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Effects of *Microcystis aeruginosa* and purified microcystin-LR on the feeding behavior of *Daphnia pulicaria*

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Abstract

We investigated the mechanisms behind the negative effects of cyanobacteria on zooplankton by comparing the effects of *Microcystis aeruginosa* single cells, colonies, and toxins on the feeding behavior of *Daphnia pulicaria* in three independent experiments. The animals were fed a mixture of *Scenedesmus* supplemented by increasing proportions (0, 20, 50, 80, 100%) of *Microcystis* or concentrations (0, 50, 500, 5,000 ng ml^{-1}) of purified microcystin-LR. The changes in feeding behavior, as indicated by the appendage beat, mandible or labrum movement rates were evaluated by a direct observation method that coupled video recording and computerized image analysis. *Daphnia* responded in a different manner to the presence of single cells and colonies. In the case of the single cells, the mandibular movement rate (MMR) declined more than appendage beat rate (ABR), suggesting that *Daphnia* have the ability to discriminate between *Microcystis* and *Scenedesmus.* Colonies, on the other hand, produced a typical feeding interference response: the animals increased their labral rejection rate (LRR) and showed starvation signs. LRR increased in the presence of both unicellular and colonial *Microcystis.* In both cases, the changes in MMR and ABR were rapidly reversible and hence unlikely to be caused by intoxication from the presence of cell-bound microcystins. In contrast, the addition of purified microcystin-LR at the concentration of 5,000 ng ml^{-1} produced a nonreversible impairment of *Daphnia* feeding behavior.

Massive growth of cyanobacteria, also called blooms, represents a serious threat to aquatic ecosystems. Besides the fact that cyanobacterial blooms contribute to the degradation of water quality and recreational value of lakes and reservoirs, some cyanobacterial species can produce a variety of potent toxins (e.g., Falconer and Humpage 1996). During the last decade, several forms of cyanobacterial secondary metabolites, mainly microcystins, have been found to be hepatotoxic or neurotoxic for a wide range of organisms, including humans (e.g., Jochimsen et al. 1998). A number of severe intoxication cases caused by cyanotoxins have since been reported worldwide (Falconer and Humpage 1996; Jochimsen et al. 1998). The phenomenon of toxic algal blooms is not limited to freshwaters, it is also a largely recognized problem in shallow coastal marine environments; however, in marine systems, dinoflagellates are the main cause of harmful algal blooms (e.g., Horner et al. 1997). Hence, it is critical to increase our understanding of the ecological and toxicological implications of these harmful algal blooms in our water bodies (Christoffersen 1996).

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The first-order effects of cyanobacterial blooms can be experienced by zooplankton communities that constitute the closest link to phytoplankton communities in aquatic systems (Haney 1987). Although several studies have reported that mechanical feeding interference (Webster and Peters 1978; DeMott et al. 2001), low nutritional quality (e.g., Müller-Navarra et al. 2000), or toxin production (Reinikainen et al. 1995; Rohrlack et al. 1999*a*) of cyanobacteria make them unsuitable food for zooplankton, other studies support an opposite view, which states that zooplankton could develop some resistance to cyanobacteria (Hairston et al. 2001) and possibly control their biomass by means of intensive grazing (e.g., Boon et al. 1994; Matveev et al. 1994). The ability of large filter feeders, such as *Daphnia*, to survive during cyanobacterial blooms is critical to the success of biomanipulation in eutrophic systems (Brett and Goldman 1996). However, controversy still surrounds the success of biomanipulation in cyanobacteria-dominated systems, mainly because the interactions between zooplankton and cyanobacteria are not well understood (e.g., Carpenter and Kitchell 1992; DeMelo et al. 1992; MacKay and Elser 1998).

Although several studies have reported a wide variety of changes in feeding behavior of daphnids after exposure to cyanobacterial cells or colonies and their toxins (Haney et al. 1995; Rohrlack et al. 1999*b*; DeMott et al. 2001; Ghadouani and Pinel-Alloul 2002), the mechanisms behind the inhibitory effects are yet to be elucidated. For example, it is not clear yet whether the feeding inhibitions observed in *Daphnia* when exposed to cyanobacteria could be attributable to their intracellular toxin content (Lampert 1981*a*; Rohrlack et al. 1999*b*) or some other substances attached to the cyanobacterial cell wall (Jungmann 1992). Previous studies reported no correlation between the concentration of cyanotoxins extracted from several laboratory and natural *Microcystis* strains and toxicity to *Daphnia* (Lampert 1981*a*; Nizan et al. 1986). Although it is well established that cladocerans, unlike copepods, have poor abilities to discriminate between food particles on the basis of taste (Kerfoot and Kirk 1991), recent studies suggest that *Daphnia* feeding can be controlled by a mechanism of chemosensory stimulation or ''bad taste factor'' (Haney et al. 1995), leading to the possibility of selective feeding by *Daphnia* in the presence of filamentous cyanobacteria (Epp 1996). In contrast, it is suspected that feeding interference by large cyanobacterial colonies and filaments could be simply the cause of feeding inhibition in *Daphnia* (Webster and Peters 1978; DeMott et al. 2001; Ghadouani et al. 2003). Whether of chemical or physical origin, feeding impairment caused by cyanobacteria could play a critical role in structuring zooplankton communities. For instance, this could possibly explain the midsummer declines in large filter-feeding daphnids, the change in size structure of zooplankton communities usually observed in natural systems following cyanobacterial blooms, or both (Threlkeld 1979; Ghadouani et al. 1998, 2003).

This study aims to investigate the mechanisms of feeding impairment experienced by *Daphnia* during cyanobacterial blooms. We evaluate in three separate experiments the effects on *Daphnia pulicaria* feeding behavior of the addition of unicellular and colonial *Microcystis aeruginosa* and a purified toxin usually produced by cyanobacterial species, microcystin-LR. We used the filtering appendage beat rate (ABR), mandibular movement rate (MMR), and labral rejection rate (LRR) as indicators of feeding behavior of *Daphnia* (Watts and Young 1980; Porter et al. 1982; Lampert 1987). Although these rates do not represent a direct measurement of food uptake, they can be indicative of changes in cladoceran feeding behavior. Pioneering studies by McMahon and Rigler (1963) have shown that ABR is a good measure of filtering rate, and any decrease of ABR at a given food level means a decrease in the amount of particles gathered in the *Daphnia* food groove, whereas MMR is a good indicator of food ingestion and could be used as a measure of feeding rate of daphnids (McMahon and Rigler 1963; Burns 1968). The mandibles and maxillules are the mouthparts responsible for the mastication and the transport of ingested food particles to the oral cavity (Starkweather 1978). Previous studies based on direct observation have shown that the mandibular movements fairly reflect feeding rate in cladocerans (Burns 1968). Declines in feeding rate were shown to be accompanied by an increase in labral and postabdominal rejection rate as the animals tried to remove the excess of food or large particle clumps, especially in high food conditions (Burns 1968; Porter et al. 1982).

In this study, we evaluate and compare the changes in feeding behavior of *D. pulicaria* when exposed to (1) unicellular *M. aeruginosa*, (2) colonies of the same species, and (3) purified microcystin-LR. To examine whether feeding inhibition changes with increasing proportion of *Microcystis* cells or colonies, we tested the response of daphnids in 0%, 20%, 50%, 80%, and 100% *Microcystis* biomass relative to *Scenedesmus obliquus*. We also tested the feeding behavior changes of daphnids in four different concentrations of dissolved microcystin (0, 50, 500, 5,000 ng ml^{-1}) added to their preferred food mixture.

Materials and methods

Experimental design—The present experiments were designed to evaluate the response of *Daphnia* to the addition of unicellular and colonial *M. aeruginosa* and purified microcystin-LR to their regular food. This study was composed of three independent experiments (Fig. 1). The behavior of the animals was recorded during four phases in experiments 1 and 2 and three phases in experiment 3. Phase 1 and 3 in experiments 1 and 2 represented the control phases, during which the animals were fed pure suspensions of *S. obliquus* (Fig. 1). The use of two successive control treatment phases was intended to evaluate whether the animals would recover their normal feeding behavior if the food mixture was changed back to the control. We assume that daphnids are displaying an intoxication reaction caused by a chemical factor (i.e., microcystin) when there is no sign of recovery of the pretreatment feeding behavior after *Microcystis* cells have been removed. Alternatively, if the daphnids recover a normal feeding behavior immediately (i.e., a few seconds) after the *Microcystis* cells have been removed, we assume that there was no intoxication reaction and that the change in feeding behavior might have been caused by other factors, such as physical interference or chemosensory stimulation, rather than

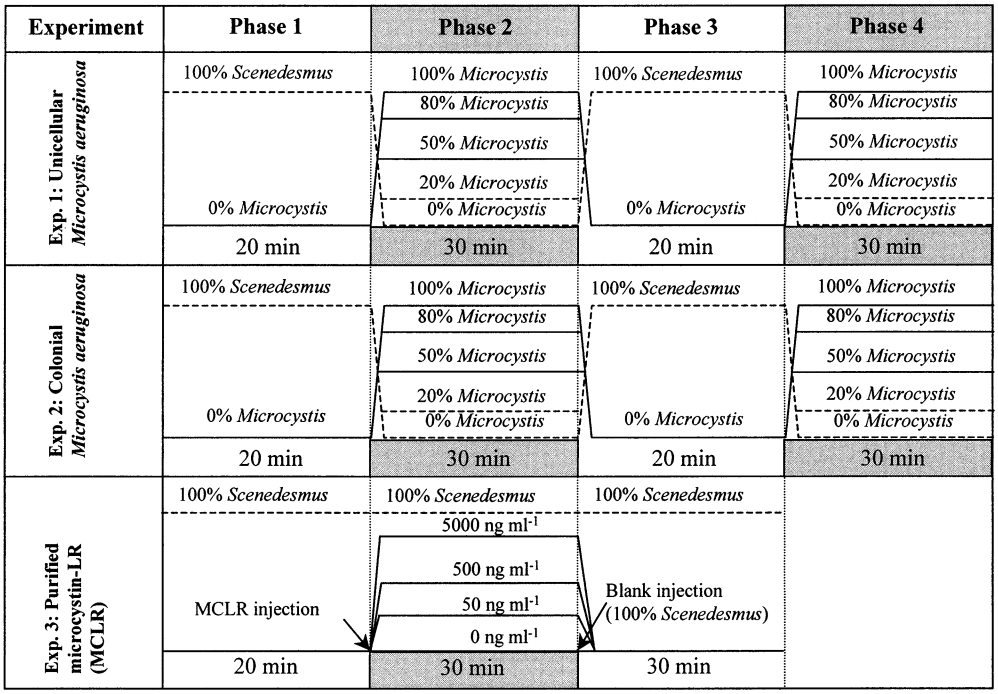

Fig. 1. Illustration of the experimental setup for the three experiments showing the conditions and duration of each experimental phase. Phase 1 represented a pretreatment or control phase in which the animals were observed in their original conditions with only *Scenedesmus obliquus* as food. At the end of phase 1, the conditions were changed by adding a proportion (0, 20, 50, 80, 100%) of unicellular or colonial *Microcystis aeruginosa* in experiments 1 and 2, respectively. Phase 3 represented a second control phase with identical conditions as in phase 1. In phase 4, the conditions were switched back to treatment conditions identical to phase 2. Experiment 3 was conducted in the same manner, except that there were only 3 phases. In this experiment, the animals were always fed the *Scenedesmus* suspension supplemented with different amounts (0, 50, 500, 5,000 ng ml^{-1}) of purified microcystin-LR during treatment phase 2. During phase 3, the conditions were switched back to the control conditions as in phase 1.

neuromotor inhibition by microcystins (Haney et al. 1995). In this sense, the use of successive control treatment phases can help gain insights into the recovery patterns of the daphnids. During phases 2 and 4, the animals were exposed to a mixture of *Microcystis* and *Scenedesmus* in different proportions (0, 20, 50, 80, 100%) according to the experimental design (Fig. 1). We also assume, from previous dual-labeling experiments (Lampert 1981*a*), that *Daphnia* would ingest both *Microcystis* and *Scenedesmus* cells without any selectivity when presented with a mixture of both species.

The high cost of the purified microcystin-LR used in experiment 3 led to a slightly modified methodology. Only one treatment phase was possible (Fig. 1). The animals were exposed all the time to *Scenedesmus* and experienced the addition of four different concentrations (0, 50, 500, 5,000 ng ml^{-1}) of purified microcystin-LR at the beginning of phase 2 followed by a blank injection at the end of phase 2 (Fig. 1). *Scenedesmus* suspensions mixed with purified microcystin-LR were injected into the observation chamber with a syringe. We intended to expose the animals for a short period of time (a few minutes) to low concentrations of dissolved microcystin, such as they would experience in natural systems. Although the reported concentrations of dissolved microcystin from natural systems vary across several orders of magnitude (0.04–25,000 $\mu g\ L^{-1}$), typical concentrations are on the order of a few micrograms per liter of water (*see* reviews in Christoffersen 1996; Chorus and Bartram 1999). It is believed that *Daphnia* is not sensitive to dissolved microcystins on the basis of the rather high 50% lethal concentration (LC_{50}) reported in the literature (DeMott et al. 1991; Jungmann 1992). In these experiments, we use a range of relatively low concentrations of microcystin similar to those usually found in natural systems to test physiological responses (i.e., feeding rate impairment), which are more ecologically relevant than lethality tests (Gliwicz and Sieniawska 1986; Hatch and Burton 1999; McWilliam and Baird 2002).

This study used the same methodological approach for the direct observation of *Daphnia* feeding behavior as was developed and tested in earlier studies (McMahon and Rigler 1963; Burns 1968). The animals were maintained in a 10-ml flow-through observation chamber (*see* Haney et al. 1995; Plath 1998) at a controlled temperature of 20°C and were fed at a rate of ~13 ml min^{-1} directly from a freshly prepared algal suspension. It was then possible to rapidly change the food mixture without disturbing the animals (Plath 1998). The suspension fed into the chamber could be

changed easily by switching from the control *Scenedesmus* to the treatment *Microcystis* according to the experimental design (Fig. 1). The animals were maintained in an upright position and could be viewed by an infrared-lighted video camera and a ×4 macro lens. Before their installation in the chamber, the animals were affixed along their dorsal carapace margin to a coverslip with a drop of petroleum jelly (Vaseline). The movements of the animals were then recorded on videotape for a continuous period of 100 min divided into four phases for experiments 1 and 2 and 80 min divided into only three phases for experiment 3 (Fig. 1). All the animals were allowed a 30-min acclimatization period before the beginning of the video recording (Burns 1968). The position of the camera allowed a ventral carapace opening view of the animals (Fig. 2a). The measurements of ABR were made by a fully automated method with an image analysis system (Medealab). The recorded tapes were viewed with a VCR connected to a computer equipped with a video image acquisition technology. The image analysis software was set to take a 5-s measurement of light intensity every 90 s within a measurement area positioned adequately to detect movement of the filtering appendages (Fig. 2a). The software produced a precise count of the light intensity peaks over the 5-s measurement time, which corresponded to the frequency of filtering appendage movement or ABR (Fig. 2b). The MMR and LRR were not counted with the automated method because of their less regular nature compared with ABR. We simply counted them visually by viewing the recorded tapes. The time taken by the animals to produce 10 mandibular movements was recorded at a 90-s interval, and the frequency was calculated by simply dividing 10 by the time in seconds. The LRR was estimated by counting the total number of rejections for the whole duration of the phase and expressed in number per minute. Postabdominal rejections were not counted in these experiments, as is commonly done in similar studies (Haney et al. 1995), because on several occasions, we noted that those movements took place out of the recording area. However, we have noted that labral (upper lip) movements were synchronized with postabdominal rejection. Hence, we used the labral rejections as an indicator of feeding interference in the mouth region. These rejections always occurred when the animal needed to clear the mouth region of excess food or large particle clumps (Burns 1968).

Daphnia *and algal cultures*—All the daphnids used in the present study belonged to a *D. pulicaria* clone maintained in the laboratory at 20°C under constant dim light at the *Daphnia* culture collection of the Max Planck Institute for Limnology, Plön, Germany. The animals used in the experiments were all descendants of one mother and were cultured in a flow-through culture system at a controlled temperature of 20°C. This system is very efficient in maintaining the same food condition for all the experimental animals as long as they were needed for the experiments. The daphnids used in these experiments were fed a suspension of *S. obliquus* equivalent to 0.5 mg C L^{-1}. The size of the animals used in the experiments ranged from 2.3 to 2.8 mm in body length (top of the head to the base of tailspine). Only large animals were used to avoid any possible allometric effects of the body length on the feeding behavior of the animals. Previous studies found no detectable effects of body length on feeding mechanisms (i.e., ABR or MMR) within the same species (Porter et al. 1982; Plath 1998).

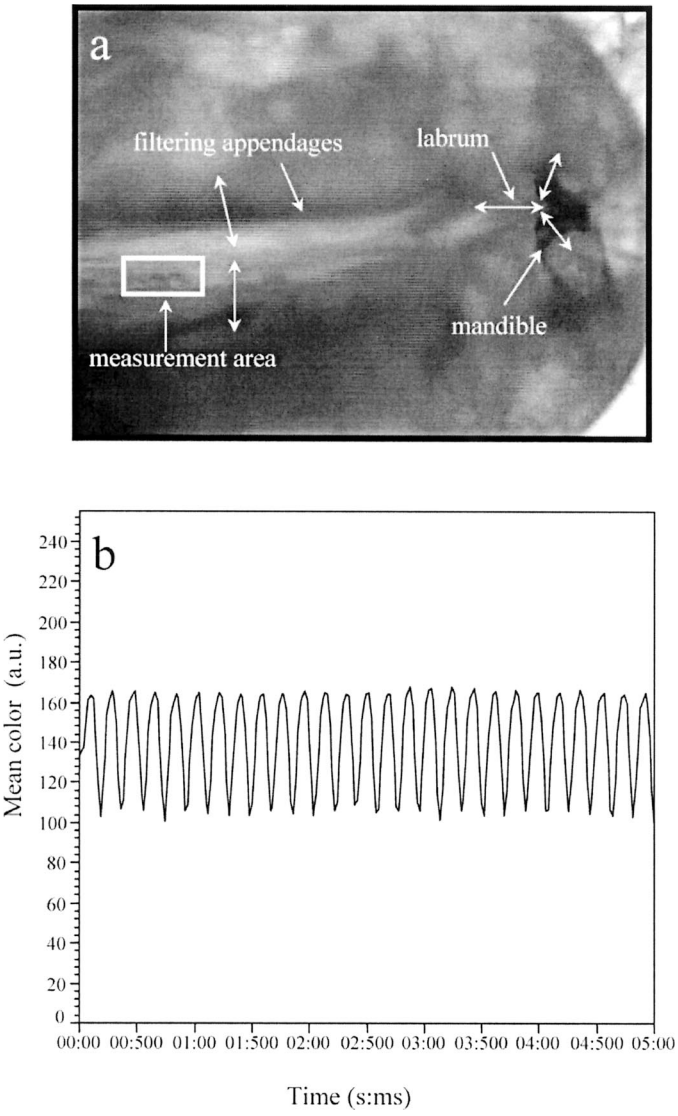

Fig. 2. An example of (a) image used for the calculation of *Daphnia pulicaria* appendage beat rate (ABR), mandibular movement rate, and labral rejection rate and (b) computerized calculation of ABR based on the change in light intensity measured by average RGB (red, green, blue) color in arbitrary units of the grayscale (a.u.) produced by the movement of the filtering appendages inside a carefully positioned measurement area shown by double arrows. The direction of *Daphnia* mandibular movements and the labral rejections are also shown by double arrows in panel a.

Scenedesmus suspensions used in the flow-through system and the control phases (i.e., phases 1 and 3) of the experiments were prepared daily by adding a volume of a fresh culture to 0.45-μm filtered lake water to make an equivalent 0.5 mg C L^{-1} on the basis of a previously established calibration curve between carbon content of *Scenedesmus* and light absorbance at 800 nm. *Scenedesmus* was cultured in chemostats in modified CHU 12 medium (Müller 1972). Two different strains of *M. aeruginosa* were used in these

experiments: a strain that grows only in single cells and that was maintained in the laboratory for years (Lampert 1981*b*) and a newly isolated strain (UTCC436) capable of growing colonies in laboratory conditions. Both strains were cultured in a BG-11 medium in low, cold-light conditions (Moss 1972). The proportion of *Microcystis* to be added to *Scenedesmus* suspension was calculated to achieve the same total biovolume as a pure suspension of *Scenedesmus* of 0.5 mg C L^{-1}. The biovolume of algal suspensions (*Scenedesmus* and *Microcystis*) was measured by a Casy I particle counter (Schärfe). On the basis of the biovolume calculation, different proportions of *Microcystis* were added to *Scenedesmus* to achieve 0%, 20%, 50%, 80%, and 100% of *Microcystis* relative biovolume. The total biovolume of the mixed *Scenedesmus–Microcystis* suspension was always maintained equivalent to a pure *Scenedesmus* suspension biovolume of 0.5 mg C L^{-1}. Hence, the animals were always exposed to the same biovolume of algal suspension, either pure *Scenedesmus* (i.e., phases 1 and 3) or a mixture of *Scenedesmus* and *Microcystis* (i.e., phases 2 and 4). The spherical equivalent diameter of *Scenedesmus* and unicellular *Microcystis* was ~5 and ~7 μm on average, respectively. The size of the colonies of *Microcystis* ranged from 10 to 90 μm in spherical equivalent diameter. Lyophilized *Microcystis* cells from both strains extracted at a concentration of 50 mg dry mass per milliliter with 70% aqueous methanol then analyzed by high-performance liquid chromatography with photodiode array detection revealed the presence of microcystins (Fastner et al. 2002). The concentration of cell-bound microcystins was ~1.5 and ~2.5 in the unicellular and the colonial strains, respectively. These concentrations are typical of microcystin-producing strains found in natural systems during bloom events (Christoffersen 1996; Chorus and Bartram 1999). However, the release of the cell-bound microcystin occurs only after lysis of the cells, and for the daphnids to be exposed to the toxins, the cells need to be ingested and fully digested. To ensure that daphnids are exposed to dissolved microcystin, as they would experience in nature after lysis of the cells, purified dried microcystin-LR provided by G. A. Codd (Dundee University, Scotland) was diluted in 1 ml of deionized water and added to *Scenedesmus* suspensions to achieve the desired concentration of 0, 50, 500, and 5,000 ng ml^{-1} (experiment 3).

Statistical analyses—Repeated-measures analysis of variance was used to test for significant differences between the different experimental phases. For each experimental condition (i.e., 0, 20, 50, 80, 100%), the comparisons were based on the observation of five independent animals (replicates). Repeated-measures analysis of variance was used to test for the effect of the treatment and also the treatment × time interaction. The interaction term gives insight as to the nature of the effect produced by the treatment over time. In our experiments, this information was as important as the treatment effect, because it allowed testing as to whether the feeding behavior changed during the treatment phase (Potvin et al. 1990). The nonparametric Wilcoxon test was considered more appropriate for the comparison of LRR because the number of observations was low compared with ABR and MMR. The less regular nature and low frequency of labral rejections, as opposed to appendage and mandibular movements, did not permit measurement of LRR in short sequences of 5 s every 90 s, resulting in a low number of observations for each experiment. Hence, only one value of LRR per phase per animal was available; consequently, the two treatment phases (2 and 4) were pooled and compared with pooled control phases (1 and 3) for the experiments with unicellular and colonial *Microcystis.* In the experiment with microcystin-LR, there was one treatment phase (2); hence, only the rates of the control period were pooled and compared with the treatment phase (2). All the statistical analyses were performed with Systat 8.0.

Results

The addition of unicellular *Microcystis* to *Daphnia* food produced only a slight decline in ABR compared with the stable behavior exhibited by the animals fed a pure suspension of *Scenedesmus* (Fig. 3). The daphnids, in the 20% to 100% *Microcystis* treatment, exhibited a pattern of response showing a slight decline during phase 2 compared with phase 1, a recovery during phase 3, and again a slight decline in phase 4, especially at high proportions of *Microcystis* (Fig. 3c–e). These changes were not strong enough to be statistically significant (Table 1). However, daphnid food ingestion as measured by MMR declined after the addition of unicellular *Microcystis* (Fig. 3). The first exposure to *Microcystis* cells (phase 2) caused a statistically significant decline in MMR (Table 2). The animals seem to have recovered the same level of MMR in phase 3 (Fig. 3), and accordingly there was no statistically significant difference between phase 1 and 3 with the exception of the 50% treatment (Table 2). After the second exposure to *Microcystis* during phase 4, the daphnids exhibited a reduction in their feeding activity consistent with the response observed in phase 2 (Fig. 3). The comparison of MMR showed a statistically significant decline in MMR from phase 3 to 4 for the 50%, 80%, and 100% *Microcystis* treatments (Table 2).

The exposure of daphnids to colonies of *Microcystis* seems to have affected their ABR in a different way compared with the effects observed with unicellular *Microcystis* (Fig. 4). In the 20%, 50%, and 80% *Microcystis* treatments, the first contact with the colonies caused a sharp decline of ABR in the first minutes, immediately followed by an increase during the remaining duration of phase 2 (Fig. 4b–d). The changes were statistically significant only for the 80% *Microcystis* treatment (Fig. 4; Table 1). It is important to note that unlike the experiment with unicellular *Microcystis*, ABR seems to change rapidly with time (Fig. 4). However, this observation was supported by the statistical analyses only for the 80% *Microcystis* treatment in which the interaction terms (treatment × time) were highly significant for phases 2 and 4 compared with their respective controls (Table 1). During phase 3 of the same treatment (80%), the animals decreased their ABR when they were given the control treatment, but as soon as they were exposed again to colonies, they rapidly increased their ABR (Fig. 4d). Surprisingly, the changes observed in the ABR of daphnids in all treatments from 20% to 80% were inexistent in the 100% *Microcystis* treatment (Fig. 4e).

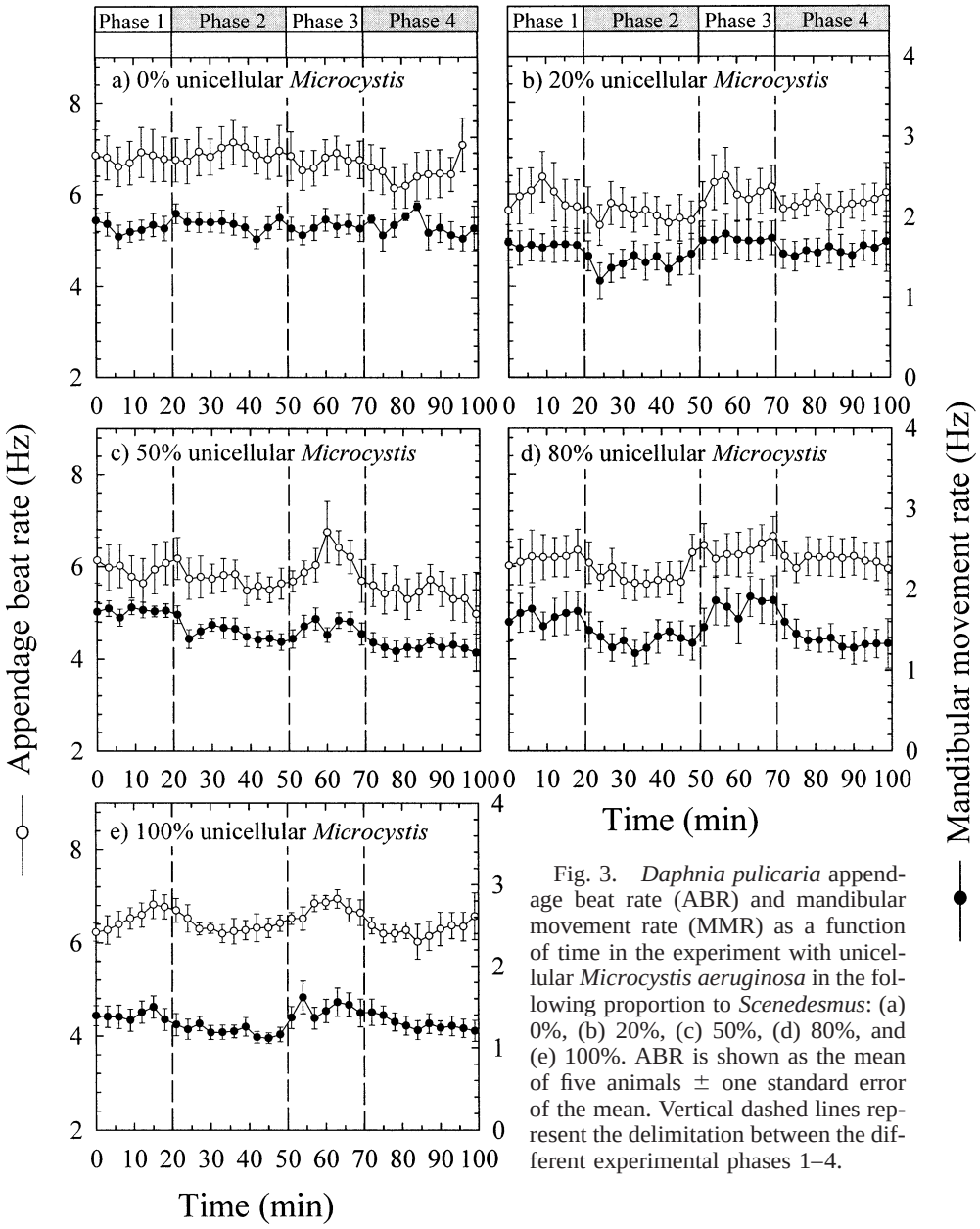

Fig. 3. *Daphnia pulicaria* appendage beat rate (ABR) and mandibular movement rate (MMR) as a function of time in the experiment with unicellular *Microcystis aeruginosa* in the following proportion to *Scenedesmus*: (a) 0%, (b) 20%, (c) 50%, (d) 80%, and (e) 100%. ABR is shown as the mean of five animals ± one standard error of the mean. Vertical dashed lines represent the delimitation between the different experimental phases 1–4.

Daphnia exposure to *Microcystis* colonies seems to have affected their ABR more than their MMR. A slight decline in MMR was observed in the 20–80% treatment at the beginning of phase 2, but the animals quickly recovered their previous MMR during the remaining duration of the experiment (Fig. 4). The only treatment in which the animals reduced significantly their MMR, and consequently their ingestion rate, was the 100% colonial *Microcystis* treatment, for which the ABR remained unchanged (Fig. 4e). The reduction in the ingestion rate was statistically highly significant (Table 2). The changes were clearly visible in phase 2; however, the animals quickly recovered their previous MMR as soon as they were again fed *Scenedesmus* suspensions in phase 3 (Fig 4e). The response of *Daphnia* during phase 4 was again a consistent decline similar to the response observed in phase 2 (Fig. 4e).

The addition of microcystin-LR at low concentrations (50 and 500 ng ml^{-1}) did not produce a detectable response in *Daphnia* feeding behavior (Fig. 5b,c). The behavior of *Daphnia* at 50 and 500 ng ml^{-1} remained similar to the controls (Fig. 5a–c). At 50 and 500 ng ml^{-1}, the comparison of *Daphnia* ABR during treatment phase 2 with phases 1 and 3 showed no statistically significant differences (Table 3). However, the daphnids exposed to 5,000 ng ml^{-1} showed a quick and sharp decline in their ABR as soon as the exposure started in phase 2 (Fig. 5d). The daphnids did not recover their previous ABR after complete removal of the toxins from the observation chamber in phase 3, and they

Table 1. Results of repeated measures ANOVAs for comparison of *Daphnia pulicaria* appendage beat rate (ABR) in different phases of experiments with different proportions (0, 20, 50, 80, 100%) of unicellular and colonial *Microcystis aeruginosa* added to *Scenedesmus obliquus.*

		P value							
		Unicellular *Microcystis*				Colonial *Microcystis*			
	df	Phase 1 vs. 2	Phase 1 vs. 3	Phase 1 vs. 4	Phase 3 vs. 4	Phase 1 vs. 2	Phase 1 vs. 3	Phase 1 vs. 4	Phase 3 vs. 4
0% *Microcystis*									
Treatment	1	0.1511	0.8005	0.2087	0.1008	0.1511	0.8005	0.2087	0.1008
Time	6	0.7678	0.4613	0.0093	0.1235	0.7678	0.4613	0.0093	0.1235
Time × Treatment	6	0.6574	0.9647	0.2489	0.0915	0.6574	0.9647	0.2489	0.0915
20% *Microcystis*									
Treatment	1	0.1161	0.4719	0.5790	0.2024	0.2547	0.0118	0.8048	0.0657
Time	6	0.0203	0.0003	0.6098	0.1062	0.4144	0.8327	0.7949	0.5633
Time × Treatment	6	0.1222	0.0941	0.0013	0.1220	0.3260	0.4578	0.3608	0.2230
50% *Microcystis*									
Treatment	1	0.4158	0.6507	0.2845	0.0685	0.1015	0.0797	0.0238	0.9933
Time	6	0.0184	0.6171	0.5103	0.0305	0.3034	0.9272	0.9563	0.8322
Time × Treatment	6	0.3502	0.0017	0.0572	0.2422	0.6978	0.3144	0.8646	0.8763
80% *Microcystis*									
Treatment	1	0.0861	0.3552	0.9775	0.3350	0.0025	0.0066	0.0225	0.5589
Time	6	0.8865	0.2423	0.8772	0.7865	0.0001	0.0003	0.0385	0.0020
Time × Treatment	6	0.1315	0.8568	0.2045	0.4219	0.0045	<0.0001	0.0401	<0.0001
100% *Microcystis*									
Treatment	1	0.1250	0.3722	0.5707	0.1424	0.9676	0.3248	0.3259	0.6091
Time	6	0.0982	0.0232	0.0105	0.6505	0.0995	0.0631	0.8828	0.0077
Time × Treatment	6	0.0537	0.1002	0.3188	0.1508	0.8861	0.2857	0.1821	0.4700

maintained their ABR ~30% lower compared with phase 1 (Fig. 5d). At 5,000 ng ml^{-1}, the comparison of phase 2 with phases 1 and 3 showed that differences were statistically significant (Table 3). In contrast, the animals showed some indication of low ingestion rate as MMR declined slightly for the 50- and 500-ng ml^{-1} treatments when compared with no addition of microcystin (Fig. 5a–c). The decline in MMR was visible during phase 3 at 50 ng ml^{-1}, as shown by the statistically significant difference between phase 3 and the other two phases (Fig. 5b; Table 3). At 500 ng ml^{-1}, there was a trend of decline in MMR during the first 9 min during phase 2; however, the animals recovered their previous MMR for the remaining duration of the experiments (Fig. 5c). This behavior was supported by statistical evidence; the interaction term (time × treatment) was highly significant when phases 1 and 2 were compared (Table 3). During phase 3, MMR remained slightly lower than the average of phase 1; however, this difference was not statistically significant (Fig. 5c; Table 3). The addition of 5,000 ng ml^{-1} produced a clear and statistically significant reduction in MMR during phases 2 and 3 compared with phase 1 (Fig. 5d; Table 3). It is important to note that the reduction in MMR occurred only 10 min after the addition of the toxins, whereas the ABR was reduced immediately after the addition of the purified toxins (Fig. 5d).

In the experiments with unicellular and colonial *Microcystis,* the daphnids responded to all the treatments from 20% to 100% by increasing their LRR significantly (Fig. 6a,b). In the absence of any *Microcystis* (cells or colonies), LRR were always <1 min^{-1} in all the experimental phases (Wilcoxon, $P > 0.05$), but as soon as the animals were exposed to the smallest proportion (20%), the LRR increased to 15 min^{-1} (Fig. 6). The difference in LRR between the treatment and the control phases was statistically significant for all the experimental conditions from 20% to 100% with unicellular and colonial *Microcystis* (Wilcoxon, $P < 0.05$). The addition of microcystin-LR produced an increase in LRR only for the highest concentration used, consistent with ABR and MMR responses (Fig. 6c). The difference between the treatment phase and the control phases was statistically significant only for the highest (5,000 ng ml^{-1}) microcystin addition (Wilcoxon, $P < 0.05$). At 5,000 ng ml^{-1}, the animals increased their LRR by a factor of five compared with the control phase in which no toxins were present. However, this increase was not as high as in the experiments with unicellular or colonial *Microcystis,* in which LLR increased by >20 times (Fig. 6a,b). It is important to note that the increase in LRR was not proportional to the amount of *Microcystis* added (Fig. 7a). Both for unicellular and colonial *Microcystis,* LRR did not increase in magnitude as the proportion of added *Microcystis* increased from 20% to 100% (Fig. 7a). However, there was a marked difference between the response of the animals to unicellular or colonial *Microcystis* additions. Overall, LRR was 33% higher (ANOVA, $P < 0.001$) in the presence of unicellular *Microcystis* (Fig. 7a). There was no sign of correlation between the overall labral

Table 2. Results of repeated measures ANOVAs for comparison of *Daphnia pulicaria* mandibular movement rate (MMR) in different phases of the experiments with different proportions (0, 20, 50, 80 100%) of unicellular and colonial *Microcystis aeruginosa* added to *Scenedesmus obliquus.*

		P value							
		Unicellular *Microcystis*				Colonial *Microcystis*			
	df	Phase 1 vs. 2	Phase 1 vs. 3	Phase 1 vs. 4	Phase 3 vs. 4	Phase 1 vs. 2	Phase 1 vs. 3	Phase 1 vs. 4	Phase 3 vs. 4
0% *Microcystis*									
Treatment	1	0.6290	0.8097	0.9105	0.3225	0.6290	0.8097	0.9105	0.3225
Time	6	0.3302	0.6514	0.1620	0.9465	0.3302	0.6514	0.1620	0.9465
Time × Treatment	6	0.1363	0.1945	0.4504	0.4723	0.1363	0.1945	0.4504	0.4723
20% *Microcystis*									
Treatment	1	0.0025	0.1871	0.2297	0.0960	0.0893	0.8094	0.1574	0.5435
Time	6	0.6518	0.9870	0.8798	0.7005	0.2743	0.8786	0.1805	0.4229
Time × Treatment	6	0.7265	0.9193	0.9752	0.9854	0.8079	0.2899	0.7871	0.2115
50% *Microcystis*									
Treatment	1	0.0006	0.0123	0.0038	0.0083	0.1237	0.6721	0.1226	0.1129
Time	6	0.4685	5639	0.0287	0.0500	0.4237	0.4675	0.9975	0.0621
Time × Treatment	6	0.4344	0.1001	0.8846	0.3050	0.9621	0.2081	0.2009	0.2981
80% *Microcystis*									
Treatment	1	0.0117	0.2425	0.0293	0.0044	0.1235	0.3321	0.0212	0.0464
Time	6	0.1129	0.0179	0.2882	0.1801	0.2787	0.3279	0.9519	0.2616
Time × Treatment	6	0.5480	0.5858	0.6324	0.1313	0.1836	0.3328	0.4632	0.8857
100% *Microcystis*									
Treatment	1	0.0033	0.3129	0.0275	0.0148	<0.0001	0.1007	<0.0001	<0.0001
Time	6	0.3439	0.2753	0.7954	0.4918	0.0013	0.9483	0.1466	0.1512
Time × Treatment	6	0.1866	0.4880	0.6282	0.1611	0.4365	0.0006	0.2317	0.1854

response and the amount of microcystin-LR added to the food mixture (Fig. 7b)

Discussion

The present experiments revealed contrasting patterns of feeding inhibition in *D. pulicaria* in the presence of single cells or colonies of *M. aeruginosa* or of purified microcystin-LR in their food. The daphnids, when presented a food mixture containing unicellular *Microcystis,* did not reduce their ABR significantly; however, food ingestion was reduced very quickly. These observations indicate that the animals were able to detect the presence of a small proportion of *Microcystis* cells (i.e., 20%) in their food suspension and to reduce their food ingestion. Because the *Microcystis* and *Scenedesmus* cells have comparable size and shape, it is unlikely that daphnids could detect *Microcystis* cells on the basis of their size or shape. Our findings suggest that *D. pulicaria* might have the ability to discriminate between food particles on the basis of their ''taste,'' which contrasts with the assumption that cladocerans are usually referred to as lacking tasting abilities compared with other zooplankton groups such as calanoids (DeMott 1986, 1988; Kerfoot and Kirk 1991). Several studies have clearly shown that daphnids filter unselectively over a wide range of particle sizes and shapes (i.e., bacteria, diatoms, or plastic beads) consistent with their passive sieving feeding mechanism; however, assimilation tends to decline for large particles (*see* review in Lampert 1987).

The fast reduction in MMR and not in ABR suggests that the toxic content of the cell might not be the reason for the change in *Daphnia* feeding behavior. A more plausible explanation of this inhibition would be the presence of a chemosensorially detectable factor (or a bad taste factor) on the surface of the cell as suggested by previous studies (Lampert 1982; Henning et al. 1991; Jungmann 1992). The inhibition of food ingestion caused by *Microcystis* single cells was easily reversible; the animals regained their previous MMR in phase 3, weakening the likelihood of the microcystin poisoning hypotheses. Our results are in line with previous studies, which found no relationship between the amount of toxins in *Microcystis* cells and their inhibitory effect on *Daphnia* (Nizan et al. 1986; Rohrlack et al. 1999*a*,*b*; DeMott et al. 2001). The presence of *Microcystis* single cells caused an important increase in LRR, confirming the feeding inhibition demonstrated by the decrease in MMR. The increase in LRR was easily reversible, suggesting that the animals were not intoxicated by the presence of the single cells. There was no apparent correlation between the proportion of *Microcystis* added and LRR, suggesting that the animals react to the presence of a small or a large number of *Microcystis* cells in the same manner. These results are consistent with previous findings by Lampert (1981*b,* 1982) in which *Daphnia* was able to rapidly detect the presence of *Micro-*

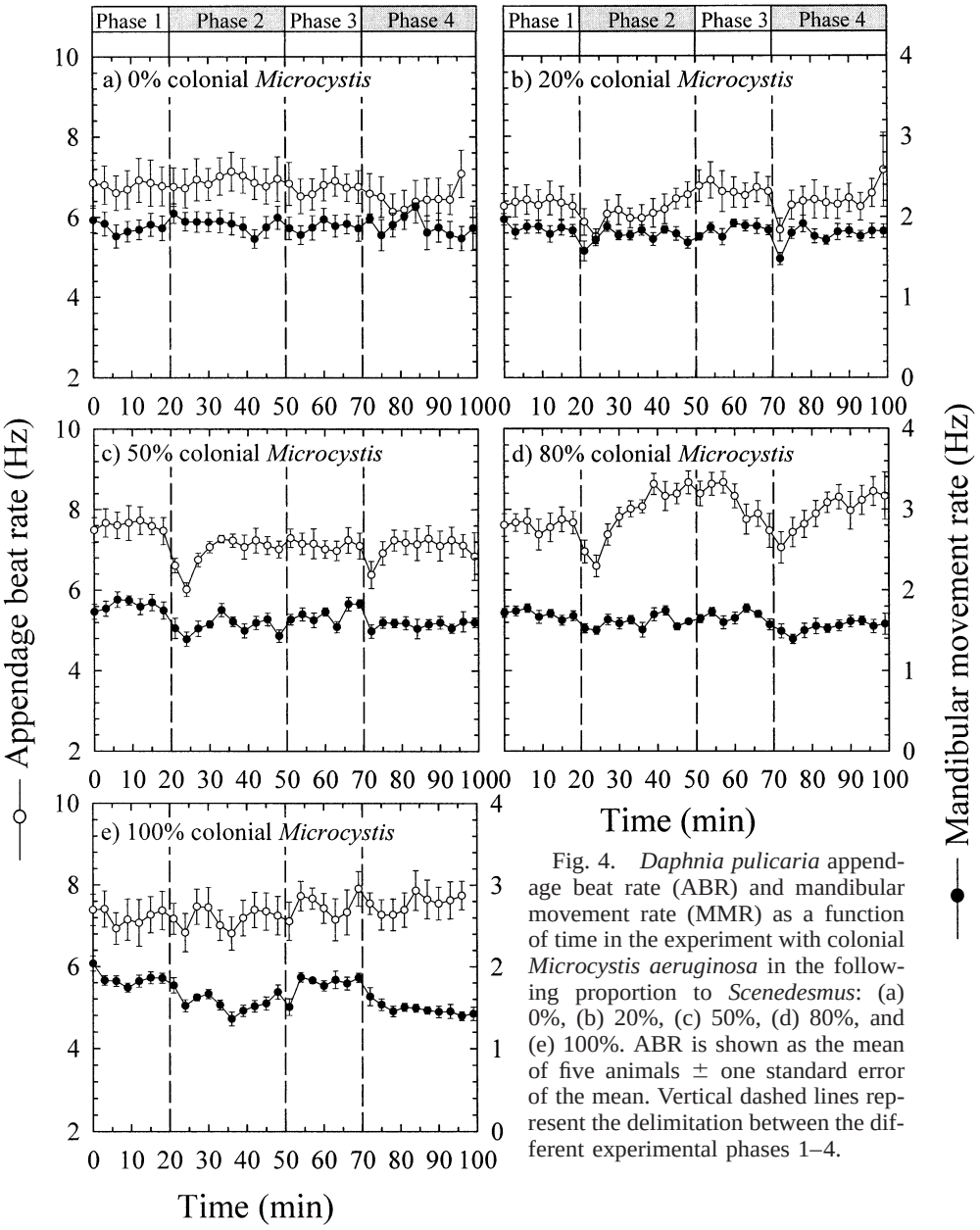

Fig. 4. *Daphnia pulicaria* appendage beat rate (ABR) and mandibular movement rate (MMR) as a function of time in the experiment with colonial *Microcystis aeruginosa* in the following proportion to *Scenedesmus*: (a) 0%, (b) 20%, (c) 50%, (d) 80%, and (e) 100%. ABR is shown as the mean of five animals ± one standard error of the mean. Vertical dashed lines represent the delimitation between the different experimental phases 1–4.

cystis and stop feeding, but eventually continue feeding activities after leaving the *Microcystis* patch. The strong reaction of *Daphnia* to the presence of unicellular *Microcystis*, even at low concentrations, confirmed the previous suspicion that the rejection reaction is chemically mediated and is based on the animal's chemosensory abilities (Lampert 1982; Porter et al. 1982). The immediate recovery displayed by the daphnids in all the cases could well be an indication of their chemosensory ability. It is unlikely that these remarkably fast responses (reaction and recovery) could be explained by the presence of microcystins in the cells. Typically, daphnids not only have to ingest the cells, but they have to digest them for the toxins to be released, which is likely to be a slow process. Furthermore, recent studies found no difference in daphnid response to the presence or absence of microcystins in a genetically engineered *Microcystis* strain (Lürling 2003).

In contrast with unicellular *Microcystis*, the presence of colonies in *Daphnia* food suspension produced a more complex pattern of feeding inhibition. The ABR decreased when in contact with colonies in the very first minutes of treatment phases 2 and 4 and increased to reach sometimes even higher ABR compared with the control phases. At the same time, the animals increased their LRR substantially. This observation suggests that an increase in the rejection rate prevented the animals from gathering food, and as the food gathering decreased, the animals were trying to compensate by increasing their ABR. The lack of reduction in MMR at 20–80% of colonial *Microcystis* might be an indication that the animals were maintaining their ingestion rate high to

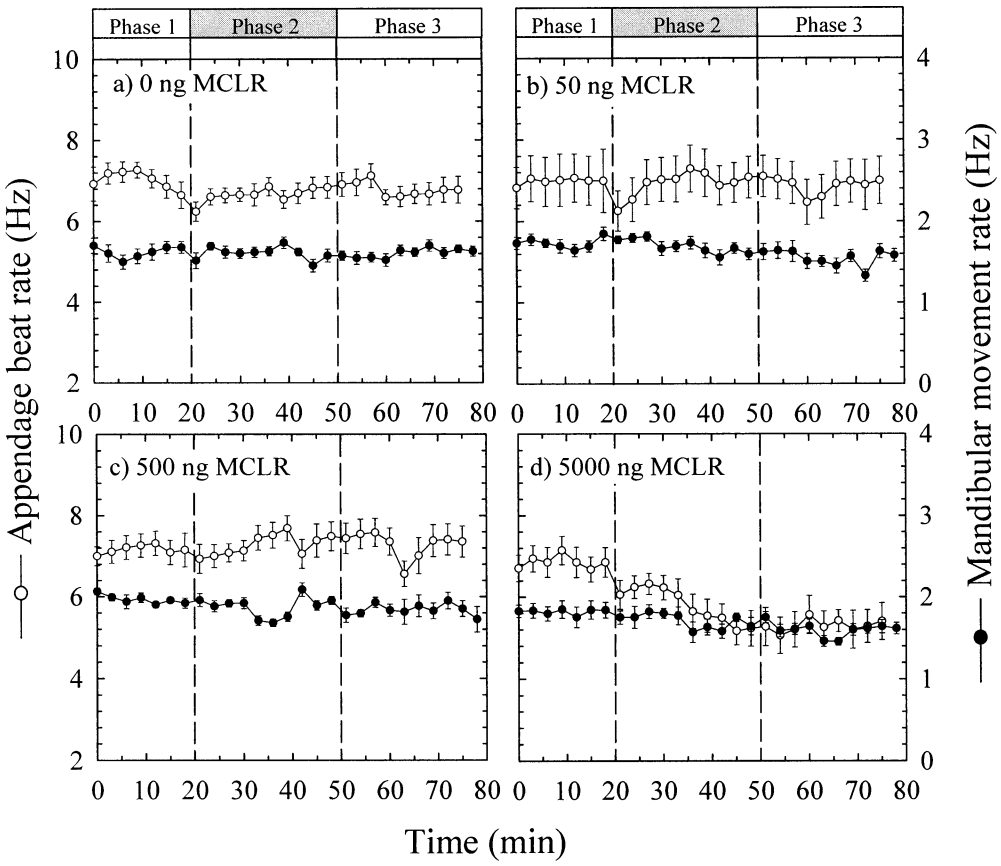

Fig. 5. *Daphnia pulicaria* appendage beat rate (ABR) and mandibular movement rate (MMR) as a function of time in the experiment with purified microcystin-LR added to *Scenedesmus* in the following concentrations: (a) 0, (b) 50, (c) 500, and (d) 5,000 ng ml^{-1}. ABR is shown as the mean of five animals ± one standard error of the mean. Vertical dashed lines represent the delimitation between the different measurement phases 1–3.

compensate for their hunger state. In pure colonial *Microcystis* suspensions, the animals did not change their ABR but rather reduced significantly their MMR, which is in contrast with their response at 20–80%. One possible explanation could be that the animals reduced their feeding as a response to the absence of edible food and started rejecting the colonies. These results suggest that the presence of *Microcystis* colonies (>30 μm) produced a typical feeding interference reaction in *Daphnia*. These patterns could not be solely attributable to the simple presence of colonies as opposed to single cells because there could be also a marked difference in the biochemical proprieties between the two strains. However, the high frequency of labral rejections is likely to prevent the animals from gathering food particle; hence, the biochemical properties might play only a marginal role. To compensate for the lack of food, the animals tried to increase their ABR to higher rates and to maintain a stable MMR. This could be clearly an indication of a state of hunger. The reversibility of this behavior points toward a feeding interference rather than an intoxication reaction of *Daphnia*. Previous studies by Webster and Peters (1978), and more recently by DeMott et al. (2001), described similar sensitivities in *Daphnia* exposed to cyanobacteria filaments.

Although there was no clear response of *Daphnia* to the addition of small amounts of microcystins (50–500 ng ml^{-1}), high concentration of 5,000 ng ml^{-1} caused a nonreversible decrease in ABR and MMR and a stimulation of LRR. These observations suggest that *Daphnia* can respond to the presence of dissolved toxins by modifying their feeding behavior. The decrease in ABR and MMR occurred only 10 min after the beginning of phase 2, suggesting that the changes are indicative of a possible intoxication reaction. It is important to note that the concentrations used in these experiments are several orders of magnitude lower than the LC_{50}s reported in the literature (DeMott et al. 1991; Jungmann 1992). In natural systems, daphnids are more likely to be exposed to relatively low concentrations of dissolved toxins, except when blooms are senescent (Christoffersen 1996). Our results provide evidence that low or nonlethal concentrations of dissolved microcystin could cause significant feeding inhibition which in turn could impair the growth, development, and survival of *Daphnia*. However, the mechanisms involved in the low-dose intoxication of *Daphnia* remain to be elucidated. There is some evidence for the possibility of inhibition after dissolved cyanotoxins come into contact with motor nerves controlling the feeding append-

Table 3. Results of repeated measures ANOVAs for comparison of *Daphnia pulicaria* appendage beat rate (ABR) and mandibular movement rate (MMR) in different phases of the experiments with different amounts of purified microcystin-LR (0, 50, 500, and 5,000 ng ml^{-1}) added to *Scenedesmus obliquus* suspensions.

		P value					
		Appendage beat rate			Mandibular movement rate		
	df	Phase 1 vs. 2	Phase 2 vs. 3	Phase 1 vs. 3	Phase 1 vs. 2	Phase 2 vs. 3	Phase 1 vs. 3
0 ng Microcystin-LR							
Treatment	1	0.1309	0.0782	0.3019	0.8945	0.9697	0.9048
Time	6	0.0650	0.1703	0.2630	0.0655	0.0272	0.2025
Time × Treatment	6	0.0810	0.0757	0.0583	0.0114	0.1354	0.0357
50 ng Microcystin-LR							
Treatment	1	0.8348	0.3267	0.6724	0.1882	0.0024	0.0002
Time	6	0.5514	0.0546	0.1171	0.0418	<0.0001	0.0219
Time × Treatment	6	0.3725	0.1798	0.2855	0.0466	0.4969	0.1177
500 ng Microcystin-LR							
Treatment	1	0.2617	0.4970	0.6796	0.1969	0.7733	0.5793
Time	6	0.0239	0.0057	0.0634	0.0003	0.0001	0.0058
Time × Treatment	6	0.4267	<0.0001	0.0002	<0.0001	0.1478	0.2906
5000 ng Microcystin-LR							
Treatment	1	0.0057	0.0015	0.0043	0.0486	0.0059	0.0020
Time	6	0.0599	0.0007	0.6125	0.2400	0.0026	0.2202
Time × Treatment	6	0.0386	<0.0001	0.4351	0.0908	0.1298	0.4381

ages (Haney et al. 1995). Our results support this hypothesis when we consider ABR and MMR, which have declined following exposure to dissolved microcystins. However, it is still unclear why these neuromotor inhibitions have not affected labral movement in the same way as the filtering appendages and the mandibles. Certainly, the persistent nature of the feeding inhibition, even after the complete removal of the toxin, supports the intoxication hypothesis. Daphnids have been shown to display similar feeding depression after short-term exposure to sublethal doses of a whole range of metal and organic contaminants (Gliwicz and Sieniawska 1986; Hatch and Burton 1999; McWilliam and Baird 2002). We should also point out that our experiments were conducted only with microcystin-LR, which is only representative of the microcystin family. Other cyanotoxins, such as anatoxin-a and saxitoxins, also produced by common cyanobacterial species, could possibly have different effects on daphnids (e.g., Haney et al. 1995).

Our results demonstrated that *D. pulicaria* reacted in different ways to the addition of *Microcystis* single cells, colonies, or secondary metabolites; however, in the three cases, the animals displayed feeding impairment. This study provided new insights into the mechanisms of feeding inhibition produced by the different forms of *Microcystis* that could be

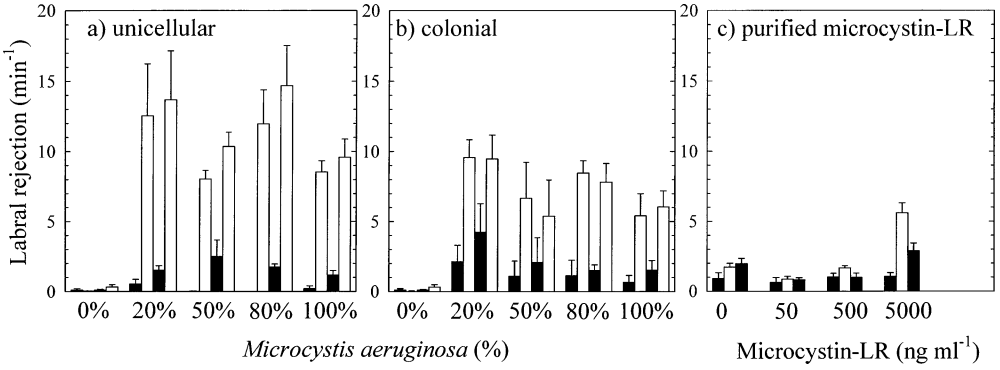

Fig. 6. *Daphnia pulicaria* labral rejection (min^{-1}) exhibited by animals exposed to (a) 0%, 20%, 50%, 80%, or 100% of unicellular *Microcystis aeruginosa* to *Scenedesmus*; (b) 0%, 20%, 50%, 80%, or 100% of colonial *M. aeruginosa* to *Scenedesmus*; and (c) 0, 50, 500, and 5,000 ng ml^{-1} of purified microcystin-LR added to *Scenedesmus.* Bars represent the mean of the labral rejection rate of five animals during phases 1–4 for the experiments with unicellular and colonial *M. aeruginosa* and phases 1–3 for the experiment with purified microcystin-LR. Error bars represent one standard error of the mean. Solid bars represent phases 1 and 3, and open bars represent phases 2 and 4.

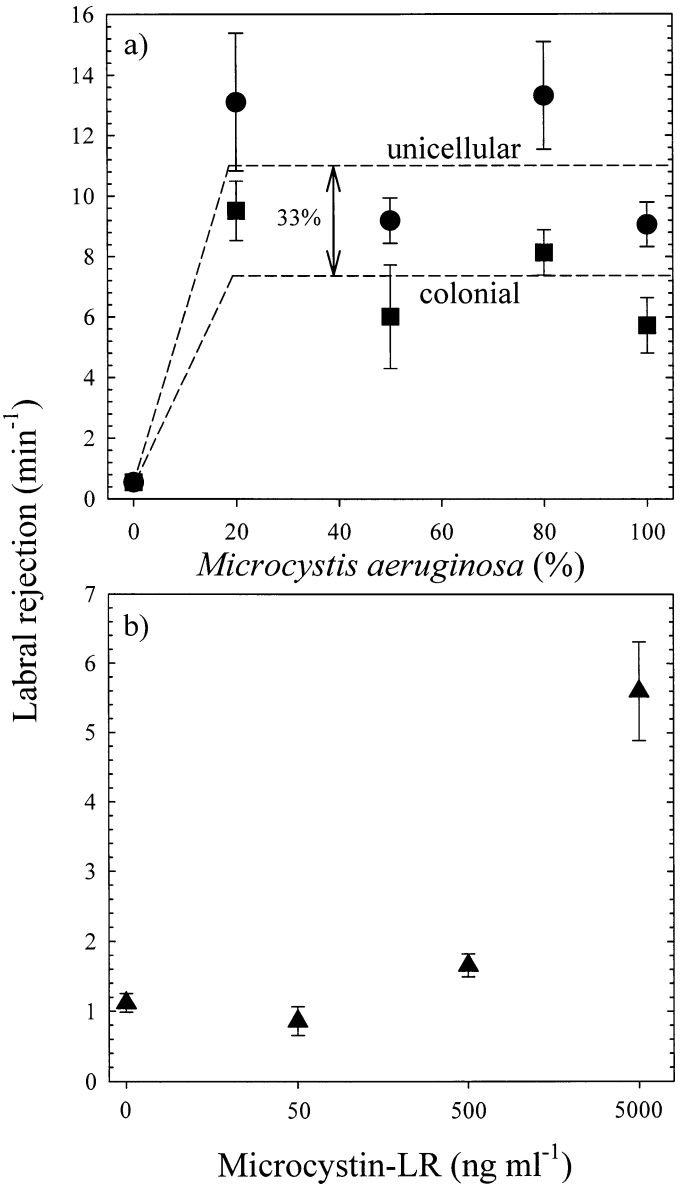

Fig. 7. Overall *Daphnia pulicaria* labral rejection rates after the addition of different proportions of (a) unicellular (solid circles) or colonial (solid square) *Microcystis aeruginosa* and (b) purified microcystin-LR. Error bars represent one standard error of the mean.

extended to filamentous and other colonial cyanobacterial species. In view of the results of this study, we speculate that the feeding inhibition might occur in steps, whereby daphnids chemosensorially detect the presence of cyanobacteria in their environment, reduce or possibly stop feeding, and actively reject the colonies, filaments, or both. In natural systems, this means that daphnids could possibly avoid cyanobacterial patches after detecting their presence, possibly through a bad taste factor independent of microcystins. Provided that the bloom is still at an early developmental stage and cyanobacteria biomass is relatively low, daphnids could possibly build dense populations between those patches, taking advantage of the typical spatially heterogeneous distribution of cyanobacteria. However, their survivorship could be compromised as the proportion of cyanobacteria increases (Reinikainen et al. 1994). Field observations and large-scale in-lake experiments have shown that populations of large cladocerans decline significantly in response to increased cyanobacterial biomass following bloom events (Ghadouani et al. 1998, 2003). From the ecological point of view, it is more likely that the chemosensorially triggered feeding inhibition would have a potentially more important (and faster) effect on daphnids populations than the toxins contained inside cyanobacterial cells. However, if the toxins are released in the water at high concentrations following the lysis of cyanobacterial cells, this study shows that the daphnids could become intoxicated. Although the negative effect of cyanobacteria on *Daphnia* is consistent across numerous studies, there is a wide variation of sensitivity between *Daphnia* species and clones to different strains. In natural systems, more than one strain of the same species is present during algal blooms. Hence, variability in the biochemical composition (including toxin forms) of those strains could contribute to the differences in sensitivity by *Daphnia.* It is also possible for *Daphnia* species to evolve some resistance to low concentrations of cyanobacteria through a mechanism of natural selection, as it has been shown recently (Hairston et al. 2001). We argue that ecophysiological studies such as this one and others (Rohrlack et al. 1999*a*,*b*; Lürling 2003), in combination with a good understanding of the ecology and evolution of zooplankton communities, would help improve our predictive ability as to the role played by harmful algal blooms in waterbodies.

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