

# Influence of phenylacetic acid pulses on anaerobic digestion performance and archaeal community structure in WWTP sewage sludge digesters

Léa Cabrol, Johana Urrea, Francisca Rosenkranz, Pablo Araya Kroff, Caroline M. Plugge, Yves Lesty and Rolando Chamy

## ABSTRACT

The effect of phenylacetic acid (PAA) pulses on anaerobic digestion (AD) performance and archaeal community structure was evaluated in anaerobic digesters treating sewage sludge from a wastewater treatment plant (WWTP). Four pilot-scale continuous stirred tank reactors were set up at a full-scale municipal WWTP in Santiago de Chile, and fed with either primary or mixed sewage sludge. AD performance was evaluated by volatile fatty acid (VFA) and biogas production monitoring. Archaeal community structure was characterized by 16S rRNA denaturing gradient gel electrophoresis and band sequencing. In the primary sludge digester, a single PAA pulse at 200 mg L<sup>-1</sup> was sufficient to affect AD performance and archaeal community structure, resulting in long-term VFA accumulation, reduced biogas production and community shift from dominant acetoclastic (*Methanosaeta concilii*) to hydrogenotrophic (*Methanospirillum hungatei*) methanogens. By contrast, AD performance and archaeal community structure in the mixed sludge digester were stable and resistant to repeated PAA pulses at 200 and 600 mg L<sup>-1</sup>. This work demonstrated that the effect of PAA pulses on methanogenic activity and archaeal community structure differed according to AD substrate, and suggests that better insights of the correlations between archaeal population dynamics and functional performance could help to better face toxic shocks in AD.

**Key words** | acetoclastic methanogens, anaerobic digestion, hydrogenotrophic methanogens, mixed sludge, phenyl acetic acid pulses, primary sludge

Léa Cabrol (corresponding author)

Johana Urrea

Francisca Rosenkranz

Rolando Chamy

Biochemical Engineering,  
Pontifical Catholic University of Valparaíso,  
Avenida Brasil 2950, Valparaíso,  
Chile  
E-mail: lea.cabrol@ucv.cl

Léa Cabrol

Fraunhofer Chile Research,  
M. Sánchez Fontecilla 310,  
Las Condes, Santiago de Chile,  
Chile

Pablo Araya Kroff

Suez Environment,  
CIRSEE. 38 Rue du Président Wilson,  
78230 Le Pecq,  
France

Caroline M. Plugge

Laboratory of Microbiology,  
Wageningen University,  
Dreijenplein 10, 6703HB Wageningen,  
The Netherlands

Yves Lesty

Aguas Andinas S.A.,  
Avenida Presidente Balmaceda, 1398  
Santiago de Chile,  
Chile

## INTRODUCTION

Anaerobic digestion (AD) technologies experienced a strong development in the last decades, because of their fundamental role in wastewater treatment coupled to sustainable energy production (Weiland 2010). AD has been applied successfully to the treatment of a high variety of wastes, including sludge from industrial and municipal wastewater treatment plants (WWTPs) (Appels *et al.* 2008). Several types of sludge are generated during the WWTP process: primary sludge (from the primary sedimentation of settleable solids), secondary sludge (from the biological conversion of organic matter in bioreactors such as activated sludge (AS)) and mixed sludge (combination of primary and secondary sludge) (Arnaiz *et al.* 2006). Each sludge presents specific and distinct physico-chemical properties, in terms

of density, moisture content, viscosity, size distribution, organic matter composition and toxic content.

The AD process is known to be inhibited by a wide range of organic compounds such as aromatic compounds, halogenated compounds, nitrogenous compounds and aliphatic compounds (Chen *et al.* 2008). Aromatic compounds, such as phenol and its derivatives, are toxic for microorganisms due to their effect on the cell membrane, and induce loss of metabolic activity in biological processes used for wastewater treatment (Veeresh *et al.* 2005; Monlau *et al.* 2014). The adverse effect of phenol on AD, and the capacity of microbial communities to adapt and resist to high phenol concentrations (up to 1,200 mg L<sup>-1</sup>), have been evidenced in laboratory-scale anaerobic sequencing

batch reactors (Rosenkranz *et al.* 2013). However, the toxicity level of aromatic compounds reported in the literature is conflicting as it also depends on other parameters such as process configuration, physico-chemical properties of the substrate and sludge characteristics (biomass concentration, cell age and microbial community composition) (Chen *et al.* 2008). At low concentrations, some phenolic compounds can even have stimulatory effect on methanogenic fermentation (Field & Lettinga 1987).

Phenylacetic acid (PAA) is a phenolic contaminant present in wastewaters from chemical facilities and agro-industrial plants like olive oil mills (Hamdi & Garcia 1993; Fiorentino *et al.* 2003; Kyriakou *et al.* 2005; Khoufi *et al.* 2006), citrus processing plants (Lane 1980) and wine distilleries (Sierra Alvarez & Lettinga 1991), swine manure (Iannotti *et al.* 1986), landfill leachate (Li *et al.* 2007) and sugar beet pulp wastewater (Retfalvi *et al.* 2013). PAA concentrations between 10 and 30 mg L<sup>-1</sup> have been reported, respectively, in olive oil mill wastewater (Kyriakou *et al.* 2005) and landfill leachate (Li *et al.* 2007), while they ranged from 25 to 345 mg L<sup>-1</sup> in swine manure (Iannotti *et al.* 1986) and reached 350–400 mg L<sup>-1</sup> in sugar beet pulp wastewater (Retfalvi *et al.* 2013). In the primary sludge of municipal WWTP 'La Farfana', Santiago, Chile, a preliminary study indicated that PAA was present at 148 mg L<sup>-1</sup> and it was identified as one of the most toxic compounds among 13 potential inhibitors in municipal wastewater entering the WWTP (Urrea *et al.* 2008). PAA can be produced by lignocellulose and tannin decomposition in plants, by the degradation of proteins containing aromatic amino acids, or from the degradation of detergents and surfactants used in personal care products and in the formulation of various chemicals (alkyl phenol ethoxylates and alkylbenzene sulfonate) (Scott & Jones 2000).

Like other phenolic compounds, PAA is considered as potentially harmful for aquatic and plant ecosystems (Scott & Jones 2000; Fiorentino *et al.* 2003). The toxicity of PAA on various aquatic organisms from different trophic levels ranged between 4.5 and 93.7 mg/L EC<sub>50</sub> (Fiorentino *et al.* 2003). PAA is also a potential inhibitor of AD from various residual substrates, involving strong process failure (Lane 1980; Khoufi *et al.* 2006), and its high toxicity on acetoclastic methanogens has been demonstrated in granular sludge batch experiments at laboratory scale (Sierra Alvarez & Lettinga 1991). During AD of high load kitchen wastes, PAA accumulated in highest concentration among other aromatic acids, even before the increase of volatile fatty acid (VFA) concentration. This makes it an early indicator of subsequent process failure (Hecht & Griehl 2009). Like

other phenolic acids, PAA can inhibit both the hydrolytic and methanogenic activities within the AD process (Iannotti *et al.* 1986). PAA inhibition thresholds on AD range from <10 to 3,000 mg L<sup>-1</sup> depending on the process configuration and substrate considered, as further detailed in the 'Results and discussion' section.

The AD process involves several groups of bacterial and archaeal populations in close interaction (Narihiro *et al.* 2007). In this symbiosis, the methanogenic populations are known to be more sensitive than the hydrolytic and acidogenic bacteria to changes in environmental and/or operational conditions (such as pH, temperature or toxic surfactant), leading to process disturbances (Garcia Morales *et al.* 2001; Appels *et al.* 2008). Optimization of anaerobic systems is complex, given that each microbial group exhibits different optimal conditions and different resistance levels to toxic compounds (Karakashev *et al.* 2005). For instance, acetate-utilizing methanogens are more sensitive to ammonia and VFA than are hydrogenotrophic methanogens (Karakashev *et al.* 2005). The recent development of molecular techniques allows in-depth characterization of microbial community structure, diversity and composition in anaerobic digesters (Talbot *et al.* 2008). These techniques have been increasingly used in order to monitor changes of microbial community in relation to changes of operating conditions and functional performance (Keyse *et al.* 2006; Rosenkranz *et al.* 2013). A better knowledge and understanding of the community changes in response to toxic compound stress could eventually help operators to better manage the AD process in the face of inhibitors.

Since PAA has been identified as a major toxic compound in WWTP sludge digestion, the objective of this study was to evaluate the effect of PAA pulses on AD performance and archaeal community structure, in four pilot-scale continuous stirred tank reactors (CSTRs) treating either primary or mixed sludge from a full-scale municipal WWTP.

## MATERIAL AND METHODS

### Experimental set-up

Four pilot-scale CSTR anaerobic digesters were set up at the WWTP La Farfana in Santiago, Chile (Aguas Andinas S.A.). This plant treats wastewater from 3.7 million population-equivalent with a capacity of 8.8 m<sup>3</sup> s<sup>-1</sup>. Each pilot-scale reactor had a total volume of 2 m<sup>3</sup> corresponding to a

working volume of 1.6 m<sup>3</sup>. All reactors were operated at 35 ± 1 °C and pH 7.4 ± 0.1 for 160 days.

The digesters were fed with real substrate coming from the WWTP itself: two reactors (R1 and R2) were fed with primary sludge, while two reactors (R3 and R4) were fed with mixed sludge. The mixed sludge was a mixture of primary/secondary sludge in the ratio 60/40% (dry weight). The total solids (TS) content of primary and mixed sludge was, respectively, 71 ± 10 g L<sup>-1</sup> and 48 ± 4 g L<sup>-1</sup>, corresponding to a volatile solids (VS) content of, respectively, 72.8% and 78% of TS (w/w). All digesters were inoculated with methanogenic sludge from the full-scale anaerobic digesters of the CSTR set up at the WWTP.

Given the differences of TS and VS contents between primary and mixed sludge, the digesters were operated at different solids retention times (SRTs) in order to maintain a similar organic loading rate (OLR) (Table 1).

### PAA pulses

Reactors 1 and 3 were exposed to an instantaneous pulse of PAA at 200 mg<sub>PAA</sub> L<sup>-1</sup> on days 58 and 49, respectively, while reactors 2 and 4 were operated as control, as shown in Table 1. In addition, reactor 3 was exposed to a second pulse of PAA at higher concentration (600 mg<sub>PAA</sub> L<sup>-1</sup> in the reactor) on day 72. PAA concentrations were chosen as representative of its detection in the WWTP under study. Given its hydrophobicity, PAA had to be diluted in ethanol for the pulse application in reactors 1 and 3. Therefore, the control reactors were submitted to a pulse of solely ethanol at a concentration equivalent to the total chemical oxygen demand (COD) concentration of ethanol+PAA used in the test reactors.

**Table 1** | Operating conditions of the anaerobic digesters

Reactor	Sludge	OLR (kg <sub>VS</sub> fee m <sup>-3</sup> d <sup>-1</sup> )	SRT (d)	PAA concentration (mg L <sup>-1</sup> )	
				Pulse 1	Pulse 2
R1	Primary	1.1 ± 0.2	51.5 ± 8.7	200 <sup>a</sup>	0
R2	Primary	1.1 ± 0.2	51.5 ± 8.7	0	0
R3	Mixed	1.5 ± 0.2	25.9 ± 8.9	200 <sup>b</sup>	600 <sup>c</sup>
R4	Mixed	1.5 ± 0.2	25.9 ± 8.9	0	0

Mean values ± standard deviations were calculated during the operation periods.

<sup>a</sup>PAA pulse applied at day 58.

<sup>b</sup>PAA pulse applied at day 49.

<sup>c</sup>PAA pulse applied at day 72.

### Analytical methods

TS, VS, pH and alkalinity were measured following *Standard Methods* (APHA 1996).

VFA were quantified daily by a gas chromatograph GC-8A (Shimadzu, Kyoto, Japan), equipped with a 30" × 4 mm ID packed column GP 60/80 Carbowax C/0.3% Carbowax 20M/0.1% H<sub>3</sub>PO<sub>4</sub> (Sigma Aldrich, St Louis, MO, USA). The analysis was carried out at 120 °C, with nitrogen as carrier gas (30 mL min<sup>-1</sup>) and a flame ionization detector (200 °C). The total VFA concentration was expressed in acetate equivalents by molar conversion.

Biogas flow rate was determined daily by monitoring the volume accumulated in a floating gasometer (Indutécnica Chacón SIC LTDA, Santiago, Chile). Methane concentration in the biogas was quantified weekly by gas chromatography using a Clarus 500 gas chromatograph (Perkin Elmer, Waltham, MA, USA) equipped with a filled Teflon column Hayesep-Q, Supelco, 4 m, 1/8" diameter, and thermal conductivity detector (120 °C). The specific biogas production rate (L kg<sub>VSS-feed</sub><sup>-1</sup> d<sup>-1</sup>) was calculated as the ratio of biogas production flow rate (L d<sup>-1</sup>) to the VS feeding rate (kg<sub>VSS-feed</sub> d<sup>-1</sup>). The sludge methanogenic activity (SMA) was assessed punctually according to the procedures described by Soto et al. (1993).

PAA concentration was determined using a high-performance liquid chromatograph (Jasco, Easton, MD, USA) equipped with a Kromasil 100 5C18 (150 × 4.6 mm<sup>2</sup>) column (AkzoNobel, Göteborg, Sweden) and UV light detector UV 2075 plus (Jasco, Easton, MD, USA). The analysis was carried out at room temperature, with 35% acetonitrile and 65% phosphate buffer (pH 3) as carrier (1.0 mL L<sup>-1</sup>).

### Characterization of the archaeal community structure

Sludge was sampled from each reactor before and after the PAA shocks (see sampling days in Figure 2) and stored at -20 °C. Genomic DNA was extracted from 0.5 mL of sludge sample using FastDNA SPIN soil kit (MP Biomedicals, Santa Ana, CA, USA) and quantified by absorbance at 260 nm (Biospec-nano, Shimadzu, Kyoto, Japan).

The V2-V3 region of archaeal 16S rRNA was amplified by polymerase chain reaction (PCR) using the primers A109f (Groskopf et al. 1998) and A515r with GC clamp at the 5' end (Lane 1991). PCR amplification was carried out in 50 µL with 1.25 U GoTaq DNA Polymerase (Promega, Madison, WI, USA), 25 mM MgCl<sub>2</sub>, 10 µM each primer, 10 µM dNTP and 10 ng template DNA, in an MJ-Mini thermocycler (Bio-Rad Laboratories Inc., Hercules, CA, USA) through 35 cycles using hybridization temperature of 56 °C.

PCR products (10  $\mu$ L) were separated by denaturing gradient gel electrophoresis (DGGE) with a linear gradient ranging from 30 to 50%, according to the protocol of [Muyzer \*et al.\* \(1993\)](#), using the DCode System (Bio-Rad Laboratories Inc., Hercules, CA, USA). Gel images were analyzed with Bionumerics software (Applied Maths, Sint-Martens-Latem, Belgium). The pair-wise similarity index between community profiles was calculated by the Bray–Curtis coefficient.

Bands of interest were cut from the gel and incubated overnight at 37 °C in 25  $\mu$ L of TE buffer. The obtained DNA fragments were PCR-amplified with the same primers as before, without GC-clamp. Amplicons were purified with DNA Clean&Concentrator-5 kit (Zymo Research, Irvine, CA, USA) and sequenced by BaseClear services (Leiden, The Netherlands). Sequence analysis was carried out with BLAST against the NCBI GenBank database to check for similarity (percentage of sequence identity). The partial 16S rRNA gene sequences were deposited in GenBank under accession numbers KM821359 to KM821363.

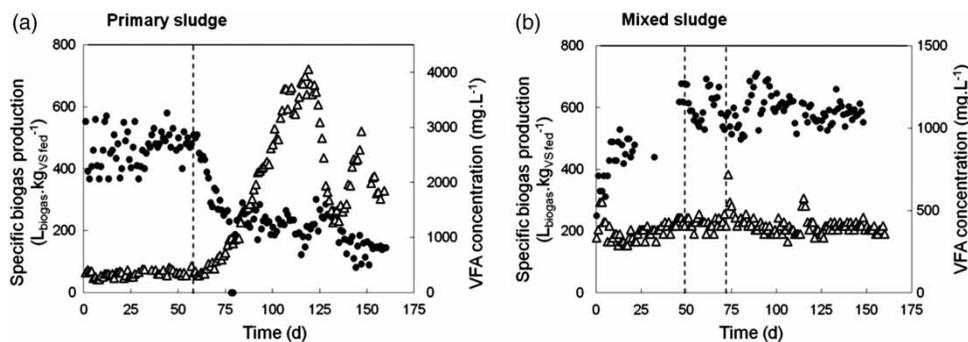
The robustness of the whole method (from DNA extraction to DGGE profile analysis) has been evaluated on duplicated sludge samples from full-scale anaerobic digesters from the WWTP.

## RESULTS AND DISCUSSION

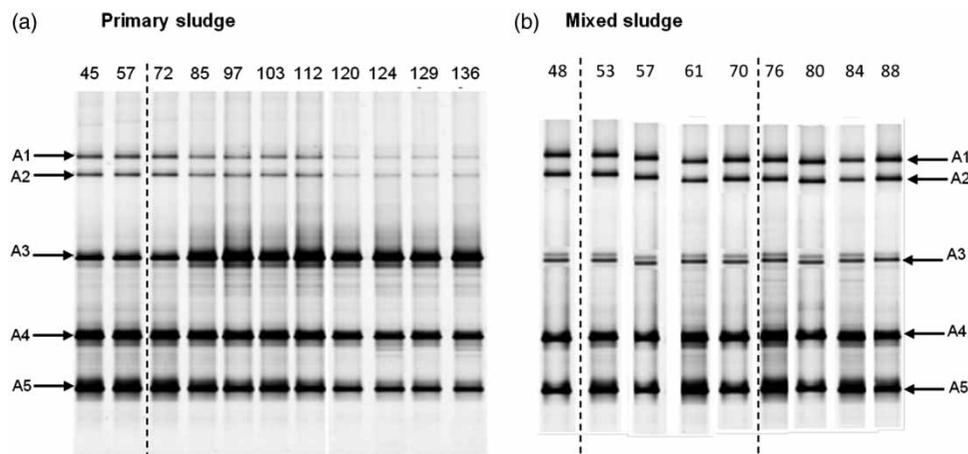
### Effect of PAA shock on the performance of primary sludge AD

After initial variability during the first 30 days, the performance of primary sludge digestion in R1 stabilized, with specific biogas production of  $460 \pm 60$  L  $\text{kg}_{\text{VS fed}}^{-1}$  and VFA concentration of  $346 \pm 56$  mg  $\text{L}^{-1}$  before the PAA pulse ([Figure 1\(a\)](#)). The methane concentration in the biogas stayed constant around  $66.0 \pm 0.8\%$ .

After the PAA pulse on day 58, the performance of primary sludge AD showed a clear destabilization compared



**Figure 1** | Evolution of specific biogas production ( $\bullet$ , L<sub>biogas</sub> kg<sub>VS fed</sub><sup>-1</sup>) and VFA concentration ( $\nabla$ , mg<sub>CH<sub>3</sub>COOH-eq</sub> L<sup>-1</sup>) in digesters fed with primary sludge (a) and mixed sludge (b). Dotted lines indicate the times of PAA pulses.



**Figure 2** | 16S rRNA-DGGE profiles of archaeal community from (a) primary sludge and (b) mixed sludge digesters over time. The sampling days are indicated above each lane. Excised and sequenced bands are indicated from A1 to A5 (see [Table 2](#) for identification). Dotted line indicates the time of PAA pulses.

to the untreated reactor, with an increase of VFA concentration up to a peak of  $4,056 \text{ mg L}^{-1}$  at day 120, and a decrease by 67% of specific biogas production, down to  $150 \pm 30 \text{ L kg}_{\text{VSS}}^{-1} \text{ fed}$  (Figure 1(a)). Similarly, the SMA of the primary sludge dropped by 63% after the PAA pulse, from  $0.16 \pm 0.01$  to  $0.06 \pm 0.01 \text{ gCOD}_{\text{methane}} \text{ g}_{\text{VSS}}^{-1} \text{ d}^{-1}$ .

After day 120, the VFA concentration tended to decrease but was still higher than expected and unstable, oscillating between  $1,272$  and  $2,928 \text{ mg L}^{-1}$ . The SMA and the specific biogas production rate remained at a low level below the biogas production level reached in the control reactor R2 and did not recover for 100 days after the PAA pulse. The methane content in the biogas stayed unchanged during the whole experiment ( $64.3 \pm 2.2\%$ ). The pH was controlled and maintained constant in the reactor during the whole study ( $7.4 \pm 0.2$ ), except between days 106 and 123 when a slight acidification occurred ( $6.9 \pm 0.1$ ).

By contrast, the control reactor R2 exhibited stable AD performance during the whole study, in terms of VFA concentration ( $327 \pm 29 \text{ mg L}^{-1}$ ), biogas production rate ( $270 \pm 40 \text{ L}_{\text{biogas}} \text{ kg}_{\text{VSS}}^{-1} \text{ fed}$ ), methane concentration in the biogas ( $66.0 \pm 0.8\%$ ) and SMA ( $0.15 \pm 0.01 \text{ gCOD}_{\text{methane}} \text{ g}_{\text{VSS}}^{-1} \text{ d}^{-1}$ ). Initial absolute performance was lower in R2 than R1 probably because of inherent substrate variability, but it was much more stable with time, leading to higher final performance. The stability of the digestion process in R2 indicates that the pulse of ethanol which occurred in the control reactor at the same time and same COD level as the PAA+ethanol pulse applied to the test reactor had no significant effect on AD performance during primary sludge digestion.

Various phenolic compounds can inhibit the anaerobic degradation of readily biodegradable organic matter, with 50% inhibition at concentrations ranging between 100 and  $600 \text{ mg g}_{\text{VSS}}^{-1}$  (Hernandez & Edyvean 2008). The level of inhibition depends on the oxidation, polarity, type, size and number of substitutions of the phenolic compounds. The concentration of the phenolic inhibitors has generally more influence on the biogas production efficiency than the pH has (Hernandez & Edyvean 2008). Among 13 potentially inhibitory compounds identified in municipal wastewater entering the WWTP La Farfana, Santiago, Chile, PAA was considered as one of the most toxic compounds, its half maximal inhibitory concentration ( $\text{IC}_{50}$ ) on methanogenesis being  $1,700 \text{ mg L}^{-1}$  at sludge concentration ranging from 1.5 to  $3.0 \text{ g}_{\text{VSS}} \text{ L}^{-1}$  (Urrea *et al.* 2008). The threshold of PAA concentration reported to inhibit methanogenic activity differs according to the process configuration and substrate considered: it ranged from  $<10$  to  $135 \text{ mg L}^{-1}$  in citrus-processing wastewater (Lane 1980), while it reached

$681 \text{ mg L}^{-1}$  (5 mM) in digestion of food industry and household waste (Karlsson *et al.* 2012),  $718 \text{ mg L}^{-1}$  in acetate-degrading consortia (Sierra Alvarez & Lettinga 1991) and up to  $3,000 \text{ mg L}^{-1}$  in anaerobic fermentation of sugar beet pulp (Retfalvi *et al.* 2013). In anaerobic fermentation of sugar beet pulp, PAA overdosing caused an extremely high accumulation of VFA, that consequently decreased the pH. This points to an indirect inhibition of methanogenesis by acidification rather than by PAA itself (Retfalvi *et al.* 2013). However, bacteria such as *Thauera aromatica* and some sulfate reducers are able to metabolize PAA under anaerobic conditions (Heider & Fuchs 1997). In pure cultures of cellulolytic rumen bacteria using *Ruminococcus albus*, the rate of cellulose digestion was even significantly improved by PAA addition, which was involved in phenylalanine biosynthesis (Stack *et al.* 1983).

### Effect of PAA shock on the performance of mixed sludge AD

After the first PAA pulse on day 49, the VFA concentration stayed remarkably stable ( $370 \pm 53 \text{ mg}_{\text{VFA}} \text{ L}^{-1}$ ) in test reactor R3, similar to the pre-pulse and control levels (Figure 1(b)). After initial increase during the start-up phase, the biogas production did not show any clear destabilization in response to the first PAA pulse, its oscillations remaining in the range of variability observed in the control digester ( $605 \pm 50 \text{ L}_{\text{biogas}} \text{ kg}_{\text{VSS}}^{-1} \text{ fed}$ ). The methane concentration in the biogas also stayed constant around  $65.0 \pm 1\%$ . Similarly, the SMA of the mixed sludge stayed relatively stable around  $0.11 \pm 0.03 \text{ gCOD}_{\text{methane}} \text{ g}_{\text{VSS}}^{-1} \text{ d}^{-1}$ , similar to the SMA level in control reactor R4. This suggests that the first PAA pulse of  $200 \text{ mg L}^{-1}$  had no significant effect on the overall performance of mixed sludge digestion.

The second PAA pulse on day 72 resulted in a sharp and transitory increase of VFA concentration in the test reactor R3, which rapidly recovered its pre-shock level. The specific biogas production rate was not affected by the second PAA pulse, and remained at the same high level until the end of the experiment, demonstrating the high resistance capacity of mixed sludge AD.

The different structural and functional stability capacities of mixed sludge and primary sludge digesters can be explained by different compositions of the feed, resulting in overall different exposures to contaminant and distinct substrate-to-microorganism ratio, which directly influences the methanogenic activity (Moreno Andrade & Buitrón 2003). The greater sensitivity of methanogens in the primary sludge has been reported for other types of

perturbations, such as thermal shock, while the indigenous methanogens present in secondary sludge were more resistant to stress (Gavala et al. 2003). The mixed sludge used in this study was a mixture of primary and AS, with globally lower methanogenic activity as indicated by SMA. Several studies revealed that primary sludge is more readily digestible than AS, which is generally hydrolysis-limited (Gavala et al. 2003, Arnaiz et al. 2006, Carrère et al. 2010). As evidenced from six full-scale municipal WWTPs in Denmark, primary sludge has greater potential of VFA production than activated and mixed sludge, because of its higher content of easily degradable organic matter. By contrast, most of the organic matter in AS is less available, accessible and biodegradable since it contains more polymers, inert materials and cell debris from AS (Ucisik & Henze 2008). The higher content of difficult-to-degrade material in mixed sludge can create a physical protection barrier against contaminants, by decreasing the contact between active methanogens and toxic compounds, which could explain the lower sensitivity of mixed sludge observed in our study. Moreover, mixed-sludge biomass might be adapted to recalcitrant compounds and less affected when exposed to PAA.

As previously reported by Hecht & Griehl (2009), the response of the overall AD process to PAA pulses cannot be generalized because it strongly depends on the substrate used and the PAA concentration. When swine manure was digested, a PAA pulse of 100 mg L<sup>-1</sup> resulted in biogas production stimulation, without VFA accumulation; a PAA pulse of 200 mg L<sup>-1</sup> resulted in a slight and transitory increase of VFA, quickly returning to basal level; whereas a PAA pulse of 400 mg L<sup>-1</sup> led to significant long-term accumulation of propionate and several other toxic

aromatic compounds, with the maximal VFA accumulation being reached around 1 month after the PAA pulse (Hecht & Griehl 2009). In contrast, when kitchen waste was used as substrate, the VFA accumulation and biogas production inhibition had already occurred at the first PAA pulse at 100 mg L<sup>-1</sup>.

### Archaeal community structure in primary sludge digesters

Despite the quantitative bias inherent in the different steps of PCR-DGGE analysis (Muyzer et al. 1993), here this methodology complies with the objective to compare the dominant archaeal community members between samples undergoing the same methodological steps. The robustness of the whole method (from DNA extraction to DGGE profile analysis) has been previously evaluated on duplicated sludge samples from full-scale anaerobic digesters from the WWTP, providing >98% similarity (Bray-Curtis index) between replicate DGGE profiles (data not shown).

### Under steady-state

Before the contaminant pulse, the archaeal community in digesters fed with primary sludge was dominated by five bands, as revealed by DGGE profiles (Figure 2(a)). In absence of perturbation, the archaeal community composition stayed stable during the whole experiment in the control digester. The most abundant bands (A4 and A5) were affiliated with 99% similarity to the acetoclast methanogen *Methanosaeta concilii* (Table 2). Bands A4 and A5 exhibited highly similar sequences and were affiliated to the same species, which confirms one of the possible DGGE

**Table 2** | Phylogenetic affiliations of the archaeal 16S DNA sequences excised from DGGE gels

DGGE Band	Closest BLAST affiliation (accession number)	Similarity (%)	Origin of the closest match	Primary sludge		Mixed sludge	
				Before shock	After shock	Before shock	After shock
A1	<i>Methanospaera stadtmannae</i> (NR_074323)	87	Human intestine <sup>a</sup>	+	-	++	++
A2	Uncultured archaeon clones (JX110157)	99	Pig manure <sup>b</sup>	+	-	++	++
A3	<i>Methanospirillum hungatei</i> (NR_074177)	89	Pure strain	++	+++	+	+
A4	<i>Methanosaeta concilii</i> GP6 (NR_102903)	99	Sewage sludge <sup>c</sup>	+++	++	+++	+++
A5	<i>Methanosaeta concilii</i> GP6 (NR_102903)	99	Sewage sludge <sup>c</sup>	+++	++	+++	+++

The accession number of the closest BLAST match is indicated in parenthesis. The intensity of each band before and after the PAA pulses in primary and mixed sludge digesters is summarized by symbols from very low intensity (-) to very high intensity (+++).

<sup>a</sup>Fricke et al. (2006).

<sup>b</sup>Barret et al. (2013).

<sup>c</sup>Patel (1984).

biases linked to multiple 16S sequences in single species. Another sub-dominant community member (band A3) was affiliated to the hydrogenotroph *Methanospirillum hungatei*, able to use either formate or H<sub>2</sub>/CO<sub>2</sub> to produce methane. A minor community member (band A1) was distantly related to the methylotroph *Methanospiraer stadtmannae* which requires methanol and hydrogen for methane production. Another minor community member (band A2) was related with 99% similarity to an uncultured methanogen isolated from various swine manure digesters, and only very distantly related (<88%) to any cultured methanogen (Table 2).

The dominance of the acetoclastic *Methanosaeta* sp. under steady-state was in accordance with previous studies reporting the dominance of acetate-mediated metabolism in mesophilic AD (McMahon et al. 2004; Karakashev et al. 2005; Tabatabaei et al. 2010), even when treating phenolic compounds such as dimethyl phthalate (Liang et al. 2009).

### Response to the PAA shock

The archaeal community composition in the primary sludge digester was significantly affected by the PAA pulse and the subsequent VFA accumulation, and was not resilient long-term. At a short time after the shock, between days 72 and 85 (i.e. when VFA concentration increased from 672 to 1,488 mg L<sup>-1</sup>), the relative abundance of bands A1, A2, A4 and A5 (affiliated with *Methanospiraer stadtmannae*, *Methanosaeta concilii* and swine-manure uncultured methanogen) decreased. Simultaneously, the relative abundance of band A3 (affiliated with *Methanospirillum hungatei*) increased strongly (Figure 2(a)).

This community shift was even stronger after day 120, i.e. after the peak of VFA concentration at 4,056 mg L<sup>-1</sup>. From day 120 to the end of the experiment, bands A1 and A2 had almost disappeared, the abundance of previously-dominant bands A4 and A5 affiliated to acetoclastic *Methanosaeta concilii* still decreased, and band A3 affiliated to the hydrogenotroph *Methanospirillum hungatei* became the dominant community member. The major changes of community composition did not occur just after the PAA pulse, but later on, when VFA accumulated. Since hydrogen was not quantified in the gas phase it is not possible to conclude if the inhibition was due to H<sub>2</sub> accumulation instead of the toxic shock itself.

The similarity between archaeal community structures from perturbed and unperturbed digesters (computed by Bray-Curtis similarity index) decreased from 99% initially to 86% at the end of the experiment, indicating the divergence of the communities as a result of PAA shock.

The acetoclastic methanogen *Methanosaeta* sp. is known for its sensitivity to environmental disturbances (Demirel & Scherer 2008). As in our system, shifts from the *Methanosaeta* dominated system towards the hydrogenotrophic-methanogen-dominated methanogenic community have also been reported in response to elevated ammonium concentration, low pH, pulse of long chain fatty acid and increased OLR, causing the accumulation of VFA, especially acetic acid (Tabatabaei et al. 2010; Shah et al. 2014). The absence of *Methanosaetaceae* has been interpreted as an indication of process instability in an overloaded maize-silage-fed digester exposed to acidification (Blume et al. 2010). In the *Methanosaeta*-dominated AD community, substrate overloading resulted in an increased functional diversity of the methanogenic community, with increased abundance of functionally distinct hydrogenotrophic and acetoclastic methanogens, which enhanced the capacity to overcome subsequent occurrences of process perturbations without performance disruption (Chen et al. 2012). An increase of acetate concentration up to a value of 3,000 mg<sub>COD</sub> L<sup>-1</sup> can be considered as the threshold for the shift in the methanogenesis pathway (Shah et al. 2014). The dominance of hydrogenotrophic methanogens was also reported in digesters treating olive oil mill effluent (Tabatabaei et al. 2010) and in a full-scale corn straw digester, where transient aromatic intermediate compounds (*p*-cresol, phenylpropionate, phenol and benzoate) were degraded by syntrophic bacteria coupled to hydrogenotrophic methanogens (Qiao et al. 2013).

The accumulation of VFA observed in the primary sludge digester could be a typical reactor response to overloading or toxic pulse. Indeed, under stress situations, metabolic shifts can result in an imbalance between VFA producers and VFA consumers (Leitao et al. 2006). It is not clear to date if high VFA concentrations are the result of the process imbalance, or the cause of reactor destabilization (Leitao et al. 2006). Therefore, the long-term disturbing effect observed at the community level in the primary sludge digester might not be attributed directly to the PAA shock but might be rather a further consequence of the VFA accumulation and system imbalance, since high VFA concentrations are known to be toxic to methanogens (Appels et al. 2008).

### Archaeal community structure in mixed sludge digesters

#### Under steady-state

The dominant archaeal members were similar between mixed- and primary-sludge digestion, but the abundances

of the minor species differed according to the substrate considered (>94% similarity). Before the PAA shock, five archaeal community members could be detected and identified in treated and untreated anaerobic digesters fed with mixed sludge (Figure 2(b)). The most dominant ones (bands A4 and A5) were affiliated with the acetoclast *Methanosaeta concilii*, similarly to what was observed in anaerobic digesters fed with primary sludge. The intensity of bands A1 and A2 (respectively, affiliated to *Methanospiraera stadtmannae* and to an uncultured methanogen) was higher in mixed-sludge-fed anaerobic digesters while they were minor members of primary-sludge-fed anaerobic digesters under initial steady-state. By contrast, the intensity of band A3, affiliated with *Methanospirillum hungatei*, was lower in mixed-sludge-fed anaerobic digesters than in primary-sludge-fed anaerobic digesters.

### Response to the PAA shock

Contrary to what occurred for primary-sludge digestion, the archaeal community structure revealed by DGGE in mixed-sludge digesters remained extremely constant over time in control and test conditions (the Bray–Curtis similarity index was always >98% between perturbed and unperturbed conditions). The same community members were maintained at similar relative abundances during the whole experiment. This suggests that the successive PAA pulses at 200 and 600 mg L<sup>-1</sup> had no significant effect on the archaeal community composition and relative abundances in the mixed-sludge-fed anaerobic digester, at least at the time-scale considered in the study. The lower proportion of hydrogenotrophic methanogens in mixed sludge might be related to a minor proportion of hydrogen production from fermentation or anaerobic oxidation processes, which may be considered as a stabilizing factor. Indeed, a high hydrogen potential substrate can be affected by toxic disturbances since a little inhibitory effect on H<sub>2</sub> consumers could affect a lot the acidogens, eventually resulting in VFA accumulation. Further studies should therefore take into account a deeper monitoring of hydrogen and VFA over time.

### CONCLUSIONS

The effect of PAA pulse on methanogenic activity and archaeal community structure differed according to the substrate of AD. The methane production efficiency and

archaeal community structure in the primary sludge digester were strongly affected by a single PAA pulse at 200 mg L<sup>-1</sup>, while the biomass was resistant to repeated PAA pulses up to 600 mg L<sup>-1</sup> in the mixed sludge digester. In the primary sludge digester, the VFA accumulation induced by the PAA pulse led to a community shift from an acetoclastic-dominated (*Methanosaeta concilii*) to a hydrogenotrophic-dominated (*Methanospirillum hungatei*) community, correlated to a loss of methanogenic activity after the perturbation. Thus, it can be assumed that: (i) the acetoclastic methanogens were responsible for most of the methane production activity under steady-state, but were the most sensitive to the PAA shock; (ii) the hydrogenotrophic methanogens were the most resistant in response to the shock but did not provide efficient methane production performance afterwards; and/or (iii) the emergence of hydrogenotrophic and formate-converting methanogens may also be determined by thermodynamics, since hydrogen consumers are needed to degrade the high VFA contents through syntrophy metabolism.

It is not clear to date if PAA is a direct inhibitor of the methanogens or if other physiological groups in the anaerobic food chain are inhibited, leading to the consecutive indirect inhibition of the methanogens inhibited (Hecht & Griehl 2009). To unravel the direct or indirect – or combined – effect of PAA on the AD community, future research should focus on evaluating the response of pure cultures of micro-organisms involved in AD in the face of contaminant pulses.

### ACKNOWLEDGEMENT

This study was supported by FONDECYT Project 1110861.

### REFERENCES

- APHA 1996 *Standard Methods for the Examination of Water and Wastewater*. 20th edn, American Public Health Association/American Water Works Association/Water Environment Federation, Washington, DC, USA.
- Appels, L., Baeyens, J., Degreè, J. & Dewil, R. 2008 *Principles and potential of the anaerobic digestion of waste-activated sludge*. *Progress in Energy Combustion Science* **34**, 755–781.
- Arnaiz, C., Gutierrez, J. C. & Lebrato, J. 2006 *Biomass stabilization in the anaerobic digestion of wastewater sludges*. *Bioresource Technology* **97**, 1179–1184.
- Barret, M., Gagnon, N., Kalmokoff, M. L., Topp, E., Verastegui, Y., Brooks, S. P. J., Matias, F., Neufeld, J. D. & Talbot, G. 2013 *Identification of Methanoculleus spp. as active methanogens*

- during anoxic incubations of swine manure storage tank samples. *Applied and Environmental Microbiology* **79**, 424–433.
- Blume, F., Bergmann, I., Nettmann, E., Schelle, H., Rehde, G., Mundt, K. & Klocke, M. 2010 Methanogenic population dynamics during semi-continuous biogas fermentation and acidification by overloading. *Journal of Applied Microbiology* **109**, 441–450.
- Carrère, H., Dumas, C., Battimelli, A., Batstone, D. J., Delgenès, J. P., Steyer, J. P. & Ferrer, I. 2010 Pretreatment methods to improve sludge anaerobic degradability: a review. *Journal of Hazardous Materials* **183**, 1–15.
- Chen, Y., Cheng, J. J. & Creamer, K. S. 2008 Inhibition of anaerobic digestion process: a review. *Bioresource Technology* **99**, 4044–4064.
- Chen, S., Zamudio Cañas, E. M., Zhang, Y., Zhu, Z. & He, Q. 2012 Impact of substrate overloading on archaeal populations in anaerobic digestion of animal waste. *Journal of Applied Microbiology* **113**, 1371–1379.
- Demirel, B. & Scherer, P. 2008 The roles of acetotrophic and hydrogenotrophic methanogens during anaerobic conversion of biomass to methane: a review. *Reviews in Environmental Science and Bio/Technology* **7**, 173–190.
- Field, J. A. & Lettinga, G. 1987 The methanogenic toxicity and anaerobic degradability of a hydrolyzable tannin. *Water Research* **21**, 367–374.
- Fiorentino, A., Gentili, A., Isidori, M., Monaco, P., Nardelli, A., Parrella, A. & Temussi, F. 2003 Environmental effects caused by olive mill wastewaters: toxicity comparison of low-molecular-weight phenol components. *Journal of Agricultural and Food Chemistry* **51**, 1005–1009.
- Fricke, W. F., Seedorf, H., Henne, A., Krüer, M., Liesegang, H., Hedderich, R., Gottschalk, G. & Thauer, R. K. 2006 The genome sequence of *Methanosphaera stadtmanae* reveals why this human intestinal archaeon is restricted to methanol and H<sub>2</sub> for methane formation and ATP synthesis. *Journal of Bacteriology* **188**, 642–658.
- García-Morales, J. L., Nebot, E., Romero, L. I. & Sales, D. 2001 Comparison between acidogenic and methanogenic inhibition caused by linear alkylbenzene-sulfonate (LAS). *Chemical and Biochemical Engineering Quarterly* **15**, 13–19.
- Gavala, H. N., Yenal, U., Skiadas, I. V., Westermann, P. & Ahring, B. K. 2003 Mesophilic and thermophilic anaerobic digestion of primary and secondary sludge. Effect of pre-treatment at elevated temperature. *Water Research* **37**, 4561–4572.
- Grosskopf, R., Janssen, P. H. & Liesack, W. 1998 Diversity and structure of the methanogenic community in anoxic rice paddy soil microcosms as examined by cultivation and direct 16S rRNA gene sequence retrieval. *Applied and Environmental Microbiology* **64**, 960–969.
- Hamdi, M. & Garcia, J. L. 1993 Anaerobic digestion of olive mill wastewaters after detoxification by prior culture of *Aspergillus niger*. *Process Biochemistry* **28**, 155–159.
- Hecht, C. & Griehl, C. 2009 Investigation of the accumulation of aromatic compounds during biogas production from kitchen waste. *Bioresource Technology* **100**, 654–658.
- Heider, J. & Fuchs, G. 1997 Anaerobic metabolism of aromatic compounds. *European Journal of Biochemistry* **243**, 577–596.
- Hernandez, J. E. & Edyvean, R. G. J. 2008 Inhibition of biogas production and biodegradability by substituted phenolic compounds in anaerobic sludge. *Journal of Hazardous Materials* **160**, 20–28.
- Iannotti, E. L., Mueller, R. E., Sievers, D. M., Georgacakis, D. G. & Gerhardt, K. O. 1986 Phenylacetic acid in an anaerobic swine manure digester. *Journal of Industrial Microbiology* **1**, 57–61.
- Karakashev, D., Batstone, D. & Angelidaki, I. 2005 Influence of environmental conditions on methanogenic compositions in anaerobic biogas reactors. *Applied and Environmental Microbiology* **71**, 331–338.
- Karlsson, A., Einarsson, P., Schnürer, A., Sundberg, C., Ejlertsson, J. & Svensson, B. H. 2012 Impact of trace element addition on degradation efficiency of volatile fatty acids, oleic acid and phenyl acetate and on microbial populations in a biogas digester. *Journal of Bioscience and Bioengineering* **114**, 446–452.
- Keyse, M., Witthuhn, R. C., Lamprecht, C., Coetzee, M. P. A. & Britz, T. J. 2006 PCR-based DGGE fingerprinting and identification of methanogens detected in three different types of UASB granules. *Systematic and Applied Microbiology* **29**, 77–84.
- Khoufi, S., Aloui, F. & Sayadi, S. 2006 Treatment of olive oil mill wastewater by combined process electro-Fenton reaction and anaerobic digestion. *Water Research* **40**, 2007–2016.
- Kyriacou, A., Lasaridi, K. E., Kotsou, M., Balis, C. & Pilidis, G. 2005 Combined bioremediation and advanced oxidation of green table olive processing wastewater. *Process Biochemistry* **40**, 1401–1408.
- Lane, A. G. 1980 Production of aromatic acids during anaerobic digestion of citrus peel. *Journal of Chemical Technology and Biotechnology* **30**, 345–350.
- Lane, D. 1991 16S/23S rRNA sequencing. In: *Nucleic Acid Techniques in Bacterial Systematics* (E. Stackebrandt & M. Goodfellow, eds). Wiley, Chichester, UK, pp. 115–175.
- Leitao, R. C., van Haandel, A. C., Zeeman, G. & Lettinga, G. 2006 The effects of operational and environmental variations on anaerobic wastewater treatment systems: a review. *Bioresource Technology* **97**, 1105–1118.
- Li, T., Li, X., Chen, J., Zhang, G. & Wang, H. 2007 Treatment of landfill leachate by electrochemical oxidation and anaerobic process. *Water Environment Research* **79**, 514–520.
- Liang, D. W., Fang, H. H. P. & Zhang, T. 2009 Microbial characterization and quantification of an anaerobic sludge degrading dimethyl phthalate. *Journal of Applied Microbiology* **106**, 296–305.
- McMahon, K. D., Zheng, D., Stams, A. J. M., Mackie, R. I. & Raskin, L. 2004 Microbial population dynamics during start-up and overload conditions of anaerobic digesters treating municipal solid waste and sewage sludge. *Biotechnology and Bioengineering* **87**, 823–834.
- Monlau, F., Sambusiti, C., Barakat, A., Quéméneur, M., Trably, E., Steyer, J. P. & Carrère, H. 2014 Do furanic and phenolic compounds of lignocellulosic and algae biomass hydrolyzate inhibit anaerobic mixed cultures? A comprehensive review. *Biotechnology Advances* **32**, 934–951.

- Moreno-Andrade, I. & Buitrón, G. 2003 Influence of the initial substrate to microorganisms concentration ratio on the methanogenic inhibition test. *Water Science and Technology* **48**, 17–22.
- Muyzer, G., Dewaal, E. & Uitterlinden, A. 1993 Profiling of complex microbial-populations by denaturing gradient gel-electrophoresis analysis of polymerase chain reaction amplified genes-coding for 16S ribosomal RNA. *Applied and Environmental Microbiology* **59**, 695–700.
- Narihiro, T. & Sekiguchi, Y. 2007 Microbial communities in anaerobic digestion processes for waste and wastewater treatment: a microbiological update. *Current Opinion in Biotechnology* **18**, 273–278.
- Patel, G. B. 1984 Characterization and nutritional properties of *Methanothrix concilii* sp. nov., a mesophilic, acetoclastic methanogen. *Canadian Journal of Microbiology* **30**, 1383–1396.
- Qiao, J. T., Qiu, Y. L., Yuan, X. Z., Shi, X. S., Xu, X. H. & Guo, R. B. 2013 Molecular characterization of bacterial and archaeal communities in a full-scale anaerobic reactor treating corn straw. *Bioresource Technology* **143**, 512–518.
- Rétfalvi, T., Tukacs-Hájos, A. & Szabó, P. 2013 Effects of artificial overdosing of p-cresol and phenylacetic acid on the anaerobic fermentation of sugar beet pulp. *International Biodeterioration and Biodegradation* **83**, 112–118.
- Rosenkranz, F., Cabrol, L., Carballa, M., Donoso-Bravo, A., Cruz, L., Ruiz-Filippi, G., Chamy, R. & Lema, J. M. 2013 Relationship between phenol degradation efficiency and microbial community structure in an anaerobic SBR. *Water Research* **47**, 6739–6749.
- Scott, M. J. & Jones, M. N. 2000 The biodegradation of surfactants in the environment. *Biochimica et Biophysica Acta* **1508**, 235–251.
- Shah, F. A., Mahmood, Q., Shah, M. M., Pervez, A. & Asad, S. A. 2014 Microbial ecology of anaerobic digesters: the key players of anaerobiosis. *The Scientific World Journal* **2014**, 1–21.
- Sierra-Alvarez, R. & Lettinga, G. 1991 The effect of aromatic structure on the inhibition of acetoclastic methanogenesis in granular sludge. *Applied Microbiology and Biotechnology* **34**, 544–550.
- Soto, M., Mendez, R. & Lema, J. M. 1993 Methanogenic and non-methanogenic activity tests. Theoretical basis and experimental set up. *Water Research* **27**, 1361–1376.
- Stack, R. J., Hungate, R. E. & Opsahl, W. P. 1983 Phenylacetic acid stimulation of cellulose digestion by *Ruminococcus albus* 8. *Applied and Environmental Microbiology* **46**, 539–544.
- Tabatabaei, M., Rahim, R. A., Abdullah, N., Wright, A. D. G., Shiraif, Y., Sakai, K., Sulaiman, A. & Hassan, M. A. 2010 Importance of the methanogenic archaea populations in anaerobic wastewater treatments. *Process Biochemistry* **45**, 1214–1225.
- Talbot, G., Topp, E., Palin, M. F. & Massé, D. I. 2008 Evaluation of molecular methods used for establishing the interactions and functions of microorganisms in anaerobic bioreactors. *Water Research* **42**, 513–537.
- Ucisk, A. S. & Henze, M. 2008 Biological hydrolysis and acidification of sludge under anaerobic conditions: The effect of sludge type and origin on the production and composition of volatile fatty acids. *Water Research* **42**, 3729–3738.
- Urrea, J., Poirrier, P., Chamy, R., Segovia, J. & Lesty, Y. 2008 Analysis of the methodology to determine anaerobic toxicity: evaluation of main compounds present in wastewater treatment plants (WWTPs). *Water Science and Technology* **57**, 857–862.
- Veeresh, G., Kumar, P. & Mehrotra, I. 2005 Treatment of phenol and cresols in upflow anaerobic sludge blanket (UASB) process: a review. *Water Research* **39**, 154–170.
- Weiland, P. 2010 Biogas production: current state and perspectives. *Applied Microbiology and Biotechnology* **85**, 849–886.

First received 30 January 2015; accepted in revised form 23 March 2015. Available online 2 April 2015