# Review of Molecular Techniques for the Identification of Bacterial Communities in Biological Effluent Treatment Facilities at Pulp and Paper Mills

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One of the processes most used in biotechnology today for handling industrial liquid wastes is biological wastewater treatment. The efficiency and quality of its operation depends on the composition and activity of the microbial community that is present. The application of traditional and molecular techniques has provided a glimpse into the "black box" and has given information to improve the wastewater treatment process. However, bleach pulp and paper mill effluents require a better understanding of the active bacterial population. For the study of these microorganisms, molecular techniques have been used for more than 15 years. However, there has been a lack of knowledge of the physiological requirements and relations with the environment, which seems to be very difficult to obtain involving profile on the diversity. Nowadays, high-throughput sequencing technology is a promising method that makes it possible to identify the entire profile of microbial communities. In combination with fingerprint methods, this approach allows the identification and analysis of the whole biodiversity of microbial communities. In this review, several identification techniques will be discussed.

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## INTRODUCTION

In nature, the size of microbial diversity is much larger and unknown than previously thought (Rondon *et al*. 1999). The microbes remain fully dominant in the earth's ecosphere. A sense of the biological dominance of microbes is given by estimates of the total number of living bacteria, roughly 4-6 x $10^{30}$ cells (Whitman *et al.* 1998; Dunlap 2001).

The vast majority of microbial species are 'uncultured', meaning that they do not grow under laboratory conditions (Lewis *et al*. 2010). Studies of microbial diversity rest on the assumption that in the microbial cultivation most organisms are recovered. However, the true extent of microbial diversity is much higher, since many microorganisms are not recovered by using the employed culture techniques (Rondon *et al.* 1999). Major limitations of used techniques are related to the culture media. More than 99% of the microorganisms in the environment, as observed through the microscope, are not cultivable (Hugenholtz 2002).

Rondon *et al.* in 1999 mention that only 1% of the cells observed in the culture of a sample can be studied. Because of the possibility that non-culturable cells must be grown under different environmental conditions, the goal of many researchers has been focused on further characterizing these uncultivable microorganisms, using molecular methods and phylogenetic analysis based on the DNA sequence information in order to understand their distribution and relationship with the environment.

Metagenomics investigations have resulted in the identification of various novel life forms in geographically distinct regions, and attempts are still underway to describe their possible role in that environment (Joshi *et al*. 2014). Microbial communities inhabiting wastewater environments are of significant interest for being applied in basic microbiology. Communities of prokaryotic microorganisms present in activated-sludge or biofilm reactors are responsible for most of the carbon and nutrient removal from sewage and thus represent the core component of every biological waste water treatment plant (WWTP) (Wagner and Loy 2002). These populations have been extensively studied for many years. However, only with the development of molecular and metagenomics approaches has it become possible to assess the true diversity of wastewater communities (Del Casale *et al.* 2011).

Biochemical activity of bacteria for the degradation of detrimental compounds is essential to reduce pollution to the environment. The knowledge of complex microbial communities developing in the treatment plants is necessary to ensure their efficient performance (Forster *et al*. 2003). It is time for wastewater microbiology to be recognized as a mature and dynamic discipline in its own right, offering a deeper understanding of life in complex microbial communities (Daims *et al.* 2006).

Molecular techniques for identification of microorganisms in several pulp and paper mill effluents have been used for more than 15 years. Techniques having names such as RISA, RT-PCR, clone library, T-RFLP, DGGE, FISH, and recently, pyrosequencing, all have allowed species identification, at least in some cases. The application of traditional and molecular techniques has allowed glimpsing into the "black box" of a wastewater treatment process, providing information that can be used to improve performance. Nevertheless, a high sample throughput is required for broad-scale community comparisons, cell lysis efficiency, and co-extraction of contaminants that may interfere with PCR. For example, extraction methods with a bead-beating tend to shear DNA and amplification of fragmented nucleic acids can generate chimeras (Wintzingerode *et al.* 1997).

Therefore, the low sequencing depth of the traditional PCR approach when compared with the vast genetic diversity present in natural systems hinders comprehensive characterization of the microbial community structure. Further, the current community analyses typically represent a mere snapshot of the dominant members, with little information on taxa with medium to low abundances (Shendure and Ji 2008). Rastogi and Sani (2011) classify these analyzes as "partial community analysis". In spite of the disadvantages of these techniques, at present they could be of great utility for dynamic population studies. In this review, the limitations and potential of the techniques in the identification of microorganisms in biological processes of pulp and paper mill effluents will be discussed.

## BACKGROUND OF TRADITIONAL MICROBIOLOGICAL TECHNIQUES

The microbial identification techniques have undergone rapid development during the $20^{th}$ and $21^{th}$ centuries. Initially, traditional or phenotypic methods were the most used. Traditional methods for microbial identification require the recognition of differences in morphology, growth, enzymatic activity, and metabolism to define genera and species (Previously isolated) (Petti *et al.* 2005). Traditional techniques to characterize microbial ecology involve isolation and characterization of microorganisms using commercial growth media such as Luria–Broth, Nutrient Agar, and Tryptic Soy Agar (Kirk *et al.* 2004). In the pulp industry, microbial communities have been explored under traditional microbiology by viable cell count and enzyme activities test.

### Viable Cell Count

The anaerobic procedures for count of aerobic/anaerobic bacteria involve growth in selective media, and the procedures are carried out in anaerobic chambers. Liss and Allen in 1992 evaluated the presence of culturable bacteria in different seasons of a lagoon that receives the effluent from a kraft pulp mill. Large aerated lagoons are the main process in the pulp and paper industry for biological wastewater treatment (Pougatch *et al.* 2007; Ordaz-Díaz *et al.* 2016). Studies revealed that variation in the culturable microbial population in a kraft pulp mill lagoon is related to process parameters: wood type and temperature. In the cited studies the differentiation between aerobic and anaerobic microorganisms was possible.

Zhang *et al.* in 1997 isolated but did not characterize five bacterial strains from bleach kraft effluents; they examined the ability of two bacterial strains to grow on

thermochemical and mechanical pulping effluent and degrade dehydroabietic acid (DHA), a resin acid commonly found in such effluents. Viable cell counts on different substrates and their correlation with the removal of DHA were performed.

Using the most probable number (MPN) and metabolic tests, Gauthier and Archibald (2001) detected the presence of numerous coliforms, especially *Klebsiella* spp., *Escherichia coli*, *Enterobacter* spp., and *Citrobacter* spp. Furthermore, the overall distribution of thermotolerant coliform bacteria in paper mill effluents and sludge's was investigated using the chromogenic broth method. The chromogenic media used were 1-Colilert Broth (Idexx Laboratories, Westbrook, ME.,) 2-LMX Broth (VWR International, Montreal, QC), and 3-Modified mTEC. For the LMX Broth, the Kovac substrate was added to the fluorescent tubes and the indole detection gave a confirmation for the presumptive *E. coli*. The population estimates were made using the most probable number MPN (Beauchamp *et al*. 2006).

## Identification by Enzyme Activities

Pattern analysis of carbon-source utilization is a method to characterize heterotrophic microbial communities. Plates contain multiple carbon substrates; these are analyzed over a short period, and the patterns are compared (Choi and Dobbs 1999). Biolog GN plate contains an array of 95 carbon sources and a tetrazolium dye (oxidation indicator), which have been developed to assist in the identification of Gram-negative microbial isolates using databases available from BIOLOG (Kaiser *et al.* 1998). This technique requires long periods of growth and yield data are skewed, because in most cases it is not possible to identify organisms at the species level.

Fulthorpe *et al.* (1993) used GN microplates for isolates. The biologic GN microplate data base contained the fingerprints of over 500 species of bacteria, but the majority of the bacterial strains from the environments sampled did not match known fingerprints. Besides, a microbial community was isolated from the discharge of effluent of pulp and paper mill, previously enriched with a minimal salt medium. It was identified by morphological, physiological, and chemotaxonomical properties as *Acinetobacter* spp., *Acidovorax* spp., *Ancylobacter aquaticus, Klebsiella* spp., *Comamonas testosteroni, Pseudomonas stutzeri, Pseudomonas fluorescens*, and *Pseudomonas putida*. Using this method, it was possible to characterize microorganisms at the species level.

The microbial identification on bleached kraft mill effluent was carried out using BIOLOG redox-base carbon-substrate utilization assay (Victorio *et al*. 1996). Characterization of separate bacteria and protozoa components of one bacterial community showed that each fraction displayed different substrate utilization patterns. This method allowed for characterizing microorganisms at the community level. The disadvantage is that microplates require a long incubation period for detecting the oxidation of bleached kraft mill effluent compounds. The use of these techniques with individual chemicals allowed for the determination of biodegradation potential (Victorio *et al*. 1996). The identification of *Microbrevis luteum* species with a commercial microplate test was used too by Singh in 2007. In addition, Chuphal *et al.* (2005) identified *Micrococcus luteus, Deinococcus radiophilus, Micrococcus diversus*, and *Pseudomonas syringae.* Predominant bacteria in paper and pulp mill effluent were evaluated and identified for the degradation efficiency of individual isolates and their combinations. The isolated bacteria were identified by colony morphology, Gram

staining, microscopic observation, and confirmation test (Saraswathi and Saseetharan 2010).

The disadvantage of traditional cultivation techniques is that they are unable to identify the majority of microorganisms in environmental samples, due to the inability of many organisms to grow on laboratory media (Mocali and Benedetti 2010). For slow-growing microorganisms, traditional phenotypic identification is difficult and time-consuming, besides, the interpretation of test results involves substantial subjective judgment (Stager and Davis 1992).

## Genomic Analysis

Usually, cloning and sequencing of genomic DNA is used when detailed and accurate phylogenetic information from environmental samples is required. In general, the method involves the extraction of DNA (of previously isolated bacteria), amplification with suitable primers, and the generation of clone libraries using sequencing vectors.

It is important to increase the knowledge of bacterial communities capable of degrading contaminates present in effluents in biological effluent treatment pulp and paper. Mishra and Thakur (2010) conducted a related study with four bacterial strains isolated from pulp and paper mill sludge having higher capability to remove color and lignin. One of them was identified as *Bacillus* sp. by 16S rDNA sequencing. Genomic DNA of bacteria was isolated using a QIAamp DNAMini Kit. The 16S rDNA gene was amplified using the primer pair P0 5'- GAGAGTTTGATCCTGGCTCAG- 3' and P6 5'- CTACGGCTACCT-TGTTACGA- 3' with a thermo cycler (Applied Biosystem, USA). The amplified DNA was purified using Qiaquick PCR Purification Kit (Qiagen). The sequences were compared in the GenBank using the standard BLAST site at NCBI server. The alignment of these sequences was done using CLUSTAL W program (version 1.8.3). From the aligned sequences neighbor-joining dendrogram was constructed with Mega 3.1 software.

Hooda *et al*. (2015) analyzed the degradation of *Brevibacillus agri* in pulp and paper mill effluent, based on its efficiency to reduce chemical oxygen demand (COD), color, adsorbable organic halides (AOX), and lignin. The genomic DNA from the bacterial isolate was extracted, and amplification of the 16 s rRNA gene was performed using universal primers, ~ 1500-bp PCR products were sequenced.

## Metagenomics

Metagenomics can be defined as "the application of modern genomics techniques to the study of communities of microbial organisms directly in their natural environments, bypassing the need for isolation and laboratory cultivation of individual species" (Chen and Pachter 2005). Metagenomics is also known by other names such as environmental genomics or community genomics, or microbial eco genomics. Essentially, metagenomics does not include methods that interrogate only PCR-amplified selected genes (*i.e.* genetic fingerprinting techniques), as they do not provide information on genetic diversity beyond the genes that are being amplified. However, metagenomics (and other cultivation independent methods) based on 16S rDNA gene copies do not distinguish between viable and dead microorganisms (Kanto-Oqvist *et al*. 2008).

Metagenomics is crucial for understanding the biochemical roles of uncultured microorganisms and their interaction with other biotic and abiotic factors. Environmental metagenomics have proved to be great resources for new microbial enzymes and antibiotics with potential applications in biotechnology, medicine, and industry (Rondon *et al*. 2000; Riesenfeld *et al.* 2004).

## MOLECULAR TECHNIQUES AND THEIR APPLICATION IN THE TREATMENT OF WASTEWATER

Today, new techniques of molecular biology are radically changing the landscape, allowing access to non-culturable microbial populations and their own fresh samples. These techniques have revolutionized the ability to link biogeochemical phenomena in microbial ecology, which is leading to novel discoveries about the taxonomic and functional diversity of microorganisms. It is now possible to proceed in the opposite direction to the traditional focus first study different functional genes, the microorganisms in which they reside, and their interactions at the community level, which can expand our knowledge of many important biogeochemical processes at local, regional and global, scale (Zak *et al.* 2006).

Since the 1980's, environmental microbiologists have observed the potential of the polymerase chain reaction (PCR) to study microbial communities, detecting or identifying microorganisms in different environments. Compared with traditional techniques (dependent on culture media) for microorganism identification, PCR makes such goals possible without depending on culture media (Albuquerque *et al.* 2009). The PCR technique simulates the process of DNA replication *in vitro*, and involves the amplification of target DNA, generating millions of copies.

Genotypic identification of microorganisms by 16S rRNA gene sequencing appeared as a more objective (although not perfect), accurate, and reliable method for bacterial identification, with the added capability of defining taxonomical relationships among bacteria (Clarridge 2004). The microbial communities study relies essentially on the detection and analysis of the small subunit ribosomal RNA molecules and genes (16S rDNA molecules for prokaryotes) (Amann *et al.* 1998).

For almost 20 years, the use of genome sequences and DNA fingerprinting techniques has overcome the need for cultivation to characterize and identify microorganisms in nature. Hence knowing the most prominent microorganisms in the population, without isolating them, has revolutionized microbial ecology opening new fields of research (Chandra *et al.* 2007).

DNA isolated from environmental samples can be hybridized with a labeled probe, cloned into a plasmid or amplified by PCR (Bitton 2005). DNA sequencing techniques, especially those targeting ribosomal RNA genes, have opened new windows for investigating uncultured bacteria in different environments (Joshi *et al.* 2014). The availability of improved DNA sequencing techniques, vastly increased databases, and more readily available kits and software, makes this technology preferred to traditional microbial identification techniques (Clarridge 2004).

## Library Clones

The use of library clones is the most widely used method to analyze the PCR products from environmental samples. It is used to clone the individual gene fragments in a vector (plasmid or phages), followed by sequencing. Later, the fragments are compared with known sequences of a database such as GenBank, RDP, and Greengenes. Commonly, the cloned sequences are a phylum, class, order, family, subfamily, or species with a degree of similarity of 80, 85, 90, 92, 94, or 97%, respectively (Rastogi and Sani 2011). A library 16S rDNA gene clone is a technique that involves cloning and analysis of isolated microbial DNA or directly from the environmental sample. Construction of the library consists of the following steps: 1) DNA extraction, 2) cloning of DNA fragments at random into a suitable vector, 3) transforming a host bacterium, and 4) detecting the clone library. Sequencing of 16s rDNA allows the identification of most microorganisms in a sample at the species level. The disadvantage is that it requires a lot of time and the technique alone does not allow quantification. Large libraries insertions of DNA fragments (100 to 200 kb) are suitable for research multigene (Rastogi and Sani 2011). This technique is demanding with respect to labor, time, and cost; however, the library of clones is considered the "gold standard" for preliminary studies of microbial diversity (DeSantis *et al.* 2007). DNA recovery of high molecular weight is, however, a prerequisite for the use of vectors with a high capacity. *Escherichia coli* are most often used as a guest option. Other hosts such as *Streptomyces lividans*, *Pseudomonas putida*, and *Rhizobium* sp., have also been used successfully. Despite the limitations mentioned above, this technique has been used in the following studies:

Clone libraries of rDNA amplicons by Yu and Mohn (2001) from paper mill effluent were constructed. The temporal differences in community structure, based on summer and winter samplings, were greater than the spatial differences during either season. Among 90 clones analyzed (30 clones from each sample), 56 phylotypes were distinguished by restriction fragment length polymorphism (RFLP). The most abrupt changes in community structure were associated with a temperature change from 35 °C to 39 °C and with increases in dissolved oxygen concentrations. A community succession was evident in the lagoon, as indicated by a progressive community transition through seven sample locations. The results of this research show the presence of *X. agilis*, *I. dechloratans*, *L. mobilis* D., *M. aerodenitrifican, P. franzmannii*, *G. ferrireducens*, *L. salivarius* subsp. *salivarilus*, *C. henricii*, *D. riboflavin*, *R. capsulatus*, *A. brasilense*, *P. manganicum*, *C. fermentans*, *F. sancti*, *R. slithyformis*, *T. maltophilum*, and *M. liquefaciens.*

Chandra *et al.* (2007) showed the ability of three aerobic bacterial strains isolated from pulp paper mill effluent sludge, to degrade lignin. DNA was extracted from the pure cultures; the PCR products were cloned and sequenced. 16S rRNA sequencing showed 95% base sequence homology, and it was identified as *Paenibacillus* sp., *Aneurinibacillus aneurinilyticus*, and *Bacillus* sp.

16S rDNA amplicons from the mixed liquor pilot plant were ligated into the pGEM®-T easy vector (Promega); then they were transformed into DH10B competent *E. coli* cells. Clones inserted were reamplified directly from the colonies, utilizing the M13/pUC universal forward and reverse sequencing primers to avoid the co-amplification of *E. coli* 16S rDNA genes, clones were differentiated by RFLP. Clones sequenced was identified as α, β, and δ-Proteobacteria and Cytophaga–Flexibacter–Bacteroides group (Reid *et al.* 2008).

## Probes

Specific microorganisms DNA probes help to hunt out and to estimate the abundance or growth rate of selected bacterial species in an environmental sample. This technique relies on the hybridization of the target DNA. The sample is subjected to heat or alkaline solution, wherein the chains are denatured (single strands produced). This is achieved by using probes, which are specific short sequences of single stranded DNA of the microorganism to be identified with 32P-labeled or fluorescent compounds (*e.g.* fluorescein isothiocyanate). The probe hybridizes with complementary DNA strand if it contains the sequence of interest (Bitton 2005).

A majority of molecular ecology studies have evaluated 16S rDNA sequences of prokaryotic microorganisms in the soil, water, and other samples found in nature and / or develop probes to determine whether an enzyme or product is present in the sample (Hunter-Cevera 1998). In 1992 Manz *et al*. located specific sequences which were evaluated as potential nucleic acid probes for the differentiation of the major subclasses of proteobacteria. They underline the importance of these probes for *in situ* monitoring of microbial communities.

The use of probes allows the *in situ* monitoring of population distribution and dynamics in microbial communities. Fortin *et al.* (1998) developed gene probes and oligonucleotide primers to monitor kraft pulp mill effluent treatment systems for the presence of key genes responsible for the dehalogenation of chloroaliphatic organics. The primer design was performed using GENEWORKS software (IntelliGenetics Inc., Cambell, Calif.). DNA probes were designed from the dhlB encoding haloacid dehalogenase, dhlA encoding haloalkane dehalogenase from *Xanthobacter autotrophicus*, $dehH_2$ encoding haloacetate dehalogenase from *Moraxella*, and mmoX encoding the soluble methane monooxygenase from *Methylococcus capsulatus*. The amplicon was transferred onto a Zeta-probe nylon membrane (BioRad Laboratories) using the LKB 2016 VacuGene vacuum blotting system (Pharmacia Biotech) and transferred to fresh prehybridization solution containing the labelled probe. After using the standard Zeta-probe membrane protocol, the membranes were exposed to X-ray films for 1 to 5 days. The plasmid transformation was performed using a modified PTZ19R *Escherichia coli* plasmid and *E. coli* NM522 competent cells.

## Real-time Reverse Transcription PCR (RT-PCR)

This technique for quantifying mRNA in biological samples have benefits such as, its sensitivity, large dynamic range, and accurate quantification (Huggett *et al.* 2005).

The procedure is as follows; firstly, primers for qPCR are designed using a software. The primers specificity is initially verified using a CHECK PROBE program provided by the RDP (Ribosomal Database Project) database analysis. To determine gene copy numbers of unknown samples, one calibration curve is run routinely with each sample set and compared with previous standard curves to check the variability of amplification efficiency. The total number of bacterial 16S rDNA genes is estimated using primers and genomic DNA is used to generate a calibration curve. The standard DNA is quantified using dye and a fluorometer, with the assistance of data analysis software. To determine the specificity of amplification, analysis of the product melting curve and PCR products are tested for the correct PCR product length by agarose gel electrophoresis, and selected PCR products are sequenced to verify primer specificity (Muttray *et al.* 2001).

Muttray *et al*. (2001) analyzed, by PCR and RT-PCR, population dynamics and metabolic activity of *Pseudomonas abietaniphila* (isolated) within pulp mill wastewater microbial communities. Besides, dominant phylogenetic groups of the domain bacteria were studied by Reid *et al*. (2008) in a model plant-based industrial wastewater treatment system (mixed liquor samples). Three important operational taxonomy units (OUT), represented with high relative abundances were analyzed by quantitative PCR to confirm their abundance in the community profile.

## Reverse Sample Genome Probing (RSGP)

This technique involves the blotting of DNA from individual strains onto filters that are then probed with DNA extracted from environmental samples. Metagenomic DNA, after it is extracted and purified, is radiolabelled and used to probe a master filter that contains chromosomal DNA from individual standard microorganism of interest bonded to a nylon membrane. The information obtained is limited to the culturable component of the microbial community. RSGP thus measures microbial diversity in the selected target environment by following the fate of selected culturable community members (Greene and Voordouw 2004). It is used to examine quickly if cultivable species were found in samples (Fulthorpe *et al.* 1993).

Gilbride and Fulthorpe in 2004 used RSGP to compare the culturable bacteria from several communities geographically separated from a pulp-mill biotreatment system. *Ancylobacter* spp., *Xanthobacter* spp., *Comomonas* spp., *Klebsiella* spp., *Pseudomonas* spp., *Sphingomonas* spp., *Blastobacter* spp., *Moraxella* spp., *Burkholderia* spp., *Ralstonia* spp., *Xanthomonas* spp., and *Acidovorax* spp. were used in this study. There was little overlap in the composition of the culturable community between mills at the genus level. The RGSP results just showed that these selected culturable isolates represent a very small percentage of the population.

## Fluorescent *in Situ* Hybridization (FISH)

The FISH procedure enables *in situ* phylogenetic identification and enumeration of individual microbial cells by whole cell hybridization with oligonucleotide probes (Amann *et al.* 1995). A large number of molecular probes targeting 16S rDNA genes have been reported at various taxonomic levels (Amann *et al.* 1995). The FISH probes are generally 18 to 30 nucleotides long and contain a fluorescent dye at the 5´ end that allows detection of probe bound to rRNA by epifluorescence microscopy. It is a relatively fast technique, if the probes are available in the market, it allows the differentiation of active microorganisms, and it does not require highly trained personnel. The disadvantages of this technique are based on the time and work required for the design of the probes, which in some cases are not as specific when taking metabolic criteria. In addition, for quantification, image analysis is often difficult.

Few experiments have been used to investigate and enumerate the different bacterial groups present at particular stages through the wastewater treatment system. For example, the diversity and abundance of three dominant genera, *Aeromonas*, *Pseudomonas*, and *Bacillus* were investigated in a pulp and paper wastewater by enrichment with $^{15}N_2$ by Addison *et al.* (2010, 2011); specific probes were used to investigate and enumerate the different bacterial groups present at particular stages through the wastewater treatment system over an extended period.

## Enterobacterial Repetitive Intergenic Consensus Polymerase Chain Reaction (ERIC-PCR)

ERIC-PCR is a powerful method for DNA fingerprinting; this method is faster, simpler, and more economical than other genomic typing methods. Repeated oligonucleotides are used as a starter of DNA synthesis when there is no need for information on the target DNA sequence, which makes it a powerful method with general applications (Eriksson *et al.* 2005). The primers are designed so that amplification occurs between copies of the ERIC sequence; if the positions of copies vary among different strains, the amplification products provide each with a unique fingerprint when run on a gel (Wilson and Sharp 2006). Unfortunately, amplification using ERIC-PCR primers indicates that ERIC sequences are not widespread among bacteria (Wilson and Sharp 2006). Therefore, it is not recommended for studies of bacterial communities because it is very specific.

Singh *et al.* (2011) studied the presence of *Enterobacter* sp. with the ability to degrade tannic acid for the treatment of effluents from pulp and paper in the shortest time, using ERIC-PCR and for identification, and 16S rDNA was sequenced.

## Repetitive Extragenic Palindromic – PCR (REP-PCR)

REP-PCR is mainly used to distinguish isolates belonging to the Enterobacteriaceae family (Zelazny *et al.* 2009). It is based on primers that are complementary to naturally occurring, highly conserved, extragenic, repetitive DNA sequences throughout the genome of most bacteria. REP sequences are found associated with 30% of bacterial operons. Amplification of DNA between REP sites produce highly reproducible fingerprints with single isolates (Rademaker and de Bruijn 1997). In this method, DNA isolated from a microorganism is extracted and then amplified with specific oligonucleotides; PCR products are separated on agarose gels 1% stained with ethidium bromide, and subsequently photographed (De Brujin 1992). There are kits in the market such as DiversiLab, which allows for automated detection, standardization, and analysis using a software (Healy *et al*. 2005).

Baker *et al*. (2003) analyzed the bacterial communities of seven treatment systems of pulp and paper wastewater. They compared RISA, RFLP, and REP-PCR methods and reported that the REP-PCR was the one that showed a higher degree of resolution, with different patterns for each sample at different times and sampling points. In this study individual members of the communities were not identified (Rademaker and de Bruijn 1997). The sensitivity of this method might potentially be used to monitor the stability of the bacterial community within a secondary treatment system (Baker *et al.* 2003). However, REP-PCR test is not an accurate tool for identifying organisms to the subspecies level (Mougari *et al.* 2014).

## Community Profiling or DNA Fingerprinting

DNA fingerprinting concerns the electrophoretic band pattern obtained through specific typing methods based upon DNA (Albuquerque *et al.* 2009). DNA fingerprinting techniques provide efficient information to monitor microbial communities. The band patterns generated from PCR amplified DNA is separated by electrophoresis. It offers the advantage of identifying non-dependent culture microorganisms. Comparison of fingerprints provides a relative measure of communities' similarity, which can be used to compare different communities or monitor a community over time (Yu and Mohn 2001).

DNA fingerprinting profiles usually involve fairly large numbers (20 to 40) of individually segregating fragments. These fingerprinting techniques are basically of two kinds: hybridization and PCR (Reineke and Devi 2013). DNA fingerprinting techniques generate a profile of microbial communities based on the direct analysis of the PCR products from environmental samples of DNA. These techniques include DGGE/ TGGE, RAPD, ARDRA, T-RFLP, and RISA. DNA fingerprinting techniques are quick and allow simultaneous analysis of multiple samples. Genetic fingerprints of different samples are compared using cluster analysis by software packages such as GelCompar (Rastogi and Sani 2011).

## Random Amplified Polymorphic DNA (RAPD)

RAPD utilizes PCR amplification with a short (usually 10 nucleotides) primer, which anneals randomly at multiple sites on the genomic DNA under low annealing temperature, typically ≤35 °C (Franklin *et al.* 1999). This method generates PCR amplicons of various lengths in a single reaction that is separated on agarose or polyacrylamide gels depending on the genetic complexity of the microbial communities. The major disadvantage to RAPD-PCR is its inability to yield reproducible polymorphism under varying laboratory conditions (Srivastava *et al.* 2012). To overcome this problem, it is necessary to develop co-dominant markers from dominant markers to avoid lengthy RAPD reaction (Li *et al.* 2010). Such markers are typical oligonucleotides designed from the sequences of the amplicon of RAPD, and they mostly have a high probability of producing polymorphic amplicons (Cheng *et al.* 2015).

Yang *et al.* (2008) isolated 38 strains of bacteria from black liquor and 11 PCR-DNA profiles. These were grouped as 11 operational taxonomy units (OTUs) using random amplified polymorphic DNA-PCR profiles (RAPD). There were 11 representative strains of each OTU, which were identified as *Halomonas* and *Bacillus* genera. Besides, Zhan *et al.* (2010) analyzed the diversity of bacterial communities in a batch reactor containing wastewater of a pulp and paper industry using the RAPD method. In this work, different primers where tested, and it was found that the optimal was S308 (CAGGGTGGA). The RAPD-PCR fingerprints showed very high polymorphism of the genetic bands (78 to 100%). Four groups of species were clustered using the un-weighted pair group method with arithmetic (UPGMA) analysis, and the genetic distance was close between the species within each group, however, were not identified. Scanning electron microscope indicated that *coccus* and *bacillus* became the dominant species in matured active sludge.

## Amplified Ribosomal DNA Restriction Analysis (ARDRA)

ARDRA is based on DNA sequence variations present in PCR-amplified 16S rDNA genes (Smit *et al.* 1997). The PCR product amplified from environmental DNA is generally digested with tetra cutter restriction endonucleases (*e.g. Alu*I, and *Hae*III), and restricted fragments are resolved on agarose or polyacrylamide gels. Although ARDRA provides little or no information about the type of microorganisms present in the sample, the method is still useful for rapid monitoring of microbial communities over time, or to compare microbial diversity in response to changing environmental conditions (Rastogi and Sani 2011). The major limitations of ARDRA is that restriction profiles generated from complex microbial communities are occasionally too difficult to determine by agarose/PAGE (Smit *et al.* 1997).

This technique has not been used in biological effluent treatment pulp and paper; however, it has been successfully used in industrial wastewater by Princič *et al.* (1998), Sarti *et al.* (2012), Gich *et al.* (2010), and Shah (2014).

## Terminal Restriction Fragment Length Polymorphism (RFLP)

RFLP analysis is a rapid, technologically simple, and highly reproducible method and consists of PCR-based amplification of a fragment of DNA, usually a gene or a part of a gene, combined with succeeding restriction digestion of the PCR product and electrophoretic analysis of RFLP. It can therefore be used to differentiate between species and strains of living organisms as a shortcut to sequence determination (Vaneechoutte and Heyndrickx 2001). In the RLFP the PCR products are digested with restriction enzymes, and terminal restriction fragments (T-RFs) are separated on an automated DNA sequencer. This method uses capillary sequencing technology allowing samples contained in 96 or 384 well plates to be sized (Thies 2007). Terminally fluorescent labeled restriction fragments are detected, thus simplifying the banding pattern and allowing analysis of complex microbial communities (Rastogi and Sani 2011).

The study of the bacterial communities from seven pulp and paper wastewater treatment systems by 16S-RFLP analysis, was performed. Community similarity coefficients were based on quantitative determinations of both the positions of the DNA bands and the band intensities in order to compare the relative differences in the populations and a dendrogram was generated for all mills. Mostly, samples from each mill formed individual clusters. Similarities with and between mills were derived from this dendrogram, *i.e*., the similarity value of the node where all samples from one mill converge and defines similarity within-mill. Similarity between 16S-RFLP profiles from mills was partially correlated to wood furnish (Baker *et al*. 2003). This method was not sensitive enough to detect differences within a mill treatment system from different locations or from different sampling times. Subsequently, Gilbride and Fulthorpe (2004) performed RFLP analysis on both whole communities and individual cultured isolates to compare and contrast the microbial populations from 10 pulp and paper mill systems. All the communities shared 60% of their DNA band pattern. Partial sequences of the 16S rRNA genes from culturable isolates were identified as *Ancylobacter aquaticus*, *Blastobacter* sp., *Comamonas* sp., *Klebsiella* sp., *Bacillus* spp., *Pseudomonas* spp., and *Xanthobacter* sp.

The bacterial community composition, functional stability, and $N_2$-fixing were investigated at the pilot plant level. DNA of mixed liquor samples was extracted and collected from pulp and paper-activated sludge wastewater treatment and was amplified up to 1.5 kb. Clones previously obtained were differentiated by RFLP with the restriction enzyme, *Hha*I (Amersham). Fragment analysis was performed using an Applied Biosystems automated DNA sequencer model 377-XL, and the sizes of the 5′ terminal restriction fragments (TRFs) and the intensities of their fluorescence emission signals (peak area) were calculated using software GelCompar II. The results suggest that the bacterial populations are distinct among treatment systems and stable from the bacterial population viewpoint. The bacterial community composition was dominated by α-Proteobacteria and β-Proteobacteria, with a lesser amount of the highly diverse bacterial phylum Bacteroidetes (Reid *et al.* 2008).

## Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE was developed and applied in microbial ecology for the first time by Muyzer *et al.* (1993), and it has been used for the rapid fingerprint analysis of the structure, diversity, and dynamics of the microbial population in complex ecosystems (Green *et al.* 2017). This method allows rapid detection of microbial community changes and information about composition (Gilbride *et al*. 2006). Joshi *et al.* (2014) mentioned that the method makes it possible to differentiate the microbial community with a simple banding pattern and allows for easy monitoring of community dynamics in a sample, based on the analysis of the relative intensity of bands. PCR products are obtained from environmental DNA using specific primers for a molecular marker (for example the 16S rDNA gene). In bacteria, the V3 hypervariable region of rRNA is the most studied. The primers used are the PRBA 338F, (ACTCCTACGGGAGGCAGCAG), and 5'PRUN 518R (5'ATTACCG-CGGCTGCTGG). One of the primers regularly contains a guanine and cytosine rich sequence (GC-clamp), which enables the double chain to remain stable until higher denaturant concentrations are reached. The polyacrylamide gel electrophoresis contains a linear denaturing gradient of DNA formed by a mixture of urea and formamide. DGGE technique is related to the difference in allelic DNA sequences of interest (amplicons generated previously) based on the differentiation of migration in a denaturing gradient gel (Rastogi and Sani 2011; Reineke and Devi 2013; Kapley and Purohit 2009). After electrophoresis, DNA fragments are excised from the gel, reamplified, and subsequently the PCR products are purified before sequencing (Eyers *et al.* 2004).

Gilbride and Fulthorpe (2004) recommend using DGGE to obtain a finer separation of the bands and thus be able to sequence and identify key species in the treatment of pulp and paper mill effluent. The studies that have been carried out in this area are as follows:

Buzzini *et al.* (2006) analyzed an anaerobic consortium collected from the reactors sludge blanket (UASB). DNA was extracted and amplified (approximately 400 bp). Formamide and urea concentrations of 40 to 60% for bacteria and 35 to 55 % for *Archaea*, were used as denaturing gradient. The running conditions were 130V for 390 min at constant temperature. Under the conditions used in this work, DGGE technique makes it possible to observe the variation of microbial populations during operation. The authors mention that bacteria were responsible for the degradation of organic matter.

Another study in this area was carried out by Yang *et al*. (2008). The black liquor was sampled for community dynamics analysis by DGGE fingerprinting during the batch treatment process. PCR amplification of the 16S ribosomal RNA gene and DGGE was used to analyze a two-stage treatment mechanism to explain the interspecies (*Halomonas* and *Bacillus*) collaboration. During this step 16S rDNA gene V3 region was amplified, a linear 30 to 55% denaturant gradient was used, and the electrophoresis was performed a constant voltage of 200 V at 60 °C for 240 min. DGGE profiles were analyzed using Quantity One (version 4.6.2, BioRad, USA). *Halomonas* isolates were important in the first stage to produce organic acids that contributed to the pH decline, while *Bacillus* isolates were involved in the degradation of lignin derivatives in the second stage under lower pH conditions. It has been mentioned that this technique does not allow the quantification of cells. However, Moura *et al.* (2009) examined DGGE patterns using two indexes, the Shannon–Weaver index and the equitability index. The diversity was calculated based on the relative intensity of DNA bands obtained from wastewater

samples from cheese industry. The effectiveness of this approach in getting new data concerning the structure, dynamics, and diversity of these communities was demonstrated.

The disadvantages of this technique are: 1) The target DNA is less than 1% of the total population and is therefore unlikely to be detected by DGGE (Muyzer *et al.* 1993). Thus, the banding profiles will be representative of microorganisms predominant in the samples. 2) This technique allows the separation of small fragments (500 to 700 bp), however, with information from short fragments belonging to variable regions, V2 and V4 regions may be sufficient for identification at the genus level (with a lower error rate) (Wang *et al.* 2007) 3) Bands cannot always be differentiated, since biases associated with PCR amplifications are also incorporated into the analysis (Green *et al.* 2017), and 4) The sequencing of excised bands is not always effective. Eyers *et al.* (2004) recommends to clone the PCR products into a cloning vector before sequencing.

## Temperature Gradient Gel Electrophoresis (TGGE)

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 sequences achieve migration to different positions of the gel. During the amplification step, a staple or "clamp" composed of guanines and cytosines (about 30 to 50 nucleotides) is added. This in order that the DNA strands do not separate completely during electrophoresis. To determine the phylogenetic identities, the gel bands must be excised, reamplified, and sequenced or transferred to nylon membranes and hybridized with specific molecular probes for different taxonomic groups (Muhling *et al.* 2008).

## Ribosomal Intergenic Spacer Analysis (RISA)

RISA involves PCR amplification of a portion of the intergenic spacer region (ISR) present between the small (16S) and large (23S) ribosomal subunits (Fisher and Triplett 1999). The ISR contains significant heterogeneity in both length and nucleotide sequence. By using primers annealing to conserved regions in the 16S and 23S rDNA genes, RISA profiles can be generated from most of the dominant bacteria existing in an environmental sample. RISA provides a community-specific profile, with each band corresponding at least to one organism of the original community. The RISA method can be used to generate more complex fingerprints than 16S-RFLP and discern differences between samples (Baker *et al*. 2003).

Publications that further illustrate the application of RISA are as follows: Yu and Mohn (2001) investigated the bacterial community structure in an aerated plug-flow lagoon treating pulp and paper mill effluent. For this research, they developed a composite method based on analyses of PCR amplicons containing the ribosomal intergenic spacer (RISA) and its flanking partial 16S rDNA gene. Community similarity percent was determined based on RISA length polymorphism. The rDNA-RIS fragments were sequenced, and phylogenetic analysis yielded organisms belonging to *Methylobacillus flagellatum*, *Azospirillum lipoferum*, *Bacteroides putredinis*, and *Reclinomonas americana.* Smith *et al.* (2003) analyzed samples from non-clarified effluent taken from the pulp and paper mill factory. From the DNA samples, rDNA-RIS fragments were amplified using the universal bacterial primers S926f and L189r, the rDNA-RIS amplicons contained approximately 600 bp of the 16S rDNA (3’ end) plus the

RIS region and approximately 190 bp of the 23S rDNA (5' end). PCR products were purified and separated on a polyacrylamide gel. Subsequently, the band patterns were observed with UV light. The library was later built and the resulting clones were digested with restriction enzymes. The profile of bands was compared and identical patterns were considered to represent one phylotype.

## ADVANCED MOLECULAR TECHNIQUES

Fingerprint methods based on the 16S rDNA gene sequence have been displaced by high throughput sequencing. Therefore, the limitations of the above-mentioned techniques seem to have been overcome. The dideoxy sequencing method developed by Sanger and Coulson (1975) has been the most commonly used DNA sequencing technique. Previous techniques usually required the cloning of DNA fragments into bacterial vectors, amplification, and purification of individual templates, followed by Sanger sequencing (Margulies *et al.* 2005). However, a long time passed before an automated and cheaper method appeared (Ronaghi *et al.* 1998; Patrick 2007).

Microbial ecologists have largely abandoned sequencing 16S rRNA genes by the Sanger sequencing method and have instead adopted new parallelized sequencing (Schloss *et al.* 2016). The advent of DNA sequencing techniques in past years has far exceeded expectations (Ansorge *et al.* 2017). Recently developed high-throughput sequencing technology is a highly efficient tool for identifying the entire profile of microbial communities (Ma *et al.* 2015) and is a promising method, as it provides enough sequencing depth to cover the complex microbial communities (Shendure and Ji 2008).

### Next-generation Sequencing

Next generation sequencing or high-throughput sequencing are the terms to describe several modern techniques such as pyrosequencing analysis, massively parallel signature sequencing (MPSS), single-cell genome sequencing *etc*., which make it possible to obtain information much faster and more cheaply. The DNA of the entire community is extracted and purified, and the specific genes such as the 16s rDNA are amplified. Each primer is encoded with short tags, as well as sequencing adapters. Thus, multiple sequences can be grouped and read simultaneously (Zhou *et al*. 2015).

Third-generation sequencing technology has been speedily developed and is capable of providing data for small genome analysis, or performing targeted screening, that promises the high quality *in novo* assembly and structural variation detection (Lu *et al.* 2016). The emergence of next generation sequencing (NGS) or massive sequencing has generated a huge number of sequences available at low cost to explore microbial structure with higher resolution (Liu *et al.* 2012). To date, fourth-generation DNA sequencing technology has the potential to quickly and reliably sequence the entire human genome. This technology allows us to further study the interplay between DNA and protein, as well as between protein and protein (Feng *et al.* 2015).

The evolution of this method may allow researchers to discover and monitor species interactions under various environmental conditions and within a replicated experimental design framework (Vacher *et al.* 2016). Despite their ability to produce only very short reads, NGS technologies have revolutionized genome analysis. The major advances are such as high speed, cell-free library construction, ability to run thousands

to millions of sequencing reactions in parallel, direct detection without the need for electrophoresis, and sequencing in real time. These technologies are becoming a potential tool for gene expression analysis, especially for those species having reference genome sequences already available time (Rajesh and Jaya 2017).

## Pyrosequencing Analysis

Pyrosequencing is a DNA sequencing technique-utilizing bioluminescence coupled to enzymatic reactions and control the incorporation of nucleotides that are accompanied by release of pyrophosphate in real time. It is the first alternative to the conventional Sanger method and is based on the detection of pyrophosphate during DNA synthesis. It has advantages of precision, flexibility, parallel processing, and easy automation. Furthermore, the technique does not need to use primers, labeled nucleotides, or gel electrophoresis (Fakruddin and Chowdhury 2012).

The method comprises binding DNA to single strands of a sphere (a strand by area), through an adapter, after which they are subjected to *in vitro* cloning. After the spheres are charged, the addition of the polymerase DNA enzymes occurs by means of sulfurylase, luciferase ATP, and apyrase, with APS and luciferin substrates. Then the reaction solution, *i.e.* one dNTP at a time, in cycles is added. The cascade begins with the release of pyrophosphate, which is converted to ATP by sulfurylase enzyme in the presence of APS. The ATP produced drives the conversion of luciferin to oxyluciferin, generating light. The emitted light is detected by a Charge Coupled Device (CCD) camera and is observed as a pic in the pyrogram, proportional to the number of nucleotides incorporated (Fakruddin and Chowdhury 2012). All these steps replace the intense labor of cloning individual DNA molecules and eliminate biases that can be introduced by cloning a population of fragments. Later, a computer records the light release, logs the sequence of the DNA in each well, and interprets the data to align smaller bits of sequence into a full genome sequence (Margulies *et al.* 2005). The method has the capability of delivering explicit information within minutes. Furthermore, is able to produce the longest reads of any NGS system, about 700 bp, and approaches the data generated by Sanger chemistry (Myllykangas *et al.* 2012).

Ketep *et al.* (2014) analyzed microbial-anodes (graphite plates) in effluents from a pulp and paper mill. The microbial communities of the six bioanodes were characterized after one month of polarization. The sequencing run was performed on a 70675 GS PicoTiterPlate by using a Genome Sequencer FLX System (Roche, Nutley, NJ) and the sequence was trimmed to Q25. The most abundant taxonomic group was Proteobacteria (40 to 50%) in all bioanodes and, among them, Deltaproteobacteria. Recently, bacterial communities were examined in a river close to the dumping sites of pulp and paper mills. The 16S RNA V5–V6 region was amplified and sequenced by a MiSeq Sequencing system (Illumina). The results indicated that bacterial communities in downstream sediments were similar to those in paper mill discharge sites. The following genera were identified: *Leadbetterella* spp., *Rheinheimera s*pp., *Rhodobacter* spp., *Thiobacillus* spp., *Algoriphagus* spp., *Polaromonas* spp., *Flavobacterium* spp., and *Psychrobacter* spp. (Guo *et al.* 2016).

## Single-cell Genome Sequencing

The field of single-cell genomics is developing rapidly. This has produced many new insights into complex biological systems, ranging from the diversity of microbial

ecosystems to the genomics (Gawad *et al.* 2016). For example, microbial communities were examined in aquatic environmental, using single-cell genomics (Brown *et al.* 2015), generating complete or near-complete genomes from many novel lineages. The steps involves: cell isolation, amplification, interrogation of whole-genome-amplification (WGA) products, overview of single-cell sequencing errors, single-cell variant calling, and determining genetic relationships between single cells. The challenges for this method are efficient separation and amplification of cells (Gawad *et al.* 2016).

### Massively Parallel Signature Sequencing (MPSS)

Massively parallel signature sequencing is a high-throughput sequencing technology, based in the combination of *in-vitro* cloning of template DNA onto microbeads by making a complex mixture of template oligonucleotide tag conjugates, followed by cycles of ligation-based DNA sequencing (Rajesh and Jaya 2017).

A mixture of adapters (that includes all possible overhangs) with a type II restriction endonuclease is annealed to the target sequence, generating a perfect complementary single adapter. Each adapter has a unique label, and the overhangs that they represent are first detected after ligation. As the numbers of oligonucleotide tags are presented at a magnitude of 100 times more than the template, it is ensured that every template sample is conjugated to a unique tag. By monitoring successful adapter ligations onto a surface of microbeads in a flow cell, millions signatures are obtained. All of microbeads are employed, and a single copy of the template is attached to each bead. The next sequencing reaction results produces of millions of signature sequences. Later, scission with a type IIs endonuclease further show other bases for identification in subsequent cycles. Whereas each microbead is subjected to successive cycles of ligation, signature identification, and cleavage, the use of the flow cell ensures that all the microbeads remain in a closely packed monolayer. Fluorescent signals from the array of microbeads are acquired onto a CCD camera leading to a digital representation of each microbead, and image-processing software then tracks the positions of fluorescent signals from individual microbeads in the flow cell (Brenner *et al.* 2000; Rajesh and Jaya 2017).

## CONCLUSIONS

Nowadays, a great variety of molecular methods are available that provide more information than the culture methods. That is, microorganisms can be identified without being seeded in culture media. These molecular methods help researchers to better understand the diversity of microorganisms and understand how biological processes occur. Quantitative assessment of microbial communities is the greatest challenge due to significant biases associated with nucleic acid isolation and amplification by PCR. It requires more advanced DNA/RNA extraction techniques for environmental samples.

NGS technologies has revolutionized genome analysis, allowing the identification of all microbial communities. NGS in combination with fingerprint methods allow the identification and analyze all biodiversity of bacterial communities. It is possible to monitor them through the debugging process (*i.e.* DGGE and T-RFLP); to characterize and measure abundance of complete bacterial communities (i.e. qPCR and NGS). In the case of ARDRA and TGGE methods, there are no reports or studies on the application of

these techniques in pulp and paper effluents for identification of microorganisms; nevertheless, ARDRA can be used to monitor specific species.

The application of traditional and molecular techniques, has allowed glimpsing into the "black box", and getting information to improve the wastewater treatment process. However, it is necessary to establish a procedure for correlating the microbiological data with the depuration process. Monitoring the changes in the microbial population as a function of the substrate composition, environmental conditions and, eventually seasonal changes.

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