

# Advances in methods for detection of anaerobic ammonium oxidizing (anammox) bacteria

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**Abstract** Anaerobic ammonium oxidation (anammox), the biochemical process oxidizing ammonium into dinitrogen gas using nitrite as an electron acceptor, has only been recognized for its significant role in the global nitrogen cycle not long ago, and its ubiquitous distribution in a wide range of environments has changed our knowledge about the contributors to the global nitrogen cycle. Currently, several groups of methods are used in detection of anammox bacteria based on their physiological and biochemical characteristics, cellular chemical composition, and both 16S rRNA gene and selective functional genes as biomarkers, including hydrazine oxidoreductase and nitrite reductase encoding genes *hzo* and *nirS*, respectively. Results from these methods coupling with advances in quantitative PCR, reverse transcription of mRNA genes and stable isotope labeling have improved our understanding on the distribution, diversity, and activity of anammox bacteria in different environments both natural and engineered ones. In this review, we summarize these methods used in detection of anammox bacteria from various environments, highlight the strengths and weakness of these methods, and also discuss the new development potentials on the existing and new techniques in the future.

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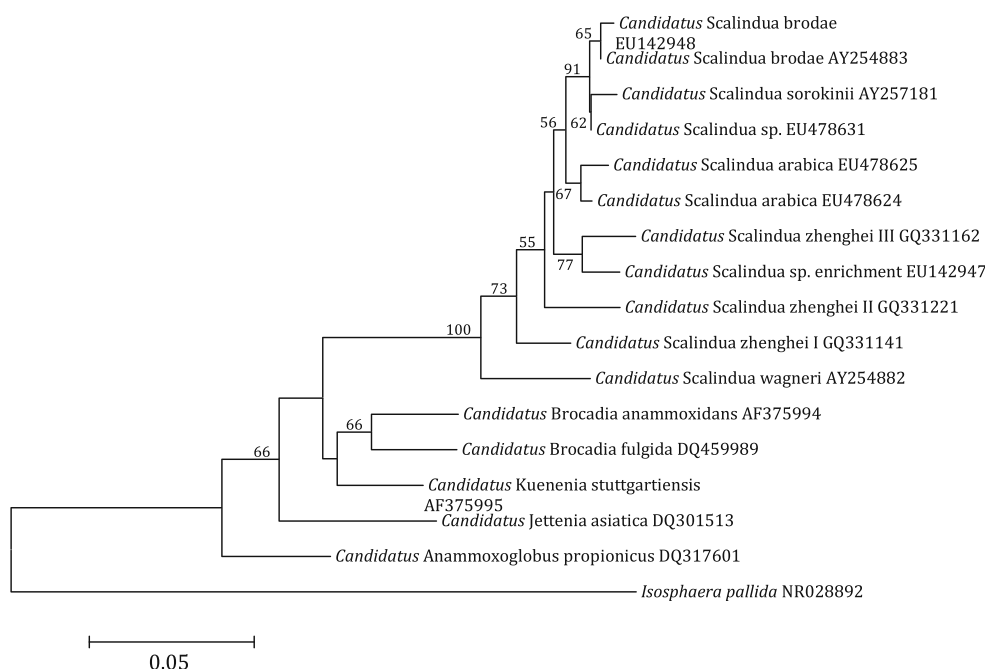
**Keywords** Anammox bacteria · Isotope pairing technique · Lipid analysis · Molecular technique · 16S rRNA gene · Functional biomarkers

## Introduction

Anaerobic ammonium oxidation (anammox) is a newly discovered biochemical pathway involved in the microbial N cycle that allows coupling between ammonium oxidation with nitrite reduction under anoxic conditions (van de Graaf et al. 1995). Since then, the applications of anammox in the removal of inorganic N species from wastewater with high ammonium concentrations had resulted in new innovations in wastewater treatments, such as combining anammox with nitrification process in a single reaction system for high ammonium removal over nitrite (Hellinga et al. 1998), completely autotrophic N removal over nitrite (Slikers et al. 2002) or oxygen-limited ammonium removal via nitrification–denitrification (Kuai and Verstraete 1998), and short-cut nitrification and denitrification (Kumar and Lin 2010). However, the first anammox bacterium named *Candidatus* “*Brocadia anammoxidans*” was firstly physically separated from a laboratory enrichment culture in bioreactor using a Percoll gradient centrifugation (Strous et al. 1999) and they display complex cellular architectures and chemical composition, including a central cellular compartment anammoxosome where the biochemical anammox process is proposed to take place and the unique ladderane lipids in the membrane (Strous et al. 1999). So far, only five anammox genera have been recognized (Fig. 1), namely *Kuenenia* (Schmid et al. 2000; Strous et al. 2006), *Brocadia* (Strous et al. 1999; Kartal et al. 2008), *Anammoxoglobus* (Kartal et al. 2007b), *Jettenia* (Quan et al. 2008), and *Scalindua* (Kuyper et al. 2003; van de Vossenberg et al. 2008; Hong et al. 2011).

While researches on the applications of anammox bacteria and their related basic biology and biochemistry have provided

**Fig. 1** A phylogenetic tree of described anammox bacteria species based their 16S rRNA genes. Phylogenetic tree was constructed by MEGA 4.0 with 1,000 times bootstraps, and the scale bar represents 5% sequence divergence



fascinating new biological information, observations made from both field and laboratory investigations have also indicated that the anammox process is a key one only recognized recently in the global N cycle (Francis et al. 2007). It has been estimated that this process alone is likely responsible for the consumption of more than 40% of the fixed N assimilated into the anoxic Black Sea (Kuypers et al. 2003), 19–35% of the total N<sub>2</sub> evolved in the anoxic water column of the coastal bay in Costa Rica (Dalsgaard et al. 2003), 24–67% of the total N<sub>2</sub> production in continental shelf sediments (Dalsgaard and Thamdrup 2002), and 1–24% of the N loss in estuarine sediments (Trimmer et al. 2003; Risgaard-Petersen et al. 2004). In a global context, anammox reaction may thus account for 30–70% of oceanic N<sub>2</sub> production, which fundamentally changes our traditional view on that denitrification is the only major N sink in the marine ecosystems (Devol 2003). Up to now, anammox process has been demonstrated in very diverse environments, including the suboxic and oxygen minimum zones of oceans (Kuypers et al. 2003, 2005), surface and subsurface sediments of deep ocean (Hong et al. 2011; Li et al. 2010b), temperate estuarine, coastal and offshore sediments (Jetten et al. 2003; Li et al. 2010b; Tal et al. 2005; Trimmer et al. 2005), lakes (Schubert et al. 2006), freshwaters (Penton et al. 2006), polar region sediments and multiyear sea ice (Rysgaard and Glud 2004; Rysgaard et al. 2004), oil reservoirs (Li et al. 2010a), and deep-sea hydrothermal vent (Byrne et al. 2009).

In the last decade of anammox research, three main categories of approaches were used to detect anammox bacteria in natural environments and wastewater treatment systems: activity measurements by the isotope pairing technique (IPT), analysis of anammox bacteria-specific lipids, and a suite of

molecular techniques from PCR amplification to quantitative PCR and reverse-transcription (RT)-PCR of anammox bacterial gene biomarkers. Several previous reviews had discussed the detection methods for anammox bacteria so far (Jetten et al. 2005, 2009; Schmid et al. 2005; Junier et al. 2010; Kartal et al. 2011), and the information improved our general understanding on distribution of anammox bacteria in various niches. However, due to the rapid advances in anammox bacteria research in recent years, especially on more in-depth information about phylogenetic groups, metabolic capability, and physiology and evolution, we would like to give an updated and collated information on methods available in detection of anammox bacteria in both environmental samples and laboratory enrichments, their strengths and drawbacks, and the future development potentials with new emerging functional gene biomarkers in the present review.

### Isotope pairing technique

IPT is a well-established <sup>15</sup>N method used in estimation of denitrification (Nielsen 1992) on many environmental samples, such as marine sediments, lake sediments, rivers, and wetland (Rysgaard et al. 1993; Stepanauskas et al. 1996; Davidsson et al. 1997; Pind et al. 1997; Dong and Tollner 2003; Trimmer et al. 2003). In 2002, IPT was used to estimate the relative contribution of anammox process to the overall total N<sub>2</sub> production through coupling with nitrate reduction in marine sediment (Thamdrup and Dalsgaard 2002). Risgaard-Petersen et al. (2003) described the detail methodology for application of IPT in sediments where anammox and denitrification coexist. Experimental

samples can be treated for a short-term incubation (e.g., 24 h) in parallel with different inorganic  $^{15}\text{N}$  species labeled substrates: (1)  $^{15}\text{NH}_4^+$  alone, (2) a mixture of  $^{15}\text{NH}_4^+$  and  $^{14}\text{NO}_2^-$ , and (3)  $^{15}\text{NO}_2^-$  alone. After incubation,  $\text{N}_2$  production from each treatment can be collected and measured on an isotope mass spectrometer for  $^{15}\text{N}$  concentrations. The first incubation is used as a control to detect any oxidation of ammonium without the addition of nitrite, while the second treatment is used to measure the anammox activity, where the production of  $^{29}\text{N}_2$  stoichiometrically is a confirmation on the oxidation of ammonium ( $^{15}\text{NH}_4^+$ ) with reduction of nitrite ( $^{14}\text{NO}_2^-$ ) through the anammox process. The third incubation is to estimate the relative contribution of anammox and denitrification collectively, where the production of  $^{29}\text{N}_2$  and  $^{30}\text{N}_2$  indicate the activities of anammox and denitrification, respectively (Risgaard-Petersen 2003; Dalsgaard et al. 2003, 2005). Experimental details of the IPT procedures for the measurement of anammox and denitrification were described by Risgaard-Petersen et al. (2003) and further modifications had been made by Ward et al. (2009). Up to now, IPT has been used as the widely accepted method to substantiate the contribution of anammox process in various environmental samples. However, new evidences had pointed out that anammox bacteria could also reduce  $^{15}\text{NO}_3^-$  to  $^{15}\text{NO}_2^-$  and then to  $^{15}\text{NH}_4^+$  under certain conditions and as a result, portions of the measured denitrification may be partitioned to the anammox reaction (Kartal et al. 2007a). In addition, IPT provides indisputable information on the specific process involved, but neither abundance nor the organisms responsible for the anammox process is available (Schmid et al. 2005).

Another isotope-labeling technique for detection the presence and activity of anammox bacteria is stable isotope probing (SIP) with  $^{13}\text{CO}_2$  and/or  $^{15}\text{N}$  labeled inorganic N species. SIP is a powerful technique when combined with current available molecular methods in revealing the anammox community composition and the associated microbial activity in environmental samples (Murrell and Whiteley 2011). This method relies on the incorporation of a substrate that is preferably enriched in a heavier stable isotope, such as  $^{13}\text{C}$  or/and  $^{15}\text{N}$  which allows the identification of an active population of microorganisms through selective recovery and analysis of isotope-enriched cellular biomolecules, such as DNA, RNA, proteins, and phosphorus lipid fatty acids (Bull et al. 2000; Murrell and Whiteley 2011). Currently, SIP has been successfully applied in the study of microbial nitrogen cycle, e.g., ammonia-oxidizing archaea and bacteria in the soils (Tourna et al. 2010; Zhang et al. 2011; Pratscher et al. 2011). In these studies,  $^{13}\text{CO}_2$  and  $^{14}\text{N}/^{15}\text{N}$  were used as substrates for microbial metabolism in the soil samples, and then total DNA, RNA, or mRNA were extracted after a period

of incubation for analysis of the relevant microbial communities and microbial abundance using relevant molecular techniques could be obtained for direct evidence on the active microbial groups and their abundance (Tourna et al. 2010; Zhang et al. 2011). In research on anammox bacteria,  $^{14}\text{C}$  labeled substrates were also used for fluorescence in situ hybridization (FISH)–microautoradiography analysis to confirm the chemolithoautotrophic biochemical pathway carried out by anammox bacteria (Strous et al. 1998; Jetten et al. 2002), and no other related reports are available. Therefore, SIP coupling with an array of molecular techniques (see below) is a promising approach for detection and quantification of anammox bacteria in the ecosystem.

### Lipid measurement

In contrast to all other known prokaryotes, anammox bacteria have special lipids in cellular membrane surrounding the anammoxosome within the anammox bacterial cells (Sinninghe Damste et al. 2002). These membrane ladderane lipids contain cyclobutane/cyclohexane ring systems, which make the anammoxosome membrane very impermeable comparing to other known non-anammox bacterial membranes (Sinninghe Damste et al. 2002; van Niftrik et al. 2004). Since the unique lipids are only found in anammox bacteria so far, the ladderane lipid can be used as an indicator and a biomarker for the presence of anammox bacteria in environmental samples (Sinninghe Damste et al. 2002, 2005; Kuypers et al. 2003). Furthermore, ladderane lipids are predominately enumerated as the core lipid derivatives (Kuypers et al. 2003, 2005; Jaeschke et al. 2007), but occurring as intact ladderane glycerophospholipids (ladderane IGP) within cells at high abundances (Boumann et al. 2006), thus the ladderane IGP, such as C20-[3]-ladderane monoalkylether–phosphocholine, may reflect living biomass more accurately than ladderane core lipids. Because of this, they are more specific biomarkers for viable anammox bacteria (Jaeschke et al. 2009). Up to now, the detection and quantification of ladderane lipids are used not only to infer the presence of anammox bacteria in environmental samples (Strous et al. 1999; Sinninghe Damste et al. 2002, 2005), but also to assess the presence of anammox bacteria associated with some of the geological events (Jaeschke et al. 2007).

In order to analyze ladderane lipids, biomass containing anammox bacteria are firstly extracted with methanol, methanol/dichloromethane, and dichloromethane substantially. The extracts are methylated with B3/methanol after removing the solvent by a rotary evaporator, and then fractionated by a small silica column using ethyl acetate as the eluent. After that, the obtained fraction is silylated with BSTFA in pyridine convert alcohols in TMS ethers for

further analysis by gas chromatography (GC) and gas chromatography/mass spectrometry (GC/MS) (Sinninghe Damste et al. 2002). Various chemicals contained in the ladderane lipids, such as fatty acids, glycerol diethers, glycerol ether-esters, and *sn*-2 glycerol monoether, would be analyzed through these procedures (Sinninghe Damste et al. 2002, 2005). Recently, a high performance liquid chromatography/atmospheric pressure chemical ionization-MS/MS method allowing determination of low levels of ladderane lipids in complex matrixes (e.g., sediments) had been developed with success, and this technique could detect as low as about 35 pg ladderane lipids, which improves the method resolution compared to the GC/MS method greatly (Hopmans et al. 2006). Although the lipids analysis provides a powerful tool to detect anammox bacteria, the following deficiencies still exist when using this technique on environmental samples: (1) extraction procedures are complicated; (2) high volume of samples is required, such as the seawaters and sediment due to the low abundance of anammox bacteria; (3) difficulties are encountered in purification when dealing with sediment materials because of other contaminants, such as humic acid and fulvic acid; and (4) these lipids may also be present in nonliving organic matter and thus may not necessarily indicate the presence of metabolically active anammox bacteria without error (Schmid et al. 2005), which limits the quantification of active anammox bacteria population with high confidence and precision.

### Molecular techniques

Since anammox bacteria have not been isolated in pure culture, culture-independent methods, specifically DNA/RNA-based molecular techniques, are the most widely used approaches available for detecting this group of microorganisms in various samples (Schmid et al. 2005). For molecular techniques, the available techniques include FISH-based techniques, PCR-based methods and quantification by quantitative PCR (qPCR) and RT-qPCR.

### FISH-based technique

FISH is a useful tool for cultivation independent in situ identification of targeted cells in environmental samples. Many investigations have used the FISH technique to collect both qualitative and quantitative data on anammox bacteria in environmental samples (Schmid et al. 2005). For this widely used technique on anammox bacteria, previous research has mainly focused on the followings.

First, the research focus is on design-specific oligonucleotide probes for anammox bacteria detection. Because different probes may specifically target different groups of

microorganisms, specificity of fluorochrome-labeled DNA oligonucleotide probe is one of the most important factors for successful application of this technique in practice (Giovannoni et al. 1988; Amann et al. 1990). To detect anammox bacteria, a number of probes are available for different genus/species of anammox bacteria, and a summary of the representatives and widely used probes for different genera of anammox bacteria are shown in Table 1. Most of the available probes used in FISH detection target the 16S rRNA genes of anammox bacteria, but probe for 23S rRNA gene has also been developed, such as the L-*\**-Amx-1900-a-A-21 (Schmid et al. 2001), and it is feasible that more specific probes will become available for anammox bacteria detection along with the fast research progress made in genomic data of anammox bacteria available currently and in the near future. However, the high divergence (<87.1% similarity) among different genera of anammox bacteria often impedes the discovery of new anammox species (Schmid et al. 2003; Jetten et al. 2009). Thus, it is essential to re-evaluate the anammox-specific probe sets available so that new probes can be used for more effective revealing of new anammox species from the environment.

Second, FISH technique can be used to quantify the abundance of anammox bacteria. FISH with specific oligonucleotide probes is the standard technique to quantify the abundance of targeted anammox bacteria in environmental samples (Kuypers et al. 2003; Risgaard-Petersen et al. 2004; Schmid et al. 2000, 2003). Up to now, FISH technique has been widely used to assess the abundance of anammox bacteria in various environmental samples, which has provided a quantitative distribution of anammox bacteria globally (Schmid et al. 2007; Zhu et al. 2010). However, it is often hampered by its detection limit (1,000 cells/ml seawater) or various interferences from the matrix of environmental samples (Kuypers et al. 2003; Pavlekovic et al. 2009). To overcome these limitations, different strategies have been used to optimize the FISH procedures for quantitatively detect anammox bacteria, such as increasing the probe signal intensity by polynucleotide FISH (Zwirgmaier 2005) and catalyzed reported deposition FISH (CARD-FISH) (Pernthaler et al. 2002; Hoshino et al. 2008), or minimizing probe penetration problems and increasing hybridization efficiencies with different probes chemistries, like peptide nucleic acid FISH (Perry-O'Keefe et al. 2001) and locked nucleic acid FISH (Kubota et al. 2006).

Thirdly, integration of FISH with other experiment approaches to gain more insights into the metabolic activity of anammox bacteria and extends the applications of FISH as a powerful technique. The first approach discussed above is to target the intergenic spacer region (ISR) between 16S and 23S rRNA genes by FISH. In general,

**Table 1** A list of commonly used probes in identifying known anammox bacteria with probe sequences, target genes, and formamide required for specific FISH technique

Name of probes	Sequence 5'–3'	Targeted gene	Specificity group	Formamide (%)/mM NaCl	References
S-P-Planc-0046-a-A-18	GACTTGCATGCCTAATCC	16S rRNA	Planctomycetes	25/159	Neef et al. (1998)
S-P-Planc-0886-a-A-19	GCCTTGCAGCCATACTCCC	16S rRNA	Isosphaera, Gemmata, Pirellula, Planctomycetales	30/112	Neef et al. (1998)
S-D-Bact-0338-b-A-18	GCAGCCACCCGTAGGTGT	16S rRNA	Specific for Planctomycetales and to be used in combination with EUBI and III	0/900	Daims et al. (1999)
S*-Amx-0368-a-A-18	CCTTTCGGGCATTGCGAA	16S rRNA	All anammox organisms	15/338	Schmid et al. (2003)
S*-Amx-0820-a-A-22	AAAACCCCTCTACTTAGTGCCC	16S rRNA	Brocadia anammoxidans Kuenenia stuttgartiensis	40/56	Schmid et al. (2000)
S-G-Sca-1309-a-A-21	TGGAGGCGAATTCAGCCTCC	16S rRNA	Scalindua	5/675	Schmid et al. (2003)
S*-Scabr-1114-a-A-22	CCCCTGGTAACTAAAAACAAG	16S rRNA	Scalindua brodae	20/225	Schmid et al. (2003)
S*-BS-820-a-A-22	TAATTCCTCTACTTAGTGCCC	16S rRNA	Scalindua wagneri Scalindua sorokinii	40/56	Kuypers et al. (2003)
S-S-Kst-0157-a-A-18	GTTCCGATTGCTCGAAAC	16S rRNA	Kuenenia stuttgartiensis	25/159	Schmid et al. (2001)
S*-Kst-1275-a-A-20	TCGGCTTTATAGGTTTCGCA	16S rRNA	Kuenenia stuttgartiensis	25/159	Schmid et al. (2000)
S-S-Ban-0162(B.anam.)-a-A-18	CGGTAGCCCAATTGCTT	16S rRNA	Brocadia anammoxidans	40/56	Schmid et al. (2000)
S*-Amx-0156-a-A-18	CGGTAGCCCAATTGCTT	16S rRNA	Brocadia anammoxidans	40/56	Schmid et al. (2000)
S*-Amx-0223-a-A-18	GACATTGACCCTCTCTG	16S rRNA	Brocadia anammoxidans	40/56	Schmid et al. (2000)
S*-Amx-0432-a-A-18	CTTAACCTCCGACAGTGG	16S rRNA	Brocadia anammoxidans	40/56	Schmid et al. (2000)
S*-Amx-0613-a-A-22	CCGCCATTCTCCGTTAAGCGG	16S rRNA	Brocadia anammoxidans	40/56	Schmid et al. (2000)
S*-Amx-0997-a-A-21	TTTCAGGTTTCTACTTCTACC	16S rRNA	Brocadia anammoxidans	20/225	Schmid et al. (2000)
S*-Amx-1015-a-A-18	GATACCGTTCGTCGCCCT	16S rRNA	Brocadia anammoxidans	60/14	Schmid et al. (2000)
S*-Amx-1154-a-A-18	TCTTGACGACAGCAGTCT	16S rRNA	Brocadia anammoxidans	20/225	Schmid et al. (2000)
S*-Amx-1240-a-A-23	TTTAGCATCCCTTTGTACCAACC	16S rRNA	Brocadia anammoxidans	60/14	Schmid et al. (2000)
S*-Bfu-0613-a-A-24	GGATGCCGTTCTCCGTTAAGCGG	16S rRNA	Brocadia fulgida	30/112	Kartal et al. (2008)
S*-Apr-0820-a-A-21	AAACCCCTCTACCGAGTGCCC	16S rRNA	Anammoxoglobus propionicus Jettnia asiatica	40/56	Kartal et al. (2007b)
L*-Amx-1900-a-A-21	CATCTCCGGCTTGAACAA	23S rRNA	Brocadia and Kuenenia	30/112	Schmid et al. (2001)
I*-Ban-0071(B.anam.)-a-A-18	CCCTACCACAAACCTCGT	ISR	Brocadia anammoxidans	10/450	Schmid et al. (2000)
I*-Ban-0108(B.anam.)-a-A-18	TTTGGGCCGCAATCTCA	ISR	Brocadia anammoxidans	10/450	Schmid et al. (2000)
I*-Ban-0222(B.anam.)-a-A-19	GCTTAGAATCTCTGAGGG	ISR	Brocadia anammoxidans	10/450	Schmid et al. (2000)
I*-Ban-0389(B.anam.)-a-A-18	GGATCAAATTGCTACCCG	ISR	Brocadia anammoxidans	10/450	Schmid et al. (2000)
I*-Kst-0031(K.stutt.)-a-A-18	ATAGAAGCCTTTTGCGCG	ISR	Kuenenia stuttgartiensis	10/450	Schmid et al. (2001)
I*-Kst-0077(K.stutt.)-a-A-18	TTTGGGCCACACTCTGTT	ISR	Kuenenia stuttgartiensis	10/450	Schmid et al. (2001)
I*-Kst-0193(K.stutt.)-a-A-19	CAGACCGGACGTATAAAAG	ISR	Kuenenia stuttgartiensis	10/450	Schmid et al. (2001)
I*-Kst-0288(K.stutt.)-a-A-20	GCGCAAAGAAATCAAACCTGG	ISR	Kuenenia stuttgartiensis	10/450	Schmid et al. (2001)

the classical FISH as outlined to deliver both qualitative and quantitative data about the bacterial or archaeal population in a sample and the FISH intensity can also be used to assess their activity for many fast-growing microorganisms (Poulsen et al. 1993). However, the ribosome contents do not decrease significantly for betaproteobacterial ammonia-oxidizing bacteria and anammox bacteria during the period of starvation (Morgenroth et al. 2000) or inhibition (Schmid et al. 2001; Wagner et al. 1995). To learn more about the in situ activity of anammox bacteria, the ISR between 16S and 23S rRNA genes has been targeted with fluorescent-labeled oligonucleotide probes, and quantitative FISH experiments with cells of *Candidatus* “*Brocadia anammoxidans*” that were inhibited by exposure to oxygen for different time periods demonstrated that the concentration of transcribed ISR reflected the activity of the cells more accurately than the 16S or 23S rRNA concentration (Schmid et al. 2001). Results have also shown that the ISR-targeted FISH (ISR-FISH) has great potential in monitoring activity changes in enrichment cultures of anammox bacteria and ecosystems (Third et al. 2001; Schmid et al. 2005). However, one disadvantage associated with this technique is that the ISR sequence lacks evolutionary pressure, which might result in two strains of the same species showing sequences very different. In addition, only two sets of ISR probes available for *Candidatus* “*Kuenenia stuttgartiensis*” and *Candidatus* “*Brocadia anammoxidans*” limit the application of ISR-FISH currently, indicating that more new probes need to be designed for wider applications of this technique (Table 1). The second category of methods using FISH in determining the metabolic activity of anammox bacteria is to combine the classical FISH with microautoradiography (FISH-MAR). The FISH-MAR has been developed more than 10 years ago, and it links to the uptake of radiolabeled substrates, such as [ $^{14}\text{C}$ ]acetate, [ $^{14}\text{C}$ ]butyrate, [ $^{14}\text{C}$ ]bicarbonate, and  $^{33}\text{P}_i$ , with specific organisms in a complex environmental sample, and it provides a unique approach for obtaining insights into the microbial community structures and functions simultaneously (Lee et al. 1999). For detection of anammox bacteria, FISH-MAR with the radiolabeled  $^{14}\text{CO}_2$  is very meaningful in demonstrating the chemolithoautotrophic pathway active in anammox bacteria (Strous et al. 1998; Jetten et al. 2002). In the co-cultures of aerobic and anaerobic ammonium oxidizers, FISH-MAR is also successfully used to measure the uptake of  $^{14}\text{CO}_2$  (Schmid et al. 2005). Although FISH-MAR can be applied successfully in anammox bacteria detection with insights of their function, a longer incubation time is necessary for sufficient incorporation of the labeled substrate, which might not always reflect the physiological state of anammox bacteria at in situ condition and/or at the time of sampling (Schmid et al. 2005). The third category of

methods for obtaining insights into the microbial community structures and functions by FISH technique is the gene-FISH (Moraru et al. 2010). This method is to relate the presence of functional gene with cell identity in the samples, and the signals can be visualized at a single cell level. This protocol, when combined with rRNA gene and in situ functional gene detections targeted by CARD-FISH, has identified that at least 30% of the marine Crenarchaeota in the Benguela upwelling system harbored the *amoA* genes, providing stronger evidence for the important role of Crenarchaea in nitrification (Moraru et al. 2010). However, no related research has been reported and thus further efforts are needed to extend this new technique for detection of anammox bacteria in various environmental systems.

Briefly, although the experimental procedures for higher resolution and elimination of the interferences from sample matrix are still needed to improve the performance of detection methods in a wide range of environment samples and the better anammox-specific probe sets are also required for further specific evaluation, FISH has been proven a powerful technique for environmental detection of anammox bacteria, which not only obtains the qualitative and quantitative basic information, but also provides new insights into the metabolic activity of anammox bacteria.

### PCR-based technique

In environmental samples, PCR amplification of sample DNA templates with specific biomarkers specific primers and subsequent phylogenetic analysis of the amplified DNA products is the method of choice to detect any previously unknown microorganisms (Amann et al. 1995). In the anammox case, this method is the most widely used and has successfully identified the presence of anammox bacteria in a wide range of samples. In addition to the commonly used 16S rRNA gene, additional gene biomarkers associated with metabolic reactions for anammox bacteria detection via PCR amplification have also been reported.

#### 16S rRNA gene

The most commonly used phylogenetic biomarker for studying microbial communities is the 16S rRNA gene. According to available information, 16S rRNA gene sequence analyses have shown that all known anammox bacteria form a monophyletic clade within the phylum Planctomycetes (Kuenen 2008). Most of the studies so far for detecting anammox bacteria in natural assemblages are often based on 16S rRNA genes (Kuenen 2008; Jetten et al. 2009); however, the initial difficulty in detection of anammox bacteria with PCR method is that anammox bacterial 16S rRNA genes are not amplified very well with the universal

bacterial primers (Schmid et al. 2005; Li et al. 2010b). This is due to a high 16S rRNA gene sequence divergence that occurs among different genera of anammox bacteria (<87.1% identity), and there are no general PCR primers for different genera of anammox bacteria (Jetten et al. 2009; Junier et al. 2010), especially in some environments where anammox bacteria constitute only a very small fraction (<1%) of the whole community population (Kartal et al. 2011). Selective PCR primer sets intended for anammox bacteria from natural environment have very low specificity; as a result, non-anammox bacteria, such as *Vibrio* species, can be amplified from coastal marine sediment (Fig. S1). The available data have shown that there are also many PCR primers with high specificity to target different genera of anammox bacteria, providing ample information about the presence of anammox bacteria from phylogenetic diversity to the community structures and to the quantitative distribution in various environments. A summary of the widely used PCR primers and their optimal PCR temperatures for amplification of the anammox 16S rRNA genes is presented in Table 2.

### Functional genes

Although PCR amplification with 16S rRNA gene is widely used in anammox bacteria detection, the main pitfall of this molecular marker is that it is not necessarily restricted to the physiological group of anammox bacteria (Junier et al. 2010). Functional gene biomarker could also provide activity information in addition to the presence of anammox bacteria when quantitative reverse-transcription-PCR is performed. Furthermore, PCR amplification for a unique functional gene target would mean a significant increase in the detection efficiency from samples with low

anammox bacteria (<1%)(Kartal et al. 2011). Therefore, the recent progress in anammox bacterial genomics and biochemistry and physiology will identify reliable functional gene targets for such use.

Based on the anammox metabolism, four core catalytic proteins appear to be the most likely promising candidates for new PCR primer design: nitrite and nitrate reductases, hydrazine hydrolase, and hydrazine dehydrogenase (HZO) (Strous et al. 2006). Currently, the HZO, also called the hydroxylamine oxidoreductase-like protein (HAO), has the highest number of available sequences in the public database. More than ten primer sets are available for the PCR amplification of gene encoding this protein (Table 3). These primers target three different clusters of HAO/HZO proteins (Klotz et al. 2008; Schmid et al. 2008), but only *hzo*-cluster 1 with two copies in each genome is considered to be the most suitable biomarker for anammox bacteria phylogenetic analysis. Up to now, *hzo* gene has been successfully used to detect anammox bacteria from various environmental samples, including wastewater treatment plants (Quan et al. 2008; Li et al. 2009; Park et al. 2010), mangrove sediment (Li et al. 2010b, 2011a), estuaries (Li et al. 2011a; Hirsch et al. 2011), coastal and deep-ocean sediments (Dang et al. 2010; Hong et al. 2011a; Hirsch et al. 2011; Li et al. 2010b), hydrothermal vents (Hirsch et al. 2011), and oil reservoirs (Li et al. 2010a). These studies strongly indicated that the *hzo* gene could be a very competitive functional biomarker for anammox bacteria detection because higher resolution for the community structure of anammox bacteria than that by 16S rRNA genes has been achieved (Li et al. 2010b).

Another functional biomarker with application in anammox bacteria detection is the *cd1* nitrite reductase (NIRS)

**Table 2** List of PCR primers for the amplification the 16S rRNA genes of planctomycetes and anammox bacteria and the quantification of anammox bacteria

Primer name	Sequences 5'–3'	Specificity group	Annealing <i>T</i> (°C)	References
Pla46F <sup>a</sup>	GACTTGCATGCCTAATCC	Planctomycetes	58	Neef et al. (1998)
Amx368F <sup>b</sup>	CCTTTCGGGCATTGCGAA	All anammox bacteria	56	Schmid et al. (2003)
Brod 541F	GAGCACGTAGGTGGGTTTGT	Scalindua sp.	60	Penton et al. (2006)
AMX809F <sup>c</sup>	GCCGTAAACGATGGGCACT	Most anammox bacteria	60	Tsushima et al. (2007)
AMX818F <sup>c</sup>	ATGGGCACTMRGTAGAGGGGTTT	Most anammox bacteria	60	Tsushima et al. (2007)
Amx694F <sup>c</sup>	GGGGAGAGTGGAACCTCGG	All anammox bacteria	60	Ni et al. (2010)
Amx820R	AAAACCCCTCTACTTAGTGCCC	Brocadia and Kuenenia	56	Schmid et al. (2000)
BS 820R	TAATTCCTCTACTTAGTGCCC	Scalindua	56	Kuypers et al. (2003)
Brod1260R	GGATTTCGCTCACCTCTCGG	Scalindua sp.	60	Penton et al. (2006)
AMX1066R <sup>c</sup>	AACGTCTCACGACAGAGCTG	Most anammox bacteria	60	Tsushima et al. (2007)
Amx960R <sup>c</sup>	GCTCGACAAGCGGTGGAGC	All anammox bacteria	60	Ni et al. (2010)

<sup>a</sup> Can be used as a forward primer with other reverse primers for PCR amplification of planctomycetes and anammox bacteria

<sup>b</sup> Can be used as a forward primer with other reverse primers for PCR amplification of anammox bacteria with higher specificity

<sup>c</sup> Designed for quantitative real-time PCR

**Table 3** List of PCR primers for the amplification of functional genes of anammox bacteria

Primer name	Sequences 5'–3'	Specificity group	Product size	Annealing T (°C)	References
hzocl1F1	TGYAAGACYTGYCAYTGG	<i>hzo</i>	470	50	Schmid et al. (2008)
hzocl1R2	ACTCCAGATRTGCTGACC				
hzocl1F	TGYAAGACYTGYCAYTGGG	<i>hzo</i>	470	53	Schmid et al. (2008)
hzocl1R2	ACTCCAGATRTGCTGACC				
hzocl2aF	GGTTGYCACACAAGGC	<i>hzo</i>	289	50	Schmid et al. (2008)
hzocl2aR1	TYWACCTGGAACATAACCC				
hzocl2aF1	GGTTGYCACACAAGGC	<i>hzo</i>	525	48	Schmid et al. (2008)
hzocl2aR2	ATATTCACCATGYTTCCAG				
hzocl2aF2	GTTGTGMTGMWTGTCATGG	<i>hzo</i>	838	48	Schmid et al. (2008)
hzocl2aR1	TYWACCTGGAACATAACCC				
Ana-hzo1	ACCTCTTCWGCAGGTGCAT	<i>hzo</i>	1,000	53	Quan et al. (2008)
Ana-hzo2R	ACCTCTTCWGCAGGTGCAT				
hzoF1	TGTGCATGGTCAATTGAAAG	<i>hzo</i>	1,000	53	Li et al. (2010b)
hzoR1	CAACCTCTTCWGCAGGTGCATG				
hzoAB1F	GAAGCNAAGGCNGTAGAAATTATCAC	<i>hzo</i>	1,150	53	Hirsch et al. (2011)
hzoAB1R	CTCTTCNGCAGGTGCATGATG				
hzoAB4F	TTGARTGTGCATGGTCTAWTGAAAAG	<i>hzo</i>	600	55	Hirsch et al. (2010)
hzoAB4R	GCTGACCTGACCARTCAGG				
Scnir372F	TGTAGCCAGCATTGTAGCGT	<i>Scalindua nirS</i>	473	60	Lam et al. (2009)
Scnir845R	TCAAGCCAGACCCATTTGCT				
AnnirS379F	TCTATCGTTGCATCGCATTT	Anammox bacteria <i>nirS</i> (except <i>Scalindua nirS</i> )	442	51	Li et al. (2011b)
AnnirS821R	GGATGGGTCTTGATAAACA				

gene, and a PCR primer set designed for the amplification of *nirS* gene of *Candidatus* “*Scalindua* sp.” is available currently (Lam et al. 2009; Li et al. 2011b)(Table 3). With this primer set, the transcription levels of anammox *nirS* genes in the Peruvian upwelling zone were reported, correlating to activity rather than the presence of anammox bacteria alone in this area (Lam et al. 2009). In addition, results also indicated that these *Scalindua-nirS* genes were fairly diverse, but all clustered with the *nirS* gene present in the *Candidatus* *Scalindua* genome assembly (73%–93% nucleotide sequence identity) and two sequences obtained from the Arabian Sea; however, they were clearly different from the typical denitrifiers' *nirS* genes (<63% sequence identity), indicating that *Scalindua-nirS* can be a candidate functional gene biomarker for anammox in environmental samples (Lam et al. 2009; Li et al. 2011). Recently, a newly designed PCR primer set (AnnirS), together with the PCR primer (ScnirS), have been successfully used to detect anammox bacterial *nirS* genes from marine sediments, and results indicated that the ScnirS primer is specific for *Scalindua nirS* gene while AnnirS primer set is possibly specific for other genera of anammox bacterial *nirS* genes (Li et al. 2011b). The results further confirmed that the anammox bacterial *nirS* gene is a suitable functional

biomarker for investigation of anammox bacteria in the environment (Li et al. 2011b).

For the other two potential functional biomarkers, nitrate reductase and hydrazine hydrolase, no PCR primer is available at this moment due to the limited sequences available, thus further research opportunity is widely open in this direction.

#### Quantitative PCR

In addition to the FISH technique, qPCR, quantifying anammox bacterial 16S DNA or RNA gene copies, is also an effective and suitable method for the estimation of the anammox bacteria. Through the qPCR technique, anammox bacteria can be quantified in wastewater treatment reactors (Bae et al. 2010), marine oxygen minimum zones (Hamersley et al. 2007; Lam et al. 2009), and marine sediments (Li et al. 2010b, 2011a, 2011b; Brandsma et al. 2011). Furthermore, combining with the RT-PCR of the functional genes, such as *hzo* and *Scalindua-nirS*, the activity of anammox bacteria could also be assessed through the qPCR technique (Lam et al. 2009; Park et al. 2010). However, the abundance of anammox bacteria using qPCR technique might not match very well with the results of FISH counts (Brandsma et al. 2011). Reasons



for this mismatch are due to different detection efficiencies with different probes used and primers for the various anammox species, such as detection cells by FISH can be a challenge because of detection limit while the qPCR reaction can be hampered by organic matter in the samples (Lindberg et al. 2007; Kallmeyer and Smith 2009; Brandsma et al. 2011). In spite of this uncertainty, qPCR, estimating anammox bacterial RNA or DNA gene abundance, is still an effective and powerful tool for quantification analysis of anammox bacteria.

## Summary

Three different kinds of methods based on the physiological, biochemical characteristics of anammox bacteria are widely used in detecting anammox bacteria in natural and man-made ecosystems. IPT technique might be best choice for a proper evaluation of the contribution of anammox to the overall nitrogen removal, while the lipids analysis and molecular techniques would contribute more specifically to the presence and abundance of anammox bacteria in a particular habitat. In addition, molecular methods, using the 16S rRNA and functional genes as biomarkers, could further elucidate the anammox bacteria from the phylogenetic diversity to quantitative distribution in various samples. By combining  $^{15}\text{N}$  labeling, FISH, qPCR, RT-PCR, and ladderane lipid and IGP analysis simultaneously, anammox in a specific niche can be analyzed comprehensively and new information on anammox bacteria may be revealed. Therefore, these methods have contributed significantly to our present knowledge on its ecology, distribution, and phylogeny and on the revision of global nitrogen cycle. Future development of these techniques, especially on the most sensitive PCR-based molecular techniques, will certainly yield a more informative picture about anammox bacteria in the natural ecosystem.

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