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Nitrifying bacterial community structures and their nitrification performance under sufficient and limited inorganic carbon conditions

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Abstract This study examined the hypothesis that different inorganic carbon (IC) conditions enrich different ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) populations by operating two laboratory-scale continuous-flow bioreactors fed with 15 and 100 mg IC/L, respectively. During this study, both bioreactors maintained satisfactory nitrification performance and stably oxidized 250 mgN/L of influent ammonium without nitrite accumulation. Based on results of cloning/sequencing and terminal restriction fragment length polymorphism targeting on the ammonia monooxygenase subunit A (*amoA*) gene, *Nitrosomonas nitrosa* lineage was identified as the dominant AOB population in the high-IC bioreactor, while *Nitrosomonas europaea* and *Nitrosomonas nitrosa* lineage AOB were dominant in the low-IC bioreactor. Results of real-time polymerase chain reactions for *Nitrobacter* and *Nitrospira* 16S rRNA genes indicated that *Nitrospira* was the predominant NOB population in the high-IC bioreactor, while *Nitrobacter* was the dominant NOB in the low-IC bioreactor. Furthermore, batch experiment results suggest that *N. europaea* and *Nitrobacter* populations are proliferated in the low-IC bioreactor due to their higher rates under low IC conditions despite the fact that these two populations have been identified as weak competitors, compared with *N. nitrosa* and *Nitrospira*, under low ammonium/nitrite environments. This study revealed that in addition to ammonium/nitrite concentrations, limited IC conditions may also be important in selecting dominant AOB/NOB communities of nitrifying bioreactors.

Keywords Inorganic carbon limitation · Ammonia-oxidizing bacteria (AOB) · Nitrite-oxidizing bacteria (NOB) · Nitrification

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Introduction

Nitrogen removal from wastewater is extremely important to the protection of water resources from pollution discharges as release of untreated wastewater can result in eutrophication of the receiving water bodies. Nitrification, an efficient nitrogen control strategy for wastewater treatment, is a series of microbial oxidation processes performed

by nitrifiers, namely, two different bacterial groups involving ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB). AOB is responsible for the oxidization of ammonia to nitrite and NOB converts nitrite to nitrate. The distribution patterns of distinct AOB/NOB species in the environments reflect the physiological properties of AOB/NOB. Among these, ammonium and nitrite concentrations are considered to be important factors for the selection of distinct AOB and NOB species, respectively. According to their physiological properties on ammonium affinity, members of *Nitrosospira* spp. and/or *Nitrosomonas oligotropha* clusters are the prevailing AOB in the environment with low ammonium, whereas *Nitrosomonas europaea* cluster is dominant in the environment that is rich in ammonium (Koops et al. 1991; Koops and Pommerening-Roser 2001; Siripong and Rittmann 2007). With respect to NOB, *Nitrobacter* species represent r-strategists with a low nitrite affinity and develop large populations at a high nitrite environment, while *Nitrospira* species, as K-strategists, have a high nitrite affinity and adapt to low nitrite concentrations (Schramm et al. 1999; Gieseke et al. 2003; Siripong and Rittmann 2007; Huang et al. 2010). In early studies, *Nitrosomonas* and *Nitrobacter* are considered to be the most important nitrifying bacteria responsible for ammonium and nitrite oxidation, respectively. However, recent observations, based on tools and analyses at a molecular level, indicate that *Nitrosospira* also are present along with well-known *Nitrosomonas* (Schramm et al. 1998; Schramm et al. 1999), and *Nitrospira* often are the dominant NOB in activated sludge systems (Schramm et al. 1998; Schramm et al. 1999; Daims et al. 2001; Dionisi et al. 2002). Although a number of studies have investigated nitrifying bacteria populations in wastewater treatment processes (Daims et al. 2001; Dionisi et al. 2002; Limpiyakorn et al. 2005; Siripong and Rittmann 2007; Whang et al. 2009; Huang et al. 2010), our understanding of linking their presence to nitrification performance at a full-scale level is still limited (Koops et al. 2006).

Previous studies have demonstrated that nitrification is feasible in full-scale wastewater treatment processes even at high ammonia levels, but nitrification failure has been frequently observed since nitrifiers can be inhibited by several environmental and engineering factors. These include low temperature, extreme pH, low dissolved oxygen concentrations, and a wide variety of chemical inhibitors (Prosser 1989). In addition to the negative effects mentioned earlier, the importance of alkalinity (Biesterfeld et al. 2003) and inorganic carbon (IC) limitations (Guisasola et al. 2007; Wett and Rauch 2003) on nitrification has been discussed. IC limitations are linked to the alkalinity requirements for buffering the acidification due to the nitrification process (Biesterfeld et al. 2003; Guisasola et al. 2007; US EPA 1993) and for cellular synthesis and growth of nitrifying bacteria (US EPA 1993). The stoichiometric relations show that per mole of ammonia removed, a substantial amount of alkalinity is consumed by the production of hydrogen ions. The accepted stoichiometric ratio for alkalinity destruction is 7.07 g of alkalinity consumed per gram of ammonia (NH_3–N) oxidized to nitrate (NO_3^-–N) in a closed system (US EPA 1993). Most nitrifying bacteria are autotrophic, and insufficient IC could result in a decrease of the nitrification performance. It has been demonstrated that carbon limitation is the main cause for the dramatic decrease in growth and activity of nitrifying bacteria below neutrality (Wett and Rauch 2003). Yet, IC limitations are often ignored due presumably to its abundance in full-scale systems generated during the biological oxidation of organic matters. Recently, wastewaters with the characteristic of low organic carbon to nitrogen ratio such as rejection waters of digested sludges have been studied for the development of optimized nitrogen removal systems (Carrera et al. 2003; Fux et al. 2002; Janus and van der Roest 1997; Lai et al. 2004; Wett and Rauch 2003), but little is known regarding the effects of IC limitation on nitrifying communities in such systems. Our previous study has observed that the addition of different buffering chemicals, NaOH or Na_2CO_3, in a bioreactor treating petrochemical wastewater may influence AOB communities (Whang et al. 2009). It is reasonable to speculate that the response to IC concentration among nitrifying populations is different, and such information is important to develop and operate a stable nitrogen removal system for wastewaters with a low carbon-to-nitrogen ratio characteristic.

This study was motivated with the goal of better understanding the influence of IC conditions on AOB and NOB community structures. Two laboratory-scale continuous-flow bioreactors were operated under IC-sufficient (high IC) and IC-limited (low IC) conditions for more than 700 days, and AOB/NOB community structures in the two bioreactors were investigated using terminal restriction fragment length polymorphism (T-RFLP) and real-time polymerase chain reaction (real-time PCR). In accordance with our previous study (Whang et al. 2009), Na_2CO_3 was added to adjust IC concentration. T-RFLP analysis for monitoring of AOB community changes has been well established with a reasonably good database for fingerprint analysis (Park and Noguera 2004). On the other hand, limitations of T-RFLP analysis for NOB community have been pointed out in a previous study (Regan et al. 2002). Therefore, real-time PCR analyses with designed primer sets were applied for NOB community. Finally, batch experiments were also performed to evaluate the dependence of nitrification kinetics on ammonium, nitrite, and IC concentrations for dominant AOB/NOB communities.

Materials and methods

Bioreactor operation

Two 5-L lab-scale continuous-flow bioreactors were operated to evaluate the nitrification performance and microbial communities of AOB and NOB acclimated under IC-sufficient (high IC) and IC-limited (low IC) conditions. For each bioreactor, 2 L of seed microorganisms was inoculated on day 1. The seed microorganisms for the bioreactors were obtained from a full-scale petrochemical wastewater bioreactor with a combining treatment mechanism of powder-activated carbon (PAC) adsorption and biodegradation by biofilm growth onto PAC (Whang et al. 2009). A total of 15 and 100 mg IC/L of sodium carbonate (Na_2CO_3) was applied to simulate low- and high-IC conditions, respectively. Ammonium chloride (NH_4Cl) was added to maintain an influent concentration of 250 mgN/L for both bioreactors. The concentrations of other nutrients were identical to the ATCC 1573 medium (Park and Noguera 2004). The influent flow rate for both bioreactors was set at 0.5 L/day to maintain a 10-day hydraulic retention time. The pH was maintained at around 7 by phosphate buffer in the media. A DO concentration above 2 mg/L was maintained through aeration using diffusers. In order to minimize the introduction of IC in bioreactors through aeration, CO_2 in the air was absorbed by 1 M NaOH solution before aeration. The bioreactor was mixed using a magnetic stirrer to avoid settling. The temperature was initially maintained at room temperature without additional control, but it was maintained at 30 °C using a thermal tape system after 190 days of operation. Samples were collected from the bioreactors twice a week for chemical analyses and every other week for microbial community analyses.

DNA extraction

Mixed liquor samples for DNA extraction were taken from the bioreactor and stored at −20 °C until DNA extraction. DNA was extracted using an UltraClean Soil DNA Isolation Kit (Mo Bio Laboratories, Solana Beach, CA, USA) in accordance with the manufacturer's instructions.

Cloning and sequencing

Primers amoA-1 F and amoA-2R (Rotthauwe et al. 1997) were used to amplify a 491-bp fragment of the bacterial *amoA* gene. Primers 8 F and 1492R (Lane 1991) were used to amplify around 1,500 bp fragment of the bacterial 16S rRNA gene. Taq DNA Polymerase Master Mix RED (Ampliqon, Copenhagen, Denmark) was used. Cycling conditions for *amoA* gene included a pre-incubation step of 10 min at 95 °C, followed by 35 cycles of 95 °C for 1 min, 60 °C for 1.5 min, and 72 °C for 1.5 min, and followed by 72 °C for 10 min. Cycling conditions for bacterial 16S rRNA gene included a pre-incubation step of 5 min at 94 °C, followed by 30 cycles of 94 °C for 1 min, 55 °C for 30 s, and 72 °C for 1.5 min, and followed by 72 °C for 10 min. PCR products were ligated to the pGEM-T Easy Vector (Promega, Madison, WI, USA) and transformed into DH5α cells. Clones were randomly selected and re-amplified with amoA-1 F/amoA-2R (for *amoA* gene) or M13F/M13R (for 16S rRNA gene) primer sets. PCR products were digested with *HhaI* and *HpaII* restriction endonucleases. The resulting fragments were analyzed on ethidium bromide-stained agarose gels. Clones were resolved into operational taxonomic units (OTUs) based on restriction fragment length polymorphism (RFLP) patterns.

Plasmids of selected clones were extracted by the Wizard® Plus minipreps DNA purification system (Promega). DNA sequencing reactions were performed using ABI Prism 3100 and 3730 genetic analyzer (Perkin-Elmer Corp., Wellesley, MA, USA). Sequence homology searches were performed at the NCBI GenBank database using the BLAST algorithm (Altschul et al. 1997). A phylogenetic tree was constructed using Molecular Evolutionary Genetics Analysis software version 4.0.2 (Tamura et al. 2007). An unrooted phylogenetic tree was generated using the neighbor-joining method, and bootstrap tests were performed with 1,000 replicates.

Terminal restriction fragment length polymorphism analysis

The 5′ ends of amoA-1 F and amoA-2R were labeled with 6-carboxyfluorescein (6-FAM) and tetrachlorofluorescein (TET), respectively (Park and Noguera 2004). PCR products were purified using a PCR product purification kit (Montage PCR Centrifugal Filter Devices, Millipore) and digested with *TaqI* restriction endonuclease for 2 h at 65 °C. Digested samples were analyzed on ABI Prism 377 genetic analyzer with GeneScan version 3.1 (Perkin-Elmer Corp.). The relative abundance of each terminal restriction fragment (T-RF) was determined by calculating the ratio between the height of each peak and the summed height from all peaks in one sample. The peaks with relative abundance of <1 % or smaller T-RF length (<50 bp) were neglected in this study due to high background noise.

Real-time polymerase chain reaction

A LightCycler 2.0 system (Roche Diagnostics, Mannheim, Germany) was employed for the real-time PCR to quantify *Nitrospira* and *Nitrobacter* 16S rRNA genes and bacterial and archaeal *amoA* genes. Primers EUB338f and Ntspa0685 M were used to quantify the *Nitrospira* 16S rRNA gene (Regan et al. 2002). Primers EUB338f and NIT3 were used

for the *Nitrobacter* 16S rRNA gene (Regan et al. 2002). The PCR mixture consisted of 10 μl of 2× SYBR® Premix Ex Taq™ (Perfect Real Time; TaKaRa Bio, Otsu, Japan), 0.2 (*Nitrospira*) or 0.1 (*Nitrobacter*) μM of each primer, and template DNA in a final volume of 20 μl. Cycling conditions for the *Nitrospira* 16S rRNA gene were as follows: 95 °C for 20 s , 40 cycles consisting of 95 °C for 10 s, 58 °C for 20 s, and 72 °C for 20 s (detection), followed by the melting curve analysis (65 to 95 °C with a heating rate of 0.1 °C/s). Cycling conditions for the *Nitrobacter* 16S rRNA gene were as follows: 95 °C for 20 s, 40 cycles consisting of 95 °C for 7 s, 60 °C for 20 s, and 72 °C for 40 s (detection), followed by the melting curve analysis. The contents of PCR mixture and cycling conditions for bacterial and archaeal *amoA* genes were the same as described previously (Fukushima et al. 2012). PCR products obtained from the cloned *Nitrospira* (accession no. AB738418) and *Nitrobacter* (AB738419) 16S rRNA genes and bacterial (HM753264) and archaeal (HM753263) *amoA* genes were used as quantitative standard. The specificity of real-time PCR for *Nitrobacter* and *Nitrospira* was evaluated based on the melting curve analysis and agarose gel electrophoresis. The specificity of real-time PCR for bacterial and archaeal *amoA* genes was evaluated based on the melting curve analysis.

Batch experiments

In order to evaluate the dependences of nitrification kinetics on ammonium, nitrite, and IC concentrations, a series of batch tests was performed using activated sludge samples taken from the two bioreactors. For each batch experiment, 330 mL of biomass and 70 mL of medium were mixed in a 1-L serum bottle. Initial concentrations of IC and a nitrogen compound (ammonium or nitrite) for each batch experiment are shown in Table 1. Initial concentrations of mixed liquor volatile suspended solids were measured as biomass concentration. Other medium components were the same as those used for the bioreactor operation. After aerating with pure oxygen for 2 min, the bottles were immediately sealed with caps. The batch tests were performed in an incubator maintained at 30 °C and 125 rpm. Samples were taken frequently for nitrogen compounds. The pH and DO were monitored during experiments. The specific rates of ammonium or nitrite oxidation for batch experiments were calculated and their dependences on the nitrogen compound or IC concentrations were evaluated.

Chemical analyses

Ammonium (NH_4^+–N) and nitrate (NO_3^-–N) were measured by ionic chromatography (DX-120, Dionex, Sunnyvale, CA, USA). Nitrite (NO_2^-–N) was measured using the colorimetric method (APHA et al. 1995). MLVSS was measured according to standard procedures (APHA et al. 1995). Total inorganic carbon was determined by Model 1020A TOC Analyzer (OI Analytical, College Station, TX, USA).

Nucleotide sequence accession numbers

The *amoA* gene sequences determined in this study were submitted to the EMBL/GenBank/DDBJ databases under accession numbers AB622973 to AB622983. The 16S rRNA gene sequences were also submitted to the database under accession numbers AB738418, AB738419, and AB740393 to AB740404.

Results

Performance of bioreactors

The two bioreactors, high- and low-IC, were operated for a period of more than 700 days. Influent and effluent concentrations of nitrogen species for both bioreactors are presented in Fig. 1. After approximately 90 days of operation, the activated sludge in both bioreactors achieved more than 95 % complete nitrification with occasionally observed nitrite accumulation. During day 170 to 190, nitrification performance of the high-IC bioreactor gradually declined due presumably to a decrease in room temperature from

Table 1 Experiment conditions for AOB and NOB batch tests using biomass in high- and low-IC bioreactors

Batch no.	Ammonia conc. (mgN/L)	Nitrite conc. (mgN/L)	Inorganic carbon conc. (mg C/L)	Initial biomass conc. (mg/L)	
				High IC	Low IC
AOB-1	300	–	0, 5, 10, 20, 50, 80, 120	35	47
AOB-2	50, 100, 300, 500, 750	–	100	45	47
NOB-1	–	40	0, 5, 10, 50, 100	43	31
NOB-2	–	5	0, 0.25, 0.5, 1, 2	43	31
NOB-3	–	5, 10, 20, 40, 100	100	43	31

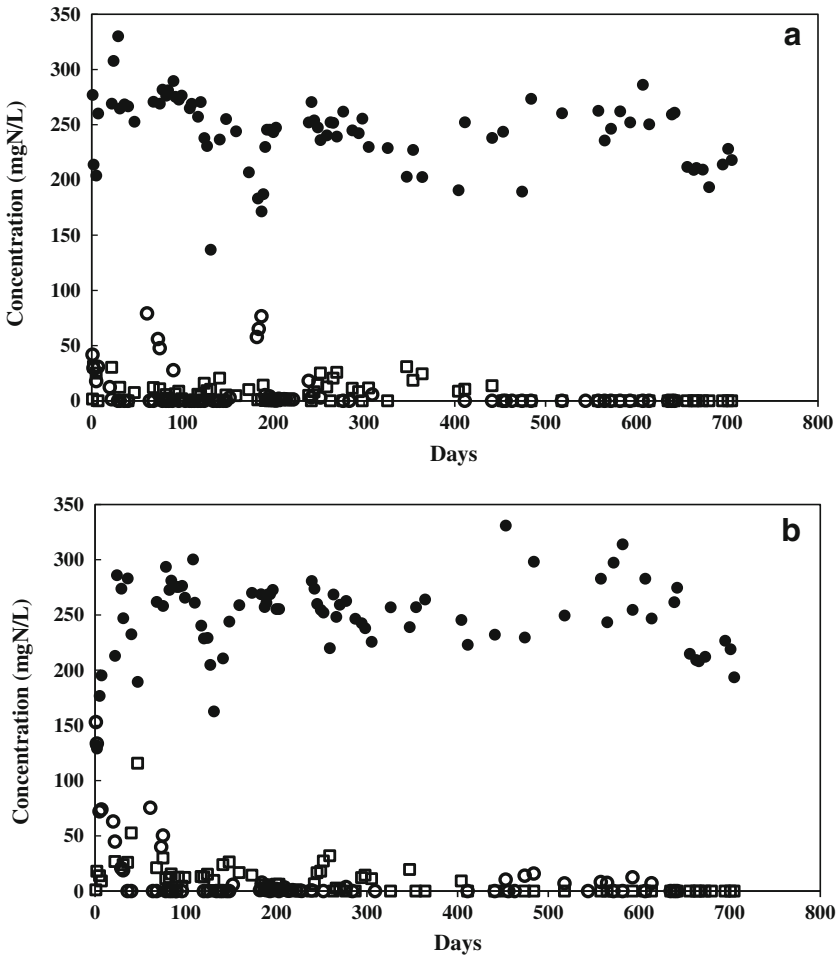

Fig. 1 Nitrification performance of high-IC (**a**) and low-IC (**b**) bioreactors. Effluent ammonium *open circle*, effluent nitrite *open square*, effluent nitrate filed circle

around 30 °C during summer time to 23 °C during winter time. Although the low-IC bioreactor experienced a temperature drop situation as well, the nitrification performance of the bioreactor, however, did not change. A thermal tape system was applied on day 190 to maintain an operational temperature of 30 °C, after which the nitrification performance of the high-IC bioreactor returned to previous levels. Unlike the high-IC bioreactor, the nitrification performance of the low-IC bioreactor was not influenced by the decreased room temperature during day 170 to 190.

AOB community structure

The bacterial and archaeal *amoA* gene copy numbers in the high- and low-IC bioreactors were determined using real-time PCR. The specificity of PCR products was confirmed by melting curve analysis (Fig. S1 of the "Electronic supplementary material"). The copy numbers of bacterial *amoA* gene in high- and low-IC bioreactors fluctuated within the range of 1.32×10^4 to 2.00×10^7 and 1.79×10^4 to 3.75×10^6 copy/mL sludge, respectively. The copy numbers of archaeal *amoA* gene in both bioreactors were below detection limit (2.50×10^3 copy/mL sludge) during most periods. The numbers were detected only after day 572 in high-IC reactor within the range of 3.83×10^3 to 6.65×10^5 copy/mL sludge. Thus, AOB was the dominant ammonia oxidizer in both bioreactors.

Cloning and sequencing of bacterial *amoA* gene fragments were performed to investigate the AOB community structure of the two bioreactors. The results indicated that all of the cloned *amoA* sequences obtained from this study were within the genus *Nitrosomonas*. A neighbor-joining phylogenetic tree is presented in Fig. 2, which graphically depicts the homologies of aligned sequences as well as the expected T-RF signatures (forward/reverse). There were 29 clones randomly selected for the seeding sludge sample, and these 29 clones were classified into two OTUs including OTUs S-1 (55 % of total clones) and S-2 (45 %). Based on phylogenetical analysis, OTU S-1 was closely related to *N. eutropha* (T-RF 491/491) lineage and S-2 was related to *N. nitrosa* (48/135) lineage. For the AOB communities in the bioreactors, 46 clones were randomly selected from each sample. OTUs H143-1 (95.7 %) and H143-2 (4.3 %) were obtained from the sludge in the high-IC bioreactor on day 143. Both OTUs were related to the *N. nitrosa* lineage, but their T-RFs were 48/135 and 48/441, respectively. Cloning and sequencing were also performed for the sample from the

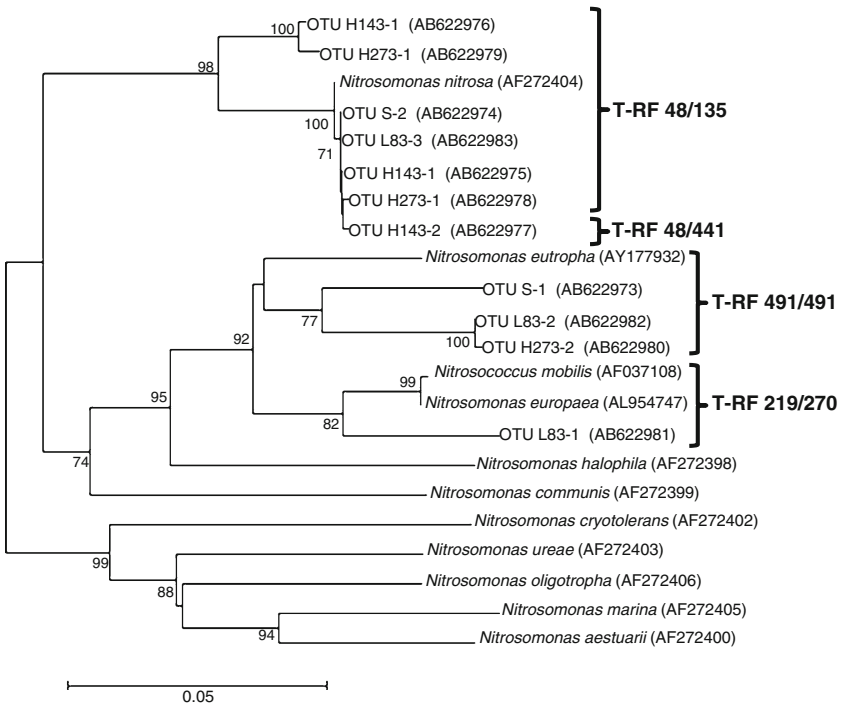

Fig. 2 Neighbor-joining tree based on *amoA* gene sequences from genus *Nitrosomonas*. Unrooted phylogenetic tree was generated using the neighbor-joining method and bootstrap tests were performed with 1,000 replicates. Boot-strap values (>60 %) are indicated at the branch points. The *scale bar* represents a 5-% sequence divergence. Accession numbers are given in *parentheses*. DNA from the seeding sludge (*S*) and sludges from high- and low-IC bioreactors (*H* and *L*) were used for cloning. OTU was decided based on RFLP analysis. Other sequences were retrieved from GenBank. The expected forward and reverse T-RF lengths after digestion with *TaqI* are indicated

high-IC bioreactor on day 273 in order to confirm the AOB community before and after temperature shock. For the day 273 sample from the high-IC bioreactor, OTUs H273-1 (95.7 %) and H273-2 (4.3 %) were obtained. OTU H273-1 was related to *N. nitrosa* (T-RF 48/135) lineage and H273-2 was related to *N. eutropha* (T-RF 491/491) lineage. This indicated that AOB community structure in high-IC bioreactor shifted back to *N. nitrosa* after the temperature was maintained at 30 °C. For the low-IC bioreactor sample taken on day 83, three OTUs were obtained, including L83-1 (71.7 %), L83-2 (26.1 %), and L83-3 (2.2 %). For the two major OTUs obtained, OTU L83-1 was closely related to *N. europaea* (T-RF 219/270) lineage and OTU L83-2 was related to *N. eutropha* (T-RF 491/491) lineage, leaving the minor OTU L83-3 which was related to *N. nitrosa* (T-RF 48/135) lineage. Cloning and sequencing of bacterial 16S rRNA gene fragments for the DNA from both bioreactors on day 442 were also performed. The total number of clones from high- and low-IC bioreactors was 86 and 112, respectively. A neighbor-joining phylogenetic tree is presented in Fig. S2 of the "Electronic supplementary material". In the high-IC bioreactor, all AOB-related OTUs (OTU HB and HC) were related to *N. nitrosa* lineage. In the low-IC bioreactor, all AOB-related OTUs (OTU LA and LB) were related to *N. europaea* lineage.

T-RFLP analysis for the *amoA* gene in high- and low-IC bioreactor sludges was performed until day 760. Three major T-RFs, 491/491, 48/135, and 219/270, were observed. Based on the cloning and sequencing results, these T-RFs were closely related to *N. eutropha*, *N. nitrosa*, and *N. europaea* lineages, respectively. Figure 3 shows the relative

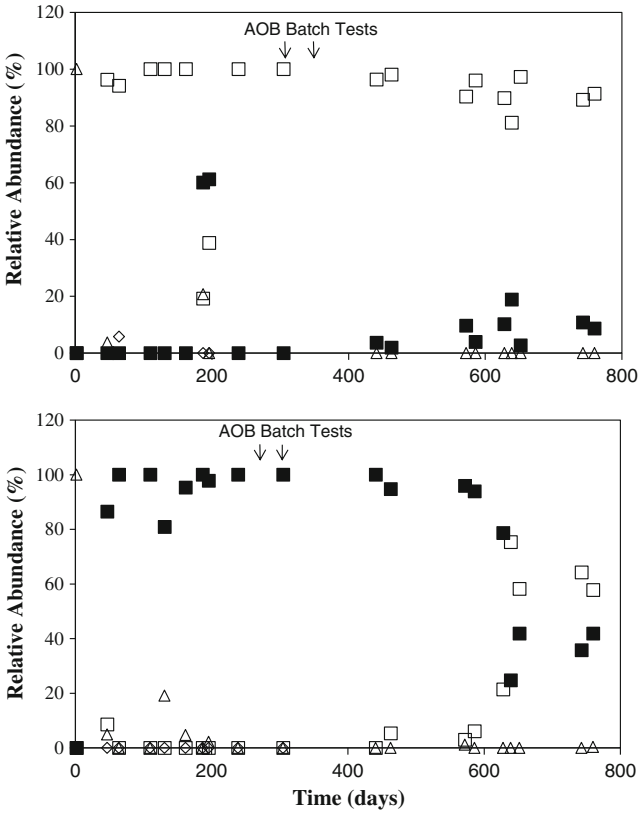

Fig. 3 Dynamics of AOB populations in the high-IC (*upper panel*) and low-IC (*lower panel*) bioreactors as determined by *amoA*-based T-RFLP. T-RF 135 *open square*, T-RF 270 *filled square*, T-RF 491 *open triangle*, T-RF for others *open diamond*. Samples were taken for ammonium oxidation batch experiments (*down arrows*)

abundances of major T-RFs as determined by the corresponding peak height from TET-labeled amoA-2R primer. On day 2, only *N. eutropha*-like AOB was observed in both bioreactors, but AOB community structures in both bioreactors shifted after day 2. In the high-IC bioreactor, *N. nitrosa*-like AOB became the dominant population after day 47. The dominant AOB shifted from *N. nitrosa*-like AOB to *N. europaea*-like AOB on day 196 when the temperature dropped, but *N. nitrosa*-like AOB came back and remained dominant after day 239. In the low-IC bioreactor, *N. europaea*-like AOB became the dominant population after day 47, and it continued to be dominant until day 630. After day 630, *N. nitrosa*-like AOB and *N. europaea*-like AOB were both dominant in the low-IC bioreactor at 60 and 40 %, respectively.

NOB community structure

The results of the phylogenetic analysis based on bacterial 16S rRNA genes (Fig. S2 of the "Electronic supplementary material") showed that NOB-related OTUs in high-IC (OTU HA and HE) and low-IC (OTU LC) bioreactors on day 442 were related to *Nitrospira* and *Nitrobacter*, respectively. The 16S rRNA copy numbers of *Nitrobacter* and *Nitrospira* in the high- and low-IC bioreactors were determined using real-time PCR. The specificity of PCR products was confirmed by both melting curve analysis and agarose gel electrophoresis (Figs. S3 and S4 of the "Electronic supplementary material"). The results indicated that significant no-primer dimer and non-target amplicon were generated in all real-time PCR results. The 16S rRNA gene copy numbers of *Nitrobacter* and *Nitrospira* are summarized in Fig. 4. In the high-IC bioreactor, the copy number of *Nitrospira* was stable. The copy number of *Nitrobacter* increased until day 463 and then remained stable. However, the copy number of *Nitrobacter* was significantly lower than that of *Nitrospira* during this study. In the low-IC bioreactor, the copy number of *Nitrospira* significantly decreased from day 2 to 47. After day 47, the number remained stable, but these values were approximately ten times lower than those observed in the high-IC bioreactor. The trend for *Nitrobacter* copy number was similar to that observed in the high-IC bioreactor. The 16S rRNA gene copy number of total NOB was estimated by the summation of the copy numbers of *Nitrospira* and *Nitrobacter*. The total NOB copy numbers of the high-IC bioreactor were stably fluctuated between 6.61×10^3 and 4.28×10^7 copy/mL sludge. In the low-IC bioreactor, the number decreased in the first 47 days, and then it fluctuated within the range of 1.29×10^3 to 1.66×10^6 copy/mL sludge. In general, the value was approximately one order of magnitude lower than that observed in the high-IC bioreactor.

The 16S rRNA gene copy number is assumed to be equivalent to the bacteria cell number for *Nitrobacter* and *Nitrospira* since these species typically possess one 16S rRNA gene operon (Dionisi et al. 2002; Navarro et al. 1992). On day 2, the relative abundance of *Nitrospira* in both bioreactors was greater than 99 %, indicating that *Nitrospira* was dominant in the seeding sludge. *Nitrospira*, with a relative abundance over 80 %, was the dominant NOB in the high-IC bioreactor during this study. In the low-IC bioreactor, the relative abundance of *Nitrospira* was higher than that of *Nitrobacter* during day 2 to 149, but the abundance of *Nitrospira* became lower than *Nitrobacter* after day 205.

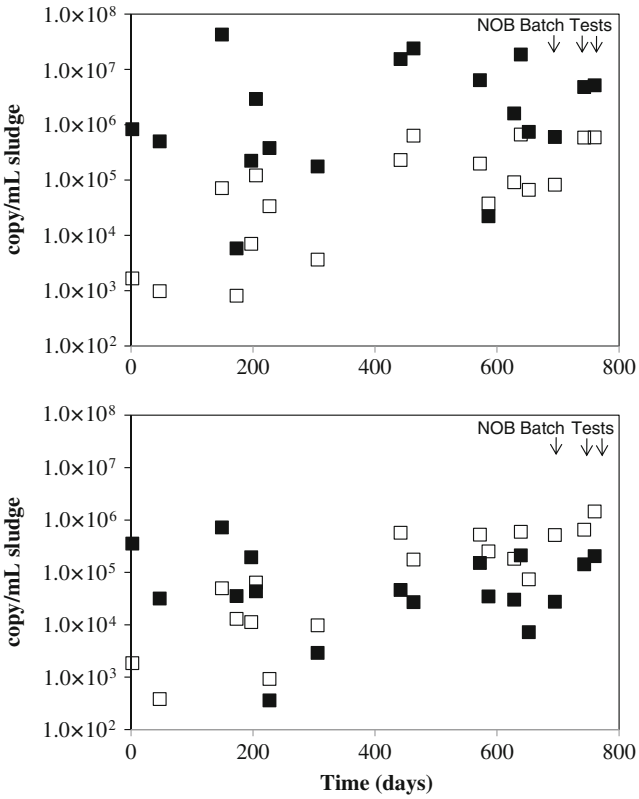

Fig. 4 The 16S rRNA gene copy numbers of *Nitrospira* and *Nitrobacter* in high-IC (*upper panel*) and low-IC (*lower panel*) bioreactors. *Nitrospira filled square*, *Nitrobacter open square*. Samples were taken for nitrite oxidation batch experiments (*down arrows*)

Evaluation of oxidation rates of nitrifying populations in the bioreactors

The dependences of specific ammonium oxidation rates on IC and ammonium concentrations for biomass taken from high- and low-IC bioreactors are summarized in Fig. 5. In all batch experiments, the specific ammonia oxidization rates of the low-IC bioreactor AOB were obviously higher than those of the high-IC bioreactor AOB. Among all experiments, the difference in specific ammonium oxidation rates between high- and low-IC AOB was minimum at an initial ammonium concentration of 50 mgN/L.

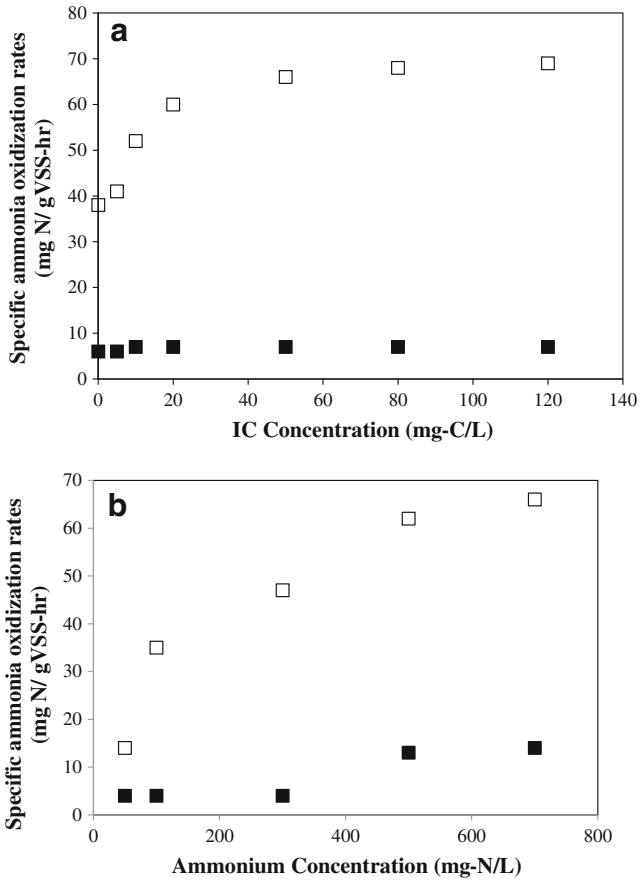

Fig. 5 Specific ammonia oxidation rates of biomass in high- and low-IC bioreactors under different IC (**a**) and ammonium (**b**) concentrations

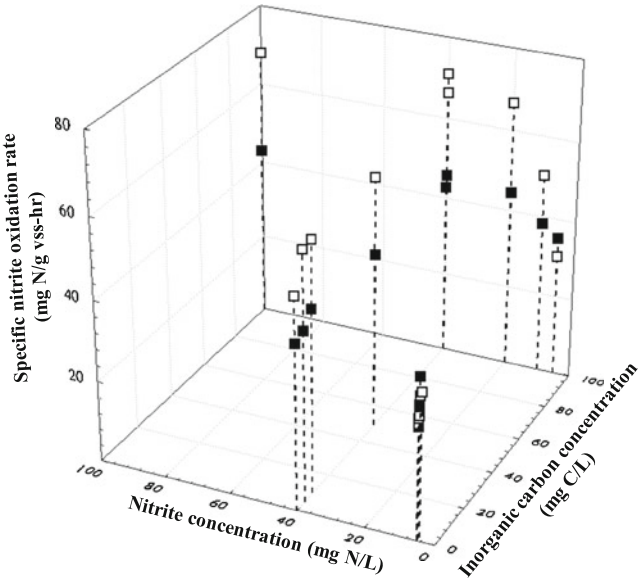

Fig. 6 Specific nitrite oxidation rates of biomass in high- and low-IC bioreactors under different IC and ammonium concentrations

The dependences of specific nitrite oxidation rates on IC and nitrite concentrations for biomass taken from high- and low-IC bioreactors were evaluated with three batch experiments as summarized in Fig. 6. In the NOB-1 batch experiment with an initial nitrite concentration of 40 mgN/L, the specific nitrite oxidization rates of the low-IC bioreactor NOB were all higher than those of the high-IC bioreactor NOB, disregarding the IC concentration applied. For the NOB-2 batch experiment with an initial nitrite concentration of 5 mgN/L, NOB in both bioreactors attained similar rates. In the NOB-3 batch experiment with an initial IC concentration of 100 mg/L, the rates for the low-IC bioreactor NOB were higher than those for the high-IC bioreactor NOB, except that at an initial nitrite concentration of 5 mgN/L the high-IC bioreactor NOB attained a slightly higher rate.

Discussion

To evaluate the effects of IC concentration on the nitrifying communities and their nitrification performance, we operated two lab-scale continuous-flow bioreactors with high and low IC concentrations, respectively. Both bioreactors achieved more than 95 % of nitrification efficiency except for the high-IC bioreactor during a period of reduced ambient temperature. In this study, we demonstrated that IC concentration did not significantly affect nitrification performance, but it affected nitrifying community structures.

The dominant AOB populations in the high- and low-IC bioreactors were of the *N. nitrosa* and *N. europaea* lineage, respectively. *N. nitrosa* has been reported for its abundance in activated sludge treating industrial wastewaters (Cabezas et al. 2009; Dionisi et al. 2002; Kim et al. 2011; Kuo et al. 2006; Layton et al. 2005). In fact, *N. nitrosa* Nm90 was isolated from an industrial sewage in Germany (Koops et al. 1991). Compared with *N. europaea*, *N. nitrosa* was reported to have a higher affinity for ammonium (Koops and Pommerening-Roser 2001; Prosser 1989), suggesting its competitiveness in a low-ammonium environment like the bioreactors operated in this study. However, the details regarding the conditions that may favor the proliferation of *N. nitrosa* over other AOB species are limited and warrant future research.

During day 170 to 190, the nitrification performance of the high-IC bioreactor gradually declined due presumably to a decrease in room temperature, while the performance of the low-IC bioreactor did not change. Schmidt et al. (2004) reported that different temperature settings had no significant effect on the growth mode of and the proteins expressed by *N. europaea*. Although the reason is unclear, the dominance of *N. nitrosa* lineage under a higher temperature has been reported (Avrahami et al. 2011; Shore et al. 2012). Those results suggest that different AOB communities present in between high- and low-IC reactors may result in different responses to temperature drop.

N. europaea has been known to be the key AOB in high-ammonium-containing wastewater treatment systems because it is the most frequently recovered AOB pure culture in nitrogen-rich culture conditions (Koops and Pommerening-Roser 2001; Prosser 1989). Previous studies have shown that members of the *N. europaea* lineage are relatively abundant among AOB species in several bioreactors (Gieseke et al. 2003; Schramm et al. 2000) due to its relatively high maximum ammonium conversion rate compared to those of other AOB populations (Koops and Pommerening-Roser 2001; Prosser 1989). The low-IC biomass dominated by *N. europaea* attained higher specific ammonia oxidization rates in all batch experiments with initial ammonium>50 mgN/L, indicating that the results of microbial community analysis agreed with the results of kinetic analysis. On the other hand, a previous study has reported that *N. europaea* was found to be dominant in a pilot-scale bioreactor buffered with NaOH under an IC-limited condition, while *Nitrosospira* became proliferated in the same bioreactor during the operation buffered with Na_2CO_3 (Whang et al. 2009). Accordingly, *N. europaea* was able to dominate under low-IC conditions, although the mechanism is not clear at this moment.

The difference in the mechanisms and characteristics of carbon fixation among AOB species may provide possible explanations for their different responses to IC concentrations. AOB fix CO_2 through the Calvin–Benson–Bassham (CBB) cycle, and one of the key enzymes is the ribulose bisphosphate carboxylase (RubisCO) (Schramm et al. 1998; Utaker et al. 2002). It has been reported for *N. europaea* that the transcription of *cbb* genes, which encode carbon-fixing enzyme RubisCO, was upregulated when the carbon source was limited (Wei et al. 2004). This property suggests that *N. europaea* may primarily distribute available resources towards key components for carbon assimilation under limited IC conditions, resulting in its competitiveness and dominance in the low-IC bioreactor. Another possibility is that *N. nitrosa* has carboxysomes, while *N. europaea* does not (Koops and Pommerening-Roser 2001). It is suggested that CO_2, rather than bicarbonate, is used as a substrate by RubisCO for microbial CO_2 fixation, and the conversion of bicarbonate to CO_2 requires another enzyme, carbonic anhydrase (Tabita 1999). Carboxysome is a microbial microcompartment that performs CO_2 fixation by co-localizing the two enzymes RuBisCO and carbonic anhydrase within the carboxysome (Yeates et al. 2008). Compared to AOB species with the carboxysome, it is likely that *N. europaea*, without the carboxysome, can fix CO_2 more efficiently, especially under an IC-limited environment. Furthermore, *N. europaea* Nm50 had a "green-like" type I RubisCO (similar to RubisCO genes found in green algae), whereas *Nitrosospira* spp. generally has a "red-like" type I RubisCO (Utaker et al. 2002). The differences in affinity of the RubisCO enzyme for $NaHCO_3$ among the AOB species (Hatayama et al. 2000) can also be attributed to their different responses to IC concentrations. Nevertheless, the exact mechanisms are not clear at this time, but the answer to whether those differences cause the different responses to varying IC concentrations among AOB species awaits future investigations.

For the NOB population, *Nitrospira* and *Nitrobacter* were dominant in the high- and low-IC bioreactors, respectively. *Nitrobacter*, an r-strategist, has been reported to be a superior competitor when nitrite is abundant, while *Nitrospira*, a K-strategist, thrives under conditions with nitrite scarcity (Blackburne et al. 2007; Huang et al. 2010; Nogueira and Melo 2006). Under high-nitrite conditions in the batch experiments, the low-IC sludge dominated by *Nitrobacter* attained higher specific nitrite oxidization rates compared to the *Nitrospira*-dominated high-IC sludge, indicating that the results of microbial community analysis agreed with the results of kinetic analysis. Kim and Kim (2006) reported that specific nitrite oxidization activities of *Nitrobacter* (93.8 mg/gNOB h) were nine times higher than that of *Nitrospira* (10.5 mg/g NOB/h). In addition, higher accumulated nitrate concentration might present an inhibitory effect to the *Nitrospira*. These may explain why low-IC biomass showed a higher activity in batch experiments even if the total NOB copy number of the low-IC biomass was lower than that of the high-IC biomass. Under low nitrite together with IC-limited conditions, the *Nitrobacter*-dominant sludge maintained similar specific nitrite oxidization rates compared to the *Nitrospira*-dominated sludge despite the fact that *Nitrospira* was considered a K-strategist. It is likely that IC limitation may favor the selection of *Nitrobacter* over *Nitrospira* even at a low-nitrite condition like the bioreactors operated in this study. By evaluating oxygen uptake rates, Guisasola et al. (2007) also observed no IC limitation effect on the nitratation process for a nitrifying sludge containing *Nitrobacter* at 5 % of the total bacterial populations, although detailed information regarding *Nitrospira* population was not mentioned in that study.

Metagenomic analyses on *Nitrobacter winogradskyi* (Starkenburg et al. 2006), *Nitrobacter hamburgensis* X14 (Starkenburg et al. 2008), and "*Candidatus* Nitrospira defluvii" (Lucker et al. 2010) indicated that different NOB populations may perform different carbon fixation mechanisms. Genes for all of the enzymes of a functional CBB cycle are present in *Nitrobacter* but not in *Candidatus* Nitrospira defluvii, suggesting that the CBB cycle does not operate in *Candidatus* Nitrospira defluvii. All genes of the reductive tricarboxylic acid cycle are instead present in *Candidatus* Nitrospira defluvii, suggesting that a different carbon fixation pathway is employed for *Nitrospira*. The difference in carbon fixation mechanisms between *Nitrobacter* and

Nitrospira is likely attributed to their different responses to IC concentrations.

In summary, under high ammonium/nitrite conditions, *N. europaea*/*Nitrobacter* was a dominant AOB/NOB disregarding the IC concentration. Compared with *N. nitrosa*/*Nitrospira*, *N. europaea*/*Nitrobacter* attained higher specific oxidation rates in such environments even at low IC conditions. Under low-ammonium/nitrite conditions, IC availability became important in selecting dominant nitrifying populations. With sufficient IC conditions like the high-IC bioreactor, *N. nitrosa*/*Nitrospira* was dominant since it was a K-strategist in terms of ammonium/nitrite, while in the low-IC bioreactor, when available IC became limited, *N. europaea*/*Nitrobacter* became important due to its efficient utilization of IC compared with *N. nitrosa*/*Nitrospira*.

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