

# Role of Microcystins in Poisoning and Food Ingestion Inhibition of *Daphnia galeata* Caused by the Cyanobacterium *Microcystis aeruginosa*

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**The effects of microcystins on *Daphnia galeata*, a typical filter-feeding grazer in eutrophic lakes, were investigated. To do this, the microcystin-producing wild-type strain *Microcystis aeruginosa* PCC7806 was compared with a *mcy*<sup>-</sup> PCC7806 mutant, which could not synthesize any variant of microcystin due to mutation of a microcystin synthetase gene. The wild-type strain was found to be poisonous to *D. galeata*, whereas the *mcy*<sup>-</sup> mutant did not have any lethal effect on the animals. Both variants of PCC7806 were able to reduce the *Daphnia* ingestion rate. Our results suggest that microcystins are the most likely cause of the daphnid poisoning observed when wild-type strain PCC7806 is fed to the animals, but these toxins are not responsible for inhibition of the ingestion process.**

Several species of cyanobacteria, including the bloom-forming freshwater species *Microcystis aeruginosa*, are able to produce several variants of microcystin, the most common cyanotoxin, which has been implicated in livestock poisoning and human poisoning (4, 5, 23). Recently, it was shown that these small cyclic peptides are synthesized nonribosomally by peptide synthetases (8). Numerous studies have been carried out in order to determine the ecological significance of the microcystins. The results obtained, however, are inconsistent (11).

One possible function of microcystins is that they play a role in the defense of *M. aeruginosa* cells against zooplankton grazing. This hypothesis is supported by the observation that exposure to *Microcystis* cells reduces the life span of daphnids (3, 10, 13, 18, 24). However, the problem seems to be more complex. The data of Jungmann (13) and Jungmann and Benndorf (14), for example, suggested that an unidentified metabolite of *Microcystis* sp. rather than microcystins was responsible for toxicity to *Daphnia*. Moreover, Nizan et al. (24) found no correlation between acute toxicity of various *M. aeruginosa* strains to daphnids and their quantitative microcystin contents. In other cases, daphnids could feed on microcystin-containing *M. aeruginosa* without suffering any harmful effects (22).

Experiments on *Microcystis* toxicity have usually been performed by comparing strains which differ in microcystin content. However, other strain-specific properties could be the source of the striking variation in the results obtained in different investigations. For example, *M. aeruginosa* strains differ in their content of potential toxic oligopeptides (25), which could strengthen or mask a toxic effect of microcystins. Also strain-specific differences in ingestibility of *M. aeruginosa* cells by daphnids may influence the dose of an endotoxin, which is determined by the ingestion rate (amount of food taken in per time unit) and by the toxin content of the cells. Indeed, the ingestion rate of daphnids depends to a large extent on the

*M. aeruginosa* strain offered as food. Some strains affect the ingestion rate of the animals, whereas others do not (10, 12, 15, 18, 19). Some authors (12, 19) have hypothesized that a perceptible factor ("bad taste") is responsible for this effect. However, the possibility that microcystins themselves are involved in the inhibition of ingestion cannot be ruled out.

The experiments described in this paper were designed to study the role of microcystins in the effect of *M. aeruginosa* on *Daphnia galeata*; both *Microcystis* toxicity and strain-dependent inhibition of ingestion were studied. To do this, we compared an *M. aeruginosa* PCC7806 mutant which was genetically engineered to knock out the production of microcystins with the microcystin-synthesizing wild-type strain. The two variants of strain PCC7806 used have the same genotype except that the mutant (*mcy*<sup>-</sup>) cells have an insertional mutation in a microcystin synthetase gene (8).

## MATERIALS AND METHODS

**Description and origins of the cyanobacterial strains used.** The unicellular strain *M. aeruginosa* PCC7806 (from Braakman Reservoir, The Netherlands) was provided by J. Weckesser (Albert-Ludwigs-University, Freiburg, Germany). This strain contains mainly microcystin MCYST-LR, (D-Asp<sup>3</sup>)MCYST-LR (7), and cyanopeptolin depsipeptides (21). The derived mutant cell line (*mcy*<sup>-</sup>) which was used in this study was obtained by performing homologous recombination. A peptide synthetase gene (*mcyB*), which occurs only in microcystin-producing *Microcystis* strains, was insertional inactivated with a chloramphenicol resistance cartridge. Cells of the mutant cell line lacked microcystins but not cyanopeptolins (8). The unicellular organism *M. aeruginosa* HUB 5-3, which was used as a reference strain in the ingestion rate analyses, was originally isolated from a water bloom on Lake Pehlitzsee (Brandenburg, Germany) in 1978. Extensive analyses of the interactions of this strain with daphnids showed that it did not have any adverse or harmful effects (12). In contrast to wild-type strain PCC7806, *M. aeruginosa* HUB 5-3 lacked microcystins. On the other hand, strains HUB 5-3 and PCC7806 (including the mutant cell line of PCC7806) did not differ significantly in cell diameter or cell ultrastructure (as determined by electron microscopic analyses performed by W. Bleiß and A. Marko, Humboldt University, Berlin, Germany).

**Culturing of cyanobacterial strains and *D. galeata*.** The different *M. aeruginosa* strains were cultured semicontinuously by using a synthetic medium as described previously (26). All of the cultures used in the experiments grew in the logarithmic phase under a light regime consisting of 12 h of light and 12 h of darkness at 20°C. The densities of the *Microcystis* suspensions were measured by using a photometric diaphragm method (16). For <sup>14</sup>C-labelling, a NaH<sup>14</sup>CO<sub>3</sub> solution (0.18 MBq per 50 ml) was added to the *M. aeruginosa* cultures, and then the cultures were incubated for 3 days. Before the ingestion rate experiments were started, the *Microcystis* cells were washed by centrifugation at 1,500 × g.

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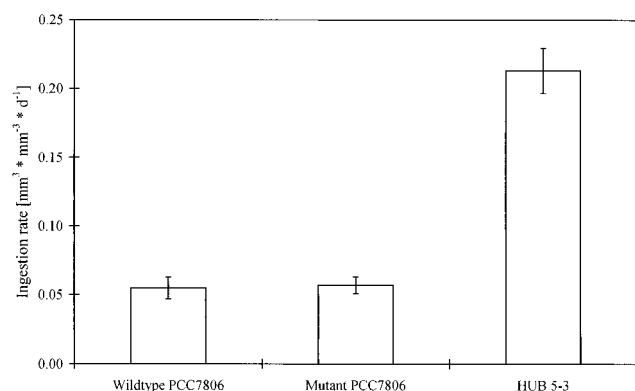


FIG. 1. Ingestion rates of the strains investigated (means  $\pm$  standard errors;  $n = 7$  for wild-type PCC7806 and the  $mcy^-$  mutant of PCC7806;  $n = 9$  for HUB 5-3). d, day.

The *D. galeata* culture used was descended from a laboratory clone culture (Humboldt University). The animals were raised on *Chlorella* cf. *minutissima* cells suspended in filtered (pore size, 0.2  $\mu\text{m}$ ; membraPure) lake water. The temperature was adjusted to 20°C. Only adult females having similar body lengths were used in this study. Before the experiments were started, the test animals were washed carefully with filtered lake water to remove all *Chlorella* cells.

**DNA isolation and PCR.** Genomic DNA of *M. aeruginosa* PCC7806 was isolated as described previously (9). A PCR was performed by using Goldstar thermostable DNA polymerase (Eurogentec) and primers Tox2p (5'GGAACA AGTTGCACAGAATCCGC3') and Tox2m (5'CCAATCCCTATCTAACACA GTAACCGG3'). The PCR procedure was initiated by a denaturation step (2 min, 95°C), which was followed by 30 cycles consisting of 20 s at 95°C, 30 s at 55°C, and 2 min at 72°C and by a final elongation step (5 min, 72°C).

**Determination of ingestion rates.** Ingestion rates were determined by using a standard radioisotope technique and  $^{14}\text{C}$ -labelled *Microcystis* cells (12). Up to 20 daphnids were placed into 210-ml incubation vessels filled with filtered (pore size, 0.2  $\mu\text{m}$ ; membraPure) lake water containing the desired cyanobacteria at a concentration of 20  $\text{mm}^3 \text{ liter}^{-1}$  (incipient limiting level of *D. galeata*, 4  $\text{mm}^3 \text{ liter}^{-1}$ ). The temperature was adjusted to 20°C. After a 50-min adaptation period,  $^{14}\text{C}$ -labelled *M. aeruginosa* cells were added at a ratio of 1:10 to the unlabelled cyanobacteria. After 15 min of feeding the animals were washed with filtered lake water and anesthetized with carbonated water. Then the body lengths were measured as a biovolume-related parameter (1). All of the animals in an incubation vessel were transferred together into a scintillation vial before a tissue solubilizer was added. The radioactivities of the *Microcystis* suspensions and the test animals were determined with a liquid scintillation analyzer (Tri-Carb; Packard). The whole experiment was repeated seven times (wild-type and mutant PCC7806) or nine times (HUB 5-3). The ingestion rates were calculated by determining the biovolume of cyanobacteria ingested (cubic millimeters) per biovolume of *Daphnia* (cubic millimeters) per day. To test whether the *Microcystis* cultures were able to affect the ingestion rate, the microcystin-producing wild type and the nonproducing  $mcy^-$  mutant of strain PCC7806 were compared with the easily ingestible standard strain HUB 5-3, which did not have any inhibitory or toxic effects on *Daphnia* in previous studies (12).

**Life table experiments.** Survival tests were carried out in 100-ml Plexiglas tubes that could be closed at both ends with gauze. These tubes were placed into 1.0-liter glass bottles that were completely filled with filtered (pore size, 0.2  $\mu\text{m}$ ; membraPure) lake water containing the desired cyanobacteria at a density of 20  $\text{mm}^3 \text{ liter}^{-1}$ . The temperature was adjusted to 20°C. Ten daphnids were transferred into each tube. The suspensions were shaken moderately. Living animals were counted and then transferred into freshly prepared *M. aeruginosa* suspensions every 24 h. In this way wild type strain PCC7806 and the strain PCC7806 mutant were tested and compared to a nonfood control (filtered lake water alone). For both strain variants (wild type and mutant) the whole procedure was repeated four times. The experiments were terminated after 5 days.

**Statistical analyses.** To compare the means of the ingestion rates obtained, Student's *t* test at the 99% level of significance was used. The microcystin toxicity experiments resulted in survival functions, which were, as usual, compared with the log rank test. For the *M. aeruginosa* cultures which were poisonous to *D. galeata*, the time needed to kill 50% of the animals was calculated as median of the Kaplan-Meier survival function estimation.

## RESULTS

**Characterization of the  $mcy^-$  mutant.** As cyanobacteria are known to contain several genome copies (2) and even apparently homozygous mutant clones may contain a few wild-type

copies, it was necessary to maintain the mutant under selective pressure with chloramphenicol. Therefore, the homozygous genotype of the mutant was proven before the experiments performed to study feeding and survival of *D. galeata* were started without chloramphenicol in the medium. The presence of the chloramphenicol resistance cartridge was checked by using primers that bound upstream and downstream of the insertion region. No wild-type gene copy was detected in the mutant cell line investigated (8). In addition, the microcystin contents of the wild type and mutant were monitored by high-performance liquid chromatography during the experiments described below (data not shown). The lack of any microcystin in the  $mcy^-$  mutant was evident over the whole period studied.

**Ingestion rate experiments.** Wild-type PCC7806 was ingested by *D. galeata* at a very low rate (Fig. 1); the ingestion rate was 75% less than the ingestion rate of HUB 5-3. In contrast, no significant difference was found between the ingestion rates of the wild type and the  $mcy^-$  mutant. Both variants of PCC7806 were ingested by *D. galeata* at nearly the same low rate.

**Life table experiments.** Life table experiments were carried out to study the toxicity of the variants of PCC7806 investigated to *D. galeata*. An *M. aeruginosa* culture was considered toxic if the animals died faster than they died due to starvation. Therefore, both variants of PCC7806, the wild type and the  $mcy^-$  mutant, were compared four times to nonfood controls. Figure 2 shows the results of a typical experiment. The results obtained in the replicate experiments were always comparable.

In all cases wild-type of *M. aeruginosa* strain PCC7806 was toxic to *Daphnia*. Thus, the survival functions observed in experiments performed with the wild type always differed significantly from the survival functions of the nonfood controls ( $P < 0.001$ , as determined by the log rank test). The time needed to kill 50% of the animals varied from 1.2 to 2.0 days. In striking contrast, no animals died in the experiments performed with the  $mcy^-$  mutant of PCC7806. Consequently, the survival functions of this variant differed significantly from those of the nonfood controls ( $P < 0.025$ ) and those of wild-type *M. aeruginosa* strain PCC7806 ( $P < 0.001$ ).

Interestingly, the animals exposed to wild-type strain PCC 7806 exhibited a significant change in their swimming behavior. Approximately 5 h after they were transferred into the suspensions containing the wild-type strain, the swimming activity of the animals decreased and the animals stayed at the bottoms of the vessels. In contrast, the daphnids fed the  $mcy^-$  mutant exhibited the normal swimming behavior.

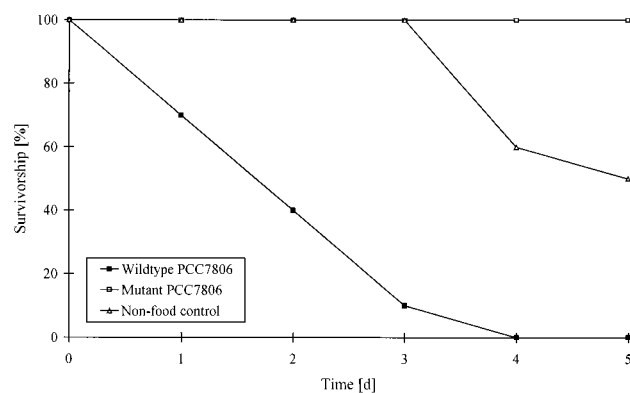


FIG. 2. Survival of *D. galeata* in suspensions containing wild-type PCC7806, the  $mcy^-$  mutant of PCC7806, and the nonfood control (results of one of the four replicate experiments). d, day.

## DISCUSSION

The results of the life table experiments performed with the original wild-type strain PCC7806 demonstrate that cells of this strain are able to poison *D. galeata* and cause the death of the animals in a comparably short time. Therefore, it is evident that there is a toxic compound in the cells. Wild-type *M. aeruginosa* PCC7806 synthesizes relatively large amounts of two microcystin variants, which are known to be potential inhibitors of *Daphnia* protein phosphatases 1 and 2A (6). Inhibition of these enzymes by microcystins plays an important role in poisoning of warm-blooded animals (20), which supports the idea that microcystins are the toxins which cause the death of daphnids. However, there has been no unequivocal evidence which supports this hypothesis until now (11). The availability of the *mcy*<sup>-</sup> mutant of PCC7806 made it possible to analyze more precisely the role of microcystins in *Daphnia* poisoning by comparing two clones of *M. aeruginosa* which have identical genotypes except for a specific mutation in a microcystin synthetase gene which leads to the inability of *mcy*<sup>-</sup> cells to synthesize microcystins (8).

While the animals fed with microcystin-containing wild-type strain PCC7806 died quickly, the *Daphnia* sp. remained alive when the *mcy*<sup>-</sup> mutant was offered as food. Therefore, we concluded that the microcystins were the most probable cause of *Daphnia* poisoning when wild-type strain PCC7806 was fed to the animals. In addition, the toxins may also have caused the significant reduction in *Daphnia* swimming activity observed some hours after the animals were transferred into the suspension containing wild-type strain PCC7806. Overall, we concluded that microcystins may be formed in order to eliminate zooplankton species, which feed on *Microcystis* spp., as previously suggested (18). The data reported here also indicates that the cyanopeptolin depsipeptides found in both the wild type and the *mcy*<sup>-</sup> mutant of PCC7806 are not responsible for the daphnid poisoning observed.

Another effect of *M. aeruginosa* PCC7806 on *D. galeata* was the inhibition of the ingestion process. Compared to the easily ingestible strain HUB 5-3, the ingestion rate of the animals was 75% lower when they were fed wild-type strain PCC7806. Interestingly, in spite of the absence of all microcystin variants, the *mcy*<sup>-</sup> mutant of PCC7806 was ingested by *Daphnia* at the same low rate as the microcystin-containing wild-type strain. Thus, there was no relationship between the presence of microcystins in the cells and the inhibition of ingestion. This means that the poisoning of *Daphnia* and the inhibition of ingestion are caused by different *Microcystis* factors.

In conclusion, our data indicates that microcystins are the cause of the toxic effects of *M. aeruginosa* on daphnids, whereas these compounds are not responsible for ingestion inhibition. Furthermore, our results support the hypothesis that one function of microcystins could be to eliminate grazers of *Microcystis* spp.

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