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Review - Environmental Sciences

Molecular Techniques to Study Microbial Wastewater Communities

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HIGHLIGHTS

- Culture-independent techniques are an optimal alternative for efficient wastewater treatment.
- Knowledge of microbial diversity is fundamental for wastewater treatment.
- Vanguard techniques provide insights into functional activities in wastewater treatment.

Abstract: wastewater treatment (WT) is of major importance on modern cities, removing wastewater pollutants resultant from anthropogenic activities. The unique abilities of microbes to degrade organic matter, remove nutrients and transform toxic compounds into harmless products make them essential players in waste treatment. The microbial diversity determines the metabolic pathways that may occur in WT and quality of treated wastewater. Therefore, understanding WT microbial community structure, distribution, and metabolic functioning is essential for development and optimization of efficient microbial engineering systems. Since cultivation methods can only detect a small fraction of the microbial diversity, the use of culture-independent molecular methods has circumvented this issue, allowing unprecedented access to genes and genomes used for microbial composition and function evaluation. Traditional approaches like RAPD, DGGE, ARDRA, RISA, SSCP, T-RFLP, and FISH and modern approaches like microarray, qPCR, and metagenomics are essential techniques for identifying and depicting the total microbial community structure and their interaction with environmental and biotic factors. Thus, this review describes traditional and state of the art molecular techniques which provide insights into phylogenetic and functional activities of microbial assemblages in a WT system.

Keywords: microbial diversity; wastewater microbiology; fingerprint techniques; qPCR; high-throughput sequencing.

INTRODUCTION

The global demand for water has been continuously rising due to population growth and socioeconomic activities increasing. In the last hundred years the world population has tripled while water consumption has increased six-fold. Currently, wastewater treatment is indispensable in modern cities, removing wastewater pollutants resultant from anthropogenic activities. Composition of wastewaters depends on their origin, but in general, major contaminants include organic compounds, xenobiotics, metals, suspended soils and nutrients (mainly nitrogen and phosphorus) [1]. The unique abilities of microbes to degrade organic matter, remove nutrients and transform toxic compounds into harmless products make them essential players in waste removal. The microorganisms present in WT are bacteria, archaea, eukaryotes (fungi, algae, protozoa and metazoa), and viruses (e.g. bacteriophages). Of those, bacteria comprises the main components of WT community [2]. Operating parameters of WT influences the microbial structures and their species composition. The microbial community structure determines the metabolic pathways that may occur in WT and the quality of treated wastewater. In this way, to investigate the relationships between microorganisms responsible for pollutant removal from wastewater, various microbial techniques have been used.

Initial investigations into the composition of wastewater microbial communities were based on culture-dependent techniques. Its methods for microbial identification require the recognition of differences in morphology, growth, enzymatic activity, and metabolism to define genera and species [3]. The mentioned traditional techniques are based in isolation and characterization of microorganisms using growth media such as Luria–Broth, Nutrient Agar, and Tryptic Soy Agar [4]. However, since the majority of bacteria cannot be easily cultivated, these culture-dependent techniques select for fast-growing heterotrophs that are able to best adapt to growth conditions and therefore culturable strains do not accurately represent the composition and diversity of natural microbial communities [5]. For example, in pulp and paper wastewaters, total microscopic bacterial counts averaged 10^{10} cells/mL while culturable counts ranged between 10^{7} and 10^{8} cells/mL [6].

In the last decades studies on microbial structure in a variety of treatment systems has been conducted through the application of culture-independent techniques such as denaturing gradient gel electrophoresis (DGGE) [7], terminal restriction fragment length polymorphism (T-RFLP) [8], cloning [9], and FISH [10]. These traditional molecular methods highlighted the dominance of the phylum Proteobacteria, followed mainly by Bacteroidetes, Chloroflexi, Actinobacteria, Planctomycetes and Firmicutes in activated sludge, one of the most broadly used technology for treating sewage [11]. These observations have been confirmed when further high throughput sequencing (HTS) techniques were applied. Furthermore, HTS allowed the identification of groups that had remained undetected with traditional molecular methods, deepening our knowledge on the diversity of activated sludge [12]. Additionally, metagenomic studies pointed out to the dominance of functional categories involved in carbohydrates, protein, amino acids derivatives and aromatic compounds metabolism [13,14].

In this review, we detailed described the traditional and emerging molecular approaches for characterizing microbial community composition and structure.

Microbial community fingerprint

In the fingerprinting techniques (Table 1) a genomic region from all community members of wastewater samples are amplified by PCR and used for identification. The profiles generated by these techniques are called DNA fingerprints.

Random Amplification of Polymorphic DNA (RAPD)

The RAPD method is a technique based on the amplification of nonspecific fragments of DNA. In general, short (8–12 nucleotides) and low annealing temperature primers are used [15]. From small quantities of DNA template, a single reaction and a random amplification various length products are generated. Depending on the microbial community complexity, different band patterns are generated during gel electrophoresis. Various length products variations in the microbial communities can be evaluated mainly by differences in the number and length of the amplicons. Although the analysis of amplicons 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 large numbers of microbial communities [16]. Unlike conventional PCR, RAPD does not require any specific knowledge about targeting organisms. Due to its feasibility, it is widely used for genetic fingerprinting of microbial communities and closely related microbial species and strains [17].

Denaturing or Temperature Gradient Gel Electrophoresis (DGGE or TGGE)

Denaturing gradient gel electrophoresis (DGGE) [18] and temperature gradient gel electrophoresis (TGGE) [19] are techniques used to separate short- to medium-length DNA fragments based on their melting characteristics. Both should be performed using a GC-clamp (CGC CGG GGG CGC GCC CCG GGC GGG GCG GGG GCA) attached to the 5′ end of one of the primers in order to not allow the complete separation of DNA strands during electrophoresis. In DGGE analysis, PCR products pass through polyacrylamide gels containing a progressive gradient of urea plus formamide (chemical denaturant). The separation of PCR products is based on the lower electrophoretic mobility of a partially melted double-stranded DNA molecule. Amplicons which has different sequence composition will migrate differently and stop at various positions, resulting in the formation of different band patterns [20]. TGGE is based on the same principle of DGGE except that a temperature gradient is applied rather than a chemical denaturalization. The sequence of different amplicons determines the melting behavior, so that fragments achieve different positions of the gel. Both techniques are been used to investigate mixed microbial communities [18–19]. In addition, for taxonomic identification, bands from DDGE or TGGE gel are excised, reamplified, and sequenced.

Amplified Ribosomal DNA Restriction Analysis (ARDRA)

The ARDRA involves amplification of the conserved region of ribosomal gene using specific primers through polymerase chain reaction followed by enzymatic digestion of the amplicons [21]. The cleaved fragments are segregated on agarose or polyacrylamide gel, and the emerging profile of bands is used for grouping the microbial community. Generally, for 16S rRNA gene amplicon, tetra cutter restriction enzymes (e.g., *RsaI*, *HaeIII*) are used. Restriction enzymes that possess the same recognition sequence should not be used [22]. Although ARDRA provides little about the type of microorganisms in the sample, the method is useful for rapid monitoring of microbial diversity over time, or to compare microbial communities in response to environmental condition changes [23].

Terminal Restriction Fragment Length Polymorphism (T-RFLP)

The T-RFLP includes fluorescent labeling of PCR products followed by restriction digestion. For amplification one or both primers should have their 5’ end labeled with a fluorochrome molecule, Rox or FAM [24]. The mixture of PCR products is subjected to restriction digestion by using one or more restriction enzymes. After the restriction digestion, fragments are separated by polyacrylamide gel electrophoresis coupled to a DNA sequencer. Different sized labeled fragments produce a unique signature of each microbial community [5]. In this technique, only fluorescently labeled terminal fragments are detected, while other unlabeled fragments are not considered.

Ribosomal Intergenic Spacer Analysis (RISA)

The RISA focus on the intergenic spacer region, called ISR [25]. The most used spacer region is between large 23S and small 16S subunit of rRNA operon as there is a significant heterogeneity in terms of nucleotide sequence and length. RISA fragments can be generated by PCR with primers, which are complimentary to 23S and 16S rRNA genes [26]. The resulting amplicons are a mixture of fragments representing the most dominant community members [27]; this methodology provides the microbial community structure, with each band corresponding at least to one microorganism of the community. RISA is used to study the microbial community structure in anaerobic treatment facilities or bioreactors [26].

Single-Strand Conformation Polymorphism (SSCP)

The SSCP is a sensitive method used to study variations in nucleotide sequences of identical length and to detect polymorphism in DNA amplicons [28]. This method allows separation of different amplicons due to their different conformation using polyacrylamide gel electrophoresis and ultimately helps in distinguishing different sequences. In SSCP, the environmental DNA is first amplified using PCR and then denatured. After denaturation, single-stranded amplicon is separated on gel electrophoresis. Amplicons having a minute

difference like single base substitution may migrate differently in non-denaturing polyacrylamide gel due to different conformation. The technique works on the principle that under non-denaturing conditions, DNA can form different secondary structures based on specific sequences [29].

Table 1. Main advantages and disadvantages of fingerprint techniques that are commonly employed in the identification of wastewater microorganisms

Technique	Advantages	Disadvantages	WT studies employing the respective technique
RAPD	Cheap and does not require prior knowledge.	It has poor reproducibility, and requires strict standardization of PCR conditions. Different final results can be obtained due to variations in DNA polymerase, DNA template and primer amount and annealing temperatures.	Municipal wastewater [30 – 31]; Pharmaceutical wastewater [32]; Industrial wastewater [17, 33 – 34].
DGGE TGGE	Good sensibility and it is possible to excise band from gel for amplification and sequencing.	Dissimilar DNA sequences of different bacteria species can display the same separation as a result of the same GC contents.	Municipal wastewater [30, 35 – 37]; Industrial wastewater [17, 38 – 39].
ARDRA	It is a fast, simple and accurate molecular tool to determine the environmental population profile.	It has lower discriminatory power compared to other fingerprinting techniques such as DDGE, TGGE, T-RFLP, RISA and SSCP.	Municipal wastewater [40]; Industrial wastewater [41–42, 85].
T-RFLP	It gives the relative amounts of bacteria of a sample with good sensibility by using fluorochome.	The identification of different bacteria depends of the restriction enzymes that are used.	Municipal wastewater [43 – 46]; Industrial wastewater [47 – 48].
RISA	It has good discriminatory power and is less likely to produce inconsistent results.	Detects only differences in ISR fragment length. Different bacteria with the same ISR length will not be discriminated.	Industrial wastewater [25–27,49–50]; Pharmaceutical wastewater [51].
SSCP	It is quick, simple and cost-effective.	There is currently no theoretical model for predicting the exact conformation of a DNA fragment under different parameters such as mutation, size of DNA fragment, G and C content, porosity of gel matrix, DNA concentration, ionic strength and pH.	Municipal wastewater [52]; Industrial wastewater [53 – 54]; Gelatinaceous wastewater [55].

Nucleic acid hybridization for microorganisms detection

Hybridization techniques (Table 2) based on the interaction between labeled single-stranded nucleic acids molecules (probes) and their complementary targets allow the determination of the relative and absolute abundance of genes and their transcriptional products.

*Fluorescent in Situ Hybridization (FISH***)**

The FISH procedure enables *in situ* phylogenetic identification and counting of individual microbial cells by culture-independent probe-based genome. A large number of molecular probes targeting 16S rRNA genes have been reported at various taxonomic levels [56]. The technique involves hybridization of oligodeoxynucleotide complementary (probe - generally 18 to 30 nucleotides long) to rRNA gene sequences that have phylogenetic group-specific sequence signatures. In laboratory, microbial cells from wastewater samples are often fixed by ethanol or paraformaldehyde treatment, and their rRNA gene is hybridized with fluorescently labeled taxon-specific rRNA-targeted probes. The abundance of rRNA gene in bacterial cell, apparently drives lack of lateral gene transfers, and a good length (e.g., 16S rRNA size is 1500 bp) serve as a basis for hybridization of group-specific fluorescent probes complimentary to rRNA gene. The FISH probes

bound to rRNA can be detected by epifluorescence microscopy because contain a fluorescent dye at the 5´ end. The advantages of this technique are: (i) it is a relatively fast, if the probes are available in the market; (ii) it allows the differentiation of active microorganisms (ii) it does not require highly trained personnel; (iv) the artifacts and bias introduced due to the DNA extraction, PCR artifacts, and cloning are avoided [57]. The disadvantages of this technique are the amount of time and work required for design the probes that in some cases are not as specific when taking metabolic criteria. In addition, for quantification, image analysis is often difficult. Few experiments have been reported to investigate and enumerate the various bacterial groups at particular stages through wastewater treatment systems.

Microarray

The microarray is based on the ability of complementary sequences of nucleic acids to hybridize one another. The technique was originally devised for studies of differential gene expression in health-related issues, but their applications goes beyond for environmental studies like differential gene expression in response to environment pollutants [58]. Usually, oligonucleotide probes targeting rRNA genes or functional genes are attached to the surface of a chemically treated glass slide (spotting). Either DNA or RNA is extracted from a wastewater sample of interest and incubated with slide under conditions where complementary sequences can hybridize. Since the hybridized material has been previously labeled with a radioactive or fluorescent group, intensity of radiation/fluorescence reflects the concentration of the specific targeted sequence [59]. The technology can allow the detection of a specific strain within an entire array of microorganisms from wastewater samples or analyze whether specific genes are turned on/ off in a particular sample [60]. The sensitivity of microarrays is always a critical factor. The advantage of this system over FISH is that hundreds of probes can be spotted on the microarray surface which can allow the detection of hundreds of specific target sequence [61].

Quantitative PCR (qPCR)

The qPCR allows the quantification of a number of target genes in wastewater samples [62]. Specific targeted DNA sequences are amplified and quantified simultaneously in real time, with the progress of amplification reaction. To determine gene copy numbers of unknown wastewater samples, a calibration curve is created. For total wastewater bacterial estimation, the number of 16S rRNA genes is quantified using specific primers and the genomic DNA purified from wastewater samples [63]. The qPCR uses either intercalating fluorescent dyes such as SYBR Green or fluorescent probes (e.g. TaqMan probes, molecular beacons, scorpion probes, etc.) in order to measure the accumulation of PCR amplicons in real time as the amplification progress. The dynamics and metabolic activity of *Pseudomonas* population from pulp mill wastewater microbial communities were studied using qPCR [64]. This methodology was used also to study dominant phylogenetic groups of the bacteria in a model plant-based industrial WT system [65–66].

Table 2. Main advantages and disadvantages of hybridization techniques that are commonly employed in the identification of wastewater microorganisms.

Technique	Advantages	Disadvantages	WT Studies employing the respective technique
FISH	Does not require special training.	Difficulty for identify targets that have low DNA copies; high time consumption and laboratory efforts.	Municipal wastewater [43, 67–70]; Industrial wastewater [39, 71–72].
Microarray	It is rapid and sensitive, and one protocol can be used to identify different targeted bacteria simultaneously on a single array.	Low signal intensity due to insufficient penetration and improper contact of probes with targeted DNA; besides the fading away of fluorochromes upon excitation can lead to inaccurate analysis.	Municipal wastewater [73–75]; Industrial wastewater [76–77].
qPCR	Simultaneously amplifies and quantifies the DNA sequence of interest.	It does not determine the number of cells but estimates the number of copies of the tagged gene.	Municipal wastewater [35, 45, 52, 65, 73, 78–80]; Industrial wastewater [48, 64, 66].

DNA sequencing for taxonomic classification

Advances in molecular biology in the last decades, together with the accessibility to high-throughput sequencing techniques (Table 3), empowered the study of environmental DNA (eDNA) [81]. The knowledge of microbial ecology is fundamental to improve bioprocesses such as wastewater treatment [82]. High-throughput sequencing techniques have the potential, not only to access the global diversity of microbiomes but also to determine the biogeography of sludge bacterial communities of wastewater treatment plants increasing the ecological knowledge of these systems [83].

Clone Library

Before next-generation/high-throughput sequence-based microbial diversity analysis, the most widely used technique was clone library-based. The construction of the library consists of the following steps: i) DNA extraction, ii) cloning of DNA fragments at random into a suitable vector, iii) transforming a host bacterium, and iv) sequencing the clone library [23]. Later, the fragments compared with known sequences of a database such as GenBank, RDP Ribosomal Database Project (RDP), Silva, Greengenes, etc. for taxonomic assignment. Based on good-quality sequence size, cloned sequences are assigned at a taxonomic level like phylum, class, order, family or genus. However, one of the limitations of this technique is being time-consuming and labor intensive. Large libraries insertions of DNA fragments (100 to 200 kb) are suitable for research multigene and are considered a powerful approach to isolate new microbial genes. DNA recovery of high molecular weight is, however, a requirement for use vectors with high capacity. This technique has been used to study microbial diversity in wastewater [84] and slaughterhouse treatment filters [85].

454 Pyrosequencing

The method 454 is based on the “sequencing by synthesis” principle. In this method the target gene, generally 16S rRNA gene is amplificated by PCR or DNA is randomly fragmented (400–600 base pairs). Adapters (short sequence of DNA) are attached to the DNA fragments, and tiny resin beads are added to the mixture. The adaptor sequences complementary bind with template DNA which helps DNA fragments to bind directly to the beads. The DNA fragments are polymerized several times by polymerase chain reaction on each bead. Beads without sequence are filtered to remove, and the remaining DNA-containing beads are placed into wells on a sequencing plate for sequencing. Nucleotides are added to the wells in turns of one type of base at time. After single base incorporation into DNA, the chemical signals, i.e., light generated by luciferase enzyme, are converted into light that is recorded by CCD camera. The intensity of light varies proportionally with the consecutive number of nucleotides [86–87]. To determine the sequence of DNA fragment sequenced, this pattern of light intensity is plotted in a graph. This technique has been widely used in recent years to analyze microbial communities from different wastewater treatment plants [88–92].

Illumina

The Illumina technology is based on sequencing-by-synthesis method using reversible dye termination nucleotides. Along with DNA polymerase, all four fluorescent label nucleotides are added consecutively to the flow cell channels to sequence millions of clusters on the flow surface. The DNA is randomly fragmented (200–600 base pairs) or 16S rRNA gene is amplificated by PCR, and adapters are linked to the end of the fragments. Unlabeled nucleotides and DNA polymerase are added to join DNA strands which create “bridges” between double-stranded DNA (dsDNA). Using heating, dsDNA is denatured into single-stranded DNA. The denaturation step leaves several millions of condensed clusters of DNAs that are produced in each flow channel. After that, sequencing cycles started by adding primer, DNA polymerase, and four labeled reversible terminators [87]. Using laser excitation, the emitted fluorescence from each cluster is captured and bases are identified. In Illumina sequencing, DNA sequence is analyzed base by base, making it a highly accurate method [93]. This technique has been an important tool for study of microbial community of waste from leather industry [66], water basin treatment [83] and WT sludge [94].

Ion Torrent

The Ion Torrent method also uses the technology of sequencing by synthesis, but its technology differs from the previous one; instead of fluorescence it measures the H^+ ion release during base incorporation [95]. Chemical signals are directly transferred into digital information in Ion Torrent sequencer. The first step in Ion Torrent workflow is the target amplification or DNA cleavage and it is binding to Ion Torrent adapters. The

library binds to beads and is amplified by emulsion PCR. Beads coated with million copies of the template are placed in chip wells. The template-loaded chip is placed in Ion Torrent sequencer [96]. Individual bases are introduced one at time and are incorporated by DNA polymerase. For each base incorporated, a proton is released that results in pH change, which is detected by ion sensor that transforms the chemical changes into digital information. The chip records two bases if voltage is doubled by detection of two identical nucleotides. Ion Torrent technique has proven to be quite versatile, having already been used to detection of silver nanoparticle residues in sludge [97], in treatment of laundry wastewater [98], food waste-recycling wastewater [99] and removal of nitrogen from urban water treatment [65].

Table 3. Main advantages and disadvantages of DNA sequencing techniques that are commonly employed in the identification of wastewater microorganisms

Technique	Advantage	Disadvantages	WT Studies employing the respective technique
Clone Library	Average read length up to 1500 bp with paired-end sequencing.	It is laborious and time-consuming. Typical gene clone libraries include fewer than 1,000 sequences.	Municipal wastewater [84, 100 – 102]; Industrial wastewater [25, 85, 103].
454 Pyrosequencing (GS FLX Titanium XL+ model)	Clonal amplification by emulsion PCR. Average read length up to 1000 bp with paired-end sequencing. Throughput up to 450 Mb.	It is an expensive technology, approximately US$ 9,500 per Gb.	Municipal wastewater [14, 90 – 91, 104 – 106]; Industrial wastewater [66, 92].
Illumina (MiSeq model)	Clonal amplification by bridge amplification. Average read length up to 300 bp with paired-end sequencing available. Throughput up to 15 Gb.	It is an expensive technology, approximately US$ 110 per Gb.	Municipal wastewater [35, 79, 83, 107 – 110]; Industrial wastewater [95, 111].
Ion Torrent (Ion S5 530 model)	Clonal amplification by emulsion PCR. Average read length up to 400 bp with paired-end sequencing available. Throughput up to 8 Gb.	It is an expensive technology , approximately US$ 475 per Gb.	Municipal wastewater [65, 112 – 115]; Industrial wastewater [97, 116 – 118].

CONCLUSION

The dynamic and composition of wastewater treatment systems microbial communities have advanced with molecular methods development and appliance. Molecular methods allowed researches glimpsing into the “black box” and getting information to improve wastewater treatment process. Almost a decade of research on metagenomic techniques showed its ability to identify novel and rare unculturable organisms and their function in maintaining biogeochemical cycles. However, conventional techniques of microbial community analysis still remain important as many findings of high-throughput studies need to be validated and substantiated using conventional techniques like qPCR, FISH, Microarray etc. For the next steps in understanding wastewater microbiomes, richer multi-omic studies will be necessary. This goal can be partially accomplished by adapting current sequencing techniques to probe under-appreciated aspects of microbial community behavior, such as strain-level phenomena, temporal dynamics and functional activity. However, a complete understanding of nature and functioning of microbial community with environmental interactions will require the development and application of alternative, high-throughput molecular biological screens. To achieve success in this field will not be possible without the widespread adoption of integrative methods for managing and exploring such data. These include basic statistical considerations, such as methods for normalizing functional activity measurements against metagenomic potential, as well as the continued application and development of supervised and unsupervised approaches for identifying patterns in large multi-omic databases.

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