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Differences in nitrite-oxidizing communities and kinetics in a brackish environment after enrichment at low and high nitrite concentrations

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ABSTRACT

Nitrite accumulation in shrimp ponds can pose serious adverse effects to shrimp production and the environment. This study aims to develop an effective process for the enrichment of ready-to-use nitrite-oxidizing bacteria (NOB) inocula that would be appropriate for nitrite removal in brackish shrimp ponds. To achieve this objective, the effects of nitrite concentrations on NOB communities and nitrite oxidation kinetics in a brackish environment were investigated. Moving-bed biofilm sequencing batch reactors and continuous moving-bed biofilm reactors were used for the enrichment of NOB at various nitrite concentrations, using sediment from brackish shrimp ponds as seed inoculum. The results from NOB population analysis with quantitative polymerase chain reaction (qPCR) show that only *Nitrospira* were detected in the sediment from the shrimp ponds. After the enrichment, both *Nitrospira* and *Nitrobacter* coexisted in the reactors controlling effluent nitrite at 0.1 and 0.5 mg-NO_2^--N/L. On the other hand, in the reactors controlling effluent nitrite at 3, 20, and 100 mg-NO_2^--N/L, *Nitrobacter* outcompeted *Nitrospira* in many orders of magnitude. The half saturation coefficients (K_s) for nitrite oxidation of the enrichments at low nitrite concentrations (0.1 and 0.5 mg-NO_2^--N/L) were in the range of 0.71–0.98 mg-NO_2^--N/L. In contrast, the K_s values of NOB enriched at high nitrite concentrations (3, 20, and 100 mg-NO_2^--N/L) were much higher (8.36–12.20 mg-NO_2^--N/L). The results suggest that the selection of nitrite concentrations for the enrichment of NOB inocula can significantly influence NOB populations and kinetics, which could affect the effectiveness of their applications in brackish shrimp ponds.

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Introduction

Nitrite accumulation is usually a concern in shrimp ponds that lack sediment, as sediment is the major source of nitrifying microorganisms, including nitrite-oxidizing bacteria (NOB). The accumulation of nitrite greater than 1 mg/L-N in shrimp ponds can cause serious adverse effects on aquaculture (Hart and O'Sullivan, 1993). Therefore, the development of techniques for controlling nitrite concentrations in shrimp ponds is essential. Bioaugmentation with NOB inocula is considered an attractive approach for resolving intermittent nitrite accumulation in shrimp ponds. Bioaugmentation for nitrification has previously been demonstrated in activated sludge systems (Yu et al., 2012; Leu and Stenstrom, 2010; Parker and Wanner, 2007). The approach has great potential to be further developed and applied in aquaculture.

NOB are groups of microorganisms that are capable of converting nitrite into nitrate, which can be applied to resolve nitrite accumulation in shrimp ponds. *Nitrobacter*, *Nitrospira*, and *Nitrotoga* are the three main genera of NOB commonly observed in nitrification systems. These groups of NOB usually thrive under different environmental conditions (Daims et al., 2001). Studies have shown that physicochemical and operational parameters, such as nitrite concentrations, dissolved oxygen (DO) concentrations, temperature, and salinity affected NOB populations (Daims et al., 2001; Huang et al., 2010; Moussa et al., 2006). In the past, *Nitrobacter* was believed to be the key NOB in wastewater treatment plants (Grady and Lim, 1988). However, *Nitrobacter*-related organisms were not detected in nitrifying activated sludge samples by fluorescence *in situ* hybridization (FISH) (Wagner et al., 1996). In addition, NOB communities in a nitrifying bioreactor were investigated using FISH, and the results revealed that *Nitrospira* spp. were the responsible NOB in nitrification systems (Schramm et al., 1999). Moreover, a recent study found that *Nitrotoga*-like bacteria were key nitrite oxidizers in full-scale wastewater treatment plants (Lücker et al., 2015). Nevertheless, until now, the occurrence and importance of *Nitrotoga* in brackish systems have not yet been reported. Therefore, *Nitrobacter* and *Nitrospira* are still considered to be the two main genera of NOB commonly observed in brackish environments.

With the kinetic characteristics of NOB, it has been suggested that *Nitrospira* are K-strategists that can adapt to low nitrite concentrations, while *Nitrobacter* are r-strategists that thrive when nitrite is at high concentrations (Daims et al., 2001; Schramm et al., 1999). *Nitrospira* generally have higher nitrite affinities (lower K_S values) compared to *Nitrobacter* (Nowka et al., 2015). Many studies have also supported the K/r hypothesis. For examples, Kim and Kim (2006) reported that the distribution of *Nitrobacter* and *Nitrospira* depended on nitrite concentrations. Nogueira and Melo (2006) studied the competition between *Nitrospira* and *Nitrobacter* in nitrite-oxidizing bioreactors. The dominance of *Nitrobacter* over *Nitrospira* appeared to be caused by the elevated nitrite concentrations in bioreactors, which confirmed the K/r hypothesis (Nogueira and Melo, 2006). These previous findings suggest that nitrite concentrations can significantly influence NOB communities. Therefore, the selection of nitrite concentrations to enrich NOB inocula is crucial since it can lead to different NOB communities and kinetics, which could affect their applications in brackish shrimp ponds.

In general, nitrite concentrations in shrimp ponds are in the range of 0.02–0.17 mg-NO_2^--N/L, in which only *Nitrospira* spp. were observed (Srithep et al., 2015). However, higher nitrite concentrations are usually used for the enrichment of NOB inocula for applications in shrimp ponds. The differences in nitrite concentrations used for the enrichment could result in different nitrite-oxidizing bacterial communities and kinetics. The effects of nitrite concentrations on NOB communities and kinetics remain unclear, especially in brackish environments. Such information would be useful for the development of NOB inocula appropriate for nitrite removal in aquaculture ponds. The objective of this study is to investigate the effects of nitrite concentrations on microbial communities and the kinetics of NOB enrichments. The approach for NOB enrichment in this study can also be further applied to develop NOB inocula suitable for aquaculture ponds.

1. Material and methods

1.1. Moving-bed biofilm sequencing batch reactors

Sediment from two outdoor brackish shrimp ponds in Chachengsao, Thailand, was collected and mixed to use as seed inoculum for the enrichment of NOB on biofilm carriers (2H-BCN 012 KLL, Kunststoff GmbH, Germany) in two moving-bed biofilm sequencing batch reactors (50 L). The biofilm carriers (Appendix A Fig. S1) had specific surface area of 859 m^2/m^3, a protected area of 704 m^2/m^3, and a weight of 150 ca. kg/m^3. The first (Reactor A) and second (Reactor B) moving-bed biofilm sequencing batch reactors were fed intermittently with nitrite at a low concentration (1 mg-NO_2^--N/L) and at a high concentration (50 mg-NO_2^--N/L), respectively. The NOB enrichment in these moving-bed biofilm sequencing batch reactors was aimed to increase the amount of NOB populations at low and high nitrite concentrations for further NOB enrichment in continuous-flow moving-bed biofilm reactors.

The synthetic wastewater used in this experiment consisted of $NaNO_2$ (1 or 50 mg/L NO_2^--N), 0.2 g/L of KH_2PO_4, 0.4 g/L of $MgSO_4{\cdot}7H_2O$, 0.1 g/L of KBr, 200 g/L of $NaHCO_3$, 35.8 g/L of Marinium™ reef sea salt (Mariscience, USA), 1 mL/L of nonchelated trace element mixture, 1 mL/L of vitamin mixture, 1 mL/L of vitamin B12 solution, 1 mL/L of vitamin B1 solution, 1 mL/L of selenite-tungstate solution (modified from Könneke et al. (2005)). The nonchelated trace element mixture, vitamin mixture, vitamin B12 solution, vitamin B1 solution, and selenite-tungstate solution were prepared according to Widdel and Bak (1992). The reactors were operated for 90 days at room temperature (28 ± 3°C). Both reactors were operated within a pH range of 7.5–8.5 and an alkalinity range of 120–150 mg-$CaCO_3$/L. DO was controlled to be greater than 4 mg-O_2/L throughout the operation. The salinity of the medium was 15 ppt. The nitrite and nitrate concentrations in both reactors were monitored.

1.2. Continuous-flow moving-bed biofilm reactors

The biofilm carriers that were enriched in Reactor A were then transferred to 2 aerobic continuous-flow moving-bed biofilm

reactors (10 L), Reactor A0.1 and Reactor A0.5. On the other hand, the biofilm carriers enriched in Reactor B were transferred to 3 aerobic continuous-flow moving-bed biofilm reactors (10 L), Reactor B3, Reactor B20, and Reactor B100. The biofilm carriers with the total volume of 5 L were placed in each reactor. The nitrite concentrations in the reactors were controlled to be 0.1 mg-NO_2^--N/L (Reactor A0.1), 0.5 mg-NO_2^--N/L (Reactor A0.5), 3 mg-NO_2^--N/L (Reactor B3), 20 mg-NO_2^--N/L (Reactor B20), and 100 mg-NO_2^--N/L (Reactor B100), respectively. The flow rate and influent concentration of the nitrite were adjusted to achieve aforementioned nitrite concentrations in the reactors. The flow rates of Reactor A0.1, Reactor A0.5, Reactor B20, and Reactor B100 were 2.88 L/day, 8.40 L/day, 3.80 L/day, and 6.00 L/day, respectively. In Reactor B3, the initial flow rate was 1.63 L/day. It was then increased to 1.74 L/day, 1.86 L/day, and 1.97 L/day on day 8, 16, and 42 of operation, respectively. The hydraulic retention time, determined by the total volume of the reactor divided by the flow rate (*V*/*Q*), of Reactor A0.1, Reactor A0.5, Reactor B20, and Reactor B100 was 3.47 days, 1.19 days, 2.63 days, and 1.67 days, respectively. For Reactor B3, the initial hydraulic retention time was 6.13 day. It then decreased to 5.75 days, 5.38 days, and 5.08 days on day 8, 16, and 42 of operation, respectively. The nitrite loading of each reactor (mg-N/day) is shown in Appendix A Fig. S2. The synthetic wastewater composition used in this study was identical to that used in the moving-bed biofilm sequencing batch reactors except that the nitrite concentrations were different. The nitrite and nitrate concentrations in the influent and effluent of the five reactors were monitored over time.

1.3. Nitrite-oxidizing bacterial population analysis

Deoxyribonucleic acid (DNA) from 0.5 g of sediment from shrimp ponds was extracted using a FastDNA® SPIN Kit for Soil (Qbiogene, USA). Then, humic acids were removed from the extracted DNA using G-50 Mini Column (Geneaid, Taiwan). Duplicates of the extracted DNA were combined and used for further molecular analysis (Srithep et al., 2015). For the bioreactor samples, biomass was washed from the biofilm carriers using deionized water then centrifuged and collected in Eppendorf tubes to obtain 2 mg of mixed liquor suspended solids (MLSS) per tube. DNA was then extracted using a FastDNA® SPIN Kit for Soil (Qbiogene, U.S.).

Quantitative polymerase chain reaction (qPCR) (qPCR, Thermo Scientific, USA) was used with Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific, USA) for nitrite-oxidizing bacterial population analysis. Primers P338f and NIT3 were used to amplify the partial 16S ribosomal ribonucleic acid (rRNA) gene of *Nitrobacter*; while NSR1113f and NSR1264r were used to amplify the partial 16S rRNA gene of *Nitrospira* (Table 1) (Regan et al., 2002; Dionisi et al., 2002). The qPCR conditions for the 16S rRNA genes of *Nitrobacter* and *Nitrospira* are summarized in Table 1. The plasmid inserts of the clones derived from the samples collected from Reactor B100 and Reactor A0.1 were used as standards for the partial 16S rRNA genes of *Nitrobacter* and *Nitrospira*, respectively. For all samples, qPCR was run at 1-, 10-, and 100-fold dilutions with duplicates.

1.4. Nitrite-oxidizing bacterial community analysis

To further investigate the microbial communities of *Nitrobacter* and *Nitrospira*, clonal libraries of the partial 16S rRNA genes of *Nitrobacter* and *Nitrospira* were constructed in certain samples. A clonal library of partial 16S rRNA genes of *Nitrobacter* was constructed for the biomass from the biofilm carriers in Reactor A0.5, B3, and B100 whereas a clonal library of partial 16S rRNA genes of *Nitrospira* was constructed for the biomass from the biofilm carriers in Reactor A0.1, B3, B100, and the shrimp pond sediment. The DNA extraction was described in the nitrite-oxidizing bacterial population analysis. The primers used for PCR are summarized in Table 1 (Regan et al., 2002; Dionisi et al., 2002). PCRs were run in a thermocycler (Takara, Japan) with Taq DNA polymerase (Takara, Japan). The PCR conditions used are summarized in Table 1. Clonal libraries were then constructed using a pGem-T Easy vector system I kit (Promega, USA) and XL1-Blue competent cells (Stratagene, USA). From each clonal library, 6 to 15 clones were randomly selected for sequencing (Macrogen Inc., Korea). A total of 35 and 51 clones were sequenced for *Nitrobacter* and *Nitrospira*, respectively. The sequences were calculated for an arrangement of operational taxonomic units (OTUs), based on 99% OTU identities using the DOTUR program (Schloss and Handelsman, 2005). Any sequences obtained from the same clonal library showing 99% similarity were defined as one OTU. The representative sequences and reference sequences were aligned and phylogenetically analyzed using the ARB program package (version 2.0; Department of Microbiology, Technische Universitat Munchen [http://www.arb-home.de]). Phylogenetic trees were constructed by adding our analyzed sequences into distance trees, which were previously constructed based on a comparison of >1200-bp sequences of reference *Nitrobacter* or *Nitrospira*.

1.5. Nitrite oxidation kinetics

The nitrite oxidation kinetics of intact biofilms obtained from Reactors A0.1, A0.5, B3, B20, and B100 were investigated in a series of batch reactors (1 L). To examine the kinetics, the biofilm carriers were directly transferred from the continuous-flow moving-bed biofilm reactors into batch reactors. The biofilm carriers from Reactors A0.1, A0.5, B3, B20, and B100 were collected for the kinetic tests on day 63, 72, 85, 25, and 35, respectively. For the biofilm carriers from each reactor, nitrite oxidation was tested at 8 initial concentrations. All nitrite oxidation tests were conducted in duplicates. The DO concentrations were in the range of 5–6 mg-O_2/L. The nitrite concentrations in the bulk liquid phase were measured over time. The initial rates of nitrite oxidation were then calculated with respect to mixed liquor volatile suspended solids (MLVSS). Plots of nitrite oxidation rates (mg-NO_2^--N/mg MLVSS · hr) *versus* initial nitrite concentrations were then constructed. The half saturation coefficients (K_s) and maximum specific rates (q_{max}) of nitrite oxidation were estimated based on the Monod equation as described in Eq. (1) using SigmaPlot software (Systat Software Inc., USA).

$$q = \frac{q_{max} S}{K_s + S} \quad (1)$$

Table 1 – Primers for the 16S rRNA genes of *Nitrobacter* and *Nitrospira* and PCR/qPCR conditions used in this study.

Target gene	Primer	Nucleotide sequence	Reference	PCR conditions		qPCR conditions	
16S rRNA genes of *Nitrobacter*	P338f	ACTCCTACGGGAGGCAGCAG	Regan et al. (2002)	Initial denaturation 5 min 95°C DNA denaturation 1.5 min 95°C Primer annealing 0.5 min 65°C DNA extension 1 min 72°C Final extension 6 min 72°C	30 cycles	Initial denaturation 5 min 95°C DNA denaturation 1.5 min 95°C Primer annealing 0.5 min 65°C DNA extension 1 min 72°C With data captured for each cycle at 80°C for 15 sec	40 cycles
	NIT3	CCTGTGCTCCATGCTCCG					
16S rRNA genes of *Nitrospira*	NSR1113f	CCTGCTTTCAGTTGCTACCG	Dionisi et al. (2002)	Initial denaturation 10 min 95°C DNA denaturation 0.5 min 95°C Primer annealing 0.5 min 60°C DNA extension 1 min 72°C Final extension 6 min 72°C	30 cycles	Initial denaturation 10 min 95°C DNA denaturation 0.5 min 94°C Primer annealing 0.5 min 60°C DNA extension 0.5 min 72°C with data captured for each cycle at 78°C for 15 sec	40 cycles
	NSR1264r	GTTTGCAGCGCTTTGTACCG					

where, q (mg-NO_2^--N/mg MLVSS · hr) is specific rate of nitrite oxidation, q_{max} (mg-NO_2^--N/mg MLVSS · hr) is the maximum specific rates of nitrite oxidation, S (mg-NO_2^--N/L) is nitrite concentration, and K_s (mg-NO_2^--N/L) is the half saturation coefficient.

1.6. Analytical measurements

Nitrite and nitrate were analyzed using the colorimetric method according to the standard methods (APHA et al., 2005). The concentrations of DO in the reactors were measured using a DO meter (Eutech model DO110). Salinity was measured using a salinity meter (Atago model S/Mill-E), and pH was monitored using a portable pH meter (Mettler-Toledo SevenGo, Schwerzenbach, Switzerland). Alkalinity was measured using alkalinity test kits (AQUA-VBC). Biomass samples were collected from the biofilm carriers *via* washing by deionized water and sonication at 40 kHz/200 W for 10 min (MS-4010, Y J Tech Ultrasonic Co., Ltd., Thailand), then the MLSS and MLVSS of biomass were measured using the gravimetric method (APHA et al., 2005). The reported MLSS and MLVSS accounted for both the suspended solids coming out from the washing process and from the sonication.

2. Results and discussion

2.1. Moving-bed biofilm sequencing batch reactors

Reactor A and Reactor B were operated for 90 days with intermittent feeding of nitrite at low and high nitrite concentrations, respectively. In Reactor A, the nitrite was intermittently fed at 1 mg-NO_2^--N/L. The average nitrite concentration in Reactor A was 0.54 mg-NO_2^--N/L. In Reactor B, the nitrite was intermittently fed at 50 mg-NO_2^--N/L, resulting in an average nitrite concentration of 25.76 mg-NO_2^--N/L during the operation period. In both reactors, nitrite was completely oxidized to nitrate. The DO, pH, alkalinity and salinity were maintained at 4–7 mg-O_2/L, 7–8.5, 120 mg-$CaCO_3$/L, and 10–20 ppt, respectively.

2.2. Continuous-flow moving-bed biofilm reactors

The biofilm carriers enriched in Reactor A and Reactor B were then used in Reactors A0.1, A0.5, B3, B20, and B100 in which the nitrite concentrations in the reactors were maintained at approximately 0.1, 0.5, 3, 20, and 100 mg-NO_2^--N/L, respectively. Fig. 1 shows the nitrite concentrations of the influent and effluent in Reactors A0.1 and A0.5, and Fig. 2 shows the nitrite concentrations of the influent and effluent in Reactors B3, B20, and B100. The average nitrite concentrations in Reactors A0.1 and A0.5 were 0.11 ± 0.01 mg-NO_2^--N/L and 0.47 ± 0.06 mg-NO_2^--N/L, respectively, during steady states of operation. In Reactors B3, B20, and B100, the average nitrite concentrations were 2.47 ± 1.04 mg-NO_2^--N/L, 21.89 ± 5.00 mg-NO_2^--N/L, and 107.91 ± 26.94 mg-NO_2^--N/L, respectively. In all of the reactors, nitrite was completely oxidized to nitrate. According to the mass balance of nitrogen, denitrification appeared to be minimal. The DO, pH, alkalinity and salinity were in the range of 5–7 mg-O_2/L, 7.2–8.5, 120–150 mg-$CaCO_3$/L, and 10–15 ppt, respectively, throughout the operation.

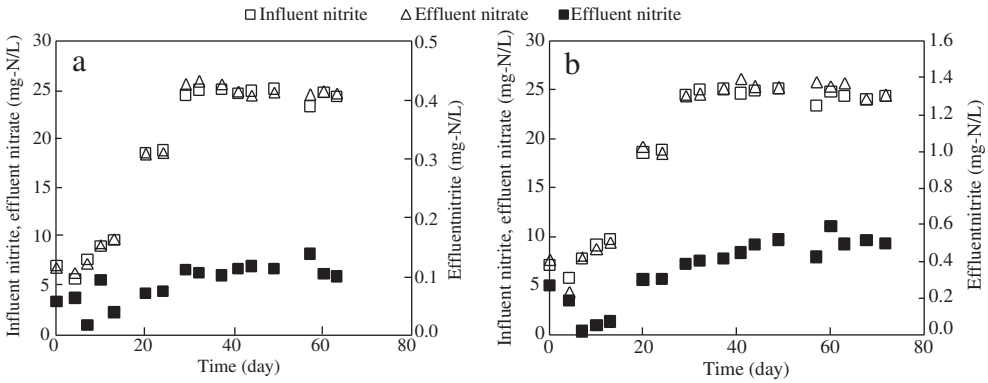

Fig. 1 – Nitrite and nitrate concentrations in the continuous-flow moving-bed biofilm reactors: (a) Reactor A0.1 and (b) Reactor A0.5.

2.3. Nitrite-oxidizing bacterial population analysis

Fig. 3 shows the nitrite-oxidizing bacterial populations (*Nitrobacter* and *Nitrospira*) in the shrimp pond sediment and biofilms of Reactors A0.1, A0.5, B3, B20, and B100. From the results, only *Nitrospira*, not *Nitrobacter*, were detected in the sediment of shrimp ponds. The results are consistent with Srithep et al. (2015), in which only *Nitrospira* were detected in all shrimp ponds where the nitrite concentrations were in the range of 0.02–0.17 mg-NO_2^--N/L. *Nitrospira* have also been found in natural habitats, such as freshwater and marine sediments (Altmann et al., 2003; Haaijer et al., 2013; Watson et al., 1986) and in biofilters in fresh, marine, and brackish recirculation aquaculture/aquarium systems (Itoi et al., 2007; Sugita et al., 2005; Tal et al., 2003; Keuter et al., 2011; Kruse et al., 2013).

In the moving-bed biofilm sequencing batch reactors intermittently fed with 1 mg-NO_2^--N/L (Reactor A) and the continuous-flow moving-bed biofilm reactors enriched at low nitrite concentrations (Reactors A0.1 and B0.5), both *Nitrospira* and *Nitrobacter* coexisted. In previous studies, *Nitrospira* spp. were reported to have a high nitrite affinity (low K_s values) and found to be predominant at low nitrite concentrations (Schramm et al., 1999; Kim and Kim, 2006). Therefore, it is rather unexpected that *Nitrobacter* were observed at low nitrite concentrations in this study. Although *Nitrobacter* were not detected in shrimp pond sediment, they were detected in the moving-bed biofilm sequencing batch reactor intermittently fed with 1 mg-NO_2^--N/L (Reactor A). *Nitrobacter* were subsequently detected in all of the continuous-flow moving-bed biofilm reactors enriched at low nitrite concentrations (0.1–0.5 mg-NO_2^--N/L), in which the biofilm carriers of these reactors were initially transferred from Reactor A. A possible explanation is that the enrichment of NOB in Reactor A intermittently fed with 1 mg-NO_2^--N/L could support the growth of both *Nitrobacter* and *Nitrospira*. Therefore, when the biofilm carriers from Reactor A were transferred to Reactor A0.1 and Reactor A0.5, *Nitrobacter* could still be observed in the biofilm carriers since inactive biomass cannot be effectively removed from the attached growth process. Although a nitrite concentration of 1 mg-NO_2^--N/L appears to be low, the kinetic results show that the nitrite concentrations resulting in the shifts in kinetic characteristics of NOB enrichments were in the range of 0.5–3 mg-NO_2^--N/L. Therefore, it is possible that Reactor A intermittently fed with 1 mg-NO_2^--N/L could support the growth of both *Nitrobacter* and *Nitrospira*. More details on the kinetics of the nitrite oxidation of these enrichments are discussed in Section 2.5. A previous study demonstrated that a relative abundance of *Nitrobacter* was positively correlated with DO whereas *Nitrospira* was better adapted to low DO (Huang et al., 2010). As excess oxygen was provided in this study, the conditions (*i.e.* oxygen availability) could have been more favorable to *Nitrobacter* than *Nitrospira*. The high concentrations of DO in the systems might also help to explain the occurrence of *Nitrobacter* during the NOB enrichment at low nitrite concentrations (Fig. 3).

In the reactors enriched at high nitrite concentrations (3–100 mg-NO_2^--N/L), *Nitrobacter* clearly outcompeted *Nitrospira* in many orders of magnitude. The results are consistent with previous studies that reported the dominances of *Nitrobacter* at high nitrite concentrations (Kim and Kim, 2006; Nogueira and Melo, 2006). The presence of *Nitrospira* at high nitrite concentrations could also be derived from the uses of attached growth processes in this study. Moreover, in the thick biofilms (*e.g.* in the reactors fed with high nitrite loadings), substrate and oxygen gradients may have developed along the biofilm depth. Oxygen and substrate limitations within the biofilm might favor the growth of *Nitrospira*. However, according to a previous study that investigated oxygen mass transfer in nitrifying biofilms (Brockmann et al., 2008), oxygen should have been transferred effectively through the biofilm considering the high level of DO (5–7 mg-O_2/L) in these reactors. The formation of microenvironments within biofilms favoring interspecies competition has been shown in Mattei et al. (2015), but competition between *Nitrobacter* and *Nitrospira* within biofilms has not been investigated until now.

Nevertheless, the results still suggest the favorability of *Nitrobacter* toward high nitrite concentrations. The amount of *Nitrobacter* greatly decreased when nitrite concentrations were below 3 mg-NO_2^--N/L. From this perspective, the enrichment of NOB at low nitrite concentrations (<3 mg-NO_2^--N/L) would help prevent the *Nitrobacter* from outcompeting while retaining the *Nitrospira* that were actually observed in brackish shrimp ponds. The NOB enrichment at low nitrite concentrations is likely to benefit further applications of NOB in actual shrimp ponds.

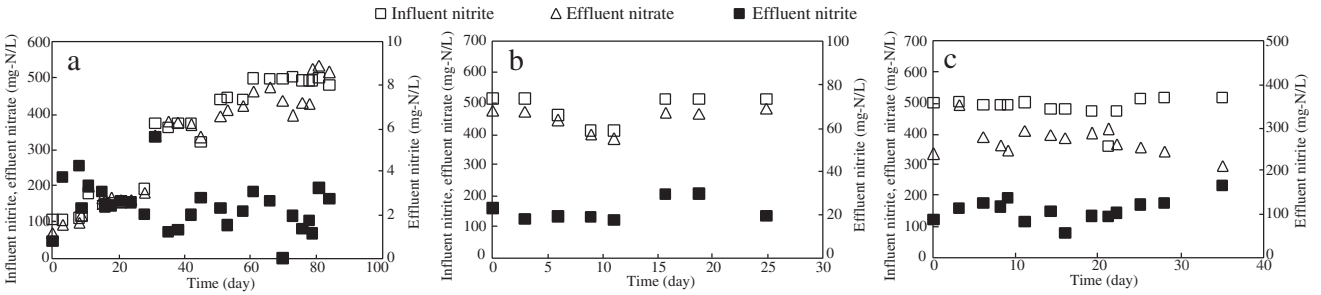

Fig. 2 – Nitrite and nitrate concentrations in the continuous-flow moving-bed biofilm reactors: (a) Reactor B3, (b) Reactor B20, and (c) Reactor B100.

Besides nitrite and DO concentrations, salinity can influence competition between *Nitrobacter* and *Nitrospira* (Moussa et al., 2006). *Nitrobacter*, not *Nitrospira*, were found at high salt levels in both long-term (one-year) and short-term (one-month) adapted nitrifying sludge (Moussa et al., 2006). However, several other studies reported the occurrence and importance of *Nitrospira* in marine environments (Haaijer et al., 2013; Keuter et al., 2011; Off et al., 2010; Watson et al., 1986). Although the nitrite concentration was the main parameter investigated in the present study, the DO concentration and salinity may also have influenced the competition between *Nitrobacter* and *Nitrospira* to some extent under the conditions used in this study.

2.4. Nitrite-oxidizing bacterial community analysis

The phylogenetic tree of the partial 16S ribosomal deoxyribonucleic acid (rDNA) of *Nitrobacter* from nitrite-oxidizing enrichments (Reactors A0.1, B3, and B100) is illustrated in Appendix A Fig. S3. All 35 sequences obtained from *Nitrobacter* clone libraries show 99% similarity of the 16S rRNA gene sequences to previously reported sequences of *Nitrobacter* in the GenBank database. The result confirmed very high specificity of the primers P338f and NIT3 to quantify and amplify *Nitrobacter* in qPCR and clone libraries. Our sequences related closely to *Nitrobacter vulgaris*, *Nitrobacter winogradskyi*, and *Nitrobacter alkalicus*.

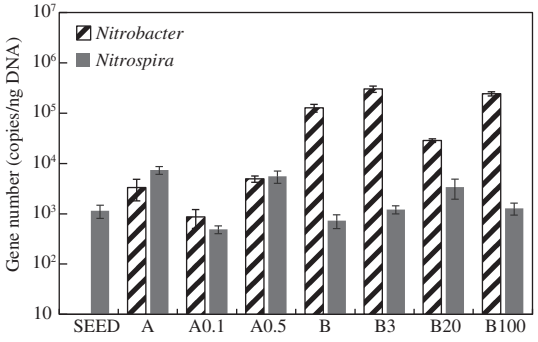

Fig. 3 – The 16S rRNA gene numbers of *Nitrobacter* and *Nitrospira* in shrimp pond sediment and the biofilms of Reactors A, A0.1, A0.5, B, B3, B20, and B100. The limits of detection (LOD) for *Nitrobacter* and *Nitrospira* were 2 gene copies/ng DNA.

The phylogenetic tree of the partial 16S rDNA of *Nitrospira* from nitrite-oxidizing enrichments (Reactors A0.1, B3, and B100) and sediment from shrimp ponds is illustrated in Appendix A Fig. S4. All sequences fell into only the *Nitrospira defluvii* lineage, suggesting that the communities of *Nitrospira* in the brackish shrimp pond sediment, A0.1, B3, and B100 samples were similar. Together with the qPCR results, it is implied that we were able to maintain NOB responsible for nitrite oxidation in the shrimp ponds in all reactors. Nevertheless, the *Nitrospira* spp. observed in this study belong to a different sublineage from those observed in the study by Srithep et al. (2015), in which *Nitrospira* spp. from the *Nitrospira moscoviensis* lineage and the *Nitrospira marina* lineage were observed in shrimp ponds.

2.5. Nitrite oxidation kinetics

The nitrite oxidation kinetics of the biofilms obtained from Reactors A0.1, A0.5, B3, B20, and B100 were investigated. The graphs of initial nitrite oxidation rates *versus* initial nitrite concentrations are plotted in Fig. 4. Table 2 summarizes the nitrite oxidation kinetic coefficients of the biofilms obtained from Reactors A0.1, A0.5, B3, B20, and B100 as described by Monod equation. The NOB enriched at low nitrite concentrations (0.1–0.5 mg-NO_2^--N/L) in Reactors A0.1 and A0.5 appear to have much lower K_s values (0.71–0.98 mg-NO_2^--N/L) than those enriched at higher nitrite concentrations (3–100 mg-NO_2^--N/L) as in Reactors B3, B20, and B100, in which the K_s values were in the range of 8.36–12.20 mg-NO_2^--N/L. Therefore, the nitrite concentrations that could result in the shifts in nitrite oxidation kinetics of the enrichments were likely to be in the range of 0.5–3 mg-NO_2^--N/L.

The results on nitrite oxidation kinetics agree well with the observed NOB populations. In general, the K_s values of *Nitrobacter* spp. (0.36–15.26 mg-NO_2^--N/L), are higher than those of *Nitrospira* spp. (0.13–1.0 mg-NO_2^--N/L) (Schramm et al., 1999; Manser et al., 2005; Laanbroek et al., 1994; Blackburne et al., 2007a,b; Vadivelu et al., 2006; Nowka et al., 2015). A recent study by Nowka et al. (2015), which compared nitrite oxidation kinetics of pure cultures of *Nitrobacter* spp. and *Nitrospira* spp., also confirmed the differences in substrate affinities of *Nitrobacter* and *Nitrospira* with the K_s values of 0.13–0.38 mg-NO_2^--N/L for *Nitrospira* and 0.67–7.6 mg-NO_2^--N/L

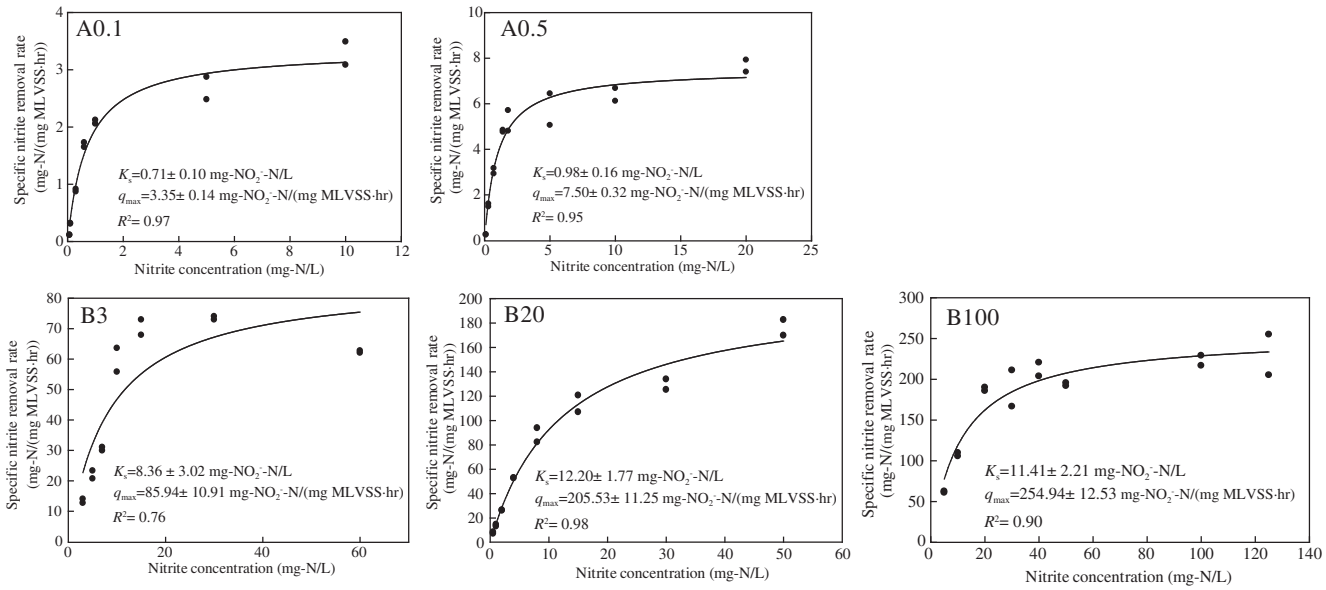

Fig. 4 – Nitrite oxidation kinetics of the biofilms from Reactors A0.1, A0.5, B3, B20, and B100 described by Monod kinetics.

for *Nitrobacter*, respectively. The NOB enrichments that possess high K_s values (Reactors B3, B20, and B100) were found to be dominated by *Nitrobacter*, while for those with low K_s values (Reactors A0.1 and A0.5), both *Nitrospira* and *Nitrobacter* coexisted. The results also support the K/r hypothesis that *Nitrobacter* tend to thrive at high nitrite concentrations due to their high K_s and q_{max} values.

A study on the metagenome of *Nitrospira defluvii*, a representative of *Nitrospira*, which was also observed in this study, has revealed that *Nitrospira* are extremely different from *Nitrobacter* in the key metabolic pathways, such as the enzyme nitrite oxidoreductase (NXR), the composition of the respiratory chain, and the pathway for autotrophic carbon fixation (Lücker et al., 2010). The different orientation of NXR attached to the cytoplasmic membranes in *Nitrobacter* and *Nitrospira* has been suggested to contribute to the differences in the substrate affinities of *Nitrobacter* and *Nitrospira* (Lücker et al., 2010; Spieck et al., 1996, 1998). Moreover, the NXR in *Nitrobacter* and *Nitrospira* appear to have vastly different evolutionary histories (Lücker et al., 2010). The comparative genomics strongly indicated an occurrence of the horizontal gene transfer of the genes involved in nitrite oxidation between Anammox organisms and *Nitrospira* (Lücker et al., 2010). In contrast, it has been proposed that *Nitrobacter* originated from photosynthetic ancestors (Teske et al., 1994). The findings on the metabolic pathways and evolution of *Nitrobacter* and *Nitrospira* have provided a strong supportive explanation on the different nitrite oxidation kinetics observed in *Nitrobacter* and *Nitrospira*.

The results from this study have clearly shown the connections between nitrite-oxidizing bacterial communities and their corresponding kinetics. To achieve certain desirable activities and kinetics, appropriate microbial communities are required. In addition, the results suggest the important roles of nitrite concentrations during the NOB enrichment, as it can significantly influence NOB populations and kinetics, which are likely to affect its applications in shrimp ponds.

3. Conclusions

Nitrite concentrations have a considerable effect on nitrite-oxidizing bacterial populations and nitrite oxidation kinetics. The NOB enriched at high nitrite concentrations

Table 2 – The half saturation coefficients (K_S) of nitrite oxidation and the maximum specific rate of nitrite oxidation (q_{max}) of biofilms from different continuous-flow moving-bed biofilm reactors.

Reactor	Effluent nitrite concentration	Half saturation coefficient, K_s	Maximum specific rate of nitrite oxidation, q_{max}	R^2
	Average ± SD (mg-NO_2^--N/L)	Average ± SD (mg-NO_2^--N/L)	Average ± SD (mg-NO_2-N/mg MLVSS-hr)	
A0.1	0.11 ± 0.01	0.71 ± 0.10	3.35 ± 0.14	0.97
A0.5	0.47 ± 0.06	0.98 ± 0.16	7.50 ± 0.32	0.95
B3	2.47 ± 1.04	8.36 ± 3.02	85.94 ± 10.91	0.76
B20	21.89 ± 5.00	12.20 ± 1.77	205.53 ± 11.25	0.98
B100	107.91 ± 26.94	11.41 ± 2.21	254.94 ± 12.53	0.90

SD: standard deviation; MLVSS: mixed liquor volatile suspended solids.

(3–100 mg-NO_2^--N/L) resulted in the predominance of *Nitrobacter* with high q_{max} and K_s (8.36–12.20 mg-NO_2^--N/L). In contrast, the NOB enriched at low nitrite concentrations (0.1–0.5 mg-NO_2^--N/L) led to the coexistence of *Nitrospira* and *Nitrobacter* with low K_s values (0.71–0.98 mg-NO_2^--N/L). According to the NOB population and kinetic results, the NOB enriched at low nitrite concentrations (<0.5 mg-NO_2^--N/L) should be more appropriate for further uses in actual shrimp ponds.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jes.2015.07.014.

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