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FILAMENTOUS GREEN ALGAE, EXTRACELLULAR ALKALINE PHOSPHATASES AND SOME FEATURES OF THE PHOSPHORUS CYCLE IN PONDS

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Filamentous green algae (FGA) may reach high biomass and play a very important functional role in productivity and nutrient cycling in the different water bodies. Their extracellular alkaline phosphatase activity may be an important player in the phosphorus cycle. Currently, there is intensive development of green algae in various freshwater and marine water bodies, which creates problems for people's activities and necessitates its investigation. Filamentous green algae in four Chinese and Crimean (Russia) shallow freshwater ponds were in focus of this study. The dissolved phosphorus fraction in pond water, algal pigment level, activity and kinetic properties of alkaline phosphatase were evaluated in water column and cell membrane of filamentous green algae. Microalgal taxa were identified in the plankton samples. Species composition and density of FGA in the studied ponds were different. Two ponds had more than 50 % coverage of a water surface by FGA and its wet biomass more than 100 g·m⁻². Two others were with wet biomass less than 2 g·m⁻². In ponds with low FGA biomass, the soluble reactive phosphorus concentration exhibited considerably low level with less than 10 µg·L⁻¹, and the dissolved organic phosphorus comprised the largest phosphorus fraction, averaging 23.1 µg·L⁻¹ and ranged from 20.8 to 25.4 µg·L⁻¹. However, in ponds with high FGA biomass, particulate phosphorus was the major component, which contributes 45.8 % and 56.7 % of total phosphorus, respectively. Size fractionation of extracellular alkaline phosphatase activity in water column expressed spatial heterogeneity, which corresponded with biomass of FGA. The response of extracellular alkaline phosphatase activity to different phosphate concentration in water column was completely distinct from that in the cell membrane of FGA, the last of which represented the significantly inhibition effect to high phosphate concentration. The significant inhibition of alkaline phosphatase activity in cell membrane of FGA by phosphate in water may validate that FGA growth was limited by phosphorus. The contradiction between a low concentration of soluble reactive phosphorus and high FGA biomass may indicate that there was high speed nutrient cycling, probably, due to the alkaline phosphatase activity. Excreting exo-alkaline phosphatases, FGA, microalgae and bacteria accelerate phosphorus cycling through different mechanisms, and this may increase their development. In ponds with high FGA biomass, many of bacteria are responsible for regeneration of nutrients, which then consuming by FGA. Those bacteria also may concurrently restrict a microalgae development, such as unicellular Chlorophyta species. As an example, *Cladophora* provides habitat for different species of epibionts (bacteria and microalgae, primarily diatoms), and sustains of strong mutualistic alga-bacterium interactions. Therefore, the problem of excessive FGA growth should not be considered in isolation, but in a whole-ecosystem context.

Ключевые слова: phosphorus cycle, exo-alkaline phosphatases, Chlorophyta, ponds, China, Crimea

Phosphorus is an essential constituent element in every known form of life; it is built-in energy storage, cell structure, and the genetic material that encodes all life on the planet. It is well known that plants absorb phosphorus from water, animals consume plants, and phosphorus is released back to water in the form of phosphate from the dead plant and animal material. Recent studies have shown that phosphorus cycle in the water bodies is more complicated thing, and the case of filamentous green algae (FGA) may demonstrate this [11, 32, 37].

High biomass of filamentous green algae (FGA) is regarded as a signal of eutrophication in aquatic ecosystems; it is the globally distributed phenomenon now [3, 9, 15, 19, 36, 39]. FGA has become more abundant during the last decades in many aquatic ecosystems, such as estuaries [13, 34], streams [24], seas [8, 14], reservoirs [10], and lakes [26, 28], creating some problems for human population. Species from the genera *Spirogyra*, *Oedogonium*, *Ulva* and *Cladophora* are most frequently encountered among them. Like other aquatic and terrestrial plants, FGA require inorganic nutrients (P, N) to support a growth in a specific range of temperature and light conditions. There is consensus that phosphorus is the growth-limiting nutrient for FGA in the lakes [27, 32]. For example, removal or abatement of phosphorus from a wastewater substantially reduced *Cladophora* biomass in Lake Huron [4], Lake Ontario [25], Lake Erie [22], and Lake Windermere [26]. The *Cladophora* growth model predicts that its growth is highly sensitive to spatial and temporal variations in soluble phosphorous concentration [31]. However, there are only few reports on nitrogen-limited FGA growth in freshwater systems but we will not discuss them here. The occurrence of heterotrophic nitrogen-fixing bacteria among *Cladophora* epibionts may be one of the reasons of this [38, 40].

FGA, as well as other algae, have mechanisms to absorb and store phosphorus for immediate needs [1]. Phosphorus uptake depends on external nutrient supply and hydrolysis of polyphosphates due to activity of alkaline phosphatase enzymes (AP). AP was found in intracellular, extracellular and cell-bound fractions [20, 38]. Activity of phosphatases, isolated from *Cladophora*, was observed to be significantly inhibited by phosphate concentration

[11]. The AP capacity of *Cladophora* was higher than other macroalgae species in Lagoon Harrington Sound (Bermuda), and AP activity (APA) was enhanced by nitrogen enrichment and suppressed by phosphorus enrichment [18]. The importance of AP for FGA growth through phosphorus regeneration is clear, but field data on the relationship between the extracellular AP and ambient phosphorus concentration are lacking. Also, the information on the relationship between AP of FGA, bacterial AP and phytoplankton AP is very scarce. A better understanding of phosphorus cycling in the aquatic ecosystems is important, as it affects aquatic food webs and, therefore, the ability of every water body to absorb atmospheric carbon dioxide.

Thus, the objectives of this study are: 1) to give further evidence of the occurrence and activity of extracellular AP produced by different FGA species; 2) to detect the kinetic property of the extracellular AP (total AP, dissolved AP in water column and AP in cell membrane of FGA); 3) to analyze the mutual relationship between FGA, microalgae and bacteria, and 4) to get deeper understanding of the FGA AP role in phosphorus cycling through the comparative study in Chinese and Crimean (Russia) freshwater ponds. Freshwater ponds may be used as a good model for better understanding of phosphorus cycle complexity in every other type of aquatic ecosystems.

MATERIAL AND METHODS

Description of sampling ponds. We studied four shallow ponds, which located nearby the Lake Yuehu, Wuhan City, China (Pond 2-C and 4-C), and in Sevastopol city, Crimea, Russia (Pond 1-U and 3-U). The dominant species were *Rhizoclonium tortuosum* and *Cladophora* sp. in Pond 3-U and 4-C, respectively, with more than 50 % coverage of a water surface and its wet biomass more than 100 g·m⁻². However, few *Spyrogyra* sp. floating mats in Pond 1-U and *Cladophora* sp. benthic mats in Pond 2-C were observed with wet biomass less than 2 g·m⁻². All these ponds have a surface area of 500–700 m² and average depth of about 1 m.

Chemical analysis. The surface water samples (0–0.5 m) and fresh FGA samples were collected in Chinese and Crimean ponds from September to October, 2008. FGA samples were put into the bottles filled with surface water. Samples were

Таблица 1. Численность и биомасса планктонных микроводорослей разных таксонов в прудах 1-U и 3-U
Table 1. Abundance and biomass of different taxa of planktonic microalgae in Pond 1-U and Pond 3-U

Taxon	Pond 1-U		Pond 3-U	
	Density, cells·L ⁻¹	Biomass, mg·m ⁻³	Density, cells·L ⁻¹	Biomass, mg·m ⁻³
Chlorophyta	22 032 200	749.7	–	–
Dinophyta	39 000	195.4	2312	43.2
Cryptophyta	–	–	–	–
Bacillariophyta	104 400	530.5	146 863	916.6
Chrysophyta	–	–	–	–
Суанопхита	397 800	15.1	2856	0.3
Total	22 893 420	1490.7	152 031	960.1

Примечание: «–» — очень мала. Note: “–” — undetectable.

transported to the laboratory within 2 hours after collection.

The water samples were filtered through pre-washed 0.45- μm filters. All samples were analyzed in triplicate. Soluble reactive phosphorus (SRP) was measured with the molybdate blue method [23]. The concentration of dissolved total phosphorus (DTP) and total phosphorus (TP) was determined by the method [12]. Dissolved organic phosphorus (DOP) was calculated as $\text{DOP} = \text{DTP} - \text{SRP}$, and particulate phosphorus (PP) was calculated as $\text{PP} = \text{TP} - \text{DTP}$.

The surface water and FGA samples were analyzed for different pigment concentration using 90 % acetone extraction and spectrophotometric measurements at 663 nm for chlorophyll *a*, at 646 nm for chlorophyll *b*, and at 470 nm for total carotenoids with correction for turbidity at 750 nm. The amount of these pigments was calculated according to the formulas [37].

Alkaline phosphatase activity (APA) in water column and in FGA samples was determined spectrophotometrically. We followed the rate of release of p-nitrophenol from model substrate p-nitro-phenylphosphate (pNPP) over 1–4 h depending on different samples. Enzyme activity was measured as the increase in absorbance at 410 nm in 1-cm cuvettes [2]. For APA of FGA, measurements had been made in pH 8.5 Tris-HCl buffer solution with freshly collected FGA.

APA was determined at 8 different concentrations of substrate (pNPP from 0.01 to 1.8 mmol·L⁻¹) for the calculation of enzymatic kinetics parameters (Michaelis constant K_m and maximum

velocity V_{max}) though the Lineweaver — Burke transformation ($1/V$ vs. $1/S$) of the Michaelis — Menten equation.

The water sample was filtered through 0.45 and 3.0 μm membrane filter. The contributions of APA to the algal and bacterial fractions were calculated as follows (formula 1, 2):

$$A = U - F(3.0) \quad (1)$$

and

$$B = F(3.0) - F(0.45) \quad (2)$$

where A — activity in algal fraction, i. e. in fraction larger than 3.0 μm ; B — activity in bacterial fraction, i. e. in fraction 0.45–3.0 μm ; U — activity of unfiltered water sample, i. e. total APA; F (3.0) — activity in water sample prefiltered through 3.0 μm ; F (0.45) — activity in water sample filtered through 0.45 μm . The final pNPP concentration (0.3 mmol·L⁻¹) was used for the size-fractionation of APA [7].

The different amount of phosphate (KH_2PO_4) was added to the unfiltered and filtered 0.45 μm water samples as well as fresh FGA immersed with Tris-HCl buffer solution to test the responses of APA to phosphate. The final phosphate concentration was from 0.02 to 0.6 mg·L⁻¹.

Biological analysis. Microalgae were sampled (1 L) in surface water and preserved by Lugol solution [35]. Samples were pre-sedimented and concentrated to no more than 20 ml for at least two weeks. After mixing, microalgal taxa were identified and their abundance was counted under light microscop

at $\times 640$ magnification. Results are expressed as density ($\text{cells}\cdot\text{L}^{-1}$) and biomass ($\text{mg}\cdot\text{m}^{-3}$). FGA samples were freshly collected in the bottles filled with surface water. Samples were thoroughly rinsed to remove all macrofauna and preserved with formalin for taxonomic identification.

Statistical analysis. Fisher's least significant difference test and independent-samples *T*-test were used to make comparisons among the parameters in different pond, using the SPSS statistical package.

RESULTS AND DISCUSSION

In Pond 1-U and Pond 2-C with low FGA biomass, the SRP concentration exhibited considerably low level with less than $10\ \mu\text{g}\cdot\text{L}^{-1}$, and the DOP comprised the largest phosphorus fraction, averaging $23.1\ \mu\text{g}\cdot\text{L}^{-1}$ and ranged from 20.8 to $25.4\ \mu\text{g}\cdot\text{L}^{-1}$. However, in Pond 3-U and Pond 4-C, PP was the major component, which contributes 45.8% and 56.7% of TP, respectively (fig. 1).

The chlorophyll *a* and *b* of microalgae in Pond 3-U and Pond 4-C had shown markedly higher value over almost ten times than the other two ones ($P < 0.01$) (fig. 2), also, the intracellular pigments of FGA including chlorophyll *a*, *b* and total carotenoid with relatively higher level in Pond 3-U and Pond 4-C were observed (fig. 3, $P < 0.05$). Species composition of microalgae and its density were different in different ponds (table 1). In Pond 1-U, Chlorophyta was a dominant taxon, which contributing 96% and 50% for total microalgal density and biomass. In Pond 3-U, Chlorophyta were not found, but Bacillariophyta were responsible for the total density and biomass (97% and 96% , respectively).

Size fractionation of APA in water column expressed spatial heterogeneity, which corresponded with biomass of FGA. In Pond 1-U and Pond 2-C, the bacterial APA represented a larger portion with 35% and 45% of total APA, respectively. However, in Pond 3-U and Pond 4-C, the algal fraction had the major contribution of 58% and 56% to total APA, even though a great abundance of bacteria below the FGA in Pond 4-C were found (our unpublished data). Moreover, the highest value for total APA in Pond 2-C and the lowest value for dissolved APA in Pond 4-C had been recorded (fig. 4). In the cell membrane of FGA, the order for the magnitude of the total APA in all studied ponds was the same as in the

dissolved fraction. In addition, it is worthy to note that, wherever in China or Crimea, the low level of dissolved APA was always accompanied with high FGA biomass, especially in Pond 4-C with more than 90% *Cladophora* coverage (fig. 5).

There seems to be more complicated to compare the kinetic characteristics for APA in water column and cell membrane of FGA. However, we may find out some trends (fig. 6): the highest V_{max} value in Pond 2-C and lower K_m value in Pond 4-C for total APA, and the higher K_m value in Pond 4-C for dissolved APA. For the APA of FGA cell membrane, lower V_{max} and K_m in Pond 2-C as well as higher V_{max} and K_m value in Pond 4-C were found that implies distinct mechanism for enzymatic hydrolysis (fig. 7). K_m value of APA, a specific parameter to characterize enzyme affinity to a substrate, in water column of Pond 2-C and Pond 4-C was significantly lower than that in cell membrane of FGA (figs. 6, 7).

The response of APA to different phosphate concentration in water column was completely distinct from that in the cell membrane of FGA, the last of which represented the significantly inhibition effect to high phosphate concentration (figs. 8, 9).

The high FGA abundance in two of all ponds is somewhat paradoxical in light of the fact that concentrations of SRP were relatively low. Along with observations of intensive FGA coverage in water areas that are relatively remote from external nutrient sources, this fact suggests that there are the special and crucial mechanisms of nutrients transfer between FGA and ambient water. Here, a hypothesis was presented that nutrient recycling with high efficiency mediated by extracellular enzymes should be responsible for FGA excessive growth. In detailed, the slightly higher SRP concentration in Pond 3-U and Pond 4-C, accompanied with relatively lower APA in water column and cell membrane of FGA, as well as higher DOP concentration in Pond 1-U and Pond 2-C in parallel with higher APA indicated that the production of AP was regulated by enzymatic depression-induction mechanisms. The "induction — repression" mechanism was previously shown in the lakes with lower SRP concentration [6].

Furthermore, the significant inhibition response of APA in cell membrane of FGA (*Cladophora*) to phosphate supplement validated that the growth of FGA was more sensitive to phosphate concentration

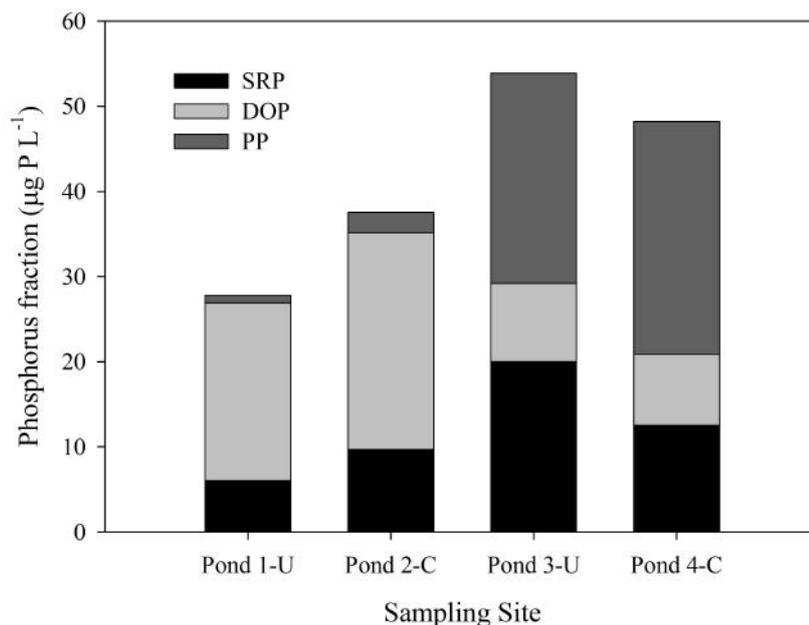


Рис. 1. The phosphorus fractions in water of different ponds

Fig. 1. Фракции фосфора в воде разных прудов

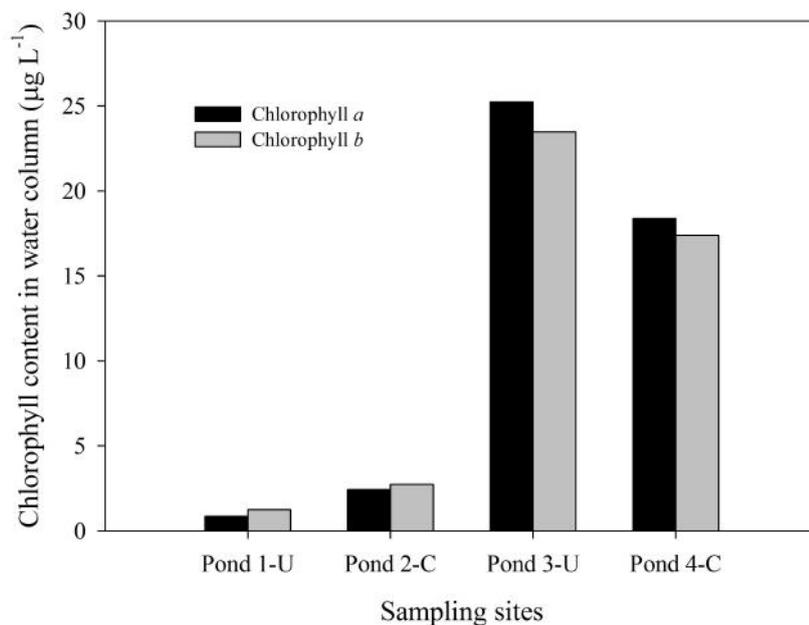


Рис. 2. The chlorophyll *a* and *b* content in water of different ponds

Fig. 2. Содержание хлорофиллов *a* и *b* в воде разных прудов

than that in microalgae plankton. For instance, in Lake Ontario, *Cladophora* tissue phosphorus concentration has declined in parallel with decreasing P concentration in water, resulting in reduced *Cladophora* biomass at all depths in the euphotic zone [21]. Also, in Harrington Sound, the APA capacity was enhanced by nitrogen enrichment and suppressed by phosphorus enrichment [18]. Thus, we

may conclude that FGA (*Cladophora*) were limited by phosphorus; this also strongly stimulates the AP secretion on cell membrane.

Additionally, comparing to the kinetic characteristics (K_m value) of APA in cell membrane of FGA (*Cladophora*) and water, we found lower K_m value in water column. This fact may indicate that the AP secreted by microalgae had a higher

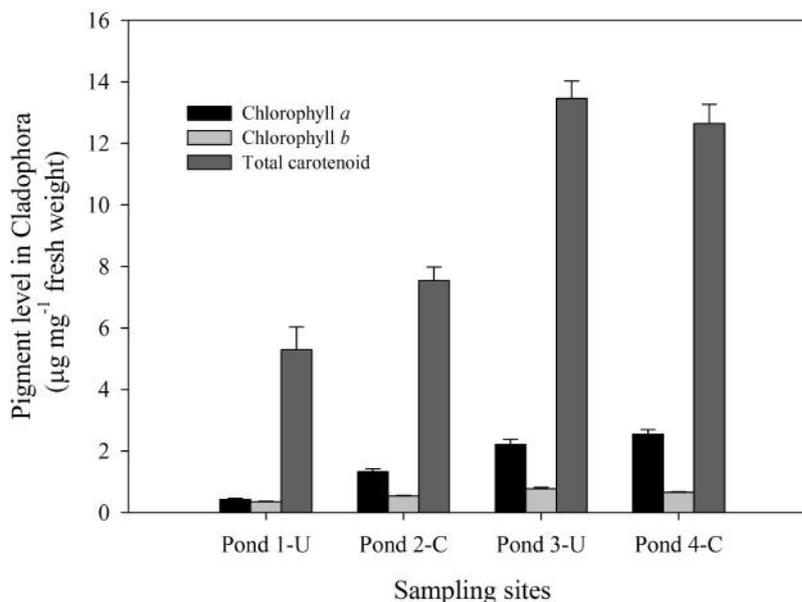


Рис. 3. The chlorophyll *a*, *b* and total carotenoid content inside filamentous green algae cells in different ponds

Fig. 3. Концентрации хлорофиллов *a*, *b* и общих каротиноидов в клетках зелёных нитчатых водорослей из разных прудов

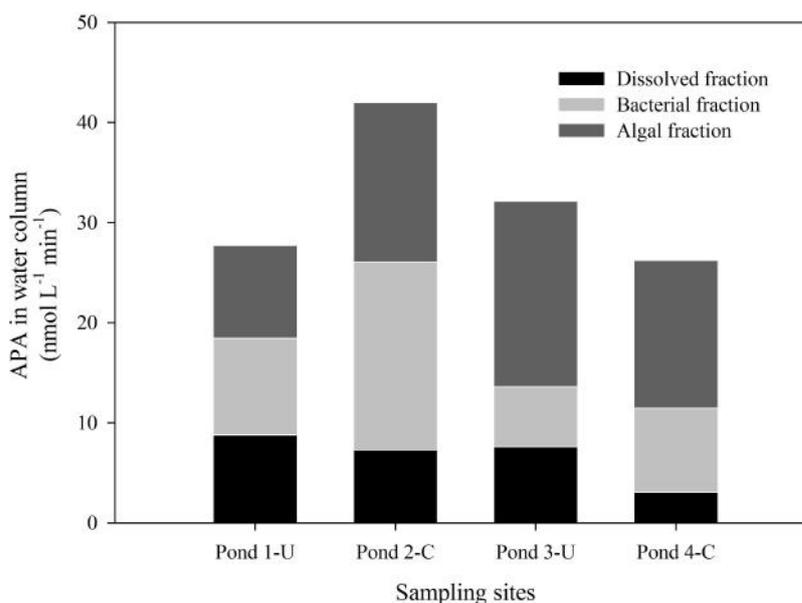


Рис. 4. Size fractionation of alkaline phosphatase activity in water of different ponds

Fig. 4. Активность щелочной экзофосфатазы в разных размерных фракциях клеток в воде разных прудов

affinity to organic phosphorus. On the other hand, there were higher V_{max} and K_m values for the APA in cell membrane of FGA in Pond 4-C than in Pond 2-C. It may be suggested that FGA in high biomass pond had the advantage in the hydrolysis velocity of organic substrate. However, in low biomass pond, FGA overcome the difficulty of phosphate limitation

through higher affinity to substrates, which is conflicted with plankton nutrient uptake mechanism [5, 38]. These facts suggested that if the enzymatic hydrolysis mechanism of FGA (*Cladophora*) and microalgae would be the same, their growth would be jointly restricted, whereas, excessive growth of FGA was observed.

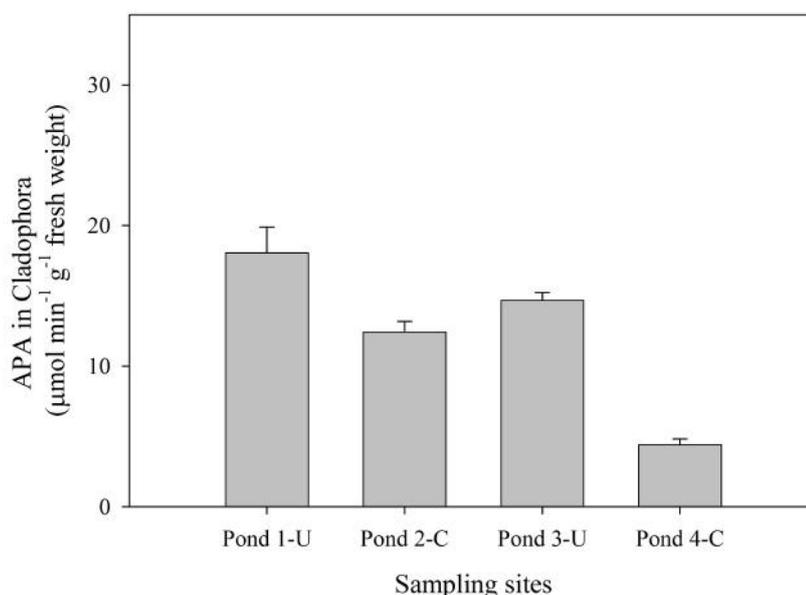


Рис. 5. The alkaline phosphatase activity in cell membrane of filamentous green algae of different ponds

Fig. 5. Активность щелочной фосфатазы в клеточных мембранах нитчатых зелёных водорослей из разных прудов

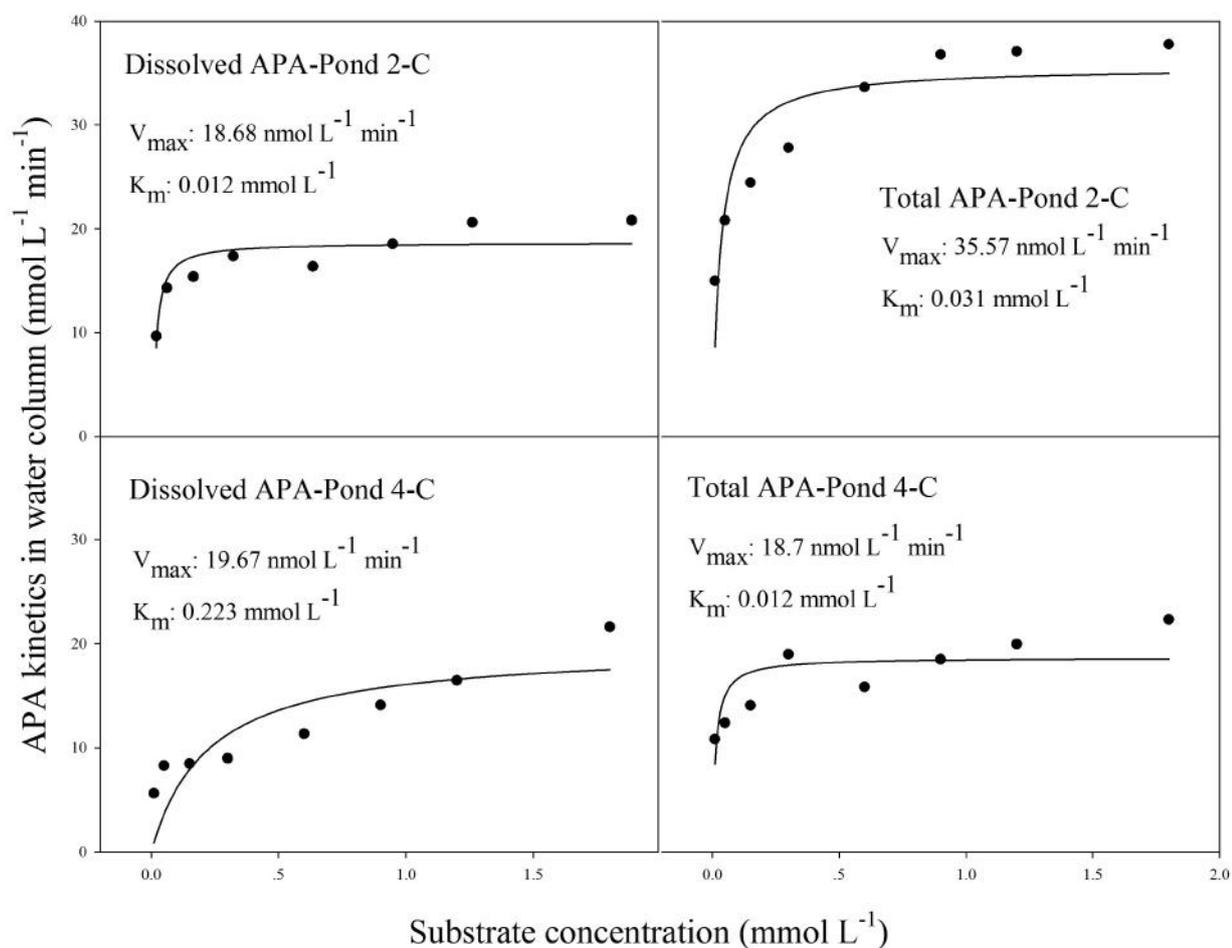


Рис. 6. Kinetic curves of dissolved and total alkaline phosphatase in water of different ponds

Fig. 6. Кинетические кривые растворённой и общей щелочной фосфатазы в воде разных прудов

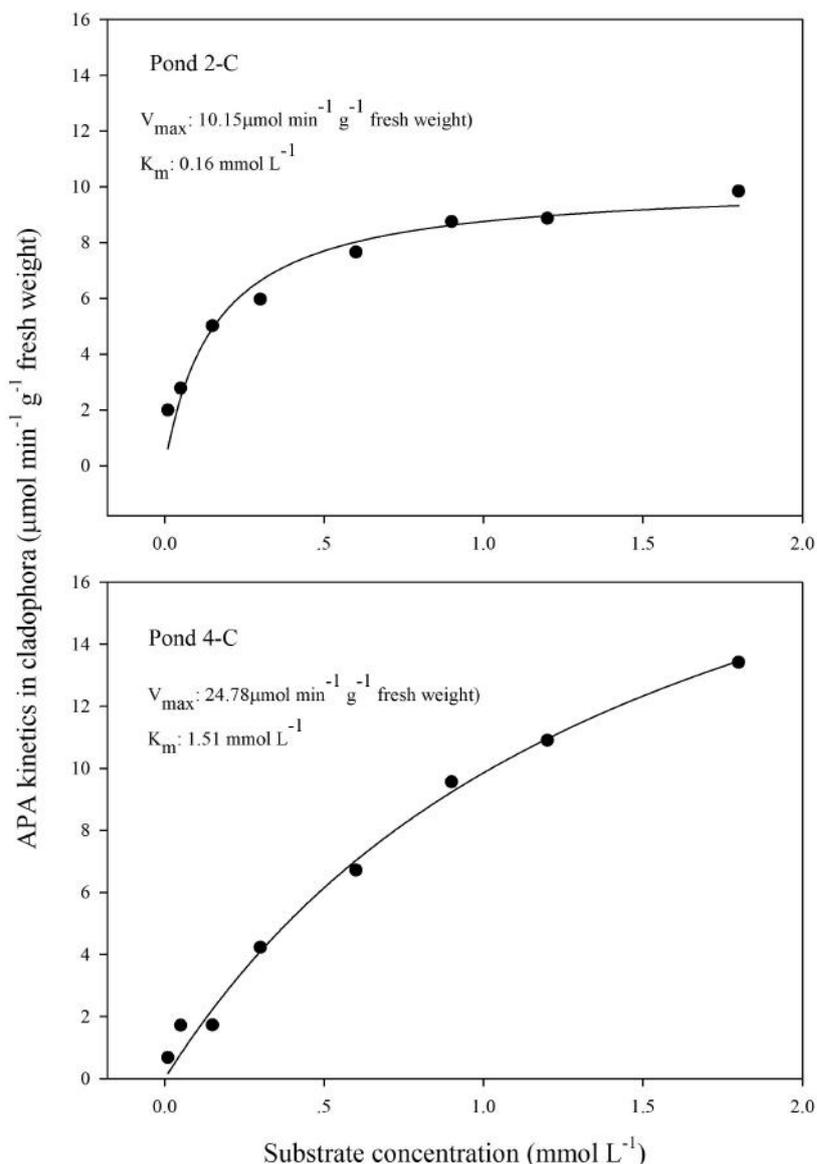


Рис. 7. The chlorophyll *a*, *b* and total carotenoid content inside filamentous green algae cells in different ponds

Fig. 7. Концентрации хлорофиллов *a*, *b* и общих каротиноидов в клетках зелёных нитчатых водорослей из разных прудов

At the same time, in Pond 4-C, high enzymatic activity of bacteria underneath FGA (*Cladophora*) mats should be responsible for the organic matter decomposition and nutrient regeneration. The higher algal APA (due to bacterial attachment) and these regenerated nutrient support the FGA growth. On the other hand, organic carbon produced by FGA, and extracted as exopolysaccharids [29], provides necessary carbon and energy source for bacteria. The similar results were observed in cyanobacterial mat community by [33]. The positive nutrient mutualism between bacteria and FGA as well as the enzymes

with high efficiency secreted by FGA, are two main reasons for excessive FGA growth [17, 30, 38].

Besides, the close coexistence between FGA and bacteria, to some extent, restricted the other microalgae development, especially Chlorophyta species. On the contrary, due to relatively low nutrient requirement or special strategy for nutrient acquisition, Bacillariophyta species can survive in ponds with high FGA biomass. Differences in APA mechanisms may play an important role in competition between species [16]. On the other hand, the mutual coupling between the dominant

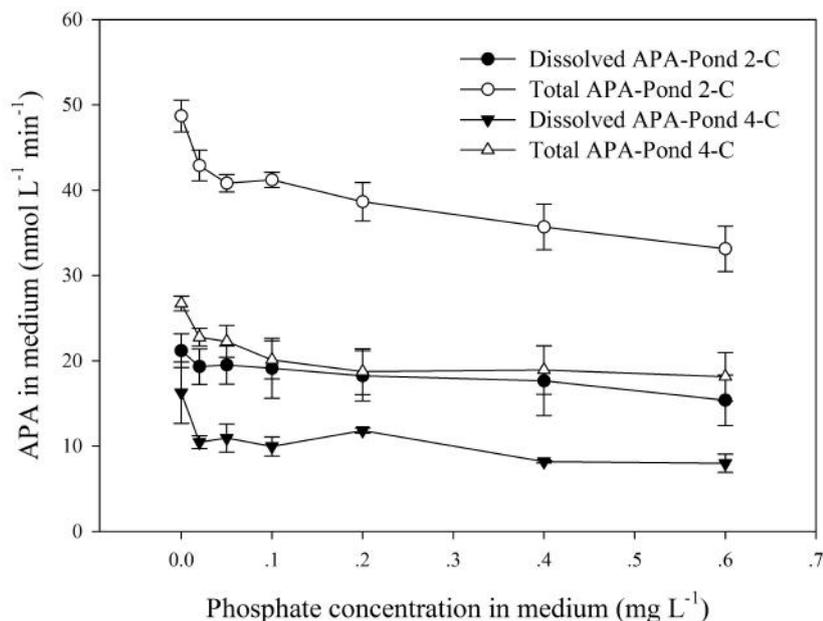


Рис. 8. The chlorophyll *a*, *b* and total carotenoid content inside filamentous green algae cells in different ponds

Fig. 8. Концентрации хлорофиллов *a*, *b* и общих каротиноидов в клетках зелёных нитчатых водорослей из разных прудов

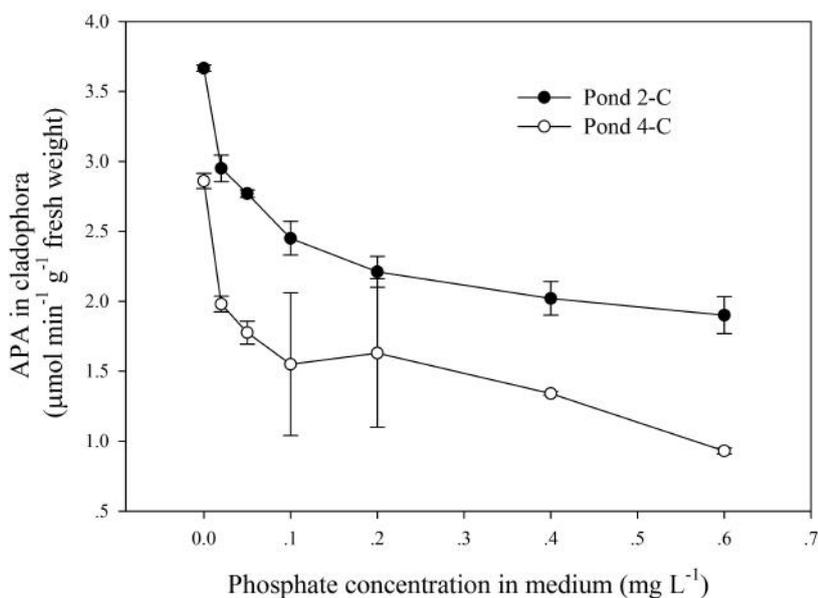


Рис. 9. Size fractionation of alkaline phosphatase activity in water of different ponds

Fig. 9. Активность щелочной экзофосфатазы в разных размерных фракциях клеток в воде разных прудов

Chlorophyta species with higher V_{max} and the dominant Bacillariophyta species with low K_m suggested different organic phosphorus hydrolysis mechanisms for these two groups [5, 6].

Finally, there are some contradictions in our results, and we need to give a reasonable explanation

of them. For example, in Pond 1-U and 3-U, microalgae density and biomass had not changed in parallel with chlorophyll *a* and *b* content in water column, which may be explained by the overestimation for chlorophyll in plankton of Pond 3-U due to the destruction of FGA. In addition, in

Pond 2-C and 4-C, the discrepancy between APA reaction velocity at single substrate concentration and V_{\max} value should be considered as a normal phenomenon because of the enzymatic specificity of different Michaelis — Menten curves. As for the dissolved AP in our results, it is still very difficult to evaluate its origins and functions in different kind of environment with FGA, even if it is consistent with the high concentration with APA in cell membrane of FGA.

FGA, microalgae and bacteria accelerate phosphorus cycling through different mechanisms, and this may increase their development. In ponds with high FGA biomass, many of bacteria are responsible for regeneration of nutrients, which then consuming by FGA [40]. Those bacteria also may concurrently restrict a microalgae development, such as unicellular Chlorophyta species. As an example, *Cladophora* provides habitat for different species of epibionts (bacteria and microalgae, primarily diatoms), and strong mutualistic alga-bacterium interactions exist [40]. Therefore, the problem of excessive FGA growth should not be considered in isolation, but in a whole-ecosystem context. This article demonstrates the complexity of the phosphorus cycle in water bodies, but it raises new questions rather than provides comprehensive answers to them.

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Нитчатые зелёные водоросли, внеклеточные щелочные фосфатазы и некоторые особенности цикла фосфора в прудах

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Зелёные нитчатые водоросли (ЗНВ) могут достигать высокой биомассы и играть важную функциональную роль в образовании продукции и круговороте биогенов в различных водоёмах. Активность внеклеточной щелочной фосфатазы ЗНВ может существенно влиять на процессы в цикле фосфора. В настоящее время наблюдается интенсивное развитие зелёных водорослей в различных пресноводных и морских водоёмах, что создаёт проблемы для деятельности людей и обуславливает необходимость исследования ЗНВ. Целью нашей работы было изучение ЗНВ в четырёх прудах Крыма и Китая. Определены показатели концентрации растворённого фосфора, уровня пигментов, степени активности и кинетических характеристик щелочных фосфатаз в воде и мембранах ЗНВ, а также выявлен состав и численность микроводорослей в планктоне. Видовой состав микроводорослей и биомасса ЗНВ в изученных прудах различались в широких пределах. Два

пруда имели более чем 50%-ное покрытие поверхности воды ЗНВ с сырой биомассой выше $100 \text{ г} \cdot \text{м}^{-2}$. Биомасса ЗНВ в двух других водоёмах составляла менее $2 \text{ г} \cdot \text{м}^{-2}$. В прудах с низкой биомассой ЗНВ концентрация растворимого реактивного фосфора находилась на низком уровне (менее $10 \text{ мкг} \cdot \text{л}^{-1}$), а содержание растворённого органического фосфора составляло максимальную долю общего фосфора (в среднем $23.1 \text{ мкг} \cdot \text{л}^{-1}$) и находилось в диапазоне от 20.8 до $25.4 \text{ мкг} \cdot \text{л}^{-1}$. Однако в прудах с высокой биомассой ЗНВ основная доля общего содержания фосфора принадлежала взвешенному фосфору и составляла 45.8 и 56.7 % соответственно. Распределение активности внеклеточной щелочной фосфатазы по размерным фракциям частиц в водном столбе и распределение биомассы ЗНВ было аналогично и характеризовалось пространственной гетерогенностью. Реакция активности внеклеточной щелочной фосфатазы на различную концентрацию фосфата в водном столбе существенно отличалась от реакции в клеточной мембране ЗНВ, последняя из которых ингибировалась высокими концентрациями фосфата. Достоверно установленное ингибирование фосфором активности щелочных фосфатаз в клеточных мембранах ЗНВ свидетельствует о том, что рост водорослей лимитируется фосфором. Несоответствие между низкой концентрацией растворённого в воде фосфора и высокой биомассой ЗНВ может быть обусловлено увеличением скорости круговорота биогенов за счёт активности щелочных фосфатаз. Выделение в среду щелочных экзофосфатаз ЗНВ, микроводорослями и бактериями может ускорять круговорот фосфора различными путями. В прудах с высокой биомассой ЗНВ массово развивающиеся бактерии могут вносить дополнительный вклад в ускорение регенерации неорганического фосфора, который впоследствии поглощается зелёными водорослями. Эти бактерии также могут одновременно ограничивать развитие микроводорослей, таких как одноклеточные виды Chlorophyta. Например, *Cladophora* обеспечивают среду обитания для различных видов эпибионтов (бактерий и микроводорослей, в первую очередь диатомовых) и поддерживают мутуалистические взаимоотношения между водорослями и бактериями. Полученные результаты обуславливают необходимость изучения процесса интенсивного развития ЗНВ в различных водоёмах в контексте общего функционирования экосистемы.

Keywords: цикл фосфора, щелочные экзофосфатазы, Chlorophyta, пруды, Китай, Крым