Transformation, CO$_2$ formation and uptake of four organic micropollutants by carrier-attached microorganisms

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Abstract

A tiered process was developed to assess the transformation, CO$_2$ formation and uptake of four organic micropollutants by carrier-attached microorganisms from two municipal wastewater treatment plants. At the first tier, primary transformation of ibuprofen, naproxen, diclofenac, and mecoprop by carrier-attached microorganisms was shown by the dissipation of the target compounds and the formation of five transformation products using LC-tandem MS. At the second tier, the microbial cleavage of the four organic micropollutants was confirmed with $^{14}$C-labeled micropollutants through liquid scintillation counting of the $^{14}$CO$_2$ formed. At the third tier, microautoradiography coupled with fluorescence in situ...
hybridization (MAR-FISH) was used to screen carrier-attached microorganisms for uptake of the four radiolabeled micropollutants. Results from the MAR-FISH screening indicated that only a small fraction of the microbial community (≤1‰) was involved in the uptake of the radiolabeled micropollutants and that the responsible microorganisms differed between the compounds. At the fourth tier, the microbial community structure of the carrier-attached biofilms was analyzed by 16S rRNA gene amplicon sequencing. The sequencing results showed that the MAR-FISH screening targeted ~80% of the microbial community and that several taxonomic families within the FISH-probed populations with MAR-positive signals (i.e. *Firmicutes*, *Gammaproteobacteria*, and *Deltaproteobacteria*) were present in both biofilms. From the broader perspective of organic micropollutant removal in biological wastewater treatment, the MAR-FISH results of this study indicate a high degree of microbial substrate specialization that could explain differences in transformation rates and patterns between micropollutants and microbial communities.

1 Introduction

The biological treatment at wastewater treatment plants (WWTPs) presents an important removal step for a broad array of organic micropollutants (Reemtsma et al., 2006). A large fraction of these compounds is at least partly biologically transformed (Wick et al., 2009; Radjenović et al., 2009). The rates at which the transformation reactions occur are, however, dependent on the chemical structure of the micropollutant (Gulde et al., 2016), the composition of the microbial community (Helbling et al., 2012; Vuono et al 2016, Torresi et al., 2016), and the operating conditions of the biological treatment process (Suarez et al., 2010; Petrie et al., 2014).
Under aerobic conditions, some micropollutants such as ibuprofen and naproxen are almost ubiquitously biotransformed, whereas others such as diclofenac and mecoprop seem to require quite specific biomasses for their transformation (Bernhard et al., 2006; Zupanc et al., 2013). Coexisting biomasses of suspended and attached origin in a hybrid biofilm-activated sludge process have previously displayed different capabilities to transform the primary structure of diclofenac (Falás et al., 2013). Further analysis of the transformation pathway of diclofenac in hybrid biofilm-activated sludge processes has shown that the enhanced removal of diclofenac in contact with carrier-attached biofilms is not due to a single transformation reaction, but to several such reactions and their combinations, leading up to 20 identifiable transformation products (Jewell et al., 2016a). However, for diclofenac and most other micropollutants, it is rarely known whether the transformation products constitute end products in biological wastewater treatment or if part of the molecule is converted to CO$_2$.

Radiolabeling can provide technical support in elucidating the transformation products and identifying the micropollutant-transforming microorganisms. The combined use of CO$_2$ traps and $^{14}$C-labeled micropollutants has confirmed the biologically mediated formation of CO$_2$ from ibuprofen, naproxen and diclofenac in soils/sediments (Löffler et al., 2005; Dodgen et al., 2014) and mecoprop in drinking- and wastewater treatment (Hedegaard et al., 2014; Casas et al., 2017), but the microorganisms responsible for these CO$_2$-forming reactions are unknown.

Microautoradiography combined with fluorescence *in situ* hybridization (MAR-FISH) is a microbial identification technique allowing microorganisms with an active uptake and degradation of a radiolabeled substrate to be detected and classified according to their taxonomic identity (Nielsen and Nielsen, 2005). The method is culture-independent and is
frequently employed for *in situ* identification of microorganisms with specific substrate uptakes (Okabe et al., 2004; Hagman et al., 2008; McIlroy et al., 2010). Despite this, MAR-FISH has only been used as a microbial identification technique for a few micropollutants such as gemfibrozil (Lolas et al., 2012) and estrone (Kurisu et al., 2015; Zang et al., 2008). Further identification of micropollutant-transforming microorganisms can ultimately improve our understanding of the reasons why biotransformation rates differ between compounds, microbial communities and operating conditions.

The objective of this study was to assess the biological degradation of four organic micropollutants (ibuprofen, naproxen, diclofenac, and mecoprop) in contact with carrier-attached microorganisms from two municipal WWTPs through a tiered approach with different transformation endpoints and microbial evaluation steps. At the first tier, multi-residue LC-tandem MS analysis was used to elucidate primary transformation rates and patterns. At the second tier, $^{14}$C-labeled micropollutants and liquid scintillation counting were used to monitor the dissipation of radioactivity in the liquid phase and the formation of radiolabeled CO$_2$. At the third tier, MAR-FISH was used to screen 13 broad microbial groups for uptake of the four micropollutants. Finally, at the fourth tier, the microbial composition of the carrier-attached biofilms was examined by 16S rRNA gene amplicon sequencing, structured according to the microbial uptake, and discussed with respect to the transformation of the four micropollutants.

**2 Materials and methods**

Information on the micropollutants, the full-scale wastewater treatment plants, and the tiered approach used in this study is summarized in Table 1 and detailed in Sections 2.1-2.4.
2.1. Wastewater treatment plants

Carriers (BiofilmChip™ M, AnoxKaldnes) and biologically treated wastewater for the lab-scale incubations were collected from two municipal hybrid biofilm-activated sludge processes: one in Bad Ragaz, Switzerland, with 25,500 person equivalents connected, and one in Klippan, Sweden, with 13,000 person equivalents connected. Incoming wastewater of mainly domestic origin is treated mechanically through screening, grit removal and sedimentation at both locations. The subsequent biological treatment processes with carriers and activated sludge consist of one or two anoxic sludge compartments, followed by an oxic sludge compartment and a final compartment with both suspended sludge and carriers (Table 1). The sludge age is maintained at 3-7 days at both plants and the fill-ratio in the final compartment is 35% at Bad Ragaz WWTP and 40% at Klippan WWTP. At the time of collecting the carriers, the wastewater temperature was low, 12-15 °C, at both locations.

2.2. Biological experiments (Tier 1-3)

Biological incubations with carriers and biologically treated wastewater from the two municipal WWTPs were performed to assess microbial transformation, CO₂ formation, and uptake of three pharmaceuticals (ibuprofen, naproxen, and diclofenac) and one herbicide (mecoprop). The general treatment conditions in the biological setups are described below and detailed in Sections 2.2.1-2.2.3.

All incubations were performed over 5 days at 25±1 °C in sealed glass reactors covered in aluminum foil with a headspace to liquid ratio of ~1:1. At the outset of the incubations, micropollutants were added without the addition of organic solvents. The reactors were placed on a shaker board (150 rpm) to maintain complete mixing and high oxygen transfer from the
head space to the liquid phase. To prevent oxygen depletion, the reactors were operated at low fill-ratios (2-4 carriers per liter; Table 1), and a daily replacement of the headspace with air.

The CO₂ formation experiments (Tier 2) and the biological uptake experiments (Tier 3) were performed with commercially available ¹⁴C-labeled micropollutants: ibuprofen[RS-carboxyl-¹⁴C], naproxen[O-methyl-¹⁴C] and diclofenac[carboxyl-¹⁴C] from American Radiolabeled Chemicals (USA), and mecoprop[Ring-u-¹⁴C] from Izotop (Hungary).

2.2.1. Primary transformation experiments (Tier 1)
Batch experiments to assess the primary transformation of the micropollutants were performed in 1 L reactors with two carriers and 500 mL biologically treated wastewater at pH 7±0.2. A pH-adjustment to pH 7 with 10 mM NaH₂PO₄ and NaOH was made in the experiment with carriers from Klippan WWTP. The micropollutants were spiked as a mixture to a concentration of 4 µg/L in the Klippan experiment and to 4 µg/L and 100 µg/L in the Bad Ragaz experiment. To test for abiotic transformation, a control reactor with tap water without carriers and a micropollutant spike of 4 µg/L was run in parallel. Samples (5 mL) for micropollutant analysis were taken after 10 min, 2 h, 4 h, 6 h, 8 h, 10 h, 12 h, 24 h, 36 h, 48 h, 72 h, 96 h and 120 h. All samples were immediately filtered (0.45 µm - Chromafil, Macherey-Nagel) and stored at -20 °C until analysis.

2.2.2 CO₂ formation experiments (Tier 2)
Batch experiments to assess biologically mediated ¹⁴CO₂ formation of the ¹⁴C-labeled micropollutants and the dissipation of radioactivity in the liquid phase were run in parallel to the primary transformation experiments. The ¹⁴CO₂ formation experiments were performed in gas-tight serum bottles (250 mL) with crimp-sealed rubber septa, containing 0.5 carrier and
125 mL biologically treated wastewater, adjusted to pH 7 with 10 mM NaH$_2$PO$_4$ and NaOH. The radiolabeled micropollutants were individually exposed in a biological active reactor and a heat-treated negative control (80 °C, 10 min). At the outset of the experiments, 0.1 µCi of the $^{14}$C-labeled micropollutants (ibuprofen[RS-carboxyl-$^{14}$C], 3.0 µg/L; naproxen[O-methyl-$^{14}$C], 3.4 µg/L; diclofenac[carboxyl-$^{14}$C], 4.3 µg/L; mecoprop[Ring-u-$^{14}$C], 4.9 µg/L) were added to the reactors. The $^{14}$CO$_2$ formed during the experiment was trapped in a glass vial containing 10 mL of 0.1 M NaOH. Samples of the wastewater (1.2 mL) and the trapped $^{14}$CO$_2$ (0.4 mL) were collected with a hypodermic needle through the rubber septum, at the same time points as for the primary transformation experiments. Liquid samples were centrifuged at 21,000×g for 8 min and stored at -20 °C until analysis. Samples from the CO$_2$ trap were stored at 4 °C without additional treatment. The headspace was replaced on a daily basis by injecting air through the rubber septum.

$^{14}$C losses due to the daily replacement of the headspace and the formation of $^{14}$CO$_3^{2-}$ species were quantitatively assessed in the Bad Ragaz experiment. Losses due to the replacement of the headspace were quantified through gentle sparging of the air in the reactor through a CO$_2$ trap, containing 5 mL 0.1 M NaOH. Losses due to carbonate precipitation during the experiment were evaluated at the end of the incubation period by acidifying the reactor liquid to pH 3.5 with HCl. The $^{14}$CO$_2$ formed was subsequently trapped in 5mL NaOH (0.1 M). The potential $^{14}$C losses were finally quantified, by relating radioactivity in the CO$_2$ traps to the initial $^{14}$C addition.

### 2.2.3 Biological uptake experiments (Tier 3)

Incubations to assess the biological uptake of the four micropollutants were performed in 1 L reactors with one carrier and 500 mL biologically treated wastewater, adjusted to pH 7 with
10 mM NaH$_2$PO$_4$ and NaOH. At the outset of the incubations, 10 µCi of the $^{14}$C-labeled micropollutants (ibuprofen[RS-carboxyl-$^{14}$C], 75 µg/L; naproxen[O-methyl-$^{14}$C], 84 µg/L; diclofenac[carboxyl-$^{14}$C], 108 µg/L; RS-mecoprop[Ring-u-$^{14}$C], 122 µg/L) were added individually to four separate reactors. Biofilm samples for MAR-FISH analysis were collected at the end of the incubation period (120 h). The samples were rinsed in tap water and divided into two aliquots, which were fixed with ethanol for gram-positive bacteria and with paraformaldehyde for gram-negative bacteria according to the FISH protocol described in Nielsen (2009). After fixation, the biomass was separated from the carriers through mechanical abrasion. In brief, each node in the carrier mesh was cut free, afterwards the carrier nodes were vortexed together to abrade the biomass from the carrier media.

2.3 Analytical methods (Tier 1-4)

2.3.1 LC-tandem MS (Tier 1)

Analysis of micropollutants and their transformation products was performed by LC-tandem MS (LC-ESI-QqQ-MS, HPLC: 1260 Infinity Series equipped with a Zorbax Eclipse Plus C18 column (2.1 by 150 mm, 3.5 µm), Agilent; MS: TripleQuad 6500+, Sciex). The analysis method is described in detail in Hermes et al. (2018). In brief, a water–acetonitrile gradient was used; for the analysis of diclofenac and its transformation products, the aqueous phase was buffered with 0.1% formic acid, while for the analysis of mecoprop, ibuprofen and naproxen and their transformation products it was buffered with 0.1% acetic acid. Analysis was performed in multiple reaction monitoring mode (MRM) using deuterium labeled surrogates as internal standards for quantification.

2.3.2 Liquid scintillation counting (Tier 2)
14C analysis of the samples from the liquid phase and the CO2 trap was performed by liquid scintillation counting. Samples (0.2 mL) were pipetted into vials together with 2 mL scintillation liquid (Ultima Gold™ XR, PerkinElmer). After mixing, the samples were measured within 4 h in a liquid scintillation counter (Packard 1600 TR, Packard) for 5 min. To avoid interference of the background radioactivity, the average liquid scintillation counts per minute of ≥5 controls, containing either wastewater or 0.1 M NaOH, were subtracted from the recorded counts per minute in the original samples.

2.3.3 MAR-FISH (Tier 3)
Mechanically abraded biofilm was homogenized with a glass tissue grinder for 1 min. Homogenized samples were distributed as a thin film on gelatin-coated cover glass slides. After drying, FISH probes targeting general bacteria (EUB338, Amann et al., 1990; EUB338-III, Daims et al., 1999) and 13 broad microbial sub-populations were applied together with their competitors at the hybridization conditions suggested in the original probe references. To test for unspecific staining, a negative control (Non-EUB338; Wallner et al., 1993) was included. Probes were either labeled with Cy3 or FLOUS, and the hybridization was performed according to the protocol in Nielsen (2009).

To link probe-labeled microorganisms to uptake of the 14C-labeled micropollutants, the FISH-slides were overlaid with a radio-sensitive film (Ilford, K5D film emulsion). The film was exposed to the 14C induced β-radiation for ~1 month before it was developed in a liquid developer (KODAK D19, 40 g/L) for 3 minutes, fixed with sodium thiosulfate (300 g/L) for 4 minutes, and rinsed in tap water. All handling of the undeveloped film was performed in a darkroom. Developed MAR-FISH slides were examined with a confocal microscope (LSM510, Carl Zeiss). Fluorescence signals were induced by light at 543 nm (Cy3), 488 nm
(FLOUS) and imaged together with MAR-signals in the overlaying film (transmitted light image).

2.3.4 Amplicon sequencing (Tier 4)

In connection with the uptake experiments, three carriers were collected for 16S rRNA gene amplicon sequencing. Biomass was separated from the carrier media as described in Section 2.2.3. DNA was extracted from the detached biomass using the FastDNA™ Spin Kit for Soil according to the manufacturer’s protocol (MP Biomedicals). DNA quantity and quality were assessed with the Qubit dsDNA BR Assay Kit (Thermo Scientific, USA) on a Qubit 2.0 fluorometer and TapeStation 2200 with Genomic DNA ScreenTapes (Agilent, USA). The V4 region of the 16S rRNA gene was amplified as previously described (Albertsen et al., 2016). Libraries were purified using AMpure XP bead protocol (Beckmann Coulter, USA), quantified and quality checked using the Quant-iT dsDNA HS Assay Kit (Thermo Fischer, USA) and D1000 ScreenTapes (Agilent, USA). The libraries were sequenced on a MiSeq (Illumina, USA) in equimolar concentrations using reagent kit v3 (2x300 PE).

High-quality reads were removed and trimmed using Trimmomatic (v0.32) (Bolger et al., 2014) and merged using FLASH (v1.2.7) (Magoč and Salzberg, 2011). Reads were then formatted for use with the UPARSE workflow and screened for chimeric sequences (Edgar, 2013). USEARCH7 was used for de-replication, Phi-X contamination removal and Operational Taxonomic Unit (OTU) clustering at 97% sequence similarity. Taxonomy was assigned using MiDAS (version 2.1.3) as the reference database (McIlroy et al., 2015).

2.4 Kinetic analysis (Tier 1-2)

Biological transformation rate constants were estimated assuming first-order kinetics and negligible sorption (Equation 1).
\[
\frac{ds}{dt} = -k_{bio} \cdot X_{Biofilm} \cdot S
\]

(Equation 1)

where \( S \) is the micropollutant concentration (µg/L) or scintillation counts per liter and minute \((\text{counts}/(L \cdot \text{min}))\), \( t \) the time (d), \( X_{Biofilm} \) the carrier biofilm concentration (g biofilm/L) and \( k_{bio} \) the removal rate constant \((L/(g_{biofilm} \cdot d))\).

Biofilm concentrations, \( X_{Biofilm} \), were calculated from the weight difference of three dried carriers (105 °C) in three replicates before and after removal of the biofilm and the total number of carriers in the reactor. The biofilm was removed through long-term soaking (~7 d) in a basic detergent (20 g/L NaOH and 10 g/L sodium dodecyl sulfate), followed by thorough brushing as described elsewhere (Laureni et al., 2016).

3. Results and discussion

3.1 Primary transformation (Tier 1)

In biological incubations with carriers, the micropollutant concentrations in the water phase can decrease through volatilization, sorption as well as abiotic and biotic transformation. However, as none of the primary micropollutants (ibuprofen, naproxen, diclofenac and mecoprop) showed a measurable concentration decrease in the abiotic control with tap water (Figure S1), it can be assumed that neither abiotic transformation (e.g. hydrolysis) nor volatilization played a major role in the biologically active reactors (Figure 1). Moreover, the low biomass concentrations in the reactors (<0.3 g/L) in combination with the low solid-water partitioning coefficients of the four micropollutants \( (K_d <0.04 \text{ L/g}_{\text{biomass}}) \) suggest that sorption to the biomass was insignificant, <1% (Table S1). Thus, biological transformation reactions
are assumed to be the main cause of the dissipation of the added micropollutants in the reactors.

In the low concentration experiments (4 µg/L), ibuprofen showed the highest transformation rate, followed by naproxen, mecoprop and diclofenac. The transformation rates of mecoprop and diclofenac were, however, practically identical within each low concentration experiment (Figure 1). Additional evaluation of the transformation of diclofenac and mecoprop at 1 µg/L with suspended and attached biomasses from the two WWTPs, showed comparable transformation rates of diclofenac and mecoprop with the carrier-attached biomasses, and no or negligible transformation with the suspended sludge (Figure S2). These observations accord well with previous reports of diclofenac being more readily transformed by attached than suspended biomasses (Falås et al., 2012; Jewell et al., 2016a), while mecoprop shows a structural analogy to a third micropollutant that has been reported to degrade faster with attached than suspended growth, namely clofibric acid (Falås et al., 2012). Mecoprop and clofibric acid have identical molecular sum formulas, C$_{10}$H$_{11}$ClO$_3$, and the same chemical structure except the position of one methyl group, which suggests that certain molecular structures are more readily targeted by attached growth systems than by suspended growth systems for municipal wastewater treatment.

The relative removal rate ($dC/(dt\cdot C_0)$) decreased for all micropollutants when the initial concentration was increased from 4 to 100 µg/L (Figure 1). One reason for this observation could be a toxic inhibition of the micropollutant-transforming microorganisms by the micropollutants. However, this explanation seems rather unlikely as the specific turnover per unit biomass (µg/(d·g$_{biomass}$)) was higher at 100 µg/L than at 4 µg/L for all micropollutants (Figure S3). Another explanation for the lower relative removal rate as well as the higher
specific turnover per unit biomass at 100 µg/L than at 4 µg/L could be the saturation degree of
the micropollutant-transforming enzymes, which can be described by Michaelis–Menten
kinetics (Equation 2)

\[ V = V_{\text{max}} \cdot \left( \frac{S}{K_m + S} \right) \]  

(Equation 2)

where \( V \) is the reaction rate (µg/(L·d)), \( V_{\text{max}} \) the maximum reaction rate (µg/(L·d)), and \( K_m \) the
Michaelis constant (µg/L) corresponding to the micropollutant concentration (S) at \( V_{\text{max}}/2 \).

According to Equation 2, the reaction order depends on the substrate concentration and the
Michaelis constant, which accounts for the saturation degree of micropollutant-transforming
enzymes in the system. At low saturation levels (\( S \ll K_m \)), the reaction rate in the Michaelis-
Menten equation would follow first-order kinetics, as expected for organic micropollutants in
biological wastewater treatment systems (Joss et al., 2006). At high saturation levels (\( S >> K_m \)), on the other hand, the reaction rate would follow zero-order kinetics, as expected for
non-rate-limiting concentrations of ammonia (20 mg/L, NH\textsubscript{4}\textsuperscript{+}-N) and bulk organics (200
mg/L, COD) in activated sludge (Kristensen et al., 1992).

A shift in kinetics from first- to zero-order was observed for naproxen when the initial
concentration was increased from 4 to 100 µg/L (Figure 1), which suggests a possible
saturation of the transformation capacity of the biomass. Similar kinetic effects have been
indicated in the literature for a few other organic micropollutants, such as ibuprofen (Collado
et al., 2012) and trimethoprim (Jewell et al., 2016b). In addition, Jewell et al. (2016b) noted a
change in the dominant transformation pathway of trimethoprim from O-demethylation to
hydroxylation with increasing trimethoprim concentrations, which suggests that some
transformation pathways are saturated at an earlier stage than others.

Five previously known transformation products of ibuprofen (Zwiener et al., 2002), naproxen
(Quintana et al., 2005), and diclofenac (Kosjek et al., 2009; Bouju et al., 2016; Jewell et al.,
2016a) were measured quantitatively (Figure 1 and Figure S4). The underlying
transformation reactions included hydroxylation, demethylation, amidation and
decarboxylation. Primary demethylation of naproxen resulted in the formation of O-
desmethylnaproxen, whereas primary hydroxylation of ibuprofen and diclofenac resulted in
the respective formation of 2-hydroxyibuprofen and 4-hydroxydiclofenac. Although O-
desmethylnaproxen was formed in both the low and high concentration experiments, there is
an analogy to a previous study with respect to a possible saturation of the demethylation
pathway. The change in the dominant transformation pathway of trimethoprim from O-
demethylation to hydroxylation with increasing trimethoprim concentrations (Jewell et al.,
2016b) accords well with the stronger response in the transformation kinetics of naproxen (O-
demethylation) with increasing micropollutant concentrations than of ibuprofen and
diclofenac, subjected to hydroxylation and other non-O-demethylating reactions.

The recorded transformation products accounted only for a fraction of the degraded
micropollutants (Figure 1 and Figure S4). There are most likely two main reasons for this
observation. First, the list of primary transformation products was probably not complete and
more than one such product can be formed from ibuprofen (Zwiener et al., 2002), as observed
for diclofenac (Figure 1 and Figure S4). Second, the primary transformation products detected
in this study were further transformed (Figure 1 and Figure S4). Several secondary and
tertiary transformation products have also been detected for diclofenac (Jewell et al., 2016a).
3.2 CO₂ formation (Tier 2)

A concurrent dissipation of the radioactivity in the liquid phase and formation of radiolabeled CO₂ was observed for the four radiolabeled micropollutants in contact with the carrier-attached microorganisms (Figure 2). This phase transfer of the ¹⁴C-labeled part of the micropollutants was predominantly biologically mediated, as indicated by the lack of trapped ¹⁴CO₂ in the heat-treated controls (80 °C, 10 min).

The dissipation rate of ¹⁴C in the liquid phase, $k_{bio, ¹⁴C}$, of the four organic micropollutants was lower, often much lower, than that of the parent compound, $k_{bio, parent}$ (Table 2). For naproxen and its ¹⁴C-labeled methyl group, the rate difference between the dissipation of the parent compound and the radiolabeled moiety was quite small (i.e. $k_{bio, parent} / k_{bio, ¹⁴C} ≈ 1.5$), which supports O-demethylation (Figure 1) as an important transformation reaction of naproxen in biological wastewater treatment systems with carriers or at least that the O-demethylation occurs at a similar rate as the degradation of the parent compound. The cleavage rate of the ¹⁴C-labeled carboxylic moiety of ibuprofen and diclofenac was, however, much lower than the dissipation rate of the parent compound (i.e. $k_{bio, parent} / k_{bio, ¹⁴C}$ was 3-4 for ibuprofen and 5-8 for diclofenac). Among the four transformation products that were observed for these two compounds, only one lacked the ¹⁴C-labeled carbon, namely diclofenac benzoic acid (Figure S4). The remaining three transformation products resulted from hydroxylation and amidation reactions without cleavage of the organic structure of the compounds. Thus, many reactions involved in the transformation of organic micropollutants are not CO₂-forming.

The measured formation of ¹⁴CO₂ did not fully account for the dissipation of ¹⁴C in the liquid phase (Figure 2). Similar observations have previously been noted for ibuprofen, naproxen,
diclofenac and mecoprop in incubations with soil (Richter et al., 2007; Dodgen et al., 2014) or biofilm carriers (Casas et al., 2017). Additional tests were performed to evaluate whether the observed discrepancy between the formation of $^{14}$CO$_2$ and the dissipation of $^{14}$C in the liquid phase could be attributed to losses of $^{14}$CO$_2$ during the daily replacement of the headspace or to the precipitation of $^{14}$CO$_3^{2-}$ species. Results from these tests showed minor $^{14}$C losses during the replacement of the headspace (<2% of the total $^{14}$C addition), minor formation of $^{14}$CO$_2$ during acidification (pH 3.5) of the reactors containing $^{14}$C-labeled ibuprofen, naproxen and mecoprop (<2% of the total $^{14}$C addition) and a notable formation of $^{14}$CO$_2$ during acidification of the reactors containing diclofenac (5-7% of the total $^{14}$C addition) (Table S2). As the $^{14}$CO$_2$ formation during acidification of the heat-treated diclofenac control was slightly higher than in the biologically active reactor, the relatively high formation of $^{14}$CO$_2$ during the acidification of the diclofenac reactors is expected to have resulted from a pH-instability of the $^{14}$C-labeled carboxyl group rather than from an elevated production of $^{14}$CO$_3^{2-}$.

During the evaluation of the potential losses of $^{14}$CO$_2$, it was noted that the number of scintillation counts per mL of sodium hydroxide in the CO$_2$ trap decreased during prolonged contact times (i.e. days) with the scintillation liquid. The decrease in scintillation counts was most likely due to a slow release of the trapped $^{14}$CO$_2$ in contact with the acidic scintillation liquid (pH 6). However, the applied scintillation liquid (Ultima Gold™ XR) has been reported to be appropriate for $^{14}$CO$_2$ applications as long as the contact time is kept below 1 d (PerkinElmer, 2008), which is much longer than the maximum exposure time of 4 h in this study.

### 3.3 Biological uptake (Tier 3)
An imaging technique, MAR-FISH, was used to evaluate whether the radiolabeled carbon in the investigated micropollutants was accumulated by the carrier-attached microorganisms. Figure 3 shows MAR and FISH images of biofilm segments in an incubation with diclofenac[carboxyl-\(^{14}\)C]. The aggregation of black silver grains in the MAR images indicates an uptake of the \(^{14}\)C-labeled carboxyl group of diclofenac by the carrier-attached microorganisms. In the corresponding FISH images, the presence of microorganisms in the MAR-positive areas with aggregating silver grains is confirmed by the universal bacterial probe set (EUB338 and EUB338-III). Semi-quantitative screening along predefined transects of the MAR-FISH slides indicated that no more than one per mille of the areas targeted by the universal bacterial probe set were MAR-positive for the four radiolabeled micropollutants.

Two of the 13 sub-populations are depicted in Figure 3. The cells targeted by the bacteroidaceal probe (BAC303) are MAR-negative, as they are detected outside the MAR-positive area, while the small cluster targeted by the deltaproteobacterial probe (DELTA495a) could be MAR-positive, as these cells are covered with silver grains. In large biofilm segments, however, there is a risk that the aggregation of silver grains in connection with a specific group of microorganisms results from an uptake of adjacent and non-targeted microorganisms. To reduce this risk, further MAR analysis of the FISH-probed subpopulations was directed towards small isolated cell clusters, as illustrated in Figure 4 and Figure S5.

According to the MAR-FISH analysis of the isolated cell clusters, the rare MAR-positive cells were not evenly distributed across the microbial community (Table 3). MAR-positive signals were observed among a few phylogenetic groups, but the majority of each of these targeted groups were MAR-negative. Moreover, the FISH-labeled populations containing MAR-
positive cells differed between the micropollutants, which suggests that the $^{14}$C-uptake of the individual micropollutants is associated with specific microorganisms.

The MAR-FISH results were quite consistent for the two carrier-attached biofilms, as both the biofilms from Switzerland and Sweden showed a similar uptake of ibuprofen by *Firmicutes*, naproxen by *Gammaproteobacteria* and diclofenac by *Deltaproteobacteria* (Figure 4 and Figure S5). MAR-positive signals were, however, only associated with a small fraction of these three phylogenetic groups and several MAR-positive cells could not be associated with any of the FISH-probed populations, which suggests additional involvement of other non-targeted microorganisms.

### 3.4 Microbial community structure (Tier 4)

At the fourth tier, 16S rRNA gene amplicon sequencing was used to evaluate the microbial coverage of the applied FISH probes and to identify taxonomic families within the MAR-positive populations (Figure 5). Approximately 80% of the biofilm communities were covered by the FISH probes. Most core phyla in activated sludge, such as *Proteobacteria, Firmicutes,* and *Nitrospira,* (Mielczarek et al., 2013) were also present in the carrier-attached biofilms. The relative abundance of *Nitrospira* in the two biofilms (10-30%) was, however, much higher than in activated sludge (0.2-6%) and membrane bioreactor sludge (1-9%) (Nielsen et al., 2010; Saunders et al., 2013). Whether *Nitrospira* tend to proliferate in the biofilm in hybrid biofilm-activated sludge processes for municipal wastewater treatment is not fully known, but results from a hybrid biofilm-activated sludge process for oil sands process-affected water has shown a higher abundance of *Nitrospira* in the biofilm (24%) than in the suspended flocs (10%) (Huang et al., 2017).
For the three populations with MAR-positive signals in the two carrier-attached biofilms, the relative abundances were 7 to 13% for *Gammaproteobacteria*, 2 to 5% for *Firmicutes*, and 1 to 3% for *Deltaproteobacteria*. Since these abundances are much higher than that of the MAR-positive cells (≤0.1%), it appears that only specific members of these phylogenetic groups were involved in the uptake of ibuprofen, naproxen, diclofenac, or mecoprop.

Thirty taxonomic families of *Gammaproteobacteria*, *Deltaproteobacteria*, and *Firmicutes* were detected and quantified (Figure 5). The majority (90%) of these families showed relative abundances of less than 1%, and 50% showed relative abundances of less than 0.1%. The presence of 21 of the 30 families belonging to *Gammaproteobacteria*, *Deltaproteobacteria* and *Firmicutes* was also observed in both biofilms. However, the results of this study do not allow the uptake of ibuprofen, naproxen, diclofenac or mecoprop to be linked specifically to a taxonomic family or species, since the MAR-FISH results were obtained at a higher taxonomic rank.

### 3.5 Implications for identification of micropollutant-transforming microorganisms

With respect to the identification of micropollutant-transforming microorganisms in biological wastewater treatment, MAR-FISH has the potential to circumvent some of the inherent limitations of *i*) cultivation methods where the enrichment and isolation may lead to characterization of environmentally irrelevant species (Dunbar et al., 1997), *ii*) inhibition methods where the applied inhibitor may be less specific than originally expected (Men et al., 2017), and *iii*) correlation methods where the observed associations between biotransformation rates and microbial abundances or activities could be false-positive and non-causal (Gaulke et al., 2008; Johnson et al., 2015). Despite these advantages, MAR-FISH has its own limitations. First, the method is time-consuming, which hampers the screening of
micropollutant-transforming microorganisms in biological wastewater treatment at low
taxonomic ranks. Second, MAR-FISH can only target micropollutant-transforming
microorganisms with an uptake and accumulation of the radiolabeled isotope. Third, the low
number of microorganisms in biological wastewater treatment systems with an uptake of the
radiolabeled isotope makes it difficult to identify a sufficient number of MAR-positive cells to
confirm their involvement in the transformation of the micropollutant. Thus, the MAR-FISH
results in this study should be considered indicative rather than conclusive. Nonetheless, these
results do represent a first step towards in situ identification of micropollutant-transforming
microorganisms in biological wastewater treatment, and an important indication that isotope-
based methods could be used to test the increasing number of micropollutant-related
associations and hypotheses arising from high-throughput sequencing.

3 Conclusions

The combined use of different transformation endpoints and microbial evaluation steps to
assess the degradation of four organic micropollutants (ibuprofen, naproxen, diclofenac and
mecoprop) by carrier-attached microorganisms from two municipal wastewater treatment
plants resulted in the following key observations:

- The primary degradation of the four micropollutants in contact with the carrier-
attched microorganisms showed decreasing relative transformation rates (dC/(dt·C₀))
at increasing micropollutant concentrations, as expected from Michaelis–Menten
kinetics, and consisted of at least four transformation reactions, namely hydroxylation,
demethylation, decarboxylation and amidation.
• Carrier-attached microorganisms from municipal wastewater treatment plants can partly mineralize ibuprofen, naproxen, diclofenac and mecoprop, which was confirmed by the formation of $^{14}$CO$_2$.

• Only a small fraction (≤1‰) of carrier-attached microorganisms from municipal WWTPs appears to be involved in the uptake of ibuprofen, naproxen, diclofenac and mecoprop or their transformation products containing a $^{14}$C-labeled moiety.

• Two carrier attached-biofilms indicated a similar $^{14}$C uptake of ibuprofen by Firmicutes, naproxen by Gammaproteobacteria and diclofenac by Deltaproteobacteria.

• Microbial transformation of organic micropollutants appears to be performed by individual specialists rather than by the majority of the members of a phylogenetic class.

• Although not fully explored, the MAR-FISH results in this study indicate a high degree of substrate specialization among micropollutant-transforming microorganisms that could cause differences in transformation rates and patterns between compounds and microbial communities in full-scale wastewater treatment plants.

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Table 1: Overview of micropollutants, wastewater treatment plants and experimental procedures.

<table>
<thead>
<tr>
<th>Micropollutants</th>
<th>Ibuprofen</th>
<th>Naproxen</th>
<th>Diclofenac</th>
<th>Mecoprop</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1" alt="Chemical Structure" /></td>
<td><img src="image2" alt="Chemical Structure" /></td>
<td><img src="image3" alt="Chemical Structure" /></td>
<td><img src="image4" alt="Chemical Structure" /></td>
<td><img src="image5" alt="Chemical Structure" /></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>WWTPs</th>
<th>Bad Ragaz WWTP</th>
<th>Klippen WWTP</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>5.4</td>
<td>5.7</td>
</tr>
<tr>
<td>SRT (d)</td>
<td>3.7</td>
<td>4.0</td>
</tr>
<tr>
<td>COD (mg/L)</td>
<td>430</td>
<td>480</td>
</tr>
<tr>
<td>NH₄⁺-N (mg/L)</td>
<td>Influent 620</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>Effluent 30</td>
<td>Influent 37</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Effluent 4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Effluent 6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Experimental overview</th>
<th>Tier 1 Transformation</th>
<th>Tier 2 CO₂ formation</th>
<th>Tier 3 Uptake</th>
<th>Tier 4 Microbial community structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Analytical method</td>
<td>LC-tandem MS</td>
<td>Liquid scintillation counting</td>
<td>NAA-RISH</td>
<td>16S rRNA gene amplicon sequencing</td>
</tr>
<tr>
<td>Incubation time (d)</td>
<td>5</td>
<td>5</td>
<td>25</td>
<td>5</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>25</td>
<td>25</td>
<td>6.7-7.2</td>
<td>25</td>
</tr>
<tr>
<td>pH</td>
<td>6.7-7.2</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Wastewater</td>
<td>Biologically treated</td>
<td>Biologically treated</td>
<td>Biologically treated</td>
<td>Biologically treated</td>
</tr>
<tr>
<td>Number of carriers per box</td>
<td>5</td>
<td>5</td>
<td>25</td>
<td>5</td>
</tr>
</tbody>
</table>

Targeted regions: 16S
Database: MGAS
Table 2: Transformation rate constants \( (k_{\text{bio,parent}}) \) and \(^{14}\text{C}\) dissipation rate constants \( (k_{\text{bio,}^{14}\text{C}}) \) in parallel experiments with non-labeled and \(^{14}\text{C}\)-labeled micropollutants.

<table>
<thead>
<tr>
<th>Micropollutant</th>
<th>k_{\text{bio,parent}} (L/(gSS·d))</th>
<th>k_{\text{bio,}^{14}\text{C}} (L/(gSS·d))</th>
<th>k_{\text{bio,parent}} / k_{\text{bio,}^{14}\text{C}}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bad Ragaz 100 µg/L</td>
<td>Bad Ragaz 4 µg/L</td>
<td>Klippan 4 µg/L</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>21 ± 4</td>
<td>38 ± 2</td>
<td>23 ± 2</td>
</tr>
<tr>
<td>Naproxen</td>
<td>n.a.</td>
<td>5.8 ± 0.8</td>
<td>9.6 ± 1.5</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>2.2 ± 0.2</td>
<td>4.5 ± 0.5</td>
<td>2.8 ± 0.2</td>
</tr>
<tr>
<td>Mecoprop</td>
<td>0.4 ± 0.0</td>
<td>5.1 ± 0.5</td>
<td>3.0 ± 0.2</td>
</tr>
</tbody>
</table>

The 95% confidence intervals are indicated by ±, and n.a. denotes not available due to zero-order kinetics.
Table 3: Summary of MAR-FISH results.

<table>
<thead>
<tr>
<th>Probe</th>
<th>Specificity</th>
<th>Reference</th>
<th>Ibuprofen</th>
<th>Naproxen</th>
<th>Diclofenac</th>
<th>Mecoprop</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALP968</td>
<td><em>Alphaproteobacteria except Rickettsiales</em></td>
<td>Neef (1997)</td>
<td>–/–</td>
<td>–/–</td>
<td>–/–</td>
<td>–/–</td>
</tr>
<tr>
<td>BET42a</td>
<td><em>Betaproteobacteria</em></td>
<td>Manz et al. (1992)</td>
<td>–/–</td>
<td>–/–</td>
<td>–/–</td>
<td>–/–</td>
</tr>
<tr>
<td>GAM42a</td>
<td><em>Gammaproteobacteria</em></td>
<td>Manz et al. (1992)</td>
<td>–/–</td>
<td>+/+</td>
<td>–/ (+)</td>
<td>–/–</td>
</tr>
<tr>
<td>DELTA495a</td>
<td>Most Deltaproteobacteria and Gemmatimonadetes</td>
<td>Lücker et al. (2007)</td>
<td>–/–</td>
<td>–/ n.a.</td>
<td>+/ +</td>
<td>+/ (+)</td>
</tr>
<tr>
<td>Ntspa662</td>
<td><em>Genus Nitrospira</em></td>
<td>Daims et al. (2000)</td>
<td>–/–</td>
<td>(+)/ n.a.</td>
<td>–/–</td>
<td>–/–</td>
</tr>
<tr>
<td>BAC303</td>
<td>Most Bacteroidaceae and Prevotellaceae, some Porphyromonadaceae</td>
<td>Manz et al. (1996)</td>
<td>–/–</td>
<td>–/–</td>
<td>–/–</td>
<td>–/–</td>
</tr>
<tr>
<td>TM7-905</td>
<td><em>Phylum candidate division TM7</em></td>
<td>Hagenholtz et al. (2000)</td>
<td>–/–</td>
<td>–/–</td>
<td>–/–</td>
<td>–/–</td>
</tr>
<tr>
<td>GNSB-941</td>
<td><em>Phylum Chloroflexi</em></td>
<td>Gich et al. (2001)</td>
<td>–/–</td>
<td>–/–</td>
<td>–/–</td>
<td>–/–</td>
</tr>
<tr>
<td>CFX1223</td>
<td><em>Phylum Chloroflexi</em></td>
<td>Björnsson et al. (2002)</td>
<td>–/ (+)</td>
<td>–/ n.a.</td>
<td>–/–</td>
<td>–/–</td>
</tr>
<tr>
<td>PLA46</td>
<td><em>Planctomycetes</em></td>
<td>Neef et al. (1998)</td>
<td>(+)/ (+)</td>
<td>–/–</td>
<td>–/–</td>
<td>–/–</td>
</tr>
<tr>
<td>HGC69a</td>
<td><em>Actinobacteria</em></td>
<td>Roller et al. (1994)</td>
<td>–/–</td>
<td>–/–</td>
<td>–/–</td>
<td>–/–</td>
</tr>
<tr>
<td>LGC354a,b,c</td>
<td><em>Firmicutes</em></td>
<td>Meier et al. (1999)</td>
<td>+/ +</td>
<td>–/ n.a.</td>
<td>–/ –/ +</td>
<td>–/–</td>
</tr>
<tr>
<td>ARCH915</td>
<td><em>Archaea</em></td>
<td>Stahl and Amann (1991)</td>
<td>–/–</td>
<td>–/–</td>
<td>–/–</td>
<td>–/–</td>
</tr>
<tr>
<td>Non-EUB338</td>
<td>Negative control</td>
<td>Wallner et al. (1993)</td>
<td>–/–</td>
<td>–/–</td>
<td>–/–</td>
<td>–/–</td>
</tr>
</tbody>
</table>

+       Containing MAR-positive cells
(+      Containing cells that could be MAR-positive
–       MAR-negative
/       Separates results from Bad Ragaz and Klippan (Bad Ragaz / Klippan)
*       Competitor used in combination with target probe
n.a.    Not available
Figure 1: Relative concentration changes for four micropollutants and three transformation products in incubations with carrier-attached microorganisms from two municipal wastewater treatment plants. The lines in the left-hand panels (parent compounds) show the fit of the first-order model whereas those in the right-hand panels (transformation products) are added to support readability. The two last data points for naproxen (Bad Ragaz 4 µg/L) are not included in the regression analysis. Note the differences in the scale of the y-axes.
Figure 2: Partitioning of added $^{14}$C between the liquid phase and the gas phase in incubations with four $^{14}$C-labeled micropollutants (~4µg/L) in contact with carrier-attached microorganisms from two municipal wastewater treatment plants and a heat-treated control. Sample losses have been accounted for, by subtracting the recorded losses from the added radioactivity at each time point.
Figure 3: Single and combined MAR and FISH images of biofilm segments from Klippan WWTP after incubation with diclofenac[carboxyl-14C]. MAR-positive signals with aggregating black silver grains indicate a microbial uptake of diclofenac. Microorganisms targeted by the universal bacterial probe set (EUB338 and EUB338-III) are colored green and microorganisms targeted by the bacteroidaceal probe (BAC303) or deltaproteobacterial probe (DELTA495a) are colored red. Circles indicate the location of cells with an active uptake of diclofenac. The scale bars represent 10 µm.
Figure 4: MAR-FISH images of isolated biofilm colonies from Bad Ragaz WWTP after incubation with ibuprofen[RS-carboxyl-¹⁴C], naproxen[O-methyl-¹⁴C], diclofenac[carboxyl-¹⁴C] or RS-mecoprop[Ring-u-¹⁴C]. MAR-positive signals with aggregating black silver grains indicate a microbial uptake of the ¹⁴C-labeled micropollutants. Microorganisms targeted by the firmicutes probes (LGC354a, b, and c), gammaproteobacterial probe (GAM42a) or deltaproteobacterial probe (DELTA495a) are colored in red. The white arrows show MAR-positive colonies and the black arrow shows a MAR-negative colony. The scale bars represent 10 µm.
Figure 5: Relative abundance of microorganisms based on amplicon sequencing of carrier-attached biofilms from Bad Ragaz WWTP and Klippan WWTP. The pie charts are structured according to the general specificity of the FISH probes. The bar charts show the abundance of microorganisms at a family level, when a MAR-positive signal for a $^{14}$C-labeled micropollutant has been connected to a microbial class/phylum through FISH. Error bars and ± signs represent standard deviations (n=3). The average number of measured OTUs per sample was 42,000 ± 400 (Bad Ragaz) and 33,500 ± 900 (Klippan).
Highlights

- Partial mineralization of organic micropollutants by carrier-attached microorganisms
- Microbial uptake of $^{14}$C-labeled micropollutants by carrier-attached microorganisms
- Only a small fraction of the microorganisms ($\leq 1 \%$) accumulated the $^{14}$C moiety
- The $^{14}$C-accumulating microorganisms differed between the micropollutants