



## Biodegradation of clofibric acid and identification of its metabolites

R. Salgado<sup>a,c</sup>, A. Oehmen<sup>a</sup>, G. Carvalho<sup>a,b</sup>, J.P. Noronha<sup>a</sup>, M.A.M. Reis<sup>a,\*</sup>

<sup>a</sup> REQUIMTE/CQFB, Chemistry Department, FCT, Universidade Nova de Lisboa, 2829-516 Caparica, Portugal

<sup>b</sup> Instituto de Biologia Experimental e Tecnológica (IBET), Av. da República (EAN), 2784-505 Oeiras, Portugal

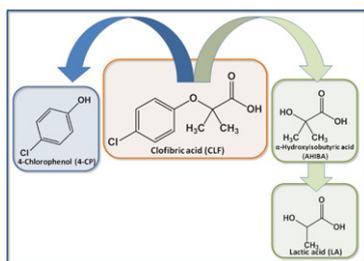
<sup>c</sup> ESTS-IPS, Escola Superior de Tecnologia de Setúbal do Instituto Politécnico de Setúbal, Rua Vale de Chaves, Campus do IPS, Estefanilha, 2910-761 Setúbal, Portugal

### HIGHLIGHTS

- ▶ Clofibric acid is biodegradable.
- ▶ Mainly heterotrophic bacteria degraded the clofibric acid.
- ▶ Metabolites of clofibric acid biodegradation were identified.
- ▶ The metabolic pathway of clofibric acid biodegradation is proposed.

### GRAPHICAL ABSTRACT

Metabolites produced during clofibric acid biodegradation.



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### ABSTRACT

Clofibric acid (CLF) is the pharmaceutically active metabolite of lipid regulators clofibrate, etofibrate and etofyllinclofibrate, and it is considered both environmentally persistent and refractory. This work studied the biotransformation of CLF in aerobic sequencing batch reactors (SBRs) with mixed microbial cultures, monitoring the efficiency of biotransformation of CLF and the production of metabolites. The maximum removal achieved was 51% biodegradation (initial CLF concentration = 2 mg L<sup>-1</sup>), where adsorption and abiotic removal mechanisms were shown to be negligible, showing that CLF is indeed biodegradable. Tests showed that the observed CLF biodegradation was mainly carried out by heterotrophic bacteria. Three main metabolites were identified, including α-hydroxyisobutyric acid, lactic acid and 4-chlorophenol. The latter is known to exhibit higher toxicity than the parent compound, but it did not accumulate in the SBRs. α-Hydroxyisobutyric acid and lactic acid accumulated for a period, where nitrite accumulation may have been responsible for inhibiting their degradation. A metabolic pathway for the biodegradation of CLF is proposed in this study.

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### 1. Introduction

Clofibric acid (CLF) is the active metabolite of the lipid regulators clofibrate, etofibrate, and etofyllinclofibrate. This drug is resistant to biodegradation and has a very high persistence in the environment [1,2]. Clofibric acid is one of the most widely and routinely reported drug metabolites found in open waters [3,4]. The presence of pharmaceutically active compounds (PhACs) and their metabolites in aquatic systems has become a concern in the past

years due to their generally persistent nature and ubiquity in the environment. However, at present, the biodegradability and ecotoxicity of many of these compounds remains unknown. CLF was detected in most aquatic systems (e.g. rivers) where pharmaceutical contaminants were monitored [2,5,6]. In a survey study of five different Portuguese wastewater treatment plants (WWTPs), CLF was consistently detected in the influent in the ng L<sup>-1</sup> to μg L<sup>-1</sup> range [7]. Peak influent concentrations of up to 41.4 μg L<sup>-1</sup> were found in another study, representing one of the most abundant of all PhACs detected [8]. Furthermore, a mass balance study suggested that CLF was mostly removed biologically in WWTPs [9]. Nevertheless, previous results revealed that clofibric acid was resistant to microbial degradation by several types of microorganisms,

\* Corresponding author. Tel.: +351 212 948 357; fax: +351 212 948 550.

E-mail address: [amr@fct.unl.pt](mailto:amr@fct.unl.pt) (M.A.M. Reis).

including WWTP biomass [10–12]. This apparent contradiction motivated the study of the conditions necessary for CLF biodegradation and its metabolic pathway. While Kosjek et al. [13] previously found that 4-chlorophenol was the only metabolite generated by an activated sludge reactor partially degrading CLF (<30%), little is known about the pathway by which CLF can be biodegraded.

The objectives of this study are to investigate the metabolism of CLF biodegradation, by (1) determining its metabolites in order to elucidate a metabolic pathway and (2) to understand whether autotrophic or heterotrophic organisms are primarily responsible for the degradation. This study necessitated a CLF degrading culture, which was not successfully obtained using activated sludge biomass as an inoculum. Therefore, a culture acclimatised to a similar chlorinated aromatic ring structure as CLF (i.e. propanil) was used as inoculum [14]. This culture was shown to convert propanil (a herbicide used on rice cultures) into dichloroaniline (DCA), ultimately degrading both compounds completely. Biodegradation studies of clofibric acid were performed after acclimatisation of this aerobic culture to clofibric acid (in the presence and in the absence of propanil) in two sequencing batch reactors (SBRs). This study gives an important contribution towards the elucidation of biodegradation mechanisms of refractory PhAC that could affect the environment after discharge.

## 2. Materials and methods

### 2.1. Chemical and reagents

HPLC-grade acetonitrile, formic acid and phosphoric acid were purchased from Panreac (Portugal). Ultrapure water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). The clofibric acid ((2-4-chlorophenoxy)-2-methylpropionic acid) standard was purchased from Sigma–Aldrich (Steinheim, Germany) and the metabolite standards,  $\alpha$ -hydroxyisobutyric acid (AHIBA), lactic acid (LA), 4-chlorophenol (4-CP), and also the derivatising reagent, *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA)  $\geq 98.5\%$  (GC grade) was purchased from Aldrich (Portugal) and *cis*-3-hexenylactate  $\geq 98.5\%$  (GC grade) was purchased from SAFC, Kosher (Portugal). The allylthiourea (ATU), sodium nitrite and sodium nitrate and the reagents used in the preparation of mineral media  $\geq 98\%$  (grade) of the reactors were all purchased from Panreac (Portugal).

### 2.2. Microbial enrichments

Microbial enrichments were initiated from a mixture of soil contaminated with several herbicides, including propanil, and soil from organic rice agriculture supplemented with  $(\text{NH}_4)_2\text{SO}_4$  and propanil [15]. These cultures were further enriched in a mixed culture that was used to inoculate a sequencing batch reactor (SBR), which was operated with a fed-batch strategy as detailed in Carvalho et al. [14]. This culture was used to seed the SBRs employed in this study, which were supplied with either clofibric acid or a mixture of clofibric acid and propanil over a period of 20 months.

### 2.3. Composition of the feed solution of the SBR

#### 2.3.1. Mineral media

The mineral media (phosphate buffer) was prepared with 2.620 g of  $\text{Na}_2\text{HPO}_4 \cdot \text{H}_2\text{O}$ ; 1.162 g of  $\text{KH}_2\text{PO}_4$  and 0.530 g of  $\text{NH}_4\text{Cl}$  dissolved in 1 L of MilliQ water. The pH was adjusted to 7.2 with NaOH, and then sterilised in an autoclave for 15 min at 120 °C.

#### 2.3.2. Nutrient solution

The nutrient solution was prepared by combining solutions A, B and C in the following proportion: 0.1 mL of A, 0.6 mL of B and

0.1 mL of C per 100 mL of solution. *Solution A (Micronutrient solution)*: 0.1 mM of  $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ ; 0.15 mM of  $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$ ; 0.5 mM of  $\text{ZnCl}_2$ ; 1 mM of  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ ; 0.1 mM of  $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ ; 0.8 mM of  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ ; 1 mM of  $\text{H}_3\text{BO}_3$  and 10 mM of HCl (25 vol%) dissolved in MilliQ water. This solution was sterilised in an autoclave for 15 min at 120 °C. *Solution B (Macronutrients solution)*: 0.135 M of  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , 0.865 M of  $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$  and 1.26 M of NaCl was dissolved in MilliQ water. This solution was sterilised in an autoclave for 15 min at 120 °C and was filtered through 0.45  $\mu\text{m}$  glass fibre membranes (GF 6, <1  $\mu\text{m}$ , diameter 47 mm from Whatman, England). *Solution C*: 0.2 M of  $\text{CaCl}_2$  dissolved in MilliQ water.

#### 2.3.3. Carbon source: clofibric acid and propanil

A clofibric acid stock solution of 1000  $\text{mg L}^{-1}$  in MilliQ water and a 100  $\text{mg L}^{-1}$  propanil solution were prepared in mineral media and stored at 4 °C to feed the reactors as carbon source.

### 2.4. Lab-scale SBR operation

#### 2.4.1. SBR start-up and operation

Two 150-mL SBRs were fed with spikes of 1  $\text{mg L}^{-1}$  clofibric acid and 3  $\text{mg L}^{-1}$   $\text{NH}_4^+\text{-N}$ . SBR A was fed with CLF as the sole carbon source and in SBR B, propanil was also added to a concentration of 3  $\text{mg L}^{-1}$  in the reactor. The hydraulic retention time (HRT) of both reactors was approximately 9.3 d, and no sludge wastage was made beyond sampling. 75% of the reactor media (supernatant after centrifugation) was replaced every 7 days (i.e. each cycle). Long HRTs and sludge retention times (SRTs) have previously been shown to promote the enrichment of microbial cultures that are suitable towards xenobiotic biodegradation [2,14,16]. The two reactors were magnetically stirred, aerated through ceramic air dispersers, and kept in an air-conditioned room at a temperature of  $25 \pm 2$  °C, while the pH was uncontrolled.

After a 6 month acclimatisation period under the aforementioned conditions, CLF was then added at 2  $\text{mg L}^{-1}$  in both reactors (between month 6 and month 12). Once per day the pH of both reactors was adjusted to  $7.0 \pm 0.1$  with sodium hydroxide (NaOH). In reactor B, propanil was spiked to get a 6  $\text{mg L}^{-1}$  concentration in the reactor three times during the cycle period of 7 days (between month 6 and month 12) in order to promote increased biomass growth. Between month 9 and month 12, both reactors A and B were supplemented with ammonium sulphate solution to get 20  $\text{mg L}^{-1}$   $\text{NH}_4^+\text{-N}$  in both reactors.

After replacing the supernatant, the biomass concentration was monitored through optical density measurements at 610 nm, and the pH was again measured in each reactor. Samples were periodically taken to monitor clofibric acid, propanil, ammonia, nitrite and nitrate and also the biodegradation metabolites of propanil and clofibric acid.

#### 2.4.2. Test with non-limiting ammonia concentration

After acclimatisation to the higher ammonia concentration, an experiment was carried out where SBR B was operated with additional ammonia spikes during the cycle to prevent limitation. The SBR initially was fed with 2  $\text{mg L}^{-1}$  of clofibric acid and 20  $\text{mg L}^{-1}$   $\text{NH}_4^+\text{-N}$ , as usual, but no propanil. The SBR was operated with pH control at  $7.0 \pm 0.1$ . Spikes of 5  $\text{mg L}^{-1}$   $\text{NH}_4^+\text{-N}$  were added on days 1 and 2 of operation and reactor performance was monitored for 5 days. Samples were taken daily to analyse for clofibric acid and metabolites, ammonia, nitrite and nitrate.

#### 2.4.3. Nitrification inhibition test

SBR B was fed with mineral media and nutrient solution and spiked with 2  $\text{mg L}^{-1}$  clofibric acid, 20  $\text{mg L}^{-1}$  N ammonia, but no propanil, as described above. Additionally, 1  $\text{mg L}^{-1}$  of allylthiourea

(ATU) was added to inhibit nitrification. The reactor's performance was monitored throughout 5 days.

#### 2.4.4. Test of adsorption to the biomass

A fraction of biomass from SBR B was centrifuged and washed with mineral media. The biomass was re-suspended with mineral media and nutrient solution in the same proportion used in the reactor, and  $2 \text{ mg L}^{-1}$  of clofibric acid was spiked to the reactor. The CLF concentrations in the supernatant and in the biomass fraction were measured in addition to the biomass concentration. This procedure was repeated after 3 days. The samples were centrifuged and the liquid was directly analysed by HPLC–DAD, while the extraction procedure detailed below was used to determine the amount of CLF and the metabolites adsorbed to the sludge.

The compounds adsorbed to the biomass were determined by the use of the modified method of ultrasonic solvent extraction described by Salgado et al. [7]. In brief,  $2 \times 2 \text{ mL}$  of methanol was added to the centrifuged biomass sample, and the extraction was carried out for 5 min in an ultrasonic bath. The solvent/biomass mixture was centrifuged, then the supernatant was collected in a vial and evaporated to 1 mL by a gentle nitrogen stream, and the compounds extracted from the sludge were analysed in HPLC–DAD.

#### 2.4.5. Abiotic CLF degradation

Two 150 mL SBRs were fed with  $2 \text{ mg L}^{-1}$  clofibric acid and  $20 \text{ mg L}^{-1} \text{ NH}_4^+\text{-N}$  in addition to mineral media, and nutrient solution. SBR A was fed with CLF as the sole carbon source and in SBR B, propanil was also added to a concentration of  $6 \text{ mg L}^{-1}$  in the reactor. No biomass was added in these tests in both reactors. The reactors' performance was monitored throughout 5 days.

### 2.5. Analytical procedures

#### 2.5.1. HPLC–DAD analysis

Samples of both reactors were centrifuged for 5 min at 10,000 rpm and injected in a high performance liquid chromatography (HPLC) for quantification of the clofibric acid, propanil and dichloroaniline (DCA, the primary metabolite of propanil degradation). Standard solutions in the range of  $0\text{--}2 \text{ mg L}^{-1}$  for clofibric acid,  $0\text{--}10 \text{ mg L}^{-1}$  for propanil and dichloroaniline, and  $0\text{--}6 \text{ mg L}^{-1} \text{ N}$  for nitrite and nitrate were used to obtain the respective calibration curves. The quantification of the CLF metabolites (AHIBA, LA and 4-CP) was carried out using the same procedure as for clofibric acid, using the metabolite standards in the concentration ranges  $1\text{--}3 \text{ mg L}^{-1}$ . HPLC–DAD was carried out in a HPLC system (Waters) coupled with a pump and controller (Waters 600), an in-line degasser (X-Act-4 channels, Jour Research), an autosampler (Waters 717 plus), and a photodiode array detector (DAD, Waters 996). Reverse-phase chromatography (LiChroCART 250-4 Purospher Star RP18 endcapped,  $5 \mu\text{m}$ , column, Merck) of the samples was performed with a  $0.6 \text{ mL min}^{-1}$  flow rate of a degassed mobile phase with 70% methanol and 30% water with pH adjusted to 3 with phosphoric acid, and diode array detection from 200 to 400 nm. The chromatograms were acquired with a MassLinx™ software data acquisition system.

Ten blank samples were analysed by HPLC–DAD to determine the lowest signal/noise ratio of each analyte. Limits of detection (LOD) for the analytes were calculated by the formula  $3 \times \text{SD}/m$ , where SD is the standard deviation of the lowest signal/noise ratio of the analyte and  $m$  is the slope of the calibration curve. Limits of quantification (LOQ) were calculated as  $10 \times \text{SD}/m$ . LOD and LOQ levels can be found in Table 1.

#### 2.5.2. GC–MS analysis of CLF metabolites

In order to identify the metabolites generated during CLF biodegradation in the SBRs, samples of both reactors were

collected to be analysed by gas-chromatography–mass spectrometry (GC–MS). Samples and standards were prepared according to the derivatisation procedure (Section 2.5.2.1) previous to GC–MS analysis.

**2.5.2.1. Derivatisation.**  $200 \mu\text{L}$  of sample of the SBR and standard solutions of the metabolites were dried with a  $\text{N}_2$  stream and then  $20 \mu\text{L}$  of the derivatisation reagent MSTFA was added to the vial. The reaction took place for 60 min at  $60^\circ\text{C}$  prior to injection in the GC–MS.

**2.5.2.2. GC–MS analysis.** GC–MS analysis was performed using an Agilent 6850 GC fitted with a 5975 VL MSD (Triple Axis Detector) Agilent mass spectrometric detector. The injection port was operated in splitless mode, during 5 min. A DB-5MS 5% phenyl- and 95% dimethylpolysiloxane capillary column ( $30 \text{ m} \times 0.25 \text{ mm i.d.}$ ,  $0.25 \mu\text{m}$  film thickness) from Agilent was used, with helium as carrier gas at a flow rate of  $1 \text{ mL min}^{-1}$ . The injection port temperature was  $250^\circ\text{C}$ . The ion source, the quadrupole and the transference line were kept at 230, 150 and  $280^\circ\text{C}$ , respectively.

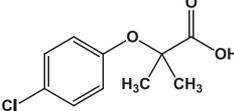
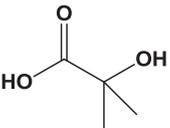
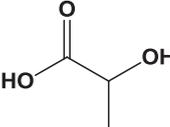
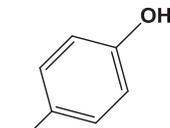
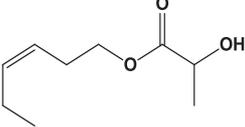
The oven temperature was maintained at  $60^\circ\text{C}$  for 3 min, programmed to  $250^\circ\text{C}$  at an increase of  $10^\circ\text{C min}^{-1}$ , then increased to  $310^\circ\text{C}$  at  $20^\circ\text{C min}^{-1}$ , and held for 13 min. The MS spectrum was obtained with electron energy 70 eV, mass range  $m/z$  40–650 and using MSD ChemStation software (Agilent).

The identification of the metabolites was performed by the use of mass spectrum database libraries of NIST (2005) and Wiley (2005) that suggest possible chemical structures for metabolites, which were confirmed by the injection of the derivatised standards. The ChemStation library of the MS search uses a probability based matching (PBM) algorithm. The PBM algorithm compares an unknown mass spectrum to the reference spectrum using a reverse search routine. A prefilter within the search routine assigns significance to each of the peaks in the unknown spectrum and uses these to find the most probable matches in the condensed reference library. The selected condensed spectra are then compared with the complete unknown spectrum. Each condensed spectrum selected from the database (reference spectrum) by the prefilter is compared with all the peaks in the unknown mass spectrum (which has been normalised). The probability is then calculated. After identifying the metabolites generated in the reactors, standards of each compound were also injected to confirm the structures suggested from the library's databases of the GC–MS. The standards previously identified in GC–MS were also analysed by HPLC–DAD to obtain the chromatographic characteristics of the standards (retention time and absorption spectra in the DAD). Calibration curves were done in order to quantify the metabolites that were generated. This was performed using the same analytical strategy as for monitoring CLF. Three of the four metabolites were possible to be quantified by HPLC–DAD (see Section 2.5.1). The limits of detection (LOD) for the analytes were determined as described in Section 2.5.1, where blank samples were analysed for the lowest signal/noise ratio of each analyte and calculated by the formula  $3 \times \text{SD}/m$ . LOD levels are found in Table 1.

#### 2.5.3. Ammonia analysis

Samples of both reactors were centrifuged for 5 min at 10,000 rpm and the ammonia concentration was measured by an ammonia selective electrode (Orion model 720A), where  $20 \mu\text{L}$  of ionic strength adjustor (ISA) solution (951211 ISA, Orion ISE series) was added to 1 mL of sample. Standard solutions of ammonia in the range of  $0\text{--}20 \text{ mg L}^{-1}$  of N were prepared to obtain the respective calibration curve.

**Table 1**  
Characteristics of chromatograms of GC–MS and HPLC–DAD analysis of CLF and metabolites.

Metabolite	$M_w$	Log $K_{ow}$	Formula	Chemical structure	GC–MS			HPLC–DAD				
					$t_r$ (min)	$m/z$ (%) <sup>a</sup>	LOD <sup>b</sup> ( $\mu\text{g L}^{-1}$ )	Match/r. match/probability with spectra (%)	$t_r$ (min)	$\lambda$ (nm)	LOD <sup>c</sup> ( $\mu\text{g L}^{-1}$ )	LOQ <sup>d</sup> ( $\mu\text{g L}^{-1}$ )
Clofibric acid	215	2.84	$\text{C}_{10}\text{H}_{11}\text{ClO}_3$		15.46	128 143 169	1.40		15.48	278	0.66	2.20
$\alpha$ -Hydroxyisobutyric acid (AHIBA)	104	−0.36	$\text{C}_4\text{H}_8\text{O}_3$		7.98	45(12), 73(100), 117(1), 131(100), 147(60), 205(20), 233(12)	3.39	91.1/97.1/60.4	3.28	250	0.09	0.29
Lactic acid (LA)	90	−0.72	$\text{C}_3\text{H}_6\text{O}_3$		8.14	45 (20), 73(100), 117(75), 147(75), 191(12), 219(6)	0.71	94.9/97.1/78.8	3.48	258	0.02	0.06
4-Chlorophenol (4-CP)	129	2.27	$\text{C}_6\text{H}_5\text{ClO}$		12.47	65 (30), 73 (18), 93(1), 100 (12), 128 (100)	0.03	81.4/86.4/62.6	8.24	281	0.04	0.14
cis-3-Hexenyl lactate	172	2.04	$\text{C}_9\text{H}_{16}\text{O}_3$		7.01	45(100), 67(69), 73(12), 82(90), 99(12), 115(2), 172(5)	2.71	65.9/72.3/40.6	7.11	277	0.55	1.84

<sup>a</sup>  $m/z$  obtained by derivatisation with MSTFA, trimethylsilyl ethers; TMSi ( $-\text{Si}(\text{CH}_3)_3$ )  $m/z$  73.

<sup>b</sup> LOD of propanil (0.17) and DCA (0.37).

<sup>c</sup> LOD of propanil (0.02) and DCA (0.03).

<sup>d</sup> LOQ of propanil (0.07) and DCA (0.09).

## 2.6. Fluorescence in situ hybridisation (FISH)

Fluorescence in situ hybridisation (FISH) was carried out according to Amann [17] in samples of SBR B for characterisation of the autotrophic bacteria. The probes (NSO1225) [18] for ammonia oxidising bacteria and (Ntspa662) [19] for nitrite oxidising bacteria, were tested against the probes for all bacteria, EUBmix (EUB338 [20], EUB338-II and EUB338-III) [21]. FISH samples were observed using an Olympus BX51 epifluorescence microscope.

## 2.7. Determination of the proposed biodegradation mechanism

The biocatalysis and biodegradation database (BBD) software developed by the University of Minnesota (UM) was used to simulate and predict the biodegradation pathway of CLF [22,23].

## 3. Results and discussion

### 3.1. Clofibric acid biotransformation

Initially, activated sludge from a WWTP was used as the inoculum for a sequencing batch reactor (SBR) fed with  $300 \mu\text{g L}^{-1}$  CLF and glucose as a supplemental carbon source to facilitate biomass growth and potentially co-metabolism of CLF. This system was operated for more than 2 months, where minimal CLF removal was observed (data not shown). At this time the CLF concentration in the feed was increased to  $1200 \mu\text{g L}^{-1}$  in order to confirm that the CLF concentration was not limiting biomass growth. However, CLF removal was still not observed after another 2 months of SBR operation (data not shown). These results show that not all activated sludge communities have the capacity to degrade CLF, which explains the contradictory biodegradation efficiencies observed in different WWTPs [9,11,12]. This motivated the use of a microbial community already adapted to chlorinated ring-based compounds.

A microbial enrichment obtained from an agricultural soil exposed to the herbicide propanil, was tested, since propanil has a similar chlorinated aromatic ring structure as CLF. This culture was shown to be able to degrade propanil as the only carbon source [14]. Two sequencing batch reactors (SBRs) were inoculated with this enriched culture. In one SBR, clofibric acid was added as the sole carbon source (SBR A) and in the other, propanil and clofibric acid (SBR B) were added together to facilitate biomass growth and potentially co-metabolism. The initial CLF and propanil concentrations were  $2 \text{ mg L}^{-1}$  and  $6 \text{ mg L}^{-1}$ , respectively. Fig. 1a and b shows the CLF biodegradation in one typical cycle from each SBR after 12 months of reactor operation, respectively.

SBR A showed lower CLF removal ( $15 \pm 1\%$ ) as compared to SBR B ( $51 \pm 2\%$ ), suggesting that the addition of propanil to the SBR increased the CLF degradation capacity. In separate sets of tests, the abiotic transformation of CLF (using the same conditions but no microbial culture added) was found to be negligible, as was the quantity of CLF adsorbed to the biomass (data not shown), suggesting that the removal observed in Fig. 1 could be attributed to CLF biodegradation. In previous work by Tran et al. [24], it was shown that the presence of acetate ( $100 \text{ mg L}^{-1}$ ) as an additional carbon source increased CLF removal from almost 10% in the absence of acetate to 35% in its presence. Similarly to our study, the addition of another carbon source induced biomass growth, leading to higher CLF removal. The biomass concentration of SBR B was more than double that of SBR A ( $1026 \text{ mg VSS L}^{-1}$  vs.  $472 \text{ mg VSS L}^{-1}$  after 11 months, respectively). Furthermore, SBR B had a superior specific CLF removal as compared to SBR A ( $1.08 \text{ mg CLF g VSS}^{-1}$  vs.  $0.67 \text{ mg CLF g VSS}^{-1}$ , respectively), suggesting that propanil stimulated the growth of organisms that are also able to degrade CLF. In SBR B, propanil biodegradation led to the temporary accumulation

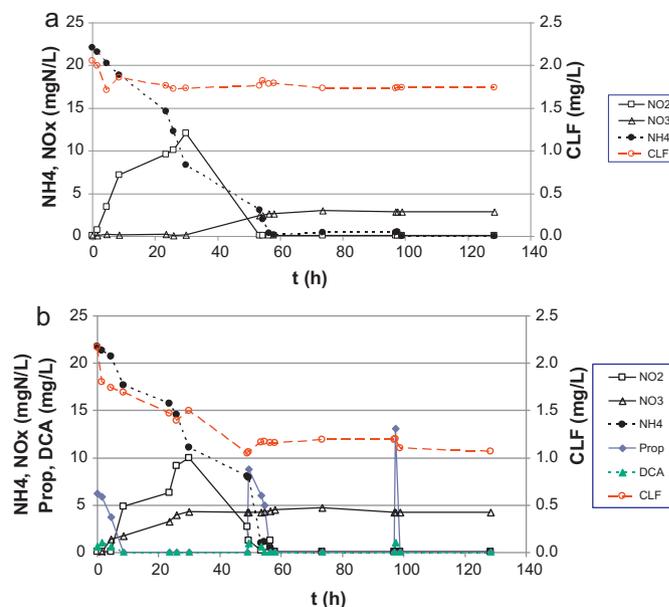


Fig. 1. Clofibric acid (CLF) degradation in SBR A (without propanil) (a) and SBR B (with propanil) (b). Propanil was added once every 48 h in (b).

of its well-known metabolite, 3,4-dichloroaniline (DCA), but it was completely removed after 1 day.

Since substantial CLF biotransformation occurred in SBR B, further analysis was performed to investigate the mechanism responsible for the biotransformations in this system. Biodegradation was observed until all of the ammonia was consumed, suggesting that either the nitrifiers were responsible for CLF biodegradation or heterotrophs were responsible for this transformation during biomass growth. Many previous studies have suggested a co-metabolic action of ammonia and aromatic compounds in nitrifying bacteria [25–28]. Yi and Harper [27] proposed that the dioxygenase enzymes are capable of mediating the co-metabolic biotransformation of aromatic compounds (for biotransformation of  $17\alpha$ -ethinylestradiol), which was not only associated with nitrifiers but also to heterotrophic bacteria.

Since nitrification to nitrite and nitrate occurred in both reactors, FISH analysis was performed to study the autotrophic microbial community. FISH confirmed that both reactors were dominated by ammonia-oxidising bacteria (AOB) (>50%), which were targeted by the FISH probe Nso1225. Each SBR also contained some nitrite oxidising bacteria (NOB) as detected by the Ntspa662 probe, for the determination of the presence of *Nitrospira* (~10%) and Nit3 for *Nitrobacter*. The high level of autotrophic nitrifiers is not surprising considering the low quantity of organic matter fed to each SBR and the relatively high content of ammonia.

In order to study whether or not nitrifiers were responsible for CLF biodegradation, an additional batch test was performed on SBR B with a higher ammonia concentration (initially  $20 \text{ mg L}^{-1}$   $\text{NH}_4^+$ -N, followed by two additional spikes of  $5 \text{ mg L}^{-1}$  each). The performance of SBR B without ammonia limitation is shown in Fig. 2. Results revealed that a similar amount of CLF biodegradation could be observed ( $41 \pm 13\%$ ) in SBR B as compared to Fig. 1b, indicating that CLF biotransformation was not linked directly with the abundance of ammonia, and that nitrifiers are not exclusively responsible for CLF degradation.

Further, nitrite accumulated (at least temporarily) in both experiments (Figs. 1 and 2). Nitrite has been frequently shown to be an inhibitor to many different types of bacteria [29]. Thus, it was investigated if nitrite inhibition represented the limiting factor towards achieving higher CLF biodegradation.

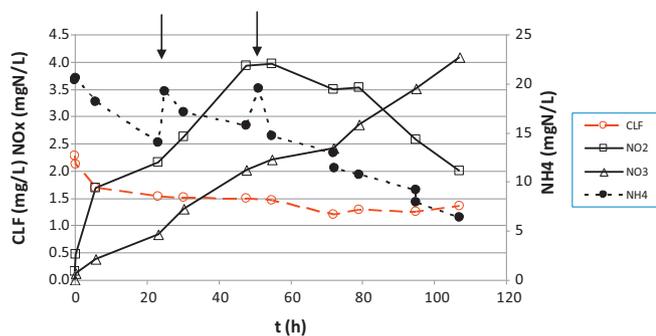


Fig. 2. Clofibric acid degradation in SBR B with no  $\text{NH}_4^+$  limitation. Arrows indicate  $5 \text{ mg L}^{-1}$   $\text{NH}_4^+$ -N spikes.

### 3.2. Nitrification inhibition

Fig. 3 shows a batch test carried out in SBR B where nitrification was inhibited by ATU. In this test, negligible ammonia was consumed and nitrite and nitrate were not produced. Nevertheless, CLF biodegradation decreased only slightly (from  $41 \pm 13\%$  to  $28 \pm 3\%$ ), suggesting that CLF removal was not principally carried out by nitrifiers, but mainly by heterotrophs. Furthermore, since nitrite was not produced in this assay, the hypothesis of nitrite inhibition leading to the interruption of CLF biodegradation was also discarded.

To the best of our knowledge, the extent of CLF biodegradation obtained in this study is higher than those previously reported in the literature. While Evangelista et al. [12] found that  $>90\%$  of a  $100 \text{ mg L}^{-1}$  CLF solution was transformed by *Rhodococcus rhodochrous* in 20 days after acclimatisation, it was mainly transformed into its parent compound ethyl clofibrate, which accumulated in the solution. Winkler et al. [2] found that CLF was not removed at an initial concentration of  $90 \mu\text{g L}^{-1}$  (over  $\sim 400$  h) and only 27% removal was obtained at  $11 \mu\text{g L}^{-1}$  (over  $\sim 95$  h) in a biofilm reactor fed with river water. In another study, the elimination of CLF was less than 30% (initial concentration:  $200\text{--}500 \mu\text{g L}^{-1}$ ) even with a high HRT of 48 h [13]. Similarly, a maximum removal of  $\sim 25\%$  was obtained for CLF with  $200 \text{ mg L}^{-1}$  N- $\text{NH}_4^+$  addition and an initial CLF concentration of  $100 \mu\text{g L}^{-1}$  after 6 days of cultivation [24].

It is not clear why CLF biodegradation proceeded until a certain value and then did not continue (Figs. 1–3). One possibility is that CLF removal is limited by an unknown nutrient present in the media or to the accumulation of an inhibitory metabolite in the culture medium. Analysis of the spectra obtained by GC–MS along the reactor cycle showed that some metabolites (see Section 3.3) temporarily accumulated in the culture media, but were later eliminated. Accumulation of these metabolites did not cause inhibition of CLF biodegradation, since even after being eliminated, the CLF concentration did not decrease further.

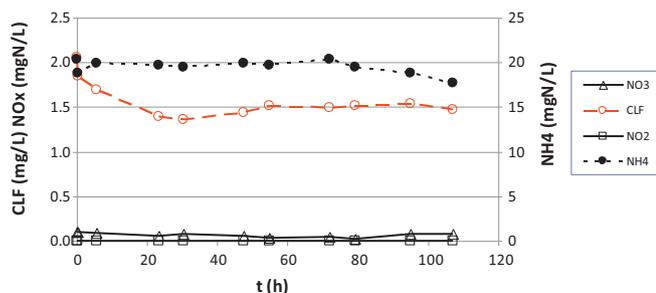


Fig. 3. Clofibric acid degradation in SBR B operated with  $1 \text{ mg L}^{-1}$  ATU for nitrification inhibition.

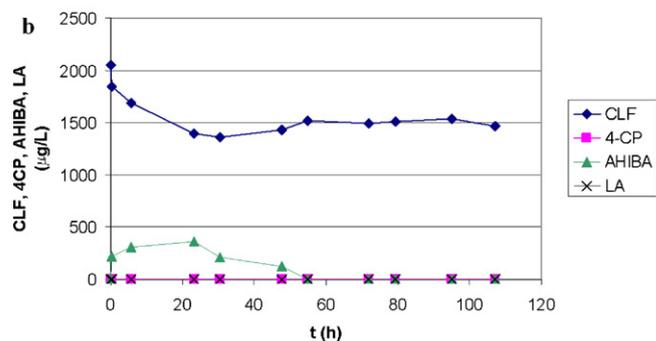
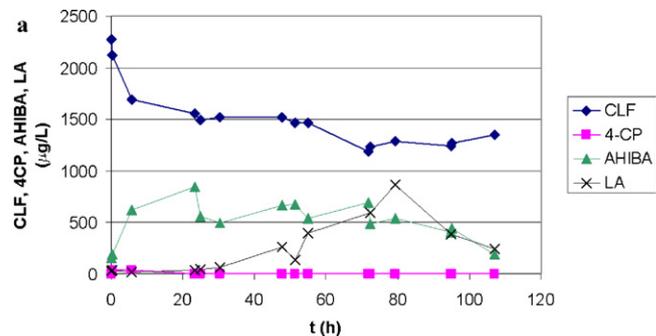


Fig. 4. Metabolites generated from CLF degradation in SBR B (with propanil) at  $20 \text{ mg L}^{-1}$   $\text{NH}_4^+$ -N (a) and with  $1 \text{ mg L}^{-1}$  of ATU (b).

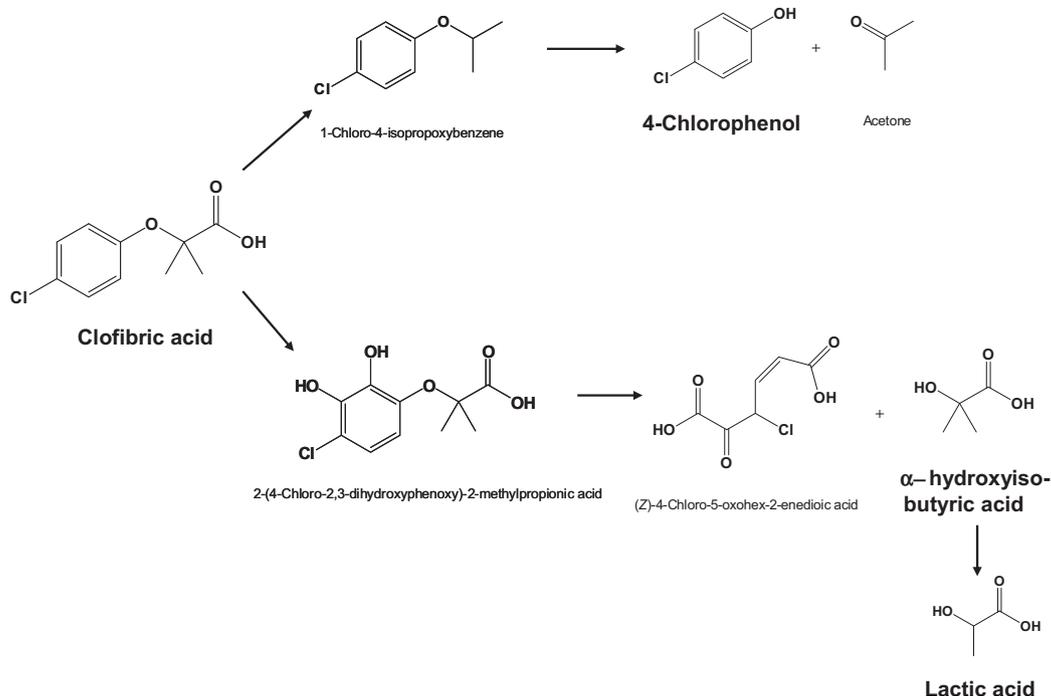
### 3.3. Products of microbial clofibric acid transformation

Identification of the metabolites produced from clofibric acid degradation was done by GC–MS analysis of the bioreactor effluent samples. Table 1 shows the chemical structures of the four identified compounds by GC–MS as well as their respective matches and similarities with the mass spectra in the NIST and Wiley databases and the reactor samples.

GC–MS analysis was able to identify 4 metabolites generated in the reactors. The similarities between the sample and the MS database spectra during GC–MS analysis were higher than 81% with the exception of the metabolite, *cis*-3-hexenylactate, which was 66%.

Three of the four metabolites were quantified by HPLC–DAD. The metabolite *cis*-3-hexenylactate did not appear in the HPLC–DAD chromatograms of the monitored reactor samples, despite the sensitivity of the technique and the detection of the corresponding standard. This suggests that this compound was unstable and/or an intermediary metabolite present at concentrations below the level of quantification (see Table 1 for LOQ). The absence of *cis*-3-hexenylactate was also in agreement with the GC–MS results, where low confidence was obtained in the identification of this compound. Neither CLF nor its metabolites were detected to be adsorbed to the sludge, which is in agreement with the low  $\log K_{ow}$  values of each compound (Table 1).

The main metabolites detected were  $\alpha$ -hydroxyisobutyric acid (AHIBA), followed by lactic acid (LA) and, in lower concentrations, 4-chlorophenol (4-CP). The quantification of these three main metabolites (by HPLC–DAD) from the biotransformation of CLF in SBR B is plotted in Fig. 4a and b. 4-Chlorophenol was generated by the cleavage of the ether bond of CLF, and has previously been recognised as a metabolite of CLF by Kosjek et al. [13]. 4-CP is known to have higher toxicity than the parent compound, CLF. In our experiments, 4-chlorophenol was temporarily detected in very low concentrations (low  $\mu\text{g L}^{-1}$ ) throughout the SBR cycle (Fig. 4a and b). 4-Chlorophenol was previously found to be biodegradable by Buitron and Moreno-Andrade [30], where after 1 h of reactor



**Fig. 5.** Clofibric acid biotransformation mechanism proposed by the biocatalysis and biotransformation database of the University of Minnesota (<http://umbbd.msi.umn.edu/predict/index.html>). The proposed metabolites that were detected in this study are highlighted in bold.

operation,  $18 \text{ mg L}^{-1}$  of 4-chlorophenol was biodegraded. To the best of our knowledge, this is the first study identifying AHIBA and LA as metabolites of CLF biodegradation in wastewater studies, although AHIBA has previously been detected in studies analysing the metabolites achieved through advanced oxidation processes, such as ozonation and fenton-based reactions [31,32].

During the biodegradation of CLF, AHIBA was the most important metabolite formed initially, and appeared almost immediately after inoculation (Fig. 4a and b). When nitrification was not inhibited, all of the metabolites were biodegraded in the SBR and a conversion of AHIBA into LA seemed to begin after 30 h (Fig. 4a). After 72 h, the biodegradation of CLF and its metabolites seemed to slow down, and LA was the metabolite accumulated in the highest abundance overall. Even so, LA and AHIBA were mostly consumed by the end of the cycle (after 107 h). The metabolite 4-CP was produced in very low concentrations and was immediately consumed thereafter.

When the nitrification was inhibited with ATU (Fig. 4b), there was a lower accumulation of metabolites detected during the experiment: only a small quantity of AHIBA was formed, which was then quickly consumed. The nitrification inhibition experiment showed that the generated metabolites were removed from the process at a higher rate, or that they were not generated in the first place. This could suggest that some of the metabolites result from partial CLF degradation concomitant to autotrophic activity, or that the nitrite produced when nitrification was uninhibited (Fig. 2) caused inhibition to the metabolite degradation, but not the degradation of the parent compound (CLF).

Fig. 5 shows the simulation of the biodegradation pathway for clofibric acid under aerobic conditions, using the UM-BBD software as described in Section 2.7. Three of the metabolites suggested by the software are in agreement with the results obtained by GC-MS and HPLC-DAD: specifically AHIBA, LA and 4-CP. Two other metabolites proposed, acetone and (Z)-4-chloro-5-oxohex-2-enedioic acid (produced by opening the aromatic ring), are hypothesised to be major compounds in the second step of the biotransformation of CLF. In the present study, acetone was not

detected either in the HPLC-DAD or the GC-MS, perhaps because it is a structure that is very easy to biodegrade. The (Z)-4-chloro-5-oxohex-2-enedioic compound was also not detected.

The experimental results obtained through HPLC-DAD (Fig. 4) show that greater AHIBA and LA accumulation was observed as compared to 4-CP, suggesting that the yield of AHIBA and LA produced per CLF under aerobic conditions is higher than 4-CP. Thus, this work contributed towards understanding the mechanism of CLF biodegradation, where the main degradation pathway for CLF appeared to be the AHIBA-LA pathway.

#### 4. Conclusions

In this study, CLF biodegradation was observed to a greater extent in a SBR fed with CLF and propanil as compared to a SBR fed with CLF as the sole carbon source. Nevertheless, the presence of propanil was confirmed not to be essential towards co-metabolism of the clofibric acid. The heterotrophic populations within the bioreactor were likely more responsible for CLF biodegradation than the autotrophic populations, as concluded after testing without ammonia limitation and with inhibition of nitrification. Combining the information from GC-MS and HPLC-DAD, it was possible to identify and quantify three main metabolites produced in the reactors, including  $\alpha$ -hydroxyisobutyric acid, lactic acid and 4-chlorophenol. The  $\alpha$ -hydroxyisobutyric acid was the first important metabolite produced and accumulated in the reactor, getting transformed into LA over time. The 4-chlorophenol was detected only in small concentrations, due either to fast biodegradation when compared to the other metabolites or due to only a small amount of CLF being channelled through the 4-CP pathway. The results of this study show that under certain conditions, CLF can indeed be biodegradable, and thus may not be refractive to biodegradation in all wastewater treatment plants.

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