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Review

# Microbial ecology of denitrification in biological wastewater treatment

*Huijie Lu* [a,*], *Kartik Chandran* [b,**], *David Stensel* [c]

[a] *Department of Civil and Environmental Engineering, University of Illinois at Urbana Champaign, 205 N Mathews, Urbana, IL 61801, USA*

[b] *Department of Earth and Environmental Engineering, Columbia University, 500 West 120th Street, New York, NY 10027, USA*

[c] *Department of Civil and Environmental Engineering, University of Washington, Seattle, WA 98195, USA*

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ABSTRACT

Globally, denitrification is commonly employed in biological nitrogen removal processes to enhance water quality. However, substantial knowledge gaps remain concerning the overall community structure, population dynamics and metabolism of different organic carbon sources. This systematic review provides a summary of current findings pertaining to the microbial ecology of denitrification in biological wastewater treatment processes. DNA fingerprinting-based analysis has revealed a high level of microbial diversity in denitrification reactors and highlighted the impacts of carbon sources in determining overall denitrifying community composition. Stable isotope probing, fluorescence *in situ* hybridization, microarrays and meta-omics further link community structure with function by identifying the functional populations and their gene regulatory patterns at the transcriptional and translational levels. This review stresses the need to integrate microbial ecology information into conventional denitrification design and operation at full-scale. Some emerging questions, from physiological mechanisms to practical solutions, for example, eliminating nitrous oxide emissions and supplementing more sustainable carbon sources than methanol, are also discussed. A combination of high-throughput approaches is next in line for thorough assessment of wastewater denitrifying community structure and function. Though denitrification is used as an example here, this synergy between microbial ecology and process engineering is applicable to other biological wastewater treatment processes.



## Contents



* *Corresponding author*. Tel.: +1 217 333 8038; fax: +1 217 333 9464.
** *Corresponding author*. Tel.: +1 212 854 9027; fax: +1 212 854 7081.
E-mail addresses: hl2409@illinois.edu (H. Lu), kc2288@columbia.edu (K. Chandran).

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## 1. Introduction

### 1.1. *Biological denitrification and denitrifying microorganisms*

Biological denitrification is the sequential reduction of nitrate or nitrite to dinitrogen gas, via the gaseous intermediates nitric oxide and nitrous oxide (Knowles, 1982). This respiratory, energy-generating process is catalyzed by four types of nitrogen reductases in sequence: nitrate reductase (Nar), nitrite reductase (Nir), nitric oxide reductase (Nor) and nitrous oxide reductase (Nos) (Zumft, 1997). Over the last century, extensive research has been conducted on denitrification for the following reasons. First, denitrification constitutes a main branch of the biogeochemical nitrogen cycle, which returns reactive nitrogen to the atmosphere and maintains the balance of the global nitrogen budget (Mike, 2008). Second, as one of the important processes to achieve biological nutrient removal (BNR), denitrification has been widely applied in engineered wastewater treatment systems for targeted water quality improvement (Grady et al., 1999). Third, biological denitrification can contribute to the global greenhouse effect through the emission of nitrous oxide ($N_2O$), approximately 300 times more potent than carbon dioxide (IPCC, 2000).

Although denitrification potentials are widely found in bacteria, archaea and some eukaryotes (e.g., fungi), nitrate reduction in natural and engineered ecosystems is primarily conducted by bacteria (Knowles, 1982; Cabello et al., 2004; Knowles, 1996; Shoun et al., 1992). Nutritionally, most denitrifying bacteria are facultative anaerobes using ionic and gaseous nitrogen oxides as electron accepters in the absence of oxygen. The electron donors can be derived from either organic (chemoorganoheterotrophs) or inorganic compounds (chemolithoautotrophs). To date, only a limited number of chemolithoautotrophs are known to be capable of denitrification (Sievert et al., 2008), whereas chemoorganoheterotrophic denitrifiers are distributed in a large variety of physiological and taxonomic groups (Knowles, 1982). The high phylogenetic diversity of heterotrophic denitrifiers is in parallel with their ubiquitous presence in soil and aquatic habitats (Verbaendert et al., 2011; Shapleigh, 2006). The microbiology and ecology of denitrifying populations in these habitats has been extensively investigated and reviewed (Knowles, 1996). Due to their important roles in wastewater treatment processes, denitrifying bacteria in engineered BNR systems are of particular interest to wastewater engineers and microbiologists as a model microbial community.

### 1.2. *Wastewater denitrification*

With increased agricultural, domestic and industrial usage of nitrogen and phosphorus, aquatic ecosystems around the world are facing severe water quality impairment caused by nutrient enrichment (Smith et al., 1999). Notwithstanding the discovery and application of novel chemolithoautotrophic denitrification processes such as anaerobic ammonia oxidation (Kuenen, 2008), chemolithoautotrophic nitrification followed by chemoorganoheterotrophic denitrification remains widely practiced for conventional biological nitrogen removal, where denitrification occurs in the anoxic zone in the presence of no or limited dissolved oxygen (ideally <0.2 mg/L). External addition of organic carbon sources (e.g., methanol and acetate) to enhance denitrification rates is commonly applied to both denitrifying activated sludge and tertiary fixed-film denitrification processes, given that the influent may not contain adequate amount of readily biodegradable

carbon sources to achieve the required nitrate reduction (Henze et al., 2008).

Traditionally, the design and operation of wastewater bioreactors was guided by empirical or "black-box" modeling, *i.e.*, models entirely based on input–output data without reflecting details of the physical or biochemical processes occurring within the reactors (Olsson and Newell, 2002). Since the mid-1980s, the Activated Sludge Models (ASM1, 2, 2D and 3) have been widely accepted as reference tools for design and control strategies in activated sludge processes (Henze et al., 2000). In these deterministic or "white-box" models, the anoxic growth of denitrifying biomass and associated substrate (chemical oxygen demand, COD and nitrate) removal are described by Monod-based equations, supplemented with a set of stoichiometric and kinetic parameters. Although these modeling tools have greatly facilitated the design and control of wastewater denitrification, this process still faces challenges in dealing with performance deficiencies, maintaining stability and reliability, improving cost-effectiveness and controlling nitrogenous gas emissions (Barnard and Steichen, 2006). During the past decade, the microbiology theory and engineering practice have been combined to address some of these challenges, primarily by determining denitrification kinetics and stoichiometry (Lee and Welander, 1996), carbon augmentation strategies (Cherchi et al., 2009), effects of inhibitors (Glass et al., 1997; Oh and Silverstein, 1999a), factors controlling nitrous oxide emissions (Kampschreur et al., 2009; Schulthess et al., 1995), composition of denitrifying communities (Lu and Chandran, 2010a; Osaka et al., 2008a) and physiology of predominant denitrifying microbes (Thomsen et al., 2007; Uzrakami et al., 1995).

In recent years, microbial ecology techniques have been brought into the ecological and biotechnological contexts of wastewater denitrification, with the goal of understanding the structure and function of target communities and elucidating the link between the two. Modern molecular approaches have enabled deeper, more accurate and routine analysis of complex ecosystems, leading to significant progress in identifying active denitrifiers (Osaka et al., 2006; Baytshtok et al., 2009), quantifying denitrifying community diversity (Lu and Chandran, 2010a), and measuring the abundance of functional denitrification genes (Geets et al., 2007). This review outlines the widely applied molecular approaches, summarizes current findings from the structural and functional ecology studies of wastewater denitrification, and highlights some new challenges associated with sustainable design and operation.

### 1.3. *Molecular techniques characterizing denitrifying communities*

Two essential parameters that define microbial community structure are the identity and abundance of the resident populations. Efforts to identify denitrifying microorganisms in various denitrifying ecosystems have traditionally relied on culture-based methods (Heylen et al., 2006), which often overlook a considerable number of unculturable bacteria and are unlikely to yield a realistic picture of the overall microbial community (Amann et al., 1995). Since the 1990s, culture-independent methods have substantially improved the identification and quantification of denitrifying populations by targeting the phylogenetic and functional biomarkers (*i.e.*, 16S rRNA gene or denitrification genes). 16S rRNA gene sequences allow more accurate taxonomic assignments, but it is almost impossible to predict from an environmentally retrieved 16S rRNA gene sequence whether a microorganism actually performs denitrification *in situ*. As a result, genes encoding the seven denitrification reductases have been tested as functional biomarkers in the detection of populations with denitrification capacities, for example, *narG* and *nosZ* (Enwall et al., 2005). Some of these genes (*narG*, *nirK* and *norB*) are also present in bacteria performing incomplete denitrification, dissimilatory nitrate to ammonia (DNRA) or autotrophic nitrification (Philippot, 2002; Chain et al., 2003). As these processes also contribute to nitrogen removal under anoxic condition, using general *narG* or *nirK* as biomarkers could capture a variety of species with nitrate/nitrite-reducing capacities.

Molecular techniques have provided valuable insights into the structure and function of wastewater denitrification communities. Specifically, DNA fingerprinting techniques, such as denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (t-RFLP), can be used to compare samples from different reactors or demonstrate the temporal or spatial variations of community composition (Supplementary information, Table S-I). Fluorescence *in situ* hybridization (FISH) and DNA-based stable isotope probing (DNA-SIP) are representative techniques that further link community structure with function by identifying and quantifying populations with specific metabolic features in wastewater denitrifying reactors. The combination of FISH with microautoradiography (FISH-MAR) can simultaneously examine the phylogenetic identity of functional groups taking up radioactively labeled substrates (e.g., nitrate or various types of electron donors) *in situ* (Lee et al., 1999). Similarly, DNA-SIP assays have been commonly used in wastewater denitrification studies for detecting specific organic carbon assimilating denitrifiers (Baytshtok et al., 2009; Ginige et al., 2004). Overall, traditional molecular techniques require pre-existing knowledge of 16S rRNA sequences and/or functional gene sequences (Table 1). There is still a lack of functional-genomics based approaches to discover novel genes, pathways and new organisms involved in denitrification. Additional limitations of these analyses include their low throughput in capturing rare species and comprehensively profiling expressions of multiple genes.

High-throughput techniques, represented by microarray and meta-omics, are in rapid development and prevalent in wastewater microbial ecology studies. Five gene families from denitrification pathways, *narG*, *nirS*, *nirK*, *norB*, and *nosZ*, are present in functional gene targeted DNA microarrays, e.g., GeoChip (He et al., 2010a). The functional gene diversity and denitrification potential of microbial communities in various environments, e.g., soil and marine, have been extensively studied based on functional gene assays (FGA) (Xie et al., 2011; McGrath et al., 2010; Wu et al., 2008). Thus far, there have been only a few microarray studies on wastewater samples (Kelly et al., 2005). The major deficiency with FGA that makes it less attractive than other high-throughput techniques (e.g.,

RNA Sequencing, as described below) is the requirement for hybridization probes of known sequences.

The meta-omics techniques include a series of high-throughput tools to study the DNA, RNA, proteins and metabolites of mixed microbial communities, referred to as metagenomics, metatranscriptomics, metaproteomics and metabolomics, respectively. Next-generation sequencing (NGS) enables targeted high-throughput amplicon sequencing (specific gene-based surveys, such as 16S rRNA genes), RNA sequencing (whole transcriptome shotgun sequencing) and large-scale shotgun metagenome sequencing of communities from wastewater treatment systems (Sanapareddy et al., 2009; Yu and Zhang, 2012; Zhang et al., 2012). Compared with metagenomics, the global gene expression level analyses as revealed by metatranscriptomics and metaproteomics are less applied due to the inherent problems with their reliability and repeatability (Wilmes et al., 2008). Although at the very early stage, innovative approaches by combining these meta-omics techniques are anticipated to accelerate the discovery of novel genes encoding specific metabolic or biodegradative capabilities (Cowan et al., 2005), whereby biodegradation of emerging pollutants in wastewater treatment systems can be better predicted.

## 2. Microbial ecology of wastewater denitrifying communities

### 2.1. Overall diversity and dominant species

Denitrification capability is widespread in both bacterial and archaeal domains, and the diversity of denitrifying communities in other environments (e.g., soil) is commonly higher than those in wastewater treatment processes (Throbäck, 2006). Despite this, *16S rRNA gene-based* studies still indicated a relatively high diversity and species richness of wastewater denitrification communities (Fig. 1). Bacterial strains isolated from denitrifying bioreactors are closely related to *Hyphomicrobium*, *Paracoccus*, *Pseudomonas* and *Comamonas* spp. in Proteobacteria (Martineau et al., 2013; Gumaelius et al., 2001; Chakravarthy et al., 2011; Su et al., 2001). However, discrepancy exists between the frequently isolated stains and those actually dominating in real wastewater denitrifying systems, such as *Azoarcus*, *Zoogloea* and *Comamonadaceae* spp., most of which are taxonomically affiliated with Proteobacteria (59%) and *Bacteroidetes* (16%, Fig. 1). Within Proteobacteria, α, β, γ and δ subclasses occur at much higher levels than ε-Proteobacteria, and this result has been verified by high-throughput metagenomics (Zhang et al., 2012). Moreover, the abundances of dominant populations in denitrifying communities also vary significantly from one treatment plant to another, depending on the influent characteristics, treatment configurations and operating conditions (Supplementary Information, Table S-I).

Different microorganisms possess complete, partial or no denitrification activities, and can be more accurately subdivided into five groups, *i.e.*, complete denitrifiers (capable of reducing both nitrate and nitrite to $N_2$), exclusive nitrite reducers (only capable of reducing nitrite but not nitrate to $N_2$), incomplete denitrifiers (reducing nitrate or nitrite to nitrogen oxide intermediates instead of $N_2$), incomplete nitrite reducers (capable of reducing nitrite to nitrogen oxide intermediates), and non-denitrifiers (not capable of nitrate or nitrite reduction). Examples of microorganisms falling into

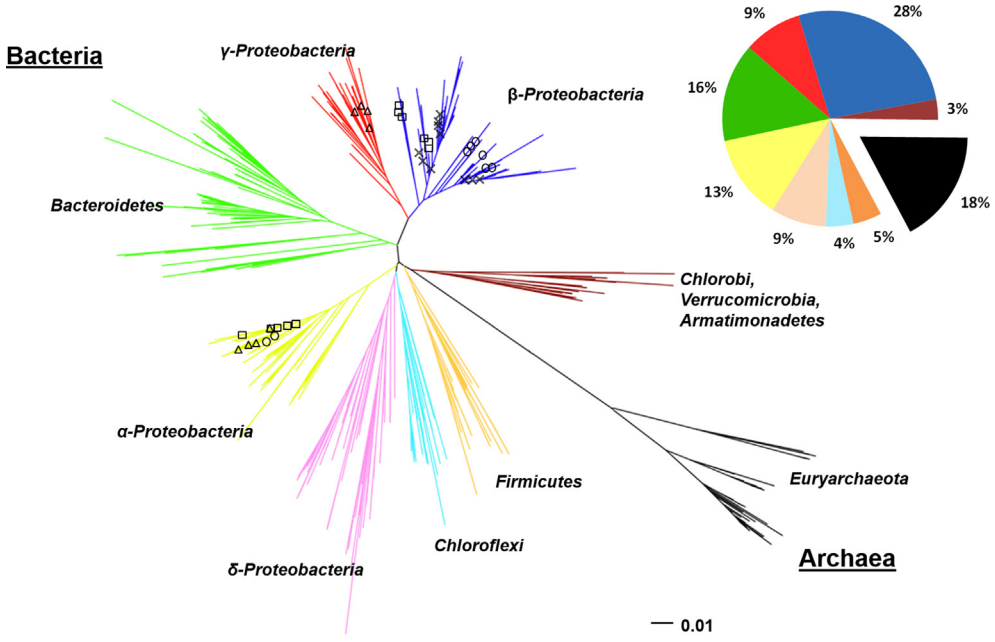


**Fig. 1 – Phylogenetic tree of the major phyla of wastewater denitrifying bacteria constructed by the neighbor-joining method on the basis of 1003 partial *16S rDNA* sequences (>500 bp) retrieved from GenBank. All available sequences cited in reviewed articles were retrieved from GenBank and aligned using the NAST web tools (DeSantis et al., 2006). Tree was built and edited using iTOL (Letunic and Bork, 2011). Special carbon assimilating populations: □ – Methanol; × – Acetate; ○ – Glycerol; △ – Methane.**

**Table 1 – Selected primers and probes applied in wastewater denitrification studies.**

| Target | Primer/probe name | Sequence (5′-3′) | Reference |
|---|---|---|---|
| *PCR primers* | | | |
| 16S rRNA | 341F/534R | F: CCTACGGGAGGCAGCAG; R: ATTACCGCGGCTGCTGG | (Muyzer et al., 1993) |
| | 968F/1401R | F: AACGCGAAGAACCTTAC; R:CGGTGTGTACAAGGCCCGGGAACG | (Heuer et al., 1997) |
| | 11F/1392R | F: GTTTGATCCTGGCTCAG; R: ACGGGCGGTGTGTRC | (Kane et al., 1993; Lane, 1991) |
| Nitrate reductase | 1960m2f/2050m2r | F: TAYGTSGGGCAGGARAAACTG; R: CGTAGAAGAAGCTGGTGCTGTT | (López-Gutiérrez et al., 2004) |
| Nitrite reductase | nirS1fF/6R | F: CCTYATGGCCGCCRCART; R: CGTTGAACTTRCCGGT | (Braker et al., 1998) |
| | cd3aF/R3cd | F: GTSAACGTSAAGGARACSGG; R: GASTTCGGSTGSGTCTTGA | (Throbäck et al., 2004; Michotey et al., 2000) |
| | nirK1F/5R | F: GGMATGGTKCCSTGGCA; R: GCCTCGATCAGRTTRTGG | (Braker et al., 1998) |
| | F1aCu/R3Cu | F: ATC ATG GTSCTGCCGCG; R: GCCTCGATCAGRTTGTGGTT | (Hallin and Lindgren, 1999) |
| Nitric oxide reductase | cnorB2F/6R | F: GACAAGNNNTACTGGTGGT; R: GAANCCCCANACNCCNGC | (Braker and Tiedje, 2003) |
| | qnorB2F/5R | F: GGNCAYCARGGNTAYGA; R: ACCCANAGRTGNACNACCCACCA | (Braker and Tiedje, 2003) |
| Nitrous oxide redutase | nosZ-F/1622R | F: CGYTGTTCMTCGACAGCCAG; R: CGSACCTTSTTGCCSTYGCG | (Throbäck et al., 2004; Kloos et al., 2001) |
| | Nos661F/1773R | F: CGGCTGGGGGCTGACCAA; R: ATRTCGATCARCTGBTCGTT | (Scala and Kerkhof, 1998) |
| *FISH probes* | | | |
| *Paracoccus* | PAR651 | ACCTCTCTCGAACTCCAG | (Theron and Cloete, 2000) |
| *Pseudomonas* | Pst67 | AAGCTCTCTTCATCGG | (Neef et al., 1996) |
| *Acidovorax* | ACI208 | CGCGCAAGGCCTTGC | (Amann et al., 1996) |
| *Thauera* | Thau646 | TCTGCCGTACTCTAGCCTT | (Lajoie et al., 2000) |
| *Azoarcus* | Azo644 | GCCGTACTCTAGCCGTGC | (Hess et al., 1997) |
| *Hyphomicrobium* | HYP1241 | GCTGCSCATTGTCACCGCC | (Layton et al., 2000) |
| Acetate denitrifiers | DEN124 | CGACATGGGCGCGTTCCGAT | (Ginige et al., 2005) |
| | DEN581 | TGTCTTACTAAACCGCCTGC | (Ginige et al., 2007) |
| | DEN444 | GAGAAGGCTTTTTCGTTCCG | (Ginige et al., 2005) |
| | DEN1454 | CCGTGGCAATCGCCCCCC | (Ginige et al., 2005) |
| Methanol denitrifiers | DEN67 | CAAGCACCCGCGCTGCCG | (Ginige et al., 2004) |

some of the above categories include *Hyphomicrobium* spp. (complete denitrifiers, (Sperl and Hoare, 1971)), *Methyloversatilis* spp. (incomplete denitrifiers, (Lu et al., 2012)) and some strains of *Pseudomonas* spp. (incomplete nitrite reducers, (Vangnai and Klein, 1974)). In wastewater denitrification systems, it is likely that bacteria with different N oxide reduction capabilities contribute together as a nitrogen sink.

### 2.2. Factors controlling community structure

#### 2.2.1. Carbon sources

Although methanol has been the most widely used carbon source for enhancing wastewater denitrification, many alternatives are also applied based on considerations such as cost, kinetics and adaptation period. For a given system, the carbon source potentially has a stronger impact on denitrifying community structure than other factors (e.g., electron acceptor, C/N ratios) (Lu and Chandran, 2010a; Baytshtok et al., 2009; Wan et al., 2011; Hagman et al., 2008; Baytshtok et al., 2008; Xia et al., 2008), as organic carbon and energy metabolism with diverse pathways forms the foundation for heterotrophic growth. Specifically, metabolism of single-carbon compounds (e.g., methanol, formate and methane) is unique to methylotrophs because of an uncommon key enzyme that oxidizes methanol to formaldehyde (Anthony, 2011). This explains why a more distinct single carbon-based denitrifying community structure than multi-carbon compound structure has been commonly observed (Lu and Chandran, 2010a; Hallin et al., 2006).

*Methanol*: Using both culture-dependent and -independent methods, populations related to *Methylophilus*, *Paracoccus*, *Methyloversatilis* and *Hyphomicrobium* spp., have been identified in various methanol-feeding denitrification systems (Baytshtok et al., 2009; Hallin et al., 2006; Claus and Kutzner, 1985), which prominently belong to β-Proteobacteria (Fig. 1). These populations can be further classified as obligate (growing on C1 compounds only) and facultative methylotrophs (growing on C1 and multi-carbon compounds). Denitrifying bacteria belonging to the former group (e.g., *Hyphomicrobium* sp.) have been detected almost exclusively in methylotrophic systems, and the latter (e.g., *Methyloversatilis* and *Paracoccus* spp.) have also been detected in denitrifying reactors fed with other carbon sources (Baytshtok et al., 2008; Timmermans and Van Haute, 1983). In general, methylotrophs have limited diversity and are metabolically distinct from non-methylotrophic denitrifying bacteria (Chistoserdova et al., 2009; Anthony, 1982). Therefore, the initial long lag phase (up to a few months) typically observed during the start-up of methanol-fed denitrifying systems results from the enrichment of methylotrophic populations and the subsequent shift in the overall denitrifying community (Nyberg et al., 1992). Methanol utilizers in lab- and full-scale reactors may actually only represent a small fraction of the overall denitrifying communities (Baytshtok et al., 2009), and the growth of other populations is potentially supported by the intermediates or products generated from methanol metabolism via cross-feeding.

*Ethanol*: Despite the extensive investigations on methanol-based denitrification, the microbial ecology of denitrifying communities enriched by alternative carbon sources are gaining increased attention. Hallin et al. performed *nirK*- and *nirS*-based RFLP analysis on active sludge samples enriched by methanol and ethanol, respectively (Hallin et al., 2006). Methanol-fed sludge possessed higher level of *nirS* diversity than that of ethanol, but the *nirK* diversity were similar for the two sludge. Moreover, dominant ethanol enriched populations revealed by *nirS* based clone libraries were related to *Paracoccus*, *Thauera*, and *Azoarcus* spp. Higher overall diversity of methanol enriched denitrifying community than that of ethanol was also observed in a lab-scale denitrifying SBR upon switching from methanol to ethanol (Baytshtok et al., 2009). The same study also identified active methanol- (*Methyloversatilis* and *Hyphomicrobium* spp.) and ethanol-assimilating populations (*Methyloversatilis* sp. only), demonstrating the substrate specificity and metabolic capabilities of the two methylotrophic bacterial populations (Table 2).

*Acetate*: Some of the acetate utilizers identified in acetate denitrifying systems were closely related to *Comamonas*, *Acidovorax* and *Thauera* spp. within the families of *Comamonadaceae* and *Rhodocyclaceae* (Osaka et al., 2006; Ginige et al., 2005). The diversity of these acetate supported populations was considerably higher than those enriched by methanol (Fig. 1), and they were found in the original denitrifying sludge that was not enriched by any carbon sources. Ginige et al. demonstrated the dominance of distinct groups of acetate-denitrifying populations under substrate excess and limited conditions (Ginige et al., 2007), and the availability of both electron donor and acceptor (e.g., acetate and nitrite) was the selective factor for acetate utilizers in the community.

*Other carbon sources*: Morgan-Sagastume et al. tested the structure of denitrifying community under acetate–and complex substrate mixture (acetate, ethanol and pyruvate)-feeding conditions (Morgan-Sagastume et al., 2008). Diverse and versatile populations identified were affiliated with $\alpha$-, $\beta$-, and $\gamma$-Proteobacteria under both conditions, and *Aquaspirillum*-related bacteria were the most abundant (20% of biovolume). *Accumulibacter* (3–7%) and *Azoarcus* (2–13%), although less abundant, may also be key denitrifying populations by utilizing more diverse carbon sources in addition to acetate, such as pyruvate and ethanol. In another study, mixtures of methanol and acetate as carbon sources enriched *Azoarcus* spp. as the main group of acetate-utilizing bacteria and the only methylotroph (Hagman et al., 2008). Glycerol-enriched denitrifying communities were studied by Lu and Chandran in a sequencing batch integrated fixed film activated sludge (SB-IFAS) reactor (Lu and Chandran, 2010a). After a one year acclimation to glycerol, *Comamonas* sp. dominated in both suspended and biofilm phases as the main glycerol-assimilating populations, and a distinct community structure was observed in the two phases. Moreover, dynamic and distinct microbial community structure profiles were also observed during the acclimation of denitrifying cultures to MicroC™ (glycerin-based byproduct of biofuel production), methanol and acetate (Cherchi et al., 2009). In solid phase denitrification systems with polyester as carbon sources, most PHA-degrading denitrifying bacteria were assigned to members of *Comamonadaceae* in $\beta$-Proteobacteria. Their rapid proliferation during the acclimation to PHA was also observed, regardless of the original sludge consortium (Khan et al., 2002; Hiraishi and Khan, 2003a).

**Table 2 – Abundant populations in denitrifying reactors with different types of wastewater influent and supplementary carbon sources.**

| Commonly found wastewater denitrifiers (genus) | Phylogenetic affiliation (class, order, family) | Abundant in different types of Wastewater: Industrial | Municipal | Seawater | Supplemental carbon sources: Methanol | Ethanol | Acetate | Others | Model strain and references |
|---|---|---|---|---|---|---|---|---|---|
| *Hyphomicrobium* | *α-Proteobacteria*, *Rhizobiales*, *Hyphomicrobiaceae* | ● | ● | ● | ● | | ● | | *H. denitrificans*, *H. zavarzinii* (Sperl and Hoare, 1971; Layton et al., 2000) |
| *Paracoccus* | *α-Proteobacteria*, *Rhodobacterales*, *Rhodobacteraceae* | | ● | ● | ● | | | | *P. denitrificans* (Baumann et al., 1996) |
| *Azoarcus* | *β-Proteobacteria*, *Rhodocyclaceae*, *Rhodocyclaceae* | ● | ● | | ● | | ● | Aromatic compounds | *A. toluvorans* KH32C, (Mechichi et al., 2002) |
| *Thauera* | *β-Proteobacteria*, *Rhodocyclaceae*, *Rhodocyclaceae* | ● | ● | | | | ● | Aromatic compounds | *T. aminoaromatica MZ1T* (Jiang et al., 2012) |
| *Methylophaga* | *β-Proteobacteria*, *Rhodocyclaceae*, *Methylophaga* | | | ● | ● | | | | *M. aminisulfidivorans* (Neufeld et al., 2007; Kim et al., 2012) |
| *Accumulibacter* | *β-Proteobacteria*, *Rhodocyclaceae*, *Rhodocyclaceae* | | ● | | | | ● | Pyruvate, propionate | *A. phosphatis* (Carvalho et al. 2007, *He et al. 2010b*) |
| *Pseudomonas* | *γ-Proteobacteria*, *Pseudomonadales*, *Pseudomonadaceae* | ● | ● | | ● | | ● | Quinolone | *P. stutzeri*, *P. putida* (Kornaros et al., 1996) |
| *Acidovorax* | *β-Proteobacteria*, *Burkholderiales*, *Comamonadaceae* | ● | ● | | | | ● | PHAs | *A. caeni* (Heylen et al. 2008) |

#### 2.2.2. *Wastewater influent*

*Industrial and municipal wastewater streams*: Domestic wastewater typically contains 10–40 mg N/L in the form of ammonia or organic nitrogen, which is converted to nitrate after complete nitrification (Tchobanoglous et al., 2003). The composition of industrial wastewater entering the denitrification stage varies significantly from industry to industry, and in general contains high nitrate levels and a large amount of other ions, such as chloride and sulfate (Davis, 2009). Thomsen et al. compared denitrifiers in plants treating industrial and municipal wastewater, and the two systems were dominated by *Azoarcus*- and *Aquaspirillum*-related bacteria, respectively (Thomsen, 2007). Overlapping dominant species in the two types of plants have also been frequently observed (Supplementary Information, Table S-I). Comparatively, municipal waste landfill leachates are complex mixtures of high-strength organic and inorganic contaminants including humic acids, ammonia, heavy metals and other inorganic salts (Christensen et al., 2001). *Thauera*, *Acidovorax* and *Alcaligenes* were isolated from an anoxic reactor treating landfill leachate rich in nitrate and aromatic compounds (Etchebehere et al., 2001). These populations have also been identified elsewhere in municipal wastewater treatment plants (Morgan-Sagastume et al., 2008; Hoshino et al., 2005).

*Salinity*: Yoshie et al. monitored the denitrifying sludge community structure during the acclimation from low- to high-salinity wastewater via t-RFLP, and identified *Halomonas* and *Marinobacterin* sp. in γ-Proteobacteria as the dominant bacterial species (Yoshie et al., 2006). In another t-RFLP based study of denitrification under low- and high-salinity conditions, acetate attained high nitrate removal at high salinity, where dominant populations were also related to *Halomonas* and *Marinobacter* sp. (Osaka et al., 2008b). In the same study, methanol was proven to be a beneficial electron donor at lower salinity (0–3%) that enriched *Azoarcus* and *Methylophaga* sp. Strains isolated from another marine methanol-fed denitrification reactor included *Hyphomicrobium*, *Phyllobacteriacea* and *Paracoccus* sp. in γ-Proteobacteria (Labbé et al., 2003). The predominance of γ-Proteobacteria in denitrifying communities has also been found in an acetate-fed denitrification reactor treating saline metallurgic wastewater (Yoshie et al., 2001).

*COD/N ratio*: Xia et al. tracked the composition and dynamics of denitrifying bacteria in a compact suspended carrier biofilm reactor, and a positive correlation was found between microbial diversity and the COD/N ratio (Xia et al., 2010). However, the relative abundance of dominant nitrifying and denitrifying populations, as detected by FISH combined with flow cytometry, was not significantly different under the three C/N ratios tested. In an anoxic reactor treating landfill leachate, enhanced denitrification capacity was observed with the increase of COD/N ratio, which coincided with the shift of primary functional denitrifiers from autotrophic *Thiobacillus* sp. to heterotrophic *Azoarcus* sp. (Sun et al., 2012).

#### 2.2.3. *Biofilm growth*

The higher chemical heterogeneity (e.g., concentration gradients of substrates, metabolic intermediates and products) within biofilm allows bacterial groups with different metabolic properties to coexist. As a result, biofilm usually enriches

more diverse communities than activated sludge (Lu and Chandran, 2010a; Stewart and Franklin, 2008). In a field-scale denitrifying fluidized-bed reactor operated with ethanol as the electron donor, the biofilm community was initially dominated by *Azoarcus* sp., which then decreased and was replaced by highly diverse *Dechloromonas*, *Pseudomonas*, and *Hydrogenophaga* populations (Hwang et al., 2006). As biofilm systems are advantageous to slow-growing bacteria such as nitrifiers, some of the N-removal biofilm reactors have been built for simultaneous nitrification and denitrification. Downing et al. applied a novel hybrid membrane biofilm process for total nitrogen removal, which also revealed that heterotrophs were predominant in bulk liquid and the community was more diverse than the nitrifier-dominated biofilm community (Downing and Nerenberg, 2007). In a four-stage biofilm Bardenpho process, denitrification mainly occurred in the post denitrification (PD) zone, and denitrifying bacteria were related to *Hyphomicrobium*, *Rhodopseudomonas*, and *Rhodobacter* spp. In comparison, nitrifying bacteria were mainly detected in the upper parts of the PD-biofilm and their abundance was generally low (Satoh et al., 2006). Moreover, a few studies have identified identical species in both fluidized- and fixed-bed reactors. For example, Yoshie et al. found *Halomonadaceae* sp. in γ-Proteobacteria predominated in both packed-bed and fluidized-bed reactors treating saline metallurgic wastewater (Yoshie et al., 2001). Labbé and colleagues monitored the bacterial colonization of fixed- and fluidized-bed denitrifying reactors with methanol as a carbon source, where *Methylophaga* sp. accounted for the majority of bacterial populations in the biofilm, with *Hyphomicrobium* sp. being established later (Labbé et al., 2003, 2007).

#### 2.2.4. *Operating conditions*

Operating parameters, such as solids retention time (SRT), pH and dissolved oxygen not only influence overall nitrogen removal and denitrification activity but also result in long-term succession of community structure and diversity (Wang et al., 2012; Hai et al., 2014). Tan et al. utilized 16S rRNA and *nirS* based t-RFLP to compare the effects of different mean cell residence time (MCRT) on the composition of microbial communities in the anoxic zones of pre-denitrification submerged MBRs (Tan et al., 2008). The fingerprints obtained under shorter MCRTs (5.0 and 8.3 d) were significantly different from those under longer MCRTs (16.7 and 33.3 d). The maximum total nitrogen removal efficiency was achieved at the longest MCRT, at which the bacterial community also had fewer species with uneven distribution, potentially being explained by the competitive exclusion theory at long MCRT (Saikaly and Oerther, 2004). Denitrification is relatively insensitive to pH variations, and values between 6.5 and 8.5 are acceptable for proper sludge floc formation. In a cloning-based phylogenetic analysis of denitrifying fluidized-bed reactor, the community composition shifted to increased community diversity and evenness as pH returned from over 9.0 to the optimal range of 6.5–7.5 (Hwang et al., 2006). Although anoxic denitrification requires near complete oxygen depletion (0.2–0.5 mg/L), aerobic denitrifying bacteria have been isolated from diverse natural and engineered ecosystems, which can tolerate as high as 5–6 mg $O_2$/L (Patureau et al., 2000a). In anoxic denitrifying reactors, transition between oxic and anoxic can effectively select aerobic denitrifiers (Patureau et al., 2000b). As most BNR systems are based on recycling biomass between aerobic and anoxic zones, there is no specific ecological niche for aerobic denitrifiers in wastewater treatment systems.

### 2.3. *Factors controlling community function and the underlying mechanisms*

The main factors controlling denitrifying community function include external carbon sources, pH, temperature, dissolved oxygen and nitrogenous oxides. Their effects on overall nitrate removal, denitrification rates and accumulation of intermediates have been extensively studied. Cellular responses to changes in these factors at functional gene expression and enzyme activity levels have also been evaluated based on pure- and mixed-culture studies. The mechanisms mediating the short- and long-term regulations of denitrifying microorganisms are of great value to reliable modeling and successful design of denitrifying bioreactors.

#### 2.3.1. *Carbon sources*

Methanol and alternative electron donors for wastewater denitrification, such as acetate, ethanol and glycerol, have been evaluated and compared largely in terms of denitrification kinetics induced and excess biomass produced. Stoichiometrically, due to the significant loss of energy during methanol assimilation, methylotrophic denitrifying bacteria typically display lower biomass yield than those grown on other carbon sources (McCarty, 2007; Minkevich, 1985). Consistent with the limited metabolic versatility of obligate methylotrophs, most denitrifying communities acclimated to methanol are incapable of utilizing other multi-carbon compounds and *vice versa* (Chistoserdova et al., 2009).

Kinetically, the specific growth rate of methylotrophic bacteria is lower than those enriched by other carbon sources, resulting in a longer adaptation period and insufficient methanol denitrification kinetics during winter (Nyberg et al., 1996; Hallin and Pell, 1998; Mokhayeri et al., 2006). Although the oxidation state of carbon sources has little influence on the synthesis of denitrification reductases (e.g., membrane-bound or periplasmic nitrate reductase) (Stewart et al., 2009), expression levels of carbon oxidases (e.g., alcohol dehydrogenase catalyzing methanol and glycerol oxidation) vary according to the carbon type (Lu et al., 2011). Therefore, differences in the denitrification kinetics potentially result from the imbalance between the overall electron supply and consumption rates. The accumulation of denitrification intermediates, such as nitrite, NO and $N_2O$, can be further attributed to the competition for available electrons among the four nitrogen reductases (Pan et al., 2012). The electron turnover rate of Nar is in general higher than Nir. Therefore, nitrate is more rapidly reduced than nitrite when the electron supply is sufficient. In practice, nitrite accumulation has been observed when specific types of carbon sources are provided (e.g., biodiesel waste) or carbon source availability is low, where the competition for available electrons between Nar and Nir is more pronounced (Oh and Silverstein, 1999b; Uprety, 2012). A few studies have attempted to elucidate the regulatory link between carbon and nitrogen metabolism in

denitrification with different carbon sources. For example, a good correlation was found between denitrification rates (induced by methanol and glycerol, respectively) and the corresponding alcohol dehydrogenase gene transcription levels, where the latter was developed as a promising biomarker for *in situ* carbon-specific denitrification activities (Lu et al., 2011). The transcription levels of 16S rRNA gene also possess the potential of indicating dynamics in denitrification efficiency (Hoshino et al., 2005).

#### 2.3.2. *pH and temperature*

The optimal pH and temperature for wastewater denitrification are 7–9 and 20–30 °C respectively, with denitrification activity falling off rapidly outside of these ranges (Grady et al., 1999). At suboptimal pH, the accumulation of denitrification intermediates, including nitrite and nitrous oxide was frequently observed (Hanaki, 1992; Glass and Silverstein, 1998). The pH dependency of $N_2O$ reduction rate was observed to be much higher than nitrate and nitrite reduction rates using a methanol enriched denitrifying culture (Pan et al., 2012). Based on a pure culture study of *Paracoccus denitrificans*, Baumann et al. found decreased denitrification activity but not *narH*, *nirS* and *nosZ* mRNA concentrations at a suboptimal pH of 6.8 (Baumann et al., 1997). Furthermore, most denitrifying bacteria are more sensitive to variations in temperature than pH. For example, the effects of pH and temperature on the denitrification gene expression were quantified by RT-PCR in *Pseudomonas mandelii* (Saleh-Lakha et al., 2009). The expression levels of *nirS* and *norB* were similar at pH = 6–8 but cells grown at 30 °C exhibited significantly higher levels of expression than those grown at 10 or 20 °C.

#### 2.3.3. *Dissolved oxygen*

Oxygen inhibits denitrification by providing a better electron acceptor for denitrifying populations to generate energy, resulting in deteriorated denitrification activity and accumulated nitrogenous intermediates at high DO concentrations. In practice, the threshold oxygen inhibition concentration can be as low as 0.1 mg $O_2$/L in wastewater denitrification systems (Oh and Silverstein, 1999a). Expression and activity of nearly all N reductases are suppressed in the presence of oxygen (Zumft, 1997; Korner and Zumft, 1989). Oxygen has an immediate and reversible inhibitory effect on nitrate respiration, with the maximal inhibition reached at as low as 0.2% oxygen saturation (Hernandez and Rowe, 1988). Nitrite reductase appeared to be less sensitive to $O_2$ than nitrate reductase, with a threshold inhibitory concentration of 2.5 mg $O_2$/L (Korner and Zumft, 1989). The activity of nitric oxide reductase is approximately 10 times higher than that of nitrite reductase, thus ensuring little accumulation of the toxic NO (Otte et al., 1996a). Nitrous oxide reductase is the most sensitive enzyme to oxygen compared with the other upstream reductases, resulting in transient $N_2O$ accumulation under aerobic conditions (Lu and Chandran, 2010b; Otte et al., 1996b).

#### 2.3.4. *Nitrogen oxides*

As nitrate and other denitrification intermediates are used as electron acceptors by the denitrification electron transport chain, they influence the expression and activity of individual reductases. In the absence of any nitrogen oxide, anaerobiosis alone cannot induce the synthesis of the four reductases to significant levels (Korner and Zumft, 1989). More specifically, as the preferred electron acceptor over other nitrogen oxides, nitrate uniformly induces all reductases. On the other hand, nitrite inhibition has more complicated impacts on denitrification as observed using both pure and mixed cultures (Glass et al., 1997; Almeida et al., 1995). There is evidence demonstrating that nitrite inhibition is actually caused by undissociated nitrous acid ($HNO_2$), with the threshold inhibitory concentration varying based on the culture condition, pH and carbon availability (Glass et al., 1997; Abeling and Seyfried, 1992). For example, accumulation of $N_2O$ and NO during denitrification in the presence of nitrite was observed with acetate-fed denitrifying cultures (Hanaki, 1992), but not in methanol- or ethanol-fed denitrifying reactors with excessive carbon source supply (Lu and Chandran, 2010b). Nitric oxide inhibits near all N reductase, and is normally detected at very low concentrations in bacterial cells (Carr and Ferguson, 1990; Kucera, 1989; Zumft, 1993). Nitrous oxide is not an inhibitor of any steps of denitrification, and preferably stimulates the synthesis of nitrous oxide reductase (Korner and Zumft, 1989).

## 3. Integrating microbial ecology information into process design, monitoring and operation

### 3.1. *Integrating microbial ecology information into denitrification models*

Although traditional wastewater treatment design based solely on chemical and physical parameters has been fairly successful, biological denitrification systems are still experiencing performance deterioration during winter, and long adaptation to methanol or newly added carbon sources (Hallin et al., 2006; US EPA, 2013). Most of these issues are related to a lack of functional groups (methylotrophic denitrifiers) in the community or a decline of their activities. In recent years, new challenges related to wastewater denitrification have been identified, e.g., greenhouse gas emission control (Flores-Alsina et al., 2011). Given the massive amount of microbial ecology data collected from different wastewater denitrifying systems, there is a crucial need to integrate this information into the biochemical denitrification models for better process design and operation.

In current denitrification models, e.g., anoxic growth in Activated Sludge Model 2 (ASM2), a single group of organisms with lumped stoichiometric and kinetic parameters are used, namely methylotrophs (Grady et al., 1999). Recent studies have elucidated the complex ecology of denitrifying bacteria assimilating different carbon sources (as summarized in Fig. 1 and Table 2). Within methylotrophic bacteria, there are also facultative bacteria that attempt to use alternate carbon sources more efficiently than methanol (Baytshtok et al., 2009). Therefore, although recent full-scale testing of novel carbon sources (e.g., biodiesel and sugar-based waste products) has been proved effective in most cases, substantial limitations and risks exist in applying methylotroph-based models to predict the performance of a

denitrifying bioreactor when carbon sources other than methanol are applied, particularly during a carbon transition (Bilyk et al., 2011). Instead, the qualitative and quantitative information of dominant denitrifying populations (obtained via DNA fingerprinting techniques) as well as their potentials in metabolizing different carbon sources (obtained via functional gene expression, kinetic or enzyme activity measurements) can be used in conjunction with traditional models to improve their accuracy in predicting system performance and stability. An example of improved simulation results by considering specific carbon utilizers in the traditional denitrification model is shown in the Supplementary Information.

The necessity and strength of coupling molecular information with ASM models has received increasing awareness, but such integration faces challenges in: 1) accurately measuring kinetic parameters for specific bacterial groups *in situ*, 2) unraveling microbial responses to environmental changes and the mechanisms, and 3) automating methods to produce rapid and reliable quantification results. To overcome these challenges, high-throughput molecular techniques need to be developed and standardized to rapidly examine and screen the composition as well as the activities of microorganisms in engineered systems (Berthiaume et al., 2004). Current progress in optoelectronics and microfluidics is also essential for improved biochemical sensing and the collection of time-resolved data (Gilbride et al., 2006).

### 3.2. *Improving system efficiency and stability*

Microbial ecology information currently has limited ability in directly guiding engineering practice, as microbial composition dynamics cannot be known *a priori*, and how microbial communities acclimates to changes in operating conditions is generally unpredictable. Nevertheless, some attempts have been made to assist the denitrification process control by tailoring community structure through bioaugmentation with both successful and failing cases reported. For example, in a denitrification bioreactor treating high strength nitrate waste, sludge augmented with the *Alcaligenes defragrans* strains displayed higher resistance to shock loading (Flores et al., 2007). In another study, bioaugmentation with *Thiobacillus denitrificans* and *Thiomicrospira denitrificans* did not enhance the efficiency of nitrate removal in a chemolithoautotrophic denitrifying bioreactor (Sanchez et al., 2008).

Significant challenges exist in engineering microbial consortia, and major problems arise in selecting and maintaining appropriate bacterial activities in biological wastewater treatment systems (Bouchez et al., 2000). Compared with bioaugmentation, protecting the plant from process deterioration using molecular tools is more feasible. Functionally important denitrifying strains have gained much attention owning to their potentials for indicating whether system operation is benign or problematic. At full-scale plants, *Hyphomicrobium* spp. have been frequently detected and isolated from the anoxic zones with methanol addition due to their abilities to degrade C1 compounds (Layton et al., 2000). Therefore, reliably monitoring *Hyphomicrobium* levels represents an effective method for optimal control and operation of methanol denitrification. Another example is maintaining the aerobic denitrifier *Pseudomonas denitrificans* in methanol-fed denitrifying systems for achieving continuous nitrate removal during transient oxidative conditions, while the denitrification potential of other denitrifiers are largely inactivated (Davies et al., 1989). Using sludge from full-scale plants, *Azoarcus* spp. were identified as the functionally important denitrifying bacterial group that utilize both methanol and acetate as carbon sources (Hagman et al., 2008). Therefore, the existence of *Azoarcus* bacteria may be beneficial for maintaining denitrification efficiency during a carbon source transition from methanol to acetate.

Different types of molecular biomarkers targeting functionally important bacterial groups have been developed to indicate denitrification performance or activity, for instance, an excellent correlation was obtained between the abundance of *nirS* and the activity of denitrifiers in a fluidized-bed reactor (Araki et al., 2006). The gene encoding the large subunit of methanol dehydrogenase, mxaF, is highly conserved among all Gram-negative methylotrophs and has been used as a functional marker to detect methylotrophs in denitrifying systems with methanol addition (Fesefeldt and Gliesche, 1997). In both long- and short-term kinetic assays, alcohol dehydrogenase gene expression represents a good biomarker of carbon-specific denitrification activity, though results may be effective for reactors fed with alcohols only (Lu et al., 2011).

Although the relationship between microbial diversity and system stability is controversial and cannot be generalized to all ecosystems (Midgley, 2012), there have been a few studies demonstrating this relationship in wastewater treatment systems (Cook et al., 2006; Fernandez et al., 2000). In addition, system stability is better correlated with functional redundancy, *i.e.,* the diversity of functionally important groups rather than the overall population diversity *per se* (Briones and Raskin, 2003). Following this principle, if two denitrifying configurations work equally efficiently, the one harboring higher functional population (or functional gene) diversity would be preferentially selected. There is also a need to continuously optimize current denitrification systems to increase their functional redundancy. The above tasks are increasingly facilitated by the modern high-throughput techniques, such as metagenomics and functional microarray, which harvest microbial ecology information in a more rapid and precise manner. However, currently, these techniques are still too expensive for large-scale use in wastewater treatment studies.

### 3.3. *Applying alternative carbon sources*

With more treatment plants facing new stringent nutrient limits, the demand for external carbon sources is expected to increase significantly. It is desirable to use carbon sources that have low cost, low biomass yield and high cold temperature kinetics. Many commercially available carbon sources can induce a higher denitrification activity than methanol (Baytshtok et al., 2009; Bodík et al., 2009). Nyberg et al. also demonstrated that dosing multi-carbon compounds (e.g., ethanol) during the start-up phase of a denitrifying reactor before switching over to methanol could potentially avoid the long lag period (Nyberg et al., 1996). Nonetheless, some of the

high-efficiency carbon sources might lead to severe nitrous oxide emissions in downstream aerobic zones (Lu and Chandran, 2010b) or consistent nitrite accumulation (Ge et al., 2012). Consequently, supplemental criteria for selecting carbon sources are required to meet goals in energy conservation and greenhouse gas mitigation.

Digester supernatant and biogas (methane and $H_2S$) are some non-conventional carbon sources generated on site, with advantages in reducing cost, energy consumption and secondary organic pollution over other commercial organic compounds. Adding readily biodegradable volatile fatty acids from an acid-phase digester supernatant is potentially promising for denitrifying domestic wastewater or landfill leachate poor in organic carbon (Elefsiniotis et al., 2004). Methane produced from anaerobically digested waste sludge (up to 70% of the total biogas) would also be an inexpensive alternative to methanol (Modin et al., 2007). Anaerobic methane oxidation coupled to denitrification (ANME-D) has been tested at lab-scale, and facultative methylotrophs can provide other denitrifies with electrons released from either aerobic or anaerobic methane oxidation (Waki et al., 2008; Houbron et al., 1999). Trace amount of poisonous and odorous hydrogen sulfide (<1%) in the biogas can be also removed by serving as an electron donor for autotrophic denitrification (Kleerebezem and Mendez, 2002). Although using biogas produced on site as an alternative carbon source leads to a reasonable cost reduction for denitrification, the inhibitory effects of several toxic compounds (e.g., siloxane and aromatics) in biogas need to be further evaluated. In recent years, solid phase denitrification using biopolymers as an electron donor are being developed, as these microbial storage materials (mainly polyhydroxyalkanoates, PHA) can be readily metabolized by a broad variety of microorganisms (Hiraishi and Khan, 2003b).

### 3.4. Nitrous oxide emission control

The emission of $N_2O$ and NO from BNR operations is gaining increased prominence, and the reported magnitude of $N_2O$ emission from biological denitrification reactors varies from 0.005% to 95% of the nitrogen load at both lab and full scales (Lu and Chandran, 2010b; Okayasu et al., 1997; Park et al., 2000; von Schulthess et al., 1994; Tallec et al., 2008). Several recognized conditions favoring $N_2O$ generation from wastewater denitrification include low pH (Focht, 1974), short solids retention time (Hanaki, 1992), organic carbon limitation (Chung and Chung, 2000), dissolved oxygen and nitrite inhibition (Schulthess et al., 1995; Park et al., 2000). Nevertheless, the above conditions cannot be generalized for all denitrifying systems, and need to be addressed on a case-specific basis. For example, a few denitrifying strains exhibit strong denitrification activity under aerobic conditions with little $N_2O$ produced, such as *Pseudomonas stutzeri* (Takaya et al., 2003).

Fundamentally, it has been postulated that electron competition among the denitrification reductases could lead to an accumulation of $N_2O$ under carbon limiting conditions. A study by Pan et al. demonstrated strong dependency of $N_2O$ reductase kinetics on pH levels (Pan et al., 2012), and it was further revealed that a gradual reduction in electrons distributed to Nos could eventually lead to $N_2O$ accumulation (Pan et al., 2013). Zeng et al. reported high $N_2O$ emissions from an anaerobic/anoxic SBR fed with artificial wastewater, where they speculated that $N_2O$ might be the major product of denitrification by glycogen accumulating organisms (GAO), as NO reductase and $N_2O$ reductase competed for limited electrons generated from storage compounds (Zeng et al., 2003). A similar mechanism was also proposed in another study based on a pure denitrifying culture (Schalk-Otte et al., 2000). Nitrifier denitrification is also recognized as an important mechanism of $N_2O$ emitted from BNR systems, as ammonia oxidizing bacteria is lack of genes encoding $N_2OR$ (Shaw et al., 2006). At the mRNA level, oxygen and all nitrogen oxides of the denitrification pathway are able to regulate *nosZ* gene transcription (Zumft, 1997), but the overall coordinated denitrification gene regulation is not yet well known. Likewise, little effort has been dedicated to investigating the effects of microbial community structure on $N_2O$ emission from complex wastewater denitrifying communities, other than the preliminary conclusion that low microbial diversity facilitated by the use of a single type of carbon source could lead to elevated $N_2O$ emissions (Lemaire et al., 2006).

Practically, several strategies have been reported to mitigate $N_2O$ emissions from wastewater denitrification, such as configuring a step-feed sequencing batch reactor, ensuring sufficient anoxic HRT, bioaugmentation with the $N_2O$-reducing denitrifier *P. stutzeri*, choosing methanol over ethanol, and adding copper ions (10–100 μg/L) (Lu and Chandran, 2010b; Park et al., 2000; Desloover et al., 2012; Yang et al., 2009; Zhu et al., 2012). An ASM-based modeling structure for estimating $N_2O$ production during wastewater denitrification has also been proposed, where simulation results were in good agreement with experimental data (Ni et al., 2011). As a generic mathematical model, it needs to be constantly updated with improved knowledge of the molecular mechanisms of $N_2O$ emissions, further calibrations against experimental data, and more precisely measured strain-specific kinetic parameters.

### 3.5. Denitrification coupled to other processes

The coexistence of different species in the same microbial matrix provides a platform for bacteria to perform respective functions synergistically. In most wastewater treatment systems, biological nitrogen and phosphate removal are integrated in a single sludge system. Enhanced biological phosphorus removal (EBPR) is typically operated in an anaerobic-aerobic sequence, where polyphosphate-accumulating organisms (PAOs) can take up excess phosphorus under aerobic conditions. As nitrate or nitrite can serve as alternative electron acceptors instead of oxygen, simultaneous denitrification and P removal is feasible and advantageous in saving aeration, reducing the demand for external carbon sources and minimizing sludge yield compared with conventional EBPR (Carvalho et al., 2007). However, when denitrifiers and PAOs coexist, the competition for limited organic carbon sources sometimes causes a failure of EBPR (Guerrero et al., 2012). Enriching denitrifying PAOs capable of performing simultaneous denitrification and anoxic phosphorus uptake is an effective method to overcome the limitation of organic carbon (Ahn et al., 2002). Microbial

communities of simultaneous P removal and denitrification process have been characterized using DGGE, 16S rRNA gene targeted (MAR)-FISH or metagenomics, where *Rhodocyclus*, *Acinetobacter* and two clades of *Candidatus Accumulibacter* spp. were recognized as key PAOs (Carvalho et al., 2007; Shoji et al., 2006; Kong et al., 2007; Flowers et al., 2013). A contentious point arose in a later metagenomic study of two EBPRs, as the respiratory nitrate reductase (but not other denitrification genes) appears to be absent from *Accumulibacter phosphatis* (Martin et al., 2006). Thus, the authors concluded that low-abundance community members contributed to nitrate reduction and would occupy a functionally important niche under nitrate respiration condition.

Within the biological nitrogen cycle, denitrification can be coupled with nitrification (Kuenen and Robertson, 1994), ANAMMOX (Kumar and Lin, 2010) and dissimilatory nitrate reduction to ammonium (Bonin et al., 1998). Simultaneous nitrification and denitrification can occur in a completely suspended reactor with controlled low DO less than 1 mg/L (Münch et al., 1996), or in aerobic granular sludge (Bassin et al., 2012) or biofilm reactors (Fu et al., 2010), where denitrifying bacteria mainly confined in the deeper anoxic layer. The co-existence of ANAMMOX and denitrification could be useful for the improved removal of nitrogen and organic carbon, as denitrifiers can take up nitrate generated by ANAMMOX and their utilization of organic carbon further reduces the inhibition to ANAMMOX (Kumar and Lin, 2010). Both groups of bacteria grow without oxygen and compete for nitrite as electron accepter. As denitrification is thermodynamically more favorable than ANAMMOX, the COD/N ratio is one of the critical parameters in ensuring the success of a coupled denitrification–ANAMMOX (Sabumon, 2007).

## 4. Conclusions

From a fundamental perspective, this review summarizes the current knowledge of structural and functional ecology of wastewater denitrification, an important process in nitrogen removal that has not been systematically reviewed in terms of its microbial ecology. Topics covered in this review include overall denitrifying community diversity and composition, dominant and functionally significant populations in lab-scale and full-scale reactors, and how they regulate their physiology under system-specific conditions. Emphasis is placed on using wastewater denitrifying community as a model to link community structure and activity, for example, identifying denitrifying populations with specific carbon utilization traits and measuring the distribution and abundance of probe-targeted denitrifiers *in situ*. Some key facts about carbon sources in wastewater denitrification include:

- Carbon source is the controlling factor for denitrifying community structure and function.
- When the carbon source is specified, lab- and full-scale reactors often possess similar dominant populations.
- Microbial populations enriched by methanol are facultative methylotrophs, e.g., *Hyphomicrobium*, *Paracoccus* and *Methylophaga*.
- Ethanol, glycerol and other alternatives could enrich more diverse and distinct microbial populations compared with methanol.
- Normally, no adaptation period is needed when the carbon source is switch from methanol to others, but not *vice versa*.
- Accumulations of N-oxide intermediates (e.g., nitrous oxide and nitrite) are often associated with multi-carbon enriched denitrifying communities.

From a practical perspective, understanding the structure and function of complex denitrifying communities contributes to resolving some emerging challenges in process design and operation. For example, integrating microbial ecology information into traditional denitrification models could add predictive abilities when novel carbon sources are applied or carbon augmentation strategies are altered. Characterizing denitrifying species enriched by specific carbon sources is important to understanding potential community shift during carbon source transition at full-scale plants. Practical applications of the microbial ecology information also include developing strategies to control intermediate accumulation (e.g., nitrite and nitrous oxide) based on the gene expression and activities of the denitrifying pathway measured in targeted systems.

In the end, it is worth noting that knowledge of microbial ecology is driven but also limited by molecular techniques. High-throughput techniques hold much promise for microbial ecology studies compared with traditional molecular techniques. As shotgun metagenomic projects proliferate and become the dominant source of publicly available sequence data, optimizing procedures for the best practices in their execution becomes increasingly important. Likewise, more functional traits of microbial communities can be explored by other omics techniques with enhanced accuracy and cost-efficiency. Future progress will be linked to advances in high-throughput molecular techniques, construction of microbial databases for denitrification processes that include both operational parameters and microbial ecology information. All of these efforts will facilitate better design and efficient operation, as well as appropriate diagnosis and correction of problems for wastewater denitrification in a more rational and scientific manner.

## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2014.06.042.

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