Bioresource Technology 138 (2013) 285–296

Contents lists available at SciVerse ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Quantitative analyses of the composition and abundance of ammonia-oxidizing archaea and ammonia-oxidizing bacteria in eight full-scale biological wastewater treatment plants

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HIGHLIGHTS

- AOB outnumbered AOA with *amoA* gene ratio varying from 2.56 to 2.41 × 10^3 in 8 WWTPs.
- AOB may play more important role than AOA in the WWTPs studied.
- *Nitrososphaera* cluster might be the worldwide distributed AOA species in WWTPs.
- There may be versatile AOA ecotypes and some AOA may be not obligate autotrophic.
- *Nitrosomonas* genus was the most dominant AOB species and showed higher diversity.

ARTICLE INFO

Article history:
Received 15 January 2013
Received in revised form 23 March 2013
Accepted 27 March 2013
Available online 4 April 2013

Keywords:
Ammonia-oxidizing archaea
Ammonia-oxidizing bacteria
amoA Gene
Wastewater treatment plants

ABSTRACT

This study investigated the diversity and abundance of AOA and AOB *amoA* genes in eight full-scale wastewater treatment plants (WWTPs). Although the process principles and system operations of the eight WWTPs were different, quantitative real-time PCR measurements showed that AOB *amoA* genes outnumbered AOA *amoA* genes with the ratio varying from 2.56 to 2.41 × 10^3, and ammonia may be partially oxidized by AOA. Phylogenetic analyses based on cloning and sequencing showed that *Nitrososphaera* cluster was the most dominant AOA species and might be distributed worldwide, and *Nitrosopumilis* cluster was few. Statistical analysis indicated that there might be versatile AOA ecotypes and some AOA might be not obligate autotrophic. The *Nitrosomonas europaea* cluster and *Nitrosomonas oligotropha* cluster were the two most dominant AOB species, and AOB species showed higher diversity than AOA species.

1. Introduction

Ammonia oxidation to nitrite is the first and rate-limiting step of nitrification in a wide variety of environments, and therefore critical to wastewater nitrogen removal and global nitrogen cycling (Pester et al., 2012). For more than 100 years it was believed that autotrophic ammonia oxidation was solely performed by bacteria, e.g., aerobic ammonia-oxidizing bacteria (AOB) affiliated with the *beta-* and *gamma-proteobacteria* (Purkhold et al., 2000), and anaerobic ammonia-oxidizing bacteria (Anammox) which were identified as members of the *Planctomycetes* (Strous et al., 1999). Based on the analysis of meta-genomic libraries from seawater (Venter et al., 2004) and soil (Treusch et al., 2005) and the cultivation of *Candidatus* Nitrosopumilus maritimus (Könneke et al., 2005), a new group of microorganism named as ammonia-oxidizing archaea (AOA) involved in ammonia oxidation was discovered and confirmed. Initially classified as mesophilic *Crenarchaeota*, comparative genomic has revealed that they form a separate and deep-branching phylum within the Archaea, named as *Thaumarchaeota*, which not only containing all known AOA but also several clusters of environmental sequences (Brochier-Armanet et al., 2008; Pester et al., 2011). More recently, based on all published available ammonia monooxygenase subunit A (*amoA*) genes sequences (status June 2010), AOA were diversified into five major clusters: *Nitrosopumilis* cluster (previously referred to as marine or I.1a AOA lineage), *Nitrososphaera* cluster (soil or I.1b AOA lineage), *Nitrosocaldus* cluster (ThAOA or HWCGIII lineage), *Nitrosotalea* cluster (group I.1a associated) and *Nitrososphaera* sister cluster (newly recognized) (Pester et al., 2012).

The microbial community (especially the microbiology of nitrification) in biological wastewater treatment plants (WWTPs), the world largest biotechnology application, has been intensively investigated based on a diverse set of methods, including terminal restriction fragment length polymorphism (Ye and Zhang, 2011), fluorescence in situ hybridization (Mußmann et al., 2011), PCR, cloning, quantitative real-time PCR (qPCR) (Limpiyakorn et al., 2011) and 454-pyrosequencing (Hu et al., 2012; Zhang et al., 2011). Community analyses based on 16S rRNA and *amoA* genes

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0960-8524/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.biortech.2013.03.176

demonstrated that generally *Nitrosomonas* was the dominant AOB in bioreactors and that *Nitrosospira* occurred only sporadically in these systems (Purkhold et al., 2000; Wells et al., 2009; Zhang et al., 2011). Compared to the studies of AOB, there have been limited studies of AOA in WWTPs. In 2006, AOA were first detected in five out of nine WWTPs in USA (Park et al., 2006). Later, Zhang et al. confirmed the detection of AOA in two Hong Kong WWTPs (Zhang et al., 2009) and three out of eight municipal WWTPs in China and Singapore (Zhang et al., 2011). And in Bai's study, AOA were present in six bioreactors (Bai et al., 2012). It seems that AOA is probably widespread in nitrifying bioreactors. However, in Muβmann's study, AOA were not widespread in the 52 municipal and industrial WWTPs in Europe, they were only abundant in a small number of industrial WWTPs (Mußmann et al., 2011). qPCR measurements of bacterial and archaeal *amoA* genes showed that the abundance of AOB seemed to exceed that of AOA by at least two to four orders of magnitude in some municipal and industrial WWTPs and lab-scale bioreactors (Jin et al., 2010; Limpiyakorn et al., 2011; Wells et al., 2009; Ye and Zhang, 2011). In contrast, significant numbers of archaeal *amoA* genes occurred in municipal WWTPs (Kayee et al., 2011; Limpiyakorn et al., 2011) and four industrial WWTPs (Mußmann et al., 2011). The recent insights into the contribution of AOA and AOB in ammonia oxidation place the abundance, composition and deterministic factors to AOA for nitrogen removal in WWTPs in doubt.

One of the objectives of this study was to compare the diversity and abundance of AOA and AOB in eight full-scale WWTPs. Another objective was to investigate the differences of the diversity and abundance of AOA and AOB among aerobic, anoxic and anaerobic tank in two out of eight WWTPs. EvaGreen based qPCR systems were used to quantify the abundance of AOA and AOB *amoA* genes. AOA and AOB community compositions were examined by cloning and sequencing their *amoA* gene clone libraries. Multivariate statistical tools were used to further elucidate the potential impact and contribution of operational parameters on the AOA and AOB community structure and abundance.

2. Methods

2.1. Description of WWTPs and sample collection

Eight full-scale WWTPs in Beijing were investigated in which nitrification was active. Relevant parameters and description about these WWTPs are summarized in Table 1. Orbal oxidation ditch process is applied in plant NKY and HC. Modified Carrousel process is applied in plant JXQ where brush aerator, not surface impeller, is equipped. Carrousel® 2000 process is used in plant CP and Carrousel® 3000 process is employed in plant YF and SY. Plant FZ operates with an anaerobic/anoxic/aerobic process (A^2O), and cilium nutrient removal technology (CNR) is used in aerobic tank to enhance nitrogen removal (A^2O + CNR). Plant BXH is equipped with an University of Cape Town (UCT) process plus Membrane BioReactor (MBR) for sludge-water separation (UCT + MBR). In Plant FZ and BXH, activated sludge samples were collected at aerobic, anoxic and anaerobic tank to compare the difference of abundance and composition of AOA and AOB. For oxidation ditch processes, the samples were collected from the outer channel where DO concentration was low. All together, there were 12 samples from eight WWTPs. After the sludge samples were collected, they were immediately kept on ice and transferred to laboratory as soon as possible, and were stored at −80 °C until DNA extraction.

2.2. DNA extraction, PCR, cloning and sequencing

0.05–0.10 g of dried sludge sample pellet was used for DNA extraction with a FastDNA SPIN kit for soil (Qiagen, CA, USA) according to the manufacturer's instructions. DNA extracted was stored at −20 °C. The concentration of extracted DNA was measured by Nanodrop Spectrophotometer ND-1000 (Thermo Fisher Scientific, USA).

Primer set amoA-1F (5′-GGGGTTTCTACTGGTGGT-3′) and amoA-2R (5′-CCCCTCKGSAAAGCCTTCTTC-3′) was used to amplify the 491-bp fragment of the *beta-proteobacteria amoA* gene. The PCR amplification was performed in a 50 μL mixture comprising 25 μL GoTaq® Green Master Mix (Promega, Madison, USA), 0.2 μM of each primer and 50–100 ng of genomic DNA. The thermo cycling steps used for PCR amplification followed the protocol of Purkhold (Purkhold et al., 2000): an initial denaturation step at 94 °C for 30 s, followed by 30 cycles of denaturation at 94 °C for 15 s, annealing at 55 °C for 20 s, and elongation at 72 °C for 40 s. Cycling was completed by a final elongation step at 72 °C for 1 min.

Primer set Arch-amoAF (5′-STAATGGTCTGGCTTAGACG-3′) and Arch-amoAR (5′-GCGGCCATCCATCTGTATGT-3′) (Francis et al., 2005) was used to amplify the 635-bp AOA *amoA* gene. PCR amplification was performed with the same reaction mixture used for the AOB *amoA* genes, except the specific primers for AOA *amoA* genes. The PCR protocol included a 5 min initial denaturation at 95 °C, followed by 30 cycles of 94 °C for 45 s, 53 °C for 60 s and

Table 1
Influent and effluent characteristics, operational parameters of eight full-scale WWTPs.

Plant	Process	Flow rate (10^3 m^3 d^{-1})	Influent (mg l^{-1})				Effluent (mg l^{-1})				MLSS (mg l^{-1})	SRT (d)	HRT (h)	DO (mg l^{-1})
			BOD_5	COD	NH_4-N	SS	BOD_5	COD	NH_4-N	SS				
NKY	Orbal	22	268	418	14	57	8	41	0	11	3185	30	20	0.4
HC	Orbal	70	150	226	58	160	15	52	11	18	4040	12	9	0.3
JXQ	Modified carrousel	212	255	561	52	226	6	47	3	12	5123	20	10	0.5
YF	Carrousel 3000	20	101	154	25	215	4	49	2	10	5022	15	10	0.3
CP	Carrousel 2000	54	273	379	49	196	18	55	3	13	3025	20	19	0.3
SY	Carrousel 2000	31	152	221	51	287	3	36	1	12	4097	14	12	0.2
FZ	A^2O + CNR	45	267	416	56	304	5	33	0	14	3106[a], 3250[b], 2912[c]	16	5[a], 3[b], 1[c]	3.5[a], 0.4[b], 0.1[c]
BXH	UCT + MBR	83	316	603	45	341	3	21	1	5	8657[a], 6851[b], 3925[c]	17	7[a], 5[b], 2[c]	1.9[a], 0.2[b], 0.0[c]

Abbreviations: NKY: Niu Kou Yu; HC: Huang Cun; JXQ: Jiu Xian Qiao; YF: Yong Feng; CP: Chang Ping; SY: Shun Yi; FZ: Fang Zhuang; BXH: Bei Xiao He; BOD_5: 5-day 20 °C biochemical oxygen demand; COD: chemical oxygen demand; SS: suspend solid; MLSS: mixed liquor suspended solids; SRT: sludge retention time; HRT: hydraulic retention time.

[a] Activated sludge in aerobic tank.
[b] Activated sludge in anoxic tank.
[c] Activated sludge in anaerobic tank.