Influence of *Daphnia* infochemicals on functional traits of *Microcystis* strains (Cyanobacteria)

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Abstract We conducted a laboratory experiment to investigate the influence of *Daphnia* infochemicals on growth rate, microcystin production, colony formation and cell size of eight *Microcystis* strains isolated from two lakes. The strains were characterized genetically by their 16S-23S rDNA ITS sequence. The experiment was composed of four treatments: (1) a control using filtered WC medium, (2) addition of *Scenedesmus obliquus* culture medium filtrate, (3) addition of *Daphnia magna* culture medium filtrate and (4) addition of sodium octyl sulphate, a commercially available *Daphnia* infochemical. Our results showed that sympatric strains differed strongly for the measured functional traits, while no correlations between traits were found. Between-strain differences in growth rate, microcystin production, colony formation and cell size were generally larger than the differences in phenotypes

observed between treatments. Despite this, several strains reacted to the infochemicals by changing functional trait values. *Daphnia* culture medium filtrate and, to a lesser extent, sodium octyl sulphate had a negative influence on the growth rate of half of the strains and stimulated microcystin production in one strain, but the latter effect was not *Daphnia*-specific as *Scenedesmus* culture medium filtrate had the same effect. *Daphnia* culture medium filtrate also induced colony formation in one strain. Our data suggest that *Daphnia* infochemicals generally have a weak influence on growth rate, microcystin production and colony formation of *Microcystis* strains as compared to the inter-strain variability, while existing inducible effects are highly strain-specific.

Keywords Colony formation · *Daphnia* · Functional traits · Infochemicals · Microcystins · *Microcystis*

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Introduction

Phytoplankton has evolved various morphological, biochemical and behavioural defense mechanisms against grazing of zooplankton. It has been shown for several phytoplankton species that herbivorous zooplankton can induce these defense mechanisms by releasing infochemicals (van Donk, 2007). In particular, the influence of zooplankton infochemicals on the morphology of the closely related green algae

Scenedesmus and *Desmodesmus* has been intensively studied (e.g. Hessen & van Donk, 1993; Lürling & van Donk, 1997; Verschoor et al., 2004). The presence of infochemicals from grazers has been shown to induce changes in cell size, colony formation and spine formation in a range of species from both genera. Other chlorococcal green algae may show a similar response (van Donk et al., 1999; Luo et al., 2006). Also, several flagellated algal species regulate their recruitment rate from the sediment depending on the presence or absence of grazers in the water column (Hansson, 1996, 2000). These adaptive changes reduce phytoplankton mortality due to herbivory, while the energetic cost of the response is lowered by the fact that the defense can be attuned depending on the presence of predators (van Donk, 2007).

Among phytoplankton, cyanobacteria are very widespread and may form nuisance blooms (Huisman et al., 2005). *Microcystis* is a common genus that forms toxic blooms, especially in eutrophic water bodies at high temperatures (Chorus & Bartram, 1999; Visser et al., 2005). There is contrasting evidence in the literature on the extent to which grazing by zooplankton can control the development of cyanobacterial blooms. For instance, the effect of *Daphnia*, a key grazer in standing waters, on *Microcystis* biomass is debated, as *Microcystis* may strongly suppress the growth of *Daphnia* (Ghadouani et al., 2003), while other studies have provided evidence that *Daphnia* may suppress developing *Microcystis* blooms depending on initial conditions and history (Christoffersen et al., 1993; Sarnelle, 2007). Probably, environmental conditions are also important for the outcome of these interactions. Understanding the mechanism of defense of *Microcystis* against predation is important to evaluate possibilities of suppressing bloom development by grazing. Previous research has shown that *Microcystis* blooms are typically composed of several genotypes that can differ in functional traits such as growth rate, production of microcystins (heptapeptides which are very toxic for eukaryotes), colony formation and cell size (Carrillo et al., 2003; Janse et al., 2004; Wilson et al., 2005, 2006; Kardinaal et al., 2007). With respect to the extent to which grazers may induce phenotypic shifts in these traits, the data of different studies lead to different conclusions. Zooplankton infochemicals seem to have no

(Yang et al., 2006a; Yang & Li, 2007) or a slightly positive impact (Jang et al., 2007, 2008) on the growth rate of *Microcystis*. Jang et al. (2003, 2007, 2008) reported that grazing or culture medium filtrate of *Daphnia magna* and *Moina macrocopa* increased microcystin production in several *Microcystis* strains. In addition, Jang et al. (2003) and Ha et al. (2004) showed that grazing or culture medium filtrate of *D. magna* and *M. macrocopa* can induce colony formation in *Microcystis* strains. However, other studies failed to find changes in this trait induced by *D. magna* (Hessen & van Donk, 1993; Yang et al., 2006b).

Given the large differences in the effects of *Daphnia* infochemicals on *Microcystis* functional traits between studies using one or a few *Microcystis* strains, there is a need for studies that compare several *Microcystis* strains. In this study, we aimed to determine whether these differences are the result of a strain-specific response to infochemicals. Therefore, we determined (1) the differences in growth rate, microcystin production, colony formation and cell size between eight *Microcystis* strains, (2) whether *Daphnia* infochemicals have an influence on these functional traits, and (3) whether the strains react differently on these infochemicals. We expected to see clear differences between the eight *Microcystis* strains, all growing under the same conditions, if reactions on *Daphnia* infochemicals in *Microcystis* are strain-specific. However, if culture conditions are the main cause of the differences between different studies, all strains should show a similar reaction.

Materials and methods

Isolation and genotypic characterization of *Microcystis* strains

Monoclonal *Microcystis* cultures were obtained by sampling single colonies with a micropipette from a bloom sample from a pond in the nature reserve Leeuwenhof (Ghent, Belgium) on 07/09/04 (25) and from a pond in the nature reserve Tiens Broek (Tienen, Belgium) on 10/08/05 (20). The colonies were grown in WC medium (Guillard & Lorenzen, 1972) at a temperature of 18°C and a light intensity of 30 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (light:dark cycle = 12 h:12 h). As the taxonomy of *Microcystis* is still largely based on

morphology and does not correspond to genetic features (Otsuka et al., 2001), it is preferable to use genetic markers, such as the 16S-23S rDNA ITS sequence, to distinguish different *Microcystis* strains from each other irrespective of morphospecies identification (Janse et al., 2004). We determined the 16S-23S rDNA ITS sequence of each strain, and submitted the sequences to GenBank under accession numbers FJ830618–FJ830625. We detected four distinct ITS types in Leeuwenhof and seven ITS types in Tiens Broek. Five ITS types were identified as microcystin producing genotypes (two toxic ITS types from Leeuwenhof named T1 and T2, and three toxic ITS types from Tiens Broek of which we randomly selected two ITS types named T3 and T4) and six as non-microcystin producing ITS types (two non-toxic ITS types from Leeuwenhof named NT1 and NT2, and four non-toxic ITS types from Tiens Broek of which we randomly selected two ITS types named NT3 and NT4), both by detecting the presence of *mcy* genes A (Hisbergues et al., 2003) and E (Vaitomaa et al., 2003) as well as by directly measuring microcystin concentrations in cultures by ELISA (Enzyme-Linked Immuno Sorbent Assay) with SDI—EnviroGard Microcystin ELISA plate kits (see below for more details). A rDNA ITS distance tree (Neighbor Joining with Jukes and Cantor correction) was constructed using the program BioNumerics 4.5 (Applied Maths BVBA).

Experimental design

All eight selected *Microcystis* strains were grown under standardized conditions in WC medium at a temperature of 23°C and a light intensity of 120 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (light:dark cycle = 12 h:12 h) for several generations. Exponentially growing cells were used as inoculum for the experiment. The experimental cultures were grown during eight days (from 13-09-06 to 21-09-06) in 50 ml flasks at an initial density of 200,000 cells ml^{-1} . Four treatments were imposed for each of the eight strains: (1) a control with addition of pure filtered WC medium, (2) addition of filtered medium from a *Scenedesmus obliquus* culture containing 250,000 cells ml^{-1} (“*Scenedesmus*” treatment), (3) addition of *Daphnia* infochemicals obtained by filtering medium from a culture in which 300 *D. magna* individuals per litre grazed for 24 h on approximately 50,000 cells ml^{-1} of *S. obliquus* (“*Daphnia*” treatment) and (4)

addition of filtered WC medium containing sodium octyl sulphate (final culture concentration of 1,000 $\mu\text{g l}^{-1}$), a commercially available *Daphnia* infochemical which can induce colony formation in *Scenedesmus gutwinskii* as described by Yasumoto et al. (2005) (“SOS” treatment). Sterile Nalgene filter units with CN membrane and pore size of 0.2 μm were used for filtration. Each culture flask contained 90% standard WC medium and 10% culture medium filtrate. New culture medium filtrate was added every second day for all four treatments because since the cultures were not axenic, infochemicals could be degraded by microorganisms (Lüring & van Donk, 1997). There were three replicate cultures for each strain $\times$ treatment combination, which resulted in 96 flasks that were gently shaken by hand daily. The position of the cultures was randomized daily. The experimental design was shown in Fig. 1.

Sample analyses

The optical density (OD) of the cultures was measured with a spectrophotometer ($\lambda = 750 \text{ nm}$) at the start of the experiment and after 2, 5, and 8 days. Growth curves showed that all eight strains in all treatments were growing exponential for the entire duration of the experiment and the growth rate of each strain was determined by calculating the slope of the regression line between time and Ln(OD) . Samples for microcystin analysis were taken after 21 h and after 8 days of growth. The total microcystin concentration ($\mu\text{g ml}^{-1}$) in the samples was determined by ELISA. For the extraction of microcystins, the cells were boiled for 20 min (van der Oost, 2007). Next, the cultures were centrifuged for 10 min at 14,000 rpm and the supernatant was used for ELISA. The ELISA-test was done according to the manufacturer’s instructions using SDI—EnviroGard Microcystin ELISA plate kits. To calculate microcystin concentration per unit biomass, cell densities were converted into biomass ($\text{pg C } \mu\text{l}^{-1}$) using the formula $\text{pg C cell}^{-1} = 0.216 \times \text{volume cell}^{0.939}$ (Menden-Deuer & Lessard, 2000). Cell biovolumes were estimated by measuring the average cell diameter of each strain using the program CellC (Selinummi et al., 2005) (see below). Microscopical pictures of the cells and colonies were taken at the start and at the end of the experiment. After 8 days of growth, 10 ml from each culture was fixed with

Fig. 1 Scheme of experimental set-up for one *Microcystis* strain (ITS type), shown in three replicate cultures. The same set-up was conducted for all eight strains. Culture medium filtrate was added every second day and the experiment was conducted for 8 days in total

formalin (end concentration 2%). Colony formation was determined quantitatively using the fixed samples by counting the unicellular cells with a counting chamber and recounting the cells after boiling the samples for 6 min to make the colonies fall apart (Joung et al., 2006). From these data, the percentage of cells in unicellular form and in colony form could be calculated. We also screened the samples for colonies and measured the size (largest diameter) of a limited number of arbitrary chosen colonies for each culture to have an idea of the size range of the colonies. In addition, 5 ml of the fixed sample was filtered on black polycarbonate filters to measure cell size by fluorescence microscopy using the program CellC.

Statistical analyses

Correlations between the studied traits were investigated by simple and partial Mantel tests using the Zt software tool (Bonnet & Van de Peer, 2002). This was done with Euclidean distance dissimilarity matrices for the four functional traits based on the mean values of the control treatment and a similarity

matrix based on sequence data (% similar nucleotides in rDNA ITS sequence) for all eight strains. To test whether there are significant differences in functional traits between strains and treatments, we carried out two-way analyses of variance (ANOVA; fixed factors: strain and treatment) followed by post hoc tests for growth rate, microcystin production and cell size, and the non-parametric Kruskal Wallis test for colony formation. Statistical analyses were performed by SPSS 15 (SPSS Inc. Chicago, USA).

Results

Figure 2 shows the rDNA ITS distance tree of the eight *Microcystis* strains, in which it is clear that strain T2 is more distantly related to the other strains and that there is no clustering per lake or toxicity.

Simple Mantel tests showed no significant correlations between traits, except for cell size, which was correlated with genetic distance ($r = -0.439$; $0.01 < P < 0.05$) and growth rate ($r = 0.417$; $0.01 < P < 0.05$). However, partial Mantel tests showed that cell size and growth rate were not correlated anymore

Fig. 2 Neighbor Joining tree of the eight studied *Microcystis* strains based on rDNA ITS (correction: Jukes and Cantor). Bootstrap values are indicated. Strains indicated with T are toxic strains and strains indicated with NT are non-toxic strains, strains numbered 1 and 2 are isolated from Leeuwenhof and strains numbered 3 and 4 are isolated from Tiens Broek

when controlling for genetic distances ($r = 0.350$; $P > 0.05$). When correlations were recalculated without T2 (this strain differs more than average from the other strains), no significant correlations between the traits were found at all ($P > 0.05$).

Results of two-way ANOVA revealed significant differences in growth rate between strains ($F = 30.94$; $P < 0.001$) and between treatments ($F = 11.31$; $P < 0.001$), but no strain $\times$ treatment interaction effect ($F = 1.56$; $P > 0.05$). Post hoc tukey tests ($P < 0.05$) revealed four groups of strains: T4-T2-NT1, T2-NT1-NT3, NT3-T1-NT2 and NT4-T3 (from slow to fast growth, no significant differences in growth rate within each group). Post hoc LSD tests showed that the *Daphnia* treatment had a significantly lower growth rate than the control for four of the eight strains ($0.001 < P < 0.01$ for T1 and NT4; $0.01 < P < 0.05$ for NT1 and NT2) and that the SOS treatment had a significantly lower growth rate than the control for two strains (T1 and NT4; $0.01 < P < 0.05$) (Fig. 3).

The four strains that did not produce microcystins at the start of the experiment (NT1-4) similarly did not produce microcystins in any of the treatments during the experiment. For each strain, the microcystin concentration after 21 h and after 8 days of growth was very similar, but the differences between the treatments were slightly larger at the end of the experiment (data not shown), so we focused on the data collected after 8 days. Two-way ANOVA revealed significant differences in microcystin production between the four toxic strains ($F = 65.73$; $P < 0.001$) and between treatments ($F = 3.39$; $P < 0.05$), and a marginal significant strain $\times$ treatment

interaction effect ($F = 2.06$; $P = 0.065$) after eight days of growth. Post hoc tukey tests of the strains showed that T2 and T4 were characterized by a significantly lower microcystin concentration than T1 and T3 ($P < 0.001$) and that T3 produced a significantly higher microcystin concentration than T1 ($0.01 < P < 0.05$). Post hoc LSD tests revealed that the microcystin concentration was significantly higher in the *Scenedesmus*, *Daphnia* and SOS treatments compared to the control for T3 ($0.01 < P < 0.05$ for *Scenedesmus* and SOS; $0.001 < P < 0.01$ for *Daphnia*) (Fig. 3). It seems that microcystin production can rapidly be influenced by *Daphnia* infochemicals as differences between treatments were already seen in T3 after 21 h of growth (data not shown).

At the start of the experiment, all strains were composed of single cells and only a few small colonies were observed. After eight days of growth in the control treatment, only NT3 formed large colonies (size ranging from a few cells to approximately 300 μm) and four strains (T1, T2, T3 and T4) were exclusively composed of single cells. T1 and T2 also failed to produce colonies in the other treatments. Kruskal–Wallis tests for each strain separately revealed that T4 had a significantly higher percentage of cells in colonies in the *Daphnia* treatment compared to the other treatments ($X^2 = 10.74$; $0.01 < P < 0.05$) indicating that colony formation was induced by *Daphnia* infochemicals in this strain. For NT1, T3 and NT4, a trend for increased colony formation in the presence of *Daphnia* infochemicals was observed, but the pattern was not significant ($0.05 < P < 0.1$) (Fig. 3).

Two-way ANOVA revealed significant differences in cell size between strains ($F = 33.35$; $P < 0.001$), but not between treatments ($F = 0.10$; $P > 0.05$), and no strain $\times$ treatment interaction effect ($F = 0.47$; $p > 0.05$). Post hoc tukey tests showed that the strains could be divided into three significantly different groups ($P < 0.05$) according to their cell size: small (T1, NT1, NT2 and NT4), medium (T3 and NT3) and large cell size (T2 and T4) (Fig. 3).

Discussion

Overall, our results confirm the large variability in functional traits among *Microcystis* strains isolated

Fig. 3 Growth rate (A), microcystin concentration (B), colony formation (C) and cell size (D) after 8 days of growth of the eight *Microcystis* strains in four treatments: control, *Scenedesmus* treatment, *Daphnia* treatment and sodium octyl sulphate

treatment (SOS). Error bars indicate the standard error of the mean. Grey asterisks indicate treatments that differ significantly ($P < 0.05$) in the studied trait from the control for the respective strain

from the same population that has been reported before (Carrillo et al., 2003; Bañares-España et al., 2006, 2007; Wilson et al., 2006). Apart from a correlation between the genetic distances and cell size, which was due to the presence of one genetically distinct strain, no significant correlations in variation among the studied traits were found across strains. This is in accordance to Wilson et al. (2006), who did not find a correlation between growth rate and microcystin production using 32 *Microcystis* strains. They also found a similar growth rate for unicellular and colony forming strains. Altogether, this indicates that microcystin production and colony formation do not influence maximal growth rate.

The differences in growth rate, microcystin production, colony formation and cell size between the eight studied strains were generally larger than the differences between the treatments for each strain. Despite this, several strains reacted to the infochemicals by changing functional trait values. In contrast to previous studies (Yang et al., 2006a, Yang & Li, 2007; Jang et al., 2007, 2008), *Daphnia* infochemicals had a specific negative influence on the growth rate of four of the eight studied strains, while sodium octyl sulphate had a similar but reduced effect on two of the strains. This growth cost may be due to investment of the strains in a defense against grazing other than colony formation and microcystin

production. Possible candidates are an increase in other metabolites harmful for *Daphnia* (Czarnecki et al., 2006), for instance microviridin J (Rohrback et al., 2004). This aspect should be investigated further. *Daphnia* culture medium filtrate induced colony formation in one strain, as reported in literature (Jang et al., 2003; Ha et al., 2004). Apparently, the infochemical involved was not sodium octyl sulphate as this substance was not effective, although the response appears to be *Daphnia*-specific because colony formation was not induced by *Scenedesmus* culture medium filtrate. In another strain, microcystin production increased in the presence of *Daphnia* culture medium filtrate, similar to Jang et al. (2003). However, the same effect was found for the *Scenedesmus* treatment indicating that the effect was not *Daphnia*-specific. Yet, sodium octyl sulphate also had a significant positive influence on microcystin production. Probably, more than one substance enhances microcystin production.

Our results clearly show that the response of *Microcystis* to zooplankton infochemicals is strongly strain-dependent, which may explain the often conflicting results reported by different studies. Inter-strain variability in growth rate and colony formation as reaction to zooplankton infochemicals was also reported in *Scenedesmus* (Lürling, 1999; Verschoor et al., 2004). Although this was not the focus of our study, the influence of zooplankton infochemicals on traits of a particular *Microcystis* strain might also vary with culture conditions, as it has been shown that several environmental factors influence growth rate and microcystin production (Lukač & Aegerter, 1993; Sivonen & Jones, 1999; Wiedner et al., 2003). As seen in another experiment we conducted (van Gremberghe et al., 2009), the functional traits of the *Microcystis* strains we studied can vary under different circumstances. In addition, Lürling & van Donk (1999) showed that temperature influenced colony induction by zooplankton infochemicals in *Scenedesmus*, and Hessen & van Donk (1993) and Lürling et al. (1997) reported on a *Scenedesmus* strain that formed colonies upon exposure to *Daphnia* infochemicals in one experiment and not in another experiment conducted under the same conditions, suggesting an influence of growth phase or physiological state of the algae. Moreover, as we did not measure and adjust the nutrient concentrations in the

cultures, we cannot rule out the possibility that the *Scenedesmus* and *Daphnia* treatments differed slightly from the control treatment in nutrient concentrations, which may also have influenced functional traits. However, we used a nutrient-rich medium and all the cultures were in exponential phase during the experiment, indicating that nutrients were not limited during the experiment. We can conclude that environmental conditions add another dimension to the complicated picture and merit further investigation in *Microcystis*. However, they do not affect the main findings of our study being (1) the large differences in functional traits between different, even sympatric, *Microcystis* strains, (2) a lack of correlation between functional traits, and (3) the fact that by *Daphnia* infochemicals induced changes in these traits are strongly strain-specific and generally smaller than differences among strains. Our data therefore also indicate that, for future research on the role of particular infochemicals on functional traits of *Microcystis*, it will be important to take inter-strain variability into account.

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