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ORIGINAL PAPER

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Metabolites from the biodegradation of pharmaceutical residues of ibuprofen in biofilm reactors and batch experiments

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Abstract The three metabolites hydroxyibuprofen (OH-Ibu), carboxyibuprofen (CA-Ibu), and carboxyhydratropic acid (CA-HA), also known from human metabolism of ibuprofen, could be identified in biodegradation experiments. Identification was based on EI mass spectra and comparison with literature data. Detection was performed by selective MS–MS measurements by GC–ion-trap MS and online methylation. Ibuprofen (Ibu), OH-Ibu, and CA-Ibu could be detected with a signal-to-noise ratio of 10:1 at a concentration of 2 nmol L^{-1}, CA-HA at 0.5 nmol L^{-1}. Degradation experiments in both biofilm reactors (BFR) and batch experiments with activated sludge (BAS) reveal OH-Ibu as the major metabolite under oxic conditions, and CA-HA under anoxic conditions. CA-Ibu was found under oxic and anoxic conditions almost only in the BAS. The metabolites together do not account for more than 10% of the initial concentration of Ibu.

Keywords Biodegradation · Pharmaceutical residues · Ibuprofen · Biofilm reactors · Sewage sludge

Introduction

Ibuprofen (Ibu) is a non-prescription drug used widely as an anti-inflammatory, analgesic, and antipyretic pharmaceutical compound in the treatment of fever and pain. Because of the large amount produced (estimated to be several kilotons per year world-wide and 100 t $year^{-1}$ in Germany) and used (dosage 600–1200 mg day^{-1}) Ibu is an environmentally relevant compound [1]. After application a large proportion of the active compound is excreted by the patient as the parent compound and in form of its metabolites hydroxyibuprofen (OH-Ibu), carboxyibuprofen (CA-Ibu), and carboxyhydratropic acid (CA-HA; Fig. 1) [2, 3]. In excretion from man the parent drug and the metabolites were found to be conjugated to glucuronic acid (so-called phase-II metabolites) [3, 4, 5]. In sewage water, sewage effluents, and surface water Ibu was detected among other pharmaceutical residues in the low μg L^{-1}-range [1, 6, 7, 8]. These findings in sewage and surface water indicate the diffuse sources of Ibu as a result of excretion and incomplete degradation during sewage treatment. Influent concentrations of Ibu are also in good agreement with overall consumption of the pharmaceutical [1]. In particular, OH-Ibu was detected in the influents of sewage treatment plants and in even higher concentrations in the effluents [6]. Starting from these data the question remained whether the elevated OH-Ibu concentrations are because of formation of this compound or because of the cleavage of the glucuronides of OH-Ibu during biodegradation in activated sludge treatment.

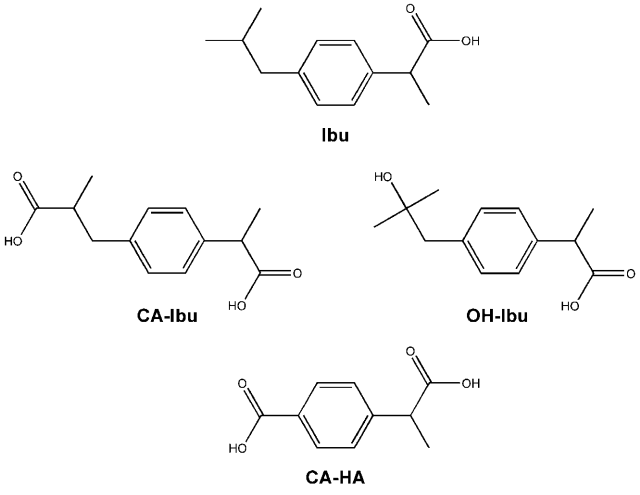

Fig. 1 The structures of Ibu and its metabolites

In this study we report metabolite formation from Ibu during oxic and anoxic biodegradation. Laboratory-scale biofilm reactors (BFR) [9] were employed as model systems for sewage treatment and the results were compared

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with those from batch experiments with activated sludge (BAS). GC with an ion-trap detector was used for selective determination by GC–MS–MS and for identification of the metabolites by GC–MS in full-scan mode.

Experimental

Standard compounds and chemicals

Ibu was purchased from Aldrich (Deisenhofen, Germany). 2,4-Dichlorobenzoic acid and trimethylsulfonium hydroxide (0.25 molar solution in methanol) were from Fluka (Buchs, Switzerland), HCl (30%, p.a.) from Merck (Darmstadt, Germany), methanol and acetone (both HPLC reagent) from Baker (Griesheim, Germany).

Sampling and sample preconcentration

Effluents from the BFR were collected directly in 25- or 50-mL flasks. Synthetic sewage water (SSW) and the BAS were sampled with a pipette from the storage jar or the flasks. Samples from the BAS were centrifuged for 20 min at 2000 g and filtered through a glass fiber filter. All samples were acidified with HCl (1 mol L^{-1}) to pH<2 and cooled immediately. They could be stored at 4 °C in the dark for about 5 days without significant changes in quality.

Extraction and pre-concentration was performed by solid-phase extraction (SPE) with LiChrolut EN cartridges (200 mg adsorbent; Merck). Cartridges were preconditioned with 10 mL acetone, 7.5 mL methanol, and 12 mL acidified water (MilliQ, pH<2). For extraction the acidified samples (25 mL to 50 mL) were sucked through the preconditioned adsorbent in cartridges at a flow rate of 3 mL min^{-1}. After the sample extraction the adsorbent was washed with 3 mL deionized water to remove salts. The wet adsorbent was dried in a stream of nitrogen for at least 30 min. Subsequently the adsorbed compounds were eluted with acetone (2×2 mL). The combined organic extracts were evaporated to dryness with a stream of nitrogen. The residue was dissolved in 0.5 mL acetone containing 2 mg L^{-1} 2,4-dichlorobenzoic acid as internal standard. Recovery of Ibu was 70% from tap-water samples and 72% from SSW (CV=5%, n=8).

Analysis

Methylation of the acidic carboxyl group was performed on-line in the hot injector of the GC by adding 50 µL trimethylsulfonium hydroxide (TMSH) solution (0.25 mol L^{-1} in methanol) to a 0.5 mL sample before injection. Measurements must be taken within one day of addition of TMSH.

Table 1 Operating conditions used for GC and MS measurements

Column:	DB5-MS 30 m×0.25 mm i.d., 0.25 µm film (J&W Scientific)
Injector:	Splitless/split injection (2 min); from 60 °C at 12 ° s^{-1} to 260 °C (15 min isothermal)
Oven:	From 60 °C (1.5 min isothermal) at 20 ° min^{-1} to 120 °C, then at 4 ° min^{-1} to 160 °C, and finally at 16 ° min^{-1} to 260 °C (5 min isothermal)
Carrier gas:	Helium 5.0, linear velocity 40 cm s^{-1}
MS:	Electron-impact ionization at 70 eV, Transfer line at 275 °C, ion source at 200 °C Scan mode: 50–350 amu, 2 scans s^{-1}
MS–MS:	Collision energy set at 1 V, isolation time 8 ms at 1 amu width, excitation time 15 ms Mass transition for IS 2,4-dichlorobenzoic acid (m/z 204→m/z 173); for Ibu and metabolites see Table 2

Analysis was performed by use of a GC–ion-trap mass spectrometer (GCQ, Finnigan MAT) equipped with a programmed temperature vaporization (PTV) injector (Optic 2, Ai Cambridge). The operating conditions used for GC and MS measurements are summarized in Table 1.

Biodegradation experiments

Degradation experiments were performed in miniaturized up-flow BFR (void volume approximately 3.2 mL; approximately 90 mg dry weight of biofilm, equivalent to approximately 28 g dry weight L^{-1}; the biofilm was grown from sludge from a municipal sewage plant). The reactors were fed with synthetic sewage water (SSW) consisting of peptone, meat extract, urea, and salts according to the German Standard DIN 38412, part 24. One reactor was run under oxic conditions (aerated at a flow rate of 0.25 mL min^{-1}), the other under anoxic conditions (denitrification conditions, i.e. the absence of oxygen and presence of nitrate). The synthetic sewage water, DOC approximately 10 mg L^{-1}, containing Ibu at concentrations between 0.5 and 2.4 µmol L^{-1} was continuously supplied at a flow rate of 1 mL min^{-1}. Elimination of DOC by the reactors was in the range 80 to 90%; it was monitored by measuring the DOC in samples from the in- and outflow of the reactors.

BAS experiments were performed with freshly sampled sewage sludge from a municipal sewage treatment plant in stirred 4-L flasks (4 g L^{-1} dry weight of sewage sludge). The oxic experiment was aerated at a flow rate of 390 mL min^{-1} to maintain an oxygen concentration in the sludge suspension of 6 mg L^{-1}. The flask of the anoxic experiment was stoppered to maintain the concentration of oxygen in the suspension below 0.5 mg L^{-1}. The sewage sludge was fed every day in two batches with synthetic sewage water, resulting in 150 mg L^{-1} DOC. Ibu was added at the start of the experiment, at a concentration of 0.86 µmol L^{-1}.

Results and discussion

Identification of metabolites

The identification of the major metabolites of Ibu, OH-Ibu, CA-Ibu, and CA-HA (the structures are given in Fig. 1) was based on the EI mass spectra of their methyl esters and comparison with literature data [1, 6]. Mass spectra similar to those described here have been reported elsewhere [1, 6]. The isomers of OH-Ibu, 1-OH-Ibu, 2-OH-Ibu, and 3-OH-Ibu could not be differentiated unequivocally by use of their mass spectra alone. The most likely metabolite described is, however, 2-OH-Ibu (structure shown in Fig. 1). Fragmentation of the molecular ion of Ibu methyl (M^+ at m/z 220) gives the major fragment ions from β-cleavage at m/z 177 (presumably $M^+–C_3H_7$) and at m/z 161 ($M^+–COOCH_3$). The fragments at m/z 91, m/z 105, and m/z 119 indicate the structure of an alkyl-substituted aromatic system. The mass spectrum of OH-Ibu methyl contains a very weak $[M+H]^+$ peak at m/z 237, because of attachment of H^+ to the OH-group; this is typical of ion-trap mass spectra. Further fragments from β-cleavage are found at m/z 178 ($M^+–COOCH_3$), m/z 118, and m/z 119 (m/z 178–C_3H_7O). Further fragments occur at m/z 161 and m/z 91. In the mass spectrum of the dimethylated CA-Ibu the M^+ ion is found at m/z 264. β-Cleavage gives the ions m/z 205 ($M^+–COOCH_3$) and m/z 177 ($M^+–CH_3CHCOOCH_3$). Further ions are found at m/z 233 ($M^+–OCH_3$; typical of dimethylated diacids) and at m/z 145 (m/z 264–$C_4H_7O_4$). For the dimethylated

Table 2 Precursor and product ions used for MS–MS detection in SRM mode for Ibu and its metabolites

Compound	Precursor ion (m/z)	Product ions (m/z) and (relative abundance)		
Ibuprofen methyl	220	161 (100)	177 (2)	
Hydroxyibuprofen methyl	178	119 (100)	159 (20)	
Carboxyibuprofen dimethyl	264	205 (100)	177 (25)	145 (60)
Carboxyhydratropic acid dimethyl	222	163 (100)	191 (4)	131 (8)

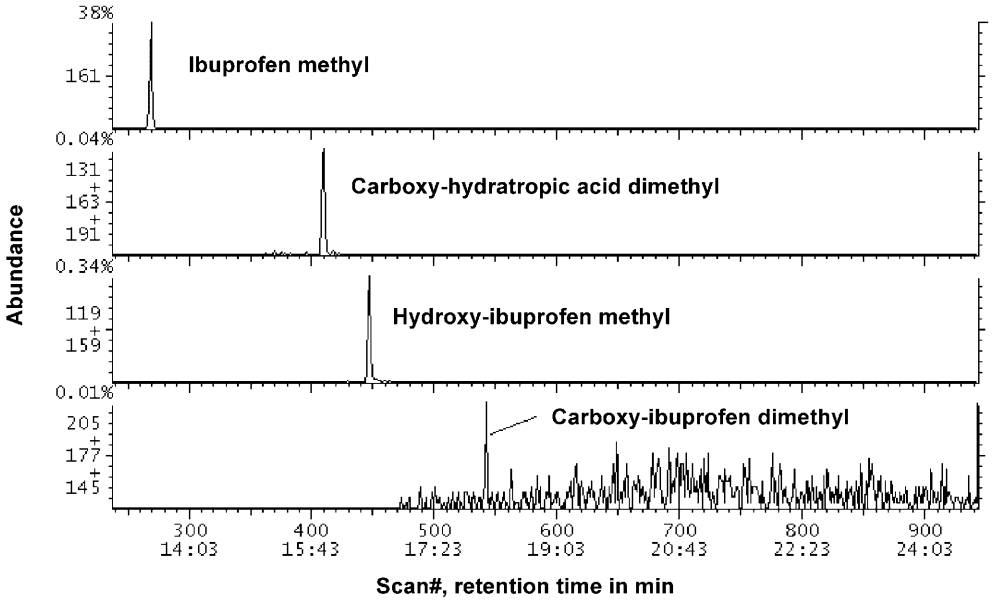

Fig. 2 SRM MS–MS chromatogram of a sample from the biodegradation of Ibu. Specific product ions of the metabolites are summed (cf. Table 2)

CA-HA the M^+ ion is found at m/z 222. Only one intense β-cleavage fragment is found at m/z 163; the ion obtained from M^+–OCH_3 is found at m/z 191.

Separation

Identification of Ibu and its metabolites in the samples was achieved by MS–MS detection, by selected reaction monitoring (SRM) of the mass transitions precursor ion→product ion. With the exception of OH-Ibu (precursor ion at m/z 178) the precursor ions were the molecular ions. The major product ions were the fragments formed by β-cleavage. One or two additional product ions were recorded to confirm peak identity (Table 2). To improve the signal intensity of the SRM traces of a sample from biodegradation of Ibu the specific product ions were summed in the SRM chromatogram (Fig. 2). The chromatogram shows the order of elution, and specific SRM detection without interferences from the biodegradation.

Quantification

Because authentic standards of the metabolites were not available the quantification was based on the SRM response of Ibu for OH-Ibu (regression equation based on peak heights relative to the IS: y=0.171x–0.016) and on the SRM response of terephthalic acid for the diacids CA-Ibu and CA-HA (regression equation based on peak heights relative to the IS: y=0.0403x–0.0017). The response of the diacids compared with Ibu is mainly dependent on the derivatization yield of the online-methylation. The different ionization cross-sections in the ion source of the MS and the different fragmentation yields were neglected. The resulting concentration values may deviate from true values, but they are sufficient for estimation of the ratio of biodegraded Ibu to the occurring metabolites. Ibu, OH-Ibu, and CA-Ibu could be detected with a signal-to-noise ratio of 10:1 at a concentration of 2 nmol L^{-1}, CA-HA at 0.5 nmol L^{-1} (with a preconcentration factor of 100).

Biodegradation in batch experiments

Batch experiments with activated sludge from a municipal sewage treatment plant were performed with (oxic) and without aeration for 51 h. After a lag phase of approximately 1 day approximately 75% of the initial Ibu was de-

Table 3 Degradation of Ibu in BAS under oxic and anoxic conditions

	Concentration of Ibu (μmol L^{-1})					
Time (h)	0	4	24	26	47	49
Oxic	0.86	0.6	0.22	0.23	0.11	0.09
Anoxic	0.86	0.68	0.66	0.63	0.57	0.59

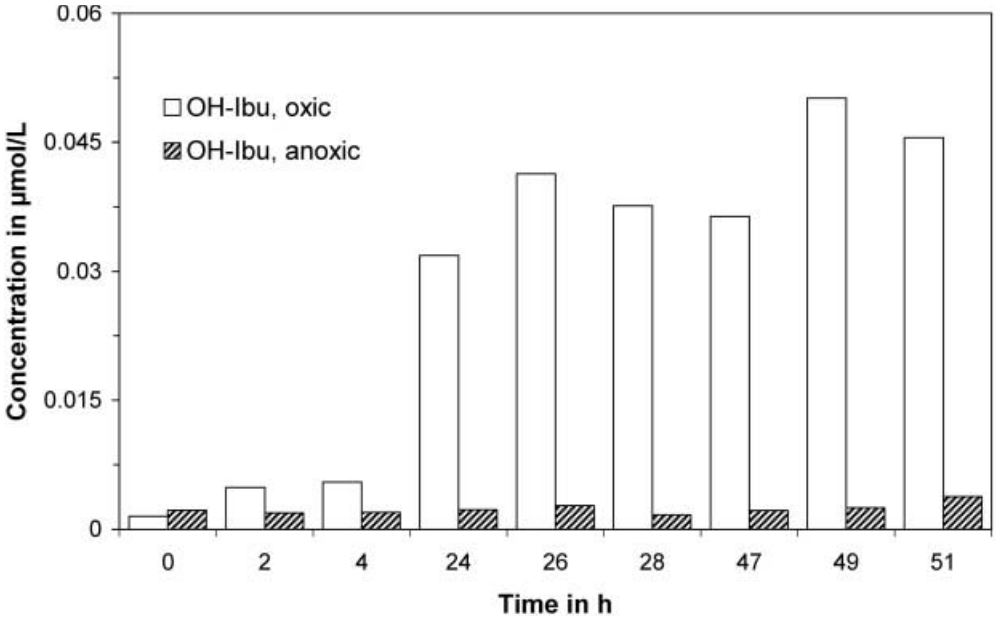

Fig. 3 Level of OH-Ibu during biodegradation of Ibu in BAS (C_0(Ibu)=0.86 µmol L^{-1})

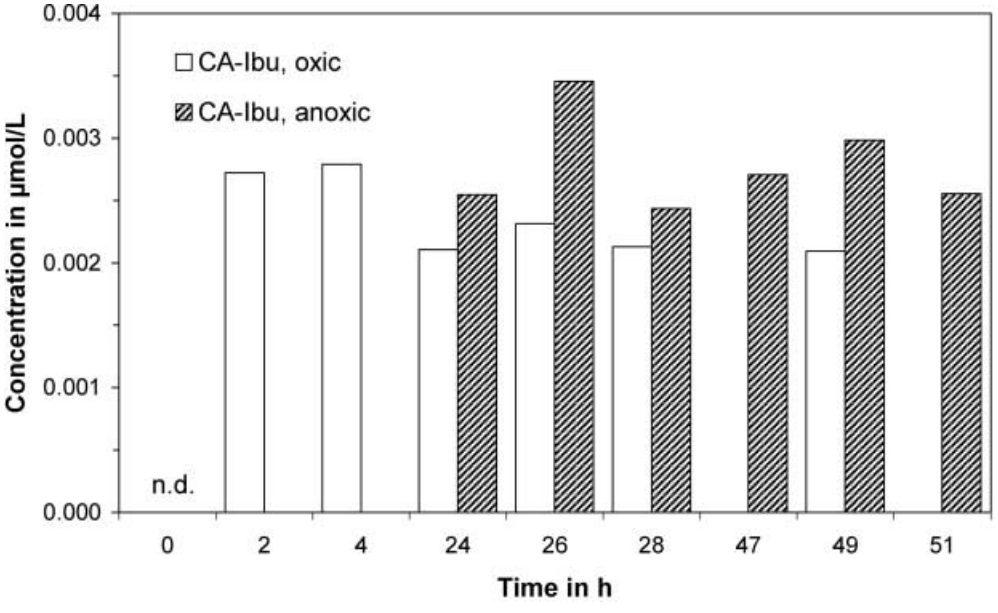

Fig. 4 Level of CA-Ibu during biodegradation of Ibu in BAS (C_0(Ibu)=0.86 µmol L^{-1})

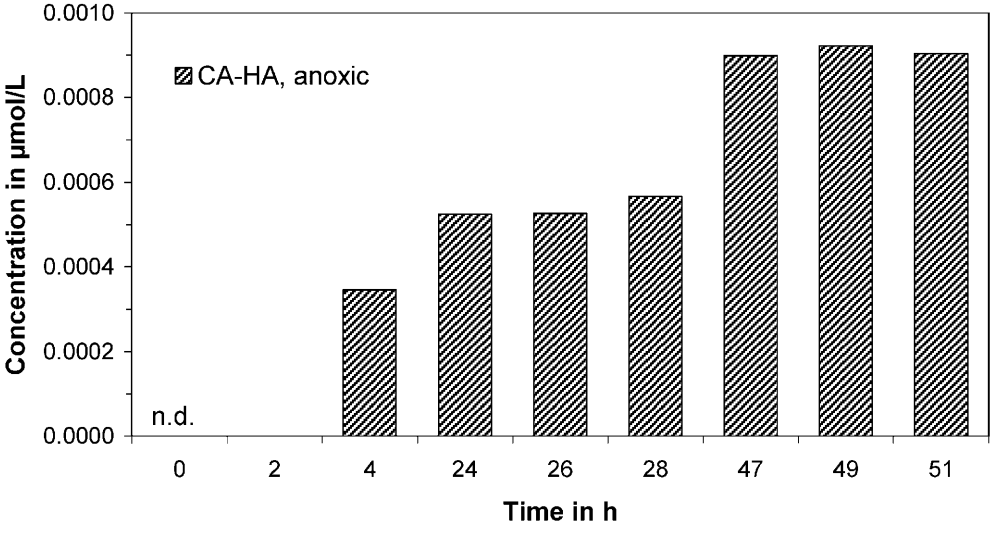

Fig. 5 Level of CA-HA during biodegradation of Ibu in BAS (C_0(Ibu)=0.86 µmol L^{-1})

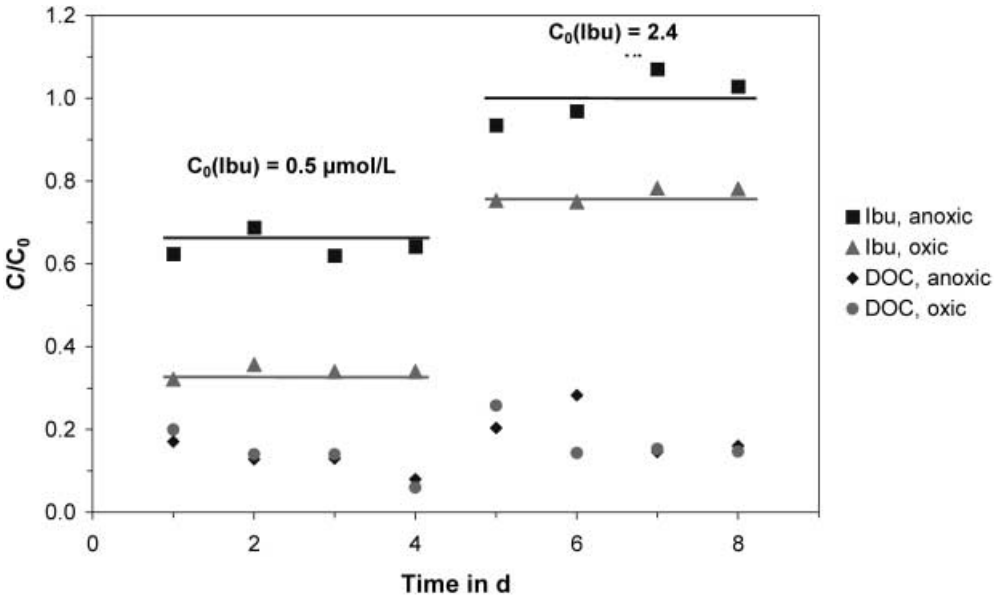

Fig. 6 Degradation of Ibu and DOC in BFR for two different initial concentrations of Ibu

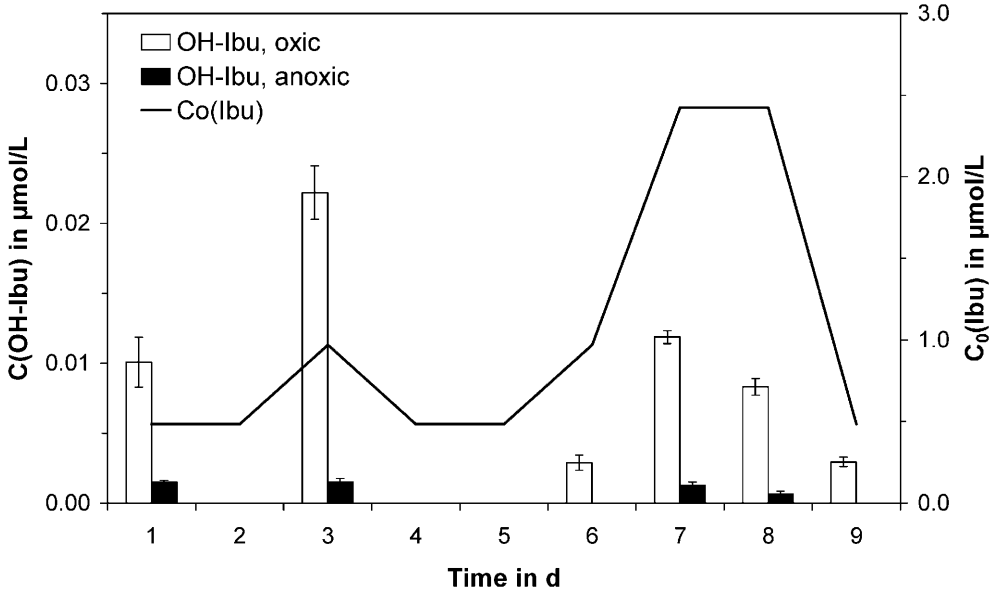

Fig. 7 Level of OH-Ibu during biodegradation of different concentrations of Ibu in BFR. (Error bars 1 s, n=4)

graded in the oxic experiment; in the anoxic experiment it was only approximately 22% (Table 3). Adsorption of Ibu on activated sludge was checked by extraction and found to be less than 3%. OH-Ibu was found as the major metabolite in the oxic batch experiment (Fig. 3). The concentration level after 0 h is the blank value of the freshly sampled sewage water. A maximum of approximately 7% of the biodegraded Ibu could be identified as OH-Ibu. In the anoxic experiment almost no OH-Ibu was formed. CA-Ibu and CA-HA were found to a much lesser extent near the quantification limit of the method. CA-Ibu was found at very low concentrations near the detection limit of 2 nmol L^{-1} at 0.4% maximum of degraded Ibu in the oxic and at 1.8% in the anoxic batch experiment (Fig. 4). In the anoxic experiment CA-Ibu seems to occur first after a lag phase of 24 h, although at concentration levels near the detection limit. In the oxic batch CA-Ibu appeared even after 2 h and could not be detected after 51 h.

CA-HA was detected in the oxic batch only below the quantification limit (<0.1% of degraded Ibu). In the anoxic experiment CA-HA occurred first at 4 h, and the amount increased with time, but accounted for only 0.3% (maximum) of degraded Ibu (Fig. 5).

Biodegradation in biofilm reactors

BFR have already proven to be reliable model systems for investigation of the biological degradation of pharmaceutical residues on the laboratory scale [9]. Major degradation of Ibu occurred in the oxic BFR. To obtain sufficient concentrations of metabolites the feed of the BFR was fortified with Ibu at concentrations between 0.5 µmol L^{-1}

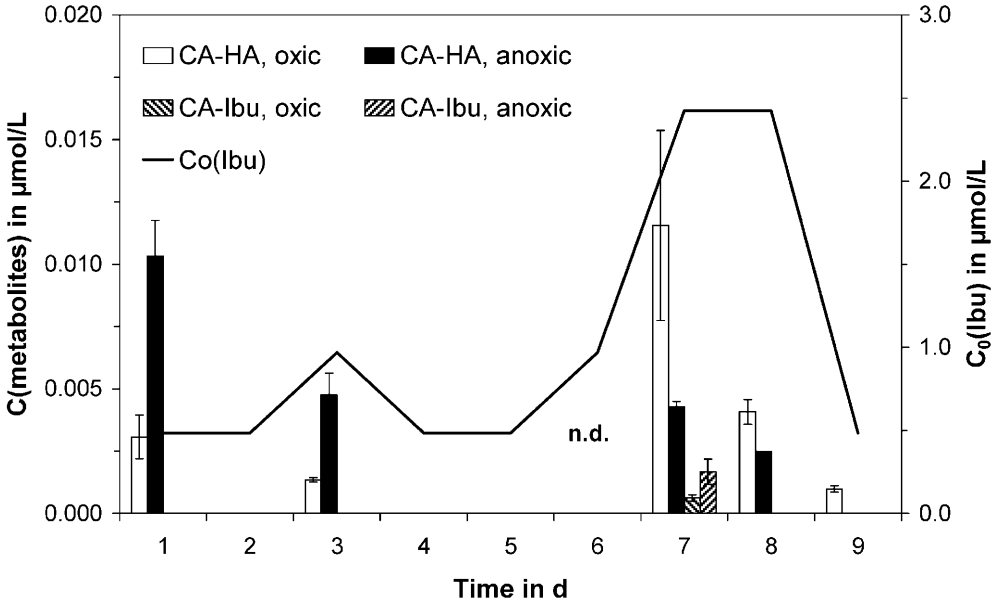

Fig. 8 Levels of CA-HA and CA-Ibu during biodegradation of different concentrations of Ibu in BFR. (Error bars 1 s, n=4)

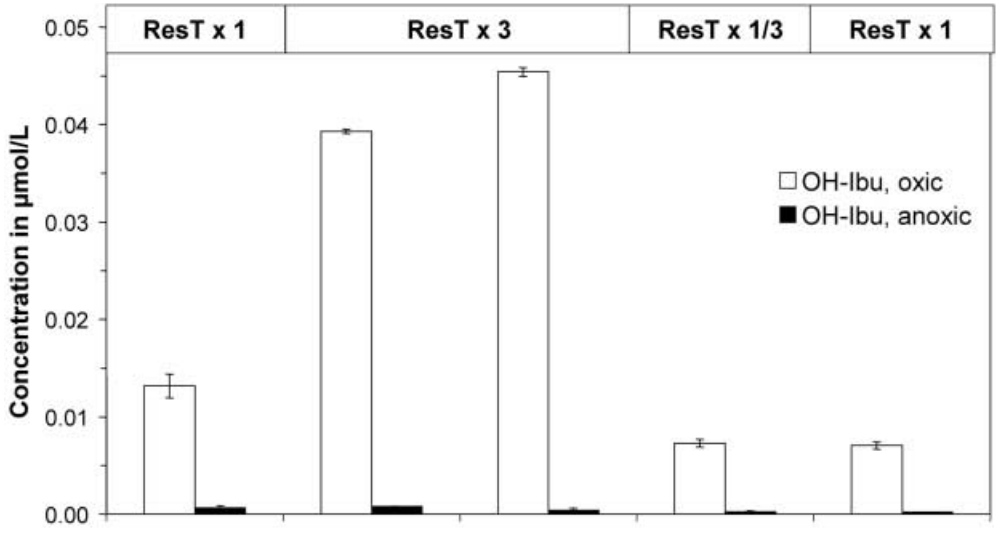

Fig. 9 Levels of OH-Ibu during biodegradation of Ibu in BFR with different residence times. ResT is the hydraulic residence time (Error bars 1 s, n=3)

and 2.4 µmol L^{-1}. The efficiency of elimination of Ibu from the oxic reactor, however, dropped from 68% removal at the initial Ibu concentration of 0.5 µmol L^{-1} to approximately 27% at 2.4 µmol L^{-1}; under anoxic conditions it even dropped from approximately 35% to almost zero. This means that the elimination capacity of the BFR was reached under the operating conditions applied and further increasing the initial concentration is not efficient. The efficiency of removal of the easy degradable DOC from synthetic sewage water was not influenced by the pharmaceutical residues (Fig. 6). Similar to the BAS, OH-Ibu again occurred preferentially in the oxic BFR at levels of 2–3% of degraded Ibu, a value little influenced by the initial Ibu concentration (Fig. 7). CA-Ibu, however, was barely detected in both the oxic and anoxic BFR, whereas CA-HA was found in the oxic and the anoxic BFR (Fig. 8). When initial concentrations of Ibu were lower (0.5 µmol L^{-1}) more CA-HA was formed in the anoxic BFR (maximum 5% of degraded Ibu) compared with the situation of overloading the BFR with Ibu (2.4 µmol L^{-1}).

The efficiency of degradation of the BFR depends on the mean hydraulic residence time (ResT) of a fluid element in the reactor. Increased residence time resulted in increased Ibu degradation, decreased residence time in decreased Ibu degradation. At the same time more OH-Ibu is formed in the oxic BFR when the residence time is increased, and vice versa (Fig. 9). This reveals that OH-Ibu is a metabolite of Ibu, and degrades much more slowly than Ibu under the conditions used.

Conclusions

In conclusion, the data reveal OH-Ibu to be the major metabolite of oxic degradation of Ibu and CA-HA to be the major metabolite of anoxic degradation. CA-Ibu oc-

curred under oxic and anoxic conditions. No more than 10% of the degraded Ibu can, however, be explained by the metabolites investigated. On the basis of our data, as a rule of thumb, during sewage treatment metabolites are expected to occur as degradation products at a level accounting for approximately 10% of Ibu input. This estimate might aid discovery of whether metabolites in sewage effluents originate from biodegradation during sewage treatment or from cleavage of glucuronides from human metabolites already introduced by the sewage water, if the Ibu concentrations in the inflow and the effluent are known.

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