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Comparison of Two Separation Methods for Biological Particles: Field-Flow Fractionation (FFF) and Sucrose Density Gradients

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COMPARISON OF TWO SEPARATION METHODS FOR BIOLOGICAL PARTICLES: FIELD-FLOW FRACTIONATION (FFF) AND SUCROSE DENSITY GRADIENTS

by

Cody E. Garrison
B.S. May 2011, Old Dominion University

A Thesis Submitted to the Faculty of Old Dominion University in Partial Fulfillment of the Requirements for the Degree of

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Sucrose gradient centrifugation and Field-flow fractionation (FFF) are two different particle separation methods that overcome the problems of similar size microorganisms clumping together during standard filtration methods. FFF separates particles based on size and density via the parabolic velocity profile of laminar flow in a ribbon-like channel. The sucrose method separates particles via centrifugation in a density gradient. Both techniques worked well in separating eukaryotic from prokaryotic microbes, with the preferred method depending on the type and relative abundance of organisms to be separated. Minicells were separated from mother cells in transformed *Escherichia coli* cultures, heterotrophic flagellates (e.g., *Diplonema papillatum*) from their bacterial prey in cultures, and various eukaryotes from prokaryotes in natural seawater communities. These separated clean fractions were further analyzed for their biochemical composition (using radiolabeling and subsequent biochemical fractionation) revealing significant differences in the bulk biochemical makeup between prokaryotic and eukaryotic microbes. Even within domains, the sucrose gradient method was able to separate prokaryotic and eukaryotic microbes by size. Variables that influence and improve these separation techniques were explored, including the effect of aldehyde fixation.
This thesis is dedicated to my mom and dad who have been there every step of the way.
ACKNOWLEDGMENTS

None of this would have been possible without the continuous guidance of my advisor and mentor Alexander Bochdansky. Also thanks to committee members Fred Dobbs and John McConaugha for their continued support. Additional thanks to my labmates Melissa Clouse and Bonnie Bailey, and to Katherine Filippino for providing environmental samples.
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INTRODUCTION

Particle separation can be challenging in the size range of microorganisms. Smaller biological particles of interest such as bacteria and protists that are similar in size cannot be effectively separated by filtration methods because of clumping and retention of particles smaller than the pore size of the filter. Removal of larger particles from a mixed sample can be successful with standard filtration, but removal of smaller particles from a mixed sample requires more complex methods. Complete separation is important where clean fractions are needed for elemental, biochemical, or genetic analysis of each group. Previously, analyses of elemental stoichiometry or biochemical composition had to be corrected for by subtracting out the influence of prokaryotes, which can sometimes be difficult (e.g., Véra et al. 2001, Chrzanowski et al. 2010). Complete separation is also desirable for tracer studies with radioisotopes where prey organisms and predators need to be separated to provide a clear uptake of a tracer over time without contamination by more numerous labeled prey items. The numerous downstream applications after successful separation can help us to better understand the ecosystem functions of marine microbes. The present study shows that with very simple methods, seemingly very similar bacterial communities can be separated into subpopulations that reflect subtle differences in cell size and biochemistry.

Particle separation techniques examined here include the field flow fractionation (FFF) method (Giddings 1966) and a modified sucrose density gradient method (Brakke 1951). The FFF technique employed in this study uses the gravitational field and the parabolic velocity profile of laminar flow. The density gradient method is based on centrifugation
of a sample in a gradient created with sucrose solutions of different concentrations. These two separation techniques were examined in detail to determine which is more effective to separate protists from prokaryotes and also to separate prokaryotes of different size classes from each other. These tests involved a wide range of organisms, reflecting different separation characteristics.

Biological particles used in this study included *Escherichia coli* mother cells and minicells that were transformed with a green fluorescent protein, a non-axenic diatom culture (*Thalassiosira weissflogii*), and three heterotrophic nanoflagellate protists: *Diplonema papillatum* (formerly *Isonema papillatum*, Porter 1973), *Neobodo designis* (formerly *Bodo designis*, Larsen & Patterson 1990), and *Cafeteria roenbergensis* (Fenchel & Patterson 1988). All flagellate cultures contained an abundance of prokaryotes that served as food. The separation techniques were also tested on environmental samples from two locations: the estuarine and eutrophic Lafayette river (Norfolk, VA), and from the oligotrophic Gulf Stream off Hatteras (NC). Heterotrophic flagellates are ubiquitous in aquatic environments, feed directly on marine prokaryotes and together with viruses play an important role in controlling prokaryote populations (Fenchel 1982, Fernandes & Mesquita 1999, Pernthaler 2005, Massana 2011).

The reason for using *E. coli* mother cells and minicells as models for separation is that minicells (ca. 0.5 \( \mu \)m diam.) are buds of their much larger mother cells (ca. 2 \( \mu \)m in length) and should be identical except for the lack of DNA (Adler et al. 1966). Assuming that the density is the same between these two cell types, it follows that any successful separation is based on size (but see Discussion for further exploration of this notion). The
three species of protists -- all having different batch culture characteristics -- are good models because it is almost impossible to obtain a clean fraction of eukaryotic cells through filtration. The protist fractions become contaminated with the much more numerous prokaryotes in these cultures. Although diatoms can be grown axenically, and clean fractions of autotrophs can be so obtained, it is of interest to analyze the biochemical composition of autotrophs in cultures in which bacteria are present, or in environmental samples. Finally, the samples from the Lafayette River and the Gulf Stream were used to test not only how well prokaryotes could be separated from eukaryotes, but also to explore to what extent further particle separation was possible within these two groups. For biochemical characterization of cells within each fraction, a very sensitive method based on $^{14}$C labeling, chemical extraction and differential solubility was employed.
METHODS

Culture Details

Minicell-producing cultures of *Escherichia coli* (Adler et al. 1967) were transformed using a green fluorescent protein (GFP) vector (Alexander B. Bochdansky, Old Dominion University, unpubl.) and stored in 30% glycerine at -80°C. A scrape of the frozen stock culture was used to inoculate 50 mL of autoclaved LB broth (Lennox) in a 125 mL culture flask with attached test tube (functioning as a spectrophotometer cuvette). The growth of the culture was monitored by filling the attached test tube with culture, and by subsequently measuring the absorption in a Fisher (S1000) spectrophotometer. The culture was incubated on a shaker table overnight at 37 °C. In the morning, spectrophotometer readings were taken until the culture reached its inflection point between the log and stationary phases. The culture was subsequently transferred into two 50 mL conical polypropylene Falcon tubes (BD) and centrifuged at 15,000x g for 15 minutes at 2 °C. The supernatant was decanted and the pellet resuspended in 10 mL of 0.2 µm filtered artificial seawater (ASW). The cells were centrifuged and resuspended two more times. The final solution containing both minicells and mother cells was aliquoted into 1 mL subsamples and stored at -80 °C.

*Thalassiosira weissflogii* was grown in f/2 medium (Guillard and Ryther 1962) (see Appendix). Cultures of *D. papillatum* were grown in an enriched *Isonema* medium (ATCC medium # 1728). The medium was created as follows: 10 mL of artificial seawater (Instant Ocean), 1% enrichment solution, and 0.1 % vitamin solution. The medium was vortexed and filtered through a 0.2 µm cartridge into a Nunclon™ 40 mL
culture container. Then 1 mL 10 % heat-inactivated horse serum and 0.4 % stock *D. papillatum* solution, generally from a culture that was no more than one month old, was added to the container. Stock cultures of *N. designis* and *C. roenbergensis* were kept alive in 10 ml ASW with a rice grain at ca. 7 °C in the dark. Working cultures of *N. designis* and *C. roenbergensis* were grown in 2% of full strength BD Difco™ marine broth in a 200 mL flask. After autoclaving and when cooled to room temperature, the media were inoculated with 1% of *N. designis* or *C. roenbergensis* stock cultures. Samples from the eutrophic Lafayette River and from the oligotrophic Gulf Stream off Hatteras Island (approximately 50 km east of the coast) containing a diverse range of autotrophic and heterotrophic eukaryotes were collected with a bucket from the surface.

**Field-Flow Fractionation**

FFF is a separation method that relies on the hydrodynamic characteristics of a laminar flow profile. For laminar flow to occur, a separation channel has to be very thin, usually in the range of 50-250 µm (Schimpf et al. 2000). If thicker, then turbulence is likely to occur and separation will not be achieved. Velocity in the thin channel is highest in the middle of the channel and lowest at the top and bottom of the channel (Fig. 1). Larger and heavier particles sink closer to the bottom of the channel and are not as susceptible to lift forces due to Brownian motion. Statistically, smaller particles are thus more likely to be exposed to the higher flow rate in the middle of the channel and exit the channel first (Reschiglian 2002) (Fig. 1). The degree of separation can be altered by changing the flow rate (Plocková & Chmelík 2000). The cell abundance per fraction gradually increases until forming a peak with the fraction containing the highest cell
abundance. The cell abundance decreases gradually after the peak, slowly tapering off to a value of zero. Complete separation occurs when the two particle groups have separate peaks with no overlap. However, results may still be useful with minimal overlap, depending on the relative abundance of the particle groups being separated and the type of the intended downstream analysis. For instance, separating only one fraction cleanly can provide information on the other fraction by subtraction as long as the signal is not overwhelming.

Fig. 1. The parabolic velocity profile of laminar flow in the Field-flow fractionation (FFF) channel exhibits the highest velocity in the middle of the channel. Smaller, less dense particles (B) are more affected by lift forces (such as Brownian motion), remain higher in the channel, and thus exit the channel before larger and more dense particles (A).
Our initial tests indicated that separation is more effective when cells were fixed probably because of the lack of cell movement that eroded their position in the laminar flow pattern (see Results). We thus fixed the sample with 2% formaldehyde for at least 30 minutes but not more than 24 hours ahead of separation to kill the cells and to prevent any cell movement within the FFF channel. Fixation, however, changes the physical properties of the cells by cross-linking double bonds of proteins and thus hardening the cell tissue (Thavarajah et al. 2012). Failure to fix the sample of interest will lead to separation results that differ from those reported here.

The FFF channel was made by sandwiching a Mylar™ sheet with a cutout channel between two polycarbonate blocks and had dimensions of 30.5 cm x 2 cm x 200 µm with a volume of 1.22 mL. It was cleaned by flushing the entire system with 70% ethanol for 10 minutes, followed by 20-30 minutes with ultrapure water (Barnstead), and 20-30 minutes with artificial seawater just before the experimental runs. The artificial seawater (ASW) was created with ultrapure water and 0.2 µm-filtered Instant Ocean™ sea salt at 35 ppt. Flushing of the FFF system before and after trials was essential because several eukaryotic cells of a different species from an earlier trial were found contaminating fractions from a later separation trial. The flow speeds ranged from 0.025 mL min⁻¹ to 0.175 mL min⁻¹ depending on the type of separation trial. The flow through the system was kept constant for the entire separation once the solvent was introduced. The samples were introduced into the flow by means of an HPLC injector (Rheodyne model 7125) with a 200 µL loop. The entire FFF setup is shown in Figure 2. It consisted of a high-pressure liquid chromatography (HPLC) pump (Rainin HPXL). The HPLC
pump acted as a delivery system, which was capable of adjusting to very precise flow rates as low as 0.025 mL min$^{-1}$. The titanium pump head was continuously washed with ultrapure water via a Fisher peristaltic pump to prevent salt buildup on the piston. The fractions were collected using an automatic fraction collector (Rainin Dynamax model FC-1). The fraction collector held up to a hundred 13 mm x 100 mm 9 mL borosilicate glass test tubes and was programmed to collect 5 drops (or 0.25 mL) per fraction using a drop counter. The test tubes were cleaned with 1.2 N HCl and deionized water, and muffled at 450 °C for 5 hours prior to each use. To ensure complete collection of both particle populations, at least 50 fractions of 5 drops (ca. 0.20 - 0.25 mL) each were collected.

**Fig. 2.** Field-flow fractionation setup for particle separation. Filtered artificial seawater acted as the carrier liquid and was pumped through the separation channel at a certain speed set by the delivery system before the sample was injected. Freshwater was constantly pumped across the delivery system pump to remove salt residue from the piston. Sample was injected simultaneously with the start of the automatic fraction collector. Between 40 and 50 fractions were collected.
Each fraction was then vortexed briefly and filtered onto a 0.2 µm polycarbonate filter, rinsing the fraction tube once by adding 1 mL of 0.2 µm filtered ASW and vortexing to remove material in the tube. A few drops of phosphate buffered saline (PBS) was then added to the filter tower to neutralize pH of the fixative, followed by a few drops of ultrapure water. The filter was then placed on a microscope slide followed by one drop of Vectashield with 4',6-diamidino-2-phenylindole (DAPI), a fluorescent stain that binds to the double-stranded DNA of a cell (Vector Laboratories), and allows for easy counting of species abundances using an epifluorescent microscope. Eukaryotic nuclei were distinguished from bacteria by size. Slides were stored in a freezer until ready for counting. Just before counting, the slides were thawed and dried at room temperature until all condensation was removed before a drop of immersion oil was placed on top of the cover slip. The slides were viewed with an ultraviolet light filter using an Olympus BX51TF epifluorescence microscope, with a 100x lens suitable for use with immersion oil. Depending on the density of cells present, 50-100 fields were counted for accurate cell abundance calculations.

**Sucrose Density Gradient fractionation**

The second separation method used in this study was the sucrose density gradient separation. The solvent for the sucrose solutions was Buffered Saline Gelatine (BSG) 10x, which consisted of ultrapure water, 0.85% NaCl, 0.03% KH₂PO₄, 0.06% Na₂HPO₄, and 0.01% Gelatine (Kemp et al. 1993). Sucrose solutions were created using 15%, 20%, 25%, 27.5%, 30%, 35%, 40%, 45%, and 50% sucrose (Mitosciences 2007). The most efficient way was to create a large stock solution of 50% sucrose and dilute individual
samples with BSG to create the full spectrum of sucrose solutions. The solutions were chilled before initial use and refrigerated during storage as well to ensure consistent viscosity of the solutions between trials. The gradient was created in 15 mL conical polypropylene Falcon tubes (BD). Solutions were transferred sequentially using an automatic pipette with the most dense solution at the bottom of the tube as shown in Figure 3. Solutions were dispensed slowly to ensure that each solution ran down the side of the tube without creating turbulence or mixing with the underlying layers of higher density (Fig. 3). During transfer, the tube was held in place with either a rod and clamp setup, or by resting it in a narrow neck Erlenmeyer flask. Volumes of each sucrose layer were as follows: 0.5 mL of 50% solution, 0.5 mL 45%, 0.5 mL 40%, 0.5 mL 35%, 0.75 mL 30%, 1 mL 27.5%, 1 mL 25%, 1 mL 20%, 1 mL 15%, and 0.5 mL of fixed sample. A weaker sucrose gradient (a gradient from 15% - 35%) was used with initial trials but was not as effective and resulted in pelleting at the bottom of the tube, therefore a stronger gradient (15% - 50%) was established. After pipetting, distinctive layers were visible within the tube. The sample was then centrifuged at 4000x g for 30 minutes at 4 °C. Sucrose separations were scaled up to 50 mL Falcon tubes (BD) in certain cases and using sample volumes of 2.5 mL (i.e., multiplying all volumes including fraction volumes by 5). An attempt was made to use the Percoll density gradient method (Amersham Biosciences) with several particle groups for comparison (data not shown), but the sucrose method appeared to produce more consistent results with higher resolution.
Initial, fractions after centrifugation were pumped out from the bottom up with a peristaltic pump and tubing inserted to the very bottom of the conical tube. Separation appeared unsuccessful with this technique likely because the gradient was disturbed when the pump tubing was inserted, and because the tubing was not thin enough to prevent turbulent flow during transport of the fractions. The more effective fraction collection method -- and the method that was followed subsequently -- was to poke a hole in the
bottom of the tube with a sewing needle and count a set number of drops into sample tubes placed in a rack. Directly after centrifugation, the cap of the sample tube was unscrewed to let air in during fraction collection. The needle was then carefully pushed into the bottom of the sample tube using a lab bench as leverage. The middle of the needle was held firmly with two fingers to prevent the needle from breaking in half. Once the needle was inserted about 2 mm into the bottom, and with the needle still in place, the tube was clamped into place using a laboratory stand (Fig. 4). Borosilicate glass test tubes for each fraction were cleaned with 1.2 N HCl and deionized water, and muffled prior to use. A test tube rack was filled with at least thirty 9 mL glass test tubes. The number of fractions collected varied slightly from trial to trial based on the size of the hole created or how far the needle was inserted. The initial volume of the gradient plus sample (7.25 mL) was divided by the number of fractions collected to calculate the exact volume of each fraction. The rack was positioned with the first tube directly under the clamped sample tube, which still had the needle stuck in place. The needle was then carefully removed once the collection tubes were in position. Each fraction consisted of 10 drops for small volume runs, or 2 mL for large volume runs, manually moving the test tube rack quickly from one tube to the next to not lose any sample. Fractions were collected in a serpentine pattern from one row to the next to avoid sample loss. Each fraction was filtered for microscope counts following the same procedure given above for FFF samples.
Fig. 4. Fraction collection setup for the sucrose method. After centrifugation, a sewing needle was inserted into the bottom of the gradient tube and positioned above the test tube rack with a clamp. Fractions were collected by removing the needle and collected manually by moving the rack to the next tube after a certain number of drops (ranging from 19 to 26) were counted.

Radiolabeling with $^{14}$C sodium bicarbonate

To label heterotrophic cells with $^{14}$C bicarbonate, we exploited the fact that all heterotrophic organisms incorporate some inorganic CO$_2$ into organic molecules. This heterotrophic carbon fixation (anapleurotic metabolism) is facilitated by carboxylases working to replenish the tricarboxylic acid cycle (e.g., Wood & Werkman 1936, Feisthauer et al. 2008, Hanson et al. 2012). The radiolabeled trials were performed with *D. papillatum* since it was the culture with the highest density of cells and the highest success rate of separation. Forty μCi of $^{14}$C sodium bicarbonate (Perkin-Elmer) were
added to roughly 11.5 ml of *D. papillatum* culture in a Nunclon™ 40 mL culture container immediately after the culture was inoculated with the stock culture of *D. papillatum* (see above). Sodium bicarbonate was preferred over other forms of $^{14}$C such as leucine because CO$_2$ was expected to be incorporated more evenly across biochemical fractions, whereas the amino acid leucine would preferentially be incorporated into the protein fraction. Inorganic $^{14}$C is incorporated into organic metabolites as heterotrophic growth requires the presence of some carbon dioxide (Wood & Werkman 1936, Hartman et al. 1972, Perez & Matin 1982, Roslev et al. 2004, Miltner et al. 2005, Alonso-Sáez et al. 2010). For instance, it is estimated that 3 to 8% of aquatic prokaryotes’ carbon demand is satisfied by these anapleurotic reactions (Romanenko 1964). This process has also been described for eukaryotic microbes such as fungi (Schinner et al. 1982), and for flagellates (Peak & Peak 1980), among many higher organisms.

By adding $^{14}$C to an exponentially growing culture, we assumed that after several generations, isotopic equilibrium would be established among all fractions (i.e., that the $^{14}$C : $^{12}$C ratio will be the same in all biochemical fractions). This is essentially the same assumption previously used in the determination of autotrophic labeling patterns in which five cell divisions were deemed sufficient for uniform labeling (Nielsen & Olsen 1989). We grew *D. papillatum* cultures for one week, over which the cultures grew into a densely packed mass of cells. Separation was then performed with the high volume (50 ml) sucrose density gradient method (see above). This method was chosen because it was more effective in separating the flagellates from the prokaryotes than the FFF method (see Results). We used the high volume sucrose protocol to ensure sufficiently large
radioactive signal in all density and biochemical fractions. Initial trials of sucrose separated cultures with 4 µCi of $^{14}$C sodium bicarbonate resulted in $D. \ papillatum$ fractions that measured 100 - 400 total disintegrations per minute (DPM). After dividing each sample into 4 biochemical fractions (see below), the DPM readings were even lower (data not shown). To increase sensitivity, the amount of $^{14}$C bicarbonate was increased from 4 µCi to 40 µCi, and the sucrose gradient volumes were increased by a factor of 5. The sample volume on top of the sucrose gradient was increased by a factor of 5 as well to 2.5 mL. Sucrose separations with these increased volumes were conducted on 4 separate $D. \ papillatum$ cultures, each after a culture growth period of 1 week.

Seventeen to eighteen fractions were collected in each run. However, the biochemical fractionation setup used in this study can handle a maximum of 8 samples per day, so only the most relevant fractions from the sucrose separations were used. Fractions 1-4 (most dense and first to come out of the gradient tube) contained the highest proportion of $D. \ papillatum$ cells relative to bacteria, and fractions 8-11 contained the highest amount of bacteria. In addition, fractions 8-11 were filtered through a 3 µm polycarbonate filter to remove any remaining $D. \ papillatum$ cells. These two sets of 4 fractions were then considered pure isolates of each group of organism. Each fraction was then divided further for several different analyses. Each fraction was diluted slightly with 0.5 mL of 0.2 µm filtered ASW to increase the total volume to 2.5 mL each; the increased volume reducing pipetting errors. To get the total level of $^{14}$C before biochemical fractionation, a subsample of 0.5 mL was filtered onto a 2.5 cm GF/F glass microfiber filter and placed in a 5 mL scintillation vial. After adding 4 mL of scintillation cocktail,
they were counted using a Perkin-Elmer Tricarb model 3110TR liquid scintillation counter. A second sub-fraction of 1 mL was filtered onto a 2.5 cm GF/F glass microfiber filter, folded in half, placed in aluminum foil, and stored in the freezer until further analysis. A third sub-fraction of 1 mL was filtered onto a 0.2 µm polycarbonate filter, placed on a microscope slide, stained with one drop of Vectashield with DAPI, protected with a cover slip and stored frozen at –20 °C until cells were enumerated using the epifluorescence microscope.

**Biochemical fractionations**

To analyze the biochemical composition of separated groups of organisms, we used the protocol given in Li et al. (1980), and modified by Rivkin (1985) and Bochdansky et al. (1999) to divide each sample into (1) proteins, (2) lipids, (3) polysaccharides and nucleic acids (P-NA), and (4) low molecular weight (LMW). The frozen GF/F filters were removed from the freezer and placed in eight separate 16 mm x 120 mm glass centrifuge tubes (15 mL). Next, 4.5 mL of a chloroform/methanol mix (1:2 by volume) was added to each tube, vortexed vigorously for 1 minute, and placed in a foam container filled with crushed ice for 20 minutes. Eight filtration towers were loaded with 2.5 cm GF/F filters in a radioactive designated fume hood. Graduated glass centrifuge tubes were placed under the filter funnels so that the stem of the filtration manifold reached into the vials and all filtrate could be collected. With the help of long forceps, the extracted filters and all of the chloroform-methanol mixtures were transferred from the centrifuge tubes into the filtration tower loaded with fresh GF/F filters. The vacuum pump was switched on and all liquid was squeezed out of the GF/F filters by gently pressing down on the filters with a glass rod. The glass rod was wiped dry between
fractions, and therefore any transfer of $^{14}$C was likely below the detection limit. Next, 1.5 mL of chloroform was added to each of the original centrifuge tubes, vortexed, and poured into filter towers to remove any residual sample. Again, remaining liquid trapped in the GF/F filters was removed using the glass rod. The GF/F filters (containing polysaccharide-nucleic acid, and protein fractions) were then removed from each filter tower and placed into 20 mL glass scintillation vials. Four mL of 5 % trichloroacetic acid (TCA) was added to each vial, and the vial was placed on a heating block at 95 °C for 1 hour. The scintillation vials were covered with glass marbles that served as lids and condensation surfaces to prohibit evaporation. It was important that the temperature did not exceed 95°C to avoid boiling over.

While the GF/F filters were extracted, the centrifuge tubes containing the filtrate (i.e., the combined lipid and low molecular weight (LMW) fractions) were removed from vacuum flasks and 3 mL of ultrapure water was added to each to create phase separation of hydrophilic and hydrophobic layers. A ground glass stopper was added to the top and the emulsion was vortexed vigorously for 1 minute. For phase separation, the tubes were centrifuged in a Fisher 225 centrifuge at 3,400x g for 10 minutes. After centrifugation, the volume of the lower chloroform layer and the upper water-methanol layer were recorded. Using a 9-inch pasteur pipette, the lower chloroform layer was removed first and placed into separate 20 mL scintillation vials (this was the lipid fraction). The remaining liquid from centrifuge vials was poured into separate 20 mL scintillation vials (this was the LMW fraction). This part of the protocol is identical to the classic lipid
extraction and purification protocol by Bligh and Dyer (1959), which is still considered the most efficient method of extraction (Sheng et al. 2011).

The hot-TCA extracted samples were removed from the heating block and the liquid and GF/F filters were transferred into filter towers containing fresh GF/F filters. As before, the filtrate was captured by glass centrifuge vials, and the remaining liquid was pressed out of the GF/F filters using a glass rod. Another 4 mL of 5% TCA was added to each of the eight scintillation vials to rinse and obtain residual material. The hot-TCA insoluble material retained by the GF/F filters was the protein fraction. The filtrate captured in the centrifuge vial was the polysaccharide – nucleic acid (P-NA) fraction. The protein fractions received 10 mL of scintillation cocktail immediately, and the other three biochemical fractions were evaporated to dryness on the heating block overnight at 60 °C. A simplified flow chart summarizing the fractionation process is presented in Figure 5.

The next day, we added 10 mL of scintillation cocktail to the lipid fractions. The P-NA and LMW fractions each received 500 µL of ultrapure water and were swirled gently to dissolve residual precipitate. Then 10 mL of scintillation cocktail was added to each of the P-NA and LMW fractions. All four biochemical fractions were finally run in the liquid scintillation counter. The DPM values of the 4 biochemical fractions of one specific sucrose fraction was summed to get total DPM. Then the DPM value for each biochemical fraction was divided by the total to get a percent value for each biochemical fraction. Note that these total sums from the four biochemical fractions were on average
about 81% of the initial total DPM values before fractionation. In other words, there was an average loss in radioactivity of approximately 19% during the biochemical fractionation procedure.

**Fig. 5.** Flowchart of the biochemical fractionation protocol. Samples were divided into four fractions according to this procedure: 1) proteins, 2) polysaccharides/nucleic acids, 3) lipids, and 4) low molecular weight compounds.

**Size determination of prokaryotes**

Cell size distributions over consecutive fractions were obtained from calibrated digital images using Image-Pro MDA for image capture and ImageJ (National Institute of Health) for analysis of cell area. Images were taken with a 100x lens and a 2x
magnification lens with the same Olympus BX51TF microscope and a QImaging cooled monochrome 12-bit camera (model Fast1394). The images were calibrated using a stage micrometer and a spatial calibration marker which was placed on each image to help calibrate the ImageJ analysis program. Threshold and bandpass filters were adjusted so that only the bacterial cells were displayed. The minimum size was adjusted to 0.08 µm² so that remaining noise was removed. Using the outline feature of ImageJ, the rendered images were compared to the original to ensure that all cells were accurately captured. Any false particles that remained were deleted from the list of particles and their sizes.

**Preconcentration procedure for natural communities of microbes**

Laboratory cultures are sufficiently dense to be separated without preconcentration steps. However, cell concentrations in environmental samples are usually too low to produce a high enough yield for each of the fractions for counting and other downstream applications. A Vivaflow 50™ tangential flow filtration (TFF) device was used to concentrate cells from large volumes (~9 L) to small volumes (2 - 3 mL). The 50 cm² polyethersulfone membrane device comes in a variety of pore sizes, but preliminary experiments (unpublished) showed that cell losses were too large using 0.2 µm membranes. Thus the next available pore size of 100,000 Daltons was used. The tangential flow device has the advantage that filtrate is excluded gently while the motion of the particles is parallel to the filter surface, reducing the amount of particle aggregation on the membrane (Fig. 6). The flow rate was adjusted so that the filtrate production was 50 ml min⁻¹ and the return flow to the sample reservoir was 150 mL min⁻¹. Over time, the sample reservoir decreased in volume while maintaining roughly the same total number
of cells. In preparation, the filtration membrane was rinsed with ultrapure water prior to use to remove trace amounts of glycerine and sodium azide that were initially present to preserve the integrity of the membrane. The concentrated sample was then fixed with formaldehyde and separated using the sucrose method. The fractions were further subdivided for counting on polycarbonate filters and biochemical fractionation on GF/F filters.

![Diagram of sample concentration procedure](image)

**Fig. 6.** Diagram of the sample concentration procedure. The initial sample was first placed in the reservoir and then pumped through a tangential flow filtration device. The filtrate from the 100,000 Dalton device was particle-free, and the concentrated particles flowed back into the reservoir of decreasing volume. The tubing in the reservoir was placed just above the bottom so a small volume remained as the concentrated sample.
To provide natural oligotrophic bacteria for biochemical fractionation, nine liters of a 5-month old Gulf Stream sample from the continental slope region off the coast of Hatteras, North Carolina (salinity of 37.2 ppt) was used. The sample was kept in the laboratory without any nutrient additions, and on microscopic examination, still appeared to be oligotrophic with low abundances of prokaryotes and small eukaryotes. Nine liters of this sample were concentrated to 58 mL using the TFF device. One mL of this first concentrated sample was fixed and separated via sucrose gradient with normal fraction volumes for ImageJ cell size analysis. The remaining concentrated sample was decanted into a 125 mL flask and inoculated with 200 µCi of $^{14}$C bicarbonate using the Wood-Werkman process (see above). The flask was wrapped in foil to prevent autotrophic growth and incubated on the lab bench for seven days. The 57 mL was fixed with 2% formaldehyde (fin. conc.) and concentrated to 2 mL using the TFF device for a second time. Half (1 mL) of the final concentrated solution was filtered for microscope counts, and the other half was separated via sucrose gradient. Twice the usual volume of fractions (20 drops) from the separation was taken to ensure sufficiently high radioactivity for the scintillation counter. Each of the sucrose fractions were further biochemically fractionated following the protocol described above. The biochemical results were directly compared to the microscope counts to validate which fractions fell in either the eukaryote peak or the bacteria peak. Cell sizes of all microbes (including eukaryotes) were analyzed using ImageJ as shown above.
RESULTS

Culture details:

*Cafeteria roenbergensis* cultures grew to a maximum density of $2 \times 10^5$ cells ml$^{-1}$ (Fig. 7). The batch cultures were short lived, peaking between 9-13 days and crashing within 18 days. Peak prokaryote numbers were $3$ and $5 \times 10^7$ cells ml$^{-1}$ in the two experiments, respectively (Fig. 7). *C. roenbergensis* cells exhibited lagged growth trends, increasing their growth rate once prokaryote concentrations were near their peaks. Then once prokaryote populations fell to as low as 15% of the peak concentration due to grazing, *C. roenbergensis* populations began to crash, followed by a slight rebound in the prokaryote population (Fig. 7). Small variability existed between the two *C. roenbergensis* cultures that were treated in the same way. *Neobodo designis* cultures grew to a maximum density of $4 \times 10^4$ cells ml$^{-1}$ (Fig. 8) and displayed great variability between two cultures treated the same way (Fig. 8). In one of the cultures *N. designis* did not begin increasing in concentration until day 13, whereas the other replicate culture exhibited growth on day 6. Prokaryotes in *N. designis* cultures peaked at $9 \times 10^7$ cells ml$^{-1}$ (Fig. 8). These prokaryotes also tended to form chains unlike the other cultures, and because individual prokaryotes were difficult to discern, the concentrations were likely even higher than that recorded. *N. designis* cultures did not exhibit the typical oscillating predator-prey abundances as observed in *C. roenbergensis* cultures. The prokaryote concentration did not fall below 45% of the peak concentration. Additionally, *N. designis* cells did not show signs of crashing within the 18 day study period. *D. papillatum* cultures, for which comparable time series data were not collected, grew to a maximum density of $7 \times 10^5$ cells ml$^{-1}$, the highest concentration of eukaryotic cells of the three
protists. Their peak concentration generally occurred after just 7 days, and all separation trials for biochemical analyses were performed on the 7th day for consistency.

\[ \begin{align*} \text{Fig. 7. Time series of two } & \text{Cafeteria roenbergensis cultures. Bacteria abundances decreased by day 7 in response to } C. \text{ roenbergensis population increase. A slight rebound in bacteria abundance was visible after } C. \text{ roenbergensis population began to decrease again.} \end{align*} \]
Fig. 8. Time series of two *Neobodo designis* cultures. Bacteria abundances decreased in response to *N. designis* population increase. Culture A exhibited a lagged growth pattern and *N. designis* did not increase in abundance until day 13. Populations of *N. designis* also did not appear to decrease in number over the time period of the study.
Separation Comparisons:

Separations using FFF

The success rate of separation for the two methods depended largely on the organisms being used. Cell size and relative abundance were the two most important factors in a successful separation. The flow rate for the FFF method was adjusted based on the difference in cell size of the organisms being separated. A smaller difference in size between the two particle groups being separated required a slower flow rate for separation. *C. roenbergensis* was the smallest eukaryote cultured for use in separations, and prokaryotic *E. coli* cells (minicells and mother cells) had the overall smallest difference in cell size. For FFF trials of *C. roenbergensis* and of *E. coli*, the slowest flow rate with this pump head of 0.025 mL min⁻¹ was used. The mother cell and minicell peak overlapped completely, and no separation occurred (Fig. 9). The peak for *C. roenbergensis* and prokaryotes also overlapped, and no separation occurred (Fig. 10). In the FFF trials of *N. designis*, some separation occurred, but there was no clear peak of *N. designis* cells (Fig. 11). Fractions containing *N. designis* were somewhat scattered and the cell abundance was too low for a significant result. Separation of *D. papillatum* from prokaryotes was successful using the FFF method (Fig. 12) at a flow rate of 0.175 mL min⁻¹. The tail ends of the two particle peaks overlapped, but the fractions at peak cell abundances were isolated from each other (Fig. 12). The peak for this eukaryote is very broad, which permits the combination of multiple fractions for a higher number of cells to work with in downstream applications.
Fig. 9. Field-flow fractionation of A) *Escherichia coli* minicells and B) *E. coli* mother cells. Particle peaks lined up exactly in the same fraction and no separation occurred.
Fig. 10. Field-flow fractionation of A) bacteria and B) *Cafeteria roenbergensis*. Particle peaks lined up exactly and no separation occurred.
Fig. 11. Field-flow fractionation of A) bacteria and B) *Neobodo designis*. Concentrations of *N. designis* were very low and no significant separation occurred.
Fig. 12. Field-flow fractionation of A) bacteria and B) Diplonema papillatum. Separation was significant and the peak of D. papillatum cells was completely separated from bacteria. The bacteria peak overlapped some D. papillatum cells, but the larger protists within those fractions could be removed using 3 µm polycarbonate filters.

A 200 µl sample from the Lafayette River during an algal bloom was processed in the FFF system at a speed of 0.08 mL min⁻¹ to assess the efficiency of separation for natural samples containing many different organisms (Fig. 13). Seven different eukaryotic particle groups were identifiable based on morphological criteria in the DAPI channel (Fig. 14). Some eukaryotes showed overlap with the prokaryote peak, however, several eukaryotic cells were present in later fractions completely separated from
prokaryotes (Fig. 13). Eukaryotes B and C were present in higher numbers and completely separated from prokaryotes.

**Fig. 13.** Field-flow fractionation of a Lafayette River (eutrophic estuary) sample. A clear peak in A) prokaryote cells (top panel) was visible, and B) eukaryote abundances were relatively low with no clear distribution pattern (lower panel). Eukaryote C (see Fig. 14 for images) exhibited a large peak in fraction 32 completely isolated from bacteria.
Fig. 14. Images of Lafayette River eukaryotes indicated in Fig. 13 and Fig. 20. Species identification was not possible.

Separations using sucrose gradients

Overall, the sucrose method proved to be much more effective than FFF in separating particle groups. A mixture of *E. coli* minicells and mother cells was placed on the top layer of the sucrose gradient and centrifuged for 20 minutes at 2,000x g and 4 °C. The lower fractions were collected first from the bottom of the tube and were higher in density (Fig. 15). The higher the fraction number, the lower the density value. Larger particles were thus found in the lower fraction numbers, reverse that of the FFF. As seen in Figure 15, cells from both particle groups were found in the top three fractions (i.e., in the highest fraction numbers), separated from the peaks of each particle group. This retention of particles at the very surface was found to occur in all sucrose separation trials, no matter the organism or size of the particles. This surface retention unfortunately
reduced the number of cells available to accumulate at the respective buoyant density values for each particle group. Using a surfactant, along with higher centrifugation speeds to break the surface tension did not ameliorate the problem (data not shown).

*C. roenbergensis* cells failed to exhibit a separate peak from the prokaryotes in sucrose trials (Fig. 16). They were also run for 20 minutes at 2,000x g and 4 °C. Their prokaryotic prey formed a clear peak below the surface, but the protists never appeared in the lower fractions where they were expected. Some *N. designis* cells appeared below the prokaryote peak, but their abundance was low and the peak was not well defined, resulting in an insufficient separation (Fig. 17). Sucrose separation of *D. papillatum* cultures resulted in clean separated peaks with high relative abundance of isolated protists. They were centrifuged for 30 minutes at 4,000x g due to their larger cell size, and at 4°C (Fig. 18). A sample of the diatom species *T. weissflogii* was also separated from the prokaryotes present in culture using the sucrose method (Fig. 19). The peaks had some overlap and the relative abundances were not nearly as high as with *D. papillatum* cultures, but the diatom cells were sufficiently isolated from prokaryotes in fraction 6. The prokaryote cell count was low enough in this fraction to be negligible.
Fig. 15. Sucrose gradient separation of A) *Escherichia coli* minicells and B) *E. coli* mother cells. An isolated minicell peak was found centered at fraction 12, and an isolated mother cell peak was found centered at fraction 5. Some residual cells always remained trapped in the surface layer (fraction 17 - 20 to the far right).
Fig. 16. Sucrose gradient separation of A) bacteria and B) *Cafeteria roenbergensis*. An isolated bacteria peak was clearly visible at fraction 13, but no *C. roenbergensis* cells were found below the surface layer.
**Fig. 17.** Sucrose gradient separation of **A**) bacteria and **B**) *Neobodo designis*. *N. designis* cells were scattered without a clear peak, and separation from bacteria did not occur.
Fig. 18. Sucrose gradient separation of A) bacteria and B) Diplonema papillatum. *D. papillatum* cells peaked in fraction 7, with negligible amounts of bacteria and separated from the main bacterial peak. Some *D. papillatum* cells were present in the bacterial peak, but the protists were removed using a 3 µm filter after separation for further analysis of the bacterial fraction.
Fig. 19. Sucrose gradient separation of A) bacteria and B) *Thalassiosira weissflogii*. The two groups were successfully separated with a *T. weissflogii* peak at fraction 6 with negligible amounts of bacteria. The bacterial peak was found in fraction 10, with some overlapping *T. weissflogii* that could be removed using filtration with a 3 µm filter.

Assuming that particles accumulate at their characteristic density values (but see Discussion for rate zonal separation mechanisms), the density values of each sucrose layer equal the density of the particles trapped in that layer. The top most layer (below the sample layer) of 15% sucrose had a density value of 1.06 g cm$^{-3}$ and the bottom most layer of 50% sucrose had a density value of 1.23 g cm$^{-3}$ (Heidcamp 2010). The prokaryote peak was found at an average buoyant density of 1.089 g cm$^{-3}$ with a range of 1.081-1.094 g cm$^{-3}$ over 5 trials, very close to the reported values of marine prokaryotes.
of 1.087 g cm\(^{-3}\) (Inoue et al. 2007). The buoyant density of \textit{D. papillatum} is unknown, but it can be estimated based on its location along the gradient. The peak of \textit{D. papillatum} cells were found at an average buoyant density of 1.129 g cm\(^{-3}\) with a range of 1.120-1.139 g cm\(^{-3}\) over 5 sucrose trials. Note that the 0.5 mL of sample containing cells that is placed on the top layer is from a culture created from artificial seawater at a density of 1.028 g cm\(^{-3}\). Density values of layers should be estimated from the bottom of the tube upward up to avoid any changes that might have occurred in the top layer due to the addition of the sample.

Eukaryotes from a Lafayette River sample were successfully separated from natural prokaryotes at their natural abundance levels using the sucrose method (Fig. 20). There were ten morphologically different eukaryotic cell types (Fig. 14) that could be distinguished using the epifluorescent microscope (both autotrophic and heterotrophic) from the separated sucrose fractions. The prokaryote peak was well defined and had several fractions without eukaryotic cell contamination. These results, together with the overall success of each separation method for each sample type are summarized in Table 1.
Fig. 20. Sucrose gradient separation and cell size regression of a Lafayette River sample. 

A. The prokaryote peak was found at fraction 16. B. Prokaryote cell size across the separated prokaryote peak (fractions 8 – 19). Cell size decreased with increasing fraction number and decreasing buoyant density (n = 8, r² = 0.799, F = 23.8, p = 0.0028).
Fig. 20 Continued. Bracketed data points indicate that these cells were outside of the main prokaryote peak, but the peak actually tapered out to near zero in fraction 8. These data points also overlapped eukaryote fractions, and could have therefore been picoeukaryotes mistaken for larger prokaryotes. C. Ten morphologically different eukaryotes (see Fig. 14) were found in sucrose separated fractions. One peak of eukaryotes was present near the surface of the gradient and a separate peak was found in the lower sucrose fractions. D. Linear regression of eukaryotes found in lower fractions. Eukaryotes increased in cell size with decreasing fraction number and increasing buoyant density \((n = 5, r^2 = 0.924, F = 36.3, p = 0.0092)\).

Table 1. Separation results for each of the two methods for different organisms. Check mark indicates successful separation between protists and bacteria. X mark indicates unsuccessful separation.

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Biochemical Analysis:

The biochemical fractionation method was primarily intended to compare differences in biochemical composition between protists and their prokaryote prey. However, the fractionation protocol was first tested on a diatom culture of *T. weissflogii*, and not separated from the prokaryotes in culture, nor fixed with formaldehyde prior to fractionation. This was done so that the results could be compared directly to literature values. The average percent $^{14}$C incorporation for *T. weissflogii* (n = 6) were: 54.6% ($\pm$ 1.5 SD) in proteins, 9.9% ($\pm$ 1.2 SD) in Polysaccharide – nucleic acid fraction (P-NA), 21.8% ($\pm$ 0.8 SD) in lipids, and 13.7% ($\pm$ 1.4 SD) in low molecular weight (LMW) compounds. Assuming isotopic equilibrium, these values reflect the relative contribution of each of the fractions to total carbon content.

Formaldehyde fixation resulted primarily in the loss of $^{14}$C from the low molecular weight fraction (ca. 90% of the radiolabel), while the other fractions were only slightly affected (Fig. 21). For this reason, LMW fractions were ignored for all subsequent analyses and total DPM for percent biomass calculations were made based on the "macromolecular" pool only (operationally defined, and also including low molecular lipids). Sucrose separations were also attempted using unfixed *D. papillatum* samples. Separation with unfixed samples was not as clean as fixed samples, and 70% of the total *D. papillatum* cells in all fractions had burst, or the nucleus was no longer intact. Therefore, all subsequent fractionations of *D. papillatum* and prokaryotes were based on formaldehyde-fixed samples.
**Fig. 21.** Comparison of non-fixed and fixed samples of *Thalassiosira weissflogii* biochemical fractions. Fixation dramatically lowered the percentage of low molecular weight (LMW) compounds, but largely maintained the labeling patterns in the macromolecular and lipid fractions. "P-NAs" stands for polysaccharides-nucleic acids.

Biochemical fractionation was performed on 4 fractions representative of the *D. papillatum* peak, and on 4 fractions representing maximum prokaryote abundances. A 2-way ANOVA (trial number and organisms as explanatory variables) revealed that the relative biochemical content (Table 2) was significantly different between experimental trials and between organisms (*D. papillatum* and prokaryotes) for proteins and lipids. P-NAs were significantly different between experimental runs, but not between organisms. (Protein: n = 32, r² = 0.832, experimental runs: F = 31.75, p <0.0001, organisms: F = 18.14, p = 0.0003) (P-NA: n = 32, r² = 0.811, experimental runs: F = 32.29, p <0.0001, organisms: F = 2.82, p = 0.106) (lipid: n = 32, r² = 0.92, experimental runs: F = 7.04, p = 0.0015, organisms: F = 236.99, p <0.0001). Protein fractions in prokaryotes were on average about 14% higher than the protein fractions in protists, and prokaryotes’ lipid fractions were on average about 64% lower than lipid fractions in protists. Figure 22 summarizes the differences between *D. papillatum* and prokaryotes with all trials pooled.
together. Differences in relative protein content were only significant when comparing within experimental trials. The differences were most apparent along the lipid axis (Fig. 22). Table 2 shows the data for each separation trial of *D. papillatum* and prokaryotes, as well as *T. weissflogii* and natural prokaryotes.

**Table 2.** Average percent composition and standard deviation of proteins, polysaccharides/nucleic acids, and lipids for *Diplonema papillatum*, *Thalassiosira weissflogii*, cultured bacteria, and Gulf Stream bacteria (n=4 for each row). Averages taken from data shown in Fig. 22.

<table>
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<th>Average % P-NA</th>
<th>Standard Deviation of P-NA</th>
<th>Average % Lipid</th>
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<tr>
<td>Bacteria trial 1</td>
<td>53.4</td>
<td>4.2</td>
<td>40.9</td>
<td>4.6</td>
<td>5.7</td>
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<td>20.6</td>
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<td>4.1</td>
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<td>Bacteria trial 3</td>
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<td>4.0</td>
<td>5.4</td>
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</tr>
<tr>
<td>Bacteria trial 4</td>
<td>62.3</td>
<td>5.3</td>
<td>32.5</td>
<td>5.3</td>
<td>5.2</td>
<td>0.4</td>
</tr>
<tr>
<td><em>T. weissflogii</em></td>
<td>52.3</td>
<td>1.7</td>
<td>17.3</td>
<td>1.3</td>
<td>30.3</td>
<td>0.7</td>
</tr>
<tr>
<td>Gulf Stream bacteria</td>
<td>56.1</td>
<td>6.3</td>
<td>26.8</td>
<td>4.3</td>
<td>17.0</td>
<td>2.8</td>
</tr>
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</table>
Fig. 22. Ternary plot showing biochemical differences between *Diplonema papillatum* (white circles), *Thalassiosira weissflogii* (white triangles), cultured bacteria (black circles), and natural bacteria (black squares). Differences in biochemical composition were most apparent along the lipid axis with the sequence: diatom > heterotrophic protist > culture bacteria. The bacteria from Gulf Stream water overlapped with the biochemical composition of *D. papillatum*. Proteins and polysaccharides-nucleic acids had a wide range of values because the data points from trial 2 were much different from the rest. See table 2 for pairwise comparisons within trials.
Overall, the sucrose density gradient centrifugation and subsequent biochemical fractionation were performed five times with *D. papillatum* cultures. An ANCOVA test was run on the biochemical data for prokaryotes on all 5 trials (Fig. 23). Table 3 shows the statistical results of the test. After testing for homogeneity of slopes, the interaction terms were all non-significant. The criterion for the analysis of covariance was thus satisfied, and a common regression slope was calculated (Underwood 1997). The effect of the covariate (i.e., fraction number / buoyant density) was significant for proteins, P-NA, and lipids (Table 3). There was a significant difference, or trend in percent $^{14}$C of proteins, P-NAs and lipids over consecutive fractions. Normality tests for each trial showed that residuals were not normally distributed, and p-values (indicated by asterisks) needed to be calculated based on customized F distributions of data randomized 10,000 times.
Fig. 23. ANCOVA regression plots for proteins, polysaccharides/nucleic acids, and lipids in cultured bacteria from 5 trials. No significant difference was found between trials for all three biochemical fractions, therefore common slopes were established.
Fig. 23 Continued. A significant trend was found in all three biochemical fractions (p-values in bold) along sucrose fractions. (Results of ANCOVA:
Proteins: n = 24, r² = 0.851, F(trial) = 17.1, p(trial) <0.0001, F(fraction) = 8.74, p(fraction) = 0.0104, F(interaction) = 0.78, p(interaction) = 0.5387.
P-NA: n = 24, r² = 0.848, F(trial) = 15.77, p(trial) <0.0001, F(fraction) = 12.59, p(fraction) = 0.0032, F(interaction) = 0.67, p(interaction) = 0.6118.
Lipids: n = 24, r² = 0.682, F(trial) = 3.71, p(trial) = 0.0293, F(fraction) = 9.7, p(fraction) = 0.0076, F(interaction) = 1.38, p(interaction) = 0.2892.)

Table 3. ANCOVA test results for biochemical trends in bacteria across sucrose gradient fractions from Diplonema papillatum cultures. Regression plots shown in Fig. 23. Asterisks indicate p-values that were calculated based on customized F distributions of data randomized 10,000 times.

<table>
<thead>
<tr>
<th></th>
<th>Protein Fractions</th>
<th>P-NA Fractions</th>
<th>Lipid Fractions</th>
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<td>n</td>
<td>24</td>
<td>24</td>
<td>24</td>
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<tr>
<td>R²</td>
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<td>F ratio (trial)</td>
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<td>15.77</td>
<td>3.71</td>
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<td>8.74</td>
<td>12.59</td>
<td>9.7</td>
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<td>F ratio (interaction)</td>
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<td>1.38</td>
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<td>p-value (trial)</td>
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<td>&lt;0.0001</td>
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<tr>
<td>p-value (fraction)</td>
<td>0.0104</td>
<td>0.0032</td>
<td>0.0076</td>
</tr>
<tr>
<td>p-value (interaction)</td>
<td>0.5387*</td>
<td>0.6118*</td>
<td>0.2892*</td>
</tr>
</tbody>
</table>

Concentrated Environmental Samples:
The Gulf Stream sample had a bacteria concentration of 2.04 x 10^6 cells mL⁻¹, with eukaryotic cell numbers below those required for use in any of the separation methods. Thus the focus was placed on the fractionation of subpopulations of bacteria. A sucrose separation was attempted with this sample, however bacteria abundance was too low to determine a clear bacterial peak from microscope counts. After TFF concentration, the final 2 mL of radiolabeled sample had 2.32 x 10^6 bacteria cells mL⁻¹ and 1.1 x 10^6 heterotrophic eukaryote cells mL⁻¹. The eukaryotic cells were very small, similar in size to C. roenbergensis, but their low abundance would likely result in a non-existent peak after dilution and separation in a sucrose gradient.
Concentrated Environmental Samples:

The Gulf Stream sample had an initial prokaryote concentration of $2.04 \times 10^5$ cells mL$^{-1}$, with eukaryotic cell numbers below those required for use in any of the separation methods. Thus the focus was placed on the fractionation of subpopulations within prokaryotes. A sucrose separation was attempted with this sample, however prokaryote abundance was too low to determine a clear prokaryote peak from microscope counts. After concentration with tangential flow filtration, the final 2 mL of radiolabeled sample had $2.32 \times 10^7$ prokaryote cells mL$^{-1}$ and $1.1 \times 10^4$ heterotrophic eukaryote cells mL$^{-1}$. The eukaryotic cells were very small, similar in size to *C. roenbergensis*, and not sufficiently abundant to be separated in the sucrose gradient. The prokaryote cells, on the other hand, were two orders of magnitude more concentrated and similar to culture abundances.

Sucrose separation of the Gulf Stream sample after a one order of magnitude increase in prokaryote cell concentration resulted in the usual surface layer accumulation of cells, followed by a clear prokaryote peak spanning eight fractions (Fig. 24). Sucrose separation of the final concentrated radiolabeled sample displayed a prokaryote peak spanning only 4 fractions due to higher volume fractions (Fig. 25). Some eukaryotic cells were found in lower fractions, but not abundant enough to register a signal from the scintillation counter after biochemical fractionation. The four prokaryote peak fractions appeared to have a trend in percent $^{14}$C of protein just like the trend from prokaryotes in culture, but with such a small sample size, a linear regression test confirmed a trend did not exist (p-value of 0.0505) (P-NA, lipid, fractions were also insignificant with a p-value
>0.05). However, when regressed with existing biochemical regressions for cultured prokaryotes (Fig. 26), the six combined trials exhibited a significant trend for protein and P-NA fractions with even lower p-values than given above for five trials (Table 3). Again testing for homogeneity of slopes, the interaction terms were all non-significant, and a common regression slope was applied (Underwood 1997). Lipid data for the Gulf Stream prokaryotes was offset most from the five culture trials. Results for protein and P-NA data are as follows:

Protein: n = 28, r² = 0.863, F(fraction) = 14.7, F(interaction) = 1.73, p(fraction) = 0.0014, p(interaction) = 0.1838).P-NA: n = 28, r² = 0.847, F(fraction) = 17.1, F(interaction) = 0.73, p(fraction) = 0.0008, p(interaction) = 0.6129). Trends in percent DPM of lipids did not exist with all p-values >0.05. The biochemical results from the Gulf Stream sample are summarized in Table 2 and compared to both prokaryotes and eukaryotes in culture.

![Fig. 24. Sucrose gradient separation of Gulf Stream prokaryotes after the sample concentration using tangential flow filtration (one order of magnitude increase). The prokaryote peak was found at fraction 19.](image-url)
Fig. 25. Sucrose gradient separation of Gulf Stream A) prokaryotes and B) eukaryotes after concentration by tangential flow filtration, radiolabeled incubation, and additional concentration after incubation (two orders of magnitude increase in total). Eukaryotes were still low in abundance, but some were found in lower fractions. A prokaryote peak was found in fraction 8.
Fig. 26. ANCOVA regression plots for proteins, polysaccharides/nucleic acids, and lipids in cultured bacteria from 5 trials and in Gulf Stream prokaryotes combined. No significant difference was found in the interaction terms for all three biochemical fractions, therefore common slopes were established.
Fig. 26 Continued. A significant trend was found along sucrose fraction (p-values in bold) in proteins and P-NA, but not in lipids. (ANCOVA results: Proteins: n = 28, r² = 0.863, F(trial) = 15.6, p(trial) < 0.0001, F(fraction) = 14.7, \textbf{p(fraction)} = 0.0014, F(interaction) = 1.73, p(interaction) = 0.1838.
P-NA: n = 28, r² = 0.847, F(trial) = 13.63, p(trial) < 0.0001, F(fraction) = 17.1, \textbf{p(fraction)} = 0.0008, F(interaction) = 0.73, p(interaction) = 0.6129.
Lipids: n = 28, r² = 0.950, F(trial) = 59.65, p(trial) < 0.0001, F(fraction) = 1.17, \textbf{p(fraction)} = 0.2948, F(interaction) = 0.98, p(interaction) = 0.462.)

Cell Size Analysis:
The average prokaryote cell size from the \textit{D. papillatum} culture (3 µm filtrate) decreased with decreasing sucrose density, or with increasing fraction number (Fig. 27). Fraction 1 represented the bottom of the tube. A linear regression indicated a significant difference in prokaryote cell size over the density gradient fractions (n = 14, r² = 0.753, F = 36.7, p = 0.0001). Prokaryotes from the \textit{N. designis} culture separated again with a sucrose gradient showed the largest average prokaryote cell size in the middle of the density gradient at fraction 6 (Fig. 28). The bottom of the tube and the top of the tube both had smaller average cell size. Piecewise regressions were significant on fractions 1-6 (n = 6, r² = 0.750, F = 12.1, p = 0.0255) showing an increasing trend, and on fractions 6-11 (n = 6, r² = 0.774, F = 13.7, p = 0.0209) with a decreasing trend in cell size.
Prokaryotes from a \textit{D. papillatum} culture that were separated using the FFF method were also analyzed for trends in cell size (Fig. 29). A linear regression test gave a p-value of 0.7072 (n =18, r² =0.009, F=0.146) indicating that there was no significant difference in prokaryote cell size over consecutive FFF fractions.
Fig. 27. Bacteria cell size regression from a sucrose gradient separation of a Diplonema papillatum culture across fraction number and buoyant density. Cell size decreased with increasing fraction number and decreasing buoyant density.

(Linear regression: $y = 0.538 - 0.022x$; $n = 14$, $r^2 = 0.753$, $F = 36.7$, $p = 0.0001$)
Fig. 28. Bacteria cell size from a sucrose gradient separation of a *Neobodo designis* culture. The highest cell size appeared in the middle of the gradient at fraction 6. No trend in size was evident across all fractions, but piecewise regressions showed a significant trend in the lower fractions, and a separate trend in the upper fractions.

Bottom fractions 1 - 6: (n = 6, r² = 0.750, F = 12.1, p = 0.0255) Top fractions 6 - 11: (n = 6, r² = 0.774, F = 13.7, p = 0.0209)
Fig. 29. Bacteria cell size from a Field-flow fractionation of a *Diplonema papillatum* culture. No trend in cell size was evident across fractions. ($n = 18$, $r^2 = 0.009$, $p = 0.7072$).

The Lafayette River sucrose separation (from Fig. 20) was also analyzed for cell size gradients. Relative abundances were low, but there was a trend of increasing cell size with increasing buoyant density for both prokaryotes and eukaryotes. Prokaryote fractions: ($n = 8$, $r^2 = 0.799$, $F = 23.8$, $p = 0.0028$). A positive trend of cell size with buoyant density was present in the lower fractions of the eukaryotes: ($n = 5$, $r^2 = 0.924$, $F = 36.3$, $p = 0.0092$), but not significant in the top fractions that remained trapped in the surface layer: ($n = 4$, $r^2 = 0.842$, $F = 10.7$, $p = 0.0824$) (Fig. 20). It was unknown whether the eukaryotic cells in the higher fractions were partitioned among their proper, respective densities or were a result of surface retention.
The Gulf Stream sucrose separation (from Fig. 24) prior to radiolabeling was analyzed for cell size gradients for comparison. A trend in prokaryote cell size was clearly visible (Fig. 30). Cell size decreased as buoyant density decreased (with increasing fraction number). A linear regression test confirmed that a significant positive trend was present in Gulf Stream prokaryote cell size over sequential sucrose gradient fractions \((n = 8, r^2 = 0.799, F = 23.8, p = 0.0028)\). Finally, pairwise ANCOVA tests were used to compare prokaryote sizes among the Lafayette River sample, Gulf Stream sample, and two \(D. papillatum\) culture samples (3 µm filtered and non-filtered, Fig. 31). The regression slope of filtered, cultured bacteria cell sizes with density were found to be significantly different from Lafayette River prokaryotes (ANCOVA test of homogeneity of slopes, \(n = 26, r^2 = 0.901, F(\text{interaction}) = 27.8, p(\text{interaction}) < 0.0001\)). The slope of filtered cultured bacteria cell sizes with density were also significantly different from Gulf Stream prokaryotes (ANCOVA test of homogeneity of slopes \(n = 22, r^2 = 0.828, F(\text{interaction}) = 7.7, p(\text{interaction}) = 0.0125\)). However, the slopes of the Lafayette River prokaryotes and Gulf Stream prokaryotes were not significantly different (ANCOVA homogeneity of slopes: \(n = 20, r^2 = 0.909, F = 0.04, p = 0.8498\)). There was a slight difference in elevation of the regression between the two environmental samples \((n=20, r^2 = 0.909, F = 6.1, p = 0.0251)\). The slopes of filtered and non-filtered cultured bacteria were not significantly different (ANCOVA homogeneity of slopes: \(n = 33, r^2 = 0.722, F = 0.45, p = 0.506\)). There was a slight difference in elevation of the regression between the two cultured samples \((n = 33, r^2 = 0.909, F = 7.85, p = 0.009)\).
Fig. 30. Bacteria cell size regression from a sucrose gradient separation of a concentrated Gulf Stream sample. Cell size decreased with increasing fraction number and decreasing buoyant density. (Linear regression: $y = 1.106 - 0.038x$; $n = 8$, $r^2 = 0.799$, $F = 23.8$, $p = 0.0028$)
Fig. 31. Comparison of prokaryote cell size from a concentrated Gulf Stream sample, a Lafayette River sample, a 3 µm filtered Diplonema papillatum culture, and a non-filtered D. papillatum culture. Pairwise ANCOVA tests (homogeneity of slopes) revealed that cultured bacteria (3 µm filtered) cell sizes were significantly different from Lafayette River bacteria (n = 26, r² = 0.901, F(interaction) = 27.8, p(interaction) < 0.0001), and from Gulf Stream bacteria (n = 22, r² = 0.828, F(interaction) = 7.7, p(interaction) = 0.0125). However, the slope of the Lafayette River bacteria was to the same as the slope in Gulf Stream bacteria but the elevations were slightly different. Homogeneity of slopes: (n = 20, r² = 0.909, F = 0.04, p = 0.8498) Difference in elevation: (n = 20, r² = 0.909, F = 6.1, p = 0.0251). Filtered and non-filtered cultured bacteria cell sizes were similar in slope. Homogeneity of slopes: (n = 33, r² = 0.722, F = 0.45, p = 0.506). Difference in elevation: (n = 33, r² = 0.909, F = 7.85, p = 0.009).
DISCUSSION

A comparison of success rates in separating different particle types allows us to better explore the limits and underlying mechanisms of the two different separation techniques. Cell numbers as found in cultures of *D. papillatum* were ideal for separation while cell numbers in the other cultures of heterotrophic nanoflagellates were too low for effective separation and any further biochemical fractionation. These cells would therefore have to be concentrated before processing. The relative abundance of prokaryotes in the *D. papillatum* culture also remained high and did not appear to be as affected by grazing as exhibited with the other protists. *D. papillatum* cells had the greatest difference in cell size from their prokaryotic prey, leading to less overlap in separation peaks in both methods. *E. coli* cells were successfully separated with the sucrose method, but not with the FFF method. This is surprising because the minicells and mother cells were thought to have the same chemical composition and therefore density, only differing in cell size. A priori, this would make FFF the better method to separate minicells from mother cells because it separates by cell size and density, whereas the sucrose method was assumed to primarily separate by density. However, the FFF method used here (with a field strength of only 1 g) seemed to be less successful overall than the sucrose method.

There are two mechanisms at work in density gradient separations that can be categorized as rate-zonal and isopycnic (Frei 2011). Rate-zonal separation depends on mass and size of the particles, but the density of the denser particle must be greater than the densest layer of the gradient (Frei 2011). Particles with larger mass move through the
density gradient more quickly than those of smaller mass. Eventually, all particles pellet if centrifuged long enough. In contrast, isopycnic separation by definition depends solely on density, and the density of the particle must fall in between density values of the gradient (Frei 2011).

The rate at which the particles descended through the sucrose gradient was governed by Stokes’ law \( V = \frac{(2r^2(\rho_p - \rho_f)g)}{9\mu} \) (Shearer & Hudson 2000), where \( V \) is the sinking velocity, \( r \) is the radius of the particle, \( \rho_p \) is the density of the particle, \( \rho_f \) is the density of the fluid, \( g \) is gravity, and \( \mu \) is the viscosity of the fluid. This equation shows that velocity is linearly proportional to the area of the particle. The observed linearity between cell area and fraction number (Figs. 27 and 30) is thus indicative of a strong rate-zonal effect during separation in these experiments. The influence of this effect was likely what allowed for separation of prokaryotes with subtle differences in size along consecutive fractions. The equation also tells us that velocity decreases when particles get closer to its respective buoyant density value, further enabling subtle differences between fractions. Therefore, while the buoyant density estimates were close to literature values, the actual buoyant density of the particles was probably slightly lower than those shown in the figures.

These gradient classifications are very loosely defined in that the classification is not determined solely by the type of gradient media. Sucrose gradients can be either rate-zonal or isopycnic, depending on the range of the gradient and the type of particles being separated (Dey et al. 1997). Our sucrose separations exhibited pelleting of protists when a
weaker gradient of up to 35% sucrose was used, but pelleting did not occur with the use of a stronger gradient up to 50% sucrose. Dey et al. (1997) suggested that sucrose might not act as an isopycnic gradient when the gradient range exceeds 40% because the high viscosity of the medium beyond 40% often inhibits particles from reaching their corresponding buoyant densities. Our *D. papillatum* cells were found near the boundary of the 30% layer and 35% layer. Therefore, it is inconclusive whether the *D. papillatum* cells were found at their respective buoyant density value, or if they were prevented from reaching their respective value due to the high viscosity.

Particle size was clearly a contributing factor due to our *E. coli* separation results, as well as the gradient in cell size found within prokaryote peak fractions. Given that the separation mechanisms do not occur in isolation, our sucrose separations were likely a combination of both types (isopycnic and rate-zonal), which increased the efficiency of separation of particles that are very similar in density and size. It is also possible that the *E. coli* minicells and mother cells had different densities because of different proportional contributions of cell wall to cell plasma constituents. The buoyant density of wild-type, non-minicell forming *E. coli* cells changes during exponential growth and stationary phase changes (Makinoshima et al. 2002), which shows that different densities are possible even within the same clone.

The majority of density gradient separations of microbes reported in the literature utilized Percoll media for gradient formation (Inoue et al. 2007, Nishino et al. 2003, Makinoshima et al. 2002, Starink et al. 1994), though we had better success with a
sucrose gradient. Percoll separations reported were strictly isopycnic without any influence of particle size (Starink et al. 1994). Gradients of buoyant density of particles across particle group peaks may in some cases coincide with a size gradient, but to our knowledge nobody has identified size gradients of particle groups using Percoll media. Some physiological and phylogenetic differences within prokaryote fractions have been identified using a Percoll gradient, but not cell size (Inoue et al. 2007, Nishino et al. 2003). Sucrose separations, as reported in our results, may be favorable over Percoll in some cases where particle size is a considerable factor. The use of cesium chloride for density gradient separations has been successfully used for decades, especially for cell constituents (Meselson et al. 1957, Schluederberg and Roizman 1962, Breedis et al. 1962) and later for stable isotope probing (Lueders et al. 2004), but gradient formation with this media requires high-speed ultra centrifugation for as long as 2-3 days (Karp 2005). Cesium chloride is capable of forming gradients of a much higher range up to density values of 1.9 g cm\(^{-3}\), but also exerts more osmotic pressure on cells than sucrose or Percoll (Dey et al. 1997).

A complicating issue with the sucrose method was the surface retention of particles at the top of the gradient. This phenomenon decreased the number of cells available for accumulation within respective buoyant density values. It prevented separation altogether for \textit{N. designis} and \textit{C. roenbergensis} cultures because they apparently had relatively low density. Had they been concentrated with tangential flow filtration prior to separation (as performed with natural particles in later experiments), the separation may have been more successful. The lack of success using surfactants and higher centrifugation speeds
may be an indication that surface tension may not be the primary cause of the issue. One possibility is that the surface cells could have been dead prior to fixation, leading to density values similar to those of the top layers of the gradient. If this were the case, then the separation of live cells from dead cells would be another strength of the sucrose method. The use of viability stains on the cells could be used in future experiments to test this hypothesis.

With the FFF method, the *C. roenbergensis* peak lined up exactly with the prokaryote peak, and the *N. designis* peak was only offset by one fraction from the prokaryote peak and could not be considered separated. Both methods were successful in separating *D. papillatum* from prokaryotes. The peak of *D. papillatum* cells in FFF separations was very broad, leading to many usable clean fractions. The volume per fraction was very small (0.25 mL), leading to a finely resolved gradient of fractions. The sucrose gradient method was a much simpler method overall, required a minimum amount of equipment, did not contaminate any equipment with radiolabel except for the centrifuge tube, and was just as successful in separation. The *D. papillatum* peak was more narrow in the sucrose gradient separation due to larger fraction volumes of 0.5 mL (or 2 mL with the high volume protocol). Thus there were fewer clean fractions to work with, but the location of the *D. papillatum* peak was consistent and reproducible. The only variable seemed to be the size of the hole produced for fraction collection, which could be easily standardized in the future. The diameter of the sewing needle tapered to a point, leading to different size holes between trials and therefore slight differences in drop sizes. This variable was accounted for by ensuring accurate drop counts, and
dividing the initial volume of the entire sucrose gradient and sample by the number of 
fractions collected to get an adjusted fraction volume. The expected location of the *D. 
papillatum* peak based on previous trials was then inferred by the adjusted fraction 
volumes. The consistency of peak locations supported the buoyant density estimates 
given in the Results. Buoyant density of an organism is important when considering both 
advective transport and vertical movement in combat with gravity (Harvey et al. 1997).

The use of a biological preservative such as formaldehyde in the physical 
separation of biological particles has both positive and negative consequences. It 
effectively kills the particles of interest, which allows for consistent separations by 
preventing the movement of the cells between gradients or within the channel. It also 
preserves and hardens the cell tissue, preventing the possibility of decomposition or cell 
body disintegration (Thavarajah et al. 2012). A side effect of formaldehyde use is that it 
slightly alters the chemical composition of the cells that it comes in contact with. It 
results in cross-linking proteins by creating methylene bridges between them (Thavarajah 
et al. 2012). As indicated by our results, this cross-linking did not lower the total protein 
content, but we have shown that radioactivity levels were lower in LMW fractions due to 
effects from formaldehyde fixation. These lower levels in turn slightly affected protein, 
P-NA and lipid percent biomass calculations by lowering the total combined DPM 
readings.

The potential osmotic effect from sucrose solutions on biological cells is also worth 
considering. Sucrose is highly osmotic in that water tends to diffuse from low solute
concentration (within the cells) to high solute concentration (the surrounding sucrose solution) (Starink et al. 1994). This effect is even stronger in solutions of higher sucrose concentration. The more dense layers of the sucrose gradient where protist cells were present likely experienced more cell dehydration than those present in less dense layers. This could have led to slightly incorrect buoyant density estimates for the protists. Despite these potential problems, however, the buoyant density estimates for the prokaryote peak were very close to literature values obtained with the use of a Percoll density gradient method and density marker beads (Inoue et al. 2007).

To determine whether the values from biochemical fractionation can be compared to protocols in other labs, the protocol was first tested on a culture of *T. weissflogii* radiolabeled with $^{14}$C bicarbonate. According to Roman (1991), $^{14}$C incorporation into biochemical fractions of *T. weissflogii* was as follows: 56% proteins, 8.6% P-NA, 21.2% lipids, and 14.4% LMW compounds. These values were within 1.4% of the values reported here for each of the four fractions. The biochemical fractionation protocol was thus reliable and reproducible. As expected, formaldehyde fixation changed the percentage biochemical makeup, especially because of losses in the low molecular weight fraction. The percentage values were thus recalculated for the three other fractions alone (protein, polysaccharide-nucleic acid, and lipids). Figure 22 shows the biochemical signature of fixed *T. weissflogii* alongside *D. papillatum*, as well as natural and cultured prokaryotes.

Grover and Chrzanowski (2006) reiterated the technical difficulties of separating
heterotrophic flagellates from prokaryotes, which has led to a lack of data on the elemental composition of these flagellates. This lack of data affects our understanding of the ecosystem functions of these flagellates. Grover and Chrzanowski (2006) suggested that another difficulty lies in the possibility of undigested prokaryotes within the food vacuoles of flagellates to significantly change the chemical composition of the predators. One potential solution to this problem could be to investigate the biochemistry of starved cultures of *D. papillatum* for comparison. But at the same time, starvation may cause more differences in biochemical composition than just the lack of undigested prokaryotes within food vacuoles. Regardless, our *D. papillatum* cells were found to be significantly different biochemically from their prey cells. Figure 22 shows the differences between them with all trials pooled together. The organisms were especially different in relative lipid content which may be a reflection of differences in membrane structures and the presence or absence of organelles. Protein content was also significantly different but only when the comparison was made within trials (Table 3).

Models of nutrient processing by heterotrophic consumers often assume homeostasis, or strictly regulated stoichiometry, for simplicity (Sterner & Elser 2002). However, Grover and Chrzanowski (2006) reported that heterotrophic flagellates did not remain homeostatic in composition when fed multiple prey differing in stoichiometry. Feeding may be selective under optimum growth conditions to prey with higher nutrient content thus enhancing fitness. In our case, multiple prey groups were not introduced and biochemical composition remained relatively constant throughout each of the eukaryotic peak fractions.
In contrast, the biochemical composition of prokaryotes varied among fractions. A variable biochemical composition is not necessarily due to differences in species, but may be the result of subpopulations at different stages of their growth cycle. Massana et al. (1997) determined that cultures containing little to no phylogenetic prokaryote diversity still have a diversity of prokaryote sizes due to differences in growth rates, activity, and grazing pressure. Marine prokaryotes have previously been successfully separated using a Percoll gradient (Amersham Biosciences) into different buoyant density subpopulations based on physiological state differences (Nishino et al. 2003) and phylogenetic differences (Inoue et al. 2007). Differences in growth phases and external stress factors such as temperature both contributed to differences in the physiological state of these bacteria. Changes in the physiological state involve changes in cellular components, which in turn lead to a change in buoyant density (Inoue et al. 2007). Nishino et al. (2003) used the separated subpopulations of bacteria to test the culturability of bacteria in different physiological states. Inoue et al. (2007) were the first to separate natural samples of prokaryotes rather than cultured samples. They concentrated natural samples using ultrafiltration, separated the sample into three large fractions, and identified phylogenetic differences in bacteria using fluorescent in situ hybridization. In contrast, the results presented here reveal differences in prokaryote distribution based on cell size and biochemical components. All biochemical fractions, excluding LMWs (due to losses from fixation), showed significant trends across consecutive density gradient fractions (see Table 3).
Simon and Azam (1989) reported that protein to carbon ratios tend to be nearly constant in planktonic marine prokaryotes, but protein to cell volume ratio tends to increase with decreasing cell size. Thus, larger prokaryote cells may not represent higher nutritional content, but simply contain more water (Simon & Azam 1989, Chrzanowski et al. 2010). In short, this would mean that smaller prokaryotes are denser. The present study shows, in contrast, that the larger the size, the greater the buoyant density, and the higher the percent protein in both cultures and environmental samples (Fig. 31). In addition, percent P-NA decreased and percent lipid increased with increasing buoyant density in prokaryotes from cultures and environmental samples. In the present study, prokaryote protein levels were inferred from levels of $^{14}$C incorporated into each biochemical fraction, while Simon and Azam (1989) determined prokaryote protein based on $^3$H-leucine incorporation and cell volume calculations using photomicrographs and a digitizer. Even taking these methodological differences into account, our results seem to contradict the findings in Simon and Azam (1989).

The regressions of prokaryote cell size versus density between cultures and environmental samples show that the two environmental samples from different sources were very similar to each other, yet clearly different from culture samples (Fig. 31). The two environmental samples collapse on top of each other with identical slopes and nearly identical intercepts (Fig. 31). The sample groups for the two environmental samples were relatively small, but they suggest that the environmental prokaryotes were slightly larger than the cultured prokaryotes. The four Lafayette River prokaryote data points that were larger than 0.6 $\mu$m$^2$ had a much smaller sample size because they were below the
prokaryote peak fractions, but still above the eukaryote peak fractions. It is possible that very small picoeukaryotes might have been mistaken for large prokaryotes in these particular counts. Filtration through a 3 µm filter did not change the relationship between cell size of the cultured prokaryotes very much so that clumping of cultured prokaryotes can be excluded to account for the differences between the environmental and field samples.

A trend in environmental eukaryote cell size was also found along consecutive density gradient fractions in the lower fractions, but they were only significant in those particle populations that made it deeper into the sucrose gradient and did not remain trapped at the surface (Fig. 20). Just like with prokaryotes, eukaryote cell sizes increased with increasing buoyant density. Assuming contribution of rate zonal separation, larger particles with larger overall mass (eukaryotes) would move faster through the viscous environment than lighter prokaryotes, consistent with Stokes’ law. Perhaps if centrifugation was performed for a longer amount of time, more eukaryotic cells could have traveled to higher density fractions. Some of the largest cells were undoubtedly dinoflagellates as the original samples contained both Gymnodinium and Amphidinium species (Katherine C. Filippino, Old Dominion University, Norfolk, Va, pers. comm.). Smaller diatoms (identified as pill-box shaped autotrophs, e.g. Euk B and D in Fig. 14) were found in slightly higher (less dense) fractions, as well as some smaller unidentified protists. All these findings suggest that rate zonal separation increased the efficiency of separation of various cell sizes.
During a significant part of the life cycle in natural environments, heterotrophic prokaryotes encounter starvation and deprivation of nutrients. Increases in nutrient availability trigger the transition from this starved stationary phase into rapid growth (Kolter et al. 1993). Our laboratory cultures were characterized by an abundance of nutrients leading to higher abundances of prokaryotes than those found in a natural setting. This means that there is likely a higher diversity of different growth phases at any one time among the prokaryotes present in cultures. Additionally, different types of proteins are synthesized by prokaryotes during different growth phases (Kolter et al. 1993). Thus, phylogenetic differences and changes in growth phase can both lead to differences in the buoyant density of prokaryotes. The radiolabeled and concentrated Gulf Stream water was not introduced to any nutrients upon incubation to maintain natural conditions. The expectation was that the increase in prokaryotes per volume would lead to higher grazing rates by any present eukaryotes. Instead, the eukaryote population was extremely scarce to begin with, and did not increase much even after concentration and incubation probably due to the strain of cross-flow filtration on the more fragile protist cells.
CONCLUSION

The success of the sucrose separation method shows that separation is consistent over many different replicate trials and sample characteristics. It requires much less mechanical equipment, effort and cost than any other separation method including FFF and Percoll gradient centrifugation. The rate zonal effect can be exploited in addition to the density gradient to increase the spread of various cell sizes among fractions. Tangential flow filtration can be applied to increase cell concentrations to perform downstream biochemical separation of $^{14}$C labeled cells. Field-flow fractionation was surprisingly effective considering that only a gravitational field of 1 was used in a relatively short channel (30.5 cm). Any additional increase in field strength, whether it is through laminar crossflow or centrifugation, would increase the effectiveness of this type of separation. However, more complicated FFF instrumentation becomes expensive very quickly. The biggest challenge in analyzing eukaryotic microbes is the minimum amount of material required for most types of biochemical and stoichiometric analyses. Radiolabeling of cell compounds is likely the most sensitive method but it has its own drawbacks such as a lack of specificity. Future work should thus focus on how to (selectively) enrich environmental samples or low density culture samples without damaging the more fragile protists. If this goal is achieved, simple sucrose gradient centrifugation as demonstrated can effectively separate eukaryotic microbes from prokaryotes, and divide eukaryotes into subpopulations based on cell sizes.
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APPENDIX

Preparations of vitamin solution and enrichment solution for f/2 medium from Guillard and Ryther (1962).

**Vitamin solution:**
Thiamine: 0.1 g
Vitamin B12: 2.0 mg
Biotin: 1.0 mg
Distilled water: 1.0 L

**Enrichment solution:**
EDTA . 2H₂O: 0.553 g
NaNO₃: 4.667 g
Na₂SiO₃ . 9H₂O: 3.000 g
Sodium glycerophosphate: 0.667 g
H₃BO₃: 0.380 g
Fe(NH₄)₂(SO₄)₂ . 6H₂O: 0.234 g
FeCl₃ . 6H₂O: 0.016 g
MnSO₄ . 4H₂O: 0.054 g
ZnSO₄ . 7H₂O: 7.3 mg
CoSO₄ . 7H₂O: 1.6 mg
Distilled water: 1.0 L
VITA

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