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Independent Effects of Temperature, Salinity, Ammonium Concentration and pH on Nitrification Rate of the Ariake Seawater Above Mud Sediment

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The Ariake Sea located in the west parts of Kyushu Island is a semi-closed and macro-tidal shallow sea, and has the largest tidal flat in Japan. A large mud tidal flat with a productive ecosystem found along the western shoreline of the sea makes this area ideal as a major production site of nori (*Porphyra yezoensis*) in Japan. We determined the independent effect of temperature, salinity, ammonium concentration and pH on nitrification rates (NR) in the Ariake seawater above the mud sediment. The NR was determined by measuring accumulation of NO_2-N production after adding sodium chlorate, an inhibitor of NO_2-N to NO_3-N oxidation. NRs were relatively high at 20-35 °C (optimum at 29.5 °C), but the rates were very low at 5, 10, and 40 °C. NRs increased sharply when increasing the salinity from 13 to 20 ppt, but it decreased drastically at salinity levels more than 35 ppt (optimum at 19 ppt). The relationship between ammonium concentration and NR showed a typical kinetic curve of enzymatic reaction with the maximum NR (*V*max) of 0.029 μM N.h^{-1} at 200 μM NH_4-N (the half saturation constant (Ks) = 35 μM NH_4-N). High NRs were determined at pH 7.5-8.0 (optimum pH 7.8). This is the first report on the independent effects of temperature, pH, salinity and NH_4-N concentration on the NR of seawater, specifically the Ariake seawater.

Keywords: nitrification, the Ariake sea, seawater, temperature, pH, ammonium, salinity

INTRODUCTION

Aerobic nitrification is a two-step process of microbial oxidation of ammonia (NH_3^-) to nitrite (NO_2^-) and subsequently to nitrate (NO_3^-). The first oxidation step is mediated by ammonia-oxidizing bacteria (AOB), while the second one is mediated by nitrite-oxidizing bacteria. In the nitrogen cycle, nitrification links N mineralization to denitrification which produces N_2 gas. Therefore, nitrification is one of the prominent biochemical processes in the global nitrogen (N) cycle and in single ecosystems. Approximately, 30% of global fixed-N loss occurs in the sediments of estuaries and of the continental-shelf (Galloway *et al*. 2004). Coupled nitrification and denitrification in the estuary ecosystem also play an important role in removal processes of approximately 10 to 80% of anthropogenic N pollution (Seitzinger 1988). Nishio *et al*. (1983) estimated that 6-70% of the N_2 produced by the denitrification process originates from nitrogenous oxides (nitrate and nitrite) which are derived from nitrification.

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In the marine ecosystems, nitrification in seawater is less studied than that taking place in the sediment. The nitrification rate (NR) of sea water has been reported by McCarthy *et al*. (1984) and Horrigan *et al*. (1990) in Chesapeake Bay, Somville (1978, 1984) in Scheldt estuary, Enoksson (1986) in the Baltic sea, Owens (1986) in Tamar estuary, Berounsky and Nixon (1993) in Narragansett Bay, Bianchi *et al*. (1997, 1999b) in the Southern ocean and the NW Mediterranean Sea, Iriarte *et al*. (1998) in the estuary of the River Nervión, Capona *et al*. (1990) in Crane Neck, de Bie *et al*. (2002) in the Schelde estuary, O'Mullan and Ward (2005) in Monterey Bay, and Miranda *et al*. (2008) in Kochi backwaters. There is no report on the independent effect of environmental parameters on NR of seawater. Nitrification has been shown to be affected by environmental factors such as substrate concentration (Kim *et al*. 2008a; Miranda *et al*. 2008), dissolved oxygen (DO) (Kemp & Dodds 2002), temperature, pH, salinity (Jones & Hood 1980; Kim *et al*. 2008a; Miranda *et al*. 2008), organic carbon (C) availability and CN ratio (Strauss & Lamberti 2000; Strauss *et al*. 2002). In this paper, we report the experimental results of independent effect of environmental parameters on

the nitrification of the Ariake seawater above mud sediment.

The Ariake Sea located in the west part of Kyushu island, Japan, is a semi-closed shallow sea with macro-tidal and several well-mixed estuaries. This sea covers 1,700 km^2 of a long inner bay with 96 km of the bay axis and 18 km of the average width. The Ariake Sea is characterized by a macro tidal range of 3-6 m, which is the largest tidal range in Japan (Kato & Seguchi 2001; Hiramatsu *et al*. 2005). A vast tidal flat area has developed in the sea, and constitutes almost 40% of the total tidal flat area in Japan. The sediments that are transported by several rivers to the bay reach around 440,000 ton per annum. Coarse sediment settles in the eastern part of the bay, but the fine sediment makes up the Ariake clay formation and mud tidal flat along the western shoreline of this area (Kato & Seguchi 2001). This tidal flat is the main area for the production of sea laver (*Porphyra yezoensis*) production in Japan, which contributes 40% of the total Japanese sea laver production (Yanagi & Abe 2005). The most important nutrient for production of *P. yezoensis* is nitrogen; either NO_3^- or NH_4^+, but NO_3^- is a better source of N in terms of growth (Hafting 1999). The uptake of these two inorganic nitrogen fractions in *Porphyra* species including *P. yezoensis* occur at similar rates (Kraemer *et al*. 2004). Moreover, the uptake is affected by the frequency and duration of cycles of immersion and exposure (Kim *et al*. 2008b), which occur naturally in the high tidal range area as found in the Ariake sea.

Although nitrogen is a crucial nutrient contributing to high productivity of the Ariake sea, there has been little research on the nitrogen cycle in this area. Moreover, water nitrification in this area has also not been studied. A report on the independent effects of environmental parameters on NR of water from the marine system, especially in the Ariake sea has not been conducted hitherto. This paper provides the experimental results of independent effects of temperature, NH_4-N concentration, pH and salinity on nitrification of the Ariake seawater.

MATERIALS AND METHODS

Water Sample. Water samples used in this study were collected in Higashi Yoka in the intertidal zone at high tide. Higashi Yoka, around 8 km south of Saga City, is located in the interior part of the Ariake sea and it has muddy sediment. This is the predominant sediment in the interior parts of the Ariake sea tidal flat (Figure 1). Samples were collected in polyethylene bottles and the samples were transported to the laboratory in a cool box.

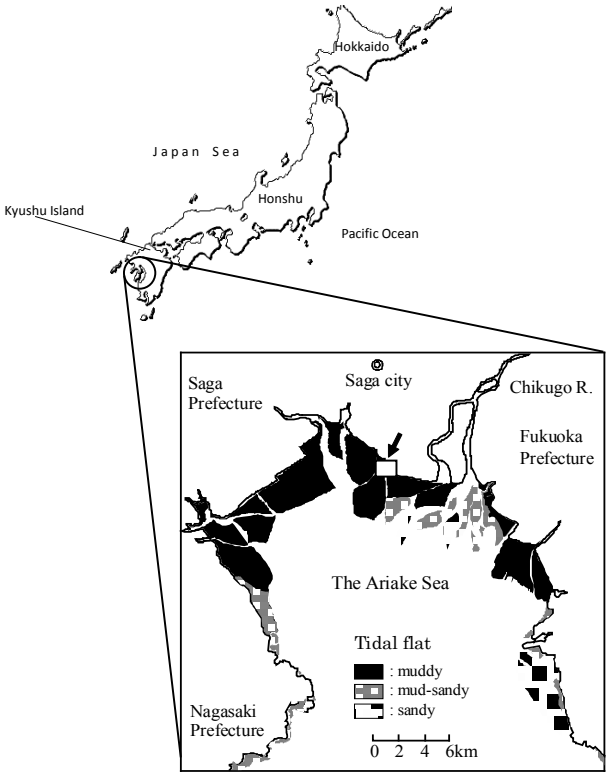

Figure 1. Map of the interior parts of the Ariake sea and distribution of sediments in its tidal flat [32]. An arrow indicates the sampling point.

Water Geochemistry. Water samples were filtered through 0.45 μm-pore-size cellulose ester filter (Advantec, Toyo Roshi Kaisha, Tokyo, Japan), and frozen immediately until analysis. NH_4-N, NO_2-N, NO_2+NO_3-N, PO_4, total nitrogen (TN) and total phosphate (TP) were analyzed by an automated water analyzer (Water auto-analyzer, swAAT, BLTEC, Tokyo, Japan). NH_4-N concentration was determined by means of the alkali phenol-hypochlorite reaction detected photometrically at 630 nm. NO_2-N concentration was analyzed by diazotizing samples with sulfanilamide and coupling with N-(1-naphthyl) ethylenediamine dihydrochloride to form a highly colored azo dye which was detected photometrically at 550 nm. NO_3-N was measured using the same method as for NO_2-N after NO_3 was reduced by the cadmium reduction process. PO_4 was determined by the ascorbic acid method at 800 nm. TN and TP concentrations were measured by peroxodisulfate oxidation (Ebina *et al*. 1983).

Density of Ammonium-Oxidizing Bacteria (AOB) in Water. 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 μL sterile medium for ammonia-

oxidizing bacteria as described by Cote and Ghena (1994). Composition of the medium was $(NH_4)_2SO_4$, 1.32 g L^{-1}; KH_2PO_4, 20 mg L^{-1}; $MgSO_47H_2O$, 0.1 g L^{-1}; $FeCl_26H_2O$, 0.014 g L^{-1}; $CaCl_22H_2O$, 0.18 g L^{-1}; $Na_2MO_42H_2O$, 100 µg L^{-1}; EDTA, 1.0 mg L^{-1}, phenol red, 0.002 g L^{-1}, dissolved in 70% artificial seawater (Tetramarin Salt Pro, USA). The medium was adjusted to pH 8 with Na_2CO_3. One hundred microliter of the sample was inoculated to the microtubes in triplicates, and serially diluted by ten fold. Incubation was carried out at 25 °C for 20 days. Any tubes that exhibited a color change from red to yellow due to acid production were further tested by adding three drops of a nitrite color reagent (sulfanilamide, 10 g L^{-1}; *n*-(1-naphtyl)-ethylenediamine 2 HCl, 0.50 g L^{-1}; concentrated HCl, 100 ml L^{-1}). Tubes that exhibited a red color after addition of the reagent were scored positive for nitrite. Bacterial density was calculated by the MPN formula in Visual Basic program (Koch 1994).

Nitrification Rate (NR). NR was determined by the method described previously (Bianchi *et al*. 1997; Welsh & Castadelli 2004; Dollhopf *et al*. 2005). Two hundreds milliliter of seawater sample was aliquoted into 300 mL Erlenmeyer flasks. Duplicate flasks from each treatment were added with sodium chlorate ($KClO_3$; 10 mM) (Wako Pure Chemical Industries Ltd.). Control flasks contained sodium chlorate, and allylthiourea (ATU; 20 mg L^{-1}) (Sigma-Aldrich, St. Louis MO, USA) (Belser & Mays 1980; Dollhopf *et al*. 2005). Flasks were capped with aluminum foil and incubated in the dark with constant stirring with a magnetic stirrer at 100 rpm. Samples were collected at intervals over the incubation time. NO_2-N in water was determined after filtering through 0.45 µm cellulose ester membrane filter (Advantec, Toyo Roshi Kaisha, Japan). As sodium chlorate is a specific inhibitor of nitrification, which blocks the oxidation of NO_2-N to NO_3-N (Belser & Mays 1980), by adding 10 mM chlorate, NR can be determined as the linear accumulation of nitrite with time (Welsh & Castadelli 2004; Dollhopf *et al*. 2005). Linear regression analyses were done after the NO_2 concentrations in all treatments were conducted by subtracting its concentration in the ATU control flask.

Effects of Temperature, Salinity, and pH on Nitrification Rate (NR). All experiments were incubated at 25 °C except for temperature experiment which was incubated at various temperatures ranging from 5 to 40 °C. Before conducting the salinity experiment, the seawater salinity was lowered by diluting the seawater sample with sterile distilled water (DW) at a ratio of seawater:DW (4:1). The lowest salinity obtained by this dilution was used as the first treatment group. The higher salinity treatments were adjusted by adding artificial seawater ingredients (Tetramarin Salt Pro, USA). The range of salinity tested in this experiment is 13-40 ppt. For pH experiment, seawater sample 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. pH and salinity was 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. Incubation was carried out for 120 h except for pH experiment, which was conducted for 60 h since the concentration of NO_2-N tended to decrease at high pH treatments when the incubation was conducted longer than 60 h.

Effect of Ammonium Concentrations on Nitrification Rate (NR). The experiment used the same method as described above with addition of NH_4-N [$(NH_4)_2SO_4$, Wako Pure Chemical Industries Ltd., Osaka, Japan] at various concentrations. Since the seawater sample naturally contained NH_4-N, the regression analyses were carried out based on the actual concentration of NH_4-N in each treatment, which was determined at the initial time of the experiment (0 h) rather than the concentrations of NH_4-N added.

RESULTS

Salinities of water samples used in all experiments were in intermediate level ranging from 13 to 22.5 ppt (Tabel 1). The salinity corresponded to concentration of NaCl, electrical conductance and resistance. pH exhibited a normal range of seawater pH at 7.5-7.6. Redox potentials of seawater samples were positive in the range of 116 to 191 mV, indicated that the water samples were in an oxidative condition. NO_3-N and NH_4-N concentrations in seawater ranged from 27.8 to 565.6 µg L^{-1} and 40.6 to 447.9 µg L^{-1}, respectively. NO_2-N concentration was relatively low ranging from 22.9 to 66.7 µg L^{-1}. Phosphate concentration was relatively constant in the range of 54.2 to 80.4 µg L^{-1}. Total nitrogen (TN) and total phosphate (TP) exhibited low fluctuation in the concentration ranges of 248.1-307.3 µg L^{-1} and 67.3-90.7 µg L^{-1}, respectively. Density of AOB had a low tendency in the range of 3.488×10^2 to 4.781×10^3 cells mL^{-1}.

In this study, nitrification was determined by adding sodium chlorate to inhibit the oxidation of NO_2-N to NO_3-N. It allows the nitrification to be determined by measuring the accumulation of NO_2-N production. Temperature significantly affected production of N measured as NO_2-N. Relatively high N productions were observed at the temperature range of 20-30 °C, but very low at 5, 10, and 40 °C. The production of N at 5 to 40 °C ranged from 0.046 to 1.586 µM N within 120 h incubation. The highest N production occurred at 30 °C, and the lowest one at 40 °C (Figure 2A). Like N production, the NRs were also significantly affected by temperature. NR of water increased drastically with rise in temperature from 15 to 30 °C, but it decreased sharply at temperatures above 30 °C (Figure 2B). The highest NR, 0.0129 µM N h^{-1} was found at 30 °C. Lower NRs, 0.0016, 0.0069, 0.0093, and 0.0048 µM N h^{-1}, were observed at 15, 20, 25, and 35 °C, respectively. The rates were very low at 5, 10, and 40 °C. The estimated maximum NR based on the curve depicted in Figure 2B, was obtained at 28 °C.

N Production was Significantly Affected by Salinity. High N productions were found at salinity 15, 20, and 25 ppt, which reached 0.026, 0.026, and 0.024 µM during 120 h incubation, respectively. Lower N productions occurred at salinity 13, 30, and 35 ppt, and even the production was drastically decreased at 40 ppt (Figure 3A). The NRs varied between 0.0009 to 0.0026 µM h^{-1} at the salinity range of 13 to 40 ppt (Figure 3B). The NRs increased drastically by increasing the salinity from 13 to 20 ppt, but it decreased sharply at salinity more than 35 ppt (Figure 3B). Based on the trend curve in Figure 3B, the optimum NR was 0.0027 µM h^{-1} at 19 ppt.

Increase in ammonium concentrations up to approximately 121 µM were significantly increased the accumulation of nitrogen production measured as NO_2-N, but the production remained relatively constant at the NH_4-N concentration ranging from 121 to 491 µM (Figure 4A). Accumulation of N production from 0.081 to 121.5 µM NH_4-N ranged from 0.0017 to 0.0288 µM N within 96 h incubation. A similar pattern was also found with NR rates, when increasing of NH_4-N concentration up to 491 µM

Table 1. Physico-chemical and microbiological properties of seawater collected from Higashi Yoka

Experiment	Salinity (ppt)	NaCl (%)	pH	Redox potential (mV)	Electrical conductance s/m	Electrical resistance Ωm	Concentration (µg/L)						Density of AOB (cells/mL)
							NO_3-N	NH_4-N	NO_2-N	PO_4	TN	TP	
Effect of salinity	14	0.372	7.5	150	2.69	ND	131.7	91.4	50.0	54.2	271.9	67.3	4.240 x 10^2
Effect of NH_4-N concentration	22.5	1.18	7.5	144	2.04	0.489	27.8	40.6	66.7	60.9	307.3	90.7	4.781 x 10^3
Effect of pH													
Effect of temperature	13	0.76	7.5	191	1.39	0.717	565.6	447.9	35.4	80.4	248.1	68.0	9.190 x 10^2
	19	1.02	7.6	116	1.74	0.599	413.4	424.2	22.9	76.3	ND	70.1	3.488 x 10^2

ND: no data, TN: total nitrogen, TP: total phosphate.

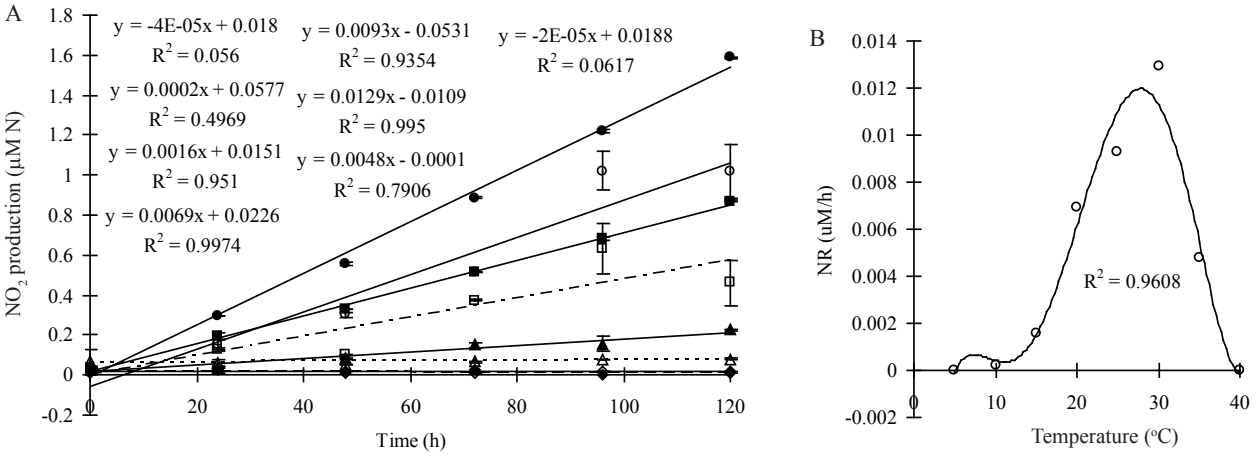

Figure 2. Nitrification rate of the Ariake seawater at various temperatures, determined by the accumulation of NO_2 production for 120 h incubation (A). Relationship between water salinities and nitrification rates of the Ariake seawater (B). ◆: 5, △: 10, ▲: 15, ■: 20, ○: 25, ●: 30, □: 35, ◇: 40, ———— Linear (5), - - - - - Linear (10), ——— Linear (15), ——— Linear (20), ——— Linear (25), ——— Linear (30), —- —- —- Linear (35), ——— Linear (40).

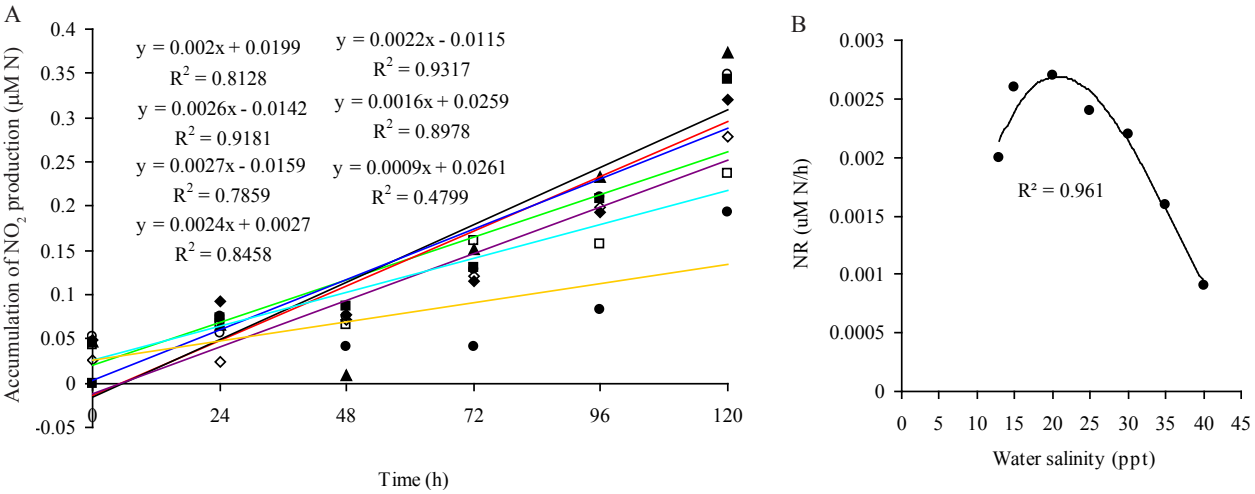

Figure 3. Nitrification rate of the Ariake seawater at various salinities, determined by the accumulation of NO_2 production for 120 h incubation (A). Relationship between water salinities and nitrification rates of the Ariake seawater (B). ◆: 13, ■: 15, ▲: 20, ○: 25, ◇: 30, □: 35, ●: 40, — Linear (13), — Linear (15), — Linear (20), — Linear (25), — Linear (30), — Linear (35), — Linear (40).

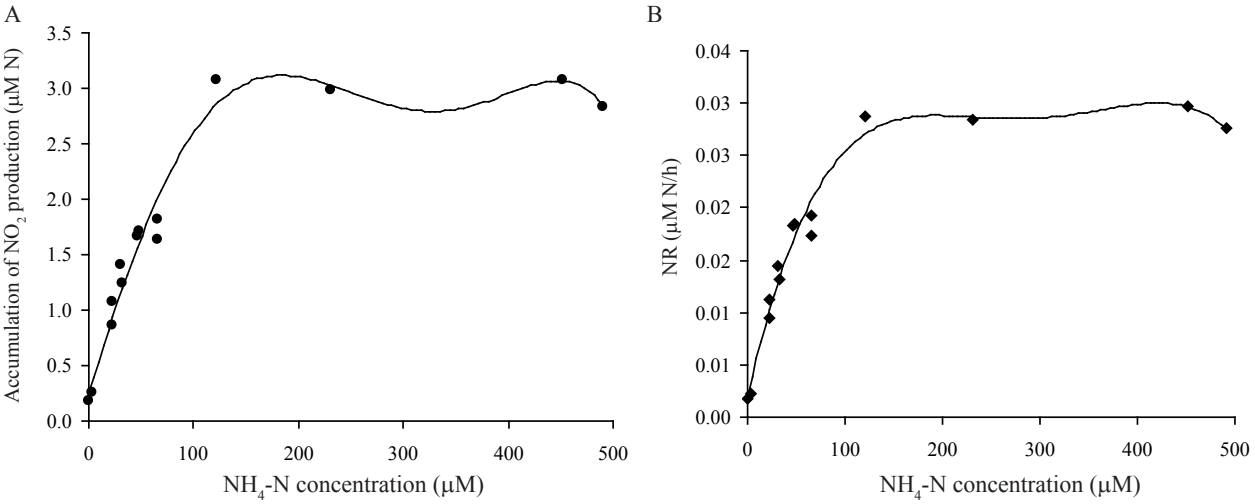

Figure 4. Accumulation of NO_2 production during 96 h incubation at various concentration of NH_4-N (A). Nitrification rate of the Ariake seawater above mud sediment at various concentrations of NH_4-N (B).

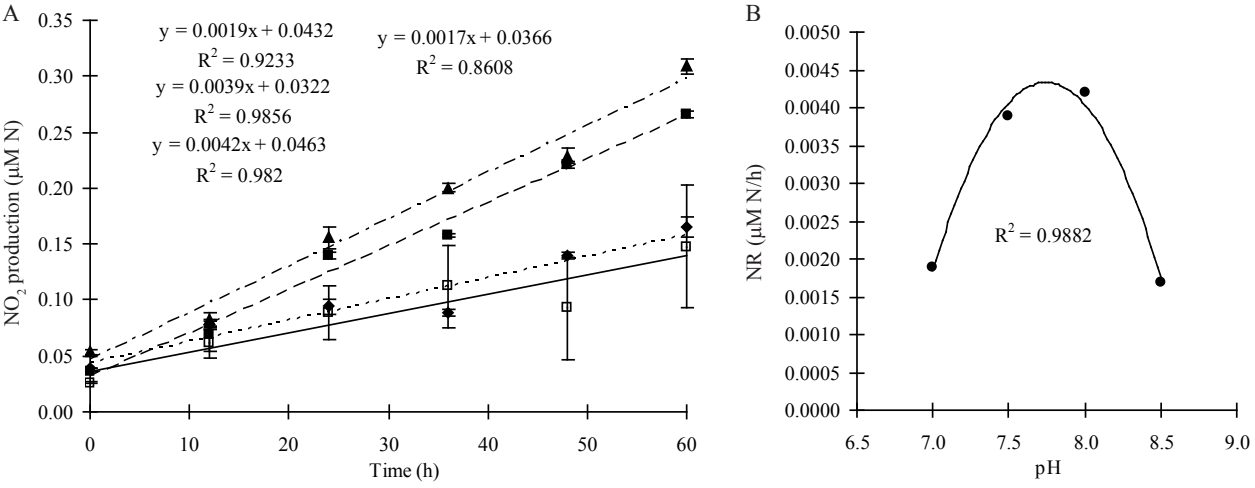

Figure 5. Nitrification rate of the Ariake seawater above mud sediment at various pHs, determined by the accumulation of NO_2 production for 60 h incubation (A). Relationship between pHs and nitrification rates of the Ariake seawater (B). ◆: 7, ■: 7.5, ▲: 8, □: 8.5, - - - - - Linear (7), – – – – Linear (7.5), —- —- —- Linear (8), ——— Linear (8.5).

(Figure 4B). The relationship between ammonium concentration and NR indicates a maximum NR rate (*V*max) of 0.029 μM N h^{-1} at NH_4-N concentration of 200 μM. The half saturation constant (*Ks*) of the nitrification was determined at 35 μM NH_4-N.

The accumulation of N production during 60 h incubation at pH 7, 7.5, 8, and 8.5 ranged from 0.148 to 0.309 μM N. The highest N production was found at pH 8 and the lowest one at pH 8.5 (Figure 5A). Relatively high NRs were found in the basic condition at pH 7.5 and 8.5, which were 0.0039 and 0.0042 μM N h^{-1}, respectively. Incubation at pH 7.0 resulted in a low NR, 0.0019 μM N h^{-1}. Based on the curve depicted in Figure 5B, the optimum NR was reached pH 7.75.

DISCUSSION

The Ariake sea is a semi-closed shallow sea with unique ecological conditions. Its productive ecosystem makes economically important as the source of around 40% of the total Japanese production of laver as well as many other fisheries products. The inner part of this area has a tidal flat dominated by mud sediment. This tidal flat plays a critical role in nutrient supply for laver (*Phorpyra* spp.) production. This seaweed takes up inorganic nitrogen in the form of nitrate and ammonium (Hafting 1999; Kraemer *et al*. 2004). Nitrogen deficiency in *Phorpyra* spp. often occurs in winter when this seaweed is cultured intensively in this area. Sharp ammonium depletion in mud sediment pore water (Koga *et al*. 2009) and in the seawater also occur in winter suggesting the two nitrogen fractions are likely to be the most critical limiting factors in producing the seaweed in the Ariake sea.

Eventhough ammonium and nitrate are critical nutrients in laver production, the nitrogen cycle in the Ariake sea is little understood. Nutrient dynamics was estimated by Hang *et al*. (2009) using a hydrodynamic model. The authors described the dynamic of dissolved inorganic nitrogen (DIN), but did not describe its fractions and cycle in this area. Koga *et al*. (2009) studied denitrification, one of the processes in the nitrogen cycle, in sediment of the Ariake sea. However, a study on the nitrification of the Ariake seawater has not been conducted hitherto.

Since nitrification is a microbiological process, its rate is dictated significantly by the nature of nitrifying bacteria, and is affected by a variety of environmental factors. The environmental factors affected nitrification are substrate concentration (Kim *et al*. 2008a; Miranda *et al*. 2008), dissolved oxygen (DO) (Kemp & Dodds 2002), temperature, pH, salinity (Jones & Hood 1980; Kim *et al*. 2008a; Miranda *et al*. 2008), organic carbon (C) availability, and CN ratio (Strauss *et al*. 2002). Most of these literatures reported the multiple effects of environmental factors in fresh water and water treatment systems. In addition, the independent effect of various environmental factors on nitrification of marine systems including seawater is little known. In this paper, we provide results of our study on the independent effects of temperature, salinity, ammonium concentration, and pH on the NR of the Ariake seawater.

Water temperature is the most prominent variable affecting nitrification, with an exponential increment produced by increasing temperature from 4 to 24 °C (Berounsky & Nixon 1990, 1993). NRs of the Ariake seawater were very low at temperatures ranging from 5 to 10 °C and at 40 °C. The rate increased drastically with the increase in temperature from 15 to 29.5 °C, but decreased at temperatures above 29.5 °C. Kim *et al*. (2008a) reported similar findings in waste water sludge, in which the ammonia oxidation rate increased significantly with the increase in temperature from 10 to 30 °C. The activation energy for ammonia oxidation was higher at lower temperature. These authors found that the activation energy of ammonia oxidation at the temperature range of 10-20 °C was significantly higher than at 20-30 °C, reported as 87.1 and 38.6 kJ mol^{-1}, respectively. Miranda *et al*. (2008) detected this same relationship between temperature and NR from field observation datasheet in Kochi backwaters at the temperature range of 28-34 °C, but did not describe its independent effect on the NR.

pH is one of the most important factors in nitrification both in freshwater and marine systems (Strauss *et al*. 2002; Miranda *et al*. 2008). However, Berounsky and Nixon (1993) indicated that pH only occasionally has a measurable effect on nitrification. In this study, pH in the range of 7.0 to 8.5 significantly affected NR of the Ariake seawater. NR increased by increasing pH from 7 to 7.8, but a negative correlation occurred above pH 7.8. This positive correlation corresponds to the increase in available NH_3 as a true substrate of oxidation (Suzuki *et al*. 1974). The relative NH_3 concentration increases by nearly a full order of magnitude by increasing each pH unit (Emerson *et al*. 1975). In contrast, 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).

The effect of pH as an isolated factor on nitrification in a marine and estuarine system has not been studied. The optimum pH for nitrification varies depending on the nature of the system. In the freshwater sediment, Strauss *et al*. (2002) determined that the maximum NR occurs at pH 7.5 over a range pH of 5.9-8.7. Antoniou *et al*. (1990) determined that maximum nitrification occurs at approximately 7.8 in the wastewater treatment sludge. Miranda *et al*. (2008) could not determine clearly the effect of pH on NR of marine sediment and seawater, but they did 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 seawater samples in this study showed a typical characteristic range of estuarine salinity (Tabel 1). NR of the Ariake seawater was high at intermediate salinity and decreased significantly at 13 ppt and high salinities. The effect of salinity on the nitrification of seawater systems is little known as many studies were conducted in the marine system without excluding other environmental factors. In this study, an experiment with a single factor of salinity was able to determine the effect on NR of seawater and its optimum level. The optimum NR, 0.0027 μM h^{-1}, occurred at 21 ppt. This finding is somewhat different from previous studies on nitrifying bacteria. Jones and Hood (1980), Helder and de Vries (1983), and MacFarlane and Herbert (1984) reported that high NR of nitrifying bacteria, increase at the salinity ranges of 5-10, 10-25, and 0-20 ppt, respectively. Somville (1984) indicated that nitrification of estuarine water samples are high at salinity range of 0-20 ppt. In addition, the optimum potential nitrification is affected by *in situ* water salinity. In the Rhone River plume, Bianchi *et al*. (1994) described that ammonium oxidation increases by increasing salinity up to 8.5 psu. Miranda *et al*. (2008) stated that NR is higher in intermediate salinity. However, Berounsky and Nixon (1993) found that salinity does not have direct measurable effect on nitrification in Narragansett Bay.

Since nitrification is an enzymatic reaction, its reaction rate is directly affected by the availability of the substrate. The difference in ammonium concentrations is responsible for the significant difference in NR at different sampling sites of Narragansett Bay in summer (Berounsky & Nixon 1993). Ammonium oxidation rates correlate well with *in situ* ammonium concentrations in the Rhone River plume (Bianchi *et al*. 1994). Moreover, in the estuarine area of the Rhone River, Bianchi *et al*. (1999a) found that 74% of the variability in nitrification can be explained by a single variable of NH_4-N availability. However, Kim *et al*. (2008a) could not determine the effect of free ammonia (NH_3-N) concentration in the range of 5.6-90.1 mg L^{-1} on the specific substrate utilization rate nor the relative nitrite accumulation in wastewater sludge.

In this study, NH_4-N concentration up to 121 μM significantly affected NR (Figure 4). In addition, the maximum NR (*V*max), 0.029 μM N h^{-1} was also succesfully determined at 200 μM NH_4-N. This is the first study to determine *V*max and *Ks* in an estuarine water sample. Cébron *et al*. (2005) previously reported NR rates for nitrifier-denitrification of mixed nitrifying bacteria populations from Seine river water. They estimated the maximum N_2O production rate (*V*max) to be 8 to 9 μg N N_2O mg^{-1} C biomass h^{-1} with *Ks* of nitrifier-denitrification 1.5 to 3 mg N-NH_4 L^{-1} for ammonium, and 1 to 4 mg N-NO_2 L^{-1} for nitrite. Ammonium concentration and NR had a positive correlation for concentrations up to 200 μM (Figure 4). However, above this concentration, the NR did not increase even at 491 μM NH_4-N. This finding reveals that the inhibition of nitrification by excessive concentration of ammonium occurred in the Ariake seawater. Such inhibition in a marine system has not been studied yet. To the best of our knowledge, it is the first report on the inhibition of nitrification by NH_4-N in seawater sample.

The inhibition effect of an excessive free ammonia concentration on ammonia oxidation has been reported at 0.1-150 mg L^{-1} in waste water samples (Anthonisen *et al*. 1976), and at free ammonia concentration over 100 mg L^{-1} in *Nitrosomonas* (Neufeld *et al*. 1980). However, ammonium at 500 to 3000 mg N L^{-1} and 500 mmol L^{-1} have no inhibitory effect on NRs in high ammonium concentration adapted-nitrifiers (Mahne *et al*. 1996), and in *Nitrosomonas europaea* prevailing at an extreme substrate (Hunik *et al*. 1992), respectively. All the above literature reported the inhibition of nitrification in freshwater and wastewater with high ammonia concentration. In the estuary system, Magalhães *et al*. (2005) found that nitrification of sandy sediment is inhibited by NH_4-N addition at 200 μM, but this concentration does not inhibit nitrification of rocky biofilm.

The extent to which ammonia concentration that inhibits nitrogen oxidation is greatly affected by the degree of adaptation of nitrifying bacteria to high ammonia concentration (Kim *et al*. 2008a). In this study, we found that nitrification inhibition occurred

at relatively low ammonium concentration. The relative low inhibition concentration of ammonia in this study was likely caused by the nitrifying bacteria in the Ariake seawater that never been adapted and exposed continuously to the extreme concentration of ammonium. Koga *et al*. (2009) reported that the annual ammonium concentrations in the Ariake sea mud sediment at sampling point used in this study ranges from 11.1 to 388.9 μM. Tabel 1 shows that the ammonium concentration ranged from 40.547 to 447.874 μg L^{-1}. The increase in ammonium level usually occurs in summer and autumn, and it becomes low in winter. As a comparison, NH_4-N concentration in the upstream part of Scheldt estuary, which is heavily polluted by industrial and domestic sewage, has been reported around 500 to 1,200 uM (Somville 1984).

The geochemistry of seawater sample used in all experiments in this study did not fluctuate significantly, except NO_3-N and NH_4-N which differed somewhat depending on sampling time. The high nitrate and ammonium concentrations in some samples might be caused by the increase in the inflow of these nitrogen fractions by increasing in water river discharge and rain water run off from agriculture and urban area. Although NH_4-N levels were low when the effect of ammonium concentration experiment was conducted, the NR was well determined since ammonium was amended at various concentrations. The AOB density in seawater samples estimated by MPN method ranged from 3.488 x 10^2 to 4.781 x 10^3 cells mL^{-1}. These densities are comparable with the results reported by Cèbron *et al*. (2003). By using PCR primers targeting the *amoA* gene, which encodes the active site of ammonia monooxygenase, a key enzyme in AOB, the authors determined the number of the gene copies in the Lower Seine River and Estuary waters ranging from 9.7 x 10^5 to 7.3 x 10^7 copies L^{-1}. Since the cells of AOB contain one to three copies of the gene (Klotz & Norton 1998; Norton *et al*. 2002), the density of AOB can be estimated only by a factor of 1 to 3 (Cébron *et al*. 2003). However, the density of AOB in the Ariake seawater may be underestimated as the culture-dependent methods usually give the density at several orders of magnitude lower than that detected by quantitative PCR (qPCR) (Hoefel *et al*. 2005) or other molecular techniques. Therefore, the use of molecular techniques is necessary to enumerate AOB in the Ariake seawater in the future.

In summary, independent effect of temperature, pH, salinity, and NH_4-N concentration on NR of the Ariake seawater were successfully determined by controlling for other factors in a laboratory experiment. The results indicated that these environmental parameters affected NR significantly. High NR occurred at the temperature, pH, and salinity ranges of 20-35 ^oC, 7.5-8.5 and 13-30 ppt, respectively. A typical kinetic of enzymatic reaction was found in the relationship between ammonium concentration and NR with the maximum NR (Vmax) of 0.029 μM N h^{-1} at 200 μM of NH_4-N. This report provides results of the first study on nitrification of the Ariake seawater, and the independent effect of several environmental parameters, thus significantly improves our understanding of nitrogen biogeochemical processes in this unique and productive ecosystem.

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