

Functional diversity and dynamics of bacterial communities in a membrane bioreactor for the treatment of metal-working fluid wastewater

Lucía Grijalbo, Carlos Garbisu, Iker Martín, Javier Etxebarria, F. Javier Gutierrez-Mañero and Jose Antonio Lucas Garcia

ABSTRACT

An extensive microbiological study has been carried out in a membrane bioreactor fed with activated sludge and metal-working fluids. Functional diversity and dynamics of bacterial communities were studied with different approaches. Functional diversity of culturable bacterial communities was studied with different Biolog™ plates. Structure and dynamics of bacterial communities were studied in culturable and in non-culturable fractions using a 16S rRNA analysis. Among the culturable bacteria, *Alphaproteobacteria* and *Gammaproteobacteria* were the predominant classes. However, changes in microbial community structure were detected over time. Culture-independent analysis showed that *Betaproteobacteria* was the most frequently detected class in the membrane bioreactor (MBR) community with *Zoogloea* and *Acidovorax* as dominant genera. Also, among non-culturable bacteria, a process of succession was observed. Longitudinal structural shifts observed were more marked for non-culturable than for culturable bacteria, pointing towards an important role in the MBR performance. Microbial community metabolic abilities assessed with Biolog™ Gram negative, Gram positive and anaerobic plates also showed differences over time for Shannon's diversity index, kinetics of average well colour development, and the intensely used substrates by bacterial community in each plate.

Key words | 16S rRNA, Biolog™, bioremediation, membrane bioreactor, metal working fluid

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INTRODUCTION

Metal working fluids (MWFs) are widely used for cooling and lubricating during machining processes. The worldwide annual usage of MWFs is estimated to exceed 2×10^9 L while the resulting waste could represent more than 10 times that volume, since MWFs have to be diluted considerably prior to use (Cheng *et al.* 2005). MWFs are divided into two main types: oil-based and water-based. Oil-based MWFs are classified into two further categories, namely straight oils and soluble oils. Water-based MWFs are divided into synthetics and semi-synthetics (Moscoso *et al.* 2012). This latter type is the most used in engineering

applications and it has resulted in increasing amounts of organic chemicals in MWF wastewater. In this respect, the complex composition of MWF wastewaters creates immense difficulties for waste disposal companies (Cheng *et al.* 2005).

At present, the majority of MWF waste is incinerated, sent to landfill sites or treated at sewage treatment plants. However, with the implementation of several proposed European Union directives (e.g. Council Directive 91/689/EEC of 12 December 1991 on hazardous waste) regulating effluent, incinerator and landfill discharges, the present cost-effective

options for the waste management of used MWF will no longer be viable (Environmental Protection Agency (EPA) 1995, 2001; European Union 2000a, b).

The membrane bioreactor (MBR) system has been widely used for the biological treatment of petrochemical wastewater (Shokrollahzadeh *et al.* 2008; Viero *et al.* 2008), and for MWF wastewater treatment since the early 1990s (Anderson *et al.* 2009). MBR consists of a suspended growth-activated sludge system that utilizes microporous membranes for solid/liquid separation instead of secondary clarifiers. The capability of MBRs for operating with long hydraulic retention time (HRT) leads to a low food/microbial ratio and provides sufficient time to micro-organisms for adapting to the presence of contaminants. Under these conditions, the micro-organisms are forced to mineralize compounds difficult to biodegrade (Jurtschko *et al.* 2002; Wever *et al.* 2007), resulting in an effluent water quality significantly higher than the one generated by conventional treatments. In consequence, the application of MBR systems in municipal and industrial wastewater treatment is becoming increasingly widespread worldwide (Judd 2007).

Bacterial communities present in activated sludge are responsible for most of the carbon and nutrient removal from the effluent and represent the major component of biological wastewater treatment plants. Thus, a better understanding of the microbial ecology in these systems is required to reveal factors influencing its efficiency and its stability, as well as to develop strategies for improved process performance (Satoh *et al.* 2013).

The objective of this study was to characterize MWF-fed MBR microbial community. To achieve this main objective, two main approaches, structural (culturable and non-culturable bacteria characterization) and functional, were followed. First, culturable bacteria were isolated in standard methods agar and in agar with MWF (to isolate adapted strains that could be used as inoculants for MWF bioremediation). Culturable bacterial community structure was then studied through a 16S rRNA partial sequencing. Moreover we studied the structure and dynamics of bacterial communities in an MWF-fed MBR using a culture-independent metagenomic 16S rRNA. Finally, functional diversity of culturable bacterial communities (community-level physiological profiles) was studied with three different Biolog™ plates.

MATERIALS AND METHODS

MWF

The MWF used in this study (Houghton Iberica S.A., Spain) was an operationally exhausted synthetic fluid, used as coolant and lubricant in large-scale continuous metal working processes machining tungsten carbide and steel. Its main chemical constituents include a formaldehyde-based biocide, alkyl benzotriazole (metal passivator), C16/C18-fatty alcohol polyglycol ether (corrosion inhibitors), isopropanolamine (lubrication agent), and 3-iodo-2-propynylbutylcarbamate. MWF is typically supplied as a concentrate, which is then diluted with water to form a 2% v/v working fluid prior to its use in machining operations. The MWF residue used in this study was kindly provided by John Deere Ibérica S.A., from its plant located in Madrid (Spain). This MWF residue was obtained after a lubrication and refrigeration process.

MBR

The MBR, provided by Gaiker (Vizcaya, Spain), was located in the Gaiker installations in Vizcaya (Spain). MBR was fed with activated sludge from Larrabetzu (Vizcaya, Spain) wastewater treatment plant (bacterial communities source), and MWF (MWF-fed MBR). Initially, the bacterial communities in the MBR were subjected to a period of adaptation (1 month) to the presence of MWF. For this, MWF was added, gradually increasing chemical oxygen demand (COD) values from 600 to around 6,000 mg/L.

MBR had a useful volume of 75 L, and HRT of 6 h, and sludge retention time of 35 d, and a flux of 12.5 LMH ($l\ m^{-2}\ h^{-1}$). In these conditions, MBR was able to reduce the COD from 6,000 to 1,750 mg/L (which is below the maximum value permitted by local law).

Immediately after the adaptation period, five sampling points (A, B, C, D, and E) were established. Sampling A was performed immediately after the adaptation period (1 operational day) and the others every 15 days: 15 operational days (B), 30 operational days (C), 45 operational days (D), and 60 operational days (E).

Measures in MWF: COD

After the adaptation period, COD of the MWF used to feed the MBR and the effluent after biological treatment were measured every 48–72 h. COD was determined by colorimetric analysis using a Merck Photometer SQ 118 with COD cuvette test kits (range 500–10,000 mg/L). Analyses were performed according to the manufacturer's instructions. This method is based on the EPA method 410.4 specifications (the determination of COD by semi-automated colorimetry; ÓDell 1993).

Isolation and phylogenetic analysis (16S rRNA) of culturable bacterial strains from the MWF-fed MBR

At each sampling point (A, B, C, D, and E), 100 mL of aerobic sludge was sampled and placed in a sterilized flask. After sonication for 7 min and subsequent homogenization (magnetic agitator) for 5 min, the sludge was allowed to decant. One millilitre of the resulting suspension (bacterial suspension) was used to prepare serial 10-fold dilutions (10^{-2} – 10^{-9}) in a final volume of 10 mL; 200 μ L of each dilution was plated on two different culture media: Standard Methods Agar (enzymatic digest of casein: 5 g/L, yeast extract: 2.5 g/L, glucose: 1 g/L, and bacteriological agar: 15 g/L) (Pronadisa, Spain) and Standard Methods Agar supplemented with MWF (COD = 6,000 mg/L). Plates were incubated for 7 days at 28 °C. From each culture medium, 10 individual colonies were randomly selected after 48 h and another 10 after 7 days of incubation, in order to select fast and slow growing strains. Isolated colonies were marked on the plate after selection. Gram staining and microscopy visualization confirmed the presence of a unique strain in each selected colony. Then, a total of 100 colonies were selected, 20 for each sampling point. DNA of each isolated strain colony was extracted with the Ultraclean Microbial DNA isolation Kit (Mo Bio, Carlsbad, CA, USA). Subsequently, 16S rRNA gene partial amplifications were carried out using 16sF (AGA GTT TGA TCC TGG CTC AG) and 16sR (AAG GAG GTG ATC CAG CCG CA) primers (Ulrike *et al.* 1989). The 25 μ L polymerase chain reaction (PCR) reactions were performed using Ultratools PCR buffer 10 \times with MgCl₂ (1 \times), dNTP Mix Ultratools (250 μ M each dNTP), Ultratools

DNA polymerase (1.4 units), primers (0.6 μ M, each), and 20 ng of genomic DNA. The reactions were incubated in a thermocycler (Applied Biosystems Gene Amp PCR system 2700) at 94 °C for 2 min and then subjected to 30 cycles consisting of 94 °C for 30 s, annealing temperature 55 °C for 30 s, and 72 °C for 1 min (increasing 5 s in each cycle from cycle 11–30). Finally, the mixtures were incubated at 72 °C for 7 min (Lucas *et al.* 2013). PCR products were purified using the QIAquick PCR purification kit (Qiagen) and sequenced in an ABI PRIMS[®] 377 DNA Sequencer (Applied Biosystems, CA, USA).

Structural diversity of MWF-fed MBR bacterial communities: culture-independent metagenomic analysis (16S rRNA)

Five 16S rRNA gene libraries were generated, one for each sampling point (A, B, C, D, and E). Total DNA extraction was performed from aerobic activated sludge, using the UltraClean[®] Soil DNA Isolation Kit (MoBio). A 16S rRNA gene partial amplification for each sampling was carried out using the primers and PCR conditions described above, except for the annealing temperature, which was set at 50 °C.

PCR products were purified using the QIAquick PCR purification kit (Qiagen) and then ligated into the pCR 4-TOPO vector (Invitrogen). *Escherichia coli* strain DH5 α (Invitrogen) was then transformed with the ligation products. One hundred colonies per treatment were randomly selected to carry out plasmid DNA extraction with the QIAprep Spin Miniprep kit (Qiagen). Fragment inserts were sequenced in an ABI PRIMS[®] 377 DNA Sequencer (Applied Biosystems, CA, USA). Sequences were screened for chimeras using the Bellerophon server (Huber *et al.* 2004).

Sequences treatments

Sequences were visualized, edited and aligned using: Sequence Scanner software v1.0, Clone Manager Professional Suite software v6.0. and MAFFT v6.0 (<http://mafft.cbrc.jp/alignment/software/>), respectively. Sequences were analysed by BLASTN 2.2.6., using the National Centre for Biotechnology Information (NCBI: www.ncbi.nlm.nih.gov/)

and Ribosomal Database Project Release 10 (RDP: <http://rdp.cme.msu.edu/>) databases.

Sequences comparisons were performed with the following statistical tools: DOTUR-1.53 (Schloss & Handelsman 2005), SONS (Schloss & Handelsman 2006) and β -LIBSHUFF (Schloss *et al.* 2004). Comparisons between libraries at the sequence level, using the DNA distance matrix (dnadist file) generated by DOTUR-1.53, were carried out with β -LIBSHUFF.

A study of shared operational taxonomic units (OTUs) was carried out with the SONS program to find out which taxa were shared and which were unique to each library. The distance between OTUs was 0.02 (2%).

Culturable bacteria sequences are available in the GenBank database with the accession numbers from JF937322 to JF937420 (see supplementary data, available in the online version of this paper).

Non-culturable bacteria sequences are available in the GenBank database with accession numbers from JN191751 to JN192053 (see supplementary data).

With the culturable bacterial sequences, an unrooted phylogenetic tree was performed with MEGA v4.0.2., from aligned sequences in MAFFT v6.0. The evolutionary distances were inferred using the Neighbor-Joining method. The bootstrap consensus tree was inferred from 500 replicates. The percentage of replicate trees in which the associated taxa clustered together in more than 50% of the 500 replicates of the bootstrap test are shown next to the branches. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option).

Functional diversity of culturable bacterial communities from the MWF-fed MBR: community-level physiological profiles with Biolog™ plates

At each sampling point, 1, 15, 30, 45, and 60 operational days (A, B, C, D, and E), culturable bacterial communities were functionally characterized according to their catabolic profiles using Gram negative (GN), Gram positive (GP) and anaerobic (AN) Biolog™ plates (BIOLOG Inc., Hayward, CA, USA) (Garland & Mills 1991; Grayston *et al.* 2004). Bacterial suspensions obtained from aerobic sludge after sonication for 7 min, homogenization 5 min, and waiting until sludge decanted from each sampling, were diluted to yield an absorbance

value of 0.15 at 420 nm. One hundred and fifty μ L per well were inoculated on the Biolog plates (Garland & Mills 1991). Plates were then incubated at 25 °C in darkness. Absorbance at 595 nm was measured at different incubation times until 262 h with an Asys High Tech Expert 96 spectrophotometer and Microwin 2000 analysis software. Anaerobic plates were measured only at 96 and 262 h of incubation. Overall colour development, expressed as average well colour development (AWCD), was calculated as the mean of the 95 blanked absorbance values (absorbance after subtracting blank absorbance). Three replicates per sampling point were performed. The blanked absorbance of each well was normalized, dividing them by AWCD in order to minimize the influence of inoculum density differences between plates (Baudoin *et al.* 2001).

For each type of Biolog plate, incubation time to calculate Shannon's diversity index and intensely utilized substrates of Biolog plates were chosen as the incubation time in which the AWCD value stops its exponential increasing. Thus, normalized absorbance values of each well at 94 h (GN plates), 166 h (GP plates) and 262 h (AN plates) of incubation were used to:

(i) calculate functional diversity as measured by the Shannon's diversity index:

$$H = \sum_{i=1}^{95} \left(\frac{ni}{N} * \text{Log} 2 \frac{ni}{N} \right)$$

where ni is the blanked absorbance value of each well from $i = 1$ to 95, and N is the sum of blanked absorbance values of all wells; and (ii) determine the intensely utilized substrates of Biolog plates. First, substrates of each Biolog plate were grouped by similar biochemical characteristics, and only groups including more than eight substrates were included. In GN plates, carboxylic acids (24 substrates), carbohydrates (32 substrates) and amino acids (20 substrates) were considered. For GP plates, polymers (11 substrates), carboxylic acids (17 substrates), carbohydrates (44 substrates) and amino acids (eight substrates) were used. For AN plates, carboxylic acids (21 substrates), carbohydrates (39 substrates) and amino acids (10 substrates) were considered. Then, the percentage of substrates in each group with a blanked absorbance value higher than the AWCD was calculated.

Longitudinal kinetics of average AWCD data (average of the three replicates) from the five sampling points were

represented and show the rate and level of growth development of the bacterial communities in the MBR.

In addition, for each sampling point, blanked absorbance data at 94 h (GN plates), 166 h (GP plates) and 262 h (AN plates) of incubation were analysed by principal component analysis (PCA).

Statistical analysis

Shannon's functional diversity values from different sampling points were compared with a one-way analysis of variance (ANOVA) analysis using Statgraphics 5.1 software. For longitudinal kinetics of AWCD values and intensely utilized Biolog substrates, two-way ANOVA were carried out (with Statgraphics 5.1 software). In kinetics of AWCD, dependent variables were incubation time and sampling points, and in the intensely utilized Biolog substrates dependent variables were biochemical compounds groups and sampling points. Significant differences between variables were considered when $P < 0.05$ and, in these cases, average values were compared by means of LSD statistic (Sokal & Rohlf 1979).

PCA was carried out with CANOCO™ v4.5 software (TerBraak & Šmilauer 1998) for each sampling point, with blanked absorbance data at 94 h (GN plates), 166 h (GP plates) and 262 h (AN plates) of incubation.

RESULTS

During 60 operational days (after the adaptation month), the COD of both the MWF used to feed the MBR and the effluent after biological treatment was measured every 3–4 days (Table 1). The MWF inlet COD varied between 4,000 and 8,600 mg/L. The MBR efficiency calculated as: $((\text{COD inlet}-\text{COD outlet}) * 100) / \text{COD inlet}$, varied between 69.37 and 95.15%. It is considered that efficiency values below 70% show a non-proper performance of the MBR.

Phylogenetic analysis (16S rRNA) of culturable bacterial strains from the MWF-fed MBR

The percentage at class level for the five sampling points is shown in Figure 1. *Alphaproteobacteria* and *Gammaproteobacteria* were the dominant classes: *Gammaproteobacteria*

Table 1 | COD values of the MWF used to feed the MBR and the effluent after biological treatment, after adaptation period (1 month)

Measure	COD inlet (mg/L)	COD outlet (mg/L)	MBR efficiency (%)
1	5,607	1,560	72.17
2	4,633	1,418	69.37
3	6,073	964	84.12
4	4,090	1,163	71.56
5	4,902	1,086	77.83
6	4,036	804	80.06
7	4,438	1,268	71.43
8	7,476	1,033	86.17
9	5,611	1,201	97.85
10	5,503	1,201	78.16
11	8,610	1,022	88.12
12	6,631	321	95.15
13	7,425	2,098	71.73
14	6,419	820	87.22

1,750 mg/L is the limit value allowed by the local law. MBR Efficiency = $((\text{COD inlet}-\text{COD outlet}) * 100) / \text{COD inlet}$.

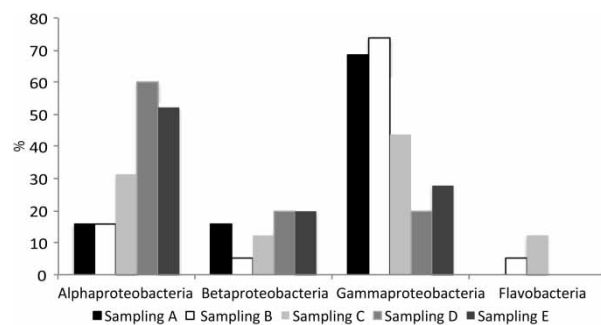


Figure 1 | Percentage of OTUs (at class level) obtained from 16S rRNA sequences of culturable bacteria in the five sampling points. Taxonomic categories were obtained from the NCBI (www.ncbi.nlm.nih.gov/) and Ribosomal Database Project Release 10 (RDP: <http://rdp.cme.msu.edu/>) databases.

in the first three sampling points (1, 15 and 30 operational days: A, B, C) and *Alphaproteobacteria* in the last two sampling points (45 and 60 operational days, respectively: D, and E). Clones of *Betaproteobacteria* and *Flavobacteria* were also identified.

The phylogenetic tree calculated using sequences from culturable bacteria of all sampling points (Figure 2) shows that, consistently with the above, *Alphaproteobacteria* and *Gammaproteobacteria* were the dominant classes.

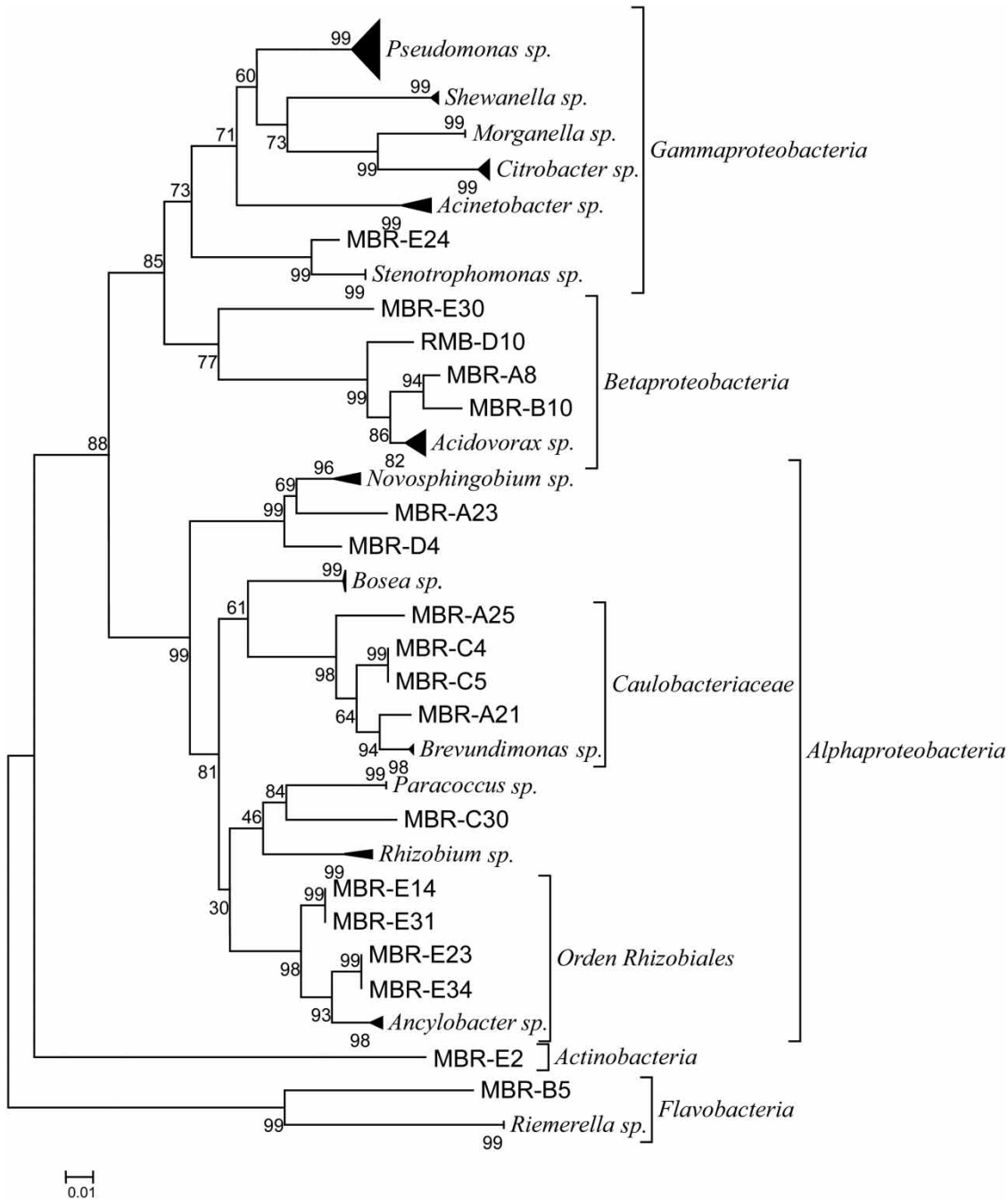


Figure 2 | Unrooted phylogenetic tree calculated using 16S rRNA sequences from culturable bacteria isolated from the MBR. The evolutionary distances were inferred using the Neighbor-Joining method. The bootstrap consensus tree was inferred from 500 replicates. The percentage of replicate trees in which the associated taxa clustered together in more than 50% of the 500 replicates of the bootstrap test are shown next to the branches. Phylogenetic analyses were conducted in MEGA4.

Within *Gammaproteobacteria* the predominant genera were *Pseudomonas*, *Citrobacter* and *Acinetobacter*. In the *Alphaproteobacteria* group predominant genera belong to the

Caulobacteriaceae family and *Rhizobiales* order. In the *Betaproteobacteria* group, *Acidovorax* was the more abundant genus.

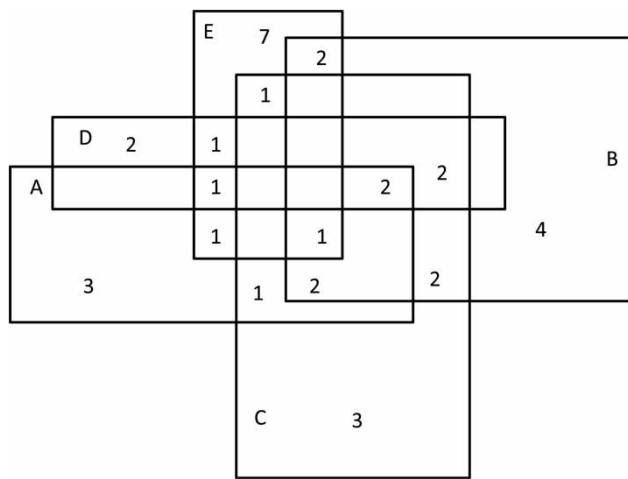
Table 2 | Comparison results for the five sampling points obtained through β -Libshuff analysis, which examines differences at sequence level (sequences of culturable bacteria) using the data provided by the distance matrix (generated by DOTUR-1.53) and provides an asymmetric matrix with *P*-values (95% confidence interval or more)

	Sampling A	Sampling B	Sampling C	Sampling D	Sampling E
Sampling A	0.0000	0.1351	0.7795	0.0001	0.4606
Sampling B	0.0028	0.0000	0.4040	0.0011	0.0000
Sampling C	0.5968	0.6808	0.0000	0.0421	0.6186
Sampling D	0.3702	0.1377	0.3223	0.0000	0.9321
Sampling E	0.0117	0.0002	0.0126	0.1894	0.0000

Significant differences are indicated in bold.

Comparative analysis of all the sequences with β -LIBSHUFF software showed significant differences between sampling points: A (1 operational day) and B (15 operational days) vs. D (45 operational days), and B (30 operational days) vs. E (60 operational days) (Table 2).

The Venn diagram displayed in Figure 3 shows the number of shared and unique OTUs (considering 0.02 distance between OTUs) for each sampling. Except for E

**Figure 3** | Venn diagram showing the shared and unique OTUs of culturable bacteria at each sampling point (A, B, C, D, and E). The distance between OTUs was 0.02 (2%).

sampling point, the number of unique OTUs was always lower than the number of shared OTUs (Table 3).

Structural diversity of MWF-fed MBR bacterial communities: culture-independent metagenomic analysis (16S rRNA)

The percentage of OTUs at class level for all five sampling points is shown in Figure 4. *Proteobacteria* was the dominant Phylum. *Alphaproteobacteria*, *Betaproteobacteria* and *Gammaproteobacteria* were found in all sampling points. Within the phylum *Proteobacteria*, *Betaproteobacteria* was the dominant class at all sampling points except for E, in which *Gammaproteobacteria* was the dominant class. Representatives of the *Sphingobacteria* class were only detected in sampling points D (45 operational days) and E (60 operational days). Within *Betaproteobacteria*, *Zoogloea* was the dominant genus in the first three sampling points (1, 15 and 30 operational days: A, B and C), and *Acidovorax* in the last two sampling points (45 and 60 operational days: D, E) (data not shown). Unclassified *Gammaproteobacteria* were dominant in the last sampling point E (60 operational days) (data not shown).

Comparative analysis of all the sequences performed with β -LIBSHUFF software, showed significant differences

Table 3 | Number of OTUs (considering 0.02 distance between OTUs) in each sampling point for culturable bacteria and total bacterial communities present in the MWF-fed MBR. The percentage of unique OTUs for each sampling is shown in brackets

No. OTUs	Sampling A	Sampling B	Sampling C	Sampling D	Sampling E
Culturable	11 (27%)	15 (26%)	14 (21%)	8 (25%)	14 (50%)
Total	40 (60%)	19 (42%)	29 (58%)	34 (55%)	23 (39%)

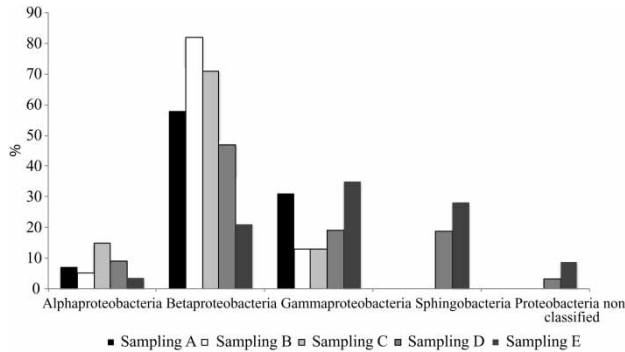


Figure 4 | Percentage of clones at class level for the five sampling points obtained from 16S rRNA sequences of total bacterial communities, present in a MWF-fed MBR. Taxonomic categories were obtained from the NCBI (www.ncbi.nlm.nih.gov/) and Ribosomal Database Project Release 10 (RDP: <http://rdp.cme.msu.edu/>) databases.

between sampling points A (1 operational day), B (15 operational days) and C (30 operational days) vs. D (45 operational days) and E (60 operational days) (Table 4).

The Venn diagram displayed in Figure 5 shows the number of shared and unique OTUs (considering 0.02 distance between OTUs) for each sampling. Except for sampling point E (60 operational days), the number of unique OTUs was always higher than the number of shared OTUs (Table 3).

Functional (Biolog™) diversity of culturable bacterial communities from the MWF-fed MBR

Metabolic rate was assessed through the study of the temporal kinetics of AWCD data with all sampling points using a two-way ANOVA analysis (Figure 6). There was a significantly higher metabolic rate in D and E sampling points with respect to the other samplings, for all Biolog™

plates used (GN, GP, AN). By contrast, the A sampling point showed lower values of metabolic rate.

Regarding Shannon's diversity (Table 5) the lowest value was found in the A sampling point (1 operational day) for all Biolog™ plates (GN, GP, AN). The highest values were obtained in C (30 operational days) and D (45 operational days) sampling points, and then decreased again in the E sampling point (60 operational days).

The percentage of intensely utilized substrates in Biolog™ plates is shown in Figure 7. In aerobic plates (GN, Figure 7(a) and GP, Figure 7(b)), amino acids were the most consumed group followed by carbohydrates and carboxylic acids. In the anaerobic plate (AN), carbohydrates were the most used compounds and amino acids the least (Figure 7(c)). In all cases significant differences between groups of compounds and between sampling points were found.

According to the PCA (Figure 8), functional community shifts were observed over time: distances were especially prominent between sampling points A (1 operational day) and B (15 operational days), as well as between sampling points C (30 operational days) and D (45 operational days). On the contrary, community shifts between sampling point B (15 operational days) and C (30 operational days) and between sampling points D (45 operational days) and E (60 operational days) were small.

DISCUSSION

Our results suggest that the MBR system used in this work could efficiently treat MWF wastewater, as it did lead to COD values below regulatory limits (regional Law 10/1993

Table 4 | Libraries comparison results for the five sampling point obtained through β -Libshuff analysis, which examines differences at sequence level (sequence of total bacterial communities) using the data provided by the distance matrix (generated by DOTUR-1.53) and provides an asymmetric matrix with *P*-values (95% confidence interval or more)

	Sampling A	Sampling B	Sampling C	Sampling D	Sampling E
Sampling A	0.0000	0.0148	0.0474	0.0046	0.0001
Sampling B	0.7834	0.0000	0.0374	0.0000	0.0000
Sampling C	0.8685	0.0058	0.0000	0.0003	0.0000
Sampling D	0.0000	0.0000	0.0000	0.0000	0.0818
Sampling E	0.0000	0.0000	0.0000	0.1058	0.0000

Significant differences are indicated in bold.

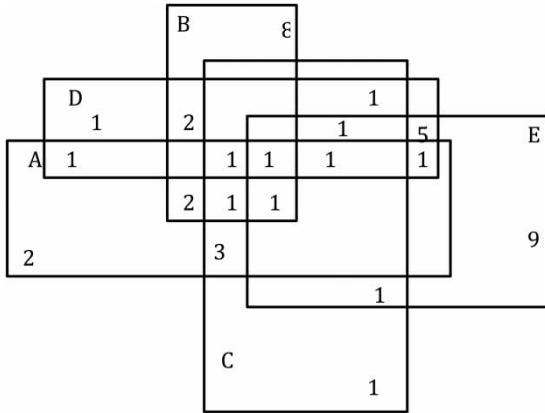


Figure 5 | Venn diagram showing the shared and unique OTUs of total bacterial communities, between sampling points (A, B, C, D, and E). The distance between OTUs was 0.02 (2%).

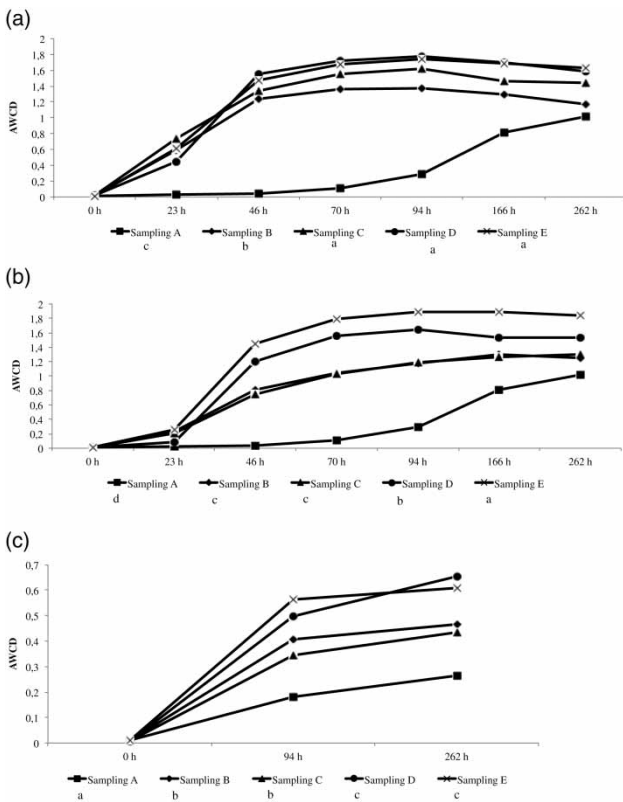


Figure 6 | Longitudinal kinetics of AWCD. (a) GN plate, (b) GP plate, and (c) AN plate. Different letters indicate significant differences between sampling points.

on industrial waste discharges into urban sanitary sewer system). All strains isolated during the experiment were able to grow in Petri dishes with MWF as the only nutrient source. Despite this, it is not possible to know if the decrease

Table 5 | Average values of functional Biolog™ diversity (Shannon's index) calculated from blanked absorbance values at 94 h (GN plates), 166 h (GP plates) and 262 h (AN plates) of incubation for each sampling point

	GN	GP	AN
Sampling A	1.894 ± 0.007 d	1.790 ± 0.006 b	1.755 ± 0.010 c
Sampling B	4.825 ± 0.061 c	1.916 ± 0.009 b	1.878 ± 0.004 b
Sampling C	6.056 ± 0.011 a	4.307 ± 0.118 a	1.912 ± 0.002 a
Sampling D	5.096 ± 0.317 b	4.709 ± 0.255 a	1.833 ± 0.008 b
Sampling E	4.546 ± 0.028 c	1.954 ± 0.001 b	1.876 ± 0.010 b

Mean values (n = 3) ± standard errors (SE). Different letters indicate significant differences between sampling points within each type of plate.

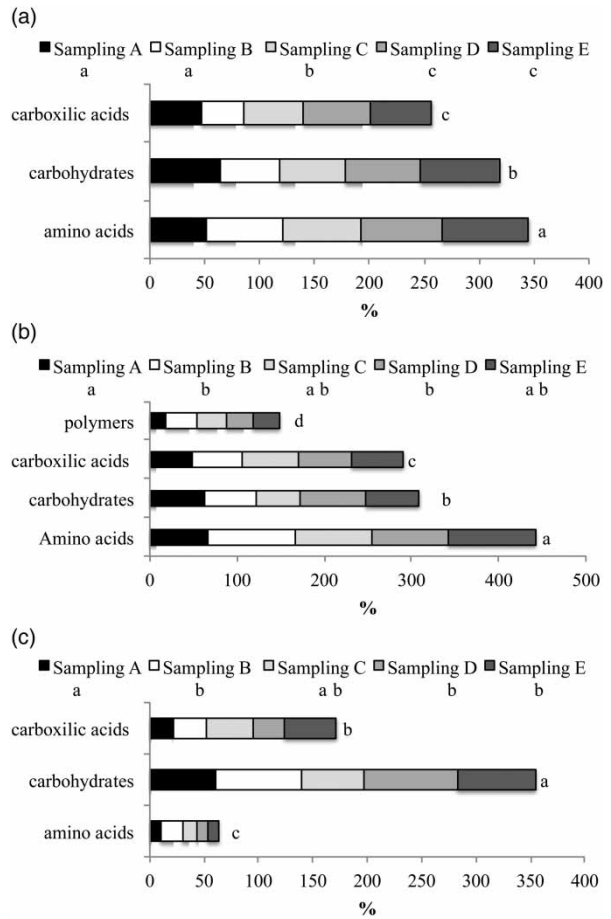


Figure 7 | Percentage of intensely used substrates by bacterial communities from the MBR in each Biolog™ plate. Substrates were grouped by similar biochemical characteristics: carboxylic acids, amino acids, carbohydrates, polymers, etc., and only those groups with more than eight substrates were used. (a) In GN plates: carboxylic acids (24 substrates), carbohydrates (32 substrates) and amino acids (20 substrates). (b) In GP plates: polymers (11 substrates), carboxylic acids (17 substrates), carbohydrates (44 substrates) and amino acids (eight substrates), and (c) in AN plates: carboxylic acids (21 substrates), carbohydrates (39 substrates) and amino acids (10 substrates).

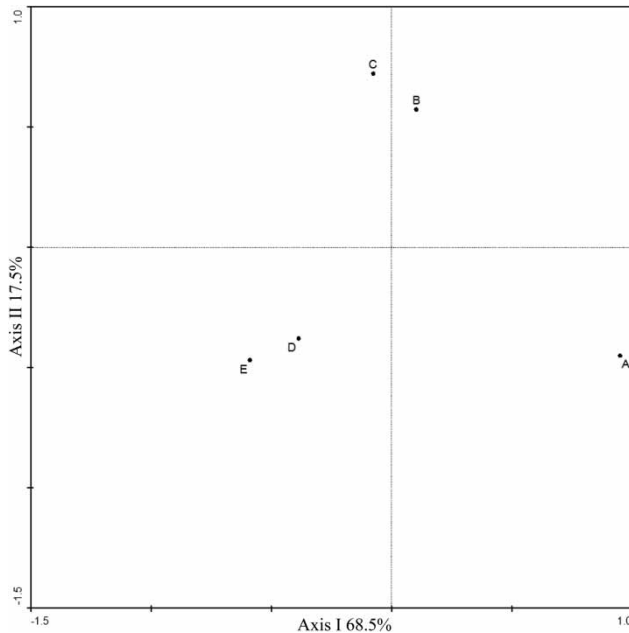


Figure 8 | PCA performed with blanked absorbance values at 94 h (GN plates), 166 h (GP plates) and 262 h (AN plates) of incubation at each sampling point (A, B, C, D, E). Percentages in axis represent the variance absorbed in each case.

of COD observed is due to bacterial community activity or to another non-biological process such as absorptions. Bacteria belonging to *Zoogloea* genus have been detected in this study, and it is known that these bacteria are able to produce extracellular polymeric substances that hold the cell aggregates together (Gerardi 2006; Bo et al. 2010), where the absorption processes are very common.

In this work, the study of the bacterial communities present in the MWF-fed MBR along time was performed from two complementary points of view: (i) a structural and functional (Biolog™) analysis of culturable bacteria; and (ii) a culture-independent approach through a metagenomic analysis of the structure of the bacterial communities. Although very useful for taxonomical, physiological and genetic studies, cultivation-based techniques are inadequate for a proper description of the composition and dynamics of real microbial communities, since it is now well known that only a small fraction (0.1–10%) of the microbial diversity in nature can be cultured in the laboratory (Tiedje & Stein 1999; Van Der Gast et al. 2003). However, culturable bacteria adapted to grow in the presence of MWF is a very interesting biological material, because it could be subsequently used as inoculants for MWF bioremediation or

phyto-rhizoremediation processes. Currently, bioreactors for MWF treatment are commonly operated with undefined microbial communities obtained from sewage sludge, a notoriously heterogeneous and potentially dangerous source of degrading bacteria, because it is likely to harbour potential pathogens (Hamer 1997). As an alternative, bioremediation or phyto-rhizoremediation processes with carefully selected strains may improve the opportunity to create a more reproducible and efficient treatment system. In fact, four bacteria isolated in this work, selected by its abundance, were used in a phyto-rhizoremediation system with maize plants growing in MWF, and it was more efficient than the other treatments. Furthermore, it seemed that these bacteria could protect plants against damage produced by MWF (Grijalbo et al. 2015).

Among the culturable bacteria, Proteobacteria was the most abundant Phylum, with *Alphaproteobacteria* and *Gammaproteobacteria* as the predominant classes: *Alphaproteobacteria* was predominant in sampling point A (1 operational day) but, in the last two sampling points (45 and 60 operational days: D and E) *Gammaproteobacteria* showed higher abundance values. *Caulobacteriaceae* and *Rhizobiaceae* were the most abundant families of *Alphaproteobacteria* class, and *Pseudomonadaceae* family of *Gammaproteobacteria* class. Van Der Gast et al. (2003, 2004) also found *Gammaproteobacteria* as the most abundant class in a MWF-fed MBR, and suggested that *Gammaproteobacteria* was responsible for the observed biocide reduction presents in MWF.

The molecular tools developed in the last decades to overcome the limitations imposed by traditional cultivation techniques have been used to study bacterial diversity in wastewater biological treatments (Wagner & Loy 2002; Chang et al. 2011). Our 16S rRNA culture-independent sequence analysis has shown that, in agreement with other studies (Silva et al. 2010; Wan et al. 2011), *Betaproteobacteria* were most frequently detected in the MBR community (Figure 5), with *Zoogloea* dominating in sampling points A, B and C, and *Acidovorax* in sampling points D and E. Bacteria of the genus *Zoogloea* have previously been reported as dominant in MBR wastewater treatment (Bo et al. 2010). Interestingly, *Zoogloea* has been suggested to play an important role in wastewater purification processes (Shin et al. 1993). Zhang et al. (2012) found this bacterium

as dominant in activated sludge from 14 sewage treatment plants, and has been assigned an important role in the degradation of petroleum hydrocarbon (Farkas *et al.* 2014), phenol (Qu *et al.* 2015), pharmaceuticals and personal care products (Zhao *et al.* 2015), malathion and a phosphonic acid (Janeczko *et al.* 2014) and benzene (Jechalke *et al.* 2013) among other contaminants.

The genus *Acidovorax* has also been found in wastewater treatment plants (Wan *et al.* 2011). In fact, the role of members of the family *Comamonadaceae*, such as *Acidovorax*, in the removal of nitrogen in wastewater treatment plants has been reported (Etchebehere *et al.* 2001; Gumaelius *et al.* 2001; Mechichi *et al.* 2003). In our study, *Acidovorax* bacteria did not appear until sampling D, that is to say, until 45 operational days. This fact has been found by other authors, such as Hoshino *et al.* (2005), who observed that these bacteria (*Acidovorax*) took up carbon mainly under aerobic conditions and that they did not play a role in denitrification until day 100.

Longitudinal shifts in the bacterial community present in the MWF-fed MBR were more markedly observed for culture-independent than for culture-dependent data (Tables 4 and 5; Figures 5 and 6). The number of unique OTUs found in each sampling was higher in the former case. This phenomenon has also been reported by other authors (García-Villaraco *et al.* 2010) using other techniques and in other ecosystems. It is known that more than 90% of bacteria are non-culturable (Tiedje & Stein 1999). This only means the nutritional requirements to cultivate them *in vitro* are not known (Vartoukian *et al.* 2010), but it does not imply that they do not have a role in ecosystem functioning.

The Biolog™ method, used in this work for the assessment of the functional diversity of the bacterial communities from the MWF-fed MBR, is a rapid assay for culturable heterotrophic organisms which has proven to be a sensitive indicator of changes in microbial functional diversity under many different conditions (Garland 1997; Grayston *et al.* 1998, 2001; Rogers & Tate 2001; Graham & Haynes 2005; Grayston & Prescott 2005; White *et al.* 2005). Similarly, kinetics of AWCD values of the three Biolog™ plates used in this work (Figure 6) showed how bacterial communities metabolic rate varied between sampling points. The evolution of the system over time implies that, at each sampling point, there are actually different conditions that were reflected in

different microbial community metabolic rates, detected with Biolog plates. In all cases, the metabolic rate increased from the first to the last sampling, suggesting an adaptation process of the MWF-exposed bacterial communities to the presence of MWF. This same phenomenon was also observed in our PCA results (Figure 8).

The results obtained in terms of the highly utilized compounds by bacteria in the Biolog™ plates also show these processes indicated above (Figure 7). In the three plates, significant differences between sampling points were observed regarding the different compounds used. Amino acids were the most used compounds in aerobically incubated plates and carbohydrates in the anaerobically incubated ones. The hydrocarbons present in MWF have a very high C/N ratio. This fact could be responsible for the high amino acid consumption in aerobically grown bacteria, since in those conditions the priority could be nitrogen but not carbon uptake. However, under anaerobic conditions, micro-organisms have to use fermentation as an energy source. Owing to that, this process is less efficient than oxidation processes in the presence of oxygen, the abundance of carbon compounds is especially important. Some authors have pointed out that the compounds used by bacteria in the Biolog™ plates should be correlated with the activity that bacteria had when they were isolated (Alisi *et al.* 2005; García-Villaraco *et al.* 2009). These studies were carried out with rhizosphere microbial communities. Rhizosphere is a very rich ecosystem that contains compounds from root exudates, some of them shared with Biolog plate compounds. In our work, the MWF composition is not as rich as a rhizosphere and therefore, it is difficult to assume this relationship. On the contrary, what we are probably observing in the Biolog plates are the nutritional deficiencies that microbial communities had at the time of being isolated.

CONCLUSIONS

Based on the results of this study, the following conclusions may be reached:

- (a) Among the culturable bacteria a clear succession process was observed during the experiment. This process was structural and functional.

- (b) Among non-culturable bacteria a succession process was also observed, but with different bacterial groups. Longitudinal shifts observed were more marked than for culturable bacteria. This indicates the important role of non-culturable bacteria in the performance of the MBR.
- (c) Strains highly adapted to live in MWF were isolated. These strains may be used to inoculate MBR fed with MWF or plants in bioremediation or phytoremediation programs to improve the remediation yield.

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