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Prof. Berrin Tansel
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Florida International University, USA

Dear Editor,

Please find enclosed the revised version of the paper "**Assesing the influence of CH$_4$ concentration during culture enrichment on the biodegradation kinetics and population structure**" co-authored by Juan C. López, Guillermo Quijano, Rebeca Pérez and Raúl Muñoz. The manuscript is submitted for publication in Journal of Environmental Management considering that is the best-suited journal for the research area of the present work (subject classification code: 100, Biofiltration), according to the mutually agreement of the four authors. The manuscript contains 5512 words including the main text and references (figures and tables were excluded).

The present work assessed the influence of the concentration of the greenhouse gas CH$_4$ during enrichment on the CH$_4$ biodegradation kinetics, microbial population structure and ability to couple CH$_4$ abatement to polyhydroxyalkanoate (PHA) production. Methanotrophic consortia with high species evenness/richness, as well as high specific biodegradation rates and affinities for CH$_4$ were enriched in the stirred tank reactors regardless of the CH$_4$ concentration. Culture enrichment under sequential N limitations did not promote the accumulation of polyhydroxybutyrate (PHB) in the cultures, with the maximum PHB cell contents achieved under exposure to higher CH$_4$/biomass ratios. Type I methanotrophs were dominant in the cultures enriched at the three concentrations tested, which explains the high CH$_4$ biodegradation potencial of the cultures and their low PHB-accumulating ability. Unexpectedly, the methanotrophic cultures with lower PHB cell contents presented a higher polyhydroxyvalerate (PHV) content.

We are confident this paper will make a very significant contribution to this journal and will attract international attention.

We look forward to your evaluation.
Best regards,

Juan C. López   Raúl Muñoz

Prof. Berrin Tansel
Civil and Environmental Engineering Department
Florida International University, USA

Ms. Ref. No. JEMA-D-14-01208 **"Assessing the influence of CH$_4$ concentration during culture enrichment on the biodegradation kinetics and population structure"** submitted for publication in **Journal of Environmental Management**.

Dear editor,

The authors would like to thank you for the attention given to our manuscript during this peer-review process. The manuscript has been carefully revised and modified in accordance to your requests and most reviewer's recommendations and comments. More specifically:
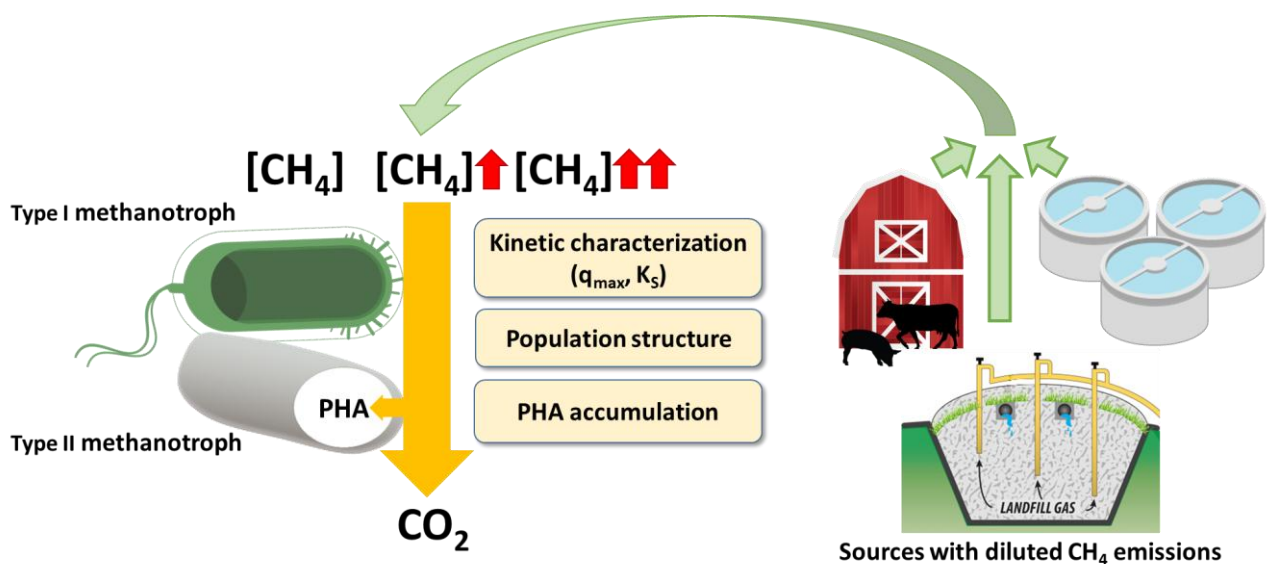
COMMENTS BY THE EDITOR

1. Due to space limitations in the printed journal, we are requesting that all authors reduce the length of their papers by at least 10% if possible. If your paper includes large tables or datasets, it is preferred that these be published as supplementary material in Science Direct rather than in print.

The length of the manuscript was reduced from 6721 to 6003 words in accordance to the Editor's request. Former Table 1 was moved to the Supplementary Material section. Former Table 2 was consequently renamed in the current version of the manuscript as Table 1.

2. When submitting your revised paper, we ask that you include the following items: Manuscript and Figure Source Files (mandatory), Highlights (mandatory), Graphical Abstract (optional).

A graphical abstract was included in the revised version of the manuscript as suggested by the Editor:

Type I methanotroph

Type II methanotroph

$[CH_4]$ $[CH_4]$⬆ $[CH_4]$⬆⬆

Kinetic characterization
(q_{max}, K_S)

Population structure

PHA accumulation

PHA

CO_2

LANDFILL GAS

Sources with diluted CH_4 emissions

*3. Highlights: Please place a period at the end of each sentence.*

A period was placed at the end of each sentence in the Highlights section.

4. References should be prepared according to the author guidelines provided at the journal web site. Please note that journal names should be abbreviated according to the ISI format. Volume numbers should be removed as appropriate. Abbreviated words should have a period. There are some inconsistencies and inaccuracies in punctuation and abbreviations for journal names in the references section. The authors should carefully check the accuracy of the references and ensure that they are prepared according to the JEM style requirements before resubmitting.

The References section was carefully revised and modified according to the author guidelines of Journal of Environmental Management.

5. Fig. 1. Some print size is too small. Fonts and font size used in figures should be legible after 1/4th reduction.

Font sizes of Figure 1 were increased resulting in a legible version for the readers:
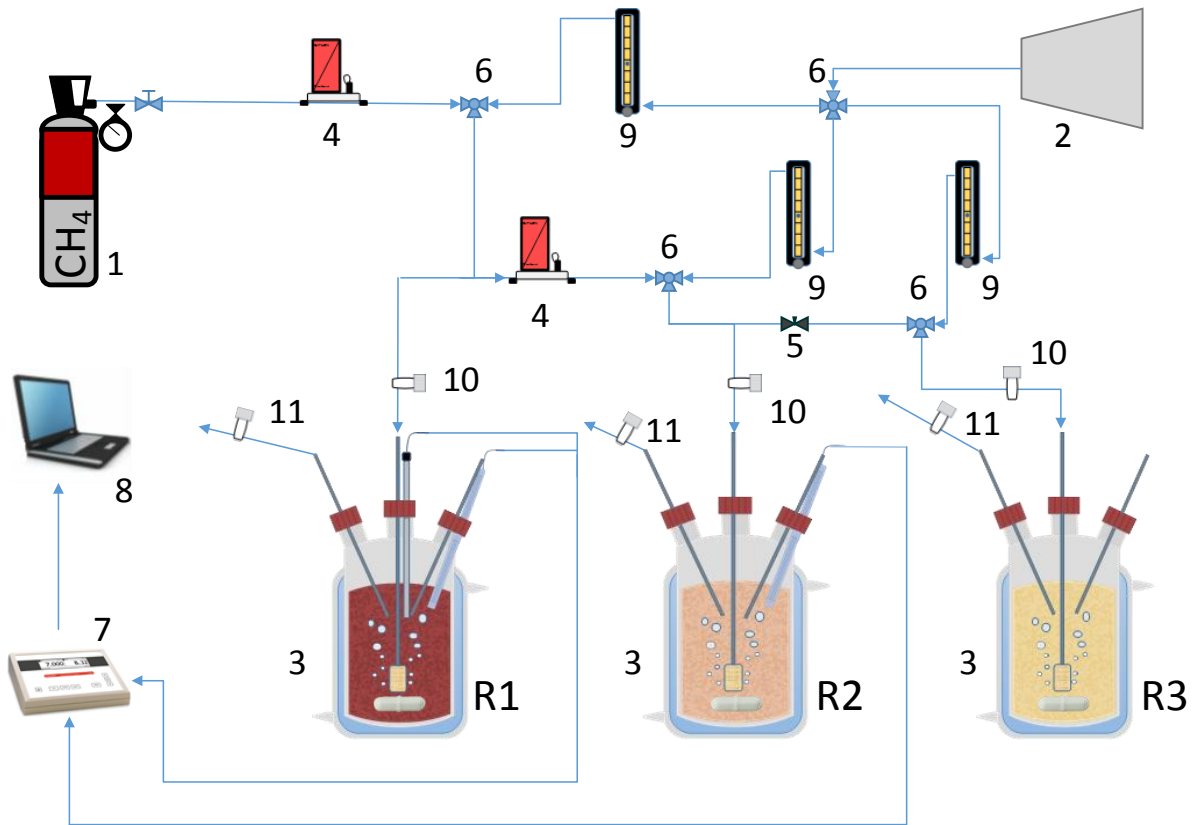
6. Tables should be prepared in 3-line format (if possible using 3 horizontal lines). Lines between the similar rows should be eliminated if they are not providing additional clarification or categorization.

The current Tables were prepared in 3-line format as recommended by the Editor.

REVIEWER 1

1. I am not sure whether all the references are actually referred to.

The match between the references cited in the text and in the References section was carefully revised and modified accordingly. In addition, the reference format was carefully checked and updated according to the JEM style requirements.

2. In the text a list of abbreviations would be helpful.

The authors agree with Reviewer 1 and included a list of abbreviations in the current version of the manuscript (current line 422):

<div style="border:1px solid">

Nomenclature

STR   Stirred tank reactor

VOC   Volatile organic compound

PHB   Poly-3-hydroxybutyrate

PHV   Poly-3-hydroxyvalerate

TSS   Total suspended solids (g L^{-1})

TOC   Total organic carbon (mg L^{-1})

H Shannon-Wiener diversity index (dimensionless)

K_S Half-saturation constant (M)

q_{max} Maximum specific biodegradation rate (gCH_4 $g^{-1}_{biomass}$ h^{-1})

</div>

3. Can you analyse in a paragraph whether the CH_4 levels you established would be typical for any type of wastewater (landfill leachate, water in sewers, …). When would one go for decomposition of CH_4, when for using it, e. g. to provide heat and/or electricity?

Please note that the CH_4 concentrations used in the three STRs (0.2, 2 and 20 g m^{-3}) are in the range of those emissions found in wastewater treatment facilities (0-0.2 g m^{-3}), mines and old landfills (up to 100 g m^{-3}) (former lines 37-40 and 64-66).

On the other hand, the authors agree with the rationale presented by Reviewer 1 regarding the fact that a CH_4 concentration limit for energy production purposes should be established. A threshold value of 400 g m^{-3} was included in current lines 47-50:

"Moreover, the gradual application of the EU landfill Directive 1999/31 will result in emissions with lower CH_4 concentrations, which will significantly restrict the implementation of CH_4 abatement technologies based on energy recovery (applied at CH_4 concentrations higher than ~400 g m^{-3})."

4. What is the realistic perspective for PHB and/or PHV recovery?

The recovery of polyhydroxyalkanoates such as PHB or PHV includes downstream processes (pretreatment, extraction and purification) which account for up to 50% of the overall production costs. A deeper analysis on the economics of PHA production from CH_4 emissions is clearly out of the scope of this work, which was more related to the acquisition of fundamental knowledge. The economics of PHB/PHV recovery should evaluate the feasibility of different downstream processes such as extraction with solvents or enzymes. Please note that no additional discussion was added in the revised version of the paper addressing this issue, since the Editor asked for reducing the length of the manuscript, and this topic was not essential to understand the main outcomes of the study.

5. Rotation speed of stirrer at 250 rpm: is this speed not a bit high? Can you explain?

Please note that the magnetic stirring provided in our setup did not cause a detrimental shear stress in the bacterial communities as the magnetic stir bar was placed at the bottom of the bioreactors (see current Figure 1). Since CH_4 is a poorly water soluble pollutant, this agitation rate was set to improve the gas/liquid mass transfer of CH_4.

6. The conclusions are very brief. Could you include:

- *Whether biodegradation changes depending on the variables under study*

- *What the adaptation of population actually means for capability to biodegrade*

- *What the relation of your findings to environmental management is: regarding climate change, regarding implications for wastewater treatment, etc.*

- *Any conclusions, how PHB, PHV production could increased: like simultaneous extraction of products via membranes, etc.*

- *Any practical or economic potential in applying your findings?*

The authors agree with Reviewer 1 and the Conclusions section was modified accordingly in order to include information about the important topics mentioned above (current lines 400-413):

"The analysis of the pair-wise similarity indexes clearly showed that CH_4 concentration during culture enrichment determined the structure of the microbial populations, which exhibited rapid dynamics and high species evenness and richness. In addition, kinetic assays revealed high specific
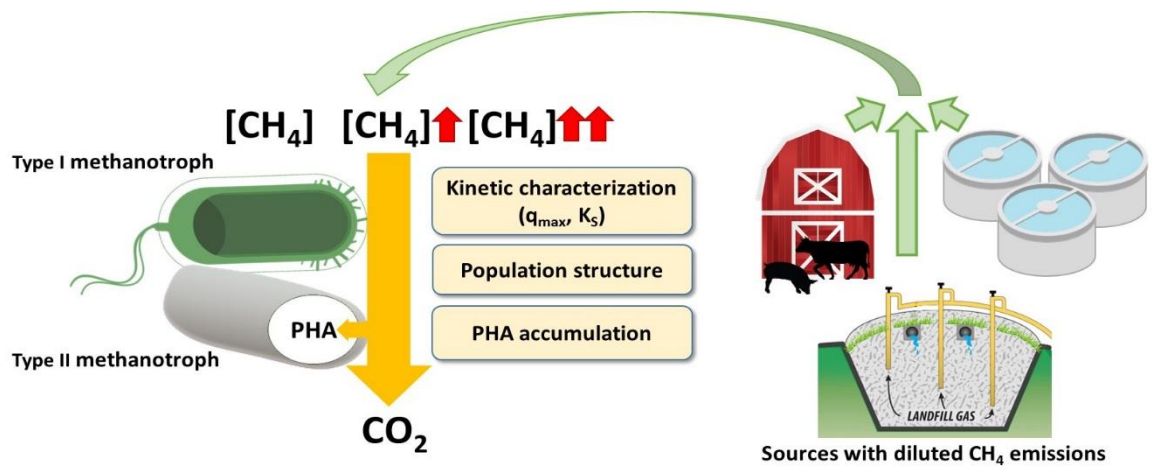
biodegradation capacities and affinities for CH_4, correlated to the dominance of type I/II methanotrophs rather than to CH_4 concentration. On the other hand, the use of sequential N limitations under continuous CH_4-laden air flow did not promote a high PHB accumulation in the cultures likely due to the low abundance of type II methanotrophs. Interestingly, the communities with lower PHB contents exhibited higher PHV contents. These findings brought new insights on the development of specific inocula not only to reduce the start-up period of bioreactors devoted to CH_4 abatement, but also to co-produce high-added value products. Although further experiments on optimizing the conditions for PHB/PHV production are still necessary, the industrial use of these high-added value products will certainly promote the application of biological processes for GHG emissions abatement.

We hope that these modifications will comply with the requests of the editor and the reviewer. Please do not hesitate to contact us at your convenience if you need further information.

Juan Carlos López   **Raúl Muñoz**

Graphical Abstract

Type I methanotroph

Type II methanotroph

$[CH_4]$ $[CH_4]$↑ $[CH_4]$↑↑

Kinetic characterization (q_{max}, K_S)

Population structure

PHA accumulation

PHA

CO_2

LANDFILL GAS

Sources with diluted CH_4 emissions

Highlights

- ✓ The enriched methanotrophic communities exhibited high species evenness and richness.

- ✓ CH_4 concentration significantly influenced the microbial population structure.

- ✓ Dominance of type I methanotrophs regardless of the CH_4 concentration.

- ✓ The enriched methanotrophs exhibited high q_{max} and low K_S values.

- ✓ Maximum PHB cell contents were achieved by increasing CH_4/biomass ratio.

1 **Assessing the influence of CH_4 concentration during culture**

2 **enrichment on the biodegradation kinetics and population structure**

3

4   Juan C. López, Guillermo Quijano, Rebeca Pérez, Raúl Muñoz[*]

5

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9

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11

12   **Abstract**

13   Methanotrophic communities were enriched in three stirred tank reactors continuously

14   supplied with CH_4-laden air at 20, 2 and 0.2 gCH_4 m^{-3} in order to evaluate the influence

15   of CH_4 concentration on the biodegradation kinetics, population structure and potential

16   polyhydroxyalkanoate production under sequential nitrogen limitations. The population

17   structure of the enriched cultures, dominated by type I methanotrophs, was influenced

18   by CH_4 concentration. No significant correlation between CH_4 concentration and the

19   maximum specific degradation rate (q_{max}) or the half-saturation constant (K_S) was

20   recorded, microorganisms enriched at 2 gCH_4 m^{-3} presenting the highest q_{max} and those

21   enriched at 20 and 0.2 gCH_4 m^{-3} exhibiting the lowest K_S. Maximum

22   polyhydroxybutyrate contents of 1.0% and 12.6% (*w/w*) were achieved at 20 and 2 g

23   CH_4 m^{-3}, respectively. Polyhydroxyvalerate was also detected at PHV:PHB ratios of up

24   to 12:1 and 4:1 in the communities enriched at 20 and 0.2 gCH_4 m^{-3}, respectively.

25

1. Introduction

Methane (CH_4) contributes to approximately 20% of the worldwide greenhouse gas (GHG) emissions, with an atmospheric concentration increase of 150% from the pre-industrial era to 2011 (EPA, 2013; IPCC, 2013). CH_4 presents a global warming potential 25 times higher than that of CO_2 (excluding additional harmful effects of water vapour production from CH_4 breakdown) and is mainly emitted from organic waste treatment activities such as landfilling, composting and wastewater treatment (122 million tn CO_2-eq in the EU-15), coal mining (6 million tn CO_2-eq in the EU-15) and livestock farming (120 million tn CO_2-eq in the EU-15) (EEA, 2013). CH_4 concentration in anthropogenic emissions greatly varies from $0 - 0.2$ gCH_4 m^{-3} in compost piles or livestock farms up to $20 - 100$ gCH_4 m^{-3} in old landfills (Nikiema et al., 2007).

Based on the urgent need to limit the increase in the global average temperature to a maximum of 2ºC 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) (EEA, 2013; IPCC, 2013). In this context, apart from the actions oriented to reduce CO_2 emissions from fossil fuel combustion, additional measurements such as an active CH_4 abatement must be considered in order to achieve these target emission cuts. Moreover, the gradual application of the EU landfill Directive 1999/31 will result in emissions with lower CH_4 concentrations, which will significantly restrict the implementation of CH_4 abatement technologies based on energy recovery (applied at CH_4 concentrations higher than ~400 g m^{-3}). Therefore, there is an urgent need to

2

51 develop cost-efficient and sustainable technologies for the active abatement of CH_4

52 diluted emissions. Biotechnologies could become, if properly tailored, a platform

53 technology for the abatement of diluted CH_4 emissions based on their proven robustness

54 and cost-effectiveness for the treatment of malodours or industrial VOC emissions

55 (Estrada et al., 2012b; López et al., 2013).

56 However, despite the fact that methanotroph-based technologies such as biofiltration or

57 biotrickling filtration have been implemented over the past 40 years for the active

58 abatement of CH_4, the performance of such conventional biotechnologies is still limited

59 by the low CH_4 mass transfer rates from the gas phase to the microorganisms and by the

60 insufficient knowledge on the microbiology underlying CH_4 oxidation (López et al.,

61 2013; Yoon et al., 2009). In this regard, microorganisms with high specific oxidation

62 rates (q_{max}) and a high affinity for CH_4 (low half-saturation constant, K_S) are desirable

63 to guarantee an efficient biocatalytic activity during the treatment of diluted CH_4

64 emissions and to reduce the start-up period of bioreactors. However, CH_4

65 biodegradation kinetic studies under non-mass transfer limiting conditions are scarce,

66 especially at the trace level CH_4 concentrations ($\sim$mg m^{-3}) often encountered under real

67 case applications (Estrada et al., 2012a; López et al., 2013). On the other hand, the

68 economic sustainability of biological CH_4 oxidation processes, often compromised by

69 the high gas residence time required to overcome mass transfer limitations, can be

70 positively impacted by the co-production of high-added value products such as

71 biopolymers (i.e. poly-3-hydroxybutyrate, PHB) (Zúñiga et al., 2011). Unfortunately,

72 the potential of methanotrophic communities to accumulate polyhydroxyalkanoates

73 during the continuous biodegradation of CH_4 at trace level concentrations has been

74 poorly explored.

3

75 This study evaluated the influence of CH_4 concentration during methanotrophic

76 community enrichment on biodegradation kinetic parameters and population structure.

77 Moreover, the influence of CH_4 concentration and the CH_4/biomass ratio on the ability

78 to accumulate PHB under nitrogen limiting scenarios was also assessed.

79

2. Materials and methods

81 *2.1. Chemicals and mineral salt medium*

82 Methane was purchased from Abelló Linde S.A. (Barcelona, Spain) with a purity of at

83 least 99.5%. Poly-3-hydroxybutyrate, chloroform (> 99.5%), phosphotungstic acid

84 solution 10% (*w/v*), uranyl acetate dihydrate ($\geq$ 98%), propylene oxide (> 99%) and

85 benzoic acid (> 99.5%) were obtained from Sigma-Aldrich® (Sigma-Aldrich, St. Louis,

86 MO, USA). Osmium tetroxide was obtained from EMS with a purity of at least 99.95%

87 (Hatfield, USA). Lead nitrate and sodium citrate were purchased from Merck

88 (Darmstadt, Germany). The Spurr resin kit TK4 4221D-1 was obtained from TAAB

89 Laboratories Equipment Ltd. (Aldermaston, England). Paraformaldehyde and ethanol

90 (96%) were purchased from AppliChem (Darmstadt, Germany). The rest of reagents

91 and chemicals were purchased from Panreac® (Barcelona, Spain) with a purity of at

92 least 99%.

93 The mineral salt medium (MSM) used for microbial enrichment and the in-vitro kinetic

94 assays was composed of (in g L^{-1}): $Na_2HPO_4 \cdot 12H_2O$ 6.15, KH_2PO_4 1.52, $MgSO_4 \cdot 7H_2O$

95 0.2, $CaCl_2 \cdot 2H_2O$ 0.0503, $NaNO_3$ 1.32 and 10 mL L^{-1} of SL4 trace element solution

96 (containing per liter: EDTA 0.5 g, $FeSO_4 \cdot 7H_2O$ 0.2 g, $ZnSO_4 \cdot 7H_2O$ 0.01 g,

97 $MnCl_2 \cdot 4H_2O$ 0.003 g, H_3BO_3 0.03 g, $CoCl_2$ 0.011 g, $CuCl_2 \cdot 2H_2O$ 0.443 g, $NiCl_2 \cdot 6H_2O$

98 0.002 g, $Na_2MoO_4 \cdot 2H_2O$ 0.003 g).

99

4

2.2. Inoculum and cultivation conditions

101 Fresh aerobic activated sludge from Valladolid wastewater treatment plant (Valladolid,

102 Spain), soil from the cover of an abandoned landfill (Almazán, Spain) and sludge from

103 an aerobic lagoon stabilizing the effluents from a full-scale anaerobic digester treating

104 swine manure (Almazán, Spain) were used as inoculum for the enrichment of

105 methanotrophs. Aliquots of the 3 microbial sources were equally mixed (on a volume

106 basis), diluted in MSM in a 1:18 ratio and then incubated at 25ºC and 150 rpm for 1 h in

107 a rotary shaker.

108

109 *2.3. Experimental set-up and operation mode*

110 Three 500 mL jacketed stirred tank reactors (STRs) (Afora S.A., Spain) initially

111 containing 380 mL of MSM were inoculated with 20 mL of the mixed inoculum above

112 described. The cultivation broth was maintained at 25ºC and magnetically agitated at

113 250 rpm with a stir bar placed at the bottom of each STR. Inert polyurethane polymers

114 (0.92 g) were introduced in each reactor in order to prevent the formation of biofilm

115 onto the reactor walls, thus avoiding the underestimation of biomass concentration. CH_4

116 was continuously supplied via aeration (400 mL min^{-1}) at 20 g m^{-3}, 2 g m^{-3} and 0.2 g m^{-3}

117 into reactors 1 (R1), 2 (R2) and 3 (R3), respectively, using 10 μm porous stainless steel

118 diffusers located at the bottom of the reactors. The concentrations of CH_4 were regulated

119 via mass flow controllers (Aalborg^{TM}, USA) by mixing an air stream with either pure

120 methane or serial dilutions of CH_4-laden air streams (Fig. 1). The pH of the enrichment

121 broths was maintained at 7.2 ± 0.2 by periodic addition of HCl (0.2 M). Distilled water

122 was added every two days to compensate for water losses by evaporation. Double

123 concentrated MSM without nitrogen was also added to compensate for sampling losses

124 and to provide enough nutrients for microbial growth. The enrichment of potential

125 PHB-accumulating methanotrophs was performed by operating the reactors under 8

126 sequential periods of N limitation (48 – 72 h per period) within the entire 310-days

127 experimentation period. $N-NO_3^-$ concentration was restored at 248.8 ± 65.3, 48.1 ± 22.9,

128 17.2 ± 6.9 mg L^{-1} in R1, R2 and R3, respectively, after each limitation period. At the

129 end of the 8th N limitation cycle, the influence of the CH_4/biomass ratio on microbial

130 PHB accumulation under N limiting conditions was assessed for a period of 18 days by

131 diluting the biomass concentration in R1 and R2 to the levels of R3.

132 *<Figure 1>*

133 Liquid samples (3 mL) were periodically drawn from the reactors to determine the

134 concentration of biomass via culture absorbance measurements (OD_{650}), dissolved total

135 organic carbon (TOC) and total nitrogen (TN). Additionally, 15 mL liquid samples were

136 drawn on days $95 - 100$ (week 14) and $130 - 135$ (week 19) to determine the CH_4

137 biodegradation kinetic parameters and periodically monitor the total suspended solid

138 concentration (TSS). Liquid samples of 3 mL were also drawn to quantify the bacterial

139 PHB content and to confirm PHB accumulation by transmission electron microscopy at

140 the end of each 3 days nitrogen limitation period. Liquid samples were also taken on

141 days 28 (week 4), 95 (week 14) and 130 (week 19) to determine the dynamics of

142 microbial population structure by denaturing gradient gel electrophoresis (DGGE). CH_4

143 and CO_2 gas concentrations were monitored by GC-TCD at the inlet and outlet of the

144 reactors.

145

146 *2.4. Kinetics of CH$_4$ biodegradation*

147 The maximum specific CH_4 biodegradation rate q_{max} (gCH$_4$ g$^{-1}_{biomass}$ h^{-1}) and the Monod

148 half-saturation constant K_S (g m^{-3}) under non-limiting N conditions were determined for

149 R1, R2 and R3 cultures on days $95 - 100$ (week 14) and $130 - 135$ (week 19) in order to

6

150   record the dynamics of CH_4 biodegradation kinetics. These assays were conducted in

151   120-mL glass bottles containing 20 mL of MSM and inoculated with fresh biomass at

152   an initial concentration of 51.7 ± 14.7 $g_{biomass}$ m^{-3}, which ensured that the kinetic

153   parameters were obtained under non-limiting mass transfer conditions (according to

154   preliminary tests). The bottles were closed with butyl septa, sealed with aluminum caps

155   and supplied with CH_4 at initial headspace concentrations of 91.5 ± 3.9 g m^{-3}, 17.9 ± 0.8

156   g m^{-3} and 4.7 ± 0.4 g m^{-3} (corresponding to initial methane aqueous concentrations of

157   3.1 ± 0.1 g m^{-3}, 0.61 ± 0.1 g m^{-3} and 0.16 ± 0.1 g m^{-3}, respectively). The bottles were

158   incubated at 25ºC and 150 rpm for 25 h. The concentrations of CH_4 and CO_2 in the

159   headspace of the bottles were periodically measured by GC-TCD. The Lineweaver-

160   Burk correlation (Equation 1) was used to determine the biodegradation kinetic

161   parameters from the initial CH_4 biodegradation rates (Walkiewicz et al., 2012):

162

163     $1/q = K_S/q_{max} \times 1/[CH_4] + 1/q_{max}$ (1)

164

165   where q represents the initial CH_4 biodegradation rate (g CH_4 m^{-3}_{liq} h^{-1}) and $[CH_4]$ the

166   methane concentration in the aqueous phase (g m^{-3}_{liq}) estimated using the dimensionless

167   Henry's law constant at 25ºC and 1 atm (29.4).

168

169   *2.5. Molecular biology analysis*

170   To evaluate the richness and composition of the microbial community, biomass samples

171   from the inoculum (A) and from R1, R2 and R3 were collected on week 4 (B, C and D,

172   respectively), 14 (E, F and G, respectively) and 19 (H, I and J, respectively) and stored

173   immediately at -20ºC. The procedures of the DNA extraction, PCR amplification,

174 DGGE analysis, sequencing and DNA sequence analysis can be found in the

175 Supplementary Data Annex.

176

2.6. Electron microscopy analysis

178 Liquid samples of 1 mL drawn from the STRs at the end of the 3rd limitation period

179 were centrifuged at 4000 rpm and 4ºC for 5 min. Subsequent biomass fixation,

180 dehydration and embedding were carried out according to Bozzola (2007). The samples

181 were finally cutted and contrasted according to Wendlandt et al. (2001). A TEM JEOL

182 JEM-1011 electron microscope (Teknolab, Indonesia) with an ES1000W Erlangshen

183 CCD camera (Gatan, Germany) was used for the analysis.

184

2.7. Measurement of PHB

186 The quantitative determination of the cellular PHB content was carried out according to

187 Zúñiga et al. (2011) using chloroform as extraction solvent.

188

2.8. Analytical procedures

190 CH_4 and CO_2 gas concentrations were determined in a Bruker 430 GC-TCD (Palo Alto,

191 USA) equipped with a CP-Molsieve 5A (15 m $\times$ 0.53 µm $\times$ 15 µm) and a CP-

192 PoraBOND Q (25 m $\times$ 0.53 µm $\times$ 10 µm) columns. The oven, injector and detector

193 temperatures were maintained at 45ºC, 150ºC and 200ºC, respectively. Helium was used

194 as the carrier gas at 13.7 mL min^{-1}. Samples for the determination of TOC/TN

195 concentrations were filtered through 0.22 µm glass fiber filters (Merck Millipore, USA)

196 prior to analysis in a TOC-VCSH analyzer (Shimadzu, Japan) coupled with a

197 chemiluminescense detection TN module (TNM-1) (Shimadzu, Japan). Culture

198 absorbance measurements at 650 nm were performed using a Shimadzu UV-2550

199 UV/Vis spectrophotometer (Shimadzu, Japan). The determination of TSS concentration

200 was performed according to standard methods (APHA, 2005). Temperature and pH

201 were on-line monitored using a multiparametric analyser C-3020 (Consort, Belgium).

202 PHB concentration was quantified in an Agilent 6890N GC-MS equipped with a DB-

203 WAX column (30 m × 0.250 mm × 0.25 μm) (J&W Scientific®, CA, USA). The

204 injector temperature was set at 250ºC. The oven temperature was initially maintained at

205 40ºC for 5 min, increased at 10ºC min^{-1} up to 200ºC, then at 5ºC min^{-1} up to 240ºC and

206 finally maintained at 240ºC for 2 min.

207

208 3. Results and discussion

209 *3.1. Methane biodegradation performance during culture enrichment*

210 CH_4 biodegradation in the three enrichment STRs was indirectly assessed by CO_2

211 production and biomass growth rather than by CH_4 consumption based on the higher

212 reliability of CO_2 measurements and the low CH_4 removal rates achieved. Since the

213 reactors were not designed to maximize CH_4 abatement, the removal rates obtained

214 were lower than the average error of the GC-FID method. In this context, a rapid CH_4

215 oxidation was recorded at the highest CH_4 concentration in R1 from the second day of

216 operation, with an increase in CO_2 production up to 104 g m^{-3} h^{-1} concomitant with a

217 rise in biomass concentration up to 4 g L^{-1} (Fig. 2a, b). However, CH_4 mineralization

218 progressively decreased to average values of 60 gCO_2 m^{-3} h^{-1} from day 40 to 60 and

219 fluctuated at 42.3 ± 17 g m^{-3} h^{-1} from day 60 onwards. Biomass concentration stabilized

220 at 4 g L^{-1} from days 33 to 45 and at 6 g L^{-1} from days 75 to 125 likely due to the

221 accumulation of both metabolites and cell lysis products in the cultivation broth. The

222 complete renewal of MSM in R1 (prior biomass centrifugation) by days 45 and 125

restored TOC concentrations at $110 - 120$ mgC L^{-1} and supported further biomass

224 growth (Fig. 2c). The CO_2 production rates in R2 and R3 underwent less variations than

225 those recorded in R1, with average values of 7.9 and 3.7 g m^{-3} h^{-1}, respectively (Fig 2a).

226 These lower CO_2 production rates were attributed to the lower CH_4 concentration

227 gradients and therefore mass transfer rates at 2 and 0.2 g m^{-3}, compared to R1. Biomass

228 growth in R2 and R3 was also significantly lower than that recorded in R1 as a result of

229 the lower CH_4 loading rates (10 and 100 times lower, respectively). Maximum biomass

230 concentrations of 2.1 and 0.7 g L^{-1} were achieved, respectively, in R2 and R3 by days

231 113 and 180, to finally decrease to 1.2 and 0.2 g L^{-1}, respectively. Likewise, TOC

232 concentrations in both reactors remained constant at ~40 mg L^{-1}, which suggests that the

233 accumulation of metabolites or cell lysis products in R2 and R3 was not significant

234 during the experimentation period (Fig. 2c). The CO_2 production rates recorded in R1

235 and R2 were similar to those reported by Rocha-Rios et al. (2010, 2009) in STRs at CH_4

236 loading rates of approximately 65 and 210 g m^{-3} h^{-1} (10 and 80 g CO_2 m^{-3} h^{-1},

237 respectively).

238 *<Figure 2>*

239

240 *3.2. Structure of the enriched communities*

241 The Shannon-Wiener diversity index (*H*) ranges typically from 1.5 to 3.5 and accounts

242 for both the number (richness) and the eveness of the species (evaluating and comparing

243 the intensity of the bands), thus allowing to obtain semi-quantitative results from the

244 DGGE analysis (McDonald, 2003). In our particular case, the complex inoculum

245 exhibited the lowest species evenness and richness among the samples analyzed as

246 demonstrated by its low *H* of 2.6. Despite the increase in microbial diversity of the

247 communities at week 4 in R1, R2 and R3 (*H* of 2.9, 3.2 and 3.1, respectively), culture

248 aging mediated a lower biodiversity at week 14 as confirmed by the decrease in H

249 values to 2.4, 3.1 and 2.8 in R1, R2 and R3, respectively. Further culture aging resulted

250 in the stabilization in microbial diversity by week 19 in R2 and R3 at H of 2.7, and at H

251 of 2.9 in R1. It is noteworthy that these H values were achieved using CH_4 as the sole C

252 and energy source and were not significantly influenced by CH_4 concentration. In

253 contrast, Estrada et al. (2012a) observed that high toluene concentrations supported

254 lower biodiversity indexes, which was attributed to the high toxicity of the model VOC

255 used in the study.

256 *<Figure 3>*

257 The analysis of the pair-wise similarity indexes revealed a low correspondence between

258 the inoculum and the cultures in the three STRs, even by week 4 (Fig. 4a). Hence,

259 similarity coefficients of 8%, 18% and 34% were recorded between the seed and the

260 cultures R1, R2 and R3 by week 4, respectively (Fig. 4b). These results confirmed the

261 rapid dynamics of the methanotrophic communities in the STRs and agreed well with

262 the differences observed among the Shannon-Wiener diversity indexes. The highest

263 similarities in the phylogenetic composition of the communities were obtained between

264 the communities on weeks 14 and 19 (88% in R1, 80% in R2 and 75% in R3) (Fig. 4a,

265 b). These empirical findings confirmed the stabilization of the methanotrophic

266 populations from week 14 onward regardless of the CH_4 concentration evaluated.

267 Moreover, the comparison of communities in the STRs by week 19 revealed that

268 cultures enriched at CH_4 concentrations differing in one order of magnitude were more

269 similar (similarities of 51% between R1 and R2, and 66% between R2 and R3) than

270 those enriched at CH_4 concentrations differing in two orders of magnitude (32%

271 between R1 and R3) (Fig. 4b). Thus, these results confirmed the significant influence of

11

272 CH$_4$ concentration during culture enrichment on the structure of the microbial

273 populations.

274 *<Figure 4>*

275 Three different phyla were retrieved according to the RDP classifier tool among the 18

276 bands sequenced from the DGGE gel (Fig. 3): *Proteobacteria* (15 bands), *Firmicutes* (1

277 band) and *Actinobacteria* (1 band), while the sequence of the last band remained

278 unclassified. The closest matches for each bacterial sequence from the NCBI database

279 are provided in the Supplementary Data Annex (Table 1) with indication of the

280 similarity percentages and sources of origin. In addition, the presence and relative

281 abundance of each band within the samples analyzed are also shown in the

282 Supplementary Data Annex (Table 1). Most bands were affiliated to the phylum

283 *Proteobacteria* and more specifically to *Alphaproteobacteria*, *Betaproteobacteria* and

284 *Gammaproteobacteria* classes. Despite CH$_4$-oxidizing bacteria belonging to type I

285 methanotrophs (*Methylosarcina*, *Methylomicrobium, Methylosoma* and *Methylobacter*

286 genera) were detected in the three STRs along the entire enrichment, type I

287 methanotrophs were more abundant in R1 and R2 (samples B, C, E, F, H and I) and

288 their abundance gradually deteriorated over time. Type II methanotrophs (*Methylocystis*

289 genus) were also present in the three STRs and gradually increased their abundance in

290 R2 and, in a lesser extent, in R3 (samples F, G, I and J). The fact that type II

291 methanotrophs exhibited a lower abundance than type I methanotrophs in the STRs can

292 be attributed to the enrichment of cultures at high Cu^{2+} concentrations. In this context,

293 preliminary quantitative-PCR results revealed the higher expression of particulate

294 methane monooxygenases (pMMO) compared to the type II-specific soluble methane

295 monooxygenases (sMMO), which also supported the predominance of type I

296 methanotrophs along the entire enrichment in the STRs (data not shown). Most

12

297   methanotrophic genera here described were previously identified in CH_4 abatement

298   bioreactors (Gebert et al., 2008; Veillette et al., 2011), which confirmed their ability to

299   degrade CH_4. Thus, the methylotrophic *Methylobacillus* and *Hyphomicrobium* genera

300   have been detected in sewers and biofilters treating CH_4, respectively (Chistoserdova et

301   al., 2007; Kim et al., 2013). In our particular study, the *Hyphomicrobium* genus (DGGE

302   band 15) was significantly present in almost all samples analyzed, while bacteria from

303   the *Methylobacillus* genus (DGGE band 12) were only found in R1 by week 19 (sample

304   H). Despite members of the *Dokdonella*, *Rhodanobacter*, *Turicibacter* and

305   *Rhodococcus* genera (DGGE fragments 10, 11, 16 and 17, respectively) were also

306   detected by week 4 in the three STRs, these microorganisms gradually disappeared

307   likely due to their incapability to assimilate CH_4, CH_4-derived metabolites or cell lysis

308   products. Bacteria from the *Rhodanobacter* genus were previously detected in a CH_4

309   abatement bioreactor, but their role in this particular microbial community was not

310   clearly identified (Veillette et al., 2011).

311

312   *3.3. Determination of kinetic parameters*

313   The highest q_{max} obtained from the Lineweaver-Burk linearization ($4.8 \times 10^{-4} \pm 8.1 \times$

314   10^{-5} gCH_4 $g_{biomass}^{-1}$ h^{-1}) was recorded at week 14 in the communities enriched in R2,

315   which was attributed to the high biodiversity encountered both for type I and II

316   methanotrophic bacteria. No significant differences were observed between the

317   communities of R1 and R3 in terms of q_{max} at week 14, with values of $2.7 \times 10^{-4} \pm 5.6 \times$

318   10^{-5} and $1.6 \times 10^{-4} \pm 1.8 \times 10^{-5}$ gCH_4 $g_{biomass}^{-1}$ h^{-1}, respectively (Fig. 5a). The q_{max} values

319   determined at week 14 in the three microbial communities were higher than those

320   previously reported in the literature, which typically ranged from 4.2×10^{-5} to 1.3×10^{-4}

321   gCH_4 $g_{biomass}^{-1}$ h^{-1} (Bender and Conrad, 1992; Gebert et al., 2003). These findings can be

322 explained by the fact that biomass concentration was optimized in the biodegradation

323 assays in order to avoid CH_4 mass transfer limiting conditions, resulting in more

324 realistic kinetic parameters. On the contrary, most kinetic studies reported for

325 methanotrophs were carried out at high biomass concentration, which did not ensure the

326 absence of mass transport limitations and, therefore, the validity of the kinetic

327 parameters obtained. At week 19, the communities of R1 and R2 exhibited lower q_{max}

328 compared to week 14 ($1.1 \times 10^{-4} \pm 3.1 \times 10^{-5}$ and $1.9 \times 10^{-4} \pm 5.4 \times 10^{-5}$ gCH_4 $g_{biomass}^{-1}$

329 h^{-1}, respectively), while q_{max} in the community of R3 remained similar. These findings

330 suggested that culture aging negatively affected the specific CH_4 biodegradation rate of

331 the microbial communities exposed to the two highest CH_4 concentrations likely due to

332 the presence of a higher inert biomass fraction.

333 *<Figure 5>*

334 On the other hand, no significant differences in the K_S of the microbial communities

335 enriched in the three STRs were recorded at week 14, exhibiting values of $1.2 \times 10^{-5} \pm$

336 1.7×10^{-6} M (Fig. 5b). K_S values at week 19 significantly decreased in the communities

337 enriched in R1 and R3 ($5.2 \times 10^{-6} \pm 1.4 \times 10^{-6}$ M and $4.8 \times 10^{-6} \pm 5.3 \times 10^{-7}$ M,

338 respectively) and remained constant in R2 ($1.6 \times 10^{-5} \pm 8 \times 10^{-7}$ M). The results here

339 obtained indicated that long-term culture exposure to CH_4 would promote the

340 enrichment of high affinity (low K_S) microorganisms, although no significant influence

341 of CH_4 concentration on K_S was observed. This empirical finding was in agreement

342 with the fact that type I (which exhibit the highest affinities for CH_4) rather than type II

343 methanotrophs were dominant in both R1 and R3. Whalen et al. (1990) reported K_S

344 values for type I methanotroph-like cultures isolated from landfill cover soils as low as

345 2.5×10^{-6} M under gas CH_4 concentrations of $1 - 1.7$ g m^{-3}, which are comparable to

346 those recorded at week 19 in R1 and R3. In contrast, K_S values of $6.8 \times 10^{-5} - 4.7 \times 10^{-4}$

14

347 M are typically reported in literature for type II methanotrophs (Delhoménie et al., 2009;

348 Hornibrook et al., 2009).

349

350 *3.4. PHB accumulation*

351 PHB was present as refractive inclusions or granules inside the methanotrophic cells,

352 which were identified in the enriched cultures by their intracytoplasmatic membranes

353 (Fig. 6). The transmission electron micrographs confirmed the microbiological

354 feasibility of coupling CH_4 abatement with the production of an added value product

355 such as PHB, which could significantly contribute to improve the economic viability of

356 the process. Polyhydroxyalkanoate accumulation in methanotrophs can be induced

357 under excess of C source and limitation in the availability of nutrients such as N, P or

358 Mg (Asenjo and Suck, 1986), N limitation being the best scenario for PHB

359 accumulation according to Wendlandt et al. (2001). Hence, the enrichment of methane-

360 oxidizing bacteria capable of accumulating PHB was performed by operating the

361 reactors under 8 sequential periods of N limitation. To the best of our knowledge, this is

362 the first systematic study assessing the influence of different CH_4 concentrations and

363 CH_4/biomass ratios on PHB accumulation by methanotrophic consortia.

364 *<Figure 6>*

365 PHB cell contents of 0.3 – 0.5% (*w/w*), 2.9 – 9.7% (*w/w*) and 0.1 – 0.8% (*w/w*) were

366 recorded following the N limitation periods in R1, R2 and R3, respectively (Table 1).

367 Despite sequential N limitations were expected to induce an increasing PHB

368 accumulation, the biopolymer content was low and neither correlated with CH_4

369 concentration nor with the time course of the enrichment. Similar results were obtained

370 by Pieja et al. (2012) in batch reactors subjected to sequential N limitations, although

15

371 sequential CH_4 and N limitations indeed promoted PHB accumulation up to 24% (*w/w*).

372 These results, compared to our particular study, were likely due to the use of the PHB-

373 producer *Methylocystis parvus OBBP* and the use of C and N feast-famine strategies.

374 Methanotroph cultivation at higher CH_4/biomass ratios following the 8th N limitation

375 episode initially resulted in the highest PHB cell contents in both R1 and R2 (1% and

376 12.6%, respectively), likely due to the higher bioavailability of the C source.

377 *<Table 1>*

378 The differences in the PHB cell content of the communities enriched in the reactors at

379 the different CH_4 concentrations can be explained by the different structure of these

380 methanotrophic communities revealed by DGGE analysis (Fig. 3; Supplementary Data

381 Annex). The key role of the type of methanotrophs on the ability to accumulate PHB

382 was recently highlighted by Pieja et al. (2011), who suggested that only type II

383 methanotrophs exhibited the ability to accumulate PHB under N limiting conditions. In

384 our particular case, the highest abundance of type II methanotrophs (*Methylocystis*

385 genus) was found in R2, which also corresponded to the community with the highest

386 PHB contents.

387 GC-MS analyses revealed also the accumulation of poly-3-hydroxyvalerate (PHV) in

388 the cultures enriched (Table 1). The highest PHV:PHB ratios were found in R1 (up to

389 12:1) and R3 (up to 4:1), which corresponded to the communities with the lowest PHB

390 contents. The low PHB cell contents detected in both reactors could be attributed to the

391 preferential microbial accumulation of PHV. In this regard, Zúñiga et al. (2013)

392 reported maximum PHV:PHB ratios of 7:11 in a STR fed with CH_4 and citrate as

393 carbon sources and highlighted that the co-production of polyhydroxyalkanoates such as

394 PHV together with PHB can improve the mechanical properties and biodegradability of

16

395 the composite biopolymers. In our particular study, the different PHV:PHB ratios

396 obtained could be attributed to the use of consortia and CH_4 instead of pure cultures and

397 CH_4/cosubstrates, as reported by Zúñiga et al. (2013).

398

4. Conclusions

400 The analysis of the pair-wise similarity indexes clearly showed that CH_4 concentration

401 during culture enrichment determined the structure of the microbial populations, which

402 exhibited rapid dynamics and high species evenness and richness. In addition, kinetic

403 assays revealed high specific biodegradation capacities and affinities for CH_4, correlated

404 to the dominance of type I/II methanotrophs rather than to CH_4 concentration. On the

405 other hand, the use of sequential N limitations under continuous CH_4-laden air flow did

406 not promote a high PHB accumulation in the cultures likely due to the low abundance of

407 type II methanotrophs. Interestingly, the communities with lower PHB contents

408 exhibited higher PHV contents. These findings brought new insights on the

409 development of specific inocula not only to reduce the start-up period of bioreactors

410 devoted to CH_4 abatement, but also to co-produce high-added value products. Although

411 further experiments on optimizing the conditions for PHB/PHV production are still

412 necessary, the industrial use of these high-added value products will certainly promote

413 the application of biological processes for GHG emissions abatement.

414

17

419 Valladolid) in the transmission electronic microscopy analyses are also gratefully

420 acknowledged.

421

Nomenclature

STR Stirred tank reactor

VOC Volatile organic compound

PHB Poly-3-hydroxybutyrate

PHV Poly-3-hydroxyvalerate

TSS Total suspended solids ($g\ L^{-1}$)

TOC Total organic carbon ($mg\ L^{-1}$)

H Shannon-Wiener diversity index (dimensionless)

K_S Half-saturation constant (M)

q_{max} Maximum specific biodegradation rate ($gCH_4\ g^{-1}_{biomass}\ h^{-1}$)

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23

522 **Figure 1.** Schematic representation of the experimental set-up. *1* CH_4 gas cylinder, *2* air

523 compressor, *3* jacketed 500-mL glass reactors, *4* mass flow controllers, *5* needle valve,

524 *6* T-connections, *7* pH data acquisition system, *8* PC data logger, *9* rotameters, *10* inlet

525 sampling points, *11* outlet sampling points.

526

527 **Figure 2.** Time course of CO_2 production rate (**a**), biomass concentration (**b**) and TOC

528 concentration (**c**) during methanotroph enrichment in R1 (▲), R2 (♦) and R3 (□).

529

530 **Figure 3.** DGGE profiles of the bacterial communities present in: *A* Inoculum, *B* R1 on

531 week 4, *C* R2 on week 4, *D* R3 on week 4, *E* R1 on week 14, *F* R2 on week 14, *G* R3

532 on week 14, *H* R1 on week 19, *I* R2 on week 19, *J* R3 on week 19. The name of the

533 samples and their Shannon-Weiner diversity indexes are shown in the upper part of the

534 gel.

535

536 **Figure 4.** Bacterial similarity dendrogram (UPGMA clustering) (**a**) and matrix (**b**) with

537 error resampling (500 resampling experiments) for: *A* Inoculum, *B* R1 on week 4, *C* R2

538 on week 4, *D* R3 on week 4, *E* R1 on week 14, *F* R2 on week 14, *G* R3 on week 14, *H*

539 R1 on week 19, *I* R2 on week 19, *J* R3 on week 19. The names of the samples in the
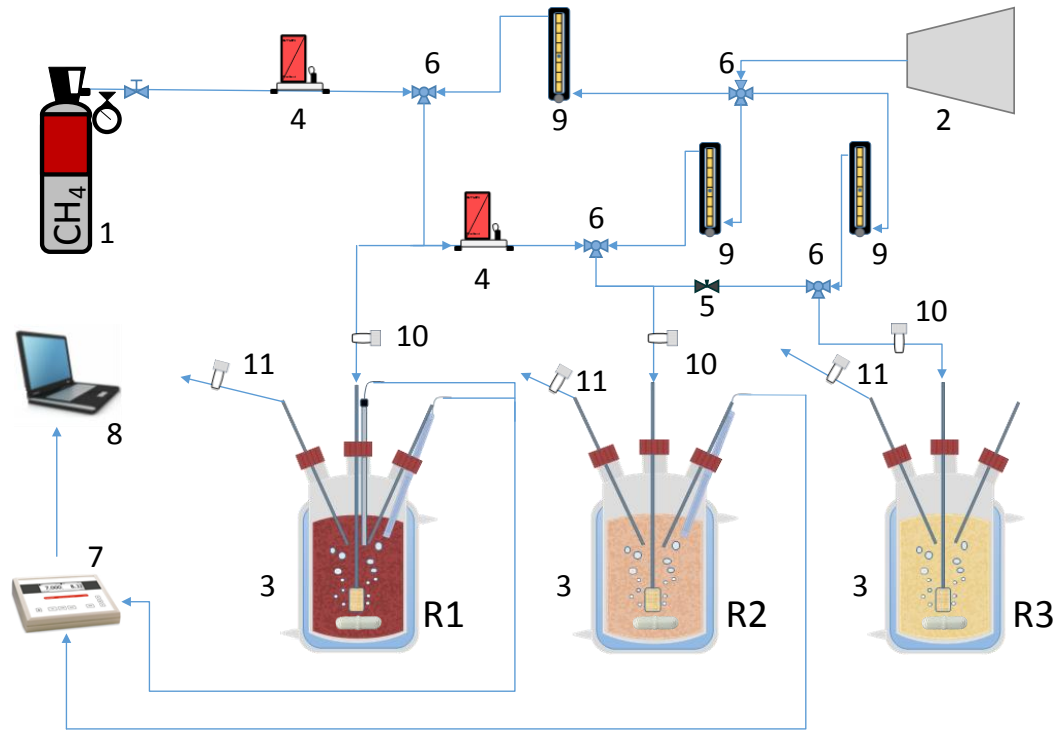
540 dendogram are shown in the lower part of the figure.

541

542 **Figure 5.** Influence of CH_4 concentration during enrichment on the CH_4 biodegradation

543 kinetic parameters q_{max} (**a**) and K_S (**b**) at week 14 (black bar) and 19 (scratched bar).

544

545 **Figure 6.** Transmission electron micrographs of methanotrophic cells containing PHB

546 enriched in R1 (**a**), R2 (**b**) and R3 (**c**) (60 000 $\times$, 120 000 $\times$ and 100 000 $\times$

547 magnification, respectively).

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552   **Figure 1.**

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554

Figure 2.

a

b

c

Time (days)

557 **Figure 3.**

558

559 **Figure 4.**

a

Dendrogram values: 22, 34, 46, 56, 68, 75, 61, 80, 88

Scale (left axis): 30, 40, 50, 60, 70, 80, 90, 100

Samples (left to right): A D B C G J E H F I

b

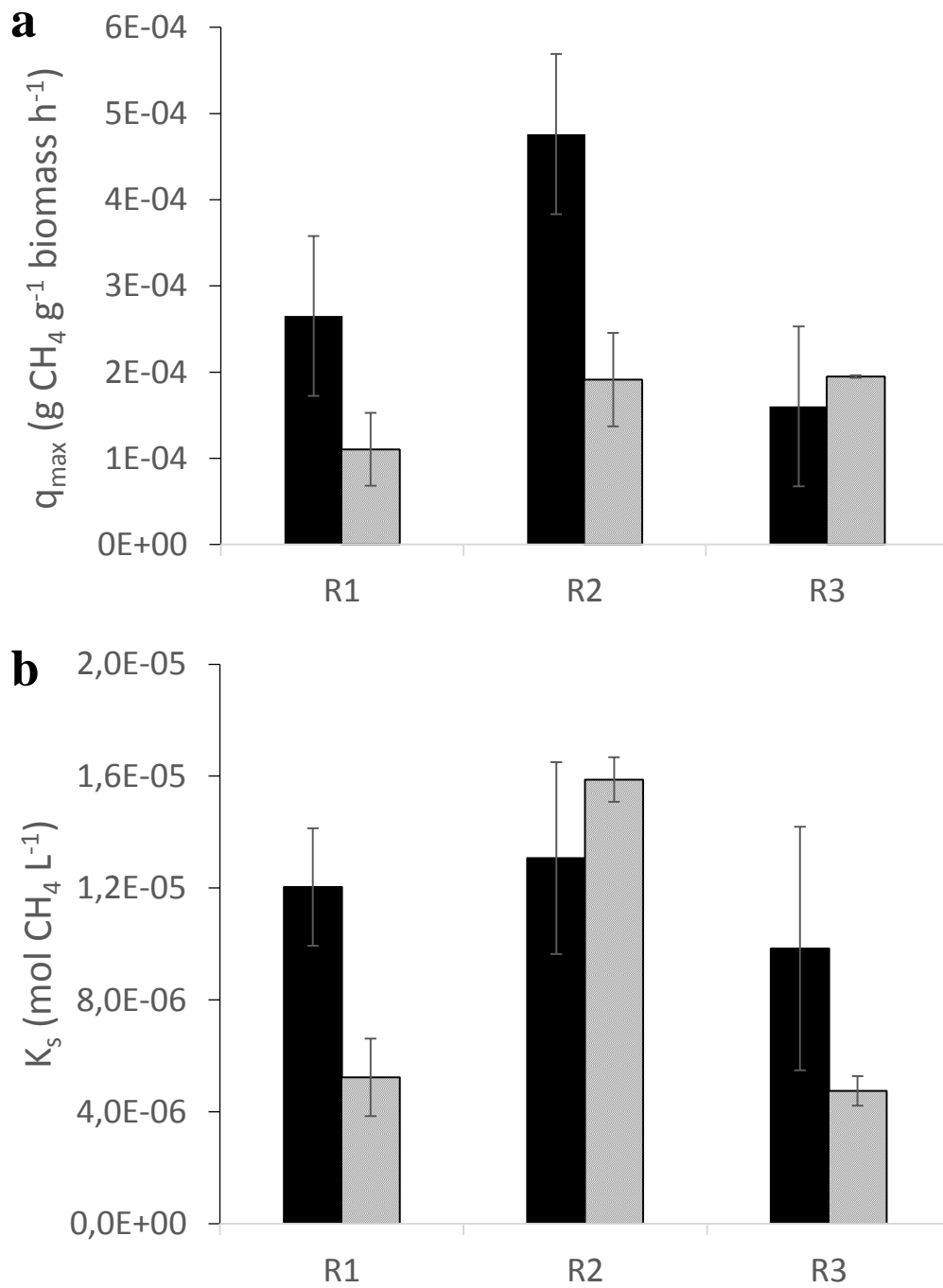| Sample	A	B	C	D	E	F	G	H	I	J
A	100	-	-	-	-	-	-	-	-	-
B	8	100	-	-	-	-	-	-	-	-
C	18	67	100	-	-	-	-	-	-	-
D	34	23	37	100	-	-	-	-	-	-
E	1	52	41	31	100	-	-	-	-	-
F	12	60	53	41	66	100	-	-	-	-
G	2	50	63	38	27	46	100	-	-	-
H	9	47	31	36	88	77	21	100	-	-
I	19	61	60	29	54	80	48	51	100	-
J	9	50	65	29	32	59	75	32	66	100

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Figure 5.

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Figure 6.

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Table 1. Polyhydroxyalkanoate content in the biomass enriched in the three STRs following N limitation

Cycle	R1		R2		R3	
	%PHB[b]	PHV:PHB	%PHB	PHV:PHB	%PHB	PHV:PHB
1	nd	nd	nd	nd	0.8 ± 0.0	3:1
2	nd	nd	3.2 ± 0.2	1:2	0.8 ± 0.0	2:1
3	nd	nd	3.1 ± 0.1	1:2	0.6 ± 0.0	2:1
4	nd	nd	9.7 ± 0.2	1:4	0.5 ± 0.1	3:1
5	nd	nd	7.5 ± 0.0	1:4	0.4 ± 0.0	4:1
6	0.3 ± 0.1	11:1	2.9 ± 0.1	1:2	0.5 ± 0.0	3:1
7	0.5 ± 0.1	10:1	4.3 ± 0.1	1:2	0.1 ± 0.0	3:1
8	0.3 ± 0.0	12:1	5.5 ± 0.2	1:3	0.5 ± 0.0	2:1
8 (3 days)	1.0 ± 0.1	3:1	12.6 ± 0.9	1:6	0.5 ± 0.0	3:1
8 (6 days)	0.6 ± 0.0	7:1	6.2 ± 0.1	1:7	0.4 ± 0.0	3:1
8 (10 days)	0.7 ± 0.0	6:1	6.4 ± 0.1	1:5	0.5 ± 0.0	4:1
8 (18 days)	0.6 ± 0.0	6:1	5.5 ± 0.3	1:5	1 ± 0.0	1:1

[a]nd: not determined

[b]%PHB = (gPHB/gTSS) × 100

Appendix A. Supplementary Data

Click here to download e-component: Appendix A. Supplementary Data.docx