



Transformation of diclofenac in hybrid biofilm–activated sludge processes



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ABSTRACT

The biotransformation of diclofenac during wastewater treatment was investigated. Attached growth biomass from a carrier-filled compartment of a hybrid-MBBR at the wastewater treatment plant (WWTP) in Bad Ragaz, Switzerland was used to test the biotransformation. Laboratory-scale incubation experiments were performed with diclofenac and carriers and high-resolution LC–QToF-MS was implemented to monitor the biotransformation. Up to 20 diclofenac transformation products (TPs) were detected. Tentative structures were proposed for 16 of the TPs after characterization by MS² fragmentation and/or inferring the structure from the transformation pathway and the molecular formula given by the high resolution ionic mass. The remaining four TPs were unambiguously identified via analytical reference standards. The postulated reactions forming the TPs were: hydroxylation, decarboxylation, oxidation, amide formation, ring-opening and reductive dechlorination. Incubation experiments of individual TPs, those which were available as reference standards, provided a deeper look into the transformation pathways. It was found that the transformation consists of four main pathways but no pathway accounted for a clear majority of the transformation. A 10-day monitoring campaign of the full-scale plant confirmed an 88% removal of diclofenac (from approximately 1.6 µg/L in WWTP influent) and the formation of TPs as found in the laboratory was observed. One of the TPs, *N*-(2,6-dichlorophenyl)-2-indolinone detected at concentrations of around 0.25 µg/L in WWTP effluent, accounting for 16% of the influent diclofenac concentration. The biotransformation of carriers was compared to a second WWTP not utilising carriers. It was found that in contact with activated sludge, similar hydroxylation and decarboxylation reactions occurred but at much slower rates, whereas some reactions, e.g. reductive dechlorination, were not detected at all. Finally, incubation experiments were performed with attached growth biomass from a third WWTP with a similar process configuration to Bad Ragaz WWTP. A similarly effective removal of diclofenac was found with a similar presence of TPs.

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1. Introduction

The non-steroidal anti-inflammatory diclofenac (DCF) belongs to the group of chemicals of emerging concern (CECs) and has an elevated environmental relevance. In addition to the well-known toxic effects of DCF to vultures (Oaks et al., 2004), renal and hepatic toxicity has also been recorded in certain fish species at concentrations in the low µg/L range (Fent et al., 2006; Triebkorn et al., 2004). As a consequence of its environmental significance, DCF has been included in the *Watch List*, which contains the candidates for a revised list of priority substances for the European

Water Framework Directive (WFD, The European Parliament and Council of the European Union (2013)). The well-documented ecotoxicological effects of DCF have resulted in the proposal of a relatively low environmental quality standard (EQS) of 0.1 µg/L as an annual average for inland surface waters (The European Commission, 2012).

It is well known that DCF is mainly discharged into the aquatic environment via WWTPs (Luo et al., 2014). A review by Verlicchi et al. (2012), of mainly European wastewater studies, reported a median concentration in raw wastewater of 0.7 µg/L with a maximum concentration of 11 µg/L. Conventional activated sludge treatment is usually rather ineffective for the removal of DCF with a median removal range of 20%–30% (Zhang et al., 2008). As a consequence, an exceedance of the EQS in surface waters is likely if the proportion of treated wastewater is higher than 10%. This has

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already been observed in European surface waters (Patrolecco et al., 2015; Nödler et al., 2010). Therefore, going forward, an overall improvement of municipal wastewater treatment would be crucial to fulfil the requirements of the revised WFD. It is noted that this applies not only to DCF but a whole range of CECs, both known and unknown, which are emitted into receiving waters due to inadequate removal.

Although a low median removal for DCF is reported, there are cases where biological treatment is able to remove this CEC. The WWTP in Bad Ragaz, Switzerland, has previously been shown to be effective at removing DCF and other, typically poorly biodegradable CECs, including trimethoprim. In addition to a conventional nitrifying/denitrifying suspended sludge treatment stage, the WWTP is equipped with a third biological compartment filled with carrier-attached biofilms (hybrid-moving bed biofilm reactor, MBBR). The carriers are small plastic disks with a mesh structure to provide a high surface area to support biofilm growth. In the previous study, the removal of DCF was attributed to biological degradation by contact with the carrier biomass, while sorption was negligible (Falás et al., 2013). MBBRs have been studied with respect to the degradation of various CECs (Hapeshi et al., 2013; Escolà Casas et al., 2015) and an improved DCF removal of MBBRs over suspended sludge was also observed in laboratory-scale reactors (Zupanc et al., 2013). However, the transformation pathway of DCF and the transformation products (TPs) which may be formed are still unknown.

One main focus of current research in the area of CECs is the elucidation of TPs during biological wastewater treatment, since this provides useful clues to elucidate i) transformation processes (Quintana et al., 2005) and ii) potential formation of stable TPs, which might pose ecotoxicological effects even after a complete removal of the parent CEC (Escher and Fenner, 2011). Several studies have previously investigated the transformation of DCF in municipal WWTPs (Vieno and Sillanpää, 2014). Hitherto identified TPs include nitro- and nitroso-derivatives of DCF (Pérez and Barceló, 2008) and an indolinone derivative resulting from intramolecular ring closure (DCF-lactam) (Kosjek et al., 2009). Studies with soil/sediment systems have found 4'-hydroxy-DCF (4HD), 5-hydroxy-DCF (5HD) and a 5HD derivative, 5HD-quinone imine (5HDQI) (Gröning et al., 2007). Another study dealing with the fate of DCF in soil identified the formation of 5HD and several isomers of dichlorobenzoic acid (Dodgen et al., 2014). The principal human metabolites of DCF are 4HD and 5HD (Stierlin et al., 1979). HDQIs are also known metabolites (Poon et al., 2001) as well as the acyl glucuronide conjugate of DCF (Seitz and Boelsterli, 1998). A study of mouse metabolism found numerous metabolites of DCF including hydroxylated DCF, the aforementioned lactam, a benzoic acid derivative resulting from decarboxylation and subsequent oxidation (DCF-BA) also referred to as DCF-carboxylic acid, as well as several different conjugates of these metabolites (Sarda et al., 2012).

The transformation pathways in biological wastewater treatment reported thus far stop mostly at the level of primary TPs (direct TPs of DCF) and do not include secondary or tertiary TPs, although the biodegradability of some primary TPs is already known (Lee et al., 2012). The slow and incomplete transformation of DCF poses a challenge to identify TPs, since long incubation periods are needed and the TP concentrations are rather low. Due to major differences in the reported DCF transformation pathways (Gröning et al., 2007; Kosjek et al., 2009), the question arises whether parts of the transformation pathway are specific to the system studied or can be generalized across all types of microbial degradation.

In this study, we investigated the biotransformation DCF in two hybrid-MBBR systems in Bad Ragaz, Switzerland and Klippan, Sweden. Specifically, the study looked at whether special primary

degradation reactions were responsible for the high degradability of DCF and if these treatment processes were capable of degrading primary TPs as well as DCF. The aims were to i) identify TPs and transformation pathways ii) measure the formation of any formed TPs in full-scale WWTPs and iii) compare the hybrid-MBBRs to conventional activated sludge systems without carriers. To model the full-scale WWTPs, laboratory-scale bioreactors were inoculated with biomass from the hybrid-MBBRs and activated sludge-based WWTPs. High-resolution mass spectrometry was employed to identify TPs and monitor transformation kinetics.

2. Methods

2.1. Chemicals

Diclofenac (DCF) was purchased from Sigma Aldrich and diclofenac-*d*₄ (CAS: 153466-65-0) was purchased from Dr. Ehrenstorfer (Teddington, UK). DCF transformation products (TPs) 4HD (4'-hydroxy-DCF, CAS: 64118-84-9), 5HD (5-hydroxy-DCF, CAS: 69002-84-2), DCF-lactam (*N*-(2,6-dichlorophenyl)-2-indolinone, CAS: 15362-40-0) and DCF-BA (DCF-Benzoic Acid, CAS: 13625-57-5) were purchased from TRC (Toronto, Canada). All chemical standards were >95% purity grade. Acetonitrile (LC-MS grade) was received from Merck (Darmstadt, Germany) and water was prepared with a Milli-Q system (Merck Millipore).

2.2. Wastewater treatment plants

The municipal WWTP in Bad Ragaz (WWTP-BR) has 25 500 PE (person equivalents) connected and an average influent load of 3200 m³/d. The WWTP is equipped with a biological activated sludge treatment stage, which is separated into a series of three compartments by low walls, i.e. cascades. The first compartment is a denitrifying compartment and the second is aerobic (2 mg/L O₂). In both compartments the biomass is suspended in sludge flocs. The third compartment is the nitrifying stage with a higher aeration rate (3 mg/L O₂). It contains carriers (Biofilm Chip M, Anox-Kaldnes, 35% filling ratio, ≈ 420 m²/m³) for biofilm growth (hybrid-moving bed biofilm reactor, MBBR). The carriers are retained in the third compartment by a screen. A recirculation of roughly 0.7 parts (ratio to influent) takes place from the 3rd to the 1st compartment.

In addition to the Bad Ragaz WWTP, two further WWTPs were studied. Both WWTPs were sampled to provide inoculant for batch experiments. One is located in Klippan, Sweden (WWTP-KL), with 13 000 PE connected and has a similar configuration to WWTP-BR (hybrid-MBBR). In one of its treatment lines, two denitrifying compartments are followed by an aerobic compartment with 2 mg/L O₂. All three are activated sludge compartments without carriers. These are followed by a fourth compartment (nitrifying) with 3 mg/L O₂ containing carriers (Biofilm Chip M, 40% filling ratio, ≈ 480 m²/m³) for biofilm growth. The third WWTP is located in Koblenz, Germany (WWTP-KO), with 220 000 PE connected and an average load of 61 000 m³/d. This plant has denitrifying, followed by nitrifying (1.5 mg/L O₂, 4 g/L), suspended sludge compartments, but does not make use of carriers in any stage of the treatment.

2.3. Aerobic laboratory incubation experiments

Incubation experiments in batch mode up to two weeks in duration were conducted in bench-top vessels using either biofilms on carriers from WWTP-BR or WWTP-KL or, for comparison, activated sludge from WWTP-KO as inoculant. In these experiments 400 mL of WWTP effluent (from WWTP-KO) was inoculated with

carriers from WWTP-BR to a concentration of 35 carriers/L ($\approx 300 \text{ m}^2/\text{m}^3$, 2.2 g/L total biomass, Section 2.3.1). Carriers were sampled from WWTP-BR on 2nd March, 18th May and 9th July 2015. The experiments were started one day after sampling due to transport time. After inoculation of the reactors the system was equilibrated for at least 3 h, after which DCF was spiked to concentrations of 5 $\mu\text{g}/\text{L}$ or 200 $\mu\text{g}/\text{L}$ and DCF-TPs were spiked to individual reactors to concentrations of 200 $\mu\text{g}/\text{L}$. The experiments with low DCF spike concentrations were conducted in triplicate. The other experiments were run at least as duplicates. During the incubation period, the vessels were constantly stirred and purged with air (flow rate: 50 mL/min). To maintain a constant pH, either by CO_2 added to the purge air mixture ($\approx 1:1000$) or NaOH solution (1 mol/L) was added drop-wise. Water samples of 2 mL were taken at defined intervals (for 200 $\mu\text{g}/\text{L}$ spike: at the start, after 2 h and 1 d and then approximately every 2 d; for 5 $\mu\text{g}/\text{L}$ spike: approximately every 3 h for the first day and then every 3 d). After sampling, the water samples were immediately filtered (0.45 μm regenerated cellulose, Whatman) and then frozen at -25°C . In a previous study, sorption of DCF to this filter material was investigated and was observed to be low, $\approx 8\%$ (Hebig et al., 2014). Sorption of DCF ($\text{p}K_a = 4.15$ (Sangster, 1997)) to sludge or biomass was estimated based on distribution coefficients for activated sludge, $K_d \approx 0.03 \text{ L/g}$ (Stevens-Garmon et al., 2011; Fernandez-Fontaina et al., 2014) and was likely to be small, $\leq 6\%$ (Schwarzenbach et al., 2005b).

Experiments with activated sludge from WWTP-KO were conducted in parallel under exactly the same conditions as those with carriers from WWTP-BR. Sludge was taken from the nitrifying reactor of WWTP-KO and diluted by a factor of two with effluent from WWTP-KO (final sludge concentration 1.6 g/L, Section 2.3.1). Incubation experiments in batch mode with carriers from WWTP-KL were conducted in 6 L vessels using 62.5 carriers/L ($\approx 525 \text{ m}^2/\text{m}^3$) and WWTP effluent as the liquid phase (4.75 g/L total biomass). These were also aerated to maintain oxic conditions. DCF was spiked to 1 $\mu\text{g}/\text{L}$. Samples were taken at defined intervals (every 2 h) during an incubation time of 24 h. The water samples from all experiments were analysed without further sample preparation (i.e. direct injection) by LC–QToF–MS as described in Section 2.5.

2.3.1. Determination of carrier-attached biomass and suspended sludge concentration

To determine the amount of carrier biomass, 5 replicates each of 5 carriers were dried overnight at 105°C then weighed. The carriers were then soaked in 2 mol/L HCl overnight, cleaned with sonication, stirring and scrubbing, twice with 2 mol/L HCl, twice with detergent and three times with Milli-Q water over the course of several days (including overnight soaking periods). Finally, the cleaned carriers were again dried overnight and weighed again. To determine the suspended sludge concentration, WWTP sludge (25 mL) was filtered onto dried and pre-weighed glass fiber filters (GF6, Whatman) followed by overnight drying at 105°C and finally weighed (5 replicates).

2.4. Monitoring campaign at WWTP Bad Ragaz and WWTP Koblenz

Flow-proportional composite samples of WWTP influent and effluent during 10 consecutive 24 h periods as well as grab samples of each reactor compartment were taken in July 2015 from WWTP-BR. During collection the samples were refrigerated at 4°C in automated sample collectors. After collection was complete the composite samples were filtered (0.45 μm regenerated cellulose, Whatman) and stored at -25°C . PP and PE plastics used in sampling and storage have been reported to not significantly sorb DCF (Hebig et al., 2014). As was the case for the batch experiments, sorption of DCF to reactor biomass was calculated to be low (15%

based on carrier biomass and suspended sludge concentrations of 4.7 g/L and 1.2 g/L (Falås et al., 2013)) and not significant for removal (see section 2.5). Most DCF-TPs contain similar structural moieties and have lower chromatographic retention times than DCF indicating they should have similar or lower sorbed fractions. TP such as DCF-lactam, which do not have a carboxylic acid moiety show slightly higher sorption, this was tested in a batch experiment with sterilized sludge. Water samples were analysed for the presence of DCF and DCF-TPs without further preparation by LC–QToF–MS as described in Section 2.5. During the sampling period there were no major rain events. The average hydraulic load was $(2800 \pm 300) \text{ m}^3/\text{d}$ with a hydraulic retention time (HRT) of 16 h for the whole system. The sludge age was 5 d, the average water temperature 21°C and the ammonium removal was $>99\%$. For WWTP-KO, time-proportional composite samples of WWTP influent and effluent were taken on 2 consecutive 3 d (72 h) periods in December 2015. These were prepared and analysed analogously to the monitoring campaign at WWTP-BR.

2.5. Analytical methods

Analysis was conducted with a high-resolution LC–QToF–MS system (HPLC: Agilent 1260 series, MS: Sciex 5600 TripleTOF). The HPLC consisted of a degasser, a binary pump to provide the gradient mobile phase flow, a second pump to provide an isocratic flow to the MS while the divert valve was in use, an autosampler with a refrigerated vial tray and a column oven. The HPLC was equipped with a ZORBAX Eclipse Plus C18 column (150 mm \times 2.1 mm, 3.5 μm , Agilent Technologies). The chromatographic method used a gradient elution with water and acetonitrile both with 0.1% formic acid buffer. The HPLC was coupled to the MS via electrospray ionization (DuoSpray Source, Sciex). Data was acquired in both positive and negative ionization modes, separate injections were used for each mode. The instrument was automatically recalibrated every 2.5 h using an automated Calibrant Delivery System (CDS) which injected a calibration solution into the MS via the APCI probe of the DuoSpray source. A divert valve was used to divert the first 1.5 min and the last 7 min of each chromatographic run to the waste. MS scans in each scan cycle included one full scan (100 u to 1200 u) and eight data-dependant MS^2 scans, which were product ion scans of the most intense peaks from the full scan (mass range of 30 u to mass of the precursor ion). An exclusion list was used to avoid acquiring MS^2 scans of background signals and a precursor mass list was used to ensure that MS^2 scans of known TP were acquired. Further details of the chromatography and acquisition method can be found in Schlüsener et al. (2015) and Nürenberg et al. (2015). For analysis of the high-resolution MS data, PeakView and MasterView software were used for the identification and structural characterization of TP and MultiQuant software was used to obtain peak areas (all provided by Sciex). Data was analysed and presented using R (R Core Team, 2015) and the ggplot2 package (Wickham, 2009). For compounds with available standards, MultiQuant was used for quantification. These were DCF and the TP 4HD, DCF-lactam and DCF-BA. The concentration of the TP 5HD was estimated based on the calibration of 4HD. DCF- d_4 was used as an internal standard (2 $\mu\text{g}/\text{L}$) and the linear calibration ranged from 5 ng/L to 5000 ng/L. Recoveries and LOQs for the method are provided in Table 1. The degradation of DCF removal during the incubation experiments was modelled by pseudo-first-order kinetics according to (Schwarzenbach et al., 2005a) (Equation (1)).

$$\frac{dC_{\text{DCF}}}{dt} = -k_{\text{biol}}C_{\text{DCF}}X_{\text{SS}} \quad (1)$$

where k_{biol} is the first-order rate constant in $\text{L}/(\text{g d})$ (g biomass or

Table 1
Recoveries and LOQs for the analysis method for DCF and TPs in wastewater samples.

	DCF	4HD	DCF-lactam	DCF-BA
Recovery influent (%)	107 ± 4	73 ± 3	114 ± 6	114 ± 2
Recovery effluent (%)	115 ± 4	93 ± 5	125 ± 5	117 ± 4
LOQ (ng/L)	10	5	10	20

suspended sludge), C_{DCF} is the DCF concentration in $\mu\text{g/L}$ and X_{SS} is the carrier-attached biomass concentration or suspended sludge concentration in g/L . A simplified model of a completely stirred tank reactor was used to estimate residual fraction of DCF (S_{out}/S_{WW}) in a full-scale reactor assuming negligible removal due to sorption (Equation (2)), (Joss et al., 2006), where θ_h is the HRT of the reactor. The removal of DCF due to excess sludge production was previously modelled by Falås et al. (2013) for WWTP-BR and found to be not significant.

$$\frac{S_{out}}{S_{WW}} = \frac{1}{1 + k_{biol}X_{SS}\theta_h} \quad (2)$$

3. Results and discussion

3.1. Transformation of diclofenac in lab-scale experiments containing carriers from a WWTP employing an MBBR

3.1.1. Elevated diclofenac concentrations (200 $\mu\text{g/L}$) to elucidate transformation products

To study the influence of an MBBR on the removal of diclofenac (DCF), laboratory-scale incubation experiments were conducted with carriers taken from WWTP-BR and WWTP-KL (see Section 3.3 for WWTP-KL experiments). In initial experiments with WWTP-BR carriers, 200 $\mu\text{g/L}$ DCF was spiked to the bioreactors and the formation of TPs was studied over a period of 12 d by analysing the aqueous phase with LC–QToF–MS. Within the first 24 h of incubation the DCF concentration was reduced by >99%. However, the elimination of DCF was concurrent with the appearance of a large number of TPs. In total, more than 20 different TPs were observed (Fig. 1). The identification of each TP was based on the high resolution ion mass and isotopic pattern, which was used to calculate a plausible molecular formula, and the MS^2 fragmentation spectrum providing structural fragments of the TP. Details of the TP identification, including the MS^2 spectrum and structural elucidation are provided in the Supplementary Data. Confidence levels (1–4) for the structures, based on the categorization proposed by Schymanski et al. (2014) are given in Fig. 1 with level 1 indicating a confirmed structure. For confidence levels 2–4 an alternative characterization method is needed to confirm their identity. However, accumulated concentrations were not high enough for the isolation of TPs.

The TPs DCF-lactam, 4HD (4'-hydroxy-DCF), 5HD (5-hydroxy-DCF) and DCF-BA (DCF-benzoic acid) were identified as primary TPs. Fortunately, they were available as analytical reference standards and hence they were also individually incubated in separate bioreactor experiments. It was found that in the lab-scale systems with carriers from WWTP-BR, the biodegradation of these four primary TPs led to the formation of all other TPs observed in the experiments where DCF was spiked. Each primary TP was transformed into several secondary TPs (Table S23 in the supplementary data) which in turn were frequently further converted. The overall outcome of these experiments was a complex web of transformation pathways, which can be predominantly explained by six reactions: a hydroxylation of the aromatic rings A and B, an intramolecular amidation/de-amidation, a sulfate conjugation of

phenolic hydroxyl groups, a reductive dechlorination of the aromatic ring A, an oxidative ring-opening of ring B and oxidations by dehydrogenation of phenolic moieties. TP structures, their time courses during the experiment and their position in the overall transformation pathway are shown in Fig. 1, along with a categorization of the reactions leading to the individual TPs. It must be noted that except for TP343, TP243 and TP287, all TPs are intermediates since they are further degraded. The subsequent TPs are unknown since no further TPs could be detected. The concentrations of TP343 and TP243 reached a plateau after a few days, while those of TP287 increased constantly until day 12. However, it was estimated from the peak areas that these did not account for a significant proportion of the transformed DCF.

The four primary TPs were formed within the first 24 h in the carrier-based, lab-scale experiment, but were quickly converted to secondary TPs. Hence, the sum of the primary TPs did not close the mass balance of DCF transformation (Fig. S23 in the Supplementary Data). DCF-lactam reached the highest concentration of the four primary TPs and had the highest peak area of all TPs (9 $\mu\text{g/L}$, 4.5% of the spiked DCF). This TP is formed by intramolecular amidation of the carboxylic acid with the primary amine. The primary TP DCF-BA is formed by a formal decarboxylation and an oxidation of the aliphatic CH_2 moiety to a carboxylic acid group. In these experiments the concentration reached 0.5 $\mu\text{g/L}$ which accounts for 0.25% of the spiked DCF. The ring hydroxylation of DCF led to 4HD and 5HD, known as both bacterial and mammalian TPs of DCF (Gröning et al., 2007; Sarda et al., 2012; Stülten et al., 2008). Both are formed and dissipated within the first 2 d of incubation and many secondary TPs are formed from these compounds.

3.1.1.1. 4HD. Six DCF-TPs formed via 4HD and are shown on the left side of Fig. 1. TP293b is formed via intramolecular amidation while TP297 is formed via decarboxylation and oxidation. A sulfate conjugation of the phenolic hydroxyl group of 4HD led to the formation of TP391b. The molecular formulas and isotopic patterns of TPs 259 and 225 show that these were formed through dechlorination reactions. A lactam structure is postulated to account for the fragmentation pattern and loss of oxygen while to account for the additional hydrogen, a reductive dechlorination reaction is proposed (further details are in the Supplementary Data Section 1.10). A dechlorination did not occur via any other primary TP, suggesting the hydroxy group on ring A is necessary for dechlorination. This may be explained by electron-donor effects or the ability to bind into an enzyme active site. Finally, TP275 could be explained by a formal oxidation by dehydrogenation of 4HD, due to the similar fragmentation spectrum. All the secondary TPs of 4HD appeared during the first day of incubation but were quickly dissipated, indicating that these were all intermediates of a larger transformation pathway leading to small molecules. However, no further TPs could be found, most likely because i) their concentrations were below the limits of detection of the instrument and ii) small, highly polar TPs were formed that are not detectable with the analytical method used or degradation proceeded to a mineralization.

3.1.1.2. 5HD. Nine TPs were formed via 5HD and are shown on the right side of Fig. 1. TP293a is formed via intramolecular amidation. A sulfate conjugation of the phenolic hydroxyl group of 5HD formed TP391a. An oxidation of 5HD by dehydrogenation was responsible for the formation of 5HDQI (hydroxy diclofenac quinone imine). 5HD was already converted into 5HDQI in ultra-pure water by an abiotic reaction (Gröning et al., 2007), and this was also observed to occur in standards of 5HD prepared for this study. However, the conversion is quite slow compared to the biological conversion. A sterile control with autoclaved carriers did not show conversion of 5HD within a 6 d incubation period (data not shown), indicating that

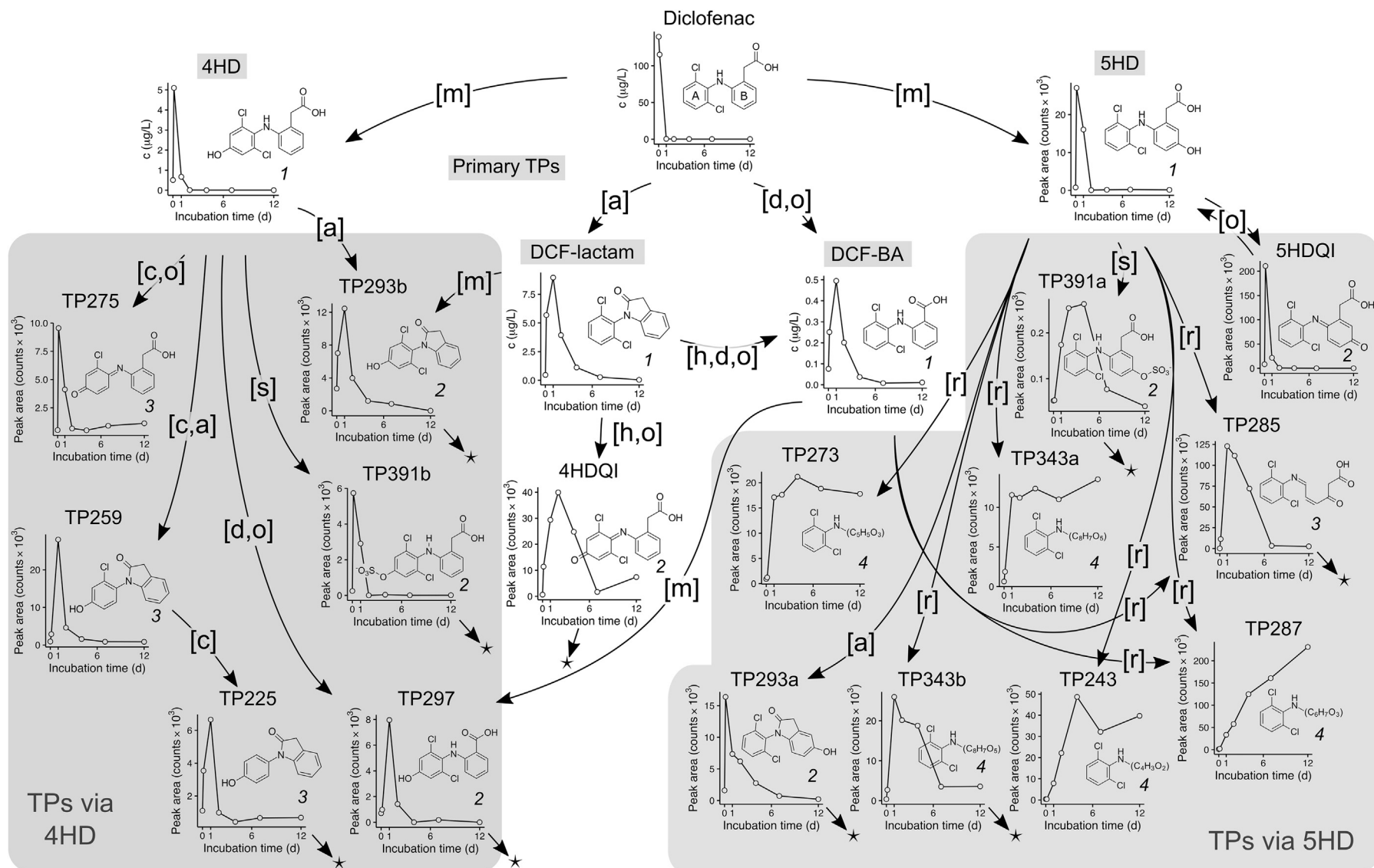


Fig. 1. Formation of TPs from DCF during the incubation with carriers showing time courses and structures. Arrows indicate pathways elucidated by separate incubations of DCF and primary TPs. Abbreviations for postulated reaction types: [m] mono-oxygenation, [o] oxidation (dehydrogenation), [a] amidation, [d] decarboxylation, [s] sulfate conjugation, [r] ring-opening reactions and [c] reductive dechlorination, [h] amide hydrolysis. Numbers at the bottom right of each structure indicate the confidence level. 1: Confirmed structure based on reference standard. 2: Proposed structure based on MS² characterization and evidenced by plausible transformation reactions. 3: Tentative structure similar to level 2 confidence, but alternative structures cannot be totally ruled out. 4: Plausible chemical structure could not be derived from the MS² spectrum. The molecular formula and only one structural moiety (ring A) could be confirmed. Transformation to unknown TPs is indicated by ★.

the biological reaction dominates in this environment. Via 5HD, six further TPs were observed whose MS² spectra indicated an oxidative opening of the non-chlorinated ring, B. This was postulated due to the large number of oxygen atoms on the right side of the molecule and consecutive CO₂, CO, and CH₂ neutral losses while ring A was left unchanged. Due to the ring opening there were several potential chemical structures based on the MS² spectra. For TP285, a plausible structure was found, while for the others the fragmentation was more ambiguous so the data could provide only the sum formula of the cleaved ring B.

3.1.1.3. DCF-lactam. The reactions of DCF-lactam are associated with an opening of the lactam ring and a subsequent reaction such as oxidation (two electron transfer) to 4HDQI or decarboxylation to DCF-BA. In addition, a hydroxylation of the chlorinated ring A led to the formation of TP293b. The 4HDQI TP was not observed as a direct TP of 4HD, but rather only via DCF-lactam. This might be due to the fast dissipation kinetics of 4HD in this environment, allowing little oxidation to take place, while DCF-lactam is more stable, allowing the formation of 4HDQI over a different route, e.g. by combined mono-oxygenation and deamination.

3.1.1.4. DCF-BA. This TP is transformed by hydroxylation into TP297 (which is also formed by decarboxylation of 4HD) as well as to two further TPs (TP285 and TP287) where the non-chlorinated ring B is oxidatively opened. Both of these TPs are also formed by the ring opening of 5HD as was discussed previously.

3.1.2. Environmental diclofenac concentrations (5 µg/L)

Lab-scale experiments at DCF concentrations of 5 µg/L with carriers from WWTP-BR exhibited similar results as obtained by spiking 200 µg/L. The DCF concentration was reduced by > 99% within the first 24 h with a similarly high degradation rate constant (Table 2). In total, 11 TPs were detected from DCF (Fig. S22 in the Supplementary Data), all of which were already seen at the 200 µg/L spike level as the most intense peaks at that level. Thus, the reduced number of detected TPs is most likely caused by the detection limits of the LC-QToF-MS measurements. It can be concluded that the results obtained with 200 µg/L DCF spike can be transferred to lower concentrations with respect to both the kinetics of DCF removal and the transformation pathways. The kinetics of TP formation and removal were in some cases different, both TP285 and 4HDQI show more persistence in these experiments suggesting these might be present in WWTP effluent.

3.2. Incubation experiments with activated sludge from WWTP Koblenz

Incubation experiments were conducted with suspended sludge from WWTP-KO, which does not use carriers anywhere on the treatment train. The removal rate of DCF in contact with this biomass was significantly slower in comparison to the incubation with carriers from WWTP-BR (Fig. 2 and Table 2). These experiments were conducted in parallel and in duplicate using the same reactor set-up and similar biomass concentrations. A long

Table 2
Pseudo-first-order rate constants of DCF in different biomass types.

Source of biomass	Concentration of DCF (µg/L)	<i>k</i> _{biot} (L/(g d))
WWTP-BR	5	1.4 ± 0.1
WWTP-BR	200	≥1.5 ^a
WWTP-KL	1	1.4
WWTP-KO	200	0.03 ± 0.01

^a Estimate, not enough data points for a precise determination.

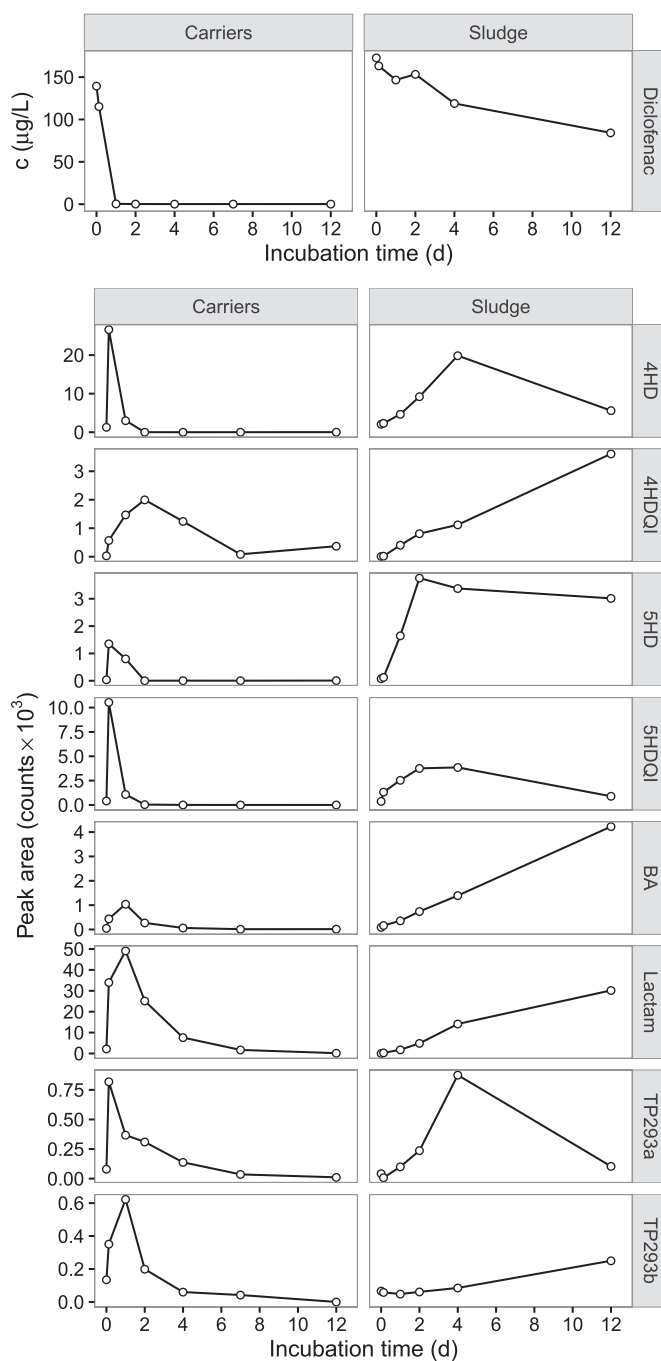


Fig. 2. Formation and dissipation of DCF-TPs in incubations with carriers from WWTP-BR (left) versus suspended sludge from WWTP-KO (right).

incubation time was chosen to be able to see a significant removal of DCF in the suspended sludge reactors. Even after 12 d only 50% of DCF was dissipated, while in experiments with carriers from WWTP-BR DCF was completely removed after 24 h.

In contact with suspended sludge, the same primary TPs (4HD, 5HD, DCF-lactam and DCF-BA) and a few secondary TPs (4- and 5HDQI and TP293) were observed as were found in the carrier-inoculated experiments. The formation rates of primary TPs were much slower, as could be expected due to the slower DCF removal rate. However, also the rates of dissipation of the primary TPs were slower compared with the incubation with carriers (Fig. 2). Reactions occurring in both systems are hydroxylation of the aromatic rings, the intramolecular amidation, decarboxylation and oxidation

reactions. Several TPs, such as TP259 (the result of dechlorination) and TP285 (the result of oxidative ring opening) are unique to the carrier-inoculated system. The TP with mass 259 might require anaerobic zones to be formed, which are more likely to occur in the dense biofilm growth on carriers than in the suspended sludge flocs. Reductive dehalogenation reactions are more commonly observed in the absence of aerobic conditions (Zhang and Bennett, 2005; de Beer et al., 1997). No TPs were found to be unique to degradation in contact with suspended sludge. A full list of the TPs formed in suspended sludge can be taken from Fig. 2.

3.3. Lab-scale experiments with carriers from WWTP Klippan

To investigate the transferability of the results from WWTP-BR to other MBBR systems, the removal of DCF was investigated in WWTP-KL, which has a similar set-up to WWTP-BR and employs a compartmentalized reactor with activated sludge and carrier-filled compartments. Lab-scale incubation experiments were conducted in lab-scale bioreactors inoculated with carriers from WWTP-KL. DCF was spiked to 1 µg/L to the bioreactors. A fast dissipation of DCF was observed, with a reaction rate constant of about 1.4 L/(g d), which was similar to that found in the lab-scale experiments with carriers from WWTP-BR (Table 2), while typical literature rate constants for DCF in activated sludge are 0.01–0.5 L/(g d) (Tran et al., 2009; Urase and Kikuta, 2005). The primary TPs DCF-lactam and DCF-BA were identified as well as TP285, which were observed in lab-scale experiments of WWTP-BR and detected in the effluent of WWTP-BR (for identification, see Table S24 in the supplementary data). Signals for the other TPs were either too weak for proper identification, or they were not observed at all. In summary, a fast degradation of DCF might be linked to hybrid-MBBR biomass, and certain TPs might be markers for these processes, e.g. the formation of TP285. The signal intensity of TP285 is nevertheless very low and is unlikely to account for the majority of DCF removal.

3.4. Monitoring campaign at WWTP Bad Ragaz

To verify the transferability of the lab-scale results to full-scale, WWTP-BR was monitored over a dry-weather period during the summer of 2015. From ten consecutive days, 24 h-composite samples of WWTP influent and effluent were analysed by LC–QToF–MS. The results for compounds for which reference standards were available are shown in Fig. 3. The DCF concentration

was reduced from a median of 1.6 µg/L in WWTP influent to 0.2 µg/L in WWTP effluent. This corresponds to an average removal of 88% during the 10-day monitoring period.

The formation of TPs was also observed in the full-scale plant. An average concentration of 0.25 µg/L DCF-lactam was detected in the effluent of WWTP-BR, accounting for about 16% of the influent DCF concentration. DCF-lactam was the most predominant TP in both WWTP effluent and in lab scale experiments where DCF-lactam reached 13% of spiked DCF (Fig. S22). The TP 4HD was present at relatively high concentrations in WWTP influent but was removed in the biological treatment as was the case in the lab-scale experiments. The TP 5HD was also found in the influent, albeit at lower concentrations and was no longer detected in the effluent. Both TPs 4HD and 5HD are human metabolites of DCF explaining their detection in WWTP influent. No evidence for the formation of these TPs during the treatment process could be obtained due to i) the high influent concentrations and ii) the fast transformation of these TPs. The TP DCF-BA was observed at low concentrations of 23 ± 8 ng/L in WWTP effluent, whereas in the influent it was below the LOQ of 20 ng/L. Thus, a minor fraction of DCF-BA might be formed. The presence of several secondary TPs could be confirmed based on their exact mass, retention time and fragmentation spectrum, which matched very well with the results of the lab-scale experiments. These were TP293b, TP259 and TP285 (for identification see Table 3). In all samples the signal intensities were relatively low, comparable with the peak areas of DCF-BA, which was present with about 20 ng/L. In the results of the lab-scale experiments, TPs 259 and 285 had very high signal intensities compared to all other secondary (or tertiary) TPs (Fig. 1), which hints at their relative importance in the transformation pathway. Since standards

Table 3

Identification of TP285 and TP259 in WWTP-BR effluent samples by comparison to lab-scale experiments.

	TP285		TP259	
	Lab-scale	WWTP	Lab-scale	WWTP
Retention time (min)	8.6	8.7	10.1	10.2
[M+H] ⁺ mass (u)	286.0035	286.0029	260.0465	260.0454
Isotope peak ratio (³⁷ Cl) (%)	67	64	37	29
Fragments of the MS ² spectrum	177.0330	177.0319	168.0800	168.0803
	242.0118	242.0138	196.0758	196.0753
			197.0840	197.0775

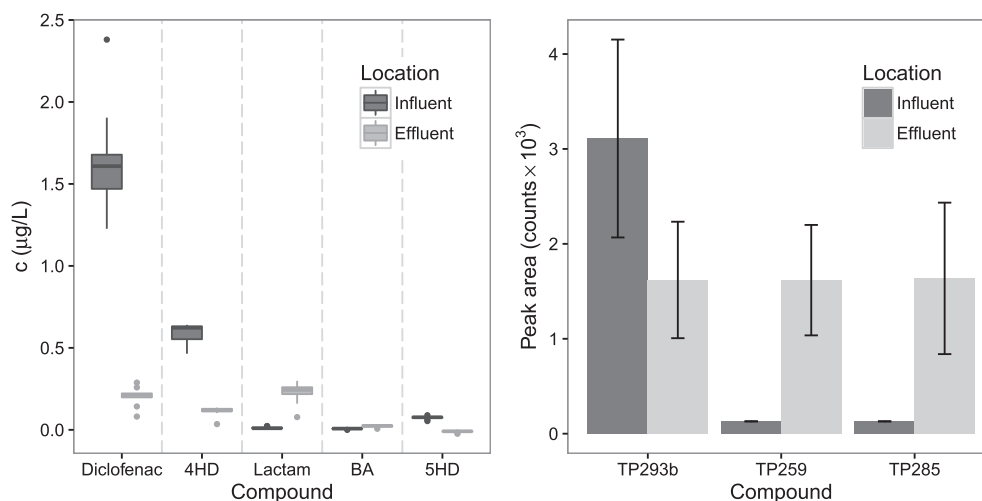


Fig. 3. Left: Analysis of samples from WWTP-BR (hybrid-MBBR) for DCF and DCF-TPs for which standards were available. Right: Comparison of concentrations of DCF-TPs in influent and effluent based on peak areas (no available standards).

of these TPs could not be obtained, peak areas in the WWTP influent and effluent were compared to estimate if a formation took place (Fig. 3). Both TP259 and TP285 were only detected in WWTP effluent, indicating that a formation is possible, whereas TP293b was detected in both WWTP influent and effluent at similar intensities. Making such comparisons between different matrices without standards should be treated as an initial estimate and the results should be taken with caution. In summary, out of 11 TPs detected in the lab, 7 TPs were detected at the WWTP, the exceptions being the two HDQIs, TP 293a and TP 287.

It can be concluded from the results that i) the laboratory incubation experiments were able to accurately represent the treatment process with respect to DCF, ii) DCF and its TPs were substantially removed by the WWTP, and low concentrations of some DCF-TPs were detected in the effluent, iii) although a wide array of TPs were found using incubation experiments, the majority of these were not detected in the WWTP, since their concentrations were probably below the limits of detection.

The influent and effluent samples of the WWTP provide only a picture of the full treatment process, which includes reactor compartments with activated sludge (no carriers) and a carrier-filled MBBR. To test if the TP formation was occurring primarily in the carrier-filled compartment, grab samples were taken from each reactor compartment. By comparing the different compartments, it was found that TPs 259, 285 and DCF-lactam showed the most intense signals in the carrier-filled compartment (Fig. 4). DCF itself had a much lower concentration in this compartment compared with the aerobic and denitrification compartments. The high concentration decrease of DCF between the influent and the denitrification stage is at least partly caused by dilution, since a recirculation of approximately 0.7 parts took place in the reactor. The expected concentration of DCF in the denitrifying stage resulting from dilution alone would be approximately 1 $\mu\text{g/L}$, which is close to the detected concentration of 0.8 $\mu\text{g/L}$. The increased concentrations of TPs in the denitrifying stage can also be explained by the recirculation. The results as a whole support the conclusion found by Falás et al. (2013), that the carrier-filled stage is mainly responsible for the DCF removal. Here this can be seen from the point of view of the TPs that are formed.

3.5. Monitoring campaign at WWTP Koblenz

To compare the relatively good removal of DCF observed at WWTP-BR with a conventional WWTP not employing an MBBR, a

monitoring campaign of WWTP-KO was carried out. In composite samples of WWTP influent and WWTP effluent no significant removal of DCF was detected (<20%), while influent concentrations were approximately 3.5 $\mu\text{g/L}$. This agrees with the slower removal of DCF observed in lab-scale incubation experiments with activated sludge not using carriers (Section 3.2 and Table 2). Using Equation (2) to model the full scale reactor, a 3% (negligible) removal would be expected based on the k_{biol} found (4 g/L sludge concentration and 6 h HRT). Examining the measured data, although no detectable removal of DCF took place, low signals for several DCF-TPs and human metabolites were observed. These included 4HD, 5HD and TP293b, which were detected in both WWTP influent and effluent, as was the case at WWTP-BR. DCF-lactam was detected in the effluent of WWTP-KO, but not in WWTP influent, indicating a small formation took place. As was the case with the lab-scale experiments, TP285 and TP259 were not detected in the effluent of WWTP-KO, which is consistent with these being unique to the degradation of DCF in contact with the carrier biomass.

4. Conclusion

DCF can be removed effectively in a cascaded hybrid moving bed biofilm reactor (hybrid-MBBR) achieving nitrification and denitrification. The degradation primarily occurred in the last compartment containing the carrier-attached biomass. In this study, a fast dissipation was observed but many TPs were formed. Due to the highly branched nature of the transformation pathway, these are mostly present at very low concentrations in the WWTP effluent. The sum of all quantifiable TPs did not explain the degraded quantity of DCF, since these were further degraded. Estimating from the peak intensities of the remaining TPs, these made up a small fraction (<5%) of the transformed DCF. Hence, although ecotoxicological studies of the complex TP mixtures formed from DCF are missing, it is likely that biological degradation of DCF results in significantly lowering its ecotoxicological impact.

After long incubation times, it was evident that the main reactions leading to DCF removal had the potential to also occur in contact with conventional activated sludge (i.e. suspended sludge with no biofilm carriers). However, based on the much slower reaction rates the transformations were not observed to occur to a significant degree in the full-scale activated sludge process. It can be concluded that the observed transformation of DCF in hybrid-MBBR systems is linked to different reaction kinetics of two very similar transformation pathways rather than one system able to

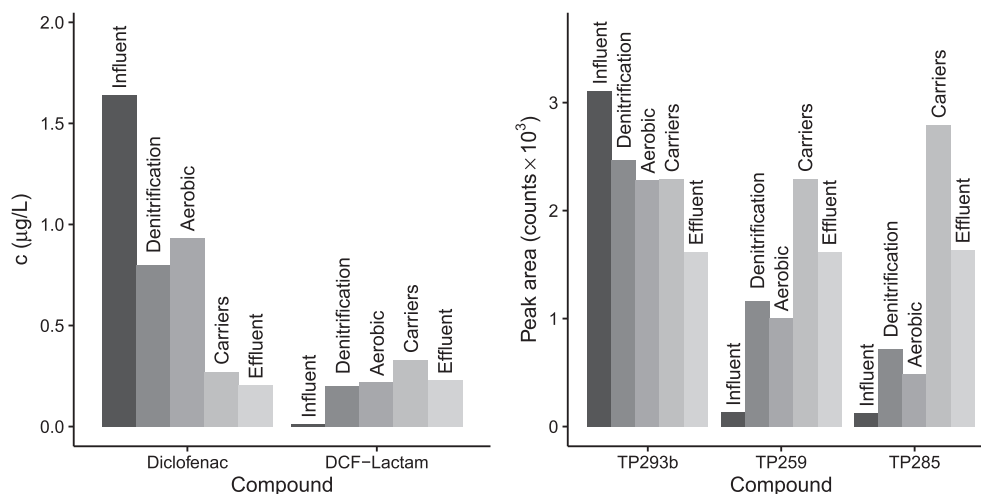


Fig. 4. Concentrations of DCF and DCF-lactam and peak areas of TP293b, TP259 and TP285 in different compartments of the biological reactor of WWTP-BR (hybrid-MBBR).

perform special reactions which the other system cannot. Some reactions forming secondary TPs were unique to the carrier systems, but these did not appear to be the main drivers for the removal of DCF. The underlying microbiological causes of the faster kinetics are still an open question.

It could also be observed that not one but all the different reaction types leading to DCF degradation were significantly faster in the hybrid-MBBR compared with conventional sludge. Therefore, it is likely that other reaction types are faster under these conditions as well, and that in combination this might lead to an improved removal of other CECs. This was indeed observed by Falás et al. (2013) for CECs such as mefenamic acid, bezafibrate and valsartan.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.watres.2016.08.002>.

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