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Ingestion of microcystins by *Daphnia*: Intestinal uptake and toxic effects

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Abstract

We investigated the intestinal uptake and adverse effects of microcystins ingested with *Microcystis* on *Daphnia galeata.* The gut structure, blood microcystin concentration, appearance, and movements of *Daphnia* fed *Microcystis* PCC 7806 or a microcystin-deficient PCC 7806 mutant were monitored over time. Microcystins were rapidly taken up from the digestive cavity into the blood. This process apparently required a preceding disruption of the gut epithelium by an as-yet-unknown *Microcystis* factor. Once microcystins entered the blood, they affected the neuromuscular communication or another life function that influences major muscle systems. Consequently, the beat rates of the thoracic legs, mandibles, and second antennae as well as the activity of the foregut decreased, whereas the midgut muscles were stimulated. Finally, the animals exhibited symptoms of exhaustion and died. The present results suggest that an ingestion of between 10.2 ng and 18.3 ng of microcystin per 1 mg of *Daphnia* body fresh weight is sufficient to kill *D. galeata* within 2 d.

Many freshwater cyanobacteria share the ability to synthesize bioactive compounds that may affect other organisms. In particular, the bloom-forming taxa, which are members of the orders Nostocales and Chroococcales, including *Microcystis, Anabaena, Aphanizomenon,* and *Planktothrix* spp., are rich sources of potent enzyme inhibitors, cellular disrupters, and compounds with a diverse range of other biological activities (Carmichael 1992; Codd 1995). Some of these metabolites present serious threats to animal and human health. Prominent examples are the microcystins, which frequently are produced by *Microcystis* sp. and have the potency to induce various harmful effects, such as an inhibition of protein phosphatases (e.g., MacKintosh et al. 1990).

Microcystins usually are cell-bound. Although *Microcystis* cells may exhibit a limited excretion of microcystins (Kaebernick et al. 2000), their internal microcystin content is often high. Microcystins therefore may harm animals that ingest *Microcystis.* Special interest has been directed to the effects on *Daphnia* sp. (e.g., Jungmann and Benndorf 1994; Reinikainen 1994), which constitute key elements of freshwater food webs (Riemann and Christoffersen 1993). *Daphnia* are also grazers of natural *Microcystis* populations (Thompson et al. 1982; Schoenberg and Carlson 1984) and, thus, may ingest considerable amounts of microcystins in nature. This may result in various adverse effects. As recently shown by Rohrlack et al. (2001) and Kaebernick et al. (2001), microcystins taken in together with *Microcystis* cells can rapidly kill five common *Daphnia* sp. The basis of this finding has been experiments with genetically engineered and spontaneous *Microcystis* mutants for microcystin production. In addition, Rohrlack et al. (2001) have presented a correlation between the microcystin ingestion rate and the survival time of various *Daphnia* sp. originating from different regions and types of waters. According to their work, differences in survival among *Daphnia* sp. exposed to *Microcystis* are caused by variation in feeding activity rather than by differences in susceptibility to food-associated microcystins. In fact, those authors demonstrated that all five tested *Daphnia* sp. shared virtually the same sensitivity to microcystins ingested with *Microcystis* cells.

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Another conclusion from the correlation between microcystin ingestion rate and survival time was that even low rates of microcystin ingestion, as may occur when *Daphnia* feed on a natural mixture of *Microcystis* and other types of phytoplankton, can result in lethal effects. Moreover, those authors found colony-forming *Microcystis* strains to be capable of poisoning daphnids and, therefore, concluded that natural, microcystin-producing *Microcystis* populations may well be toxic to *Daphnia.* In contrast to the work by Rohrlack et al. (2001), other studies, including one by DeMott (1999), have found *Daphnia* sp. and clones to differ in their response to microcystin-producing *Microcystis.* An explanation for this deviation in results may be that Rohrlack et al. (2001) used pure *Microcystis* cultures in their experiments, whereas DeMott (1999) exposed *Daphnia* to a mixture of *Microcystis* and green algae.

The studies mentioned above established the toxicity of food-associated microcystins, but they failed to clarify the actual mechanisms of the toxic microcystin effects. Poisoning by microcystins that are ingested as constituents of *Microcystis* cells probably requires an intestinal uptake of the toxins (i.e., a transport from the digestive cavity across the gut epithelium into the blood). To our knowledge, however, no data have been published that establish the mechanisms of such a process in *Daphnia.* Therefore, conditions leading to an intestinal uptake of microcystins into the blood and, consequently, to toxic effects are widely unknown. This lack of knowledge also prevents an estimation of the lethal microcystin uptake dose, a value that is essential for predicting fatal effects caused by microcystins. Another unsolved problem is the lack of information regarding target organs of microcystins within the daphnid body. This prevents the identification of factors (e.g., all kinds of bioactive compounds, including a diverse range of metabolites produced by *Microcystis* or coexisting cyanobacteria) that affect the same organs as microcystins and, thus, may either strengthen or weaken their effects on *Daphnia.*

Here, we report on the intestinal uptake and adverse effects of microcystins ingested with *Microcystis* cells and, in doing so, reveal mechanisms and conditions enabling the compounds to kill *Daphnia.* To that end, long-term feeding experiments with *Microcystis aeruginosa* and *D. galeata,* a common species in lakes with cyanobacterial dominance, were conducted. During these experiments, the structure of the daphnid gut epithelium as well as the microcystin concentration of *Daphnia* blood were monitored over time. In addition, symptoms characterizing microcystin poisoning (i.e., changes in appearance and activity of *Daphnia*) were recorded by continuous video observations and used to identify putative targets of microcystins within the daphnid body. Most experiments were based on a comparison of the microcystin-producing *Microcystis* strain PCC 7806 and its mutant, which has been genetically engineered to knock out the synthesis of microcystins (Dittmann et al. 1997). Because these variants of PCC 7806 differ only in their ability to produce microcystins, the effects caused by these compounds could be clearly distinguished from those triggered by other factors.

Methods

Origin and description of Microcystis *and* Scenedesmus *spp.*—The unicellular growing *M. aeruginosa* strain PCC 7806 was kindly provided by J. Weckesser (Albert-Ludwigs-University, Freiburg, Germany). *Microcystis* PCC 7806 produces two microcystins, MCYST-LR and (D-Asp3)MCYST-LR, and several other bioactive compounds, such as cyanopeptolin depsipeptides (Martin et al. 1993). The mutant cell line of PCC 7806 was obtained by transformation of the original strain with a mutated version of one of the microcystin synthetase genes that included recombinative replacement of the wild-type copy of this gene. For that, the peptide synthetase gene *mcyB* was insertionally inactivated using a chloramphenicol-resistance cartridge. This resulted in a highly specific and complete knockout of microcystin production, whereas the synthesis of other oligopeptides was unaffected (Dittmann et al. 1997). The PCC 7806 wild type and mutant have the same genotype except for the mutated *mcyB* gene. 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 food source for *D. galeata* cultures, grows in small aggregates consisting of up to 16 cells.

Culturing of Microcystis *and* Scenedesmus *spp.*—The PCC 7806 wild type and mutant were grown in Z8 medium (Kotai 1972) as nonaxenic, semicontinuous cultures, which were maintained under continuous light supplied by warm-white fluorescent-lamps (25 μmol photons m^{-2} s^{-1}, measured outside of the culture vessels). The temperature was kept at 20 $\pm$ 1 (range) °C. All *Microcystis* cultures were diluted daily to a final cell-biovolume concentration of 100 mm^3 L^{-1}, which corresponds to 14 mg C L^{-1} (calculated from Rocha and Duncan 1985) or 3.41×10^9 cells L^{-1}. The cell-biovolume concentration was determined using a calibration curve between light absorbance at 800 nm and the biovolume concentration. *Scenedesmus* was grown under similar conditions except for the light intensity (32 μmol photons m^{-2} s^{-1}) and the cell-biovolume concentration (50 mm^3 L^{-1}, corresponding to 7 mg C L^{-1} [calculated from Rocha and Duncan 1985] or 2.76×10^8 cells L^{-1}) to which the cultures were diluted daily. Cyanobacterial and *Scenedesmus* cultures were grown for at least 3 weeks at a constant rate before use in experiments or as food, respectively. This time span corresponds to more than six cell divisions and should ensure a complete adaptation to the culture conditions described. Cyanobacteria and algae were harvested by centrifugation for 10 min at 500 $\times$ g.

Origin and culturing of Daphnia *sp.*—All experiments were conducted with a *D. galeata* clone isolated from the lake Großer Müggelsee (Berlin, Germany) and kindly provided by M. Henning (Humboldt-University, Berlin, Germany). *Daphnia* were cultured in a synthetic zooplankton medium (Klüttgen et al. 1994) with *Scenedesmus* as sole food. Before an experiment, newborn animals were harvested from well-fed stock cultures and transferred into 0.5-liter glass vessels containing a suspension of 5 mm^3 L^{-1} of *Scenedesmus.* Each culture vessel received 10 animals. Cultures

were kept under indirect, continuous light (5 μmol photons m^{-2} s^{-1}) at 20°C ± 1 (range) °C. Every second day the food suspension was exchanged and any offspring were removed. The animals were maintained under these conditions for at least 2 weeks and served afterward as mothers for the animals used in the experiments. These experimental animals were taken as offspring born within 24 h from the mother cultures and were kept under the described conditions for a further 5 d (blood and video experiments) or 20 d (histological studies).

Effects of PCC 7806 wild type and mutant on the midgut epithelium of Daphnia *sp.*—The gut of *Daphnia* possesses three sections: foregut, midgut, and hindgut (Schultz and Kennedy 1976). Of these sections, the midgut is the location of food digestion and nutrient absorption. It consists mainly of the midgut epithelium, a single layer of tightly attached cells, that constitutes the only barrier between the midgut cavity (i.e., the actual location of food digestion) and the open blood circuit. Results of several studies indicate that microcystins or other metabolites of *Microcystis* can have a disruptive effect on the intestinal cells of mosquito larvae (Saario et al. 1994), fish (Fischer and Dietrich 2000), and birds (Falconer et al. 1992). If the same applies to *Daphnia,* then feeding on *Microcystis* would disrupt the midgut epithelium, destroying the only barrier between the digestive cavity and the blood. Consequently, microcystins ingested with *Microcystis* cells may rapidly enter the blood. The feasibility of this scenario was tested in the present study by investigating whether the PCC 7806 wild type or mutant can cause a disruptive effect on the daphnid midgut epithelium.

At the beginning of an experiment, six glass vessels each received 250 ml of a 10 mm^3 L^{-1} (corresponds to 1.4 mg C L^{-1} [calculated from Rocha and Duncan 1985] or 3.41 × 10^8 cells L^{-1}). The PCC 7806 wild-type suspension was prepared with zooplankton medium and eight animals. Afterward, the vessels were closed, placed on a plankton wheel (one rotation per min), and incubated for 0, 3, 6, 9, 12, or 15 h. These time points represent the period during which all major symptoms of microcystin poisoning occur (*see* Results). Temperature and mean light intensity were kept constant at 20°C ± 1 (range) °C and 5 μmol photons m^{-2} s^{-1}, respectively. At the end of an incubation period, the eight animals from one vessel were transferred into 1 ml of Boiun fixative. Later, the preserved individuals were enclosed in paraffin and sectioned (thickness, 5–7 μm) by means of an ultramicrotome (RM2035, Leica-Jung). Subsequently, the sections were stained with hematoxylin/eosin or Mallory's stain. Microscopic analyses were carried out using a Leica DMLB microscope at magnifications up to ×1,000. The entire procedure was repeated with the PCC 7806 mutant as food.

Intestinal uptake of microcystins into the blood of Daphnia *sp.*—These experiments were run to test both when and to what extent microcystins ingested with PCC 7806 wild-type cells are taken up from the midgut into the blood of *Daphnia.* To that end, *Daphnia* were fed PCC 7806 wild type, and blood samples were taken at several time points. At the beginning of an experiment, 300-ml glass vessels each received 250 ml of a 10 mm^3 L^{-1} PCC 7806 wild-type suspension prepared with zooplankton medium and up to 20 animals. The vessels were then closed and placed on a plankton wheel. Temperature and mean light intensity were kept constant at 20°C ± 1 (range) °C and 5 μmol photons m^{-2} s^{-1}, respectively. Triplicate vessels were incubated for 0, 3, 6, 9, 12 or 15 h.

At the end of an incubation period, all individuals of a replicate group were transferred into 100 ml of particle-free tap water in which the animals were allowed to stay undisturbed for at least 1 min. This procedure aimed to remove all attached PCC 7806 wild-type cells and was repeated three times. Afterward, the animals were fastened individually on glass slides with grease placed dorsally on the carapax. Attached water was carefully removed using paper tissue. Blood samples were obtained by introducing glass microcapillaries (tip diameter, ~50 μm) into the upper body of the experimental animals. All movements of the capillaries were steered by a stereomicroscope equipped with a micromanipulator (M33, Unisense). Special attention was paid so as not to impale the midgut or water-filled body parts and, thus, to avoid contamination or extensive dilution of the blood samples, respectively. The sample volume was determined by measuring the inner dimensions of that part of a capillary, which was filled with *Daphnia* blood. These measurements were done microscopically. The samples were collected in 1-ml glass vials containing 100 μl of deionized water each and stored at −18°C until analysis. Each glass vial received the blood samples taken from animals of one replicate group.

The microcystin concentration of blood samples was determined using a commercial enzyme-linked immunosorbent assay (EnSys). This analysis included addition of diluted samples to microtiter wells precoated with polyclonal antibodies, followed by addition of an enzyme-linked substrate (Chu et al. 1989). The antibodies were specific to microcystins at very low concentrations. Solutions of MCYST-LR were used as external standard. Mean values of the blood microcystin concentration were compared by analysis of variance, followed by Student's *t*-test at the 95% level of significance.

Effects of PCC 7806 wild type and mutant on the appearance and activity of Daphnia *sp.*—The objective here was to obtain an overview of symptoms that characterize microcystin poisoning and help to identify target organs of microcystins within the daphnid body. To start an experiment, a single *Daphnia* organism was fastened in a 5-ml, flow-through chamber (made of polycarbonate and glass) with grease placed dorsally on the carapax. The animal was positioned to allow observations of the entire body without disturbing the functions or movements of any body part. Once a *Daphnia* was fastened, the chamber was filled with zooplankton medium, closed with a plane-glass slide, and mounted on a stereomicroscope equipped with a high-resolution video camera. Afterward, a suspension of either PCC 7806 wild type or mutant was pumped continuously through the chamber at a flow rate of 1 ml min^{-1}. The food suspension, which had a cell-biovolume concentration of 10 mm^3 L^{-1}, was prepared fresh every day using artificial zooplank-

ton medium. The temperature was kept constant at 20°C ± 1 (range) °C. All reactions of the animal were continuously recorded in real time on videotape. For each food type, the entire procedure was repeated with five individual *Daphnia* organisms. Experiments with the PCC 7806 wild type were terminated after death had occurred (criterion for death was complete halt of heart beat). Tests with the PCC 7806 mutant were stopped after molting.

The video material of each individual was evaluated in two steps. First, the material was inspected in its entirety to detect alterations in the animal's appearance. Second, changes in frequency of movements, which occur regularly and fulfill essential life functions, were quantified. For that purpose, the beat rates of the thoracic legs (second through fourth legs were observed; rhythmic movements essential for ventilation and food acquisition), mandibles (beats causing food transport toward the foregut), foregut (peristaltic movements effecting intake of food particles), heart (contractions effecting blood circulation), and second antennae (beats responsible for swimming) were determined at several time points distributed over the entire experimental period. The maximal length of the interval between two time points was either 2 h (first 12 h of an experiment) or 4 h (period from 12 h to the end of an experiment). The length of the interval between two time points had been decreased during periods with significant changes in the activity of one of the body parts analyzed. At each time point, all movements of the thoracic legs, heart, and second antennae were counted 5 times for 10 s (slow motion), whereas movements of the mandibles and foregut were counted 5 times for 60 s (original speed). Afterward, mean values were calculated.

The statistical significance of changes in beat rates of a given individual was tested by calculating the Spearman rank correlation coefficient (test for changes in beat rate of a particular organ over time). All tests were done at the 95% level of significance.

Results

Animals that were not exposed to *Microcystis* (0-h treatment) showed a midgut epithelium with simple cells arranged in a single layer. Following the midgut in the posterior direction, the shape of epithelial cells changed from columnar to cubical. All epithelial cells were tightly attached to each other and did not show intercellular spaces (Fig. 1). Overall, the midgut structure closely resembled that described by Schultz and Kennedy (1976) for *D. pulex.*

Despite differences in microcystin content, both PCC 7806 wild type and mutant affected the cohesion of the midgut epithelium. After 6 h of exposure to PCC 7806 wild type or mutant, an increasing number of midgut epithelial cells lost contact with their neighbors. This loss in cell–cell contacts reached its maximal frequency and severity after 9 h of feeding on *Microcystis.* At that time, intercellular spaces had occurred all along the midgut (Fig. 1).

Microcystins ingested with *Microcystis* PCC 7806 wild-type cells entered the blood of *Daphnia* quickly. Traces were already found after 3 h and 6 h of feeding on the PCC 7806 wild type (Fig. 2). Between 6 h and 9 h of exposure to the

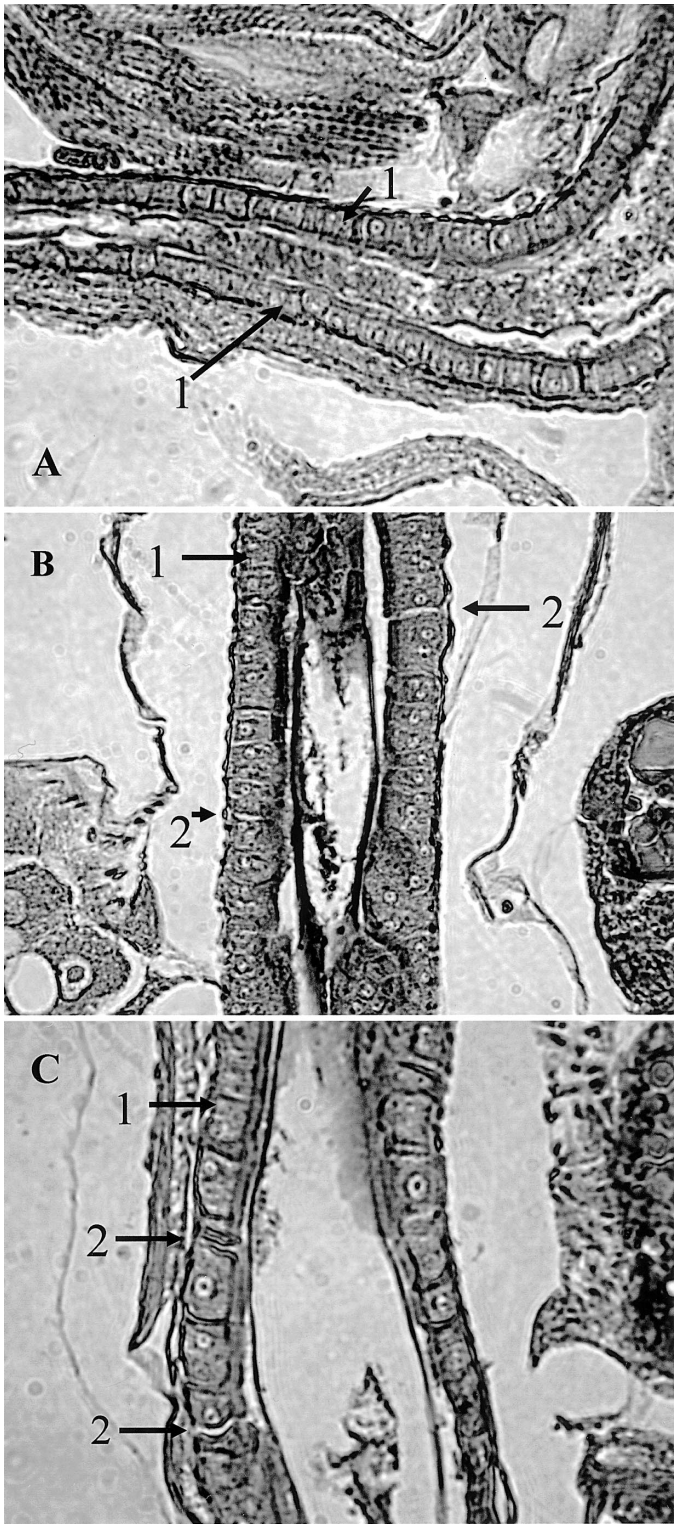

Fig. 1. Sections of *D. galeata* midgut (A) before and after exposure to (B) microcystin-producing PCC 7806 wild type (exposure for 9 h) or (C) microcystin-lacking PCC7806 mutant (exposure for 9 h). Markings within the pictures: 1, midgut epithelium; 2, areas where midgut epithelial cells lost contact to each other.

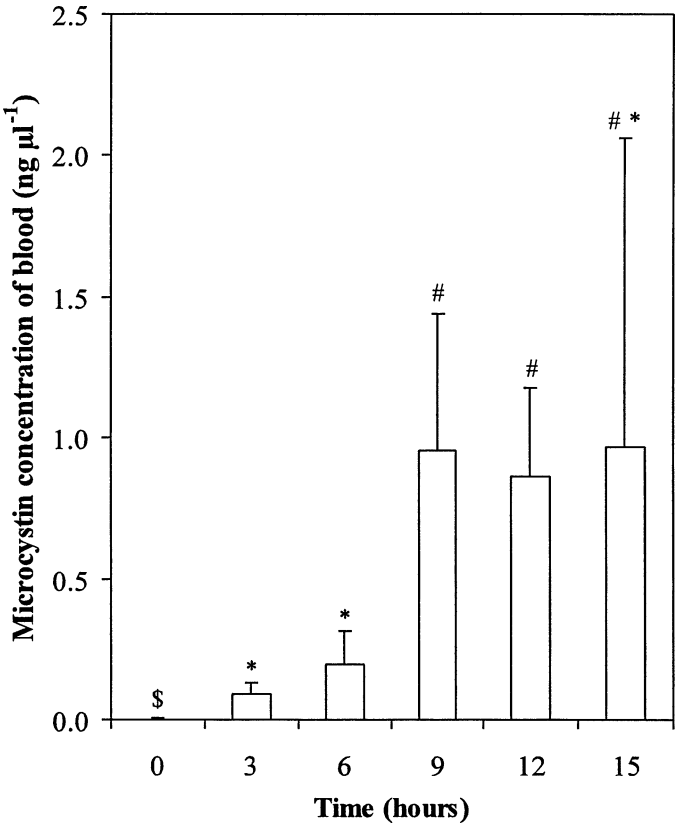

Fig. 2. Microcystin concentration of daphnid blood after feeding on *Microcystis* PCC 7806 wild type for 0–15 h. Bars represent the mean values of three replicates and the respective standard deviations. Mean values, which differ significantly from each other, are marked with different symbols (ANOVA followed by Student's *t*-test at 95% level of significance, all data show normal distribution).

cyanobacterial cells, the microcystin concentration of the blood increased significantly from 0.25 to almost 1 ng μl^{-1}. Afterward, the concentration leveled off, and no significant differences were found among blood samples taken after 9, 12 and 15 h of feeding on PCC 7806. However, variation among replicates was high.

All animals exhibited similar changes in appearance and activity when fed the microcystin-containing PCC 7806 wild type. In all cases, ingestion of the microcystin-producing PCC 7806 wild-type cells resulted in lethal poisoning, the time course of which can be described as follows: During the first 5–9 h of exposure to PCC 7806 wild type cells, the thoracic legs, mandibles, foregut, heart, and second antennae of all animals moved normally and at rates that showed no significant changes over time (*see* example in Fig. 3, left column). *Microcystis* cells were swallowed evenly, and ingested cyanobacterial cells were transported along the gut by strong peristaltic waves created by gut muscles. This caused the food particles to accumulate in the posterior midgut region and hindgut, from which they were transported outward by periodic contractions of the hindgut. After 5–9 h of feeding on PCC 7806 wild type, the beat rates of the thoracic legs, mandibles, foregut, and second antennae decreased simultaneously and significantly (*see* example in Fig. 3, left column; mean values of all test animals fed PCC 7806 wild type are shown in Table 1). Also, the peristaltic movements of the midgut stopped entirely. Instead, three sections of the midgut (gastric ceca, right below the heart, and upper posterior region) contracted, which points to a permanent contractile effect of the midgut ring muscles. This caused the sequestered food particles to accumulate in the anterior midgut. Initially, the heart beat rate remained constant or even increased; later, it gradually decreased. A few hours before death, the mandibles and second antennae started to move convulsively. Finally, all movements stopped entirely. Only the heart retained some of its activity, but it rested for periods of increasing length. After 32–41 h of exposure to the PCC 7806 wild type, the heart beat stopped completely, and the body started to contract. This was interpreted as a clear sign of death.

Animals fed the microcystin-lacking mutant of PCC 7806 did not show any of the symptoms described above. Instead, the heart beat rate increased significantly during the entire experiment (*see* example in Fig. 3, right column; mean values of all test animals fed PCC 7806 mutant are shown in Table 2). Some animals also exhibited an increasing activity of the foregut. The thoracic legs, mandibles, and second antennae showed, despite some variation, a constant beat rate. The entire gut functioned normally, and no permanent contractions of any gut section were detected. All experiments were ended by molting of the *Daphnia.* The animals had no problems shedding their old integument and swam away in a normal manner.

Discussion

Using *D. galeata* and the cyanobacterial strain PCC 7806 as model organisms, we have shown that microcystins, once ingested with *Microcystis* cells, are rapidly taken up into *Daphnia* blood. As a result, microcystins accumulate in the blood and, thus, gain access to the entire daphnid body. Moreover, our findings demonstrate that intake of microcystin-containing *Microcystis* cells causes lethal malfunctions of multiple organs, whereas ingestion of the same cyanobacterial cells without microcystins allows *Daphnia* to maintain its normal appearance and activity. These results confirm the toxicity of microcystins taken in by *Daphnia* when feeding on *Microcystis* (Kaebernick et al. 2001; Rohrlack et al. 2001).

Based on all available data, the intestinal uptake of microcystins in *Daphnia* fed *Microcystis* can now be described in more detail. Microcystins are ingested with *Microcystis* cells, which are collected in the midgut, the location of digestion and nutrient assimilation (Schultz and Kennedy 1976). The enzymatic digestion of *Microcystis* causes liberation of microcystins into the midgut cavity. There, the toxins probably accumulate until they are either broken down or transported across the midgut epithelium to enter the blood directly (shown for other compounds by Fryer 1970). The present findings indicate that this transport from the midgut cavity into the blood may require a preceding loss of cell–cell contacts within the midgut epithelium: In

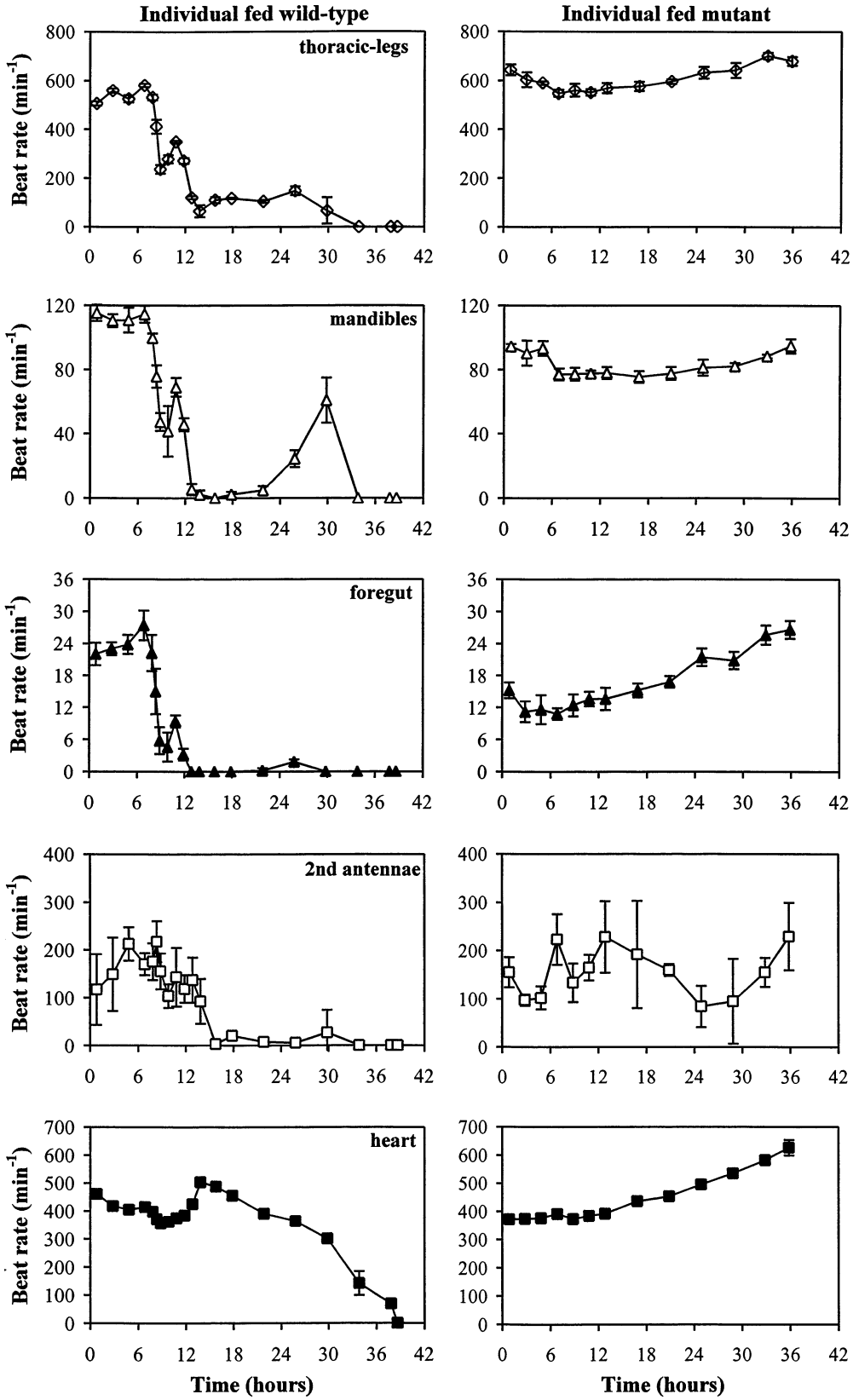

Fig. 3. Time course of beat rates of essential organs. Shown are data from two typical individuals, one fed the microcystin-containing wild type (left column) and one exposed to the microcystin-lacking mutant of PCC 7806 (right column). For each individual, the beat rates of the thoracic legs, mandibles, foregut, second antennae, and heart are shown from the first contact to *Microcystis* to the death (individual fed wild type) or molting (individual fed mutant). Each time point represents the mean of five determinations equally distributed over a period of 5 min and the respective standard deviation.

Table 1. Mean beat rates of essential body parts of five individual *D. galeata* continuously exposed to PCC 7806 wild type (mean values in beats per minutes±SD; only selected time points between the first exposure to *Microcystis* and death of *Daphnia* are shown).

	After exposure to PCC 7806 wild type for				
	1 h	10 h	20 h	30 h	38 h
Thoracic legs	537.5±30.2	252.2±106.9	136.7±44.4	68.6±50.7	0
Mandibles	103.3±11.4	41.0±25.6	21.7±22.8	37.6±28.9	0
Foregut	18.6±5.3	4.2±3.8	0.1±0.1	1.9±4.0	0
Second antennae	161.8±51.8	85.8±34.4	77.8±87.5	15.6±13.7	0
Heart	451.3±38.8	460.7±80.6	445.1±36.7	320.8±104.4	85.7±90.9

experiments with the PCC 7806 wild type, 6–9 h of feeding on *Microcystis* led to the formation of intercellular spaces among midgut epithelial cells. This probably caused an increase in permeability of the midgut epithelium (Schultz and Kennedy 1976) and, therefore, deteriorated its ability to shield the blood from the microcystin-loaded midgut cavity. At the same time, the microcystin concentration of the blood exhibited a sudden and significant increase. Taken together, these observations suggest that feeding on *Microcystis* results in a loss of cell–cell contacts within the daphnid midgut epithelium, which in turn allows a rapid uptake of microcystins into the blood. The basis of this fast uptake might be transport via newly formed intercellular spaces. Such transport would be supported by the steep osmotic gradient between the midgut, which is constantly refilled with water by oral and anal drinking (Fryer 1970), and the hypertonic blood.

The above scenario not only suggests a totally different microcystin uptake mechanism than that described for vertebrates (Falconer et al. 1992; Bury et al. 1998) but also may have significant implications for the occurrence of lethal microcystin effects in *Daphnia.* According to present morphological analyses, both the PCC 7806 wild type and its microcystin-lacking mutant cause a loss of cell–cell contacts within the midgut epithelium. Therefore, the structural alterations in the midgut, which pave the way for a microcystin uptake, likely are at least partially caused by an unknown factor that is not a microcystin and is not correlated to the production of microcystins. Thus, the occurrence of this factor may be a condition for intestinal uptake and lethal effects of microcystins ingested with *Microcystis* cells. Moreover, the midgut-disrupting factor may play a decisive role in the intestinal uptake of bioactive *Microcystis* metabolites other than microcystins.

The data presented here also allow an estimation of the oral microcystin dose causing rapid death of *D. galeata*. In continuous feeding experiments with the PCC 7806 wild type, activity of the foregut remained constant during the first 5–9 h. This indicates a constant feeding rate during that period. Afterward, the activity of the foregut and, thus, probably also the feeding rate declined rapidly. Shortly thereafter, a permanent contraction of the midgut made all ingestion of additional food impossible. Hence, feeding on PCC 7806 for 5–9 h was sufficient to gain a lethal oral microcystin dose. An estimate of that dose can be obtained by multiplying the effective feeding time on PCC 7806 (5–9 h) with the microcystin ingestion rate of *D. galeata* (2.03 ng h^{-1} mg fresh $weight^{-1}$ when fed PCC 7806 wild type; Rohrlack et al. 2001). According to that calculation, an ingestion of 10.2–18.3 ng of microcystin per 1 mg of body fresh weight causes rapid death of *D. galeata.* Similar values have been found for mammals (Yoshida et al. 1997; Fawell et al. 1999), suggesting that daphnids and vertebrates possess comparable susceptibilities to orally administered microcystins.

In the present study, the symptoms and time course of microcystin poisoning were studied by comparing the microcystin-producing PCC 7806 wild type and its microcystin-lacking mutant in long-term feeding experiments with continuous video observation. Because the PCC 7806 wild type and mutant differ only in their ability to produce microcystins, effects caused by the wild type alone can be linked to microcystins. In experiments with PCC 7806, the first visible symptoms of microcystin poisoning included an inhibition of movements (thoracic legs, mandibles, foregut, second antennae) and a stimulation of gut muscles, leading to a permanent contraction of midgut sections. In a given individual, these effects occurred simultaneously and during the same period as a significant increase in microcystin concentration in the blood. Altogether, these findings suggest that microcystins, as soon as they are taken up into the blood, cause an adverse effect on a central life function that

Table 2. Mean beat rates of essential body parts of five individual *D. galeata* continuously exposed to PCC 7806 mutant (mean values in beats per minutes±SD; only selected time points between the first exposure to *Microcystis* and the molting of *Daphnia* are shown).

	After exposure to PCC 7806 mutant for				
	1 h	10 h	20 h	30 h	35 h
Thoracic legs	640.8±49.2	650.0±85.9	618.4±81.7	624.6±24.6	671.4±11.03
Mandibles	102.2±8.9	95.0±15.8	90.0±12.6	86.0±5.6	104.7±14.0
Foregut	21.5±5.6	25.3±10.1	24.1±6.4	23.2±3.4	30.4±5.4
Second antennae	134.4±28.8	144.2±28.0	351.6±271.5	319.8±318.2	470.4±341.1
Heart	418.0±40.1	455.2±62.6	541.2±103.3	529.8±7.6	636.6±14.4

influences or controls major muscle systems in *Daphnia* (*see* Martin 1992 for a description of the daphnid muscle system).

One likely explanation for the symptoms described above is a disturbance of the neuromuscular communication by microcystins. Daphnids possess inhibitory and stimulatory neurons (Martin 1992). Thus, a disturbance of the neuromuscular communication would account for the coincidence of muscle inhibition and stimulation as observed in the present study. Moreover, rapid and concurrent changes in the activity of diverse muscle systems indicate an adverse effect on neuromuscular communication as well. In addition, neurotoxicity of microcystins has already been found in humans accidentally exposed to the toxins (Pouria et al. 1998), and microcystins can affect the L-type calcium channels (Leiers et al. 2000) that play a vital role in crustacean neuromuscular communication (Weiss et al. 2001).

Unfortunately, a complete causal analysis of the symptoms described above could not be achieved by the present study. The main problem is that the inhibitory effect of microcystins on daphnid protein phosphatases (DeMott and Dhawale 1995) enables the toxins to interfere with numerous processes involved in neuromuscular communication (e.g., Herzig and Neumann 2000). The present data are insufficient to establish one of these potential effects as the actual cause for the symptoms described above. However, our data do suggest an extracellular effect as the cause for these symptoms. A significant uptake of microcystins into cells other than those of the gut epithelium or mammalian liver requires a very high microcystin concentration in the blood ($>$18.75 ng μl^{-1}) and a long exposure time (at least 72 h; Chong et al. 2000). These conditions were not fulfilled in experiments with *D. galeata* and *Microcystis* PCC 7806 wild type. Therefore, it is unlikely that microcystins could have affected intracellular life processes directly.

The described effects of microcystins on the activity of muscle systems probably contribute to the eventual breakdown of *Daphnia* metabolism. The observed decrease in the beat rate of thoracic legs, for example, might finally result in a lack of oxygen (Pirow et al. 1999). Serious problems may also arise from the microcystin-induced contractile effect on midgut muscles, leading to a permanent contraction of the midgut. The daphnid midgut is a multifunctional organ that is involved in digestion, nutrient assimilation, and uptake of ions (Martin 1992) as well as water (Fryer 1970). A constant contraction of the midgut likely interferes with these functions, leading to starvation, lack of ions, and even more critically, a failure in osmoregulation. A combination of these secondary microcystin effects and the lack of oxygen may lead to a general exhaustion of *Daphnia* and, therefore, to death. This idea is supported by the occurrence of exhaustion symptoms (gradual decrease in all movements, irregular and convulsive movements, and heart beat that rested for periods of increasing length) observed during the last phase of experiments with the PCC 7806 wild type. However, direct effects of microcystins on the daphnid metabolism cannot be excluded as a reason for death.

From the present results and those of our previous laboratory studies (Kaebernick et al. 2001; Rohrlack et al. 2001), several common *Daphnia* species clearly will be acutely poisoned by microcystins if *Microcystis* cells are ingested in sufficient amounts. This remains true when *Daphnia* are exposed to *Microcystis* growing in its natural colony form (Rohrlack et al. 1999). Thus, we have no reason to believe that acute toxic effects caused by food-associated microcystins are unusual under natural conditions. However, in nature, ingestion of *Microcystis* and, consequently, intake of microcystins may be influenced by various factors that were not considered in the present study, such as *Microcystis* colony size, occurrence of alternative food, temperature, health of the animals, and so on. The present study therefore leaves open the question of under which conditions natural *Daphnia* populations can ingest lethal amounts of microcystins, although the present study provides, to our knowledge for the first time, the toxicological tools (estimate of lethal dose, time course, and symptoms of microcystin poisoning) to search for these conditions. Further investigations are needed to understand the effects caused by sublethal microcystin doses (Lürling 2003), because they may be taken in by *Daphnia* feeding on phytoplankton communities with a low share of microcystin-producing *Microcystis.*

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