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Author(s)	Naito, Kanako; Tanabe, Ayako; Itakura, Shigeru; Yamaguchi, Mineo; Imai, Ichiro
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Instructions for use

Evaluation of major nutrients regulating the growth of diatoms in Harima-Nada, the Seto Inland Sea, Japan

Kanako Naito[*1], Ayako Tanabe[2], Shigeru Itakura[3], Mineo Yamaguchi[3] and Ichiro Imai[*4]

Abstract

In the eastern Seto Inland Sea, dissolved inorganic nitrogen has decreased due to the reduction of nutrient inputs, and there have been changes in the total cell density and the composition of the phytoplankton assemblages.   The major phytoplankton component was diatoms, and the contribution was always over 90 percent of total phytoplankton since the 1990s.   In order to elucidate growth characteristics and nutrient consumption of diatom species, we conducted major nutrients-enrichment bottle incubation experiments on shipboard using the surface waters in Harima-Nada, eastern part of the Seto Inland Sea.   Seawater incubation experiments demonstrated that nutrient environments for phytoplankton are in a state of nitrogen-limitation in Harima-Nada.   The addition of nitrate to surface waters of this coastal region promotes blooms of large chain-forming diatom species.   These results suggest that diatom blooms cause depletion of the nutrients (mainly nitrogen) in Harima-Nada.

Key words : Phytoplankton, Diatom bloom, Nitrogen depletion, Harima-Nada

Introduction

In Japanese coastal areas, the most significant problem by diatom blooms is the bleachings of *Porphyra* thalli in aquaculture (Imai et al., 2006).   Lowering the quality and price of Nori (*Porphyra*, red alga) products has reached the damage amount of the order of billion yen (>10 million dollars) commonly (Nishikawa, 2007).   Decrease of nutrient concentrations in the coastal waters is reported to be one of the leading causes of the bleaching of *Porphyra* thalli (Matsuoka et al., 2005 ; Fujiwara and Komai, 2009).   Diatom blooms have usually been observed between autumn and spring in the coastal waters of western Japan, such as Ariake Sea and the Seto Inland Sea, where they have big aquaculture industries of the red alga *Porphyra yezoensis* Ueda (Miyahara et al., 1996 ; Sasaki and Kito, 2003).   The occurrences of diatom blooms have caused the deprivation of nutrients in seawater for Nori cultivation during the seaweed-growing season (Watanabe, 2009).   The resultant nutrient deficiency has sup-

pressed the growth of Nori and lowered its quality due to the bleaching of *Porphyra* thalli.

In Harima-Nada, eastern part of the Seto Inland Sea, Nori cultivation is one of key fisheries industry, which accounts for about half of the fisheries production volume and 40 percent of the production value (Nishikawa, 2007).   In this sea area, the problem of bleaching of *Porphyra* thalli by diatom blooms has occurred almost every year since the mid 1980s, depending mainly on the decrease of dissolved inorganic nitrogen (DIN) concentration (Harada et al., 2009).   It is thought that massive occurrences of large diatoms *Chaetoceros densus* (Cleve) Cleve, *Coscinodiscus wailesii* Gran, *Eucampia zodiacus* Ehrenberg and *Thalassiosira diporocyclus* Hasle significantly related to the problem in this area (Manabe and Ishio, 1991 ; Miyahara et al., 1996 ; Nagai, 2000 ; Nishikawa et al., 2007 ; Oyama et al., 2008).

Nutrients have been considered as one of the major factors controlling the composition and abundance of phytoplankton community, and also the occurrences of the blooms.   Dis-

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[1] *Department of Environment Sciences, Faculty of Life and Environmental Sciences, Prefectural University of Hiroshima, Shobara, Hiroshima 727-0023, Japan*
(県立広島大学生命環境学部環境科学科)

[2] *Laboratory of Marine Environmental Microbiology, Division of Applied Biosciences, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan*
(京都大学大学院農学研究科応用生物科学専攻海洋環境微生物学分野)

[3] *Harmful Algal Bloom Division, National Research Institute of Fisheries and Environment of Inland Sea, Fisheries Research Agency, 2-17-5 Maruishi, Hatsukaichi, Hiroshima 739-0452, Japan*
((独) 水産総合研究センター瀬戸内海区水産研究所赤潮環境部)

[4] *Laboratory of Marine Biology (Plankton Laboratory), Division of Marine Bioresource and Environmental Science, Graduate School of Fisheries Sciences, Hokkaido University, Hakodate, Hokkaido 041-8611, Japan*
(北海道大学大学院水産科学研究院海洋生物資源科学部門海洋生物学分野浮遊生物学領域)

 * *Corresponding author*
Kanako Naito (naito@pu-hiroshima.ac.jp), Ichiro Imai (imai1ro@fish.hokudai.ac.jp).

solved silicate and DIN play important roles in population dynamic and bloom formation of diatoms (Egge and Aksnes, 1992 ; Martin-Jézéquel et al., 2000 ; Wang et al., 2006). Different diatom species show various nutrient requirements (Riegman, 1998 ; Granéli et al., 2008 ; Heisler et al., 2008). Hence, it is important to determine the hierarchy of nutrient limitation to diatom growth, which would provide information on the parameter in simulation models of nutrients and the bloom dynamics in the region. As an investigation strategy, nutrient enrichment incubation experiment is an effective means for examining what is important nutrient for the region's ecosystem (Zou et al., 2001).

In the present study, major nutrients (nitrate, phosphate and silicate)-enrichiment bottle incubation experiments were performed using in situ phytoplankton assemblages to determine the nutrients controlling the growth of diatoms in Harima-Nada.

Materials and methods

Samplings were carried out at three stations in Harima-Nada (Fig. 1), eastern part of the Seto Inland Sea in September 2005 using the research vessel "Shirafuji-Maru". At three stations, OY3 (34°38.6′N, 134°13.7′E, 8.2 m depth), St4 (34°14.1′N, 134°31.5′E, 41.0 m depth) and HG3 (34°32.8′N, 134°32.1′E, 42.7 m depth), samples of surface

Fig. 1. Location of sampling stations (OY3, HG3 and St4) for the nutrient-addition experiment in Harima-Nada, eastern part of the Seto Inland Sea

water were collected and filtered through 100 μm-nylon mesh for eliminating large zooplankton. Eight hundred milliliters of filtered sea water was poured into 1-liter volume polycarbonate acid-cleaned bottles. The bottles were spiked with 10 μM sodium nitrate, 1 μM sodium dihydrogenphosphate dehydrate, 50 μM sodium silicate nonahydrate, respectively, or no addition as controls, and were incubated in a shipboard cistern (water temperature 26-28°C) with running seawater for 3 days. The cistern was covered with a blackout screen (18 : 00-6 : 00) to block ship light during night. The sub-samples were taken from duplicate incubation bottles every day for shipboard measurements of phytoplankton growth and nutrients.

Phytoplankton growth was determined by measuring *in vivo* fluorescence using a fluorometer (Turner Designs, TD-700R) (Brand et al., 1981). Chlorophyll *a* samples were collected on 47-mm GF/F filters (Whatman), extracted with 90% acetone and determined by fluorometry. The identification and enumeration of phytoplankton were done at the first and the last days of the experiments. Nutrients were measured by standard techniques using an auto-analyzer or spectrophotometer. Concentration of dissolved inorganic nitrogen (DIN, NO_3-N+NO_2-N+NH_4-N) was determined using an Auto-Analyzer II (Technicon). Concentrations of phosphate and silicate were analyzed with a spectrophotometer (SHIMADZU, UV-1700) according to colorimetric methods for chemical analysis of seawater (Murphy and Riley, 1962 ; The Japan Society for Analytical Chemistry, Hokkaido Branch, 2005).

Results

Figure 2 shows the phytoplankton growth measured as chlorophyll *a* (μg l^{-1}) in nutrient-addition experiments. The initial chlorophyll *a* concentrations were in order of Sts. OY3>St4>HG3. At all the stations, nitrate addition markedly promoted chlorophyll *a* increase as compared with the control (Fig. 2). Phytoplankton biomass in water of St. OY3 with nitrate addition reached the maximum earlier (only one day after the start of incubation) (Fig. 2a). On the other hand, there was no remarkable difference of the fluorescence in the phosphate or silicate-enriched bottle as compared with the control (Fig. 2).

Nutrient concentrations in sea waters of nutrient-addition experiments are shown in Figures 3 (DIN), 4 (phosphate) and 5 (silicate). In the first 3 days, significant decrease of DIN was observed in the nitrate-enriched bottles at all the stations (Fig. 3). The DIN concentrations varied from 9.9 to 0.62 μM at OY3, from 10.6 to <0.2 μM at St4, and from 15.5 to 3.3 μM at HG3. Phosphate concentrations decreased significantly from 1.04 to 0.35 μM in the phosphate-enriched bottle at OY3, but only slightly decreased in the nitrate-enriched bottles at St4 and HG3 (Fig. 4). Silicate concentra-
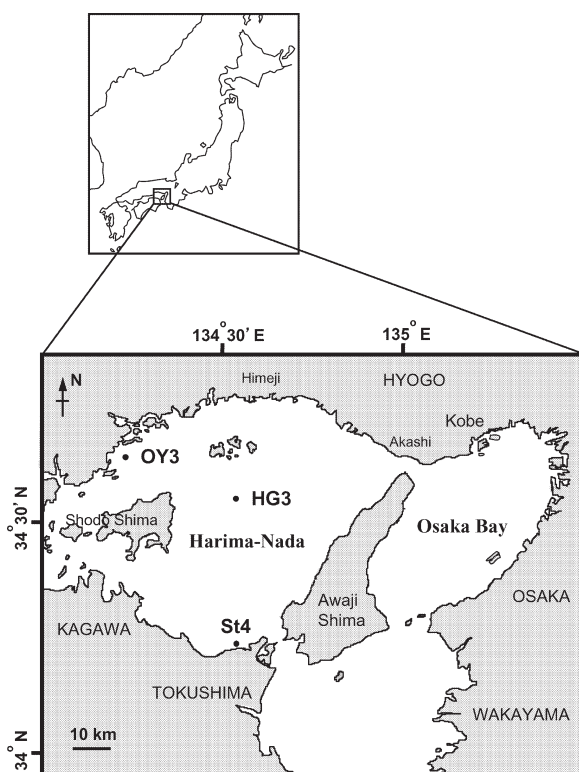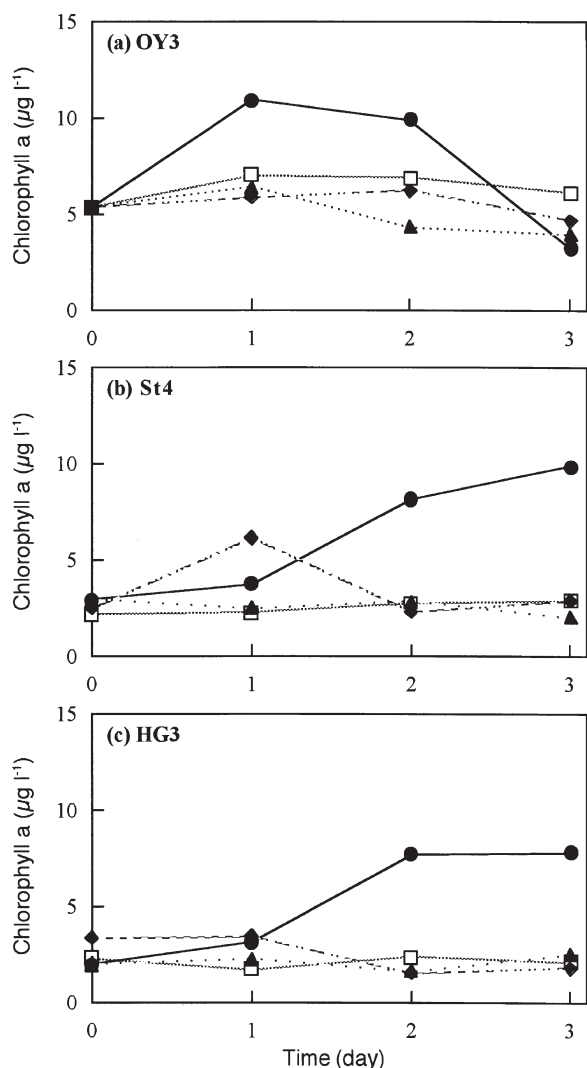
Fig. 2. Changes in concentrations of chlorophyll *a* in seawater of OY3 (a), St4 (b) and HG3 (c) for nutrient-addition experiment. Open squares, control (no nutrient addition) ; filled circles, bottle with 10 μM nitrate addition ; filled triangles, with 1 μM phosphate addition ; filled rhombi, with 50 μM silicate addition. Data are means of duplicate incubation bottles
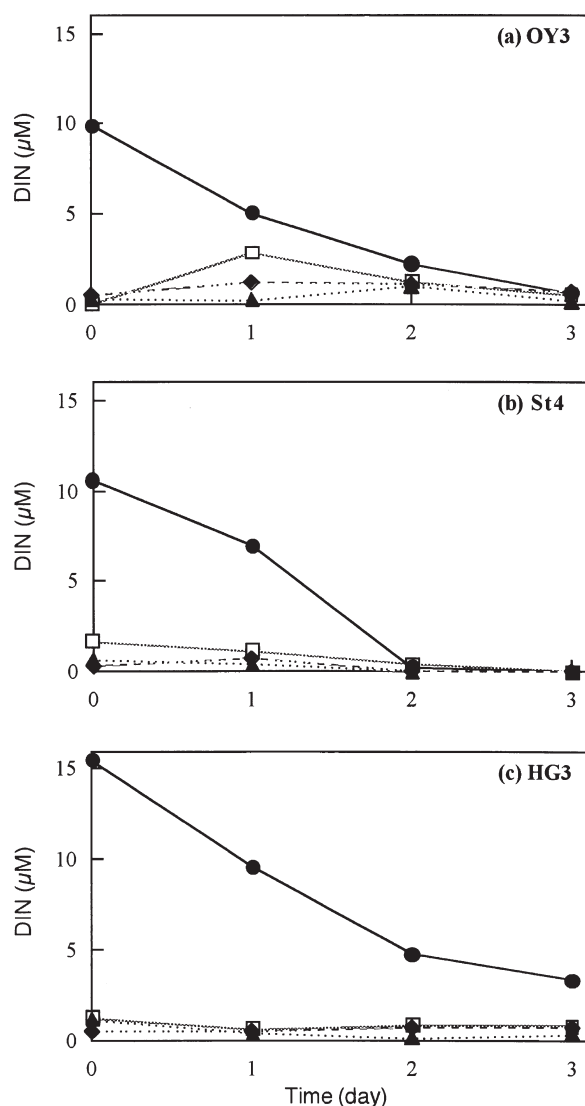

Fig. 3. Changes in concentrations of dissolved inorganic nitrogen in seawater of OY3 (a), St4 (b) and HG3 (c) for nutrient-addition experiment. Open squares, control (no nutrient addition) ; filled circles, bottle with 10 μM nitrate addition ; filled triangles, with 1 μM phosphate addition ; filled rhombi, with 50 μM silicate addition. Data are means of duplicate incubation bottles

tions decreased in all bottles except for silicate-enriched bottles at St4 and HG3 (Fig. 5). Especially in nitrate-enriched bottle, there was a noticeable decrease in silicate concentration (Fig. 5). The silicate concentrations varied from 12.6 to 3.0 μM at OY3, from 12.9 to 2.8 μM at St4, and from 7.6 to 1.5 μM at HG3.

Diatoms were the dominated phytoplankton group in surface waters of three stations at our sampling cruise. The diatom species observed in surface water of sampling stations are shown in Table 1. Typhoon No. 14 passed through a few days before the water samplings, and centric diatoms were the most dominant group in surface waters at three stations. The highest abundance was found at St. OY3 compared to other stations (Fig. 6). At St. OY3, the dominant diatoms were

Skeletonema spp. and made up 66% of the total diatoms (Fig. 6a). *Chaetoceros* (*C. curvisetus*, *C. didymus* and other *Chaetoceros* species) accounted for 13% of the total diatom cells (Fig. 6a). Nutrient enrichments induced substantial growth of *Chaetoceros curvisetus, C. didymus*, other *Chaetoceros* species (*Chaetoceros* spp.), *Leptocylindrus danicus*, *Nitzschia* spp., *Pseudo-nitzschia* spp., *Skeletonema* spp. and *Thalassiosira* spp. at OY3. At Sts. St4 and HG3, significant increases in abundance were noticed with nitrate addition as compared to control (no enrichment), while there was no positive effects on the growth of diatoms with the additions of phosphate and silicate (Fig. 6b, c). At St4, *C. curvisetus* was dominant species (41% of total) and *Skeletonema* spp. occu-
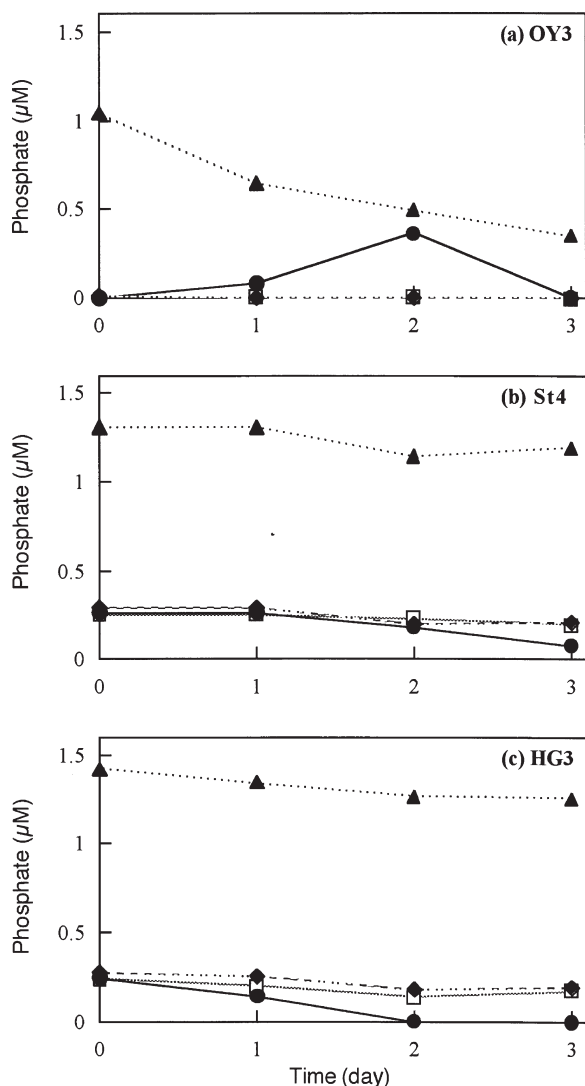
Fig. 4. Changes in concentrations of dissolved inorganic phosphate in seawater of OY3 (a), St4 (b) and HG3 (c) for nutrient-addition experiment. Open squares, control (no nutrient addition); filled circles, bottle with 10 μM nitrate addition; filled triangles, with 1 μM phosphate addition; filled rhombi, with 50 μM silicate addition. Data are means of duplicate incubation bottles
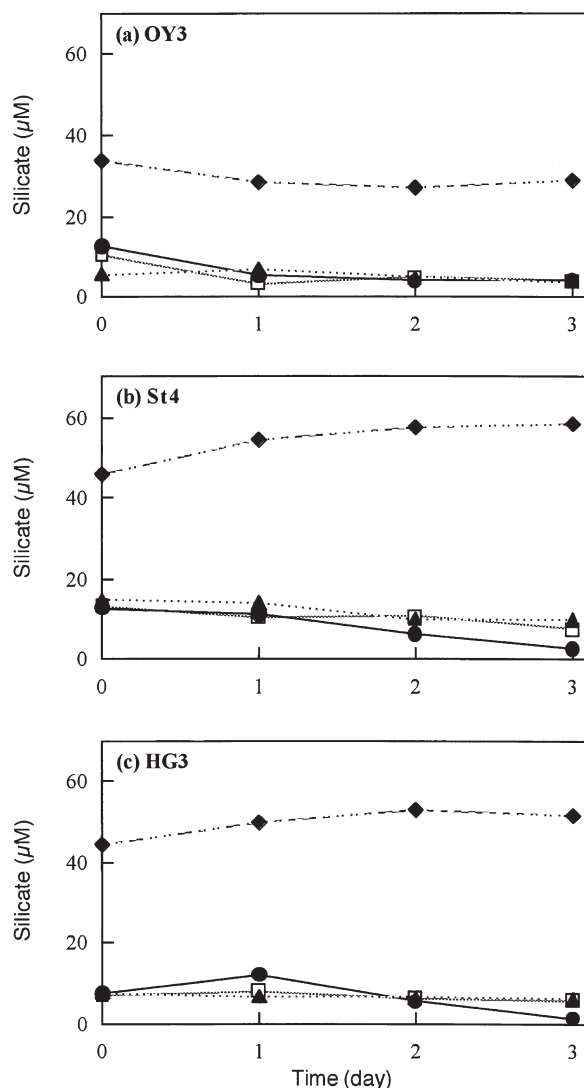
Fig. 5. Changes in concentrations of dissolved silica in seawater of OY3 (a), St4 (b) and HG3 (c) for nutrient-addition experiment. Open squares, control (no nutrient addition); filled circles, bottle with 10 μM nitrate addition; filled triangles, with 1 μM phosphate addition; filled rhombi, with 50 μM silicate addition. Data are means of duplicate incubation bottles

pied 23% of total diatoms (Fig. 6b). The order of cell abundance with nitrate addition were *C. curvisetus* (29%) > *Skeletonema* spp. (15%) > *L. danicus, C. didymius, Pseudo-nitzschia* spp. (9, 8, 8%, respectively) > *Thalassiosira* spp. (5%) at St4 (Fig. 6b). There were marked increases in growth of *C. curvisetus, C. didymus,* other *Chaetoceros* species, *Pseudo-nitzschia* spp. and *Skeletonema* spp. with the enrichment of nitrate as compared to the control (Dunnett's test, *P*<0.05) at St4 (Fig. 6b). At HG3, *C. didymus* (24% of total) and *Skeletonema* spp. (22% of total) dominated (Fig. 6c). The order of cell abundance with nitrate addition were *Skeletonema* spp. (29%) > *C. didymus* (17%) > *Nitzschia* spp., *C. curvisetus, L. danicus, Pseudo-nitzschia* spp. (8, 7, 7, 7%, respectively) > *Thalassiosira* spp. (4%) at

HG3 (Fig. 6c). There were marked increases in growth of *C. didymus,* other *Chaetoceros* species, *L. danicus, Nitzschia* spp., *Skeletonema* spp. and *Thalassiosira* spp. with the enrichment of nitrate as compared to the control (Dunnett's test, *P*<0.05) at HG3 (Fig. 6c).

Discussion

At all the three stations of OY3, St4 and HG3 in Harima-Nada, the results of nitrate addition experiments revealed promoted chlorophyll *a* increase and the synchronized DIN decrease, leading to a conclusion that nitrogen is the primary limiting factor for phytoplankton growth (Figs 2-5). Diatoms predominated numerically in surface waters of Harima-
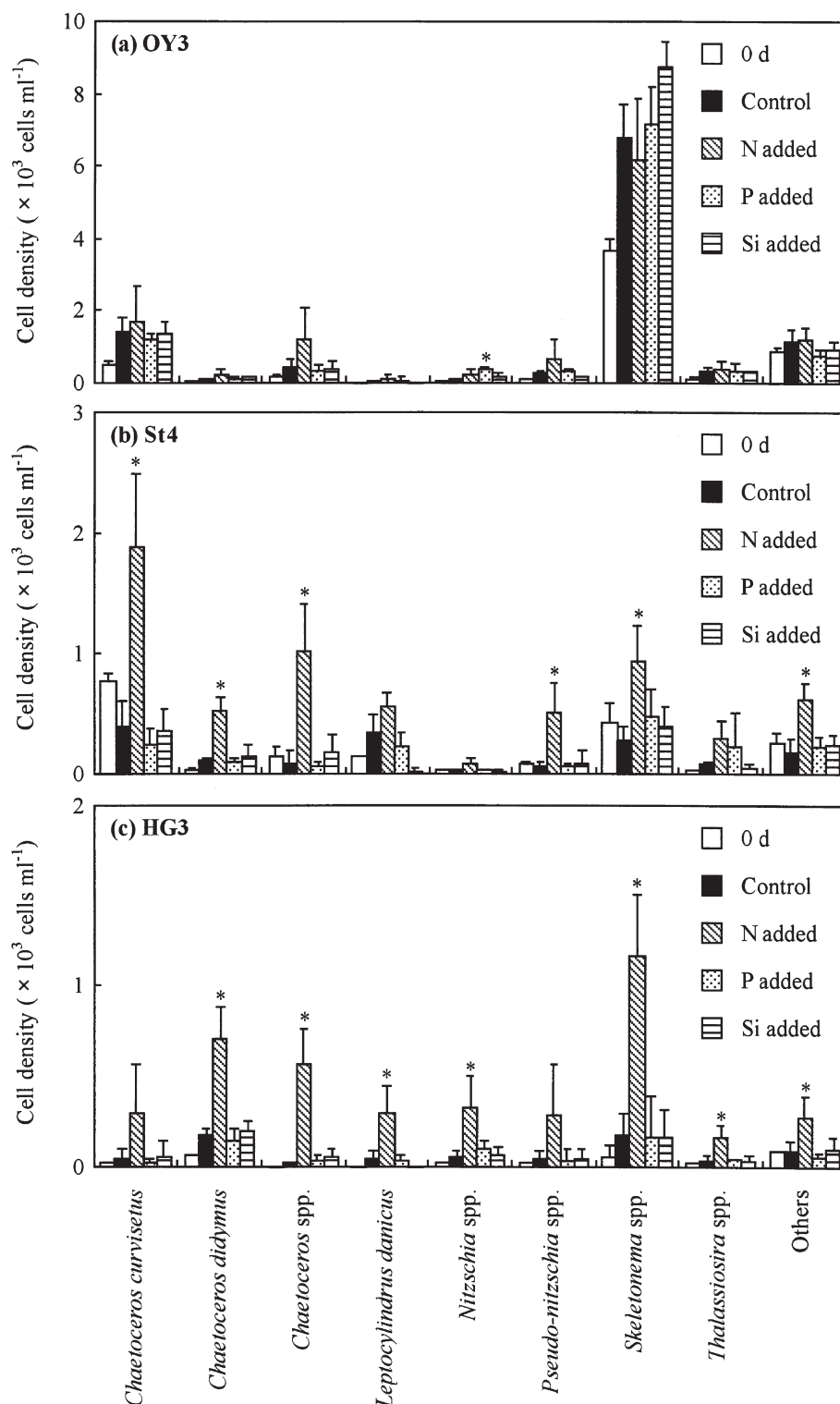
Fig. 6.　Cell densities of diatom species at the first day (0 d) and the last day (3 d) in sea water of OY3 (a), St4 (b) and HG3 (c) for nutrient-addition experiment.　Data are means±standard deviations of duplicate incubation bottles.　Asterisks (*) show the significantly higher growth yields than control according to Dunnett's test ($P<0.05$)

Nada and the significant decreases of NO_3-N concentration were found in the nitrate-enriched bottles, clearly demonstrating that there was nitrate utilization by diatoms.　There were two significant long-term trends in water quality parameters in Harima-Nada from 1973 to 2007 : the rise in winter water temperatures and the decrease in DIN (Nishikawa et al., 2010).　Average DIN concentrations had fluctuated between 5 and 10 μM in the 1970s, decreased to <5 μM from the late

Table 1. Observed diatom species in surface waters of three stations, OY3, St4 and HG3 in Harima-Nada,eastern part of the Seto Inland Sea in September 2005.

Asterionellopsis glacialis	*Eucampia zodiacus*
Bacillaria paxillifer	*Leptocylindrus danicus*
Bacteriastrum hyalinum	*Neodelphineis* spp.
Chaetoceros affinis	*Nitzschia longissima*
Chaetoceros curvisetus	*Nitzschia* spp.
Chaetoceros danicus	*Odontella scinensis*
Chaetoceros decipiens	*Pleurosigma* spp.
Chaetoceros didymus	*Pseudo-nitzschia* spp.
Chaetoceros distans	*Rhizosolenia* spp.
Chaetoceros lorenzianus	*Skeletonema tropicam*
Chaetoceros pseudocurvisetus	*Skeletonema* spp.
Chaetoceros salsugineum	*Stephanopyxis palmeriana*
Chaetoceros spp.	*Streptotheca thamensis*
Coscinodiscus spp.	*Thalassionema nitzschioides*
Ditylum brightwellii	*Thalassiothrix frauenfeldii*
Diploneis spp.	*Thalassiosira* spp.

Table 2. Growth rates and initial nutrient ratios in incubation bottles of nutrient-addition experiment. Data are means of duplicated incubation bottles.

Treatment	Growth rate (d^{-1})	DIN/P	DIN/Si
OY3			
Control	0.27	2.04	0.04
N added	0.71	55.0	0.79
P added	0.18	0.24	0.02
Si added	0.09	2.69	0.01
St4			
Control	0.20	2.64	0.05
N added	0.77	39.7	0.82
P added	0.12	0.51	0.05
Si added	0.89	2.31	0.02
HG3			
Control	0.29	4.94	0.17
N added	0.90	62.0	2.03
P added	0.43	0.79	0.14
Si added	0.15	4.18	0.03

1970s to the mid 1980s, and then gradually increased to over 5 μM up to the early 1990s and have gradually decreased thereafter (Nishikawa et al., 2010). Nishikawa et al. (2010) also suggested that the decrease in nutrient concentrations, especially DIN, is one of the most important factors relating to the trend of decrease in the total phytoplankton cell densities in Harima-Nada.

Table 2 summarizes phytoplankton growth rates estimated with the changes in Chlorophyll *a* concentrations and initial nutrient ratios (DIN/P, DIN/Si) in incubation bottles of nutrient-addition experiment. In three stations of September 2005, ratios of DIN/P and DIN/Si were well below the Redfield ratios (DIN/P=16, DIN/Si=1) (Redfield, 1958 ; Brzezin-

ski, 1985). DIN concentrations were also low (Fig. 3) and NO_3-N addition triggered increases in phytoplankton growth rates and silicate consumption (Table 2, Fig. 5). And, the silicate concentrations in surface waters were found to be 10, 12 and 7 μM at Sts. OY3, St4 and HG3, respectively, which was still higher than the 2 μM reported threshold concentration for diatoms (Egge and Aksnes, 1992). Our results of shipboard seawater incubation experiments distinctly support the idea that phytoplankton biomass production has been nitrogen-limited in Harima-Nada since the 1990s (Nishikawa et al., 2010).

Diatoms dominated phytoplankton biomass in surface waters of the three stations in Harima-Nada in September 2005. It has been reported that the contribution of diatoms was always >90% in Harima-Nada, after the mid 1980s (Nishikawa et al., 2010). It is considered that nutrient ratios are key factors associated with the shifts in predominance of phytoplankton species (Smayda, 1990 ; Hori et al., 1998). Nutrients addition experiments indicated that the change of dominant species comes from differences of nutrient requirement by diatom species. Previous study showed that *Skeletonema* spp. occurred in waters with high DIN/DIP ratio, and *Chaetoceros curvisetum* and *C. affinis* did with low DIN/DIP ratio in Harima-Nada (Hori et al., 1998). Relationships between the dominant diatom species and DIN/DIP ratio in our nutrient-addition experiments (Table 2, Fig. 6) are consistent with those observed by Hori et al. (1998). Consequently, there is a possibility of changing phytoplankton abundance and community composition by control of nutrient ratios with nutrients input into Harima-Nada.

In recent years, recurrent winter diatom blooms have caused a large-scale reduction in the amount and quality of Nori products in Harima-Nada, due to bleachings of *Porphyra* thalli by depleting dissolved inorganic nitrogen from coastal waters (Nishikawa, 2007 ; Harada et al., 2009). The main causative species is *Eucampia zodiacus*, which is thought to have a holoplanktonic life cycle (Nishikawa et al., 2007). *Eucampia zodiacus* tend to dominate under conditions relatively low concentration of inorganic nutrients as compared with *Skeletonema* spp., *Chaetoceros* spp. and *Thalassiosira* spp. that have a meroplanktonic life cycle (Nishikawa et al., 2007). In northern part of Harima-Nada, there was a dramatic shift from the predominance of *Skeletonema* (which dominates under relatively high nutrient levels) to *Chaetoceros* (which dominates under relatively low nutrient levels) since the mid 1980s (Imai et al., 1990 ; Itakura et al., 2008 ; Nishikawa et al., 2010). This shift in diatom species may be attributed to differences in the life cycle of *Skeletonema* and *Chaetoceros* such as the conditions inducing formation of resting stage cells, and the physiological response to the decrease in DIN concentration (Itakura and Yamaguchi, 2007). Therefore, the elucidation of changes in members of diatom blooms should be based on both their

growth characteristics and their life history.

Seawater incubation experiments demonstrated that nutrient environments for phytoplankton are in a state of nitrogen-limitation in Harima-Nada. Diatom blooms could cause bleaching of the edible seaweed *Porphyra* by consuming the nutrients (mainly nitrogen) needed for the growth of *Porphyra* thalli in aquaculture of Harima-Nada (Nishikawa et al., 2007 ; Harada et al., 2009). The complete consumption of DIN by diatom species such as *E. zodiacus* would enforce more severe and poorer nitrogen environment to the culturing *Porphyra* in Harima-Nada (Nishikawa, 2007 ; Nishikawa et al., 2007). It is absolutely essential to understand the growth physiology of red tide causative diatom species in order to understand the mechanism of bleaching of the seaweed *Porphyra* in aquaculture. Some of the important causative diatoms species such as *E. zodiacus* have a difficulty in establishing cultivation in chemically defined artificial medium. For future studies, it is essential to develop a new artificial medium for causative diatoms, and clarify the nutrient requirements and physiology of their growth with laboratory culture experiments to understand occurrence mechanisms of diatom blooms in coastal areas.

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