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Isolation of cyanophage CrV infecting *Cylindrospermopsis raciborskii* and the influence of temperature and irradiance on CrV proliferation

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ABSTRACT: A lytic virus that infects a European strain of the freshwater filamentous cyano-bacterium *Cylindrospermopsis raciborskii* was isolated from a lake in the Netherlands and partially characterised. With a genome size of 110 ± 15 kb, an icosahedral capsid of 65 ± 1 nm (n = 22) and a long non-contractile tail of 612 ± 31 nm (n = 15), this dsDNA cyanophage CrV appears to belong to the *Siphoviridae* family. CrV was highly host specific, not infecting other filamentous cyano-bacteria species isolated from the same lake, nor 4 Australian strains of *C. raciborskii*. The latent period of this cyanophage was 20–24 h. Varying the irradiance affected cyanophage–host interactions: at low light (20 µmol quanta m^{-2} s^{-1}) the latent period was 1.3 times longer compared with at mid light (90 µmol quanta m^{-2} s^{-1}); burst size at mid light was 332 CrV per lysed host cell, at low light it was halved (48 %) and at high light (250 µmol quanta m^{-2} s^{-1}) the burst size was further reduced to only 14 % of that of mid light. Temperature also affected the virus growth characteristics: the CrV latent period at high temperature (30°C) was reduced to just 11 % (compared with a mid temperature of 22°C), but still the burst size increased to 541 CrV per lysed host cell; at low temperature (15°C) the latent period was prolonged 1.3-fold and the burst size was reduced to 43 %. Our findings indicate that ecologically relevant environmental factors can affect the extent of viral lysis of *C. raciborskii*, advancing our understanding of the spread of this invasive cyano-bacterium across Europe.

KEY WORDS: Invasive cyanobacteria · Cyanophage · Freshwater · Latent period · Burst size · Temperature · Light

INTRODUCTION

The potentially toxic *Cylindrospermopsis raciborskii* is classified as a tropical to subtropical species. In recent years, the geographic range of the species has increased to include new areas (Briand et al. 2004), particularly in temperate regions, e.g. Europe, New Zealand, Japan and Canada (Wood & Stirling 2003, Hamilton et al. 2005, Wiedner et al. 2007, Zarenezhad et al. 2012). The spread of *C. raciborskii* is likely related to increased temperatures due to climate change, and *C. raciborskii* blooms have been reported in Europe from Greece to Germany since 1990 (Sinha et al. 2012). Blooms of this species can

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decrease water clarity and harm macrophyte and algal communities, thereby negatively impacting the functioning of the aquatic food web (Paerl & Paul 2012). In the Netherlands, *C. raciborskii* was first observed in 2001 (Mooij et al. 2005), but thus far, (dense) blooms of *C. raciborskii* have not yet been reported. In the future, temperatures and precipitation are predicted to increase in the Netherlands (Mooij et al. 2005). *C. raciborskii* has specialised dormant resting cells (akinetes) which allow the species to survive lower temperatures over winters in higher latitudes, only germinating when the bottom temperatures are again warm enough (Sinha et al. 2012). With its high temperature tolerance (Sinha et al. 2012) and the ability to use and store phosphorus highly efficiently and to fix nitrogen (Padisak 1997), *C. raciborskii* may outcompete native species during nutrient-depleted summer seasons.

In freshwaters, viruses have been discovered which lyse cyanobacterial species and have been linked to bloom termination (Tucker & Pollard 2005, Yoshida et al. 2008a, Tijdens et al. 2008a, Wu et al. 2009, Jacquet et al. 2013). The morphology of each virus type is described in detail in Ackermann & Prangishvili (2012). Cyanophages from the *Myoviridae* family infecting *Microcystis aeruginosa* were found to increase concomitantly with declining host abundance (Yoshida et al. 2008a). Cultures of *M. aeruginosa* isolated from a reservoir in Australia were lysed by the addition of cyanophages belonging to the *Podoviridae* family (Ma-LB) that originated from 0.22 µm pore-size filtered water from the lake (Tucker & Pollard 2005). Another lytic cyanophage from the *Podoviridae* family, infecting *Arthrospira platensis* (Spirulina), caused the sudden collapse of cultures grown for commercial harvesting (Jacquet et al. 2013). Similarly, in microcosms, *Anabaena flos-aquae* was lysed within 3 d of the addition of its isolated cyanophage from the *Myoviridae* family (Wu et al. 2009). Tailless viruses infecting *Synechococcus* and rod-shaped viruses infecting *Nostoc pruniforme* and *Gloeotrichia pisum* have also been characterised (Safferman & Morris 1967, Jensen & Bowen 1970). Viruses collected from lakes in the Netherlands and added back to whole lake water incubations were able to lyse a high proportion of filamentous cyanobacteria (Tijdens et al. 2008a). In the marine environment, the addition of the cyanophage isolate AaV reduced bloom populations of *Aureococcus anophagefferens* by greater than 60% in *in situ* incubations in both New York and Maryland (USA) (Gobler et al. 2007). East of New Zealand, quantitative PCR targeting the *g20* of cyanomyoviruses found that the abundance of cyanophages declined after the termination of a bloom of the *Synechococcus* host (Matteson et al. 2013).

Cylindrospermopsis raciborskii has also been shown to be sensitive to viral infection; in 1967, isolates from plaque assays repeatedly lysed cultures of *C. raciborskii* (then known as *Anabaenopsis raciborskii*) (Singh & Singh 1967). Recently, a virus from the *Siphoviridae* family was shown to infect and kill an Australian strain of *C. raciborskii* (Pollard & Young 2010). Substantial viral lysis of *C. raciborskii* under natural conditions may slow its spread; however, it may also promote the distribution of *C. raciborskii* as viral-mediated lysis was shown to fragment the filaments (Pollard & Young 2010). How environmental factors influence freshwater cyanophage–host interactions is still largely unknown. Seasonal or global climate change variations in host growth-limiting factors (phosphorus, pCO_2, light and temperature) have been shown to affect marine virus proliferation, as reviewed by Mojica & Brussaard (2014). As an essential first step, we brought into culture and partially characterised a lytic cyanophage for the species *C. raciborskii*. Subsequently, we studied the influence of temperature and irradiance, 2 important host growth-controlling factors, on cyanophage propagation.

MATERIALS AND METHODS

Isolation and culturing

Cylindrospermopsis raciborskii was discovered within blooms of *Aphanizomenon skujae* in the Reeuwijkse Lakes (52° 01′ 51.49″ N and 4° 44′ 09.18″ E) in the Netherlands. *C. raciborskii* was isolated from water collected in October 2010, and the cyanophage was isolated from water collected in August 2012. The complex of 12 adjoining and linked lakes has a total surface area of 700 ha, with an average and maximum depth of 2 and 7 m, respectively (Wijmans & Beekman 2003). Water samples (500 ml) from 30 cm below the surface were quickly transported (in the dark and at an ambient water temperature of 15°C) to the laboratory, where they were kept at 22°C under a 16 h:8 h light:dark photoperiod at 140 µmol quanta m^{-2} s^{-1} light intensity until further analysis.

Separate *C. raciborskii* trichomes (filaments) were isolated from the water sample collected in October 2010 by concentrating the sample with a 20 µm mesh sieve to remove smaller phytoplankton species, followed by isolation using an inverted light microscope

(2010: Leica DMI 4000B). Each filament was selected and isolated using a micropipette method, whereby a glass Pasteur pipette with a tip stretched thin over a flame was used to suck up the filament and wash it in serial drops of CHU-10 medium (Stein 1973). The isolated filaments were placed in wells (one filament per well) of a 96-well plate (Cellstar, Greiner Bio-One) in CHU-10 medium and kept at 22°C under 16 h:8 h light:dark conditions at 140 µmol quanta m^{-2} s^{-1} light intensity. When growth was observed the culture was transferred to 50 ml polystyrene cell culture flasks (TPP, Trasadingen), and it was then maintained in the culture collection by sub-culturing every 2 to 3 wk. Two other species of cyanobacteria used in this study (*Aphanizomenon skujae* and *Anabaenopsis cunningtonii*) and another strain of *C. raciborskii* were isolated (at the same times as the cynophage) in the same way (except by using a Zeiss Axiovert 200 inverted light microscope) from the same lake in August 2012, whereupon the medium was changed to the similar MLA medium (Bolch & Blackburn 1996). All strains isolated in this study were identified by light microscopy by the phycology group at the Department of Botany, University of South Bohemia, České Budějovice, Czech Republic (J. Komárek pers. comm.).

For cyanophage isolation, a 200 ml sample was first pre-filtered through 20 µm mesh to remove larger organisms, after which the filtrate was filtered through a 0.45 µm pore-size cellulose acetate sterile syringe filter unit and then again through a 0.2 µm cellulose acetate sterile syringe filter unit (both filters were purchased from Whatman). The 200 ml of filtrate was then concentrated using a 30 kDa Amicon Ultra-15 centrifugal filter (Millipore), whereby the retentate was collected and 1% v/v was added to exponentially growing cultures of *C. raciborskii* (strain Cr2010). Upon lysis (clearing) of the culture within 5 d, the lysate was 0.2 µm filtered (to remove cell debris of lysed host cells) and added to a new exponentially growing host culture. The infectious agents did not pass through a 0.02 µm filter (Anotop syringe units, Whatman), nor did they stay infective upon autoclaving (tested by adding treated lysates to exponentially growing *C. raciborskii* cultures and checking for lysis after 3, 7 and 14 d).

Following isolation, an end-point dilution procedure was performed to obtain a clonal cyanophage isolate (Middelboe et al. 2010), using 10-fold serial dilutions (12 concentrations) in 96-well multiwell plates (Cellstar, Greiner Bio-One). Plaque assays were not used as the filamentous cyanobacteria did not form a uniform lawn on agar. The lysate from cleared wells with the highest dilution was collected, 0.2 µm filtered and added again to a new serial dilution in a well plate containing exponentially growing host culture, a process that was repeated 3 times to ensure a clonal cyanophage isolate. Finally, the lysate from the most diluted lysed well was selected for further characterisation. The cyanophage CrV (viral collection of the NIOZ) was propagated on exponentially growing host cultures at 1 to 2 weekly intervals.

Host and viral abundances

Cyanobacterial cells and filaments were counted using a Fuchs-Rosenthal haemocytometer counting chamber (Hausser Scientific) under bright field at ×100 magnification using an epifluorescent microscope (Zeiss Axioplan2). Cell abundance was estimated by measuring the length of each filament and dividing the length of the filament by the average cell lengths, which was determined by measuring 120 cells in both virally infected and control cultures. At least 200 cells were counted in a minimum of 10 fields, or where all cells were lysed, the whole chamber area was scanned for cells.

Viruses were enumerated from 0.5% final concentration glutaraldehyde-fixed (EM grade, Sigma-Aldrich) and flash-frozen samples using a bench-top Becton Dickinson FACSCalibur flow cytometer (FCM) equipped with a 488 nm argon laser, according to Brussaard et al. (2010). Thawed samples were diluted with TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8) and stained with the green fluorescent nucleic acid-specific dye SYBR Green I (10 000× concentrate in DMSO, Molecular Probes, Invitrogen, Life Technologies) at a 0.5× final concentration, in darkness in a water bath at 80°C for 10 min. A test was performed to see whether staining at room temperature gave different results from staining at 80°C, and there was no difference in the outcome for this cyanophage. Viruses were discriminated based on their fluorescent signature in bivariate scatter plots of green fluorescence versus side scatter (Fig. 1) (Brussaard 2004). The cluster absent from the non-infected control cultures was identified as the CrV cyanophage, and had a signature comparable to that of cyanophages infecting the filamentous freshwater *Arthrospira platensis* (Jacquet et al. 2013) and a single-celled marine *Synechococcus* sp. (Brussaard 2004). Heterotrophic bacteriophages were excluded by using exponentially growing host cultures with minimal heterotrophic bacterial contamination. Furthermore, we tested old dying non-infected control cultures for

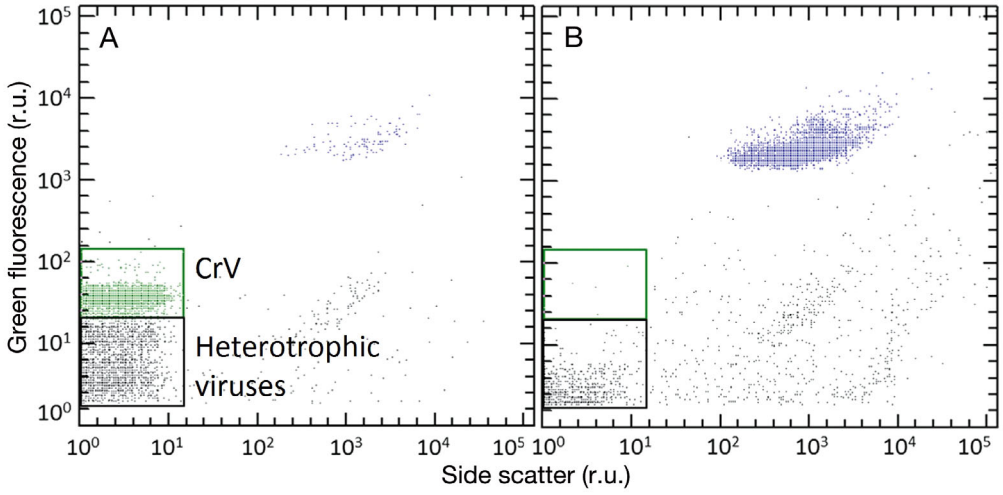

Fig. 1. Cytogram showing CrV and bacteriophages upon staining with the nucleic-acid-specific dye SYBR Green I. (A) The cluster with the highest green fluorescence (green coloured box) contains the cyanophage CrV. (B) Heterotrophic bacteriophages in old, dying, non-infected control cultures of *Cylindrospermopsis raciborskii*. The additional virus group in the infected cultures was conservatively considered to belong to the heterotrophic phage pool. Both plots show 6000 events in total

heterotrophic bacteriophages, and the staining flow cytometric signature of the cyanophages was not present. Moreover, the cyanophage flow cytometric signature was only seen in the infected cyanobacteria cultures, and the timing of the first cyanophage signatures seen was concurrent with the lysis of the hosts. The signatures of heterotrophic bacteria and, subsequently, their bacteriophages were seen many hours after the cyanobacterial lysis events.

Samples of lysing virally infected and non-infected cyanobacterial filaments were examined at ×1000 magnification using an epifluorescent microscope (Zeiss Axioplan2). A 40 µl glutaraldehyde (final concentration 2%; Grade II, Sigma-Aldrich) preserved sample was stained with SYBR Gold (10 000× concentrate in DMSO, Molecular Probes, Invitrogen, Life Technologies; at a 5× final concentration) for 15 min in darkness at room temperature (Patel et al. 2007), after which it was filtered through a 0.02 µm pore-size Anodisc aluminium oxide filter (13 mm diameter; Whatman) (Budinoff et al. 2011). Filters were examined using UV light with a blue filter (excitation <490/emission >515 nm), and were photographed using an Axiocam camera with Axiovision acquisition software (see Fig. 5).

Cyanophage visualisation

The newly isolated cyanophage was partially characterised before experimental studies were performed, i.e. particle shape and size, genome type and size, and host range were determined. Samples of virally infected and non-infected cyanobacteria were examined at ×1000 magnification using an epifluorescent microscope (EFM; Zeiss Axioplan2). A 40 µl unpreserved sample was stained with a 5× final concentration of SYBR Gold (10 000× concentrate in DMSO, Molecular Probes, Invitrogen, Life Technologies) for 15 min in darkness (Patel et al. 2007), after which it was filtered through a black 0.2 µm polycarbonate filter (25 mm diameter; Whatman). Filters were examined using UV light with a blue filter (excitation <490/emission >515 nm), and were photographed using an Axiocam camera with Axiovision acquisition software. To confirm that the viruses contained dsDNA, counts were also made of lysate stained with the dsDNA-specific dye (4′,6-diamidino-2-phenylindole (DAPI; Invitrogen, Life Technologies) and compared with SYBR Gold counts. Viral samples were stained with 1 µg ml^{-1} final concentration DAPI for 30 min, after which they were filtered through a 0.02 µm pore-size Anodisc aluminium oxide filter (13 mm diameter; Whatman) (Budinoff et al. 2011).

The presence of cyanophages within cyanobacterial host cells was confirmed with transmission electron microscopy (TEM). Infected cells were collected and pelleted using low-speed centrifugation (3000 × *g*, 10 min, using an A-4-62 swing-out rotor in a 5810R centrifuge, Eppendorf). The cell pellet was resuspended in TE buffer, transferred to 2 ml microcentrifuge tubes (Eppendorf) and fixed with 4% final concentration glutaraldehyde (EM grade, Sigma-Aldrich) for 1 h at room temperature, post-fixed in 1% OsO_4 in 0.1 M cacodylate buffer for 1 h at 4°C, dehydrated in a graded ethanol series, and embed-

ded in epon. Ultrathin sections (100 nm) were post-stained with uranyl acetate and lead citrate. Lysate from infected cultures containing cyanophages was absorbed for 1 min onto freshly glow-discharged, carbon-coated pioloform grids. After blotting, the samples were negatively stained with a 2% PTA solution for 1 min. Both ultrathin sections and negative stained cyanophages were viewed with a Tecnai 12 electron microscope (FEI) at 120 kV, equipped with a 4K Eagle CCD Camera (FEI).

Host range and thermostability

The host specificity of the cyanophage was tested using (1) another strain of *C. raciborskii* isolated from the same lake and 4 strains of *C. raciborskii* isolated in Australia (CSIRO, strains CS-505, CS-506, CS-509 and CS-510), (2) other cyanobacteria species, including 2 different filamentous species from the same lake (*Aphanizomenon skujae* and *Anabaenopsis cunningtonii*), and (3) a filamentous marine cyanobacterial species (*Geitlerinema* sp.) isolated from an intertidal benthic microbial mat in Denmark. Freshly produced 0.2 µm filtered viral lysate (sterile cellulose acetate syringe filter units, Whatman) was added to exponentially growing algal cultures at a ratio of 10% v/v lysate to culture, in a 24-well plate using 3 replicate wells and 3 controls per species, and kept at the culture conditions described above in the section 'Isolation and culturing'. The wells were monitored for host cell lysis for 2 wk using an inverted microscope (Zeiss Axiovert 200) to view wells. Cultures that did not lyse were considered resistant to the cyanophage CrV.

To determine appropriate storage methods while maintaining viable viruses, the sensitivity of CrV to heat and freezing treatments was tested, with fresh viral lysate treated for over 1 h at 40, 4, −20, −80 and −196°C (liquid nitrogen) as per Brussaard et al. (2004). CrV lysate was added to 24-well multiwell plates (Cellstar, Greiner Bio-One) of the *C. raciborskii* culture at a ratio of 10% v/v. Three replicates of control and cyanophage-treated culture were used. Cultures were checked daily for host cell lysis for up to 2 wk using an inverted microscope as described above.

Viral genome size and primer tests

To estimate the genome size of CrV, lysate was 0.2 µm filtered (syringe cellulose acetate filter units, Whatman), followed by concentration using 30 kDa cut-off Amicon Ultra-15 centrifugal filter units (Millipore). Agarose plugs were prepared for pulsed field gel electrophoresis (PFGE) according to Baudoux & Brussaard (2005). Equal volumes (50 µl) of retentate and molten 1.5% (w/v) agarose (InCert, Cambrex Bioscience) were dispensed into plug moulds (Bio-Rad, article number: 70-3713) and left to solidify for 3 min at −20°C. The plugs were incubated overnight at 30°C while placed in microtubes containing 800 µl of lysis buffer (250 mM EDTA, 1% SDS v/v, 1 mg ml^{-1} proteinase K, all from Sigma-Aldrich). After decanting and washing the plugs for 30 min in TE 10:1 buffer (10 mM Tris–Base, 1 mM EDTA, pH 8.0), they were stored at 4°C in TE 20:50 (20 mM Tris, 50 mM EDTA, pH 8.0) until analysis. The viral plugs and Lambda concatamer marker plugs (Bio-Rad) were loaded onto a 1% SeaKem GTG agarose gel (Cambrex Bioscience) prepared in 1× TBE gel buffer (90 mM Tris–Borate and 1 mM EDTA, pH 8.0). Wells of the gel were overlaid with 1% molten agarose, after which the samples were electrophoresed in 0.5× TBE tank buffer (45 mM Tris–Borate and 0.5 mM EDTA, pH 8.0) using a Bio-Rad DR-II CHEF Cell Unit (Bio-Rad) operating at 6 V cm^{-1} with pulse ramps of 20 to 45 s at 14°C for 22 h. The gel was stained for 1 h with SYBR Green I (1× final concentration) (10 000× concentrate in DMSO, Molecular Probes, Invitrogen, Life Technologies), destained for 10 min in autoclaved Milli-Q water, and digitally analysed for fluorescing bands using a FluorS (Bio-Rad).

CrV DNA was extracted from concentrated viral lysate using the MoBio PowerWater DNA Isolation Kit (MoBio Laboratories) and gene *g20* and *g23* primers were tested; CSP1 and CSP8 cyanophage primers were used to target the *g20* gene encoding the viral capsid structure of cyanomyovirus of the marine *Synechococcus* (Zhong et al. 2002), and CAP1 and CAP2 cyanophage primers were used to target the *g23* gene encoding the capsid of myoviruses and siphoviruses of the marine filamentous cyanobacterium *Nodularia* sp. (Jenkins & Hayes 2006). For a positive control, cyanomyovirus M3 of *Synechococcus* DC2 was used. Each 50 µl PCR reaction contained 1× Qiagen PCR buffer (1.5 mM $MgCl_2$), 200 µM of each dNTP (Eurogentec), 5 µM of each primer, 0.4 µg $µl^{-1}$ BSA, 10 µl Q-solution, 1 unit Qiagen *Taq* and 5 µl template. A gradient PCR was carried out with primers CSP1 and CSP8. After an initial denaturation step at 94°C for 4 min, the PCR programmes followed 40 cycles at 94°C for 30 s, annealing at 36–50°C for 30 s, extension at 72°C for 60 s and a final extension step at 72°C for 420 s. The positive control virus M3 of *Synechococcus* DC2 gave product (592 bp) at all

annealing temperatures tested, and we choose annealing at 36°C for 30 s for subsequent PCRs of gene *g20*. When the positive control was added to our virus concentrate (internal control) the intensity of the bands was slightly reduced indicating low inhibition. For PCRs on gene *g23* we tested annealing at 42°C (Jenkins & Hayes 2006) and a lower temperature, 38°C. Products were not formed from either the positive control *Synechococcus* virus or our *Cylindrospermopsis* virus concentrates or purified DNA extracts; therefore, while the result was negative, the PCR on gene *g23* cannot be verified because of the absence of amplification from the control.

Virus growth cycle at different temperatures and irradiance

A one-step cyanophage growth cycle was determined for CrV under the standard host culture incubation conditions, i.e. 22°C and 90 µmol quanta m^{-2} s^{-1}. Triplicate cultures of exponentially growing *C. raciborskii* (1.4×10^4 cells ml^{-1}) received 1.5% v/v freshly prepared 0.2 µm pre-filtered (sterile cellulose acetate syringe filter units; Whatman) viral lysate, which resulted in a multiplicity of infection of approximately 500. The cultures were infected 6 h after the beginning of the light period. The non-infected control cultures received equal volumes of medium. As progeny cyanophages were not released before 16 h post-infection (p.i.) during preliminary virus growth curves, sampling for host and cyanophage counts was performed after this point. Cyanobacterial cells and filaments were counted using a Fuchs-Rosenthal haemocytometer counting chamber as previously described, and cyanophages were enumerated using flow cytometry (see earlier section 'Host and viral abundances'; Brussaard 2004).

Additionally, experiments were conducted to test the effects of reduced and increased temperature (15 and 30°C for low and high treatments, respectively, compared with 22°C for the mid treatment) and light (low 20 and high 250 µmol quanta m^{-2} s^{-1}, compared with mid 90 µmol quanta m^{-2} s^{-1}) on the proliferation of CrV. The temperature levels are comparable to a natural average yearly water temperature in the Reeuwijkse Lakes of 25°C in 2013 (Vermaat et al. 2016). The light levels correspond to a natural irradiance range in the Netherlands of very overcast to extreme, clear sunshine (Velds et al. 1992). Exponentially growing cultures of *C. raciborskii* were acclimated to both conditions for at least 6 mo before the experiments were performed. Maximum growth rates of *C. raciborskii* were obtained from low abundance (to prevent growth limitation) cultures over time by fitting the natural logarithm regression curve for each culture.

At the start of the experiments the virally infected cultures received lysate from a mid temperature and light treatment (standard host growth conditions), at a concentration of 7–38 viruses per host cell to guarantee a one-step virus growth cycle. The non-infected control cultures received an equal volume of medium. All experiments were performed in triplicate in 48-well multiwell plates (Cellstar, Greiner Bio-One). Samples for viral enumeration (as above) were taken from 2–3 wells at regular intervals until 72 h p.i., apart from the high temperature (30°C) treatment, where the culture lysed much earlier. Cyanobacterial lysis was determined by fluorescence measurements of phycocyanin pigments (excitation 655 nm, emission 680 nm) at every sampling point (Gemini XPS Fluorescence Microplate Reader, Molecular Devices). Cell abundance could be estimated from the relative fluorescence of the culture, using a calibration curve (of cell counts at different autofluorescence units) determined prior to the experiments. However, despite having the same autofluorescence (25 r.u.), differing light conditions had different cell abundances, i.e. 3.0 ± 0.6, 2.1 ± 0.6 and 5.2 ± 1.1 × 10^5 cells ml^{-1} for low, mid and high light, respectively. Therefore, cell abundance was estimated separately for each light level. Cyanophages were enumerated by flow cytometry and CrV burst sizes were estimated by dividing the number of cyanophages produced by the number of lysed host cells.

RESULTS

Cyanophage initial characterisation

In TEM thin-sections of virally infected *Cylindrospermopsis raciborskii* cells, virus-like particles of 59 ± 1.3 nm (mean ± SE, n = 22) diameter with icosahedral capsids were observed in the cytoplasm of the host cell, while no such particles were present in non-infected cells from the control culture (Fig. 2A,B). TEM analysis of the negatively stained lysate showed predominantly one type of virus with long non-contractile tails (average length of 612 ± 31 nm, n = 15) and icosahedral capsids with diameters of around 65 ± 1 nm (n = 22; Fig. 2C,D). It is typical for viruses in thin section to be smaller than when negatively stained due to the difference in preparation methods (Field 1982).

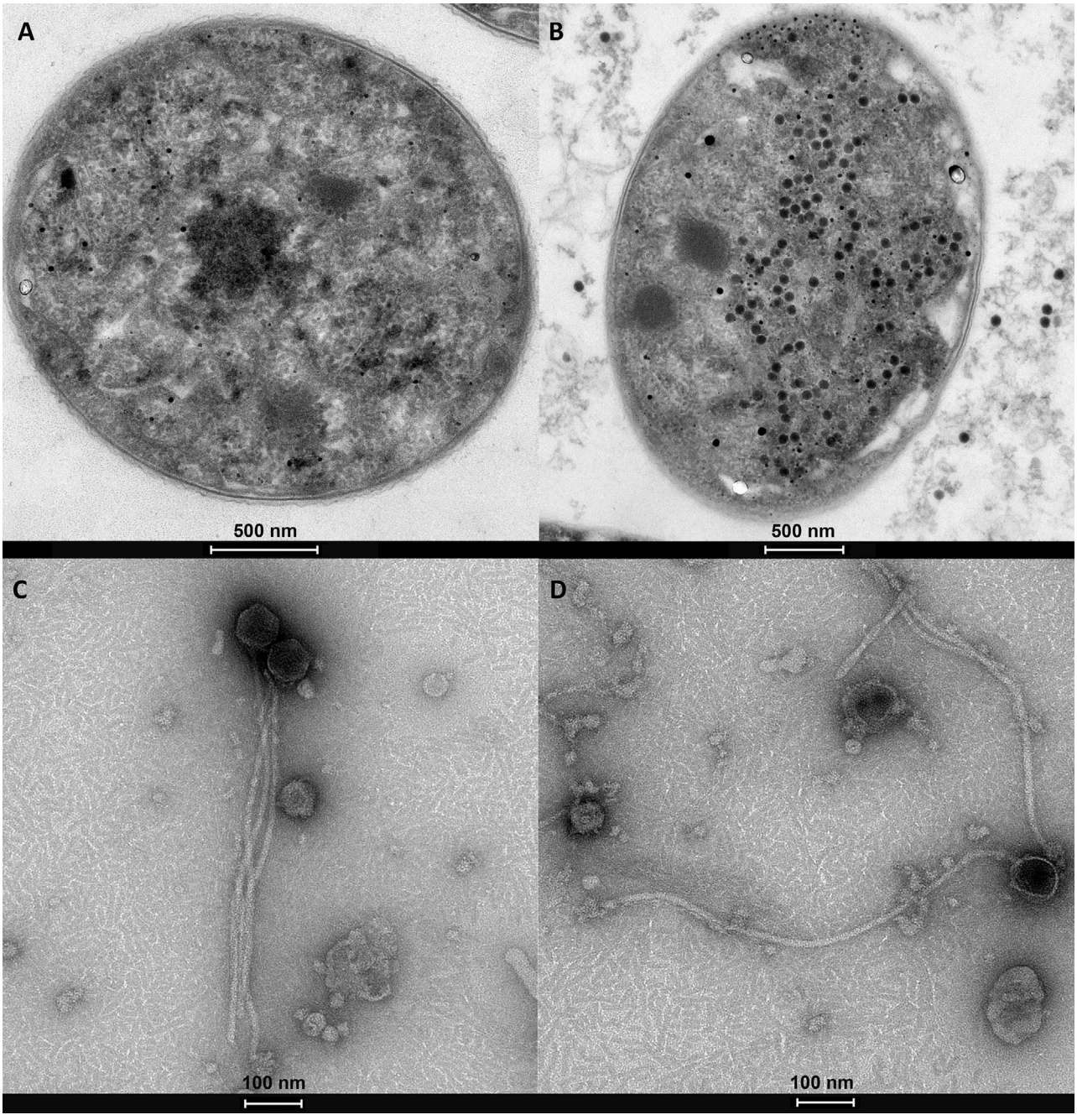

Fig. 2. Transmission electron micrographs of virally infected *Cylindrospermopsis raciborskii* cells. Thin sections show (A) cross-section view of a healthy cell in the control culture, (B) cross-section views of a virally infected cell containing virus-like particles with icosahedral capsids, and (C,D) negatively stained viral particles from CrV lysate with icosahedral capsids and long non-contractile tails. In C there appear to be 3 tails, which may be due to a detached tail becoming intertwined with the 2 viruses, or the capsid may be obscured in the micrograph

The CrV had a dsDNA genome with a size of 110 ± 15 kb (Fig. 3). The freshwater CrV did not amplify with the CSP1–CSP8 primers used to target the *g20* gene encoding the capsid structure of marine *Synechococcus* cyanomyovirus.

The host range tests showed that CrV had a restricted host range as none of the tested species (*Aphanizomenon skujae, Anabaenopsis cunningtonii*, *Geitlerinema* sp.) and strains (CS-505, 506, 509, 510 and Cr2012) lysed upon addition of the

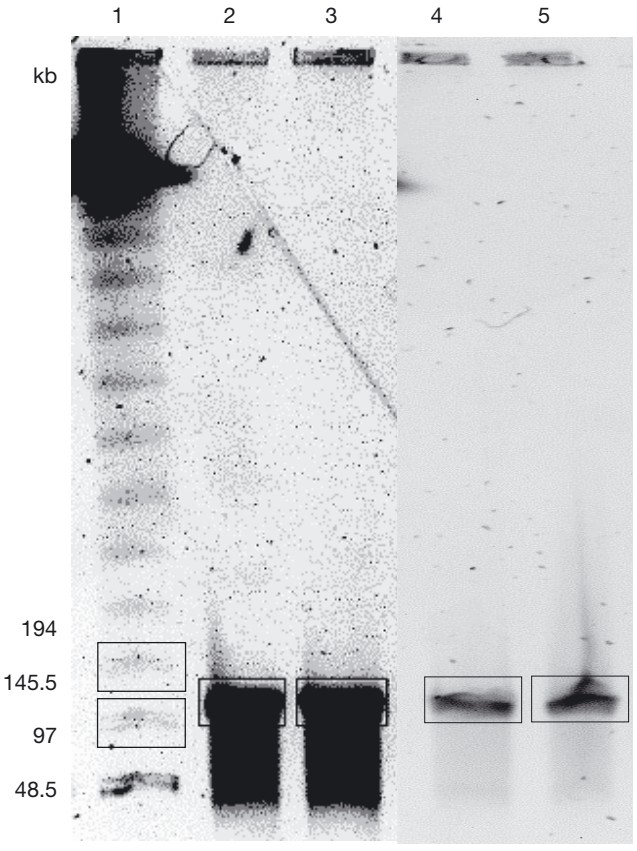

Fig. 3. Pulsed field gel electrophoresis gels showing as marker Lambda ladder (lane 1) and duplicates of the CrV genome (lanes 2–5). Lanes 4 and 5 have 10 times less DNA added. The boxes in the gel image indicate the bands in the ladder and in the lanes

viral lysate, including another strain of *C. raciborskii* that was isolated from the same lake at the same time the virus was isolated (strain Cr2012). The cyanophages remained equally infective after exposure to all temperatures tested (40, 4, −20, −80 and −196°C).

CrV growth cycles

Under the standard growth conditions of the host (22°C, 90 µmol quanta m^{-2} s^{-1}), the virally infected cultures of *C. raciborskii* showed full lysis by 44 h p.i., while the non-infected control cultures increased in cell numbers (Fig. 4A). The latent period (time point until first progeny viruses are released from infected host cell) of the cyanophage CrV was 20–24 h p.i. (Fig. 4B). Host lysis was accompanied by a strong drop in the number of cells per filament (Fig. 4C). The host cells in the filaments were individually infected and, consequently, this resulted in breaking

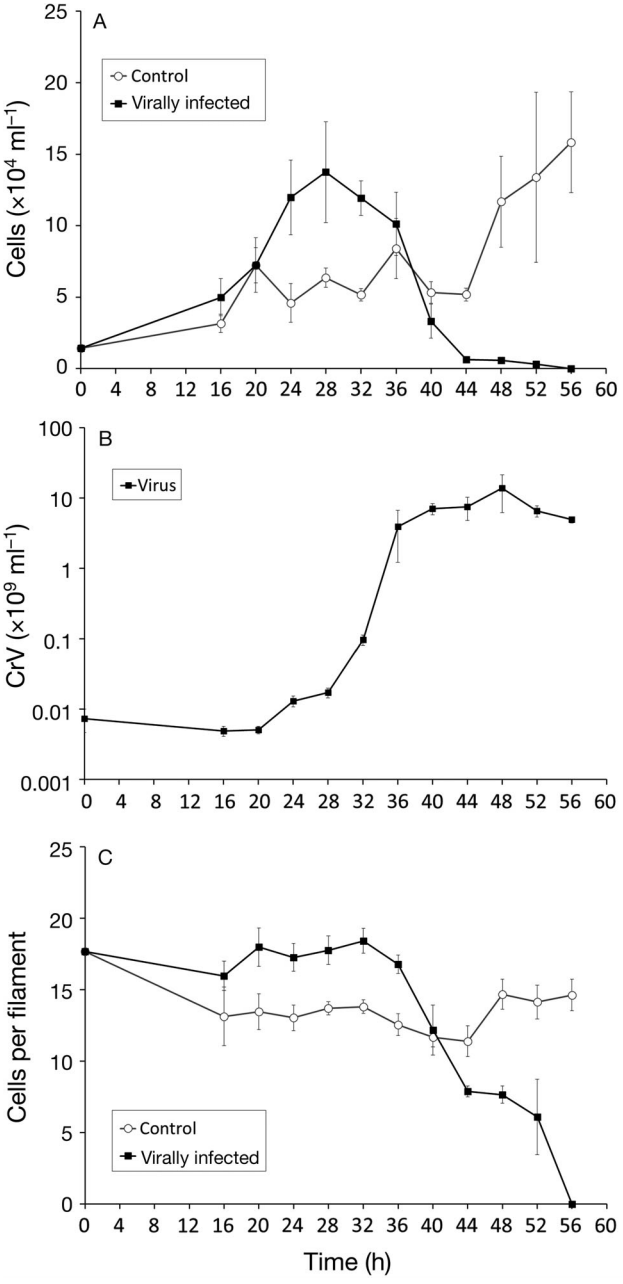

Fig. 4. Temporal dynamics of (A) cell abundance of non-infected control (open symbols) and virally infected (filled symbols) *Cylindrospermopsis raciborskii*, (B) CrV abundance and (C) number of *C. raciborskii* cells per filament. CrV was not detected in the non-infected control cultures. Error bars show the standard error (n = 3)

up of the filament upon lysis of the cell (Fig. 5). We also observed filaments with only the heterocysts remaining after all other cells lysed (Fig. 5C). Furthermore, we observed that the lengths of the infected host cells just before lysis were on average 30% longer than the non-infected cells (9.2 and 6.3 µm, respectively).

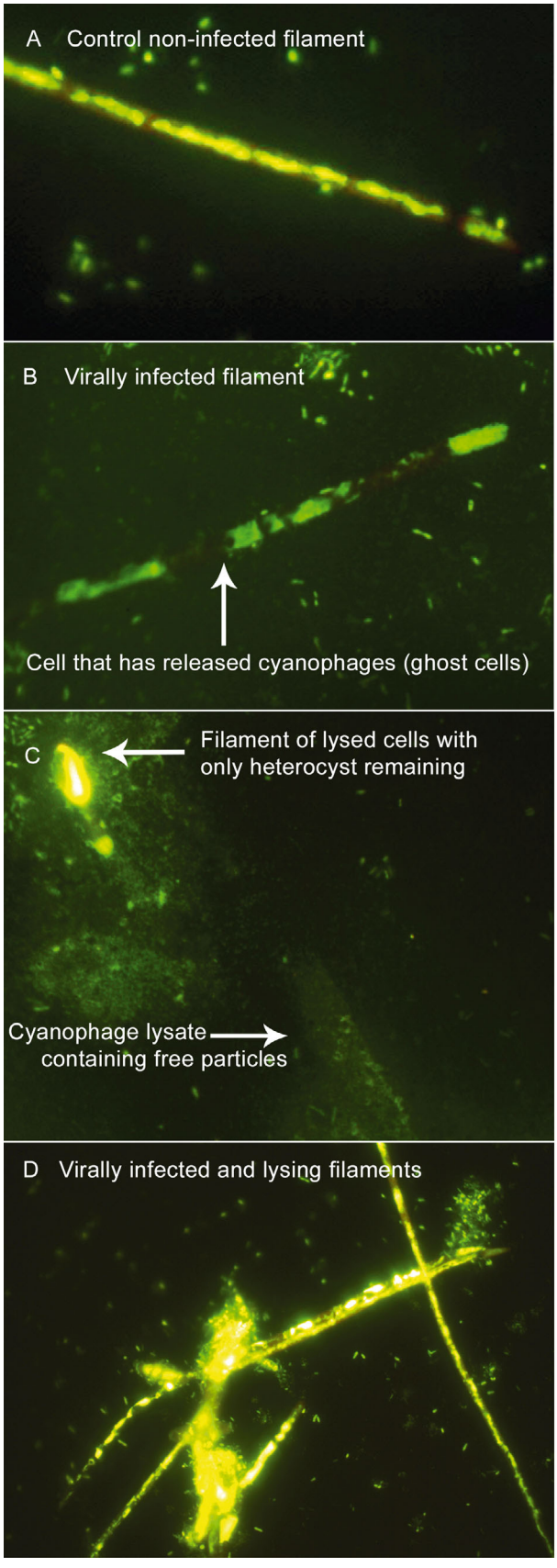

Fig. 5. Visualisation of the *Cylindrospermopsis raciborskii* filaments stained with SYBR Gold using epifluorescence microscopy in (A) non-infected control culture, and (B,C) virally infected cultures. (B) An infected filament with empty lysed 'ghost' cells (arrow). (C) Cells in a filament that has just lysed, leaving only the heterocyst. (D) A group of infected and lysing filaments

The maximum growth rates (μ_{max}) of the exponentially growing cyanobacterial host prior to experiments were 0.20, 0.43 and 0.51 d^{-1} for low, mid and high irradiance, respectively. For low, mid and high temperature the μ_{max} was 0.34, 0.43 and 0.58 d^{-1}, respectively.

The irradiance level strongly affected lysis dynamics of infected *C. raciborskii* cultures. The time point that the infected cultures phycocyanin autofluorescence started to deviate from the control cultures (Fig. 6A) was 1.5-fold longer under low light (36 h) compared with mid light (24 h), but 3-fold shorter under high light (8 h) compared with mid light. The latent period of CrV at low light was 1.3-fold longer than at mid and high light (Fig. 6B). The estimated CrV burst sizes at low and high irradiance were reduced to 48 and 14% of the 332 CrV per lysed host cell at mid light, respectively (Fig. 6C).

Temperature also strongly affected the CrV growth characteristics. The time at which the infected and control host phycocyanin autofluorescence started to deviate was slightly longer at low than at mid temperature (Fig. 7A), but was much shorter at high temperature (3-fold reduced). The low temperature treatment resulted in a 1.3-fold longer CrV latent period, while at high temperature it was shortened to just 11% of mid temperature (Fig. 7B). Despite the shorter latent period, the high temperature had a 1.6-fold higher burst size at 541 CrV per lysed host cell (Fig. 7C). At low temperature the CrV burst size was reduced to 43% of that of mid temperature (142 CrV per lysed host cell).

DISCUSSION

We present the initial characteristics of a novel lytic dsDNA cyanophage, CrV, infecting the filamentous freshwater cyanobacteria species *Cylindrospermopsis raciborskii*, invasive to Dutch lakes. The TEM image of negatively stained viral particles were of comparable size and identical shape to the virus within thin sections of infected host cells, and these

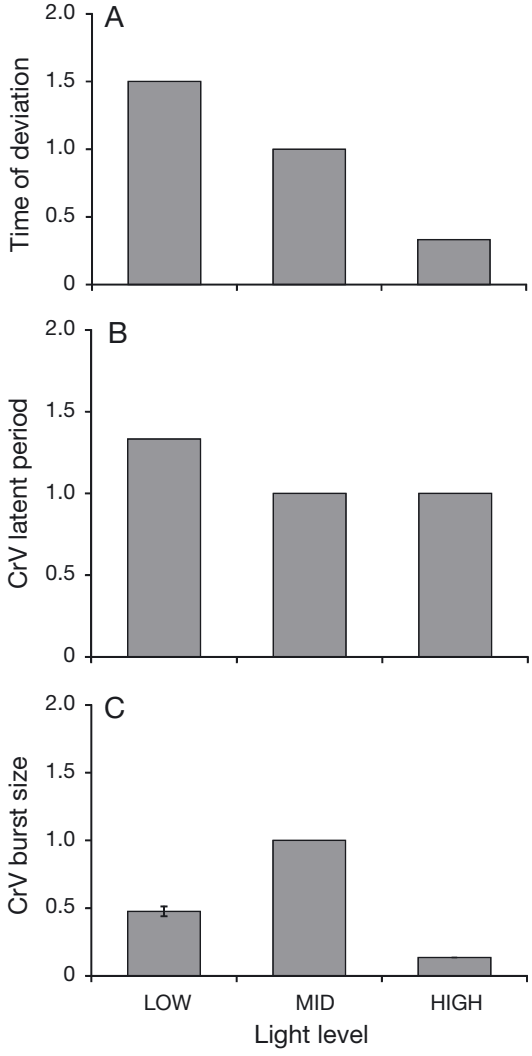

Fig. 6. Effect of light level on (A) the time point at which CrV-infected cultures start to deviate in their phycocyanin autofluorescence (or cell abundance) from the non-infected control cultures, (B) CrV latent period and (C) CrV burst size. All values are normalised to the mid treatment (i.e. irradiance level of 90 µmol quanta m^{-2} s^{-1}), in which the time of deviation was 24 h, the CrV latent period was 20–24 h and the burst size was 332 per lysed host cell. Low and high irradiance was 20 and 250 µmol quanta m^{-2} s^{-1}, respectively. Error bars show the standard error (n = 2)

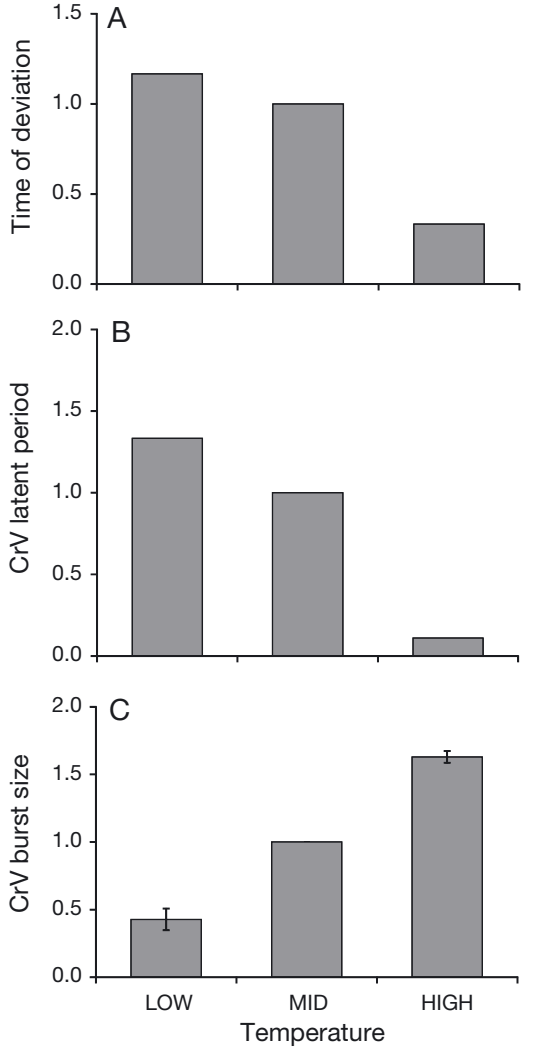

Fig. 7. Effect of temperature on (A) the time point at which CrV infected cultures start to deviate in their phycocyanin autofluorescence (or cell abundance) from the non-infected control cultures, (B) CrV latent period and (C) CrV burst size. All values are normalised to the mid treatment (i.e. a temperature of 22°C). Low and high temperature was 15 and 30°C, respectively. See Fig. 6 legend for further details

particles were observed in higher numbers after lysis of the cyanobacterial host cells. With its long, non-contractile tail, CrV appears to belong to the family *Siphoviridae.* For future research, sequencing of the entire CrV genome is recommended to obtain a more extensive molecular characterisation. The approximately 110 ± 15 kb genome size of CrV falls within the range of published freshwater cyanophages, such as the freshwater Ma-LMM01 of *Microcystis aeruginosa* (162 kbp) (Yoshida et al. 2008b), and the tail-less PaV-LD of *Planktothrix agardhii* (95.3 kbp) (Gao et al. 2009). The tails of the cyanophage were not seen in the thin section of infected cells, but this is not to be expected unless the slice through the embedded pellet contained a longitudinal section of a cyanophage tail. CrV showed a highly species- and strain-specific host range, similar to other freshwater cyanophages infecting filamentous cyanobacteria such as siphoviruses isolated from the freshwater cyanobacteria *Anabaena lemmermannii* and *Anabaena solitaria* (Deng & Hayes 2008). We observed that the length of the virally infected cells increased

prior to lysis, which may be due to the accumulation of viruses 'swelling' the cell. Therefore, estimating the cell abundance by dividing the filament length by the average cell length in infected cultures is only accurate when the increase in infected cell length is accounted for; otherwise, there may be an overestimate in cell abundance in the virally infected culture compared with the control.

Viral infection did not seem to be spreading rapidly from cell to cell within the filaments, as we observed frequent breaking of the filaments in the infected cultures when single cells within the filaments were lysed, leaving neighbouring cells uninfected. Breaking of filaments upon lysis of infected cells has been proposed as a process that enhances the dispersal rate of the host species by spreading the remaining non-infected cells in the broken-up filaments to new areas (Pollard & Young 2010).

As Australian strains of *C. raciborskii* have been found to be lysogenic (Steenhauer et al. 2014), we tested this strain for lysogeny using mitomycin C (1 µg ml^{-1}, according to the protocol by Paul & Weinbauer 2010) to induce prophages (the experiment was performed under standard culture conditions only). We found a small (4 %) increase in phages with a concurrent lysis of the host population, which suggests that there may be a temperate virus in the population (authors' unpubl. results). Decreasing the temperature (from 37 to 25°C) during viral infection of the tropical *Burkholderia pseudomallei* resulted in a shift from lytic to lysogenic infection (Shan et al. 2014). If a similar pattern is true for *C. raciborskii*, the ability to form lysogens at lowered temperatures may potentially also provide a means of virus survival at suboptimal growth conditions, such as those found in the temperate regions this species invades.

The prolonged time of deviation in host growth dynamics between infected and control cultures, the prolonged latent period and the reduced burst size for both the low light and low temperature treatments was most likely due to the reduced maximum growth rates of *C. raciborskii* under these conditions prior to infection (i.e. 0.20 and 0.34 d^{-1} for low light and low temperature, respectively, as compared with 0.43 d^{-1} for mid light and mid temperature). In contrast to these sub-optimal growth conditions, high light and high temperature stimulated the maximum growth rate of *C. raciborskii* (0.51 and 0.58 d^{-1}, respectively). However, only for high temperature did this result in a shortened latent period and higher burst size. To our knowledge, no information is available about the effect of light or temperature on the growth characteristics of freshwater viruses infecting cyanobacteria or eukaryotic photoautotrophs. A prolonged latent period at reduced light was, however, consistent with findings by Baudoux & Brussaard (2005) studying the marine eukaryotic phytoplankton *Phaeocystis globosa*. Lowering of burst size under reduced light conditions was also observed for *P. globosa* by the same authors. Also, the burst size of the AS-1 cyanophage infecting the marine cyanobacterium *Synechococcus elongatus* was strongly reduced under low light (Kao et al. 2005). Delayed lysis under low light was observed for the virally infected brown-tide-causing algae *Aureococcus anophagefferens* (Gobler et al. 2007). Finally, Nagasaki & Yamaguchi (1998) showed that temperature affected the time until lysis of the marine *Heterosigma akashiwo* (infected with HaV) in a similar way to that shown for *C. raciborskii* in the present study.

Reduced light conditions are common in the majority of Dutch lakes where eutrophication has caused turbid, cyanobacterial-dominated conditions with transparencies ranging from 0.25 to 0.5 m. Furthermore, as they are shallow, the lakes are permanently mixed with high particle loads due to wind-induced re-suspension (Mooij et al. 2005). Under such conditions, our results indicate that the impact of viral infection will be relatively low (longer latent period and reduced burst size). High light conditions at the start of the growing season, during sunny days and when cells grow at the water surface, will also reduce the viral production. Thus, *C. raciborskii* could potentially be released from the impact of viral mortality due to irradiance extremes. However, as the season progresses, temperature increases may counterbalance the lessened viral mortality effects of the light extremes (strongly reduced latent period and greater CrV burst size). Therefore, it will also be necessary to determine the consequences for viral production if both light and temperature changes are coupled, increasing or decreasing in concert. Due to global warming, temperature is predicted to increase 0.3 to 4.8°C by 2100, with more frequent hotter periods (IPCC 2013), thereby relatively quickly increasing the average temperature of the typically shallow (1–5 m) Dutch lakes (Mooij et al. 2005). In these water bodies, an increase in viral production with temperature will result in stronger viral control of the cyanobacterial host population, which may (partly) compensate for the enhanced growth of *C. raciborskii* at higher temperatures (present study).

Worldwide, populations of *C. raciborskii* are monitored for human health because of the species' potential toxicity and detrimental effects on indigenous species and ecosystems by outcompeting native spe-

cies, growing to excessive biomass and creating hypoxic dead zones due to excess bacterial respiration in decomposing populations. Models are used to predict its growth in relevant water bodies. However, the extent of viral control on the host population is an unknown factor that needs to be quantified, and as we have shown, it will vary depending on environmental conditions. Closer examination of the effects of environmental variables on viral infection of *C. raciborskii* will allow insights into the role of viral control on the host population dynamics of this invasive species and will enhance our ability to predict the spread of this potentially toxic cyanobacterium. Moreover, it will be of ecological importance to test how the growth characteristics of CrV will be affected when the host grows under the control of multiple stressors; for example, low irradiance in combination with higher temperature.

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LITERATURE CITED

Ackermann HW, Prangishvili D (2012) Prokaryote viruses studied by electron microscopy. Arch Virol 157:1843–1849

Baudoux AC, Brussaard CPD (2005) Characterization of different viruses infecting the marine harmful algal bloom species *Phaeocystis globosa*. Virology 341:80–90

Bolch C, Blackburn S (1996) Isolation and purification of Australian isolates of the toxic cyanobacterium *Microcystis aeruginosa* Kütz. J Appl Phycol 8:5–13

Briand JF, Leboulanger C, Humbert JF, Bernard C, Dufour P (2004) *Cylindrospermopsis raciborskii* (Cyanobacteria) invasion at mid-latitudes: selection, wide physiological tolerance, or global warming? J Phycol 40:231–238

Brussaard CPD (2004) Optimization of procedures for counting viruses by flow cytometry. Appl Environ Microbiol 70:1506–1513

Brussaard CPD, Noordeloos AAM, Sandaa RA, Heldal M, Bratbak G (2004) Discovery of a dsRNA virus infecting the marine photosynthetic protist *Micromonas pusilla*. Virology 319:280–291

Brussaard CPD, Payet JP, Winter C, Weinbauer MG (2010) Quantification of aquatic viruses by flow cytometry. In: Wilhelm SW, Weinbauer MG, Suttle CA (eds) Manual of aquatic viral ecology. American Society of Limnology and Oceanography, Waco, TX, p 102–109

Budinoff CR, Loar SN, LeCleir GR, Wilhelm SW, Buchan A (2011) A protocol for enumeration of aquatic viruses by epifluorescence microscopy using Anodisc™ 13 membranes. BMC Microbiol 11:168–173

Deng L, Hayes PK (2008) Evidence for cyanophages active against bloom-forming freshwater cyanobacteria. Freshw Biol 53:1240–1252

Field AM (1982) Diagnostic virology using electron microscopic techniques. Adv Virus Res 27:1–69

Gao EB, Yuan XP, Li RH, Zhang QY (2009) Isolation of a novel cyanophage infectious to the filamentous cyanobacterium *Planktothrix agardhii* (Cyanophyceae) from Lake Donghu, China. Aquat Microb Ecol 54:163–170

Gobler CJ, Anderson OR, Gastrich MD, Wilhelm SW (2007) Ecological aspects of viral infection and lysis in the harmful brown tide alga *Aureococcus anophagefferens*. Aquat Microb Ecol 47:25–36

Hamilton PB, Ley LM, Dean S, Pick FR (2005) The occurrence of the cyanobacterium *Cylindrospermopsis raciborskii* in Constance Lake: an exotic cyanoprokaryote new to Canada. Phycologia 44:17–25

IPCC (2013) Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press, Cambridge

Jacquet S, Zhong X, Parvathi A, Ram A (2013) First description of a cyanophage infecting the cyanobacterium; *Arthrospira platensis* (Spirulina). J Appl Phycol 25:195–203

Jenkins CA, Hayes PK (2006) Diversity of cyanophages infecting the heterocystous filamentous cyanobacterium *Nodularia* isolated from the brackish Baltic Sea. J Mar Biol Assoc UK 86:529–536

Jensen TE, Bowen CC (1970) Cytology of blue-green algae. II. Unusual inclusions in the cytoplasm. Cytologia 35: 132–152

Kao CC, Green S, Stein B, Golden SS (2005) Diel infection of a cyanobacterium by a contractile bacteriophage. Appl Environ Microbiol 71:4276–4279

Matteson AR, Rowe JM, Ponsero AJ, Pimentel TM, Boyd PW, Wilhelm SW (2013) High abundances of cyanomyoviruses in marine ecosystems demonstrate ecological relevance. FEMS Microbiol Ecol 84:223–234

Middelboe M, Chan AM, Bertelsen SK (2010) Isolation and life cycle characterization of lytic viruses infecting heterotrophic bacteria and cyanobacteria. In: Wilhelm SW, Weinbauer MG, Suttle CA (eds) Manual of aquatic viral ecology. American Society of Limnology and Oceanography, Waco, TX, p 118–133

Mojica KDA, Brussaard CPD (2014) Factors affecting virus dynamics and microbial host–virus interactions in marine environments. FEMS Microbiol Ecol 89:495–515

Mooij WM, Hülsmann S, De Senerpont Domis LN, Nolet BA and others (2005) The impact of climate change on lakes in the Netherlands: a review. Aquat Ecol 39:381–400

Nagasaki K, Yamaguchi M (1998) Effect of temperature on the algicidal activity and the stability of HaV (*Heterosigma akashiwo* virus). Aquat Microb Ecol 15:211–216

Padisak J (1997) *Cylindrospermopsis raciborskii* (Woloszynska) Seenayya et Subba Raju, an expanding, highly adaptive cyanobacterium: worldwide distribution and review of its ecology. Arch Hydrobiol 107(Suppl): 563–593

Paerl HW, Paul VJ (2012) Climate change: Links to global expansion of harmful cyanobacteria. Water Res 46: 1349–1363

Patel A, Noble RT, Steele JA, Schwalbach MS, Hewson I, Fuhrman JA (2007) Virus and prokaryote enumeration from planktonic aquatic environments by epifluorescence microscopy with SYBR Green I. Nat Protoc 2: 269–276

Paul JH, Weinbauer MG (2010) Detection of lysogeny in marine environments. In: Wilhelm SW, Weinbauer MG, Suttle CA (eds) Manual of aquatic viral ecology. American Society of Limnology and Oceanography, Waco, TX, p 30–33

Pollard PC, Young LM (2010) Lake viruses lyse cyanobacteria, *Cylindrospermopsis raciborskii*, enhances filamentous-host dispersal in Australia. Acta Oecol 36: 114–119

Safferman RS, Morris ME (1967) Observations on the occurrence, distribution, and seasonal incidence of blue-green algal viruses. Appl Microbiol 15:1219–1222

Shan J, Korbsrisate S, Withatanung P, Adler NL, Clokie MRJ, Galyov EE (2014) Temperature dependent bacteriophages of a tropical bacterial pathogen. Front Microbiol 5:599

Singh R, Singh P (1967) Isolation of cyanophages from India. Nature 216:1020–1021

Sinha R, Pearson LA, Davis TW, Burford MA, Orr PT, Neilan BA (2012) Increased incidence of *Cylindrospermopsis raciborskii* in temperate zones — Is climate change responsible? Water Res 46:1408–1419

Steenhauer L, Pollard PC, Brussaard CPD, Säwström C (2014) Lysogenic infection in sub-tropical freshwater cyanobacteria cultures and natural blooms. Mar Freshw Res 65:624–632

Stein JR (1973) Handbook of phycological methods: culture methods and growth measurements, Vol 2. Cambridge University Press

Tijdens M, van de Waal DB, Slovackova H, Hoogveld HL, Gons HJ (2008a) Estimates of bacterial and phytoplankton mortality caused by viral lysis and microzooplankton grazing in a shallow eutrophic lake. Freshw Biol 53: 1126–1141

Tucker S, Pollard P (2005) Identification of cyanophage Ma-LBP and infection of the cyanobacterium *Microcystis aeruginosa* from an Australian subtropical lake by the virus. Appl Environ Microbiol 71:629–635

Velds CA, Van der Hoeven P, Koopstra J, Raaf W, Slob W (1992) Zonnestraling in Nederland. In, Klimaat van Nederland 3. KNMI, Baarn

Vermaat JE, Bos B, Van Der Burg P (2016) Why do reed beds decline and fail to re-establish? A case study of Dutch peat lakes. Freshw Biol 61:1580–1589

Wiedner C, Rücker J, Brüggemann R, Nixdorf B (2007) Climate change affects timing and size of populations of an invasive cyanobacterium in temperate regions. Oecologia 152:473–484

Wijmans PADM, Beekman J (2003) Visstandbeheerplan Reeuwijkse Plassen 2003–2013. Visserijpool Reeuwijkse Plassen, Nieuwegein

Wood SA, Stirling DJ (2003) First identification of the cylindrospermopsin-producing cyanobacterium *Cylindrospermopsis raciborskii* in New Zealand. N Z J Mar Freshw Res 37:821–828

Wu W, Zhu Q, Liu X, An C (2009) Isolation of a freshwater cyanophage (F1) capable of infecting *Anabaena flos-aquae* and its potentials in the control of water bloom. Int J Environ Pollut 38:212–221

Yoshida M, Yoshida T, Kashima A, Takashima Y, Hosoda N, Nagasaki K, Hiroishi S (2008a) Ecological dynamics of the toxic bloom-forming cyanobacterium *Microcystis aeruginosa* and its cyanophages in freshwater. Appl Environ Microbiol 74:3269–3273

Yoshida T, Nagasaki K, Takashima Y, Shirai Y and others (2008b) Ma-LMM01 infecting toxic *Microcystis aeruginosa* illuminates diverse cyanophage genome strategies. J Bacteriol 190:1762–1772

Zarenezhad S, Sano T, Watanabe MM, Kawachi M (2012) Evidence of the existence of a toxic form of *Cylindrospermopsis raciborskii* (Nostocales, Cyanobacteria) in Japan. Phycol Res 60:98–104

Zhong Y, Chen F, Wilhelm SW, Poorvin L, Hodson RE (2002) Phylogenetic diversity of marine cyanophage isolates and natural virus communities as revealed by sequences of viral capsid assembly protein gene *g20*. Appl Environ Microbiol 68:1576–1584

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