A novel growth method for diatom algae in aquaculture waste water for natural food development and nutrient removal

Xiao-li Li, Thomas Kiran Marella, Ling Tao, Liang Peng, Chao-feng Song, Li-li Dai, Archana Tiwari and Gu Li

ABSTRACT

Diatom algae are known to play an important role as primary producers in many diverse ecosystems, including artificial aquaculture ponds where they also aid in maintaining water quality by consuming excess nutrients. But factors influencing their growth are still poorly understood. In the present study the effect of micronutrients, N:P ratio and silica concentration on benthic diatom Synedra sp. grown in fish pond waste water was studied along with nutrient removal efficiency. We have studied nine different treatments, of which addition of micronutrient mixture Nualgi along with adjusted N:P to 6:1 resulted in highest cell density, followed by silicate enrichment, whereas only N:P adjustment and Nualgi addition had no significant effect on diatom growth. At the end of the growth experiment, the N removal efficiencies of treatment groups (50.23%–65.44%) were significantly higher ($P < 0.05$) than that of the control group (43.56%), whereas phosphate removal efficiency was significantly higher ($P < 0.05$) with Nualgi and N:P adjustment (53.37%–68.98%). The silicate consumption was significantly higher in the control group, at 63.87%, than in other experimental groups. These results will give us a new insight into important factors influencing beneficial algae growth and simultaneous nutrient removal from aquaculture waste water.

Key words | aquaculture, diatom (Synedra), N:P ratio, nutrient removal, Nualgi, silicate

INTRODUCTION

In recent years, with the increasing demand of aquatic products, aquaculture has been developing rapidly. In 2014, China’s freshwater aquaculture products reached up to 28.02 million ton, of which the pond aquaculture output reached 19.89 million ton (Bureau of Fisheries 2014). However, in the course of breeding, along with the feeding of artificial bait and drug use, the farming environment has deteriorated, which has led to a phytoplankton community dominated by non-beneficial blue-green algae species. Due to this ecological imbalance there was an increased risk of diseases, resulting in reduced production quality. Therefore, there is an urgent need to explore environmentally friendly and sustainable initiatives to improve cultured species production and water quality.

Microalgae are an important part of the aquaculture water environment; they are necessary to maintain the normal function of the pond ecosystem and stabilize the pond environment. The community structure, population density of the algae and physical and chemical factors of water are closely related. At the same time, the species and quantity of microalgae can directly influence the change of physical and chemical factors of water (Johnston & Santillo 2002; Chuntapa et al. 2003). Beneficial microalgae like diatoms can promote the decomposition and transformation of nutrient salt in water, and reduce and eliminate the concentration of ammonia nitrogen, nitrite nitrogen, organic pollutants and other toxic substances in the process of their growth. They can also increase the dissolved oxygen by
photosynthesis and promote the oxidation and decomposition of organic matter in the aquaculture water. N and P fertilization can result in blue-green algae blooms (Boyd 1973) which can result in shallow stratification, resulting in depleted O₂ levels, and blue-green algae may also produce toxins and earthy smelling substances which can cause fish kills and off-flavor in fish (Safferman et al. 1967). The phytoplankton community in fish ponds is dominated by epipellic microalgae especially diatoms. Epipelon diversity studies in fish pond habitats was studied (Round 1955), but comparison of epipelic algae growth with nutrient and environmental factors is largely unexplored (Pouličková et al. 2008). So factors controlling growth of beneficial phytoplankton like diatoms need further studies to optimize their production in aquaculture ponds.

Diatom algae are considered as beneficial algae in aquaculture ponds due to their biochemical composition, as they do not contain cellulose but are rich in sterols, polyunsaturated fatty acids, calcium, magnesium, iron and other inorganic salts and a variety of vitamins, and can be well digested by aquatic animals. At the same time, they have a good effect on the purification of water and the ecological balance of the water body. Therefore, water bodies with diatom-dominated phytoplankton communities have been considered to be one of the best water environments in freshwater aquaculture.

Diatoms' growth is influenced by environmental and chemical factors, and the concentration of silicon is an important factor as diatoms require silicon to form their cell wall (Tréguer & Pondaven 2000). The concentration of silicon directly affects the density of diatoms in natural habitats but optimum Si concentration for diatom growth in freshwater habitats has been rarely reported. Along with silica, metal ions such as Fe, Co, Ni, and Bo play an important role in diatom growth (Martin & Fitzwater 1990). Nualgi is a patented (PCT/IN05/00195 US patent application no. 0070275856) nano-silica-based micronutrient mixture which has been used successfully in shrimp ponds in southern India to promote diatom blooms. But its effect on the growth of diatoms grown in aquaculture water has not reported.

Many studies have shown that nitrogen and phosphorus ratio affect the growth of diatom species. Compared with blue-green algae, diatoms need lower N:P ratio. A molar N:P < 15 can result in cyanobacterial blooms (Smith 1985). But in the aquaculture water, we found that the N:P usually cannot meet the needs for optimum diatom growth (Li et al. 2012, 2015). So in this study we have studied the effect of N:P, which is known to promote diatom growth according to the Redfield ratio (Redfield 1958).

Synedra sp. is one of the common diatom species in freshwater bodies (Reynolds 2006) and it is known to be a dominant species in freshwater aquaculture ponds. So we have used this diatom species as a model organism to study its growth and nutrient removal potential. Graham et al. (2012) studied the effect of silica concentration on Synedra sp. growth using artificial waste water but factors like N:P and micronutrients affecting its growth in aquaculture waste water and simultaneous nutrient removal were not studied.

Accordingly, this study examined the effects of the micronutrient enrichment using Nualgi, reduction of N:P ratio, and Si concentration on growth of a common benthic diatom isolate, Synedra sp. This work provides basic data for optimized nutrient concentrations that influence diatom growth in freshwater aquaculture ponds and their effect on nutrient removal. This information will be useful in creating optimum conditions for diatom growth in freshwater aquaculture ponds as live feed and for water quality maintenance.

MATERIAL AND METHODS

Microalgae and culture conditions

The diatom Synedra sp. FACHB-1712 was obtained from Freshwater Algae Culture Collection of the Institute of Hydrobiology, Wuhan, China. Culture conditions were maintained at 25 ± 1 °C under 12 h dark/12 h light cycles with a light intensity of 100 μmol m⁻² s⁻¹ using a light incubator (Shanghai Miao GZX-150BS-III, Shanghai, China). Cultures in the exponential phase were centrifuged at 3,500 g for 5 min, and the cell pellet was re-suspended in medium and used as inoculum in all experiments. Growth experiments were carried out for 9 days as Synedra sp. reached stationary phase by that time. Cultures were kept in 1,500 mL Erlenmeyer flasks containing 1,000 mL medium and were manually shaken thrice daily during growth experiments. Growth rate was assessed by cell counting done on days 1, 3, 6 and 9 using a BX-53 biological microscope (Olympus Co., Ltd, Shanghai, China) with a haemocytometer with 1 mm depth. Relative growth rates of diatoms were calculated according to the formula given by Furnas (1990).

Experimental water characterization

The raw water samples for this study were collected from the freshwater aquaculture pond at Ecological Engineering Technology Research Center of Chinese Academy of Fishery
with Nualgi micronutrients, adjustment of N:P ratio to 6:1, and Si enrichment.

### Statistical analysis

The growth rates used for statistical analyses were the means ± standard deviation (SD). All statistical analyses were carried out using SPSS version 21.0 software (SPSS Inc., Chicago, IL, USA). A one-way analysis of variance was used to compare the significance levels of variations in mean between groups. Statistical values of $P < 0.05$ were considered significant, rejecting the null hypothesis between groups.

### RESULTS

#### Growth studies

The effect of nine different treatments on growth of *Synedra* sp. is shown in Figure 1. The growth curve of *Synedra* sp. followed a general pattern, where algal cells attained exponential phase from day 1 without any lag phase. Maximum growth rate was observed on day 1 in all the treatments. A maximum cell density of $58.18 \times 10^4$ cells mL$^{-1}$ was attained in treatment VII in which cultures were enriched with Nualgi and N:P was adjusted. In treatments II and III a maximum cell density of $17.38 \times 10^4$ cells mL$^{-1}$ and $35.06 \times 10^4$ cells mL$^{-1}$ was reached on day 6, respectively. Notably, maximum cell density reached in each group was different.

On day 6, cell density of $17.38 \times 10^4$ cells mL$^{-1}$ was attained in treatment II, which was enriched with Nualgi. In treatments IV–VI phosphorus was added to improve the N:P ratio to 6:1. It can be seen that the cell growth trend was completely consistent in control (I) and treatments IV–VI with a gradual increase of cell density with culture time and reached stationary phase on day 9. Compared with the control group, the cell density of group IV was significantly higher after 3 days of culturing, achieving a maximum cell density of $31.26 \times 10^4$ cells mL$^{-1}$ on day 9. Addition of silicate along with the Nualgi and N:P adjustment triggered better growth in *Synedra* sp., especially in the early stages of growth (i.e. day 3). The cell growth density of groups

### Experimental setup

Nualgi micronutrients were purchased from Nualgi Nanobitech, Karnataka, India. Analytical reagents KPO$_4$·H$_2$·3H$_2$O and Na$_2$SiO$_3$·9H$_2$O were purchased from Shanghai Reagent Factory and were made into a concentration of 10 mg L$^{-1}$ and 2 g L$^{-1}$ solution respectively with double distilled water before their addition to the medium. A mono-species culture of diatom *Synedra* sp. was used in this study, instead of mixed species of naturally occurring diatoms from the pond water, for growth studies, as assessing the growth of mixed species is time consuming and different species have different nutrient requirements, so it can lead to irregular growth patterns.

Test Erlenmeyer flasks with pond water were divided into nine treatments and enriched with different nutrients as mentioned in Table 2. All treatments were set up in triplicate. Different treatments were comprised of enrichment

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Total N</th>
<th>NH$_4^+$-N</th>
<th>NO$_3^-$-N</th>
<th>Total P</th>
<th>PO$_4^{3-}$-P</th>
<th>SiO$_2^{3-}$-Si</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration (mg/L)</td>
<td>8.67 ± 9.52</td>
<td>1.35 ± 0.01</td>
<td>0.48 ± 0.02</td>
<td>0.33 ± 0.00</td>
<td>0.03 ± 0.00</td>
<td>6.90 ± 0.04</td>
</tr>
</tbody>
</table>

### Table 1 | Initial nutrient concentration levels of aquaculture pond water used for *Synedra* sp. growth and nutrient removal studies (mean ± SD)

The growth rates used for statistical analyses were the means ± standard deviation (SD). All statistical analyses were carried out using SPSS version 21.0 software (SPSS Inc., Chicago, IL, USA). A one-way analysis of variance was used to compare the significance levels of variations in mean between groups. Statistical values of $P < 0.05$ were considered significant, rejecting the null hypothesis between groups.

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On day 6, cell density of $17.38 \times 10^4$ cells mL$^{-1}$ was attained in treatment II, which was enriched with Nualgi. In treatments IV–VI phosphorus was added to improve the N:P ratio to 6:1. It can be seen that the cell growth trend was completely consistent in control (I) and treatments IV–VI with a gradual increase of cell density with culture time and reached stationary phase on day 9. Compared with the control group, the cell density of group IV was significantly higher after 3 days of culturing, achieving a maximum cell density of $31.26 \times 10^4$ cells mL$^{-1}$ on day 9. Addition of silicate along with the Nualgi and N:P adjustment triggered better growth in *Synedra* sp., especially in the early stages of growth (i.e. day 3). The cell growth density of groups
Table 3 | Specific growth rate ($\mu$) of Synedra sp. during growth period under different treatments (mean ± SD). Letters in bold indicate the maximum growth rate achieved in each treatment. See Table 2 for other information.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Cultivation time (Days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>I</td>
<td>0.06 ± 0.00</td>
</tr>
<tr>
<td>II</td>
<td>0.14 ± 0.25</td>
</tr>
<tr>
<td>III</td>
<td>0.47 ± 0.08</td>
</tr>
<tr>
<td>IV</td>
<td>0.37 ± 0.10</td>
</tr>
<tr>
<td>V</td>
<td>-0.07 ± 0.32</td>
</tr>
<tr>
<td>VI</td>
<td>0.40 ± 0.04</td>
</tr>
<tr>
<td>VII</td>
<td>0.63 ± 0.01</td>
</tr>
<tr>
<td>VIII</td>
<td>0.58 ± 0.05</td>
</tr>
<tr>
<td>IX</td>
<td>0.51 ± 0.18</td>
</tr>
</tbody>
</table>

**Figure 1** | Growth curve of Synedra sp. under nine different treatments, I: Pond water (PW) + Synedra, II: PW + Nualgi, III: PW + Nualgi + Synedra, IV: PW + N:P + Synedra, V: PW + N:P + Nualgi, VI: PW + N:P + Nualgi + Synedra, VII: PW + N:P + Nualgi + Synedra + Si (20 mg L$^{-1}$), VIII: PW + N:P + Nualgi + Synedra + Si (30 mg L$^{-1}$), IX: PW + N:P + Nualgi + Synedra + Si (40 mg L$^{-1}$). Error bars represent SD. 

VII–IX was 15.97–22.81 × 10$^4$ cells mL$^{-1}$, which was significantly higher than that of group VI and the control group ($P < 0.05$). However, by the end of the experiment, the higher cell density caused by the addition of silicate had stagnated, and remained at 38.68–42.75 × 10$^4$ cells mL$^{-1}$, which was significantly lower than that of group VI with 58.18 × 10$^4$ cells mL$^{-1}$ ($P < 0.05$). Growth was assessed by also studying specific growth rate ($\mu$) (Table 3). Maximum specific growth rate was observed during initial days of culturing from day 1 to day 3 in all the treatments. Cultures with micro-nutrient, N:P adjustment and Si enrichment showed higher $\mu$, in the range 0.40–0.63 $\mu$. Highest specific growth rate of 0.65 $\mu$ was achieved in treatment group VI, which was significantly higher than control group. Of the nine different treatments studied, the significant factors affecting Synedra sp. growth were, in order of priority, Si + Nualgi + N:P > Nualgi + N:P > Nualgi > N:P > Si.

**Nutrient removal studies**

The N removal efficacy was studied by monitoring nitrate and ammonia concentration during the culture experiments under different treatments, the results are shown in Figure 2. The NO$_3^-$-N removal was maximum in treatment VI which was enriched with Nualgi and N:P adjustment closely followed by Si-enriched cultures (Figure 2(a)). The changes of NO$_3^-$-N in the experiment group were closely correlating with growth. The final NO$_3^-$-N concentration after treatment was in the range of 0.01–0.65 mg L$^{-1}$. The difference in the nitrate nitrogen removal efficiency was not significant ($P > 0.05$) in all groups tested at the end of the experiment; the removal efficiency was in the range of 66.88%–91.31%. Of the nine different treatments studied, the significant factors affecting NO$_3^-$-N removal were, in order of priority, Si + Nualgi + N:P > Si > Nualgi > N:P.

The ammonia removal efficiency is shown in Figure 2(b). Addition of Nualgi and Si enrichment showed higher NH$_4^+$ removal efficiency when compared with other treatments. The initial values of ammonia nitrogen in each treatment group were different, but the basic trend of change was the same with a sharp decrease during initial cell proliferation and then remaining stable after cell growth has stopped; the final ammonia concentration was in the range 0.65–2.15 mg L$^{-1}$. At the end of the experiment (day 9), the NH$_4^+$-N removal efficiency of treatment groups (50.23%–65.44%)
was significantly higher than those of the control group (43.56%) except for the N:P adjusted group (49.89%). Of the nine different treatments studied, the significant factors affecting NH₄⁺-N removal were, in order of priority, Si + Nualgi > N:P > Nualgi > N:P > Si.

The changes in phosphate observed in group II, which involved the addition of Nualgi, and group III, which involved the addition of both Nualgi and Synedra, were both initially lower than control but increased over time. The lowest values for groups II and III were observed on day 6 and day 3, respectively, and the values then gradually increased. At the end of treatment, final P concentration was in the range of 0.12–0.52 mg L⁻¹. Figure 3(a) shows phosphorus removal efficiency for the different treatments. The P removal efficiency was significantly correlated with growth rate of Synedra sp., with an increase in cell number being directly proportional to P removal efficiency. In the control group, there was no significant change in the phosphate content. After adding phosphate in treatment groups IV to IX for N:P adjustment there was an increase in P removal efficiency. Si addition has a slight negative impact on P removal percentage compared with Nualgi and N:P adjusted groups but there was no significant difference between treatment IV with phosphate addition and treatments V and VI with addition of both phosphate and Nualgi (P < 0.05).

Effect of diatom growth on Si removal efficiency is shown in Figure 3(b). In treatment groups with no Si addition (I–VI) there was a higher Si removal efficiency except in group II and V where there was no Si removal observed. The final Si concentration after treatments in groups where no Si was added was in the range of 2.49–6.90 mL L⁻¹ whereas in the rest of the groups it was slightly higher (6.06–9.80 mg L⁻¹). At the end of the experiment, the
silicate consumption of the control group was 63.87%, while that of the N:P group and the adding 40 mg L\(^{-1}\) silicate group was 48.83% and 49.64%, respectively, which was significantly higher than that of the other experimental groups (~0.57%–38.30%)\((P < 0.05)\).

DISCUSSION

There are many reports on the effects of nutrient concentration and proportion on the growth of planktonic diatoms, especially in the case of the N:P ratio (Werner 1977; Smith & Geider 1985). These reports suggested that nutrient concentrations and ratios have a significant impact on the metabolic activity and growth rate of microalgae. Schöllhorn & Granéli (1996) have shown that N:P of 6–7 is optimal for diatom growth. Additional studies by Su et al. (2001) have shown that freshwater planktonic diatom have an optimum nitrogen to phosphorus ratio of 7.3:1 for the fastest rate of growth. In this study, the nitrogen to phosphorus ratio was 6:1; the cell number of Synedra in the experimental group was significantly higher than that of the control group, suggesting that the effect of the N:P ratio has an influence on the growth of Synedra sp. However, in addition to adjusted N:P, addition of Nuagli has also shown positive effect on diatom growth. Justić et al. (1995) proposed a method for assessing the nutrient limitation standards: if Si:P > 22 and N:P > 22, there will be phosphate limitation; if N:P < 10 and Si:N > 1, dissolved inorganic nitrogen limitation results; and if Si:P < 10 and Si:N < 1, there will be dissolved inorganic silicon limitation. Sun et al. (2007) found that when water is limited by silicate, the enrichment of silicate can promote the growth of diatoms and the other algal species and change the dominance of a few cyanobacteria and chlorophyte species. Therefore, silicate can elevate the algal biodiversity of an aquatic ecosystem and weaken cyanobacterial blooms to a certain degree. In this study, the initial concentration of silicon and nitrogen are 2.73 mg L\(^{-1}\), 3.99 mg L\(^{-1}\) with an Si:N ratio equal to 1, and thus silicon is not limiting, and the addition of more silicate has shown no effect on diatom growth.

Nuagli could trigger diatom growth in open waters (Kiran et al. 2015), and this could be due to the relatively high quantities of iron in the formulation and the trace metals being in a readily available form on a nano-scale in the product (claimed by the manufacturers). Nuagli was found to significantly boost growth in Cylindrotheca sp. when used in lieu of a conventional micronutrient mix used in f/2 Si medium (Suman et al. 2012). In this experiment, compared with the control group, the addition of Nuagli had some influence on the growth of Synedra sp., but at the end of the test, the difference was not significant between the two groups. A similar growth trend was also shown in the cell density of group III, which involved addition of Nuagli. This implies that Nuagli had a certain role in promoting the growth of diatoms and nutrient removal. Similar studies conducted by Marella et al. (2016) in a eutrophic pond have shown that addition of Nuagli increased diatom density and nutrient removal. In the present study the positive influence of Nuagli on growth and nutrient removal can be attributed to the low nutrient ratio in water, as diatoms cannot continue to thrive without the proper availability of nutrients. This result showed that trace elements could affect the growth of diatoms on condition that macronutrients (such as nitrogen and phosphorus) are present in the required ratio and concentration.

Due to the growing demand for aquaculture products, there is an exponential increase in inland aquaculture which has led to increased risk of eutrophication in freshwater habitats due to untreated aquaculture effluents, leading to unwanted algal growth. Diatom algae are known to be beneficial in aquaculture ponds as they are known to contain good nutrient value for farmed species and can consume excess nutrients quickly due to their fast growth rate over varied environmental conditions. Therefore, growing diatoms, which are the most diverse microalgae group, has become more beneficial and advantageous. Specific needs for nutrients vary among species, even within the same environment, so studying these conditions is essential to determine species-specific requirements rather than using the same fertilization regime for all types of aquaculture ponds. In this study, we report for the first time the optimization of major nutrient concentrations and N:P for the growth of diatom and nutrient removal from aquaculture waste water using the common benthic diatom isolate Synedra sp. Future work on the use of freshwater diatoms for natural food development and simultaneous nutrient removal from aquaculture waste water can build upon this study of optimized conditions for diatom growth.

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