Growth responses of coastal phytoplankton to continuous supply of low nitric oxide concentrations during the light period

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Abstract

As an important signaling molecule, nitric oxide (NO) plays an essential role in regulating several physiological processes. In marine environments, NO is produced in surface seawater through the photochemical reactions of nitrite. It has been hypothesized that NO affects phytoplankton growth, especially in coastal eutrophic waters abundant in nitrite; however, conducting ecological assessments is challenging due to the short lifetime and high reactivity of NO in natural surface waters. In this study, a variable-volume repeated fed-batch culture system was employed to simulate the NO supply rate in the laboratory and determine the growth responses of phytoplankton culture strains isolated from Japanese coastal waters. The growth rates of the diatom *Skeletonema marinoi-dohrnii* complex and raphidophyte *Chattonella marina* were almost completely inhibited at average NO supply rates of 28 and 1.4 pM s^{-1}, respectively, indicating species differences in sensitivity to NO. However, when NO supply was terminated, the growth rates of both cultures returned to approximately the same levels as the NO-free control within 1–2 days, indicating that the inhibitory effect of NO is temporary. These results suggest that phytoplankton growth may be sufficiently inhibited by NO produced through photochemical reactions in surface seawater. Therefore, in eutrophic waters with relatively high nitrite concentrations, the effect of NO on altering the abundance of phytoplankton species as well as the occurrence and disappearance of red tides should be considered.

Key words: phytoplankton, nitric oxide, growth, fed-batch culture, coastal waters

As a key signaling molecule, nitric oxide (NO) plays an important role in regulating the growth, development, maturation, and aging of several organisms.[1, 2] Higher plants and microalgae not only respond to exogenous NO; they also produce NO themselves.[3-5] Despite the importance of NO in mediating physiological processes *in vivo*, little is known about its biological role in marine ecosystems because NO concentrations are lower than the concentrations of other dissolved inorganic nitrogen species, and it is difficult to control experimental systems to reproduce NO concentrations under natural conditions, as NO is a highly reactive chemical species.

In the marine nitrogen cycle, NO is among the intermediate products of microbial nitrification and denitrification; meanwhile, in the ocean surface layer, NO is mainly produced via photochemical formation reactions from nitrite [NO_2^- + HOH + *hv* (UV) → NO + OH + OH^-].[6] It has been reported that approximately 2–15% of nitrite can be converted to NO per day in the equatorial Pacific Ocean, and ultraviolet (UV) light intensity and nitrite concentration are related factors of variation.[6-8] As NO photoformation rates are positively correlated with nitrite concentrations in seawater,[9-11] NO formation reactions are more active in coastal eutrophic waters than in the open ocean because the nitrite concentrations in the surface layer of eutrophic water (Osaka Bay) can reach as high as 5.6–19 μM.[11] For example, the NO production rate (0.7–39 pM s^{-1}) in the surface waters of the Seto Inland Sea in Japan is 10 times higher than that in the equatorial Pacific Ocean. However, NO produced in seawater is scavenged rapidly by reacting with dissolved oxygen and other substances as a free radical molecule. In the surface layer of the Seto Inland Sea, the lifetime of NO is approximately 1.8–20 s, and its steady-state daytime concentration, determined by the balance between NO production and scavenging, is 3–320 pM.[9-11] Owing to its high reactivity, the short lifetime of NO in seawater makes it difficult to assess the effects of NO on the physiological ecology of phytoplankton, which is responsible for primary production in the ocean surface layer.

In the past, experiments have been conducted to compare the growth of marine and freshwater phytoplankton by adding a high concentration (nM or μM) of NO solution only once at the beginning of culture or 1–2 times a day; moreover, an NO donor [*S*-nitroso-*N*-acetyl-$_{DL}$-penicillamine (SNAP) or sodium nitroprusside] may be introduced to maintain a certain NO concentration for several hours or longer.[12-16] The results of these experiments revealed that phytoplankton growth might be enhanced by low NO concentrations or inhibited by high NO concentrations. However, it is not clear whether similar phytoplankton responses are observed in the actual marine environment because the NO concentrations used in these experiments were much higher than those in the natural environment, and the exposure time to NO was brief.

To reproduce the NO supply derived from the photochemical reaction of nitrite in surface seawater, it is necessary to continuously add a small amount of NO close to the NO concentration in the natural environment during the light phase of the light–dark cycle of phytoplankton cultures. Therefore, a variable-volume repeated fed-batch (VRFB) culture system is suitable for

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this purpose because it facilitates the continuous supply of NO to the culture medium and prevents the flushing of phytoplankton cells from the culture system.

The aim of this study was to verify the role of NO in regulating the growth of phytoplankton in a realistic marine surface environment. Laboratory culture experiments using the VRFB culture system were conducted on two representative phytoplankton species that form red tides along the coast of Japan. The findings of this study contribute to the body of knowledge regarding the regulatory role of NO on phytoplankton growth as well as the development of programs aimed at mitigating eutrophication and red tides.

Materials and Methods

Procurement of phytoplankton samples

Axenic culture strains of the diatom *Skeletonema marinoi-dohrnii* complex (NIES-323, collected on January 21, 1985 from Osaka Bay, Japan; 34.505°N, 135.305°E) and the raphidophyte *Chattonella marina* (NIES-113, collected on July 30, 1982 from Seto Inland Sea, Japan; 34.410°N, 134.000°E) were provided by the Microbial Culture Collection at the National Institute for Environmental Studies through the National BioResource Project of the MEXT, Japan. Stock cultures at the exponential growth phase were maintained in sterile polystyrene cell culture flasks with vented cap (Suspension Culture Flask 1103-025; Iwaki, Tokyo, Japan) containing 50 mL of autoclaved f/2 medium[17] at 20°C under 100 μmol m^{-2} s^{-1} photon flux density with a 14:10 light–dark cycle.

All manipulations of culture strains and media were performed on a clean bench (VST-1000A; NK Systems, Tokyo, Japan). Except for pre-sterilized equipment (e.g., cell culture flasks, filter filtration units, and pipette tips) that were used as supplied by the manufacturer, all materials were first cleaned with detergent, acid-washed with 1.2 M HCl, and rinsed with ultrapure water (≤ 18 MΩ cm^{-1} Milli-Q water, Merck Millipore, Burlington, MA, USA) prior to use.

Preparation of NO feed solution

A NO feed solution was prepared by adding a fixed volume of a NO-saturated aqueous solution to a filter-sterilized f/2 medium (500 mL) that had been purged with nitrogen gas for 60 min to remove dissolved oxygen. Short and long Teflon tubes with three-way cocks were attached to the mouth of a 500-mL glass bottle containing the f/2 medium via silicone rubber stoppers, and a 1-L Tedlar Bag filled with nitrogen gas was connected to the short tube to supply nitrogen gas when the liquid level dropped due to feeding. The NO-saturated aqueous solution was prepared as follows. A small glass vial with a Teflon-coated rubber stopper was filled with 20 mL of Milli-Q water and purged with nitrogen gas for 30 min, followed by purging with pure NO gas for 10 min. The pressure of these gases was adjusted to 0.025 MPa for nitrogen and 0.02 MPa for NO, and impurities were removed through a 0.01-μm pore size air filter and 10% KOH solution in a Teflon gas-cleaning bottle. The temperature of the NO-saturated solution was adjusted to 20 °C in a thermostatic water bath. The NO-saturated aqueous solution was added to 500 mL of deoxygenated f/2 medium via a three-way cock using a 1-mL gastight syringe (Hamilton, Reno, NV, USA) at three levels: low (0.4 mL), medium (1.7 mL), and high (6.6 mL).

In the *C. marina* culture experiment, another NO feed solution was also prepared by adding 0.8 mL of the NO-saturated aqueous solution. The NO feed solution was pumped through 0.5-mm i.d. Ethylene Tetrafluoroethylene (ETFE) tubing and Viton pump tubing (Masterflex® 96412-13; Cole-Parmer, Vernon Hills, IL, USA) into the culture vessel at a flow rate of 0.5 mL min^{-1} using a peristaltic pump (Masterflex® L/S 7550-50; Cole-Parmer, Vernon Hills, IL, USA).

As the NO in the feed solution was not stable, and its concentration tended to decrease gradually even in the glass bottle with nitrogen gas, it was necessary to examine the temporal changes in the NO concentration in the NO feed solution supplied to the culture vessel. Therefore, in addition to the plankton culture experiments, the NO feed solutions (low, medium, and high) that flowed out of the outlet of the pumping tube connected to the culture vessel were collected in an amber glass vial every hour. The mouth of the vial was covered with parafilm, and its interior was purged with nitrogen gas. The volume of the collected solution was increased to 15 mL by temporarily increasing the pump flow rate to 6 mL min^{-1}. Immediately afterwards, the NO concentration in the collected solution was measured using a free-radical analyzer (TBR4100; World Precision Instruments, Sarasota, FL, USA) equipped with a NO sensor (ISO-NOPF200; World Precision Instruments, Sarasota, FL, USA). The NO sensor was calibrated with NO liberated from a SNAP donor using $CuCl_2$ as a catalyst, according to the NO Microsensor Instruction Manual from World Precision Instruments. The NO sensor had a sensitivity of 10 pA nM^{-1} and a detection limit of 0.328 nM. NO concentration was calculated using a calibration curve by subtracting the blank current values of the deoxygenated f/2 medium before NO addition from the measured values.

The VRFB culture system

The VRFB culture system used in this study consisted of the aforementioned pumping system of the NO feed solution and a 1-L glass spinner flask with two angled sidearms (4500-1L; Corning, Corning, NY, USA) that held the phytoplankton cell culture medium (Fig. 1). The flask was filled with 1 L of f/2 medium sterilized using a 0.1-μm filtration unit (Nalgene rapid-flow sterile

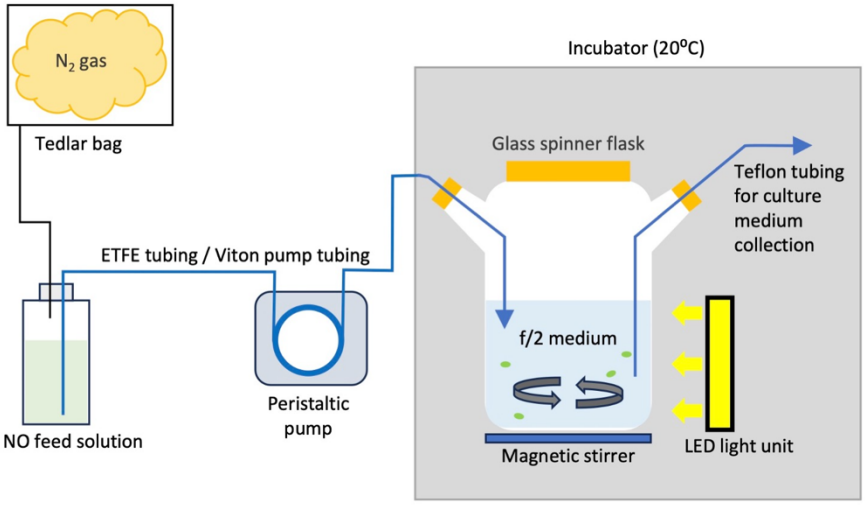

Fig. 1 Schematic diagram of the variable-volume repeated fed-batch (VRFB) culture system.

disposable filter units with a polyethersulfone membrane 567-0010; Thermo Fisher Scientific, Waltham, MA, USA) and then inoculated with a certain amount of plankton stock culture in the exponential growth phase. Afterwards, the flask was placed in an incubator (ICB-142L; Iwaki, Tokyo, Japan) at 20°C. The flask was equipped with an impeller that was rotated using a magnetic stirrer at 16 rpm. Lighting in the incubator was provided by a white light-emitting diode unit for plant research (ISL-150X150-WW-SN; CCS, Kyoto, Japan), with a 14:10 light–dark cycle and photon flux density of 100 μmol m^{-2} s^{-1}. The NO feed solution was replenished daily before the start of the light period and fed from the sidearm at a flow rate of 0.5 mL min^{-1} for 14 h during the light period, and the pump was stopped at the end of the light period. At the end of the subsequent dark period, 420 mL of culture medium, equivalent to the volume of NO feed solution added to the flask during 14 h, was withdrawn through a Teflon tube attached to the other sidearm and used for cell counting. This means that a 1-L portion of the culture medium was used as the seed culture in the same culture vessel. The pumping of the NO feed solution during the light period and collection of the culture medium at the end of the dark period were repeated thrice.

NO supply rates

Culture experiments for *S. marinoi-dohrnii* complex were performed at three NO levels: low, medium, and high. The average NO supply rates at the low, medium, and high NO levels were 1.4, 5.4, and 28 pM s^{-1}, respectively (see Results section). In the *C. marina* culture experiments, 2.5 pM s^{-1} NO supply rate was used instead of 28 pM s^{-1} because no growth was observed at 5.4 pM s^{-1}. In both experiments, a treatment feeding deoxygenated f/2 medium without NO was used as the control. Two VRFB culture systems were prepared, and culture experiments were simultaneously performed at two different NO supply rates; however, replicated experiments at each NO level were not performed because of the difficulty in preparing numerous experiments.

Monitoring phytoplankton growth

To monitor phytoplankton growth, the number of cells in the culture medium was counted daily under an inverted optical microscope (CKX41; Olympus, Tokyo, Japan) with a 1-mm ruled cell-counting glass slides at 100x magnification. *In vivo* chlorophyll fluorescence was measured using a laboratory fluorometer (Trilogy, Turner Designs, San Jose, CA, USA) equipped with a chlorophyll *in-vivo* module (data for *C. marina* are not shown due to low algal biomass). These values were corrected for the dilution factor due to the supplied NO feed solution. The specific growth rate (d^{-1}) was determined from the slope of the exponential fitting of the relationship between cell number and incubation duration. The statistical analysis tool in Excel software (Microsoft) was used to test for differences in specific growth rate.

Results

Changes in NO concentration in the feed solution flowing into the culture vessel

At all three NO levels, the NO concentration in the feed solution entering the culture vessel gradually decreased over time during the 14-h feeding period; however, at the high NO level, the NO concentration was almost similar to its initial value for the first 3 h (Fig. 2). The average NO concentrations and standard deviations (SD) at the low, medium, and high NO levels were 186 ± 141, 713

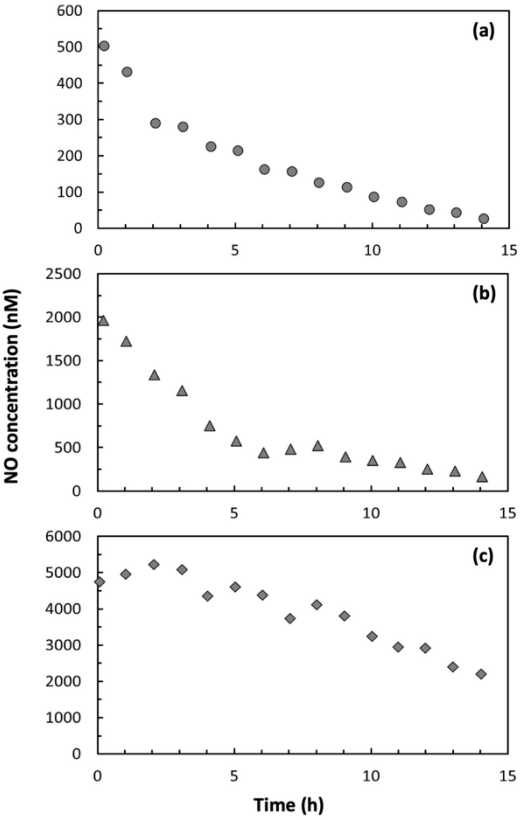

Fig. 2 Temporal changes in NO concentration in the feed solution flowing into the culture vessel at three different NO levels: (a) low, (b) medium, and (c) high.

± 566, and 3,920 ± 978 nM, respectively.

Based on the pumping flow rate of the NO feed solution (0.5 mL min^{-1}), the hourly NO concentration and the total volume of culture medium in the culture vessel at a given time [initial volume of the culture medium (1 L) + accumulated volume of the NO feed solution], the NO supply rates (the 14-h average ± SD) at the low, medium, and high levels were 1.4 ± 1.2, 5.4 ± 4.8, and 28 ± 9.6 pM s^{-1}, respectively. As the NO supplied to the culture medium induces secondary reactions within a few seconds, the steady-state concentration of dissolved NO was determined by the balance between NO supply and loss in the culture medium. Assuming that the total scavenging rate constant of NO is 0.25 s^{-1}, which is the average value observed in the Seto Inland Sea,[10] the NO steady-state concentrations (the 14-h average ± SD) in the culture medium at the low, medium, and high levels were 5.6 ± 4.8, 21 ± 19, and 110 ± 38 pM, respectively. The NO lifetime at the high level was 4 s.

Phytoplankton growth response to NO supply in the VRFB culture system

The *S. marinoi-dohrnii* complex was cultured in the VRFB culture system for 3 days, and its growth response to average NO supply rates of 1.4, 5.4, and 28 pM s^{-1} were compared with that of the control. The cell count decreased with increasing NO supply rate, and growth was strongly inhibited at NO supply rates ≥ 5.4 pM s^{-1} ($P < 0.05$) (Fig. 3a). From Days 1 to 3 of incubation, the specific growth rates at NO supply rates of 1.4, 5.4, and 28 pM s^{-1} were 93%, 38%, and 8% of that of the control, respectively. The *S. marinoi-dohrnii* complex culture almost stopped growing at the NO supply rate of 28 pM s^{-1} (Fig. 3b).

Meanwhile, the cell number of *C. marina* cultured for 3 days at NO supply rates of 1.4 and 2.5 pM s^{-1} did not change from the beginning of the experiment. Its cell number only decreased at 5.4 pM s^{-1} NO supply rate; hence, its specific growth rate became negative (Fig. 4).

These results indicate that increasing NO concentrations and NO supply rates strongly inhibited the growth of the *S. marinoi-dohrnii* complex and *C. marina*, and the threshold for growth inhibition was lower for *C. marina* than for the *S. marinoi-dohrnii* complex.

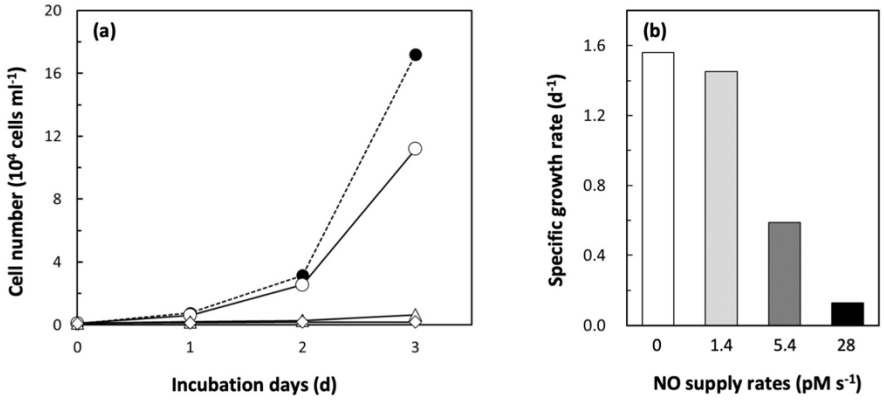

Fig. 3 Growth response of *Skeletonema marinoi-dohrnii* complex under different nitric oxide (NO) supply rates. (a) Time-course of cell growth: 0 pM s^{-1} (black circles), 1.4 pM s^{-1} (white circles), 5.4 pM s^{-1} (triangles), and 28 pM s^{-1} (diamonds). (b) Specific growth rates from Day 1 to 3 of incubation.

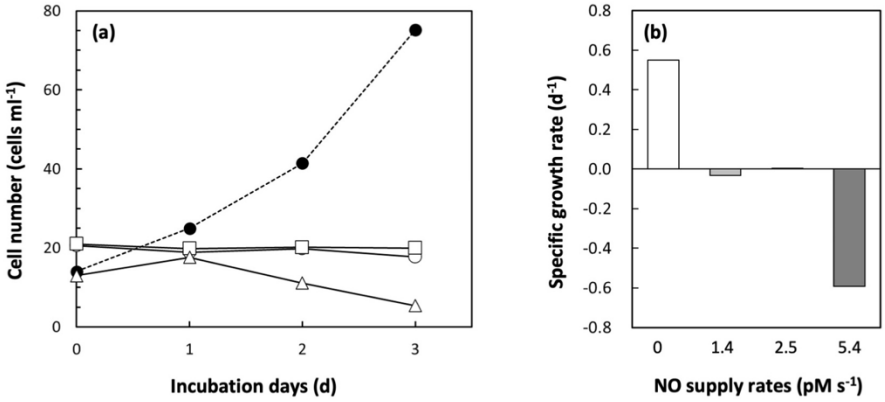

Fig. 4 Growth response of *Chattonella marina* under different nitric oxide (NO) supply rates. (a) Time-course of cell growth: 0 pM s^{-1} (black circles), 1.4 pM s^{-1} (white circles), 2.5 pM s^{-1} (squares), and 5.4 pM s^{-1} (triangles). (b) Specific growth rates from Day 1 to 3 of incubation.

Recovery of phytoplankton growth after termination of NO supply

To examine the recovery from NO-induced growth inhibition, phytoplankton growth was monitored for several days after the termination of 3-day NO supply. The growth rate of the *S. marinoi-dohrnii* complex exposed to NO supply rates of 5.4 and 28 pM s^{-1} clearly increased after Day 5 of incubation, that is, the 2nd day after the termination of NO supply (Fig. 5a). In particular, the growth rate of cells subjected to 28 pM s^{-1} NO supply rate was greater than that of the control. Similarly, the cell growth rate of *C. marina* began to increase within 1–2 days after the NO supply was terminated; the growth rate of the cells exposed to 5.4 pM s^{-1} NO supply recovered to a level comparable to that of the control (Fig. 5b). These results indicate that the cells recovered from NO-induced growth inhibition within a short period of time.

Discussion

Status of NO supply levels in the VRFB culture system

In the VRFB culture system used in this study, the NO feed solution was continuously supplied to the culture vessels during the 14-h light period; the NO supply rate was highest at the start of the feeding period, then it gradually decreased over time (Fig. 2). The average NO supply rate was 1.4–28 pM s^{-1}, and the maximum NO supply rate at the beginning of the feeding period was maintained at 4.2–41 pM s^{-1}. Photochemical NO production rates in surface waters are approximately

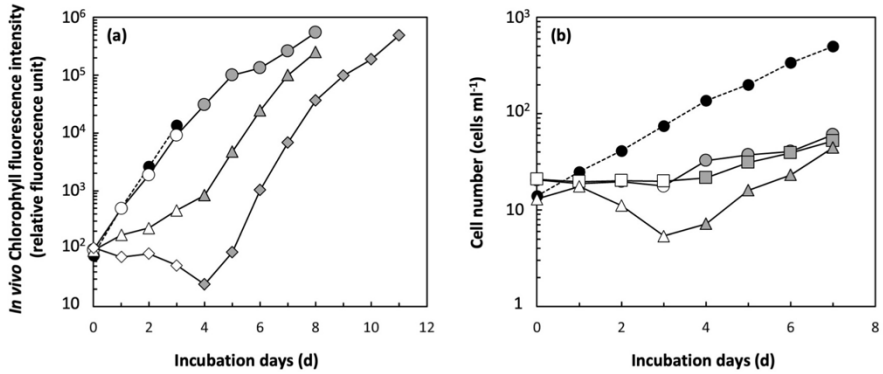

Fig. 5 (a) Time-course of *in vivo* chlorophyll fluorescence intensity of the *Skeletonema marinoi-dohrnii* complex. Each culture was exposed to NO supply rates of 0 pM s^{-1} (black circles), 1.4 pM s^{-1} (white circles), 5.4 pM s^{-1} (triangles), and 28 pM s^{-1} (diamonds) for 3 days, after which NO supply was terminated, and batch cultures were continued thereafter (gray symbols). (b) Time-course of cell growth of *Chattonella marina*. Each culture was exposed to NO supply rates of 0 pM s^{-1} (black circles), 1.4 pM s^{-1} (white circles), 2.5 pM s^{-1} (squares), and 5.4 pM s^{-1} (triangles) for 3 days, after which NO supply was terminated, and batch cultures were continued thereafter (gray symbols).

0.7–39 pM s^{-1} in the Seto Inland Sea,[9-11] 1.5 pM s^{-1} in coastal waters off Qingdao,[18] ≥ 0.4–1.2 pM s^{-1} in the central equatorial Pacific,[8] and 0.5 pM s^{-1} in the northwest Pacific.[19] In addition, the NO concentrations in various surface waters from the coast to the open ocean range from 12 to 260 pM,[8-11, 18-22] which corresponded to the NO concentrations used in this study; thus, the goal of reproducing natural NO concentration levels in the experimental system was achieved.

In previous experiments that examined the effects of NO on phytoplankton growth, high NO concentrations (nM or μM as the final concentration) were added on a one-shot basis.[12-14] Another commonly used method is introducing a NO donor (SNAP or sodium nitroprusside) to generate NO for several hours;[15, 16] however, there are concerns on the effects of the coexisting substance (N-acetyl-$_D$-penicillamine) and decomposition product (cyanide) generated during the reaction. Unlike in the past experiments, the natural ocean surface conditions are more appropriately reflected in the present study. Nevertheless, the diurnal pattern observed in the natural environment—NO concentration increases after sunrise, reaches its highest concentration around noon, and decreases toward sunset in response to changes in solar radiation intensity during the day—was not reproduced in this study. If real-time monitoring of NO concentration in the feed solution becomes possible with high sensitivity in the future, it may be possible to automatically control the pumping flow rate of the NO feed solution to more accurately reproduce natural fluctuations in NO concentration; thus, this system has a potential for further improvements in the future.

Effects of continuous low-level NO supply on phytoplankton growth

The continuous supply of low NO concentrations (at pM levels) during the light period induced a growth-inhibitory effect on the *S. marinoi-dohrnii* complex and *C. marina* (Figs. 3 and 4). The threshold NO supply rate that induced cell growth inhibition was lower for *C. marina* than for the *S. marinoi-dohrnii* complex, confirming that NO sensitivity differs among phytoplankton species. Consistent with the results of this study, in previous single NO addition experiments, phytoplankton growth was inhibited at high NO concentrations.[12-14] However, the growth inhibitory effect of a single NO addition was observed only at extremely high NO concentrations at the μM level, which is considerably different from the NO concentrations in natural surface seawater. This study confirms for the first time that phytoplankton growth is inhibited by continuous NO supply at pM levels, similar to that found in natural environments, indicating that NO cannot be ignored as a regulator of phytoplankton growth in the ocean. Although environmental conditions such as strong sunlight and abundant nutrients, including nitrite, are favorable for phytoplankton photosynthesis, they also promote photochemical NO production in the ocean surface layer. Therefore, a balance between the positive effects of light and nutrients and the negative effects of NO on phytoplankton growth should be considered in eutrophication mitigation programs. In such situations, differences in sensitivity to NO among algal species may be one of the factors that influence changes in the composition and abundance of the phytoplankton community as well as the evolution of red tide blooms.

NO is a free radical that readily diffuses across biological membranes and reacts with biomolecules such as metal complexes, radicals, nucleic acids, proteins, and lipids.[23] The reactivity of NO with reactive oxygen species in plant cells is a direct source of toxicity; thus, excessively high NO concentrations are toxic to cells.[24] Moreover, NO damages key enzymes, disrupts cell membranes, and induces cell leakage.[3] Yamasaki[25] pointed out that the molecular mechanism behind the NO inhibition of photosynthesis is not clear; nonetheless, the decline in net photosynthetic activity caused by NO could be explained by its inhibitory effect on the Calvin–Benson cycle. Meanwhile, it has been hypothesized that NO inhibits the activity of enzymes involved in the secretory pathway, such as glyceraldehyde-3-phosphate dehydrogenase, via *S*-nitrosylation of cysteine residues and, consequently, modulates cell growth in green algae.[15] Meanwhile, Omar *et al.*[26] investigated the genomes and transcriptomes of diverse marine phytoplankton and identified sequences of nitric oxide dioxygenase (NOD) involved in NO scavenging in eukaryotic pennate diatoms, haptophytes, and dinoflagellates. However, NOD sequences have not been annotated or are absent in *Prochlorococcus*, most green algae, and most centric diatoms, and the capacity to scavenge NO may increase with increasing cell size and may be affected by flagella and colony formation. Although the cell size of the centric diatom *S. marinoi-dohrnii* complex was smaller than that of *C. marina*, both may lack NOD activity. As NO inhibits diatom adherence to substrates,[27] which may be related to the fact that several pennate diatoms have genes encoding NO-scavenging enzymes,[26] it is worthwhile to compare the NO sensitivity of centric and pennate diatoms using the VRFB culture system. However, the mechanisms through which NO inhibits phytoplankton growth and the factors that contribute to the sequestration of NO remain poorly elucidated.

Although growth inhibition was apparent when the threshold NO supply was exceeded, phytoplankton growth recovered within 1–2 days after the NO supply was terminated, regardless of the degree of growth inhibition (Fig. 5). Therefore, the growth inhibition caused by continuous NO supply at the pM level is likely temporary and does not cause serious damage to cell metabolism. As NO was supplied only during the light period, it is thought that NO does not affect cell metabolism during the dark period. Nevertheless, the fact that growth was suppressed throughout the 3-day period of repeated NO supply suggests that NO has certain effects on the photosynthetic system of phytoplankton. It has been reported that the addition of low NO concentrations may promote growth. However,

the response of phytoplankton to NO supplied only during the dark period has not yet been determined. The specific growth rate of the *S. marinoi-dohrnii* complex during the recovery period after the termination of NO supply at 28 pM s^{-1} was greater than that in the control (Fig. 5a). This may be related to the observation in previous studies that a single addition of low NO concentrations promoted the growth of phytoplankton.[12-14] A possible mechanism for such growth promotion could be that NO at low concentrations enhances periplasmic membrane fluidity or regulates photosynthetic electron transport, thereby increasing the rate of photosynthesis.[3, 28, 29] Although the underlying mechanisms that promote the growth of phytoplankton after exposure to low NO concentrations remain unclear, the results of this study suggest that NO inhibition may be involved in the rapid development of algal blooms in coastal waters. However, differences in NO sensitivity and ability to recover from NO inhibition among species may complicate interspecific competition in phytoplankton communities.

Significance of NO in marine phytoplankton ecology

The findings of this study indicate that NO is a key regulator of phytoplankton growth in coastal waters with high nitrite concentrations and active photochemical NO production. If phytoplankton growth does not increase in the presence of sufficient light, nutrients, and well-developed stratification, it may be worthwhile to examine the state of nitrite concentration as an indicator of photochemical NO production.

Aside from coastal eutrophic waters, other possible areas where NO may influence phytoplankton growth, such as the upwelling area where subsurface waters rich in nitrite are lifted, should be considered. Offshore nitrite concentrations often exhibit a subsurface maximum level (up to 4.5 μM) at the base of the euphotic zone;[30] hence, the exposure of nitrite in upwelling water to strong sunlight near the sea surface is likely to stimulate NO production. Phytoplankton whose growth is inhibited by NO in upwelling areas are expected to be temporarily unable to utilize the abundant nutrients contained in the upwelling water, resulting in delayed bloom formation.

High-nitrate, low-chlorophyll (HNLC) waters, where phytoplankton growth is limited by iron deficiency, are considered potential sites for NO inhibition of phytoplankton growth. Diatoms take up nitrate from seawater into their cells and first reduce it to nitrite; however, nitrite reductase, which reduces nitrite into ammonium, is suppressed when iron supply is limited. Therefore, the nitrite accumulated in the cells is released back into the seawater.[31] The released nitrite can be converted into NO by photochemical reactions, especially in areas with strong UV radiation, such as the equatorial Pacific. Applying the correlation equation between the nitrite concentration in seawater and the photochemical NO production rate provided by Olasehinde *et al.*[9], the NO production rate is estimated to be approximately 5–8 pM s^{-1} when the nitrite concentration in the surface water is 0.1–0.2 μM (a value commonly observed in HNLC waters). Based on the results of this study, this level of NO production may be sufficient to inhibit phytoplankton growth. Hence, in addition to the growth limitation caused by iron deficiency, it is possible that phytoplankton in the HNLC waters are subjected to growth inhibition by NO. Moreover, as NO coordinates with iron ions and organic iron complexes in seawater,[32] it can alter the chemical speciation of iron as well as the bioavailability of iron to phytoplankton. Another important factor influencing phytoplankton biomass in HNLC waters is grazing pressure by zooplankton. The production of oxylipin molecules, which inhibit the reproduction and development of copepods and decrease microzooplankton growth rates, is linked to NO signaling in diatoms and provides a plausible mechanism for inducible predator deterrence in diatoms.[33] Therefore, further studies on the effects of photochemical NO production on zooplankton grazing are required.

Conclusion

In this study, a VRFB culture system was constructed to simulate photochemical NO production from nitrite at pM s^{-1} levels in coastal surface waters. The NO feeding solution was continuously supplied to the experimental system during the light period. Growth inhibition was observed in both the diatom *S. marinoi-dohrnii* complex and raphidophyte *C. marina* during 3 days of VRFB culture at average NO supply rates of 1.4–28 pM s^{-1}. There were species differences in sensitivity to NO, with the threshold for near-cessation of growth being lower for *C. marina* than for *S. marinoi-dohrnii*. After the NO supply was terminated, the growth rate of both cultures returned to almost the same level as that of the NO-free control within 1–2 days. These results indicate that phytoplankton growth may be temporarily inhibited by the NO produced by photochemical reactions in coastal surface waters. Therefore, it is necessary to consider the possibility that NO is a new factor in the transition of dominant species and the onset and disappearance of red tides in eutrophic coastal waters where nitrite concentrations are relatively high. As differences in NO sensitivity by species may explain the dominance of certain algal species in coastal phytoplankton assemblages, comparative studies of NO sensitivity in a broader range of phytoplankton species are important for further research. In addition, the mechanism by which NO suppresses phytoplankton growth is suspected to be related to the photosynthetic system; however, this should be clarified by comprehensive gene expression analysis in phytoplankton and other methods in the future.

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References

1) Feldman P, Griffith O, Stuehr D. The surprising life of nitric oxide. *Chem. Eng. News* 1993; **71**: 26–38.
2) Wendehenne D, Pugin A, Klessig D, Durner J. Nitric oxide: comparative synthesis and signaling in animal and plant cells. *Trends Plant Sci.* 2001; **6**: 177–183.
3) Leshem YY. Nitric oxide in biological systems. *Plant Growth Regul.* 1996; **18**: 155–159.
4) Mallick N, Rai LC, Mohn FH, Soeder CJ. Studies on nitric oxide (NO) formation by green alga *Scenedesmus Obliquua* and the diazotrophic cyanobacterium *Anabaena doliolum*. *Chemosphere* 1999; **39**: 1601–1610.
5) Zhang ZB, Liu CY, Wu ZZ, Xing L, Li PF. Detection of nitric oxide in culture media and studies on nitric oxide formation by marine microalgae. *Med. Sci. Monit.* 2006; **12**: BR75-85.
6) Zafiriou OC, McFarland M, Bromund RH. Nitric oxide in seawater. *Science* 1980; **207**: 637–639.
7) Zafiriou OC, True MB. Nitrite photolysis in seawater by sunlight. *Mar. Chem.* 1979; **8**: 9–32.
8) Zafiriou OC, McFarland M. Nitric oxide from nitrite photolysis in the central equatorial Pacific. *J. Geophys. Res.* 1981; **86**: 3173–3182.
9) Olasehinde EF, Takeda K, Sakugawa H. Photochemical production and consumption mechanisms of nitric oxide in seawater. *Environ. Sci. Technol*. 2010; **44**: 8403–8408.
10) Anifowose AJ, Sakugawa H. Determination of Daytime Flux of Nitric Oxide Radical (NO•) at an Inland Sea-Atmospheric Boundary in Japan. *J. Aquat. Pollut. Toxicol.* 2017; **1**: 10.
11) Adesina AO, Sakugawa H. Photochemically generated nitric oxide in seawater: The peroxynitrite sink and its implications for daytime air quality. *Sci. Total Environ.* 2021; doi.org/10.1016/j.scitotenv.2021.146683.
12) Zhang ZB, Lin C, Liu C, Sun M, Ding H. The effect of nitric oxide on the growth of marine phytoplankton. *J. Ocean Univ. China* 2003; **2**: 185–188.
13) Zhang ZB, Lin C, Liu C, Xing L, Wu ZZ, Sun F. Study on patterns and chemical features of NO effect on marine phytoplankton growth. *Sci. China, Ser. B: Chem.* 2005; **48**: 376–384.
14) Zhang ZB, Wu ZZ, Liu CY, Xing L. The study on the effect of NO on the growth of *Chaetoceros curvisetus*. *Chin. J. Oceanol. Limnol.* 2006; **24**: 413–420.
15) Lehner C, Kerschbaum HH, Lütz-Meindl U. Nitric oxide suppresses growth and development in the unicellular green alga *Micrasterias denticulata*. *J. Plant Physiol.* 2009; **166**: 117–127.
16) Li PF, Zhao M, Liu CY, Yang GP. Effects of nitric oxide on the growth of marine microalgae and carbonate chemistry parameters. *Mar. Biol.* 2022; doi.org/10.1007/s00227-021-03988-8.
17) Guillard RRL, Ryther JH. Studies of marine planktonic diatoms. I. *Cyclotella nana* Hustedt, and *Detonula confervacea* (Cleve) Gran. *Can. J. Microbiol.* 1962; **8**: 229–239.
18) Liu CY, Feng WH, Tian Y, Yang GP, Li PF, Bange HW. Determination of dissolved nitric oxide in coastal waters of the Yellow Sea off Qingdao. *Ocean Sci*. 2017; **13**: 623–632.
19) Tian Y, Yang GP, Liu CY, Li PF, Chen HT, Bange HW. Photoproduction of nitric oxide in seawater. *Ocean Sci*. 2020; **16**: 135–148.
20) Tian Y, Xue C, Liu CY, Yang GP, Li PF, Feng WH, Bange HW. Nitric oxide (NO) in the Bohai Sea and the Yellow Sea. *Biogeosciences* 2019; **16**: 4485–4496.
21) Tian Y, Wang KK, Yang GP, Li PF, Liu CY, Ingeniero RCO, Bange HW. Continuous chemiluminescence measurements of dissolved nitric oxide (NO) and nitrogen dioxide (NO_2) in the ocean surface layer of the East China Sea. *Environ. Sci. Technol.* 2021; **55**: 3668–3675.
22) Gong JC, Jin H, Li BH, Tian Y, Liu CY, Li PF, Liu Q, Ingeniero RCO, Yang GP. Emissions of Nitric Oxide from Photochemical and Microbial Processes in Coastal Waters of the Yellow and East China Seas. *Environ. Sci. Technol.* 2023; **57**: 4039–4049.
23) Wink DA, Mitchell JB. Chemical biology of nitric oxide: insights into regulatory, cytotoxic and cytoprotective mechanisms of nitric oxide. *Free Radical Biol. Med.* 1998; **25**: 434–456.
24) Beligni MV, Lamattina L. Is nitric oxide toxic or protective? *Trends Plant Sci.* 1999; **4**: 299–300.
25) Yamasaki H. Nitrite-dependent nitric oxide production pathway: implications for involvement of active nitrogen species in photoinhibition *in vivo*. *Philos. Trans. R. Soc., B* 2000; **355**: 1477–1488.
26) Omar NM, Fleury K, Beardsall B, Prášil O, Campbell DA. Genomic capacities for reactive oxygen species metabolism across marine phytoplankton. *PLoS ONE* 2023; doi.org/10.1371/journal.pone.0284580.
27) Thompson SEM, Taylor AR, Brownlee C, Callow ME, Callow JA. The role of nitric oxide in diatom adhesion in relation to substratum properties. *J. Phycol.* 2008; **44**: 967–976.
28) Wang X, Xu Y, Zhong Y, Jing B. P30: Effects of nitric oxide on photosynthesis rate of *Phaeodactylum tricornutum*. *Nitric Oxide* 2013; **31**: S25–S26.
29) Misra AN, Vladkova R, Singh R, Misr M, Dobrikova AG, Apostolova EL. Action and target sites of nitric oxide in chloroplasts. *Nitric Oxide* 2014; **39**: 35–45.
30) Lomas MW, Lipschultz F. Forming the primary nitrite maximum: Nitrifiers or phytoplankton? *Limnol. Oceanogr*. 2006; **51**: 2453–2467.
31) Milligan AJ, Harrison PJ. Effects of non-steady-state iron limitation on nitrogen assimilatory enzymes in

the marine diatom *Thalassiosira weissflogii* (Bacillariophyceae). *J. Phycol*. 2000; **36**: 78–86.

32) McCleverty JA. Chemistry of nitric oxide relevant to biology. *Chem. Rev.* 2004; **104**: 403−418.

33) Johnson MD, Edwards BR, Beaudoin DJ, Van Mooy BA, Vardi A. Nitric oxide mediates oxylipin production and grazing defense in diatoms. *Environ. Microbiol.* 2020; **22**: 629–645.

明期における一酸化窒素の低濃度連続供給に対する沿岸性植物プランクトンの増殖応答

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一酸化窒素（NO）は，重要なシグナル伝達分子として，複数の生理的プロセスの制御に重要な役割を果たしている。海洋環境では，NO は亜硝酸塩の光化学反応によって表層海水中で生成されるため，特に亜硝酸塩が豊富な沿岸の富栄養海域において，植物プランクトンの増殖に NO が影響を及ぼす可能性は高いと考えられる。しかし，NO は反応性が高く，海水中での寿命が短いため, その生態学的な役割を評価することは容易ではない。本研究では, 海洋表層における NO 生成を実験室内で再現するため，NO 供給系を備えた培養液量可変の反復式流加培養実験システムを構築して，沿岸性植物プランクトン培養株 2 種の増殖応答を調べた。珪藻 *Skeletonema marinoi-dohrnii* complex とラフィド藻 *Chattonella marina* の増殖速度は，明暗周期の明期における平均 NO 供給速度がそれぞれ 28 pM s^{-1} と 1.4 pM s^{-1} の条件でほぼ完全に抑制され，NO に対する感受性には種差が認められた。また，NO 供給を停止すると，両培養株の増殖速度は 1-2 日以内に NO 無添加の対照区とほぼ同じレベルに戻ったことから，NO の増殖阻害効果は一時的なものであることが示された。これらの結果は，光化学反応による亜硝酸塩からの NO 生成が活発な沿岸域において，NO が植物プランクトン増殖の重要な調節因子の一つになっており，赤潮の発生と消滅などに関与していることを示唆している。