

ASSESSING THE CAPABILITY OF CH₄ BIODEGRADATION UNDER VARIED CONCENTRATIONS: KINETICS AND POLYHYDROXYBUTYRATE PRODUCTION

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ABSTRACT

CH₄ concentrations are likely to influence the biodiversity of methanotrophs and, consequently, their kinetic characteristics. In the present study, microbial cultures of methane-oxidizing bacteria were enriched in three stirred tank reactors under different CH₄ concentrations in order to assess the kinetic parameters q_{\max} and K_s , and their capability to couple CH₄ biodegradation with the production of polyhydroxyalkanoates (PHA) under sequential N limitations. Culture enrichment at CH₄ concentrations of 20 (R1), 2 (R2) and 0.2 (R3) g m⁻³ supported biomass concentrations of 9, 1.7 and 0.26 g L⁻¹ by day 120, respectively. The microorganisms enriched in R2 presented the highest q_{\max} values (4.76×10^{-4} gCH₄ g_{biomass}⁻¹ h⁻¹) at week 14, while the microorganisms enriched in R1 and R3 exhibited at week 19 the lowest K_s values (5.2×10^{-6} and 4.75×10^{-6} M, respectively). Culture aging resulted in a gradual decrease in q_{\max} . Furthermore, culture enrichment under periodic N limitation episodes resulted in increasing levels of PHB, thus reaching percentages of up to 0.36% and 9.46% (w/w) in R1 and R2, respectively. Polyhydroxybutyrate and polyhydroxyvalerate accumulation during CH₄ abatement, as here recorded, could significantly contribute to improve the economic viability of the treatment process.

Keywords: CH₄ biodegradation kinetics, influence of CH₄ concentration, polyhydroxybutyrate, polyhydroxyvalerate, sequential N limitations.

INTRODUCTION

Methane (CH₄) contributes to approximately 14% of the worldwide greenhouse gas (GHG) emissions, with an increase in its atmospheric concentration of 158% from the pre-industrial era to 2010 (IPCC 2007; EPA 2013). CH₄ presents a global warming potential 21 times higher than that of CO₂ and is mainly emitted from organic waste treatment activities such as landfilling, composting and wastewater treatment (122 million tn CO₂-eq in the EU-15), coal mining (6 million tn CO₂-eq in the EU-15) and livestock farming (120 million tn CO₂-eq in the EU-15) (EEA 2013; EPA 2013). CH₄ concentrations in those emissions greatly vary from 0-0.2 g CH₄ m⁻³ for compost piles or animal houses up to 20-100 g CH₄ m⁻³ in old landfills (Nikiema et al. 2007).

Based on the urgent need to limit the increase in the global average temperature to only 2°C above pre-industrial levels, the EU committed itself under the upgraded Kyoto Protocol to reduce its GHG emissions by 20% in 2020 (compared to 1990) (IPCC 2007; EEA 2013). Moreover, the gradual application of the EU landfill Directive 1999/31 will result in emissions with lower CH₄ concentrations, which will significantly limit the implementation of CH₄ abatement technologies based on energy recovery and will require the development of cost-efficient and sustainable methods for the abatement of these diluted emissions. In this regard, biotechnologies can become a platform technology for the active abatement of diluted CH₄ emissions based on their proven robustness and cost-effectiveness for the treatment of malodours or industrial VOC emissions (Estrada et al. 2012b; López et al. 2013).

Despite the fact that methanotrophs-based technologies such as biofiltration or biotrickling filtration have been implemented for the past 40 years for an active abatement of CH₄ emissions, the performance of such conventional biotechnologies is nowadays still limited by CH₄ mass transfer from the gas phase to the microorganisms and by the insufficient knowledge on the microbiology underlying CH₄ oxidation (Yoon et al. 2009; López et al. 2013). In this context, the presence of microorganisms with high specific oxidation rates (q_{\max}) and a high affinity for CH₄ (low half-saturation constant, K_s) is desirable to guarantee an efficient biocatalytic activity under mass transfer limitations and to reduce the start-up period of bioreactors. However, kinetic studies of CH₄ oxidation under non-mass transfer limiting conditions are scarce, especially at the trace level CH₄ concentrations (~mg m⁻³) where biotechnologies can become competitive if properly tailored (Roch and Alexander 1997; Estrada et al. 2012a; López et al. 2013). In addition, the economical sustainability of biological CH₄ oxidation processes, often compromised by the high gas residence time required to overcome mass transfer limitations, can be positively impacted by the co-production of high-added value products such as biopolymers (i.e. poly-3-hydroxybutyrate, P3HB) (Helm et al. 2006; Zúñiga et

al. 2011). Unfortunately, little is known about the methanotrophic populations capable of simultaneously abating methane and producing polyhydroxyalkanoates at trace level CH₄ concentrations and continuous gas flow.

This study evaluated the influence of CH₄ concentration on the methanotrophic communities enriched in terms of process kinetic parameters, biodiversity and ability to oxidize CH₄ in stirred tank reactors. Moreover, the ability of the resulting enriched consortia to produce PHB from CH₄ under nitrogen limiting scenarios was also evaluated.

MATERIALS AND METHODS

Chemicals and mineral salt medium

Methane was purchased from Abelló Linde S.A. (Barcelona, Spain) with a purity of at least 99.5%. Poly-3-hydroxybutyrate, chloroform (> 99.5%), phosphotungstic acid solution 10% (w/v), uranyl acetate dihydrate (~ 98%) propylene oxide (> 99%) and benzoic acid (> 99.5%) were obtained from Sigma-Aldrich® (Madrid, Spain). Osmium tetroxide was obtained from EMS with a purity of at least 99.95% (Hatfield, USA). Lead nitrate and sodium citrate were purchased from Merck (Darmstadt, Germany). Spurr resin kit TK4 4221D-1 was obtained from TAAB Laboratories Equipment Ltd (Aldermaston, England). Paraformaldehyde and ethanol (96%) were purchased from AppliChem (Darmstadt, Germany). The rest of reagents or chemicals were purchased from Panreac® (Barcelona, Spain) with a purity of at least 99%.

The mineral salt medium (MSM) used for microbial enrichment and the in-vitro kinetic assays was composed of (in g L⁻¹): Na₂HPO₄·12H₂O 6.15, KH₂PO₄ 1.52, MgSO₄·7H₂O 0.2, CaCl₂·2H₂O 0.0503, NaNO₃ 1.32 and 10 mL L⁻¹ of SL4 trace solution (containing per liter: EDTA 0.5 g, FeSO₄·7H₂O 0.2 g, ZnSO₄·7H₂O 0.01 g, MnCl₂·4H₂O 0.003 g, H₃BO₃ 0.03 g, CoCl₂ 0.011 g, CuCl₂·2H₂O 0.443 g, NiCl₂·6H₂O 0.002 g, Na₂MoO₄·2H₂O 0.003 g) (Brunner et al. 1980).

Inoculum and cultivation conditions

Fresh aerobic activated sludge from the Valladolid wastewater treatment plant (Valladolid, Spain), soil from an abandoned landfill cover (Almazán, Spain) and sludge from an aerobic lagoon stabilizing the effluents from the anaerobic digestion of swine manure (Almazán, Spain) were used as inoculum for the enrichment of methanotrophs. Aliquots of the 3 microbial sources were equally mixed (on a volume basis), diluted in MSM in a 1:18 ratio and then incubated at 25°C and 150 rpm for 1 h in a rotary shaker.

Experimental set-up and operation mode

Three 500 mL jacketed stirred tank reactors (STRs) (Afora S.A., Spain) initially containing 380 mL of MSM were inoculated with 20 mL of inoculum. Small fragments of inert polyurethane polymer (0.92 g) were introduced in each reactor in order to prevent the formation of biofilm onto the walls, thus avoiding the underestimation of biomass concentration. The cultivation broth in each STR was agitated magnetically at 250 rpm and maintained at 25°C via a thermostatic water bath. CH₄ was continuously supplied via aeration (400 mL min⁻¹) at approximately 20 g m⁻³; 2 g m⁻³ and 0.2 g m⁻³ into reactors 1 (R1), 2 (R2) and 3 (R3), respectively, using porous steel diffusers of 10 µm pore size located at the bottom of the reactors. The concentrations of CH₄ were regulated via mass flow controllers (Aalborg™, USA) by mixing an air stream with either pure methane or serial dilutions of CH₄-laden air streams (Fig. 1).

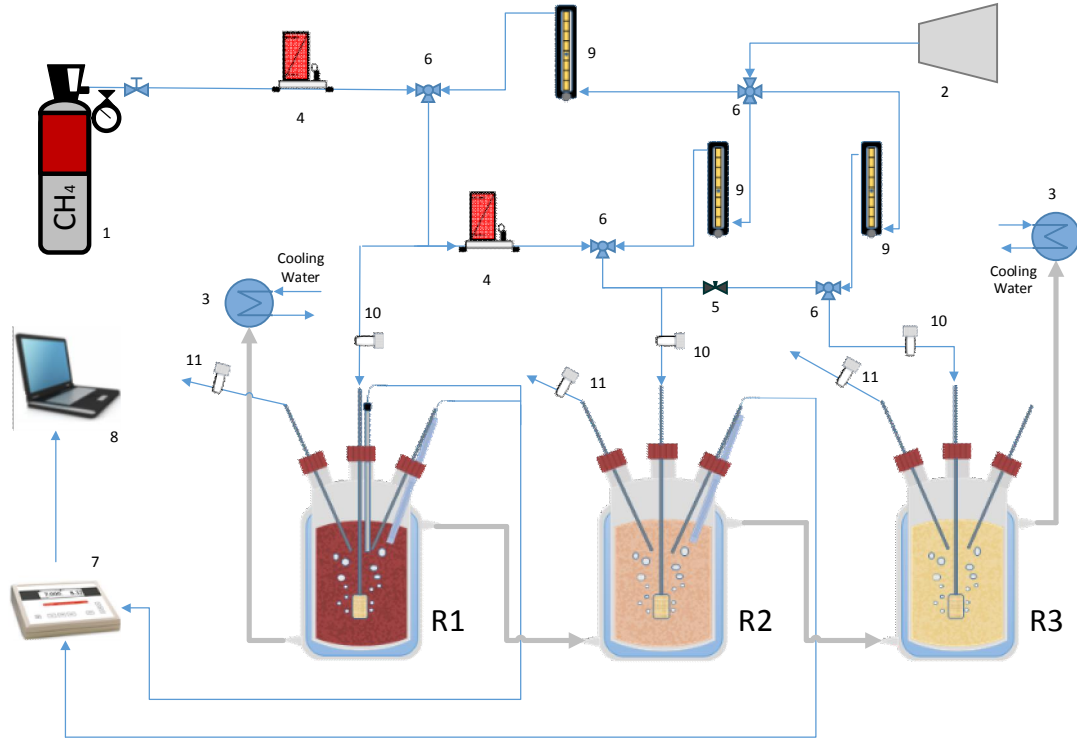


Figure 1. Schematic representation of the experimental set-up. 1 CH₄ gas cylinder, 2 air compressor, 3 thermostatic bath connection, 4 mass flow controllers, 5 needle valve, 6 T-connections, 7 pH data acquisition system, 8 PC data logger, 9 rotameters, 10 inlet sampling points, 11 outlet sampling points.

The pH of the enrichment broth was maintained at 7.2 ± 0.2 by periodic addition of HCl (0.2 M). Distilled water was added every two days to compensate for water losses by evaporation. MSM without nitrogen source and containing double concentration of the remainder components was also added after sampling to compensate for volume medium losses and to provide enough nutrients to the microorganisms. Liquid samples (3 mL) were periodically drawn to determine biomass concentration via culture absorbance measurements (OD_{650}), the concentration of dissolved total organic carbon (TOC) and total nitrogen (TN). Additionally, 10 mL samples were drawn on weeks 14 and 19 to perform the determination of kinetic parameters and to measure periodically the total suspended solid concentration (TSS). Liquid samples of 3 mL were also drawn for PHB determination by GC-MS and transmission electron microscopy after 3 days of nitrogen limitation ($0 - 5 \text{ mg L}^{-1}$) in each bioreactor. Liquid samples were also drawn on weeks 1, 4, 8, 14 and 19 to elucidate the dynamics of microbial populations by DGGE and PCR analysis. CH₄ and CO₂ concentrations were monitored by GC-TCD using gas-tight syringes of 100 μL (Hamilton, USA) at the inlet and outlet sampling points of the reactors.

Biodegradation performance

Process performance in the STRs was described by the volumetric CH₄ elimination capacity (EC), the volumetric CO₂ production rate (RCO_2) and the removal efficiency (RE) defined by Equations 1 - 3:

$$EC = \frac{Q}{V} (CH_{4inlet} - CH_{4outlet}) \quad (1)$$

$$RCO_2 = \frac{Q}{V} (CO_{2inlet} - CO_{2outlet}) \quad (2)$$

$$RE = \frac{CH_{4inlet} - CH_{4outlet}}{CH_{4inlet}} \times 100 \quad (3)$$

Where C_{CH_4inlet} , $C_{CH_4outlet}$, C_{CO_2inlet} and $C_{CO_2outlet}$ represent the average inlet and outlet gaseous CH_4 and CO_2 concentrations in the STRs ($g\ m^{-3}$), respectively. Q and V stand for the CH_4 laden air flow rate ($m^3\ h^{-1}$) and the liquid volume in the system (m^3), respectively.

Kinetic parameter determination

The maximum specific CH_4 biodegradation rate q_{max} ($g\ CH_4\ m^{-3}liq\ h^{-1}$) and the half-saturation constant ($g\ m^{-3}$) of the Monod model K_s were determined for the 3 reactors in the third and fourth month of enrichment. The assays were conducted in 120-mL glass bottles containing 20 mL of mineral salt medium and inoculated with fresh biomass from the bioreactor at an initial OD_{650} of 0.1085 ($51.2 \pm 12.1\ g_{biomass}\ m^{-3}$), optimized to avoid mass transport limitations. The bottles were closed with butyl septa, sealed with aluminum caps and supplied with methane at initial headspace concentrations of $91.4 \pm 3.9\ g\ m^{-3}$, $17.9 \pm 0.8\ g\ m^{-3}$ and $4.7 \pm 0.4\ g\ m^{-3}$ (corresponding to initial methane aqueous concentration of $3.1 \pm 1.3\ g\ m^{-3}$, $0.6\ g\ m^{-3}$ and $0.2\ g\ m^{-3}$, respectively). The bottles were incubated at 25°C and 150 rpm for 25 h. CH_4 and CO_2 concentrations in the gas phase were periodically measured by GC-TCD. The CH_4 concentrations in the liquid phase were estimated by considering the dimensionless Henry's law constant for CH_4 of 29.25.

The Lineweaver-Burk approach (Equation 4) was used to determine the kinetic parameters of methane oxidation from the initial CH_4 biodegradation rates (Stein et al. 2006; Steenbergh et al. 2010):

$$\frac{1}{q} = \frac{K_s}{q_{max}} \times \frac{1}{[CH_4]} + \frac{1}{q_{max}} \quad (4)$$

Where q is the initial CH_4 biodegradation rate ($g\ CH_4\ m^{-3}liq\ h^{-1}$) and $[CH_4]$ is the CH_4 concentration in the liquid phase ($g\ m^{-3}liq$). K_s and q_{max} were estimated from the slope and intercept of Equation 4.

Electron microscopy analysis

Liquid samples of 1 mL drawn from R1 and R2 were centrifuged at 4000 rpm and 4°C for 5 minutes. The supernatant was discarded and the biomass pellets were fixed with a solution composed of 2% glutaraldehyde, 2% paraformaldehyde and 0.1 M phosphate buffered saline (PBS), and maintained in the dark at 4°C. The samples were then washed with PBS 1X and resuspended in a PBS-ethanol solution (1:1 v/v). This cell suspension was centrifuged at 4000 rpm for 5 minutes, resuspended in 500 μ L OsO_4 1% in the dark, washed with PBS 1X and dewatered sequentially with ethanol at increasing concentrations. The samples were then resuspended in 500 μ L of propylene oxide, centrifuged at 4000 rpm for 3 minutes, immersed in a propylene-Spurr resin solution (1:1 v/v) for 2 h and maintained only in Spurr resin overnight. Fresh resin was then used to embed the samples for 24 h at 60°C.

The samples were finally cut using an ultramicrotome and contrasted with phosphoric tungsten acid, uranyl acetate and lead citrate according to Reynolds (1963) and Wendlandt et al. (2001). A TEM JEOL JEM-1011 electron microscope (Teknolab, Indonesia) with an ES1000W Erlangshen CCD camera (Gatan, Germany) was used for analysis.

Measurement of PHB

The quantitative determination of PHB was carried out as reported in the literature (Braunegg et al. 1978; Riis and Mai 1988; Zúñiga et al. 2011). In brief, 3 mL of cultivation broth were centrifuged at 10000 rpm for 10 min, discarding the supernatant and resuspending the pellet with 1 mL of acidic propanol (propanol:HCl 80%/20% v/v). Then, 2 mL of chloroform and 50 μ L of a benzoic acid solution in propanol ($40\ g\ L^{-1}$) were added before incubation at 100°C for 4 hours. After cooling at room temperature, 1 mL of deionized water was added and the mixture was vigorously agitated. The organic phase containing the PHB was filtered and analyzed by GC-MS.

Analytical procedures

CH_4 and CO_2 gas concentrations were determined in a Bruker 430 GC-TCD (Palo Alto, USA) equipped with a CP-Molsieve 5A ($15\ m \times 0.53\ \mu m \times 15\ \mu m$) and a CP-PoraBOND Q ($25\ m \times 0.53\ \mu m \times 10\ \mu m$) columns. The oven, injector and detector temperatures were maintained at 45°C, 150°C and 200°C, respectively. Helium was used as the carrier gas at $13.7\ mL\ min^{-1}$.

Samples for the determination of TOC/TN concentrations were filtered through 0.22 μm glass fiber filters (Merck Millipore, USA) prior to analysis in a TOC-VCSH analyzer (Shimadzu, Japan) coupled with a chemiluminescence detection TN module (TNM-1) (Shimadzu, Japan). Culture absorbance measurements at 650 nm were performed using a Shimadzu UV-2550 UV/Vis spectrophotometer (Shimadzu, Japan). Biomass concentration was determined by periodical measurements of TSS according to standard methods.

Temperature and pH were on-line monitored using a multiparametric analyser C-3020 (Consort, Belgium). The temperature was controlled at 25°C via a Frigiterm-10 refrigerated recirculation bath (JP Selecta S.A., Barcelona).

PHB was quantified in an Agilent 6890N GC-MS equipped with a DB-WAX column (30 m \times 0.250 mm \times 0.25 μm) (J&W Scientific®, CA, USA). The injector temperature was set at 250°C. The oven temperature was initially maintained at 40°C for 5 min, increased at 10°C min^{-1} up to 200°C and finally increased at 5°C min^{-1} up to 240°C (maintained for 2 min).

RESULTS AND DISCUSSION

Methane biodegradation performance

CH_4 biodegradation was recorded in the three STRs from the second day of operation on. R1 reached a CO_2 production rate of $\sim 70 \text{ g m}^{-3} \text{ h}^{-1}$ within the first 20 days, confirming the CH_4 oxidation by the microbial community (Fig. 2a). The increase in CO_2 production was concomitant with the increase of the biomass concentration, reaching values of 2 gTSS L^{-1} by day 20 (Fig. 3a). A further biomass growth in R1 from day 20 onwards resulted in an increase in the CO_2 production rate up to an average value of approximately $85 \text{ g m}^{-3} \text{ h}^{-1}$. However, the CO_2 production progressively decreased from day 40 to 60 to an average value of $40 \text{ g m}^{-3} \text{ h}^{-1}$ and remained constant until the end of the experiment. The TOC concentration increased from average values of approximately 50 mg L^{-1} , recorded at day 20, to average values of 175 mg L^{-1} by day 30 (Fig. 3b). This rapid increase in TOC concentration was correlated with the increase in the biomass concentration, which reached a value of approximately 4 gTSS L^{-1} by day 30. Afterwards, TOC concentration continued increasing up to 300 mg L^{-1} by day 45 despite the stabilization in biomass concentration. The increase in the TOC concentration was likely due to the accumulation of both metabolites and cell lysis products, and likely hindered microbial growth and CH_4 biodegradation. Thus, the liquid broth of R1 was centrifuged at day 45 and the biomass was resuspended in fresh MSM. Thereafter, the TOC concentration dropped to approximately 110 mg L^{-1} following MSM renewal, which triggered the increase in biomass concentration to almost 7 g L^{-1} from day 50 to 78. Nonetheless, this increase in biomass concentration did not correlate to an increase in the CO_2 production during this period. From day 75 onwards the TOC concentration increased again and reached values of up to 400 mg L^{-1} by day 125. This increase hindered biomass growth from day 80 to 125, which remained constant at stable concentration of approximately 6 g L^{-1} from day 80 to 120. Therefore, the liquid broth of R1 was once more centrifuged at day 125 and the biomass was resuspended in fresh MSM. Following this second liquid medium renewal, the TOC concentration dropped to roughly 100 mg L^{-1} , which caused an increase in biomass concentration to an average value of 9 g L^{-1} by day 130. The stimulation of biomass growth after MSM renewal suggested that biomass production was hindered by the accumulation of metabolites or cell lyses products in the culture broth. However, the stimulation of biomass growth was neither correlated to the CO_2 production rate nor to CH_4 oxidation performance in R1.

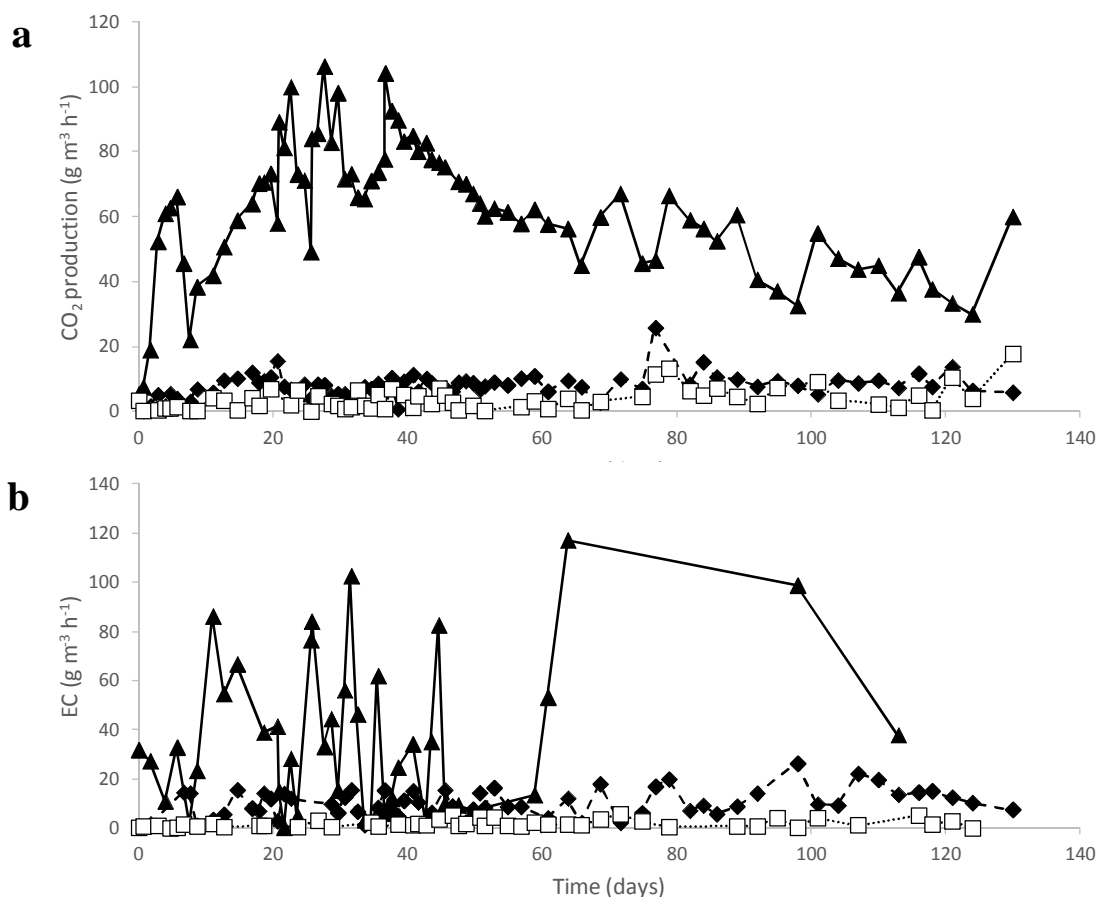


Figure 2. Time course of (a) CO₂ production rate and (b) elimination capacity during methane biodegradation in R1 (□), R2 (◊) and R3 (▲).

The CO₂ production rates in R2 and R3 showed less variations than those recorded for R1, with average values of 7.8 and 3.7 g m⁻³ h⁻¹, respectively (Fig. 2a). Likewise, TOC concentrations in both reactors remained roughly constant and below 50 mg L⁻¹, which suggested that the accumulation of metabolites or cell lyses products in R2 and R3 was not significant during the experimental time compared to the TOC accumulation recorded for R1 (Fig. 3b). The biomass growth in R2 and R3 was significantly lower than that recorded in R1, which was likely due to the fact that the methanotrophic consortia in R2 and R3 were exposed to 10 and 100 times lower CH₄ loading rates compared to R1. Nonetheless, a constant growth was observed for both reactors, thus reaching values of 1.7 and 0.26 g L⁻¹ for R2 and R3 by day 120, respectively (Fig. 3a).

R1 showed considerable variations in the CH₄ elimination capacity (EC) during the whole experimental time, with values ranging from 0 to 120 g m⁻³ h⁻¹ (Fig. 2b). These large variations in EC were likely due to the experimental errors associated to the measurements of EC at the high CH₄ loading rates of 1200 g m⁻³ h⁻¹ applied to R1. Thus, a typical experimental error of 5% in the measurement of the CH₄ concentration at the outlet of the reactor would entail variations in EC of approximately 60 g m⁻³ h⁻¹, which could explain the recorded values. However, it must be stressed that the degradation of CH₄ was clearly demonstrated by both the high CO₂ production rate and the biomass growth. These results suggest that the precise quantification of CH₄ removal performance in R1 could not be assessed due to the nature of the experimental set-up, the poor CH₄ mass transfer from the gas to the aqueous phase and the typical errors in the CH₄ concentration measurement. On the other hand, EC values were more stable in R2 and R3 (~10.1 g m⁻³ h⁻¹ for R2 and 1.7 g m⁻³ h⁻¹ for R3) (Fig. 2b). The results obtained in R2 were similar to those reported by Rocha-Rios et al. (2010, 2011), who reported ECs of up to 10-15 g m⁻³ h⁻¹ for loading rates of approximately 60 g m⁻³ h⁻¹ in stirred tank reactors. In this context, EC values as high as 22 and 75 g m⁻³ h⁻¹ have been also recorded at loading rates of 80 and 200 g m⁻³ h⁻¹, respectively (Zúñiga et al. 2011; Rocha-Rios et al. 2009). However, the optimization of the CH₄ abatement performance was not the main objective of this work, which was devoted to elucidate the influence of CH₄ loading on the characteristics of the methanotrophic population.

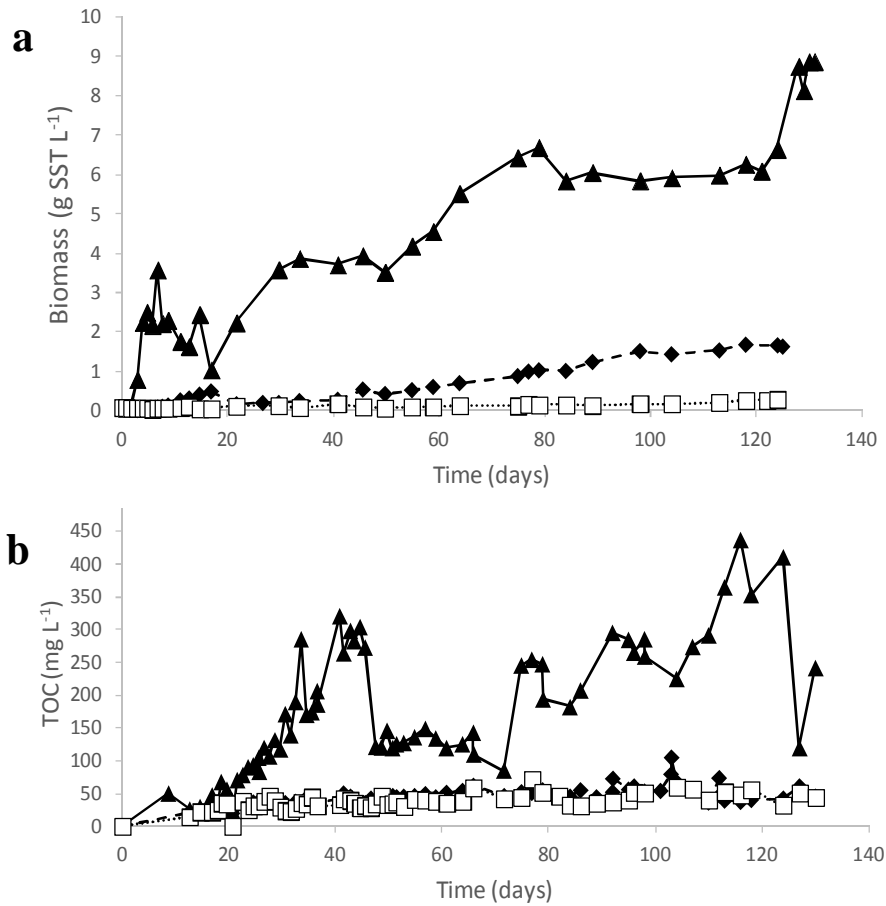


Figure 3. Time course of biomass concentration (**a**) and TOC concentration (**b**) in R1 (), R2 () and R3 ().

Determination of q_{max} and K_S

The kinetic parameters q_{max} and K_S of the microbial communities established in the reactors were estimated using the Lineweaver-Burk linearization of the Monod equation. The kinetic assays were performed at weeks 14 and 19 of operation in order to record the dynamics of the kinetic characteristics in time (Fig. 4).

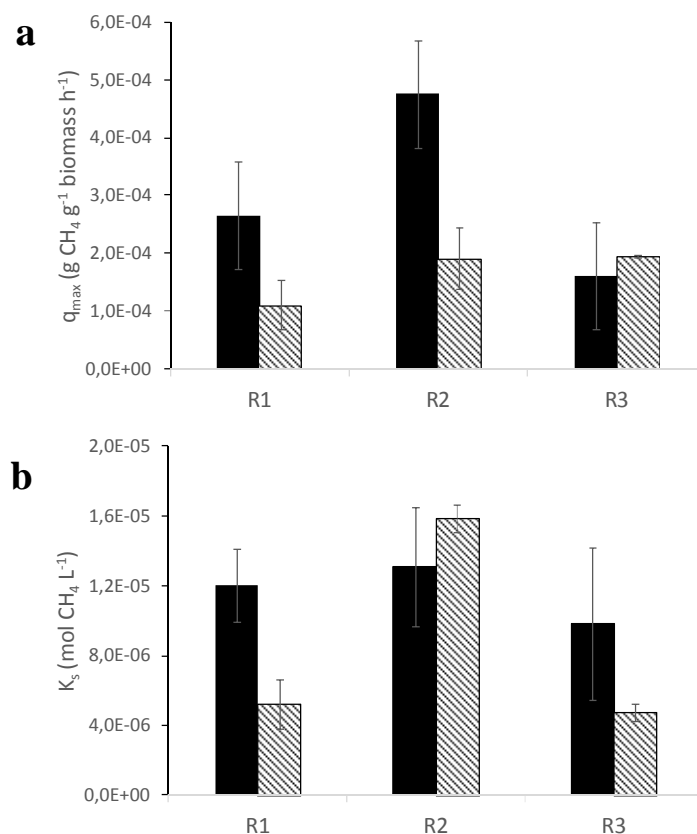


Figure 4. Determination of the kinetic parameters q_{\max} (a) and K_s (b) of the microbial communities established in R1, R2 and R3 at week 14 (black bar) and week 19 (scratched bar).

The highest q_{\max} value (4.76×10^{-4} gCH₄ g_{biomass}⁻¹ h⁻¹) was recorded at week 14 for R2. No significant differences were observed among R1 and R3, with values of 2.65×10^{-4} and 1.6×10^{-4} gCH₄ g_{biomass}⁻¹ h⁻¹ determined at week 14, respectively (Fig. 4a). Likewise, q_{\max} determination at week 19 showed lower values for R1 and R2 (1.1×10^{-4} and 1.9×10^{-4} gCH₄ g_{biomass}⁻¹ h⁻¹, respectively), while q_{\max} for R3 remained similar compared to the value obtained at week 14. These findings suggest that culture aging negatively affected the specific CH₄ biodegradation rate of the microbial consortia established in R1 and R2. In contrast, the CH₄ biodegradation performance of R3 remained roughly constant over the experimental time. The q_{\max} values obtained at week 14 for the three reactors were higher than those previously reported in the literature, which typically ranged from 4.16×10^{-5} to 1.28×10^{-4} gCH₄ g_{biomass}⁻¹ h⁻¹ (Bender and Conrad 1992; Figueroa 1993; Gebert et al. 2003).

On the other hand, the K_s determination at week 14 showed values of $\sim 1 \times 10^{-5}$ M without significant differences among the three STRs (Fig. 4b). Nonetheless, the K_s values at week 19 were significantly lower in R1 and R3 (5.2×10^{-6} and 4.75×10^{-6} M, respectively), these values being the lowest recorded during the whole experimental period. The K_s value for R2 at week 19 was similar to that recorded at week 14 (1.58×10^{-5} M). The results here obtained suggest that the microorganisms enriched in both R1 and R3 supported a higher affinity for CH₄ compared to those enriched in R2, allowing for a faster growth at low CH₄ concentrations in the liquid phase. Similar results were obtained by Buchholz et al. (1995), who reported K_s values in the range of 4.38×10^{-6} . 9.38×10^{-6} M for sediment pore water samples from Lake Michigan exposed to trace levels of CH₄ in the gas phase (~ 2.4 g m⁻³). Whalen et al. (1990) reported K_s values for samples from landfill cover soils as low as 2.5×10^{-6} M under CH₄ concentrations around 1 . 1.7 g m⁻³ in the gas phase, which are in the same magnitude order than those obtained at week 19 for R1 and R3. However, K_s values of up to 6.8×10^{-5} and 4.7×10^{-4} M have also been reported in the literature for methanotrophs (Hornibrook et al. 2009; Delhom nie et al. 2009).

This work constitutes, to the best of our knowledge, one of the few studies assessing the kinetic parameters of CH₄ biodegradation of cultures enriched under a wide range of CH₄ concentrations, including trace levels. The values obtained for both parameters, q_{\max} and K_s , were in agreement with the values reported in the

literature and ranked among the best ones in terms of specific biodegradation rate and affinity towards CH₄. Therefore, microbial isolation under varied CH₄ concentrations indeed results in the enrichment of inocula with specific characteristics that could improve the performance of biological GHG treatment systems by reducing process start-up and the abatement performance.

PHB detection by electron transmission microscopy

Transmission electron micrographs of an ultra-thin section of cells from R1 and R2 at the end of the third cycle of N limitation were taken. PHB was present as refractive inclusions or granules inside the methanotrophic microorganisms, which can be identified in the enriched cultures of R1 and R2 by their intracytoplasmic membranes (Fig. 5). Therefore, these electron micrographs confirmed the technical feasibility of coupling of CH₄ abatement with the production of an added value product such as PHB, which can significantly contribute to improve the economic viability of the treatment process.

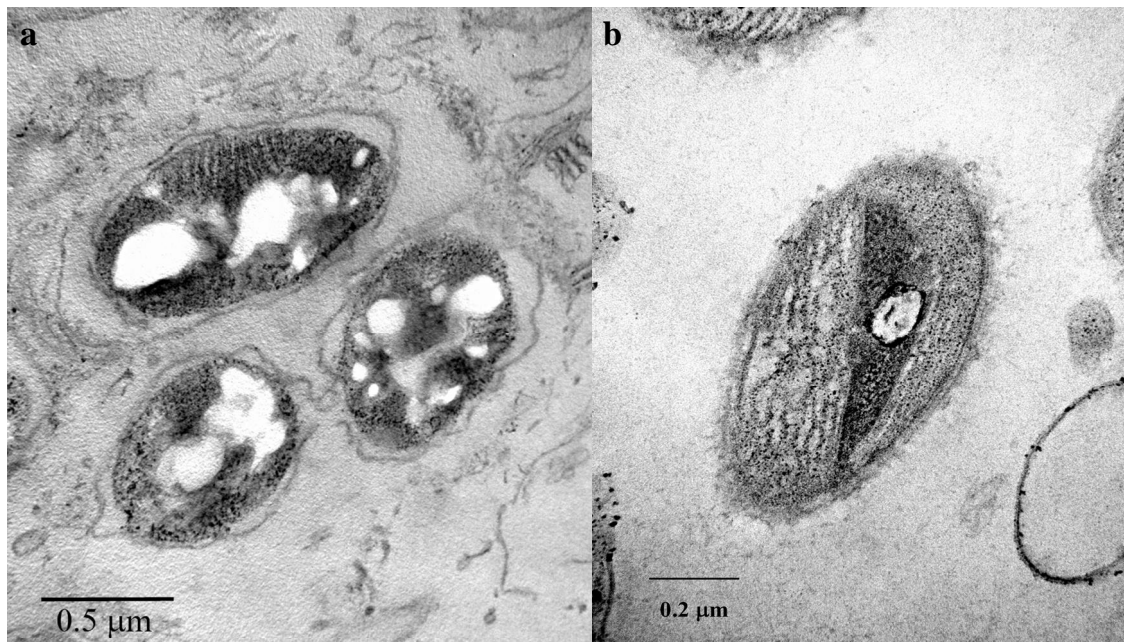


Figure 5. Transmission electron micrograph of the methanotrophic consortia cells of R1 (a) and R2 (b) containing PHB (60 000 × magnification).

PHB quantification

Polyhydroxyalkanoate production in methanotrophs can be induced under nutrient limiting conditions in N, P or Mg (Asenjo and Suck 1986), N limitation being apparently the best condition for PHB accumulation according to Wendlandt et al. (2001). Hence, the isolation of methane-oxidizing bacteria capable of producing PHB was performed by operating the reactors under sequential periods of N limitation. The enrichment of the microbial communities included periodic N limitation cycles of 48-72 h, and after each cycle the N concentration was restored. The total enrichment period was 135 days and the particular N uptake rate in each reactor allowed the occurrence of 7, 4 and 0 N-limitation periods in R1, R2 and R3, respectively. The TN concentration in R3 remained still at 39 mg L⁻¹ by day 130 (Fig. 6) and thus, PHB quantification for the biomass enriched in R3 was not conducted.

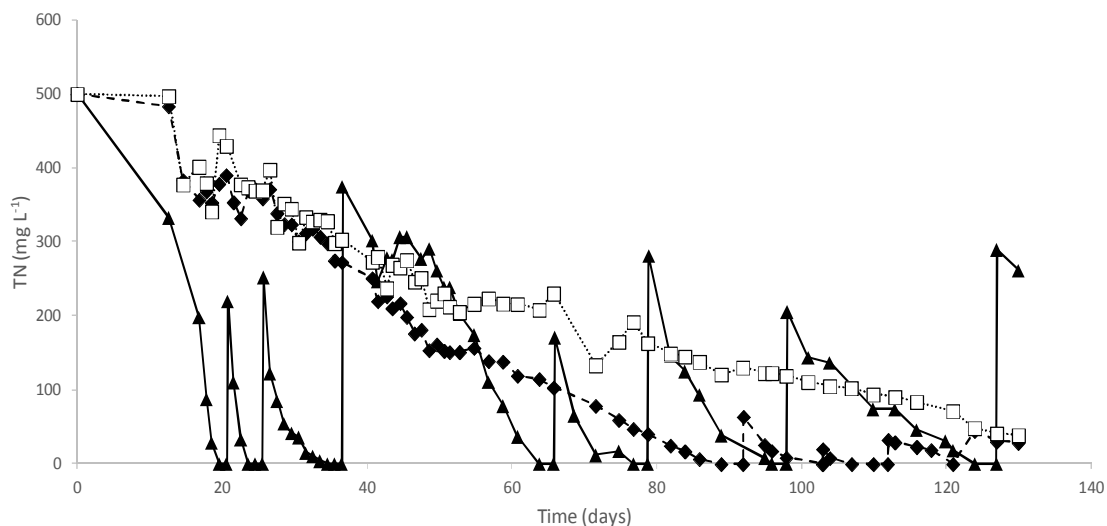


Figure 6. Time course of dissolved TN concentration in R1 (□), R2 (◆) and R3 (▲).

GC-MS analysis showed that the PHB content of biomass reached up to 0.36% (w/w) in R1 during the limitation cycle 7 and up to 9.46% (w/w) in R2 during the limitation cycle 4 (Table 1), despite the fact that higher C/biomass ratios in R1 were expected to induce a higher PHB accumulation. In this regard, Pieja et al. (2012) observed that CH₄ limitations alternated with N limitations indeed increased PHB production in methanotrophic communities.

Table 1. PHB Content in the biomass of R1 and R2 determined during the N limitation cycles.

Cycle	R1			R2		
	PHB (mg L ⁻¹)	%PHB ^b	%PHB ^c	PHB (mg L ⁻¹)	%PHB ^b	%PHB ^c
1	nd ^a	nd	nd	nd	nd	nd
2	nd	nd	nd	77.05	3.3	4
3	nd	nd	nd	93.95	3	3.6
4	nd	nd	nd	244.26	9.46	10.4
5	nd	nd	nd	nd	nd	nd
6	22.19	0.25	0.28	nd	nd	nd
7	35.53	0.36	0.41	nd	nd	nd
8	nd	nd	nd	nd	nd	nd

^a nd: not determined.

$${}^b \%PHB = \frac{gPHB}{gTSS} \times 100$$

$${}^c \%PHB = \frac{gPHB}{gVSS} \times 100$$

The differences in the PHB contents in R1 and R2 can be also explained by the fact that different methanotrophic communities were enriched in R1 and R2 as revealed the denaturing gradient gel electrophoresis (DGGE) analysis (data not shown). The relevance of the type of methanotrophs enriched on the PHB accumulation was highlighted by Pieja et al. (2011), who demonstrated that only type II methanotrophs have the capability to produce PHB under N limiting conditions. Rostkowski et al. (2013) evaluated the PHB production capacity of *Methylosinus trichosporium* OB3b and *Methylocystis parvus* OBBP in serological glass bottles. They observed that *M. parvus* OBBP accumulated 22% more PHB than *M. thichosporium* under optimal accumulation conditions. Furthermore, the PHB production seems to vary not only among the type of methanotroph but also among the culture conditions. Zúñiga et al. (2011) evaluated the PHB content of *Methylobacterium organophilum* both in microcosm experiments and in a stirred tank reactor, reaching up to 57% (w/w) and 39% (w/w), respectively. Moreover, Pfluger et al. (2011) measured PHB accumulation levels lower than 10% (w/w) in a fluidized bed reactor, while accumulations of up to 20-40% (w/w) were recorded in external test bottles using the same biomass.

Finally, it must be stressed that GC-MS analysis revealed the accumulation of poly-3-hydroxyvalerate (PHV) in the cultures enriched in R1 and R2. The available data from cycle 7 in R1 and cycle 4 in R2 showed PHB:PHV ratios of 1:12 and 4:1, respectively. These findings suggest that low percentages of PHB in R1 can be attributed in some extent to the preferential formation of PHV in comparison to R2. However, PHV content was not quantified during the experimental time and thus, further analysis focused on PHV quantification must be performed.

CONCLUSIONS

The results obtained showed that differences in the CH₄ concentration during culture enrichment resulted in microbial communities with different kinetic characteristics. The microorganisms enriched in R2 and R3 presented the highest q_{max} values (~1.9 · 2 × 10⁻⁴ gCH₄ gbiomass⁻¹ h⁻¹) at the end of the experiment, while the microorganisms enriched in R1 and R3 exhibited the lowest K_S values (5.2 × 10⁻⁶ and 4.75 × 10⁻⁶ M, respectively). Hence, the microorganisms isolated at trace levels of CH₄ (0.2 g m⁻³ in the gas phase) presented suitable kinetic characteristics in terms of biodegradation capacity and affinity for CH₄, thus allowing for the reduction of the start-up period in CH₄ abatement biotechnologies.

Moreover, the capability of the microbial communities established in R1 and R2 to couple CH₄ oxidation with the production of high added-value products such as PHB or PHV was demonstrated. The enrichment of methanotrophs using sequential N limitation cycles to induce PHB production successfully resulted in increasing levels of the biopolymer.

As future work, further experiments assessing the performance of these inocula in different CH₄ abatement technologies such as biofilters or biotrickling filters should be carried out. In addition, enrichment of type II methanotrophs should be further investigated in order to achieve higher percentages of PHB and PHV.

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