International Scholars Journals

African Journal of Ecology and Ecosystems ISSN 2756-3367 Vol. 8 (3), pp. 001-006, March, 2021. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

Comparison diversity and distribution of ammonia-oxidizing bacteria in five different water treatment processes

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Accepted 05 November, 2020

A better understanding of the biology and ecology of the microbial populations in biological reactor systems is the key to developing and optimizing efficient and economic reactor systems. In the study, we have analyzed the diversity and community components of ammonia oxidizing bacteria (AOB) in five different water treatment processes. All results indicated that the diversity and abundance of AOB were influenced by distinct influent characteristics and system operation. The phylogenetic analysis suggested that the distribution pattern of AOB among different processes showed that genetic types of *amo*A could separate into three which were municipal wastewater treatment pattern, food industrial wastewater treatment pattern and raw water treatment pattern.

Key words: Ammonia oxidizing bacteria, ammonia monooxygenase gene, activated sludge, granular activated carbon, water treatment processes.

INTRODUCTION

Eutrophication was recognized as a pollution problem all over the world (ILEC/Lake Biwa Research Institute, 1988-1993). It is commonly caused by human activities such as the release of sewage, urban storm water run-off, and releasing excess fertilizers into natural waters. On the other hand, water quality deterioration of surface water is also one of major problem of drinking water treatment processes (Li and Chu, 2003). Nitrification is an important process in global nitrogen cycling, in controlling effluent toxicity, in wastewater treatment and is also a critical step in many wastewater treatments. Likewise, nitrification is also important for reducing toxicity in drinking water. Until recently, biological treatment processes of sewage and drinking water are very common. This process involves two groups of microorganisms, one is an ammonia oxidizing organism; the other is nitrate oxidizing organism. Ammonia-oxidizing bacteria (AOB), which are known as principal ammonia oxidizer, are primarily responsible for the first step in nitrification (Wei et al., 2011).

Consequently, abundance and physiological activity of nitrifying bacteria in wastewater treatment reactors are considered the rate-limiting parameters for the biocon-version of nitrogen in sewage (Wagner and Loy, 2002), as well as in drinking water treatment reactors. Activated sludge systems and biofilm on granular activated carbon are capable of nitrification in WWTP (wastewater treatment plant) and drinking water treatment plant res-pectively. Some previous studies have documented that one of AOB genus "*Nitrosomonas*" dominates wastewater treatment systems (Dionisi et al., 2002; Layton et al., 2005). As well, *Nitrosomonas oligotropha* lineage dominates in drinking water treatment biofilm reactor and distribution system (Qin et al., 2007; Regan et al., 2003). Because of the slow growth rates and poor yields of the

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Abbreviations: AOB, Ammonia oxidizing bacteria; **SBR,** sequencing batch reactor; **GAC,** granular activated carbon; **WWTP,** wastewater treatment plant; **SRT,** sludge retention time; **COD,** chemical oxygen demand; **COM,** chemical oxygen material.

organisms involved, nitrification is generally regarded as the rate limiting step of ammonia nitrogen removal efficiencies in the nitrogen removal process (Coelho et al., 2000). Engineers continuously search for the most efficient, optimal, and stable way to maintain the populations and biological activities of nitrification. Consequently, the better understanding of ecology of the microbial populations in full-scale wastewater treatment bioreactors is the key of developing and optimizing efficient and economic reactor systems (Whang et al., 2009). However, the description of AOB community structure and diversity comparison among different water treatment processes, as well as potable water treatment process, in more detail, is still little.

METHODOLOGY

In the current study, measures of AOB presence other than just *amo*A-based assays, such as DNA sequencing and real-time PCR assay, were used. We collected samples from sewage and drinking water treatment processes. Municipal WWTPs and industry WWTPs were also considered. The goal of this study is to provide a better understanding of the linkage between dominant AOB communities and differences in system operation, influent characteristics in full-scale processes bioreactors. Here we present the results of AOB communities' diversities and community's distribution in five water treatment processes.

RESULTS AND DISCUSSION

Abundance of AOB in different processes

We chose 6 kinds of biological nitrogen removal processes for water treatment which were oxidation ditch, A2/O, sequencing batch reactor (SBR), contact oxidation, suspended plastic media and granular activated carbon filter. Two WWTPs, which are treating mainly municipal wastewater in Xianmen, employ oxidation ditch process and A2/O process respectively. The average flow capacities of two municipal WWTPs are 45,000 m^3/day for WWTP-A and 60,000 m^3/day for WWTP-B. SBR process are employed for treating soybean and slaughter sewage by a food producer company in Xiamen China, with an average flow capacity of 1,800 m^3/day. Contact oxidation process samples were taken from a brewery company in Xiamen. Suspended plastic media and GAC filter samples were taken from a drinking water plant in south China, which employs suspended plastic media pretreatment for partial removal of NH_4^+-N from raw water and GAC filter for further removal of COM and NH_4^+-N. The water flow capacity of the plant was 40,000 m^3/day. All plants were going properly for many years when we sampled. All of them could remove NH_4^+-N effectively and water could discharge under standard (Table 1). The parameters and diversity index of all treatment processes were also present in Table 1. The Sludge Retention Time (SRT) of SBR system was much longer than other sewage treatment processes, and the removal efficiencies of ammonia nitrogen had no significant difference among the processes except the contact oxidation process. The total cost of SBR and suspended plastic media processes were higher than others probably because of the higher power consumption.

Ammonia-oxidizing bacteria in samples were quantified in DNA extracts using real-time PCR methods targeting the *amo*A gene. DNA of biofilm attached on suspended plastic media and GAC samples was extracted following patented methods (number: ZL200910258259 China) which was designed by our group. DNA of activated sludge samples was extracted directly from samples using OMIGA soil DNA extraction kit (OMIGA^{TM}). The total numbers of ammonia-oxidizing bacteria varied among the samples studied. The *amo*A gene copy numbers in treatments suspended plastic media process (sample ID: TL) were significantly higher than other processes, which approximated to 3.32×10^{13} copies/L. No significant differences were found in the inner treatments process (Figure 1). In soybean SBR process (sample ID: XL2), *amo*A number approximated to 1.63×10^{11} copies/L. In slaughter SBR process (sample ID: XL1), the copy number was a little less than in soybean sample. In oxidation ditch and A2/O processes, *amo*A copy numbers in aerobic stages were both about twice as much as anaerobic stages. The lowest concentration of *amo*A was in the anaerobic stage of A2/O process.

Interestingly, the concentration of *amo*A in GAC filter was so high that it could compare with oxidation ditch process or SBR process. There was abundant *amo*A gene copy about 1.74×10^{11} copies/L. Harms et al. (2003) found that the average *N. oligotropha*-like AOB abundance were about $7.5(\pm6.0)\times10^{9}$ copies/L in single stage reactors of a municipal WWTP by using *amo*A probes, and it was very stable during the year. In another study, researchers considered that stable concentrations of AOB's 16S rRNA genes was $1.37(\pm1.56)\times10^{11}$ /L in the activated sludge in an industrial WWTP as determined by real-time PCR assays (Layton et al., 2005). The range of abundance of our results were from 10^8 to 10^{13} copies/L in different processes. It is clearly that our results were comparable with previous researches. Assuming that one cell of AOB has 2 copies of the *amo*A gene (Klappenbach et al., 2001) the range number of AOB measured in the samples were from 10^7 to 10^{13} cells/L. The result of AOB on suspended plastic media treatment tank was much higher than others. It was probably because of the high destiny of plastic media in the tank, and the process was designed to remove ammonia; so the AOB could be the major component of the bacterial community in the suspended plastic media process. The cost of suspended plastic media process was the highest of all, due to the process needs of higher oxygen content (Table 1). It reveals that higher oxygen content may also cause the higher number of AOB. The number of AOB in GAC filter

Table 1. Description of all sampled treatment processes.

Sample ID	JW1-JW3	XL1-XL3	YX1-YX2	QP	TL	D5-D9
Process types	Oxidation ditch	A2/O	SBR	Contact oxidation	Suspended Plastic Media	GACfilter
SRT /d	14. 9	16.7	32. 0	15. 5	-	-
Removal efficiencies of ammonia nitrogen	93.8%	90.6%	93.6%	75. 8%	98. 3%	90. 2%
Removal efficiencies of COD	91.2%	86.6%	87.6%	84.0%	26. 6%	-
Total cost (China Yuan)	0.85	0.82	1.23	0.65	1.22	0.73
Shanon diversity index of AOB community	1.46179	2.28277	1.1709	0.467652	1.1205	1.07463

All the samples were collected before and after December 25th, 2010 and the collected volume of each sample was 1 L. The profile of GAC samples contained 0, -10, -20, -40 and -60 cm layers. Samples ID were D5, D6, D7, D8 and D9 respectively. JW1, JW2 and JW3 were collected in oxidation ditch process aerobic stage, anaerobic stage and anoxic phase respectively. Meanwhile, sample ID of three stages (aerobic, anoxic and anaerobic) of A2/O process were XL1, XL2 and XL3. YX1 was from soybean reactor and YX2 was from slaughter reactor. All the system operation parameters were collected from the operation records of the plants. Shanon diversity index was performed by using DOTUR (Schloss and Handelsman, 2005) from the results of the *amo*A gene DNA cloning libraries of each samples.

suggested that in an efficient drinking water treating filter, the concentration of AOB was compared with which in the activated sludge, despite the influence NH_4^+ was lower.

It also revealed that the AOB may increase the copy of *amo*A gene in each cell when the concentration of NH_4^+ in the surrounding was lower. The removal efficiencies of ammonia nitrogen of all processes have no significant difference, but the AOB abundances are difference. It suggested there is no significant correlation between the concentration of AOB and removal efficiencies of ammonia nitrogen in different processes. The AOB abundances in SBR process system are significant higher than AOB in other sewage treatment system (Figure 1). Considering the sludge retention time of SBR process is much longer than other processes (Table 1), it reveals that the SRT will influence the abundance of AOB in the activated sludge. The previous study also considered that the system operation will influence the abundance of AOB in the activated sludge (Limpiyakorn et al., 2005). The AOB communities on the solid media, such as suspended plastic media and GAC have much longer retention time than other samples. Similarly, the AOB abundances are higher in these samples than other samples (Figure 1).

Diversity and community structure analysis of AOB

To measure the diversity and to compare the community structures of AOB in all samples, fifteen *amo*A gene DNA cloning libraries were constructed by using purified PCR products. Triplicate PCR products were mixed for minimizing PCR bias and cloned with the pMD18-T cloning kit (TaKaRa^{TM}). More than thirty clones from each library were randomly selected and the recombinant plasmid DNA was extracted and sequenced on ABI 3730 automated sequencer (Applied Biosystems). The *amo*A sequences of bacterial achieved in this study have been submitted to the GenBank database under accession numbers GU248715 to GU249150.

For AOB diversity comparison, the Shanon diversity index of all fifteen cloning libraries were calculated by using DOTUR program (Schloss and Handelsman, 2005) (Table 1). It was clear that AOB had higher diversity in activated sludge than on solid carriers. The highest diversity of AOB was in aerobic stage of A2/O process. It was also clear that plants which treat municipal wastewater had higher AOB diversity than others. To determine the phylogenetic structures of AOB in those processes, all 436 sequences of 15 cloning libraries were clustered by DOTUR based on 5% nucleotide acid difference. Then 30 OTUs were gained for constructing phylogenetic tree (Figure 2). The AOB community structures in municipal treating activated sludge were also similar with each other. AOB attached on solid media always had a lower diversity but a higher abundance. The diversity of AOB in two SBR tanks were almost the same, though the influent sewage are totally different. Further-more, they had similar AOB community structures too. Other diversity indices, as well as, Chao1 and ACE richness estimators also indicated that A2/O process had the highest AOB richness and contact oxidation process had the lowest. Diversities of AOB in GAC filter were also lower, the highest was on a 60 cm layer and the lowest was on a 20 cm layer respectively. It might have been caused by the detachment of AOB from upper layers of the filter. Diversity of AOB on suspending plastic media was similar to SBR. Interestingly, it was contrary to the abundance of AOB. Which samples had higher AOB abundance, the diversity of AOB in the samples were lower. It revealed that longer retention time probably made the low diversity of AOB community in the process system.

Diversities of AOB in different process types were different, as well as the community structures. All *amo*A cloning libraries from the same process could be grouped

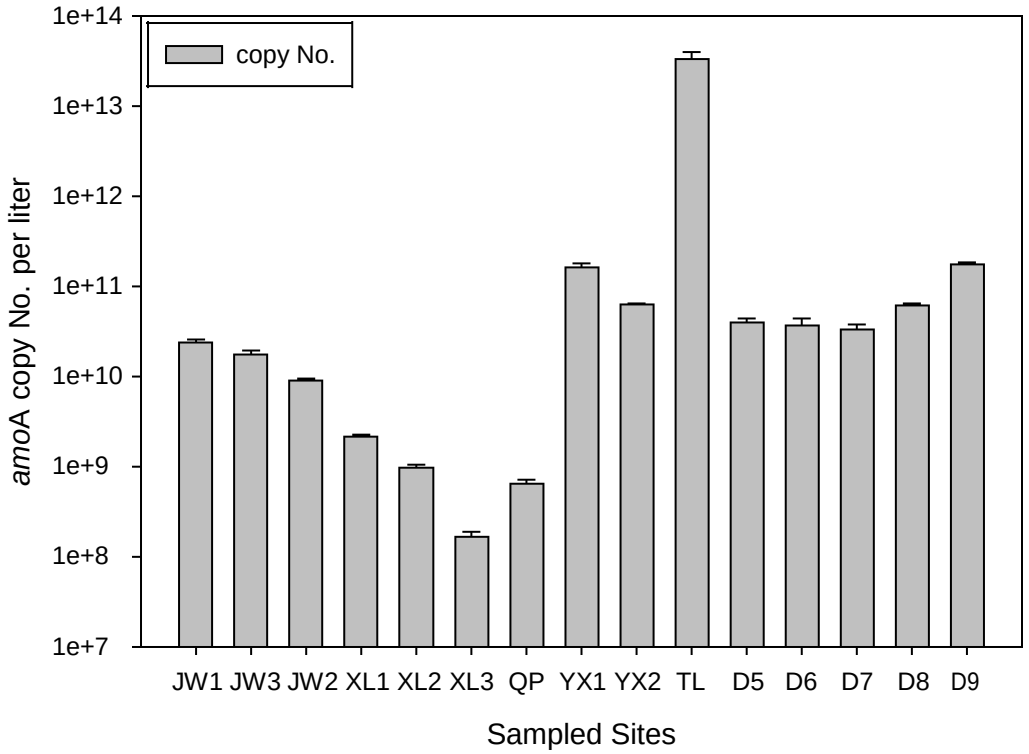

Figure 1. Abundance of bacterial *amo*A genes per liter mixture of fifteen samples. Bacterial *amo*A primer pair *amo*A1F and *amo*A2R (Urakawa et al., 2006) were used for bacterial *amo*A gene real-time PCR assays. The quantity was based on the SYBR-Green I dye method, which can bind to double-stranded DNA during PCR reaction as the previous study (Urakawa et al., 2006). The R^2 of standard curves was >0.999.

together. Other researchers had found similar results in the nitrifying biofilm (Schramm et al., 2000). All results indicated that the community structures of AOB were different among different water treatment processes. It indicated that the influent quality and the treatment processes both influence the AOB diversity. This finding corresponded to several previous studies (Limpiyakorn et al., 2005; Qin et al., 2007). All *amoA* OTUs could be clustered into 4 mainly clades as shown in Figure 2. Almost all reference sequences similar to sequences of the present study were from various soil environments. Clade *Nitrosomonas europaea* was the biggest branch and contained half number of the OTUs. It also contained the most *amo*A clones which was 204 clones of 436. It was a little different from that of Park et al. (2002), compared with Hallin et al. (2005). Some previous studies suggested that *N. europaea* always dominated the environments with high ammonia concentration (Otawa et al., 2006). The subgroup, which contained OTU1 to OTU7, was a widespread group. Especially OTU4 was almost present in every process but in SBR. OTUs 8 to 15 which belonged to the other subgroup contained *N. europaea* and *N. eutropha* as reference sequences of the clade were mainly present in SBR and A2/O processes. The reference sequence gb|ACM62204.1| was from an ANNAMOX reactor. The most similar OTU of our results was OTU9 which was from two SBR libraries. It suggested that AOB in activated sludge of SBR and ANNAMOX might share the same OTUs and the communities in SBR activated sludge were totally different from the municipal WWTP.

The second clade was an unknown clade because no known sequences were homologous from these two OTUs (OTU16 and 17). They were both unique and retrieved from A2/O and GAC filter respectively. Eight OTUs belonged to the third clade, *Nitrosomonas urea* clade, which contained 184 clones. OTU18 in the clade was the largest OTU which contained 116 clones of all, and was also a widespread OTU. OTU23 and OTU24, which were in the subgroup of *N. urea*, were only recovered from WWTPs treating municipal wastewater. Because some previous studies considered that *N. urea* clade was often dominant where there is low ammonia concentration (Kelly et al., 2005; Otawa et al., 2006). The remaining OTUs were clustered into another unknown clade which had a reference sequence that was retrieved from soil environment by PCR amplification. These OTUs were also merely found in WWTP samples, except OTU30 which was also amplified from GAC filter. The NH_4-N concentrations in those environments were lower than in food industrial wastewater treatment reactors in this study. Therefore, the unknown clade was also

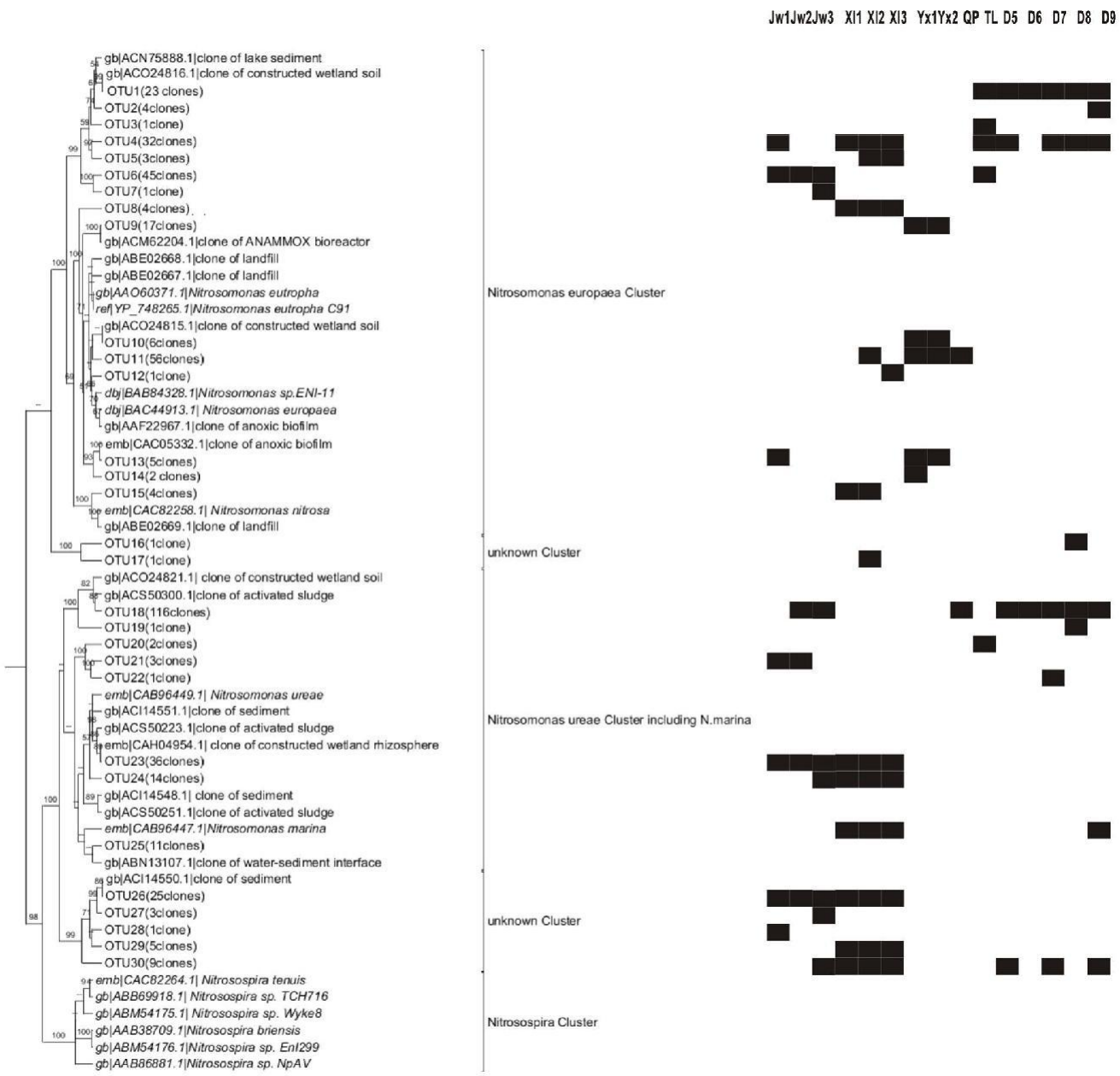

Figure 2. Phylogenetic and distribution analysis of bacterial *amo*A. Trees were constructed by Bayesian methods. Topologies of trees were supported by more than 50% MCMC posterior probability. Relevant phylogenetic sequences were extracted from GenBank by us ing Unifrac (Lozupone et al., 2006) programs. Nucleotide alignments were generated by using clastalx (Thompson et al., 1997) and checked manually. Corrected distances for nucleotide and amino acid were chosen by employing Modeltest and Proftest (Abascal et al., 2005) programs respectively. Sequences obtained in this study are indicated by bold type font. Scale bars 0.1% estimated sequence divergence. Colored bulks meant that the OTU was present in the environments which it corresponding.

suitable for low ammonia concentration environments.

The distribution pattern of AOB among different processes showed that genetic types of amoA could separate into three, which were municipal wastewater treatment pattern, food industrial wastewater treatment pattern and raw water treatment pattern. In some previous studies, the phylogenetic relationships of AOB in WWTP were different (Wagner and Loy, 2002; Layton et al., 2005; Sanapareddy et al., 2009), as well as the present study. It has been already proven that solids retention time (SRT) (Layton et al., 2005), NH_4-N (Francis et al., 2007), and season (Daims et al., 2001) could influence the species of AOB in water treatment processes. However, our investigation revealed that

the influent quality could influence the community composition of AOB (Figure 2). We had not recovered members of the *N. oligotropha* cluster from all samples, and they comprised the majority of the bands analyzed. Although the *N. oligotropha* lineage were often recovered from oligotrophic environments, including drinking water distribution systems (Lipponen et al., 2004), freshwater (C´ebron et al., 2003) and wastewater treatment systems with low-ammonia influents (Limpiyakorn et al., 2005).

In the present study, we mainly discussed the ammonia oxidizing bacteria found in activated sludge of sewage treatment systems and drinking water treatment systems. We noted the effects of influent characteristics, system operation and ammonia concentration on the AOB numbers and the AOB communities. We showed that the ammonia oxidizing bacteria observed in these systems differ and are influenced by distinct influent characteristics and system operation. However, the removal efficiencies of ammonia are different while the AOB numbers and the AOB communities are different in those systems. Further studies are necessary to develop and to better clarify the roles of these ammonia oxidizers in sewage treatment systems and drinking water treatment systems environments.

ACKNOWLEDGEMENTS

We thank Ye Zhilong and Lei Xiaojin for preparing samples. The research was supported by technological Consultation project of ademician of china engineering academy "The study of pollution prevention and Ecological remediation approach of Typical Estuary and marine in the economic zone on the western coast of the Taiwan strait.

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