Assessment of active methanogenic archaea in a methanol-fed upflow anaerobic sludge blanket reactor

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ABSTRACT

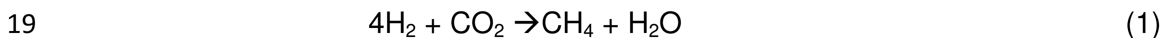
Methanogenic archaea enrichment of a granular sludge was undertaken in an upflow anaerobic sludge blanket reactor (UASB) fed with methanol in order to enrich methylotrophic and hydrogenotrophic methanogenic populations. A microbial community assessment, in terms of microbial composition and activity –throughout the different stages of the feeding process with methanol and acetate– was performed using specific methanogenic activity assays (SMA), quantitative real-time polymerase chain reaction (qPCR), and high throughput sequencing of *16S* rRNA genes from DNA and cDNA. Distinct methanogenic enrichment was revealed by qPCR of *mcrA* gene in the methanol-fed community, being two orders of magnitude higher with respect to the initial inoculum, achieving a final *mcrA*/*16S r*RNA ratio of 0.25. High throughput sequencing analysis revealed that the resulting methanogenic population was mainly composed by methylotrophic archaea (*Methanomethylovorans* and *Methanolobus* genus), being also highly active according to the RNA-based assessment. SMA confirmed that the methylotrophic pathway, with a direct conversion of methanol to CH_4, was the main step of methanol degradation in the UASB. The biomass from the UASB, enriched in methanogenic archaea, may bear great potential as additional inoculum for bioreactors to carry out biogas production and other related processes.

Keywords

UASB, biogas upgrading, gene expression, RNA, methanogenic biomass enrichment

Introduction

Biogas production in anaerobic digestion plants is spreading due to its potential as an alternative to fossil fuels. This renewable energy carrier can be stored and used in different applications, such as heating or electricity production, or upgraded to biomethane to inject into the grid or use as transport fuel. Raw biogas consists mainly of methane (CH_4, 40-75%) and carbon dioxide (CO_2, 15-60%), and trace amounts of other components such as water (H_2O, 5-10%), hydrogen sulphide (H_2S, 0.005-2%) or ammonia (NH_3, <1%) (Ryckebosch et al. 2011). In order to transform biogas into biomethane, a cleaning and upgrading process should be performed. Upgrading consist in the adjustment of the calorific value of the biogas –separating CH_4 from CO_2– generally performed in order to meet the standards required to use it as vehicle fuel or for injection in the natural gas grid. After biogas transformation, applying techniques for biogas upgrading such as pressure swing adsorption, membrane separation or chemical CO_2-absorption, the final product obtained typically contains 95-97% of CH_4 and 1-3% of CO_2 (Ryckebosch et al. 2011). An alternative to these enrichment techniques, focused on CO_2 removal without changing CH_4 mass, is biological methane enrichment using hydrogenotrophic methanogenic populations capable of using CO_2 as a carbon source and H_2 as an energy source, and convert them to CH_4 (Equation 1) (Strevett et al. 1995), or even capable of obtaining these electrons directly from the cathode in a process known as electromethanogenesis (Cheng et al. 2009).

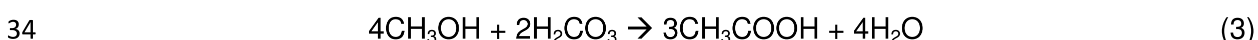


$$4H_2 + CO_2 \rightarrow CH_4 + H_2O \quad (1)$$

Hydrogenotrophic methanogens belong to the orders *Methanobacteriales*, *Methanococcales*, *Methanomicrobiales* and *Methanosarcinales* (Karakashev et al. 2005). Thus, obtaining a biomass rich in these microorganisms to be used as inoculum could accelerate the start up of biogas production, and of other related bioreactors processes.

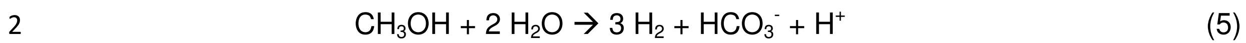
Upflow anaerobic sludge blanket reactors (UASB) are suitable for enriching methanogenic archaea because they can be operated at low hydraulic retention times (HRT). Bhatti et al. (1996) investigated the feasibility of methanolic waste treatment in an UASB reactor and demonstrated that methanol can be converted to methane via at least three routes. Later, Vavilin (2010) developed a model for explaining the metabolic pathways for methanol degradation with ^{13}C-labeled methanol. Methanol can either be i) directly converted to methane by methylotrophic methanogens (Equation 2), ii) generated via the intermediate formation of acetate (acetogenesis) and later converted to methane by acetoclastic methanogens (Equations 3 and 4), iii) or by hydrogenotrophic methanogens, with the use of H_2 and CO_2 (Equation 5 and 6).

$$4CH_3OH \rightarrow 3CH_4 + CO_2 + 2H_2O \quad (2)$$

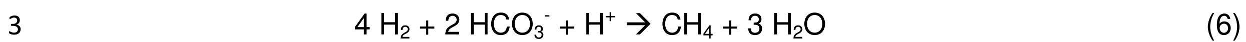


$$4CH_3OH + 2H_2CO_3 \rightarrow 3CH_3COOH + 4H_2O \quad (3)$$

$$CH_3COOH + H_2O \rightarrow CH_4 + H_2CO_3 \quad (4)$$



$$CH_3OH + 2\ H_2O \rightarrow 3\ H_2 + HCO_3^- + H^+ \quad (5)$$



$$4\ H_2 + 2\ HCO_3^- + H^+ \rightarrow CH_4 + 3\ H_2O \quad (6)$$

Methanol was used in a previous study to favour the emergence of hydrogenotrophic methanogens when treating domestic wastewater at low to moderate temperatures (<20 ºC), and improve methane generation (Saha et al. 2015). Therefore, methanol feeding can be an alternative to CO_2/H_2 gassing or cultivation in an electrochemical bioreactor, to enrich an inoculum with hydrogenotrophic methanogenic archaea.

The main aim of this study was to assess the utilization of a methanol-fed UASB as a system for enriching a granular sludge in methanogenic archaea and characterise the evolution of the microbial community when shifting from acetate to methanol substrate, in terms of composition and activity, using quantitative real-time polymerase chain reactions (qPCR) and high throughput sequencing of *16S rDNA* and *16S rRNA*. Specific methanogenic activity tests (SMA) were also performed so as to corroborate the results obtained through the microbial community analysis on active methanol routes in the UASB.

Materials and methods

Experimental set-up

A lab-scale UASB reactor with a working volume of 0.5 L was used. The reactor was constructed with glass and equipped with a water jacket to keep the temperature at mesophilic temperature range (35 ºC). Peristaltic pumps were used to control the influent feed rate and the recirculation rate. The reactor was inoculated with 100 mL of anaerobic granular sludge (volatile suspended solids content, VSS, of 59.60 g kg^{-1}) taken from a full-scale UASB reactor processing fruit juice wastewater (Mollerussa, Spain). The inoculum was stored at 4 ºC until its utilization in this study.

Reactor operation

The UASB was fed in continuous mode with a mineral medium, with a hydraulic retention time (HRT) fixed at 6 h. The reactor was operated for 416 days in 3 different phases (Table 1). The UASB was initially fed with an acetate influent for 214 days, increasing the organic loading rate (OLR) from 3 to 10 $kg_{DQO}\ m^{-3}\ d^{-1}$ in order to activate the biomass and acclimate it to a high OLR (Phase 1). Having achieved high operational performance, the feed was progressively changed to a methanol substrate during 21 days (Phase 2). And finally, only methanol was used as substrate for another 180 days in order to promote the enrichment in methanogenic archaea biomass (Phase 3). The mineral medium contained acetate and/or methanol as organic carbon source, in

concentrations shown in Table 1 for each phase, and (per litre of deionised water): NH_4Cl, 1.33 g; $CaCl_2$, 0.04 g ; KH_2PO_4, 3 g; Na_2HPO_4, 6 g; $MgSO_4$ 0.25 g; yeast extract, 0.1 g and 1 mL of a trace mineral solution. The trace mineral solution contained (per litre of deionised water): $FeCl_3 \cdot H_2O$, 1.50 g; H_3BO_3, 0.15 g; $CuSO_4 \cdot 5H_2O$, 0.03 g; KI, 0.18 g; $MnCl_2 \cdot 4H_2O$, 0.12 g; $Na_2MoO_4 \cdot 2H_2O$, 0.06 g; $ZnSO_4 \cdot 7H_2O$, 0.12 g; $CoCl_2 \cdot 6H_2O$, 0.15 g; $NiCl_2 \cdot 6H_2O$, 0.023 g; EDTA, 10 g (Lu et al. 2006).

Specific methanogenic activity (SMA)

SMAs of the anaerobic granular sludge used as inoculum, and those at the end of phase 1 (acetate operation) and 3 (methanol operation) were evaluated at 36 ºC in serum bottles (120 mL), in duplicate (Angelidaki et al. 2009; Silvestre et al. 2015; Soto et al. 1993). Acetate, a VFA mix (acetate/propionate/butyrate, 70/20/10), methanol and H_2 were used as substrates. The serum bottles were filled with a 50 mL solution of the granular sludge (5 g_{VSS} L^{-1}), substrate (5 g_{COD} L^{-1}), macronutrients, micronutrients and bicarbonate (1 $g_{NaHCO3-}$ $g_{CODadded}^{-1}$). A control duplicate without the medium was included in the setup. The bottles were sealed with rubber stoppers and capped with aluminium crimp caps. The headspace was purged for 5 min with N_2 in order to remove O_2. Methane production was monitored periodically taking a gas sample (0.2 mL) from the head space with a gas-tight syringe, and analysing the gas composition by gas chromatography. The SMA was calculated from the linear increase in the CH_4 concentration at the beginning of the experiments – when no lag phase was observed– divided by the amount of VSS.

Analytical methods and calculations

Reactor head space methane content, soluble chemical oxygen demand (CODs) and pH of the UASB effluent were used as control parameters for each experimental condition. Volatile suspended solids (VSS), CODs and pH (CRISON 2000 pH electrode) were determined according to Standard Methods 5220 (APHA 1999). CODs removal efficiency was calculated from the difference between influent and effluent concentrations, divided by the influent concentration. Biogas composition (CH_4, CO_2) was analysed using a VARIAN CP-3800 (Varian, USA) gas chromatograph equipped with a thermal conductivity detector (TCD).

Microbial community analysis

A microbial community assessment in the initial UASB inoculum and in the granular sludge at the end of Phase 1 (acetate feed) and 3 (methanol feed) was performed using both culture-independent molecular techniques –quantitative real-time polymerase chain reactions (qPCR) and high throughput sequencing (MiSeq, Illumina) of 16S rDNA and 16S rRNA. Nucleic acid extracts such as DNA, RNA, and cDNA were stored frozen at -80 ºC.

1 ***Nucleic acid extraction and complementary DNA (cDNA) synthesis***

2 Simultaneous total genomic DNA and RNA (including rRNA) were extracted from triplicate
3 independent samples at each sampling event from known weights (granular biomass) by means of
4 PowerMicrobiome™ RNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, CA, USA), according to
5 the manufacturer's instructions. Purified mRNA and rRNA were obtained by removal of co-
6 extracted DNA with DNase I incubation (provided in the kit) at 25 ºC for 10 min, and inactivation of
7 DNase I with EDTA 50 mM (Thermo Scientific Fermentas, USA) at 75 ºC for 5 min. A reverse
8 transcription step PCR (RT-PCR) for cDNA synthesis from the obtained RNA was performed using
9 a PrimeScript™ RT Reagent Kit (Takara Bio Inc., Japan). The reaction was carried out in a final
10 volume of 30 µL which contained 15 µL of purified RNA, 6 µL of PrimeScript™ buffer, 1.5 µL of
11 retrotranscriptase mix, 1.5 µL of Random 6 mers, and 6 µL of RNase Free dH_2O. Henceforth, the
12 term cDNA or 16S rRNA is used to refer to the extracted RNA or 16S amplicons from cDNA, as a
13 measure of gene expression and microbial activity, whereas DNA or 16S rDNA terms will be used
14 to refer to the extracted genomic DNA and 16S amplicons from DNA.

15

16 ***Quantitative PCR assay (qPCR)***

17 Total and expressed gene copy numbers of eubacterial *16S rRNA* gene and *mcrA* gene
18 (methanogenic archaeal methyl coenzyme-M reductase) were quantified by means of quantitative
19 real-time PCR (qPCR). Each sample was analysed in triplicate by means of the three independent
20 DNA and RNA (cDNA) extracts. The analysis was carried out with the Brilliant II SYBR Green
21 qPCR Master Mix (Stratagene, La Jolla, CA, USA) in a Real-Time PCR System Mx3000P
22 (Stratagene) following the protocol described elsewhere (Cerrillo et al. 2016).
23 Standard curve parameters of the qPCRs show that the reactions performed, highly efficient, were
24 as follows (for *16S* rRNA and *mcr*A, respectively): slope of -3.515 and -3.558; correlation
25 coefficient of 0.999 and 0.996; efficiency of 93 and 91%.

26

27 ***High throughput sequencing of 16S rDNA and 16S rRNA and data analysis***

28 Simultaneous extracts of DNA and RNA (cDNA), obtained from the initial inoculum and the UASB
29 granular sludge, were assessed by RT-qPCR analysis and high throughput 16S-sequencing
30 purposes. The specific steps followed during the MiSeq analysis of massive libraries of 16S rDNA
31 and 16S rRNA both for eubacteria and archaea were carried out as follows. Massive bar-coded
32 *16S rRNA* gene libraries (16S rDNA and 16S rRNA), targeting eubacterial region V1-V3 *16S rRNA*
33 and archaeal region V3-V4, were sequenced utilizing MiSeq equipment (Illumina, San Diego, CT,
34 USA). Each DNA or cDNA was amplified separately (16S rDNA and 16S rRNA respectively) with
35 both *16S*-based eubacteria and archaea set of primers. For eubacteria libraries the primers set
36 was 27F (5'-AGRGTTTGATCMTGGCTCAG–3') and 519R (5'-GTNTTACNGCGGCKGC TG-3'),
37 while the archaeal set of primers was 349F (5'-GYGCASCAGKCGMGAAW-3') and 806R (5'–

GGACTACVSGGGTATCTAAT-3'). The sequencing step was performed at MR DNA (www.mrdnalab.com, Shallowater, TX, USA) on a MiSeq instrument following the manufacturer's guidelines. The obtained reads were compiled in FASTq files for further bioinformatics processing, following the steps described elsewhere (Sotres et al. 2016). OTUs were then taxonomically assigned using the Ribosomal Database Project (RDP training set 14) Naïve Bayesian Classifier (http://rdp.cme.msu.edu), and compiled into each taxonomic level with a bootstrap cutoff value of 80% (Cole et al. 2009; Wang et al. 2007).

The data obtained from sequencing datasets were submitted to the Sequence Read Archive of the National Center for Biotechnology Information (NCBI) under study accession number SRP071847 for eubacterial and archaeal populations.

Diversity indices estimators (Shannon (*H'*), *inverted Simpson* and sampling Goods *coverage*) were calculated with the defined OTUs table (shared.file) using MOTHUR software, version 1.34.4, for each sample, normalising the number of reads of all samples to those of the sample with the lowest number of reads (http://www.mothur.org) (Schloss et al. 2009). Statistical multivariate analysis by means of correspondence analysis (CA) on the OTUs abundance matrix of Eubacterial *and* Archaeal OTUs distribution was performed. The obtained samples and predominant OTUs were depicted in a 2D biplot (relative abundance above 1%). Statistical multivariate correspondence analysis of MiSeq data was performed by means of XLSTAT 2014 software (Addinsoft, Paris, France).

Results

Operation performance

Average COD removal efficiencies and methane content in the biogas for each phase are shown in Table 2. The COD removal efficiency by the end of Phase 1 was of 82±12%, gradually increasing during the acetate shift to methanol, up to an average value of 97±1% by the end of Phase 3. Methane content in the head space of the reactor increased from 68±14%, when using acetate as feed, to 85±1% during the methanol fed phase. The low methane content during Phase 1 was partly due to operational problems with the outlet of the reactor, which led to air flowing into the head space.

Metabolic pathways and granular sludge activity

To better understand the metabolic pathways of methanol in the UASB (Vavilin 2010), and assess the activity of the biomass in the UASB reactor, both the SMA of the granules used as inoculum and from the samples taken at the end of Phase 1 –acetate activated–, and Phase 3 –methanol feed–, were determined with different substrates (VFA mix, acetate, H_2, and methanol). Table 3 shows that at the end of Phase 3, the granular sludge had a high SMA for methanol, acetate and

VFA mix (470; 239 and 220 mg COD_{CH4} g^{-1} VSS d^{-1}, respectively), while being 20-40 fold lower for H_2 (12 mg COD_{CH4} g^{-1} VSS d^{-1}). Nevertheless, this latter value increased 6 times with respect to the one corresponding to the acetate feeding phase (2 mg COD_{CH4} g^{-1} VSS d^{-1}). Again, the VFA mix and acetate SMA showed values 70 times higher than those of the H_2 assay.

Microbial community assessment

The microbial community structure and the activity of the samples taken from the initial inoculum and the biomass in the UASB by the end of Phase 1 (acetate feeding) and 3 (methanol feeding) were characterised by means of qPCR technique and high throughput sequencing (MiSeq) of the *16S* rRNA gene –of the total and active eubacteria and archaea.

Quantitative analysis by qPCR

qPCR results of the 3 samples, regarding DNA (present microorganisms) and cDNA (active microbial populations), for *16S* rRNA (eubacteria) and *mcr*A (methanogenic archaea) gene copy numbers showed a progressive increase in *mcr*A gene copy numbers from the inoculum to the biomass sample by the end of Phase 3 (Figure 1). This result correlates with the observed increase in methane content in the biogas in the UASB. An increase of two orders of magnitude of *mcrA* gene (at DNA level) in Phase 3, in comparison to the initial inoculum ($1.13 \cdot 10^{10}$ and $1.25 \cdot 10^{8}$ gene copy numbers g^{-1}, respectively) was revealed; while the *mcrA* expression (cDNA level) in Phase 3 was twice the one obtained in Phase 1 ($4.76 \cdot 10^{8}$ and $2.46 \cdot 10^{8}$ gene copy numbers g^{-1}, respectively). On the contrary, *16S* rRNA gene copy numbers remained in the same order of magnitude in both Phases and the initial inoculum. As a consequence, the highest *mcr*A/*16S* rRNA gene ratio achieved was of 0.25, by the end of Phase 3.

Sequencing results for eubacteria and archaea

During high throughput sequencing analysis (MiSeq) 2,770 and 483 OTUs were detected for eubacteria and archaea, respectively, with 50,466-64,777 reads for eubacteria, and 66,226-121,706 reads for archaea. Figure 2a shows the relative abundance of eubacterial *phyla* in the inoculum and the anaerobic granular sludge of the UASB at the end of Phase 1 (acetate feeding) and 3 (methanol feeding), both at DNA and RNA (cDNA) level. Although *Proteobacteria* was the predominant *phylum* in the inoculum (39%), *Bacteroidetes*, *Firmicutes* and *Synergistetes* grew into the most abundant ones in Phase 1 (40, 26 and 14%, respectively) and Phase 3 samples (61, 14 and 8%, respectively). At gene expression level (cDNA), the relative abundance of the predominant *phylum* was consistent with the one obtained for DNA, except for an increase to 22% in *Proteobacteria* in Phase 1 sample and a general reduction of *Synergistetes phylum*. At family level, between 24% and 74% of the OTUs were unclassified, Phase 3 sample showing the highest

1 values (Figure 2b). Of the classified OTUs, *Pseudomonadaceae* accounted for 37% of the relative
2 abundance in the inoculum, it being below 1% in the granular sludge of the UASB in Phase 1 and
3 Phase 3. *Porphyromonadaceae*, *Ruminococcaceae* and *Synergistaceae* were the predominant
4 families in Phase 1 (28, 13 and 14%, respectively), which were less abundant in Phase 3 (14, 6
5 and 8%, respectively). The first family, *Porphyromonadaceae,* maintained its predominance as an
6 active group (cDNA level) in Phase 1 (19%), and *Desulfobulbaceae* revealed itself as a highly
7 active family (15%) in spite of its low relative abundance (2%) at DNA level. Finally, in Phase 3
8 sample, no clear dominant active families were highlighted, due to the high number of unclassified
9 OTUs (74%).
10 For archaea population, Figure 3 shows a clear *Methanosarcinaceae* family enrichment in the
11 UASB, particularly in Phase 3, both in community composition and activity (52 and 64% of relative
12 abundance, respectively). On the contrary, *Methanotrichaceae*, an acetotrophic family formerly
13 known as *Methanosaetaceae*, was clearly reduced during Phase 3 due to methanol feeding.
14 Although maintaining 19% of relative abundance at DNA level, it solely represented 3% of all OTUs
15 at cDNA level.
16
17 ***Biodiversity analysis***
18 Table 4 shows the results for the biodiversity analysis performed on UASB granular sludge
19 samples. The Inverted Simpson and Shannon indices for archaea population decreased
20 throughout the entire operation time of the UASB, and when the change from acetate to methanol
21 feeding was carried out. The inoculum was the most diverse sample, followed by Phase 1 and
22 Phase 3 samples. This biodiversity reduction in Phase 3 is observed not only at community
23 composition level, but also at activity level. For eubacterial population, both indices showed that
24 the inoculum sample was the least diverse community. The highest biodiversity was harboured by
25 the Phase 1 sample, according to the Shannon index but, according to the Inverted Simpson index,
26 it was harboured by the Phase 3 sample. On the contrary, both indices were the highest in Phase 1
27 when it comes to gene expression (cDNA).
28
29 ***Correspondence analysis***
30 Correspondence analysis results for eubacteria community are shown in Figure 4a. A clear
31 evolution in population was evidenced with the change of feed, from the inoculum to Phase 1
32 sample, using acetate, and from Phase 1 to Phase 3 sample, with methanol as a substrate. DNA
33 (16S rDNA) and cDNA samples (16S rRNA) for each phase were clustered together, suggesting
34 that few differences could be found between existing and active microorganisms. Therefore, the
35 distribution of the samples agrees with the discussion of the sequencing results. In archaea
36 correspondence analysis, Phase 1 sample remained near to the inoculum when looking at DNA

composition but moved away when looking at gene expression. Phase 3 samples, as in the case of eubacteria community, were clustered together and far from the 3 other samples.

Discussion

COD removal efficiency by the end of Phase 3 (97±1%) was in the range of the 86-98%, obtained with a similar OLR as the one previously described by Badshah et al. (2012). These values are also comparable or even higher than those described in previous studies with higher OLR, such as 97.1% and 92.5% with an OLR of 30 and 48 kg_{DQO} m^{-3} d^{-1}, respectively (Kobayashi et al. 2011; Lu et al. 2015); showing high adaptation of the UASB biomass to the methanol feeding.

The results obtained from the SMA of the granules used as inoculum and from the samples taken at the end of Phase 1 –acetate activated–, and Phase 3 –methanol feed– (Table 3) suggest that in spite of the long term operation of the reactor with methanol as the sole carbon source (180 days), the granular sludge did not completely lose its acetate utilisation capacity. Other studies have reported the loss of acetic activity after long periods of methanol feeding (Paulo et al. 2003). A recent study stated that after operating an UASB with methanol for 143 days, the granules presented an acetate SMA of 150 mg COD_{CH4} g^{-1} VSS d^{-1}, losing completely this capacity after 300 days of operation (Lu et al. 2015). In this same study, higher SMA values were achieved for H_2 and methanol (0.08 and 2.11 g COD_{CH4} g^{-1} VSS d^{-1}, respectively) than those obtained in the present study. From the SMA results, it can be concluded that the main pathway in the UASB reactor for methanol conversion to methane was more likely methylotrophic methanogenesis, although the conservation of acetic activity suggests that the acetogenesis-acetoclastic route may be taking place as well. Finally, such a low hydrogenotrophic activity indicates that the methanol oxidation followed by hydrogenotrophic methanogenesis was not promoted in the operational conditions applied in the UASB, the hydrogenotrophic methanogenic enrichment not taking place. Nevertheless, these hypotheses should be confirmed by a microbial community assessment simultaneously performed.

qPCR results prove that a progressive enrichment in methanogenic archaea was taking place in the reactor biomass, and that its activity was coincident with an enhancement of methane production. Since the ratio between methanogenic archaea and eubacteria in the biomass clearly increased during the methanol feeding phase of the UASB, it may harbour a great potential as inoculum for biogas production and other related bioreactor processes.

High throughput sequencing showed that no clear dominant active eubacteria family was highlighted in Phase 3 (Figure 2b), due to the high number of unclassified OTUs (74%). 78% of the unclassified OTUs obtained in Phase 3 cDNA sample corresponded to *Bacteroidetes phyla*, and 21% to *Firmicutes*. These OTUs which cannot be assigned to a known family could be novel taxa or perhaps still poorly defined in the RDP database. As for archaea, the presence of *Methanotrichaceae* in the granular sludge (Figure 3) is relevant since it correlates well with the

results obtained in the SMA test, in which the acetic activity of the granular sludge was high when acetate was used as feed. The fact that the *Methanotrichaceae (Methanosaeta)* family was still active after 180 days of methanol feeding as sole carbon source suggests that the homoacetogenic route may be responsible of methanol transformation to acetate. Bicarbonate plays an important role in the anaerobic conversion of methanol, as a required co-substrate in the acetogenic breakdown. Although bicarbonate was not added to the medium used in this assay –in order to avoid the acetogenic route–, it is produced when methanol is converted into methane (Equation 4). According to stoichiometry, up to one third of the methanol can potentially be consumed by acetogens from the endogenous methanogenic supplied bicarbonate (Florencio et al. 1997). Indeed, the methylotrophic acetogenic eubacteria *Sporomusa* was active in the granular sludge with a low relative abundance (0.2%), and may be involved in the conversion of methanol to acetate. In Phase 1, the *Methanotrichaceae* family accounted for the highest relative abundance at cDNA level (60%), demonstrating its high activity during acetate feeding in spite of presenting a lower relative abundance at DNA level (39%). However, according to a recent study, it seems that *Methanothrix* (*Methanosaeta*) is capable of accepting electrons via direct interspecies electron transfer (DIET), to reduce carbon dioxide to methane (Rotaru et al. 2014) and, not being strictly aceticlastic, to what extend it could have a role in the hydrogenotrophic route in Phase 3 should be analysed in depth. *Methanobacteriaceae* and *Methanoregulaceae* –families where most of its members obtain energy from the reduction of CO_2 with H_2– decreased their relative abundance during Phase 3 (10% and not detected, respectively), thus suggesting that the enrichment in the hydrogenotrophic methanogenic group, aim of this work, was not achieved. Conversely, genus *Methanomethylovorans* and *Methanolobus*, both part of the *Methanosarcinaceae* family and defined as methylotrophs (Jiang et al. 2005; Mochimaru et al. 2009), were the predominant and most active groups. These were followed by the *Thermoplasmatales* genus (*Methanossiliicoccaceae* family), which is also capable of using methanol as a substrate (Poulsen et al. 2013). The predominance of methylotrophic groups agrees with the results of the SMA test, which showed a high activity with methanol substrate. These results disagree with the ones obtained in a previous study where methanol was dosed to an UASB treating domestic wastewater at low to moderate temperatures (<20 ºC). In that case, it was stated that methanol directly induced hydrogenotrophic methanogens (*Methanobacteriales*) and also indirectly induced the acetoclastic methanogens (*Methanosetaceae*), due to the demand of H^+ created by *Methanobacteriales* (Saha et al. 2015). Differences observed compared to the present study may be due to the lower temperature used by Saha et al. (2015), which favours the emergence of hydrogenotrophic methanogens.

Although methanol is a simple compound with only one carbon, it can support a very complex food chain under anaerobic conditions (Florencio et al. 1994). The methylotrophic population enriched in this study may have been favoured by the pH in operation, which was maintained between 6.9 and

7.0. A slightly more acidic pH would have stimulated the hydrogenotrophic pathway, according to Bhatti et al. (1996), who established that at pH values close to 7.0, methanol will either be converted directly to methane (by methylotrophic methanogens), via the intermediate formation of acetate (by acetoclastic methanogens), or through a combination of both. Hydrogenotrophic methanogens will be mainly responsible for this conversion, by utilizing H_2 and CO_2, only with pH values between 5.0 and 6.0.

The decrease of Inverted Simpson and Shannon indices for archaea population throughout the entire UASB operation and the change of feed from acetate to methanol suggest that Phase 3 promoted the enrichment of certain groups of methanogenic microorganisms, reducing the biomass biodiversity of the granular sludge. This reduction in biodiversity in Phase 3 is observed at community composition level and also at activity level. In eubacteria population a reduction in biodiversity was also observed when looking at gene expression (cDNA), suggesting that acetate feeding promoted more eubacteria species to be active in the granular sludge than when using methanol as feed. Thus it can be concluded that the use of methanol as carbon source induced a reduction in the biomass biodiversity due to the high predominance of the methylotrophic route for its degradation. In the same way, results obtained from the Correspondence Analysis confirm that a clear population shift in UASB microbial communities was promoted during the operation of the reactor, obtaining specialised acetotrophic and methylotrophic communities in Phase 1 and Phase 3, respectively, due to the different feeding strategies applied.

In this study, the anaerobic granular sludge used as inoculum in the UASB was activated during the acetate feeding phase, and later progressively adapted to a methanol substrate, achieving high COD removal efficiencies (97±1%). From the different metabolic pathways known for methanol, the methylotrophic methanogenic (by the genus *Methanomethylovorans* and *Methanoglobus*) was the predominant pathway by the end of the UASB operation, followed by the aceticlastic one (by the genus *Methanothrix (Methanosaeta)*), while the hydrogenotrophic route presented a low activity. The ratio between methanogenic archaea and eubacteria in the biomass showed a distinct increase during the methanol feeding phase of the UASB, so it may harbour a great potential as inoculum for biogas production and other related bioreactor processes.

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Compliance with ethical standards

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Conflict of interest

The authors declare that there are no conflicts of interests.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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2 Tables

3

4 **Table 1.** Operational conditions of the UASB reactor

Phase	Length (d)	OLR (kg_{COD} m^{-3} d^{-1})	Acetate concentration (g L^{-1})	Methanol concentration (g L^{-1})	Aim of the phase
1	135	3.25	1.02	0	Start-up, activation of the biomass and acclimatization to high OLR
	7	6.05	1.89	0	
	7	8.44	2.64	0	
	65	10.08	3.15	0	
2	7	10.08	2.01	0.53	Acclimatization of the biomass to methanol feeding
	7	10.08	1.34	1.06	
	7	10.08	0.67	1.58	
3	180	10.08	0	2.11	Enhancement of the biomass enrichment

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2 **Table 2.** Average performance of the UASB reactor through the different operational phases
3 (mean±standard deviation).

Phase	Carbon source (% COD)		OLR (kg_{COD} m^{-3} d^{-1})	COD removal efficiency (%)	Biogas CH_4 content (%)
	Acetate	Methanol			
1	100	0	3.25	73±9	22±8
			6.05	74±1	41±1
			8.44	82±3	32±1
			10.08	82±12	68±14
2	75	25	10.08	70±16	70±0
	50	50	10.08	93±13	81±8
	50	75	10.08	96±1	81±3
3	0	100	10.08	97±1	85±1

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2 **Table 3.** Specific methanogenic activity (SMA) of the inoculum, Phase 1 and Phase 3 granular sludge, fed
3 with different substrates (mean±standard deviation). ND: not determined.

Phase	SMA (mg COD_{CH4} / g VSS d)				
	AGV Mix	Acetate	H_2	Methanol	Blank
Inoculum	107±25	125±3	40±5	ND	6±2
1	138±3	149±27	2±1	ND	0.4±0.1
3	220±0	239±90	12±1	470±65	3±0

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2 **Table 4.** Diversity index for Eubacteria and Archaea communities for the inoculum and biomass in the UASB
3 by the end of Phase 1 (acetate feeding) and Phase 3 (methanol feeding) for DNA and cDNA samples
4 (mean±standard deviation). Data normalised to the sample with the lowest number of reads (50466 and
5 66226 for eubacteria and archaea, respectively).
6

	Coverage	**Inverted Simpson**	**Shannon**
Eubacteria			
Inoculum	1.00±0.00	8.35±0.03	3.66±0.00
Phase 1-DNA	0.99±0.00	15.06±0.00	4.01±0.00
Phase 3-DNA	0.99±0.00	15.27±0.04	3.75±0.00
Phase 1-cDNA	0.99±0.00	9.80±0.02	3.66±0.00
Phase 3-cDNA	0.99±0.00	5.44±0.02	2.94±0.01
Archaea			
Inoculum	1.00±0.00	5.92±0.02	2.33±0.00
Phase 1-DNA	1.00±0.00	4.12±0.01	1.95±0.00
Phase 3-DNA	1.00±0.00	2.83.±0.00	1.63±0.00
Phase 1-cDNA	1.00±0.00	3.37±0.01	1.99±0.01
Phase 3-cDNA	1.00±0.00	2.97±0.01	1.85±0.01

7

Figure captions

Figure 1 Gene copy numbers for *16S* rRNA and *mcr*A genes, and DNA ratio, of the initial inoculum and the biomass in the UASB by the end of Phase 1 (acetate feeding) and Phase 3 (methanol feeding).

Figure 2 Taxonomic assignment of sequencing reads from the Eubacterial community of the initial inoculum and biomass of the UASB by the end of Phase 1 (acetate feeding) and Phase 3 (methanol feeding) for genomic DNA and RNA (cDNA) level, at a) phylum b) family levels. Relative abundance was defined as the number of reads (sequences) affiliated with any given taxon, divided by the total number of reads per sample. Phylogenetic groups with a relative abundance lower that 1% were categorized as “others”

Figure 3 Taxonomic assignment of sequencing reads from Archaeal community of the initial inoculum and biomass of the UASB by the end of Phase 1 (acetate feeding) and Phase 3 (methanol feeding) for genomic DNA and RNA (cDNA) at family level. Relative abundance was defined as the number of reads (sequences) affiliated with any given taxon, divided by the total number of reads per sample. Phylogenetic groups with a relative abundance lower that 1% were categorized as “others”.

Figure 4 Correspondence Analysis of the initial inoculum and biomass of the UASB by the end of Phase 1 (acetate feeding) and Phase 3 (methanol feeding) for DNA and cDNA samples regarding (a) Eubacteria and (b) Archaea communities.

Figure 1

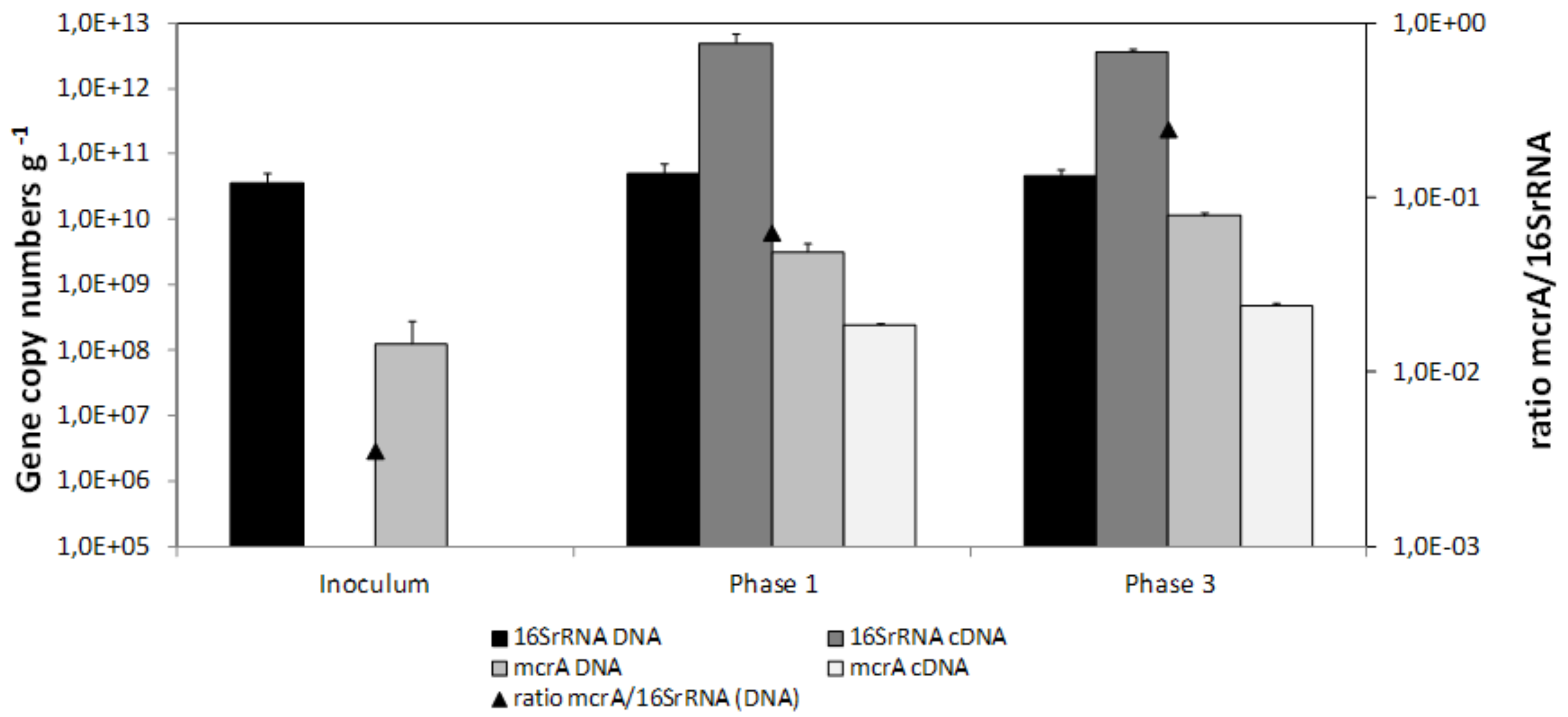
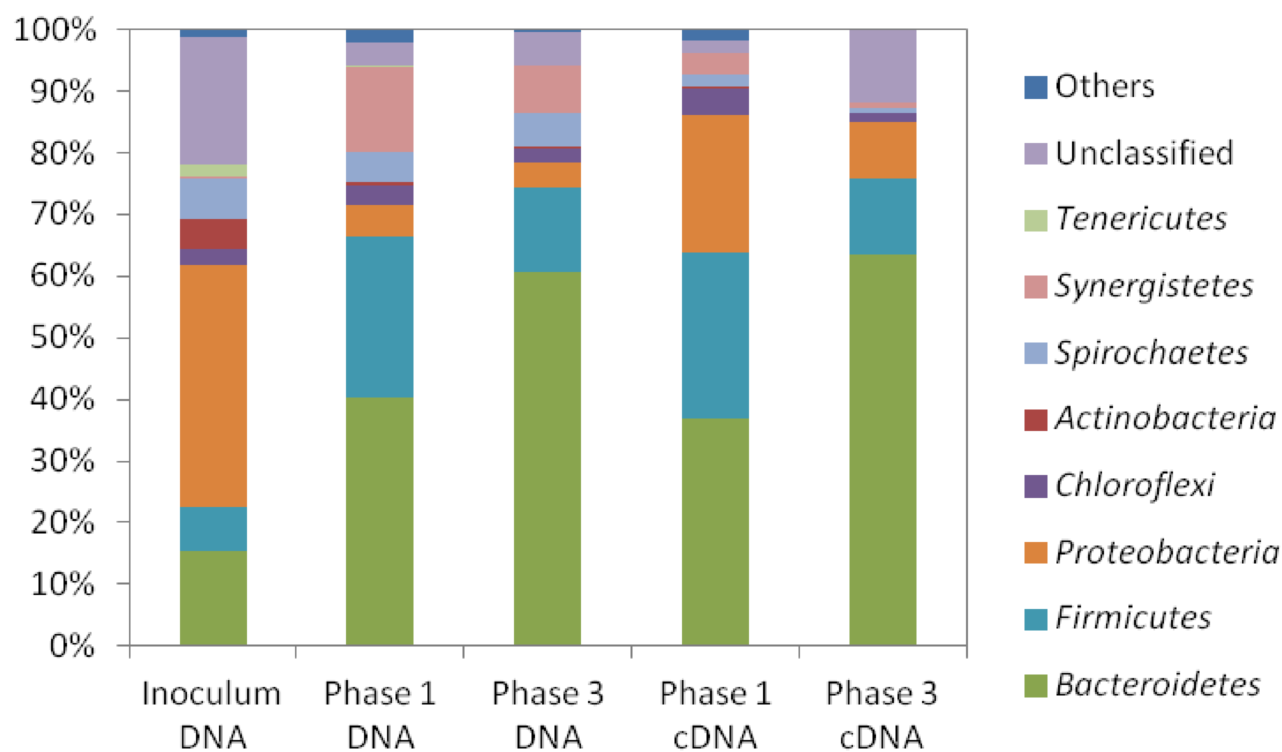


Figure 2



b

100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%

Inoculum DNA
Phase 1 DNA
Phase 3 DNA
Phase 1 cDNA
Phase 3 cDNA

Unclassified
Others
Spirochaetaceae
Alcaligenaceae
Ruminococcaceae
Peptococcaceae 2
Desulfobulbaceae
Clostridiales_Incertae Sedis XI
Anaerolineaceae
Actinomycetales
Synergistaceae
Pseudomonadaceae
Porphyromonadaceae

Figure 3

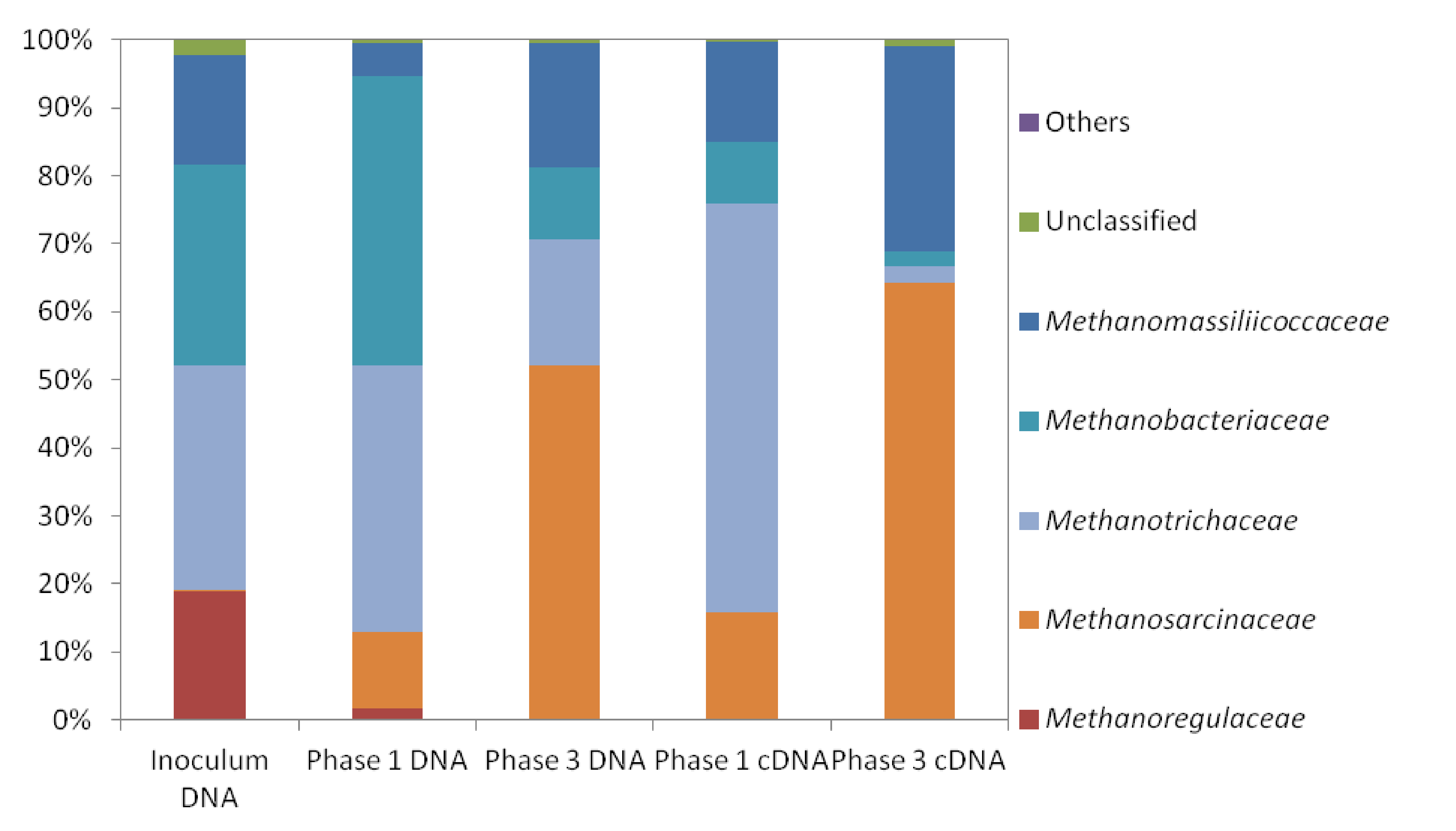


Figure 4