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Effect of the Pulsed Addition of Phosphorous on the Growth and Toxin Production of the Bloom-Forming Cyanobacterium *Chrysosporum ovalisporum* (Forti) Zapomelová et al.

Sicheng Yin [1,†], Ruohan Huang [1,†], Ni Wang [1], Changxing Li [1], Erik Jeppesen [2,3,4], Liqing Wang [1,5,*] and Wei Zhang [1,5,*]

[1] Centre for Research on Environmental Ecology and Fish Nutrient of the Ministry of Agriculture, Shanghai Ocean University, Shanghai 201306, China
[2] Department of Ecoscience, Aarhus University, C.F. Møllers Allé 4, 8000 Aarhus, Denmark
[3] Sino-Danish Centre for Education and Research (SDC), University of Chinese Academy of Sciences, Beijing 100190, China
[4] Limnology Laboratory, Department of Biological Sciences, Centre for Ecosystem Research and Implementation, Middle East Technical University, 06800 Ankara, Turkey
[5] Engineering Research Center of Environmental DNA and Ecological Water Health Assessment, Shanghai Ocean University, Shanghai 201306, China
* Correspondence: lqwang@shou.edu.cn (L.W.); weizhang@shou.edu.cn (W.Z.)
† These authors contributed equally to this work.

Abstract: Eutrophication and global warming boost the outbreak of cyanobacterial blooms and the spread of invasive cyanobacterial species such as *Chrysosporum ovalisporum*. Different from the often steady-state conditions under laboratory cultivation, the external input of nutrients in natural water is often intermittent and pulsed, which may affect the response of cyanobacteria. In this study, we cultured *C. ovalisporum* under two phosphorus treatment patterns: a one-time treatment and a five-time pulsed treatment with the same total dosing. Our results showed that phosphorus deficiency in the water inhibits the growth of *C. ovalisporum*, thereby significantly reducing its biomass and photosynthetic activity ($p < 0.05$). In addition, phosphorus deficiency led *C. ovalisporum* to secrete more exopolysaccharides and alkaline phosphatase. Compared with the one-time treatment, the multiple pulses promoted the absorption of phosphorus by *C. ovalisporum* and inhibited the synthesis of alkaline phosphatase but had no significant effect on the release of cylindrospermopsin (CYN). We also found that multiple pulses had a more significant growth-promoting effect on *C. ovalisporum* under low phosphorus concentrations. Our results indicated the overall strong adaptability of *C. ovalisporum* to dynamic changes in phosphate levels in the water column and provide new insight into the outbreak and dispersal strategies of *C. ovalisporum*.

Keywords: *Chrysosporum ovalisporum*; pulse input; alkaline phosphatase; Cylindrospermopsin

Citation: Yin, S.; Huang, R.; Wang, N.; Li, C.; Jeppesen, E.; Wang, L.; Zhang, W. Effect of the Pulsed Addition of Phosphorous on the Growth and Toxin Production of the Bloom-Forming Cyanobacterium *Chrysosporum ovalisporum* (Forti) Zapomelová et al. *Water* **2023**, *15*, 351. https://doi.org/10.3390/w15020351

Academic Editor: Armah A. de la Cruz

Received: 11 December 2022
Revised: 11 January 2023
Accepted: 12 January 2023
Published: 14 January 2023

1. Introduction

With the intensification of eutrophication, the frequency and intensity of cyanobacteria blooms in freshwater habitats have increased globally, and some invasive cyanobacteria species have spread to new aquatic systems [1]. Invasive cyanobacteria, such as *Cylindrospermopsis*, and *Chrysosporum*, employ flexible strategies to respond to environmental changes [2]. Yang et al. [3] found that the growth of *Raphidiopsis raciborskii* (Woloszynska) Aguilera et al. 2018 (formerly known as *Cylindrospermopsis raciborskii* (Woloszynska) Seenayya and Subba Raju 1972) was not vulnerable to transient nutrient fluctuations, whereas nitrogen fluctuations can significantly alter the synthesis and release of cylindrospermopsin (CYN). Another study showed that the synthesis and degradation of *R. raciborskii* cyanophycin are evolutionarily conserved to support the proliferation in N-fluctuating and/or deficient conditions [4]. These invasive cyanobacteria also have a high

affinity for phosphorus uptake and storage capacity. They can perform coordinated and complex cellular and physiological responses to environmental P fluctuations [5]. They can secrete exopolysaccharides (EPS) that rapidly adsorb phosphate from the water column followed by uptake into the cells [6]. They can also store inorganic phosphorus in their cells as polyphosphate in response to possible phosphorus-deficient conditions [7]. However, in studies of the relationship between nutrient levels and cyanobacteria blooms, nutrients are often added only once. The impact of more dynamic changes in nutrient levels on the invasion and bloom of cyanobacteria has not been clarified.

Some phytoplankton species can adjust their nutrient absorption under the variable frequency, intensity, and duration of pulsed nutrient input, thereby promoting their growth [8]. Amaral et al. [9] found that compared with a one-time pulse, the growth rate of *Cylindrospermopsis raciborskii* was 2–3 times higher with multiple phosphorus pulses. Yang et al. [3] revealed that although nitrogen pulsing had little effect on the growth of *C. raciborskii*, it significantly changed its toxigenic characteristics. Since the exogenous input of nutrients in natural waters has obvious pulse input characteristics, e.g., during extreme rain events, understanding the effects of the growth response of cyanobacteria to variable nutrients may help to predict and ultimately prevent the changes in cyanobacterial blooms.

Chrysosporum ovalisporum, formerly known as *Aphanizomenon ovalisporum*, is a filamentous bloom cyanobacterium with nitrogen fixation and cylindrospermopsin-producing abilities. *C. ovalisporum* is mainly distributed along the Mediterranean coast of Europe and the Queensland region of Australia [10,11]. In recent years, this species has invaded many areas and is considered an invasive species with strong dispersal ability [12]. Hadas et al. [13] studied the response of *C. ovalisporum* to various environmental factors in laboratory experiments to predict its bloom, and they found that the concentration of available phosphorus in the water was of key importance for its growth. Bar-Yosef et al. [14] recorded that *C. ovalisporum* could stimulate other phytoplankton to produce extracellular alkaline phosphatase (APA) through the production of CYN, and it could thereby maintain its growth in the case of reduced phosphorus levels in the water. Compared with other cyanobacteria, only a few studies have focused on the absorption of phosphorus by *C. ovalisporum* from water [13].

In this study, a pulsed phosphate input was used to simulate the fluctuation of phosphate levels in the water column and to study the effects of different phosphate levels and input patterns on the growth, phosphate storage, and toxin production of *C. ovalisporum*. We hypothesized that the pulsed input would promote the uptake of phosphate by *C. ovalisporum* and stimulate its growth and CYN production. Our study provides new insight into the outbreak and dispersal strategies of *C. ovalisporum*.

2. Materials and Methods

2.1. Strains and Culture Conditions

C. ovalisporum CFWA01007 was isolated from Lake Dishui, Shanghai, China. Pure cultures of *C. ovalisporum* were maintained in BG11 medium (pH 7.5) at 25 °C in a 12/12 h light/dark cycle with a constant white light intensity of 2500 Lx. The cultures were manually shaken twice daily during incubation until the exponential phase. We centrifuged *C. ovalisporum* at 2655× *g* for 8 min, then washed the cultures three times and resuspended them with sterile P-free BG11 medium, which removed K_2HPO_4 from the formula. The resuspended cultures were then further incubated for 7 days to create a P deficiency before the experiments.

2.2. Experimental Design

The experiments included two phosphate pulse treatments. One treatment was a one-time treatment: phosphate-dependent growth was tested at four initial levels (0.02, 0.05, 0.1, and 0.5 $mg \cdot L^{-1}$), expressed as 0.02—1, 0.05—1, 0.1—1, and 0.5—1. The other treatment method was a five-time treatment: an amount of phosphate was divided equally into five accretions, each applied every 30 min, expressed as 0.02—5, 0.05—5, 0.1—5,

and 0.5—5. The different nutrient treatments were obtained by adding the phosphate (KH_2PO_4) in a P-free BG11 medium. We set the frequency and time of phosphate pulses according to Aubriot and Bonilla [8], Amaral et al. [9], and Yang et al. [3]. Their studies showed that during a sequence of phosphate pulses applied to phytoplankton, it took about 20–30 min for the dynamic change and energy optimization of phosphate uptake to occur. The initial N concentration (1500 mg·L^{-1}, same as BG11 medium) in the medium was the same for all treatments. The experiments for the phosphate pulses with *C. ovalisporum* were conducted in 500 mL Erlenmeyer flasks with 400 mL of medium (the starting dry weight was 10 mg·L^{-1}).

2.3. Growth Measurement

A 3 mL sample was measured at 750 nm by a spectrophotometer and converted to biomass (dry weight) according to the standard curve. The standard curve was drawn following Wang et al. [15]. Dry weight was calculated using the equation:

$$y = 0.9041x - 0.007 \quad (1)$$

where y is the biomass and x is the OD_{750} for *C. ovalisporum*.

The specific growth rate (μ) was calculated using the equation:

$$\mu = (\mathrm{InC2} - \mathrm{InC1})/(t2 - t1) \quad (2)$$

where C is the biomass at time t for *C. ovalisporum.*

2.4. Photosynthetic Activity Parameters

A 3 mL algal cell suspension was taken to measure the maximum optical quantum yield (Fv/Fm), actual optical quantum yield (Yield), and chlorophyll-a with a pulse-amplitude-modulated fluorescence monitoring system (PAM, Walz, Effeltrich, Germany).

2.5. Intracellular Phosphate

The extraction of intracellular phosphate was performed according to Zhou et al. [6]. The filter- and thermal-based methods were used to extract the intracellular phosphate from *C. ovalisporum* cells. Firstly, a 5 mL sample was filtered through a cellulose acetate membrane (pore size = 0.45 um) to remove all medium. We then collected the cells from the membrane and resuspended them in their original volumes using distilled water. In order to destroy the cell member fragments completely, we heated the suspension in a boiling-water bath for 30 min (temperature about 100 °C). When the cell lysis had released its contents into the boiling-water bath, we filtered the sample again through a cellulose acetate membrane (pore size 0.45 μm) to remove cell member fragments. The dissolved total phosphorus (DTP) and soluble reactive phosphorus (SRP) contents in the filtrate were determined using the method of Xu et al. [16].

2.6. Exopolysaccharides

The determination of EPS included two parts, water-soluble polysaccharides (RPS) and extracellular gum sheath polysaccharides (CPS). We first centrifuged a 2 mL sample at 5204× *g* at 4 °C for 15 min and collected the supernatant, which contained the RPS. We collected the pellets and resuspended them in their original volumes using 0.05% sodium chloride solution. The suspensions were heated at 60 °C for 30 min and then centrifuged at 5204× *g* at 4 °C for 15 min; the collected supernatant contained the CPS. The determination of RPS and CPS was performed according to Wang et al. [17].

2.7. Alkaline Phosphatase

APA was determined using the colorimetric method using p-Nitrophenyl phosphate (pNPP, Sigma-Aldrich, Shanghai, China) as the substrate [18]. We first mixed 0.5 mL of algal cell suspension, 0.4 mL of 3.6 mM pNPP, 0.1 mL of 2.0 mM $MgCl_2$, and 2 mL of 0.2 M

Tris-HCl buffer solution in a centrifuge tube and heated the mixed samples at 37 °C for 2 h. We then added 0.3 mL of 4M NaOH and centrifuged the samples at 5204× *g* for 4 min. The supernatant was measured at 405 nm using a spectrophotometer. APA was expressed in micromoles of p-nitrophenol released per microgram of chlorophyll per hour at 37 °C, unit: μmol ρNP released μg Chl-a·h^{-1}. APA was calculated using the equation:

$$y = (0.1816x - 0.0142)/(2 \times C), \quad (3)$$

where y is the APA, x is the OD_{405} for the supernatant, and C is the chlorophyll-a at the time for *C. ovalisporum.*

2.8. Cylindrospermopsin (CYN)

A 2 mL algal cell suspension was filtered with 0.45 μm cellulose acetate filters (Bkmam, China), and the determination of extracellular CYN in the filtrate was made by LC/MS (Thermo_Q Exactive, Waltham, MA, USA), with a detection limit of 0.02 μg·L^{-1}. The methods applied for CYN detection were those reported by Zhu and Li [19]. CYN contents were normalized to dry weight. CYN standards were purchased from Enzo Life Science (Enzo Biochem, Farmingdale, NY, USA).

2.9. Statistical Analysis

All experiments were conducted in triplicate. Data are presented as mean ± standard deviation. Statistical differences were evaluated by ANOVA and Tukey's post hoc comparison test in SPSS 23.0 software for Windows at a significance level of $p < 0.05$. All polyline diagrams were made in Origin Pro 2016 (OriginLab, San Francisco, CA, USA).

3. Results

3.1. Biomass and Specific Growth Rate

At 0.02, 0.05, and 0.1 mg·L^{-1} P, the biomass of *C. ovalisporum* increased rapidly at the beginning of the experiment but became stable after 10 days of incubation. The difference in biomass between the two treatment methods at the same phosphate levels was not significant during the entire experiment ($p > 0.05$). After 8 days, the biomass at 0.5 mg·L^{-1} P was significantly higher than at the other phosphate levels ($p < 0.05$). The biomass of *C. ovalisporum* in the five-time treatment was lower than in the one-time treatment during the experiment at 0.5 mg·L^{-1} P; the difference between the two treatment methods was significant in the middle and at the end of the experiment ($p < 0.05$, Figure 1a).

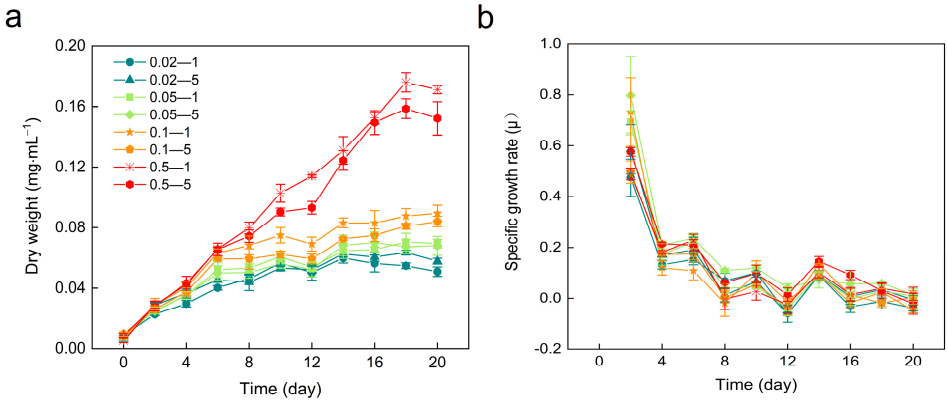

Figure 1. The changes in biomass (**a**) and specific growth rate (**b**) of *C. ovalisporum* in the different treatments.

The specific growth rate of *C. ovalisporum* decreased during the experiment. At the same *p* levels, the specific growth rate in the five-time pulse treatment method was slightly higher than in the one-time treatment method, but the difference was not significant ($p > 0.05$, Figure 1b).

3.2. Chlorophyll-a

Chl-a at 0.02, 0.05, and 0.1 mg·L^{-1} P increased rapidly during the first 8 days of the experiment and then decreased. Chl-a at 0.5 mg·L^{-1} P increased rapidly again after a brief decrease during days 8–12, showing a trend of secondary growth. After 14 days, Chl-a at 0.5 mg·L^{-1} P was significantly higher than at the other phosphate levels ($p < 0.05$). Maximum Chl-a in the five-time treatment at 0.02 mg·L^{-1} P was 1.3 times higher than in the one-time treatment, and the difference was significant ($p < 0.05$). At the end of the experiment, Chl-a in the one-time treatment at 0.5 mg·L^{-1} P was significantly higher than in the five-time treatment ($p < 0.05$, Figure 2).

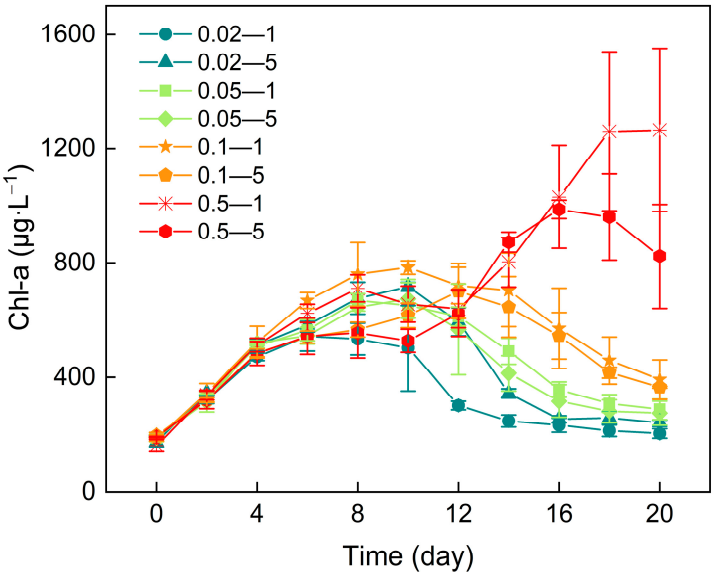

Figure 2. The changes in the Chl-a of *C. ovalisporum* in the different treatments.

Two-way ANOVA tests showed that the effect of the pulse treatments on Chl-a was significant ($p < 0.05$) on days 6–8, and after 12 days, the Chl-a of *C. ovalisporum* was more significantly influenced by the phosphate level ($p < 0.05$, Table S4).

3.3. Photosynthetic Activity Variations

The changing trends in Fv/Fm and Yield were similar for all treatments. At 0.02, 0.05, and 0.1 mg·L^{-1} P, the Fv/Fm and Yield decreased in the late stage of the experiment, while they remained stable at 0.5 mg·L^{-1} P throughout the entire experiment. At 0.02 mg·L^{-1} P, the Fv/Fm and Yield in the five-time treatment were significantly lower than in the one-time treatment in the middle of the experiment ($p < 0.05$) but significantly higher at the end of the experiment ($p < 0.01$). At 0.05, 0.1, and 0.5 mg·L^{-1} P, the difference in Fv/Fm and Yield between the two P treatment methods did not differ significantly ($p > 0.05$, Figure 3).

3.4. Intracellular Dissolved Total Phosphorus (DTPint) and Soluble Reactive Phosphorus (SRPint)

The DTP_{int} and SRP_{int} of *C. ovalisporum* reached a maximum on day 2 and then continued to decline. On days 2–16, DTP_{int} at 0.5 mg·L^{-1} P was significantly higher than at the other phosphate levels ($p < 0.05$). However, at the end of the experiment, no significant difference in the treatments was observed ($p > 0.05$, Figure 4a). SRP_{int} at 0.5 mg·L^{-1} P was significantly higher than at the other phosphate levels on day 4 ($p < 0.05$, Figure 4b). There were no significant differences between the five-time treatment and the one-time treatment for all P levels ($p > 0.05$, Figure 4).

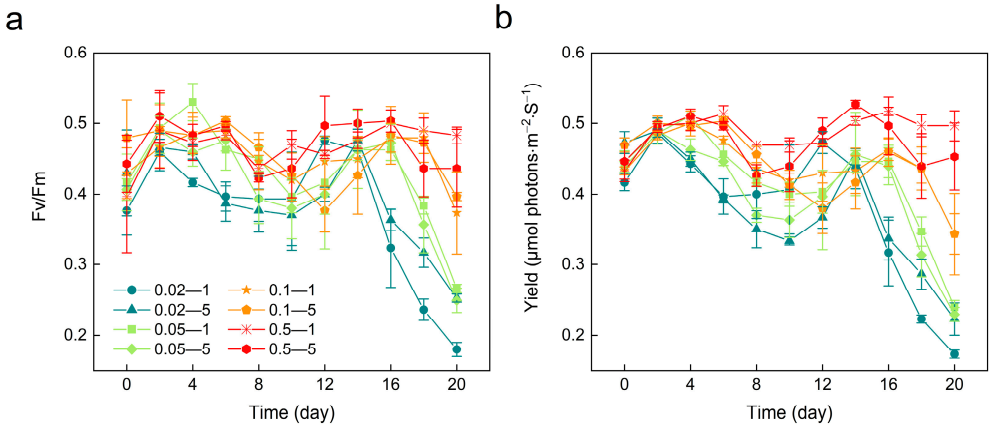

Figure 3. The changes in Fv/Fm (**a**) and the yield (**b**) of *C. ovalisporum* in different treatments.

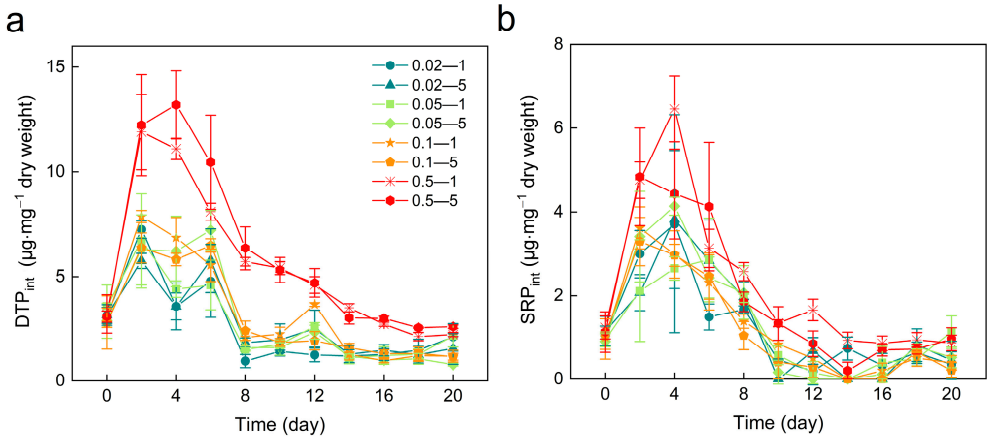

Figure 4. The changes in DTP_{int} (**a**) and SRP_{int} (**b**) of *C. ovalisporum* in the different treatments.

3.5. Exopolysaccharides

RPS decreased with increasing phosphorus levels, but there was no significant difference between the treatments on day 10 ($p > 0.05$). At the end of the experiment, RPS at 0.5 mg·L^{-1} P was significantly lower than at 0.02 mg·L^{-1} P ($p < 0.05$, Figure 5a). There was no significant difference in CPS between the treatments on days 10 and 20 ($p > 0.05$, Figure 5b). At the same phosphorus level, there were no significant differences in RPS and CPS between the two P treatment methods ($p > 0.05$, Figure 5).

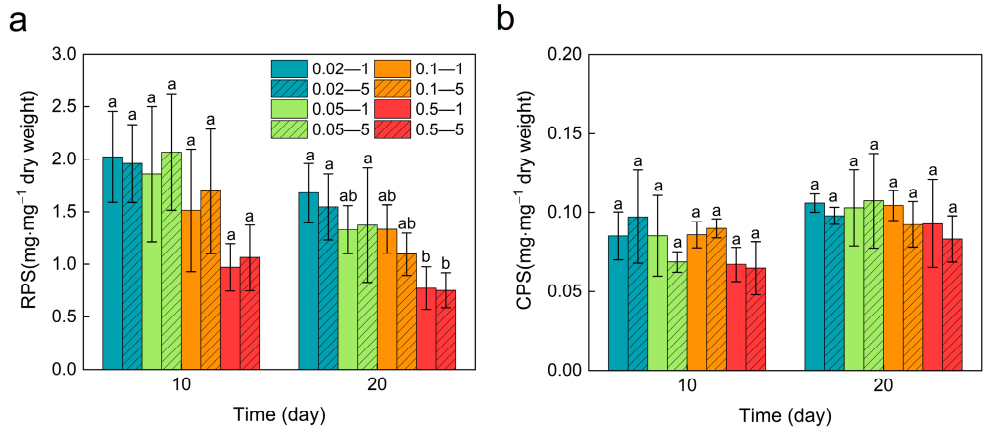

Figure 5. The changes in RPS (**a**) and CPS (**b**) of *C. ovalisporum* in the different treatments. All the values are means ± S.D. Identical letters denote no significant difference ($p < 0.05$).

3.6. Alkaline Phosphatase Activity

At 0.02, 0.05, and 0.1 mg·L^{-1} P, APA continued to increase throughout the experiment, and RRS decreased with increasing phosphorus levels. At 0.5 mg·L^{-1} P, APA generally remained unchanged for the duration of the experiment and was significantly lower than at the other phosphorus levels after day 8 ($p < 0.05$). At the end of the experiment, the difference between all phosphorus levels was significant ($p < 0.05$). At the same phosphorus level, the differences in APA were not significant between the two p treatment methods ($p > 0.05$, Figure 6).

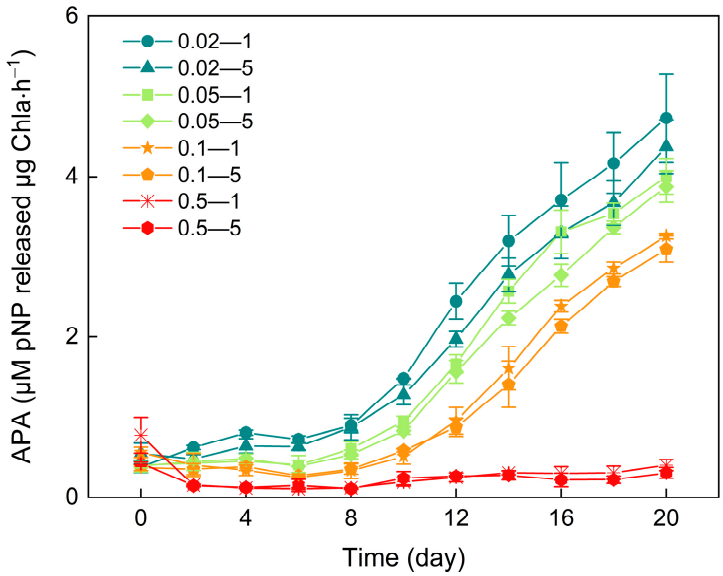

Figure 6. The changes in APA of *C. ovalisporum* in the different treatments.

3.7. Cylindrospermopsin

At 0.02, 0.05, and 0.1 mg·L^{-1} P, CYN rapidly increased and remained stable after 12 days. At 0.5 mg·L^{-1} P, CYN gradually increased during the experiment and was significantly lower than at the other phosphorus levels after 8 days of incubation ($p < 0.05$). The difference in CYN was not significant between the two P treatment methods at the same phosphorus level ($p > 0.05$, Figure 7).

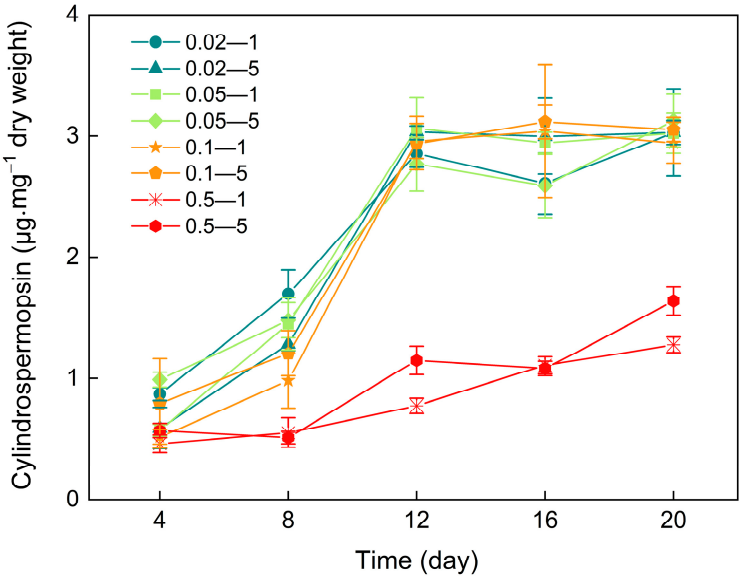

Figure 7. The CYN dynamics of *C. ovalisporum* in the different treatments.

4. Discussion

The availability of phosphorus in the water column may affect the spread of cyanobacteria species [20]. Our results showed that the effect of phosphorus level on the growth of *C. ovalisporum* was more significant than on how it was supplied (one-time treatment or five-time pulsed). With the increase in phosphate levels, the growth stress response of *C. ovalisporum* decreased significantly, and its growth was promoted. First, we found that *C. ovalisporum* could not maintain its growth at 0.02 and 0.05 $mg \cdot L^{-1}$ P in the later stages of the experiment (Figures 2 and 3), which is in accordance with field experiments showing a threshold of 0.05 $mg \cdot L^{-1}$ for *C. ovalisporum* growth [21]. We further found that low phosphorus levels promoted the secretion of exopolysaccharides and APA by *C. ovalisporum* (Figures 4 and 5). APA can hydrolyze phosphorus compounds in the water into orthophosphate, and this is an important way in which algae utilize phosphorus in phosphorus-limited conditions. We found that the APA content was negatively correlated with phosphate concentration, which is consistent with previous studies [22]. Under phosphorus-deficient conditions, *C. ovalisporum* stimulates other algae to secrete APA by secreting CYN, thereby improving their phosphate supply [7]. CYN may have toxic effects on a variety of animals and plants and is a cyanotoxin with a wide range of toxicity [23]. Some studies suggest that phosphorus is associated with CYN production, but the results are ambiguous. Thus, Bar-Yosef et al. [14] found that phosphorus deficiency increased the intracellular CYN content of *C. ovalisporum*, while Bacsi et al. [24] revealed that phosphorus deprivation decreased Q_{CYN} in *C. ovalisporum*. In our study, lower levels of phosphate enhance the release of CYN. However, the rapid rise of CYN at low phosphorus levels after 8 days of incubation most likely reflected the stress response (e.g., cell damage) of *C. ovalisporum* (Figures 1 and 2). EPS are microbe-produced solids located outside of the cell and are composed of proteins, carbohydrates, and other organic components [25]. EPS can quickly adsorb and store phosphorus and is an important channel for inorganic phosphorus to enter the phytoplankton cells [6]. Studies have shown that a lack of nutrients will enhance the EPS content in algae, e.g., *Merismopedia* [26] and *Anabaena* [27]. We found that at the end of the experiment, the RPS content had increased significantly at low phosphate levels (Figure 5), consistent with previous studies [28]. Our results suggest that *C. ovalisporum* can improve the efficiency of phosphorus uptake by secreting more EPS. In addition, studies have shown that phosphate deficiency leads cyanobacteria to produce large amounts of ROS and an upregulation of the expression of their antioxidant system [29]. The increase in EPS can be related to cell damage and growth stress due to nutrient deficiency [30].

The effect of phosphate pulsed treatments on *C. ovalisporum* is mainly reflected in the uptake and storage of phosphate. Phytoplankton shows "extravagant" absorption of phosphate by absorbing a large amount of phosphate in the environment and storing it in cells to maintain normal growth at low phosphorus concentrations [2]. We found that *C. ovalisporum* had converted most of the absorbed inorganic phosphorus into phosphorus complexes on day 2 of the experiment. Afterwards, the phosphate content gradually decreased over time, probably as a result of consumption during *C. ovalisporum* growth (Figure 3). In the early stage of the experiment, the intracellular phosphate content of *C. ovalisporum* in the five-time treatment was higher than in the one-time treatment, indicating that multiple pulses contributed more effectively to the uptake of phosphate by *C. ovalisporum*. The efficient uptake of phosphorus by *C. ovalisporum* under phosphorus deficiency depends on its high-affinity Pi transport system, and multiple pulses may prolong the duration of the high-affinity Pi transport system in *C. ovalisporum*. We also found that multiple pulses reduced the synthesis and secretion of alkaline phosphatase by *C. ovalisporum* (Figure 6), indicating that at the same total input, multiple pulses can more effectively alleviate phosphorus deficiency [31]. We did not find that the treatments had a significant effect on the extracellular CYN content in *C. ovalisporum*, which may perhaps reflect that phosphorus pulses do not stimulate the expression of genes related to toxigenicity; albeit evidence of this is lacking.

We found that the five-time pulse treatment stimulated the growth of *C. ovalisporum* more than the one-time treatment but not at the higher phosphorus levels (>0.1 mg L^{-1}). Accordingly, Amaral et al. [9] found that the pulsed addition of phosphoric acid prolonged the exposure of cyanobacteria to phosphate, thereby increasing their growth rate. Our study indicated that nutrient-poor or mildly eutrophic water bodies are more likely to exhibit increasing *C. ovalisporum* growth and blooms after receiving a nutrient pulse, such as during rainfall than at more constant phosphorus concentrations.

5. Conclusions

Our experiments indicated that phosphorus deficiency in the water inhibited the growth of *C. ovalisporum*, leading it to secrete more exopolysaccharides and alkaline phosphatase. Compared with a one-time treatment, multiple pulses promoted the absorption of phosphorus by *C. ovalisporum* and reduced the synthesis of alkaline phosphatase, whereas it had no significant effect on the release of CYN. Our results show an overall strong adaptability of *C. ovalisporum* to dynamic changes in phosphate levels in the water column.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/w15020351/s1, Table S1: Results of multiple comparisons of the different treatments presented in Figure 1a; Table S2: Results of multiple comparisons of the different treatments presented in Figure 1b; Table S3: Results of multiple comparisons of the different treatments presented in Figure 2; Table S4: Effect of pulse treatments and phosphate on the Chl-a of *C. ovalisporum* by two-way ANOVA; Table S5: Results of multiple comparisons of the different treatments presented in Figure 3a; Table S6: Results of multiple comparisons of the different treatments presented in Figure 3b; Table S7: Results of multiple comparisons of the different treatments presented in Figure 4a; Table S8: Results of multiple comparisons of the different treatments presented in Figure 4b; Table S9: Results of multiple comparisons of the different treatments presented in Figure 6; Table S10: Results of multiple comparisons of the different treatments presented in Figure 7.

Author Contributions: S.Y.: experiment, analysis, visualisation, writing—original draft, and writing—review and editing. R.H.: experiment, analysis, visualisation, modification draft, and writing—review and editing. N.W.: experiment. C.L.: experiment. E.J.: writing—review and editing. L.W.: supervision and project administration. W.Z.: supervision, project administration, and writing—review and editing. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the National Natural Science Foundation of China (41901119). E.J. was supported by the TÜBITAK program BIDEB2232 (project 118C250).

Data Availability Statement: Experimental data that support the findings of this study are available from the corresponding author upon request.

Acknowledgments: We thank Anne Mette Poulsen for her valuable English editing.

Conflicts of Interest: The authors declare no conflict of interest.

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