Midsummer decline of a *Daphnia* population attributed in part to cyanobacterial capsule production

MAGGIE TRABEAU, ROBERTA BRUHN-KEUP, COLLEEN McDERMOTT*, MELANIE KEOMANY, ANNE MILLSAPS, ANISSA EMERY[1] AND BART DE STASIO JR[1]

DEPARTMENT OF BIOLOGY AND MICROBIOLOGY, UNIVERSITY OF WISCONSIN-OSHKOSH, OSHKOSH, WI, USA AND [1]DEPARTMENT OF BIOLOGY, LAWRENCE UNIVERSITY, APPLETON, WI, USA

*CORRESPONDING AUTHOR: mcdermot@uwosh.edu

A midsummer decline in a Daphnia population has been documented in Lake Winnebago, Wisconsin, USA, similar to that which typically occurs in other freshwater lakes throughout the world. We investigated the role of two products of the cyanobacterium Microcystis aeruginosa (microcystin and polysaccharide capsular matrix) in contributing to this population decrease by utilizing laboratory and lake studies. Feeding on either the microcystin-producing, minimally encapsulated M. aeruginosa strain PCC 7820 or the highly encapsulated, non-microcystin-producing strain C3-40, resulted in decreased survival when compared with animals fed chlorophytes. The survivorship of Daphnia fed C3-40 cells washed to remove the capsule increased by 30% over that seen in animals fed encapsulated C3-40. Feeding purified microcystin or capsule alone decreased Daphnia survival to a greater degree than did starvation. Physiological studies conducted while Daphnia were exposed to these food sources showed that ingestion of capsular material resulted in increased post-abdominal claw movements and decreased mandibular movements. Concurrently, elevated respiration rates were measured in Daphnia, implying that capsule increased the energy expended by these animals through increased attempts to reject the material and decreased food intake. Lake studies reflected the results of the laboratory experiments. The midsummer decline of the Daphnia population occurs as the Microcystis biovolume increases and both microcystin and capsular matrix levels rise. While both cyanobacterial products may contribute to the midsummer decline in Daphnia pulicaria, laboratory studies suggest that encapsulation may play the greater role.

INTRODUCTION

One of the most widely reported features of the population dynamics of large cladocerans is a midsummer decline in abundance [e.g. (Threlkeld, 1979; Sommer *et al.*, 1986; Boersma *et al.*, 1996)]. Following population growth in spring and early summer many *Daphnia* populations decline rapidly and remain at low or undetectable densities throughout the summer. Most studies have examined changes in biological conditions during midsummer declines of *Daphnia*. The decline has been attributed to a number of factors, including poor food availability (Threlkeld, 1985) or quality (Lampert *et al.*, 1986; Hein *et al.*, 1993), a combination of poor feeding conditions and increased invertebrate predation (Luecke *et al.*, 1990; De Stasio *et al.*, 1995; Mehner *et al.*, 1998), or increased predation by fish (Mills and Forney, 1983; Rudstam *et al.*, 1993). No single mechanism for this phenomenon has yet been ascertained, but one ubiquitous factor appears to be the increase of cyanobacterial populations during the period of midsummer declines (Sommer *et al.*, 1986). *Microcystis aeruginosa*, a unicellular cyanobacterium, is a major species of the summer phytoplankton assemblages that typically occur in lakes throughout the world (Reynolds, 1984, 1997; Graham and Wilcox, 2000). While some cladocerans are known to be able to feed and survive when *M. aeruginosa* is abundant (Fulton, 1988; Hanazato, 1991; Epp, 1996), *Daphnia* feeding abilities, reproduction and survivorship are typically severely decreased in the presence of *Microcystis* (Porter, 1977; Hietala *et al.*, 1995; Reinikainen *et al.*, 1995; Rohrlack *et al.*, 1999b; Ferrao-Filho *et al.*, 2000).

Many genera of cyanobacteria produce either hepato-toxic or neurotoxic secondary metabolites. Some strains of *M. aeruginosa* produce potent hepatotoxins, the microcystins (Carmichael, 1988a). Microcystins, contained within cyanobacterial cells and usually only released upon lysis or changing cell wall permeability, have the ability to inhibit protein phosphatases in a variety of mammalian cells. These cyanotoxins have been responsible for acute liver necrosis and deaths in wildlife, domesticated animals, and humans (Gorham and Carmichael, 1988; Carmichael *et al.*, 2001). The effects of microcystins on zooplankton grazers are less well understood. Different strains of cyanobacteria within the same species may have differing toxic effects on grazers (DeMott *et al.*, 1991; Schaffner *et al.*, 1994; Ferrao-Filho *et al.*, 2000). In addition, some 'toxic' strains of cyanobacteria may not be toxic to all populations of the same species of zooplankton such as *Daphnia* (Gilbert, 1990; Epp 1996; Ferrao-Filho *et al.*, 2000). The ingestion rate of *M. aeruginosa*, as well as its cellular microcystin content, may play an important role in the degree of damage that occurs in *Daphnia* (Rohrlack *et al.*, 1999a). In addition, there may exist an even more complicated relationship between predator and prey given the possibility of evolved physiological resistance to toxins by local populations or clones of daphnids (Epp, 1996; Walls and Ventela, 1998).

The morphology of phytoplankton cells and arrangements of cells into filaments or colonies has been shown to affect grazing rates by zooplankton significantly, especially *Daphnia* (Porter, 1977; Lampert, 1987; Hein *et al.*, 1993). Filamentous algae, in particular, cause mechanical interference with feeding by clogging the filtering combs, necessitating clearing of the combs by a sweeping motion of the post-abdominal claw [i.e. the abreptor (Porter and Orcutt, 1980; Lampert, 1987; Fryer, 1991)]. Non-filamentous colonial algae may also interfere with feeding mechanics as a result of the production of a gelatinous mucus, consisting of a polysaccharide matrix (Porter, 1977). Production of a polysaccharide capsule matrix has been documented in many cyanobacteria (Prescott, 1970, 1978), with different sugar residues comprising the capsule matrix depending on genus (Bender *et al.*, 1994; Nicolaus *et al.*, 1999; De Philippis *et al.*, 2000). Environmental factors, such as metal ion concentrations, may change the amount of polysaccharide capsule produced by cyanobacteria (Bender *et al.*, 1994). *Microcystis* species produce capsules composed of galacturonic acid, rhamnose, xylose, and other minor sugar components (Nakagawa *et al.*, 1987). Differences in sugars have been shown to accompany differences in capsule morphology (Plude and Parker, 1991). The amount of capsule produced also differs between members of this genus. Capsule may surround *Microcystis*

unicells or may aggregate numerous unicells into a colonial structure (Prescott, 1970; Doers and Parker, 1988).

The role of the polysaccharide capsule of aquatic micro-organisms has been debated, and is probably diverse. In photosynthetic organisms the capsule can function as a light transmitter, providing light energy to organisms located deep within a microbial mat (Flemming and Wingender, 2001). The capsule may be involved in the sequestration of nutrients and allow these colonial single-celled organisms to function as a multi-cellular unit (Flemming and Wingender, 2001). The presence of this 'sticky' polysaccharide covering may also affect the ability of zooplankton grazers to ingest and assimilate cyanobacteria. Algae and bacteria surrounded by a polysaccharide capsule are less efficiently digested than non-encapsulated cells (Porter, 1977; Decho and Lopez, 1993), at least for some strains of bacteria (Plante, 2000). Colony-forming strains of *Microcystis* may mechanically hinder the maxillules of *Daphnia*, decrease the maxillary beat rate, decrease swallowing, and impede food transport efficiency (Rohrlack *et al.*, 1999b).

The polysaccharide capsule has also been postulated to have an interaction with microcystin in producing toxicity to *Daphnia galeata*. It has been shown that microcystin-producing *M. aeruginosa* strains that also produce a mucilage sheath are toxic to *Daphnia* (Rohrlack *et al.*, 1999a). This implies that microcystin absorption is not impeded by the presence of the polysaccharide capsule.

Our study investigates the effects of the polysaccharide capsule matrix surrounding 'colonial' cyanobacteria as well as the biotoxin microcystin on *Daphnia pulicaria* survivorship, physiology, and behaviour. First, a series of laboratory feeding studies was utilized to determine the detrimental or beneficial effects of polysaccharide capsule and/or microcystin on *D. pulicaria* from Lake Winnebago, WI, USA. Second, seasonal data were collected from Lake Winnebago to determine if production of these cyanobacterial substances was correlated with the midsummer decline in *Daphnia* that has been observed in this water system every year for the past decade (Steeves, 1996; De Stasio *et al.*, 2000). In both instances, the polysaccharide capsule produced by the cyanobacteria *Microcystis* appears to be linked with decreases in the *Daphnia* population, while microcystin has a lesser effect on *Daphnia* survival.

METHOD

Laboratory studies

Cyanobacteria cultures
Cultures of *M. aeruginosa* PCC 7820 and *M. aeruginosa* C3-40 were maintained in modified Jansen's medium (Parker,

1982) for use in capsule harvest and feeding experiments. All cultures were kept at ambient temperature ($\sim$25°C) under cool white fluorescent lighting at $\sim$15 μmol m^{-2} s^{-1} and manually swirled daily to ensure gaseous exchange. *Microcystis aeruginosa* PCC 7820 is known to produce the cyanobacterial toxin, microcystin (Codd and Carmichael, 1982). However, this strain produces very little polysaccharide capsule matrix under these culture conditions (unicellular). *Microcystis aeruginosa* strain C3-40, in contrast, has never been shown to produce microcystin, but does produce large amounts of polysaccharide capsule (colonial) (Plude and Parker, 1991).

Polysaccharide capsule harvest

Cultures of *M. aeruginosa* strain C3-40 were centrifuged at 21 500 g for 20 min at 4°C. The cell pellet was resuspended in sterile deionized water to a volume that approximated the original volume of the culture, and held overnight at 4°C. Resuspended cells were centrifuged as above and the supernatant was saved. Washing was repeated until capsule could no longer be observed adhering to subsamples of the cells when examined with an India ink negative stain. Supernatants were pooled and filtered through a 5.0 μm nylon filter (MSI, Westborough, MA) and then through a 0.45 μm nylon filter. A 0.0274 M solution of cetyltrimethylammonium bromide (CTAB) (Sigma Chemicals, St Louis, MO) was added dropwise to the filtered capsule suspension until no additional precipitate was observed (Isobe *et al.*, 1992). Precipitated capsule was resuspended in 1.71 M NaCl, then reprecipitated in 95% ethanol. Precipitate was dialysed (3500 MW cut-off, Spectrum, Los Angeles, CA) with sterile deionized water at 4°C in the presence of 100-mesh Chelex resin (contained in 3500 MW cut-off dialysis tubing) (BioRad, Hercules, CA) to aid in the removal of divalent cations. Purified capsule was then lyophilized for storage until needed.

Daphnia cultures

Two sets of survivorship experiments were conducted. In 1999 *D. pulicaria* used in the survivorship studies were from laboratory cultures started with individuals collected from the lower Fox River (Appleton, WI, USA), 11 km below the Menasha Dam at the outflow of Lake Winnebago. Experiments conducted in 2000 used animals from cultures established with individuals from Lake Winnebago. Cultures were maintained at 22°C with a 14:10 h light:dark cycle. Experiments in 1999 employed individual *Daphnia* held in the laboratory for at least 48 h, whereas in 2000 stocks were maintained in the laboratory for at least two generations prior to the experiments. Previous studies have shown no significant differences in *Daphnia* size, feeding rates or genetic

structure between animals collected from the lower Fox River site and from Lake Winnebago [(Berman and Richman, 1974; Steeves, 1996) De Stasio, unpublished data]. Animals in culture were fed a mixture of chlorophytes daily at a concentration of $\geq$10^5 cells mL^{-1}. In 1999 animals were fed a mixture of *Chlamydomonas reinhardi* (+) and *Scenedesmus quadricauda*, while in 2000 animals received a mixture of *C. reinhardi* and *Ankistrodesmus falcatus*. Algae were cultured in Bristol's solution (Starr, 1964) and grown under a 12:12 h light:dark regime in a controlled temperature environment at 22°C. Only cultures in the exponential phase of population growth were used in experiments. Culture medium was changed on alternate days, and population growth dynamics were monitored by cell counts using an Elzone (Model 282PC) electronic particle counter.

Survivorship studies

A series of feeding studies with adult *D. pulicaria* was conducted to determine the effects of cyanobacterial polysaccharide capsule, cyanobacterial cells and the cyanobacterial toxin microcystin on *Daphnia* survivorship in the laboratory. Treatments for the survivorship studies are shown in Table I. *Daphnia* were fed a variety of combinations of food sources with and without microcystin, and with and without polysaccharide capsule. These combinations were comprised of chlorophytes (*Chlamydomonas* or *Ankistrodesmus*) to serve as 'well-fed' controls, *M. aeruginosa* PCC 7820 (non-encapsulated, known microcystin-producing strain), *M. aeruginosa* C3-40 (non-microcystin producing, highly encapsulated strain), or no cyanobacteria or chlorophytes ('starved' controls). *Daphnia* were starved for 3 h prior to each experiment. Twenty animals were placed into 200 ml filtered (GF/C) lake water in glass beakers using a large-bore sterile Pasteur pipette. Individual treatment food supplies were added to each of three beakers containing *Daphnia*. *Daphnia* exposed to a treatment were kept at room temperature ($\sim$25°C) for 48 h. Dead *Daphnia* (not moving) were counted at the end of the experiment and percent survivorship was calculated. The starvation condition was assigned a per cent survival of zero, and results for all other treatments are reported as per cent differences from the starvation condition.

Daphnia physiological measurements

Measurements of *Daphnia* feeding behaviour, rejection rate and respiration rate were determined while exposed to a subset of the treatment conditions listed in Table I. Each animal ($n = 5$) was tethered to a fine dissecting pin during filming using 3M Pronto CA40 Instant Adhesive. Tethered individuals were held in 50 mL of each treatment solution and filmed with a Sony CCD Colour

Table I: Survivorship experiment treatment conditions

Food treatment	Pure microcystin[a] added	Polysaccharide capsule removed[b]	Purified polysaccharide capsule added[c]	Number of replicates (*n*)
None (starved)	No	–	No	11
None (starved)	No	–	Yes	11
None (starved)	Yes	–	No	5
M. aeruginosa[d] strain C3-40	No	No	No	8
M. aeruginosa[d] strain C3-40	No	Yes	No	11
M. aeruginosa[d] strain C3-40	Yes	No	No	5
M. aeruginosa[d] PCC 7820	No	–	No	11 / 3
M. aeruginosa[d] PCC 7820	No	–	Yes	11
Chlamydomonas control[e]	No	–	No	11 / 5
Chlamydomonas control[e]	No	–	Yes	8
Chlamydomonas control[e]	Yes	–	No	5

Daphnia pulicaria were exposed for 48 h to the treatments detailed in the table. Treatments were run in triplicate, unless otherwise noted.
[a]Purified microcystin–LR was purchased from Calbiochem, CA. Microcystin–LR was added to give a final concentration of 100 μg L^{-1}.
[b]Polysaccharide capsule was removed from encapsulated organism as described in Methods under Capsule harvest.
[c]Purified polysaccharide capsule was obtained from *Microcystis aeruginosa* strain C3-40 by deionized water washes and CTAB precipitation (see Methods: Capsule harvest). The amount of purified capsule added to each treatment was equivalent to the average amount of capsule removed from 1×10^6 cells of *M. aeruginosa* C3-40 growing under culture conditions.
[d]Twenty *Daphnia* were fed 10^9 cells L^{-1} (200 mL volume) of either *M. aeruginosa* PCC 7830 or *M. aeruginosa* strain C3-40.
[e]Well-fed controls (20 *Daphnia*) were fed *Chlamydomonas* at a concentration of 10^9 cells L^{-1} (200 mL volume).

Video Camera (Model DXC-107) attached to a horizontal Wild M-5A dissecting microscope. A Panasonic Multiplex Video Cassette Recorder using S-VHS mode was used to record behaviour, and activity time was recorded with a frame counter running at 30 frames s^{-1}. Animals were acclimated under experimental conditions for 5 min prior to filming sessions. Between feeding trials, the chamber was flushed with filtered (0.8 μm Millipore), aged tap water. Animals were filmed for 5 min on each food treatment and under starvation conditions (i.e. filtered aged water). To assess feeding behaviour, 1 min segments of feeding were analysed for number of mandible movements. Five minute segments of video were examined for post-abdominal claw (i.e. abreptor) movements as a measure of rejection rate. Mandible and abreptor rates are expressed as per cent difference from starved treatment rates.

Respiration rate was determined to evaluate differences in energy use when feeding on the different treatment conditions. Oxygen consumption was measured in 60 mL bottles with 20 adult female *Daphnia* exposed to each treatment using the ENDECO Pulsed Dissolved Oxygen System. In each experiment oxygen changes were determined simultaneously on duplicate treatment bottles and duplicate control bottles. Controls contained only the food suspension and were used to correct for algal and bacterial oxygen production and consumption. Changes in oxygen concentration were determined every 15 min over a 6 to 8 h period. Bottles were incubated in the dark at 22°C during the experiments.

Dry weight of *Daphnia* biomass in each respiration bottle was determined by removing all individuals with a pipette, rinsing animals through four successive containers of filtered, aged tap water, and then pipetting them onto pre-weighed and dried filter paper disks. Animals were dried at 55°C for 24 h before determining mass using a Cahn Ultramicrobalance (Model C-35). Respiration rates in each food treatment were determined from regression analysis of changes in dissolved oxygen concentrations in the chambers. Rates were expressed as mL O$_2$ g^{-1} animal h^{-1} using corrected oxygen consumption rate and animal biomass estimates for each bottle.

Lake studies (Lake Winnebago, WI)

Study site

Lake Winnebago is a large, shallow body of water located in northeastern Wisconsin (surface area 557 km^2, maximum depth 6 m, mean depth 4.7 m). Each year since 1992 the lake was sampled at least biweekly, but usually weekly, from May through August and less frequently during September and October. Sampling was conducted at an established sampling location near the northern end of the lake (N44°11′18″, W88°23′30″). The sampling site has a maximum depth of 5.2 m and is located at least 2 km from shore. On each sampling date physical and chemical limnological data were collected at 0.5 m intervals from the surface to the bottom of the lake. A Hydrolab DataSonde multiprobe was employed to measure depth, temperature, dissolved oxygen concentration, pH, conductivity, and turbidity. The multiprobe was calibrated weekly prior to each sampling event. Light penetration was measured as photon flux density determined at each depth with a LI-COR Model LI-1000 datalogger with 2π underwater quantum sensor and deck sensor. Water clarity was determined with a 20 cm diameter black and white Secchi disk.

Integrated water samples for phytoplankton identification and enumeration were collected with a 4 m length of plastic tubing (inner diameter 2.5 cm). The weighted end was lowered to a depth of 4 m and the upper end was fitted with an expanding stopper to create a vacuum. The lower end was quickly pulled to the surface with an attached rope, and the integrated sample was collected in a 1.5 L container. Phytoplankton samples were preserved in Lugol's iodine solution or 4% sugar–formalin solution. The zooplankton community was sampled with a Clark–Bumpus sampler fitted with a 153 µm mesh net. Replicate oblique tows were performed between 0 and 4 m depth, ensuring that at least 400 L water was filtered. Zooplankton samples were preserved in 4% sugar–formalin solution and examined at ×25 to ×50 magnification in a circular counting tray. Replicate subsamples were enumerated using a Wild M-5A dissecting microscope. Crustacean zooplankton were identified to species using standard taxonomic references (Balcer *et al.*, 1984; Pennak, 1989). *Daphnia* were identified using Hebert (Hebert, 1995), employing cellulose acetate electrophoresis for determination of *D. pulicaria* identity (De Stasio *et al.*, 2000).

Polysaccharide capsule measurements

The integrated samples of water from Lake Winnebago collected biweekly from June 9, 1999 to September 1, 1999 were preserved with Lugol's iodine solution to produce a final concentration of 1% (v/v) Lugol's. The preserved water sample was then centrifuged at 1650 *g* for 15 min to pellet the cells. Centrifugation plus the addition of Lugol's iodine causes the cyanobacterial gas vacuoles to burst and allows these organisms to sink. Samples of cyanobacteria were removed from the pellet and observed with a negative staining technique. A wet mount of the cyanobacteria was made using a 10% (v/v) India ink solution in a 1:1 ratio of cyanobacterial pellet to India ink solution. The wet mount was observed with a light microscope with a Whipple grid eyepiece. Capsule is observed as a clear area surrounding the green cyanobacterial cells (the area outside the encapsulation is grey because of the India ink). Five wet mount slides were prepared for each sample date. Each slide was observed in a predetermined pattern to view the entire area under the cover slip.

The amount of mucilage (capsule) surrounding the cells was calculated after detailed drawings (to scale) were made. The capsule area was determined by measuring the length and width of the encapsulations at their greatest distances (excluding any solitary finger-like protrusions). Encapsulation was expressed as the average area of encapsulations (µm^2) around the cyanobacteria present in 1 mL of integrated lakewater sample.

Cyanobacterial biovolume determination

Preserved water samples, as described above, were placed in an Ütermohl settling chamber (Phyco Tech, St Joseph, MI, USA) and organisms were allowed to settle for 48 h. At this time organisms were viewed with an inverted microscope and identified. Biovolume was determined by standard methods (American Public Health Association, 1998). Cell volume was determined by assigning the simplest geometric shape that best matched the shape of the cell (sphere, cylinder, etc.). Cyanobacterial biovolume was calculated by multiplying the average cell biovolume for that species by the number of that species mL^{-1}.

Microcystin production

The cyanobacterial biotoxin, microcystin, is an intracellular toxin released into water only when the cyanobacterium dies and/or lyses. When investigating the effects of microcystin on *Daphnia*, it is necessary to measure not only the toxin found in cell-free water but also the toxin that is located within viable cyanobacteria (potential for consumption of cyanobacterial whole cells by *Daphnia*). Intracellular microcystin was measured from aliquots of the cyanobacterial pellet obtained from integrated water samples. Cyanobacterial cell pellets were sonicated, lyophilized and weighed. An aliquot was then extracted with 0.833 M glacial acetic acid to free microcystin (Martin *et al.*, 1990). Microcystin concentration was determined by immunoassay, and was expressed as µg L^{-1} microcystin in lake water from which the pellet

was obtained. The immunoassay was a direct, competitive enzyme-linked immunosorbent assay (ELISA) utilizing anti-microcystin antibodies prepared by Chu *et al.* (Chu *et al.*, 1989). In some cases, a commercially available ELISA kit (EnviroLogix, Portland, ME, USA) for the detection of microcystin was used.

Statistical analysis

Data were examined for deviations from normality using normal probability plots of raw and transformed data. The square root transformation was applied to all data prior to statistical analysis. Data were back-transformed for graphical display. Single factor analysis of variance was employed to test the null hypothesis of equality of mean responses to each treatment. In cases where there was a significant effect of treatment on responses, Tukey's honestly significant difference (HSD) test was used for making multiple comparisons (Zar, 1984) and the statistical significance is indicated in parentheses. In cases where statistical significance was not found, but a trend was seen, no P-value is indicated. Data analyses were performed using SYSTAT (version 10).

RESULTS

Laboratory studies

Daphnia feeding studies
Daphnia fed green algae showed the greatest survivorship (117–172% greater than starved; Figure 1). Animals fed *M. aeruginosa* C3-40, a non-toxin producing, encapsulated cyanobacterium, also showed survivorship greater than that of starved animals (41.5% difference from starved). *Daphnia* fed *M. aeruginosa* PCC 7820 (the toxin-producing strain) survived better than did those exposed to the starvation condition (54% difference from starved). One-way analysis of variance of survivorship experiment 1 (Figure 1A) indicated that there was no significant effect of food type on survivorship ($F_{2,30} = 1.817$, $P = 0.1799$). In the second experiment (Figure 1B), there was a significant effect of food type on survivorship ($F_{4,15} = 64.230$, $P < 0.0001$). Survivorship in the three treatments employing microcystin was significantly lower than in the well-fed control (Tukey's test, df = 15, $P < 0.0001$ for each). Those animals fed purified microcystin alone (Figure 1B) had a lower survivorship than did the starved control animals (-22.5% difference from starved animals). Adding purified microcystin to the green algae treatment or to the non-toxic cyanobacteria (*M. aeruginosa* C3-40) resulted in a dramatic reduction in survivorship (-14 and -15.5% difference from starved, respectively).
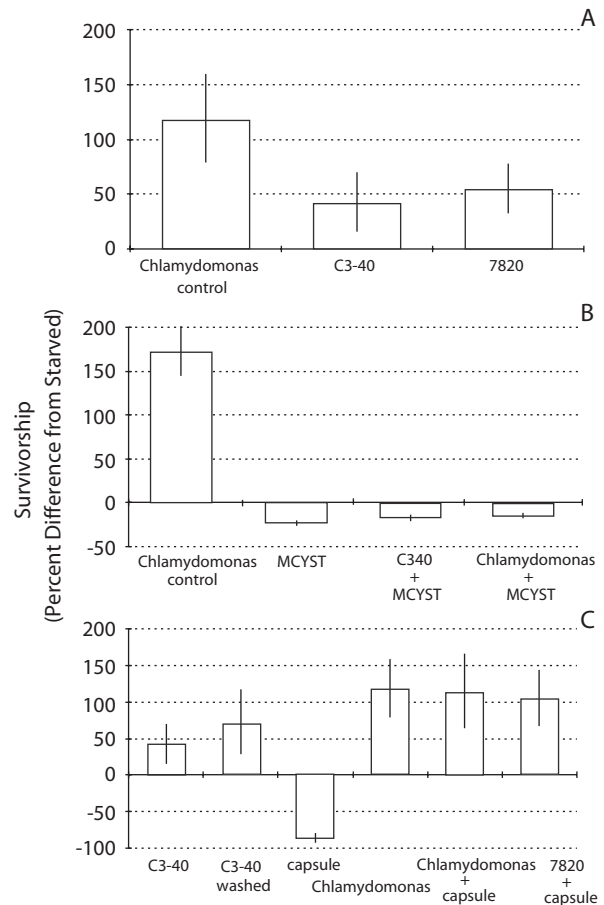
Fig. 1. Survivorship of *Daphnia pulicaria* exposed to various food sources for 48 h. The green alga *Chlamydomonas* was utilized as a 'well-fed' control, the cyanobacterium, *Microcystis aeruginosa* strain C3-40 was a non-toxic, highly encapsulated food source, and the *M. aeruginosa* PCC 7820 strain was a toxic, minimally encapsulated food source (**A**). Purified microcystin (MCYST) was added to non-toxic food sources, or fed alone (**B**). Purified polysaccharide capsule was removed from or added to food sources (**C**). In all instances, survivorship is expressed as percent difference from the starvation condition.

Not only did exposure to microcystin cause detrimental effects on *Daphnia* survival, but exposure to the polysaccharide capsule matrix also decreased the animals' ability to survive. *Daphnia* exposure to *M. aeruginosa* C3-40 with and without capsule present resulted in a significant effect of feeding treatment on survivorship (Figure 1C, $F_{6,64} = 8.791$, $P < 0.0001$). Feeding *M. aeruginosa* C3-40 washed to remove capsule resulted in an increase in the survivorship of *Daphnia* when compared with those fed encapsulated *M. aeruginosa* C3-40 (70% difference from starved vs. 41.5% difference from starved). Adding purified capsular material to the chlorophyte food resulted in a slight decrease in the survivorship of *Daphnia* (111.5% difference from starved) when compared to survivorship associated with chlorophyte feeding alone (117% difference from starved). Adding purified capsular material to the

M. aeruginosa PCC 7820 feeding did not cause a further decrease in survivorship of *Daphnia*. Instead, addition of capsule to the microcystin-producing cyanobacterium resulted in an increase in *Daphnia* survival compared to that associated with feeding *M. aeruginosa* PCC 7820 alone, or compared to the starvation condition. The most dramatic decrease in *Daphnia* survival resulted from feeding purified capsular material alone (−86.5% difference from starved). Based on Tukey's multiple comparison test, only animals fed purified capsule exclusively exhibited significantly lower survivorship compared to those in the well-fed treatment (df = 64, $P < 0.001$).

Daphnia physiological studies

Analysis of *Daphnia* feeding mechanics in the experiments demonstrated that the presence of capsule caused a decrease in feeding rate and an increase in movements associated with clearing capsule from the feeding apparatus (Figure 2). There was a significant effect of feeding treatment on both mandible rates ($F_{5,39} = 3.96$, $P = 0.005$) and abreptor sweeps ($F_{5,39} = 3.55$, $P < 0.01$). The number of mandible movements was significantly higher for animals feeding on the green alga *Chlamydomonas* compared to starved animals (>100% greater; df = 13, $P < 0.001$; Figure 2A). Feeding on the encapsulated *M. aeruginosa* C3-40 resulted in a significant decrease in mandible rate compared to animals fed *Chlamydomonas* (df = 11, $P < 0.001$). Separation of the capsule from the C3-40 cells demonstrates that the decrease in mandible rate was due to the capsule itself. Mandible rates for animals feeding on washed C3-40 were not significantly different from those of the *Daphnia* fed *Chlamydomonas* (df = 12, $P = 0.336$). However, feeding on purified capsular material alone resulted in significantly lower mandible rates compared to the *Chlamydomonas* treatment (df = 11, $P < 0.0001$), and even lower rates on average than those for starved animals (Figure 2A). *Daphnia* fed the toxic strain of *M. aeruginosa* PCC 7820 also exhibited decreased mandible rates compared to animals fed *Chlamydomonas* (df = 11, $P < 0.001$).

Consistent with these effects on mandible rates, the presence of capsular material resulted in increased use of the post-abdominal claw to clear the feeding groove (Figure 2B). Rate of abreptor sweeping when feeding on *Chlamydomonas* was not significantly different from that for starved animals (df = 18, $P = 0.25$). When feeding on the encapsulated *M. aeruginosa* C3-40, animals exhibited higher rates of abreptor sweeping than when feeding on *Chlamydomonas* (df = 8, $P = 0.057$). Removal of the capsule from C3-40 cells significantly decreased abreptor rates compared to those fed *Chlamydomonas* (df = 6, $P = 0.011$), as well as compared to the rates when animals were fed non-encapsulated cells of *M. aeruginosa* PCC 7820 (df = 11, $P = 0.013$).
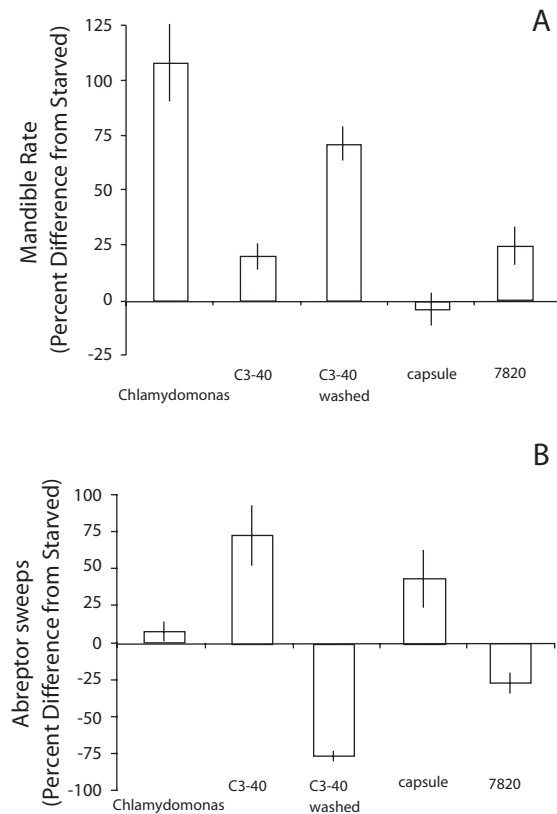

Fig. 2. Physiological studies of *Daphnia pulicaria* exposed to toxic and/or encapsulated *Microcystis aeruginosa* strains. Number of mandible movements (measure of feeding behaviour) was determined in 1 min segments of feeding (**A**). Abreptor sweeps (measure of rejection rate of food source) were counted for 5 min segments (**B**). In all instances, counts are expressed as per cent difference from the starvation condition. C3-40 indicates *M. aeruginosa* strain C3-40 (highly encapsulated) and 7820 indicates *M. aeruginosa* PCC 7820 (toxin producer). *Chlamydomonas* was fed to *Daphnia* to represent the 'well-fed' condition.

Respiration rates of *Daphnia* fed the encapsulated strain of *M. aeruginosa* C3-40 were significantly higher than those when animals were fed single-celled green algae (*Ankistrodesmus falcatus*; df = 2, $P = 0.013$; Figure 3). Removal of the capsular material resulted in significantly lower respiration rates compared to when animals were feeding on encapsulated cells (*M. aeruginosa* C3-40 vs. washed *M. aeruginosa* C3-40; df = 2, $P = 0.05$). Respiration rate when feeding on the washed *M. aeruginosa* C3-40 was not significantly different than when fed *A. falcatus* (df = 2, $P = 0.68$).

Lake studies (Lake Winnebago, WI)

Daphnia pulicaria population dynamics

The population of *D. pulicaria* in Lake Winnebago increased during the spring period in each year that was studied and then underwent a relatively rapid
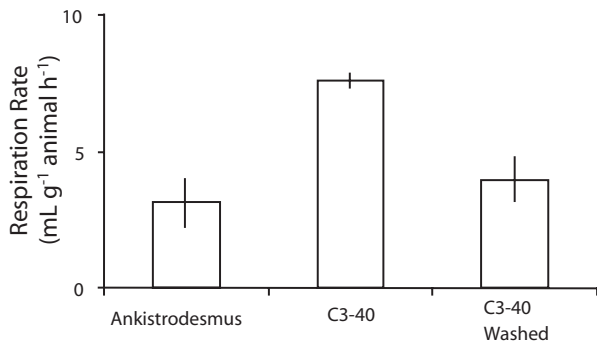
Fig. 3. Respiration rates of *Daphnia pulicaria* exposed to encapsulated or non-encapsulated *Microcystis aeruginosa* strain C3-40. Changes in oxygen concentration were determined every 15 min for 6–8 h. Notice that removal of polysaccharide capsule from *M. aeruginosa* strain C3-40 results in a decrease in respiration rate, when compared to the non-washed, encapsulated cyanobacteria.

decrease in abundance during late June or early July (Figure 4). In 1995, the population increased during the spring and reached a maximum of over 60 individuals (ind.) L^{-1} in late May (Figure 4B). After that time abundance decreased through June, dropping to $\sim$1–2 ind. L^{-1} through July. By the second week of August *Daphnia* abundances were below detection limits and no animals were found in samples for the remainder of the summer. A similar pattern occurred in 1999 (Figure 4B). The population abundance peaked at $\sim$25 ind. L^{-1} during the middle of June, and then quickly declined in early July to <1 ind. L^{-1}. By the end of July no *Daphnia* could be found in samples.

Microcystis capsule abundance
The average area of *Microcystis* encapsulation was relatively low from June 9 through June 29, 1999 (3802 μm^2 mL^{-1}, 3998 μm^2 mL^{-1}, and 6015 μm^2 mL^{-1}, respectively; Figure 5), but increased $\sim$10-fold by July 13, 1999 (56 501 μm^2 mL^{-1}). Degree of encapsulation remained relatively high throughout the remainder of the sampling season.

Cyanobacteria population dynamics
Microcystis abundance increased at the same time as the *D. pulicaria* population decreased in Lake Winnebago in both 1995 and 1999 (Figure 4). *Microcystis* was not detected in samples during early May of 1995, but increased throughout late May and June, reaching an initial peak in late June (Figure 4A). Following a decrease in abundance in mid-July, *Microcystis* increased again during late July and early August. A similar pattern occurred in 1999, except that the population continuously increased throughout the summer, reaching a maximum in late August (Figure 4B).

Cyanobacterial population assemblage was identified from lakewater samples collected from Lake Winnebago,
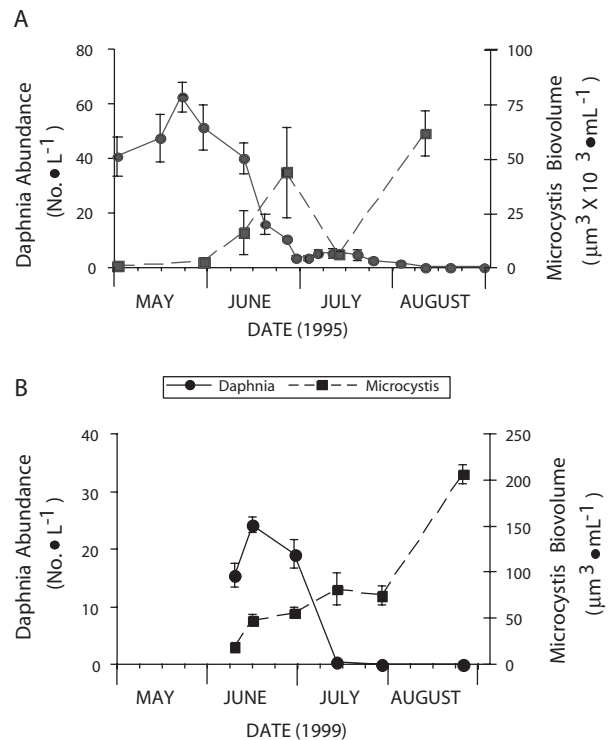

Fig. 4. *Daphnia pulicaria* abundance and *Microcystis aeruginosa* biovolume in Lake Winnebago, WI during summers 1995 and 1999. In midsummer (mid-June through mid-July) a decline in *Daphnia* abundance occurs. This decline appears to occur at the same time that the *Microcystis* biovolume increases.
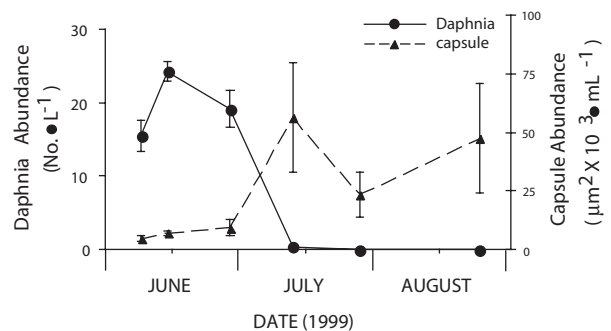
Fig. 5. *Daphnia pulicaria* and cyanobacterial capsule abundance in Lake Winnebago, WI in summer 1999. The midsummer decline in *Daphnia* abundance (early–mid July) occurs simultaneously with an increase in cyanobacterial capsule production.

WI in 1995 and 1999 (Figure 6). Biovolume of cyanobacteria was low in early summer and increased throughout the summer season. In both years four main genera of cyanobacteria were observed; *Anabaena*, *Aphanizomenon*, *Microcystis*, and *Oscillatoria* (currently *Planktothrix*). *Aphanizomenon* constituted the majority of the cyanobacterial biovolume in both years, although
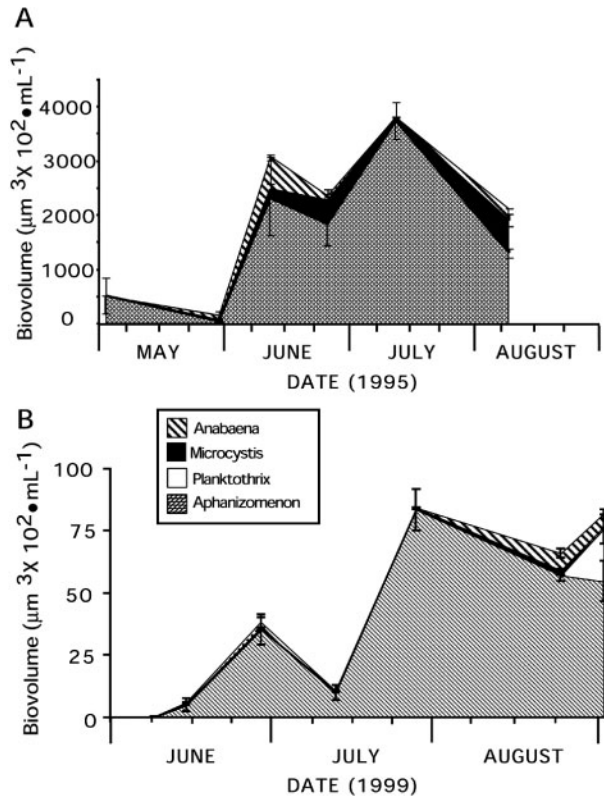
Fig. 6. Biovolume of cyanobacteria found in Lake Winnebago, WI in summers 1995 (**A**) and 1999 (**B**). In 1995, *Daphnia* abundance decline was observed concurrently with a sharp rise in both *Microcystis* and *Aphanizomenon* biovolumes. In 1999, however, only a rise in *Microcystis* biovolume appears negatively correlated with the *Daphnia* population decline.

numbers fluctuated throughout the season. *Microcystis* populations were observed throughout the seasons, but were most significant in mid–late summer (Figure 6), with peaks in populations in August. *Anabaena* was most predominant in June in 1995 and in August in 1999. *Oscillatoria* was primarily observed in late summer. Total cyanobacterial biovolume was substantially higher in 1995 (peak >3000 $\mu m^3 \times 10^2 \ mL^{-1}$), than in 1999 (peak ~75 $\mu m^3 \times 10^2 \ mL^{-1}$), but relative abundances of organisms were similar in both years (*Aphanizomenon* was in greatest abundance, followed by *Microcystis* and *Anabaena*; *Planktothrix* was in lowest abundance and appeared last).

DISCUSSION

A midsummer decline in *Daphnia* populations has been documented in many freshwater systems (Threlkeld, 1979; Sommer *et al.*, 1986; Benndorf *et al.*, 2001) and a multitude of causative factors have been linked to this

decline. The *D. pulicaria* population in Lake Winnebago, WI, USA, has undergone a midsummer decline in abundance every summer from 1992 to 1999. We found that the decline is concurrent with increases in the abundance of the cyanobacteria *Microcystis* and also with its production of gelatinous capsule and the biotoxin microcystin. Our laboratory experiments, in which the effects of capsule and microcystin were isolated and tested individually, demonstrate that capsule plays a more important role than microcystin in affecting the *D. pulicaria* from this system. The presence of capsule significantly decreases survivorship of *D. pulicaria* in standard 48 h bioassay tests, affects feeding behaviour by decreasing mandibular movements and increasing food bolus rejection rates, and significantly increases respiration rates of animals compared to individuals feeding on chlorophytes.

Environmental factors such as spring and early summer water temperature have been shown to influence midsummer dynamics of *Daphnia* populations in some other lakes [e.g. (George *et al.*, 1990)]. In Lake Winnebago there is no clear relationship between *Daphnia* population declines and water temperature (B. T. De Stasio Jr, unpublished data). However, the abundance of *Microcystis* does generally increase in June and early July when water temperature increases, as has been demonstrated in other lake systems (Reynolds, 1997). Our laboratory experiments demonstrate that when temperature is held constant there is still a significant effect on the *Daphnia* from this system of the gelatinous capsule produced by *Microcystis*. Although we did not test for temperature effects directly in the laboratory, the field data suggest that there is little direct effect of temperature on *D. pulicaria* dynamics in Lake Winnebago.

A potential indirect effect of temperature on the *Daphnia* could be through the direct effect of temperature on the population dynamics of planktivores in the system (Benndorf *et al.*, 2001). Temperature has been shown to affect growth rates of young-of-the-year (YOY) fish in Lake Winnebago (Weber and Les, 1982; Staggs and Otis, 1996). However, increases in YOY fish abundances in the past have occurred in early July in Lake Winnebago, later than the initiation of the *Daphnia* declines in most years (Otis and Staggs, 1988). In addition, the lake is well known for its prize trophy fishery for walleye (*Stizostedion vitreum vitreum*). The large population of this piscivore apparently keeps planktivorous fish populations at relatively low levels [primarily yellow perch, *Perca flavescens*, and white bass, *Morone chrysops* (Otis and Staggs, 1988; Staggs and Otis, 1996)]. This check on planktivorous fish populations, coupled with low light penetration in this highly eutrophic lake, indicate that mortality from fish predation probably does not account for the dramatic decline in *Daphnia* observed in

late June and early July, similar to what has been documented in other eutrophic lakes (Boersma *et al.*, 1996). Size structure shifts in the *Daphnia* population likewise do not support fish predation as a significant cause of the midsummer decline in *Daphnia* numbers. Large-sized *Daphnia* persist through the midsummer decline (maximum size over 3.0 mm), and egg-stage analysis for this population does not support the conclusion that size-selective predation by visual predators plays an important role in determining the declines in the *Daphnia* population (B. T. De Stasio Jr, unpublished data). The invertebrate predator *Leptodora kindtii* does occur in Lake Winnebago, but two aspects of *Leptodora* population dynamics argue against *Leptodora* predation as an important factor causing *Daphnia* declines. The *Leptodora* population is initiated each year by the hatching of resting eggs and the initial cohort does not become large enough to prey upon *Daphnia* until after the midsummer decline occurs (B. T. De Stasio Jr, unpublished data). Also, the *Leptodora* population either stayed relatively constant or simultaneously declined during periods when *Daphnia* declined over the past 10 years in Lake Winnebago. In summary, predation upon *Daphnia* by invertebrates or by YOY fish does not seem to be an important factor for the *Daphnia* decline in Lake Winnebago, yet more recent studies of planktivory in this system would be helpful for furthering our understanding of this complex ecosystem.

Food quality and abundance, therefore, become the most likely factors able to explain the *Daphnia* declines observed in Lake Winnebago. *Daphnia* have ample food supplies in Lake Winnebago, a hypereutrophic system (Sloey and Blum, 1972). The summer phytoplankton community is typically dominated by *Microcystis* and *Aphanizomenon*, both of which have traditionally been thought to create poor feeding conditions for *Daphnia* (Richman and Dodson, 1983; Lampert *et al.*, 1986, Gliwicz and Lampert, 1990). Reasons that these groups are considered a poor food source for *Daphnia* include their production of toxins, size- and shape-related constraints on ingestion, poor nutritional quality and mechanical interference of filaments and colonies with the collection of other food sources (Hanazato, 1996).

Recent studies have suggested that strictly mechanical interference when feeding on filamentous algae may not be as important as previously suspected, raising the question of the relative role of these processes in controlling *Daphnia* populations (Kurmayer, 2000, 2001). This factor may certainly affect the *Daphnia* in Lake Winnebago given the abundance of *Aphanizomenon* in most years. However, in Lake Winnebago *Aphanizomenon* typically forms long, flexible filaments, and consequently may be fed upon by *Daphnia* during the summer. This may explain why the observed decreases in *Daphnia* abundance in 1995 and

1999 do not consistently coincide with increases in *Aphanizomenon* abundance (Figures 4 and 6). In contrast, *Daphnia* dynamics were seen to reflect changes in *Microcystis* populations in both years, suggesting the potential importance of *Microcystis* in affecting the *Daphnia* population in Lake Winnebago.

Microcystis aeruginosa is commonly found in high numbers in Lake Winnebago during most summers [(McDermott *et al.*, 1995); B. T. De Stasio Jr, unpublished data]. This organism has been shown to produce a toxin, microcystin, able to increase *Daphnia* mortality and decrease offspring production (Hietala *et al.*, 1995; DeMott *et al.*, 1991). In addition, this organism produces a polysaccharide capsule matrix that surrounds unicells or causes unicells to aggregate into colonial forms (Prescott, 1970; Doers and Parker, 1988). Both products of *M. aeruginosa* were studied in this investigation.

Laboratory feeding trials revealed that both microcystin and polysaccharide capsule decrease *Daphnia* survivorship. Animals fed microcystin-producing *M. aeruginosa* (PCC 7820) showed significantly lower survivorship than did those fed chlorophytes ($P < 0.0001$). Animals fed purified microcystin alone survived less well than did the starvation control animals. Likewise, addition of purified microcystin to the chlorophyte feeding treatment or to the non-toxic culture of *M. aeruginosa* (C3-40) resulted in decreased survivorship (Figure 1A, B). These data confirm for the Lake Winnebago population what has been shown by others (DeMott *et al.*, 1991; Rohrlack *et al.*, 1999a,b,c; Rohrlack *et al.*, 2001), that microcystin is capable of killing *Daphnia* and could be responsible for declines in *Daphnia* populations in lake systems when *Microcystis* is abundant.

Polysaccharide capsule was also shown to adversely affect *Daphnia* survivorship in feeding trials. When encapsulated *M. aeruginosa* C3-40 was washed to remove the capsular matrix and fed to *Daphnia*, survivorship in *Daphnia* increased ~30% over that seen in animals fed encapsulated *M. aeruginosa* C3-40 (Figure 1C). Feeding purified capsule alone caused the most dramatic decline in *Daphnia* survivorship when compared to the chlorophyte feeding treatment ($P < 0.001$) or to starved animals (−86% difference from starved *Daphnia*). These data indicate that the polysaccharide capsule could also play a role in the seasonal declines observed in lake populations of *Daphnia*.

Behavioural studies in the laboratory also indicate the important effect of the polysaccharide capsule and microcystin on *D. pulicaria* (Figure 2). Feeding of highly encapsulated *M. aeruginosa* (C3-40 strain) or purified capsular material to *Daphnia* resulted in the greatest increases in post-abdominal claw movements (abreptor sweeps) when compared to feeding chlorophytes, or non-encapsulated *M. aeruginosa* (PCC 7820 strain or washed

C3-40 strain). This implies that capsular polysaccharides increase the rate of rejection of the food source. In addition, feeding of capsular material results in decreased rates of mandible movements when compared to the other food sources. Lastly, feeding encapsulated *M. aeruginosa* resulted in elevated respiration rates in *Daphnia* when compared with chlorophytes or non-capsulated *M. aeruginosa* feeding (washed C3-40 strain; Figure 3). Excessive energy is expended when *Daphnia* are forced to make numerous abreptor sweeps (Hein *et al.*, 1993). These data indicate that feeding on encapsulated cyanobacteria may cause *Daphnia* to expend more energy than can be extracted from the food source.

Lake studies reflected the findings of the laboratory experiments. In both years examined, there was a dramatic midsummer decline in *Daphnia* numbers during early or mid-July (Figure 4). Concurrent with the fall in *Daphnia* numbers in 1995 was a sharp rise in *Microcystis* and *Aphanizomenon* biovolumes (Figures 4 and 6). In 1999, however, only a rise in *Microcystis* biovolume was negatively correlated with the *Daphnia* population decline. The *Daphnia* decline occurred about 2 weeks earlier in 1995 than in 1999, but the same relationship existed between increasing *Microcystis* and declining *Daphnia* populations.

In 1999, we measured the degree of encapsulation of cyanobacteria isolated from Lake Winnebago (Figure 5). Similar to the observed relationship between *Daphnia* and *Microcystis* population numbers, the *Daphnia* population declines when capsule abundance around *Microcystis* increases. This suggests that polysaccharide capsule plays an important role in the midsummer decline of *Daphnia* in Lake Winnebago, WI.

Microcystin production in Lake Winnebago was not determined in 1999, but has been documented for other years in the lake (McDermott *et al.*, 1995). In 2000, a significant correlation between microcystin concentrations and *M. aeruginosa* biovolume was identified over the *Microcystis* biovolume range observed in 1999 ($y = 0.0002x + 0.1028$, $r^2 = 0.4088$; where y = MCYST and x = total *Microcystis* biovolume; C. M. McDermott, unpublished data). Other investigators have shown similar correlations (Carmichael *et al.*, 1988b; Watanabe *et al.*, 1988). From these relationships it can be assumed that in summer 1999 microcystin concentrations increased as *Microcystis* biovolume increased, and that the decline in *Daphnia* populations can be attributed in part to death associated with microcystin toxicity. Both capsule and microcystin logically could play a role in *Daphnia* population decreases in the lake system. Laboratory studies discussed above, however, suggest that presence of cyanobacterial capsule may be a more important factor leading to *Daphnia* declines by causing the animals to expend excessive amounts of energy

sweeping polysaccharide capsule material from the feeding groove and preventing the animals from consuming adequate volumes of food. This energy expenditure without compensatory energy gain probably results in an earlier death of the animals. For this reason, it is suggested that cyanobacterial polysaccharide capsule, in addition to microcystin, plays a large role in triggering the midsummer decline of *Daphnia* in Lake Winnebago, WI.

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