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Elucidating the key environmental parameters during the production of ectoines from biogas by mixed methanotrophic consortia

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ABSTRACT

Anaerobic digestion (AD) is a robust biotechnology for the valorisation of organic waste into biogas. However, the rapid decrease in renewable electricity prices requires alternative uses of biogas. In this context, the engineering of innovative platforms for the bio-production of chemicals from CH_4 has recently emerged. The extremolyte and osmoprotectant ectoine, with a market price of ~1000€/Kg, is the industrial flagship of CH_4-based bio-chemicals. This work aimed at optimizing the accumulation of ectoines using mixed microbial consortia enriched from saline environments (a salt lagoon and a salt river) and activated sludge, and biogas as feedstock. The influence of NaCl (0, 3, 6, 9 and 12 %) and Na_2WO_4 (0, 35 and 70 μg L^{-1}) concentrations and incubation temperature (15, 25 and 35 °C) on the stoichiometry and kinetics of the methanotrophic consortia was investigated. Consortia enriched from activated sludge at 15 °C accumulated the highest yields of ectoine and hydroxyectoine at 6 % NaCl (105.0 ± 27.2 and 24.2 ± 5.4 $mg_{extremolyte}$ $g_{biomass}^{-1}$, respectively). The consortia enriched from the salt lagoon accumulated the highest yield of ectoine and hydroxyectoine at 9 % NaCl (56.6 ± 2.5 and 51.0 ± 2.0 $mg_{extremolyte}$ $g_{biomass}^{-1}$, respectively) at 25 °C. The supplementation of tungsten to the cultivation medium did not impact on the accumulation of ectoines in any of the consortia. A molecular characterization of the enrichments revealed a relative abundance of ectoine-accumulating methanotrophs of 7–16 %, with *Methylomicrobium buryatense* and *Methylomicrobium japanense* as the main players in the bioconversion of methane into ectoine.

1. Introduction

Anaerobic digestion (AD) biotransforms multiple sources of residual organic matter such as high-strength industrial wastewater, sewage sludge, livestock waste, municipal solid waste or food waste (Nguyen et al., 2019) into biogas (mainly composed of 45–70 % CH_4 and 35–55 % CO_2) (Angelidaki et al., 2019). In the past decade, Europe has become the world's leading region in the field of AD with >18k digesters in operation by the end of 2018 (Grobrügge, 2019). Despite the effectiveness of AD to treat residual organic matter, its economic sustainability is limited by the low price of natural gas and decreasing prices of renewable electricity from wind or solar farms. Therefore, alternatives that confer higher added-value to biogas (than electricity and heat) are needed to guarantee the cost-competitiveness of AD.

In this context, the development of innovative technology platforms for the bio-production of chemicals from the CH_4 contained in biogas has emerged as a potential alternative to overcome the above-mentioned limitations of AD (Kleerebezem et al., 2015). Thermochemical processes for biogas conversion involve energy intensive methods based on chemical catalysts, high temperature and high pressure. On the other hand, biotechnologies are less energy intensive, operate under atmospheric pressure, ambient temperature and a wide range of environmental conditions (*e.g.*, O_2/CH_4 concentrations, temperature, pH, salinity and nutrient requirements), which induce the synthesis of a broad portfolio of bio-products (Becker and Wittmann, 2020).

Ectoine, an extremolyte and osmoprotector, first detected in *Halorhodospira halochloris*, is the industrial flagship of CH_4-based biochemicals (Galinski et al., 1985). Ectoine is a compatible compound

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that allows stable water-water interactions between microorganisms and their environment by shielding proteins and cell membranes. In addition, ectoine protects human cells from allergens, heat, dryness and UV light, making ectoine a valuable compound in the cosmetic industry. In the pharmaceutical sector, ectoine is used as a component of lung inhalation fluids and nasal spray products (Pastor et al., 2010). This makes ectoine the most valuable product bio-synthesized by any microorganism nowadays (~1000€/Kg) (Cantera et al., 2020). Ectoine can be chemically (*i.e.*, abiotically) synthetized but this method implies the use of expensive precursors such as diaminobutyric acid (Lentzen and Neuhaus, 2009). Current industrial production of ectoine is based on a pure culture of *Halomonas elongata* ATCC 33173 in a process originally denominated "bacterial milking" (Sauer and Galinski, 1998). Ectoine production is based on a high-cell-density fermentation fed with the costly substrate glucose, subsequent biomass harvesting and an osmotic shock of the microorganisms that release ectoine to the extracellular medium (Kunte et al., 2014). Although industrial ectoine production is based on the use of glucose, CH_4 contained in biogas stands out as an attractive feedstock from the point of view of obtaining an added-value product from waste (*i.e.*, the anaerobic digestion process) considering the metabolic similarity among ectoine-producing microorganisms. When comparing the metabolic pathways of two well-known ectoine producing microorganisms, *Methylomicrobium alcaliphilum* 20Z (Cantera et al., 2019) and *Chromohalobacter salexigens* (Pastor et al., 2010), one can see that the metabolic pathway of *M. alcaliphilum* 20Z is similar to the one of a halophilic/halotolerant heterotroph that uses glucose as raw material. Both known metabolic pathways employ the following three enzymes: diaminobutyric acid (DABA) aminotransferase (EctB), DABA acetyltransferase (EctA), and ectoine synthase (EctC). Together, these enzymes catalyze the conversion of the precursor aspartate into ectoine.

On the other hand, the accumulation of ectoine's derivative hydroxyectoine was firstly reported in *Streptomyces parvulus* (Inbar and Lapidot, 1988) and later observed in the halophilic microorganism *Marinococcus* sp. M52 (Frings et al., 1995). Hydroxyectoine possesses an even greater stabilization capacity with similar physicochemical properties to ectoine. Unfortunately, the industrial-scale production of hydroxyectoine has not been yet developed. In this context, the production of ectoine and hydroxyectoine from biogas will eventually decrease the operating costs and environmental impacts compared to glucose-based fermentations. Additionally, the use of biogas as feedstock will enhance the cost-competitiveness of AD by creating a new added-value chain from organic waste.

Methanotrophs (CH_4-oxidizing microorganisms) have recently emerged as a platform for the bio-production of value-added products such as ectoine and hydroxyectoine from biogas (AlSayed et al., 2018). The ability of the alkaliphilic methanotroph *Methylomicrobium alcaliphilum* 20Z to synthesize ectoine from pure CH_4 has been investigated at varying CH_4, Cu^{2+} and NaCl concentrations under batch (Cantera et al., 2016) or continuous (Cantera et al., 2017) operation in classical stirred tanks (with accumulations of 67 and 37 $mg_{ectoine}\ g_{biomass}^{-1}$, respectively) or high-mass-transfer bioreactors (109 $mg_{ectoine}\ g_{biomass}^{-1}$) (Cantera et al., 2020). Interestingly, very few studies have targeted the production of ectoine (94 $mg_{ectoine}\ g_{biomass}^{-1}$) from biogas to date (Cantera et al., 2018). In this context, the enrichment of novel halophilic methanotrophic consortia capable of synthesizing ectoine and hydroxyectoine from a low-cost feedstock such as biogas stands out as a highly attractive alternative for the production of these added-value bio--chemicals due to the intrinsic resilience of the process from a microbiological perspective and the non-requirement of sterile conditions. Pure cultures of methanotrophic microorganisms seems to accumulate less amount of ectoine and are less robust when compared to methanotrophic consortia (Cantera et al., 2020). However, downstream operation might be facilitated by the presence of a lower spectrum of metabolites. Furthermore, there is a limited understanding of the influence of environmental conditions (*i.e.*, pH, temperature, nutrients requirements, NaCl content, etc.) on the biosynthesis of these extremolytes from biogas, which supports the need for further research. With respect to CH_4 concentration, its influence at 2, 4 and 20 % has previously been tested in *Methylomicrobium alcaliphilum* 20Z and a direct correlation with ectoine production has been found (Cantera et al., 2016).

A strategy to boost ectoines accumulation is based on the study of micronutrients in the cultivation broth, which are known to influence primary methane oxidation in methanotrophs (Semrau et al., 2018). Among them, tungsten has recently been shown to activate formate dehydrogenase in *Methylomicrobium alcaliphilum* 20Z (Akberdin et al., 2018b). The lack of this compound causes the accumulation of formic acid in the medium, resulting in a lower biomass yield and potential inhibitory effects. In this context, tungsten supplementation could eventually increase the productivity of ectoines in methanotrophic cultures. The effect of environmental parameters on ectoine synthesis is species specific and must be evaluated for each novel consortium (Cantera et al., 2016). Table S1 shows a compilation of tested environmental parameters for the accumulation of ectoines using methane contained in biogas as feedstock.

This work aimed at optimizing ectoine and hydroxyectoine productivity from biogas as a feedstock using novel mixed methanotrophic consortia enriched from natural saline environments and activated sludge. The influence of i) NaCl and ii) tungsten concentrations and iii) incubation temperatures on biomass yield, ectoine and hydroxyectoine content, O_2:CH_4 consumption ratios, and the volumetric and specific CH_4 biodegradation rate was investigated. Finally, a molecular characterization of the key microbial communities present in the enrichments was conducted.

2. Materials and methods

2.1. Reagents

All chemicals and reagents were purchased from either PANREAC, Sigma Aldrich or Merck, with a purity higher than 99.0 %. The synthetic biogas mixture, composed of CH_4 (70%) and CO_2 (30 %), was purchased from Air Products and Chemicals, Inc (Spain). *Real biogas was not available at the facilities where these experiments were carried out. However, the future scaling-up of the technology will be tested with real biogas in the framework of the European DEEP-PURPLE (No. 837998) project.*

2.2. Mineral salt medium

Mineral salt medium (MSM) was used in all experiments unless stated otherwise. 1 L of MSM contained 20 mL of phosphate buffer, 45 mL of $NaHCO_3$ buffer, 100 mL of NMS2 (10 ×) solution and 2 mL of trace elements solution. The NMS2(10 ×) solution contained (per L): 2.0 g $MgSO_4{\cdot}7H_2O$, 0.13 g $CaCl_2{\cdot}2H_2O$ and 10.0 g KNO_3. The phosphate buffer solution contained (per L): 5.44 g KH_2PO_4 and 6.25 g $Na_2HPO_4{\cdot}2H_2O$. The $NaHCO_3$ buffer contained (per L): 84 g $NaHCO_3$. The trace elements solution contained (per L): 5.0 g Na_2EDTA, 0.076 g $CuCl_2{\cdot}2H_2O$, 2.0 g $FeSO_4{\cdot}7H_2O$, 0.1 g $ZnSO_4{\cdot}7H_2O$, 0.02 g $NiCl_2{\cdot}6H_2O$, 0.2 g $CoCl2{\cdot}6H_2O$, 0.035 g $Na_2MoO_4{\cdot}2H_2O$, 0.03 g $MnCl_2{\cdot}4H_2O$ and 0.03 g H_3BO_3(Atlas, 2010).

2.3. Bioreactors for methanotrophs cultivation

Gas-tight custom-made glass bioreactors of 1200 mL of total volume, with external diameter of 107 mm, height of 214 mm and nozzle 14/23 were used for cultivation of methanotrophs (Afora S.A., Spain). Gas-tightness was preserved via plastic screw caps and butyl septa. Bioreactors were filled up with 490 mL MSM, 10 mL of inoculum and 700 mL of a mixture of air and synthetic biogas with a final composition of 109 g O_2 m^{-3} (18 %) and 54 g CH_4 m^{-3} (9 %). These O_2 and CH_4 headspace concentrations were obtained by filling the bioreactors with

the above-described volumes of MSM and inoculum. Then, the headspace of the bioreactors was flushed with air for 5 min and firmly closed with air-tight butyl septa and plastic screw caps. Then, 100 mL of the air headspace were removed with a calibrated 100 mL gas-tight syringe (Model 1100 SL SYR, Hamilton, USA) and replaced with 100 mL of synthetic biogas contained in a Tedlar gas-tight bag (Supelco, USA). Unless otherwise specified, a constant temperature of 25 °C was used for incubation under continuous magnetic agitation at 300 rpm. Bioreactors were operated in fed-batch modus. O_2 and CH_4 were daily monitored via gas chromatography. Once CH_4 was completely exhausted from the headphase, the previously described mixture of air and synthetic biogas was again replenished.

2.4. Inocula

Three locations in Spain were selected to collect two samples of salty sediments, Poza de la Sal, (Burgos); and Medinaceli, (Soria) and one sample of activated sludge from Estiviel WWTP, (Toledo) (Table 1).

2.5. Enrichment of halotolerant methanotrophs

An enrichment was initially carried out for each environmental sample in the bioreactors described in section 2.3 and outlined in Fig. S1. A concentration of 3 % NaCl was used during the batch enrichments of halotolerant methanotrophs. Aliquots of 10 mL from the previous culture were sequentially transferred 4 times to new bioreactors with 490 mL of fresh MSM and 700 mL of a headspace composed of 109 g O_2 m^{-3} (18 %) and 54 g CH_4 m^{-3} (9 %). During the last enrichment, the bioreactor headspace was sequentially replaced 3 times following CH_4 depletion. The headspace of the bioreactors was periodically sampled using 100 μl gas-tight syringes to monitor O_2 and CH_4 consumption by GC-TCD. The methanotrophic enriched consortia with the highest ectoines content (one per location) were selected and used as inocula for further assays.

2.6. Influence of the incubation temperature, NaCl and tungsten concentrations on extremolytes production and microbial growth

For each enriched methanotrophic consortium Assays with NaCl concentrations of 0, 3, 6, 9 and 12 %, incubation temperatures of 15, 25 and 35 °C, and tungsten (provided as $Na_2WO_4{\cdot}H_2O$) concentrations of 0, 35 and 70 μg W L^{-1} were carried out in duplicate as described above (using 10 mL of inoculum and 490 mL of MSM) and depicted in Fig. 1. The headspace of the gas-tight bioreactors, which was replaced 4 times with the initial air/biogas atmosphere, was periodically monitored by GC-TCD using 100 μl gas-tight syringes. These data were used to estimate the O_2:CH_4 consumption ratios and volumetric CH_4 consumption rates. Liquid samples were drawn at the end of each assay in order to determine biomass yields, ectoines yields, specific CH_4 consumption rates and microbial population structure. With respect to headspace composition, different CH_4 and CO_2 concentrations could have been also studied as part of the experimental design. However, CH_4 and CO_2 concentrations were kept constant at the beginning as above detailed. The influence of CH_4 (Cantera et al., 2016) and CO_2 (Cantera et al., 2020) on ectoines accumulation has elsewhere been studied. Regarding CO_2, the authors have found that no significant effect of the gas headspace composition was observed on the production of ectoine with or without this compound. On the other side, the influence of CH_4 has been tested by using different concentrations (*e.g.*, 2, 4 and 20 %) and showing a direct correlation of CH_4 with ectoine accumulation.

Table 1
Environmental samples used for the enrichment of halotolerant methanotrophs.

Code	Location	Samples	Coordinates	Description
Activated sludge	Toledo	2	39°52′30.8″N 4°07′23.3″W	Activated sludge collected at Estiviel WWTP
Salt lagoon	Burgos	4	42°40′08.9″N 3°30′11.1″W	Sediments sampled from a salt lagoon
Salt river	Soria	2	41°09′14.8″N 2°24′36.4″W	Sediments sampled from a salt river

2.7. Extremolytes extraction procedure

Culture broth was concentrated 3 fold by centrifugation of 2 mL at 9000×*g* for 10 min in an Eppendorf tube as previously reported (Cantera et al., 2020). The final pellet was washed twice with a solution of 6 % NaCl in Milli-Q water to avoid any interference of the salts present in the MSM during the analysis of the extraction broth by HPLC-UV (*i.e.*, overlapping of peaks). An aliquot of 2 mL of ethanol at 70 % and 25 ± 5 mg of 0.1-mm-diameter zirconia/silica beads (BioSpec, Spain) were added to the Eppendorf tube containing the pellet in order to extract hydroxyectoine and ectoine from microbial cells. Cell disruption was performed in a Mini-BeadBeater-16 (BioSpec, Spain) at 1048×*g* for 1 min. The supernatant of the suspension was centrifuged at 9000×*g* for 15 min and filtered through 0.22 mM filters prior analysis by HPLC-UV. The intra-cellular hydroxyectoine and ectoine concentration (in $mg_{extremolyte}$ $g_{biomass}^{-1}$) was calculated using the final biomass concentration estimated as Volatile Suspended Solids (in g_{VSS} L^{-1}) of the corresponding cultivation broth.

2.8. Analytical procedures

HPLC-UV was used to measure hydroxyectoine and ectoine in a 717 plus auto-sampler coupled with a UV Dual λ Absorbance detector at 220 nm and 40 °C using a LC-18 AQ þ C Supelcosil column and a C18 AQ + pre-column (Supelco) as reported elsewhere (Cantera et al., 2020). A phosphate buffer (0.8 mM $K_2HPO_4{\cdot}3H_2O$ and 6.0 mM Na_2HPO_4 $12H_2O$) was used as mobile phase at 25 °C and a flow rate of 1 mL min^{-1}. The quantification of both extremolytes was carried out using external standards of commercially available hydroxyectoine ((4S, 5S)-5-Hydroxy-2-methyl-1,4,5,6-tetrahydropyrimidine-4-carboxylic acid, purity ≥ 95 %, Sigma Aldrich) and ectoine ((S)-b-2-methyl-1,4,5, 6-tetrahydro pyrimidine-4-carboxylic acid, purity ≥ 95 %, Sigma Aldrich) prepared in Ethanol-70 %. Retention times of hydroxyectoine and ectoine ranged from 1.91 to 1.94 and from 2.32 to 2.35 min depending on the column pressure, respectively. The headspace composition (mainly CH_4, CO_2, O_2 and N_2) of the gas-tight bioreactors was determined using a Varian CP-3800 GC-TCD (Palo Alto, USA) coupled with a thermal conductivity detector and equipped with a CP-Molsieve 5A (15 m × 0.53 mm × 15 μm) and CP-PoraBOND Q (25 m × 0.53 mm × 15 μm) columns. Injector and detector temperatures were maintained at 150 and 175 °C, respectively. Helium at 13.7 mL min^{-1} was used as the carrier gas. Total (TSS) and Volatile (VSS) suspended solid concentrations were measured according to standard methods (Rice et al., 2012).

2.9. Data treatment

The apparent biomass yield ($Y_{Biomass}$, gVSS gCH_4^{-1}) was calculated by dividing the final biomass mass (in gVSS) in batch assay tests by the total mass of methane consumed (in gCH_4) in the four cycles of incubation. The volumetric methane consumption (q_{CH4}, gCH_4 m^{-3} h^{-1}) was computed from the slope of the time course plot of methane concentration within the linear range of the batch cultivation tests carried out. O_2:CH_4 consumption ratios (dimensionless) were obtained from the slope of the graph of methane versus oxygen concentrations (in mol basis). Finally, the specific CH_4 biodegradation rate (SBDR, g CH_4 $gVSS^{-1}$ h^{-1}) was calculated according to Equation 1 from the slope of the time course plot of methane concentration within the linear range in the last 4th cycle of the batch cultivation test carried out divided by the final biomass concentration recorded at the end of test (Cantera et al.,

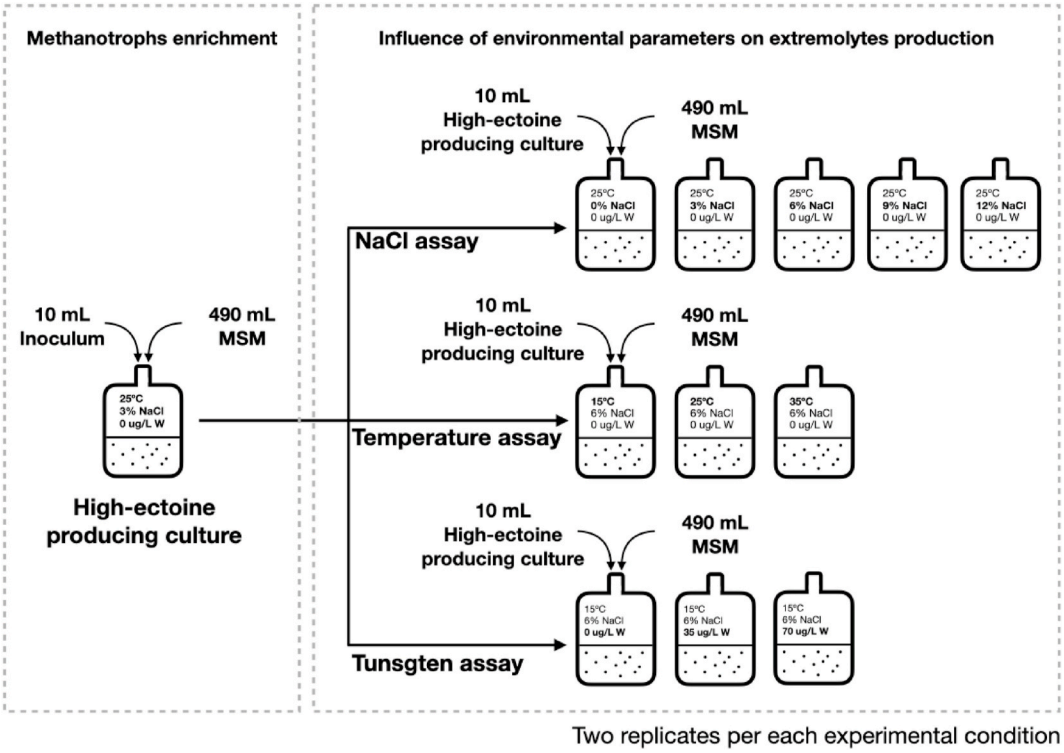

Fig. 1. Overview of the experimental procedures used for the evaluation of the influence of environmental parameters on extremolytes productivity in mixed methanotrophic cultures.

2016). An example of the calculation of these parameters is outlined in Fig. S5. Equation 1.

$$SBDR = \frac{q_{CH4}\left(g\ m^{-3}\ h^{-1}\right)\ V_{Headspace}(m^3)}{Biomass\left(g\ L^{-1}\right)\ V_{Liquid}(L)}$$

2.10. Bacterial community analysis

From the 36 different experimental conditions tested for the accumulation of extremolytes (i.e., incubation temperatures and NaCl/Tungsten concentrations) using 3 enriched methanotrophic consortia only a total of 9 representative samples were selected for bacterial community analysis (Fig. S2). The selection considered those microbial consortia showing the highest ectoines accumulation (1 sample per location, i.e., Activated sludge, Sal lagoon and Salt river): 3 samples from the enrichment phase, 3 samples from the NaCl assay and 3 samples from the incubation temperature assay. No samples from the tungsten assay were selected for bacterial community analysis due to the lack of significant effects of this trace element on extremolytes accumulation.

A total of 50 ng of DNA was amplified following the 16S Metagenomic Sequencing Library Illumina 15044223 B protocol (Illumina, Inc.). In the first amplification step, primers were designed containing: i) a universal linker sequence allowing amplicons for incorporating indexes and sequencing primers by Nextera XT Index Kit (Illumina, Inc.); and ii) 16S rRNA gene universal primers (Klindworth et al., 2013). In the second and last assay, amplification indexes were included. 16S based libraries were quantified by fluorimetry using the Quant-iT™ PicoGreen™ dsDNA Assay Kit (Thermofisher Scientific). Libraries were pooled prior to sequencing on the MiSeq platform (Illumina, Inc.), 250 cycles paired reads configuration. The size and quantity of the pool were assessed on the Bioanalyzer 2100 (Agilent Technologies, Inc.) and with the Library Quantification Kit for Illumina (Kapa Biosciences), respectively. PhiX Control Library (v3) (Illumina, Inc.) was combined with the amplicon library (expected at 20 %). Sequencing data were available within approximately 56 h. Image analysis, base calling and data quality assessment were performed on the MiSeq instrument (MiSeq Control Software, MCS v3.1). Raw sequences, forward and reverse, were merged in order to obtain the complete sequence using the "pear v0.9.6" software. The ends of the sequences can be overlapped to obtain complete sequences using this approach. The amplification primers from the sequences obtained in the sequencing step were trimmed to reduce the bias in the annotation step, with "cutadapt v1.8.1" and parameters by default. Once the primers have been removed, sequences lower than 200 nts were removed from the analysis. It should be stressed that short sequences have a higher chance of generating erroneous taxonomical group associations. After obtaining the clean complete sequences, a quality filter was applied to them to delete poor quality sequences. Those bases in extreme positions that did not reach Q20 (99 % well incorporated base in the sequencing step) or a greater phred score were removed. Subsequently, sequences whose average quality did not surpass the Q20 threshold, as a mean quality of the whole sequence, were also deleted. These FASTQ files were converted to FASTA files and the UCHIME program version 7.0.1001 was used in order to remove chimeras that could arise during the amplification and sequencing step. Those clean FASTA files were BLAST27 again NCBI 16s rRNA database using blastn version 2.2.29+. The resulting XML files were processed using a phyton script developed by the company Lifesequencing S. L.-ADM in order to annotate each sequence at the different phylogenetic levels (Phylum, Family, Genus and Species). The nucleotide sequence dataset obtained in this study can be accessed from the public database Sequence Read Archive with the accession number PRJNA743851 (http://www.ncbi.nlm.nih.gov/bioproject/743851).

3. Results and discussion

3.1. Enrichment of halotolerant methanotrophs capable of biogas conversion into ectoine and hydroxyectoine

O_2 and CH_4 consumption, concomitantly with CO_2 production,

occurred during all the enrichments carried out, which confirmed the presence of aerobic halotolerant methanotrophs in the salt lagoon, Estiviel WWTP and salt river. CH_4 was initially depleted within 5 and 6 days in the first enrichments from the Salt lagoon and Salt river, respectively, while 12 days were required for a complete CH_4 consumption in the bioreactors inoculated with Activated sludge. No significant differences in CH_4 and O_2 consumption were recorded among enrichments during the sequential replacements of the bioreactor headspace in the last phase of the enrichment procedure. The bioconversion of CH_4 into extremolytes was later confirmed by HPLC-UV. Thus, maximum ectoine concentrations of 25.44, 18.88 and 15.37 mg $g_{biomass}^{-1}$, and hydroxyectoine concentrations of 7.62, 7.03 and 13.77 mg $g_{biomass}^{-1}$ were recorded in enrichments from Estiviel WWTP (Sludge 1), salt lagoon (Poza 3) and salt river (Medi 7), respectively (Fig. S1). Apparent biomass yields of 0.38, 0.46 and 0.37 $g_{biomass}$ g_{CH4}^{-1} were recorded from the activated sludge, the salt lagoon, and the salt river enrichments. No correlation was observed between extremolytes accumulation and apparent biomass yields. A more detailed discussion about the influence of saline stress, incubation temperature and the presence of micronutrients such as tungsten on biomass yield from methane consumption is given below. Hereinafter, the term inoculum refers to the enriched consortia.

3.2. Influence of NaCl concentration

The three methanotrophic enriched consortia used as inocula, from now on referred as activated sludge, salt lagoon and salt river, accumulated increasing extremolytes concentrations when increasing NaCl concentrations from 0, to 3 and 6% (Fig. 2). Salt lagoon was the only enrichment that showed growth and extremolytes accumulation at 9 % NaCl. None of the inocula showed either O_2 or CH_4 consumption or extremolytes accumulation at 12 % NaCl, which indicated that such extreme saline conditions entailed a prohibitive osmotic stress for the enriched microbial communities. The inoculum with the highest extremolyte production capacity was salt lagoon at a NaCl concentration of 9 % (56.6 ± 2.5 $mg_{ectoine}$ $g_{biomass}^{-1}$ and 51.0 ± 2.0 $mg_{hydroxyectoine}$ $g_{biomass}^{-1}$) (Fig. 2A). Indeed, the environmental sample resulting in the inoculum salt lagoon was collected from a salt lagoon exploited since Roman times. The second and third highest ectoine-producing inocula were activated sludge (34.1 ± 0.1 $mg_{ectoine}$ $g_{biomass}^{-1}$) and salt river (31.9 ± 4.9 $mg_{ectoine}$ $g_{biomass}^{-1}$) at a NaCl concentration of 6 % (Fig. 2A). In a similar experimental approach (Cantera et al., 2016), evaluated the bioconversion of CH_4 into ectoine by a pure culture of the well-known ectoine-producing methanotroph *Methylomicrobium alcaliphilum* 20Z under varying concentrations of CH_4 (%), Cu^{2+} (uM) and NaCl (%) and incubation temperatures. The ectoine yields herein obtained at 6 % NaCl and 25 °C (~30 $mg_{ectoine}$ $g_{biomass}^{-1}$ regardless of the inocula used) were lower than those obtained by (Cantera et al., 2016) under similar environmental conditions (67 $mg_{ectoine}$ $g_{biomass}^{-1}$). It is worth noting that (Cantera et al., 2016) tested the accumulation of this extremolyte with a pure culture of *M. alcaliphilum* 20Z as inoculum and 20 % CH_4 instead of 9 % CH_4. The use of a specialized strain in the bioconversion of CH_4 into ectoine and the higher CH_4 concentration (20 %) could explain the higher ectoine yields obtained by (Cantera et al., 2016). On the other hand, the enrichment from the salt lagoon also produced the highest amount of hydroxyectoine per gram of biomass at 9 % NaCl (51.0 ± 2.0 $mg_{hydroxyectoine}$ $g_{biomass}^{-1}$) followed by the enrichment from the salt river at 6 % NaCl (25.5 ± 1.9) and from Estiviel WWTP at 6 % NaCl (13.5 ± 2.12 $mg_{hydroxyectoine}$ $g_{biomass}^{-1}$) (Fig. 2B). Overall, the accumulation of ectoine was higher than that of hydroxyectoine.

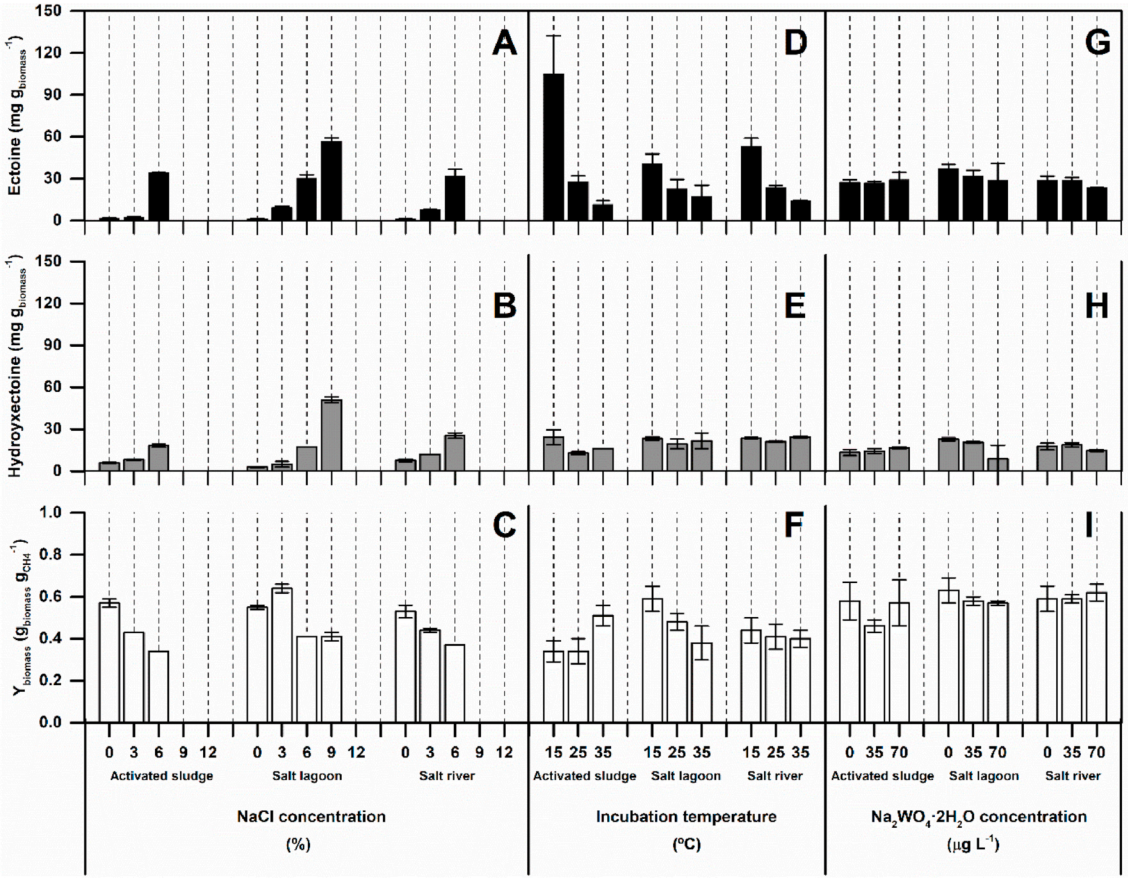

Fig. 2. Influence of NaCl concentration (A–C), incubation temperature (D–F) and tungsten concentration (G–I) on ectoine and hydroxyectoine intracellular contents and apparent biomass yields ($Y_{biomass}$).

However, the higher market price of this osmolite makes hydroxyectoine a promising product to be industrially obtained from biogas. The extremolyte concentrations herein reported correspond only to the metabolites extracted from the intracellular content of methanotrophs, since it was previously shown that the amount of extracellular ectoine content was negligible (Cantera et al., 2016). Organic osmoprotectors are kept inside cells under high salinity conditions due to the salt dependent expression of ATP-binding cassette transporters (ABC transporters), which are able to pump organic molecules against their concentration gradient with ATP consumption. This salt dependent expression of ABC transporters has been observed in RNA sequencing experiments with *Methylomicrobium alcaliphilum* 20Z (Bordel et al., 2020b). Activation and deactivation of ABC transporters is also at the basis of ectoine extraction by bio-milking (Czech et al., 2016).

Halophilic microorganisms use two strategies to grow in high salinity environments: the so-called salt-in and salt-out strategies (Czech et al., 2018). The salt-in approach relies on the accumulation of K^+ and Cl^- ions inside the cells and the excretion of Na^+ ions. This strategy is energetically favourable but less useful in environments where salinity fluctuates. On the other hand, the salt-out strategy is based on the accumulation of compatible solutes such as ectoine and hydroxyectoine inside the cell. These extremolytes, besides compensating the differences in osmotic pressure between the cytosol and the cellular environment, also form and stabilize protective water layers around macromolecules and cell structures (Becker and Wittmann, 2020), which allows them resisting conditions of high molecular crowding without getting denatured. Thus, the intracellular ectoine concentration is expected to follow a linear correlation with NaCl concentration (*i.e.*, the higher the salt concentration, the higher the extremolyte accumulation) since ectoine is produced as a response to salinity. The results herein obtained indicate that such correlation is valid as long as the salt content in the cultivation broth allows the proper growth of halotolerant methanotrophs. In our particular study, the salinity range at which the methanotrophs were able to grow was different among the three enrichments tested. Halotolerant methanotrophs able to grow in biogas as a carbon and energy source up to a salinity of 9 % NaCl were herein enriched. However, such high salinity of 9 % NaCl is expected to cause a severe corrosion in bioreactors during the continuous production of ectoines, which should be considered during process scale-up.

Overall, an inverse correlation between the apparent biomass yields and the extremolytes accumulation or NaCl concentration was observed (Fig. 2C). Indeed, the lower the biomass yields the higher the amount of extremolytes accumulated. Apparent yields of 0.57 ± 0.02, 0.43 ± 0.00 and 0.34 ± 0.00 $g_{biomass}\ g_{CH4}^{-1}$ and 0.55 ± 0.01, 0.64 ± 0.02 and 0.41 ± 0.00 $g_{biomass}\ g_{CH4}^{-1}$ were recorded at 0, 3 and 6% NaCl in the enrichments from activated sludge and the salt lagoon, respectively. Similarly, the apparent yields of the enrichments from the salt river at 0, 3 and 6% NaCl averaged 0.53 ± 0.03, 0.44 ± 0.01 and 0.37 ± 0.00 $g_{biomass}\ g_{CH4}^{-1}$. These $Y_{biomass}$ were well in agreement with those found in the literature for either pure (0.45–1.20 $g_{biomass}\ g_{CH4}^{-1}$) or mixed (0.70–0.82 $g_{biomass}\ g_{CH4}^{-1}$) cultures of methanotrophs (Akberdin et al., 2018b; Bordel et al, 2019a, 2019b, 2020a, 2019b; Carere et al., 2019; Fergala et al, 2018a, 2018b, 2018c, 2018a; Hakobyan et al., 2020) (see also Table S2). The high osmotic stress caused by the high salinity in the cultivation broth, along with the additional energy costs for extremolyte synthesis and operation of sodium pumps such as the Na (+)-translocating NADH-quinone reductase (NQR), are responsible for the lower biomass yields observed. Hence, the methanotrophs within the consortia herein enriched used an important fraction of the methane consumed in the energy yielding reactions (generating CO_2) and in the production of either ectoine or hydroxyectoine, which reduced the share of methane available for biomass build-up.

The complete oxidation of 1 mol of CH_4 requires 2 mol of O_2 in the absence of biomass formation (Cantera et al., 2016). The ratio O_2:CH_4 consumed has been recently used as a proxy to elucidate the CH_4 oxidation mechanism in methanotrophs (Bordel et al., 2019a). No significant correlation was found between the O_2:CH_4 ratio and NaCl concentration, with values ranging from 1.18 to 1.25, 1.00–1.45 and 1.40–1.43 in the enrichments from activated sludge and the salt lagoon and salt river, respectively. These values are below 1.5, which is the theoretical ratio for methanotrophs using the so-called redox arm mechanism of CH_4 oxidation, characteristic of type II methanotrophs such as those of the genus methylocystis (Bordel et al., 2019a). This is consistent with the predominance of methanotrophic organisms using the direct coupling oxidation mechanism. This was further confirmed by the 16S analysis, which revealed the presence of *Methylomicrobium buryatense* in many of the consortia. The concentration of NaCl was negatively correlated with the volumetric CH_4 biodegradation rates in the salt river and salt lagoon enrichments. Hence, the increase from 3 to 6% NaCl resulted in a decrease in q_{CH4} from 0.92 ± 0.06 to 0.56 ± 0.00 g $CH_4\ m^{-3}\ h^{-1}$ in the salt lagoon enrichment and from 0.89 ± 0.00 to 0.42 ± 0.03 g $CH_4\ m^{-3}\ h^{-1}$ in the salt river enrichment, respectively. However, no significant differences in q_{CH4} were found when NaCl concentrations increased from 0 to 3%. The increase in NaCl concentration mediates a decrease in the aqueous solubility of gases, which might not have determined the q_{CH4} values recorded. The lack of a consistent correlation between qC_{H4} and NaCl concentration in the three enrichments tests suggests that the deterioration in microbial activity under high salinity conditions likely governed the volumetric CH_4 consumption rates. Finally, the specific CH_4 biodegradation rates, which ranged between 6.17 ± 0.07 and 9.99 ± 0.03 $mg_{CH4}\ g_{biomass}^{-1}\ h^{-1}$, did not exhibit any significant correlation with NaCl concentrations likely due to the absence of a consistent correlation between qC_{H4} and NaCl concentration (Fig. 3G).

3.3. Influence of the incubation temperature

An accumulation of extremolytes concomitant with CH_4 biodegradation occurred at the three tested incubation temperatures independently of the inocula used (Fig. 2D and E). An increase in ectoine concentration was observed at decreasing incubation temperatures regardless of the enrichment tested. Thus, 105.0 ± 27.2, 27.9 ± 4.0 and 11.5 ± 2.8 $mg_{ectoine}\ g_{biomass}^{-1}$ and 40.8 ± 6.9, 22.7 ± 6.8 and 16.9 ± 8.4 $mg_{ectoine}\ g_{biomass}^{-1}$ were achieved at 15, 25 and 35 °C in activated sludge and salt lagoon enrichments, respectively. Likewise, the cultivation of the enrichments from the salt river at 15, 25 and 35 °C induced ectoine concentrations of 53.0 ± 6.1, 23.7 ± 1.3 and 14.2 ± 0.5 $mg_{ectoine}\ g_{biomass}^{-1}$, respectively. This correlation has consistently been shown in literature (García-Estepa et al., 2006; Kuhlmann et al., 2008; Onraedt et al., 2005). For instance, the accumulation of ectoine at lower temperatures in a pure culture of *Virgibacillus pantothenticus* was mediated by the increased transcription of the ectABC operon, which is known to induce the biosynthesis of ectoine in multiple microorganisms (Kuhlmann et al., 2008). In this context (Cantera et al., 2016), did not observe a significant increase in ectoine concentration at decreasing temperatures in *Methylomicrobium alcaliphilum*, although a narrower temperature range was evaluated (25–35 °C). Interestingly, hydroxyectoine concentration did not exhibit a significant correlation with temperature, with values ranging from 16.0 ± 0.2 to 24.2 ± 5.4, 19.4 ± 3.5 to 23.3 ± 1.1 and 21.1 ± 0.5 to 24.4 ± 0.6 $mg_{hydroxyectoine}\ g_{biomass}^{-1}$ in activated sludge, salt lagoon and salt river enrichments, respectively. On the other hand, temperature did not exert a consistent impact on the apparent biomass yields, which decreased from 0.59 ± 0.06 to 0.38 ± 0.08 $g_{biomass}$ g CH_4^{-1} when the incubation temperature increased from 15 to 35 °C in salt lagoon enrichments, but remained constant at 0.40–0.44 $g_{biomass}$ g CH_4^{-1} in the salt river enrichments (Fig. 2F).

The increase in the incubation temperature resulted in higher O_2:CH_4 consumption ratios. Thus, O_2:CH_4 consumption ratios of 1.10 ± 0.08, 1.35 ± 0.04 and 1.38 ± 0.03 were recorded in the activated sludge enrichment, 1.05 ± 0.06, 1.42 ± 0.01 and 1.52 ± 0.11 in the salt lagoon enrichment, and 1.08 ± 0.15, 1.50 ± 0.04 and 1.56 ± 0.02 in the salt river enrichment at 15, 25 and 35 °C, respectively. The decrease in

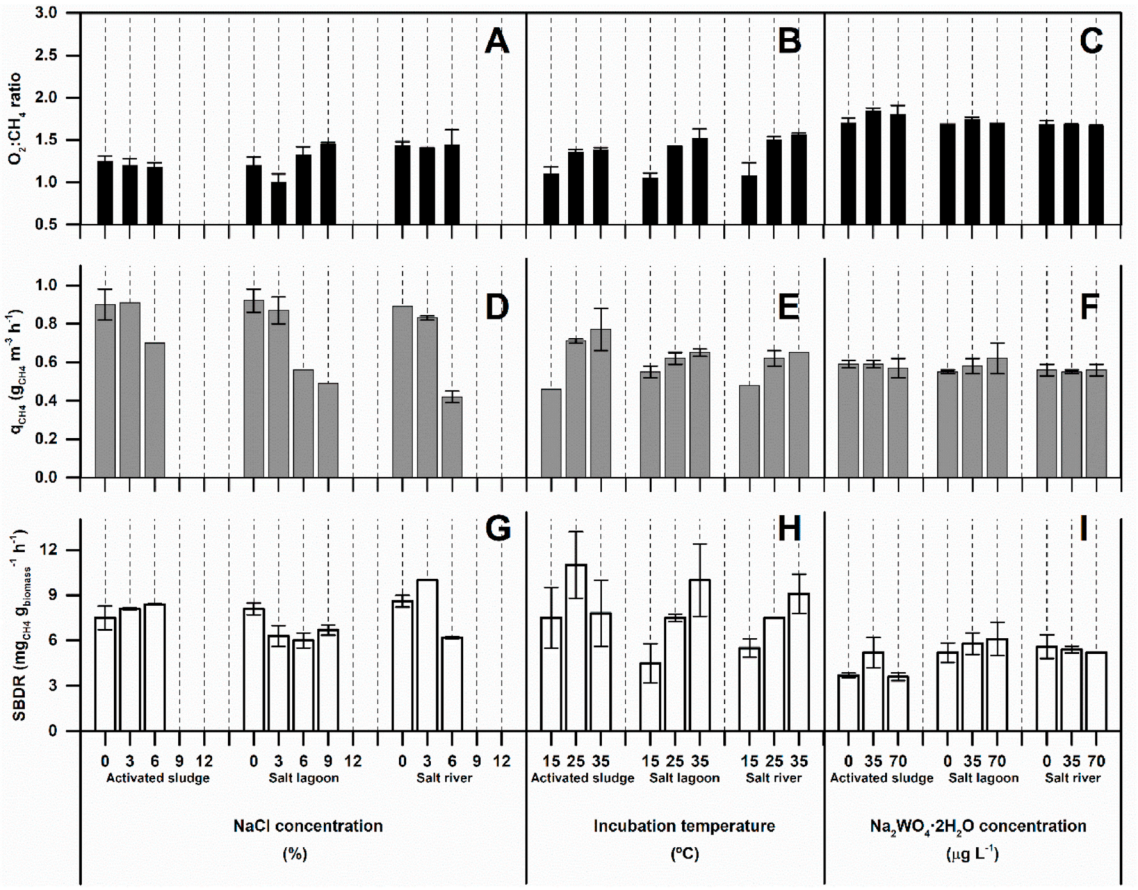

Fig. 3. Influence of the NaCl and tungsten concentrations and temperature on A-C) Oxygen and methane consumption ratio, D-F) Volumetric methane consumption rate and G-I) Specific methane biodegradation rate.

metabolic activities non associated to CH_4 biodegradation in non methanotrophic bacterial groups might be associated with the lower O_2:CH_4 consumption ratios observed at the lower temperatures. A similar correlation was observed between qCH_4 and the temperature of incubation. Volumetric CH_4 biodegradation rates of 0.46 ± 0.00, 0.71 ± 0.01 and 0.77 ± 0.11 g CH_4 m^{-3} h^{-1} were recorded in the activated sludge enrichment, 0.55 ± 0.03, 0.62 ± 0.03 and 0.65 ± 0.02 g CH_4 m^{-3} h^{-1} in the salt lagoon enrichment, and 0.48 ± 0.00, 0.62 ± 0.04 and 0.65 ± 0.00 g CH_4 m^{-3} h^{-1} in the salt river enrichment at 15, 25 and 35 °C, respectively. Despite the fact that higher temperatures entail lower CH_4 gas-liquid mass transfers as a result of the lower CH_4 aqueous solubility, the increase in methanotrophic activity mediated the higher CH_4 biodegradation rates observed regardless of the enrichment. The consistent enhancement in q_{CH4} and decrease $Y_{biomass}$ at increasing temperatures of incubation supported a positive linear correlation between the specific CH_4 biodegradation rates and temperature in the enrichments obtained from the salt lagoon and salt river. Thus, specific CH_4 biodegradation rates of 4.48 ± 1.31, 7.52 ± 0.25 and 10.19 ± 2.40 mg_{CH4} $g_{biomass}^{-1}$ h^{-1} and 5.55 ± 0.62, 7.48 ± 0.01 and 9.06 ± 1.26 mg_{CH4} $g_{biomass}^{-1}$ h^{-1} were recorded in the salt lagoon and salt river enrichments, respectively, at 15, 25 and 35 °C (Fig. 3H). No consistent trend between the specific CH_4 biodegradation rates and temperature was observed in the enrichment from Estiviel WWTP.

3.4. Influence tungsten concentration

The presence of microelements in the cultivation broth is known to influence primary methane oxidation in methanotrophs (Semrau et al., 2018). For instance, three metabolic-switches have been described in methanotrophs: i) a copper-switch that regulates the activity of methane oxidation, ii) a tungsten-switch that controls formate oxidation and iii) a lanthanum-switch for conversion of methanol into formate (Akberdin et al., 2018a). Tungsten is not typically added to methanotrophs cultivation media, but its addition has been recently reported to increase biomass yield up to 7–8% and reduce formate driven inhibition (Akberdin et al., 2018b). In this context, tungsten addition could eventually increase the productivity of ectoines. However, the addition of tungsten at 35 and 70 µg W L^{-1} did not impact ectoine or hydroxyectoine accumulation. Hence, ectoine contents ranging from 26.9 to 29.2, from 28.6 to 37.3 and from 23.7 to 28.9 $mg_{ectoine}$ $g_{biomass}^{-1}$ were recorded in the activated sludge, salt lagoon and salt river enrichments, respectively (Fig. 2 G). Similarly, hydroxyectoine contents ranging from 13.4 to 16.7, from 8.7 to 22.8 and from 14.7 to 18.8 $mg_{hydroxyectoine}$ $g_{biomass}^{-1}$ were achieved in the activated sludge, salt lagoon and salt river enrichments, respectively, with no significant influence of tungsten concentration (Fig. 2H). The apparent biomass yields did not change significantly in the presence of tungsten and remained in the range of 0.46 ± 0.03 to 0.63 ± 0.06 $g_{biomass}$ g_{CH4}^{-1} (Fig. 2I). Tungsten has been shown to activate formate dehydrogenase in *Methylomicrobium alcalyphilum* 20Z (Akberdin et al., 2018b). The lack of this compound causes the accumulation of formic acid in the medium, resulting in a lower biomass yield and potential inhibitory effects. In a complex consortium, other organisms are likely to use the excreted formate as a substrate for growth, which could explain the lack of effects of tungsten in the studied consortia.

No significant correlation was found between the O_2:CH_4 consumption ratio and tungsten concentration, with values ranging from 1.70 ± 0.06 to 1.84 ± 0.04, 1.69 ± 0.00 to 1.74 ± 0.03 and 1.67 ± 0.01 to 1.68 ± 0.05 in the enrichments from activated sludge, salt lagoon and salt river, respectively. Similarly, the volumetric CH_4 consumption rates and

the specific CH_4 biodegradation rates were not significantly influenced by the tungsten concentration. Interestingly, all enrichments showed similar q_{CH4} values ranging from 0.55 ± 0.01 to 0.62 ± 0.08 g_{CH4} m^{-3} h^{-1} and specific CH_4 biodegradation rates ranging from 3.63 ± 0.25 to 6.13 ± 1.07 mg_{CH4} $g_{biomass}^{-1}$ h^{-1} (Fig. 3I). Finally, it should be highlighted that that the evaluation of the influence of tungsten during methanotrophic enrichment is worth trying and it should be considered in future experiments.

3.5. Structure of the enriched methanotrophic consortia

The molecular analysis addressed first the presence of methanotrophs with a relative abundance ≥1 % in order to elucidate their contribution to the bioconversion of methane to ectoines. Proteobacteria, Bacteroidetes, Actinobacteria, Chlamydiae, Balneolaeota and Planctomycetes were the most abundant phyla (Fig. S4). Interestingly, Type I and II methanotrophs are present in the Gamma- and Alphaproteobacteria phylum (Murrell, 2010). *Methylomicrobium buryatense* (abundance *ca.* 2–9%) and *Methylomicrobium japanense* (abundance *ca.* 4–15 %) from the *Methylococcaceae* family were identified as the main methanotrophs present in the cultivation broths of the samples analysed (Fig. 4) (Dedysh et al., 2004). The well-known ectoine-producing methanotroph *M. buryatense* was isolated for the first time from the sediments of Southeastern Transbaikal soda lakes (pH 9.5–10.5) (Kaluzhnaya et al., 2001). Strains of *M. buryatense* were described as bacteria able to grow at temperatures ranging from 15 to 37 °C, pH from 6.0 to 11.0 and NaCl salinities from 0.2 to 5 % (w/v). The growth conditions reported in literature for *M. buryatense* matched those used in the assays herein conducted and explain its presence in 6 of the 9 cultivation broth selected (Fig. 4). On the other side, the slightly halophilic methanotroph *M. japanense* was first reported as a bacterium isolated from marine sediments able to grow between 0 and 9 % NaCl (optimally below 6 % NaCl) (Kalyuzhnaya et al., 2008). The optimal temperature range for *M. japanense* is 15–37 °C, negligible growth being detected below 4 °C or above 45 °C. These optimal growth conditions also support the occurrence of *M. japanense* in 6 of the 9 microbial consortia here selected for analysis (Fig. 4). It is worth noticing that *M. buryatense* and *M. japanense* were only simultaneously detected with a relative abundance ≥1 % in the sludge enrichment cultivated at 6 % NaCl and 15 °C (exhibiting 105.0 ± 27.2 $mg_{ectoine}$ $g_{biomass}^{-1}$), and in the enrichment from the salt river cultivated at 6 % NaCl and 25 °C. In the rest of the samples analysed only one of these methanotrophs, either *M. buryatense* or *M. japanense*, was present with a relative abundance value ≥ 1 % (Fig. 4).

The contribution of microorganisms with a relative abundance lower than 1 % might be of importance in any biotechnological processes devoted to the production of added-value molecules from waste (Rafrafi et al., 2013). In particular, those microorganisms that may use inhibitory intermediates of methane oxidation excreted by methanotrophs into the medium might play a key role in process stability (Doronina et al., 2003a,b). Besides *Methylomicrobium buryatense* described earlier, other ectoine-producing methanotrophs were detected in the selected samples although with an abundance <1 %. An extensive bibliographic screening revealed that 10 microbial species herein identified are able to accumulate ectoine (Table S3). For instance, several microorganisms belonging to the *Methylomicrobium* genus such as *M. kenyense*, *M. alcaliphilum* and *M. album* were present in the analysed samples. An interesting finding was the detection in all selected samples of *Methylophaga alcalica*, a microorganism belonging to the genus *Methylophaga* (typically aerobic, halophilic and non-methane-utilizing methylotrophs) (Kim et al., 2007). However, *M. alcalica* has been consistently shown to consume methane and accumulate the extremolyte ectoine (N. Doronina et al., 2003a,b). Methane oxidation by other members of the *Methylophaga* genus has been inferred by their contribution to the consumption of methane released at oil spills (Gutierrez and Aitken, 2014). Other 13 methanotrophs have also been detected in the selected samples but at

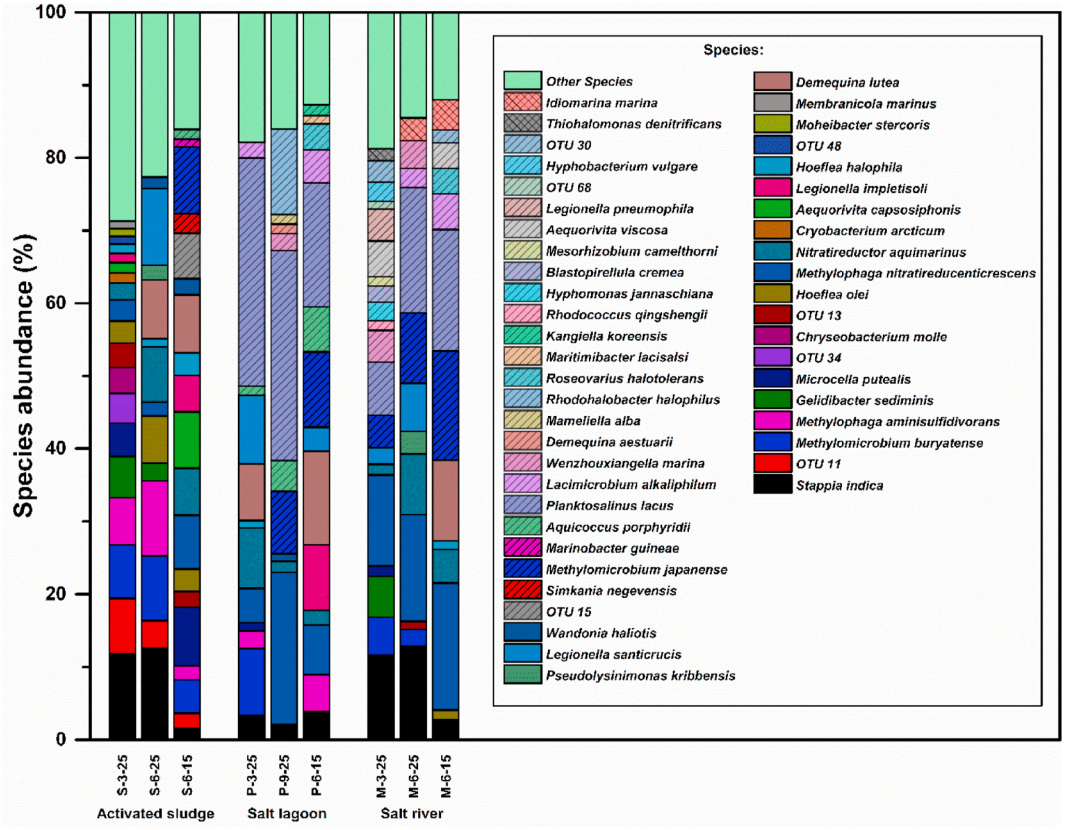

Fig. 4. Community structure at species level in the selected samples.

very low relative abundances (Table S3). On the other hand, 21 microbial species with the ability to use intermediates of the methane-oxidation metabolism were detected at a low abundance value (≤1 %) (Table S3). Although methane oxidation has not been experimentally confirmed in these bacteria, two microorganisms within the *Methylophaga* genus (*M. muralis* and *M. lonarensis*) and one within the *Methylobrevis* genus (*M. pamukkalensis*) are able to accumulate ectoine (Antony et al., 2012; Poroshina et al., 2015; Shmareva et al., 2018).

Finally, it is worth noticing that the total amount of methanotrophs, independently of their relative abundance, ranged between 7 and 16 % (Table S3), while bacteria using intermediates of methane oxidation accounted for 7–19 % of the total bacterial population. In this context, these bacteria were capable of converting up to a 3 % of the available CH_4 into ectoine despite their abundance did not exceed 19 % of the total sample (Fig. S3).

The molecular analysis of the communities selected also revealed the presence of non-methanotrophs or bacteria without a known evident connection to a methanotrophic metabolism but with a relative abundance >1 %. These bacteria have been reported to grow under the tested experimental conditions of saline concentrations (0–12 % NaCl) and temperature (15-35 °C) (Table S4). Besides these microbiological growth characteristics, no specific function within the consortium was possible to be assigned for these bacteria. The 10 most abundant microorganisms detected were *Stappia indica*, *Gelidibacter sediminis*, *Microcella putealis*, *Chryseobacterium molle*, *Hoeflea olei*, *Nitratireductor aquimarinus*, *Cryobacterium arcticum*, *Aequorivita capsosiphonis*, *Legionella impletisoli* and *Hoeflea halophila*. The saline conditions used during the assays here reported might have triggered the accumulation of the extremolytes ectoine and hydroxyectoine in these species. Most of the species do not have a common ecological niche but have been found in locations with similar alkali-/saline-characteristics (Table S4): i) sediments collected from the sea, ii) Soda lakes, iii) oil contaminated waters, iv) soils within a saline environment and v) sewage sludge.

4. Practical applications and future research prospects

Bioconversion of greenhouse gases such as methane has several practical important implications, not only from the point of view of the proper bioresource management (either solid or liquid waste), via its valorisation into biogas but also from the point of view of the production of added value molecules from biowaste in the framework of a circular bioeconomy scheme.

The future research prospects in the field of ectoine production from non-conventional feedstocks such as biogas are two. The first one involves bringing the technology readiness level of biogas bioconversion into ectoines to an industrial scale where the main bottleneck of the technology are properly assessed. In this context, ectoine production yields from biogas bioconversion should be comparable to the only commercial industrial fermentation process that uses glucose as substrate and the halotolerant microorganism *Halomonas elongata*. The second research prospect involves unravelling the metabolic pathways that allows the biotransformation into value-added products (and thus, their mitigation) of two of the greenhouse gases with a significant contribution to climate change: CH_4 and CO_2. This requires increasing fundamental and applied research efforts.

Overall, the technology here described represents an alternative and promising means of bioresource valorisation beyond the well-established technology of anaerobic digestion for biogas production. The high cost of ectoine entails an economically feasible process that can boost the use of anaerobic digestion as waste stabilization technology in developing countries.

5. Conclusions

This work aimed at optimizing the accumulation of ectoine and hydroxyectoine from biogas using mixed microbial consortia enriched from saline environments and activated sludge. Ectoines's accumulation was positively correlated with NaCl concentration, regardless of the enrichment. Salt lagoon's enrichment grown at 9 % NaCl exhibited the highest content of ectoine and hydroxyectoine. Interestingly, the lower the temperatures of incubation, the higher the ectoine yields and the lower the volumetric CH_4 biodegradation rates. The micronutrient tungsten did not entail a significant change in the extremolite yields or biomass yields. The relative abundance of known ectoine-accumulating methanotrophs ranged from 7 to 16% in all analysed samples, with *M. buryatense* and *M. japanense* as dominant species. This paper confirmed the feasibility of biotransforming the methane contained in biogas and oxygen into ectoines using mixed microbial consortia enriched with methanotrophs obtained from multiple environments.

Credit author statement

Alessandro A. Carmona-Martínez: Methodology, Validation, Formal analysis, Investigation, Data Curation, Writing - Original Draft, Writing - Review & Editing, Visualization. Eva Marcos-Rodrigo: Validation, Formal analysis, Investigation, Data Curation, Writing - Review & Editing. Sergio Bordel: Conceptualization, Methodology, Validation, Writing - Review & Editing, Supervision. David Marín: Investigation, Writing - Review & Editing. Raquel Herrero-Lobo: Investigation, Writing - Review & Editing. Pedro A. García-Encina: Writing - Review & Editing. Raúl Muñoz: Conceptualization, Validation, Resources, Writing - Original Draft, Writing - Review & Editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jenvman.2021.113462.

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