The crucial influence of trophic status on the relative requirement of nitrogen to phosphorus for phytoplankton growth

Mengqi Jiang[1*], Shin-ichi Nakano[1,]

[1] Center for Ecological Research, Kyoto University, 520-2113 Shiga, Japan

[*] Corresponding author

Email addresses: mqjiang@ecology.kyoto-u.ac.jp (M.J.), nakano@ecology.kyoto-u.ac.jp (S.N.)

Keywords: nitrogen; phosphorus; trophic status; phytoplankton; nitrogen-to-phosphorus ratio; limiting factor

Send correspondence to: [Mengqi Jiang]

[Center for Ecological Research, Kyoto University, 520-2113 Shiga, Japan, 080-5711-6059, mqjiang@ecology.kyoto-u.ac.jp]

Authorship statement

MJ conceived the study design, performed experiments, analysed data, and wrote the first draft of the manuscript; SN contributed to data analysis and manuscript writing. Both authors contributed substantially to the manuscript and gave final approval for publication.

Highlights

1. The relative N:P requirement for growth was explored for eight phytoplankton species.
2. Trophic status is negatively related to the N:P ratio required for optimal growth.
3. Both N and P are important for phytoplankton growth in eutrophic waters.
4. Phytoplankton tend to require higher N relative to P under low-nutrient conditions.
5. The results contribute to the ongoing debate of N vs. P limitation in freshwaters.

Abstract

Clarifying the pattern of relative nitrogen (N)-to-phosphorus (P) requirements for phytoplankton growth is of great significance for eutrophication migration and management of aquatic systems. Relative N-to- P requirement for phytoplankton growth is considered an essential trait determining species dominance within ecosystems and explaining phytoplankton response to nutrient availability. These requirements vary with environmental trophic statuses, though this variation remains unclear. Here, we evaluated the relative N-to-P requirements under different absolute nutrient levels using previous and current experimental data on eight phytoplankton species (three studied by us and five extrapolated from the previous studies). Results showed that relative N-to-P requirements for phytoplankton growth decreased as absolute nutrient levels increased. Thus, N may be crucial for enhancing phytoplankton growth under low nutrient conditions, whereas P may be the primary limiting factor of phytoplankton growth under sufficient nutrient conditions. This result applies to single species as well as species assemblages, which are independent of species shifts occurring along water N:P gradients. The response observed in our large trophic status level gradient may help elucidate the relative importance of N and P reductions in mitigating the impact of eutrophication on ecosystems.

1. Introduction

Nitrogen (N) and phosphorus (P) are two key macronutrients utilized in the biochemical functions of phytoplankton and are the main nutrients limiting phytoplankton growth in aquatic ecosystems (Elser *et al.* 2007; Paerl 2009; Abell *et al.* 2010). The relative importance of the N-to-P supply (i.e., the supply N:P ratio) for phytoplankton growth remains an important subject in research on waterbodies with various trophic statuses, including research on controlling nuisance phytoplankton blooms in eutrophic waters (Lewis *et al.* 2011; Paerl *et al.* 2011; Paerl *et al.* 2016) and enhancing primary production (and thus enhancing zooplankton biomass and fish production) in oligotrophic waters (Budy *et al.* 1998; Reeder 2017). However, the relative importance of these two elements for phytoplankton growth remains controversial. P is generally considered the main factor responsible for controlling phytoplankton growth (Carpenter 2008; Schindler *et al.* 2008) largely because the nitrogen fixation of some cyanobacterial species can fulfill their N requirements (Smith 1990). Contrastingly, N limits phytoplankton growth during bloom conditions in some eutrophic lakes (Chaffin *et al*. 2013). Accordingly, a dual nutrient control (N & P) strategy is considered more effective in controlling phytoplankton growth than either nutrient alone (Elser *et al.* 2007; Lewis *et al.* 2011; Paerl *et al.* 2016). Regardless, related studies have mainly focused on the relative N:P ratios and have seldom mentioned the influence of absolute nutrient levels, which vary greatly. As P accumulates faster than N in freshwaters exposed to anthropogenic impact, the

66 relationship between total nitrogen (TN) and total phosphorus (TP) is relevant to the
67 trophic status of a waterbody, that is, high TN:TP ratios in oligotrophic waters and
68 low TN:TP ratios in eutrophic waters (Downing & McCauley 1992; Yan *et al* 2016).
69 However, absolute nutrient concentrations may have a greater impact on
70 phytoplankton growth than relative nutrient ratios because phytoplankton tend to be
71 insensitive to resource stoichiometry during their fast-growing phase (Klausmeier *et*
72 *al*. 2004; Hilebrand *et al*. 2013; Yang *et al.* 2020). Accordingly, if absolute nutrient
73 concentrations are not considered, discussions regarding N:P ratios become largely
74 inaccurate. Therefore, establishing situations where various N:P ratios with different
75 nutrient levels is a prerequisite for evaluating the relative importance of N:P ratios for
76 phytoplankton growth.
77 Most studies on relative N:P requirements are based on observations of how
78 phytoplankton respond to various supply N:P ratios (Liu & Vyverman 2015; Rasdi &
79 Qin 2015; Thrane *et al.* 2016, 2017; Kelly *et al*. 2021). However, the importance of
80 trophic status cannot be reflected by a single supply N:P ratio series. Specifically,
81 phytoplankton biomass would increase with the increase in supply N:P ratio when N
82 was limiting, and decrease with the increase in supply N:P ratio when P was limiting.
83 A peak of phytoplankton biomass could be achieved at the supply N:P ratio where N
84 and P are co-limiting, which has been termed the “optimal supply N:P ratio” (the red
85 line in Fig. 1a and b) (Sperfeld *et al*. 2012; Sperfeld *et al*. 2016; Tilman 1980). The
86 optimal supply N:P ratio is a definitive value derived from a gradient of supply N:P

ratios and an indicator for assessing the relative importance of N-to-P requirements (Thrane *et al.* 2016, 2017). However, the definitive optimal supply value has various possibilities. For example, if a nutrient supply of 16 μmol N L^{-1} and 1 μmol P L^{-1} was calculated as the optimal supply N:P ratio for the growth of a certain phytoplankton species, we cannot easily generalize that 16 is the optimal supply N:P ratio in any environmental condition. Although the nutrient supply of 16 μmol N L^{-1} and 1 μmol P L^{-1} was optimal in a specific series of N:P ratios, we cannot tell whether a nutrient combination of 32 μmol N L^{-1} and 2 μmol P L^{-1} or 8 μmol N L^{-1} and 0.5 μmol P L^{-1} would also be optimal among different supply N:P ratio gradients, despite having a ratio of 16. That is, the relative N:P requirement for phytoplankton may not be equivalent for higher or lower trophic statuses.

A definite optimal N:P ratio indicates that the relative importance of N-to-P requirement for phytoplankton growth remains unchanged under different trophic statuses (red line in Fig. 1a and b). Yet, N and P limitations affect phytoplankton cell physiology in significantly different ways. Protein is the largest N-containing component in phytoplankton cells, whereas P is distributed among phospholipids, adenosine triphosphate, and nucleic acids, especially ribosomal ribonucleic acid (rRNA) (Geider & La Roche 2002). N limitation hampers phytoplankton photosynthesis by causing large declines in the availability of photosynthetic pigments and Rubisco pools (Geider *et al.* 1993; Geider *et al.* 1998). Meanwhile, P limitation greatly suppresses phytoplankton growth because of the large investment of

P in rRNAs and the growth dependence of protein synthesis driven by P-rich ribosomes (Sterner & Elser 2002; Loladze & Elser 2011). Owing to these differences in the physiological functions of N and P, the relative importance of N:P requirements for phytoplankton growth may vary temporally among different environmental conditions. Phytoplankton can regulate the contents of cellular complexes (i.e., pigments and ribosomes) to acclimate to different irradiance or temperature conditions, which can further influence their relative N:P requirement (Thrane *et al.* 2016, 2017). Furthermore, Galbraith and Martiny (2015) emphasized that the cellular P of phytoplankton has greater plasticity than cellular N, resulting in high phytoplankton N:P ratios in oligotrophic waterbodies. Phytoplankton may have a basic and steady requirement for N, whereas their demand for P may be much more flexible and dependent on phytoplankton growth and nutrient availability.

Herein, we propose that optimal supply N:P ratios would be flexible under different trophic statuses and would decline with an increase in absolute nutrient levels (Fig. 1b and c). We assumed that the response of N-fixing cyanobacterium to supply N:P ratios may differ from other phytoplankton species due to its higher tolerance to N starvation. We tested these hypotheses using previous and present experimental data on eight phytoplankton species. Most of the phytoplankton species tested in the present study, including a N-fixing cyanobacterium, yielded similar results supporting our hypothesis: trophic status is negatively related to the N:P ratio required for optimal growth.

2. Materials and methods

2.1 Biological material

We used three phytoplankton species: the green algae *Chlorella vulgaris* NIES-2172, the cyanobacteria *Anabaena variabilis* NIES-2093 (N-fixing cyanobacterium), and *Microcystis aeruginosa* NIES-44. All axenic stock cultures were obtained from the Microbial Culture Collection of the National Institute for Environmental Studies, Tsukuba, Japan. Stock cultures were transferred into a modified BG-11 medium, added with 0.1 μmol L^{-1} vitamin B_{12} and 0.1 μmol L^{-1} biotin. All stock cultures were grown under controlled conditions with a light intensity of 15 μmol photons m^2 s^{-1} and a temperature of 24 °C for a two-transfer acclimation period.

2.2 Experimental design

To maximize the absolute nutrient concentration and the supply N:P ratio gradients, we set the concentrations of N and P to have 12 levels each (Table S1 and Figure S1). According to trophic state classification criteria, the nutrient levels for N and P range from oligotrophic to hypereutrophic (Schlesinger & Bernhardt 2020). Forty-eight combinations of N and P were prepared for each of the three phytoplankton species, with each level of N or P replicated four times (Fig. S1). The concentration of all other nutrients was determined based on the composition of the BG-11 medium, which reduced the risk of nutrient limitation by factors other than N and P.

To test whether the effects of the N:P ratio on phytoplankton growth would

change under different trophic statuses, we categorized the combinations of resource stoichiometries into a series of nutrient levels. Accordingly, we defined the nutrient levels of different resource stoichiometries by assigning weights to N and P. The nutrient supply level (NSL) was defined as the combined result of the absolute concentrations of N and P and expressed as $NSL = (C_N + 16C_P) / 32$, where C_N and C_P represent the concentrations of N and P resources (μmol L^{-1}), respectively. The weight allocated to P was defined as 16 times N based on the Redfield ratio (the average N:P ratio of 16:1 observed in marine phytoplankton; Redfield 1934); thus, the NSL of a nutrient resource combination with 16 μmol L^{-1} N and 1 μmol l^{-1} P would be calculated as 1. All N and P combinations (white dots) on decreasing diagonal lines shared the same NSL value, and the value increased across the X-Y plane (Fig. S1). This method ensured that each specific N:P ratio value could be found for any NSL.

2.3 Experiment execution

We conducted the experiment using 48-well microplates (AGC Techno Glass Co., Ltd., Shizuoka, Japan), with each well containing 750 μL medium prepared based on the modified BG-11 medium described above. Prior to the experiment, exponentially growing cultures were harvested through centrifugation (4000 × g for 8 min), washed with ultrapure water, transferred into BG-11 medium modified to be N-free ($NaNO_3$ replaced with an equimolar equivalent of NaCl, and ferric ammonium citrate substituted with ferric citrate) and P-free (K_2HPO_4 replaced with an equimolar equivalent of KCl), and cultivated under the aforementioned conditions for three days

171 to reduce the effects of N and P stored in phytoplankton cells (Huang *et al.* 2014; Ren
172 *et al.* 2017).

173 At the start of the experiment (day 0), each well was inoculated with 40 µL
174 phytoplankton stock culture (initial optical density at 595 nm [OD_{595}] < 0.05;
175 background OD_{595} of the BG-11 medium was approximately 0.03–0.04). All
176 microplates were kept in a climate-controlled room at 24 °C with a 16:8 h light:dark
177 cycle of 15 µmol photons s^{-1} m^{-2}. Irradiance was measured using an LI-1400 data
178 logger (Li-Cor, Lincoln, NE, USA). All microplates were covered with a gas-
179 permeable sealing membrane (Breathe-Easy; Sigma-Aldrich, St. Louis, MO, USA) to
180 prevent evaporation and microbial contamination. The microplates were randomly
181 rearranged daily to ensure comparable light conditions during the experiment. The
182 experiments were initiated in batch cultures. After one week of cultivation, we shifted
183 the batch cultures into semi-continuous cultures. The dilution rate was 0.15 d^{-1}, which
184 was achieved by replacing 15% of the well volume with fresh culture medium under a
185 laminar flow cabinet every day to refresh the culture media and extend the steady-
186 state growth phase. Each experiment was terminated when the respective culture
187 reached the steady-state growth phase.

188 ***2.4 Monitoring phytoplankton growth***

189 To determine whether the phytoplankton cultures had reached a quasi-steady
190 state, the OD_{595} in each well was measured daily using a microplate reader (Infinite
191 F200 PRO; Tecan Group Ltd., Männedorf, Switzerland). OD_{595} has been widely used

as a proxy for monitoring phytoplankton growth (Huesemann *et al.* 2009; Kapoore *et al.* 2019); we demonstrated that OD_{595} is highly correlated with the cell density of the three phytoplankton species (Fig. S2a–c). However, OD measurements for phytoplankton biomass are susceptible to errors due to pigment interference during different growth phases (Griffiths *et al.* 2011). Therefore, to better evaluate the culture biomass, a colorimetric analysis was performed according to our preparatory work (Jiang & Nakano, 2021). Immediately before cell enumeration of the three phytoplankton species (day 14 for *A. variabilis*, day 16 for *M. aeruginosa*, and day 21 for *C. vulgaris*), RGB (red, green, and blue) color information for the phytoplankton cultures was collected using a free mobile application called "Color-Meter" (https://apps.apple.com/us/app/color-meter/id1512406137; accessed on 1st December 2021). The RGB color information was then converted into the HSI (hue, saturation, intensity) color space for subsequent analysis (Jiang & Nakano, 2021). The results indicated that variations in the hue of the experimental phytoplankton cultures were limited (Fig. S3a), thereby verifying the validity of the optical and colorimetric analyses. Additionally, the measurements of the phytoplankton biomass via OD_{595} and color intensity were highly correlated (Fig. S3b–d). We used the results of the optical and colorimetric analyses to confirm that the cultures reached a quasi-steady state, as the timing varied depending on the cultivation conditions (Fig. S4). However, the steady state of the phytoplankton in some experimental units could not be maintained for an extended period due to nutrient limitation (Fig. S4); thus, it was difficult to

accurately and timely judge whether the cultures reached a steady state during the experiments. Therefore, the steady-state OD_{595} was estimated as the mean of the OD_{595} values greater than 95% of the maximum OD_{595} during the growth period for each experimental unit. We counted the cell density during the quasi-steady state or near the peak of the growth curves with a Neubauer hemocytometer (Brand, Wertheim, Germany) under an optical microscope (Olympus BX51; Olympus, Tokyo, Japan). The steady-state phytoplankton cell density ($C_{\text{steady-state}}$) was calculated as:

$$C_{\text{steady-state}} = C_{\text{count}} \frac{\text{OD}_{595(\text{steady-state})} - \text{OD}_{595(\text{BG-11})}}{\text{OD}_{595(\text{count})} - \text{OD}_{595(\text{BG-11})}}$$

where $OD_{595(\text{count})}$ represents the OD_{595} of the counted subsamples, $OD_{595(\text{steady-state})}$ represents the steady-state OD_{595}, C_{count} represents the phytoplankton cell density counted on the sampling day, and $OD_{595(\text{BG-11})}$ is the background OD_{595} value of the BG-11 medium, which was estimated to be 0.035 using a microplate reader (Infinite F200 PRO; Tecan, Austria).

2.5 Determining the transitions between N and P requirements

Each phytoplankton species contained 48 experimental units, which were classified into several different NSLs (Fig. S1). We established relationships between the supply N:P ratio and the steady-state phytoplankton cell density for four NSLs (0.91, 1.24, 1.58, and 1.91; Figs. 2 and S5). The transitions between N and P limitation at each NSL were calculated using one-dimensional locally weighted sequential smoothing method (1D-LOESS; *loess* function in Base R) by drawing "peak-shaped" relationship curves (Fig. 2). The parameter *span*, which controls the

234 degree of smoothing in 1D-LOESS, was accepted at its default value of 0.75.

235 The 1D-LOESS method provided results for limited NSLs because it can only
236 estimate the relationships between supply N:P ratios and phytoplankton biomass
237 based on experimental units sharing the same NSL value (Fig. 2). To obtain a
238 continuous relationship between the optimal N:P ratio and the NSL, we estimated the
239 relationships between two related variables (N and P concentrations) and an outcome
240 (phytoplankton cell density during the steady-state phase) using a two-dimensional
241 LOESS curve with a default *span* of 0.75 (2D-LOESS; *loess* function in Base R). By
242 fitting the available 48 experimental data points to the 2D-LOESS model, the steady-
243 state cell density could be calculated for any possible combination of N and P
244 resource concentrations. Using the *predict* function in Base R, the estimates for each
245 combination of N and P concentrations were generated into a matrix and visualized
246 through contour plots to show a smoothing relationship between the resource
247 stoichiometry and phytoplankton biomass (Fig. 3a–c). Each value of the
248 phytoplankton density on the 2D-LOESS contours was estimated according to pre-
249 defined (N, P) locations (https://stat.ethz.ch/pipermail/r-help/2007-
250 February/125269.html, accessed on 1st December 2021).

251 The phytoplankton density at the intermediate NSLs (0.74–1.94) was calculated
252 with an interval of 0.12 based on the estimates from the 2D-LOESS contours. For
253 each NSL, the cell density of each supply N:P ratio was extracted at an interval of less
254 than 0.01, generating smoothing curves that described the relationships between the

supply N:P ratio and phytoplankton density (Fig. S6). We sorted through all phytoplankton density estimates with different supply N:P ratios and extracted the maximum value whose N:P ratio was regarded as the most optimal for that NSL (Fig. 3d–f). The analytical process for determining the optimal supply N:P ratios of different nutrient levels using 2D-LOESS is shown in Figure S7.

2.6 Literature dataset analyses

Published datasets from previous studies were compared with the results of the present study. Datasets were selected based on the four following criteria: (1) phytoplankton monocultures were cultivated under conditions with different absolute and relative N and P concentrations; (2) the combinations of the supply N and P concentrations were sufficient for calculating the optimal N:P ratios for at least two NSLs; (3) the cultures were cultivated through the steady-state phase, and the steady-state biomass was determined; and (4) N and P were the only limiting factors for phytoplankton growth during cultivation. Although studies have focused on the influence of supply N:P ratios on phytoplankton growth, the majority of the obtained datasets did not cover two or more NSLs. Nevertheless, datasets derived from Frank *et al*. (2020) and Kunikane *et al*. (1984) fulfilled the aforementioned criteria.

For Frank et al. (2020), we applied the 2D-LOESS method described above to determine the effects of the NSL on the optimal supply N:P ratios during the steady state for the same four phytoplankton species in monoculture (*Ankistrodesmus* sp., *Chlamydomonas reinhardtii*, *Scenedesmus obliquus*, and *Staurastrum* sp.) (Fig. 4a-d)

and in admixture (Fig. 4e). Optimal supply N:P ratios at the NSLs from 1.7 to 4.7 were estimated with an interval of 0.1, and only the NSLs resulting in a unimodal curve with a peak (the curve describes the relationship between steady-state phytoplankton biomass and the supply N:P ratios; Fig. S7) were applied to assess the impacts of the NSL on the optimal supply N:P ratio (Fig. 4f-j, Fig. S7).

In Kunikane *et al.* (1984), higher dilution rates led to higher available N and P concentrations in the medium during the steady-state phase, which was equivalent to the high NSLs used in the present study. We calculated the transitions between N and P requirements using smoothing spline curves in GraphPad Prism (GraphPad, San Diego, CA, USA) (Fig. 4k and l).

The dataset in Frank *et al.* (2020) was directly obtained from the authors, whereas the data presented in Figure 4 of Kunikane *et al.* (1984) was estimated using the open-access digitizing software Engauge Digitizer version 12.1 because we were unable to contact the authors.

3. Results

For most of the NSLs of the three phytoplankton species, the steady-state phytoplankton density followed a significant unimodal relationship with the supply N:P ratio, with the exceptions of *C. vulgaris* and *A. variabilis* cultures at the NSL of 1.91 (Fig. 2). The optimal ratios estimated using 1D-LOESS generally decreased with an increased NSL (Fig. 2). 2D-LOESS predictions of the relationships between the supply N:P ratio and the NSL were obtained from all 48 experimental units rather than

297 only the experimental units having the same NSL (Fig. 3a–c), and this method
298 generated more detailed relationships between the optimal supply N:P ratio and the
299 NSL. The results of the 2D-LOESS models showed the same pattern as the 1D-
300 LOESS: the optimal supply N:P ratio decreased with the increase in NSL for all three
301 studied phytoplankton species (Fig. 3d–f).
302 The datasets obtained from the literature also supported this trend. Three of the
303 four phytoplankton species studied by Frank *et al.* (2020) were found to have the
304 decrease in optimal supply N:P ratio with an increasing NSL (Fig. 4f–i). *C. reinhardtii*
305 was the only exception, as its optimal supply N:P ratios were distributed in a narrow
306 range (mean ± SD: 23.9 ± 1.2; Fig. 4i). In addition, the results of the mixed culture in
307 Frank *et al*. (2020) showed a similar trend of a decreasing optimal supply N:P ratio
308 with NSLs (Fig. 4j). The calculated results of the data from Kunikane *et al.* (1984)
309 indicated that the optimal N:P ratios of *S. dimorphus* decreased with the increase in
310 dilution rate (Fig. 4l). At the steady state observed in the chemostat, the growth rate of
311 the phytoplankton density was equal to the dilution rate. In other words,
312 phytoplankton with high growth rates tended to require more P than N. Overall, seven
313 out of the eight analyzed phytoplankton species exhibited a decreasing optimal supply
314 N:P ratio with the increase in nutrient availability. This trend was achieved under
315 different culture conditions (i.e., semi-continuous culture in the present study, batch
316 cultures in Frank *et al.* [2020], and chemostat cultures in Kunikane *et al.* [1984]) and
317 methods for regulating the absolute nutrient concentrations (i.e., varying input

318 nutrients in the present study and Frank *et al.* [2020], varying dilution rates in
319 Kunikane *et al.* [1984]).

320 Although the optimal supply N:P ratio generally decreased with the increase in
321 NSL, the downward trend was not immutable, especially for high NSLs. The decline
322 tended to level off at high NSLs (or high dilution rates in the chemostat experiment),
323 which was observed in half of the studied species: *C. vulgaris* and *M. aeruginosa*
324 cultures in the present experiments (Fig. 3), *S. obliquus*, *Staurastrum* sp. and mixed
325 cultures in Frank *et al.* (2020), and *S. dimorphus* in Kunikane *et al.* (1984) (Fig. 4).

326 **4. Discussion**

327 N and P requirements for phytoplankton growth seem to be flexible among
328 various trophic statuses, as reflected by the decrease of the optimal supply N:P ratio
329 with the increase in NSL. The optimal supply N:P ratio was defined as the supply N:P
330 ratio in a medium where the transition from N limitation to P limitation for
331 phytoplankton growth occurred. We found only one exception (*C. reinhardtii*) among
332 the eight studied species (Fig. 4d and 4i), while stable ranges of the optimal supply
333 N:P ratio were observed in half of the other phytoplankton cultures at high NSLs.
334 Therefore, we cannot identify whether the optimal supply N:P ratio for *C. reinhardtii*
335 was truly stable over all NSLs, or whether the NSLs studied by Frank *et al.* (2020)
336 were too high to cover the decreasing ranges of the optimal supply N:P ratio. Despite
337 this exception, a significant proportion of the phytoplankton species supported the
338 conclusion that phytoplankton has higher N requirements than P requirements at low

NSLs, or vice versa at high NSLs. Although we focused mainly on monocultures in the present study, it is noteworthy that mixed cultures studied in Frank *et al*. (2020) produced similar results that support our hypothesis (Fig. 4j). The trend of decreasing optimal supply N:P ratio with NSLs was unaffected by species shifts within the mixed cultures. All four species in the mixed cultures belonged to a single phylum (Chlorophyta); however, we have not yet determined whether our hypothesis is also applicable to mixed cultures comprising various taxa.

To the best of our knowledge, this study is the first to demonstrate that trophic status is negatively related to the relative N:P requirement for phytoplankton growth. Changes in the optimal N:P ratio at different NSLs could be explained by the growth-rate hypothesis, which states that the variation in cellular N:P ratios is largely attributed to cellular protein and rRNA contents (Elser *et al.* 2000; Sterner & Elser 2002), as proteins are the largest cellular N pool, and rRNAs are the largest contributor of P to phytoplankton cells (Geider & La Roche 2002; Sterner & Elser 2002). For phytoplankton cultures under the same supply N:P ratio but at different NSLs, higher NSLs (or high dilution rates in the chemostat experiment) likely increased nutrient availability and promoted phytoplankton growth, resulting in higher biomass (Fig. 2–4). Phytoplankton growth enhancement would increase the rRNA requirement, resulting in a higher requirement of P relative to N. It is also possible that the changes in the optimal N:P ratios were related to certain adaptive responses of phytoplankton to low nutrient supplies. In low P environments, phytoplankton would

selectively increase the synthesis of P-free compounds rather than P-containing compounds to reduce the P demand, such as by replacing phospholipids in cell membranes with sulfonated lipids (Snyder *et al.* 2009; Wurch *et al.* 2011). However, N seems to be more essential for phytoplankton photosynthesis than P. N limitation in phytoplankton can cause a substantial decline in the content of the major photosynthetic pigment, chlorophyll *a*, and increase the amount of non-photosynthetic pigments (Herrig & Falkowski 1989; Geider *et al.* 1993). However, the effects of P limitation on cellular chlorophyll *a* are relatively smaller than those of N limitation (Geider *et al.* 1998). In addition, N limitation may greatly reduce the efficiency of photosystem II and the relative abundance of Rubisco in phytoplankton cells (Falkowski *et al.* 1989; Geider *et al.* 1993), whereas P limitation moderately affects phytoplankton photosystems (Geider *et al.* 1998). Accordingly, we assumed that phytoplankton may have a steady N requirement because of its importance for photosynthesis, while P requirement may be more flexible via adaptation and acclimation responses. This assumption is consistent with the results of Galbraith and Martiny (2015), who found that the cellular abundance of N in phytoplankton tends to be less plastic than the P content. Phytoplankton under dual N and P limitation, therefore, would show a higher requirement for N than P.

The decreasing curve of the optimal N:P ratio tended to level off with increasing NSL, as observed in half of the studied phytoplankton species (Figs. 3 and 4). For datasets derived from experiments with a factorial design (the present study and Frank

et al. [2020]), high NSLs were only studied with limited experimental units, and the supply N:P ratios of these units were distributed in a narrow range. As shown in Figure 2, the peaks of the unimodal curves became indistinct at high NSLs, and the optimal supply N:P ratios for *C. vulgaris* and *A. variabilis* at the NSL of 1.91 (molar supply N:P ratios distributed from 7.71 to 33.29) could not be identified using 1D-LOESS method. Hence, the disappearance of the decreasing trend may be partly explained by the factorial design used in the present study and by Frank *et al.* (2020). The data of Kunikane *et al.* (1984) showed similar results, as the optimal N:P ratio became stable at high absolute NSLs (Fig. 4l); however, this trend could not be explained by the same reason because NSLs were regulated by dilution rates instead of nutrient input. Thus, stable optimal N:P ratios at high NSLs may not be a coincidence. Stable optimal N:P ratios at high NSLs may result from the decrease in nutrient affinity alongside the increase in absolute nutrient concentrations (Smith *et al.* 2009; Bonachela *et al.* 2011). Phytoplankton cells can increase the number of surface uptake sites (proteins that incorporate nutrients from the cell membrane into the cytoplasm) to increase the encounter with nutrients in low-nutrient waterbodies (Smith *et al.* 2009; Bonachela *et al.* 2011), leading to a high nutrient affinity for phytoplankton cells. With the increase in nutrient availability, phytoplankton growth is no longer limited by extracellular nutrients, and the nutrient uptake rates are thus controlled by intracellular enzymes that assimilate the encountered nutrients (Smith *et al.* 2009). Therefore, we can find explicitly decreasing curves of optimal N:P ratios

under low NSLs due to the high nutrient affinity in this phase, and the curves become stable at high NSLs because of the decrease in nutrient affinity (Fig. 5). For high NSLs, the optimal supply N:P ratios can be calculated using the 2D-LOESS method (Fig. 3), but cannot always be estimated using 1D-LOESS method (Fig. 2). The optimal N:P ratios at high NSLs derived from the 2D-LOESS method were calculated using experimental units of both high NSLs and low NSLs. So, the values calculated on the smoothing curves of high NSLs could be regarded as an inertial extension of values estimated at low NSLs. Thus, although the optimal N:P ratios at high NSLs could be calculated mathematically, the ecological significance of the calculated N:P ratios would be negligible (Fig. 5).

The cyanobacterium *A. variabilis* is capable of nitrogen fixation (Thiel *et al.* 2014), which is thought to have the potential to offset the decline in resource N supply. The results indicate that the optimal supply N:P ratios for *A. variabilis* cultures at low NSLs are much higher than those at high NSLs (Fig. 2b and 3d). In other words, nitrogen fixation in *A. variabilis* cells did not offset the decline in the N supply, which supports the idea that, although nitrogen fixation could stimulate cyanobacterial growth, cyanobacterial N fixers do not fully compensate for N deficiency (Lewis Jr & Wurtsbaugh 2008; Scott & McCarthy 2010).

Experimental setups and culture systems varied between the experiment in the present study and those of previous studies from which datasets were used in the present study (Kunikane *et al*. 1984; Frank *et al*. 2020). NSLs calculated from batch

cultures in Frank *et al*. (2020) were almost 2.5-fold higher than NSLs calculated using data derived from the present semi-continuous cultures in the present study (Figs. 3 and 4) because the initial nutrient level of the batch cultures was higher than that of the semi-continuous cultures. Additionally, both values and changing ranges of optimal supply N:P ratios calculated from the present semi-continuous cultures were lower and narrower than those in previous studies (Kunikane *et al*. 1984; Frank *et al*. 2020), which could be attributed to the differences in experimental setups and phytoplankton taxa among the experiments. Additionally, light intensity applied in Frank *et al*. (2020) (40.45 ± 15.00 μmol photons m^2 s^{-1}) was much higher than that in our study (15 μmol photons m^2 s^{-1}), which could explain the broader optimal supply N:P ratio ranges in Frank *et al.* (2020).

5. Conclusions

The response curves depicting the relationships between the optimal supply N:P ratio and trophic status varied among the eight tested phytoplankton species, but seven of them showed similar declining trends. Our results support the idea that the transition from N requirements to P requirements is not a definite boundary, but it varies depending on trophic status, with lower nutrient supply leading to higher N requirements than P requirements (Fig. 5). The N vs. P limitation for controlling phytoplankton growth has been discussed for decades, but it still remains an open question (Schindler *et al.* 2008; Conley *et al.* 2009; Xu *et al.* 2010; Paerl *et al*. 2016). The present study assumed that neither N nor P was superior in controlling

phytoplankton growth. P was considered to have high priority in conditions with sufficient N and P supplies (i.e., phytoplankton blooms in eutrophic waterbodies), which is consistent with studies showing that P is more effective in controlling eutrophication than N (Carpenter 2008; Schindler *et al.* 2008). In contrast, we suggest that the importance of N to phytoplankton productivity is greater in environments with a low nutrient supply (i.e., in oligotrophic waterbodies). Generally, our results could be instructive for the control of phytoplankton biomass and eutrophication mitigation in aquatic ecosystems.

Acknowledgements

The authors thank Dr. Yukiko Goda for assistance with the experimental setup and Dr. Koichi Ito for valuable comments on statistics. Further, the authors thank Maren Striebel and Michael Danger for valuable comments on the manuscript. This work was partly supported by KAKENHI, Grants-in-Aid for Scientific Research, grant number 19H03302 and 21J15473 from the Japan Society for the Promotion of Science.

References

Abell, J.M., Özkundakci, D. & Hamilton, D.P. (2010). Nitrogen and Phosphorus Limitation of Phytoplankton Growth in New Zealand Lakes: Implications for Eutrophication Control. *Ecosystems*, 13, 966-977.

Bonachela, J.A., Raghib, M. & Levin, S.A. (2011). Dynamic model of flexible

phytoplankton nutrient uptake. *Proceedings of the National Academy of Sciences*, 108, 20633-20638.

Budy, P., Luecke, C. & Wurtsbaugh, W.A. (1998). Adding Nutrients to Enhance the Growth of Endangered Sockeye Salmon: Trophic Transfer in an Oligotrophic Lake. *Transactions of the American Fisheries Society*, 127, 19-34.

Carpenter, S.R. (2008). Phosphorus control is critical to mitigating eutrophication. *Proceedings of the National Academy of Sciences*, 105, 11039-11040.

Chaffin, J.D., Bridgeman, T.B., & Bade, D.L. (2013). Nitrogen Constrains the Growth of Late Summer Cyanobacterial Blooms in Lake Erie. *Advances in Microbiology*, 3, 16-26

Conley, D.J., Paerl, H.W., Howarth, R.W., Boesch, D.F., Seitzinger, S.P., Havens, K.E. *et al.* (2009). Controlling Eutrophication: Nitrogen and Phosphorus. *Science*, 323, 1014.

Downing, J.A. & McCauley, E. (1992). The nitrogen : phosphorus relationship in lakes. *Limnology and Oceanography*, 37, 936-945.

Elser, J.J., Bracken, M.E.S., Cleland, E.E., Gruner, D.S., Harpole, W.S., Hillebrand, H. *et al.* (2007). Global analysis of nitrogen and phosphorus limitation of primary producers in freshwater, marine and terrestrial ecosystems. *Ecology Letters*, 10, 1135-1142.

Elser, J.J., Sterner, R.W., Gorokhova, E., Fagan, W.F., Markow, T.A., Cotner, J.B. *et al.* (2000). Biological stoichiometry from genes to ecosystems. *Ecology Letters*, 3, 540-550.

Falkowski, P.G., Sukenik, A. & Herzig, R. (1989). Nitrogen limitation in Isochrysis galbana (Haptophyceae). II. Relative abundance of chloroplase proteins. *Journal of Phycology*, 25, 471-478.

Frank, F., Danger, M., Hillebrand, H. & Striebel, M. (2020). Stoichiometric constraints on phytoplankton resource use efficiency in monocultures and mixtures. *Limnology and Oceanography*, 65, 1734-1746.

Galbraith, E.D. & Martiny, A.C. (2015). A simple nutrient-dependence mechanism for predicting the stoichiometry of marine ecosystems. *Proceedings of the National Academy of Sciences*, 112, 8199-8204.

507 Geider, R. & La Roche, J. (2002). Redfield revisited: variability of C:N:P in marine
508 microalgae and its biochemical basis. *European Journal of Phycology*, 37, 1-17.
509
510 Geider, R., Macintyre, Graziano, L. & McKay, R.M. (1998). Responses of the
511 photosynthetic apparatus of Dunaliella tertiolecta (Chlorophyceae) to nitrogen
512 and phosphorus limitation. *European Journal of Phycology*, 33, 315-332.
513
514 Geider, R.J., La Roche, J., Greene, R.M. & Olaizola, M. (1993). Response of the
515 photosynthetic apparatus of Phaeodactylum tricornutum (Bacillariophyceae) to
516 nitrate, phosphate, or iron starvation. *Journal of Phycology*, 29, 755-766.
517
518 Griffiths, M.J., Garcin, C., van Hille, R.P. & Harrison, S.T.L. (2011). Interference by
519 pigment in the estimation of microalgal biomass concentration by optical
520 density. *Journal of Microbiological Methods*, 85, 119-123.
521
522 Herrig, R. & Falkowski, P.G. (1989). Nitrogen limitation in Isochrysis galbana
523 (Haptophyceae). I. Photosynthetic energy conversion and growth efficiencies.
524 *Journal of Phycology*, 25, 462-471.
525
526 Hillebrand, H., Steinert, G., Boersma, M., Malzahn, A., Meunier, C.L., Plum, C. *et al*.
527 (2013). Goldman revisited: Faster growing phytoplankton has lower N:P and

lower stoichiometric flexibility. *Limnology and Oceanography*. 58, 2076–2088.

Huang, W., Bi, Y. & Hu, Z. (2014). Effects of Fertilizer-Urea on Growth, Photosynthetic Activity and Microcystins Production of Microcystis aeruginosa Isolated from Dianchi Lake. *Bulletin of Environmental Contamination and Toxicology*, 92, 514-519.

Huesemann, M.H., Hausmann, T.S., Bartha, R., Aksoy, M., Weissman, J.C. & Benemann, J.R. (2009). Biomass Productivities in Wild Type and Pigment Mutant of Cyclotella sp. (Diatom). *Applied Biochemistry and Biotechnology*, 157, 507-526.

Jiang, M. & Nakano, S. (2021). Application of image analysis for algal biomass quantification: a low-cost and non-destructive method based on HSI color space. *Journal of Applied Phycology*, 33, 3709–3717.

Klausmeier, C. A., Litchman, E. & Levin, S.A. (2004). Phytoplankton growth and stoichiometry under multiple nutrient limitation. *Limnology and Oceanography*. 49, 1463–1470.

Kapoore, R.V., Huete-Ortega, M., Day, J.G., Okurowska, K., Slocombe, S.P., Stanley,

549 M.S. *et al.* (2019). Effects of cryopreservation on viability and functional
550 stability of an industrially relevant alga. *Scientific Reports*, 9, 2093.
551
552 Kelly, P.T., Taylor, J.M., Andersen, I.M., Stovall, J. and Scott, J.T. (2021). Highest
553 primary production achieved at high nitrogen levels despite strong
554 stoichiometric imbalances with phosphorus in hypereutrophic experimental
555 systems. *Limnology and Oceanography*. 66, 4375-4390.
556
557 Kunikane, S., Kaneko, M. & Maehara, R. (1984). Growth and nutrient uptake of green
558 alga, Scenedesmus dimorphus, under a wide range of nitrogen/phosphorus
559 ratio—I. Experimental study. *Water Research*, 18, 1299-1311.
560
561 Lewis Jr, W.M. & Wurtsbaugh, W.A. (2008). Control of Lacustrine Phytoplankton by
562 Nutrients: Erosion of the Phosphorus Paradigm. *International Review of*
563 *Hydrobiology*, 93, 446-465.
564
565 Lewis, W.M., Wurtsbaugh, W.A. & Paerl, H.W. (2011). Rationale for Control of
566 Anthropogenic Nitrogen and Phosphorus to Reduce Eutrophication of Inland
567 Waters. *Environmental Science & Technology*, 45, 10300-10305.
568
569 Liu, J. & Vyverman, W. (2015). Differences in nutrient uptake capacity of the benthic

570 filamentous algae Cladophora sp., Klebsormidium sp. and Pseudanabaena sp.
571 under varying N/P conditions. *Bioresource Technology*, 179, 234-242.
572
573 Loladze, I. & Elser, J.J. (2011). The origins of the Redfield nitrogen-to-phosphorus ratio
574 are in a homoeostatic protein-to-rRNA ratio. *Ecology Letters*, 14, 244-250.
575
576 Paerl, H.W. (2009). Controlling Eutrophication along the Freshwater–Marine
577 Continuum: Dual Nutrient (N and P) Reductions are Essential. *Estuaries and*
578 *Coasts*, 32, 593-601.
579
580 Paerl, H.W., Scott, J.T., McCarthy, M.J., Newell, S.E., Gardner, W.S., Havens, K.E. *et*
581 *al.* (2016). It Takes Two to Tango: When and Where Dual Nutrient (N & P)
582 Reductions Are Needed to Protect Lakes and Downstream Ecosystems.
583 *Environmental Science & Technology*, 50, 10805-10813.
584
585 Paerl, H.W., Xu, H., McCarthy, M.J., Zhu, G., Qin, B., Li, Y. *et al.* (2011). Controlling
586 harmful cyanobacterial blooms in a hyper-eutrophic lake (Lake Taihu, China):
587 The need for a dual nutrient (N & P) management strategy. *Water Research*, 45,
588 1973-1983.
589
590 Rasdi, N.W. & Qin, J.G. (2015). Effect of N:P ratio on growth and chemical

composition of Nannochloropsis oculata and Tisochrysis lutea. *Journal of Applied Phycology*, 27, 2221-2230.

Redfield, A. (1934). On the proportions of organic derivatives in sea water and their relation to the composition of plankton. In Daniel, R.J. (ed James Johnstone Memorial Volume). University Press of Liverpool, 177–192.

Reeder, B.C. (2017). Primary productivity limitations in relatively low alkalinity, high phosphorus, oligotrophic Kentucky reservoirs. *Ecological Engineering*, 108, 477-481.

Ren, L., Wang, P., Wang, C., Chen, J., Hou, J. & Qian, J. (2017). Algal growth and utilization of phosphorus studied by combined mono-culture and co-culture experiments. *Environmental Pollution*, 220, 274-285.

Saito, M.A., Goepfert, T.J. & Ritt, J.T. (2008). Some thoughts on the concept of colimitation: Three definitions and the importance of bioavailability. *Limnology and Oceanography*, 53, 276-290.

Schindler, D.W., Hecky, R.E., Findlay, D.L., Stainton, M.P., Parker, B.R., Paterson, M.J. *et al.* (2008). Eutrophication of lakes cannot be controlled by reducing nitrogen

612 input: Results of a 37-year whole-ecosystem experiment. *Proceedings of the*
613 *National Academy of Sciences*, 105, 11254-11258.
614
615 Schlesinger, W.H. & Bernhardt, E.S. (2020). Chapter 8 - Inland Waters. In:
616 *Biogeochemistry (Fourth Edition)* (eds. Schlesinger, WH & Bernhardt, ES).
617 Academic Press, pp. 293-360.
618
619 Scott, J.T. & McCarthy, M.J. (2010). Nitrogen fixation may not balance the nitrogen
620 pool in lakes over timescales relevant to eutrophication management.
621 *Limnology and Oceanography*, 55, 1265-1270.
622
623 Smith, S.L., Yamanaka, Y., Pahlow, M. & Oschlies, A. (2009). Optimal uptake kinetics:
624 physiological acclimation explains the pattern of nitrate uptake by
625 phytoplankton in the ocean. *Marine Ecology Progress Series*, 384, 1-12.
626
627 Smith, V.H. (1990). Nitrogen, phosphorus, and nitrogen fixation in lacustrine and
628 estuarine ecosystems. *Limnology and Oceanography*, 35, 1852-1859.
629
630 Snyder, D.S., Brahamsha, B., Azadi, P. & Palenik, B. (2009). Structure of
631 Compositionally Simple Lipopolysaccharide from Marine Synechococcus.
632 *Journal of Bacteriology*, 191, 5499-5509.

Sperfeld, E., Martin-Creuzburg, D. & Wacker, A. (2012). Multiple resource limitation theory applied to herbivorous consumers: Liebig's minimum rule vs. interactive co-limitation. *Ecology Letters*, 15, 142-150.

Sperfeld, E., Raubenheimer, D. & Wacker, A. (2016). Bridging factorial and gradient concepts of resource co-limitation: towards a general framework applied to consumers. *Ecology Letters*, 19, 201-215.

Sterner, R.W. & Elser, J.J. (2002). How to Build an Animal: The Stoichiometry of Metazoans. In: *Ecological Stoichiometry: Biology of Elements from Molecules to the Biosphere*. Princeton University Press, pp. 135-178.

Thiel, T., Pratte, B.S., Zhong, J., Goodwin, L., Copeland, A., Lucas, S. *et al.* (2014). Complete genome sequence of Anabaena variabilis ATCC 29413. *Standards in Genomic Sciences*, 9, 562-573.

Thrane, J.-E., Hessen, D.O. & Andersen, T. (2016). The impact of irradiance on optimal and cellular nitrogen to phosphorus ratios in phytoplankton. *Ecology Letters*, 19, 880-888.

654 Thrane, J.-E., Hessen, D.O. & Andersen, T. (2017). Plasticity in algal stoichiometry:
655 Experimental evidence of a temperature-induced shift in optimal supply N:P
656 ratio. *Limnology and Oceanography*, 62, 1346-1354.
657
658 Tilman, D. (1980). Resources: A Graphical-Mechanistic Approach to Competition and
659 Predation. *The American Naturalist*, 116, 362-393.
660
661 Wurch, L.L., Bertrand, E.M., Saito, M.A., Van Mooy, B.A.S. & Dyhrman, S.T. (2011).
662 Proteome Changes Driven by Phosphorus Deficiency and Recovery in the
663 Brown Tide-Forming Alga Aureococcus anophagefferens. *PloS one*, 6, e28949.
664
665 Xu, H., Paerl, H.W., Qin, B., Zhu, G. & Gaoa, G. (2010). Nitrogen and phosphorus
666 inputs control phytoplankton growth in eutrophic Lake Taihu, China. *Limnology*
667 *and Oceanography*, 55, 420-432.
668
669 Yan, Z., Han, W., Peñuelas, J., Sardans, J., Elser, J.J., Du, E. *et al*. (2016). Phosphorus
670 accumulates faster than nitrogen globally in freshwater ecosystems under
671 anthropogenic impacts. *Ecology Letters*, 19, 1237–1246.
672
673 Yang, Y., Pan, J., Han, B.-P. & Naselli-Flores, L. (2020). The effects of absolute and
674 relative nutrient concentrations (N/P) on phytoplankton in a subtropical

reservoir. *Ecological Indicators*, 115, 106466.

Figures

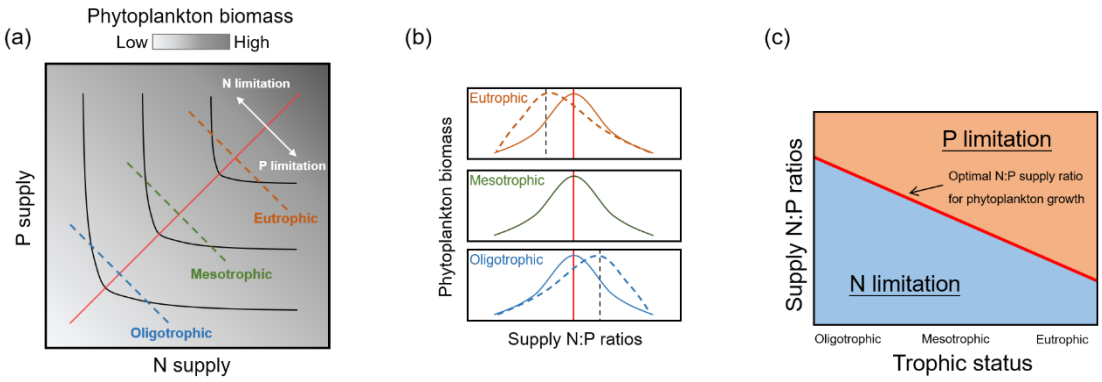

Figure 1 Hypothesis of the experiment. (a) Hypothetical response of phytoplankton growth under simultaneous co-limitation of nitrogen (N) and phosphorus (P) (Saito *et al*. 2008; Sperfeld *et al*. 2012; Sperfeld *et al*. 2016). Resource-dependent growth isoclines (black lines) indicate equal growth at varying resource availabilities. Colored dash lines represent the trophic statuses. The red line indicates the optimal supply N:P ratio. (b) Unimodal relationship between supply N:P ratios and phytoplankton biomass under different trophic statuses. Solid colored curves represent the hypotheses of previous studies that the position of the peaks are fixed. Dashed colored curves represent the hypothesis of the present study that the peaks will shift with trophic status (i.e., that higher trophic statuses have lower optimal supply N:P ratios, and lower trophic statuses have higher ratios). (c) The predicted development of the optimal N:P ratios, which have a negative relationship with trophic status. Phytoplankton tend to be more P-limited than N-limited at high trophic statuses, and vice versa at low trophic statuses.

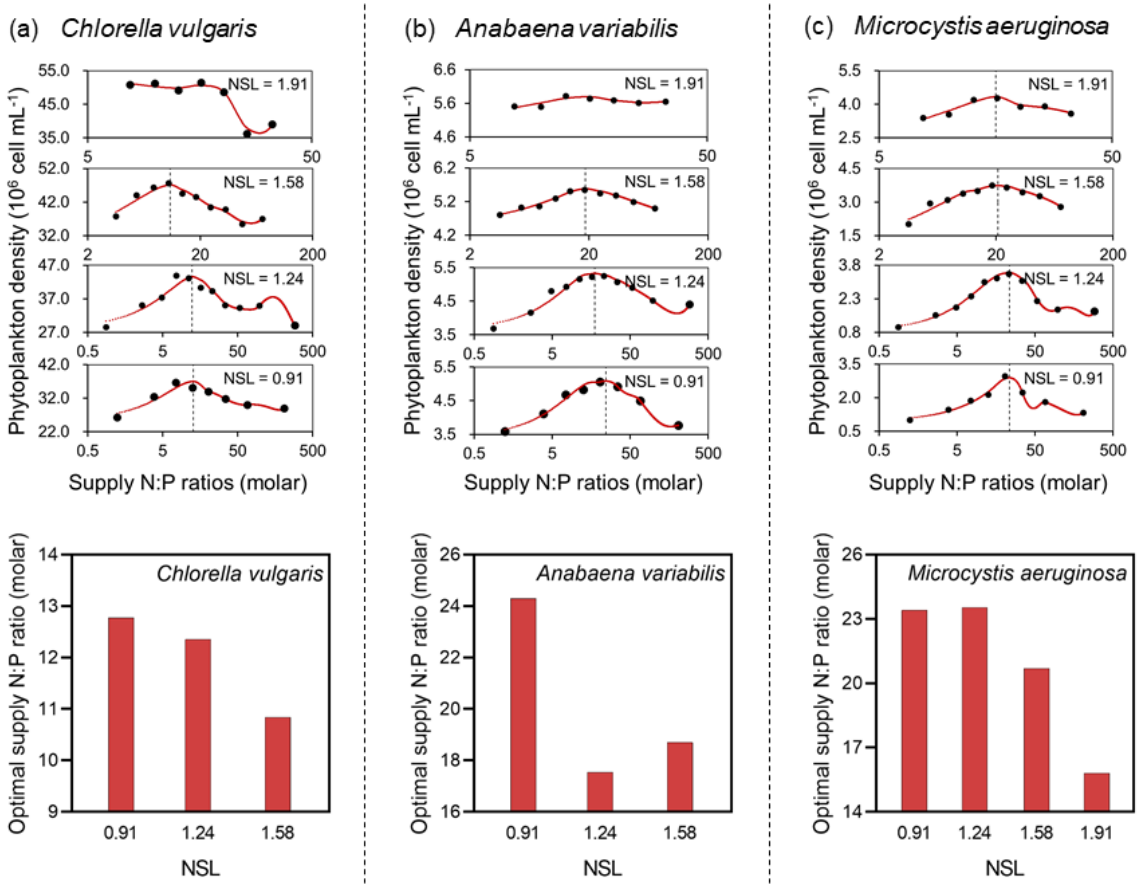

Figure 2 Analytical results of the one-dimensional locally weighted sequential smoothing method (1D-LOESS). Response curves of steady-state phytoplankton biomass (cell density) of (a) *C. vulgaris*, (b) *A. variabilis*, and (c) *M. aeruginosa* to the supply N:P ratios under four NSLs (0.91, 1.24, 1.58, and 1.91). Dashed lines represent the position of the optimal supply N:P ratios calculated using 1D-LOESS fits. The bar graphs summarize the calculated optimal supply N:P ratios under each NSL for the corresponding phytoplankton species.

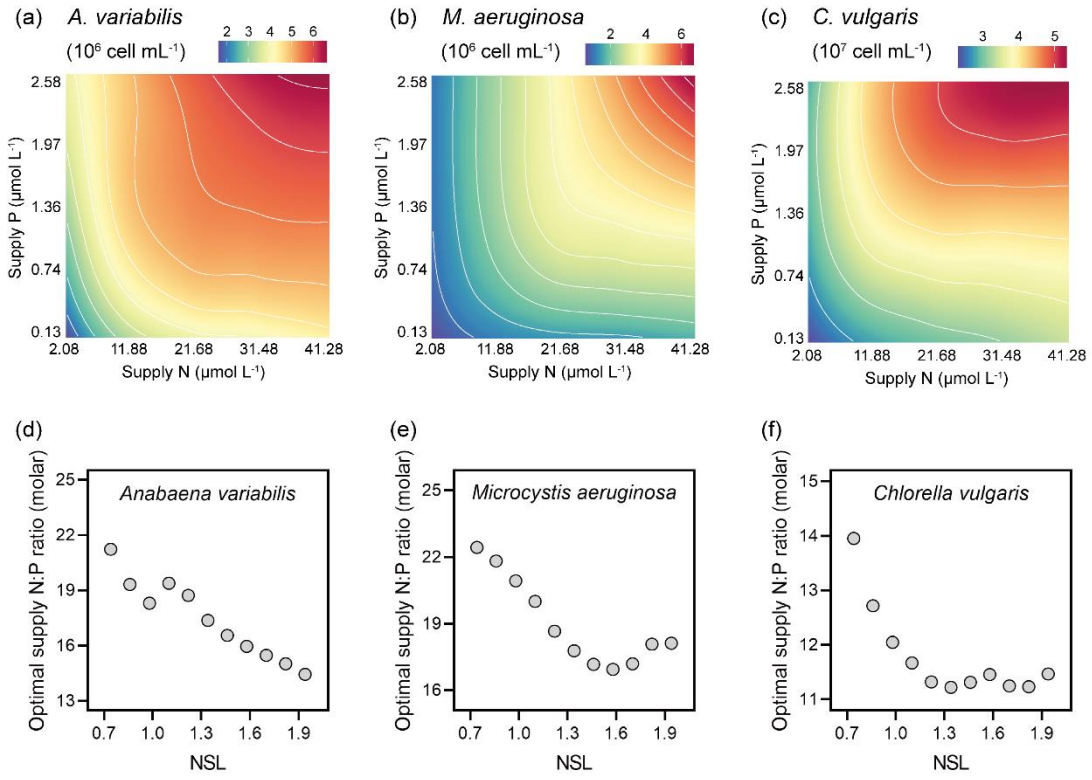

Figure 3 2D-LOESS response surface contours of the effects of nitrogen (N) and phosphorus (P) on the steady-state phytoplankton biomass of (a) *C. vulgaris*, (b) *A. variabilis*, and (c) *M. aeruginosa*. (d–f) Summary of the relationships between the optimal supply N:P ratios and the NSLs for the three phytoplankton species. Results were derived from the 2D-LOESS response surface contours.

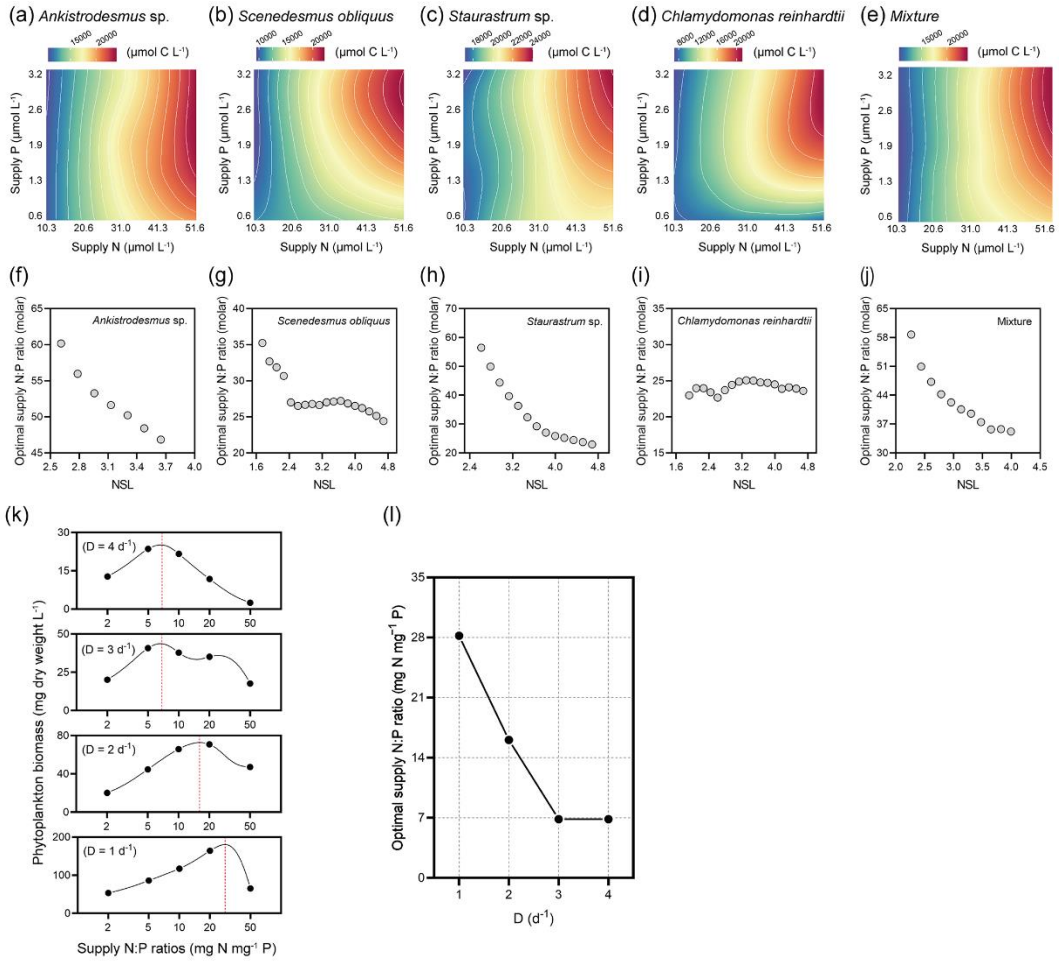

Figure 4 Literature data review. 2D-LOESS response surface contours were applied to the data of Frank *et al.* (2020) to assess the effects of N and P on the steady-state phytoplankton biomass of (a) *Ankistrodesmus* sp., (b) *Scenedesmus obliquus*, (c) *Staurastrum* sp., (d) *Chlamydomonas reinhardtii*, and (e) mixed cultures. Relationships between the optimal supply N:P ratios and NSLs were determined for (f) *Ankistrodesmus* sp., (g) *S. obliquus*, (h) *Staurastrum* sp., (i) *C. reinhardtii*, and (j) mixed cultures, which were derived from the corresponding 2D-LOESS response surface contours (a–e). With the data of Kunikane *et al*. (1984), we examined the (k) response curves of the steady-state biomass of *Scenedesmus dimorphus* to the supply N:P ratios under four dilution rates (D, d^{-1}), and (l) the change in the optimal supply N:P ratios at different levels of D.

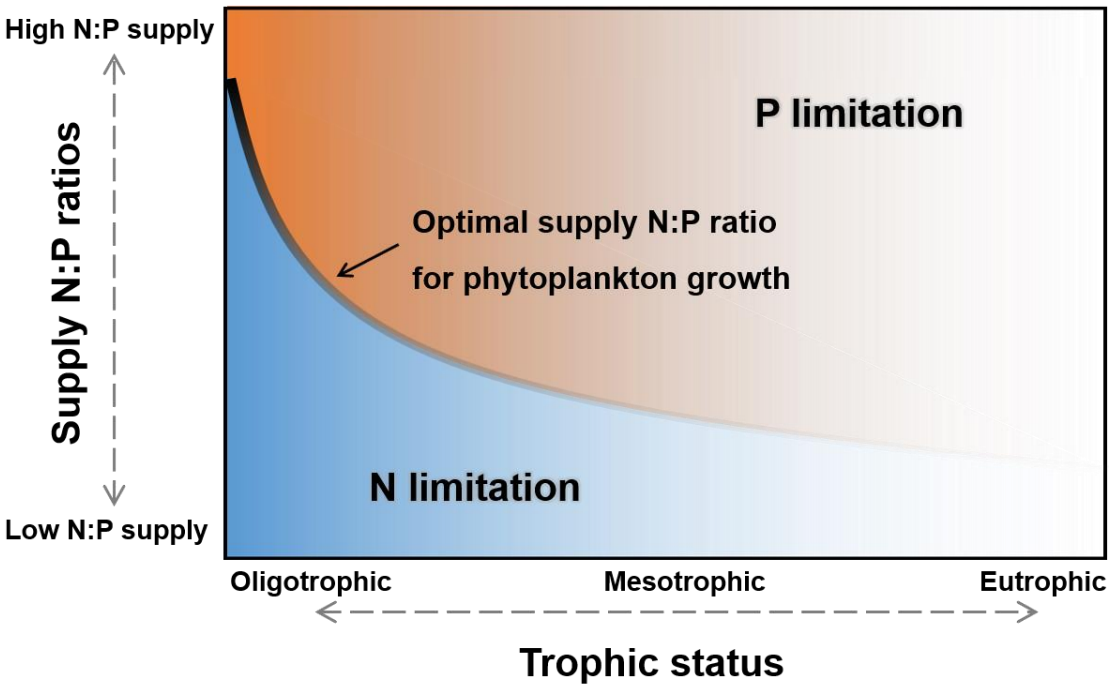

Figure 5 Schematic of the effects of trophic status on the relative N:P limitations for phytoplankton growth. The threshold between N limitation (blue area) and P limitation (orange area) is determined by the curve of the optimal supply N:P ratio. The curve of the optimal supply N:P ratio decreases with the rise of the trophic status. The decreasing curve is steep during low trophic phases and becomes shallow at high trophic phases. Additionally, the threshold between the N and P limitations for phytoplankton may become indistinct at high trophic phases due to the decreased affinity for nutrients.