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## Nitrification Potential of Mud Sediment of the Ariake Sea Tidal Flat and the Individual Effect of Temperature, pH, Salinity and Ammonium Concentration on its Nitrification Rate

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**Abstract:** The aims of this study were to know the nitrification potential of the mud sediment of the Ariake sea tidal flat, and to investigate the individual influence of environmental parameters such as temperature, pH, salinity and ammonium concentration on its nitrification rate. The Ariake Sea is a semi-closed shallow sea with macro-tidal and the largest tidal flat in Japan. Nitrification Potential Rate (NPR) and Nitrification Rate (NR) of the tidal flat mud sediment were determined by amending sodium chlorate to inhibit the oxidation of NO<sub>2</sub>-N to NO<sub>3</sub>-N. NPRs of the sediment were 91.4, 71.2 and 64.8 nmol N/g Dry Weight Sediment (DWS)/h at the depth of 0-3, 3-6 and 6-9 cm, respectively. NRs were relatively high in the temperatures range of 20-35°C with the optimum temperature of 29.3°C. Relatively high NRs were determined in the range of pHs 7.5-8 with the optimum pH 7.7. The optimum salinity for nitrification was 15 ppt. NRs increased drastically in the range of salinity 10 to 15 ppt, but it decreased sharply at 15 to 20 ppt. Relationship between ammonium concentration and NR resulted a hyperbolic Michaelis-Menten type curve ( $R^2 = 0.98$ ) with the maximum NR rate ( $V_{max}$ ) 32.5 nmol N/g DWS/h at NH<sub>4</sub>-N concentration 3,000 mM and the half saturation constant ( $K_s$ ) 700 mM NH<sub>4</sub>-N. To our knowledge, this is the first report on nitrification of mud sediment of the Ariake sea tidal flat, and its optimal temperature, pH, salinity and NH<sub>4</sub>-N concentration as a single parameter.

**Key words:** Environment parameter, mud sediment, nitrification, the Ariake sea, tidal flat

### INTRODUCTION

The Ariake Sea located in the west parts of Kyushu island, Japan, is a semi-closed shallow sea with macro-tidal (in the range of 3-6 m) (Kato and Seguchi, 2001; Hiramatsu *et al.*, 2005) and several well-mixed estuaries. This sea covers 1,700 km<sup>2</sup> of a long inner bay with 96 km of the bay axis and 18 km of the average width. A vast tidal flat area that covers almost 40% of the total tidal flat in Japan develops in this sea. The sediments that are transported by several rivers to the bay reach around 440,000 ton per annum. The coarse sediment settles in the eastern parts of the bay, but the fine sediment develops the Ariake clay formation and mud tidal flat along the western shoreline of this area (Kato and Seguchi, 2001).

The unique ecosystem of the Ariake sea with estuaries and tidal flat area provides nutrients supply (i.e., nitrite, nitrate, ammonium, organic nitrogen and phosphorus) which are responsible for its productivity.

This tidal flat is the main area for sea lavers (*Porphyra* sp.) production in Japan, which contributes 40% of the total Japanese sea Lavers production (Yanagi and Abe, 2005).

The most important nutrient for production of *Porphyra* sp. is nitrogen either NO<sub>3</sub><sup>-</sup> or NH<sub>4</sub><sup>+</sup>, but NO<sub>3</sub><sup>-</sup> is a better source of N in terms of growth (Hafting, 1999). NO<sub>3</sub><sup>-</sup> and NH<sub>4</sub><sup>+</sup> uptakes in six *Porphyra* species occur at similar rates (Kraemer *et al.*, 2004). Nitrogen uptake in *Porphyra* is also affected by the frequency and duration of immersion and exposure (Kim *et al.*, 2008), which occur naturally in the high tidal range area as found in the Ariake sea.

Nitrification is the microbial oxidation of ammonia (NH<sub>3</sub><sup>-</sup>) to nitrite (NO<sub>2</sub><sup>-</sup>) and subsequently to nitrate (NO<sub>3</sub><sup>-</sup>). Rate of the ammonium oxidation is significantly affected by the nature of nitrifying bacteria and a variety of environmental factors. However, little report on the single effect of environmental factors on nitrification especially in the marine system is found. The overall

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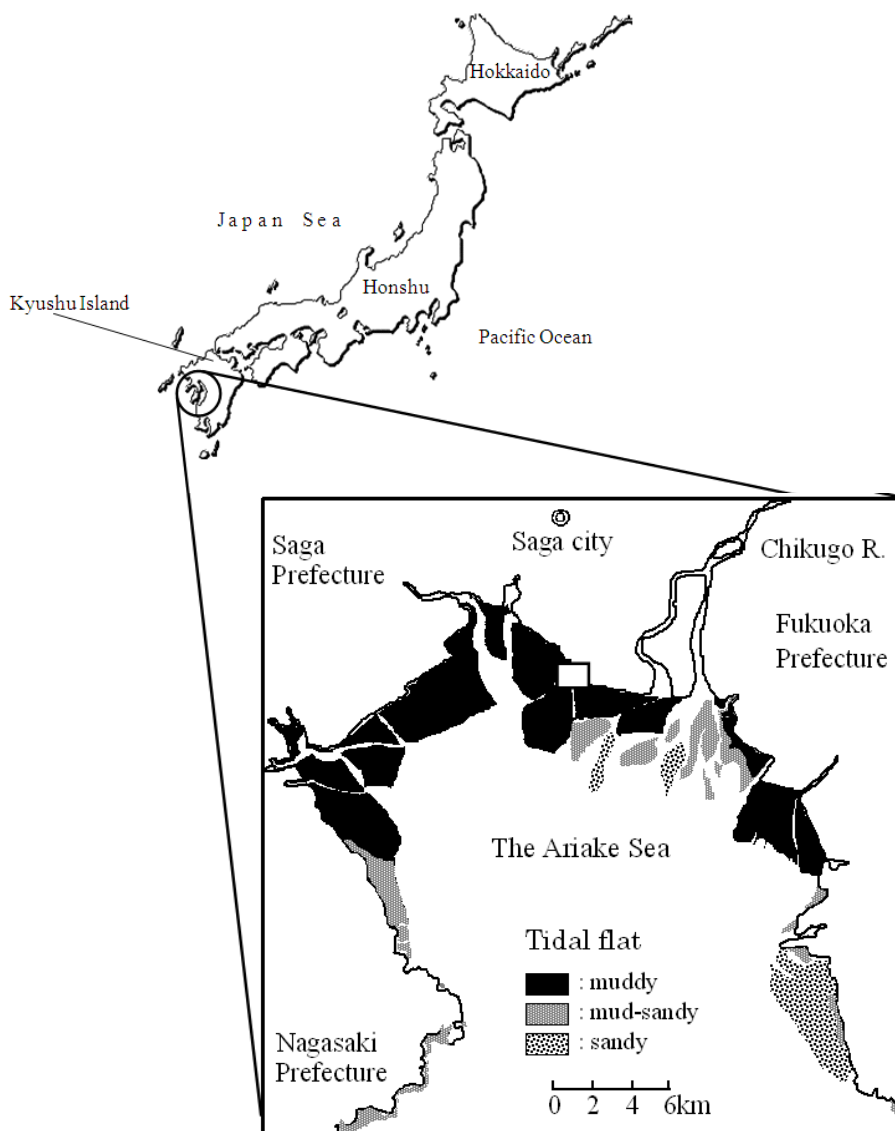


Fig. 1: Distribution of sediment in the Ariake sea tidal flat (Koga *et al.*, 2009). The sampling point is indicated by an open square

objective of this study was to investigate the individual effect of environmental factors such as temperature, pH, salinity as well as ammonium ( $\text{NH}_4\text{-N}$ ) concentration on the heterotroph/chemoliterotroph nitrification kinetic of mud sediment of the Ariake sea as the major tidal flat sediment in the inner part of this area.

## MATERIALS AND METHODS

**Sediment sampling:** Mud sediment samples used in this study were collected in Higashi Yoka in the intertidal zone at low tide, on December 2, 2008 for NPR experiment, and between January 29 and April 22, 2009 for NR experiments. Higashi Yoka was located around 8

km south of Saga City (Fig. 1). Sample was taken by mean of an acrylic core with the inner diameter of 85 mm. The cores were transported to the laboratory in a cool box.

**Pore water geochemistry:** The sediment sample was placed into a polypropylene centrifuge tube and tightly capped. After centrifugation at 4,000 g for 15 min, the supernatant was collected, filtered through 0.45  $\mu\text{m}$ -pore-size cellulose ester filter (Advantec, Toyo Roshi Kaisha, Tokyo, Japan), and frozen immediately until analysis (Lerat *et al.*, 1990; Nissenbaum *et al.*, 1990; Magni and Montani, 2006).  $\text{NH}_4\text{-N}$ ,  $\text{NO}_2\text{-N}$ ,  $\text{NO}_2+\text{NO}_3\text{-N}$ ,  $\text{PO}_4$ , Total Nitrogen (TN) and Total Phosphate (TP) were analyzed by an automated water analyzer (Water auto-analyzer,

swAAT, BLTEC, Tokyo, Japan).  $\text{NH}_4\text{-N}$  concentration was determined by the method of the alkali phenol-hypochlorite reaction detected photometrically at 630 nm.  $\text{NO}_2\text{-N}$  concentration was analyzed by diazotizing with sulfanilamide and coupling with N-(1-naphthyl) ethylenediamine dihydrochloride to form a highly colored azo dye and detected photometrically at 550 nm.  $\text{NO}_3\text{-N}$  was determined by the same method for  $\text{NO}_2\text{-N}$  after  $\text{NO}_3$  was reduced by the cadmium reduction process.  $\text{PO}_4$  was determined by the ascorbic acid method at 800 nm. TN and TP concentrations were measured by peroxodisulfate oxidation (Ebina *et al.*, 1980).

**Density of ammonium-oxidizing bacteria (AOB) in mud samples:**

Density of ammonium-oxidizing bacteria was determined by the Most Probable Number (MPN) method in 1.5 mL sterile microtubes. The microtubes were filled with 900 mL sterile medium for ammonia-oxidizing bacteria as described by Cote and Gherna (1994). The medium was composed with  $(\text{NH}_4)_2\text{SO}_4$ , 1.32 g/L;  $\text{KH}_2\text{PO}_4$ , 20 mg/L;  $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$ , 0.1 g/L;  $\text{FeCl}_2\cdot 6\text{H}_2\text{O}$ , 0.014 g/L;  $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$ , 0.18 g/L;  $\text{Na}_2\text{MO}_4\cdot 2\text{H}_2\text{O}$ , 100 mg/L; EDTA, 1.0 mg/L, phenol red, 0.002 g/L, dissolved in 70% artificial sea water (Tetramarin Salt Pro, USA). The medium was adjusted to pH 8 with  $\text{Na}_2\text{CO}_3$ . One gram of mud was suspended with 9 mL sterile ASW, and 100 mL of the suspension was used to inoculate the microtube in triplicates, and serially tenfold diluted. Incubation was carried out at 25°C for 20 days. The tubes which exhibited the color change from red to yellow due to acid production, were tested by adding three drops of a nitrite color reagent (sulfanilamide, 10 g/L; *n*-(1-naphthyl)-ethylenediamine 2 HCl, 0.50 g/L; concentrated HCl, 100 mL/L). Tubes that exhibited a red color after addition of the reagent were scored positive for nitrite. The bacterial density was calculated by the MPN formula in Visual Basic program (Koch, 1994).

**Nitrification potential rate (NPR):** NPR was determined by the method described previously (Bianchi *et al.*, 1997; Welsh and Castadelli, 2004; Dollhopf *et al.*, 2005). Core samples were divided into three sections, 0-3, 3-6 and 6-9 cm of sediment depth intervals. The samples were homogenized and slurried (20.0 g of wet sediment/200 mL of sterile artificial seawater) and placed into 300 mL Erlenmeyer. Duplicate flasks from each depth were amended with ammonium  $((\text{NH}_4)_2\text{SO}_4$ ; 500 mM) (Wako Pure Chemical Industries Ltd., Osaka, Japan) and sodium chlorate ( $\text{KClO}_3$ ; 10 mM) (Wako Pure Chemical Industries Ltd.). Control flasks contained ammonium, sodium chlorate, and allylthiourea (ATU; 20 mg/L) (Sigma-Aldrich, St. Louis MO, USA) (Belser and Mays, 1980; Dollhopf *et al.*, 2005). Flasks were capped with aluminum foil and incubated in the dark at 25°C with

constant stirring with a magnetic stirrer at 100 rpm for 72 h. Samples were collected at intervals over the incubation time.  $\text{NO}_2\text{-N}$  was determined in interstitial waters of the slurry samples after filtering through 0.45  $\mu\text{m}$  cellulose ester membrane filter (Advantec, Toyo Roshi Kaisha, Japan). Since sodium chlorate is a specific inhibitor of nitrification, which blocks the oxidation of  $\text{NO}_2\text{-N}$  to  $\text{NO}_3\text{-N}$  (Belser and Mays, 1980), in the presence of 10 mM chlorate, NPR can be determined as the linear accumulation of nitrite with time (Welsh and Castadelli, 2004; Dollhopf *et al.*, 2005). The linear regression analyses were done after the  $\text{NO}_2$  concentrations in all treatments were corrected by subtracting its concentration in the ATU control flask.

**Effects of temperature, pH, and salinity on Nitrification Rate (NR):**

The sample used in these experiments was mud obtained from cores with 0-3 cm of sediment depth. The experiments were carried out by the method as used in the NPR experiment without emendation of  $(\text{NH}_4)_2\text{SO}_4$ . Incubation was carried out at 25°C except for temperature experiment, which were incubated at 5, 10, 15, 20, 25, 30, 35 and 40°C. For pH experiment the artificial seawater was buffered with 0.1 mM tris-HCl buffer (Kanto Chemical Co. Inc., Tokyo, Japan) and the pHs were adjusted at 7, 7.5, 8, 8.5 and 9. Salinity experiment was conducted by adjustment the salinity of artificial seawater at 10-38 ppt. pH and salinity were measured by a pH meter (ION meter IM-20E, TOA electronics Ltd., Tokyo, Japan) and a hand refractometer (ATC-S/Mill-E, ATAGO Co. Ltd, Tokyo, Japan), respectively.

**Effect of ammonium concentrations on nitrification rate (NR):**

The experiment used the same method as described above with  $\text{NH}_4\text{-N}$  addition at various concentrations. Since the mud sample contained  $\text{NH}_4\text{-N}$ , the regression analyses were carried out based on the actual concentration of  $\text{NH}_4\text{-N}$  in each treatment determined at the initial time of the experiment (0 h) rather than the concentrations of  $\text{NH}_4\text{-N}$  added.

## RESULTS

$\text{NO}_3\text{-N}$  and  $\text{NO}_2\text{-N}$  concentrations in pore water were low with averages of 0.1 and 1.7  $\mu\text{g/L}$ , respectively (Table 1).  $\text{NH}_4\text{-N}$  that was the dominant fraction of dissolved inorganic nitrogen in the pore water ranged from 22.5 to 232.6  $\mu\text{g/L}$  with an average concentration of 66.3  $\mu\text{g/L}$ . The average concentration of phosphate was 23.0  $\mu\text{g/L}$  with the maximum concentration 49.1  $\mu\text{g/L}$  sediment. Density of AOB ranged from  $7.17 \times 10^3$ - $1.64 \times 10^5$  cells/g of wet sediment. The density had a tendency to be increase in the end of April 2009.

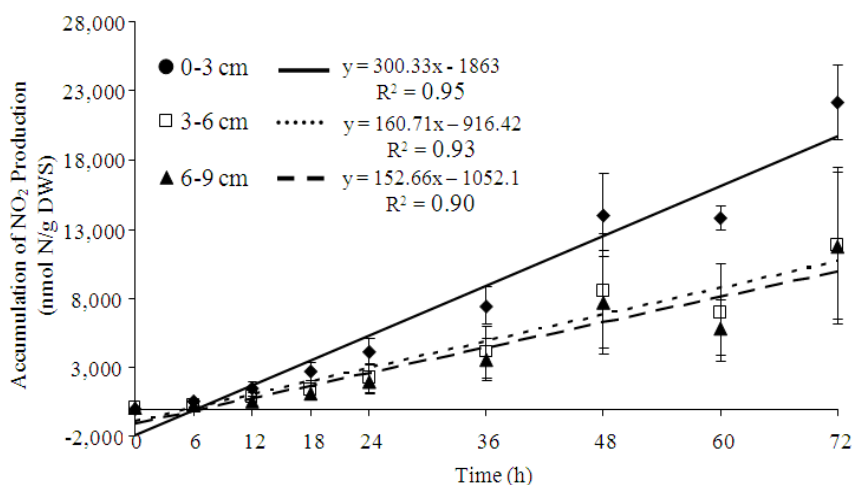


Fig. 2: Nitrification potential rate of the Ariake mud sediment at various core depths, determined by the accumulation of NO<sub>2</sub>-N production for 72 h incubation

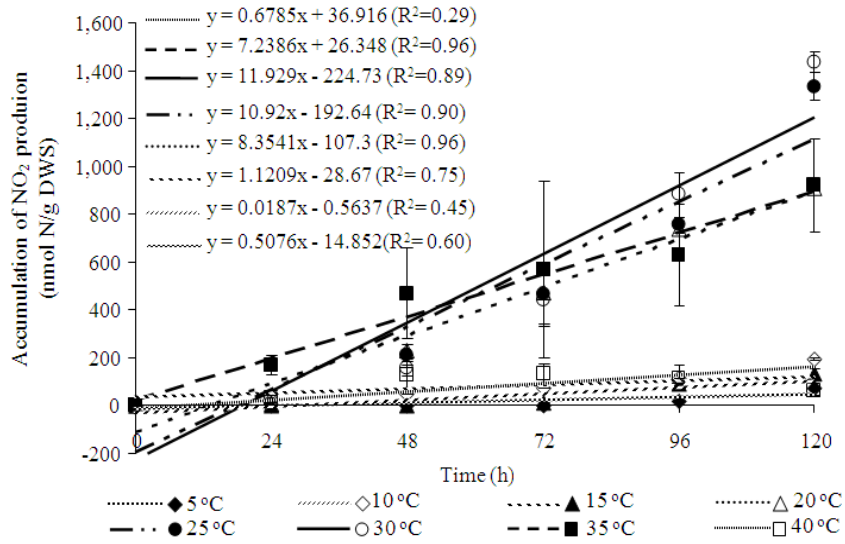
Table 1: Pore water geochemistry and ammonium-oxidizing bacteria density of the Ariake sea mud sediment collected from Higashi Yoka

	Average	Range
NO <sub>3</sub> -N (µg/L)	0.1	Undetectable-0.2
NO <sub>2</sub> -N (µg/L)	1.7	1.1-3.8
NH <sub>4</sub> -N (µg/L)	66.3	22.5-232.6
PO <sub>4</sub> (µg/L)	23.0	11.9-49.1
Total Nitrogen (µg/L)	1100.8	579.8-1401.2
Total Phosphate (µg/L)	101.1	85.4-128.4
pH	8.3	8.2-8.5
Salinity (ppt)	25.81	8.0-38.0
Density of ammonium-oxidizing bacteria (cells/g of wet sediment)*	6.09×10 <sup>4</sup>	7.17×10 <sup>3</sup> -1.64×10 <sup>5</sup>

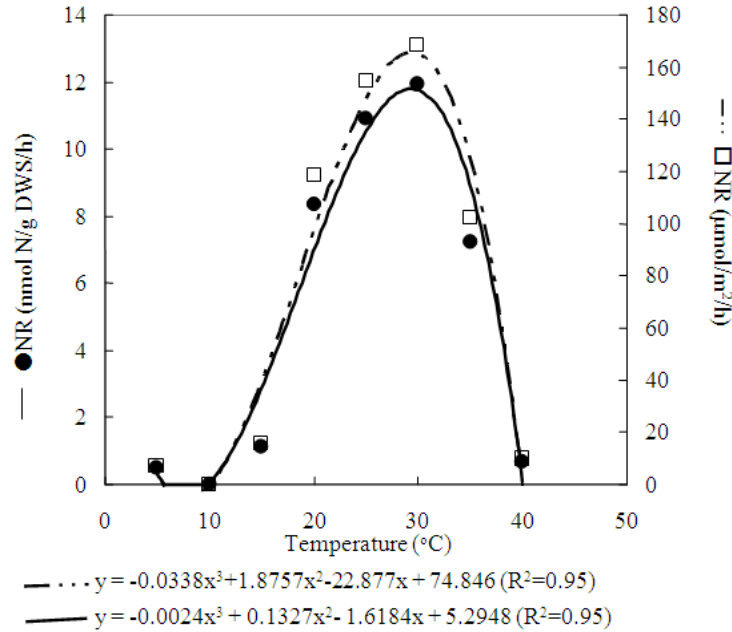
In this study, sodium chlorate was used to inhibit the oxidation of NO<sub>2</sub>-N to NO<sub>3</sub>-N. It allows the nitrification could be determined by measuring the accumulation of NO<sub>2</sub>-N production. NPR that measures the NR without limitation of ammonium and O<sub>2</sub> differed in the range of sediment depths of 0-3, 3-6 and 6-9 cm. The upper layer of the sediment showed higher NR, 91.4 nmol N/g dry weight sediment (DWS)/h (Fig. 2). The lower layers of sediment at the depth of 3-6 and 6-9 cm showed lower NPR, 71.2 and 64.8 nmol/g DWS/h, respectively. NO<sub>2</sub>-N as the product of nitrification at all the range of sediment depths was linearly accumulated indicating no growth of ammonium-oxidizing bacteria during 72 h incubation. The NO<sub>2</sub> accumulation and incubation time also showed a high correlation indicated by linier regression lines with coefficient correlation (R<sup>2</sup>) ranging from 0.86 to 0.95. The accumulation of nitrogen measured as NO<sub>2</sub> reached 6.7±1.7, 5.2±2.0 and 5.2±0.9 mmol N/g DWS during 72 h incubation for the depth of 0-3, 3-6 and 6-9 cm, respectively. Production of NO<sub>2</sub>-N was significantly affected by temperature. Relatively high NO<sub>2</sub>-N productions were observed at the temperature range of 20-35°C. The highest NO<sub>2</sub>-N production occurred at 30°C

which reached 1436.8 nmol N/g DWS for 120 h incubation (Fig. 3A). The NO<sub>2</sub>-N production was very low in the range of 3.1 to 133.5 nmol N/g DWS at 5-15°C and 40°C. As indicated by the NO<sub>2</sub>-N production, the NRs were also clearly affected by temperature. The highest NR, 11.9 nmol N/g DWS/h equivalent to 168.6 mmol N/m<sup>2</sup>/h, was also obtained at 30°C. Incubation at 20, 25 and 35°C gave lower NRs, 8.4, 10.9 and 7.2 nmol N/g DWS/h, respectively. The NRs were very low ranging from 0.02 to 1.12 nmol N/g DWS/h when the incubation was carried out at 5, 10 and 40°C (Fig. 3B). Based on the curve in Fig. 3B, the maximum NR, 11.4 nmol N/g DWS/h equal to 164.6 mmol N/m<sup>2</sup>/h, was obtained at 29.3°C.

The accumulation of NO<sub>2</sub>-N productions during 120 h (Fig. 4A) incubation at pH 7, 7.5, 8, 8.5 and 9.0, and their linier regression indicated that the NRs were greatly affected by pH. High NRs were found at pH 7.5 and 8.0, which reached 4.9 and 6.6 nmol N/g DWS/h, respectively. The later was the highest NR within the pH range of 7.0 to 9.0. Incubation at pH 7.0 and 8.5 resulted similar NRs, approximately 2.2 nmol N/g DWS/h. A lower NR was obtained at pH 9. The estimated maximum NR based



(A)



(B)

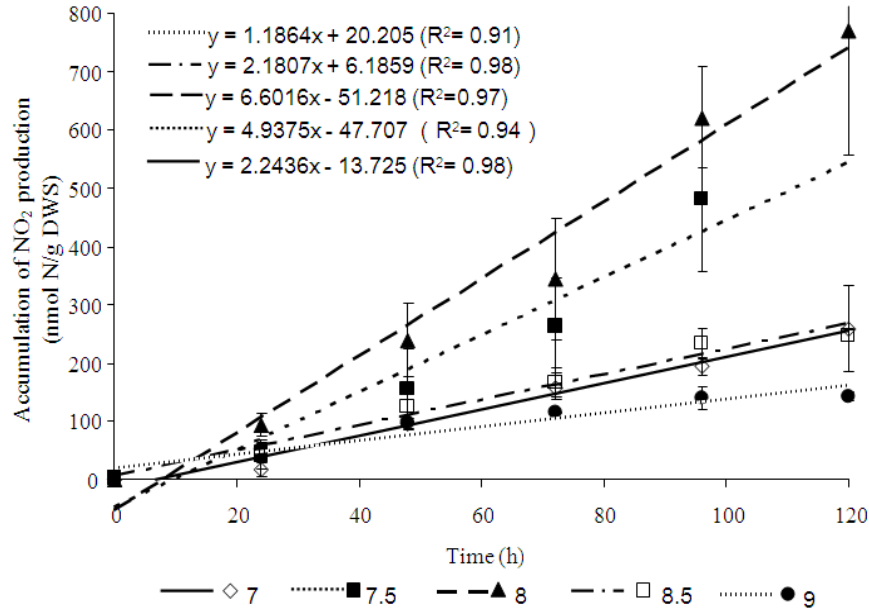
Fig. 3: Nitrification rate of the Ariake mud sediment at various temperatures, determined by the accumulation of  $\text{NO}_2$  production for 120 h incubation (A). Relationship between temperatures and nitrification rates of the Ariake sea mud sediment (B)

on the curve depicted in Fig. 4B, was 5.97 nmol N/g DWS/h equivalent to 81.6 mmol N/m<sup>2</sup>/h obtained at pH 7.7.

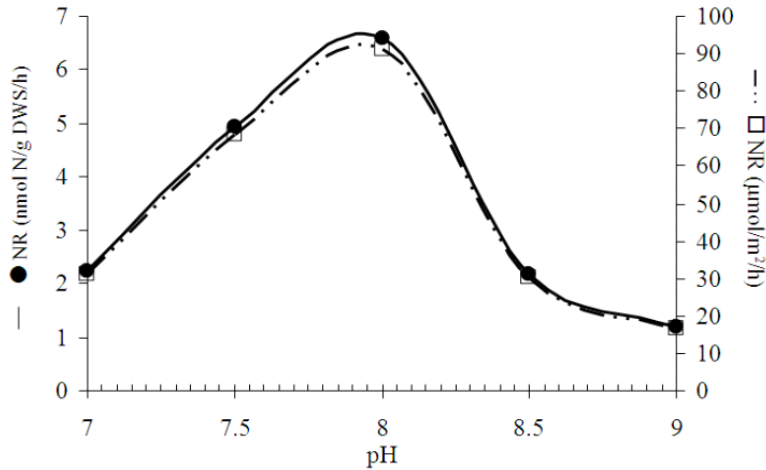
Salinity significantly affected the NR. The NRs varied between 3.12 to 14.5 nmol N/g DWS/h at the salinity range of 10 to 38 ppt (Fig. 5A). The highest NR occurred at 15 ppt, which reached 14.5 nmol N/g DWS/h.

NRs increased drastically in the salinity range of 10 to 15 ppt, but it decreased sharply in the range of 15 to 20 ppt. Relatively stable low NRs ranging from 3.1 to 4.5 nmol N/g DWS/h were observed at the salinity range of 20 to 38 ppt (Fig. 5B).

Ammonium concentrations up to approximately 1,500 mM increased exponentially the accumulation of



(A)



(B)

Fig. 4: Nitrification rate of the Ariake mud sediment at various pHs, determined by the accumulation of  $\text{NO}_2$  production for 120 h incubation (A). Relationship between pHs and nitrification rates of the Ariake sea mud sediment (B)

$\text{NO}_2$ -N production. The accumulation rate was lower at  $\text{NH}_4$ -N concentration around 1,500 to 2,800  $\mu\text{M}$ . The production was relatively constant at the  $\text{NH}_4$ -N concentration above 2,800  $\mu\text{M}$  (Fig. 6). The similar pattern was also found in the NR by the increase of this concentration. The highest NR, 33.8 nmol/g DWS/h, was found when the ammonium concentration was 3,043  $\mu\text{M}$ . Relationship between ammonium concentration and NR resulted a hyperbolic Michaelis-Menten type curve ( $R^2 = 0.98$ ) (Fig. 6) with 32.5 nmol N/g DWS/h of the

maximum NR rate ( $V_{\text{max}}$ ) at  $\text{NH}_4$  concentration 3,000  $\mu\text{M}$ . The half saturation constant ( $K_s$ ) of the nitrification was determined at  $\text{NH}_4$  concentration 700  $\mu\text{M}$ .

## DISCUSSION

Nitrogen is often to be the limiting factor of primary production in marine environments (Howarth and Marino, 2006). Since nitrate and ammonium are two nitrogen fractions to be up taken by *Porphyra* (Hafting, 1999;

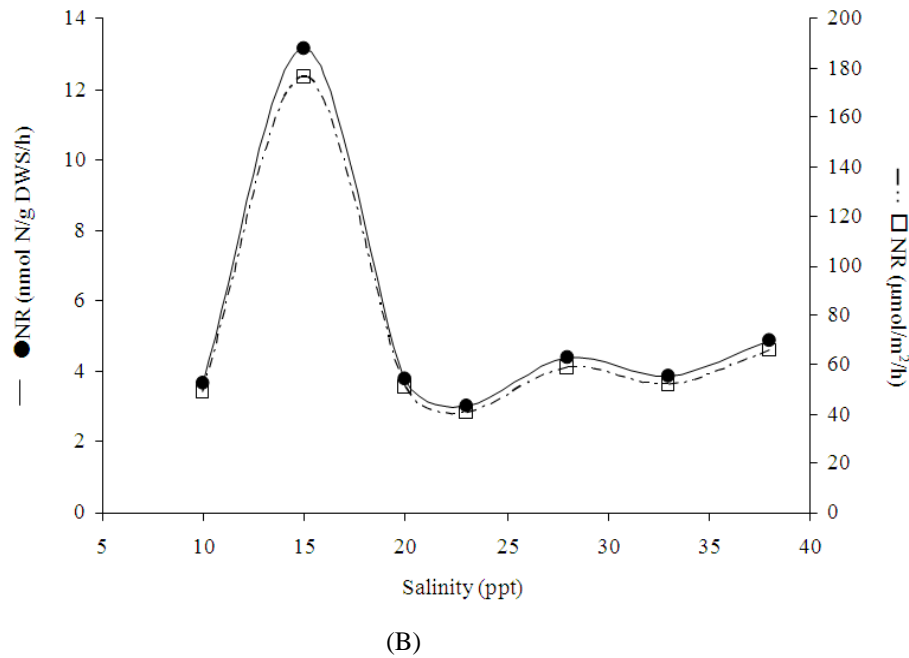
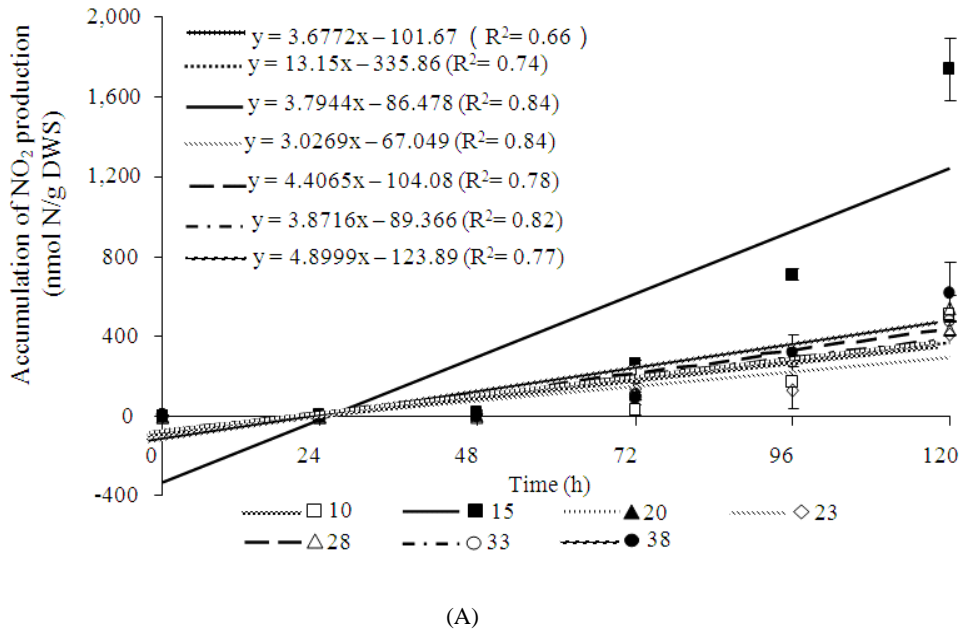


Fig. 5: Nitrification rate of the Ariake mud sediment at various water salinities, determined by the accumulation of  $\text{NO}_2$  production for 120 h incubation (A). Relationship between water salinities and nitrification rates of the Ariake sea (B)

Kraemer *et al.*, 2004), these fractions are likely to be the most critical limiting factors in producing the seaweed in the Ariake sea. Sharp ammonium depletion in mud sediment pore water (Koga *et al.*, 2009) and the sea water, and nitrogen deficiency in *Porphyra* often occur in winter when this seaweed is cultured intensively in this area. Low ammonia concentration in the Ariake sea water and

pore water in winter (Hayami *et al.*, 2009; Koriyama *et al.*, 2009) indicates that the uptake of this inorganic nitrogen is likely predominated by *Porphyra*. The high uptake of ammonium increases the diffusion of ammonium from sediment into the water layer. The other factors are low ammonification rate in sediment at a low temperature in winter and low inflow of river and rain

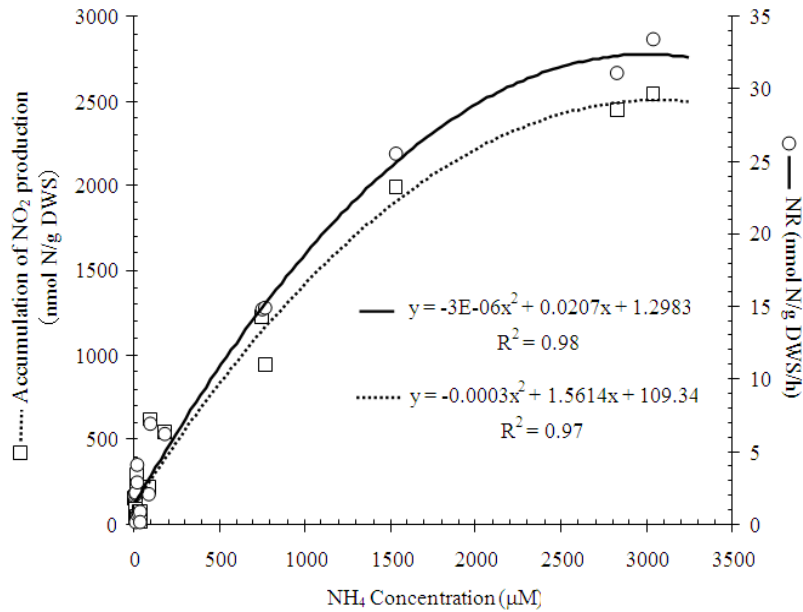


Fig. 6: Accumulation of NO<sub>2</sub> production during 120 h incubation at various concentration of NH<sub>4</sub>-N (solid line), Nitrification rate of the Ariake Sea mud sediment of various concentration of NH<sub>4</sub>-N (broken line)

water (run off) from surrounding terrestrial area in this season (Yamamoto and Yokohama, 2005).

As ammonium and nitrate are the substrate and final product of nitrification, respectively, nitrification is likely to be one of the important processes for supplying nitrate in *Porphyra* culture and generally in the nitrogen cycle in the Ariake sea. Inorganic nitrogen in pore water of the Ariake mud sediment is dominated by ammonium (Table 1). Ammonium is uptaken by phytoplankton and predominantly by *Porphyra*. On the other hand, ammonia is also oxidized to nitrate by nitrification. Nitrate is the best source of N for the growth of *Porphyra* (Hafting, 1999). Hence, nitrification in the Ariake sea area is responsible to clean up and detoxify ammonia as well as generate nitrate for better growth of the seaweed.

The density of Ammonium-Oxidizing Bacteria (AOB) in the Ariake sea mud sediment estimated by MPN method ranged from  $7.17 \times 10^3$  to  $1.64 \times 10^5$  cells/g of wet sediment. This result is comparable to the bacterial density determined by ammonia monooxygenase (*amoA*) gene copy numbers in salt marsh sediments, which ranges from  $5.6 \times 10^4$  to  $1.3 \times 10^6$  cells/g of wet sediment (Dollhopf *et al.*, 2005). Hoefel *et al.* (2005) reported that the densities of AOB determined by MPN method are several orders of magnitude lower than that of detected by quantitative PCR (qPCR). It suggests that the molecular techniques are necessary to enumerate AOB in the mud sediment of the Ariake sea, as the techniques are more sensitive than culture-dependent technique.

Besides nitrogen, phosphate is considered to be the major limiting nutrient for the growth of algae and phytoplankton. Iwasaki and Matsudaira (1956) indicated that the uptake of phosphate by *Porphyra tenera* increases by increasing the phosphate concentration in seawater up to 10 µg/L, but the rate decreases at higher phosphate concentration. The narrow range of phosphate concentration that increases its uptake is likely caused by the inability of *Porphyra* to store the excessive phosphate concentration (Hafting, 1999). Furthermore, the addition of potassium phosphate (monobasic) and β-glycerophosphate at 1 mg/L increase slightly its photosynthesis rate (Iwasaki and Matsudaira, 1957). Different physiology of phosphate uptake was observed by Gordillo *et al.* (2002) in a brown alga, *Fucus serratus* showing double Michaelis-Menten curves with 0.21 and 2.15 µmol/g fresh weight/h of Vmax (maximum uptake rate) and 19 and 65 µM soluble reactive phosphate of K<sub>0.5</sub> (semi-saturation constant). A narrow range of phosphate concentration from 0.3 to 0.5 pM that increases the growth rate of a macroalga, *Sargassum baccularia* has also been reported by Schaffelke and Klumpp (1998). Although we have not found any reference describing the phosphate requirement of *Porphyra*, phosphate concentration in the Ariake sea is unlikely to be the limiting factor for the growth of this macroalga. Hayami *et al.* (2009) reported the fluctuation of PO<sub>4</sub>-P in inner parts of the Ariake sea is in the range of 20 to 175 µg/L. The PO<sub>4</sub>-P concentration is much higher in the sediment pore water (Koriyama *et al.*, 2009).



NPR, a reliable indicator of nitrifying bacteria population (Jenkins and Kemp, 1984) indicates the NR without limitation of ammonia and oxygen concentrations. NPR can be used to estimate actual in situ NR, when the depth of oxygen penetration into the sediment is known (Henriksen *et al.*, 1981). In this study, ammonia was amended by adding  $(\text{NH}_4)_2\text{SO}_4$ , and oxygen was supplied by continuous stirring. NPRs of mud sediment of the Ariake sea varied in the different sediment depths of 0-3, 3-6 and 6-9 cm, which reached 91.4, 71.2 and 64.8 nmol/g DWS/h, respectively. Relatively high NPRs in the mud sediment suggest the high population of nitrifying bacteria.

The nitrification still occurs even in the sediment depth of 6-9 cm indicating the presence of the bacteria in the sample. There are at least three reasons to figure out nitrification in the lower depth sediment, which is usually unoxygenated well. Firstly, nitrifying bacteria are able to tolerate and actively grow under a very low oxygen condition (Caffrey *et al.*, 2003). Secondly, there is perturbation that allow the oxygen penetrate to the deeper sediment. In the Ariake sea, the bioturbation in the mud sediment is likely contributed mainly by the population of burrowing organisms such as mud skipper (Japanese: *mutugoro*) (*Boleophthalmus pectinirostris*), worm and several species of crabs. Thirdly, physical disturbance due to the high tidal range and the exposure to the air of this tidal flat allow penetration of oxygen to the deeper sediment. Henriksen *et al.* (1981) also found nitrifying bacteria in anoxic sandy and muddy sediments. Diverse community of ammonia-oxidizing bacteria population including *Nitrosomonas* and *Nitrospira* in anoxic marine sediment have reported by Freitag and Prosser (2003). Infaunal borrows also play an important role in  $\text{O}_2$  transport to lower layer of sediment. Real-time quantitative PCR analysis indicates that AOB 16S rRNA gene copy numbers in the burrow walls were comparable with those in the sediment surfaces. Furthermore, higher  $\text{NH}_4^+$  consumption activity at the burrow wall than those at the surrounding sediment (Satoh *et al.*, 2007). Nitrification potential rate is strongly correlated and enhanced by macrofaunal burrow abundance and activity (Dollhopf *et al.*, 2005).

In the last decade, anoxic nitrification with manganese oxide as the terminal electron acceptor has been reported (Hulth *et al.*, 1999; Mortimer *et al.*, 2002, 2004; Bartlett *et al.*, 2008). Bartlett *et al.* (2008) also proved that this anoxic nitrification is a microbially mediated reaction, and its significance and persistence depend on the frequency and magnitude of sediment perturbation. This anoxic nitrification is necessary to be investigated in the Ariake mud sediment in the further study.

The rate of nitrification is significantly affected by the nature of nitrifying bacteria and a variety of environmental factors, such as substrate concentration

(Kim *et al.*, 2008; Miranda *et al.*, 2008), dissolved oxygen (DO) (Kemp and Dodds, 2002), temperature, pH, salinity (Jones and Hood, 1980, Kim *et al.*, 2008; Miranda *et al.*, 2008), organic carbon (C) availability and CN ratio (Strauss and Dodds, 1997; Strauss *et al.*, 2002). Most of these literatures reported the effect of environment factors in fresh water and water treatment systems. In addition, the individual effect of various environmental factors on nitrification of marine sediment is little known. In this study, we investigated the single effects of temperature, pH, salinity and substrate concentration on the NR of the Ariake sea mud sediment.

NRs of the Ariake sea mud sediment were very low at temperatures ranging from 5 to 15°C and at 40°C. The rate increased drastically by increasing temperature from 15 to 29.3°C, but decreased by increasing temperature above 29.3°C (Fig. 3). Kim *et al.* (2008) reported similar finding, in which ammonia oxidation rate increases significantly with the increase in temperature from 10 to 30°C. However, a higher optimum temperature, 40°C was reported for an estuarine isolate of nitrifying bacterium, *Nitrosomonas* (Jones and Hood, 1980). The activation energy of ammonia oxidation at the temperature ranges of 10-20°C (87.1 kJ/mol) is significantly higher than at 20-30°C (38.6 kJ/mol) (Kim *et al.*, 2008).

pH is one of the most major factors in nitrification both in freshwater and marine systems (Strauss *et al.*, 2002; Miranda *et al.*, 2008). In this study, effect of pH was clearly noticeable in the pH range of 7.0 to 9.0. The positive correlation between pH and NR was clearly detected in the pH range of 7 to 7.7, but a negative correlation occurred above pH 7.7. This positive correlation is related to the increase in available  $\text{NH}_3$  as a true substrate of oxidation (Suzuki *et al.*, 1974). Emerson *et al.* (1975) stated that the relative  $\text{NH}_3$  concentration increases by nearly a full order of magnitude by increasing each pH unit. The negative correlation between pH and nitrification above the optimum pH is likely caused by the negative effect of increasing pH on enzyme activity (Strauss *et al.*, 2002). Effect of pH as a single factor on nitrification of a marine and estuarine system has not been studied. Miranda *et al.* (2008) could not determine clearly the effect of pH on NR of marine sediment, but detect a tendency, though weak, that nitrification increases by increasing pH. The authors also suggested the positive relationship between pH and nitrification without defining the range of pH clearly. The optimum pH for nitrification varies depends on the nature of the system. In the freshwater sediment, Strauss *et al.* (2002) have determined that the maximum NR occurs at pH 7.5 over a pH range of 5.9-8.7. Antoniou *et al.* (1990) determined the maximum nitrification occurs at pH approximately 7.8 in the wastewater treatment sludge (Table 2).

Salinity of pore water samples in this study had a typical characteristic of estuarine salinity with a wide

Table 2: Comparison of pH effect on nitrification rate reported in this study and in references

Sample	Nitrification Rate	Reference
An estuarine isolate of nitrifying bacterium	Maximum at pH 8	Jones and Hood (1980)
Freshwater sediment	Maximum at pH 7.5	Strauss <i>et al.</i> (2002)
The wastewater treatment sludge	Maximum at pH 7.8	Antoniou <i>et al.</i> (1990)
Mud Sediment of the Ariake sea	Maximum at pH 7.7.	This study

Table 3: Comparison of salinity effect on nitrification rate reported in this study and in references

Sample	Nitrification Rate (NR)	Reference
An estuarine isolate of nitrifying bacterium	Maximum NR at 5-10 ppt	Jones and Hood (1980)
Nitrifying bacterium	Maximum NR at 10 ppt	MacFarlane and Herbert (1984)
River and estuarine water	High NR at 0-17 Cl <sup>-</sup> /L depend on the <i>in situ</i> salinity	Somville (1984)
River and estuarine water	High NR at 20-28 ppt.	Berounsky and Nixon (1993)
River and sea water	High NR at 7-10 psu	Bianchi <i>et al.</i> (1994)
Estuarine sediment	Decrease by increasing salinity	Rysgaard <i>et al.</i> (1999)
Intertidal sandy sediments and rocky biofilms	Increase by increasing salinity from 0 to 15 ppt.	Magalhães <i>et al.</i> (2005)
Estuarine water	High NR at intermediate salinities	Miranda <i>et al.</i> (2008)
Sediments from saline shallow coastal lagoons	Decrease by increasing salinity from 0 to 30 ppt	Santoro and Enrich-Prast (2009)
Mud Sediment of the Ariake sea	Optimum at 15 ppt	This study

fluctuation from 18-38 ppt. Although the nature of nitrifying bacteria in this estuarine are adaptable to this fluctuation, the NR is greatly affected by salinity. High NRs of mud sediment of the Ariake sea were found at intermediate salinity and drastically decreased at low and high salinities. Previous studies on nitrifying bacteria indicated that high NR occurs at intermediate salinities. Jones and Hood (1980) and Helder and de Vries (1983) found high NR of the bacteria at 5-10 ppt and 10-25 ppt, respectively. A maximum NR is determined at 10 ppt (MacFarlane and Herbert, 1984). NRs of marine and estuarine samples are also higher at low or intermediate salinities (Somville, 1984; Berounsky and Nixon, 1993; Bianchi *et al.*, 1994; Magalhães *et al.*, 2005; Miranda *et al.*, 2008) (Table 3).

In this study, the optimum salinity was obtained at 15 ppt with NR 14.5 nmol N/g DWS/h equivalent to 193.9  $\mu\text{mol N/m}^2/\text{h}$ . Somville (1984) reported that the optimum salinity for NR of estuarine samples is affected by the *in situ* water salinity. In contrast, Rysgaard *et al.* (1999) indicated that the optimum salinity for NR is not influenced by the *in situ* salinity with the highest NR at 0 ppt. The finding reported by the later authors is supported by Santoro and Enrich-Prast (2009), who reported that NPRs of sediment from saline shallow coastal lagoons have negative correlation to the increase of salinity ranging from 0 to 30 ppt.

Since nitrification is an enzymatic reaction, its reaction rate is directly affected by the availability of the substrate. Bianchi *et al.* (1999) found that 74% of the variability in nitrification in the estuarine area of the Rhone River can be explained by a single variable,  $\text{NH}_4\text{-N}$  availability. Kim *et al.* (2008) could not determine the effect of free ammonia ( $\text{NH}_3\text{-N}$ ) concentration in the range of 5.6-90.1 mg/L on the specific substrate utilization rate as well as the relative nitrite accumulation.

In this study, we used much higher ammonium concentration to know the kinetic of ammonium oxidation and to know the possibility of construction of Michaelis-Menten curve since the reaction is an enzymatic reaction. To obtain an asymptotic curve, the ammonium concentration was increased to some levels, which were much higher than its field concentration.  $V_{\text{max}}$  and  $K_s$  were found at high concentrations of ammonium over its actual concentration in the field. This finding will be relevant to predict the rate of nitrification at various  $\text{NH}_4$  concentrations.

In this study, the effect of  $\text{NH}_4\text{-N}$  concentration ranging from 11.7 to 3043.3  $\mu\text{M}$  on NR could be determined very well (Fig. 6). Furthermore, the maximum NR ( $V_{\text{max}}$ ), 32.5 nmol N/g DWS/h was also succeeded to be determined at  $\text{NH}_4\text{-N}$  concentration 3,000  $\mu\text{M}$  with  $K_s$  700  $\mu\text{M NH}_4\text{-N}$ . It is the first study determining  $V_{\text{max}}$  and  $K_s$  of estuarine sediment sample of the Ariake sea. Cébron *et al.* (2005) previously reported NR of nitrifier-denitrification of mixed nitrifying bacteria populations from Seine river water. They estimated the maximum  $\text{N}_2\text{O}$  production rate ( $V_{\text{max}}$ ) to be 8 to 9  $\mu\text{g N-N}_2\text{O}^-/\text{mg C biomass/h}$  with  $K_s$  of nitrifier-denitrification 1.5 to 3 mg  $\text{N-NH}_4/\text{L}$  for ammonium, and 1 to 4 mg  $\text{N-NO}_2/\text{L}$  for nitrite. The ammonium concentration and NR had a positive correlation in the concentrations range of 11.7 to 3,000  $\mu\text{M}$  (Fig. 6). However, above the later concentration, the correlation became negative suggesting the nitrification inhibition by excessive ammonia concentration.

Inhibition of nitrification by excessive concentration of ammonium in a marine system is little studied. The inhibition effect of an excessive free ammonia concentration on ammonia oxidation has been reported by Anthonisen *et al.* (1976). The ammonia concentration that inhibits its oxidation is greatly affected by degree of

adaptation of nitrifying bacteria to high ammonia concentration. Kim *et al.* (2008) described that the nitrifying bacteria and enzyme involve in the nitrification process are more resistant to high concentration of ammonia when they are adapted to the high ammonium concentration in advance, while the significant inhibition of nitrification often occurred in a system with low ammonium concentration. For example, the nitrification inhibition occurs at  $\text{NH}_3$  concentration ranging from 0.1 to 150 mg/L (Anthonisen *et al.*, 1976), activity of ammonia oxidizing bacteria, *Nitrosomonas* are significantly inhibited at free ammonia over 100 mg/L (Neufeld *et al.*, 1980), NRs of high ammonium concentration adapted-nitrifiers are not inhibited by ammonium concentration ranging from 500 to 3000 mg N/L (Mahne *et al.*, 1996), and *Nitrosomonas europaea* prevailing at an extreme substrate is not inhibited by substrate concentration up to 500 mmol/L (Hunik *et al.*, 1992). All the above literatures reported the inhibition of nitrification in freshwater and wastewater with high ammonia concentration. In this study, we found that nitrification inhibition occurred at relatively low ammonium concentration comparing to the inhibition reported in the above references. The relative low inhibition concentration of ammonia in this study is likely caused by the nitrifying bacteria in the Ariake mud sediment are never adapted and exposed to the extreme concentration of ammonium. Koga *et al.* (2009) reported that annual ammonium concentrations in the Ariake sea mud sediment at sampling point used in this study ranges from 11.1 to 388.9  $\mu\text{M}$ . The increase in ammonium level usually occurs in summer, and it becomes low in winter. As a comparison,  $\text{NH}_4\text{-N}$  concentration in the upstream part of Scheldt estuary which is heavily polluted by industrial and domestic sewage has reported to be 500 to 1,200  $\mu\text{M}$  (Somville, 1984). The nature of sediment also affects the inhibition concentration of  $\text{NH}_4\text{-N}$ . Magalhães *et al.* (2005) found that nitrification of sandy sediment is inhibited by  $\text{NH}_4\text{-N}$  addition at 200  $\mu\text{M}$ , but the same concentration does not inhibit nitrification of rocky biofilm.

In summary, the NPR of the Ariake sea mud sediment is lower in the deeper sediment, but it was still noticeable even at 6-9 cm sediment depth suggesting the important role of perturbation and possible occurrence of anoxic nitrification. In this study, individual effect of temperature, pH, salinity and  $\text{NH}_4\text{-N}$  concentration on NR of the Ariake sea mud sediment were succeeded to be evaluated by controlling other factors in laboratory experiment. The results indicated that these environmental parameters affected NR significantly. High NR occurred at 20 to 35°C, pH 7.5-8, and salinity around 15 ppt. The effect of ammonium concentration on NR exhibited typical kinetics of enzymatic reaction with Michaelis-Menten curve. This report represents the first study on nitrification of the Ariake sea mud sediment and the

single effect of several environment parameters, which significantly improves the understanding of nitrification kinetic in this area.

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