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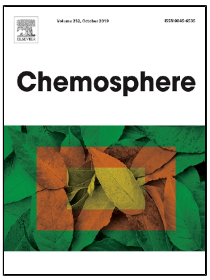


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# Anaerobic methane oxidation coupled to sulfate reduction in a biotrickling filter: reactor performance and microbial community analysis

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## Abstract

The aim of this work was to evaluate the performance of a biotrickling filter (BTF) packed with polyurethane foam and pall rings for the enrichment of microorganisms mediating anaerobic oxidation of methane (AOM) coupled to sulfate reduction (SR) by activity tests and microbial community analysis. A BTF was inoculated with microorganisms from a known AOM active deep sea sediment collected at a depth of 528 m below the sea level (Alpha Mound, Gulf of Cadiz). The microbial community analysis was performed by catalysed reporter deposition - fluorescence in situ hybridization (CARD-FISH) and 16S rRNA sequence analysis. The AOM occurrence and rates in the BTF were assessed by performing batch activity assays using $^{13}C$-labelled methane ($^{13}CH_4$). After an estimated start-up time of ~20 days, AOM rates of ~0.3 mmol $l^{-1}$ $day^{-1}$ were observed in the BTF, values almost 20 times higher than previously reported in a polyurethane foam packed BTF. The microbial community consisted mainly of anaerobic methanotrophs (ANME-2, 22% of the total number of cells) and sulfate reducing bacteria (SRB, 47% of the total number of cells). This study showed that the BTF is a suitable reactor configuration for the enrichment of microbial communities involved in AOM coupled to SR at ambient pressure and temperature with a relatively short start-up time.


## Highlights

- A biotrickling filter inoculated with deep-sea sediment performed anaerobic oxidation of methane coupled to sulfate reduction
- A short start-up (20 days) and high conversion rate (0.3 mmol $l^{-1}$ $day^{-1}$) were achieved
- Anaerobic methanotrophs and sulfate reducing bacteria were considerably enriched (~70% of the total community) after 200 days of operation

**Keywords:** Biotrickling filter; anaerobic oxidation of methane; sulfate reduction; anaerobic methanotrophs; sulfate reducing bacteria; deep sea sediment.

## 1. Introduction

Anaerobic oxidation of methane (AOM) coupled to sulfate reduction (SR) is a biological process occurring in anoxic environments, especially in marine sediments (Reeburgh, 2007; Knittel and Boetius, 2009; Scheller et al., 2016). AOM contributes to the removal of methane, thereby controlling its emission to the atmosphere (Raghoebarsing et al., 2006; Reeburgh, 2007). Methane is a well-known greenhouse gas and its presence in the atmosphere at high concentrations has large implications for future climate change (Forster et al., 2007). Many terrestrial and aquatic surfaces are possible methane sources, thus, it is important to understand the processes and mechanisms involved in its consumption pattern in the environment (Kirschke et al., 2013).

AOM coupled to SR (AOM-SR) is a process mediated by anaerobic methanotrophs (ANME) and sulfate reducing bacteria (SRB). ANME-1, ANME-2 and ANME-3 are the three different types of ANME (Knittel and Boetius, 2009; Bhattarai et al., 2017a). All ANME are phylogenetically related to various groups of methanogenic archaea; it was recently shown how the ANME metabolism can be reversed to methanogenesis by only a few changes in carbon-metabolizing enzymes in ANME (McGlynn et al., 2017; Timmers et al., 2017). ANME are usually associated with different types of SRB, namely *Desulfosarcina/Desulfococcus* (DSS) or *Desulfobubaceae* (DBB) (Schreiber et al., 2010). AOM-SR is a well-known phenomenon occurring in anaerobic environments (Reeburgh, 2007; Knittel and Boetius, 2009; Scheller et al., 2016). However, the mode of cooperative interaction between ANME and SRB is still under debate and the difficulty in enriching the ANME under laboratory conditions hampers the investigation of the mechanism of this process. The main challenges for enrichment of ANME-SRB consortia are their slow

growth rates (doubling time of ~2 to 7 months), the low solubility of methane in water at standard atmospheric pressure and they are strictly anaerobic (Deusner et al., 2009; Meulepas et al., 2009a; Zhang et al., 2011; Wegener et al., 2016). Besides the biogeochemical implications of AOM, this microbial process can have biotechnological applications for the removal of sulfate from wastewater streams low in electron donors, using methane which is less costly and readily available. Moreover, the AOM process can be applied for nitrogen removal in wastewater with low levels of biodegradable organic matter. Sidestreams produced from anaerobic digestion for the production of biogas are rich in ammonium and lack organic matter (Rikmann et al., 2018; Zekker et al., 2018). Therefore, methane can be used as an electron donor and AOM can be applied to reduce nitrogen levels (Zhu et al., 2016) and for nutrient removal (e.g. nitrate and phosphorous) from wastewater (Mandel et al., 2019; Klein et al., 2017).

The enrichment of ANME can be enhanced by the use of different types of bioreactor configurations such as a high pressure reactor (Deusner et al., 2009; Zhang et al., 2011), membrane reactor (Meulepas et al., 2009a; Timmers et al., 2015a) or a biotrickling filter (Cassarini et al., 2017; Bhattarai et al., 2018b; Cassarini et al., 2018). However, the SR rates reported so far (~0.6 mmol $l^{-1}$ $day^{-1}$) with methane as the electron donor are more than 100 times lower than the rates achieved with other electron donors, such as hydrogen or ethanol (Suarez-Zuluaga et al., 2014; Bhattarai et al., 2017b). Moreover, a long start-up period is required for the bioreactor to perform AOM-SR, i.e. ~400 days for a membrane bioreactor inoculated with Eckenförde Bay sediment (Meulepas et al., 2009a).

In a recent study, Cassarini et al. (2018) operated a biotrickling filter (BTF) for 230 days with sediment collected from the Alpha Mound (Gulf of Cadiz, Spain) as inoculum. A BTF packed with polyurethane foam cubes was chosen to mimic the natural habitat of ANME and SRB to promote

their growth. These microorganisms have been found in carbonate-minerals, which is a porous matrix similar to the polyurethane foam (Marlow et al., 2014). The polyurethane foam provides good biomass retention, low shearing force, and high gas-liquid mass transfer (Cassarini et al., 2017). The high methane-liquid mass transfer will help retain the poorly soluble methane in the polyurethane pores (Aoki et al., 2014; Estrada et al., 2014).

Cassarini et al. (2018) showed that ANME and SRB obtained from deep sea conditions (528 m below sea level) can be enriched in a 0.4 l BTF at ambient pressure and temperature with a start-up time of 42 days. In that study, sulfate was reduced at a maximum rate of 0.3 mmol $l^{-1}$ $day^{-1}$ and ANME-2 was enriched (7% of the total visualized archaea), but the AOM rate was ~10% lower than the SR rate. However, the reactor start-up period was comparatively long for BTF reactors used for waste-gas treatment (Pérez et al., 2016), i.e. ~1–2 weeks for a BTF used to treat methane emissions under oxic conditions (Avalos Ramirez et al., 2012; Estrada et al., 2014).

The aim of this study was, therefore, to further enrich this biomass from deep sea sediment in a 6.6 l BTF packed with polyurethane foam cubes and pall rings to obtain higher AOM rates and thus, SR rates closer to the rates achieved with other electron donors, such as hydrogen or ethanol, ~60 mmol $l^{-1}day^{-1}$ (Suarez-Zuluaga et al., 2014; Bhattarai et al., 2017b). Batch activity assays using $^{13}C$-labelled methane ($^{13}CH_4$) were used to determine the AOM rate, whereas the growth of ANME and SRB cells was determined using catalyzed reporter deposition-fluorescence in situ hybridization (CARD-FISH) and analysis of 16S rRNA gees.

## 2. Material and methods

### *2.1. Source of biomass and composition of the artificial seawater medium*

The enriched biomass after 230 days of incubation in a polyurethane sponge packed BTF (Cassarini et al., 2018) was used as inoculum for this study. The original sediment was obtained

from the Alpha Mound (35°17.48'N; 6°47.05'W, water depth ca. 525 m), Gulf of Cadiz (Spain), during the R/V Marion Dufresne Cruise MD 169 MiCROSYSTEMS to the Gulf of Cadiz in July 2008. The characteristics of the sediment have been described in Cassarini et al. (2017). In brief, the Alpha Mound showed evidence for the presence of a shallow sulfate-methane transition zone driven by substantial methane fluxes and temperature variating between 10 and 15 °C (Wehrmann et al., 2011).

The artificial seawater mineral medium was composed of and prepared as described by Bhattarai et al. (2017a) with 10 mM of $SO_4^{2-}$ added as $Na_2SO_4$ in its anhydrous form (Fisher Scientific, Landsmeer, the Netherlands) as described previously (Cassarini et al. 2018). . Briefly, the vitamins and trace element mixtures were prepared according to Widdel and Bak (1992). 0.01 mM of $Na_2S$ was added as the reducing agent to the seawater mineral medium and 0.5 g $l^{-1}$ resazurin solution was added as the redox indicator. The pH of the seawater medium was adjusted to 7.0 with sterile 1 M $Na_2CO_3$ or 1 M $H_2SO_4$ solution. The medium was maintained under anoxic conditions with the purging of nitrogen until it was recirculated to the BTF.

All the chemicals were purchased as lab grade from Fisher Scientific (Landsmeer, the Netherlands).

*2.2. BTF setup and operation*

The BTF (Figure 1) was a custom made cylindrical glass reactor (height: 62 cm, internal diameter: 12 cm, total volume: 6.6 l), sealed air-tight to prevent leakages or air intrusion during its operation. The BTF was equipped with three sampling ports for the gas phase (inlet and outlet), the liquid phase and for the biomass (Figure 1). The filter bed volume of the BTF was 5.6 l which was packed with 76 g of polyurethane foam cubes of 1 $cm^3$ (0.98 void ratio and a density of 28 kg $m^{-3}$) and 230 g of polypropylene pall rings (0.87 void ratio and a density of 115 kg $m^{-3}$). Two circular

acrylic sieve plates (pore size of 3.5 mm) were placed at the top of the BTF to facilitate homogeneous sprinkling of the medium and at the bottom to hold the polyurethane foam cubes (Figure 1).

The BTF was operated in sequential fed-batch mode for the trickling of artificial seawater medium, recirculating at a flow rate of 10 ml $min^{-1}$ using a Masterflex S/L peristaltic pump (Metrohm Netherlands B.V, Schiedam, the Netherlands). The gas-phase methane (99.5% methane, Linde gas, Schiedam, the Netherlands) was supplied to the BTF by a gas sparger placed at the bottom of the BTF. The influent methane flow was measured and controlled by a Smart Thermal Mass Flow Controller (Brooks Instrument, Model SLA5850, Veenendaal, the Netherlands) at a constant flow rate of 0.5 ml $min^{-1}$, with a methane molar flow rate of 29.6 mmol $d^{-1}$, methane residence time with packing material of 8.7 days and methane loading rate of 4.7 mmol $l^{-1}$ $d^{-1}$. The outlet gas containing hydrogen sulfide, carbon dioxide and residual methane left the BTF via a gas cleaning bottle, which was filled with a 0.5 M $ZnCl_2$ solution to selectively retain the hydrogen sulfide. The sulfide concentration in the bottle was measured once every 2 weeks. The pH of the BTF was monitored by a sulfide resistant pH electrode (Prosense, Oosterhout, The Netherlands).

550 ml of biomass (0.07 ± 0.01 g volatile suspended solids), pre-enriched by incubation for 230 days in a 0.4 l acrylic BTF (Cassarini et al., 2018) was used to inoculate the BTF. The BTF was operated for 238 days in the dark, at atmospheric pressure and room temperature (~20 ± 2 ºC) throughout the experimental period. The artificial seawater medium containing 10 mM sulfate was replaced periodically and the different operational phases of the BTF were defined by the medium replacement: days 0–61 (I), days 61–119 (II), days 119–160 (III), days 160–214 (IV) and days 214–238 (V).

*2.3. Sampling*

Both gas (inlet and outlet) and liquid samples were collected twice a week from the sampling ports (Figure 1). pH, sulfate and sulfide concentrations were measured in samples collected from the liquid medium, while methane and carbon dioxide were analyzed from the gas samples collected at the inlet and outlet of the BTF. Biomass samples were collected in triplicate on day 112 and at the end of the BTF operation (day 238) for carrying out activity assays and analysis of microbial diversity by sequence analysis of 16S rRNA genes.

*2.4. Activity assays*

The occurrence of AOM and the estimation of the methane oxidation rates were determined from the $^{13}C$-labeled carbon dioxide produced during batch tests with 5% $^{13}CH_4$ following the procedure described by Cassarini et al. (2018). The polyurethane foam cubes containing the enriched inoculum were collected from the BTF at the end of its operation. The tests were performed in triplicate to evaluate the standard deviation. Control batch experiments were prepared under nitrogen atmosphere (without methane), in the absence of the electron acceptor and without the biomass (fresh polyurethane foam cubes were added).

The bottles were placed on an orbital shaker (Cole-Parmer, Germany) at 100 rpm in the dark at the operation temperature of the BTF (20 ± 2 $^{o}$C) for 42 days. Sampling was performed once a week for both gas and liquid phase analysis as well as CARD-FISH analysis (only 4 time points: 0, 21, 28 and 42 days).

*2.5. Chemical analysis*

Samples for analysis of pH, sulfate, thiosulfate and dissolved sulfide concentrations were analyzed at least in duplicate. The pH, sulfate, thiosulfate, methane and carbon dioxide concentrations were analyzed according to the procedure described in Cassarini et al. (2017). The total dissolved sulfide

concentrations ($H_2S$, $HS^-$ and $S^{2-}$) in the BTF were analyzed spectrophotometrically using the methylene blue method (Acree et al., 1971). The volumetric AOM, SR and the total dissolved sulfide production rates were determined for each phase of the BTF. The data points of the different compound (methane, sulfate and total dissolved sulfide) concentrations were fitted to a zero-order rate equation using the least square method ($R^2 > 0.97$). The mass balance of carbon and sulfur was calculated on the basis of the concentration of carbon/sulfur compounds entering and leaving the BTF during every operational phase. The mass balance was calculated in terms of mM of carbon/sulfur recovered and also in fractions of different sulfur/carbon compounds formed during the different phases.

The stable carbon isotope composition of methane and carbon dioxide were determined using a gas chromatography - isotope ratio mass spectrometer (GC-IRMS, Agilent 7890A) as described by Herrmann et al. (2010). Ratios of stable carbon isotopes ($^{13}C/^{12}C$) were estimated as described by Dorer et al. (2016). Measurements of the stable isotope composition of methane and carbon dioxide were performed in triplicate and the standard deviation was observed to be less than 0.5 δ-units. For quality assurance, standard gas mixtures of methane and carbon dioxide were measured periodically during the entire isotopic analysis. The AOM rate was estimated on the basis of the dissolved inorganic carbon (DIC) produced from $^{13}CH_4$ during the activity assays as described by Cassarini et al. (2018).

The volatile suspended solids (VSS) were estimated before inoculation on the basis of the difference between the dry and ash weights of the sediment according to the procedure outlined in Standard Methods (APHA 2012).

*2.6. DNA extraction*

202 DNA was extracted using a FastDNA® SPIN Kit for soil (MP Biomedicals, Solon, OH, USA)
203 following the manufacturer's protocol. Approximately 0.5 g of the sediment was used for DNA
204 extraction from the initial inoculum and ~0.5 ml of liquid obtained by washing the polyurethane
205 foam packing with nuclease free water was used for extracting the DNA from the BTF biomass.
206 The extracted DNA was quantified and its quality was checked according to the procedure outlined
207 by Bhattarai et al. (2017a).

208 *2.7. Polymerase chain reaction (PCR) amplification for 16S rRNA genes and Illumina Miseq data*
209 *processing*

210 The DNA was amplified using the bar coded archaea specific primer pair Arc516F (forward) and
211 Arc855R (reverse). The primer pairs used for bacteria were forward bac520F 5 ′-3 ′ AYT GGG
212 YDT AAA GNG and reverse Bac802R 5′ -3′ TAC NNG GGT ATC TAA TCC (Song et al., 2013).
213 The PCR reaction mixture was prepared as described by Bhattarai et al. (2017a), whereas the PCR
214 amplification for bacteria and archaea was performed as described by Cassarini et al. (2019).

215 After checking the correct band size, 150 µl of PCR amplicons were loaded in 1% agarose gel and
216 electrophoresis was performed for 120 min at 120 V. The gel bands were excited under UV light
217 and the PCR amplicons were cleaned using the E.Z.N.A.® Gel Extraction Kit by following the
218 manufacturer's protocol (Omega Biotek, USA). The purified DNA amplicons were sequenced by
219 an Illumina HiSeq 2000 (Illumina, San Diego, USA) and analyzed according to the detailed
220 analytical procedure described in Bhattarai et al. (2017a).

221 A total of 40,000 (± 20,000) sequences were assigned to archaea and bacteria by examining the
222 tags assigned to the amplicons for at least duplicate (one sample did not contain enough number
223 of sequences, i.e. < 10,000, for the analysis) DNA samples extracted from the BTF biomass at the

224 start and the end of the BTF operation. More details on the analytical procedure have been
225 described by Bhattarai et al. (2017a). After eliminating the chimeras, the sequences for archaea
226 and bacteria were analyzed and classified in MOTHUR (Schloss and Westcott, 2011). Briefly
227 stating, the faulty sequences with mismatch tags or primers and with a size <200 bp were removed
228 by using the shhh.flows command. Therefore, the putative chimeric sequences were identified and
229 removed by the chimera.uchime command using the most abundant reads in the respective
230 sequence data sets as references. Assembled reads that passed the chimera checking were clustered
231 into the new operational taxonomic units (OTUs) at a cut off of 97% sequence similarity. Finally,
232 the sequence reads were classified according to the SILVA taxonomy database version 121
233 (Pruesse et al., 2007) using the classify.seqs command and the relative sequence abundance of
234 each phylotype was assessed. These sequence data were submitted to the NCBI GenBank database
235 under the BioProject accession number PRJNA415004 (direct link:
236 http://www.ncbi.nlm.nih.gov/bioproject/415004).F

237 *2.8. Cells visualization and counting by CARD-FISH*

238 Analysis of single cells in biomass samples collected from the BTF and from the activity assays
239 were performed by CARD-FISH as described by Cassarini et al. (2017) and Cassarini et al. (2018).
240 For dual-CARD-FISH, peroxidases of initial hybridizations were inactivated according to the
241 procedure described in Holler et al. (2011). Tyramide amplification was performed using the
242 fluorochromes Oregon Green 488-X and Alexa Fluor 594, which were prepared according to the
243 procedure outlined in Pernthaler et al. (2004).

244 The microorganisms were visualized using archaeal and bacterial HRP-labeled oligonucleotide
245 probes ARCH915 (Stahl and Amann, 1991) and EUB338-I-III (Daims et al., 1999), respectively.
246 The probes DSS658 (Manz et al., 1998) and ANME-2 538 (Schreiber et al., 2010) were used for

247 the detection of DSS and ANME-2 cells, respectively. Oligonucleotide probes were purchased
248 from Biomers (Ulm, Germany).
249 Total cells were counterstained with 4',6'-diamidino-2-phenylindole (DAPI) and visualized using
250 an epifluorescence microscope (Carl Zeiss, Germany). Cell counting was performed as described
251 by Cassarini et al. (2019), 700-1,000 DAPI-stained cells and their corresponding probe fluorescent
252 signals for each probe per incubation (triplicate) were counted as described previously in the
253 literature (Musat et al., 2008; Kleindienst et al., 2012). Cell counts are indicated as the average ±
254 standard deviation of triplicate samples. The statistical data analysis was performed using SPSS
255 25.0 (IBM, USA). Significant differences were determined by one-way ANOVA and post-hoc
256 analysis for multiple group comparison (Tukey HSD). Differences were considered to be
257 significant at $p \leq 0.05$. The homogeneity of the variance of the parameters was evaluated using a
258 Levene test.

259 **3. Results and discussion**

260 *3.1. Performance of the BTF*

261 *3.1.1. Start-up time of the BTF*

262 The long start-up time of the AOM-SR process is one of the major drawbacks for its application
263 in the treatment of wastewaters and groundwater (Meulepas et al., 2009a). In previous studies,
264 start-up periods of ~400 days (Meulepas et al., 2009a) and ~365 days (Aoki et al., 2014) have been
265 reported in a membrane bioreactor and hanging sponge reactor, respectively. The estimated AOM-
266 SR start-up time in this study was only 20 days (Figure 2), much shorter than what was previously
267 reported and comparable to the start-up period (~ 1-2 weeks) of a BTF used to treat methane
268 emissions under aerobic conditions (Avalos Ramirez et al., 2012: Estrada et al., 2014, Pérez et al.,
269 2016). Recently, Cassarini et al. (2018) showed that the polyurethane foam packed BTF (without

pall rings) is a suitable bioreactor for the enrichment of slow growing microorganisms reducing the start-up time (120 days) to more than half of what was previously reported (~400 days). This study showed that the start-up period of AOM-SR BTF can be further reduced by using an enriched AOM-SR biomass and a mix of polyurethane foam and pall rings as the packing material.

*3.1.2. Sulfate reduction rates*

The maximum SR rates obtained (0.3 mmol $l^{-1}$ $day^{-1}$) were similar to the rates previously achieved in BTFs packed with only polyurethane foam (Cassarini et al., 2018; Bhattarai et al., 2018b). In phase I, 3 mM of sulfate was consumed after 20 days of reactor operation (start-up period), while sulfide was only scarcely produced (0.9 mM) towards the end of phase I (Figure 2B). In the first two phases (phases I and II), the SR and sulfide production rates were low (Figure 2B and Table 1) and not all the sulfate reduced was recovered as sulfide, i.e. 33% (phase I) and 61% (phase II). As previously described (Cassarini et al., 2018), part of the dissolved sulfide could have been precipitated as metal sulfide or could have been oxidized to elemental sulfur due to the presence of iron oxides in the Alpha Mound sediment (Wehrmann et al., 2011). Other sulfur compounds, such as polysulfides, could have also been formed, which were not analyzed in this study.

The highest SR rates were obtained in the last three phases of the BTF operation (Figure 2B and Table 1) and around 80% of the sulfate was reduced during each phase. The SR and sulfide production rates in the last three phases are not significantly different from each other (one-way ANOVA: $p > 0.050$). In phase III, the SR and sulfide production rates were 0.29 mmol $l^{-1}$ $day^{-1}$ and 0.17 mmol $l^{-1}$ $day^{-1}$, respectively. On day 133, almost all sulfate reduced was recovered as total dissolved sulfide (96%). However, the total dissolved sulfide concentration decreased abruptly from 4.7 to 2.1 mM on day 147 until the end of phase III, while the concentration of sulfate kept decreasing. This is probably due to polysulfides formation during this period: the

abrupt increase in the dissolved sulfide concentration on day 140 and the alkaline pH (8.3) favored polysufide formation (Finster et al., 1998). This assumption is supported by the change of color of the mineral medium (yellow) observed from day 150 until the replacement of fresh medium (160 days).

In phases IV and V, the total dissolved sulfide concentration increased to values as high as 6.6 mM. The SR rate ranged between 0.26 and 0.29 mmol $l^{-1}$ $day^{-1}$ during the last two phases of the BTF operation, similarly to phase III. The sulfide production rate was 0.17 mmol $l^{-1}$ $day^{-1}$ in phase IV, the same as in phase III. Differently in phase V, the sulfide production rate was lower: 0.15 mmol $l^{-1}$ $day^{-1}$. The pH of the BTF increased from 7.5 to 8.3 in all the five phases of the reactor operation (Figure 2A). The pH was brought down to 7.5 at each starting phase due to the mineral medium replacement, but increased to 8.3 due to the sulfide production. pH changes can cause disturbance on the microbial community mediating the target process (Tenno et al., 2018). The optimum pH for ANME and SRB is between 7 and 8 (Meulepas et al., 2009b). A pH higher than 9 or lower than 6.5 can substantially decrease the SR rate. However, in the last three phases of the BTF operation the pH ranged from 7.5 to 8.4 (in phase V), but the SR rate did not significantly change.

Nonetheless, the performance of the BTF can be further improved and customized to treat sulfur compounds other than sulfate, or other unwanted compounds such as selenium for future biotechnology applications.

*3.1.3. Methane oxidation rates*

As reported in Table 1 and shown in Figure 2C, differently than the SR rate, the methane consumption rate was higher than previously reported in BTF systems performing AOM, in which

the AOM rates were ten times lower than the SR rates (Bhattarai et al., 2018b; Cassarini et al., 2018). The methane consumption rate was the highest in the last three phases (III, IV and V) of the BTF operation, similar to the SR rates, but in phase IV and V the AOM rate was significantly higher than the SR in the corresponding phases (determined by one-way ANOVA: $p = 0.011$ and $p = 0.001$, respectively), showing that the stoichiometry of the reaction for AOM-SR (Eq. 1) was not followed. During the BTF operation, the reported methane consumption rate corresponds to the net methane consumption but it does not correspond to the net sulfate dependent-AOM. Previous studies showed that if net methane production (methanogenesis) occurs, trace amounts of methane will be concomitantly oxidized (Timmers et al., 2015b), leading to higher AOM rates.

$$CH_4 + SO_4^{2-} \rightarrow HCO_3^- + HS^- + H_2O \qquad \text{Eq.1}$$

Similar to the SR rates, the AOM rates did not significantly change during the last three phases of the BTF operation (one-way ANOVA: $p > 0.050$), showing that, from day 54 until the end of the operation the BTF reactor, the AOM-mediating microorganisms performed AOM-SR at similar volumetric rates at ambient temperature and pressure in the BTF.

*3.1.4. Carbon dioxide production*

The amount of carbon dioxide produced was the highest in phase III and IV (Figure 2C), when sulfide production was also the highest (6.6 mM). However, this amount corresponds to the net carbon dioxide concentration and does not account for the carbon dioxide production from sources other than methane or for its utilization by microorganisms. ANME, especially ANME-1, were found to utilize carbon dioxide as a carbon source (Treude et al., 2007; Holler et al., 2011). However, hardly any ANME-1 sequences were retrieved by 16S rRNA gene sequence analysis, as further discussed in the following section.

The carbon mass balance showed that the carbon recovery for each phase of the reactor operation was between 76 and 99% (Table 1), the majority of the methane entering the BTF escaped as methane-outgas from the BTF due to its low solubility in the artificial seawater medium (1.3 mM in seawater at 20ºC). Only a small amount of carbon (around 3%) accounts for the dissolved methane, which was mostly converted to carbon dioxide by AOM-SR. In order to account for the potential formation of methane and trace methane oxidation due to methanogenesis and carbon dioxide production from sources other than methane, batch activity assays with the enriched biomass after 238 days of BTF operation were performed, and the results are shown and discussed in section 3.3.

*3.2. Archaeal and bacteria diversity in the BTF*

Figure 3 shows the microbial community profiles obtained at the start and at the end of BTF operation (238 days). The highest percentages of archaeal 16S rRNA reads are shown in Figure 3A. Among the ANME clades, ANME-2a/b comprised 37% of the archaeal reads at the start and 60% at the end of the BTF operation. Other ANME clades, such as ANME-1, were also retrieved but the relative abundance was very low, from 0.08 to 0.01%, while the relative abundance of ANME-3 increased from 0.09 to 0.5%. More than 60% of the retrieved archaeal sequences belong to the ANME group, which reflects the high AOM rate obtained in the BTF. ANME-2 was the most abundant ANME type as in the previously reported studies using Alpha Mound sediment in a BTF (Cassarini et al., 2017; Cassarini et al., 2018), but also in other AOM studies performed in continuous bioreactor enrichments either at ambient (Meulepas et al., 2009a) or elevated (Zhang et al., 2011) pressure. However, ANME-1 cells were enriched in a similar BTF performing AOM by the Gingsburg Mud Volcano sediment (Bhattarai et al., 2018b). Different factors can influence the growth of ANME types, such as the availability of the substrates (e.g. methane and sulfate),

360 their mode of transport, the temperature and pressure, which can influence their availability.
361 However, the microbial and chemical composition of the inoculum also plays a major role and can
362 reflect the growth of the different microorganisms.

363 The bacterial community was more diverse at the start, where the relative abundance of other
364 different bacteria ("other bacteria" in Figure 3B) was 19%, much higher than at the end (238 days
365 of BTF operation), where the abundance of "other bacteria" was 0.1 % (Figure 3B). Bacteria that
366 could not compete in the AOM-SR environment in the BTF almost disappeared, such as
367 *Marinobacter* (Figure 3B). A high sulfate reducing activity was recorded in the BTF,
368 corresponding to a high percentage of bacterial 16S rRNA reads belonging to the order of
369 *Desulfobacterales* (Figure 3B), especially the relative percentage of *Desulfosarcina* increased
370 (from 36% to 58%). Other bacterial sequences from the *Deltaproteobacteria* class, such as
371 *Desulfarculus* and *Desulfuromonas*, were retrieved as well (Figure 3B). The relative percentage of
372 the *Desulfarculus* increased from less than 0.01 to 11% and the relative percentage of
373 *Desulfuromonas* increased from less than 1 to 10%. *Desulfuromonas* are elemental sulfur and
374 polysulfides reducers (Schauer et al., 2011), which gives a further indication of possible formation
375 of polysulfides in the BTF and their further reduction.

376 *3.3. AOM activity*

377 During the activity assays with $^{13}CH_4$ using the biomass from the BTF, 95% of the reduced sulfate
378 was recovered as total dissolved sulfide (Figure 4A). In the control incubation without biomass
379 (Figure 4A, dashed lines), sulfide was not produced, while in the batch incubations without
380 methane (Figure 4A, dotted lines), sulfide was produced only until day 20 (1.7 mM).

In the first 20 days of activity assays, the SR and sulfide production rate for the samples and control without methane were not significantly different (Table 1). Enriched biomass entrapped in the polyurethane foam cubes from the BTF operated for 238 days with a constant supply of methane was used for all the batches and control without methane during the activity assays. Most likely the enriched microorganisms mediating AOM-SR continued to reduce sulfate from endogenous carbon sources, even without the further addition of methane for 20 days.

The total DIC produced from methane increased only in the incubations with the biomass from the BTF and in the presence of methane in the headspace to 5.36 mM (Figure 4B). The calculated AOM was 0.16 mmol $l^{-1}$ $day^{-1}$, which was higher than the SR (0.10 mmol $l^{-1}$ $day^{-1}$) and sulfide (0.09 mmol $l^{-1}$ $day^{-1}$) production rates during the activity assays (Table 1). No methane was produced in the control without methane, confirming that no methanogenic activity occurred in the batches. Therefore, 0.16 mmol $l^{-1}$ $day^{-1}$ is the net AOM occurring in the activity assays, excluding carbon dioxide from sources other than methane and trace methane oxidation. In 42 days, 5 mM of sulfate were consumed and 5 mM of DIC were produced following the reaction stoichiometry (Eq. 1). The AOM rate found in this study was almost 20 times higher than that previously found after enrichment in a polyurethane foam packed BTF for 230 days (Cassarini et al., 2018), due to the further enrichment of ANME from the Alpha Mound sediment (Table 2).

*3.4. Cell numbers of ANME-2 and DSS*

ANME-2 and DSS were the most abundant microorganisms mediating AOM-SR in a previously reported BTF incoulated with Alpha Mound sediment (Cassarini et al., 2017; Cassarini et al., 2018). In this study, higher numbers of ANME-2 and DSS (22% and 55%, respectively) compared to previous studies (7% of ANME-2 and 46% of DSS) were found after 238 days in the BTF packed with polyurethane foam and pall rings and after 42 days of activity assays (Figure 5).

404 The cocci-shaped ANME-2 cells were mostly visualized in the form of aggregates (Figure 5A and
405 5C), but also ANME-2 clusters without bacterial partner were visualized (Figure 5B). The DSS
406 cells were present in different shapes, either as cocci usually surrounded with ANME-2 (Figure
407 5A, 5C and 4D), e) or vibrio shaped, usually visualized without archaeal partner (Figure 5B). The
408 cooperative interaction between ANME-2 and DSS is suggested in this study by their more
409 common visualization in aggregates (Figure 5A, 5C and 5D). However, ANME-2 have been
410 visualized without bacterial partner before, as more rarely found in this study (Figure 5B),
411 suggesting that this ANME type is supporting a metabolism independent of an obligatory bacterial
412 association (Ruff et al., 2016). The cooperative interaction between the ANME-2 and DSS is still
413 under debate: Milucka et al. (2012) stated that a syntrophic partner might not be required for
414 ANME-2 and that they can be decoupled by using external electron acceptors (Scheller et al.,
415 2016), whereas recent studies have shown direct electron transfer between the two (ANME-2 and
416 DSS) partners (McGlynn et al., 2015; Wegener et al., 2015).

417 The number of cells stained with specific CARD-FISH probes were counted along the incubation
418 time during the activity assays (Figure 5E). The number of cells stained with the bacterial CARD-
419 FISH probe (EUB 338-I-II-III) was always higher than the number of stained archaeal cells (Figure
420 5E). A significant decrease in the bacterial population was registered from the start of the activity
421 assays till day 21 (one-way ANOVA: $p = 0.047$) and till day 28 (one-way ANOVA: $p = 0.003$).
422 The samples were successfully hybridized with the DSS probe and with the ANME-2 probe,
423 separately. The cells hybridized with the DSS probe were always significantly more abundant than
424 the ANME-2 cells. However, there was no significant difference in the DSS population from the
425 start till the end of the activity assays (one-way ANOVA: $p > 0.050$). The DSS were ~42 % of the
426 total number of the stained cells during the activity assays (Figure 5E).

The archaeal population did not change significantly during the activity assays (one-way ANOVA: $p > 0.050$) and the number of ANME-2 visualized and counted is close to the total number of archaea. However, ANME-2 significantly increased (22% of the total number of stained cells) from the start of the activity assays till day 28 (one-way ANOVA: $p = 0.000$) and from the beginning till day 42 (one-way ANOVA: $p = 0.020$).

The visualization and quantification of ANME-2 and DSS, supposedly mediating AOM-SR, confirmed that further enrichment of the anaerobic methantrophs, specifically ANME-2, occurred during the BTF operation with polyurethane foam and pall rings as packing material following the increase in the AOM rate compared to the previously reported BTF (Cassarini et al., 2018).

## 4. Conclusions and implications

This study indicated that a BTF packed with polyurethane foam and pall rings inoculated with enriched biomass showed a rapid start-up (~20 days) and a better performance in terms of AOM-rates (0.4 mmol $l^{-1}$ $day^{-1}$) and ANME-2 (22%) and DSS (50%) enrichment. This is the highest AOM rate reported so far in a BTF system inoculated with deep sea sediment. However, the SR rate should still be 100 times higher than what was found in this study to be competitive with the SR rates achieved for desulfurization of wastewater with other electron donors, such as hydrogen or ethanol.

The BTF technology is widely used for the treatment of industrial waste gases containing volatile organic and inorganic pollutants, however, its main disadvantage is clogging due to the accumulation of excess biomass. The BTF used in this study did not pose any operational problem with respect to clogging or channeling, and the biomass was actively maintained during its long term operation (230 days). The BTF design for AOM-SR can be nevertheless further optimized.

The majority of the methane entering the BTF escaped as methane outgas from the BTF due to its low solubility in the artificial seawater medium. A gas-recycling strategy could be used to enhance the $CH_4$ gas to liquid-phase mass transfer even at low concentrations of $CH_4$. In the BTF for AOM-SR, recycling the non-oxidized $CH_4$ to achieve its complete removal might be a good approach. However, unwanted or toxic compounds, such as hydrogen sulfide, need to be stripped out prior recirculation.

For future applications, naturally occurring materials (e.g. sandstone, lava rocks) or inert materials such as plastic rings or resins can be tested in a BTF for AOM-SR. Based on the knowledge gained from this work, ANME and SRB can be enriched in a BTF. The enriched community can be further used to understand its mechanisms and the BTF design can be further improved and controlled for future biotechnological applications of AOM-SR.

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## References

Acree, T.E., Sonoff, E.P., Splittstoesser, D.F., 1971. Determination of hydrogen sulfide in fermentation broths containing $SO_2$. Appl. Microbiol. 22, 110-112.

Aoki, M., Ehara, M., Saito, Y., Yoshioka, H., Miyazaki, M., Saito, Y., Miyashita, A., Kawakami, S.,Yamaguchi, T., Ohashi, A., Nunoura, T., Takai, K., Imachi, H., 2014. A long-term cultivation of an anaerobic methane-oxidizing microbial community from deep-sea methane-seep sediment using a continuous-flow bioreactor. Plos One 9, e105356.

APHA., 2012. Standard methods for the examination of water and wastewater, in: American Public Health Association, 19th ed. Washington DC, USA. pp.1325.

Avalos Ramirez, A., Jones, J.P., Heitz, M., 2012. Methane treatment in biotrickling filters packed with inert materials in presence of a non-ionic surfactant. J. Chem. Technol. Biotechnol. 87, 848-853.

Bhattarai, S., Cassarini, C., Gonzalez-Gil, G., Egger, M., Slomp, C.P., Zhang, Y., Esposito, G., Lens, P.N.L., 2017a. Anaerobic methane-oxidizing microbial community in a coastal marine sediment: anaerobic methanotrophy dominated by ANME-3. Microb. Ecol. 74, 608-622.

Bhattarai, S., Cassarini, C., Naangmenyele, Z., Rene, E.R., Gonzalez-Gil, G., Esposito, G., Lens, P.N.L., 2017b. Microbial sulfate reducing activities in aoxic sediment from Marine Lake Grevelingen. Limnology. https://doi.org/10.1007/s10201-017-0516-0.

Bhattarai, S., Cassarini, C., Rene, E.R., Kümmel, S., Esposito, G., and Lens, P.N.L., 2018a. Enrichment of ANME-2 methanotrphy from cold seep sediment in an external ultrafiltration tration membrane bioreactor. Eng. Life Sci. 18, 368.

Bhattarai, S., Cassarini, C., Rene, E.R., Zhang, Y., Esposito, G., and Lens, P.N., 2018b. Enrichment of sulfate reducing anaerobic methane oxidizing community dominated by ANME-1 from Ginsburg Mud Volcano (Gulf of Cadiz) sediment in a biotrickling filter. Bioresour. Technol. 259, 433-441.

Cassarini, C., Bhattarai, S., Rene, E.R., Vogt, C., Musat, N., Esposito, G., Lens, P.N.L., 2018. Enrichment of anaerobic methanotrophs in biotrickling filters using different sulfur compounds as electron acceptor. Environ. Eng. Sci. https://doi.org/10.1089/ees.2018.0283.

Cassarini, C., Rene, E.R., Bhattarai, S., Esposito, G., Lens, P.N.L., 2017. Anaerobic oxidation of methane coupled to thiosulfate reduction in a biotrickling filter. Bioresour. Technol. 240, 214-222.

Cassarini, C., Zhang, Y., Lens, P.N. L., 2019. Pressure selects dominant anaerobic methanotrophic phylotype and sulfate reducing bacteria in coastal marine lake Grevelingen sediment. Front. Environ. Sci. 6, 162.

Daims, H., Brühl, A., Amann, R., Schleifer, K.-H., Wagner, M., 1999. The domain-specific probe EUB338 is insufficient for the detection of all Bacteria: development and evaluation of a more comprehensive probe set. Syst. Appl. Microbiol. 22, 434-444.

Deusner, C., Meyer, V., Ferdelman, T., 2009. High-pressure systems for gas-phase free continuous incubation of enriched marine microbial communities performing anaerobic oxidation of methane. Biotechnol. Bioeng. 105, 524-533.

Dorer, C., Vogt, C., Neu, T.R., Stryhanyuk, H., Richnow, H.H., 2016. Characterization of toluene and ethylbenzene biodegradation under nitrate-, iron (III)-and manganese (IV)-reducing conditions by compound-specific isotope analysis. Environ. Pollut. 211, 271-281.

Estrada, J.M., Dudek, A., Muñoz, R., Quijano, G., 2014. Fundamental study on gas-liquid mass transfer in a biotrickling filter packed with polyurethane foam. J. Chem. Technol. Biotechnol. 89, 1419-1424.

Finster, K., Liesack, W., Thamdrup, B., 1998. Elemental sulfur and thiosulfate disproportionation by *Desulfocapsa sulfoexigens* sp. nov., a new anaerobic bacterium isolated from marine surface sediment. Appl. Environ. Microbiol. 64, 119-125.

Forster, A., Schouten, S., Baas, M., Sinninghe Damsté, J.S., 2007. Mid-Cretaceous (Albanian-Santonian) sea surface temperature record of the tropical Atlantic Ocean. Geology 35, 919.

Herrmann, S., Kleinsteuber, S., Chatzinotas, A., Kuppardt, S., Lueders, T., Richnow, H.-H., Vogt, C., 2010. Functional characterization of an anaerobic benzene-degrading enrichment culture by DNA stable isotope probing. Environ. Microbiol. 12, 401-411.

Holler, T., Widdel, F., Knittel, K., Amann, R., Kellermann, M.Y., Hinrichs, K.-U., Teske, A., Boetius, A., Wegener, G., 2011. Thermophilic anaerobic oxidation of methane by marine microbial consortia. ISME J. 5, 1946-1956.

Kirschke, S., Bousquet, P., Ciais, P., Saunois, M., Canadell, J.G., Dlugokencky, E.J., Bergamaschi, P., Bergmann, D., Blake, D.R., Bruhwiler, L., Cameron-Smith, P., Castaldi, S., Chevallier, F., Feng, L., Fraser, A., Heimann, M., Hodson, E.L., Houweling, S., Josse, B., Fraser, P.J., Krummel, P.B., Lamarque, J.-F., Langenfelds, R.L., Le Quéré, C., Naik, V., O'Doherty, S., Palmer, P.I., Pison, I., Plummer, D., Poulter, B., Prinn, R.G., Rigby, M., Ringeval, B., Santini, M., Schmidt, M., Shindell, D.T., Simpson, I.J., Spahni, R., Steele, L.P., Strode, S.A., Sudo, K., Szopa, S., van der Werf, G.R., Voulgarakis, A., van Weele, M., Weiss, R.F., Williams, J.E., Zeng, G., 2013. Three decades of global methane sources and sinks. Nat. Geosci. 6, 813-823.

Klein, K., Kivi1, A., Dulova, N., Zekker, I., Mölder, E., Tenno, T., Trapido, M., Tenno, T., 2017. A pilot study of three-stage biological–chemical treatment of landfill leachate applying continuous ferric sludge reuse in Fenton-like process. Clean Techn. Environ. Policy 19, 541-551.

Kleindienst, S., Ramette, A., Amann, R., Knittel, K., 2012. Distribution and in situ abundance of sulfate-reducing bacteria in diverse marine hydrocarbon seep sediments. Environ. Microbiol. 14, 2689-710.

Knittel, K., Boetius, A., 2009. Anaerobic oxidation of methane: progress with an unknown process. Annual. Rev. Microbiol. 63, 311-334.

Mandel, A., Zekker, I., Jaagura, M., Tenno, T., 2019. Enhancement of anoxic phosphorus uptake of denitrifying phosphorus removal process by biomass adaption. Int. J. Environ. Sci. Technol. https://doi.org/10.1007/s13762-018-02194-2.

Manz, W., Eisenbrecher, M., Neu, T.R., Szewzyk, U., 1998. Abundance and spatial organization of gram-negative sulfate-reducing bacteria in activated sludge investigated by in situ probing with specific 16S rRNA targeted oligonucleotides. FEMS Microbiol. Ecol. 25, 43-61.

Marlow, J.J., Steele, J.A., Case, D.H., Connon, S.A., Levin, L.A., Orphan, V.J., 2014. Microbial abundance and diversity patterns associated with sediments and carbonatesfrom the methane seep environments of Hydrate Ridge, OR. Front. Mar. Sci.1, 1-16.

McGlynn, S.E., 2017. Energy metabolism during anaerobic methane oxidation in ANME archaea. Microbes Environ. 32, 5-13.

McGlynn, S.E., Chadwick, G.L., Kempes, C.P., Orphan, V.J., 2015. Single cell activity reveals direct electron transfer in methanotrophic consortia. Nature 526,531-535.

Meulepas, R.J.W., Jagersma, C.G., Gieteling, J., Buisman, C.J.N., Stams, A.J.M., Lens, P.N.L., 2009a. Enrichment of anaerobic methanotrophs in sulfate-reducing membrane bioreactors. Biotechnol. Bioeng. 104, 458-470.

Meulepas, R.J.W., Jagersma, C.G., Khadem, A.F., Buisman, C.J.N., Stams, A.J.M., Lens, P.N.L., 2009b. Effect of environmental conditions on sulfate reduction with methane as electron donor by an Eckemförde Bay enrichment. Environ. Sci. Technol. 43, 6553-6559.

Milucka, J., Ferdelman, T.G., Polerecky, L., Franzke, D., Wegener, G., Schmid, M., Lieberwirth, I., Wagner, M., Widdel, F., Kuypers, M.M.M., 2012. Zero-valent sulphur is a key intermediate in marine methane oxidation. Nature 491, 541-546.

Musat, N., Halm, H., Winterholler, B., Hoppe, P., Peduzzi, S., Hillion, F., Horreard, F., Amann, R., Jørgensen, B.B., Kuypers, M.M.M., 2008. A single-cell view on the ecophysiology of anaerobic phototrophic bacteria. PNAS 105, 17861-17866.

Pérez, M., Álvarez-Hornos, F., Engesser, K., Dobslaw, D., Gabaldón, C., 2016. Removal of 2-butoxyethanol gaseous emissions by biotrickling filtration packed with polyurethane foam. New Biotechnol. 33, 263-272.

Pernthaler, A., Pernthaler, J., Amann, R., 2004. Sensitive multi-color fluorescence in situ hybridization for the identification of environmental microorganisms, in: Kowalchuk, G., de Bruijn, F., Head, I., Akkermans, A., van Elsas, J.D. (Eds.), Molecular Microbial Ecology Manual. Springer, Dordrecht, pp. 711-726.

Pruesse, E., Quast, C., Knittel, K., Fuchs, B.M., Ludwig, W., Peplies, J., Glöckner, F.O., 2007. SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucleic Acids Res. 35, 7188-7196.

Raghoebarsing, A.A., Pol, A., van de Pas-Schoonen, K.T., Smolders, A.J.P., Ettwig, K.F., Rijpstra, W.I.C., Schouten, S., Damsté, J.S.S., Op den Camp, H.J.M., Jetten, M.S.M., Strous, M., 2006. A microbial consortium couples anaerobic methane oxidation to denitrification. Nature 440, 918-921.

Rikmann, E., Zekker, I., Tenno, T., Saluste, A., Tenno, T., 2018. Inoculum-free start-up of biofilm- and sludge-based deammonification systems in pilot scale. Environ. Sci. Technol. 15, 133-148.

Reeburgh, W.S., 2007. Oceanic methane biogeochemistry. Chem. Rev. 107, 486-513.

Ruff, S. E., Kuhfuss, H., Wegener, G., Lott, C., Ramette, A., Wiedling, J., Knittel, K., Weber, M., 2016. Methane seep in shallow-water permeable sediment harbors high diversity of anaerobic methanotrophic communities, Elba, Italy. Front. Microbiol. 7, 374.

Schauer, R., Røy, H., Augustin, N., Gennerich, H.-H., Peters, M., Wenzhoefer, F., Amann, R., Meyerdierks, A., 2011. Bacterial sulfur cycling shapes microbial communities in surface sediments of an ultramafic hydrothermal vent field. Environ. Microbiol. 13, 2633-2648.

Scheller, S., Yu, H., Chadwick, G.L., McGlynn, S.E., Orphan, V.J., 2016. Artificial electron acceptors decouple archaeal methane oxidation from sulfate reduction. Science 351, 703-707.

Schloss, P.D., Westcott, S.L., 2011. Assessing and improving methods used in operational taxonomic unit-based approaches for 16S rRNA gene sequence analysis. Appl. Environ. Microbiol. 77, 3219-3226.

Schreiber, L., Holler, T., Knittel, K., Meyerdierks, A., Amann, R., 2010. Identification of the dominant sulfate-reducing bacterial partner of anaerobic methanotrophs of the ANME-2 clade. Environ. Microbiol. 12, 2327-2340.

Song, Z.-Q., Wang, F.-P., Zhi, X.-Y., Chen, J.-Q., Zhou, E.-M., Liang, F., Xiao, X., Tang, S.K., Jiang, H.C., Zhang, C.L., Dong, H., Li, W.J., 2013. Bacterial and archaeal diversities in Yunnan and Tibetan hot springs, China. Environ. Microbiol. 15, 1160-1175.

Stahl, D.A., Amann, R.I., 1991. Development and application of nucleic acid probes, in: Stackebrandt, E., Goodfellow, M. (Eds.), Nucleic Acid Techniques in Bacterial Systematics. John Wiley & Sons Ltd, Chichester, UK, pp. 205-248.

Suarez-Zuluaga, D.A., Weijma, J., Timmers, P.H.A., Buisman, C.J.N., 2014. High rates of anaerobic oxidation of methane, ethane and propane coupled to thiosulphate reduction. Environ. Sci. Pollut. Res. 22, 3697-3704.

Tenno, T., Rikmann, E., Uiga, K., Zekker, I., Mashirin, A., Tenno, T., 2018. A novel proton transfer model of the closed equilibrium system $H_2O$–$CO_2$–$CaCO_3$–$NH_X$. P. Est. Acad. Sci. 67, 26-270.

Timmers, P.H., Gieteling, J., Widjaja-Greefkes, H.A., Plugge, C.M., Stams, A.J., Lens, P.N.L., Meulepas, R.J., 2015a. Growth of anaerobic methane-oxidizing archaea and sulfate-reducing

bacteria in a high-pressure membrane capsule bioreactor. Appl. Environ. Microbiol. 81, 1286-1296.

Timmers, P.H., Suarez-Zuluaga, D.A., van Rossem, M., Diender, M., Stams, A.J., Plugge, C.M., 2015b. Anaerobic oxidation of methane associated with sulfate reduction in a natural freshwater gas source. ISME J. 10, 1400-1412.

Timmers, P.H., Welte, C.U., Koehorst, J.J., Plugge, C.M., Jetten, M.S.M., Stams, A.J.M., 2017. Reverse methanogenesis and respiration in methanotrophic archaea. Archaea 2017,1654237.

Wegener, G., Krukenberg, V., Riedel, D., Tegetmeyer, H.E., Boetius, A., 2015. Intercellular wiring enables electron transfer between methanotrophic archaea and bacteria. Nature 526,587-603.

Wegener, G., Krukenberg, V., Ruff, S.E., Kellermann, M.Y., Knittel, K., 2016. Metabolic capabilities of microorganisms involved in and associated with the anaerobic oxidation of methane. Front. Microbiol. 7, 46.

Wehrmann, L.M., Risgaard-Petersen, N., Schrum, H.N., Walsh, E.A., Huh, Y., Ikehara, M., Pierre, C., D'Hondt, S., Ferdelman, T.G., Ravelo, A.C., Takahashi, K., Zarikian, C., 2011. Coupled organic and inorganic carbon cycling in the deep subseafloor sediment of the north-eastern bering sea slope. Chem. Geol. 284, 251-261.

Widdel, F., Bak, F., 1992. Gram negative mesophilic sulfate reducing bacteria, in: Balows, A., Truper, H., Dworkin, M., Harder, K., Schleifer, H. (Eds.), The prokaryotes: a handbook on the biology of bacteria: ecophysiology, isolation, identification, applications. Springer-Verlag, New York, USA, pp. 3352-3378.

Zekker, I., Kivirüüt, Rikmann, E., Mandel, A., Jaagura, M., Tenno, T., Artemchuk, O., Rubin, S., Tenno, T., 2018. Enhanced efficiency of nitritating-anammox sequencing batch reactor achieved at low decrease rates of oxidation–reduction potential. Environ. Eng. Sci. https://doi.org/10.1089/ees.2018.0225.

Zhang, Y., Maignien, L., Zhao, X., Wang, F., Boon, N., 2011. Enrichment of a microbial community performing anaerobic oxidation of methane in a continuous high-pressure bioreactor. BMC Microbiol. 11, 1-8.

Zhu, J., Wang, Q., Yuan, M., Tan, G.-Y.A., Sun, F., Wang, C., Wu, W., Lee, P.-H, 2016. Microbiology and potential applications of aerobic methane oxidation coupled to denitrification (AME-D) process: a review. Water Res. 90, 203-215.

## Tables

**Table 1.** Sulfide production, SR and AOM rates, and the sulfur and carbon balance for each different phase in the BTF and for the activity tests with $^{13}CH_4$, controls without $CH_4$ but with $N_2$ in the headspace and controls without biomass.

| Incubation description and duration | | Sulfide production rate (mmol $l^{-1}$ $day^{-1}$) | SR rate (mmol $l^{-1}$ $day^{-1}$) | Sulfur balance | AOM rate (mmol $l^{-1}$ $day^{-1}$) | Carbon balance |
|---|---|---|---|---|---|---|
| **BTF (238 days)** | | | | | | |
| Phase I 0–61 days | | 0.00 ± 0.00 | 0.05 ± 0.00 | 0.33 | 0.19 ± 0.09 | 0.76 |
| Phase II 61–119 days | | 0.08 ± 0.00 | 0.12 ± 0.00 | 0.61 | 0.28 ± 0.02 | 0.88 |
| Phase III 119–160 days | | 0.17 ± 0.01 | 0.29 ± 0.03 | 0.96 | 0.34 ± 0.06 | 0.99 |
| Phase IV 160–214 days | | 0.17 ± 0.01 | 0.26 ± 0.03 | 0.98 | 0.37 ± 0.03 | 0.97 |
| Phase V 214–238 days | | 0.15 ± 0.01 | 0.26 ± 0.04 | 0.98 | 0.31 ± 0.02 | 0.87 |
| **Activity assays (42 days)** | | | | | | |
| Incubation with $^{13}CH_4$ | 0-42 days | 0.09 ± 0.01 | 0.10 ± 0.03 | 0.99 | 0.16 ± 0.01 | 0.99 |
| Incubation without $^{13}CH_4$ | 0-20 days | 0.08 ± 0.02 | 0.05 ± 0.03 | 0.99 | Not detected | 0.99 |
| | 20-42 days | Not detected | Not detected | 0.99 | | |
| Incubation without biomass | 0-42 days | Not detected | Not detected | 0.99 | Not detected | 0.99 |

651 **Table 2.** Overview of inocula and bioreactor types used in AOM studies.

652

| Inoculum | Bioreactor type | Operation time (d) | AOM rate ($\mu mol\ l^{-1}\ day^{-1}$) | Reference |
|---|---|---|---|---|
| Alpha Mound, Gulf of Cadiz | Polyurethane foam and pall rings packed BTF | 238 | 160 | This study |
| Alpha Mound, Gulf of Cadiz | Polyurethane foam packed BTF | 248 | 8.4 | Cassarini et al., 2018 |
| Gingsburg mud volcano, Gulf of Cadiz | Polyurethane foam packed BTF | 200 | 10 | Bhattarai et al., 2018b |
| Nankai Trough | Down-flow hanging sponge (DHS) bioreactor | 2013 | 0.375 $\mu mol\ g_{dw}^{-1} d^{-1}$ | Aoki et al., 2014 |
| Ginsburg mud volcano, Gulf of Cadiz | External ultrafiltration membrane bioreactor | 726 | 1.2 $\mu mol\ g_{dw}^{-1} d^{-1}$ | Bhattarai et al., 2018a |

653

654 **Figure legends**

655 **Figure 1.** Schematic of a biotrickling filter (BTF) configuration for anaerobic methane oxidation
656 coupled to thiosulfate reduction. The filter bed (5.6 l) is packed with polyurethane foam cubes and
657 polypropylene pall. SP: sampling port.

658 **Figure 2.** Profiles of different process parameters monitored during the operation of the BTF with
659 methane as electron donor and sulfate as electron acceptor: (A) pH, (B) sulfate and sulfide, and
660 (C) carbon dioxide concentration measured as [carbon dioxide outlet – carbon dioxide inlet]. The
661 vertical lines represent the different phases in bioreactor operation; I: days 0–61, II: days 61–119,
662 III: days 119–160, IV: days 160–214, V: days 214–238. The start of each phase indicates the days
663 at which the mineral medium was replaced.

664 **Figure 3.**

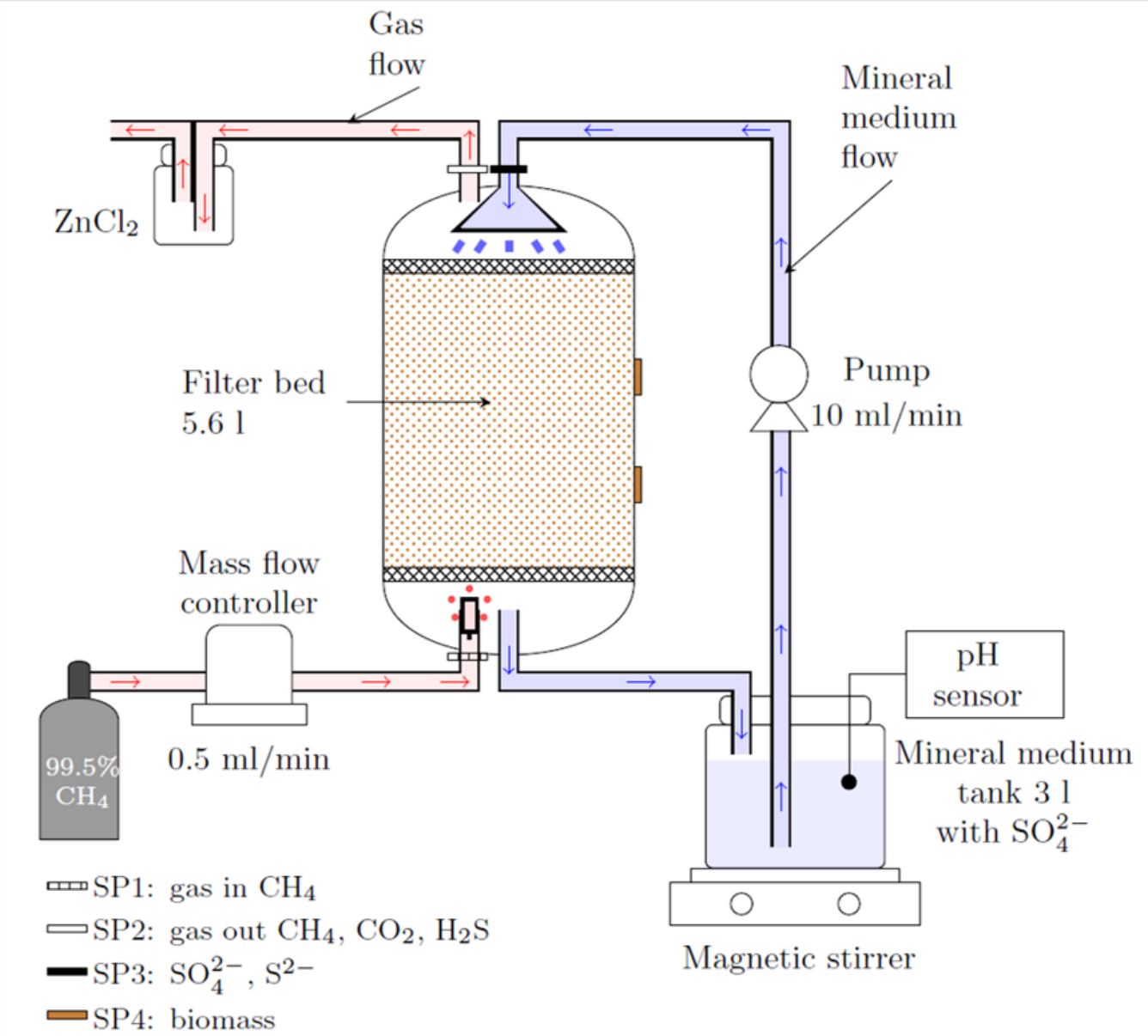
665 Topmost abundant 16S rRNA sequences showing the phylogenetic affiliation up to gene level as
666 derived by high-throughput sequencing of archaea (a) and bacteria (b) at the start (t=0) and at the
667 end (t = 238 d) of the BTF operation.

668 **Figure 4.** Batch activity assay profiles for the BTF with sulfate as electron acceptor and methane
669 as the sole electron donor during the activity test for the batches incubated with $^{13}CH_4$ (triplicates)
670 and controls. *Dotted lines* show the controls without $CH_4$, but with $N_2$ in the headspace and *dashed*
671 *lines* showed controls without biomass. (A) Sulfide and sulfate profiles and (B) the total dissolved
672 inorganic carbon (DIC) production calculated from the produced $^{13}CO_2$. Error bars represent the
673 standard deviation of triplicate measurements.

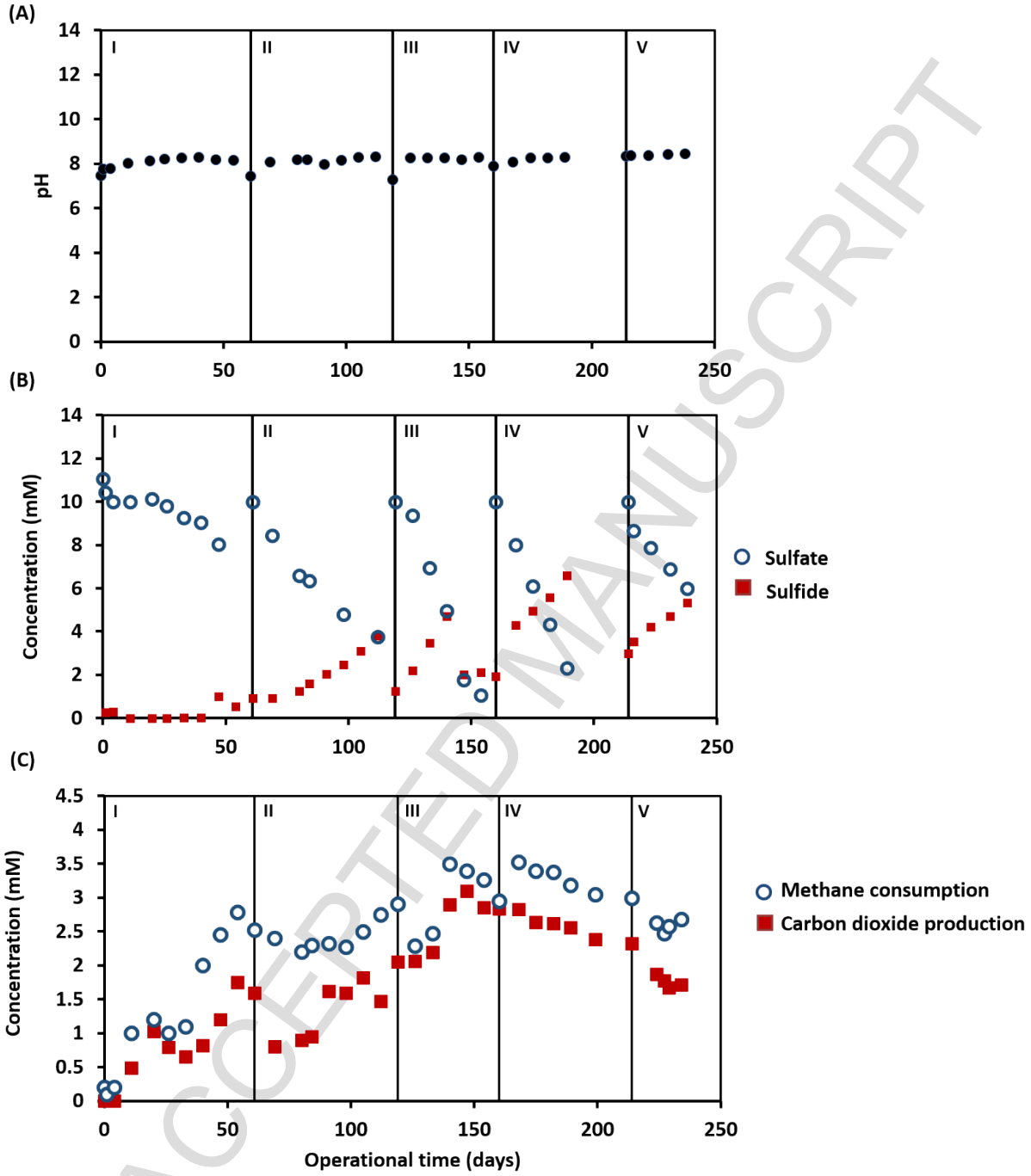
674 **Figure 5.** Catalyzed reporter deposition - fluorescence *in situ* hybridization (CARD-FISH)
675 analysis during the activity assays. CARD-FISH images (A–D) from ANME-2 in red color and

676 *Desulforsarcina*/*Desulfococcus* group (DSS) in green after 42 days of incubation with $^{13}CH_4$.
677 White scale bar representing 10µm. (E) The number of single cells stained by specific CARD-
678 FISH probe over DAPI (4', 6-diamidino-2-phenylindole, all cells) stained cells during the activity
679 assays incubated with $^{13}CH_4$ (triplicates). Error bars indicate the standard deviation (n=3). CARD-
680 FISH probes used: ARC 915 (for all archaea), EUB 338-I-II-III (for all bacteria), ANME-2 538
681 (for ANME clade: ANME-2) and DSS658 (for a specific gorup of SRB: *Desulfosarcina* /
682 *Desulfococcus*, DSS).

**Figure 1**

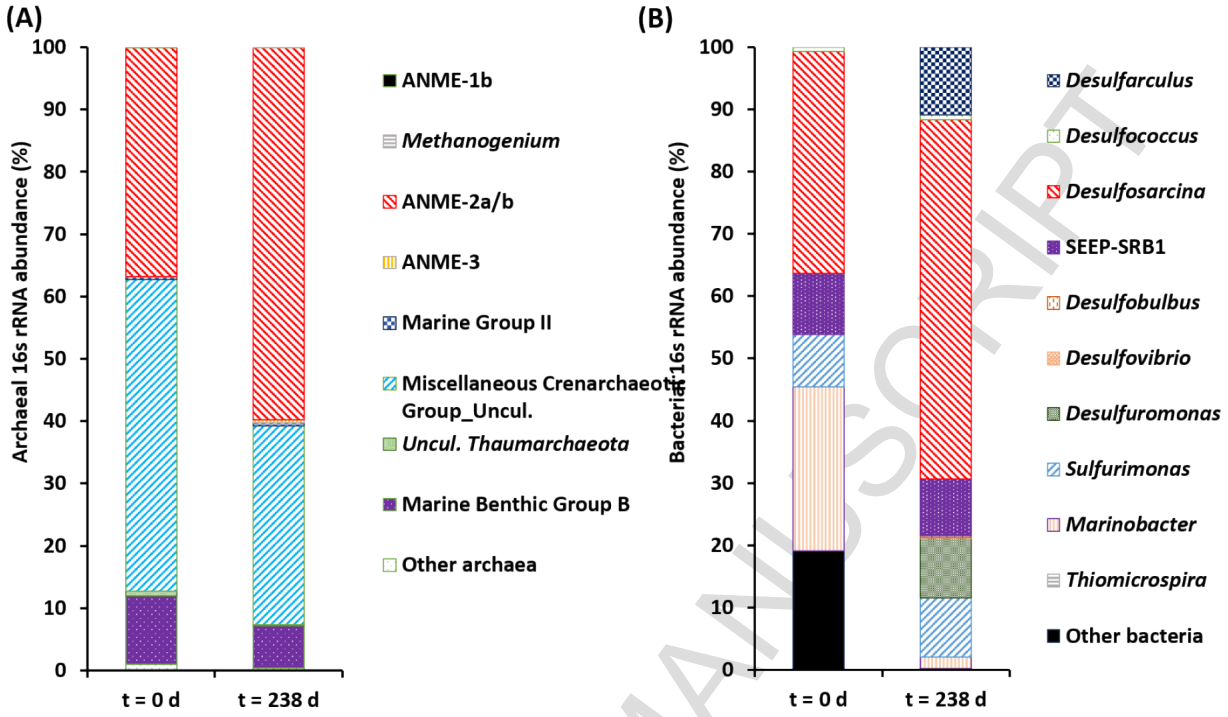


686 **Figure 2**



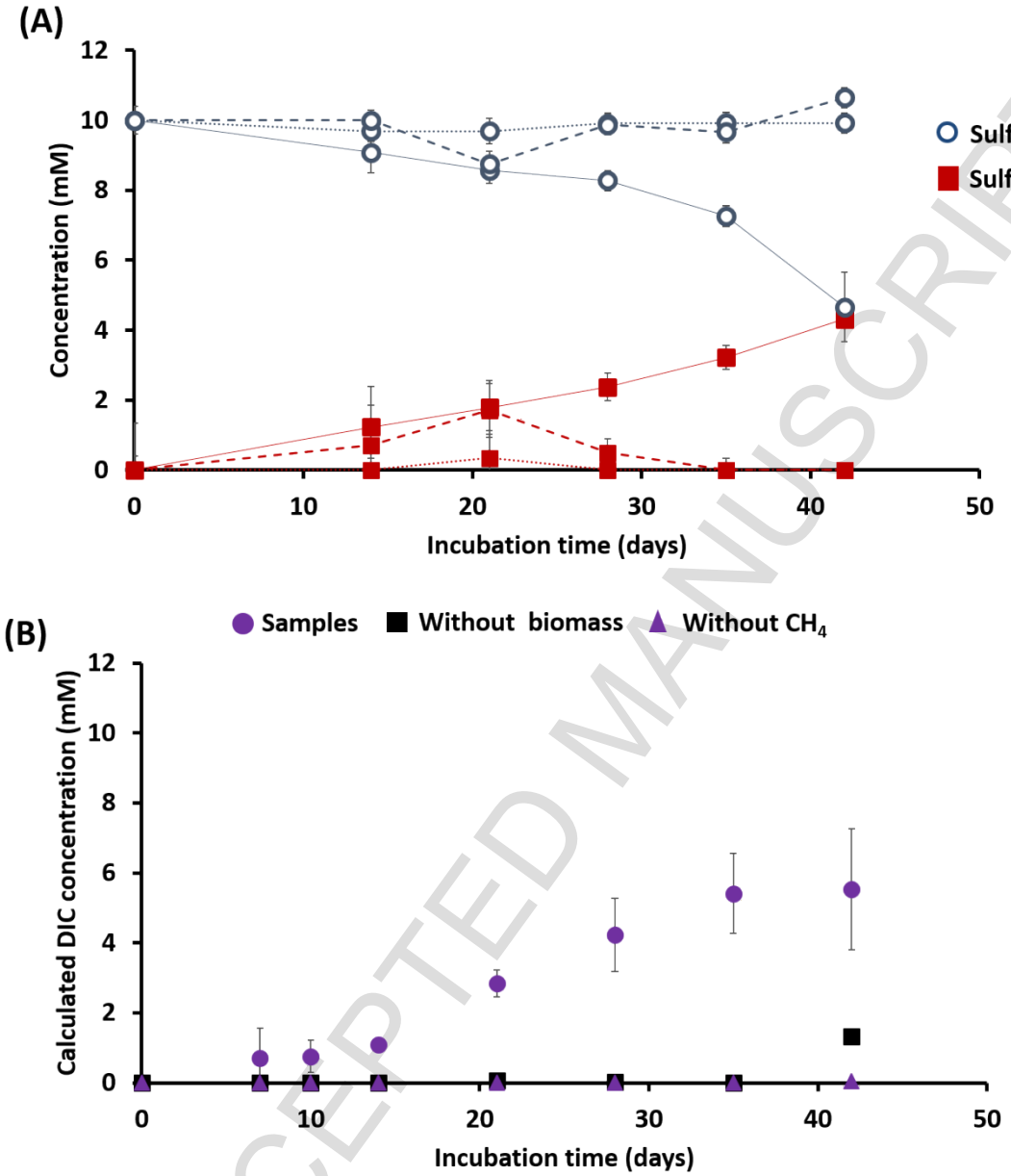

687

688 **Figure 3**



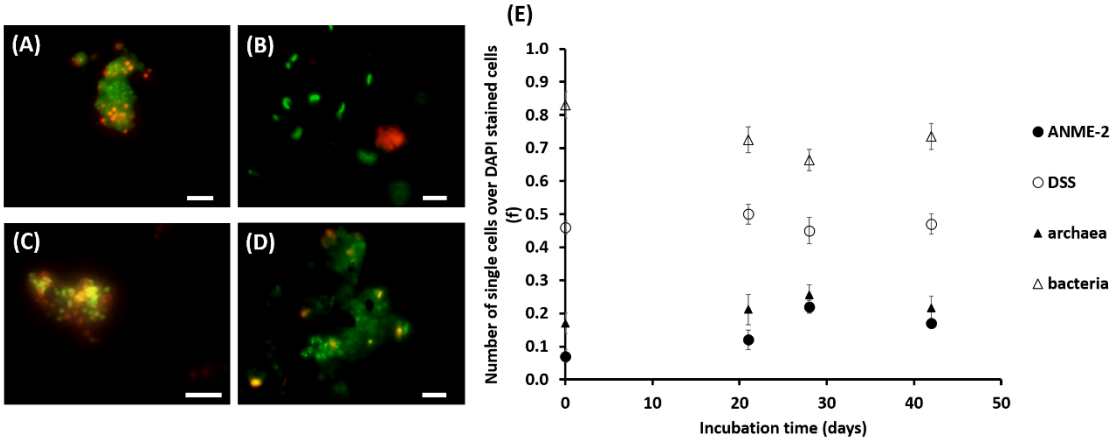

689

690 **Figure 4**




691

692 **Figure 5**

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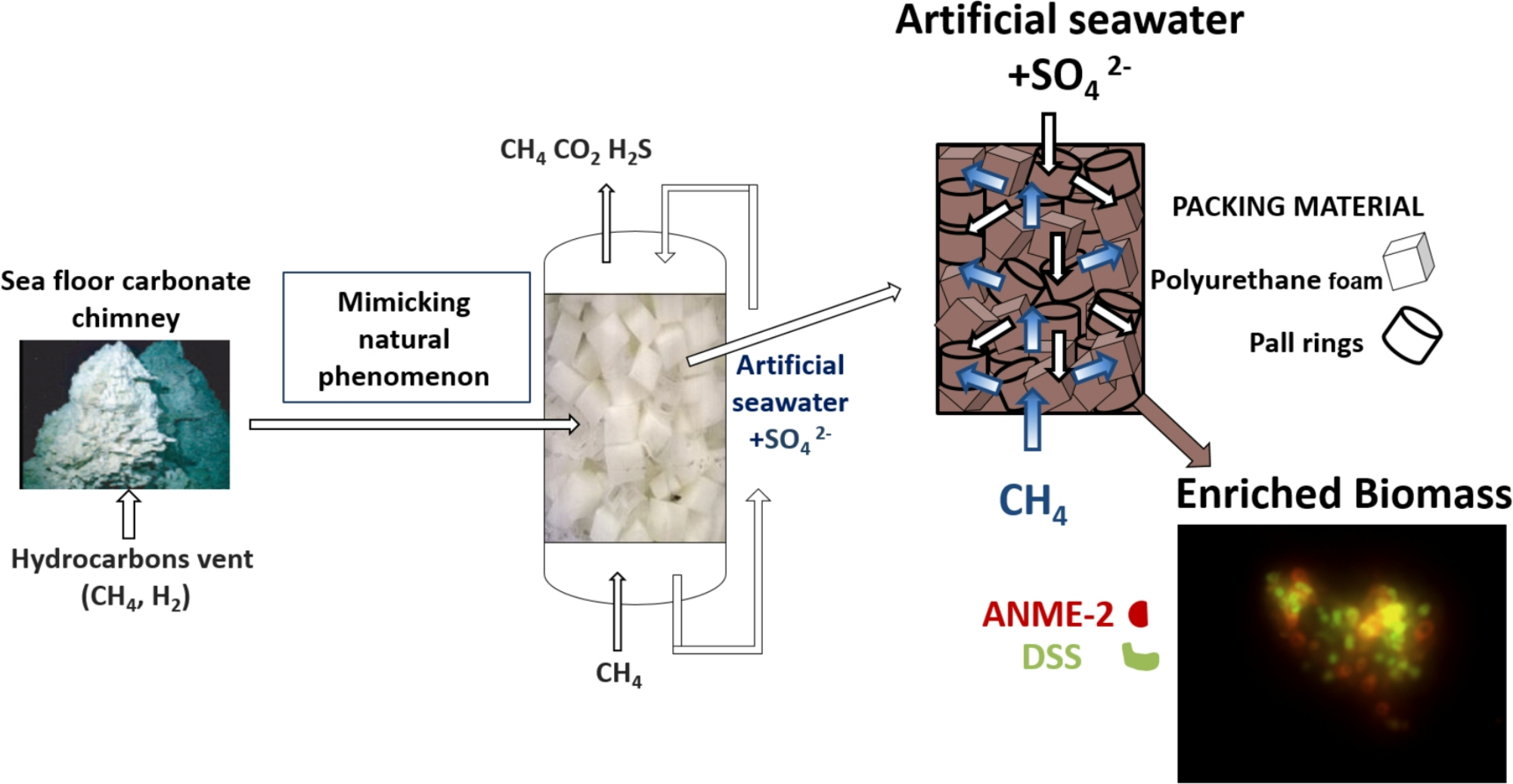
Artificial seawater
+SO4 2-
CH4 CO2 H2S
PACKING MATERIAL
Polyurethane foam
Pall rings
Sea floor carbonate chimney
Mimicking natural phenomenon
Artificial seawater +SO4 2-
CH4
Enriched Biomass
Hydrocarbons vent (CH4, H2)
CH4
ANME-2
DSS