Community analysis of a denitrifying reactor treating landfill leachate

C. Etchebehere a,*, M.I. Errazquin a, P. Dabert b, L. Muxi a

a Cátedra de Microbiología, Facultad de Química y Facultad de Ciencias, Universidad de la República, General Flores 2124, Montevideo, Uruguay
b Institut National de la Recherche Agronomique, Laboratoire de Biotechnologie de l’Environnement, Narbonne, France

Received 6 August 2001; received in revised form 10 January 2002; accepted 14 January 2002

First published online 24 April 2002

Abstract

The bacterial community of a denitrifying reactor from a system for landfill leachate decontamination was studied applying cultivation methods, denitrifying activity measurements and characterisation of community 16S rDNA. The sludge presented a high denitrifying activity but a relatively low number of denitrifying bacteria as determined by most probable number. Over 50% of the sequences retrieved in the molecular analysis were related to genera with the capacity to denitrify in the α- and β-subdivisions of the Proteobacteria. Fifteen percent of the DNAs were related to not yet cultured organisms belonging to the green non-sulphur phylum. High similarity values between sequences from isolates and clones were observed.

Keywords: Denitrification; Denitrifier; Amplified ribosomal DNA restriction analysis; Community analysis; Landfill leachate

1. Introduction

The leachate generated in sanitary landfills is a highly contaminated fluid that should be treated before disposal. In the case of protein-rich solid wastes, high ammonium concentrations as well as high carbon contamination may occur, thus requiring both carbon and nutrients decontamination. A combination of the biological processes of methanogenesis, aerobic treatment and denitrification may be used to perform C and N decontamination of this waste [1]. Although denitrification is a well documented process, the compositions of the microflora of few denitrifying ecosystems have been studied, and for denitrifying reactors in particular, information is still very fragmentary. As for other physiological groups [2] it has been shown that isolation techniques only recover a low proportion of the total denitrifying population. Furthermore, the phenotypic characterisation of denitrifiers is complicated by the great diversity of bacteria showing this ability and by the fact that many of them belong to the β-subdivision of the Proteobacteria and do not have the ability to utilise sugars, resulting in low identification profiles when using commercial kits [3–5].

A molecular, cultivation-free approach has been successfully used to determine microbial community structure in complex waste water treatment systems like activated sludge [6–8], a trichlorobenzene (TCB) transforming reactor [9] or phosphate removal reactors [10]. However, limitations and pitfalls in PCR-based methods have been reported [11]. Since such methods can lead to bias in the proportion of the 16S rDNA sequences recovered, the approach must be considered qualitative rather than quantitative. In addition, it is often not possible to infer the physiological role of the organisms based only on their phylogenetic positions. To overcome these problems, a combined approach using conventional cultivation methods and molecular cultivation-free methods has been applied to anoxic-paddy soil ecosystems [12,13] and to a seawater ecosystem in the Red Sea [14].

Denitrifying activity measurements, and enumeration and isolation of culturable denitrifiers, were used in a previous study, to gain insight into the composition of the denitrifying microbiota of the anoxic reactor of a system used for C and N decontamination of landfill leachate in the city of Montevideo. In that work, cultivation methods resulted in an underestimation of the denitrifying community present in the reactor [3]. In the present study,
we focused on the same bacterial community by applying, to one sample of reactor sludge, a cultivation method for counting and isolating denitrifiers, denitrifying activity measurements and characterisation of 16S rDNA of the community. By this combined approach, we obtained a complementary view of the structural and functional composition of the community that was enriched during the operation of the anoxic reactor.

2. Materials and methods

2.1. Sludge samples

Sludge samples were obtained from an anoxic UASB denitrifying reactor (working volume 4.6 l). This reactor was part of a laboratory scale combination of three reactors – methanogenic, anoxic and aerobic – for carbon and nitrogen removal from the leachate of the sanitary landfill in the city of Montevideo [15]. During this work, the denitrifying reactor was fed with leachate and effluent from the methanogenic reactor and nitrate at a C/N ratio of 7 g COD (g N-NO$\textsubscript{3}$)$^{-1}$ for 28 months [15]. It was sampled at different times of operation: sample 1: 14 months operation; sample 2: 16 months; sample 3: 19.5 months; sample 4: 23 months; sample 5: 24 months; sample 6: 29 months. Samples 1, 2, 3, 4 and 5 were taken during full operation (mean nitrate removal 75%, with values between 60 and 90%; mean carbon removal 35%, with values between 30 and 40%). Sample 6 was taken after the reactor had not been fed for 1 month. Sample 5 (24 months of operation) was selected for the microbial characterisation.

2.2. Specific denitrifying activity tests

The specific denitrifying activity of the sludge was determined in duplicate by measuring the rate of N$\textsubscript{2}$O production after acetylene inhibition of N$\textsubscript{2}$O reduction to N$\textsubscript{2}$, as previously described [3]. Acetate (40 $\mu$mol) or raw leachate (equivalent to 6400 $\mu$g COD) were tested as substrates for the assay. Specific denitrifying activity of pure cultures was measured in duplicate using a modification of the method described by Mahne and Tiedje [16], as previously described [17].

2.3. Most probable number (MPN) count, isolation and physiological characterisation of nitrate-reducing bacteria

Denitrifiers were enumerated by MPN in a basal medium (BC) supplemented with yeast extract (0.5 g l$^{-1}$), potassium acetate (1.84 g l$^{-1}$) and potassium nitrate (0.72 g l$^{-1}$) (BCY.Acetate), as previously described [18]. Acetylene (purity: > 99.9%, AGA 10% v/v) was added to the headspace and tubes were considered positive for denitrification when accumulation of N$\textsubscript{2}$O occurred in the headspace. Denitrifiers were isolated in tryptic soy agar (Difco) from the positive tubes of the highest dilutions of the MPN enumeration. The ability of pure cultures to denitrify was confirmed in BCY.Acetate. A preliminary characterisation was performed by Gram stain, catalase and oxidase tests [19] and by commercial identification kits API 32GN and VITEK GN+ (bioMérieux). Enumeration of ammonia producing nitrate reducers (ammonifiers) was performed in a medium with glucose, tryptone and yeast extract as electron donors and carbon source, as previously described [20].

2.4. 16S rDNA analysis of the microbial community

Total DNA from sludge sample 5 was extracted, purified, and used for 16S rDNA PCR amplification with universal primers for the domain Bacteria, according to Fernandez et al. [7]. An Escherichia coli library was obtained by cloning the PCR products within a pGEM-T plasmid using TA cloning kits and blue–white screening (Invitrogen). Inserts from the positive clones were amplified using primers corresponding to the plasmid sequence and screening was accomplished by determining the ARDRA profiles of the clones using HhaI and HaeIII restriction enzymes (Amersham) [7]. Restriction patterns were normalised and compared by GelCompar software (version 4.1; Applied Maths, Kortrijk, Belgium). Pattern clustering was done by the UPGMA method applying the Dice coefficient. A maximum tolerance of 3.0 ± 0.5% was used for band positions. Clones that represented different ARDRA profiles were selected and the 16S rDNA PCR product corresponding to each clone was purified and partially sequenced. Sequences were determined with the dye-terminator cycle sequencing ready reaction kit, with AmpliTaq DNA polymerase FS (Perkin-Elmer) using internal primers. Sequence reaction mixtures were run on the ABI model 373A sequencer (Applied Biosystems, Perkin-Elmer). The 16S rDNA sequences were compared with all accessible sequences in databases using the BLAST server at NCBI (National Centre of Biotechnology Information).

The sequences were aligned with those belonging to representative organisms of the domain Bacteria. Alignments were performed using Clustal W and manually corrected using the Seq-pup sequence editor software. Only unambiguously aligned positions were used to construct an unrooted tree using DNADIST (Jukes and Cantor algorithm) and Neighbour-Joining programs contained in the PHYLYP Phylogeny Inference Package, version 3.5 [21]. A bootstrap analysis of 100 replicates was also performed using programs included in the same package. Sequences were deposited at the EMBL nucleotide sequence database with accession numbers as follows: AJ412668 for clone 112; AJ412669 for clone 19; AJ412670 for clone 23; AJ412671 for clone 31; AJ412672 for clone 3; AJ412673 for clone 44; AJ412674 for clone 87; AJ412675 for clone...
86: AJ412676 for clone 92; AJ412678 for clone 81; AJ412679 for clone 71; AJ412680 for clone 94; AJ412681 for clone 101; AJ412682 for clone 95; AJ412683 for clone 78; AJ412684 for clone 77.

2.5. Characterisation of the isolates

The bacterial isolates were differentiated by determining their ARDRA profiles. ARDRA was carried out as previously described [7], with primers specific for the 16S rRNA gene of the domain Bacteria, using HhaI and HaeIII restriction enzymes (Amersham). Strains with different ARDRA profiles were selected for 16S rDNA sequencing. Sequencing and phylogenetic analyses were performed as described previously. Sequences were deposited at the EMBL nucleotide sequence database with the following accession numbers: AJ412685 for strain H; AJ412686 for strain I; AJ412687 for strain L; AJ412688 for strain B.

2.6. Analytical procedures

N₂O was measured by gas chromatography (Chrompack CP900001) with an electron capture detector operating at 300°C, with a Porapak Q 80100 (Chrompack) column. The carrier gas was N₂ and the oven temperature was 55°C. Total N₂O content was calculated from the headspace concentration as described by Christensen and Tiedje [22]. Protein was measured using the method described by Lowry with bovine serum albumin as standard [23].

3. Results

3.1. Specific denitrifying activity and enumeration of denitrifiers in sludge sample 5

The specific denitrifying activity of sludge sample 5 was 82 ± 5 nmol N₂O ml⁻¹ min⁻¹ with acetate, and 230 ± 2 nmol N₂O ml⁻¹ min⁻¹ with leachate. The number of denitrifiers, as determined by MPN, was 2.4 × 10⁷ denitrifiers ml⁻¹. No ammonifiers were detected in the MPN count.

3.2. Molecular characterisation of the community in sample 5

A 16S rDNA library was obtained from sludge sample 5, using primers specific for the domain Bacteria. Eighty positive clones were selected and screened by determining their insert ARDRA profiles. According to the GelCompar analysis these inserts grouped in 37 different profiles, and each pattern was defined as an operational taxonomic unit (OTU). Partial 16S rDNA sequences were determined for one clone representing each OTU. For sequences showing low similarity values with the closest relatives in

Table 1: Clones affiliation according to their 16S rDNA sequence, using BLAST search at NCBI database

<table>
<thead>
<tr>
<th>OTU</th>
<th>Frequency (%)</th>
<th>Clone selected for sequencing</th>
<th>Length sequence (bp)</th>
<th>Closest taxonomic unit (NCBI accession number)</th>
<th>Similarity (%)</th>
<th>Phylum</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>12.6</td>
<td>clone 31</td>
<td>152</td>
<td>clone SJA101 (AJ009480)</td>
<td>87</td>
<td>green non-sulphur bacteria</td>
<td>15</td>
</tr>
<tr>
<td>5</td>
<td>2.5</td>
<td>clone 71</td>
<td>158</td>
<td>clone SJA104 (AJ009485)</td>
<td>88</td>
<td>β-Proteobacteria</td>
<td>40</td>
</tr>
<tr>
<td>17</td>
<td>10.1</td>
<td>clone 86</td>
<td>1579</td>
<td>R. eutropha st. JS705 (AF027407)</td>
<td>98</td>
<td>β-Proteobacteria</td>
<td>16</td>
</tr>
<tr>
<td>28</td>
<td>3.4</td>
<td>clone 94</td>
<td>379</td>
<td>Thauera selenatis (Y17591)</td>
<td>98</td>
<td>γ-Proteobacteria</td>
<td>3</td>
</tr>
<tr>
<td>18</td>
<td>0.9</td>
<td>clone 91</td>
<td>1573</td>
<td>Acidovorax sp. IMI (AJ019736.3)</td>
<td>99</td>
<td>Cytophagaceae</td>
<td>12</td>
</tr>
<tr>
<td>22</td>
<td>0.9</td>
<td>clone 81</td>
<td>1596</td>
<td>Bordetella bronchiseptica (X57026)</td>
<td>99</td>
<td>Flavobacteraeum M. bronchiseptica BX811 1</td>
<td>12</td>
</tr>
<tr>
<td>21</td>
<td>0.9</td>
<td>clone 105</td>
<td>1550</td>
<td>clone PRO-HE36 (AF314417)</td>
<td>99</td>
<td>α-Proteobacteria</td>
<td>1</td>
</tr>
<tr>
<td>30</td>
<td>5</td>
<td>clone 112</td>
<td>1573</td>
<td>Acinetobacter lwoffii (AJ005816)</td>
<td>99</td>
<td>β-Proteobacteria</td>
<td>1</td>
</tr>
<tr>
<td>31</td>
<td>3.8</td>
<td>clone 19</td>
<td>1530</td>
<td>Acinetobacter lwoffii (AF013557)</td>
<td>96</td>
<td>green sulphur bacteria</td>
<td>4</td>
</tr>
</tbody>
</table>

Clones represent 75.4% of the library. Profiles which were found only once were not included.

Clones were sequenced using BLAST search at NCBI database.

Sequences were not included.
the databases, further sequencing was performed. The distribution of the most frequent OTUs (each OTU including at least two clones within the same profile) and their phylogenetic affiliation according to their 16S rDNA sequence are shown in Table 1. A dendrogram, constructed with the sequences of the clones representing the different OTUs, with lengths higher than 500 bp, and of their closest relatives, is shown in Fig. 1.

To test the method of clone screening by ARDRA profiling, two clones with identical profiles, affiliated with the green non-sulphur phylum, and two clones with identical profiles, affiliated with the β-Proteobacterium subdivision, were sequenced. In both cases the divergence in sequences for the clones with the same ARDRA profile was less than 0.5%, showing that clone screening by ARDRA profiles did not underestimate rDNA diversity. Similar results were previously reported for the screening of clones from a methanogenic reactor [7].

3.3. Characterisation of the denitrifying isolates

During operation of the reactor, 16 denitrifying bacterial strains were isolated from the highest dilutions of the MPN enumeration of six samples of the sludge, taken at different times as indicated in Table 2. All the isolates were Gram-negative rods and presented positive results in oxidase and catalase tests. The conventional identification performed by API 32GN or VITEK GN+ (bioMérieux)
identification kits presented profiles of low acceptability (data not shown). The distribution of the ARDRA profiles of the isolates and the affiliation to the different phyla according to the 16S rDNA sequence based phylogenetic analysis are also shown in Table 2. Some of the denitrifying bacterial strains were described in previous reports [3,17], and are included in this work to present a complete overview of the community. Strains 23310, K and 6 were previously described as Comamonas nitrativorans, a new species of Comamonas with the ability to denitrify [17]. In this work we present the characterisation of strains B, C, D, F, H, I, L and 2477 by ARDRA and 16S rDNA phylogenetic analysis (Table 2, Figs. 2 and 3). Most of the isolates presented high denitrifying activities when tested in acetate and nitrate (values ranging between 42% to 98% of N₂O (g protein⁻¹ min⁻¹) except for strains H, I and N which showed a very low rate of N₂O production, not measurable in the conditions of the assay. In tryptic soy broth (TSB), the specific denitrifying activity of strains H, I and N was 70% to 70% of N₂O (g protein⁻¹ min⁻¹), respectively. No organisms capable of dissimilatory nitrate reduction to ammonia or fermenting bacteria were detected by the cultivation methods in any of the six samples.

4. Discussion

In this work denitrification rate measurements, cultivation-dependent counting and isolation and molecular 16S rDNA inventory were applied to the same denitrifying community. The combined molecular and conventional cultivation approach gave a more complete view of the composition of the community. Although molecular methods were applied to evaluate the communities from methanogenic, phosphate removal, and activated sludge wastewater treatment systems, this was, to our knowledge, the first time that a community from a continuously anoxic denitrifying reactor was evaluated by 16S rDNA molecular methods.

Both the molecular and cultivation-based methods suggested that a physiologically specialised community developed in the reactor under study. More than 50% of the sequences retrieved by the molecular cloning methods were related to genera with the capacity of denitrify (Ralstonia, Thauera, Comamonas, Azotobacter in the β-subdivision, and Paracoccus and Bradyrhizobium in the α-subdivision of Proteobacteria; Table 1). Furthermore, sludge sample 5 showed a higher specific denitrifying activity with acetate as substrate than did either activated sludge samples [24] or methanogenic sludge [3]. The number of denitrifiers, as determined by MPN, and the specific denitrifying activity were similar to those previously reported for samples taken from the same reactor at other times and from other denitrifying reactors in full operation [3]. In addition, no bacteria able to perform dissimilatory ni-
trate reduction to ammonia were detected by the molecular or cultivation techniques, suggesting that the community was mostly enriched with denitrifying microorganisms during the operation of the reactor. These results were in agreement with the data on reactor performance that indicate that the carbon flow occurred mostly via denitrification [15]. On the other hand, the fact that the rate of denitrification was higher for leachate than for acetate suggests that the number of organisms with the ability to use acetate was lower than the number of denitrifiers that use other substrates in the leachate. As the enumeration was performed in a medium with acetate, the method may have resulted in an underestimation of the denitrifying population.

The 16S rDNA approach clearly showed the high diversity of the microbial composition of this particular ecosystem and suggests the prevalence of the β-Proteobacteria (40% of the clones).

4.1. Organisms affiliated to β-subclass of Proteobacteria

Nine different OTUs were affiliated with the β-Proteobacteria subdivision (Table 1, Fig. 1), within the genera Thauera, Comamonas, Acidovorax, Azoarcus, Bordetella and Ralstonia. Cloned DNAs belonging to this phylum were also reported as making the predominant group in phosphate-removing and non-phosphate-removing activated sludge libraries from SBR reactors [10] and from a library obtained from an intermittently aerated activated sludge with a high nitrifying-denitrifying capacity [25]. Fourteen out of the 16 strains isolated at different sampling times were also affiliated with the β-subdivision of Proteobacteria, and presented a relatively high diversity (eight profiles in 14 isolates). Strain B was very closely related to Brachymonas denitrificans, a denitrifier positioned in the family Comamonadaceae within the β-subdivision of Proteobacteria [26] (Table 2 and Fig. 2). Strains H, N and I grouped within the Alcaligenaceae family, but the 16S rDNA similarity between the sequences and their closest relatives was not high enough to infer a good identification (Table 2). The three strains showed a very low denitrification rate for acetate as electron donor, which could not be determined in the conditions of the assay. However, in TSB the denitrification rates were similar to
those previously determined for other *Alcaligenes* strains [16]. These isolates were retrieved from samples taken 10 and 4 months before the one used for the molecular analysis. No clones were affiliated with this family, indicating that probably they did not persist in the reactor in an amount to be detected by the molecular techniques. The low denitrification rate from acetate may explain the inability to persist in the reactor.

The phylogenetic tree (Fig. 2), constructed including the sequences of the clones and isolates of the β-subdivision of *Proteobacteria*, showed high similarity values between strains and clones. Several sequences, both of clones and isolates, were positioned in the *Comamonadaceae* family, indicating a prevalence of organisms affiliated with this family in the reactor. Clone 86 showed 97.0% similarity with strain 23310, which had an identical ARDRA profile to that of strain 2476, isolated from sludge sample 5. These strains were previously described as members of a novel denitrifying species, *C. nitrativorans* [17]. Several sequences from clones and isolates were affiliated to the genus *Thauera* indicating a prevalence of organisms belonging to this genus in the reactor. In particular, clone 23 presented high similarity with strains 1917 and O (99.2 and 97.3%, respectively), affiliated with the genus *Thauera* [3]. On the other hand, none of the isolates grouped with the most frequent OTU within the β-*Proteobacteria* (OTU 17, represented by clone 87) related to *Ralstonia eutropha* (Table 1). This indicates that the organisms retrieved as the most abundant by the cultivation methods (present in a number of 10⁶–10⁷ per ml according to the MPN count) were not the same as represented by the most frequent clones detected in the sample.

4.2. Organisms affiliated to the α-subdivision of *Proteobacteria*

Four clones (16% of the library), representing four different OTUs, and two isolates (strains C and L) with the same ARDRA profile (S3) were affiliated with the α-subdivision of *Proteobacteria*. Clone 112 clustered with the *Rhodobacter* group, related to the environmental clone 49523, retrieved from activated sludge, and presented 94.4% sequence similarity with strain L. Strain L was very closely related (Table 2, Fig. 3) to *Paracoccus versutus*, suggesting that it may belong to this species. Members of the genus *Paracoccus* are frequently isolated denitrifiers.

![Phylogenetic Dendrogram](https://example.com/phylogenetic_tree.png)

Fig. 3. Phylogenetic dendrogram showing the position of the clones and isolate sequences in the α-subdivision of *Proteobacteria*. The dendrogram was constructed using 508 unambiguously aligned positions (from position 539 to 1042 according to *E. coli* numbering). The scale bar represents 10 nucleotide substitutions per 100 nucleotides. The sequence from *Comamonas terrigena* (AF078772) was used as outgroup. The numbers next to the nodes represent the bootstrap values in 100 replicates (only values of 70 and above are shown).
The other three clones in the α-subdivision (92, 78 and 77, Table 1), representing 11.3% of the library, were affiliated with the *Rhizobiales* family, related to the genera *Bradyrhizobium*, *Afipia* and *Mesorhizobium*. These organisms are frequently isolated nitrogen-fixing organisms, however their ability to denitrify is also well documented [27].

4.3. Organisms affiliated to the green non-sulphur bacteria and green sulphur bacteria

Fifteen percent of the clones, represented by two OTUs, were affiliated with the green non-sulphur phylum and one OTU (number 3) comprising 4% of the clones, grouped with the green sulphur bacteria (Fig. 1). However, no denitrifiers with sequences belonging to these phyla were isolated, either because these microorganisms are not easily recovered by cultivation methods or because they do not have the ability to denitrify. In particular, clone 31, representing the most frequent OTU (number 4) in the library, clustered in a sub-branch of the green non-sulphur phylum which includes a high number of environmental clones and only two cultured bacteria, *Dehalococcoides ethenogenes*, capable of reductive dechlorination of chloroethene [28], and the recently isolated strain CBDB1, a highly specialised bacterium that dechlorinates chlorobenzenes [29]. Several environmental clones retrieved from anoxic ecosystems which perform reductive dechlorination [9,30,31] are positioned in this phylogenetic sub-branch. The level of 16S rDNA sequence similarity between the sequence of clone 31 and the sequence of the closest relative in the phylogenetic analysis, clone SHA 105, was low (85.3%), indicating a moderately distant relationship of these organisms (Fig. 1). It has been suggested that organisms represented by sequences positioned in this branch within the green non-sulphur division may play an important role in the degradation of chlorinated compounds [9]. Nothing is known about the role of the organisms affiliated with this phylum detected in the denitrifying reactor sludge, nevertheless a role in the decontamination of recalcitrant compounds could be suggested. Although the chemical composition of the leachate used in this work was not determined, previous reports show the presence of aromatic recalcitrant compounds in landfill leachate [32]. More physiological studies on the sludge and on the isolated strains are necessary to confirm this hypothesis.

Within the green sulphur phylum, clone 19 was closely related to clone PHO-HE36, retrieved from a reactor performing phosphate and nitrate removal [33], suggesting that these sequences belong to microorganisms with a role in denitrifying ecosystems (Fig. 1).

4.4. Organisms affiliated to the γ-Proteobacteria

Surprisingly, no sequences belonging to the γ-Proteobacteria were retrieved either by the molecular or the cultivation approach, suggesting the absence of organisms belonging to this phylum in the community, in an amount to be detected by the techniques used. In particular no sequences related to the genus *Pseudomonas* were found, indicating the difference between the denitrifying microbiota present in this reactor and the most frequently isolated denitrifiers from soil [34]. Furthermore, the overall composition of the microflora, as determined by the molecular approach, revealed differences with activated sludge, where clones representing the α-, γ- and ε-subdivisions as well as clones corresponding to Gram-positive bacteria and members of the phyla *Chlamydia* and *Planctomycetes* [6,35] were also reported.

Interestingly, several clones positioned in different branches of the phylogenetic tree presented sequences closely related to sequences retrieved from a TCB transforming reactor (SJA, SHA clones) [9,31] (Fig. 1). Although nothing is known about the physiology of the bacteria represented by the clones, the environmental conditions of the latter reactor suggest that these organisms may play an important role in decontamination of recalcitrant compounds such as TCB. The presence of isolated strains related to clones retrieved from this TCB transforming reactor was previously reported for samples from the same leachate-treating reactor [3].

The results of the present work indicate that a diverse denitrifying community developed in the reactor under study, in spite of the stable function shown during a long period of operation [1,15]. This may be explained by possible changes in the composition of the influent, due to the variability in the degree of fermentation of the leachate, and in the composition of the municipal wastes.

Although sequences with high similarity between clones and isolates were retrieved, the phylogenetic position of the clones did not exactly match that of the strains, even for those isolated from the same sample used for the molecular analysis. This fact may be due to bias in the 16S rRNA-based identification method (DNA polymerase errors, bias in sequencing methods, the presence of several copies of 16S rRNA genes, [36–39]) or to the existence of a highly phylogenetically related population in the ecosystem. This situation has been reported for other environmental ecosystems, and it has been suggested that highly related populations could coexist by adaptation to environmental parameters [7,40]. Further research is needed to determine if the observed differences in sequence between clones and isolates are due to the existence of numerous highly related organisinal populations or to diversity of 16S rRNA operons. As functional genes show higher evolutionary rates than 16S rRNA, an analysis of the diversity of nitrite reductase-encoding genes [41] in the ecosystem may contribute to evaluate diversity of phylogenetically closely related organisms. Such research will contribute...
to elucidate the role of microdiversity on functional stability of reactors.

Acknowledgements

This investigation was financially supported by International Foundation for Science (IFS), by PEDECIBA-Quimica (a foundation for the development of basic science in Uruguay) and by CONICYT (Consejo Nacional de Investigación Científica) fondo Clemente Estable.

References


