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Full Length Research Paper

Biological wastewater treatment: Microbiology, chemistry, and diversity measurement of ammonia oxidizing bacteria

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Nitrification is an important biological process in nitrogen cycling and has a significant effect on effluent quality in wastewater treatment. Nitrification occurs in two steps by two types of chemoautotrophic bacteria, the ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB). The ammonia oxidizing bacteria is responsible for the oxidation of ammonia with oxygen into nitrite and is often the rate-limiting step in nitrification in wastewater treatment plants. Hence, a better understanding of the ecology, microbiology and chemistry of ammonia oxidizing bacteria in biological wastewater treatment systems is necessary in order to enhance treatment performance and control. A detailed review of various biological wastewater treatment processes, ammonia oxidizing bacteria and archaea; economic importance, problems, various molecular techniques for the investigation of the diversity and community structure, as well as the isolation of ammonia oxidizing bacteria were discussed.

Key words: Wastewater, activated sludge process, nitrification, ammonia oxidizing bacteria, ammonia oxidizing archaea, nitrite oxidizing bacteria, PCR primers.

INTRODUCTION

Wastewater treatment is the process of taking wastewater and making it suitable for discharge back into the environment. Wastewater results from a number of different activities, including industrial activities, rainwater runoff and domestic activities (Van der Hoek, 2004; Thomas, 2005). No matter where the wastewater comes from, this water is full of bacteria, chemicals, and other contaminants. Wastewater treatment therefore reduces the contaminants to acceptable levels so as to be safe for discharge into the environment. There are two types of wastewater treatment systems: a biological treatment plant and a physical/chemical treatment plant. Either of the treatment plants are utilized depending on the nature and components of pollution but some other pollution will require a combination of both wastewater treatment systems. When considering either a chemical or biological wastewater treatment for a particular application, it is very important to understand the sources of the wastewater generated, typical wastewater composition, discharge requirements, events and practices within a facility that can affect the quantity and quality of the wastewater, and pretreatment ramifications. Consideration of these factors will allow for maximization of the benefits the treatment plant will gain from effective wastewater treatment.

The various wastewater treatment processes have the following objectives: To confer and preserve the inherent physical, chemical and biological qualities of water of different origins which make it suitable for specific uses such as water for drinking and for use in productive processes, to protect the public from health risks without causing any damage to the environment and to confer and preserve those characteristics of water in its natural

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environment which are necessary for the conservation and development of fauna and aquatic vegetation, and for provision of drinking water for cattle and wild animals or for recreational and aesthetic purposes (Boari et al., 1997).

Ammonia wastewater treatments

Nitrogen appears in wastewater as ammonia, nitrite, nitrate and organic nitrogen (Sotirakou, 1999). With the advancement in technology, there have been various biological and chemical wastewater treatments meant to address the problems of ammonia in wastewater (Jorgensen and Weatherley, 2003). The various treatment processes for treating ammonia include; lagoon systems, membrane bioreactor, fixed film treatment processes etc.

Lagoon systems are not expensive and are much easier to operate than mechanical wastewater treatment systems. Its components use little concrete and built through excavation. However, the effluent quality can become substandard because it is hard to control the wastewater's temperature, return rate, and oxygen level inside the system (Middlebrooks et al., 1999).

Membrane bioreactor (MBR) technology which combines biological-activated sludge process and membrane filtration has become more popular, abundant, and accepted in recent years for the treatment of many types of wastewaters, whereas the conventional activated sludge (CAS) process cannot cope with either composition of wastewater or fluctuations of wastewater flow rate (Jelena et al., 2007). The MBR has three essential components - the anoxic basin, the pre-aeration basins, and the MBR basin. The raw wastewater is poured into a fine screen for filtration, before placing it inside the anoxic basin. In the anoxic basin, the mixed liquor gushes into the pre-aeration basins until it reach the MBR basins. The fluids will then pass through the membranes of the MBR basins, wherein the membrane that pushes the effluent for disinfection is connected. The effluent must be sent first to the disinfection stage to ensure the quality of wastewater prior to release. The membranes lessen the repeat clarification process, making wastewater treatment more practical and convenient (Churchouse, 1997; Maryam et al., 2009). The MBR treatment process is known for its high effluent quality. Unlike other treatments, MBR treatment does not need additional filtration or clarification because the membranes are designed to clear the impurities in wastewater, and removes the total nitrogen from wastewater. The MBR machine is also less cumbersome and fits to small areas compared to other wastewater treatment machines. The costs involved in operating MBR are thus much higher compared to other wastewater systems.

BOD removal and biological nitrification can also be achieved using fixed film treatment (Park et al., 1996). This technique involves the use of microorganisms such as the ammonia oxidizing bacteria (AOB) to treat wastewater. It uses a trickling filter/activated sludge treatment process wherein a plastic media contains microorganisms, which will grow inside a tower where the wastewater is placed for treatment. It is then followed by activated sludge process. The trickling filters/activated sludge treatment process harness the best quality of wastewater. Trickling filters are more energy efficient and reduce the production of low quality effluent. The disadvantages of fixed film treatment include high solid retention and foul odour.

Activated sludge process

The activated sludge process is a system used for the treatment of sewage and industrial wastewaters that involves the mixture of biological mass and wastewater (Beychok, 1967). It is a complex biological wastewater treatment system that is currently designed for removing carbon (Akpor, 2011), phosphorus (Metcalf and Eddy, 1991; Henze, 1996) and/or nitrogen constituents (Metcalf and Eddy, 1991; Larsdotter, 2006) in the wastewater. In activated sludge process, organic waste is fed to the system and leaves the process depending on the desired treatment efficiency set by the operator. The process begins by mixing the biological waste present in industrial wastewater or sewage with an aerobic bacterial culture in the reactor and air. This mixture is known as the mixed liquor. Once in the reactor, the mixed liquor is aerated for a particular period of time in order to ensure that this solution is fully mixed. This mixture therefore undergoes separation through the gravity clarifier, where the waste activated sludge is removed from the treatment and mixed with primary treated wastewater before it is recycled back to the beginning of the process in order to maintain the desired concentration of organisms and sludge. Lastly, the sludge goes through further treatment and the result of all this process is the treated wastewater that can be safely disposed to nature. A generalize schematic diagram of an activated sludge process is as shown in Figure 1.

There are basically three types of activated sludge processes, they are conventional activated sludge processes (Brucculeri et al., 2005; Marcos, 2007), contact stabilization processes (Gujer and Jenkins, 1975) which uses two separate aeration processes, and the extended aeration processes (Lowe and Gaudy, 1989; Sotirakou et al., 1999). The different activated sludge processes all accomplish the biochemical reduction of organics using aeration basins and the return and waste sludge systems. It is the detention times, mixed liquor suspended solids (MLSS), and food/microorganism ratio (F:M) loadings that are different. Other modifications are oxidation ditch, complete mix activated sludge process,

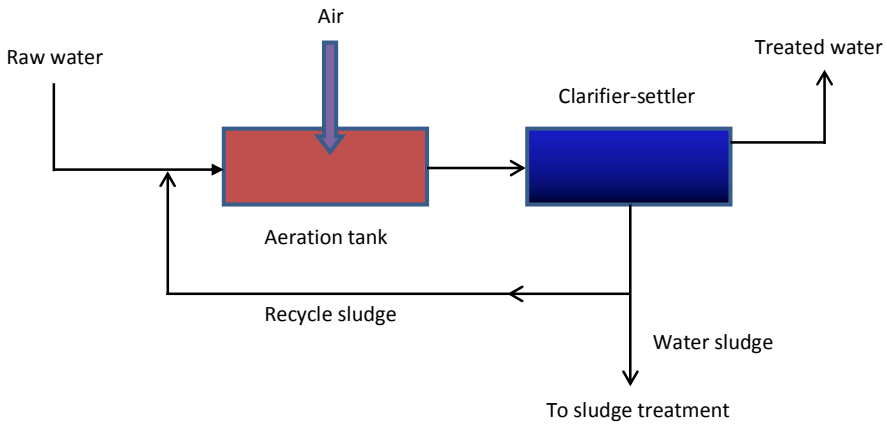

Figure 1. A generalize schematic diagram of an activated sludge process.

step feed, tapered aeration and Kraus process. The different types of activated sludge plants include: Package plants, oxidation ditch, deep Shaft and surface-aerated Basins/Lagoons (Beychok, 1967).

Ammonium oxidizing bacteria and archaea

Ammonia in water environments is toxic to fish and other aquatic life at high concentrations, and also contributes to eutrophication. Biodegradation and elimination of ammonia in wastewater is thus one of the main functions of wastewater treatment plants (WWTPs) and can be achieved by nitrification. Nitrification is the biological oxidation of ammonia with oxygen into nitrite followed by the oxidation of these nitrites into nitrates and can be represented by equation 1 and 2, respectively. A chemolithotrophic nitrification is a two-step process and is carried out by two different groups of organisms, the AOB and the nitrite oxidizing bacteria (NOB) (Bin et al., 2009). AOB are primarily responsible for the first step which is the oxidation of ammonia with oxygen into nitrite and often the rate-limiting step in nitrification. It is this process that is essential for the removal of ammonia from the wastewater.

$$NH_3 + O_2 \rightarrow NO_2^- + 3H^+ + 2e^- \quad (1)$$

$$NO_2^- + H_2O \rightarrow NO_3^- + 2H^+ + 2e^- \quad (2)$$

It is generally accepted that ammonia (NH_3) and not ammonium (NH_4^+) is used as substrate, and the ammonia/ammonium ratio may therefore affect the growth of AOB. AOB are obligatory chemolithoautotrophs and can be found among the beta-proteobacteria and gamma-proteobacteria (Purkhold, 2000). Most AOB are phylogenetically closely related to other activated sludge bacteria within the beta subdivision class of *Proteobacteria* (Harms, 2003). AOB are generally rod-shaped, spherical, spirillar, or lobular, typically Gram-negative, and flagellation of motile cells is polar to sub-polar or peritrichous. Most species are aerobic but can grow at reduced oxygen partial pressure. Studies suggest that there are physiological and ecological differences between the different AOB genera and lineages and that environmental factors such as salinity, pH, and concentrations of ammonia and suspended particulate matter select for certain species of AOB (Kowalchuk and Steven, 2001; Bin et al., 2009). The physiological activity and abundance of AOB in wastewater processing is important in the design and operation of waste treatment systems, particularly since these organisms display low growth rate and high sensitivity to environmental disturbances and inhibitor (Okabe, 1999).

Ammonia oxidizing archaea

Autotrophic ammonia/ammonium oxidation was initially assumed to be restricted to aerobic AOB and anaerobic ammonium oxidizing (Anammox) bacteria until recent molecular and culture-dependent evidence showed that autotrophic ammonia oxidation also occurs in the domain Archaea. The first strain of ammonia oxidizing archaea (AOA), *Nitrosopumilis maritimus*, was isolated from the rocky substratum of a tropical marine aquarium tank (Erguder et al., 2009). Some evidences showed that AOA are more abundant than AOB in marine, lake waters and soil environments (Sonthiphand and Limpiyakorn, 2010). AOA have also been detected in activated sludge bioreactors by using specific PCR primers targeting archaeal *amo*A gene. However, AOA abundances seemed to be much lower (four orders or more) in most cases than AOB based on analysis of *amoA* gene copy number (Jin et al., 2010; Limpiyakorn et al., 2011). The factors influencing the presence or/and dominance of AOA in different environments are ammonium levels, salinity, temperature, organic carbon, dissolved oxygen (DO) levels, pH, sulphide levels, and phosphate levels.

Ammonia monooxygenase

Ammonia monooxygenase (AMO) is a membrane-bound enzyme in *Nitrosomonas europaea* and other autotrophic AOB of the beta and gamma-subclasses of *Proteobacteria*. The enzyme contains multiple subunits; *amoA, amoB* and *amoC*. All the three AMO genes have been cloned and sequenced from several AOB (McTavish et al., 1993). AMO is responsible for the conversion of ammonia to hydroxylamine. Hydroxylamine is then oxidized to nitrite by hydroxylamine oxidoreductase (HAO) in an endergonic reaction. HAO is an unusual enzyme with a highly complex structure, located as a soluble enzyme in the perplasmic space, but anchored in the cytoplasmic membrane. AMO and HAO enzymes are necessary for energy conversion during the oxidation of ammonia. The initial oxidation of ammonia, which yields hydroxylamine as a reduced product, is an O_2-dependent reaction catalyzed by AMO:

$$NH_3 + O_2 + 2e^- + 2H^+ \rightarrow NH_2OH + H_2O \quad (4)$$

Hydroxylamine is further oxidized to nitrite by HAO:

$$NH_2OH + H_2O \rightarrow NO_2^- + 5H^+ + 4e^- \quad (5)$$

Two of the four electrons generated from hydroxylamine are used to support the oxidation of additional ammonia molecules; the other two enter the electron transfer chain and are used for CO_2 reduction and ATP biosynthesis (Wood, 1986).

Ammonia monooxygenase subunit A (*amoA*) gene and 16S rRNA genes has been widely used to analyze the diversity and abundance of AOB in various samples. Based on comparative analysis of 16S rRNA and *amoA* gene sequences, it was found that 16S rRNA gene is more conserved than *amoA* gene and the suggested similarity thresholds of 16S rRNA and *amoA* genes to define different AOB species are 97 and 80%, respectively (Ye and Zhang, 2011).

Economic importance of ammonium oxidizing bacteria

Nitrogenous wastes are increasing as a result of the expansion of animal husbandry, nitrogen-producing industries, and human activities and have therefore become a critical factor in environmental management. The removal of nitrogen from wastewater treatment is of extreme environmental importance. This is because the release of untreated waste can result to toxic effect on aquatic animals and can lead to eutrophication of the environment. Even in cases where treatment does not lead to successful denitrification, nitrification helps to avoid environmental contamination with potentially toxic ammonia salts (Painter, 1986). The broad specificity of the AMO complex common to all AOB often permits the co-oxidation of numerous recalcitrant aliphatic, aromatic, and halogenated molecules. AOB may also play a role in methane oxidation and biofilter systems. Biofilter systems have been used for the elimination of odours associated with waste treatment and composting (Bohn, 1992) and also for purposes as providing long-term filtering capacity suitable for manned spacecraft (Joshi et al., 2000). The reduction of ammonia released into the environment reduces the risk of local oxygen depletion.

Problems associated with ammonia oxidizing bacteria

AOB can lead to the production of the ozone-depleting gas NO or the greenhouse gas N_2O either at low or high levels via partial-denitrification processes under reduced oxygen conditions (Cho and Kim, 2000). The process of ammonia oxidation leads to a net acidification of the environment. The acidification of forest soils may thus have a detrimental effect on tree, and high levels of nitrification may intensify problems involving the effects of acid rain. Nitrogen transformations that lead to an increased proton load can lead to the release of metals such as aluminum, which can contribute to root damage and forest decline. AOB may generate elevated levels of nitrous acid and can lead to corrosion of natural stones, historical monuments, and building materials (Meincke et al., 1989).

Diversity, abundance and community structure measurement

A better understanding of the microbial ecology of AOB and AOA in wastewater treatment systems could potentially enhance the treatment performance and control, and would also help engineers to utilize the functional characteristics of the microbial population to model and improve the design and operation of the systems (Wang et al., 2010). The development of culture independent molecular techniques has enhanced the ability of researchers to analyze environmental samples.

Denaturing gradient gel electrophoresis (DGGE)

DGGE is a molecular fingerprinting method that separates polymerase chain reaction (PCR)-generated DNA products. The technique enables sequence diversity of PCR-amplified genes from a large number of samples to be compared in one gel to reveal changes in community structure over time or space. This approach separates DNA sequences and allows 95% of single base sequence differences to be detected. This approach also has the ability to separate genomic sequences differing by more than one base (Muyzer et al., 1993).

DGGE is a useful method for bacterial community

profiling by targeting the 16S rRNA and/or *amoA* genes of AOB (Nicolaisen and Ramsing, 2002). This method is less time consuming for comparing AOB communities than conventional analysis by cloning and sequencing.

Hornek et al. (2006) reported the communities of AOB in activated sludge of a municipal wastewater treatment plant (WWTP) located in Linz (Austria) by the use of DGGE technique. DGGE analyses of PCR products generated by the *amoA* primers; amoA-1F, amoA-2R, amoAf-I, and amoAr-I were performed with the D-gene system. Selected bands were recovered, sequenced and subsequently submitted to BLAST to allocate to available partial *amoA* sequences. Sequences were aligned using the program ClustalX 1.81. The application of the primer set amoA-1F in combination with amoA-2R and amoAr-I was suggested for a rapid PCR-DGGE analysis, because they seem to complement each other to screen for present AOB in the environment.

The microbial community composition and dominant bacterial populations in anoxic-oxic activated sludge from a full-scale WWTP in Liaoning, China were investigated with PCR-DGGE coupled with sequence analysis of 16S rRNA gene fragments from dominant bands by Ding et al (2011). PCR amplification of bacterial 16S rRNA gene fragments was performed using primer 968F-GC (5'-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC-3') and primer 1401R (5'-CGG TGT GTA CAA GGC CCG GGA ACG-3') for the Domain Bacteria, corresponding to positions 968 and 1401 in the 16S rDNA of *Escherichia coli*, with a 40 bp-GC-rich sequence (5'- CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G - 3') attached to the 5' end of the forward primer to stabilize the melting behavior of the DNA fragments. DGGE of the PCR amplified 16S rDNA was carried out using the DCodeTM Universal Mutation Detection System. The prominent DGGE bands were selected and excised for nucleotide sequence determination. The sequences obtained from the DGGE were then analyzed in comparison with the 16S rDNA sequences in the GenBank database by using the basic local alignment search tool (BLAST). The alignment was calculated by the neighbor-joining method using Clustal X. The phylogenetic affiliation of the sequences was further analyzed and a phylogenetic tree was plotted by Mega 3.1 program.

Because of high similarity of the 16S rDNA sequences that makes it difficult to identify closely related ammonia oxidizing species, some difficulties have been experienced in studying ammonia oxidizing bacterial diversity using DGGE of 16S rDNA gene sequence. Another method based on the same principle is the temperature gradient gel electrophoresis (TGGE).

Real-time PCR

Real-time PCR is a highly sensitive technique that facilitates amplification and quantification of a specific DNA sequence with the detection of the PCR product in real time. Quantification of DNA targets can easily be achieved by determination of the cycle when the PCR product can first be detected. Thus, Real-time PCR technique is reliable and reproducible for AOB and for evaluating correlations between microbial activities, cell numbers and population changes in time and space. Real-time PCR analysis has been applied to numerous environmental samples to reveal the comparative abundance of AOA and AOB. In many cases, according to Caffrey et al. (2007), the archaeal *amoA* gene outnumbered that of AOB.

The abundance of *amoA* genes of AOB and AOA in activated sludge of seven full-scale wastewater treatment plants in Thailand was investigated by Limpiyakorn (2011). Quantitative real-time PCR was performed with duplicate sets of extracted DNA. Each set of extracted DNA was prepared by pooling the DNA extracted in triplicate, then diluted for four different 10-fold dilutions and a quantitative real-time PCR was carried out for each dilution in duplicate with a Brilliant II SYBR Green QPCR Master Mix in an Mx3005P instrument. Archaeal *amoA* genes were quantified using the primers Arch-amoAF (5'-STA ATG GTC TGG CTT AGA CG-3') and Arch-amoAR (5'-GCG GCC ATC CAT CTG TAT GT-3'), the quantification of bacterial *amoA* genes was performed using the primers amoA 1F (5'-GGG GTT TCT ACT GGT GGT-30) and amoA 2R (5'-CCC CTC KGS AAA GCC TTC TTC-3') while AOB 16S rRNA gene was quantified using the primers CTO 189A/Bf (5'-GGA GRA AAG CAG GGG ATC G-3'), CTO189Cf (5'-GGA GGA AAG TAG GGG ATC G-3'), and CTO 654r (50-CTA GCY TTG TAG TTT CAA ACG C-3'). To confirm the single target fragment of the PCR amplified products, dissociation curves were analyzed and plotted at the end of every quantitative real-time PCR reaction and to verify the correct amplification of the target microorganisms' DNA, few clones from the clone libraries constructed from the real-time PCR amplified products were randomly selected for sequencing and the results for every reaction tested verified the correct amplification of the target microorganisms' DNA.

Terminal restriction fragment length polymorphism (T-RFLP)

T-RFLP is a molecular biology technique for profiling of microbial communities based on the position of a restriction site closest to a labeled end of an amplified gene. The method is based on the digestion of a mixture of PCR amplified variants of a single gene using one or more restriction enzymes and detecting the size of each of the individual resulting terminal fragments using a DNA sequencer. The result is a graph image where the X axis represents the sizes of the fragment and the Y axis represents their fluorescence intensity.

Wang et al. (2010) investigated the communities of AOB in activated sludge collected from eight wastewater treatment systems in Beijing using polymerase chain reaction (PCR) followed by T-RFLP, cloning, and sequencing of the α-subunit of the *amoA* gene. The primers amoA-1F (5'-GGG GTT TCT ACT GGT GGT-3') and amoA-2R (5'-CCC CTC KGS AAA GCC TTC TTC-3') were used to amplify a 491 base pairs (bp) fragment of the *amoA* gene of AOB. A Clone library was constructed and the software Clustal X 1.81 was used to align sequences of the recovered clones with other published *amoA* sequences and software Mega 4.0 was used to generate a phylogenetic tree using the neighbor joining method. Their results (T-RFLP fingerprint analysis) showed that the different wastewater treatment systems harbored distinct AOB communities and that the source of influent affected the AOB community with the WWTPs treating domestic wastewater containing a higher AOB diversity than those receiving mixed domestic and industrial wastewater. A combination of the results of sequencing the *amoA* gene and the T-RFLP profiles of clones clearly indicated which species each peak represented.

The AOB populations in Marshall WWTP (an aerated-anoxic Orbal process treating 900 ~ 1,300 m^3/day of domestic wastewater) and Nine Springs WWTP (a variation of the UCT process and treats 150,000 ~ 200,000 m^3/day of domestic wastewater) with T-RFLP was investigated by Park et al (2001). Primers amoA-1F and amoA-2R were used to amplify a 491-bp fragment of the *amoA* gene.

The analysis was complemented by cloning and sequencing the *amoA* gene fragment to detect and identify AOB.

TRFLP is one of several molecular methods aimed to generate a fingerprint of an unknown microbial community (Liu et al., 1997). Because of its relatively high resolution and reproducibility, T-RFLP has been widely used to assess the AOB community (Osborn et al., 2000).

Other molecular techniques are fluorescent *in situ* hybridization (FISH), restriction fragment length polymorphism (RFLP), amplified ribosomal DNA restriction analysis (ARDRA), singlestranded-conformation polymorphism (SSCP), randomly amplified polymorphic DNA (RAPD) or DNA amplification fingerprinting (DAF), bisbenzimide-polyethyleneglycol (Bb-PEG) electrophoresis, etc.

Fluorescent in situ Hybridization (FISH)

FISH (fluorescent *in situ* hybridization) is a cytogenetic technique developed by Christoph Lengauer that is used to detect and localize the presence or absence of specific DNA sequences on chromosomes. The technique is one of the methods that have been described for direct visualization of AOB. Hybridization techniques generally use directly extracted DNA as a "probe" for specific detection of various microorganism species. The application of *In situ* Hybridization approaches to AOB has been most effective in detecting AOB that dominate total bacterial community in environment such as the sewage treatment plants (Wagner et al., 1995).

Restriction fragment length polymorphism (RFLP)

RFLP is a technique that exploits variations in homologous DNA sequences. Ribotyping (RFLP of rRNA genes) combines restriction enzyme digestion of the total genomic DNA with a Southern analysis, in which rRNA gene-specific DNA probes are used. The hybridization pattern obtained is a characteristic of each organism. RFLP determines the multiplicity, the arrangement and the relative location of rRNA genes in bacterial genomes and also contain taxonomic information. Aakra et al. (1999) in their study, ribotyped 12 isolates of AOB, and the sequences of the 16s-23s rDNA intergenic spacer region (ISR) were determined and used in a phylogenetic study.

Amplified ribosomal DNA restriction analysis (ARDRA)

ARDRA is a DNA fingerprint technique based on PCR amplification of 16S ribosomal DNA using primers for conserved regions, followed by enzyme digestions and agarose gel electrophoresis (Smit el al., 1997).

T-RFLP is different from ARDRA and RFLP in that only the terminal fragments (i.e. the labeled end or ends of the amplicon) are read and all other fragments ignored whereas all restriction fragments are visualized for ARDRA and RFLP.

Singlestranded-conformation polymorphism (SSCP)

With SSCP, DNA fragments such as PCR products obtained with primers specific for the 16S rRNA gene, are denatured and directly electrophoresed on a non-denaturing gel. Separation is based on differences in the folded conformation of single-stranded DNA, which influences the electrophoretic mobility.

Randomly amplified polymorphic DNA (RAPD) and DNA amplification fingerprinting (DAF)

RAPD and a similar approach termed DAF use short (5-10 nucleotides) random primers, which anneal at different sites of the genomic DNA, generating PCR products of various lengths. The products are separated on agarose

Table 1. PCR primers.

Primers	Sequences (5'→3')	Target	Reference
AmoA-1F-Clamp[a]	GGGGTTTCTACTGGTGGT	amoA	Nicolaisen and Ramsing (2002)
AmoA-2R-Clamp[a and b]	CCCCTCKGSAAAGCCTTCTTC	amoA	Nicolaisen and Ramsing (2002)
AmoA-2R-KS[b]	CCCCTCKGSAAAGCCTTCTTC	amoA	Nicolaisen and Ramsing (2002
AmoA-2R-TS[b]	CCCCTCTGSAAAGCCTTCTTC	amoA	Nicolaisen and Ramsing (2002)
AmoA-2R-GS[b]	CCCCTCGGSAAAGCCTTCTTC	amoA	Nicolaisen and Ramsing (2002)
AmoA-2R-TC	CCCCTCTGCAAAGCCTTCTTC	amoA	Nicolaisen and Ramsing (2002)
AmoA-2R-TG	CCCCTCTGGAAAGCCTTCTTC	amoA	Okano et al. (2004)
AmoA-2R-GC	CCCCTCGGCAAAGCCTTCTTC	amoA	Nicolaisen and Ramsing (2002)
AmoA-2R-GG	CCCCTCGGGAAAGCCTTCTTC	amoA	Nicolaisen and Ramsing (2002)
Cren-amoAF	ATGGTCTGGCTAAGACGMTGTA	amoA	Hallam et al. (2006)
Arch-amoAR	GCGGCCATCCATCTGTATGT	amoA	Francis et al. (2005)
CTO189f-Clamp[a and b]	GGAGRAAAG**Y**AGGGGATCG	16s rDNA	Kowalchuk et al. (1997)
CTO654r[b]	CTAGC**Y**TTGTAGTTTCAAACGC	16s rDNA	Kowalchuk et al. (1997)
6R	AGAAAGGAGGTGATCCAGCC	16s rDNA	Dorsch and Stackebrandt (1992)
7F	GCCTTGTACACACCGC	16s rDNA	Lane et al. (1985)
9F	GAGTTTGATCCTGGCTCAG	Bacteria	Dionisi et al. (2003)
11F	TGGCGAAGGCGGCCCCCTGGA	16s rDNA	Edwards et al. (1989)
13R	GCCAAGGCATCCACCACATG	23s rDNA	Gurtler and Stanisich (1996)
23SF	CCGAATGGGGAAACC	23s rDNA	Gurtler and Stanisich (1996)
23SR	CCTTTCCCTCACGGTA	23s rDNA	Gurtler and Stanisich (1996)
1393R	ACGGGCGGTGTGTAC	Bacteria	Dionisi et al. (2003)
1055F	ATGGCTGTCGTCAGCT	Bacteria	Dionisi et al. (2003)
341F[c]	TACGGGAGGCAGCAG	Bacteria	Lopez-Gutierrez et al. (2004)
518R	ATTACCGCGGCTGCTGG	Bacteria	Lopez-Gutierrez et al. (2004)

[a]5' GC-Clamp (CGCCGCGCGGCGGGCGGGGCGGGGGC); [b]Degeneracies are shown in bold; [c]5' GC-clamp (CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG).

or acrylamide gels, and visualized by ethidium bromide or silver staining (Muyzer, 1999).

Bb-PEG Electrophoresis

Electrophoresis is performed in agarose gels containing the DNA ligand bisbenzimide to which long chains of polyethyleneglycol (PEG) are covalently coupled. Bisbenzimide binds to adenine and thymine (A+T) rich sequence motifs in the DNA. Therefore, being loaded with the Bb-PEG conjugate, the A+T-rich DNA molecules are more retarded in the gel than the molecules which are low in A+T, and so separation is achieved (Muyzer, 1999).

PCR primers

Primers are strands of nucleic acid that function as starting point for DNA synthesis. Some other PCR primers used in several other studies are presented in Table 1. Primers are required for DNA replication because the enzymes DNA polymerases, which catalyze the process can only add new nucleotides to an existing strand of DNA. The polymerase therefore starts replication at the 3'-end of the primer, and copies the opposite strand.

Isolation of ammonia oxidizing bacteria

AOB can be isolated in most aerobic environments where ammonia is available through the mineralization of organic matter or anthropogenic nitrogen sources, such as fertilizers and waste. AOB pure cultures are also obtained by picking colonies from a solid medium or by the use of dilution methods in liquid culture (Ford et al., 1980; Schmidt and Belser, 1982). The selective medium used must be free of organic carbon sources and contain inhibitors of heterotrophic organisms, an ammonia source, and essential trace elements (MacDonald and Spokes, 1980). AOB are very difficult to handle as a result of their slow growth and low maximum growth yield, making their isolation and maintenance in pure culture difficult and time-consuming. Culture-dependent techniques such as selective plating and the most probable number (MPN) method have been used for the

enumeration of AOB; however, such techniques are thought to underestimate actual cell numbers. In addition to medium selectivity and bias, MPN underestimation may also stem from inadequate suspension of cells from solid substrates in the environmental sample or dispersal of flocks and microcolonies. Cell damage due to rigorous disruption methods or osmotic shock and the possible dependence on inter- or intraspecies interactions for growth may also generate inaccuracies (Kowalchuk and Steven, 2001).

Conclusion

Ammonia has been found to be very dangerous to water environment especially to fishes and other aquatic life, its high concentration is mostly responsible for this. It is therefore necessary to convert ammonia to other nitrogen compounds that will have little or no effect on water environment. An example of microorganisms widely used for this purpose are the AOB, they are used for the oxidation of ammonia into nitrite in biological WWTPs. AOB are ecologically important, being the only group of organisms that oxidize ammonia to nitrite in significant amounts, and they appear to be present in all environments in which nitrogen is mineralized (Aakra et al., 1999). Because these microorganisms display low growth rate and high sensitivity to environmental disturbances and inhibitor, the physiological activity and abundance of AOB in wastewater processing is important in the design and operation of waste treatment systems. AOB can be isolated in most aerobic environments as well as low-oxygen environments. The different techniques for the investigation of diversity and community structure of AOB are: denaturing gradient gel electrophoresis (DGGE), Real-Time PCR, fluorescent *in situ* hybridization (FISH), terminal restriction fragment length polymorphism (T-RFLP), restriction fragment length polymorphism (RFLP), amplified ribosomal DNA restriction analysis (ARDRA), singlestranded-conformation polymorphism (SSCP), randomly amplified polymorphic DNA (RAPD) or DNA amplification fingerprinting (DAF), and bisbenzimide-polyethyleneglycol (Bb-PEG) electrophoresis. Among the various techniques, DGGE appears to be the most frequently used community fingerprinting method. It allows a rapid comparison of the microbial communities between the samples and is generally used to detect shifts in microbial population under different environmental conditions. DGGE technique based on 16S rDNA gene enables the investigation of the spatial and temporal variability of the population in environment, provide information on the predominant species in a community and analyze multiple samples simultaneously.

However, it should also be noted that none of these methods is absolutely perfect; they all have their advantages and disadvantages (Muyzer, 1999). Hence, only a polyphasic approach combining different molecular biological techniques, microbiological methods, and methods to determine the environmental parameters will lead to an unbiased understanding of the role of microorganisms in their environment.

REFERENCES

Aakra A, Utaker JB, Nes IF (1999). RFLP of rRNA genes and sequencing of the 165-235 rDNA intergenic spacer region of ammonia-oxidizing bacteria: a phylogenetic approach. Int. J. Syst. Bacteriol., 49: 123-130.

Akpor OB (2011). Wastewater effluent discharge: Effects and treatment processes. 3rd International Conference on Chemical, Biological and Environmental Engineering, IACSIT Press, Singapore.

Beychok MR (1967). Aqueous wastes from petroleum and petrochemical plants (1st ed.). John Wiley & Sons Ltd. LCCN 67019834.

Bin Z, Baosheng S, Min J, Huina L (2009). Population dynamic succession and quantification of ammonia-oxidizing bacteria in a membrane bioreactor treating municipal wastewater. J. Hazardous Mater., 165: 796–803.

Boari G, Mancini IM, Trulli E (1997). Technologies for water and waste water treatment. Ciheam-Options mediteraneene, pp 261-287.

Bohn H (1992). Consider biofiltration for decontaminating gases. Chem. Eng. Prog., 88:34–40.

Brucculeri M, Bolzonella D, Battistoni P, Cecchi F (2005). Treatment of mixed municipal and winery wastewaters in a conventional activated sludge process: a case study. Wat. Sci. Technol., 51: 89-98.

Caffrey JM, Bano N, Kalanetra K, Hollibaugh JT (2007). Ammonia oxidation and ammonia oxidizing bacteria and archaea from estuaries with differing histories of hypoxia. ISME J., 1: 660–662.

Cho JC, Kim SJ (2000). Increase in bacterial community diversity in subsurface aquifers receiving livestock wastewater input. Appl. Environ. Microbiol., 66: 956–65.

Churchouse S (1997). Membrane bioreactors for wastewater treatment - operating experiences with the Kubota submerged membrane activated sludge process. Membrane Technol., 83: 5-9.

Ding L, Zhou Q, Wang L, Zhang Q (2011). Dynamics of bacterial community structure in a fullscale wastewater treatment plant with anoxic-oxic configuration using 16S rDNA PCR-DGGE fingerprints. Afri. J. Biotechnol., 10: 589-600.

Dionisi HM, Hamrs G, Layton AC, Gregory IR, Parker J, Hawkins SA, Robinson KG, Sayler GS (2003). Power analysis for real-time PCR quantification of genes in activated sludge and analysis of the variability introduced by DNA extraction. Appl. Environ. Microbiol., 69: 6597-6604.

Dorsch M, Stackebrandt E (1992). Some modifications in the procedure of direct sequencing of PCR amplified 16s rDNA. J. Microbiol. Methods 16: 271-279.

Edwards U, Rogall T, Blocker H, Emde M, Bottger EC (1989). Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16s ribosomal RNA. Nucleic Acids Res., 17: 7843-7853.

Erguder TH, Boon BN, Wittebolle L, Marzorati M, Verstraete W (2009). Environmental factors shaping the ecological niches of ammonia oxidizing archaea. FEMS Microbiol. Rev., 33: 855–869.

Ford DL, Curchwell RL, Kachtick JW (1980). Comprehensive analysis of nitrification of chemical processing wastewaters. J. Water Pollut. Control Fed., 52: 2726–45.

Francis CA, Roberts KJ, Beman JM, Santoro AE, Oakley BB (2005). Ubiquity and diversity of ammonia-oxidizing archaea in water columns and sediments of the ocean. Proc. Natl. Acad. Sci., 102: 14683–14688.

Gujer W, Jenkins D (1975). The contact stabilization activated sludge process—oxygen utilization, sludge production and efficiency. Water Res., 9: 553-560.

Gurtler V, Stanisich VA (1996). New approaches to typing and identification of bacteria using the 16s-23s rDNA spacer region. Microbiol., 142: 3-16.

Hallam SJ, Mincer TJ, Schleper C, Preston CM, Roberts K, Richardson PM, DeLong EF (2006). Pathways of carbon assimilation and ammonia oxidation suggested by environmental genomic analyses of marine crenarchaeota. PLoS Biol., 4: e95.
Harms G, Layton AC, Dionisi HM, Gregory IR, Garrett VM, Hawkins SA, Robinson KG, Sayler GS (2003). Real-time PCR quantification of nitrifying bacteria in a municipal wastewater treatment plant. Environ. Sci. Technol., 37: 343–351.
Henze M (1996). Biological phosphorus removal from wastewater: processes and technology. Water Quality International, pp 32-36.
Hornek R, Pommerening-Roser A, Koops HP, Farnleitner AH, Kreuzinger N, Kirschner A, Mach RL (2006). Primers containing universal bases reduce multiple amoA gene specific DGGE band patterns when analysing the diversity of beta-ammonia oxidizers in the environment. J. Microbiol. Methods 66: 147–155.
Jelena R, Marin M, Ivan M, Mira P, Damià B (2007). Membrane Bioreactor (MBR) as an Advanced Wastewater Treatment Technology. Hdb. Env. Chem., 5: 37–101.
Jin T, Zhang T, Yan Q (2010). Characterization and quantification of ammonia-oxidizing archaea (AOA) and bacteria (AOB) in a nitrogen-removing reactor using T-RFLP and qPCR. Appl. Microbiol. Biotechnol., 87: 1167–1176.
Jorgensen TC, Weatherley LR (2003). Ammonia removal from wastewater by ion exchange in the presence of organic contaminants. Water Res., 37: 1723-1728.
Joshi JA, Hogan JA, Cowan RM, Strom PF, Finstein MS (2000). Biological removal of gaseous ammonia in biofilters: space travel and earth-based applications. J. Waste Manage. Assoc., 50: 1647–54.
Kowalchuk GA, Stephen JR (2001). Ammonia-oxidizing bacteria: a model for molecular microbial ecology. Annu. Rev. Microbiol., 55: 485–529.
Kowalchuk GA, Stephen JR, De Boer W, Prosser JI, Embley TM, Woldendorp JW (1997). Analysis of ammonia-oxidizing bacteria of the beta subdivision of the class *Proteobacteria* in coastal sand dunes by denaturing gradient gel electrophoresis and sequencing of PCR-amplified 16S ribosomal DNA fragments. Appl. Environ. Microbiol., 63: 1489–1497.
Lane DJ, Pace B, Olsen GJ, Stahl DA, Sogin ML, Pace NR (1985). Rapid determination of 16s ribosomal RNA sequences for phylogenetic analyses. Proc. Natl. Acad. Sci., 82: 6955-6959.
Larsdotter K (2006). Microalgae for phosphorus removal from wastewater in a Nordic climate. Doctoral Thesis from the School of Biotechnology, Royal Institute of Technology, Stockholm, Sweden, ISBN: 91-7178-288-5.
Limpiyakorn T, Sonthiphand P, Rongsayamanont C, Polprasert C (2011). Abundance of amoA genes of ammonia-oxidizing archaea and bacteria in activated sludge of full-scale wastewater treatment plants. Biores. Technol., 102: 3694–3701.
Liu W, Marsh T, Cheng H, Forney L (1997). Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. Appl. Environ. Microbiol., 63: 4516-4522.
Lopez-Gutierrez JC, Henry S, Hallet S, Martin-Laurent F, Catroux G, Philippot L (2004). Quantification of a novel group of nitrate-reducing bacteria in environment by real-time PCR. J. Microbiol. Methods 57: 399-407.
Lowe WL, Gaudy AF (1989). Modified extended aeration process for removal and recovery of cadmium from wastewaters. Biotechnol. Bioeng., 34: 600-608.
MacDonald RM, Spokes JR (1980). A selective and diagnostic medium for ammonia oxidizing bacteria. FEMS Microbiol. Lett., 8: 143–45.
Marcos S (2007). Activated sludge and aerobic biofilm reactors. Biological Wastewater Treatment Series, IWA Publishing, Alliance House, 12 Caxton Street, London SW1H 0QS, UK, pp 1-313.
Maryam TR, Tahereh K, Ali K (2009). Application of membrane separation processes in petrochemical industry: a review. Desalination 235: 199–244.
McTavish H, Fuchs JA, Hooper AB (1993). Sequence of the gene coding for ammonia monooxygenase in Nitrosomonas europaea. J. Bacteriol., 175: 2436-2444.
Meincke M, Kreig E, Bock E (1989). Nitrosovibrio spp., the dominant ammoniaoxidizing bacteria in building stones. Appl. Environ. Microbiol., 55: 2108–10.
Metcalf, Eddy (1991). Wastewater Engineering. Treatment, disposal, reuse. 3rd edition, McGraw-Hill Int. Ed., Singapore.
Middlebrooks EJ, Sherwood CR, Abraham P, Adams VD (1999). Nitrogen removal in wastewater stabilization lagoons, Presented at 6th National Drinking Water and Wastewater Treatment Technology Transfer Workshop Kansas City, Missouri 64105. p. 38.
Muyzer G (1999). Genetic fingerprinting of microbial communities – present status and future perspectives. Proceedings of the 8th International Symposium on Microbial Ecology Bell CR, Brylinsky M, Johnson-Green P (ed) Atlantic Canada Society for Microbial Ecology, Halifax, Canada, 10pp.
Muyzer G, De Waal EC, Uitterlinden AG (1993). Profiling of complex microbial population by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol., 59: 695-700.
Nicolaisen MH, Ramsing NB (2002). Denaturing gradient gel electrophoresis (DGGE) approaches to study the diversity of ammonia-oxidizing bacteria. J. Microbiol. Meth., 50: 189-203.
Okabe S, Satoh H, Watanabe Y (1999). In situ analysis of nitrifying biofilms as determined by in situ hybridization and the use of microsensors. Appl. Environ. Microbiol., 65: 3182–3191.
Okano Y, Hristova KR, Leutenegger CM, Jackson LE, Denison RF, Gebreyesus B, Lebauer D, Scow KM (2004). Application of real-time PCR to study effects of ammonium on population size of ammonia-oxidizing bacteria in soil. Appl. Environ. Microbiol., 70: 1008–1016.
Osborn AM, Moore ERB, Timmis KN (2000). An evaluation of terminal-restriction fragment length polymorphism (TRFLP) analysis for the study of microbial community structure and dynamics. Environ. Microbiol., 2: 39–50.
Painter HA (1986). Nitrification in the treatment of sewage and wastewaters. Spec. Publ. Soc. Gen. Microbiol., 20: 185–213.
Park HD, Regan JM, Noguera DR (2001). Molecular analysis of ammonia-oxidizing bacterial populations in aerated-anoxic orbal processes. IWA Conference on microorganisms in activated sludge and biofilm processes, Rome.
Park TJ, Lee HK, Kim SD, Kim WC (1996). Petrochemical wastewater treatment with aerated submerged fixed-film reactors (ASFFR) under high organic loading. Water Sci. Technol., 34: 9-16.
Purkhold U, Pommerening-Roser A, Juretschko S, Schmid MC, Koops HP, Wagner M (2000). Phylogeny of all recognized species of ammonia oxidizers based on comparative 16S rRNA and amoA sequence analysis: implications for molecular diversity surveys. Appl. Environ. Microbiol., 66: 5368–5382.
Schmidt EL, Belser LW (1982). Nitrifying bacteria. in Methods of soil analysis, part 2. Chemical and microbiological properties. ed Page A. (American Society of Agronomy, Inc., Crop Science Society of America, Inc., and Soil Science Society of America, Inc. Madison, Wis), pp 1027–1042.
Smit E, Leeflang P, Wernars K (1997). Detection of shifts in microbial community structure and diversity in soil cause by coper contamination using amplified ribosomal DNA restriction analysis. FEMS Microbiol. Ecol., 23: 249-261.
Sonthiphand P, Limpiyakorn T (2010). Communities of ammonia-oxidizing archaea and bacteria in enriched nitrifying activated sludge. World Acad. Sci. Eng. Technol., 64: 425-428.
Sotirakou E, Kladitis G, Diamantis N, Grigoropoulou H (1999). Ammonia and phosphorus removal in municipal wastewater treatment plant with extended aeration. Global Nest: the Int. J., 1: 47-53.
Thomas ES (2005). Biological wastewater treatment. Chemical engineering magazine, Access Intelligence, LLC, pp 1-6.
Van der Hoek W (2004). A frame work for a global assessment of the extent of wastewater irrigation: The need for a common wastewater typology. In: Scott CA, Faruqui NI, Raschid-Sally L (eds.). Wastewater use in irrigated agriculture: Confronting the livelihood and environmental realities. CABI Publishing, Wallingford, IWMI, Colombo, IDRC, Ottawa, pp 11-24.
Wagner M, Rath G, Amann R, Koops HP, Schleifer K (1995). *In situ* identification of ammonia oxidizing bacteria. Syst. Appl. Microbiol., 18: 251-264.
Wang X, Wen X, Criddle C, Wells G, Zhang J, Zhao Y (2010). Community analysis of ammonia oxidizing bacteria in activated

sludge of eight wastewater treatment systems. J. Environ. Sci., 22: 627–634.
wastewater stabilization lagoons, Presented at 6th National Drinking Water and Wastewater Treatment Technology Transfer Workshop Kansas City, Missouri 64105. 38pp.
Wood PM (1986). Nitrification as a bacterial energy source, Nitrification Spec. Publ. Soc. Gen. Microbiol., IRL Press, Oxford. 20: 39-62.
Ye L, Zhang T (2011). Ammonia-oxidizing bacteria dominates over ammonia-oxidizing archaea in a saline nitrification reactor under low DO and high nitrogen loading. Biotechnol. Bioeng,, 108: 2544-2552.