



# Potential role of the cellular matrix of *Aphanizomenon* strains in the effects of cylindrospermopsin—an experimental study

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## Abstract

Some literature data suggest that one of the possible roles of the cyanotoxin cylindrospermopsin (CYN) is forcing other phytoplankton species in the environment to produce alkaline phosphatase, which enables the cyanobacterium to take up the enzymatically liberated phosphate. In this study, cultures of a planktonic green alga, *Scenedesmus obtusus* (Chlorophyta, Sphaeropleales), were treated with CYN producer *Aphanizomenon* (Cyanobacteria, Nostocales) crude extract (C+), with non-CYN producer *Aphanizomenon* crude extract (C–), and with non-CYN producer *Aphanizomenon* crude extract supplemented with CYN (C–+C). The results showed that C+ treatment induced both acidic and alkaline phosphatases of the studied cosmopolitan green alga, which otherwise was neither sensitive to the relatively high CYN concentration, nor to phosphate limitation. In cases of C– and C–+C treatments, these phenomena were not observed. Several studies suggest that additional compounds may support CYN action. The results presented here suggest in a more direct way that other components present in the cellular matrix of the producer organism itself are involved in the effects of CYN, activation of phosphatases (not only alkaline ones) among them. These other components are absent in C– crude extract or cannot actively contribute to the effects of exogenously added CYN.

**Keywords** Cylindrospermopsin · *Aphanizomenon* crude extracts · Phosphatases · *Scenedesmus*

## Introduction

Cyanobacteria are extensively studied organisms, mainly because of their ability of producing a wide variety of biologically active metabolites, cyanotoxins among them. Despite the increasing number of studies, the possible roles of the cyanotoxins in the producer organisms and in their environment are still unanswered questions (Omidi et al. 2018); this is especially true to cylindrospermopsin (CYN; Rzymiski and Poniedzialek 2014).

CYN is a tricyclic alkaloid produced by a number of filamentous cyanobacteria from the orders Nostocales and Oscillatoriales. The first CYN producer strains were reported from tropical and subtropical areas, but nowadays CYN-producing cyanobacteria show wide geographical distribution, including temperate and arid regions (Poniedzialek et al. 2012a). Moreover, next to aquatic species, the soil cyanobacterium *Hormoscilla pringsheimii* also has been reported to be a CYN producer (Bohunická et al. 2015). It is important to emphasize that CYN-producing ability could be different within the same species: there are CYN-producing and non-CYN-producing strains of the same species. CYN is absent, if only one gene is missing from the gene cluster responsible for CYN production (Rzymiski and Poniedzialek 2014). It is hard to show a clear correlation between CYN production ability and geographical distribution: CYN producer *C. raciborskii* strains are reported from Asia and Australia but not from Europe and Africa. At the same time, CYN-producing *Aphanizomenon* and *Anabaena* species are described from all over the world (Rzymiski and Poniedzialek 2014).

CYN has many negative effects both to photosynthetic and heterotrophic organisms, because it is able to interfere with several metabolic pathways: it can cause DNA damage

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(Humpage et al. 2000; Shen et al. 2002) and irreversibly inhibit glutathione and protein synthesis (Terao et al. 1994; Runnegar et al. 1995; Frosco et al. 2001, 2003, 2008). CYN has a general cytotoxic effect, so it can affect any kind of exposed cells, although it seems that the metabolism of this compound may increase its toxicity; thus, CYN is considered mainly as hepatotoxin (Bernard et al. 2003; Fastner et al. 2003; Saker et al. 2003). More recently, the effects of CYN on the different cells of immune systems also have been reported (Poniedziałek et al. 2012a, b, 2014a, b). It seems that the toxicity of CYN is mediated through cytochrome P450 (Pearson et al. 2010), and oxidative stress (Rymuszka and Sieroslawska 2014; Poniedziałek et al. 2015), which is followed by all the abovementioned phenomena.

The reason of the toxin production, the role of CYN in producing organisms, and in their environment is still not well known. Several studies have been conducted for understanding the possible role of the toxin in nature. The few available data related to eukaryotic algae show that the effects of CYN or CYN-containing cyanobacterial extracts depend on concentration and on target organism (Campos et al. 2013; Pinheiro et al. 2013; Rzymiski et al. 2014; B-Béres et al. 2015). According to some studies, low CYN concentrations may stimulate algal growth (*Chlorella vulgaris*, 0.005–0.179  $\mu\text{g mL}^{-1}$  purified CYN; Campos et al. 2013, *Chlamydomonas reinhardtii*, *C. vulgaris*, and *Nannochloropsis* sp., 0.025–0.5  $\mu\text{g mL}^{-1}$  semi-purified CYN; Pinheiro et al. 2013), but crude extracts with 0.032 and 0.333  $\mu\text{g mL}^{-1}$  CYN concentrations inhibited *C. vulgaris* significantly (Campos et al. 2013).

Some authors have suggested that cyanobacterial metabolites may play a crucial role in allelopathy, which can be an important factor in the organization and formation of algal assemblages in all types of surface waters, especially in the case of those with low water velocity or standing waters (Leflaive and Ten-Hage 2007). Toxic cyanobacterial species can affect negatively the other members of assemblages both by their presence (e.g., by shading and nutrient uptake) and by allelopathic compounds, although it is still not clearly stated whether cyanotoxins can be considered as allelochemicals (Leão et al. 2009; B-Béres et al. 2012).

Bar-Yosef et al. (2010) suggested allelopathic effects of CYN-producing *Aphanizomenon ovalisporum* on eukaryotic algae in the field, influencing their presence, growth, and alkaline phosphatase (APase) activity. They reported strong correlation between *A. ovalisporum* abundance in Lake Kinneret and APase activity; their results suggest that members of phytoplankton are forced to APase secretion by CYN producer strains (Bar-Yosef et al. 2010). Similar phenomena were observed when laboratory cultures of *C. reinhardtii* and *Debarya* sp. were treated with purified CYN or CYN-containing cyanobacterial extract (Bar-Yosef et al. 2010). Upregulated APase activity was also reported recently in a *Microcystis aeruginosa* strain, and the study also

indicated that CYN may inhibit microcystin production (Rzymiski et al. 2014). In contrast to enzyme stimulation, and affected toxin production, the applied lower concentrations of CYN (0.001 and 0.005  $\mu\text{g mL}^{-1}$ ) caused only slight growth inhibition of the unicellular cyanobacterium (Rzymiski et al. 2014).

Modelling the possible roles of toxic metabolites in the environment is quite complicated. Application of purified metabolites is required to specify exact effects, although use of them may lead weaker responses than using crude extracts (Bar-Yosef et al. 2010; Campos et al. 2013). The reason of this phenomenon is the presence of other metabolites in the extracts beside the toxins, which probably can interact with the toxins influencing their effect on algal species (Bittencourt-Oliveria et al. 2015). The use of cyanobacterial extracts instead of purified toxins may seem to be an environmentally relevant approach in modelling certain circumstances (e.g., collapse of a toxic bloom; Bittencourt-Oliveria et al. 2015, 2016). On the other hand, application of crude extracts is not the best way for studying allelopathic interactions, since extracts contain compounds, which are not actively released by intact cells, but only due to cell lysis, and allelopathic reactions are mediated by living (and not lysed) organisms (Leflaive and Ten-Hage 2007). However, despite the fact that several studies suggest important environmental roles of CYN, it is still a question whether CYN producers really compete with other phytoplankton species due to their CYN-producing ability. The dominance of CYN producer strains in the environment has been reported several times and the involvement of CYN in competitive advantages is proved in certain cases (Soares et al. 2009; Bar-Yosef et al. 2010; Karadžić et al. 2013; Rzymiski et al. 2014). However, in many other cases, it seems that the dominance of CYN producers would be hard to explain exclusively with their CYN production ability (Rzymiski and Poniedziałek 2014; Burford et al. 2016; Aguilera et al. 2017; Zhang et al. 2017).

CYN occurs in the habitats of aquatic algal assemblages either actively excreted or released during cell lysis. Although the potential synergistic role of other simultaneously produced bioactive compounds (i.e., that the cellular matrix affects the toxicity of CYN) is suggested by several studies (reviewed by Rzymiski and Poniedziałek 2014), there are no studies—at least according to our knowledge—dealing more directly with this question. Previous work of our laboratory showed that the inhibitory effects of crude extract of CYN-producing cyanobacterium depend on cell debris presence: cell debris-free crude extracts caused stronger growth inhibition than cell debris-containing extracts. Those results suggest already that cellular matrix could have a significant role in the effect of CYN (B-Béres et al. 2015).

In this present study, effects of CYN producer *Aphanizomenon* crude extract (C+), non-CYN producer *Aphanizomenon* crude extract (C-), and non-CYN producer

*Aphanizomenon* crude extract supplemented with CYN (C−+C) on the planktonic green alga *Scenedesmus obtusius* were investigated. We assumed that (i) growth of the green algal cultures will be inhibited and (ii) phosphatase activity will be induced by CYN in C+ and C−+C treatments, while the C− extract will have opposite effects. Preliminary experiments proved that phosphatases of *S. obtusius* have pH optima at pH 5 and 9; therefore, effects of the different crude extracts on phosphatase activities were measured both at pH 5 and 9. The former measurements could provide new results about the effects of CYN on eukaryotic algal metabolism.

## Materials and methods

### Strains and culturing conditions

The CYN producer *Aphanizomenon* strain (ACCDH-UD1001; C+) is the derivative of BGSD-423, which is derived from ILC-164 isolated in 1994 from Lake Kinneret, Israel. The non-CYN producer *Aphanizomenon* strain (ACCDH-UD1304; C−) was isolated in 2012 from a recreational lake in Debrecen, Hungary. The C− strain was identified as *Aphanizomenon* on the basis of morphological characteristics using Komárek (2013). Light microscopic observations were done with an Olympus BX50F-3 microscope at ×400 magnification, and measurements were carried out using an Olympus DP80 digital camera and cellSens Standard software.

The cosmopolitan, eukaryotic green alga *S. obtusius* strain (ACCDH-UD1310) was isolated in 2013 from a small pond of pond sliders in Debrecen, Hungary. The strain was identified on the basis of morphological characteristics using Hindák (1990), and microscopic observations were carried out using the same equipment as described above.

The strains are maintained in the Algal Culture Collection, Department of Hydrobiology, University of Debrecen as standing and sterile air-bubbled cultures under a 14-h light (40  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) and 10-h dark photoperiod at 24 °C.

### Preparation of cyanobacterial crude extracts and experimental design

For the preparation of C+ and C− cyanobacterial crude extracts, *Aphanizomenon* strains were cultured in 4-L Erlenmeyer flasks for 10 days, under the conditions described above. The 10-day-old cultures (with a density  $1.12 \pm 0.06 \text{ mg dry weight mL}^{-1}$ ) were centrifuged (6000×g, 10 min, Beckman Avanti J-25). The supernatants were removed and the cells were disrupted by freezing, thawing, and sonication (5 min, Bandelin Sonorex RK 103 H ultrasonic bath) at least

three times. This material was centrifuged again, and the clear blue supernatants were used as crude extracts.

Exact concentration of CYN of the toxic crude extract was measured by capillary electrophoresis (PrinCE-C 700, fused silica capillary with 80 cm total length and 50  $\mu\text{m}$  i.d.; 100 mbar 0.15 min hydrodynamic injection, +25 kV voltage, 20 min running time). CYN standard was purified in the laboratory of the Department of Botany, University of Debrecen according to Vasas et al. (2002).

For C+ treatments, crude extract of CYN-producing *Aphanizomenon* strain was added to the *S. obtusius* cultures to reach 1.0, 1.5, 2.0, and 2.5  $\mu\text{g mL}^{-1}$  CYN concentration (marked as 1.0, 1.5, 2.0, and 2.5 C+). For C− treatments, the crude extract of the non-CYN-producing *Aphanizomenon* strain was added to the *S. obtusius* cultures in equivalent amount with the C+ one. Required volumes were calculated on the basis of dry mass (marked as 1.0, 1.5, 2.0, and 2.5 C−). In the case of treatments with C− crude extracts supplemented with CYN (C−+C), the amounts of C− crude extract were calculated similarly to that of C− treatments, and purified CYN was added from stock solution with known concentration to reach 1.0, 1.5, 2.0, and 2.5  $\mu\text{g mL}^{-1}$  CYN concentration (marked as 1.0, 1.5, 2.0, and 2.5 C−+C). For quantification of CYN content of the cultures, 3 mL of culture samples was centrifuged (16,200×g, 5 min; 24 °C, Heraeus Fresco 17 centrifuge) and the pellets and supernatants were lyophilized separately. Lyophilized supernatants were treated as described in B-Béres et al. (2015). Limit of detection (LOD) was 1  $\mu\text{g mL}^{-1}$  and limit of quantification (LOQ) was 2.5  $\mu\text{g mL}^{-1}$  for the applied method. Maximum tenfold concentrations were applicable in the case of the supernatants, so 0.1 and 0.25  $\mu\text{g mL}^{-1}$  were the minimum amounts of CYN for detection and quantification, respectively (B-Béres et al. 2015).

The experiments were carried out in shaken cultures (SOH-D2 shaker, 90 rpm), in Jaworski's medium (CCAP Media Recipes n.d.) in 100-mL Erlenmeyer flasks with 50 mL final volume. Cultures were kept in a 14-h light (40  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) and 10-h dark photoperiod at 24 °C. The time of exposure was 14 days. Phosphate starvation was achieved under the same conditions in Jaworski's medium lacking phosphate.

So-called negative control cultures containing only cyanobacterial crude extracts (not inoculated with the green alga) were also prepared to check the chlorophyll content and phosphatase activity of the cyanobacterial crude extracts.

### Measurement of the growth of the cultures

Growth of the cultures was followed by counting the number of coenobia and by measuring chlorophyll-*a* content. Coenobia numbers were counted from 10  $\mu\text{L}$  samples in a Bürker chamber using an Olympus BX50F-3 microscope at ×400 magnification. Structural composition of coenobia

(single cells, two- and four-celled coenobia) was also recorded. Samples of 1 mL were collected on 0, 7th, and 14th days for chlorophyll-*a* content measurements. Samples were centrifuged (16,200×*g*, 5 min, Heraeus Fresco 17), and supernatants and pellets were separated and stored at −20 °C before further processing. Chlorophyll-*a* contents were measured from the pellets spectrophotometrically (Hach Lange DR 6000 UV-VIS spectrophotometer) after methanolic extraction according to the method of Felföldy (1987). To give EC<sub>50</sub> value, the extents of growth inhibitions (%; considered that control shows 100% growth) were plotted as functions of CYN concentrations and trend lines were fitted. The concentrations causing 50% inhibition were calculated from the equations of the trend lines.

### Measurement of phosphate uptake

Samples of 1 mL were collected on every second day, samples were centrifuged (16,200×*g*, 5 min, Heraeus Fresco 17), and the supernatants were removed and stored at −20 °C before further processing. Inorganic dissolved phosphate concentrations were measured from 200 μL aliquots of the supernatants by the acidic molybdate method (MSZ EN ISO 6878 2004). On the basis of the amounts of remaining phosphate, phosphate uptake was calculated to a unit (10<sup>6</sup>) of coenobia.

### Measurement of phosphatase activity

Preliminary experiments showed that phosphatase enzymes of *S. obtusus* had maximal activity at pH 5 and pH 9. Therefore, the reaction mixtures were buffered to pH 5 and 9 with potassium hydrogen phthalate and sodium tetraborate, respectively. The measurements were based upon the modified methods of Tabatabai and Bremner (1969) and Ihlenfeldt and Gibson (1975). The reagent mixtures contained 400 μL of sample, 500 μL pH 5 or 9 buffer, and 400 μL 8 mM *p*-nitrophenyl-phosphate (pNPP). The reaction mixtures were incubated for 60 min at 24 °C in darkness. The reaction was stopped by adding 500 μL of 0.2 M Na<sub>2</sub>HPO<sub>4</sub> in 1 M NaOH. The reaction mixtures were centrifuged for 1 min at 1000×*g* (Heraeus Fresco 17) and the amounts of the liberated *p*-nitrophenol (pNP) were measured at 400 nm (Hach Lange DR 6000 UV-VIS spectrophotometer). The complete reaction system stopped at zero time served as blank. Enzyme activities were calculated as μmol pNP 10<sup>6</sup> coenobia<sup>−1</sup> h<sup>−1</sup>.

### Statistical analysis

All experiments were done in triplicate. One-way analysis of covariance (ANCOVA) was used to check the significances among tendency differences of curves of control and treated cultures for growth and phosphate uptake (Zar 1996; Hammer et al. 2001). For statistics of chlorophyll-*a* content changes

and phosphatase activity changes, data were subjected to analysis of variance (two-way repeated measure ANOVA for treatment and time). Tukey's test as multiple comparison procedure was used to show the significant differences between means at the 5% level. PAST software was used for statistical analysis (Hammer et al. 2001).

## Results

### Growth of the *S. obtusus* cultures

There were no significant differences among the growth tendencies of the *Scenedesmus* strain in control, in C+ treated, or in phosphate-limited cultures (Fig. 1a). However, the growth of the treated cultures was differently affected in the different phases of the exposure. Growth of the cultures was stimulated by the C+ crude extracts on the first week, then growth inhibition was observed after the ninth day of cultivation (Fig. 1a). The extent of inhibition increased with the increasing CYN concentration of the C+ crude extract. Since the growth inhibition exceeded 50% only at the 14th day of exposure, effective concentration of CYN causing 50% growth inhibition (EC<sub>50</sub>) could be calculated only for 14-day-old cultures, which was 2.85 μg mL<sup>−1</sup>.

Phosphate depletion caused no growth inhibition within the timeframe of the experiment (Fig. 1a).

Growth tendencies differed significantly ( $p < 0.05$ ) in cases of control vs 1.5, 2.0, and 2.5 C− treatments (Fig. 1b). EC<sub>50</sub> for the C− crude extract could not be calculated because growth inhibition was not observed; in contrast, growth stimulation occurred. The extent of stimulation increased with the increasing amount of the C− crude extract (Fig. 1b), and the same phenomena occurred also in C−+C treatments (Fig. 1c).

Comparing the C+, C−, and C−+C treatments, significantly higher ( $p < 0.05$ ) numbers of coenobia were observed in certain C− and C−+C-treated cultures than in C+ treated ones. There were no significant differences among the coenobia numbers in C− and C−+C treatments on the 14th day (Fig. 1d). The treatments did not cause significant changes in coenobial structure of the *S. obtusus* isolate (data not shown).

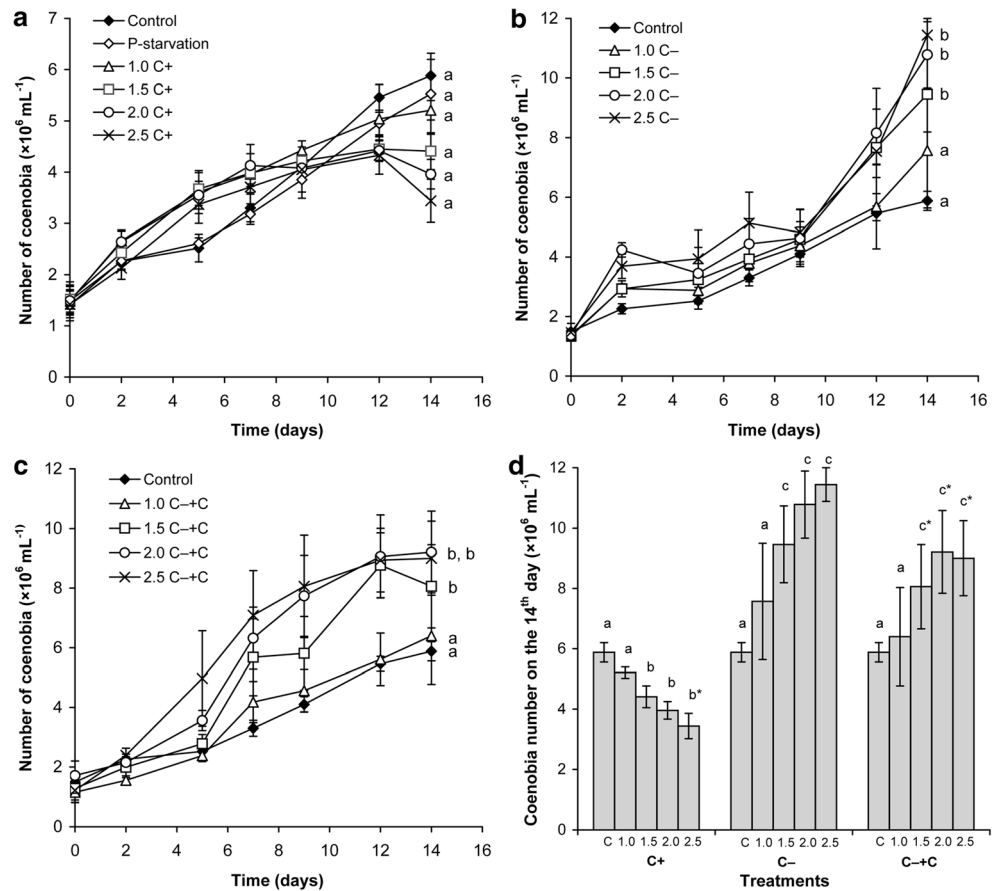
C− and C−+C treatments contained significantly ( $p < 0.05$ ) higher amounts of chlorophyll than C+ treatments on the seventh day in the case of all applied concentrations, while there was significantly higher chlorophyll content only in the case of 1.5 C−+C treatment on the 14th day. Nevertheless, chlorophyll content increased with the increasing amounts of the cyanobacterial crude extracts in all cases (Fig. 2).

### Phosphate uptake of the *S. obtusus* cultures

A decreasing trend in phosphate uptake of a unit of coenobia was observed both in the case of control and phosphate-



**Fig. 1** Coenobia number changes in *Scenedesmus obtusius* cultures in different treatments. **a** Cultures treated with CYN-producing *Aphanizomenon* crude extract (C+), **b** cultures treated with non-CYN-producing *Aphanizomenon* crude extract (C−), and **c** cultures treated with non-CYN-producing *Aphanizomenon* crude extract supplemented with CYN (C−+C). **d** Coenobia number at the end (on the 14th day) of the experiments. Numbers (1.0–2.5) refer to CYN content in  $\mu\text{g mL}^{-1}$  and equivalent amounts of C− crude extract. Mean values ( $n = 3$ ) and standard deviations are plotted. Significant differences ( $p < 0.05$ ) among the growth tendencies and coenobia number values of different treatments are indicated with different lowercase letters



depleted cultures (Fig. 3a). Although the statistical analysis did not show significant differences among phosphate uptake tendencies of control and differently treated cultures, there was significantly higher phosphate uptake in C+ crude extract-treated cultures than in C− and C−+C crude extract-treated cultures on the 12th–14th days of the experiments (Fig. 3b, c).

### Phosphatase activities in the *S. obtusius* cultures

Acidic phosphatase activity of control, phosphate-deficient, and C+–treated cultures increased over time. Furthermore, increasing activities were observed with the increasing amount of the crude extract. Activities on the 7th and 14th days were significantly higher ( $p < 0.005$ ) than on the 0 day in each cultures (Fig. 4a).

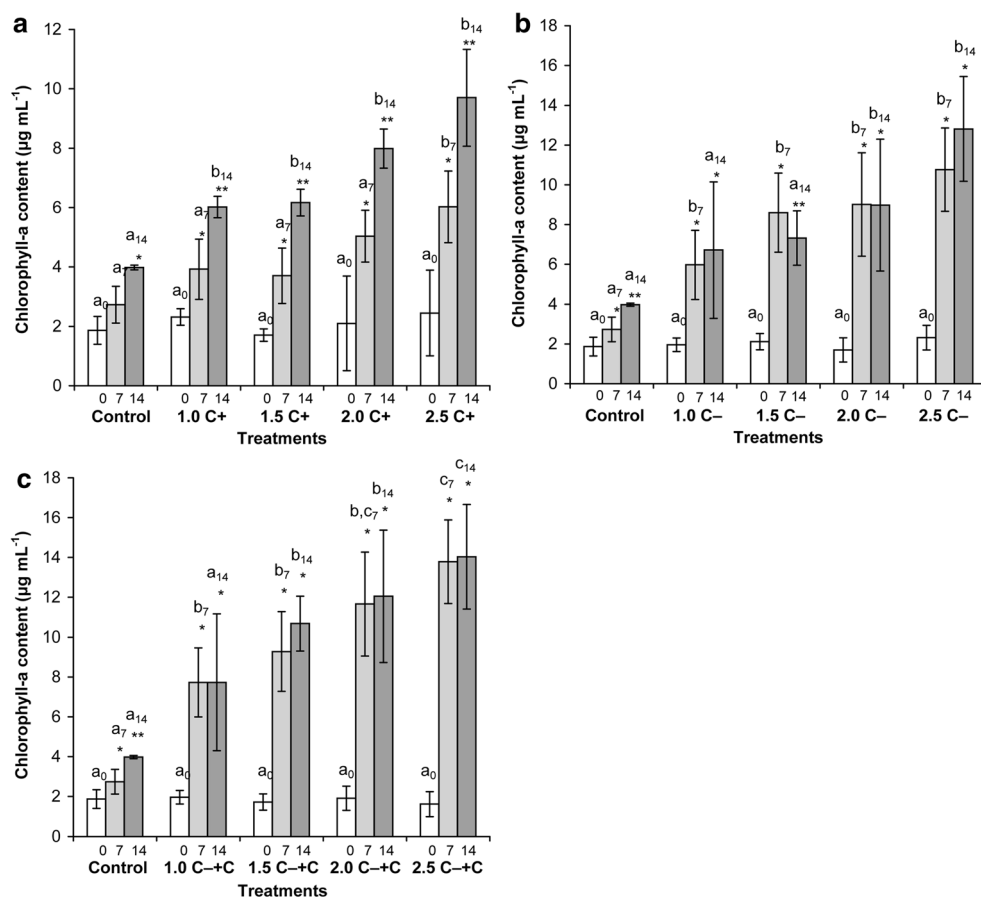
Acidic phosphatase activities were lower in the C−–treated cultures than in C+ treatments on each sampling day. Furthermore, acidic phosphatase activities in the 1.5, 2.0, and 2.5 C− treatments were significantly lower on the 7th and 14th days than in control. Acidic phosphatase activities did not change significantly over time in any C− treatments (Fig. 4b).

Increasing acidic phosphatase activities were observed in C−+C–treated cultures both in time and with the increasing concentration of added CYN, similarly to C+ treatments (Fig. 4c). The measured values were significantly higher ( $p < 0.05$ ) than in the control (Fig. 4c).

Comparing the C+, C−, and C−+C treatments on the same days, it can be said that acidic phosphatase activities of C+ treatments were the highest, significantly higher ( $p < 0.005$ ) than in the C−– and C−+C–treated cultures on the 7th and 14th days. Significantly higher ( $p < 0.05$ ) activities were measured in C−+C–treated cultures than in C−–treated ones on the 14th day.

Alkaline phosphatase activity was significantly higher ( $p < 0.001$ ) in the phosphate-starved culture than in control on every sampling days. In case of C+ treatments, the highest alkaline phosphatase activities were measured on the seventh day in the treated cultures; however, these activities decreased both in time (to the 14th day) and with the increasing amount of C+ crude extract (Fig. 5a). It has to be emphasized that alkaline phosphatase activities were significantly higher than in control both on the 7th and on the 14th days in every C+ treatment. The highest activity was measured in 1.0 C+ treatment on the seventh day, which latter was significantly higher even than the activity in phosphate starved culture (Fig. 5a).

**Fig. 2** Chlorophyll-*a* content changes in differently treated *Scenedesmus obtusius* cultures. **a** Cultures treated with CYN-producing *Aphanizomenon* crude extract (C+), **b** cultures treated with non-CYN-producing *Aphanizomenon* crude extract (C−), and **c** cultures treated with non-CYN-producing *Aphanizomenon* crude extract supplemented with CYN (C−+C). Numbers (1.0–2.5) refer to CYN content in  $\mu\text{g mL}^{-1}$  and equivalent amounts of C− crude extract. Numbers 0, 7, and 14 refer to the sampling days. Mean values ( $n = 3$ ) and standard deviations are plotted, and significant differences ( $p < 0.05$ ) among 0, 7th, and 14th days within a certain treatment are indicated with asterisks (\*, \*\*). Significant differences ( $p < 0.05$ ) among the different concentrations on a given day are indicated with different lowercase letters (with the given day (0, 7, 14) in subscript)



Alkaline phosphatase activities in the C−-treated cultures were not higher than in the control; moreover, activities on the 7th and 14th days were significantly lower ( $p < 0.05$ ) than in control on the same days (Fig. 5b).

Alkaline phosphatase activity increased in the C−+C-treated cultures during the 14 days of the experiments (Fig. 5c). Tendencies were similar to that of control, highest activities were measured on the 14th day, significant difference ( $p < 0.005$ ) was shown only in the case of 1.0 C−+C treatment on the 14th day (Fig. 5c).

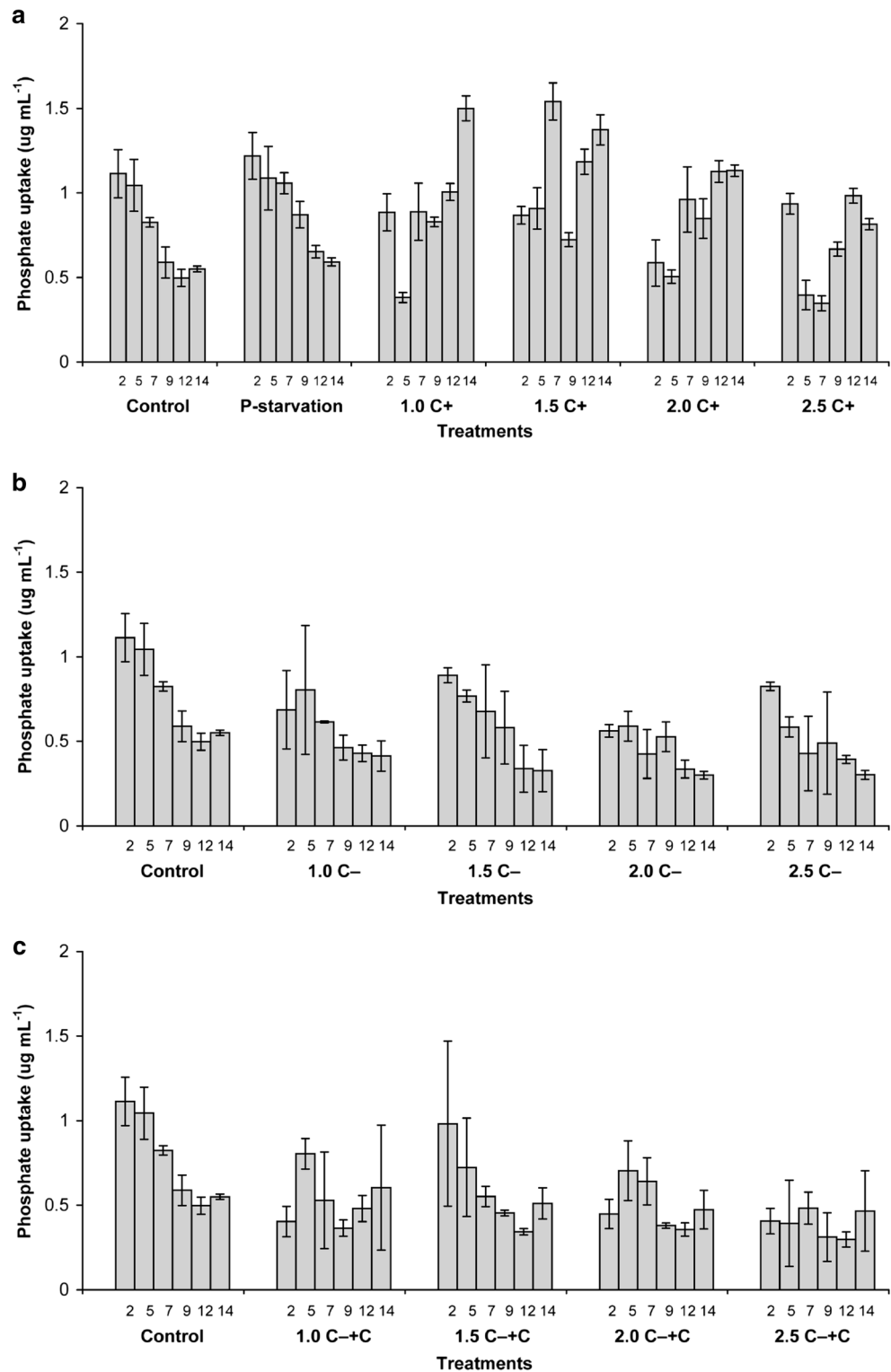
Comparing the alkaline phosphatase activities of the different treatments, there were significantly higher measured values ( $p < 0.005$ ) in all C+ treatments than in C− and C−+C treatments on the seventh day.

## Discussion

**Growth** It was assumed that growth of the green algal cultures would be inhibited in C+ and C−+C treatments, while the C− extract would have opposite effects. This assumption was only partially justified. CYN-producing *Aphanizomenon* crude extract (C+) caused slight growth inhibition only from the ninth day of the experiment. In contrast to our expectation, C−+C

crude extracts stimulated growth and no growth limitation was observed during the whole timeframe of the experiment (similarly to C− treatments), regardless of the extract that was supplemented with CYN. The growth stimulation can be explained by the presence of nutrients and hormone-like compounds in the crude extracts, which could stimulate growth and compensate for the negative effects of CYN (Sergeeva et al. 2002; Stirk et al. 2002; Tsavkelova et al. 2006; Hussain et al. 2010). At the same time, growth stimulation of eukaryotic algae by low CYN concentrations of cyanobacterial crude extracts has been reported already in the case of a few other green algal species (*C. reinhardtii*, *C. vulgaris*, and *Nannochloropsis* sp.; Pinheiro et al. 2013).  $EC_{50}$  could be calculated only for the 14th day and it was  $2.85 \mu\text{g mL}^{-1}$  CYN in the case of the investigated *Scenedesmus* strain. This value is much higher than reported by Campos et al. (2013) for *C. vulgaris*, which was  $0.333 \mu\text{g mL}^{-1}$  CYN-containing crude extract caused 48% decrease in growth rate after 3 days of exposition. Pinheiro et al. (2013) also reported more than 50% growth rate inhibition by  $2.5 \mu\text{g mL}^{-1}$  CYN-containing cyanobacterial crude extract in the case of *C. reinhardtii*, *C. vulgaris*, and *Nannochloropsis* sp. already after 4 days of exposure. These results and the lack

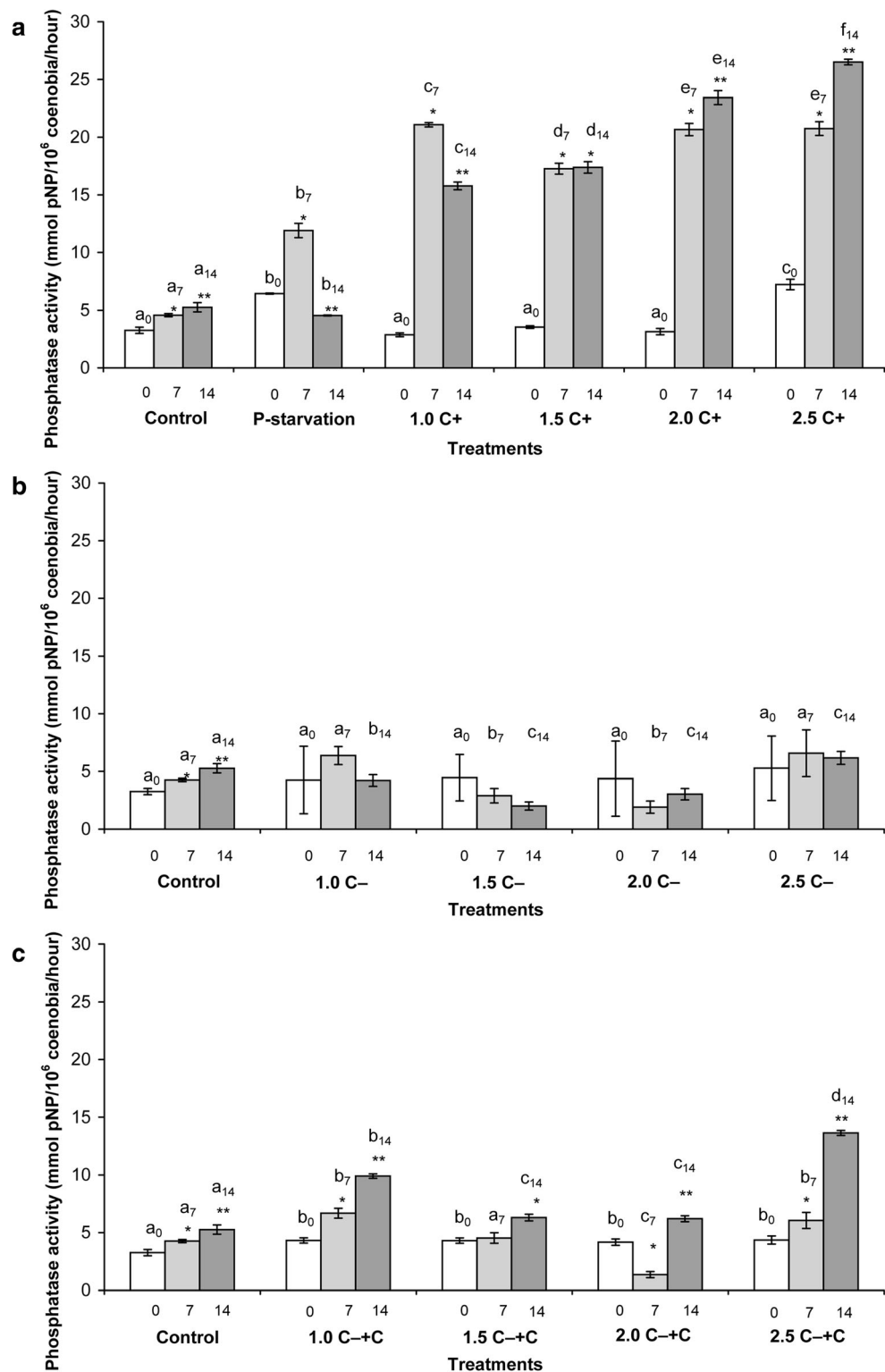
**Fig. 3** Phosphate uptake changes in differently treated *Scenedesmus obtusus* cultures. **a** Cultures treated with CYN-producing *Aphanizomenon* crude extract (C+), **b** cultures treated with non-CYN-producing *Aphanizomenon* crude extract (C–), and **c** cultures treated with non-CYN-producing *Aphanizomenon* crude extract supplemented with CYN (C–+C). Numbers (1.0–2.5) refer to CYN content in  $\mu\text{g mL}^{-1}$  and equivalent amounts of C– crude extract. Numbers 2–14 refer to the sampling days. Mean values ( $n = 3$ ) and standard deviations are plotted



of coenobial structural changes clearly show that the used *Scenedesmus* strain was not sensitive to the relatively high concentration of CYN. Moreover, as the results showed, this strain also was not sensitive to phosphate limitation, which phenomenon could be due to the presence of polyphosphate bodies (PBI) in the cells (Rhee 1973).

The lack of growth inhibition during C– treatments suggests that CYN probably had a role in the growth inhibition of cultures treated with C+ crude extract. At the same time, the similar lack of growth inhibition in the case of CYN-supplemented C– crude extract treatments (C–+C-treated cultures) shows that supplement of the crude extract of the phenotypically similar,

**Fig. 4** Acidic phosphatase activities in differently treated *Scenedesmus obtusius* cultures. **a** Cultures treated with CYN-producing *Aphanizomenon* crude extract (C+), **b** cultures treated with non-CYN-producing *Aphanizomenon* crude extract (C−), and **c** cultures treated with non-CYN-producing *Aphanizomenon* crude extract supplemented with CYN (C−+C). Numbers (1.0–2.5) refer to CYN content in  $\mu\text{g mL}^{-1}$  and equivalent amounts of C− crude extract. Numbers 0, 7, and 14 refer to the sampling days. Mean values ( $n = 3$ ) and standard deviations are plotted, significant differences ( $p < 0.05$ ) among 0, 7th, and 14th days within a certain treatment are indicated with asterisks (\*, \*\*). Significant differences ( $p < 0.05$ ) among the different concentrations on a given day are indicated with different lowercase letters (with the given day (0, 7, 14) in subscript)



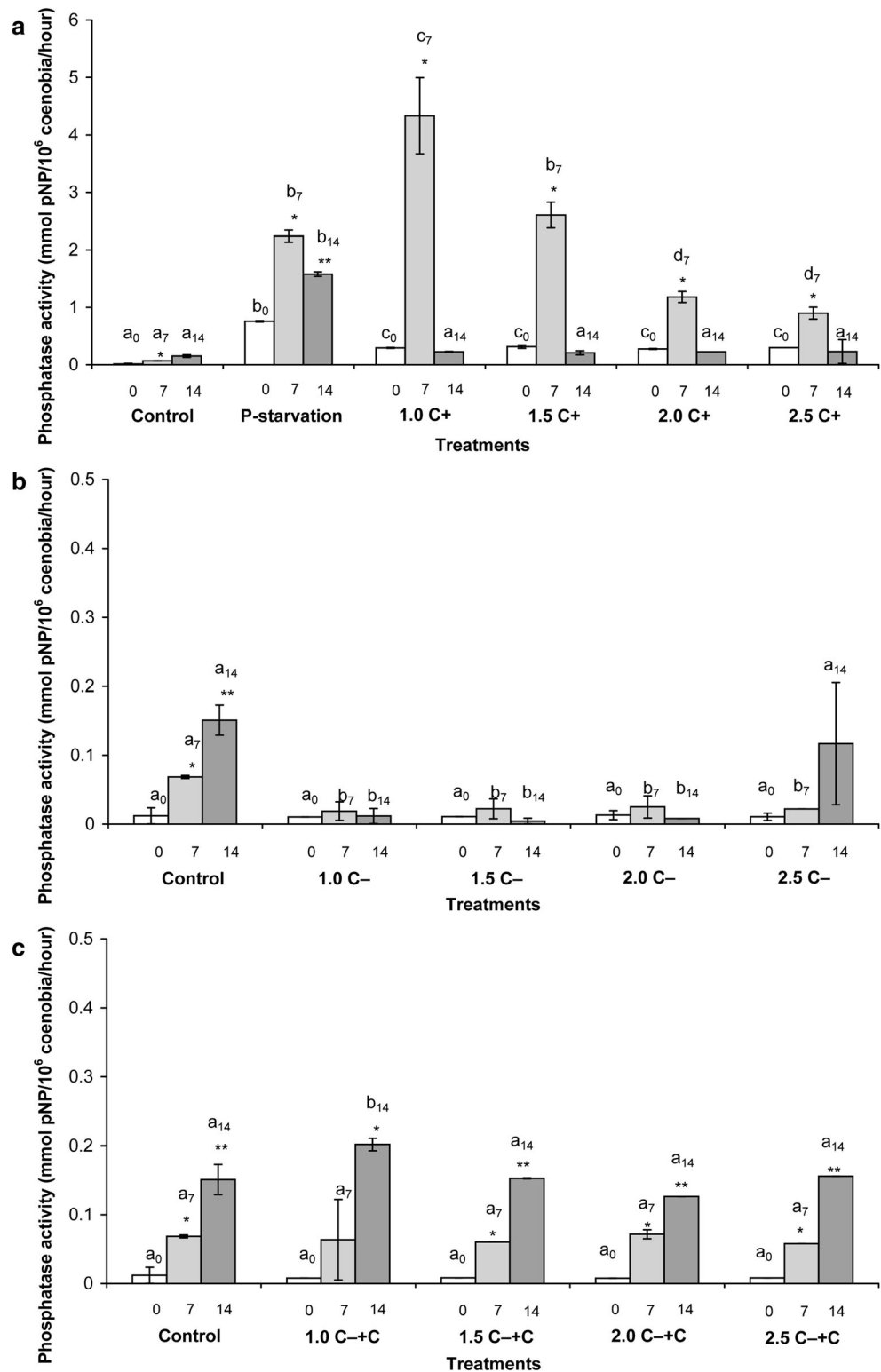
non-CYN-producing *Aphanizomenon* with CYN did not lead to similar phenomena. This suggests that the contribution of molecules in the cellular matrix of the producer organism to the effects of CYN is potentially plausible.

Chlorophyll content of cultures increased independently of the toxin content of the crude extracts. The strong blue color of

the crude extracts resulted in a strong shading effect; thus, the increasing chlorophyll content could be a compensatory reaction to the lower amount or changed wavelength of light (Carvalho et al. 2009; Bonente et al. 2012; He et al. 2015; Ferreira et al. 2016). The lack of inhibition of chlorophyll synthesis also supports the insensitivity of the used



**Fig. 5** Alkaline phosphatase activities in differently treated *Scenedesmus obtusius* cultures. **a** Cultures treated with CYN-producing *Aphanizomenon* crude extract (C+), **b** cultures treated with non-CYN-producing *Aphanizomenon* crude extract (C−), and **c** cultures treated with non-CYN-producing *Aphanizomenon* crude extract supplemented with CYN (C−+C). Numbers (1.0–2.5) refer to CYN content in  $\mu\text{g mL}^{-1}$  and equivalent amounts of C− crude extract. Numbers 0, 7, and 14 refer to the sampling days. Mean values ( $n = 3$ ) and standard deviations are plotted, and significant differences ( $p < 0.05$ ) among 0, 7th, and 14th days within a certain treatment are indicated with asterisks (\*, \*\*). Significant differences ( $p < 0.05$ ) among the different concentrations on a given day are indicated with different lowercase letters (with the given day (0, 7, 14) in subscript)



*Scenedesmus* strain to CYN and highlights that important phenomena could be lost if growth is investigated exclusively on the basis of chlorophyll content.

**Phosphate uptake** Phosphate uptake slightly increased with the increasing amount of C+ crude extract compared both to

control and to all the other treatments (including phosphate starvation). Since there were fewer coenobia in C+ crude extract-treated cultures from the ninth day, the phenomenon means that the fewer coenobia took up more phosphate in the case of C+ treatments. Although more intense phosphate uptake does not require necessarily higher phosphatase activity,

these results are in accordance with the results of alkaline phosphatase in C+ treatments. It could be possible that CYN contribute to more intense phosphate uptake beside the appearance of the high-affinity  $P_i$  transporter in the CYN-producing cyanobacterium, and without the producer organism (the living cyanobacterium), the toxin slightly induced the higher phosphate uptake of the target green alga. Of course, the proof or disproof of these assumptions definitely requires further investigations. Although the statistical analysis did not show significant differences in phosphate uptake of the different treatments, based on the phenomena detailed above, it can be presumed that CYN affects phosphate uptake of the studied green alga but only when it is the original component of the crude extract (e.g., intermediates of its synthesis or degradation could be present), and similar effects of the added CYN (if any) are masked by other components during the C+ C treatments.

**Phosphatase activity** It was assumed that phosphatase activity of the green algal cultures would be induced in C+ and C+ C treatments, while the C- extract will have opposite effects. This assumption was justified in the case of acidic phosphatase activity. Acidic phosphatase activities increased in the C+ crude extract-treated cultures compared to control on each sampling day. Moreover, activities increased with the increasing amount of the crude extract. Acidic phosphatases are mostly intracellular enzymes and are necessary for the mobilization of intracellular phosphate storages (polyphosphate bodies; Bowen and Bryant 1978; DuBois et al. 1984). One possible explanation of increasing acidic phosphatase activity during C+ treatments could be the increased stress. It is reported that acidic phosphatase activity may increase when cells are exposed to any external stress factors (e.g., dark periods in case of *Nostoc* sp., DuBois et al. 1984; limiting or low nitrogen and phosphate concentration in the environment, Kruskopf and Du Plessis 2004). Shading effect of the crude extract and the myriads of organic molecules present could mean a stressful environment. The results of phosphorous uptake measurements and the fact that elevated acidic phosphatase activities were measured only in the presence of CYN exclude the possibility of limited phosphorous bioavailability caused by the chemical matrices of the crude extracts. The possibility that CYN not induce higher expression of acidic phosphatase directly but via limitation of P transport also can be excluded based on the results of phosphorous uptake measurements. Although the measured activities were significantly lower, trends in acidic phosphatase activities in C+ C treatments were more similar to that of C+ treatments. This phenomenon and the significantly higher acidic phosphatase activities on the 14th day in C+ C-treated cultures than in C- treated ones suggest that CYN has a role in the elevated acidic phosphatase activities. According to our knowledge, the phenomenon has not been described before and it is not clear why

and how CYN induce acidic phosphatases. Elevated acidic phosphatase activities might be connected to signal transduction processes initiated by CYN (Freitas et al. 2015); however, the exact explanation undoubtedly requires further investigation. Acidic phosphatase activity increased also in the phosphate starved culture, and it was significantly higher on every sampling day. The phosphate-starved culture obviously was not able to take up enough external phosphate; therefore, it started to consume its internal polyphosphate storages which required a higher acidic phosphatase activity.

The assumption about induced phosphatase activity was partially justified in the case of alkaline phosphatase. The liberation of bound phosphate (e.g., hydrolysis of phosphoester bonds) is the primary role of the alkaline phosphatases (Kuenzler 1965; Kuenzler and Perras 1965; Cembella et al. 1984); thus, elevated alkaline phosphatase levels are a general phenomenon among phosphate-limited circumstances. High alkaline phosphatase activity was detected on the seventh day in the case of C+ treatments. This activity was higher in 1.0 C+ treatment than in the phosphate starved culture and decreased with the increasing amount of crude extract but remained higher than in control even on the 14th day. Higher alkaline phosphatase activity was also shown in *C. reinhardtii* cultures, which were inoculated to *A. ovalisporum* spent medium ( $7\text{--}8 \mu\text{g L}^{-1}$  CYN) or were treated with purified CYN ( $50 \mu\text{g mL}^{-1}$ , Bar-Yosef et al. 2010). Increasing alkaline phosphatase activity was also observed in *Microcystis panniformis* cultures in the presence of *A. ovalisporum* (Zhang et al. 2016). Rzymiski et al. (2014) did not observe higher alkaline phosphatase activity in *M. aeruginosa* cultures treated with their highest ( $0.05 \mu\text{g mL}^{-1}$ ) CYN concentration than in control, which is in accordance with our observations about decreasing alkaline phosphatase activity with increasing CYN concentration. This phenomenon suggests that higher CYN concentration may inhibit more physiological processes, for example protein synthesis (Froschio et al. 2001, 2003, 2008).

However, these effects were not observed in C- experiments. There were increasing trends of alkaline phosphatase activities in C+ C experiments, and there were significantly higher values of alkaline phosphatase activity than in C- treatments, although alkaline phosphatase activity was significantly higher than in control only in 1.0 C+ C treatment on the 14th day. The slight alkaline phosphatase induction in C+ C treatment suggests the presence of other metabolites in the extract originally containing CYN (C+ crude extract), which exert synergistic effect. This is in accordance with the results of Bar-Yosef et al. (2010): enzyme activity was higher in case of cyanobacterial media (containing  $7\text{--}8 \mu\text{g mL}^{-1}$  CYN), than in case of purified toxin ( $50 \mu\text{g mL}^{-1}$ ). Existence of molecules able to induce alkaline phosphatases is also suggested by the results of Rzymiski et al. (2014), which indicates that non-CYN-producing *C. raciborskii* strains are able to produce

different extracellular compounds with a similar mode of action than CYN.

## Conclusions

In this study, effects of crude extracts of phenotypically closely related *Aphanizomenon* strains were introduced on growth, phosphate uptake, and phosphatase activities of a green alga *S. obtusus*. Responses of the green alga in phosphate-limited circumstances were also investigated. Our results show that the *Scenedesmus* strain is sensitive neither to the relatively high concentration of CYN nor to phosphate limitation. Nonetheless, alkaline phosphatase activity of algal cells was significantly higher during C+ treatments; therefore, the concept that CYN is forcing other phytoplankton species in the environment to produce alkaline phosphatase was confirmed also in the case of an insensitive species. Acidic phosphatase activity also increased during C+ treatments. The lack of growth inhibition and phosphatase induction in C− treatments strongly support the role of CYN in these phenomena. At the same time, the lack of growth inhibition and the weaker effects on phosphatases in the case of C−+C treatments highlight the possible role of synergistic metabolites in originally CYN-containing crude extract. Our results also suggest that these metabolites together with CYN contribute to external phosphate uptake, since in the lack of the living CYN producer (*Aphanizomenon*), the phosphate uptake of the treated green alga increased. Currently, it is unknown how these theoretical additional compounds may support CYN action. To assess whether they directly contribute to the effects of CYN or rather they influence CYN bioavailability requires further studies. The results presented here suggest that CYN, together with other molecules of its producer, could affect significantly even non-sensitive phytoplankton species, thus could affect the processes in algal assemblages.

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**Author contribution** Experiments were performed by D.D. and V.B.-B. (Figs. 1, 2, 3, 4, and 5). S.G. and G.V. provided the purified CYN and contributed to the preparation of crude extracts and instrumental analytical measurements. S.A.N. provided additional financial support for the experiments. D.D., G.V., and I.B. related to conception and design of the study, acquisition of data, analysis and interpretation of data, and drafting the article. I.B. supervised the whole work and finalized the manuscript.

## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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