Genotype-dependent interactions among sympatric *Microcystis* strains mediated by *Daphnia* grazing

Ineke van Gremberghe, Pieter Vanormelingen, Bart Vanelander, Katleen Van der Gucht, Sofie D'hondt, Luc De Meester and Wim Vyverman

I. van Gremberghe (ineke.vangremberghe@ugent.be), P. Vanormelingen, B. Vanelander, K. Van der Gucht, S. D'hondt and W. Vyverman, Laboratory of Protistology and Aquatic Ecology, Ghent Univ., Krijgslaan 281-S8, BE-9000 Ghent, Belgium. – L. De Meester, Laboratory of Aquatic Ecology and Evolutionary Biology, Katholieke Univ. Leuven, Charles DeBériotstraat 32, BE-3000 Leuven, Belgium.

Natural populations of the bloom forming cyanobacterium *Microcystis* are typically composed of several distinct genotypes. Using *Microcystis* strains that differ in growth rate, microcystin production and colony formation, we conducted a laboratory experiment in the presence and absence of a grazer, the water flea *Daphnia*, to investigate whether interactions among strains can be predicted from functional traits, and whether the outcome of competition between strains is influenced by a grazer. Two toxic and two non-toxic *Microcystis* strains, isolated from a single lake, were grown during four weeks as single strains, in all possible combinations of two strains and all together, in the presence and absence of *Daphnia magna*. The relative abundance of strains in the populations was assessed using denaturing gradient gel electrophoresis, and the growth rate of each strain in mixed populations was compared to its growth rate in monoculture to determine interactions between strains. The observed interactions were strain-specific, and the relative abundances of strains in mixed populations could be partially explained by taking toxicity and colony formation into account. Importantly, some of the interactions were strongly altered by the presence of *Daphnia*. *Daphnia* induced colony formation in one strain, which then became a better competitor. *Daphnia* grazing also caused a higher evenness in the populations, both through a weakening of competitive interactions as well as by facilitation effects. Strong facilitation effects were due to non-toxic strains benefiting from the protection offered by toxic strains in the presence of predation.

Overall, our results emphasize the presence of strong competitive interactions between *Microcystis* strains in the absence of grazing, whereas indirect positive interactions are prevalent in the presence of a generalist grazer. Our results suggest that differences in functional traits and grazer-mediated facilitation effects may enhance coexistence of *Microcystis* strains, including toxic and non-toxic strains.

Species interactions are among the main factors shaping communities. Interactions among members of the same guild can be negative (resource competition and allelopathy) or positive (facilitation and niche complementarity) and depend on particular physiological traits of the species (Callaway and Walker 1997, Suikkanen et al. 2004, Agawin et al. 2007). Additionally, the strength and sign of these interactions depend upon the ecological context, as the presence of predators or competitors can alter a species' behaviour or morphology and this can influence interspecific interactions (i.e. trait-mediated interactions) (Abrams 1995, Relyea and Yurewicz 2002). In studies on plant community structure, negative effects are mainly caused by resource competition, whereas positive effects can be directly caused by habitat amelioration or indirectly by predator-mediated facilitation. Increased environmental severity (abiotic stress or herbivory) tends to increase the importance of positive interactions relative to negative interactions (Bertness and Callaway 1994, Callaway and Walker 1997, Brooker et al. 2008). For instance, Callaway

et al. (2005) and Graff et al. (2007) showed that palatable plants could be protected against herbivory when growing near unpalatable plants. While in the absence of herbivores the two plant species showed strong competitive interactions, the unpalatable plant species had a strong indirect positive effect on the palatable plant species in the presence of herbivores by acting as a grazer deterrent. While these sometimes complex interactions are well known in plant communities (Hambäck et al. 2000, Rousset and Lepart 2000, Milchunas and Noy-Meir 2002, Baraza et al. 2006), much less is known about interactions in communities of micro-organisms, especially in combination with predation (Hulot et al. 2001, Fox 2004, Jiang 2007).

Bacteria are genetically and biochemically very diverse and complex interactions may occur among strains within morphospecies. This is indicated by recent research on the cyanobacterium *Microcystis*, a cosmopolitan micro-organism that forms toxic blooms under warm weather conditions in eutrophic water bodies (Chorus and Bartram 1999, Visser et al. 2005). Naturally occurring *Microcystis* populations are

typically composed of several genotypes with different ecophysiological traits (Wilson et al. 2005, 2006, Bañares-España et al. 2006, Kardinaal et al. 2007a), but little is known about the mechanisms through which this diversity is maintained. The toxicity of a *Microcystis* bloom is to a large extent determined by the production of the heptapeptide microcystin, which depends on the strain composition, as not all strains possess microcystin synthetase genes (Janse et al. 2004, Kardinaal et al. 2007a). Therefore, it is expected that the structure of *Microcystis* populations and the interactions between strains can have a strong impact on other components of the food web (Hansson et al. 2007). Ecologically relevant traits like growth rate, microcystin production and colony formation may be important for the sign and strength of the interactions among strains as well as with other species. For instance, non-toxic *Microcystis* strains may be better competitors for light than toxic *Microcystis* strains, as suggested by Kardinaal et al. (2007b). In contrast, in *Planktothrix*, toxic strains dominate over non-toxic strains under conditions unfavourable for growth, including low light availability (Briand et al. 2008). Additionally, the ability of *Microcystis* to form colonies, which are generally formed by cells clustering together in a mucilaginous sheet after division, although merging of already existing single cells and colonies may also occur (Reynolds et al. 1981, Burkert et al. 2001), entails advantages such as protection against predation (Fulton and Paerl 1987), the ability to float and shade unicellular strains, a higher photosynthetic efficiency, and enhanced stress tolerance compared to unicellular strains (Zhang et al. 2007, Wu and Song 2008). Previous studies have shown that microcystin production and colony formation appear to have no direct growth rate costs (Wilson et al. 2006) and can both be influenced by zooplankton (Jang et al. 2003, Ha et al. 2004).

In this study, we investigated interactions between four naturally co-occurring *Microcystis* strains that differ in functional traits (growth rate, microcystin production and colony formation) in experimental populations with and without a key grazer, the large-bodied cladoceran *Daphnia magna*. We tested the hypothesis that interactions are strain-specific and influenced by functional traits such as toxin production and colony formation. In addition, we hypothesized that the presence of a grazer changes among-strain interactions substantially. More specifically, we predicted less strong negative interactions among strains, and a facilitation effect of toxic strains on non-toxic strains in the presence of *Daphnia*. If interactions are strain-dependent and involve both competition and facilitation as predicted, then this provides a strong mechanism for the maintenance of strain polymorphism in natural populations of *Microcystis*, including the coexistence of toxic and non-toxic strains.

Material and methods

Isolation and genotypic characterization of *Microcystis* strains

Lake Leeuwenhof (a lake of 7 ha, part of a nature reserve in Ghent, Belgium, 51°04'01"N, 3°39'23"E) was sampled on 6 September 2004 when a very dense *Microcystis* bloom

was present. Twenty-five monoclonal *Microcystis* cultures were obtained by picking up single colonies with a micropipette and growing them in WC medium (Guillard and Lorenzen 1972, but without pH adjustment) 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* genotypes from each other irrespective of morphospecies identification (Janse et al. 2004). We determined the 16S-23S rDNA ITS sequence of each strain, and the sequences were submitted to GenBank under accession numbers FJ830618-FJ830621. Four ITS genotypes could be distinguished and one strain of each ITS type was used in the experiment. Two ITS genotypes were identified as microcystin producing genotypes (toxic genotypes, named T1 and T2) based on the presence of *mcy* genes A (Hisbergues et al. 2003) and E (Vaitomaa et al. 2003) and through direct measurement of microcystin concentrations by ELISA (enzyme-linked immuno sorbent assay). Two strains were identified to be non-microcystin producing genotypes (non-toxic genotypes, named NT1 and NT2) based on the absence of *mcy* genes and negative results in the ELISA measurement. Only T2 formed colonies before the start of the experiment.

Experimental design

Before the start of the experiment, the four *Microcystis* strains were grown 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 two weeks. Culture conditions were the same throughout the study. Exponentially growing cells (growth phase was determined by making growth curves based on optical density measurements with a spectrophotometer, see also below) were used as inocula for the experiment. The four *Microcystis* strains were grown in triplicate in monoculture, in all possible combinations of two strains, and in a four strain mixed culture in 200 ml WC medium in E-flasks during four weeks. All population treatments were grown with and without addition of four individuals of the large-bodied zooplankton species *Daphnia magna*. Our design resulted in a total of 11 populations $\times$ 2 grazing treatments $\times$ 3 replicate observations = 66 experimental units. All cultures were shaken by hand and their position randomized daily. *Microcystis* cell density was estimated in the experiment by converting optical density, measured at 750 nm with a spectrophotometer, into cell density (cells ml^{-1}) using two standard curves made in a pilot experiment. Cell density in the pilot experiment was determined by cell counting using the program Cell C (Selinummi et al. 2005). One curve was fitted for strains T1, NT1 and NT2 ($R^2 = 0.996$), and one for strain T2 ($R^2 = 0.998$) as this strain is characterized by slightly larger cells than the other ones. Cultures without *Daphnia* ('no predation', 'NP') were inoculated at a total density of 50 000 cells per ml, whereas the cultures with *Daphnia* ('predation', 'P') were inoculated at a total density of 500 000 cells per ml. We used two different inoculation

densities in the two predation treatments because a pilot experiment indicated that this was the best compromise to obtain comparable densities towards the end of the experiment (3–4 weeks), when most samples were taken. This reflects the strong impact of *Daphnia* grazing on these cultures, resulting in a collapse of *Microcystis* populations unless inoculated at relatively high densities. Within each mixed population, all strains had the same initial relative abundance. *Daphnia* individuals were obtained from a multiclonal population isolated from different water bodies (fed *Scenedesmus obliquus* in the laboratory) to ensure that the observed effects of grazing represented an average effect of *Daphnia* on *Microcystis*. Before adding *Daphnia* to the *Microcystis* cultures, they were starved for at least three hours. During the experiment, the *Daphnia* individuals were systematically replaced by new individuals every 1–3 days in order to keep densities the same across cultures. Replacement was done after three days during the first week, after two days during the second week and after one day during the last two weeks. The replacements were consistent across all cultures. *Daphnia* survival was low in some of the cultures, especially from the third week of the experiment onwards. At the end of the third week, *Daphnia* survival was monitored in all cultures as the number of individuals alive 24 h after inoculation of four fresh *Daphnia* individuals.

To determine *Microcystis* cell density and microcystin concentrations, a weekly subsample (1 ml) was taken from each culture, which was homogenized first by manual shaking. Also, just before the optical density measurement with a spectrophotometer, the subsample was homogenized again by rapidly pipetting it up and down. After optical density measurement, the sample was stored at -20°C until measurement of microcystin concentrations. Once a week, colony formation was checked microscopically, and pictures of the cells and colonies were taken at the start of the experiment, after two and after four weeks. For single cell and colony counting, 6 ml of each culture was fixed at the end of the experiment with formalin (end concentration 2%) and stored at 4°C . Samples for DNA extraction (2 ml) were taken after two, three and four weeks and stored at -20°C .

Sample analyses

To test to what extent a particular relative quantity of cells corresponds to an equal relative band intensity as determined by denaturing gradient gel electrophoresis (DGGE), a pilot experiment was carried out in which, for all possible two-strain combinations, the two strains were mixed in a gradient of relative cell densities (10:1, 5:1, 2:1, 1:1, 1:2, 1:5 and 1:10). The relative abundance of each strain in the mixtures was determined by DGGE using the protocol of Janse et al. (2003, 2004). DNA from the samples was extracted as described by Zwart et al. (1998), which includes beat-beating with phenol extraction and ethanol precipitation. The rDNA ITS sequence was amplified using the primers (GC)-CSIF and ULR, and a denaturing gradient of 34–40% denaturant was used. Digitalized DGGE images were analysed using the software package BioNumerics 4.5. A matrix was compiled based on band

intensities, and absolute values were converted into relative values. The results clearly showed that DGGE worked well to determine the relative abundance of *Microcystis* ITS genotypes (average R^2 of the correlations between the known relative abundance and relative abundance determined by DGGE in the mixtures is 0.96, data not shown), which is in agreement with the observations of Kardinaal et al. (2007b). We used the same method as described above to study the relative abundance of the strains in the mixed populations during the experiment. The population structure after two and three weeks of growth was comparable with the population structure at the end of the experiment, although evenness gradually declined in all mixed populations. We only present the data after four weeks of growth. The relative abundances of the strains in the populations at the end of the experiment were used to determine which strains are superior and which strains inferior competitors in particular mixtures. *Microcystis* cell density of the monocultures was estimated by converting the optical density into cell density (cells ml^{-1}) using the standard curves mentioned above. *Microcystis* cell density of each strain in the mixtures was estimated by converting the optical density into cell density using the standard curves and combining this with the relative abundance of each strain as determined by DGGE. Pielou's evenness (Pielou 1966) was calculated using Primer 5 (Clarke and Gorley 2001) based on cell densities of the strains in the mixtures at the end of the experiment. Following Loreau (1998), we defined interactions between strains to be present when the growth rate of a particular strain in a mixture departs from the growth rate in monoculture, implying that the presence of other strain(s) influences the growth rate of that particular strain positively or negatively. Growth curves of the monocultures using the weekly density measurements showed an exponential increase of *Microcystis* cell density over the full four-week period, confirming that the growth rate was exponential during the whole experiment. We performed the experiment until the end of the exponential phase. When in stationary phase, few further changes in strain composition would probably occur, as the strains hardly grow. This might be rather similar to a natural situation during bloom build-up. We determined the growth rate of each strain in monoculture and in the mixtures using the formula:

$$[\ln(\text{cells ml}^{-1})_2 - \ln(\text{cells ml}^{-1})_1]/(t_2 - t_1)$$

with t_1 = start of the experiment and t_2 = after four weeks of growth.

For the cultures with *Daphnia* only the net growth rate was actually known (= cell growth rate minus grazing rate).

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 (SDI-EnviroGard Microcystin ELISA plate kit). To calculate the microcystin concentration per biomass, the cell density was 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 and Lessard 2000)

Cell biovolumes were estimated by measuring the average cell diameter of each strain using the program CellC (Selinummi et al. 2005).

Colony formation in the monocultures was determined quantitatively using the fixed samples by counting the cells in unicellular form with a counting chamber and recounting the cells after boiling the samples for 6 minutes to make the colonies fall apart (Joung et al. 2006). This way, the percentage of cells as single cells and colonies could be calculated. In addition, we screened the samples for colonies and measured the size (largest diameter) of a limited number of arbitrary chosen colonies (ten on average) for each culture to have an idea of the size range of the colonies rather than to perform a detailed quantitative analysis of colony size.

Statistical analyses

As our design involved a difference in inoculation densities among predation treatments, a direct comparison of total cell densities and growth rates among predator treatments cannot be interpreted as a biological effect. Within predator treatments, however, total cell densities and growth rates can be compared among monocultures and mixtures. Moreover, we carried out two-way ANOVA (fixed factors: population and predation) on total densities in the cultures as a methodological test to determine whether final densities (fourth week) in the predation treatments differed. As there was no main effect of predation treatment on total final cell densities ($F = 0.449$; $p = 0.506$), we conclude that we can meaningfully compare predation treatments for variables that were measured during the fourth week of our experiment.

One-way ANOVA (fixed factor: strain) was used to test for significant differences in monoculture growth rate between the four strains for each predation treatment separately. Post hoc Tukey tests were performed to identify which strains differed significantly in growth rate in monoculture. A Kruskal-Wallis test followed by post hoc Dunn tests were used to test for significant differences in *Daphnia* survival when grazing on the four *Microcystis* strains in monoculture. Two-way ANOVA (fixed factors: strain and predation) was used to test for significant differences in microcystin production between the two toxic strains and between predation treatments after four weeks of growth. To know whether the presence of a non-toxic strain influenced microcystin production of a toxic strain, two-way ANOVA (fixed factors: presence non-toxic strain and predation) was also performed on the microcystin concentrations of T1 and T2 separately in monoculture and with NT1 and NT2 after four weeks of growth. One-way ANOVA (fixed factor: predation) was used to test for an effect of predation on colony formation (percentage cells in colony form) in T2 after four weeks of growth.

Concerning the mixed populations, one-way ANOVA (fixed factor: population) for each predation treatment separately was used to determine significant differences between total cell densities after four weeks of growth. One-tailed t-tests with Bonferroni correction were used to

determine whether the total cell density of the different mixtures after four weeks of growth was significantly higher than the total cell density of the respective monoculture with the highest yield (referred to as 'overyielding', Loreau 1998). Two-way ANOVA (fixed factors: mixed population and predation) and post hoc LSD tests were performed to test for significant differences in evenness in the mixed populations between the predation treatments after four weeks of growth. Only the results of post hoc tests were shown. One-way ANOVA (fixed factor: competing strain) and post hoc LSD tests were performed for each strain separately to test for significant differences between the monoculture growth rate and the growth rate in each of the mixed populations for each predation treatment separately. Only the results of post hoc tests were shown.

All statistical analyses were performed in SPSS 15.

Results

Strain characteristics in the absence and presence of *Daphnia* grazing

A summary of the traits of the four strains grown in monoculture in the absence and presence of predation is given in Table 1. The growth rate differed significantly between the four strains (Table 1, 2) in both predation treatments. T2 had a lower growth rate than the other three strains, and T1 and NT1 grew slower than NT2 in the absence of predation. In the presence of predation, NT1 had a lower growth rate (around zero) than the other three strains (Fig. 1), and T2 and NT2 grew slower than T1 (Table 1, 2).

The different *Microcystis* populations differentially affected mortality in the grazing *Daphnia*. When grazing on non-toxic strains, the *Daphnia* individuals were in good condition and were found to reproduce asexually. When grazing on toxic strains (or toxic strains together with non-toxic strains), the *Daphnia* showed high mortality. In the last week of the experiment they often died within 24 h, i.e. before they were replaced, and the *Microcystis* strains reached high densities at that time. Figure 2 shows that *Daphnia* exhibited high survival rates when grazing on only non-toxic strains, low survival rates in cultures containing T2 but without T1, and very high mortality in cultures containing T1. The survival of *Daphnia* grazing on the monocultures of NT1 and NT2 was significantly higher than when grazing on T1 ($X^2 = 8.23$, $p = 0.041$) after three weeks of growth.

As expected, no microcystin was detected in the populations with only non-toxic strains. There was no effect of grazing on microcystin production of these two strains, but T1 had a significantly higher microcystin production than T2 after four weeks of growth (Table 1, 3). Total microcystin concentrations increased towards the end of the experiment together with the strong increase in *Microcystis* biomass, while microcystin concentrations per unit biomass of T1 and T2 decreased, especially for T1 (size range of mean microcystin concentrations: for T1, NP = 25.7–17.9, P = 27.3–14.0 and for T2, NP = 5.4–2.8, P = 7.1–3.9 pg per ng C). There were no significant differences in microcystin production for T1 and T2 between monocultures and in presence of NT1 or NT2 (all $p > 0.05$).

Table 1. Summary of the mean value and standard error (between parentheses) of growth rate, microcystin production and colony formation of monocultures of the four *Microcystis* strains in the absence (NP) and presence (P) of predation by *Daphnia*. Growth rate was measured over a period of four weeks, whereas the reported values of microcystin concentration and colony formation were quantified after four weeks of growth.

	T1			T2			NT1			NT2		
	NP	P		NP	P		NP	P		NP	P	
Growth rate (day ⁻¹)	0.212 (±0.001)	0.143 (±0.002)		0.187 (±0.003)	0.108 (±0.006)		0.213 (±0.003)	0.000 (±0.001)		0.227 (±0.002)	0.105 (±0.017)	
Microcystin concentration (pg per ng C)	17.9 (±4.5)	14.0 (±2.3)		2.8 (±0.7)	3.9 (±0.8)		0 (±0)	0 (±0)		0 (±0)	0 (±0)	
Colony formation (% cells in colony)	0 (±0)	0 (±0)		37.5 (±10.9)	39.7 (±7.6)		0 (±0)	0 (±0)		0 (±0)	90 (±9.9)	

No colony formation was detected in T1 and NT1 during the experiment. T2 formed large, loose colonies (size ranging from a few cells to large colonies of approximately 1 mm in diameter) both in presence and absence of predation. There were no significant differences in the percentage of cells in colony form in the presence and absence of predation ($p > 0.05$) after four weeks of growth for this strain (Table 1). NT2 formed large, dense colonies after two weeks of growth in the presence of predation (size ranging from a few cells to large colonies of approximately 1 mm diameter) and kept its colony form until the end of the experiment. In the absence of predation, only single cells were seen in cultures of this strain (Fig. 1, Table 1).

Interactions between *Microcystis* strains in the absence and presence of *Daphnia* grazing

Figure 3 shows the total cell density of the monocultures and mixed populations as well as the relative abundances of the strains in the mixed populations after four weeks of growth. In both predation treatments, the populations differed significantly in total cell density (NP: $F = 24.91$; $p < 0.001$, P: $F = 11.61$; $p < 0.001$). In the absence of predation, none of the mixed populations had a total cell density that was significantly higher than the highest respective monoculture cell density ($p > 0.05$), indicating the absence of overyielding. However, in the presence of predation, total cell densities of the mixtures T1 + NT2 and T2 + NT1 were significantly higher than the monoculture cell densities of T1 and T2, respectively ($p < 0.05$), resulting in significant overyielding in these combinations of toxic and non-toxic strains.

In many of the mixed populations, one of the strains was clearly dominant, whereas in other mixtures, the different strains were co-dominant. Predation by *Daphnia* tended to reduce dominance of specific strains in the mixed populations, resulting in a higher evenness (Fig. 3). A significant higher evenness in the presence than in the absence of *Daphnia* was seen for mixtures T1 + NT2, T2 + NT2 and the four-strain mixtures ($p < 0.05$). A significant lower evenness in the presence than in the absence of *Daphnia* was seen in the mixture involving the two non-toxic strains NT1 + NT2 ($p < 0.05$), where *Daphnia* grazing induced dominance of NT2.

The growth rate of strains was often influenced by the presence of other strains. As a result, the dominance of a strain in a mixture could not be explained by differences in monoculture growth rate alone. Overall, we detected complex and strain-dependent patterns of suppression and facilitation of growth in the mixed populations (see Fig. 4 for significant results). For instance, T1 never suppressed another strain but was suppressed by all other strains in the absence of *Daphnia* (very strongly by NT2), and moderately suppressed by T2 in the presence of *Daphnia*. T2 strongly suppressed NT2 in the absence of predation. NT1 weakly suppressed T1 and NT2 in the absence of grazing and was never suppressed by another strain, except in the four-strain cultures in the absence of grazing. T1 was never facilitated by any other strain, but it facilitated all other strains in both grazed and non-grazed populations. It very strongly facilitated both non-toxic strains in the presence of

Table 2. ANOVA table on the differences in growth rate of monocultures of the four *Microcystis* strains in the absence (NP) and presence of *Daphnia* (P). Results of Tukey post hoc tests are given (p-values) for all strain combinations (significant p-values are in bold).

Effect	DF	MS	F	p	Post hoc	NP	P
Strain (NP)	3	0.001	44.45	<0.001	T1/T2	0.001	0.003
Strain (P)	3	0.012	187.77	<0.001	T1/NT1	0.972	<0.001
					T1/NT2	0.009	0.039
					T2/NT1	<0.001	<0.001
					T2/NT2	<0.001	0.274
					NT1/NT2	0.017	<0.001

predation. T2 was facilitated by all other strains, except by NT1 in the absence of grazing, and NT1 was facilitated by both toxic strains in the presence of *Daphnia* and by NT2 in the absence of *Daphnia*. Generally, strong negative interactions among strains were detected in the absence of predation, whereas strong positive interactions were especially seen in the presence of predation (Fig. 4).

In Fig. 4, suppression and facilitation are quantified relative to growth rates in monocultures. For an assessment of the fitness of strains and a prediction of their occurrence in nature, absolute growth rates in the presence of other strains are also important (Fig. 4 and resulting population densities in Fig. 3). Taking both absolute growth rates as well as competition/facilitation effects into account, it is clear that strain T1 is well-adapted to grow in isolation in both the presence and absence of grazers. In the absence of grazers, it did not grow faster than the non-toxic strains, but in the presence of grazers, it was by far the best growing strain. This strain seems, however, sensitive to competition, and especially to competition from strain NT2. T2, which had a lower monoculture growth rate than the other three strains in the absence of predation, was co-dominant or dominant in all mixed populations. This strain seems very well adapted to co-occur with other strains. In the four-strain cultures, this strain completely outcompeted all other strains in the absence of grazers. Both non-toxic strains

performed well in the absence of grazers, but were sensitive to strain-specific interactions: NT1 was very sensitive to the combined interaction with the other three strains, whereas NT2 was very sensitive to T2. In the presence of grazers, NT1 performed very poorly when grown in isolation but was able to grow in mixed cultures with the toxic strains and then became even co-dominant or dominant. NT2 could grow in the presence of grazers, and gained additional advantage from co-occurrence with T1.

Discussion

Our experiment revealed strong negative as well as positive interactions between *Microcystis* strains in mixed cultures, and these interactions were modulated by the presence of *Daphnia magna*. Allelopathic interactions as well as resource competition may lead to negative interactions, and additional experiments are needed to distinguish between these mechanisms. In the filamentous cyanobacterium *Planktothrix*, it was recently shown that growth inhibition in mixed cultures was at least partly caused by allelopathic interactions among strains, and that these interactions are strain-specific (Oberhaus et al. 2008). The interactions observed in our experiment are highly strain-specific too, and several other functional traits than the ones we quantified are

Figure 1. Photographs of the four *Microcystis* strains in monoculture at the end of the experiment, and for strain NT2 microscopic photographs of the cells and colonies showing colony induction in the presence of *Daphnia magna* (scale bar = 100 µm). NP = no predation, P = predation.

Figure 2. *Daphnia* individuals alive after 24 h grazing on *Microcystis* cultures grown for three weeks and total microcystin concentration in the *Microcystis* cultures at the third week. Error bars indicate the standard error of the mean.

probably involved. Yet, many of the interactions observed are in line with expectations based on among-strain variation in toxin production and colony formation and their association with competitive strength. Strain T1 confirmed the lower competitive strength of toxic strains compared to non-toxic strains when light becomes limited (Kardinaal et al. 2007b) and probably grows well at low cell densities early in the growing season or under pioneer conditions. Its relatively fast growth in the presence of predation may be due to its high microcystin production, however the toxicity is mainly determined by the microcystin variants produced and these were not measured.

Strain T2 exhibited a striking dominance in competition. This may be related to the capacity of T2 to form colonies, which may convey a strong advantage in competition because of shading effects of the floating colonies, thus reducing light availability for competing strains. Alternatively, strain T2 may exhibit traits that we did not quantify and that strongly interact with other strains in a strain-specific manner. The latter is not unlikely, given that T2 did not suppress strain NT1 to the same degree as it did strain NT2. Colony formation may also explain other instances of competitive dominance in our experiment, such as the dominance of T2 over T1 in both presence and absence of grazing, and the dominance of NT2 over NT1 in the presence of grazing.

Table 3. ANOVA table on the effects of strain and predation on microcystin production of monocultures of T1 and T2 after four weeks of growth (significant p-values are in bold).

Effect	DF	MS	F	p
Strain	1	4.794	24.332	0.001
Predation	1	0.056	0.283	0.609
Strain × predation	1	0.180	0.912	0.367

We observed striking facilitation and/or niche complementarity in the mixed *Microcystis* populations, as growth rate of some of the strains was stimulated by the presence of other strains. These interactions too were strain-specific. We observed a tendency for more facilitation in the presence than in the absence of predation, which is largely due to very strong facilitation of non-toxic strains by toxic strains in the presence (three cases) but not in the absence of predation (two cases of mild facilitation).

One of the key results of our experiment was indeed the strong impact of grazing by *Daphnia* on the interactions among *Microcystis* strains. When taking absolute growth rates into account, it appears that non-toxic strains have an equally high or higher growth rate than toxic strains in the absence of grazing, but are less buffered against grazing when growing in isolation. Additionally, colony formation conveys an advantage in the presence of grazing. Admittedly, these conclusions are based on observations on just a few strains, but are largely consistent with expectations from literature (Fulton and Paerl 1987, Kardinaal et al. 2007b, Zhang et al. 2007, Wu and Song 2008). Predators can diminish strong competitive interactions within a prey community directly by grazing more on the dominant species, or indirectly by the recycling of limiting nutrients and the secretion of growth-promoting substances (Vanni and Layne 1997, Elser and Urabe 1999, Vanni 2002). Although in our experiment the effect of *Daphnia* on the population structure of *Microcystis* can be caused by grazing and/or through the excretion of nutrients that are taken up by the *Microcystis* strains, the strain-specific effects we observed rather point to specific mechanisms and facilitation. First, *Daphnia* induced colony formation in one non-toxic strain (NT2), and this resulted in a protection against predation, which gave the strain a competitive advantage over the non-toxic unicellular strain NT1. The

Figure 3. Total cell density of *Microcystis* monocultures and mixed populations of the four strains after four weeks of growth, with indication of the relative contribution of each strain in the mixed populations (lower graphs) and Pielou's evenness (J') of the mixed populations after four weeks of growth (upper graphs), (A) in the absence of predation, (B) in the presence of predation. Error bars indicate the standard error of the mean.

occurrence of colony induction in *Microcystis* by *Daphnia* has been reported before (Jang et al. 2003, Ha et al. 2004), although other studies did not observe colony induction (Hessen and van Donk 1993, Yang et al. 2006). Our results confirm that induction of colony formation is strain-specific as only one strain responded to the presence of *Daphnia* by colony formation. Colonies of *Microcystis* can reduce the filtering rate of cladocerans by mechanical interference with the filtering system (Fulton and Paerl 1987), and *Daphnia* may avoid ingestion of large colonies by narrowing the carapace gape (Lampert 1987). In natural systems, it is commonly believed that *Microcystis* strains are present in colony form, which may be caused by the presence of predators. Second, grazing by *Daphnia* caused very strong

indirect facilitation effects (Callaway et al. 2005, Graff et al. 2007) in some of the mixed populations. More specifically, non-toxic strains benefited from the presence of toxic strains because *Daphnia* suffered severely from the microcystins. This protection offered by toxic strains is parallel to herbivory protection as reported in plants (Callaway et al. 2005, Graff et al. 2007). Once protected, it is conceivable that the non-toxic strains are even stimulated in their growth by the nutrients that *Daphnia* excrete (Vanni 2002). Our results do not allow, however, testing this idea. The fact that *Daphnia* cannot distinguish between toxic and non-toxic strains and is poisoned by microcystins has been extensively studied (DeMott 1999, Rohrlack et al. 1999, 2001, Lotocka 2001). As expected, the production of microcystins seemed to influence the survival rate of *Daphnia*. However, as shown previously, negative influences of *Microcystis* strains on *Daphnia* are not exclusively due to microcystin production and tend to be strain-specific (Lüring 2003, Tillmanns et al. 2008). Contrary to Jang et al. (2003), we did not find an increased microcystin production in the presence of *Daphnia*. In accordance with Orr and Jones (1998), the decrease of microcystin concentration per unit biomass towards the end of the experiment might be due to decreasing light intensities and nutrient concentrations resulting in a slight decrease in growth rate.

One may argue that we had to replace the *Daphnia* in our grazing treatment and thereby artificially increasing the impact of grazing by zooplankton compared to the natural situation. We replaced the grazers, as we wanted to investigate what the impact of continued grazing is on mixed populations that involve both toxic and non-toxic strains of *Microcystis*. In natural systems, at the beginning of a bloom, the biomass of *Microcystis* is low and other (more edible) algae are present, and *Daphnia* will, as a consequence, not die very fast. Moreover, *Daphnia* can increase its tolerance to *Microcystis* toxins through maternal effects (Gustafsson et al. 2005) and microevolution (Hairston et al. 1999). The effect of *Daphnia* on *Microcystis* biomass is debated, as *Microcystis* may strongly suppress 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). Our experiment suggests that it is indeed possible that *Daphnia* prevents *Microcystis* bloom formation, depending on the initial genetic composition of the *Microcystis* population, and the relative initial densities of *Microcystis* and *Daphnia*.

We did not find direct evidence for populations with a higher diversity to have a generally higher production (biomass), as reported in several studies that have evaluated the relationship between biodiversity and ecosystem functioning (Loreau and Hector 2001, Hooper et al. 2005, Reusch et al. 2005). However, non-toxic unicellular strains cannot grow in the presence of grazers, and while toxic strains decreasing grazing pressure are needed to obtain a high total biomass of *Microcystis* in the presence of *Daphnia*, it is the combination of defended toxic strains and fast growing non-toxic strains that yields the highest total biomass under grazing. In natural circumstances, grazers are usually present, and a higher genotypic diversity in *Microcystis* populations will increase the likelihood that

Figure 4. Growth rate of the four *Microcystis* strains in monoculture and in mixed populations in the absence (no predation) and presence (predation) of predation. Hatched lines indicate the growth rate in monoculture. Zero-values for the growth rate mean that the growth rate was zero or negative. Asterisks indicate significant differences (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$) between the growth rate of the strain in the mixed population and the growth rate in monoculture. Error bars indicate the standard error of the mean (non-visible error bars are smaller than symbols).

toxic strains and/or strains forming large colonies are present, resulting in a higher probability of *Microcystis* blooms. In a meta-analysis of the impact of grazers on periphyton biomass, Hillebrand and Cardinale (2004) also found evidence that a higher diversity of prey decreases consumer effects. We also followed the population dynamics and total density of the populations in time, as it is known that interactions and diversity effects on productivity can change over time (Fox 2004, Weis et al. 2007). However, we did not find considerable changes in the structure or relative total density of the different populations after two, three and four weeks of growth (unpubl.). This indicates that strain interactions do not seem to be strongly influenced by the cell densities used in our experiment.

Despite the fact that our study is based on only four strains and that more research involving a broader set of genotypes and environmental conditions would be most welcome to elucidate how interactions among strains structure *Microcystis* populations in nature, our study allows some general conclusions. First, our results show that complex strain-specific interactions occur among *Microcystis* strains, and that the nature and strength of these interactions depends on the presence of grazers. Also, it is difficult to predict the responses entirely on the growth characteristics of the strains in isolation. Knowledge on toxicity and colony formation appears very useful to predict competitive dominance in mixed cultures, but does not lead to perfect predictions. Our observations suggest that strain polymorphism in populations of *Microcystis* can be maintained by strain-specific interactions that are modulated by

differences in key functional traits, and by interactions with environmental variation. In the absence of grazing, polymorphism is mediated by specific interactions that partly involve tradeoffs between growth characteristics in isolation and in mixtures. In the presence of grazing, polymorphism is enhanced by facilitation effects.

Our results suggest that complex strain-specific interactions and predator-mediated facilitation effects may contribute to our understanding of the so-called ‘paradox of plankton’ (Hutchinson 1961, Huisman et al. 2001, Scheffer et al. 2003). It is difficult to directly translate the results from our experiment to natural circumstances as different environmental conditions (abiotic conditions or predators) may strongly influence the sign and strength of the interactions observed. However, our experiment does show the kind of and complexity of interactions that can be expected between sympatric strains and how these are mediated by predators. Another notable observation is that toxic and non-toxic strains can coexist both in the absence and presence of intense *Daphnia* grazing. From our observations on pure cultures, one would expect that *Daphnia* grazing would promote toxic strains over non-toxic strains, whereas non-toxic strains would be expected to predominate in the absence of grazing. Yet, all but two of the two-strain mixtures that involved toxic and non-toxic strains showed an appreciable fraction of the population to consist of the toxic strain in the absence of *Daphnia*, whereas all mixed populations had relatively high abundances of the non-toxic strain in the presence of *Daphnia*. The latter observation is especially striking and reflects

strong facilitation effects of non-toxic strains being protected by the presence of toxic strains.

The development and characteristics of *Microcystis* populations should be viewed in the context of both environmental conditions and strain composition, and account for environment-strain interactions. Whether the relationship between strain composition and environmental conditions is predictable in terms of lineage sorting (Baas Becking 1934, Finlay and Clarke 1999, Finlay 2002, Van der Gucht et al. 2007) or impacted by colonization effects and dispersal limitation requires further study. Our results, however, indicate that lineage sorting, if prevalent, may not operate in a simple manner, but on combined traits of strains in relation to both environmental variation and strain composition of the resident population. It might be a fruitful approach to view *Microcystis* populations as communities of functionally different strains and analyze their ecology (e.g. occurrence, blooms and toxicity) from that perspective.

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