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Monitoring the Bacterial Community Dynamics in a Petroleum Refinery Wastewater Membrane Bioreactor Fed with a High Phenolic Load

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The phenolic compounds are a major contaminant class often found in industrial wastewaters and the biological treatment is an alternative tool commonly employed for their removal. In this sense, monitoring microbial community dynamics is crucial for a successful wastewater treatment. This work aimed to monitor the structure and activity of the bacterial community during the operation of a laboratory-scale continuous submerged membrane bioreactor (SMBR), using PCR and RT–PCR followed by denaturing gradient gel electrophoresis (DGGE) and 16S rRNA libraries. Multivariate analyses carried out using DGGE profiles showed significant changes in the total and metabolically active dominant community members during the 4-week treatment period, explained mainly by phenol and ammonium input. Gene libraries were assembled using 16S rDNA and 16S rRNA PCR products from the fourth week of treatment. Sequencing and phylogenetic analyses of clones from the 16S rDNA library revealed a high diversity of taxa for the total bacterial community, with predominance of *Thauera* genus (ca. 50%). On the other hand, a lower diversity was found for metabolically active bacteria, which were mostly represented by members of Betaproteobacteria (*Thauera* and *Comamonas*), suggesting that these groups have a relevant role in the phenol degradation during the final phase of the SMBR operation.

Keywords: Sludge, bacterial community, DGGE, 16S rRNA gene library, multivariate analysis

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Phenol and its derivatives are known to be the most common toxic environmental pollutants found in many industrial effluents, such as cellulose source plants, industrial resin manufacturing, petrochemical, pharmaceutical, plastic, and varnish industries [8, 39]. These compounds are toxic to animals at relatively low concentrations (5–25 mg/l) [6, 41]. Recent environmental regulations have become more and more restrictive, making the efficient removal of such compounds from industrial aqueous effluents of great concern [41]. Biological treatments have been the most employed strategy for phenol removal in effluents, mainly because of their relatively low costs when compared with physicochemical methods and also of their potential to promote the complete mineralization of such toxic compounds [39].

It is well known that the degradation efficacy of a biological treatment depends on the stability of the microbial community involved in the decontamination process [24]. Recently, bacterial phenol degradation in bioreactor conditions has been successfully demonstrated by several authors [1, 4, 7, 33]. However, despite the great relevance of biological treatments to industrial wastewater plants, most of the related studies have focused mainly on treatment performance and associated parameters, without giving proper attention to the microbial community involved. The understanding of the ecology, structure, and dynamics of those populations are major points for the success of the wastewater treatment and may provide a basis for future application of methods for *in situ* analyses of the function of specific microorganisms, helping to establish the links between microbial biodiversity and process stability in wastewater treatment plants [22].

Methods based on 16S rDNA analysis provide extensive information about the composition, spatial distribution, and relative abundance of taxonomical groups present in a specific environment [32]. Fingerprint techniques, such as denaturing gradient gel electrophoresis (DGGE), have proved to be fast and straightforward tools for monitoring shifts in microbial communities submitted to pollutant impact, and can be used to evaluate the genetic diversity and the temporal or spatial dynamics of the community [25].

This study aimed to monitor the dynamics of the bacterial community present in a submerged membrane bioreactor (SMBR) fed with high phenolic load, by using PCR and RT–PCR followed by DGGE, in an attempt to establish correlations between shifts in bacterial community and the phenolic stream and evaluate a possible impact on specific populations. The assembly and analysis of 16S clone libraries were subsequently employed for a detailed characterization of the bacterial community composition.

MATERIAL AND METHODS

Membrane Bioreactor and Operation

Experiments were carried out in a laboratory-scale continuous submerged membrane bioreactor (SMBR), consisting of an acrylic tank with volume of 4.4 l with a polyetherimide hollow fiber membrane skein (2.78×10^{-2} m^2 area) inserted. Filtration was alternated with air backwashing in order to keep the permeate flux constant. Air was used as the oxygen source, supplied through a porous stone diffuser placed in the bottom of the tank. Further details on the membranes, equipment, and operating conditions were reported elsewhere [38]. Before seeding the bioreactor, sludge was sampled from a Petrobras oil refinery in Brazil, and settled to achieve the total suspended solids concentration of 10 g/l. Acclimatization of the sludge took place for 30 days, when the SMBR processed the oily stream (OS) only. After the acclimatization step, phenolic wastewater (PW) was added to the oily stream (1:6, PW:OS), to increase the concentration of recalcitrant compounds, resulting in an average phenol feed of 86±2 mg/l, as previously described in Viero *et al.* [38]. The oily wastewater was pre-treated in a sand filter to reduce the free oil and grease content and, further, sodium tripolyphosphate was added as a phosphorus source to improve the microbial activity. Both wastewater and sludge were sampled in the same oil refinery.

Sampling and Nucleic Acid Extraction

Sludge samples were collected weekly during the 30-day period of the SMBR operation. The four sludge samples were collected in triplicate using sterilized microtubes and submitted to co-extraction of DNA and RNA by the TRIzol method (Invitrogen, Grand Island, NY, U.S.A.), according to the manufacturer's instructions. Aliquots of 150 µl of each nucleic acid sample were treated with DNase (Fermentas, U.S.A.) for 1 h at 37°C, in order to remove contaminant DNA for RT–PCR assays. The remaining nucleic acid solution, 50 µl, was used in PCR reactions for subsequent analysis of total bacterial community.

PCR and RT–PCR Amplification

PCR and RT–PCR targeting 16S rDNA and rRNA, respectively, were performed using the universal primers 968f (attached to a 40-nucleotide GC-rich sequence) and 1401r [13], which are homologous to conserved bacterial 16S rDNA regions. Reverse transcription of 16S rRNA and subsequent PCR amplification were performed after checking the absence of DNA in the total RNA extract. The first cDNA strand was synthesized in a 50-µl reaction containing 0.1 mM dNTP mix, 7.5 U of SuperScript (Invitrogen, Grand Island, NY, U.S.A.), 5 µl of 10× buffer (GIBCO-BRL, Grand Island, NY, U.S.A.), 25 pmol of p(dN)$_6$ random hexamer primers (GE Healthcare, U.S.A.), and 2 µg of extracted RNA. The reaction was incubated at 42°C for 1 h, followed by 1 min at 70°C, to promote the enzyme denaturation. The RT–PCR and PCR amplifications were performed in 50-µl reactions containing 1 µl of cDNA and 30 ng of DNA, respectively, 5 µl of 10× Tris-HCl reaction buffer, 1.5 mM MgCl$_2$, 0.4 µM primers 968f and 1401r, 0.2 mM dNTP mix, and 2 U *Taq* DNA polymerase (Invitrogen, Grand Island, NY, U.S.A.). The PCR amplifications were done using an initial denaturation step of 5 min at 94°C, 10 cycles of 1 min at 94°C, 30 s at 58°C, decreasing 1°C each cycle, and 2 min at 72°C, followed by another 25 cycles of 1 min at 94°C, 30 s at 53°C and 2 min at 72°C. DNA-free RNA was used as the control in the PCR reactions to check the presence of contaminating DNA in the RNA preparations. The amplicons were first checked on 1.2% agarose gels prior to DGGE analyses.

DGGE and Statistical Analyses

DGGE analysis of RT–PCR and PCR products was carried out in the D-Code Universal Mutation Detection System (Bio-Rad, U.S.A.) using a linear denaturing gradient of urea and formamide ranging from 45% to 65% [100% denaturant corresponding to 7 M urea and 40% (v/v) deionized formamide]. Gels (6% polyacrylamide) containing about 250 ng of the PCR or RT–PCR products for each sample, in triplicate, were run at 50 V and 60°C for 16 h in 1× TAE buffer. Gels were stained with SYBR Green 1× solution and documented under UV light.

DGGE patterns were analyzed using GelCompar v. 4.1 (Applied Maths, Kortrijk, Belgium). Gels were normalized and UPGMA-based dendrograms constructed from Pearson (product-moment) correlation coefficient matrices [26]. In addition, band relative surfaces were estimated and used to allow comparison between different samples.

The multivariate analyses were conducted using Canoco (Canoco v. 4.5; Biometrics, Wageningen, The Netherlands). Input data consisted of combining the table containing band relative surfaces with the table containing values for chemical compound removal. Bands were considered as bacterial groups and the relative areas as the frequency of group occurrence. The chemical removal percentages for phenol, chemical oxygen demand (COD), and ammonium (NH_4^+-N) were determined for the same SMBR samples and used to construct the table of environmental factors.

Prior to multivariate analyses, bacterial group tables were generated from DGGE patterns based on DNA and RNA. They were then subjected to a detrended correspondent analysis (DCA) to estimate the data distribution and choose the appropriate model for further correlations between bacterial groups and chemical data. Values of data distribution around 3.0 led to the choice of the redundancy analysis (RDA) and values above 4.0 led to the choice of canonical correlation analysis [2, 35].

16S rDNA and 16S rRNA Libraries and Sequencing

Two libraries, 16S rDNA and 16S rRNA, were constructed for the fourth-week bioreactor samples. The 16S rDNA library was constructed following the amplification of partial 16S rRNA genes from total community DNA, using 27f and 1100r primers [20]. The PCR products were gel-purified using GFX PCR DNA and the Gel Band Purification Kit (GE Healthcare, U.S.A.), according to the manufacturer's recommendations, and used for cloning. The 16S rRNA library was constructed using the region excised from the RT–PCR–DGGE gel that showed the greatest number of dominants bands. Briefly, one block of acrylamide gel containing fragments of interest were excised and DNA was extracted by using the "crush and soak" method [28]. Pellets were suspended in 15 µl of Milli-Q water for subsequent PCR and cloning, using 968f and 1400r primers, without CG-clamp.

After purification, 16S rDNA- and 16S rRNA-derived PCR products (50 ng) were ligated into pGEM T-Easy Vector (Promega, Madison, WI, U.S.A.) according to the manufacturer's instructions and transformed into Escherichia coli JM109 competent cells. The 16S rRNA gene inserts were amplified from selected clones using the universal M13 forward and reverse primers. The PCR products were purified as previously described for subsequent automated sequencing in the MegaBACE DNA Analysis System 1000 (GE Healthcare). The sequencing was carried out using M13 primers and the DYEnamic ET Dye Terminator Cycle Sequencing Kit for automated MegaBACE 500 system (GE Healthcare), in accordance with the recommendations of the manufacturer.

Phylogenetic Analysis

Partial 16S rRNA gene sequences obtained from clones were assembled in a contig using the phred/Phrap/CONSED program [11, 12]. The identification was achieved by comparing the contiguous 16S rRNA gene sequences obtained with data from reference and type strains, as well as environmental clones, available in the public databases GenBank (http://www.ncbi.nlm.nih.gov) and RDP (Ribosomal Database Project - Release 10; http://rdp.cme.msu.edu/) using BLASTn and Classifier routines, respectively. The sequences were aligned using the CLUSTAL X program [36] and analyzed with MEGA software Version 4.0 [34]. Evolutionary distances were derived from the sequence-pair dissimilarities, calculated as implemented in MEGA using the DNA substitution model reported by Kimura [19]. The phylogenetic reconstruction was done using the neighbor-joining (NJ) algorithm [27], with bootstrap values calculated from 1,000 replicate runs, using the routines included in the MEGA software.

GenBank Accession Numbers

Sequences obtained from the clone libraries were deposited in the GenBank database under the accession numbers FJ438995 to FJ439118.

Analytical Methods

Standard Methods [31] procedures were used to measure COD (chemical oxygen demand) and ammonium concentration (NH_4^+-N). TOC (total organic carbon) determination was performed in a TOC analyzer (Model 5000, Shimadzu, Japan). Phenol concentrations were
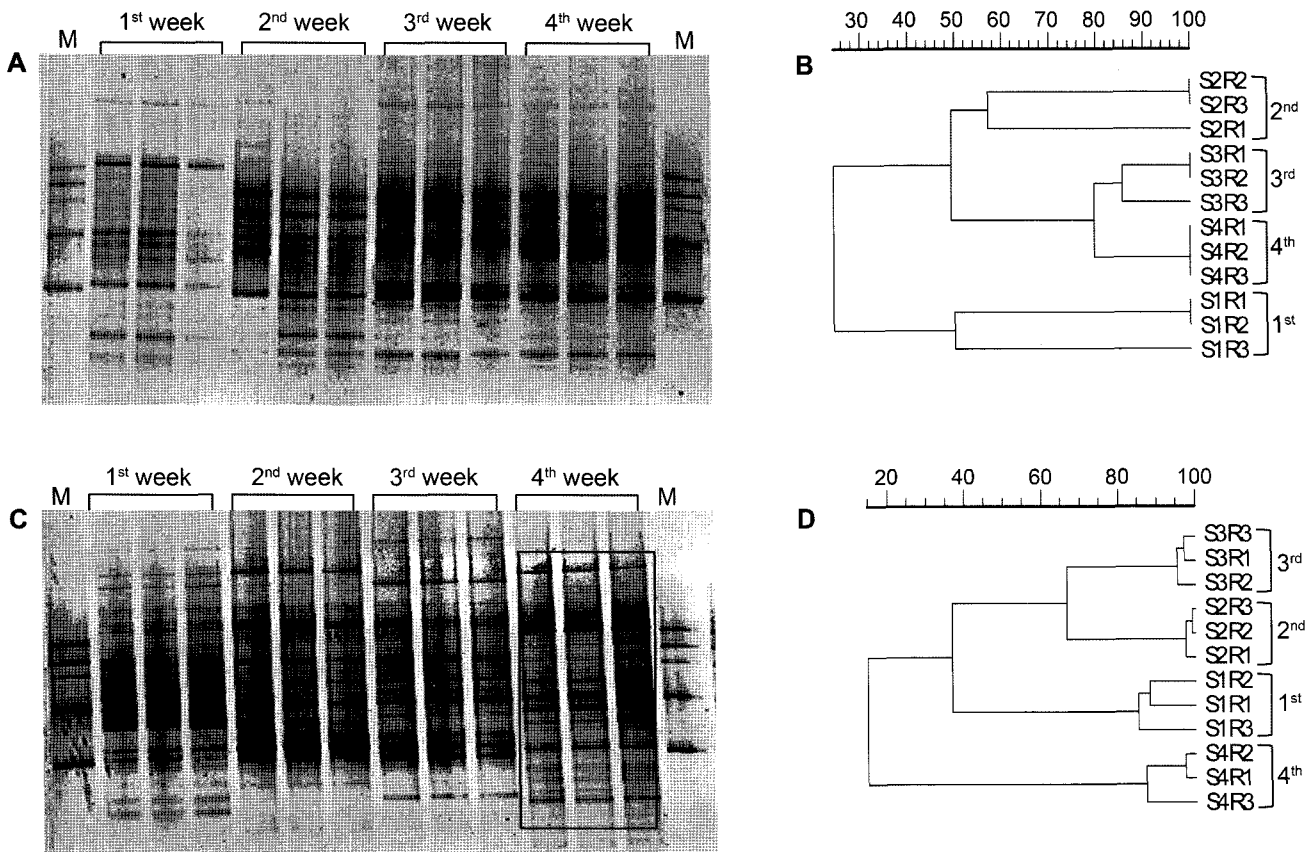

Fig. 1. UPGMA clustering by pattern similarity of DGGE bands obtained from MBR sludge samples during four weeks with phenolic feeding. A. 16S rDNA; B. 16S rRNA. Lanes: M ladder. Square: DGGE prominent bands excised for library construction of the metabolically active bacterial community.

analyzed with R-8012 kits supplied by Chemetrics, Inc. The analyses were performed weekly.

RESULTS

The structure and activity of dominant populations of the SMBR sludge were monitored, respectively, by PCR–DGGE and RT–PCR–DGGE during 30 days of bioreactor operation with high phenolic load feed. The extraction of nucleic acids and PCR amplifications, including RT-PCR, were successful for all samples. RNA yielded no PCR products when subjected to amplification reaction, indicating the absence of residual DNA in the RNA preparations (data not shown).

The DGGE profiles revealed pronounced changes in the diversity of total and metabolically active bacterial communities during the evaluated period (Fig. 1A and 1C). Cluster analyses of total community fingerprints yielded two main clusters presenting <30% similarity with each other (Fig. 1B). The first cluster corresponded to the 1st-week replicate samples, and the second group corresponded to the 2nd-, 3rd-, and 4th-week samples. The 2nd week samples showed around 50% similarity with the 3rd- and 4th-week samples, which in turn showed 80% similarity between each other, revealing a progressive differentiation of the bacterial populations along the bioreactor operation period (Fig. 1A and 1B).

The DGGE fingerprints of the metabolically active community revealed three main clusters (Fig. 1D). The first cluster was formed by the samples of the 2nd and 3rd

Table 1. Percentage of chemical removal in the SMBR bioreactor evaluated during the four weeks of the experiment.

Removals	1st week	2nd week	3rd week	4th week
Ammonium	75.0%	45.5%	40.0%	72.0%
Phenol	99.6%	99.5%	99.6%	98.9%
COD	61.0%	61.2%	65.6%	68.2%
TOC	78.5%	70.2%	70.0%	75.3%

weeks, which clustered with 66% similarity. The 1st-week replicate samples formed the second cluster, which showed 35–40% similarity with the first cluster. The third cluster was formed by the 4th-week samples, which showed <20% similarity with the other two clusters. The band profiles observed in the RT–PCR–DGGE gel indicated a different dynamics for the metabolically active populations and more profound changes along the 4 weeks of monitoring, based on similarity values, when compared with the total bacterial populations of the sludge samples.

According to the results of COD, TOC, ammonium, and phenol analyses, for feed and permeate samples, the SMBR biomass showed good efficacy in the removal of compounds, especially phenol, present in the wastewater during a 30-day operation (Table 1). Canonical ordination was applied in order to evaluate our hypothesis that the high phenol load might be determining the variations observed for both total and metabolically active bacterial populations in the SMBR. Values of DCA, 2.87 and 3.17, for DNA- and RNA-based DGGE, respectively, revealed the linear distribution of data, leading to the application of RDA as the best model for multivariate analysis [35].
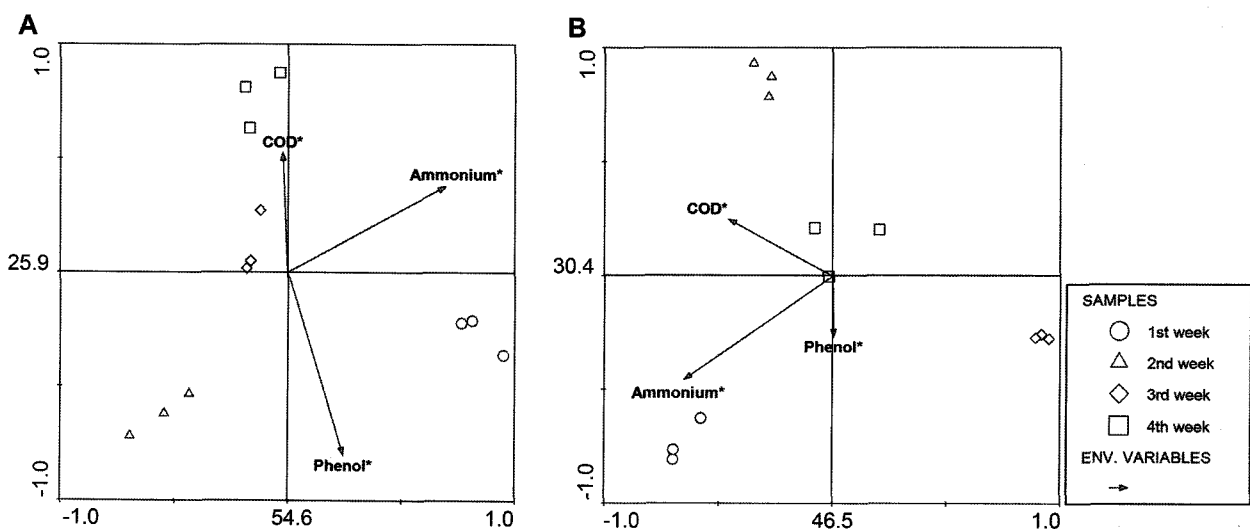

Fig. 2. Redundancy analyses (RDA) based on DNA (**A**) and RNA (**B**) DGGE patterns correlations with the removing of chemical compounds.

Values in axes indicate the percentage of species-variable correlation. Chemical quantifications are shown (environmental variables), and the indication with * mark these significant factors according to the Monte Carlo permutation test with 499 unrestricted permutations under full model selection. COD means chemical oxygen dissolved.

RDA showed that NH_4^+-N, phenol, and COD were the major factors explaining data variation. Higher NH_4^+-N and phenol concentrations were mainly related with total and metabolically active communities of the 1st week. Higher COD factor was related with the total community of the 3rd and 4th weeks and also with the metabolically active community of the 1st and 2nd weeks (Fig. 2).

The Monte Carlo permutation test, based on the RDA model, showed that p values for the removal of phenol, ammonium, and COD were significant in both DNA- and RNA-based DGGE analyses (Table 2). Lambda1 values indicated that, in DNA-based analysis, environmental factors explained data variation as follows: ammonium>phenol>COD. In the case of RNA-based analysis, the variation was explained as ammonium>COD>phenol (Table 2).

The 16S rDNA and 16S rRNA libraries, assembled from DNA and RNA templates, respectively, from the 4th-week samples, allowed the identification of the bacterial populations selected in the last phase of the SMBR operation. A total of 117 clone sequences of the 16S rDNA library presented good quality for subsequent sequence alignment and phylogenetic inference. BLASTn analyses revealed that clones were affiliated to the phyla Bacteroidetes (2.5%), Nitrospirae (2.5%), and Proteobacteria (95%), the latter being represented mainly by the class Betaproteobacteria (ca. 83% of all clones). The betaproteobacteria were affiliated to the orders Rhodocyclales, Nitrosomonadales, Hydrogenophilales, and Burkholderiales. The first one was the most abundant (56% of all clones) and exclusively represented by Thauera spp.-related clones (Fig. 3A).

For the 16S rRNA library, 80 clones were subjected to phylogenetic analyses, which showed relationships with the phyla Planctomycetes, Bacteroidetes, and Proteobacteria. Similarly to 16S rDNA library results, the clones were mainly affiliated to the class Betaproteobacteria (61.3%), which was represented by the orders Rhodocyclales and Burkholderiales. Clones belonging to the order Rhodocyclales were related to the genus Thauera (20% of all clones) and the ones belonging to the order Burkholderiales were related to Comamonas spp. (29%). The phylum Bacteroidetes was also well represented, accounting for 25% of all clones in the 16S rRNA library (Fig. 3B). The comparison of the

bacterial composition between the two libraries revealed that, except for a few clones affiliated to Planctomycetes, the taxonomic groups observed in the 16S rRNA library consist of a subset of the 16S rDNA library (Fig. 4).

DISCUSSION

Biological treatment has been usually preferred for large-scale phenol removal in wastewaters, since it allows for phenol degradation to innocuous low molecular weight compounds. In the petrochemical industry and oil refineries, the presence of high concentrations of phenol reduces significantly the biological degradation of the other components, in addition to the significant environmental toxicity hazard [6].

In this study, it was verified that a SMBR biomass was able to degrade almost all phenol added in the bioreactor. Studies have revealed that the use of the membrane bioreactor process (MBR) has been effective in the biological treatment of wastewater with high concentration of phenol [1, 6]. The main advantage of MBR is that it can achieve complete sludge retention (including the floating populations), resulting in high sludge concentrations and long sludge retention times (SRTs) [42].

Molecular approaches based on PCR have helped in the elucidation of population dynamics and the development of mechanisms to improve the control of the biological process [16, 21, 23]. In this study, the monitoring of the SMBR biomass was done by DGGE fingerprints, which clearly distinguished between the profiles of total and metabolically active communities, assessed by DNA and RNA, respectively. The results suggested a greater change for the active populations during phenol degradation, probably because the responses at the RNA level are more rapid and have greater amplitude than those at the DNA level [10]. Similar results were found by Ibekwe and Lyon [15], who observed distinct clustering of DNA- and RNA-derived DGGE profiles when evaluating alterations in the bacterioplankton community composition of a drinking water aquifer.

Redundancy analysis was applied in order to test the correlation between community structure and physicochemical factors of the bioreactor. COD, phenol, and NH_4^+-N removal results were shown to be significantly related with the changes in the bacterial communities. The phenol and NH_4^+-N were related mainly with the total and active bacterial communities of the 1st week. Possibly, during this week, the bacterial populations had to cope with the initial selective pressure owing to high load of phenol and the presence of NH_4^+-N in the influent. During the other three weeks, the populations were progressively differentiated, what might reflect a gradual acclimation to the new conditions of the bioreactor.

Table 2. Significance of chemical compounds variation on the diversity of the bacterial community estimated by multivariate analysis.

	DNA-based		RNA-based	
	Lambda1	p value	Lambda1	p value
Phenol	0.21	0.002*	0.16	0.030*
Ammonium	0.30	0.006*	0.33	0.018*
COD	0.16	0.002*	0.24	0.002*
TOC	0.30	0.422	0.26	0.098

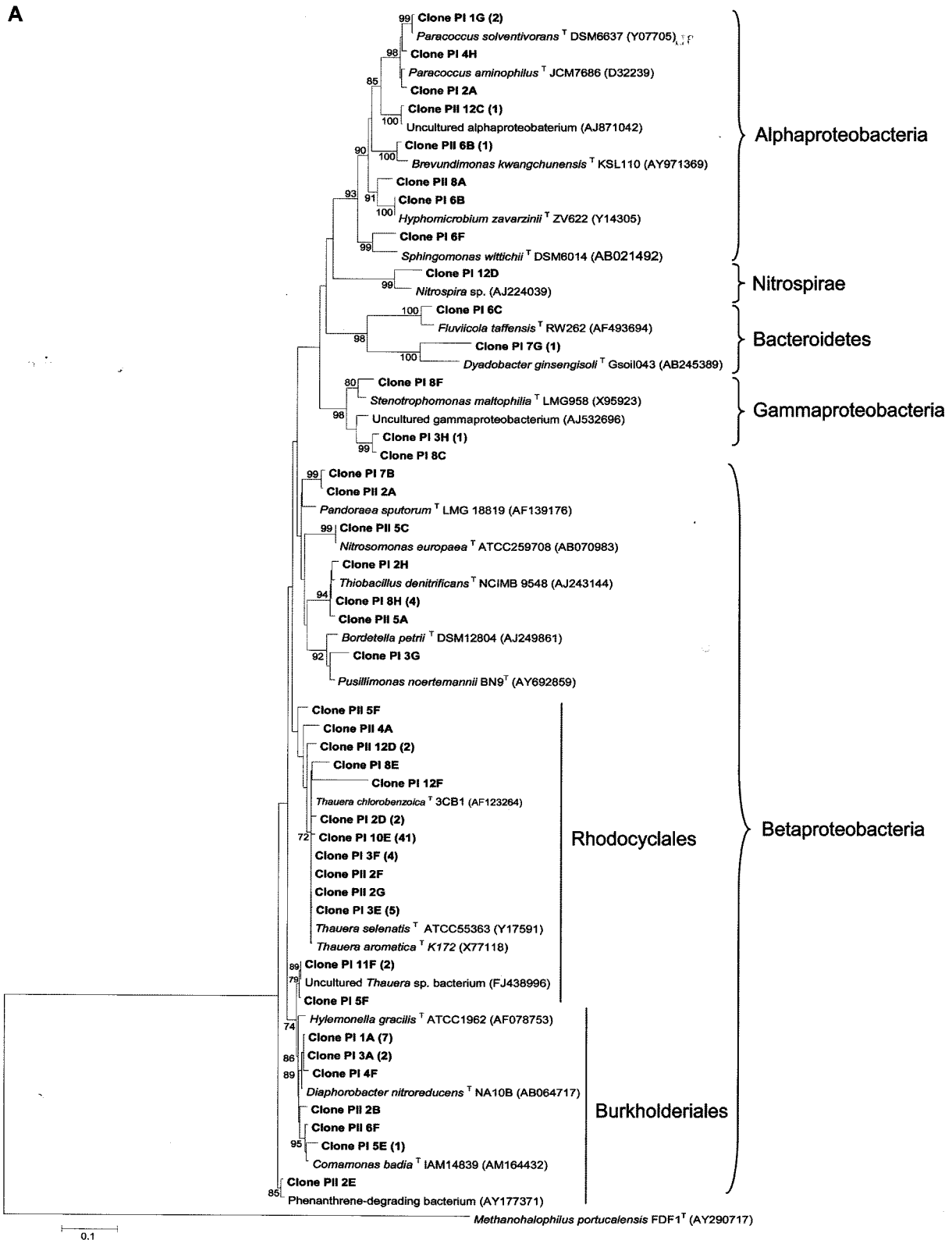*Significance is considered for values of $p < 0.05$.

A

Fig. 3. Phylogenetic analysis of partial 16S rRNA gene sequences from fourth-week MBR bioreactor obtained with (**A**) PCR systems and (**B**) RT–PCR–DGGE systems.

Evolutionary distances were based on the Kimura 2p model and tree reconstruction on the neighbor-joining method. Bootstrap values (1,000 replicate runs, shown as %) greater than 70% are listed. The number of clones in parenthesis showed ≥97% similarity with the clone represented in the branch. GenBank accession numbers are listed after species names. *Methanohalophilus portucalensis* was used as the outgroup.

B


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         71 ┌─ Acidovorax konjaci ᵀ ATCC 33996 (AF078760)  ┐
            └─ Acidovorax anthurii ᵀ CFBP 3232 (AJ007013)   │
         96 ├─ Clone 1A (9)                                 │  Bulkholderiales
        100 └─ Variovorax dokdonensis ᵀ DS-43 (DQ178978)    │
                    ┌─ Clone 6D2 (22)                        │
         94  100 ─┤└─ Comamonas badia ᵀ IAM 14839 (AB164432) │                    Betaproteobacteria
                  ┌─ Clone 3C (15)                           │
              100 ├─ Thauera aromatica ᵀ K172 (X77118)       │
        100 ──────┤  Thauera selenatis ᵀ ATCC 55363 (Y17591) │  Rhodocyclales
                  └─ Thauera chlorobenzoica ᵀ 3CB-1 (AF123264)│
     84    ┌─────── Clone 2A2 (5)     A                       │  Alphaproteobacteria
        100 └── Sandaracinobacter sibiricus ᵀ RB16-17 (Y10678)
              ┌── Uncultured Planctomycete GMD14H10 (AY162122)│  Planctomycetes
         87  └──── Clone 2C (4)
              ┌── Flavobacterium denitrificans ED5 (AJ318907)
     81  ┌── Clone 9A (3)
     91  └─ Flavobacterium aquatile ᵀ (M62797)                │  Bacteroidetes
              ┌──────── Clone 4B2 (15)
              └── Uncultured Bacteroidetes bacterium (AF364862)
  ──────────────────────── Methanohalophilus portucalensis ᵀ FDF1 (AY290717)
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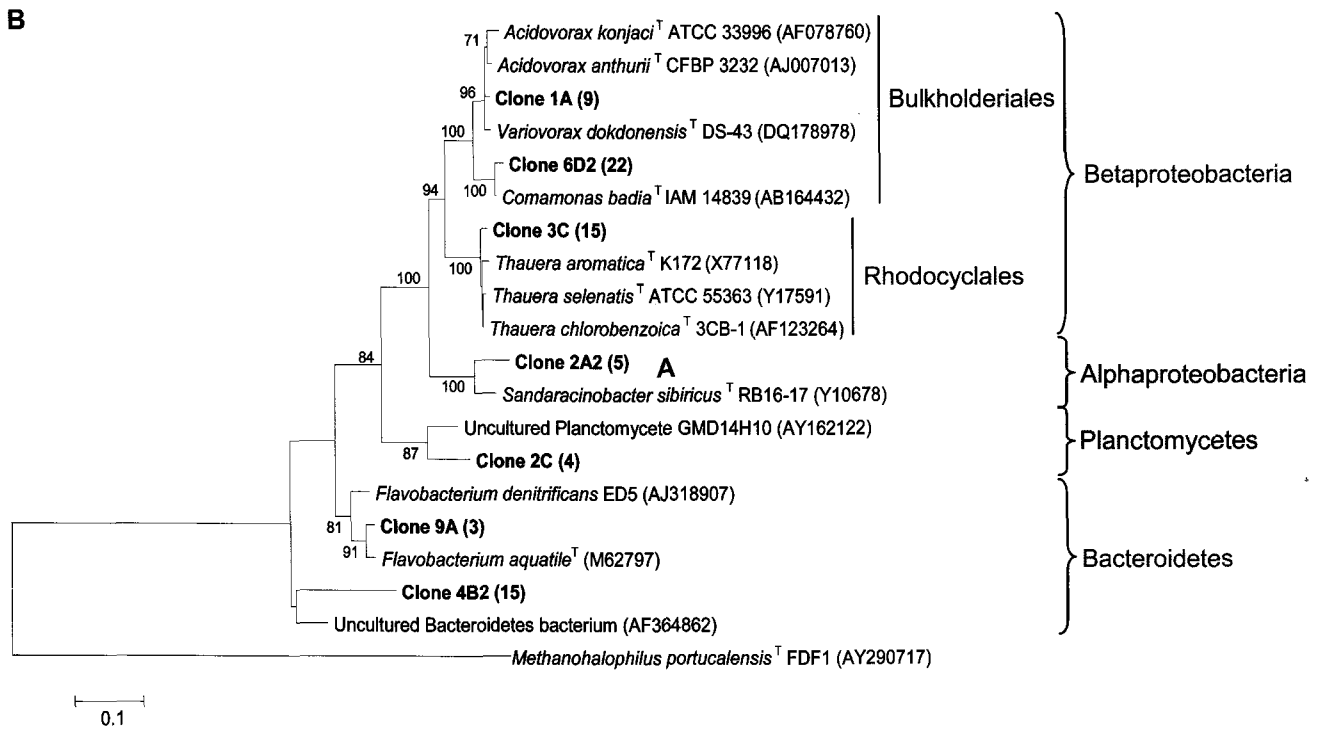
├───────┤
   0.1

Fig. 3. Continued.

The COD is used as an index of the total organic carbon in a sample, and may be used to quantify all organic substances with potential to be used as nutrients by microorganisms [4]. The COD factor showed a higher correlation with the variation in the RNA-based patterns of the 1st and 2nd weeks, suggesting that metabolic responses such as the degradation of organic matter are first noticed at the rRNA level than at the rDNA level.

Amongst the factors analyzed, the NH_4^+-N results explained more than those of phenol did the data variation of total and metabolically active populations. This is
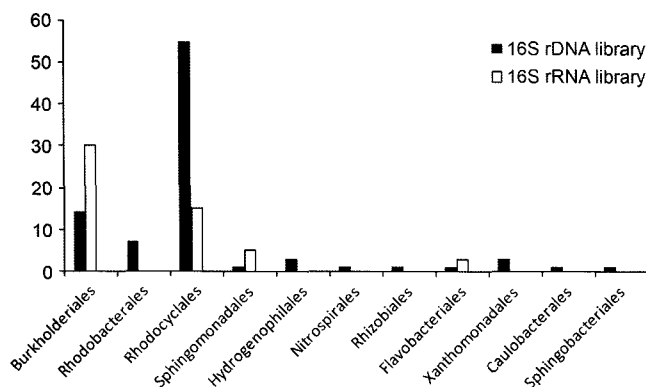

Fig. 4. Abundance of clones belonging to each bacterial order in libraries based on DNA or RNA extracted from a SMBR bioreactor at the 4th sampling.

probably due to the relevant role of ammonium in biological processes conducted by bacterial communities inside the bioreactor. The ammonium is important for all microorganisms, since it is used in a variety of metabolic pathways related to energy metabolism and biosynthesis [40]. Nonetheless, the higher correlation of the NH_4^+-N factor with the biological data does not exclude the important role of phenol in modulating the composition of bacterial communities present in the bioreactor.

Phylogenetic analyses of 16S rDNA and 16S rRNA clone libraries revealed that bacterial communities of the fourth week were mostly represented by members of the class Betaproteobacteria, which were related to *Thauera* spp. and *Diaphorobacter* spp. in the total community and *Thauera* spp. and *Comamonas* spp. in the active community. The relevance of the last two genera in phenol metabolism is broadly supported by literature data. *Thauera* spp. are commonly found in biological treatment systems and are able to utilize different kinds of aromatic compounds, such as phenol [28], polyphenol [9], toluene [29], and halobenzoate [30] as carbon source. Functional studies based on SIP-RNA have revealed that members of *Thauera* genus dominated the phenol degradation process in bioreactor sludges [23, 37], corroborating our findings. Additionally, studies based on culturing and/or DGGE fingerprint analyses have showed that *Comamonas* populations can be involved with the metabolism of aromatic compounds, including phenol [3, 5, 16], and can even supplant *Thauera*

populations as the dominant functional members of an industrial wastewater treatment reactor during phenol degradation in response to the presence of N-acyl-L-homoserine lactones, suggesting that both organisms are responsible for phenol metabolism. [37].

Members of the *Diaphorobacter* genus have already been related with ammonium metabolism in wastewater treatment plants [14, 17], occasionally displaying simultaneous nitrification and denitrification by aerobic conversion of ammonium to N_2 gas [18].

Sequencing of clones from both libraries indicated that dominant taxonomic groups (Rhodocyclales and Burkholderiales) observed in the RNA-derived library were essentially the same verified in the DNA-derived library, suggesting that these bacterial populations play essential roles in the metabolic processes that take place in the final phase of the bioreactor operation. These results are in agreement with those reported by Duineveld *et al.* [10], who observed that numerically dominant populations were responsible for most of the metabolic activity in the community. Some bacterial groups were observed only in one of the 16S libraries, such as the orders Hydrogenophilales, Nitrospirales, and Rhizobiales, found in the rDNA library, as well as the phylum Planctomycetes, found exclusively in the rRNA library. In the first case, those bacteria could represent cells of high abundance and/or with multiple operons, although with low metabolic activity [15]. In the second case, the Planctomycetes-related bacteria could represent active members of the complex community with more ribosomes present in their cells and/or low cellular abundance [15]. This could also explain why the most abundant clone in the 16S rRNA library (clone 6D2) is poorly represented in the 16S rDNA library.

Although populations related with ammonium metabolism were found in the 16S rDNA library, such as *Diaphorobacter* spp., *Nitrospira* spp., and *Nitrosomonas* spp., these populations do not appear in the metabolically active library, corroborating the low value of ammonium removal observed in the bioreactor. In contrast, populations related with phenol degradation were found in high abundance in both libraries and seemed not to be affected by the NH_4^+-N concentration in the SMBR wastewater.

Finally, investigating bacterial populations responsible for the degradation of toxic compounds may contribute to our understanding of the community dynamics and to increasing the efficiency of biological treatment wastewater plants. The combined use of DGGE, multivariate analysis, and 16S rRNA gene libraries was shown to be a relatively rapid and efficient approach in monitoring the changes of bacterial communities in response to exogenous perturbation. This work certainly helps to provide a future direction for the functional analyses of specific microorganisms of the community.

Acknowledgments

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