World J Microbiol Biotechnol (2011) 27:2893–2902
DOI 10.1007/s11274-011-0771-x

ORIGINAL PAPER

Study of the microbial diversity in a full-scale UASB reactor treating domestic wastewater

Rodrigo Mendonça de Lucena · Sávia Gavazza · Lourdinha Florencio · Mario T. Kato · Marcos Antonio de Morais Jr

Received: 1 March 2011 / Accepted: 27 April 2011 / Published online: 12 May 2011

Abstract The microorganisms diversity in a full-scale UASB reactor treating domestic sewage was studied by molecular techniques, with the objective of identifying the population differences associated with the specific methanogenic activity (SMA) of the sludges. Samples were collected at levels A (0.8 m; bottom), B (1.3 m), C (1.8 m), D (2.3 m) and E (2.8 m). *Actinobacteria* was dominant at the three lower points and should have been primarily responsible for the degradation of organic matter. DNA sequences belonging to *Methanomicrobiales* order of *Archaea* domain was detected in all five levels with the majority producing methane from hydrogen and carbon dioxide. Points A and E showed similar bacteria variety. The SMA of point A was the highest (0.374 g COD-CH_4/g SSV.d); however, the point E showed much lower value, probably due to the predominance of *Proteobacteria* phylum, including sulfate-reducing bacteria. In the overall, the results obtained can be considered important because data from full-scale UASB reactors treating domestic sewage remain scarce.

Keywords *Archaea* · Domestic sewage · Microbial diversity · Full-scale UASB reactor · rRNA-16S library

R. M. de Lucena · M. A. de Morais Jr
Interdepartment Reserch Group on Metabolic Engineering, Federal University of Pernambuco, Recife, Brazil

S. Gavazza
Laboratory of Environmental Engineering, Academic Center of Agreste, Federal University of Pernambuco, Recife, Brazil

L. Florencio · M. T. Kato
Laboratory of Environmental Sanitation, Department of Civil Engineering, Federal University of Pernambuco, Recife, Brazil

M. A. de Morais Jr (✉)
Laboratory of Microbial Genetics, Department of Genetics, Federal University of Pernambuco, Av. Moraes Rego, 1235, Cidade universitária, 50670-901 Recife, Brazil
e-mail: marcos.morais@pq.cnpq.br
URL: www.ufpe.br/nem

Introduction

Brazil is a tropical country with an average temperature of 25°C and has environmental conditions that favor the use of anaerobic technology. In the last 15 years, several wastewater treatment plants that use anaerobic processes as their core technology have been implemented in Brazilian municipalities. Some examples include the domestic wastewater treatment plants (WWTP) such as the following with the correspondent population: Mangueira (Recife PE), 18,000 inhabitants; the Onça (Belo Horizonte MG), one million inhabitants; the Piçarrão (Campinas SP), 200,000 inhabitants; and the Atuba Sul (Curitiba PR), 370,000 inhabitants (Jordão et al. 2007). The Mangueira upflow anaerobic sludge blanket (UASB) 810 m^3 reactor with eight parallel cells has been in operation since 1997. The reactor has been monitored since the beginning of the operation, and it has proven to be a robust and stable reactor (Florencio et al. 2001; Morais et al. 2004; Barros et al. 2008).

An evaluation of the microorganisms diversity is important to understand their behavior in a UASB reactor that operates under full-scale conditions. Most studies have been conducted under bench-scale testing conditions (Pholchan et al. 2010; Fernández et al. 2009); when conducted at full-scale conditions, the diversity that is discussed only includes specific groups or conditions (Angenent et al. 2002; Werner et al. 2011).

In general, microorganisms belonging to the domains *Archaea* and *Bacteria*, which are present in anaerobic reactors used for wastewater treatment, are difficult to grow in the laboratory. Therefore, the identification of bacterial populations has been accomplished through the use of molecular tools, such as the cloning and sequencing of conserved genes like rRNA, gel electrophoresis denaturing gradient (DGGE), fluorescent in situ hybridization (FISH), and recently, through metagenomic analysis (Krause et al. 2008; Schlüter et al. 2008). Because of this, the presence of certain species in the sludge can be determined by 16S rRNA coding sequences without the need to cultivate the microorganisms.

The aim of this work was to learn more about the microorganisms present in a full-scale WWTP. Our objective was to identify microorganisms belonging to the domains *Bacteria* and *Archaea* that were present in a UASB reactor and to evaluate the population differences occurring along several levels of a sludge bed in combination with specific methanogenic activity.

Materials and methods

Sludge sampling

The anaerobic sludge used in this study was obtained from a full-scale UASB reactor, which is located at the Mangueira WWTP in Recife, Pernambuco state, Brazil. The reactor operated with an 8-h hydraulic retention time (HRT). The sludge was collected at five sampling points in the reactor (from bottom to top): A (0.8 m), B (1.3 m), C (1.8 m), D (2.3 m) and E (2.8 m). At the time of the sampling, the reactor had not been subjected to excess sludge discharge during the previous 8 months.

Specific methanogenic activity (SMA)

The SMA was determined in accordance with the works of Florencio et al. (1993) and Field et al. (1987). A mixture of acetic, propionic and butyric acids at a ratio of 1:1:1 on COD, a solution of macro and micronutrients and sludge were added into 500-mL reaction flasks with 450 mL as the working volume, resulting in a final concentration of 4 g COD L^{-1}, 200 mL L^{-1} and 5 g VSS L^{-1}, respectively.

Physico-chemical parameters

The parameters were determined according to APHA (1992) twice a week to monitor the reactor temperature, pH, suspended solids (total, fixed and volatile), COD (total and soluble), alkalinity (total and partial), volatile acids and redox potential. The parameters were determined in reactor influent and effluent for 3 months prior to the collection of sludge samples.

Total DNA extraction and PCR

The extraction protocol proposed by Moreira et al. (2005) was used for DNA extraction. The PCR reaction for the 16S rRNA gene was performed according to Nielsen et al. (1999) and Kudo et al. (1997) for the domains *Bacteria* and *Archaea*, respectively. For the domain *Bacteria*, the following primers were used:

(i) 968f (5′-AACGCGAAGAAC CTTAC-3′);
(ii) 1392r (5′-ACGGGCGGTGTGTAC-3′).

For the domain *Archaea*, the following primers were used:

(i) 1100F (5′-AACCGTCGACAGTCAGGYAACGAGC GAG-3′);
(ii) 1400r (5′-CGGCGAATTCGTGCAAGGAGCAGGG AC-3′).

The amplicons were purified with Wizard SV Gel and a PCR Clean-Up System kit (Promega Co.) in accordance with the manufacturer's instructions.

Cloning and sequencing

The purified amplicons that corresponded to portions of the 16S rRNA gene were cloned in a pGEM-T vector (Promega Co.) in accordance with the manufacturer's instructions; recombinant plasmids were introduced into the *E. coli* TOP10 strain by using the calcium chloride method. The transformed cells were selected on an LB + ampicillin medium that was supplemented with IPTG and X-gal (Sambrook and Russell 2001). White recombinant colonies were grown in an LB + ampicillin medium, and plasmids were extracted by the miniprep method. The presence of the insertion of the 16S rRNA gene was verified by the NotI restriction enzyme (New England Biolabs) in accordance with manufacturer's instructions. The plasmid DNA (200–500 ng) was used as a PCR labeling template by using the BigDye® Terminator Cycle Sequencing kit (Applied Biosystems), with 3.2 pmol of the M13 primer in 10 μL of the final volume. The steps included initial denaturation at 94°C for 2 min, followed by 40 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 10 s and extension at 60°C for 4 min. The amplification products were precipitated with ethanol/EDTA/sodium acetate and resuspended in formamide according to the manufacturer's instructions. The sequencing of both strands was done on an ABI Prism 3100 Genetic Analyzer device (Applied Biosystems).

16S rRNA gene sequence analysis

The BioEdit 7.0.5 program and the Staden Package set of programs (http://staden.sourceforge.net/) were used to assess the quality of the sequences and the consensus sequences assembly. The consensus sequences were compared with the database of the Ribosomal Database Project–RDP (http://rdp.cme.msu.edu/) by using the Classifier program (confidence threshold = 95%) and the BLASTn program in their default settings for the nucleotide nr/nt collection GeneBank database from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/). For species identification, a 97% minimum similarity between sequences was followed.

Results and discussion

UASB reactor performance and sludge methanogenic activity

The results presented in Table 1 indicate a strong relationship between the solids profile and the lengthy previous period without excess sludge discharge. The amount of fixed solids was significantly high and represented approximately 50% of the total solids, whereas the VSS concentrations (16.9–40.2 g L^{-1}) were relatively low compared to values of around 50–60 g VSS L^{-1} found in other full-scale UASB reactors that treat domestic sewage (van Haandel et al. 2006). The concentrations of VSS tended to decrease from A to C and to increase again upwards. The high accumulation of solids possibly caused a short circuit inside the reactor, and it is possible that the withdrawal of the sludge samples was not uniform due to some sampling difficulties that were caused by pipe clogging. The accumulation of inert material inside the reactor can be attributed to the WWTP's inefficient removal of a large amount of grit entering the sewerage. The population that is served by the WWTP lives in a low-income area where the streets are not paved and the region's precipitation is very high (above 2,000 mm annually).

Nevertheless, the average COD removal efficiency (based on total influent and filtered effluent COD) was 83%, which confirms the robustness of the reactor and the previous results (Florencio et al. 2001; Morais et al. 2004; Barros et al. 2008). However, the average removal efficiency was only 23% for the suspended solids, which is much lower than the previously obtained value of 75% (Morais et al. 2004) due to solids washout.

Regardless of the VSS concentrations, the SMA values apparently decreased from the bottom to the top, although the differences were not significant. The exception is perhaps at point E, which showed a small loss of activity probably due to the presence of less substrates and the older age of the sludge.

Reactor population belonging to the domain *Bacteria*

16S rRNA libraries were produced by cloning the amplification fragments generated with primers 968f and 1392r at different levels and bacterial clones were subjected to DNA sequencing. Over 54 different nucleotide sequences were produced ranging from 93 to 100% similarity to sequences deposited in the RDP database and NCBI database. Bacterial diversity was quite similar among the reactors levels (Table 2). However, for identification the minimum similarity accepted was 97%. The five identified bacterial phyla were distributed as follows: 42% belonged to the *Actinobacteria* phylum, 13% to the *Proteobacteria* phylum, 9% to the *Chloroflexi* phylum, 6% to the *Firmicutes* phylum and 4% to the *Bacteroidetes* phylum. The 26% of the remaining sequences did not fit in any of the phyla (Fig. 1).

Actinobacteria are a group of Gram-positive bacteria that play an important role in the decomposition of organic matter and in the carbon cycle (Stackebrandt et al. 1997). Representatives of this phylum were found in all levels of the reactor. Members of the family *Nocardioidaceae*, such as *Nocardioides dokdonensis* and *Propionicimonas paludicola*, were found at levels A and B, respectively. *Nocardioides dokdonensis* is a species that is still not well known and belongs to a genus that was initially identified

Table 1 Temperature, pH, solids and UASB reactor sludge SMA values

Sludge sampling point (Level)[a]	Temp. (°C)	pH	Suspended solids (g L^{-1})			SMA (gCOD-CH_4 g^{-1} VSS day^{-1})
			Total (TSS)	Fixed (FSS)	Volatile (VSS)	
E (2.8 m)	30.7	7.2	58.5	30.5	28.0	0.29
D (2.3 m)	30.8	7.4	48.7	27.7	22.0	0.34
C (1.8 m)	30.8	7.4	35.9	19.0	16.9	0.34
B (1.3 m)	30.8	7.3	62.4	34.5	27.9	0.30
A (0.8 m)	31.2	7.2	89.9	49.7	40.2	0.37

[a] Refers to the height of the sludge collection point, from bottom (A) to top (E)

Table 2 Clones representative of the different nucleotides sequences produced by the amplification of sludge total DNA with primers for bacteria

Clone	Description	ID[a] (%)	Access[b]	e-value
A1	Uncultured *Nocardioidaceae* bacterium	98	EU266885.1	2e-169
A4	*Propionibacteriaceae* bacterium DA02	98	EU541469.1	2e-160
A5	*Tessaracoccus* sp. KSS-17Se	97	FM178840.1	8e-169
A6	Uncultured gamma proteobacterium	96	EF141974.1	7e-139
A7	*Nocardioides dokdonensis* strain FR1436	98	EF633986.1	0.0
A8	*Cellulomonas* sp. d20	93	AJ298927.1	5e-156
A9	*Succiniclasticum ruminis*	96	X81137.1	2e-165
A11	Uncultured bacterium clone R5 E8	90	DQ462739.1	6e-105
A12	Uncultured bacterium clone C16	100	EU234255.1	0.0
A13	Uncultured bacterium clone 6E6_cons	100	EF688250.1	9e-173
A14	Uncultured low G + C Gram-positive bacterium	99	AY261810.1	1e-177
A15	*Brachymonas denitrificans* strain a44	98	EU434445.1	1e-142
A16	Uncultured bacterium clone 4D11_cons	100	EF688195.1	0.0
B2	*Propionibacteriaceae* bacterium WR061	98	AB298731.2	0.0
B3	*Brooklawnia cerclae* strain BL-34	97	DQ196625.1	0.0
B4	*Cellulomonas* sp. d20	97	AJ298927.1	0.0
B5	Uncultured bacterium clone 4D11_cons	99	EF688195.1	0.0
B7	*Brooklawnia cerclae* strain BL-34	97	DQ196625.1	0.0
B9	Uncultured bacterium clone ORSFAB_b12	99	EF393231.1	0.0
B10	*Cellulomonas* sp. d20	97	AJ298927.1	0.0
B11	Uncultured bacterium clone Eb48	98	EF063623.1	0.0
B13	Uncultured bacterium clone Eb48	99	EF063623.1	0.0
B14	*Propionicimonas paludicola*	100	AB078859	0.0
B15	Uncultured bacterium gene	99	AB291424.1	7e-165
C1	*Tessaracoccus* sp. KSS-17Se	99	FM178840.1	3e-163
C3	Uncultured bacterium clone Eb48	99	EF063623.1	0.0
C5	*Propionibacteriaceae* bacterium FH044	100	AB298766.2	0.0
C6	Bacterium enrichment culture clone MB2_3	100	AM933660.1	0.0
C8	Uncultured bacterium clone 4D10_cons	98	EF688194.1	4e-127
C11	*Desulfovibrio* sp. A45	100	AB081579.1	2e-153
C15	Uncultured *Thiobacillus* sp.	100	AB425223.1	1e-121
C16	Uncultured bacterium clone Eb48	97	EF063623.1	0.0
D2	*Megasphaera elsdenii*	100	AY038994.1	0.0
D3	Uncultured anaerobic bacterium clone B-1AG	99	AY953154.1	2e-170
D4	*Propionibacteriaceae* bacterium FH044	100	AB298766.2	3e-179
D7	Uncultured bacterium partial	98	CR933321.1	4e-112
D8	Uncultured bacterium clone 4D11_cons	99	EF688195.1	0.0
D10	Uncultured anaerobic bacterium clone B-1AI	98	AY953155.1	0.0
D11	Uncultured bacterium clone 4D11_cons	98	EF688195.1	0.0
D12	*Propionibacteriaceae* bacterium WR061	98	AB298731.2	1e-161
D13	Uncultured bacterium clone Eb48	97	EF063623.1	1e-172
D15	*Pseudonocardia petroleophila*	98	X80596.1	2e-150
D16	Uncultured bacterium clone 4D11_cons	100	EF688195.1	0.0
E2	Uncultured bacterium clone 4D11_cons	98	EF688195.1	0.0
E3	Uncultured bacterium clone Flyn1_9	95	DQ256687.1	0.0
E5	Uncultured epsilon proteobacterium PD-UASB-2	99	AY261811.1	0.0
E6	Uncultured bacterium	98	CR933321.1	0.0
E8	Uncultured bacterium clone 3C7_cons	99	EF688178.1	0.0

Table 2 continued

Clone	Description	ID[a] (%)	Access[b]	e-value
E10	Uncultured bacterium clone LTR-R13	99	EU722381.1	0.0
E11	Uncultured bacterium clone 013C-F4	99	DQ905431.1	0.0
E12	*Tessaracoccus* sp. KSS-17Se	98	FM178840.1	0.0
E15	*Propionivibrio limicola* strain GolChi1 T	98	AJ307983.1	0.0
E16	Uncultured bacterium clone C16	99	EU234255.1	0.0

[a] Sequence similarity

[b] Access at NCBI of the sequence with similarity to the clones in this work

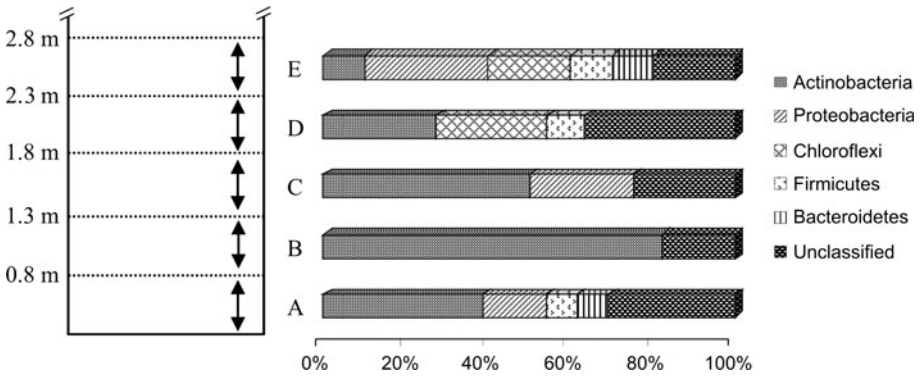

Fig. 1 Bacteria domain phyla distribution scheme for levels A, B, C, D and E of the UASB reactor. Representatives found: (▓) ACTINOBACTERIA—*Nocardioides dokdonensis*, *Propionicimonas paludicola*, *Brooklawnia cerclae* and *Tessaracoccus* sp., *Cellulomonas* sp. *Pseudonocardia petroleophila*. (▨) PROTEOBACTERIA—*Brachymonas denitrificans*, *Propionivibrio limicola*, *Thiobacillus* sp., *Desulfovibrio* sp. (▩) CHLOROFLEXI—class *Anaerolineae*. (▩) FIRMICUTES—*Succiniclasticum ruminis*, *Megasphaera elsdenii*. (▥) BACTEROIDETES—order *Bacteroidales*. (▩) Unclassified

in soil, where many species are known to degrade hydrocarbons present in raw oil (Schippers et al. 2005). *Propionicimonas paludicola* is a facultative bacterium, but it grows best under anaerobic conditions by fermenting organic matter to propionate (Akasaka et al. 2003).

Members of the family *Propionibacteriaceae*, such as *Brooklawn cerclae* and *Tessaracoccus* sp. KSS-17Se, were found in all levels of the reactor. *Brooklawn cerclae* was isolated from groundwater that was contaminated with mixtures of chlorinated solvents and polycyclic aromatic hydrocarbons. They are mesophilic and produce mainly propionate and acetate from glucose (Bae et al. 2006). *Tessaracoccus* sp. KSS-17Se belongs to a genus of facultative anaerobes that form tetrads of cocci. An example is *Tessaracoccus bendigoensis,* which uses various substrates in its metabolism, such as glucose, sucrose, fructose, mannose, trehalose, valeric acid, butyric acid and propionic acid. These microorganisms are mesophilic and were previously isolated from aerobic-activated sludge systems (Maszenan et al. 1999).

Three sequences that show similarities to *Cellulomonas* sp. d20 were found in levels A and B of the reactor. This genus includes hydrolytic fermentative bacteria that produce acetate, ethanol and formate from complex polysaccharides, such as cellulose (Guyot 1986; An et al. 2005).

A similarity to the D3bac sequence at Genebank, which was only detected at level D, was found for the bacterium *Pseudonocardia petroleophila*. This genus was described by Henssen in 1957 as belonging to a group of biochemically versatile bacteria; some species may oxidize hydrocarbons, whereas others turn sulfide into sulfate or degrade cellulose (Huang et al. 2002). However, the sequence D3bac showed only 89% similarity to that bacterium. A search for similarity in RDP by using the program Classifier was also conducted. In this database, isolated D3bac was classified at the level of the phylum *Actinobacteria*. These results indicate that D3bac probably corresponds to a bacterium that has not yet been described but is comparable to *Pseudonocardia petroleophila*.

It is also worth mentioning that bacteria belonging to the *Actinobacteria* group were prevalent at levels A, B and C. As described, these microorganisms are mostly fermentative hydrolytic and fermentative; they are probably the most responsible for the degradation of complex organic matter that is fed into the reactor, which leads to the generation of precursor compounds for methane formation.

The emergence of microorganisms that are involved in the degradation of hydrocarbons and raw oil may be the result of different substrates entering the reactor. The presence of some oily effluents or other leakages in the ground should be noted. The streets and sidewalks were not paved, and there were informal commercial shops and small-scale industrial activities in the study area. The liquid waste was able to reach the sewerage due to an inefficient drainage system that could not separately collect the rain water.

There was great diversity in the phylum *Proteobacteria*, which was represented by aerobic, anaerobic, photoautotrophic, photoheterotrophic and chemolithotrophic bacteria (Oren 2004). This large group of cultured bacteria is divided into five major classes: *α-Proteobacteria*, *β-Proteobacteria*, *γ-Proteobacteria*, *δ-Proteobacteria* and *ε-proteobacteria*. In this study, representatives of all of the classes were found, except *α-Proteobacteria*. In the class *β-Proteobacteria*, *Brachymonas denitrificans* was found at level A of the reactor. Initially isolated from the aerobic system (Hiraishi et al. 1995), this strain has the ability to remove phosphorus from the system under aerobic conditions and can remove nitrogen under anoxic conditions (Shi and Lee 2007). It is possible that these microorganisms were using the small amounts of oxygen that entered the reactor because they were found only at the bottom portion of the reactor.

Propionivibrio limicola strain GolChi1 T was found only in level E. It is a mesophilic anaerobic bacterium and was isolated in culture-metabolizing hydroaromatic compounds, such as hydro-quinic acid and shikimic acid. These acids are important precursors to the biosynthesis of lignin and tannins in plants, which produce propionate and acetate (Brune et al. 2002). In this work, the easily degradable organic matter that could have reached the upper part of the reactor had probably already been removed earlier. Therefore, the only remaining compounds were those that were more difficult to degrade, which explains the occurrence of this bacterium.

A clone from the *γ-proteobacteria* class that is 100% similar to *Thiobacillus* sp. was found at level C of the reactor. This organism is related to the oxidation of hydrogen sulfide in the treatment of wastewaters in reactors and of sulfur springs (Visser et al. 1997; Ravichandra et al. 2007). The presence of this bacterium in the UASB is interesting because it has been described as chemoautotrophic aerobic, which indicates that oxygen should occasionally be available in the influent that is being consumed inside the reactor. The sulfate-reducing bacteria (SRB) *Desulfovibrio* sp. A45 from the *δ-Proteobacteria* class was found at level C of the UASB. These bacteria are a group of microorganisms that live in various anaerobic environments, which include soil, domestic, industrial and mining wastewaters (Chang et al. 2001). The SRB can use many substrates, which include hydrogen, lactate, formate, malate, fumarate, pyruvate, alcohols, volatile fatty acids and various types of hydrocarbons and phenolic compounds (Rueter et al. 2002). Both sulfate and the previously described substrates are commonly present in anaerobic reactors. During complex organic matter degradation at level C of the UASB reactor, those substrates became available and could be more easily used as electron donors for the existing SRB.

A search for the D8bac, D9bac, D11bac, E6bac and E10bac sequences in the Genebank database showed maximum similarity with non-cultivable bacteria. A search in the RDP Classifier indicated that the sequences belonged to the phylum *Chloroflexi* and class *Anaerolineae*. Studies of the phylum *Chloroflexi* have shown that its members play an important role in the formation of granules in UASB reactors (Yamada et al. 2005).

Members of the phylum *Firmicutes* were found at levels A, D and E of the reactor. *Succiniclasticum ruminis*, which was found at level A, was first isolated from cow rumen. It is a gram-negative mesophilic bacterium that ferments succinate to propionate (van Gylswyk 1995). *Megasphaera elsdenii* was found at level D. It comprises a group of bacteria that live in the rumen and intestine, which are also found in anaerobic reactors. They can ferment glucose and lactate that produce valerate, propionate and butyrate (Bouallagui et al. 2004).

It is worth mentioning that the top and bottom levels showed the greatest diversity of microorganisms belonging to domain *Bacteria* and the highest VSS concentrations (Table 1). Layer B showed the least diversity of bacteria, despite having a VSS content comparable to that of layer E. This indicates that the reactor's hydrodynamic regime probably affected the selection of these populations because the lowest diversity was expected to occur at layer E. At this level, the substrates should be less diversified and more difficult to degrade. It is possible that preferential paths associated with biogas and liquid movement along the height of the reactor resulted in substrate diversity at the top of the reactor. However, it is also important to consider the limitations of this work when looking for these statements since only one sample from each reactor level was taken.

Archaea population in the reactor

16S rRNA libraries were produced by cloning the amplification fragments generated with primers 1100F and 1400R at different levels and bacterial clones were subjected to DNA sequencing. Over 56 different nucleotide sequences were produced ranging from 94 to 100% similarity to sequences deposited in the RDP database and NCBI database. *Archaea* diversity was quite similar among the reactors

Table 3 Clones representative of the different nucleotides sequences produced by the amplification of sludge total DNA with primers for Archaea

Clone	Description	ID[a] (%)	Access[b]	e-value
A2	Uncultured *Methanosaeta* sp. clone D_E05	99	AY454756.1	1e-161
A4	Uncultured *Methanomicrobiales archaeon*	100	AB236107.1	5e-109
A6	Uncultured *Methanolinea* sp.	97	AB434764.1	6e-51
A7	*Methanobacterium* sp. F	98	AB302952.1	3e-118
A8	Uncultured *Methanomicrobiales archaeon*	100	AB236107.1	3e-92
A9	Uncultured *Methanomicrobiales archaeon*	98	AB236107.1	3e-137
A15	Uncultured *Methanosaeta* sp. clone D_G10	99	AY454768.1	2e-155
A16	*Methanobacterium* sp. T01	98	AB288275.1	8e-139
B2	Uncultured *Methanomicrobiales archaeon*	99	AB236107.1	6e-140
B3	Uncultured *Methanomicrobiales archaeon*	96	AB236107.1	8e-123
B4	Uncultured *Methanomicrobiales archaeon*	99	AB236107.1	1e-126
B5	Uncultured *Methanomicrobiales archaeon*	100	AB236107.1	1e-150
B6	Uncultured *Methanomicrobiales archaeon*	100	AB236107.1	1e-155
B7	*Methanobacterium* sp. T01	100	AB288275.1	6e-125
B11	Uncultured *Methanomicrobiales archaeon*	100	AB236107.1	3e-152
B14	Uncultured *Methanomicrobiales archaeon*	100	AB236107.1	1e-150
B16	Uncultured *Methanomicrobiales archaeon*	96	AB236107.1	3e-138
C1	Uncultured *Methanomicrobiales archaeon*	100	AB236107.1	7e-124
C2	Uncultured *Methanosaeta* sp.	100	AM491934.1	2e-133
C3	Uncultured *Methanosaeta* sp. clone D_E05	100	AY454756.1	4e-172
C4	Uncultured *Methanomicrobiales archaeon*	100	AB236107.1	5e-135
C5	Uncultured *Methanomicrobiales archaeon*	99	AB236107.1	2e-155
C6	Uncultured *Methanomicrobiales archaeon*	99	AB236107.1	1e-135
C9	Uncultured *Methanosaeta* sp.	98	AB434763.1	6e-72
C10	Uncultured *Methanosaeta* sp.	99	AY454758.1	1e-121
C11	*Methanobacterium* sp. T01	99	AB288275.1	4e-146
C15	Uncultured *Methanosaeta* sp.	99	AB288619.1	5e-150
C17	Uncultured *Methanomicrobiales archaeon*	99	AB236107.1	3e-122
D1	Uncultured *Methanosaeta* sp. clone DI_G06	100	AY454766.1	2e-129
D2	Uncultured *Methanomicrobiales archaeon*	99	AB236107.1	9e-158
D3	Uncultured *Methanosaeta* sp.	99	AY454761.1	5e-161
D4	Uncultured *Methanomicrobiales archaeon*	99	AB236107.1	7e-149
D5	*Methanobacterium* sp.	99	AB368917.1	2e-139
D6	Uncultured *Methanospirillum* sp.	97	DQ903695.1	6e-160
D8	*Methanobacterium* sp. T01	99	AB288275.1	7e-129
D9	*Methanobacterium beijingense* strain 4-1	94	AY552778.3	6e-110
D10	Uncultured *Methanosaeta* sp. clone DI_C03	99	AY454761.1	5e-161
D11	Uncultured *Methanosaeta* sp.	99	AB434763.1	8e-149
D12	Uncultured *Methanosaeta* sp. clone D_G10	99	AY454768.1	5e-166
D13	Uncultured *Methanomicrobiales archaeon*	99	AB236107.1	2e-159
D14	Uncultured *Methanomicrobiales archaeon*	100	AB236107.1	4e-152
D15	Uncultured *Methanomicrobiales archaeon*	99	AB236107.1	2e-159
D16	*Methanobacterium* sp. T01	96	AB288275.1	3e-127
E1	Uncultured *Methanosaeta* sp.	99	AB288619.1	3e-137
E2	Uncultured *Methanosaeta* sp.	99	AB288619.1	1e-156
E3	Uncultured *Methanosaeta* sp. clone DI_G06	99	AY454766.1	2e-170
E4	Uncultured Methanomicrobia archaeon	98	AB236066.1	9e-128
E5	Uncultured Methanomicrobia archaeon	98	AB236066.1	7e-149

Table 3 continued

Clone	Description	ID[a] (%)	Access[b]	e-value
E6	Uncultured *Methanosaeta* sp.	98	AB434766.1	4e-94
E7	Uncultured *Methanosaeta* sp. clone D_G10	99	AY454768.1	3e-75
E8	Uncultured *Methanobrevibacter* sp. clone EI_G11	99	AY454736.1	9e-107
E9	Uncultured *Methanosaeta* sp. clone D_G10	95	AY454768.1	6e-104
E10	Uncultured *Methanomicrobiales archaeon*	100	AB236107.1	4e-141
E12	Uncultured *Methanobrevibacter* sp. clone DI_B05	100	AY454733.1	8e-153
E13	Uncultured *Methanosaeta* sp. clone D_G10	99	AY454768.1	1e-120
E15	Uncultured *Methanosaeta* sp. clone DI_G06	99	AY454766.1	2e-154

[a] Sequence similarity

[b] Access at NCBI of the sequence with similarity to the clones in this work

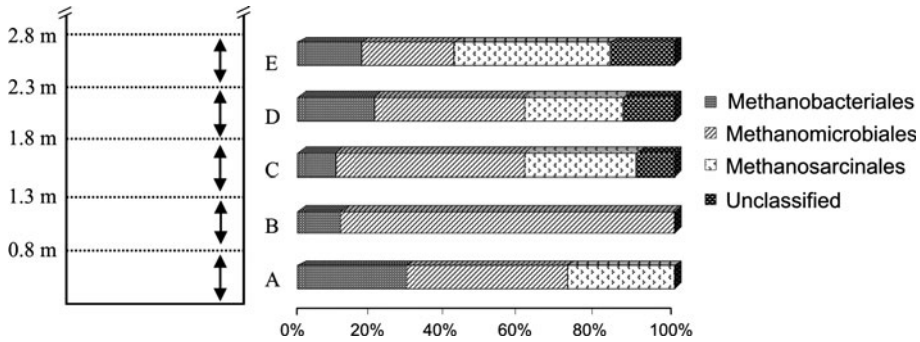

Fig. 2 Archaea domain order distribution scheme for levels A, B, C, D and E of the UASB: (▨) METHANOMICROBIALES—*Methanospirillum* sp.; (■) METHANOBACTERIALES—*Methanobacterium* sp., *Methanobrevibacter* sp., *Methanobacterium beijingense;* (▩) METHANOSARCINALES—*Methanosaeta* sp.; (■) Unclassified

levels (Table 3). According to the Classifier program, 42% of the obtained sequences belonged to the *Methanomicrobiales* order, 17% to the *Methanobacteriales* order and 12% to the *Methanosarcinales* order (Fig. 2). The other 17% were grouped with confidence within the phylum *Euryarchaeota*. However, when comparing the sequences of this last group with the Genebank database,we found organisms belonging to the genera *Methanobacterium*, *Methanosaeta*, *Methanospirillum*, *Methanobrevibacter,* and *Methanolinea* and the species *Methanobacterium beijingense*.

Methanobacterium sp. was found at levels A, B, C and D of the reactor but not in the highest level. *Methanospirillum* sp. and *Methanobrevibacter* sp. were only found at levels D and E, respectively. These hydrogenotrophic genera were also found in anaerobic reactors that were used for treating various effluents, such as those from breweries, paper mills, food manufacturing plants, phenolic compounds, whiskey production and domestic sewage (Leclerc et al. 2004). They can use H_2, CO_2 and formate in their metabolism for energy and CH_4 production, which may have occurred in this study because these substances were detected in the upper part of the reactor.

Methanosaeta sp. was found at levels A, C, D and E of the reactor. This *Archaea* plays an important role in removing carbon from the system, and it can use acetate, methanol and methylamines as energy source (Laloui-Carpentier et al. 2006). This genus has also been identified in various reactors that were used for the treatment of sewage, effluent from slaughterhouses and the production of wine and paper (Leclerc et al. 2004). The fact that this genus was not detected in level B may have been caused by the hydrodynamic behavior of the reactor because the preferred substrates for this group of organisms should be available at this level of the reactor.

A new species identified as *Methanolinea tarda* was recently isolated from an anaerobic culture that was enriched with propionate in domestic sewage sludge in Japan. The strain uses H_2 and formate as energy source and produces CH_4 (Imachi et al. 2008). One of the sequences obtained in this study was observed only at level A of the reactor and showed 97% similarity to the genus *Methanolinea*.

Methanobacterium beijingense was found in an anaerobic reactor that was used to treat effluents from the production of beer in Beijing, China. This organism also uses H_2 and formate as an energy source for the production of CH_4 (Ma et al. 2005). The sequence D15arc that was found at level D showed 94% similarity to this *Archaea*. The relatively low percentage of similarity was mitigated by the

high quality level (PHRED values between 35 and 49), which suggests that this may be a sequence of a bacterium that has not yet been described but is closely related to *Methanobacterium beijingense.*

We observed the marked presence of sequences that were similar to a clone of the order *Methanomicrobiales,* which is related to a non-cultivable organism. This clone was found at all levels of the reactor and represents 42% of all the sequences of *Archaea*; eight of the nine sequences were identified at level B. By using the RDP Classifier, 20 sequences with 87% similarity to the family *Methanomicrobiaceae* were found, and some of them showed little similarity to the genus *Methanoculleus*. It is notable that the sample from level B showed the lowest specific methanogenic activity and the lowest diversity level of microorganisms belonging to the domain *Archaea*. We also detected a reduced level of bacteria diversity at this level.

Conclusion

The results for the domain *Archaea* confirm the occurrence of major groups of methane-producing microorganisms in the reactor used in this study. The traditional process via acetate and H_2/CO_2 must have been used by microorganisms to produce methane.

The wide diversity of *Bacteria* and *Archaea* was higher in full-scale UASB upper and bottom layers showing the highest VSS levels, with middle's less diversity and lowest SMA values. Reactor hydrodynamics influenced microorganisms selection and substrate transport. *Actinobacteria* hydrolysing complex organics dominated the lower layers. *Archaea* was well detected but with the highest SMA (0.37 g $CODCH_4$/g SSV.d, based on VFA mixture) in the bottom. The majority of *Archaea* indicated predominance of methane production pathway from H_2–CO_2. Despite similar bottom's diversity, the top SMA was lower due the predominance of *Proteobacteria* like the sulfate reducers that competed with methanogens for hydrogen and acetate.

Acknowledgments The authors express their special thanks to the colleagues of the Laboratory of Environmental Sanitation of the Federal University of Pernambuco for the analytical help, sampling and field activities. This work has been done with grants from the Brazilian agencies Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Financiadora de Estudos e Projetos (FINEP) and Fundação de Amparo a Ciência e Tecnologia do Estado de Pernambuco (FACEPE). The public companies COMPESA and URB-Recife are also acknowledged.

References

Akasaka H, Ueki A, Hanada S, Kamagata Y, Ueki K (2003) *Propionicimonas paludicola* gen nov sp nov a novel facultatively anaerobic gram-positive propionate-producing bacterium isolated from plant residue in irrigated rice-field soil. Int J Syst Evol Microbiol 53:1991–1998. doi:101099/ijs002764-0

An DS, Im WT, Yang HC, Kang MS, Kim KK, Jin L, Kim MK, Lee ST (2005) *Cellulomonas terrae* sp. nov a cellulolytic and xylanolytic bacterium isolated from soil. Int J Syst Evol Microbiol 55:1705–1779. doi:101099/ijs063696-0

Angenent LT, Sung S, Raskin L (2002) Methanogenic population dynamics during startup of a full-scale anaerobic sequencing batch reactor treating swine waste. Wat Res 36:4648–4654

APHA/AWWA/WEF (1992) Standard methods for the examination of water and wastewater, 19th edn, Washington

Bae HS, Moe WM, Yan J, Tiago I, Da Costa MS, Rainey FA (2006) *Brooklawnia cerclae* gen nov sp nov a propionate-forming bacterium isolated from chlorosolvent-contaminated groundwater. Int J Syst Evol Microbiol 56:1977–1983. doi:101099/ijs064317-0

Barros KK, Gavazza S, Florencio L, Kato MT (2008) Performance of UASB reactors under excess sludge discharge at different periods of time. In: Proceedings of the 9th Latin American workshop and symposium on anaerobic digestion, pp 572–578 Easter Island Chile (*in Portuguese*)

Bouallagui H, Torrijos A, Godon JJ, Moletta R, Ben Cheikh R, Touhami Y, Delgenes JP, Di AH (2004) Two-phases anaerobic digestion of fruit and vegetable wastes: bioreactors performance. Biochem Eng J 21:193. doi:101016/jbej200405001

Brune A, Ludwig W, Schink B (2002) *Propionivibrio limicola* sp. nov a fermentative bacterium specialized in the degradation of hydroaromatic compounds reclassification of *Propionibacter pelophilus* as *Propionivibrio pelophilus* comb nov and amended description of the genus *Propionivibrio*. Int J Syst Evol Microbiol 52:441–444. doi:101099/ijs002082-0

Chang YJ, Peacock AD, Long PE, Stephen JR, Mckinley JP, Macnaughton SJ, Hussain AK, Saxton AM, White DC (2001) Diversity and characterization of sulfate-reducing bacteria in groundwater at a uranium mill tailings site. Appl Environ Microbiol 67:3149–3160. doi:101128/AEM6773149-3160 2001

Fernández N, Sierra-Alvarez R, Amils R, Field JA, Sanz JL (2009) Compared microbiology of granular sludge under autotrophic mixotrophic and heterotrophic denitrification conditions. Wat Sci Technol 59:1227–1236. doi:102166/wst2009092

Field JA, Lettinga G, Geurts M (1987) The methanogenic toxicity and anaerobic degradability of potato starch wastewater phenolic amino acids. Biol Wastes 21:37–54. doi:101016/0269-7483(87)90145-5

Florencio L, Jenicek P, Field JA, Lettinga G (1993) Effect of cobalt on anaerobic degradation of methanol. J Ferment Bioeng 75:368–374. doi:101016/0922-338X(93)90136-V

Florencio L, Kato MT, Morais JC (2001) Domestic sewage treatment in Mangueira full-scale UASB plant at Recife Pernambuco. Wat Sci Technol 44:71–78

Guyot JP (1986) Role of formate in methanogenesis from xylan by *Cellulomonas* sp. associated with methanogens and *Desulfovibrio* vulgaris: inhbition of the aceticlastic reaction. FEMS Microbiol Lett 34:149–153

Hiraishi A, Shin YK, Sugiyama J (1995) *Brachymonas denitrificans* gen nov sp nov an aerobic chemoorganotrophic bacterium which contains rhodoquinones and evolutionary relationships of rhodoquinone producers to bacterial species with various quinone classes. J Gen Appl Microbiol 41:99–117

Huang Y, Wang L, Lu Z, Hong L, Liu Z, Tan GY, Goodfellow M (2002) Proposal to combine the genera Actinobispora and *Pseudonocardia* in an emended genus *Pseudonocardia* and description of *Pseudonocardia zijingensis* sp nov. Int J Syst Evol Microbiol 52:977–982. doi:101099/ijs001977-0

Imachi H, Sakai S, Sekiguchi Y, Hanada S, Kamagata Y, Ohashi A, Harada H (2008) *Methanolinea tarda* gen nov sp nov a methane-producing archae on isolated from a methanogenic digester sludge. Int J Syst Evol Microbiol 58:294–301. doi:101099/ijs065394-0

Jordão EP, Volschan Jr I, Alem Sobrinho P (2007) Secondary WWTP preceded by UASB reactors–an excellent Brazilian experience. In: Proceedings of the 10th IWA specialised conference on design operation and economics of large wastewater treatment plants September 9–13 2007 Vienna Austria

Krause L, Diaz NN, Edwards RA, Gartemann K-H, Krömeke H, Neuweger H, Pühler A, Runte KJ, Schlüter A, Stoye J, Szczepanowski R, Tauch A, Goesmann A (2008) Taxonomic composition and gene content of a methane-producing microbial community isolated from a biogas reactor. J Biotechnol 36:91–101

Kudo Y, Nakajima T, Miyaki T, Oyaizu H (1997) Methanogen flora of paddy soils in Japan. FEMS Microbiol Ecol 22:39–48. doi:101111/j1574-69411997tb00354x

Laloui-Carpentier W, Li T, Vigneron V, Mazéas L, Bouchez T (2006) Methanogenic diversity and activity in municipal solid waste landfill leachates. Antonie Van Leeuwenhoek 89:423–434

Leclerc M, Delgènes JP, Godon JJ (2004) Diversity of the archaeal community in 44 anaerobic digesters as determined by single strand conformation polymorphism analysis and 16S rDNA sequencing. Environ Microbiol 6:809–819. doi:101111/j1462-2920200400616x

Ma K, Liu X, Dong X (2005) *Methanobacterium beijingense* sp. nov a novel methanogen isolated from anaerobic digesters. Int J Syst Evol Microbiol 55:325–329. doi:101099/ijs063254-0

Maszenan AM, Seviour RJ, Patel BK, Schumann P, Rees GN (1999) *Tessaracoccus bendigoensis* gen nov sp nov a gram-positive coccus occurring in regular packages or tetrads isolated from activated sludge biomass. Int J Syst Bacteriol 49:459–468. doi:101099/00207713-49-2-459

Morais JC, Gavazza S, Florencio L, Kato MT (2004) Six-year monitoring period of a full-scale UASB reactor treating domestic sewage under tropical conditions. In: Proceedings of the 10th world congress on anaerobic digestion montreal, vol 1, pp 1–4

Moreira JL, Mota RM, Horta MF, Teixeira SM, Neumann E, Nicoli JR, Nunes AC (2005) Identification to the species level of Lactobacillus isolated in probioticprospecting studies of human animal or food origin by 16S–23S rRNA restriction profiling. BMC Microbiol 23:15. doi:101186/1471-2180-5-15

Nielsen AT, Liu WT, Filipe C, Grady L Jr, Molin S, Stahl DA (1999) Identification of a novel group of bacteria in sludge from a deteriorated biological phosphorus removal reactor. Appl Environ Microbiol 65:1251–1258

Oren A (2004) Prokaryote diversity and taxonomy: current status and future challenges. Phil Trans R Soc Lond B 359:623–638

Pholchan MK, Baptista JD, Davenport RJ, Curtis TP (2010) Systematic study of the effect of operating variables on reactor performance and microbial diversity in laboratory-scale activated sludge reactors. Wat Res 44:1341–1352. doi:101016/jwatres200911005

Ravichandra P, Mugeraya G, Rao AG, Ramakrishna M, Jetty A (2007) Isolation of *Thiobacillus* sp. from aerobic sludge of distillery and dairy effluent treatment plants and its sulfide oxidation activity at different concentrations. J Environ Biol 28:819–823

Rueter P, Rabus R, Wilkest H, Aeckersberg F, Rainey FA, Jannasch HW, Widdel F (2002) Anaerobic oxidation of hydrocarbons in crude oil by new types of sulphate-reducing bacteria. Nature 372:455–458. doi:101038/372455a0

Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual, 3rd edn. Cold Spring Harbor Lab Press, Cold Spring Harbor

Schippers A, Schumann P, Spröer C (2005) *Nocardioides oleivorans* sp. nov a novel crude-oil-degrading bacterium. Int J Syst Evol Microbiol 55:1501–1504. doi:101099/ijs063500-0

Schlüter A, Bekel T, Diaz NN, Dondrup M, Eichenlaub R, Gartemann K-H, Krahn I, Krause L, Krömeke H, Kruse O, Mussgnug JH, Neuweger H, Niehaus K, Pühler A, Runte KJ, Szczepanowski R, Tauch A, Tilker A, Viehöver P, Goesmann A (2008) The metagenome of a biogas-producing microbial community of a production-scale biogas plant fermenter analysed by the 454-pyrosequencing technology. J Biotechnol 136:77–79

Shi HP, Lee CM (2007) Phosphate removal under denitrifying conditions by *Brachmonas* sp. strain P12 and Paracoccus denitrificans PP15. Can J Microbiol 53:727–737. doi:101139/W07-026

Stackebrandt E, Rainey FA, Ward NL (1997) Proposal for a new hierarchic classification system Actinobacteria classis nov. Int J Syst Evol Microbiol 47:479–491. doi:101099/00207713-47-2-479

van Gylswyk NO (1995) *Succiniclasticum ruminis* gen. nov. sp. nov., a Ruminal Bacterium converting succinate to propionate as the sole energy-yielding mechanism. Int J Syst Evol Microbiol 45:297–300. doi:101099/00207713-45-2-297

van Haandel A, Kato MT, Cavalcanti PFF, Florencio L (2006) Anaerobic reactor design concepts for the treatment of domestic wastewater. Rev Environ Sci Biotechnol 5:21–38. doi:101007/s11157-005-4888-y

Visser JM, Robertson LA, Van Verseveld HW, Kuenen JG (1997) Sulfur production by obligately chemolithoautotrophic *Thiobacillus* species. Appl Environ Microbiol 63:2300–2305

Werner JJ, Knights D, Garcia ML, Scalfone NB, Smith S, Yarasheski K, Cummings TA, Beers AR, Knight R, Angenent LT (2011) Bacterial community structures are unique and resilient in full-scale bioenergy systems. Proc Natl Acad Sci USA 108:4158–4163. doi:101073/pnas1015676108

Yamada T, Sekiguchi Y, Imachi H, Kamagata Y, Ohashi A, Harada H (2005) Diversity localization and physiological properties of filamentous microbes belonging to Chloroflexi subphylum I in mesophilic and thermophilic methanogenic sludge granules. Appl Environ Microbiol 71:7493–7503. doi:101128/AEM71117493-75032005