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# Investigation into the effect of high concentrations of volatile fatty acids in anaerobic digestion on methanogenic communities

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ABSTRACT

A study was conducted to determine whether differences in the levels of volatile fatty acids (VFAs) in anaerobic digester plants could result in variations in the indigenous methanogenic communities. Two digesters (one operated under mesophilic conditions, the other under thermophilic conditions) were monitored, and sampled at points where VFA levels were high, as well as when VFA levels were low. Physical and chemical parameters were measured, and the methanogenic diversity was screened using the phylogenetic microarray ANAEROCHIP. In addition, real-time PCR was used to quantify the presence of the different methanogenic genera in the sludge samples. Array results indicated that the archaeal communities in the different reactors were stable, and that changes in the VFA levels of the anaerobic digesters did not greatly alter the dominating methanogenic organisms. In contrast, the two digesters were found to harbour different dominating methanogenic communities, which appeared to remain stable over time. Real-time PCR results were inline with those of microarray analysis indicating only minimal changes in methanogen numbers during periods of high VFAs, however, revealed a greater diversity in methanogens than found with the array.



## 1. Introduction

The anaerobic digestion (AD) of organic wastes is a sustainable waste management strategy that is gaining significance due to the increasing costs of fossil fuels and the need to mitigate anthropogenic global warming. Biogas production from various types of raw materials (e.g. manure, sewage sludge, food waste) has been shown to be a source of renewable energy that can occur sustainably in many different countries around the world (Bond and Templeton, 2011). The process produces a sludge of agricultural value, as well as biogas, which can be used to generate electricity and heat (Lastella et al., 2002; Insam and Wett, 2008). The efficient conversion of organic matter to methane in an anaerobic digester is dependent on the mutual and syntrophic interactions of functionally distinct microorganisms (Akuzawa et al., 2011). However, despite a continuously increasing interest and popularity of AD, the process remains inefficient and enigmatic. This is because of a lack of knowledge linking microbial community content, dynamics, and activity with reactor performance (Lee et al., 2009; Pycke et al., 2011).

The initial step in the AD process is the hydrolysis of the organic materials. During this step, macromolecules, such as proteins, carbohydrates and fats, are broken down to amino acids, sugars and fatty acids, respectively, by bacteria (Nielsen et al., 2007) and fungi (Leis et al., 2014). In the second stage of the process, acidogenic bacteria convert sugars, amino acids, and fatty acids to organic acids, alcohols and ketones, acetate, $CO_2$, and $H_2$. Acetogenic bacteria then convert fatty acids and alcohols into acetate, $H_2$ and $CO_2$, products used by methanogenic archaea to form biogas (typically 60% methane, 38% carbon dioxide and 2% trace gases). Archaea thus hold the key position in the methanisation. Methane can be produced by both acetotrophic and hydrogenotrophic methanogens, and differences in environmental conditions as well as reactor operating conditions (pH, temperature, hydraulic retention time, input material) have been reported to affect the composition of these communities (Demirel and Scherer, 2008; Akuzawa et al., 2011; Kim et al., 2013). Because the degradation phases are all closely connected with each other, an imbalance between the bacterial and archaeal communities can cause a deterioration in reactor performance, and thus changes in the amount of methane produced (Demirel and Yenigün, 2002; Rastogi et al., 2008; Akuzawa et al., 2011).

Reactor acidification through reactor overload is one of the most common reasons for process deterioration in anaerobic digesters (Akuzawa et al., 2011). This occurs because of a build-up of volatile fatty acids (VFAs) which are produced by acidogenic and acetogenic

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bacteria, and reflects a kinetic uncoupling between the acid producers and consumers (Ahring, 1995). High VFA concentrations cause pH values to decrease, and result in toxic conditions in the reactor. In anaerobic digesters with low buffering capacity, pH, partial alkalinity and VFAs are reliable indicators for process imbalance, however, in highly buffered systems, pH changes can be small, even when the process is extremely stressed, and only VFAs can be considered reliable for process monitoring (Murto et al., 2004). Various VFAs exist in ADs, and they have different and co-operative effects on bacteria and archaea. Wang et al. (2009) reported that acetic acid and butyric acid concentrations of 2400 and 1800 mg $L^{-1}$, respectively, resulted in no significant inhibition of the activity of methanogens, while a propionic acid concentration of 900 mg $L^{-1}$ resulted in significant inhibition of the methanogens. Opinions vary regarding which VFA is the best indicator for impending reactor failure, with different authors suggesting i-butyric, i-valeric, propionic acid, or the ratio of propionic:acetic acid as the most appropriate indicator (Boe, 2006). Nonetheless, it does not appear to be possible to define VFA levels to indicate the state of an anaerobic process, as different systems have their own levels of VFAs that can be considered 'normal' for the reactor, and conditions that cause instability in one reactor do not cause problems in another reactor (Angelidaki et al., 1993).

Other chemical compounds which are known to cause toxic effects in biogas reactors and can lead to a complete failure of methanogenesis are ammonia and hydrogen sulfide (Kayhanian, 1994; Chen et al., 2008), as well as accumulations of hydrogen and acetate, excess tannins, salts and heavy metals (Karri et al., 2006; Panyadee et al., 2013).

The aim of this study was to investigate the hypothesis that changes in the VFA levels of anaerobic digester plants can influence indigenous methanogenic communities. Archaeal communities present in two different anaerobic digesters were monitored using the ANAEROCHIP microarray. This microarray, which targets the 16S rRNA gene, offers the possibility to analyse an entire array of methanogens concerning their presence or absence in a particular sludge sample in a single experiment (Franke-Whittle et al., 2009a). Methanogens detected using the microarray were quantified using real-time PCR to determine exact numbers.

## 2. Materials and methods

### 2.1. Sampling of sludge from biogas producing reactors

Anaerobic sludges were collected from two AD plants (Inzing and Neustift) in Tirol, Austria, at various times (May 18, 2009; August 5, 2009; September 16, 2009 and October 27, 2009; for Neustift reactor, no samples for the August sampling were available). The Inzing reactor (I) was run under mesophilic conditions and had a reactor volume of 173 $m^3$, an organic loading rate (OLR) of 2.8 kg VS $m^{-3}$ $d^{-1}$, a hydraulic retention time (HRT) of 57 d and a combined heat and power plant (CHP) with 20 kW electrical performance. The Neustift reactor (N) was run under thermophilic conditions, had a reactor volume of 110 $m^3$, an OLR of 5.2 kg VS $m^{-3}$ $d^{-1}$, a HRT of 26 d and a CHP with 25 kW electrical performance. Both plants produced sludges that were used for agricultural purposes.

Information on the AD plants is listed in Table 1. Three bulked samples (each about 0.5 L) were collected from the reactors through the sampling ports.

### 2.2. Physical–chemical parameters

Temperature and biogas production were measured online in the reactors, and gas quality ($CH_4$ [%], $CO_2$ [%]) of the reactor was analysed with a portable Biogas Check BM 2000 instrument (Geotechnical Instruments, Warwickshire, UK). pH and electrical conductivity (EC) were measured in sludge samples at the time of collection using a portable multi-parameter meter Multi 340i (WTW, Weilheim, Germany). Total solids (TS) were calculated as the amount of solids remaining after oven-drying the samples overnight (105 °C). Volatile solids (VS) were calculated as the loss of weight after igniting the oven-dried residue at 550 °C for 5 h. Sample preparation for HPLC analysis was performed using dialysis and the method of Wagner et al. (2012). Following sample collection, a dialysis tube filled with 10 ml of distilled water was submerged into the liquid sample. The bottle was shaken three times and stored at 4 °C overnight in order to reach a total equilibrium in the dialysate. The tubing was removed, washed with a minimum volume of distilled water and opened. Dialysate (0.5 ml) was subjected to high performance liquid chromatography (HPLC) analysis on an Aminex HPX-87H column (Bio-Rad, Hercules, USA). A 5 mM $H_2SO_4$ mobile phase run at 0.7 ml $min^{-1}$ and a detection wavelength of 210 nm were used. The detection limit for VFAs was >1 mmol $l^{-1}$. Ammonium nitrogen ($NH_4$–N) was measured photometrically after appropriate dilution with distilled water using the colorimetric tube test from Macherey-Nagel (Düren, Germany). $NH_3$–N was calculated from $NH_4$–N concentrations according to the formula of Calli et al. (2005). The PASW-SPSS 17.0 software was used to determine correlations between physical–chemical parameters and methanogenic genera present in sludges.

### 2.3. DNA extraction

The PowerSoil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, California) was used to extract genomic DNA from sludge samples according to the instructions of the manufacturer, with the exception that the sample after being subjected to lysis buffers and mixing was exposed to three freeze–thaw cycles (30 min at −80 °C followed by 5 min at 65 °C). This was done in order to improve the efficiency of cell lysis. DNA extractions were conducted in triplicate from the well mixed bulked sludge samples. Extracted DNA was subjected to electrophoresis in a 1% agarose gel in 1 X TAE buffer, and DNA concentration was determined by fluorescence using a PicoGreen® dsDNA quantitation kit (Molecular Probes Inc., Oregon, USA) and a fmax Fluorescence Microplate Reader (Molecular Devices, CA, USA), as described by the manufacturer.

### 2.4. ANAEROCHIP microarray analysis

The 109F and 934R primers (Grosskopf et al., 1998) were used to amplify the 16S rRNA gene of methanogens in the sludge samples by PCR, as described by Franke-Whittle et al. (2009b). Single-stranded Cy5-labeled PCR product was generated using Lambda exonuclease and 500 ng of single-stranded DNA was hybridised on an ANAEROCHIP microarray at 55 °C for 4 h (Franke-Whittle et al., 2009b). Arrays were washed after hybridisation, and a ScanArray Gx microarray scanner (Perkin Elmer, MA, USA) was used to scan hybridised microarray slides. The ScanArray Gx software (Perkin Elmer, MA, USA) was used to analyse fluorescent images, as described by Franke-Whittle et al. (2009b). For all spots, the median foreground and background signals were determined. The signal-to-noise ratio (SNR) for all spots was calculated using the following calculation, as described by Loy et al. (2002):

$SNR = [I_p\text{-}(I_{np}–I_{bnp})]/I_{bp}$ where $I_p$ is median intensity of fluorescence of the probe, $I_{np}$ is the median intensity of fluorescence of the nonbinding control probe, $I_{bnp}$ is the median intensity of fluorescence of the background area around the nonbinding control probe, and $I_{bp}$ is the median intensity of fluorescence of the background area around the probe. Signals were treated as positive if a SNR value of $\geqslant 2$ was obtained (Loy et al., 2002).