Elsevier Editorial System(tm) for Journal of Environmental Management
Manuscript Draft

Manuscript Number:

Title: Two-liquid phase partitioning biotrickling filters for methane abatement: exploring the potential of hydrophobic methanotrophs

Article Type: Research Paper

Keywords: Biotrickling filter; Greenhouse gas; Mass transfer; Methane; Two-liquid phase partitioning bioreactor; Silicone oil

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Dear Editor,

Please find enclosed our original paper **"Two-liquid phase partitioning biotrickling filters for methane abatement: exploring the potential of hydrophobic methanotrophs"** co-authored by Raquel Lebrero, Laura Hernández, Rebeca Pérez, Jose Manuel Estrada and Raúl Muñoz. The paper is submitted for publication in **Journal of Environmental Management** with the agreement of all coauthors.

While biotrickling filters (BTF) are one of the preferred biological techniques for air pollution control, the efficient removal of hydrophobic volatile organic compounds such as methane still remains challenging due to mass transfer limitations. The addition of a non-aqueous phase to conventional BTFs may overcome this limitation by enhancing CH_4 transport from the gas to the microorganisms. Moreover, the development of a hydrophobic community growing inside the non-aqueous phase may boost the performance of conventional BTFs as a result of the exploitation of the full mass transfer potential of the non-aqueous phase. However, the application of two-phase partitioning BTFs for methane removal has been scarcely investigated. The work here submitted assessed the performance of two BTFs supplemented with silicone oil using two different inocula: *Methylosinus Sporium* and a hydrophobic microbial consortium. The addition of silicone oil increased the elimination capacity of both BTFs compared with conventional BTFs while mediating a low metabolites concentration in the recycling aqueous media. Finally, the biodiversity and richness of the microbial communities developed in both bioreactors, along with their phylogenetic composition, were also determined to further explain the macroscopic performance of the BTFs.

We look forward to your evaluation.
Best regards,

Raquel Lebrero Raúl Muñoz

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Highlights (for review)
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Research Highlights

- Two-liquid phase biotrickling filters were investigated for methane abatement
- The use of hydrophobic methanotrophs may overcome mass transfer limitations
- Enhanced methane removal was observed due to the addition of the non-aqueous phase
- Silicone oil mediated a reduced metabolites concentration in the aqueous phase
- Water and nutrient diffusion into the silicone oil hindered bioreactor performance

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Two-liquid phase partitioning biotrickling filters for methane abatement: exploring the potential of hydrophobic methanotrophs

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Abstract

The potential of two-liquid phase biotrickling filters (BTFs) to overcome mass transfer limitations derived from the poor aqueous solubility of CH_4 has been scarcely investigated to date. In this context, the abatement of diluted methane emissions in two-liquid phase BTFs was evaluated using two different inocula: a type II methanotrophs culture in BTF 1 and a hydrophobic microbial consortium capable of growing inside silicone oil in BTF 2. Both BTFs supported stable elimination capacities above 45 g m^{-3} h^{-1} regardless of the inoculum, whereas no improvement derived from the presence of hydrophobic microorganisms compared to the type II metanotrophs culture was observed. Interestingly, the addition of silicone oil mediated a reduced metabolites concentration in the recycling aqueous phase, thus decreasing the needs for mineral medium renewal. Moreover, a 78% similarity was recorded between the microbial communities enriched in both BTFs at the end of the experimental period in spite of the differences in the initial inoculum structure. The results obtained confirmed the superior performance of two-liquid phase BTFs for CH_4 abatement compared with conventional biotrickling filters.

Keywords: biotrickling filter, greenhouse gas, mass transfer, methane, two-liquid phase partitioning bioreactor, silicone oil

1. Introduction

The atmospheric concentration of greenhouse gases (GHGs) has increased from preindustrial levels at unnatural and unprecedentedly high rates, which is expected to raise the average global temperature by 0.6 – 4 ºC by the end of this 21st century (IPCC, 2013). Methane is ranked as the second most detrimental GHG, with a global warming potential 21 times higher than that of CO_2 and an atmospheric lifetime of approximately 9 years (Bocka et al., 2012; Wuebbles and Hayhoe, 2002). CH_4, exhibiting an atmospheric concentration increase of 0.2-1% year^{-1}, accounts nowadays for ~ 16% of the total GHG emissions (Karakurt et al., 2012). In this context, anthropogenic emissions represent approximately 60% of the total CH_4 emitted, the main sources of CH_4 being organic waste treatment activities such as landfilling, composting and wastewater treatment (102 million tons CO_2 equivalent in the EU in 2011), and livestock farming (168 million tons CO_2 equivalent in the EU in 2011) (EPA; EEA). These emissions are increasingly characterized by their low CH_4 concentrations: 0-200 mg CH_4 m^{-3} in compost piles or animal houses, 0.7-7 g m^{-3} in coal mines and up to 20-100 g CH_4 m^{-3} in old landfills (Ahn et al., 2011; Limbri et al., 2013; Scheutz et al., 2009). Unfortunately, these low concentrations are not suitable for either energy recovery or treatment by flaring, which hinders their cost-effective abatement. In this particular scenario of highly diluted CH_4-laden emissions and increasing enforcement of GHG emissions legislation, biotechnologies emerge as a promising end-of-the-pipe solution for methane abatement, biotrickling filtration (BTF) being one of the most advantageous configurations due to its robustness and cost effectiveness (Estrada et al., 2012; Lopez et al., 2013).

However, despite the well-known potential of biotechnologies for waste gas treatment, the poor aqueous solubility of CH_4 hinders their application for the abatement of this GHG due to its limited mass transfer from the gas phase to the microbial community. The addition to the off-gas treatment bioreactors of a non-aqueous phase (NAP) with high affinity for the target gas pollutant has been consistently shown as an efficient approach to improve the performance of biological process in the so called two-liquid phase partitioning bioreactors (TLPPB). The NAP supports an increase in both the pollutant and O_2 mass transfer from the gas phase to the microorganisms, while buffering the process against high pollutant or metabolite concentrations potentially toxic for the microbial community. TLPPBs have been successfully implemented for the degradation of hydrophobic VOCs such as hexane or alpha-pinene, gaining importance in recent years as a result of their superior performance compared to conventional bioreactors (Lebrero et al., 2013; Muñoz et al., 2013). Moreover, recent studies have demonstrated the ability of certain microbial communities to grow inside the NAP, which can boost the performance of conventional bioreactors by a factor of 17 (Muñoz et al., 2013; MacLeod and Daugulis, 2005). The promising results obtained to date certainly encourage the application of TLPPBs for the abatement of highly hydrophobic gas pollutants such as methane. However, there is a lack of systematic studies assessing the performance of two-liquid phase BTFs for methane abatement, which limits their potential scale-up. Therefore, more research is needed in order to elucidate the methane removal capacity of TLPPBs operated with hydrophobic microbial consortia.

This work was devised to determine the potential of two-liquid phase BTFs constructed with silicone oil and inoculated with either the methanotroph *Methylosinus Sporium* or a microbial consortium capable of growing inside silicone oil for the treatment of diluted

CH_4 air emissions. The influence of CH_4 inlet loading rate on the performance of both BTFs together with the structure and composition of the microbial communities present in both bioreactors were also assessed.

2. Materials and Methods

2.1 Chemicals

The mineral salt medium (MSM) employed consisted of (g L^{-1}): $Na_2HPO_4 \cdot 12\ H_2O$, 6.15; KH_2PO_4, 1.52; $NaNO_3$, 0.61; $MgSO_4 \cdot 7\ H_2O$, 0.2; $CaCl_2 \cdot 2\ H_2O$, 0.05; EDTA, 0.005; $FeSO_4 \cdot 7H_2O$, 0.002; H_3BO_3, 0.0003; $CoCl_2 \cdot 6H_2O$, 0.0002; $ZnSO_4 \cdot 7H_2O$, 0.0001; $Na_2Mo_4 \cdot 2H_2O$, 0.00003; $MnCl_2 \cdot 4H_2O$, 0.00003; $NiCl_2 \cdot 6H_2O$, 0.00002; $CuCl_2 \cdot 2H_2O$, 0.00001. In addition, 6 mL L^{-1} of a $CuSO_4$ stock solution (1 g L^{-1}) were supplemented to the MSM (final Cu^{2+} concentration of 10 µM) in order to avoid copper limitations in methanotrophic activity. The final pH of the medium was 7. All chemicals were purchased from Panreac (Spain) with a purity higher than 99.0%. Methane (99.5% purity) was supplied by Abello-Linde, S.A. (Spain), while silicone oil 200 cSt (99.9% purity) was purchased from Sigma Aldrich (USA).

2.2 Inoculum preparation

The inoculum used in the first continuous CH_4 biodegradation experiment in BTF 1 was a methanotrophic *Methylosinus Sporium* culture (17706, Type strain) purchased from DSMZ (Germany). The strain was initially incubated under non-sterile conditions for three days in 1200 mL air-tight glass bottles containing 100 mL of silicon oil and 300 mL of MSM agitated in an orbital shaker (MaxQ 4000, Thermo Scientific, USA) at 150

rpm and 25ºC, under a 11% (~72.1 g m^{-3}) CH_4 air atmosphere. Microbial growth at the aqueous-organic interphase was observed after 3 days of incubation (Figure 1). Previous studies have tentatively identified this strain as partially hydrophobic, preferentially growing at the interphase between oil droplets and the water phase or even inside oil phase (Han et al., 2009).

A hydrophobic bacterial community (capable of growing inside silicone oil) from a stirred tank bioreactor degrading hexane was acclimated to CH_4 biodegradation prior to use in a second continuous CH_4 biodegradation experiment in BTF 2 (Muñoz et al., 2013). Two hydrophobic inocula were incubated in 1200 mL glass bottles containing 100 mL of MSM and 50 mL of silicone oil, in an orbital shaker (MaxQ 4000, Thermo Scientific, USA) at 150 rpm and 25 ºC. The inocula were incubated for 50 and 33 days under an air atmosphere containing CH_4 at 1.6 and 3% (10.5 and 19.7 g m^{-3}), respectively. The MSM was replaced every two days with fresh MSM in order to promote the enrichment of a microbial community capable of growing in the organic phase, while CH_4 was supplied to the headspace at the corresponding concentration after depletion. The hexane degrading community was able to completely degrade the methane present in the bottle's headspace after 6 days of acclimation, concomitantly with a decrease in oxygen concentration and an increase in CO_2 concentration.

2.3 Experimental Set-up

The laboratory scale BTFs consisted of cylindrical jacketed PVC columns with a total working volume of 4 L (1 m height, 0.08 m diameter) packed with 1 cm^3 polyurethane foam (PUF) cubes (Filtren TM 25280, Recticel Iberica S.L.) at an initial height of 0.56

m (Figure 2). The silicone oil from the corresponding inoculum was separated from the MSM and impregnated in the polyurethane foam for inoculation.

A 1 L min^{-1} CH_4 laden air emission containing 14.3 ± 0.5 g CH_4 m^{-3} (~ 2%) was fed in countercurrent mode at the bottom of the BTFs by mixing a pure CH_4 stream (mass flow controller, Aalborg, USA) with a pre-humidified air flow, resulting in an empty bed residence time (EBRT) of 4 min. The BTF recycling liquid consisted of a mixture of MSM and silicon oil 200 cSt at a ratio of 1:4 v/v. The recycling solution was magnetically stirred at 300 rpm (Agimatic S., P. Selecta, Spain) in an external 1 L holding tank at 300 rpm, and recycled at 6.0 m h^{-1} (~ 500 mL min^{-1}) by means of a peristaltic pump (Milton Roy Serie G, Spain). The total volume of the liquid phase was maintained at 1.8 L throughout the experimentation. BTF recycling liquid replacement (only aqueous phase) with fresh MSM was conducted in order to remove accumulated metabolites and to replenish nutrients. Indeed, 300 mL of recycling liquid were daily withdrawn, the NAP being recovered from the withdrawn liquid and returned to the reactor in order to maintain a constant NAP/MSM ratio.

The BTFs were operated at a constant inlet load (IL) of 410 ± 35.8 g m^{-3} h^{-1} in BTF 1 and 440.9 ± 45.6 g m^{-3} h^{-1} in BTF 2 for 56 and 34 days, respectively. The IL was then progressively decreased by decreasing the inlet CH_4 concentration at a constant EBRT of 4 min in order to determine the elimination capacity (EC) vs. IL curves of each BTF. Finally, in order to elucidate the limiting step during methane biodegradation in BTF 1, a mass transfer test was carried out by increasing the inlet CH_4 concentration (thus the IL) by approximately three times and recording the concomitant change in EC. The temperature and humidity of the CH_4-laden air emission remained constant at 25 ± 1°C and 70.0 ± 6.2%, respectively, throughout the experiments.

The CH_4 and CO_2 gas concentrations at the inlet and outlet of the BTFs were daily analyzed by GC-TCD. A 50 mL liquid sample was periodically drawn from the stirred tank in order to determine the pH, and the concentration of total organic carbon (TOC) and total nitrogen (TN) in the aqueous phase previously separated by decantation from the organic phase. Culture absorbance in the aqueous medium of BTF 2 was also monitored. Additionally, the pressure drop and packing material height were daily recorded. The real packed bed volume, which determined the effective EBRT, was used for EC calculation.

2.4 Analytical procedures

CH_4 and CO_2 gas concentrations were determined in duplicate in a Bruker 430 gas chromatograph coupled to a TCD detector (Palo Alto, USA). The GC oven, injector and detector temperatures were set at 45, 150 and 200 °C, respectively. The carrier and make up gas was helium at 6 mL min^{-1} and 24 mL min^{-1}, respectively. The GC was equipped with a BR-Molesieve 5A (15 m × 0.53 mm diameter, USA) and a CP-Porabond capillary column (25 m × 0.53 mm diameter, USA).

Temperature and humidity in the inlet air were determined using a Testo 605-H1 detector (Testo AG, Germany). Pressure drop was monitored by means of a differential pressure meter using water as the manometric fluid. The pH was measured with a pH meter Basic 20 (Crison, Spain). TOC and TN concentrations were analyzed with a total organic carbon analyzer (Shimadzu, Japan) coupled with a total nitrogen module (Shimadzu, Japan). Culture absorbance was determined in a spectrophotometer UV-Visible 2550 (Shimadzu, Japan) at λ = 650 nm.

2.5 Microbiological procedures

Biomass samples from both inocula, from the recycling liquids and from the biofilms at the end of the operation of both BTFs were collected and stored at -20ºC in order to evaluate the richness and composition of the bacterial communities. The genomic DNA was extracted according to Lebrero et al. (2011). The PCR mixture (50 μL) was composed of 25 μL of BIOMIX ready-to use 2× reaction mix (Bioline, Ecogen) containing reaction buffer, magnesium chloride, deoxynucleotide triphosphates (dNTPs), Taq polymerase and additives, l or 2 μL of the extracted DNA, PCR primers 968-F-GC and 1401-R (10μM) (Sigma- Aldrich, St. Louis, MO, USA) for bacterial 16S rRNA gene amplification, and Milli-Q water up to a final volume of 50 μL. The PCR thermo-cycling program used was previously described in Lebrero et al. (2011). The DGGE analysis of the amplicons was performed with a D-Code Universal Mutation Detection System (Bio Rad Laboratories) using 8% (w/v) polyacrylamide gel with a urea/formamide denaturing gradient from 45 to 65%. The DGGE running conditions were applied according to Roest et al. (2005). The gels were stained with GelRed Nucleic Acid Gel Stain (biotium) for 1 h and the obtained DGGE patterns processed using the GelCompar IITM software (Applied Maths BVBA, Sint-Martens-Latem, Belgium). After image normalization, bands were defined for each sample using the band search algorithm within the program. Similarity indices of the compared profiles were calculated from the densitometric curves of the scanned DGGE profiles by using the Pearson product–moment correlation coefficient (Häne et al., 1993). The peak heights in the densitometric curves were also used to determine the Shannon–Wiener diversity index (H). The most relevant bands were excised from the DGGE gel in order to identify the bacteria present in the samples above described. The procedure was previously described in Lebrero et al. (2011). The taxonomic position of the sequenced

DGGE bands was obtained using the RDP classifier tool (50% confidence level) (Wang et al., 2007). The closest matches to each band were obtained using the BLAST search tool at the NCBI (National Centre for Biotechnology Information) (McGinnis and Madden, 2004). Sequences were deposited in GenBank Data Library under accession numbers KM058887- KM058913.

3. Results and Discussion

3.1 Two-liquid phase BTF inoculated with a type II methanotrophs culture

The EC rapidly increased up to 24.6 g m^{-3} h^{-1} within the first 3 days following BTF 1 inoculation , fluctuating afterwards from 7.4 to 30.3 g m^{-3} h^{-1} until day 16 (Figure 3A). During this initial start-up period (~ 10 days) no clear biomass growth was observed in the packing bed. From day 16 on, the EC steadily increased up to 60.1 ± 4.6 g m^{-3} h^{-1} by day 34, the maximum EC recorded in BTF 1 over the entire operational period. This increase in EC was associated to an intense accumulation of biomass in the packed bed and even in the upper part of the reactor wall not filled with packing material. From day 35 onward, a roughly stable EC of 48.5 ± 7.2 g m^{-3} $h^{-1}$was obtained. The BTF achieved a RE = 12.0 ± 0.5% during steady state operation, with a maximum value of 14.6% recorded by day 50.The CO_2 production rate (PCO_2) also increased concomitantly with the initial EC increase, and continued increasing up to average values of 111.1 ± 9.5 g m^{-3} h^{-1} despite the EC stabilized by day 30. This could be attributed to the accumulation of biomass in a mass transfer-limited system, which resulted in a steady increase in endogenous respiration. This was further confirmed by a specific mass transfer limitation test (see below).

By day 57, the IL was progressively decreased and both the EC and RE were determined during steady state operation for each of the six different IL values tested (Figure 3B). An increase in the RE was observed when decreasing the IL, with a maximum value of 39.8 ± 0.9% at an IL of 16.5 ± 1.1 g m^{-3} h^{-1}, corresponding to an EC = 6.8 ± 0.4 g m^{-3} h^{-1}.

Methane mineralization initially oscillated from 30.6 to 75.6% (days 1 to 20), corresponding to CO_2 yield coefficients (YCO_2) from 0.84 up to 2.08 g CO_2 (g CH_4)$^{-1}$. From days 20 to 43, a steady mineralization of 72.6 ± 7.5% (YCO_2 = 2.0 ± 0.2 g CO_2 (g CH_4)$^{-1}$) was recorded, increasing by the end of the experiment to 90.4 ± 12% (which corresponded to a CO_2 yield coefficient of 2.5 ± 0.3 g CO_2 (g CH_4)$^{-1}$). A comparable CH_4 mineralization was reported by Estrada et al. (2014) in a similar BTF operated without silicone oil (about 89%), suggesting that the addition of the NAP did not affect the CO_2 yield. This high mineralization ratio could be associated to a low biomass production, with accumulated biomass not assimilating methane structurally but contributing to CO_2 production by CH_4 oxidation for maintenance purposes.

The aqueous TOC concentration initially increased from ~ 32 mg L^{-1} to final values of ~ 63.5 mg L^{-1}, with a maximum recorded TOC concentration of 79.3 mg L^{-1} by day 31. In this context, Estrada et al. (2014) found a TOC-mediated inhibition at concentrations ~100 mg L^{-1}. The low TOC concentrations here recorded throughout the entire experimentation period ruled out the occurrence of a potential microbial inhibition and confirmed that the implemented liquid dilution rate (0.17 d^{-1}) was adequate to prevent TOC accumulation. This liquid dilution rate was considerably lower compared to that of Estrada et al. (2014) in a similar single-phase BTF (CH_4 inlet concentration = 15.3±0.5 g CH_4 m^{-3}, EBRT = 4 min), who required up to 0.27 d^{-1} to achieve sub-inhibitory TOC levels even at lower ECs. This suggests that the presence of silicone oil in the BTF

mediated a reduced metabolites concentration in the aqueous phase, which entailed significant savings in terms of fresh mineral medium supply. This effect could be attributed to the development of an efficient bacterial community either in the aqueous phase or in the organic-aqueous interphase able to degrade the hydrophilic intermediates excreted. On the other hand, the total nitrogen concentration remained at 109.7 ± 25.7 mg L^{-1} until day 32, with a slight decrease to 73.8 mg L^{-1} by day 34. A N-concentrated MSM (2.44 g L^{-1} of $NaNO_3$) was supplemented from day 35 in order to avoid nitrogen limitations, which resulted in a steady increase in TN concentration, finally stabilizing at 223.0 ± 9.9 mg L^{-1} from day 48 onward, while no variation in the EC was recorded.

The pressure drop of the packed bed remained at 1.2 ± 1.1 Pa m^{-1}, sporadically increasing up to 3.7 Pa m^{-1}. These low pressure drop values were recorded in spite of the high compaction experienced by the packing material, up to 54% by the end of the experimentation period. Higher pressure drops (ranging from 53 to ~80 Pa m^{-1}) were recorded in a full scale BTF packed with PUF cubes when intermittent trickling was applied. However, even these values were far below the maximum tolerable pressure drop in biofiltration, estimated according to economic criteria at 980 Pa m^{-1} (Gabriel and Deshusses, 2003; Lebrero et al., 2011).

The result of the mass transfer limitation test showed that the system was clearly limited by the transport of CH_4 to the degrading community, since a ×2.9 increase in the IL resulted in a ×2.3 increase in the EC and a concomitant increase of ×1.3 in the CO_2 production (Figure 4). The ability of the bacterial community to degrade the additional mass flow of methane supplied ruled out a potential biological limitation.

The steady state ECs here obtained were in agreement with previous studies of two-liquid phase BTFs treating CH_4. For instance, Avalos et al. (2012) reported ECs ranging

from 3.9 to 21 g m^{-3} h^{-1} in a biofilter packed with stones and operated with a non-ionic surfactant at an EBRT of 4.25 min; while an EC of 51 g m^{-3} h^{-1} was recorded by Rocha-Rios et al. (2009) in a two-liquid phase BTF (10% v/v silicone oil) packed with PUF and operated at an EBR of 4.8 min and an average methane IL of 140 g m^{-3} h^{-1}.[24] Moreover, an identical BTF operated in our laboratory under identical conditions but without silicone oil reached a maximum EC of 35 g m^{-3} h^{-1}.[21] Thus, the addition of the non-aqueous phase resulted in a remarkable improvement in the BTF performance by approximately 40%, derived from the higher CH_4 partition coefficient in silicone oil than in water: $K_{SO} = C_G/C_{SO} = 3.2 \pm 0.3$ vs. $K_W = C_G/C_W = 33.5$ at 30ºC (where C_G, C_{SO} and C_W are methane concentrations in the gas, silicone-oil and water phases, respectively) (Rocha-Rios et al., 2009). Comparative studies in varied bioreactor configurations operating with and without a NAP reported improvements in CH_4 removal performance of 102-131% due to the presence of the NAP (Avalos et al., 2012; Rocha-Rios et al., 2009). This enhanced performance of the two-liquid phase partitioning bioreactor can be attributed to an improved CH_4 availability either due to a better mass transfer from the CH_4-laden air emission to the cells or to a direct methane uptake from the organic phase.

Direct substrate uptake from the organic phase has been previously proposed to enhance substrate mass transfer of hydrophobic gas pollutants from the gas phase, and therefore bioreactor performance (Muñoz et al., 2013). In this context, a microscopic analysis of the inoculum used in BTF 1 revealed that methanotrophs were capable of growing at the silicone oil-aqueous interphase, as the bacterial aggregates were apparently on the organic side of the interphase rather than inside the silicone oil (Figure 1). Biomass confinement in organic solvents has been previously reported by several authors. For example, interfacial uptake mechanisms have proven to be of paramount importance in

the biodegradation of many polycyclic aromatic hydrocarbons (PAHs), since the organisms adhered to organic solvents often have significantly higher PAH degradation rates than those suspended in the aqueous phase of a TLPPB (MacLeod and Daugulis, 2005). Muñoz et al. (2013) reported a stable and increased degradation when using a cell-loaded NAP in a stirred tank for hexane removal. On the basis of these results, a hydrophobic inoculum consisting of a silicone-oil-embedded microbial community was tested in order to overcome the mass transfer limitations recorded in BTF 1.

3.2 Two-liquid phase BTF inoculated with a hydrophobic microbial consortium

After the acclimation of the hexane-degrading bacterial community to CH_4, the organic phase of both cultures was inoculated to the BTF 2 recycling liquid. No CH_4 removal was observed until day 4, while the EC steadily increased from day 6 onward, reaching a maximum of 53.2 g m^{-3} h^{-1} by day 23 and stabilizing afterwards at 44.4 ± 4.7 g m^{-3} h^{-1} (Figure 5A). Similarly, the RE progressively increased to steady state values of 9.4 ± 1.0% from day 17 onward. The EC vs. IL curve showed a maximum RE of 18.4 ± 0.2% at an IL = 53.0 ± 0.4 6 g m^{-3} h^{-1}, which corresponded to an EC of 9.7 ± 0.0 g m^{-3} h^{-1} (Figure 5B).

The mineralization yield coefficient increased progressively up to 88.3% within the first 9 days of operation, corresponding to an YCO_2 = 2.43 g CO_2 (g CH_4)$^{-1}$). After this initial increase, a roughly steady mineralization (70.4 ± 7.9%) was recorded for methane biodegradation, which represented a CO_2 yield coefficient of 1.9 ± 0.2 g CO_2 (g CH_4)$^{-1}$. This value was slightly lower compared to the mineralization recorded in BTF 1, but it is still high when compared with typical CO_2 yields reported for methanotrophic bacteria (1.0 – 1.3 g CO_2 (g CH_4)$^{-1}$) (Rocha-Rios et al., 2009).

Total organic carbon accumulated in the recycling liquid of BTF 2 during the first 15 days of experimentation, stabilizing along with the stabilization of EC at 53.0 ± 2.4 mg L^{-1}. These low values ruled out any potential inhibition as a result of TOC accumulation and confirmed the better performance of TLPPBs towards TOC buildup in the aqueous phase. Total nitrogen concentration remained constant at 102.7 ± 19.0 mg L^{-1} until day 27, when TN increased up to 225 mg L^{-1} as a result of a decreasing bacterial activity.

A slightly higher pressure drop was recorded in BTF 2 compared to BTF 1, with average and maximum values of 3.1 ± 1.2 and 5.3 Pa m^{-1}, respectively, probably as a result of the different hydrodynamic properties of the trickling silicone oil with biomass embedded. A lower bed compaction was observed after 33 days of operation in BTF 2 (33% height reduction) compared with BTF 1at the same operation time (48% height reduction).

The results obtained did not confirm the initial hypothesis of an improved system performance when using microorganisms capable of growing inside the NAP. A limited nutrient and water diffusion into the bacteria growing inside the silicone oil might explain the limited enhancement in EC compared to the conventional two-liquid phase BTF treating CH_4. As a matter of fact, an experimental study performed in our Department (data not published) demonstrated the low transfer rate of nitrate or phosphorous from an aqueous to an organic phase. This low nutrient availability inside the NAP could also have mediated the migration of the bacterial community to the aqueous phase. In this context, the analysis of the optical density of the liquid phase revealed microbial growth and accumulation in the aqueous phase during the first 15 days, decreasing afterwards probably as a result of biomass growth in the organic phase and in the BTF packing media (Supplementary Material). A subsequent increase in the optical density from day 24 onward confirmed the dynamic nature of the growth and

detachment of microbial community in two-liquid phase partitioning packed bed reactors.

3.3 Microbiological analysis

The structure of the bacterial communities in both BTFs was elucidated by sequencing 11 bands from the DGGE gel of BTF 1 (Figure 6A), and 16 from BTF 2 (Figure 6B). The analysis of the DGGE bacterial sequences in BTF 1 showed the presence of members belonging to the phyla *Proteobacteria* (8 bands), *Actinobacteria* (2 bands) and *Deinoccocus-Thermus* (1 band). Likewise, the bacterial population in BTF 2 was composed of the phyla *Proteobacteria* (10 bands), *Actinobacteria* (4 bands), *Verrucomicrobia* (1 band) and *Deinoccocus-Thermus* (1 band).The closest matches for each band, along with its similarity percentage and reported sources, are shown in Tables 1 and 2 (Supplementary Material).

The Shannon-Wiener diversity indices revealed an increase in bacterial diversity in BTF 1, with both liquid recycling and biofilm exhibiting high index values (2.9 and 2.7, respectively) compared to the inoculum (2.3). This inoculum was a commercial strain cultivated under non-sterile conditions for a short period, thus resulting in this low diversity. Conversely, a high bacterial diversity was observed in the inoculum of BTF 2 (2.9), which remained constant by the end of the operation in the biofilm but decreased to 2.2 in the recycling liquid. The hydrophobic nature of the microbial community developed in BTF 2, preferentially adhered to the NAP and packing material, could have mediated the poor diversity in the recycling liquid.

A low similarity was observed between the inoculum and the communities established by the end of the experiment in the biofilm and in the recycling liquid (4.9% and 6.8%

similarity, respectively). A significant evolution of the communities was also observed in BTF 2, with similarity values of 35.4% and 42.5% between the inoculum and the samples retrieved from the biofilm and the recycling liquid by the end of the experiment, respectively. Surprisingly, despite the 25% similarity found between the inocula of both BTFs, the analogous operating conditions resulted in a similar bacterial population structure in both BTFs at the end of the experimentation regardless of the inoculum, with similarity coefficients of 68% between both recycling liquid samples and 78% between biofilm communities in BTF 1 and BTF 2.

Bacteria from the *Proteobacteria* phylum were predominant in both BTFs, which are closely related to methane oxidizing cultures previously retrieved from methane oxidation reactors or from environments exposed to CH_4 (Heyer et al., 2002; Knief and Dundfield, 2005; López et al., 2014). DGGE bands 8 and 3 in BTF 1 and 2, respectively, were affiliated to the *Methylocystis* genus (type II methanotrophs), previously found in bioreactors treating methane (Hatamoto et al., 2010).

In BTF 1, bacteria from the *Methylocystaceae* family and *Methylocystis* genus (bands 7 and 8), belonging to the *Proteobacteria* phylum, were the only microorganisms present in the inoculum that remained in BTF 1 with a significant intensity. Bacteria from the *Proteobacteria* phylum were also the most abundant in BTF 2, the *Methylocystis* genus (band 3) and the *Comamonadaceae* family (band 6) being present along the entire experimentation period. Similarly to *Methylocystis* related bacteria, *Comamonadaceae* communities have been also identified in methane oxidation systems (Liu et al., 2014).

The similar operating conditions of both BTFs resulted in the enrichment of two new phyla in both the liquid recycling and biofilm (*Actinobacteria* and *Deinococcus-Thermus)* and in the maintenance of type II methanotrophs. These phyla have been

associated to the biodegradation of aliphatic hydrocarbons (Militon et al., 2010), and *Deinococcus-thermus* bacteria have been also identified in air treatment bioreactors from landfill sites (Li et al., 2013). However, *Verrucomicrobia* related bacteria were only retrieved in the inoculum, while this phylum was not maintained throughout the experiment.

4. Conclusions

The addition of a non-aqueous phase (25% v/v silicone oil) to a conventional BTF resulted in an enhanced methane removal, supporting ECs ~45 g m^{-3} h^{-1} when operated at an EBRT of 4 min and a liquid recycling rate of 6 m h^{-1}. Moreover, the presence of silicone oil decreased the accumulation of metabolites in the aqueous media, thus reducing the requirements for liquid media replacement. However, no improvement derived from the inoculation of a hydrophobic, silicone-oil embedded community was recorded, likely due to the reduced water and nutrients supplementation to the microorganisms. Despite the low similarity found between the inocula of both BTFs, the analogous operating conditions mediated a similar bacterial population structure at the end of the experiments, with type II methanotrophs and *Proteobacteria* predominating in both systems.

Acknowledgements

This research was supported by the Spanish Ministry of Economy and Competitiveness (BES-2010-030994 grant and CTQ2012-34949 project) and the European Union through the FEDER Funding Program.

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Figure captions

Figure 1. Microscopic images of *Methylosinus sporium* after incubation with silicone oil during inoculum preparation using a phase contrast microscope Leica DM4000B at 40× magnification: A) focus on the organic phase, B) focus on the aqueous phase, C and D) microorganisms at the organic/aqueous interphase.

Figure 2. Schematic of the biotrickling filter set up

Figure 3. (A) Time course of the IL (×), EC (○, continuous line) and PCO2 (□, dashed line) during methane degradation in BTF 1. (B) Influence of the IL on the EC (○, continuous line) and RE (×).The straight line represents 100% RE.

Figure 4. Time course of the IL (black continuous line), the EC (○, continuous line) and the PCO_2 (□, dashed line) during the mass transfer limitation test.

Figure 5. (A) Time course of the IL (×), EC (○, continuous line) and PCO_2 (□, dashed line) during methane degradation in BTF 2. (B) Influence of the IL on the EC (○, continuous line) and RE (×).The straight line represents 100% RE

Figure 6. Bacterial DGGE profile of the inocula (1), the recycling liquids (2) and the biofilms (3) of BTF 1 (A) and BTF 2. (B) The Shannon-Wiener diversity indices are indicated in the upper part of the gel. The sequenced bands are indicated by "►" and the corresponding number of each band.

Figure 1
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Figure 1.

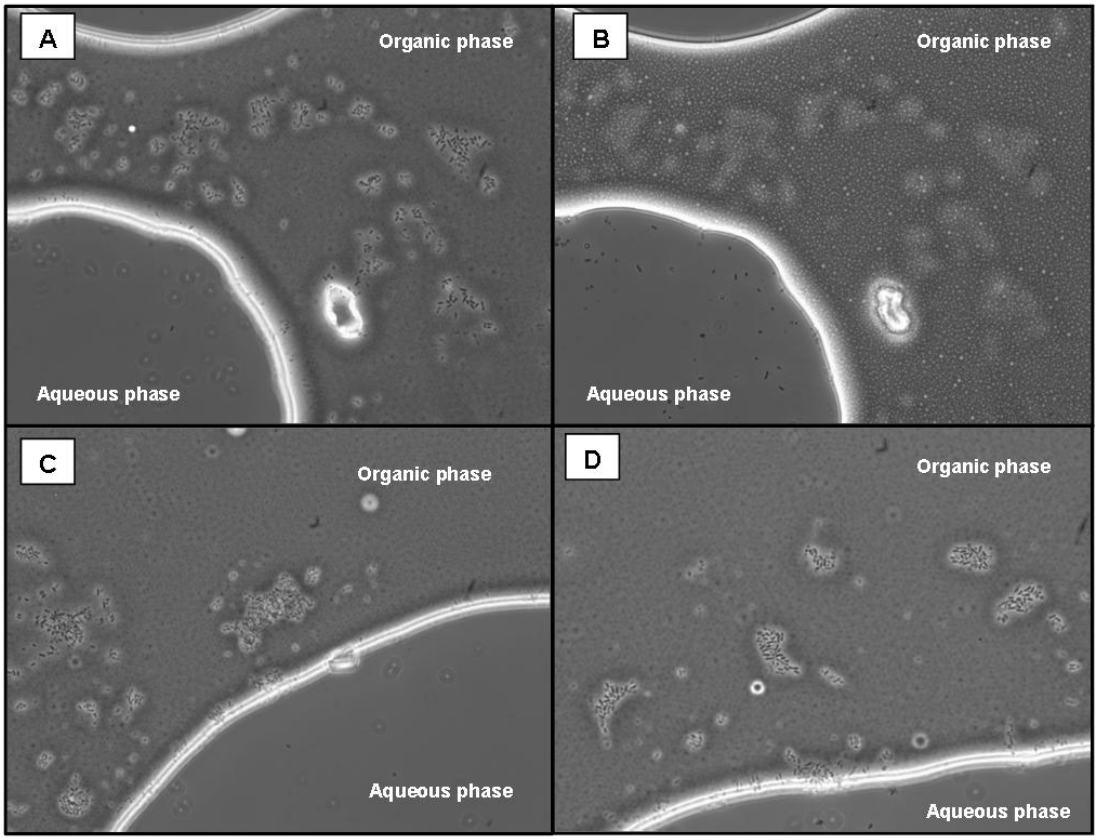

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Figure 2
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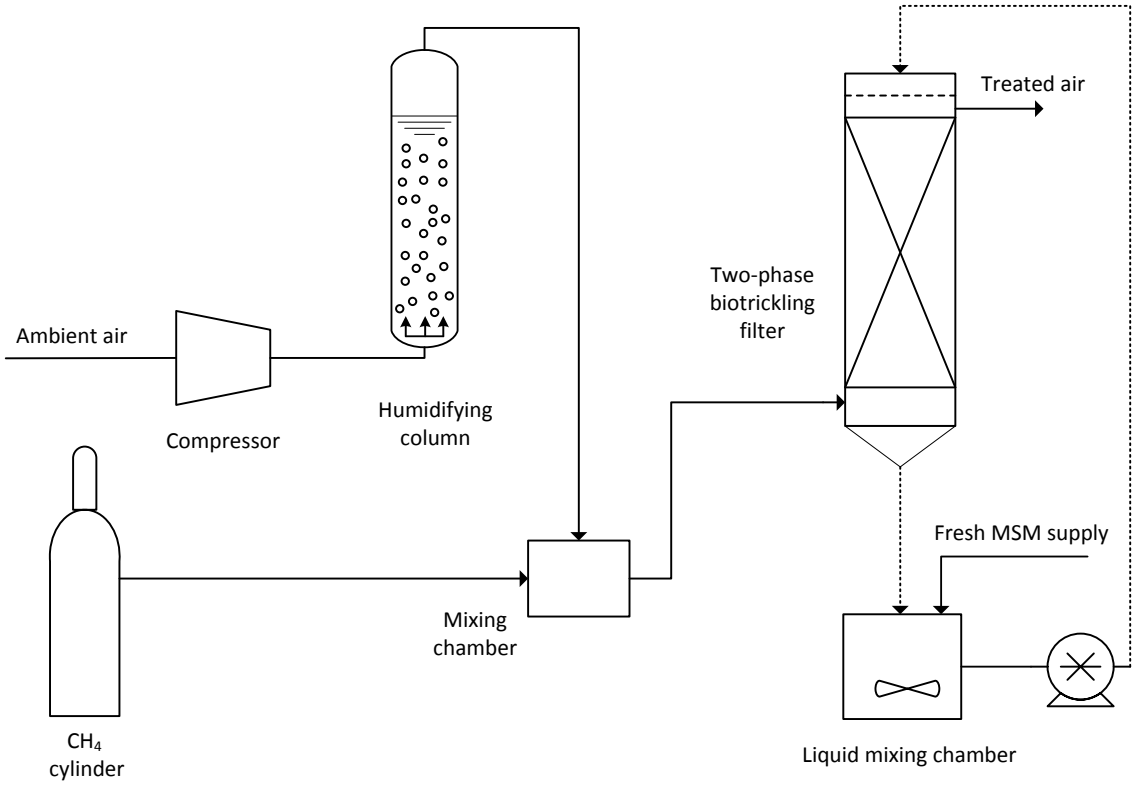

Schematic of the biotrickling filter set up

Figure 3
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Figure 3.

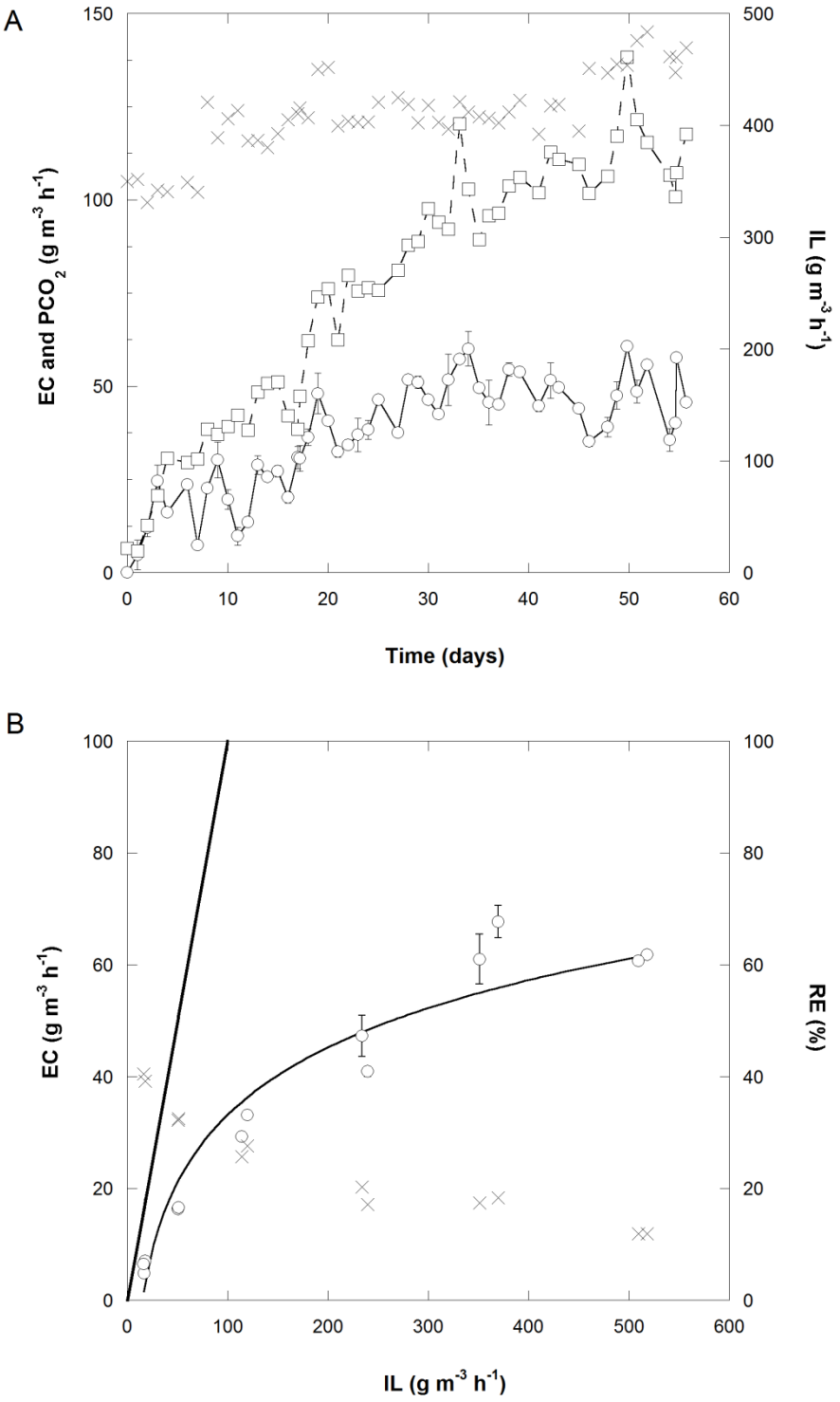

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Figure 4
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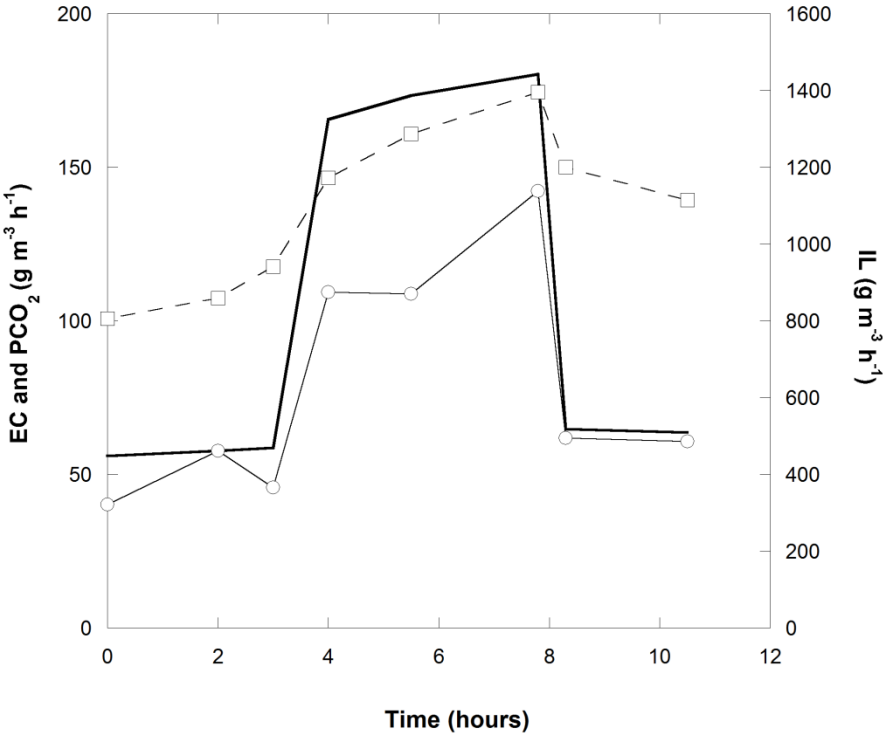

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Figure 5
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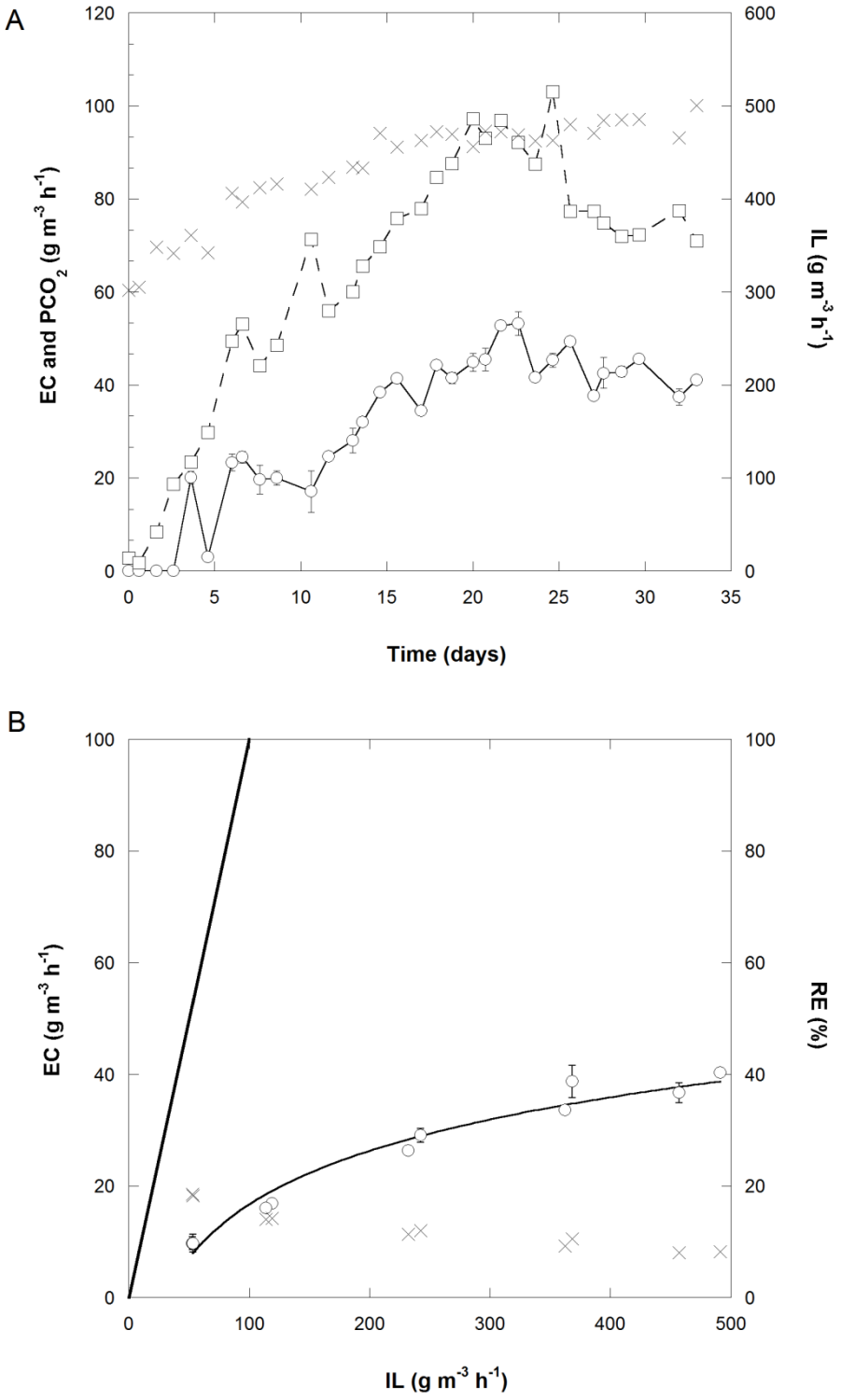

(A) Time course of the IL (×), EC (○, continuous line) and PCO_2 (□, dashed line) during methane degradation in BTF 2. (B) Influence of the IL on the EC (○, continuous line) and RE (×).The straight line represents 100% RE

Figure 6
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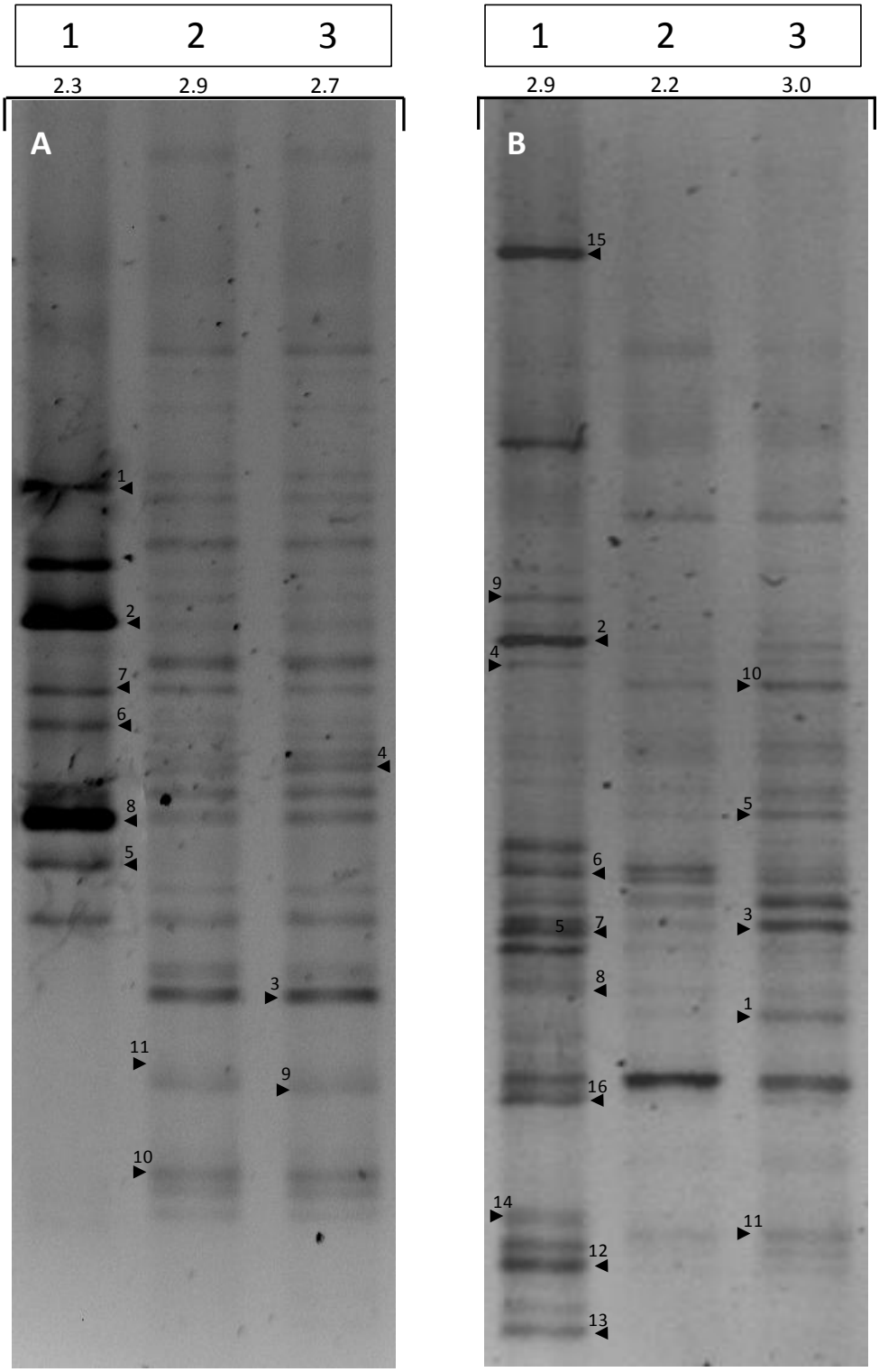

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Supplementary material

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