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Research article

Evaluation of the influence of methane and copper concentration and methane mass transport on the community structure and biodegradation kinetics of methanotrophic cultures

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ABSTRACT

The environmental conditions during culture enrichment, which ultimately determine its maximum specific biodegradation rate (q_{max}) and affinity for the target pollutant (K_s), play a key role in the performance of bioreactors devoted to the treatment of methane emissions. This study assessed the influence of Cu^{2+} and CH_4 concentration and the effective CH_4 supply rate during culture enrichment on the structure and biodegradation kinetics of methanotrophic communities. The results obtained demonstrated that an increase in Cu^{2+} concentration from 0.05 to 25 μM increased the q_{max} and K_s of the communities enriched by a factor of ≈3, even if the Cu^{2+} concentration did not seem to have an effect on the enzymatic "copper switch" and only pMMO was detected. In addition, high Cu^{2+} concentrations supported lower diversity coefficients (Hs ≈ 1.5× lower) and apparently promoted the growth of more adapted methanotrophs such as *Methylomonas*. Despite no clear effect of CH_4 concentration on the population structure or on the biodegradation kinetics of the communities enriched was recorded at the two low CH_4 concentrations studied (1 and 8%), a higher agitation rate increased the q_{max} by a factor of ≈2.3 and Ks by a factor of ≈3.1.

1. Introduction

Methane (CH_4) is the second most relevant greenhouse gas (GHG) emitted by anthropogenic activities, representing more than 20% of the total worldwide GHG emissions. CH_4 atmospheric concentration increases yearly at 0.2–1% its current tropospheric level exceeding preindustrial concentrations by ≈150%. Anthropogenic emissions are mainly attributed to agriculture, livestock farming, waste management and energy production, which combined represent over 60% of the total CH_4 emissions worldwide (European Environmental Agency, 2013; IPCC, 2013; United States Environmental Protection Agency, 2013). In addition, the impact of CH_4 on climate change is ≈34 times more detrimental than that of carbon dioxide (CO_2) in a 100-y horizon (European Environmental Agency, 2013; IPCC, 2013; Scheutz et al., 2009). This scenario has caused a raised governmental and public awareness, which has promoted both the enforcement of multidisciplinary political initiatives and an increased research on CH_4 abatement technologies (European Environmental Agency, 2013; IPCC, 2013; Scheutz et al., 2009). Although emissions with CH_4 concentrations above 20% are suitable for energy recovery by incineration or low-cost treatment by flaring, more than 50% of anthropogenic emissions contain CH_4 at concentrations below 3% (old landfills (0–20%), ventilated coal mines (0.1–1%), covered liquid manure storage tanks (0–3%), etc.) (Nikiema et al., 2007; Scheutz et al., 2009). CH_4 abatement at such low concentrations using conventional physical/chemical technologies is either inefficient or too costly, and often entails a large CO_2 footprint (Estrada et al., 2014; Nikiema et al., 2007). In this context, biological treatment technologies can become, if appropriately optimized, a low-cost and environmentally friendly alternative for the treatment of CH_4 due to their already proven effectiveness and low operating costs during the abatement of malodors and volatile organic compound emissions (López et al., 2013).

However, the cost-effective implementation of current biotechnologies for the treatment of CH_4 is still limited by the understanding of the communities underlying CH_4 biodegradation (Li et al., 2014; López et al., 2013). In this regard, there is still a need for studies assessing the effect of environmental factors during culture

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enrichment on the performance and characteristics of methanotrophic communities. Among them, CH_4 gas concentration, O_2 gas concentration and Cu^{2+} concentration in the aqueous media have been identified as the main parameters influencing microbial CH_4 abatement due to their key role on the nature and level of expression of CH_4 monooxygenases (MMO), and therefore on the population structure of methane oxidizing bacteria (MB) (Li et al., 2014; Murrel et al., 2000; Semrau et al., 2010). However, most studies assessing the role of CH_4 and Cu^{2+} concentrations have focused only on the physiological and enzymatic aspects of CH_4 biodegradation (Murrel et al., 2000). On the contrary, studies on the macroscopic performance and characteristics of the microbial communities have not found a clear effect of these parameters on CH_4 abatement (Estrada et al., 2014; Ho et al., 2013; Li et al., 2014). Likewise, the influence of the effective CH_4 supply rate to the microbial communities during culture enrichment on the kinetics of CH_4 biodegradation, which can differ at similar gas CH_4 concentrations depending on the bioreactor configuration, has often been disregarded (López et al., 2014, 2013; Yoon et al., 2009). In this context, there is a limited understanding of the effect of CH_4 and Cu^{2+} concentration and of the effective CH_4 supply rate during culture enrichment on the characteristics and structure of methanotrophic communities (Semrau et al., 2010), which could shed light on the optimal operating conditions leading to an enhanced performance of CH_4 abatement biotechnologies.

The present study aims at systematically elucidating the influence of CH_4 and Cu^{2+} concentrations and of the effective CH_4 supply rate (governed by the CH_4 mass transfer to the microbial community) during the enrichment of MB communities on CH_4 biodegradation kinetic parameters (q_{max} and Ks) and community structure in order to identify the optimum environmental factors supporting an efficient CH_4 abatement and high biodiversity in full scale bioreactors.

2. Materials and methods

2.1. Chemicals and mineral salt medium

The mineral salt medium (MSM) utilized during the enrichment of the methanotrophic communities and in the kinetic assays was a modified Brunner medium (Lopez et al., 2014) containing (g L^{-1}): $Na_2HPO_4 \cdot 2H_2O$, 3.17; KH_2PO_4, 1.50; $NaNO_3$, 5.28 (used instead of $(NH_4)_2SO_4$ to avoid the inhibition of methanotrophs by NH_4 (Carlsen et al., 1991)); $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2 \cdot 2H_2O$, 0.05; EDTA, 0.005; $FeSO_4 \cdot 7H_2O$, 0.002; H_3BO_3, 0.0003; $CoCl_2 \cdot 6H_2O$, 0.00011; $ZnSO_4 \cdot 7H_2O$, 0.0001; $Na_2Mo_4 \cdot 2H_2O$, 0.00003; $MnCl_2 \cdot 4H_2O$, 0.00003; $NiCl_2 \cdot 6H_2O$, 0.00002. Cu^{2+} was added to the MSM as $CuCl_2 \cdot 2H_2O$ at two Cu^{2+} concentrations (0.05 μM and 25 μM). The final pH of the MSM was 7. All chemicals and reagents were procured from Panreac (Barcelona, Spain) with a purity higher than 99.0%. CH_4 was purchased from Abello-Linde, S.A. (Barcelona, Spain) with a purity of at least 99.5%. Silicone oil 200 cSt was obtained from Sigma-Aldrich (Madrid, Spain).

2.2. Inoculum

The inoculum used for the enrichment of the methanotrophic communities was a mixture (50/50%/% on a volume basis) of aerobic activated sludge ($\approx$6 g L^{-1}) from a denitrification-nitrification wastewater treatment plant (Valladolid, Spain) and fresh cow manure from a dairy farm (Cantabria, Spain) 10× diluted in MSM.

2.3. Microbial community enrichments

Eight enrichment series (45 days/enrichment) were carried out at two different mixing ratios of CH_4 (1% and 8%) and Cu^{2+} (0.05 μM and 25 μM) under two different magnetic stirring rates (300 rpm and 650 rpm). The series were performed in duplicate (Table 1).

In each enrichment series, 1.2 L batch gas-tight reactors containing 100 mL of MSM at its corresponding Cu^{2+} concentration and 50 mL of silicone oil (in order to support a high CH_4 mass transfer from the headspace to the microbial community), were inoculated with 10 mL of the inoculum previously described. The reactors were closed with gas-tight butyl septa and plastic screw caps, and monitored daily for O_2, CO_2 and CH_4 concentrations in the headspace. O_2 was daily supplied via air flushing of the reactor headspace prior injection of pure CH_4 using a calibrated 100 mL gas tight syringe to obtain the desired headspace concentration (before methane injection the same volume of air was removed from the reactors to avoid overpressure). The enrichment batch reactors were maintained under agitation at 25 °C. A dilution rate of 0.25 ± 0.05 day^{-1} was set in order to maintain the pH at ≈7.0, to replenish essential nutrients and, to remove any potential inhibitory metabolite accumulated in the medium. Silicone oil was not removed from the batch reactors during the enrichment series. Biomass samples from the aqueous phase were drawn with a liquid sampling syringe at the end of each enrichment series to determine the microbial population structure by denaturing gradient gel electrophoresis (DGGE)-sequencing, the presence of soluble or particulate methane monoxygenases (sMMO or pMMO, respectively), and the CH_4 biodegradation kinetics constants (q_{max} and Ks). The concentration of CH_4 and CO_2 in the headspace of the enrichment bottles was periodically measured by GC-TCD.

2.4. Structure of the enriched communities

The 16 samples from the 8 duplicate enrichment series stored at −20 °C were thawed and centrifuged at 5000 rpm for 15 min. The biomass pellets were resuspended in 10 mL of phosphate buffered saline (PBS) medium at pH 7 by vortexing.

The total DNA of these samples was extracted by the Fast® DNA Spin Kit for Soil (MP Biomedicals, LLC) and its quantity and quality were evaluated by spectrophotometry using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) (English et al., 2006). The DNA was amplified with polymerase chain reaction (PCR) universal bacterial primers for 16S rRNA gene amplification (968-F-GC and 1401-R (10 μM)) (Sigma- Aldrich, St. Louis, MO, USA) according to Lopez et al. (2014). The PCR products of the bacterial 16S rRNA fragments from the samples were separated by DGGE according to Lopez et al. (2014). The gels were stained for 60 min with GelRed Nucleic Acid Gel Stain (biotium). Specific PCR-DGGE bands were detected by a transilluminator, UV wavelength, 254–312 nm (Sigma-Aldrich) and carefully cut from the gel. The DNA contained in each band was extracted by incubation in 50 mL of sterile water at 63 °C for 70 min. The last PCR cycle was accomplished without the GC-clamp attached to the primer 968-F. The PCR products obtained were sequenced by

Table 1
Cultivation conditions during microbial community enrichments.

Test series (TS)	CH_4 headspace (%)	Cu^{2+} (μM)	Agitation (rpm)
TS 1	8	25	300
	8	25	650
TS 2	8	0.05	300
	8	0.05	650
TS 3	1	0.05	300
	1	0.05	650
TS 4	1	25	300
	1	25	650

Secugen S.L. (Madrid, Spain). RDP classifier tool was used to analyze the taxonomic classification of the DGGE bands sequenced (70% confidence level) (Wang et al., 2007). DECIPHER search tool was used to analyze the presence of chimeras (Wright et al., 2012). The NCBI BLAST search tool was used to compare the DNA sequences obtained with nucleotide sequences from the NCBI database (McGinnis and Madden, 2004). The sequences of the bacteria identified were deposited in the NCBI GenBank. The DGGE band pattern present in each gel was compared using the GelCompar IITM software (Applied Maths BVBA, Sint-Martens-Latem, Belgium) and the Pearson product-moment correlation coefficient as well as, the Shannon–Wiener diversity index (H_s) were calculated according to Lopez et al. (2014). In addition, a multiple sequence alignment was performed. Sequences alignment (Clustal X 2.1) and phylogenetic analysis were obtained using the MEGA software (version 6.06). The phylogenetic trees were constructed using the neighbor-joining method (1000-fold bootstrap analysis) (Hall, 2013).

2.5. Detection of pMMO and sMMO

After DNA extraction, the specific primer set *A189f/mb661r*, targeting the *pmoA* gene (which encodes the *β-subunit* of particulate MMO) (Costello and Lidstrom, 1999; Erwin et al., 2005), and the primer set *mmoX* f882/*mmoX* r1403, targeting the *mmoX* gene (which encodes the *α-subunit* of the hydroxylase component of sMMO) (McDonald et al., 1995; Newby et al., 2004) were used for PCR amplification. The PCR mixture (50 μL) consisted of 25 μL of BIOMIX (Bioline, Ecogen), 2 μL of the extracted DNA, 2 μL of the specific primer set depending of the gene amplified and Milli-Q water up to a final volume of 50 μL. The annealing temperature used was 58 °C for *mmoX* and 56 °C for *pmoA*. The PCR conditions were as follows: 94 °C for 5 min; *Taq* polymerase added; 58/56 °C for 1 min; 72 °C for 1 min; 40 cycles consisting of 94 °C for 1 min, 58/56 °C for 1 min, and 72 °C for 1 min; and a final cycle consisting of 94 °C for 1 min, 58/56 °C for 1 min, and 72 °C for 5 min.

2.6. CH_4 biodegradation kinetics

Kinetic assays were performed in duplicate to elucidate the influence of CH_4 and Cu^{2+} concentration and the agitation rate (i.e. the effective CH_4 supply rate) during culture enrichment on the maximum specific CH_4 biodegradation rate q_{max} (g CH_4 $g^{-1}_{biomass}$ h^{-1}) and the Michaelis–Menten half-saturation constant, K_s (g m^{-3}) for CH_4 of the enriched microbial communities. In-vitro kinetic assays were performed in 120 mL glass serum bottles. Each bottle contained 20 mL of MSM and was inoculated with the corresponding enriched methanotrophic community at a concentration of 98 ± 34 g $_{biomass}$ m^{-3}. Preliminary assays carried out under the same conditions in our laboratory (CH_4 concentration = 25.8 ± 2.3 g m^{-3}) showed that even a total suspended solids (TSS) concentration of 900 ± 70 g $_{biomass}$ m^{-3} ensured the absence of CH_4 mass transfer limitations (Data non published).

The glass bottles were sealed with butyl septa and aluminum crimp seals. CH_4 was initially added at headspace concentrations of 131 ± 6 g m^{-3}, 99 ± 6 g m^{-3}, 60 ± 5 g m^{-3}, 21 ± 0.9 g m^{-3} and 2.4 ± 0.3 g m^{-3}. CH_4 aqueous concentrations were 4.3 ± 0.2 g m^{-3}, 3.3 ± 0.8 g m^{-3}, 2.0 ± 0.2 g m^{-3}, 0.7 g m^{-3} ± 0.03 and 0.08 ± 0.001 g m^{-3}, according to a CH_4 Henry's law constant at 25 °C and 1 atm of 29.4. The glass bottles were agitated at 650 rpm (25 °C) for 13 h. A GC-TCD was used to monitor the headspace concentration of CH_4 and CO_2 every 1 h. The kinetic parameters K_s and q_{max} were obtained by fitting the initial specific CH_4 biodegradation rate (q, g CH_4 $g^{-1}_{biomass}$ h^{-1}) at each aqueous CH_4 concentration ([CH_4], g m^{-3}) to the Michaelis–Menten equation (Equation (1)) by nonlinear regression using Microsoft Excel Solver (Microsoft Office Professional Plus 2013) (Sakač and Sak-Bosnar, 2012):

$$q = q_{max}\frac{[CH_4]}{[CH_4] + K_s} \quad (1)$$

2.7. Analytical procedures

CH_4, O_2 and CO_2 headspace concentrations were measured in a Bruker 430 GC-TCD (Palo Alto, USA) according to Estrada et al. (2014). The culture optical density was measured at 650 nm with a Shimadzu UV-2550 UV/Vis spectrophotometer (Shimadzu, Japan) and correlated to dry biomass concentrations, which were determined as TSS (American Water Works Association, 2012).

2.8. Statistical analysis

The statistical data analysis was performed using SPSS 20.0 (IBM). The results are given as the average ± standard deviation. The homogeneity of the variance of the parameters was evaluated using a Levene test. Significant differences were analyzed by Student's t-test for two group comparisons, and ANOVA and post-hoc analysis for multiple group comparisons. Differences were considered to be significant at $p \leq 0.05$.

3. Results

3.1. Microbial population structure

The Shannon–Wiener diversity index revealed that Cu^{2+} was the main factor determining bacterial diversity (Fig. 1). Hence, an increase in Cu^{2+} concentration from 0.05 to 25 μM at a CH_4 concentration of 8% entailed a statistically significant decreased in bacterial diversity from 2.7 ± 0.05 to 1.4 ± 0.15 at 300 rpm and from 2.8 ± 0.1 to 1.8 ± 0.15 at 650 rpm. Similarly, H_s decreased from 2.1 ± 0.05 to 1.9 ± 0.05 at 300 rpm and from 2.3 ± 0.05 to 1.8 ± 0.05 at 650 rpm when Cu^{2+} concentration was increased from 0.05 to 25 μM at a CH_4 concentration of 1%.

The influence of Cu^{2+} on the Pearson similarity coefficients was consistent with the effect on the Shannon–Wiener indexes. Thus, the similarities found between the communities enriched at 0.05 and 25 μM at a CH_4 concentration of 8% were 42.5 ± 3.7% and 37.5 ± 3.8% at 300 and 650 rpm, respectively, while the similarities obtained in the cultures grown at 1% were slightly higher (76.0 ± 4.0% at 300 and 65.8 ± 6.9% at 650 rpm).

The phylogenetic analysis based on the 16S rRNA sequences obtained from the DGGE gels at 650 rpm represented by the phylogenetic tree (Fig. 2) further supported the effect of Cu^{2+} on the similarity among the bacterial populations enriched.

3.2. Microbial community profile

A total of 37 bands were sequenced from all conditions tested (18 for 300 rpm; 19 for 650 rpm). These 16S rRNA sequences were classified in three different phyla: *Proteobacteria*, *Lentisphaerae* (at 650 rpm) and *Chlamydiae* (at 300 rpm). The closest matches for each band, and its similarity percentage and sources, are shown in Tables 2 and 3. The phylum *Proteobacteria* was predominant in all cultures enriched (DGGE bands 2–13, Table 2, and DGGE bands 1–18, Table 3). Most of these *Proteobacteria* were closely related (>90% sequence identity) to aerobic CH_4 oxidizing bacteria within the class *Gammaproteobacteria*. They belonged to the type I

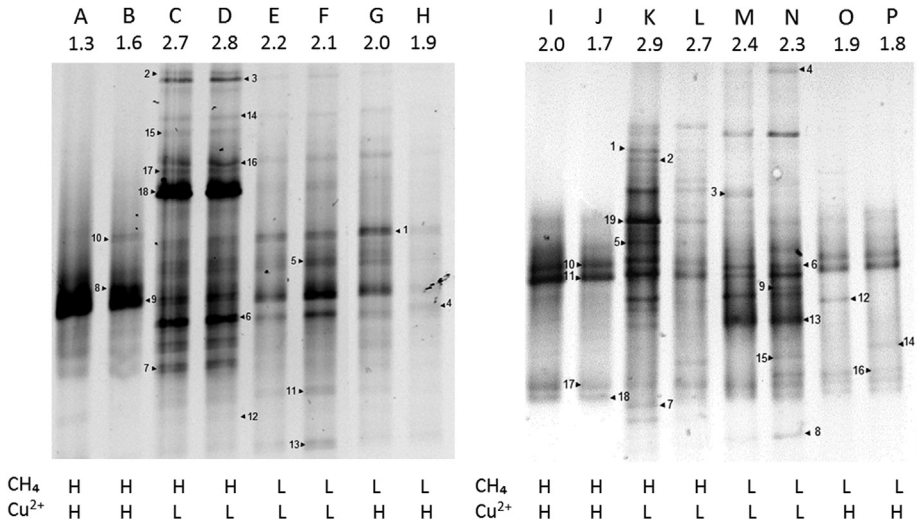

Fig. 1. DGGE profile of the cultures enriched at 300 rpm (A, B, C, D, E, F, G, H) and 650 rpm (I, J, K, L, M, N, O, P). *L* and *H* refer to low and high CH_4 and Cu^{2+} concentrations, respectively. The Shannon–Wiener diversity indexes are indicated in the upper part of the gel. The sequenced bands are indicated by "►" and the corresponding number of each band.

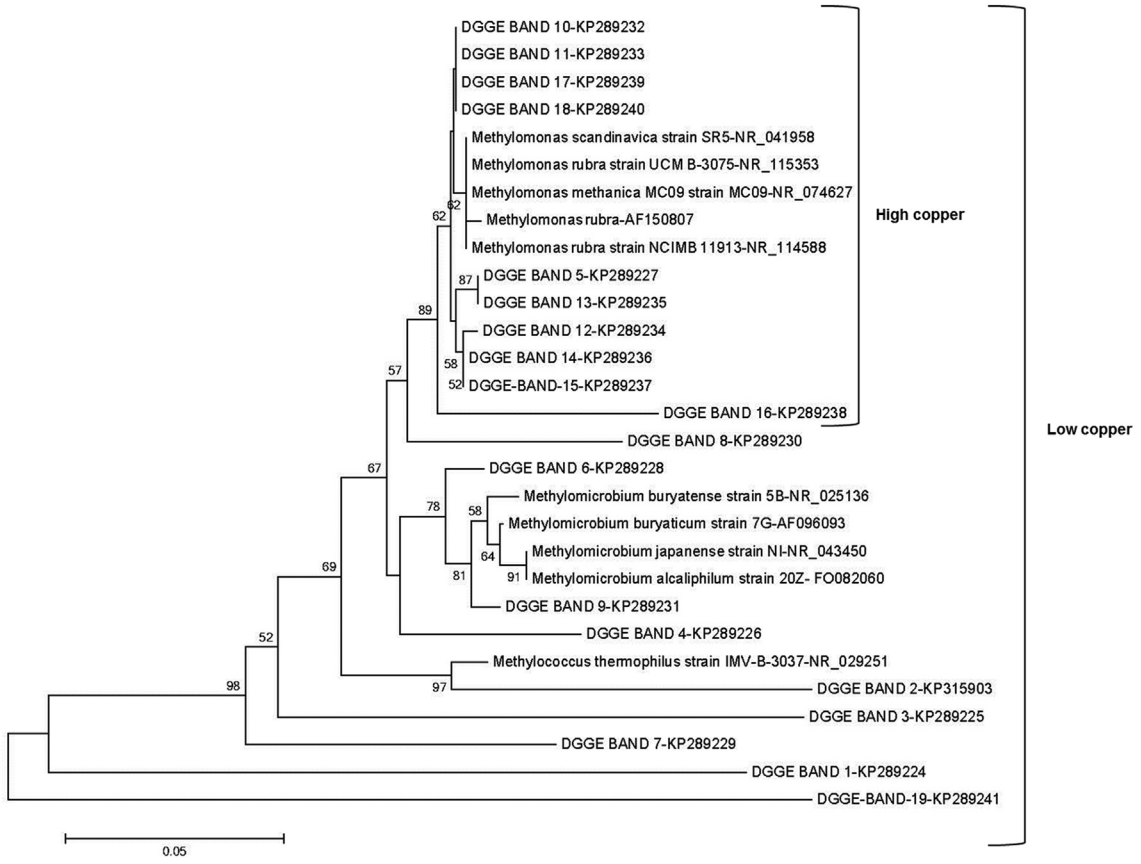

Fig. 2. Bacterial phylogenetic tree based on neighbor-joining analysis of the 16S rRNA sequences from the enriched populations at 650 rpm detected by PCR-DGGE and their closest relatives (similarity >96%) in GenBank obtained by the Blast search tool. Accession numbers are indicated. Numbers on the nodes indicate bootstrap values (1000 replicates). The scale bar indicates 10% sequence difference.

methanotroph genera *Methylomonas*, *Methylobacter* and *Methylomicrobium* (which were always present in the microbial community regardless of the enrichment conditions) and to the type X methanotroph genus *Methylococcus*. A Cu^{2+} concentration of 25 μM supported the dominance of *Methylomonas* (particularly at a CH_4 concentration of 8%), while *Methylobacter* was never detected in the cultures enriched at the highest Cu^{2+}concentration. On the other hand, despite being more representative in the presence of 0.05 μM Cu^{2+}, *Methylomicrobium* appeared in the enrichments conducted at both Cu^{2+} concentrations at a CH_4 concentration of 8% (Figs. 1 and

Table 2
RDP classification of the bacterial DGGE bands, sequences and corresponding matches (Standard Nucleotide BLAST) of cultures enriched at 300 rpm using the NCBI database, including similarity percentages and sources of origin.

Taxonomic placement (70% confidence level)	Band no	Acc. No	A	B	C	D	E	F	G	H	Closest relatives in blast name (accession number)	Similarity (%)	Source of origin
Kingdom Bacteria	1	KP289206			x	x	x	x	x	x	*Uncultured bacterium YC7*(KJ734895)	95	Compost of cow dung
Phylum *Proteobacteria*													
Class Deltaproteobacteria													
Order *Bdellovibrionales*													
Family *Bdellovibrionaceae*													
Genus Bdellovibrio	2	KP289207			x	x					*Bdellovibrio bacteriovorus* (CP002190)	97	High loaded MBR
											Bdellovibrio sp. (DQ302728)	97	Soil
	3	KP289208			x	x	x	x			*Uncultured bacterium* (EU050708)	99	Production water of an oil field
											Bdellovibrio sp. (DQ302728)	98	Soil
Class Gammaproteobacteria	4	KP289209							x	x	*Methylomonas rubra* (AF150807)	92	Lake sediment
											Methylomonas methanica (NR074627)	93	Marine sediment
	5	KP289210			x	x	x	x			*Uncultured Methylococcaceae* (JX505396)	94	Soil
Order *Methylococcales*													
Family *Methylococcaceae*	6	KP289211			x	x	x	x	x	x	*Methylomicrobium agile* (NR_116197)	94	Soil
	7	KP289212			x	x					*Uncultured Methylomicrobium sp.* (HQ044145)	90	Tailing pond
Genus *Methylomonas*	8	KP289213	x	x							*Methylomonas rubra* (NR_114588)	99	Sea sample
											Methylomonas methanica (NR074627)	99	Marine sediment
	9	KP289214	x	x	x	x	x	x	x	x	*Methylomonas rubra* (NR_115353)	99	Coal mine drainage
											Methylomonas methanica (NR074627)	99	Marine sediment
	10	KP289215		x							*Methylomonas methanica* (NR074627)	99	Marine sediment
											Methylomonas rubra (AF150807)	99	Lake sediment
Order *Xanthomonadales*													
Family *Xanthomonadaceae*	11	KP289216	x		x	x	x	x	x	x	*Uncultured Gammaproteobacteria* (HM238179)	96	Biofilter treating waste gas
Genus *Dokdonella*	12	KP289217			x		x	x			*Uncultured bacterium* (KM886284)	98	Biofilter for CH_4 abatement
											Uncultured Dokdonella sp. (KJ486363)	97	Sludge from membrane bioreactor
	13	KP289218	x		x	x	x	x	x	x	*Uncultured Dokdonella sp.* (KJ486363)	97	Sludge from membrane bioreactor
Phylum Chlamydiae													
Class Chlamydiia													
Order *Chlamydiales*													
Family *Parachlamydiaceae*													
Genus *Neochlamydia*	14	KP289219			x	x	x	x	x		*Neochlamydia hartmannellae.* (NR_025037)	99	Water public conduit system
											Neochlamydia endosymbiont of Acanthamoeba (KF924593)	97	river sediment
	15	KP289220									*Neochlamydia hartmannellae* (NR_025037)	91	Water public conduit system
	16	KP289221			x	x	x	x	x	x	*Neochlamydia hartmannellae* (NR_025037)	95	Water public conduit system
	17	KP289222			x	x					*Neochlamydia hartmannellae* (NR_025037)	98	Water public conduit system
											Neochlamydia endosymbiont of Acanthamoeba (KF924590)	97	river sediment
	18	KP289223			x	x	x	x			*Neochlamydia hartmannellae* (NR_025037)	99	Water public conduit system
											Neochlamydia sp. (EU683885)	97	Water reservoir sediment

2). These results further confirmed the influence of Cu^{2+} concentration on the community profile. The type X genus *Methylococcus* was found at 650 rpm under low Cu^{2+} and high CH_4 concentrations (Fig. 1).

Non methanotrophic genera of the phylum *Proteobacteria* such as *Dokdonella* (bands 12–13) and *Bdellovibrio* (bands 2–3) were also detected at 300 rpm. However, while *Dokdonella* was present in all enrichments, *Bdellovibrio* was only detected at 0.05 μM Cu^{2+}. In addition, in the cultures enriched at 300 rpm, bands 14–18 were affiliated with the phylum *Chlamydiae*. All of them were closely related with the genus *Neochlamydia* (>90% sequence identity) and were found under all enrichment conditions but 8% CH_4 and 25 μM Cu^{2+}. Finally, all bacteria enriched at 650 rpm detected by the DGGE were methanotrophs regardless of the Cu^{2+} and CH_4 concentration, except for band 19 which was an associated heterotroph classified within the phylum *Lentisphaerae*. Bacteria corresponding to band 19 were classified in the genus *Victivallis* (>90% sequence identity) and observed under all enrichment conditions regardless of the concentration of Cu^{2+} and CH_4.

3.3. Detection of mmoX and pmoA genes

The gene *pmoA* was present in the enrichments obtained under all tested conditions regardless of the concentration of Cu^{2+} or CH_4 or the agitation rate (Fig. 3). The gene *pmoA* was more abundant at 650 rpm due to the higher concentration of biomass and DNA extracted under this enrichment condition (Fig. 4). The gene *mmoX* was only detected in the inoculum and the cultures enriched at 8% of CH_4 and 0.05 μM Cu^{2+} at 650 rpm (Fig. 3).

3.4. Biodegradation kinetics

The kinetic parameters q_{max} and K_s differed up to one order of magnitude among the cultures enriched at 300 and 650 rpm

Table 3
RDP classification of the bacterial DGGE bands sequences and corresponding matches (Standard Nucleotide BLAST) of cultures enriched at 650 rpm using the NCBI database with indication of the similarity percentages and sources of origin.

Taxonomic placement (70% confidence level)	Band no	Acc. No	I	J	K	L	M	N	O	P	Closest relatives in blast name (accession number)	Similarity (%)	Source of origin
Kingdom Bacteria													
Phylum *Proteobacteria*	1	KP289224			x	x	x	x			*Uncultured bacterium* (EF648097)	98	Aerobic activated sludge
Class Gammaproteobacteria	2	KP315903			x	x					*Methylococcus thermophilus* (NR_029251)	97	Soil
Order *Methylococcales*													
Family *Methylococcaceae*	3	KP289225					x	x			*Methylobacter* sp. (HF565143)	91	Landfill cover soil
	4	KP289226					x	x			*Uncultured bacterium* (JX434234)	93	Water mine
	5	KP289227			x				x	x	*Uncultured Methylomicrobium sp.* (HQ044206)	94	Tailing pond
	6	KP289228					x	x			*Methylomicrobium buryatense* (NR_025136)	96	Soda lake
											Methylomicrobium japanese (NR_043450)	96	Marine sediment
	7	KP289229			x	x	x	x			*Methylomicrobium sp.* (DQ496230)	90	Soda lake
	8	KP289230					x	x			*Methylomonas methanica* (NR_074627)	93	swamp soil
											Methylobacter sp. (AF131868)	91	
Genus Methylomicrobium	9	KP289231						x			*Methylomicrobium buryaticum* (AF096093)	97	Soda lake
											Methylomicrobium alcaliphilum (FO082060)	96	Saline environment
Genus *Methylomonas*	10	KP289232	x	x	x	x			x	x	*Methylomonas methanica* (NR_074627)	99	Marine sediment
											Methylomonas rubra (AF150807)	98	Lake sediment
	11	KP289233	x	x	x	x	x	x	x	x	*Methylomonas rubra* (NR_115353)	99	Coal mine drainage
											Methylomonas methanica (NR_074627)	99	Seawater
	12	KP289234						x			*Methylomonas rubra* (NR_114588)	98	Sea sample
											Methylomonas methanica (NR_074627)	98	Marine sediment
	13	KP289235	x		x		x	x			*Methylomonas methanica* (NR_074627)	98	Marine sediment
											Methylomonas rubra (NR_115353)	98	Coal mine drainage
											Methylomonas scandinavica (NR_041958)	97	Igneous rocks
	14	KP289236	x	x					x		*Methylomonas methanica* (NR_074627)	98	Marine sediment
											Methylomonas rubra (AF150807)	97	Lake sediment
	15	KP289237	x			x		x			*Methylomonas rubra* (AF150807)	94	Lake sediment
											Methylomonas methanica (NR_074627)	94	Marine sediment
	16	KP289238						x	x	x	*Methylomonas rubra* (AF150807)	95	Lake sediment
											Methylomonas methanica (NR_074627)	95	Marine sediment
	17	KP289239	x	x	x	x	x	x	x	x	*Methylomonas rubra* (AF150807)	99	Lake sediment
											Methylomonas methanica (NR_074627)	99	Marine sediment
	18	KP289240	x	x			x	x	x	x	*Methylomonas rubra* (AF150807)	99	Lake sediment
											Methylomonas methanica (NR_074627)	99	Marine sediment
Phylum Lentisphaerae													
Class Lentisphaeria													
Order *Victivallales*													
Family *Victivallaceae*													
Genus Victivallis	19	KP289241	x	x	x	x	x	x	x	x	*Uncultured Victivallaceae* (JQ724358)	91	Biofilm in a microbial cell fuel

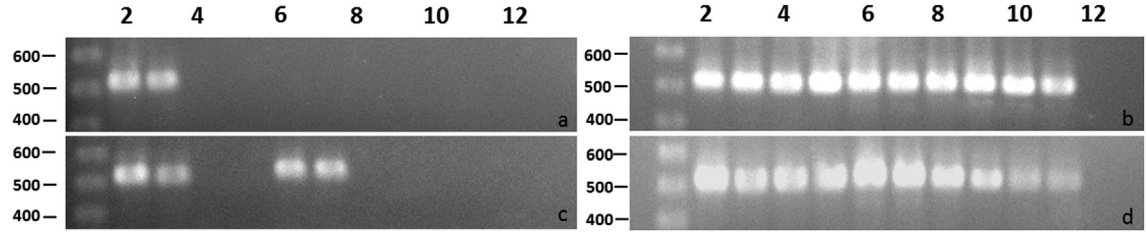

Fig. 3. PCR profile for the gene *mmoX* of the cultures enriched at 300 (a) and 650 rpm (c). Lanes: 1) HiperLadder II (50–2000 bp), 2) *Methylosinus sporium*, *Methylocystis* sp. (commercial strains mixed), 3) Inoculum (A mixture (50/50%/% on a volume basis) of fresh aerobic activated sludge and fresh cow manure), 4/5) High CH_4-High Cu^{2+}, 6/7) High CH_4-Low Cu^{2+}, 8/9) Low CH_4-Low Cu^{2+}, 10/11) Low CH_4-High Cu^{2+}, 12) C^- (negative control without DNA). PCR profile for the gene *pmoA* of the cultures enriched at 300 (b) and 650 rpm (d). Lanes: 1) HiperLadder II (50–2000 bp), 2) *Methylomonas* and *Methylobacter* sp. mixed culture, 3) Inoculum, 4/5) High CH_4-High Cu^{2+}, 6/7) High CH_4-Low Cu^{2+}, 8/9) Low CH_4-Low Cu^{2+}, 10/11) Low CH_4-High Cu^{2+}, 12) C^- (negative control without DNA).

(Table 4), with the highest q_{max} and K_s obtained at 650 rpm.

No statistical differences among the kinetic parameter values were found in the cultures enriched at 1% and 8% of CH_4 under similar Cu^{2+} concentrations regardless of the agitation rates. However, a higher Cu^{2+} concentration increased both q_{max} and K_s in the low range of CH_4 concentration tested (Table 4). Hence, the highest values of q_{max} were recorded at 25 μM Cu^{2+} regardless of the CH_4 concentration during enrichment. The q_{max} ranged from $7.7 \pm 0.21 \times 10^{-4}$ to $6.3 \pm 0.4 \times 10^{-4}$ g CH_4. $g_{biomass}^{-1}$ h^{-1} at 650 rpm and from $3.6 \pm 0.3 \times 10^{-4}$ to $2.4 \pm 0.24 \times 10^{-4}$ g CH_4. $g_{biomass}^{-1}$ h^{-1} at 300 rpm (Table 4). Likewise, the highest half saturation constants were also recorded at 25 μM Cu^{2+} regardless of the CH_4 concentration and the agitation rates during enrichment. The average Ks values of the cultures enriched at a CH_4 concentration of 1 and 8% increased from 8×10^{-5} to 1.7×10^{-4} at 650 rpm and from 2.3×10^{-5} to 6.0×10^{-5} at 300 rpm when Cu^{2+} increased from 0.05 to 25 μM (Table 4).

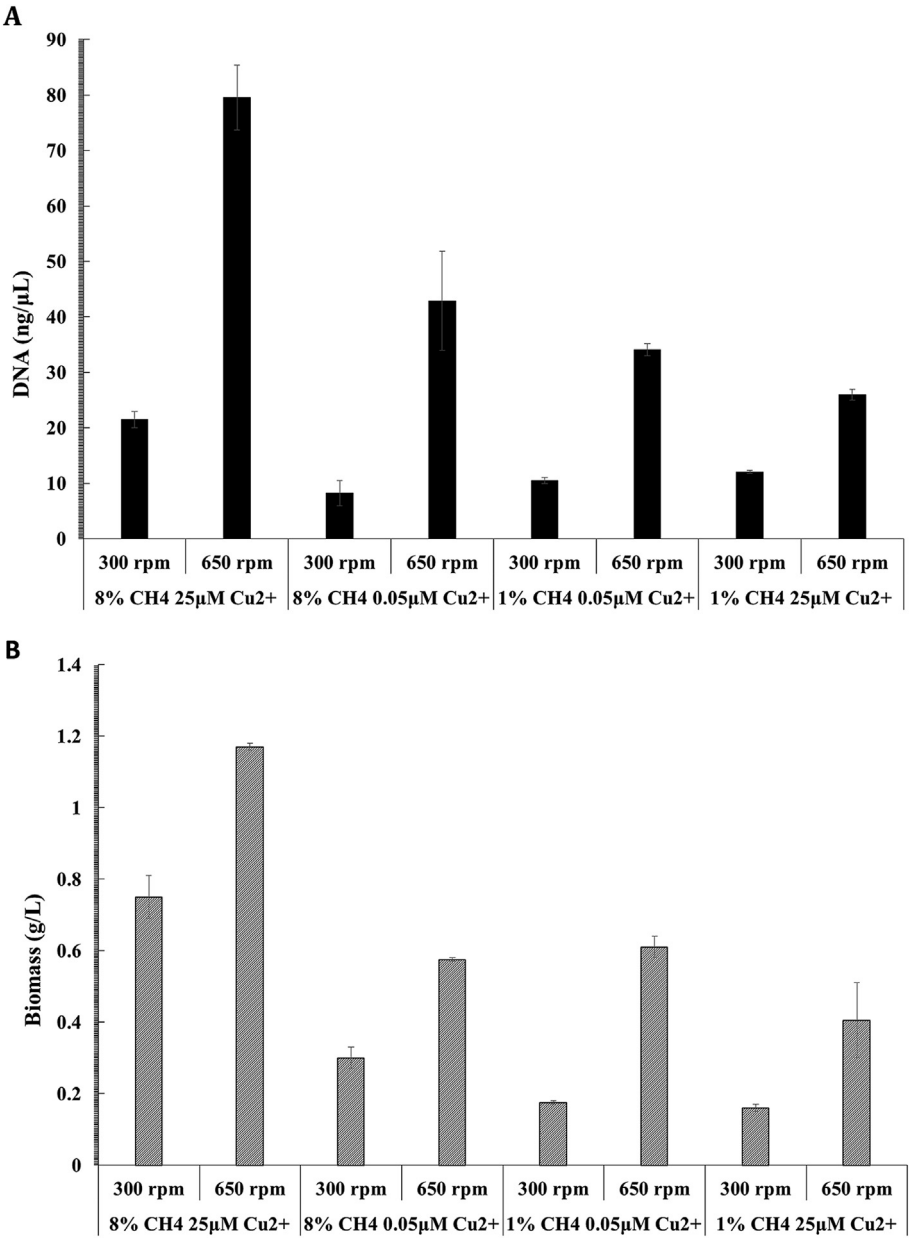

Fig. 4. Extracted DNA (A) and biomass (B) concentration from the cultures enriched under each particular condition tested. Error bars represent the standard deviations from duplicate samples.

Table 4
Kinetic parameters of the communities obtained at the different conditions tested.

	300 rpm		650 rpm	
	K_s (M)	q_{max} (g CH_4·g biomass^{-1} h^{-1})	K_s (M)	q_{max} (g CH_4·g biomass^{-1} h^{-1})
TS1				
8% CH_4, 25 µM Cu^{2+}	$6.4 \times 10^{-5} \pm 1.2 \times 10^{-6a}$	$3.6 \times 10^{-4} \pm 3.0 \times 10^{-5a}$	$1.9 \times 10^{-4} \pm 5.0 \times 10^{-6a}$	$7.7 \times 10^{-4} \pm 2.1 \times 10^{-5a}$
TS2				
1% CH_4, 0.05 µM Cu^{2+}	$1.7 \times 10^{-5} \pm 1.2 \times 10^{-6b}$	$9.7 \times 10^{-5} \pm 7.1 \times 10^{-6b}$	$6.6 \times 10^{-5} \pm 6.5 \times 10^{-6b}$	$1.9 \times 10^{-4} \pm 1.0 \times 10^{-5b}$
TS3				
8% CH_4, 0.05 µM Cu^{2+}	$2.9 \times 10^{-5} \pm 4.4 \times 10^{-7bc}$	$9.3 \times 10^{-5} \pm 4.7 \times 10^{-7b}$	$9.4 \times 10^{-5} \pm 3.5 \times 10^{-6bc}$	$2.8 \times 10^{-4} \pm 1.4 \times 10^{-5bc}$
TS4				
1% CH_4, 25 µM Cu^{2+}	$5.7 \times 10^{-5} \pm 3.5 \times 10^{-6ac}$	$2.4 \times 10^{-4} \pm 2.4 \times 10^{-5a}$	$1.5 \times 10^{-4} \pm 2.0 \times 10^{-5ac}$	$6.3 \times 10^{-4} \pm 4.0 \times 10^{-5ac}$

Mean values and standard deviations for each duplicate. Kinetic parameters followed by different superscript letters were significantly different at $p < 0.05$.

4. Discussion

4.1. Microbial population diversity and community profile

This study demonstrated the key role of Cu^{2+} concentration on the diversity and bacterial composition during the enrichment of CH_4 degrading microbial communities. CH_4 concentration did not entail significant differences on either the microbial diversity or community structure. The Shannon–Wiener diversity index revealed that low Cu^{2+} concentrations at similar CH_4 supply rates resulted in a higher diversity. This result was mainly due to a negative effect of high Cu^{2+} concentrations in some of the ecosystem communities.

The analysis of the Pearson similarity coefficients and the phylogenetic trees also confirmed that the enriched methanotrophic bacteria shifted differently depending on the Cu^{2+} concentration. While *Methylomonas*, *Methylobacter* and *Methylomicrobium* were always present in all enrichments, a clear influence of Cu^{2+} concentration on the relative abundance and dominance of these species was observed. This was more evident in *Methylomonas*, which became dominant in the presence of high Cu^{2+} concentrations and outcompeted other microorganisms. Indeed, this genus was present at different positions in the DGGE gel and the phylogenetic tree, thus representing either different species of *Methylomonas* or distinct fragments of the same rich species. This fingerprint pattern was comparable with that obtained in batch bioreactor cultures operated at low CH_4 concentrations, where *Methylomonas* became the dominant microorganism when Cu^{2+} concentration was increased by a factor of 10 (Estrada et al., 2014; Van Der Ha et al., 2013).

On the contrary, the DGGE profiles of the communities enriched under the 8 conditions tested showed that both Cu^{2+} concentration and the cross effect of CH_4 and Cu^{2+} concentration exhibited a minor impact on the structure of the associated heterotrophs. In this sense, the genus *Bdellovibrio* was the single identified genus associated to a low copper concentration at 300 rpm. Interestingly, other authors have found using stable isotope probing (SIP) that *Bdellovibrio* is labeled in methanotrophic enrichments, suggesting that these might be parasites or predators of methanotrophs (McDonald et al., 2005; Semrau et al., 2010). In this regard, the high Cu^{2+} concentration used could have been toxic for *Bdellovibrio*, which suggest that the addition of Cu^{2+} could be used as a potential predator-parasite control strategy. Despite previous studies suggested that changes on Cu^{2+} concentration can induce a positive effect on the dominant species, to the best of our knowledge no clear influence of Cu^{2+} on the main bacterial communities and on the microbial diversity was reported to date. This is probably due to the narrow range of concentrations tested in environmental studies simulating natural soils but not taking into account the possible biostimulation effect derived from the addition of copper in landfill cover soils (Estrada et al., 2014; Ho et al., 2013; Nikiema et al., 2013).

The effective CH_4 supply rate was determined by the agitation rate set under similar CH_4 headspace concentrations, the lower stirring rates supporting a limited transfer of CH_4 to the microbial community. The agitation rate exhibited a strong influence not only on the structure of the methanotrophic community (*Methylomonas* presence was significantly higher at 650 than at 300 rpm) but also on that of the associated heterotrophic population, which can play a key role on CH_4 oxidation by avoiding a metabolite-mediated inhibition of MB or by excreting substances that stimulate MB activity (Hrsak and Begonja, 2000; Van Der Ha et al., 2013). The diversity of associated heterotrophs was higher at 300 rpm. In this context, *Dokdonella* (bands 12–13) and *Bdellovibrio* (bands 2–3), two heterotrophs belonging to the *Proteobacteria* phylum that have been retrieved in bioreactors treating waste gases (Kallistova et al., 2014; Lebrero et al., 2013), were detected. In addition, the phylum *Chlamydiae*, typically found in bioreactors inoculated with aerobic activated sludge, was present under low agitation rates (Estrada et al., 2014). However, the main bacteria present in the cultures enriched at 650 rpm were methanotrophic bacteria. Therefore, a more efficient transfer of CH_4 favored the presence of dominant methanotrophs, while a lower CH_4 effective supply rate promoted the occurrence of non-specialized organisms.

4.2. Methane monoxygenase

Copper is one of the most important factors controlling the relative expression of the two forms of monooxygenases in methanotrophs, sMMO and pMMO, and therefore influences the type of MB enriched (Murrel et al., 2000; Semrau et al., 2010). Previous enzymatic assays have shown that sMMO is synthesized at low Cu^{2+} concentrations (below 0.8 μM (Semrau et al., 2010)), while *pmoA* is always expressed at a significant level regardless of the Cu^{2+} concentration and such expression increases at increasing copper concentrations (Hakemian and Rosenzweig, 2007; Murrel et al., 2000). However, other studies assessing the influence of copper on MB structure and MMO expression in natural ecosystems did not observe sMMO or type II MB at concentrations as low as 0.1 μM (Chen et al., 2007; Ho et al., 2013; Semrau et al., 2010). Thus, the mechanisms governing both the environmental distribution of both enzymes and the cross-effect of multiple parameters on the type of methanotrophs enriched or the enzyme expression are still unclear. In fact, additional factors such as the inorganic nitrogen concentration have been claimed to determine the type of methanotroph enriched. For example, Type II methanotrophs are often dominant in environments exposed at high CH_4 concentrations and low inorganic nitrogen levels, due to their nitrogen fixing ability (Graham et al., 1993; Li et al., 2014; López et al., 2013). In this study, the high concentration of nitrogen (62 mM) together with the low CH_4 concentrations (<8%) treated could explain the enrichment of type I methanotrophs.

On the other hand, the presence of both *mmoX* and *pmoA* genes in the inoculum confirmed that both enzymes were initially present, which suggests that the three types of methanotrophs were present in the inoculum but were gradually overcame by type I MB (Fig. 3A), with only the type X *Methylococcus* being detected at high CH_4 and low Cu^{2+} concentrations. pMMO was thus the main enzyme present in the cultures enriched, even at lower Cu^{2+} concentrations than the thresholds set by previous enzymatic studies (Hanson and Hanson, 1996; Murrel et al., 2000; Semrau et al., 2010). This finding could be due to the presence of a Cu^{+2} uptake mechanism, such as that driven by methanobactin, that sensed and concentrated Cu^{2+}, thus supporting the correct activity of pMMO (Kalidass et al., 2015; Semrau et al., 2010). At this point it should be noted that despite some research provided insight into the role of copper using enzymatic and biochemistry studies, real scale operation is far more complex and requires the elucidation of the role of Cu^{2+} on the enzyme expression in natural mixed cultures.

4.3. Biodegradation kinetics

No significant effect of Cu^{2+} concentration on the CH_4 biodegradation rates were reported to date in literature for methane abatement technologies (Estrada et al., 2014; Nikiema et al., 2013).

The results obtained in this study showed that differences in Cu^{2+} concentration of 500× supported significant variations on CH_4 biodegradation kinetics. In this sense, the effect of Cu^{2+} on the population was consistent with the changes observed on the biodegradation kinetics parameters. Interestingly, the values of q_{max} at 25 μM of Cu^{2+} were higher than those previously reported in the literature (typically ranging from 4.2×10^{-5} to 1.3×10^{-4} $gCH_4\ g_{biomass}^{-1}\ h^{-1}$ (Bender and Conrad, 1992; Gebert et al., 2003)). However, K_s values were within the ranges determined for low-term culture exposure to diluted CH_4 emissions (López et al., 2014). In addition, a higher mass transfer of CH_4 during bacterial enrichment (resulting in a higher CH_4 availability) favored the growth of those communities with lower affinity for CH_4 but with higher specific degradation rates. The superior specific CH_4 biodegradation rates of the communities grown under higher CH_4 transfer rates (650 rpm) was also confirmed by the higher biomass and DNA concentration obtained (Fig. 4).

In summary, high copper concentrations and an enhanced methane transfer (higher agitation rate) to the community during microbial enrichment resulted in communities with superior specific methane biodegradation rates (based on a different specialization of the MB community). Despite the large differences in Cu^{2+} concentration, only Type I methanotrophs were preferentially enriched as confirmed by the absence of sMMO. In addition, the present study demonstrated the key role of enrichment cultivation conditions to develop microbial communities capable of maintaining a high efficiency and robustness, overcoming the limitations of current biotechnologies and improving methane abatement performance.

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