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REVIEW

Ecophysiology of nitrifying communities in membrane bioreactors

O. O. Awolusi · S. K. S. Kumari · F. Bux

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Abstract Membrane bioreactors (MBRs) are rapidly becoming the technology of choice over conventional activated sludge treatment systems due to their smaller footprint, reduced sludge production, rapid start-up of biological processes, complete removal of suspended solids and better effluent quality. The retention of sufficient amount of slow-growing nitrifiers makes it feasible for the MBRs to achieve strong tolerance against the shock loads with stable and highly efficient nitrogen removal. Various studies have focused on the ecophysiology of nitrifiers in MBRs as well as their distinctive operational parameters as well as their impact on the selection and activity of nitrifying community. Several techniques have been employed over the years to understand the nitrifying community and their interaction within the MBR system, which led to its modification from the initial design. This review focuses on the identification of optimal operational and environmental conditions for efficient nitrification in MBRs. The advantages and limitations of different techniques employed for investigating the nitrifying communities in MBRs are also emphasized.

Keywords Ammonia-oxidizing archaea · Proteobacteria · Nitrification · Ammonia-oxidizing bacteria · Nitrite-oxidizing bacteria · Activated sewage sludge

O. O. Awolusi · S. K. S. Kumari (✉) · F. Bux
Institute for Water and Wastewater Technology,
Durban University of Technology, P. O. Box 1334,
Durban 4000, South Africa
e-mail: sheenas@dut.ac.za

Introduction

Wastewater in its raw and untreated form usually contains a heavy load of nutrients, chemicals and pathogens, which results in pollution and widespread waterborne diseases when discharged into the receiving aquatic environments (Holeton et al. 2011; Uan et al. 2013). Inorganic nitrogen (ammonia and nitrate) and phosphates at a higher level (>0.05 mg/L) may stimulate eutrophication (WEF 2009; Chuai et al. 2012). Even at low concentrations (<0.2 mg/L), the unionized ammonia has been reported to be acutely toxic to fish (Yang et al. 2010; Chen et al. 2012). Globally, there are national environmental agencies which regulate and oversee compliance with the effluent discharge limits which includes dissolved organic carbon (biological or chemical oxygen demand) as well as nitrogen compounds and phosphates (Holeton et al. 2011).

The epidemics in London between 1831 and 1866, which resulted from water pollution, necessitated the requirement of specific regulations on wastewater treatment and discharge. This in turn prompted the construction and operation of wastewater treatment plants (WWTPs) and the eventual development of advanced wastewater treatment technologies (Glicksman and Batzel 2010; Sciampacone 2013). Although there are biological and chemical WWTPs, the former are usually the preferred choice as they are more environmentally friendly (Akpor and Muchie 2010). Activated sludge, membrane bioreactors (MBRs), trickling filters, up-flow anaerobic sludge blanket reactors, lagoons and artificial wetlands are the most commonly used biological treatment processes for both industrial and domestic wastewaters (Akpor and Muchie 2010; Heffernan et al. 2011). However, the design and operation of treatment systems are constantly being improved for better efficiency and robustness. The current

drive is towards using submerged MBRs for treating wastewater, which combine membrane filtration with a biological reactor (Liang et al. 2010; Marti'n-Pascual et al. 2013).

The submerged MBR systems use membrane interception to separate activated sludge from treated water, thereby controlling and increasing the solid retention time independently from the hydraulic retention time within the bioreactor (Lesjean et al. 2011; Lin et al. 2011; Sarp et al. 2011; Yu et al. 2011; Zaw et al. 2011). Under these conditions, nitrifiers, which are naturally slow growers, are intercepted by the membrane and prevented from being washed out of the reactor. This allows them to have sufficient concentration of slow-growing microorganisms such as the nitrifiers and helps them to achieve a strong tolerance of shock loads with stable and highly efficient nitrification (Yu et al. 2011). Membrane retention greatly influences the bioprocesses and the microbial community structure development within the MBR (Wan et al. 2011). A complete nitrification was reported in MBR systems, when operated with longer sludge retention time (SRT) (Kumar et al. 2012). There is a need to understand the links between the microbial dynamics, wastewater composition and the stability of the biological system in order to design a sustainable treatment process (Gentile et al. 2007). Moreover, operating MBR systems efficiently remains controversial due to insufficient information on the development and activity of microorganisms (Li et al. 2006; Du et al. 2008).

This review focuses on understanding the ecophysiology of nitrifying bacteria and their impact on nitrification process in MBRs. The possible role of ammonia-oxidizing archaea (AOA) in MBR nitrification process was highlighted. The nitrification efficiency in MBRs was compared to the conventional activated sludge systems. Finally, advantages and limitations of the current detection methods for nitrifiers have also been highlighted.

Nitrification and nitrifying community structure of MBR

Nitrification process

Nitrification involves the biological conversion of ammonia/ammonium to nitrite by ammonia-oxidizing bacteria (AOB) followed by the conversion of nitrite to nitrate by nitrite-oxidizing bacteria (NOB) (Bae et al. 2013). In a wastewater treatment process, most of the organic nitrogen contained in raw sewage in the form of urea and faecal material will be converted to ammonia by hydrolysis through anaerobic processes while travelling through the sewer pipes. Various authors have noted that nitrification can be carried out by organisms other than bacteria such as protozoa, algae and fungi, however, at a very low level (1,000–10,000 times less than the rates associated with bacteria) (Gerardi 2002; Nicol and Schleper 2006). The nitrifying bacteria therefore represent an important group in the global nitrogen cycle. The nitrifying bacteria are aerobes and chemolithoautotrophs, obtaining their energy by oxidation of either NH_3 or NO_2^- (Daims and Wagner 2010; Jin et al. 2010). Some bacteria can also oxidize NH_3 under anaerobic conditions in a process known as anammox (anaerobic ammonium oxidation). These organisms use NH_4^+ as their energy source and NO_2^- as electron acceptor with the production of hydrazine in an intermediate process (Xiao et al. 2013).

Although all nitrifiers are known to be slow growers, NOB has a lower specific growth rate than the AOB (Daims and Wagner 2010). Among these, AOB uses the enzyme ammonia monooxygenase (*amoA*) to catalyse the oxidation of ammonia to hydroxylamine (NH_2OH)

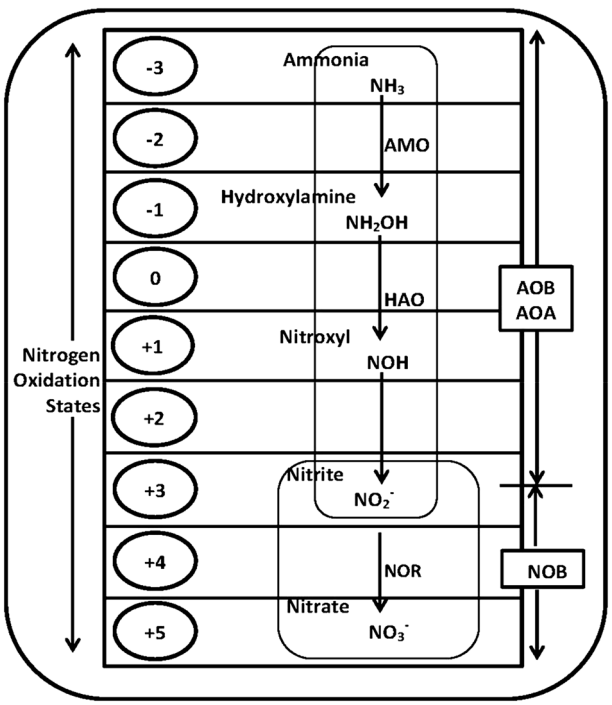

Fig. 1 The nitrification pathway incorporating the AOA [adapted from Alleman and Preston (2010)]. AMO: ammonia monooxygenase; HAO: hydroxylamine oxidoreductase; NOR: nitrite oxide

(Bahadoorsingh 2010), and the enzyme hydroxylamine oxidoreductase converts it to NO_2^- (Canfield et al. 2010). The NOB further oxidizes NO_2^- to NO_3^- using nitrite oxidoreductase enzyme (Bahadoorsingh 2010) (Fig. 1). Thus, a successful nitrification process requires a balance in the linked activity of the two nitrifying bacterial groups involved. However, there is a difference in the effect of environmental factors that influence these two groups of nitrifiers as they are phylogenetically different and prefer specific conditions for their growth. It has been reported that factors such as high ammonia concentration and low dissolved oxygen (DO) level can result in the disruption of the equilibrium between these two nitrification steps, resulting in significant reduction in the activities of nitrite oxidizers which can lead to toxic nitrite build-up and a subsequent failure of nitrification process (Mbakwe et al. 2013). According to Graham et al. (2007), there is a delicate and vulnerable AOB–NOB mutualism present which makes the process prone to chaotic behaviour and incomprehensible failure at times.

The nitrification process in wastewater is therefore limited by the AOB and NOB population densities and their physiological activities in different types of WWTPs, including conventional activated sludge treatment systems and MBR (Graham et al. 2007; Huang et al. 2010b; Zhang et al. 2011b). In both cases, the nitrification efficiency seems to be controlled by the prevalent operational and environmental conditions (Manser et al. 2005; Bahadoorsingh 2010). In two different MBRs investigated (one was adapted with biofilm support medium), Liang et al. (2010) noted that high nitrification rates correlated with the high species richness of nitrifying bacteria. Various authors have reported 96–99 and 92–98 % ammonia–nitrogen and COD removals, respectively, in MBRs investigated (Liang et al. 2010; Yu et al. 2010; Ozdemir et al. 2011). Although a higher nitrogen removal is being reported in MBR by various authors in comparison with conventional activated sludge systems, MBRs seem not to have an edge in terms of specific nitrification rates. Specific ammonia oxidization rate and nitrite oxidization rates were also reported to vary with no particular pattern in MBRs (Liang et al. 2010; Yu et al. 2010; Ozdemir et al. 2011). Nevertheless, the major advantage of MBR over CAS is the ability to retain a more diverse nitrifying community including slow-growing species, which makes it relatively more resilient in the face of operational and environmental fluctuations (Zhang et al. 2009a; Yu et al. 2011).

Nitrifying bacterial population (AOB and NOB)

The great majority of nitrifiers in wastewater remains uncultivable, and thus, only very few strains of AOB (25 species) and NOB (8 species) have so far been identified and classified based on conventional cultivation techniques (Egli et al. 2003; Wojnowska-Baryla et al. 2010). The growth rate of autotrophic bacteria (nitrifiers) is five times slower than that of heterotrophic bacteria in WWTPs (Ozdemir et al. 2011). Thus, the nitrifiers form only 3–10 % of the total bacteria in activated sludge (Gerardi 2002), which makes the isolation of nitrifiers difficult. However, the successful application of molecular techniques to the complex environmental samples has helped to unravel the complexity and diversity of these groups in nature. The 16S rDNA sequences revealed that these two groups of nitrifying bacteria (AOB and NOB) are phylogenetically distinct (Daims and Wagner 2010). All ammonia oxidizers can be classified in the β-subclass of Proteobacteria with the exception of *Nitrosococcus*, which belongs to a distinct branch of the γ-subclass. The NOB can be found within the α- and γ-subclasses of Proteobacteria, with the exceptions of *Nitrospira*, which has its own distinct phylum (Duan et al. 2013), and *Nitrospina*, which belongs to the δ-subclass of Proteobacteria (Zeng et al. 2012) (Fig. 2).

Due to their low specific growth rate and sensitivity to stress from environmental and operational factors, their population and physiological activities can limit the rate of biotransformation of nitrogen in many WWTPs. The characteristically higher mixed liquor suspended solids (MLSS) and long SRT in MBRs have been reported to favour the slow-growing nitrifiers compared to the conventional activated sludge plants (Cerrone et al. 2013). There have been contradictory reports on community structure of CAS and MBRs. Luxmy et al. (2000) based on a denaturing gradient gel electrophoresis (DGGE) analysis reported significant difference in the nitrifying community structures of conventional activated sludge system compared with pilot-scale MBR, while Manser et al. (2005) observed only a minor difference in the nitrifying community structures of MBR and CAS studied. However, a higher AOB and NOB fraction (7.19 %) of the total bacteria was reported in MBRs compared to the CAS (Zhang et al. 2011b). A study conducted by Ozdemir et al. (2011), on nitrification in MBRs (operating at an MLSS of 4.62 g/L), observed NOB (*Nitrospira* sp.) as the most abundant with a population about

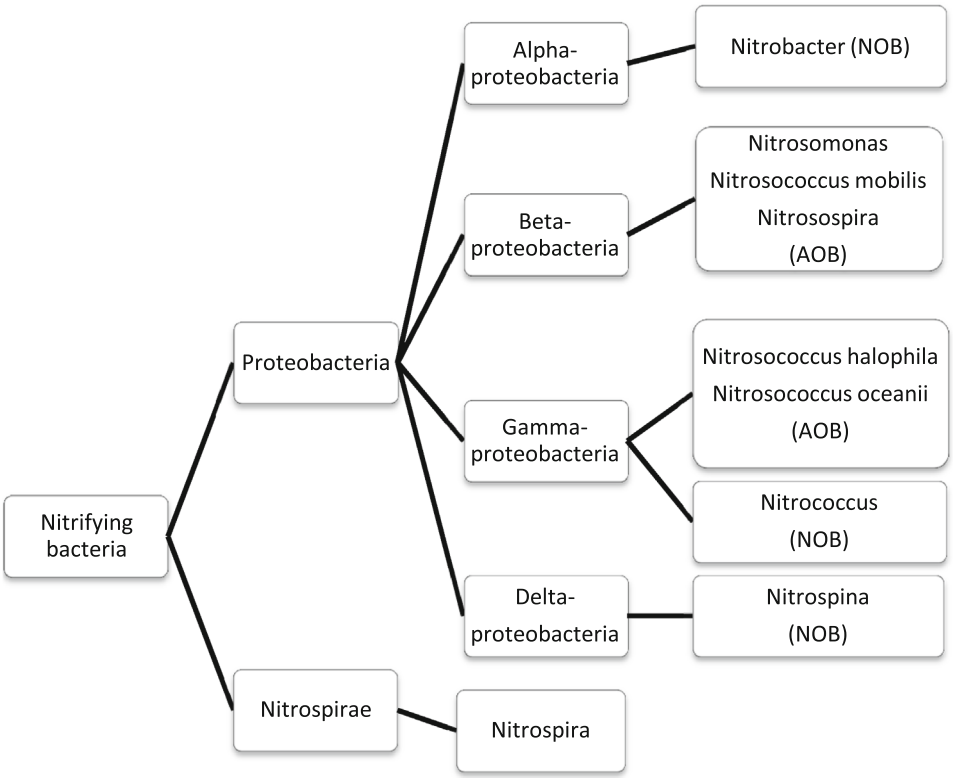

Fig. 2 Schematic representation of the nitrifying bacterial community in wastewater [adapted from Daims et al. (2001), Kowalchuk and Stephen (2001)]

10 times higher than AOB as the system proceeded towards the high MLSS. Ozdemir et al. (2011) also observed that the MBR was diverse in AOB richness, with different members of the *Nitrosomonas* and *Nitrosospira* lineage, whereas *Candidatus Nitrospira defluvii* was the only NOB detected throughout the study. Similarly, a study conducted by Zhang et al. (2009a), on AOB community on MBR, treating municipal wastewater, reported a shift in AOB dominance from initial *Nitrosomonas* spp. to *Nitrosospira* spp. throughout the later part of that study. These results indicate a possibility in the predominance of K-strategists group of nitrifiers (organism with high affinity for substrate, low growth rate and thrive at low substrate concentration) in MBRs compared to CAS owing to its more stable environment and a higher MLSS concentration (Chiellini et al. 2013).

Ammonia-oxidizing archaea (AOA): the new player in MBR nitrification

The developments in molecular biology techniques have helped us to understand the diversity, distribution and abundance of possible functional archaea in engineered systems such as WWTPs (Hatzenpichler 2012). In recent times, the contribution of archaea to nitrification in WWTPs is being acknowledged by various researchers; however, very little information is available on the physiology and activity of ammonia-oxidizing archaea in the environment. Among the different AOA, *Candidatus Nitrosopumilus maritimus* was the first member of the group to be isolated (Stahl and de la Torre 2012). This species is reported to have the same growth and cell production rates as those of AOB and is capable of using ammonia as the sole energy source for growth (You et al. 2009). AOA can tolerate environment with oxygen concentration over the range of <3.1 μM–0.2 mM; however, the environment with low oxygen may select them in contrast to AOB (Limpiyakorn et al. 2011). The AOB uses the Calvin cycle to fix their carbon, whereas the AOA rely on the 4-hydroxybutyrate pathway or citric acid cycle (Hatzenpichler 2012). The AOA was previously grouped under phylum *Crenarchaeota* (Jin et al. 2010; Zhang et al. 2011a) but have recently been reclassified under phylum *Thaumarchaeota* (Stahl and de la Torre 2012). Ozdemir et al. (2011) in their study on MBR nitrification reported the

presence of a small fraction of AOA among other nitrifiers. An observation of interest about AOA is that they are less sensitive to plant operational conditions (e.g. DO and ammonia loading) compared to AOB (Jin et al. 2010; Sonthiphand and Limpiyakorn 2010). The fact that in natural environments AOA are associated with significant role in nitrification contrary to what is currently known about engineered systems leaves the question of whether the optimum conditions for their ecological functioning have been adequately understood or replicated in such systems. If the assertion by some authors that AOA are tolerant to fluctuations in operational conditions (Jin et al. 2010; Sonthiphand and Limpiyakorn 2010) is valid, then it may be desirable to harness such potential for a stable and efficient wastewater treatment. There would be need for the development of engineered systems that can adequately replicate the conditions which support AOA's optimal growth as obtained in the natural environment.

The role and efficiency of AOA in nitrification remains unclear, since their isolation and cultivation is still largely unsuccessful. The archaeal membrane lipids (isoprenoid glycerol dialkyl glycerol tetraethers) have been suggested as a bioindicator for investigating the ecophysiology of AOA in wastewater treatment bioreactors. These lipids have been observed to correlate with archaeal amoA gene copies (You et al. 2009). Recently, AOA that were deficient in both carbon fixation and NH_3 oxidation abilities despite possessing amoA gene were reported (Mussmann et al. 2011; Stahl and de la Torre 2012). This indicates that AOA importance in nitrification cannot be determined based on mere presence or abundance of arch-amoA gene. Various authors observed that the AOA are able to utilize amino acids as a carbon source akin to heterotrophs (You et al. 2009; Bouskill et al. 2011). Recently, studies based on radiocarbon and genomic analyses suggest that the AOA either include both heterotrophs and autotrophs, or they are single population of mixotrophs (Bouskill et al. 2011).

Factors affecting the activity of nitrifiers and optimal nitrification in MBR

Nitrification, like any other process that hinges on microbial physiology, is subject to failure whenever there is a shift in operational conditions or in the presence of inhibitors (Ducey et al. 2010). This shift may result in loss of microbial populations which need to be replenished in order for the system to recover its functional ability (Kim et al. 2011). Thus, efficient nitrification in engineered systems such as MBR depends on a combination of environmental and operational parameters which include pH, temperature (Gerardi 2002; Kim et al. 2011) DO and substrate concentration (Bae et al. 2002; Liu et al. 2010a) and the presence of inhibitory or toxic substances (Çeçen et al. 2010).

Environmental factors

pH and temperature

Traditionally, efficient nitrification has been reported at a pH ranging from 7.5 to 8.5 (Sajuni et al. 2010; Fulweiler et al. 2011). A higher pH value of 8–9 was reported to favour elevated nitrite accumulation, thereby affecting the optimal nitrification process (Bae et al. 2002). A study conducted by Bae et al. (2002) in conventional activated sludge plants reported an increase in the specific ammonium oxidizing rate (SAOR) when the pH increased from 7 to 8; however, the rate dropped as the pH reached 9 and yielded the lowest activity at pH 10 (Bae et al. 2002). In the same experiment, the specific nitrite oxidizing rate (SNOR), on the other hand, correlated with an increase in pH from 7 to 9, but was decreased at pH 10. Similarly, He et al. (2009), in their study on MBR nitrification, noted that pH plays a major role in NH_3 and total nitrogen (TN) removal in MBR. When the influent pH was acidic (approximately 4.8), the NH_3 removal rate was 56 %, whereas that of TN was 45 %. An increased removal of NH_3 up to 99 % was observed, while that of TN rose to 91 % when the pH was neutral (7.2), and a decrease to 75 % (NH_3) and 60 % (TN) was noted when the pH increased to about 9.7. These findings by He et al. (2009) show that efficient nitrification within the MBRs still falls within known traditional pH range of 7.5–8.5.

Temperature typically has a significant effect on nitrifiers and their nitrification efficiency (Ducey et al. 2010). According to earlier studies, the optimal temperature range for nitrifiers was observed to be between 15 and 30 °C (Chandra and Sathasivan 2011). Colliver and Stephenson (2000) reported that most nitrifiers will grow optimally in a temperature range of 25–30 °C. A study conducted by Huang et al. (2010a) showed that the optimal temperature regime that supported *Nitrobacter* growth was between 24 and 25 °C, whereas higher range of 29–30 °C favoured *Nitrospira*. The activity of the AOB is generally faster than that of NOB because of their different activation energy, which is between 72 and 60 kJ mol^{-1} for AOB, whereas it is from 43 to 47 kJ mol^{-1} for NOB (Hulle 2005). In a MBR system, Kim et al. (2008) found that when the temperature increased from 20 to 30 °C, oxidation of ammonia proceeded from 0.253 to 1.33 g N/g VSS d (5.3-fold

increase) whereas nitrite oxidation was just by a multiple of 2.6 times (0.45–1.18 g N/g VSS d), thereby indicating a high correlation of temperature with ammonia oxidation. These various findings therefore indicate that environmental conditions influence differently the various nitrifying bacterial groups (AOB and NOB).

Recent reports on activity of nitrifiers at very low temperature and DO level indicate that nitrifiers are capable of adapting to extreme conditions such as low temperature. Zhang et al. (2011a) observed that *Nitrosospira* spp. thrive at lower temperatures (4–10 °C) compared to other nitrosifyer bacteria. Ducey et al. 2010 reported a high nitrification rate of 11.2 mg N/g MLVSS/h at low temperatures (5 °C) by an acclimatized nitrifying community, which is far above the optimum 1.71–2.0 mg NO_3-N/gVSS-h reported by Fan et al. (2000) and Kornboonraksa et al. (2009). These observations show that nitrifiers are capable of adaptation in response to environmental and operational conditions; however, a sudden shift in environmental factors would certainly affect the nitrifiers' activity.

Plant operational conditions

Dissolved oxygen

A DO concentration of between 3 and 4 mg O_2 L^{-1} has been described as optimum for AOB and NOB growth in MBR systems (Hulle 2005). However, Sarioglu et al. (2009) observed a higher nitrogen removal of about 85–95 % in an MBR treating domestic wastewater when the DO level was maintained at low level (1.5 mg O_2 L^{-1}). Niche-specific adaptation to DO concentration has been observed within the NOB, with *Nitrospira* demonstrating a negative correlation to DO concentrations ($r = -0.46$, $P < 0.01$), whereas *Nitrobacter* exhibited a positive correlation ($r = 0.38$, $P < 0.01$) (Huang et al. 2010b). *Nitrobacter* population was also found to increase in winter (low temperatures) and high DO levels (Huang et al. 2010a). This shows that nitrifiers can be highly specialized, exhibiting niche-specific adaptation in response to environmental and operational conditions. The microbial ecology of nitrifiers reveals that *Nitrospira* thrives optimally in an environment with a combination of low nitrite and oxygen levels, whereas *Nitrobacter* requires an environment with elevated levels of nitrite and oxygen. This makes them K- and r-strategists, respectively, based on r–K selection theory (Bahadoorsingh 2010). Studies on the relationship between NOB (*Nitrospira* and *Nitrobacter*) populations and their sensitivity to environmental/operating factors that favours good nitrification (under high DO and limited NH_3 conditions) are necessary to understand their effect on plant performance (Huang et al. 2010a).

Sludge retention time

It has been reported that longer SRT could impact the biological activities negatively including nitrification rate of MBR (Yu et al. 2010). A study conducted by Yu et al. (2010), observed a negative correlation between SRT and the nitrifier activities, i.e. for both SAOR and specific nitrate formation rate (SNFR). The MBR system operated at a shorter SRT of 30 days showed a higher SAOR and SNFR (0.22 kg NH_4^+-N/kg MLSS/day and 0.13 kg NO_3-N/kg MLSS/day, respectively), compared to the system operated for a longer SRT of 90 days (0.12–0.14 kg NH_4^+-N/kg MLSS/day and 0.068–0.042 kg NO_3-N/kg MLSS/day, SAOR and SNFR, respectively). This reflects earlier reports which also indicated a negative correlation of SRT to SAOR/SNFR in MBR (Li et al. 2006). Similarly, Huang et al. (2001) reported that SRT has no significant influence on the biological activity of the MBR when operated at SRT of less than 40 days. Cicek et al. (2001) reported that when a pilot MBR was operated at an increased SRT up till 30 days, there was no significant effect on nitrification. MBR can be operated at relatively longer SRTs compared to CAS, which confers the advantage of retention of a larger diversity of nitrifiers on them. However, from the above findings, SRTs above 40 days should not be encouraged since they influence nitrification adversely.

Substrate concentration

Research findings have shown that nitrifiers get inhibited by free ammonia and unionized nitrous acid (Gil and Choi 2001). Increased accumulation of NH_3 in biotreatment systems occurs whenever toxicant or any inhibitory factor disrupts the nitrifiers' functional ability. This increased NH_3-N level often gets to inhibitive level, which can result in loss of nitrification that can last for several days to months. Concentration of NH_3-N above a threshold of 200 mg/L has been reported to inhibit nitrification efficiency (Mordorski 1987; Kim and Kim 2003). Optimizing the C:N ratio is also essential for efficient nitrogen removal in waste treatment systems. A low C:N ratio favours nitrification, whereas a higher ratio supports the heterotrophs (Fu et al. 2010). In a study of membrane-aerated biofilm reactor, nitrification efficiency of 93 % was achieved at C:N ratio 5; however, at C:N ratio of 6,

Table 1 Summary of techniques that have been employed for biodiversity studies on nitrifiers

Type of reactor	Sample type	Method of analysis	Population detected	Reference
Lab scale: single aerobic zone MBR; submerged hollow fibre	Synthetic wastewater	FISH	Nitrospira sp., nitrosomonas sp., nitrobacter sp.	Li et al. (2006)
Lab scale: single aerobic zone MBR; submerged hollow fibre	Synthetic wastewater	Quinone profiling	α, β, and γ *Proteobacteria*	Li et al. (2006)
Full scale: anaerobic reactor, anoxic reactor, aerobic reactor and submerged hollow fibre membrane tank	Municipal sewage	FISH	AOB and nitrobacter sp.	Yu et al. (2011)
Full scale: anaerobic reactor, anoxic reactor, aerobic reactor and submerged hollow fibre membrane tank	Municipal sewage	PCR-DGGE	Nitrosomonas sp.,	Yu et al. (2011)
Full scale: anaerobic reactor, anoxic reactor, aerobic reactor and submerged hollow fibre membrane tank	Municipal sewage	RFLP	Nitrosomonas sp.,	Yu et al. (2011)
Lab scale: single aerobic zone MBR; submerged hollow fibre	Synthetic wastewater	FISH	β-*Proteobacteria* (nitrobacter)	Yu et al. (2010)
Lab scale: single aerobic zone MBR; submerged hollow fibre	Synthetic wastewater	PCR-DGGE	Nitrosomonas sp.; nitrosospira sp.; nitrospira sp.	Yu et al. (2010)
Lab scale: single aerobic zone MBR; submerged hollow fibre	Synthetic wastewater	RFLP	Nitrosomonas sp.; nitrosospira sp.	Yu et al. (2010)
Lab scale: single aerobic zone MBR coupled with fibre anaerobic packed-bed biofilm reactor	Synthetic wastewater	FISH	β-*Proteobacteria*; nitrobacter sp.	Zhang et al. (2009b)
Lab scale: (a) anoxic–aerobic module; (b) anoxic–aerobic module incorporated with biofilm support plastic medium. Both have hollow fibre membrane	Synthetic wastewater	T-RFLP	Nitrosomonas sp.; nitrospira sp.; nitrobacter sp.	Liang et al. (2010)
Full scale: activated sludge system	Industrial wastewater	Microarrays	Nitrosomonas sp.	Kelly et al. (2005)
Full scale: activated sludge system	Municipal wastewater	Microarrays	Nitrospira sp.	Siripong et al. (2006)

FISH fluorescent in situ hybridization, *PCR–DGGE* polymerase chain reaction–denaturing gradient gel electrophoresis, *RFLP* restriction fragment length polymorphism, *T-RFLP* terminal restriction fragment length polymorphism, *MBR* membrane bioreactor

increased heterotrophic bacteria growth was observed with resultant inhibition of nitrifiers (Liu et al. 2010b). According to Fu et al. (2010) also, it was observed that AOB and NOB showed negative correlation with C:N ratio.

Techniques applied in investigating the biodiversity of nitrifiers: the pros and cons

Culture-dependent methods have been relied upon solely in the past to study microorganisms present in the natural environment including wastewater ecosystems. Only a few bacterial species were thought to be involved in the process based on these laboratory culture techniques, whereas in reality, a great diversity of the organisms involved is nonculturable. The advent of molecular techniques has brought about a better understanding of the structure and functions of microbial communities including nitrifiers in wastewater treatment systems. These, however, still have limitations when applied in full-scale WWTPs. Summary of these techniques used for detecting nitrifiers from wastewater ecosystem is presented in Tables 1 and 2. Some advantages and disadvantages of these commonly used techniques are highlighted below.

Among these, quinone profiling, a chemotaxonomic method, is being commonly used by researchers for microbial community structure analysis from environmental samples (Kurisu et al. 2002). This technique is based on the presence of specific respiratory quinone as an

Table 2 Summary of techniques that have been employed for quantitative studies on nitrifiers

Type of MBR	Influent	Method of analysis	Estimated population	Reference
Lab scale: single aerobic zone MBR; submerged hollow fibre	Synthetic wastewater	FISH	AOB (% among total bacteria) ≈23–57 %; Nitrosomonas sp. (% among AOB) ≈50–90 %	Li et al. (2006)
Lab scale: single aerobic zone MBR; submerged hollow fibre	Synthetic wastewater	MPN	AOB ≈10^7–10^9 L^{-1}; NOB ≈10^5–10^8 L^{-1}	Li et al. (2006)
Full scale: anaerobic reactor, anoxic reactor, aerobic reactor and submerged hollow fibre membrane tank	Municipal sewage	FISH	AOB ≈1.9–4.5 %; NOB ≈0.9–2.8 %	Yu et al. (2011)
Pilot scale: anaerobic-anoxic–aerobic zones	Domestic wastewater	q-PCR	amoA AOB ≈1.15–4.05 %; Nitrobacter ≈0.04–1.17 %; Nitrospira ≈8.23–13.01 %; amoA AOA ≈0.05–0.09 %	Ozdemir et al. (2011)
Lab scale: single aerobic zone MBR; submerged hollow fibre	Synthetic wastewater	MPN	AOB ≈1.5×10^7–3.4×10^7 cells g^{-1} MLSS; NOB ≈2.7×10^4–1.4×10^7cells g^{-1} MLSS	Yu et al. (2010)
Lab scale: (a) anoxic–aerobic module; (b) anoxic–aerobic module incorporated with biofilm support plastic medium. Both has hollow fibre membrane	Synthetic wastewater	q-PCR	AOB ≈10^8 cells/L	Liang et al. (2010)

FISH fluorescent in situ hybridization, *MPN* most probable number, *q-PCR* quantitative real-time PCR, *MBR* membrane bioreactor, *AOB* ammonia-oxidizing bacteria, *NOB* nitrite-oxidizing bacteria, *MLSS* mixed liquor suspended solids, and *amoA* ammonia monooxygenase

indicator of a particular bacterial population. However, since some bacterial groups that are phyletically different share similar quinone groups, this technique is inadequate for analysis beyond the phylum level (Kurisu et al. 2002). In a study on MBR by Li et al. (2006), ubiquinones belonging to UQ-8 (β-*Proteobacteria*), UQ-9 (α-*Proteobacteria*) and UQ-10 (γ-*Proteobacteria*) were recovered. However, the authors noted the difficulty in reconciling the NOB with the quinone profiles. The entire analysis based on the quinone profiling is characterized by assumptions and would require more specific techniques to complement it. Thus, the species specificity was a limiting factor for this technique when applying to complex environmental samples.

Fluorescent in situ hybridization, a widely used molecular method, involves the binding of fluorescent oligonucleotide probes (probes available for nitrifiers are listed in Tables 3, 4, 5) to ribosomal ribonucleic acid (Nielsen 2009; Junier et al. 2010; Xia et al. 2010b; Yu et al. 2011). This method can be employed for both identification and quantification of specific bacterial groups directly from the environment even up to the species level (Li et al. 2006) (Tables 3, 4, 5). However, the major limitations of this technique include the lack of availability of probes, inefficient cell permeability, inadequate or difference in ribosome content which can lead to low signal intensity, loop and hairpin formation of rRNA structure, as well as rRNA-protein interactions which hinders hybridization, auto-fluorescence and non-specific bindings (Nielsen 2009). Unlike the fast-growing microorganisms, the cellular rRNA content of anammox and β-*Proteobacterial* ammonia oxidizers do not really reflect the physiological activity of these organisms, especially during starvation and inhibition periods (Schmid et al. 2005). Thus, correlation of the nitrifier population to its physiological activity can be biased (Schmid et al. 2005). Witzig et al. (2002) observed that due to the low food-to-microorganisms conditions in MBRs with resultant low rRNA molecules for the organisms, less than half of the population were detectable by FISH whereas 80 % in CAS. However, few researchers have reported a direct correlation between nitrifier population and specific ammonium and nitrite oxidation rate using FISH probes (Yu et al. 2011). Yu et al. (2011) in a study using FISH observed a direct correlation between the nitrifier population and the specific ammonium and nitrite utilization rate. Thus, in spite of all the above-mentioned limitations, FISH is still considered important as it provides information about the presence, abundance, morphology

Table 3 rRNA-targeted oligonucleotide probes for detecting AOB in the activated sludge

Probe name	Target	Sequence (5′–3′)	FA (%)	Reference
Nso1225	β-*Proteobacterial* ammonia-oxidizing bacteria	CGC CAT TGT ATT ACG TGT GA	35	Bassin et al. (2012)
Nse1472	*Nitrosomonas europea*, *N. halophila*, *N. eutropha*, Kraftisried-Isolate Nm103	ACC CCA GTC ATG ACC CCC	50	Bassin et al. (2012)
Nsc825	β-*Proteobacterial* ammonia-oxidizing bacteria	CCC TCC CAA CGT CTA GTT	ND	Siripong et al. (2006)
Nsm 156	*Nitrosomonas* sp., *Nitrosococcus mobilis*	TAT TAG CAC ATC TTT CGA T	5	Liu et al. (2010a)
Nsv443	*Nitrosospira* sp.	CCG TGA CCG TTT CGT TCC G	30	Liu et al. (2010a)
Nso 190	Betaproteobacterial ammonia-oxidizing bacteria	CGA TCC CCT GCT TTT CTC C	55	Liu et al. (2010a)
NEU	Most halophilic and halotolerant *Nitrosomonas* sp.	CCC CTC TGC TGC ACT CTA	35/ 40	Bassin et al. (2012); Cui et al. (2013)
NmIV	*Nitrosomonas cryotolerans* lineage	TCT CAC CTC TCA GCG AGC T	35	Bellucci and Curtis (2011)
NmII	*Nitrosomonas communis* lineage	TTA AGA CAC GTT CCG ATG TA	25	Bellucci and Curtis (2011)
NmV	*Nitrosococcus mobilis*	TCC TCA GAG ACT ACG CGG	35	Bassin et al. (2012)
Cluster 6a192	*Nitrosomonas oligotropha lineage* (Cluster 6a)	CTT TCG ATC CCC TAC TTT CC	35	Gilmore et al. (2012)

FA formamide, *ND* not determined

Table 4 rRNA-targeted oligonucleotide probes for detecting NOB

Probe name	Target	Sequence (5′–3′)	FA (%)	Reference
Ntspa662	Genus *Nitrospira*	GGA ATT CCG CGC TCC TCT	35	Li et al. (2013)
NIT3	Genus *Nitrobacter*	CCT GTG CTC CAT GCT CCG	40	Wu et al. (2013)
Ntspa1431	*Nitrospira* sub-linage I	TTG GCT TGG GCG ACT TCA	35	Raszka et al. (2011)
Ntspa1151	*Nitrospira* sub-linage II	TTC TCC TGG GCA GTC TCT CC	35	Raszka et al. (2011)
Ntspa 1026	*Nitrospira moscoviensis*, activated sludge clones A4 and A11	AGC ACG CTG GTA TTG CTA	20	Hauzmayer (2010)
Nsr1156	*Nitrospira moscoviensis*, freshwater *Nitrospira* sp.	CCC GTT CTC CTG GGC AGT	30	Mota et al. (2012)
Nspmar62	*Nitrospiramarina*-related *Nitrospira*	GCC CCG GAT TCT CGT TCG	40	Daims and Wagner (2011)
NTG840	*Nitrotogaarctica*	CTA AGG AAG TCT CCT CCC	10–20	Daims and Wagner (2011)
Ntspa712	Phylum *Nitrospitae*	CGC CTT CGC CAC CGG CCT TCC	35/50	Morales et al. (2013)

FA formamide, *ND* not determined

and spatial distribution of microorganisms in its natural habitat. Due to these advantages, researchers have come up with new ideas for its improvement, viz catalysed reporter deposition–FISH, microautoradiography combined with FISH, FISH–confocal scanning laser microscope, and combinatorial labelling and spectral imaging–FISH (Egli et al. 2003; Daims et al. 2006; Valm et al. 2012).

Denaturing gradient gel electrophoresis, a PCR-based method, is a common method of choice by researchers and

Table 5 rRNA-targeted oligonucleotide probes to be used in detecting AOA

Probe name	Target	Sequence (5′–3′)	FA (%)	Reference
CREN499	Most crenarchaeota		0	Xia et al. (2012)
CREN537	Crenarchaea	TGA CCA CTT GAG GTG CTG	20	Bleijswijk et al. (2013)
CREN569	Most environmental crenarchaeota	GCT ACG GAT GCT TTA GG	0	Radax et al. (2012)

FA formamide, *ND* not determined

is based on generating a genetic profile or "fingerprint" of the microbial community of complex environmental samples (Li et al. 2006; You et al. 2009). The species richness of the microbial community being examined is revealed by the different base pair sequences in the amplicons (Gao and Tao 2012). This method has been employed extensively by researchers to evaluate the microbial community composition of different wastewater treatment samples (Boon et al. 2002; Xia et al. 2010b; Zhang et al. 2010) or "shifts" in microbial community composition over time (Wan et al. 2011; Yu et al. 2011; Zhang et al. 2009a). Yu et al. (2011) in their study of MBR using a combination of PCR-DGGE and clone library analysis established *Nitrosomonas* sp. as the dominant AOB. This technique has also been used successfully by researchers to study the shift in the dominance of different species of nitrifiers in MBR (Yu et al. 2011). The sensitivity of this method is high, and its main advantage is that the individual DNA bands, or fragments from the gel can be excised and phylogenetically analysed. However, since the DGGE analysis can only be performed for shorter PCR amplicons ($\leq$500 bp), the sequences of the bands obtained from a gel correspond to only short fragments of DNA (200–500 bp), and thus, the phylogenetic relations are less constantly established using DGGE bands (Sanz and Köchling 2007; Gao and Tao 2012). Analysis from DGGE technique can also be influenced adversely by the following limitations: the difficulty of DNA extraction and PCR amplification, depending on the nature of the samples, the variations in DNA copy number after PCR, depending on the abundance of the specific microorganisms and the intensity of the band obtained on a DGGE gel (Sanz and Köchling 2007; Gao and Tao 2012). The non-specific amplification of the PCR primers (primers available for amplifying nitrifiers are shown in Table 6) and the presence of duplex molecules of DNA can also introduce error into the results obtained by this method (Guler 2006; Li et al. 2006).

Terminal restriction fragment length polymorphism (T-RFLP) is one of the latest molecular techniques which are being used by the researchers to monitor the microbial shift based on the restriction banding pattern. This technique involves cleavage of terminally labelled PCR-amplified gene by the restriction enzymes (Sanz and Köchling 2007; Gao and Tao 2012). The technique can be employed to investigate the shift in both the spatial and temporal microbial community composition from a given natural or engineered ecosystem (Yang et al. 2011). It is a highly sensitive technique and can be used for semi-quantitative analysis of microbial populations in a particular microbial ecological system as an alternative to PCR-DGGE (Liu et al. 2010b). The fingerprints from T-RFLP are usually inadequate for identification of individual taxonomic units (Yang et al. 2011). Nonetheless, it is possible to sequence and identify the dominant organisms via comparison of the fragments generated with a sequence from a public database or a related clone library (Yang et al. 2011). However, same like any other PCR-based techniques, the biases related to DNA isolation steps and amplification also can affect the accuracy of this method (Sanz and Köchling 2007). Liang et al. (2010) used the T-RFLP technique successfully to investigate the difference in nitrifier population from two different MBR systems.

The quantitative real-time PCR (qRT PCR) is the most commonly used and accepted technique in the recent years to quantify microbes from natural and engineered environments. This technique can be used to quantify the particular gene copies of target organisms from a complex environment using species-specific primers. It is an efficient and rapid technique regarded as more sensitive than FISH (Haarman and Knol 2005; Fukushima and Bond 2010). However, according to Zhang et al. (2009a), the application of either AOB 16S rDNA or the functional gene amoA for the analysis usually has their different shortcomings of false positives and false negatives, respectively. A combination of the two assays is therefore usually a way of overcoming and compensating for the disadvantages when applied to AOB detection and quantification. Using qRT-PCR, Ozdemir et al. (2011) investigated nitrifiers in MBR and found that the NOB (*Nitrospira*

Table 6 Available specific primers for AOB, AOA and NOB

Primers	Target	Sequence (5′–3′)	Reference
amoA-1F	Ammonia monooxygenase	GGGGTTTCTACTGGTGGT	Yu et al. (2010)
amoA-2R	Ammonia monooxygenase	CCCCTCKGSAAAGCCTTCTTC	Yu et al. (2010)
FGPS872	*Nitrobacter*	CTAAAACTCAAAGGAATTGA	Ozdemir et al. (2011)
FGPS1269	*Nitrobacter*	TTTTTTGAGATTTGCTAG	Ozdemir et al. (2011)
NSR1113F	*Nitrospira*	CCTGCTTTCAGTTGCTACCG	Wang et al. (2011)
NSR1264R	*Nitrospira*	GTTTGCAGCGCTTTGTACCG	Wang et al. (2011)
CTO189fAB	β-*Proteobacteria* ammonia oxidizers	GGAGRAAAGCAGGGGATCG	Yu et al. (2011)
CTO189fC	β-*Proteobacteria* ammonia oxidizers	GGAGGAAAGTAGGGGATCG	Yu et al. (2011)
CTO654r	β-*Proteobacteria* ammonia oxidizers	CTAGCYTTGTAGTTTCAAACGC	Yu et al. (2011)
Arch-amoAF	Archaeal ammonia monooxygenase	STAATGGTCTGGCTTAGACG	Lopez-Legentil et al. (2010)
Arch-amoAR	Archaeal ammonia monooxygenase	GCG GCC ATC CAT CTG TAT GT	Lopez-Legentil et al. (2010)
CRENamo_F	Archaeal ammonia monooxygenase	ATGGTCTGGCTAAGACGMTGTA	Jin et al. (2010)
CRENamo_R	Archaeal ammonia monooxygenase	CCCACTTTGACCAAGCGGCCAT	Jin et al. (2010)

sp.) population was 5–10 times higher than that of AOB. This is in contrast to various reports on conventional activated sludge systems, which usually indicate AOB as the dominant population (Guo et al. 2010) in wastewater treatment. Among the NOB population, *Nitrospira* sp. was also observed to have 16S rRNA gene copy/cell about 100 times more than *Nitrobacter* (Ozdemir et al. 2011). This difference in the amount of rRNA/cell for different species could lead to erroneous results.

Pyrosequencing is a real-time DNA sequencing technique that monitors DNA synthesis through a series of linked enzymatic processes (Ronaghi 2000; Ronaghi and Elahi 2002). It is a promising and relatively new technique that has the advantage of being rapid and highly accurate (Rastogi and Sani 2011). Unlike other alternatives, it does not require cloning, gel electrophoresis, size separation, labelled oligonucleotides or labelled primers (Fakhrai-Rad et al. 2002; Clarke 2005; Prosser and Nicol 2008). The technique can be employed to detect, identify and type bacteria (Clarke 2005; Sanapareddy et al. 2009). Pyrosequencing was first employed for investigating activated sludge community in a study to reveal the plasmid metagenome and the antimicrobial resistance tendencies of the microbial community present (Hu et al. 2012). Ye et al. (2011) in his study on activated sludge community noted that the commonly employed molecular techniques can underestimate the complex populations in wastewater, but pyrosequencing has the potential of a more reliable estimation and better understanding of such communities. However, this technology is relatively new and consequently only few studies of microbial community structure and nitrifiers in wastewater have so far been recorded (Xia et al. 2010a; Ye et al. 2011; Zhang et al. 2011a). In a study of nitrifying communities in WWTPs, Ye et al. (2011) using pyrosequencing identified *Nitrosomonas* spp., *Nitrospira* spp., *Nitrospira* spp., *Nitrosococcus* spp. and *Nitrobacter* spp. They noted that apart from *Nitrosomonas* spp. and *Nitrospira* spp., other nitrifiers did not have significant contribution in the nitrification process (Ye et al. 2011). Zhang et al. (2011a) in a study observed an incongruity in the results when nitrifying communities in different wastewater bioreactors were analysed using quantitative PCR and pyrosequencing. Majority of the nitrifiers identified with high-thoroughput pyrosequencing were related to *Nitrosomonas* spp. (Zhang et al. 2011a).

Some other less frequently applied techniques for wastewater samples include microarray and most probable number (MPN). Among these, microarray is a multiplex technique that harnesses the characteristics of DNA or RNA to bind to their complementary sequences (Gilbride et al. 2006). Siripong et al. (2006) in their study on WWTPs noted that the microarray technique was able to confirm the presence of nitrifiers; however, due to insufficient fluorescence intensity, it failed to differentiate adequately between matched and mismatched sequence. This indicates a significant shortcoming of this technique. Kelly et al. (2005) investigated nitrifiers in samples from a wastewater treatment facility and observed that the microarray technique could detect nitrifiers directly without any need for complementary PCR amplification. However, they observed that other

methods, especially T-RFLP, were sensitive enough to confirm the presence of more diversity of nitrifiers (*Nitrospira* sp. and *Nitrobacter* sp.) apart from only *Nitrosomonas* sp. that microarray could detect (Kelly et al. 2005). The MPN involves samples being incubated in a mineral medium selective for nitrifiers. This method usually involves biasness because the synthetic medium and laboratory conditions cannot truly reproduce the complex ecological interactions which apply in the activated sludge environment (Hirooka et al. 2009; Xia et al. 2010a; Ayanda and Akinsoji 2011). The cells are sometimes bound within the complex matrix called floc, and some microbes interdepend on others for their metabolic activities, which can only be achieved in a complex ecosystem such as the activated sludge system (Ducey et al. 2010). In effect negligible diversity and amount of the nitrifiers are enumerated using the MPN (Ayanda and Akinsoji 2011; Xia et al. 2010a). Li et al. (2006) investigated nitrifiers' population dynamics in MBR using MPN and FISH, and they observed that FISH correlated more with the specific nitrification rate analysis than the MPN.

Conclusion

In this review, the ecophysiology of the various nitrifying populations in MBR ecosystem was examined. The AOA have been identified to play significant role in nitrification in other environments; however, there is still need for more studies in order to fully understand their metabolic pathway and actual contribution to nitrification in MBRs. The impacts of the various operational and environmental conditions on nitrifiers in MBR have been highlighted. The MBRs have characteristic operational higher MLSS and long SRT as advantage over the conventional activated sludge system. This encourages high diversity of microbial communities which makes the system relatively more tolerant to environmental and operational shocks. However, SRT higher than 40 days can impact nitrification negatively. Likewise, the low food-to-microorganisms conditions in MBRs and the resultant low rRNA molecules for the organisms lead to poor estimation of its microbial community. The molecular biology techniques have contributed immensely to the elucidation of the nitrifying community structure in MBRs; however, limitations and biases of the individual techniques still pose challenges to the accuracy of the results.

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