INTRODUCTION

In freshwater ecosystems, the community composition of bacterioplankton shows strong seasonal patterns, particularly in temperate lakes (Pernthaler et al. 1998, Hofle et al. 1999, Van der Gucht et al. 2001, Zwisler et al. 2003, Kent et al. 2004, Wu & Hahn 2006). Changes in freshwater bacterial community composition may be driven by bottom-up factors, such as quality and quantity of organic matter (Crump et al. 2003), top-down factors, such as protozoan grazing or viral infection (Weinbauer & Hofle 1998), and environmental factors, such as temperature, pH or water residence time (Lindstrom et al. 2005). Since the discovery of high viral abundance in aquatic ecosystems, virioplankton in particular have

Optimization of viral profiling approaches reveals strong links between viral and bacterial communities in a eutrophic freshwater lake

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ABSTRACT: The goals of this 18 mo field study were to examine temporal changes in viral community composition in a temperate eutrophic freshwater lake (Lake Matoaka) and to identify potential drivers of seasonal changes in microbial community composition. We show that analyses of aquatic viral community composition can be streamlined by concentrating viruses from small water samples without compromising the resolution of the community profile data. The use of small-volume viral concentrates paired with randomly amplified polymorphic DNA (RAPD) polmerase chain reaction (PCR) generated significantly richer and more complex banding patterns than the use of pulsed-field gel electrophoresis (PFGE) and the large-volume concentrates that PFGE requires. Thus, the small-volume RAPD-PCR approach was used in the subsequent analyses. Viral and bacterial communities in Lake Matoaka were highly dynamic, exhibiting strong seasonal shifts. However, repeating annual patterns were not detected among the bacterial or viral communities. Temperature was the most important factor explaining changes in viral and bacterial abundance. Viral and bacterial abundance were also significantly correlated to each other. Multivariate analysis indicated weak relationships between temperature and bacterial community composition and between temperature and viral community composition. Mantel tests revealed a strong and significant correlation between viral and bacterial community composition. Taken together with the correlation between viral and bacterial abundance, these data suggest that phages dominate the virioplankton of Lake Matoaka and indicate tightly linked phage–host dynamics.

KEY WORDS: Virioplankton · Bacterioplankton · RAPD-PCR · tRFLP · Eutrophic lake · Community dynamics

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come to be regarded as key players in aquatic ecosystems, and there is strong interest in characterizing the roles of viruses in aquatic food webs and in driving aquatic microbial dynamics (Fuhrman 1999, Miki et al. 2008).

Viral lysis can be a major cause of bacterial mortality in freshwaters, accounting for up to 100% of bacterial biomass turnover (Weinbauer & Hofle 1998, Sime-Ngando & Ram 2005, Jacquet et al. 2007, Simek et al. 2007, Berdjeb et al. 2011). This has important implications in the microbial dynamics of freshwater ecosystems. If viruses are host-specific, it would be expected that changes in viral community composition would reflect seasonal patterns of change in their host communities. As bacterial species increase in abundance, the contact rate between virus and host increases, leading to higher infection rates and eventual decline of the dominant host species (Thingstad 2000, Miki et al. 2008). It is likely that the majority of aquatic viruses are bacteriophages that infect heterotrophic bacteria (Wommack & Colwell 2000). However, the relationships between aquatic viral assemblages, autotrophs (both eukaryotic algae and cyanobacteria) and heterotrophic bacterioplankton vary across studies, and seasonal changes in viral abundance and community composition may not reflect those of bacterial heterotrophs in all aquatic ecosystems (Clasen et al. 2008).

Relatively little is known regarding temporal change in viral community composition in fresh waters. Change in viral community composition in marine ecosystems has been characterized over spatial and temporal scales using pulsed-field gel electrophoresis (PFGE) with a great deal of success (Wommack et al. 1999, Steward et al. 2000, Fuhrman et al. 2002, Riemann & Middelboe 2002, Jiang et al. 2003, Hewson et al. 2006, Parada et al. 2008). Despite its wide use, PFGE is not without its drawbacks. In particular, its high limit of detection necessitates concentration of viruses from large sample volumes, in some cases, up to 80 l (Wommack et al. 1999). In freshwaters, a few studies have explored the dynamics of specific viruses and their hosts (e.g. Hewson et al. 2011, Short et al. 2011), and a handful of studies have used PFGE to examine changes in total virioplankton community composition (Auguet et al. 2006, Filippini & Middelboe 2007, Tijdens et al. 2008). However, a qualitative comparison of existing studies shows that PFGE band richness and resolution is much poorer for freshwater samples than for marine samples.

Randomly amplified polymorphic DNA (RAPD) polymerase chain reaction (PCR) has recently been developed as an alternative means for fingerprinting marine viral communities (Winget & Wommack 2008, Helton & Wommack 2009, Winter & Weinbauer 2010) and holds several advantages over PFGE in the analysis of viroplankton community composition. In particular, RAPD-PCR generates more bands per sample than PFGE, increasing the likelihood of detecting changes in viral community composition. However, to our knowledge, RAPD-PCR has not yet been applied to the characterization of freshwater viral assemblages.

The main goals of the present work were to improve understanding of microbial dynamics and to identify potential drivers of seasonal changes in virio- and bacterioplankton community composition in freshwater ecosystems. To accomplish this goal, an initial task was to develop and optimize viral concentration procedures for analyzing changes in freshwater viral community composition using RAPD-PCR. Once optimized, RAPD-PCR was used in a 18 mo field study to examine temporal changes in viral community composition in a temperate eutrophic freshwater lake (Lake Matoaka), with co-variance in the bacterioplankton community quantified using terminal restriction fragment length polymorphism (tRFLP).

**MATERIALS AND METHODS**

**Study site and sample collection**

Lake Matoaka is a 16 ha freshwater (salinity ≥0.2 ppt) impoundment located on the campus of the College of William and Mary in Williamsburg, VA (Pensa & Chambers 2004). Its average depth is 2.5 m (max. depth 4.57 m), and the water residence time is 75 d (turnover 1.3% d⁻¹). The lake is fed by 3 main tributaries (College Creek, Pogonia Creek and Strawberry Creek) and discharges into the James River and ultimately the Chesapeake Bay. For analysis of seasonal change in the microbial community, water samples were collected at a boat-launch pier adjacent to the Keck Environmental Field Laboratory (37°16’13.7”N, 76°43’22”W). Surface samples (<1 m, collected by hand in polypropylene carboys) were collected monthly from May 2009 to November 2011, with the exceptions of May 2010 and January 2011. For the comparison of viroplankton concentration methods, surface water samples were collected at the Lake Matoaka Inlet (37°16’31.9”N, 76°43’27.4”W), Pier and Spillway (37°15’49.1”N, 76°43’20.3”W). For samples collected from March 2009 through July
2009, viral community composition was analyzed using PFGE. For samples collected from August 2009 through November 2010, analysis of viral community composition was performed using RAPD-PCR. To compare results of RAPD-PCR with marine versus freshwater samples, marine surface samples (salinity 22 ppt) were collected from the Chesapeake Bay (Gloucester Point, VA; 37°14’51.2”N, 76°30’01.6”W) on 7 March 2011 and 10 June 2011.

**Viral and bacterial abundance**

Water samples (2 ml) were dispensed into cryovials, flash frozen in liquid nitrogen and stored at −80°C until processing (Wen et al. 2004). Viral and bacterial abundance were determined based on methods devised by Noble & Fuhrman (1998). Aliquots (100 to 200 µl) of thawed water samples were suspended in 1 ml total volume in sterile Milli-Q water, loaded onto a 25 mm, 0.02 µm pore-size Anodisc (Whatman) and drawn through the filter under vacuum. Anodiscs were stained with 100 µl of 2.5× SYBR Gold (diluted from 10 000× stock; Invitrogen) in the dark for 15 min. After drawing off the stain, the Anodiscs were air-dried and mounted onto a glass slide using a drop of antifade (50 mg p-phenylene diamine; 2 ml of 1× PBS; 2 ml 1.0 M Tris, pH 8.0; 46 ml 80% glycerol).

Bacterial cells and virus particles were visualized using an Olympus BX-51 microscope outfitted with a mercury arc lamp and a fluorescein isothiocyanate filter. A total of 10 to 20 digital photographs (>200 cells and 200 virus particles per sample) were taken for each raw water sample using a Hamamatsu CCD camera. Bacterial and viral abundances were determined using the Metamorph Basic software package (Olympus).

**Water chemistry and chlorophyll a**

Temperature was measured using a standard mercury thermometer at the time samples were taken. The pH was measured in the laboratory using an UltraBasic pH meter (Denver Instruments). Nutrient levels (NO₂ + NO₃, NH₄ and PO₄) were determined using colorimetric assays with water filtered through Whatman glass fiber filters (GF/F) (Parsons et al. 1984). Chlorophyll a (chl a) was determined spectrophotometrically (Lorenzen 1967). Briefly, 250 ml of whole water was filtered through 47 mm diameter Whatman GF/F filters. Filters were extracted with 90% acetone for 24 h at 4°C before reading absorbance in a Genesys 20 spectrophotometer (Thermo-Spectronic). Calculations were corrected for the presence of phaeopigments. Monthly rainfall data was obtained from the Williamsburg Government website: www.williamsburgva.gov/index.aspx?page=242.

**Bacterioplankton processing**

Surface water samples (6 l) were pre-filtered through a 10 µm capsule filter (GeoTech Environmental) to remove debris, followed by a series of 142 mm diameter nylon filters: a 5 µm and 3 µm filter (Pall) were used to remove protozoa, zooplankton and phytoplankton. Bacterioplankton were captured on a 0.22 µm ExpressPlus membrane filter (Millipore). The filters were folded, placed in sterile Falcon tubes and stored at −80°C until DNA extraction (Rusch et al. 2007).

**Comparison of viral concentration methods**

To compare the impact of concentration factor on viral fingerprints obtained by RAPD-PCR, aquatic viruses were concentrated by 3 different methods.

**20 l tangential flow filtration.** Samples were processed similar to the method used by Suttle et al. (1991). Surface water samples were collected in 20 l polypropylene carboys and pre-filtered through 10 µm capsule filters (GeoTech Environmental). Bacteria were removed using a 0.22 µm tangential flow filtration (TFF) cartridge (Millipore Prep/Scale CDUF006LT, 6 ft² [0.557 m²]) to a volume of ~100 ml. This primary concentrate was further concentrated using centrifugal filtration devices (Millipore Centricron Plus 70, 30 kDa MWCO) to a volume of ~2 ml (Wiget & Wommack 2008). The final viral concentrate was washed 3 times with 50 ml TMG buffer (10 mM Tris-Cl, 10 mM MgSO₄ and 1% w/v gelatin), and sodium azide was added (0.1% final conc.) to inhibit growth of any potential bacterial contamination. Viral concentrates were stored at 4°C.

**6 l tangential flow filtration.** Surface water samples were collected and pre-filtered as described above. To remove bacteria, water was filtered through a series of 142 mm diameter membranes of decreasing pore size as described above (Rusch et al. 2007). Virus
particles in the 0.22 µm filtrate were concentrated using a 30 kDa TFF cartridge (Millipore Prep/Scale CDUF006LT, 6 ft² [0.557 m²]) to a volume of ~100 ml (Suttle et al. 1991) and further concentrated using centrifugal filtration devices as described above. The final viral concentrate was washed 3 times with 50 ml TMG buffer and stored as above.

50 ml ultracentrifugation. Surface water samples were collected using a sterile 50 ml Falcon tube and filtered through a 0.22 µm Sterivex syringe filter (Millipore) into polyallomer ultracentrifuge tubes (Beckman). Samples were spun at 22 000 rpm (82 000 \(\times\) g) for 2 h at 4°C in a Beckman SW41Ti rotor. Supernatants were carefully decanted to avoid disturbing viral pellets, and the pellets were resuspended in 20 µl of TMG buffer and combined in a sterile 1.5 ml microcentrifuge tube. Viral concentrates were amended with azide and stored as above.

Analysis of viral community composition by PFGE

Agarose plugs were prepared by mixing 40 µl 1.5% molten In-Cert agarose (Bio-Rad) with 40 µl of viral concentrate, diluted such that each plug contained \(2 \times 10^9\) virus particles. The mixture was dispensed into 80 cc plastic plug molds and allowed to solidify at 4°C for 30 min. Plugs were then released from molds into 2 ml of digestion buffer (250 mM EDTA, 1% SDS, 10 mg ml\(^{-1}\) proteinase K) and incubated overnight at 37°C. The digestion buffer was carefully decanted, and the plugs were washed 3 times, 30 min each, with 1x TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0) and stored at 4°C in 2x TE buffer (Wommack et al. 1999).

PFGE was performed with a CHEF DR-II system (Bio-Rad) using Mid-Range PFG molecular weight markers (New England BioLabs). Plugs containing virus concentrates and molecular weight markers were loaded into 0.8% pulsed-field certified agarose gel (ISC BioExpress) in 0.5x TBE buffer, run at 4 V cm\(^{-1}\). Gels were stained with 1x SYBR Safe (Invitrogen) in 0.5x TBE buffer for 1 h prior to visualization of bands using a Kodak Gel Logic 100 imaging system. Banding patterns were analyzed using ImageQuant TL software to perform densitometry analysis and converted to binary matrix format. Cluster analysis (Dice coefficient) was used to construct a dendrogram following unweighted pair group method with arithmetic means.

Bacterial DNA extraction

Bacterial genomic DNA was extracted from cells captured on the 0.22 µm filters using methods adapted from Rusch et al. (2007), with the following modifications. After cell lysis, crude DNA extracts were precipitated with isopropanol and transferred to 1.5 ml tubes. The DNA was then purified using phenol/chloroform extraction on a smaller scale in
the 1.5 ml tubes. After precipitating purified DNA with isopropanol and washing with 70% ethanol, DNA pellets were resuspended in 50 µl of 1× TE Buffer. DNA was quantified using a NanoDrop® 1000 Spectrophotometer (Thermo Scientific) and stored at −20°C until use.

16S rRNA PCR and purification of PCR products

A fragment of the 16S rRNA gene was amplified by PCR for each sample. Primers were obtained from Integrated DNA Technologies and included 519r (5’-GWA TTA CCG CGG CKG CTG-3’) and 27f-HEX (5’-HEX-AGA GTT TGA TCC TGG CTC AG-3’) (Lane 1991). PCR products were then run on a 0.8% agarose in 1× TAE Buffer at 90 V for 1 h. Following the run, gels were stained in 400 ml of 1× TAE buffer with 25 µl of 10 000× SYBR Safe (Invitrogen) in the dark at 22°C for 1 h. Gels were visualized using a Kodak Gel Logic 100 Imaging System with Kodak Molecular Imaging Software (Eastman-Kodak). PCR products from 4 replicate reactions per sample were pooled and purified using a QIAquick PCR purification kit (Qiagen).

tRFLP analysis

Bacterial community composition was characterized using tRFLP based on 2 different restriction enzymes (HinfI and MspI) to generate a profile of the bacterial community in the lake and to track changes over time (Lymer et al. 2008b). The reproducibility of the tRFLP data was measured by running 5 different samples in duplicate using the HinfI enzyme. Cluster dendrograms showed that replicate restriction digests of pooled 16S rRNA-PCR amplicons generated highly similar (≥90%) T-RF patterns (see Fig. S2 in the supplement).

To remove terminal overhangs on HEX-labeled 16S rRNA gene amplicons, purified PCR products were treated with 1 µl of mung bean nuclease (New England BioLabs) for 30 min at 37°C. The mung bean nuclease was removed by phenol/chloroform extraction. DNA was precipitated with ethanol, and the pellet was resuspended in 60 µl of sterile de-ionized water (Liu et al. 1997, Lymer et al. 2008b).

A mixture of 12 µl of HI-DI formamide and 0.25 µl of 500-ROX size standard (Applied Biosystems) were added to the restriction fragments, which were then loaded in a 96 well plate for analysis in an ABI3130 Avant Gene Sequencer (Applied Biosystems) with Pop7 polymer. Each run was performed in a 36 cm capillary with dye set DS-30 (ROX, 6FAM, HEX and NED) as a matrix standard. The resulting chromatograms from the above gene fragment analysis were analyzed using GeneMapper Software Version 4.0 (Applied Biosystems). Replicate tRFLP runs were aligned by manual inspection of peak shift. Peak thresholds were set using a minimum peak height of 50 fluorescence units. The light-smoothing algorithm available in the GeneMapper software was applied to the peaks to reduce background noise or artifacts in peak detection. Peaks were sorted into bins of 1 to 50 base pairs, and the data from each chromatogram were converted to presence/absence matrices.

Statistical analyses

All environmental data were log-transformed to obtain normal distributions. To test for relationships between bacterial and viral richness, bacterial and viral abundance, and environmental data, Pearson’s correlation analysis and Bonferroni post-tests were performed in Prism 5 (GraphPad Software). The presence/absence matrix data for the bacterial HinfI and MspI tRFLP profiles and viral RAPD profiles were converted into similarity matrices (Dice similarity coefficient) and analyzed by non-metric multidimensional scaling (NMDS) in PAST (Hammer et al. 2001). This type of ordination analysis was used to identify overarching patterns that may help explain similarities (or dissimilarities) across samples. In essence, the closer 2 samples are to each other in the NMDS plot, the more similar their community composition (as determined by RAPD banding pattern); the further away 2 samples are, the more dissimilar. BioEnv tests (Clarke & Ainsworth 1993) were performed using the vegan package in R (Version 2.12.1; R Development Core Team 2012) to identify potential environmental factors that best explain shifts in both bacterial and viral community composition. To test for co-variance between bacterial (HinfI and MspI profiles) and viral community profile shifts, Mantel statistics were computed in R based on Spearman rank correlation and 9999 random permutations.
RESULTS

Water chemistry

The surface water temperature of Lake Matoaka was highest in August 2009 and September 2010 and lowest in December 2009 and February 2010, when a thin layer of ice covered shallower sections of the lake (Table 1). Chl a levels were highest in the summer months, with maxima in August 2009 and July 2010, corresponding with abundant algal biomass in the lake. Levels of dissolved nitrogen compounds also appeared to follow a cyclical trend and were highest in August and September 2009 and October and November 2010. Dissolved inorganic phosphate levels varied only slightly over the sample period, averaging 0.73 µM. The pH varied between 6.96 and 8.01 over the 18 mo observation period, with an average value of 7.45.

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<th>NH₄ (µM)</th>
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³Average of duplicate samples

Bacterial and viral abundance

Bacterial and viral abundance in Lake Matoaka followed a cyclical trend over the 18 mo sampling period (Fig. 1). Viral abundance was consistently higher than bacterial abundance by a factor of ~30. Both viral and bacterial abundance were highest in the summer months and lowest in the winter months. Abundance decreased drastically between the summer and winter of 2009. However, this pattern of sharp decrease in abundance trends was not observed between the summer and winter of 2010. Viral abundance and bacterial abundance were significantly correlated (r = 0.932; p = 4.21 × 10⁻⁷; Table 2)
Both viral and bacterial abundance were significantly correlated with surface water temperature (viruses: $r = 0.869$, $p = 0.000026$; bacteria: $r = 0.857$, $p = 0.000045$; Table 2). However, none of the other measured parameters, including pH, dissolved nutrients and chl $a$, were found to have biologically meaningful correlations with viral or bacterial abundances.

### Comparison of viral concentration methods

To determine whether viral community profiles were sensitive to the manner in which viral concentrates (VC) are generated, 3 methods of virus concentration were compared: concentration from either 6 or 20 l of water via TFF and concentration from 50 ml of water via ultracentrifugation (UCF). The 6 l TFF-processed concentrates yielded the highest viral particle concentrations, followed by UCF and the 20 l TFF concentrates (Table 3). The concentration efficiency of each method (calculated by comparing the measured viral concentration to the theoretical viral yield based on viral abundance in the starting sample and VC volume) was consistently highest for UCF and lowest for the 20 l TFF concentrates (Table 3). This may be partly due to poor recovery of virus particles adsorbed to filters. Alternatively, the increased processing times involved in TFF may expose viral particles to greater...

physical agitation, increasing the likelihood of loss due to thermal decay. In UCF, these sources of loss are reduced as the viral particles are processed for 2 h at 4°C.

Assessment of virioplankton community composition by PFGE

Change in viral community composition was initially assessed using PFGE from March 2009 to July 2009 for water sampled from 3 discrete locations in Lake Matoaka. However, the resulting bands (see Fig. S3 in the supplement) revealed very little change over time or space, when bands could be resolved. The high limit of detection for PFGE was identified as a key issue as the freshwater samples in the present study required at least \(10^9\) virus particles per lane to resolve bands (see Fig. S4 in the supplement). These results prompted the further refinements in application of RAPD-PCR to study freshwater viral assemblages.

Assessment of virioplankton community composition by RAPD-PCR

To ensure that amplicons generated by RAPD-PCR originated from viral DNA templates and not from free DNA, several viral concentrates generated by UCF were subjected to DNase treatment (see Fig. S5 in the supplement). Addition of DNase resulted in negligible changes to banding patterns relative to untreated samples (83 to 93.6% similarity), indicating that RAPD templates did not originate from free DNA. To test the reproducibility of RAPD-PCR, all reactions were performed in duplicate. Banding patterns generated from duplicate reactions were on average 93% similar, ranging from 87 to 100% (Fig. 2; Fig. S6 in the supplement). Most differences arose from band binning as visual inspection of profiles revealed identical patterns in almost all cases.

RAPD-PCR of freshwater (Lake Matoaka) and marine (Chesapeake Bay) viral concentrates processed by each of the 3 concentration approaches successfully produced DNA amplicons. Unlike PFGE, RAPD-
PCR produced distinct bands with as few as $10^4$ viruses per lane (e.g., Fig. 2b). Furthermore, RAPD-PCR generated anywhere from 15 to 22 distinct bands per sample (Fig. 2, Table 3) compared to the 3 to 4 diffuse bands per sample typically generated by PFGE (see Fig. S3 in the supplement). In general, the UCF viral concentrates yielded the highest numbers of bands compared to corresponding 6 l and 20 l TFF concentrates generated from the same samples (Table 3). The exception was the CB March 2011 sample, wherein the 20 l TFF replicates had the highest number of bands at 21 compared to the other viral concentrates at 19 (Table 3). Comparison of RAPD-PCR banding patterns generated from freshwater and marine viral concentrates revealed that the mean similarity between banding patterns produced by TFF and UCF viral concentrates for any given sample was >85% (Fig. 2c,d; Fig. S6 in the supplement).

**Seasonal change in Lake Matoaka viral community composition**

RAPD-PCR band richness varied between 11 (February 2010) and 28 (November 2011) distinct bands (Fig. 3). In 2009, the summer months (July and August) yielded the highest numbers of bands, with slightly decreasing band richness into winter, while in 2010, slight peaks in RAPD band richness were observed in March, July, and November (Fig. 3). With the exception of the low richness observed in February 2010, viral richness generally fluctuated between 20 and 28 bands during the 18 mo observation period. No significant relationship was observed between viral abundance and viral richness ($r = 0.401, p = 0.138$; Table 2). For example, viral richness in November 2009 was equal to that in June of 2010, despite the fact that viral abundance was ~10-fold higher in June than in November (Fig. 1). Pearson correlations revealed a weak relationship between viral richness and temperature ($r = 0.665; p = 0.007$; Table 2). However, no significant correlations were found between viral richness and any other measured environmental parameters (Table 2).

NMDS analysis of changes in RAPD banding patterns revealed an interesting pattern of monthly succession among the virioplankton in Lake Matoaka (Fig. 4). The viral community composition was fairly stable through the summer and fall of 2009, with the samples from July, August and October clustering tightly together and September and November forming another tight clustering. Through the winter and spring of 2010, the viral community composition underwent dramatic shifts (December 2009 and February and March 2010). The summer of 2010 also showed relatively large changes in viral community composition, evidenced by the distances between the April, June and July samples. These patterns suggest that the virioplankton community can be fairly static over monthly timescales (e.g. summer 2009) but that large and dramatic shifts are also possible over the same timescales (e.g. winter 2009 and 2010; Fig. 4).

BioEnv tests were performed to determine the relationships between the viral community composition data and the environmental data (Clarke & Ainsworth 1993). This test was based on Dice dissimilarity matrices for the viral RAPD-PCR banding patterns and Euclidean distances of environmental data (Melo.
2009) and identified environmental factors that best explain the variation observed in the biological data. The resulting Spearman rank correlations (r-statistics) are a measure of the explanatory power of an environmental variable in accounting for the variation in the biological (i.e. community composition) data. Results from BioEnv tests indicated that surface water temperature was the most important factor related to shifts in viral community composition. However, the Spearman rank correlation was only 0.5602 (see Table S2 in the supplement), suggesting that temperature is still not a particularly strong factor in driving viral community shift.

**Seasonal change in Lake Matoaka bacterial community composition**

Bacterial richness (number of tRFLP peaks) varied between 4 (July 2010, MspI profile) and 48 (September 2010, MspI profile) OTU. In general, bacterial richness as estimated from both HinfI and MspI profiles showed peaks in mid-summer of 2009, winter of 2010 and late fall of 2010 (Table 1). It is quite interesting that bacterial richness in the more productive summer months and that richness generally bottomed out in the late summer/early autumn. This perceived decrease in bacterial richness might be due to dominance by fewer bacterial species, whereas the increased richness in winter may actually be due to an increase in evenness among bacterial taxa in the lake. Bacterial richness data obtained from the HinfI and MspI tRFLP profiles were positively correlated with each other ($r = 0.791$, $p = 0.00045$; Table 2). However, no significant correlations were found between bacterial richness and any of the measured environmental variables, including viral and bacterial abundance.

Analysis of bacterial profiles by NMDS revealed a dynamic pattern of bacterial community succession (Figs. 5 & 6), though little in the way of repeating annual patterns. Based on NMDS analysis of HinfI profiles, the bacterial community forms a fairly tight cluster from July through September of 2009 then undergoes a larger shift into the winter and maintains a looser clustering through early summer (November 2009 through June 2010). In the late summer and fall of 2010, the bacterial community appears to go through large shifts in composition each month (August through November 2010)
October 2010 appeared to be an outlier, shifted furthest away from all other samples in the NMDS plot. One potential explanation for this community shift is elevated rainfall in that month (360.4 mm; Table 1).

NMDS analysis based on MspI profiles revealed a different pattern of change, with a high degree of similarity between the bacterial communities observed in December 2009, February 2010, November 2010 and September 2010 (Fig. 6). This spread displays some degree of grouping based on season. The winter months of December 2009 and February 2010 form a tight group in the upper left of the NMDS plot. Samples from the summer, spring and fall months appear to be intermingled, though samples from the summer months (July and August of 2009 and June and August of 2010) form a fairly tight cluster (Fig. 6). October 2009 was an outlier in this data set, though it is difficult to determine the cause as there were no corresponding changes in rainfall, temperature, pH, bacterial richness or other measured environmental factors during that month (Table 1).

NMDS analysis based on MspI profiles revealed a different pattern of change, with a high degree of similarity between the bacterial communities observed in December 2009, February 2010, November 2010 and September 2010 (Fig. 6). This spread displays some degree of grouping based on season. The winter months of December 2009 and February 2010 form a tight group in the upper left of the NMDS plot. Samples from the summer, spring and fall months appear to be intermingled, though samples from the summer months (July and August of 2009 and June and August of 2010) form a fairly tight cluster (Fig. 6). October 2009 was an outlier in this data set, though it is difficult to determine the cause as there were no corresponding changes in rainfall, temperature, pH, bacterial richness or other measured environmental factors during that month (Table 1).

Results from BioEnv analysis indicated that temperature was the single best environmental factor for explaining the changes in bacterial community composition, with a Spearman rank correlation of 0.5676 and 0.5660 for the Hinfl and MspI tRFLP patterns, respectively (see Table S2 in the supplement).

### DISCUSSION

#### Methodological considerations

To our knowledge, the present study is the first to compare profiles of freshwater virioplankton communities using RAPD-PCR. Previous concerns regarding RAPD-PCR focused on the assumptions that only viral templates are being amplified and that the banding patterns produced from this amplification are reproducible. In the present study, as with previous studies using RAPD-PCR on viral assemblages (Winget & Wommack 2008, Helton & Wommack 2009, Winter & Weinbauer 2010), the purity of VC and lack of microbial contamination was confirmed by epifluorescence microscopy (see Fig. S7 in the supplement). Twenty fields were examined for each VC and confirmed to be free of bacterial cells before proceeding with further analysis. Furthermore, treatment of VC with DNase did not result in changes in banding patterns (Fig. S5 in the supplement). Both of these observations support the conclusion that RAPD banding patterns originated from viral templates. Replicate RAPD-PCR reactions yielded banding patterns of ≥80% similarity (Fig. 2; Fig. S5 in the supplement), consistent with the reproducibility cited by other studies (Winget & Wommack 2008, Helton & Wommack 2009).

As an amplified fragment polymorphism technique, RAPD-PCR suffers from the same issues as 16S rRNA gene-based profiling methods, such as tRFLP and denaturing gradient gel electrophoresis (DGGE). The main concerns are that some templates may be preferentially amplified over others (Polz & Cavanaugh 1998) and that some genomes may contain multiple copies of the template sequence.
(Crosby & Criddle 2003). However, these biases are largely accepted in the use of 16S rRNA gene-based data to examine microbial communities, and it is therefore difficult to reject RAPD-PCR on these grounds alone. A recent paper investigated the ecological interpretation of RAPD-PCR results in the study of aquatic viral communities and provides a reasonable assessment of potential biases introduced by this technique (Winter & Weinbauer 2010). Unlike the 16S rRNA gene, no universal sequence target exists among the viruses. Thus, it is important to note that the primer favored in most RAPD-PCR studies, CRA-22, only captures the richness and dynamics of some unknown subset of the viral community. That said, RAPD-PCR remains a powerful tool for documenting changes in viral community composition and for identifying relationships between viral community change and other biological and environmental factors (Winter & Weinbauer 2010). In the present study, we found that RAPD-PCR is highly sensitive to changes in viral community composition and holds several advantages over the more commonly used viral profiling technique, PFGE.

We examined more closely the utility of RAPD-PCR for profiling freshwater viral communities largely because preliminary field studies using PFGE yielded unsatisfactory results. Pulsed-field gels of freshwater viral assemblages from the present study (see Fig. S3 in the supplement) and others (Auguet et al. 2006, Filippini & Middelboe 2007, Tijdens et al. 2008) tend to produce relatively few, often highly diffuse bands compared to those originating from marine concentrates, reducing the discriminatory power of PFGE in analyzing freshwater viral communities. In addition, large numbers of virus particles must be loaded into gels to resolve bands. Thus, most PFGE-based analyses of aquatic viral community composition have used TFF to concentrate viruses from water samples, using volumes from 12 to 80 l (Auguet et al. 2006, Filippini & Middelboe 2007, Parada et al. 2007, Winget & Wommack 2008). While previous reports have indicated that the sensitivity of PFGE can be as low as $10^6$ viruses per band (Wommack et al. 1999, Filippini & Middelboe 2007), viral concentrates from Lake Ma-toaka required a minimum of $10^6$ viruses per lane to resolve any bands (Fig. S4 in the supplement). Even a 10-fold reduction in virus concentration dramatically reduced the number of detectable bands by PFGE.

Current investigations using RAPD-PCR to analyze changes in aquatic viral community composition have relied upon TFF to concentrate viruses from large sample volumes prior to amplification (Winget & Wommack 2008, Winter & Weinbauer 2010). However, given that successful RAPD-PCR of aquatic viral templates requires ~$10^5$ particles per reaction (Srinivasiah et al. 2008) and that overloading of DNA templates frequently reduces the complexity of RAPD banding patterns (see Fig. S1 in the supplement), TFF concentration of viruses from large sample volumes may not be necessary. Our results indicate that for water samples containing an ambient viral abundance of at least $10^6$ viruses ml$^{-1}$, UCF of a 50 ml sample produces viral concentrates sufficient to generate RAPD banding patterns from 1 µl of VC. Furthermore, the use of smaller sample volumes to generate VC did not lead to any losses in the ability to detect changes in viral richness or viral community composition by RAPD-PCR (Figs. 2 & 3). In fact, viral richness (number of RAPD band types) was slightly higher when UCF concentrates were used compared to larger volume TFF concentrates (Table 3). This may be due to a slightly higher efficiency of UCF to concentrate viruses as compared to TFF, which has been observed elsewhere (Takashima et al. 2007).

It is important to note that PFGE remains a viable option for assessing aquatic viral community composition, particularly for marine samples. Furthermore, TFF is still advantageous if field work necessitates the processing of large-volume water samples shipboard. However, for freshwater studies (particularly where lab access is available), the application of UCF viral concentrates in tandem with RAPD-PCR can increase sample throughput and allow more information to be obtained regarding viral dynamics using smaller sample volumes. For example, in 1 microcosm study performed by Auguet et al. (2006), fingerprints of viral communities in 4 l batch experiments could not be obtained due to the volume constraints of PFGE (requiring at least 10 l of water to generate bands by PFGE). Due to this constraint, potential changes in viral community composition could not be compared with contemporaneous bacterial community data. Preparation of viral concentrates by ultracentrifugation of 50 ml samples and subsequent analysis of changes in viral community composition by RAPD-PCR could have enabled such comparisons.

Finally, recent studies have shown that particle-associated microbial communities may be quite different from planktonic communities (Mari et al. 2007, Riemann & Grossart 2008, Luef et al. 2009). However, it is important to note that due to the filtration steps involved in obtaining bacterial assemblages and viral concentrates, the present study focuses exclusively on the planktonic fraction of the lake’s microbial community. While most previous aquatic studies have included a 0.22 µm filtration step to remove...
bacterial cells prior to generating viral concentrates, we cannot ignore the possibility that this filtration step has excluded certain large viruses from the viral community analysis in the present study. While several large algal viruses, such as *Chlorella* viruses (e.g. PBCV-1, 190 nm diameter) (Van Etten et al. 1991) and diatom viruses (e.g. EhV, 180 nm diameter) (Jacquet et al. 2002), should be able to pass through a 0.22 µm filter, larger viruses, such as *Cafeteria roenbergensis* virus (CroV, 300 nm diameter) (Fischer et al. 2010), would likely be retained on a 0.22 µm filter. Although CroV infects a marine alga and no freshwater algal viruses of this size have been yet reported, it is possible that some unknown fraction of large algal viruses were eliminated from our analysis, thus skewing the results toward phages. However, we temper this possibility with the fact that electron microscopic observation of aquatic virioplankton suggests that the majority are tailed phages (Wommack & Colwell 2000, Weinbauer 2004).

**Ecological considerations**

**Abundance**

Bacterial dynamics were similar to those observed in other freshwater systems, with minimal abundances in winter and maximal abundances observed in spring (2009) or early autumn (2010; Fig. 1). The average bacterial abundance in Lake Matoaka is somewhat low compared to shallow eutrophic lakes in Europe (Lymer et al. 2008b, Tijdens et al. 2008, Personnic et al. 2009), although within the range of reported values for several shallow eutrophic lakes in China (Cheng et al. 2010).

Viral abundances in Lake Matoaka were within the range reported for other freshwater ecosystems (Wommack & Colwell 2000), albeit somewhat low compared to other eutrophic lakes. Temperature was strongly correlated with both bacterial and viral abundance in Lake Matoaka, consistent with many other studies (Jiang & Paul 1994, Allgaier & Grossart 2006, Boucher et al. 2006, Tijdens et al. 2008). However, viral abundance in Lake Matoaka was not correlated to dissolved nutrient levels, as has been observed in other lakes (Peduzzi & Schiemer 2004, Lymer et al. 2008a, Personnic et al. 2009).

While several studies have found viral abundance in freshwater lakes to be strongly correlated to chl a levels (Madan et al. 2005, Liu et al. 2006, Tijdens et al. 2008), other studies have found viral abundance in certain lakes (Personnic et al. 2009, Cheng et al. 2010) and many marine systems (Maranger & Bird 1995) to be significantly correlated with bacterial abundance. These differences could indicate differences in the dominance of certain types of virioplankton (bacteriophages vs. cyanophages or algal viruses, for example) among different ecosystems. Alternatively, the differences in correlation could be due to sampling interval or the ways in which samples are compared. For example, a study comparing 7 sampling sites across 2 lakes found that viral abundance was significantly correlated to bacterial abundance based on temporal variation within each sampling site. However, when correlations were based on spatial variation across different sampling sites, viral abundance was not related to bacterial abundance but instead strongly related to chl a, total N and total P (Cheng et al. 2010). In the present study, the strong correlations between viral and bacterial abundance in Lake Matoaka suggest that viral and bacterial dynamics are tightly linked and support the hypothesis that the majority of the lake virioplankton are bacteriophages.

**Community composition**

Marked seasonal shifts in bacterial community composition appear to be a common feature of temperate freshwater lakes (Hofle et al. 1999, Van der Gucht et al. 2001, Lymer et al. 2008b). In spite of numerous studies that have explored freshwater microbial community composition, few common factors have been identified that best explain community composition change in all freshwater ecosystems (Hiorns et al. 1997, Lindström 2000, Boucher et al. 2006, Newton et al. 2011).

Previous studies have demonstrated that annual variations in microbial community composition are not generally repeated over annual cycles in eutrophic lakes (Lindström 2000, Boucher et al. 2006). Repeating seasonal trends have, however, been identified in humic and oligotrophic lakes (Yannarell et al. 2003, Lymer et al. 2008b). In eutrophic lakes, microbial community composition appears to be highly variable over time (Lindström 2000) and may even change rapidly on relatively short timescales (Lymer et al. 2008b). Such observations are consistent with the dynamic bacterial community and lack of repeating annual cycles in bacterial community composition in the present study.

Using multivariate statistical approaches, different studies have identified different factors as potential drivers of bacterial community composition changes...
in freshwater lakes. In 1 study that examined 3 different lakes, temperature was shown to account for 43 to 60% of community composition change over time using canonical correspondence analysis (Lymer et al. 2008b). A separate study comparing 15 temperate lakes ranging from oligomesotrophic to hypereutrophic found that temperature, pH and water residence time were the primary factors that determined bacterial community composition, with the 3 variables in aggregate accounting for 64% of the variance. However, when considered separately, temperature alone was a relatively weak explanatory factor, with an eigenvalue of –0.3887 (Lindström et al. 2005).

In the present study, a similarly weak relationship was identified between temperature and temporal change in bacterial community composition in Lake Matoaka (r = 0.5602, Hinfl profile; r = 0.5660,MspI profile) (see Table S2 in the supplement). However, as mentioned above, temperature was strongly correlated with both viral and bacterial abundance. This combination of a stronger relationship between microbial abundance and temperature but a weak or no relationship between temperature and community composition has been reported in several other freshwater studies (Allgaier & Grossart 2006, Boucher et al. 2006, Tijdens et al. 2008). We noted that bacterial richness was generally lowest in the late summer/early autumn and that richness was not significantly correlated to bacterial abundance. We hypothesize that the decrease in bacterial richness was due to dominance by fewer bacterial species in the summer, whereas the increased richness in winter was due to an increase in evenness among bacterial taxa in the lake. This hypothesis is supported by a separate study in which nutrient enrichment of an Antarctic lake induced the growth of specific bacterial taxa, resulting in a net increase in total bacterioplankton abundance but a decrease in overall richness (Pearce et al. 2005).

pH has also been identified as a significant driver of community composition change. In addition to the study by Lindström et al. (2005), a separate study found significant clustering of bacterial communities by pH across 5 different lakes, with a Pearson’s correlation between pH and bacterial richness of 0.785 (Allgaier & Grossart 2006). In the present study, no meaningful correlation was observed between community composition and pH (r = 0.316; p = 0.251). However, it is important to note that the study by Allgaier et al. (2006) examined 5 lakes with different properties (i.e. different trophic statuses and humic contents), and while pH was the only explanatory factor common to all 5, different environmental factors were found to drive community change in each of the 5 lakes (Allgaier & Grossart 2006). As the present study focused on 1 lake and the pH within the lake fluctuated only moderately (Table 1), this may explain why pH did not have any explanatory power in accounting for bacterial community composition change.

Indeed, the general lack of strong correlations between environmental and biological data may be explained by the fact that most of the measured environmental variables were fairly stable over the sampling period (Table 1). The weak correlation with temperature has been observed in other freshwater studies and may be explained by thermal selection for species with different growth kinetics (Hall et al. 2008). However, since temperature can influence patterns in other organisms such as aquatic macrophytes (Bornette & Puijalon 2011), algae and cyanobacteria (Elliott 2012), all of which contribute to the quantity and quality of dissolved organic matter, the relationship between temperature and bacterial and viral community composition may also be indirect.

Viruses are obligate genetic parasites and thus engage in predator–prey type oscillations with their hosts in terms of abundance and community composition (Fuhrman 1999, Thingstad 2000, Weinbauer 2004). While viral community composition has been investigated in several marine settings (Wommack et al. 1999, Steward et al. 2000, Fuhrman et al. 2002, Riemann & Middelboe 2002, Jiang et al. 2003, Hewson et al. 2006, Parada et al. 2008), relatively few studies have quantitatively analyzed viroplankton community composition in freshwater lakes. Of these existing freshwater studies, few explanatory factors have been identified that account for observed changes in virus community composition. An annual study of viroplankton using PFGE in 3 Scandinavian lakes identified dissolved nutrients (dissolved organic carbon [DOC], soluble reactive phosphorus and total nitrogen) as the best factors explaining shifts viral community composition, but with weak eigenvalues of <0.3 (Lymer et al. 2008b). This differs from Lake Matoaka, where temperature was the best explanatory factor accounting for changes in viral community composition. However, it is important to point out that DOC was not measured in the present study; thus, potential relationships between DOC and biological parameters, such as abundance and community compositions, could not be determined for Lake Matoaka. Similar to results reported by Lymer et al. (2008b), the relationship between environmental factors (temperature) and viral community composition in Lake Matoaka was weak (r = 0.5602;
Table S2 in the supplement). In their study, the application of Mantel tests by Lymer et al. (2008b) revealed that bacterial community composition and viral community composition in the Scandinavian lakes were also linked, though the correlation coefficient was fairly weak ($r = 0.48$).

In a separate study of virioplankton composition in a Dutch lake, PFGE indicated that viral assemblages were largely stable over the observation period, with samples differing primarily in band intensity (Tijdens et al. 2008). This is reminiscent of our preliminary analysis of Lake Matoaka using PFGE, in which very little change in banding patterns was observed over 5 mo (see Fig. S3 in the supplement), and underscores the high likelihood that PFGE may not be sensitive enough to capture important fluctuations in freshwater viral community composition. While significant correlations were found between viral abundance and bacterial community composition as well as bacterial abundance and viral community composition, no significant relationship was identified between bacterial and viral community composition. The authors speculated that ‘more specific techniques with a higher resolution might be necessary to reveal virus–host interactions in natural assemblages’ (Tijdens et al. 2008).

Intersection of methods development and ecological data

A recent study of viral community dynamics in the Mediterranean Sea serves to illustrate the utility of RAPD-PCR as just such a high resolution technique (Winter & Weinbauer 2010). Using RAPD-PCR, researchers identified a strong relationship ($r = 0.82$) between bacterial richness and viral richness in the epipelagic zone. Weaker relationships were observed between viral and bacterial richness in the mesopelagic ($r = 0.6$) and bathypelagic zones ($r = −0.64$). Most interestingly, Mantel tests revealed that changes in bacterial community composition were strongly linked to changes in the viral community composition, particularly in the bathypelagic zone ($r = 0.61$), indicating a highly responsive and dynamic viral community. That study was the first to employ RAPD-PCR to document temporal and spatial change in viral community composition, and to compare viral RAPD data with contemporaneous bacterial profiles. The results clearly demonstrated the utility of RAPD as a sensitive and high-resolution technique for revealing virus–host interactions in natural assemblages.

Results from the present study support the idea that RAPD-PCR provides higher resolution data than PFGE for analyzing freshwater virioplankton dynamics. The use of RAPD-PCR to analyze viral community composition of Lake Matoaka revealed an extremely strong link between viral and bacterial communities ($r = 0.9975$, $p = 0.0001$, HinfI profile; $r = 0.9976$, $p = 0.0001$,MspI profile). The strength of this correlation is much higher than any reported so far and may be a result of the increased sensitivity of RAPD-PCR to changes in the viral community relative to PFGE. Since it has been established that the CRA-22 primer captures some unknown subset of total viral richness, it is possible that the virioplankton in Lake Matoaka have a high frequency of primer binding sites. This would increase the sensitivity of our methods for detecting changes in Lake Matoaka virioplankton community structure. This seems unlikely, however, since the RAPD band richness in our samples were comparable to those found in the Mediterranean study (Winter & Weinbauer 2010). Regardless, the observed strong correlation between viral and bacterial community composition, in conjunction with the strong relationship between viral and bacterial abundance, indicates that viral and bacterial dynamics in Lake Matoaka are very tightly linked. However, much work remains to determine the directionality of this relationship as well as the ultimate drivers of both viral and bacterial community succession.

Based on community assembly patterns observed in other freshwater ecosystems, it is highly likely that freshwater bacterial communities are impacted by the introduction or invasion of terrestrial bacteria (Lindström et al. 2005). The same may be true of viruses; however, no known studies have directly investigated the impacts of terrestrial viruses on aquatic bacterial dynamics. In addition to microbial inputs, high levels of terrestrial DOC inputs are a prominent feature of many freshwater lakes (Jansson et al. 1999, Cole et al. 2007) and can be a significant factor impacting bacterial community composition (Judd et al. 2006, Jones et al. 2009). Due to lack of instrumentation and support for DOC analysis, this was not included in the present study. However, DOC may well be an important factor explaining the observed changes in bacterial and viral community composition. Thus, future work will test for relationships between DOC quantity and quality and changes in virio- and bacterioplankton dynamics in Lake Matoaka. In addition, we will also explore the contribution of allochthonous terrestrial viruses and bacteria in driving shifts in community composition.
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Editorial responsibility: Gunnar Bratbak, Bergen, Norway

Submitted: April 3, 2012; Accepted: July 6, 2012
Proofs received from author(s): August 29, 2012