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Research Paper

Unravelling the contribution of nitrifying and methanotrophic bacteria to micropollutant co-metabolism in rapid sand filters

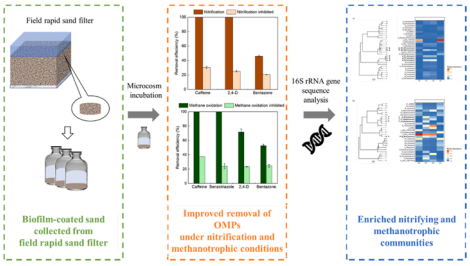
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HIGHLIGHTS

- Nitrifying and methanotrophic communities from field RSF co-metabolize OMPs.
- The removal of caffeine, 2,4-D and bentazone were improved under nitrification.
- Methane oxidation enhanced the removal of caffeine, benzotriazole, 2,4-D, bentazone.
- *Nitrosomonas* and *Nitrospira* dominated nitrifying community.
- Methanotrophic community contained *Methylobacter*, *Methylomonas* and *Methylotenera*.

GRAPHICAL ABSTRACT



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ABSTRACT

The presence of organic micropollutant (OMP) in groundwater threatens drinking water quality and public health. Rapid sand filter (RSF) rely on biofilms with nitrifying and methanotrophic bacteria to remove ammonia and methane during drinking water production. Previous research observed the partial removal of OMPs with active nitrification and methane oxidation due to co-metabolic conversion of OMPs. However, the contribution of indigenous nitrifying and methanotrophic communities from RSF has yet to be fully explored. Accordingly, experiments were carried out with biofilm-covered sand collected from field-scale RSF, to assess the removal of nine OMPs by nitrifying and methanotrophic bacteria. Results indicated that stimulating nitrification resulted in significantly more removal of caffeine, 2,4-dichlorophenoxyacetic acid and bentazone. Stimulating methanotrophic conditions enhanced the removal of caffeine, benzotriazole, 2,4-dichlorophenoxyacetic acid and bentazone. Microbial community analysis based on 16 S rRNA gene sequencing revealed *Nitrosomonas* and *Nitrospira* are the dominant genus in the community under nitrifying conditions. The three genera *Methylobacter*, *Methylomonas* and *Methylotenera* were enriched under methanotrophic conditions. This study highlights that nitrifying and methanotrophic bacteria play important roles during OMP removal in field RSF. Furthermore, results suggest that bioaugmentation with an enriched nitrifying and methanotrophic culture is a promising approach to improve OMP removal in RSF.

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1. Introduction

Groundwater is an important resource for drinking water production. However, many organic micropollutants (OMPs) including pesticides and pharmaceuticals are widely detected in groundwater at trace concentrations from the low ng/L to the μg/L range (Albers et al., 2015; Phillips et al., 2015). These OMPs can adversely affect the quality and safety of drinking water, due to their recalcitrance and suspected toxicity (Jones et al., 2005; Schwarzenbach et al., 2006; Zuehlke et al., 2007). Thus, various treatment technologies are employed at drinking water treatment plants (DWTPs) to effectively remove OMPs, such as activated carbon adsorption (Westerhoff et al., 2005; Kim and Kang, 2008; Piai et al., 2020), membrane filtration (Košutić et al., 2005) and advanced oxidation (Westerhoff et al., 2005). However, these treatments usually have high investment and energy costs (Vandermaesen et al., 2016), and could possibly produce large amounts of toxic transformation products (Müller et al., 2012; Pereira et al., 2011). Therefore, more economic and sustainable approaches should be exploited to remove OMPs from drinking water at DWTPs.

Rapid sand filter (RSF) is a widely applied technology for treatment of groundwater at DWTPs, where different pollutants can be removed by an interplay of biological and chemical processes. Organic and inorganic particles are retained in a sand bed (Teunissen et al., 2008; Craft, 1966), while ammonia and methane are oxidized by nitrifying and methanotrophic bacteria (Gülay et al., 2016; Hu et al., 2020; Papadopoulou et al., 2019). In addition, Fe(II), Mn(II) and As(III) can be removed by biological or/and chemical oxidation (van Beek et al., 2012; Vries et al., 2017; Gude et al., 2016). In addition to these traditional RSF treatment functions, recent studies have also reported the potential of RSF to remove OMPs (Zearley and Summers, 2012; Hedegaard et al., 2014). Vandermaesen et al. (2016) found that 34% of 2,6-dichlorobenzamide (BAM) and 63% of 2,6-dichlorobenzoic acid (2,6-DCBA) were degraded by microorganisms growing in full-scale RSF. In addition, a column-scale RSF achieved at least 50% of removal of four pesticides, seven pharmaceuticals and one biological algal metabolite (Zearley and Summers, 2012). However, the majority of studies using field-collected RSF sand examine overall OMP removal efficiency but provide no insight into OMP removal mechanism (Zearley and Summers, 2012; Hedegaard et al., 2014).

Due to the limited OMP adsorption onto sand material, biodegradation of OMPs by autochthonal microbial populations is the most important removal mechanism of OMPs in RSF (Ho et al., 2006; Hedegaard et al., 2014; Wang et al., 2021). Microorganisms can degrade OMPs via primary metabolism in which heterotrophic bacteria can utilize OMPs as their growth substrates, such as carbon sources (Zearley and Summers, 2012; Benner et al., 2013). Furthermore, co-metabolism results in the transformation of OMPs by nonspecific enzymes produced by microorganisms using a different substrate for their primary metabolism (Alexander, 1981; Benner et al., 2015). Nitrifying and methanotrophic bacteria are able to co-metabolically degrade OMPs though the activity of their ammonia monooxygenase enzyme (AMO) (Roh et al., 2009; Rattier et al., 2014) and methane monooxygenase enzyme (MMO) (Jiang et al., 2010; Semrau et al., 2010; Papadopoulou et al., 2019), respectively. Rod et al. (2009) reported that AMO can not only promote ammonia oxidation, but also degrades a number of hydrocarbon compounds as well. In addition, one nitrifying bacterium, *Nitrosomonas europaea*, was shown to co-metabolically transform OMPs, including estrogens, triclosan and bisphenol A (Roh et al., 2009). Similarly, Benner et al. (2015) used methanotrophic cultures enriched from different inocula to degrade a variety of OMPs, indicating that MMO was capable of oxidising sulfamethoxazole and benzotriazole. Moreover, recent studies provided evidence of co-metabolic bentazone and phenoxy acids biodegradation in lab-scale RSF (Hedegaard et al., 2018; Papadopoulou et al., 2019).

To our knowledge, few studies have reported on the individual roles of autochthonous nitrifying and methanotrophic microorganisms from field RSF in OMP removal, especially for various OMPs with different properties. In addition, the communities of nitrifying bacteria and methanotrophic bacteria related with OMP biodegradation in field RSF remain unclear. Therefore, this study used biological sand sampled from full-scale RSF to assess the contribution of nitrifying and methane oxidizing biomass to the removal of a mixture of five pesticides, three pharmaceuticals and one industrial compound. The abundance and diversity of nitrifying and methanotrophic bacteria were determined by qPCR and 16S rRNA gene sequence analysis. This investigation verified the capability of autotrophic communities in field RSF to biodegrade the OMPs via co-metabolic mechanisms. Promisingly, our study revealed the feasibility of employing nitrifying and methanotrophic bacteria in full-scale RSF for OMPs removal at DWTPs, and also gave suggestions for enhancing OMP removal.

2. Materials and methods

2.1. Sand samples

Sand material was collected from different groundwater-fed RSF operated by Oasen Water Company and Evides Water company (Rotterdam, the Netherland). The sand was stored at 4 ℃ until use. Sand material properties and intake water quality of each RSF are listed in Table 1. The raw water of two field RSFs located in Oasen and Evides, from which nitrifying and methanotrophic inocula were collected, respectively contained large amounts of ammonia and methane. At the Evides location, the majority of methane was removed by an aeration tower where the activity of methanotrophic bacteria should be higher than the ones in RSF. Therefore, the sand collected from Evides was firstly soaked in the aeration water until use to get more methanotrophic inocula.

2.2. Chemicals and reagents

Five pesticides (2,6-dichlorobenzamide (BAM), mecoprop, 2,4-dichlorophenoxyacetic acid (2,4-D), chloridazon and bentazone), three pharmaceuticals (salicylic acid, caffeine and ioxitalamic acid) and one industrial chemical (benzotriazole) were selected as model compounds in this study, as listed in Table S1. These selected compounds have been detected in groundwater in Europe, and are regarded as priority OMPs regularly observed in the source water used for drinking water production (Hedegaard et al., 2014; Vandermaesen et al., 2016). All the OMPs were commercially purchased from Sigma-Aldrich Chemie N.V. (the Netherlands), except for salicylic acid and ioxitalamic acid, which were purchased from Matrix Scientific (Columbia, USA) and Merck Schuchardt (Hohenbrunn, Germany), respectively. A mixture stock

Table 1
Characterization of sand material and raw water quality of field scale RSF.

Locations	Flow rate (m/h)	Sand size (mm)	Raw water quality (mg/L, except for As, which is μg/L)[a]							Bed height (m)	Filter surface (m^2)	Sand mass (kg)	Empty bed contact time (min)
			NH_4^+	CH_4	Fe	Mn	As	DOC	PO_4^{3-}				
Oasen	3.5	2–3.15	5.9	0.23	4.5	0.42	4.1	3.6	3.6	2	30	95,400	34
Evides	5.3	1.5–2.5	1.2	22.5[b]	2.4	0.13	<0.5	6.1	N.D.[c]	2	18.5	58,830	24

[a] For Oasen location, the raw water refers the influent of RSF;
[b] Most of CH_4 is removed by aeration tower, the concentrations of CH_4 after aeration and after RSF are 0.0769 and 0.0061 mg/L, respectively.
[c] N.D. refers to not detected.

solution of nine compounds (2 g/L) was prepared in methanol and stored at −20 ℃. In subsequent experiments, the stock solution was diluted to initial concentrations (approximately 400 μg/L) with MilliQ water (Millipore, USA) and prepared media. The composition of medium solutions is given in Table S2. All the chemicals used in this study were of analytical purity.

2.3. Microcosm experiments

2.3.1. Micropollutants biodegradation by nitrifying communities

Microcosm experiments were performed in 250 mL serum bottles to study the roles of nitrifying bacteria on OMP biodegradation (Fig. S1). In all batches, RSF sand (30 g) and 120 mL of prepared medium were added to each bottle, in which the headspace was flushed and set at 100% pressurized air (1.5 bar) by a gas exchanger system. Batch bottles were sealed with a rubber plug and an aluminium cap, and placed on a shaker (125 rpm) at 20 ℃ for 31 days. All the bottles were wrapped in aluminium foil to avoid OMP photodegradation. Ammonia was spiked in vials with initial concentrations of 21.3 ± 0.14 and 66.5 ± 1.13 mg NH_4^+-N/L at day 0 and day 11, respectively, to support the growth of nitrifying bacteria. In non-nitrification controls, allylthiourea (ATU, 5 mg/L) was used to suppress nitrification. Abiotic controls used chemical biocides to inhibit all biological activity (1.3 g/L NaN_3 and 0.3 g/L $HgCl_2$). In addition, blank control was incubated without OMPs. ATU and biocides were respiked at day 10 and day 20 to continuously inhibit nitrification and microbial activity, respectively. Equal amounts of Milli-Q water were spiked in batches without the addition of ATU or biocides to obtain the same working volume and OMP concentration in all bottles. All OMPs were added in similar concentrations (approximately 400 μg/L). Every test was performed in triplicate and under the same conditions. Results presented here are from duplicate analyses, the third bottle was operated parallelly as backup.

Since the purpose of this study is not to assess the OMP removal capacity of the whole autochthonal microbial community on RSF sand, the inoculum sands were not directly used for testing OMP removal. Although we cannot completely exclude that other populations are non-specifically affected by the inhibitor (ATU), the comparison of OMP removal between the stimulated and inhibited groups can still show the contribution of nitrifying bacteria to OMP co-metabolism.

2.3.2. Micropollutants biodegradation by methanotrophic communities

Batches to study the capacity of methanotrophic bacteria for OMP biodegradation were performed similarly as described in Section 2.2 (Fig. S1). However, the bottles contained 20% CH_4 and 80% pressurized air (1.5 bar) in headspace to support the growth of methanotrophic bacteria. Once the percentage of CH_4 or O_2 was below 3% (v/v), the headspace was refreshed with the same initial composition of CH_4 and pressurized air. In this case, the headspace in methanotroph active bottles was re-flushed by every day. In non-methanotrophic control bottles, methanotrophic bacteria were inhibited by acetylene (approximately 2% v/v) in the headspace (Benner et al., 2015). Acetylene can rapidly and irreversibly inactivate MMO by formation of reactive intermediates where the active hydroxylase subunit will disappear (Prior and Dalton, 1985). Acetylene were respiked at day 10 and day 20 to inhibit methanotrophic activity.

Table 2
Sand samples for molecular analysis.

Nitrification Group		Methanotrophic Group	
Sample name	Condition	Sample name	Condition
A0	Inoculum[a]	M0	Inoculum[a]
A1	Nitrification without OMPs	M1	Methanotrophic without OMPs
A2	Nitrification with OMPs	M2	Methanotrophic with OMPs
A3	Nitrification inhibited	M3	Methanotrophic inhibited
A4	Abiotic	M4	Abiotic

[a] The inoculum samples were taken from two field RSFs without addition of OMPs.

2.4. Molecular analysis

The sand samples were collected from triplicate bottles that all showed similar physiological characteristics in the experiment, and then mixed in one tube for DNA extraction. DNA was extracted by DNeasy PowerSoil Kit (QIAGEN, Hilden, Germany) from the raw RSF sand (the inoculum) and the collected sand samples at the end of experiment (approximately 1 g). The information of each sample was listed in Table 2. Quantative PCR was used to measure the copy numbers of total bacterial 16S rRNA genes, *amo*A genes coding for AMO (representing nitrifying bacteria) (Fowler et al., 2018), and *pmo*A genes coding for MMO (representing methanotrophic bacteria) (Papadopoulou et al., 2019). Each sample was assayed in technical triplicates by using a C1000 Thermal Cycler (CFX384 Real-Time system, Bio-Rad Laboratories, USA) with iQTM SYBR Green Supermix (Bio-Rad Laboratories, USA). The V4 variable region of the prokaryotic 16S rRNA gene was amplified, in which each sample was specified with a unique barcode at the 5-terminal of the consensus forward primer (515F: 5′-GTGCCAGC [AC]GCCGCGGTAA-3′) and reverse primer (806R: 5′-GGACTAC[ACT] [ACG]GGGT[AT]TCTAAT-3′) (Caporaso et al., 2011). The collected amplicons were pooled with negative (no template) and positive (synthetic Mock community of known composition) control samples, and pools were further sequenced by Hiseq2000 (GATC-Biotech, Germany). The output raw data was analysed by NG-Tax 2.0 pipeline (Poncheewin et al., 2020). The generated Biological Observation Matrix (BIOM) and tree files were included together to create the phyloseq object (McMurdie and Holmes, 2013), which was used for analysing the alpha and beta diversity, and microbial compositions. The NG-Tax 2.0 processed data was rarefied stochastically according to the lowest number of reads and then used for the comparisons between samples. In addition, several related R packages were used for downstream analysis, including ggplot2, microbiome, phyloseq and ggtree (McMurdie and Holmes, 2013). The 16S rRNA amplicon sequencing data have been uploaded to BioProject PRJEB48767 repository.

2.5. Analytical methods

2.5.1. Conventional chemical analysis

Liquid samples (1.5 mL) were taken from batch bottles at day 0, 1, 2, 3, 5, 7, 10, 13, 15, 18, 21, 25, 31. Samples were centrifuged at 15,000 rpm for 10 min. The supernatants were stored at − 20 ℃ before measurement. The concentrations of NH_4^+-N were measured by Ammonia TNTplus® chemistry and a spectrophotometer (DR3900, Hach, USA). Concentrations of NO_2^--N and NO_3^--N were analysed by ion chromatography (Dionex ICS-2100, Thermo, USA). CH_4, O_2 and CO_2 in the headspace of batch bottles were detected by gas chromatography (GC-2010, Shimadzu, Japan). The instrument was operated with a parallel combination column: Porabond Q (50 m x 0.53 mm; 10 μm) and Molsieve 5 A (25 m x 0.53 mm; 50 μm). The carrier gas was helium and set at 0.95 bar. Column temperature, detector and injection temperatures were 80 °C, 150 °C, and 120 °C, respectively. Pressure in the serum bottles was measured by a digital pressure meter (GMH 3151, Greisinger) for calculating the amounts of gases.

2.5.2. Quantification of OMPs

OMPs were measured by an ultra-performance liquid chromatography (UPLC) coupled with a diode array detector (DAD) (Dionex ultimate 3000, Thermo Fisher Scientific, USA). The column used was a CSH Phenyl-Hexyl column (1.7 μm, 2.1 × 150 mm) (Waters, USA). The mobile phase was a mixture of water with 0.1% (v/v) formic acid (solvent A) and acetonitrile with 0.1% (v/v) formic acid (solvent B) with a

flow rate at 0.3 mL/min and changing ratio through time. The column compartment temperature was 40 °C and the sample injection volume was 50 µL. Standards were prepared for a calibration range from 100 µg/L to 900 µg/L. The results of standards showed a good linearity (R^2 > 0.99). The results were automatically quantified by using Chromeleon™ software (version 7.2, Thermo, USA).

2.6. Evaluation of autotrophic biodegradation of OMPs in field RSF

To evaluate the potential co-metabolism of OMPs in field RSF, we calculated the expected OMP removal rates. The transformation yield, T_y, expresses the OMP removal rate over primary substrates (ammonia or methane) removal rate. However, the concentrations of OMPs and primary substrates in the present study are different from the field-relevant condition. Hedegaard et al. (2018) suggested to normalize the transformation yield with consideration of the concentration ratio between secondary and primary substrates. Therefore, we firstly calculated the normalized substrate preferences of selected compounds, SP_{OMP}, based on the data from batch test and the following equation (adjusted from Hedegaard et al., 2018):

$$SP_{OMP} = \frac{\frac{C_{OMP}}{C_{pri}}}{T_y} = \frac{\frac{C_{OMP}}{C_{pri}}}{\frac{r_{OMP}}{r_{pri}}} \quad (1)$$

where SP_{OMP} (-) is the normalized OMP substrate preference; T_y (-) is the transformation yield, which is the ratio of OMP removal rate, r_{OMP} (mg/L/h), versus primary substrate (ammonia or methane) removal rate, r_{pri} (mg/L/h); C_{OMP} (mg/L) and C_{pri} (mg/L) are the concentrations of OMP and primary substrates, respectively.

Then, the total decrease in concentration of OMPs by co-metabolic biodegradation under field condition, $\Delta C'_{OMP}$ (mg/L), can be expressed as:

$$\Delta C'_{OMP} = r'_{OMP} \times t = \frac{r'_{pri} \times \frac{C'_{OMP}}{C'_{pri}}}{SP_{OMP}} \times t \quad (2)$$

Thus, the total removal efficiency of OMPs by co-metabolism under field condition, R_{OMP} (%), can be calculated by the below Eq. (3):

$$R_{OMP} = \frac{\Delta C'_{OMP}}{C'_{OMP}} = \frac{\frac{r'_{pri}}{C'_{pri}}}{SP_{OMP}} \times t = \frac{R_{pri}}{SP_{OMP}} \quad (3)$$

where r'_{OMP} (mg/L/h) and r'_{pri} (mg/L/h) are the removal rate of the primary substrate (ammonia or methane) in field RSF; C'_{OMP} (mg/L) and C'_{pri} (mg/L) are the initial concentrations of OMPs and primary substrates in intake water of field RSF, respectively, t (h) is the empty bed contact time (EBCT), R_{pri} (%) is the total removal efficiency of primary substrate in field RSF.

3. Results and discussion

3.1. Biodegradation of OMPs during nitrification

Batches were incubated under four conditions (Fig. S1 and Table 2) to elucidate the role of nitrification in OMP biodegradation. Nitrification activity was assessed during incubation by the cumulative consumption of NH_4^+-N and production of NO_2^--N and NO_3^--N in liquid. The addition of ATU successfully inhibited nitrification, as observed by the absence of NH_4^+-N consumption and NO_2^--N and NO_3^--N production (Fig. S2). The nitrification rates were similar with or without OMP addition, suggesting that the nitrifying community was not directly affected by the presence of OMPs (Fig. S2). Under abiotic conditions, there was no consumption of NH_4^+-N and no production of NO_2^--N and NO_3^--N (Fig. S2). In addition, all the OMP compounds were not significantly removed (Fig. S5).

In the presence of nitrification activity, improved removal of caffeine, bentazone and 2,4-D, was observed as compared to nitrification inhibited tests (Fig. 1). However, different removal kinetics were observed for these compounds. Caffeine and 2,4-D were completely removed within 161 h and 600 h, while only 46% of bentazone was removed at the end of incubation (737 h) (Fig. 1a). Furthermore, the removal rate of 2,4-D increased after 425 h, which corresponded to an increased nitrification activity (Fig. S2). Removal of caffeine, 2,4-D and

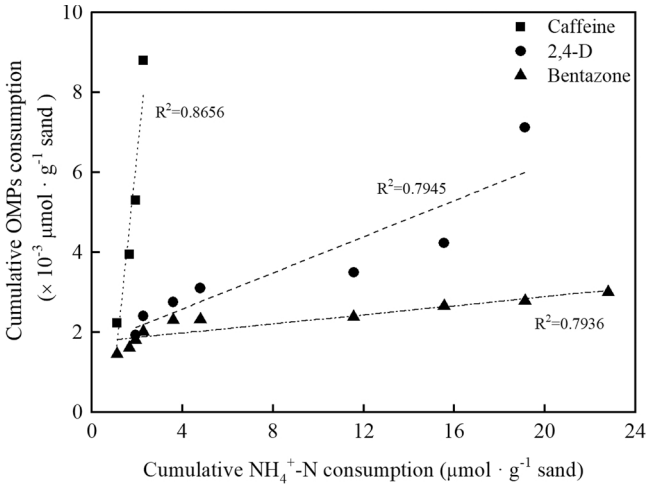


Fig. 2. Correlation between OMP biodegradation and ammonia oxidation. The data points where both OMPs and ammonia removal occurred was used for the correlation analysis. The selected incubation period was 41–161 h for caffeine, 113–593 h for 2,4-D and 41–737 h for bentazone.

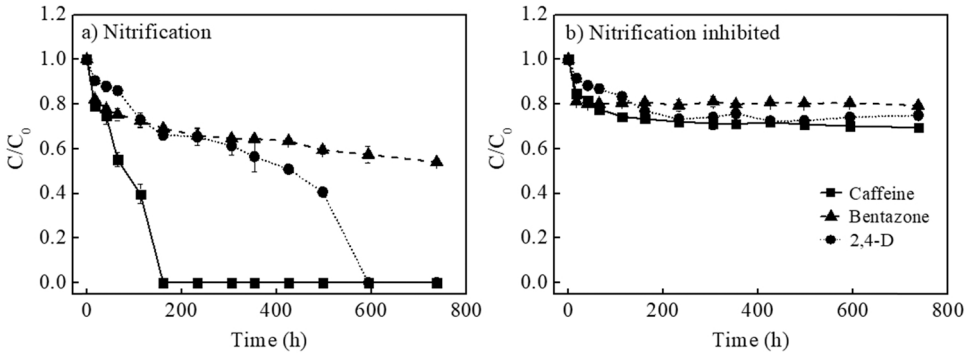


Fig. 1. Influence of nitrification on the biodegradation of caffeine, 2,4-D and bentazone in batches. a) Nitrification: addition of NH_4^+-N and OMPs; b) Nitrification inhibited: addition of NH_4^+-N, OMPs and allylthiourea (ATU). Each point is average of two independent samples from duplicate bottles ± standard deviation.

bentazone were linearly correlated with ammonia removal (Fig. 2), which indicated that degradation of caffeine, 2,4-D and bentazone coincided with the nitrification activity. As shown in Fig. 1a, with same nitrification activity, the order of OMP degradation was caffeine >2,4-D >bentazone.

Nitrification activity thus enhanced the removal of caffeine, bentazone and 2,4-D. However, nitrification did not stimulate the biodegradation of other compounds, suggesting that other OMPs are not susceptible to transformation by AMO (Fig. S5). Previous research showed that AMO enzymes have specific affinity to certain functional groups (Fernandez-Fontaina et al., 2016). AMO can easily perform nucleophilic attack and hydroxylate hydrocarbons, especially for secondary and tertiary carbons in linear alkyl chains, which were available in bentazone. However, some OMP functional groups can impede biodegradation by AMO, such as halogens, nitrogen-heterocyclic aromatic rings and primary and secondary amines and amides (Fernandez-Fontaina et al., 2016). Thus, the absence of biodegradation of BAM, chloridazon, mecoprop, ioxitalamic acid and benzotriazole by AMO could be explained by the presence of the aforementioned functional groups in these compounds (Table S1). Although 2,4-D contains impeding functional groups (chloride), the aerobic pathway of 2,4-D biodegradation can start with the hydroxylation of the acetic acid side chain (Sinton et al., 1986). Similarly, caffeine contains both affinitive and impeding functional groups (Table S1), but the three outside alkyl groups are firstly degraded during caffeine biodegradation (Dash and Gummadi, 2006). Thus, the removal of 2,4-D and caffeine could still able to be enhanced by AMO (Fig. 1).

3.2. Biodegradation of OMPs during methane oxidation

Field collected RSF sand samples containing methanotrophic biofilms were incubated under four conditions to determine their effects on OMPs removal (Fig. S1 and Table 2). CH_4 consumption rate increased after the first 3 days of incubation during the experiment (Fig. S3). The addition of acetylene suppressed the methanotrophic activity, as it is shown by the CH_4 consumption inhibition (Fig. S3). In abiotic batches, there was no consumption of CH_4 and O_2, suggesting that the bioactivity was successfully inhibited. In addition, the presence of OMPs also did not affect the methanotrophic activity (Fig. S3). There was no significant removal of all compounds in abiotic condition (Fig. S5).

The removal of caffeine, benzotriazole, 2,4-D and bentazone was enhanced in the presence of methanotrophic activity (Fig. 3). However, those compounds were removed at different rates and overall efficiencies. Caffeine and benzotriazole were completely removed within 113 h and 425 h, respectively, while only 53% of bentazone and 72% of 2,4-D were removed by the end of the incubation (Fig. 3a). The removal of these compounds was limited after 17 h in methane oxidation inhibited batches (Fig. 3b). Linear correlation analysis showed the

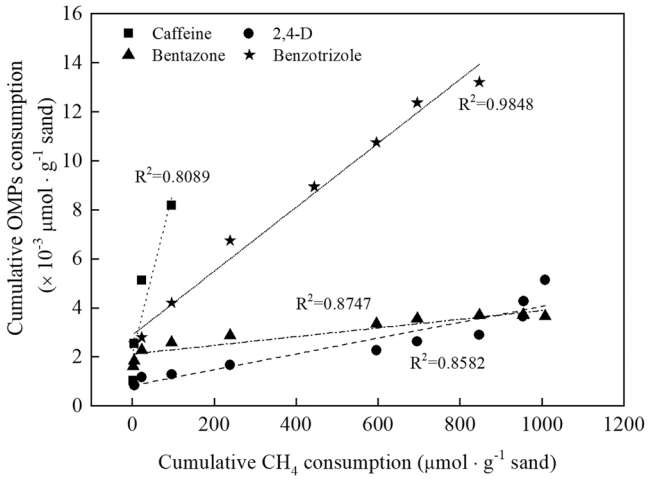


Fig. 4. Correlation between OMP biodegradation and methane oxidation. The data points where both OMPs and methane removal occurred was used for the correlation analysis. The selected incubation period was 17–113 h for caffeine, 41–425 h for benzotriazole, 41–737 h for 2,4-D and 17–737 h for bentazone.

degradation of these four compounds coincided with methane consumption (Fig. 4). The order of OMP degradation was caffeine > benzotriazole > 2,4-D / bentazone (Fig. 3a). This can be explained by the different affinities of MMO towards different functional groups. In addition, the removal of other compounds were not improved by methanotrophs, such as ioxitalamic acid, BAM, chloridazon, and mecoprop (Fig. S5).

Methanotrophs can produce both soluble MMO (sMMO, the catalytic subunit encoded by *mmoX*) and particulate MMO (pMMO, the catalytic subunit encoded by *pmoA*) (Nielsen et al., 1997). sMMO can oxidize broader organic carbon substrates, such as alkanes, alkenes, aromatic and alicyclic compounds. In contrast, pMMO cannot oxidize aromatic or alicyclic compounds (Burrows et al., 1984). In this study, sMMO production and activity might drive the co-metabolic transformation of OMPs by methanotrophs. Since benzotriazole is a typical aromatic compound without any side alkanes or alkenes (Table 1), the enhanced removal of benzotriazole in the presence of methane oxidation suggests degradation by sMMO. In addition, the alkanes or/and aromatic ring in caffeine, 2,4-D and bentazone can be easily oxidized by sMMO. Hedegaard et al. (2018) found that MMO could hydroxylate alkanes and the aromatic ring in bentazone, then degrade bentazone into three transformation products, isopropyl-OH-bentazone, 8-OH-bentazone and 6-OH-bentazone. A more recent study indicated that methanotrophic enrichment can improve the removal of dichlorprop, a chlorophenoxy herbicide similar in structure to 2,4-D, where sMMO stimulated its

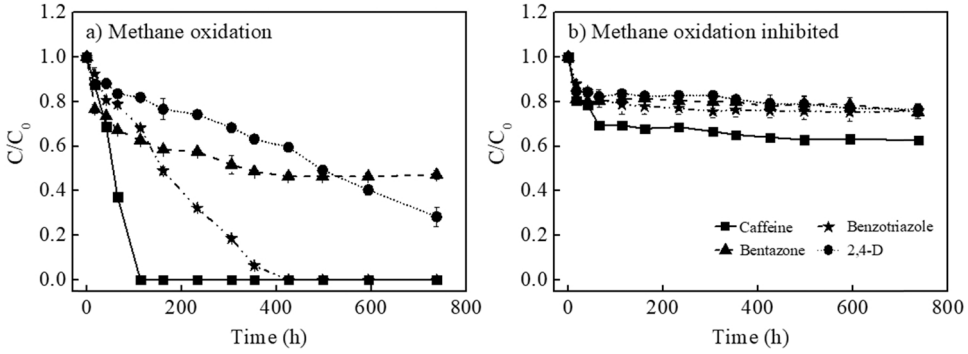


Fig. 3. Influence of methane oxidation on the biodegradation of caffeine, benzotriazole, 2,4-D and bentazone in batches. a) Methane oxidation stimulated addition of CH_4 and OMPs; b) Methane oxidation inhibited: addition of CH_4, OMPs and acetylene. Each point is average of two independent samples from duplicate bottles ± standard deviation.

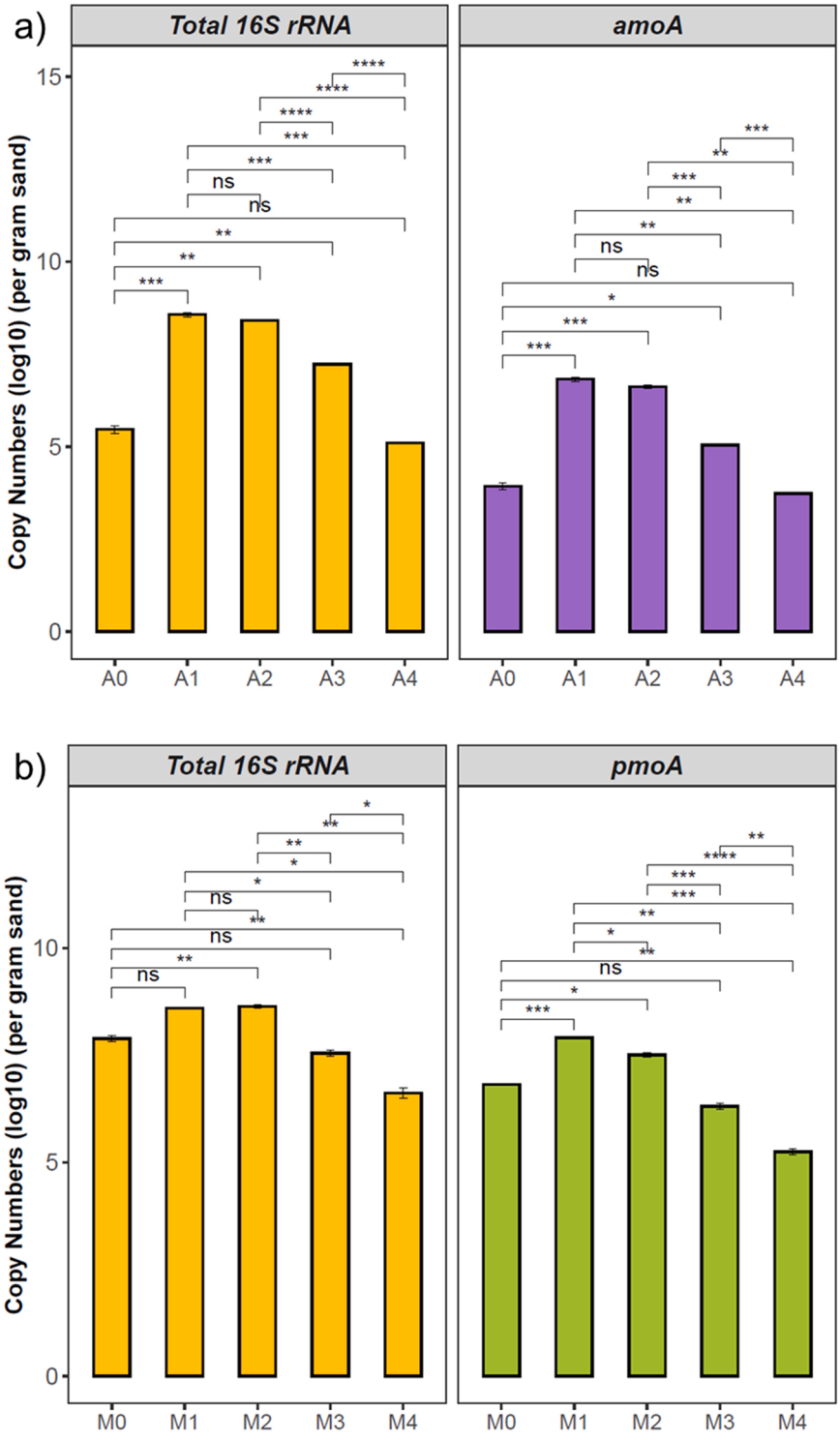


Fig. 5. Bacterial density measured by qPCR under different microcosm conditions. a) The abundance of nitrifying bacteria was assessed by quantification of *amoA* gene copies; b) the abundance of methanotrophic bacteria was assessed by quantification of *pmoA* gene copies. The abundance of total bacteria was assessed targeting 16 S rRNA genes copies. The qPCR measurements were performed in technical triplicate. Student's test (*t*-test) was used to check the significance of each paired group, "ns" indicated no significant difference with p-value > 0.05; *p-value is 0.01–0.05; **p-value is 0.001–001; ***p-value is 0.0001–0001; ****p-value is 0.00001–00001.

ring-hydroxylation (Papadopoulou et al., 2019). Therefore, we speculated that hydroxylation of alkanes, alkenes and the benzene ring was the main mechanism in OMP degradation by MMO in the present study.

3.3. Abundance and diversity of nitrifying and methanotrophic bacteria under different conditions

In this study, the abundances of total bacteria, nitrifying bacteria and methanotrophic bacteria incubated under different conditions were quantified by calculating the copy numbers of total bacterial 16S rRNA genes, *amoA* genes and *pmoA* genes. As mentioned before, the activities of nitrification and methane oxidation were not affected by the addition of OMPs, as seen in the similar copy numbers of *amoA* in nitrification without OMPs (A1) and with OMPs (A2) conditions (Fig. 5a) and the similar copy numbers of *pmoA* in methanotrophic without OMPs (M1) and with OMPs (M2) conditions (Fig. 5b). In condition A2 and M2, the addition of ammonia and methane resulted in increased copy numbers of *amoA* and *pmoA* genes, respectively, compared to the RSF inoculum (A0 and M0) (Fig. 5). Furthermore, these genes decreased after the addition of the respective inhibitors in condition A3 and M3,

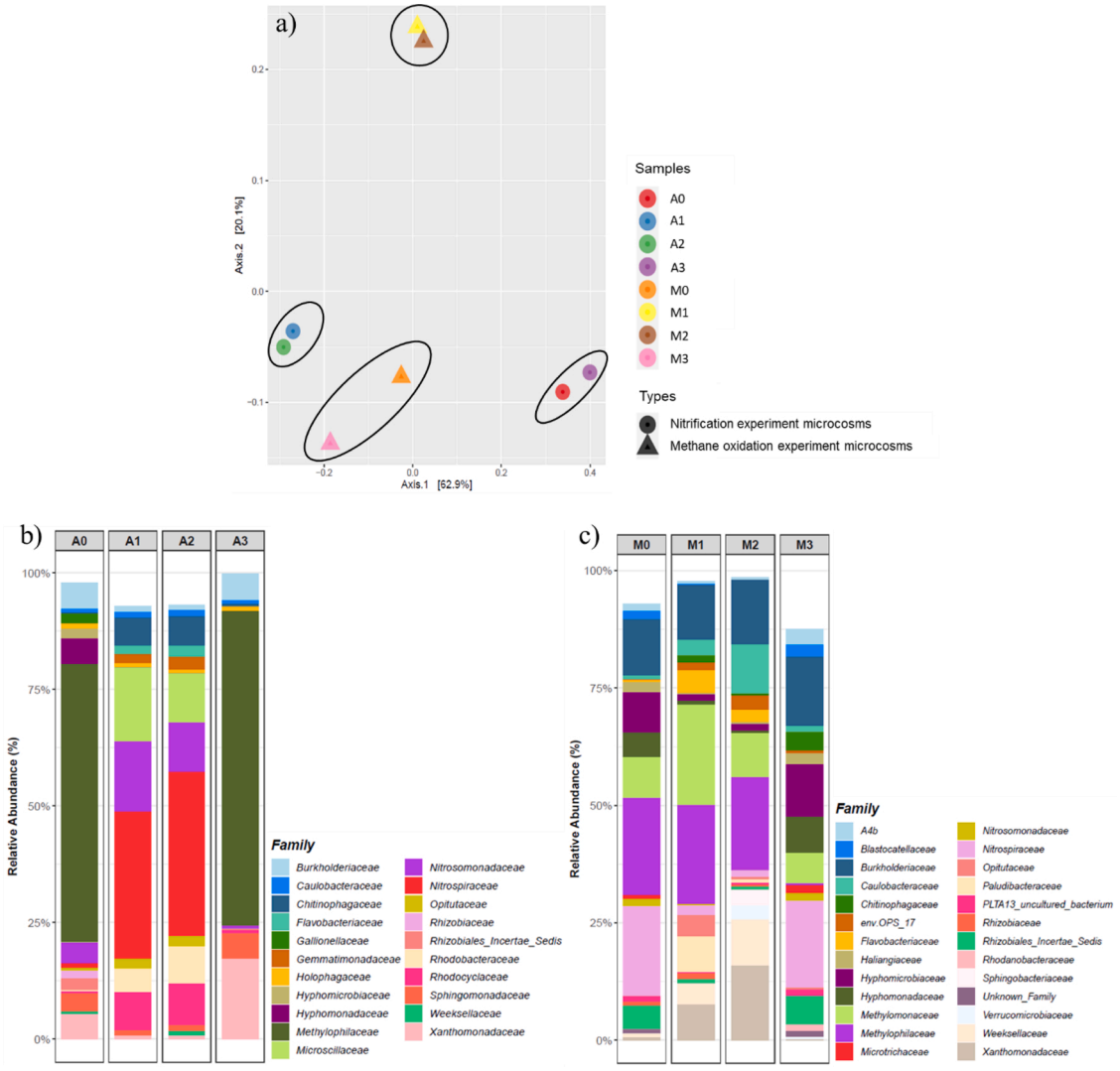


Fig. 6. Microbial community analysis of biofilms growing on different sand samples. a) beta diversity analysis by Principal Coordinates Analysis (PCoA, also termed Multidimensional scaling, MDS) of Weighted UniFrac distances. b) microbial composition at family level in nitrification experiment microcosms; c) microbial composition at family level in methane oxidation experiment microcosms. The relative abundance cut-off was set at 1%.

respectively (Fig. 5). As mentioned above, the presence of nitrifying and methanotrophic activity enhanced the biodegradation of certain OMPs compared to the inhibited conditions (Figs. 1 and 2). This fact, combined with the improved removal of OMPs in A2 and M2 conditions, could indicated the important roles of nitrifying bacteria (quantified by *amoA*) and methanotrophic bacteria (quantified by *pmoA*) in co-metabolic biodegradation of OMPs.

Microbial community composition was analysed based on the 16S rRNA gene sequence data. Addition of OMPs did not influence microbial community composition, as reflected in similar beta diversities with and without OMP addition (Fig. 6a). Interestingly, the inhibition of nitrification and methane oxidation did not reshape the microbial communities from the inoculum (Fig. 6a).

In nitrification conditions, *Nitrosomonadaceae* (4.35%) and *Nitrospiraceae* (1.06%) were detected in the RSF inoculum of nitrifying bacteria (A0) (Fig. 6b). Stimulation of nitrification resulted in enrichment of *Nitrosomonadaceae* (10.5%) and *Nitrospiraceae* (35.26%) regardless of OMP addition (Fig. 6b). Inhibition of nitrification resulted in a decrease in both families to 0.5% and 0% respectively (Fig. 6b). A previous investigation reported the presence of families *Nitrosomonadaceae* and *Nitrospiraceae* in full-scale RSFs at DWTP, and *Nitrospiraceae* dominated the nitrifying bacterial community (Poghosyan et al., 2020). At genus- and species-level (OTU), *Nitrosomonas* and *Nitrospira* dominated in the nitrifying community, which were classified into *Nitrosomonadaceae* and *Nitrospiraceae,* respectively (Fig. 7a). Consistent with previous research (Poghosyan et al., 2020), the genera *Nitrosomonas* and *Nitrospira* were widely identified in the field RSF. In addition, Hu et al. (2020) found that *Nitrospira* was the genera carrying the comammox *amoA* genes, which accounts for 64.0% of *amoA* genes in average in RSF, emphasizing the important role of *Nitrospira* in microbial ammonia oxidation in RSF (Poghosyan et al., 2020; Hu et al., 2020). In addition, previous investigations reported that the *Nitrosomonas europaea* was able to degrade steroidal estrogens, triclosan and bisphenol A (Shi et al., 2004; Roh et al., 2009), suggesting that the nitrifying bacteria found in this study were capable of OMP biodegradation.

Two methanotrophic families *Methylomonadaceae* (8.51%) and *Methylophilaceae* (20.62%) were discovered in the RSF inoculum of methanotrophs (M0) (Fig. 6c). This is in agreement with the results of Poghosyan et al. (2020), who revealed that these two families were the main methanotrophs in the field RSF. With the periodic feeding with methane in M1 and M2 condition, the relative abundance of *Methylophilaceae* were still maintained at around 20%, whereas *Methylomonadaceae* relative abundances further increased to be 21.19% and 9.27%, respectively (Fig. 6c). Furthermore, the relative abundance of

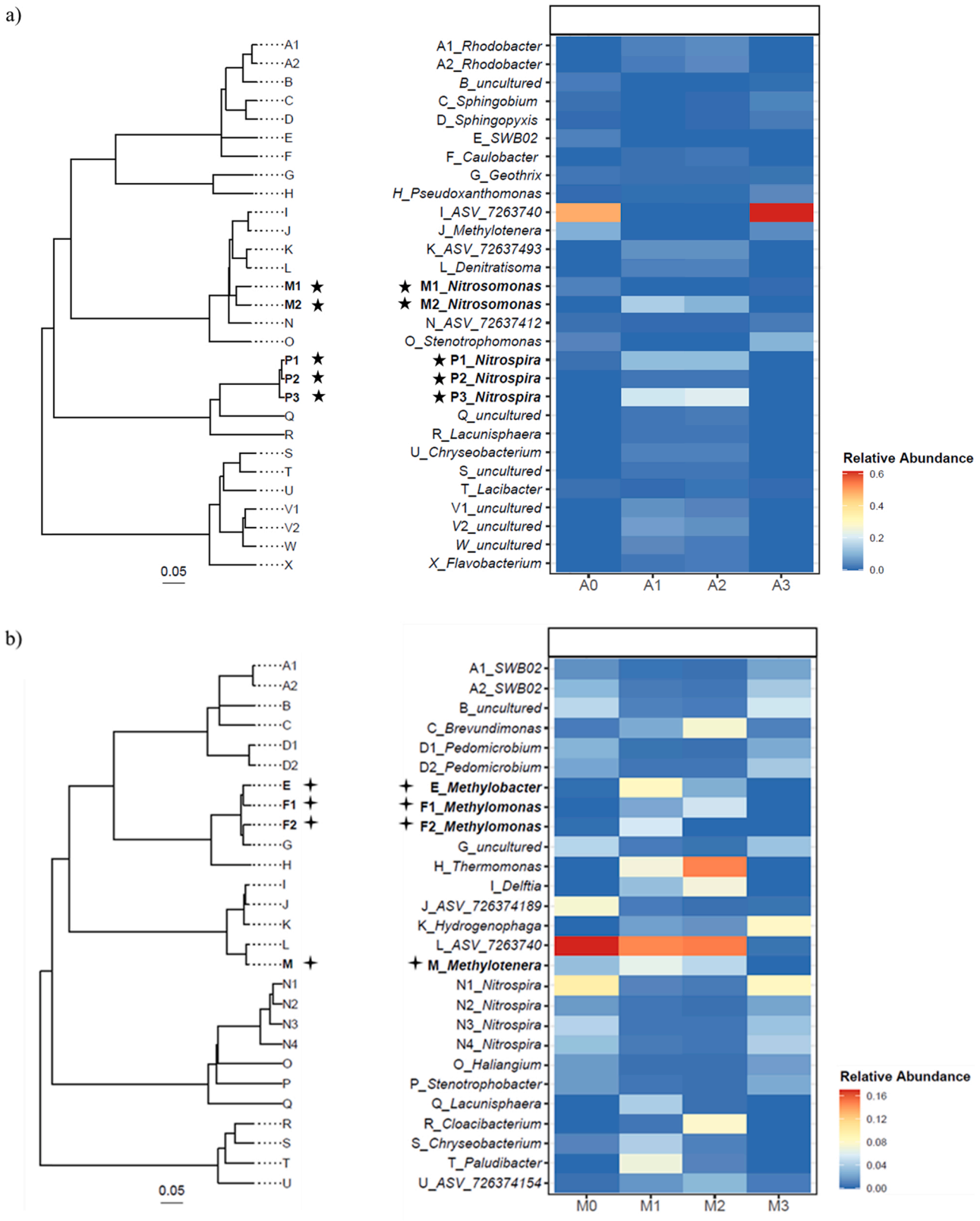


Fig. 7. Microbial composition at genus and OTU levels. a) Nitrification experiment microcosms. Nitrifying bacteria are indicated by stars, and OTU relative abundance cut-off is 0.5%; b) Methane oxidation experiment microcosms. Methanotrophic bacteria are marked by stars, and OTU relative abundance cut-off is 1%. OTU: Operational Taxonomic Unit (equally to the species-level amplicon sequencing variants, ASV).

both *Methylomonadaceae* and *Methylophilaceae* decreased with the addition of methanotroph inhibitor in condition M3 (Fig. 6c). *Methylophilaceae* was reported as one novel methanotrophic family, which was previously assumed to only include methylotrophic bacteria that were incapable of growing on methane, but can just use methane-oxidised intermediates (e.g., methanol and formate) as growth substrates

(Poghosyan et al., 2020). However, recent genomic studies indicated that some members of *Methylophilaceae* could also encode MMO, suggesting their methanotrophic potential (Vekeman et al., 2016; Poghosyan et al., 2020). The phylogenetic analysis further revealed the methanotrophic community contained three genera, namely *Methylobacter* and *Methylomonas* and *Methylotenera* (Fig. 7b). The relative abundance of these three genera in M1 and M2 was higher than in condition M0 and M3, indicating that methanotrophic bacteria were enriched (Fig. 7b). *Methylobacter* and *Methylomonas* belonging to *Methylomonadaceae*, were previously reported as the major methanotrophic bacteria colonizing sand granules in RSF (Poghosyan et al., 2020). In addition, recent metagenomic analysis showed that all assembled *Methylomonadaceae* metagenome-assembled genomes (MAGs) contained the operon encoding pMMO, and some of them can additionally encode sMMO (Poghosyan et al., 2020). The relative abundance of *Methylotenera* also increased in M1 and M2, which was the member of *Methylophilaceae* family and likely contributes to methane oxidation. Previous metagenomic study indicated the presence of *Methylotenera* in field scale RSF (Poghosyan et al., 2020). Although the lack of molecular evidence indicating the production of sMMO by *Methylotenera*, the previous study discovered that one of *Methylophilaceae* MAGs possessed the whole operon to encode sMMO (Poghosyan et al., 2020). Accordingly, these findings suggest sMMO presence in our microcosm. sMMO-containing bacteria in the methanotrophic community are likely to contribute to OMPs removal (Burrows et al., 1984). Taken together, we infer from the microbial community that methanotrophic bacteria bearing sMMO might be contributing to the removal of OMPs.

Besides the well-characterized nitrifying and methanotrophic families, some synergistic genera involved with nitrification and methane oxidation were discovered (Figs. 6b and 6c). In nitrification experiment microcosms, the temporary accumulation of nitrite has been detected in A1 and A2 conditions (Fig. S2b). This probably served as the substrate to support the growth of *Gemmatimonadaceae* (Fig. 6b), since several *Gemmatimonadaceae* species have been reported to convert nitrite to nitric oxide via the *nirk* gene (Decleyre et al., 2016). In addition, the co-existence of *Gemmatimonadaceae* and nitrifying bacteria, like *Nitrospiraceae*, were also found in some soil (Aanderud et al., 2018). A polyphosphate accumulating organism, *Rhodocyclaceae* bacterium was enriched, most likely due to the presence of phosphate buffer in batch bottles (Hesselmann et al., 1999). In methane oxidation experiment microcosms (M1 and M2), *Caulobacteraceae*, *Xanthomonadaceae* and *Flavobacteriaceae* were enriched. These families were previously associated with methanotrophic bacteria and regarded as important non-methanotrophic bacteria under methane oxidation condition(Ho et al., 2016; Oshkin et al., 2015) . Furthermore, *Nitrospiraceae* was also detected in M0, which might be due to the presence of ammonia in the raw water (Table 1). With the ammonia limitation, the relative abundance of *Nitrospiraceae* largely decreased in microcosms M1 and M2 (Fig. 6c). Thus, the incubation conditions in microcosms not only enriched nitrifying and methanotrophic families, but also selected the eco-physiology-associated families, and consequently reshaped microbial community for nitrification and methane oxidation.

3.4. Proposed removal mechanisms of OMPs with field RSF sand

Our results showed that caffeine, bentazone, 2,4-D and benzotriazole could be removed under nitrifying or methane oxidizing conditions. The main removal mechanisms might be reaction or interaction with specific functional groups in chemical structures (Table S3). It has been shown that AMO and MMO from nitrifying and methane oxidizing bacteria are able to attack and hydroxylate hydrocarbons, especially alkyl groups and aromatic ring (Fernandez-Fontaina et al., 2016; Burrows et al., 1984). However, some functional groups in those four compounds may also adversely affect enzymic biodegradation, such as halogens, nitrogen-heterocyclic aromatic rings, amines and amides (Fernandez--Fontaina et al., 2016). Thus, the exact biodegradation processes by AMO or MMO still need to be confirmed by the detection of related transformation products in future research.

In contrast to caffeine, 2,4-D, bentazone and benzotriazole, whose degradation dependent on nitrification and methane oxidation, salicylic acid was completely removed in all biotic microcosms (Fig. S5). Salicylic acid is readily biodegradable (Combarros et al., 2014; Hack et al., 2015), and therefore degradation is not dependent on autotrophic activity. This shows that field RSF sand has natural potential to biodegrade certain easily biodegradable compounds (Hedegaard et al., 2014). Although previous investigations also indicated that caffeine and 2,4-D were easily biodegradable compounds in different biological treatment systems (He et al., 2018; Mangat and Elefsiniotis, 1999), we did not observe their removal when nitrification or methane oxidation were inhibited. This seems to indicate that field RSF in our study did not contain specialized microbial communities to degrade caffeine and 2,4-D.

Except for salicylic acid, the removal of other compounds in nitrification / methane oxidation inhibited conditions were similar to the abiotic conditions (Fig. S5), suggesting the potential for abiotic removal of OMPs in RSF. In addition, all the compounds showed a similar removal efficiency of approximatively 20% (Fig. S5). The pre-test showed that there was no OMP removal by rubber stopers and glass batch bottles (data not shown), that was consisted with a previous study (Piai et al., 2019). In addition, all the bottles were wrapped in aluminium foil to avoid OMP photodegradation. Thus, we concluded that the abiotic removal of OMPs was due to the adsorption by sand materials. Although sand itself is not a good adsorbent for OMPs, our field collected sand granules were coated with natural biofilms and iron and manganese oxides, which could contribute to the absorption of OMPs (Hedegaard et al., 2014). However, the limited OMP removal efficiency indicated that the abiotic removal was not the governing removal process.

3.5. Perspectives of OMP co-metabolic biodegradation in field condition

To assess the potential of co-metabolic biodegradation for OMP removal in field RSF, we calculated the substrate preference (SP) values of caffeine, bentazone, 2,4-D and benzotriazole (Table S4). The concentrations and removal rates of OMPs and primary substrates (ammonia and methane) and are listed in Table S5. According to Eq. (2), with the same concentrations and removal rates of primary substrates, the removal of OMP ($\Delta C'_{OMP}$) is negatively related with SP value of OMP (SP_{OMP}) in field RSF. Additionally, the theoretical OMP removal efficiencies in the field RSF were calculated by Eq. (3), which showed that a higher removal efficiency of primary substrate (R_{pri}) could improve the total removal of OMP (R_{OMP}). Thus, the compounds with higher SP_{OMP} may require higher bacterial activity to support effective co-metabolic biodegradation, such as benzotriazole, 2,4-D and bentazone.

As mentioned before, by incubating with ammonia and methane, the nitrifying and methanotrophic bacteria were further enriched. The resulting microbial community showed better OMP removal activity. We therefore suggest bioaugmentation as a strategy to improve OMP removal in RSF. Previous investigation suggested that bioaugmentation with enriched nitrifier was able to successfully shorten the star-up time of the nitrification process in RSF (Albers et al., 2018). Moreover, Papadopoulou et al. (2019) reported that an enriched methanotrophic culture improved the removal of phenoxy acid compared to raw sand collected from an RSF. Thus, the field RSF sand can be sampled and incubated with ammonia and methane to stimulate the growth of nitrifying and methanotrophic bacteria. After that, RSF can be bioaugmented with field sand with enriched nitrifying and methanotrophic communities to improve co-metabolic biodegradation of those recalcitrant compounds. Although the actual removal of methane in the field RSF already reached 92.06%, the theoretical removal of benzotriazole, 2,4-D and bentazone were still lower than 12% (Table S4). Thus, bioaugmentation with nitrifying and methanotrophic bacteria might be able to further enhance the biodegradation of those compounds RSF. A

main challenge for such a bioaugmentation strategy is maintaining autotrophic activity under real RSF conditions. Thus, future studies should examine bioaugmentation longevity with RSF-relevant concentrations of ammonium, methane, OMPs and other nutrients. Furthermore, retention of bioaugmentation under the fast flow and backwashing regimes of real RSF must be explored. Priming the biomass with biocarrier could be a promising method to support and maintain bioaugmented biomass in RSF (Horemans et al., 2017). Although in this study the effect of OMP addition on nitrifying and methanotrophic activities was negligible (Fig. S2 and S3), the inhibitive effects of other single or mixed OMP(s) on microbial activities still need to be studied in a real water matrix. In addition, the stimulated nitrifying and methanotrophic cultures showed a limited capacity to biotransform iox-italamic acid, chloridazon, BAM and mecoprop in this study (Fig. S5). Bioaugmentation of RSF with a specific hetertrophic degrader may help with the removal of these recalcitrant compounds (Albers et al., 2015). Otherwise, downstream treatment steps are required to treat the contaminated source water.

4. Conclusions

This study aimed to understand OMP co-metabolism by nitrifying and methanotrophic bacteria in full scale groundwater-fed RSF. Enhanced biodegradation of caffeine, bentazone and 2,4-D was observed with nitrification activity. In addition to these three compounds, methanotrophic activity also improved the removal of benzotriazole. AMO and MMO activity could be responsible for co-metabolic biodegradation of OMPs, where the target functional groups, such as side alkyl chains or aromatic rings, could be hydroxylated by AMO and MMO. By incubating with ammonia and methane, *Nitrosomonas* and *Nitrospira* dominated in the nitrifying community, while the methanotrophic community contained *Methylobacter*, *Methylomonas* and *Methylotenera*. Bioaugmentation of RSF with nitrifying and methanotrophic bacteria could be a promising approach to enhance the removal of certain OMPs in RSF from drinking water. However, the future research is required to explore feasible bioaugmentation strategies to support sustained OMP removal in conjunction with nitrification and methane oxidation. This study also showed that some OMPs cannot be biotransformed by nitrifying and methanotrophic bacteria. Thus, bioaugmentation with specific degraders or other downstream treatments are required for removing those recalcitrant OMPs.

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CRediT authorship contribution statement

Jinsong Wang: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Visualization, Project administration. **Chen Zhang:** Methodology, Software, Formal analysis, Investigation, Writing – original draft, Visualization. **Baptiste A.J. Poursat:** Formal analysis, Writing – review & editing. **David de Ridder:** Resources, Writing – review & editing, Supervision. **Hauke Smidt:** Resources, Writing – review & editing. **Albert van der Wal:** Conceptualization, Resources, Writing – review & editing, Supervision, Funding acquisition. **Nora B. Sutton:** Conceptualization, Methodology, Resources, Writing – review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2021.127760.

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