Effect of Pollutant Concentration During Isolation on the CH$_4$ Biodegradation Kinetics, Population Structure and PHB Accumulation

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Methane-oxidizing bacteria were enriched in three stirred tank reactors continuously supplied with CH$_4$-laden air at 20, 2 and 0.2 g CH$_4$ m$^{-3}$ to assess the effect of pollutant concentration on the biodegradation kinetics, population structure and polyhydroxyalkanoate (PHA) production under sequential nitrogen limitations. CH$_4$ concentration influenced the population structure of the enriched cultures, which were mainly constituted by type I and, in a lesser extent, by type II methanotrophs. Microorganisms enriched at 2 g CH$_4$ m$^{-3}$ presented the highest maximum specific degradation rate ($q_{\text{max}}$) and those enriched at 20 and 0.2 g CH$_4$ m$^{-3}$ exhibited the lowest half-saturation constant ($K_S$), which ruled out a potential correlation between CH$_4$ concentration and kinetic parameters. Maximum polyhydroxybutyrate (PHB) contents of 1.0, 12.6 and 1% (w/w) were obtained at 20, 2 and 0.2 g CH$_4$ m$^{-3}$, respectively. Polyhydroxyvalerate (PHV) was also detected at PHB:PHV ratios of up to 12:1 and 4:1 in the cultures enriched at 20 and 0.2 g CH$_4$ m$^{-3}$, respectively.

1. Introduction

Methane (CH$_4$) contributes to approximately 20% of the worldwide greenhouse gas (GHG) emissions and increased its atmospheric concentration by 150% from the pre-industrial era (IPCC 2013). CH$_4$ exhibits a global warming potential 25 times higher than that of CO$_2$ and is mainly emitted from organic waste treatment activities such as landfilling, composting and wastewater treatment (122 million t CO$_2$-eq in the EU-15), coal mining (6 million t CO$_2$-eq in the EU-15) and livestock farming (120 million t CO$_2$-eq in the EU-15) (EEA 2013). Its concentration in anthropogenic emissions varies in the range of 0-0.2 g CH$_4$ m$^{-3}$ for compost piles or livestock farms, and of 20-100 g CH$_4$ m$^{-3}$ for old landfills (Nikiema et al. 2007). Considering the gradual application of restrictive legislations on CH$_4$ emissions, cost-efficient and sustainable methods such as biotechnologies must be properly implemented for an active abatement of CH$_4$ on-site (Copelli et al. 2012). However, conventional biotechnologies such as biofilters or biotrickling filters are prone to CH$_4$ mass transfer limitation from the gas to the liquid phase (and consequently to the microorganisms) due to the low CH$_4$ solubility in water (dimensionless Henry’s law constant of 30). Besides, there is a lack of knowledge on the kinetics and the microbiology involved, especially at the trace level CH$_4$ concentrations (~mg m$^{-3}$) encountered under real applications (López et al. 2013). In this context, microorganisms with high specific biodegradation rates ($q_{\text{max}}$) and affinity towards CH$_4$ (low half-saturation constant, $K_S$) are desirable to guarantee an efficient abatement and a reduction of the start-up period during the implementation of biotechnologies. On the other hand, the potential of methanotrophic bacteria to co-produce high-added value products such as biopolymers (i.e. PHB) could positively impact the economic sustainability of biological CH$_4$ treatment, although this technological alternative has been poorly explored (Zúñiga et al. 2013). The present work evaluated the effect of CH$_4$ concentration on their biodegradation kinetic parameters and population structure. Furthermore, the influence of the CH$_4$...
concentration and the CH4/biomass ratio on the ability to accumulate PHB under sequential nitrogen limitations was assessed.

2. Materials and methods

2.1 Experimental set-up

Three 500-mL jacketed stirred tank (STRs) with mineral salt medium were initially seeded with a mixed inoculum in order to start the experiment with a high diversity of methanotrophs. The inoculum contained fresh aerobic activated sludge from a wastewater treatment plant, soil from an abandoned landfill cover and sludge from an aerobic lagoon stabilizing effluents from a full-scale anaerobic digester (working liquid volume of 400 mL). Diffusers located at the bottom of the reactors 1 (R1), 2 (R2) and 3 (R3) were used to continuously supply CH4 (diluted in air) via aeration (400 mL min⁻¹) at 20 g m⁻³, 2 g m⁻³ and 0.2 g m⁻³, respectively. Figure 1 depicts the set-up and summarizes the key experimental conditions used during microbial enrichment. The reactors were operated under 8 sequential periods of N limitation (48 – 72 h per period) in order to promote the production of PHB in the methanotrophic cultures. The N-NO₃⁻ concentration was restored to 249 ± 65, 48 ± 23, 17 ± 7 mg L⁻¹ in R1, R2 and R3, respectively, after each limitation period. At the end of the 8th N limitation cycle, the influence of the CH4/biomass ratio on microbial PHB accumulation under N limiting conditions was assessed for a period of 18 days by diluting the biomass concentration in R1 and R2 to the levels of R3. Liquid samples were periodically drawn to measure culture absorbance (OD650) and the concentration of dissolved total organic carbon (TOC), total nitrogen (TN) and total suspended solids (TSS). Liquid samples were drawn on weeks 14 and 19 to determine the biodegradation kinetic parameters. At the end of each 3 days nitrogen limitation period, liquid samples were also drawn to quantify bacterial PHB content. CH4 and CO2 gas concentrations were monitored by GC-TCD at the inlet and outlet of the reactors.

2.2 Kinetics of CH4 biodegradation

The determination of the maximum specific CH4 degradation rate qmax (g CH4 g⁻¹ biomass h⁻¹) and the Monod half-saturation constant KS (g m⁻³) in R1, R2 and R3 cultures was carried out in 120-mL bottles containing 20 mL of MSM and biomass at an initial concentration of 51.7 ± 14.7 g biomass m⁻³, which ensured that the kinetic parameters were obtained under non-limiting CH4 mass transfer conditions. CH4 was supplied at initial headspace concentrations of 91.5 ± 3.9 g m⁻³, 17.9 ± 0.8 g m⁻³ and 4.7 ± 0.4 g m⁻³. The bottles were incubated at 25°C under orbital agitation at 150 rpm for 25 h. The concentrations of CH4 and CO2 in the headspace of the bottles were periodically measured by GC-TCD. The Lineweaver-Burk correlation (Eq. 212)
(1) was used to determine the biodegradation kinetic parameters from the initial CH₄ biodegradation rates (Walkiewicz et al., 2012):

\[
\frac{1}{q} = \frac{K_3}{q_{\text{max}}} \cdot \frac{1}{[\text{CH}_4]} + \frac{1}{d_{\text{max}}}
\]

where \( q \) represents the initial CH₄ biodegradation rate (g CH₄ m⁻³ liq h⁻¹) and [CH₄] the CH₄ concentration in aqueous phase (g m⁻³ liq) estimated using the dimensionless Henry's law at 25ºC and 1 atm.

2.3 Molecular biology analysis

To evaluate the richness and composition of the microbial community, biomass samples from the inoculum (A) and from R1, R2 and R3 were collected on week 4 (B, C and D, respectively), 14 (E, F and G, respectively) and 19 (H, I and J, respectively) and stored immediately at –20ºC. DNA extraction, PCR and qPCR amplifications, DGGE analysis, sequencing and DNA sequence analysis were carried out according to Lebrero et al. (2013). The sequences were deposited in GenBank Data Library under accession numbers KF957448 – KF957465. Similarity and Shannon-Wiener diversity (H) indexes were also determined according to Lebrero et al. (2013).

2.4 Measurement of PHB

The quantitative determination of PHB was carried out according to Zúñiga et al. (2011) using chloroform as extraction solvent and a GC-MS for quantification.

3. Results and discussion

3.1 Structure of the enrichment communities

The lowest Shannon-Wiener diversity index was found in the seed (H = 2.6). The samples retrieved from the STRs on week 4 onwards exhibited a higher microbial diversity as a result of the continuous CH₄ supply, although no significant differences were found among communities growing at different CH₄ concentrations (Table 1). In contrast, Estrada et al. (2012) determined for toluene that high concentrations of the pollutant supported lower H indexes, which was attributed to the high toxicity and water solubility of this aromatic compound. The analysis of the pair-wise similarity indexes revealed a low correspondence between the inoculum and the cultures in the three STRs. Hence, similarity coefficients of 8%, 18% and 34% were recorded between the seed and R1, R2 and R3 by week 4, respectively, thus confirming the rapid dynamics of the methanotrophic communities in the STRs. The highest similarity coefficients were obtained between the communities on weeks 14 and 19 (88% in R1, 80% in R2 and 75% in R3), which confirmed the stabilization of the methanotrophic populations from week 14 onward regardless of the CH₄ concentration evaluated. Moreover, the comparison of communities in the STRs by week 19 revealed that cultures enriched at CH₄ concentrations differing in one order of magnitude were more similar (similarities of 51% between R1 and R2, and 66% between R2 and R3) than those enriched at CH₄ concentrations differing in two orders of magnitude (32% between R1 and R3). Thus, these results confirmed the significant influence of CH₄ concentration during culture enrichment on the structure of the microbial populations. DGGE analysis revealed the presence of three different phyla in the STRs (Proteobacteria, Firmicutes, Actinobacteria), with a dominance of type I methanotrophs (Methylosarcina, Methylococcoides, Methylosoma and Methylobacter genera) over the rest of microorganisms (Table 1). Type II methanotrophs (Methylocystis genus) were also present in the three STRs and gradually increased their abundance in R2 and, in a lesser extent, in R3 (samples F, G, I and J). The fact that type II methanotrophs exhibited a low abundance in the three STRs can be attributed to the enrichment of cultures at high Cu²⁺ concentrations. In this context, preliminary quantitative-PCR results revealed the higher expression of particulate methane monooxygenase (pMMO) compared to the type II-specific soluble methane monooxygenases (sMMO), which was predominantly detected in R2 and, in a lesser extent, in R3 (Figure 2). Methylo trophic bacteria belonging to the Methylo bacteriaceae and Hyphomicrobiaceae genera were also significantly detected among the samples, which agreed well with previous findings in biofilters treating CH₄ (Kim et al. 2013). Moreover, bacteria from the Dokdonella, Rhodanobacter, Turicibacter, Acidibacterium and Rhodococcus were initially detected in the STRs and gradually disappeared, likely due to their incapability to consume CH₄ or CH₄-derived metabolites.
Table 1: Microbiological analysis of the enrichment communities in the STRs

<table>
<thead>
<tr>
<th>Source of origin</th>
<th>H index</th>
<th>Significant similarities</th>
<th>Dominant genera (closest relatives in Blast with similarity ≥ 94%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>2.6</td>
<td>34% D, &lt;19% rest</td>
<td>Acidobacterium, Turicibacter, Methylocystis</td>
</tr>
<tr>
<td>B</td>
<td>2.9</td>
<td>52% E, 67% C</td>
<td>Methylomicrobium, Dokdonella, Rhodanobacter, Methylocystis, Methylosoma</td>
</tr>
<tr>
<td>C</td>
<td>3.2</td>
<td>53% F, 37% D</td>
<td>Methylomicrobium, Methylocystis, Dokdonella, Acidobacterium, Rhodococcus</td>
</tr>
<tr>
<td>D</td>
<td>3.1</td>
<td>38% G, 23% B</td>
<td>Methylobacter, Acidobacterium, Turicibacter, Methylocystis</td>
</tr>
<tr>
<td>E</td>
<td>2.4</td>
<td>88% H, 66% F</td>
<td>Methylosarcina, Methylomicrobium, Hyphomicrobium, Methylocystis</td>
</tr>
<tr>
<td>F</td>
<td>3.1</td>
<td>80% I, 46% G</td>
<td>Methylobacter, Methylosoma, Methylocystis, Methylomicrobium, Hyphomicrobium</td>
</tr>
<tr>
<td>G</td>
<td>2.8</td>
<td>75% J, 27% E</td>
<td>Methylocystis, Dokdonella, Hyphomicrobium, Methylomicrobium, Methylosoma</td>
</tr>
<tr>
<td>H</td>
<td>2.9</td>
<td>47% B, 51% I</td>
<td>Methylosarcina, Methylomicrobium, Methylobacillus, Hyphomicrobium</td>
</tr>
<tr>
<td>I</td>
<td>2.7</td>
<td>60% C, 66% J</td>
<td>Methylomicrobium, Methylocystis, Methylosoma, Hyphomicrobium</td>
</tr>
<tr>
<td>J</td>
<td>2.7</td>
<td>29% D, 32% H</td>
<td>Methylocystis, Methylosoma, Hyphomicrobium, Methylomicrobium</td>
</tr>
</tbody>
</table>

Figure 2: sMMO (upper gel) and pMMO (lower gel) expression profiles of the bacterial communities present in the STRs. The size of the amplified fragments and the names of the samples are shown in the left and the upper parts of the gels, respectively.

3.2 Determination of kinetic parameters

Table 2 summarizes the CH₄ biodegradation kinetic parameters qₘₐₓ and Kₛ obtained at weeks 14 and 19. No significant differences were found in terms of maximum specific biodegradation rate between the enrichment cultures of R1 and R3 at week 14, while the community of R2 exhibited the highest qₘₐₓ obtained during the whole experiment (4.8 × 10⁻⁴ ± 0.8 × 10⁻⁴ gCH₄ gbiomass⁻¹ h⁻¹), possibly due to the high biodiversity for both type I and II methanotrophs revealed by the DGGE analysis. The qₘₐₓ values here recorded were higher than those previously reported in literature, which typically ranged from 4.2 × 10⁻⁵ to 1.3 × 10⁻⁴ gCH₄ gbiomass⁻¹ h⁻¹. These differences can be explained by the fact that in the present study biomass concentration was optimized in order to avoid CH₄ mass transfer limitations, thus resulting in a more realistic parameter estimation. The qₘₐₓ values for the communities of R1 and R2 significantly decreased at week 19 compared to those obtained at week 14 (1.1 × 10⁻⁴ ± 0.3 × 10⁻⁴ and 1.9 × 10⁻⁴ ± 0.5 gCH₄ gbiomass⁻¹ h⁻¹, respectively), while qₘₐₓ in R3 remained constant. These results suggested that culture aging negatively influenced the biodegradation capacity of the communities exposed to 20 and 2 gCH₄ m⁻³.
Table 2: Influence of CH₄ concentration during enrichment on the CH₄ biodegradation kinetic parameters \( q_{\text{max}} \) and \( K_S \) at weeks 14 and 19

<table>
<thead>
<tr>
<th>Kinetic parameter</th>
<th>R1 (week 14)</th>
<th>R2 (week 14)</th>
<th>R3 (week 14)</th>
<th>R1 (week 19)</th>
<th>R2 (week 19)</th>
<th>R3 (week 19)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( q_{\text{max}} ) (gCH₄ gbiomass(^{-1}) h(^{-1}))</td>
<td>2.7 ± 0.6 × 10(^{-4})</td>
<td>4.8 ± 0.8 × 10(^{-4})</td>
<td>1.6 ± 0.2 × 10(^{-4})</td>
<td>1.1 ± 0.3 × 10(^{-4})</td>
<td>1.9 ± 0.5 × 10(^{-4})</td>
<td>1.9 ± 0.0 × 10(^{-4})</td>
</tr>
<tr>
<td>( K_S ) (gCH₄ L(^{-1}))</td>
<td>19.2 ± 3.2 × 10(^{-5})</td>
<td>20.8 ± 4.8 × 10(^{-5})</td>
<td>16 ± 6.4 × 10(^{-5})</td>
<td>8 ± 1.6 × 10(^{-5})</td>
<td>25.6 ± 1.28 × 10(^{-5})</td>
<td>8 ± 0.7 × 10(^{-5})</td>
</tr>
</tbody>
</table>

Moreover, the \( K_S \) values of the bacterial communities obtained at week 14 showed no significant differences among the samples (Table 2). In contrast, \( K_S \) values recorded at week 19 significantly decreased in the methanotrophic consortia of R1 and R3 (0.5 × 10\(^{-5}\) ± 0.0 M in both cases), which represents an enrichment of high affinity (low \( K_S \)) microorganisms mediated by the long-term exposure to CH₄. These findings were in agreement with the fact that type I (which often present the highest affinities for CH₄) were dominant over type II in both R1 and R3 during almost the whole enrichment. In this regard, Whalen et al. (1990) reported \( K_S \) values as low as 4 × 10\(^{-5}\) gCH₄ L\(^{-1}\) for type I-methanotroph like cultures, while Delhoménie et al. (2009) obtained higher \( K_S \) values (108.8 × 10\(^{-5}\) – 75.2 × 10\(^{-4}\) gCH₄ L\(^{-1}\)) for type II methanotrophs.

3.3 PHB accumulation

Considering the feasibility of coupling CH₄ abatement to PHA production under nutrient limiting conditions (Wendlandt et al. 2001; Zúñiga et al. 2011), the reactors were operated under 8 sequential N limitations in order to evaluate the influence of different CH₄ concentrations and CH₄/biomass ratios on PHB accumulation by methanotrophic consortia. The PHB cell contents in R1, R2 and R3 following the N limitation periods varied within the ranges 0.3-0.5%, 2.9-9.7% and 0.1-0.8%, respectively (Figure 3). The biopolymer content was low and neither correlated with CH₄ concentration nor with the time course of the enrichment, possibly due to a lack of naturally CH₄ accumulating methanotrophs or a low bioavailability of the C source. The highest PHB cell contents in R1 and R2 were achieved when the CH₄/biomass ratio was increased at the end of the 8th N limitation, likely due to a higher C availability.

Figure 3: Specific PHB content in the biomass enriched in R1 (▲), R2 (♦) and R3 (○) following N limitation.

The differences in PHB cell content among the communities enriched agreed well with the different structure of the methanotrophic communities revealed by the DGGE analysis. In this regard, Pieja et al. (2011) confirmed that only type II methanotrophs exhibit the ability to produce PHB under nutrient limiting conditions. In the present study, the highest PHB contents were achieved in R2, which presented the highest abundance of type II methanotrophs (Methylocystis genera). GC-MS analyses also revealed the
production of PHV in the enrichment cultures. The highest PHV:PHB ratios were obtained in R1 (up to 12:1) and R3 (up to 4:1), which interestingly corresponded to the reactors where the lowest PHB contents were achieved. These results suggested that the low PHB cell contents detected in both reactors could be attributed to the preferential accumulation of PHV by the methanotrophic cultures. In this context, Zúñiga et al. (2013) found maximum PHV:PHB ratios of 7:11 for *Methylobacterium organophilum* in a STR fed with CH₄ and citrate as a co-substrate. However, as far as the authors know, this is the first work on PHA accumulation from CH₄ removal performed in continuous mode without additional carbon sources. Thus, comparisons with previous reports must be carefully done.

4. Conclusions

The pair-wise similarity indexes revealed that CH₄ concentration during enrichment influenced the structure of the bacterial populations. The cultures were characterized by a rapid population dynamics and a high species evenness and richness. Kinetic assays showed that the communities exhibited high specific biodegradation capacity (high q_{max}) and affinity (low Kₛ) for the pollutant regardless of the CH₄ concentration, possibly due to a higher abundance of type I over type II methanotrophs. PHB accumulation was not enhanced by using sequential N limiting conditions and a continuous CH₄-air feed, which was attributed to the low abundance of type II methanotrophs in the STRs. However, it must be stressed that those cultures with the lowest PHB contents presented the highest PHV contents.

References


IPCC (Intergovernmental Panel on Climate Change), 2013, Fifth assessment report: Climate change 2013, the physical science basis <www.climatechange2013.org> accessed 21.01.2014.


