Polyhydroxyalkanoates production from methane emissions in *Sphagnum* mosses: assessing the effect of temperature and phosphorus limitation

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Abstract: The isolation of highly efficient methanotrophic communities is crucial for the optimization of methane bioconversion into products with a high market value such as polyhydroxyalkanoates (PHA). The research here presented aimed at enriching a methanotrophic consortium from two different inocula (*Sphagnum* peat moss (Sp) and *Sphagnum* and activated sludge (M)) able to accumulate PHA while efficiently oxidizing CH_4. Moreover, the effect of the temperature and phosphorus limitation on the biodegradation rate of CH_4 and the PHA accumulation potential was investigated. Higher CH_4 degradation rates were obtained under P availability at increasing temperature (25, 30 and 37 ºC). The biomass enriched from the mixed inoculum always exhibited a superior biodegradation performance regardless of the temperature (a maximum value of 84.3 ± 8.4 mg CH_4 h^{-1} g $biomass^{-1}$ was recorded at 37 ºC). The results of the PHB production showed

that phosphorus limitation is required to promote PHB accumulation, the highest PHB content being observed with the *Sphagnum* inoculum at 25 ºC (13.6 ± 5.6%).

The differential specialization of the microbial communities depending on the enrichment temperature supported the key role of this parameter on the results obtained. In all cases after the completion of the enrichment process and of the P limitation tests, *Methylocystis,* a type II methanotroph known for its ability to accumulate PHA, was the genus that became dominant (reaching percentages from 16 to 46 % depending on the enrichment temperature). Thus, the results here obtained demonstrated for the first time the relevance of the temperature used for the enrichment of the methanotrophic bacteria to boost PHA production yields under P limiting condition, highlighting the importance of optimizing culture conditions to improve the cost-efficiency of bioprocesses based on using methane as the primary feedstock for the PHA industrial market.

Keywords: Greenhouse gases mitigation, Methane abatement, Methanotrophs, Phosphorus limitation, Polyhydroxyalkanoates, *Sphagnum* mosses

1- Introduction

During the first decade of the 21st century, the average global surface temperature was 0.8 ºC warmer compared to that of the 20th century, with the most significant warming being recorded over the past three decades (NRC, 2010). The increase in the concentration of greenhouse gases (GHGs) in the atmosphere is directly linked to this increase in the average temperature of the planet. Among them, methane (CH_4) concentration in the atmosphere (the

second most important GHG (Desai, M. P., & Harvey, 2017)) increased at a yearly rate of 0.2-1 %, mainly due to anthropogenic activities (landfilling, agriculture, livestock farming, waste management and energy production) ((EPA), 2017). Efficient energy recovery from CH_4-laden emissions is restricted to CH_4 concentrations higher than 20 %, while more than 56 % of the anthropogenic sources emit CH_4 at a concentration below 5 % (Estrada et al., 2011; López et al., 2013). These diluted emissions are commonly directly released into the atmosphere, contributing to the greenhouse effect and the subsequent environmental and health damage (Awe et al., 2017; Muñoz et al., 2015; Shuman, 2011). In this context, the biological treatment of diluted methane emissions in combination with the co-production of high-added value products such as polyhydroxyalkanoates (PHA) represents a feasible alternative to enhance the economic sustainability of current CH_4 abatement processes (Cal et al., 2016; Pieja et al., 2017; Strong et al., 2016).

PHA such as poly(3-hydroxybutyrate) (PHB) are the only bioplastics totally produced by microorganisms, which exhibit mechanical and thermal properties similar to those of synthetic polyesters (polyethylene, polypropylene) (Strong et al., 2016). Additionally, PHA are biodegradable and have the potential to be produced from renewable sources (Koller et al., 2017; Kourmentza et al., 2017). Type II methanotrophs responsible of CH_4 biodegradation are able to divert the flux of carbon associated with CH_4 assimilation to the production of intracellular PHA under very specific cultivation conditions (Pieja et al., 2017). Unfortunately, there is limited knowledge about the optimal temperature conditions for the cultivation of type II methanotrophs for the production of PHA. In this sense, tailoring different operational parameters such as pH, temperature, CH_4/O_2 ratio, or the concentration of sodium, copper or citrate, has been reported as a key strategy for the enrichment of type II methanotrophs with the ability to synthetize PHA (Pieja et al., 2011; Scheutz et al., 2009;

Semrau et al., 2013). Therefore, the elucidation of these particular operating conditions to promote continuous bioplastics production is of paramount importance to support a cost-efficient production of PHA from CH_4 (García-Pérez et al., 2018; López et al., 2014). Unfortunately, to date, no study has systematically addressed the influence of these parameters during enrichment of methanotrophs.

On the other hand, pure methanotrophic strains are frequently used for the accumulation of PHA using CH_4 as the carbon source, while enrichment of a methanotrophic culture from a mixed consortium might provide higher community resilience. In this context, different studies have identified *Sphagnum* mosses as ecosystems able to reduce CH_4 emissions (Kip et al., 2010; Raghoebarsing et al., 2005), being described as the bryophytes with the highest richness in type II methanotrophs (Kip et al., 2011; Stępniewska and Kuźniar, 2014). These characteristics make *Sphagnum* mosses a potential new source of effective PHA-accumulating microorganisms (Zhao et al., 2007).

The present study aimed to systematically elucidate the influence of temperature and phosphorus limitation on the CH_4 biodegradation rate and PHA accumulation potential of two enriched methanotrophic communities from two different inocula: *Sphagnum* sp. and a mixture of *Sphagnum* sp. and activated sludge. The influence of the temperature on the evolution of the structure of the bacterial communities was also assessed.

2. Materials and Methods

2.1-Inoculum and mineral salt medium

Two inocula were used for the enrichment of microorganisms able to degrade CH_4 under different conditions of temperature: i) *Sphagnum* sp. (Sp) selected from living mixed

Sphagnum peat moss (between 150-350 species) from *Plantas Carnívoras*, (Madrid, Spain) and ii) a mixture of *Sphagnum* sp. + activated sludge (M) obtained from the Valladolid wastewater treatment plant (Valladolid, Spain).

The mineral salt medium (MSM) used for the enrichment was modified from (Mokhtari-Hosseini et al., 2009). The MSM was composed of (g L^{-1}): 2.25 $NaNO_3$, 0.1 $MgSO_4 \cdot 7H_2O$, 0.02 $CaCl_2 \cdot 2H_2O$, 0.68 KH_2PO_4, 6.14 $Na_2HPO_4 \cdot 12H_2O$, 1.3×10^{-3} $FeSO_4 \cdot 7H_2O$, 3.5×10^{-3} $MnCl_2 \cdot 4H_2O$, 1.5×10^{-3} $ZnSO_4 \cdot 7H_2O$, 0.04×10^{-3} $Na_2MoO_4 \cdot 2H_2O$, 0.04×10^{-3} $CuSO_4 \cdot 5H_2O$, 0.32×10^{-3} $CoCl_2$, and 0.2×10^{-3} H_3BO_3. All chemicals needed for MSM preparation were purchased from PANREAC (Barcelona, Spain).

CH_4 ($\geq$ 99.5 %) and O_2 ($\geq$ 99 %) were purchased from Abelló Linde S.A. (Barcelona, Spain). Poly [(R)-3-hydroxybutyric acid-*co*-(R)-3-hydroxyvaleric acid] (molar ratio 88/12, $\geq$ 99.99 %) was obtained from Sigma-Aldrich® (St. Louis, MO, USA).

2.2- Culture enrichment at different temperatures

The first enrichment was performed batch wise in 1250 mL serum bottles initially containing either 200 mL of MSM and 20 g (dry weight) of *Sphagnum* sp. or 190 mL of MSM, 10 mL of activated sludge and 10 g (dry weight) of *Sphagnum* sp. The bottles were closed with butyl septa and plastic caps, and the headspace was flushed with pure O_2 for 15 min in order to completely eliminate the N_2. CH_4 was then supplied at an initial headspace concentration of 198.0 ± 2.7 g m^{-3} for inoculum Sp and 196.6 ± 5.2 g m^{-3} for inoculum M. The bottles were incubated in an orbital shaker MaxQ 4000 (Thermo Scientific, USA) at 25 ºC and 200 rpm. CH_4 and CO_2 concentrations in the headspace were periodically analyzed. Upon CH_4 depletion, the biomass was harvested and used as inoculum for a subsequent enrichment at different temperatures (25, 30, 37 and 45 ºC). Five CH_4 degradation cycles were carried out

until the end of the enrichment period, with an initial headspace concentration of 195 ± 7 g m^{-3}, which lasted between 27 and 45 days, except for batch tests at 45 ºC which showed no CH_4 degradation. Headspace concentration was periodically monitored until complete CH_4 consumption and then replenished to start a new cycle. No replacement of the cultivation medium was performed during the cycles.

2.3. Influence of temperature on CH_4 biodegradation kinetics

The biodegradation kinetics study was carried out batch wise in 1250 mL serum bottles initially containing 200 mL of MSM and ~7.3 mg of the TSS (total suspended solids) from the second enrichment. The bottles were closed with butyl septa and plastic caps, and the headspace was flushed with O_2 for 15 min. CH_4 was then supplied at an initial headspace concentration of 196.8 ± 9.8 g m^{-3} and the bottles were incubated in an orbital shaker at 25, 30 or 37 ºC and 200 rpm until complete CH_4 consumption. All the assays were carried out in duplicate. The CH_4, O_2 and CO_2 composition of the headspace and the biomass concentration (measured as TSS) in the cultivation broth were periodically monitored. The specific CH_4 biodegradation rates were estimated from the ratio of the specific growth rates and the observed biomass yields in each experiment.

2.4. Influence of phosphorus limitation on CH_4 biodegradation kinetics and PHA accumulation at different temperatures

The influence of P on CH_4 biodegradation and PHA accumulation was also evaluated batch wise at different temperatures: 25, 30 and 37 ºC. The study was performed in 1250 mL serum bottles initially containing 200 mL of MSM and ~7.3 mg of the TSS of the second

enrichment. The bottles were closed with butyl septa and plastic caps, and the headspace was flushed with O_2 for 15 min in order to eliminate the remaining N_2. CH_4 was then supplied at an initial headspace concentration of 161.0 ± 13.0 g m^{-3} and the bottles were incubated in an orbital shaker at 25, 30 or 37º C and 200 rpm until complete CH_4 consumption. The biomass was centrifuged and resuspended in 1250 mL serum bottles initially containing 200 mL of P-free MSM. The glass bottles were sealed with butyl septa and plastic caps, washed with pure O_2 and supplemented with CH_4 to the headspace both in the growth and accumulation stages at an initial concentration of 177.0 ± 7.1 g m^{-3}. All assays were carried out in duplicate. The CH_4, O_2 and CO_2 composition of the headspace, and the biomass concentration as TSS in the cultivation broth were periodically monitored. Samples for the determination of the PHA concentration were also withdrawn throughout the limitation tests. Samples for the determination of the biomass composition were withdrawn by the end of the limitation tests.

2.5. Analytical procedures

CH_4, O_2, and CO_2 gas concentrations were measured in a Bruker 430 GC-TCD (Palo Alto, USA) equipped with a CP-Molsieve 5A column (15 m × 0.53 μm × 15 μm) and a CP-PoraBOND Q column (25 m × 0.53 μm × 10 μm). 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}. TSS concentration was determined according to the standard methods (American Public Health Association (APHA) et al., 2005). Total nitrogen (TN) concentration was quantified following sample filtration (0.45 μm) in a TOC-VCSH analyzer (Shimadzu, Japan) coupled with a chemiluminescence detection TN module (TNM-1) (Shimadzu, Japan). PHB accumulation was quantified by GC–MS (GC System 7820A MSD 5977E, Agilent Technologies, Santa Clara, USA) using a DB-wax column

(30 m × 250 µm × 0.25 µm) according to Frutos et al., (2017). The determination of the C, H, N and S content of the bacterial biomass was conducted in a LECO CHNS-932 analyzer.

2.6-DNA/RNA extraction, illumina library preparation and pyrosequencing

Samples for the determination of the microbial communities' structure were withdrawn by the end of the first enrichment and of the P limitation tests, and immediately stored at - 80 ºC for subsequent analysis. Total genomic DNA of the initial inocula was extracted from 500 µL samples using the FastDNA Spin kit for soil (MP Biomedicals, USA) according to the manufacturer's instructions. Total RNA of the remaining samples was extracted using the RNeasy Plus Mini kit from Quiagen Iberica SL according to the manufacturer's instructions. Subsequently, the cDNA synthesis was performed with the iScriptTM Adv cDNA Kit for RT-qPRC from BIO-RAD. DNA concentration was estimated in a Qubit fluorometer (Invitrogen), and the final concentration of the DNA sample was normalized to 5 ng μL^{-1}. Amplicon sequencing was carried out targeting the 16S V3 and V4 regions (464 bp, Escherichia coli based coordinates) with the bacterial primers S-D-Bact-0341-b-S-17 and S-D-Bact-0785-a- A-21 (forward and reverse, respectively), which were selected according to Klindworth et al., (2013). Illumina adapter overhang nucleotide sequences were added to the gene-specific sequences, thus resulting in the following full-length primers for the analysis: 5´TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG (16S amplicon PCR forward primer), and 5´ GTCTCGTGGGCTCGGAGATGTGTATAAGAGAC-AGGACTACHVGGGTATCTAATCC (16S amplicon PCR reverse primer). Microbial genomic DNA (5 ng μL^{-1} in 10 mM Tris pH 8.5) was used to initiate the protocol. After 16S

186 rDNA gene amplification, the mutiplexing step was performed using Nextera XT Index Kit
187 (FC-131-1096). It used to run 1 μL of the PCR product on a Bioanalyzer DNA 1000 chip to
188 verify the size. The expected size on a Bioanalyzer trace is ~550 bp. After size verification
189 the libraries were sequenced using a 2x300 bp paired-end run (MiSeq Reagent kit v3 (MS-
190 102-3001)) on a MiSeq Sequencer according to manufacturer's instructions (Illumina). The
191 pyrosequencing analysis was carried by the Foundation for the Promotion of Health and
192 Biomedical Research of Valencia Region (FISABIO, Spain).
193 After demultiplexing, only reads that had quality value scores ≥ 20 for more than 99 % of the
194 sequence were extracted for further analysis. All sequences with ambiguous base calls were
195 discarded. Quality assessment was performed by a prinseq-lite program (Schmieder and
196 Edwards, 2011). After quality assessment, paired-end reads were joined together with the
197 FLASH program (Magoc and Salzberg, 2011). Once eventual chimeras belonging to PCR
198 artifacts among the sequences were discarded using the Usearch program (Edgar, 2010),
199 taxonomic affiliations have been assigned using the Naive Bayesian classifier integrated in
200 quiime2 plugins and database used for this taxonomic assignation was the SILVA release132
201 (Caporaso et al., 2010) and RDP- Classifier from the Ribosomal Database Project (Cole et
202 al., 2009; Wang et al., 2007) which is available from the RDP website
203 (http://rdp.cme.msu.edu/ classifier/).
204
205 *2.7-Statistical Analyses*
206 All analyses were performed using biomass obtained from two biological replicas for each
207 condition. Error bars represent standard errors from duplicates. Bioinformatic analysis was
208 obtained using an *ad-hoc* pipeline written in RStatistics environment (Varriano-Marston and
209 Omana, 1979) utilizing several Open Source libraries such as *gdata* and *vegan* (Oksanen et

al., 2017). The sequence data were analyzed using qiime2 pipeline originally cited in Caporaso et al (2010).

3. Results

3.1. Influence of temperature on CH_4 biodegradation

During the first enrichment series, a similar influence of the temperature on the degradation of CH_4 was observed for both inocula (i.e. *Sphagnum* and *Sphagnum* + activated sludge), with the highest degradation recorded at 30 ºC (Figure 1). Nevertheless, the *Sphagnum* showed faster degradation rate regardless of the temperature than those observed for the mixed inoculum. It is also worth noting that microbial activity was inhibited at the highest temperature tested of 45 ºC, with no CH_4 degradation being observed (no further experiments were therefore performed at this temperature).

<Figure1>

On the contrary, the second enrichment series, consisting on five consecutive CH_4 degradation cycles, showed a negligible effect of the temperature on the specific CH_4 biodegradation rates of the biomass enriched from *Sphagnum*: 58.7 ± 5.9, 60.8 ± 0.3 and 65.1 ± 6.5 mg-CH_4 h^{-1} g-biomass^{-1} at 25, 30 and 37 ºC, respectively (Table 1). However, the biomass enriched from the mixed inoculum exhibited a significant difference among the specific CH_4 biodegradation rates at the temperatures tested (71.6 ± 0.4, 79.9 ± 3.6 and 84.3 ± 8.4 mg-CH_4 h^{-1} g-biomass^{-1} at 25, 30 and 37 ºC, respectively). Moreover, the degradation rates recorded for the M inoculum were ~12 % higher for all temperatures compared to those of the Sp inoculum. Accordingly, higher specific CO_2 production rates were obtained at

increasing temperatures for both inocula, with higher values observed for inoculum M at 37 ºC (366.2 ± 36.6 mg-CO_2 h^{-1} g-biomass^{-1}) (Table 1).

<Table1>

3.2 Influence of phosphorus on CH_4 biodegradation and biomass composition

Under phosphorus limitation, no significant influence of the temperature or the initial culture composition on CH_4 biodegradation was observed, with values ranging from 8.8 up to 14.9 mg-CH_4 h^{-1} g-biomass^{-1} (Figure 2). Moreover, the biomass enriched from both the M and Sp inocula exhibited lower specific CH_4 biodegradation rates under P limitation compared to regular P supplementation.

<Figure 2>

Biomass composition (C, H, N and S) was determined under phosphorus limitation for both inocula (Figure 3). Interestingly, the same effect of the temperature on carbon content was observed for the Sp and the M enriched biomass, with lower C concentrations at 37 ºC compared to 25 and 30 ºC (48.3 ± 1.2, 46.2 ± 0.5 and 45.5 ± 0.0 % and 49.3 ± 0.0, 46.3 ± 0.5 and 45.6 ± 0.7 % in the Sp and M enriched biomass at a 25, 30 and 37 ºC, respectively). Conversely, the concentration of nitrogen increased from 6.1 ± 0.1 % at 25 ºC to 7.9 ± 0.3 % at 37 ºC in the M biomass, remaining roughly constant for the Sp consortia. Moreover, sulphur content increased from 0.5 ± 0.2 to 0.9 ± 0.0 % when increasing the enrichment temperature of the Sp biomass from 25 to 37 ºC, and remained at ~ 0.8 % in the M biomass regardless of the temperature tested. Finally, no impact of the temperature on the hydrogen concentration was observed for any inoculum, with values of ~ 6.7 %.

<Figure3>

254

255 *3.3 PHA accumulation under phosphorus limitation*

256 When phosphorus was supplemented to the MSM, the enriched biomass showed PHA
257 concentrations < 1 % at the tested temperatures. However, PHA accumulation was detected
258 in all samples under phosphorus limiting conditions, with PHB as the dominant PHA in all
259 the samples analysed. In this sense, the biomass enriched at 25 ºC was able to accumulate
260 12.4 ± 5.3 and 11.3 ± 0.6 % of PHB in Sp and M enriched biomass, respectively. Lower
261 accumulation of PHB was recorded at 30 and 37 ºC in the Sp tests (9.5 ± 1.6 and 9.0 ± 0.5
262 %, respectively), while similar concentrations of PHB were recorded at 30 ºC in the M
263 biomass (11.0 ± 2.7 %) (Figure 4). The amount of 3-hydroxyvalerate (PHV) detected was
264 negligible regardless of the temperature and the inoculum (maximum detected concentrations
265 of 0.032 %).

266 <*Figure4*>

267

268 *3.4 Influence of the enrichment temperature and P limitation on the structure of the*
269 *microbial communities*

270 Effective bacterial sequences from the active communities of the samples were affiliated to
271 a total of 15 phyla. Among them, the most dominant phyla (≥ 1 % abundance) in the inoculum
272 Sp were *Proteobacteria* (62.1 %), *Bacteroidetes* (31.7 %), *Verrucomicrobia*. (2 %) and
273 *Parcubacteria* (1 %); with 6 predominant phyla being detected in the inoculum M:
274 *Bacteroidetes* (39.8 %), *Proteobacteria* (35.7 %), *Parcubacteria* (1.4 %), *Actinobacteria* (1.3

%), *Verrucomicrobia*. (1.1 %) and *Chloroflexi* (1 %); and 16.3 % of the reads not classified (Figure 5a).

The activity of *α*-, *β*-, *γ*- and *δ*-*Proteobacteria* within the *Proteobacteria* phylum was evenly represented (Figure 5b). The *Methylocystaceae* (*a-Proteobacteria*) and *Methylococcaceae* (*γ-Proteobacteria*) families are taxonomically clustered as type I and type II methanotrophs, respectively (Knief, 2015). Surprisingly, their presence in both inocula samples was scarce. In this sense, the *Methylococcaceae* family represented 1.94 % of the whole population (mainly included in the genus *Methylomonas*) in the inoculum Sp and 16.48 % in the inoculum M (including the genus *Methylobacter*, *Methylomonas* and *Methylosarcina*), while only 0.24 and 0.08 % of the bacterial population were affiliated to the genus *Methylocystis* within *Methylocystaceae* family in the inoculum Sp and M, respectively (Figure 5c).

<*Figure5*>

The complete enrichment process (including the first and second enrichment with 5 CH_4 degradation cycles, the 2 subsequent growth series and the last PHB accumulation stage under phosphorus limitation) resulted in the presence of only one methanotrophic genus, *Methylocystis*, in the enriched communities regardless of the inoculum source and temperature (with abundance percentages ranging between 16 and 46 %) (Figure 6). The higher proportion of *Methylocystis* was obtained at 25 ºC in the biomass enriched from *Sphagnum* (46 %), decreasing its abundance to 27 and 30 % at 30 and 37 ºC, respectively. The genus *Methylocystis* was less abundant in the community enriched from *Sphagnum* and activated sludge (~ 27 and 34 % at 25 and 30 ºC, respectively, decreasing to 17 % at 37 ºC). In addition, the biomass enriched from both inocula contained a significant percentage of the

297 methylotroph *Methylobacterium* (38 and 31 % of the community enriched at 30 ºC in Sp and
298 M, respectively). The results here obtained revealed that temperature was a strong selective
299 pressure for the enrichment of PHB accumulating bacteria.
300
301 *<Figure6>*
302
303 **4. Discussion**
304 An enhanced CH_4 degradation was obtained at 30 ºC for the biomass enriched from both
305 inocula, with lower values obtained at 25 and 37 ºC.
306 No significant difference was observed between the specific CH_4 biodegradation rates of the
307 biomass enriched from *Sphagnum* and that enriched from *Sphagnum* and activated sludge at
308 25, 30 and 37 ºC. Moreover, the limitation of phosphorus resulted in lower specific CH_4
309 degradation rates regardless of the inocula and the enrichment temperature. Although a lack
310 of carbon has been proposed as the main limiting factor for bacterial growth in soil (Alden et
311 al., 2001), other reports have also pointed out the importance of nutrients availability (such
312 as nitrogen or phosphorus) on the limitation of microbial respiration (Ilstedt and Singh,
313 2005). In our particular study, phosphorus was identified to play a key role in the growth of
314 microorganisms from *Sphagnum* mosses.
315 The highest accumulation of PHB was recorded at 25 ºC under phosphorus limitation,
316 although the maximum values here obtained (~ 18 %) were considerably lower compared to
317 the PHB production capacity observed by previous authors. For instance, López et al.
318 obtained PHA accumulations up to 45 % under N-limiting conditions in a pure culture of the
319 type II methanotroph *Methylocystis hirsuta* (López et al., 2018a). Similarly, Zhang et al.

(2016) reported accumulations of ~ 45 % of PHB in a mixed culture enriched from sewage sludge under N limitation and Cu surplus, whereas the absence of Cu resulted in a reduced PHB synthesis (12 % – 18 %).

These results suggest that, among the several nutrient limitations that might promote PHB production, N-limitation results in higher PHB content compared to P-limitation. However, given the limited number of studies conducted on phosphorus limitation, further investigation is required to support these preliminary conclusions.

In addition, the only type II methanotroph found in this study under phosphorus limitation was *Methylocystis*, which was likely responsible for the synthesis of PHA (Asenjo and Suk, 1986). Nevertheless, further studies will be necessary in order to corroborate the influence of P limitation on the PHB synthesis capacity of pure and mixed methanotrophic cultures.

The composition of the microbial communities in the inoculum Sp was similar to that reported in previous studies with *Sphagnum* species (Bragina et al., (2012)), whereas a slightly different microbial composition was obtained in the inoculum M, with predominant phyla commonly found in activated sludge (López et al., 2018b). Moreover, a different evolution of the methanotrophic bacteria was observed in both inocula depending on the enrichment temperature (Figure 6). In this context, and to the best of the authors knowledge, the effect of the temperature on the PHA accumulation potential has not been previously studied. The differences observed in the overall microbial composition under P limitation at the tested temperatures could explain the influence of this parameter on the PHB production results. For example, the enrichments conducted at 25 ºC from the inoculum Sp, which resulted in higher PHB accumulations, showed a higher percentage of *Methylocystis* than those conducted at 30 and 37 ºC. A similar result was observed for the inoculum M, but in

this case the enrichments conducted at 25 and 30 ºC, which resulted in higher PHB accumulations, exhibited a higher percentage of *Methylocystis* than those conducted at 37 ºC.

5. Conclusion

This research demonstrated that *Sphagnum* is an appropriate inoculum for the enrichment of the genus *Methylocystis*, a type II methanotroph able to accumulate PHA (and more specifically PHB) using CH_4 as the only carbon source under phosphorus limitation. In addition, the temperature was identified as a key operating parameter influencing both the specific CH_4 biodegradation rate (with highest values recorded at 30 ºC) and the PHB accumulation capacity. In this sense, the differential specialization of the microbial communities depending on the enrichment temperature could explain this finding. The genus *Methylocystis* increased its abundance from 0.24 % up to 45 % in the biomass enriched from Sp at 25 ºC, with a lower contribution observed at 30 and 37 ºC. This likely mediated the higher PHB accumulations obtained at 25 ºC. On the contrary, *Methylocystis* was less abundant in the community enriched from the mixed inoculum, which exhibited the higher abundance of *Methylocystis* at 25 and 30 ºC, also resulting in higher PHB production at these temperatures.

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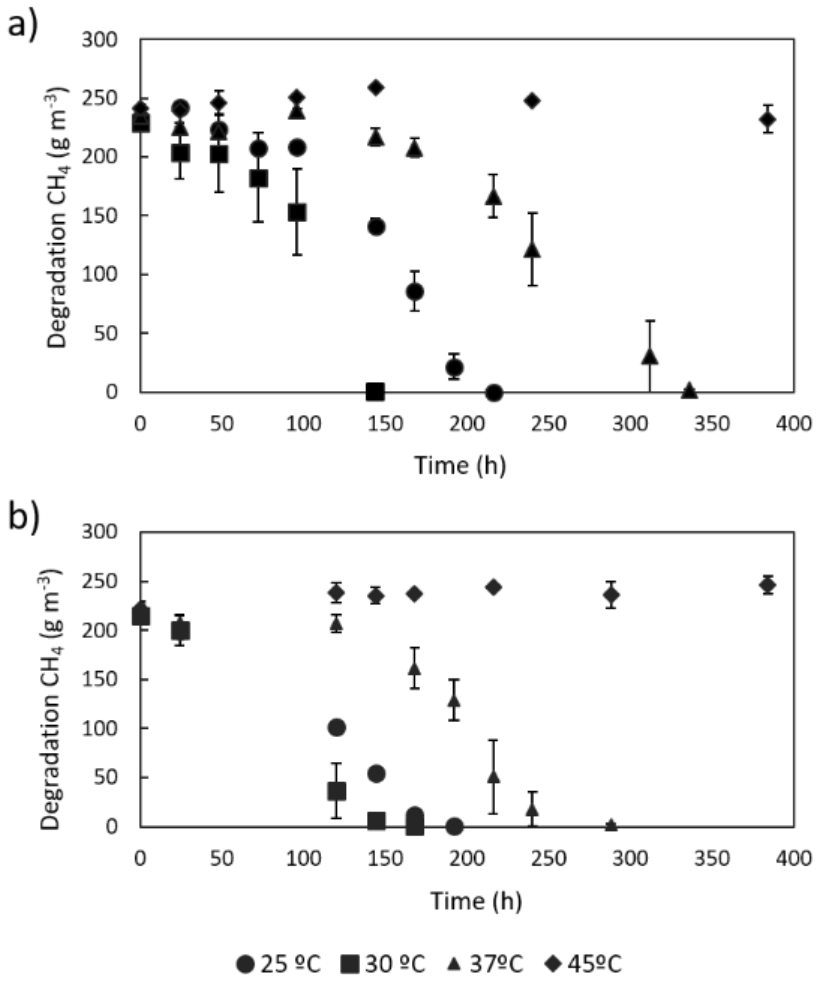
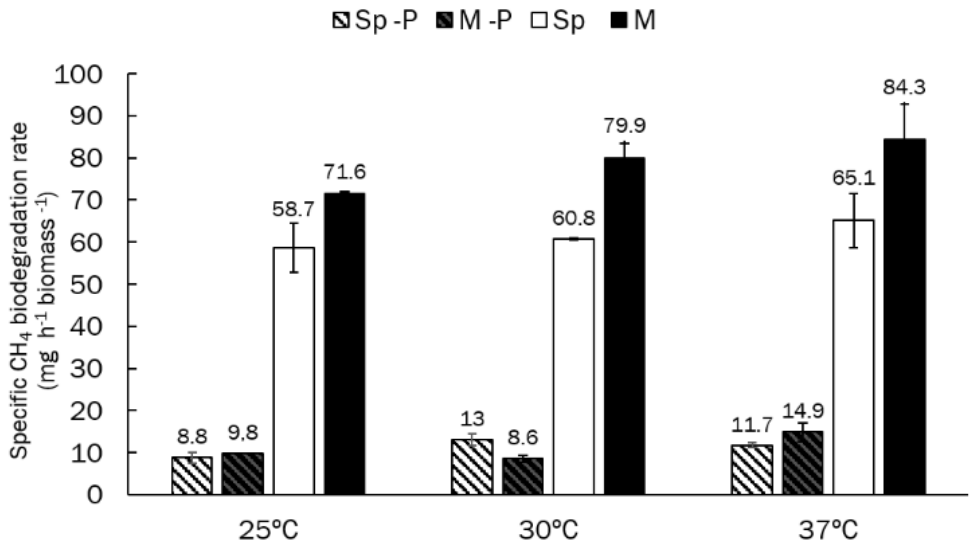


Figure 1. Time course of CH_4 concentration during the enrichment of (a) *Sphagnum* (Sp) and b) *Sphagnum* + activated sludge (M) inocula at 25 (●), 30 (■),37 (▲) and 45 (♦) ºC. Error bars represent standard errors (n=2).

Table 1. Specific CH_4 biodegradation and CO_2 production rates

		Specific CH_4 biodegradation (mg-CH_4 h^{-1} g-biomass^{-1})		**Specific CO_2 production** (mg-CO_2 h^{-1} g-biomass^{-1})	
		Inoculum Sp	Inoculum M	Inoculum Sp	Inoculum M
Temperature	**25ºC**	58.7 ± 5.9	71.6 ± 0.4	255.2 ± 35.5	316.6 ± 9.4
	30ºC	60.8 ± 0.3	79.9 ± 3.6	281.6 ± 10.2	311.3 ± 2.1
	37ºC	65.1 ± 6.5	84.3 ± 8.4	349.1 ± 34.9	366.2± 36.6



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545 **Figure 2.** Influence of the temperature on the specific CH_4 biodegradation rate for the
546 communities enriched from *Sphagnum* (white bars) and *Sphagnum* + activated sludge (black
547 bars) with phosphorus (non-striped bars) and under phosphorus limitation (striped bars).
548 Error bars are standard errors (n=2).

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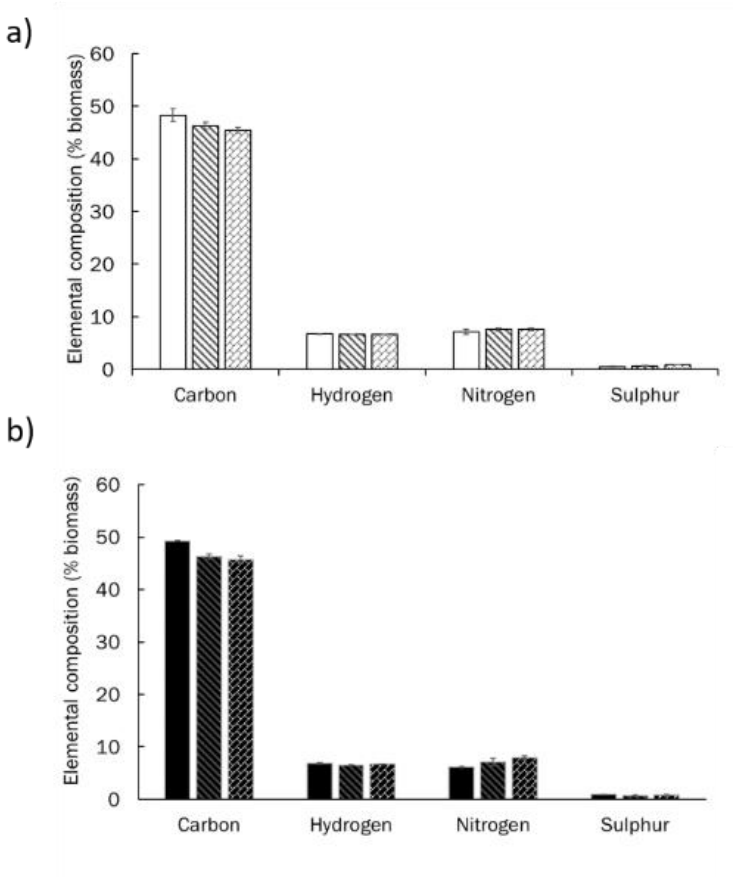

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554 **Figure 3**. Elemental biomass composition (% of carbon, hydrogen, nitrogen and sulphur) of
555 the communities enriched from (a) *Sphagnum* and (b) *Sphagnum* + activated sludge under
556 phosphorus limitation at 25 (first blank bars), 30 (second dashed bars) and 37 ºC (third
557 meshed bars). Error bars are standard errors (n=2).

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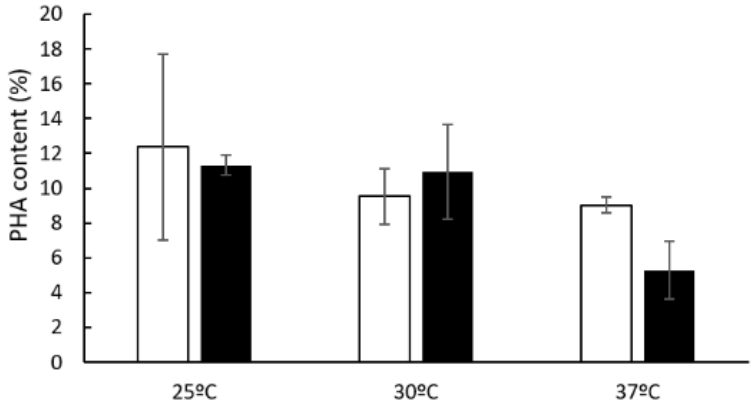

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562 **Figure 4.** Influence of the temperature on the PHB content of the biomass enriched from
563 *Sphagnum* (white bars) and *Sphagnum* + activated sludge (black bars) under phosphorus
564 limitation at 25, 30 and 37 ºC. Error bars are standard errors (n=2).

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a)

PHYLUM	Sp	M
Acidobacteria	0,7	0,7
Actinobacteria	0,1	1,3
Armatimonadetes	0,3	0,8
Bacteroidetes	31,7	39,8
candidate division WPS-1	0,1	0
Candidatus Saccharibacteria	0	0,5
Chlamydiae	0,2	0,1
Chloroflexi	0	1
Cyanobacteria/Chloroplast	0,6	0,3
Firmicutes	0	0,3
Lentisphaerae	0	0,4
Parcubacteria	1	1,4
Planctomycetes	0,2	0,2
Proteobacteria	62,1	35,7
Verrucomicrobia	2	1,1
Unclassified Bacteria	0,7	16,3

b)

***PROTEOBACTERIA* CLASS**	Sp	M
Alphaproteobacteria	6,0	29,1
Betaproteobacteria	9,0	12,5
Deltaproteobacteria	0,6	0,1
Gammaproteobacteria	19,5	20,4
Unclassified *Proteobacteria*	0,6	0,1

c)

METANOTROPHIC GENUS (*Methylocystaceae* family)	Sp	M
Methylocystis	0,08	0,24

METANOTROPHIC GENUS (*Methylococcaceae* family)	Sp	M
Methylobacter	8,23	0,02
Methylomonas	3,80	1,74
Methyloparacoccus	0,03	0,00
Methylosarcina	1,17	0,00
Methylovulum	0,06	0,00
Unclassified *Methylococcaceae*	3,19	0,18

Figure 5. a) Community composition at a phylum level from *Sphagnum* (Sp) and *Sphagnum* + activated sludge (M). b) Abundance of *α*-, *β*-, *γ*- and *δ*-*Proteobacteria* within the *Proteobacteria* phylum in Sp and M. c) Percentage of methanotrophic genera in Sp and M. The abundance is presented in terms of percentage in total effective bacterial sequences in a sample, classified using RDP Classifier.

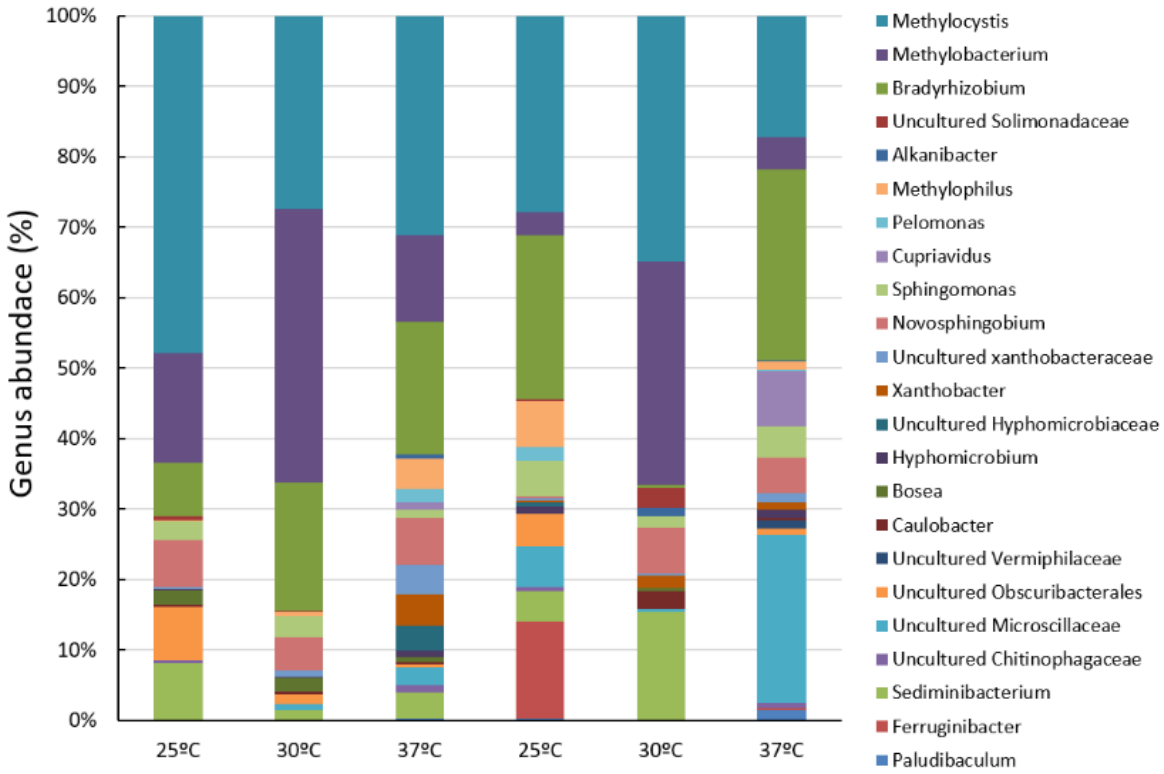

Figure 6. Influence of the enrichment temperature on the community composition at a genus level of the biomass enriched from *Sphagnum* (first three rows) and *Sphagnum* + activated sludge (last three rows) under phosphorus limitation at 25, 30 and 37 ºC. The abundance is presented in terms of percentage using SILVA classifier. Taxa represented occurred at a threshold abundance > 1 % in at least one sample.

Note: This figure will appear in color