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NEW APPROACHES TO UNDERSTANDING MICROBIAL DIVERSITY IN WASTEWATER, LANDFILLS AND LEACHATE TREATMENT

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ABSTRACT

Population growth is accompanied by an alarming increase of solid and liquid waste production worldwide. The maintenance of soil and water reservoirs as well as their protection against pollution effects are of extreme importance for human civilization. Microorganisms are directly involved in biogeochemical cycles, being key drivers of the degradation of many carbon sources, as pollutants, which when properly known and managed provide a wide range of applications, including in wastewater, landfills and leachate treatment. Recent advancements in molecular tools to study the diversity and function of microbial communities are driving and contributing to a better understanding of microbial ecology, and researchers apply this knowledge to manage and understand biotechnological processes. In wastewater and leachate treatment, as well in landfills, microbial ecology techniques have been applied to study the behavior of microbial community during the process. This article will briefly outline the most widely used and the newest tools presenting their potential and limitations.
Keywords: Microbial ecology, cultivation independent methods, waste, pyrosequencing.

RESUMO

NOVOS MÉTODOS PARA ANÁLISE DA DIVERSIDADE MICROBIANA EM SISTEMAS DE TRATAMENTO DE RESÍDUOS SÓLIDOS E LÍQUIDOS. O crescimento cada vez maior da população mundial é acompanhado pelo aumento alarmante da produção e deposição de resíduos sólidos e líquidos no mundo inteiro. A manutenção de reservatórios de água e de solos, assim como a proteção desse ambientes contra os efeitos da poluição, são essenciais para a manutenção da civilização humana. Os microrganismos estão diretamente envolvidos nos ciclos biogeoquímicos sendo os responsáveis pela degradação de inúmeras fontes de carbono, como muitos poluentes e, quando devidamente conhecidos e manejados, podem ser utilizados em uma vasta gama de aplicações biotecnológicas, incluindo tratamento de esgotos, aterros sanitários e lixiviados. Avanços recentes no desenvolvimento de técnicas moleculares utilizadas no estudo da diversidade e função de comunidades microbianas vêm possibilitando e/ou contribuindo de forma expressiva para um melhor conhecimento da ecologia microbiana, e os pesquisadores aplicam esse maior conhecimento para entender, desenvolver e aperfeiçoar processos biotecnológicos. Em sistemas de tratamento de esgoto e lixiviados, assim como em solos de cobertura de aterros sanitários, essas técnicas vêm sendo aplicadas no estudo da estrutura e do perfil das comunidades microbianas durante o processo de tratamento desses resíduos. O presente trabalho irá apresentar resumidamente as técnicas mais utilizadas atualmente no estudo de comunidades microbianas nesses ambientes, e relatará algumas novas e promissoras técnicas, com suas potenciais aplicações e limitações.
Palavras-chave: Ecologia microbiana, técnicas independentes de cultivo, resíduos, pirosequenciamento.

RESUMEN

NUEVAS APROXIMACIONES AL ENTENDIMIENTO DE LA DIVERSIDAD MICROBIANA EN AGUAS RESIDUALES, RELLENOS SANITARIOS Y TRATAMIENTO

DE LIXIVIADOS. El crecimiento de la población viene acompañado por el aumento alarmante de la producción de residuos sólidos y líquidos en todo el mundo. La manutención del suelo y de los cuerpos de agua y su protección contra los efectos de la contaminación son esenciales para la civilización humana. Los microorganismos están directamente relacionados con los ciclos biogeoquímicos, como agentes responsables de la degradación de varias fuentes de carbono, como numerosas sustancias contaminantes y, cuando son conocidos y manejados adecuadamente, proveen un amplio rango de aplicaciones biotecnológicas, incluyendo el tratamiento de aguas residuales, rellenos sanitarios y lixiviados. Avances recientes en el desarrollo de técnicas moleculares para el estudio de la diversidad y función de las comunidades microbianas están contribuyendo a un mejor entendimiento de la ecología microbiana y los investigadores aplican este conocimiento para entender, desarrollar y perfeccionar procesos biotecnológicos. Estas técnicas de ecología microbiana son aplicadas para estudiar la estructura y el perfil de las comunidades microbianas durante el proceso de tratamiento de aguas residuales y lixiviados, así como en rellenos sanitarios. El presente trabajo documenta brevemente las herramientas más utilizadas actualmente para el estudio de comunidades microbianas en estos ambientes, y presenta algunas nuevas y prometedoras técnicas, con sus aplicaciones potenciales y limitaciones.
Palabras clave: Ecología microbiana, métodos independientes del cultivo, residuos, pirosecuenciamiento.

LANDFILL AND LEACHATE MICROBIOLOGY

One of the most serious global environmental problems in the current world, concerning to the production of waste which is inherent in the human condition. The population growth has been contributing to increase the quantity and variety of waste. Collection, transport and handling of the waste must also be properly dealt with. If not, the waste creates a number of problems, many of which are related of human health and environment (Bitton 1999). The major part of municipal solid waste (MSW) disposal is land filling, and in developing countries municipal dumps represent a large part of the waste disposal system. Actually, a considerable amount of the MSW is disposed in open dumps or poorly managed landfills even in industrialized countries (Christensen *et al.* 1992).

According to the National Survey of Basic Sanitation in 2000 in Brazil the forms used for final destination of waste were: open dumps, landfills, recycling and incineration plants. The same study describes that from a total of 8,381 cities which have urban cleaning services and/or collection of garbage, about 71% have open dump and approximately 22% have controlled landfills, which are the forms of allocation of waste frequently used in the country (IBGE 2000).

Leachate, a complex mixture of chemicals, is a liquid product of MSW degradation. When waste is land applied, leachate can pose a major environmental risk, especially in high demographic densities. After leachate formation, the liquid can either percolate through the soil profile, or run off site. Current landfill technology aims to prevent and control leachate release to the environment. Leachate can then be treated chemically or biologically, depending upon the nature of the leachate. The most widely used method in reducing the pollution load of landfill leachate is by the utilization of biological treatment either separately or together with sewage (Ding *et al.* 2001).

Little is currently known about the biological pathways that lead to the degradation of solid waste and the microorganisms responsible for that degradation. Huang *et al.* (2005) analyzed the phylogenetic composition of bacterial community in the effluent leachate of a full-scale recirculation landfill using a 16s rDNA clone library. The authors recovered many bacterial sequences with low levels of similarity to any other previously reported rDNA sequences, indicating the limited available information about the diversity of microorganisms associated with solid waste. Such lack of knowledge about diversity, and consequently about the processes of biodegradation of the solid waste, are obstacles for optimization of leachate biological treatment processes.

WASTE WATER MICROBIOLOGY

Wherever there is human presence, there is constant demand for fresh drinking water, and a concomitant production of wastewater. Therefore, wastewater treatment is crucial to sustainable water use and reuse. Wastewater treatment facilities are important biotechnological applications, preventing the pollution of natural ecosystems and the spread of sewage-borne diseases such as cholera (Crockett 2007). The microbes used wastewater treatment systems play a primordial role in environmental water sustainability (Bitton 1999). Their diverse metabolic capabilities have allowed societies to recycle and reuse water after treatment. Our knowledge about the diversity and function of microbial communities in wastewater treatment systems can aid in the removal of harmful components of wastewater, including nutrients, pathogens, pharmaceuticals, toxins, and other chemicals (Mara 2003).

The use of molecular tools independent of the cultivation of microorganisms has also been expanding the knowledge about wastewater microbial diversity, as well as fundamental microbial processes such as nitrification and denitrification. By using cultivation independent methods, many bacteria of importance to wastewater treatment are being identified, such as bacteria involved in biological phosphorus removal (Bond *et al.* 1999, Jeon *et al.* 2003, Seviour *et al.* 2003), nitrifiification (Coskuner & Curtis 2002, Otawa *et al.* 2006) and denitrification (Beline *et al.* 2001). During this report we will discuss and illustrate some molecular tools, which aimed to extend the knowledge of microbial ecology in landfill biocover soil, leachate, and wastewater treatment environments.

CULTIVATION INDEPENDENT METHODS TO STUDY MICROBIAL DIVERSITY AND ECOLOGY

Microorganisms have a long evolutionary history (circa 3.5 billion years) and are present in very complex communities. The total number of prokaryotic cells on earth has been estimated at 4 $x10^{30}$–6 $x10^{30}$ (Whitman *et al.* 1998). These microorganisms can be accessed by a classical approach, involving culturing the microorganism by solid or liquid growth medium containing appropriate carbon and electron acceptor sources and a range of other physiological conditions to promote microbial growth. However, general culture conditions impose a selective pressure, preventing the growth of many "uncultivable" microorganisms. Some studies suggest that only a small fraction (1–15%) of microbial genomes are cultivable under laboratory conditions and more than 85% have never been studied, which means culturing techniques provide only a narrow vision of the actual microbial community (Amann *et al.* 1995, Pace 1997).

It is well accepted that the number of known prokaryotic species (including Bacteria and Archaea) is very small compared to the real microbial diversity (Whitman *et al.* 1998). One possible method to address this problem is to use molecular biology approaches. The application of molecular tools to study microbial ecology in natural environments has been practiced since the mid-1980s, and many new insights into the composition of uncultivated microbial communities have been acquired. There are groups of organisms that are only known from molecular sequences and that are now believed to be very significant in many environments (Pace 1997). Nowadays, the use of molecular tools in microbial ecology is taken as essential and classic microbiology and molecular microbiology are not longer easily consider as separated sciences (Peixoto *et al.* 2008).

The molecular approaches are mainly based on the RNA of the small ribosomal subunit (16S rRNA for prokaryotes) or their corresponding genes, considering it as a 'molecular clock'. This molecule was chosen because it presents some specific features, such as its universal distribution among all organisms, and some highly conserved and other highly variable regions. This allows comparison of organisms within the same domain, as well as differentiation of strains of the same species. The size of the gene sequence is large enough to generate data that can be statistically compared (Gutell *et al.* 1994, Amann *et al.* 1995). Therefore, it is possible to make a comprehensive survey of the microbial diversity of a natural habitat in a relatively simple and more extensive way than those provided by cultivation techniques.

EXPLORING THE UNCULTIVABLE WORLD

It is important to remember that the first and fundamental step to explore molecular microbial

ecology is to obtain the nucleic acids (DNA or RNA, depending on the aim of the work). This step can be developed by different protocols available in literature (Ogram *et al.* 1987, Rosado & Duarte 2002, Courtois *et al*. 2003), and the choice of which protocol to use for each approach will depend on many variables, as the type of sample, the type of subsequent analysis and the available material of each laboratory. The quantity and quality of nucleic acids recovered from environmental sample will be critical for the success of any molecular study.

Subsequent molecular biology techniques most often applied to wastewater and leachate treatment would include: denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP), fluorescent in *situ* hybridization (FISH), and cloning of 16s rDNA (Sanz & Kochling 2007). Bellow, we will briefly describe some of these applications available in the literature.

DENATURANT GRADIENT GEL ELECTROPHORESIS (DGGE)

Denaturing gradient electrophoresis is a molecular fingerprinting technique which separates polymerase chain reaction (PCR) DNA products based on the variation in the electrophoretic mobility profile of this molecule under denaturing gradient according to its sequence of base pairs, generating band patterns that can reflect the genetic biodiversity of a given sample (Muyzer *et al.* 1993). In other words, DGGE provide a pattern or a profile of the genetic diversity in a microbial community, where different bands, generally, corresponds to different gene sequences, generating a fingerprint analysis of the target sample.

Since the first report which applied the DGGE tool to analyze complex microbial populations (Muyzer *et al.* 1993), many authors reported the use of DGGE in a wide range of habitats, and is possible to observe a increasing number of works that have used the DGGE in environmental microbiology (Rosado *et al.* 1998, Peixoto *et al.* 2002, Peixoto *et al.* 2006, Aboim *et al.* 2008, Alves *et al.* 2009, Hardoim *et al.* 2009). For certain purposes simple analysis of band patterns may be sufficient, for example, Mehmood *et al.* (2009) assessed the efficiency of leachate treatment by microbial oxidation in four connected on-site aerated lagoons at a landfill site by 16S - DGGE band pattern analysis. Additional analysis can be performed by extracting DNA fragments represented by DGGE bands from gels and sequenced to identify representatives of the microbial community, being not only a comparative tool but allows for downstream community member identification. Moura *et al.* (2007) used a 16S-DGGE approach to estimate bacterial diversity and to monitor community changes in two aerated lagoons from a wastewater treatment plant receiving urban and industrial effluents. Pronounced shifts between bacterial communities collected in winter-spring and summer-autumn months were detected by the authors. Temperature, dissolved oxygen (DO) and pH were the variables that most influenced this shift. Sequencing of predominant DGGE bands demonstrated phylogenetic affiliations to *Cytophaga–Flexibacter–Bacteroides* (CFB) group, *Firmicutes*, and β- and ε-*proteobacteria*.

Santegoeds *et al.* (1998) evaluated sulfate-reducing bacteria with DGGE and, concomitantly, in situ hybridization. In this case, the authors identified specific microorganisms that are crucial to the process of sulfate reducing by evaluating DGGE band patterns. With predominant band sequences information, the authors searched for these members designing a specific probe, which allowed the quantification of the candidate confirming the results obtained by DGGE.

In a recent study, the profile and changes in the bacterial communities at two acclimation stages (with and without ultrasound) in a small ultrasound-enhanced anaerobic reactor for treating carbazole-containing wastewater were analyzed by PCR-DGGE combined with real-time PCR (qPCR) (Tan & Ji 2009). Real-time PCR provides quantitative information of specific groups of microorganisms (Higuchi *et al.* 1992) and in this case it was possible after predominant DGGE bands were sequenced. The authors identified *Pseudomonas sp*., *Comamonas sp*., and *Diaphorobacter sp*. as being able to utilize carbazole as a carbon source, and detected that the total bacterial density in the stages with-ultrasound was 10 times higher than without-ultrasound treatment. The proportion of *Pseudomonas* was relatively stable in both treatments, which indicates that *Pseudomonas* can flourish and promote carbazole degradation either with and without-ultrasound.

The DGGE approach was also used to evaluate methanotrophic diversity in a landfill with passive

methane oxidation biocover (PMOB-1) in Quebec, Canada, and the microbial profiles were compared with those obtained for the existing landfill cover and to a reference soil (RS). The results indicated no differences between methanotroph diversity from the PMOB-1 and RS what about existing, and the authors praised that the PMOB was found to be a good technology to enhance methane oxidation, as its performance was clearly better than the starting soil that was present in the landfill site (Ait-Benichou *et al.* 2009).

In Brazil there are two available studies that describe the utilization of DGGE to evaluate microbial diversity in wastewater treatment. The first (Clementino *et al.* 2007) evaluated Archaeal diversity in several impacted and non-impacted environments from Rio de Janeiro, including Guanabara Bay (GB) water, halomarine sediment (HS), municipal landfill leachate, agricultural soil and wastewater treatment plant (WWTP) system. Their results showed that archaeal communities of impacted environments seem to be specific of ecosystems with similar physicochemical properties, while communities from natural environments appear to be widely distributed. The second work (Rosa *et al.* 2009) described the effect of a lipase-rich fungal enzymatic preparation, produced by a *Penicillium sp*. in an anaerobic digester treating dairy wastewater with 1200 mg of oil and grease/L. The PCR-DGGE analysis of the Bacteria and Archaea domains were able to reveal remarkable differences in the microbial profiles in trials conducted with and without the oil and grease step.

The main advantage of this method is monitoring dynamic changes in microbial communities, especially when many samples have to be processed (Sanz & Kochling 2007). It is a very reproducible technique, which can provide the clustering of samples. In the example given above (unpublished data), 7 samples in duplicates were taken from an experimental wastewater treatment plant (Federal University of Brazil/RJ) along the treatment steps and procedures to DGGE analysis of 16s rDNA gene fragment. Computation of DGGE matrix was carried out using Pearson correlation, and UPGMA (unweighed pair group method with arithmetic mean) was selected as a clustering method for the presentation of the results. The resulting dendrogram (Figure 1) showed that the samples were clustered according to the steps of the treatment (different steps represented by different colors).

The most important limitations of DGGE we can include PCR bias, and the limitations on fragments size that can be separated, up to 500 base pairs of difference (Muyzer *et al.* 1993). This limits the amount of sequence information for phylogenetic inferences as well as for probe design. Also, depending on the

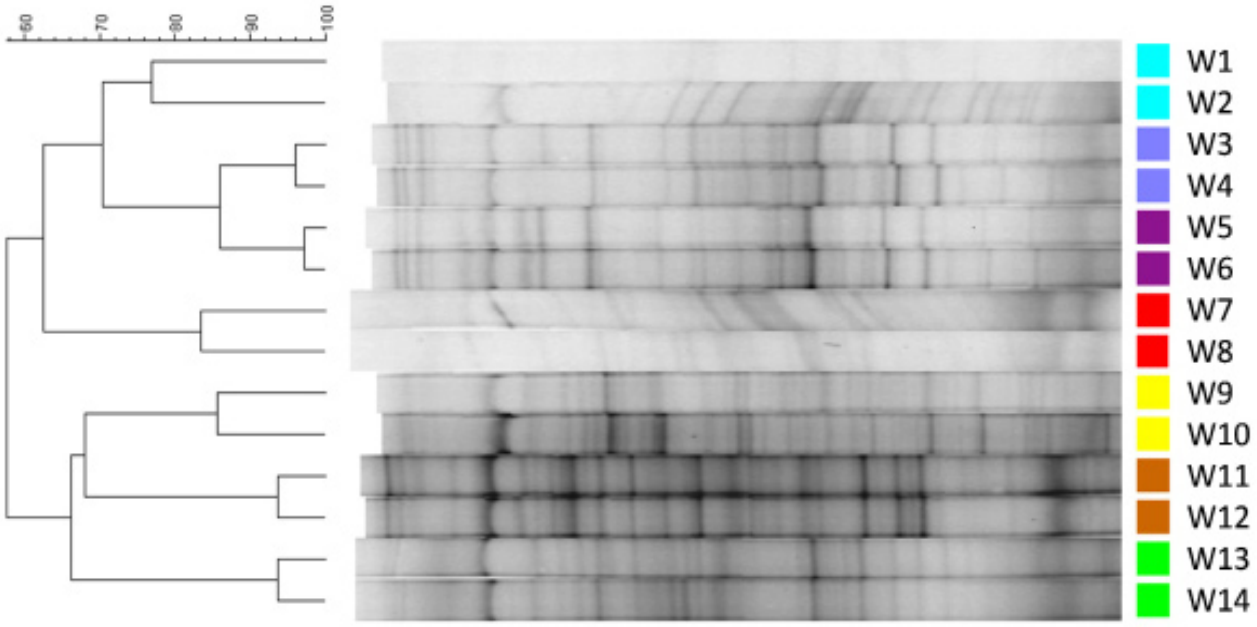

Figure 1. DGGE fingerprints and dendrogram of 16S rDNA gene fragments amplified from DNA samples extracted from experimental WWTP (Federal University of Brazil/RJ) along the treatment steps and analyzed by Bionumerics Software version 5.1 (Applied Maths). W1 and W 2, are duplicate samples from sample point 1, and every other sample is also presented with its duplicate sample (different steps representing by different colors).

nature of the sample, extraction and amplification of representative genomic DNA can be difficult.

TERMINAL RESTRICTION FRAGMENT LENGTH POLYMORPHISM (T- RFLP)

Alternatively to DGGE as a fingerprinting method, terminal restriction fragment length polymorphism (T-RFLP) can be applied to monitor changes in the structure and composition of microbial communities (Liu *et al.* 1997). This technique is also PCR-based but in T-RFLP the gene of interest is amplified with primers, one or both of them being fluorescently labeled, and the product is digested with one or more restriction enzymes. The resulting fluorescently labeled gene amplicons are then analyzed by an automated DNA sequencer. Since differences in sequence will generate labeled amplicons with different sizes, it will be possible to cluster the groups of populations of organisms that are phylogenetically different (Liu *et al.* 1997).

Some authors presented the application of T-RFLP to evaluate the microbial community of waste treatment systems. Briones *et al.* (2009) monitored by T-RFLP the microbial community of bacteria and archaea in a multi-compartment anaerobic bioreactor. They found that the less stable reactor did not produce granular biomass, and they observed a shift in electron flow from butyrate to propionate as a consequence of the predominance of bacterial populations such as butyrate-producing clostridia. On the other hand, the stable upflow anaerobic sludge blanket (UASB) reactor developed and retained granules and maintained a relatively stable archaeal community. Sulfate perturbation led to the selection of a novel bacterial group (*Thermotogaceae*), which was most likely well adapted to the increasingly sulfidogenic conditions in the bioreactor.

Wen *et al.* (2008) simultaneously detected nitrifying- and denitrifying-bacteria in a reactor by T-RFLP and described that the composition and number of both AOB (ammonia-oxidizing bacteria) and NOB (nitrite-oxidizing bacteria) changed with the position in the reactor and operating time. Another good example of T-RFLP is the work led by Uz *et al.* (2003). These authors reported the characterization of methanogenic and methanotrophic bacteria compositions in samples taken from two regions of a municipal solid waste landfill that varied in age.

To characterize the effects of pentachlorophenol (PCP) on the microbial community performance and structure in an aerobic granular sludge in sequencing batch reactor (SBR), Liu *et al.* (2008) used T-RFLP and qPCR. The authors concluded that the quantity of ammonia-oxidizing bacteria (AOB) remained constant, although the number of bacteria species decreased with the increase of PCP concentration. They also observed significant shifts in bacterial community structure within aerobic granular sludge at different PCP stresses, generating an understanding of the microbial community structure under this stress and its relationship with the performance for wastewater treatment by aerobic granular sludge.

T-RFLP has proven to be a useful molecular tool and the technology is continuing to be improved statistically. Recent studies are describing tools to improve the statistical analysis of T-RFLP profiles, allowing better interpretation of generated data, such as the introduction of objective procedures to distinguish between signal and noise, the alignment of T-RFLP peaks between profiles, and the use of multivariate statistical methods to detect changes of the microbial communities due to spatial and temporal variation or treatment effects (Schutte *et al.* 2008). Real-time PCR and T-RFLP can also be combined in one step, called real-time terminal restriction fragment length polymorphism (real-time-T-RFLP) assay. Such kind of approach can be applied for simultaneous determination of microbial diversity and abundance within a complex community and was proposed by Yu *et al.* (2005). The assay was validated by using a model microbial community containing three specific strains. This study strongly suggested that the real-time-T-RFLP assay can be a powerful and efficient molecular tool for gaining insight into microbial communities in various engineered systems and natural habitats, providing a quantitative fingerprinting molecular assay.

As observed in DGGE, it is important to stress that data interpretation must be taken carefully, taken in account the limitations related to the introduction of bias with PCR amplification or DNA extraction (Schutte *et al.* 2008).

FLUORESCENT IN SITU HYBRIDIZATION (FISH)

Fluorescent *in situ* hybridization (FISH) is a molecular technique for RNA- or DNA- specific

sequences detection of microbial cells within their natural environments allowing direct visualization of non-cultured microorganisms (Amann *et al.* 2001). It is one of the most popular molecular techniques applied in wastewater and leachate treatment research, perhaps because it is relatively easy and fast, if the required probes are available (a wide array has already been described) (Nielsen *et al.* 2009).

This method uses fluorescent probes which are generally short sequences of DNA (16–20 nucleotides) labeled with a fluorescent dye that recognizes RNA and DNA sequences in fixed cells with which it hybridizes *in situ*, being possible to detect specific representatives of different taxonomic level (Sanz & Kochling 2007). Wagner *et al.* (1993) performed the first analysis of the FISH application (using specific probes for the detection of α-, β- and γ-*proteobacteria*) to analyze microbial communities in activated sludge (aerobic treatment) which validated the technique. Studies of anaerobic digesters are also being conducted by using FISH. Raskin *et al.* (1994) presented many of the probes currently used to identify methanogenic microorganisms at different taxonomic levels (order, family, and genus). The application of FISH technology proved so important in wastewater research that there is a recently published book which provides all required information for the user to be able to identify and quantify important microorganisms specifically in activated sludge by FISH and epifluorescence microscopy (Nielsen *et al.* 2009).

FISH studies have increased knowledge about microbial communities that grow in activated sludge systems, however this approach cannot describe function, only phylogeny of communities (Sanz & Kochling 2007). One such study is described by Juretschko *et al.* (2002) and showed that the FISH data confirmed the results obtained by clone library method (described below) indicating that members of β-*Proteobacteria* were the dominant members of the microbial community of the nitrifying-denitrifying activated sludge analyzed. By the other hand, Davenport *et al.* (2000) utilized quantitative FISH to evaluate the relationships of Mycolic-acid-containing *Actinomycetes* and foaming in activated sludge plants. The authors concluded that quantitative FISH use is feasible and that quantification is a prerequisite for rational investigation of foaming in activated sludge.

One advantage of FISH is that it allows for the observation of the spatial distribution of microbes in their environment. Diaz *et al*. (2003) evaluated the microbial structure of anaerobic sludge, combining FISH and both transmission (TEM) and scanning (SEM) electron microscopy. The authors praised the efficiency of FISH, with TEM and SEM applications concluding, between other results, that the granules have a multi-layer structure and that in the outer layers only bacteria were present. The microbial ecology of nitrifying bacteria in different types of wastewater treatment processes and the dynamic response of a biofilm community were investigated using FISH (Aoi *et al.* 2000). The results indicated that the dynamics of the spatial distribution of ammonia-oxidizing bacteria and heterotrophic bacteria caused by a gradual change in substrate composition was successfully monitored by FISH analysis.

Disadvantages of FISH would include the need to know the ecosystem being studied before hand, the possible difficulty in designing certain new specific and unambiguously restrictive probes and in optimizing the conditions for hybridization (Sanz & Kochling 2007). Even with the known limitations observed to all molecular tools, the advantages provided by such techniques are much more representative than the disadvantages, since the data generated are expressively improving our knowledge about microbial world.

CLONING OF 16S rDNA

Cloning and sequencing of 16S rDNA have been increasingly used in molecular microbial ecology studies. This technique began a revolutionary modern era in microbial ecology, which started with the pioneering work of Pace *et al.* (1986). They evaluated the taxon composition of a microbial community through ribosomal RNA molecules analysis by shotgun cloning.

Clone libraries are developed from the extraction of genomic DNA from a given sample followed by amplification and cloning of the 16S rRNA genes (or others genes, as functional genes of interest), to further identification of the isolated clones. The cloning step is required in order to separate the different copies from the mixed template of 16S rDNA present in genomic DNA extracted from microbial communities

(Sanz & Kochling 2007). Once cloned, the 16S rRNA gene library can be screened by a variety of methods, such as hybridization of colonies with gene–specific probes, colony PCR with specific primers to confirm the insertion of the cloned PCR products, and DNA sequencing followed by phylogenetic evaluations about microbial diversity of the original sample (Olsen *et al.* 1991, Cole *et al.* 2003).

There are only a few studies which use cloning to describe the microbial diversity of wastewater, landfills, or leachate treatment processes. For instance, cloning was employed to establish phylogenetic analyses and *in situ* identification of bacteria in activated sludge. In this work, Snaidr *et al.* (1997) examined the microbial composition and structure of activated sludge of a large municipal wastewater treatment plant. In their 16S rDNA clone libraries, they found the sequences of several previously undetected and uncommon microorganisms, as well as others that were confirmed to be associated with the process by FISH analysis. Interestingly, they also found potentially pathogenic representatives of the genus *Arcobacter* in significant numbers (4%) in the activated sludge sample examined.

Monitoring microbial community shifts based on 16S rRNA associated with denitrification was done by Hoshino *et al.* (2005). Combination of the results of from T-RFLP analysis and 16S rRNA clone library indicated that the bacteria belonging to the genera *Hydrogenophaga* and *Acidovorax* play an important role in denitrification.

Zhang *et al.* (2009) compared the ammonia-oxidizing archaea (AOA) community in activated sludge from different WWTPs by clone libraries utilizing different sets of specific primers for ammonia mono-oxygenase alpha-subunit (amoA) gene. The results showed a diverse set of amoA genes within individual samples. The results also allowed the distinction between the AOA communities from activated sludge from different WWTPs.

The clone libraries are useful not only identification by 16S, but can allow further detection and/or quantification of a certain organism (or group of microorganisms), or functional genes, as described by Zhu *et al.* (2007). They investigated communities of ammonia-oxidizing bacteria (AOB) and denitrifying bacteria associated with the leachates from three MSW disposal sites by examining the diversity of the ammonia monooxygenase structural gene amoA and the nitrous oxide reductase gene nosZ, respectively. The data generated in this work indicated that functional genes revealed novel and similar groups of prokaryotes involved in nitrogen cycling in the leachates with different chemical compositions.

In a multidisciplinary work, Hery *et al.* (2008) used a comprehensive range of different molecular techniques, including clone libraries, DGGE, SIP (Stable Isotope Probe, described below) and microarrays, in order to determine the composition of the active methanotroph community and its interactions with earthworms in a landfill biocover soil. These authors quote previous reports which described that Methanotrophic bacteria present in landfill biocovers can significantly reduce methane emissions, oxidizing up to 100% of the methane produced, and that Earthworm-mediated bioturbation has been linked to an increase in methanotrophy in a landfill biocover soil. Their results suggested that the earthworm-mediated increase in methane oxidation rate in the landfill soil was more likely to be due to the stimulation of bacterial growth or activity than to substantial shifts in the methanotroph community structure. A Bacteroidetes-related bacterium was identified only in the active bacterial community of earthworm-incubated soil but its capacity to actually oxidize methane has to be proven.

DGGE and cloning libraries have in common at least one step of DNA or RNA extraction. This step represents one of the most significant limitations. The release of nucleic acids from microbial cells depends mainly on the structure of the peptidoglycan and outer membranes of bacteria (Head *et al.* 1998). Recently, Feinstein *et al.* (2009) conducted multiple extractions on subsamples of clay, sand, and organic soils. Bacterial and fungal ribosomal gene copies were measured by different molecular techniques during the successive extractions. They found that the relative abundances of sequences from rarely cultivated groups such as *Acidobacteria*, *Gemmatimonades*, and *Verrucomicrobia* were higher in the first extraction than in the sixth but that the reverse was true for *Proteobacteria* and *Actinobacteria*. The authors suggest that bias can be adequately reduced in many situations by pooling three or more successive extractions. Bias introduced by PCR amplification includes differences in the specificity of polymerases,

inhibition of the reaction by interfering substances, differential PCR amplification and PCR artifacts.

IMPROVEMENTS TO STUDY MICROBIAL FUNCTION

Currently, a very important challenge to microbial ecologists is to develop and/or improve tools to evaluate microbial function in natural environments. As discussed before, the only methodology within the methods mentioned, that allows this kind of approach is FISH. Stable isotope probing is a second approach that makes it possible to study the function and activity of microorganisms in their natural environment, overcoming FISH problems.

Briefly, stable isotope probing (SIP) is a technique that is used to identify the microorganisms in environmental samples that use a particular growth substrate, and was described by Radajewski *et al.* (2000). This method is based on the incorporation of an enriched substrate with a stable isotope, such as ^{13}C, and the following selective recovery and identification of active microorganisms by isotope-enriched cellular components analysis. DNA and rRNA ^{13}C-labelled molecules can be separated from unlabelled nucleic acid by density-gradient centrifugation (Radajewski *et al.* 2000). Thus, SIP coupled with 16S rDNA cloning library, or DGGE and T- RFLP fingerprinting analysis, is one approach to more directly explore which organisms use a specific substrate (Dumont & Murrell 2005). The combination of these techniques was used to identity active methanotrophs in landfill cover soil (Cebron *et al.* 2007), determine the composition of the active methanotroph community and to investigate the interactions between earthworms and bacteria in this landfill biocover soil (Hery *et al.* 2008) as described before; and more recently to identify anaerobic phenol-assimilating bacteria present in activated sludge (Sueoka *et al.* 2009). The major problem in this technique is the dependence on the commercial availability of compounds that are highly enriched in ^{13}C.

Another tool recently applies to wastewater research is microarray analysis. In 1997, Guschin *et al*. first applied microarray approach in environmental microbiology and since them many approaches have been developed. This methodology, initially described for *Arabdopsis* cells transcriptome analysis (Schena *et al.* 1995), consists in miniaturized platforms with thousands of DNA probes targeting genes or gene products for highly parallel hybridization reactions with labeled target nucleic acids. The hybridization signal of each probe can be simultaneously recorded with a detector (Wagner *et al.* 2007).

Hesselsoe *et al.* (2009) addressed the diversity and ecophysiology of *Rhodocyclales* in activated sludge from a full-scale wastewater treatment plant using SIP, 16s cloning library, quantitative FISH and DNA microarray. This work gives us a very good example of how the combination of these techniques could provide a wide vision of the microbial community at the target environment. They found that most detected *Rhodocyclales* groups were actively involved in nitrogen transformation, but varied in their consumption of propionate, butyrate, or toluene, and thus in their ability to use different carbon sources in activated sludge. The authors concluded that the functional redundancy of nitrate reduction and the functional versatility of substrate usage are important factors governing niche overlap and differentiation of diverse *Rhodocyclales* members in the activated sludge.

For more details about microarray analysis, Wagner *et al.* (2007) reviewed a survey of microarray applications during the last decade and highlighted the advances of this methodology and also innovative combinations of microarrays combined with other molecular technologies to study microbial structure and function of microbial communities. These called microbial functional genomics studies are becoming possible thanks to the development and improvement of DNA sequencing and microarray technologies and more recently by transcriptomics based on high-throughput DNA-sequencing technologies (van Vliet 2009).

NEW SEQUENCING TECHNOLOGIES

Microbial ecology is driven by the tools available to sample. Molecular biology and genomic technologies are under constant development and improvement being marked by the introduction of new technologies, their rapid uptake, and then a steady state or slow decline in use. Subsequent technologies are then developed (Kahvejian *et al.* 2008).

The increasing availability of molecular techniques, as well the consequent generation of important data about microbial communities is demanding even more comprehensive techniques. This is contributing for an explosion of the development of promising and amazing methodologies, which are very powerful to describe DNA sequences of microbial cells or microbial diversity from an environmental sample faster and more efficiently. The new technologies must to attend to some principal goals, such as increase the throughput of sequencing significantly without loss the high quality of data produced by the current approach (Rogers & Venter 2005).

Since Frederick Sanger (Sanger & Coulson 1975) began sequencing by electrophoretic size separation, many improvements allowed the upgrade of data from DNA sequencing, which mainly include: the use of fluorescent tags instead of radioactive labels to detect the terminal ladders, the use of capillary electrophoresis, and the development of paired-end sequencing protocols incorporating template sizes to provide improved sequence context and orientation. To increase the throughput of DNA sequencing, 454 Life Sciences sequencing platform (454; Branford, CT, USA, Roche) initiated the next generation movement by pioneering solutions to two limitations; library preparation and labor-intensive Sanger method (Mardis 2008)

The goal of this next generation of sequencers is the concept of sequencing by synthesis presented for the first time by Nyren *et al.* (1993). The approach was chosen by 454 platforms was the technology that sequencing is done by detecting pyrophosphate release with enzymatic cascade ending in luciferase and detection of emitted light (Margulies *et al.* 2005). Indeed, pyrosequencing has been available to the scientific community since mid-1990s as a genotyping tool; however, it was not considered powerful enough for standard sequencing needs because of the short read lengths generated (Ronaghi *et al.* 1996).

Margulies *et al.* (2005) based the 454 platform on miniaturizing a pyrosequencing reaction and moving both the template preparation step and pyrosequencing chemistry to the solid phase. The developing the solid-phase sequencing and optimizing read-lengths in the microwells (immobilization the reaction in small wells) contributed substantially to improved accuracy and read-length.

Pyrosequencing is based on the detection of light produced whenever a nucleotide is incorporated, being independent of a physical separation process to resolve the next base in the DNA strand, allowing this methodology to be able to analyze any reaction volume that generates detectable levels of light (Margulies *et al.* 2005). The results achieved by these authors was a highly parallel system capable of sequencing 25 million bases in a four-hour period, which means about 100 times faster than the current Sanger sequencing and capillary based electrophoresis platform, allowing an entire genome sequencing in only few days (Rogers & Venter 2005). Furthermore, this methodology also allows the sequencing of regions with technical obstructions (e.g. due to strong secondary structure or high GC content) and coverage regions difficult to cloning in *Escherichia coli* (Sorek *et al.* 2007).

The sample preparation starts with fragmentation of the genomic DNA or amplification of desire gene, followed by the attachment of adaptor sequences to the ends of the DNA pieces. In some cases it is interest to combine the selectivity of primer – based PCR and use one run for different samples for economical reasons. For that purpose PCR products can be processed simultaneously thanks Binladen *et al.* (2007). The idea is quite simple.

In theory a pool of different PCR products at equimolar concentration should generate an equal number of sequences from each PCR product. Thus, initial PCR primers are 'tagged' with short nucleotide sequences (barcode) in such a way that unique tagged primer combination can be applied to each specific DNA template source. In this method, samples from different origins can be mixed in one run and after sequencing their data can be separated according to their barcode (Meyer *et al.* 2007, Parameswaran *et al.* 2007), which also contributes to decrease the cost per sample. For example, from using 454 sequencers 400,000 reads can usually be generated, and from using 80 barcodes, 5,000 reads per sample can be obtained, representing at least one order of magnitude higher than those from traditional clone libraries (Cardenas & Tiedje 2008).

Follow the fragmentation of the genomic DNA or amplification of desire gene, the adapters are linked to the ends of the DNA fragments which allow them to bind to tiny beads (around 28μm in

diameter) and the procedure conditions restrict that only one piece of single strand DNA binds to each bead. The beads are isolated and compartmentalized in the droplets of a PCR-reaction-mixture-in-oil emulsion and PCR amplification occurs within each droplet, resulting in beads each carrying ten million copies of a unique DNA template. The oil droplets form part of an emulsion so that each bead is kept apart from its neighbor, ensuring the amplification is uncontaminated (Margulies *et al.* 2005).

To perform the sequencing reaction, the emulsion is broken and the DNA strand are denatured. The DNA-template-carrying beads are loaded into the picolitre reactor wells of a fiber – optic slide (each well having space for just one bead) and then smaller beads carrying immobilized enzymes required for the pyrophosphate sequencing reaction are deposited into each well. The incorporation of the complementary base at the growing DNA chain generates inorganic pyrophosphate (PPi), which is converted to ATP by the sulfurylase. This ATP is used by luciferase to convert luciferin to oxyluciferin, producing light. Sequential washes of each of the four possible nucleotides are run over the plate, and a detector senses which of the wells emit light with each wash to determine the sequence of the growing strand. This is the solid phase (Nyren *et al.* 1993, Margulies *et al.* 2005).

Three additional next-generation technologies have become available, beside as described by Margulies *et al.* (2005). The first technology to follow 454 was Illumina's (Hayward, CA, USA) Genome Analyzer, developed by Solexa (Cambridge, UK); the second technology was based on the work of (Shendure *et al.* 2005)and available by Applied Biosystems (Foster City, CA, USA) as the SOLiD system; the third system from Helicos Biosciences (Cambridge, MA, USA) does not require PCR amplification of template material, eliminating PCR bias and limitations and enabling true single-molecule sequencing. For a more detailed review refer to Mardis (2008).

One of the major advantages of the high-throughput sequencing is the substantial reduction of cost, allowing massive gene surveys as the discovery of new microbes and genes, what is especially useful for the study of microbial communities by 16S rRNA gene analysis. Combining primers that target hypervariable regions of the 16S rRNA gene, which are short enough (100–350 bases) to be covered by some of the new sequencing technologies and at same time are long enough to be informative for classification by the current rRNA databases (Wang *et al.* 2007), it is possible to survey environments with thousands of sequences at the time. The RDP (ribosomal data project) has reported accuracy in classification of partial sequences to the genus level for 400-base reads and to the family level for 200-base reads (Cardenas & Tiedje 2008).

To survey gene expression of a given microbial community, analysis of transcriptome (all the mRNA molecules transcribed from the genome or microbial community) has been successfully applied, being such approach limited to microarray technology and random cloning methodologies. This methodology is also being improved by new sequencing technologies, since probe design, cloning, and hybridization steps involved in traditional transcriptomic analysis can all be outlined by massively parallel direct sequencing of cDNA followed by mapping, generating the called "Metatranscriptomics".

The study of microbial communities on basis of its genetic material has been named Metagenomics (Riesenfeld *et al.* 2004) and has expanded the way for discovery of new genes, proteins and biochemical pathways. Metagenomics has proved more valuable in recovering complete genomes in systems with low diversity or in highly enriched cultures, such as the first genome of the annamox (anaerobic ammonia oxidation) group, *Kuenenia stuttgartiensis* (Strous *et al.* 2006). This is very important because anammox has become a main focus in oceanography and mainly in wastewater treatment. When applied to more diverse environments, such as soils, the yield of assembled genomes was poor (James Tiedje, personal communication). However, assembly is not necessary to make some important inferences; metagenome can be the basis for metranscriptomic and metaproteomics (Gilbert *et al.* 2008).

As an improvement of metagenomic, it should be possible to clone ^{13}C-labelled DNA from a DNA-SIP experiment to generate a library of microorganisms in an environment that have incorporated a specific substrate, linked to a target function. Such an approach enables the selection of organisms that are involved in a specific metabolic process, reducing the number of clones to be screened for a particular set of genes, and aids in the reconstruction of a smaller number

of targeted genomes in a microbial population. However, the required conditions to SIP experiment (as ultra- centrifugation) can compromise the quality and quantity of the labeled DNA thus constituting a limitation of SIP metagenomic libraries (Dumont & Murrell 2005).

Few studies have been published using these new sequencing technologies to draw the picture of microbial ecology in wastewater and leachate treatment. Sanapareddy *et al.* (2009) applied pyrosequencing technology to probe the molecular diversity of the aerobic basin of a wastewater treatment plant. Their observed extremely high levels of diversity and found that substantial regions of the genomes of the most prevalent microbes in the wastewater treatment plant are poorly described by existing sequence databases, showing how the microbial population of wastewater treatment plants remains inadequately characterized. In another example, plasmid metagenome nucleotide sequence data were obtained from WWTP bacteria with reduced susceptibility to selected antimicrobial drugs by applying the 454-sequencing technology (Schluter *et al.* 2008, Szczepanowski *et al.* 2008).

When we talk about microbial populations participating in the biological degradation in solid waste landfills is still limited, as discussed before. Our group in collaboration with professor James Tiedje (Michigan State University, USA) is performing a study using 454 platform to evaluate whether there are significant differences in the microbial community in spite of the physicochemical variations found in leachate from different landfills, and if it is possible to pinpoint the parameter that exerts a greater influence on this community. To that end, samples were collected from different sites, five within the metropolitan region of Rio de Janeiro (Brazil) and one in Pernambuco, northwestern Brazil.

The new sequences technologies create a scenario where the limitation is not the ability to produce sequence data but the ability to store and analyze it in new revealing ways. Databases and software tools are essential to deal with the growing of metagenomic and metatranscriptomic data. Their development represents new challenge (Shendure & Ji 2008).

UNDERSTANDING THE DATA

Although there have been huge contributions by rDNA/rRNA and other genes to elucidate information about microbial ecology, it is not practical or recommended to focus on a single molecule. It is also essential to evaluate and integrate the biological data with physiochemical variables to describe interactions and effects between microbial diversity and environmental conditions. For this reason, the use of adequate numerical tools to further analyze the data for statistical significance is extremely important. Statistical approaches, which have been developed by ecologists to work on distribution and diversity patterns of plants and animals, can also be applied to microbial ecology. Specifically, the number of species or operational taxonomic units (OTU) and gene presence or absence or polymorphism can be measured.

For example, in DGGE fingerprinting interpretation, one assumes that one band refers to a unique sequence type or phylotype for a bacterial population present at the sample. Furthermore, the band intensity is a consequence of the density of corresponding bacterial phylotypes within the sample (Murray *et al.* 1996). Thus, the total number of bands and their relative intensities in each sample can be used to calculate well-known diversity indices such as the Shannon – Weaver index (Nübel *et al.* 1999). Additionally, similarity coefficients such as Jaccard (Diez *et al.* 2001) or distance coefficients such as the Euclidean measure (McSpadden Gardener & Lilley, 1997) or the Pearson correlation (Rölling *et al.* 2001, Smalla *et al.* 2001) can also be measured.

Similarity and distance matrices can be visualized as a dendogram and can be extended to clustering and ordination methods. Clustering techniques, such as unweighed pairing using arithmetic averages (UPGMA) offer a simply way to view the DGGE profiles, especially when the goal is to identify samples generated similar patterns (Fromin *et al.* 2002). Ordination methods can be used to correlate diversity patterns to environmental parameters. Common ordination methods that have proven useful in ecological studies include cluster analysis, principal component analysis (PCA), principal coordinate analysis (PCoA), correspondence analysis (CA), and non metric multidimensional scaling (NMDS) (Ramette, 2007).

Given the high number of cells present in environmental samples (estimates include 10^9 cells per gram of biomass), a complete census of every cell in a given sample is impossible even with current

high-throughput sequencing technologies. However, a growing number of statistical approaches have been successful in describing and comparing microbial communities that can be sequenced (Schloss 2008).

Three sets of approaches have been used to analyze the large amounts of short read data from high throughput sequencing efforts. The first approach was to assign operational taxonomic units (OTUs) based on the genetic distance between sequences. Subsequently, the abundance of sequences among OTUs provides the parameters necessary to estimate the richness, evenness and ecological diversity of individual communities as well as the richness of OTUs shared between communities. This is the principle behind the software DOTUR (Schloss *et al.* 2009) and a newer version called MOTHUR. The second approach introduced by Cole and collaborators (2007) and used in the software called Library Compare, compares two communities by using reference databases. The final approach to analyzing large sequencing projects involves the use of Monte Carlo testing procedures to evaluate differences between communities. Examples using this approach include LIBSHUFF (Singleton *et al.* 2001, Scholoss *et al.* 2004), TreeClimber (Martin 2002, Schloss & Handelsman 2006), UniFrac (Lozupone & Knight 2005, Lozupone *et al.* 2006) and the analysis of molecular variance – AMOVA (Martin 2002). All three of these methods are based on using sequence similarities to assess qualitative and quantitative descriptions of microbial communities and are often used complementarily to understand the microbial ecology in different habitats.

However, current approaches to understanding microbial communities with high throughput sequencing are limited, since a large number of sequences are necessary to minimize the underestimation of richness due to insufficient sampling and because the available database is still poor (Scholoss 2008). Nonetheless, there is a wide array of molecular technologies and analytical tools available which can be useful to test well-defined hypotheses.

Microbial ecology lives now the era of "Meta" where massive sequencing, metagenomics, metaproteomics and metatranscriptomics will allow us to survey a comprehensive range of data about microbial diversity, ecology, and function. Our next limitations will be how to analyze and integrate all the generated data and how to use all the information to improve biotechnological development as well environmental maintenance.

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