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ENVIRONMENTAL MICROBIOLOGY - REVIEW

Wastewater microbial diversity versus molecular analysis at a glance: a mini-review

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Abstract

Microorganisms play a vital role in biological wastewater treatment by converting organic and toxic materials into harmless substances. Understanding microbial communities' structure, taxonomy, phylogeny, and metabolic activities is essential to improve these processes. Molecular microbial ecology employs molecular techniques to study community profiles and phylogenetic information since culture-dependent approaches have limitations in providing a comprehensive understanding of microbial diversity in a system. Genomic advancements such as DNA hybridization, microarray analysis, sequencing, and reverse sample genome probing have enabled the detailed characterization of microbial communities in wastewater treatment facilities. This mini-review summarizes the current state of knowledge on the diversity of microorganisms in wastewater treatment plants, emphasizing critical microbial processes such as nitrogen and phosphorus removal.

Keywords Wastewater microbial diversity · Molecular techniques · Metaproteomics · Nitrogen removal · Phosphorus removal

Introduction

Wastewater treatment (WWT) involves removing toxic chemicals and suspended solid particles from contaminated water to produce environmentally and ecologically safe effluent. One of the most extensively studied approaches in WWT is the use of microbial flora [1]. Thus, the waste-degrading communities of microorganisms are the core components of wastewater treatment plants (WWTPs). They were subjected to many studies as the effectiveness and standard of the treatment processes depend on the makeup and capability of microbial communities [2]. Conventional microbial diversity analysis methods in WWTPs are community-level physiological profiling, plate counts, and fatty acid analysis [3]. These methods work mainly on the presumption that culture-dependent techniques aid in the isolation of most organisms present in the sample, leaving the fact that the actual extent of microbial diversity is much beyond expectations. This is one of the significant disadvantages of the conventional methods, making them inappropriate for diversity studies in natural and engineered ecosystems [4]. Before the arrival of culture-independent techniques, the leading players in WWT processes were barely known, and this situation was reversed in the last decade with the advancements in molecular techniques [5].

The current state of knowledge on the diversity of microorganisms in WWTPs is summarized in this article, focusing on critical microbial processes carried out by specific types of microbes, such as nitrogen and phosphorus removal bacteria.

Molecular techniques for microbial diversity analysis—a summary

Polymerase chain reaction (PCR) is one of the significant contributions of molecular biology that has been practiced for decades to study the detection, expression, and diversity of genes coding for ribosomes and proteins in natural and engineered ecosystems [6]. It facilitates the detection of essential microbes involved in different processes like

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ammonia oxidation, carbon degradation, and phosphorus removal [7]. However, the primer-based problems and inherent PCR amplifications can limit the quantitative data extracted from these methods [8]. Nucleotide hybridization approaches like fluorescent in situ hybridization (FISH) use target-specific oligonucleotide probes that can overcome PCR-related biases when applied to find the dominant group of microbes in WWTPs [9]. Other molecular methods such as denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP), random amplified polymorphic DNA (RAPD), and amplified ribosomal DNA restriction analysis (ARDRA) have been used to investigate microbial structure and diversity in different treatment systems [10]. Major molecular techniques used to analyze microbial diversity in WWTPs and their advantages and disadvantages are summarized in Table 1.

Besides the typical applications of molecular techniques, the commencement of the omics era has become a milestone in the study of functional and phylogenetic diversity in WWTPs [22]. The first attempt to understand the functional capacities of microorganisms relevant to WWT was the whole-genome sequencing of isolated pollutant-degrading bacteria (Table 2).

Table 1 Molecular techniques in microbial diversity analysis and their advantages and limitations

Techniques	Advantages	Disadvantages	Ref
Microarray	It allows the simultaneous detection of many species It is quick and accurate	There may be low signal intensity due to improper probe and DNA interaction	[11]
FISH	Easy detection and analysis It does not require professional trainers to conduct experiments	It is difficult to find targets with low DNA copies Laborious and time-consuming method	[12]
qRT PCR	It allows real-time monitoring of the amplification	Rather than determining the number of cells, it calculates the number of copies of the tagged gene	[13]
DGGE	Sensitivity is good, and bands may be excised from gels for amplification and sequencing	Dissimilar DNA sequences from distinct bacterial species may show the same separation due to equal GC contents	[14]
ARDRA	It is a simple and accurate method Rapid and cost-effective Compatibility with other techniques, such as PCR	When compared to other fingerprinting techniques, it has a weaker discriminatory power	[15]
RISA	It has a high degree of discrimination and is less prone to provide inconsistent findings	Only changes in ISR (Intergenic Spacer Region) fragments are detected Bacteria with the same ISRs will be unable to be distinguished	[16]
T-RFLP	Sensitive and reliable method Fluorochrome-based detection system provides accuracy	Restriction enzymes are specific and may therefore vary for different bacteria Limited taxonomic resolution Difficulty in fragment identification Lack of sequence information Limited insights into functional potential	[12]
454 pyrosequencing	With paired-end sequencing, the average read length can reach 1000 bp	High-cost technology It can only sequence short-length nucleotide sequences	[17]
MPSS	The level of unique gene expression is represented by the count of transcripts present per million molecules	Several transcripts can be lost due to a lack of restriction enzyme recognition sites and ambiguity in tag annotation	[18]
Illumina sequencing	Bridge amplification for clonal amplification With paired-end sequencing, the average read length can reach 300 bp	High-cost technology GC bias Short read lengths	[19]
Iron torrent method	Emulsion PCR for clonal amplification With paired-end sequencing, the average read length can reach 400 bp	High-cost technology Short read lengths compared to Illumina and other sequencing methods Limited scalability Inaccuracy in indels detection	[20]
Single-cell genome sequencing	It helps to detect heterogeneity among individual cells	Initial isolation and culturing of single cells are required	[21]

Table 2 Wastewater microbes for which the whole-genome sequencing was completed

Function	Organism	Refer
Nitrogen removal	*Nitrosomonas europaea*	[23]
	Nitrobacter hamburgensis	
	Paracoccus denitrificans	
	Nitrospina gracilis	[24]
Carbon and toxic removal	*Anaerobaculum mobilis*	[25]
	Thauera sp.	
Phosphorus removal	*Tetrasphaera jenkinsii*	

Major processes in WWT and identification of key players using molecular techniques

In the last decade, studies on microbial community structure in various WWTPs were initially conducted with the applications of molecular methods such as T-RFLP, cloning, DGGE, and FISH. These studies reported the dominance of Proteobacteria and the presence of some other groups, such as Actinobacteria, Firmicutes, Bacteroidetes, and Planctomycetes, in activated sludge samples. Furthermore, these findings have been confirmed with the help of HTS (high-throughput sequencing) technologies [26]. For instance, Oueslati et al. (2022) [9] assessed bacterial diversity in three water qualities: industrial poultry wastewater, tap water, and a mixture of both. They employed culture-independent techniques to analyze the microbial composition, including DGGE-PCR and sequencing. The study yielded a collection of 44 strains, out of which 25 were identified through sequencing. The dominant group among the isolated bacteria was Proteobacteria, accounting for 76% of the strains. Specifically, Gamma-Proteobacteria represented the majority, comprising 68% of the isolates. Other identified strains belonged to the phyla Firmicutes (8%), Bacteroidetes (12%), and Actinobacteria (8%).

Furthermore, metagenomic studies also help to determine the dominant functional microbial groups and key drivers of protein, carbohydrate, lipid, and aromatic hydrocarbon metabolism in WWTPs [27]. These approaches have proved that bacteria are responsible for most carbon removal, while Archaea serves less. Sharma et al. (2021) [28] conducted a study on the characterization of microbial communities present in wastewater from the pulp and paper industry. By analyzing the sequence alignment of the 16S rRNA V3-V4 variable regions with the Illumina MiSeq platform, they identified a total of 25,356 operational taxonomic units (OTUs). The major phyla detected in the wastewater comprised Proteobacteria, Bacteroidetes, Firmicutes, Chloroflexi, Actinobacteria, Spirochetes, Patesibacteria, and Acidobacteria, and other miscellaneous phyla that included unidentified microorganisms. Similarly, the analysis of wetland samples by pyrosequencing revealed the presence of a wide range of microbial phyla, such as Verrumicrobia, Planctomycetes, Nitrospirae, Cyanobacteria, and Gemmatimonadetes, apart from Proteobacteria. When these results were analyzed with metagenomic tools, all these studies affirmed the prevalence of Proteobacteria in all the wastewater processes [29]. The major processes and key players of WWTPs so far identified using molecular tools are discussed below.

Nitrogen removal

The primary nitrogen removal mechanisms, nitrification and denitrification, are crucial in wastewater processes. Ammonia and nitrate contribute to eutrophication and cause a significant threat to aquatic life. Some aerobic ammonia-oxidizing bacteria (AOB) (proteobacterial ammonium oxidizers) and anaerobic ammonia oxidizers can oxidize ammonia in wastewater [30]. Depending on oxygen availability, these bacteria oxidize ammonia aerobically or anaerobically (anammox). They are divided into Betaproteobacteria ammonia oxidizers (*Nitrosomonas* and *Nitrosospira*) and Gammaproteobacteria ammonium oxidizers (*Nitrosococcus mobilis*). Under aerobic conditions, they produce nitrite, while nitric oxide, dinitrogen, and nitrite are produced during anaerobic conditions. Zhu et al. (2019) [31] studied the microbial diversity of three sequential bioreactor (O_1/H/O_2) systems during the removal of ammonia using high-throughput MiSeq sequencing by examining the 16S rRNA genes. Results revealed a contrasting microbial composition among the activated sludge samples of the three sequential bioreactors. The β-Proteobacteria-related sequences dominated in the O_1-activated sludge with a relative abundance of 56.44%, while 7.53% of the sequences were assigned to *Thiobacillus*. *Rhodoplanes*-related sequences dominated in the bioreactor H and O_2-activated sludge with the relative abundance of 8.86% and 8.92%, respectively.

Anaerobic oxidation of ammonia (AOA) is a relatively new and understudied process controlled by a class of bacteria known as Planctomycetes. Ali et al. (2013) [32] identified five genera of bacteria (*Candidatus anammoxoglobus*, *Candidatus kuenenia*, *Candidatus scalindua*, *Candidatus brocadia*, and *Candidatus jettenia*) in the phylum Planctomycetes involved in anaerobic nitrogen removal by culture-independent techniques.

Similarly, some heterotrophic and Archaea bacteria were also reported for ammonia oxidation [33]. The role of Archaea bacteria in WWTPs has not been extensively studied. Moreover, the results obtained by comparing the quantity of AOA and AOB during WWT using molecular techniques were contentious [34]. They showed either the equal or minimal dominance of AOA over AOB or the complete absence of AOA [35]. These findings do not

allow for a definitive conclusion. The role of Archaea bacteria in ammonia oxidation and the conditions under which they thrive must thus be better understood.

The aerobic nitrite-oxidizing bacteria, which generally include members of the genera *Nitrospira*, *Nitrococcus*, and *Nitrobacter*, execute the second step of oxidation, i.e., nitrite oxidation (nitrite to nitrate) [36]. However, little information about complex nitrite-oxidizing microbial communities and their ecological niche variations is available. In a diversity study integrating multiple molecular approaches, Gruber Dorninger and colleagues (2015) [37] have identified distinctive *Nitrospira* clusters with functional distinctions involving spatial co-aggregation with other AOB. They also detected 121 nxrB (encode the nitrite oxidoreductase beta subunit) OTUs of *Nitrospira* at the species level, exhibiting incredible diversity. In recent years, molecular methods like DGGE [9], metagenomics [38], FISH [8], and others have aided in identifying microorganisms participating in nitrification during nitrogen removal (Table 3).

Denitrification, or the conversion of nitrate or nitrite into nitrogen or nitrous oxide (N_2 and N_2O), is a phylogenetic capacity found in many organisms. Different types of bacteria, such as chemoorganotrophic, litho-autotrophic, and phototrophic bacteria, and other microbes like Archaea and fungi participate in this process. Environmental conditions such as high nitrogen oxide and low oxygen levels activate this facultative character. The structure and function of these wastewater denitrification communities were studied using molecular methods [43]. Denitrification is carried out by different genera such as *Pseudomonas*, *Bacillus*, *Alcaligenes*, *Paracoccus*, and *Methylobacterium*, and members of the order Rhodocyclales [36]. Novel mechanisms involving non-conventional types of denitrification have recently been discovered using genetic analysis. Non-denitrifying species, such as *Anaeromixobacter dehalogenans*, can catalyze the reduction of N_2O to N_2 via an atypical nitrous oxide reductase [44], while other microbes, like *Candidatus methylomirabilis oxyfera*, have a unique mechanism of combining N_2 synthesis with the anaerobic methane oxidation [45].

Table 3 Some molecular studies in activated sludge where nitrifying microbes are detected

Method	Nitrifying bacteria	Ref
Metagenomics	*Nitrosomonas*, *Nitrosovibrio*	[39]
454 pyrosequencing	*Nitrosomonas*	[40]
FISH, DGGE	*Nitrosomonas*-like species	[41]
Cloning, FISH	*Nitrospira*-like species *Nitrococcus*	
DGGE, 16S rRNA gene cloning	*Nitrosomonas*	[42]

Phosphorous removal

Phosphorus (P) can also cause eutrophication in receiving waterways, prompting the development of biological and chemical treatments to remove it. Enhanced biological phosphorus removal (EBPR) is a biological technique for removing P. Polyphosphate accumulating organisms (PAOs) are the major participants in this phosphorus removal activity. These microbes collect polyphosphate intracellularly and then waste P-rich sludge to eliminate phosphorus from the system [46]. Jena et al. (2016) analyzed the diversity of phosphate-removing bacteria in high-strength wastewater treated in an anoxic–aerobic sequencing batch reactor. The results revealed Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, and *Paracoccous* as the prominent phylum, class, order, family, and genus, respectively [47]. Modern molecular and metagenomic techniques appear to have become critical in identifying the key players involved in EBPR [48]. Recent studies highlight that EBPR reactors were selectively enriched in *Rhodocyclus*-related organisms [49]. The FISH technique detected the uncultivated bacterium *Candidatus accumulibacter phosphatis* as a major PAO [50]. Furthermore, EPBR enriched with *C. phosphatis* was subjected to metagenomic analysis to study its metabolic versatility. Several studies have been published emphasizing the usefulness of *–omics* methods in providing unique biological insights into WWT and elucidating the function of distinct community members [46]. These studies validated the presence of many genes involved in nitrogen metabolism in the *Accumulibacter* genome. They indicated that *Accumulibacter* clades had similar metabolic properties regarding phosphorus and carbon consumption. Many other bacteria have been identified as possible EPBR species using culture-independent approaches in addition to *C. phosphatis* and *Accumulibacter*. For instance, Myeong and his coworkers (2013) [51] identified many *Dechloromonas* genus (Betaproteobacteria) bacteria as possible PAOs. They accumulated polyphosphate and contributed to denitrification during the EPBR processes.

Metaproteomics

Metaproteomics, a subset of proteomics, has swiftly established itself as a critical tool for the worldwide functional evaluation of the microbiome system. The analysis of all protein samples collected from environmental sources is called metaproteomics (community proteomics or community proteogenomics) [52]. It allows the large-scale characterization of the whole proteins of ambient microbiota at a given point in time [53]. Metaproteomics can also provide information on microbiome composition by measuring the biomass contributions of diverse species. Since protein constitutes the

vast majority of cellular material in most bacteria, proteins discovered and quantified by metaproteomics can be used to calculate species biomass [54]. Metaproteomics principles are based on advances in proteomics techniques, such as 2D gel electrophoresis for identifying proteins and peptides in a specific microbial population. Mass spectrometry (MS) coupled with liquid chromatography (LC–MS) or capillary electrophoresis (CE-MS) techniques is commonly used for protein identification and analysis. In these approaches, the separated proteins are ionized and introduced into the mass spectrometer for detection and characterization. Matrix-assisted laser desorption/ionization (MALDI) is used to identify and separate proteins while being combined with TOF–MS (time-of-flight mass spectrometry) to produce superior results. When high-performance liquid chromatography (HPLC) and liquid chromatography (LC) for peptides and proteins are combined with computer techniques to sequence them, metaproteomics procedures become more advanced [55]. Wilmes and his colleagues (2008) [56] have successfully used metatranscriptomics for the functional analysis of microbes in activated EPBR and EPBR sludge during WWT in a bioreactor. The 2D gel protein analysis found that the uncultured polyphosphate-accumulating organism *C. phosphatis* dominated the microbial communities. Similarly, Li et al. (2019) [52] analyzed the bacterial community structure of wastewater sludge by using mass spectrometry coupled with 2D protein profiles. The results demonstrated that most proteins exhibiting differential expression profiles during the process were derived from *Burkholderiales*populations. Unstable species distribution, a wide range of protein expression levels among microorganisms, and substantial genetic heterogeneity within microbial communities are the challenges to metaproteomics investigations. Despite these obstacles, metaproteomics offers enormous potential for linking microbial community, genetic diversity, and activity to their impact on ecosystem function [57].

Future directions of microbial diversity studies in WWTPs

Even though we have various methods to identify and characterize microbes in wastewater processing, numerous issues still need to be resolved. One such problem is understanding the interactions between different microbes during a specific process and determining whether these interactions are beneficial. Molecular-omics techniques may provide ecological insights into these questions. The combination of metagenomics, transcriptomics, and comparative gene expression studies helps describe the pattern of gene expression during a particular process, but such studies are currently scarce. Sequences encoding hypothetical proteins must be thoroughly investigated to characterize them, requiring tedious experimental work. More effort should be dedicated to resolving these issues to enhance our knowledge of the functional capabilities of microbial communities in WWTPs.

Conclusion

This minireview highlights the current status of techniques used for biodiversity studies in various WWTPs, which harbor dynamic and diverse microbial populations with a wide range of critical metabolic processes. Molecular techniques are rapidly emerging as the preferred method for studying microbial communities, providing a foundation for various applications, particularly in connecting metabolic potential with gene function and regulatory mechanisms. By integrating existing molecular techniques and expected advancements in new procedures, novel ecological methods can be developed to unravel the mysteries of structural and functional correlations in wastewater microbiology.

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Data Availability All data are presented in this manuscript.

Declarations

Conflict of interest The authors declare no competing interests.

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