The fate of ^{14}C-radiolabelled diclofenac and 4′-hydroxydiclofenac in membrane bioreactor treatment of wastewater

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ABSTRACT

This study aimed at enhancing knowledge on the fate of diclofenac (DF), together with its main human metabolite 4′-hydroxydiclofenac (4′OHDF), during wastewater treatment by using a laboratory-scale membrane bioreactor (MBR). The reactor was fed continuously with non-radiolabelled diclofenac for a one month period prior to a single pulse of a ^{14}C-radiolabelled solution of DF and 4′OHDF. The solution spike contained approximately 25% 4′OHDF and 65% DF, which corresponds to the ratio observed in municipal sewage, as well as traces of two other metabolites. The radioactivity was monitored for a total of twelve days in the various output streams. The calculation of the complete mass balance in the system demonstrated that the major part of the radioactivity left the reactor with the permeate (88.7%), while 2.1% was recovered in the excess sludge. Negligible amounts were recovered in the off-gas traps and on the membranes. Chromatographic analyses of effluent samples, by means of HPLC-MS coupled in parallel to a radiodetector, displayed a different pattern than the one of the spiked solution. It showed the occurrence of three additional metabolites.

Key words | diclofenac, fate study, membrane bioreactor, wastewater

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INTRODUCTION

The last decades have seen a growing concern about organic micropollutant removal from wastewater. Indeed, increasing knowledge on the negative effects of micropollutants on ecosystems has emphasized the absolute need to tackle this problem.

Diclofenac (2-(2-(2,6-dichlorophenylamino)phenyl)acetic acid) is one of the most widely prescribed non steroidal anti-inflammatory drugs (Gonzalez *et al*. 2006). The reported European consumption rates can reach up to 100 tons per year (Zwiener & Frimmel 2003). In fact, 6.2% of the oral dose of DF is excreted in human urine subject to normal renal function, while 16% is found as 4′-hydroxydiclofenac, 6.1% as 5-hydroxydiclofenac, 2% as 3′-hydroxydiclofenac and 0.009% as 3′-hydroxy-4′-methoxy diclofenac (Sawchuk *et al*. 1995). The occurrence of hydroxylated metabolites of DF in raw wastewater and treated effluents was reported recently (Perez & Barcelo 2008; Stülten *et al*. 2008; Scheurell *et al*. 2009).

DF is only partially removed from raw wastewater. Various studies reported strongly diverging results with removal rates ranging from 0% (Clara *et al*. 2004; De Wever *et al*. 2007) to 69% (Ternes 1998) with the highest distribution between 21 and 40% (Zhang *et al*. 2008). Higher loads and concentrations in the treated effluent with respect to the influent were also observed (Roberts & Thomas 2006; Zorita *et al*. 2009). The latter papers reported the possible deconjugation of glucuronidated or sulfated DF conjugates and/or desorption from sludge particles, which would explain the detected increased load of DF in the final effluent. No direct correlation between sludge retention time (SRT) and removal rate could be clearly established (Strenn *et al*. 2004). The same authors reported the impossibility to determine an explicit behaviour. In fact, while some scientists have observed a really slow degradation of DF (Urase & Kikuta 2005), others did not evidence any microbial degradation in batch experiments (Quintana *et al*. 2005; Gonzalez *et al*. 2006; Joss *et al*. 2006). Nevertheless, attention has to be paid to the difficulties encountered with the analysis of trace contaminants in complex matrixes,

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such as wastewater. As highlighted previously, the disappearance of a substance does not prove its degradation (Quintana *et al.* 2005). To circumvent these issues, the use of ^{14}C-radiolabelled substances has demonstrated its great potential to detect biodegradation products, as well as to give the possibility to calculate an accurate mass balance of trace compounds in various wastewater treatment systems (Ivashechkin *et al.* 2005; Cirja *et al.* 2006, 2007; Junker *et al.* 2006).

Regrettably, the fate of metabolites is often neglected and there is a knowledge gap concerning the fate of DF and metabolites thereof in WWTP, although the assessment of their fate is necessary to understand degradation and interconversion mechanisms during wastewater treatment.

The present study was aimed at the determination of DF and 4′OHDF removal pathways, especially to distinguish between biodegradation, adsorption and volatilization in a laboratory-scale MBR. In order to circumvent analytical difficulties in the complex wastewater matrix the ^{14}C- radiolabelled forms of both compounds were applied to the wastewater treatment system.

MATERIAL AND METHODS

Reactor design

This study was carried out in a 1.5 L glass reactor, hermetically sealed and developed for experiments under radioactive conditions as described elsewhere (Cirja *et al.* 2006) (Figure 1). The filtration unit consisted of 6 polyvinylidene fluoride (PVDF) flat sheet membranes (Microdyn-Nadir GmbH, Germany) of 40 cm^2 each (4 × 10 cm), with a nominal pore size of 0.20 μm. Filtration was operated in sequence of 5 minutes filtration at a rate of 2.9 mL/minute and 1 minute backwashing at a rate of 4 mL/minute.

To consider the radioactivity contained in the gas phase in the radioactivity balance, off-gas was washed in three successive traps containing respectively 500 mL of monoethylene glycol for Volatile Organic Compounds (VOC) trapping followed by two alkaline traps containing each 500 mL of 5 mol/L sodium hydroxide for CO_2 trapping.

The reactor was operated under continuous aeration using a porous diffuser made of stainless steel, and mixing was performed by a magnetic stirrer.

It was inoculated with 1.1 L of sludge sampled from the Sequencing Batch Reactor of ARA Birsfelden WWTP, Switzerland. After a stabilization phase of twice the SRT, the reactor was operated under constant conditions at a SRT of 28 days and a HRT of 8 hours. The total solids concentration was 10 g/L on average.

In order to operate the reactor under strictly controlled conditions, the MBR was fed by means of a sterile synthetic medium, which was enriched with 800 ng/L of non-radiolabelled diclofenac sodium (GE Healthcare, Amersham) for one month prior to the ^{14}C-radiolabelled substances, single spike. The composition of the synthetic feed was adapted from DIN ISO 11733 and had the following average composition: 772 ± 22 mg O_2/L of Chemical Oxygen Demand (COD), 41 ± 2 mg TN/L of Total Nitrogen (TN),

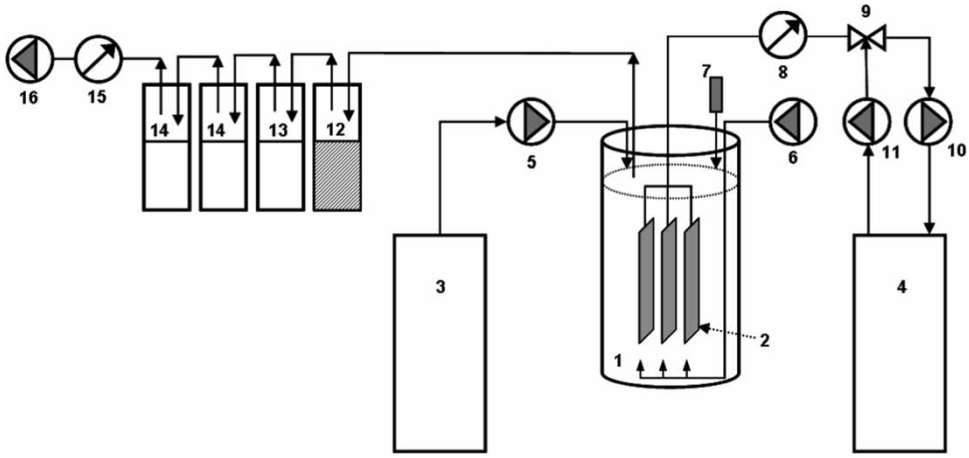

Figure 1 | Scheme of the laboratory scale membrane bioreactor. 1 – Bioreactor vessel; 2 – Membrane plate modules; 3 – Influent tank; 4 – Effluent tank; 5 – Influent peristaltic pump; 6 – Air pump; 7 – Level controller; 8 – Digital pressure control; 9 – Controlling valve; 10 – Effluent peristaltic pump; 11 – Backwashing peristaltic pump; 12 – Reactor overflow; 13 – Mono ethylene glycol flask for VOC trapping; 14 – NaOH flasks for CO_2 trapping, 15 – Digital pressure control; 16 – Vacuum pump.

and 8.5 ± 0.6 mg TP/L of Total Phosphorus (TP). The MBR achieved good COD removal (97.8 ± 0.7%), and TN removal (42.0 ± 10.3%) comparable to that observed during long term aerobic MBR operation (Holakoo *et al.* 2007).

Radiolabelled compounds

Radiolabelled[phenyl acetic acid ring- U- ^{14}C] diclofenac sodium was purchased from GE Healthcare, UK, and radiolabelled 4′OHDF was synthesized by incubating ^{14}C-diclofenac sodium with CYP2C9 pool from human liver containing the NADPH depending P450 enzyme (S9 from human liver, Sigma Aldrich) over 24 hours at 37 °C. The phenyl acetic acid ring- U- ^{14}C-labelling chosen is noteworthy as it enables the radiodetection of all initial degradation products reported so far in the literature.

The reaction mixture was then analysed and characterized by means of HPLC–DAD–MS coupled to a radiodetector. The mass spectrum of the biologically-produced 4′OHDF matched the one reported by Stülten *et al.* (2008). In addition, the incubation mixture contained also traces of two other metabolites. Due to their low amount and the limited sensitivity of the MS measurement, their identification was not possible.

After a stabilization period of twice the SRT, the solution of ^{14}C-labelled DF and 4′OHDF was spiked in the reactor as a single pulse. The solution was composed of 108.4 μg DF and 41.4 μg 4′OHDF dissolved in methanol (Ultra gradient HPLC grade, J.T. Backer) for a total radioactivity amounting to 1.19 MBq. This corresponded to a mixture composition consisting of 24.6% of 4′OHDF and 64.3% of DF with respect to all the detected substances, which was similar to that reported in domestic wastewaters by Stülten and collaborators (Stülten *et al.* 2008).

Sampling and analytical methods

Permeate sampling and sample preparation

Permeate samples were continuously collected by using a fraction collector (SuperFrac, Pharmacia Biotech). Sampling was performed over periods ranging from 30 to 150 minutes in order to collect sufficient amounts of radioactivity in each sample.

For HPLC analyses, permeate samples were extracted using Chromabond (C18, 1,000 mg, Macherey and Nagel) SPE cartridges, pre-conditioned with 2 × 5 mL methanol and 2 × 5 mL Millipore water at pH 6.3. Each sample contained 1 mol/L citric acid solution to adjust the pH to 6.3 to enable a good reproducibility. Percolation was performed at a constant rate of 10 mL/minute. The cartridges were then washed with 2 × 5 mL Milllipore water (pH 6.3) and dried under vacuum for 15 minutes. Finally, extracts were eluted with 3 × 2 mL methanol and reduced under a nitrogen-flow. The average ($n = 3$) recovery rate for the total radioactivity was of 91.8%.

Liquid scintillation counting (LSC) analyses

Each sample was analysed by means of LSC (Perkin Elmer 2800TR Tri Carb Liquid scintillation analyzer). A 0.1 to 10 mL aliquot of the samples was taken and placed into a 20 mL polypropylene LSC vial. A scintillation cocktail (Lumasafe plus, PerkinElmer Life & Analytical Sciences B.V.) was added up to a total volume of 20 mL. Samples were then placed in the LSC analyzer where the total radioactivity was measured.

HPLC analyses

HPLC analyses were carried out using Agilent Tech. HPLC 1200 Series coupled to a radioisotope detector 'Ramona Star' (Raytest, Germany) and an Agilent Tech. 6320 Ion Trap mass spectrometer. A Nucleodur C18 Pyramid column (150 × 4 mm, 3 μm particle size, Macherey and Nagel) was thermostated at 40 °C and the flow-rate was set at 1 mL/minute. The mobile phase consisted of nanopure water containing 0.1% of formic acid (Solvent A) and acetonitrile (Ultra gradient HPLC grade, J.T Backer) containing 0.1% of formic acid (Solvent B). The separation gradient was applied as follows: linear gradient from 2 to 40% B for 5 minutes, then a linear gradient from 40 to 67% B for 15 minutes and finally a linear gradient from 67 to 98% B for 5 minutes. The system was then maintained at initial conditions for 5 minutes.

Biomass extraction

MLSS samples were extracted daily according to the following protocol in order to determine the distribution of the total radioactivity in the different fractions. The total radioactivity was measured, together with pH. First, the bioavailable fraction was measured, it corresponded to the radioactivity recovered in the aqueous phase, which consisted of the supernatant collected after centrifugation at 2,500 g for 15 minutes, and that collected after rinsing the pellet with 5 mL distilled water and centrifugation at 4,500 g for 5 minutes. Then, the radioactivity associated to

solids in suspension (extractable) was extracted by a sequence of four washing steps using 5 mL of a sodium hydroxide solution at 0.5% volume and centrifugation at 4,500 g for 5 minutes. Finally, the solid phase, i.e. pellets recovered after the extraction steps (non-extractable fraction), was combusted in a 307 PerkinElmer Sample Oxidizer. Samples were placed on micro-crystalline cellulose contained in the combusto-cone and then combusted for 2.5 minutes. During the combustion cycle, ^{14}C was oxidized to $^{14}CO_2$, and the latter was subsequently trapped in carbon dioxide absorbent (Carbo-Sorb® E) and later mixed with the scintillation cocktail (Permafluor E+). The ratio absorbent/scintillation cocktail was equal to 5 mL/15 mL.

RESULTS AND DISCUSSION

Radioactivity monitoring

The radioactivity was monitored for 12 days in the permeate, the MLSS, and the off-gas (Figure 2). It was observed that most of the radioactivity left the reactor with the permeate. Forty-three hours after the pulse, the cumulative percentage of total applied radioactivity collected in the permeate reached 80%. These results were similar to those obtained during a previous study without any acclimation to DF, where 87% of the total applied radioactivity was collected in the permeate after 48 hours (Bouju *et al*. 2009).

The rapid release of radioactivity from the system indicated a poor tendency of DF and metabolites thereof to adsorb to the biomass. The quantity of radioactivity associated to the 40 mL excess sludge removed daily from the reactor confirmed this tendency. Indeed, excess sludge contributed to the removal of only 2.1% of the total applied radioactivity. After twelve days, a total of 88.7% of the applied radioactivity was recovered in the permeate. Finally, the negligible percentage of radioactivity recovered in the off-gas, 0.12%, highlights the absence of remarkable mineralization. Moreover, DF and its metabolite did not exhibit any tendency to adsorb to the membranes, as only 0.03% of the total applied radioactivity was recovered on them.

Radioactivity was almost immediately detected in the permeate as 0.04 MBq were recovered in the first half-an-hour sample. In the first two days, radioactivity concentration dropped dramatically in the effluent, from 530 to 88 Bq/mL within 24 hours, down to 9 Bq/mL after 48 hours.

HPLC analyses

Permeate sample extracts were analysed by means of HPLC–LSC–MS. The spiked solution contained four substances (Figure 3(a)). The first one with a retention time (RT) in the HPLC–LSC column of 10.9 min was identified

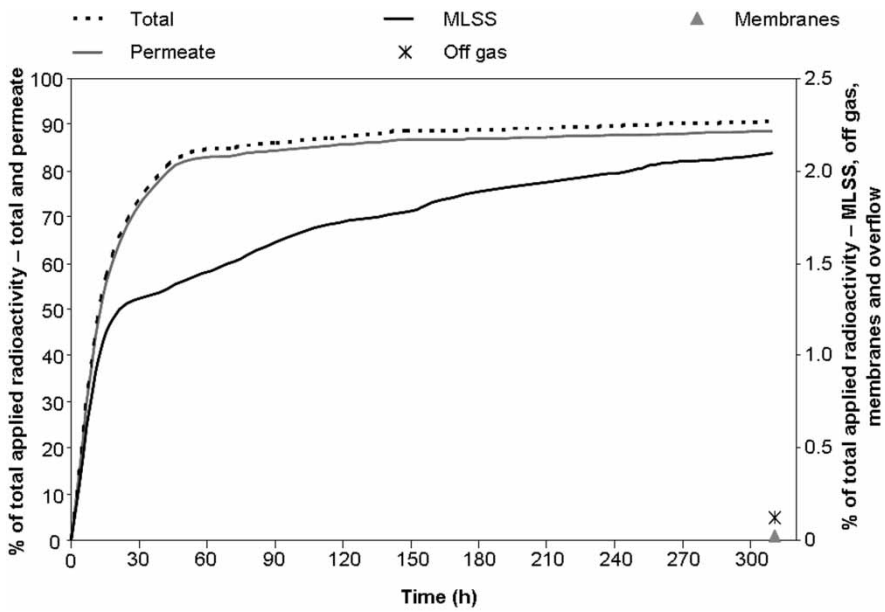

Figure 2 | Cumulative radioactivity collected in the overall system and the permeate (left axis), the excess sludge, the off-gas, and the membranes (right axis). Values are expressed relatively to the initially applied radioactivity.

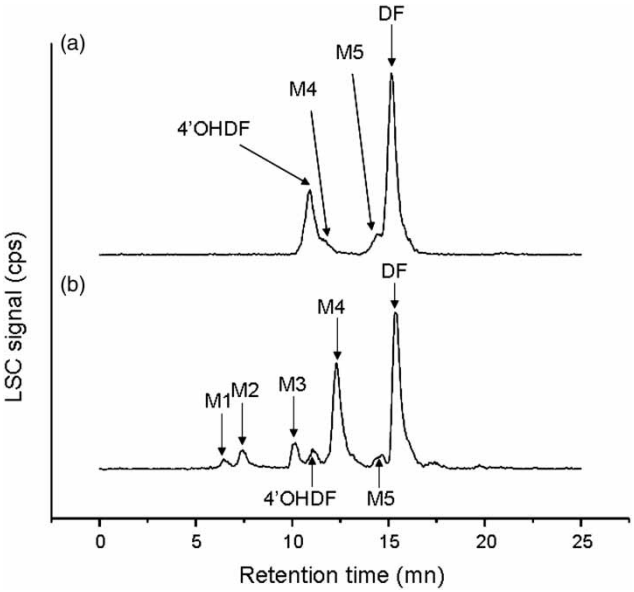

Figure 3 | Chromatograms of the spiked solution (a) and the permeate sample after 12 hours (b).

as 4′OHDF, two unidentified traces at RT 11.9 min (M4) and 14.5 min (M5), and finally, DF at 15.2 min. These four substances were all detected in the first permeate samples.

Twelve hours after spiking (1.5 HRT), three more polar substances containing ^{14}C-radiolabelled atoms were detected at 6.1 minutes (M1), 7.0 minutes (M2) and 9.9 minutes (M3) RT (Figure 3(b)).

After 48 hours, the total radioactivity level was too low to accurately quantify the substances; however, DF was still detected. Nevertheless, the concentration course in the permeate highlights a fast decrease of DF and 4′OHDF concentrations. The first eight hours (equivalent to 1 HRT) exhibited inconsistent results probably due to a short cut inside the reactor. Nevertheless, after this period, the concentration course in the permeate highlights a relatively fast decrease of DF and 4′OHDF concentrations. Indeed, in 7.9 hours, 4′OHDF concentration decreased from 44 to 6 nmol/L (13.6 to 1.9 μg/L), while DF concentration dropped from 81 to 32 nmol/L (24.0 to 9.7 μg/L). Figure 4 represents the molar concentration profile of the various detected substances.

The faster drop of 4′OHDF concentration, with respect to that of DF led to the hypothesis that the increased concentrations of the five unidentified metabolites are likely to be the results of 4′OHDF degradation, rather than biological transformation of DF. However, the results do not allow the validation of this hypothesis and the elucidation of the fate of these five detected metabolites. This would require applying a spike of radiolabelled DF together with non-radiolabelled 4′OHDF.

MLSS analyses

Besides permeate analyses, the distribution of radioactivity in MLSS samples was also assessed. Radioactivity was measured in the aqueous phase (bioavailable fraction), in the extractable fraction, as well as the non-extractable fraction remaining with the pellet (Figure 5).

In the first two samples, the bioavailable fraction remained constant and represented approximately 45% of

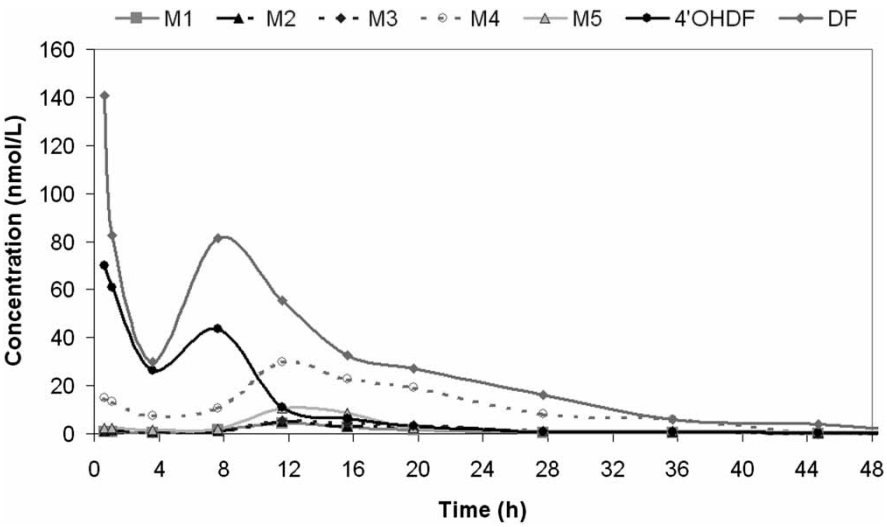

Figure 4 | Diclofenac, 4′-hydroxydiclofenac and detected metabolites concentration profile in the permeate.

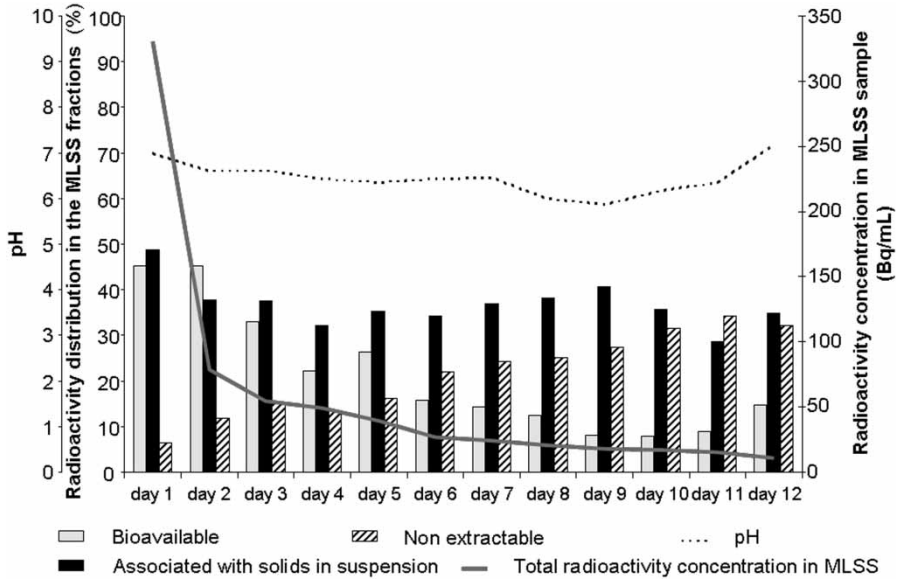

Figure 5 | Radioactivity distribution in the MLSS fractions, expressed as a percentage of radioactivity measured in the samples before extraction (left axis) and pH (left axis), radioactivity concentration in the MLSS sample (right axis).

the radioactivity associated to the MLSS. Then it progressively decreased down to 8%. In the mean time, the fraction accounting for the non-extractable fraction increased slowly up to 32%, while the percentage of radioactivity associated with the solids in suspension seemed to remain more or less constant after a slight decrease on the second day.

These results showed that the radioactivity sorbed to the biomass (non-extractable fraction) originates apparently from the bioavailable fraction. Nevertheless, it is not clear whether the radioactivity associated to the solids comes from residues strongly bound, i.e. covalently linked parent compound and reactive degradation intermediates thereof, or assimilated by the microorganisms or from the original compounds sequestered in the total suspended solids (TSS) (Li *et al*. 2007). A study with ^{13}C-radiolabelled substances would allow the elucidation of such bonds.

The same behaviour was observed without acclimation of the biomass, confirming the poor tendency of diclofenac and its metabolite to adsorb to the biomass (Buser *et al*. 1998; Bernhard *et al*. 2006).

CONCLUSIONS

This study highlighted the poor biodegradability of diclofenac and 4′-hydroxydiclofenac in the activated sludge processes. As a consequence, no remarkable mineralization was observed. In addition, the three additional metabolites are likely to originate from 4′OHDF, rather than DF. Moreover, though the logK_{ow} of both substances are higher than 2, they did not exhibit a strong tendency to adsorb to the biomass.

With metabolites detection and characterization being rather complicated at environmentally relevant concentrations, the use of ^{14}C-radiolabelled substance is of great help. Indeed, not less than three additional metabolites were detected in permeate samples, however, a higher resolution mass spectrometry device is required for identification of newly formed metabolites.

In order to confirm these results obtained with a single pulse following a one month acclimation phase, experiments with continuous feeding of ^{14}C-radiolabelled DF and non-radiolabelled 4′OHDF would be of interest. This could help in better appraising the reactions of interconversion of DF and 4′OHDF into further metabolites. Understanding this complex metabolic pattern requires making a distinction between human produced metabolites and microbial degradation products.

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