Effects of Nutrient Addition on Phytoplankton Growth in the Coastal Waters of Kagawa Prefecture, Eastern Seto Inland Sea, Japan

Theppitak Vipulakom[1*], *Lapassakorn* Srisomros[1], and *Hitomi* Yamaguchi[2]

[1]Faculty of Food Biotechnology and Innovation, Assumption University of Thailand, Bangkok, Thailand
[2]Faculty of Agriculture, Kagawa University, Kagawa, Japan

Abstract. This research explores the effects of nutrient addition on phytoplankton growth. In marine ecosystems, nutrients refer to dissolved inorganic nitrogen (DIN), phosphorus (DIP) and dissolved silica (DSi). These nutrients often directly limit the growth of phytoplankton—the main primary producer and the fundamental food source for higher trophic organisms in marine ecosystems. This study conducted nutrient addition experiments on phytoplankton growth in the Seto Inland Sea, an area currently facing oligotrophication due to rapid seawater quality improvement, which may be affecting phytoplankton species composition and contributing to a decrease in fisheries yields. Seawater was incubated in enrichment experiments over 48 hours for each nutrient, with chlorophyll *a* measured as an indicator of phytoplankton biomass. Results showed increased phytoplankton growth only in nitrogen-enriched (nitrate-enriched) samples, indicating that DIN was insufficient when compared to the other essential nutrients. This research indicates nitrogen as the limiting nutrient in the studied area.

1 Introduction

Ecosystems rely on primary producers to generate the initial energy that sustains all other organisms in the food chain by converting sunlight into chemical energy. In marine ecosystems, this role is chiefly carried out by phytoplankton [1]. When phytoplankton thrive, the entire ecosystem benefits; when their growth stagnates, the consequences cascade throughout the food web. It has been demonstrated that the fishery yield per unit area in estuarine and marine ecosystems is tightly related to phytoplankton primary production in the area [2,3].

According to the Liebig's Law of the Minimum, growth is not determined by the total amount of resources available, but by the scarcest one [4]. In the case of coastal phytoplankton communities, the primary limiting nutrients are dissolved inorganic nitrogen (DIN), dissolved inorganic phosphorus (DIP), and dissolved silica (DSi). In many coastal areas, phytoplankton are mainly composed of diatoms which require nutrients in an

* Corresponding author: takvipulakom@gmail.com

approximate atomic ratio of N: P: Si = 16:1:16 [5,6]. The limiting nutrient for phytoplankton growth may therefore be roughly estimated from the N: P: Si ratio of the ambient seawater, while direct investigation on the limiting nutrient for phytoplankton growth has been less frequently conducted.

In this study, we investigated the effects of each nutrient addition on phytoplankton growth in the eastern Seto Inland Sea in Japan. The aim is to clarify the limiting nutrient for phytoplankton growth in the target area through conducting nutrient enrichment experiments and observing the growth responses.

2 Materials and Methods

2.1 Sampling Site

This study focused on the Seto Inland Sea in Japan (Fig. 1), an area that faces the reversal trend of eutrophication, in relation to the reduction in the terrestrial nitrogen and phosphorus inputs into the Sea [7]. In the Seto Inland Sea, concerns have been raised that rapid water quality improvements may be affecting phytoplankton dynamics and causing a decrease in fishery yield [8].

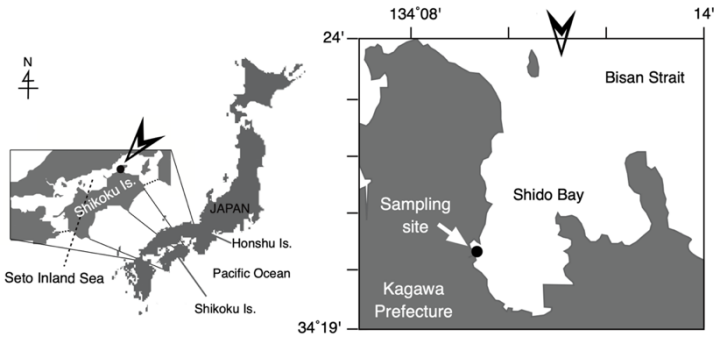

Fig. 1. Geographical location of the sampling site in Shido Bay, along the coast of Kagawa Prefecture, eastern Seto Inland Sea, Japan

A sampling site was set in Mure Port, Shido Bay, along the coast of Kagawa Prefecture (34°20'22.7"N 134°09'22.5"E), eastern Seto Inland Sea. (Fig. 1). Surface seawater (~0.4 m depth) was collected in the morning using a Heyroth water sampler and taken to the laboratory within 3 hours after the sample collection. Seawater samples for nutrient addition experiments (section 2.2) were collected on 30 April, 7 May, 12 May, and 19 May, 2025, for a total of 4 sampling events.

2.2 Laboratory Experimental Method

2.2.1 Nutrient Enrichment and Incubation

On each date of the nutrient addition experiment (i.e., 30 April, 7 May, 12 May, and 19 May), intact seawater and nitrogen-enriched (+25 μM nitrate), phosphorus-enriched (+2.5 μM phosphate), and silicon-enriched (+25 μM silica) seawaters were prepared. The added nutrient concentrations were varied accordingly to meet the approximate stoichiometric requirements of N: P: Si = 16:1:16, based on the Redfield ratio for nitrogen and phosphorus

[5] and its extension to include silicon for diatoms [6], ensuring that nutrient addition reflected biologically relevant proportions. Each sample was incubated in a screw-cap test tube and an Erlenmeyer flask at ambient seawater temperature (approximately 16ºC) for 48 hours using an incubator (LH-350SP, NK System). A moderate level of light (approximately 150–200 µmol m^{-2} sec^{-1}) was continuously maintained throughout the incubation period. During incubation, chlorophyll fluorescence of the capped test tubes was measured using a fluorometer (10-AU, Turner Designs) at 0, 3, 6, 24, 29, 48 hours to roughly monitor relative changes in phytoplankton biomass (i.e., chlorophyll *a*). The samples in Erlenmeyer flasks were used for the determination of chlorophyll *a* after 48 hours of incubation. The incubation period was limited to 48 hours to capture the short-term physiological response to nutrient enrichment while minimizing secondary effects such as nutrient recycling and changes in community structure. For each experimental date, all incubations were performed in singlicate (n = 1).

2.2.2 Determination of Chlorophyll a

At the end of cultivation, samples from the Erlenmeyer flasks were filtered through a GF/F filter (0.7 µm pore size). Each filter was placed in a screw-cap tube containing 10 ml of 90% acetone. The tubes were covered with aluminum foil to prevent light exposure and stored at 4ºC for 24 hours to allow for the pigment extraction. Prior to the measurement, the tubes were placed in an ultrasonic bath for 10 minutes. The acetone extract was filtered through a PTFE filter (0.45 µm pore size) to remove any suspended solids. The concentration of the extracted chlorophyll *a* in the acetone was then determined by the fluorometric acidification method [9] using a fluorometer (10-AU, Turner Designs).

3 Results and Discussion

Temporal variation of chlorophyll fluorescence (arbitrary unit; RFU) for both intact and nutrient-enriched samples over 48 hours on each experimental date is illustrated in Fig. 2. Chlorophyll fluorescence represents excess energy absorbed by phytoplankton but not utilized for photosynthesis. This was used as a non-invasive analytical technique to estimate changes in relative phytoplankton biomass in real time. During the first 24 hours of incubation, initial fluorescence (0 h fluorescence) either remained stable (Fig. 2a and d) or slightly declined over time (Fig. 2b and c), regardless of sample (i.e., intact, nitrogen-, phosphorus-, or silicon-enriched samples). However, in three of the four experiments (Fig. 2a–b and d), fluorescence in the nitrogen-enriched samples increased between 24 and 48 hours, whereas phosphorus- and silicon-enriched samples followed a declining trend similar to the intact sample. In the 12 May experiment (Fig. 2c), the fluorescence in the nitrogen-enriched sample continued to decrease over time, but the decline was more gradual compared to the other samples.

Changes in initial (0 h) chlorophyll fluorescence incubated after 24 and 48 hours were calculated and expressed as mean ± SD on the four experimental dates (Fig. 3). At 24 h, treatments exhibited a decline in fluorescence relative to the initial measurement. The largest decrease was observed in the silicon-enriched sample (-0.36 ± 0.39) followed by phosphorus-enriched (-0.29 ± 0.39), intact (-0.28 ± 0.33), and nitrogen-enriched (-0.25 ± 0.25), though there was no significant difference among the four samples (one-way ANOVA, $p = 0.972$).

At 48 h, nitrogen-enriched samples exhibited a positive change in fluorescence (0.24 ± 0.79), surpassing the initial reading, although with high variability (CV = 3.17). In contrast, the remaining treatments showed further decline, with the intact sample decreasing to -0.70 ± 0.35, phosphorus-enriched at -0.55 ± 0.49, and silicon-enriched to -0.79 ± 0.45. A one-way

ANOVA indicated that the difference in fluorescence among the four samples approached statistical significance ($p = 0.075$).

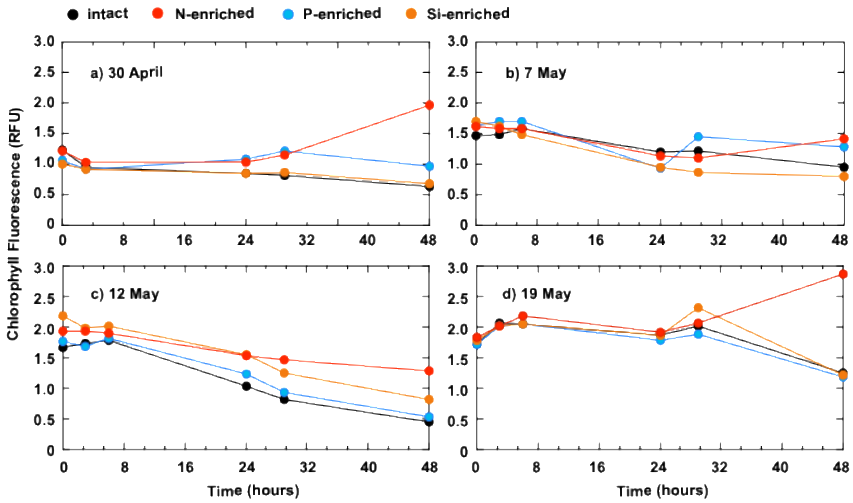

Fig. 2. Chlorophyll fluorescence (RFU) trend of intact and nutrient-enriched samples over 48 hours on a) 30 April, b) 7 May, c) 12 May, and d) 19 May. Black, red, blue, and orange circles denote intact, nitrogen-enriched, phosphorus-enriched, silicon-enriched sample, respectively

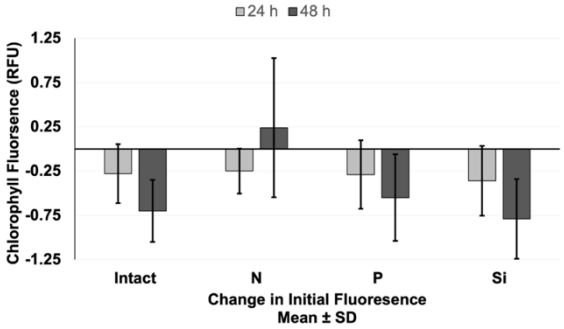

Fig. 3. Mean ± SD change in initial chlorophyll fluorescence at 24 and 48 h (n = 4). Light and dark gray denotes after 24 hours incubation and 48 hours of incubation, respectively

Figure 4 illustrates the concentrations of chlorophyll *a* in each sample after 48 hours of incubation. The measurements of chlorophyll *a* reflected a similar trend to the fluorescence readings, with nitrogen-enriched samples showing increased biomass (1.30 ± 0.75 µg/L) compared to intact samples at 0.40 ± 0.19 µg/L, phosphorus-enriched at 0.40 ± 0.23 µg/L and silicon-enriched samples at 0.36 ± 0.20 µg/L. A one-way ANOVA with post-hoc Tukey HSD test indicated that there was a significant difference among the four treatments (p = 0.020) and nitrogen-enriched samples was higher than the intact, phosphorus- and silicon-enriched samples (p = 0.034–0.043). This pattern indicates that nitrogen-enriched (nitrate-enriched) samples increased the growth rate of phytoplankton assemblages compared to the other treatments.

Phytoplankton assemblages in coastal areas are mainly composed of diatoms which are generally regarded as requiring nutrients in the average atomic ratio of N: P: Si = 16:1:16 [5,6]. At the sampling site, day-to-day variations of DIN, DIP and DSi concentrations in the surface seawater have been monitored since 2022 (Yamaguchi et al., unpublished data).

According to the results from April to May 2025 (i.e., the study period of the present study), the DIN: DIP ratio of the surface seawater was continuously less than 5, suggesting that DIN was relatively insufficient compared to DIP during the studied period. In addition, the DSi: DIN ratio was consistently higher than 1, suggesting that DIN was insufficient compared to both DSi and DIP. Yamaguchi et al. [10] also reported that DIN was considered to be the most insufficient nutrient in Shido Bay year-round, judging from the mean nutrient uptake ratio of diatom-dominated phytoplankton assemblages (N: P: Si = 16: 1: 16). The information on the atomic ratio of nutrients in the ambient seawater suggests that DIN is the limiting nutrient for phytoplankton growth in Shido Bay, although the evidence to support this possibility was previously insufficient. On the other hand, the results of the present study (Fig. 4) clearly demonstrate that DIN indeed limits the growth of phytoplankton—as predicted by the ambient nutrient balance—at least during spring.

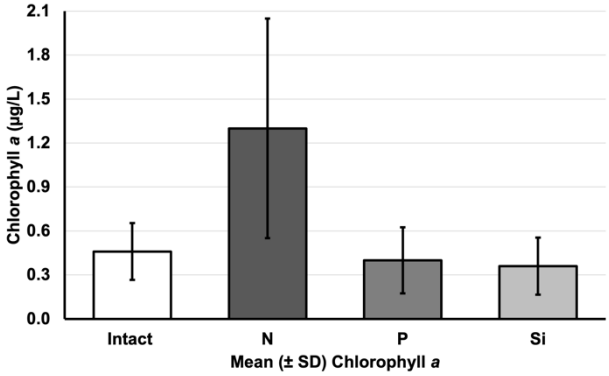

Fig. 4. Mean ± SD of chlorophyll *a* (n = 4) after 48 hours incubation

Nishikawa et al. [11] reported that DIN has decreased consistently after the 1990s in Harima Nada, a region adjacent to Shido Bay in the eastern Seto Inland Sea; however, such a trend remains unclear for DIP and DSi. Integrating the results of Nishikawa et al. [11] with the present study suggests that oligotrophication has been primarily driven by the decline in DIN at least since the 1990s. Moreover, it is also expected that nitrogen limitation for phytoplankton growth in the eastern Seto Inland Sea, including Shido Bay, is becoming increasingly severe over time. This may be a contributing factor to the recent decline in fisheries yield in the Seto Inland Sea. However, other factors such as climate change, prey-predator dynamics, loss of seaweed and seagrass beds are also suspected to contribute to this decline. Hence, in future studies, it is important to quantitatively evaluate the role of nitrogen limitation in the reduction of fisheries yield.

4 Conclusion

The results of the present study show that seawater samples collected from Shido Bay responded positively to nitrogen enrichment (nitrate enrichment) by increasing phytoplankton growth, whereas phosphorus- and silicon-enriched samples showed no observable increase. This finding provides direct evidence that DIN is the primary limiting nutrient in Shido Bay, in the eastern Seto Inland Sea.

This study was supported in part by JSPS KAKENHI Grant Number 23K11449.

References

1. T.R. Parsons, M. Takahashi, and B. Hargrave, *Biological Oceanographic Processes* (Pergamon Press, Oxford, 1984)
2. S.W. Nixon, Physical energy inputs and the comparative ecology of lake and marine ecosystems. Limnol. Oceanogr. **33,** 1005–1025 (1988)
3. J.H. Ryther, Photosynthesis and fish production in the sea. Science. 166, 72–75 (1969)
4. E.P. Odum, *Fundamentals of Ecology* (Toppan Company, Tokyo, 1971)
5. A.C. Redfield, B.H. Ketchum, and F.A. Richards, The influence of organisms on the composition of sea water, in *The Sea*, Vol. 2, edited by M.N. Hill (Interscience Publishers, New York, 1963), pp. 26–77
6. M.A. Brzezinski, Si:C:N ratio of marine diatoms: interspecific variability and the effect of some environmental variables. J. Phycol. **21**, 347–357 (1985)
7. T. Yamamoto, The Seto Inland Sea—eutrophic or oligotrophic? Mar. Pollut. Bull. **47**, 37–42 (2003)
8. K. Abo, T. Yamamoto, Oligotrophication and its measures in the Seto Inland Sea, Japan. Bulletin of Japan Fisheries Research and Education Agency, **49**, 21–26 (2019)
9. O. Holm-Hansen, C.J. Lorenzen, R.W. Holmes, J.D.H. Strickland, Fluorometric determination of chlorophyll. Journal du Conseil International Pour l'Exploration de La Mer, **30**, 3–15 (1965)
10. H. Yamaguchi, N. Koga, K. Ichimi, K. Tada, Seasonal variations in phytoplankton productivity in a shallow cove in the eastern Seto Inland Sea, Japan. Fish. Sci. **86**, 1067–1078 (2020)
11. T. Nishikawa, Y. Hori, S. Nagai, K. Miyahara, Y. Nakamura, K. Harada, M. Tanda, A. Manabe, K. Tada, Nutrient and phytoplankton dynamics in Harima-Nada, eastern Seto Inland Sea, Japan, during a 35-year period from 1973 to 2007. Estuar. Coasts. **33**, 417–427 (2010)