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Cynophages Which Impact Bloom-Forming Cyanobacteria

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ABSTRACT

Various mesotrophic and eutrophic freshwater environments in the state of Florida were surveyed for the existence of cyanophages. Cyanophages were discovered which infect and kill four common bloom-forming species of cyanobacteria (i.e. blue-green algae); *Lyngbya birgei, Anabaena circinalis, Anabaena flos-aquae,* and *Microcystis aeruginosa*. These cyanophages are being maintained in the laboratory at titers around 10^7 PFU/ml. The potential use of these cyanophages to control blooms of these cyanobacteria is discussed.

Key words: biocontrol, algae blooms, *Microcystis, Anabaena, Lyngbya.*

INTRODUCTION

Viruses specific to cyanobacteria (*i.e.* blue-green algae) occur widely in the natural environment (1-11). The best known of these groups are the LPP 1 and LPP 2 viruses which infect members of the cyanobacterial genera *Lyng-*

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bya, Phormidium and *Plectonema*. The existence of cyanophages presents the possibility of using them to control blooms of specific cyanobacteria (4, 12-17). The concept of using cyanophages as biocontrol agents has not been extensively pursued, however, recent increases in anthropogenically driven eutrophication of aquatic systems has heightened interest in the problem of controlling the growth of nuisance algae.

Cyanobacteria are among the most problematic groups of aquatic weeds in the state of Florida. *Microcystis aeruginosa* Kutz. emend. Elenkin., *Anabaena flos-aquae* Breb ex Born. et Flah., *Anabaena circinalis* Rabenh. ex. Born. et Flah., and the benthic species *Lyngbya birgei* G. H. Smith are four of the most common bloom-forming species in Florida. *L. birgei* is a filamentous non-heterocystous cyanobacterium which can form dense blooms of massive proportions in freshwater lakes (18). It is characterized by its foul odor and related species have been reported to produce noxious and even toxic substances (19). Although public demand for the control of this species is widespread in Florida, *Lyngbya* is resistant to currently registered herbicides. Hence the more common mechanisms for control of aquatic weeds are limited in their effectiveness against *Lyngbya*. For this reason we propose that viral agents may be an alternative means of controlling blooms of this species.

The three plankton forms of cyanobacteria named above have all been observed to form extensive surface scums in Florida and around the world. *A. flos-aquae* and *A. circinalis* are heterocystous species common throughout North America. *M. aeruginosa* is the unicellular species most commonly associated with surface scums of cyanobacteria in Florida. *A. flos-aquae* and *M. aeruginosa* have also been associated with the periodic production of toxic substances (20). The possibility of targeting these species for control, without impacting less noxious forms, is a potential alternative to the more general action of herbicides.

This paper describes the discovery of new cyanophages which infect and kill *L. birgei, A. flos-aquae, M. aeroguinosa* and *A. circinalis* under laboratory conditions.

MATERIALS AND METHODS

Water samples for the isolation of cyanophages were

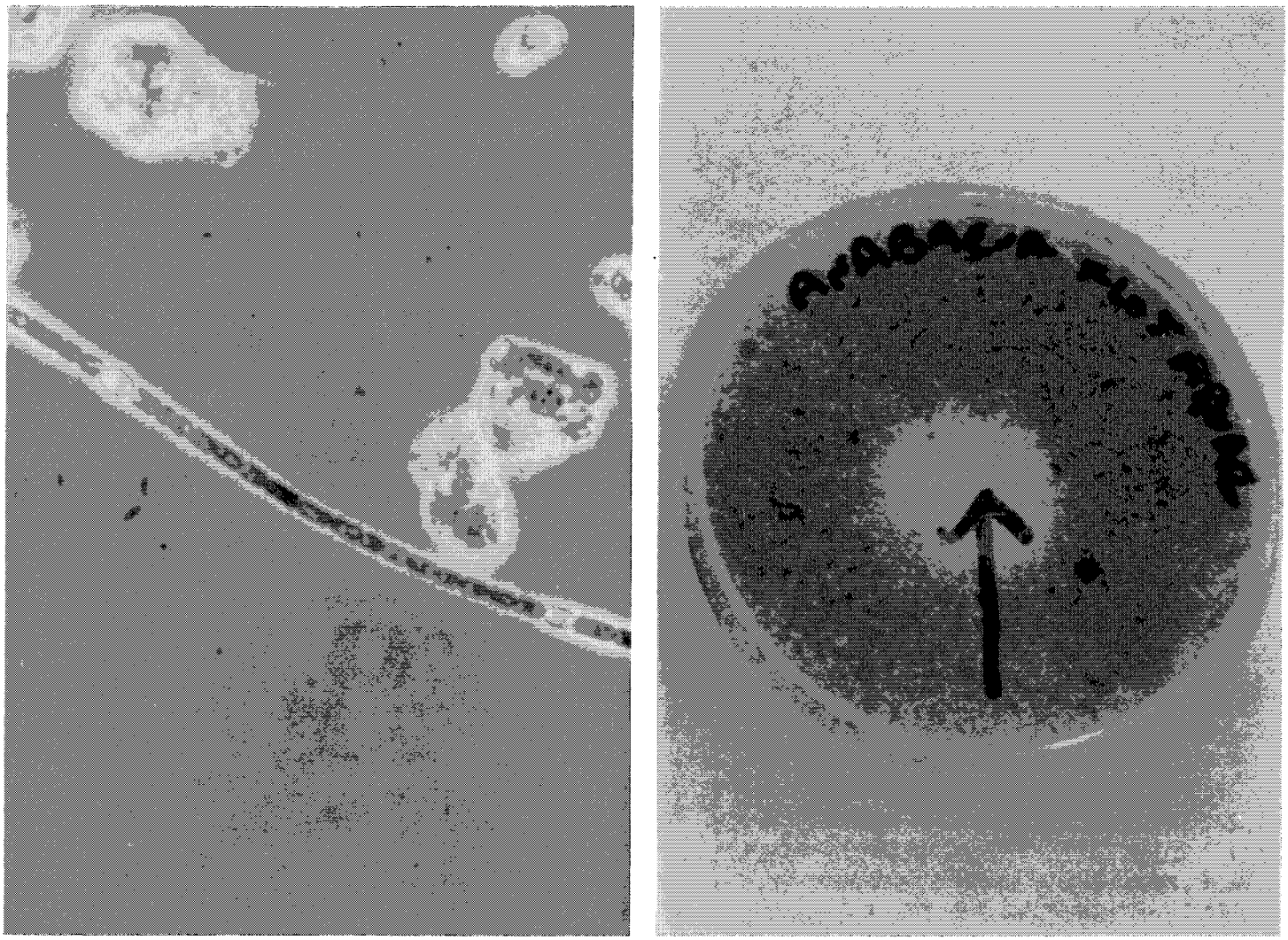

Figure 1. *Anabaena flos-aquae* (X400) (left) and viral plaques on lawns of this cyanobacterium (right).

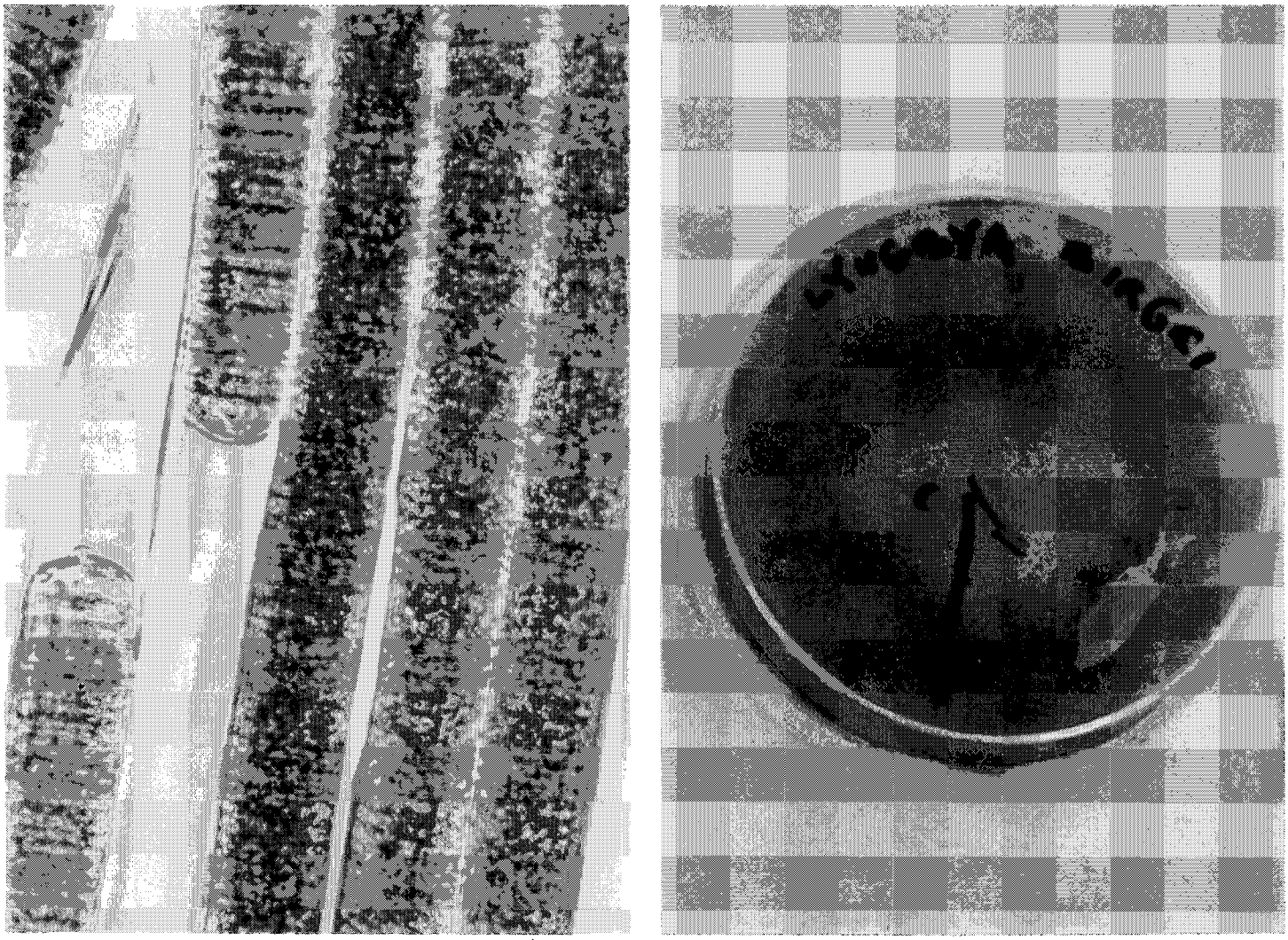

Figure 2. *Lyngbya birgei* (X400) (left) and viral plaques on lawns of this cyanobacterium (right).

collected in 1 L bottles from the surface of the water column and from the bottom, which included sediment, and kept at 4C until processing in the laboratory. Samples were collected from the University of Florida sewage treatment lagoon in Gainesville, Florida and the following Florida lakes; Lake Orange, Alachua county,; Lake Lochloosa, Alachua county; Lake Apopka, Orange county; Lake Alice, Alachua county; Lake Bivens Arm, Alachua county; Fish Eating Creek, Glades county; Lake Okeechobee, Glades county. Ten samples were taken per water body in the winter and summer of 1986/87 and 1987/88.

Two known cyanophages, LPP1 (#18200-B1) & LPP2 (#18200-B2), were obtained from the American Type Culture Collection (ATCC), Rockville, Maryland, for verification of assay methods.

Cyanophages were isolated using the chloroform extraction method reported by Safferman & Morris (21). Each water sample was shaken vigorously and a 50 ml subsample was removed and centrifuged at 10,000 g for 20 minutes. A 20 ml aliquat of supernatant was pipeted into a separatory funnel and 0.4 ml chloroform added. The funnel was stoppered and vigorously shaken for 30 seconds. The chloroform-sample was allowed to separate for 30 minutes and then the lower chloroform phase was discarded. The extracted sample phase was transferred to sterile 15x150 mm glass test tubes and aseptically sealed with a rubber serum sleeve stopper. Each sample was degassed for 10 minutes to eliminate any chloroform residual. All extracted samples were stored at 4C for later assays. Distilled water blanks were also extracted for use as controls.

The presence of cyanophages was assayed by spot inoculating sample extracts on petri plate lawns of axenic cyanobacteria cultures. Cyanobacteria species tested as host for cyanophage activity were *Lyngbya bergei* (local isolate), *Anabaena circinalis* (local isolate), *Microcystis aeruginosa* (local isolate), *Anabanea flos-aquae* (ATCC #22664), *Plectanema boryanum* (ATCC #18200). Host cyanobacteria lawns were cultured as follows (21, 22): 1. Agar-media base plates were prepared by making a 1% agar solution in a modified Hoaglands media (FW1) consisting of Tris 1g, KNO_3, $NaHCO_3$, 0.042g, K_2HPO_4 0.02g, H_3BO_3 0.014g, Na_2SiO_4 3mg, $FeSO_47H_2O$ 1.96mg, $MnCl_24H_2O$ 1.72mg, $Na_2MoO_42H_2O$ 0.4mg, $ZnCl_2$ 0.0128mg, Thiamine 5μg,

$CuSO_4$ 1.2μg, $CoCl_26H_2O$ 0.56μg, B12 0.05μg, Biotin 0.05μg in 1 liter of deionized water. The agar-media was autoclaved, cooled to 45°C and poured in 60 X 15mm petri plates. 2. Host cyanobacterial species, cultured in FW1 media, were mixed 1:1 with 45°C 1% agar-FW1 media and immediately poured on previously prepared base plates. *Lyngbya* was finely minced, aseptically, before mixing to make a uniform lawn. Lawns were incubated for 1 week prior to assay.

One drop of viral extract was spotted on a marked location on the host lawn. Lawns were then incubated at 28C for 4 to 6 weeks. The spot assays were done in triplicate for each sample and host lawn type. The formation of a plaque on lawns of cyanobacteria hosts were used as indication of cyanophage activity. Control lawns were inoculated with chloroform extracted distilled water.

Following initial observation of lytic activity the titer of the phage was enhanced. Agar containing the inhibition zone was aseptically removed from the plate and placed into 250 ml erlenmeyer flasks containing rapidly growing axenic cultures of the host species in 100 ml FW1 liquid media. The inoculated cultures were incubated until significant mortality of the host species was visually apparent, usually 2 to 6-weeks. The contents of the flask were then centrifuged at 10,000 G for 20 minutes. The supernatent was chloroform extracted as previously described using 0.2 ml chloroform/10 ml supernatent.

Titer cultures were done in 100 X 15 mm petri plates on a base of 1% agar made with FW#1 media. Cyanophage extracts displaying the greatest virulence were selected for titer evaluation. A serial dilution series of 1:1, 1:10, $1:10^2$, $1:10^3$, $1:10^4$ and $1:10^5$ was made for each extract. Lawns of the host species were prepared by mixing 0.5 ml extract dilution with a 2.0 ml culture of host cyanobacteria in FW# liquid media. 2.5 ml of 1% agar-FW#1 media was added to this mixture, autoclaved and cooled to 45°C. The combined mixture was immediately poured onto the base plates. Titer assays were done in triplicate for each dilution series and host lawn type. After incubation the number of plaques per plate were counted and the concentration of cyanophages calculated.

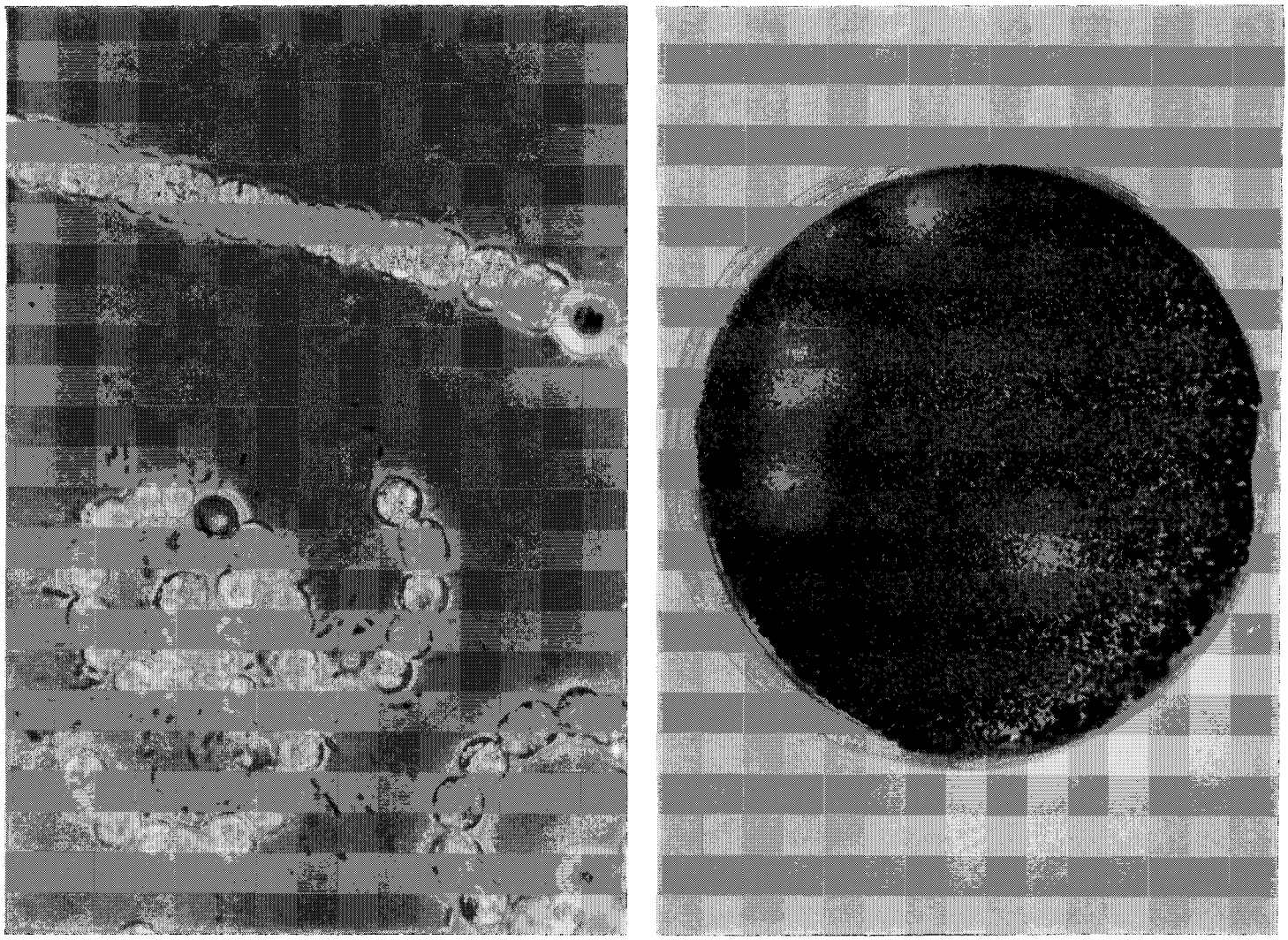

Figure 3. *Anabaena circinalis* (X400) (left) and viral plaques on lawns of this cyanobacterium (right).

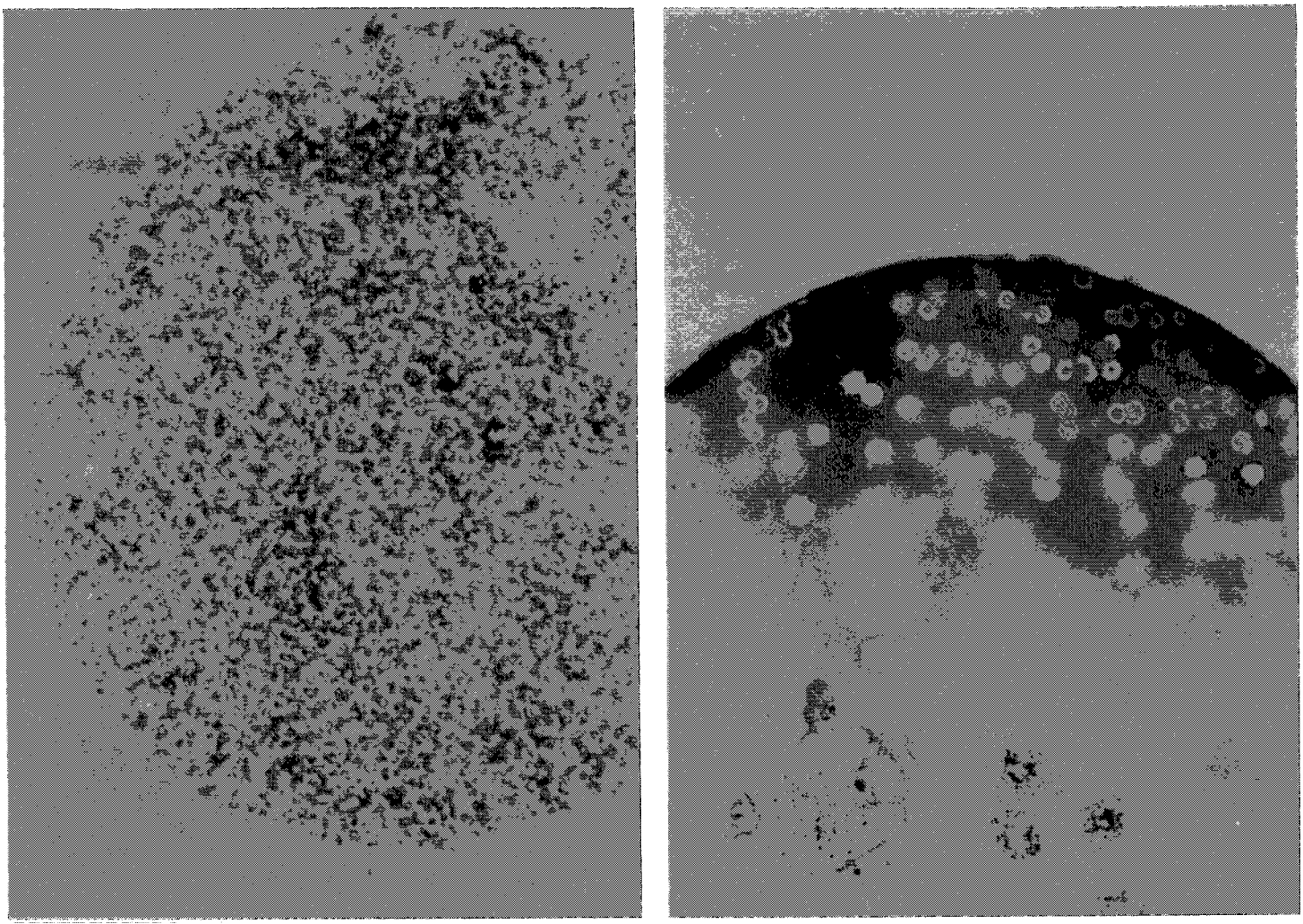

Figure 4. *Microcytis aeruginosa* (X400) (left) and viral plaques on lawns of this cyanobacterium (right).

RESULTS AND DISCUSSION

L. birgei is a member of the general group of cyanobacteria designated "LPP-type". This group includes genera *Lyngbya, Plectonema* and *Phormidium*. Viruses which infect species like *Plectonema boryanum* have been isolated and stored in culture collections for over two decades. Two of these cyanophages, LPP 1 and LPP 2, were tested for virulence with *L. birgei*. Neither exhibited activity against this species, although they were effective against *Plectonema boryanum*. Similarly these two cyanophages did not kill *A. flos-aquae*.

A survey was made of aquatic environments around Florida for the existence of local viruses which act against *L. birgei* and *A. flos-aquae*. The initial sets of water samples collected during the winter of 1986/87 did not provide any evidence of viral activity. Samples taken in the spring and summer of 1987 did test positive in plaque assays. Plaques were observed on lawns of *A. flos-aquae* (Figure 1) and *L. birgei* (Figure 2). These cyanophages were designated AF 1 and LB 1, respectively. Confirmation of activity was made by excising plaques from plates and placing them into 100 ml batch cultures of actively growing cyanobacteria. These combined cultures were incubated for a month. This incubation period enhanced the viral titers. In 1988 further collection efforts yielded cyanophages which were effective in killing *A. circinalis* (Figure 3) and *M. aeruginosa* (Figure 4). These cyanophages were maintained in the laboratory at titer levels around 10^7 PFU/ml.

The discovery of cyanophages specific to *L. birgei* (LB 1), *A. circinalis* (AC 1), *M. aeruginosa* (MA 1) and *A. flos-aquae* (AF 1) provides the working material for testing a biocontrol hypothesis. The success of these screening efforts indicate the potential for finding additional viral agents specific to other bloom-forming species. The problem of cyanobacterial blooms is becoming more prominent in Florida as the population of the state grows. Nuisance blooms are not restricted to *L. birgei*, but include a host of other species, including the planktonic species mentioned above. While the concept of viral control of algae has not received widespread attention to date there are preliminary indications that such an approach may be viable. Martin (15, 16) reported success in controlling cyanobacteria in experimental field enclosures using cyanophages and cyanophage/bacteria blends. Similarly, Desjardins and

Olsen (13) reported success in controlling populations of *Plectonema* using the LPP cyanophage in experimental ponds. There is also a growing body of evidence pointing toward an important role for natural cyanophages in the dynamics of cyanobacterial populations (2, 4, 8, 9, 11, 12, 14).

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