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Yujie He[1,2], Alette A.M. Langenhoff[1]*, Rob N.J. Comans[3], Nora B. Sutton[1], Huub H.M. Rijnaarts[1]

1. Department of Environmental Technology, Wageningen University and Research, P.O. Box 17, 6700 AA Wageningen, The Netherlands

2. State Key Laboratory of Pollution Control and Resource Reuse, School of the Environment, Nanjing University, 163 Xianlin Avenue, 210023 Nanjing, China

3. Department of Soil Quality, Wageningen University and Research, P.O. Box 47, 6700 AA Wageningen, The Netherlands

* Corresponding author. Email: alette.langenhoff@wur.nl; Tel.: +31 (0)317 480254; Fax.: +31 (0)317 482108.

Abstract

Natural dissolved organic matter (DOM) and nitrification can play an important role in biodegradation of pharmaceutically active compounds (PhACs) in aerobic zones of constructed wetlands (CWs). This study used an enrichment culture originating from CW sediment to study the effect of DOM and nitrification on aerobic biodegradation of seven PhACs. The enriched culture degraded caffeine (CAF), metoprolol (MET), naproxen (NAP), and ibuprofen (IBP) with a consistent biodegradability order of CAF > MET > NAP > IBP. Biodegradation of propranolol, carbamazepine, and diclofenac was insignificant (<15%). CAF biodegradation was inhibited by the easily biodegradable DOM. Conversely, DOM enhanced biodegradation of MET, NAP, and IBP, potentially by contributing more biomass capable of degrading PhACs. Nitrification enhanced biodegradation of NAP and IBP and mineralization of the PhAC mixture as well as less biodegradable DOM, which may result from co-metabolism of ammonia oxidizing bacteria or

enhanced heterotrophic microbial activity under nitrification. MET biodegradation was inhibited in the presence of nitrification. DOM and nitrification effects on PhAC biodegradation in CWs gained from this study can be used in strategies to improve CW operation, namely: designing hydraulic retention times based on the biodegradability order of specific PhACs; applying DOM amendment; and introducing consistent ammonium streams to increase removal of PhACs of interest.

Keywords

Pharmaceuticals; Aerobic biodegradation; DOM; Nitrification; Constructed wetland; Microbial degradation preference

1. Introduction

The occurrence and accumulation of PhACs become a growing concern with the extensive detection of pharmaceutically active compounds (PhACs) in wastewater effluent, surface water, groundwater and sediment (Hernando et al., 2006). Some PhACs are beginning to be associated with adverse effects in aquatic organisms at environmentally relevant concentrations, i.e. ng/l to µg/l (Fatta-Kassinos et al., 2011). The conventional wastewater treatment plants (WWTPs) could be a key barrier to eliminate PhAC discharge into the surface water, but are at this moment not designed to treat PhACs sufficiently (Li et al., 2014). Therefore, development of post-treatment processes in addition to conventional WWTPs are important ways to further remove PhACs from domestic effluents.

An alternative technique that could be coupled to WWTP is a constructed wetland (CW), which is sustainable, inexpensive in construction and easy to operate and maintain (Meng et al., 2014). In recent years CWs have shown promising functionality for PhAC removal (Zhang et al., 2014).

PhAC removal in CWs involves various mechanisms such as photodegradation, sorption, biodegradation, and phytoremediation (Hijosa-Valsero et al., 2016). Of those mechanisms, biodegradation plays a main role in the transformation and mineralization of PhACs (Li et al., 2014). PhAC biodegradation from wastewater is favored by aerobic conditions (Zhang et al., 2014). Oxygen supporting aerobic biodegradation of PhACs can be present in CWs throughout the water column in free water surface flow CWs, in the upper sediment layer, and in rhizosphere area of CWs (Eger, 1994). Therefore, it is of importance to study the aerobic biodegradation of PhACs in CWs functioning as contaminant remediation systems. Compared to related studies with soil and river sediment (Koba et al., 2016; Lin and Gan, 2011; Zhang et al., 2013), aerobic biodegradation of PhACs in CW sediment or using enrichment cultures originating from CW sediment is hardly investigated. A rare study of aerobic biodegradation of carbamazepine (CBZ) and ibuprofen (IBP) in CW sediment focused on the biodegradation kinetics of these PhACs (Conkle et al., 2012).

CW sediments contain a large and potentially reactive pool of dissolved organic matter (DOM), and DOM plays an important role in the bioavailability and fate of contaminants in CWs (Chin et al., 1998). DOM is a complex mixture of organic compounds varying widely in molecular weight, from small molecules like hydrophilic organic acids to intermediate polymers like hemicellulose, and large humic substances such as humic acids (HA) and fulvic acids (FA) (Khadka et al., 2016). DOM is believed to interact with aerobic microbial communities influencing the biodegradation of organic contaminants through various mechanisms, including catabolic processes, redox cycling, and nutrient bioavailability (Van Trump et al., 2006). The effect of DOM on the biodegradation of pesticides, herbicides, and polycyclic aromatic hydrocarbon are well investigated (Van Trump et al., 2006). However, studies on the DOM impact on aerobic biodegradation of PhACs as emerging contaminants are limited.

Nitrification is an important redox reaction under aerobic conditions. Nitrification is known to enhance PhAC removal in various systems, e.g. nitrifying activated sludge (NAS), and membrane bioreactors (Batt et al., 2006; De Gusseme et al., 2009; Tran et al., 2009). It is believed that the ammonia monooxygenase (AMO) enzyme in ammonia oxidizing bacteria (AOB) can co-metabolize non-target PhACs in biochemical processes (Benner et al., 2013; Yi and Harper, 2007). Nevertheless, the co-metabolism occurs for some PhACs, but is ineffective for others. For example, the biodegradation rate of 17α-ethinylestradiol (EE2) significantly decreased after AOBs were inhibited, whereas trimethoprim biodegradation was not influenced by AOB inhibition in the same NAS systems (Khunjar et al., 2011). Moreover, contradicting findings of nitrification on PhAC biodegradation were reported in different treatment systems. Fernandez-Fontaina et al. (2016) showed that AOB enhanced biodegradation of IBP in NAS, whereas AOB was ineffective for IBP biodegradation in biofiltration columns packed with anthracite (Rattier et al., 2014). To date, there is no information available on the role of nitrification in biodegradation of PhACs in CW sediment microbial culture, especially for a group of PhACs with different physiochemical properties.

This study applied a microbial enrichment culture originating from CWs to investigate the effect of DOM and nitrification on the aerobic biodegradation of a mixture of seven PhACs with different physiochemical properties. This research aims to elucidate the role of DOM and nitrification in CWs on PhAC biodegradation under aerobic conditions.

2. Materials and methods

2.1. Chemicals and reagents

PhACs were purchased commercially: (±)-metoprolol (+)-tartrate salt (MET, ⩾98%), caffeine (CAF, ⩾99%), (±)-propranolol hydrochloride (PRO, ⩾99%), CBZ (⩾99%), naproxen (NAP, ⩾98%), IBP (⩾98%), and fenoprofen calcium salt (FEN, ⩾97%) from Sigma Aldrich Chemie B.V (the Netherlands). PhAC mixture stock (20 mg/L) was prepared with MilliQ water (Millipore, USA). Acetonitrile and water with 0.1% formic acid (Biosolve B.V., the Netherlands) were used for ultra-performance liquid chromatography (UPLC) analysis. All other chemicals used were of analytical grade.

2.2. Batch experiments

Batch experiments were performed to study the effect of DOM and nitrification on the aerobic biodegradation of a mixture of 7 PhACs in a microbial enrichment culture. The enriched culture is originally from CW sediment microbial culture, which was acclimated aerobically with PhAC mixture (1 mg/l each) for 22 months in growth medium (Table S1 of the Supplementary Materials). The spiked PhAC concentration is above the environmental relevant concentrations of PhACs to enrich PhAC-degrading cultures with PhACs as the sole carbon source. The mixed sediment was collected from two CWs that are applied as post-treatment processes for two Dutch WWTPs.

15 g sediment inoculum was mixed with 120 ml growth medium for the incubation. Batch bottles sealed with rubber stoppers (250 ml) were incubated on a shaker (120 rpm) at 20 °C and covered with aluminum foil to avoid photodegradation of PhACs. The gas phase was filled with over-pressured air (1.3 bar) and was exchanged to maintain the O_2 supply (>10% of the gas phase) during the incubation. During 22 months of incubation, the inoculum was amended with PhAC mixture repeatedly once easily biodegradable PhACs (CAF, MET, NAP, IBP) were almost depleted to enrich a PhAC-degrading microbial community. Afterwards, 10% of the enrichment

(11 ml) was transferred to fresh aerobic medium (110 ml) spiked with PhAC mixture, and incubated for 2 weeks to obtain sediment free enrichment culture. After repeating the transfer twice, 10% of the active PhAC degrading enrichment was used as inoculum for our batch experiments. TOC analysis confirmed that no residual organic matter (< 0.3 mg/l, detection limit) was transferred from the original sediment inoculum to our batch experiments.

Table 1 Designed experimental conditions to investigate the effect of DOM and nitrification on removal of PhACs.

Conditions	PhACs (1 mg/l each)	DOM (22.8 mg/l in carbon)	Nitrification (ammonium, 41.7 mg/l)
A	√	×	×
B	√	√	×
C	√	×	√
D	√	√	√
E	×	√	×

For the experiments, five batch conditions were designed as shown in Table 1. Nitrification was inhibited by spiking with allylthiourea (ATU, 5 mg/l) in both conditions A and B. Condition E was only amended with DOM to determine its biodegradability in the microbial enrichment culture. For each condition, biotic batches were conducted in triplicate and abiotic controls in duplicate. Abiotic bottles were prepared by autoclaving for 20 min at 120 °C and adding chemical inhibitors (0.3 g/L $HgCl_2$ and 1.3 g/L NaN_3) after cooling down. Milli-Q water was added to batches without DOM, ATU or chemical inhibitor addition to maintain the same liquid volume (131.9 ml) and PhAC concentration in all batches.

The batch experiments lasted for 35 days and liquid samples were withdrawn at day 0, 1, 2, 4, 7, 10, 14, 22, 28, and 35. Samples were pre-treated by centrifugation at 10000 rpm for 5 min prior to UPLC and ion chromatography (IC) analyses. DOM samples were taken at day 0 and 35 from

triplicate batches and mixed to compare the composition variation during the biodegradation. 50 ml liquid samples were filtered using 0.2 µm Isopore^{TM} membrane filters (Milipore, Ireland) and stored in centrifuge tubes at -20°C for DNA extraction. Based on pre-test of ATU efficiency, at day 23 ATU was re-spiked to maintain nitrification inhibition.

2.3. DOM extraction, fractionation and characterization

DOM was extracted from the sediment (together with litters) in the CW to obtain natural organic matter. The sediment was mixed with Milli Q water (1:1, wet w/w) by shaking at 180 rpm for 1 h. The mixture was centrifuged at 3750 rpm for 15 min and the supernatant obtained was further centrifuged at 10000 rpm for 20 min. Afterwards the supernatant was filtered through 0.45 µm mixed cellulose ester filters (ME25/21, Whatman, USA) to collect the DOM. For the 35 days batch experiments, DOM samples were collected at day 0 and 35 and fractionated to get the fractions including HA, FA, hydrophilic compounds (Hy), and hydrophobic neutrals (HON), according to the procedure reported by Van Zomeren and Comans (2007). Work-flow of the procedure is summarized in Fig. S1. Recovery of the fractionation procedure was 84-96% in this work. For characterization, the DOM samples were first freeze-dried to solid material by an alpha 2-4 LD+ freeze dryer (Martin Christ GmbH, Germany) and then characterized by using a Bruker Tensor 27 Fourier transform infrared spectrometer (FTIR) equipped with a Platinum ATR accessory (Bruker, Germany).

2.4. Chemical analysis

Concentrations of PhACs were measured by a UPLC (Ultimate 3000, Thermo, USA) equipped with diode array detection, as previously described (He et al., 2016). Detection limits of the PhACs analyses were 50 µg/l. Quantification of PhACs was performed based on the internal standard (FEN) and external calibration standards. Concentrations of NO_2^- and NO_3^- were

155 analyzed by an IC (Dionex ICS-2100, Thermo, USA) as described in the work of Wilt et al. (2018).
156 Concentrations of O_2 and CO_2 were followed by gas chromatography (GC-2010, Shimadzu,
157 Japan) according to the method described previously (Wilt et al., 2018). A Sievers™ 900 Series
158 TOC Analyzer (GE Analytical Instrument, USA) was used to quantify the concentrations of DOM
159 and its fractions.

160 2.5. Molecular analysis

161 DNA was extracted from the filtered samples using PowerSoil® DNA Isolation Kits (MoBio
162 Laboratories, USA) according to the manufacturer's protocol. Concentration and purity of the
163 extracted DNA were estimated by a Nanodrop spectrophotometer (Denovix, DS-11, USA). The
164 DNA extracts were stored at -20 °C until further analysis. Quantitative PCR (qPCR) was used to
165 quantify the abundances of bacterial 16S rRNA gene and AOB amoA genes. The qPCR
166 measurements were performed in triplicate by a CFX384 touch real-time PCR detection system
167 (Bio-Rad, Veenendaal, the Netherlands). In the reaction mixture, 2 µl diluted sample (10 times
168 dilution) was mixed with 2 µl master mix comprising of iQ™ SYBR® Green (Bio-Rad), primers
169 (forward and reverse), and DNase-free water (50:2:2:26). Standards and blanks were prepared
170 similarly to the samples. Standard curves were obtained by using serial dilutions of a known
171 amount of plasmid DNA containing a fragment of the respective genes. Detailed information of
172 the primers and cycling conditions used for qPCR analyses are summarized in Table S2.
173 Abundance of amoA was represented in absolute concentration and 16S rRNA gene-normalized
174 values.

175 **3. Results and discussion**

176 3.1. Biodegradation of PhACs

177 Biodegradation of the seven PhACs tested showed remarkable differences. For example, in
178 condition C with nitrification present, half of MET was removed and CAF, NAP, and IBP were
179 completely removed within 35 days (Fig. 1c). This can be ascribed to biodegradation as no
180 obvious removal was found in abiotic controls (Fig. S2). Our study obtained comparable or
181 higher removal for the biodegradation of CAF, MET, and NAP than biodegradation observed in
182 microbial cultures of soil and river sediments (Koba et al., 2016; Lin and Gan, 2011; Zhang et al.,
183 2013). A CW sediment-based research presented that 50% of IBP can be removed within nine
184 days resulting from sorption onto sediment and biodegradation by natural microbial population
185 (Conkle et al., 2012). In comparison, our sediment-free enrichment cultures gave a 50%
186 biodegradation approximately within 22 days. Similar with our findings (Fig. S3), PRO, CBZ, and
187 DFC were reported to be persistent during biodegradation tests (Tixier et al., 2003; Xu et al.,
188 2016; Zhang et al., 2008). The same conclusions can be drawn for the biodegradability of the
189 seven PhACs in conditions A (nitrification inhibition), B (nitrification inhibition and DOM
190 addition), and D (with both DOM and nitrification present), as indicated in Fig. 1. Especially, in
191 conditions B and D with DOM addition, no obvious sorption of PhACs onto the extracted DOM
192 was found in the abiotic controls (Fig. S2). In Fig. 1 the notable increase of IBP concentrations in
193 between may be attributed to detection deviations. PRO, CBZ, and DFC will not be discussed in
194 the following content considering their insignificant biodegradation (<15%, Fig. S3).

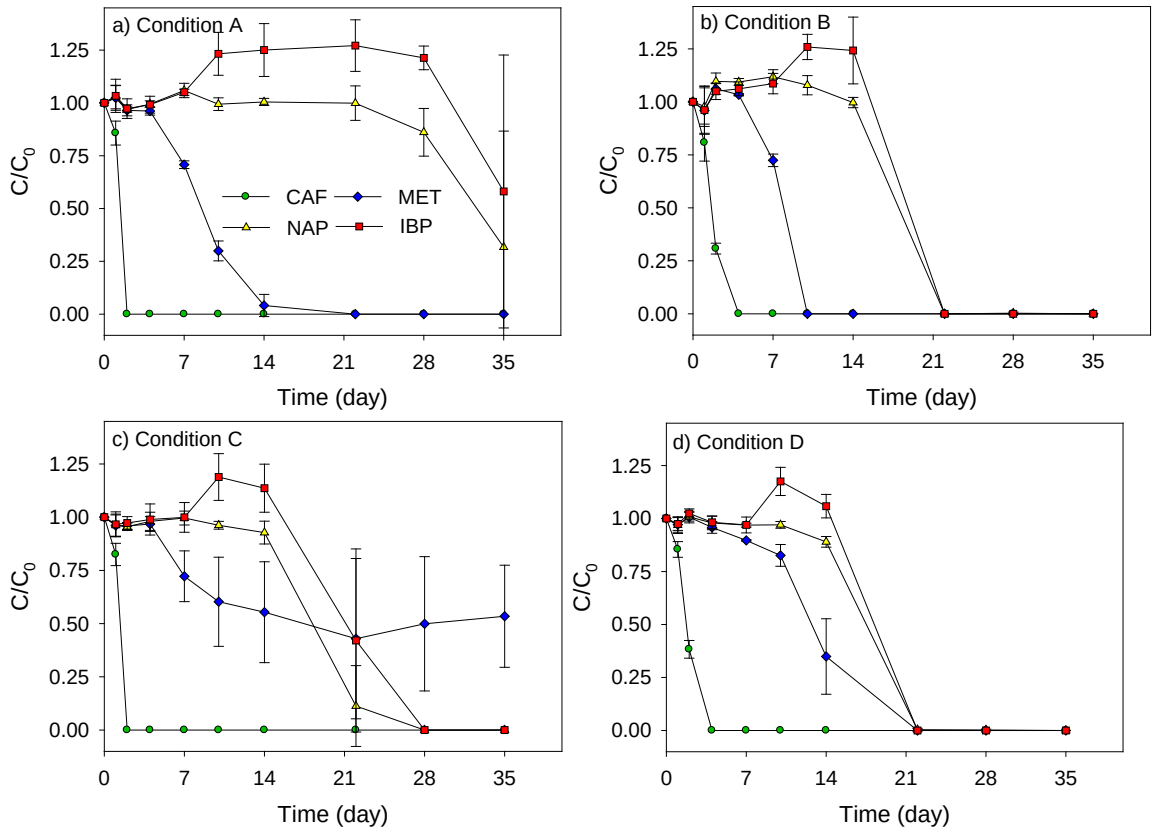


Fig. 1. Biodegradation of a mixture of seven PhACs in the microbial enrichment culture under different conditions: conditions A (nitrification inhibition), B (nitrification inhibition and DOM addition), C (with nitrification present), and D (with both DOM and nitrification present). Results are mean values ± standard deviation (n = 3).

Microorganisms seem to have a preferential order when biodegrading PhACs, i.e. less biodegradable PhACs were only biodegraded once the readily degradable PhACs were removed. As shown in Fig. 1c, CAF was first biodegraded during day 0-2 followed by MET during day 2-14, and finally NAP and IBP during day 14-28 in condition C. The notable standard deviation of MET biodegradation was due to different biodegradation activity in our triplicate batches, but the biodegradation tendency in the triplicates were consistent where MET was not further biodegraded after day 14 (Fig. S4a). The microbial biodegradation preference was also observed for the other conditions: CAF > MET > NAP > IBP (Fig. 1). Therefore, our study demonstrates a

clear microbial preference in the order of PhACs biodegraded regardless of conditions with or without DOM and nitrification.

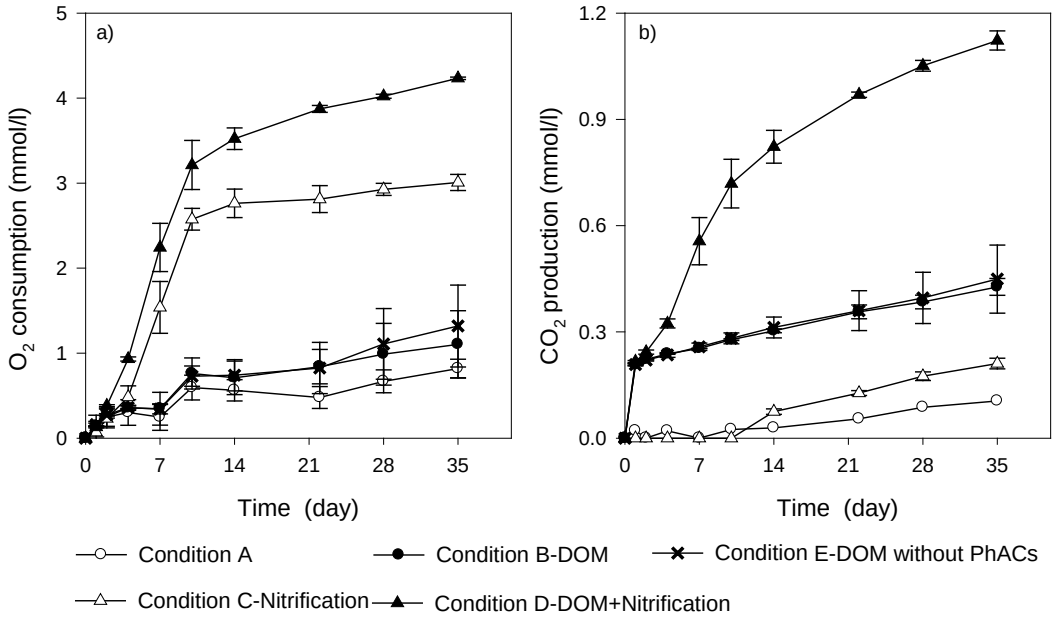


Fig. 2. Microbial respiration in the five conditions: a) consumption of O_2 and b) production of CO_2. Results are mean values ± standard deviation (n = 3).

PhACs could be a sole carbon substrate to support microbial growth, as seen in condition A. Looking at the microbial respiration, O_2 was consumed and CO_2 was produced from PhACs in condition A (Fig. 2). Moreover, the amount of total bacteria in this condition increased from 1×10^4 to 4.7×10^4 copies/ml after 35 days incubation (Fig. S5a). Both the microbial respiration and growth supported by PhACs indicate that PhACs could be used as a sole carbon substrate, and results in a selective pressure to enrich microorganisms capable of biodegrading PhACs. Similarly, ketoprofen was reported to act as a sole source of carbon and energy for microorganisms in a membrane bioreactor (Quintana et al., 2005). These findings demonstrate the possibility to enrich microorganisms capable of biodegrading PhACs for the use of enhanced biodegradation technologies such as bioaugmentation. Iasur-Kruh et al. (2011) successfully

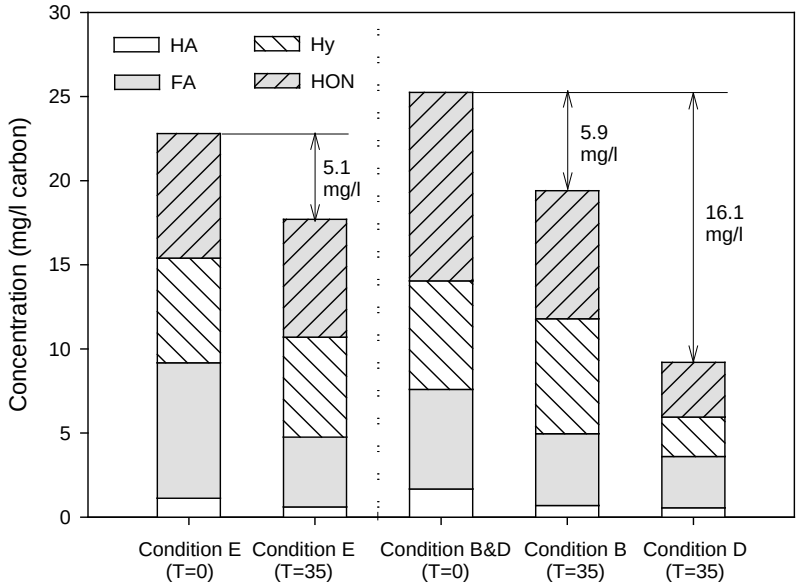
enriched and isolated a pure culture of *Novosphingobium JEM-1* from CW sediment and bioaugmented the culture to CW sediment film to achieve 40% removal of estron (E1) and 17-estradiol (E2) in batch experiments.

3.2. Effect of DOM on PhAC biodegradation

First, the characteristics of the DOM extracted from CW sediment were investigated. The DOM fractions comprised 5% HA, 35% FA, 27% Hy, and 33% HON (Fig. 3, condition E with only DOM present). As presented in the FTIR spectra (Fig. S6), the main absorption bands of the extracted DOM corresponded to the following functional groups: 1) 3390 cm^{-1} (O-H stretching of inter- and intra- molecular hydrogen bonds); 2) 1639 cm^{-1} (aromatic C=C skeletal vibrations, asymmetric stretching of C=O, symmetric stretching of COO-); 3) 1359 cm^{-1} (C-O asymmetric stretching of COOH conditions); 4) 1105 cm^{-1} (C-O stretching of alcohols and carbohydrate) (Chen et al., 2002; Traversa et al., 2014).

Second, the biodegradability of the DOM was evaluated by microbial respiration and fractionation studies. DOM was rapidly mineralized to CO_2 in the first two days followed by a relatively slow mineralization rate in the following days (Fig. 2b, condition E). A similar rapid CO_2 production was also found in conditions B and D with DOM present. Furthermore, we confirmed that the significant CO_2 production in the first two days was related to DOM mineralization rather than DOM breakdown due to abiotic process (results not shown). The observed fast production of CO_2 may indicate that the microbial enrichment culture first rapidly consumed relatively easily biodegradable DOM, followed by utilization of the less biodegradable DOM with a slower rate. This microbial preference towards carbon substrate was reported in previous work, where the authors found that Gram-negative bacteria in the soil prefer to use fresh plant-derived carbon while Gram-positive bacteria use more older soil organic matter-derived carbon

247 independent on the soil depth (Kramer and Gleixner, 2008). The microbial biodegradation
248 preference for DOM compositions is similar to the microbial preference found among the PhACs.



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250 Fig. 3. Composition of DOM fractions at day 0 and 35 in conditions with DOM present (HA =
251 humic acid, FA= fulvic acid, Hy = hydrophilic compounds, HON = hydrophobic neutrals).

252 In terms of the microbial respiration, the easily biodegradable DOM contributes to half of the
253 CO_2 production and the less biodegradable DOM is responsible for the other half. In total 22%
254 DOM was consumed during 35 days incubation, with HA and FA being the main metabolized
255 DOM fractions (Fig. 3, respectively 46% and 49% consumed). This is in line with a previous
256 modelling study, where HA and FA are mentioned as significant DOM fractions for microbial
257 respiration (Straathof, 2015). The functional groups of 3390 and 1639 cm^{-1} in DOM disappeared
258 after 35 days biodegradation (Fig. S6), which indicates that the microbial enrichment culture is
259 capable to transform DOM with those structures.

260 3.2.1. DOM inhibits biodegradation of CAF

The effect of DOM on PhAC biodegradation was identified by comparing the biodegradation efficiencies of PhACs between conditions A and B as well as between conditions C and D. In the presence of DOM, CAF biodegradation was delayed (Fig. 1). CAF in conditions A and C without DOM addition was completely biodegraded within 2 days. This coincides with the time to consume the easily biodegradable DOM (Fig. 2). Furthermore, CAF contains functional groups of asymmetric stretching of C=O (1639 cm^{-1}) (Gunasekaran et al., 2005). These functional groups are also present in our extracted DOM and they disappeared during 35 days of incubation due to biodegradation (Fig. S6). Therefore, we assume that the easily biodegradable DOM competed with the readily biodegradable CAF resulting in a delay in CAF biodegradation till day 4 in the presence of DOM (Fig. 1b and 1d). The competition between the easily biodegradable DOM and CAF also indicates a microbial biodegradation preference, which is similar to the preferences found among PhACs and among DOM compositions.

3.2.2. DOM enhances biodegradation of MET, NAP, and IBP

As opposed to CAF, biodegradation of MET, NAP, and IBP was enhanced by DOM addition. After the easily biodegradable DOM and the readily biodegradable CAF was utilized, microorganisms started degrading the other PhACs. A faster biodegradation of MET, NAP, and IBP was found in conditions B and D compared to conditions A and C in general (Fig. 1). As with MET in condition C, the standard deviation of IBP biodegradation at day 22 was caused by different biodegradation activity in triplicate batches (Fig. S4b), but in general the IBP biodegradation in this condition was slower than in condition D (Fig. 1c and 1d).

More biomass was found in the conditions with DOM addition (Fig. S5a, B > A, D > C). Furthermore, the extracted DOM can act as a substrate or a co-substrate together with PhACs for the microbial enrichment culture as indicated by the increased CO_2 production in conditions

B and D compared to conditions A and C (Fig. 2b). The observed higher biomass production and higher microbial respiration with DOM addition indicate a more active microbial community that may have increased PhAC biodegradation capacity. Rauch-Williams et al. (2010) hypothesized that removal of organic contaminants including NAP and IBP was caused by a specialized microbial community growing on hydrophobic acids, i.e. HA and FA, as in our case.

There appears to be other hypotheses to explain this DOM enhanced PhAC biodegradation. First, DOM may act as structural analogues to enhance PhAC biodegradation. By biodegrading DOM with similar molecular structures to PhACs, microorganisms may activate more enzymes that can readily biodegrade PhACs with a similar structure. This structural analogue theory has been applied for the biodegradation of organic compounds such as pesticides, or cyclic ethers (Foght et al., 2001; Kim et al., 2008). Second, DOM can act as electron shuttles to stimulate microorganisms to degrade PhACs. DOM is a complex mixture of organics with many functional groups that could be potentially functional in redox reactions (Van Trump et al., 2006). For example, quinones in DOM (especially in HA) can act as redox-mediating functional groups to stimulate the electron transfer between electron donors and electron acceptors and thus enhance redox reactions (Martinez et al., 2013).

It has been confirmed that biodegradation of certain PhACs are dependent on the availability and concentrations of DOM (Lim et al., 2008; Maeng et al., 2011). However, there are no studies that explicitly describe the mechanism of DOM in the enhancement of PhAC biodegradation. Further research efforts need be dedicated to understand the underlying mechanisms of these processes.

3.3. Effect of nitrification on PhAC biodegradation

306 Nitrification occurred in conditions C and D whereas it was inhibited in conditions A and B by the
307 addition of ATU. 70 % of the ammonium in batches was converted to NO_3^- in conditions C and D
308 along with biodegradation of PhACs. NO_3^- was not formed in the other two conditions (Fig. S7).
309 In the q-PCR tests, amoA abundance was higher in conditions C and D as compared to the other
310 two conditions, indicating an enrichment of AOBs (Fig. S5b). The detection efficiency of amoA
311 absolute abundance in conditions B and D might be influenced by the presence of humic acids
312 due to inhibition of enzymatic reaction (Fig. S5b), as reported by Schriewer et al. (2011).
313 However, this effect in those two conditions was absent after normalization for abundance
314 relative to the 16S rRNA gene abundance (Fig. S5b). In our nitrification conditions, nitrification
315 plays an important role in microbial respiration as it stoichiometrically accounts for 39% and 38%
316 of the O_2 consumption for NO_2^- and NO_3^- production in conditions C and D, respectively.

317 3.3.1. Nitrification enhances biodegradation of NAP and IBP

318 The effect of nitrification on PhAC biodegradation is seen by comparing the biodegradation
319 efficiencies between conditions A and C and between conditions B and D. Nitrification enhanced
320 the biodegradation of NAP and IBP. Significantly higher biodegradation of NAP and IBP was
321 found in condition C than in condition A (Fig. 1). No difference was observed between conditions
322 B and D, which were both amended with DOM.

323 The enhancement by nitrification is likely a result of AOB driven co-metabolism. As
324 demonstrated by Fernandez-Fontaina et al. (2016), biodegradation of NAP and IBP was
325 enhanced by AOB instead of contribution from heterotrophic microorganisms. AOB containing
326 AMO can easily hydroxylate linear alkyl carbons especially for the secondary and tertiary
327 carbons in the chain, due to its low specificity and broad substrate spectrum (Fernandez-
328 Fontaina et al., 2016). Such structures can be found in NAP and IBP (Fig. 5, circles). Co-metabolic

function of AOB was reported for many other PhACs in nitrifying bioreactors and membrane bioreactors (Roh et al., 2009; Yi and Harper, 2007). It has been demonstrated that sludge-based wastewater treatment processes with significant nitrification showed higher removal efficiencies than those without nitrification for 90% of the investigated PhACs (Xu et al., 2016). Comparable with sludge-based microbial cultures, our findings demonstrate a positive effect of nitrification on PhAC biodegradation also in the CW sediment derived microbial cultures.

In our case, nitrification not only enhanced biodegradation of NAP and IBP but also enhanced mineralization of PhACs, as illustrated in the increased CO_2 production in condition C compared to condition A (Fig. 2b). In addition to increase PhAC mineralization, nitrification also stimulated mineralization of less biodegradable DOM. DOM fractionation reveals that condition D with nitrification showed more biodegradation of HA, Hy, and HON, compared to conditions B (Fig. 3). The more biodegraded DOM in condition D was mainly mineralized to CO_2, as illustrated by comparing the carbon balances between conditions D and B. In condition D, more DOM was consumed than in condition B (equal to 0.85 mmol CO_2/l medium, Fig. 3) and more CO_2 was produced (0.70 ± 0.04 mmol/l medium, Fig. 2b). The enhanced biodegradation of less biodegradable DOM might be due to co-metabolism via AOB and/or enhanced heterotrophic microbial activity. Microbial CO_2 production was higher in conditions C and D with nitrification than in conditions A and B, respectively (Fig. 2b), which indicates higher heterotrophic microbial activity under nitrifying conditions. The higher heterotrophic microbial activity might contribute to the higher mineralization of PhACs and less biodegradable DOM under nitrifying conditions.

3.3.2. Nitrification inhibits biodegradation of MET

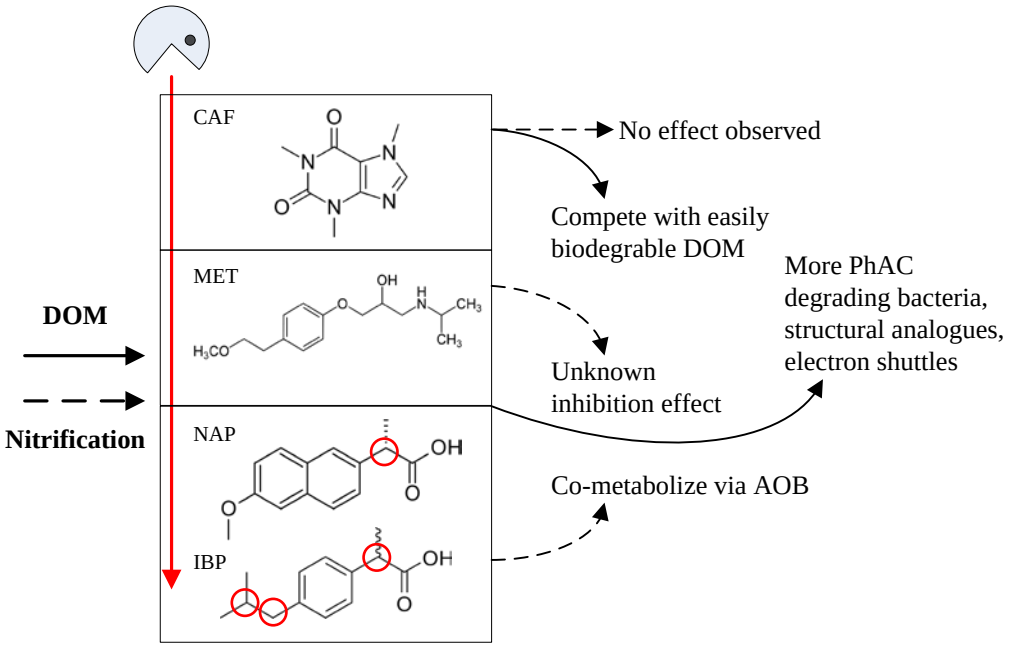
MET biodegradation was inhibited in the presence of nitrification, as indicated in Fig 4. To date, there is no literature referring to inhibition of PhAC degradation in nitrifying cultures; only

352 enhanced PhAC biodegradation has been reported, as described at 3.3.1, or no effect on PhAC
353 biodegradation has been observed (Khunjar et al., 2011). To confirm the unexpected finding, we
354 repeated our experiment with MET and the same conclusion was obtained (Table S3). The
355 inhibitory effect of nitrification on MET is not yet well understood. Further research is needed to
356 elucidate the observed discrepancies of nitrification effect on NAP and IBP, and MET.

357 Compared to the other three biodegradable PhACs, biodegradation of CAF was not obviously
358 influenced by nitrification. The reason might be that CAF was readily biodegradable and thus an
359 effect of nitrification could not be observed.

360 3.4. Implications for theory and practice

361 In summary, the effect of DOM and nitrification on the biodegradation of PhACs is shown in Fig.
362 5. Translating our findings to practice, first the clear biodegradability order among PhACs can
363 provide an operational strategy for designing hydraulic retention time (HRT) for removing
364 specific PhACs. Second, DOM addition could be a strategy to enhance the aerobic
365 biodegradation of less biodegradable PhACs in CWs. Instead of harvesting dead plants
366 seasonally and transporting them for special disposal, dead plant biomass can be (partially)
367 disposed directly into a CW to obtain more natural DOM. Third, nitrification is beneficial for
368 biodegradation of PhACs with certain structures. However, ammonium concentration is
369 normally low in the influent of CWs used as post-treatment, resulting in limited nitrification. A
370 sub-stream of wastewater influent of WWTPs rich in ammonium could be introduced to CWs to
371 strengthen the biodegradation of PhACs of interests.



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373 Fig. 5. Proposed mechanisms of the effect of DOM and nitrification on biodegradation of
374 caffeine, metoprolol, naproxen, and ibuprofen. DOM pathway represented by solid lines;
375 nitrification pathways represented by dashed lines. Directions of arrows show enhancement
376 (up), inhibition (down), and no effect (horizontal). The red line represents the biodegradability
377 order of the microbial enrichment culture. Red circles on the structure of NAP and IBP label the
378 secondary and tertiary carbons in linear alkyl chains for hydroxylation by ammonia oxidizing
379 bacteria (AOB).

380 **4. Conclusions**

381 This study contributes to the understanding of the impact of DOM on aerobic biodegradation of
382 PhACs and the role of nitrification in PhAC biodegradation in enrichment cultures originating
383 from CW sediment.

384 • Our enrichment culture showed different biodegradation capacities for the seven PhACs
385 that all had different physiochemical properties. Aerobic microbial communities appear

to have a biodegradation preference for the biodegradable PhAC in the order of CAF > MET > NAP > IBP. It is feasible to use PhACs as a sole carbon substrate as a selective pressure to enrich microorganisms capable of biodegrading PhACs for the use of enhanced biodegradation technologies.

- With DOM present, the easily biodegradable DOM was rapidly consumed followed by less biodegradable DOM. Biodegradation of CAF competed with easily biodegradable DOM during the first two days. In contrast, DOM enhanced the biodegradation of MET, NAP, and IBP, potentially by either contributing more biomass capable of degrading PhACs, acting as structural analogues, or acting as electron shuttles to stimulate microorganisms to metabolize or co-metabolize PhACs. The exact mechanisms need to be further identified.
- Nitrification enhanced the biodegradation of NAP and IBP which may result from AOB driven co-metabolism. MET biodegradation was inhibited in the presence of nitrification. In comparison, no difference was observed for the readily biodegradable CAF in the presence or absence of nitrification.
- For application, the clear biodegradability preference found among PhACs can offer a strategy to design different HRTs to remove corresponding PhACs. From the operational perspective, DOM additions and introducing consistent ammonium streams may be used to increase the potential of CWs to achieve higher PhAC biodegradation as post-treatment processes.

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