COMPARISON OF PHYTOPLANKTON SUCCESSIONS IN TWO EUTROPHIC LAKES BY FLOW CYTOMETRY

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ABSTRACT

Flow cytometry is an effective tool for the study of algal ecology, physiology and toxicology. It allows for multi-parameter analysis of individual cells on the basis of light scattering properties, indicating cellular complexity, and fluorescence. Flow cytometry has been widely used in oceanography to quantify and sort heterogeneous algal populations and more recently in the development of bioassays for marine and freshwater algae using toxicity tests based on growth inhibition. Fluorescence emission characteristics may be especially useful in classifying blue-green algae (cyanobacteria) as they contain phycoerythrin (550-600 nm emission), chlorophyll *a* (650-700 nm emission) and allophycocyanin (660 nm emission). The basis of this work was to assess the utility of flow cytometric analysis for the rapid identification and sorting of freshwater algae and cyanobacteria species, with particular emphasis on the Rotorua lakes. Using a selection of laboratory cultured freshwater algae and cyanobacteria species, this study demonstrated unique light scatter and fluorescent characteristics for each species examined. These properties allowed for rapid species identification and sorting of mixed populations of laboratory cultures and Rotorua lakes water samples. Further analysis of lake water samples collected over time demonstrated changes in abundance and community composition that may be useful in future examination of seasonal changes in lake dynamics.

KEYWORDS

Flow cytometry; phytoplankton; cyanobacteria; Rotorua lakes; eutrophic;

1   INTRODUCTION

Several lakes within the Rotorua Lakes District in the central North Island of New Zealand have undergone rapid eutrophication and experience regular cyanobacterial blooms over the summer months, with marked but still relatively poorly understood successional sequences associated with these bloom periods.

Disturbances in aquatic waters are caused by a number of factors such as changes in thermal stratification, pollution levels and predation pressure (Figueredo & Giani 2001) and phytoplankton blooms are a prime agent of water quality deterioration. (Paerl H.W. et al. 2001). Phytoplankton communities are strongly influenced by both nutrient bioavailability and temperature (Abrantes et al., 2006). Cyanobacteria in particular are known to possess a strong temperature-dependence for growth (Hense and Beckmann, 2006) and may respond more strongly than diatoms and chlorophytes to rising temperature (Domis et al, 2007). Imai et al. (2009) have recently demonstrated that growth rates between two related cyanobacteria species (*Microcystis aeruginosa* and *M. Wesenbergii*) differed significantly at higher temperatures (30 and 35°C) but not lower temperatures (20 and 25°C) suggesting that temperature changes during summer are a key factor controlling succession, at least in this group. In addition, many bloom-forming cyanobacteria possess the ability to fix atmospheric N_2, enabling them to capitalise under conditions of excess phosphorus and low nitrogen (Pearl et al. 2001). Thus, in eutrophic systems, chlorophytes may be rapidly replaced by cyanobacteria as temperature increases and nitrogen becomes limited between the spring and summer months (Pearl et al., 2001; Ke et al., 2008).

Up until the early 1900's most of Rotorua's lakes were nitrogen limited and a number have remained this way. However the increased supply of nitrogen from anthropogenic activities has led to a number of lakes becoming phosphorus limited, and in some cases nitrogen and phosphorus limited. (Burns N., McIntosh J., & Scholes P. 2005). The soils of these lakes have high allophone content and tend to be phosphorus absorbing, but the cold springs entering the watersheds tend to be high in phosphorus from minerals dissolved in the underlying geology. Progressive nutrient enrichment resulting from conversion to pasture during the 1950's and 1970's (McColl R.H.S. 1972) (Jolly V.H. 1968) has led to the eutrophication of several of these lakes.

Cyanobacteria grow better in warmer temperatures (above 25ºC) giving them a competitive advantage over green algae and diatoms. (Paerl H.W., Fulton R.S., Moisander P.H., & Dyble J.2001). Drought conditions can inhibit surface and groundwater seepage into water bodies thereby increasing water residence time in lakes and favouring bloom formation. Often these blooms take place after elevated spring rains followed by extended drought periods. (Paerl H.W., Fulton R.S., Moisander P.H., & Dyble J.2001). Dry summers also create an effect on nitrogen and phosphorus loading in hypoxic sediments. Lowered runoff favours nitrogen fixing organisms as the N:P ratio becomes more phosphorus rich.

Turbulence regulates bloom dynamics and cyanobacterial are particularly sensitive to water column stability and vertical stratification. (Paerl H.W., Fulton R.S., Moisander P.H., & Dyble J.2001). Vertical stratification in lakes is strengthened by global warming thereby reducing vertical mixing and the resulting early stratification and late destratification provides optimal growth conditions for phytoplankton.

Gentle mixing of waters promotes phytoplankton growth however excessive turbulence can cause disintegration of aggregates and thereby reduced growth. Some cyanobacteria are also able to counter turbulence via their own internal buoyancy.

Numerous methods for measuring the relative abundance and composition of phytoplankton species exist, though when it comes to species discrimination, most have severe limitations. Chlorophyll-a (*chl-a*) is commonly used as a proxy for total phytoplankton biomass (Gregor & Marsβlek 2004) (Aminot A & Rey F 2000). After a simple extraction step *chl-a* can be easily measured fluorometrically or spectrophotometrically. However, monitoring chl-*a* provides little information on phytoplankton population dynamics and therefore potentially misses important shifts in community composition often associated with changes in lake processes (i.e., cyanobacterial domination during stratification). Light microscopy has traditionally been used to identify and enumerate phytoplankton. Distinguishing genera or species using morphological characteristics remains difficult, laborious, and requires a great deal of expertise and experience. In the past decade alternative detection methods based on molecular assays have been developed including; denaturing gradient gel electrophoresis (DGGE) (Janse et al., 1993), microarrays (Castiglioni et al., 2004) and qPCR (e.g., Bowers *et al*, 2000; Kurmayer and Kutzenberger 2003). Molecular techniques have the limitation that they only detect organisms from specific phyla (e.g., Castiglioni et al., 2004; developed a micro-array specific for cyanobacteria) or that the number of targets that can be detected in a single assay is minimal (i.e., <3 in qPCR). Thus, the ability to monitor multiple phyla of algae and therefore track phytoplankton community dynamics is not practical using these techniques.

Flow cytometry is a potentially powerful tool for measuring mixed phytoplankton communities in freshwater and marine environments (Marie et al., 2005). The advantage of flow cytometry over traditional methods arises from the ability to rapidly and simultaneously investigate multiple optical characteristics such as size, structure and fluorescence. Furthermore, some flow cytometers are capable of cell sorting which offers the ability to sort and culture subpopulations to enhance identification by other methods (Reckermann, 2000). The autofluorescing compounds, or photosynthetic pigments, are especially valuable for discriminating mixed algal populations e.g. chlorophyll (green algae), phycoerythrin (red algae) and phycocyanin (cyanobacteria) and for species that are often difficult to identify using light microscopy due to their size (e.g. picophytoplankton). The spectra of the same types of phycobiliproteins from different species can also differ (Shapiro H.M. 2003). This combination of multi-parameter analysis is especially useful for the discrimination of algal species.

Despite the increasing popularity of flow cytometry in algal toxicity testing (e.g. Stauber et al. 2002), quantification of sorting of marine algal populations (Wood et al. 2002; Crosbie et al. 2003; Marie et al. 2005; van den Engh et al. 2009) and the enumeration of freshwater algal species (Becker et al. 2002; Yew-Hoong Gin and Yee Neo 2005), there has been no attempt to understand phytoplankton succession using this tool. The purpose of this study was to assess the phytoplankton dynamics in two of Rotorua's most anthropogenically degraded (eutrophic) lakes using modern flow cytometric techniques coupled with traditional light microscopy.

2   STUDY SITES

Figure 1. Lakes in the Rotorua district
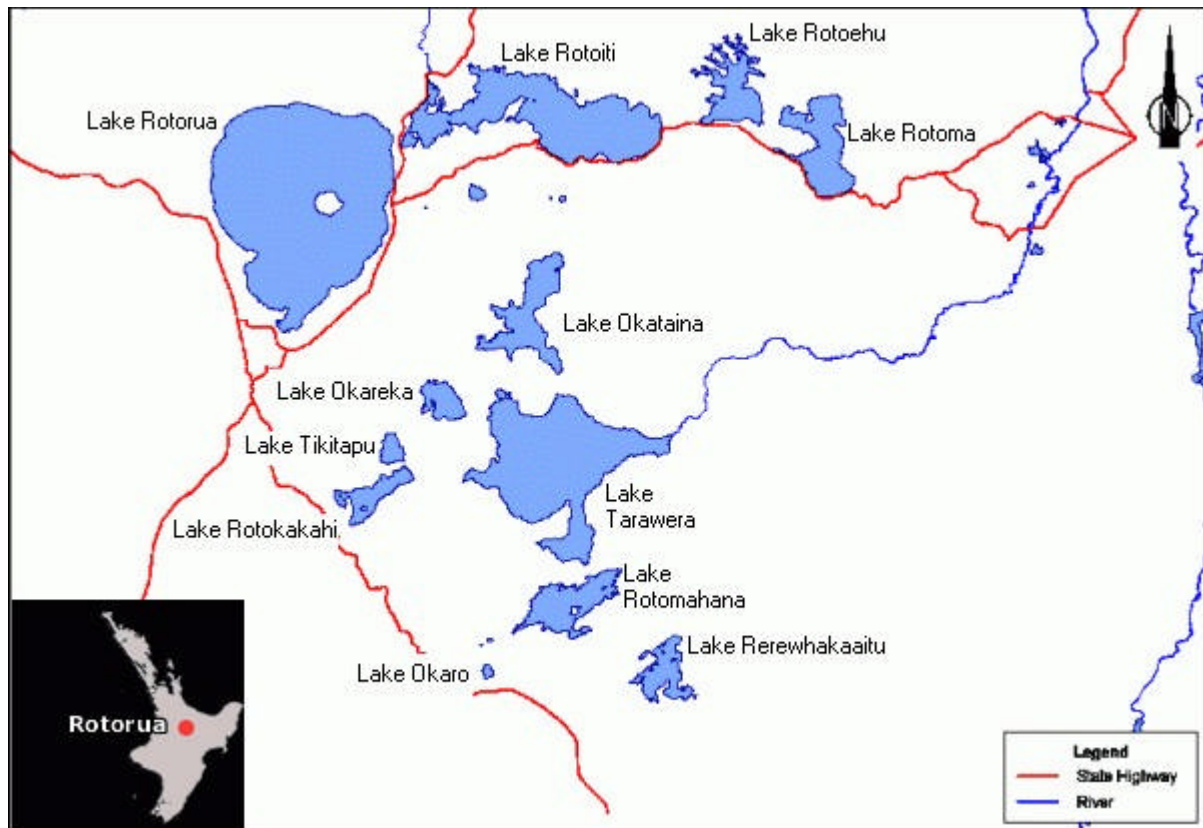
2.1   LAKE OKARO

Lake Okaro is a highly eutropic lake located southeast of Rotorua between the Waimangu thermal reserve and the township of Rotomahana. It is a small explosion crater lake, $0.32km^2$ in area formed about 900 years ago by hydrothermal eruptions (McColl R.H.S.1972). The lake has a maximum depth of 18m with water entering via a small stream on the north-west of the lake and draining from the south-east. High nutrient loading into Lake Okaro is due to geothermal activity in the area, rural runoff and seepage to groundwater, and remobilization from the bottom sediments. The soils around Lake Okaro are influenced by Rotomahana mud and heavier clay soils. (McColl R.H.S.1972). Lake Okaro has poor water quality and suffers from recurrent algal blooms in the summer months. It is the subject of ongoing applications of a modified zeolite compound designed to create a phosphorus absorbing cap to limit phosphorus release from the sediment layer and ongoing monitoring of ecological, physiological and toxilogical effects. (Environment Bay of Plenty 2009)

2.2   LAKE ROTOEHU

Lake Rotoehu is located 35km north-east of Rotorua and is the smallest of a chain of three lakes lying on the edge of the Okataina caldera. The lake has an area of $8.1km^2$ and maximum depth of 13.4m. There are no major inflows or outflows and the water source is from small streams and sub-terranian flow. The lake has a series of arms at its northern end and geothermal springs located on the south east shore. It is possible that the south west section of the lake may receive thermal input. (Kitsios A 2009)The exit for the water is via a sink hole along the shoreline of one of the northern arms (Kitsios A2009). The soil around Lake Rotoehu is a light

pumice and mainly basaltic Tarawera ash. (McColl R.H.S.1972). Lake quality deteriorated significantly in 1994 with the main impact on the lake originating from runoff to surface waters and soakage to groundwater from surrounding pastoral land. Lake Rotoehu suffers significant algal blooms in the summer months and severe hornwort (*Ceratophyllum demersum)* infestation in the southern corner (Wikipedia 2009).

3   METHODS

3.1   FIELD SAMPLE COLLECTION

Water samples from Lake Okaro were collected on a weekly basis between October 2008 and February 2009. One litre samples were collected into glass Schott bottles using a Schindler-Patalas trap at depths of 1m, 3m, 5m, 7m and 9m. Sampling took place from the same location (at a central point in the lake) and at approximately the same time of day each week. Samples were immediately transported back to Scion for analysis.

3.2   PURE CULTURE COLLECTION

Pure algal samples were obtained from Waikato University and Cawthron Institute. All samples were kept at $20^{o}C$ under cool-white fluorescent lamp with an intensity of 3200lux and diurnal conditions. Cultures were maintained in MLA media (Bolch & Blackburn 1996) (Gorham P. et al. 1964) apart from *Trachelomonas* which was maintained in modified G.P. Media. (Loeblich A.R. & Smith V.E. 1968).

3.3   PREPARATION

Samples were prepared for microscopy and Flow Cytometry within 4 hours of collection to avoid degradation of samples and possible ingestion by zooplankton collected with the sample. Each sample was vigorously shaken to disperse colonial algal species then sieved through a 40µm sieve to remove zooplankton and large debris to prevent clogging of the flow cell. 350ml samples were then centrifuged at 5000rpm for 10 minutes to concentrate algal populations, the supernatant discarded and a resulting 4ml of concentrated sample retained for microscopic analysis and Flow Cytometry. The samples were concentrated to give an optimal cell count of 1000 cells per minute for Flow Cytometry.

3.4   MICROSCOPY

Morphological analysis at Scion was carried out on unpreserved samples using an Olympus BX61 at 400x magnification. Photographs were taken using an Olympus Colourview digital camera under bright field. Some species identifications were made by reference to Baker and Fabbro (2002).1000µL of concentrated sample was preserved with Lugols iodine and sent to Cawthorn Institute for cell counts and identification.

3.5   FLOW CYTOMETRY

Concentrated samples were analysed unstained using a FACSVantage SE DiVa (BD Biosciences, USA) flow cytometer. The sheath fluid was 1xPBS and a daily calibration was undertaken using Becton Dickinson Calbrite Beads. Measured parameters were size (forward scatter), granularity (side scatter), and the fluorescence of chlorophyll (red) or phycoerthrin (PE-C5-A) (orange) for phytoplankton, and phycocyanin (PEA) and allophycocyanin (APC) for cyanobacteria fluorescence. Species/population discrimination was obtained using a dual gating procedure based on two dimensional cytograms of Allophycocyanin - Phycoerthrin.

3.5.1   CELL COUNTS

Cell counts were obtained using an internal standard of diluted TruCOUNT™tubes (BD BioSciences). A fluorescent bead stock was prepared by adding 1mL of MLA growth medium to a TruCOUNT tube containing a known quantity of beads (51088 beads). 100µL of bead stock was added to 1000µL of algal sample and the sample then counted directly on the flow cytometer. Absolute cell counts were determined by the following equation:

No. of beads in 100µL bead stock   **x** No. of events per population **x** concentration factor   (1)
　　　1mL　　　　　　　　　　No. of events in bead population

3.5.2　SORTING

For sorting the frequency was set at approximately 39kHz and the drop delay set at between 14 and 16. The sheath fluid was retained as 1xPBS. Sort criteria were defined by the populations which had been distinguished in the original cell counting and set up and the sort optimized for yield. The gated cells were collected into 2mL of MLA medium. The collected sort was then aseptically transferred into 50mL prepared MLA medium (Gorham P., McLachlam J., Hummer V., & Kim W.1964) (Bolch & Blackburn1996) in a sterile specimen container for growth studies and subsequent identification.

(Baker P.D & Fabbro L 1999)

4　RESULTS

4.1　SPECIES DISCRIMINATION

Pure cell cultures of phytoplankton were used for optimization of flow cytometer operation and for species discrimination. Cultures were processed by flow cytometry and the resulting dot plot patterns used as reference plots for identifying species in mixed cultures.

Figure 1. Dot plot and cell counts from 9m deep Lake Rotoehu
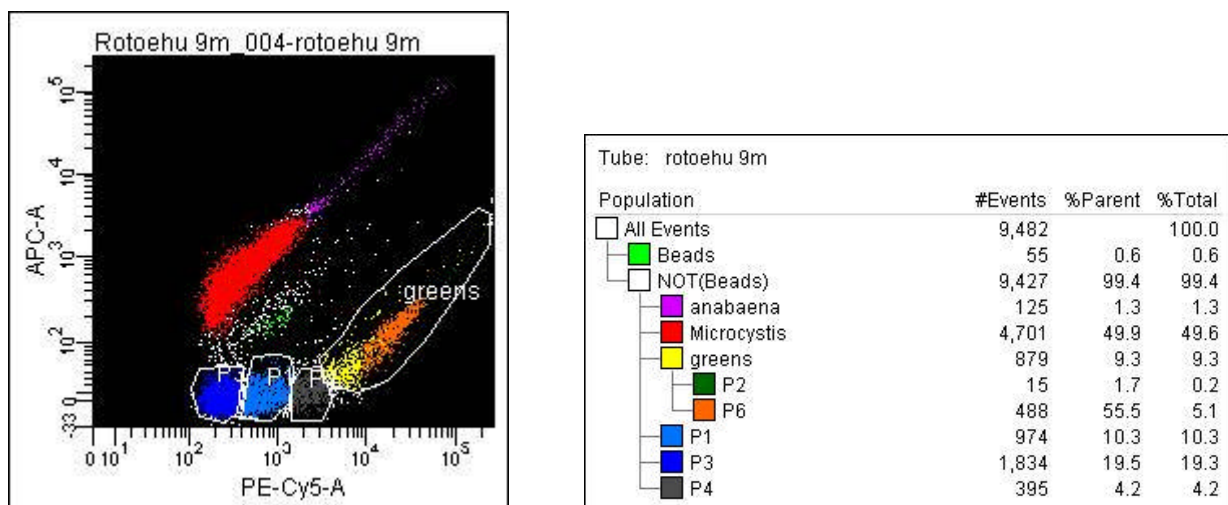
4.2　ALGAL COUNTS

Algae were classified as either Cyanobacteria or Eukaryotes with the Eukaryotes further classified as green algae, Euglenophyta, Cryptophyta, Dinoflagellates or Diatoms.

Table 1. Phytoplankton characteristics

	Structure	Cell Wall	Pigment
Eukaryotes			
Green algae	unicellular or colonial		chlorophyl a & b
Euglenophyta	unicellular		chlorophyl a & b
Cryptophyta	unicellular		chlorophyl a & c 2, phycoerythrin and phycocyanin
Dinoflagellates	unicellular with flagella	armour (theca)	chlorophyl a & c
Diatoms	unicellular or colonial	silica cell wall (frustule)	carotenoids
Cyanobacteria			
	unicellular or colonial colonies may form filaments		chlorophyl a, phycoerethrin, phycocyanin and allophycocyanin

4.2.1   LAKE OKARO

Algal counts as cells per milliliter were correlated for the sampling period and graphed to show the relative abundance of species and to show changes in total algal counts. Temperature data for the surface waters was included.

Figure 2. Seasonal phytoplankton counts for Lake Okaro at varying depths.
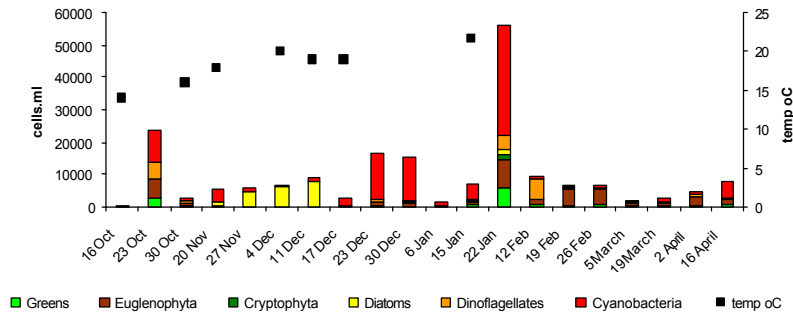
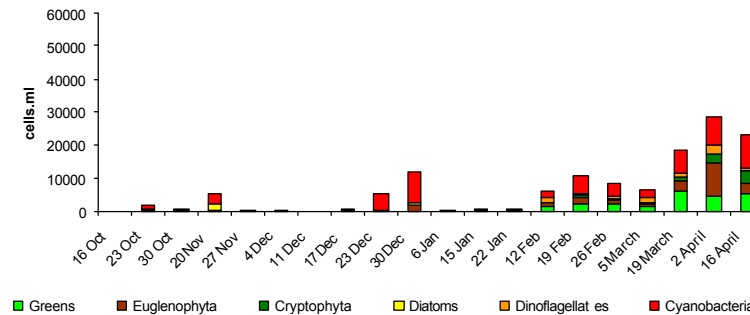*(a) 1metre deep*
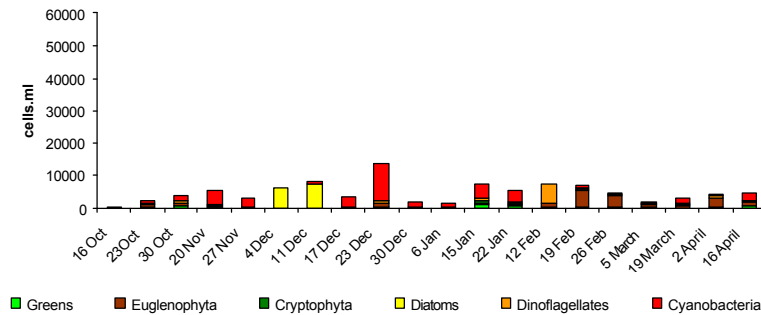
(b) 3metre deep
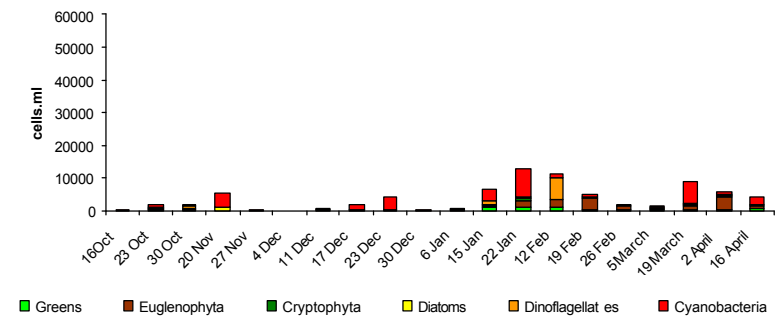
(c) 5metre deep
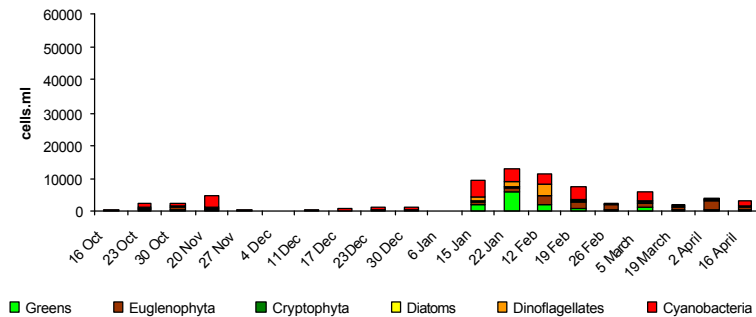
(d) 7metre deep

(e) 9metre deep

Lake Okaro experienced some bloom episodes during the season, particularly in the top water layer. Cell counts increased at the end of October and again dramatically at the end of January, coinciding with increases in water temperature. However these blooms dissipated and cell counts were considerably less at the end of the sampling period in April. Between the three and seven metre mark cell counts were reasonably stable with no major peaks in total cell numbers. There were shifts in the type of phytoplankton present with the emergence of greater numbers of green algae toward the end of the season. Diatoms were only present in large numbers at the beginning of the season and appeared to dominate in the top layers around the December period before giving way to cyanobacteria domination. Euglenophyta also became more abundant toward the end of the season at all levels in the lake and may have contributed to the brown hue to the lake. These phytoplankton are favored by a highly eutrophic environment and are often found in ponds and ditches near bovine pastures (University of Maryland 2009). Sampling at the end of January saw a fish kill at the boat ramp and a foul stench emitting from the 7 metre mark in the lake. *Peridinium* (dinoflagellate) were detected in samples at this time and this organism is know to cause odors and is responsible for fish kills. The end of the season saw a shift in the distribution of total phytoplankton numbers from the top layer into the bottom 9m depth. Not only were there less total phytoplankton on the surface but there was an increase in all species at the lower level. Lake Okaro is a stratified lake and tends to become anoxic at the end of a stratified period (Burns N., McIntosh J., & Scholes P.2005). This anoxia may cause increases in anoxically regenerated nutrients and therefore may be the reason for the increased cell counts of phytoplankton as this low depth. As the surface waters have supported high numbers of phytoplankton over the season they have become somewhat nutrient deficient and phytoplankton growth has then concentrated on the lower depths where there may be increased availability of nutrients for growth through nutrient regeneration.
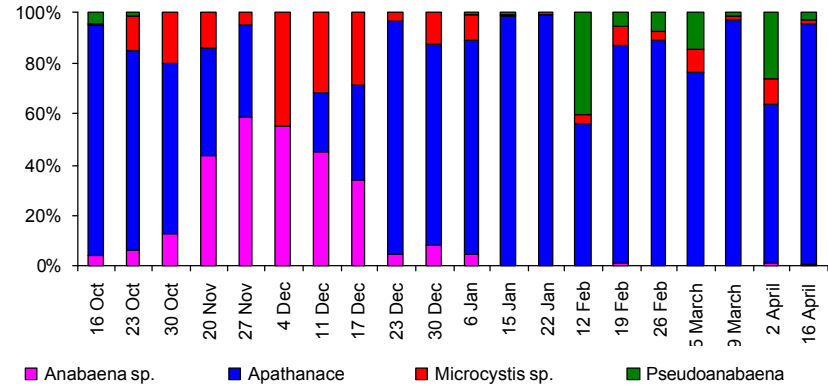
Cyanobacterial populations were split into four major groups, *Anabaena sp., Apathance, Microcystis sp.* and *Psendoanabaena sp.,* and the dominance of each species graphed.

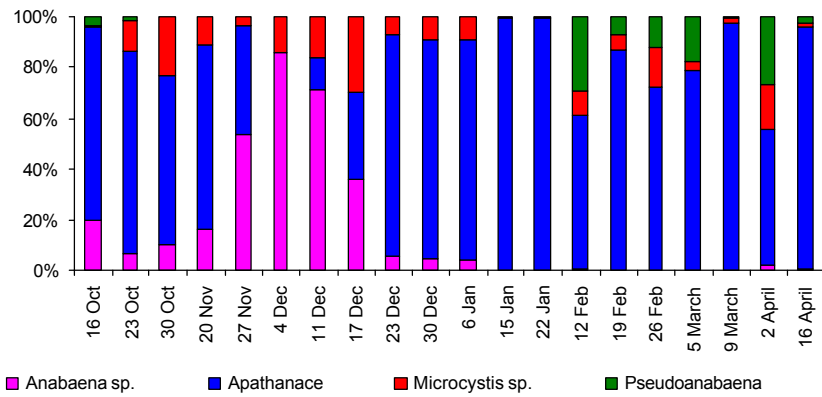Figure 3: Cyanobacteria species breakdown at varying depths in Lake Okaro
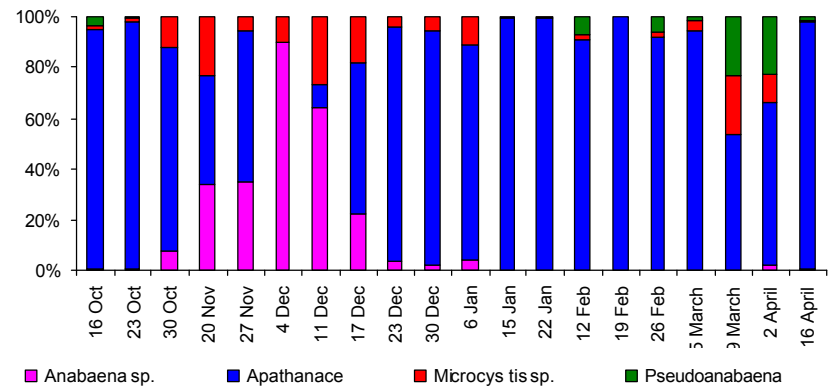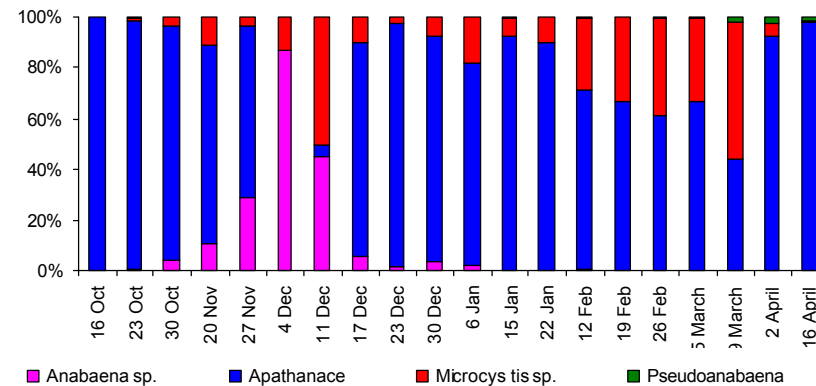
(a) 1 metre deep
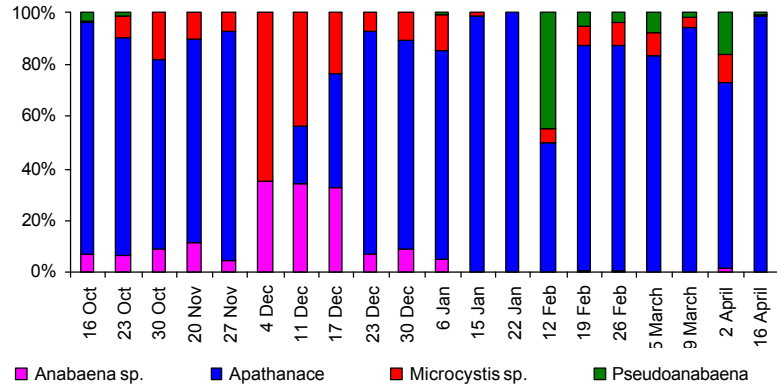
(b) 3metre deep
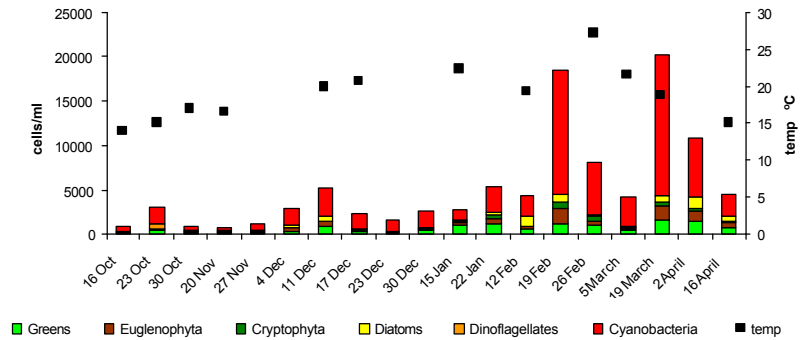
(c) 5 metre deep

(d) 7 metre deep

(e) 9 metre deep

The dominating cyanobacteria in Lake Okaro was recognized as *Apathance* apart from a short period between November and December when *Anabaena sp.* were the dominant cyanobacteria. *Anabaena sp.* are able to fix nitrogen in nitrogen limited conditions and prefer conditions where there is water column stability and increased underwater radiance (Havens 2008). Lake conditions in November and December may have supported the growth of this cyanobacteria with less available nitrogen in the lower lake levels prior to anoxic conditions later in the season. *Pseudoanabaena* are rarely recognized as a dominant cyanobacterial species but can form blooms in eutrophic waters (Acinas et al. 2008) and this is borne out in the appearance of a small bloom late in February which was concentrated in the upper water levels. *Microcystis sp.* also bloomed during December in the upper waters and then increasingly at the lower depths in March. This cyanobacteria is non-nitrogen fixing and generally surface dwelling dominating in waters with no nutrient limitations (Paerl H.W., Fulton R.S., Moisander P.H., & Dyble J.2001). Therefore its emergence at lower depths seems to indicate that anoxic conditions in deeper waters have favored its growth.
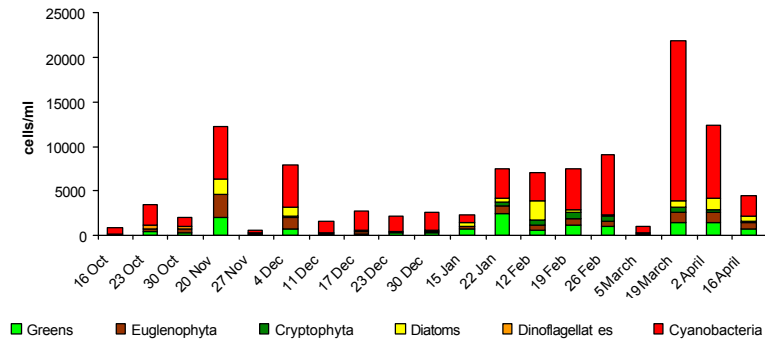
4.2.2   LAKE ROTOEHU

Total algal counts for Lake Rotoehu were similar to those observed in Lake Okaro with peak blooms reaching counts of around 20,000 cells per ml.

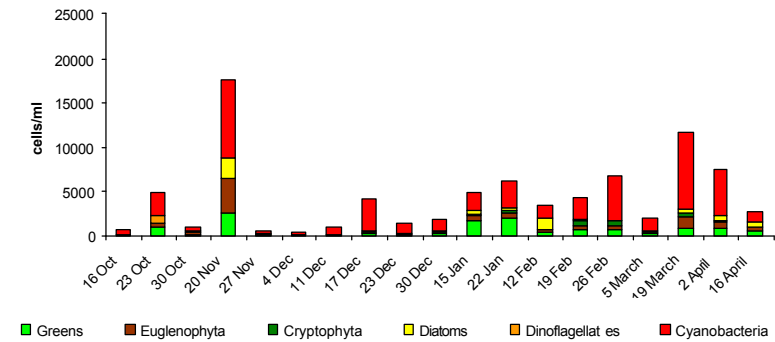*Figure 4. Seasonal phytoplankton counts for Lake Rotoehu at varying depths*
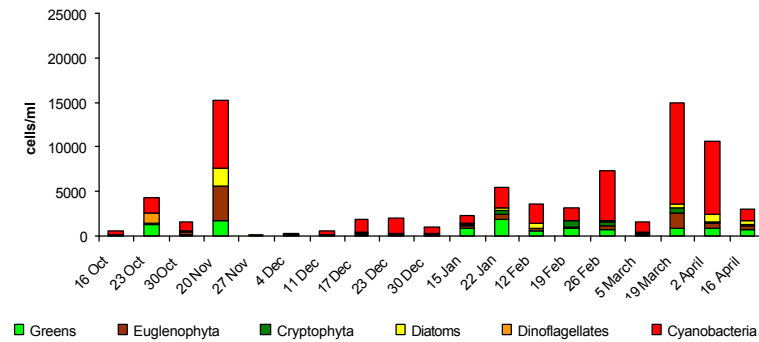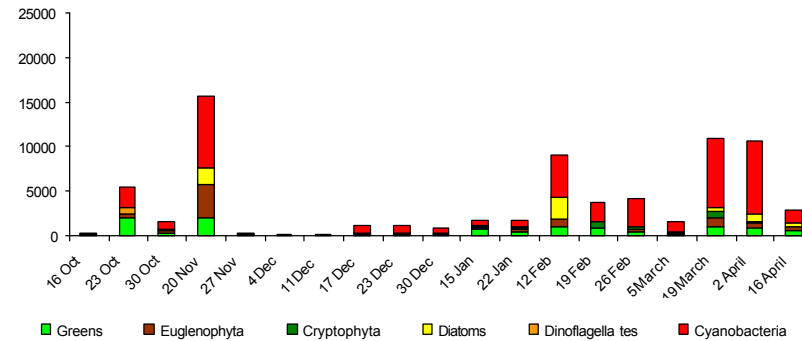
(a) 1 metre deep

(b) 3 metre deep

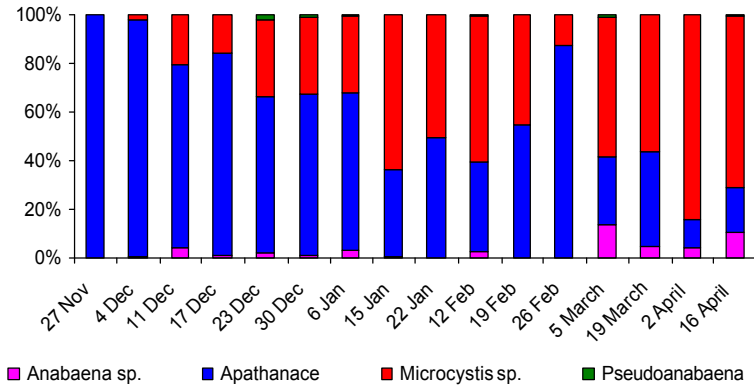*(c) 5 metre deep*

(d) 7 metre deep
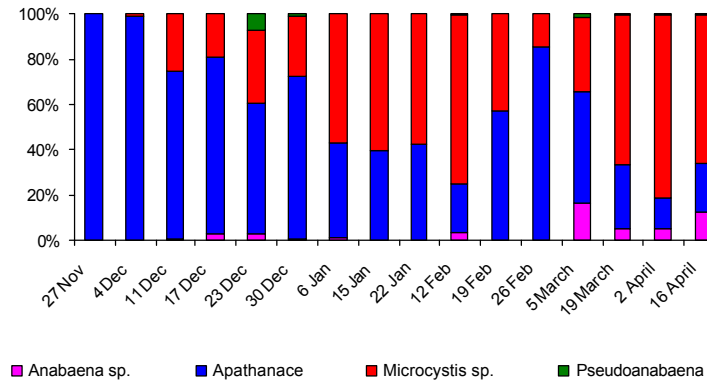
(e) 9 metre deep

Cyanobacteria have dominated throughout the sampling period at all depths in the lake. Green algae have also been present in high numbers at all depths throughout the season. High chlorophyte (green algae) density is indicative of eutrophying waters (Paerl H.W., Fulton R.S., Moisander P.H., & Dyble J.2001) and they often co-dominate with cyanobacteria. Total algal numbers are similar at all depths in Lake Rotoehu, an indication that the lake is well mixed and not stratified. A significant bloom appeared late in November but strangely it was not associated with the top layer of the lake. The lack of surface bloom at a time when all other depths of lake were exhibiting bloom conditions may have been due to surface mixing which can inhibit aggregation of cells and may interfere with buoyancy regulation. All depths showed a gradual increase in algal cell counts corresponding with an increase in water temperature and a corresponding tailing off of cell count numbers as the temperature dropped at the end of the season. Euglenophyta and diatoms were present at all depths throughout the season also. The middle of February saw a huge deterioration in the lake quality (data not presented here) with observed rotting horn weed in the lake, significant floating blooms of *Microcystis sp*. and fish kills around the shore and in the lake body. These observed events correlated with the increase in algal cell counts at this time. The fish kill may have been in response to either toxin production or oxygen depletion. Floating blooms were also observed at the beginning of January but corresponding bloom counts were not observed at the 1 metre depth so the bloom may have only been concentrated on the surface. Interestingly the lake began to take on a brown hue around March and at this time euglenophyta and dinoflagellate numbers increased.

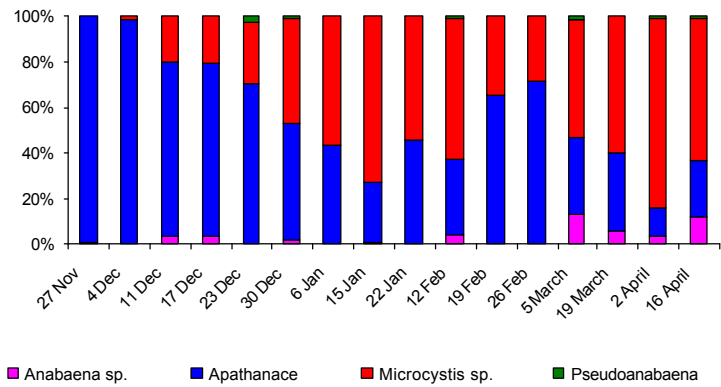*Figure : Cyanobacteria species breakdown at varying depths in Lake Rotoehu*
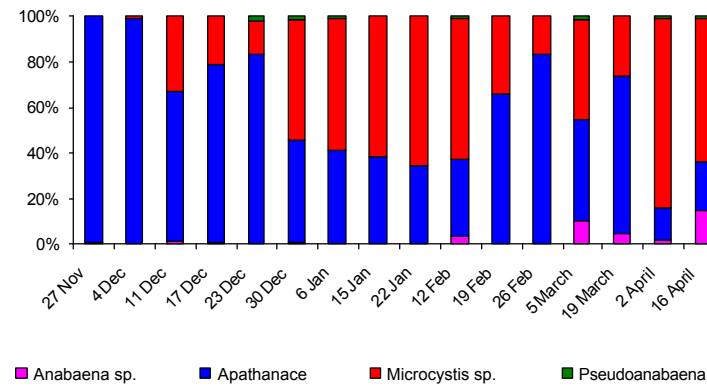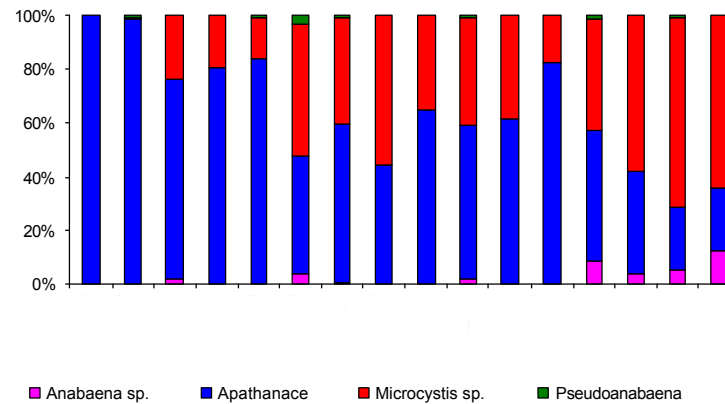
(a) 1 metre deep

(b) 3 metre deep

(c) 5 metre deep

(d) 7 metre deep

(e) 9 metre deep

■ Anabaena sp.　■ Apathanace　■ Microcystis sp.　■ Pseudoanabaena

Cyanobacterial populations in Lake Rotoehu were dominated by *Apathanace* at the onset of sampling with later domination by *Microcystis sp.* toward the end of the season. Microcystis is a non-nitrogen fixing, surface dwelling, bloom forming cyanobacteria (Paerl H.W., Fulton R.S., Moisander P.H., & Dyble J.2001). It favours conditions where there are no nutrient limitations and it's growth is only inhibited by non-nutient limitations such as mixing, residence time, light etc. Lake Rotoehu is rich in both nitrogen and phosphorus so provides optimal growth conditions for this species. There were very small numbers of anabaena sp. in this lake which may be due to the nitrogen rich conditions deselecting for this species. As was seen in the total cell counts, cyanobacterial numbers were evenly distributed over all depths in Lake Rotoehu.

5 DISCUSSION

Traditional manual methods for phytoplankton enumeration are time consuming and fraught with human error. Flow cytometry offers a rapid alternative for species classification and enumeration. By utilizing the natural fluorescence of phytoplankton and using multi-parameter analysis of size, density and fluorescence properties freshwater algal populations can be easily quantified and sorted for analysis. This methodology has been used in this study to demonstrate changes in abundance of phytoplankton populations and to suggest relationships between community composition and environmental conditions.

The target phytoplankton groups investigated in this study were chlorophyta (green algae), euglenophyta, dinoflagellates, diatoms, chryptophyta and cyanobacteria Each group has its own unique pigmentation and thus natural fluorescence which was able to be utilized for classification by flow cytometry.

Each group also has specific requirements for growth and dominance in aquatic environments and the study has highlighted changes in abundance during a season and between two differing lakes which can possibly be explained by their nutritional and environmental requirements. Members of the chlorophyta (green algae) favor nutrient rich conditions and high chlorophyte density is indicative of eutrophying conditions. These organisms were found in both lakes but in greater numbers in Lake Rotoehu. These organisms are also able to co-dominate with cyanobacteria which we also observed in both lakes.

Dinophyta are planktonic and prefer well illuminated surface waters but are able to migrate between nutrient rich deeper waters and light rich surface waters to satisfy both their nutrient and light requirements. They were however more abundant in Lake Okaro perhaps where they did not have to compete with high numbers of cyanobacteria and were able to utilize their ability to migrate according to their growth requirements.

Cryptophyta are motile planktonic organisms which were found in both lakes showing their preference for nutrient rich environments.

Diatoms have broad growth requirements and while they never dominated the phytoplankton populations in either lake, they were present throughout the sampling period and appeared to co-exist with the other phytoplankton species.

Cyanobacteria are by far the most diverse of the phytoplankton studied here as some are able to utilize atmospheric nitrogen and some may also take up phosphorus in excess (luxury consumption) and store it for later assimilation in phosphorus limited conditions. Thus they are able to exploit both nutrient rich and nutrient deficient conditions. (Paerl H.W., Fulton R.S., Moisander P.H., & Dyble J.2001). It is not surprising therefore that both lakes studied exhibited dominance by cyanobacterial populations. The greater diversity of cyanobacterial species seen in Lake Okaro may be explained by the environmental conditions which differ between the two lakes. Lake Okaro is often stratified whereas Lake Rotoehu only intermittently stratifies (Burns N., McIntosh J., & Scholes P.2005) Lake Okaro also suffers from anoxia at the end of a stratification period, whereas Lake Rotoehu is still oxic. These differences along with turbulence and mixing which regulates colony formation and buoyancy capability, are regulators for optimal cyanobacterial growth and can therefore predict whether a nitrogen fixing or non-nitrogen fixing cyanobacterial species will dominate. Dominance by the nitrogen-fixing *Microcystis sp*. in Lake Rotoehu may be explained by environmental conditions that were observed during this season. Water levels in Lake Rotoehu were extremely low (data not presented here) toward the end of the season due to drought conditions in the previous year and subsequent low groundwater runoff this season. This led to an increase in residence time which will have favored nitrogen-fixing organisms as waters became nitrogen limited and phosphorus rich.

The focus of this study was to assess the ability of flow cytometric analysis to determine the ability to differentiate freshwater algae based on autofluorescence and cellular complexity. Through the use of pure cell cultures discreet reference plots were able to be collected for known phytoplankton species and mixed culture sorting and enumeration achieved by comparison. From this data a detailed seasonal succession of phytoplankton in two eutrophic Rotorua lakes has provided valuable information as to the dynamics of community composition and seasonal algal variation in these lakes. Thus flow cytometry has proven itself to be

a powerful fool for the quantification and sorting of freshwater algal blooms, allowing for rapid identification and enumeration of algal species.

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