


# Asymmetrical response of anaerobic digestion microbiota to temperature changes

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**Abstract** In natural settings, anaerobic digestion can take place in a wide temperature range, but industrial digesters are usually operated under either mesophilic (~35 °C) or thermophilic (~55 °C) conditions. The ability of anaerobic digestion microbiota to switch from one operating temperature to the other remains poorly documented. We therefore studied the effect of sudden temperature changes (35 °C/55 °C) in lab-scale bioreactors degrading <sup>13</sup>C-labelled cellulose. An asymmetric behaviour was observed. In terms of methane production, after an adaptation period, mesophilic inoculum exhibited a functional resistance to temperature increase but no functional resilience when temperature was reset to 35 °C, while thermophilic inoculum methanogenic activity strongly decreased under mesophilic conditions but partially recovered when temperature was reset to 55 °C. Automated ribosomal intergenic spacer analysis community fingerprints evidenced a strong influence of temperature on microbial diversity, particularly pronounced and persistent for *Archaea*. Key phylotypes involved in <sup>13</sup>C-cellulose degradation were identified with a coupled stable isotope probing (SIP)-16S rDNA pyrotag sequencing approach, suggesting that the hydrolytic and fermentative metabolic functions could be maintained thanks to functional redundancy between members of the class

*Clostridia*, whereas methanogenic activity primarily relied on specialized groups affiliated either to genus *Methanosarcina* (mesophilic conditions), *Methanothermobacter* or *Methanoculleus* (thermophilic conditions) that were irreversibly modified by temperature increase.

**Keywords** Methanization · Cellulose · Stable isotope probing · ARISA · 16S

## Introduction

Whether natural or anthropogenic, anaerobic digestion can take place in a wide temperature range. However, studies at different temperatures and in various media have identified three main categories of systems, according to their operating temperature: psychrophilic (5–20 °C), mesophilic (20–45 °C) and thermophilic (45 to 65 °C) systems (Van Lier et al. 1997). Even though the major degradation steps of organic molecules are similar for the three ranges of temperature, metabolic pathways may vary. Populations of microorganisms present at the three temperatures are not identical and seem to be affected by the temperature, as well as kinetics and yields of degradation (Donoso-Bravo et al. 2009).

In particular, temperature levels and changes can have important consequences on the overall performance and functioning of industrial anaerobic digesters (Levén et al. 2007; Lindorfer et al. 2008). These bioprocesses, treating organic waste to produce biogas that can be valued energetically, are an important economic issue. Their operating temperature can be easily controlled to optimize their efficiency. Consequently, temperature impact on the performance of industrial anaerobic digesters has been widely studied and various data supporting mesophilic or thermophilic operating conditions are found in

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the literature. For example, digestion at high temperature could shorten the latency and increase the efficiency of degradation of organic matter (Van Lier et al. 2001), resulting in a higher total production of biogas and a more complete digestion. Moreover, digestion at high temperatures could promote the inhibition of the growth of pathogens present in the waste (Bagge et al. 2005). However, mesophilic processes, using less energy for heating, seem generally less affected by the inhibitory effects of ammonia released during the mineralization of proteins than thermophilic processes.

These studies were often limited to the interpretation of physicochemical data, and the effect of temperature on the microbial communities has not been systematically studied as molecular tools have seldom been used. However, the studies conducted on that topic showed that the impact of temperature on microbial communities in anaerobic digestion processes is very important (Levén et al. 2007; Pender et al. 2004). Different microbial communities, adapted to different temperatures, catalyse specific metabolic pathways (Levén et al. 2007; Li et al. 2009; Wu et al. 2006). Temperature modifications are another important issue that can occur when environmental conditions change or for example in case of heating failure or variation of operating conditions of anaerobic digesters. Existing studies show both positive and negative effects of temperature changes on performance degradation, probably because of the complexity of microbial groups involved in anaerobic digestion (Conrad et al. 2009).

In this context, our study was designed to assess the impact of sudden changes in the operating temperature of anaerobic bioprocesses degrading cellulose. Modifications of microbial ecosystems, associated pathways and degradation performances were evaluated. In particular, we evaluated how the anaerobic digestion microbiota adapted to the new temperature conditions and if the changes induced by the temperature modification were temporary or permanent. Cellulose was chosen as a representative substrate. Indeed, lignocellulose is a major component of the biosphere, an important amount of this bioresource is available and it is also the most voluminous waste produced by our society (Lynd et al. 2002).

## Materials and methods

Two similar experiments were set up in parallel with respectively a thermophilic and mesophilic inoculum (TI and MI experiment). Each experiment was divided into three successive steps, corresponding to three temperature conditions: expected temperature for the inoculum (55 or 35 °C), change to another temperature condition (35 or 55 °C) and return to the initial temperature (55 or 35 °C) (Table 1). The efficiency of [<sup>13</sup>C]-cellulose degradation was tracked by monitoring gas production and the accumulation of metabolites with gas chromatography combustion isotope ratio mass spectrometry (GC-

C-IRMS). These results were linked to the monitoring of the relative dynamics of *Bacteria* and *Archaea* (automated ribosomal intergenic spacer analysis (ARISA) method (Fisher and Triplett 1999)) and to the identification of microorganisms that assimilated <sup>13</sup>C (coupled stable isotope probing (SIP) (Radajewski et al. 2000)-16S rDNA pyrotag sequencing approach).

## Experimental setup and inoculum

Anaerobic batch digesters (glass plasma bottles, total working volume 150 mL) were set up using mesophilic or thermophilic municipal solid waste (MSW) anaerobic digester sludge (0.9 g of volatile matter introduced in each bottle). Sludge was recovered from full-scale household waste digesters at Varennes-Jarcy, France (mesophilic digester), and at Graincourt-lès-Havrincourt, France (thermophilic digester). It was filtered to remove solids bigger than 3 mm, incubated in anaerobic condition at 35 or 55 °C for several days until gas production was over, centrifuged (10,000×g, 10 min) and stored at -80 °C until use (mesophilic sludge characteristics: pH = 8.1, C<sub>[wt% dry solids]</sub> = 24.6 %, N<sub>[wt% dry solids]</sub> = 1.3 %; thermophilic sludge characteristics: pH = 8.3, C<sub>[wt% dry solids]</sub> = 22.9 %, N<sub>[wt% dry solids]</sub> = 1.5 %). One hundred millilitres of supernatant originating from the centrifugation (13,000×g, 15 min) of MSW leachate (from a closed MSW landfill of Vert-le-Grand, France) was used to suspend the sludge (pH = 8.2, DOC = 1.9 g/L, DIC = 1.7 g/L, VFA < 10 mg/L). MSW leachate was used because it contained a low amount of dissolved organic carbon and had a high buffering capacity. Bottles were hermetically sealed with a screw cap and a rubber septum and headspaces were flushed with nitrogen gas.

Ten bioreactors in total were set up as described in Table 1. Experiments were divided into three successive steps during which bioreactors were incubated either at 35 or 55 °C as described in Table 1. Five bioreactors (TI1, TI2, TI3, TIc1 and TIc2) were inoculated with thermophilic inoculum. Another five bioreactors (MI1, MI2, MI3, MIc1 and MIc2) were inoculated with mesophilic inoculum. TIc1, TIc2, MIc1 and MIc2 were used as controls with no temperature. Cellulose was introduced in the bioreactors at the beginning of each step and headspaces were flushed with nitrogen gas. As the volume of liquid in the bioreactors evolved during the experiment (liquid samplings), the amount of cellulose added at the beginning of each step was adjusted to the volume of the remaining liquid in the bioreactors to bring a constant concentration of cellulose (2.7 g/L, synthesized from glucose by *Acetobacter xylinum* at CERMAV-CNRS, Grenoble, France, according to the procedures described in Gagnaire and Taravel (1980). [<sup>13</sup>C]-labelled cellulose and unlabelled cellulose (same amount and type) were used alternatively as shown in Table 1 to identify functional microorganisms and track

**Table 1** Description of the different batch bioreactors set up and sampling dates for molecular characterization

	Name of the incubation	Inoculum	Step 1	Step 2	Step 3
TI experiment	TI1	Thermophilic	<b>[<sup>13</sup>C]-cellulose</b>	Unlabelled cellulose	<b>Unlabelled cellulose</b>
	TI2	Thermophilic	<b>Unlabelled cellulose</b>	[ <sup>13</sup> C]-cellulose	<b>Unlabelled cellulose</b>
	TI3	Thermophilic	<b>Unlabelled cellulose</b>	Unlabelled cellulose	<b>[<sup>13</sup>C]-cellulose</b>
	TIc1	Thermophilic	<b>Unlabelled cellulose</b>	<b>Unlabelled cellulose</b>	<b>Unlabelled cellulose</b>
	TIc2	Thermophilic	<b>Unlabelled cellulose</b>	<b>Unlabelled cellulose</b>	<b>Unlabelled cellulose</b>
	Sampling dates			Days 4, 8, 21 and 32	Days 11, 32, 48 and 68
MI experiment	MI1	Mesophilic	[ <sup>13</sup> C]-cellulose	<b>Unlabelled cellulose</b>	Unlabelled cellulose
	MI2	Mesophilic	Unlabelled cellulose	<b>[<sup>13</sup>C]-cellulose</b>	Unlabelled cellulose
	MI3	Mesophilic	Unlabelled cellulose	<b>Unlabelled cellulose</b>	[ <sup>13</sup> C]-cellulose
	MIc1	Mesophilic	Unlabelled cellulose	Unlabelled cellulose	Unlabelled cellulose
	MIc2	Mesophilic	Unlabelled cellulose	Unlabelled cellulose	Unlabelled cellulose
	Sampling dates			Days 9, 17, 28 and 44	Days 4, 22, 34, 47 and 76

Data in bold has an incubation temperature of 55 °C

All other data in normal font has an incubation temperature of 35 °C

degradation metabolites. [<sup>13</sup>C]-labelled cellulose was synthesized from [<sup>13</sup>C]-labelled glucose, 99 % enriched (Aldrich). Time zero samples were taken and the reactors were incubated under anaerobic conditions, without agitation, in the dark. Gas production and composition were measured and analysed every 2 days. The different steps were considered finished when no more gas had been produced for at least 10 days, i.e. after 68 days for MI step 1, 76 days for MI step 2, 65 days for MI step 3, 45 days for TI step 1, 68 days for TI step 2 and 42 days for TI step 3. Liquid samples (6 mL) were regularly collected through the septum and centrifuged at 12,000×g for 10 min. The pellets and supernatant thus obtained were stored separately at −20 °C for analysis of biomass and chemical parameters, respectively.

### Analytical methods

Biogas accumulation in the headspace was measured using a differential manometer (Digitron 2082P). Headspace gas analysis was performed using a micro GC (CP4900, Varian) exactly as described in Chapleur et al. (2014). This data was used to calculate gas production and composition, at standard temperature and pressure, taking into account the extracted volume of gas and liquid samples. For calculations, biogas was assimilated to an ideal gas. The ratio of cellulose conversion to biogas was calculated by dividing the measured biogas production (CH<sub>4</sub> and CO<sub>2</sub>) by the theoretical biogas production if all carbon of cellulose was converted into biogas. Gas samples for analysis of percentage of <sup>13</sup>C in CH<sub>4</sub> were periodically collected with a syringe and transferred into 7-mL vacuumed serum tubes. The analyses were performed using a Trace GC Ultra (Thermo Electron Corporation) attached to a Delta V Plus isotope ratio mass spectrometer via a GC Combustion

III (Thermo Electron Corporation). Typical uncertainties, quantified by replicate measurements of different samples, were ±0.2 %.

The concentrations of formic, acetic, propionic, butyric, valeric and lactic acids were measured by conductometric detection, using a Dionex 120 equipped with an IonPac ICE-AS1 column (9 mm × 250 mm). Isotopic composition of volatile fatty acids (VFA) was measured with a Trace GC Ultra (Thermo Electron Corporation) attached to a Delta V Plus isotope ratio mass spectrometer via a GC Combustion III (Thermo Electron Corporation) as described in Chapleur et al. (2014).

### DNA extraction and ARISA fingerprinting

Total DNA was extracted using a Power Soil DNA Isolation Kit (Mobio Laboratories Inc., Carlsbad) according to the manufacturer's instructions. Extracted DNA was quantified by Qubit (dsDNA HS Assay Kit, Invitrogen, Eugene), and DNA integrity was checked by EtBr staining and electrophoresis on 1 % agarose gel.

The ARISA method (Fisher and Triplett 1999) was applied to analyse *Bacteria* and *Archaea* from the samples. PCR was performed with two different sets of primers. The bacterial primer set ITSf/ITSr (5'-GTCGTAACAAGGTAGCCGTA-3'/5'-GCCAAGGCATCCACC-3') (Cardinale et al. 2004) was used to amplify the 16S-23S ITS region of *Bacteria*, while the primer set 71R/1389F (5'-TCGCAGCTTRSCACGYCCTTC-3'/5'-CTTGACACACCGCCCGT C-3') was used for the 16S-23S ITS region of *Archaea* (García-Martínez and Rodríguez-Valera 2000). Primer 1389F was adapted from a FISH probe described in Loy et al. (2002). The bacterial 16S-23S ITS DNA region was amplified in a 25-μL reaction mixture containing 1× PCR buffer,

0.75 U of Thermo-Start Taq DNA polymerase (ABGene Ltd, Epsom, UK), 0.4 mM dNTP (each), 1.5 mM MgCl<sub>2</sub>, 0.2 μM ITSF and ITSReub (each) and 1 μL extracted DNA (2 to 25 ng). The mixture was held at 94 °C for 15 min, followed by 35 cycles of 94 °C for 1 min, 55.2 °C for 1 min and 72 °C for 2 min, and a final extension step at 72 °C for 10 min. The PCR protocol that was used to amplify the archaeal 16S-23S ITS DNA region was similar except for the annealing temperature, which was set at 54.5 °C. To ensure a fast processing of samples, ARISA was performed with a capillary electro-phoresis bioanalyser (2100 Electrophoresis Bioanalyzer, Agilent Technologies, Santa Clara) using the Agilent DNA 1000 Kit (Agilent Technologies).

### DNA ultracentrifugation and pyrosequencing

CsCl density gradient ultracentrifugation was performed in a 2-mL Quick-Seal polyallomer tube, using a TLA-120.2 rotor (Beckman) for the separation and collection of <sup>13</sup>C-DNA and <sup>12</sup>C-DNA fractions as described previously (Radajewski et al. 2000). Five hundred nanograms of total DNA was loaded to CsCl gradient and centrifuged at 150,000g for 20 h. After ultracentrifugation, 100-μL fractions were recovered from the bottom of the tubes by pumping water in the top of the tubes. Samples from unlabelled experiments were always analysed in parallel as negative controls. Each fraction was subjected to DNA quantification with Qubit (dsDNA HS Assay Kit, Invitrogen, Eugene) and density measurement (using an Arias 500 Reichert refractometer). After fraction collection, DNA was purified by a MicroCon YM-30 column (Millipore) to remove CsCl and recovered in 30 μL of Tris (pH 7.5) buffer.

Pyrosequencing was performed as described in (Smith et al. 2010). Archaeal and bacterial tag-encoded FLX 454 amplicon pyrosequencing (bTEFAP) was performed by the Research and Testing Laboratory (Lubbock, TX), based upon RTL protocols ([www.researchandtesting.com](http://www.researchandtesting.com)) using primers 28F (5'-TTTGATCNTGGCTCAG-3') and 519R (5'-GTNTTACNGCGGCKGCTG-3') for *Bacteria* and ARCH 349F (5'-GYGCASCAGKCGMGAAW-3') and ARCH 806R (5'-GGACTACVSGGGTATCTAAT-3') for *Archaea*. The raw reads of both runs (*Bacteria* and *Archaea*) have been deposited in the sequence read archive section of GenBank with the following accession numbers for each sample: SRX345903, SRX345904, SRX345907, SRX345912, SRX345936, SRX345938, SRX345939, SRX345948, SRX345950 and SRX345912.

### Data analysis

Statistical tools were used to compare all the ARISA profiles with one another. ARISA electropherograms in ASCII format were imported into the StatFingerprints R package (Michelland et al. 2009). ARISA profiles were aligned and the area under each curve was normalized to 1 using the

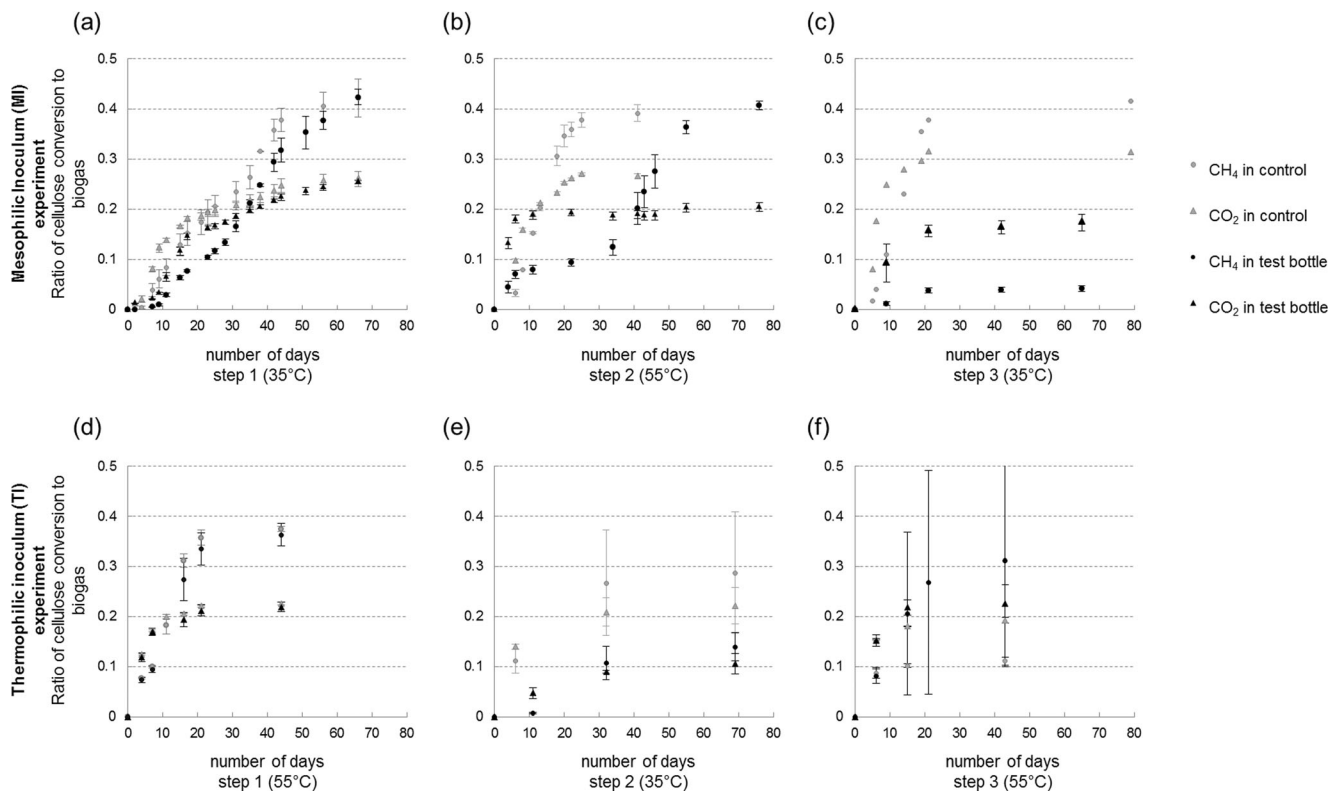
StatFingerprints package. A matrix containing data from all aligned and normalized profiles was obtained and statistically analysed with the ADE4 R package (Thioulouse et al. 1997). Centred, not scaled, principal component analyses (PCAs) were performed on both bacterial and archaeal ARISA profile datasets. PCAs were plotted with colour scales corresponding to different steps of the experiments in order to understand the grouping of the profiles within the PCA. Figures were produced with the Adegraphics R package. Archaeal and bacterial diversities were analysed separately.

Pyrosequencing data analyses were realized with the open-source software package QIIME (Caporaso et al. 2010b). Library de-multiplexing and quality filtering were first performed to remove low-quality reads: the reads with an average base quality below 25, the reads containing homopolymers longer than 6 bases or ambiguous bases, and the reads shorter than 150 bases after trimming were discarded. The remaining reads were aligned with PyNAST (Caporaso et al. 2010a) using as template the Silva 108 database core-aligned set formatted for QIIME (<http://www.arb-silva.de/download/archive/qiime/>). The putative chimeric sequences were identified with ChimeraSlayer using the same Silva 108 reference set and discarded. The remaining sequences were used for operational taxonomic unit (OTU) picking at the 0.97 threshold (~species-level) with uclust. The taxonomic assignment was done with the RDP classifier (Cole et al. 2009; Wang et al. 2007) at the 0.8 bootstrap cutoff value, using one representative sequence for each OTU (longest sequence) and the Silva 108 reference set. OTUs not corresponding to the expected phylogenetic domain were discarded. Rarefaction curves were determined based on the OTUs belonging to the correct phylogenetic domain. After resolving based upon these parameters, the percentage of each bacterial/archaeal taxon was individually analysed for each sample, providing relative abundance information based upon relative numbers of reads within a given sample. Centred and scaled PCAs were computed with ADE4 R package, as described for ARISA, to compare the results of sequencing. They were plotted with the same colour scale. Correlation circles were drawn to see how the different OTUs influenced the distribution of the samples within the PCA.

## Results

### Mesophilic inoculum performances were resistant to temperature increase but not resilient

During the first step of the experiment (Fig. 1a), cellulose was converted into biogas composed of circa one third of CO<sub>2</sub> and two thirds of CH<sub>4</sub>. After the first temperature change (step 2), CO<sub>2</sub> production of experimental incubations of the MI experiment (Fig. 1b) reached 80 % of controls' production



**Fig. 1** Ratio of CH<sub>4</sub> (circles) and CO<sub>2</sub> (triangles) production (mg of C) vs amount of cellulose introduced (mg of C) during time (number of days) for step 1 (a), step 2 (b) and step 3 (c) of the experiment with mesophilic inoculum (MI) and step 1 (d), step 2 (e) and step 3 (f) of the experiment

with thermophilic inoculum (TI). Black markers correspond to triplicate experimental incubations with temperature changes (mean value). Grey markers correspond to controls without temperature changes (mean value). Error bars represent standard deviation

and the CH<sub>4</sub> production level was similar. Nevertheless, the pattern of CH<sub>4</sub> production was different from that of controls: two steps of production were observed, separated by a latency of 12 days, and CH<sub>4</sub> only was produced in the second step. Performances were resistant on the basis of the total production, but not on the basis of the kinetics, suggesting that the overall process of cellulose degradation was affected. Finally, after temperature was reset to the initial value in step 3, CH<sub>4</sub> and CO<sub>2</sub> productions of experimental incubations of the MI experiment (Fig. 1c) were significantly reduced compared to those of the controls. Nearly no CH<sub>4</sub> was produced.

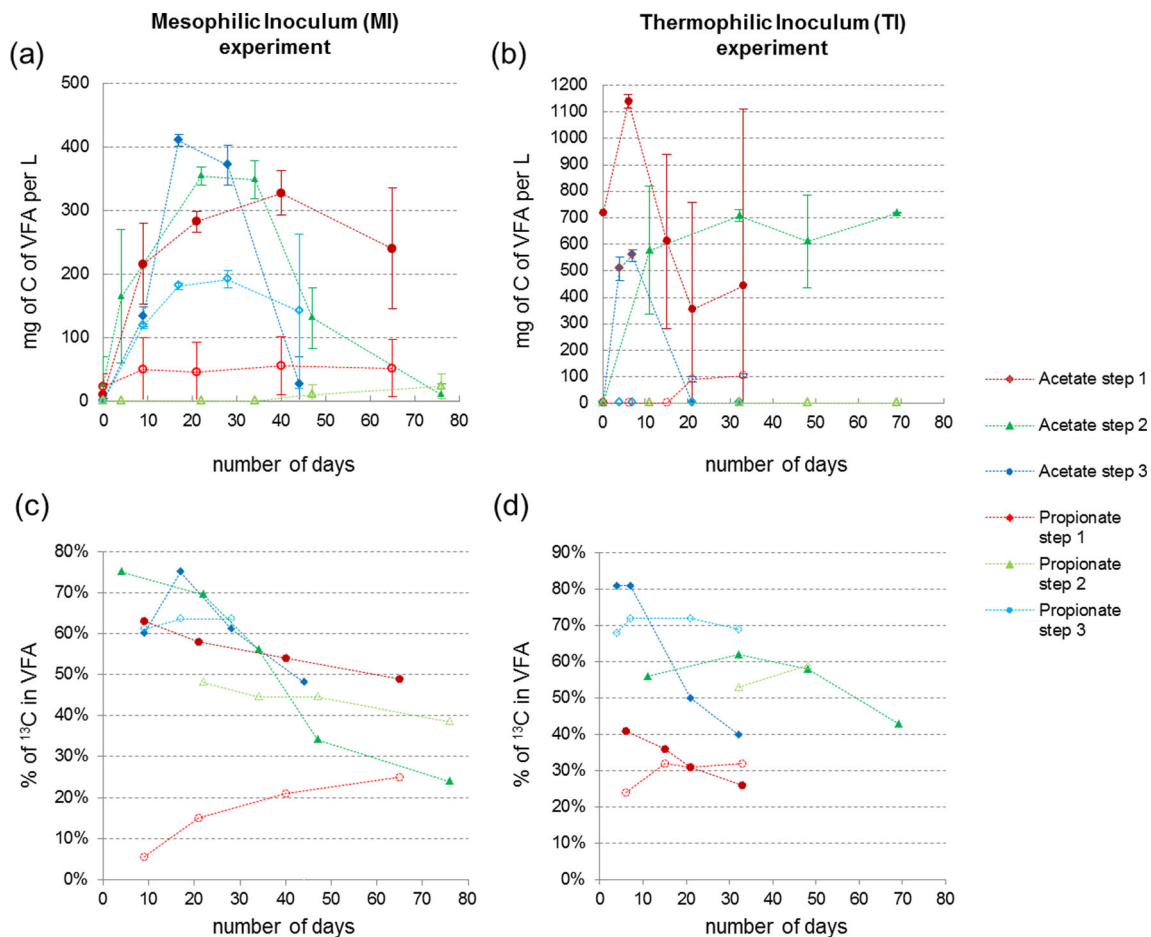
Patterns of VFA accumulation and degradation (Fig. 2a) were consistent with patterns of gas production in terms of date. Both acetate and propionate accumulated up to resp. 400 and 200 mg of C/L during step 1 and were consumed as biogas was produced. Acetate was consumed first. After temperature change (step 2), only acetate accumulated, up to 350 mg of C/L, and was degraded while biogas was produced. Finally, in step 3, even if a very low amount of biogas was produced, both acetate and propionate accumulated, up to resp. 325 and 50 mg of C/L, but were not degraded.

Isotopic composition of VFA (Fig. 2c) and gas (Fig. 3a) was measured in bottles with [<sup>13</sup>C]-cellulose to estimate the amount of metabolites coming from cellulose. In step 1,

acetate was 75–80 % enriched and propionate was 65–70 % enriched. The level of acetate enrichment remained stable in steps 2 and 3. The metabolites were not 100 % <sup>13</sup>C enriched, signifying that biomass was partly degraded or possibly that a part of acetate could have been synthesized from CO<sub>2</sub> by homoacetogenic bacteria. In step 1, the level of <sup>13</sup>C enrichment of CH<sub>4</sub> of MI was first similar to CO<sub>2</sub> enrichment. After 20 days, it increased importantly and nearly reached the acetate enrichment value at the end of the incubation. It suggested that hydrogenotrophic methanogenesis dominated at the beginning of degradation and was followed by acetoclastic methanogenesis or syntrophic oxidation of acetate after day 20. CO<sub>2</sub> enrichment did not increase as quickly as CH<sub>4</sub> enrichment because CO<sub>2</sub> coming from the degradation of acetate was diluted with the inorganic carbon contained in the liquid phase (1.7 g/L at the beginning of the incubation). No data were obtained for MI experiment steps 2 and 3 (analysis failed).

#### Thermophilic inoculum performances were not resistant to temperature decrease but partially recovered at return to the initial temperature condition

During the first step of the experiment, the total amount of production and biogas composition (Fig. 1d) was similar to



**Fig. 2** Volatile fatty acid accumulation (mg of C per L) and isotopic enrichment (atom % of  $^{13}\text{C}$ ) during time (number of days) for experimental incubations of experiments with mesophilic inoculum (MI) (a, c) and with thermophilic inoculum (TI) (b, d). Black markers correspond to acetate accumulation (mean value for panels a and b, data

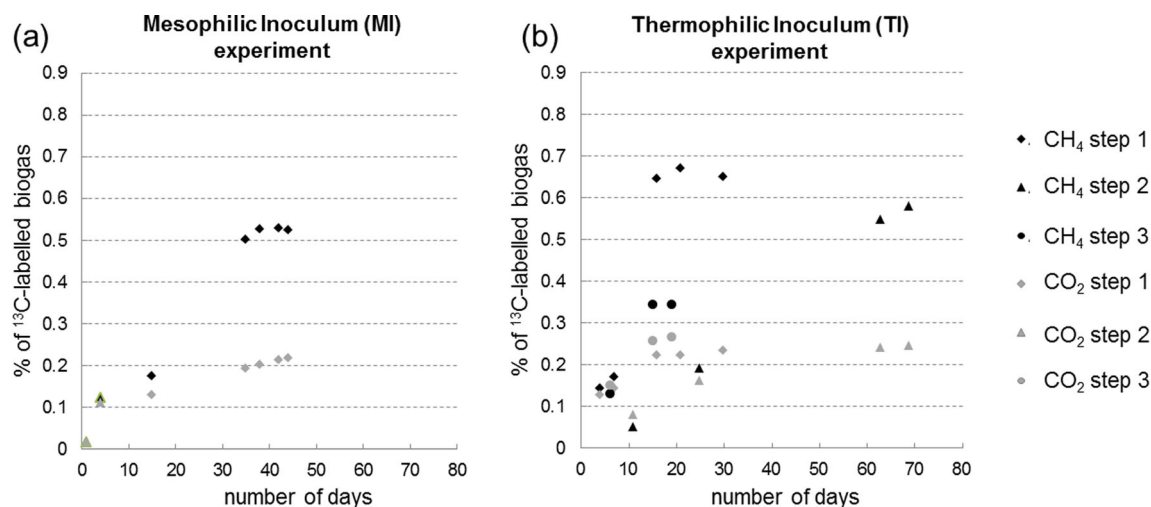
for incubation with  $^{13}\text{C}$ -cellulose for panels c and d). Grey markers correspond to propionate (mean value for panels a and b, data for incubation with  $^{13}\text{C}$ -cellulose for panels c and d). Squares, triangles and circles respectively correspond to steps 1, 2 and 3. Error bars represent standard deviation

the one observed with mesophilic inoculum, even if production was quicker in thermophilic conditions. After the first temperature change (step 2), biogas production (Fig. 1e) was strongly reduced compared to controls. Finally, after the temperature was reset to the initial value in step 3, biogas production was significant for experimental incubations of experiment TI (Fig. 1f), with a mean production higher than the production observed in controls. However, important standard deviations, up to 40 % of the biogas production, were measured between triplicates.

Patterns of VFA accumulation and degradation were in accordance with patterns of gas production in terms of date (Fig. 2b). Only acetate accumulation was observed. Five hundred and fifty milligrams of C per litre accumulated in step 1 and was degraded quickly while biogas was produced. In step 2, even if biogas production was very low, acetate accumulated up to 700 mg of C/L but was not consumed and was still

present at the beginning of step 3. Five hundred and fifty milligrams of C per litre of acetate was newly formed during step 3, and all the acetate was finally consumed and converted into biogas.

Acetate was 75–80 % enriched during step 1 (Fig. 2d). Isotopic enrichments diminished across the different steps. In particular, for step 3, the low enrichment of VFA can be explained by an important amount of  $^{12}\text{C}$  not-degraded VFA remaining at the end of step 2. As observed with mesophilic inoculum, in step 1, the level of  $^{13}\text{C}$  enrichment of  $\text{CH}_4$  (Fig. 3b) was first similar to  $\text{CO}_2$  enrichment and then increased importantly and nearly reached the acetate enrichment value at the end of the incubation, suggesting that hydrogenotrophic methanogenesis was followed by acetoclastic methanogenesis or syntrophic oxidation of acetate. Enrichment values of  $\text{CH}_4$  and  $\text{CO}_2$  for TI step 3 were close, suggesting a predominant hydrogenotrophic methanogenesis.



**Fig. 3** Biogas isotopic enrichment (% of  $^{13}\text{C}$ -labelled gas) during time (number of days) for experimental incubations of experiments MI (a) and TI (b). Black markers correspond to methane. Grey markers correspond

to carbon dioxide. Squares, triangles and circles respectively correspond to steps 1, 2 and 3. Data for the late dates of experiment MI step 2 and for MI step 3 were not obtained

### Effect of temperature changes on microbial community structure

Dynamics of microbial communities were studied in a first approach with ARISA. ARISA gives a qualitative and general view of the total diversity contained in the samples and does not provide specifically information on the microorganisms degrading cellulose, contrary to the coupled SIP-16S sequencing approach, but it made it possible to process a large number of samples efficiently in order to obtain a well-replicated dataset. This large dataset was appropriate to correctly identify ecological patterns (Prosser 2010) and correlate patterns of temperature with microbial community structure. In total, 81 samples were processed for both *Archaea* and *Bacteria*. They correspond to four or five different sampling dates per step for all triplicates of bioreactors. As it was impossible to compare all the profiles visually, they were compared statistically using the R Cran software. PCA was used to visualize the proximity and differences between the different samples analysed. Figure 4 presents the PCA performed on all archaeal and bacterial ARISA profiles from the experiments, together with profiles from the inocula.

PCA performed on *Archaea* profiles clearly distinguished two groups of profiles. Profiles of samples corresponding to the mesophilic inoculum and to the first step of the MI experiment clustered together at the left of the factorial plane (Fig. 4a). All the other profiles, i.e. profiles of samples of the TI experiment and of MI experiment steps 2 and 3, namely all the samples of digesters incubated at least once in thermophilic conditions, clustered on the right of the factorial plane. It suggested a strong influence of thermophilic conditions on the archaeal community that was irreversibly modified.

PCA performed on *Bacteria* profiles was less contrasted. Distinct groups corresponding to the different steps of the

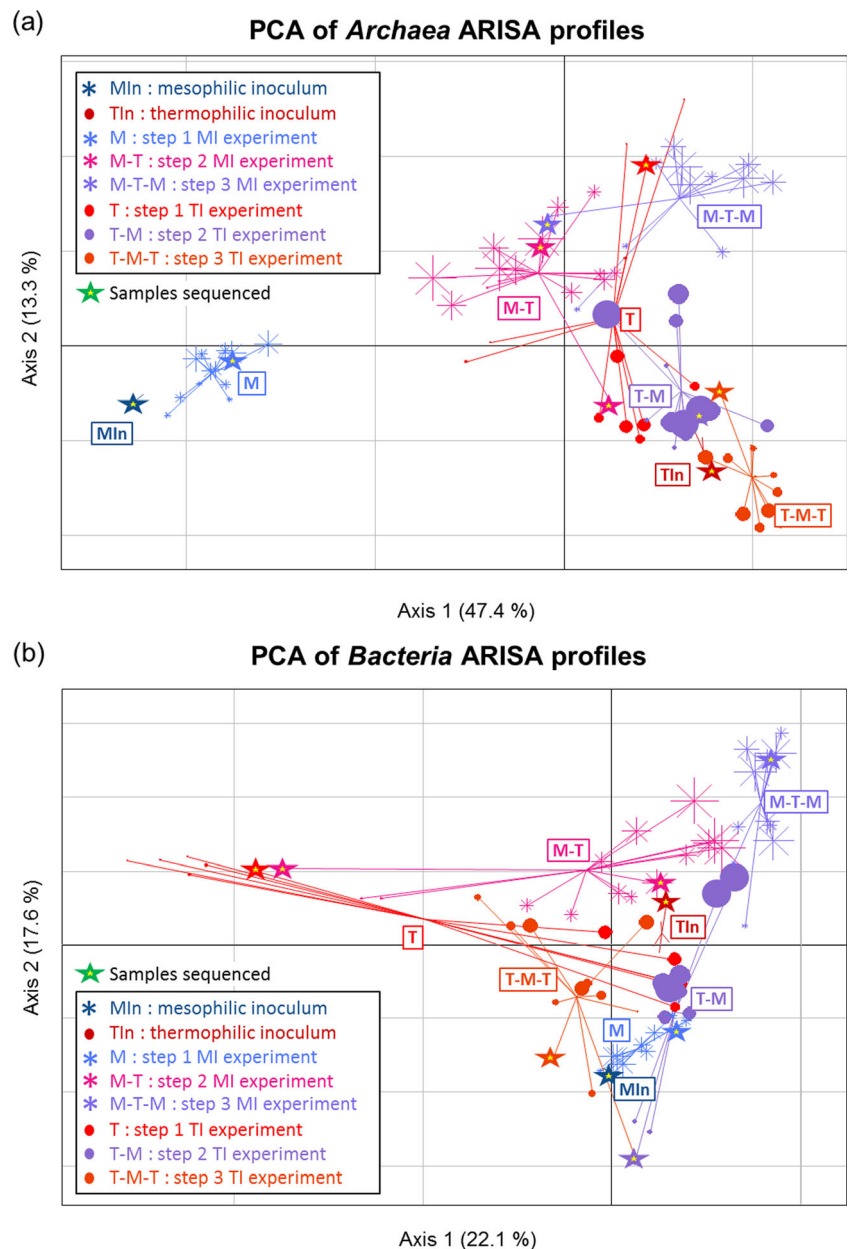
experiments were however observed. Moreover, samples taken in bioreactors incubated in thermophilic conditions (in red) were recovered on the left of the factorial plane while samples taken in bioreactors incubated in mesophilic conditions (in blue) were recovered on the right of the factorial plane. It suggested that the bacterial community was also dependant on temperature changes, even if its modifications were not as radical as those observed with *Archaea* and obviously not irreversible. Important dispersion observed within several groups was explained by the date of sampling.

### Functional phylotypes assimilating $^{13}\text{C}$ from cellulose under different temperature conditions

One sampling date was selected for each experiment step to be centrifuged in CsCl gradient. These dates corresponded to the most intense gas production period of the experiments and represented to our eyes the best available compromise to clearly identify the microbes involved in the processes whereas at the same time minimizing possible cross-feeding biases. The corresponding samples are shown with a star on Fig. 4.

After buoyant density gradient ultracentrifugation, DNA density profiles from labelled experiments were compared with unlabelled experiments, as negative controls (see Fig. S1). Control DNAs extracted from incubations with unlabelled substrates exhibited a buoyant density ranging from 1.68 to 1.71 g/mL, while DNA from the experiment with [ $^{13}\text{C}$ ]-cellulose resulted in the detection of a heavier peak. This peak was recovered in three different fractions (light, medium and heavy DNA fractions), corresponding respectively to a mean density of 1.69, 1.71 and 1.73 g/mL. Efficiency of the separation was checked with ARISA performed on the different fractions of DNA recovered (Fig. S2). It showed that the microbial communities recovered in the different fractions

**Fig. 4** Principal component analysis (PCA) of archaeal (a) and bacterial (b) microbial diversity profiles generated by ARISA. A colour scale was used to distinguish the profiles depending on the experiment and step number, and each group's centroid was represented. Within each group of colour, the size of the markers increases with the date of sampling. For *Archaea*, the first and second axes provided the clearest separation of ARISA profiles, with resp. 47.4 and 13.3 % of the total variance. For *Bacteria*, the first and second axes provided the clearest separation of ARISA profiles, with resp. 22.1 and 17.6 % of the total variance

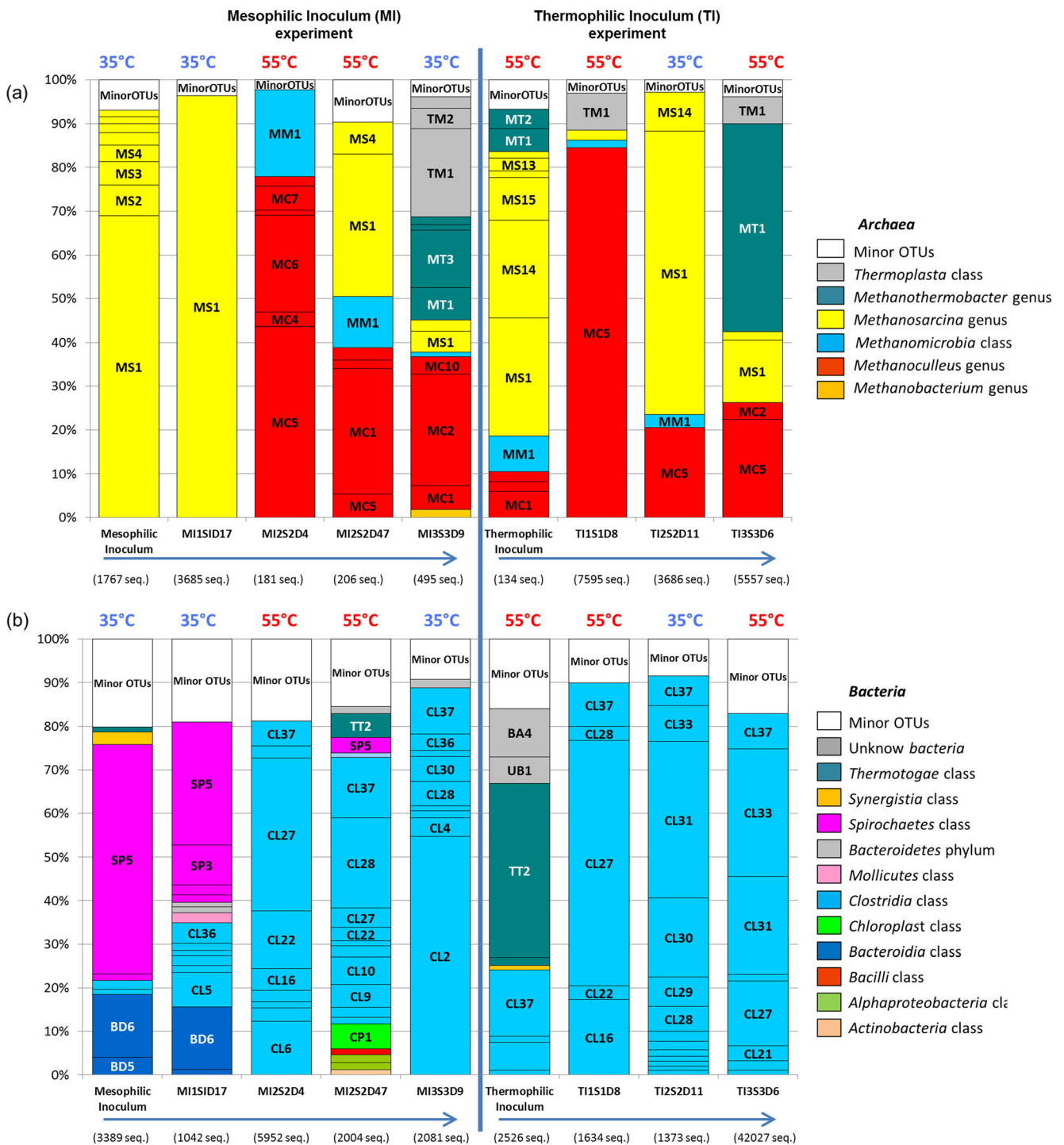


were different and that fractionation had been efficient. Light and heavy fractions of DNA as well as initial inocula were sequenced. Several thousands of sequences were obtained and identified for *Archaea* and *Bacteria*. Figure 5a, b summarizes the results obtained for heavy fractions and inocula respectively for *Archaea* and *Bacteria*. A comparison of the heavy and light fractions of several samples is detailed in the supplementary material (Fig. S3). It also confirmed that fractionation had been efficient to isolate the DNA of microorganisms involved in  $^{13}\text{C}$ -labelled substrate degradation. The name (used in the following paragraphs) and detailed taxonomic assignment of each OTU are presented in the supplementary material (Table S1). In the following paragraphs, microorganisms are described at the species, genus or class level, according to the

precision of the taxonomic information obtained. Two sampling dates were selected for MI step 2 (day 4 and day 47), corresponding to both biogas production periods observed (before 10 days and after 40 days). Cross-feeding may have occurred for day 47 of MI step 2, especially for *Bacteria*; however, as most of the biogas was produced between day 40 and day 55, we supposed that  $^{13}\text{C}$ -labelled DNA signal could give important information on the second biogas production period.

Figure 5a gives a general view of the composition of the archaeal functional biomass in the different samples. Mesophilic inoculum was only composed of OTUs belonging to the genus *Methanosarcina*, as well as a sample of the first step of the MI experiment (dominated by MS1 OTU, see





**Fig. 5** Archaeal (a) and bacterial (b) functional community structure in mesophilic and thermophilic inocula and in heavy fractions of DNA obtained after ultracentrifugation. Heavy DNA sample names are composed of the bioreactor name (MI1, MI2, MI3, TI1, TI2 or TI3),

incubation step (S1, S2 or S3) and number of days since the beginning of the step (D0, D1, etc.). The number of sequences (seq.) analysed and OTU ID are indicated on the graphs. Archaeal genus or bacterial class of OTUs is presented with a colour scale

Table S1 or Fig. 5). Just after temperature change, in step 2, at day 4, during the first biogas production, the functional archaeal community structure changed totally and was dominated by *Archaea* from the *Methanoculleus* genus, *Methanoculleus thermophilus* species (mainly MC5 and

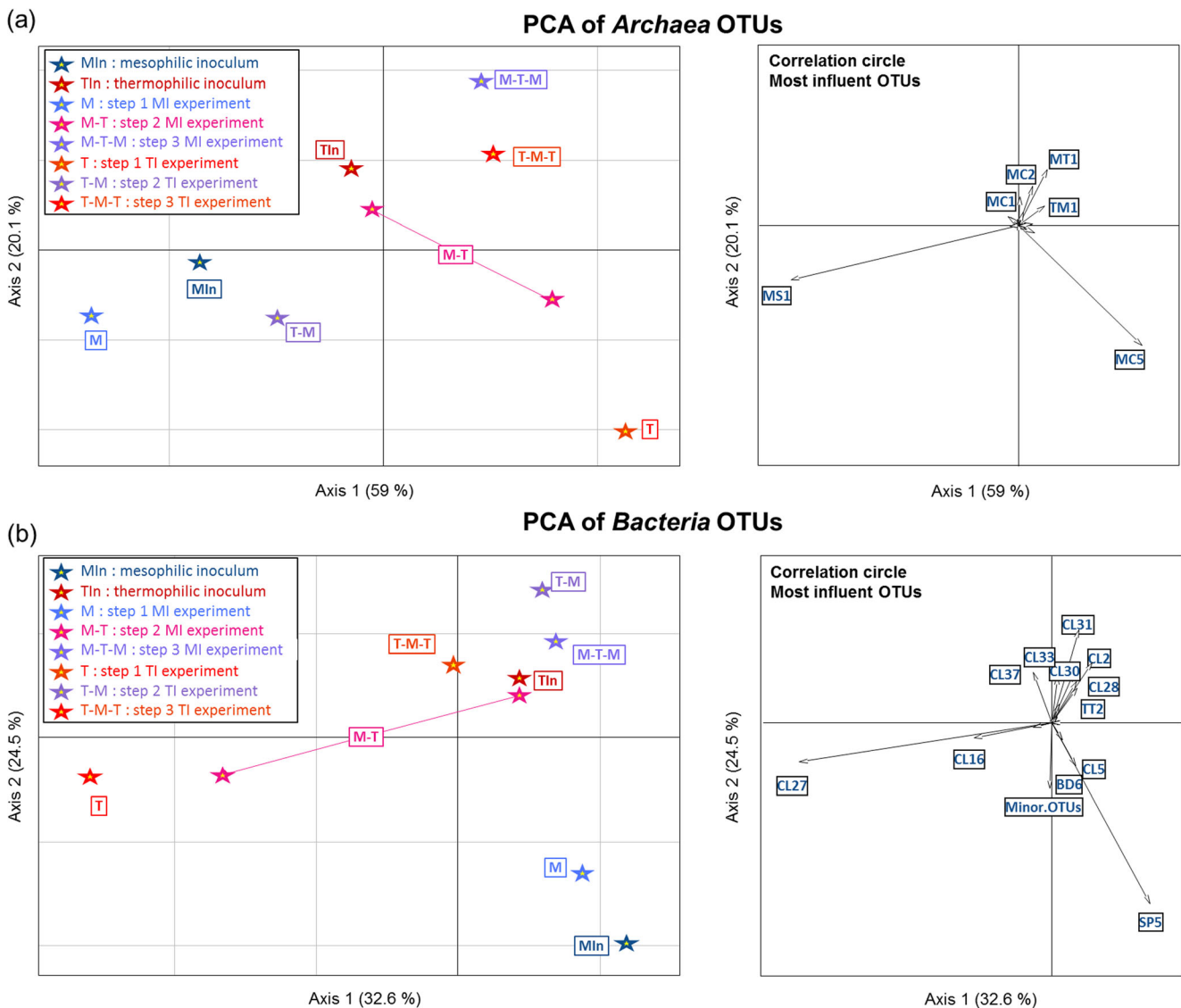
MC6 OTUs). The archaeal community then evolved during the second biogas production (day 47) to a more heterogeneous community consisting of other members of the *Methanomicrobia* class from the *Methanoculleus* and *Methanosarcina* genera (mainly MC1 and MS1 OTUs). After

the temperature was set back to the initial value in step 3, new OTUs from the *Thermoplasta* class and *Methanothermobacter* genera (mainly TM1 and MT3 OTUs) were recovered in the heavy fraction of DNA, together with OTUs of the *Methanosarcina* and *Methanoculleus* genera (mainly MC2 and MS1) found at the end of step 2.

The *Methanosarcina* genus also dominated the archaeal population in thermophilic inoculum (mainly MS1 and MS14 OTUs), which was also composed of members of the *Methanoculleus* and *Methanothermobacter* genera (mainly MC1, MT1 and MT2 OTUs). During the first step of the TI experiment, an OTU of *M. thermophilus* species gained the upper hand (MC5), even if it was a minor OTU in the

inoculum. After the first temperature change, in step 2, two OTUs from the *Methanosarcina* genus already found in the thermophilic inoculum gained in importance (MS1 and MS14), while an OTU of *M. thermophilus* species dominant in step 1 only accounted for 20 % of the sequences (MC5). Finally, in step 3, the archaeal community evolved to a more diverse community, composed of *Archaea* from the *Methanothermobacter* genus (MT1) and of the main OTUs of step 2.

A PCA computed with the OTU data enabled to identify the OTUs differentiating the most the different samples (Fig. 6a). The most discriminating archaeal OTUs were MS1 from the *Methanosarcina* genus; MC5, closely related to



**Fig. 6** Principal component analysis (PCA) of archaeal (a) and bacterial (b) functional community structure in mesophilic and thermophilic inocula and in heavy fractions of DNA obtained after ultracentrifugation. The colour scale represents the incubation steps. The influence of the different OTUs on the PCA is shown with the arrows in the correlation

circles. For *Archaea*, the first and second axes provided the clearest separation of ARISA profiles, with resp. 59.0 and 20.1 % of the total variance. For *Bacteria*, the first and second axes provided the clearest separation of ARISA profiles, with resp. 32.6 and 24.5 % of the total variance

*M. thermophilus* species; and MT1, from the *Methanothermobacter* genus. MS1 was recovered in nearly all the samples but was especially abundant in bioreactors incubated in mesophilic conditions, and its abundance decreased with temperature. On the contrary, MC5 was mostly abundant in thermophilic conditions. MT1 was the dominant OTU in the last step of the TI experiment.

The same analysis was realized with *Bacteria* (Figs. 5b and 6b). Mesophilic inoculum was mainly composed of *Bacteria* from *Spirochaetes* and *Bacteroidia* classes (mainly SP5 and BD6 OTUs). During the first step of the MI experiment, *Spirochaetes* and *Bacteroidia* classes were still found (the same OTUs, but not the only; in particular, OTUs from the *Treponema* genus were found), and the *Clostridia* class accounted for nearly 20 % of total *Bacteria* (*Caldicoprobacter* genus but not the only). After the temperature was changed in step 2, the *Clostridia* class quickly became the dominant functional bacterial class (day 4), with no major OTUs from other classes (but OTUs different from step 1, for example from *Halocella*, *Tepidimicrobium*, and *Opb54* genera). At the end of step 2 (day 47), *Clostridia* remained the dominant class (with common and different OTUs from day 4, the main genera were recovered, *Opb54* became the dominant one and one OTU from *Anaerobranca* appeared), with less than 25 % of *Bacteria* from other classes (mainly *Thermotogae*, *Spirochaetes* and *Chloroplast*). After the temperature was set back to the initial value in step 3, the *Clostridia* class still dominated, even if major OTUs were different from OTUs of step 2. In particular, one OTU from an *incertae sedis* genus became dominant.

In thermophilic inoculum, the dominant bacterial OTUs belonged to *Thermotogae* and *Clostridia* classes (mainly TT2, CL37, CL17 OTUs). During the three steps of the TI experiment, only OTUs from the *Clostridia* class were observed. However, the composition of OTUs among this class evolved a lot, especially between steps 1 and 2. Unfortunately, comparison at the genus level was not possible due to the missing taxonomic assignation.

A PCA computed with the OTUs was used to visualize how samples were differentiated between each other and which OTUs were responsible for the main differences (Fig. 6b). Mesophilic and thermophilic samples were separated on the factorial plane of the PCA. OTUs CL27 and to a lesser extent CL16, from the *Clostridia* class, were mainly recovered in thermophilic incubations of both experiments, even if they were not detected in the inocula. CL16 belonged to the *Halocella* genus but no precise taxonomic assignation could be obtained for CL27. On the contrary, OTUs SP5 (*Spirochaetes* class, *W27* genus) and to a lesser extent BD6 (*Bacteroidia* class) were dominant in the mesophilic inoculum and in the first step of the MI experiment. The other samples were less differentiated within the PCA and mainly distinguished by different abundances of OTUs from the *Clostridia*

class and various genera (CL2 and CL33 from the *incertae sedis* genus, CL28 and CL37 from the *Opb54* genus, CL30 and CL31, undetermined genus).

## Discussion

The first step of the experiments showed that the overall efficiency of cellulose anaerobic degradation was similar in mesophilic and in thermophilic incubations inoculated with an appropriate inoculum. However, the microbial population carrying out the degradation was totally different, but in keeping with previously described results (Chin et al. 1999; Levén et al. 2007; Li et al. 2009). *Spirochaetes*, *Bacteroidia* and *Clostridia* were the dominant bacterial classes in mesophilic conditions, while only the *Clostridia* class was recovered in thermophilic conditions. The *Methanosarcina* genus was recovered in mesophilic conditions, while the *M. thermophilus* species was the dominant methanogen in thermophilic conditions. Different types of modifications were observed as the temperature was increased or decreased, but in general terms, temperature increase was always associated with an effective biogas production—sometimes after an adaptation of several days or weeks—while temperature decrease was always associated with a strong decrease of biogas production—even after waiting several days or weeks.

In the MI experiment, even if the final level of biogas production remained unchanged after temperature increase, a temporary destabilization was observed, as indicated by the high level of VFA accumulation and a delayed biogas production. It was also noticed by several authors with different substrates during increase of temperature (Bouskova et al. 2005; Choorit and Wisamwan 2007; Lindorfer et al. 2008; Pap et al. 2015). In response to temperature stress, important rearrangements of the microbial communities occurred, the archaeal community being dominated by *M. thermophilus* sp. and the bacterial community by the *Clostridia* class. Non-adapted microorganisms were replaced by mesophilic thermotolerant microorganisms or thermophilic mesotolerant microorganisms that took over released ecological niches. A delay between the archaeal community and hydrolysing bacterial ecosystem rearrangement could explain the temporary destabilization of the system: *Archaea*, being more sensitive to temperature variations than *Bacteria*, were inhibited immediately or adapted less quickly, which led to the accumulation of acetate produced by *Bacteria* (Pap et al. 2015). Acetate was finally consumed after 40 days. From this point on, mainly methane was produced, suggesting that hydrogenotrophic methanogenesis was dominant, as confirmed by sequencing results, as *Archaea* from the *Methanoculleus* genus are hydrogenotrophic methanogens (Schnürer et al. 1999). Acetate was probably consumed by microorganisms carrying out the oxidation of acetate (Zinder 1994), and the products of this

reaction were used in the hydrogenotrophic methanogenesis to produce methane. Several authors showed that this syntrophy was predominant in disturbed systems (McHugh et al. 2004; Noll et al. 2010) and that *Archaea* of the *Methanoculleus* genus could be involved (Schnürer et al. 1999). Similarly, a rapid adaptation of acetotrophs associated with an increase of hydrogenotrophic microorganisms during the transition from mesophilic sludge to thermophilic conditions has also been observed by Cabirol et al. (2003) and an increased importance of syntrophic acetate-oxidizing bacteria under thermophilic or stressed conditions was described by Pap et al. (2015).

In the TI experiment, the yield and rate of methane production were much lower after temperature decrease. However, not all degradation steps were stopped. An accumulation of acetate was observed, indicating that the bacterial community was still active even if it was modified through the *Clostridia* class. Unfortunately, a comparison of the genera involved was not possible due to the missing taxonomic assignment. Microorganisms of step 1 were replaced by microorganisms that were more efficient in mesophilic conditions. Acetate isotopic enrichment was lowest than enrichment measured in step 1, suggesting that a fraction of the biomass died and was recycled. *Archaea* did not adapt well even if sequencing after SIP showed an evolution of the functional community composition, maybe not relevant. Indeed, as *Archaea* were not very active, they were not highly  $^{13}\text{C}$  enriched. Consequently, we suggest that the heavy fraction of DNA after fractionation probably contained a low level of  $^{13}\text{C}$ -enriched archaeal DNA (compared to bacterial DNA) but also residual not enriched DNA badly resolved during SIP and amplified during the sequencing.

The same observations were made during temperature decrease in step 3 of the MI experiment. After incubation in thermophilic conditions, the bioreactors were not able to reach a normal operation in mesophilic conditions, even if the inoculum initially came from a mesophilic digester. It suggested that temperature increase in step 2 irreversibly selected thermotolerant microorganisms, as for example *Bacteria* from *Clostridia* class. The growth rate of mesophilic microorganisms dropped in thermophilic conditions, maybe due to denaturation of key proteins, and they were eliminated from the biomass of the bioreactors. Thermotolerant microorganisms that remained in the bioreactors in step 2 were not necessarily efficient at low temperature in step 3, explaining the low biogas production. Re-inoculation could be necessary to restore the digestion process (Conrad et al. 2009). Methanogens were more influenced than *Bacteria*, maybe because their functional redundancy was less important or because their physiology was more sensitive to temperature.

Per contra, temperature decreases induced important pathways and flora modifications, especially within *Bacteria*, but did not permanently affect microorganisms' functionality.

Indeed, after temperature increase in step 3 of the TI experiment, degradation of cellulose occurred, signifying that thermophilic microorganisms had survived in mesophilic conditions. However, for the first time in the experiment, replicate bioreactors behaved differently. Reduced activity in step 2 probably modified microbial diversity balance within the triplicates, especially thermophilic minority members that survived in the bioreactors. When temperature was increased in step 3, a dissimilar and stochastic evolution of these microorganisms occurred and resulted in various patterns of cellulose degradation. The selection imposed by the mesophilic conditions was less strict than selection imposed by thermophilic conditions, but resulted anyway in strongly modified microbial communities.

We observed an asymmetric behaviour toward temperature modifications. We think that thermophilic conditions probably selected thermotolerant microorganisms that were not necessarily efficient in mesophilic conditions. Selection imposed by mesophilic conditions was less strong, but the temperature stress during temperature decrease enabled a modification of the microbiota equilibrium and of the dominant microorganisms. *Archaea* were particularly temperature sensitive, maybe because their functional redundancy was lower than that of *Bacteria*.

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

**Conflict of interest** The authors declare that they have no competing interests.

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