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Effects of Cell-Bound Microcystins on Survival and Feeding of *Daphnia* spp.

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The influence of cell-bound microcystins on the survival time and feeding rates of six *Daphnia* clones belonging to five common species was studied. To do this, the effects of the microcystin-producing *Microcystis* strain PCC7806 and its mutant, which has been genetically engineered to knock out microcystin synthesis, were compared. Additionally, the relationship between microcystin ingestion rate by the *Daphnia* clones and *Daphnia* survival time was analyzed. Microcystins ingested with *Microcystis* cells were poisonous to all *Daphnia* clones tested. The median survival time of the animals was closely correlated to their microcystin ingestion rate. It was therefore suggested that differences in survival among *Daphnia* clones were due to variations in microcystin intake rather than due to differences in susceptibility to the toxins. The correlation between median survival time and microcystin ingestion rate could be described by a reciprocal power function. Feeding experiments showed that, independent of the occurrence of microcystins, cells of wild-type PCC7806 and its mutant are able to inhibit the feeding activity of *Daphnia*. Both variants of PCC7806 were thus ingested at low rates. In summary, our findings strongly suggest that (i) sensitivity to the toxic effect of cell-bound microcystins is typical for *Daphnia* spp., (ii) *Daphnia* spp. and clones may have a comparable sensitivity to microcystins ingested with food particles, (iii) *Daphnia* spp. may be unable to distinguish between microcystin-producing and -lacking cells, and (iv) the strength of the toxic effect can be predicted from the microcystin ingestion rate of the animals.

Apart from the significance of being widespread and often bloom forming, many cyanobacterial species share the ability to produce bioactive compounds (4, 6). The microcystins, mainly produced by *Microcystis* spp., are among the most dominant of these bioactive substances and have been characterized as cyclic heptapeptides. The bioactivity of microcystins is mainly based on an inhibition of eukaryotic protein phosphatases (12, 27). As a result, microcystins may, once they have entered tissues or cells, interfere with numerous processes essential for life and are thus potentially harmful for higher organisms and humans (19). However, despite the broad attention given them and many advances in their molecular and biochemical characterization, the ecological significance of microcystins is still controversial.

Microcystins are usually cell bound. Although *Microcystis* cells may have possibilities for an outward transport (22), the intracellular microcystin concentration is typically high. Microcystins may therefore harm organisms that feed on *Microcystis* cells. Special interest has been applied to the effects on *Daphnia* spp., which are a key component of freshwater food chains (e.g., see reference 35). *Daphnia* spp. can consume considerable amounts of the phytoplankton biomass and are large enough to feed on *Microcystis* colonies (e.g., see references 40 and 41).

The presence of *Microcystis* in the diet can affect *Daphnia* in different ways. Several *Microcystis* strains, for example, cause a nonmechanical feeding inhibition and, once ingested, direct toxic effects (7, 21, 25, 31, 42). Experiments performed to test whether or not microcystins are the cause of these effects have produced an array of inconsistent data. DeMott and Dhawale (8), for example, have shown that purified microcystins inhibit the in vitro activity of *Daphnia*'s protein phosphatases 1 and 2A and may thus have various adverse effects if assimilated into the body of the animals. This is in good agreement with the finding that dissolved microcystins are toxic to several *Daphnia* spp. (reviewed in reference 5). Based on survival tests with cyanobacterial cells and cell extracts, Jungmann (20) and Reinikainen (34) have, on the other hand, suggested that toxicity to *Daphnia* is not due to microcystins. Matveev et al. (29) have even proposed an ineffectiveness of microcystins to harm *Daphnia carinata*, and Pflugmacher et al. (33) have described an in vitro detoxification mechanism that, if also active in living *Daphnia*, may result in a resistance to microcystins.

The role of microcystins in feeding inhibition is unclear as well. As mentioned, many *Microcystis* strains, though not all, slow down the feeding activity of *Daphnia* in a non mechanical way (7, 16, 21, 25, 31). The responsible factor is yet unknown, but it may be a perceptible structure or substance located in the outer cell compartments (26, 37). Since microcystins can possibly pass through the cell wall of *Microcystis* (22), they may well be involved in the feeding inhibition. Several studies have been conducted to test that idea, but the results obtained are, as for the case of the toxic effect, contrary (16, 21, 25, 26).

The main problem in studying the impact of microcystins seems to be their cell-bound character. Since *Daphnia* spp. ingest microcystins together with living cells, it is difficult to distinguish the potential microcystin effects from those caused

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TABLE 1. Origin and mean length of the *Daphnia* clones used in the experiment

Daphnia clone[a]	Origin	Characteristics of original habitat	Length of 5- to 6-day-old animals (mm [mean ± SD])
D. galeata (clone A)	Lake Müggelsee, Germany	Shallow, hypertrophic lake, with summer plankton dominated by cyanobacteria; microcystins are common	1.60 ± 0.14
D. galeata (clone B)	Alte Donau, Austria	Eutrophic water; potential microcystin producers (*Microcystis*) are present	1.69 ± 0.15
D. hyalina	Lake Zürichsee, Switzerland	Mesotrophic lake; potential microcystin producers (*Oscillatoria rubescens*) are present	1.49 ± 0.15
D. pulicaria	Lake Constance, Germany	Mesotrophic lake; cyanobacteria may be present	1.93 ± 0.20
D. pulex	Gadekæret, Zackenberg, Northeast Greenland	Ultra oligotrophic pond of the high arctic region; no cyanobacteria in the plankton	1.72 ± 0.18
D. magna	Langedam, Denmark	Eutrophic pond; cyanobacteria are present from time to time	2.11 ± 0.22

[a] *Daphnia* clones were kindly supplied by the following: *D. galeata* clone A, M. Henning (Humboldt-University, Berlin, Germany); *D. galeata* clone B and *D. hyalina*, R. Kurmayer (Institut für Wasser-, Boden- und Lufthygiene, Berlin, Germany); *D. pulicaria*, W. Lampert (Max Planck Institute for Limnology, Plön, Germany); *D. pulex* and *D. magna*, laboratory cultures of the Freshwater Biological Laboratory (Hillerød, Denmark).

by other components. To solve that problem, Rohrlack et al. (39) have compared the effects of the microcystin-producing *Microcystis* strain PCC7806 and its mutant, which has been genetically engineered to knock out microcystin production (11). That line of study has been combined with an analysis of the relationship between the microcystin ingestion rate by *Daphnia* and its survival time (38). It turned out that the toxic effect of *Microcystis* spp. could be explained by microcystins, while the feeding inhibition seems to be due to another factor. However, Rohrlack et al. (38, 39) have based their experiments on a single *D. galeata* clone only and so it is uncertain if the results obtained can be generalized and used to clarify the role of microcystins in the effects of *Microcystis* on other *Daphnia* spp. Furthermore, it is unknown if *Daphnia* spp. can differ in their sensitivity to cell-bound microcystins and if microcystins ingested with food particles are toxic to all species. Another important but still unanswered question is whether or not it is possible to find a simple dose-response relationship for microcystin effects. Such a relationship may be useful to estimate the possible impact of *Microcystis* on *Daphnia* populations and to distinguish microcystin effects from those of other bioactive compounds.

Therefore, aims of the present study were (i) to examine the effects of cell-bound microcystins on six *Daphnia* clones belonging to five common species, (ii) to compare the sensitivity of these *Daphnia* clones to microcystins ingested with food particles, and (iii) to test if it is possible to predict the strength of microcystin effects. To that end, feeding and survival of *Daphnia* were tested with either the microcystin-producing *Microcystis* strain PCC7806 or its genetically engineered, microcystin-lacking mutant (11) as sole food. Additionally, the relationship between the microcystin ingestion rate of the six *Daphnia* clones and their survival time was analyzed.

MATERIALS AND METHODS

Origin, description, and culturing of *Microcystis* and *Scenedesmus*. The strain *Microcystis aeruginosa* PCC7806 was kindly provided by J. Weckesser (Albert-Ludwigs-University, Freiburg, Germany). It was originally isolated from the Braakman Reservoir (The Netherlands) in 1972 and grows as single cells without any sign of a mucilaginous envelope (transmission electron microscopic analysis by W. Bleiß and A. Marko, Humboldt-University, Berlin, Germany). *Microcystis* strain PCC7806 produces several bioactive compounds, mainly the microcystins MCYST-LR, (D-Asp^3)MCYST-LR (10, 20), and cyanopeptolin depsipeptides (28). Cells of this strain usually cause a marked feeding inhibition in *Daphnia* (7) and have a moderate to strong toxic effect (21, 38, 39). The mutant cell line of PCC7806 was obtained by transformation of strain PCC7806 with a mutant version of one of the microcystin synthetase genes, including recombinative replacement of the wild-type copy of this gene. The peptide synthetase gene *mcyB*, which is known to be an essential part of the microcystin synthetase gene cluster (43), was insertionally inactivated using a chloramphenicol resistance cartridge. The insertion resulted in a highly specific and complete knockout of microcystin synthesis while it did not affect the production of other oligopeptides, such as cyanopeptolines (11). Wild-type and mutant cells of PCC7806 have the same genotype except for the insertion region.

The stock culture of *Scenedesmus acutus* was kindly supplied by W. Lampert (Max Planck Institute for Limnology, Plön, Germany). This green alga, which served as a food source for *Daphnia* cultures and a control in feeding experiments, grows in small aggregates consisting of up to 16 cells.

Both variants of *Microcystis* strain PCC7806 were grown in Z8 medium (24) as nonaxenic, semicontinuous cultures. The cultures were maintained under continuous light (25 μmol of photons $m^{-2} \cdot s^{-1}$) supplied by cool white fluorescent lamps and were shaken twice a day. The temperature was kept at 20 ± 1°C. The cultures were diluted daily at the same time to a final concentration of 100 mm^3 $liter^{-1}$ (a cell biovolume of 1 mm^3 of PCC7806 [wild-type or mutant] is equal to 3.413×10^7 cells or 0.14 mg of C [calculated from reference 36], and under the described growth conditions the cell diameter of both PCC7806 variants was 3.83 ± 0.40 μm [mean ± standard deviation]). The cell density was determined using a calibration curve of the light absorbance at 800 nm and the biovolume concentration. Under these growth conditions the wild-type and mutant PCC7806 strains exhibited the same growth rate, which was about 0.30 day^{-1}. This is comparable to the rates given for nutrient-saturated turbidostat cultures of *Microcystis* (30). Both variants of PCC7806 were clearly light- but not nutrient limited, since an increase in light intensity accelerated growth, while the addition of major nutrients (N, P) had no significant effect. *Scenedesmus* was grown using the same conditions and procedures except for the light intensity (32 μmol of photons $m^{-2} \cdot s^{-1}$) and the density (50 mm^3 $liter^{-1}$; 1 mm^3 of *Scenedesmus* is equal to 5.53×10^6 cells and 0.14 mg of C [calculated from reference 36], and cell dimensions under the described growth conditions were 5.61 ± 0.65 by 10.85 ± 1.62 μm [means ± standard deviations]) to which the cultures were diluted daily. The mean growth rate was about 0.5 day^{-1}.

Cyanobacterial and algal cultures were grown for at least 3 weeks at a constant rate before use in experiments. That time corresponds to nine or more cell divisions and should ensure a complete adaptation to the culture conditions described. Algae and cyanobacteria were harvested by means of centrifugation for 15 min at 500 × *g*. To produce radiolabeled *Microcystis* or *Scenedesmus* cells, 0.36 MBq of $NaH^{14}CO_3$ was added to 100-ml cultures, which were then grown under the described conditions for two further days.

Origin and culturing of *Daphnia* clones. Six *Daphnia* clones belonging to five species were used in the experiments. They were isolated from different kinds of waters, ranging from small ponds to large lakes, and different locations, including the temperate zone of Central Europe and the high arctic region of Northeast Greenland. The main idea was to include animals that strongly differed in their genotype and that thus represent the diversity of the genus *Daphnia*, at least to some extent (Table 1).

All *Daphnia* clones were cultured under the same conditions in a synthetic zooplankton medium (23) with *Scenedesmus* as the sole food source. Prior to an

experiment 7 to 10 newborn animals were harvested from well-fed stock cultures and transferred into 0.5-liter glass vessels completely filled with a suspension of 5 mm^3 of *Scenedesmus* $liter^{-1}$. The culture vessels were kept under indirect, continuous light (5 μmol of photons $m^{-2} \cdot s^{-1}$) and a constant temperature of 20 ± 1°C. The food suspension was changed and any offspring were removed every second day. The animals were maintained under these conditions for at least 2 weeks and then served as "defined mothers" for the animals used in the experiments. The experimental animals were taken as offspring born within 24 h from the mother cultures. They were kept under the described conditions for five further days and were then ready for use (see Table 1 for mean body lengths).

DNA isolation, PCR, and microcystin analyses. As cyanobacteria are known to contain several genome copies (2), it was necessary to prove the homozygous genotype of the mutant before starting the experiments with *Daphnia*. Remaining wild-type copies of the *mcyB* gene might undergo amplification under the nonselective culture conditions (medium without chloramphenicol) applied during this study and lead to a restoration of microcystin production. The absence of all wild-type *mcyB* gene copies in the mutant was therefore checked by PCR using primers binding upstream and downstream of the mutated region. Genomic DNA of *Microcystis* strain PCC7806 was isolated as described previously (14). The PCR was performed using Goldstar thermostable DNA polymerase (Eurogentec) and primers Tox2p (5′GGAACAAGTTGCACAATCCG C3′) and Tox2m (5′CCAATCCCTATCTAACACAGTAACTCGG3′). The PCR procedure was initiated by a denaturation step (2 min, 95°C), followed by 30 cycles consisting of 20 s at 90°C, 30 s at 55°C, and 2 min at 72°C and by a final elongation step (5 min, 72°C).

Microcystins were extracted from cyanobacterial cells collected on glass fiber filters. A culture volume corresponding to 5 mm^3 of cell biovolume was filtered through a GF/F filter (47 mm), which was then kept frozen at −18°C. Prior to the extraction procedure the filters were thawed and refrozen three times to break down the cell structures. Afterwards, each wet filter was transferred into a glass vial filled with 3 ml of 100% methanol, which was then sonicated (indirect sonication in a water bath in order to lyse and dislodge cyanobacterial cells; Bransonic model 2210, 20°C) for 15 min and shaken for a further 20 min. The filter was then squeezed repeatedly with a forceps and eventually removed from the extract. The whole procedure was repeated three times. The liquid phases from all extraction steps of a sample were combined, filtered (GF/F filter), and evaporated at 50°C overnight. The dried extract was reconstituted in 20% acetonitrile, shaken for 30 min, and transferred into a high-performance liquid chromatography (HPLC) autosampler vial.

The reversed-phase HPLC analysis of microcystins (Waters 600 PDA detector, 717 autosampler, 600E controller, symmetry C_{18} 5-μm, 3.9- by 150-mm column) used a linear gradient starting with 20% acetonitrile in 10 mM ammonium acetate solution (pH 5.0) and ending after 30 min with 28% acetonitrile. That gradient allows a separation and quantification of all microcystin variants produced by *Microcystis* strain PCC7806. The column temperature was 40°C, the detector was set at 239 nm, and the flow rate was set at 1 ml min^{-1}. The microcystins were quantified using a microcystin-LR standard supplied by G. Codd (University of Dundee, Dundee, Scotland). The microcystin content of the PCC7806 wild-type and mutant strains was analyzed for all cultures which were used in the experiments. The microcystin content is given as micrograms per cell biovolume (in cubic millimeters) of the cyanobacteria.

Determination of feeding rate and calculation of microcystin ingestion rate. Feeding rates were measured by using a radioisotope technique and ^{14}C-labeled *Microcystis* or *Scenedesmus* cells. The experiments were run in 300-ml glass vessels at 20 ± 1°C and a constant light intensity of about 5 μmol of photons $m^{-2} \cdot s^{-1}$. At the beginning of an experiment each container received 200 ml of an unlabeled suspension of either wild-type *Microcystis* strain PCC7806, the PCC7806 mutant, or *Scenedesmus* and up to 10 animals of one of the *Daphnia* clones. The food suspensions were prepared with synthetic zooplankton medium. The particle concentration was always 10 mm^3 $liter^{-1}$. After an adaptation period of 1 h, ^{14}C-labeled material of the respective food source was added at a ratio of 1:3 to the unlabeled food. The animals were exposed to the radioactive food suspension for 10 min, after which they were removed from the containers, washed with culture medium, and measured to calculate their biovolume (1). All animals from one incubation vessel were then collected in a scintillation vial to which 100 μl of a tissue solubilizer was added. These vials were then incubated at room temperature overnight. To measure the specific radioactivity of the food, two 10-ml samples of food suspension were taken from each container at the beginning of the 10-min feeding time. These samples were individually filtered through 0.45-μm-pore-size cellulose nitrate filters, which were then separately transferred into scintillation vials. To all vials (animal and food samples) 10 ml of a liquid scintillation cocktail (Ultima-Gold; Packard) were added and the vials were then shaken for 12 h. The radioactivity of the samples was measured using a liquid scintillation counter (Rackbeta 1219; LKB Wallac) and external standards. The whole experimental procedure was repeated five times for all food types and *Daphnia* clones. Feeding rates were calculated as biovolume of ingested food (in cubic millimeters) per biovolume of *Daphnia* (in cubic millimeters) per hour.

The microcystin ingestion rate describes the amount of microcystins which the animals have taken in together with food particles per time unit (see reference 38). The value was calculated by multiplying the cellular microcystin content of a *Microcystis* strain (sum over all microcystin variants) with the feeding rate of *Daphnia* on that particular cyanobacterial strain. The microcystin ingestion rate is given in nanograms of microcystin per cubic millimeter of animal biovolume per hour.

Survival experiments. Survival experiments were carried out in autoclaved 300-ml glass bottles (Schott, Mainz, Germany) at 20 ± 1°C and a mean constant light intensity of about 5 μmol of photons $m^{-2} \cdot s^{-1}$. The bottles were placed on a plankton wheel (one rotation per minute) to ensure a homogenous distribution of the food particles. At the beginning of an experiment each bottle received 300 ml of a suspension of either wild-type *Microcystis* strain PCC7806 or its mutant and 10 animals of one of the *Daphnia* clones. The food suspensions were prepared using synthetic zooplankton medium. The food particle concentration was always 10 mm^3 $liter^{-1}$. In addition to the *Microcystis* suspensions, nonfood controls were run to evaluate the effect of starvation. Survivors were counted every 12 h. Animals were considered dead if they did not show any movement during 30 s of intensive disturbance. The food suspensions (or medium in the case of nonfood controls) and bottles were changed daily. The whole procedure was repeated three (*D. magna, D. galeata, D. hyalina*) or four (*D. pulex, D. pulicaria*) times for all food types, the nonfood control, and the *Daphnia* clones. The experiments were terminated when all animals were dead or after 10 days.

Statistics. Student's *t* test was used to compare the means of feeding rates for the different food types (data showed normal distribution). The significance of possible differences between survival functions was tested by the log-rank test. In order to quantify the effect of *Microcystis* or starvation on survival, the time needed to kill 50% of the animals (LT_{50}) was calculated as the median of the Kaplan-Meier survival function estimation (44). Correlation and regression analyses were carried out using the SPSS regression program package. All statistical tests were performed at the 95% level of significance unless something different is stated.

RESULTS

Characterization of the PCC7806 mutant and microcystin analysis. Before any experiment was started, the absence of wild-type gene copies in the PCC7806 mutant was checked by the described PCR procedure. The results clearly proved the lack of wild-type *mcyB* gene copies in the mutant cells and thus their inability to produce microcystins (data not shown). In addition, all cultures of the PCC7806 mutant used in the experiments were analyzed by HPLC. The lack of any microcystin was evident in all cases. The wild-type strain PCC7806, on the other hand, produced considerable amounts of MCYST-LR (0.27 ± 0.04 μg mm^{-3} [mean ± standard deviations]; $n = 22$) and (D-Asp^3)MCYST-LR (0.60 ± 0.06 μg mm^{-3}). Other microcystin variants were not detected. The total microcystin content was the same in all cultures used in the experiments.

Feeding rates. All six *Daphnia* clones ingested the wild-type strain PCC7806 at rates that were 75 to 95% less than those measured for *Scenedesmus* (Fig. 1). It is thus obvious that the animals somehow avoid feeding on PCC7806 cells. The strength of that effect differed slightly among the *Daphnia* clones tested. *D. magna* showed the strongest effect, and although the feeding rate was different from zero, the animals of this clone almost refused to feed on *Microcystis* strain PCC7806. *D. galeata* clone B, on the other hand, exhibited a comparatively high feeding activity on wild-type PCC7806 cells. The most important finding is, however, that the microcystin-lacking cells of the PCC7806 mutant were ingested at

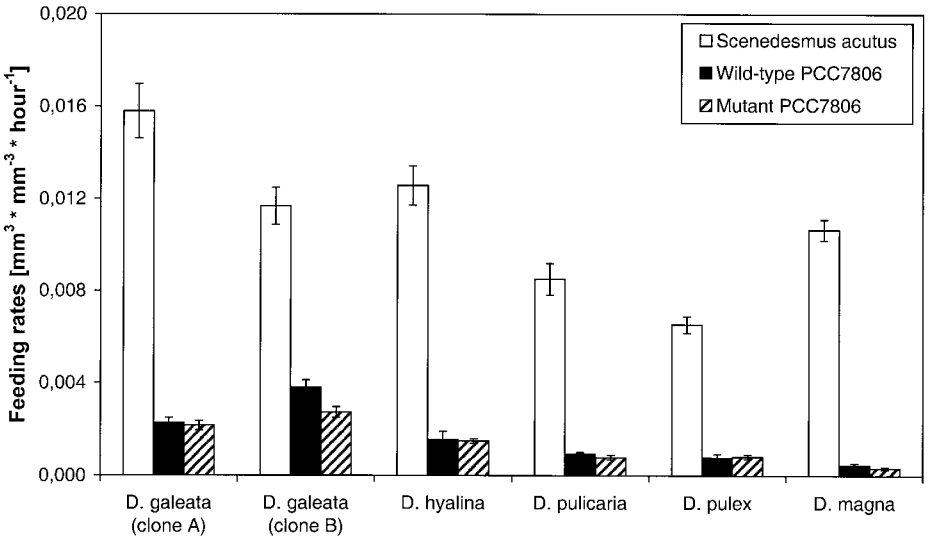

FIG. 1. Feeding rates of *Daphnia* clones on *Scenedesmus*, wild-type PCC7806, and mutant PCC7806. The data represent mean values of five replicates and the respective standard errors (SEs).

the same low rate as the microcystin-producing cells of the wild-type PCC7806 (Fig. 1). A slight, though significant, difference was only found for *D. galeata* clone B, which ingested wild-type cells at a higher rate than the microcystin-lacking cells of the mutant.

Survival tests. Life table experiments showed that all *Daphnia* clones survived significantly longer in suspensions of the microcystin-lacking mutant than in those of the microcystin-producing wild-type strain PCC7806 (Fig. 2). The differences in the median survival time (LT_{50}) between animals fed with either mutant or wild-type cells ranged from 2 days (*D. pulex*) to more than 8.5 days (*D. galeata* clone A) (Table 2).

Animals fed with the wild-type PCC7806 died significantly faster than the starved animals of the nonfood controls. The only exception was *D. magna*, which showed no significant differences in survivorship when fed either with wild-type PCC7806 or nothing at all (nonfood control). Animals fed with the mutant, on the other hand, survived either longer (*D. galeata, D. hyalina, D. magna*) or as long (*D. pulex, D. pulicaria*) as starving animals (Table 2). Before death all animals fed with the wild type showed malfunctional symptoms such as sudden stops in swimming and filtering activity, remaining quiet at the bottom of the bottle unless touched, and incomplete molting. In contrast, animals fed with the mutant exhibited clear starvation symptoms such as getting pale, lack of oil drops in the body, and a gradual decrease in swimming and filtering activity.

The *Daphnia* clones differed in their survival times when fed with wild-type cells. The LT_{50} values range from approximately 1.5 days (*D. galeata* clone A) to almost 6 days (*D. magna*) (Table 2). Furthermore, the LT_{50} is closely related to the feeding rate on wild-type PCC7806 cells and thus is also related to the respective microcystin ingestion rate. The relationship between microcystin ingestion rate and LT_{50} follows a reciprocal power function (Fig. 3). A regression analysis of this function revealed that 71% of the variation in LT_{50} among *Daphnia* clones can be explained by differences in the microcystin ingestion rate of the animals. Moreover, the reciprocal power function also describes the results of experiments performed with *D. galeata* and four *Microcystis* strains which produce different microcystin variants (data taken from reference 38). The equation for the whole data set is $LT_{50} = 3.28 \cdot \text{microcystin ingestion rate}^{-0.58}$ ($r = 0.92$, $F = 42.1$, $P < 0.001$).

There were also clone-specific differences in the survival time of animals fed with the mutant of PCC7806. Animals

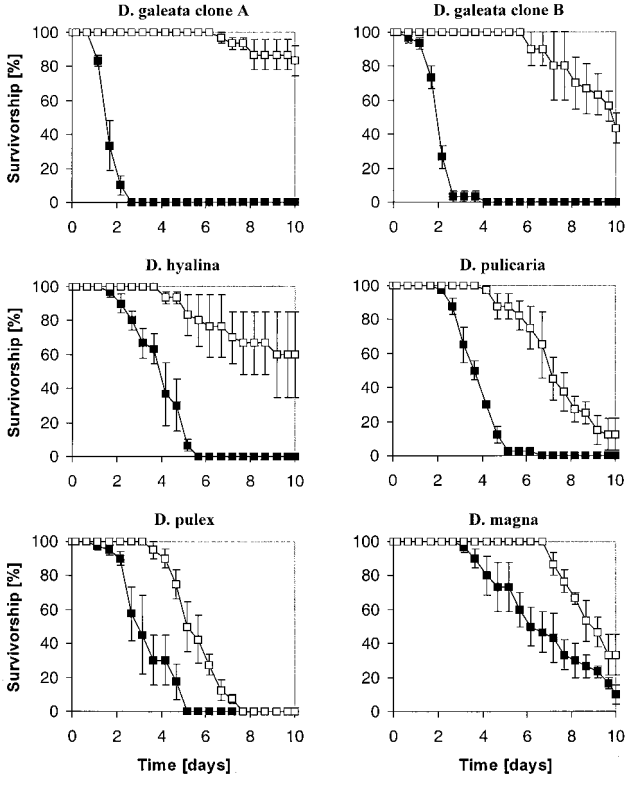

FIG. 2. Survivorship of *Daphnia* clones fed with either the wild-type PCC7806 (black squares) or the mutant (open squares). The data represent mean values of three (*D. galeata* clone A and B, *D. hyalina, D. magna*) or four (*D. pulex, D. pulicaria*) replicates and the respective SEs.

TABLE 2. LT_{50}s of animals fed with either the wild-type PCC7806, mutant PCC7806, or nothing at all (nonfood control)

Daphnia clone	LT_{50} values (days [± SE])		
	Wild-type PCC7806	Mutant PCC7806	Nonfood control
D. galeata (clone A)	1.48 ± 0.09	>10	5.49 ± 0.40
D. galeata (clone B)	1.91 ± 0.10	9.12 ± 1.74	5.88 ± 0.17
D. hyalina	3.87 ± 0.18	>10	5.15 ± 0.26
D. pulicaria	3.58 ± 0.28	6.89 ± 0.22	5.24 ± 1.73
D. pulex	2.91 ± 0.32	5.07 ± 0.17	4.78 ± 1.70
D. magna	5.83 ± 0.52	8.29 ± 0.43	7.41 ± 0.27

which exhibited a higher feeding rate tended to live longer (*D. galeata* clone A and B, *D. hyalina*) than those which ingested the mutant with a comparatively low rate (*D. magna, D. pulex, D. pulicaria*) (Table 2). However, this tendency was not statistically significant.

DISCUSSION

Most *Microcystis* strains can poison *Daphnia* spp. in a way that usually causes a die-off faster than that due to starvation (21, 25, 31). Based on the data presented here, it seems evident that microcystins are the major source of acute *Daphnia* toxicity caused by *Microcystis*. It seems furthermore obvious that microcystins ingested with living cyanobacterial cells are poisonous to most or maybe all *Daphnia* spp. The main support for these conclusions comes from experiments performed with the microcystin-producing *Microcystis* strain PCC7806 and its mutant. Toxicity tests with both variants of PCC7806 have clearly shown that wild-type cells are acutely toxic to clones of five common *Daphnia* spp. while cells of the mutant allow a significantly better survival. The most likely explanation for these different effects on survival is the lack of any microcystins in the mutant. A further clue to the significance of microcystins comes from the analysis of the relationship between survival time and microcystin ingestion rate of the *Daphnia* clones.

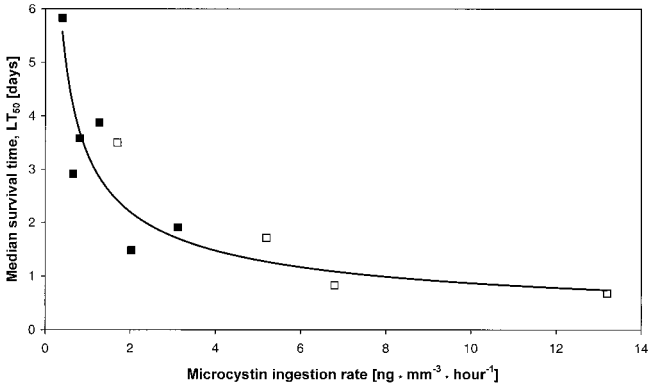

FIG. 3. Regression analyses of the relationship between microcystin ingestion rate and LT_{50}. Black squares represent data obtained with different *Daphnia* clones, with the wild-type PCC7806 as food ($r = 0.84$, $F = 9.3$, $P < 0.04$). The open squares correspond to data obtained with *D. galeata* and four microcystin-producing *Microcystis* strains (data from reference 38). The equation for the whole data set is $LT_{50} = 3.28 \cdot \text{microcystin ingestion rate}^{-0.58}$ ($r = 0.92$, $F = 42.1$, $P < 0.001$).

Both values are closely correlated, which probably means that daphnids die faster the more microcystins they ingest with food particles.

According to widespread opinion, *Daphnia* spp. and clones differ strongly in their sensitivity to microcystins. Several authors reported, for example, striking species- or clone-specific variations in the 50% lethal concentration or 50% effective concentration for dissolved, purified microcystins (reviewed in reference 5). Others found differences in survival and growth among species and clones fed with microcystin-containing *Microcystis* cells (7, 13, 15). At least one paper reported an apparent resistance to microcystins (29). However, the observed differences in response to microcystins contained in solutions or cyanobacterial cells can have various causes. These differences can be due to variations in sensitivity to microcystins but also to variations in toxin uptake. Our studies with the wild-type PCC7806 strongly suggest the latter. The results clearly show that 71% of the differences in survival among six *Daphnia* clones can be explained by differences in microcystin intake which are due to variations in feeding activity on the microcystin-producing cells. The microcystins themselves, once ingested, probably affect all *Daphnia* clones in a comparable way. This is somewhat surprising, since the tested daphnids belong to different species, they were isolated from various habitats and geographical regions, and not all of them came from waters with toxic cyanobacteria. Explanations for this may include a loss of resistance or detoxification mechanisms during the culturing process or the notion that the microcystins affect *Daphnia* in a way which limits the possible extent of an adaptation to the toxin. It is also possible that the toxic effect of microcystins is based on an interference with life processes, which are expressed in all *Daphnia* spp. in a similar way. The latter idea finds some support in the fact that microcystins inhibit protein phosphatases 1 and 2A (8), which contribute to many basic and essential life functions (e.g., see references 32 and 45).

Since microcystins are ingested together with living cyanobacterial cells, the toxicity of a *Microcystis* strain depends, as shown, not only on its cellular microcystin content but also on the rate with which *Daphnia* feeds on that particular strain. Thus, variations in feeding activity will in turn influence the toxic effect of *Microcystis*. In short-term experiments, for example, different *Microcystis* strains usually are ingested at different rates. Strains like PCC7806 cause a feeding inhibition and are thus only slightly ingested (7, 21, 25, 31), while other strains are consumed at high rates (16). However, our data strongly suggest that the feeding inhibition and the resulting *Microcystis* strain-specific differences in consumption by *Daphnia* are neither caused by nor related to microcystins. The feeding experiments with the wild-type PCC7806 and its mutant have indeed shown that *Daphnia* ingests *Microcystis* strain PCC7806 cells at low rates regardless of whether the cyanobacterial cells contain microcystins or not. The microcystin content of *Microcystis* cells and the feeding activity of *Daphnia* on these cells are thus independent values which nevertheless both determine the rate of microcystin intake. This may explain why several authors failed to find a correlation between microcystin content and toxicity of *Microcystis* cells (21, 31, 38). Furthermore, it emphasizes the fact that differences in inges-

tion rate must always be considered when evaluating the toxicity of *Microcystis* cells.

This can be done by calculating the microcystin ingestion rate, which may serve as a gross estimation of the maximal microcystin dose taken up per time. As long as it is impossible to determine the microcystin assimilation directly, the microcystin ingestion rate is actually one of the easiest ways to estimate microcystin uptake and maybe to predict the microcystin-based toxicity of *Microcystis*. As shown here, the relationship between microcystin ingestion rate and survival time of *Daphnia* fits well to a reciprocal power function. That function describes the effects of ingested microcystins and may thus help to distinguish these effects from those of additional toxins. The function may also help to understand the mechanisms of microcystin intoxication. It turns out that survival of *Daphnia* is strongly affected if the microcystin ingestion rate is higher than approximately 0.4 ng $mm^{-3} \cdot h^{-1}$. Microcystins are thus effective even at low intake rates. The toxins can possibly accumulate in the animals until a lethal dose is reached. Microcystin ingestion rates higher than 6 ng $mm^{-3} \cdot h^{-1}$, however, do not further accelerate the time of death and so LT_{50} values lower than approximately half a day seem unlikely. This may indicate that intoxication by microcystins is based on an interference with life processes, the malfunction of which does not kill *Daphnia* immediately.

The results presented here indicate that microcystins may have an ecological significance to *Microcystis*. Microcystins are, as shown, effective toxins which most likely affect not only daphnids but also other grazers of *Microcystis*. The toxins are efficient at low intake rates and kill, once ingested, within hours or a few days. Furthermore, the occurrence of microcystin-producing *Microcystis* cells may induce shifts in the zooplankton community, since grazers of *Microcystis* eventually die or are impaired, while animals that can select other food sources survive and reproduce. Future studies should show if these frequently observed changes in the zooplankton community (e.g., see reference 26) could play a decisive role in the formation of *Microcystis* blooms by shifting the grazing pressure from the cyanobacterium to its potential competitors.

The present study suggests that microcystins are very effective and potent *Daphnia* toxins produced by *Microcystis*, but there may be additional poisonous substances produced. *Microcystis* produces many other bioactive compounds (4, 6) which could potentially harm *Daphnia*. Jungmann (20), for instance, isolated and partially characterized a toxic substance from cell extracts of *Microcystis* that was not a microcystin. Another toxin has been found by Reinikainen (34). Protease inhibitors like cyanopeptolines (17, 28) frequently occur in *Microcystis* and may interfere with the digestion process of *Daphnia*. At least one such substance has been shown to be toxic to *Daphnia* (18). However, all these compounds have been tested in a purified form only. It remains unknown if these compounds are also effective when taken in with living cyanobacterial cells as has so far been shown exclusively for microcystins.

The occurrence of additional toxins would, nevertheless, explain why some *Daphnia* clones fed with the microcystin-free PCC7806 mutant died almost as fast as animals of the nonfood controls. A more likely explanation is that PCC7806 does not provide enough resources for the survival of the animals. The feeding rate on PCC7806 is, indeed, very low, and as cyanobacteria are also of poor nutrient value (3, 9, 26), *Daphnia* may thus die of starvation. The observation of clear hunger effects supports this hypothesis.

In summary, the present study strongly suggests that microcystins ingested with living cyanobacterial cells are acutely toxic to *Daphnia* spp. in general. This shows that our previously published findings of experiments performed with a single *D. galeata* clone (38, 39) can be generalized. Moreover, the present study demonstrates that toxicity of *Microcystis* to several *Daphnia* spp. and clones can be estimated from a simple parameter like the microcystin ingestion rate. Microcystin-based toxicity may thus be predictable not only for laboratory *Daphnia* cultures but also for heterogeneous mixtures of different clones and species. The results and methods of this study may furthermore help to perform experiments to clarify the ecological role of microcystins in *Microcystis-Daphnia* interactions in nature.

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