsible Editor: Vítor Pais Vilar

Electronic supplementary material The online version of this article (doi:10.1007/s11356-016-7602-4) contains supplementary material, which is available to authorized users.

✉ Roberto Candal
rjcandal@gmail.com

[1] 3iA-UNSAM, Instituto de Investigación e Ingeniería Ambiental, CONICET, Universidad Nacional de San Martín, Campus Miguelete, Av. 25 de mayo y Francia, B1650HMP General San Martín, Provincia de Buenos Aires, Argentina

[2] IMPAM-UBA, Laboratorio de Investigaciones en Mecanismos y Resistencias a Antibióticos, CONICET, Universidad Nacional de Buenos Aires, Paraguay 2155, C1121ABG, Ciudad de Buenos Aires, Argentina

Introduction

Imazalil (IMZ) is a postharvest pesticide widely used for the control of fungi that attack citrus and other vegetables and fruits. It is usually commercialized in the form of aqueous emulsions containing the active compound and coadjutants. IMZ, $C_{14}H_{14}Cl_2N_2O$, belong to the family of the imidazole; it is moderately soluble in water and persistent in soils. The United States Environmental Protection Agency (US-EPA) had classified this fungicide as "likely to be carcinogenic in humans" (EPA 1999; Santa Cruz Biotechnology 2010). The presence of IMZ was detected in postharvest wastewater (Santiago et al. 2013) and in influent and effluent of secondary wastewater treatment plants. IMZ is only partially removed in sewage treatment plants. (Campo et al. 2013). As a consequence, it is important to use effective treatment methods for the removal of this agrochemical from wastewater before its discharge in water bodies. Our own studies with effluents

Published online: 17 September 2016

produced by citrus packaging companies in Argentina indicate that concentrations in the range 2–10 mg/L of IMZ can be found in the effluents that discharge in the receptor water course. The treatment of high volume of wastewater containing low concentration of pesticide may be impractical and expensive. An alternative that may help to facilitate water treatment, avoiding the management of high amount of water, is the treatment of the solutions containing high concentration of pesticide before it is mixed with water without pesticide (coming from other steps of the packaging process). The highest concentration of COD and IMZ was found in effluents coming from the drencher, where the concentration of COD was approximately 6000 mgO_2/L. Effluents with high concentration of pollutants are also found in the washing of pesticides containers. A strategy of treatment can be focused in the degradation of the pesticide present in the concentrate solution before it reaches the main effluent stream.

Aqueous effluents containing pesticides can hardly be decontaminated by conventional biological treatment (Santiago et al. 2013). However, due to the low cost and versatility of this process, there are important efforts dedicated to find microorganisms that independently or associated in consortium display the capacity to degrade recalcitrant and/or toxic compounds. Several bacterial strains have been proved to be useful for the removal of pesticides. *Pseudomonas* sp., *Pseudomonas putida*, *Pseudomonas aureofaciens*, *Micrococcus lylae*, *Achromobacter* sp., *Sphingomonas* sp., and *Acetobacter liquefaciens* are some of the bacterial strains which are capable of degrading several persistent pesticides like malathion, phenylurea, DDT, carbofuran, lindane, and *p*-nitrophenol (Rathore and Nollet 2012). However, none of the species mentioned present IMZ degradation capacity. In the case of fungicides, it was recently reported that very low concentration of IMZ (80 μg/L) can be partially degraded by biotreatment in immobilized biomass reactor (Jiménez-Tototzintle et al. 2015). White root fungi (as *Trametes versicolor*, *Pleurotus ostreatus*, and *Phanerochaete chrysosporium*) has the capability to degrade IMZ (Karas et al. 2011). However, the use of fungi in biological treatment systems is a challenging task owing to their limited competitiveness and low capacity to degrade other fungicides used in the fruit packaging industry, such as tiabendazol (Perruchon et al. 2016).

In this context, the search for microorganisms (or microbial consortium) that display the capacity to degrade toxic and recalcitrant compounds as IMZ is of current interest. In the case of growth-linked biodegradation, concentration of the substrate is generally too low to sustain an adequate microbial growth. In both cases, it is necessary to introduce a high concentration of a co-substrate that can be readily metabolized to produce enough biomass to catalyze the degradation of the target compound. Given this constraint of biological treatment, it is of interest to investigate sequential or coupled degradation methods where biological processes are coupled with other technologies such as advanced oxidation. This type of coupling has been successfully used in different systems (see for example: Ballesteros Martín et al. 2009; Lapertot et al. 2007; Mantzavinos and Psillakis 2004; Zapata et al. 2010a; Oller et al. 2007; Jiménez-Tototzintle et al., 2015).

Advanced oxidation processes (AOPs) are considered one of the most promising technologies for the elimination of recalcitrant pollutants present in water effluents. AOPs involve the generation of the powerful oxidants hydroxyl radicals ($HO^{\bullet}$), which act as a no specific oxidant leading to mineralization, or partial oxidation of several organic compounds dissolved in water. These radicals can be generated by different processes as TiO_2-photocatalysis, UVC-H_2O_2, Fenton, photo-Fenton, and other related processes (Malato et al. 2009; Soon and Hameed 2011; Ballesteros Martín et al. 2009; Hincapié Pérez et al. 2006).

Fenton process is based on the catalytic decomposition of H_2O_2 in the presence of Fe(II), being $HO^{\bullet}$ and Fe(III) generated as products. Fe(III) can be reduced by H_2O_2 to Fe(II), entering the catalytic cycle. Fenton process can be described by the following set of reactions:

$$Fe(II)+H_2O_2 \rightarrow \quad Fe(III)+HO^{\bullet}+HO^{-} \tag{1}$$

$$Fe(III)+H_2O_2 \rightarrow \quad Fe(III)-OOH^{2+}+H^{+} \tag{2}$$

$$Fe(III)-OOH^{2+} \rightarrow \quad Fe(II)+HO_2^{\bullet} \tag{3}$$

The rate of the process is limited by reaction (3) which lead to the regeneration of Fe(II), which is necessary for the continuity of the cycle. However, Fe(II) can be quickly produced by the photodecomposition of $Fe(HO)^{2+}$, which is generated by the hydrolysis of Fe(III) in water:

$$Fe(III)+H_2O \rightarrow Fe(OH)^{2+}+H^{+} \tag{4}$$

$$Fe(OH)^{2+}+h\nu \rightarrow Fe(II)+HO^{\bullet} \tag{5}$$

This process is called photo-Fenton and is produced under illumination with light in the range 365–420 nm (Perez et al. 2002; Pignatello et al. 1999; Babuponnusami and Muthukumar 2014). Several works showed that AOPs are effective to degrade IMZ, both in low (μg/L) and in high concentrations (mg/L) (Jiménez et al. 2015; Santiago et al. 2013). Besides, in the oxidation processes, organic acids are formed, which are more biodegradable than the original compound. (Hazime et al. 2012; Santiago et al. 2015).

Although AOPs have great potential for water decontamination if they were applied to reduce the chemical oxygen demand (COD) in effluents with high content of organic matter to acceptable levels, the consumption of the needed chemicals may be too high and the treatment results unaffordable. In these cases, the combination of AOPs with biological treatment may help to reduce costs.

The key to appropriately combine chemical with bio-oxidation is to find the adequate chemical concentration and necessary chemical treatment time to obtain an effluent containing substances bio-degradable enough to be mostly eliminated during bioremediation. The optimum time is related with the biodegradability and toxicity of the byproducts generated during chemical oxidation. Long treatment times consume high quantities of H_2O_2 producing highly oxidized byproducts; these oxidized compounds possess low yield for the formation of biomass (biomass obtained/consumed carbon), a fact that can lead to the need of introducing additional carbon source in the system to support the growth of the microorganisms. However, the lower biomass generation involves less sludge formation in the process of removing organic carbon, helping to reduce the cost of sludge disposal. By the other hand, short oxidation times produce byproducts with molecules quite similar to the pesticide, which are also toxic and recalcitrant.

Specific treatment methods or a combination of different methods can be used to eliminate recalcitrant products from water effluents. Taking into account the limited information available regarding the biodegradation of IMZ and its practical implication for the depuration of wastewaters from the fruit packaging industry, the objectives of this work were (i) to obtain a microbial consortium able to degrade aqueous solutions of the commercial pesticide containing high concentrations of IMZ and coadjutants, (ii) to partially oxidize the pollutants by photo-Fenton treatment optimizing H_2O_2 concentration and doses, and (iii) coupling photo-Fenton and biodegradation in order to develop a system for wastewater depuration with application in fruit packaging industries.

Materials and methods

Chemicals

In all the experiments, the commercial fungicide Xedrel 50® (IMZ 50 g/100 mL, Magan) was used as simulated effluent with IMZ and coadjutants. The concentration of the active principle is 50 %, in the form of emulsifiable concentrate, partially soluble in water but soluble at pH 3 or lower, with a TOC value of 50 %. This commercial product applied via waxing, dipping, and sprinkling treatments, with its maximum concentration in the water used for the treatment of fruits being 1 and 5 g/L in the wax. For analytical purposes, pure IMZ (PESTANAL® analytical grade, Sigma-Aldrich) was used. Analytical grade hydrogen peroxide 30 %, ferric chloride, sodium sulfite, sodium hydroxide, sulfuric acid, and HPLC grade acetonitrile were also used.

Analytical determinations

The IMZ concentration was determined by high-performance liquid chromatography (HPLC) with UV detector, using modular Shimadzu equipment coupled to a Shim-Pack VP-ODS, reverse phase column (250 × 4.6 mm long, 4.6 μm particle size, and 12 nm pore size). A mixture of acetonitrile and water (70:30) was used as eluent (flow: 1 mL min^{-1}) and the wavelength monitoring was 220 nm. Total organic carbon (TOC) analysis was carried out by combustion-infrared method with TOC-L Shimadzu. The H_2O_2 concentration (Pupo Nogueira et al. 2005) and chemical oxygen demand (COD) (Pivinski 1999) were colorimetrically determinated. Organic acids were quantified with kit from Nanocolor® Organic Acids 3000, Ref. 985 050; results are expressed in mg acetic acid/L (mg aa/L).

Culture media and conditions

Two culture media were used to grow the microbial consortium in the reactors. The *rich culture medium* contains 5.0 glucose, 2.0 g/L $(NH_4)_2SO_4$, 1.0 g/L K_2HPO_4, 0.1 g/L $MgSO_4$, 0.01 g/L $CaCl_2$. The *basal culture medium* was the same as the rich medium but without organic carbon. Different concentrations of glucose and IMZ from Xedrel 50® were added to the media in the different experiments. Incubation was carried out in shake flasks at constant temperature (25 °C). The pH was periodically measured during the experiment and adjusted at 7.0 by addition of small quantities of NaOH (or H_2SO_4). Biomass was quantified by turbidimetry and by dry weight. Measurements of optical density were made at 620 nm by a UV-Vis spectrophotometer PG Instruments Ltd. To obtain dry weight, biomass was separated from the medium by centrifugation and then it was washed and dried at 80 °C until constant weight. Biomass concentration (x) expressed in g/L was related to optical density (y) by the equation $y = 2.951x$ ($R^2 = 0.996$).

Experimental set-up

Resistant consortium enrichment Sludge coming from a fruit packaging company wastewater treatment plant was exposed to a selection process by incubation in a rich culture medium with increasing concentration of Xedrel 50®. In the first step, 2.5 % of sludge was incubated in rich medium with 50 mg/L of IMZ (from Xedrel 50®) at neutral pH, 25 °C for 3 days. In the second step, 1 % of the enriched consortium was subcultured in rich media with increasing IMZ concentration, up to 500 mg/L. The consortium, named *consortium 1*, was maintained by periodical subculturing in rich medium with 500 mg/L of IMZ. The same procedure was carried out with pre-oxidized effluent. IMZ-resistant consortium obtained before was exposed to pre-oxidized effluent, and a new

consortium named *consortium 2* was generated. The main cultivable microorganisms in both consortia were characterized as follows.

Isolating microorganisms from consortium 1 and 2 Serial dilutions of cultures were incubated on rich medium agar which consisted of 5.0 g/L glucose, 2.0 g/L $(NH_4)_2SO_4$, 1.0 g/L K_2HPO_4, 0.1 g/L $MgSO_4$, 0.01 g/L $CaCl_2$, 15 g agar, and 1000 mL distilled water. The final pH was 7.0. For some plates, aliquots of a filter-sterilized aqueous solution of IMZ (500 mg/L final concentration) were added after autoclaving and cooling the media. Isolation was made picking and subculturing colonies with morphologic differences.

Identification of isolated microorganisms by 16S rDNA analysis Bacterial strains were grown aerobically in rich medium from a single colony. Total genomic DNA was extracted with Highway® ADN Puriprep-S kit according to the manufacturer's instructions. 16S rDNA gene was amplified by PCR using the forward-primer FD2: 5′-AGAGTTTGATCATG GCTCAG-3′ and the reverse-primer RP2: 5′-ACGG CTACCTTGTTACGACTT-3′. The pair of primers used, amplifying nearly the full 16S rDNA gene (1300 bp), was used for phylogenetic characterization of the isolated bacteria. Amplifications were carried out in 25-μL reactions containing 1 U of GoTaq polymerase, 0.4 μM of each primer, and 200 μM of each dNTP. The thermal cycling conditions were 95 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 h 30 min, with a final extension of 72 °C for 5 min. The purified PCR product was sequenced, on both strands. The determined sequence was compared with those available in the GenBank/EMBL database using the BLAST program. The full 16S rRNA gene was used for phylogenetic characterization of the isolated. Phylogenetic tree was constructed by neighbor-joining method using the MEGA package. The bar represents distance values calculated in MEGA, and values at nod represent percentage of 500 bootstrap replicates. The reference used was *Desulfos porosinus acidophilus*.

Photo-Fenton treatment Water solutions containing 500 mg/L of IMZ, 500 mg/L of TOC, and c.a. 1350 mg/L of COD from Xedrel 50® were partially oxidized by photo-Fenton process. $FeCl_3{\cdot}6H_2O$ (0.15 mM) and H_2O_2 in different doses (3 × 0.1 g/L (that means three successive additions of H_2O_2, each one corresponding to 0.1 g/L in the bulk, added after H_2O_2 was consumed), 0.3 g/L, 3 × 0.3 g/L, 0.9 g/L, 1.2 g/L, 1.4 g/L, 3 × 0.6 g/L, 1.8 g/L, and 2.7 g/L) were incorporated to the water solutions. In all the cases, the oxidant was below the stoichiometric amount necessary to completely mineralize the organic carbon present in the solutions; the pH was adjusted at 3.0 using H_2SO_4. The reaction was carried out in a 600 mL borosilicate glass batch reactor, with top illumination (black light Interelec®—UVA—20 W, 35 W/m^2), and magnetically stirred. The temperature of the reactors was maintained at 25 °C with the help of a water bath. The volume of test solution was 250 mL. The reaction kinetics was studied by taking aliquots at several times, determining for each one the IMZ concentration, TOC, COD, organic acids, H_2O_2, and pH. Sodium sulfite for TOC (2 M Na_2SO_3, 1:2 *v*/*v*) or acetonitrile (1:2 *v*/*v*) for IMZ was used to quench the reaction in the samples. The photo-Fenton treatment was performed until the complete consumption of H_2O_2 because the presence of H_2O_2 may damage the bacterial cells, hindering the biological process. The treated solutions were supplemented with inorganic nutrients ($SO_4(NH_4)_2$, K_2HPO_4, $MgSO_4$, and $CaCl_2$) until reaching the basal medium concentrations and were used in biodegradation assays as described below.

Biodegradation assays Biological assays with planktonic cells were carried out in batch mode, in 100 mL Erlenmeyer flasks incubated at 25 °C in an orbital shaker at 100 rpm, using basal culture medium under three different pH conditions: (i) initial pH = 7.0 without pH control; (ii) initial pH = 3.0 without pH control; (iii) constant pH = 7.0, fixed at 7.0 during the experiment by periodical addition of NaOH solution. All the assays were run in duplicate. One set of experiments was run in the presence of 500 mg/L IMZ (from Xedrel 50®) supplemented with glucose (8.5, 4.5 g/L) and inoculated with consortium 1. Control experiments without IMZ or glucose were also run (using consortium 1 as inoculum). Another set of experiments was run with pre-oxidized solutions of IMZ in Xedrel 50®. The pH was adjusted at 7.0 with NaOH and controlled all throughout by addition of $NaOH/H_2SO_4$; no external carbon source was added, and the systems were inoculated with consortium 2. In all liquid culture experiments, fresh cultures growing at the logarithmic phase were used as starting inoculum.

The following parameters were measured in samples taken at different reaction times: pH, biomass, TOC, COD, and IMZ. The yield biomass to substrate $Y^x/_s$ was calculated by

$$Y_{X/S} = \frac{(X_f - X_i)}{(S_i - S_f)} \tag{6}$$

where X and S are dry biomass and the substrate concentration, respectively, expressed in g/L.

The average oxidation state (AOS) is a measure used to know the average oxidation degree of organic components in a complex mixture. AOS was calculated from the following formula:

$$\mathrm{AOS} = 4\frac{(\mathrm{TOC} - \mathrm{COD})}{\mathrm{TOC}} \tag{7}$$

where TOC and COD are expressed in moles of C/L and moles of O2/L, respectively. AOS takes values between +4

for CO2, the most oxidized state of C, and −4 for CH4, the most reduced state of C (Parra et al. 2000). In the middle, the simplest alcohol (CH_4O) has AOS = −2; the simplest aldehyde (CH_2O) has AOS = 0, and the simplest organic acid (CH_2O_2) has AOS = +2.

The AOS of the mineralized fraction in the biological treatment (AOSmfb) was calculated by

$$\mathrm{AOSmfb} = 4\frac{(\Delta(\mathrm{TOC})-\Delta(\mathrm{COD}))}{\Delta(\mathrm{TOC})} \quad (8)$$

Results and discussion

Isolation and identification of microorganisms in consortia 1 and 2

The isolated microorganisms were resistant to high concentrations of IMZ and/or oxidation intermediaries. Four different colony morphologies were clearly identified in the isolation plates: yellow, fawn, white, and pink. The fawn colony was notoriously predominant in consortium 1. DNA from each colony was extracted and subjected to PCR with 16S rDNA primers in order to identify the genus and/or the species. The results are presented in Table 1. According to different authors, these microorganisms are usually found in soil contaminated with hydrocarbons (Owsianiak et al. 2009, Zhang et al. 2007; Vandamme et al. 1994).

It is remarkable that in the absence of IMZ but in the presence of the pre-oxidized IMZ solution (consortium 2), the growth of higher number of species was promoted. This result suggests that the byproducts formed after application of photo-Fenton are less toxic than IMZ to the other microorganisms present in the consortium (different than *Burkholderia cepacia* (*fawn colonies*)).

Figure 1 shows the phylogenetic tree of bacteria typically found in soils containing pesticides. The figure shows how distant are the microorganisms isolated in this work, such as *Chryseobacterium sp.*, *Burkholderia cepacia*, *Burkholderia sp.*, and *Staphylococcus sp.*, with respect to those found in literature (Rathore and Nollet 2012). Besides, the distance between each isolated strain indicates a big genetic diversification. This diversity that is presented by the consortium would give it a high flexibility in stress situations.

IMZ biodegradation assays

The influence of IMZ on the growth kinetics and feasibility to degrade IMZ by an adapted microbial consortium (consortium 1), in a water solution prepared from a commercial formulation, was studied in the presence and absence of added carbon source (glucose) with pH control (pH = 7.0) by incorporation of NaOH or H_2SO_4. Previous experiments run without pH control showed that pH can decrease at values close to 3.0. At this pH, the biomass growth stopped before all the glucose was consumed (see Fig. S1 in supplementary material). This pH diminution is typically a consequence of the assimilation of ammonium when this is the main source of N. Figure 2a–c shows, respectively, the evolution of biomass, TOC, and IMZ in experiments run under different initial conditions. In the control system without IMZ and 4.5 g/L glucose, the maximum amount of biomass was produced after 3 days of incubation, producing 1.0 g/L of biomass. The biomass to substrate yield $Y_{x/s}$ value was $0.22_{gx/gs}$. In the case of the samples containing IMZ 500 mg/L with 4.5 and 8.5 g/L glucose, $Y_{x/s}$ were $0.24_{gx/gs}$ and $0.22_{gx/gs}$, respectively. These values of $Y_{x/s}$ were very similar to those obtained in the control without IMZ; hence, $Y_{x/s}$ is not affected by IMZ. This fact suggests that the consortium do not need to spend extra maintenance energy in cultures with high IMZ concentrations. Figure 2b shows that in samples containing Xedrel 50®, TOC decreased until approximately 500 mg/L, suggesting that TOC from IMZ was not degraded. In the case of the control experiment without IMZ, TOC decreased to values lower than 100 mgC/L. Figure 2c shows the evolution of IMZ in systems with 0.0, 4.5, and 8.5 g/l of glucose and 500 mg/L of IMZ. Only in the cases with added glucose, where higher amounts of biomass were generated, IMZ concentration decreased at values close to 50 % of the initial concentration. Adsorption experiments indicated that IMZ was only slightly adsorbed by the biomass in the range of concentrations used in this work (see Fig. S2 in supplementary material). Other authors also reported that IMZ is not adsorbed by biomass in slurries (Jiménez-Tototzintle et al. 2015). These results suggest that the reduction in IMZ concentration was due to degradation and that another substrate should be added to the culture as carbon and energy source to reach and sustain the necessary biomass to catalyze the process. In the presence of a high amount of biomass, TOC decreased only until values that correspond with the TOC

Table 1 Identification and characterization of isolated microorganisms

Colony	Highest match	Homology (%)	Coverage (%)	Accession number
Yellow	*Chryseobacterium sp.*	99	100	KC479152.1
Fawn	*Burkholderia cepacia*	99	100	KT281920.1
Pink	*Burkholderia sp.*	99	99	KP687355.1
White	*Staphylococcus sp.*	99	100	KT306679.1

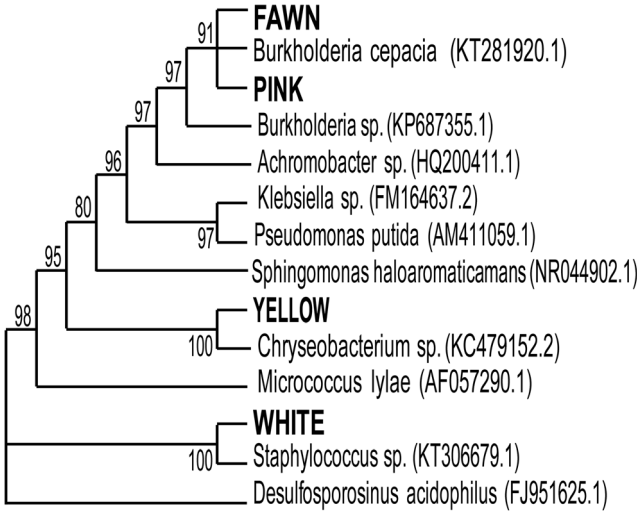

Fig. 1 Phylogenetic tree. Phylogenetic analysis of the isolated microorganisms

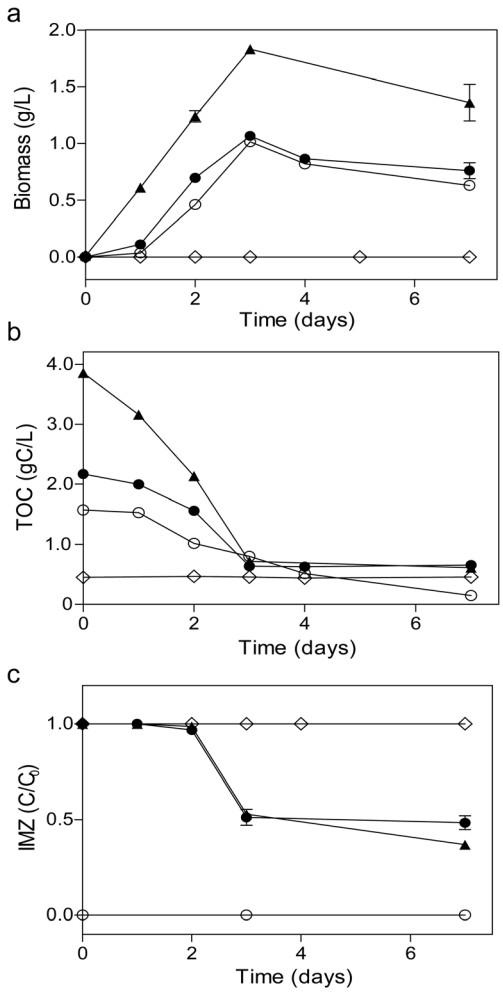

Fig. 2 **a**–**c** Evolution of biomass, TOC, and IMZ in the solution during the biological treatment at controlled pH = 7.0. Without glucose, 500 mg/L IMZ (*white diamond*); 4.5 g/L glucose, 500 mg/L IMZ (*black circle*); 8.5 g/L glucose, 500 mg/L IMZ (*black triangle*); 4.5 g/L glucose, without IMZ (*white circle*)

provided for the pesticide. This result may indicate that IMZ is not mineralized by the biomass but converted in other species. As mentioned previously, some fungi probed to be able to degrade IMZ despite its toxicity, but in this work, we showed a bacteria consortium capable to resist and partially degrade up to 500 mg/L of IMZ. This value is more than 10 times higher than the concentration previously reported (Karas et al. 2011). The amount of IMZ degraded was close to 250 mg/L with a biomass concentration of 1–2 g/L. A detailed study of the biodegradation mechanism is out of the scope of this work; however, data suggest an unspecific attack of the target compound due to enzymatic action, being necessary to reach a biomass concentration threshold for achieving a significant degradation. It is known that the oxidative enzyme laccase in the presence of a reaction mediator (a laccase/mediator system) degrades the postharvest fungicide imazalil (Maruyama et al. 2007) and that the enzymatic complex peroxidase-laccase is involved in unspecific degradation of several pesticides (Karas et al. 2011). There is bibliographic evidence that the bacterial strains found here presented laccase activity (Kellner et al. 2008; Bugg et al. 2011). Besides, bioinformatic analysis reveals high diversity of bacterial genes for laccase-like enzymes (Ausec et al. 2011). Further studies are in course in order to obtain more details on the degradation mechanism.

Photo-Fenton process

The results discussed above showed that the presence of a rich organic substrate to generate a high amount of biomass was necessary to reduce the concentration of IMZ in solutions containing 500 mg/L of this pesticide. The total elimination of IMZ was not accomplished. As an alternative, a previous oxidation treatment via photo-Fenton process was studied.

Figure 3a, b shows, respectively, the evolution of IMZ and H_2O_2 during the photo-Fenton process. Different initial H_2O_2 concentration and dosage were tested in order to determine the more efficient use of the oxidant. In all cases, IMZ was completely degraded in approximately 4 h, except in the system with 3 × 0.1 g/L of H_2O_2 because the first H_2O_2 dose was not enough to complete the process. IMZ degradation followed pseudo-first order kinetics with half times in the range 1.5–0.4 h depending on the initial concentration of hydrogen peroxide. Control experiments were performed: one without Fe(III), another without H_2O_2, a third one without light, and the last one without Fe(III), light, and H_2O_2. In such cases, IMZ degradation was not detected. The H_2O_2 consumption profile shown in Fig. 3b can be described in two steps with different slopes. The first step displayed a smooth slope and extends from the beginning of the irradiation until the elimination of IMZ. The second step displayed a steep slope and extends from the elimination of IMZ until the consumption of all the remaining H_2O_2. This phenomenon may be associated

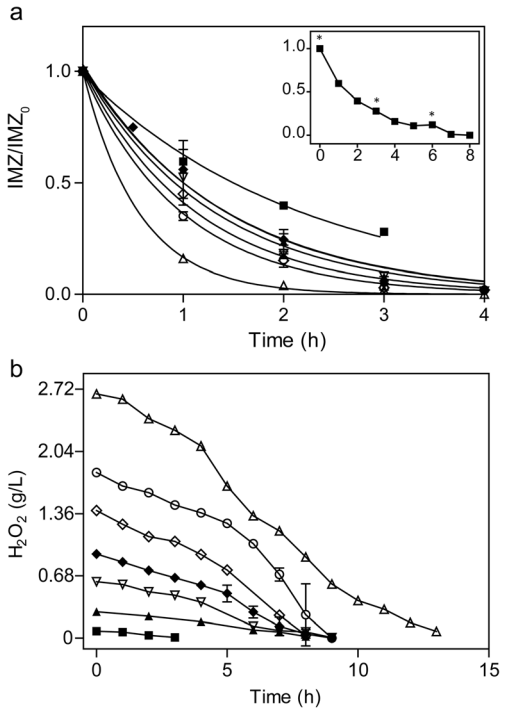

Fig. 3 **a** Evolution of IMZ concentration in the solution when exposed to the photo-Fenton treatment. **b** Evolution of H_2O_2 concentration in each system. 0.1 g/L (*black square*), 0.3 g/L (*black triangle*), 0.6 g/L (*inverted white triangle*), 0.9 g/L (*black diamond*), 1.4 g/L (*white diamond*), 1.8 g/L (*white circle*), and 2.7 g/L (*white triangle*). 3 × 0.1 g/L (*black square*) is shown in the *insert*. *Asterisks* indicate the times at which H_2O_2 doses were added

with the formation of carboxylic acids after IMZ degradation. It was reported that carboxylates can complex Fe(III) and Fe(II). Fe(III) carboxylates photolyze rapidly, reducing Fe(III) to Fe(II). Fe(II) carboxylates react faster with H_2O_2 than Fe(II) (Faust and Zepp 1993). Both effects might lead to a rapid decomposition of H_2O_2 after IMZ degradation.

TOC, COD, and organic acids were measured in all the cases immediately after H_2O_2 was completely consumed. As shown in Table 2, H_2O_2 concentration increased the percentage of mineralization and oxidation also increased in all the systems. The reduction in COD was notably higher than TOC diminution indicating that the organics present in water were oxidized with respect to the original compounds. In systems with 0.3 g/L total H_2O_2 concentration (B and C), mineralization was negligible indicating that H_2O_2 concentration was not enough to mineralize some compound from Xedrel 50®. In some experiments, H_2O_2 was incorporated in doses added after total consumption of the previous one. When H_2O_2 was incorporated as three sequential additions (C, E, and I), COD decrease was higher than for the systems with the same amount in a single dose (B, D, and H). Similar results were observed with respect to TOC. Incorporation of H_2O_2 in doses resulted more efficient towards mineralization and oxidation than the incorporation of the same final quantity of H_2O_2 in a single dose. Similar results were reported by other authors in

Table 2 Mineralization and oxidation percentages, calculated from changes in the TOC and COD values, and organic acids produced in photo-Fenton treatment

Assay	H_2O_2 (g/L)	Mineralization (%)	Oxidation (%)	Organic acids (mg aa/L)
A	0	0	0	0
B	0.3	0	22	172
C	3 × 0.1	0	28	224
D	0.9	4	48	485
E	3 × 0.3	5	49	519
F	1.2	7	52	494
G	1.4	10	55	475
H	1.8	27	67	466
I	3 × 0.6	47	74	357
J	2.7	59	81	279

different systems (Primo et al. 2008). This effect could be related with the scavenging of hydroxyl radicals by H_2O_2 when applying a large initial dose of H_2O_2. This process produces less active $HO_2^{\bullet}$ as indicated in Eq. 9 (Pouran et al. 2015):

$$H_2O_2 + HO^{\bullet} \rightarrow HO_2^{\bullet} + H_2O \quad (9)$$

In all the cases, organic acids were formed in the range 172–519 mg aa/L (Table 2). The concentration of organic acids increased with the concentration of H_2O_2, reaching a maximum value when H_2O_2 concentration was 0.9 g/L (systems D and E). Higher concentrations of H_2O_2 lead to higher mineralization, COD reduction, and lower concentration of organic acids. These results indicated that the organic acids were oxidized to CO_2 with H_2O_2 concentrations higher than 0.9 g/L with the consequent reduction of its concentration and increment in mineralization. Final pH was in the range 2.7–2.2; the diminution in pH may be associated with the production of organic acids as oxidation byproducts. The degradation pathway of pure IMZ in dilute solutions by advanced oxidation was recently studied by several authors. (Hazime et al. 2012, 2013, 2014; Santiago et al. 2013, 2014, 2015). In early stages, several oxidized byproducts, produced by hydroxylation of the aromatic ring or the carbon-carbon double bound, were detected. In the final stage, before complete mineralization, the main products were carboxylic acid. Santiago D.E. and coworkers reported the presence of formic, acetic, and oxalic acid after the oxidation of 50 mg/L of IMZ by Fenton or photo-Fenton process. IMZ degradation by advanced oxidation has a complex degradation mechanism, where the different byproducts display different degradation degree and H_2O_2 consumption rates. Complexation of Fe(III) by the oxidized byproducts cannot be disregarded, because it is known that carboxylic acids form complexes with ferric iron (Zapata

et al. 2010b). In particular, Fe(III)-oxalate complex has an active role in photo-activated oxidation processes and may help to the degradation of organic in solution (Santiago et al. 2015).

Coupled photo-Fenton—biological treatment

Based on the previous results and in order to explore the effect of photo-Fenton treatment on biodegradability of this pesticide, Xedrel 50® water solutions were photo-Fenton treated until all IMZ and H_2O_2 disappeared. The remaining solutions were submitted to biological treatment in batch reactors containing the previously isolated consortium 2. In this way, both photo-Fenton and biological treatments were coupled. The samples that were submitted to biological treatment correspond to assays A, B, C, D E, H, I, and J, indicated in Table 2. The eight solutions were adjusted for biological treatment: pH was raised to 7.0 and the solutions were supplemented with inorganic nutrients like basal culture medium. The solutions (100 mL) were inoculated with the consortium 2 and incubated at 25 °C.

Figure 4 shows the temporal evolution of TOC during biotreatment of solutions obtained after different photo-Fenton treatments. Consortium showed biodegradation capability in all the systems containing IMZ byproducts. At the end of biological treatment, the TOC considerably decreased indicating that the photo-Fenton oxidation treatment produced byproducts more easy to mineralize than the fungicide. The TOC degradation rate is different depending on the degree of photo-Fenton pretreatment. The initial degradation rate increased with the total concentration of H_2O_2 used in the photo-Fenton pretreatment (the slope increased from systems B–C with 0.3 g/L H_2O_2 to systems H–I with 1.8 g/L H_2O_2 total concentration). This fact suggests the presence of many byproducts, from the easily and faster biodegradable to the more recalcitrant. With low doses of H_2O_2, byproducts are likely similar to components of Xedrel 50® with a high percentage of recalcitrant organic compounds. With higher doses of H_2O_2, formed byproducts are likely different and more biodegradable than Xedrel 50®; however, a higher fraction of TOC was mineralized in the photo-Fenton treatment; therefore, there were less energy and carbon source available in the biological process.

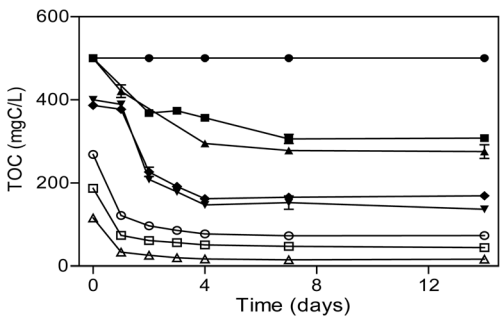

Fig. 4 Evolution of TOC during the biological treatment of the oxidized samples. Without photo-Fenton treatment (*black circle*), 0.3 g/L (*black triangle*), 3 × 0.1 g/L (*black square*), 0.9 g/L (*black diamond*), 3 × 0.3 g/L (*inverted black triangle*), 1.8 g/L (*white circle*), 3 × 0.6 g/L (*white square*), 2.7 g/L (*white triangle*)

There are three clear tendencies regarding H_2O_2 concentrations used in the photo-Fenton treatment, previous to the biotreatment. The amount of carbon mineralized in biological systems is higher in systems D, E, and H, which showed between 308 and 340 mg of mineralized carbon (representing between 60 and 80 % of the input to biological treatment). Pretreatments at lower and higher doses of H_2O_2 showed lower mineralized carbon (between 192 and 235 mg). In the first case (systems B and C), oxidation was not enough to produce biodegradable compounds, and the percentage of mineralization was low (40–46 %). In others (systems I and J), although the percent of mineralization was high (83–93 %), the total amount of mineralized carbon was low due to the low carbon input to the bioreactor. Table 3 shows AOS values of solutions obtained after photo-Fenton treatment (*final Photo*), after the biological treatment (*final Coupled*) and the AOS of the mineralized fraction in the biological treatment (*AOSmfb*). These values indicate that when the reaction ends up in the biological treatment, the AOS of the remained byproducts displayed values between 0.6 and 0.7 (under "acid" level but above "aldehyde" level). This fact indicates that, for this system, byproducts with these AOS are non-biodegradable. When the photo-Fenton pretreatment was stronger (systems I and J), the biological reaction ends up leaving more reduced compounds (−1.4 and −3.2). These results suggest that a more energetic photo-Fenton pretreatment in addition to mineralizing a larger percentage of TOC would be capable to degrade the pesticide to more oxidized byproducts. In this particular case, these byproducts turned out to be more biodegradable than the original compound. At the end of the coupled treatment, only the most reduced and recalcitrant compounds which were not oxidized at any stage of treatment remained.

The AOSmfb (calculated using TOC and COD consumption by the biomass in the biotreatment, see Eq. 8) were almost constant around 2.5–2.9 suggesting that these byproducts oxidized in the photo-Fenton process are easily mineralized in the biological treatment (except for systems with low amount of H_2O_2 in photo-Fenton treatment, assays B and C). The pH of the culture rises all along the assay for the seven conditions tested; probably due to the consumption of organic acids generated in photo-Fenton treatment. Due to the small amount of carbon source, a significant increase in biomass in these experiments is not expected; therefore, the effect of acidification by metabolism of the nitrogen source is not significant.

Table 3 shows the ratio of mol of mineralized organic carbon by mol of consumed H_2O_2 (*molTOC/mol* H_2O_2) in photo-

Table 3 AOS after each treatment, AOS mfb, and efficiency in the use of H_2O_2 in the treatments

Assay	AOS final photo	AOS final coupled	AOS mfb	Eff. photo (molTOC/molH_2O_2)	Eff. coupled (molTOC/molH_2O_2)
A	0.15	0.15	–	–	–
B	0.56	0.31	0.86	0	1.92
C	1.21	0.71	1.98	0	2.83
D	1.91	0.65	2.48	0.07	1.08
E	1.94	0.61	2.63	0.12	1.08
F	2.00	–	–	0.11	–
G	2.00	–	–	0.13	–
H	2.31	0.67	2.69	0.22	0.67
I	2.13	−1.44	2.87	0.38	0.75
J	2.23	−3.23	2.88	0.32	0.5

Fenton treatment and after the coupled treatment. Figure 5 shows the overall oxidation percentage for the combined systems. The efficiency for TOC elimination by H_2O_2 unit consumed decreased as the dose of H_2O_2 in the photo-Fenton process increased (*Eff Coupled*, Table 3). Once IMZ was degraded into byproducts, the biological treatment took care for mineralization. However, even if minimization of photo-Fenton treatment time helped to save in expensive reagents, decreasing H_2O_2 dose leads to a lesser degree of refining of wastewater, since in the biological treatment byproducts with AOS lower than 0.4–0.6 were not easily degraded. The incorporation of H_2O_2 in doses notably increased the percentage of mineralization after both photo-Fenton and biological treatment at low total H_2O_2 concentration (for example, 44 % for assay B vs. 59 % for assay C). The effect was less notable at higher total H_2O_2 concentration (for example, 86 % for assay H vs. 91 % for assay I). These results can be consequence of the important increment in AOS after photo-Fenton treatment observed at low total H_2O_2 concentration (compare in Table 3, AOS values for assays B and C, with respect to assays H and I).

A compromise between the efficiency of H_2O_2 utilization, and the overall efficiency of the process should be taken. There is not a universal guideline to effluents discharge due that each place has characteristics that make it unique. In the view of this fact, the obtained results are compared with the Argentine legislation, particularly the legislation from Entre Rios Province where this commercial product is frequently used (Ley Provincial N°6260). Limits for COD are <700 mgO_2/L for discharge into the sewage, <400 mgO_2/L for discharge into the Paraná River, <250 mgO_2/L for discharge into smaller rivers of the Province, and <50 mgO_2/L for discharge into the streams. Consequently, all the effluents with the coupled treatments cover in B to I could be discharged into the sewage. If the effluent is discharged to Paraná River, the treatments D and E reach the limit. Treatments H, I, and J could be used to discharge in smaller rivers.

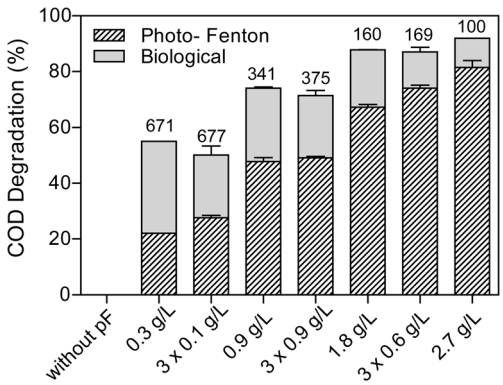

Fig. 5 Contribution of photo-Fenton and biological treatment to oxidation of the different systems (%COD diminution). Final COD (mg O_2/L) values are indicated *above the bars*

Conclusions

Consortia with capability to resist and degrade high concentrations of IMZ or its oxidation byproducts in water were isolated from sludge coming from a fruit packaging wastewater treatment plant. These consortia can resist and degrade high concentration of IMZ. However, the complete elimination of IMZ could not be accomplished. Photo-Fenton treatment was an alternative for the total elimination of IMZ in a shorter time, but mineralization percentage was low. However, if the solution was oxidized by photo-Fenton until AOSmfb values were in the range 2.5–2.9, the application of a biological treatment by inoculation with consortium 2 (adapted to the presence of oxidized IMZ byproducts) was effective for the mineralization of the organics dissolved in water. H_2O_2 concentration and dosage have an important role in determining the AOS of the oxidized solutions. Dosage of H_2O_2 resulted more efficient for IMZ oxidation than the incorporation of all the oxidant in only one dose. Oxidized IMZ solutions were less toxic than pure IMZ solutions; the number of different colonies isolated from consortium 2 was higher than in consortium 1. The concentration of H_2O_2 could be

adjusted to values that allow reducing COD until values appropriate for discharge in rivers of the Entre Rios Province (Argentina). Higher concentrations lead to a useless consumption of the oxidant. Lower concentrations were not enough to achieve the allowed levels of COD. The combination of photo-Fenton with biological treatment (using specialized consortium) was a successful procedure for the elimination of IMZ and COD reduction, in concentrated solutions of commercial fungicides.

Acknowledgments This work was supported by project FSNano-08 (ANPCyT, Argentina) and PICT 2014-2386. GC, DC, and RC are members of CONICET. ELL, FA, and MSM are Doctoral fellows of CONICET.

References

Ausec L, Zakrzewski M, Goesmann A, Schlüter A, Mandic-Mulec I (2011) Bioinformatic analysis reveals high diversity of bacterial genes for laccase-like enzymes. PLoS One 6(10):e25724

Babuponnusami A, Muthukumar K (2014) A review on Fenton and improvements to the Fenton process for wastewater treatment. Journal of Environmental Chemical Engineering 2(1):557–572

Ballesteros Martín M, Pérez JS, López JC, Oller I, Rodríguez SM (2009) Degradation of a four-pesticide mixture by combined photo-Fenton and biological oxidation. Water Res 43(3):653–660

Bugg TD, Ahmad M, Hardiman EM, Singh R (2011) The emerging role for bacteria in lignin degradation and bio-product formation. Curr Opin Biotechnol 22(3):394–400

Campo J, Masiá A, Blasco C, Picó Y (2013) Occurrence and removal efficiency of pesticides in sewage treatment plants of four Mediterranean river basins. J Hazard Mater 263:146–157

EPA (1999). Draft guidelines for carcinogenic assessment. Environmental Protection Agency, United States.

Faust BC, Zepp RG (1993) Photochemistry of aqueous iron (III)-polycarboxylate complexes: roles in the chemistry of atmospheric and surface waters. Environmental Science & Technology 27(12): 2517–2522

Hazime R, Ferronato C, Fine L, Salvador A, Jaber F, Chovelon JM (2012) Photocatalytic degradation of imazalil in an aqueous suspension of TiO_2 and influence of alcohols on the degradation. Appl Catal B Environ 126:90–99

Hazime R, Nguyen QH, Ferronato C, Huynh TKX, Jaber F, Chovelon JM (2013) Optimization of imazalil removal in the system UV/TiO_2/$K_2S_2O_8$ using a response surface methodology (RSM). Appl Catal B Environ 132:519–526

Hazime R, Nguyen QH, Ferronato C, Salvador A, Jaber F, Chovelon JM (2014) Comparative study of imazalil degradation in three systems: UV/TiO_2, UV/$K_2S_2O_8$ and UV/TiO_2/$K_2S_2O_8$. Appl Catal B Environ 144:286–291

Hincapié Pérez M, Peñuela G, Maldonado MI, Malato O, Fernández-Ibáñez P, Oller I, Malato S (2006) Degradation of pesticides in water using solar advanced oxidation processes. Appl Catal B Environ 64(3):272–281

Jiménez M, Ignacio Maldonado M, Rodríguez EM, Hernández-Ramírez A, Saggioro E, Carra I, Sánchez Pérez JA (2015) Supported TiO_2 solar photocatalysis at semi-pilot scale: degradation of pesticides found in citrus processing industry wastewater, reactivity and influence of photogenerated species. J Chem Technol Biotechnol 90(1): 149–157

Jiménez-Tototzintle M, Oller I, Hernández-Ramírez A, Malato S, Maldonado MI (2015) Remediation of agro-food industry effluents by biotreatment combined with supported TiO_2/H_2O_2 solar photocatalysis. Chem Eng J 273:205–213

Karas PA, Perruchon C, Exarhou K, Ehaliotis C, Karpouzas DG (2011) Potential for bioremediation of agro-industrial effluents with high loads of pesticides by selected fungi. Biodegradation 22(1):215–228

Kellner H, Luis P, Zimdars B, Kiesel B, Buscot F (2008) Diversity of bacterial laccase-like multicopper oxidase genes in forest and grassland Cambisol soil samples. Soil Biol Biochem 40(3):638–648

Lapertot M, Ebrahimi S, Dazio S, Rubinelli A, Pulgarin C (2007) Photo-Fenton and biological integrated process for degradation of a mixture of pesticides. J Photochem Photobiol A Chem 186(1):34–40

Ley Provincial 6260 Anexo I "De prevención y control de la contaminación por parte de las industrias y Decreto Reglamentario N° 5837"

Malato S, Fernández-Ibáñez P, Maldonado MI, Blanco J, Gernjak W (2009) Decontamination and disinfection of water by solar photocatalysis: recent overview and trends. Catal Today 147(1):1–59

Mantzavinos D, Psillakis E (2004) Enhancement of biodegradability of industrial wastewaters by chemical oxidation pre-treatment. J Chem Technol Biotechnol 79(5):431–454

Maruyama T, Komatsu C, Michizoe J, Sakai S, Goto M (2007) Laccase-mediated degradation and reduction of toxicity of the postharvest fungicide imazalil. Process Biochem 42(3):459–461

Oller I, Malato S, Sánchez-Pérez JA, Maldonado MI, Gassó R (2007) Detoxification of wastewater containing five common pesticides by solar AOPs–biological coupled system. Catal Today 129(1):69–78

Owsianiak M, Chrzanowski Ł, Szulc A, Staniewski J, Olszanowski A, Olejnik-Schmidt AK, Heipieper HJ (2009) Biodegradation of diesel/biodiesel blends by a consortium of hydrocarbon degraders: effect of the type of blend and the addition of biosurfactants. Bioresour Technol 100(3):1497–1500

Parra S, Sarria V, Malato S, Péringer P, Pulgarin C (2000) Photochemical versus coupled photochemical–biological flow system for the treatment of two biorecalcitrant herbicides: metobromuron and isoproturon. Appl Catal B 27(3):153–168

Perez M, Torrades F, Domenech X, Peral J (2002) Fenton and photo-Fenton oxidation of textile effluents. Water Res 36(11):2703–2710

Perruchon C, Patsioura V, Vasileiadis S, Karpouzas DG (2016) Isolation and characterisation of a Sphingomonas strain able to degrade the fungicide ortho-phenylphenol. Pest Manag Sci 72(1):113–124

Pignatello JJ, Liu D, Huston P (1999) Evidence for an additional oxidant in the photoassisted Fenton reaction. Environmental Science & Technology 33(11):1832–1839

Pivinski JT (1999) Chemical oxygen demand. In: Clesceri LS, Greenberg AE, Eatona AD (eds) Standard Methods for the Examination of Water and Wastewater, 20th edn. American Public Health Association, American Water Works Association, Water Environment Association, Washington DC, Section 5220

Pouran SR, Aziz AA, Daud WMAW (2015) Review on the main advances in photo-Fenton oxidation system for recalcitrant wastewaters. J Ind Eng Chem 21:53–69

Primo O, Rivero MJ, Ortiz I (2008) Photo-Fenton process as an efficient alternative to the treatment of landfill leachates. J Hazard Mater 153(1):834–842

Pupo Nogueira RF, Oliveira MC, Paterlini WC (2005) Simple and fast spectrophotometric determination of H_2O_2 in photo-Fenton reactions using metavanadate. Talanta 66(1):86–91

Rathore HS, Nollet LML (eds) (2012). Pesticides: evaluation of environmental pollution. CRC Press

Santa Cruz Biotechnology, Inc. (2010). MSDS Imazalil, USA.

Santiago DE, Araña J, González-Díaz O, Alemán-Dominguez ME, Acosta-Dacal AC, Fernandez-Rodríguez C, Doña-Rodríguez JM (2014) Effect of inorganic ions on the photocatalytic treatment of

agro-industrial wastewaters containing imazalil. Appl Catal B Environ 156:284–292

Santiago DE, Araña J, González-Díaz O, Henríquez-Cárdenes E, Ortega-Méndez JA, Pulido-Melián E, Pérez-Peña J (2015). Treatment of wastewater containing imazalil by means of Fenton-based processes. Desalin Water Treat: 1–13.

Santiago DE, Doña-Rodríguez JM, Araña J, Fernández-Rodríguez C, González-Díaz O, Pérez-Peña J, Silva AM (2013) Optimization of the degradation of imazalil by photocatalysis: comparison between commercial and lab-made photocatalysts. Appl Catal B Environ 138:391–400

Soon AN, Hameed BH (2011) Heterogeneous catalytic treatment of synthetic dyes in aqueous media using Fenton and photo-assisted Fenton process. Desalination 269(1):1–16

Vandamme P, Bernardet JF, Segers P, Kersters K, Holmes B (1994) NOTES: new perspectives in the classification of the Flavobacteria: description of Chryseobacterium gen. nov., Bergeyella gen. nov., and Empedobacter nom. rev. Int J Syst Evol Microbiol 44(4):827–831

Zapata A, Malato S, Sánchez-Pérez JA, Oller I, Maldonado MI (2010a) Scale-up strategy for a combined solar photo-Fenton/biological system for remediation of pesticide-contaminated water. Catal Today 151(1):100–106

Zapata A, Oller I, Rizzo L, Hilgert S, Maldonado MI, Sánchez-Pérez JA, Malato S (2010b) Evaluation of operating parameters involved in solar photo-Fenton treatment of wastewater: interdependence of initial pollutant concentration, temperature and iron concentration. Appl Catal B Environ 97(1):292–298

Zhang RL, Huang GQ, Lian JY, Li XG (2007) Degradation of MTBE and TEA by a new isolate from MTBE-contaminated soil. J Environ Sci 19(9):1120–1124

All figures were made using Prisma 5 (GraphPad Software, Inc.) and edited using Adobe Illustrator (Adobe Systems, Inc.).