

Article

Alteration of Dissimilatory Nitrate Reduction Pathways in the Intertidal Sediment during Macroalgae Blooms

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Abstract: To elucidate the effect of macroalgae blooms on dissimilatory nitrate reduction pathways (denitrification, anammox, and DNRA) in sediments of the hypereutrophic Yatsu tidal flat, eastern Japan, sediment denitrification, anammox, and DNRA rates were measured using a ¹⁵N tracer technique at two sites affected and unaffected by macroalgae (*Ulva*) blooms and in incubation experiments with and without *Ulva*. Anammox was insignificant at both sites and in both experiments. The denitrification rate was consistently higher than the DNRA rate, and its contributions to the total dissimilatory nitrate reduction were 82% and 85% at sites affected and unaffected by *Ulva*, respectively. In a sediment incubation experiment with *Ulva*, the contribution of DNRA had increased to approximately 30% on day 7, which is when the sulfide concentration was the highest. Sulfide produced by sulfate reduction during macroalgae blooms inhibited denitrification and did not change the DNRA, and consequently increased the DNRA contribution. On day 21, after reaching the peak sulfide concentration during the late macroalgae collapse, the DNRA contribution decreased to 15%. These results indicated that the DNRA contribution was greater during the macroalgae blooms than at the collapse, although denitrification dominated DNRA regardless of the macroalgal status. Therefore, vigorous macroalgae cover and sulfide production under the macroalgae cover had an important impact on the nitrogen dynamics.

Keywords: DNRA; denitrification; sulfide; macroalgae blooms; hypereutrophic intertidal ecosystem



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1. Introduction

Blooms of drift green macroalgae in coastal areas have increased both in scale and frequency over the last few decades in response to elevated levels of coastal eutrophication [1,2]. The macroalgae blooms occur mainly in the North Temperate Zone, with America, Europe, and the Asia-Pacific area being the most seriously affected [1]. Macroalgae have diverse ecology and can be widely distributed [1–3]. For example, *Ulva prolifera* has four main propagation methods [1]. Additionally, opportunistic and drift macroalgae, such as *Ulva* sp., take up large amounts of nutrients, resulting in explosive blooms within short periods [1,2,4]. Macroalgae cover on surface sediment attenuates sunlight penetration and decreases sediment-attached periphytic algae. In addition, macroalgal decomposition promotes anoxic conditions and provides a source of organic carbon and sulfide [4–6]. Therefore, large mats of algae have the potential to greatly affect biogeochemical cycles in the coastal sediment, particularly nitrogen dynamics, which involve heterotrophic and autotrophic microorganisms.

Nitrogen is a limiting nutrient in coastal ecosystems. Under aerobic conditions, organic nitrogen is mineralized to ammonium (NH₄⁺), which is continuously oxidized to nitrate (NO₃[−]) via nitrification. Therefore, NO₃[−] and NH₄⁺ are generally the dominant

forms of nitrogen nutrients that are delivered from rivers to coastal ecosystems [7]. Dissimilatory nitrate reduction processes, including denitrification, anaerobic ammonium oxidation (anammox), and dissimilatory nitrate reduction to ammonium (DNRA), play important roles in controlling the NO_3^- and NH_4^+ dynamics and fate in estuaries and coastal environments [8]. Denitrification generally results from the respiratory reduction of NO_3^- to nitrogen gas (N_2) in response to the oxidation of electron donors such as organic matter [9,10]. However, some chemolithoautotrophic denitrifying organisms use inorganic matter, such as reduced sulfur compounds and hydrogen, instead of organic matter [11]. In anammox, NH_4^+ is oxidized to N_2 , with NO_3^- or NO_2^- serving as the electron acceptor under anaerobic conditions [12]. Therefore, the processes of denitrification and anammox remove NO_3^- from coastal ecosystems. In contrast to these processes, DNRA does not remove NO_3^- from coastal ecosystems in the form of N_2 but instead reduces it to NH_4^+ [13,14]. DNRA is performed by both heterotrophic organisms, which use organic carbon as the electron donor (fermentative), and chemolithoautotrophic organisms, which use nitrate to oxidize sulfide or other reduced inorganic substrates [15]. The balance between denitrification, anammox, and DNRA determines whether bioavailable nitrogen is removed as nitrogen gas (N_2) or recycled as NH_4^+ [16]. Therefore, an understanding of nitrogen transformation is required to assess the nitrogen fate and control nitrogen pollution in aquatic ecosystems [8]. Despite its importance, alterations in sediment denitrification, anammox, and DNRA during macroalgae blooms remain unclear.

The Yatsu tidal flat in eastern Japan, which extends over an area of approximately 40 ha, was isolated from Tokyo Bay by the construction of concrete dikes in the 1970s. Seawater containing excessive nutrients from intrudes in Tokyo Bay enters the Yatsu tidal flat via two small channels. As a result, eutrophication in the tidal flat has progressed over the past few decades [4,17,18]. Macroalgae blooms were first observed in the Yatsu tidal flat in 1995 and gradually continued to develop [19]. Recently, macroalgae blooms of *Ulva* spp. (*Ulva*), including *Ulva pertusa* and *U. ohnoi*, were observed [17–20]. Previous studies conducted in the Yatsu tidal flat reported that denitrification rather than anammox is the major nitrogen removal process [18], and *Ulva* blooms promote sulfide release via sulfate reduction and alter sulfur dynamics from sulfate reduction to sulfide oxidation [4].

In this study, to elucidate the effect of macroalgae blooms on dissimilatory nitrate reduction in sediments of the hypereutrophic Yatsu tidal flat, denitrification, anammox, and DNRA rates were measured in sediments that were affected and unaffected by *Ulva* blooms using a ^{15}N tracer technique. In addition, in sediment incubation experiments with and without *Ulva*, the changes in denitrification, anammox, and DNRA rates were observed when the macroalgae status shifted from vigorous macroalgae cover to macroalgal collapse. Moreover, we hypothesized that sulfide production in the presence of macroalgae would influence the dissimilatory NO_3^- reduction pathways. To understand the effect of sulfide concentration on denitrification, the denitrifying activity was measured with different concentrations of sulfide using the acetylene block method.

2. Materials and Methods

2.1. Study Site and Sampling

Sampling was performed monthly during low tide at sites C ($35^\circ 40' 34''$ N, $140^\circ 00' 22''$ E) and S ($35^\circ 40' 29''$ N, $140^\circ 00' 27''$ S) of the hypereutrophic Yatsu tidal flat, eastern Japan (Figure 1). At St. C near the center of the tidal flat, the surface sediment was frequently affected by *Ulva* cover, while at St. S, located at the southeastern edge of the tidal flat, the sediment was unaffected by the *Ulva* cover [18].

To examine the denitrification, anammox, and DNRA rates using a ^{15}N tracer technique, surface sediments (0–5 cm depth) at Sts. C and S were collected in November 2017. Sediment sampled at St. C was also used for sediment incubation experiments with and without *Ulva*. In November 2017, there were almost no macroalgae at Sts. C and S of the Yatsu tidal flat. The sediment temperature and pH were measured using a sensor (IQ170, IQ Scientific Instruments, Carlsbad, CA, USA). Additionally, the surface sediments sampled

at St. C in October 2017 were used for the effect of sulfide on the denitrifying activity. All seawater was collected in the tidal creek, and *Ulva* samples for sediment incubation experiments were collected near St. C.

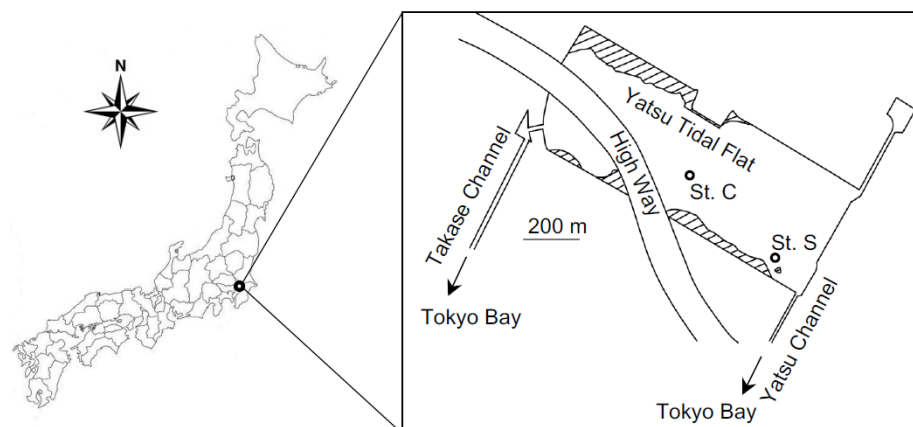


Figure 1. Location of the Yatsu tidal flat, eastern Japan, and sampling Sts. C ($35^{\circ}40.34' \text{ N}$, $140^{\circ}00.22' \text{ E}$) and S ($35^{\circ}40.29' \text{ N}$, $140^{\circ}00.27' \text{ E}$) within the tidal flat.

2.2. Sediment Incubations

2.2.1. Denitrification, Anammox, and DNRA Rates in Sediments with and without *Ulva*

Ulva samples were rinsed with tap water and then washed several times with sterile seawater. Approximately 250 mL of sediment and 200 mL of seawater were transferred to 500 mL glass flasks. The flasks were mixed overnight to oxidize and remove the sulfide. A 35 g wet *Ulva* sample (0.6 kg DW m^{-2} of *Ulva* biomass) was added to each flask. Control flasks (without *Ulva*) were also prepared. The flasks with and without *Ulva* were prepared in triplicate. The flasks were incubated for 21 days at 25°C under a 12 h:12 h light:dark cycle. Seawater and sediment were collected from the flasks at 0, 7, and 21 days to measure the denitrification, anammox, and DNRA rates using a ^{15}N tracer technique. Nutrients and sulfide concentrations in the seawater were also measured.

2.2.2. Effect of Sulfide on Denitrifying Activity

The effect of sulfide on denitrification was examined using the acetylene block method [21,22]. This method is suitable for clarifying the effect of sulfide on denitrification only. Approximately 5 g of wet sediment and 15 mL of seawater were transferred to a 70 mL glass vial. The vial was then sealed with a butyl-rubber stopper and an aluminum cap, after which the air in the headspace was replaced with N_2 . A potassium nitrate (KNO_3) solution and acetylene (C_2H_2) were then added to give final concentrations of 5 mg-N L^{-1} and 6 kPa, respectively.

A sodium sulfide (Na_2S) solution was prepared by bubbling argon in water. The Na_2S solution was added to the vials to attain concentrations ranging from 0 to 200 mg-S L^{-1} before adding a KNO_3 solution and C_2H_2 . Six vials were prepared for each sulfide concentration treatment. Sulfide concentrations in the slurry phase of three vials were measured immediately (initial sulfide concentration). The remaining three vials were incubated under anaerobic conditions for 20 h at 20°C in the dark. After incubation, N_2O in the gas phase of the vial was measured to determine the denitrifying activity. Additionally, the sulfide concentration was measured immediately after the N_2O measurement (final sulfide concentration).

2.3. Chemical Analysis

Denitrification, Anammox, and DNRA Rates Measured Using a ^{15}N Tracer Technique

The denitrification and anammox rates were measured using a ^{15}N tracer technique [23–27]. A sediment slurry (10% w/v) was dispensed into glass vials after sterilizing the seawater

via autoclaving at 121 °C for 15 min, and the vials were tightly capped with butyl-rubber stoppers and purged with helium. The vials were incubated at 20 °C in the dark, and the slurry was continuously stirred using a magnetic stirrer. The incubation was performed strictly anaerobically with combinations of ^{15}N -labeled or unlabeled ammonium chloride (NH_4Cl) and KNO_3 as reactive substrates for N_2 production. The sediment suspension was incubated with 5.6 mg-N L^{-1} NH_4Cl and 28 mg-N L^{-1} KNO_3 in the following combinations: (i) $^{15}\text{NH}_4\text{Cl} + \text{KNO}_3$, (ii) $\text{NH}_4\text{Cl} + \text{K}^{15}\text{NO}_3$, (iii) $^{15}\text{NH}_4\text{Cl}$ without NO_3^- (negative control), and (iv) no NH_4^+ or NO_3^- (negative control). The N_2 isotopologues ($^{14}\text{N}^{15}\text{N}$, $^{15}\text{N}^{15}\text{N}$) in the headspace gas of each vial were collected periodically using a gas-tight syringe under a helium stream and were quantified using a quadrupole gas chromatography–mass spectrometry system (6890N-5973 inert, Agilent Technologies, Santa Clara, CA, USA). The denitrification and anammox rates were estimated by quantifying two N_2 isotopologues. See Senga et al. [18] for a more detailed description of the procedure.

The DNRA rate was measured using $^{14}\text{NH}_4^+ / ^{15}\text{NH}_4^+$ analyses via sequential conversion to N_2O [28]. After measurements of denitrification and anammox rates, a water sample in a 20 mL vial was immediately collected via centrifugation (4 °C, 3000 rpm, 5 min). Then, 5 mL of water sample was filtered and transferred to a new vial. Magnesium oxide and a polytetrafluoroethylene (PTFE sealing tape, Sigma-Aldrich, St. Louis, MI, USA) envelope containing an acidified glass fiber filter (Whatman GF/D; GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) were added to the vial. After sealing the vial with a butyl-rubber stopper and an aluminum cap, it was shaken at 150 rpm for 3 h at 4 °C to trap NH_4^+ in the water sample on the acidified glass fiber filter into the envelope as an ammonium salt. After shaking, the PTFE envelope was removed from the vial and opened to remove the glass fiber filter. The glass filter was transferred to an 11 mL screw cap test tube containing a persulfate-oxidizing reagent. The tube was autoclaved for 1 h at 121 °C to oxidize NH_4^+ to NO_3^- . After the NO_3^- converted from NH_4^+ was oxidized to N_2O via the denitrifier method, the amounts of $^{45}\text{N}_2\text{O}$ and $^{46}\text{N}_2\text{O}$ in the headspace gas were determined using quadrupole GC/MS. The DNRA rate was calculated by quantifying the two N_2O isotopologues. See Kuroiwa et al. [28] for a more detailed description of the procedure.

2.4. Other Quantifications

To determine the sediment water content, approximately 5 g of wet sediment was weighed (wet weight), dried for 24 h at 105 °C to a constant weight, and weighed again. The dry sediment was then homogenized and combusted at 450 °C for 12 h to determine the loss on ignition (ignition loss).

The pore water in the sediment at St. C was extracted from a sediment sample via centrifugation at $3000 \times g$ for 10 min. Insufficient pore water for analysis was extracted from the sediment at St. S due to the low water content. The supernatant was filtered through a pre-combusted (450 °C) glass fiber filter (Whatman GF/F, GE Healthcare) and analyzed for NO_3^- , NH_4^+ , NO_2^- , and PO_4^{3-} concentrations. Seawater was filtered through a pre-combusted glass fiber filter (Whatman GF/F) to determine the NO_3^- , NH_4^+ , NO_2^- , and PO_4^{3-} concentrations. The NO_3^- , NO_2^- , NH_4^+ , and PO_4^{3-} concentrations were measured using the methods described by Wood et al. [29], Bendschneider and Robinson [30], Sagi [31], and Murphy and Riley [32], respectively. The sulfide concentration in seawater was analyzed following the in situ extraction method of Davison and Lishman [33] and Sugahara et al. [34]. All analytical procedures were performed in a sealed system of glass syringes to prevent the volatilization of sulfide and sulfide oxidation by air. Approximately 20 mL of seawater was added to a graduated glass syringe, which was filtered using a 0.45 μm Millex filter (Millipore, Carrigtwohill, Ireland) into an empty 10 mL syringe (syringe–filter–syringe system). The sulfide concentration of the filtrate was spectrophotometrically determined using the methylene blue method [35]. Hereafter, sulfide refers to the sum of the dissolved H_2S gas and its dissociated forms, namely, HS^- and S^{2-} .

The N_2O in the acetylene block method was measured using a gas chromatograph with an electron capture detector (GC-ECD) (6890, Agilent Technologies, Palo Alto, CA, USA). The amount of dissolved N_2O was calculated using the solubility formula of Weiss and Price [36].

3. Statistical Analysis

Significant changes in denitrification and DNRA rates; NH_4^+ , NO_2^- , NO_3^- , and sulfide concentrations with and without *Ulva*; and denitrification activities with different sulfide concentrations were identified using analysis of variance (ANOVA). Student's *t*-test was used to compare the initial and final sulfide concentrations in the experiment to determine the effect of sulfide on denitrification. The statistical analyses were performed using R statistical software [37].

4. Results

4.1. Denitrification, Anammox, and DNRA Rates in Sediments Affected and Unaffected by *Ulva*

Anammox was not detected in November 2017 in the surface sediments at Sts. C and S (Table 1). The contributions of denitrification and DNRA to total dissimilatory NO_3^- reduction (i.e., denitrification + DNRA) at St. C were 82% and 18%, respectively. On the other hand, at St. S, the contributions of denitrification and DNRA were 85% and 15%, respectively. The DNRA rates at both sites did not exceed the denitrification rates. The denitrification and DNRA rates at St. C were higher than at St. S.

Table 1. Temperature ($^{\circ}\text{C}$); pH; water content (%); ignition loss (%); NH_4^+ , NO_2^- , NO_3^- , and PO_4^{3-} concentrations ($\mu\text{g-N}$ or $-\text{P L}^{-1}$); and denitrification, anammox, and DNRA rates ($\mu\text{g-N g-dry}^{-1} \text{ day}^{-1}$) in the surface sediments at the Yatsu tidal flat in November 2017.

	St. C	St. S
Temperature	17.3	17.9
pH	7.63	6.83
Water content	39.7	18.4
Ignition loss	1.4	0.9
NH_4^+	4549	
NO_2^-	5	
NO_3^-	5	
PO_4^{3-}	164	
Denitrification	10.4	5.9
Anammox	nd	nd
DNRA	2.3	1.0

Notes: The NH_4^+ , NO_2^- , NO_3^- , and PO_4^{3-} concentrations at St. S could not be measured due to the low water content. The denitrification, anammox, and DNRA rates were the averages of three measurements.

4.2. Denitrification, Anammox, and DNRA Rates in Sediment Incubations with and without *Ulva*

The biomass of *Ulva* samples added to the flasks (0.6 kg DW m^{-2}) was similar to the maximum biomass of the blooms in May 2016 [18]. It was visually confirmed that the *Ulva* biomass decayed greatly after 7 days. The *Ulva* debris looked like sludge at 21 days (late *Ulva* collapse). Whitish and pink particles above the *Ulva* at the air/water interface were observed after day 7.

All concentrations in the seawater phase of the flasks, except for NH_4^+ and sulfide without *Ulva*, varied significantly with time (ANOVA, $p < 0.05$; Figure 2). The concentration of NH_4^+ with *Ulva* increased with time, while almost no NH_4^+ was detected in the flask without *Ulva* (Figure 2a). In the flask with *Ulva*, the NO_2^- concentration measured on day 0 (ca. $100 \mu\text{g-N L}^{-1}$) was completely consumed by day 7 (Figure 2b). The NO_2^- concentration in the flask without *Ulva* peaked on day 7 and then decreased to almost nil by day 21. In both the presence and absence of *Ulva*, the initial concentration of NO_3^- (ca. $260 \mu\text{g-N L}^{-1}$) on day 0 had largely decreased by day 7, and was completely consumed by day 21 (Figure 2c). The concentration of sulfide in the seawater phase of flasks containing

Ulva peaked on day 7 and then decreased until day 21, while sulfide was not detected in the flask without Ulva (Figure 2d).

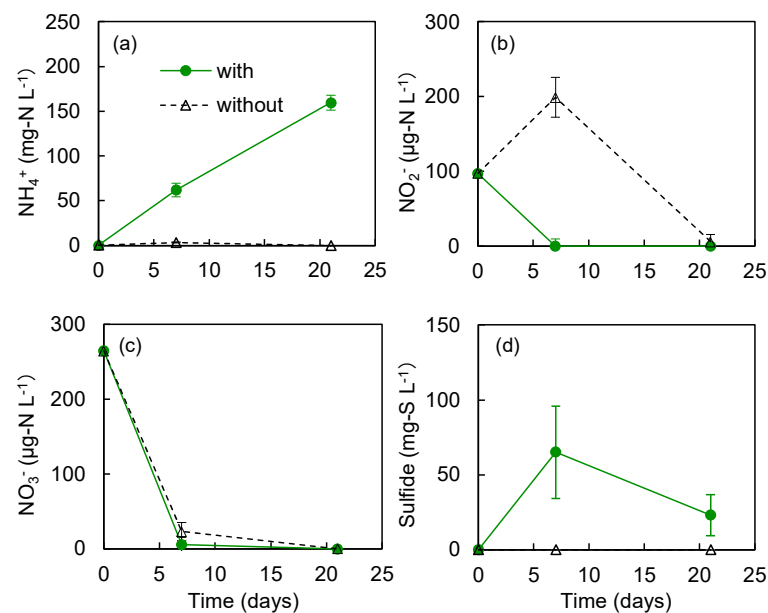


Figure 2. Changes in NH_4^+ (a), NO_2^- (b), NO_3^- (c), and sulfide (d) concentrations in flasks with and without Ulva.

The rate of denitrification in the presence of Ulva varied significantly with time (ANOVA, $p < 0.01$; Figure 3a). The denitrification rate with Ulva decreased slightly on day 7 and then increased on day 21. The rates of denitrification in the absence of Ulva did not vary significantly with time (Figure 3b). The DNRA rates in the presence and absence of Ulva tended to slightly increase and decrease over time, respectively, but not significantly.

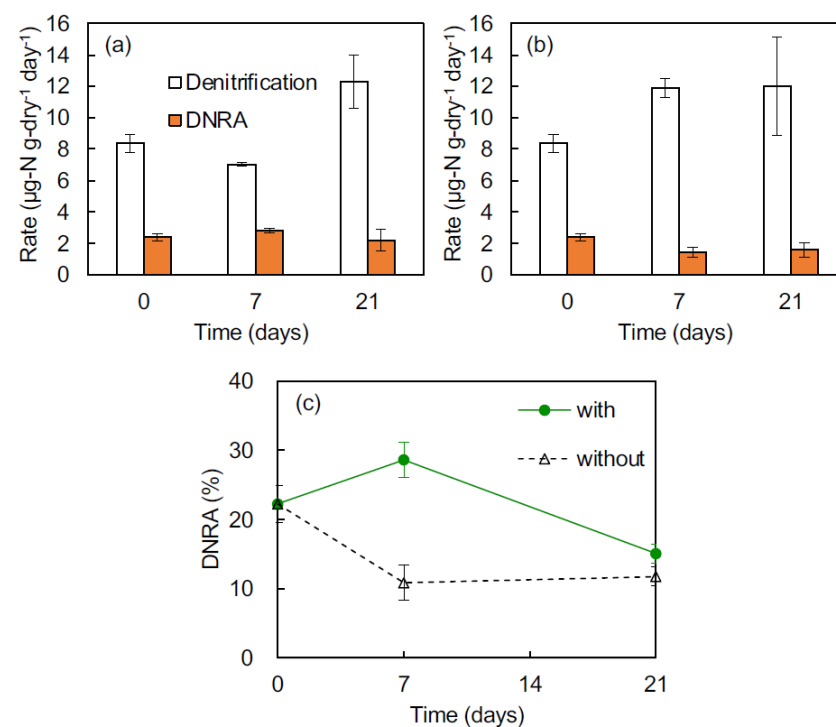


Figure 3. Changes in denitrification and DNRA rates in flasks with Ulva (a) and without Ulva (b) and contributions of DNRA to the total dissimilatory NO_3^- reduction in each treatment (c).

There were significant differences between times regarding the contributions of DNRA to the total dissimilatory NO_3^- reduction with and without Ulva (Figure 3c; ANOVA, $p < 0.05$). The contribution of DNRA with Ulva significantly increased from approximately 22% on day 0 to 29% on day 7, and then significantly decreased to 15% on day 21. On the other hand, the contribution of DNRA without Ulva significantly decreased to 11% on day 7 and was constant between days 7 and 21.

4.3. Effect of Sulfide on Denitrifying Activity

The effect of sulfide concentration on denitrification in the surface sediment at St. C was quantified using the acetylene block method. Although sulfide was added at 0, 5, 20, 50, 100, and 200 mg-S L^{-1} , the sulfide concentrations measured in the seawater at the beginning of the incubation were 0, 4, 21, 55, 108, and 212 mg-S L^{-1} , respectively (Figure 4 and Table 2). The denitrifying activities varied significantly with sulfide concentration (ANOVA, $p < 0.001$; Figure 4). The denitrifying activities drastically decreased between 0 and 108 mg-S L^{-1} and were constant between 108 and 212 mg-S L^{-1} sulfide concentrations (Figure 4). After the incubation, there were significant differences between the initial and final sulfide concentrations for all treatments (Student's t -test, $p < 0.01$; Table 2). All of the final sulfide concentrations decreased by $>40\%$.

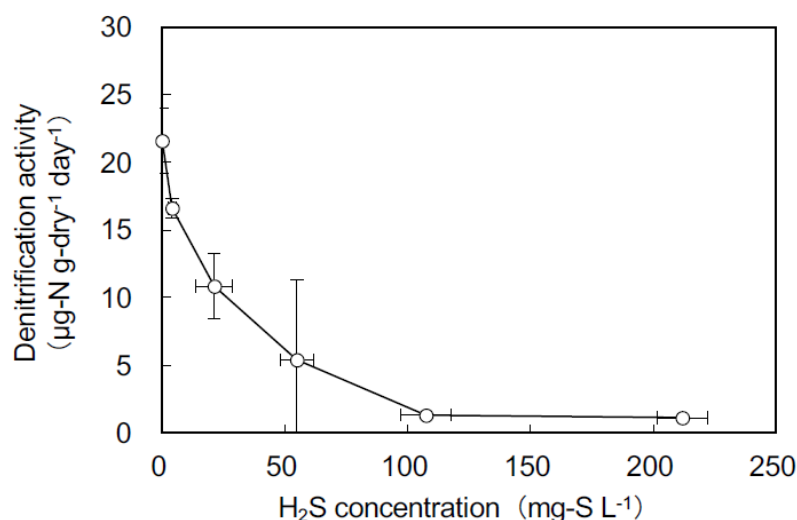


Figure 4. The effects of different sulfide concentrations on denitrification activities in the sediment at St. C measured using the acetylene blockage method.

Table 2. Estimated, initial, and final sulfide concentrations (mg-S L^{-1}) to determine the effect of sulfide on denitrification. The initial and final concentrations were measured on days 0 and 21, respectively.

Estimated Concentration	Initial Concentration	Final Concentration
0	0.0 ± 0.0	0.0 ± 0.0
5	4.1 ± 1.7	0.0 ± 0.0
10	21.1 ± 7.3	0.0 ± 0.0
50	54.9 ± 6.8	15.1 ± 2.7
100	107.5 ± 10.4	65.5 ± 2.0
200	211.9 ± 10.5	95.8 ± 32.0

5. Discussion

5.1. Dissimilatory NO_3^- Reduction Pathways in the Yatsu Tidal Flat

In this study, anammox was negligible at Sts. C and S. In a previous study, we reported that anammox at Sts. C and S was consistently low compared with denitrification, accounting for $<7\%$ of the total N_2 production in September 2015 and November 2016 [18].

This result suggested that anammox played a limited role in NO_3^- removal. Therefore, the principal pathway of nitrogen removal in the surface sediment at the hypereutrophic Yatsu tidal flat was denitrification. Anammox generally plays a limited role in regulating NO_3^- reduction in eutrophic coastal ecosystems [6,7,38].

The rates of denitrification and DNRA were approximately twofold higher at St. C than at St. S (Table 1). Ulva cover on the surface sediment was frequently found at St. C, but not at St. S. Ignition loss, as a measure of organic content, at St. C was higher than at St. S, probably due to the presence of Ulva (Table 1). Additionally, at St. C, sulfide accumulation under macroalgal mats was reported [4]. There are two ways to enhance denitrification and DNRA: a heterotrophic (fermentative) process with organic carbon as an electron donor and a chemolithoautotrophic process with sulfide or Fe (II) as an electron donor [7,15,39]. Therefore, because there was a rich source of electron donors to drive denitrification and DNRA, the rates of denitrification and DNRA were higher at St. C than at St. S. From August 2012 to January 2017, the denitrifying activity found using the acetylene block method was also found to be higher at St. C than at St. S [18]. A key factor that regulates denitrification in the sandy sediment at St. S is the organic carbon content, while the key factor at St. C is nitrogen substrates due to competition with macroalgae for nitrogen.

There was no significant difference in the contribution of DNRA to the total dissimilatory NO_3^- reduction between Sts. C and S. Unfortunately, on November 2017 when sediment was collected from the Yatsu tidal flat, no macroalgae blooms were found at St. C. Therefore, the effect of macroalgae cover or its collapse on the dissimilatory NO_3^- reduction could not be determined. However, even in the absence of macroalgae, there were differences in the rates of denitrification and DNRA between Sts. C and S, but there were no significant differences in the ratios. The surface sediment at the Yatsu tidal flat had a 10–20% DNRA potential even without the impact of Ulva cover. The estimated contribution of DNRA in this study was in the range of values previously reported values for other intertidal sediments. In the intertidal permeable sediments of the eutrophied Wadden Sea, DNRA also accounted for 10–20% of the NO_3^- consumption [40]. The DNRA in the intertidal sediments of the Yangtze Estuary and Colne Estuary accounted for 3–45% and 11–60% of the total NO_3^- consumption, respectively [8,41]. The DNRA is of major importance in most shallow coastal sediments [15].

5.2. Influence of Macroalgae Blooms on Dissimilatory NO_3^- Reduction Pathways

Although sulfide in flasks without Ulva was not detected in seawater, the presence of Ulva accelerated the sulfide production via sulfate reduction in the sediment until day 7, and the sulfide concentration decreased thereafter (Figure 2d). Sulfide is oxidized both chemically and microbially [42,43]. Since whitish and pink particles were observed in the flasks containing Ulva after day 7, most of the sulfide was expected to have been oxidized microbially. Namely, these particles might be sulfide-oxidizing bacteria (purple sulfur bacteria) or elemental sulfur. These results are consistent with the results obtained in the incubation experiment of the previous study in which sulfate reduction would be shifted to sulfur oxidation after the peak of sulfide production [4]. In this study, the sulfide was produced largely by sulfate reduction in flasks containing Ulva on day 7 and was transformed to elemental sulfur by purple sulfur bacteria between days 7 and 21. Under such a change in sulfur speciation, the NO_3^- and NO_2^- concentrations were almost undetectable between days 7 and 21, while the NH_4^+ concentration increased over time (Figure 2a–c), suggesting that the anaerobic environment was conserved in flasks containing Ulva throughout the experiment, and there would be virtually no nitrification in the presence of macroalgae.

The rate of denitrification decreased in the flasks containing Ulva from days 0 to 7. The sulfide concentration was the highest on day 7. The denitrification rate was reduced with increasing sulfide concentration, as sulfide inhibited the reduction of N_2O to N_2 [39,44]. In the experiment using sediment from the Yatsu Tidal Flat, negative effects of $>4 \text{ mg-S L}^{-1}$ on denitrifying activity were observed (Figure 4). Therefore, sulfide production under

macroalgae covers negatively affected denitrification in the Yatsu tidal flat. The DNRA rates were unchanged on day 7; therefore, DNRA was not affected by sulfide. Previous studies reported that treatment with high concentrations of sulfide results in either an increase or no difference in the DNRA rate [6,16,44,45]. In the surface sediment at the Yatsu tidal flat, sulfide produced in the presence of vigorous macroalgae negatively influenced denitrification but had no significant effect on the DNRA rate. Consequently, the DNRA contribution to total dissimilatory NO_3^- reduction increased from 20% on day 0 to 30% on day 7. A similar result was reported in the sediment of the invasive *Spartina alterniflora* marshland in the Yangtze River Estuary, China [46]. The invasion of *Spartina alterniflora* increased DNRA by altering the SO_4^{2-} concentration and pH in the sediment.

The denitrification rate in the flasks with *Ulva* increased, while the DNRA rate was unchanged between days 7 and 21. On day 21, the sulfide concentration had decreased greatly from day 7, and elemental-sulfur-like particles were observed above the *Ulva*. In the experiment examining the effect of the sulfide concentration on the denitrifying activity, the sulfide concentration decreased by 40–100% after 20 h (Table 2). This result suggested that sulfide produced in the presence of *Ulva* might be consumed via sulfur oxidation by microorganisms. Autotrophic denitrification, sulfide oxidation coupled with nitrate reduction was found in the salt marsh at the Plum Island Ecosystem Long-Term Ecological Research site in Rowley, USA [16]; the Gulf of Thailand [47]; and the Yarra River Estuary, Australia [48]. We hypothesized that sulfide produced beneath *Ulva* mats inhibits general heterotrophic denitrification and accelerates sulfide-oxidizing denitrification, but does not affect DNRA. The macroalgae status alters sulfur dynamics and affects dissimilatory NO_3^- reduction pathways. The details of the relationship between sulfur dynamics and the dissimilatory NO_3^- reduction pathways in the Yatsu tidal flat need to be explored using molecular microbiology techniques.

The contribution of DNRA in the presence of *Ulva* decreased on day 21 to 15% due to an increase in the denitrification rate and was similar to the DNRA observed on day 21 without *Ulva* (12%). This result indicated that DNRA was greater during the blooms than at the collapse. The amount of NO_3^- removed by denitrification decreased and the proportion of N that remained as NH_4^+ consequently increased during the macroalgae blooms. This result suggested that macroalgae controlled the removal versus retention of bioavailable nitrogen within the sediment under anaerobic conditions. This is very important in terms of the physiology of *Ulva* because *Ulva* in the Yatsu tidal flat preferentially uptakes NH_4^+ over NO_3^- [4]. An increase in ammonification is considered to provide a strong advantage to the subsequent generation of *Ulva* blooms.

In the flasks containing *Ulva*, NH_4^+ was likely produced via DNRA, but also via *Ulva* decomposition, especially in the late experiment (Figure 2a). NH_4^+ is reported to account for a major proportion of the total nitrogen efflux during the decomposition of *Ulva* sp. [49]. NH_4^+ produced via decomposition is typically greater than that produced via DNRA in coastal ecosystems [50,51]. In the anaerobic sediment where DNRA and decomposition occur, nitrification was not able to progress; consequently, denitrification did not occur. Thus, it was expected that the large proportion of nitrogen present as NH_4^+ would not transform further and would be recycled by macroalgae such as *Ulva* in these ecosystems.

The behaviors of NH_4^+ , NO_2^- , and NO_3^- indicated that nitrification occurred in the flasks without *Ulva* (Figure 2a–c). Sulfide, which inhibits denitrification and nitrification [52], was not produced in the flasks without *Ulva* (Figure 2d). Additionally, the rate of denitrification in the absence of *Ulva* was higher from days 7 to 21 than on day 0 (Figure 3b). Nitrogen removal pathways, i.e., nitrification–denitrification, appeared to proceed at a constant rate in the flasks without *Ulva* for 21 days. However, DNRA progressed slightly, and its contribution was 12% (Figure 3c). This contribution was in the range of those measured at Sts. C and S (Table 1). Therefore, in the sediment at the Yatsu tidal flat, the DNRA potential was in the range of 10–20% and increased to approximately 30% only when there were macroalgae blooms.

6. Conclusions

This study demonstrated the important role of the dissimilatory nitrate reduction pathway in nitrogen dynamics in the sediment hypereutrophic Yatsu tidal flat. Denitrification was a major pathway for dissimilatory nitrate reduction regardless of the macroalgal status. Macroalgae cover on the sediment accelerated sulfide production, which inhibited denitrification and enhanced the DNRA contribution (30%). During the late collapse of macroalgal blooms, sulfate reduction shifted to sulfide oxidation. Consequently, the denitrification rate increased and the DNRA contribution returned to its original value in the absence of macroalgae (10–20%). During macroalgal blooms, NH_4^+ tends to accumulate because NH_4^+ is produced by macroalgal decomposition and DNRA, and nitrification is not able to proceed in the reductive sediment under macroalgal mats. NH_4^+ accumulation was very important for sustaining the growth of macroalgal blooms, as well as for the progress of eutrophication.

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