FLOW CYTOMETRIC METHODS FOR ASSESSING WATER QUALITY

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ABSTRACT

Several Rotorua/Te Arawa lakes have undergone eutrophication and exhibit prolonged periods of poor water quality and cyanobacterial blooms. Sediment capping aimed at inhibiting the release of phosphorus from bottom sediments has been trialled on highly eutrophic Lake Okaro, as part of an ongoing remediation of water quality in the Te Arawa lakes. A modified zeolite product, Z2G1, developed by Scion as a phosphorus binding agent, was applied in September 2007 and August 2009, to inhibit the release of phosphorus from the sediment that occurs seasonally in the hypolimnion under anoxic conditions. While there has been considerable work on phytoplankton assemblages in eutrophic lakes, there has been relatively little work undertaken on other microbial components of eutrophic lake ecosystems, particularly bacteria and viruses. These micro-organisms have potential effects on algal community dynamics and nutrient cycling. The aim of this study was to investigate the possible inter-relationships amongst viruses, bacteria and algae, and the environmental factors that affect these relationships. Flow cytometry was used a rapid and effective method to assess the potential relationships between these micro-organisms and their effect on nutrient cycling in Lake Okaro.

KEYWORDS

Bacteria; eutrophication; phytoplankton; flow cytometry; lake remediation

INTRODUCTION

Bacteria and viruses are a major component of aquatic microbial communities. Viruses are responsible for 20 to 50% of daily prokaryotic mortality, providing a major source of dissolved organic material (Duhamel and Jacquet, 2006). Viral activity is reliant on a number of factors including water depth, trophic status, oxygen concentration, temperature, light penetration, suspended particulate matter and bacterial activity (Bettarel et al., 2006). Balanced algal and microbial growth in fresh water lakes is therefore reliant on the total microbial makeup and the influence of external and internal factors into these systems. While extensive work has been reported on the algal composition of eutrophic lakes, the abundance and composition of viral and bacterial elements in these systems, and their effect on cell lysis and potential nutrient cycling, has received considerably less attention (Lymer et al., 2008a, 2008b, Tijdens et al., 2008).

Viral reproduction is via cell lysis or by replication in a host cell. Cell lysis involves the attachment of a phage to a host cell with the phage redirecting the host metabolism toward production of new phages which are released when the host cell dies. In lysogenic reproduction the phage genome remains dormant in the host cell and replicates with the host cell until a lytic cycle is induced. Lysis results in both particulate and dissolved organic matter becoming available to bacteria for consumption and recycling. Eutrophic ecosystems generally

exhibit an increase in bacterial cell growth, which provides increased resources for phage development (Wilhelm and Matteson, 2008). The most significant viral impacts have been observed in aquatic systems with low productivity, where viral lysis plays a major part in carbon recycling (Sawstrom et al., 2007). Essential nutrients such as phosphorus may be released from lysed host cells and may either be in a form readily available for consumption by plants and organisms, or may require enzymatic release. Nutrient availability can select for particular organisms, which may in turn affect community composition.

To obtaining meaningful bacterial and viral data sets that allow spatial and temporal analysis high numbers of samples must be analyzed (Vives-Rego et al., 2000). Previous methods for bacterial and viral enumeration have included transmission electron microcoscopy (TEM) and epifluorescence microscopy (EFM). Both methods have their advantages and disadvantages. Transmission electron microscopy is thought to underestimate viral abundance, while EFM does not provide any distinction in genome size or viral morphology.

Cellular–base analysis of viruses in aquatic samples has also been hindered by the size and lower specific macromolecules (DNA, RNA and proteins) of viruses compared with pure mammalian cultures (Vives-Rego et al., 2000). Most commercially available fluorocromes have been designed for use with mammalian cells, while prokaryote cells are less permeable than mammalian cells and fitted with very efficient efflux pumps which rapidly expel dyes prior to interaction with the target molecule. Viruses have a significantly smaller amount of nucleic acid in comparison to even the smallest prokaryotic cells (Polson, 2010), making it necessary to use large volumes and often complex preparation methods for analysis.

In contrast to the above methods, flow cytometry (FC) can be used to carry out cell counts rapidly and has the capability to sort cells for culture, identification and subsequent analysis. High speed multi-parameter data acquisition and multivariate data analysis are possible, using cell size and complexity, and fluorocromes as discriminators (Duhamel and Jacquet, 2006). Mixed populations of phytoplankton have been analyzed according to their size and physiological status and mixed populations of bacteria and viruses by their DNA content (Balfoort et al., 1992, Becker et al., 2002, Brussaard et al., 2001, Brussaard, 2004, Li and Dickie, 2001, Marie et al., 2005). With FC there is also no need to pre-concentrate viruses and no detection limit for enumeration of viruses by FC (Weinbauer, 2006).

This study describes the development and application of a flow-cytometry method for rapid multi-parameter analysis of bacteria and viruses in aquatic systems. Phytoplankton, bacteria and viral counts were obtained for a large number of samples from Lake Okaro, a highly eutrophic lake in the Rotorua area which has been subjected to multiple remediation strategies.

One remediation strategy trialed on Lake Okaro was the addition of an aluminium-based P-inactivation agent Z2G1 (AQUAL-P), which uses zeolite clay as a carrier for the aluminium. This material was developed as a capping agent by Scion, Rotorua, and manufactured by Blue Pacific Minerals Ltd., Matamata, New Zealand. Approx 100 tonnes of modified zeolite product Z2G1 (500 g m^{-2}) was added to Lake Okaro as a granular (1-3 mm) product using a fertilizer spreader from a floating barge in September 2007. Sediment coverage was highly variable with patches of high- and low-sediment loading and some incorporation of Z2G1 granules into the sediment in places (Gibbs, 2010).

In August 2009 further dosing of Z2G1 was undertaken by mixing the Z2G1 (grain size 0.1-0.4 mm) with lake water (1:1 weight:volume). The resultant fine slurry was injected through fertilizer spreader nozzles 2-3 m below the lake surface at a rate of 166 g m^{-2} (total of 44 tonnes) from a barge, using GPS positioning to ensure even coverage of the lake sediment (Gibbs, 2010).

Remedial action taken to date on Lake Okaro appears to be having a positive effect on dissolved reactive phosphorus levels (Figure 1.) Comprehensive data sets provided by Environment Bay of Plenty show a gradual regression in DRP levels at the hypolimnion.

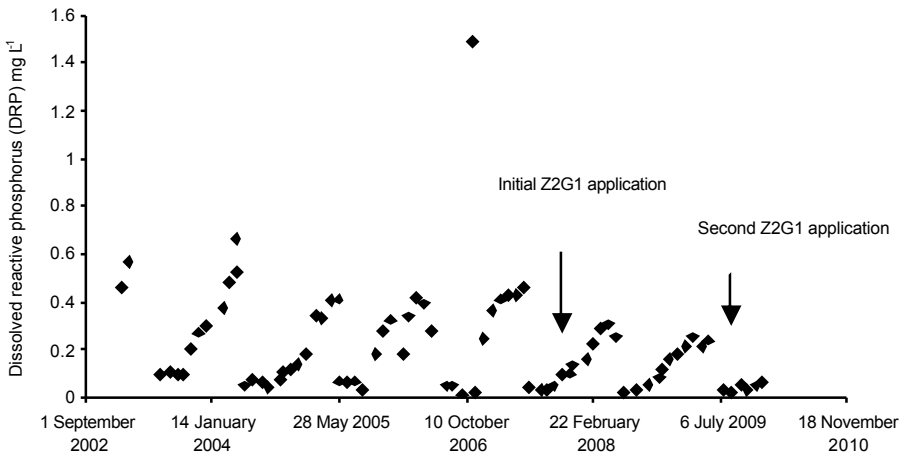


Figure 1. Dissolved reactive phosphorus levels at the hypolimnion in Lake Okaro from past ten years. Data courtesy of Environment Bay of Plenty

METHODS

1 STUDY SITE

Lake Okaro is a small explosion crater lake, 0.32 km^2 in area with a maximum depth of 18 m and mean depth 12.1 m. It is monomictic, and is continuously stratified between 4 and 8 m for around 8 months commencing in spring (c. September). Water enters the lake via a small stream on the north-west of the lake and drains from an outflow in the south-east. Previous high nutrient loading into Lake Okaro was due primarily to pastoral land use, as 95% of the lake catchment is used for pastoral farming (mostly dairy), and there is substantial remobilization of nutrients from the bottom sediments due mostly to prolonged anoxia of bottom waters associated with seasonal stratification (Hickey and Gibbs, 2009, McColl, 1972, Özkundakci et al. 2010). Lake Okaro has poor water quality, and has experienced recurrent blooms of *Microcystis* and *Anabaena* since the late 1970s (Dryden, 1986). The lake is an important recreational lake in the region and is the subject of ongoing studies aimed at improving the water quality through the use of catchment and in-lake remediation techniques (Gibbs and Özkundakci, 2010).

2 PURE CULTURES

2.1 PURE PHYTOPLANKTON CULTURES

Cultures representing the groups Bacillariophyta (diatoms), Chlorophyta (green algae), Chrysophyta (chrysopytes), Cryptophyta (cryptophytes), Cyanophyta (cyanobacteria), Dinophyta (dinoflagellates) and Euglenophyta (euglenophytes) were sourced from the Cawthron Institute (Nelson, NZ) culture collection and maintained in 50 mL plastic pottles (Biolab, NZ) containing 30 mL of MLA enrichment medium (Gorham et al., 1964, Bolch and Blackburn, 1996) at 300 $\mu mol\ m^{-2}\ s^{-1}$ on a 12:12 h light:dark cycle at 20 °C.

2.2 PURE BACTERIAL CULTURES

Cultures of *Pseudomonas putida* (Landcare Research, Canterbury, NZ), *Escherichia coli* (John Innes Centre Norwich, UK) and *Novosphingobium nitrogenifigens* (Scion, Rotorua, NZ) were grown on nutrient agar (Fort

Richard Laboratories Ltd, NZ) and cultured in nutrient broth (Fort Richard Laboratories Ltd, NZ) overnight. Samples showing exponential growth were fixed in 2% formaldehyde.

2.3 PURE PHAGE CULTURES

Pure phages, coliphage (infecting *E. coli*) and NZRM 2331 (infecting *P. aeruginosa*) were obtained from the Cawthron Institute (Nelson, NZ) and Environmental Science and Research (Christchurch, NZ). Phages were shipped as freeze-dried mass and reconstituted in nutrient broth prior to regeneration with host bacteria by a double-layer agar method (Adams, 1959).

3 SAMPLE COLLECTION

Water sampling began 13 August 2009, which was the week prior to the August 2009 Z2G1 application. Samples, were initially collected on a monthly basis and this shifted to weekly during stratification (11 November 2009 to 29 June 2910). Conductivity, temperature and depth (CTD) vertical profiles, also including photosynthetically active radiation, dissolved oxygen, pH and fluorescence, were taken from a station at the deepest point in Lake Okaro (38°180.14S, 176°236.35E) prior to water sampling . Temperature, light, dissolved oxygen, pH and fluorescence were visualized on a laptop immediately following the casts to ascertain the depth of the anoxic-oxic layer. Samples were then taken using a Schindler-Patalas trap from the epilimnion and, hypolimnion. Discrete depth samples were taken in the anoxic-oxic layer for bacterial and viral analysis using a modified sampling apparatus which enabled samples to be taken at 10 cm intervals throughout this layer.

3.1 PHYTOPLANKTON

Samples from the epilimnion and hypolimnion were immediately transferred into PVC containers containing 1% acidified Lugol's Iodine and kept in the dark for phytoplankton identification (results not reported here). Further samples were transferred into glass Schott bottles (ThermoFisher Scientific, NZ) and kept on ice for flow cytometry (FC) for phytoplankton identification and enumeration.

3.2 BACTERIA

Samples for enumeration by epifluorescence and FC were transferred immediately into prepared 15 mL Falcon tubes (Raylab, NZ) containing paraformaldehyde/PBS at a final concentration of 2% (m/v). Tubes were snap-frozen in liquid nitrogen and held at -80 °C.

3.3 VIRUSES

Sub-samples for enumeration by FC were transferred immediately into prepared 15 mL Eppendorf tubes containing paraformaldehyde/PBS at a final concentration of 2% (m/v). Tubes were snap-frozen in liquid nitrogen and held at -80 °C.

4 BACTERIAL ENUMERATION BY EPIFLOURESENCE MICROSCOPY

Frozen samples for bacteria were thawed at 35 °C and 2 mL vacuum-filtered through 0.22 µm MF-Millipore Membrane filter (ThermoFisher Scientific, NZ). The filter was dried with a Kimwipe (ThermoFisher Scientific, NZ) and placed sample-side up on a clean Petri dish (Raylab NZ). The filter was then stained with 300 µL of 2X SYBR Gold (Invitrogen Molecular Probes, USA) and incubated at room temperature in the dark for 15 minutes. Residual stain was vacuum filtered through the filter and excess moisture removed with a Kimwipe. The filter was then mounted onto a clean slide prepared with a drop of mounting solution (50% glycerol:50% TE buffer) followed by a drop of mounting oil. A clean cover slip was applied and the slide examined at 1000× magnification under blue-green excitation and oil immersion using an Olympus BX61 microscope equipped with analysis LS Research software (Olympus, USA). Photographs were taken using an Olympus Colourview (Olympus, USA) digital camera under bright field. Bacterial counts were undertaken using Image J particle counting software (National Institute Health, USA) with at least 300 cells counted from 20 randomly selected fields of views.

5 FLOW CYTOMETRY

Samples were analysed using a FACSVantage™ SE flow cytometer (Becton Dickinson (BD) Biosciences, USA) equipped with the BD FACSDiVa™ digital processing electronics and software option. The sheath fluid was phosphate-buffered saline solution (PBS) (Lorne Laboratories Ltd., UK), adjusted to pH 7.0 and delivered through a 70 µm nozzle at 131 kPa. Daily instrument optimization was performed using SPHERO™ fluorescent beads (BD BioSciences, USA). A target number of 1000 cells per second was used to obtain optimal cell counts by FC.

5.1 CELL COUNTS

Cell counts were obtained using an internal standard of diluted TruCOUNT™ beads (BD BioSciences, USA). A fluorescent bead stock was prepared by adding 1 mL of TE-Buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8) 10:1 for bacterial and viral enumeration and 1mL of PBS (polyphosphate buffer solution) for algal enumeration, to a TruCOUNT tube containing a known quantity of beads (n = 51 088). A sub-sample (100 µL) of bead stock was added to 1 000 µL of sample and a fixed number (n = 100) of beads counted directly by FC. Absolute cell counts were determined by using the following equation:

$$\text{cell count} = \frac{\text{number of beads in 100uL bead stock}}{x} \times \frac{\text{number of events counted per population}}{\text{number of events counted in bead population}}$$

where x = volume of sample (including dilution factors)

5.2 ALGAL ENUMERATION

Samples were vigorously shaken to disperse algal aggregates then passed through a 40 µm sieve (Falcon, USA) to remove zooplankton and large debris and prevent clogging of the flow cell. Samples (350 mL) were centrifuged (4 000 rpm, 10 minutes) to concentrate phytoplankton populations. The supernatant was discarded and 2 mL of concentrated sample retained for FC. A target number of 1 000 cells per second was used to obtain optimal cell counts by FC. Measured parameters included forward scatter (FSC), side scatter (SSC), chlorophyll fluorescence (650-700 nm), phycoerythrin fluorescence (575 nm), indicative of phytoplankton, and allophycocyanin (APC) fluorescence (660 nm), indicative of cyanobacteria. Cell populations were resolved using a dual gating procedure based on two-dimensional cytograms of APC-PE fluorescence.

5.3 VIRAL AND BACTERIAL ENUMERATION

Frozen samples for viral and bacterial enumeration were thawed at 35 ºC and the viral samples were diluted 1:10 in TE buffer in order to avoid coincidence (Shapiro, 2003, Duhamel and Jacquet, 2006). Bacterial samples were analysed undiluted. Samples were incubated with SYBR Green I (Invitrogen Molecular Probes, USA) (final concentration of 0.5×10^{-4} of the commercial stock) at room temperature in the dark for 5 minutes followed by incubation at 80.5 ºC in the dark for 10 minutes. Samples were cooled for 5 minutes then analysed by FC. Triggering was set on green fluorescence with a sample event rate below 1000 events s^{-1} and cell populations were resolved using a dual gating procedure based on two-dimensional cytograms of green fluorescence and SSC.

A regenerated phage culture of NZRM2331 was used to refine viral identification and to set detection limits and instrumentation parameters for FC and to infect bacterial samples of *P.pudita* for FC analysis. Field samples were then run under the same parameter settings.

RESULTS

Epilimnion temperatures ranged between 8.4 °C (17 August 2009) and 22.6 °C (16 February 2010) and hypolimnion temperatures between 7.8 °C (17 August 2009) and 10.0 °C (29 June 2010).

Lake Okaro was completely mixed (< 0.1 °C temperature difference between top and bottom) until late August 2009 (Figure 2). The first seasonal temperature differential was observed at a depth of ca. 9 m (7 September 2009) (Figure 2). The thermocline depth reduced throughout the sampling period to a minimum of 5 m (19 January 2010) before increasing again to the point where the lake was completely mixed at the end of June 2010.

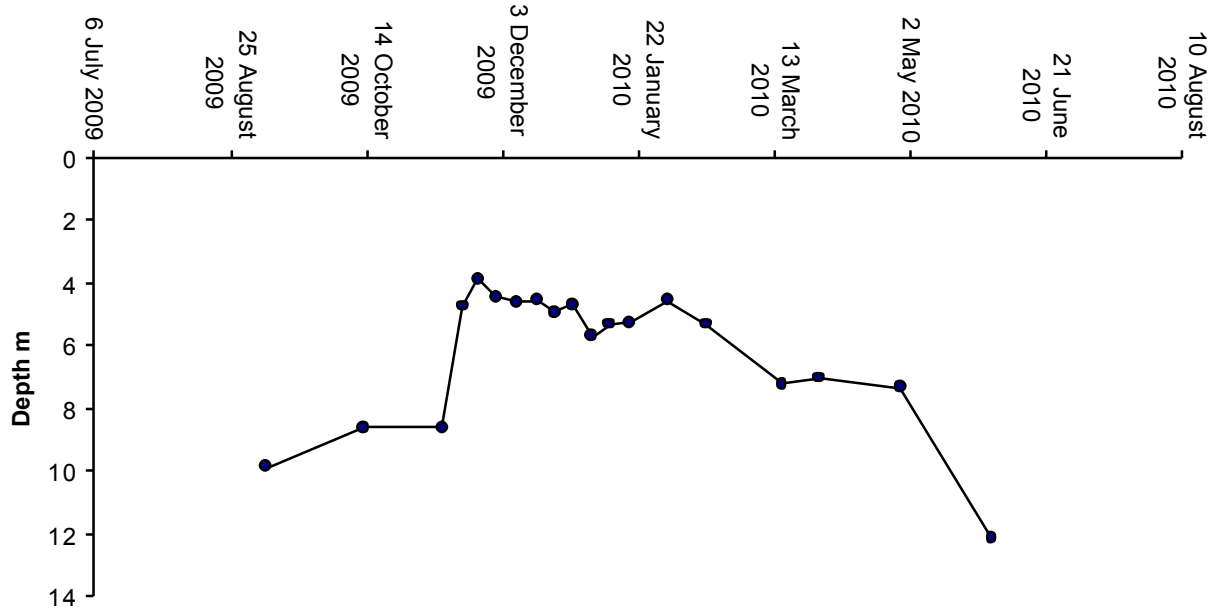


Figure 2. Thermocline depth as determined by temperature differential (> 2.5ºC temperature difference between top and bottom) in Lake Okaro between August 2009 and July 2010.

Dissolved phosphorus concentrations (DRP) in the hypolimnion were variable at the beginning of the sampling period and more stable toward the end (Figure 3). They were also below the values reported by Environment Bay of Plenty in previous years continuing the regressional behaviour plotted over the past eight years (Figure 1).

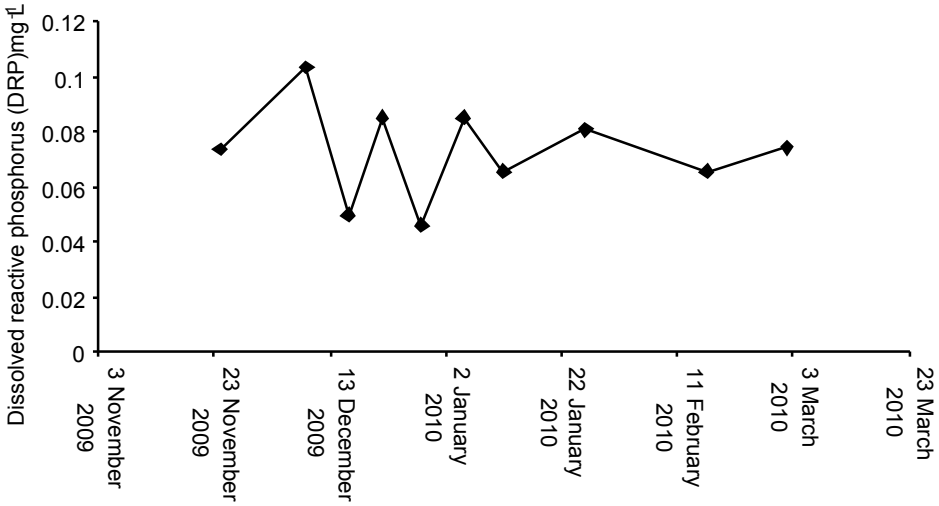
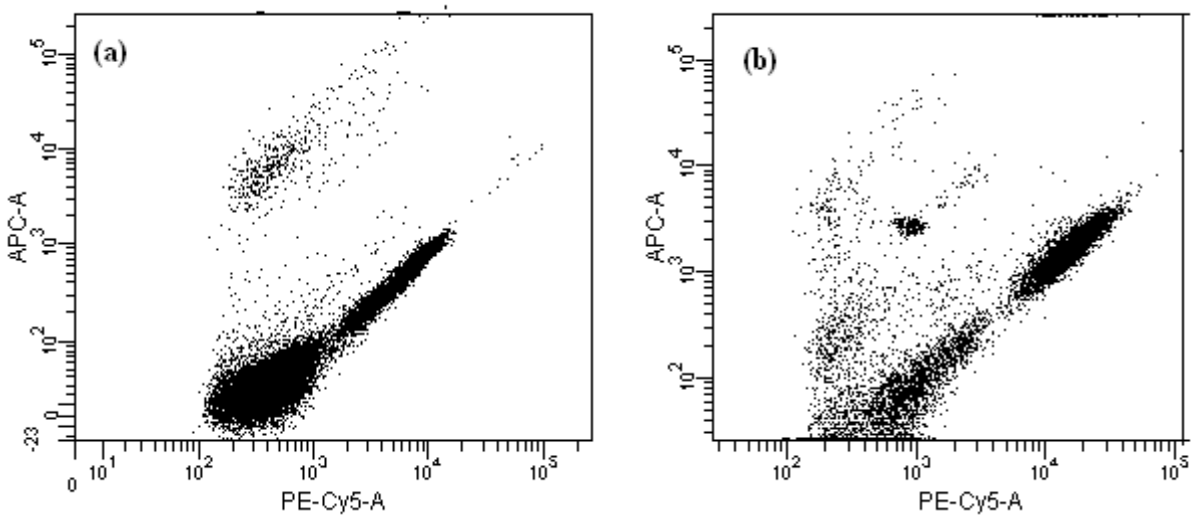


Figure 3. Preliminary dissolved reactive phosphorus (DRP) levels in the hypolimnion of Lake Okaro from monthly samples collected between August 2009 and June 2010.

Unialgal cultures were used to produce a template for determining phytoplankton counts in the lake samples. Cultures representative of the phyla Euglenophyta, Cryptophya, Bacillariophyta, Cyanophyta, Dinophyta and Chlorophyta were processed both separately and as mixed cultures by FC, and displayed as a bivariate scatterplots of APC-PECY5 fluorescence. The discrete clusters formed by the unialgal cultures were used to discriminate the composition of field samples collected from Lake Okaro.



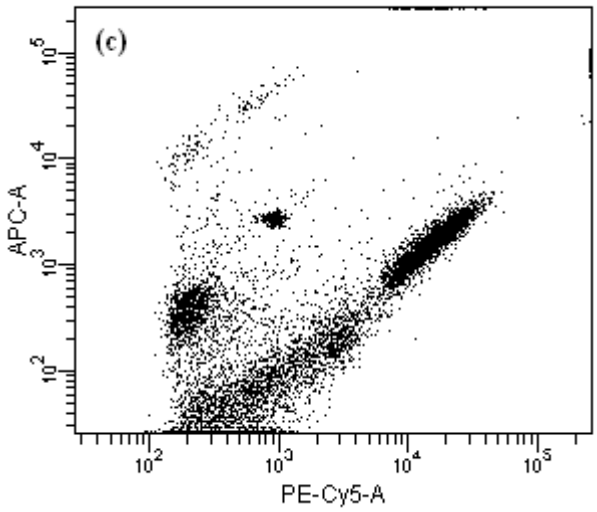


Figure 4. Example of scatterplots of allophycocyanin (APC) versus chlorophyll (PE-Cy5) fluorescence from (a) mixed unialgal culture, (b) Okaro epilimnion and (c) Okaro hypolimnion.

Fluoresence levels in Lake Okaro peaked on 19 January 2009 at 148 (Figure 5) at a depth of 6.6 m (thermocline depth was 5.3 m) indicating that this was the period when phytoplankton numbers were at their greatest and located just above the anoxic-oxic layer.

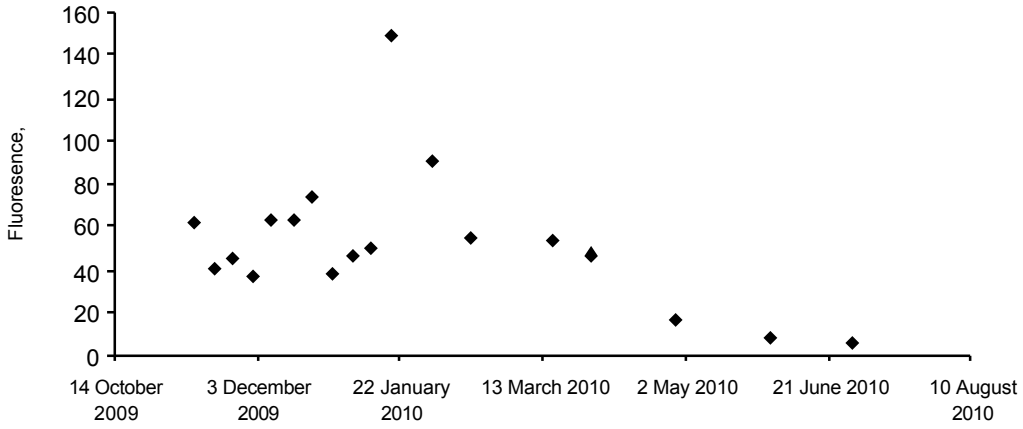


Figure 5. Maxiumum fluoresence values in Lake Okaro between October 2009 and June 2010.

Epifluoresence counts of bacteria at the epilimnion and hypolimnion were maximal at 3.04 x 10^6 cells ml^{-1} (date) and 4.89 x 10^6 cells ml^{-1} (date), respectively. Minimum bacterial counts were 4.53 x 10^5 cells ml^{-1} (date) and 1.61 x 10^5 cells ml^{-1} (date), respectively. There was considerable variation between FC and epifluorescence counts (Figure 6.)

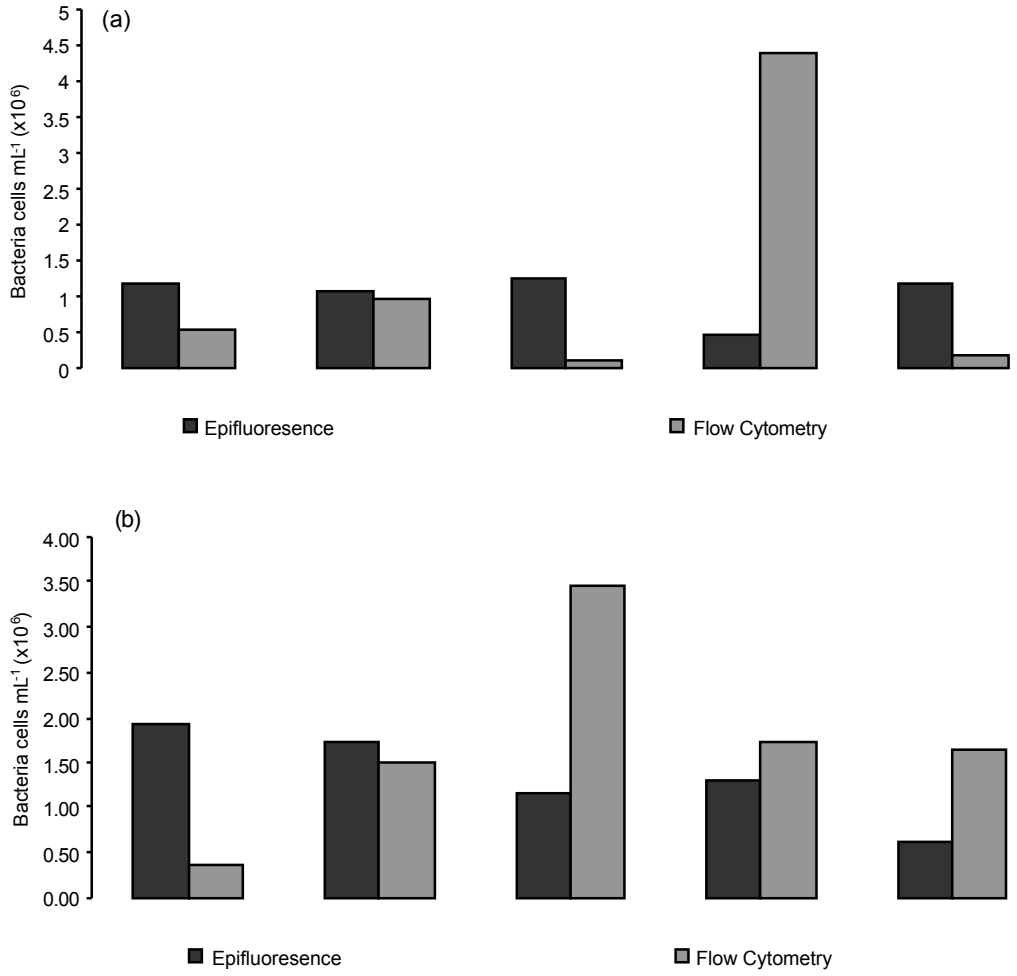


Table 1. Bacterial counts from the a) epilimnion and b) hypolimnion by epifluorescence and flow cytometry (FC) between 13 August 2009 and 29 June 2010.

Scatterplots of viral counts by FC show a distinct difference between pure cultures and samples from Lake Okaro (Figure 4). Figure 4a shows the scatter plot of *Pseudomonas pudita* infected with a phage NZRM2331 and shows the discrimination of two viruses indicated by red circles. However the more complex scatterplot obtained from a Lake Okaro sample shows a number of discriminatory groupings indicating the presence of a several phages with differing nucleic acid makeup.

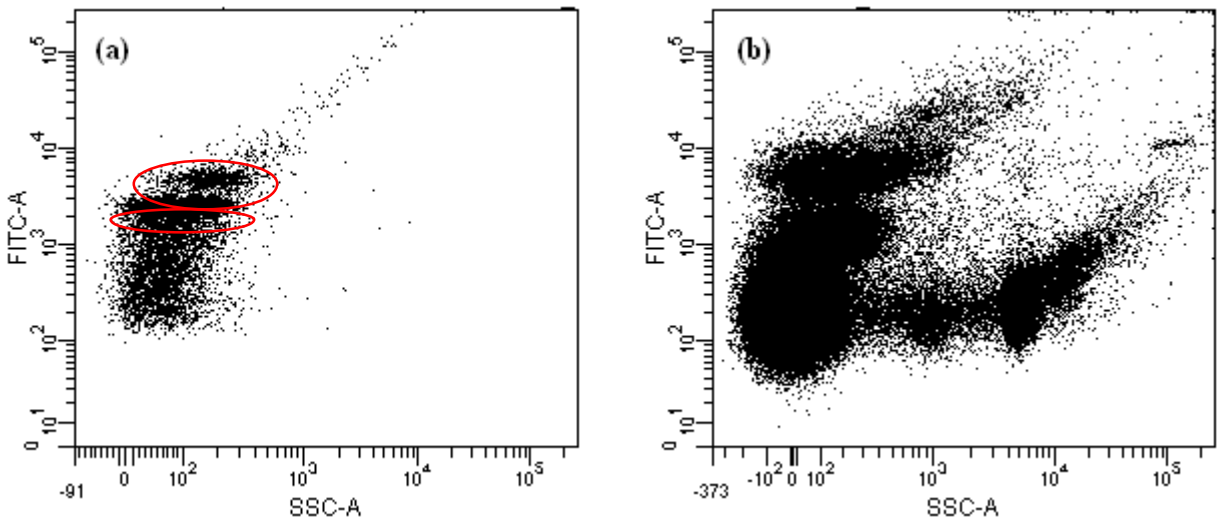
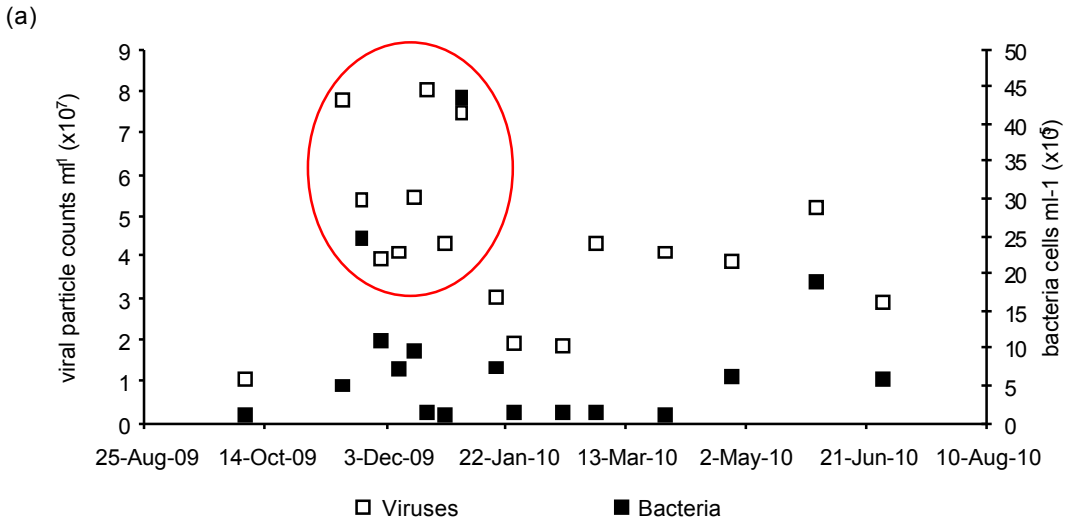


Figure 4. Scatter plots of a) *Pseudomonas pudita* infected with NZRM2331 (1000× dilution) and b) Lake Okaro surface water from November 2009 (undiluted) stained with SYBR Green, side scatter (SSC) vs. green fluorescence (FITC).

Viral and bacterial counts (determined by FC) varied throughout the sampling period (Figure 5a and b). In the epilimnion there was a marked increase in viral-like particles (VLP) in December. Bacterial concentrations were more variable during this period, with high (43.7 x 10^5 on 5 January 2010) and low concentrations (0.96 x 10^5 on 29 December 2009) occurring. In the hypolimnion bacterial concentrations were relatively stable apart from a period of sustained elevated levels in December (14.9 x 10^5 on 16 December 2009 to 34.7 x 10^5 on December 29). Viral-like particles followed a similar trend.



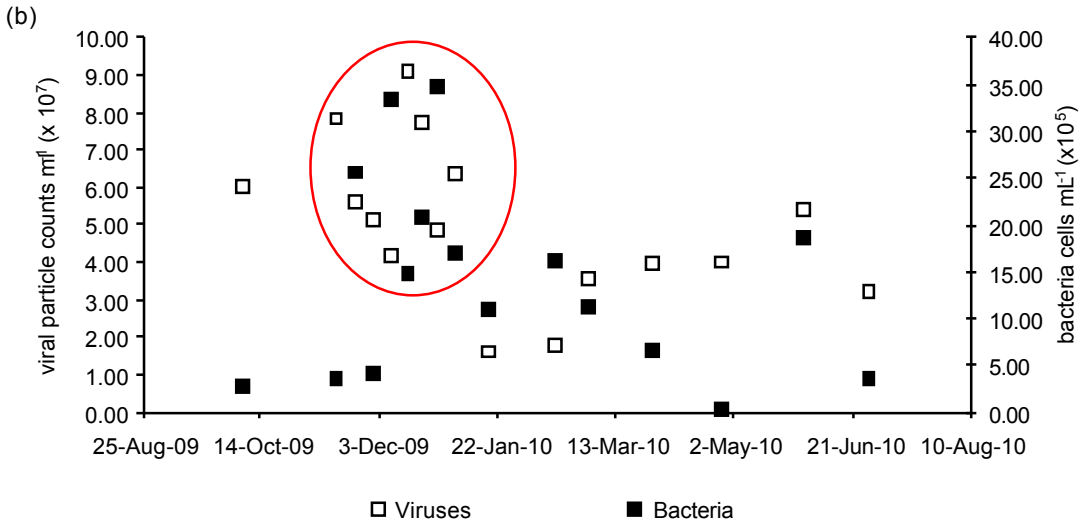


Figure 5. Bacterial and viral particle counts by flow cytometry (FC) as particles mL^{-1} in the a) epilimnion and b) hypolimnion of Lake Okaro between September 2009 and June 2010. Red circles indicate periods of marked increases in viral and/or bacterial concentrations.

6 CONCLUSIONS

The FC methods used in this study enabled a large number of samples to be processed, allowing relationships between, bacteria and VLPs in to be investigated in Lake Okaro.

Analysis of this data has shown that marked changes in bacteria and VLP occur on seasonal time frames. Viral concentrations across freshwater and marine aquatic systems have been reported at less than 10^4 to greater than 10^8 viral like particles mL^{-1} with some eutrophic lakes ranging between 10^7 to 10^8 viral like particles mL^{-1} (Bettarel et al., 2006,Middleboe et al., 2008). Viral abundance in Lake Okaro falls within the range reported by Bettarel at 1 to 8 x 10^7 viral particles mL^{-1}. The peaks in VLP and bacteria in December appear to be correlated with DRP availability at this time indicating the importance of studying the interplay between physical, chemical and biological parameters when study lake dynamics. Further in-depth analysis of this dataset will be undertaken as additional samples are fully analysed and characterized.

Epifluoresence microscopy counts of bacteria were comparable to FC, with counts neither higher nor lower by either method but equally variable. Epifluoresence microscopy methodology requires the counting of a portion of the filter to ensure a total count of only 300 cells, whereas FC counts a much larger number of cells and therefore a more representative population. There are also the inherent problems associated with EFM through the loss of fluorescence due to exposure to light. Bacterial and viral enumeration cannot be performed on a common sample due to the high VBR ratio. Sample dilution for viral analysis is imperative to avoid coincidence caused by the high number of particles passing through the laser.

The bacterial concentrations reported here are also very variable, ranging from 9 x 10^4 up to 5 x 10^6 cells mL^{-1}. A high degree of variability would also be expected spatially through the lake due to the varying physical conditions caused by stratification which in turn creates extreme differences in lake chemistry and thus bacterial activity and composition. Virus to bacteria ratios (VBR) have also been reported as highly variable, with an average value of 20 across all aquatic systems. Our findings in this study are significantly higher than this ratio, with a VBR ranging between 17 to 450. This may be a reflection of the trophic status of the lake. Although nutrient concentrations continue to decline following remedial treatments in the lake the overall trophic status continues to support increased microbial growth which in turn provides for viral infection leading to cell lysis and further nutrient cycling.

The methods reported in this paper provide only for enumeration of microorganisms. Our future research will combining FC counting and sorting with molecular techniques, greatly enhancing the capability of the methodology. Through the utilisation of cell sorting there is potential for culture of phytoplankton and amplification of bacterial and viral DNA and RNA for quantification and analysis of community structure and composition. The attachment of fluorochromes or oligonucleotides can also be used in the determination of community heterogeneity as well as indicating cell health and physiological state.

A greater understanding of the role of microbial elements of a freshwater system can be gained by using FC to investigate the spatial and temporal distribution of these organisms and to gain further insight into the community structure and diversity of these systems. There is still little information on viral genome sizing and sequencing in environmental samples and on community structure of micro-organisms of functional significance in freshwater systems. The use of FC in conjunction with molecular methods will provide a broader understanding of the structure and function of the microbial communities of lakes of ranging trophic status.

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