Nutrient Absorption and Biomass Production by the Marine Diatom *Chaetoceros Muelleri*: Effects of Temperature, Salinity, Photoperiod, and Light Intensity

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ABSTRACT

The marine diatom *Chaetoceros muelleri* is commonly used for aquacultural feed and is well known for its fast growth and easy maintenance. In order to evaluate the potential of *C. muelleri* to be used for the nutrient removal and biomass production from eutrophic saline wastewaters, the algae were cultured under a wide range of temperature, salinity, photoperiod, and light intensity. The optimum temperature for the biomass production was observed at 30°C, but the algae could maintain at least 66% of the highest production between 20°C and 35°C. The optimum salinity for the biomass production was 25, but the algae could maintain at least 22% of the highest production between 10 and 30. Both light intensity and photoperiod affected the algal biomass production, and the minimum light requirement was considered 100 μmol m^{-2}·s^{-1} for 6 hours to maintain the biomass production and nitrogen (N) and phosphorus (P) absorption. Throughout all the experiments, the N and P absorption increased with the biomass production, but the ratio of N and P to the biomass exponentially decreased with the biomass production. These results showed that *C. muelleri* is tolerant to the wide range of environmental conditions, absorbing nutrients and producing organic matter. *C. muelleri* has a great potential to be introduced in the water treatment processes, especially where the temperature and salinity fluctuate.

Keywords: microalgae, bioremediation, wastewater, eutrophication, estuary, brackish water, aquaculture

INTRODUCTION

The effects of human activities on aquatic environmental pollution have been a growing concern due to the disposal of wastewaters from various sources [Ummalyma et al. 2018]. In order to minimize the risk of aquatic ecosystem deterioration, wastewater treatments are the essential process before the water is discharged to the natural environment. Because conventional physical and chemical water treatments come with the problems of expensive cost and high energy demand [Mccarty et al. 2011], the bioremediation technologies have been developed as an alternative, eco-friendly approach. In order to efficiently remove nutrients, organic pollutants, and heavy metals from wastewaters, the potential of various aquatic organisms (e.g., fungi, bacteria,

microalgae, macroalgae) for bioremediation has been evaluated [Kues 2015; Mondal et al. 2019; Zhi et al. 2019; Tanaka et al. 2020]. Compared with bacteria or fungi, microalgae and macroalgae have the absolute advantage of quickly absorbing inorganic nutrients from water, producing the algal biomass from photosynthesis. The produced algae can be easily collected and separated from the water because of its larger size than bacteria or fungi.

Among all the known sources of biomass energy, microalgae show the highest potential as a feedstock for the production of biofuel because they have higher biomass production than macroalgae or vascular plants, accumulating up to 20–50% lipid content [Chisti 2007; Driver et al. 2014; Klein-Marcuschamer et al. 2013; Rai et al. 2015]. The production of biofuel from microalgae

has been studied well, but because of the continuous requirement of nutrient and CO_2 supplies, the microalgae-derived biofuel production is still too expensive to industrialize at present [Pate et al. 2011; Yang et al. 2011; Hong et al. 2013]. Therefore, cultivating microalgae for obtaining not a single but multiple benefits simultaneously is receiving growing attention [Mandal, Mallick 2010; Guo et al. 2013; Caporgno et al. 2015; Aslam et al. 2020]. One of the most hopeful combinations is the production of the algal biomass for biofuel and the removal of nutrients from wastewater [Ansari et al. 2017; Abinandan et al. 2018]. However, most of previous studies evaluated the freshwater microalgae such as *Chlorella* [Li et al. 2011; Yang et al. 2011; Ansari et al. 2017; Abinandan et al. 2018], but not focused on the potential of salt-tolerant microalgae such as marine diatoms, though many types of wastewater or eutrophic environmental water contain salts [Gengmao et al. 2010; Datta et al. 2019].

Chaetoceros muelleri is a marine diatom that is ubiquitously distributed worldwide. Because of its fast growth and easy maintenance, *C. muelleri* has been used in aquaculture as feed for zooplankton and shrimp larvae at commercial hatcheries in many regions [Wang et al. 2014; Malibari et al. 2018], making this species as one of the most important microalgae in the aquacultural industry [Lopez-Elias et al. 2005; Wang et al. 2014; Rahmadi et al. 2020]. Another reason that *C. muelleri* has been frequently used in aquaculture is its adaptation to salinity change [Fujii et al. 1995; Barros et al. 2014; Rahmadi et al. 2020], which is likely to often occur in the land-based aquacultural ponds. Though the growth of *Chaetoceros* spp. has been studied under various conditions from ecological and aquacultural aspects [Harrison et al. 1990; Leonardos and Geider 2004; Magnotti et al. 2016], the potential of *C. muelleri* to be used for bioremediation and biomass production has been scarcely studied [Rahmadi et al. 2020].

The present study aimed to evaluate the potential of *C. muelleri* to be used for the bioremediation (nutrient removal) and biomass production from saline and brackish wastewaters. From the aspects of the double benefits, the algae were cultured to study the effects of fundamental environmental factors (salinity, temperature, photoperiods, and light intensity) under laboratory conditions. This study would be the first that tried to apply *C. muelleri*, which has been recognized well in aquaculture, to the saline and brackish wastewater treatment for wider applications.

MATERIALS AND METHODS

The marine diatom, *Chaetoceros muelleri*, was supplied by the Broodstock Development Centre (Department of Fisheries, Ministry of Primary Resources and Tourism) in Brunei Darussalam in December 2019. The diatom had been kept in the center for many months as feed used for the shrimp aquaculture. Natural seawater, which had a salinity of 30, was collected from the coastal sea of Brunei Darussalam and was sterilized before use. The diatom was inoculated in sterilized natural seawater supplemented with the f/2 medium [Guillard, Ryther 1962] and was maintained at a room temperature (approximately 22−24°C) under fluorescent lights (the light intensity: 100 μmol m^{-2}·s^{-1}) with the light:dark (L:D) cycles of 12:12 hours in the laboratory of Universiti Brunei Darussalam. This algal stock solution was renewed every week for the following experiments by transferring a portion of the algal solution to a new seawater medium.

Temperature

The sterilized natural seawater (salinity 30) was enriched with the f/2 medium, and ammonium chloride (NH_4Cl) and sodium bicarbonate ($NaHCO_3$) were also added to elevate its concentration by 100 μmol·L^{-1} and 4.8 mmol·L^{-1}, respectively, to promote the growth of the algae. The seawater medium (225 mL) and the algal stock solution (25 mL) were added to each culture bottle (total volume 250 mL), and 20 culture bottles were prepared in total. These 20 bottles were randomly allocated to five water baths (n = 4 for each treatment bath), where the temperature was controlled at 20, 25, 30, 35, and 40°C, using submersible heaters. The cultures were subjected to the light intensity of 100 μmol m^{-2}·s^{-1} using white LED lights and 12:12 hours of L:D cycles for 7 days. For all of the experiments in the present study, the underwater aeration was not provided, and all bottles were shaken manually at least twice per day to keep the algae suspended in the medium.

Salinity

After the optimum temperature range was determined in the above-mentioned experiment, the next set of 20 bottles were prepared similarly to test the effects of salinity: sterilized natural seawater (salinity 30) was mixed with distilled water to prepare culture media with five different salinities (10, 15, 20, 25 and 30, n = 4 for each treatment). The culture medium (225 mL) and the algal stock solution (25 mL) were added to each culture bottle (total volume 250 mL). The cultures were maintained at the most optimum temperature, using submersible heaters, under the light intensity of 100 µmol m^{-2}·s^{-1} using white LED lights and 12:12 hours of L:D cycles for 7 days.

Photoperiod

After an optimum salinity was determined in the above-mentioned experiment, new culture media with the most optimum salinity were prepared, and 12 mL of the algal solution and 238 mL of the sterilized seawater were mixed to have a total volume of 250 mL for each bottle. Twenty bottles were prepared in total and were placed under four different L:D cycles (24:00, 18:06, 12:12 and 06:18, n = 5 per treatment) with the light intensity of 100 µmol m^{-2}·s^{-1} for 7 days using white LED lights. Photoperiods were managed automatically using a timer switch. The cultures were maintained at 25°C for 7 days in the same way as above.

Light intensity

New culture media with the most optimum salinity were prepared, and 8 mL of the algal solution and 242 mL of the sterilized seawater were mixed to have a total volume of 250 mL for each culture bottle. Twenty bottles were prepared in total, and they were placed under white LED lights, having four different light intensities (10, 50, 100 and 170 µmol m^{-2}·s^{-1}, n = 5 per treatment) for L:D cycles of 12:12 h. The light intensity was measured with a light meter (LI-COR LI-250) and was adjusted by changing the distance between the light and culture bottles. The cultures were maintained at 25°C for 7 days in the same way as above.

Sampling and analysis

After 7 days of cultivation, the cultured solutions were filtered through a combusted and pre-weighted glass fiber filter (Whatman GF/F, diameter 47 mm, pore size 0.7 µm). The filter paper was rinsed with distilled water to remove salts and placed on a sterile petri dish using clean forceps. Then, the filter papers were completely dried in an oven at 50°C for 6 hours or until a constant weight was achieved. The filters were cooled to a room temperature and the dry weight was measured using an analytical balance with a precision of 0.1 mg. The dry weight of the algal biomass was then calculated by subtracting the initial dry weight of the filter and was normalized to a unit volume of water (mg L^{-1}). The algal biomass was also measured for the initial algal stock solution. The production of the algal biomass during the culture experiment was calculated as follows:

$$\text{Biomass production} = \frac{DW_e - DW_i}{t}, \text{mg L}^{-1} \cdot \text{d}^{-1}$$

where: DW_i and DW_e are the dry algal biomass (mg L^{-1}) at the start (day 0) and end (day 7) of the culture experiment, respectively, and t is the duration of the culture (7 days).

Because the algal biomass was measured only for the stock solution at day 0, the initial algal biomass (DW_i) in the culture bottles was calculated from the dilution ratio of the algal solution.

In order to determine the nitrogen (N) and phosphorus (P) content in the microalgae, two small disks were cut out from each sample filter using a hole punch for papers. Each disk contained 2% of the whole biomass on the filter. Each disk was then placed into a 10 mL glass tube with 10 mL of the oxidizing reagent (1% $K_2S_2O_8$), and the solution was autoclaved at 121°C for 1 hour to oxidize all N and P into nitrate and phosphate, respectively [Raimbault et al. 1999; Miyajima et al. 2005]. As standards, the solutions of potassium nitrate (KNO_3) and potassium dihydrogen phosphate (KH_2PO_4) were also prepared and processed at the same time. After the solutions were cooled to a room temperature, 5 mL were used to measure the nitrate concentration from the absorbance at 220 nm [Collos et al. 1999], using a spectrophotometer (Shimadzu UV-1800). The

phosphate concentration was determined using the molybdate and ascorbic solutions, according to Hansen and Koroleff [1999]. After determining the amount of N and P in each disk, total N and P content in the whole filter were calculated and then, the percent ratio of the N and P content (mg) to the total algal dry weight (mg) was calculated (N% and P%, respectively). The N and P absorption rates were calculated by multiplying the biomass production by N% and P%, respectively.

Statistical analyses

The relationships between the treatment factors (temperature, salinity, photoperiod, and light intensity) and the algal parameters (biomass production, N%, P%, N:P, N and P absorption) were tested by the regression analyses. The linear, quadratic, hyperbolic, and exponential regression analyses were performed for each parameter using SigmaPlot 12.5 (Systat Software, San Jose, CA). Only for the salinity treatment, the Loretzian regression was applied to the biomass production, N%, and P%, because other regressions did not fit well, obviously. The regression analysis that resulted in the lowest root mean squared error (RMSE) and that was statistically significant ($p < 0.05$) is shown in the results and figures.

RESULTS AND DISCUSSION

From the experiment on temperature, the algal biomass production was highest at approximately 30°C and lower at higher or lower temperatures (Fig. 1a). The maximum biomass production was estimated to be 31 mg $L^{-1} \cdot d^{-1}$ at 28°C from the quadratic regression (Table 1). Though the algal production drastically declined to 10 mg $L^{-1} \cdot d^{-1}$ at 40°C, it could maintain at least 66% of the maximum production between 20°C and 35°C, which shows a strong tolerance of the algae to the wide range of temperature. Most tropical microalgae decline their growth above 30°C or 33°C, but only *Chaetoceros* sp. was reported to be tolerant to a higher temperature (33−35°C) [Renaud et al. 2002], which is consistent with the present observation. Even at 40°C, the present algae were able to steadily increase the biomass with time, which was estimated from the time-course change of the optical density of the cultured water at 750 nm (data not shown).

The algal N% and P% showed a contrasting pattern to the biomass production (Fig. 1b, c). The reduction of N% and P% with the increasing biomass production would be attributed to the relative increase in the storage compounds such as carbohydrates in the algae because of the enhanced photosynthetic C fixation under optimum temperature conditions. Similar contrasting patterns were observed for *Chaetoceros* sp. [Renaud 2002], *Chaetoceros calcitrans* [Raghavan et al. 2008], and *Spirulina maxima* and *Spirulina platensis* [Oliveira et al. 1999]. On the other hand, the N:P ratio and the N and P absorption were not affected by the temperatures, and the mean values were 6.4, 0.40 mg $L^{-1} \cdot d^{-1}$, and 0.14 mg $L^{-1} \cdot d^{-1}$, respectively (Fig. 1d-f). These results showed that the algae were able to keep synthesizing the functional compounds (e.g., proteins and phospholipids) under the studied range of temperature.

From the experiment on salinity, the algal biomass production was highest at salinity 25 and lowest at salinity 10 (Fig. 1g). Using the regression model with a peak, the maximum biomass production was estimated to be 39 mg $L^{-1} \cdot d^{-1}$ at salinity 27, while the lowest production was 8.5 mg $L^{-1} \cdot d^{-1}$ at salinity 10 (Table 1). The range of optimum salinity observed in the present study (25−30) was consistent with the previous reports on the same algal species [Barros et al. 2014; Rahmadi et al. 2020]. Microalgae expend energy to maintain the turgor pressure in their cells at salinity below and above the optimum, which results in a reduction in photosynthesis, biomass productivity, or cell division [Salama et al. 2014]. However, it should be noted that though the growth declined, the algae could maintain 36%, 25%, and 22% of the maximum biomass production at salinity 20, 15, and 10, respectively (Fig. 1g). The growth reduction was almost negligible at salinity 15, compared to 25 in a recent study using a continuous photobioreactor [Rahmadi et al. 2020]. This tolerance to the wide range of salinity would be advantageous for bioremediation or nutrient recovery from the saline or brackish wastewater, which has been scarcely studied so far [Gengmao et al. 2010].

Along with the experiment on temperature, the algal N% and P% showed a contrasting pattern to the biomass production in the experiment on salinity (Fig. 1h, i). However, unlike the temperature experiment, the algal N:P ratio
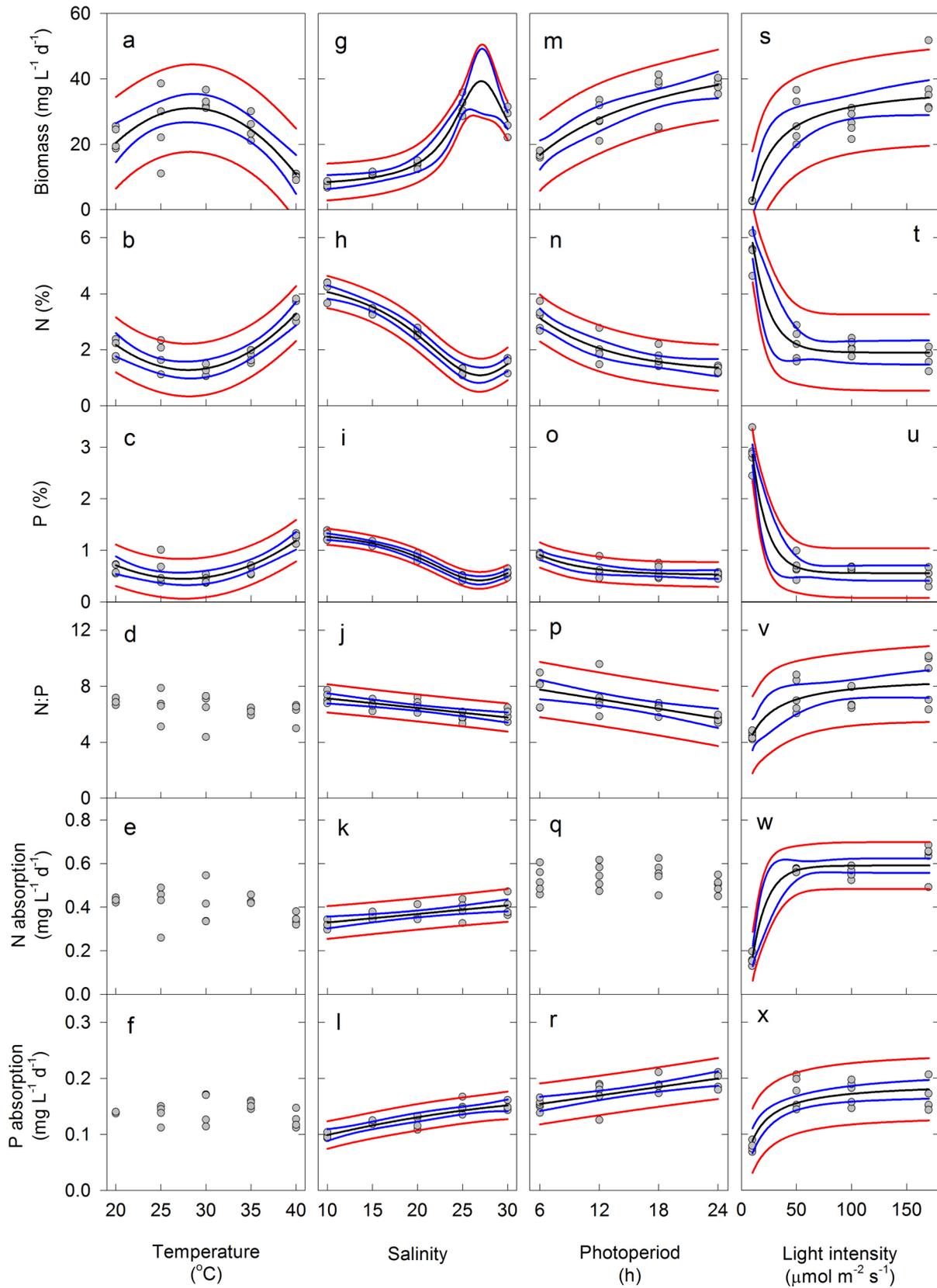
Fig. 1. The effects of temperature (a-f), salinity (g-l), photoperiod (m-r), and light intensity (s-x) on the biomass production, nutrient content (N%, P%, N:P), and nutrient absorption by *Chaetoceros muelleri*. Black, blue, and red lines indicate the regression line, 95% confidence bands, and 95% prediction bands, respectively. The formulae of the regression lines are listed in Table 1.
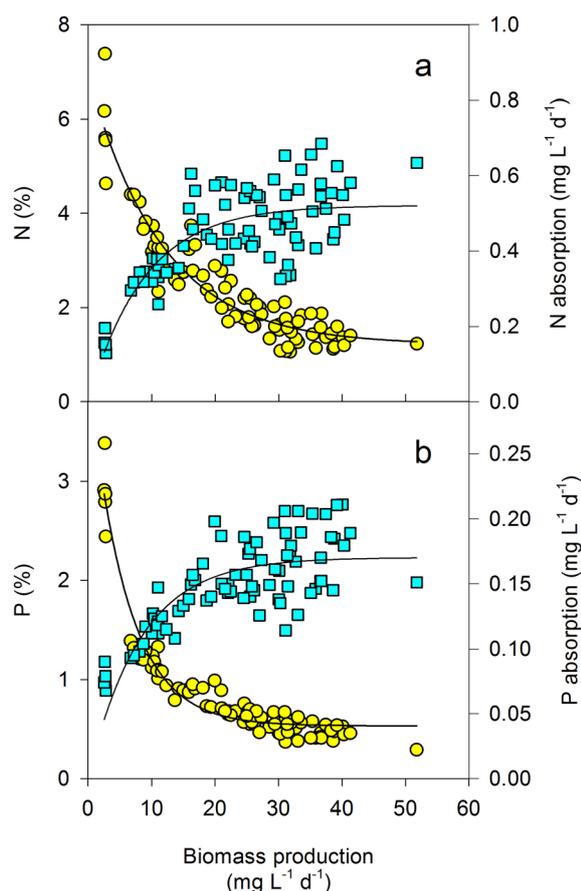
Fig. 2. The relationship (a) between the biomass production and N content (circle) and absorption (square) as well as (b) between the biomass production and P content (circle) and absorption (square)

N (%): $y = 5.73e^{-0.0859x} + 1.21$ ($r^2 = 0.90$, $p < 0.0001$)

N absorption: $y = 0.521 (1 - e^{-0.114x})$ ($r^2 = 0.61$, $p < 0.0001$)

P (%): $y = 3.59e^{-0.168x} + 0.532$ ($r^2 = 0.95$, $p < 0.0001$)

P absorption: $y = 0.170 (1 - e^{-0.122x})$ ($r^2 = 0.60$, $p < 0.0001$)

slightly increased and the N and P absorption decreased along with salinity (Fig. 1j-l, Table 1). These results show that the biochemical composition of the algal cells was altered by low salinity stress; for example, a relative decrease in phospholipids to proteins with decreasing salinity [Renaud and Parry 1994; Bartley et al. 2013; Benavente-Valdés et al. 2016]. However, the lowest N and P absorption (salinity 10) still maintained 80% and 64% of the highest absorption (salinity 30), respectively, which suggests that the capability of the algae to absorb N and P is more resistant to low salinity stress than its biomass production (Fig. 1g).

Different photoperiods and light intensities caused similar effects on the algal biomass production and nutrient content: the biomass production was described with a hyperbolic regression (Fig. 1m, s), and the N% and P% increased exponentially with decreasing light availability (Fig. 1n, o, t, u). Though the cycles of light and dark conditions, instead of continuous lighting, have a potential to mitigate photoinhibition [Krzeminska et al. 2014], photoinhibition was not observed in the present experiments. The low half-saturation constant for the light intensity (13 μmol m^{-2} s^{-1}) would be due to the acclimation of the algae to the laboratory conditions for many months before the experiment.

It should be noted that the algal N:P ratio increased under the short photoperiod of 6 h, but contrastingly decreased under the low light intensity of 10 μmol m^{-2}·s^{-1} (Fig. 1p, v). The decline of the N:P ratio at the extremely low light intensity indicates that the nutrient uptake, especially the N uptake (Fig. 1w), was almost stopped because of the lack of light-derived metabolic energy and consequently, only minimum proteins or enzymes were maintained in the algal cells [Harrison et al. 1990; Meseck et al. 2005]. On the other hand, the N uptake was maintained constant (mean for all photoperiods: 0.53 mg L^{-1}·d^{-1}; Fig. 1q) even under the shortest photoperiod, while the P absorption slightly decreased, which might have resulted in an increase in the proportion of proteins [Harrison et al. 1990; Leonardos and Geider 2004] as seen in the increasing N:P ratio with decreasing photoperiods (Fig. 1p-r). Thus, the irradiation of 100 μmol m^{-2}·s^{-1} for 6 h would have been sufficient for the algae to maintain the N uptake and metabolism, though photosynthetic C fixation and P absorption declined (Fig. 1m, q, r).

Throughout all of the present experiments, the N% and P% exponentially declined with the biomass production, while the N and P absorption exponentially rose to the maximum rate with the increasing biomass production (Fig. 2). These relationships indicate that the nutrient uptake might be a limiting step for the biomass production, causing a reduction of the N and P content in the algae. Though the culture waters were not continuously shaken in the present laboratory experiments, continuous movement of culture waters, such as using aeration in the bottle, might enhance the algal N and P uptake by thinning a nutrient diffusion layer around the algal cell [Sobczuk et al. 2006]. However, this mass transfer limitation becomes less important than kinetic control

Table 1. Regression analyses of the relationship between the treatments (x: temperature, salinity, photoperiod, and light intensity) and algal parameters (y: biomass production, N%, P%, N:P, and N and P absorption) for *Chaetoceros muelleri*.

Parameters	Regression	p	r^2
Temperature			
Biomass production	$y = -0.151x^2 + 8.55 - 90.3$	0.0002	0.63
N%	$y = 0.014x^2 - 0.786x + 12.3$	<0.0001	0.77
P%	$y = 0.005x^2 - 0.259x + 4.01$	<0.0001	0.73
N:P	Not significant	-	-
N absorption	Not significant	-	-
P absorption	Not significant	-	-
Salinity			
Biomass production	$y = 32.4/(1 + ((x - 27.1)/3.77)^2) + 6.98$	<0.0001	0.95
N%	$y = -3.79/(1 + ((x - 26.9)/8.88)^2)$	<0.0001	0.96
P%	$y = -1.03/(1 + ((x - 26.9)/7.81)^2)$	<0.0001	0.96
N:P	$y = -0.068x + 7.81$	0.0001	0.56
N absorption	$y = 0.004x + 0.290$	0.0016	0.44
P absorption	$y = -5.29 \times 10^{-5}x^2 + 0.005x + 0.056$	<0.0001	0.79
Photoperiod			
Biomass production	$y = 65.2x/(9.43 + x) - 8.60$	<0.0001	0.78
N %	$y = 4.38e^{-0.136x} + 1.19$	<0.0001	0.81
P %	$y = 1.40e^{-0.214x} + 0.525$	<0.0001	0.70
N:P	$y = -0.114x + 8.45$	0.0011	0.46
N absorption	Not significant	-	-
P absorption	$y = 0.003x + 0.139$	0.0002	0.55
Light intensity			
Biomass production	$y = 63.0x/(13.4 + x) - 24.2$	<0.0001	0.81
N%	$y = 7.58e^{-0.065x} + 1.90$	<0.0001	0.90
P%	$y = 4.83e^{-0.073x} + 0.556$	<0.0001	0.96
N:P	$y = 6.77x/(16.9 + x) + 1.99$	0.0002	0.63
N absorption	$y = 0.904(1 - e^{-0.074x}) - 0.314$	<0.0001	0.94
P absorption	$y = 0.193x/(12.1 + x)$	<0.0001	0.72

under the nutrient-enriched conditions [Sanford and Crawford 2000]. Because the present study used a common f/2 medium, nutrient concentration was high enough and therefore, the N and P absorption in the present study might have been largely controlled by the kinetic reaction in the algal metabolism.

Overall, the present study showed a tolerance of *C. muelleri* to a wide range of temperature, salinity, photoperiod, and light intensity for the nutrient absorption and biomass production. This high tolerance to various environmental conditions would be useful in the water treatment processes, especially for estuaries, where these environmental conditions often fluctuate due to mixing with seawater and river water. For the wastewaters from land-based aquaculture or from sewage treatment plants, many species of seaweeds (e.g., *Gracilaria caudate*, *Gracilaria crassa*, *Ulva fasciata*, *Ulva lactuca*, *Ulva ohnoi*, and *Ulva reticulata*) have been tested for removing nutrients from the water, but the removal performance of the seaweeds was strongly affected by salinity, temperature, and nutrient concentration [Msuya and Neori 2002; Marinho-Soriano et al. 2009; Lawton et al. 2013; Sode et al. 2013]. For example, *G. caudate* decreased its growth by more than 80% when the salinity decreased to 24 [Marinho-Soriano et al. 2009], which is a much drastic reduction, compared to the response of *C. muelleri* observed in the present study. Microalgae have also been tested for removing nutrients from the wastewater of freshwater aquaculture [Ansari et al. 2017], but rarely of the seawater aquaculture [Magnotti et al. 2016]. It would be required to further evaluate the potential of *C. muelleri* to purify eutrophicated saline water, producing the organic biomass, by conducting larger-scale experiments in the future.

Acknowledgments

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