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Molecular monitoring of microbial diversity in an UASB reactor

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Abstract

The dynamics of the microbial community structure of a full-scale upflow anaerobic sludge blanket reactor has been analyzed using culture-independent 16S rRNA-based methods. During the start-up of the process, denaturing gradient gel electrophoresis of bacterial rDNA showed significant changes in the structure of the bacterial community, but only a slight increase in the bacterial diversity. Associated with the shifts in bacterial populations, significant changes in the relative abundance of different methanogenic species are revealed by taxon-specific dot-blot experiments. The ratio of archaeal 16S rRNA to bacterial 16S rRNA, but none of the individual microbial taxons, correlated with the maximum allowable daily organic loading rate. The results argue against the notion that many anaerobic wastewater treatment systems fail due to the lack of adequate inoculum.

1. Introduction

Anaerobic technologies have been applied for the treatment of a variety of industrial wastewaters. While both aerobic and anaerobic degradation treatments can remove complex organics from wastewater, the use of anaerobic treatment for industrial wastewater with high organic content are particularly attractive because the energy required for operating the system is minimal compared to the aeration energy required for aerobic processes. The anaerobic treatment has additionally an obvious advantage in that it produces methane, a combustible gas with a reasonably good calorific value (24 MJ/m^3). Aerobic processes in contrast produce no biogas. Finally, due to the lower growth rate of anaerobic microorganisms, much less excess sludge is produced. Excess sludge post-treatment, i.e. stabilization, dewatering and disposal, involves considerable expense and high energy consumption (Van Lier et al., 2001).

A break-through in the application of anaerobic wastewater treatment system arrived with the introduction of new configurations in which the immobilization of biomass allowed uncoupling of solids retention time and hydraulic retention time. In the system which enjoys the widest application, the upflow anaerobic sludge blanket (UASB) reactor (Lettinga et al., 1980), sludge develops in particular granular or flocculent form, with good settling properties. The highly active granules make a blanket through which the effluent flows up the reactor. The substrate present in the effluent diffuses into the sludge granules, where it is degraded.

More extensive application of anaerobic processes in the degradation of organic substrates has been hampered by the doubts on many aspects of its operation and stability and the limited understanding of the interdependence of microorganisms within the complex structure of the anaerobic microbial community. Anaerobic metabolism is fundamentally different from aerobic metabolism in that the concerted action of many different microbial species is often required for the effective bioconversion of organic substrates (Lettinga, 1995). There are two distinct phases in anaerobic treatment: in the first phase, called acidogenesis, facultative acid-forming *Bacteria* reduce complex organic matter to organic acids. In the second phase, called methanogenesis, methane-forming microorganisms convert the acids to methane gas and carbon dioxide, called biogas. Some of the carbon dioxide produced also is converted to methane gas through biological methanation, thus increasing methane yield (Novaes, 1986). Variations in the composition of one trophic level due to changes in one or more operating

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conditions (e.g., substrate composition, temperature, solid retention time and other operating parameters) influence the entire microbial community structure, affecting the bioreactor performance. Complete insight into the microbial ecology of anaerobic processes is therefore essential to make effective and reliable control of the bioreactor performance possible.

One of the alleged disadvantages of anaerobic treatment is the long start-up when inoculum with adequate microbial composition is not available. However, reports of reactors that started up successfully in the absence of highly active biomass (Seghezzo et al., 1998) confirm that the actual effect of the type and quantity of the seed on the initial performance of anaerobic biological reactors has not been elucidated. We have addressed this issue using 16S rRNA-dependent methods targeting at different phylogenetic levels (Amann et al., 1998). The use of molecular techniques allows the comparison of microbial species composition, consisting of both cultivable and non-cultivable microorganisms, at different stages during the start-up of the bioreactor, eliminating the restrictions of cultivation studies. DGGE analyses provided data on the presence and extent of sequence diversity whereas the targeting of communities by rRNA probing allowed analysis of the actual distribution of components groups within the identified communities.

2. Materials and methods

2.1. UASB reactor

The current study was performed on a full-scale upflow anaerobic sludge bed reactor constructed for the treatment of wastewater from a cheese factory in Charlone, Province of Buenos Aires, Argentina. The 150 m^3 reactor was seeded with 40 m^3 of granular sludge from an UASB reactor treating slaughterhouse wastewater (Santa Fe, Argentina). The scarcity of granular anaerobic sludge for the inoculation of anaerobic reactors for wastewater treatment is a problem in most regions of Argentina. The reactor temperature was maintained between 26°C and 30°C. The design daily flow was 50 m^3/d of cheese whey, with a chemical oxygen demand (COD) of 60 and 1 g/l of suspended solids, resulting in an average daily load of 3000 kg COD/d, at a hydraulic retention time of 3 d. During start up, the alkalinity was maintained at a constant value by adjusting the daily input flow. In this way, the reactor operated at pH between 7.0 and 7.2 and the COD removal efficiency, based on influent-total versus effluent-soluble was 95%.

2.2. Sampling and DNA extraction

2 ml sludge samples were collected from a sampling point located at the upper layer of the UASB reactor, i.e. far from the wastewater entry source. DNA was extracted by a direct-lysis procedure involving physical disruption of cells, using acid-washed glass beads in a reciprocating shaker (bead-beater, BioSpec Products, Bartlesville, OK) and purified by the CTAB method (Eichner et al., 1999).

2.3. PCR amplification

The primers F341 (5′-CCTACGGGAGGCAGCAG), and R534 (5′-ATTACCGCGGCTGCTGG), specified for conserved bacterial 16S rDNA were used for amplification of a fragment corresponding to positions 341 to 534 of the *E. coli* numbering. A GC-rich sequence was attached to the 5′ end of the forward primer, in order to prevent complete melting of the PCR products during subsequent DGGE analysis (Muyzer et al., 1993). PCR amplification was performed in a total volume of 50 μl in a DNA thermocycler PTC 100 (MJ Research, Waltham, MA). Each PCR mixture contained 10 ng of template DNA, 2.5 mM $MgCl_2$, 1.25 U of Taq polymerase, 100 μM of each dNTP and 0.2 μM of each primer. Amplification was performed for 25 cycles according to a touch-down protocol (Don et al., 1991), with the annealing temperature decreasing 1°C every second cycle from 66°C down to 56°C.

2.4. Denaturing gradient gel electrophoresis (DGGE)

DGGE analysis was performed by loading 8 μl of PCR-amplified DNA product onto 8% (wt/v) polyacrylamide gel containing a denaturant gradient of 20–60% (100% denaturant consisted of 7 M urea and 40% [v/v] formamide) parallel to the direction of electrophoresis (Muyzer et al., 1993). Gels were run at 60°C at a constant voltage of 50 V for 18 h. After the electrophoresis, the gels were soaked for 60 min in SYBR green I nucleic acid stain (1:10,000 dilution [pH = 8.0], Molecular probes, Eugene, OR). The stained gel was immediately photographed on an UV transillumination table with a camera module (Image Master VDS, Amersham Pharmacia Biotech, Uppsala, Sweden). Gels were scanned with a GS-700 Imaging Densitometer (Bio-Rad, Hercules, CA). Densitometric curves were calculated for each gel track in scanned gels using the NIH Image 1.61 software, a public domain image processing and analysis program, developed by the National Institute of Mental Health, NIMH, Bethesda, MD) (http://rsb.info.nih.gov/nih-image).

As a parameter of structural diversity of the microbial community, the Shannon–Weaver index of structural diversity (H) was calculated by using the function $H = -\sum P_i \log P_i$ is the importance probability to find a band in a particular track, and is calculated as $P_i = n_i/N$, where n_i is the height of the i peak and N represents the sum of all peak heights in the densitometric curve (Eichner et al., 1999).

Table 1
Oligonucleotide probes used in dot-blot experiments

Probe	Sequence	Target group	Reference	T_d (°C)
UNIV1392	ACGGGCGGTGTGTRC	All organisms	Novaes (1986)	54
EUB338	GCTGCCTCCCGTAGGAGT	Bacteria	Amann et al. (1990)	54
ARC915	GTGCTCCCCCGCCAATTCCT	Archaea	Stahl et al. (1988)	58
EUK516	ACCAGACTTGCCCTCC	Eucarya	Amann et al. (1990)	47
MC1109	GCAACATAGGGCACGGGTCT	Methanococcales	Raskin et al. (1994)	55
MB310	CTTGTCTCAGGTTCCATCTCCG	Methanobacteriales	Raskin et al. (1994)	57
MG1200	CGGATAATTCGGGGCATGCTG	Methanogenium	Raskin et al. (1994)	53
MSMX860	GGCTCGCTTCACGGCTTCCCT	Methanosarcinaceae	Raskin et al. (1994)	60
MS1414	CTCACCCATACCTCACTCGGG	*Methanosarcina*	Raskin et al. (1994)	58
MS821	CGCCATGCCTGACACCTAGCGAGC	*Methanosarcina*	Raskin et al. (1994)	60
MX825	TCGCACCGTGGCCGACACCTAGC	*Methanosaeta*	Raskin et al. (1994)	59

2.5. RNA extraction, dot-blot hybridization and quantification

Total RNA was extracted by mechanical disruption on a reciprocating shaker with glass beads (diameter 100 μm). Samples were applied in a total volume of 400 μl to nylon membranes (Hybond N+, Amersham Biosciences, Uppsala, Sweden) by using a Minifold I dot-blot device (Schleicher & Schuell, Dassel, Germany) under slight vacuum. A 0.5–20 μg/ml sample of RNA was spotted for hybridization to all probes (Table 1). Prehybridization was performed in solutions containing 0.9 M NaCl, 50 mM NaPO4, 5 mM EDTA, 10 × Denhardt's solution. The oligonucleotide probes were labeled with [γ^{32}P] ATP by using T4 polynucleotide kinase (Promega, Madison, WI). The unincorporated nucleotide was removed from the labeled probes by using a spin-column containing Biogel P4 (Bio-Rad, Hercules, CA). Hybridization was performed in the same prehybridization solution containing 5 pmol of the ^{32}P-labeled with probes specific for different groups of microorganisms (Table 1) for 16 h at 50°C. The membranes were washed twice with 2 × SSC − 0.1% SDS at hybridization temperature for 10 min. Final wash temperatures were done at each corresponding T_d (Stahl et al., 1988).

The abundance of specific groups of organisms (genera, families, orders or domains) are expressed as percentages of total 16S rRNA in the sample, or archaeal 16S rRNA, as indicated. The total 16S rRNA was quantified with a universal probe (Table 1) in combination with RNA standards. Specific 16S rRNA concentrations, were calculated from the blot intensities, using calibration curves prepared with reference series of RNAs extracted from pure cultures.

2.6. Chemical analyses

Bicarbonate alkalinity in the reactor was determined by titration to pH 5.75, at which 80% of bicarbonate ion is converted to CO_2 (pK = 6.4). At this pH, less than 20% of volatile fatty acids (VFA) will have contributed to alkalinity, because of their lower pKs. The dominant acetic acid and propionic acid have pK's = 4.7 and 4.9, respectively. The total bicarbonate alkalinity S_{TB} was calculated as

$$S_{TB} = 1.25 S_{5.75},$$

where the subscript 5.75 indicates the endpoint pH of the titration (Jenkins and Moore, 1977). Other chemical analyses were conducted in accordance with Standard Methods (APHA, 1998).

3. Results and discussion

The UASB reactor was monitored over a total period of 9 months. During this time, pH and alkalinity were used as control techniques, limiting the input organic load to the reactor accordingly, to keep its performance steady and stable. Already 2 months after the start-up, efficient degradation of wastewater was achieved. Effluent COD, as a parameter of reactor performance, decreased from values in the range of 5000–6000 mg/l at month 2 to 3600 mg/l at month 5 and 2500 mg/l at month 9.

Fig. 1 illustrates the result of the analysis of temporal changes occurring in the microbial community during start-up period performed with domain-specific probes. During the first 9-month period of the bioreactor, a decrease in the relative concentration of bacterial 16S rRNA (EUB338) from 75.9% to 49.9% occurred. Conversely, the relative concentration of archaeal 16S rRNA (ARC915) increased from 24.1% to 46.7%. As expected, the relative concentration of eucaryal 18S rRNA (EUK516) remained low (< 3%) (Fig. 1). It is possible that the domain Bacteria may have been underestimated, considering that Daims et al. observed Bacteria that do not hybridize with the probe EUB338 (Daims et al., 1999). On the other hand, overestimation of archaeal concentration may have occurred if ARC915 hybridized to some Eubacteria, as suggested recently by Pernthaler et al. (Pernthaler et al., 2002).

DGGE analysis of the samples corresponding to months 2, 5 and 9 after start-up demonstrated the presence of many distinguishable bands, derived from at least as many different bacterial species (Fig. 2). Apart from a few dominant

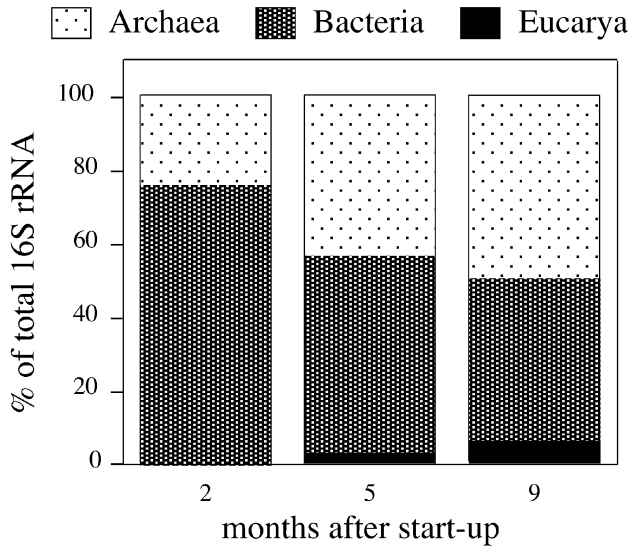

Fig. 1. Comparison of microbial composition of the samples from the UASB reactor during start-up period, as determined by dot-blot of RNA using domain-specific 16S rRNA-targeted oligonucleotides. The domain-specific DNA oligonucleotide probes used were ARC915 for Archaea (⚃), EUB338 for Bacteria (▩) and EUK516 for Eucarya (■).

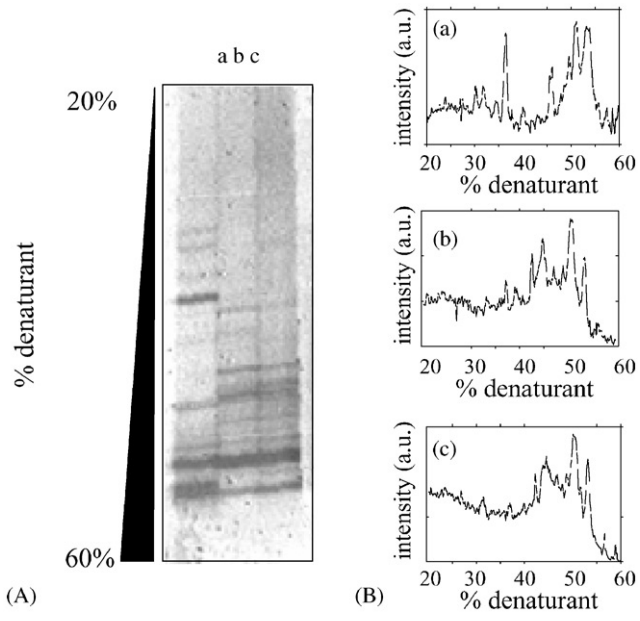

Fig. 2. Dynamics of bacterial population of the UASB reactor determined by DGGE of PCR-amplified 16S rRNA fragments: (A) DGGE of samples taken at (a) 2 months after start-up, (b) 5 months after start-up, (c) 9 months after start-up; (B) densitometric analysis of DGGE lanes from part (A). The Shannon index diversity analysis of banding patterns resulted in values of $H = 0.96, 1.06$ and 1.08, respectively.

Bacteria species, the high number of weak bands resulted in a smear, a characteristic feature of highly diverse ecosytems, e.g. activated sludge (Boon et al., 2002). The DGGE banding pattern revealed significant changes in the structure of the bacterial community, but only a slight increase in the bacterial diversity was observed. The Shannon–Weaver index of structural diversity increased from a value of 0.96 2 months after start-up to 1.06 at month 5 and to 1.08 at month 9 after start-up (Fig. 2). These results show that the functional heterogeneity needed to degrade the complex substrate composition can be achieved by highly diverse albeit different bacterial community structures .

For the quantification of the domain *Archaea* we have used groups of radioactive probes targeting different phylogenetic levels in a nested manner, such that the sum of probe conferred signals cover all known methanogens (Table 1). Fig. 3a shows that the increase in the relative activity of Archaea is accompanied by a population shift from hydrogenotrophic to aceticlastic microorganisms (MSMX860). Methanobacteriales (MB310) were the most abundant hydrogenotrophic methanogens. Methanococcales (MC1109) appeared initially at significant concentration, but decreased to very low levels at month 9 (Fig. 3a). Methanogenium relatives (MG1200) were at trace levels.

In this case a syntrophic population of acetate oxidizing organisms and hydrogenotrophic methanogens was changed to an aceticlastic population producing methane from acetate degradation. A similar switch was reported to occur in anaerobic digesters treating sewage sludge, when mixing caused disruption of spatial juxtaposition of synthropic Bacteria and their methanogenic partners (McMahon et al., 2001). In this case low levels of hydrogenotrophic methanogens were also observed in anaerobic wastewater reactors operated under changing wastewater composition and solid retention times (SRT) (Merkel et al., 1999).

Among the family Methanosarcinae, *Methanosarcina* and relatives species dominated (MS1414). Interestingly, the ratio between *Methanosarcina* and *Methanosaeta* (MX825) species remained almost invariant during the period under study (Fig. 3b). This apparent stability is likely to be a trade-off between the rapid growth rate and the broader substrate versatility of *Methanosarcina*, and the lower threshold for acetate utilization by *Methanosaeta* species. Other studies have shown that the outcome of the competition for common substrate among different microorganisms is more complex than that suggested by pure culture studies. Examples of seed complex populations include: 1) a codigester treating municipal solid waste and biosolids (McMahon et al., 2001); 2) a laboratory chemostat operating at high retention time, where *Methanosarcina* species were dominant at high acetate levels (Raskin et al., 1994); 3) the rumen, where differences in ATP yield and maintainance requirements influence the competition between microorganisms (Stahl et al., 1988).

Reactor performance was controlled by adjusting on a daily basis the organic loading rate, up to a maximum that prevented substrate overload. It was also controlled by maintaining constant alkalinity, so that consistent COD reduction was achieved without external pH regulation. We have established a link between the loading rates and the composition of microbial populations, showing that maximum

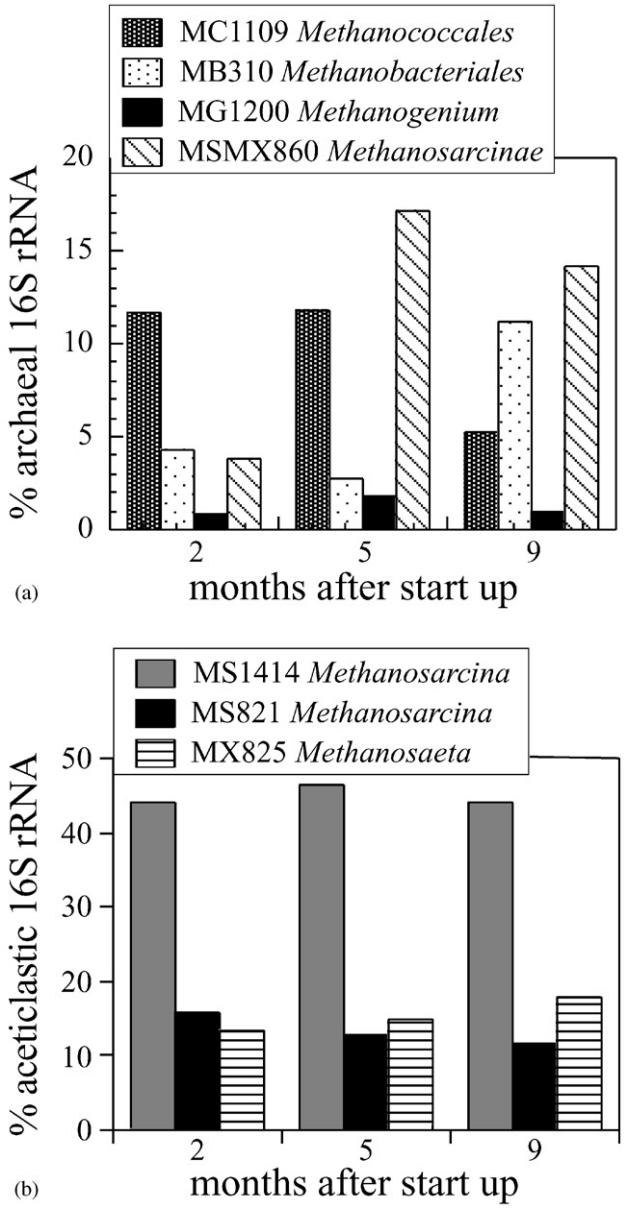

Fig. 3. Methanogenic composition of the samples from the UASB as determined by dot-blot of RNA using taxon-specific 16S rRNA-targeted oligonucleotides (see Table 1 for oligonucleotide specificity): (A) Proportion of methanogenic belonging to different families in relation to total archaeal 16S rRNA: (B) Proportion of different aceticlastic *Archaea* in relation to total aceticlastic methanogenic 16S rRNA.

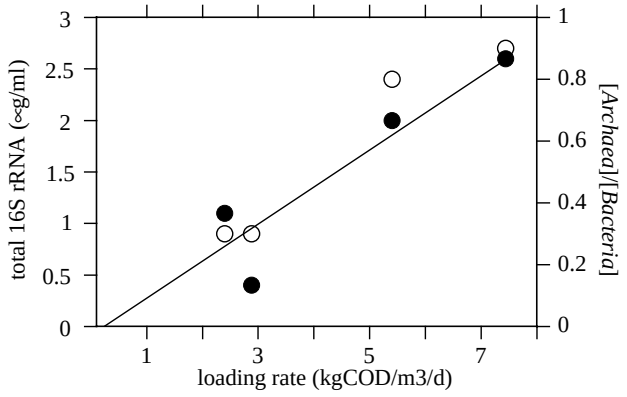

Fig. 4. Correlation between the volumetric loading rate and total 16S rRNA (open circles) and ratio of archaeal 16S rRNA/bacterial 16S rRNA). Each 16S rRNA was measured by dot-blot of RNA using the corresponding domain-specific 16S rRNA-targeted oligonucleotides.

allowable organic load correlated with both total 16S rRNA (probed with a universal probe) and the ratio between archaeal 16S rRNA and bacterial 16S rRNA (Fig. 4). No statistically significant correlation has been observed between maximum allowable organic loading rate and any of the individual archaeal taxon probed (not shown).

The rRNA content of cells is related not only to the abundance of the microorganisms, but also to the metabolic activity of microbial populations (Poulsen et al., 1993). Therefore, the loading capacity of the anaerobic wastewater treatment system is dictated by the amount of total active biomass retained in the reactor, but more importantly by the ratio between metabolically active methanogens and acidogens. This ratio reflects the concerted activity of acetogenic hydrogen-producing Bacteria and methanogenic Archaea required for anaerobic degradation of fermentable substrates. Since the average growth rate of the methanogens is much lower than that of acidogens, the overall rate of the biomethanation process is therefore controlled by the methanogenic step. At low methanogenic activity, the unstable response of anaerobic systems is due to the reduced use by methanogens of acetic acid and H_2 generated by the fermenting populations, causing accumulation of volatile fatty acids (VFA) and a sharp decrease in the pH.

On the other hand, the rate at which fatty acids are taken up by the methanogens is an inherent property of the organisms and the environmental conditions within the reactor. The uptake of VFA depends on their specific growth rate and cannot be increased beyond a certain value, setting a high limit for the organic load that can be applied to the reactor. Since acetogenic hydrogen-producing Bacteria and methanogenic Archaea have optimum microbiological environments that differ substantially from each other, optimum performance in a single-phase anaerobic reactor is difficult to achieve.

We cannot exclude the possibility that given the relatively small representation of the archaeal population and the presence of sulfate reducing Bacteria (not shown), SRB may be competing for available electrons with methanogenic microorganisms, particularly at high loading rates (Oude Elferink et al., 1994). Dairy cheese has a low sulfate content (< 0.35 ppm). However, Raskin et al. (1996) have shown that a methanogenic reactor contained up to 15% sulfate-reducing *Bacteria*, even though sulfate was not present in the influent of the reactor (Raskin et al., 1996). The major competitors for hydrogen with these SRB in the absence of sulfate were *Methanobacteriales*-species, which

were the most abundant hydrogenotrophic methanogens that we have detected in our system. A more thorough analysis of the competition between SRB and methanogenic Archaea using fluorescence in situ hybridization is currently under way.

Although the complexity of an open system precludes a rigorous quantitative analysis of microbial diversity (Godon et al., 1997), our work indicates that important changes in the structure of the microbial community may take place during the establishment of a functionally competent ecosystem. An increase in the species number during the reactor start-up suggests that the initial inoculum does not determine the structure of the microbial composition at later steps of operation. On the other hand, consistent performance can be achieved with a highly dynamic microbial structure showing little or no dependence on specific taxon at the archaeal level. Therefore, these results do not support the idea that many anaerobic wastewater treatment systems fail due to the lack of an adequate inoculum.

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