CURRENT MOLECULAR BIOLOGIC TEHNIQUES FOR ANAEROBIC AMMONIUM OXIDIZING (ANAMMOX) BACTERIA

Ozgun D[1], Basak S[2], Cinar O[3]

1 Department of Bioengineering and Sciences, Kahramanmaras Sutcu Imam University, Turkey
2 Department of Environmental Engineering, Kahramanmaras Sutcu Imam University, Turkey
3 Biological Sciences and Bioengineering Program, International University of Sarajevo, Bosnia and Herzegovina

ABSTRACT

Anaerobic Ammonium Oxidation (Anammox) is an alternative process to conventional nitrification-denitrification bacteria, which oxidize ammonium with nitrite as electron acceptor via Anammox bacteria. These chemoautotrophic bacteria have very slow generation time (10-30 days).

Anammox bacteria may not isolate from pure culture, because of their low growth rate (0.0027 h-1). Therefore, using molecular techniques for the bacteria identification have more advantages than traditional microbial techniques, which are preferred as cultivation especially for detection and diversity of Anammox bacteria. Molecular techniques include PCR-based methods (fingerprinting techniques), like SSCP, DGGE and T-RFLP, are available in order to show the variety of genetic profiling. PCR amplification of DNA template with specific biomarker (16S rRNA gene) in environmental sample is necessary to detect any unknown microorganisms. The other important molecular technique is FISH-based method for identification and quantification with fluorescent signal probes of Anammox bacteria. In this article is purposed, comparison of molecular methods commonly used for microbial diversity, and also it is emphasized strengths or weakness of the methods.

Keywords: Anammox, Denaturant gradient gel electrophoresis (DGGE), Terminal restriction fragment length polymorphism (T-RFLP), Fluorescent in-situ hybridization (FISH).

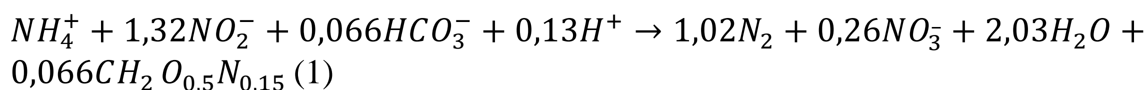
1. INTRODUCTION

Nitrogenous compounds like ammonium (NH_4^+) are dominant in many wastewaters, which can cause serious problems especially oxygen depletion, methemoglobinemia, and eutrophication (excessive growth of green algae and cyanobacteria), if they are not treatment prior to discharge (Jin et al., 2008). These compounds are removed by biological and physicochemical processes. Biological treatment processes consists of two steps called conventional nitrification-denitrification process. Nitrification process is oxidation of ammonium (NH_4^+) to nitrite (NO_2^-), and following nitrite to nitrate

(NO_3^-) in aerobic condition by ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB). Denitrification process is reduction of nitrate to nitrite, and after dinitrogen (N_2) to nitrite in anoxic condition by facultative anaerobic heterotrophic denitrifying bacteria (Terada et al., 2011). Biological treatment to remove nitrogen from wastewater is cost effective and more efficient than physicochemical treatment and therefore has been used more often to succeed nitrogen removal from wastewaters (Li et al., 2010). However, nitrogen removal by biological processes used in wastewater has generally high NH_4^+ but low carbon content. Hence, the carbon in wastewaters is inadequate for the conventional denitrification process, an extra carbon source such as acetate or glucose must be added. These carbon sources are increase the cost of operation condition (Noophan et al., 2009).

Approaches for biological nitrogen removal processes have been brought as a new and cost effective way to treat high nitrogen-loaded wastewater with low organic matter content (Mulder et al., 1995). These processes have been termed partial nitrification; Anammox (Anaerobic Ammonium Oxidation) , Sharon (Single reactor system for High Activity Ammonia Removal Over Nitrite), Canon (Complete Autotrophic Nitrogen removal Over Nitrite), and Oland (Oxygen Limited Autotrophic Nitrification and Denitrification) with shortcomings of conventional treatments (Suneethi and Joseph, 2011).

Under anaerobic conditions, Anammox is an alternative process to conventional nitrification-denitrification processes, which oxidize ammonium with nitrite as electron acceptor with hydrazine as an intermediate and carbon dioxide is used for growth of the Anammox microorganisms (van de Graaf et al., 1995; Li et al., 2010). Stoichiometry conversion of NH_4^+ and NO_2^- to N_2 in Anammox reaction was indicated in Equation (1) (Strous et al., 1998).



$$NH_4^+ + 1{,}32NO_2^- + 0{,}066HCO_3^- + 0{,}13H^+ \rightarrow 1{,}02N_2 + 0{,}26NO_3^- + 2{,}03H_2O + 0{,}066CH_2\,O_{0,5}N_{0,15} \quad (1)$$

Discovery of Anammox bacteria was found pilot plant treating wastewater with a fluidized bed reactor in Delft, the Netherlands in 1995 (Mulder et al., 1995). However, it was already estimated to exist 30 years ago (Van Hulle et al., 2010). Compared with conventional biological nitrogen treatment process, Anammox process can be save up to 90% of operation cost because external organic carbon source and aeration could be excluded (Jetten et al., 2001; Chamchoi and Nitisoravut, 2007). Nevertheless applications of Anammox are limited by a long start-up period because of low growth rates (0.0027 h^{-1}) of Anammox bacteria with the generation time of 1-30 days (Van Niftrik et al., 2004).

Chemolithoautotrophic Anammox bacteria, being very slowly growing microorganisms, identified as member of *Planctomycetes* phylum was firstly isolated though Percoll centrifugation and characterized in 1999 as Candidatus "*Brocadia anaommoxidans*" (Kartal et al., 2004; Jetten et al., 1999; van de Graaf et al., 1996; Wang et al., 2011). Following, Anammox bacteria are more identified with molecular

techniques than traditional microbiological techniques of cultivation (Qin and Zhou, 2009).

Application of molecular techniques to detect and define these bacteria by molecular markers, such as 16S rRNA or functional markers, are more and more frequently used to explore the microbial diversity and to investigate the structure of microbial community. For this purpose the cloning approach is useful but it is time-consuming and labor excessive, and thus not practical for multiple sample analysis (Muyzer and Smalla, 1998). However, cloning is used lately because these problems is probably extirpate.

This study only focus on specifically to fluorescent in-situ hybridization (FISH) technique and to genetic fingerprinting techniques like single-strand conformation polymorphism (SSCP), denaturant gradient gel electrophoresis (DGGE), and terminal restriction fragment length polymorphism
(T-RFLP). In conclusion is purposed comparison of available molecular methods commonly used for the detection and diversity of Anammox bacteria, and also are emphasized advantages and disadvantages of the methods.

2. PCR-BASED METHODS

The genetic fingerprinting techniques are widely used in exploration the structure and dynamics of microbial community. The target genes in different species, or more higher taxonomy are amplified via polymerase chain reaction (PCR) with universal primers, and following the different sequences are illustrated as different bands or peaks such as the SSCP, DGGE, and T-RFLP (Zhao et al., 2008). These techniques are the most widely used and have successfully identified in the Anammox bacteria.

2.1 16S rRNA

16S rRNA is used the most commonly as phylogenetic marker for studying microbial communities. 16S rRNA gene sequence analyses of Anammox bacteria show that they generate in phylum *Planctomycetes* (Junier et al., 2010) (Table 1). Nonspecific 16S rRNA gene primers have been used to characterize environmental communities with high abundance of Anammox bacteria, in samples with low agent of Anammox but clone libraries are not a good choice (Junier et al., 2010). Hence, some specific primers predominantly derived from FISH probes (Schmid et al., 2005). Anammox bacteria, the 16S rRNA, the 23S rRNA, and an intergenic sequence between them have as an operon. This intergenic sequence has been used for designing FISH probes, which may be used for the specific detection of Anammox bacteria (Junier et al., 2010). FISH has been especially beneficial for the study of these bacteria because of their characteristic granular shape generated by the presence of the “anammoxosome” (Junier et al., 2010). Up to now been found only in Anammox bacteria the anammoxosome is an intracytoplasmic compartment bounded by a single ladderane lipid-containing membrane (Van Niftrik et al., 2004).

Table 1. List of PCR primers for the amplification the 16S rRNA genes of *Planctomycetes* phylum and Anammox bacteria and the quantification of Anammox bacteria (modified Li and Gu, 2011)

Primer name	Sequences 5′–3′	Specificity group	References
Pla46F[a]	GACTTGCATGCCTAATCC	*Planctomycetes*	Neef et al. (1998)
Amx368F[b]	CCTTTCGGGCATTGCGAA	All Anammox bacteria	Schmid et al. (2003)
Brod 541F	GAGCACGTAGGTGGGTTTGT	*Scalindua sp.*	Penton et al. (2006)
AMX809F[c]	GCCGTAAACGATGGGCACT	Most Anammox bacteria	Tsushima et al. (2007)
AMX818F[c]	ATGGGCACTMRGTAGAGGG GTTT	Most Anammox bacteria	Tsushima et al. (2007)
Amx694F[c]	GGGGAGAGTGGAACTTCGG	All Anammox bacteria	Ni et al. (2010)
Amx820R	AAAACCCCTCTACTTAGTGC CC	*Brocadia* and *Kuenenia*	Schmid et al. (2000)
BS 820R	TAATTCCCTCTACTTAGTGC CC	*Scalindua*	Kuypers et al. (2003)
Brod1260R	GGATTCGCTTCACCTCTCGG	*Scalindua* sp.	Penton et al. (2006)
AMX1066 R[c]	AACGTCTCACGACACGAGCT G	Most Anammox bacteria	Tsushima et al. (2007)
Amx960R[c]	GCTCGCACAAGCGGTGGAG C	All Anammox bacteria	Ni et al. (2010)

[a] Can be used as a forward primer with other reverse primers for PCR amplification of *Planctomycetes* phylum and Anammox bacteria
[b] Can be used as a forward and reverse primers for PCR amplification of Anammox bacteria with higher specificity
[c] Can be used as primers of quantitative real-time PCR

2.2Functional marker

The basic hardship of the 16S rRNA gene as a molecular marker is that it is not definitely related to the physiology of the target organisms. Functional markers can provide activity information together with presence of Anammox bacteria. Moreover, PCR amplification for a matchless functional gene target would mean substantial increase in the detection efficiency from <1% samples to low Anammox bacteria (Kartal et al., 2011; Junier et al., 2010). Hence, functional gene targets will identify reliable in Anammox bacterial genomics, biochemistry, and physiology. Potentially common functional markers for Anammox bacteria are hydrazine oxidoreductase (HZO). Also *hzo* gene successfully used to detect Anammox bacteria from several environmental samples such as wastewater treatment plants, coastal and deep-ocean sediments (Dang et al., 2010; Li and Gu, 2011).

Anammox bacteria have in addition to HZO nitrite and nitrate reductases, hydrazine hydrolase, which are the most likely candidates for new PCR primer design (Strous et al., 2006). The other functional markers with application in Anammox bacteria detection is cd1 nitrite reductase (NIRS) gene and PCR primer set designed for the amplification of *nirS* gene. In Table 2 is shown, that list of PCR primers for the amplification of functional genes of Anammox bacteria (Li and Gu, 2011).

Table 2. List of PCR primers for the amplification of functional genes of Anammox bacteria (modified Li and Gu, 2011)

Primer name	Sequences 5′–3′	Specificity group	References
hzocl1F1 hzocl1R2	TGYAAGACYTGYCAYTGG ACTCCAGATRTGCTGACC	*hzo*	Schmid et al. (2008)
hzocl1F hzocl1R2	TGYAAGACYTGYCAYTGGG ACTCCAGATRTGCTGACC	*hzo*	Schmid et al. (2008)
hzocl2AF hzocl2AR	GGTTGYCACACAAGGC TYWACCTGGAACATACCC	*hzo*	Schmid et al. (2008)
hzocl2AF1 hzocl2AR2	GGTTGYCACACAAGGC ATATTCACCATGYTTCCAG	*hzo*	Schmid et al. (2008)
hzocl2AF2 hzocl2aR1	GTTGTGMTGMWTGTCATGG TYWACCTGGAACATACCC	*hzo*	Schmid et al. (2008)
Ana-hzo1 Ana-hzo2R	ACCTCTTCWGCAGGTGCAT ACCTCTTCWGCAGGTGCAT	*hzo*	Quan et al. (2008)
hzoF1 hzoR1	TGTGCATGGTCAATTGAAAG CAACCTCTTCWGCAGGTGCATG	*hzo*	Li et al. (2010)
hzoAB1F hzoAB1R	GAAGCNAAGGCNGTAGAAATT ATCAC CTCTTCNGCAGGTGCATGATG	*hzo*	Hirsch et al. (2011)
hzoAB4F hzoAB4R	TTGARTGTGCATGGTCTAWTGA AAG GCTGACCTGACCARTCAGG	*hzo*	Hirsch et al. (2010)
Scnir372F Scnir845R	TGTAGCCAGCATTGTAGCGT TCAAGCCAGACCCATTTGCT	*Scalindua nirS*	Lam et al. (2009)
AnnirS379F AnnirS821R	TCTATCGTTGCATCGCATTT GGATGGGTCTTGATAAACA	Anammox bacteria *nirS*	Li et al. (2011a)

2.3Denaturant gradient gel electrophoresis (DGGE)

DGGE is commonly used to define phylogenetic relationships between bacteria since it can supply precise and abundant information on genetic diversity of a targeting ecosystem. This technique is often appointed to investigate changes in microbial community structures (Gao and Tao, 2011). DGGE can be separated different base-pair sequences (around 200-700 base pair). However these sequences have to DNA fragments in the same length that they cannot be separated on agarose or nondenaturing acrylamide gels. Separation in DGGE with denaturants mixture of urea and formamide is based on electrophoresis of PCR amplified rDNA gene fragments the in polyacrylamide gels having a low to high denaturant gradient (Muyzer et al., 1993;

Nocker et al., 2007). DGGE is used more than other genetic fingerprinting techniques. Besides, temperature gradient gel electrophoresis (TGGE) has similar assignment with DGGE but gradient is obtained from temperature instead of denaturant.

DGGE/TGGE is generally used in determination of Anammox bacteria. Because identification of these bacteria is required DNA extraction, amplification of PCR with 16S rRNA gene of total bacterial community, DGGE, and DNA sequencing respectively. Ultimately phylogenetic tree is detected of Anammox bacteria. Also cloning is recently studied after PCR amplification.

2.4Single-strand conformation polymorphism (SSCP)

SSCP using separation of PCR products of similar length like DGGE/TGGE is an electrophoresis method adapted to the analysis of microbial communities. However, the separation is based on single-stranded DNA separation of PCR products in contrast to current double-stranded DNA in DGGE/TGGE. Nondenaturing acrylamide gel is put account in this technique after strand separation is acquired under denaturing conditions. Secondary structures which single-stranded DNA is adopted a folded are used to separate between products from different phylotypes even if they have the same molecular weight. Different configurations generate different migration behaviors and motilities in the gel, enabling the separation of complex mixtures of culture (Lee et al., 1996). In the present case of short PCR products, a single mutation can markedly alter the secondary structure of the single-stranded DNA, hence leading to different migration velocities and separation in the gel. Accordingly, DGGE or TGGE, different bands of interest can be isolated and sequenced after extraction from the acrylamide gel. In SSCP is not required that GC clamped primers or restriction digestions, compared to DGGE/TGGE and T-RFLP, but reannealing of single-stranded DNA is been high during electrophoresis. This is especially take place in high DNA concentrations, which may be required for analysis of high diversity communities (Nocker et al., 2011). Another problem of SSCP is typically appearance of three bands two DNA single strands and one double-stranded DNA molecule after electrophoresis, but in one gel is found coexist several conformations of one product. Finally, as already described with DGGE/TGGE, the formation of heteroduplex DNA from PCR products with similar sequences occurs mostly (Schwieger and Tebbe, 1998).

2.5Terminal restriction fragment length polymorphism (T-RFLP)

T-RFLP is used to analyze microbial community with restriction digest of double stranded fluorescently dye end-labeled PCR fragments. Compared with other molecular fingerprinting techniques such as DGGE/TGGE and SSCP, it has advantages due to higher throughput, faster analysis but has disadvantages with some factors as time-consuming, expensive and underestimation of microbial diversity. Although all these, T-RFLP has been widely used in the research both large-scale plants and laboratory-scale reactors (Gao and Tao, 2011).

DGGE is more widely employed than T-RFLP. Because it has both longer applications history and more cost-effective and appropriate obtaining sequence

information by separating DGGE bands. Also, T-RFLP needs more steps such as achieving fluorescently dye PCR products and using restriction enzymes (Gao and Tao, 2011). However, the studies about the application of T-RFLP indicated functional microbial communities in research wastewater such as AOB, NOB, and Anammox bacteria. In the same time, these studies focused on the species composition of microbial community and variation (Gao and Tao, 2011).

DGGE and T-RFLP are compared some approaches.
1) The size of DGGE bands is usually less than 500 base pair (bp). Therefore, the DNA sequence information obtained from bands is limited and phylogenetic identification may be poor in the case of novel sequences having less than 85% identity to known sequences (Muyzer et al., 1993).
2) DGGE gel is lesser sensitivity limiting to ensure reproducibility and detection of minor populations and small changes but the identification of T-RFLP peaks can be directly obtained.
3) DGGE has more miss information than T-RFLP in terms of no dominant species.
4) DGGE profile is only detected about 14 bands whereas peaks of T-RFLP method are detected approximately 34 peaks. Samples which are less than 14, DGGE is more efficient than T-RFLP, on the contrary the samples too much it is inefficiency.
This is because, there are limitations to the number of DNA samples for each methods, and that for DGGE is always much less than T-RFLP (Gao and Tao, 2011).

Over prediction of environmental samples variety usually happen using DGGE since microorganisms have target genes with different sequences, and the PCR fragments appear more different sites of DGGE bands. Quantity each band of a DGGE profile which the most widely used software for distinguish is always influenced the results by annealing temperature, electrophoresis conditions, pigmentation process and mechanical problems of bio-imaging system. In conclusion, DGGE has weaker and much potential biases for the following procedures as statistical analysis (Gao and Tao, 2011). In Table 3 is illustrated that comparison of the molecular fingerprinting such as DGGE,
T-RFLP, and SSCP techniques using identification of bacteria.

Table 3. Comparison of the molecular fingerprinting techniques using identification of bacteria (modified Gao and Tao, 2011; Nocker et al., 2007)

Name	Sensitivity	Advantages	Disadvantages	Cost
DGGE	Limited sensitivity	Bants of interest can be excised from gel for sequencing	Primer GC clamp decreases yield and favors primer dimers	Medium cost
T-RFLP	High sensitivity	High throughput and short run times	Restriction digestion can result in pseudo-T-RFs	Medium high cost
SSCP	High sensitivity	Not GC clamp and restriction digestion	High rate of reannealing of single strands with high DNA concentration	Medium cost

3. FISH-BASED METHOD

3.1Fluorescent in-situ hybridization (FISH)

FISH is a useful tool for in situ identification of targeted cells in environmental samples. Anammox bacteria in environmental samples have widely been used the FISH technique to collect both qualitative and quantitative data (Schmid et al., 2005). FISH is an effective and fast technique to identify environmental microorganisms and microbial community structure. In contrast to the other molecular biologic techniques, it can identify environmental microorganisms at any desired taxonomical level. FISH, which depends on the specificity of the used fluorochrome-labeled DNA oligonucleotide probes, has been commonly used to analyze the phylogenetic composition and distribution of microbial communities in nature due to high sensitivity, simpleness and rapidity. FISH in combination with 16S rRNA-targeted technique are used for studying the community structure and detecting activity dynamics of specific microorganisms in wastewater treatment such as nitrifying bacteria, sulfate-reducing bacteria, polyphosphate accumulating organisms and Anammox bacteria in different wastewater treatment systems. In detect Anammox bacteria, probes are widely used available for the representatives and for different genera of Anammox bacteria are shown in Table 4. More specific probes related to this technique will become available for new Anammox bacteria identification along with the fast research available currently and in the near future (Li and Gu, 2011). However, it is often obstruct by its defining limit from the matrix of environmental samples. Accordingly different strategies have been used to optimize the FISH procedures for detect Anammox bacteria, such as increasing the probe signal intensity by polynucleotide FISH and catalyzed reported deposition FISH (CARD-FISH), or minimizing probe penetration problems and increasing hybridization efficiencies with different probes chemistries, like peptide nucleic acid FISH, and locked nucleic acid FISH (Li and Gu, 2011). Also, metabolic activity of Anammox bacteria has approaches to gain more insights detection with FISH as a powerful technique compare to other technique (Li and Gu, 2011).

Although all these advantage the step of fixation and the reduction of probe penetration problem are very significant FISH process. Therefore, the result is directly influenced with the design and assessment of the probes. In FISH detection is not suitable the cells being low ribosome content (Gao and Tao, 2011).

Table 4. A list of commonly used probes in identifying known Anammox bacteria with probe sequences, and target genes specific FISH technique (modified Li and Gu, 2011)

Name of probes	Sequence 5′–3′	Targeted gene	Specificity group	References
S-P-Planc-0046-a-A-18	GACTTGCATGCCTAATCC	16S rRNA	*Planctomycetes*	Neef et al. (1998)
S–P–Planc– 0886–a– A–19	GCCTTGCGACCATACTCCC	16S rRNA	*Isosphaera, Gemmata, Pirellula, Planctomycetales*	Neef et al. (1998)
S-D-Bact-0338-b-A-18	GCAGCCACCCGTAGGTGT	16S rRNA	*Planctomycetales* and to be used in combination with EUBI and III	Daims et al. (1999)
S-*-Amx- 0368-a-A-18	CCTTTCGGGCATTGCGAA	16S rRNA	All Anammox	Schmid et al. (2003)
S-*-Amx- 0820-a-A-22	AAAACCCCTCTACTTAGTGCCC	16S rRNA	*Brocadia anammoxidans, Kuenenia stuttgartiensis*	Schmid et al. (2000)
S-G-Sca- 1309-a-A-21	TGGAGGCGAATTTCAGC CTCC	16S rRNA	*Scalindua*	Schmid et al. (2003)
S-*-Scabr-1114-a-A-22	CCCGCTGGTAACTAAAAACAAG	16S rRNA	*Scalindua brodae*	Schmid et al. (2003)
S-*-BS- 820-a-A-22	TAATTCCCTCTACTTAGTGCCC	16S rRNA	*Scalindua wagneri*, *Scalindua sorokinii*	Kuypers et al. (2003)
S-S-Kst-0157-a-A-18	GTTCCGATTGCTCGAAAC	16S rRNA	*Kuenenia stuttgartiensis*	Schmid et al. (2001)
S-*-Kst- 1275-a-A-20	TCGGCTTTATAGGTTTCGCA	16S rRNA	*Kuenenia stuttgartiensis*	Schmid et al. (2000)
S-S-Ban 0162(B. anam.)-a-A-18	CGGTAGCCCCAATTGCTT	16S rRNA	*Brocadia anammoxidans*	Schmid et al. (2000)
S-*-Amx- 0156-a-A-18	CGGTAGCCCCAATTGCTT	16S rRNA	*Brocadia anammoxidans*	Schmid et al. (2000)
S-*-Amx- 0223-a-A-18	GACATTGACCCCTCTCTG	16S rRNA	*Brocadia anammoxidans*	Schmid et al. (2000)
S-*-Amx- 0432-a-A-18	CTTAACTCCCGACAGTGG	16S rRNA	*Brocadia anammoxidans*	Schmid et al. (2000)
S-*-Amx- 0613-a-A-22	CCGCCATTCTTCCGTTAAGCGG	16S rRNA	*Brocadia anammoxidans*	Schmid et al. (2000)
S-*-Amx- 0997-a-A-21	TTTCAGGTTTCTACTTCTACC	16S rRNA	*Brocadia anammoxidans*	Schmid et al. (2000)
S-*-Amx- 1015-a-A-18	GATACCGTTCGTCGCCCT	16S rRNA	*Brocadia anammoxidans*	Schmid et al. (2000)
S-*-Amx- 1154-a-A-18	TCTTGACGACAGCAGTCT	16S rRNA	*Brocadia anammoxidans*	Schmid et al. (2000)
S-*-Amx- 1240-a-A-23	TTTAGCATCCCTTTGTACCAACC	16S rRNA	*Brocadia anammoxidans*	Schmid et al. (2000)
S-*-Bfu- 0613-a-A-24	GGATGCCGTTCTTCCGTTAAGCGG	16S rRNA	*Brocadia fulgida*	Kartal et al. (2008)
S-*-Apr- 0820-a-A-21	AAACCCCTCTACCGAGTGCCC	16S rRNA	*Anammoxoglobus propionicus, Jettnia asiatica*	Kartal et al. (2007)
L-*-Amx- 1900-a-A-21	CATCTCCGGCTTGAACAA	23S rRNA	*Brocadia and Kuenenia*	Schmid et al. (2001)
I-*-Ban- 0071(B. anam.)-a-A-18	CCCTACCACAAACCTCGT	ISR	*Brocadia anammoxidans*	Schmid et al. (2000)
I-*-Ban- 0108(B. anam.)-a-A-18	TTTGGGCCCGCAATCTCA	ISR	*Brocadia anammoxidans*	Schmid et al. (2000)
I-*-Ban- 0222(B. anam.)-a-A-19	GCTTAGAATCTTCTGAGGG	ISR	*Brocadia anammoxidans*	Schmid et al. (2000)
I-*-Ban- 0389(B. anam.)-a-A-18	GGATCAAATTGCTACCCG	ISR	*Brocadia anammoxidans*	Schmid et al. (2000)
I-*-Kst- 0031(K.stutt.)- a-A-18	ATAGAAGCCTTTTGCGCG	ISR	*Kuenenia stuttgartiensis*	Schmid et al. (2001)
I-*-Kst- 0077(K.stutt.)- a-A-18	TTTGGGCCACACTCTGTT	ISR	*Kuenenia stuttgartiensis*	Schmid et al. (2001)
I-*-Kst- 0193(K.stutt.)- a-A-19	CAGACCGGACGTATAAAAG	ISR	*Kuenenia stuttgartiensis*	Schmid et al. (2001)
I-*-Kst- 0288(K.stutt.)- a-A-20	GCGCAAAGAAATCAAACTGG	ISR	*Kuenenia stuttgartiensis*	Schmid et al. (2001)

4. SUMMARY

Identification of Anammox bacteria has significantly accelerated since discovery of this reaction. In that Anammox has been important on field microbial ecology, biotechnology, and microbiology. Much microbial research has identified genetic elaborate and biosynthesis of Anammox bacteria, which has been clue the behavior of Anammox bacteria in natural environments and in engineered systems. Also, the particular techniques using Anammox microorganisms have provided positive effect to studies on environmental samples (Li and Gu, 2011). These techniques fundamentally contain PCR-based methods (community fingerprinting techniques) such as DGGE, T-RFLP, SSCP, ribosomal intergenic spacer analysis (RISA) or automated rRNA intergenic spacer analysis (ARISA), random amplified polymorphic DNA (RAPD), amplified rDNA restriction analysis (ARDRA), length heterogeneity polymerase chain reaction, microarray analysis (Geochip), metagenomics, serial analysis of ribosomal sequence tags (SARST) and non-PCR-based molecular methods like phospholipid fatty acid (PLFA), stable isotope probing (SIP), community level physiologic profiling (CLPP), combination techniques of FISH and microautoradiography (MAR) (Gao and Tao, 2011).

This review has been focused the most used from molecular techniques in Anammox bacterial density and diversity. These techniques are FISH from non-PCR-based molecular methods and SSCP, DGGE, T-RFLP from PCR-based methods. After PCR amplification of DNA template with 16S rRNA gene, DGGE is more used common than other techniques in order to show the variety of genetic profiling in order to detect Anammox bacteria species, genus or higher taxonomy. However, genetic fingerprinting techniques based on nucleic acid extraction and amplification by PCR is not quantitative. Hence, FISH is important identification for qualitative and quantification with fluorescent signal specific probes of Anammox bacteria. Once optimized FISH technique, it is easy and will be more sensitive and quantitative (Sanz and Köchling, 2007). Also this technique provides new perspective into metabolic activity of these bacteria. In future with development of these techniques will identify different species or genus of Anammox bacteria.

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