Microbial dynamics of the main bacterial groups in an anaerobic pond treating landfill leachate

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Abstract Landfill leachate, that comes from the percolation of rain water and the water released during waste decomposition process, has been identified as a potential source of soil, surface, and ground water pollution, with strong variations in its composition and quality. Stabilization ponds, due to their low cost of implantation, maintenance and operation, have been widely used for landfill leachate treatment. An anaerobic pond of a pilot system with three stabilization ponds (anaerobic + facultative + maturation) was evaluated from December, 2007 until May 2008. Microbial diversity in the anaerobic pond was analyzed every 15 days, using fluorescent *in situ* hybridization (FISH), polymerase chain reaction (PCR), sequencing and phylogenetic analysis. Therefore its occurrence was compared with physical-chemical variation. Results revealed the predominance of *Pseudomonas*, *Desulfovibionaceae,* archaea and nitrificants bacteria. Besides this, the results were negative for *Anammox* and there were only a few cells of nitrifying bacteria.
Keywords Bacteria, FISH, sequencing and phylogenetic analysis.

INTRODUCTION

Sanitary landfills are a widely used method for solid waste disposal around the world. In many Brazilian sanitary landfills containing urban solid wastes, operational, implementation, and management criteria have not been adopted. Landfill leachate is a wastewater released from sanitary landfills treating a variety of municipal and industrial solid wastes. Due to the anaerobic conditions and the long retention time prevailing in sanitary landfills, landfill leachate normally contains high concentrations of organic matter, nutrients, pathogens and heavy metals which, if not correctly collected and treated, can cause serious pollution problems to nearby surface and groundwater sources (Polprasert and Sawaittayothin, 2006).

Several physical, chemical and biological processes can be applied in the treatment of these types of effluents, being most common publications including biological and physical-chemical treatment. Stabilization ponds technology is one of the most important natural methods for wastewater treatment and has been used in all Brazilians regions and it is also used to treat leachate in many sanitary landfills (Hamada and Matsunaga, 2000). In these systems the treatment occurs by biochemical, biological and physical-chemical processes regulated by a diversity of organisms which are responsible for the natural organic matter and nutrient recycling. This kind of system is sensitive to climatic conditions that regulate the activity of bacteria and algae involved in the treatment process.

The knowledge of microbiology and ecology of microorganisms involved in biological processes for landfill leachate depuration becomes relevant for treatment control and efficiency because it allows to evaluate their interaction with the environment and others organisms. The identification

and physiology knowledge can show the role of each one in different treatment steps as well as individualize it for a specific effluent. Fluorescence *in situ* hybridization (FISH) and Polymerase Chain Reaction (PCR) are widely used to describe bacterial community composition and, sometimes, to describe the cell physiological state. These methodologies are now used to describe the temporal and spatial distribution of aquatic bacteria and the specific roles of microbes in biogeochemical cycles and food dynamics (Bouvier and Giorgio, 2003).

The present work studied one pond of a serial pilot system, which includes anaerobic, facultative, and maturation ponds. The research aimed to characterize the microbial community in anaerobic pond, for 6 months, and relate them to physical-chemical parameters.

MATERIALS AND METHODS

Pond system and samples

The experiment was conducted at the LARESO (Laboratory of Research in Solid Wastes) from Federal University of Santa Catarina – UFSC, Florianópolis - SC/Brazil between December, 2007 and May, 2008. This study used the first (anaerobic) pond of a serial pilot system that includes anaerobic (AP), facultative (FP) and maturation (MP) fiberglass like ponds. The ponds were fed with raw effluent (leachate) from a sanitary landfill that receives urban solid waste from 14 cities around Florianópolis. The influent flow rate is 200 L/day. The system has been in operation since August, 2007. Table 1 summarizes the characteristics of the anaerobic pond.

Table 1. Physical characteristics of the anaerobic pond.

Dimensions	Anaerobic Pond
Depth (m)	2.0
Diameter	1.85
Volume (m^3)	5.0
Hydraulic Detention Time (d)	25

The samples were collected, every 15 days, at 02:00 pm. Dissolved oxygen (DO) mg/L, pH, potential of oxide reduction (ORP) mV, conductivity mS/cm and temperature ^oC, were measured *in situ* using multiparameters equipment YSI 6820 V2. In the laboratory ammonium, Chemical Oxygen Demand (COD), Biochemical Oxygen Demand (BOD_5) and Suspended Solids (SS) were measured according to Standard Methods (2005).

Fluorescence *in situ* hybridization (FISH) analysis

The evolutionary relationships (based on 16S rDNA phylogeny) of the nitrogen cycle chemolithoautotrophs are relevant to wastewater treatment, because 16S rDNA-based probing of these organisms population has been remarkably successful. Such 16S rDNA probes have been used to quantify the amounts of nitrifiers or anaerobic ammonia oxidizers in wastewater treatment plants (Gieseke *et al*., 2001; Schmid *et al.,* 2001). The probes were used to measure the *in situ* growth rates of relevant organisms in the actual plant

The FISH method involves oligonucleotide probes application to permeabilized whole microbial cells and, specifically hybridized the cells with their complementary target sequence in the ribosomes. In this study, the samples obtained in anaerobic pond were determined in steady state condition (02:00 pm). The collected samples were put in sterile microtubes and transported to the laboratory for FISH analyses.

The samples were fixed overnight in 4% paraformaldehyde-phosphate-buffered saline at 4°C. Fixed cells were spotted on gelatin-coated 0.6% gelatin and 0.06% $KCr(SO_4)_2$ glass slides (10 slides; 10µL of sample) and allowed to dry in a sterile hood (Raskin *et al.*, 1994). Hybridizations were performed at 46°C for 2 hours with a hybridization buffer (5M NaCl, 1M Tris/HCl, pH 8.0, 0.01% SDS) containing each labeled probe (50ng/µL for Cy3). For bacteria identification, probes were used according to table 2. Formamide was added to the final concentrations to ensure the optimal hybridization stringency. After hybridization, unbound oligonucleotides were removed by rinsing with washing buffer containing the same components as the hybridization buffer except for the probes. For detection of all DNA, 4,6-diamidino-2-phenylindole (DAPI) was added to the wash buffer (10µL of 0.1% DAPI). Slides were subsequently incubated at 48°C for 20 min with washing buffer, rinsed briefly with distillated H_2O and immediately air-dried. Antifading solution was used to prevent photo bleaching. The slides were examined with an Olympus BX40 microscope.

Table 2. Probe sequences used for *in situ* hybridization.

Probe name	Sequence	Reference
EUB mix (I+II+III)[1]	5'-GCTGCCTCCCGTAGGAGT-3' 5'-CAGCCACCCGTAGGTGT-3' 5'-CTGCCACCCGTAGGTGT-3'	[1]Polprasert and Sawaittayothin (2006)
NEU[1]	5'-CCCCTCTGCTGCACTCTA-3'	
AMX820[1]	5'-AAAACCCCTCTACTTAGTGCCC-3'	
Eury 499[1]	5'-CGGTCTTGCCCGGCCCT-3'	
DSV 407[1]	5'-CCGAAGGCCTTCTTCCCT-3'	
ARC 915	5'-GTGCTCCCCCGCCAATTCCT-3'	Stahl et al. (1991)
NSO 190	5'-CGATCCCCTGCTTTTCTCC-3'	Mobarry et al. (1996)

DNA extraction

The DNA extraction was performed as previously described (Leão *et al.*, 1999) using 400µL of TE/Triton 1% (10mM de Tris-EDTA pH 8.0, 1% de TRITON 100X) following three alternate cycles of boiling (100°C) and freezing (-80°C) for 10 minutes each.

Amplification, DNA sequencing and phylogenetic analysis

The DNA was amplified by PCR technique performed as followed: 3ng of DNA, 10% PCR buffer (Invitrogen), 2mM $MgCl_2$, 2mM each deoxyribonucleotide, 10pmoles each primer (forward 436For 5'- GAGCGGTGAAATGCG-3' and reverse 436Rev 5'-GGGTTTCGCTCGTTA-3'), 2.5 U Taq DNA polimerase and ultra pure water q.s.p. 25µL. PCR was carried out according to the following parameters: a denaturation step at 95ºC for 3 minutes, followed by 30 cycles of denaturation at 95°C for 45 seconds, annealing at 57°C for 1 minute, extension at 72°C for 1 minute and a final extension step at 72°C for 7 minutes. The reaction was analyzed by 1% agarose gel electrophoresis stained with ethidium bromide (0.5µg/mL), visualized under UV light (Sambrook et al., 2001). The amplified DNA was purified with GFX PCR and Gel DNA purification kit (GE Healthcare, Giles, United Kingdom). Sequencing was carried out with the DYEnamic ET terminators sequencing kit (GE Healthcare, Giles, United Kingdom) following the manufacturer´s protocol. Sequence determination was performed in a 3130 XL automatic sequencer (Applied Biosystems).

The quality of DNA sequences was checked and overlapping fragments were assembled using the BioEdit package 7.0.5 (Hall, 1999), Vector NTI 8.0, AlignX and ContigExpress (InforMax, Inc.). Assembled sequences with high quality were aligned using Clustal W (Thompson et al., 1994) with default gap penalties. Homologies analyses were performed with the NCBI database and BLAST (Altschul *et al.*, 1997).

Phylogenetic relationship between these protein sequences was performed with MEGA 4.0.2, neighbor-joining trees were constructed from Kimura-2 parameters and calculated using pairwise deletion. Bootstrap was resampled as a test of phylogeny using 250 replications.

RESULTS AND DISCUSSION

Table 3 shows the comparing results between raw influent and pond effluent during the studied period. Temperature, pH, DO and ORP had no significant variation. Suspended Solids had reduced values (from 438 to 352 mg/L). Mean temperature was 26.6ºC which is a good condition for biological process development in liquid material. However, the COD and BOD_5 parameters had a little decrease, while ammonium had a depressible reduction. These results are in accordance with the expected and are related to the microbial community present, however the effluent needs an aerobic treatment, aiming to reduce physical-chemical parameters, like ammonium.

Table 3. Parameters results of raw landfill leachate and pond. The results were expressed by mean ± Standard Deviation.

Parameters	Mean ± SD	
	RAW	AP
Suspended Solids (mg/L)	438 ± 206	352 ± 133
T (ºC)	25.9 ± 2.95	26.6 ± 3.09
pH	8.92 ± 0.17	9.17 ± 0.08
DO (mg/L)	0.75 ± 0.25	0.63 ± 0.13
ORP (mV)	-292 ± 34	-302 ± 26
COD (mg/L)	3251 ± 950	2518 ± 636
BOD_5 (mg/L)	1356 ± 608	827 ± 306
$N\text{-}NH_4$ (mg/L)	932 ± 236	918 ± 98

In the hybridization FISH assay with specific probes, Eubacteria group was found in high amounts (ratio of cells hybridized with EUB MIX probes and the cells stained with DAPI) during sampling, with mostly rod and colonial shapes. In the initial months, the average Eubacteria proportion was 80% (EUB/DAPI = 80/100). In the subsequent period, this percentage decreased to 30%. This relationship (EUB/DAPI) indicates a decrease in metabolic activity of the bacteria present in biological treatment systems (Amann *et al.,* 1995).

Nitrificant bacteria (*Nitrosomonas*, *Nitrobacter* and beta-proteobacteria), were found in small percentage, with maximum value of 10% until February, maintaining since then rare occurrence, this is probably related to low dissolved oxygen (near or equal to 0 mg/L). This bacteria rare occurrence can be also explained because the high pH found (9.2) increases free ammonia concentration (Table 3) which inhibits nitrifying organisms (Boothe *et al.*, 2001).

Archaea and methanogenic Archaea groups (*Methanosarina*, *Methanosaeta* and other groups Methanomicrobiales), were found around 35% of DAPI in January. Methanogens are strict anaerobes which share a complex biochemistry for methane synthesis as part of their energy metabolism. Methanogens exhibit rods, regular and irregular cocci, spirilla, sarcina and irregular unusual flattened plates. They can be Gran positive or negative even within members of the same genera (Beveridge and Schultze-Lam, 1996). This fact coincides with the low occurrence of Eubacteria, determining an inversely proportional relationship between these two major microbial groups. *Anammox* microrganisms were not detected in this study.

Sulfate-reducing bacteria (SRB) groups from genera *Desuifovibionaceae* were also found. Sulfate reduction is carried out by anaerobic, sulfate-reducing bacteria. SRB need sources of organic carbon (for biomass) and sulfates as electron acceptors for their metabolism. They oxidize organic compounds or H_2 with the sulfate reduction, producing sulfide. Generally, SRB do not grow well at pH values below 5.5 and prefer higher levels of alkalinity, with 6.6 being optimal (Govind *et al.*, 1999).

As the *Anammox* was not detected by FISH technique, we tested other molecular methodologies. A pair of specific primers was used to amplify bacterial 16S rDNA region with samples collected from May to June 2008. Sequencing and phylogenetic analysis of sample named APBG (Anaerobic Pond Bacterial Group) showed similarity with *Pseudomonas* sp. No Anammox bacteria like was detected by such procedures above mentioned (Figure 1).

Pseudomonas genera belongs to the gamma subgroup of *Proteobacteria.* They are rod Gran-negatives, heterotrophic, do not need a big amount of nutrient to survive and can be found on soil working as denitrifying organisms, in marine and freshwater (Ulbrich, 2004). Denitrification is the reduction of oxidized nitrogen compounds like nitrite or nitrate to nitrogen gas. This process is performed by various microrganisms, especially under oxygen-reduced or anoxic conditions. During the denitrification alkalinity production occurs. The carbonic acid (H_2CO_3) is converted to bicarbonate (HCO_3^-) as a result of the nitrate conversion to nitrogen gas. Favorable denitrification conditions are pH around 8.0, temperature around 35°C, nitrates or nitrites presence and sources of carbonaceous matter of fast degradation. These characteristics show that the microorganisms present in the system are contributing to inicial leachate treatment, reducing main raw matter indicators parameters (COD and BOD_5), however it is a low reduction (Table 3).

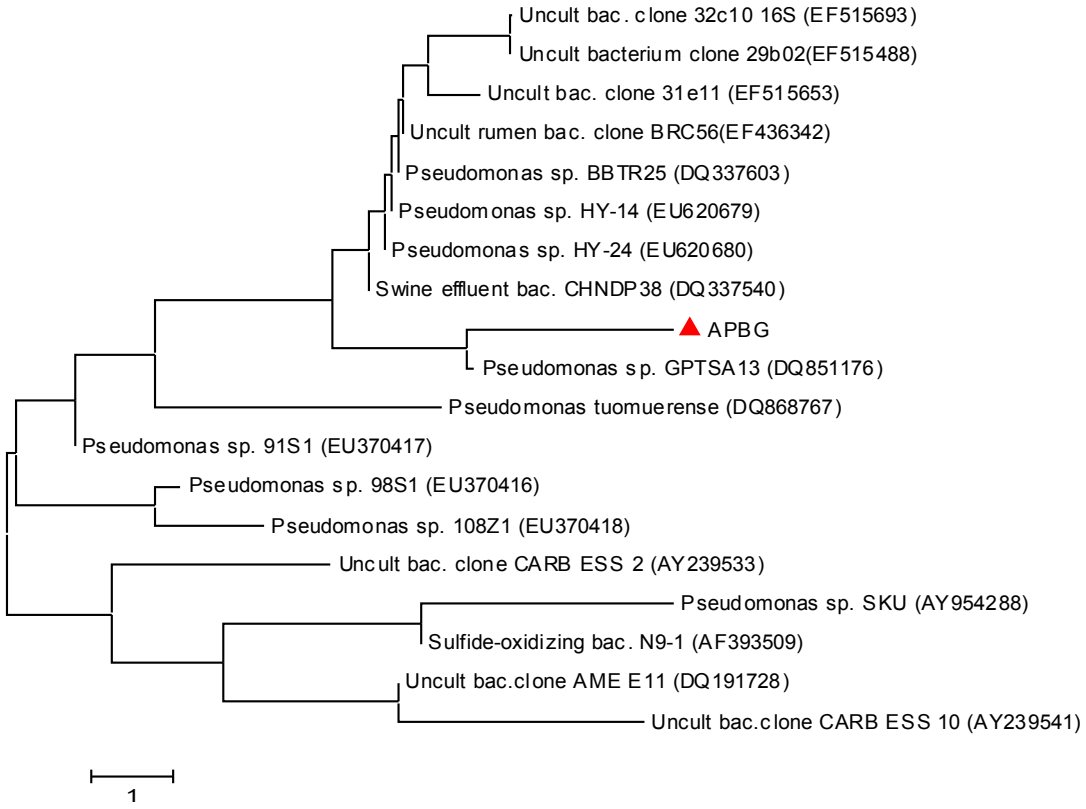

Figure 1. Phylogenetic neighbor-joining tree based on 16S rDNA. Only sequences with more than 98% of similarity were included. The sequences found in the present study are represented by a red triangle (▲). The GenBank accession numbers are indicated in the parentheses.

CONCLUSIONS

The overall experiment results demonstrate that landfill leachate, which has been treated in anaerobic pond, presents favorable physical-chemical characteristics and can be transferred to a second biological treatment stage (aerobic). By using the FISH technique and phylogenetic analyses, different organisms were identified during the studied period. A variety of bacterial species developed spontaneously and are involved in the anaerobic degradation process of organic matter, including hydrolytic, fermentative, acidogenic, acetogenic, sulfate-reducing, and methanogenic bacteria. No Anammox bacteria like was detected in the present study.

In this context, studies of microbial dynamics associated to physical-chemical oscillations, can contribute to validate more suitable reactor type, loading criteria, oxygen and nutrients requirement, control of filamentous organism as well as effluent characteristics to improve future system management. However, for this we recommend continuous and longer studies on landfill leachate treatment methods.

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