Journal of Hazardous Materials 172 (2009) 1061–1068

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

Identification of intermediates and assessment of ecotoxicity in the oxidation products generated during the ozonation of clofibric acid

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ARTICLE INFO

Article history:
Received 24 April 2009
Received in revised form 5 July 2009
Accepted 24 July 2009
Available online 4 August 2009

Keywords:
Ozonation
Toxicity
Kinetics
Titanium dioxide
Clofibric acid
D. magna
V. fischeri

ABSTRACT

The degradation of an aqueous solution of clofibric acid was investigated during catalytic and non-catalytic ozonation. The catalyst, TiO_2, enhanced the production of hydroxyl radicals from ozone and raised the fraction or clofibric acid degraded by hydroxyl radicals. The rate constant for the reaction of clofibric acid and hydroxyl radicals was not affected by the presence of the catalyst. The toxicity of the oxidation products obtained during the reaction was assessed by means of *Vibrio fischeri* and *Daphnia magna* tests in order to evaluate the potential formation of toxic by-products. The results showed that the ozonation was enhanced by the presence of TiO_2, the clofibric acid being removed completely after 15 min at pH 5. The evolution of dissolved organic carbon, specific ultraviolet absorption at 254 nm and the concentration of carboxylic acids monitored the degradation process. The formation of 4-chlorophenol, hydroquinone, 4-chlorocatechol, 2-hydroxyisobutyric acid and three non-aromatic compounds identified as a product of the ring-opening reaction was assessed by exact mass measurements performed by liquid chromatography coupled to time-of-flight mass spectrometry (LC-TOF-MS). The bioassays showed a significant increase in toxicity during the initial stages of ozonation following a toxicity pattern closely related to the formation of ring-opening by-products.

1. Introduction

The presence of environmental xenobiotics such as pharmaceuticals and personal care products in surface and groundwaters has become a major cause for concern due to their effects on aquatic life and potential impact on human health. Pharmaceutical compounds such as analgesics, antibiotics, β-blockers or lipid regulators have a widespread distribution in the environment due to their continuous release. Municipal wastewaters are important sources of micropollutant discharges into the environment. In particular, hospitals may constitute a major contributor of disinfectants and biologically active pharmaceuticals to the bulk wastewater discharges [1,2]. Ellis also noted the importance of episodic events associated with stormwaters and non-point sources that tend to increase with urbanization [3]. Many drugs pose environmental risks not only because of their acute toxicity, but also the development of pathogen resistance and endocrine disruption [4]. The presence of these compounds in aqueous streams exposes aquatic organisms to multigenerational exposure with a risk of accumulative effects leading to changes that may remain undetected until irreversible damage has been caused [5]. Most drugs are persistent and many have even been designed to resist metabolic degradation. Although some compounds are not persistent, their continuous discharge to the environment ensures that they are often present at measurable levels in receiving waters. Another effect related to the environmental effects of pharmaceuticals is the large variety of metabolites and degradation products they may originate and the complexity of mixtures originated in wastewater.

Clofibric acid is the primary metabolite of clofibrate, a drug used as a lipid regulator which remains in the environment for a long time [6]. Due to its polar character, clofibric acid does not significantly adsorb in soil and can easily spread in surface and groundwater. Its biological effects are not completely understood, but it has been associated with endocrine disruption through interference with cholesterol synthesis [7]. The presence of clofibric acid in WWTP has been reported repeatedly since Ternes measured up to 1.6 μg/L of clofibric acid in the effluent of a German treatment plant [8]. Heberer and Stan [9] and Heberer [10] found clofibric acid in drinking water samples from the Berlin area at concentrations of up to 270 ng/L; this was associated with the practices of bank filtration and artificial groundwater enrichment. Zuccato et al. reported values of various nanograms per liter in drinking water in Lombardy, Italy [11] and Weigel et al. measured over 1 ng/L in different samples taken in the North Sea [12]. Boyd et al. reported over 100 ng/L of clofibric acid in samples taken at the inlet of a drinking water treatment plant [13]. Andreozzi et al. and Tauxe-Wuersch et

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doi:10.1016/j.jhazmat.2009.07.110

al. found concentrations of several hundred nanograms per liter of clofibric acid in the effluent of WWTP [14,15].

Ozonation has been studied with a view to transforming dissolved organic compounds into a more biodegradable form that can eventually be removed by conventional methods [16,17]. Recently, it has been shown that ozonation may release oxidation intermediates with enhanced toxicity for aquatic life [18,19]. Dantas et al. monitored the biodegradability and toxicity of a solution of sulfamethoxazole during an ozonation treatment in conditions of moderate Total Organic Carbon (TOC) removal [20]. The results showed a considerable increase in biodegradability accompanied by a rise in acute toxicity during the first 30 min of ozonation. This problem can be overcome by advanced oxidation processes. These processes include alkaline ozonation and the combination of ozone and hydrogen peroxide; they involve the generation of hydroxyl radicals, a highly reactive and unselective species, in sufficient amounts to oxidize dissolved organics [21]. A major drawback of AOP is the relatively high cost of reagents and energy, which forces a balance to be struck between the target degree of mineralization and the quality required for the effluent. A toxicity assessment of partially oxidized mixtures would allow optimal low severity ozonation treatments to be designed.

The aim of this study was to assess the toxicity of the oxidation products obtained during the catalytic and non-catalytic ozonation of clofibric acid. The catalytic reactions were performed using a commercial TiO_2 catalyst usually employed in photocatalysis whose ability to decompose dissolved ozone and to improve ozonation reactions has been previously studied elsewhere [22–24]. The acute toxicity of the intermediates was assessed by means of *Vibrio fischeri* (*V. fischeri*) and *Daphnia magna* (*D. magna*) bioassay tests and results were related to the evolution of TOC, specific ultraviolet absorption at 254 nm ($SUVA_{254}$) and the concentrations of carboxylic acids of low molecular weight. The presence of several reaction intermediates was assessed by LC-TOF-MS.

2. Material and methods

2.1. Materials

Clofibric acid, atrazine, and tert-butanol (t-BuOH) were high-purity analytical grade reagents supplied by Sigma–Aldrich. MiliQ ultrapure water with a resistivity of at least 18 MΩ cm at 25 °C was obtained from a Milipore system. pH adjustments were made with analytical grade sodium hydroxide or hydrochloric acid from Merck. The heterogeneous catalyst used was titanium dioxide Degussa P25, a 80/20 mixture of anatase/rutile. The catalyst is a powder whose primary particles have a size of about 20 nm; that in water forms aggregates of several hundred nanometers. The catalyst's point of zero charge (PZC) was pH_{PZC} 6.6, a result that has been reported elsewhere [23]. The specific BET surface was $52 \pm 2\ m^2/g$ as determined by nitrogen adsorption.

2.2. Ozonation procedure

The ozonation of clofibric acid was performed in a 1 L glass jacketed reactor whose temperature was controlled by a Huber Polystat cc2 thermostatic regulator and recorded by means of a Pt100 RTD sensor. pH was measured using a Crison 5052 electrode connected to a Eutech αlpha-pH100 feed-back control device that delivered a solution of sodium hydroxide by means of a LC10AS Shimadzu pump. The pH control system allowed pH to be controlled with ±0.1 units. The experiments were carried out at pH 1, 3 and 5. The mixture of ozone and oxygen was produced by a corona discharge ozonator (Ozomatic, SWO100) fed by an AirSep AS-12 PSA oxygen generation unit; the mixture was bubbled into the liquid by means of a porous glass diffuser. The concentration of ozone in the gas was essentially constant at $27 \pm 1\ g\ m^{-3}$. Details of the experimental set-up are given elsewhere [23,24]. Ozonation experiments were conducted during 1 h using a concentration of clofibric acid of 25–100 mg/L (116–466 μM), necessary to analyze reaction intermediates and to assess the evolution of toxicity during treatment. t-BuOH, 10 mM, has been added in some runs to suppress the contribution of the radical reaction due to its well-known role of radical scavenger. Atrazine has been added at a concentration of 1 mg/L as reference compound to use competition kinetics to determine rate constants [25]. Although the use of p-chlorobenzoic acid is widespread, we preferred atrazine because, being a weak base, it does not dissociate nor adsorbs significantly on positively charged surfaces (<3% in 1 h at pH 5 for the conditions used in this work). In the samples withdrawn for analysis, dissolved ozone was removed by bubbling nitrogen. In samples taken during catalytic runs, and after removing ozone, the pH was raised to >8.5 with NaOH and kept under stirring for at least 30 min prior to filtering using 0.45 μm Teflon filters. The reason was to force the desorption of ionized acidic substances by raising pH over the point of zero charge of the surface. The role of anion exchangers played by positively charged surfaces ($pH < pH_{PZC}$, pH_{PZC} 6.6) with dissociated acids has been widely recognised [26]. Comparative runs have also been carried out with the catalyst being immediately filtered from samples.

2.3. Toxicity bioassays

Toxicity tests were performed with the photo-luminescent bacteria *V. fischeri* and the planktonic crustaceans *D. magna*. During and incubation period of 15 min, the bioassay with *V. fischeri* measures the decrease in bioluminescence induced in the cell metabolism by the presence of a toxic substance and was carried out in accordance with ISO 11348 standard protocol [27]. The bacterial assay used the commercially available Biofix Lumi test (Macherey-Nagel, Germany). The bacterial reagent was supplied freeze-dried (*Vibrio fischeri* NRRL-B 11177) and was reconstituted with a growth medium (NaCl, 2%) and incubated at +3 °C for 5 min before use. Tests were performed at 15 °C and light measurements were taken by an Optocomp luminometer. The effect of toxics was measured as percentage of inhibition with respect to the light emitted under test conditions in the absence of any toxic influence. Acute immobilization tests with *D. magna* were conducted following the standard protocol described in the European Guideline [28]. The *D. magna* bioassay used a commercially available test kit (Daphtoxkit F™ magna, Creasel, Belgium). The dormant eggs were incubated in standard culture medium imitating natural freshwater at 20 ± 1 °C under continuous illumination of 6000 lx in order to induce hatching. Between hatching and test steps, the daphnids were fed with the cyanobacteria *Spirulina* to avoid mortality during tests. The pH of samples was adjusted so that if fell within the tolerance interval of the test organisms [29]. Test plates with *D. magna* neonates were incubated for 48 h in the dark at 20 °C. Acute toxicity was assessed by observing the effects of the test compounds on the mobility of *D. magna*. The neonates were considered immobilized if they lay on the bottom of the test plate and did not resume swimming within a period of 15 s. Acute toxicity is expressed in this test as the median effective concentration (EC_{50}) leading to the immobilization of 50% of the daphnids after the prescribed exposure time. All bioassays were replicated with different ozonation batches.

2.4. Analytical methods

The concentration of ozone dissolved in the liquid was continuously monitored using an amperometric Rosemount 499AOZ analyzer equipped with Pt 100 RTD temperature compensation and calibrated against the Indigo Colorimetric Method (SM 4500-O_3 B).

The signal from the electrode was transmitted to an Agilent 34970 Data Acquisition Unit by means of a Rosemount 1055 SoluComp II Dual Input Analyser. The Data Acquisition unit digitalized the signals from the concentration of dissolved ozone, pH and temperature with a sampling period of 5 s. The concentration of ozone in gas phase was determined using a non-dispersive UV Photometer Anseros Ozomat GM6000 Pro, tested against a chemical method. Total Organic Carbon (TOC) was determined by means of a Shimadzu TOC-VCSH total carbon organic analyzer equipped with an ASI-V autosampler. COD measurements were performed using the Standard Method 5220D. Carboxylic acids were determined in dissociated form using a Dionex DX120 Ion Chromatograph with a conductivity detector and an IonPac AS9-HC 4 mm × 250 mm analytical column (ASRS-Ultra suppressor). The eluent was 9.0 mM Na_2CO_3 with a flow of 1.0 mL/min and the sample loop volume was 1 μL. Ultraviolet absorbance at 254 nm was recorded by means of a Shimadzu SPD-6AV spectrophotometric detector. Specific ultraviolet absorbance ($SUVA_{254}$) was obtained by calculating the ratio of ultraviolet absorbance at 254 nm while the total organic carbon of the sample in mg/L. $SUVA_{254}$ provided an indirect measure of the aromaticity of the dissolved organic matter and was calculated in accordance with the protocol of the US Environmental Protection Agency [30]. The analyses of clofibric acid and atrazine were performed by HPLC using a Hewlett Packard 1100 apparatus (Agilent Technologies, Palo Alto, USA) equipped with a reversed-phase Zorbax C18 analytical column of 3 mm × 250 mm, 5 μm particle size. The mobile phase was a mixture of water containing 4 mL/L of phosphoric acid and 50 mL/L of methanol and acetonitrile (40:60) with an isocratic flow of 1.0 mL/min at room temperature. The UV detection was carried out at 230 nm. High accuracy mass analyses of the ozonation products were performed using an Agilent 1100 G1354A chromatograph coupled with an Agilent 6210 ESI/MS time-of-flight mass spectrometer (LC-TOF-MS) that used the same column as indicated above. The mobile phase was a mixture of 0.1% formic acid and 5% Milli-Q water in acetonitrile as mobile phase A and 0.1% formic acid in water as mobile phase B (pH 3.5) at a flow rate of 0.4 mL min^{-1}. A linear gradient progressed from 10% A (initial conditions) to 100% A in 30 min, and then remained steady at 100% A for 5 min. The injection volume was 20 μL. TOF acquisition parameters were used: capillary 4000 V, nebulizer 340 kPa, drying gas 10 L min^{-1}, gas temperature 350 °C, and skimmer voltage 60 and 4000 V.

3. Results and discussion

3.1. Ozonation of clofibric acid

In order to ensure a slow gas–liquid kinetic regime, all measurements were made with ozone in solution with at least 1/10th of its equilibrium concentration calculated from that of the ozone in the gas phase. These conditions ensure Hatta numbers lower than 0.20 even at pH 5. In contrast, for pH > 6, the kinetic regime was not slow and that the model outlined above cannot be applied. The ozonation of a given organic compound, M, is the consequence of second-order parallel reactions with dissolved ozone and hydroxyl radicals. The hydroxyl radicals originate from the decomposition of ozone in a reaction initiated by the hydroxyl anion. Following Elovitz and von Gunten [31], a constant ratio of hydroxyl radicals to ozone existed at any time, leading to the following expression:

$$-\frac{dc_M}{dt} = k_{HO^\bullet} c_{HO^\bullet} c_M + k_{O_3} c_{O_3} c_M$$
$$= (k_{HO^\bullet} R_{ct} c_{O_3} + k_{O_3} c_{O_3}) c_M = k_R c_{O_3} c_M \quad (1)$$

The presence of 0.1 mM t-BuOH, a substance acting as radical scavenger, eliminates the contribution of the radical reaction and, therefore, the direct ozonation constant k_{O_3} can be derived from the integrated form of Eq. (1):

$$-\ln\left(\frac{c_{M,o}}{c_M}\right) = k_{O_3} \int c_{O_3}\, dt \quad (2)$$

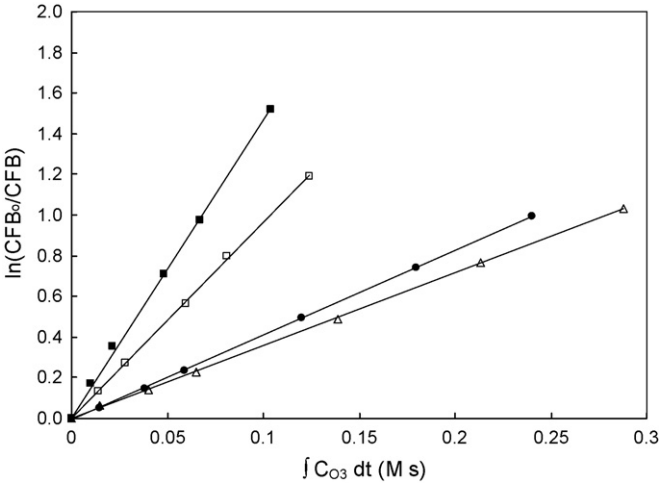

Fig. 1. Logarithmic decay of clofibric acid as a function of the integral ozone exposure during non-catalytic ozonation runs in the presence on t-BuOH 10 mM at pH 1 (△) and 5 (□). Filled symbols correspond to catalytic ozonation at pH 3 (●) and 5 (■) using 1 g/L of P25 TiO_2.

Runs performed at different pH values and, therefore, with a different degree of dissociation of clofibric acid ($pK_a = 3.2$), allowed the calculation of the direct ozonation constant for the protonated (pH 1) and dissociated form (pH 5). Fig. 1 shows the experimental values of the logarithmic decay of the concentration of clofibric acid as a function of the integral ozone exposure. The results evidenced an increase in the rate constant from $3.5 \pm 0.5\ M^{-1}\ s^{-1}$ at pH 1 to $14.3 \pm 1.6\ M^{-1}\ s^{-1}$ at pH 5, the last corresponding to the deprotonated form. Direct ozonation rate constants typically depend on speciation. This result agrees with observations reporting that deprotonated species react faster with the electrophilic ozone molecule [32].

A competition kinetics method using atrazine as reference compound, R, was used to determine the rate constant for the reaction with hydroxyl radicals. Eq. (1) can be applied for both compounds yielding the following expression:

$$\ln\left(\frac{c_{M,o}}{c_M}\right) = \frac{k_{R(M)}}{k_{R(R)}} \ln\left(\frac{c_{R,o}}{c_R}\right) = \frac{k_{R(M)}}{k_{O_3(R)} + R_{ct} k_{OH(R)}} \ln\left(\frac{c_{R,o}}{c_R}\right) \quad (3)$$

The fitting of experimental data to Eq. (3) was performed using the apparent rate constants for clofibric acid, k_R, previously evaluated at pH 3 ($8.16\ M^{-1}\ s^{-1}$) and 5 ($177\ M^{-1}\ s^{-1}$) with the same catalyst load [24] and the values reported in the literature for the rate constants of atrazine ozonation, $k_{O_3} = 4\ M^{-1}\ s^{-1}$ and $k_{OH} = 2.7 \times 10^{-9}\ M^{-1}\ s^{-1}$ [33]. R_{ct} values of 2.9×10^{-8} (pH 5) and 7.5×10^{-10} (pH 3) have been derived. The rate constant for the reaction of clofibric acid with hydroxyl radical, $k_{OH,}$ can be calculated from the integrated form of Eq. (1) yielding $5.5 \times 10^9 \pm 8 \times 10^8\ M^{-1}\ s^{-1}$. Huber et al. [34] and Packer et al. [35] published second-order rate constants for the reaction of O_3 ($<20\ M^{-1}\ s^{-1}$) and $HO^\bullet$ ($4.7 \times 10^9\ M^{-1}\ s^{-1}$) with clofibric acid at pH 7. Razawi et al. [36] recently reported a value of $6.98 \times 10^9 \pm 1.2 \times 10^8\ M^{-1}\ s^{-1}$ for the bimolecular reaction rate constants with $HO^\bullet$. All these values are in good agreement with those reported here. These figures also show that at pH 5, 90% of the oxidation of clofibric acid takes place by reaction with hydroxyl radicals, while at pH 3 only half of clofibric acid reacted through a direct ozonation route.

Catalytic ozonation has been shown to follow the same kinetic expression by making simple assumptions such as the adsorption equilibrium of organic compounds and reaction with hydroxyl radicals from the bulk [37]. In a previous work, we reported pseudo-homogeneous rate constants at pH 3 ($21.7\,M^{-1}\,s^{-1}$) and 5 ($680\,M^{-1}\,s^{-1}$) for a bulk catalyst concentration of 1 g/L [24]. Using these data and with the same procedure described before, we obtained R_{ct} values of 4.5×10^{-9} (pH 3) and 1.2×10^{-7} (pH 5). They represent about a four- to six-time increase respectively in the efficiency of the production of hydroxyl radicals from ozone. Using k_{O3} determined in catalytic runs performed in the presence of t-BuOH, $4.2\,M^{-1}\,s^{-1}$ at pH 3 and $14\,M^{-1}\,s^{-1}$ at pH 5, it was also possible to derive the rate constant for the reaction with hydroxyl radical by means of Eq. (1). These values were 4.0×10^{-9} (pH 3) and 5.7×10^{-9} (pH 5), both essentially coincident with the same rate constant obtained in the absence of catalyst. These results suggest that the catalyst surface plays a significant role in the production of hydroxyl radicals but the interaction between solid surface and organics seems to be limited. At least, the kinetic data obtained in this work did not reveal a significant change in $k_{OH^\bullet}$ as a consequence of such interaction.

3.2. Efficiency of mineralization

The mineralization of reaction intermediates was monitored by simultaneously determining TOC and COD in samples taken during the experiments. The decay of TOC was relatively rapid during the first part of the runs. Thereafter, it took place a second period marked by a much lower rate of mineralization and in which we measured important amounts of simple carboxylic acids. The catalyst, TiO_2 P25, increased the rate of TOC decay during the first period, in which still persisted a certain amount of unreacted clofibric acid, with almost no effect on the final part of the run. The evolution of TOC has been extensively treated in a previous work [24]. We determined the dissolved organic carbon in the form of clofibric, oxalic (OXA), acetic (ACE), and formic (FOR) acids, in all samples collected during the runs. The organic carbon belonging to non-quantified compounds was defined as:

$$TOC_{oc} = TOC - TOC_{CFB} - TOC_{OXA} - TOC_{ACE} - TOC_{FOR} \quad (4)$$

The relative amount of TOC_{oc} in catalytic and non-catalytic runs performed at pH 3 and 5 is shown in Fig. 2. Non-catalytic reactions clearly produced a higher amount of intermediates other than carboxylic acids, at least during the first part of the ozonation. The effect of the introduction of catalyst can also be computed by using the mean oxidation number of carbon (MOC). The increment of this parameter, ΔMOC, is expressed as follows in molar units [25]:

$$\Delta MOC = 4\left(\frac{COD_o}{TOC_o} - \frac{COD}{TOC}\right) \quad (5)$$

Also plotted in Fig. 2 are the values of ΔMOC for representative samples. The graph shows a steady increase in the oxidation number of carbon associated with the formation of oxidized derivatives but without reaching a stationary state in terms of the carbon oxidation number after 1 h. Another parameter used to reflect the mineralization efficiency of an oxidation system is the partial oxidation efficiency, *E*, defined in Eq. (6) with units expressed in mg/L [38]:

$$E = \left(\frac{COD_o}{TOC_o} - \frac{COD}{TOC}\right)\left(\frac{TOC}{COD_o - COD}\right) \quad (6)$$

E represents the percentage of COD still not mineralized at a given time. In the catalytic runs performed in this study, *E* reached an almost constant value with a mineralization fraction that approached 65% after 20–30 min. In all cases the degree of mineralization obtained was not particularly high and was consistent with the accumulation of partially oxidized intermediates in the reaction mixture.

3.3. Identification of oxidation intermediates

The two main simple carboxylic acids detected by ionic chromatography were acetic and oxalic acids. Oxalic acid was the only oxidation product that accumulated in all runs irrespective of pH. As indicated in Fig. 3, its concentration increased with the severity of treatment, reaching about 15% of the remaining dissolved carbon. Jointly, oxalic and acetic acid accounted for over one-third of the total organic carbon at the end of catalytic and non-catalytic runs. Both a pH increase and the addition of catalyst improved the formation of simple carboxylic acids up to a certain point; beyond that point their rate of mineralization was greater than that of their formation, mostly due to the mineralization of acetic acid [24].

The identification of more complex oxidation by-products was performed by LC-TOF-MS carried out on reaction samples collected during non-catalytic runs performed with the highest concentration of clofibric acid. The measurements allowed elemental compositions to be proposed with precision for the molecular ions of the compounds detected as well as for their characteristic fragments and sodium adducts. The high resolution of TOF

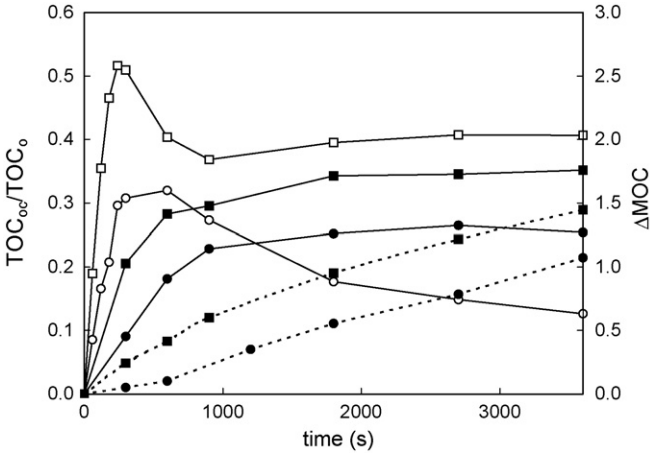

Fig. 2. Relative amount of organic carbon in non-quantified compounds at pH 3 (circles) and 5 (squares) during catalytic (filled symbols and solid lines) and non-catalytic (empty symbols). Also MOC (dashed lines and right scale) for catalytic runs at pH 3 (●) and 5 (■).

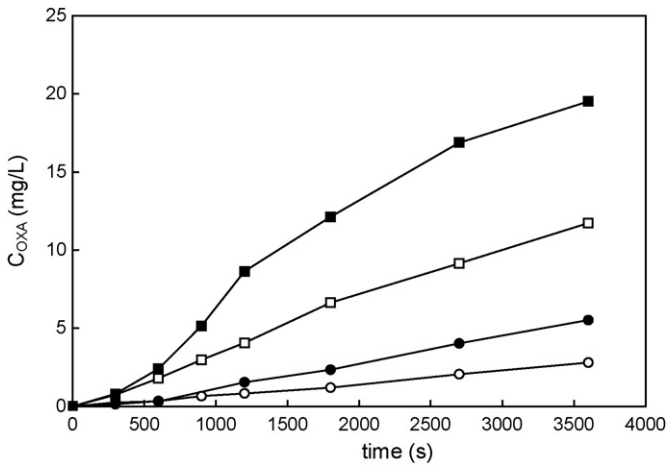

Fig. 3. Concentration of oxalic acid in runs at pH 3 (circles) and 5 (squares) during catalytic (filled) and non-catalytic (empty) runs. Initial concentration of clofibric acid: 100 mg/L.

Table 1
Mass measurements obtained by LC-TOF-MS for clofibric acid and its identified ozonation products. Compound numbers refer to Fig. 4.

No.	Name	Molecular formula	Detected ion	Ionization mode	Experimental mass (m/z)	Calculated mass (m/z)
1	Clofibric acid	$C_{10}H_{11}ClO_3$	$(C_{10}H_9ClO_3)^+$	Negative	213.0312	213.0317
2	4-Chlorophenol	C_6H_5ClO	$(C_6H_4ClO)^-$	Negative	126.9971	126.9951
		C_6H_5ClO	$(C_6H_6ClO)^+$	Positive	129.0098	129.0107
3	2-Hydroxyisobutyric acid	$C_4H_8O_3$	$(C_4H_7O_3)^-$	Negative	103.0391	103.0395
4	Hydroquinone	$C_6H_6O_2$	$(C_6H_5O_2)^-$	Negative	109.0284	109.0290
5	4-Chlorocatechol	$C_6H_5ClO_2$	$(C_6H_4ClO_2)^-$	Negative	142.9913	142.9900
6	P6	$C_9H_{10}O_8$	$(C_9H_9O_8)^-$	Negative	245.0292	245.0297
7	P7	$C_8H_{10}O_6$	$(C_8H_{10}O_6Na)^+$	Positive	225.0371	225.0375
8	P8	$C_7H_8O_7$	$(C_7H_9O_7)^+$	Positive	205.0359	205.0348

instruments yields accurate mass measurements and permits elemental compositions to be proposed for the fragment ions of the detected compounds. As indicated in Table 1, errors were below 10 ppm except for 4-chlorophenol detected in negative mode, a compound also detected in positive mode with less error. Although this information does not provide full certainty with regard to chemical structures, a structure assignment was proposed based on these measurements and on the chemistry of ozone reactions. Some of the intermediates detected coincided with those reported elsewhere. Sirés et al. [39] studied the oxidation of clofibric acid using Fenton systems and proposed a reaction scheme in which clofibric acid is first oxidized to 4-chlorophenol by the breaking of the C(1)–O bond, thus also yielding 2-hydroxyisobutiric acid. Further hydroxyl attack on C4 position of 4-chlorophenol yields hydroquinone, whereas the attack on C(2) position leads to 4-chlorocatechol. The formation of 4-chlorocatechol from 4-chlorophenol may take place as a result of a direct ozone attack or by the selective attack of hydroxyl radical on the *ortho*-position of 4-chlorophenol [40]. Doll and Frimmel [41] investigated the catalytic photodegradation products of clofibric acid and proposed the same pathway together with a parallel dechlorination reaction which supposedly yields 2-(4-hydroxyphenoxy)-isobutyric acid. This reaction was not detected in the present study. 4-Chlorophenol, 4-chlorocatechol and hydroquinone were detected in most samples tested but in very low amounts and, therefore, they have not been quantified. A fragment ion with m/z 103.0391 was also detected in EI negative mode with signals decreasing with time and intensity of treatment. The signal was attributed to the empirical formula $[C_4H_7O_3^-]$ corresponding to 2-hydroxyisobutyric acid, also detected by Doll and Frimmel [41]. These results suggest that the breaking of the C(1)–O bond to yield 4-chlorophenol, characteristic of HO· attack, is a minor degradation route in ozonation in acidic conditions. The corresponding reaction scheme in shown in Fig. 4a. The relatively large amount of acetic acid detected during the runs was possible the outcome of the oxidation of 2-hydroxyisobutiric acid.

The three other oxidation products, identified as P6, P7 and P8 in Table 1, were assumed to correspond to the ring-cleavage of clofibric acid though a normal ozonation mechanism. The ion fragment at m/z 245.0292 ($C_9H_9O_8^-$, −2.04 ppm error) was assigned to the neutral empirical formula $C_9H_{10}O_8$, an oxidation and dechlorination product from a ring opened structure still preserving the methylpropionic group from clofibric acid. The extracted ion chromatogram (XIC) at m/z 245.029 clearly shows four isomers with the same exact mass; these were assumed to be the products of the ozonation of different positions in the aromatic ring. A probable reaction pathway leading to four isomers has been depicted in Fig. 4b with an initial ozone attack on C2–C3 and C4–C5 bonds. The proposed pathway starts with the ring-opening by ozone cycloaddition and includes a double $HO^{\bullet}$ addition to the double bond followed by the oxidation and decarboxylation of the α-keto acid. This sequence leads to four isomers, the Z–E forms of the structures labelled as 6 in Fig. 4b. Compound 6 may undergo further decarboxylation to yield 7, whose proposed structure was confirmed by the presence of the corresponding sodium adduct m/z 225 ($C_8H_{10}O_6Na^+$, −1.78 ppm error). By following an oxidative sequence such as that indicated in Fig. 4c, 7 could generate 8. Compound 8 showed a nominal mass m/z 204 ($C_7H_8O_7$, 5.36 ppm) and

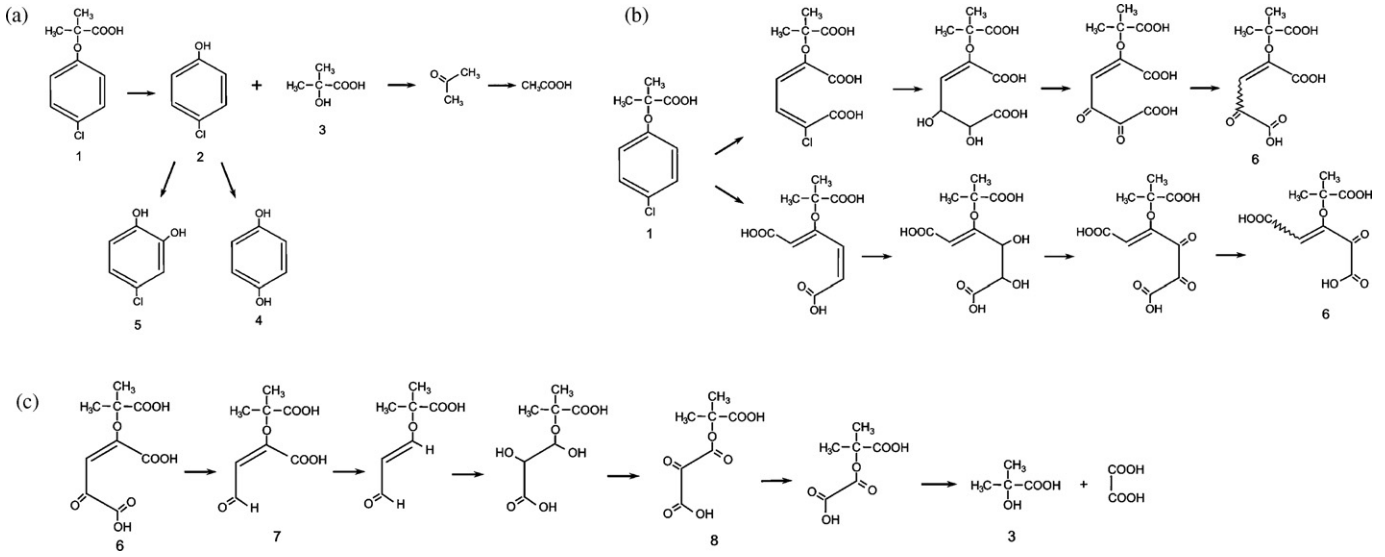

Fig. 4. Suggested reaction scheme for the ozonation of clofibric acid.

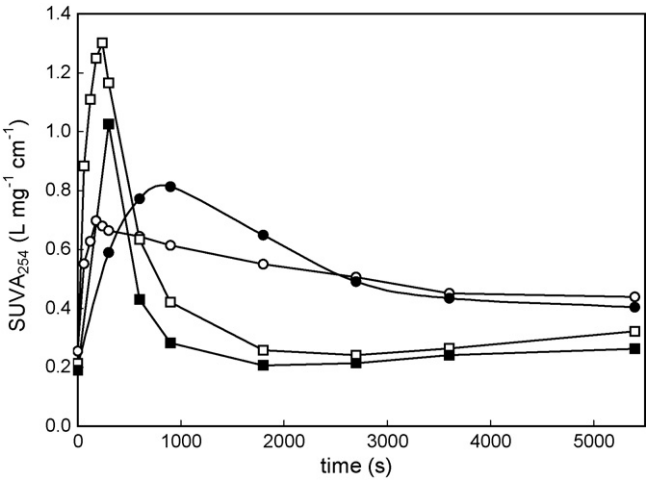

Fig. 5. Specific ultraviolet absorption at 254 nm ($SUVA_{254}$) for the ozonation of clofibric acid at pH 3 (◯) and 5(□) in the absence of catalyst and at pH 3 (●) and 5 (■) using 1 g/L of TiO_2 as catalyst.

its oxidation products should be 2-hydroxyisobutyric and oxalic acids, thus completing the oxidative chain. It is worth noting that prior to this study, none of these three ring-opening products from the ozonation of clofibric acid had been previously reported.

3.4. Toxicity of ozonated samples

The evolution of $SUVA_{254}$ with reaction time is shown in Fig. 5. The pattern was similar in all cases, with a maximum reached during the first few minutes that was more pronounced at higher pH. The catalyst always reduced the absorbance during the first part of the run, even though for runs at pH 3, $SUVA_{254}$ reached higher maximum values for intermediate reaction times in the presence of catalyst. These data seem to reflect shifts in the absorbance of aromatic derivatives of clofibric acid which tended to accumulate in the reaction mixture at pH 3 probably as a consequence of the greater interaction of the positively charged catalyst with clofibric acid. UV-absorbing compounds like 4-chlorophenol and other probable products not detected in this work are supposed to be responsible for the $SUVA_{254}$ peak at the beginning of the run. The non-saturated character of these reaction products was expected to be linked to an increase in the toxicity of the reaction mixture even if TOC diminished considerably. A certain similarity between $SUVA_{254}$ profiles and the relative TOC in non-quantified compounds, especially for non-catalytic runs, also supported this assumption.

The toxicity of reaction mixtures consisting of partially oxidized reaction intermediates was assessed on *V. fischeri* and acute *D. magna*. The inhibition observed in *V. fischeri* bioassay of an untreated solution of 100 mg/L of clofibric acid in ultrapure water was 21% for a contact time of 15 min, consistent with the EC_{50} value obtained in this work, namely, 258 ± 34 mg/L. The toxicity obtained was considerably lower than that obtained by Ferrari et al. for a 30 min Microtox assay with a reported EC_{50} value of 91.8 mg/L [42]. The experimental value of EC_{50} for clofibric acid in 48 h *D. magna* toxicity test was 91 ± 9 mg/L, in good agreement with data presented by Henschel et al. who reported 89 mg/L [43].

The results of toxicity bioassays of samples taken during the runs at different reaction times area shown in Figs. 6 and 7 and represent the average of two replicate runs. The results obtained at pH 5 (Fig. 7) follow a pattern similar to the $SUVA_{254}$ profile represented in Fig. 5 with a significant increase of toxicity during the initial stages of ozonation. This phenomenon is particularly marked for *D. magna* tests, which reached near 100% immobilization during

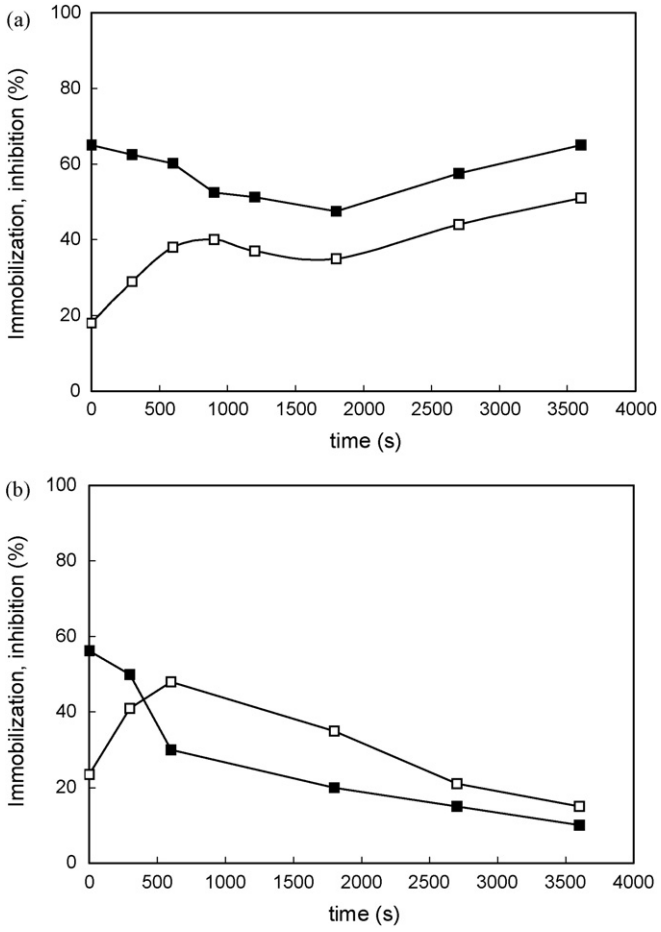

Fig. 6. Toxicity assessed by inhibition of *V. fischeri* (□) and immobilization of *D. magna* (■) in the reaction mixture from the ozonation of 100 mg/L of clofibric acid in non-catalytic (a) and catalytic (b) runs at pH 3. The catalyst used was 1 g/L of TiO_2.

the first 5–10 min. This period of increased toxicity lasted considerably longer than the maximum observed in $SUVA_{254}$. As indicated below, the discrepancy with the $SUVA_{254}$ profile could be attributed to the accumulation of reaction products from the breaking of the aromatic ring. These compounds may account for a substantial part of the relatively high amount of dissolved carbon, up to 40% of the initial TOC that remained in solution after 1 h in the form of compounds different from the three simple carboxylic acids that were accurately monitored in this work.

A toxicity increase in treated samples due to the formation of by-products that can cause greater toxicity than the parent compound has been previously observed [44]. Closely related to this work, Shang et al. [18] reported a toxicity increase during the early stages of the ozonation of chlorophenols that was attributed to the formation of chlorocatechols, chloromuconic acids and other hydroxylated or chlorinated compounds. In the present study, the identified oxidation products with higher toxicity were hydroquinone and 4-chlorophenol with EC_{50} values one order of magnitude lower for *V. fischeri* than for *D. magna*. Reported EC_{50} for *V. fischeri* (15 min) are 1.2 mg/L for 4.chlorophenol [44] and 0.041 mg/L for hydroquinone [45]. As for *D. magna* (48 h), the corresponding figures are 12.8 mg/L [46] and 0.15 mg/L [47]. In the present study the observed toxicity was in general higher for *D. magna* than for *V. fischeri*, suggesting a major effect associated with the ring-opening products rather than the aforementioned aromatic compounds. In support of this hypothesis, the sum of chromatographic areas from peaks corresponding to compounds

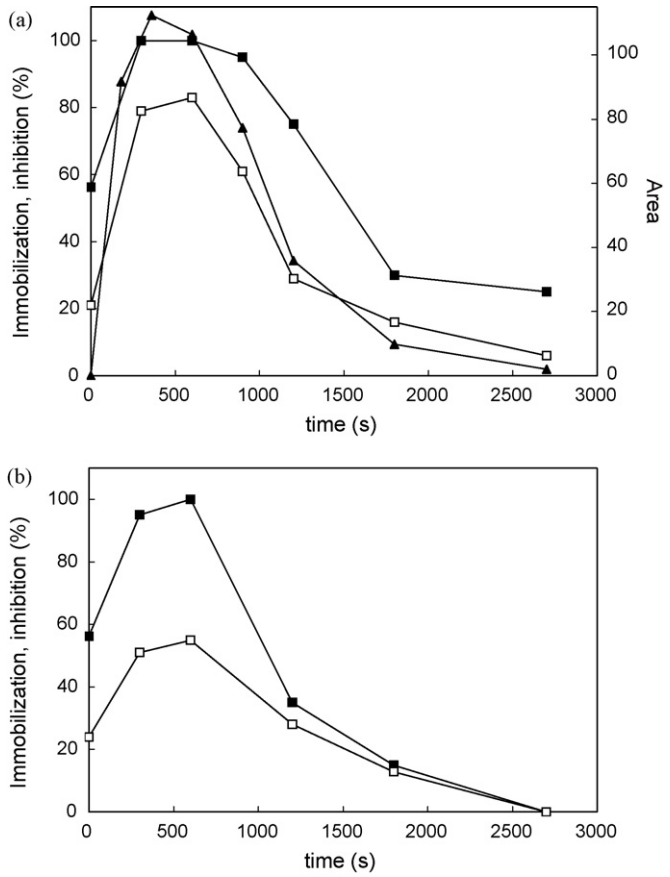

Fig. 7. Toxicity assessed by inhibition of *V. fischeri* (□) and immobilization of *D. magna* (■) in the reaction mixture from the ozonation of 100 mg/L of clofibric acid in non-catalytic (a) and catalytic (b) runs at pH 5. In Fig. 7a the area corresponding to compounds 6, 7 and 8 in Table 1 is also shown in arbitrary units ((▲) right scale). The catalyst used was 1 g/L of TiO_2.

6, 7 and 8, closely following the experimental toxicity pattern, are shown in Fig. 7a.

The significantly different evolution of toxicity observed at pH 3 with respect to pH 5 is probably due to the lower rate of accumulation on oxidized intermediates. Low molecular weight carboxylic acids might also play a role in the significantly lower immobilization of *D. magna* during the first part of ozonations at pH 3. These compounds were preferentially produced during the first part of runs performed at pH 5 and were markedly more toxic for *D. magna* than for *V. fischeri* [45,48]. As for formic acid, at pH 5, its concentration showed a maximum of about 20 mg/L after 15 min of ozonation, whereas it accumulated continuously at pH 3 with little difference between catalytic and non-catalytic runs.

The considerably lower toxicity observed in catalytic runs indicates that the catalyst reduces the accumulation of oxidation intermediates in the mixture. As shown before, the catalyst enhanced the ozonation of clofibric acid, which suggests that the same mechanism could operate with acidic intermediates, particularly acidic compounds from the ring-opening of clofibric acid. The interaction of a catalyst with organic molecules in aqueous solution is governed by the pH of the medium and the point of zero charge (PZC) of the solid, which is the pH at which the surface is neutral. The adsorption of neutral compounds on oxides in aqueous solutions is hindered by the competitive adsorption of water molecules, but the adsorption of dissociated acidic compounds is relatively favoured as the surface is positively charge below pH_{PZC} and may behave as anion exchanger [32]. Clofibric acid and other compounds dissociated in considerable amounts at pH 3–5 can adsorb on TiO_2.

The aromatic intermediates formed in the ozonation of clofibric acid were not dissociated in the operational conditions established in the present study as the pK_a of hydroquinone is 10.35 and that of 4-chlorophenol 9.38. The results of *D. magna* immobilization, particularly low at pH 3, might be explained on the basis of the interaction of the catalyst with acidic substances generated during the run. The aromatic compounds, not affected by the presence of catalyst are more toxic for *V. fischeri*, and would be perceived selectively by this bioassay.

4. Conclusions

The rate constant for the homogeneous direct ozonation of clofibric acid increased from $3.5 \pm 0.5\,M^{-1}\,s^{-1}$ at pH 1 to $14.3 \pm 1.6\,M^{-1}\,s^{-1}$ at pH 5 due to the speciation of the acid. The rate constant for the reaction of hydroxyl radicals and clofibric acid was calculated using the competitive method of kinetic analysis yielding $5.5 \times 10^9 \pm 8 \times 10^8\,M^{-1}\,s^{-1}$. At pH 3, 50% of the oxidation of clofibric acid takes place by reaction with hydroxyl radicals, a figure that increases to 90% at pH 5. The depletion of clofibric acid during ozonation was considerably enhanced by the presence of titanium dioxide with at least a four-time increase in the efficiency of the production of hydroxyl radicals from ozone. The catalyst raised the fraction or clofibric acid degraded by hydroxyl radicals to 85% (pH 3) and 98% (pH 5). The estimation of the rate constant for the reaction of clofibric acid with hydroxyl radicals in catalytic reactions yielded values essentially coincident with homogeneous ozonation. Both facts suggest that even if the catalytic surface plays a significant role in the production of hydroxyl radicals, the interaction of surface sites and organics is probably limited.

A simultaneous increase in the mean oxidation number of carbon was observed with ΔMOC of up to 1.5 at pH 5 for a 1-h treatment in conditions of about 30% mineralization. The reaction produced simple carboxylic acids as end-products but only oxalic acid tended to accumulate in the reaction mixture in reflection of its more refractory character under the non-alkaline ozonation conditions used in the present study. In all cases, a significant 20–40% of the dissolved organic carbon was in the form of reaction intermediates which differed from the simple carboxylic acids that represented the final reaction step before mineralization.

The identification of oxidation by-products was performed by LC-TOF-MS and a structure assignation was proposed for those whose exact mass could be clearly assessed. Minor amounts of 4-chlorophenol, hydroquinone and 4-chlorocatechol were detected and are probably associated with the maximum for $SUVA_{254}$ observed during the first part of the ozonation runs. These intermediates were assumed to be the product of breaking the C(1)–O bond, the corresponding hydroxyl radical attack constituting the primary reaction which led in turn to the depletion of clofibric acid. Accordingly, 2-hydroxyisobutyric acid was also detected throughout the run in amounts that decreased with reaction time. Three more compounds, whose occurrence had not been reported previously, were identified by accurate mass measurements. Tentative structures were proposed for these products that correspond to the ring-cleavage of clofibric acid though a normal ozonation mechanism followed by a sequence of oxidation and decarboxylation reactions. The results showed that this group of compounds retained the methylpropionic group from clofibric acid and at least one of them ($C_9H_{10}O_8$) was a mixture of four isomers that accumulated in relatively large amounts during reaction.

The results of toxicity bioassays on samples taken during the runs showed a significant increase of toxicity during the initial stages of ozonation both for *V. fischeri* and *D. magna* tests, but particularly for the latter. The accumulation of ring-opened acidic

structures from clofibric acid was the most likely origin of the increased toxicity of treated samples. The products of catalytic runs exhibited considerably lower toxicity. There was a particular fall in the immobilization of *D. magna* at pH 3, a result that was thought to reflect the interaction of the positively charged catalyst surface with the dissociated acidic intermediates generated during the run.

Acknowledgements

The authors wish to express their gratitude to the Ministry of Education of Spain (CTM2005-03080/TECNO and CSD2006-00044) and the Dirección General de Universidades e Investigación de la Comunidad de Madrid, Research network 0505/AMB-0395.

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