

Assessing multi-dimensional physical fractionation of phytoplankton as a means to identify uncultivated virus-host systems

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Abstract

Novel marine viruses are regularly being identified using molecular techniques. However, the role of these viruses in marine ecology, and by extension biogeochemical processes in the ocean, is not fully understood. To fully appreciate their role in marine processes it would be helpful to identify the host species that these viruses infect. Although culturing has been utilized to successfully identify virus host systems, most of the new viruses identified by molecular methods have yet to be cultivated. One possible approach to identifying virus-host pairs without cultivation would be to physically separate populations of cells and identify the viruses specifically associated with a given cell population. For this approach to work, populations of cells must be cleanly separated from one another and from any free viruses. For my thesis project, I evaluated the effectiveness of various methods of physically fractionating plankton communities, both individually and in series, and tested whether specific viruses could be detected in association with the individual cell fractions. The results of the three trials suggest that bulk physical separation of planktonic cells can be used to partially purify individual populations of cells, and that viruses can be detected in association with those cells. Further refinement of the fractionation procedures to achieve pure cell populations may ultimately allow for definitive linkages between uncultivated plankton and the viruses that infect them. The plankton fractionation procedures tested in this study may also prove useful for other types of population-specific analyses of uncultivated plankton.

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Introduction

The oceans cover >70% of the Earth's surface and, through biotic and abiotic activities, make significant contributions to the biogeochemical processes that sustain life (Karl, 2007). Microorganisms (protists, bacteria, and archaea) dominate the biology of the ocean; they make up the majority of the plankton biomass and directly drive major elemental cycles that influence global climate and productivity. The marine microbial contribution to total global net photosynthesis (oxygen production), for example, is roughly 50%, which implies that the oxygen in one out of every two breaths of air we inhale derives from the activity of marine microorganisms (Field et al., 1998). Marine microbes are also involved in the cycling of carbon, nitrogen, phosphorus, and many trace elements essential for life (Bertilsson et al., 2003). These processes are carried out by a very diverse suite of microorganisms, each having specialized functions. Understanding how the ocean works ecologically and biogeochemical requires an understanding of what controls the growth and loss of these microbes.

Two major loss terms for plankton are grazing and viral lysis, which have very different consequences. Grazing results in the transfer of carbon and energy in cells to higher trophic levels, but viral lysis results in the transformation of cells into dissolved and colloidal material (Fuhrman, 1999). This makes the material unavailable to grazers, but the carbon and energy in the lysate is very efficiently used by bacteria (Bratbak et al., 1998, Middelboe et al., 1996). Virus-host relationships are also more specific than most predator-prey relationships between plankton and grazers, with viruses displaying complex and overlapping host ranges (Moebus and Nattkemper, 1981, Wichels et al., 2002). Each individual plankton species appears to be infected by many genetically distinct viruses, which has led some to suggest that viruses are the most diverse members of the plankton (Suttle, 2007). Viruses also contribute to the maintenance of diversity among the plankton by providing a selective pressure for cells to evolve resistance to infection and by mediating the exchange of genetic material (Wommack and Colwell, 2000). The diversity of plankton and viruses are interdependent phenomena and the

interactions between them contribute to the structure and function of marine microbial communities (Rohwer and Thurber, 2009).

Studies of plankton diversity using single-gene sequencing surveys have made it clear that the species presently in culture are a poor representation of the breadth and depth of diversity in the ocean (Caron et al., 2004, Rappe and Giovannoni, 2003). Likewise, the diversity of viruses present in the ocean is far greater than the limited number that have been isolated by cultivation and, more important, many of these are so divergent, that the hosts that these viruses infect cannot be inferred from sequence data alone. This has been observed in local waters. For example, many novel protistan viruses in the family *Phycodnaviridae* (double-stranded DNA-containing) and Picorna-like viruses (RNA-containing) have been detected in Kaneohe Bay, HI (Culley and Steward, 2007, Culley et al., 2008). The persistence of some of these viruses suggests that they are an integral part of the ecology in the bay, but many of these viruses have no close relatives that have been cultivated, so it is impossible to know what types of plankton these viruses infect.

Cultivating phytoplankton from Kaneohe Bay and challenging them with viral concentrates could eventually lead to isolation and identification of specific virus-host pairs, but cultivation is a time-consuming process. An alternative to studying the properties of phytoplankton at the population level is to isolate cells of a certain type based on their physical properties, rather than by cultivation. The ability to physically separate distinct phytoplankton populations from a complex community could facilitate some types of studies that would normally require cultivation. It could allow, for example, measurements of tracer incorporation into specific plankton groups or improvement in genomic analyses (Steward and Rappe, 2007). Since viruses spend a portion of their life cycle replicating within their host cell, it might also be possible to infer virus host relationships by determining the viral types associated with specific populations of cells.

The goal of my thesis work was to evaluate methods for physically fractionating phytoplankton populations from complex communities and to determine whether it might

be possible to specifically associate uncultivated viral phylotypes with the isolated populations. A number of different fractionation methods have been used to separate and concentrate phytoplankton populations, including filtration (Runge and Ohman, 1982), density gradients (Lammer, 1967), centrifugation, continuous flow centrifugation (Pomponi and Cucci, 1989) and flow cytometry (Tijdens et al., 2007, Larsen et al., 2001). However, each of these methods by itself has drawbacks including, the damaging of cells through osmotic shock and pelleting, changes in cell physiology over long separation time, low yield of cells of interest and the inability to resolve small differences in size or density.

Another method for fractionating cells is centrifugal elutriation (Bird and Quinn, 1986, Gerath and Chisholm, 1989, Grabske, 1978). Like continuous flow centrifugation, centrifugal elutriation separates cells based on their sedimentation rate; however, unlike continuous flow centrifugation and density gradient sedimentation, it does not require the use of a density gradient medium, which may result in osmotic shock to cells of interest. Also, the use of relatively low rotor speeds increases the chances of maintaining cell integrity during the separation and concentration process of centrifugal elutriation. This method is common in cell biology (Anonymous, 1990), but has rarely been applied to plankton communities (Pomponi and Cucci, 1989).

I hypothesized that by combining existing fractionation methods, including elutriation, it would be possible to separate distinct populations of phytoplankton from field samples. I further hypothesized that, if some portion of the population is infected by a virus, the viral phylotype could be tracked through fractionation procedures eventually linking the virus to the host. For my Master's thesis, I performed three trials in which I assessed the viability of several different protocols for phytoplankton fractionation and attempted to associate specific host and virus phylotypes without cultivation.

Materials and Methods

Overview

This work is arranged as three separate trials that involve distinct samples processed by different combinations of fractionation procedures. The objectives of the fractionation procedures in all cases were to 1) wash phytoplankton cells to remove as many free viruses as possible and 2) separate phytoplankton populations as effectively as possible from one another. The goal was to identify cell fractions in which a viral signal could be detected using group-specific primers (suggesting the presence of infected cells), and in which only a single cell type could be detected (suggesting that that cell type is the host for the virus).

Trial I – Filtration and Flow Cytometry

Collection and filtration: A 500 ml sample of surface sea water was collected from Kaneohe Bay's Lilipuna Pier (21° 25' 46.80" N, 157° 47' 31.51" W) and filtered through a 53 µm nylon mesh to remove larger cells and particles that could clog the flow cytometer.

Concentration and washing: The pre-screened seawater sample was concentrated to 5 ml by gravity filtration through a 0.6 µm pore size, 47 mm diameter, polycarbonate filter. A gentle vacuum (< 5 mm Hg) was applied close to the end of concentration when the drip rate became too slow. The 5 ml concentrate was washed three times by adding 45 ml of 0.02 µm-filtered seawater and re-concentrating to 5 ml after each addition. During all filtration steps, the surface of the filter was flushed with jets of sample using a pipette to help keep cells in suspension. The final volume collected was 5 ml.

Flow cytometric sorting: Cells in the concentrate were analyzed and sorted using a Cytopeia Influx flow cytometer. Sheath fluid (0.5×) was made by mixing 250 ml of sheath solution concentrate (BioSure, Inc.) with 4 L of deionized water. Five distinct cell populations were discriminated based on red fluorescence intensity and forward scatter. Several thousand cells from each population were sorted into 5 tubes. One portion of

each sorted population (10 μ l) was fixed with formaldehyde for microscopic examination and the remaining $\sim$ 4 μ l was used for molecular analyses.

Microscopy: Formaldehyde-fixed subsamples were stained with DAPI (1 μ g ml⁻¹ final concentration) for $\geq$ 10 min, then cells were collected on the surface of a 25 mm, 0.2 μ m pore size, polycarbonate filter using a vacuum filter frit and a glass fiber filter (GF/F, Whatman) as a backing filter. All samples were deposited onto different areas of the same filter by drawing small circles on the filter surface with a permanent marker and depositing each sample one drop at a time from a micropipette into one of the circles with the vacuum on. The filter was mounted in oil on a glass slide with cover slip and examined by epifluorescence microscopy to determine the morphologies of cells present in each sorted fraction.

Extraction: Nucleic acids were extracted from the remaining portion of each sorted cell sample using a total nucleic acid extraction kit (MasterPure, Epicentre) following the manufacturer's instructions for liquid samples. The extract was stored at -80 °C.

PCR: To determine the identities of phytoplankton in the sorted cell populations, a fragment of the 18S rRNA gene was amplified from each of the total nucleic acid extracts using the primers listed in Table 1. The samples were also interrogated with the Algal Virus Specific (AVS) primer set (Chen et al., 1996), and following cDNA synthesis, the Marine Picornalike subclade 1 (MPL-sc1) DNA virus primer set and the Marine Picornalike subclade 2 (MPL-sc2) primer set (Culley and Steward, 2007).

PCR was conducted using the Invitrogen Platinum[®] Taq DNA Polymerase High Fidelity kit in 50 μ l reactions. The initial denaturing temperature for each assay was 94 °C for 2.25 min followed by 30 cycles of 45 sec denaturing at 94 °C, 45 sec annealing, and 45 sec extension at 72 °C, followed by a final elongation step at 72 °C for 14.25 min for all assays. The annealing temperature, primer, and MgCl₂ concentrations, and template volume added varied among reactions (Table 1). For each set of reactions, a positive control and a blank were run. For positive controls, purified PCR products of the target

gene for each primer set was added as template. For negative controls, nuclease-free water was added instead of template.

PCR clean-up and Sequencing: After amplification, samples were loaded into wells of a 1% agarose gel and run for 45 min at 100 V in 0.5× TBE. After confirming that a single amplicon of the correct size was present on the gel, the PCR product was cleaned and concentrated using a silica-adsorption-based spin column, (MiniElute PCR cleanup kit, Qiagen). Amplicons were ligated into pSMART vector (Lucigen) and used to transform *E. coli* by electroporation. Transformed cells were plated on LB medium with kanamycin (50 ug ml⁻¹) and screened for inserts by colony PCR with the SL1 and SR2 vector-specific primers. Amplicons from insert-positive colonies were sequenced in 10 µl reactions using 1.5ul template and the SR2 primer by Sanger cycle-sequencing with dye-terminator chemistry analyzed by capillary electrophoresis (ABI 3730).

Sequence Analysis: Primers and low-quality bases were trimmed from the ends of the sequences in GeneiousTM Pro (Drummond AJ, 2011), and NCBI's Basic Local Alignment Search Tool (BLAST; (Altschul et al., 1990) was used to compare sequences with other sequences in the NCBI database.

Trial II – Filtration and Buoyant Density Fractionation

Collection: Two liters of surface seawater was collected from a boat in Kaneohe Bay (South Bay) using a polycarbonate bottle. A portion (450 ml) of the sample was filtered through a 0.02 µm filter to make virus-free filtrate, which was used to wash cell fractions prepared later. Another portion (100 ml) was filtered onto a 0.02 µm aluminum oxide filter (Anotop, Whatman) and stored at -80 °C for molecular analysis of the total viral and phytoplankton community.

Concentration, Fractionation and Extracellular Virus Washing: The remaining seawater (1,550 ml) was filtered through a 100 µm nylon mesh, and the filtrate was collected. The filtrate was further fractionated by passing sequentially through a 53 µm nylon mesh and a 0.8 µm pore size, 47 mm diameter polycarbonate filter. For each

filtration, the volume was reduced to 5 ml and the retained cells were washed four times with 50 ml of virus-free (i.e., 0.02 μm -filtered) seawater. A subsample of 100 μl from each fraction was filtered onto a 0.02 μm Anotop filter and stored at -80°C .

Buoyant Density Fractionation: Linear density gradients were formed by loading OptiprepTM (Axis-Shield), a sterile iodixanol solution (60 % w/v), and SM buffer in the two chambers of a GM-40 Gradient Maker connected to the Auto Densi-Flow (Labconco) and depositing into sterile polypropylene tubes. The density gradients ranged from 1.1 g ml^{-1} to 1.32 g ml^{-1} . A 3 ml aliquot of each 5 ml cell concentrate was gently loaded at the top of a gradient by pipetting the sample onto the wall of the tube above the gradient. The gradients were centrifuged for 20 min at $3000 \times g$ and one-ml fractions were removed from each gradient using the Auto Densi-Flow to aspirate from the top down. A total of 14×1 ml fractions were collected from each buoyant density gradient.

Buffer Exchange: A subsample (300 μl) of each 1 ml buoyant density fraction was loaded into a centrifugal ultrafiltration device (30 kDa cut-off, Amicon) and centrifuged in a microcentrifuge for 10-min increments at $14,000 \times g$ until all the OptiprepTM media was removed from the reservoir. The cells were recovered by adding $1 \times \text{TE}$ to the column, inverting it, and centrifuging the tube at $14,000 \times g$ for 5 min. The remaining 700 μl of each buoyant density fraction was fixed with 100 μl of formaldehyde (0.02 μm -filtered, 37 % w/v stock) and stored at 4°C for microscopic analysis.

Extraction: Total nucleic acids were extracted from the cells and recovered in $1 \times \text{TE}$ using a selective precipitation protocol (Masterpure Complete DNA and RNA Purification kit, Epicentre). The extracts were stored at -80°C .

Molecular Analysis: Amplification, cloning, sequencing and analysis of 18S rRNA and viral group-specific genes was performed as described in Trial 1, with the exception that fractions were screened with two additional viral primer sets specific for the major capsid protein of phycodnaviruses (MCP), and the DNA polymerase of giant mimi-like viruses (GVpolB) using parameters shown in Table 1. To determine the number of distinct phylotypes in each sample and their distribution among samples, sequences were grouped

with the clustering software, MOTHUR (Schloss et al., 2009), using a threshold 98 % identity. Sequences with ≥ 98 % sequence identity were considered to be the same phylotype. Phylogenetic trees were constructed using SILVA[®] alignment (Pruesse et al., 2007) and the Geneious[™] Pro (Drummond AJ, 2011) tree building function to show the nearest known neighbors of the eukaryotic and viral sequence in trials II and III. The eukaryotic sequences in trial II are labeled with II and a lower case letter. The eukaryotic sequences in trial III are labeled with III and a lower case letter and the viral sequences are labeled with a capital 'V' followed by a lower case letter. Some of the sequences were too short, or from a different region of the 18S rRNA gene and were not included in the tree. In those cases, nearest neighbors were identified using Geneious[™] Pro sequence search (nucleotide BLAST) (Table 2).

Optimizing Elutriation

Since elutriation has rarely been used to fractionate plankton communities, some initial tests were conducted to evaluate its performance and determine the procedures to be applied in the final trial. The elutriation system consisted of an Avanti J-26XP centrifuge (Beckman) with a JE-5.0 rotor equipped with a 4 ml capacity elutriation chamber. Samples were dynamically loaded, washed, and unloaded using a peristaltic pump. To minimize pulsation, the tubing was split and run through two stacked pump heads with occlusion offset, then rejoined to a single line.

Size selection by elutriation: A one-liter sample of seawater was collected from the head of the Ala Wai Canal (21° 16' 31.84" N, 157° 49' 4.33" W) and filtered through 100 μm nylon mesh. The < 100 μm sample was loaded at a flow rate of 27 ml min^{-1} and a rotor speed of 5000 rpm. Under these conditions, cells ≥ 5 μm in diameter are supposed to be retained (idealized spherical cells under standard conditions). Cells retained in the chamber were then washed by switching the inlet to a reservoir of 0.02 μm -filtered seawater and maintaining same flow rate. The wash flow through (405 ml) was collected and saved. Cells were then eluted into five nominal size fractions: 5-10 μm , 10-20 μm , 20-40 μm , 40-60 μm and 60-80 μm by sequentially changing the pump and/or rotor speed

according to the nomogram supplied with the rotor. After each change in conditions, 250 ml was collected to ensure all cells of the targeted size would be collected. After the five fractions were collected 2×250 ml volumes of flow through were again collected.

A subsample of each fraction (50 ml) was transferred to a sterile polypropylene conical tube and stained and fixed with 5% Lugol's solution then stored at 4 °C for microscopy. Microscopy was done by filling wells of a 96-well plate with 200 μ l of the stained sample and letting the cells settle for 20 min before doing microscopy counts with an inverted microscope. The cells were first sized and counted, then classified by cell type.

Effect of cell motility on elutriation: To determine whether highly motile cells may elute anomalously from the elutriation chamber, a live and a sodium azide-poisoned sample were run sequentially and the cell elutriation profiles compared. Prior to the run, an initial test was performed to determine the minimum concentration of sodium azide needed to inhibit motility in a reasonable amount of time (≤ 30 min). Surface seawater was collected from the Ala Wai Canal and 15 ml subsamples were transferred to six polypropylene tubes. The samples were centrifuged for 10 min at $4000 \times g$. The supernatant was removed leaving 2 ml of concentrated cells. A one-ml subsample of each concentrate was loaded into a well of a 24-well plate containing 0.5 ml each of sodium azide at different concentrations yielding final azide concentrations of 0.5%, 0.25%, 0.1%, 0.01%, and 0.001% (w:v). Samples were examined by light microscopy after 20, 30, 40, 60, and 70 min to evaluate the number of motile cells.

For the elutriation experiment, two one-liter samples were collected from the Ala Wai Canal (head of the Canal) and filtered using a 100 μ m nylon mesh filter. The first one-liter sample was loaded into the elutriation chamber at a rotor speed of 5000 rpm and a pump flow rate of 26 ml min⁻¹. The loaded sample was washed with 0.02 μ m-filtered seawater for 11.5 min, with a rotor speed of 5000 rpm, at a pump flow rate of 20 ml min⁻¹ (approximately 230 ml). Five nominal size fractions were collected from the loaded sample (5-10 μ m, 10-20 μ m, 20-40 μ m, 40-50 μ m, and 50-80 μ m). A 50 ml subsample of each size fraction was fixed and stained with 2.5 ml of 5% Lugol's solution. A 10 ml

subsample of the pre-fractionation flow through was fixed with 100 µl of 37% w/v, 0.02 µm-filtered formaldehyde and stored at -80°C. The elutriation chamber was then washed with one liter of Nanopure[®] water. Approximately 70 min prior to loading the second one-liter sample, 10 ml of 25% sodium azide was added to the sample (final concentration of 0.25%). The sample was loaded, elutriated and sampled using the same conditions as for the control. Lugol's stained samples were examined by light microscopy on an inverted microscope to determine the size distribution of cells in each fraction.

Trial III – Filtration, Elutriation, and Buoyant Density Fractionation

Collection: A two-liter sample of seawater was collected from Ala Wai in the same location as for Trial II. The sample was filtered through a 100 µm nitex mesh, and a one-ml subsample was preserved with 3.7% w:v, 0.02 µm-filtered formaldehyde for bacteria and virus counts. Another 150 ml subsample of the < 100 µm sample was filtered onto a 0.02 µm Anotop filter and stored at -80°C for extraction.

Elutriation and Concentration: One-and-a-half liters of the filtered sample was loaded into the elutriator at a rotor speed of 5000 rpm and a pump flow rate of 13.5 ml min⁻¹, which theoretically results in retention of cells ≥ 4 µm in diameter. At the end of loading period the sample was washed with 500 ml of 0.02 µm-filtered seawater while maintaining the same rotor speed and flow rate. Two samples (10 ml each) were collected during the loading and three samples (10 ml each) during the course of the wash. Rotor speed was then reduced to 500 rpm and flow rate increased to 28 ml min⁻¹ to elute 250 ml of the nominal 4-50 µm fraction. Flow rate was then increased to 120 ml min⁻¹, while maintaining the same rotor speed to elute the nominal 50-100 µm fraction in 250 ml. Subsamples (10 ml) of all fractions were fixed with formaldehyde for microscopy. Cells in the remaining 190 ml of the two elutriated fractions were further concentrated by centrifugal ultrafiltration in Centricon plus 70 filters (30 kDa cut-off; Millipore). Since the filter reservoir has a 70 ml capacity, each sample was concentrated by sequential loading in the same filter unit, emptying the filtrate collection cup as needed. For each round of concentration, filter units were centrifuged for 20 min at 2500

× g. The cells from each concentrate were recovered in a final volume of 5 ml and saved for further fractionation on a buoyant density gradient.

Buoyant Density Fractionation: Subsamples (3 ml) of the concentrated cells from each elutriation fraction (4-50 µm and 50-100 µm) were separated on pre-formed OptiPrep buoyant density gradients. The gradients were prepared and centrifuged, and the fractions collected, as described in Trial II, except that only 13 fractions were obtained from the gradient that was loaded with the nominal 50-100 µm elutriator fraction. Buffer exchange and nucleic acid extraction of each fraction was performed as described in Trial II.

PCR: The Roche Expand High Fidelity PLUS DNA Polymerase kit was used for the primer sets in this trial. Reactions were in a final volume of 50 µl with buffer and enzyme concentrations according to the manufacturer's instructions and 1.5 µl of bovine serum albumin (BSA) added to all reactions. Primers and other variable components are as indicated in Table 1. Other reaction conditions are as described in Trial I. In addition to the primers used in Trials I and II, two additional viral primers sets, Marine Picorna-like subclade 4 (MPL-sc 4) and Marine Picorna-like subclade 5 (MPL-sc 5) were used. For each assay, a positive control and blanks (nuclease free water instead of template) were included.

Gel Electrophoresis and PCR clean-up: Samples were separated on agarose gels as described in Trial II. After gel confirmation the PCR product was cleaned and concentrated using a protocol from the MiniElute[®] PCR cleanup kit.

Cloning and Sequencing: Ligation and transformation of the PCR product in preparation for sequencing was done using the TOPO TA cloning kit (Life Technologies) with cells plated on LB plate with kanamycin (50 µg ml⁻¹). The eukaryotic host PCR product and the viral PCR product were submitted for Sanger sequencing to the Advanced Studies in Genomics, Proteomics and Bioinformatics (ASGPB) laboratory at the University of Hawai'i at Mānoa.

Sequence editing: The sequences were edited and analyzed by BLAST using GeneiousTM Pro.

Results

Trial I

Flow cytometry of the concentrated Kaneohe Bay plankton sample revealed five chlorophyll-containing populations that were discriminated based on their optical signatures (Fig 1). None of the viral gene primer sets tested resulted in detectable amplification product from the sorted cell populations. However, two of the five samples were successfully amplified with the 18S rRNA primers. Cloning and sequencing of the products indicated that the genes were most closely related to those from the diatoms *Thalassiosira tenera* and *Thalassiosira nordenskioeldii*. Observation of the sorted cells by microscopy confirmed that diatoms were present in these samples.

Trial II

Seven viral gene sequences (Va, Vb, Vc, Vd, Ve, and Vf, Vg) were successfully amplified in the < 100 µm-screened fraction, the washed 0.8-10 µm bulk fraction and in buoyant density fraction 8. Four of the viral sequences (Va, Vb, Vd, and Vg) were most closely related to a virus that infects a prasinophyte, the *Pyramimonas orientalis* virus (PoV) (Fig 2). The other three sequences were most closely related to the *Phaeocystis globosa* virus (Vc) and Organic Lake virus (Ve and Vf). A progressive reduction in diversity of viral sequences was shown with each successive step in the fractionation. In addition, the proportion of sequences most similar to PoV increased from the < 100 µm community to buoyant density fraction 8 (Fig 3). There was a progressive enrichment in the Vb phylotype from <10% (detection limit) to 40%, to > 75%. The detection limit was determined by the number of samples (12) of each amplified sequence submitted for sequencing.

Eight eukaryotic gene sequences were identified in the 0.8-10 μm bulk fraction and buoyant density fraction 8. One of the sequences (II d) was identified as a prasinophyte (Fig 4). The closest known neighbors of the other sequences included phytoplankton and one nekton (Table 2). A progressive reduction in diversity, and an increasing representation of the prasinophyte, from the 0.8-10 μm fraction to buoyant density fraction 8 was demonstrated using NCBI BLAST hits (Fig 5).

Elutriation Tests

Initial Elutriation Experiment – Results from the initial elutriation experiment were variable. Enrichment of cells in the eluted fractions based on cell size was inconsistent. In the nominal 5-10 μm size fraction only 14.3% of the cells were of the appropriate size. Likewise, in the nominal 10-20 μm , 20-40 μm , 40-60 μm size, and 60-80 μm size fraction only 7.3%, 52.2%, 22% and 3.5% of the cells were of the appropriate size, respectively. Fractions were also evaluated to determine whether fractionation occurred among cell morphotypes that differed in aspects other than simply cell diameter. The nominal 5-10 μm fraction was enriched with an approximately 20 μm long, chlorophyll containing, oblong cell (cell type A; Fig 6), eluted size fraction 10-20 μm was enriched with ciliates, eluted size fraction 20-40 μm was enriched with dinoflagellates, eluted size fraction 40-60 μm was not enriched with any particular cell type, and eluted size fraction 60-80 μm was enriched with a dinoflagellate in the genus *Gymnodinium*.

Motility Experiment – Treatment of natural plankton communities with varying concentrations of sodium azide indicated that the motility of nearly all cells could be eliminated within 30 minutes at concentrations $\geq 0.25\%$. A concentration of 0.25% was therefore used in subsequent elutriation experiments. Pre-treatment with sodium azide had a varying effect on elutriation results based on cell type. For ciliates and an elongated dinoflagellate (~25 μm width) with a pointed protrusion at one end (dinoflagellate A) (Fig 6), two examples of motile cells in the sample, there was a shift in the distribution over the range of fractions. The ciliate population showed a shift from primary enrichment in the 10-20 μm fraction in the control to the 50-80 μm fraction in the sodium azide-treated sample (Fig. 7). For dinoflagellate A there was a restriction of the

distribution in sodium azide treated sample to the 50-80 μm size fraction (Fig 7). The distribution of dinoflagellate A is also narrower in the sodium azide-treated sample relative to the control. For diatoms there was a shift in enrichment from the 10-20 μm and 40-50 μm fractions in the non-treated sample to the 50-80 μm fraction in the sodium azide-treated sample, along with a restriction in the distribution across the range of size fractions. For *Gymnodinium* there was no change in the primary size fraction of enrichment. For both the non-treated and sodium azide-treated sample, the 10-20 μm size fraction was enriched, while there was a shift in cells present in the 40-50 μm and 50-80 μm size fractions between treatments (Fig 7). For all cell types, with one exception – a rectangular chlorophyll containing cell (cell type F; Fig 6), there were losses in the number of cells per cell type, but the fraction of cells lost varied among cell types (Fig. 8; Fig 9).

Trial III

The concentration of extracellular viruses eluting from the chamber dropped following the washing steps and remained low in the 4-50 μm and 50-100 μm bulk fractions (Fig. 10). The ratio of cells to viruses also increased (Fig 11). A progressive reduction in diversity of eukaryotic cell types was achieved through each subsequent fractionation step (Fig. 12). Sequencing and clustering results showed that at least 3 buoyant density fractions (4, 7, & 10) were enriched with specific cell types (Fig. 12). This was confirmed by microscopy (Fig 13). No viral gene sequences were amplified with the viral primer sets that were used to interrogate the various fractions.

Discussion

Flow cytometry showed some promise as a method for sorting individual phytoplankton host populations for molecular analysis, as it easily distinguished > 5 optically distinct chlorophyll-containing cell populations. The 18S rRNA gene was successfully amplified from some, but not all of the sorted populations, suggesting that there may have been some inhibition of the PCR in some samples, or that the PCR assay was near the

detection limit with the conditions used. The failure to amplify any of the targeted viral genes may also have been a result of inhibition in some cases, but suggests that those viruses may simply have been absent or present at concentrations below the practical detection limit. The sensitivity of this approach for detecting infected cells could be improved by sorting much larger numbers of cells. This could be achieved by concentrating cells from a larger volume prior to flow cytometry. Ultimately, though, flow cytometry is a relatively low-throughput sorting method, since cells are sorted one at a time and the recovered biomass is low. Flow cytometry could be well suited to studies targeting one or two populations, but is less suitable for sorting large numbers of many different types of cells.

For this study, the populations of interest (those with a significant fraction of the cells infected) are not known ahead of time. It was therefore decided that bulk fractionation of plankton communities, would be a useful first approach to the problem, because it would allow screening a larger number of cells, thereby increasing the chances of detecting intracellular viruses. Microscopic and molecular analyses of samples taken before and after bulk fractionation using a combination of filtration and separation on a buoyant density gradient demonstrated that these procedures could effectively separate different cell populations and that viruses could be successfully detected in those fractions using PCR with group-specific primers. An enrichment in cells most similar to a prasinophyte (*Pyramimonas orientalis*) in one of the final fractions together with an enrichment in viruses most similar to a known prasinophyte-infecting virus (*Pyramimonas orientalis* Virus, or PoV) suggests that the viruses may have been associated with those cells (Fig 3; Fig 5). This was promising, but the presence of other cell types in the fraction means that the relationship could not be determined unequivocally.

In an attempt to improve the resolution of the fractionation, elutriation was evaluated as another dimension that could be used for cell separation. Elutriation separates cells based on their sedimentation rate. As described by Stoke's law sedimentation rate is directly proportional to a cell's shape, diameter and specific gravity:

$$dx/dt = SD^2(d_c - d_m)*a/ku,$$

where S is the shape of the cell, D is its diameter, d_c is its specific gravity, d_m is the specific gravity of the medium the cell is in, u is viscosity, a is acceleration of the medium, and k is a constant (Pomponi and Cucci, 1989). Although sedimentation rate is partially dependent on cell density, it is also strongly influenced by cell size and shape. It may therefore be combined in series with buoyant density fractionation to improve resolution among cell types. A major advantage of elutriation over simple size fractionation by filtration is that cells are maintained in suspension during the procedure, allowing separation of cells that are more easily damaged.

The initial test of the elutriator performance showed that free viruses (and bacteria) could be partially removed from natural plankton populations by washing with 0.02 μ m-filtered seawater after loading the community. However, many of the phytoplankton cell types did not elute according to the nominal diameters predicted by the nomogram. This is not too surprising, since the nomogram is based on idealized spherical cells of a specific density, whereas the plankton have widely varying shapes and densities. The latter point was demonstrated by the buoyant density fractionation results from Trial II. With this in mind, the effectiveness of the initial elutriation experiment was evaluated based on its ability to separate various cell morphotypes, rather than its ability to separate based on diameter alone. That analysis demonstrated that some cell types could be effectively separated from one another.

Although generally effective, it was noted that ciliates eluted in a nominal size fraction much smaller than their observed diameter, even though their shape did not differ dramatically from an idealized sphere. Since ciliates are motile and the sample was run live through the elutriator, it was hypothesized that motility (even if directionally random) might be altering the elutriation profiles. Random motility could, for example, broaden the zone of equilibrium in the chamber, causing cells to elute earlier and over a broader nominal size range. A test of the effect of motility on elutriation profiles showed that, at least in the case of one dinoflagellate, poisoning the sample to eliminate motility

improved the results as predicted: the cells eluted in a narrower, more pronounced peak at a nominal size fraction that matched their observed size and with good recovery (Fig. 8). Although the elution profile of the diatoms also showed a narrower dominant peak in the 50-80 μm size fraction, the sizes of the diatoms in that fraction varied widely. For many other cell types, including the ciliates, the effect of poisoning on the distribution of cells among size fractions was not apparent. What was obvious, however, was that the numbers of cells recovered was dramatically reduced for many cell types (up to 75% in one case). Since poisoning improved the elution profile for only one of many cell types analyzed, and because it caused substantial losses for many others, it was decided that poisoning would not be used as a routine procedure.

Both buoyant density fractionation and elutriation were found to be at least partially effective in separating cell populations, so it was decided that the two techniques be run in series in an attempt to further improve resolution. Quantification of bacteria, viruses, and protists by microscopy showed that elutriation was able to remove some bacteria and viruses from cells in the chamber, but the loss of cells was also substantial. Only a small fraction of the total loaded cells eluted in the 20-40 μm size fraction, but this may be in line with the number of cells in that size range. The chamber walls accumulated a visible film during the course of processing, suggesting that many of the cells unaccounted for may have adsorbed to the chamber. In any case, the 6- to 10-fold increase in the ratio of cells to viruses in the eluted fractions indicated that free bacteria and viruses were removed in greater proportion to eukaryotic cells as required for a successful wash (Fig 11).

Molecular analysis of the types of protists present in the initial sample after elutriation, and after buoyant density fractionation, indicated that there was progressive enrichment of specific cell phylotypes with each of the fractionation steps. One buoyant density fraction in particular (Fraction 7) was highly enriched in dinoflagellates as determined by both microscopy and by 18S rRNA analysis (Fig 13). Although the sequencing depth was low, all of the sequences retrieved in that fraction appeared to derive from dinoflagellates, with sequences most similar to members of the genus *Karlodinium* being

most abundant. Sequence III a is a representative of the fraction 7 (Table 2). This result was encouraging and showed that separation of cell types might be improved by multi-dimensional fraction. Unfortunately, none of the fractions screened in Trial III yield a positive signal for the specific viruses being targeted (phycodnavirus- and mimivirus-like). It is possible that there were actually infected cells among the fractions, but the viruses may have been below the practical PCR detection limit or the cells were infected with other types of viruses that were not detectable by these primers.

Conclusion

The experiments described in this thesis demonstrate that some phytoplankton populations can be greatly enriched without cultivation from initially complex plankton communities based on their physical properties. Both buoyant density fractionation and elutriation are promising, but underutilized, methods that can improve cell separation compared to the more common method of size fractionation with filters. The data also demonstrated that viral gene sequences can be detected in association with washed and fractionated cell populations, presumably as a result of intracellular viruses in infected cells. The reduction in diversity of both the viruses and the hosts detected in successively more fractionated populations in one of the trials suggests that this approach of identifying intracellular viruses may be useful for identifying uncultivated virus-host pairs. With additional development, the bulk cell fractionation procedures evaluated here might also be useful for enriching cells for cultivation, examining physical associations between different types of cells (e.g., host-symbiont relationships) and performing other population-specific assays that require large amounts of biomass, such as stable isotope analyses.

Appendix

Table 1. Primer names and PCR reaction details

Primer names, trials in which they were used, targets, sequences, annealing temperatures, and variable reaction components used for PCR amplification of eukaryotic and viral gene targets. Where two numbers are given, the first applies to conditions in Trials I and II, and the second to Trial III.

Primer	Trials	Target	Forward Primer Sequence	Reverse Primer Sequence	Annealing Temp (°C)	MgCl ₂ (ul)	Forward primer (ul)	Reverse Primer (ul)	Template (ul)
18S rRNA	I, II, III	Eukaryotes	acc tgg ttg atc ctg cca g	tga tcc ttc ygc agg ttc ac	54	1.5/3	1/5	1/5	2/3
Mplsc1.1	I, II, III		tct tti aia arg atg aas	ytc ctt wtc rgs cat kgt a	50	3/6	5	5	5/3
Mplsc2.1	I, II, III	Protistan RNA	ttw gci ggi gat twc a	cky ttc aar aaw tca gca tc	50/43	3/6	5	5	5/3
Mpl chop. 1	I	viruses	tgc ccg tat gaa rga yga r	ata ccc aat gcc tyt tia tra a	44	3	5	5	5
Mplsc4_cdh	III		ctt ctg cgc ctg cny naa rga yga	cca acc gcc agt cc kyt tha tra a	55	6	5	5	3
Mplsc5_cdh	III		ctt cca gga ctt cgt gaa rga yga m	ggg gtc tcc tgc tgg byy tcn gtn gt	55	6	5	5	3
AVS	I, II, III	Protistan DNA	gar ggi gci aci gti yi gev gc	gci gci tai cky thy ti swr ta	45	1.5/6	1	3	2/3
MCP	I, II, III	Viruses	ggv ggy car cgy att ga	tgi ayy tgy tcr ayt agg ta	45	1.5/6	2.5	2.5	5
GV PolB	I, II, III		att gta tta aaa agg mgn gev aay gc	tct atc agc taa tac yti ttg ngc dat	57	3	5	5	5

Table 2. Nearest neighbor of sequences not in figures 2 and 4

The nearest neighbors of the sequences not included in figures 2 and 4 were determined by GeneiousTM Pro sequence search (nucleotide BLAST).

Trial II		
Eukaryotic sequences		
Sequences	Nearest neighbor	E-value
II h	<i>Kephyrion sp.</i>	0
II i	<i>Paracyclops nana</i>	0

Trial III		
Eukaryotic sequences		
Sequences	Nearest neighbor	E-value
III a	<i>Gymnodinium simplex</i>	0
III b	Zebra fish	2.83E-06
III c	<i>Leishmania mexicana</i>	5.23E-07
III d	No match	
III e	No match	
III g	<i>Rimostrombidium lacustris</i>	0
III h	<i>Ciliate sp.</i>	0
III k	<i>Gyrodinium galatheanum</i>	0
III m	<i>Karlodinium micrum</i>	2.13E-166
III n	<i>Karlodinium micrum</i>	0
III p	<i>Katablepharis japonica</i>	0
III q	<i>Chroomonas vectensis</i>	1.74E-127
III r	<i>Tetraselmis chuii</i>	0

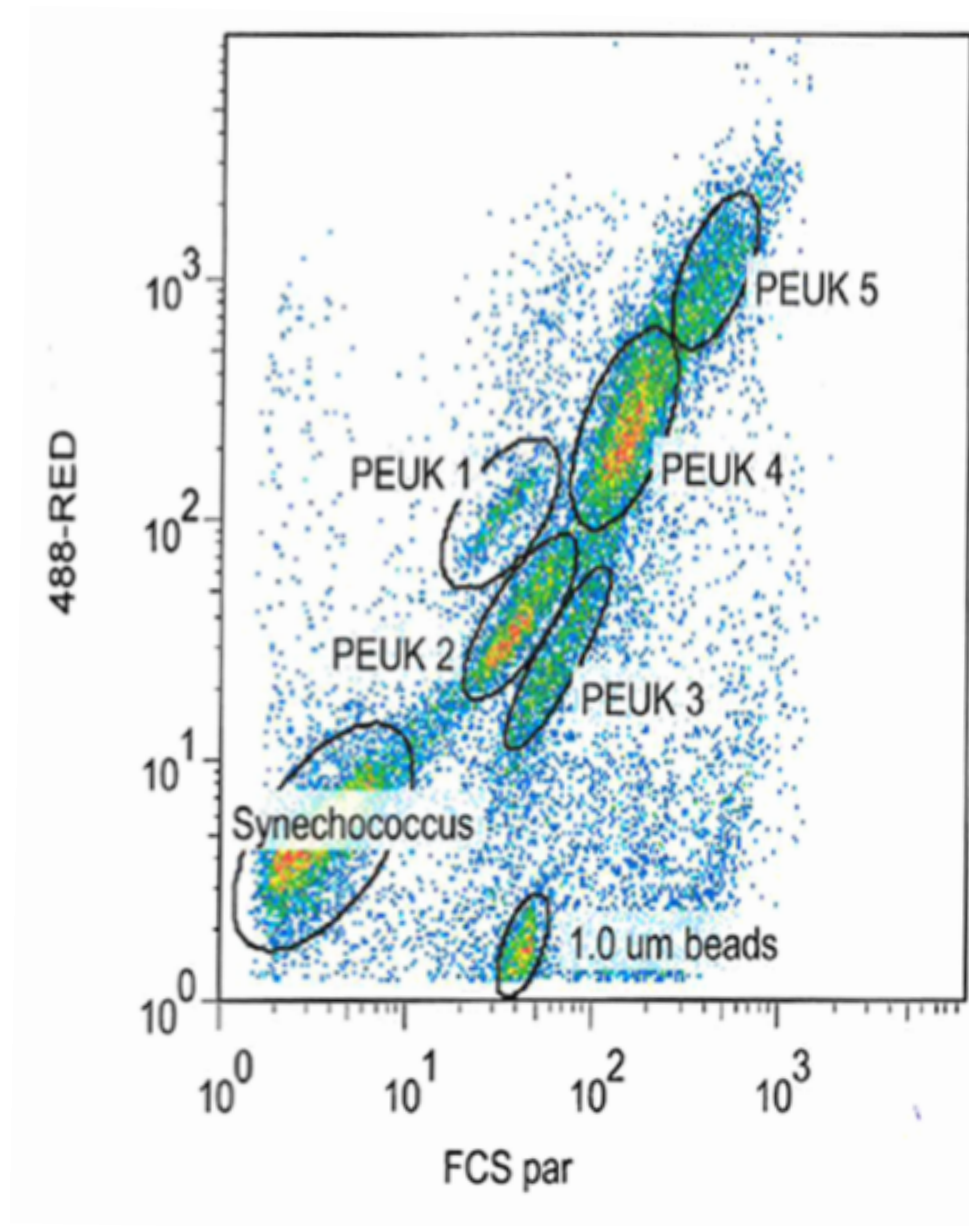


Figure 1. Flow cytogram

The flow cytogram shows five sorted chlorophyll-containing populations of picoeukaryotes (PEUK1-5), with *Synechococcus* and 1.0 μm as size references.

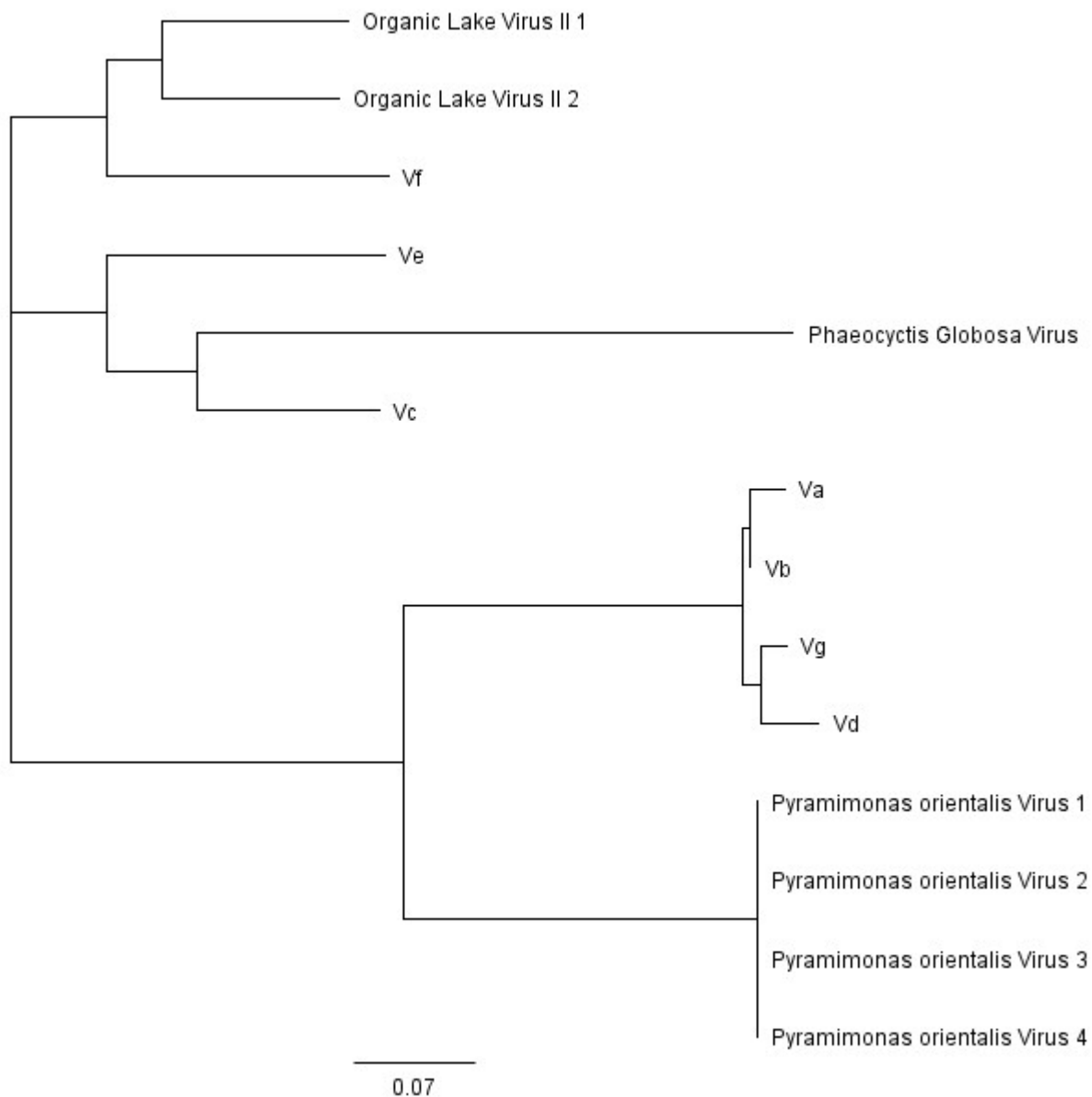


Figure 1. Viral sequences: Nearest known neighbor

The Neighbor-Joining method was used to create a phylogenetic tree that shows the closest known neighbors of the viral sequences from Trial II. The close clustering of sequences Va, Vb, Vd and Vg with known *Pyramimonas orientalis* sequences supports the NCBI BLAST results for Trial II.

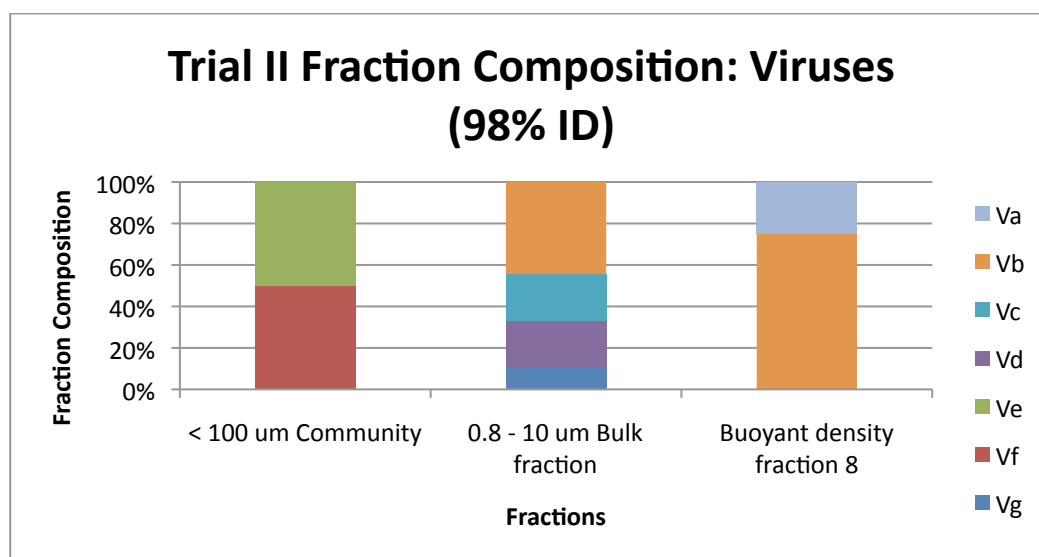


Figure 2. Viral phylotype clusters

In Trial II a 98% pairwise identification criterion was used to group viral phylotypes. The clusters show that there was a progressive enrichment of the Vb viral phylotype from the < 100 μm community, to the washed cellular fraction (0.8 μm – 10 μm) and then to buoyant density fraction 8. The apparent absence of Vb in the < 100 μm community may be due to it being below the detection limit.

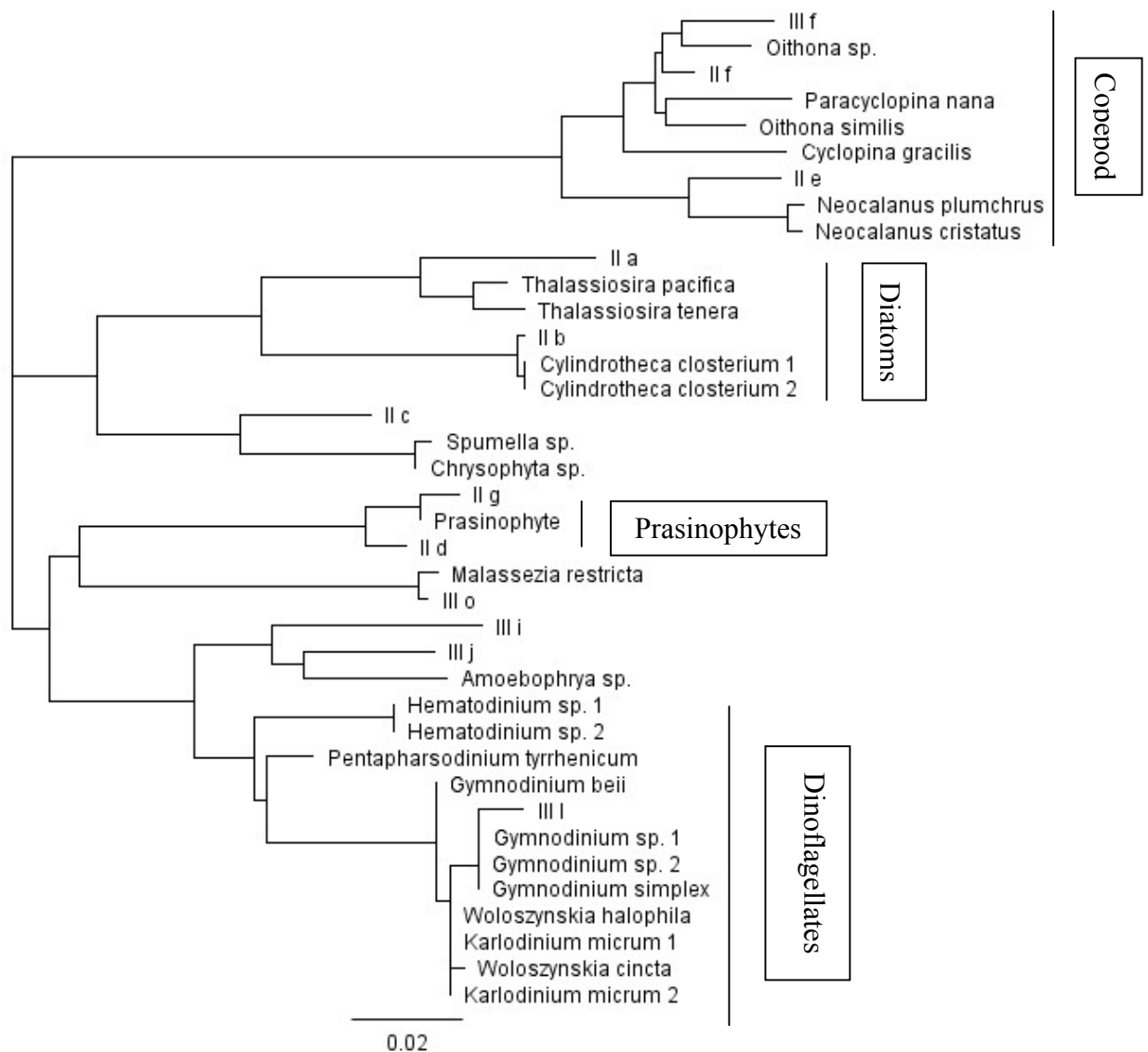


Figure 2. Eukaryotic sequences: Nearest known neighbors

The Neighbor-Joining method was used to create a phylogenetic tree that shows the closest known neighbors of the eukaryotic sequences from Trial II and Trial III. The clustering of sequence II d with a known prasinophyte sequence supports the NCBI BLAST results for Trial II. The following 18S rRNA sequences from Trial II are not included in the tree – II h and II i. The following 18S rRNA sequences in Trial III are not included in the tree – III (a – e, g, h, k, m, n, p – r) (Table 2).

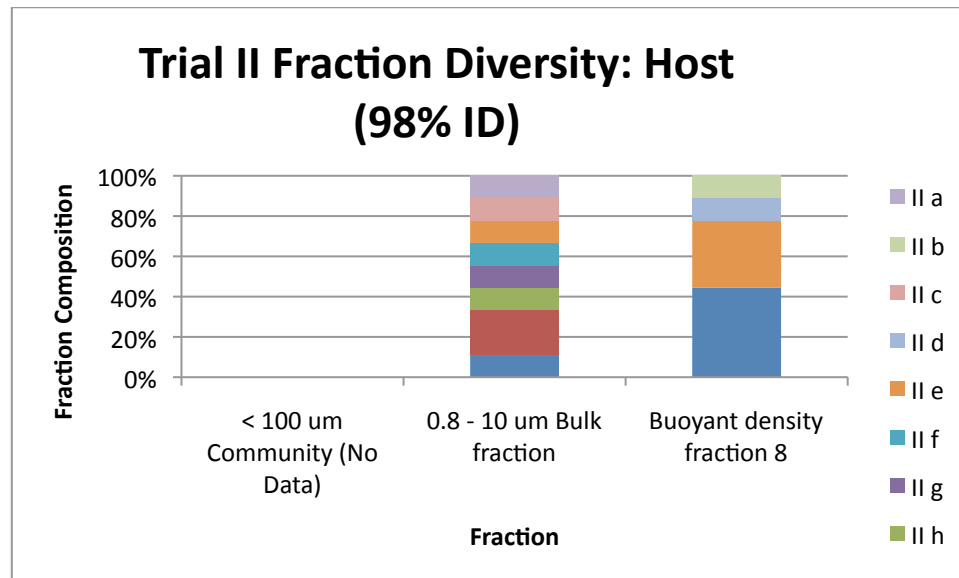


Figure 3. Eukaryotic phylotype clusters

In Trial II a 98% pairwise identification criteria was used to group eukaryotic genotypes. No data was collected for the 100 μm community. The clusters show that there is a reduction in diversity of genotypes from the washed cellular fraction (0.8 μm – 10 μm) to buoyant density fraction 8.

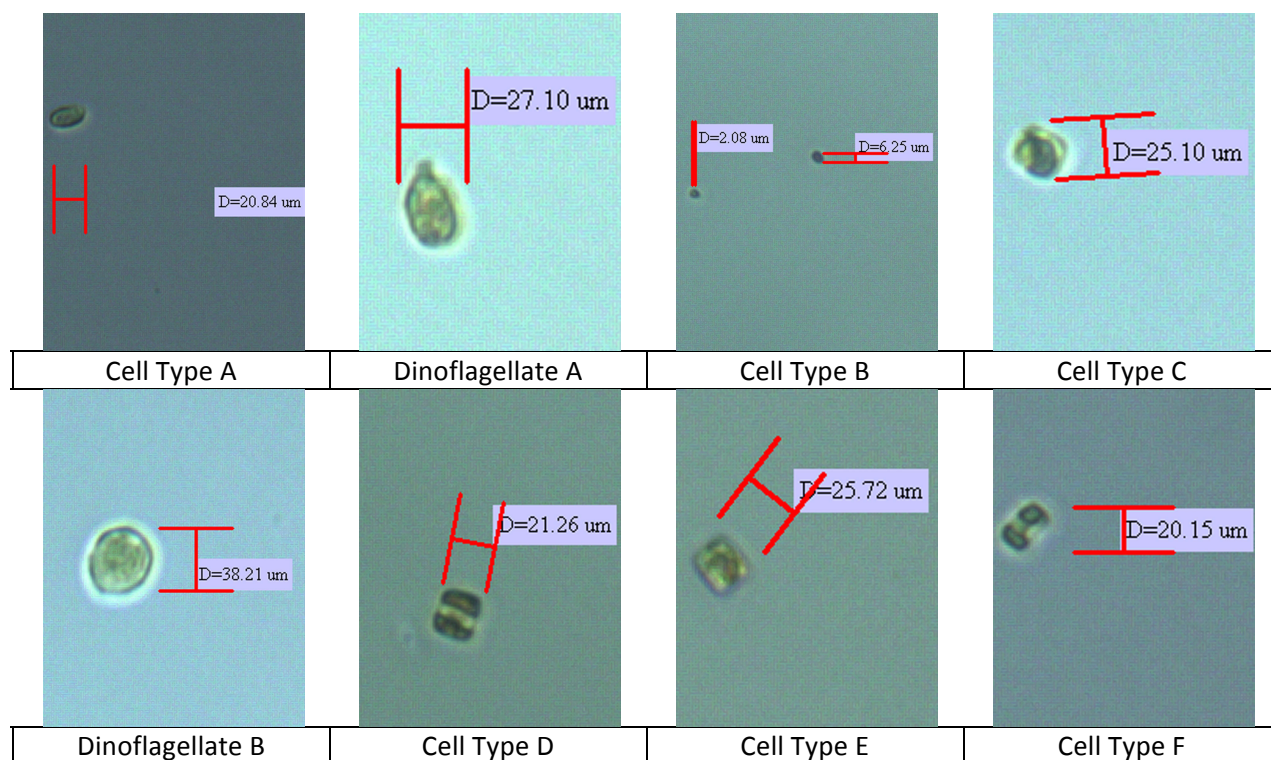


Figure 4. Cell type images

The cells types were imaged using light microscopy at 40x magnification. Dinoflagellate A is one of the cell types identified by its general morphology in the elutriation test and referenced in Figure 5. Dinoflagellate B and Cell types A – F are cells identified in the motility experiment by their general morphology and referenced in figures 7, 8, and 9..

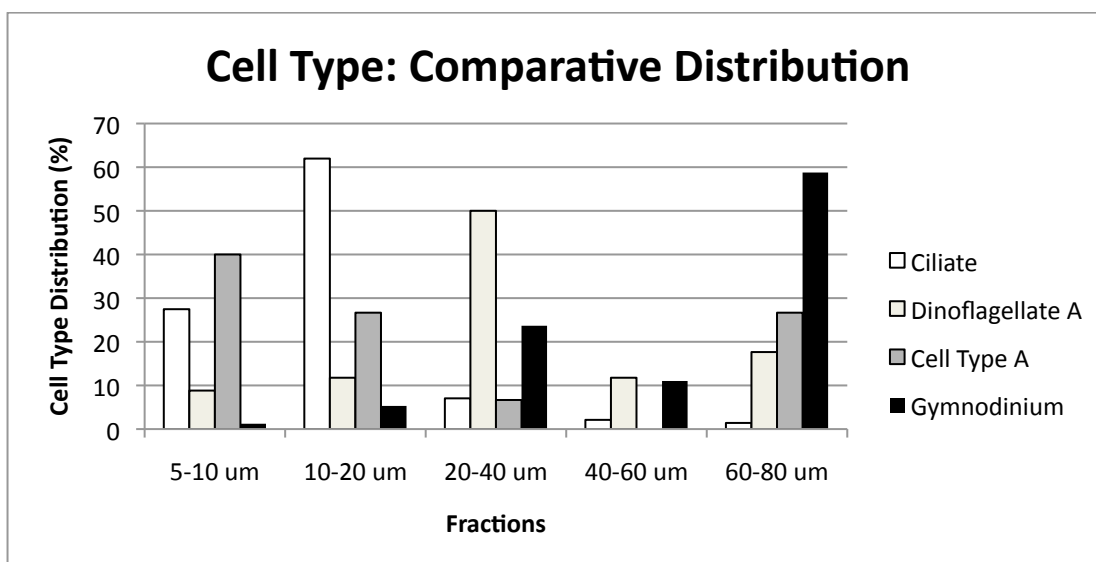


Figure 5. Comparative distribution of cell types

The distribution of cell types based on their morphology shows that four of the five size fractions were enriched with specific cell types. With the exception of the 40 μm – 60 μm fraction, each cell type has a main peak in one of the size fractions; for example, the 5 μm – 10 μm fraction was enriched with cell type A, the 10 μm – 20 μm was enriched with ciliates, the 20 μm – 40 μm fraction was enriched with dinoflagellate A, and the 60 μm – 80 μm fraction was enriched with *Gymnodinium*.

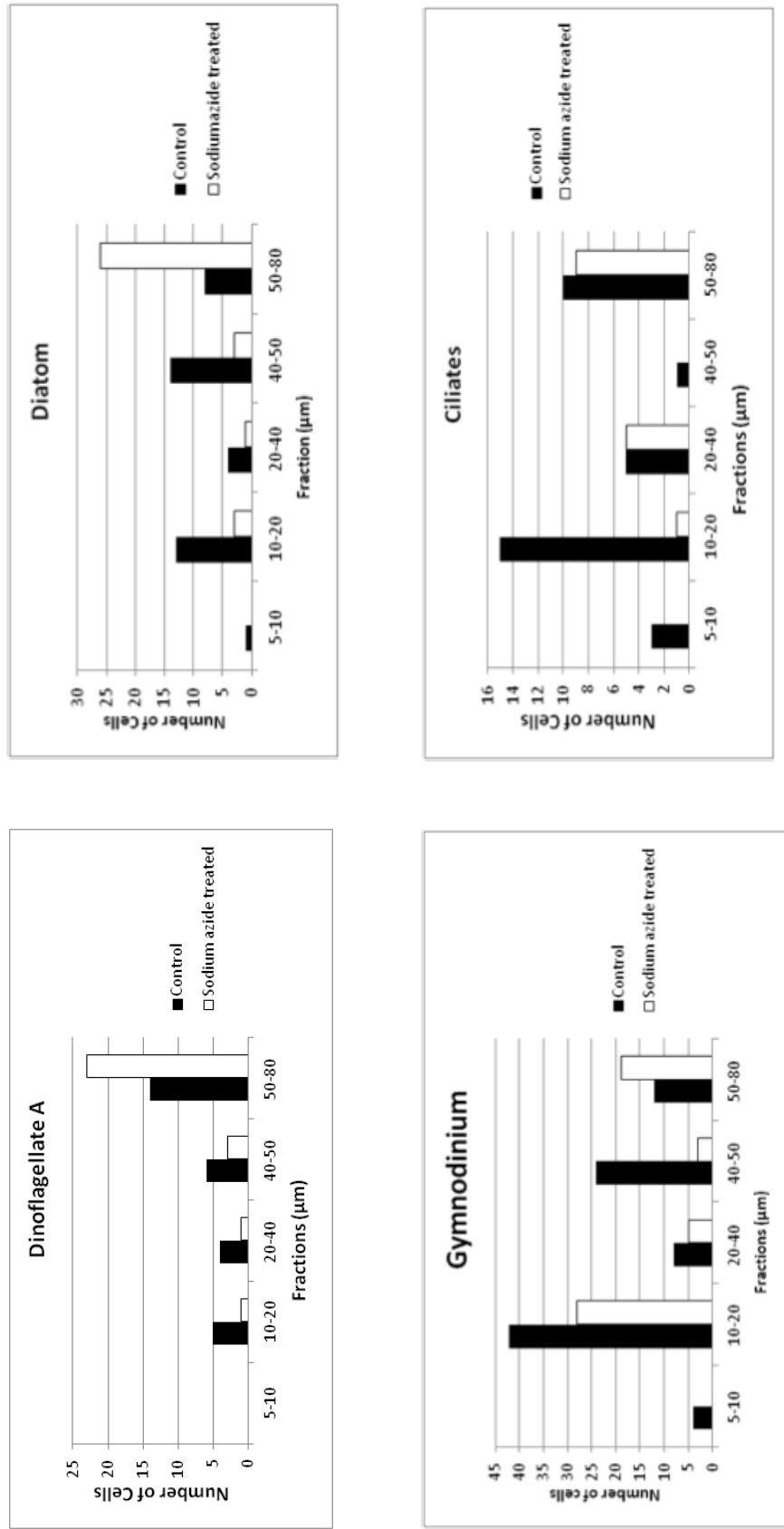


Figure 6. Motility experiment: Cell type distribution

Sodium azide was used as a poison (metabolic inhibitor) to test whether motility had an influence on the elution profile of different cell types. As the four examples show, the results were varied. For the dinoflagellates and diatoms the distribution profile narrowed to one size fraction (50 μm – 80 μm); however, for the ciliates and *Gymnodinium* the distribution of the treated sample was still relatively broad compared to the control.

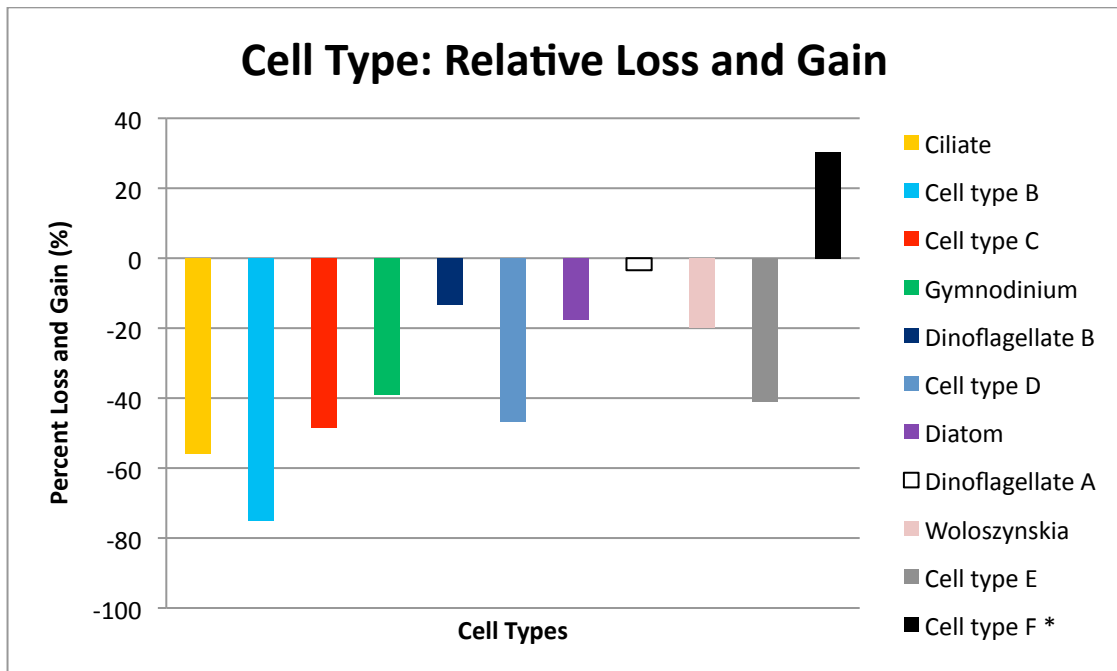


Figure 6. Motility Experiment: Percent loss and gain

(-) numbers
represent loss

With the exception of cell type F, the use of sodium azide resulted in the loss of cells. For each cell type the amount of loss varied. For example, there was < 10% loss for dinoflagellate A, but > 70% loss for cell type B.

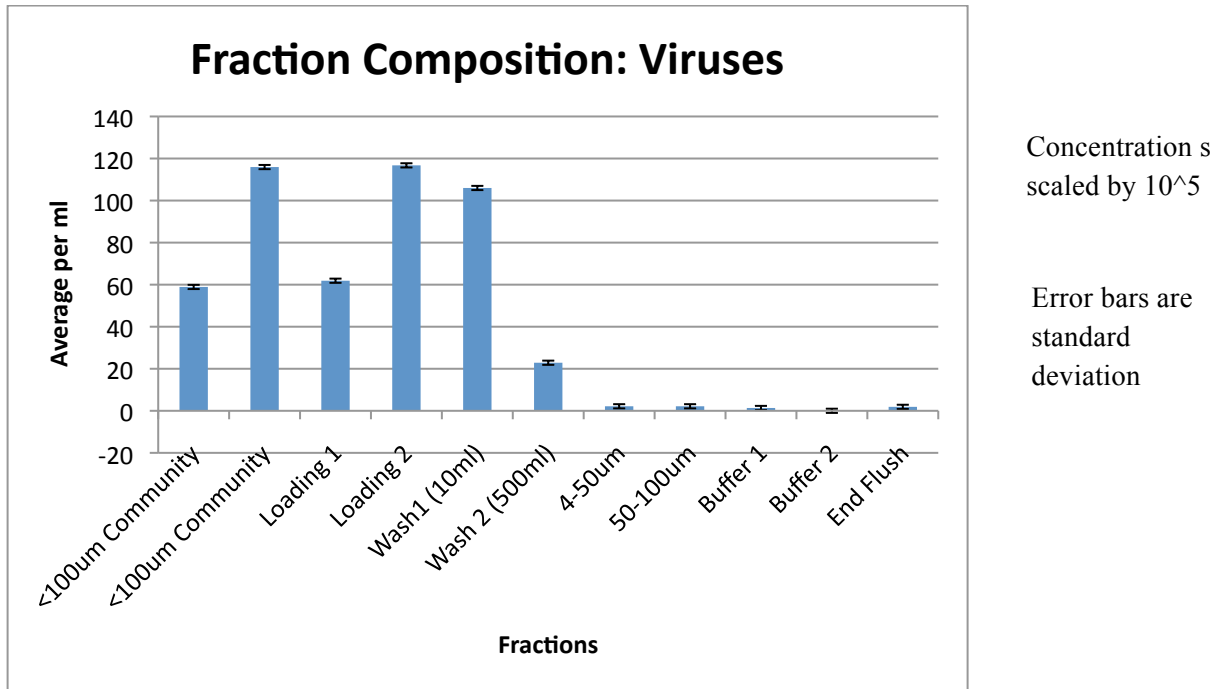


Figure 6. Trial III: Sample wash

For trial III, viral counts were done for each fraction to see if the elutriation process could be used as a method to wash extra cellular viruses from a sample. As the plot indicates, there is a significant reduction in viruses in the sample after wash 2.

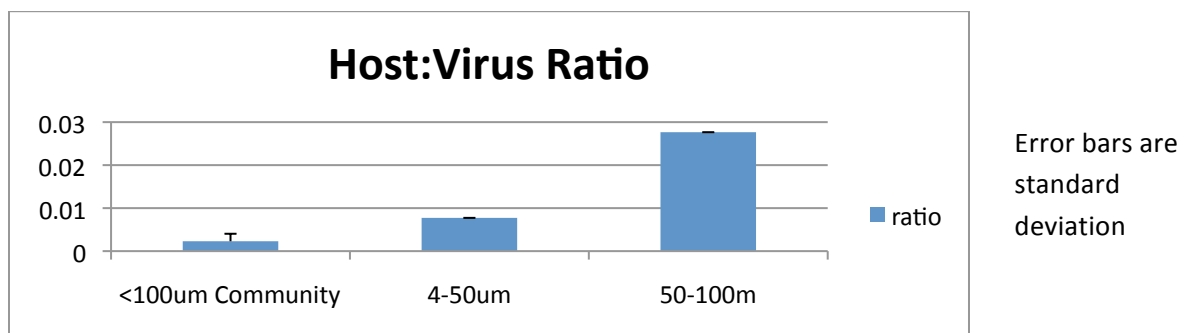


Figure 6. Eukaryotic and virus ratio

Although eukaryotic cells (potential hosts) were lost during the elutriation process, there was a significant increase in the ratio of cells to viruses from the $< 100 \mu\text{m}$ community to the two bulk fractions. The plot shows that the number of viruses lost to washing was greater than the number of cell lost.

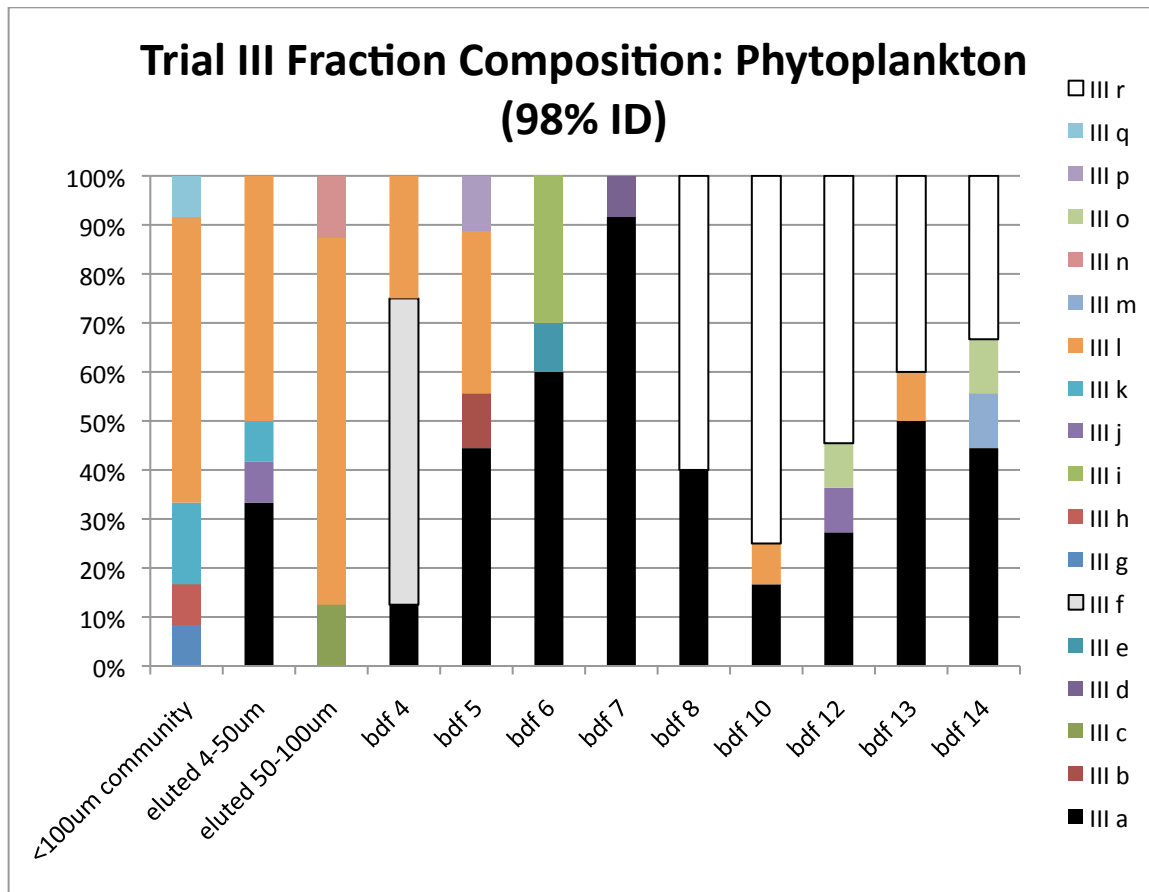


Figure 7. Trial III: Phylogenetic clusters

In trial III a 98% pairwise identification criteria was used to group eukaryotic genotypes. The clusters show that there is a reduction in diversity of genotypes from the < 100 μm community, to the two bulk fractions (4 – 50 μm & 50 – 100 μm), to the buoyant density fractions. In particular, buoyant density fraction 4, 7 and 10 show enrichment of specific genotypes.

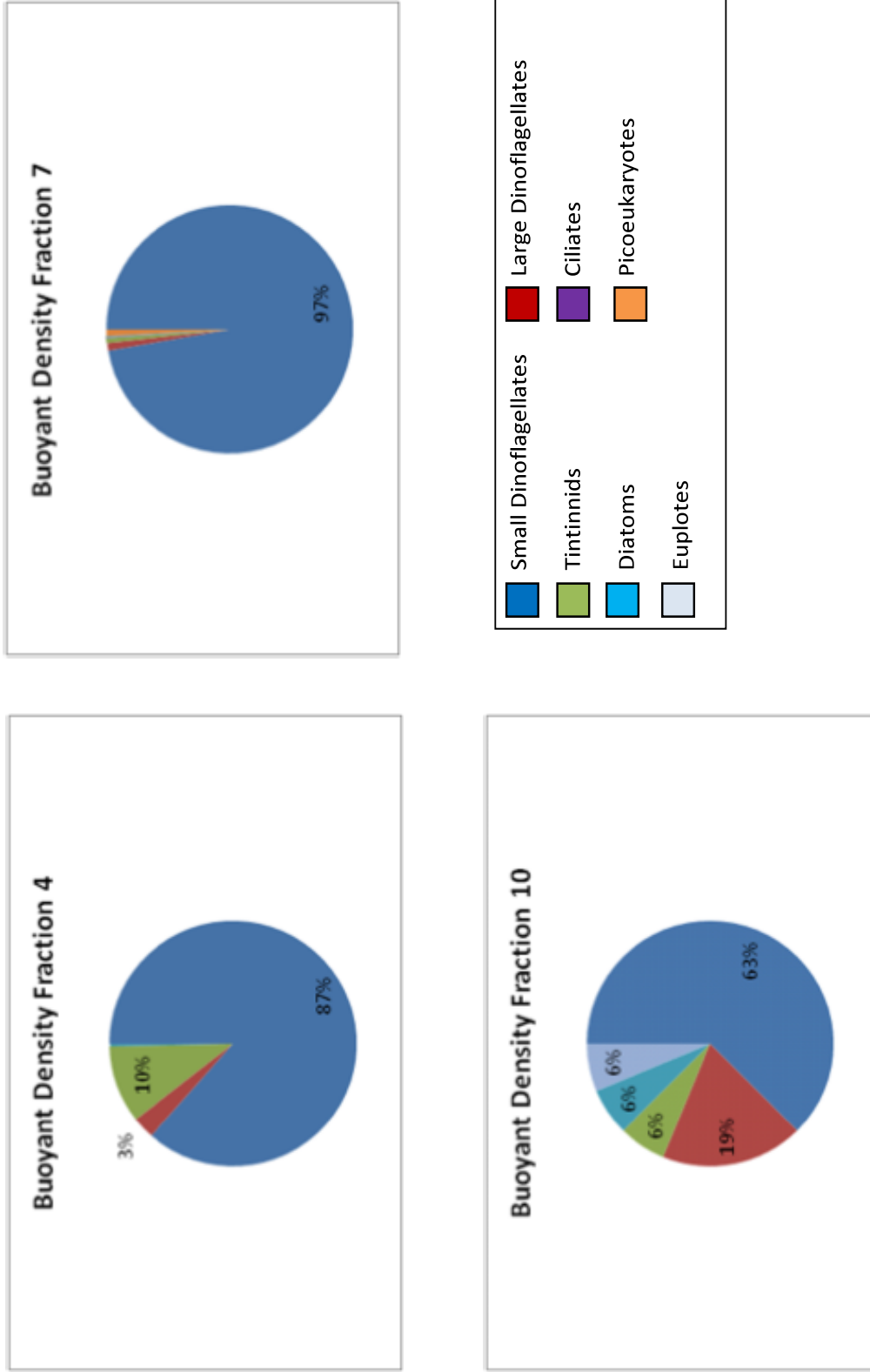


Figure 8. Trial III Microscopy: Buoyant density Fractions (4, 7 and 10)

Microscopy was done to confirm the molecular analysis of buoyant density fractions 4, 7 and 10. Microscopic analysis showed that each fraction was enriched with a dinoflagellate.

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