Available online at www.sciencedirect.com

SciVerse ScienceDirect

journal homepage: www.elsevier.com/locate/watres

Industrial activated sludge exhibit unique bacterial community composition at high taxonomic ranks

Federico M. Ibarbalz [a], *Eva L.M. Figuerola* [a], *Leonardo Erijman* [a,b,*]

[a] *Instituto de Investigaciones en Ingeniería Genética y Biología Molecular (INGEBI-CONICET), Vuelta de Obligado 2490, C1428ADN Buenos Aires, Argentina*

[b] *Departamento de Fisiología, Biología Molecular y Celular, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria, Pabellón 2, C1428EHA Buenos Aires, Argentina*

ARTICLE INFO

Article history:
Received 7 December 2012
Received in revised form
7 April 2013
Accepted 9 April 2013
Available online 17 April 2013

Keywords:
Biological wastewater treatment
Industrial activated sludge
Pyrosequencing
Bacterial community structure
High bacterial taxa

ABSTRACT

Biological degradation of domestic and industrial wastewater by activated sludge depends on a common process of separation of the diverse self-assembled and self-sustained microbial flocs from the treated wastewater. Previous surveys of bacterial communities indicated the presence of a common core of bacterial phyla in municipal activated sludge, an observation consistent with the concept of ecological coherence of high taxonomic ranks. The aim of this work was to test whether this critical feature brings about a common pattern of abundance distribution of high bacterial taxa in industrial and domestic activated sludge, and to relate the bacterial community structure of industrial activated sludge with relevant operational parameters. We have applied 454 pyrosequencing of 16S rRNA genes to evaluate bacterial communities in full-scale biological wastewater treatment plants sampled at different times, including seven systems treating wastewater from different industries and one plant that treats domestic wastewater, and compared our datasets with the data from municipal wastewater treatment plants obtained by three different laboratories. We observed that *each* industrial activated sludge system exhibited a unique bacterial community composition, which is clearly distinct from the common profile of bacterial phyla or classes observed in municipal plants. The influence of process parameters on the bacterial community structure was evaluated using constrained analysis of principal coordinates (CAP). Part of the differences in the bacterial community structure between industrial wastewater treatment systems were explained by dissolved oxygen and pH. Despite the ecological relevance of floc formation for the assembly of bacterial communities in activated sludge, the wastewater characteristics are likely to be the major determinant that drives bacterial composition at high taxonomic ranks.

1. Introduction

Biological treatment of industrial and municipal wastewater by activated sludge (AS) relies in the self-assembly of an active microbial community, which is able to form flocculent aggregates that are separated from the treated effluent by gravity settling. Past studies have been valuable in showing that AS systems contain highly diverse and dynamic microbial consortia, where bacteria are the dominant organisms responsible for the removal of most of the oxygen-demanding

* *Corresponding author*. Instituto de Investigaciones en Ingeniería Genética y Biología Molecular (INGEBI-CONICET), Vuelta de Obligado 2490, C1428ADN Buenos Aires, Argentina. Tel.: +54 11 4783 2871; fax: +54 11 4786 8578.
E-mail addresses: erijman@dna.uba.ar, erijman@gmail.com (L. Erijman).

0043-1354/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.watres.2013.04.010

pollutants and nutrients. Even though fluctuations in bacterial composition in wastewater treatment processes may occur without affecting performance (Kaewpipat and Grady, 2002), it is generally accepted that bacterial community structure and diversity influences wastewater treatment stability and robustness (Wagner and Loy, 2002; Werner et al., 2011). Yet consensus has still not been reached on the principles underlying the assembly of activated sludge microbial communities. It has been proposed that both neutral processes and species sorting act simultaneously during the assembly of bacterial communities (Ayarza and Erijman, 2011; Ayarza et al., 2010; Langenheder and Szekely, 2012; Ofiteru et al., 2010; Valentin-Vargas et al., 2012).

Recent high-throughput studies showed that bacterial communities of municipal WWTP reactors operated under diverse configurations at different geographic locations are highly similar at the phylum level (Hu et al., 2012; Wang et al., 2012a; Xia et al., 2010; Zhang et al., 2012). The suggestion that similarities in the distribution of bacterial phyla among diverse wastewater treatment plants reflect similar life strategies under most configurations is consistent with the recent proposition that bacterial groups of high taxonomic ranks exhibit ecological coherence (Philippot et al., 2010). This concept refers to the fact that members of a taxon share functional traits, which distinguish them from members of other taxa.

Fewer diversity surveys have been performed in activated sludge systems treating industrial wastewater (Bramucci and Nagarajan 2006; Degenaar et al., 2008; Figuerola and Erijman, 2007; Juretschko et al., 2002). Despite the increasing trend towards combining industrial and domestic wastes for treatment in municipal plants, the need for industrial wastewater treatment remains a critical issue worldwide. Industrial wastewaters comprise a wide spectrum of diverse organic compounds, characteristic of the different industrial categories and their production schemes. Similar to domestic sewage, most industries rely on the activated sludge process to treat their wastewater, after a suitable pretreatment when needed (Orhon et al., 2009). The characteristics of the wastewater can have a decisive influence as a driving force behind the bacterial community dynamics. It was observed that in activated sludge reactors subjected to a gradient of increasing industrial wastewater concentrations the deterministic component of community assembly increased with the concentration of industrial wastewater (van der Gast et al., 2008). Earlier studies of industrial activated sludge showed a numerical dominance of major bacterial phyla, i.e. *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, and *Actinobacteria* (Bramucci and Nagarajan 2006; Degenaar et al., 2008; Figuerola and Erijman, 2007; Juretschko et al., 2002). These patterns do not differ markedly from those found in municipal wastewater treatment plants. However, those early molecular studies of biological industrial wastewater treatment systems have been limited to the analysis of only several dozens of clones, and no attempt has been made to compare bacterial community structures of industrial activated sludge in relation to wastewater characteristics and operational parameters.

Metagenomic approaches based on 'next generation sequencing' have the capacity of providing a deeper insight into the participating bacterial populations. In this work we have investigated seven full-scale industrial and one municipal activated sludge plant using barcoded 454 pyrosequencing of 16S rRNA gene and compared our datasets with the data from municipal wastewater treatment plants obtained by three different research groups. The objectives of this work were: 1) to test whether the similarity in the abundance distribution patterns of high bacterial taxa, which have been observed in municipal WWTPs, holds for industrial wastewater treatment systems as well, and 2) to examine the taxa distribution of industrial WWTPs in relation to process parameters.

2. Materials & methods

2.1. *Wastewater treatment plants and sampling*

Activated sludge samples were taken from the aeration basins of eight full-scale WWTPs located in Argentina. Six of the plants were sampled twice, at times separated by periods of two months to almost three years. The other two WWTPs, which treated wastewater from two different pet food industries, were sampled once. The type of wastewater, main operating conditions and times of sampling are given in Table 1 and Table S1. Textile dyeing (TXT) wastewater contained unreacted dyestuffs and other auxiliary chemicals that are used at the various stages of dyeing, such as organic acids, fixing reagents, anti-foaming agents, oxidizing and reducing (sodium dithionite) reagents, sodium hydroxide and diluents. Petroleum refinery wastewater (REF) was characterized by high concentrations of aliphatic and aromatic hydrocarbons, phenolic compounds and ammonia. The pharmaceutical industry evaluated in this study (PHA) manufactures dosage-form products by mixing active ingredients and excipients, such as binders, fillers, flavoring and bulking agents, preservatives and antioxidants. Many different products are manufactured, resulting in large variations in wastewater composition. Compounds present in wastewater are usually found in low concentrations, and include organic acids, carbohydrates, phospholipids, vitamins and inorganic minerals. No antibiotics are manufactured or processed in this industrial facility. Pet food industries (PF1 and PF2) generate high-strength wastewater containing high animal fat concentration, protein, carbohydrates and vitamins, in addition to various cleaning and sanitizing compounds. Organic components of wastewater from the whey processing plant (WHY) consist mostly of lactose and milk serum proteins, generated upon the use of automated cleaning-in-place systems (CIP) for cleaning of equipments and pipelines. The polymer wastewater treatment plant (PLM) receives wastewater-containing monomers (alkanes, alkenes and other aliphatic hydrocarbons) from the manufacturing of basic plastics (polyethylene) and performance plastics (polyurethane).

The operational parameters from each wastewater treatment plant were obtained from the respective staff members. Data were averaged over the month prior to sampling. Phosphorus deficiency in the refinery wastewater and nitrogen deficiency in the textile dyeing wastewater were corrected by the addition of phosphoric acid and urea, respectively. Nutrients were in excess in all the other WWTPs. With only one

Table 1 – Characteristics and average process parameters for the surveyed WWTPs. Data were averaged over the month prior to sampling.

Wastewater	Code	ABV[a] (m^3)	MLSS[b] (mg/L)	SRT[c] (d)	Org. Load (kgBOD/d)	F/M	pH	DO[d] (mg/L)	Temp (°C)
Textile dyeing	TXT-08	3500	3594	68	1165	0.12	8.6	0.7	33
	TXT-11	3500	6563	40	1008	0.05	8.4	0.6	28
Petroleum refinery	REF-09	5430	4850	86	2402	0.11	7.6	1.9	34
	REF-11	5430	4425	69	2778	0.14	7.1	3.9	35
Acrylic Polymer	PLM-08	200	4350	70	13.3	0.02	7.4	8.1	23
	PLM-09	200	3313	53	11	0.02	7.1	8.6	23
Pharmaceutical	PHA-5.11	40	2788	16	18	0.20	6.8	3.2	21
	PHA-7.11	40	3113	15	22	0.22	6.9	2.9	22
Sewage	SWG-08	200	4469	18	81	0.11	7.4	3.4	21
	SWG-09	200	5038	23	76	0.09	7.2	2.9	19
Whey Processing	WHY-10	5000	8163	28	3018	0.09	7.7	0.9	22
	WHY-11	5000	6531	33	3148	0.12	7.7	0.8	20
Pet food 1	PF1-08	125	3875	32	112	0.29	6.8	1.8	22
Pet food 2	PF2-11	160	8125	5	546	0.53	7.5	0.8	22

a ABV: Total aeration basin volume.
b MLSS: Mixed Liquor Suspended Solids.
c SRT: Solids Retention Time.
d DO: Dissolved Oxygen concentration.

exception, the WWTPs were not designed for nutrient removal. The WWTP treating wastewater from the petroleum refinery contains an anoxic basin for denitrification. However, since no carbon is added as electron donor for denitrification, nitrogen removal efficiency was very low.

After sampling, sludge samples were transported within 2 h to the laboratory in plastic flasks at room temperature with a large air chamber in order to avoid anaerobic conditions, and stored at −20 °C, except the sludge samples from the whey-processing factory, which were transported in ice and stored at −20 °C within 6 h.

2.2. DNA extraction

Pellets derived from centrifugation of 1 mL of sludge were resuspended in 500 μL of TE buffer (pH 8.0) and transferred to 2 mL screw cap tubes with 0.5 g zirconia silica beads (BioSpec Products, OK). 50 μL of 10% sodium dodecyl sulfate (SDS) were added, and the tubes were immediately filled with equilibrated phenol [pH = 8.0] and chloroform–isoamyl alcohol (25:24:1). Cells were physically disrupted by shaking for 3 cycles of 30 s in a cell homogenizer (Precellys 24, Bioamerica) at 5000 rpm. The aqueous phase was transferred to a clean tube and re-extracted twice with chloroform-isoamyl alcohol (24:1). Nucleic acids were precipitated with 2 volumes of ethanol 100% and 0.1 volumes of sodium acetate and washed twice with 70% ethanol. The pellets were resuspended in 50 μL of nuclease free water.

2.3. PCR and sequencing

Variable V1-V3 regions of the 16S rRNA gene were amplified in triplicate with barcoded bacterial universal primers F27 (5′-GAGTTTGATCMTGGCTCAG-3′) and R518 (5′-WTTACCGCGGCT-GCTGG-3′). PCR reaction mix consisted of 0.3 μM of each barcoded primer, 15 ng template DNA, 2× reaction buffer, 0.2 mM dNTPs, 1 mM $MgSO_4$ and 1U Platinum Pfx DNA polymerase (Life Technologies Corporation). Barcodes sequences 10 bp long, located at the 5′ end, were added to enable the association of individual PCR products to each specific sample. Amplification conditions were as follows: 2 min at 94 °C for initial denaturation, 40 cycles of 30 s at 94 °C, 30 s at 50 °C and 30 s at 68 °C, and a final extension at 68 °C for 10 min. The obtained amplicons were gel-purified, extracted using AxyPrep™ DNA Gel Extraction Kit (Axygen Biosciences), quantified by UV–vis spectroscopy (NanoDrop-1000, Thermo Scientific, Wilmington, DE, USA), pooled in an equimolar mix and sent to Macrogen Inc. (Seoul, Republic of Korea) for the final steps in the Roche 454 GS-FLX pyrosequencing process: amplicon library construction, emPCR and sequencing. Pyrosequencing raw reads were deposited in the NCBI Short-Read Archive under accession number SRA061963.

2.4. Data analysis

Mothur v.1.22.2 was used to denoise, trim, filter and align sequences, find chimeras, assign sequences to operational taxonomic units (at 97% and 95% similarity), and describe alpha-diversity (Schloss et al., 2009), following the standard operation procedure suggested by the program's author. Briefly, after extracting fasta, flow and quality files from the raw Standard Flowgram Format (sff) file, the data was denoised with the AmpliconNoise algorithm (Quince et al., 2011). Quality-filtered sequences (minimum length 200 bp, with no ambiguous bases and no more than 1 and 2 mismatches to the barcode and primer, respectively, and homopolymers of 8 bp as a maximum) were separated by primer and barcode, and then trimmed (Schloss et al., 2011). Forward primer sequences were aligned to the SILVA-database reference alignment v 102. Sequences outside the most represented alignment space were removed. Chimeras identified with the 'uchime' algorithm were also removed. For the remaining sequences, the trimmed raw FASTA reads were classified against 16S rRNA RDP database, training set 9, with a bootstrap cutoff of 50%. Because the comparison of our data with datasets from other labs could only be based on classified

taxa, the confidence threshold used for classification of the sequences was kept at 50% as a trade-off between maintaining relatively high assignment accuracy and maximizing the number of classifiable sequences.

The filtered reads were also used for generating a distance matrix and then clustered into OTUs (defined at 3% and 5% cutoff) by average neighbor linkage. The program built a table indicating the abundances of OTUs in each sample. For normalization, 5000 sequences were randomly subsampled from each group using Mothur's *sub.sample* function, except sample corresponding to the oil refinery in year 2011 (REF-11), which only had 4753 sequences left. The sequence processing profile is shown in Table S2.

Rarefaction curves were built with a sampling iteration of 1000. Shannon-Weaver index was calculated in a collecting sampling way, every 100 sequences, and then plotted against sampling effort. OTUs defined at 3 and 5% cutoff were also used to calculate Good's coverage, Chao1 and ACE indices.

Data from municipal wastewater treatment plants from three different laboratories were included for comparison with the data from this study. These were a full-scale anaerobic/anoxic/oxic (A/A/O) plant in Guangzhou, China (CN-GZ-DT) (Zhang et al., 2012), a CAS + MBR full-scale plant in Singapore (SG-SG-UP) (Zhang et al., 2012), a full-scale A/A/O in Wuxi, China (Wang et al., 2012a), a full-scale A/A/O + MBR plant in Wuxi, China (Wang et al., 2012a), and a A/A/O pilot plant for domestic sewage in Korea (Kim et al., 2013a). Whereas the latter study sequenced the same region of the 16S rRNA gene that we did (hypervariable region V1-V3), the former two studies used the V4 region of the 16S gene. The datasets of Zhang et al. (2012) and Kim et al., 2013a, whose raw sequences were available in the NCBI Short-Read Archive (SRA026842.2 and SRA050633, respectively), were downloaded and processed through the Mothur pipeline together with the rest of the samples from this study. On the other hand, since the dataset of the work by Wang et al. (2012a) was not publicly available, we have reconstructed the high rank information based on the tables of relative abundance of classified genera. Comparison of taxa distribution between samples were next performed using only classified sequences.

The square root transformed data of abundance of bacterial phyla, classes and genera were loaded in the program PAST to perform a hierarchical cluster analysis using Bray–Curtis similarity measure, UPGMA linkage algorithm and a bootstrap of $N = 100$.

Ordination analyses were performed using the 'vegan' package version 1.15-1 (Department of Statistics, Iowa State University, Ames, IA, USA) in R 2.15.1, using default parameters. Rare classes, with less than 0.5% abundance, and unclassified bacteria were excluded from the analysis. A square root transformation was applied to the classes relative abundances to down-weight the effect of dominant taxa.

Principal Coordinates Analysis (PCoA) was used for the visualization of distances between samples. We have applied the Constrained Analysis of Principal Coordinates (CAP) to visualize overall patterns of dispersion among communities from the different WWTPs and to examine whether bacterial communities correlate with the main process operational variables. CAP is a constrained ordination method similar to Redundancy Analysis, which is gaining attention in community ecology because it can be applied to non-Euclidean dissimilarity indices, like Jaccard, Bray-Curtis or phylogenetic distances (Anderson and Willis, 2003). It has recently been used to explain observed phylogenetic differences between anaerobic bioreactors on the basis of a subset of measured environmental gradients (Werner et al., 2011). For this analysis we have used the Bray–Curtis distance, a common metric in community ecology, which takes into account the presence and abundance of taxa. Because our data were obtained using the V1–V3 variables region of the 16S rRNA gene and the data from the papers by Zhang et al. (2012) and Wang et al. (2012a) were obtained using the variable V4 region, we could not evaluate variations in phylogenetic structure between samples using phylogenetic distances.

Statistical comparisons of Shannon index and Chao1 were performed using analysis of variance (ANOVA). Because the raw dataset from Wang et al was not available, those data were not included in this analysis.

3. Results

3.1. Analysis of high bacterial taxa

Table 1 and Table S1 describe the main characteristics of the seven industrial and one domestic activated sludge plants, and contain the operational data averaged over one month prior to sampling. Six of the WWTPs were sampled two times several months apart (see Table S1 for details). A total of 156.988 sequences distributed among the 14 samples remained available for analysis after filtering. To ensure even coverage across samples, a set of 5000 sequences per sample, ranging between 200 and 530 bp, with an average length of 423 bp, was used for taxonomical classification.

Even though the value of Good's coverage indicated that all samples were sequenced at high depth (Table S2), rarefaction curves do not approach saturation at a cut-off value of 97% similarity (Fig S1), and hence some bacterial taxa still may have remained undetected, especially those present as minor populations. Samples from industrial activated sludge exhibited significantly lower diversity than samples from the domestic activated sludge (Fig S2).

Fig. 1 and Fig. 2 show the analysis of bacterial composition at the phylum and class levels. Each activated sludge sample had a characteristic distribution of high rank taxa, according to their origin. No single phylum exhibited similar abundance across all the samples. The class *Sphingobacteria* within the phylum *Bacteroidetes*, was highly represented in most samples (10–50%), but was only a minor component of the sludge from the polymer industry (ca. 1%). In opposition, members of the phyla *Acidobacteria* (Gp4 group) were dominant in the WWTP from polymer industry (10 and 56%), relatively abundant in the textile dyeing WWTP (up to 10%), but much less abundant in the rest of the examined plants. Within the Proteobacteria, the class *Betaproteobacteria* was highly represented in several samples (12–40%), but had a rather low abundance in textile and whey processing AS (1–9%). The orders *Burkholderiales*, *Hydrogenophilales* and *Rhodocyclales* account for the majority of reads within this class. *Gammaproteobacteria* fluctuated between the dominance observed in the polymer industry plant

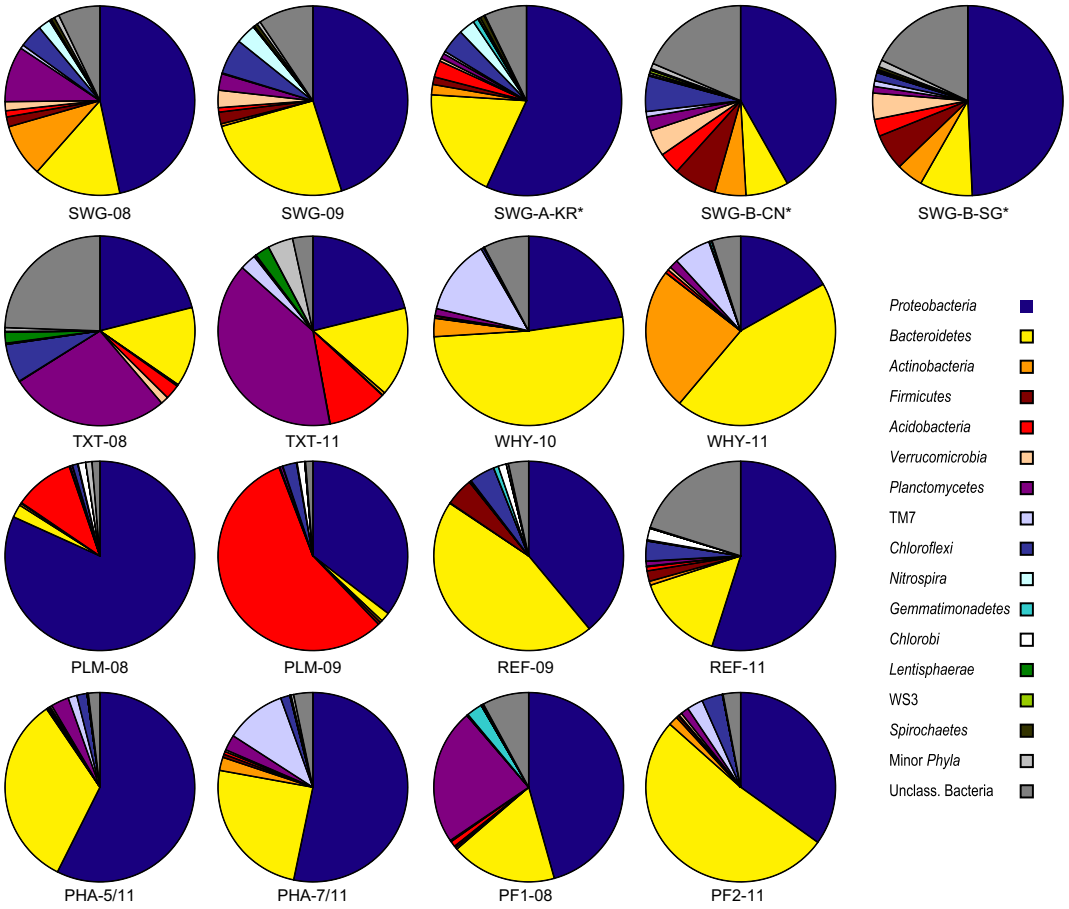

Fig. 1 – Pie chart showing the pattern of bacterial distribution in activated sludge systems. Each pie chart represents the relative abundance of different bacterial phyla at the WWTP indicated above. Rare phyla with less than 1% abundance were grouped as "Minor Phyla". SWG samples marked with * were taken from Kim et al. (2013a) (SWG-A-KR) and Zhang et al., 2012 (SWG-B–CN: CN-GZ-DT, SWG-B-SG: SG-SG-UP).

(27%) and the poor representation found in textile and pharmaceutical samples (1–3%), whereas in the remaining WWTPs analyzed showed moderate abundance (3–14%). *Actinobacteria* were detected in all WWTPs but presented particularly high abundance values at single samples of sewage and whey AS (9 and 24%). *Planctomycetes* were frequently detected in the textile dyeing plant (27 and 40%), and had moderate abundance in others, such as sewage (3 and 10%), pharmaceutical (2%) and pet food (21%). TM7 was over-represented in the whey processing activated sludge (6 and 13%), and was also present in the pharmaceutical plant (2 and 10%), and in one of the pet food plants (3%).

Other phyla occurred only at specific sites. This is the case of *Verrucomicrobia* and *Spirochaetes*, which occurred in sewage treatment facilities (2–3% and 1%, respectively), and *Chlorobi*, which was detected AS treating petrochemical wastewater: oil refinery (1–2%) and polymer synthesis wastewater (1%). Lastly, a rare phylum called *Lentisphaerae* was found with moderate abundance (2–3%), only in the WWTP of the textile dyeing facility.

Dendrograms prepared by using the Bray–Curtis similarity index show that at the class level samples clustered with high bootstrap values according to WWTP identity (Fig. 2). Also included in Fig. 2 are the data from municipal sewage treatment plants published by three different laboratories, including two full-scale WWTPs in China and Singapore (Zhang et al., 2012), two full-scale WWTPs in China (Wang et al., 2012a) and a pilot A/A/O process in Korea (Kim et al., 2013a). The five samples clustered with the domestic WWTP evaluated in this study.

The dendrogram of phyla is showed in Supplementary Fig S3. The low bootstrap values revealed uncertain branching patterns. We deem that our ability to distinguish the actual patterns at this taxonomic level is limited because only a few phyla dominate microbial communities in wastewater treatment plants.

3.2. *Analysis of bacterial community structure at the genus level*

The number of classified sequences varied greatly between samples, ranging from 34 to 41% in samples from the petroleum refinery to 72–89% in the samples from the acrylic polymer industry (Table S3). Sequences classified at the genus level were compared using the Bray-Curtis similarity index. The values where then used to build a second dendrogram that is shown integrated with a heatmap of the most

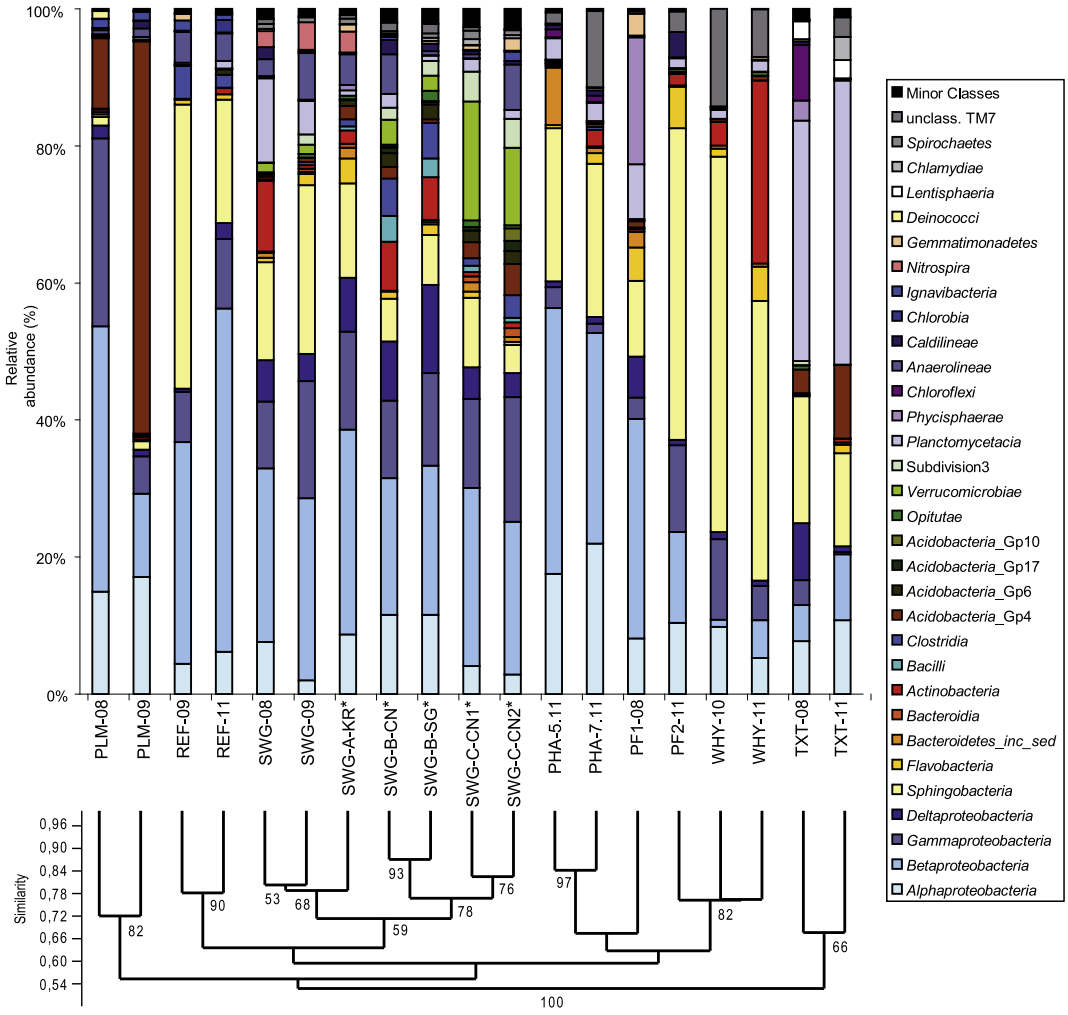

Fig. 2 – Patterns of bacterial classes distribution in activated sludge samples. Cluster analysis was performed based on Bray-Curtis similarity index and average neighbor linkage method, without including unclassified bacteria. Square root of relative abundance was used as input. Bootstrap values above 50 are shown. Data with an asterisk were taken from Kim et al (2013a), Zhang et al. (2012), and Wang et al. (2012a) and renamed (see Fig. 1; SWG-C-CN1: D2, SWG-C-CN2: E1).

abundant genera (Fig. 3). The similarity between samples decreased going from higher to lower taxonomic levels. However, the clustering by WWTP was still supported, although with lower bootstrap values and a clear separation between municipal samples according to the variable region targeted for sequencing (Kumar et al., 2011).

At the genus level we could not detect a core of taxa common to all the investigated systems. There were, though, common genera, such as *Haliscomenobacter* or *Thauera*, shared by several treatment plants. Some genera were shared by several communities, but were particularly abundant in some of the systems. Among these were Gp4, of the *Acidobacteria*, which was abundant in the polymer synthesis and textile dyeing WWTPs. Other genera were present in particular systems, in which they displayed high abundance. These were the case for *Lewinella*, *Micropruina* and *Olivibacter* and *Tesarococcus*, which were abundant in the WWTP of the whey processing industry, *Thiobacillus* found in the activated sludge treating oil refinery wastewater and *Rudaea*, an abundant genus in the polymer synthesis activated sludge.

3.3. *Relationship between operational parameters and bacterial community structure*

Principal Coordinate Analysis (PCoA) together with Canonical Analysis of Principal Coordinates (CAP) was used to visualize overall patterns of dispersion among communities from the different WWTPs and to examine whether bacterial communities correlate with critical process operational variables (Fig. 4). In the unconstrained PCoA the two first ordination axis explained 45.7% of the variability observed in bacterial community structure (Fig. 4A), whereas the first two axes of the constrained coordinates of the same data (Fig. 4B and C) explained only 30.3% of the variation.

In Fig. 4D one sample from each of the six WWTPs was randomly included in the analysis to avoid violating the assumption of independence. Importantly, Fig. 4D maintains a similar ordination pattern as that of Fig. 4A and B, which were obtained using the complete set of samples. Bacterial structures of municipal wastewater treatment plants formed a relatively close cluster clearly separated along one or both

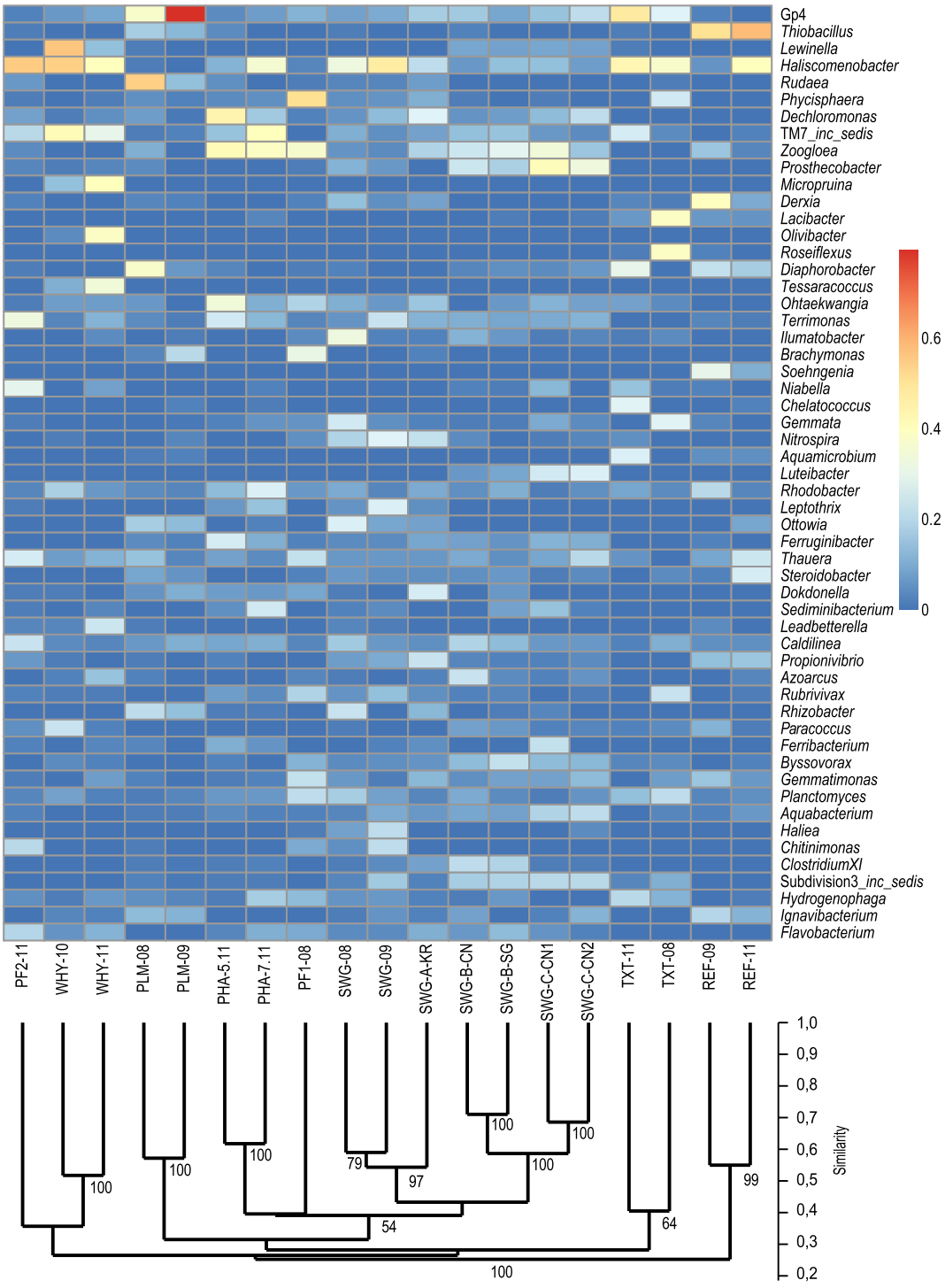

Fig. 3 – Heat map of the most abundant bacterial genera found in the full-scale activated sludge samples. Cluster analysis was performed on the 184 most represented genera (with abundance >0.5%), using the Bray-Curtis similarity index and average neighbor linkage method. Only the top 55 genera were used to build the heatmap.

axes from WWTPs treating industrial wastewater. According to the permutation test the significant underlying operational variables were the average levels of dissolved oxygen ($p = 0{,}01$) and pH ($p = 0.02$).

From Fig. 4C and Fig S4 it is also possible to infer the association of high rank taxa to particular sites. The phyla *Lentisphaera*, and to a lesser extent the class *Planctomycetacia*, are mostly confined to the WWTP treating textile wastewater. The phyla *Chlorobi*, were distinctive to the refinery wastewater treatment sludge. The class *Acidobacteria*-Gp4 was relatively associated to the WWTP that treats wastewater from the polymer synthesis plant.

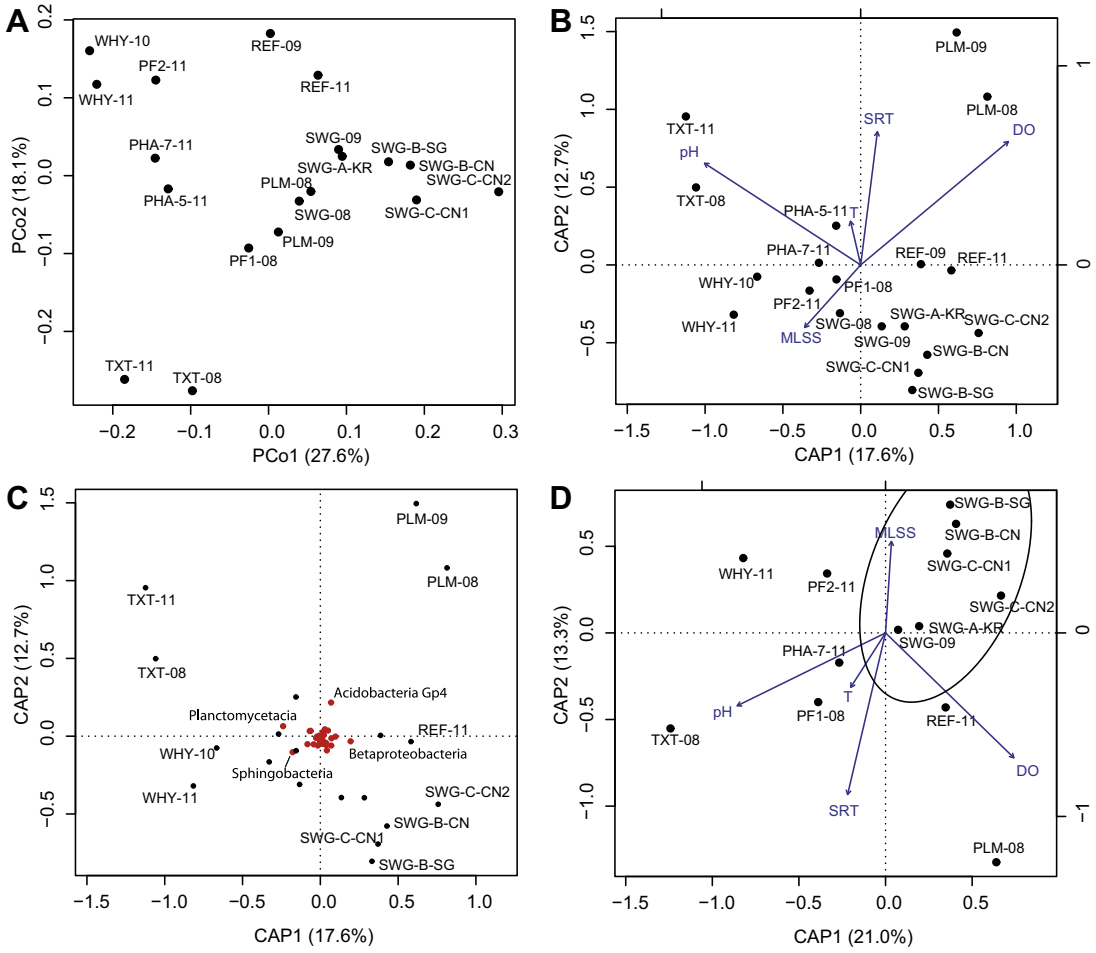

Fig. 4 – Ordination analysis of pyrosequencing data and operational variables in the activated sludge (AS) samples. A) Principal Coordinates Analysis (PCoA) using Bray-Curtis distance, based on the bacterial classes relative abundances. B) and C) Constrained Analysis of Principal Coordinates (CAP) using bacterial classes relative abundances and five measured operational parameters: pH, DO, temperature, SRT and MLSS. Arrows indicate the direction and magnitude of the operational variables association with bacterial community structures. For clarity, taxa were represented as red dots, and AS samples as black dots, and only high-score dots were labeled. The three-letter abbreviation indicates the origin of wastewater treated by the AS, which is followed by the year of sampling (see Table 1 and legends of figures 1 and 2). D) The same analysis as in B and C, in which only one sample from each of the six WWTPs was included. The ellipse indicates the 95% confidence interval for the sewage treatment plants. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

4.1. *Bacterial community structure in industrial activated sludge*

This work shows that industrial activated sludge systems do not share the characteristic profile of high bacterial rank commonly observed in municipal plants, but exhibit unique bacterial community composition. Earlier analysis of activated sludge performed in full-scale and lab-scale wastewater treatment plants indicated that bacterial communities were dominated by the phylum *Proteobacteria* and *Bacteroidetes*, followed by *Actinobacteria* and *Firmicutes* (Eschenhagen et al., 2003; Snaidr et al., 1997; Wagner and Loy, 2002). More recently, several high-throughput 16S rRNA gene-sequencing surveys executed worldwide confirmed the results of those previous studies (Sanapareddy et al., 2009; Valentin-Vargas et al., 2012; Wang et al., 2012a, 2012b; Xia et al., 2010; Yang et al., 2011; Ye et al., 2011; Yu and Zhang, 2012; Zhang et al., 2012). Remarkably, the distribution of bacteria in most municipal wastewater treatment plants was highly consistent at high taxonomical levels of organization, across a wide range of geography and reactor configurations. These observations are highly consistent with the concept of ecological coherence of bacterial groups at taxonomic ranks higher than the species (Philippot et al., 2010).

Our results also show close similarity between the bacterial composition of samples from a small WWTP treating domestic wastewater in Argentina and the published data of five municipal plants in Singapore, China and Korea (Kim et al., 2013a; Wang et al., 2012a; Zhang et al., 2012). It is extremely

interesting to note that this is true even when the data originated from multiple laboratories and the methods used for DNA extraction and the region of the 16S rRNA that were sequenced differed between the studies.

The distinction between community structures of municipal and industrial activated sludge clearly perceived at the class levels might be extended to lower taxonomic ranks as well, even though at the genus level biogeographical distribution of genera may be likely. Adding to the large differences in the percentage of classified genera between samples, and the uncertainty of the low bootstrap support, the clustering of genera appeared influenced by the 16S variable region targeted for sequencing, which may mask other effects on sample grouping (Kumar et al., 2011).

The formation of a biological floc that settles in a downstream basin is a characteristic ecological feature that distinguishes activated sludge communities. Therefore, following the notion of ecological coherence, one could have anticipated that the similar distribution of phyla and classes observed in geographically distributed biological wastewater treatment plants under varied configurations would be also maintained in industrial activated sludge. Yet no such pattern was found. On the contrary, each community clearly diverged according to their origin. High rank taxa occurred in a reproducible manner within each type of plant, even in samples separated by almost three years.

The tables of taxa abundance distribution mark a distinct preference of selected bacterial groups to particular plants, also visualized in the ordering of phyla in CCA (Fig S4). The WWTP treating textile-dyeing wastewater was distinguished by a considerable abundance of *Lentisphaera*, *Chlamydiae* and *Planctomycetes*, three phyla belonging to the monophyletic group referred as the PVC superphylum (Wagner and Horn, 2006). The poorly characterized phylum *Lentisphaera* have been related to the degradation of lignocellulose and/or conversion lignocellulolytic products within an anaerobic mesophilic lignocellulolytic consortium (Yan et al., 2012). Similarly, *Chlamydiae* were found to be part of a cultivated consortium adapted to grow on dried ground switchgrass as the sole carbon source (DeAngelis et al., 2010). This is remarkable because lignin-degrading enzymes are able to degrade a wide range of azo, heterocyclic and polymeric dyes (Nyanhongo et al., 2002). Further exploration of these understudied groups of bacteria may aid in the mining of genetic novelty with potential biotechnological application.

The characteristically high abundance of TM7 in samples of the whey processing industry and in pet food industry wastewater treatment activated sludge is consistent with the previous description of members of this phylum as specialized protein hydrolyzing organisms, a function that links them to protein turnover in activated sludge plants (Xia et al., 2007).

The fact that the phylum *Chlorobi* was only detected in WWTPs treating wastewater from petrochemical processes (petroleum refinery and acrylic polymers) can be related to the presence of sequences binned to *Chlorobi* in the recently published whole gene amplified (WGA) metagenome from a hydrocarbon-contaminated groundwater (Abbai et al., 2012). Relatively high proportion of *Acidobacteria* has already been associated to petrochemical wastewater treatment (Figuerola et al., 2007).

Understandably, this does not imply that reactor configuration and operation are unrelated with bacterial community composition. Recent high throughput studies aimed at defining the operational and environmental parameters that influence microbial community composition in activated sludge treating municipal wastewater (Kim et al., 2013b; Pholchan et al., 2010; Wells et al., 2011). It appeared that influent BOD, dissolved oxygen (Kim et al., 2013b; Wells et al., 2011), as well as reactor configuration (Pholchan et al., 2010) can be important factors shaping bacterial community structure at low taxonomic ranks. We have also observed that dissolved oxygen and pH are variables that may explain, to some extent, the bacterial composition in activated sludge. We note that the distribution of WWTPs along the ordination plot would be also compatible with a gradient of the biodegradability of influent wastewater. Unfortunately, data were not available for all WWTPs at the time of sampling. However, scattered data of the ratio of BOD/COD, a proxy for biodegradability, follows the increasing trend from textile dyeing and polymer synthesis WWTPs to whey processing plant.

The activated sludge treating domestic wastewater harbored higher diversity than industrial activated sludge. Yet to assess how the bacterial diversity affects the overall system performance is still less straightforward, as diversity and performance are not necessarily associated (Pholchan et al., 2010). As the dominant taxa in most samples were unique to each plant, we did not find a common population core between industrial and municipal systems. In opposition, it appears that there was higher consistency in the composition of bacterial community structure between geographical distant municipal WWTPs than between activated sludge treating different type of wastewater in proximate geographical areas.

The two samples from pet food industries examined in this study were not closely related. Major differences in the characteristics of the raw material used and the processes applied preclude us from drawing any conclusion about the observed divergence in bacterial composition.

The results of this work suggest that bacterial community structure is for the most part influenced by the wastewater type rather than by geographical distances or operation conditions. Performing a thorough chemical characterization of each industrial wastewater is not straightforward, as wastewater is largely a complex mixture of many different organic compounds. Yet a deeper characterization of the wastewater generated will be necessary to interpret the differences and similarities of bacterial community structure of several treatment plants for each industrial category.

On the other hand, the question of 'who is there' should naturally lead to the question of 'what can they do'. Whether the observed taxonomic differences point to a concomitant divergence in metabolic functions will require further investigation on the ensuing metagenomic profile of each individual system.

4.2. *Implications for industrial activated sludge design and operation*

Mechanistic models, like the Activated Sludge Model No. 1 (ASM1) or ASM3 are valuable tools for the representation of the biological processes taking place in bioreactors, critical for the design and evaluation of the activated sludge process

(see (Hauduc et al., 2013) for a recent review) For successful application in process optimization, calibration of the models requires the experimental determination of relevant data on process stoichiometry and kinetics obtained from the full-scale WWTP under study (Sin et al., 2005). Similarly, respirometry is commonly used for the experimental assessment of wastewater characteristics as well as of the active heterotrophic biomass, a significant component of the model (Hauduc et al., 2013). For the design of new plants, default parameters set for municipal cases are typically used (Insel et al., 2012). When using ASM models for biological treatment of industrial wastes, design engineers are usually aware of the differences in composition between industrial and domestic wastewater, including the higher incidence of inhibitors in the former (Boursier et al., 2004; Stricker and Racault, 2005; Ubay Cokgor et al., 2009). However, the critical differences in the composition of the population of heterotrophs that grow aerobically and are responsible for BOD removal in municipal and industrial wastewater treatment plants might not be well recognized. We suggest that major differences in biomass composition in industrial activated sludge, compared to municipal activated sludge may be relevant because they could be reflecting changes in the kinetics of substrate consumption and storage products formation.

5. Conclusions

The following conclusions can be drawn from this study:

1. The activated sludge treating domestic wastewater harbored higher diversity than industrial activated sludge.
2. No common population core was found between industrial and municipal systems.
3. High rank taxa occurred in a reproducible manner within each plant in samples separated by up to three years.
4. There was higher consistency in the composition of bacterial community structure between geographical distant municipal WWTPs than between activated sludge treating different type of wastewater in proximate geographical areas.
5. There is a distinct preference of selected bacterial groups to particular wastewater treatment plants.
6. Differences in the bacterial community structure between industrial wastewater treatment plants were partly explained by dissolved oxygen and pH.
7. Major differences in the composition of the population of heterotrophs responsible for BOD removal in industrial activated sludge might be worth considering for experimental assessment of process kinetics.

Acknowledgments

We thank staff members in all the wastewater treatment plants for making available for us the samples and the operational data. This work was partly funded by FONCyT (PICT .2008 No. 1950) and UBACYT. F.M.I. is a fellow, and E.L.M.F. and L.E. are investigators from the Consejo Nacional de Investigaciones Científicas y Técnicas of Argentina (CONICET).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2013.04.010.

REFERENCES

Abbai, N.S., Govender, A., Shaik, R., Pillay, B., 2012. Pyrosequence analysis of unamplified and whole genome amplified DNA from hydrocarbon-contaminated groundwater. Molecular Biotechnology 50, 39–48.

Ayarza, J.M., Erijman, L., 2011. Balance of neutral and deterministic components in the dynamics of activated sludge floc assembly. Microbial Ecology 61, 486–495.

Ayarza, J.M., Guerrero, L.D., Erijman, L., 2010. Nonrandom assembly of bacterial populations in activated sludge flocs. Microbial Ecology 59, 436–444.

Anderson, M.J., Willis, T.J., 2003. Canonical analysis of principal coordinates: a useful method of constrained ordination for ecology. Ecology 84, 511–525.

Boursier, H., Beline, F., Paul, E., 2004. Activated sludge model no. 1 calibration for piggery wastewater treatment using respirometry. Water Science and Technology 49, 389–395.

Bramucci, M., Nagarajan, V., 2006. Bacterial communities in industrial wastewater bioreactors. Current Opinion in Microbiology 9, 275–278.

DeAngelis, K.M., Gladden, J.M., Allgaier, M., D'haeseleer, P., Fortney, J.L., Reddy, A., Hugenholtz, P., Singer, S.W., Vander Gheynst, J.S., Silver, W.L., Simmons, B.A., Hazen, T.C., 2010. Strategies for enhancing the effectiveness of metagenomic-based enzyme discovery in lignocellulolytic microbial communities. Bioenergy Research 3, 146–158.

Degenaar, A.P., Ismail, A., Bux, F., 2008. Comparative evaluation of the microbial community in biological processes treating industrial and domestic wastewaters. Journal of Applied Microbiology 104, 353–363.

Eschenhagen, M., Schuppler, M., Roske, I., 2003. Molecular characterization of the microbial community structure in two activated sludge systems for the advanced treatment of domestic effluents. Water Research 37, 3224–3232.

Figuerola, E.L., Erijman, L., 2007. Bacterial taxa abundance pattern in an industrial wastewater treatment system determined by the full rRNA cycle approach. Environmental Microbiology 9, 1780–1789.

Hauduc, H., Rieger, L., Oehmen, A., van Loosdrecht, M.C., Comeau, Y., Heduit, A., Vanrolleghem, P.A., Gillot, S., 2013. Critical review of activated sludge modeling: state of process knowledge, modeling concepts, and limitations. Biotechnology and Bioengineering 110, 24–46.

Hu, M., Wang, X., Wen, X., Xia, Y., 2012. Microbial community structures in different wastewater treatment plants as revealed by 454-pyrosequencing analysis. Bioresource Technology 117, 72–79.

Insel, G., Guder, B., Gunes, G., Ubay Cokgor, E., 2012. Are standard wastewater treatment plant design methods suitable for any municipal wastewater? Water Science and Technology 66, 328–335.

Juretschko, S., Loy, A., Lehner, A., Wagner, M., 2002. The microbial community composition of a nitrifying-denitrifying activated sludge from an industrial sewage treatment plant analyzed by the full-cycle rrna approach. Systematic and Applied Microbiology 25, 84–99.

Kaewpipat, K., Grady, C.P.L., 2002. Microbial population dynamics in laboratory-scale activated sludge reactors. Water Science and Technology 46, 19–27.

Kim, B.C., Kim, S., Shin, T., Kim, H., Sang, B.-I., 2013a. Comparison of the bacterial communities in anaerobic, anoxic, and oxic chambers of a pilot A_2O process using pyrosequencing analysis. Current Microbiology 66, 555–565.

Kim, T.S., Jeong, J.Y., Wells, G.F., Park, H.D., 2013b. General and rare bacterial taxa demonstrating different temporal dynamic patterns in an activated sludge bioreactor. Applied Microbiology and Biotechnology 97, 1755–1765.

Kumar, P.S., Brooker, M.R., Dowd, S.E., Camerlengo, T., 2011. Target region selection is a critical determinant of community fingerprints generated by 16S pyrosequencing. PLoS ONE 6, e20956.

Langenheder, S., Szekely, A.J., 2012. Species sorting and neutral processes are both important during the initial assembly of bacterial communities. The ISME Journal 5, 1086–1094.

Nyanhongo, G.S., Gomes, J., Gubitz, G.M., Zvauya, R., Read, J., Steiner, W., 2002. Decolorization of textile dyes by laccases from a newly isolated strain of *Trametes modesta*. Water Research 36, 1449–1456.

Ofiteru, I.D., Lunn, M., Curtis, T.P., Wells, G.F., Criddle, C.S., Francis, C.A., Sloan, W.T., 2010. Combined niche and neutral effects in a microbial wastewater treatment community. Proceeding of the National Academy of Sciences USA 107, 15345–15350.

Orhon, D., Babuna, F.G., Karahan, O., 2009. Industrial Wastewater Treatment by Activated Sludge. International Water Association, London, United Kingdom.

Philippot, L., Andersson, S.G., Battin, T.J., Prosser, J.I., Schimel, J.P., Whitman, W.B., Hallin, S., 2010. The ecological coherence of high bacterial taxonomic ranks. Nature Reviews Microbiology 8, 523–529.

Pholchan, M.K., Baptista Jde, C., Davenport, R.J., Curtis, T.P., 2010. Systematic study of the effect of operating variables on reactor performance and microbial diversity in laboratory-scale activated sludge reactors. Water Research 44, 1341–1352.

Quince, C., Lanzen, A., Davenport, R.J., Turnbaugh, P.J., 2011. Removing noise from pyrosequenced amplicons. BMC Bioinformatics 12, 38.

Sanapareddy, N., Hamp, T.J., Gonzalez, L.C., Hilger, H.A., Fodor, A.A., Clinton, S.M., 2009. Molecular diversity of a north carolina wastewater treatment plant as revealed by pyrosequencing. Applied and Environmental Microbiology 75, 1688–1696.

Schloss, P.D., Gevers, D., Westcott, S.L., 2011. Reducing the effects of PCR amplification and sequencing artifacts on 16S rRNA-based studies. PLoS ONE 6, e27310.

Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., Lesniewski, R.A., Oakley, B.B., Parks, D.H., Robinson, C.J., Sahl, J.W., Stres, B., Thallinger, G.G., Van Horn, D.J., Weber, C.F., 2009. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Applied and Environmental Microbiology 75, 7537–7741.

Sin, G., Van Hulle, S.W., De Pauw, D.J., van Griensven, A., Vanrolleghem, P.A., 2005. A critical comparison of systematic calibration protocols for activated sludge models: a SWOT analysis. Water Research 39, 2459–2474.

Snaidr, J., Amann, R., Huber, I., Ludwig, W., Schleifer, K.H., 1997. Phylogenetic analysis and in situ identification of bacteria in activated sludge. Applied and Environmental Microbiology 63, 2884–2896.

Stricker, A.E., Racault, Y., 2005. Application of activated sludge model no. 1 to biological treatment of pure winery effluents: case studies. Water Science and Technology 51, 121–127.

Ubay Cokgor, E., Sozen, S., Insel, G., Orhon, D., 2009. Respirometric evaluation of biodegradation characteristics of dairy wastewater for organic carbon removal. Environmental Technology 30, 1169–1176.

Valentin-Vargas, A., Toro-Labrador, G., Massol-Deya, A.A., 2012. Bacterial community dynamics in full-scale activated sludge bioreactors: operational and ecological factors driving community assembly and performance. PLoS ONE 7, e42524.

van der Gast, C.J., Ager, D., Lilley, A.K., 2008. Temporal scaling of bacterial taxa is influenced by both stochastic and deterministic ecological factors. Environmental Microbiology 10, 1411–1418.

Wagner, M., Loy, A., 2002. Bacterial community composition and function in sewage treatment systems. Current Opinion in Biotechnology 13, 218–227.

Wagner, M., Horn, M., 2006. The Planctomycetes, Verrucomicrobia, Chlamydiae and sister phyla comprise a superphylum with biotechnological and medical relevance. Current Opinion in Biotechnology 17, 241–249.

Wang, X., Hu, M., Xia, Y., Wen, X., Ding, K., 2012a. Pyrosequencing analysis of bacterial diversity in 14 wastewater treatment systems in china. Applied and Environmental Microbiology 78, 7042–7047.

Wang, X., Wen, X., Criddle, C., Yan, H., Zhang, Y., Ding, K., 2012b. Bacterial community dynamics in two full-scale wastewater treatment systems with functional stability. Journal of Applied Microbiology 109, 1218–1226.

Wells, G.F., Park, H.D., Eggleston, B., Francis, C.A., Criddle, C.S., 2011. Fine-scale bacterial community dynamics and the taxa-time relationship within a full-scale activated sludge bioreactor. Water Research 45, 5476–5488.

Werner, J.J., Knights, D., Garcia, M.L., Scalfone, N.B., Smith, S., Yarasheski, K., Cummings, T.A., Beers, A.R., Knight, R., Angenent, L.T., 2011. Bacterial community structures are unique and resilient in full-scale bioenergy systems. Proceeding of the National Academy of Sciences USA 108, 4158–4163.

Xia, S., Duan, L., Song, Y., Li, J., Piceno, Y.M., Andersen, G.L., Alvarez-Cohen, L., Moreno-Andrade, I., Huang, C.L., Hermanowicz, S.W., 2010. Bacterial community structure in geographically distributed biological wastewater treatment reactors. Environmental Science and Technology 44, 7391–7396.

Xia, Y., Kong, Y., Nielsen, P.H., 2007. In situ detection of protein-hydrolysing microorganisms in activated sludge. FEMS Microbiology Ecology 60, 156–165.

Yan, L., Gao, Y., Wang, Y., Liu, Q., Sun, Z., Fu, B., Wen, X., Cui, Z., Wang, W., 2012. Diversity of a mesophilic lignocellulolytic microbial consortium which is useful for enhancement of biogas production. Bioresource Technology 111, 49–54.

Yang, C., Zhang, W., Liu, R., Li, Q., Li, B., Wang, S., Song, C., Qiao, C., Mulchandani, A., 2011. Phylogenetic diversity and metabolic potential of activated sludge microbial communities in full-scale wastewater treatment plants. Environmental Science and Technology 45, 7408–7415.

Ye, L., Shao, M.F., Zhang, T., Tong, A.H., Lok, S., 2011. Analysis of the bacterial community in a laboratory-scale nitrification reactor and a wastewater treatment plant by 454-pyrosequencing. Water Research 45, 4390–4398.

Yu, K., Zhang, T., 2012. Metagenomic and metatranscriptomic analysis of microbial community structure and gene expression of activated sludge. PLoS ONE 7, e38183.

Zhang, T., Shao, M.F., Ye, L., 2012. 454 pyrosequencing reveals bacterial diversity of activated sludge from 14 sewage treatment plants. The ISME Journal 6, 1137–1147.