

## Effect of wastewater composition on archaeal population diversity

Alper T. Akarsubasi<sup>a</sup>, Orhan Ince<sup>b</sup>, Betül Kirdar<sup>c</sup>, Nilgun A. Oz<sup>a</sup>, Derin Orhon<sup>b</sup>,  
Thomas P. Curtis<sup>d</sup>, Ian M. Head<sup>d</sup>, Bahar K. Ince<sup>a,\*</sup>

<sup>a</sup>Institute of Environmental Sciences, Bogazici University, 34342 Istanbul, Turkey

<sup>b</sup>Department of Environmental Engineering, Istanbul Technical University, 34469 Istanbul, Turkey

<sup>c</sup>Department of Chemical Engineering, Bogazici University, 34342 Istanbul, Turkey

<sup>d</sup>School of Civil Engineering and Geosciences and Center for Molecular Ecology, University of Newcastle upon Tyne, NE1 7RU, Newcastle upon Tyne, UK

Received 26 April 2004; received in revised form 29 November 2004; accepted 13 December 2004

Available online 7 March 2005

### Abstract

Distribution and occurrence of *Archaea* and methanogenic activity in a laboratory scale, completely mixed anaerobic reactor treating pharmaceutical wastewaters were investigated and associated with reactor performance. The reactor was initially seeded with anaerobic digester sludge from an alcohol distillery wastewater treatment plant and was subjected to a three step feeding strategy. The feeding procedure involved gradual transition from a glucose containing feed to a solvent stripped pharmaceutical wastewater and then raw pharmaceutical wastewater. During the start-up period, over 90% COD removal efficiency at an organic loading rate (OLR) of  $6 \text{ kg COD m}^{-3} \text{ d}^{-1}$  was achieved with glucose feeding, and acetoclastic methanogenic activity was  $336 \text{ ml CH}_4 \text{ gTVS}^{-1} \text{ d}^{-1}$ . At the end of the primary loading, when the feed contained solvent stripped pharmaceutical wastewater at full composition, 71% soluble COD removal efficiency was obtained and acetoclastic methanogenic activity decreased to half of the rate under glucose feed ( $166 \text{ ml CH}_4 \text{ gTVS}^{-1} \text{ d}^{-1}$ ). At the end of secondary loading with 60% (w/v) raw pharmaceutical wastewater, COD removal dropped to zero and acetoclastic methanogenic activity fell to less than  $10 \text{ ml CH}_4 \text{ gTVS}^{-1} \text{ d}^{-1}$ . Throughout the course of the experiment, microbial community structure was monitored by DGGE analysis of 16S rRNA gene fragments. Five different archaeal taxa were identified and the predominant archaeal sequences belonged to methanogenic *Archaea*. Two of these showed greatest sequence identity with *Methanobacterium formicicum* and *Methanosaeta concilii*. The types of *Archaea* present changed little in response to changing feed composition but the relative contribution of different organisms identified in the archaeal DGGE profiles did change.

© 2005 Elsevier Ltd. All rights reserved.

**Keywords:** Anaerobic treatment; Archaeal diversity; Acetoclastic methanogenic activity; Completely stirred tank reactor; Pharmaceutical wastewater; 16S rRNA gene

### 1. Introduction

Biological treatment systems are widely used to achieve high quality effluent for environmental disposal. Performance of biological treatment systems may be

\*Corresponding author. Tel.: +90 212 359 70 16;  
fax: +90 212 257 50 33.

E-mail address: [bahar.ince@boun.edu.tr](mailto:bahar.ince@boun.edu.tr) (B.K. Ince).

related to the composition and activity of microbial populations they contain. The types of organisms present and their relative population levels in reactor biomass depend on wastewater characteristics as well as operational conditions maintained in an anaerobic reactor (McHugh et al., 2003). Anaerobic treatment systems are favorable for medium to high strength wastewaters such as industrial wastes including those from bulk and fine chemical manufacture (Malina and Pohland, 1992). Wastewaters from the chemical synthesis of pharmaceuticals contain a wide variety of organic chemicals, xenobiotic chemicals including both readily biodegradable and relatively non-biodegradable solvents. These complex wastes can present difficulties for biological treatment systems due to temporal changes in manufacturing processes that result in heterogeneous wastewater composition. Furthermore, the potential toxicity of some of the chemicals present, which may not be readily metabolized by the microbial population in the bioreactors, can lead to severe problems in the efficiency of treatment. The composition, distribution and dynamics of the microbial population are, therefore, of particular importance in pharmaceutical wastewater treatment.

Improvements in the understanding of both the microbial communities and processes in anaerobic reactors are essential to design and control anaerobic systems effectively. Application of molecular methods such as fluorescence in situ hybridization (FISH) (Amann et al., 1995) and denaturant gradient gel electrophoresis (DGGE) (Muyzer et al., 1993, 1998) has led to new insights into microbial processes in biological reactors. Now, both qualitative and quantitative analysis can be made and the microbial population dynamics and the species responsible for a specific degradative function within the treatment system can be identified to a certain extent. This may make it possible to design better anaerobic treatment processes, in terms of degradation capacity with higher biogas production.

In this study, archaeal community structure was monitored in a lab-scale anaerobic completely stirred tank reactor (CSTR), under conditions of changing influent wastewater composition. Archaeal diversity was investigated using DGGE analysis of PCR-amplified 16S rRNA gene fragments and comparative sequence analysis.

## 2. Materials and methods

### 2.1. Bioreactor operation

A lab-scale anaerobic CSTR with an active volume of 7.5 l was operated for approximately 190 days at mesophilic ( $35 \pm 1^\circ\text{C}$ ) temperature. Before the operation, the CSTR was initially flushed with inert nitrogen

gas for 15 min to maintain anaerobic conditions in the reactor. All gas outlets and ports were sealed with silicone grease to ensure airtight seals. A rubber sampling septum was present in the gas line to enable samples to be taken for gas analysis. The pH and mixing in the CSTR were maintained at 6.8–7.2 and 90 rpm, respectively throughout the study. The seed sludge was obtained from an upflow anaerobic sludge blanket (UASB) reactor of an alcohol distillery treatment plant. The granule size of the sludge was in a range of 1.2–2.7 mm. The CSTR was inoculated with 45% (v/v) of the sludge and operated for 104 days (start-up) at which point flow through operation was initiated to give a hydraulic retention time (HRT) of 2.5 days. Stronach et al. (1986) recommended a start-up strategy for pharmaceutical wastewater treatment involving gradual replacement of readily degradable substrates with the industrial effluent. Initially, the CSTR was fed with artificial wastewater containing glucose. Nutrients (nitrogen and phosphorus as  $(\text{NH}_2)_2\text{CO}$  and  $\text{KH}_2\text{PO}_4$ , respectively) were added to the glucose solution to give a COD:N:P ratio of 400:5:1. Organic loading rate (OLR) was increased in a stepwise mode from 1 to  $6 \text{ kg COD m}^{-3} \text{ d}^{-1}$  by increasing the influent glucose concentration from  $3000 \text{ mg l}^{-1}$  up to  $16000 \text{ mg l}^{-1}$ . Chemical Oxygen Demand (COD) removal efficiency was over 90%. During the start-up period with glucose, the food to microorganism (F/M) ratio was 0.43 with a HRT of 2.5 days. Following the start-up period, the glucose containing feed was gradually replaced with increasing amounts of pharmaceutical wastewater (Stronach et al., 1986) which was initially pre-aerated to strip residual solvent from the wastewater and then flushed with nitrogen before being fed to the reactor. The proportion of solvent stripped pharmaceutical wastewater was increased to 10% (w/v), 30% (w/v), 70% (w/v) and then 100% (w/v) on days 105, 113, 120 and 129, respectively (Table 1). At this point the solvent stripped pharmaceutical wastewater in the feed was gradually replaced with raw pharmaceutical wastewater in increasing proportions of 10% (w/v), 30% (w/v), 60% (w/v) on days 163, 170 and 177, respectively.

### 2.2. Characteristics of pharmaceutical wastewater

The wastewater was from a chemical-synthesis based pharmaceutical processes producing mainly bacampicillin and sultamicillin tosylate. The general characteristics of the wastewater are given in Table 2. The production processes generate wastewater containing high concentrations of solvents such as n-butyl acetate, ethyl acetate, methylene chloride, dimethyl formamide, and isopropyl alcohol. These are extracted and recovered as part of the wastewater treatment conducted in the plant. However, considerable amounts of solvent can remain even after this treatment due to the malfunction

Table 1  
Summary of operational schedule with feeding strategy applied to the CSTR

Period/Stages	Operation	Time (days)	Feeding strategy
Start-up			
1	Initial studies	001–104	Glucose (HRT = 2.5 day)
Primary loading			
2	Steady-State	105–112	10% aerated wastewater, 90% glucose (HRT = 2.5 day)
3	Steady-State	113–119	30% aerated wastewater, 70% glucose (HRT = 2.5 day)
4	Steady-State	120–128	70% aerated wastewater, 30% glucose (HRT = 2.5 day)
	Steady-State	129–152	100% aerated wastewater (HRT = 2.5 day)
5	Steady-State	157–162	100% aerated wastewater (HRT = 3.5 day)
Secondary loading			
6	Steady-State	163–169	10% raw, 90% aerated wastewater (HRT = 3.5 day)
7	Steady-State	170–176	30% raw, 70% aerated wastewater (HRT = 3.5 day)
8	Steady-State	177–190	60% raw, 40% aerated wastewater (HRT = 3.5 day)

Table 2  
Characteristics of a chemical synthesis based pharmaceutical wastewater

Parameter	Concentration (mg l <sup>-1</sup> )
COD	39 000–60 000
COD <sub>aerated</sub> (after 48 h of aeration)	25 000–30 000
TKN	1000–1575
PO <sub>4</sub> -P	3–6
SS	800–1000
VSS	500–690
pH	7–8

of extraction unit. The wastewater was therefore subjected to aeration for about 48 h to strip residual solvents from the wastewater and nitrogen gas flushed before being fed to the CSTR. This procedure decreased the COD by approximately 50% from ca. 50 000 mg l<sup>-1</sup> to ca. 25 000 mg l<sup>-1</sup>.

### 2.3. Analytical methods

During the operation of the CSTR, temperature, pH, gas production rate were monitored daily. Feed and effluent samples were taken for the analysis of COD, alkalinity and volatile fatty acids (VFA) once every other day. Suspended solids (SS)/volatile suspended solids (VSS) in the effluent, total solids (TS)/total volatile solids (TVS) in the CSTR and gas composition were carried out weekly. VFAs were measured using a HP Model 5890 Series II gas chromatograph (GC) (HP FFAP Column, 10 m × 530 μm × 1 μm) while gas composition was determined using a HP 6850 GC (HP Plot Q column, 30 m × 0.53 mm). All analyses were carried out according to APHA (1995).

### 2.4. Specific methanogenic activity test unit

A fully computerized Specific Methanogenic Activity (SMA) test unit originally developed by Monteggia (1991) and modified by Ince et al. (1995) was used to determine acetoclastic methanogenic activity. The SMA test results are expressed as ml CH<sub>4</sub> g TVS<sup>-1</sup> d<sup>-1</sup>. The details of SMA test unit and the laboratory routine is published elsewhere (Ince et al., 1995).

### 2.5. Feed and seed sludge for SMA tests

Acetate was used as feed during SMA tests, since approximately two-thirds or more of methane formed during anaerobic degradation of complex substrate results from acetic acid (Zinder, 1993). Acetate concentrations of 1000, 2000, 3000 and 4000 mg l<sup>-1</sup> were used to obtain a maximal potential methane production (PMP) rate and 3000 mg l<sup>-1</sup> acetate concentration was found to be optimum.

### 2.6. Sampling and DNA extraction

Duplicate samples of 50 ml were taken from the reactor when steady-state had been reached following changes in the influent composition. Samples were stored at -20 °C until DNA extraction. DNA was extracted from 1 ml aliquots of the stored samples using a method modified from Curtis and Craine (1998). Instead of bead-beating the cells were physically sheared for 1 min using a vortex mixer, due to the granular characteristics of samples glass beads were not used.

### 2.7. Polymerase chain reaction (PCR)

Partial 16S rRNA genes of *Archaea* and eubacteria were amplified from the extracted genomic DNA by

Table 3  
Oligonucleotide primers used for PCR amplification of archaeal and bacterial 16S rRNA gene fragments

Primer	Annealing site <sup>a</sup>	Annealing temp. (°C)	Sequence 5'–3' <sup>a</sup>	Reference
Arch 46F	46-61	40	YTA AGC CAT GCR AGT	Øvreas et al. (1997)
Arch1017R	1017-999	40	GGC CAT GCA CCW CCT CTC	Barns et al. (1994)
Arch344F-GC	344-358	53	GC <sup>b</sup> -GAC GGG GHG CAG CAG GCG CGA	Raskin et al. (1994)
Univ522R	522-504	53	GWA TTA CCG CGG CKG CTG	Amann et al. (1995)
EubacVF-GC	341-357	55	GC <sup>b</sup> -GGC CTA CGG GAG GCA GCA G	Muyzer et al. (1993)
Vr	518-534	55	ATT ACC GCG GCT GCT GG	Muyzer et al. (1993)

<sup>a</sup>*E. coli* numbering according to Brosius et al. (1978).

<sup>b</sup>GC-clamp: CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG.

PCR using a GeneAmp PCR System 9600 thermal cycler (Perkin Elmer Cetus, USA). A nested amplification procedure was used for *Archaea*. Primers Arch 46F and Arch 1017R were used in the first round of amplification followed by Arch 344FGC and Univ 522R in the second round (Table 3). For the second round of amplification, product from the first round reaction was diluted 1 in 10 and 1 µl used as template. The first round of amplification comprised initial denaturation at 95 °C for 3 min, followed by 35 cycles of 95 °C for 1 min, 40 °C for 1 min, 72 °C for 1 min, with a final elongation step at 72 °C for 7 min. Conditions for the second round of amplification were identical except that an annealing temperature of 53 °C was used. Primers Vf-GC and Vr were used to amplify the V3 region of eubacterial 16S rRNA genes (Table 3). The following thermocycler program was used for amplification of bacterial rRNA gene fragments, 95 °C for 3 min followed by 35 cycles of 95 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min and 72 °C for 7 min final elongation step. All PCR reactions were performed in a total volume of 50 µl. PCR reactions contained 10 µM of each primer, 0.2 mM dNTPs, 1U BioTaq enzyme in the buffer provided by the manufacturer (Bioline, London, UK) and 1 µl DNA template. PCR products were stored at 4 °C prior to DGGE analysis.

## 2.8. Denaturant gradient gel electrophoresis (DGGE)

DGGE was performed with the Bio-Rad DCode™ system (Bio-Rad, Hercules, CA, USA). PCR products were loaded on 0.75 mm thick 10% (w/v) polyacrylamide (37.5:1 acrylamide: bisacrylamide) gels containing a 30–70% linear denaturant gradient for archaeal PCR products and 30–55% for bacterial PCR products (100% denaturant is 7 M urea and 40% (v/v) deionized formamide). Archaeal gels were run at 60 °C and 80 V for 760 min in 1 × TAE buffer (40 mM Tris-acetate, 1 mM Na-EDTA, pH:8.0). Whereas, gels for the

bacterial PCR products were run at 60 °C and 200 V for 270 min in 1 × TAE buffer. Gels from which bands were excised for sequencing were stained in 1 × TAE buffer containing SYBR Green I (Sigma-Aldrich, Inc., USA; 1:1000 diluted) and images were taken using a Fluor S™ imaging system (Bio-Rad, Hercules, CA, USA). All other gels were stained using a modified Silver Stain (Felske et al., 1996), dried and the gels were scanned using a flatbed scanner.

## 2.9. Analysis of DGGE patterns

Gel images were analyzed using Quantity One Software Vers.4.1 (Bio-Rad, Hercules, CA, USA) and SPSS 10 (SPSS Inc., Chicago, USA). Similarity between DGGE profiles was calculated using Pearson product moment correlation coefficients (densitometric curve based) and similarities in band patterns were measured as Dice coefficients (unweighted data based on band presence or absence) mean similarities and associated standard deviations were calculated by selection of similarity values from matrices of all pairwise similarities between DGGE profiles.

## 2.10. Sequencing

For sequence determination, bands were excised from DGGE gels, eluted into 50–200 µl of TE buffer and 1 µl was used as template in a PCR using primers Arch344F and Univ522R. The PCR products were purified using a QIAquick™ PCR Purification Kit (QIAGEN GmbH, Germany) according to the manufacturer's instructions. After purification, PCR products were sequenced with primer Univ522R (3 pmol/µl). Sequencing was performed using the ABI prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit and an ABI Prism 377 DNA sequencer (Applied Biosystems, USA).

### 3. Results

#### 3.1. Bioreactor performance

Initially, the CSTR was fed with glucose up to an OLR of  $6 \text{ kg COD m}^{-3} \text{ d}^{-1}$  corresponding to an F/M ratio of 0.43 with a HRT of 2.5 days. Soluble COD removal efficiency was 92% and a methane yield of  $0.32 \text{ m}^3 \text{ CH}_4 \text{ kg COD}_{\text{utilized}}^{-1}$  was achieved (data not shown). Initially, the seed sludge had a maximum potential methane production (PMP) rate of  $446 \text{ ml CH}_4 \text{ gTVS}^{-1} \text{ d}^{-1}$ . After 104 days of start-up operation, SMA test results showed that the maximum PMP rate decreased to  $336 \text{ ml CH}_4 \text{ gTVS}^{-1} \text{ d}^{-1}$ . Thereafter, the glucose-containing artificial wastewater was gradually replaced with solvent stripped pharmaceutical wastewater in the following proportions; 10% (w/v), 30% (w/v), 70% (w/v) and then 100% (w/v). During this stage, there was a gradual decrease in COD removal efficiency to 71% (Fig. 1), methane yield to  $0.28 \text{ m}^3 \text{ CH}_4 \text{ kg COD}_{\text{utilized}}^{-1}$  (Fig. 2) and an increase in VFA concentration from  $56 \text{ mg l}^{-1}$  (as acetic acid) to  $1474 \text{ mg l}^{-1}$  (primarily acetic acid, 86%) at an HRT of 3.5 days. Acetoclastic methanogenic activity was found to be  $166 \text{ ml CH}_4 \text{ gTVS}^{-1} \text{ d}^{-1}$  indicating a decrease of approximately 47% compared to initial value. After this stage, raw pharmaceutical wastewater diluted with solvent stripped wastewater was fed to the CSTR in increasing ratios of 10% (w/v), 30% (w/v) and 60%

(w/v). When the proportion reached 60% (w/v), there was a dramatic deterioration in performance of the CSTR in terms of COD removal efficiency (almost none) and acetoclastic methanogenic activity (less than  $10 \text{ ml CH}_4 \text{ gTVS}^{-1} \text{ d}^{-1}$ ) and a significant increase in VFA concentration reaching over  $9000 \text{ mg l}^{-1}$  (45% acetic acid, 34% butyric acid). The CSTR was operated for 10 more days and no improvement was observed in the performance and the granular structure of sludge was destroyed; thereafter the experiment was discontinued.

#### 3.2. Microbial community dynamics

Archaeal and bacterial community structure at each steady-state were initially screened by DGGE analysis of PCR amplified 16S rRNA gene fragments. In terms of the bands present in archaeal DGGE profiles the taxa present in the CSTR changed little over the course of the experiment (Fig. 3). DGGE profiles were compared using unweighted DICE coefficients and when data from all of the archaeal profiles were compared, a similarity of  $70.75\% \pm 4.4\%$  was obtained. Interestingly, although the composition of the DGGE profiles was similar throughout the experiment, the relative intensity of different bands changed with time (Fig. 3). When the data from different periods of operation were considered separately (start-up period, primary loading—glucose with solvent stripped wastewater and secondary loading—raw wastewater with solvent stripped

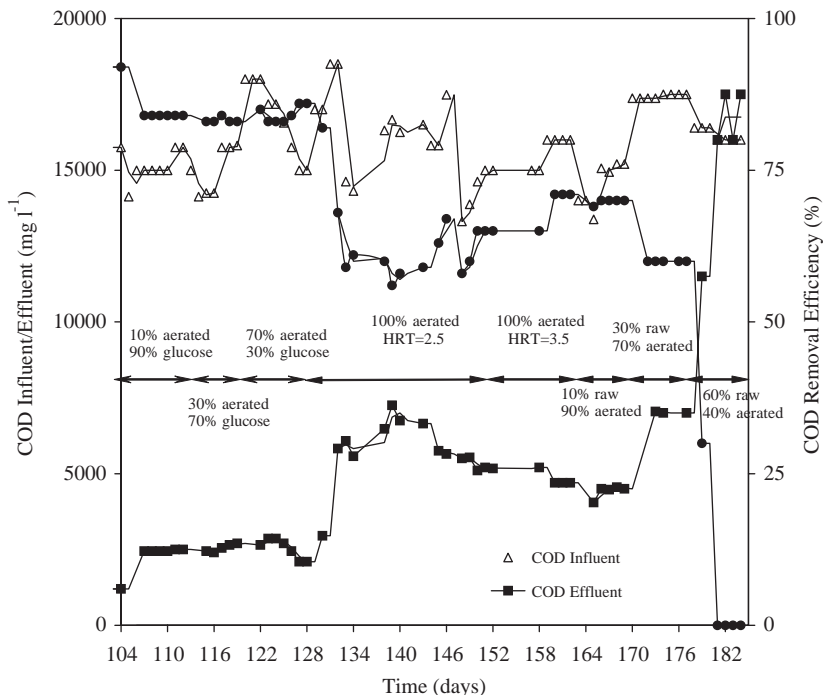


Fig. 1. Changes in COD removal efficiency with respect to feeding regime.

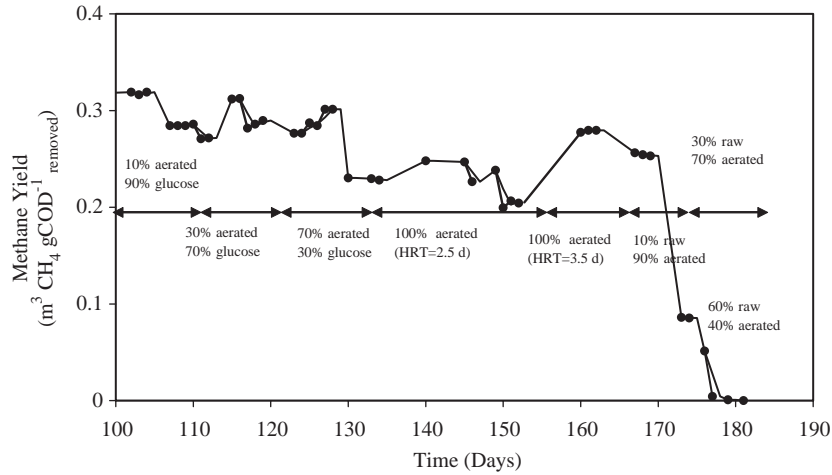


Fig. 2. Changes in methane yield with respect to feeding regime.

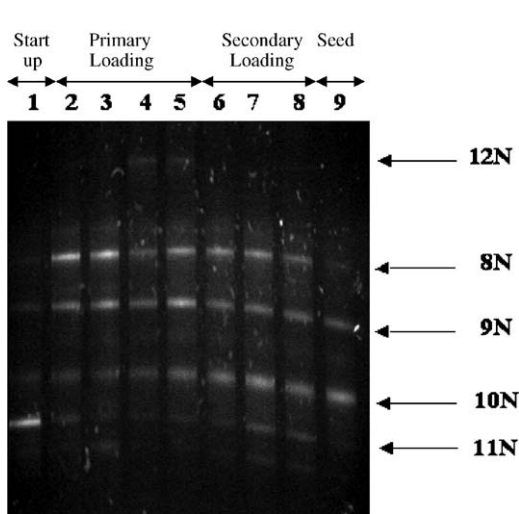


Fig. 3. Archaeal DGGE profiles (lanes 1–8 corresponds to feeding regime in Table 1 and lane 9 seed sludge).

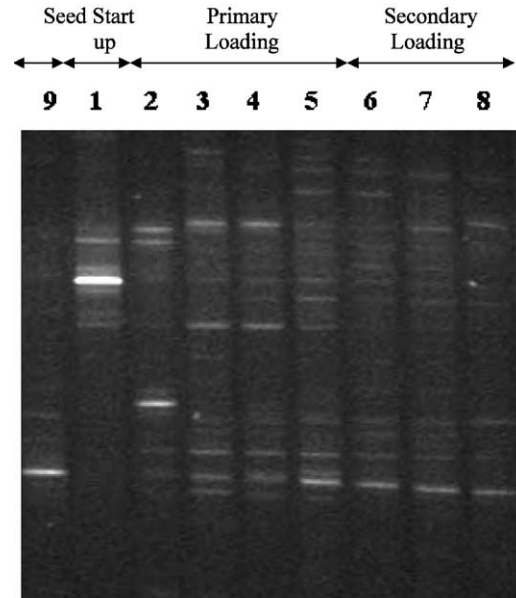


Fig. 4. Eubacterial DGGE profiles (lanes 1–8 corresponds to feeding regime in Table 1 and lane 9 seed sludge).

wastewater) higher similarities were obtained. The DGGE profiles from the primary loading period had a similarity of  $88.88\% \pm 2.7\%$  whereas samples from the secondary loading period had a similarity of  $96.22\% \pm 0.6\%$ . This contrasts with the bacterial population which varied significantly ( $46.44\% \pm 3.9\%$  similarity across all samples) during the course of the experiment (Fig. 4). The bacterial DGGE profiles had a similarity of  $42.38\% \pm 2.0\%$  during the primary loading and  $68\% \pm 0.8\%$  during the secondary loading indicating that there was less change in community composition during the second period of operation.

The identity of organisms represented by bands in the DGGE profiles was determined by sequencing of bands excised from the gels and re-amplified. Almost all excised bands from DGGE profiles generated with primers Arch344F-GC/Univ522R yielded good-quality sequence data. A total of five sequences was determined (designated 8–12N). Comparison of the sequences against the GenBank database using Fasta3 analysis showed that they were most closely related to methanogenic *Archaea* (Table 4). Sequences 8 and 10N were most

Table 4

Phylogenetic sequence affiliation of amplified 16S rRNA gene sequences (~170 bp) excised from DGGE gels

Order	Sequence	Closest FASTA match	Identity (%)	Reference
<i>Methanobacteriales</i>	8N	<i>Methanobacterium formicicum</i> AF169245	98	Jarvis et al. unpublished (1997)
<i>Methanobacteriales</i>	9N	Uncultured archaeal clone. ASDS 9 U81775	100	McHugh et al. (2003)
<i>Methanosarcinales</i>	10N	<i>Methanosaeta concilii</i> X51423	100	Lin and Miller (1998)
<i>Methanococcales</i>	11N	Uncultured archaeal clone. ASDS 5 AF424769	86	McHugh et al. (2003)
<i>Thermoplasmatales</i>	12N	Uncultured <i>Euryarchaeota</i> clone. P3Ar9 AF293578	96	Friedrich et al. (2001)

similar to the 16S rRNA of *Methanobacterium formicicum* and *Methanosaeta concilii* (Table 4) respectively. Sequences 9 and 11N also fall within the *Archaea* (100% and 86% sequence identity to their nearest neighbors respectively). The most similar sequences in the database come from uncultured archaeal clones from within the *Methanobacteriales* radiation (McHugh et al., 2003). One of these (9N) had highest identity with members of the order *Methanobacteriales* and was most closely related to *Methanobacterium formicicum* (McHugh et al., 2003). Sequence 11N has lower identity with database sequences and was distantly related to archaeal sequences related to *Methanococcus* species, identified previously in a study of an anaerobic hybrid reactor (AHR) (McHugh et al., 2003). These represented approximately 43% of the archaeal clones recovered from the AHR sludge which was treating a volatile fatty acid mixture (McHugh et al., 2003). In contrast, sequence 12N was unusual in that it was most closely related to sequences from the order *Thermoplasmatales*. The *Thermoplasmatales* form an isolated cluster which branches between the *Methanobacteriales* and the *Methanomicrobiales*/Halophiles (Reysenbach, 2001). The sequences generated were too short (~170bp) to be used in robust phylogenetic analysis, however the very high sequence identity (except 11 and 12N) with methanogens over this short stretch of sequence supports the notion that the sequences genuinely originated from methanogens.

#### 4. Discussion

Efforts to assess the microbial communities of anaerobic treatment processes have primarily examined classical parameters (such as VSS) or used microscopic or culture-based counts (MPN, autofluorescence for methanogens) which are informative but may not be sufficient. An important parameter, for the efficiency of anaerobic treatment systems is acetoclastic methanogenic capacity, which can be determined by measuring SMA (Ince et al., 2002). However, none of these parameters can explain biological treatment systems

where failures often remain unexplained, partly due to lack of information about the constituent microorganisms. Determining the underlying principles of the structure and function of microorganisms that govern biological treatment processes may help in the design of optimized biological treatment systems with lower rates of failure. Recently, more research has been conducted to relate the efficiency of biological treatment systems to their microbial community using molecular techniques such as analysis of cloned 16S rRNA gene fragments, DGGE and/or FISH (Curtis et al., 2003; Fernandez et al., 1999; Godon et al., 1997; Pereira et al., 2002).

Analysis of DGGE data from an anaerobic reactor treating pharmaceutical wastewater indicated that the composition of archaeal communities likely changed little during changes to the reactor feed. However, despite having quite similar archaeal DGGE profiles, the reactor exhibited different levels of methanogenesis when fed with different proportions of pharmaceutical wastewater. Although not robustly quantitative, marked differences in band intensity were observed when the reactor was fed wastewater containing increasing amounts of pharmaceutical waste. This suggested that the feeding regime affected the relative abundance of the different *Archaea*. Given the limitations of quantitative interpretation of DGGE profiles, the fact that the differences in band intensity were reproducible within replicate samples and between samples from the reactor operated under different conditions suggests that changes in relative intensity of bands in the DGGE profiles may reflect real differences in the relative abundance of different components of the archaeal community. In a study on bacterial and archaeal dynamics in an anaerobic digester a shift in the route of methanogenesis could be detected through 16S rRNA monitoring but not by monitoring 16S rRNA genes. This was probably due to low growth rate of *Archaea* which results in a slower adjustment of the DNA level. However, it was also reported that changes in 16S rRNA gene abundance usually correlated with the 16S rRNA changes but the adjustments happened more gradually and were of lower magnitude (Delbes et al., 2001).

In the seed sludge, archaeal DGGE profiles exhibited predominance of *Methanosaeta concilii*-like sequences (Fig. 3, 10N, Lane 9) which have also been widely reported in mesophilic anaerobic reactors. These microorganisms are regarded as being important for the formation and maintenance of granular sludge (Rocheleau et al., 1999). In the initial period of operation with glucose, the archaeal community DGGE profile was dominated by sequences related to hydrogenotrophic *Methanococcales* (Fig. 3, 11N, Lane 1). During this period, acetoclastic methanogenic capacity decreased by approximately 25% from 446 ml CH<sub>4</sub> gTVS<sup>-1</sup> d<sup>-1</sup> in the seed sludge to 336 ml CH<sub>4</sub> gTVS<sup>-1</sup> d<sup>-1</sup> in the reactor on day 104. This could be attributed to the shift in the community structure from acetoclastic methanogens related to *Methanosaeta concilii* (10N), to hydrogenotrophic methanogens of *Methanococcales* (11N). Following the first 104 days operation, bands representative of *Methanosaeta*-like organisms returned to high relative abundance (Fig. 3, Lane 2 to 8). Sequences 8 and 9N corresponded to members of the order *Methanobacteriales* (*M. formicum*-like species, which mainly use H<sub>2</sub>, CO<sub>2</sub>, formate, 2-propanol and 2-butanol as a substrate) were found to be predominant during the primary loading period unlike bands corresponding to *Methanosaeta*-like species had relatively lower intensity (Fig. 3, Lane 2 to 4). Throughout the feeding regime with raw pharmaceutical wastewater in increasing ratios (10–60% w/v), bands corresponding to sequences 8, 9 and 10N were all detected in DGGE profiles and each had similar intensity. However, even though bands representative of *Methanosaeta*-like *Archaea* remained prevalent in the DGGE profiles during the period of 105–190 days (Fig. 3, Lane 2 to 8), the SMA values and performance of the reactor gradually decreased with an increase in the proportion of raw pharmaceutical wastewater from 10% (w/v) to 30% (w/v) and collapsed when the feed contained 60% (w/v) raw wastewater. This could be explained by the destruction of the granular sludge which occurred shortly after the proportion of raw pharmaceutical wastewater was increased to 60% resulting in higher exposure of acetoclastic methanogens (*Methanosaeta*-like species, 10N) mostly found in the core of granular sludges (Rocheleau et al., 1999) to the inhibitory components of raw pharmaceutical wastewater.

## 5. Conclusion

Contrary to the belief that variations in reactor design, operating conditions and feed composition would result in changes in the microbial populations present in anaerobic digestion systems. In this study, all archaeal taxa identified by DGGE analysis could be detected throughout the course of the experiment where

the wastewater composition changed significantly. This could be beneficial from the point of view of process engineering and the development of engineered biological processes. Consistent and reproducible changes in the relative intensity of bands representing different *Archaea* in the DGGE profiles however strongly indicated that changes in the relative abundance of key methanogens occurred in response to the changing wastewater composition. Future studies, should therefore not only focus on identification of particular methanogen taxa but also their quantification using reliable quantitative analyses such as FISH or real-time PCR. Assessment of activity and the interactions between the component organisms will also be important for the design and control of specific anaerobic biological reactors.

## Acknowledgements

Authors would like to acknowledge the project coded 01M101, Research Fund of Bogazici University and TBAG-1935 (100T054) of TUBITAK. Authors wish to thank particularly the staff of University of Newcastle upon Tyne, Department of Civil Engineering, UK for their kind cooperation. Author Alper T. Akarsubasi would like to acknowledge TUBITAK NATO-A2 fund and also Bogazici University Foundation Dr. Fahri Ilter Scholarship for their support to complete this study.

## References

- Amann, R.I., Ludwig, W., Schleifer, K.H., 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol. Rev.* 59 (1), 143–169.
- APHA, 1995. Standard Methods for the Examination of Water and Wastewater, nineteenth ed. American Public Health Association, Washington, DC.
- Barns, S.M., Fundyga, R.E., Jeffries, M.W., 1994. Remarkable archaeal diversity detected in a yellowstone-nationalpark hot spring environment. *Proc. Natl. Acad. Sci.* 91 (5), 1609–1613.
- Brosius, J., Palmer, J.L., Kennedy, J.P., Noller, H.F., 1978. Complete nucleotide sequence of a 16S ribosomal gene from *Escherichia coli*. *Proc. Natl. Acad. Sci.* 75, 4801–4805.
- Curtis, T.P., Craine, N.G., 1998. The comparison of the diversity of activated sludge plants. *Water Sci. Technol.* 37, 71–78.
- Curtis, T.P., Head, I.M., Graham, W.D., 2003. Theoretical ecology for engineering biology. *Environ. Sci. Technol.* 37 (3), 65A–70A.
- Delbes, C., Rene, M., Godon, J.J., 2001. Bacterial and archaeal 16S rDNA and 16S rRNA dynamics during an acetate crisis in an anaerobic digester ecosystem. *FEMS Microbiol. Ecol.* 35, 19–26.

- Felske, A., Engelen, B., Nubel, U., 1996. Direct ribosome isolation from soil to extract bacterial rRNA for community analysis. *Appl. Environ. Microbiol.* 62 (11), 4162–4167.
- Fernandez, A., Huang, S., Seston, S., Xing, H., Hickey, R., Criddle, C., Tiedje, J., 1999. How stable is stable? Function versus community composition. *Appl. Environ. Microbiol.* 65 (8), 3697–3704.
- Friedrich, W.M., Wagner-Schmitt, D., Lueders, T., Brune, A., 2001. Axial differences in community structure of *Crenarchaeota* and *Euryarchaeota* in the highly compartmentalized gut of the soil-feeding termite *Cubitermes orthognathus*. *Appl. Environ. Microbiol.* 67 (10), 4880–4890.
- Godon, J.-J., Zumstein, E., Dabert, P., Habouzit, F., Moletta, R., 1997. Molecular microbial diversity of an anaerobic digester as determined by small-subunit rDNA sequence analysis. *Appl. Environ. Microbiol.* 63 (7), 2802–2813.
- Ince, O., Anderson, G.K., Kasapgil, B., 1995. Control of organic loading rate using the specific methanogenic activity test during start-up of an anaerobic digestion system. *Water Res.* 29 (1), 349–355.
- Ince, O., Oz, N.A., Ince, B.K., Kocaarslan, B., 2002. Determination of potential loading capacity of a full-scale anaerobic reactor treating alcohol distillery effluents. Fifth Specialized Conference on IWA Small Water and Wastewater Treatment Systems, Istanbul, Turkey.
- Lin, C., Miller, T.L., 1998. Phylogenetic analysis of *Methanobrevibacter* isolated from faeces of humans and other animals. *Archiv. Microbiol.* 169, 397–403.
- Malina, J.F., Pohland, F.G., 1992. Design of Anaerobic Processes for Treatment of Industrial and Municipal Wastes, vol. VII. Technomic Publication, Pennsylvania, USA, pp. 43.
- McHugh, S., Carton, M., Mahony, T., O'Flaherty, V., 2003. Methanogenic population structure in a variety of anaerobic reactors. *FEMS Microbiol. Lett.* 219, 297–304.
- Monteggia, L., 1991. The use of a specific methanogenic activity test controlling anaerobic reactors. Ph.D. Thesis, University of Newcastle upon Tyne.
- Muyzer, G., de Waal, E.C., Uitterlinden, A.G., 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis, analysis of polymerase chain reaction amplified genes encoding for 16S rRNA. *Appl. Environ. Microbiol.* 59, 695–700.
- Muyzer, G., Brinkhoff, T., Nubel, U., Santegoeds, C., Schafer, H., Wawer, C., 1998. Denaturing gradient gel electrophoresis (DGGE) in microbial ecology. In: Akkermans, A.D.L., van Elsas, J.D., de Bruijn, F.J. (Eds.), *Molecular Microbial Ecology Manual*. Kluwer Academic Publishers, Dordrecht, pp. 1–27.
- Øvreas, L., Forney, L., Daae, F.L., Torsvik, V., 1997. Distribution of bacterioplankton in meromictic Lake Saelevanet, as determined by denaturant gradient gel electrophoresis of PCR-amplified gene fragments coding for 16S rRNA. *Appl. Environ. Microbiol.* 63, 3367–3373.
- Pereira, M.A., Roest, K., Stams, A.J., Mota, M., Alves, M., Akkermans, A.D.L., 2002. Molecular monitoring of microbial diversity in expanded granular sludge bed (EGSB) reactors treating oleic acid. *FEMS Microbiol. Ecol.* 41, 95–103.
- Raskin, L., Stromley, J.M., Rittmann, B.E., Stahl, D.A., 1994. Group-specific 16S rRNA hybridization probes to describe natural communities of methanogens. *Appl. Environ. Microbiol.* 60 (4), 1232–1240.
- Reysenbach, S.L., 2001. Order Thermoplasmatales ord nov. In: Garrity, G. (Ed.), *Bergey's Manual of Systematic Bacteriology*, second ed., vol. 10. Springer, New York, pp. 35.
- Rocheleau, S., Greer, C.W., Lawrence, J.R., Cantin, C., Laramée, L., Guiot, S.R., 1999. Differentiation of *Methanosaeta concilii* and *Methanosarcina barkeri* in anaerobic mesophilic granular sludge by fluorescent in situ hybridization and confocal scanning laser microscopy. *Appl. Environ. Microbiol.* 65 (5), 2222–2229.
- Stronach, S.M., Rudd, T., Lester, J.N., 1986. *Anaerobic Processes in Industrial Wastewater Treatment*. Springer, Berlin.
- Zinder, S.H., 1993. Physiological ecology of methanogens. In: Ferry, J.G. (Ed.), *Methanogenesis: Ecology, Physiology, Biochemistry and Genetics*. Chapman & Hall, New York, pp. 128–206.