

Virus-like particles associated with *Lyngbya majuscula* (Cyanophyta; Oscillatoriaceae) bloom decline in Moreton Bay, Australia

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ABSTRACT: Expansive blooms of the toxic cyanobacterium *Lyngbya majuscula* were observed in 2 shallow water regions of Moreton Bay, Australia. The rapid bloom decline (8 to <1 km² in <7 d) prompted an investigation of the role of cyanophage viruses in the ecophysiology of *L. majuscula*. Virus-like particles produced by decaying *L. majuscula* were observed using electron microscopy. The virus-like particles were similar in morphology to viruses in the genus *Cyanostyloviridae*. The effect of viruses on *L. majuscula* photosynthesis was investigated by: (1) creating a virus concentrate using tangential-flow ultrafiltration of seawater surrounding *L. majuscula*; (2) inoculating *L. majuscula* with the concentrate; and (3) measuring photosynthetic response using a pulse-amplitude modulated fluorometer. Virus concentrate addition resulted in decreased initial fluorescence, decreased photochemical efficiency and decreased electron transport rate in rapid light curves after 5 d. Viruses present within *L. majuscula* filaments may play an important role in the bloom dynamics of this ecologically important cyanobacterium.

KEY WORDS: *Lyngbya majuscula* · Cyanophage · Virus · Lysogeny

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INTRODUCTION

Marine viruses are now recognised as being both ubiquitous and abundant in aquatic environments (Proctor et al. 1988, Bergh et al. 1989). The typical surface water abundance of virus-like particles (VLP), which are viruses that have not been cultured to determine host specificity, is 10¹⁰ VLP l⁻¹ (Fuhrman 1999). Substantial portions of marine virus communities are viruses specific to unicellular cyanobacteria (cyanophage) in oceanic phytoplankton. Approximately 25% of all free VLP in the Gulf of Mexico are specific to *Synechococcus* spp. (Suttle & Chan 1993). Few cyanophages specific to macroscopic, filamentous cyanobacteria have been identified (Ohki & Fujita 1996, Ohki 1999). Only temperate cyanophages

that remain within the host genome until induced by environmental stimuli (Wilson & Mann 1997) have been reported in marine non-heterocystous cyanobacteria. Ohki & Fujita (1996) isolated a temperate cyanophage specific to the tropical cyanobacterium *Phormidium persicinum*, while Ohki (1999) isolated a temperate phage specific to *Trichodesmium* spp. The isolation of these cyanophages has caused review of the role of viruses in the demise of cyanobacterial blooms (Suttle 2000).

Large benthic mats of the filamentous cyanobacterium *Lyngbya majuscula* have recently formed during summer months in northern waters of Moreton Bay, Queensland, Australia (27° S, 153° E; Fig. 1) (Dennison & Abal 1999). *L. majuscula* produces a powerful suite of toxins including dermatitis-causing compounds and tumour promoters, and aerosolisation of dried *L. majuscula* has been linked with asthma-like symptoms in humans (Dennison et al. 1999). *L. majuscula* is characterised by wide cells (30 to 40 µm wide and 5 µm thick), a thick, gelatinous sheath and a distinct dark

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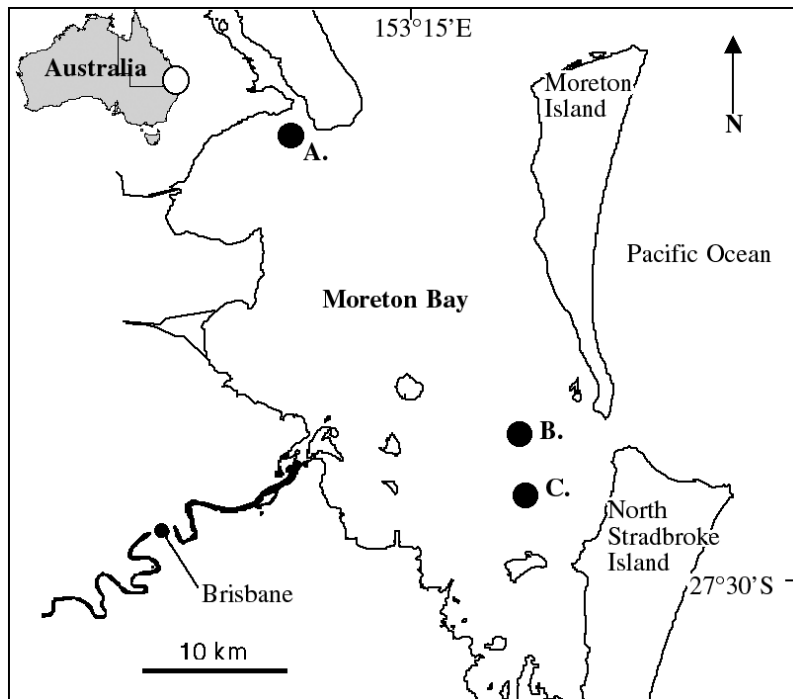


Fig. 1. Map of Moreton Bay, Australia, showing location of (A) Deception Bay bloom in austral summer 2000, (B) site of decomposing *Lyngbya majuscula* during Amity Banks bloom in austral spring 2000 and (C) site of healthy *L. majuscula* collection on Amity Banks

colouration (olive to green-grey; Fig. 2). *L. majuscula* is non-heterocystous; however, high nitrogen fixation rates have been recorded in association with *L. majuscula* (Dennison et al. 1999).

A large bloom of *Lyngbya majuscula* was observed in northern Deception Bay (a northern embayment that forms part of Moreton Bay) during late austral summer 2000. The bloom disintegrated rapidly (<7 d) after onset of decay symptoms, which included chlorosis, lack of photosynthesis and the colonisation by heterotrophic bacteria, e.g., *Beggiatoa* spp. A larger second bloom of *L. majuscula* formed later in mid-austral spring 2000 on Amity and Moreton Banks in eastern Moreton Bay. The second bloom did not collapse as rapidly as the first, but the cyanobacteria were observed to form extensive mats in deeper (2 to 3 m water depth) areas, which displayed similar symptoms to the decomposing cyanobacteria at Deception Bay. The second bloom eventually disappeared after 4 to 6 wk.

The rapid decline of the Deception Bay *Lyngbya majuscula* bloom suggests that cyanophages may play a role in the ecophysiology and bloom dynamics of *L. majuscula*. This study describes the observation of virus production from decaying *L. majuscula* and the effects of these viruses on the physiology of healthy cyanobacteria.

MATERIALS AND METHODS

Observation of viruses produced by decaying *Lyngbya majuscula*. *L. majuscula* was collected in plastic bags by SCUBA divers at the Deception Bay site (Fig. 1, point A; approximately 3 m water depth) at the first observation of bloom decline, and samples were transported at ambient temperature to the University of Queensland in 30 l black polycarbonate drums containing seawater collected at the bloom site.

Viruses, bacteria and phytoplankton were removed from 100 ml aliquots of Deception Bay seawater by filtration through Whatman (Maidstone, UK) 0.02 μm Anodisc filters while filtrate was retained in sterile 90 mm plastic Petri dishes. Sections of *Lyngbya majuscula* were blotted dry on tissue paper and gently placed using forceps into the open end of a 5 ml cut-off syringe. A volume of *L. majuscula* (~1 ml measured in the syringe) was then placed into the 0.02 μm filtered seawater (50 ml). Care was taken to minimise time (<20 s) between blotting and submersion in virus-free seawater.

Small pieces of *Lyngbya majuscula* were blotted dry, immersed in virus-free seawater and observed under epifluorescence microscopy to confirm the absence of associated bacteria and protists. Samples were prepared according to the protocols of Noble & Fuhrman (1998). Briefly, a small volume of water (2 ml) containing *L. majuscula* filaments was filtered through a Whatman Anodisc 0.02 μm Al_2O_3 filter, dried on the surface of tissue paper and stained with 1:100 000 diluted SYBR Green I (Molecular Probes Inc., Eugene, OR, USA). Filters were then dried once more before mounting on a glass slide using 50:50 phosphate-buffered saline and glycerol containing 0.1% w/v *p*-phenylenediamine as a combined mountant and anti-fade solution. Slides were observed using epifluorescence microscopy at 1000 \times magnification under blue light excitation.

To determine whether temperate cyanophages inducible through UV light irradiation were present in *Lyngbya majuscula* filaments, 3 replicates of cyanobacteria were subjected to UV light radiation for 60 s using an Oliphant UV lamp (λ ~ 250 to 350 nm) (Oliphant UV Products, Arndell Park, NSW, Australia). Petri dishes (including 3 replicates not subjected to UV light treatment) were placed on a shaker table for 4 h and incubated at a temperature of ~25°C

(approximate water temperature at the bloom site). After agitation, *L. majuscula* was removed from the Petri dishes, and the lysate was filtered through Whatman 0.7 μm GF/F filters and subsequently through Durapore 0.22 μm polycarbonate (low-binding properties) filters to remove single cellular debris. The filtered lysate was concentrated using Amicon Centriprep centrifugal ultrafilters (Millipore Corp., Bedford, MA, USA) following the manufacturer's recommendations. Approximately 300 ml were concentrated to a final volume of 3 ml for both controls and for UV light-treated samples. The concentrate was immediately fixed in 3.5% electron microscopy-grade glutaraldehyde and stored at 4°C until examination by electron microscopy.

Suspended particles in concentrates were harvested directly by ultracentrifugation in a Beckman airfuge (Beckman Coulter, Fullerton, CA, USA) at $100\,000 \times g$ for 40 min onto carbon stabilised formvar-coated 200-mesh copper grids. These were rinsed once in 0.02 μm filtered Milli-Q water to remove salts and stained with 4% w/v NH_4MO_4 for 30 s. Grids were air dried and observed using a JEOL 1010 electron microscope at 80 kV accelerating voltage and $100\,000\times$ magnification.

Effects of viruses produced by decaying *Lyngbya majuscula* on healthy cyanobacteria. Seawater was collected from an area of the Amity Banks bloom site (Fig. 1, Point B) that displayed symptoms of bloom decay using sterile opaque 1 l polypropylene containers. Air-filled bottles were submerged to a depth of 3 m and opened near mats of decomposing *Lyngbya majuscula*, then capped underwater, before being transported to the Moreton Bay Research Station.

Viruses were concentrated in several steps: (1) seawater collected around the Amity Banks bloom was prefiltered through a 0.7 μm Whatman GF/F filter to remove filaments of *Lyngbya majuscula* and large phytoplankton; (2) samples were then filtered through a 0.22 μm Durapore low-protein binding filter (Millipore Corporation, Bedford, MA, USA) to remove bacteria; and (3) filtrate was concentrated using a Vivaflow 200 tangential-flow ultrafilter (Vivascience,

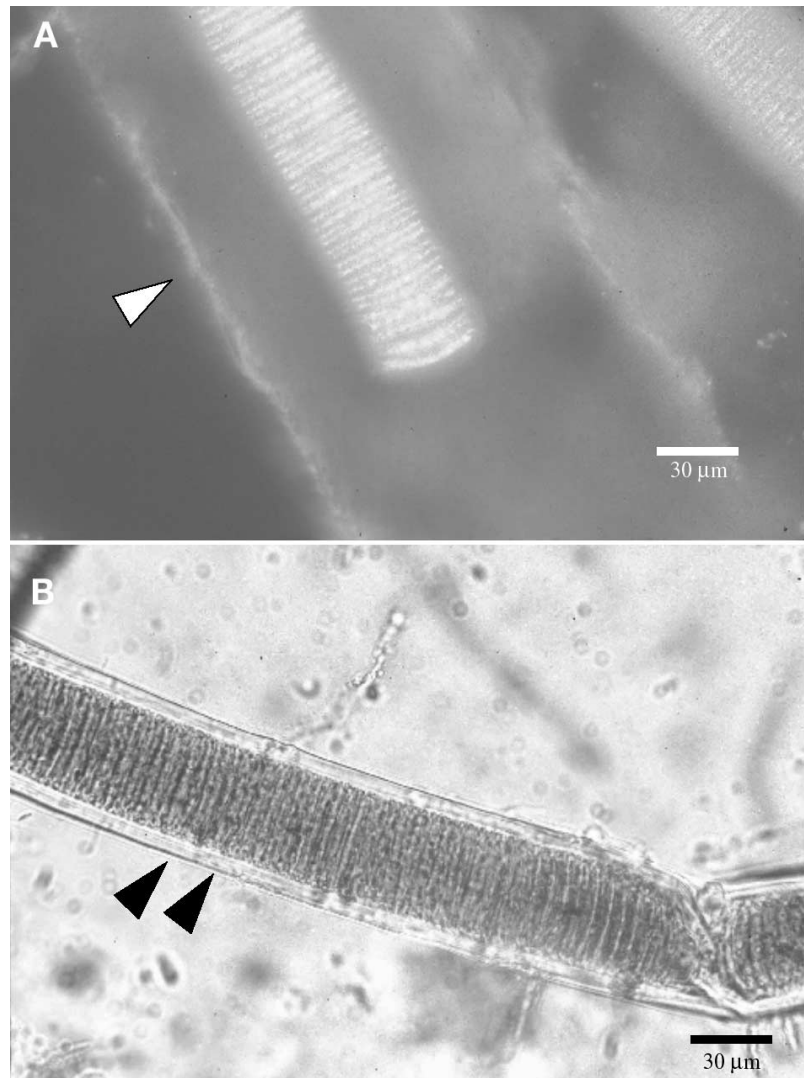


Fig. 2. *Lyngbya majuscula* (A) stained with SYBR Green I under epifluorescence microscopy and (B) under bright field microscopy. Arrowheads indicate sheath that is covered in heterotrophic bacteria

Hannover, Germany) driven by a peristaltic pump. Approximately 3 l of Amity Banks seawater were concentrated to a final volume of 6 ml. Concentrates were observed under epifluorescence microscopy using protocols of Noble & Fuhrman (1998) as previously described.

Small sections (~1 ml volume) of *Lyngbya majuscula* displaying no symptoms of decay were collected at a site approximately 2 km from decomposing cyanobacterial mats (Fig. 1, Point C) and were measured volumetrically as described above. *L. majuscula* sections were extruded into six 50 ml polypropylene tissue culture flasks containing 50 ml seawater from Amity Banks. Three flasks were inoculated with virus concentrates (2 ml in each flask) while nothing was added

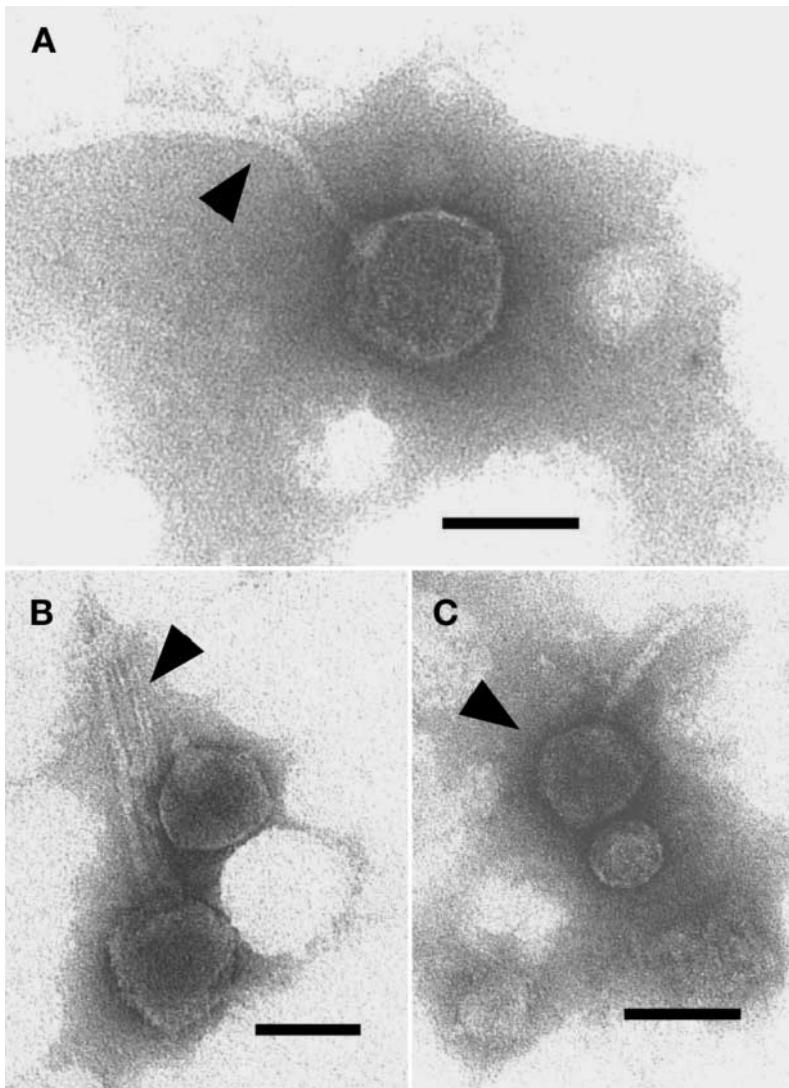


Fig. 3. (A) Electron micrograph of negatively stained phage particles from lysate of *Lyngbya majuscula* from Deception Bay. The long tail that appears to be flexible (arrowhead). (B) On the left is an intact particle, while on the right the tail of the particle has been broken off (arrowhead). (C) Electron micrograph of negatively stained virus-like particles from Amity Banks seawater concentrates. On the top is a virus-like particle morphologically similar to the cyanobacterial lysate (arrowhead) while below is an icosahedral virus-like particle. Scale bars = 50 nm

to the remaining 3 flasks (controls). Flasks were incubated in outdoor flow-through aquaria at 25°C and subjected to ambient light (typical maximum intensity of 800 to 1700 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$).

Photosynthesis and fluorescence of *Lyngbya majuscula* were measured using a pulse-amplitude modulated fluorometer (Walz Mess- and Regeltechnik, Bremen, Germany) (White & Critchley 1999). Flasks were dark adapted for 15 min, before initial fluorescence (F_0) and photochemical efficiency (F_v/F_m , where F_m is the maximum excitable fluorescence and F_v is the

maximum minus initial fluorescence) were measured. Rapid-light (photosynthesis – irradiance) curves were generated at ambient light (which was approximately 1000 $\mu\text{mol quanta m}^{-2} \text{s}^{-2}$ each day sampled) at random positions on cyanobacterial filaments. Three replicate measurements of fluorescence and photosynthesis were generated on *L. majuscula* from each flask at 1, 3 and 5 d after virus addition.

RESULTS

Virus morphology

Lyngbya majuscula collected was not colonised by heterotrophic bacteria. Blotted-dry filaments of decaying *L. majuscula* were devoid of associated bacteria, protists and metazoa.

VLP were observed in the lysate of UV-light-treated and -untreated *Lyngbya majuscula* from Deception Bay (Fig. 3a,b). VLP were predominantly binial with isomorphic heads 40 to 50 nm in diameter and tails 80 to 120 nm in length. Tails appeared bent or broken off in several VLP, indicating that they may have been flexible (Suttle 2000). On the basis of their morphology, the VLP belong to the family *Siphonoviridae* and genus *Cyanostylovirus* according to descriptions in Suttle (2000). Control lysate contained visibly fewer VLP than UV-treated samples; however, reliable enumeration of both treatments was not possible due to the low number of VLP per grid division, which seldom exceeded 30.

Effects of elevated virus abundance on *Lyngbya majuscula*

Viral concentrates added to *Lyngbya majuscula* contained no bacteria and approximately 2.3×10^{10} VLP ml^{-1} , while unconcentrated seawater contained approximately $7.2 \times 10^6 \pm 4.8 \times 10^5$ VLP ml^{-1} and the abundance of bacteria at the site of collection was $3.6 \times 10^5 \pm 1.7 \times 10^4$ cells ml^{-1} . It is estimated that addition of virus concentrate elevated virus abundance in the flasks by 25% above ambient levels.

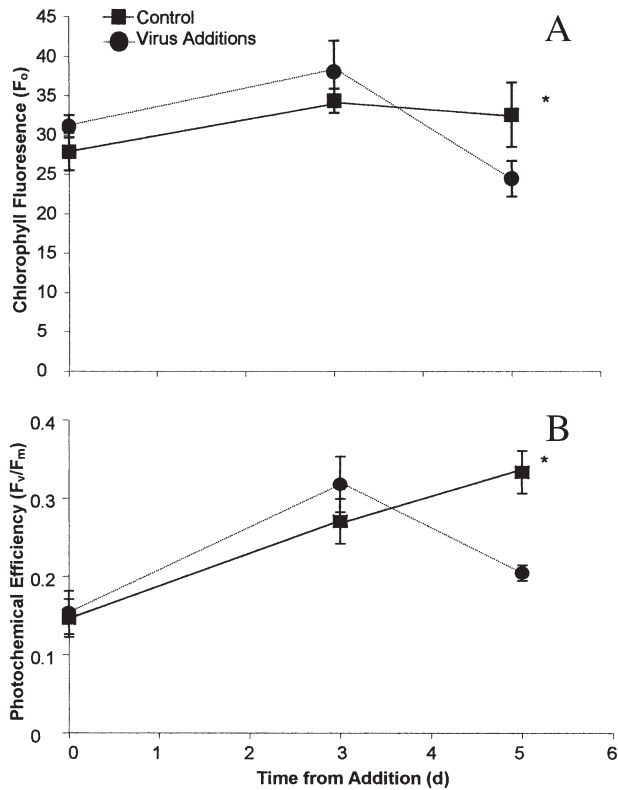


Fig. 4. Effect of virus concentrates on the state of PSII as measured by (A) chlorophyll fluorescence (F_0 : initial fluorescence) and (B) photochemical efficiency (F_v/F_m , where F_m is the maximum excitable fluorescence and F_v is the maximum minus initial fluorescence). *Significant differences at $p < 0.05$

Viral concentrate additions affected *Lyngbya majuscula* photosynthesis and the state of PSII, which indicates virus infection (Suttle et al 1990, Balanchandran et al. 1997). Evidence of viral infection was decreased maximum photosynthetic rate and photoinhibition of *L. majuscula* in virus additions after 5 d (Fig. 4); increased F_0 after 3 d and then subsequent decrease after 5 d; and decreased F_v/F_m after 5 d (Fig. 5). The first indication of virus infection and onset of cell lysis was an increase in dark-adapted *in vivo* fluorescence after 3 d (concomitant with the release of red pigments into the culture flasks in virus additions, which was most likely phycoerythrin from lysed cells), followed by a decrease in F_0 after cell lysis at 5 d. This phenomena is due to the breakdown of the electron transport chain between PSII and PSI, which results in photochemical quenching of excess energy through fluorescence. After destabilisation of PSII-dependant protein turnover and degradation of photosynthetic membranes, F_0 decreases as chlorophyll *a* degrades (Balachandran et al. 1997). Concomitant with the decline in F_0 at 5 d, photochemical efficiency was reduced, indicating reduced light use by damaged or degraded photosynthetic tissues.

DISCUSSION

Viruses have been implicated for some time as agents of bloom decline of freshwater cyanobacteria (reviewed in Martin & Benson 1988) and eukaryotic phytoplankton (Bratbak et al. 1990, Nagasaki et al 1994). However, the role of viruses in bloom decline of macroscopic cyanobacterial species has not been previously investigated.

Cyanostylovirus observed in the lysate of *Lyngbya majuscula* are morphologically similar to cyanophages isolated from several freshwater cyanobacterial genera including *Synechococcus elongatus* (S-2L, SM-2), *Microcystis aeruginosa* (S-1) and LPP (*Lyngbya*, *Phormidium* and *Plectonema*) group cyanobacteria (LPP-1, LPP-2, LPP-3A) (reviewed in Martin & Benson 1988) (Table 1). The tail structure of the *L. majuscula* virus is substantially shorter than the temperate cyanophage of *Phormidium persicinum* (Ohki & Fujita 1996), another marine filamentous cyanobacteria with a *Cyanostylovirus* cyanophage.

The infection in *Lyngbya majuscula* may be by either lytic or temperate cyanophages. VLP were observed in greatest abundance after UV light treatment of *L. majuscula*. It is therefore proposed that the virus parti-

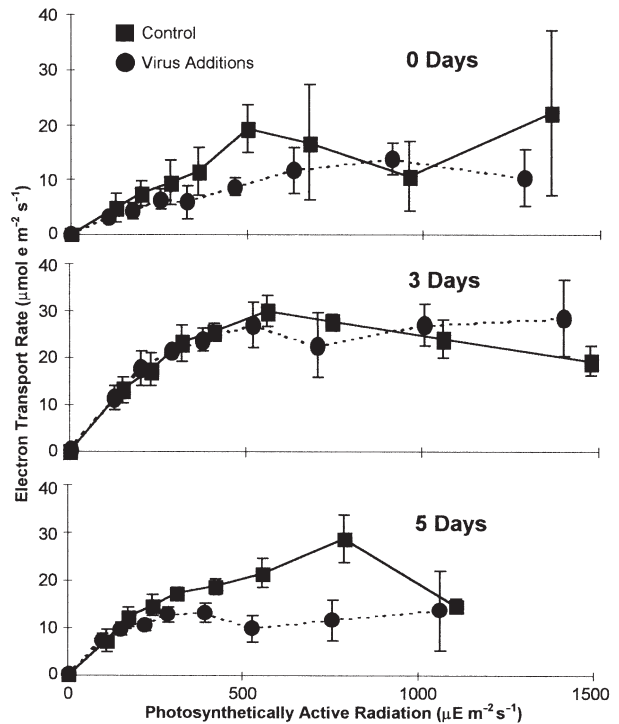


Fig. 5. Changes in photosynthesis of *Lyngbya majuscula* in the presence of elevated virus abundances as measured by pulse-amplitude modulated fluorescence. Data points are the mean of 3 measurements made at random points on filaments. Error bars indicate SD from means

Table 1. Virus-like particles observed in *Lyngbya majuscula* lysate compared with other isolated cyanophages. diam.: diameter

Cyano-phage	Host	Capsid diam. (nm)	Tail morphology	Source
	<i>Lyngbya majuscula</i>	50 to 60	Contractile, 80 to 120 nm long	Present study
S-1	<i>Microcystis aeruginosa</i>	50	Noncontractile, 140 nm long	(Adolph & Haselkorn 1973)
S-2L	<i>Synechococcus</i> sp.	56	Noncontractile, 120 nm long	(Khudyakov 1977)
SM-2	<i>Synechococcus elongatus</i>	50 to 55	Noncontractile, 130 to 140 nm long	(Fox et al. 1976)
LPP-1	<i>Lyngbya</i> , <i>Phormidium</i> and <i>Plectonema</i>	60	Contractile, short	(Safferman & Morris 1963)
LPP-2	<i>Lyngbya</i> , <i>Phormidium</i> and <i>Plectonema</i>	60	Contractile, short	(Safferman et al. 1969)
	<i>Phormidium persicinum</i>	40	Noncontractile, 300 nm	(Ohki & Fujita 1996)

cles observed were primarily temperate and induced by the UV light treatment; however, lytic cyanophage infection cannot be discounted as a possible cause of cyanobacterial disease since some VLP were produced in non-UV controls.

The presence of lytic virus particles around decomposing mats of the cyanobacterium can be inferred by comparing the photosynthetic response of non-decaying cyanobacteria after 3 d with cyanobacteria in the presence of virus populations augmented with lysate of decaying *Lyngbya majuscula*. High molecular weight concentrates (which are rich in viruses with capsids >2 nm) of seawater surrounding decaying *L. majuscula* reduced photosynthetic rate and caused photoinhibition in healthy *L. majuscula* after 5 d incubation. The maximum electron transport rate of virus-enriched cyanobacterium was approximately 70% lower than that of *L. majuscula* with no viral addition (Fig. 5). This is similar to reduced rates reported for other cyanobacteria and microalgae in the presence of virus concentrates (Suttle et al. 1991, Suttle 1992).

The possible existence of temperate cyanophages of *Lyngbya majuscula* may help explain differences in toxicity previously noted within this species (Orjala et al. 1995). Cyanophages have been shown to be important in genetic exchange among prokaryotes (Jiang & Paul 1998), and genes responsible for toxin production may be a result of the inclusion of a temperate cyanophage genome in host DNA, as is observed in other bacteria (Waldor & Mekalanos 1996). Additionally, it has been shown that toxic strains of *Microcystis aeruginosa* have temperate inducible phage, while non-toxic strains do not have temperate cyanophage (Vance 1977).

This study emphasises the need for further research into the consequences of potential cyanophage-induced collapse of *Lyngbya majuscula* blooms. In particular, the understanding of *L. majuscula* host-cell resistance dynamics and decay rates of cyanophage under bloom conditions is essential to the complete understanding of the role of cyanophage in bloom disintegration.

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