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Absence of evidence for viral infection in colony-embedded cyanobacterial isolates from the Curonian Lagoon*

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The aim of the present study was to assess the frequency of viral infections in colony-embedded cells of the cyanobacteria *Aphanizomenon flos-aquae* and *Microcystis aeruginosa* collected from the brackish Curonian Lagoon. Natural and mitomycin C-treated *A. flos-aquae* and *M. aeruginosa* samples were examined for the presence of viruses and lysis by a combination of light-, epifluorescence and transmission electron microscopy techniques. Here we report a lack of evidence for virus infection, progeny formation and cell lysis in colony-embedded cells of *A. flos-aquae* and *M. aeruginosa*. These results indicated that viruses contribute little to the mortality of these cyanobacteria when the latter occur in colonies. Consequently, the results supported the hypothesis that colony formation can, at least temporarily, provide an efficient strategy for protection against virus-induced mortality. Finally, assuming that grazing has a negligible effect on colony-embedded cells in the Curonian Lagoon, we propose that most of the cyanobacterial biomass produced is lost from the pelagic food web by sedimentation.

Communication

Cyanobacterial blooms frequently occur in fresh and brackish waters of the coastal lagoons of the Baltic Sea. Filament and/or colony formation prevents the grazing of cyanobacteria populations by other organisms (Callieri 2010, Yang & Kong 2012), eventually leading to depressed ecotrophic efficiency of the microbial food web during conditions that favour bloom formation (Sellner et al. 1994, Jürgens & Güde 1994). Although colony formation has also been proposed as a strategy that enables populations to escape viral attacks (Hamm et al. 1999, Jacobsen et al. 2007), some studies based on isolated phage-host systems indicate that viruses are capable of successfully infecting and lysing embedded colonies and mucus-producing cells (Baudoux & Brussaard 2005) by means of, for example, phage enzyme activity (Hughes et al. 1998). Cell lysis may also occur in cells of embedded colonies upon induction of lysogenic cells (Hewson et al. 2004). In the present study, the colony-embedded cyanobacteria *Aphanizomenon flos-aquae* and *Microcystis aeruginosa* were isolated from the Curonian Lagoon, and natural and mitomycin C-treated samples were examined for virus infection and virus production.

In eutrophic aquatic ecosystems, cyanophages (viruses that infect cyanobacteria) contribute significantly to the control of cyanobacterial blooms (Jassim & Limoges 2013). For example, Coulombe & Robinson (1981), based on long-term observations, argued that viruses are among the key factors that terminate blooms of *A. flos-aquae* in nutrient-rich lake

ecosystems. Furthermore, Granhall (1972) reported that bloom collapse of *A. flos-aquae* in the eutrophic Lake Erken (Sweden) coincided with increased numbers of podo-like viruses in thin sections of its cells. Although those viruses that infect *Microcystis* have been studied in more detail (Deng & Hayes 2008, Yoshida et al. 2008b, Kimura et al. 2012), there is still a paucity of evidence for the susceptibility of cells of *M. aeruginosa* colonies to viral infection (Yoshida et al. 2006).

The Curonian Lagoon (55°30′N, 21°15′E) is a temperate and highly eutrophic body of water characterised by the massive re-occurrence of two species of cyanobacteria, *Aphanizomenon flos-aquae* and *Microcystis aeruginosa*, during summer and autumn (Gasiūnaitė et al. 2005). The rate of grazing on colony-embedded *A. flos-aquae* and *M. aeruginosa* present in the Curonian Lagoon appears to be negligible (Gasiūnaitė & Olenina 1998), probably because of the inhibitory effect of cyanobacterial colonies on zooplankton populations (Łotocka 2001). Although the correlation between myoviruses and chlorophyll *a* concentration during intensive bloom formation of *A. flos-aquae* has previously been demonstrated (Sulcius et al. 2011), the extent to which viruses contribute to the regulation of cyanobacterial blooms and the interactions between viruses and planktonic colony-embedded cells in the Curonian Lagoon are still poorly understood.

Colonies of *A. flos-aquae* and *M. aeruginosa* were isolated separately by means of a microcapillary-capturing technique and resuspended in virus-free lagoon water. Virus-free water was prepared by the filtration of water samples through 100 000 kDa PES (polyethersulphone) filters (Sartorius) using a tangential flow filtration system (VivaFlow 200, Sartorius). In order to remove attached bacteria, colonies were further washed with 300 ml of virus-free water. Filtration and washing resulted in the removal of 99% and 92% of bacteria-like and virus-like particles respectively (calculated by scoring through a microscope). Triplicates of 50 colonies each of *A. flos-aquae* and *M. aeruginosa* were transferred to incubation bottles containing 50 ml of virus-free lagoon water.

Natural or mitomycin C-treated samples (Sigma-Aldrich) were incubated for 24 h in situ by immersing the incubation bottles beneath the surface water layer, thereby subjecting them to natural solar radiation levels and water temperature conditions (~ 18°C). The mitomycin C method was used in order to maximise the number of induction events (Paul & Weinbauer 2010). This method produces a greater percentage of lysogens, as compared with other physical and chemical induction agents (Weinbauer & Suttle 1999). The final mitomycin C concentration was increased to 20 μg ml^{-1}, as recommended by Dillon & Parry (2008). Aliquots (1 ml) for analysis of lytic and lysogenic virus production were sampled every 3 h

and treated as described in Patel et al. (2007). Samples were fixed with glutaraldehyde (Sigma-Aldrich, Grade I) to a final 2% concentration and kept in the dark at +4°C for 30 min. Slides for epifluorescence microscopy were prepared immediately after fixation following SYBR Green I staining protocol and stored frozen (−20°C) until analysis (Patel et al. 2007). Thin sections of natural and mitomycin C-treated colonies were prepared for transmission electron microscopy in order to assess the presence of virus-like structures both within the cells and the surrounding mucus layer. Colonies for thin sections were collected by centrifugation at 5000 × g for 10 min and fixed with Karnovsky's fixative (2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2)) for 24 h (at +4°C). Postfixation was done with 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.2) for 1 h (at +4°C) followed by washing with 0.1 M cacodylate buffer (pH 7.2). Samples were then dehydrated in ethanol (30 to 70%), transferred to 2% of uranyl acetate in 70% ethanol for 12 h and subsequently incubated in 90% and 100% ethanol, ethanol:propylene oxide (1:1 w/w) for 30 min and in propylene oxide. Samples were embedded in standard single-mix 'Epon' embedding media as described in Luft (1961) with benzyldimethylamine (BDMA) accelerator instead of DMP-30 (Glauert & Lewis 1998) and ultrathin sections (∼ 70 nm thickness) were stained with a lead salt mixture according to Sato (1968). Light microscopy was used to determine the morphological characteristics of both colonies and trichomes pre- and post- incubation. Several microscopy techniques were employed in order to minimise the potential limitations of the methodology when observing phage production and lysis.

The results for both natural and mitomycin C-treated samples of colony-embedded cells of *A. flos-aquae* and *M. aeruginosa* (Figure 1) did not indicate any significant increase in virus abundance following an incubation period of 24h. These findings were consistent with transmission electron microscopy observations. Although some virus-like structures were found in the mucus layer that surrounds colonies of *A. flos-aquae*, no viruses were detected in thin sections of the cells. Thus, neither epifluorescence nor transmission electron microscopy analyses revealed either the presence of virus-infected cells or lytic virus production and mitomycin C-induced prophages. Pollard & Young (2010) showed that when lysis occurs, trichomes break into smaller fragments and the morphology of the colony changes. However, light microscopy showed no obvious changes in colony morphology either pre- or post-incubation, thus indicating the absence of cell damage.

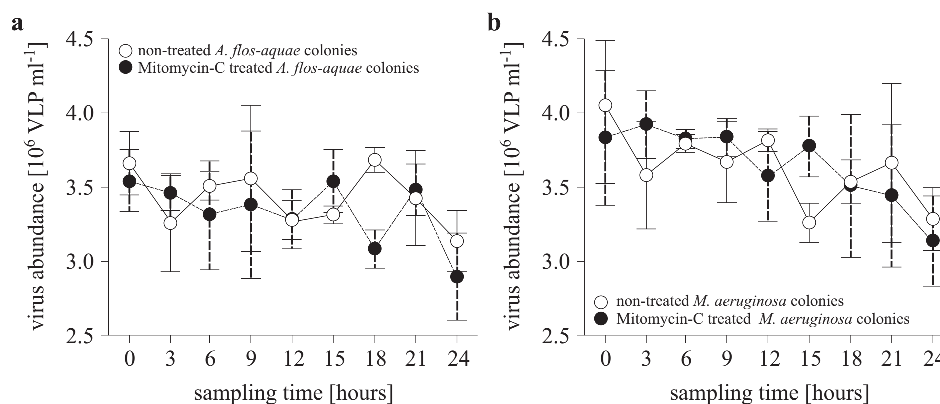

Figure 1. Box plot representing changes in the abundance of virus-like particles (VLP) during the 24 h incubation period in non-treated (open circles) and mitomycin C (closed circles) treated samples of a) *Aphanizomenon flos-aquae* and b) *Microcystis aeruginosa* colonies. Error bars show standard deviation

The combined results indicated that colony-embedded cyanobacterial isolates were not subject to viral attack in the Curonian Lagoon, or at least, not during the period of study. The absence of virus infection and lysis in our samples may be associated with structural differences between free-living single cells and those that occur in colonies. It has been suggested that the colony matrix forms a physical barrier that prevents the host population from coming into contact with virus particles, whereas increased colony size reduces the probability of successful viral infections (Jacobsen et al. 1996, Hamm et al. 1999, Ruardij et al. 2005, Baudoux et al. 2006, Brussaard et al. 2007). Brussaard et al. (2005) and Jacobsen et al. (2007) have shown that viruses fail to prevent the formation of blooms in *Phaeocystis globosa* and *P. pouchetii* when most of the *P. globosa* and *P. pouchetii* cells occur in colonies, suggesting an efficient strategy of cells of embedded colonies for protection against virus-induced mortality (Hamm et al. 1999, Ruardij et al. 2005). This also suggested that viral infection, and thus progeny production, can be avoided even at the initial stages of bloom formation and this, in turn, may explain why no virus could be detected within the embedded cells of older colonies. Moreover, since cyanobacterial colonies were isolated randomly, presumably at different stages following infection, the stage of bloom development at the time of sampling and the length of incubation during the experiments may also influence the detection of viral lysis. For example, if the latent period exceeds 24 h and phages are visible only in the last phase of infection ($\sim$10% of the pre-lysis period; Waterbury & Valois 1993), a longer incubation period would be required in order to detect cell lysis and virus production. Indeed, even if adsorption of the virus

to the cell surface of colony-embedded cells were possible, the actual rates of infection at the initial and exponential phases of bloom development would generally be low, increasing significantly only during the bloom termination phase (Granhall 1972, Coulombe & Robinson 1981). Therefore, assuming that only a small fraction of colonies in the exponential phase (data not shown) was isolated from the natural population, it is possible that the actual infection and lysis rate of colony-embedded cyanobacteria in the Curonian Lagoon is under-represented in the results.

Hewson et al. (2004) have demonstrated prophage induction in colonies of *Trichodesmium*. However, the absence of mitomycin C-inducible prophages in isolated colonies of *A. flos-aquae* and *M. aeruginosa* may indicate that lysogeny is not the main strategy of viral attack in these species. On the other hand, not all prophages are induced by mitomycin C or by other inducing agents such as UV radiation, intense light, heat, chemicals etc. (Paul & Weinbauer 2010). It has also been shown that colony formation may lead to antibiotic resistance, including resistance to mitomycin C (Martínez & Rojo 2011 and references therein). Furthermore, some studies indicate that a seasonal pattern of lysogeny may exist that depends on the prevailing temperature conditions (Cochran & Paul 1998, McDaniel et al. 2002). For example, Cochran & Paul (1998) have shown that prophage induction occurs only when the water temperature exceeds 19°C, which is greater than the temperature used in the present study. To date, there is but scanty information on the *M. aeruginosa* prophage (Yoshida et al. 2008a) and no investigations have yet demonstrated that the *A. flos-aquae* genome contains known prophage sequences (Cao et al. 2014). Collectively, these factors all have the potential to frustrate the detection of lysogeny in our samples.

This study provides only a snapshot of cyanobacterial populations in time. However, the absence of virus-infected cells, together with the lack of evidence for lytic and lysogenic virus production in *A. flos-aquae* and *M. aeruginosa* colonies, may have important implications for the development of bloom and community structure in the Curonian Lagoon. If colony formation is able to prevent cyanobacteria from being grazed or from being infected by viruses, even if only temporarily (Hamm et al. 1999, Jacobsen et al. 2007, Yamamoto et al. 2011), then one would expect a relatively greater number of single-celled bacteria to be removed from the water column both by viral lysis and predation (Tang 2001, Brussaard et al. 2007). This could further indirectly enhance the emergence of grazing and virus-resistant morphotypes (Šimek et al. 2007). A lack of control of cyanobacterial colonies by virus and grazing would also affect the flow of materials and energy within the ecosystem, since most of the biomass

produced would be lost from the pelagic zone due to increased sedimentation (Lürling & Van Donk 2000). Previous studies have suggested that grazing has an insignificant effect on the mortality of colony-embedded *A. flos-aquae* and *M. aeruginosa* occurring in the Curonian Lagoon (Gasim̄naitė & Olenina 1998, Pilkaitytė & Razinkovas 2006). In parallel with the observations presented in this study, this may result in a greater quantity of organic matter (accumulated within *A. flos-aquae* and *M. aeruginosa* during the intensive growth period) entering the benthic food web owing to colony sedimentation. The high chlorophyll *a* concentration observed in the surface sediment layer during the summer-autumn period (Zilius et al. 2012) would indirectly support this hypothesis.

To conclude, this study is the first attempt to detect virus production in two globally important colony-forming cyanobacteria occurring in a eutrophic temperate lagoon of the south-eastern Baltic Sea. The application of a range of different methods was not able to confirm virus infection, progeny formation or lysis in the embedded cells of *A. flos-aquae* and *M. aeruginosa* colonies. Despite the limitations of this study, we demonstrated for a particular stage of bloom development that colony-embedded cyanobacteria were free from virus infections. This supports the hypothesis of colony resistance to phage infection and agrees with the results of previous studies that have investigated physical, rather than biological control of cyanobacterial bloom dynamics (Gasiūnaitė & Olenina 1998, Pilkaitytė & Razinkovas 2006). Thus, a lack of viral control of potentially toxic cyanobacteria that occur in the Curonian Lagoon could have major implications in terms of bloom management, eutrophication issues and climate change perturbations.

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