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

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Abstract

A few 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 can not 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; Rzymski and Poniedziałek, 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 (Poniedziałek et al. 2012). Moreover, next to aquatic species, the soil cyanobacterium *Hormoscilla pringsheimii* was also 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 (Rzymski and Poniedziałek, 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. In the same time, CYN-producing *Aphanizomenon* and *Anabaena* species are described from all over the world (Rzymski and Poniedziałek, 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 (Humpage et al. 2000; Shen et al. 2002) and irreversibly inhibits glutathione and protein synthesis (Terao et al. 1994; Runnegar et al. 1995; Froscio 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

70 compound may increase its toxicity, thus CYN is considered mainly as hepatotoxin (Bernard
71 et al. 2003; Fastner et al. 2003; Saker et al. 2003). Most recently the effects of CYN on the
72 different cells of immune systems were also reported (Poniedziałek et al. 2012a,b; 2014a,b). It
73 seems that the toxicity of CYN is mediated through cytochrome P450 (Pearson et al. 2010),
74 and oxidative stress (Rymuszka and Sieroslawska 2014; Poniedziałek et al. 2015), which is
75 followed by all the above mentioned phenomena.
76 The reason of the toxin production, the role of CYN in producing organisms and in their
77 environment is still not well known. Several studies were conducted for understanding the
78 possible role of the toxin in nature. The few available data related to eukaryotic algae show
79 that the effects of CYN or CYN containing cyanobacterial extracts depend on concentration
80 and on target organism (Campos et al. 2013; Pinheiro et al. 2013; Rzymski et al. 2014; B-
81 Béres et al. 2015). According to some studies, low CYN concentrations may stimulate algal
82 growth (*Chlorella vulgaris,* 0.005-0.179 μg mL^{-1} purified CYN; Campos et al. 2013,
83 *Chlamydomonas reinhardtii*, *Chlorella vulgaris,* and *Nannochloropsis* sp., 0.025-0.5 μg mL^{-1}
84 semi-purified CYN; Pinheiro et al. 2013), but crude extracts with 0.032 and 0.333 μg mL^{-1}
85 CYN concentrations inhibited *Chlorella vulgaris* significantly (Campos et al. 2013).
86 Some authors suggested that cyanobacterial metabolites may play a crucial role in allelopathy,
87 which can be an important factor in the organization and formation of algal assemblages in all
88 types of surface waters, especially in the case of those with low water velocity or standing
89 waters (Leflaive and Ten-Hage 2007). Toxic cyanobacterial species can affect negatively the
90 other members of assemblages both by their presence (e.g. by shading and nutrient uptake)
91 and by allelopathic compounds, although it is still not clearly stated whether cyanotoxins can
92 be considered as allelochemicals (Leão et al. 2009; B-Béres et al. 2012).
93 The report of Bar-Yosef et al. (2010) suggested allelopathic effects of CYN-producing
94 *Ahanizomenon ovalisporum* on eukaryotic algae in field, influencing their presence, growth,

and alkaline phosphatase (APase) activity. They reported strong correlation between *A. ovalisorum* 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 *Chlamydomonas reinhardtii* and *Debarya* sp. were treated with purified CYN or CYN containing cyanobacterial extract (Bar-Yosef et al. 2010). Up-regulated APase activity was also reported recently in a *Microcystis aeruginosa* strain, furthermore, the study also indicated that CYN may inhibit microcystin production (Rzymski et al. 2014). In contrast to enzyme stimulation, and affected toxin production, the applied lower concentrations of CYN (0.001 and 0.005 $\mu g \cdot mL^{-1}$) caused only slight growth inhibition of the unicellular cyanobacterium (Rzymski 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-Oliviera 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-Oliviera 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 that CYN-producers really compete with other phytoplankton species due to their CYN-producing ability. The

dominance of CYN producer strains in the environment was reported several times, and the involvement of CYN in competitive advantages is proved in certain cases (Soares et al. 2009b; Bar Yosef et al. 2010; Karadžić et al. 2013; Rzymsky et al. 2014). However, in many other cases it seems that the dominance of CYN producers would be hard to be explained exclusively with their CYN production ability (Rzymski and Poniedziałek, 2014; Burford et al. 2016; Aguilera et al. 2017; Zhang et al. 2017).

Anyway, 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 Rzymski 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 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 obtusus* 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. obtusus* have pH optima at pH 5 and pH 9, therefore effects of the different crude extracts on phosphatase activities were measured both at pH 5 and pH 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, measurements were carried out using an Olympus DP80 digital camera and cellSens Standard software (Olympus Corporation).

The cosmopolitan, eukaryotic green alga *Scenedesmus obtusus* 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), 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 14 hours light (40 µmol photons m^{-2} s^{-1}) - 10 hours 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-liter Erlenmeyer flasks for 10 days, under the circumstances described above.

The 10-day-old cultures (with a density 1.12 ± 0.06 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 μm 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 *Ahanizomenon* strain was added to the *Scenedesmus obtusus* cultures to reach 1.0, 1.5, 2.0 and 2.5 μg 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 *Ahanizomenon* strain was added to the *Scenedesmus obtusus* 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 μg mL^{-} 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 were 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 μg·mL^{-1}, limit of quantification (LOQ) was 2.5 μg·mL^{-1} for the applied method. Maximum ten-fold concentrations were applicable in the

194 case of the supernatants, so 0.1 and 0.25 $\mu g \cdot mL^{-1}$ were the minimum amount of CYN for
195 detection and quantification, respectively (B-Béres et al. 2015).
196 The experiments were carried out in shaken cultures (SOH-D2 shaker, 90 rpm), in Jaworski's
197 medium (CCAP Media Recipes) in 100 mL Erlenmeyer flasks with 50 mL final volume.
198 Cultures were kept on 14 hours light (40 μmol photons m^{-2} s^{-1}) - 10 hours dark photoperiod at
199 24 °C. The time of exposition was 14 days. Phosphate starvation was achieved under the same
200 conditions in Jaworski's medium lacking phosphate.
201 So called "negative control" cultures containing only cyanobacterial crude extracts (not
202 inoculated with the green alga) were also prepared to check the chlorophyll content and
203 phosphatase activity of the cyanobacterial crude extracts.
204
205 Measurement of the growth of the cultures
206
207 Growth of the cultures was followed by counting the number of coenobia and by measuring
208 chlorophyll-a content. Coenobia numbers were counted from 10 μL samples in Bürker
209 chamber, using an Olympus BX50F-3 microscope at 400× magnification. Structural
210 composition of coenobia (single cells, two- and four-celled coenobia) was also recorded.
211 Samples of 1 mL were collected on zero, 7^{th} and 14^{th} days for chlorophyll-a content
212 measurements. Samples were centrifuged (16200 ×g, 5 min, Heraeus Fresco 17), supernatants
213 and pellets were separated and stored at -20 °C before further processing. Chlorophyll-a
214 contents were measured from the pellets spectrophotometrically (Hach Lange DR 6000 UV-
215 VIS spectrophotometer) after methanolic extraction according to the method of Felföldy
216 (1987). To give EC_{50} value, the extents of growth inhibitions (%, considered that control
217 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 2nd day, samples were centrifuged (16200 ×g, 5 min, Heraeus Fresco 17), 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 (10^6) of coenobia.

Measurement of phosphatase activity

Preliminary experiments showed that phosphatase enzymes of *Scenedesmus obtusus* had maximal activity at pH 5 and pH 9. Therefore the reaction mixtures were buffered to pH 5 and pH 9 with potassium hydrogen phthalate and sodium tetraborate, respectively. The measurements were based upon the modified methods of Tabatabai and Bremner (1969) and Inhlenfeldt and Gibson (1975). The reagent mixtures contained 400 µL of sample, 500 µL pH 5 or pH 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_2HPO_4 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 10^6 number of coenobia $^{-1}$ hour^{-1}.

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 *Scenedesmus 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 (Figure 1a). However, the growth of the treated cultures was differently affected in the different phases of the exposition. Growth of the cultures was stimulated by the C+ crude extracts on the first week, than growth inhibition was observed after the 9th day of cultivation (Figure 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 exposition, effective concentration of CYN

causing 50% growth inhibition (EC_{50}) could be calculated only for 14-day-old cultures, which was 2.85 μg mL^{-1}.

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

Growth tendencies differed significantly ($p< 0.05$) in cases of control vs 1.5, 2.0 and 2.5 C– treatments (Figure 1b). EC_{50} 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 (Figure 1b), the same phenomena occurred also in C–+C treatments (Figure 1c).

Comparing the C+, C– and C–+C treatments, significantly higher ($p<0.05$) number 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 (Figure 1d). The treatments did not cause significant changes in coenobial structure of the used *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 7th 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 (Figure 2).

Phosphate uptake of the *Scenedesmus obtusus* cultures

A decreasing trend in phosphate uptake of a unit of coenobia was observed both in the case of control and phosphate depleted cultures (Figure 3a). Although the statistical analysis did not show significant differences among phosphate uptake tendencies of control and differently

291 treated cultures, there were significantly higher phosphate uptake in C+ crude extract treated
292 cultures than in C– and C–+C crude extract treated cultures on the 12^{th} – 14^{th} days of the
293 experiments (Figure 3b,c).
294
295 Phosphatase activities in the *Scenedesmus obtusus* cultures
296
297 Acidic phosphatase activity of control, phosphate deficient and C+ treated cultures increased
298 over time, furthermore, increasing activities were observed with the increasing amount of the
299 crude extract. Activities on the 7^{th} and 14^{th} days were significantly higher ($p<0.005$) than on
300 the zero day in each cultures (Figure 4a).
301 Acidic phosphatase activities were lower in the C– treated cultures than in C+ treatments on
302 each sampling days, furthermore, acidic phosphatase activities in the 1.5, 2.0 and 2.5 C–
303 treatments were significantly lower on the 7^{th} and 14^{th} days than in control. Acidic
304 phosphatase activities did not changed significantly over time in any C– treatments (Figure
305 4b).
306 Increasing acidic phosphatase activities were observed in C–+C treated cultures both in time
307 and with the increasing concentration of added CYN, similarly to C+ treatments (Figure 4c).
308 The measured values were significantly higher ($p<0.05$) than in control (Figure 4c).
309 Comparing the C+, C– and C–+C treatments on the same days, it can be said that acidic
310 phosphatase activities of C+ treatments were the highest, significantly higher ($p<0.005$) than
311 in the C– and C–+C treated cultures on the 7^{th} and 14^{th} days. Significantly higher ($p<0.05$)
312 activities were measured in C–+C treated cultures than in C– treated ones on the 14^{th} day.
313 Alkaline phosphatase activity was significantly higher ($p<0.001$) in the phosphate starved
314 culture than in control on every sampling days. In case of C+ treatments, the highest alkaline
315 phosphatase activities were measured on the 7^{th} day in the treated cultures, however, these

316 activities decreased both in time (to the 14th day) and with the increasing amount of C+ crude
317 extract (Figure 5a). It has to be emphasized that alkaline phosphatase activities were
318 significantly higher than in control both on the 7th and on the 14th days in every C+
319 treatments. The highest activity was measured in 1.0 C+ treatment on the 7th day, which latter
320 was significantly higher even than the activity in phosphate starved culture (Figure 5a).
321 There were no higher alkaline phosphatase activities in the C– treated cultures than in control,
322 moreover, activities on the 7th and 14th days were significantly lower ($p<0.05$) than in control
323 on the same days (Figure 5b).
324 Alkaline phosphatase activity increased in the C–+C treated cultures during the 14 days of the
325 experiments (Figure 5c). Tendencies were similar to that of control, highest activities were
326 measured on the 14th day, significant difference ($p<0.005$) was shown only in the case of 1.0
327 C–+C treatment on the 14th day (Figure 5c).
328 Comparing the alkaline phosphatase activities of the different treatments, there were
329 significantly higher measured values ($p<0.005$) in all C+ treatments than in C– and C–+C
330 treatments on the 7th day.
331
332 **Discussion**
333
334 Growth
335
336 It was assumed that growth of the green algal cultures would be inhibited in C+ and C−+C
337 treatments, while the C− extract would have opposite effects. This assumption was only
338 partially justified. CYN-producing *Aphanizomenon* crude extract (C+) caused slight growth
339 inhibition only from the 9th day of the experiment. In contrast to our expectation, C−+C crude
340 extracts stimulated growth and no growth limitation was observed during the whole

timeframe of the experiment (similarly to C− treatments), regardless of the extract 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 the negative effects of CYN (Sergeeva et al. 2002; Stirk et al. 2002; Tsavkelova et al. 2006; Hussain et al. 2010). In the same time, growth stimulation of eukaryotic algae by low CYN concentrations of cyanobacterial crude extracts was reported already in the case of a few other green algal species (*Chlamydomonas reinhardtii, Chlorella vulgari*s and *Nannochloropsis* sp.; Pinheiro et al. 2013). EC_{50} could be calculated only for the 14^{th} day and it was 2.85 μg mL^{-1} CYN in the case of the investigated *Scenedesmus* strain. This value is much higher than reported by Campos et al. (2013) for *Chlorella vulgaris*, which was 0.333 μg mL^{-1} CYN containing crude extract caused 48% decrease in growth rate after 3 days exposition. Pinheiro et al. (2013) also reported more than 50% growth rate inhibition by 2.5 μg mL^{-1} CYN containing cyanobacterial crude extract in the case of *Chlamydomonas reinhardtii*, *Chlorella vulgari*s and *Nannochloropsis* sp. already after 4 days exposition. These results and the lack 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, the strain was sensitive neither 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. In 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, non-CYN producing *Aphanizomenon* with CYN did not lead to similar phenomena. It suggests that contribution of molecules in the cellular matrix of the producer organism to the effects of CYN is potentially plausible.

366 Chlorophyll content of cultures increased independently of the toxin content of the crude
367 extracts. The strong blue colour of the crude extracts resulted in a strong shading effect, thus
368 the increasing chlorophyll content could be a compensatory reaction to the lower amount or
369 changed wavelength of light (Carvalho et al. 2009; Bonente et al. 2012; He et al. 2015;
370 Ferreira et al. 2016). The lack of inhibition of chlorophyll synthesis also support the
371 insensitivity of the used *Scenedesmus* strain to CYN, and highlights that important
372 phenomena could be lost if growth is investigated exclusively on the basis of chlorophyll
373 content.
374
375 Phopshate uptake
376
377 The phosphate uptake slightly increased with the increasing amount of C+ crude extract
378 compared both to control and to all the other treatments (including phosphate starvation).
379 Since there were less coenobia in C+ crude extract treated cultures from the 9^{th} day, the
380 phenomenon means that less coenobia took up more phosphate in the case of C+ treatments.
381 Although more intense phosphate uptake does not require necessarily higher phosphatase
382 activity, these results are in accordance with the results of alkaline phosphatase in C+
383 treatments. It could be possible, that CYN contribute to more intense phosphate uptake beside
384 the appearance of the high-affinity P_i transporter in the CYN producing cyanobacterium, and
385 without the producer organism (the living cyanobacterium), the toxin slightly induced the
386 higher phosphate uptake of the target green alga. Of course, the proof or disproof of these
387 assumptions definitely requires further investigations. Although the statistical analysis did not
388 show significant differences among the phosphate uptake of the different treatments, based on
389 the phenomena detailed above, it can be presumed that CYN affects phosphate uptake of the
390 studied green alga, but only when it is the original component of the crude extract (e.g.

391 intermediates of its synthesis or degradation could be present), and similar effects of the
392 added CYN (if any) are masked by other components during the C−+C treatments.
393
394 Phosphatase activity
395
396 It was assumed that phosphatase activity of the green algal cultures would be induced in C+
397 and C−+C treatments, while the C− extract will have opposite effects. This assumption was
398 justified in the case of acidic phosphatase activity. Acidic phosphatase activities increased in
399 the C+ crude extract treated cultures compared to control on each sampling day. Moreover,
400 activities increased with the increasing amount of the crude extract. Acidic phosphatases are
401 mostly intracellular enzymes, they are necessary for the mobilization of intracellular
402 phosphate storages (polyphosphate bodies; Bowen and Bryant 1978; DuBois et al. 1984). One
403 possible explanation of increasing acidic phosphatase activity during C+ treatments could be
404 the increased stress. It is reported that acidic phosphatase activity may increase when cells are
405 exposed to any external stress factors (e.g. dark periods in case of *Nostoc* sp., DuBois et al.
406 1984; limiting or low nitrogen and phosphate concentration in the environment, Kruskopf and
407 Du Plessis 2004). Shading effect of the crude extract and the myriads of organic molecules
408 present could mean a stressful environment. The results of phosphorous uptake measurements
409 and the fact that elevated acidic phosphatase activities were measured only in the presence of
410 CYN exclude the possibility of limited phosphorous bioavailability caused by the chemical
411 matrices of the crude extracts. The possibility that CYN not induce higher expression of
412 acidic phosphatase directly but via limitation of P transport also can be excluded based on the
413 results of phosphorous uptake measurements. Although the measured activities were
414 significantly lower, trends in acidic phosphatase activities in C−+C treatments were more
415 similar to that of C+ treatments. This phenomenon and the significantly higher acidic

phosphatise 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 phosphatise activities. According to our knowledge, the phenomenon was not 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, 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 from 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 phospho-esther 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 general phenomenon among phosphate limited circumstances. High alkaline phosphatase activity was detected on the 7th 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 *Chlamydomonas reinhardtii* cultures, which were inoculated to *Aphanizomenon ovalisporum* spent medium (7-8 μg L^{-1} CYN) or were treated with purified CYN (50 μg mL^{-1}, Bar-Yosef et al. 2010). Increasing alkaline phosphatase activity was also observed in *Microcystis panniformis* cultures in the presence of *Aphanizomenon ovalisporum* (Zhang et al. 2016). Rzymski et al. (2014) did not observe higher alkaline phosphatase activity in *Microcystis aeruginosa* cultures treated with their highest (0.05 μg 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 (Froscio 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 7-8 μg mL^{-1} CYN), than in case of purified toxin (50 μg mL^{-1}). Existence of molecules able to induce alkaline phosphatases is also suggested by the results of Rzymski 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 *Scenedesmus 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 were significantly higher during C+ treatments; so 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. In 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 (*Ahanizomenon*), 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, require 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. (Figures 1-5). S.G. and G.V. provided the purified CYN, contributed to the preparation of crude extracts and instrumental analytical

491 measurements. S.A.N provided additional financial support for the experiments. D.D., G.V.
492 and I.B. related to conception and design of the study, acquisition of data, analysis and
493 interpretation of data, and drafting the article. I.B. supervised the whole work and finalised
494 the manuscript.
495
496 **Conflict of Interest**
497
498 The authors declare that they have no conflict of interest.
499
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732

Figure Legends

Figure 1 Coenobia number changes in *Scenedesmus obtusus* 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 μg 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.

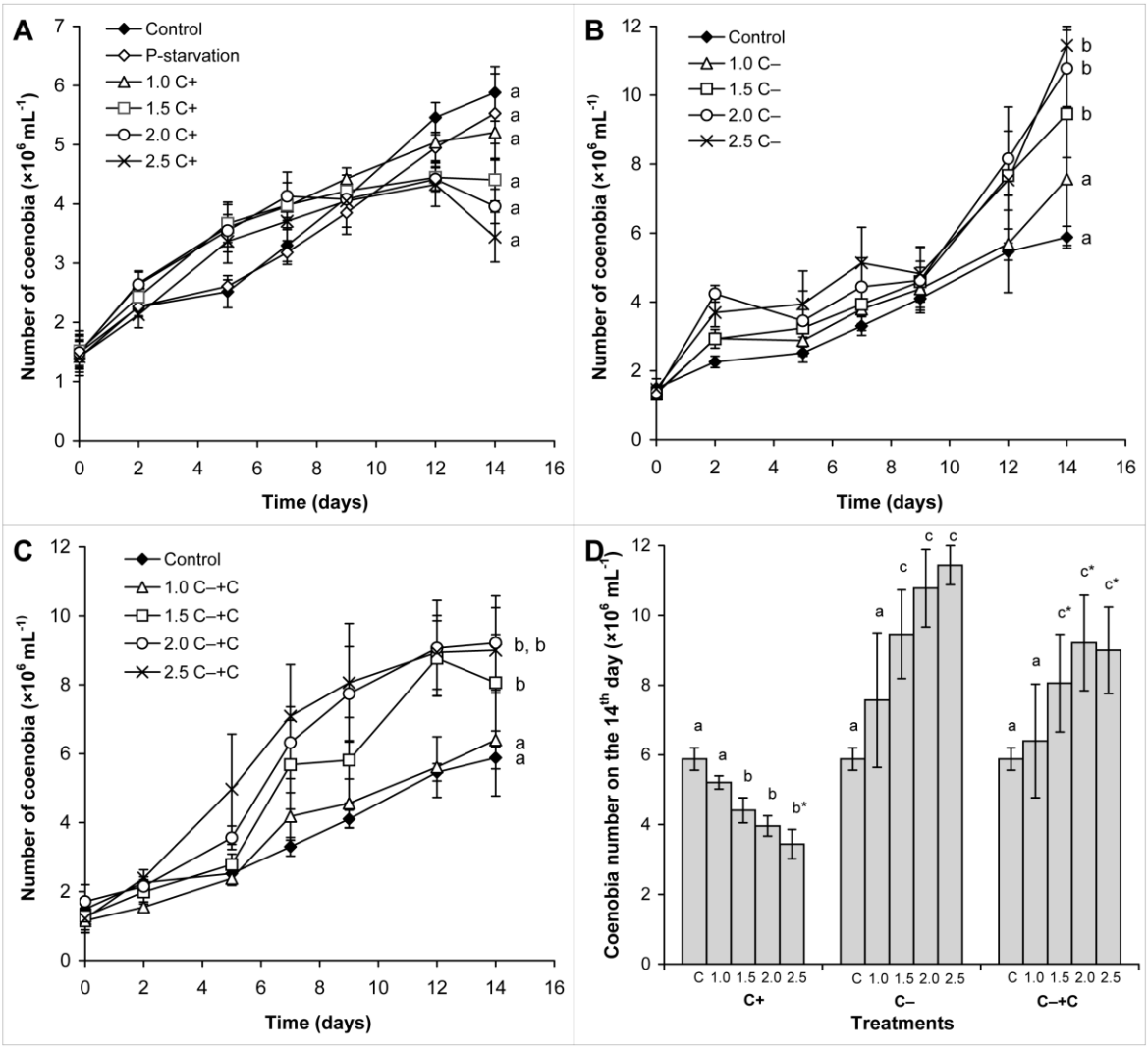

Figure 2 Chlorophyll-a content changes in differently treated *Scenedesmus obtusus* cultures. A: cultures treated with CYN-producing *Aphanizomenon* crude extract (C+), B: cultures treated with non-CYN producing *Aphanizomenon* crude extract (C–) and C: cultures treated with non-CYN producing *Aphanizomenon* crude extract supplemented with CYN (C–+C).. Numbers (1.0-2.5) refer to CYN content in µg mL^{-1}, and equivalent amounts of C– crude extract. Numbers 0, 7, 14 refer to the sampling days. Mean values (n=3) and standard deviations are plotted, significant differences ($p<0.05$) among zero, 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).

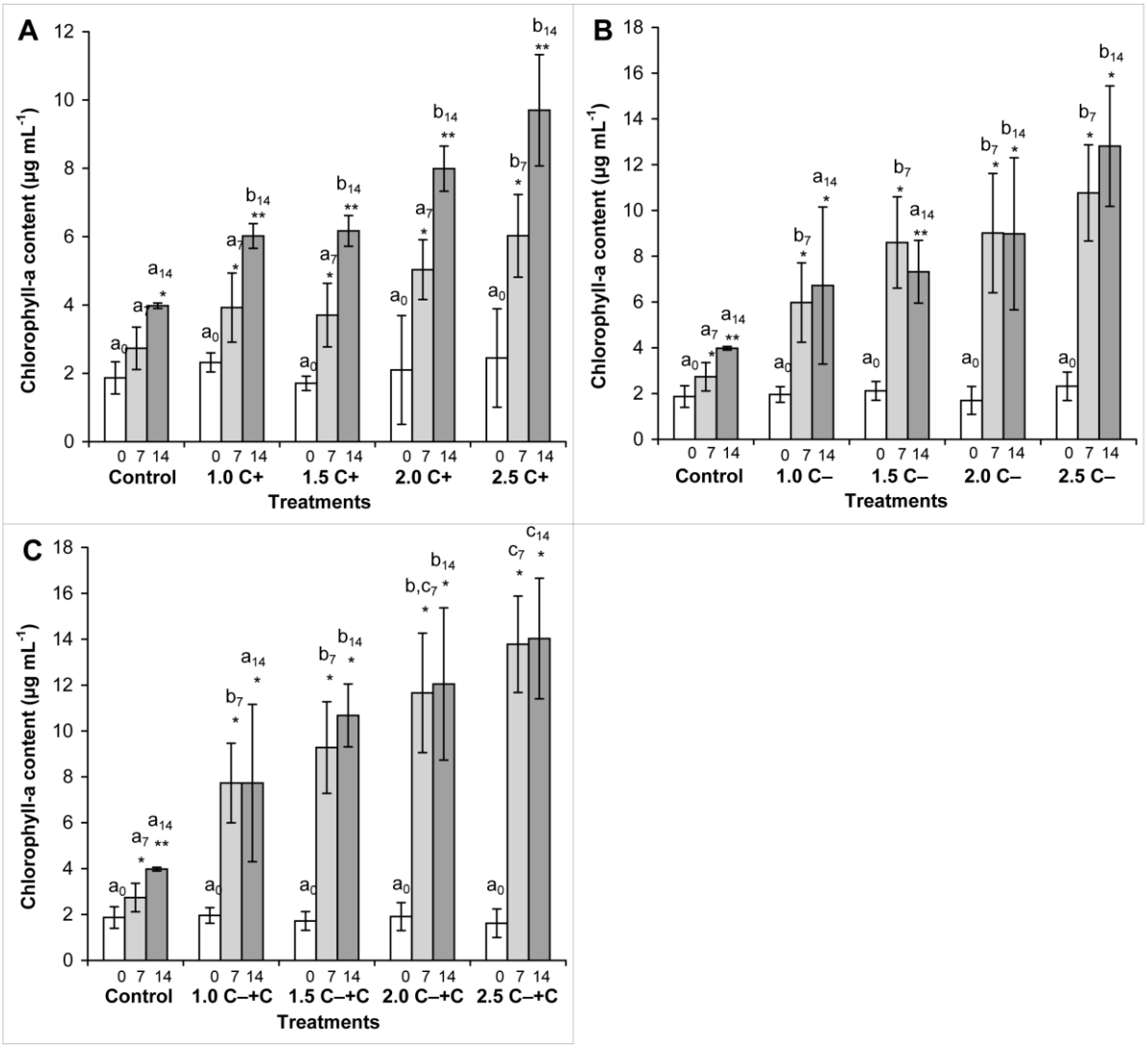

Figure 3 Phosphate uptake changes in differently treated *Scenedesmus obtusus* cultures. A: cultures treated with CYN-producing *Aphanizomenon* crude extract (C+), B: cultures treated with non-CYN producing *Aphanizomenon* crude extract (C–) and C: cultures treated with non-CYN producing *Aphanizomenon* crude extract supplemented with CYN (C–+C). Numbers (1.0-2.5) refer to CYN content in µg 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.

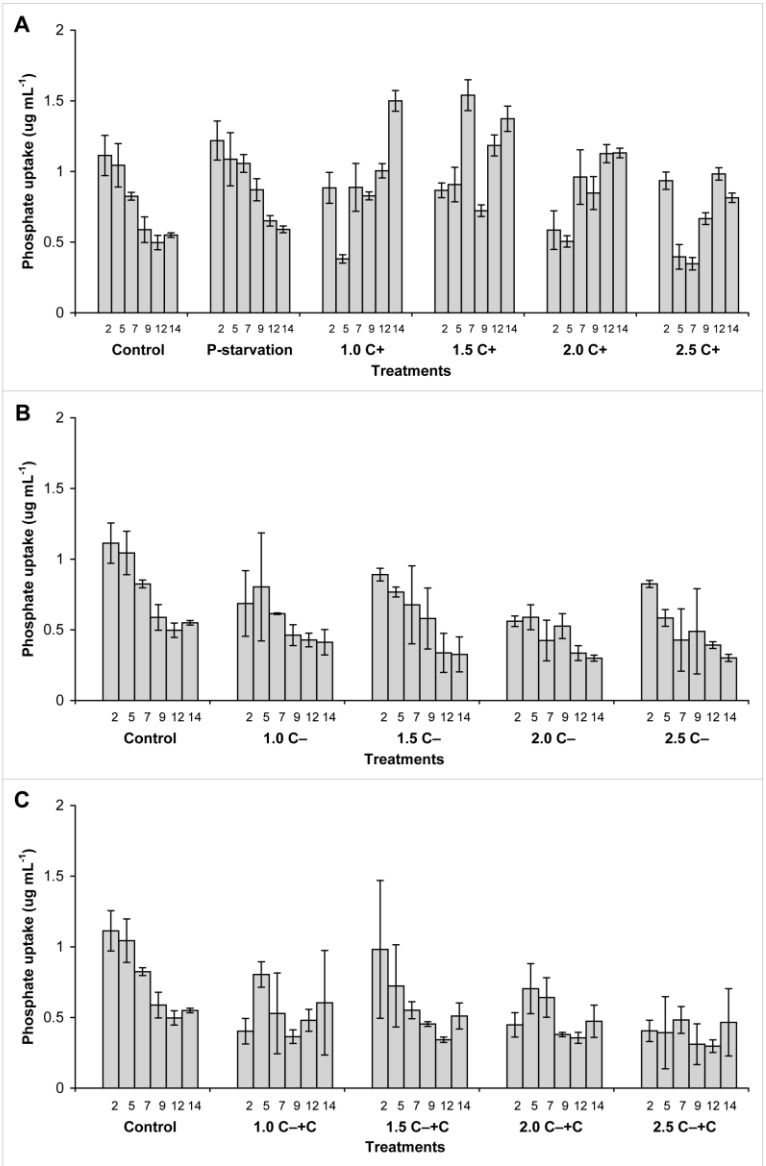

Figure 4 Acidic phosphatase activities in differently treated *Scenedesmus obtusus* cultures. A: cultures treated with CYN-producing *Aphanizomenon* crude extract (C+), B: cultures treated with non-CYN producing *Aphanizomenon* crude extract (C–) and C: cultures treated with non-CYN producing *Aphanizomenon* crude extract supplemented with CYN (C–+C). Numbers (1.0-2.5) refer to CYN content in µg mL^{-1}, and equivalent amounts of C– crude extract. Numbers 0, 7, 14 refer to the sampling days. Mean values (n=3) and standard deviations are plotted, significant differences ($p<0.05$) among zero, 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).

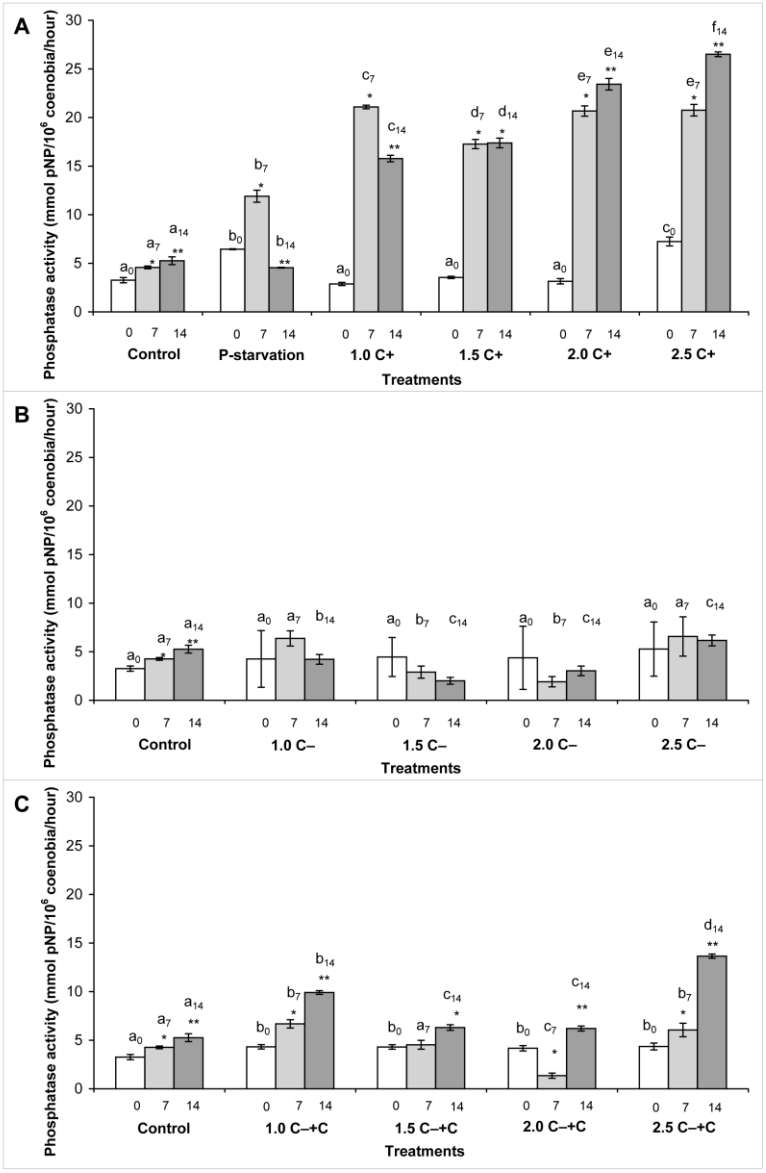

Figure 5 Alkaline phosphatase activities in differently treated *Scenedesmus obtusus* cultures. A: cultures treated with CYN-producing *Aphanizomenon* crude extract (C+), B: cultures treated with non-CYN producing *Aphanizomenon* crude extract (C–) and C: cultures treated with non-CYN producing *Aphanizomenon* crude extract supplemented with CYN (C–+C). Numbers (1.0-2.5) refer to CYN content in µg mL^{-1}, and equivalent amounts of C– crude extract. Numbers 0, 7, 14 refer to the sampling days. Mean values (n=3) and standard deviations are plotted, significant differences ($p<0.05$) among zero, 7^{th} and 14^{th} 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).