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Microbial Ecology of the Denitrification Process and Its Application in Wastewater Treatment: Challenges and Opportunities

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Abstract: Denitrification is a critical microbial process for nitrogen removal in wastewater treatment, offering a cost-effective and sustainable alternative to conventional chemical and physical methods. This review synthesises current knowledge on the microbial ecology of denitrification, focusing on the diversity, physiology, and community dynamics of denitrifiers in biofilms and activated sludge systems. Key bacterial genera, including Pseudomonas, Paracoccus, Hyphomicrobium, Comamonas, and Azoarcus, play dominant roles, with carbon sources such as methanol, ethanol, acetate, and waste-derived substrates strongly shaping community structure and function. Advances in molecular approaches—such as PCR-based techniques, stable isotope probing, fluorescence in situ hybridisation, metagenomics, and transcriptomics—have provided new insights into microbial diversity, gene expression, and metabolic pathways, linking ecological patterns with treatment performance. Applications of denitrification span conventional activated sludge processes, biofilm reactors, and emerging autotrophic methods such as anammox, which enhance nitrogen removal efficiency. Despite these advances, operational challenges remain, including incomplete denitrification, seasonal failures, greenhouse gas emissions, and limited predictability of microbial responses to environmental shifts. Integrating molecular data into process models and optimising carbon source utilisation represent key strategies for future improvement. This review highlights the opportunities and challenges in bridging microbial ecology with engineering practices, ultimately advancing wastewater treatment technologies toward greater sustainability and resilience.

Keywords: Denitrification, Microbial Ecology, Wastewater Treatment, Carbon Sources, Molecular Techniques

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1. Introduction

For thousands of years, medicinal plants have served as an integral part of human health care, and nitrogen is known to be an important part of life for everyone. But an excess of it in some parts of the environment can pose serious environmental issues. It is true for some species of nitrogen in the environment and in aquatic and terrestrial environments. Nitrate has been one of the vital environmental challenges due to its implications for animal and human health. Nitrate enters the body through drinking water from the ground and causes a lot of health issues, like gastric cancer, goitre, methemoglobinemia, hypertension, and birth defects, when it is consumed in high amounts through groundwater for drinking [1]. Since nitrate is a problem, it is important to remove nitrate from the water resources to avoid environmental damage. Many nitrogen removal processes are employed, such as reverse osmosis (RO), ion exchange, air stripping, nitrification, breakpoint chlorination, and denitrification. This paper is centred on microbial denitrification because it is one of the most economical and cost-effective processes compared to other processes used in the removal of nitrogen in drinking water. It is a practical method as well on a large scale. Biological

denitrification involves the reduction of nitrates using various enzyme reactions. Most bacteria can multiply and proliferate by regulating ionic oxides to gaseous components [2].

The bacteria respirate on nitrate, which converts it to nitrogen gas via various reactions. The terminal acceptor of electrons involved in this respiratory process is nitrites or nitrates and not oxygen. This is what is called denitrification. Reduction of nitrate requires a source of carbon, and this can be oxidised as a source of electrons. Liquid, solid, and gaseous sources of carbon, such as methanol, ethanol, fatty acids, acetic acid, wheat straw, newspaper, sugar, unprocessed carbon fibre, sugarcane, synthetic granules of polyester, biodegradable water-insoluble polymers, and natural organic components (bark of a variety of trees, straw, etc.) are abundant [1]. The denitrification process is carried out by the facultative aerobic bacteria that use nitrite during respiration without oxygen as a terminal electron acceptor. The majority of the procedures of microbial denitrification occur on heterotrophic bacteria, which require a natural carbon substrate. A typical carbon compound is generally employed, such as methanol, ethanol, or acetate. It is, however, necessary to consider more readily accessible and inexpensive sources of carbon [2]. It is the purpose of this paper to review the process of denitrification of microbial communities and nitrogen, other than the microbiology of this process. Briefly, molecular methods employed in the denitrification of microbial compounds will also be discussed [4].

Phosphorus, as well as nitrogen, is essential to the growth of microbes, and the growth of animals and plants is known as bio-stimulants or nutrients. Some minerals, such as iron, are also needed in small quantities to grow biologically. Phosphorus and nitrogen are essential nutrients in the majority of situations. Nitrogen is an important essential protein component. It may be important to treat the waste because a lack of nitrogen can render it valuable. The growth of nitrogen or algae in the water should be put in check. Before discharging, it is worth reducing or eliminating the nitrogen in wastewater [3]. A significant amount of wastewater contains nitrogen, including industrial wastewater and municipal wastewater, as well as stormwater runoff, particularly agricultural and urban runoff. Nevertheless, in a storm, waters are extremely erratic and dispersed in their origin, and therefore, any problem is not receptive. Sodium nitrate, nitrogenous animal and plant investments, and wild nitrogen are some of the typical nitrogen sources. The chemistry of nitrogen is extremely complex because of the multiple oxidation conditions that can be assumed by nitrogen, as well as because oxidation is supposed to alter [5].

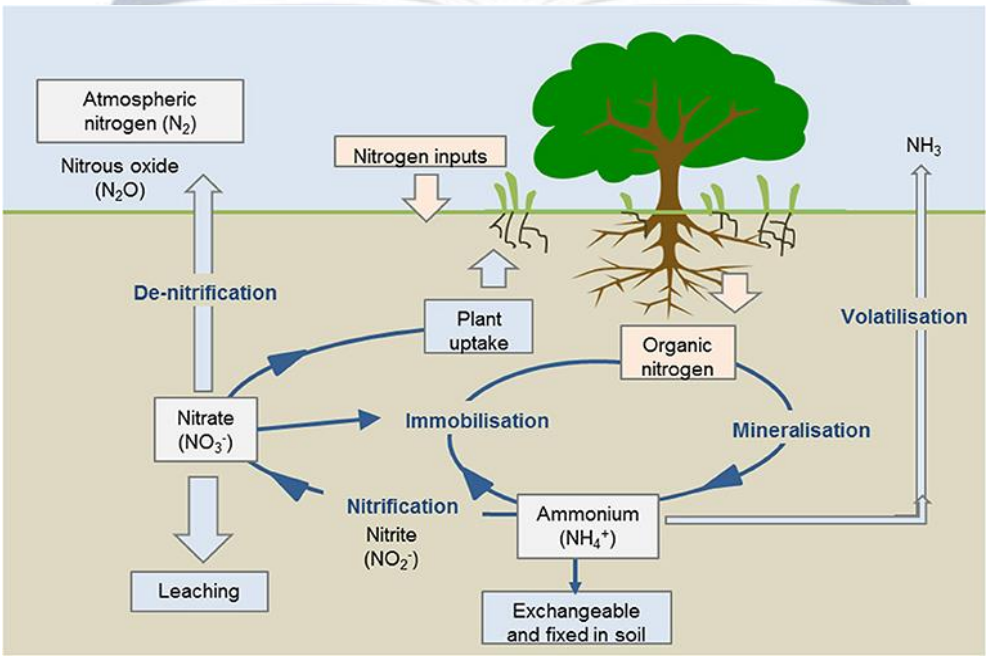

Figure 1: Nitrogen Cycle [4]

Along with N_2, nitrogen compounds in the atmosphere in all states of oxidation can cause environmental challenges. Nitrogen is changed well in oxidation, which adds to the complexity and in the form of chemicals with biological, natural, photochemical, and chemical processes. From one oxidation state to another, transformations can be done by living organisms in the environment. Relations between these transformations and nitrogen forms are usually expressed easily in the form of the "Nitrogen Cycle" illustrated in Figure 1. The nitrate, which is formed in the environment, may act as a fertiliser for plants. Excess nitrate is transferred to the water percolating through the soil, as the soil cannot hold nitrate. It releases a very high amount of nitrate in groundwater. Nitrate is extracted into nitrite under anaerobic conditions. This process is termed "denitrification" [4].

2. Biological Denitrification for Nitrogen Removal and Denitrifying Microorganisms

Biological denitrification is the most versatile and promising method to study. This process is very effective and has been used on a large scale in wastewater treatment for ages. This process is best used for nitrate removal. This process is highly efficient and can easily beat any other method for 100% nitrate reduction. The major con of this process is that treated water can be contaminated with bacteria. Subsequent disinfection and treatment are needed to meet the existing standards for drinking water. Without having a proper supply of oxygen which has dissolved molecules to be used in respiration, a lot of heterotrophic bacteria can be turned into the use of nitrate as an alternate acceptor of electrons.

This denitrification process reduces nitrates to nitrite and then turns it into a gaseous form. Energy yield is known to be lower than that from the respiration of oxygen. This way, denitrification works only at low concentrations of dissolved oxygen. An external source of carbon is also needed for the optimum rate of reaction, even though the process proceeds by "endogenous respiration" at a lower rate. Some of the carbon sources used are carbohydrate wastes and settled wastewater. Nitrogen can also be removed with methanol, as it has been quite cost-effective, easily available, and provides easier control over the process. Here is the simple formula of reactions when methanol is used as the source of carbon –

$$6NO_3^- + 2CH_3OH \rightarrow 6NO_2^- + 2CO_2 + 4H_2O \quad (1)$$

$$6NO_2^- + 3CH_3OH \rightarrow 3N_2 + 3CO_2 + 3H_2O + OH^- \quad (2)$$

Total reaction of energy would be like -

$$6NO_3^- + 5CH_3OH \rightarrow 6N_2 + 5CO_2 + 7H_2O + OH^- \quad (3)$$

In order to improve water quality in a lab study using biological denitrification, Singh et al. [5] reduced nitrate nitrogen to 0.57 mg 1-1 from 50.79 mg 1-1 with a special "Pseudomonas stutzeri" culture. Denitrification of completely nitrified effluent can be possible by giving a zone where effluent has contact with a huge biomass having "heterotrophic microorganisms", for example, which are usually found in several aerobic processes of treatment in anoxic settings, and with a suitable source of exogenous carbon. The reaction will go ahead by cellular material of "endogenous decay", but at a significantly lower rate. There are cases when it is possible to accept partial denitrification to achieve up to 80% of nitrogen with settled sewage as the source of carbon. It can be used to nitrify "activated sludge plants" with well-placed anoxic zones, like midway and inlet end, apart from the aeration tanks. There is a vast capacity of denitrification in both archaeal and bacterial domains, and denitrifying communities are diverse in other environments, and they are higher than those in the processes of wastewater treatment [6]. The richness of species and high diversity of "denitrification communities" are still shown in "165 rRNA gene-oriented studies

(Figure 2). Bacterial strains separated from "denitrifying bioreactors" have close relations with "Pseudomonas", "Hyphomicrobium", "Paracoccus", and "Comamonas spp." in Proteobacteria [8-11].

However, there is a discrepancy between the widely separated stains and the ones ruling in the true denitrification systems like "Azoarcus, Zoogloea and Comamonadaceae spp." Most of them are affiliated taxonomically with "Bacteroidetes (16%)" and "Proteobacteria (59%)" (Figure 2). The "a, b, d, and g subclasses" take place in proteobacteria at very high levels as compared to "ε-Proteobacteria", and it has been verified with high levels of metagenomics [12]. Some of the techniques which further combine "community structure" with function by quantifying populations with particular metabolic elements in "wastewater denitrifying reactors" are "DNA-based stable isotope probing (DNA-SIP)" and "Fluorescence in situ hybridisation (FISH)". The phylogenetic presence of functional groups considering "radioactively-labelled substrates (like different types of electron donors or nitrate)" in situ can be examined by combining "FISH and Microautoradiography (FISH-MAR)" [13].

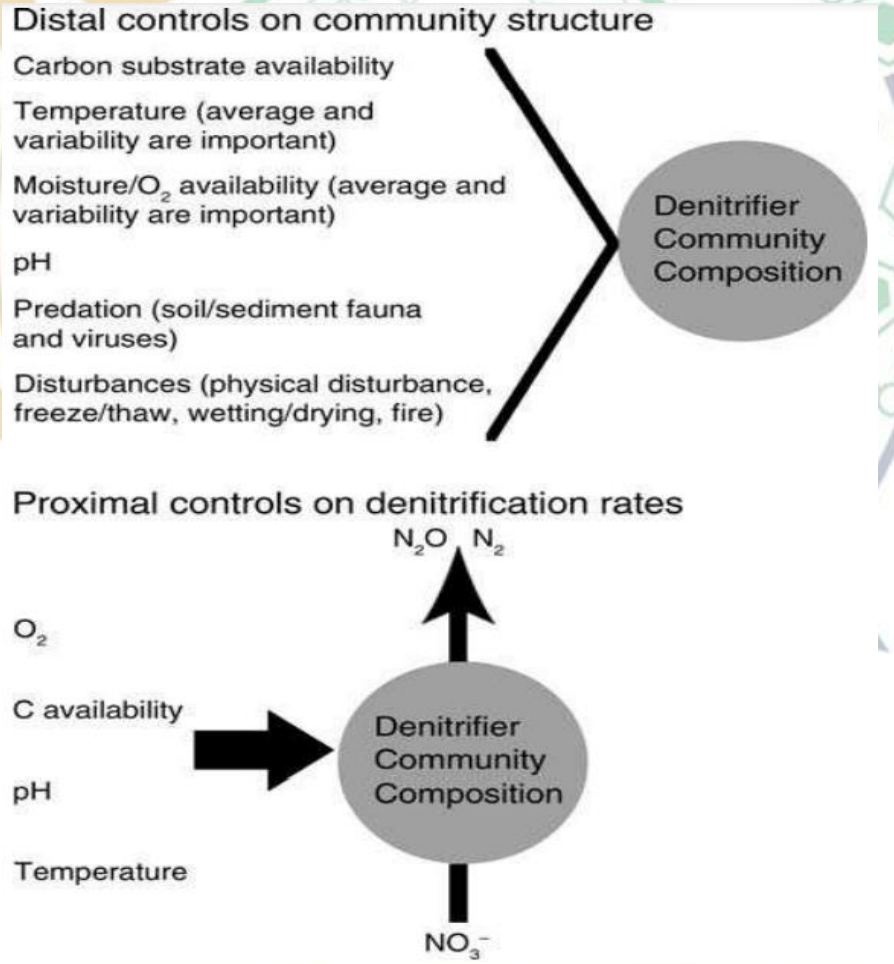

Figure 2: Proximal and Distal Controls on Denitrification and Denitrifiers [7]

At the same time, there has been a widespread use of "DNA-SIP assays" in studies related to wastewater denitrification to detect specific denitrifiers that can assimilate specific organic carbon [14][15]. Conventional molecular processes need existing information of "functional gene sequences" or 165 rRNA sequences for molecular techniques (Table 1). More approaches are needed related to functional genomics to look for new pathways, genes, and organisms in denitrification. Some of the issues in these analyses are a lack of throughput when it comes to recording rare species and profiling of several genes completely.

Table 1 – Application of Probes and Primers in Denitrification-related studies

Primer/probes	Sequences	Source
"968F/1401R"	"F: AACGCGAAGAACCTTAC; R: CGGTGTGTACAAGGCCCGGGAACG	[16]
"1960m2f/2050m2r"	"F: TAYGTSGGGCAGGARAAACTG; R: CGTAGAAGAAGCTGGTGCTGTT"	[17]
"341F/534R"	"F: CCTACGGGAGGCAGCAG; R: ATTACCGCGGCTGCTGG"	[18]
"11F/1392R"	"F: GTTTGATCCTGGCTCAG; R: ACGGGCGGTGTGTRC"	[19][20]
"nirS1fF/6R"	"F: CCTYATGGCCGCCRCART; R: CGTTGAACTTRCCGGT"	[21]
"cd3aF/R3cd"	"F: GTSAACGTSAAGGARACSGG; R: GASTTCGGSTGSGTCTTGA"	[22][23]
"Nos661F/1773R"	"F: CGGCTGGGGGCTGACCAA; R: ATRTCGATCARCTGBTCGTT"	[24]
"DEN67"	"CAAGCACCCGCGCTGCCG"	[25]

Various microorganisms have partial, complete or no activities of denitrification and there are five groups of it – "complete denitrifiers" (which can reduce both nitrite and nitrate to nitrogen), "complete nitrite reducers" (which can reduce nitrite to Nitrogen, but not nitrate), "incomplete denitrifiers" (which can reduce nitrite/nitrate to "nitrogen oxide intermediates" rather than nitrogen), "non-denitrifiers" (which cannot reduce nitride/nitrate), and "incomplete nitrite reducers" (which can reduce nitrite to "nitrogen oxide intermediates"). Some of the common microorganisms in some of the aforementioned categories are "Methyloversatilis spp. (incomplete denitrifiers)" and Hyphomicrobium spp. (complete denitrifiers) [26][27]. There are chances that bacteria with varied capacities to reduce nitrogen oxides play a role in sinking nitrogen in the wastewater denitrification process.

3. Microbial Ecology and Diversity of Denitrifying Microbial Communities

Secondary-treated wastewater that comes from a municipality usually consists of the right amounts of nutrients and oxidised nitrogen, which cause a serious risk of the "eutrophication" process in drinking water. In addition, an increased amount of nitrate was supposed to have both non-lethal and lethal impacts on several aquatic species that are commercially relevant [28]. "Denitrifying biological filter (DNBF)" is widely known to be an economical, effective, feasible, and stable technology to manage oxidised nitrogen from "secondary effluents" of wastewater treatment units used by municipalities [29]. The denitrifying process is conducted by DNBF with biological transformation of oxidised nitrogen and organics without oxygen by biofilms, which are fixed on granular media, while removing suspended particles by the filtration of the media. In the biofilm, denitrifying bacteria are vital to turn nitrate into nitrogen gas, while organic carbon is an important

factor to conduct the proper denitrification process as denitrifying donor of electrons. In secondary effluents, organic matters are usually low in meeting electron donor demands for anoxic energy and denitrification for cellular maintenance and growth [30].

Hence, outer organic carbon is needed for the process of "wastewater tertiary denitrification" to prevent the accumulation of nitrite and incomplete denitrification. It always consists of acetate, methanol, and ethanol (widespread sources of organic carbon) and alternative sources of carbon (such as hydrolysis products of solid waste and sludge) [31,32]. Under the specific hydraulic load, plant size, operation conditions and influent quality of water in denitrification, there are significant impacts of external organic carbon, bio-kinetics, and denitrifying [33]. Those efforts could be credited to various donors of electrons, i.e., external sources of carbon, which lead to various denitrifying ecosystems of microbes [34]. Additionally, types of carbon influence the expression levels of oxidation of carbon [35]. Using molecular techniques has been playing a vital role to determine exogenous source of carbon as one of the major factors defining the function and structure of the "denitrifying microbial community" during "anoxic denitrification" [40-42]. With the help of "stable-isotope probing", "fluorescence in situ hybridisation-micro-autoradiography (FISH-MAR)" and "full-cycle rRNA analysis" [36], the "acetate-fed and methanol-fed denitrifying community" is characterized into batch reactors. It is observed that bacteria named "Methylophilales" were prominent denitrifiers in the "methanol-fed denitrifying sequencing batch reactor", while other dominant denitrifiers were "Rhodocyclaceae" and "Comamonadaceae", were prominent in the "acetate-fed reactor". The microbial community differences have been characterised by Osaka et al. [43] between methanol and acetate-fed active sludge reactors as an external source of carbon with "terminal restriction fragment length polymorphism (T-RFLP)" and "cloning analysis".

According to Baytshtok et al. [14], "*Hyphomicrobium*" and "*Methyloversatilis*" were the common methylotrophic bacteria in the reactor and the level of "*Hyphomicrobium*" is reduced drastically when it comes to switch donor of electrons to ethanol from methanol with stable probing of ^{13}C 16S rRNA gene and quantitative, real-time chain reaction polymerase assays. Additionally, the impact of various sources of carbon on "denitrifying community structure" was also conducted with PCR techniques or techniques giving high throughput [40,41]. Molecular techniques definitely provide valuable details on the microbial denitrification community. However, a lot of studies related to the effects of various carbon sources on microbial systems were based on the suspension of sludge systems. In addition, more diverse communities are enriched by biofilm reactors than active sludge, but there are still significant knowledge gaps in biofilm systems [42]. Srinandan et al. [43] conducted a study to investigate the impact of various carbon sources, such as glucose, acetate, ethanol, and methanol, on the structure of biofilm. It is observed that the efficiency of nitrate removal was low in biofilm fed by ethanol, but there was a high number of denitrifying bacteria.

4. Factors Affecting the Structure and Functions of Denitrifying Microbial Communities

The importance of sources of carbon is known to dictate the form and functionality of denitrifying microbial communities as they have a direct effect on growth kinetics, adaptation period and metabolic routes. Although methanol has historically been the most actively used carbon feedstock to augment denitrification in wastewater treatment systems, many alternatives have also been examined with regard to the specific needs of the system. The source of carbon was found to have a more direct influence on the denitrifying community composition as compared to other factors, including C/N ratios or availability of electron acceptors [44]. The alternative energy pathways permit a diversified way of energy usage and organic carbon use, so it defines the initiation of the heterotrophic growth. Specifically, single-carbon compounds like methane, methanol and formate have different metabolic routes in methylotrophic organisms, in part because of the availability of enzymes to oxidise methanol, which are yet to be fully described [45]. Such biochemical variability is one of the reasons a more diverse microbial community pattern is frequently found in methanol-based systems

compared to systems based on multi-carbon compounds [46]. Both culture-dependent and culture-independent studies have continually identified organisms, including Hypha microbium spp, Paracoccus, Methyloversatilis and Methylophilus, as dominant members of methanol-fed denitrifying systems [47]. They are mainly related to 2-Proteobacteria and have been diagrammed using phylogenetic techniques, such as a neighbour-joining tree based on over 1003 partial 16S rDNA sequences (>500 bp) of the GenBank database, demonstrating their patterns of clustering (Figure 3) [48,49]. Other carbon sources, such as ethanol, have also received a lot of research. As an example, Hallin et al. [33] used nirK- and nirS-based restriction fragment length polymorphism (RFLP) testing on active sludge samples supplemented with ethanol and methanol to reveal different profiles of microbial community. Equally, acetate has found extensive use in denitrification systems, where microbial communities have been dominated by species in the families Rhodocyclaceae and Comamonadaceae, comprising such species as Thauera spp., Comamonas and Acidovorax [43]. Furthermore, various more complicated carbon sources have also been explored, e.g. Morgan-Sagastume et al. [50] tested the structure of the denitrifying community in conditions of mixed substrates and acetate addition, again highlighting the heterogeneity and flexibility of denitrifying microorganisms to carbon sources.

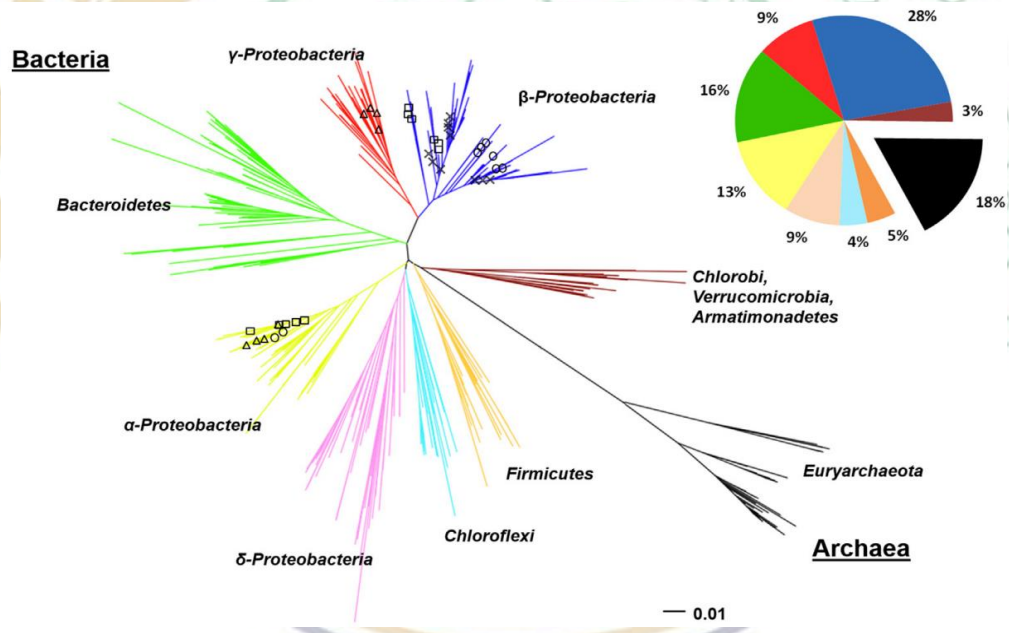

Figure 3: "Phylogenetic tree" of denitrification bacteria built by neighbour-specific method as per "1003 partial 165 rDNA sequencing" (>500bp) from GenBank [48,49]

Carbon populations – "□ Methanol; × Acetate; ○ Glycerol; △ Methane"

4.1 Wastewater Influent

Usually, domestic wastewater has around 10-40 mg N/L of organic nitrogen, or ammonia turned into nitrate when nitrification is done [51]. The industrial wastewater composition, which enters the stage of denitrification, varies drastically as per the industry and generally has a high amount of nitrate and other ions like sulfate and chloride [52]. Thomsen et al. [53] have done the comparison of denitrifiers in municipal and industrial wastewater treatment plants, and "Aquaspirillum-related and Azoarcus-related bacteria" were prevalent in both types of wastewaters, respectively.

4.2 Biofilm buildup

The higher heterogeneity of chemicals like metabolic intermediates, substrate gradients and products in biofilm enables bacterial groups to coexist with various metabolic strengths. Hence, biofilm basically builds up more diverse communities than the sludge, which is activated [54]. Since biofilm

systems are helpful to slow-growth bacteria like nitrifiers, some "N-removal" biofilm reactors are developed for denitrification and nitrification to be done at the same time. A novel process of a hybrid membrane was applied for the absolute removal of nitrogen by Downing et al. [54]. It was also found that heterotrophs were the major component in the bulk liquid, and there was a more diverse community than the biofilm, overwhelmed by nitrifiers.

4.3 Operating environments

"Solids Retention Time (SRT)", dissolved oxygen, pH, and other operating parameters affect the overall removal of nitrogen and denitrification and ensure a prolonged succession of diversity and community structure [56, 57]. Tan et al. [58] used "nirS-reliant t-RFLP and 165 rRNA" for comparing the impact of various "mean cell residence time (MCRT)" when composing microbial communities in anoxic areas of "pre-denitrification submerged MBRs".

5. Microbial Ecology and Diversity of Denitrifying Communities

The molecular strategies have been critical in showcasing our insight into the microbial ecology and diversity of the denitrifying communities, particularly in wastewater systems. PCR-based methods are commonly used where genomic regions are amplified using environmental DNA, and DNA fingerprints are constructed, which are used as community profiles to compare across samples. Several approaches have been implemented on their merits and demerits. As an example, Random Amplification of Polymorphic DNA (RAPD) is a low-cost technology with the disadvantage that it is sensitive to PCR conditions, allowing it to be used without the need to know any genomic background but being badly reproducible; however, RAPD has been successfully employed with pharmaceutical [59], municipal [60], and industrial wastewater [61,62]. A basic and accurate community profile is achieved using Amplified Ribosomal DNA Restriction Analysis (ARDRA), although not as efficient as subsequent sequencing tools, and has been used in industrial [63,64] and municipal wastewater [65]. In other methods of fingerprinting, Denaturing Gradient Gel Electrophoresis (DGGE) and Temperature Gradient Gel Electrophoresis (TGGE) are popular, separation techniques that separate DNA fragments on the basis of melting temperature and provide distinctive banding patterns to use in sequence analysis, but have limited species discriminatory capability. These have been applied in industrial [66,67] and municipal wastewater [68]. Terminal Restriction Fragment Length Polymorphism (T-RFLP) that integrates PCR amplification with fluorescent labelling and restriction digestion offers good sensitivity to quantify relative bacterial abundance, but findings are highly dependent on the restriction enzyme used, as demonstrated on industrial [69,70] and municipal wastewater [71]. Another method that has been used to differentiate populations based on intergenic spacer regions is Ribosomal Intergenic Spacer Analysis (RISA), but due to its limited discriminating power against closely related organisms, it has been used in pharmaceutical [75] and industrial wastewater [76]. Single-Strand Conformation Polymorphism (SSCP) has likewise been practical in the detection of nucleotide polymorphisms at relatively low cost; however, it also has limitations due to the lack of predictable models of the DNA fragment conformations. They have been used in gelatinaceous wastewater [72], industrial wastewater [73], and municipal wastewater [74].

RAPD specifically has been used to produce random pieces of DNA with low annealing temperature primers, resulting in the formation of many band patterns on a gel electrophoresis, reflecting the complexity of the microbial community [77]. Changes in amplicon length and quantity can be observed using these patterns, and the differing groups of microbes in the denitrification systems can be identified. The DGGE and TGGE are also known to separate the short and medium-length fragments of DNA based on their melting behaviour [78,79]. In DGGE, DNA that has been amplified via PCR is electrophoresed through gels that contain chemical denaturants such as urea and formamide, and when the partly melted molecules differ in their migration, they form a characteristic banding pattern that may subsequently be sequenced to determine the denitrifiers [80].

T-RFLP, on the other hand, utilises fluorochrome-labelled primers to amplify DNA fragments, which are subsequently digested by restriction enzymes. The fragment separations through electrophoresis generate community-specific signatures that are comparable between samples [81,82]. This renders T-RFLP especially appropriate in the comparative analysis of denitrifying communities in environmental research, especially of large scale.

Likewise, ARDRA is based on amplifying conserved ribosomal regions (typically the 16S rRNA gene), then digesting the product with tetra-cutter restriction enzymes to produce fragment profiles [83,84]. The banding pattern generated by this method is indicative of microbial composition and is an inexpensive, useful tool of community analysis despite its lower resolution than that of next-generation sequencing. RISA further discriminates where the ribosomal intergenic spacer region between the 16S and 23S rRNA genes is targeted, as this region is different in length and sequence. This region can be amplified using PCR to form fragments of various sizes, and these fragments can be separated and viewed to determine the diversity of microbes, but comparable ISR lengths can prevent resolution [85,86]. The other technique, SSCP, is powerful, which enables the identification of DNA polymorphisms based on conformational differences in single-stranded DNA molecules produced under PCR conditions. Such strands assume distinct secondary structures based on their sequence, and their electrophoretic mobility is based on such conformations, so distinguishing amplicons of the same length but differing nucleotide composition [87]. Taken together, these types of molecular studies have played a significant role in showing the ecological richness, adaptability and functionality of denitrifying microbial communities in diverse wastewater systems.

Table 2 – Molecular Techniques used to detect microbial communities

Techniques	**Pros**	**Cons**	**Samples Used in Studies**
RAPD	Cost-effective. No prior knowledge needed.	Extreme standardization needed in PCR environments. Poor reproducibility. Final result varies as per the DNA template, the DNA polymerase, and the amount of primer.	Wastewater in pharmaceuticals [59], municipal wastewater [60], industrial wastewater [61,62]
ARDRA	Simple, fast, precise molecular technique to define population profile.	Not very efficient in defining compared to other techniques.	Industrial wastewater [63,64] Municipal wastewater [65]
DGGE/TGGE	Highly sensitive and capable to form band for sequencing and strengthening from the gel.	DNA sequences are not similar among various species of bacteria.	Industrial wastewater [66,67] Municipal wastewater [68]
T-RFLP	Decent sensitivity to give a relative quantity of bacteria with a	Restriction enzymes used are vital to identify various bacteria.	Industrial wastewater [69,70] Municipal wastewater

	fluorochrome.		[71]
SSCP	Simple, quick and cheap	Lack of a hypothetical model to determine the actual conformation of a DNA fragment in various parameters, such as DNA fragment size, mutation, concentration of DNA, pH, etc.	Gelatinaceous wastewater [72] Industrial wastewater [73] Municipal wastewater [74]
RISA	Good at discriminating and less risk of inconsistent outcomes.	Only changes in the ISR fragment are detected. Not possible to discriminate between different bacteria having similar ISR strength.	Pharmaceutical wastewater [75] Industrial wastewater [76]

6. Applications of Denitrification in Industrial Affluent Treatment

The conventional process of biological denitrification relies on nitrification, denitrification, and ammonification processes, and the microorganisms related to them can be covered as activated sludge (Figure 4).

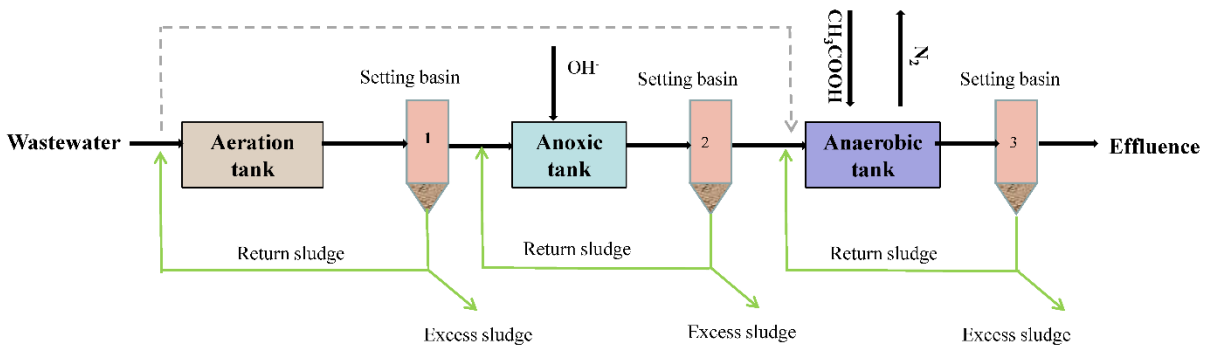

Figure 4: Biological Denitrification of Activated Sludge [88]

The effluent enters after precipitation into the nitrification tank, where conversion from NH4+-N to NO3--N is done. An acid is needed in the nitrification reaction to reduce the pH level in the reactor. The NO3--N then lowered to N2 in the process of denitrification, which needs the sources of organic carbon like glucose and methanol. To be specific, organic carbon-containing wastewater is blended with effluent [89]. Along with the given process, the "recurring denitrification or anerobic-aerobic process (A/O)" is also useful to get rid of nitrogen. The previous organic compounds can also be used well in wastewater and control air input in the "A/O process", and this process also helps in removing the reflux system and intermediate tank [90]. The operation and construction costs can be significantly reduced in this process. The "anerobic/aerobic/anoxic (A2/O)" process is optimised based on the A/O process to conduct dephosphorization and denitrification processes, which can be synchronised in a reactor. At the same time, it gets rid of phosphorus. Hence, conventional bio wastewater treatment has been cost-effective and powerful [91].

The microbiota is examined in the process of nitrogen removal. Some of the most common microbial genera are "Thauera, Dechloromonas, Nitrospira, and Ignavibacterium" in A2/O sludge [92,93]. In

addition, the key taxa identified for oxidation of nitrite are “Nitrosomonas, Nitrospira, and Nitrobacter” [94,95]. Denitrification is mainly done by “Truepera, Paracoccus, and Denitratisoma” [96]. The systems used to remove autotrophic nitrogen are anammox, PN, and PN/A processes in one or two bioreactors. These are some of the cheap ways to treat the wastewater full of NH4+ [97]. The anammox process was developed for the treatment of industrial wastewater in China [98]. The anammox microbes were found to be “Nitrosomonas, Stuttgartiensis, and Candidatus Kuenenia” for the treatment of synthetic wastewater [90]. The production of vitamin B2 can be treated in anammox process, and the main microorganisms are “Nanaocystis and Ca. Kuenenia” [99]. Along with it, sulfate and ammonium can be removed while treating wastewater with sulfate-specific anammox bacteria and new species of anammox bacteria like “Bacillus benzoevorans [100] and Anammoxoglobus sulfate [101]”.

7. Molecular Techniques in Denitrification Research

Molecular approaches have made tremendous contributions to our knowledge on denitrification, especially on the definition of the main microbial communities, gene activities, and control mechanisms in the process of reduction of nitrates to nitrogen gases. Conventional culture-dependent techniques tended to ignore microbial diversity, and molecular techniques, including polymerase chain reaction (PCR)-based gene amplification, quantitative PCR (qPCR), metagenomics, and transcriptomics, have enabled a greater understanding of microbial complexity of the denitrifying communities [102]. The most common molecular marker that is employed to assess the abundance and activity of denitrifiers in different ecosystems as an indicator of nitrite reduction is genes that encode nitrite reductase (nirK and nirS), nitric oxide reductase (norB), and nitrous oxide reductase (nosZ) [103]. Such functional gene markers not only enable identification of taxa in the microbes but also indicate differences in the gene expression, which affect denitrification efficiency in the environment under varied conditions [104].

High-throughput sequencing technologies have transformed the study of denitrification since they allow the profiling of the microbial community structures as never before. In metagenomic investigations, it can be seen that denitrification is not confined to classical denitrifiers (like Pseudomonas and Paracoccus), but it is a common metabolic property across a wide range of bacterial and archaeal taxa [105]. Metatranscriptomic studies also indicate that gene expression is different based on oxygen concentration, pH, availability of organic carbon and also nitrate loading, indicating the dynamic regulation of denitrification pathways [106]. Moreover, other methods, such as stable isotope probing (SIP) coupled with molecular methods, have been utilised to specifically track the assimilation of denoted nitrogen substrates, and have thus been able to associate functional genes with microbial groups specifically involved in denitrification [107].

Recent developments in next-generation sequencing (NGS) and omics-based methods have enabled scientists to combine genomic, proteomic, and metabolomic information into complete denitrification network models [108]. As an example, proteomic studies of denitrifiers have been used to study important enzymes in response to adverse stresses, like low oxygen or elevated salinity, and so offer a mechanistic understanding of adaptation and regulation [109]. Equally, shotgun metagenomics has identified new combinations of the nosZ gene, broadening our knowledge of nitrous oxide-reducing microorganisms, which are important in alleviating the impact of greenhouse gas emissions [110]. In addition, there are molecular fingerprinting techniques like denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP) and fluorescent in situ hybridisation (FISH), which have been extensively utilised to determine community dynamics and space distribution of denitrifiers both in natural and engineered habitats [111].

New molecular genetic techniques, including CRISPR-based gene editing and single-cell genomics, are starting to illuminate how the denitrification process is regulated on the cellular level [112]. Such

techniques can also be used to manipulate or monitor individual denitrification genes in vivo and have the potential to enhance the efficiency of nitrogen removal in wastewater treatment plants and in agricultural soils [113]. Moreover, metabolic networks, gene-interaction predictions, and consortia design with bioinformatics and systems biology tools are becoming common to optimally model and predict the behaviour of microbial consortia to design improved denitrification [114]. There is also growing interest in using machine learning with omics data, which can provide predictive information on the potential of denitrification under varying environmental conditions [115]. Molecular methods have revolutionised denitrification studies, in general, and the future of these technologies is likely to increase our ability to track and regulate the processes of nitrogen cycling by improving our ability to understand the diversity, activity, and regulation of microbial communities [116].

8. Future Research

Future research should prioritise cost-effective, low-emission carbon sources, such as biogas and biodegradable polymers, alongside improved strategies for controlling nitrous oxide release. High-throughput molecular approaches, including metagenomics and functional gene assays, are expected to refine predictions of community dynamics and system stability. Developing microbial biomarkers and integrating them into real-time monitoring and process control will enhance reactor resilience. Ultimately, linking microbial ecology with engineering practice will enable the design of adaptive, sustainable, and energy-efficient wastewater treatment systems that meet growing environmental challenges.

9. Conclusions

Denitrification remains one of the most efficient biological approaches for nitrogen removal in wastewater treatment. Its success relies heavily on microbial community diversity, carbon source availability, and reactor conditions. Advances in molecular tools have improved our ability to characterise functional communities and link microbial ecology with process performance. However, operational bottlenecks such as nitrous oxide emissions and variability in community adaptation emphasise the need for better integration of ecological insights into engineering models.

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