Chapter 6
Molecular Biology Techniques Applied to the Study of Microbial Diversity of Wastewater Treatment Systems

João Paulo Bassin, Márcia Dezotti, and Alexandre Rosado

6.1 Introduction

6.1.1 Microbial Diversity

These are extraordinary times for the field of microbiology given the rapid development in scientific knowledge and the existence of an enormous biodiversity, which is only just beginning to be characterized. Microorganisms have evolved over approximately four billion years, and two billion years ago, they were the only forms of life on earth. They are able to exploit a wide spectrum of energy sources and to live in almost all habitats. In the course of their existence, all of the basic biochemistry of life has evolved, and all of the other living forms developed from the ancestral microorganisms. They represent the richest repertoire of chemical and molecular diversity in nature. In addition, they comprise the foundation of basic environmental processes, such as biogeochemical cycles and food chains, and also maintain vital and complex relations between themselves and with higher organisms (ROSADO and DUARTE 2002).

Diversity is a critical subject which involves all levels of biological organization, from molecular to global. In general, biological diversity programs have emphasized studies on plants and animals, and little attention has been given to microorganisms. The diversity of microorganisms is as vast as it is unknown (ROSADO and DUARTE 2002). The study of microbial diversity is also important with regard to

J.P. Bassin (✉) • M. Dezotti
Chemical Engineering Program, COPPE, Federal University of Rio de Janeiro, Rio de Janeiro, Rio de Janeiro, Brazil
e-mail: jbassin@peq.coppe.ufrj.br; mdezotti@peq.coppe.ufrj.br

A. Rosado
Institute of Microbiology Prof. Paulo de Goés, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil

M. Dezotti et al., *Advanced Biological Processes for Wastewater Treatment*,
DOI 10.1007/978-3-319-58835-3_6

advances in biotechnology. The new technologies developed, mainly those related to nucleic acid analysis, bioinformatics, analytical chemistry, and the sampling and characterization of ecosystems, have placed microbial diversity in a prime position in the scientific arena.

The detection and identification of bacteria were traditionally carried out based on their main forms of obtaining carbon and energy, their nutritional demands, and the culture medium for their growth, besides direct observation by microscopy (KENNEDY 1999; HERBERT 1990).

The tradition methods to describe microorganisms are not normally directly applicable to microbial communities; the microorganisms are generally described and classified by their phenotype. The phenotype is a very broad term which encompasses the observable traits of the cell, such as the morphology, physiological activity, structure of the cell components, and, in some cases, the ecological niche which the cell occupies. Unfortunately, these traits provide limited information regarding the evolutionary relationships of microorganisms, which, theoretically, should serve as a base for any classification system (HUGENHOLTZ and PACE 1996). In the case of microbial systematics based on phenotype, many tests to determine phenotypic traits require information on cellular function or growth. In this context, plating and cultivation in culture media is required in order to obtain pure cultures of the microorganisms or for direct observation by microscopy to establish their phenotype (HUGENHOLTZ and PACE 1996).

Conventional microbiological techniques based on the isolation of pure cultures and on morphological, metabolic, biochemical, and genetic tests have provided extensive information with respect to the diversity of microbial communities both in natural and engineered systems. However, this information is limited and thus needs to be further refined (ZAK et al. 1994). In most cases information is not available in relation to the physiological needs (nutritional and physicochemical) of the microorganisms under study, and the complexity of the syntrophic and symbiotic relationships, abundant in nature, contributes to the difficulties associated with obtaining pure cultures of most microorganisms which live in a wide diversity of environments (SANZ and KÖCHLING 2007).

The limitations of traditional techniques for the detection and identification of microorganisms are even greater when the aim is to study the diversity of microorganisms associated with a certain environment. Bacteria display a higher diversity than any other group of organisms, and each culture medium is, to a greater or lesser extent, selective to certain microbial groups, favoring the growth of some of these groups, while the development of others, also present in the original sample, is inhibited. Even on using a medium which is selective toward a certain target organism, some strains in a non-cultivatable state in the environment will be excluded from the analysis (COUTINHO et al. 1999). Thus, in many cases, microorganisms isolated using conventional methods which employ a culture medium may not represent those which are actually present in larger numbers.

According to PACE (1996), only a tiny proportion of the microorganisms present in the environment, equivalent to 1%, can be cultivated using standard cultivation and plating techniques, which highlights the limitations of conventional methods. Moreover, cell counts obtained by microscopy represent only a quantitative

evaluation of the microbial population and provide little information regarding the diversity of organisms in the sample (PICKUP 1991). In addition, it is important to note that knowledge regarding prokaryotic organisms is limited. Their small size and often the absence of distinguishable phenotypic traits means that most of these organisms cannot be cultivated (PACE 1997).

As alternatives to the traditional methods and seeking to overcome the scarcity of information provided by these methodologies, several techniques have been developed, notably those based on nucleic acids. In this regard, molecular biology has arisen as an alternative approach to overcome the intrinsic limitations of conventional techniques in the study of the diversity of microorganisms. The limitations of traditional methods, together with the technological advances in the area of molecular biology, have led to molecular techniques being widely used for the study of microbial diversity (VAN ELSAS et al. 1998). Thus, the elucidation of the microbial world in various biological systems has become more interesting.

6.1.2 Basic Concepts of Genetics

Prior to describing the principles and concepts of the main molecular biology techniques employed to study microbial diversity, it is worth looking at the origin of these tools. Thus, a brief summary of the fundamental concepts of genetics will be outlined, encompassing information related to the nucleic acids (deoxyribonucleic acid, DNA, and ribonucleic acid, RNA), their composition, and the important stages of duplication/replication, transcription, and translation involving these biomolecules which carry genetic information.

All cells of organisms, from bacteria to humans, contain one or more sets of a collection of DNA characteristic of the species. This DNA profile is known as the genome of living beings, where all of the genetic information required for their existence is contained. This information held in the DNA is expressed in cells and is perpetuated in the progeny. Thus, DNA can be considered to have the function of storing and transmitting genetic information (genetic code).

The genome can be subdivided into chromosomes, each containing one continuous and very long molecule of DNA. The chromosomes, in turn, are composed of millions of regions called genes (segments of DNA that codify the information required for the production of a certain polypeptide or a segment of RNA), which are distributed to each daughter cell at the moment of cell division. Thus, the cell needs to make a copy of its genes in order to divide them equally between its daughter cells. The position of the genes on the chromosome is known as the locus.

DNA is a long, unbranched polymer, composed of subunits called deoxyribonucleotides, grouped into four types. Each type subdivides into smaller molecules that include nitrogen bases (dNTPs) which can be purines (adenine-A and guanine-G) or pyrimidines (thymine-T or cytosine-C), a pentose sugar (deoxyribose) and a phosphate group. The bases A and T and the bases G and C are complementary and are linked to the pentose by one bond on the carbon 1′ of the latter, and the phosphate is associated with the carbon 5′ of the same pentose and the carbon 3′ of the pentose

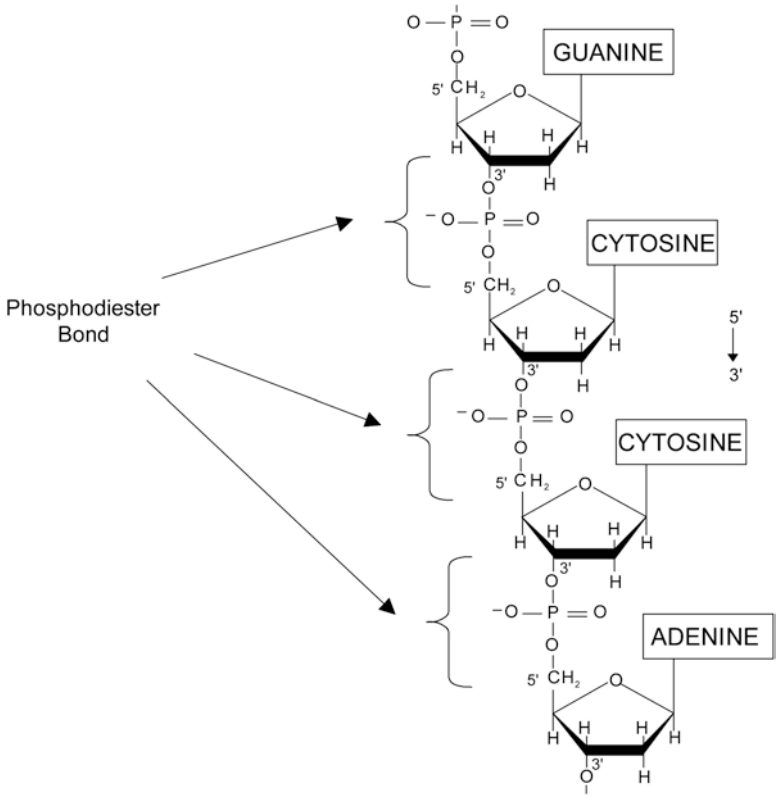

Fig. 6.1 Schematic representation of phosphodiester bond between two nucleotides (adapted from LEHNINGER 1985)

of the adjacent nucleotide. This latter bond is called the phosphodiester bond and constitutes the strand of nucleotides. This type of bond confers polarity on the DNA molecule which plays a role in the replication and transcription, called the 5′ → 3′ direction, as shown in Fig. 6.1. The direction of the 3′ and 5′ phosphodiester bonds of one strand is the opposite to that of the other strand. Thus, these strands are commonly referred to as antiparallel. As a result, at each extremity of the molecule, one of the polynucleotides ends in a 3′ and the other in a 5′.

DNA is comprised of two strands of nucleotides, and the association between them is carried out by the pairing of nitrogen bases. As previously mentioned, adenine associates with thymine and cytosine with guanine. This configuration is achieved through hydrogen (H) bridge bonds, as shown in Fig. 6.2, which are responsible for the stability of the helix. There are two possibilities for a bridge between adenine and thymine (involving the nitrogen of one base and the oxygen of the complementary base) and three between cytosine and guanine (involving two nitrogen with oxygen and one nitrogen with nitrogen). This scenario is universal in living organisms, since the structure of DNA is the same for bacteria, fungi, plants, and animals. In fact, the differentiation lies in the sequence in which the nucleotides are associated.

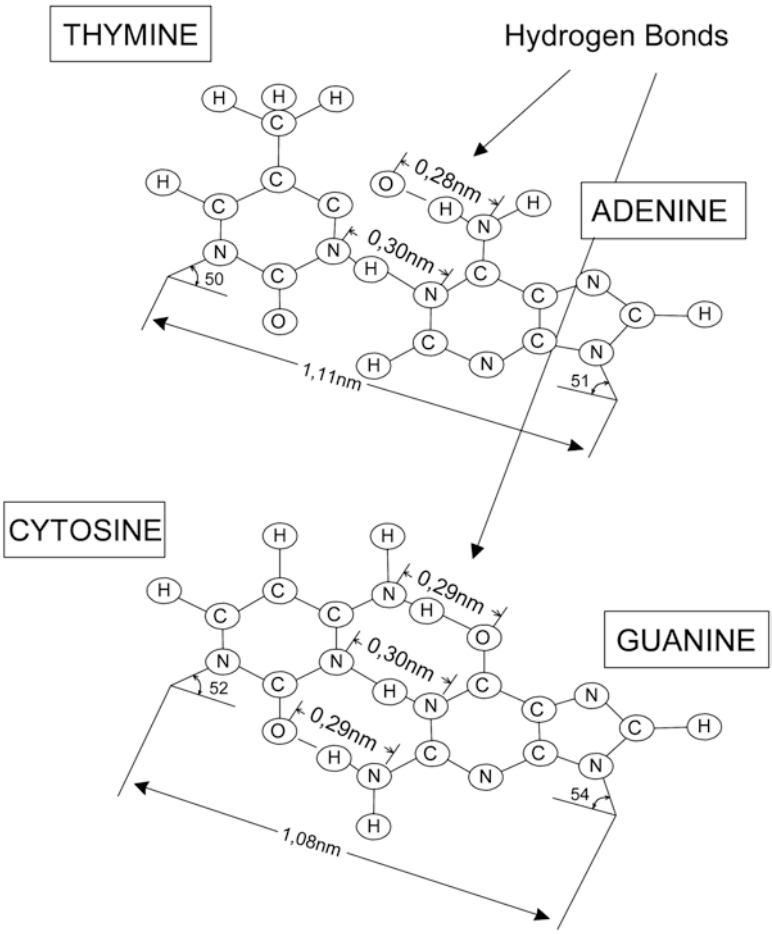

Fig. 6.2 Hydrogen bridge bonds between adenine and thymine or cytosine and guanine (adapted from LEHNINGER 1985)

The denaturation through the rupture of the hydrogen bridges can be complete or partial and occurs earlier in the case of the A = T bonds, which have two hydrogen bridges in contrast to the C ≡ G bonds, which are more resistant, since they have three hydrogen bridges. The partial denaturation allows the zones rich in AT and the zones rich in CG to be identified.

Biochemical analysis of DNA preparations of different species shows that, although the composition of nucleotides is very varied, there is a general quantitative rule that the number of adenine bases is equal to the number of thymine bases (A = T) and likewise for guanine and cytosine (G = C). The constructed model revealed that the effective number of hydrogen bridges which can be formed between G and C or between A and T was greater than for any other combination.

The double-helix model for DNA, proposed by Watson and Crick in the 1950s, perfectly explains the quantitative biochemistry.

With the discovery of the double-helix structure of DNA, the transfer of genetic information between cells can be explained. Since each strand of DNA contains a sequence of nucleotides which is exactly complementary to the nucleotide sequence of the other strand, both strands carry the same genetic information. Thus, if we designate the two strands as A and A′, strand A can serve as a template for creating a new A′ and vice versa. Therefore, the genetic information can be copied through a process in which strand A is separated from strand A′ and each one serves as a template for the production of a new complementary strand. This process is known as semiconservative replication, which enables the perpetuation of the DNA molecule through cell division (mitosis or meiosis).

Based on this understanding, the scientists could conclude that with the base pairing mechanism, it becomes evident that DNA carries information by way of the linear sequence of its nucleotides. Each nucleotide can be considered as one letter of an alphabet of four letters, used to "write" biological messages. Organisms differ from each other because their respective DNA molecules carry different nucleotide sequences (ALBERTS et al. 1994).

The enzyme which catalyzes the formation of the 5′ → 3′ phosphodiester bond between the new nucleotide and the strand being formed is called DNA polymerase. After the formation of this bond, the DNA polymerase moves along the old nucleotide of the template strand, repositioning itself to promote the binding of a new dNTP to the growing strand. It is worth noting that DNA polymerase, responsible for the polymerization of the DNA molecule, can only catalyze the growth of a new polynucleotide in the 5′ → 3′ direction, considering that it is only able to add nucleotides at the free 3′-OH end of a growing strand. This mechanism ensures that the new nucleotide provides the energy required for its own incorporation, that is, the energy comes from the breaking of the linkages between the phosphates bound to the carbon 5′ of the triphosphate of the nucleotide to be incorporated and not by the end of the growing strand.

The addition of a nucleotide to a strand via DNA polymerase only occurs when it is correctly aligned with the corresponding nucleotide of the template strand. If this is not the case, the DNA polymerase removes the mismatched nucleotide from the free 3′-OH end, a property known as 3′ → 5′ exonucleotide activity, in the opposite direction to the growth of the strand. This property is in fact a correction mechanism which enables DNA polymerase to verify the last nucleotide incorporated, correcting its own incorporation errors as it moves along the template DNA molecule. The capacity for auto-correction of DNA polymerase is crucial to ensure a high degree of fidelity in the DNA replication which, in turn, provides genetic stability over generations.

If the errors which occur during the DNA replication process are not corrected by the DNA polymerase enzyme, they will perpetuate in the form of mutations. Furthermore, DNA is continually damaged by physical and chemical external agents, and although cells have sophisticated repair mechanisms, some damage will remain and will also be expressed in the form of mutations. However, low mutation

rates are important for evolution, bearing in mind that new alleles (alternative forms of the gene which occupy the same place on homologous chromosomes) are formed from mutations.

The other known nucleic acid is RNA (ribonucleic acid), formed from the transcription of DNA (reproduction of a DNA strand in a complementary RNA sequence). The transcription process is catalyzed by the enzyme RNA polymerase and begins in certain regions of the DNA known as promoters, this being the site where the RNA polymerase binds (initiation stage). The RNA polymerase unwinds the DNA, initiating the synthesis of RNA which, as in the case of DNA, occurs in the $5' \rightarrow 3'$ direction (elongation stage). Finally, the termination of the polynucleotide strands of the synthesized RNA occurs.

In contrast to DNA, RNA is comprised of a single strand of nucleotides which are linked through phosphates ($5' \rightarrow 3'$ direction). Furthermore, RNA is smaller and less stable molecule in comparison with DNA. The bases which constitute RNA are adenine, uracil, cytosine, and guanine. RNA can be observed in the cell nucleus and cytoplasm.

From the functional and structural point of view, three major varieties of RNA can be identified: transfer RNA (tRNA), messenger RNA (mRNA), and ribosomal RNA (rRNA). The main function of the different types of RNA is the synthesis of proteins (a process called translation), which are the final compounds of the gene expression of a genetic code stored in DNA. Proteins have structural and catalytic functions.

The messenger RNA carries genetic information directly from the DNA, present in the nucleus, transporting it to the ribosomal sites. It enables the transcription of the genetic code to be carried out. The mRNA molecule is much larger than the protein which it forms, considering that three nucleotides are required to codify one amino acid. In prokaryotic cells, in particular, mRNA molecules can be even larger, since in bacteria a long mRNA molecule can be translated starting from different locations, giving rise to more than one protein, depending on the location at which the translation was initiated.

Ribosomal RNA represents the locus of protein synthesis in the cytoplasm. It is found free in the cytoplasm or associated with rough endoplasmic reticulum. When combined with proteins, it forms particles called ribosomes which are easily observed by electron microscopy. When bound to messenger RNA filaments, ribosomes form polyribosomes, where protein synthesis occurs. Ribosomal RNA is much more abundant than other types of RNA, accounting for 80% of the cellular RNA. Ribosomes are formed of two subunits, one large and one small, with different structural and functional characteristics. The subunits bind in a reversible way at the beginning of the synthesis of the protein molecule and separate when the protein is completed. The large subunit of ribosomes in eukaryotic cells contains three types of RNA, with sedimentation coefficients of 28S, 5.8S, and 5S. On the other hand, the large subunit of ribosomes in prokaryotic cells contains two types of RNA, one with a sedimentation coefficient of 23S and the other 5S. The small subunit is comprised of only one type of RNA (18S in eukaryotic cells and 16S in prokaryotic cells). As will be described in Sect. 6.2.1, this is the subunit most commonly used in

studies which employ molecular biology techniques for the identification and quantification of microorganisms.

tRNA presents the smallest molecule of the three types of ribonucleic acid, with a structure in the form of a cloverleaf. Its function is to bind specific amino acids required for protein synthesis and transfer them to the correct positions on the polypeptide chains under formation in the complexes formed by ribosomes and messenger RNA (polyribosomes). Thus, one property of tRNA is that it can combine with amino acids and recognize certain locations of the mRNA molecule comprised of a sequence of three bases. These sequences, which are distinct for each amino acid, are called codons. The correspondence between a nucleotide triplet and an amino acid is known as the genetic code. The sequence of three bases in the tRNA molecule, which recognizes the codon, is called the anticodon. For each amino acid there is at least one tRNA. It should be noted that tRNA can recognize more than one codon and thus the code is known as degenerate. This characteristic is important to studies conducted in the area of genetic engineering, for instance, the amplification of gene sequences starting from degenerate primers, which are based on a sequence of proteins.

6.2 Principles and Concepts of Molecular Biology Techniques Applied to the Study of Microbial Diversity

6.2.1 Introduction to Molecular Biology Techniques

The best form of biological classification is based on phylogenetic or evolutionary relationships. Genotypic information, that is, the sequences of semantic molecules (genes or transcripts, DNA, RNA, and proteins), constitutes the history of evolution, and the determination of the DNA sequence not only enables the evolutionary relationship between sequenced molecules to be measured but also provides an insight into the evolution of organisms which gave origin to the generation of these sequences.

The comparison of organisms to determine their evolutionary relationships can be carried out by comparing the nucleotide sequences of their genomes. However, this information is difficult to obtain and to interpret and generally is not a practical approach. Nevertheless, genotypes have been compared in a limited way using DNA-DNA hybridization to determine the degree of kinship between cultivated microorganisms (STACKEBRANDT et al. 1993). Studies on DNA reassociation have also been carried out to estimate the complexity of microbial communities. In this analysis DNA extracted from a certain community is denatured and then reassociated. The rate of reassociation is a measure of the magnitude of the number of individual genotypes in the environmental sample (TORSVIK et al. 1990). The conclusion drawn from these studies is that natural microbial ecosystems are extremely complex. A small sample of soil, for instance, can contain thousands of different

genotypes. Evolutionary relationships can also be inferred through comparative analysis of the individual gene sequences of the genome (ZUCKERKANDL and PAULING 1965). The evaluation of the phylotype of an organism provides considerable information regarding its characteristics. Some properties of an organism can be predicted based on the properties of related organisms. It is expected that all of the representatives of a certain phylogenetic group have the same properties which occur frequently in the group.

Given the difficulty associated with using the complete primary structure of DNA to carry out these comparisons, protein-codifying gene sequences, amino acid sequences, and genes which have a sufficient degree of conservation for the species analyzed (e.g., ribosomal RNA genes) are compared (HILLIS et al. 1996).

In this context, molecular biology techniques are frequently based on ribonucleic acids (RNAs) of small ribosomal subunits (16S rRNA for prokaryotes) or their corresponding genes (rDNAs), considered to be the biopolymers most suitable for biodiversity studies. The sequences which codify for 16S ribosomal RNA have been used most extensively to classify phylogenetically the diversity of life (WOESE 1987; WOESE et al. 1990).

This molecule in particular was selected because of its universality and its abundance in all living beings (10^3–10^5 ribosomes per cell) and also because it derives from a common ancestor and is genetically stable and its size is compatible with amplification by polymerase chain reaction (PCR) (described in Sect. 6.2.2). Besides these characteristics, the rRNA molecules have regions which are extremely well conserved among all organisms which share that species of rRNA, since, given its rigid structure, needed to maintain its function, the rate of mutation during the evolutionary process is very low when compared with other genes (ROSADO et al. 1997). At the same time it has regions which are highly variable and the degree of variation in these regions can vary from one taxon to another. The presence of these variable regions offers great possibilities for the design of domain-specific, genus-specific, or even species- or strain-specific probes (WOESE 1987; ROSADO et al. 1996).

These specific characteristics of the rRNA molecule allow the comparison of organisms within a single domain and also enable strains within the same species to be differentiated and the microbial diversity to be phylogenetically classified. Furthermore, the gene sequence is sufficiently long for the data generated to be statistically relevant, and it can be easily sequenced using current technology. In this context, differences in the sequences of ribosomal nucleic acids have led to an expansion in knowledge in the area of phylogeny (construction of phylogenetic trees) and quantitative schematic representations of the evolutionary diversity (HILLIS et al. 1996; WOESE 1987; OLSEN et al. 1994). The construction of phylogenetic trees, in turn, is the most incisive way to infer phylogenetic relationships from molecular sequencing data.

On analyzing information gathered using techniques based on the RNA molecule, it was possible, for the first time, to evaluate the biodiversity of a natural habitat in a complete and relatively simple manner (SANZ and KÖCHLING 2007). One significant advantage of using information on rRNA sequencing is its availability in

databases (RDP, GenBank, EMBL) which, in most cases, can be accessed free of charge, allowing the comparison of new sequences obtained with others already recorded in the databanks (COUTINHO et al. 1999).

Studies on the molecular diversity of microorganisms should focus on defined functional groups. The analysis of ribosomal sequences obtained directly from environmental samples can reveal new species, break established paradigms, and even lead to the restructuring of the taxonomy of functional groups. For example, the sequencing of fragments of 16S rDNA of a group of ammonium-oxidizing bacteria amplified from soil and sediment samples revealed that the vast majority of sequences were new, indicating that the three cultivated genera of this group (*Nitrosomonas*, *Nitrosospira*, and *Nitrosococcus*) were not dominant in any of the samples analyzed (STEPHEN et al. 1996). The results of this study redefined this functional group, previously considered to be of low diversity, and highlighted the importance of the molecular approach in studies to evaluate and monitor microorganisms in the natural environment (ROSADO and DUARTE 2002).

The perfecting of molecular biology techniques together with advances in the area of bioinformatics has led to specialization within environmental microbiology to such an extent that an area called molecular microbial ecology has emerged (ROSADO et al. 1997).

In environmental biotechnology, bioreactors destined for wastewater treatment processes can be considered as one of the most elaborate niches created with the main objective of cleaning up water (OKABE and KAMAGATA 2010). These engineered systems have been applied for more than a century in order to minimize the impact of anthropogenic activity on the environment. Over this period, the design of bioreactors was gradually changed to improve its performance in terms of pollutant (e.g., organic matter and nutrients) removal efficiency, leading to a more stable operation of the treatment system.

Despite the extensive use of bioreactors for more than a century in the wastewater treatment sector and all of the innovations related to these biological systems, many plants still face a variety of problems such as bulking sludge, excess foaming, and unsatisfactory nitrification and phosphate removal (MARTINS et al. 2004; SEVIOUR et al. 2000). The reasons for these operational problems are diverse and can be attributed to inappropriate reactor design, inadequate operation of the reactor, exposure to inhibitors, and shock loads of organic compounds, toxic substances, and salt. However, it should be noted that most problems related to functioning and performance of the bioreactors are associated with changes in the structure of the microbial communities.

Biological wastewater treatment processes harbor a great variety of microorganisms. In the activated sludge process, for instance, which is one of the most applied technologies, several types of microorganisms are present, such as bacteria, protozoans, fungi, micrometazoans, and algae. Of the whole microbial population, 95% correspond to bacteria (JENKINS et al. 1993), which play a crucial role in the biodegradation of pollutants.

Despite the importance of biological processes for the treatment of wastewaters, information on the microbial ecology of these systems is scarce. For a long time, biological processes have been regarded as a “black box,” without complete

elucidation of the microbiological phenomena taking place in bioreactors. This was mainly due to the lack of analytical techniques capable of determining the identity of microorganisms playing a key role in the biodegradation processes. Therefore, in most cases, the interest has been centered entirely on the removal efficiency of certain pollutants, and the main objective is to adequate the wastewater to the requirements established by the environmental legislation. In this context, the powerful metabolic potential of the microbial communities was exploited without a detailed knowledge of the organisms responsible for the treatment.

In the 1900s, research on the microbiology of the biological wastewater treatment processes had many methodological limitations. Methods which were dependent on cultivation, such as plate counts or most probable number (MPN), were the preferred choice for the detection and quantification of bacteria in wastewater treatment systems. In fact, despite the limitations of these techniques, they are still used in some cases for the quality control of effluents, particularly with regard to pathogens and several indicator organisms.

The introduction of different culture-independent molecular techniques and new insights into microbiology (notably in relation to wastewater treatment) in the past decade have aided the improvement of the design, operation, control, and hence the performance of new generations of bioreactors. The molecular methods allowed to identify the most important microorganisms in the treatment system (particularly the prokaryotes) and contributed to gain an understanding of the mechanisms of these processes. Therefore, it became possible to determine precisely the composition, structure, function, and dynamics of complex microbial communities present in biological wastewater treatment plants.

Microbial ecology and biotechnology are hence intrinsically related. The former generates a theoretical foundation for carrying out biotechnological processes, whereas in environmental biotechnology, the concepts and tools of microbial ecology are applied in order to better manage the processes. When managed appropriately, a wide range of benefits can be obtained.

On contemplating the possibility of identifying the microorganisms present in a certain treatment system, a question may arise: why is it important to determine which types of bacteria are present in each functional group of a certain wastewater treatment plant? The answer to this question may be given taking into consideration that the identification of the key microorganisms that are involved in degradation processes taking place in biological treatment reactors, be it related to organic matter, nutrients (nitrogen and phosphorus), or even micropollutant and pathogen removal, is of great importance since these organisms are responsible for the maximum utilization of a certain substrate. Thus the identification of the organisms playing important role in the treatment is essential for obtaining high removal efficiency of pollutants, which is the main objective of all wastewater treatment plants. Furthermore, molecular tools can aid the identification of bacteria not yet cultivated, some of which are important for the formation of floc structures and others responsible for problems occurring during the operation of the bioreactors, such as bulking sludge (commonly attributed to filamentous bacteria) and the excessive formation of foam (WAGNER and LOY 2002). Therefore, the importance of the identification and analysis of both beneficial and harmful microorganisms in the systems is clear,

as the efficiency and robustness of a wastewater treatment plant depend on the composition and activity of its entire microbial community.

In summary, the possibility of relating the biological system performance to the composition of the microbial community present in treatment systems and the functions of each bacterial population, individually, is edging ever closer to reality. The ways in which this information will be used to gain better control over biological processes are being revealed continuously as knowledge on this topic is gained by microbiologists and environmental engineers.

Several functional groups of bacteria involved in the most commonly employed treatment processes have already been clearly identified and described. These groups are represented, predominantly, by the bacteria involved in nitrification and to some extent by those involved in denitrification, by several bacteria involved in enhanced biological phosphorous removal (EBPR) and by most of the bacteria which cause problems related the sludge sedimentation (bulking) and the formation of foam and scum. In each functional group, for example, nitrifiers, a limited number of phylogenetic lineages (<10) are found in nitrifying plants, in general, with few dominant populations (3–5) being present in one plant (NIELSEN et al. 2009a).

Besides the importance of identifying key microorganisms associated with the degradation of a variety of pollutants in wastewater treatment systems, attention should be given to the appearance of new and interesting sustainable solutions. These include the recovery of nutrients (e.g., P) from wastewaters or the conversion of the components present in wastewaters into compounds which can be used as, for instance, bioplastics (polyhydroxyalkanoates, PHAs). The conversion of the components in wastewater into energy through the production of methane via anaerobic digestion has been used for decades, and these processes have begun to be developed together with other energy-generating processes such as microbial fuel cells (NIELSEN et al. 2009a). In order to reach these objectives, it is clear that the management of these complex engineered biological systems requires knowledge on the microbial populations and the factors which affect their activity. This knowledge does not relate only to the identification, which needs to be reliable and precise, but also to the ecophysiology, ecology, and population dynamics of the microbial communities involved.

It should be emphasized that the composition of the microbial community at a certain treatment plant is strongly dependent on the composition of the wastewater, the design of the process, and the mode of operation of the plant. Clearly, certain functional groups are dominant only when specific processes are included in the treatment plant design (for instance, nitrogen removal or EBPR). However, little is known regarding the factors which control the composition of a microbial community. Thus, studies on the microbiology of wastewater treatment plants are important in order to observe and record factors which may be decisive for the presence of different species.

An activated sludge system aimed solely at the removal of organic matter requires only aerobic tanks (besides the settling tank). However, when denitrification/EBPR stages are also included, anoxic/anaerobic tanks are also required. The presence of anoxic/anaerobic tanks substantially modifies the structure of the microbial community.

The sludge age (sludge retention time) is another determinant parameter for the selection of the dominant bacterial populations in the bioreactor. Low values for this parameter (less than 5–10 days) may prevent the occurrence of nitrification due to the low growth rates of the nitrifying microorganisms. On the other hand, a high value for sludge age (more than 20–30 days) is crucial for obtaining complete N and P removal in temperate climates. In plants which operate at high temperatures (over 40 °C), microbial communities which are less common are generally selected (NIELSEN et al. 2009a).

As mentioned previously, the composition of the influent wastewater is another decisive factor for the bacterial growth, and it has a notable influence on the determination of the dominant populations in certain systems. Industrial and domestic wastewaters differ considerably. The former may contain many substances which adversely affect the microbial development in biological treatment processes (organic/inorganic substances which have an inhibitory effect on microorganisms). Moreover, they may be lacking in important nutrients, such as P, N, and other micronutrients. Depending on their origin, industrial wastewaters can contain high salt concentrations, which affect the microbial ecosystem. On the other hand, domestic wastewaters provide a more balanced relationship between organic compounds and nutrients and also contain a higher number of microorganisms. These microorganisms entering the bioreactor with the influent wastewater can also affect the composition of the microbial community.

Another important factor to be taken into consideration, which has also been little explored and is poorly understood, is the reactor configuration used to carry out a specific process, such as nitrification. The investigation of whether different reactor configurations, operating in continuous or batch modes, receiving the same wastewater, lead to development and growth of different microorganisms is a research topic which should be highlighted. Table 6.1 summarizes some factors which can determine the microbial community in wastewater treatment systems.

Studies describing the complete composition of the community of microorganisms in wastewater treatment plants have been published. The work of JURETSCHKO et al. (2002), who studied the activated sludge process for the treatment of industrial wastewater aimed at removing C and N, and a study by KONG et al. (2007), who investigated the biological removal of N and P from a mixture of domestic and industrial wastewaters, are examples. Several studies have also been carried out on specific populations in several (laboratory and full-scale) treatment systems, considering, for instance, only nitrifying and/or denitrifying microorganisms, polyphosphate-accumulating organisms, or filamentous bacteria. In Sect. 6.3.2 some studies are described, each related to specific microorganisms in different reactor configurations. Table 6.2 shows the most common species and genera of bacteria found in wastewater treatment plants.

To highlight the importance of the molecular approach to studies regarding the biological nitrogen removal process, particularly the nitrification step, for decades the main microorganisms responsible for this process were considered to be *Nitrosomonas* and *Nitrobacter*. However, with the application of molecular techniques, it became evident that other microorganisms are also important. The same

Table 6.1 Overview of important factors which determine the structure of microbial communities in wastewater treatment systems (adapted from NIELSEN et al. 2009a)

Factors	Description of factors
Process performance	• Removal of C, removal of C and nitrification • Removal of C and N (nitrification/denitrification) • Removal of C and N and EBPR • Chemical precipitation of P • Sludge age
Plant operation	• Oxygen concentration • Cell retention time • Addition of chemical agents (Fe/Al salts, polymers) • External addition of C (methanol) • Biomass concentration
Type of treatment plant	• Activated sludge (continuous or batch regime) • Biofilters (percolation filters) • Membrane bioreactors • Moving bed biofilm reactors • Aerobic granular sludge reactors
Wastewater composition	• Industrial/domestic • Soluble fractions/particulates (C, N and P) • Specific organic compounds • Micronutrients • Toxic substances • Salinity • Alkalinity • pH

occurred for the microorganisms responsible to biological phosphorus removal, which are able to accumulate polyphosphate. *Acinetobacter* spp. were known as examples of such organisms. However, several other microorganisms were identified with the use of molecular microbial ecology to be the main responsible for the bio-P removal process (e.g., polyphosphate-accumulating organisms).

Another example of information which has been obtained from molecular tools is related to ammonium-oxidizing bacteria (AOB) in wastewater treatment systems. The studies suggest that different plants have different AOB populations and different levels of species richness. OKABE et al. (1999), for instance, observed that a laboratory-scale biofilm reactor for the treatment of domestic wastewater was dominated by *N. europaea*. SCHRAMM et al. (1998) and JURETSCHKO et al. (1998) observed that the populations of AOB in laboratory and full-scale plants were dominated by *Nitrosospira* and *N. mobilis*, respectively. However, based on the gene *amoA*, it has been observed that the *Nitrosospira* species are not important AOBs in the full-scale treatment systems (WAGNER and LOY 2002).

It is important to mention that the knowledge and information acquired with the use of molecular techniques can, in some cases, be minimal and their usefulness for engineers and technicians responsible for the design and operation of bioreactors is not well defined. Thus, it should be considered how knowledge obtained applying molecular techniques can be used to improve the performance of treatment systems. Questions regarding how important it is to know the exact composition of the

Table 6.2 Microorganisms commonly observed in wastewater treatment systems (adapted from NIELSEN et al. 2009a)

Functional group	Populations commonly reported
Nitrifiers	
Ammonium-oxidizing bacteria (AOB)	Genus *Nitrosomonas* (*N. europaea*, *N. eutropha*, *N. mobilis*, and *N. oligotropha* (class *β-Proteobacteria*) Genus *Nitrosospira* (class *β-Proteobacteria*)
Nitrite-oxidizing bacteria (NOB)	Genus *Nitrospira* (sublineage 1 and 2) (phylum *Nitrospirae*) Genus *Nitrobacter* (class *α-Proteobacteria*)
Anammox bacteria	Lineage *Brocadia*, *Kuenenia*, *Scalindua* and *Anammoxoglobus* (phylum *Planctomicetos*)
Denitrifying bacteria	Genus *Candidatus* Accumulibacter (class *β-Proteobacteria*) Genus *Azoarcus* (class *β-Proteobacteria*) Genus *Curvibacter* (class *β-Proteobacteria*) Genus *Thauera* (class *β-Proteobacteria*) Genus *Zoogloea* (class *β-Proteobacteria*)
Polyphosphate-accumulating organisms (PAOs)	Genus *Candidatus* Accumulibacter (class *β-Proteobacteria*) Genus *Tetrasphaera* (phylum *Actinobacteria*)
Glycogen-accumulating organisms (GAOs)	Genus *Candidatus* Competibacter (class *γ-Proteobacteria*) Genus *Defluviicoccus* (classe *α-Proteobacteria*)
Filamentous bacteria	Species of class *α-Proteobacteria* Genus *Sphaerotilus* (class *β-Proteobacteria*) Genus *Thiothrix* (*Theiothrix* spp and type 021N) (class *γ -Proteobacteria*) *Candidatus* Microthrix parvicella (phylum *Actinobacteria*) Genus *Skermania* (phylum *Actinobacteria*) Genus *Gordonia* (phylum *Actinobacteria*) Genus *Rhodococcus* (phylum *Actinobacteria*) Genus *Dietzia* (phylum *Actinobacteria*) Species of phylum and class *Chloroflexi* Genus *Haliscomenobacter* (phylum *Bacteroidetes*)

microbial community in order to improve the performance of the process need to be answered, in order to take advantage of the enormous bank of information provided by the molecular tools. Further information regarding the relationship between a certain microbial community and the operation and stability of biological treatment systems is still needed. The future perspective is that, based on the reliable identification of microbial populations by way of molecular techniques, these questions will be answered.

Clearly, it is not necessary to have specific information on the phylogenetic position or taxonomic classification of each organism acting in a certain biological system in order to design a wastewater treatment plant. However, knowledge gained from molecular techniques can aid a better understanding of the biological processes and break certain paradigms which have been established over time (SANZ and KÖCHLING 2007). The use of conventional methods combined with molecular tools should also be encouraged, since it has been demonstrated that both methodologies leave gaps when used individually, but these can be filled when they are used concomitantly (MUYZER and SMALLA 1998).

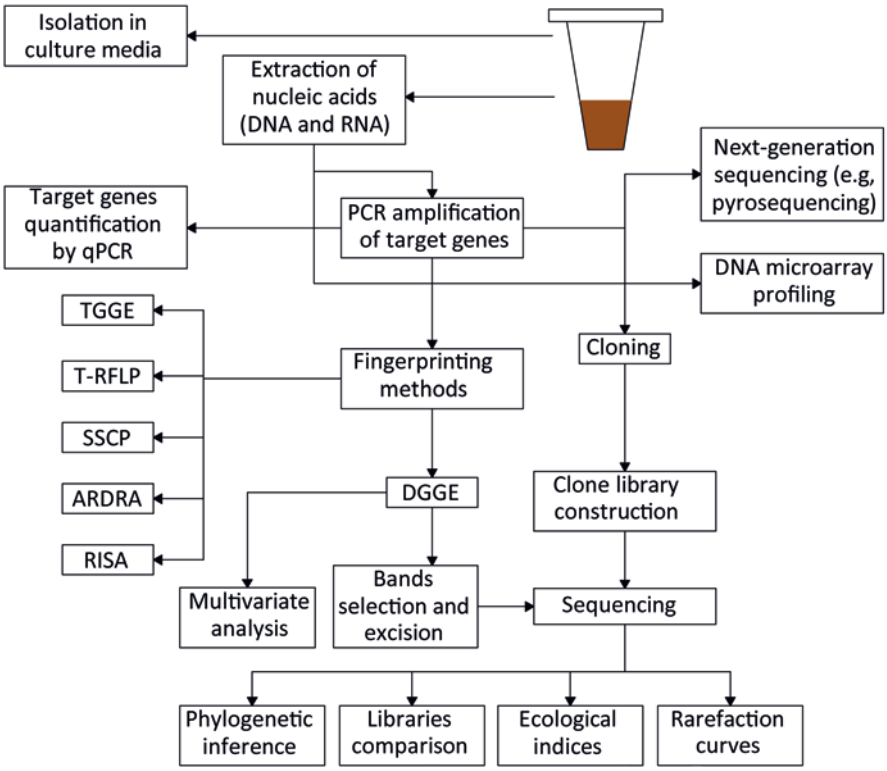

Fig. 6.3 Schematic representation of several molecular techniques used for the characterization of microorganisms from different environments (adapted from ANDREOTE et al. 2009)

The schematic diagram in Fig. 6.3 shows some molecular techniques used to study the genetic diversity of environmental samples of microorganisms. In general, the strategy used to collect the samples and the method employed for DNA/RNA extraction are important factors which determine the success of the experiment.

To complement the information given in Fig. 6.3, Fig. 6.4 provides an illustrative summary of the main procedures used in molecular microbial ecology, many of them used to assess the microbial community of wastewater treatment bioreactors.

In both natural and engineered environments (e.g., biological wastewater treatment reactors), there is a high diversity of microorganisms which are present in different numbers. This high diversity represents the variety of different populations which are organized into different communities. In general, these are structured in a similar way, being comprised of many populations represented by a few individuals and a few dominant populations with many individuals.

On carrying out a survey of a certain microbial community to determine the species and the number of individuals present in each species, it will be difficult to sample all of the populations present. Thus, in order to obtain data which are closer to the reality, some steps must be respected before the sampling. The first step

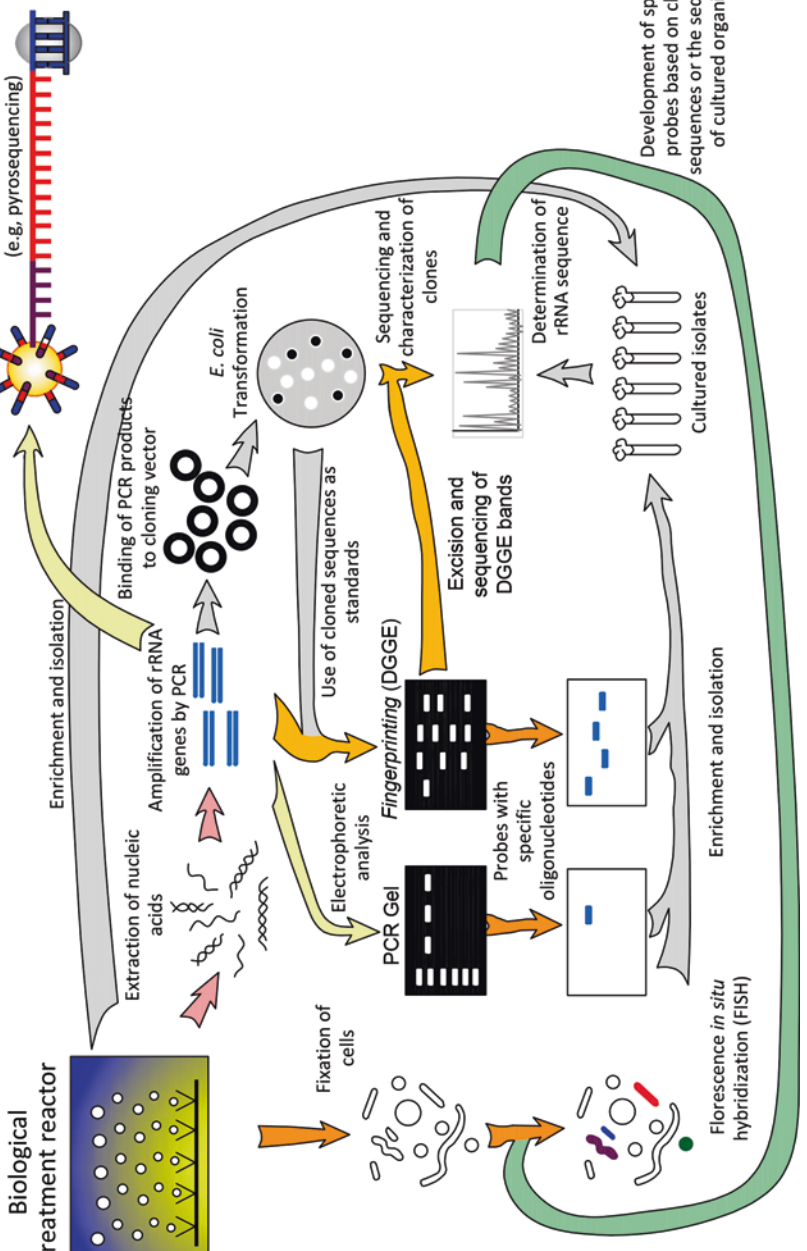

Fig. 6.4 Illustration of the main techniques used in molecular microbial ecology of engineered wastewater treatment systems (adapted from HEAD et al. 1998)

before beginning any study is to formulate a hypothesis to be proved. The objectives to prove this hypothesis can then be outlined after which the methodology can be defined. This last step is very important since not only the analysis to be carried out needs to be defined but the entire sampling process to be conducted. Issues such as the sample number and frequency have to be carefully addressed since they provide the statistical support for the study. If the aim is to describe the community, the samples must be collected randomly from the environment under investigation (e.g., biological reactors for wastewater treatment) since different populations can be distributed in the form of aggregates (in the case of biofilms), uniformly or even randomly.

Once the sampling strategy has been defined, the collection can be carried out. The highest degree of care must be taken to avoid contamination at this stage so that the sample obtained faithfully represents the number or activity of the populations of the environment under investigation. Specific equipment can be used depending on the sample to be collected. The preservation of samples until processing is also of great importance. They should be kept in a water bath with ice at a temperature of between 0 and 4 °C and sheltered from light. In some cases the freezing of samples can be harmful. However, for molecular analysis, freezing the sample at −20 °C immediately after collection is recommended. The preservation time also has to be appropriate. Several studies have demonstrated that the earlier a sample is processed the closer the results will be to the reality. Thus, ideally, the sample should be processed immediately after collection.

The processing, and also the analysis, will be dependent on the type of sample and the microbial group to be studied. However, in all cases the samples should be homogenized prior to processing. The sample may be diluted or concentrated depending on the environment sampled. Both qualitative and quantitative analyses need to be carried out.

With the development of molecular microbial ecology, investment in DNA extraction methods for different types of organisms from different samples has become very important and consists of an essential step in research using molecular techniques (ROSADO et al. 1996). The choice of the best method for the isolation of DNA or RNA from different environments is dependent on the type of nucleic acid to be isolated and the type of environment from which it will be extracted. Different methods are available for the extraction of nucleic acids from samples originating from different sources, and each case should be considered individually.

Several protocols have been described for the extraction of nucleic acids from different environments (OGRAM et al. 1987; SMALLA et al. 1993; VAN ELSAS et al. 1997). The extraction has been carried out based on two approaches: direct lysis in situ (OGRAM et al. 1987) or cell extraction followed by cell lysis (HOLBEN et al. 1988). These methods originate from pioneering studies which served as a starting point for the development and perfecting of other simpler and faster protocols (TREVORS and VAN ELSAS 1995).

Most techniques currently used for the extraction of nucleic acids from an environment involve direct DNA and/or RNA extraction. This methodology enables a greater total DNA yield to be obtained (OGRAM 2000). The identification of the

most efficient method for the study of bacterial diversity should take into consideration the extraction of nucleic acids from highly differentiated cell types, such as bacterial spores and Gram-positive and Gram-negative cells (NIEMI et al. 2001). These methods, in general, involve the use of mechanical disaggregation of the cells. In this case, the main problem is the excessive fragmentation of the nucleic material obtained. Fragmentation inhibits studies on the hybridization of the total DNA of a community and can lead to artifacts on applying the PCR technique (NIEMI et al. 2001).

The extraction of DNA from water samples basically involves four different stages. In the first method, the lysis of microbial cells is carried out by chemical methods using, for instance, SDS and phenol (OELMÜLLER et al. 1990). The second method involves mechanical cell lysis (FERRIS et al. 1996). In the other methods the DNA is extracted from the samples using heat generated in the thermocycler during PCR. KIRCHMAN et al. (2001) proposed that a small fragment of the membrane used in the sample filtrations is placed in the amplification tube to serve as a template in the reaction. ØVREÅS et al. (1997) used a concentrated suspension of non-lysed cells as a template. According to the authors only 3% of the cells were lost using this methodology.

In this context the selection of an extraction protocol will be dependent on the material to be used as well as the application of the nucleic acid, which will require different degrees of purity. Several commercial kits for DNA extraction are available on the market, each one containing a specific protocol to be followed. For each specific sample the best method available should be selected according to sample origin.

After extracting the genetic material, target genes can be amplified by PCR (in the case of DNA) or reverse transcription polymerase chain reaction (RT-PCR) (in the case of RNA). The target DNA molecules can also be quantified during amplification by means of quantitative real-time PCR (qPCR), which provides the number of molecules produced per cycle (Fig. 6.3).

The PCR or RT-PCR product can be analyzed by molecular fingerprinting methods, such as denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) (Sect. 6.2.3), terminal restriction fragment length polymorphism (T-RFLP), amplified fragment length polymorphism (AFLP), ribosomal intergenic spacer analysis (RISA), and single-stranded conformation polymorphism (SSCP) (Sect. 6.2.6), and/or can be cloned and sequenced (Sect. 6.2.4) for identification of the microorganisms. These sequences can be used for phylogenetic inference and to characterize the diversity and richness of microbial populations by means of several ecological indices. Furthermore, the obtained sequences can be very useful for designing oligonucleotide probes to be applied in fluorescence in situ hybridization (FISH—Sect. 6.2.5), a technique which does not require the extraction of DNA (Fig. 6.4).

The identity of PCR products can also be directly revealed by next-generation high-throughput sequencing technologies. These powerful miniaturized and parallelized platforms can generate hundreds of mega- to gigabases of nucleotide sequence data. Recently developed technologies can also analyze single molecules and do not

require previous PCR amplification. Further details on the next-generation sequencing approaches are given in Sect. 6.2.7.

DNA microarray (Sect. 6.2.6.7) is also a powerful molecular technique which may be used to determine the expression of thousands of genes from a certain biological sample in a single experiment. It usually makes use of cDNA obtained from RT-PCR of the RNA extracted from the sample. Thus, information regarding the degradation potential of the microbial communities from wastewater bioreactors can be obtained.

The schematic representation in Fig. 6.4 highlights the importance of combining conventional techniques of culture and isolation with molecular methods, particularly when aiming to better understand the microbial communities present in a certain environment. The representative diagrams of each technique shown in Fig. 6.4 will be better understood as the reader progresses through this chapter.

There follows a description of the main molecular biology techniques used both for the identification and quantification of microorganisms in various biological systems and to study the dynamics of microbial populations under different environmental conditions. Some techniques applied with less frequency, but with a considerable potential for use, will also be described.

6.2.2 PCR

The first molecular techniques employed after the total extraction of nucleic acids from microbial communities of environmental samples were DNA hybridization, the determination of the percentage of GC content in the communities, DNA reassociation, and RFLP. The major limitation of these techniques is the need for a large quantity of relatively pure DNA (FRANKLIN et al. 1999). The molecular techniques currently employed to study microbial diversity in a wide variety of biological systems are based on the principle of amplifying a target sequence through the polymerase chain reaction (PCR). Described by MULLIS and FALOONA (1987), PCR is a powerful tool which revolutionized molecular biology practices, enabling the development of highly sensitive and specific protocols for the detection and/or quantification of microorganisms, plasmids, transposons, or genes (ROSADO et al. 1996).

The polymerase chain reaction can be carried out based on an understanding of the DNA duplication mechanism and is based on several factors associated with the mechanism, such as the fact that DNA serves as a template for its own duplication and that the enzyme DNA polymerase catalyzes the formation of a new strand of DNA. Through PCR it is possible to amplify selectively in vitro a single DNA or RNA molecule millions of times within only a few hours, using the basic elements of the natural DNA replication process. This makes it possible to detect and analyze specific gene sequences of a sample, without cloning or the need for hybridization (OSTE 1988; VAN DER ZEE and HUIS 1997). Disadvantages of this technique include the need for prior knowledge regarding the target DNA sequences which need to be copied and infidelity in the DNA replication (STRACHAN and READ 2002).

In summary, the method is based on the capacity of specific primers for a certain DNA region of an organism to anneal only to the desired sequence via the complementarity of bases. The primers, which are able to locate a single site in a highly complex genome, are short oligonucleotides generally of 10 or 20 base pairs. They promote a perfect hybridization with the opposite filaments of the target sequence (base pairs of the DNA strand under study) and trigger the synthesis of the complementary DNA sequence with the aid of the enzyme DNA polymerase. This enzyme is responsible for the extension of the target DNA, recognizing the complex formed by the primer and the strand of the template DNA, which results in the simultaneous copying of both directions of the DNA segment flanked by the two annealed primers (OSTE 1988).

In order for the PCR procedure to reproduce, in the laboratory, the natural DNA replication process, the DNA polymerase needs to tolerate the thermal cycles used during this procedure. Thus, this enzyme is commonly extracted from *Thermus aquaticus*, a bacterial species which survives at high temperatures (SAIKI et al. 1988). When extracted from this microorganism, the enzyme is referred to as Taq DNA polymerase.

As a result of the PCR, parts of the gene, generally the exons of the DNA, can be rapidly amplified using known specific primers. The PCR procedure involves a succession of three stages (representing one cycle), which are determined by different temperatures:

- Thermal denaturation of the double-stranded DNA of the sample which will serve as a template, generating two single strands. This stage involves rapid incubation (30 s to 1 min) at a temperature of 90–95 °C.
- Hybridization of oligonucleotide primers or specific annealing by complementarity with a target DNA sequence by cooling. The primers flank the DNA ends of interest on the two strands resulting from the previous step (denaturation). The annealing occurs at position 5′ of the single strand of the template DNA. The ideal temperatures for this stage vary between 45 and 60 °C, and it lasts approximately 1 min. The temperature is selected according to the size and composition of oligonucleotide primers.
- Amplification or elongation of oligonucleotides with the aid of the enzyme Taq DNA polymerase, carried out at 70–72 °C, for a period of 1–3 min. This stage is characterized by the extension or polymerization and is initiated when the primer has bound to the complementary segments of the template strand. The DNA polymerase binds the nucleotides together, completing the single strand and transforming it into a double strand, promoting its extension and producing an exact copy of the target sequence.

In the following cycles, a chain reaction subsequently occurs in which the recently synthesized DNA strands are separated from the original strands in the denaturation stage and each strand again serves as a template in the annealing and extension stages for the formation of new strands, which guarantees an exponential increase. Figure 6.5 shows a schematic representation of the principle of the polymerase chain reaction.

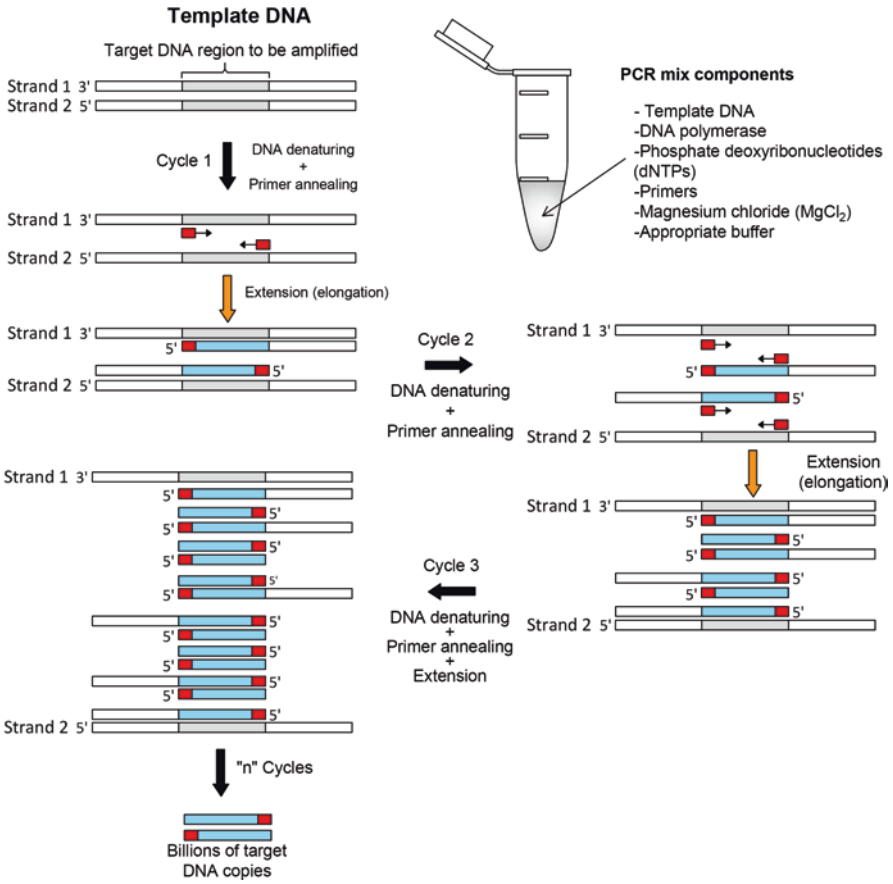

Fig. 6.5 Principle of the PCR in which small specific DNA sequences (primers) are used to link target DNA strands denatured by heating. The extension of the molecule for the formation of a complementary strand is carried out through the annealing of primers, via the thermostable enzyme DNA polymerase

The denaturation, annealing, and extension processes can be carried out in a cyclic form, due to the thermostability of the enzyme DNA polymerase, until the target sequence is found in detectable quantities. These processes are usually repeated for 30–40 cycles. Since all of the components are present in the sample from the beginning of the reaction, the procedure used to carry out the PCR can be automated and conducted on an instrument known as a thermocycler, programmed by adjusting the time, temperature, and number of cycles, which are mainly dependent on the objective to be accomplished (OSTE 1988; VAN DER ZEE and HUIS 1997).

Before the first cycle in the thermocycler is started, the oligonucleotide primers, DNA polymerase, phosphate deoxyribonucleotides (dNTPs—dATP, dTTP, dCTP, dGTP), magnesium chloride ($MgCl_2$), and an appropriate buffer are mixed in a PCR tube. Some commercial buffers for PCR already contain $MgCl_2$. This component

Table 6.3 Some components of the PCR reaction and their most common concentrations (adapted from LORENZ 2012)

Component	Final concentration
Template DNA	10–100 ng
PCR buffer	One-tenth of reaction volume (buffer 10× concentrate)
$MgCl_2$	1.5 mM
dNTPs (dATP, dCTP, dGTP, dTTP)	200 µM
Primers (forward and reverse)	20 µM = 20 pmol/µL
Enzyme (Taq DNA polymerase)	2.5 units
Sterile nuclease-free water	Until completing the final volume (50 µL)

provides Mg^{2+} divalent cations which act as a cofactor for type II enzymes such as restriction endonucleases and polymerases. The reaction volume generally varies from 25 to 100 µL, and the most common concentrations (for a 50 µL reaction) are given in Table 6.3. These conditions allow the amplification of most samples, but can be modified for each new PCR reaction as required. The temperature of each stage, the duration of each cycle, and the number of cycles must be adjusted in order to increase the reaction yield. The annealing temperature determines the degree of specificity of the reaction, with higher temperatures increasing the specificity of the primer/template DNA.

Theoretically, considering that the sequence flanked by the primers doubles with each cycle (Fig. 6.6), after 30 cycles a theoretical factor of one billion copies of the target sequence can be reached.

The quantity of DNA amplified during the polymerase chain reaction (PCR) can be analyzed by electrophoresis in agarose or polyacrylamide gel, a technique used to separate and characterize the DNA fragments obtained. Thus, it can be observed whether or not the amplification occurred. Electrophoresis is a basic procedure in all analysis carried out on nucleic acids. This technique is based on the fact that the nucleic acid molecules have a negative electric charge in aqueous solution, which is originated from the ionization of the phosphate groups present in the nucleic acid molecules. The negatively charged molecules can migrate in a solid support (agarose or polyacrylamide) submitted to an electric field. The nucleic acids migrate in the direction of the positive pole and separate according to their molecular weight. Small molecules tend to migrate at higher velocities compared to large molecules.

Acrylamide gel, in particular, is commonly used for DNA fragments of less than 1 kb. The gel can later be stained with dyes (e.g., ethidium bromide and SYBR® Safe or SYBR® Green) and visualized directly or using light (HIRATA and HIRATA 1991). Figure 6.7 shows an agarose gel, visualized by staining with SYBR® Safe in a transilluminator and the bands resulting from the PCR product. In this case the primers used were Bac341f (5′-CCTACGGGAGGCAGCAG-3′) and Bac907r (5′-CCC CGT CAA TTC CTT TGA GTT-3′) (MUYZER et al. 1993), yielding a PCR fragment of around 550 base pairs.

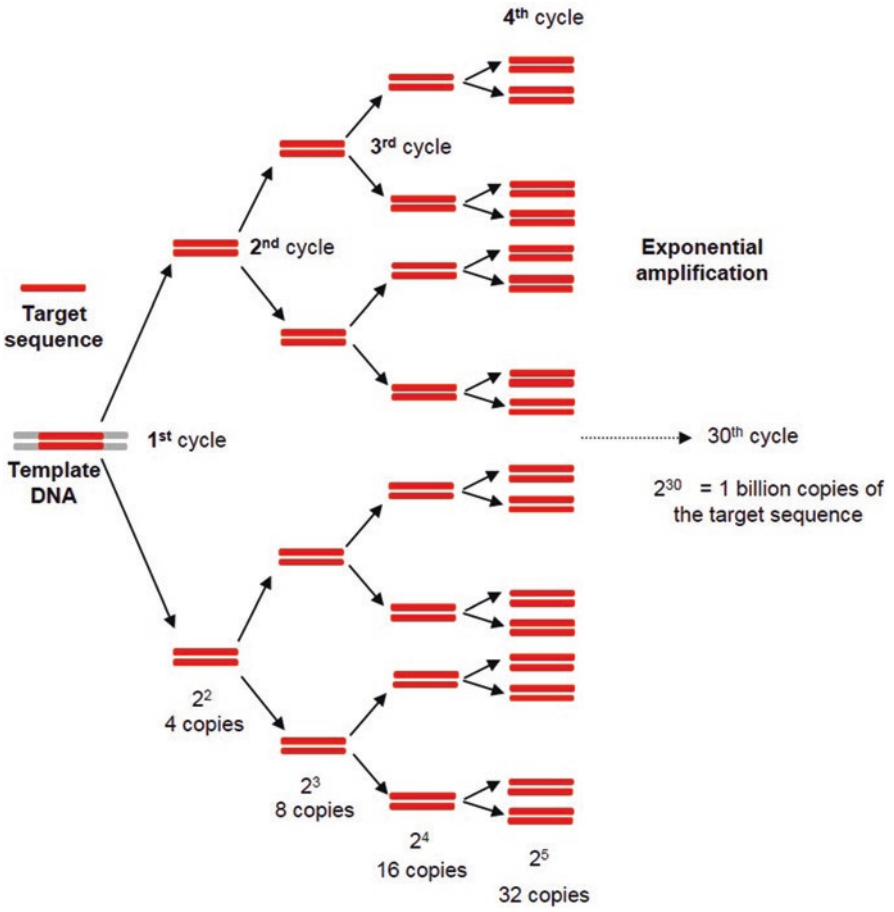

Fig. 6.6 Schematic diagram illustrating the duplication of the sequence flanked by the oligonucleotide primers

The primers binding to the region of the template DNA are important for the enzyme DNA polymerase to perform its polymerization function, since amplification of the DNA will only occur if there is hybridization of the primer with a DNA segment of the sample. The efficiency and specificity of the amplification must be taken into account when the primer is designed. In addition, it is crucial that there is no complementarity between the two 3′ ends of the primers to avoid the formation of dimers which would reduce the formation of the target sequence.

The primer design generally represents the most critical parameter in the development of PCR protocols, bearing in mind that in many cases it determines the success of the DNA fragment amplification. There is no region which guarantees the amplification of the desired segment, although some strategies can be used in the designing of the primer pairs. Examples of these strategies include that the primer length should be around 15–30 base pairs and the content of GC residues should be

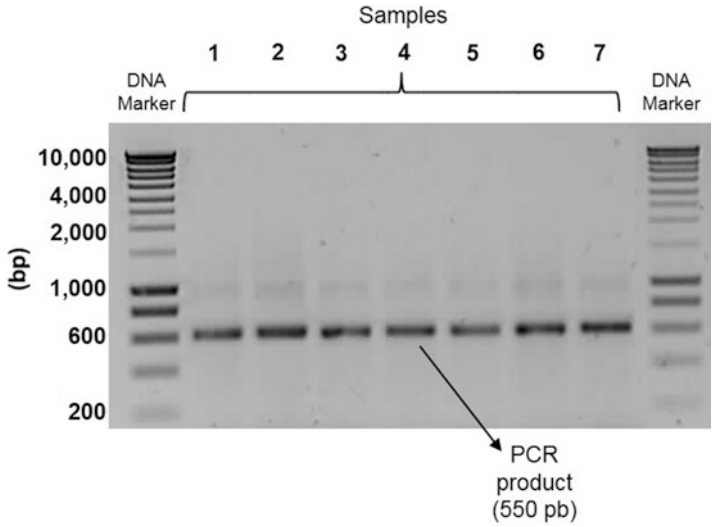

Fig. 6.7 Electrophoresis in agarose gel of PCR products

around 50%. Besides that, these nucleotides must be randomly distributed in the primer. The designing of the primers, when carried out with care, can allow savings in terms of time and costs, considering that they consist of the most expensive components in the PCR technique.

One fault which has been observed in the PCR technique is the preferential amplification of sequences caused by the nonspecific pairing of some regions of the DNA molecule, which hinders the binding of the PCR primers with the target region, generating nonspecific products. Another factor which needs to be taken into consideration in PCR studies on complex samples is the preferential amplification caused by the different sizes and concentrations of the genes in the sample. One way to prevent the selective amplification in these samples is to create conditions which facilitate the denaturation of the DNA strands, with the addition of alkalis, cosolvents, or acetamide to the reaction (REYSENBACH et al. 1992).

The choice of the DNA region located between two primers which is specifically and selectively amplified in an exponential manner strongly influences the degree of specificity of the PCR procedure and is dependent on the purpose of the test. The specificity is dependent on the degree of homology between the target sequence and the DNA of different genera and species.

As described above, highly conserved regions of a universal gene are normally chosen as target to carry out the PCR test for the identification of microorganisms. This strategy is based on the use of universal sequences as targets for amplification, as, for instance, the sequences of ribosomal RNA (rRNA). The rRNA genes are well conserved both functionally and with regard to their sequence, besides being associated with regions of moderate variation (WOESE 1987), which makes the comparison of rRNA sequences a very useful tool not only for identification but also for understanding the phylogenetic and evolutionary relationships of the organisms

(RODÍCIO and MENDOZA 2004). In fact, the first applications of techniques based on nucleic acids in the study of microbial ecology were related to the phylogenetic relationships between microorganisms determined through the analysis of the sequence of 16S rRNA (MACRAE 2000). Furthermore, other techniques were developed based on the use of these markers, such as in situ hybridization and ARDRA (KOZDRÓJ and VAN ELSAS 2000), which will be described in Sects. 6.2.5 and 6.2.6, respectively.

The small subunit of rRNA, that is, 16S, is a molecule which is widely used for phylogenetic analysis in prokaryotes. The 16S rRNA is a polyribonucleotide of approximately 1500 nucleotides, codified by the rrs (rDNA) gene. The 16S rRNA, as with any single-chain sequence, folds into a secondary structure which intercalates single chains and double chains, as shown in Fig. 6.8 (RODÍCIO and MENDOZA 2004).

23S rRNA has also been gaining attention since it has a higher number of variable regions, allowing phylogenetic analysis at the species and subspecies levels, and thus it can be used to complement 16S rRNA (CHRISTENSEN et al. 1998). Although 23S rRNA has approximately 3000 nucleotides, presenting therefore twice as much information and greater accuracy in relation to phylogenetic inferences in comparison with 16S rRNA, the latter has become a reference because it can be sequenced more easily. However, this does not diminish the importance of the use of 23S rRNA as a supplement for the data generated from 16S rRNA in studies on closely related organisms (STAHL 1997).

The spacing region between the ribosomal genes 16S and 23S of rRNA has been applied not only for the identification of species, but it has also been the target of many studies seeking to characterize and develop molecular markers in prokaryotes and eukaryotes (JENSEN et al. 1993). The space between genes 16S and 23S of rDNA is known as the internal transcribed spacer (ITS), and it has a higher degree of variability compared with regions 16S and 23S. These variations have been used to differentiate bacterial species and for phylogenetic analysis (LEBLOND-BOURGET et al. 1996).

In summary, interest in the conserved region of rRNA originates from two factors. The first relates to the principle that the three genes which codify the ribosomal subunits are contained in a single locus, which occurs several times in one genome. The second factor is associated with the presence of variable regions flanked by codifying regions, known as intergenic spacer (IGS) and internal transcribed spacer (ITS) regions, both of which are very useful for the differentiation of species and genera (EDEL 1998). In general, the use of well-conserved regions enables the identification of a set of related microorganisms and not an individual genus or species. The use of highly variable regions provides greater specificity, which allows the identification of a particular genus or species. The greater the variability of the region the more discriminatory the PCR will be (MITCHELL et al. 1993).

Besides the use of universal primers, PCR can also be carried out with functional genes encoding enzymes which catalyze specific reactions. If the process to be monitored involves a characteristic biochemical reaction, such as the biological degradation of a certain organic compound in a wastewater treatment reactor, the gene which encodes for the enzyme involved in the degradation can be amplified by

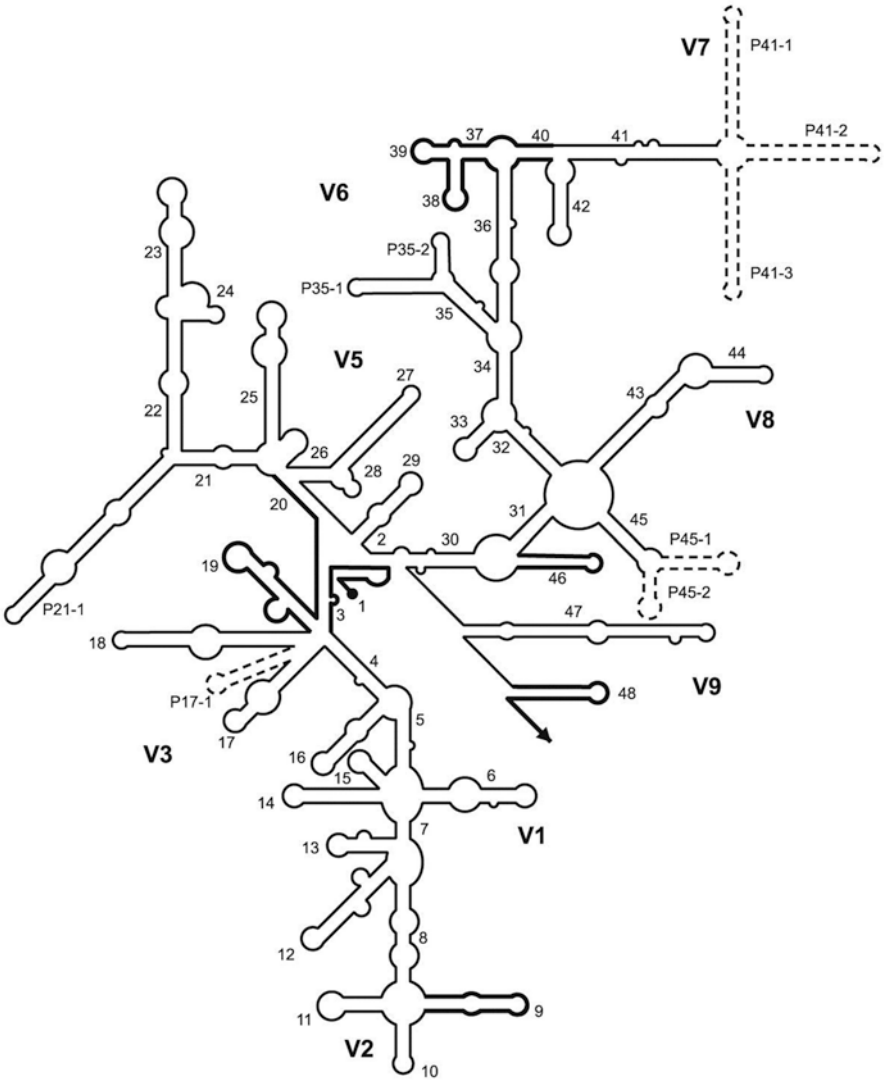

Fig. 6.8 Schematic representation of the secondary structure of 16S rRNA. The conserved regions are represented by the *bold lines* and the variable regions (V1–V9) by *fine lines* (adapted from RODÍCIO and MENDOZA 2004)

PCR and the products submitted to fingerprinting methods (e.g., DGGE) and/or sequencing. In this PCR approach where the gene of interest is targeted, unimportant bacteria are excluded from the analysis, and classification at lineage level is possible. However, information regarding the overall profile of bacterial community is not provided. Furthermore, microorganisms which may contribute to the process functioning but do not have the genes of the target enzyme are not detected.

The PCR technique is still evolving and innovations are frequently incorporated into this procedure. Some operational problems are prone to occur, for instance, extreme sensitivity and contamination, which leads to false-positive results, and samples containing substances which inhibit the amplification, giving false negatives.

Since PCR technique can be used to amplify DNA from a small number of molecules, the contamination of any component of the mixture with exogenous DNA can lead to the simultaneous amplification of another species of DNA. In addition, the presence of a PCR product and primers from previous amplifications can also act as a source of contamination. The inclusion of a negative control in each PCR experiment is one way to detect contamination in the reaction. This control should contain the same components used in the other samples, except the target DNA molecule.

Another point to be noted is the importance of also including a positive control to observe the occurrence of false negatives, which can originate from problems with the thermocycler, with a reagent used or with the enzyme Taq DNA polymerase. The false negatives can also originate from the choice of primers with a high concentration of pairs of guanine-cytosine nucleotides (GC greater than 50%). This bond is comprised of a triple-hydrogen bridge, characterizing a stronger bond, and thus it is more difficult to denature, requiring a longer time to reach the required temperature to break this bond. These factors can lead to incomplete denaturation, reducing the possibility for hybridization and consequently causing a decrease in the PCR product yield (HIRATA and HIRATA 1991).

To avoid the occurrence the false positives, the reagents used must be carefully selected and dosed, in order to maintain their quality and purity, together with standardized concentrations and equilibrated pH, parameters which should remain constant from one reaction to another. Disequilibrium in the PCR system can lead to the appearance of dimers, which can be avoided by maintaining standardized procedures in carrying out all of the manual stages, as well as using special initial annealing techniques in the thermocycler program. False-positive results can also originate from contamination by amplicons, which can be avoided by following some specific procedures for the appropriate handling of samples (DEGRAVE et al. 1994).

Other modifications to the PCR technique aimed at improving the sensitivity and specificity have been proposed. One of these is known as nested PCR, where initial amplifications are carried out with a pair of primers without a high degree of specificity (e.g., universal primers). The product amplified by PCR is then submitted to a second round of amplification with another set of primers located more internally in relation to the first pair, increasing the sensitivity and specificity of the method (DIEFFENBACH et al. 1993).

Reverse transcription polymerase chain reaction (RT-PCR) technique is another variant of PCR, often used to qualitatively detect RNA expression through formation of a double-stranded complementary DNA (cDNA) from single-stranded RNA (e.g., messenger RNA—mRNA). The reverse transcription of the RNA of interest into its DNA complement is mediated by the enzyme reverse transcriptase (KAWASAKI 1990). Subsequently, the synthesized cDNA is amplified by means of traditional PCR.

There is also in situ PCR, which combines the high sensitivity and specificity of the PCR with the capacity of in situ hybridization to detect small quantities of nucleic acids inside cells, which allows rare RNA and DNA sequences to be located.

Finally, an important PCR variant is the quantitative real-time PCR (also referred to as q-PCR), which allows for simultaneous amplification and detection of targeted DNA molecules during PCR by means of fluorescent dyes. The quantity of initial target DNA molecules can be estimated by way of comparison with an internal standard (CROSS 1995; LOGAN et al. 2009). q-PCR can also be used to quantify RNA. In this case, the RT-PCR and qPCR are combined giving rise to the technique often known as RT-qPCR or even real-time RT-PCR. This approach is very useful for analyzing gene expression.

Real-time PCR requires a thermocycler which has a fluorescence emission monitoring system. This technique has the advantage of being quantitative, since it can be used to evaluate the number of molecules produced in each cycle, enabling the monitoring of the reaction and providing more precise and faster results compared with conventional PCR, which generates only qualitative data.

The most relevant characteristics of real-time PCR are speed, specificity, sensitivity, and quantification. The method involves basically three phases: baseline phase, where the PCR products are not sufficient for fluorescence detection; exponential (log) phase, where the quantity of PCR products doubles for each cycle; and finally the plateau phase, reached when the number of products no longer increases. Characteristics such as precision and good reproducibility in the quantification of the nucleic acids associated with real-time PCR arise from the fact that the values are determined during the exponential phase of the reaction.

The instrumentation required to perform real-time PCR includes a thermocycler, equipped with an optical system for the fluorescence excitation and emission collection and a computer with software for data acquisition and the final analysis of the reaction.

The point at which the cycle in the reaction reaches the threshold of the exponential phase is called the cycle threshold (C_T), as shown in Fig. 6.9. The C_T point enables precise and reproducible quantification in the fluorescence analysis. The emission of the fluorescence compounds generates a signal which increases as a function of the quantity of PCR product. The fluorescence intensity, which reflects the quantity of product amplified, is recorded during each cycle.

There are two main approaches to the quantification in real-time PCR: nonspecific detection and specific detection. SYBR® Green is the most commonly used example of nonspecific detection, in which fluorophores (molecules which absorb and emit light at a specific wavelength) bind to the double strand of the DNA and, with the excitation by light emitted by the optical system of the thermocycler, emit green fluorescence (Fig. 6.10a).

At the beginning of the amplification, the reaction mixture contains denatured DNA, the primers, and the SYBR® Green. The non-bound SYBR® Green molecules have weak fluorescence, producing a minimal signal which is subtracted during the computer analysis. After the primers have been recognized during the polymerization process catalyzed by the enzyme DNA polymerase, the SYBR® Green binds to

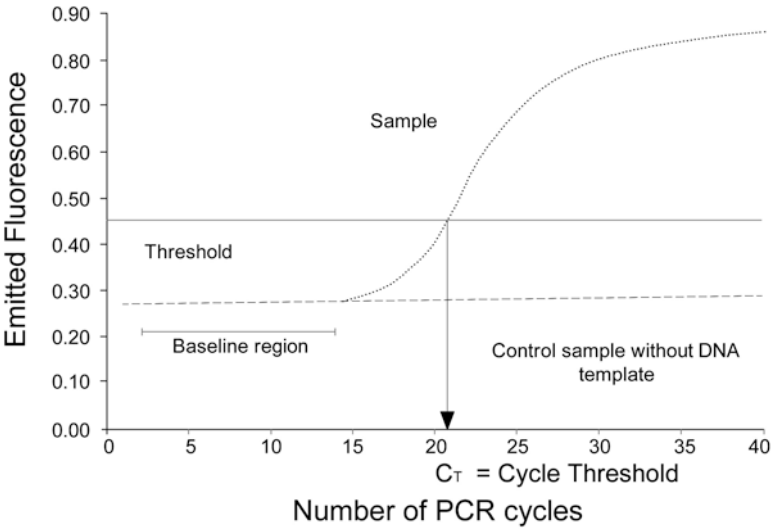

Fig. 6.9 Amplification curve of real-time PCR showing three distinct phases: baseline, log, and plateau phases

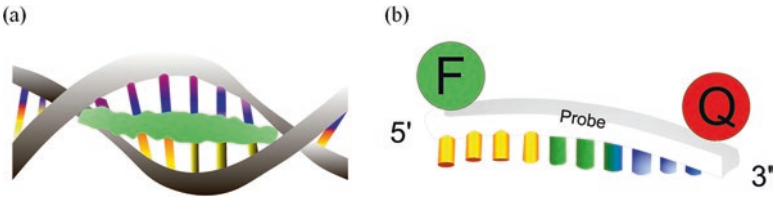

Fig. 6.10 SYBR® Green between the double strands of DNA (**a**) and TaqMan probe (**b**)

the recently synthesized double strand of DNA, enhancing the fluorescence. Thus, the reaction is monitored in continuous mode, and the fluorescence increase is observed in real time. In the subsequent cycle, in the DNA denaturation stage, the SYBR® Green molecules are released, and there is a drop in the fluorescence signal. The fluorescence detected at the end of the extension phase of each PCR cycle enables the monitoring of the increasing quantity of amplified DNA (VITZTHUM et al. 1999). The first significant increase in the quantity of PCR product can therefore be related to the initial quantity of target DNA. The higher the amount of target DNA present at the beginning of the PCR reaction the lower the number of cycles required to reach the point at which the fluorescence signal is initially detected.

SYBR® Green offers advantages such as low cost, ease of use, and sensitivity. The disadvantage is that it binds to all of the double strands of DNA which appear during the reaction, including the primer dimers and other nonspecific products, which can lead to the overestimation of concentration of the target fragment.

Besides SYBR® Green, another two methods are TaqMan and molecular beacons, both able to carry out hybridization generating energy transfer for quantification.

These latter two options consist of fluorescent probes which are highly specific for the target sequence, releasing fluorescence only when the PCR product of interest is present, i.e., when hybridization of the probe with its complementary sequence occurs. Furthermore, these specific probes allow to perform multiple reactions.

Functioning as a probe, TaqMan is a labeled DNA fragment used for the hybridization of another DNA molecule, employed to detect specific sequences of the DNA fragments amplified in the PCR. As shown in Fig. 6.10b, this probe has a fluorophore at one end and at the other end there is a quencher, that is, a molecule which accepts the energy of the fluorophore in the form of light and dissipates it in the form of light or heat.

During real-time PCR the TaqMan probe hybridizes with the target complementary single-stranded DNA sequence for amplification. In this final stage, the TaqMan is degraded due to the 5′ → 3′ exonuclease activity of the DNA polymerase, separating the quencher from the fluorescent molecule during the extension phase. With the separation of the fluorophore from the quencher, the fluorescence intensity increases, and thus during the amplification process the light emission increases exponentially. This increase in the fluorescence occurs only when the probe hybridizes and when the amplification of the target sequence is established (HEIDM et al. 1996).

Molecular beacons are nucleotides used as single-stranded probes which form a secondary structure between the 5′ and 3′ ends, known as a stem-and-loop structure. The loop contains a sequence which is complementary to the target sequence, and the stem is formed by annealing of the complementary sequences which are located at the ends. A fluorophore is bound to one end and a quencher is bound to the other, both through covalent bonds (Fig. 6.11a).

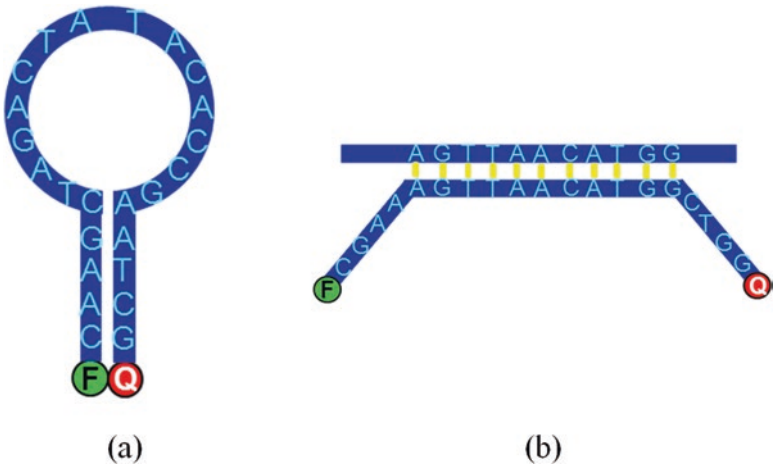

Fig. 6.11 (**a**) Oligonucleotide used as probe is synthesized so as to allow the formation of a secondary structure at the 5′ and 3′ ends. (**b**) Every strand formed during the amplification is a target for the annealing of the molecular beacon, increasing the fluorescence intensity

When there are no targets, the oligonucleotide remains free in solution and does not emit fluorescence since the quencher is close to the fluorophore, capturing energy. When the molecular beacon finds its target hybridization occurs, which results in a reorganization of its structure (change in the conformation), the fluorophore is dissociated from the quencher, thus emitting fluorescence (Fig. 6.11b).

It is important to mention that the molecular beacons can be synthesized with fluorophores of different colors and thus tests which require the detection of several targets can be carried out in the same reaction. The high level of specificity means that target sequences which differ by only a single nucleotide can be discriminated.

6.2.3 DGGE

Denaturing gradient gel electrophoresis (DGGE) has been widely applied in microbial ecology to study the phylogeny of microbial communities (MUYZER et al. 1993). It consists of a molecular fingerprinting method since it is able to generate "fingerprints" or "bar codes" of microbial communities. The DGGE technique is based on the electrophoresis of DNA fragments, previously amplified by PCR in polyacrylamide gel with a linear denaturing gradient. These fragments are submitted to an environment with increasing levels of denaturing chemical agents (urea and formamide), which promote conformational changes in the molecule, reducing its migration (SIGLER et al. 2004; ERCOLINI 2004). When the denaturing agent is temperature, the technique is called temperature gradient gel electrophoresis (TGGE).

In the case of DGGE, the concentration of polyacrylamide in the gel is constant, the urea and formamide have an increasing concentration gradient, and the temperature is fixed at around 60 °C. On the other hand, in TGGE the separation of the DNA fragments is obtained with an increasing temperature gradient and a high constant concentration of urea and formamide.

The principle of dissociation is based on the fact that the electrophoretic mobility of DNA in a polyacrylamide gel is sensitive to the secondary structure of the DNA molecule, with respect to its conformation, which can be helicoidal, partially denatured, or as a single strand. The partially denatured molecules, comprised partially of a double helix and partially a single strand, move more slowly in the gel compared with double or single strands (MUYZER et al. 1998).

When the DNA is submitted to electrophoresis under increasing conditions of denaturation (chemical or thermal), the fragments remain as double strand until the conditions required for the denaturation of molecular domains, called melting domains, are reached. These domains have identical denaturation temperatures, and thus at a certain temperature there is a complete denaturation of these domains, which are intercalated along the molecule. When a domain is denatured, the conformation of the molecule changes from helicoidal to partially denatured. Under these conditions, the migration of the molecule in the gel almost ceases, and it occupies a particular position in the gel, as shown in Fig. 6.12.

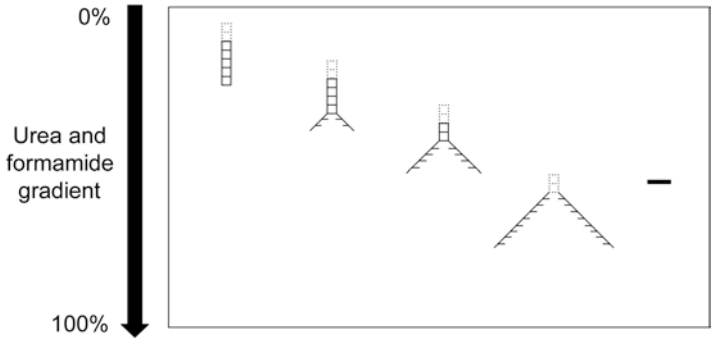

Fig. 6.12 Schematic representation of a parallel gel containing a gradient of denaturing agents (adapted from ROSADO and DUARTE 2002)

Variations in the nucleotide sequences of these domains lead to different denaturation conditions, and the molecules with different sequences stop migrating at different positions in the gel, resulting in their separation (ROSADO and DUARTE 2002).

The determination of the melting behavior of the DNA fragments is important to be determined before their analysis by DGGE/TGGE, so that the ideal gradient and duration of electrophoresis can be applied (MUYZER et al. 1998). With prior knowledge on the nucleotide sequence of one or more DNA molecules which will be analyzed in the denaturing gels, it is possible to theoretically predict the optimum gradient for the separation of different products. In this regard, computer programs can be employed, such as MELT95 (Ingeny, Leiden, the Netherlands) and several others which can predict the denaturation behavior of DNA molecules (ROSADO and DUARTE 2002).

The gradient and time period which provide the best separation of the PCR products are generally determined using a perpendicular gel and/or a parallel gel containing different gradients, where the samples are applied for varying time periods. In the perpendicular gel, there is an increasing concentration of denaturing agents located in a position perpendicular to the direction of electrophoresis. The DNA is applied in a lane which occupies almost all of the gel width. After the DNA staining, a sigmoidal curve can be observed, and on the left side of the gel (containing lower concentrations of denaturants), the PCR products have greater electrophoretic mobility, since the DNA remains as a double strand. On the other hand, on the right side of the gel (with a higher concentration of denaturants), the migration of the DNA is suddenly interrupted by the high concentration of denaturing agent (urea/formamide or temperature). In the intermediate range of denaturant concentrations, where the PCR products have different mobilities, a strong transition in the mobility occurs when the denaturant concentration reaches that required for the initiation of the separation of the double strands in a certain domain (the domain in which denaturation occurs at the lowest temperature or under the mildest chemical condition). In order to determine the best gradient for the separation of specific PCR products, various parallel gels containing different gradients can be used (MUYZER et al. 1998).

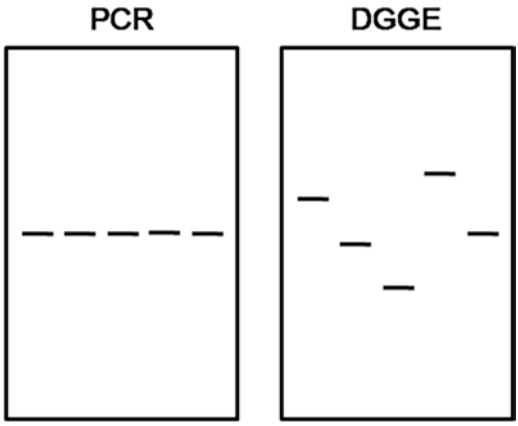

Fig. 6.13 Illustration showing PCR products with different nucleotide sequences analyzed in agarose (PCR) and through DGGE in polyacrylamide with urea/formamide denaturing gradient (adapted from ROSADO and DUARTE 2002)

The optimum time for the electrophoresis is determined through the electrophoresis in gels containing parallel gradients. Gels containing a parallel gradient have an increasing gradient of denaturing agents (chemical and physical), which increases from the beginning to the end of the gel. They are used to analyze multiple samples in the same gel. Before analyzing the samples in the parallel gel, the duration of the electrophoresis must be determined in order to obtain the maximum resolution between the different PCR products (DNA fragments). To achieve this, the individual samples are applied in a parallel gel, for the same period of time, in experiments called "time travel" (MUYZER et al. 1993).

The PCR products generally consist of fragments of the same size but with different nucleotide base sequences. Thus, they will present different electrophoretic patterns in the DGGE, that is, different migration profiles in the denaturing gel, which enables the differentiation of each microorganism (MUYZER et al. 1993; COUTINHO et al. 1999; ERCOLINI 2004). In this way, by providing a "bar code" pattern for the microbial community of the samples studied, DGGE complements the PCR information, since this latter technique only provides a single band which is not very descriptive, as discussed previously in Sect. 6.2.2.

As in the case of the PCR, the bands obtained by DGGE/TGGE can be visualized after staining with ethidium bromide, silver, or SYBR® Green. Figure 6.13 shows a schematic diagram of a situation in which fragments of the same size, obtained after amplification by PCR and visualized in agarose gel, can be separated by DGGE since they have different nucleotide sequences. It should be noted that the migratory behavior of the DNA fragment in DGGE is not only determined by the nucleotide composition (G + C content) but also by the interactions between nucleotides in the molecule (BRESLAUER et al. 1986; SUGIMOTO et al. 1996).

As a result, different DNA sequences, originating from different bacteria, denature in different concentrations of denaturant, which results in a pattern of bands where, theoretically, each band represents a specific population of organisms in the same sample. Thus, an overall view of the predominant species in the sample analyzed can be obtained. In DGGE or in TGGE, it is possible to detect approximately

50% of sequence variations in DNA fragments of up to 500 base pairs (MYERS et al. 1985). This percentage can be increased to almost 100% through the attachment of a segment rich in GC (GC clamp) to one side of the DNA fragment.

This clamp, containing 30–50 bp, when attached to the 5′ end of a primer, is amplified by PCR together with the DNA and introduced into the amplified DNA, acting as a domain with high resistance to denaturation, hindering the disassociation of the double strand of the DNA into single strands (SHEFFIELD et al. 1989). Thus, the occurrence of a loss in the definition of the electrophoretic patterns is avoided (SHEFFIELD et al. 1989; ERCOLINI 2004). The addition of this clamp increases the capacity of the DGGE to detect changes in a single base in the DNA (SHEFFIELD et al. 1989).

Some authors have used psoralen as a chemical clamp, which consists of a photoactive compound which acts as a base intercalator, marking one of the primers. This specific type of clamp has the advantage of not altering the length of the primers, although it has the drawback of not allowing the direct reamplification of PCR products. Besides that, it increases the DNA degradation rate on exposure to UV light (ROSADO and DUARTE 2002). Bipolar clamps, which are those attached to the two ends of the PCR products, have been recommended in order to increase the sensitivity of the gels with temperature as the denaturant in TGGE (GILLE et al. 1998).

Once generated, the fingerprints can be sent to the database in which the similarity between the different banding patterns can be accessed to determine the structural resemblances and differences which exist between different environments or treatments. Figure 6.14 shows the DGGE gel obtained from the PCR product amplified using primers specific to 16S rRNA, representative of the microbial community of two nitrifying reactors (SBR_1 and SBR_2) submitted to different salinity regimes (BASSIN et al. 2012). Analysis of the gel was carried out using the software program BioNumerics (Applied Maths). A dendrogram is shown in the upper part of Fig. 6.14 in which the similarity between the banding patterns can be observed. This actually represents the similarity between the microbial communities present in the samples. Besides considering the number of different bands to estimate the diversity of the microbial community, some authors consider the intensity of each band as indicative of the abundance of a specific population represented by the band (NÜBEL et al. 1999). However, this interpretation should be done with caution.

According to MUYZER et al. (1993), DGGE is a technique which is not only useful to characterize microbial communities with a high degree of complexity but also to infer the phylogeny of the community members, test the purity of microbial lineages, and monitor the isolation of microorganisms from different samples. Since it adopts band patterns, DGGE also allows the monitoring of the dynamics of specific populations as a function of environmental impacts (the introduction of stress-causing agents such as chemical products or other pollutants) and also the variations in the conditions of the feeding and the operating system (for instance in the case of treatment bioreactors). This enables the study, in a relatively rapid and simple manner, of the spatial-temporal variability of the microbial populations. Furthermore, in view of the different PCR primers available, DGGE can also be used to investigate an infinite number of phylogenies or specific target organisms.

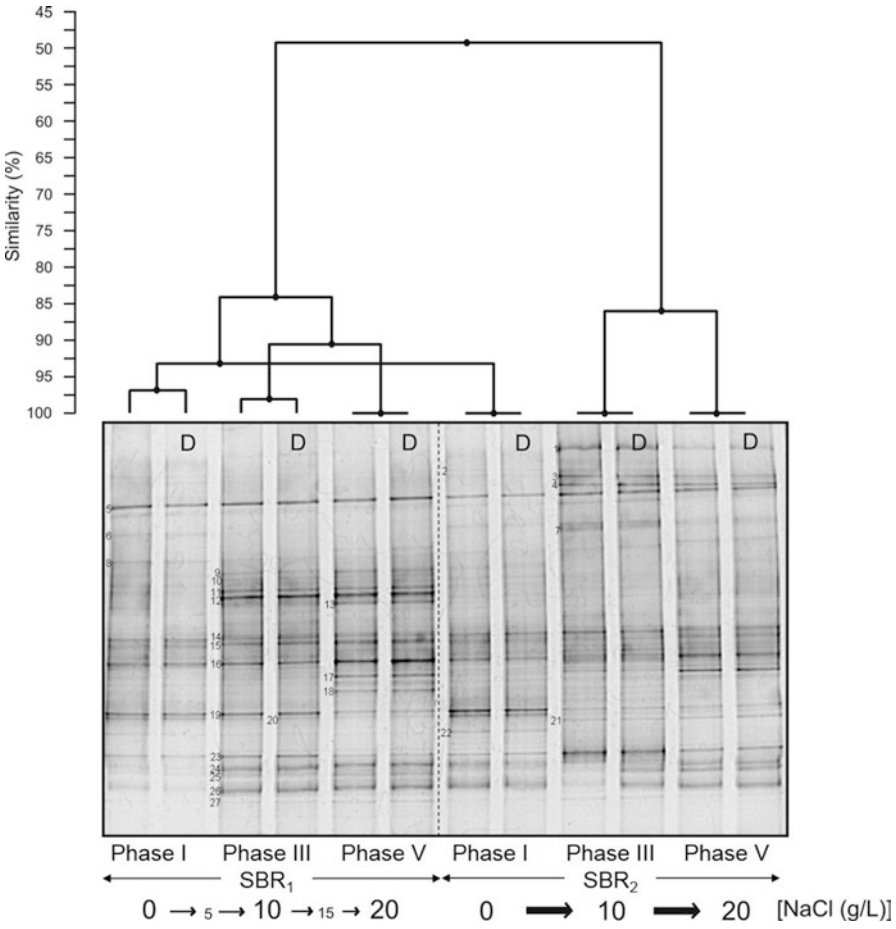

Fig. 6.14 DGGE of PCR products amplified with primers specific to the 16S rRNA gene. An analysis of the grouping showing the similarities between the different fingerprints is presented in the form of a dendrogram. D indicates the duplicate for each sample, while the numbers indicate individual bands which were excised from the gel for sequencing (BASSIN et al. 2012)

The DGGE technique also allows the excision of bands in the gels for the sequencing of DNA fragments contained in the bands. The fingerprinting obtained with the DGGE technique, when associated with band sequencing and phylogenetic analysis, has been widely employed in studies on the structural diversity of microbial communities and the phylogenetic placement of their members (MUYZER and SMALLA 1998; MUYZER et al. 1996). The sequencing of the bands present in the gel can be submitted to a global databank, thus allowing comparison with the results of different research groups worldwide. Concomitantly, the sequences obtained can be reanalyzed as the databank contents increase, providing the phylogenetic updating of the results. The DNA present in the gel can also be transferred to nylon membranes and hybridized with specific probes for the determination of certain microbial

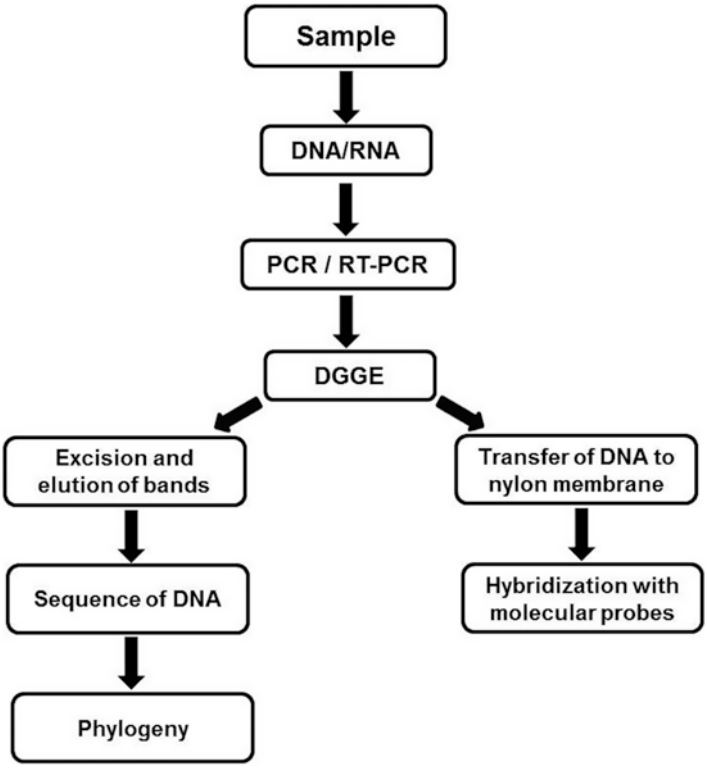

Fig. 6.15 Schematic representation of the stages and possibilities in DGGE analysis of DNA extracted from different samples

groups. Figure 6.15 shows a diagram of the different stages and possibilities which exist when working with gels containing denaturation gradients.

Another attractive feature of DGGE is the possibility to compare and analyze different samples in a single gel, in contrast to cloning. However, a drawback associated with this technique is that very long sequences may not be separated efficiently. The PCR product should not surpass 500 bp, which can limit the information required for the inference of the phylogeny (MUYZER and SMALLA 1998).

There are other aspects which should be taken into account in studies on microbial communities using techniques based on PCR-DGGE. Care should be taken regarding the fact that some samples contain several distinct species even though the G + C% contents are very similar, hindering the analysis of certain groups of organisms, referred as operational taxonomic units (OTUs). JONES and THIES (2007) proposed a complementary method for DGGE, coupling bidimensional analysis in polyacrylamide gel in order to separate PCR fragments generated by microbial communities, to avoid the superposition of the fragments.

With regard to the environmental microbiology, DGGE has been widely employed as a tool to obtain the profile of complex microbial populations without

the need for their cultivation (MUYZER and SMALLA 1998). 16S rRNA is the region most commonly used in DGGE, although the use of other specific primers has been recently proposed, particularly for analysis involving closely related microorganisms. In fact, it has been demonstrated that other regions of the bacterial genome, when compared with the region 16S rRNA, allow better results to be obtained in the differentiation of organisms of the same species (ERCOLINI 2004; YERGEAU et al. 2005; TACÃO et al. 2005).

6.2.4 Cloning and Sequencing

The cloning and sequencing of the gene which codifies for 16S rRNA is considered to be the most powerful method for the exploration of microbial diversity and is widely employed in the field of molecular microbial ecology (MUYZER 1999).

Cloning basically involves cleaving a certain gene sequence of the genome and inserting it into another DNA known as the vector, which is normally a plasmid (small DNA fragments of approximately 3 kb which can be naturally found in bacteria and are resistant to a certain antibiotic). This cleavage process followed by insertion became possible as the knowledge regarding restriction enzymes and the ligases evolved.

It should be noted that besides the control genes (resistance to antibiotics), the vectors have a multiple cloning site with several sites of enzyme restriction and initiating sequences. This allows the rapid sequencing of the cloned fragment. Fragments of up to 5 kb can be inserted into plasmids. After the cleavage and insertion of the fragment (or gene) into the vector, this can be placed into bacterial cells employing several methods such as the use of calcium chloride, electroporation, and thermal shock. Once in the cells the plasmids can multiply and produce many plasmids which contain the cloned fragment. Thus, the cloning enables an organism to contain and express a gene which is not naturally present in its structure. Figure 6.16 represents, schematically, the cloning process.

As mentioned above, gene fragments which codify for 16S rRNA (or other functional genes) present in the genomic DNA of complex samples can be amplified through the polymerase chain reaction (PCR). A genomic library derived from the amplification of these samples is produced using the cloning method. The cloning stage allows the separation of the different copies of the target 16S rDNA sequence present in the DNA extracted from microbial communities. Once the cloning has been carried out, the clone library for the 16S rRNA gene can be accessed in different ways, such as hybridization of colonies with specific probes to determine the gene, PCR with specific primers to confirm the insertion of the cloned PCR products, or sequencing to identify the fragments. After the identification, a phylogenetic evaluation of the microbial community representative of the original sample can be carried out with the aid of phylogeny programs (OLSEN et al. 1994; COLE et al. 2003). Another way to carry out this type of molecular characterization is cloning and sequencing of cDNA transcribed from 16S rRNA with the use of the enzyme reverse transcriptase (AMANN et al. 1995).

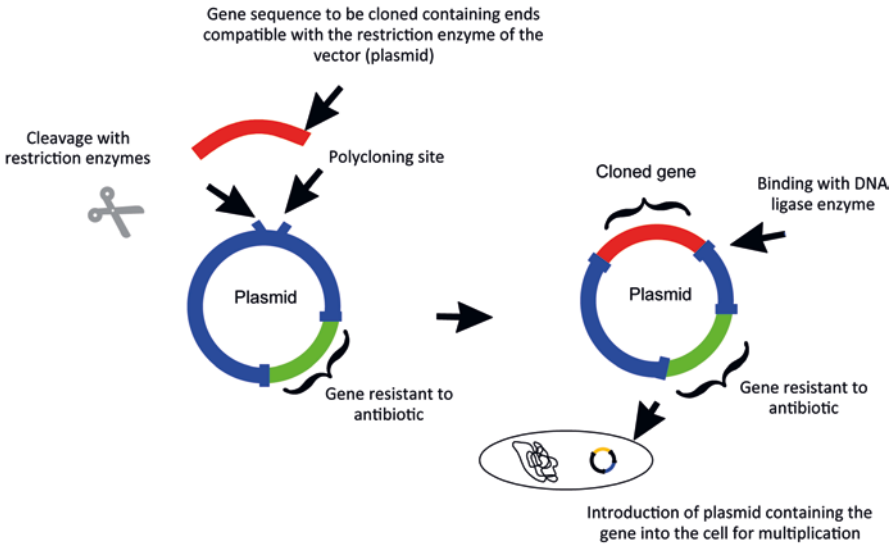

Fig. 6.16 Schematic representation of the cloning stages

While the amplicons generated from pure bacterial cultures can be directly sequenced, in the case of genomic DNA extracted from microbial communities the cloning stage needs to be included to separate the different copies of 16S rRNA, since a mixture of DNA from different sources cannot be directly sequenced.

In general, the cloning and construction of an rRNA gene library has been applied in combination with other techniques in wastewater treatment studies. The cloning of the complete gene allows the obtainment of more precise phylogenetic information in comparison with other molecular techniques such as FISH or DGGE, providing very precise taxonomic studies and phylogenetic trees of high resolution. The identification of microorganisms which have still not been cultivated or identified is also possible applying the cloning technique. In addition, it should be mentioned that cloning has potential for use as a tool for the designing of new specific primers for PCR and molecular probes for the detection and/or quantification of certain microbial groups by fluorescence in situ hybridization (SANZ and KÖCHLING 2007).

The stage of selecting clones from a library is crucial for the process of metagenomic sequencing. The selection methodologies can be based on sequence or on function. The analysis through sequence is based on the use of conserved DNA regions to design probes or PCR primers employed in the selection of clones which contain a certain sequence of interest (SCHLOSS and HANDELSMAN 2003) and is not dependent on the expression of a certain characteristic by the host. Furthermore, it is based on reliable analysis methods, such as PCR, hybridization of colonies, and microarray. As a disadvantage, the method is not selective for complete genes, and thus only genes with partial sequences of the chosen genes can be selected (KNIETSCH et al. 2003). In the case of analysis by function, this is dependent on the capacity of testing a large number of clones in the library for the production of

the desired enzyme or metabolite. Therefore, it is necessary to standardize the tests on large scale, bearing in mind that most tests are not suitable for a simple and rapid evaluation of a large quantity of clones. Also, there are some cases where the clone selection stage is not carried out, such as the random sequencing of DNA, in which the whole metagenomic library, normally of small inserts, is sequenced (PEIXOTO et al. 2008).

The creation of gene libraries allows the identification of a substantial part of the microbial species present in a sample, although a complete panorama of the microbial habitat, besides being very time consuming, is laborious to obtain. The fact is that several clones need to be sequenced in order to ensure that most of the individual species in the sample have been taken into account. It should be remarked that cloning is not a quantitative technique and PCR can favor certain species due to the differences in the accessibility of the target DNA (SANZ and KÖCHLING 2007).

The fact that the cloning method is laborious and time-consuming makes it impractical when dealing with a large number of samples. This hinders the monitoring of changes in microbial communities, particularly when several sample points are required. On the other hand, when time and effort are not limiting factors, cloning encompasses the majority of microorganisms, including minority groups, which are difficult to detect with fingerprinting methods.

Another point which is worth noting is the problems associated with the metagenomic libraries related to the low frequency with which the desired clone is found (SCHLOSS and HANDELSMAN 2003). In this context, it is common to adopt sample enrichment strategies to increase the chance of recovering a clone with the desired characteristic (PEIXOTO et al. 2008). One enrichment strategy is the creation of a library starting with an environment where the desired characteristic is present in large quantities. Another enrichment method consists of the addition of a substrate such that the microorganisms which have the desired characteristic can multiply to the detriment of the others. Also, a stable-isotope probe (SIP) can be used, in which a substrate marked with ^{13}C is added to the sample. Microorganisms which can utilize the substrate will incorporate ^{13}C into their DNA. On extracting the nucleic acid of the sample, it is then possible to separate the marked from the non-marked material by ultracentrifugation with a density gradient (RADAJEWSKI and MURRELL 2002).

Despite their broad application in the study of microbial communities in natural habitats, cloning techniques are less widespread in research on wastewater treatment processes. This lack of popularity may be due to the need for specialized personnel and equipment, which are not always available in engineering or chemistry departments. This technology can involve considerable complexity, which often restricts it to a support tool, even though it has enormous potential for application in the area of wastewater treatment (SANZ and KÖCHLING 2007).

After the cloning step, sequencing procedures are used to determine the nitrogen base sequence corresponding to a fragment or gene. There are several methodologies for DNA chain sequencing. These include the chemical method and the dideoxy method with radiation or fluorescent labeling (based on the Sanger method).

One of the most commonly used methods is automated dideoxy with fluorescent labeling, in which the sequence under study is amplified using mixtures, each containing one of the dNTPs (A, T, C, or G). In these mixtures, besides the normal nucleotide, dideoxynucleotides (A, T, C, or G) are present. These do not have hydroxyls in the pentose, which hinders the binding of a new nucleotide to them. At this point in the amplification, the DNA chain growth is interrupted. Bearing in mind that there is competition between the dideoxy and the normal dNTP, the polymerization is interrupted at different times. In addition, it is possible to obtain different-sized fragments. There are two ways to carry out this reaction. In the first, a primer containing a fluorescent label (tetramethylrhodamine) is placed into four tubes, each containing one of the dideoxy nucleotides and the other normal nucleotides. The second procedure is carried out by adding one of the four nucleotides containing a different fluorescent label (fluorescein; NDB-4-chloro, 7-nitrobenzene, 2-oxal-diazol, tetramethylrhodamine, and Texas Red) and the appropriate dideoxynucleotide in four separate reactions. Thus, for each sequence studied, four polymerase chain reactions (PCRs) analyzed by gel electrophoresis are carried out, in which the fragments are identified through their molecular size by means of fluorescent labeling.

In the second procedure, one analytical run can be carried out with all samples in a single gel slot, since the fragments have different labels. The final result is organized sequentially on a computer, which provides the product which corresponds to the correct sequence of the bases comprising the gene or the expressed sequence. Automated sequencing is able to provide information on sequences of up to 1 kb per reaction, allowing the accumulation of large quantities of information.

6.2.5 FISH

Fluorescence in situ hybridization (FISH) is one of the simplest and fastest techniques for the identification, localization, and quantification of species of microorganisms present in a wide variety of samples. This technique has also emerged as an alternative to the traditional methods for the identification of microorganisms which are based on isolation through cultivation in appropriate culture media. Basically, in situ hybridization promotes the interaction of knowledge on cell biology, cytogenetics, and molecular genetics (SPECTOR et al. 1998). Regarding the microbiology of wastewater treatment systems, the FISH technique has been shown to be very useful, allowing to detect microorganisms with high detection sensitivity.

The FISH technique is based on the existence of known and specific rRNA sequences of an organism, which allow complementary sequences to be designed. This specific sequence is known as a probe. The fluorescence probe is commonly a short sequence (15–30 nucleotides) of single-stranded DNA, bound to fluorophores which recognize complementary sequences of rRNA (16S and 23S subunits in the case of bacteria) in previously fixed cells (permeable to the probe). As a consequence, an in situ hybridization occurs (DNA-RNA combination). Thus, both the

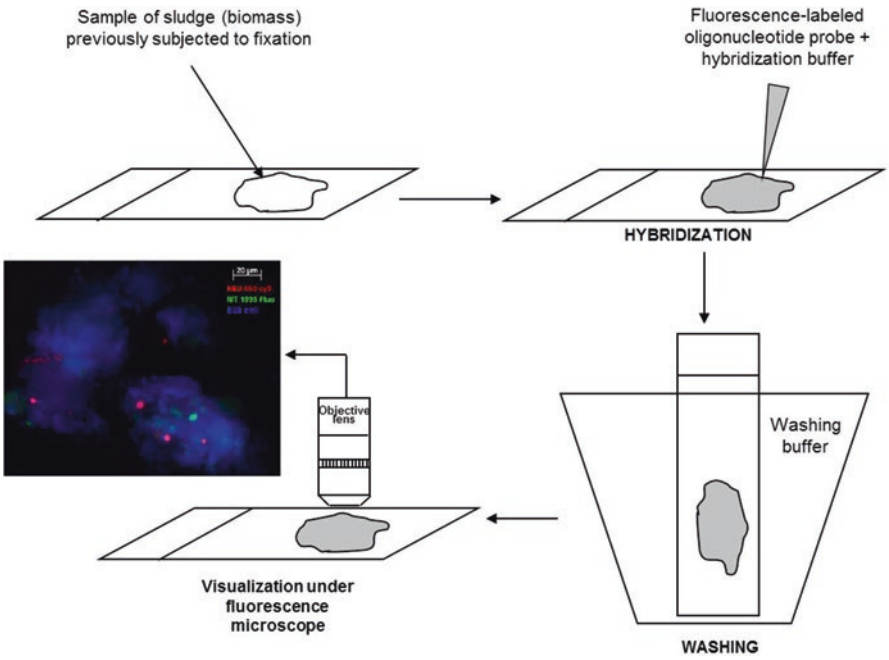

Fig. 6.17 Simplified schematic representation of the steps involved in the FISH technique for identification of microorganisms in a sludge sample (adapted from NIELSEN 2009)

location and the length of the target sequence, which, in fact, correspond to different microorganisms representative of different taxonomic levels, can be accessed by fluorescence microscopy (AMANN et al. 1990). Figure 6.17 shows schematically the procedure involved in the characterization of samples by FISH.

No probe will hybridize with cells which do not contain the target sequence. On the other hand, cells containing the desired sequence will retain the hybridized probe, and, due to the high number of ribosomes within the active cells, they become fluorescently labeled.

The FISH methodology becomes even simpler and faster when the required probes are available, allowing the direct visualization of the non-cultivated microorganisms. One of the advantages of this technique is the possibility to observe the spatial distribution of the microorganisms in the different environments in which they are found. The quantification of the specific microbial groups is also possible, which is not the case with conventional techniques (most probable number, plate counts) or with other molecular techniques. There is no need for highly trained or specialized personnel, since basic knowledge of microscopy and some laboratory experience are sufficient.

The DNA probe can be labeled directly through the incorporation of a fluorescence-labeled nucleotide precursor or indirectly through the incorporation of a nucleotide containing a reporter molecule (biotin or digoxigenin). After the incorporation

Table 6.4 Fluorescent dyes most commonly used to label oligonucleotide probes in FISH analysis (adapted from NIELSEN et al. 2009a)

Fluorochrome	Color	Maximum excitation λ (nm)	Maximum emission λ (nm)	Extinction coefficient (M^{-1} cm^{-1})
Cy3	Red	552	565	150,000
Cy5	Red[a]	649	670	250,000
SYBR Green	Green	494	520	73,000
DAPI	Blue	350	456	27,000
FLUOS	Green	494	523	74,000
TAMRA	Red	543	575	65,000
Alexa-488	Green	493	517	71,000
Alexa-546	Red	562	573	104,000
Alexa-350	Blue	343	441	19,000

[a]Emission in the infrared region, which requires a digital camera which detects infrared light and image analysis to enable the observation

in DNA, this reporter molecule is bound, through affinity, to a fluorescence-labeled molecule. Table 6.4 shows the fluorescent dyes most commonly used to label oligonucleotides in fluorescence in situ hybridization analysis.

The specificity of the probes allows the detection/identification at any taxonomic level required, from the domain to a resolution which enables the differentiation of very specific phylogenetic groups, such as species. In general, a probe aimed at the domain of bacteria is used in combination with other more specific probes.

One point to be considered relates to the fact that, although the FISH technique has allowed a better understanding of the dominant microbial communities in different biological systems, this methodology does not describe the aspects related to the function of microorganisms, but only to the phylogeny of the microbial communities.

The main disadvantage of this technique is the limited availability of specific probes for certain groups of bacteria. Although it is possible, in theory, to develop the most appropriate probe for each application in virtue of the growth of databases for the rRNA sequence (16/18S and 23/28S rRNA), in some cases it is almost impossible to develop a probe which detects specifically certain microbial groups which share the same metabolic properties, for instance, those involved in nitrification and sulfate reduction.

Some factors which hinder the use of the FISH technique are poor cell permeability, insufficient ribosome content, and inaccessibility of the ribosomes. Another point which should be noted is the fact that the FISH technique can produce false-positive (autofluorescence of the sample) and false-negative results, and both methodological and environmental factors can influence the performance of this technique. The choice of the probe and the fluorochrome, the operating conditions and the protocols used, the hybridization temperature, the fact that some microorganisms possess autofluorescence, the specific type of ecosystem, and the physiological state of the target cells can significantly influence the efficiency of this molecular tool (OUVERNEY and FUHRMAN 1997; DAIMS et al. 1999; MOTER

Table 6.5 Oligonucleotide probes aimed at the 16S rRNA gene regions used to detect ammonia-oxidizing bacteria (AOB) in nitrifying activated sludge and biofilm systems (adapted from DAIMS et al. 2009; LIPSKI et al. 2001)

Probe	Target organisms	Sequence	Reference
Nso1225	Ammonia-oxidizing bacteria (subclass *β-Proteobacteria*)	CGC CAT TGT ATT ACG TGT GA	MOBARRY et al. (1996)
Nso190	Ammonia-oxidizing bacteria (subclass *β-Proteobacteria*)	CGA TCC CCT GCT TTT CTC C	MOBARRY et al. (1996)
Nsm156	*Nitrosomonas* spp., *Nitrosococcus mobilis*	TAT TAG CAC ATC TTT CGA T	MOBARRY et al. (1996)
Nsv443	*Nitrosospira* spp.	CCG TGA CCG TTT CGT TCC G	MOBARRY et al. (1996)
NSM1B	*Nitrosomonas*	TCT GTC GGT ACC GTC AT	HOVANEC and DELONG (1996)
NEU	Halophilic and halotolerant *Nitrosomonas* spp.	CCC CTC TGC TGC ACT CTA	WAGNER et al. (1995)
Nmv	*Nitrosococcus mobilis*	TCC TCA GAG ACT ACG CGG	JURETSCHKO et al. (1998)
Cluster 6a192	*Nitrosomonas oligotropha* (*Cluster* 6a)	CTT TCG ATC CCC TAC TTT CC	ADAMCZYK et al. (2003)

and GOBEL 2000). Therefore, all these factors need to be taken into consideration in order to obtain reliable and comparable information.

It is important to note that the selection and improvement of the hybridization conditions for a new probe is a laborious and relatively complicated process, which requires experience and dedication, since the results are often unsatisfactory. The quantification can be laborious and subjective (manual counting) or complex (image analysis). The structural analysis of the aggregates (granular sludge and biofilms) requires a confocal microscope and an image analysis environment, which increases the costs and the need for specialist personnel (SANZ and KÖCHLING 2007).

Additionally, some prior knowledge regarding the microorganisms expected to be present in the sample is generally required for the successful application of this method, which leads to the need to use other techniques. For the identification and quantification of a particular species, a specific probe needs to be available, or its 16S rRNA sequence must be known (if a probe has not been previously published) (SANZ and KÖCHLING 2007).

The use of oligonucleotide probes aimed at the 16S rRNA region represents a revolution in microbial ecology, both for carrying out research and for practical applications. In studies regarding biological wastewater treatment, in particular, the hybridization techniques are by far the most commonly used.

Tables 6.5, 6.6, 6.7, 6.8, 6.9, and 6.10 provide information on some of the probes most commonly used for the identification of several bacteria found in wastewater treatment plants. The bacteria are divided into the following functional groups: nitrifiers (ammonia-oxidizing bacteria, AOB, and nitrite-oxidizing bacteria, NOB), anammox bacteria, denitrifiers, polyphosphate-accumulating organisms (PAOs), and glycogen-accumulating organisms (GAOs). This set of microorganisms can be found in aerobic or aerobic/anoxic/anaerobic activated sludge or biofilm systems.

Table 6.6 Oligonucleotide probes aimed at the 16S rRNA gene regions used to detect nitrite-oxidizing bacteria (NOB) in nitrifying activated sludge and biofilm systems (adapted from DAIMS et al. 2009; LIPSKI et al. 2001)

Probe	Target organisms	Sequence	Reference
NIT2	*Nitrobacter*	CGG GTT AGC GCA CCG CCT	WAGNER et al. (1996)
NIT3	*Nitrobacter*	CCT GTG CTC CAT GCT CCG	WAGNER et al. (1996)
Nb1000	*Nitrobacter*	TGC GAC CGG TCA TGG	MOBARRY et al. (1996)
NBAC2	*Nitrobacter*	GCT CCG AAG AGA AGG TCA CA	HOVANEC and DELONG (1996)
Ntspa662	*Nitrospira*	GGA ATT CCG CGC TCC TCT	DAIMS et al. (2001)
Ntspa1026	*Nitrospira*—sublineages I and II	AGC ACG CTG GTA TTG CTA	JURETSCHKO et al. (1998)
Ntspa1431	*Nitrospira*—sublineage I	TTG GCT TGG GCG ACT TCA	MAIXNER et al. (2006)
Ntspa1151	*Nitrospira*—sublineage II	TTC TCC TGG GCA GTC TCT CC	MAIXNER et al. (2006)
Nsr1156	*Nitrospira*—sublineage II	CCC GTT CTC CTG GGC AGT	SCHRAMM et al. (1998)
Nspmar62	*Nitrospira marina*	GCC CCG GAT TCT CGT TCG	FOESEL et al. (2008)

Table 6.7 Oligonucleotide probes aimed at the 16S rRNA gene regions used to detect anammox bacteria in activated sludge and biofilm systems

Probe	Target organisms	Sequence	Reference
Pla46	All *Planctomycetes*	GAC TTG CAT GCC TAA TCC	NEEF et al. (1998)
Amx368	All anammox bacteria	CCT TTC GGG CAT TGC GAA	SCHMID et al. (2003)
Amx820	*Brocadia anammoxidans* *Candidatus Kuenenia stuttgartiensis*	AAA ACC CCT CTA CTT AGT GCC C	SCHMID et al. (2000)
Kst157	*K. Candidatus Kuenenia stuttgartiensis*	GTT CCG ATT GCT CGA AAC	SCHMID et al. (2001)
Amx1015	*Brocadia anammoxidans*	GAT ACC GTT CGT CGC CCT	SCHMID et al. (2000)
Bfu613	*Candidatus Brocadia fulgida*	GGA TGC CGT TCT TCC GTT AAG CGG	KARTAL et al. (2008)
Apr820	*Candidatus Anammoxoglobus propionicus*	AAA CCC CTC TAC CGA GTG CCC	KARTAL et al. (2007)
BS820	*Candidatus Scalindua wagneri*	TAA TTC CCT CTA CTT AGT GCC C	KUYPERS et al. (2003)
Scabr1114	*Scalindua brodae*	CCC GCT GGT AAC TAA AAA CAA G	SCHMID et al. (2003)

Table 6.8 Oligonucleotide probes aimed at the 16S rRNA gene regions for the identification of potential denitrifying organisms (adapted from NIELSEN and HANSEN 2009)

Probe	Target organisms	Sequence	Reference
PAR651	Genus *Paracoccus*	ACC TCT CTC GAA CTC CAG	NEEF et al. (1996)
G_Rb	*Rhodobacter*, *Roseobacter*	GTC AGT ATC GAG CCA GTG AG	GIULIANO et al. (1999)
HyphoCII-654	*Hyphomicrobium denitrificans*, *H. methylovorum*, *H. facilis*	CCC ACC TCT ATC GGA CTC	LAYTON et al. (2000)
Curvi997	*Curvibacter*	CTC TGG TAA CTT CCG TAC	THOMSEN et al. (2004)
PAOmix	Most of the *Accumulibacter*	PAO462, PAO651 and PAO846	CROCETTI et al. (2000)
AZA645	Most members of *Azoarcus cluster*	GCC GTA CTC TAG CCG TGC	HESS et al. (1997)
THAU646	*Thauera*	TCT GCC GTA CTC TAG CCT T	LAJOIE et al. (2000)
ACI208	*Acidovorax* spp.	CGC GCA AGG CCT TGC	AMANN et al. (1996)
ZRA23a	Most members of the *Zoogloea* lineage	CTG CCG TAC TCT AGT TAT	ROSSELLÓ-MORA et al. (1995)
AT1458	*Azoarcus-Thauera* cluster	GAA TCT CAC CGT GGT AAG CGC	RABUS et al. (1999)
Pae997	Most of the *Pseudomonas* spp.	GCT GGC CTA GCC TTC	AMANN et al. (1996)

6.2.6 Alternative Methods Applied to the Study of Microbial Diversity

6.2.6.1 Terminal Restriction Fragment Length Polymorphism (T-RFLP)

An alternative method to DGGE to investigate the microbial diversity of different environments is terminal restriction fragment length polymorphism (T-RFLP). T-RFLP consists of a quantitative molecular method for the rapid analysis of complex microbial communities, with a high number of species (LIU et al. 1997). This technique is also based on PCR, although the procedure differs from the techniques of PCR/DGGE and PCR/cloning.

In T-RFLP, the 16S rRNA gene is amplified with universal primers, one of which is labeled with fluorochromes, which allow labeling of the PCR products. These are digested with restriction enzymes, also known as restriction endonucleases, which are "molecular scissors" able to recognize specific sequences of bases in double-stranded DNA and cleave both the double-stranded fragments at specific points (STRYER 1996). Assuming that each species present in the sample shows differences in the amplified gene sequence, the terminal restriction fragment will have a different length and can thus be separated through gel electrophoresis.

Table 6.9 Oligonucleotide probes aimed at the 16 rRNA gene regions for the identification of potential phosphate-accumulating organisms (PAOs) (adapted from NIELSEN et al. 2009b; LIPSKI et al. 2001)

Probe	Target organisms	Sequence	Reference
PAO462	*Accumulibacter*	CCGTCATCTACWCAGGGTATTTAAC	CROCETTI et al. (2000)
PA0651	*Accumulibacter*	CCCTCTGCCAAACTCCAG	CROCETTI et al. (2000)
PAO846	*Accumulibacter*	GTTAGCTACGGCACTAAAAGG	CROCETTI et al. (2000)
PAOmix	*Accumulibacter*	PAO462, PAO651 and PAO846	CROCETTI et al. (2000)
S-G-RHX-0991-a-A-18	*Candidatus Accumulibacter phosphatis*	GCTCTCTTGCGAGCACTC	HESSELMANN et al. (1999)
RHC439	*Rhodocyclus/Accumulibacter*	CNATTTCTTCCCCGCCGA	HESSELMANN et al. (1999)
RHC175a	*Rhodocyclaceae*	TGCTCACAGAATATGCGG	HESSELMANN et al. (1999)
PAO462b	*Rhodocyclus tenuis*	CCGTCATCTRCWCAGGGTATTAAC	ZILLES et al. (2002)
PAO846b	*Rhodocyclus tenuis*	GTTAGCTACGGYACTAAAAGG	ZILLES et al. (2002)
actino1011	*Tetrasphaera japonica*	TTGCGGGGCACCCATCTCT	LIU et al. (2001)
HGC69a	Gram-positive bacteria with high G + C—*Actinobacteria*	TATAGTTACCACCGCCGT	ROLLER et al. (1994)
Actino221	Potential PAOs—*Actinobacteria*	CGCAGGTCCATCCCAGAC	KONG et al. (2005)
Actino658	Potential PAOs—*Actinobacteria*	TCCGGTCTCCCCTACCAT	KONG et al. (2005)
MP2	*Microlunatus phosphovorus*	GAGCAAGCTCTTCTGAACCG	KAWAHARASAKI et al. (1998)

Table 6.10 Oligonucleotide probes aimed at the 16 rRNA gene regions for the identification of potential glycogen-accumulating organisms (GAOs) (adapted from NIELSEN et al. 2009a, b)

Probe	Target organisms	Sequence	Reference
GAM1019	*Gammaproteobacteria*	GGTTCCTTGCGGCACCTC	NIELSEN et al. (1999)
GAM1278	*Gammaproteobacteria*	ACGAGCGGCTTTTTGGGATT	NIELSEN et al. (1999)
GAOQ431	*Gammaproteobacteria*	TCCCCGCCTAAAGGGCTT	CROCETTI et al. (2002)
GAOQ989	*Competibacter* (some)	TTCCCCGGATGTCAAGGC	CROCETTI et al. (2002)
GB	*Competibacter* (most)	CGATCCTCTAGCCCACT	KONG et al. (2002)
TFO_DF218	Organisms related to *Defluviicoccus*	GAAGCCTTTGCCCCTCAG	WONG et al. (2004)
TFO_DF618	Organisms related to *Defluviicoccus*	GCCTCACTTGTCTAACCG	WONG et al. (2004)

In addition, it is possible to sequence and identify the terminal restriction fragments (T-RFs) generated by comparing them with a sequence database. The length of these fragments can also be determined and their quantity can be estimated. The different fragments obtained are distinguished by laser-induced fluorescence detection. The fluorescence data are converted into electropherograms.

Based on the terminal location of the fluorescent marker in the PCR products, it is possible to ensure that only terminal fragments are measured. The abundance of different species in the microbial communities is estimated through the determination of the number of terminal fragments, observed with the digestion of the total rDNA of the communities amplified by PCR.

Therefore, the T-RFLP pattern obtained function as a true fingerprint of the microbial communities and is a set of the number of fragments with the same length. The observation of different peaks is indicative of the presence of fragments of different lengths, and the relative abundance of each fragment is reflected by the area under the peaks on the electropherogram. The samples are run in polyacrylamide gel. Each fluorescent band corresponds to a single population, and the intensity of each band is directly proportional to the quantity of the PCR product, which provides an approximate indication of the abundance of the population in the community (REIS JR et al. 2002). This characteristic should be observed with caution, in the same way as the band intensity of a gel pattern in DGGE (SANZ and KÖCHLING 2007). Bearing in mind that differences in the sequence will generate amplicons of different lengths, it is possible to carry out clustering of groups of microorganisms which are phylogenetically different (LIU et al. 1997).

The advantage of the T-RFLP technique is its capacity to detect even rare members of the microbial community. However, some studies have indicated that pseudo-terminal restriction fragments can be formed, causing an overestimation of the microbial diversity (EGERT and FRIEDRICH 2003). Moreover, ENGEBRETSON

and MOYER (2003) have demonstrated that the T-RFLP technique is very useful for estimating the diversity of communities characterized by low or medium diversity, but not for complex microbial populations.

On carrying out RFLP studies with complete bacterial DNA (whole genome), a huge quantity of fragments is generated. Thus, the differences in the fragment lengths cannot be visualized directly in the gel, since innumerous fragments resulting from the treatment with the restriction enzyme produce a continuous "drag" effect in the gel.

In order to detect the RFLP markers, the gel fragments are transferred to a nylon or nitrocellulose membrane through a process known as Southern blot. The polymorphic fragments can be visualized through hybridization with DNA probes which have sequences homologous to the DNA immobilized on the membrane (FERREIRA and GRATTAPAGLIA 1998). This method has greater resolution when the target gene is present in multiple copies and is highly conserved (MELO et al. 2002).

The T-RFLP technique has been shown to be a useful molecular tool and is constantly being statistically improved. Recent studies have described methods to improve the statistical analysis of the T-RFLP profiles, enabling a better interpretation of the data generated and allowing researchers to select more appropriate methodological and statistical options for each particular study. These new methodologies include the introduction of procedures aimed at distinguishing between signals and noise, the alignment of the T-RFLP peaks, and the use of multivariate statistical methods able to detect changes in the microbial communities due to specific reasons (SCHUTTE et al. 2008).

The T-RFLP technique can also be combined with real-time PCR, a molecular method known as real-time T-RFLP. This approach was proposed by YU et al. (2005) and can be applied to the simultaneous determination of the diversity and abundance of the microorganisms present in a complex microbial community. Validated using a model microbial community containing three specific microbial lineages, it has been observed that the real-time T-RFLP technique is an efficient molecular tool for greater discrimination of microbial communities in various natural or engineered systems, providing a means to carry out quantitative molecular fingerprinting.

6.2.6.2 Amplified Fragment Length Polymorphism (AFLP)

The AFLP method is a combination of PCR and RFLP based on restriction pattern analysis (RFLP) followed by amplification via PCR of DNA fragments selected from the restriction fragments obtained. The restriction is carried out using two enzymes which produce DNA fragments containing different ends. Short oligonucleotides (adaptors) are coupled to these ends of known sequences, which will serve as a template for the PCR. The selective amplification is carried out using two primers which are complementary to the adaptors, containing some bases complementary to the restriction ends (REIS JR et al. 2002).

6.2.6.3 Single-Stranded Conformation Polymorphism (SSCP)

The single-stranded conformation polymorphism (SSCP) technique is used to detect small alterations in the DNA sequence or mutations (ORITA et al. 1989). Under non-denatured conditions, DNA has a folded structure which is determined by its nucleotide sequence. Some changes in this sequence, for instance, simple nucleotide substitutions, can alter this structure and, consequently, its electrophoretic mobility in the gel. A change in the mobility can be identified in the shape of the profile generated by the single-stranded bands which stop at distinct positions in the gel, depending on the secondary structure assumed by the molecule according to its folding. Thus, the sensitivity of the SSCP technique is dependent on the way in which the mutations affect the folding of the molecule and how the folding affects the electrophoretic mobility of the mutant sequence (ROSADO and DUARTE 2002). This technique was first used to study microbial communities by LEE et al. (1996), for the analysis of complex environmental communities.

6.2.6.4 Random Amplification of Polymorphic DNA (RAPD)

The RAPD method requires small quantities of DNA and is able to reveal a high level of polymorphism. It is a rapid technique which is easily automated (FUNGARO and VIEIRA 1998). This method is considered to be simpler and cheaper than RFLP and is based on the amplification of nonspecific fragments of DNA. Primers are used as arbitrary nucleotide sequences, which generate amplification products via PCR which are analyzed. The ability to amplify specific fragments can be affected by the insertion or deletion of large fragments between the recognition regions of the primers, as well as by the quality of the DNA and other factors related to the PCR reaction (ELLSWORTH et al. 1993). In general, primers with around 10 bp are used in the RAPD method, with annealing at low temperatures, and the variations regarding the amplification of regions close to the introns can be studied with semi-random primers (DOWLING et al. 1996).

Since they are generated arbitrarily, the primer sequences allow the observation of the RAPD profiles with various amplification products, due to the presence of several sites homologous to these primers found at various points in the genome (FUNGARO and VIEIRA 1998). The variations in the DNA sequence of the fragments can be evaluated mainly regarding the variations in the number, length, and conformation. Although the analysis of fragments has a lower resolution compared with nucleotide sequences, in many cases analysis by RAPD has been used as an efficient and economically viable technique for the analysis of a large number of microbial populations (MELO et al. 2002). Molecular phenotypes, generated by RAPD, can be used to differentiate at various taxonomic levels and can even discriminate at the intraspecies level (REIS JR et al. 2002).

It should be mentioned that the RAPD technique is most commonly used for isolates in pure cultures. However, there are some studies reporting the use of RAPD-PCR along with 16S rRNA PCR combined with DGGE to monitor changes in the structure of activated sludge microbial communities during adaptation to the influent wastewater (Li et al. 2010).

6.2.6.5 Ribosomal Intergenic Spacer Analysis (RISA)

Ribosomal intergenic spacer analysis (RISA) was developed by Borneman and Triplett (1997) and was firstly applied to the study of microbial diversity in soils.

Identification of microorganisms is based on the 16S rRNA gene; other DNA regions, such as those which carry information for specific genes of interest or even specific intergenic regions, can also be used. The spacing between the 23S rRNA (large subunit of the rRNA in prokaryotes) and 16S rRNA (small subunit of the RNA in prokaryotes) genes is used as a target of PCR in RISA. This region is highly variable both in terms of its length, extending from 50 bp to over 1.5 kb, and also its nucleotide sequences.

In the RISA technique, the observed polymorphism is related to the degree of heterogeneity. The amplification products are separated in the polyacrylamide gel based on their different lengths and visualized after staining with silver. This tool has been used successfully to obtain fingerprints of microbial communities, where each band corresponds to at least one microorganism (DORIGO et al. 2005).

The RISA technique offers higher resolution than analysis of the 16S rRNA gene, considering that the spacer region is not as evolutionarily conserved as the ribosomal genes. Differences in the length of this sequence and in the base composition allow a fast monitoring method and also serve as a tool to distinguish between microorganisms at the subspecies level (JENSEN et al. 1993).

FISHER and TRIPLETT (1999) developed an automated version of the RISA technique, called ARISA, in order to access the microbial diversity in a faster and more efficient manner. The amplification by PCR of the 16S–23S region is carried out using a primer labeled by fluorescence, which enables the detection of amplicons by automated capillary electrophoresis. The total number of different fluorescence peaks obtained with the ARISA technique for a certain sample is taken as an estimate of the species diversity, and the lengths of the fragments can be compared with those present in, for instance, the GenBank database.

Despite the interesting features of the RISA technique, it has been scarcely used for assessing the microbial community of wastewater treatment systems. CIESIELSKI et al. (2013) employed RISA to monitor changes in the community of methanogenic Archaea in an anaerobic digester used for sludge treatment. Different variants of RISA (standard and automated) were tested. All provided good results. The automated approach enabled rapid analysis of the archaeal community, whereas the conventional was more favorable for identification of microorganisms.

6.2.6.6 Phospholipid Ester-Linked Fatty Acid (PLFA) Analysis

The PLFA method reveals the presence of certain microbial groups through the detection of their phospholipid ester-linked fatty acid molecules, thus enabling the identification without depending on genes and on their amplification (ROONEY-VARGA et al. 1998).

Although there are some previous works reporting the use of PLFA analysis for monitoring the microbial community structure of sediments (Salomonová et al. 2003), and freshwater reservoirs (SUSHCHIK et al. 2003), this technique has been little used to investigate microbial communities of biological wastewater treatment reactors. CHANG et al. (2011) investigated the effects of intermittent aeration on the bacterial community profile of a submerged membrane bioreactor. They observed the dominance of both monounsaturated and saturated PLFA types in activated sludge biomass, followed by branched PLFAs.

6.2.6.7 DNA Microarray

It is currently known that the data obtained through the sequencing of DNA molecules, although relevant, are limited, since it is necessary to investigate both the processes used for the transcription of the information contained in the sequences obtained and their products, i.e., the proteins. In this context, the technological strategy called DNA microarray has revolutionized the capacity to collect information in the area of functional genomics.

Representing one of the most recent advances in molecular methods, DNA microarray (also known as DNA microchip) has great potential for the elucidation of a great variety of aspects related to the microbial ecology of different environments. These aspects include the identification of variations in the structure of a microbial community present in different samples, the identification of phylogenetic groups which can be active or non-active during a certain period, or even the identification of differences between lineages isolated from different environments. In addition, this technique has boosted research on the functional genomics of different organisms (OGRAM 2000; GUSCHIN et al. 1997).

The most common use of DNA microarray is for the determination of gene expression, aimed at measuring the levels of expression of different transcripts simultaneously, which are detected by hybridization. In other words, DNA microarrays allow to obtain the complete profile of the genes which are expressed in any type of cell or even in different samples offering, in this case, important markers for the evaluation of the environment from which the samples were obtained. The capacity to determine the differential expression of thousands of genes in a single experiment greatly contributes to the increase in the attractiveness of this technique (CHEUNG et al. 1999).

The methodology allows, simultaneously, the hybridization with large gene-specific probes and the detection of the phylogenetic or metabolic characteristics. In addition, it enables the hybridization with probes specific for DNA regions which

encode enzymes, providing important information with regard to the degradation characteristics of the microbial communities under study (DENNIS et al. 2003).

Microchips consist of predefined arrays of DNA molecules (fragments of genomic DNA, cDNAs, or oligonucleotides) prepared with oligonucleotide probes immobilized in an ordered manner and in specific areas (probe cells) in a polyacrylamide gel matrix attached to a solid surface, generally glass plates (microscope slides) coated with compounds which provide a positive charge (YERSHOV et al. 1996). Also, they can be prepared in positively charged nylon membranes. For use in the microchips, the oligonucleotides synthesized must be purified by gel electrophoresis or high-performance liquid chromatography. This requirement increases the control of the stringency quality, which ensures a high specificity.

In a single microchip, thousands of different oligonucleotides can be immobilized, which allows the simultaneous detection of a great variety of microorganisms in a sample. In a single chip almost all of the genes of the genome of a series of organisms can be present. Furthermore, a microchip, with washing using distilled water, can be used up to 30 times without a reduction in the hybridization signal (GUSCHIN et al. 1997).

The use of microarrays is associated with the detection and quantification of nucleic acids originating from biological samples, which are able to be hybridized, through the complementarity of bases, with the DNA fixed in the array. This in turn presents sequences similar to the genes of interest and complementary to the mRNA or complementary DNA (cDNA). The DNA or RNA samples are labeled with fluorochromes (cyanine 3, Cy3, or cyanine 5, Cy5) when glass microarrays are used and with isotope 33-P when the microarrays are produced with a nylon membrane. In both cases, a hybridization image needs to be generated. This is obtained using a laser reader (scanner) for fluorochromes or using phosphorus readers for the isotope 33-P (DALMA-WEISZHAUSZ et al. 2006; CHEUNG et al. 1999).

A typical experiment consists of comparing the levels of gene expression for two different test conditions, such as case-control study, pre- and posttreatment, or with or without a certain experimental manipulation. More specifically, the procedure using a microarray platform which is most commonly used internationally (i.e., GeneChip®, Affymetrix Inc., Santa Clara, CA, USA) includes the conversion of the total RNA obtained from the biological sample into double-stranded cDNA through a reverse transcriptase reaction. Subsequently, the cDNA serves as a template for an in vitro transcription reaction in the presence of oligonucleotides labeled with fluorophores, resulting in labeled complementary RNA (cRNA). These labeled molecules are hybridized on the microarray slide and may or may not bind to the array probes, depending on whether the sequences are complementary or not. The hybridized slide is then stained and placed in a scanner connected to software which enables the data analysis and the quantification of the fluorescence intensity at each point. The signal generated represents the binding of the cRNA of the sample to the probe in the array and is proportional to the abundance of cRNA present, up to a certain concentration of transcripts. The quantification of the signal allows the expression of thousands of genes to be compared under different experimental conditions (LOCKHART et al. 1996; CHEUNG et al. 1999). Figure 6.18 shows a simplified scheme of a DNA microarray.

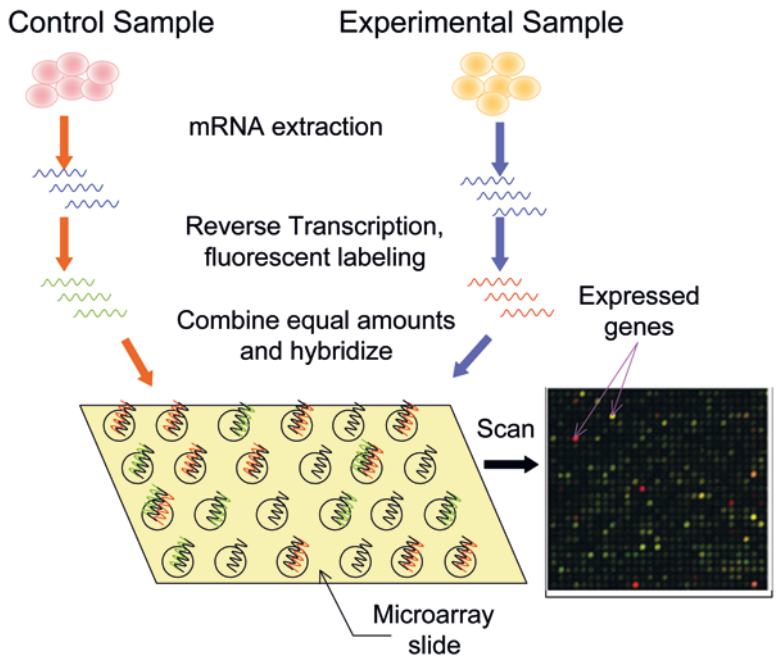

Fig. 6.18 General representation of the DNA microarray technique. The synthetic DNA probes are attached to different spots of the microarray slide and can hybridize to complementary sequences, enabling the measurement of the gene expression (adapted from http://bitesizebio.com/7206)

For comparison purposes, Table 6.11 summarizes the main characteristics of some alternative and complementary molecular methods addressed in Sect. 6.2.6. It should be noted that these methods are viable options for the study of microbial diversity, although their current state of development combined with the limited availability of references in the area of biological wastewater treatment makes them less commonly used compared with more traditional methods, such as DGGE and FISH.

6.2.7 Next-Generation High-Throughput Sequencing Methods

Research on biological wastewater treatment continuously grows in the world given the importance of this subject in the environmental protection context. A more comprehensive understanding of the mechanisms underlying the biological treatment process often requires knowledge of the microbial community responsible for the removal of the main classes of pollutants. For a complete overview of the ecology of the degradation processes mediated by microorganisms, several samples collected from the bioreactors should be analyzed and their identity revealed by sequencing methods.

Table 6.11 Main characteristics of some alternative and complementary methods used to study microbial diversity (adapted from SANZ and KÖCHLING 2007)

Method	Description	Advantages	Disadvantages
T-RFLP	Monitoring method which takes into consideration the 16S rRNA sequence, based on differences in the restriction behavior, (polymorphism)	– Relatively simple procedures – Makes use of intensity of fluorescence signal, allows an approx. Estimate of the species abundance	– Heterogeneity in the fragment length makes the phylogenetic analysis less reliable – Has no advantages compared with DGGE, which is the most commonly used method
RISA	Phylogenetic profiling based on the intergenic region between the 23S and 16S rRNA sequences	High sensitivity, detecting even subspecies	Small database for comparative analysis in the comparison of 16S rRNA sequences
PLFA	Profile of microorganisms obtained via characteristic fatty acid content	– Molecular characterization of the microorganisms without the involvement of genes – Complementary information for tests based on 16S	– Not considered a good option when used as the only standard method – Need for a chromatograph/mass spectrometer for the identification
DNA microarray	Hybridization method for several samples	– Appropriate for analysis of large number of samples – Parallel analysis of different parameters	– High cost of equipment – Difficult procedure to perform

Recent advances in science and technology are leading to a revision and reorientation of the molecular methodologies, allowing old and current issues to be approached under a new perspective. The greater availability of molecular approaches allows the generation of increasingly relevant data on microbial communities. This not only requires more sophisticated analysis techniques but has also contributed to an explosion in the development of promising methodologies to describe the DNA sequences of microbial cells or the microbial diversity of a certain environment in a more rapid and efficient manner.

The classic Sanger sequencing method (described in Sect. 6.2.4) was the dominant approach for DNA sequencing for the last 30 years. However, the technological advances observed in distinct areas and the need for obtaining fast and reliable sequencing results for multiple samples at limited cost and time pushed the development of many advanced and high-throughput sequencing methods by the research

community (Voelkerding et al. 2009). A great variety of these technologies emerged in the 1900s and became available in the 2000s. The commercial launch of the massively parallel sequencing platforms was a breakthrough in DNA sequencing, which revolutionized the field and established a new era of genomic analysis (Tan et al. 2015).

Also referred to as "next-generation sequencing" or "second-generation sequencing," the high-throughput sequencing approaches allow obtaining massive information regarding the identity of specific microorganisms and microbial communities living in a wide range of biological systems, such as those employed for wastewater treatment. These methods make use of miniaturized and parallelized platforms which differ from each other in many aspects, such as sequencing mechanism and configuration. However, they have in common the concept of massively parallel sequencing of either clonally PCR-amplified products or single DNA molecules which are spatially separated in a flow cell. Such design is different from the classic Sanger sequencing method, based on electrophoretic separation of chain-termination products generated in individual sequencing reactions (Voelkerding et al. 2009; Bayés et al. 2012; Tan et al. 2015).

The recent sequencing technologies enable quick generation of great amount of data at a relatively lower cost compared to the Sanger method. Whereas the cost has been decreased to less than 4–0.1%, time has been reduced by a factor of 100–1000 based on daily throughput (Kircher and Kelso 2010).

The principle of the recently developed methods is based on the concept of sequencing by synthesis, whereby millions of relatively short reads are generated from amplified single DNA fragments using a repetition of cycles of nucleotide extensions mediated by polymerase or ligase (Voelkerding et al. 2009; Bayés et al. 2012). The procedure starts by placing single DNA molecules into individual reaction vessels (sequencing library preparation by random fragmentation of DNA) where amplification takes place either in liquid (emulsion PCR) or solid phase (bridge PCR on a planar solid surface) (Diehl et al. 2006; Bentley et al. 2008; Shendure and Hanlee 2008). As the DNA templates are clonally amplified, millions of DNA clones are sequenced in a microfluidic device coupled with a high-resolution imaging system. Data acquisition is performed by imaging of the fluorescently labeled nucleotides incorporated by the corresponding enzyme at each cycle. Finally, the images are converted into sequence reads (Shendure and Hanlee 2008; Morozova and Marra 2008; Bayés et al. 2012).

Given the massively parallel sequencing process, the next-generation methods are capable of generating hundreds of mega- to gigabases of nucleotide sequence data in a single run of the equipment, depending on the platform (Voelkerding et al. 2009). In comparison with old times when the limiting factor was the generation of sequencing data, the challenge in current days is the storage, handling, analysis, and interpretation of the great amount of data obtained by means of the high-throughput sequencing platforms. Such technologies provide hundreds of millions of short DNA sequence reads which should be properly handled to render reliable and high-quality results in a relatively short time span. In this context, specialized bioinformatics tools and knowledge are mandatory. This makes the analysis of the data the main obstacle to be faced in sequencing projects.

Apart from the computer resources a hardware infrastructure (e.g., computing power, disk storage capacity, and memory) needed to store and process all sequencing datasets, it is necessary to count on knowledgeable bioinformaticists capable of analyzing the large amount of sequence data released by the next-generation sequencing methods (Bayés et al. 2012; Rodríguez-Ezpeleta et al. 2012). Moreover, the need for specific software packages for data analysis makes this task even more complex, as most programs rely on considerable number of parameters which are dependent on the biological system under study, type of data, and experimental design (Rodríguez-Ezpeleta et al. 2012).

A great variety of bioinformatics tools available for analysis and interpretation of next-generation sequence data can be found in the review carried out by (Shendure and Hanlee 2008). Their functions fall within several categories, such as (1) alignment of the obtained raw sequence reads (often with 50–400 base pairs) to known (reference) microbial genomes, (2) base calling and variant calling, (3) de novo assembly from paired or unpaired reads piecing together the resulting sequence reads into longer continuous stretches of sequence (contigs), and (4) filtering, genome browsing, and annotation (Shendure and Hanlee 2008; Dolled-Filhart et al. 2013; Ekblom and Wolf 2014).

While Basic Local Alignment Search Tool (BLAST) is a largely employed alignment solution for long reads generated by conventional sequencing, the analysis of large sets of short reads provided by next-generation methods requires rapid and efficient alignment algorithms and tools (Shendure and Hanlee 2008). Moreover, the assembly of short sequencing read is also a challenge. The use of mate-paired sequences (also designated as mate pairs) can be used to facilitate de novo assembly and obtain a more complete picture of the genome (Wetzel et al. 2011).

The next-generation sequencing methods consist of a rapidly evolving field. Many technologies are commercially available, while other new methodologies and instruments are constantly being released on the market. Currently, the most used next-generation sequencers are 454 pyrosequencing (Roche Applied Science, Basel, Switzerland), Illumina/Solexa genome sequencer (Illumina, San Diego, CA, USA), SOLiD (Applied Biosystems, Foster City, CA, USA), and the Single Molecule Real-Time platform (Pacific Biosciences, Menlo Park, CA, USA). Table 6.12 presents the most well-known platforms developed in recent years and their particular features. It should be emphasized that the data here provided quickly changes and becomes out of date as a result of the advancement in the sequencing technologies. In this sense, it is recommended that the reader should always consult updated literature information. A detailed description of the high-throughput sequencing analysis and the principle behind the main methods and equipment used for this purpose can be found elsewhere (Shendure and Hanlee 2008; Bayés et al. 2012; Rodríguez-Ezpeleta et al. 2012).

Some of the next-generation platforms (HiSeq 2000 and 5500x1 SOLiD) generate short length sequences (much shorter than conventional sequencing methods) (Shendure and Hanlee 2008), which may limit bacterial identification. Furthermore, short sequence reads provide less phylogenetic information compared to full-length 16S rRNA gene sequences. Therefore, the identification of bacteria using these

Table 6.12 Examples of next-generation sequencing methods and their particular features (adapted from Morozova and Marra 2008; Voelkerding et al. 2009; Kircher and Kelso 2010; Bayés et al. 2012)

Platform/company name/website	Characteristics								
	Amplification approach—library preparation)	Sequencing mechanism	Reads per run	Read length	Output data per run	Time per run	Accuracy	Advantages	Disadvantages
ABI3730x1 (Applied Biosystems)	PCR/cloning	Automated Sanger method (dideoxy chain termination)	1536	400–900 bp	1.3 Mb	20 min–3 h	99.999%	High quality, long read length	High cost, low throughput
Genome Sequencer (GS) FLX (454 Life Sciences, Roche) http://www.454.com/	Clonal—emulsion PCR	Pyrosequencing	1 M	400–700 bp	500–700 Mb	10–24 h	99.9%	Read length, fast	Error rate with polybase more than 6, high cost, low throughput
HiSeq 2000 (Illumina, Solexa) http://www.illumina.com/	Solid-phase amplification (bridge PCR)	Sequencing by synthesis using reversible dye terminators	1000–3000 M[a]	50–100 bp	600 Gb[a]	3–10 days	98%	High throughputs	Short read assembly
5500x1 SOLiD (Life Technologies) http://www.appliedbiosystems.com/	Clonal—emulsion PCR	Massively parallel sequencing by ligation and two-base coding	1200–2400 M[a]	35–50 bp	120 Gb–300 Gb[a]	4–7 days	99.94%[b]	Accuracy	Short read assembly
PacBio RS (Pacific Biosciences) http://www.pacb.com/	No PCR amplification (single molecule)	Phospholinked Fluorescent Nucleotides	100 GB	30 pb	NA	<1	–		

[a]From two flow cells or slides
[b]Raw data

sequencers has been mainly accomplished based on 16S rRNA gene hypervariable regions, which are highly informative and may enable analysis of lower taxonomic levels (e.g., genera and species) in spite of the short length of the sequence (Nasidze et al. 2009).

The new technologies need to satisfy some main objectives, such as a significant increase in the yield without a loss in the quality of the data produced by the technique used (Rogers and Venter 2005). Hence, the accuracy of data is also of great concern in the new sequencing platforms, which are often assumed to be less accurate than the Sanger-based sequencing technologies. However, it should be taken into account that the next-generation sequencers are still evolving, and great improvements in such platforms are expected over time (Shendure and Hanlee 2008).

In general, the choice of the most appropriate sequencing platform is project specific. Sometimes, combination of different technologies can be technically and economically advantageous. This stimulates the establishment of companies which provide sequencing services on demand. Nevertheless, despite the third-party services, the construction of libraries and analysis of a great amount of data will still be dependent on the research laboratory personnel (Morozova and Marra 2008).

The application of high-throughput sequencing methods has a significant impact on the study of the microbial diversity of wastewater treatment bioreactors. The use of these powerful sequencing tools in order to get the most information possible about the microbial diversity of these biological systems is becoming an increasingly routine activity (Hu et al. 2012; Zhao et al. 2015; Ng et al. 2015). In general, genomic DNA is extracted from the reactor samples at different operating conditions, converted to a next-generation sequencing library and finally sequenced. The sequencing results obtained are then aligned to known microbial reference genomes. Besides the qualitative genomic information, the relative abundance of the sequence reads may be used to obtain quantitative data regarding individual microbial species.

With a large amount of data available, the challenge will be the extraction of useful insights from the DNA sequencing results to better understand the biological processes taking place in the engineered wastewater treatment reactors. The combination of metagenomics, metatranscriptomics, and other approaches to explore microbial interactions in biological wastewater treatment processes will be crucially important for obtaining a wealth of information regarding the functioning of such microbial systems.

6.3 Application of Molecular Biology Techniques to Wastewater Treatment

6.3.1 Introduction

Several studies reported in the literature regarding the application of molecular biology techniques to the study of the microbial diversity of wastewater treatment systems, on both laboratory and full scale, are described in this section. The main aspects of each study will be highlighted, including the most important results

obtained in each case. For more details about a specific study, the reader is referred to the corresponding full version of the published articles cited here. Bearing in mind that the overall objective of this book is not restricted to molecular tools, in many cases short discussions involving the biological treatment process are provided.

6.3.2 *Application of Molecular Biology Techniques in Studies to Characterize the Microbial Communities of Wastewater Treatment Systems*

BOND et al. (1995) compared the microbial communities of two activated sludge sequencing batch reactors (SBRs): one with (SBR_1) and one without (SBR_2) phosphate removal. The reactors were fed with wastewater containing on average a COD of 370 mg/L, while the average soluble phosphate concentration was 18 mg/L. Immediately after the reactor start-up, the feed of the SBR_1 was supplemented with sodium acetate to increase the COD by 100 mg/L. This procedure was carried out to favor the development of the microorganisms responsible for the bio-P removal only in SBR_1, thus obtaining two systems with different phosphate removal capacities.

After 3 weeks of operation, it was observed that the phosphate removal in SBR_1 was greater than that in SBR_2. The values for the release of phosphate during the anaerobic phase in SBR_1 and SBR_2 were 35.4 and 5.2 mg/L, respectively. The effluent phosphate concentrations in SBR_1 and SBR_2 were 1.5 and 12.0 mg/L, respectively. These data suggested notable differences in the microbial communities present in the two reactors, particularly with respect to the biochemical transformation of phosphate (BOND et al. 1995).

Clone libraries with partial sequences of the 16S rRNA gene of the bacterial populations of the sludge were constructed. In order to identify the differences which could indicate groups or genera which are important in the biological removal of phosphorus (a process known as enhanced biological phosphorous removal, EBPR), the structures of the communities were determined through phylogenetic analysis of the partial 97 and 92 sequences of the clones of SBR_1 and SBR_2, respectively. For both reactors, the predominant bacterial group with which the clones were affiliated was the subclass β-*Proteobacteria* (28%). Bacteria belonging to this subclass have been observed by FISH as being dominant in activated sludge communities (WAGNER et al. 1993, 1994). Given their numerical dominance, it is probable that the representatives of the β subclass play an important role in the degradation of organic matter and nutrients and the formation of the floc structure (BOND et al. 1995).

Other main groups were those belonging to the subclass α-*Proteobacteria*, the phylum *Planctomycetes*, and the group related to *Flexibacter-Cytophaga-Bacteroides* (particularly in SBR_2). In addition, several clone groups not affiliated with known bacterial groups were identified in the clone libraries. *Acinetobacter* spp., reported in the past as one of the most important microorganisms in phosphate removal from activated sludge according to the methods dependent on cultivation

(WENTZEL et al. 1988), were poorly represented in the clone sequences in both libraries (approximately 2%). Differences in the community structure were observed on comparing the two reactors, in particular in relation to the *Rhodocyclus* group within the β subclass, which was extensively present in the microbial community of SBR_1. This finding may be related to the fact that this microbial group, in particular, is associated with an important function in the removal of phosphate. The same authors also report that the high species diversity in SBRs and the limited number of clones analyzed make it difficult to identify one or more bacterial species which may play a crucial role in phosphate removal (BOND et al. 1995).

SCHRAMM et al. (1998) investigated, using the FISH technique coupled with confocal laser scanning microscopy (CLSM), the distribution of ammonium-oxidizing lithoautotrophic bacteria belonging to the genera *Nitrosomonas* and *Nitrobacter* in the nitrifying biofilm of a trickling filter employed in a water recirculation system for aquiculture. The ammonium concentration varied from 0.3 to 7 mM. The hybridization of fixed cells was carried out with 16S rRNA oligonucleotide probes. It was observed that the ammonium-oxidizing bacteria (AOB) formed a dense layer of cellular aggregates in the upper part of the biofilm (aerobic part), while the nitrite-oxidizing bacteria (NOB) formed less dense colonies with lower numbers compared with the AOB. The CLSM revealed the coexistence of AOB and NOB in neighboring regions, which is related to the sequential metabolism of ammonia to nitrate, via nitrite, promoted by the joint action of these two microbial groups. Due to this spatial arrangement, the diffusion pathway of *Nitrosomonas* (AOB) to *Nitrobacter* (NOB) is very short and facilitates the efficient transfer of the intermediate nitrite. Neither species is restricted to the oxic region of the biofilm, both being detected, although in much lower numbers, in anoxic layers. Some NOB colonies, in particular, have been found in upper anoxic layers, at a depth of 100–200 μm, as well as occasionally at the bottom of the biofilm.

SNAIDR et al. (1997) investigated the structure of bacterial communities at a large wastewater treatment plant (activated sludge process) based on the 16S rRNA gene. Almost complete genes which encode the 16S rRNA subunit (rDNA) were amplified by PCR and subsequently cloned. The clones were classified by dot-blot hybridization with group-specific oligonucleotide probes. The phylogenetic affiliations of the clones were compared with the results obtained with an original sample by means of in situ hybridization, in which specific rRNA oligonucleotide probes were used. It was observed that the data were in agreement. A total of 25 16S rDNA clones were completely sequenced, 11 were almost completely sequenced (>80%) and 27 were partially sequenced.

Comparative analysis of the sequences was carried out and most of the clones examined (35) were affiliated with the β subclass of the class *Proteobacteria*. The γ and α subclasses were represented by 13 and 4 clones, respectively. Eight clones were affiliated with the ε-subclass of *Proteobacteria*, and five clones were grouped with Gram-positive bacteria characterized by DNA with a low GC content. The 16S rRNA gene of two clones showed similarity with the 16S rRNA genes of the members of the phyla *Chlamydiae* and *Planctomyces*. Oligonucleotide probes were designed and used for the enumeration of the respective bacteria. Interestingly,

pathogenic representatives of the genus *Arcobacter* were present in significant numbers (4%) in the activated sludge sample examined.

BURRELL et al. (1998), in order to identify the nitrite-oxidizing bacteria found in wastewater treatment plants, operated a sequencing batch reactor (SBR) fed with a solution of inorganic salts, in which nitrite was the only source of energy. With the use of microscopy, cultivation methods, and molecular techniques, the biomass in the nitrite-oxidizing system was investigated after a period of 6 months of reactor operation. Molecular biology methods have also been used to characterize the sludge used to inoculate the SBR, and this was then compared with the biomass present inside the reactor. It was observed that the SBR biomass was comprised of a complex community with a high diversity containing Gram-negative and Gram-positive bacteria.

The methods based on cultivation in culture medium (micromanipulation, sample dilution, and plating) identified only heterotrophic bacteria, and no autotrophic microorganisms were isolated. 16S rDNA clone libraries of two microbial communities revealed that the inoculation sludge presented a complex microbial community dominated by *Proteobacteria* (29% β subclass and 18% γ subclass) and a large quantity of high GC Gram-positive bacteria (10%). In relation to the inserts of 77 clones of the library corresponding to the inoculum sludge, it was observed that most of the clone sequences were grouped into the phylum *Proteobacteria* (56%), while 4% (three clones) were grouped into the phylum *Nitrospira*, specifically *Nitrospira moscoviensis*. On the other hand, *Nitrobacter* was not identified. The inserts of 102 clones of the clone library corresponding to the biomass present in the reactor, examined through analysis with restriction enzymes, were classified into 13 different operational taxonomic units (OTU). A total of 90 clones (88%) were grouped into one particular OTU, while the remaining 12 OTUs were composed of individual clones, each one corresponding to 1% of the total number of clones. The biomass inside the SBR had a predominance of bacteria related to *Nitrospira moscoviensis*, and two clone sequences were similar to that of the genus *Nitrobacter*. Based on the results obtained, the authors concluded that the "unknown" nitrite-oxidizing bacteria (referred to in this way since they were not easily isolated through conventional cultivation techniques) present in the activated sludge systems belong to the phylum *Nitrospira* (BURRELL et al. 1998).

OKABE et al. (1999) investigated the presence and distribution of ammonium-oxidizing (AOB) and nitrite-oxidizing (NOB) bacteria in the biofilm of a rotating disk bioreactor fed with both a domestic wastewater and a synthetic medium (autotrophic nitrifying biofilm). The authors employed microsensors and FISH with 16S rRNA-specific oligonucleotide probes. The combination of these techniques allowed the microbial activity to be directly related in situ to the occurrence of populations of nitrifying bacteria. FISH analysis revealed that bacteria belonging to the genus *Nitrosomonas* represented the numerically dominant AOB in both biofilms. However, bacteria belonging to the genus *Nitrobacter* were not detected. *Nitrospira* was the main genus representative of the NOB in both biofilms, mainly found in the intermediate part of the biofilm. *Cells of Nitrospira* lineages were aggregated in irregular shapes comprised of microcolonies which were grouped around the AOB.

While most of the AOB were present throughout the biofilm, the NOB were restricted to the zones where nitrite oxidation would actively occur, located in the internal parts of the biofilm. Through measurements taken with microelectrodes, it was possible to observe that the active zone of ammonium oxidation was located in the external part of the biofilm, while the active zone of nitrite oxidation was located directly below the ammonium oxidation zone, confirming the observations obtained by FISH.

AOI et al. (2000) applied the FISH technique with 16S rRNA-specific oligonucleotide probes to investigate the microbial ecology of nitrifying bacteria in several types of wastewater treatment processes. They also evaluated the dynamic response of microorganisms in different types of biofilms and suspended flocs, as well as the distribution of ammonium-oxidizing bacteria (AOB) and other heterotrophic bacteria. Samples from three types of nitrifying reactors were analyzed: (1) biofilm from a fluidized bed reactor fed with an influent rich in ammonia without the addition of organic compounds or micronutrients, (2) activated sludge from a reactor in which the inorganic influent contained micronutrients and had a low rate of ammonia oxidation, and (3) the biofilm of another fluidized bed reactor fed with organic wastewater with a high concentration of ammonia and organic compounds.

The same authors observed that the nitrifying bacteria exhibited different forms of organization according to the operational conditions imposed, particularly in relation to the composition and concentration of the substrate. The AOB were found to be dominant in inorganic wastewaters rich in ammonium. In the case of organic wastewater, both heterotrophic bacteria and AOB were distributed in different positions in the biofilm. The samples of reactor 1 revealed a predominance of bacteria of the genus *Nitrosomonas*, as well as *Nitrosococcus mobilis*. In reactor 2, members of the genus *Nitrosomonas* and *N. mobilis* were detected as well as other AOB such as *Nitrosospira*, *Nitrosovibrio*, and *Nitrosolobus*. These microbial groups only appeared in reactor 2. The authors argue that this result is probably related to the lower ammonia oxidation rates (0.3 kgN-NH_4^+/(m^3 day)) observed in this system compared with the other two reactors (1 and 3). *Nitrosomonas* and *N. mobilis* exhibited greater activity in the presence of high ammonia concentrations, while for *Nitrosospira*, *Nitrosovibrio*, and *Nitrosolobus*, the activity was greater when the ammonia concentration was low, indicating the strong dependence of the microbial ecology on the operation conditions. This makes the observation and control of ammonium-oxidizing microbial groups of great importance in the maintenance of stable nitrifying activity (AOI et al. 2000).

In reactor 3, fed with organic influent, heterotrophic bacteria were detected in external region of the biofilm, whose thickness was 100 μm. AOB were also found, particularly in the interior of the biofilm. According to the authors, the AOB and heterotrophic bacteria tend to be located in different positions in the biofilm due to the fact that the heterotrophic population has a higher growth rate than the autotrophic population, and thus, initially, these bacteria occupy the external region of the biofilm in which the dissolved oxygen (DO) and substrate concentrations are high enough to allow their growth. On the other hand, the AOB are able to spread both individually and in the form of groups within the interior of the biofilm due to their

low growth rate, even though in this region the environment is unfavorable for growth in terms of the DO concentration. As in the case of reactor 1, *Nitrosomonas* was the predominant genus of AOB in reactor 3.

AOI et al. (2000) also evaluated the dynamic response of microbial communities to changes in the influent composition, particularly in relation to a gradual reduction in the TOC/N-NH_4^+ ratio. It was observed that with a decrease in this ratio, the AOB, which were previously only located in the interior of the biofilm, extended their area of growth to the more external part, which had previously been occupied exclusively by heterotrophic bacteria. The decrease in the concentration of organic compounds limited the growth of heterotrophic microorganisms, which consequently allowed the development of the nitrifying consortium (particularly AOB) in the external part of the biofilm. When the TOC/N-NH_4^+ ratio was 0 (no organic carbon in the influent), the AOB were still present in large numbers throughout the biofilm, while the heterotrophic bacteria had practically disappeared. After a certain period of time under this condition, the nitrifying activity increased, and the biofilm thickness, which at the beginning of this regime without organic carbon had been decreasing, remained stable. The results indicated that effective control of the microbial ecology can be obtained by controlling the operating conditions of the reactor, highlighting the possibility of obtaining dense and highly active biofilms containing a high diversity of nitrifying bacteria with a gradual reduction in the TOC/N-NH_4^+ ratio.

In order to evaluate the relation between excessive foaming in wastewater treatment plants and actinomycetes, microorganisms containing mycolic acid (family *Mycolata*) and generally considered to cause this phenomenon, DAVENPORT et al. (2000) developed a specific probe for these bacteria as well as a protocol to permeabilize these microorganisms and a method for their statistical quantification. The quantitative FISH technique was used to investigate the relation between the production of foam and the concentration of actinomycetes in an activated sludge system, completely mixed, with a volume of 20 m^3. The authors observed that the formation of foam occurred when the number of actinomycetes belonging to the family *Mycolata* exceeded a certain value, which was estimated to be around 2×10^6 cells/mL or 4×10^{12} cells/m^2.

The nitrification process can occur in different reactors with different configurations, although the way in which the nitrifying microbial communities act in the different configurations is unknown. Recent studies on ammonium-oxidizing bacteria (AOB) in wastewater treatment systems have suggested that different plants support different populations and different levels of species richness. In this context, ROWAN et al. (2003) compared the diversity and the community structure of AOB, belonging to the subclass β-*Proteobacteria*, in two reactor configurations operated in full scale: an aerated biological filter (ABF) and two trickling filters (TF) in series (primary and secondary). The reactors were fed with the same mixed wastewater, originating mainly from an industrial source (70% industrial/30% domestic), previously treated in a high-rate activated sludge system and then mixed with domestic wastewater (2:1 ratio—domestic/pretreated industrial). Different molecular techniques were employed to carry out the study. PCR was used for the amplification of

the 16S rRNA gene fragments, using primers selective for AOB (β-*Proteobacteria*). The use of DGGE allowed the analysis of the fragments amplified by PCR, and the profiles obtained were analyzed to compare the dominant AOB populations in the reactors. In addition, a selection of the 16S rRNA gene fragments amplified by PCR and cloned was sequenced to determine the phylogenetic affiliation of the sequences representative of each species of dominant AOB.

The results obtained from the visual comparison of the DGGE profiles shown by the 16S rRNA fragments originating from the ABF and TF revealed distinct populations in different sections of each reactor and also differences between the reactors. The ABR was comprised of three interlinked layers, each one associated with a different biodegradation process: denitrification (anoxic), organic matter removal (oxic), and nitrification (oxic). A change in the aeration level appeared to have an effect on the bacterial population present in the reactor. Also, significant differences were observed in the results obtained by DGGE for the different TF depths and the primary and secondary TF. More bands were detected in the sample from the bottom of the primary filter and in the secondary filter compared with the top of the primary filter (ROWAN et al. 2003).

Results obtained by the same authors revealed that although both reactors (ABF and TF) were fed with the same wastewater, they harbored different bacterial populations. ABF had a lower diversity of AOB when compared with TF. The sample originating from the latter presented some bands on the DGGE gel which did not appear in the gel run with samples originating from the ABF. However, both reactors appeared to have a predominant population in common (ROWAN et al. 2003).

Clone libraries of the 16S rRNA gene of AOB were constructed from the samples of each of the two reactors, and those which had a greater diversity based on the DGGE profiles were selected. The nucleotide sequences were determined for each type of clone from the clone libraries and were compared with the GenBank database. The detailed analysis of the sequences recovered from the ABF and TF revealed that all of them derived from non-cultivated β-*Proteobacteria* or γ-*Proteobacteria*. In addition, most of the sequences (97% from the ABF, 80% from the top of the TF, and 85% from the bottom of the TF) showed the strongest similarity with the AOB (β-*Proteobacteria*). The remainder of the sequences recovered was related to *Thauera* spp. and *Dechlorimonas agitatus* (ROWAN et al. 2003).

The sequences obtained from the nitrification unit of the ABF and the top and bottom of the TF were identified as belonging to the genus *Nitrosomonas*. The clone library of the ABF had a preponderance of *Nitrosomonas mobilis*, while the TF samples showed greater diversity. The predominant clones of this system belonged to *N. mobilis* and *Nitrosomonas* spp. The sequences of *N. mobilis* were found in all of the reactors, but they were more abundant in the clone library of the ABF (90% of the sequences) than that of the TF (33% from the top of the secondary filter, 27% from the bottom of the secondary filter) (ROWAN et al. 2003).

PERSSON et al. (2002) investigated the composition of a biofilm adhered to the plastic supports (surface area of 240 m^2/m^3) of a full-scale nitrifying trickling filter (NTF) treating municipal wastewater. Fluorescence in situ hybridization (FISH) with 16S rRNA-targeted oligonucleotide probes in combination with confocal laser

scanning microscopy was used to characterize the microbial community. In all of the samples taken from different depths of the NTF, it was observed that the ammonium-oxidizing bacteria (AOB), with the exception of two marine species of the genus *Nitrosococcus*, belonged to the subclass β-*Proteobacteria*, in particular the genus *Nitrosomonas*. It was also observed that none of the bacteria were detected with the probes specific for *Nitrosospira* spp. Furthermore, *Nitrosococcus mobilis* and *Nitrosomonas europaea* were not detected in any of the samples by specific probes.

The results showed a tendency for a decrease both in the proportion of AOB in relation to the total bacteria and in the total quantity of AOB in the different layers of the biofilm (30 ± 23% less in the more external layers compared with the inner layers). This total quantity of AOB also showed a progressive decrease with depth, that is, from the top to the bottom of the trickling filter. Measurements and simulations of the ammonium oxidation activity also showed a decrease in the activity with an increase in the depth of the NTF, which was generally operated with complete nitrification (PERSSON et al. 2002).

DIONISI et al. (2002), using the principle of competitive PCR, developed tests to quantify the ammonium-oxidizing bacteria (AOB) belonging to the species *Nitrosomonas oligotropha* and nitrite-oxidizing bacteria (NOB) belonging to the genus *Nitrospira*. The specificities of the primers used, which were designed for two target regions (*amoA* and 16S rRNA genes of *Nitrospira*), were verified by DNA sequencing. Both tests were optimized and applied to samples from an activated sludge system. The results showed that the AOB represented 0.0033% ± 0.0022% of the total bacterial population in the treatment plant. It was observed that the AOB belonging to the species *N. oligotropha* were not detected in the sample. *Nitrospira* spp. represented 0.39% ± 0.28% of the bacterial population present in the biomass. Competitive PCR analysis showed that the number of *Nitrospira* spp. cells in the sample collected from the treatment plant was 62 times greater than the number of *N. oligotropha* cells. Nevertheless, the results confirmed that the fraction of autotrophic nitrifying biomass present in the treatment plants was small, as also observed in some studies reported in the literature.

JURETSCHKO et al. (2002) evaluated the composition of the microbial community of a nitrifying-denitrifying activated sludge system treating domestic wastewater by means of molecular techniques targeting the 16S rRNA gene. The influent wastewater contained high ammonium concentrations (around 500 mg/L). Short-chain fatty acids (mainly acetate, but also butyrate and propionate) were also found in significant quantities in the influent. In order to carry out nitrification and denitrification in a single tank, the system was aerated intermittently (30 min of aeration and 15 min under anoxic conditions). During the sampling period, over 90% of the nitrogen compounds were converted to nitrogen gas through nitrification and denitrification.

Ninety-four clones of the 16S rRNA gene (almost complete length) were phylogenetically analyzed. Of these, 59% were affiliated with the class *Proteobacteria*, grouped in the β (29 clones)-, α (24 clones)-, and δ (2 clones)-subclasses. Fifteen clones were grouped among green non-sulfurous (GNS) bacteria, and 11 clones

belonged to *Planctomycetes*, *Verrucomicrobia*, *Acidobacteria*, *Nitrospira*, *Bacteroidetes*, *Firmicutes*, and *Actinobacteria*, each represented by one to five clones. Interestingly, the greatest species richness measured as the number of operational taxonomic units (OTUs) was found to be within the subclass α-*Proteobacteria*, followed by *Planctomycetes*, subclass β-*Proteobacteria*, and GNS bacteria (JURETSCHKO et al. 2002).

The same authors also studied the composition of the microbial community of an activated sludge system, determined quantitatively using the FISH technique combined with confocal laser scanning microscopy. Thirty-six oligonucleotide probes, group, subgroup, and OTU specific, targeting the rRNA gene were used in this study. It was observed that 89% of all of the bacteria detected by FISH with probes specific for bacterial cells were related to specific microbial groups. Consistent with the data generated in the clone libraries constructed from the 16S rRNA gene, members of the subclass β-*Proteobacteria* were dominant in the microbial community and represented almost half of the biovolume of all of the bacteria detected by FISH. In relation to the β subclass, 98% of the cells were identified using genus- or OTU-specific probes, and a high abundance of commonly known denitrifying bacteria related to *Zoogloea* and *Azoarcus* was observed (JURETSCHKO et al. 2002).

In aerobic-anoxic systems, in which small additions of oxygen are supplied to the anoxic reactor to induce simultaneous nitrification and denitrification, ammonium oxidation in the anoxic zone may correspond to 30–50% of the total nitrification of the reactor, even when the dissolved oxygen concentration is, generally, below the detection limit. In order to investigate whether the nitrification efficiency in the aerobic/anoxic processes is related to the presence of specialized ammonium-oxidizing bacteria (AOB), PARK et al. (2002) analyzed the population of these bacteria in aerobic/anoxic Orbal processes and in conventional nitrogen removal processes. They carried out phylogenetic analysis based on the ammonia monooxygenase (*amoA*) gene. The T-RFLP analysis revealed that organisms such as *Nitrosospira* were mainly responsible for the oxidation of ammonium in the anoxic/aerobic Orbal reactor. However, populations of *Nitrosospira* and *Nitrosomonas* were not constant and showed seasonal variability. The cloning and comparison of sequences of fragments of the *amoA* gene showed that most of the AOB in the Orbal process belonged to the lineages *Nitrospira* spp. and *Nitrosomonas oligotropha*. The abundance of organisms such as *Nitrosospira* in aerobic/anoxic reactors is significant and quite surprising, since this group of AOB have not been often associated with nitrification in wastewater treatment plants.

CHEN et al. (2003) evaluated the dynamic response of cultures of nitrifying activated sludge, cultivated in batch regime, on increasing the chloride concentration up to 30,000 mg/L. The specific nitrification rate and the dominant nitrifying species were monitored. In one of the cultures, the chloride increase was carried out gradually, whereas in the other cases the increase was applied in the form of a pulse (stepwise increase). The species of ammonium-oxidizing (AOB) and nitrite-oxidizing (NOB) bacteria were examined employing the fluorescence in situ hybridization (FISH) technique, using 16S rRNA-specific oligonucleotide probes. The experiments, for both cultures, were divided into three different operational phases

in terms of the chloride increase: Phase 1, with a duration of 70 days and an increase in the chloride concentration up to 10,000 mg/L; Phase 2, with a duration of 70 days and an increase in the chloride concentration from 10,000 to 20,000 mg/L; and Phase 3, with a duration of 60 days and an increase in the chloride concentration from 20,000 to 30,000 mg/L. In the culture submitted to a stepwise increase, the chloride concentration was fixed at a certain value in each phase, while for the culture submitted to a gradual increase the chloride concentration was increased by 2000 mg/L every 10 days.

It was observed that concentrations below 10,000 mgCl^-/L (Phase 1), in both experiments (i.e., with the gradual and stepwise chloride increase) did not affect the specific rate of nitrification of the microbial cultures. Actually low chloride concentration such as 2500 mgCl^-/L provoked an increase in the nitrification rate. On applying the FISH technique, it could be observed that the AOB groups in the culture submitted to increasing chloride concentrations included *N. marine*, *N. oligotropha*, *N. europaea*, *N. eutropha*, *N. halophila*, *N. mobilis*, *N. communis*, and *N. cryotolerans*. With the gradual or stepwise increase from 10,000 to 20,000 mgCl^-/L in Phase 2, there was a significant variation in the two cultures, with an initial drop in the specific rate of nitrification, reflecting the inhibition of AOB and NOB by the chloride. However, after a few days, the specific nitrification rate began to increase again, even with the increased salinity. This finding is associated with a change in the dominant AOB species. In fact, the species which are not resistant to salinity, such as *Nitrosomonas europaea* and *Nitrosomonas eutropha*, were replaced by bacteria which are resistant to saline conditions, such as *Nitrosococcus mobilis*. The specific nitrification rate of the culture submitted to a stepwise increase in the chloride concentration was lower than that reached by the culture submitted to a gradual increase, which implies that in the latter case the adaptation and development of AOB resistant to saline conditions were favored. It was also observed that *Nitrobacter* was the only dominant genus of the NOB when the chloride concentration was lower than 10,000 mg/L, while no NOB were detected when the chloride concentration was ≥18,000 mg/L. Finally, in Phase 3 (30,000 mgCl^-/L), the nitrification rate remained constant for the culture in which the chloride content was gradually increased and showed a slight increase in the case of the culture subjected to a stepwise increase (CHEN et al. 2003).

Making use of some molecular techniques, such as PCR, DGGE, cloning, and sequencing, TAL et al. (2003) investigated the microbial consortium in a moving bed bioreactor (MBBR) connected to a marine aquaculture system with recirculation. The authors aimed at obtaining more detailed information regarding the functioning of different bacterial species, both anaerobic and aerobic, in the nitrogen removal process.

After 4 months of operation of the MBBR submitted to high organic loading rate, some of the supports present in the reactor were transferred to an experimental system operated under aerobic conditions without the addition of organic load and with a hydraulic retention time similar to that of the original reactor. This system served as a source of supports submitted to low organic loads for later studies. In this way, the different biological processes for nitrogen transformation were carried out through

short incubation procedures, in a batch regime, with supports submitted to low organic loads (originating from the abovementioned experimental system) as well as to high organic loads (originating from the original MBBR) (TAL et al. 2003).

Nitrosomonas cryotolerans and *Nitrospira marina* were identified as representatives of ammonia-oxidizing and nitrite-oxidizing bacteria, respectively. Heterotrophic bacteria were also detected, including *Pseudomonas* spp., *Aquaspirillum metamorphum*, and *Sphingomonas* spp. An interesting observation made by the authors was the presence of two PCR products with sequences which were very similar to those of two representatives of *Planctomycetes* spp. These organisms were detected in the system submitted to high organic loads, suggesting anaerobic ammonia oxidation (anammox) capacity. The sequencing of PCR products also revealed sequences very similar to those of sulfite-oxidizing microorganisms, such as *Sulfitobacter* spp., suggesting the establishment of anaerobic conditions which allowed the reduction of sulfate to sulfite in the MBBR. In this study, due to the methodology used for the microbial analysis (DGGE based on PCR products), it was not possible to determine the relative abundance of the different bacterial species adhered to the biofilm, although an estimate of the dominant species in the different tests (high or low organic load applied) was obtained based on the conversion processes (TAL et al. 2003).

TSUNEDA et al. (2005) investigated the formation of nitrifying granules in an aerobic upflow fluidized bed (AUFB) reactor applied for nitrification of synthetic wastewater containing 500 mgN/L of ammonium. The formation of spherical, pseudocubic, and elliptical granules, with diameters of around 346 μm, was observed in the bottom of the reactor after an operating period of 300 days. Sequencing of the gene fragments removed from the DGGE gels did not allow a perfect combination of the sequences obtained with those registered in the databases, suggesting that members of ammonium-oxidizing bacteria (AOB), which have not yet been characterized, were present in the granules. The phylogenetic analysis derived from the sequencing of a particular DGGE band revealed a nucleotide sequence with a strong similarity with *Nitrosococcus mobilis*. Other sequences derived from other bands have shown high similarity with *Nitrosomonas marina*. The authors suggested that the high ammonium loads applied to the AUFB reactor led to the predominance of the genus *Nitrosomonas*, which is consistent with other literature studies.

The analysis of the spatial distribution of the bacteria using the FISH technique revealed that the AOB were present at a depth of 100 μm from the granule surface. Groups of AOB presented diameters in the range of 10–20 μm. Using NOB-specific probes, it was observed that these bacteria were also located close to the granule surface. However, the number of NOB was well below that of AOB, result attributed by the authors to the greater specific nitrate production of the *Nitrobacter* (5.1–42 fmol/(cell h)) in comparison with the nitrite production rate of *Nitrosomonas* (0.9–42 fmol/(cell h)) (PROSSER 1989 *apud* TSUNEDA et al. 2005). Bacteria belonging to the genus *Nitrospira*, although frequently observed in nitrifying activated sludge systems (SCHRAMM et al. 1998), were not detected in the AUFB reactor using genus-specific FISH probes. The absence of *Nitrospira* was probably related to the high concentration of ammonium fed to the system (500 mg/L), a

value much higher than those commonly reported for conventional activated sludge systems, which can reach 100 mgNH_4^+-N/L (TSUNEDA et al. 2005).

EGLI et al. (2003a) operated a rotating biological contactor (RBC) and observed high nitrogen removal in the tertiary treatment of a leachate containing a high concentration of ammonium (100–500 mgN-NH_4^+/L). This leachate had been previously treated in a preliminary RBC, in which the TOC removal of 88% had been achieved. In addition, 6% of the TOC had been removed through adsorption onto activated carbon applied before the first RBC. This previous treatment ensured high removal of organic compounds (such as chlorinated hydrocarbons, phenols, and anilines) in the first RBC. Thus, the effluent of the first RBC basically contained a high ammonium concentration, which was sent to the second RBC, submitted to a maximum ammonium load of 30 kgN-NH_4^+/day.

The spatial structure of the microbial community in the RBC biofilm was analyzed, focusing on the presence of ammonium-oxidizing (AOB), nitrite-oxidizing (NOB) bacteria and anammox bacteria. The composition of the microbial community present in the biofilm was determined using different molecular tools. Initially, a 16S rRNA clone library was constructed for the RBC biofilm samples based on 16S rDNA fragments amplified by PCR. The sequencing revealed the presence of an unusual number of 16S rDNA sequences, with few sequences related to well-known AOB and NOB species. The DNA inserts of 26 clones were sequenced, 9 of which were different. Two different 16S rRNA sequences had 93% similarity between them, but both had at least 97% similarity with the 16S rRNA sequence of *Sphingomonas* spp. Another sequence belonged to an organism related to the genus *Staphylococcus*. Moreover, a 16S rDNA region of an AOB supposedly related to the genus *Nitrosomonas* was cloned, which was the only sequence present in the clone library related to the AOB group. Other sequences were observed, with similarity of less than 85% with the class *Sphingobacteria*, with the phylum *Actinobacteria* and with the phylum *Planctomycetes*. The 16S rRNA sequences of the anammox bacteria or NOB were not detected in the clone libraries (EGLI et al. 2003a).

From the analysis of biofilm samples using the FISH technique with known probes and by dot-blot hybridization with the same probes for total purified RNA, it was observed that four bacterial groups represented the largest populations in the biofilm samples, three of these being involved in the nitrogen conversion process according to their phylogenetic classification. The main groups of microorganisms present in the biofilm included *Nitrosomonas europaea* and *Nitrosomonas eutropha*, anammox bacteria of the type "*Candidatus Kuenenia stuttgartiensis*," NOB of the genus *Nitrospira*, and filamentous bacteria of the phylum *Bacteroidetes*. Ammonium-oxidizing bacteria corresponded to approximately 20–30% of the biomass, while members of the phylum *Cytophaga-Flavobacterium-Bacteroides* (CFB) represented 7%. According to FISH, NOB were present in relatively small amounts, representing around 5% of the microbial community. Besides those mentioned, no other AOB groups were found. According to the author, these results may be related to the high ammonium concentrations (up to 35 mM) and high salt concentrations (10 g/L), which may favor the selection of specific AOB groups resistant to the conditions imposed (i.e., *Nitrosomonas europaea/eutropha*. The presence of anammox bacteria

suggested that both the operating conditions of the treatment system and the specific type of wastewater treated provided conditions appropriate for the inoculation and spontaneous enrichment of these bacteria (EGLI et al. 2003a).

Besides being investigated by the FISH technique, the relative abundance of phylogenetically similar groups was evaluated by dot-blot hybridization. Compared with the intensity of the hybridization signal obtained with the general bacterial probe (considered as 100%), the quantity of rRNA labeled with the *Nitrosomonas*-specific probe was 23%. The same relative quantity was obtained with a more general probe for AOB. The dot-blot hybridization results indicated that filamentous bacteria of the phylum *Bacteroidetes* accounted for around 40% of the microbial population. NOB corresponded to 14% of the total bacterial community. Anammox bacteria were detected in very low quantities (around 1% of the microbial population), although FISH suggested a relatively high number for this group in the biofilm. The FISH methodology allowed an analysis of the biofilm structure, clearly showing that a very high amount of aerobic nitrifying microorganisms was located in the outer layer of the biofilm. The anammox bacteria were exclusively present in a dense inner layer of the biofilm, in which oxygen diffusion was limited, while filamentous CFB bacteria were distributed throughout the biofilm (EGLI et al. 2003a).

EGLI et al. (2003b) operated continuous stirred-tank reactors (CSTR), inoculated with activated sludge, to investigate the conditions required to promote the establishment of partial nitrification of ammonium and nitrite (nitritation) under oxic conditions. The reactors were submitted to different conditions of pH, temperature, and dilution rate, fed with either synthetic medium or the supernatant of a sludge digester (both containing 50 mM of ammonium). In all of the biological systems, stable nitritation was reached within 10–20 days after inoculation. FISH analysis using 16S rRNA-specific fluorescent probes conducted on samples from different reactors showed that the nitrite-oxidizing bacteria (NOB) of the genus *Nitrospira* were only active at the beginning of the reactor operation, that is, immediately after the inoculation with activated sludge. These bacteria gradually lost their activity and were almost completely washed out of the reactors. According to the FISH and the RFLP analysis of the *amoA* gene (which encodes the subunit of the active sites of the enzyme ammonia monooxygenase), the community of ammonium-oxidizing bacteria (AOB) showed an alteration between the first 15–20 days of reactor operation, from a very diverse group of populations in the inoculation sludge, represented by members of *Nitrosomonas communis* and *Nitrosomonas oligotropha* and of *Nitrosomonas europaea-Nitrosomonas eutropha*, to a smaller group inside the reactor.

The authors also observed that the RFLP patterns in the reactors submitted to pH 7.0 and 30 °C differed considerably from those of reactors submitted to pH 7.5 and 30 °C and to pH 7.5 and 25 °C. The reactors operated at pH 7.5 and 30 °C showed the predominance of one *amoA* RFLP type from the group *N. europaea-N. eutropha*. In the reactors operated at pH 7, which presented diversified communities, transitory changes in the population occurred. The reactors maintained at pH 7.5 and 25 °C developed communities which were indistinguishable using the FISH probes applied, although they differed in terms of the *amoA* RFLP patterns.

Furthermore, under these conditions, the increase in AOB was less pronounced than at 30 °C. On the other hand, the NOB community did not stabilize at any time, showing a decline and being no longer detectable after 10 days (EGLI et al. 2003b).

Sludge samples of these systems were very similar to those of the biomass coming with the sludge digester supernatant. This finding revealed that these reactors were constantly inoculated with the biomass from the sludge digester, allowing these systems to be operated with greater dilution rates (0.75/day) when compared with the reactors fed with synthetic medium (0.2/day). The communities of the reactors fed with digester supernatant showed a high degree of similarity, although they differed considerably from those of the reactors fed with synthetic medium. Despite the performance of the different reactors being similar with regard to the parameters evaluated, the structures of the communities differed, which probably influenced the stability during disturbances (EGLI et al. 2003b).

By means of FISH and electron microscopy, DÍAZ et al. (2003) evaluated the diversity, in qualitative and quantitative terms, of the microorganisms present in anaerobic granular sludge fed with different substrates. The granules presented a structure with several layers, in which slightly or densely packed microcolonies were observed. In the external region of the granules, only bacteria were found. On the other hand, in the center of the granules, both archaea and bacteria were present. Although the cell density of the granules was high (over ten cells per gram, labeled with DAPI), only a small fraction of the cells were hybridized with the specific probes used. The authors observed significant differences in the microbial composition of the granules fed with different substrates (formate, acetate in high and low concentrations, propionate, sucrose, amide, and peptone). Active *Proteobacteria* were in low numbers in the granules fed with volatile fatty acids. Bacteria of the genus *Syntrophobacter* dominated when the granules were fed with propionate. On the other hand, methanogenic archaea, particularly species of the genus *Methanosaeta*, were predominant when the granular reactor was fed with complex substrates or acetate in low concentrations. When high concentrations of acetate or formate were used as substrate, bacteria of the genus *Methanosarcina* and members of the order Methanobacteriales were dominant, respectively.

KIM et al. (2004) used the FISH technique to analyze nitrifying microbial communities in an activated sludge reactor (ASR) and in a fixed-bed reactor (FBR) used for the treatment of wastewater generated at a pig farm. Heterotrophic oxidation and nitrification occurred simultaneously in the ASR. The COD removal and nitrification efficiencies were observed to be dependent on the applied loads. In the FBR, the nitrification efficiency was also related to the ammonium load applied to the reactor. Nitrite accumulation was observed when the concentration of free ammonia was greater than 0.2 mgNH_3-N/L. With the use of FISH, it was observed that the ammonium-oxidizing bacteria (probe NSO1225) and the denitrifying bacteria (probe RRP1088) were less abundant than other bacteria (probe EUB338) in the ASR. Subsequent analysis of nitrifying bacteria in the FBR showed that species of *Nitrosomonas* (probe NSM156) and *Nitrospira* (probe NSR1156) were, respectively, the dominant ammonium-oxidizing and nitrite-oxidizing bacteria in the pig farm wastewater treatment system.

LIMPIYAKORN et al. (2005) investigated the ammonium-oxidizing bacteria (AOB) in activated sludge samples originating from 12 sewage treatment systems, subjected to low ammonium concentrations. These systems have shown different ammonium removal efficiencies and were operated according to different treatment processes: anaerobic/anoxic/aerobic process (A2O), anaerobic/aerobic process (AO), and a conventional activated sludge process (AS). The samples were collected in three different seasons (summer, autumn, and winter). In this study, real-time PCR was used to determine the total number of bacteria and AOB. AOB were also studied through PCR amplification, followed by DGGE, cloning, and sequencing of the 16S rRNA genes. The total number of bacteria and of AOB was in the ranges of 1.6×10^{12}–2.4×10^{13} and 1.0×10^{9}–9.2×10^{10} cells/L, respectively. These values varied according to the season of the year and to the influent BOD/NH_4^+-N ratio. The ammonia oxidation activity per cell of AOB varied between 0 and 49.6 fmol/(cell h), a value which was dependent on the season of the year. The highest values for this parameter were observed during summer, followed by those obtained during autumn and winter.

Although periodic variations in the number of AOB were observed, this was not the case for the composition of the AOB communities, which showed very stable behavior, even with the temperature varying over the seasons. Most of the bands excised from the DGGE gel and sequenced were related to the group *Nitrosomonas* spp. The highest number of the bands analyzed has shown high sequence similarity to *Nitrosomonas oligotropha*, which was found in all of the samples. *Nitrosomonas europaea-Nitrosococcus mobilis* (frequently considered to be halophilic microorganisms able to support high salt concentrations) and unknown species of *Nitrosomonas* appeared only in the anaerobic/anoxic/aerobic (A2O) system. It was observed that the solid retention time influenced the total number of AOB, while the dissolved oxygen concentrations affected the ammonium oxidation activity per cell of AOB. It was not possible to determine a relation between the different activated sludge systems and the presence of *Nitrosospira*, which was only recovered from one sample (LIMPIYAKORN et al. 2005).

Employing the T-RFLP technique, HOSHINO et al. (2005) monitored the microbial dynamics at the time in which the denitrification process started to occur in an activated sludge reactor with intermittent aeration. The terminal restriction fragments (T-RF) which increased due to the starting of denitrification were determined and identified through analysis of the 16S rRNA sequences obtained by cloning. It was observed that the bacteria belonging to the genera *Hydrogenophaga* and *Acidovorax* were present in greater numbers after the denitrification had begun.

CORTÉS-LORENZO et al. (2005) used PCR-TGGE to evaluate the microbial diversity of a submerged filter reactor employed for the removal of ammonium and phenol from a wastewater with a high salt concentration (30 g/L) originated from a pharmaceutical plant. The influent ammonium concentration was 340 mg/L and the phenol content was equivalent to 1000 mg/L. The system operated in pre-denitrification mode and therefore consisted of two separate columns (one anoxic and the other aerobic), with a connection between them.

The spatial diversity of the microbial communities in the biofilm was analyzed by collecting samples at four different heights in the system. The TGGE profiles for the sequences of the 16S rRNA gene (V3 hypervariable region) amplified by PCR showed significant variations in the microbial diversity, mainly due to the variation in the dissolved oxygen concentration along the reactor. Several bands obtained from TGGE were reamplified and sequenced in order to reveal the composition of the microbial communities present in the biofilm. Most of the sequenced bands (10 out of 13) were related to the 16S rRNA gene of marine α-*Proteobacteria*, mainly assigned to the genus *Roseobacter*. The remainder of the sequences which were related to the α-*Proteobacteria* was assigned to the order *Rhizobiales*. Organisms phylogenetically related to the group *Roseobacter* were found in several locations and are important primary colonizers of surfaces in coastal environments, consisting of precursors for the formation of biofilms (CORTÉS-LORENZO et al. 2005). Other sequences were related to γ-*Proteobacteria*, *Nitrospira marina* (nitrite oxidizer present only in the aerobic part of the system), and the anaerobic bacteria of the family *Desulfobacteraceae*, which is responsible for the degradation of phenol (CORTÉS-LORENZO et al. 2005).

OTAWA et al. (2006) investigated in the microbial community diversity in a sequencing batch reactor (SBR_1) with intermittent aeration and in 12 plants treating wastewater comprised of animal residues (8 SBR and 4 conventional activated sludge systems). In order to investigate the seasonal variation of microbial communities, techniques such as RT-PCR followed by DGGE were used along with the construction of clone libraries for 16S rRNA and ammonia monooxygenase (*amoA*) genes. In SBR_1, the dominant bacteria were represented by uncultured *Bacteroidetes* and *Proteobacteria*. The percentages of the bands related to these two groups in relation to the total sequenced bands corresponded to 47.4% and 34.2%, respectively. The DGGE profiles showed that the bacterial communities were stable during a certain treatment cycle, although there were seasonal variations. In relation to the AOB communities, two phylotypes (*Nitrosomonas ureae-oligotropha-marina*) were dominant in SBR_1 during the all seasons. Although the phylotypes of the AOB from the 13 treatment plants presented differences, the dominance of one or two groups was observed at all plants.

Sequence of DGGE bands revealed that *amoA* sequences belonging to the group *Nitrosomonas europaea-eutropha* were dominant at 11 plants, in which the influent wastewater contained high concentration of ammonium. On the other hand, at the plants where the ammonium concentration was relatively low, there was a predominance of the groups *Nitrosomonas urea-oligotropha-marina*. The authors also observed that, although the removal efficiency remained stable during the study period, the bacterial community varied during the year, indicating that the stability of the treatment efficiency does not necessarily reflect stability in the bacterial community. In fact, variations in the influent characteristics and in the reactor temperature during the process can lead to changes in the community structure of the bacteria (OTAWA et al. 2006).

FENG et al. (2007) investigated the diversity of the dominant microbial communities during the start-up of a granular sludge sequencing batch reactor, in which

partial removal of nitrogen was observed via the simultaneous nitrification/denitrification (SND) process. This process is favored by the irregular distribution of dissolved oxygen and by the anoxic microenvironment. These conditions often lead to the accumulation of nitrite (YOO et al. 1999). The start-up process of the reactor was divided into three stages: the first stage (day 1–15) was characterized by low nutrient removal rates and large fluctuations; in the second stage (day 16–35), there was an increase in the ammonium concentration with the consequent accumulation of nitrite; and in the third stage (day 36–60), the accumulation of nitrite remained constant and the pollutant removal rate reached stable values.

The DGGE method was used to obtain a fingerprint of the communities of α-*Proteobacteria*, β-*Proteobacteria*, ammonium-oxidizing bacteria (AOB), and *Nitrospira* during the start-up of the reactor. The results obtained with this technique showed that the profiles for the microbial community changed during the nitrite accumulation. Substantial modifications were observed during the initial period, particularly between days 25 and 35, although the structure of the microbial community became stable after 45 days of operation. The diversity analysis based on the DGGE patterns was carried out by calculating the Shannon index (*H*). For the samples collected on days 25 and 35, the *H* value was higher for β-*Proteobacteria* compared with the α-*Proteobacteria*, although the latter had higher values for the last two samples collected (days 45 and 55). This indicates that α-*Proteobacteria* have shown a greater diversity when the stability of the SND process was reached. During the experimental period, a decrease in the *H* index occurred both for the β-*Proteobacteria* and for *Nitrospira*. On the other hand, this index remained almost stable for α-*Proteobacteria* and for ammonium-oxidizing bacteria, which suggests that some species of β-*Proteobacteria* are more sensitive to free ammonia (FA) than the α-*Proteobacteria*. In fact, inhibition by free ammonia can result in the elimination of some species of β-*Proteobacteria* and *Nitrospira* (FENG et al. 2007).

The use of real-time PCR, determining the 16S rDNA copies, allows the quantification of the different microorganisms present in the system. From day 25 to 35, an apparent change was observed in the size of the populations of α- and β-*Proteobacteria*, which increased from 6.7×10^{11} cells/L to 1.0×10^{12} cells/L in the first day of the SND process. AOB presented a very similar growth behavior. On the other hand, the *Nitrospira* population decreased at the beginning of the SND process and with the accumulation of nitrite. The sizes of the AOB and *Nitrospira* populations were 8.7×10^{9}–2.4×10^{10} cells/L and 1.7×10^{10}–2.1×10^{10} cells/L, respectively. The proportions of AOB and *Nitrospira* in relation to the total bacteria in the stable stage were 2.1–2.4% to 0.8–1.2%, respectively. The same authors also observed, through the Pearson correlation coefficient, a relation between the nitrifying activity, diversity, and the size of the AOB and *Nitrospira* populations. It was observed that the size of the population had a greater influence on the degradation capacity compared to the diversity (FENG et al. 2007).

LIU et al. (2007) investigated the structure of the bacterial community of two sewage treatment plants with different processes and performances. For this purpose, DGGE was used to analyze the fragments of the 16S rRNA gene amplified by nested PCR using group-specific primers. In one of the plants (plant A), an

anoxic-anaerobic-aerobic process (A2O) was adopted, and in the other plant (plant B), an anoxic/aerobic process (AO) was used. The two plants showed different sludge characteristics, particularly in relation to foaming and settleability, as well as different pollutant removal efficiencies.

Samples of raw sewage, treated effluent, and activated sludge were amplified using PCR for further evaluation of the bacterial community structure by DGGE. Analysis of the community of ammonium-oxidizing bacteria and actinomycetes was also carried out to investigate the relation between the structures of specific populations and the system performance. The samples of raw sewage from both plants were located in a cluster in a dendrogram due to their similarity (Dice coefficient equivalent to 73%), which can be attributed to the similarity of the sewage constituents. Furthermore, the similarity between the influent sewage composition of the plants may have led to the similarity observed between the activated sludge samples of the two treatment systems (Dice coefficient of 60%).

The Dice coefficients obtained on comparing the similarity between the raw sewage and activated sludge samples from the two plants were very low, 27 and 29%, respectively. In fact, many of the bacterial populations of raw sewage do not appear in the activated sludge samples, suggesting that the dominant bacterial populations in the raw sewage do not play an important role in the biological treatment. Most of the microbial populations present in the raw sewage originate from sanitary wastes or soil. The bacteria from sanitary wastes are intestinal microorganisms which result from human or animal excretion and generally do not survive in unsuitable environments, such as wastewater treatment plants (MARSHALL 1980 *apud* LIU et al. 2007).

As previously described, the two plants showed high similarity in the microbial community structure of the raw sewage and activated sludge, although the microbial populations of their respective effluents differed (Dice coefficient of 25%). The difference between the sludge settleability of the two systems may be the main reason for the structural differences in bacterial communities. The sludge volume index (SVI) of plant B was 197 L/g, whereas that of plant A was 71 L/g. The relatively poor settleability of the activated sludge in plant B when compared with plant A may have led to washout of the bacteria, without the selection of one or another specific group. This may lead to a high degree of similarity between the populations of the effluent and the activated sludge (Dice coefficient of 79%). On the other hand, only some of the bacterial species were selectively washed out of plant A, which resulted in a low degree of similarity between the structures of the bacterial communities of the effluent and activated sludge (Dice coefficient of 26%) (LIU et al. 2007).

The same authors carried out a comparative study on the structure of the AOB communities in the two treatment plants. Plant A showed more bands in the DGGE in comparison with plant B. In addition, the two systems presented different AOB community structures. The greater ammonium removal obtained at plant A can be attributed to its greater richness of AOB species. The main bands of the DGGE gel related to AOB were excised and sequenced. The analysis of the sequences of the 16S rRNA genes revealed that all of the bands were closely related to the genus *Nitrosomonas* spp. The sequencing results showed that the main populations present in the raw sewage were non-cultivated bacteria, while in the activated sludge the

predominant populations belonged to the subclass β-*Proteobacteria*. Their numerical dominance suggests that the representatives of this subclass play an important role in the activated sludge process (removal of organic matter and nutrients as well as the formation of floc structure) (LIU et al. 2007).

Considering that the specific growth rates and activity of the ammonium-oxidizing bacteria (AOB) are very reduced at low temperatures, ISAKA et al. (2007) developed a technology for the immobilization of nitrifying bacteria on a support in the form of a polyethylene glycol (PEG) gel. By adopting this procedure, it was possible to reach high nitrification rates (0.71 kgN/(m^3 day)) in the treatment of leachate even at low temperatures (10 °C). The PEG supports were added to a 1.2 L air-lift reactor in which the filling fraction (ratio between the filling volume and total volume of the reactor) was 15% (180 mL). According to the authors, the immobilization of AOB is a key factor in the development of a nitrification process under low temperature conditions. The ammonium concentration of the effluent varied from 16 to 35 mg/L independently of the nitrogen load. Moreover, accumulation of nitrite was observed. In this study, in particular, the nitrite accumulation was not related to the low level of dissolved oxygen, which was greater than 7 mg/L, but may have been associated with the loss of activity of the nitrite-oxidizing bacteria (NOB) due to other factors, such as inhibition by free ammonia (ANTHONISEN et al. 1976). On applying the DGGE technique, four bands corresponding to ammonium-oxidizing bacteria were observed. The phylogenetic tree based on the sequences of the 16S rRNA gene from DGGE bands revealed that three of these bands were similar to those of *Nitrosomonas* and one band was similar to that of *Nitrosospira* spp. (95% similarity). Based on the DGGE results obtained, FISH was employed to identify the presence of these AOB. *Nitrosomonas* spp. and *Nitrosospira* spp. were detected. Nevertheless, direct cell counts showed that *Nitrosomonas* spp. was the dominant AOB group.

MONTRÀS et al. (2008) investigated the microbial community of the biofilm adhered to the spherical expanded polystyrene (Biostyr®) supports of a packed bed reactor containing two autotrophic species (*Nitrosomonas europaea* and *Nitrobacter winogradskyi*). The analysis were carried out after 4.8 years of continuous operation of reactor, which was achieving complete nitrification. It was observed that these two nitrifying species were, respectively, the only ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) present in the reactor. Quantitative real-time PCR (Q-PCR) was used to quantify the two abovementioned species along the vertical axis of the reactor (divided into eight fractions F_0 to F_7, from the bottom to the top), and the authors observed a spatial segregation of these two microbial lineages. The Q-PCR results showed a high degree of similarity at F_0 (89.7 ± 7.8% *N. europaea*) and F_1 (91.3 ± 7.1% *N. europaea*) positions. The relative quantities of *N. europaea* in F_2 (57.3 ± 8.3% *N. europaea*) and F_3 (58.7 ± 5.3% *N. europaea*) samples were also very similar. There was a gradual increase in *N. winogradskyi* with an increase in the bed height, as demonstrated by the relative quantities obtained at positions F_4 (61.0 ± 2.6% *N. winogradskyi*), F_5 (70.1 ± 1.3% *N. winogradskyi*), and F_6 (70.0 ± 1.0% *N. winogradskyi*).

In the last fraction of the packed bed (F_7), an increase in the *N. europaea*/*N. winogradskyi* ratio was observed, as the relative quantity of the latter species reduced by 53.9 ± 1.9% at this position in the reactor. The results have shown that spatial distribution of AOB and NOB occurred along the bed height, similarly to that observed in other nitrifying bioreactors with unidirectional flow, for instance, nitrifying trickling filters or nitrifying plug-flow bioreactors (HOLBEN et al. 1998 *apud* MONTRÀS et al. 2008).

MERTOGLU et al. (2008) monitored the changes in a population of nitrifying bacteria submitted to a cadmium load in a continuous flow nitrification system, fed with synthetic effluent. The authors used molecular microbiology tools, such as slot-blot hybridization (for the detection of nitrite-oxidizing bacteria), DGGE, and real-time PCR followed by melting curve analysis (for the detection of ammonium-oxidizing bacteria), cloning, and sequencing (for the identification of microbial species).

When the system was fed with doses equivalent to 1 mgCd/L, no decrease in the ammonium removal rate was observed, and even a stepwise increase to 10 mgCd/L did not cause inhibition of the microbial activity. However, after 15 days of operation at this concentration, a decrease in the ammonium removal efficiency from 99 to 10% was observed, which was accompanied by a reduction in the biomass concentration. On observing these results, the addition of cadmium was interrupted in order to allow the recovery of the microbial activity. Under these conditions, the inhibitory effect ceased. With a second application of cadmium at the same concentration (10 mg/L), it was observed that the biomass adapted to this metal and the inhibition reached 60%. In the next step, when the cadmium concentration was increased to 15 mg/L, the ammonium removal efficiency was 50% (MERTOGLU et al. 2008).

A change in the diversity of the ammonia-oxidizing bacteria was observed after the first application of 10 mg/L of Cd. The species resistant to this metal became dominant, and *Nitrosomonas* and *Nitrosococcus* spp. were replaced by *Nitrosospira* spp. This latter lineage was found to be resistant to high cadmium loads and gradually became dominant in the system. The results obtained indicated that the level of nitrification inhibition was not only related to the metal concentration and the quantity of microorganisms but was also dependent on the specific type of species present in the system (MERTOGLU et al. 2008).

DYTCZAK et al. (2008) observed that a laboratory-scale nitrifying sequencing batch reactor (SBR_1), operating under alternating anoxic/aerobic conditions, reached nitrification rates twice as high as those of another reactor (SBR_2), with the same dimensions and operational conditions, except that it was strictly aerobic. The microbial populations in the two reactors were investigated using the FISH technique, which revealed the predominance of rapid nitrifying microorganisms, such as *Nitrosomonas* and *Nitrobacter* (79.5% of the nitrifying population), in the anoxic/aerobic reactor, while in the strictly aerobic reactor, slow nitrifiers, such as *Nitrosospira* and *Nitrospira*, were predominant (78.2% of total nitrifiers). The proportion of ammonium-oxidizing bacteria (AOB) in relation to the total cells identified by DAPI was greater in SBR_1 (25.9%) compared with SBR_2 (19.9%), although the most significant difference was observed on comparing the AOB popu-

lations (particularly of *Nitrosomonas*) present in each reactor. In SBR_1, these microorganisms reached 81% of the total AOB, while in SBR_2 this percentage was only 19%. Similar behavior was observed for the NOB population in relation to the total bacteria, with a higher proportion in SBR_1.The largest discrepancy was related to the population of *Nitrobacter* in relation to the total NOB in the reactors, which was 76% in SBR_1 and 27% in SBR_2. In summary, both the AOB and the NOB were more abundant in SBR_1, which also had a greater AOB/NOB ratio.

The reactor SBR_2 (strictly aerobic) runs at the maximum rate and was negatively affected by ammonium and nitrite, while the nitrification rates obtained in the reactor submitted to alternating conditions (anoxic/aerobic) were proportional to the ammonium and nitrite concentrations. It was observed that the alternating conditions were more favorable for nitrification, since they allowed a more rapid selection of nitrifying microorganisms, characterized by higher oxidation, growth, and decay rates. These results are very useful for the operation of biological reactors aimed at nitrification, which are generally designed based on the growth rate of nitrifiers determined under strictly aerobic conditions (DYTCZAK et al. 2008).

MUNZ et al. (2008) compared a pilot-scale membrane bioreactor (MBR) with a conventional activated sludge (AS) system, in order to evaluate the global efficiency of the treatment, the presence and distribution of Gram-negative bacteria, and the kinetics of the nitrifying bacteria. Both systems were fed with industrial tannery wastewater and subjected to the same operating conditions. The MBR, in comparison with the AS reactor, presented a higher COD removal and a more stable nitrification process. These results were attributed to the differences in the composition and distribution of the microbial community.

The Gram-negative bacteria were detected by means of FISH. Phylogenetic probes specific for α-, β-, and γ-*Proteobacteria* of the main ammonia-oxidizing (AOB) and nitrite-oxidizing (NOB) bacteria belonging to the genera *Nitrobacter* and *Nitrospira* were used. The results showed that the main differences between the two reactor systems in terms of the biomass composition were the high abundance of α- and β-*Proteobacteria* in the MBR and the presence of aggregates of AOB only on the surface of the flocs present in this reactor. It should be noted that there was a predominance of α-*Proteobacteria* in the samples originating from the two reactor configurations and not of β-*Proteobacteria* as frequently reported in the literature. This finding may be related to the specific industrial wastewater fed to the system. The differences observed in the quantitative distribution of the three subdivisions of *Proteobacteria* (α, β, and γ) in the samples collected from the MBR and the AS reactor suggest that the biomass separation process, by filtration (MBR) or sedimentation (AS reactor), affected the selection of the specific microorganisms (MUNZ et al. 2008).

WEN et al. (2008) operated a fluidized bed reactor (FBR) without a separation between the aerobic and anoxic zones and achieved simultaneous nitrification-denitrification (SND) through control of the dissolved oxygen concentration. In this study, the efficiency and composition of the nitrifying bacteria were investigated. The volumetric nitrogen load varied between 0.12 and 0.20 kgN/(m^3 day). The ammonium removal efficiency was greater than 80% and the DO

concentration, maintained within the range of 1–5 mg/L, did not appear to influence this performance. When the DO concentration was below 3 mg/L, the nitrogen removal efficiency was greater than 50%. With the use of the T-RFLP technique based on fragments of 16S rRNA, it was possible to characterize the diversity and distribution of the ammonium-oxidizing (AOB) and nitrite-oxidizing (NOB) bacteria in the FBR. The results indicated that the composition and quantity of AOB and NOB varied according to the position inside the reactor and time of operation. The dominant species of the AOB community were represented by *Nitrosomonas* spp. Also, *Nitrobacter* spp. were detected in the system. According to the authors, the SND mechanism in FBR was associated with the vertical stratification of the active populations. However, the possible presence of microenvironments within the biofilm was not discarded (WEN et al. 2008).

MOURA et al. (2007) employed the DGGE technique to estimate the bacterial diversity and monitor the microbial communities in two aerated lagoons (lagoon 1 and lagoon 2) of a plant treating urban and industrial wastewaters. Significant changes were detected on comparing the samples collected in the winter/spring and autumn/summer months. Thus, the microbial community showed seasonal variations. As previously described, OTAWA et al. (2006) also observed a pattern of seasonal variability in the bacterial community present in a sequencing batch reactor operated with intermittent aeration.

The variables which had the greatest impact on the bacterial communities were temperature, dissolved oxygen, and pH. The authors carried out a numerical analysis of the DGGE patterns based on two indicators, each one describing a different aspect of the diversity of the community of microorganisms. The diversity indices were obtained taking into consideration the relative intensity of each band of the DGGE gel. The reproducibility of the results was obtained by conducting replicates of the experiments (MOURA et al. 2007).

The Shannon index (H), used to calculate the diversity of microbial communities, varied from 0.717 to 1.042 and from 0.784 to 1.121 in lagoons 1 and 2, respectively. The highest H values were recorded in the months of August and March, indicating a greater number of species and higher relative abundance during these months. Another parameter evaluated was the equitability index (E), which can vary from values close to 0 (indicating the pronounced dominance of some microbial groups) to values close to 1 (indicating complete equality of abundance of all species). The analysis of the DGGE patterns provided values for the equitability index in the ranges 0.756–0.886 and 0.796–0.968 for lagoons 1 and 2, respectively. These values indicate a high degree of uniformity in the distribution of taxons between samples in terms of abundance in both lagoons (MOURA et al. 2007).

The phylogenetic affiliation of the predominant groups was determined through the sequencing of 16S rDNA fragments excised from the DGGE gel. The results for the phylogenetic analysis allowed classifying the microorganisms in the groups *Cytophaga-Flexibacter-Bacteroides* (CFB), *Firmicutes*, and β- and ε-*Proteobacteria* (MOURA et al. 2007).

Recently, it was discovered that the autotrophic oxidation of ammonia is not restricted only to the domain bacteria (Könneke et al. 2005; TREUSCH et al. 2005).

The ammonium-oxidizing archaea (AOA) called *Nitrosopumilus maritimus* was isolated from a marine aquarium (Könneke et al. 2005) and was the first representative of AOA. These microorganisms, as in the case of ammonium-oxidizing bacteria (AOB), grow under chemolithotrophic conditions oxidizing ammonium to nitrite. Characteristics such as high diversity, high nitrification rate, and low demand for dissolved oxygen mean that AOA can be important agents in the partial nitrification process.

In this context, ZHANG et al. (2009) conducted a study to characterize the AOA community both in an activated sludge bioreactor designed for nitrogen removal and several wastewater treatment plants. A pair of primers specific for the α subunit of ammonia monooxygenase (*amoA*) of archaea was used in the analysis. This study confirmed the occurrence of AOA in the systems evaluated. In total, 18 sequences of the functional gene *amoA* were recovered and compared with previously reported sequences. A comparison between the clone libraries obtained with the samples collected from one of the plants and from the laboratory scale bioreactor indicated that the community of AOA varied significantly only after 30 days of enrichment. The authors also observed that the community of AOA of the treatment plants studied was similar to those found in sediments and soils, but distinct from those present in other activated sludge systems. Finally, they also mentioned that for the detection of the different AOA communities present in environmental samples, a combination of different primers specific for the *amoA* gene is required (ZHANG et al. 2009).

Bassin et al. (2011) studied the dynamics of the microbial community structure of an aerobic granular sludge subjected to high salt concentrations. The bacterial population was evaluated by applying PCR-DGGE of 16S rRNA and *amoA* genes. The PCR products obtained from both genomic DNA and rRNA after reverse transcription were compared to determine the bacteria present as well as the metabolically active fraction of the bacteria. Fluorescence in situ hybridization (FISH) was used to validate the PCR-based results and to quantify the dominant bacterial populations. The authors observed that salt concentrations up to 33 gNaCl/L did not affect the ammonium removal efficiency. Conversely, a high accumulation of nitrite was observed above 22 gNaCl/L, which coincided with the disappearance of the only nitrite-oxidizing bacteria detected in the sludge (*Nitrospira* sp.). The increase in salt concentration had a strong impact on the phosphorus removal. Under steady-state operation conditions at 33 gNaCl/L, polyphosphate-accumulating organisms (PAOs) (*Candidatus Accumulibacter phosphatis*) were no longer detected by PCR-DGGE or by FISH. As a consequence, no P-release or P-uptake was observed at this salinity level. Glycogen-accumulating organisms (GAOs) became the dominant microorganisms at increasing salt concentrations, especially at 33 gNaCl/L. From the comparative analysis of the diversity (DNA-derived pattern) and the activity (cDNA-derived pattern) of the microbial population, it was observed that the microorganisms with the highest metabolic activity were those related to ammonium (*Nitrosomonas* spp.) and phosphate (*Candidatus Accumulibacter*) removal (Bassin et al. 2011).

6.4 Final Considerations

This chapter has demonstrated the large amount of information that can be provided by the molecular biology techniques. These approaches allow the study of the diversity of the microbial communities present in a variety of different environments, such as biological wastewater treatment systems.

A period in which an enormous and increasing quantity of information will become available has only just begun, and this will enable the evaluation of the taxonomy and the functional diversity of complex communities of microorganisms. This information is of particular interest since establishing a link between the experimental measurements and the microbiological analysis will elucidate the relations between the biodiversity and the functioning of natural or engineered ecosystems.

In the particular case of wastewater treatment systems, new techniques will be very useful for the diagnosis of the system and identification of problems related to its operation, such as bulking sludge and foaming. Currently, the treatment plants may operate with good or poor performance, and in most cases the reason for this is unknown.

Although a certain level of understanding has been gained regarding the structure of activated sludge and biofilm processes, we are still a long way from a complete understanding of the structure of microbial communities involved in these processes. The objective is to elucidate the origin of certain microorganisms, the factors and mechanisms which lead to changes in the bacterial populations (microbial population dynamics) and their relation with the performance of biological processes. In other words, it is necessary to gain an understanding of microbial populations and how they interact with the wastewater components. For this purpose, it is necessary to link a wide range of areas from the microbiology of a single cell to studies in laboratory scale. A better understanding of the microbiology and ecology of various groups of bacteria would provide insights essential for improving the performance and control of the processes, revealing the factors which lead to their proper functioning. This understanding represents the bridge which unites fundamental knowledge on microbial communities and the wastewater treatment processes.

Studies seeking to enhance this knowledge are already underway in several research centers around the world, and many are being carried out in laboratory systems, providing a rapidly growing understanding of the most important microorganisms involved in the wastewater treatment processes and the factors which alter their numbers and activity. However, there is a wide gap in the information available, particularly due to the fact that few studies have been carried out in full scale and the difficulty associated with extrapolating information gained in laboratory studies to full-scale plants.

With the aid of molecular biology techniques, it is hoped that it will be possible to manipulate and control the populations of microorganisms, to select only those which function under certain operational conditions, and thus to achieve high pollutant removal efficiencies and stable operation. Looking even further forward, real-time monitoring of biological treatment systems can be imagined, in which the

diversity and activity of the microbial communities associated with certain operating conditions are obtained at any given time.

Therefore, it is necessary to encourage the process engineers to better appreciate the valuable information obtained at the molecular level when designing treatment plants, rather than simply considering the chemical and physical parameters. With this approach, the operation of these systems will be carried out in a less empirical way, contributing to the beginning of a new era of novel and advanced biological treatment processes.

References

ADAMCZYK, J., HESSELSOE, M., IVERSEN, N., HORN, M., LEHNER, A., NIELSEN, P. H., SCHLOTER, M., ROSLEV, P., WAGNER, M. The isotope array, a new tool that employs substrate-mediated labeling of rRNA for determination of microbial community structure and function. *Applied and Environmental Microbiology*, v. 69, p. 6875-6887, 2003.

ALBERTS, B., BRAY, D., LEWIS, J., RAFF, M., ROBERTS, K., WATSON, J. D. *DNA and RNA structure*, 1994. In: Molecular biology of the cell. Third edition. New York: Garland Science, p. 100-109.

AMANN, R. I., KRUMHOLZ, L., STAHL, D. A. Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. *The Journal of Bacteriology*, v. 172, p. 762-770, 1990.

AMANN, R. I., LUDWIG, W., SCHLEIFER, K. H. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiological Reviews*, v. 59, p. 143-169, 1995.

AMANN, R., LUDWIG, W., SCHULZE, R., SPRING, S., MOORE, E., SCHLEIFER, K. H. rRNA-targeted oligonucleotide probes for the identification of genuine and former pseudomonads. *Systematic and Applied Microbiology*, v. 19, p. 501-509, 1996.

ANDREOTE, F. D., AZEVEDO, J. L., ARAÚJO, W. L. Assessing the diversity of bacterial communities associated with plants. *Brazilian Journal of Microbiology*, v. 40, n. 3, p. 417-432, 2009.

ANTHONISEN, A. C., LOHER, R. C., PRAKASAM, T. B. S., SRINATH, E. Inhibition of nitrification by ammonia and nitrous acid. *Journal Water Pollution Control Federation*, v. 48, n. 5, p. 835-852, 1976.

AOI, Y., MIYOSHI, T., OKAMOTO, T., TSUNEDA, S., HIRATA, A., KITAYAMA, A., NEGAMUNE, T. Microbial Ecology of Nitrifying Bacteria in Wastewater Treatment Process Examined by Fluorescence *In Situ* Hybridization. *Journal of Bioscience and Bioengineering*, v. 90, n. 3, p. 234-240, 2000.

BASSIN, J.P., KLEEREBEZEM, R., MUYZER, G., ROSADO, A.S., VAN LOOSDRECHT, M.C.M., DEZOTTI, M. Effect of different salt adaptation strategies on the microbial diversity, activity, and settling of nitrifying sludge in sequencing batch reactors. *Applied Microbiology and Biotechnology*, v. 93, n. 3, p. 1281-1294, 2012.

BASSIN, J.P., PRONK, M., MUYZER, G., KLEEREBEZEM, R., DEZOTTI, M., VAN LOOSDRECHT, M.C.M. Effect of elevated salt concentrations on the aerobic granular sludge process: linking microbial activity with microbial community structure. *Applied and Environmental Microbiology*, v. 77, n. 22, p. 7942-7953, 2011.

BAYÉS, M., HEATH, S., GUT, I.G. Applications of Second Generation Sequencing Technologies in Complex Disorders. Current topics in behavioral neurosciences. In: CRYAN, J.F., REIF, A. Behavioral Neurogenetics. Springer-Verlag, Berlin Heidelberg, 2012.

BENTLEY, D.R., BALASUBRAMANIAN, S., SWERDLOW, H.P., SMITH, G.P., MILTON, J., BROWN, C.G., HALL, K.P., EVERS, D.J., BARNES, C.L., BIGNELL, H.R., et al. Accurate whole human genome sequencing using reversible terminator chemistry. *Nature*, v. 456, p. 53–59, 2008.

BOND, P. J., HUGENHOLTZ, P., KELLER, J., BLACKALL, L. L. Bacterial community structures of phosphate-removing and non-phosphate-removing activated sludges from sequencing batch reactors. *Applied and Environmental Microbiology*, v. 61, n. 5, p. 1910-1916, 1995.

BORNEMAN J., TRIPLETT, E. Molecular microbial diversity in soils from eastern Amazonia: evidence for unusual microorganisms and microbial population shifts associated with deforestation. *Applied and Environmental Microbiology*, v. 63, p. 2647-2653, 1997.

BRESLAUER, K. J., FRANK, R., BLOCKER, H., MARKY, L.A. Predicting DNA duplex stability from the base sequence. *Proceedings of the National Academy of Sciences - USA*, v. 83, p. 3746-3750, 1986.

BURRELL, P. C., KELLER, J., BLACKALL, L. L. Microbiology of a Nitrite-Oxidizing Bioreactor. *Applied and Environmental Microbiology*, v. 64, n. 5, p. 1878-1883, 1998.

CHANG, J.-J., LIANG, W., XIAO, E.-R., WU, Z.-B. Effect of intermittent aeration on the microbial community structure of activated sludge in a submerged membrane bioreactor. *Water and Environment Journal*, v. 25, p. 214-218, 2011.

CHEN, G. H., WONG, M. T., OKABE, S., WATANABE, Y. Dynamic response of nitrifying activated sludge batch culture to increased chloride concentration. *Water Research*, v. 37, p. 3125-3135, 2003.

CHEUNG, V. G., MORLEY, M., AGUILAR, F., MASSIMI, A., KUCHERLAPATI, R., CHILDS. Making and reading microarrays. *Nature Genetics*, v. 21, p. 15-19, 1999.

CHRISTENSEN, H., NORDENTOFT, S., OLSEN, J.E. Phylogenetic relationships of Salmonella based on rRNA sequences. *International Journal of Systematic Bacteriology*, v. 48, p. 605-610, 1998.

CIESIELSKI, S., BULKOWSKA, K., DABROWSKA, D., KACZMARCZYK, D., KOWAL, P., MOZEIKO, J. Ribosomal intergenic spacer analysis as a tool for monitoring methanogenic *Archaea* changes in an anaerobic digester. *Current Microbiology*, v. 67, n. 2, p. 240-248, 2013.

CORTÉS-LORENZO, C., MOLINA-MUÑOZ, M. L., GÓMEZ-VILLALBA, B., VILCHEZ, R., RAMOS, A., RODELAS, B., HONTORIA, E. GONZÁLEZ-López, J. Analysis of community composition of biofilms in a submerged filter system for the removal of ammonia and phenol from industrial wastewater. *The 11th Nitrogen Cycle Meeting*, p. 165-168, 2005.

COUTINHO, H. L. C., OLIVEIRA, V. M., MANFIO, G. P., ROSADO, A. S. Evaluating the microbial diversity of soil samples: methodological innovations. *Anais da Academia Brasileira de Ciências*, v. 71, p. 491-503, 1999.

CROCETTI, G. R., BANFIELD, J. F., KELLER, J., BOND, P. L., BLACKALL, L. L. Glycogen-accumulating organisms in laboratory-scale and full-scale wastewater treatment processes. *Microbiology*, v. 148, p. 3353-3364, 2002.

CROCETTI, G. R., HUGENHOLTZ, P., BOND, P. L., SCHULER, A., KELLER, J., JENKINS, D., BLACKALL, L. L. Identification of polyphosphate-accumulating organisms and design of 16S rRNA-directed probes for their detection and quantitation. *Applied and Environmental Microbiology*, v. 66, p. 1175-1182, 2000.

CROSS, N. C. P. Quantitative PCR techniques and applications. *British Journal of Haematology*, v. 89, p. 693-697, 1995.

DAIMS, H., BRUHL, A., AMANN, R. SCHLEIFER, K. H., WAGNER, M. The domain-specific probe EUB338 is insufficient for the detection of all bacteria: development and evaluation of a more comprehensive probe set. *Systematic and Applied Microbiology*, v. 22, p. 434-444, 1999.

DAIMS, H., MAIXNER, F., SCHMID, M. C. The nitrifying microbes: ammonia oxidizers, nitrite oxidizers, and anaerobic ammonium oxidizers. In: NIELSEN, P. H., DAIMS, H., LEMMER, H. FISH Handbook for Biological Wastewater treatment: Identification and Quantification of Microorganisms in Activated Sludge and Biofilms by FISH. London: IWA Publishing, 2009.

DAIMS, H., NIELSEN, J. L., NIELSEN, P. H., SCHLEIFER, K. H., WAGNER, M. In situ characterization of *Nitrospira*-like nitrite oxidizing active in wastewater treatment plants. *Applied and Environmental Microbiology*, v. 67, p. 5810-5818, 2001.

DALMA-WEISZHAUSZ, D. D., WARRINGTON, J., TANIMOTO, E. Y., MIYADA, C. G. The affymetrix GeneChip platform: an overview. *Methods in Enzymology*, v. 410, p. 3-28, 2006.

DAVENPORT, R. J., CURTIS, T. P. GOODFELLOW, M., STAINSBY, F. M., BINGLEY, M. Quantitative use of fluorescent in situ hybridization to examine relationships between mycolic acid-containing actinomycetes and foaming in activated sludge plants. *Applied and Environmental Microbiology*, v. 66, p. 1158-1166, 2000.

DEGRAVE, W., FERNANDES, O., CAMPBELL, D., BOZZA, M., LOPES, U. Use of molecular probes and PCR for detection and typing of *Leishmania* – a mini-review, *Memórias do Instituto Oswaldo Cruz*, v. 89, p. 463-469, 1994.

DENNIS, G., SHERMAN, B. T., HOSACK, D. A., YANG, J., GAO, W., LANE, H. C., LEMPICKI, R. A. DAVID: Database of annotation, visualization, and integrated discovery. *Genome. Biology*, v. 4, p. R60, 2003.

DÍAZ, E., AMILS, R., SANZ, J.L. Molecular ecology of anaerobic granular sludge grown at different conditions. *Water Science and Technology*, v. 48, n. 6., p. 57-64, 2003.

DIEFFENBACH, C. W., LOWE, T. M. J., DVEKSLER, G. S. General concepts for PCR *primer* design. *Genome Research*, v. 3, p. 30-37, 1993.

DIEHL, F., LI, M., HE, Y. KINZLER, K.W., VOGELSTEIN, B., DRESSMAN, D., BEAMing: single-molecule PCR on microparticles in water-in-oil emulsions. *Nature Methods*, v. 3, p. 551–559, 2006.

DIONISI, H. M., LAYTON, A. C., HARMS, G., GREGORY, I. R., KEVIN, G. R., SAYLER, G. S. Quantification of *Nitrosomonas oligotropha*-like ammonia-oxidizing bacteria and *Nitrospira* spp. from full-scale wastewater treatment plants by competitive PCR. *Applied and Environmental Microbiology*, v. 68, n. 1, p. 245-253, 2002.

DOLLED-FILHART, M.P., LEE, M. JR., OU-YANG, C.W., HARAKSINGH, R.R., LIN, J.C. Computational and Bioinformatics Frameworks for next-generation whole exome and genome sequencing. Scientific World Journal, Article ID 730217 p. 1-10, 2013.

DORIGO, U., VOLATIER, L., HUMBERT, J.F. Molecular approaches to the assessment of biodiversity in aquatic microbial communities. *Water Research*, v. 39, n. 11, p. 2207-2218, 2005.

DOWLING, T. E., MORITZ, C., PALMER, J. D., RIESEBERG, L. H. Nucleic acids III: Analysis of fragments and restriction sites, p. 249-320, 1996. In: HILLIS, D. M., MORITZ, C., MABLE, B. K. (Eds.). *Molecular systematic*, 2nd ed. Sunderland: Sinauer Associates Inc, 655p.

DYTCZAK, M. A., LONDRY, K. L., OLESZKIEWICZ, J. A. Activated sludge operational regime has a significant impact on the type of nitrifying community and its nitrification rates. *Water Research*, v. 42, p. 2320-2328, 2008.

EDEL, V. PCR in Mycology: an Overview. In: BRIDGE, P.D., ARORA, D.K., REDDY, C.A., ELANDER, R.P. (eds.) Applications of PCR in Mycology. CAB International, Wallingford, UK, p. 1–20, 1998.

EGERT, M., FRIEDRICH, M. W. Formation of pseudo-terminal restriction fragments, a PCR-related bias affecting terminal restriction fragment length polymorphism analysis of microbial community structure, *Applied and Environmental Microbiology*, v. 69, p. 2555-2562, 2003.

EGLI, K., BOSSHARD, F., WERLEN, C., LAIS, P., SIEGRIST, H., ZEHNDER, A. J. B., VAN DER MEER, J.R. Microbial Composition and Structure of a Rotating Biological Contactor Biofilm Treating Ammonium-Rich Wastewater without Organic Carbon. *Microbial Ecology*, v. 45, p. 419-432, 2003a.

EGLI, K., LANGER, C., SIEGRIST, H-R., ZEHNDER, A. J. B., WAGNER, M., ROELOF, J., VAN DER MEER, J. R. Community Analysis of Ammonia and Nitrite Oxidizers during Start-Up of Nitritation Reactors. *Applied and Environmental Microbiology*, v. 69, n. 6, p. 3213-3222, 2003b.

EKBLOM, R., WOLF, J.B.W. A field guide to whole-genome sequencing, assembly and annotation. *Evolutionary Applications*, v. 7, n. 9, p. 1026-1042, 2014.

ELLSWORTH, D. L., RITTENHOUSE, K. D., HONEYCUTT, R. L. Artifactual variation in randomly amplified polymorphic DNA banding patterns. *Biotechniques*, v. 14, p. 214-217, 1993.

ENGEBRETSON, J. J., MOYER, C. L. Fidelity of select restriction endonucleases in determining microbial diversity by terminal-restriction fragment length polymorphism. *Applied and Environmental Microbiology*, v. 69, n. 8, p. 4823-4829, 2003.

ERCOLINI D. PCR-DGGE fingerprinting: novel strategies for detection of microbes in food. *Journal of Microbiological Methods*, v. 56, p. 297-314, 2004.

FERREIRA, M. E., GRATTAPAGLIA, D. Introducción al uso de marcadores moleculares em el análisis genético. Brasilia: Embrapa-Cenargen, 1998, 200p.

FERRIS, M.J., MUYZER, G., WARD, D.M. Denaturing gradient gel electrophoresis profiles of 16S rRNA-defined populations inhabiting a hot spring microbial mat community. *Applied and Environmental Microbiology*, v. 62, p. 340–346, 1996.

FENG, W., SI-QING, X., YI, L., XUE-Song, C., JUN, Z. Community analysis of ammonia and nitrite oxidizers in start-up of aerobic granular sludge reactor. *Journal of Environmental Sciences*, v. 19, p. 996-1002, 2007.

FISHER, M. M., TRIPLETT, E. Automated approach for ribosomal intergenic spacer analysis of microbial diversity and its application to freshwater bacterial communities. *Applied and Environmental Microbiology*, v. 65, p. 4630-4636, 1999.

FOESEL, B. U., GIESEKE, A., SCHWERMER, C., STIEF, P., KOCH, L., CYTRYN, E., TORRÉ, J. R., VAN RIJN, J., MINZ, D., DRAKE, H. L., SCHRAMM, A. *Nitrosomonas* Nm143-like ammonia oxidizers and *Nitrospira marina*-like nitrite oxidizers dominate the nitrifier community in a marine aquaculture biofilm. *FEMS Microbiology Ecology*, v. 63, p. 192-204, 2008.

FRANKLIN, R., TAYLOR, D., MILLS, A. Characterization of microbial communities using randomly amplified polymorphic DNA (RAPD). *Journal of Microbiology Methods*, v. 35, p. 225-235, 1999.

FUNGARO, M. H. P., VIEIRA, M. L. C. Aplicações da PCR em ecologia molecular, 1998. In: MELO, I. S., AZEVEDO, J. L. (Ed.). *Ecologia microbiana*. Jaguariúna: Embrapa-CNPMA, p. 205-228.

GILLE, C., GILLE, A., BOOMS, P., ROBINSON, P. N. NURNBERG, P. Bipolar clamping improves the sensitivity of mutation detection by temperature gradient gel electrophoresis. *Electrophoresis*, v. 19, p. 1347-1350, 1998.

GIULIANO, L., DE DOMENICO, M., DE DOMENICO, E., HOFLE, M. G., YAKIMOV, M. M. Identification of culturable oligotrophic bacteria within naturally occurring bacterioplankton communities of the Ligurian sea by 16S rRNA sequencing and probing. *Microbial Ecology*, v. 37, p. 77-85, 1999.

GONZÁLEZ, J.M., MORAN, M. A. *Applied and Environmental Microbiology*, v. 63, p. 4237-4242, 1997.

GUSCHIN, D. Y., MOBARRY, B. K., PROUDINIKOV, D., STAHL, D. A., RITTMANN, B., IZARBEKOV, A. D. Oligonucleotide microchips as genosensors for determinative and environmental studies in microbiology. *Applied and Environmental Microbiology*, v. 63, p. 2397-2402, 1997.

HEAD, I. M., SAUNDERS, J. R., PICKUP, R. W. Microbial evolution, diversity, and ecology: a decade of ribosomal RNA analysis of uncultivated microorganisms. *Microbial Ecology*, v. 35, p. 1-21, 1998.

HERBERT, R. A. Methods for enumerating microorganisms and determining biomass in natural environments. *Methods in Microbiology*, v. 19, p. 1-40, 1990.

HESS, A., ZARDA, B., HAHN, D., HANER, A., STAX, D., HOHENER, P., ZEYER, J. In situ analysis of denitrifying toluene- and m-xylene-degrading bacteria in a diesel fuel-contaminated laboratory aquifer column. *Applied and Environmental Microbiology*, v. 63, p. 2136-2141, 1997.

HESSELMANN, R. P., WERLEN, C., HAN, D., VAN D.M., Jr., ZEHNDER, A. J. Enrichment, phylogenetic analysis and detection of a bacterium that performs enhanced biological phosphate removal in activated sludge. *Systematic and Applied Microbiology*, v. 22, p. 454-465, 1999.

HEIDM C.A., STEVENS, J., LIVAK, K.J., WILLIAMS, P.M. Real time quantitative PCR. *Genome Research*, v. 6, n. 10, p. 986-994, 1996.

Hillis, D. M.; Mable, B. K.; Moritz, C. Applications of molecular systematics: The state of the field and a look to the future. *Molecular Systematics*, p. 515-543, 1996. In: Molecular systematics. 2nd ed. D. M. Hillis, C. Moritz, and B. K. Mable (eds.). Sinauer Associates, Inc., Sunderland, MA.

HIRATA, M. H., HIRATA, R. D. C. Aplicação da PCR em laboratório clínico e medicina forense. In: Curso de Atualização, São Paulo. Laboratório de Biologia Molecular Aplicada ao Diagnóstico do Departamento de Análises Clínicas e Toxicológicas da Faculdade de Ciências Farmacêuticas da Universidade de São Paulo, p. 28-37, 1991.

HOLBEN, W. E., JANSSON, J. K., CHELM, B. K., TIEDJE, J. M. DNA probe method for the detection of specific microorganisms in the soil bacterial community. *Applied and Environmental Microbiology*, v. 54, p. 703-711, 1988.

HOLBEN, W. E., NOTO, K., SUMINO, T., SUWA, Y. Molecular analysis of bacterial communities in a three-compartment granular activated sludge system indicates community-level control by incompatible nitrification processes. *Applied and Environmental Microbiology*, v. 64, v. 7, p. 2528-2532, 1998.

HOSHINO, T., TERAHARA, T., TSUNEDA, S., HIRATA, A., INAMORI, Y. Molecular analysis of microbial population transition associated with the start of denitrification in a wastewater treatment process. *Journal of Applied Microbiology*, v. 99, p. 1165-1175, 2005.

HOVANEC, T. A., DELONG, E. F. Comparative analysis of nitrifying bacteria associated with freshwater and marine aquaria. *Applied and Environmental Microbiology*, v. 62, p. 2888-2896, 1996.

HUGENHOLTZ, P., PACE, N. R. Identifying microbial diversity in the natural environment: a molecular phylogenetic approach. *Trends in Biotechnology*, v. 14, p. 190-197, 1996.

HU, M., WANG, X., WEN, X., XIA, Y. Microbial community structures in different wastewater treatment plants as revealed by 454-pyrosequencing analysis. *Bioresource Technology*, v. 117, 72-79, 2012.

ISAKA, K., YOSHIE, S., SUMINO, T., INAMORI, Y., TSUNEDA, S. Nitrification of landfill leachate using immobilized nitrifying bacteria at low temperatures. *Biochemical Engineering Journal*, v. 37, p. 49-55, 2007.

JENKINS, D.; RICHARD, M. G.; DAIGGER, G. T *Manual on the causes and control of activated sludge bulking and foaming*. 2nd edition, Michigan, 1993.

JENSEN, M. A., WEBSTER, J. A., STRAUS, N. Rapid identification of bacteria on the basis of polymerase chain reaction-amplified ribosomal DNA spacer polymorphisms. *Applied and Environmental Microbiology*, v. 59, p. 945-952, 1993.

JONES, C. M., THIES, J. E. Soil microbial community analysis using two-dimensional polyacrylamide gel electrophoresis of the bacterial ribosomal internal transcribed, *Journal of Microbiological Methods*, v. 69, p. 256-267, 2007.

JURETSCHKO, S., LOY, A., LEHNER, A., WAGNER, M. 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*, v. 25, p. 84-99, 2002.

JURETSCHKO, S., TIMMERMANN, G., SCHMID, M., SCHLEIFER, K.H., POMMERENING-ROSER, A., KOOPS, H. P., WAGNER, M. Combined molecular and conventional analyses of nitrifying bacterium diversity in activated sludge: *Nitrosococcus mobilis* and *Nitrospira*-like bacteria as dominant populations. *Applied and Environmental Microbiology*, v. 64, n. 8, p. 3042-3051, 1998.

KARTAL, B., RATTRAY, J., VAN NIFTRIK, L. A., VAN DE VOSSENBERG, J., SCHMID, M. C., WEBB, R. I., SCHOUTEN, S., FUERST, J. A., DAMSTÉ, J. S., JETTEN, M. S., STROUS, M. *Candidatus* "Anammoxoglobus propionicus" a new propionate oxidizing species of anaerobic ammonium oxidizing bacteria. *Systematic and Applied Microbiology*, v. 30, p. 39-49, 2007.

KARTAL, B., VAN NIFTRIK, L., RATTRAY, J., VAN DE VOSSENBERG, J. L., SCHMID, M. C., SINNINGHE, D. J., JETTEN, M. S., STROUS, M. *Candidatus* 'Brocadia fulgida': an autofluorescent anaerobic ammonium oxidizing bacterium. *FEMS Microbiology Ecology*, v. 63, p. 46-55, 2008.

KAWAHARASAKI, M., KANAGAWA, T., TANAKA, H., NAKAMURA, K. Development and application of 16S rRNA-targeted oligonucleotide probe for detection of the phosphate-accumulating bacterium *Microlunatus phosphovorus* in an enhanced biological phosphorus removal process. *Water Science and Technology*, v. 37, p. 481-484, 1998.

KAWASAKI, E. S. *Amplification of RNA*. In: INNIS, M.A.; GELFAND, D.H.; SNINSKY, J.J.; WHITE, T.J. (Ed.) PCR protocols: a guide to methods and applications. San Diego: Academic Press, p. 21-27, 1990.

KENNEDY, A. C. Bacterial diversity in agroecosystems. *Agriculture, Ecosystems and Environment*, v. 74, p. 65-76, 1999.

KIM, D. J., KIM, T. K., CHOI, E. J., PARK, W. C., KIM, T. H., AHN, D. H., YUAN, Z., BLACKALL, L., KELLER, J. Fluorescence *in situ* hybridization analysis of nitrifiers in piggery wastewater treatment reactors. *Water Science and Technology*, v. 49, n. 5-6, p. 333-340, 2004.

KIRCHER, M., KELSO, J. High-throughput DNA sequencing—concepts and limitations. *Bioessays*, v. 32, n. 6, p. 524-536, 2010.

KIRCHMAN, D. L., YU, L., FUCHS, B. M., AMANN, R. Structure of bacterial communities in aquatic system as revealed by filter PCR. *Aquatic Microbial Ecology*, v. 26, p. 13-22, 2001.

KNIETSCH, A.; WASCHKOWITZ, T.; BOWIEN, S; HENNE, A.; DANIEL, R. Construction and screening of metagenomic libraries derived from enrichment cultures: generation of a gene bank for genes conferring alcohol oxidoreductase activity on *Escherichia coli*. *Applied and Environmental Microbiology*, v. 69, p. 1408-1416, 2003.

KONG, Y., NIELSEN, J. L., NIELSEN, P. H. Identity and ecophysiology of uncultured actinobacterial polyphosphate-accumulating organisms in full-scale enhanced biological phosphorous removal plants. *Applied and Environmental Microbiology*, v. 71, p. 4076-4085, 2005.

KONG, Y., XIA, Y., NIELSEN, J. L.; NIELSEN, P. H. Structure and function of the microbial community in a full-scale enhanced biological phosphorous removal plant. *Microbiology*, v. 153, p. 4061 - 4073, 2007.

KONG, Y. H., BEER, M., REES, G. N., SEVIOUR, R. J. Functional analysis of microbial communities in aerobic-anaerobic sequencing batch reactors fed with different phosphorous/carbon (P/C) ratios. *Microbiology-Sgm*, v. 148, p. 2299-2307, 2002.

Könneke, M., Bernhard, A.E., DE LA TORRE, J. R., WALKER, C. B., WATERBURY, J. B., STAHL, D.A. Isolation of an autotrophic ammonia-oxidizing marine archaeon. Nature, v. 437, p. 543-546, 2005.

KOZDRÓJ, J., VAN ELSAS, J. D. Response of the bacterial community to root exudates in soil polluted with heavy metals assessed by molecular and cultural approaches. *Soil Biology & Biochemistry*, v. 32, p. 1405-1417, 2000.

KUYPERS, M. M., SLIEKERS, A. O., LAVIK, G, SCHMID, M., JORGESEN, B. B., KUENEN, J. G. Anaerobic ammonium oxidation by anammox bacteria in the Black Sea. *Nature*, v. 422, p. 608-611, 2003.

LAJOIE, C. A., LAYTON, A. C., GREGORY, I. R., SAYLER, G. S., TAYLOR, D. E., MEYERS, A. J. Zoogleal clusters and sludge dewatering potential in an industrial activated-sludge wastewater treatment plant. *Water Environment Research*, v. 72, p. 56-64, 2000.

LAYTON, A. C., KARANTH, P. N., LAJOIE, C. A., MEYERS, A. J., GREGORY, I. R., STAPLETON, R. D., TAYLOR, D. E., SAYLER, G. S. Quantification of Hyphomicrobium populations in activated sludge from an industrial wastewater treatment system as determined by 16S rRNA analysis. *Applied and Environmental Microbiology*, v. 66, p. 1167-1174, 2000.

LEBLOND-BOURGET, N., PHILIPPE, H., MANGIN, I., DECARIS, B. 16S rRNA and 16S to 23S internal transcribed spacer sequence analyses reveal inter- and intraspecific *Bifidobacterium* phylogeny. *International Journal of Systematic Bacteriology*, v. 46, p. 102-111, 1996.

LEE, D.-H., ZO, Y.-G.., KIM, S.-J. Nonradioactive method to study genetic profiles of natural bacterial communities by PCR-single-strand-conformation polymorphism. *Applied and Environmental Microbiology*, v. 62, p. 3112-3120, 1996.

LEHNINGER, A.L. *Princípios de bioquímica*. São Paulo: Sarvier, 1985.

LI, J, JIN, Z., YU, B. Changes in the structure and diversity of bacterial communities during the process of adaptation to organic wastewater. Canadian Journal of Microbiology, v. 56, n.4, p. 352-355, 2010.

LIPSKI, A., FRIEDRICH, U., ALTENDORF, K. Application of rRNA-targeted Oligonucleotide Probes in Biotechnology. Applied Microbiology and Biotechnology, v. 56, n. 1-2, p. 40-57, 2001.

LIU, W. T., MARSH, T. L., CHENG, H., FORNEY, L. J. Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. *Applied and Environmental Microbiology.*, v. 63, p. 4516-4522, 1997.

LIU, W. T., NIELSEN, A. T., WU, J. H., TSAI, C. S., MATSUO, Y., MOLIN, S. In situ identification of polyhydroxyalkanoate-accumulating traits for microbial populations in a biological phosphorous removal process. *Environmental Microbiology*, v. 3, p. 110-122, 2001.

LIMPIYAKORN, T., SHINOHARA, Y., KURISU, F., YAGI, O. Communities of ammonia-oxidizing bacteria in activated sludge of various sewage treatment plants in Tokyo. *FEMS Microbiology,* v. 54, p. 205-217, 2005.

LIU, X.-C., ZHANG, Y., YANG, M., WANG, Z.-Y., LV Q.-Z. Analysis of bacterial structures in two sewage treatment plants with different sludge properties and treatment performance by nested PCR-DGGE method. *Journal of Environmental Sciences*, v. 19, p. 60-66, 2007.

LOCKHART, D.J.; DONG, H.; BYRNE, M.C.; FOLLETTIE, M.T.; GALLO, M.V.; CHEE, M.S., 1996. Expression monitoring by hybridization to high-density oligonucleotide arrays. *Nature Biotechnology*, v. 14, p. 1675-1680.

LOGAN, J., EDWARDS, K., SAUNDERS, N. Real-time PCR: current technology and applications. Caister Academic Press, 2009.

LORENZ, T. C., 2012. Polymerase chain reaction: basic protocol plus troubleshooting and optimization strategies. Journal of Visualized Experiments, v. 63, e 3998, p. 1-15.

MACRAE, A. The use of 16S RDNA methods in soil microbial ecology, *Brazilian Journal of microbiology*, v. 31, p. 77-82, 2000.

MAIXNER, F., NOGUERA, D. R., ANNESER, B., STOECKER, K., WEGL, G., WAGNER, M., DAIMS, H. Nitrite concentration influences the population structure of *Nitrospira*-like bacteria. *Environmental Microbiology*, v. 8, p. 1487-1495, 2006.

MARSHALL, K. C. *Adsorption of microorganisms to soils and sediments*. In: BRITTON, G., MARSHALL, K. C., ed. Adsorption of microorganisms to surfaces. New York, N.Y. Wiley. 317-329, 1980.

MARTINS, A. M. P., PAGILLA, K., HEIJNEN, J. J., VAN LOOSDRECHT, M. C. M. Filamentous Bulking Sludge. A Critical Review. *Water Research*, v. 38. p. 793-817, 2004.

MELO, I.S.; SCHNEIDER, R.P.; INGLIS, P.W.; VALADARES-INGLIS, M.C. Biomarcas e Biossensores microbianos. In: Melo, I.S.; VALADARES-INGLIS, M.C.NASS, L.L.; VALOIS, A.C.C. (Ed.). *Recursos Genéticos e Melhoramento – Microrganismos*, Jaguariúna: Embrapa Meio Ambiente, p. 164-169, 2002.

MERTOGLU, B., SEMERCI, N., GULER, N., CALLI, B., CECEN, F., SAATCI, A. Monitoring of population shifts in an enriched nitrifying system under gradually increased cadmium loading. Journal of Hazardous Materials, v. 160, p. 495–501, 2008.

MITCHELL, T. G., FREEDMAN, E. Z., MEYER, W., WHITE, T. J., TAYLOR, J. W. PCR detection of *Cryptococcus neoformans*. In: PERSING, D. H., SMITH, T. F., TENOVER, F. C., WHITE, T. J. (Ed.) Diagnostic molecular microbiology: principles and applications. Washington: American Society for Microbiology, p. 431-436, 1993.

MOBARRY, B. K., WAGNER, M., URBAIN, V., RITTMANNM B. E., STAHL, D. A. Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria. *Applied Environmental and microbiology*, v. 62, p. 2156-2162, 1996.

MONTRÀS, A., PYCKE, B., BOON, N., GODIA, F., MERGEAY, M., HENDRICKX, L. Distribution of *Nitrosomonas europaea* and *Nitrobacter winogradskyi* in an autotrophic nitrifying biofilm reactor as depicted by molecular analyses and mathematical modeling. *Water Research*, v. 42, pp. 1700-1714, 2008.

MOROZOVA, O., MARRA, M.A. Applications of next-generation sequencing technologies in functional genomics. *Genomics*, v. 92, p. 255-264, 2008.

MOTER, A., GOBEL, U.B. Fluorescence in situ hybridization (FISH) for direct visualization of microorganisms. *Journal of Microbiological Methods*, v. 41, p. 85-112, 2000.

MOURA, A. TACAO, M., HENRIQUES, I., DIAS, J., FERREIRA, P., CORREIA, A. Characterization of bacterial diversity in two aerated lagoons of a wastewater treatment plant using PCR-DGGE analysis. *Microbiological Research*, v. 164, p. 560-569, 2007.

MULLIS, K. B., FALOONA, F. A. Specific synthesis of DNA *in vitro* via a polymerase-catalyzed chain reaction. *Methods in Enzymology*, v. 55, p. 335-350, 1987.

MUNZ, G., GUALTIERO, M., SALVADORI, L., CLAUDIA, B., CLAUDIO, L. Process efficiency and microbial monitoring in MBR (membrane bioreactor) and CASP (conventional activated sludge process) treatment of tannery wastewater. *Bioresource Technology*, v. 99, p. 8559-8564, 2008.

MUYZER, G. DGGE/TGGE a method for identifying genes from natural ecosystems, *Current Opinion in Microbiology*, v. 2, p. 317-322, 1999.

MUYZER, G., DE WAAL, E. C., UITTERLINDEN, A. G. Profiling of complex microbial by denaturing gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology*, v. 59, p. 695-700, 1993.

MUYZER, G., HOTTENTRAGER, S., TESKE, A., WAWER, C. In: AKKERMANS, A.D.L; VAN ELSAS, J.D; BRUJIN, F.J. Denaturing gradient gel electrophoresis of PCR-amplified 16S rDNA - A new molecular approach to analyze the genetic diversity of mixed microbial communities (2nd ed.), *Molecular Microbial Ecology Manual*, Kluwer, Dordrecht, The Netherlands, p. 3.4.4.1-3.4.4.22, 1996.

MUYZER, G., SMALLA, K. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology, Mini review. *Antonie van Leeuwenhoek*, Dordrecht, v. 73, p. 127-141, 1998.

MUYZER, G., BRINKHOFF, T., NUBEL, U., SANTEGOEDS, C., SCHAFER, H., WAWER, C. Denaturing gradient gel electrophoresis (DGGE) in microbial ecology. In AKKERMANS, A.D.L., VAN ELSAS, J.D., DE BRUIJN, F.J. Molecular Microbial Ecology Manual (eds.), p. 1–23. The Netherlands: Kluwer Academic, 1998.

MYERS, R. M., FISHER, S. G., LERMAN, L. S., MANIATIS, T. Nearly all single base substitutions in DNA fragments joined to a GC-clamp can be detected by denaturing gel electrophoresis. *Nucleic Acids Research*, v.13, p. 3131-3145, 1985.

NASIDZE, I., LI, J., QUINQUE, D., TANG, K., STONEKING, M., Global diversity in the human salivary microbiome. *Genome Research*, v. 19, n. 4, p. 636-643, 2009.

NEEF, A., AMANN, R., SCHLESNER, H., SCHLEIFER, K. H. Monitoring a widespread bacterial group: in situ detection of planctomycetes with 16S rRNA-targeted probes. *Microbiology*, v. 144, p. 3257-3266, 1998.

NEEF, A., ZAGLAUER, A., MEIER, H., AMANN, R., LEMMER, H., SCHLEIFER, K. H. Population analysis in a denitrifying sand filter: conventional and in situ identification of *Paracoccus* spp. in methanol-fed biofilms. *Applied and Environmental Microbiology*, v. 62, p. 4329-4339, 1996.

NG, K.K., SHI, X., ONG, S.L., NG, H.Y. Pyrosequencing reveals microbial community profile in anaerobic bio-entrapped membrane reactor for pharmaceutical wastewater treatment. *Bioresource Technology*, v. 200, p. 1076-1079, 2015.

NIELSEN, A. T., LIU, W. T., FILIPE, C., GRADY, L. Jr., MOLIN, S., STAHL, D. A. Identification of a novel group of bacteria in sludge from a deteriorated biological phosphorus removal reactor. *Applied and Environmental Microbiology*, . 65, p. 1251-1258, 1999.

NIELSEN, J. L. Protocol for Fluorescence in situ hybridization (FISH) with rRNA-targeted oligonucleotides. In: NIELSEN, P. H., DAIMS, H., LEMMER, H. FISH Handbook for Biological Wastewater treatment: Identification and Quantification of Microorganisms in Activated Sludge and Biofilms by FISH. London: IWA Publishing, 2009.

NIELSEN, J. L., HANSEN, A. A. Identification of denitrifying microorganisms in activated sludge by FISH. In: NIELSEN, P. H., DAIMS, H., LEMMER, H. FISH Handbook for Biological Wastewater treatment: Identification and Quantification of Microorganisms in Activated Sludge and Biofilms by FISH. London: IWA Publishing, 2009.

NIELSEN, P. H., DAIMS, H., LEMMER, H. Introduction. In: NIELSEN, P. H., DAIMS, H., LEMMER, H. FISH Handbook for Biological Wastewater treatment: Identification and Quantification of Microorganisms in Activated Sludge and Biofilms by FISH. London: IWA Publishing, 2009a.

NIELSEN, P. H., NGUYEN, H. T. T., McILROW, S. J., MIELCZAREK, A. T., SEVIOUR, R. Identification of polyphosphate-accumulating and glycogen-accumulating organisms by FISH. In: NIELSEN, P. H., DAIMS, H., LEMMER, H. FISH Handbook for Biological Wastewater treatment: Identification and Quantification of Microorganisms in Activated Sludge and Biofilms by FISH. London: IWA Publishing, 2009b.

NIEMI, R., HEISKANEN, I., WALLENIUS, K., LINDSTRÖM, K., Extraction and purification of DNA in rhizosphere soil samples for PCR-DGGE analysis of bacterial consortia. *Journal of Microbiology Methods*, v. 43, p. 155-165, 2001.

NÜBEL, U., GARCIA-PICHEL, F., KÜHL, M., MUYZER, G. Quantifying microbial diversity: morphotypes, 16S rRNA genes, and carotenoids of oxygenic phototrophs in microbial mats. *Applied and Environmental Microbiology*, n. 65, p. 422-430, 1999.

OELMÜLLER, U., FRÜGER, N., STEINBÜCHEL, A. FRIEDRICH, G. C. Isolation of prokaryotic RNA and detection of specific mRNA with biotinylated probes. *Journal of Microbiology Methods*, v. 11, p. 73-84, 1990.

OGRAM, A. Soil molecular microbial ecology at age 20: methodological challenges for the future. *Soil Biology and Biochemistry*, v. 32, p. 1399-1504, 2000.

OGRAM, A., SAYLER, G. S., BARKAY, T. J. The extraction and purification of microbial DNA from sediments. *Journal of Microbiological Methods*, v. 7, p. 57-67, 1987.

OKABE S, KAMAGATA Y. Wastewater treatment. In: Liu WT, Jansson JK, editors. Environmental Molecular Microbiology. Norfolk, UK: Caister Academic Press; 2010. pp. 191–210.

OKABE, S., SATOH, H., WATANABE, Y. In situ analysis of Nitrifying biofilms as determined by in situ hybridisation and the use of microelectrodes. *Applied and Environmental Microbiology*, v. 65, n. 7, p. 3182-3191, 1999.

OLSEN, G. J., WOESE, C. R., OVERBEEK, R. The winds of (evolutionary) change: breathing new life into microbiology. *Journal of Bacteriology*, v. 176, p. 1-6, 1994

ORITA, M., IWAHANA, H., KANAZAWAT, H., HAYASHI, K., SEKIYA, TAKAO. Detection of Polymorphisms of Human DNA by Gel Electrophoresis as SSCPs. *Proceedings of the National Academy of Sciences of the United States of America*, v. 86, p. 2766-2770, 1989.

OSTE, C. Product application focus: polymerase chain reaction. *BioTechniques*, v. 6, n. 2, p. 162-167, 1988.

OTAWA, K., ASANO, R., OHBA, Y., SASAKI, T., KAWAMURA, E., KOYAMA, F., NAKAMURA, S., NAKAI, Y. Molecular analysis of ammonia-oxidizing bacteria community in intermittent aeration sequencing batch reactors used for animal wastewater treatment. *Environmental Microbiology*, v. 8, n. 11, p. 1985-1996, 2006.

OUVERNEY, C. C., FUHRMAN, J. A. Increase in fluorescence intensity of 16S rRNA in situ hybridization in natural samples treated with chloramphenicol. *Applied and Environmental Microbiology*, v. 63, p. 2735-2740, 1997.

ØVREÅS, L., FORNEY, L., DAAE, F. L., TORSVIK, V. Distribution of Bacterioplankton in Meromictic Lake Sælenvannet, as Determined by Denaturing Gradient Gel Electrophoresis of PCR-Amplified Gene Fragments Coding for 16S rRNA. *Applied and Environmental Microbiology*, v. 63, p. 3367-3373, 1997.

PACE, N. R. A molecular view of microbial diversity and the biosphere. *Science*, v. 276, p. 734-740, 1997.

PACE, N. R. New perspective on the natural microbial world: molecular microbial ecology. *ASM News*, v. 62, p. 463-470, 1996.

PARK, H. D., REGAN, J. M., NOGUERA, D. R. Molecular analysis of ammonia-oxidizing bacterial populations in aerated-anoxic Orbal processes. *Water Science Technology*, v. 46, n. 1-2, p. 273-280, 2002.

PEIXOTO, R. S., ROSADO, A. S., TAKETANI, R. G. *Bioprospecção da diversidade microbiana cultivável e não cultivável*. In: Mello, I.S.; AZEVEDO, J.L. (Org.), Microbiologia Ambiental, 2. ed.. Jaguariúna: Embrapa Meio Ambiente, v. 1, p. 83-106, 2008.

PERSSON, F., WIK, T., SORENSSON, F., HERMANSSON, M. Distribution and activity of ammonia oxidizing bacteria in a large full-scale trickling filter. *Water Research*, v. 36, p. 1439-1448, 2002.

PICKUP, R. W. Development of molecular methods for the detection of specific bacteria in the environment. *Journal of General Microbiology*, v. 137, p. 1009-1019, 1991.

PROSSER, J. I. Autotrophic nitrification in bacteria. *Advances in Microbial Physiology*, v. 30, p. 125-181, 1989.

RABUS, R., WILKES, H., SCHRAMM, A., HARMS, G., BEHRENDS, A., AMANN, R., WIDDEL, F. Anaerobic utilization of alkylbenzenes and n-alkanes from crude oil in an enrichment culture of denitrifying bacteria affiliating with the beta-subclass of Proteobacteria. *Environmental Microbiology*, v. 1, p. 145-157, 1999.

RADAJEWSKI, S., MURRELL, J. C. Stable isotope probing for detection of methanotrophs after enrichment with $^{13}CH_4$, *Methods in Molecular Biology*, v. 179, p. 149-157, 2002.

REIS JUNIOR, F. B., MENDES, I.C., TEIXEIRA, K. R. S.; REIS, V. M. Uso de Ferramentas Moleculares em Estudos da Diversidade de Microrganismos do Solo. Planaltina, DF: Embrapa Cerrados, 33p, 2002.

REYSENBACH, A., GIVER, L., WICKMAN, G. & PACE, N. Differential amplification of rRNA genes by polymerase chain reaction. *Applied and Environmental Microbiology*, v. 58, p. 3417-3418, 1992.

RODÍCIO, M. R., MENDOZA, M. C. Identificación bacteriana mediante secuencación del ARNr 16S: fundamento, medología y aplicaciones en microbiología clínica. *Enferm. Infecc. Microbiol. Clin.*, v. 22, n. 4, p. 238-245, 2004.

RODRÍGUEZ-EZPELETA, N., HACKENBERG, M., ARANSAY, A.M. (Eds.) Bioinformatics for High Throughput Sequencing. Springer, 2012.

ROGERS, Y. H., VENTER, J. C. Genomics: massively parallel sequencing. *Nature*, v. 437, p. 326-327, 2005.

ROLLER, C., WAGNER, M., AMANN, R., LUDWIG, W., SCHLEIFER, K. H. In situ probing of Gram-positive bacteria with high DNA G+C content using 23S rRNA-targeted oligonucleotides. *Microbiology*, v. 140, p. 2849-2558, 1994.

ROONEY-VARGA, J. N., GENTHNER, B. R. S, DEVEREUX, R., WILLIS, S. G., FRIEDMAN, S. D., HINES, M. E.. Phylogenetic and physiological diversity of sulphate-reducing bacteria isolated from a salt marsh sediment. *Systematic and Applied Microbiology*, v. 21, p. 557-568, 1998.

ROSADO, A. S., DUARTE, G. F. *Utilização da eletroforese em gel com gradiente de desnaturantes (DGGE) e gel com gradientes de temperatura (TGGE) para estudar diversidade microbiana*. In: MELO, I.S.; VALADARES-INGLIS, M.C.; NASS, L.L.; VALOIS, A.C.C. Recursos genéticos e melhoramento – microrganismos. Jaguariúna: Embrapa Meio Ambiente, p. 97-128, 2002.

ROSADO, A. S., DUARTE, G. F.; SELDIN, L., VAN ELSAS, J. D. Molecular Microbial Ecology: a minireview. *Brazilian Journal of Microbiology*. v. 28, p. 135-147, 1997.

ROSADO, A. S., SELDIN, L., WOLTERS, A. C., VAN ELSAS, J.D. Quantitative 16S rDNA-targeted polymerase chain reaction and oligonucleotide hybridization for detection of *Paenibacillus azotofixans* in soil and the wheat rhizosphere. *FEMS Microbial Ecology*, v. 19, p. 153-164, 1996.

ROSSELLÓ-MORA, R. A., WAGNER, M., AMANN, R., SCHLEIFER, K. H. The abundance of *Zoogloea ramigera* in sewage treatment plants. *Applied and Environmental Microbiology*, v. 61, p. 702-707, 1995.

ROWAN, A. K., SNAPE, J. R., FEARNSIDE, D., BARER, M. R., CURTIS, T. P., HEAD, I. M. Composition and diversity of ammonia-oxidizing bacterial communities in wastewater treatment reactors of different design treating identical wastewater. *FEMS Microbiology Ecology*, v. 43, p. 195-206, 2003.

SAIKI, R. K., GELFAND, D. H., STOFFEL, S., SCHARF, S. J., HIGUCHI, R., HORN, G. T., MULLIS, K. B., ERLICH, H. A. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science*, v. 239, n. 4839, p. 487-491, 1988.

Salomonová, S., LAMAČOVÁ, J., RULÍK, M., ROLČÍK, J., ČÁP, L., BEDNÁŘ, P., BARTÁK, P. Determination of phospholipid fatty acids in sediments. *Acta Universitatis Palackianae Olomucensis. Facultas Rerum Naturalium*, v. 42, p. 39-49, 2003.

SANZ, J. L., KÖCHLING, T. Molecular biology techniques used in wastewater treatment : An overview. *Process biochemistry*, v. 42, n. 2, p. 119-133, 2007.

SCHLOSS, P. D., HANDELSMAN, J. Biotechnological prospects from metagenomics. *Current Opinion in Biotechnology*, v. 14, p. 303-310, 2003.

SCHMID, M., SCHMITZ-ESSER, S., JETTEN, M., WAGNER, M. 16S-23S rDNA intergenic spacer and 23S rDNA of anaerobic ammonium-oxidizing bacteria: implications for phylogeny and in situ detection. *Environmental Biotechnology*, v. 3, p. 450-459, 2001.

SCHMID, M., TWACHTMANN, U., KLEIN, M., STROUS, M., JURETSCHKO, S., JETTEN, M., METZGER, J. W., SCHLEIFER, K. H., WAGNER, M. Molecular evidence for genus level diversity of bacteria capable of catalyzing anaerobic ammonium oxidation. *Systematic and Applied Microbiology*, v. 23, p. 93-106, 2000.

SCHMID, M., WALSH, K., WEBB, R., RIJPSTRA, W. I., PAS-SCHOONEN, K, VERBRUGGEN, M. J., HILL, T., MOFFETT, B., FUERST, J., SCHOUTEN, S., DAMSTEM, J. S., HARRIS, J., SHAW, P., JETTEN, M., STROUS, M. *Candidatus* "Scalindua brodae" sp. nov., *Candidatus* "Scalindua wagneri", sp. nov., two new species of anaerobic ammonium-oxidizing bacteria. *Systematic and Applied Microbiology*. v. 26, p. 529-538, 2003.

SCHRAMM, A., DE BEER, D., WAGNER, M., AMANN, R. I. Identification and activity in situ of *Nitrospira* and *Nitrospira spp*. as dominant populations in a nitrifying fluidized bed reactor, *Applied and Environmental Microbiology.*, v. 64, n. 9, p. 3480-3485, 1998.

SCHUTTE, U. M., ABDO, Z., BENT, S. J. Advances in the use of terminal restriction fragment length polymorphism T-RFLP analysis of 16S rRNA genes to characterize microbial communities. *Applied Microbiology Biotechnology*, v. 80, p. 365-380, 2008.

SEVIOUR, R. J., SODDELL, J. A., LIU, J.-R., McKENZIE, C. M., SAINT, C. The filamentous bacteria causing bulking and foaming in activated sludge plants. *Australian Biotechnology*, v. 10, n. 3, p. 24-29, 2000.

SHEFFIELD, V. C., COX, D. R., MYERS, R. M. Attachment of a 40bp G+C rich sequence (GC-Clamp) to genomic DNA fragments by polymerase chain reaction results in improved detection of single-base changes. *Proceedings of the National Academy of Sciences USA*, v. 86, p. 232-236, 1989.

SHENDURE, J., HANLEE, J. Next-generation DNA sequencing. *Nature Biotechnology*, v. 26, p. 1135–1145, 2008.

SIGLER, W. V., MINIACI, C., ZEYER, J. Electrophoresis time impacts the denaturing gel electrophoresis-based assessment of bacterial community structure. *Journal of Microbialogical Methods*, v. 57, p. 17-22, 2004.

SMALLA K., CRESWELL, L. C., MENDOÇA-HAGLER, L.C.; WOLTERS, A., VAN ELSAS, J.D. Rapid DNA extraction protocol from soil for polymerase chain reaction-mediated amplification. *Journal of Applied Bacteriology*, v. 74, p. 78-85, 1993.

SNAIDR, J., AMANN, R., HUBER, I., LUDWIG, W., SCHLEIFER, K.-H. Phylogenetic analysis and in situ identification of bacteria in activated sludge. *Appl Environmental Microbiology*, v. 63, p. 2884-2896, 1997.

SPECTOR, D. L., GOLDMAN, R. D., LEINWAND, L.A. *Cells: A Laboratory Manual.* Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 1998.

STACKEBRANDT, E., LIESACK, W., GOEBEL, B. M. Bacterial diversity in a soil sample from a subtropical Australian environment as determined by 16S rDNA analysis. *FASEB Journal*, v. 7, p. 232-236, 1993.

STAHL, D. A. Molecular approaches for the measurement of density, diversity and phylogeny. In: McINERNEY, M. J., WALTER, M. V., STETZENBACK, L. D. (Eds.) *Manual of environmental microbiology,* p. 102-114. ASM Press, Washington, D.C, 1997.

STEPHEN, J. R., MCCAIG, A. E., SMITH, Z., PROSSER, J. I.; EMBLEY, T. M. Molecular diversity of soil and marine 16S rRNA gene sequences related to β-subgroup ammonia-oxidizing bacteria. *Applied and Environmental Microbiology*, v. 62, n. 11, p. 4147-4154, 1996.

STRACHAN, T., READ, A. P. Genética Molecular Humana. Porto Alegre: Artmed, p. 119-122, 2002.

SUGIMOTO, N., NAKANO, S., YONEYAMA, M., HONDA, K. Improved thermodynamic parameters and helix initiation factor to predict stability of DNA duplexes. *Nucleic Acids Res.*, v. 24, p. 4501-4505, 1996.

SUSHCHIK, N. N., GLADYSHEV, M. I., KALACHOVA, G. S., KRAVCHUK, E. S., DUBOVSKAYA, O. P., IVANOVA, E. A. Particulate Fatty Acids in Two Small Siberian Reservoirs Dominated by Different Groups of Phytoplankton. *Freshwater Biology*, v. 48, n. 3, 394-403, 2003.

STRYER, L. *Explorando os genes*. In: Bioquímica (4ª ed.), New York: FREEMAN, W.H. & Company, 1996.

TACÃO, M., MOURA, A., ALVES, A., HENRIQUES, I., SAAVEDRA, M.J., CORREIA, A. Evaluation of 16S-rDNA and gyrB-DGGE for typing members of the genus *Aeromonas*. *FEMS Microbiology Letters*, v. 246, n.1, p. 11-18, 2005.

TAL, Y., WATTS, J. E. M., SCHREIER, S. B., SOWERS, K. R., SCHREIER, H. J. Characterization of the microbial community and nitrogen transformation processes associated with moving bed bioreactors in a closed recirculated mariculture system. *Aquaculture*, v. 215, p. 187-202, 2003.

TAN, B., NG, C., NSHIMYIMANA, J.P., LOH, L.L., GIN, K.Y.-H, THOMPSON, J.R., Next-generation sequencing (NGS) for assessment of microbial water quality: current progress, challenges, and future opportunities. Frontiers in Microbiology, v. 6., Article 1027, p. 1-20, 2015.

THOMSEN, T. R., NIELSEN, J. L., RAMSING, N. B., NIELSEN, P. H. Micromanipulation and further identification of FISH-labelled microcolonies of a dominant denitrifying bacterium in activated sludge. *Environmental Microbiology*, v. 6, p. 470-479, 2004.

TORSVIK, V., GOKSOYR, J., DAAE, F.L. High diversity in DNA of soil bacteria. *Applied And Environmental Microbiology*, v. 56, p. 782-787, 1990.

TREUSCH, A.H., LEININGER, S., KLETZIN, A., SCHUSTER, S.C., KLENK, H.P., SCHLEPER, C. Novel genes for nitrite reductase and Amo-related proteins indicate a role of uncultivated mesophilic Crenarchaeota in nitrogen cycling. *Environmental Microbiology*, v. 7, n. 12, p. 1985-1995, 2005.

TREVORS, J. T., VAN ELSAS, J. D. Nucleic Acids in the Environment: Methods and Applications. Heidelberg, Springer-Verlag, 1995.

TSUNEDA, S., MIKAMI, M., KIMOCHI, Y., HIRATA, A. Effect of salinity on nitrous oxide emission in the biological nitrogen removal process for industrial wastewater, *Journal of Hazardous Materials*, v. 119, p. 93-98, 2005.

VAN DER ZEE, H., HUIS, J. H. J. Rapid and alternative screening methods for microbiological analysis. *Journal of AOAC International*, v. 80, p. 934-940, 1997.

VAN ELSAS, J. D., DUARTE, G. F., ROSADO, A. S., SMALLA, K.. Microbial and molecular biological methods for monitoring microbial inoculants and their effects in the soil environment. *Journal of Microbiological Methods*, v. 32, p. 133-154, 1998.

VAN ELSAS, J.D., WELLINGTON, E. M. H., TREVORS, J. T. In: Modern Soil Microbiology. New York, Marcel Dekker, 1997.

VITZTHUM, F., GEIGER, G., BISSWANGER, H., BRUNNER, H., BERNHAGEN, J. A quantitative fluorescence-based microplate assay for the determination of double-stranded DNA using SYBR Green I and a standard ultraviolet transilluminator gel imaging system. *Analytical Biochemistry*, v. 276, p. 59-64, 1999.

VOELKERDING, K.V., DAMES, S.A., DURTSCHI, J.D. Next-generation sequencing: from basic research to diagnostics. *Clinical Chemistry*, v. 55, n. 4, p. 641-658, 2009.

WAGNER, M, LOY, A. Bacterial community composition and function in sewage treatment systems. Current Opinion Biotechnology, v. 13, p. 218-227, 2002.

WAGNER, M., AMANN, R., LEMMER, H., SCHLEIFER, K.-H. Probing activated sludge with oligonucleotides specific for proteobacteria: inadequacy of culture-dependent methods for describing microbial community structure. *Applied and Environmental Microbiology*, v. 59, p. 1520-1525, 1993.

WAGNER, M., AβMUS, B., HARTMANN, A., HUTZLER, P., AMANN, R. In situ analysis of microbial consortia in activated sludge using fluorescently labelled, rRNA-targeted oligonucleotide probes and confocal laser scanning microscopy. *Journal of Microscopy*, v. 176, p. 181-187, 1994.

WAGNER, M.; RATH, G.; AMANN, R.; KOOPS, H. P.; SCHLEIFER, K. H. In situ identification of ammonia-oxidizing bacteria. *Systematic and Applied Microbiology*, v. 18, p. 251-264, 1995.

WAGNER, M., RATH, G., KOOPS, H. P., FLOOD, J., AMANN, R. In situ analysis of nitrifying bacteria in sewage treatment plants. *Water Science and Technology*, v. 34, p. 237-244, 1996.

WENTZEL, M. C., LOEWENTHAL, R. E., EKAMA, G. A., MARAIS, G. R. Enhanced polyphosphate organism cultures in activated sludge systems. Part 1. Enhanced culture development. Water S. A. (Pretoria), v. 14, p. 81-92, 1988.

WETZEL, J., KINGS, C., POP, M. Assessing the benefits of using mate-pairs to resolve repeats in de novo short-read prokaryotic assemblies. BMC Bioinformatics, v. 12, n. 95, p. 1-14, 2011.

WOESE, C.R. Bacterial evolution. *Microbiology Review*, v. 51, p. 221-271, 1987.

WOESE, C. R., KANDLER, O.; WHEELIS, M. L. Towards a natural system for organisms: proposal for the domains Archaea, Bacteria and Eukarya. *Proceedings of National Academy of Sciences USA*, v. 87, p. 4576-4579, 1990.

WONG, M. T., TAN, F. M., NG, W. J., LIU, W. T. Identification and occurrence of tetrad-forming *Alphaproteobacteria* in anaerobic-aerobic activated sludge processes. *Microbiology-Sgm*, v. 150, p. 3741-3748, 2004.

YERGEAU, E., FILION, M., VUJANOVIC, V., ST-ARNAUD, M. A PCR-denaturing gradient gel electrophoresis approach to assess *Fusarium* diversity in asparagus. *Journal of Microbiological Methods*, v. 60, p. 143-154, 2005.

YERSHOV, G., BARSKY, V., BELGOVSKIY, A., KIRILLOV, E., KREINDLIN, E., IVANOV, I., PARINOV, S., GUSHIN, D., DROBISHEV, A., DUBILEY, S., MIRZABEKOV, A. *Proceedings of the National Academy of Sciences,* v. 93, p. 4913-4918, 1996.

YOO, H., AHN, K.H., LEE, H.J., LEE, K.H., KWAK, Y.J., SONG, K.G. Nitrogen Removal from synthetic wastewater by simultaneous nitrification and denitrification (SND) via Nitrite in an Intermittently-Aerated reactor. *Water Research*, v. 33, n. 1., p. 145–154, 1999.

YU, C.P., AHUJA, R., SAYLER, G, CHU, K.H. Quantitative molecular assay for fingerprinting microbial communities of wastewater and estrogen-degrading consortia. *Applied and Environmental Microbiology*, v. 71, p. 1433-1444, 2005.

ZAK, J. C., WILLIG, M. R., MOORHEAD, D. L., WILDMAN, H. G. Functional diversity of bacterial communities: a quantitative approach. *Soil Biology & Biochemistry*, v. 26, p. 1101-1108, 1994.

ZHANG, T., JIN, T., YAN, Q. Occurrence of ammonia-oxidizing Archaea in activated sludges of a laboratory scale reactor and two wastewater treatment plants. *Journal of Applied Microbiology*, v. 107, p. 907-977, 2009.

ZHAO, X., CHEN, Z., WANG, X., LI, J., SHEN, J., XU, H. Remediation of pharmaceuticals and personal care products using an aerobic granular sludge sequencing bioreactor and microbial community profiling using Solexa sequencing technology analysis. *Bioresource Technology*, v. 179, p. 104-112, 2015.

ZILLES, J. L., PECCIA, J., KIM, M. W., HUNG, C. H., NOGUERA, D. R. Involvement of *Rhodocyclus*-related organisms in phosphorous removal in full-scale wastewater treatment plants. *Applied and Environmental Microbiology*, v. 68, p. 2763-2769, 2002.

ZUCKERKANDL, E., PAULING, L. Molecules as documents of evolutionary history. *Journal of Theoretical Biology*, v. 8, p. 357-366, 1965.