Nutrient Stoichiometry and Cellular Allocation of *Micromonas* Strain RCC299

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ABSTRACT

The flux of nutrients throughout the biosphere is one of the main drivers of global biogeochemical cycling. Some important drivers of these global biogeochemical cycles lie within the picoeukaryotes that live within the surface regions of our oceans (Arrigo 2005). Although the Redfield ratio is a standard assumption for the elemental composition of phytoplankton cells use, research has shown that the ratio between C:N:P can vary greatly between different taxa of phytoplankton (Arrigo 2005). The variability in elemental composition and stoichiometry between different phytoplankton and how they use those elements towards important macromolecules within the cell is poorly understood. The focus of this project is to better understand and provide empirical data for the plasticity in cellular quotas of *Micromonas* strain RCC299, as well as to cellular allocate the amount of protein and pigment content within the cells when they are depleted of important inorganic nutrients such as Nitrate and Phosphate.

INTRODUCTION

The flux of important elements such as Carbon, Phosphorus, and Nitrogen is what drives biogeochemical cycling throughout our biosphere (Diel et al. 2005). One of the most important drivers of biogeochemical cycling includes the utilization of these basic elements in the form of inorganic nutrients used by phytoplankton (Arrigo 2005). Phytoplankton are small microscopic organisms that live within the surface regions of our oceans worldwide that can be thought of as small microscopic plants (Arrigo 2005). In 1934, Alfred Redfield discovered that throughout the world's oceans, the C, N, and P content in all regions were very similar with the sea water having a C:N:P ratio of 105:15:1, and the stoichiometric ratio of phytoplankton cells to be 106:16:1 (Redfield 1934). However, research now encourages that there can be variability between different taxa of plankton for their stoichiometric ratios. Very little research is done on the nutrient stoichiometry of specific picoeukaryotic species. There is also very little empirical data for the flexibility and plasticity of cellular quotas of phytoplankton. A well-established and interesting ecological theory in Arrigo, 2005 suggested that phytoplankton can have either high N:P ratios or low N:P ratios for being either a survivalist, bloomer, or a generalist. For example, a bloomer would have a low N:P ratio because it is utilizing most of the phosphorus and putting it towards important macromolecules such as RNA and DNA to build proteins, to build more cells quickly. A survivalist would have a high N:P ratio because it is utilizing a lot of Nitrogen and putting it towards pigments and proteins within the cell. This raises the question of whether or not *Micromonas* RCC299 could be plastic throughout the duration of the growth curve. For example, the cells could be bloomers during the exponential phase and survivalists during the stationary phase. A better understanding of the nutrient stoichiometry as well as the elemental ratios for each of these taxa of phytoplankton will help shed light on a better understanding of global biogeochemical cycling, and can therefore lead to more accurate models and predictions for global climate change. Specifically, this study is analyzing the genus *Micromonas* strain RCC299 and analyzing the nutrient stoichiometry as well as the amount of protein and pigmentation within the cell under specific limiting factors within their media. During this internship, I wanted to gain a better understanding of the elemental ratios of the Nitrogen and Phosphorus within *Micromonas* strain RCC299, as well as understand

how much of the Nitrogen and Phosphorus are being used towards important macromolecules within the cell such proteins, and pigments within chlorophyll a, which is a major component of the photosynthetic machinery of the cell.

MATERIALS AND METHODS

Dilution Experiment

In addition to determining the concentration gradient for the phosphorus and nitrogen limiting medias, the most minimum cell density that we could inoculate the cells to for the beginning of the experiment needed to be determined. the most minimum cell density was determined by doing a dilution experiment with four different dilutions and two replicates for each dilution. The experiment was performed in order to determine the most minimum cell abundance we could inoculate. The dilutions for the dilution experiment were $5x10^5$, $2.5x10^5$, $1.25x10^5$, and $6.0x10^4$ cells/mL. These cells were then grown out so we could determine whether or not the lowest concentration of cells inoculated would survive the duration of entire cell growth curve. The results show that the cells were able to survive the lowest concentration of cells, so we did not repeat the experiment to test for a lower concentration since we thought that this would be a viable low concentration to use for a large volume grow-out experiment since time we had a time constraint with this being a summer internship.

Axenic culturing of *Micromonas* strain RCC299

Determining the right concentration of Phosphorus and Nitrogen to use in L1 media was determined by inoculating three different sets of cultures of both Nitrogen and Phosphorus with two duplicates of axenic RCC299 for each concentration. The concentration gradients for phosphorus used were $1.8x10^{-6}$, $3.6x10^{-6}$, and $5.4\ x10^{-6}$ M. The replete Phosphorus concentration in the L1 media is $3.62\ x10^{-5}$ M. For Nitrogen, the concentration gradients were $44.1\ x10^{-6}$, $88.2\ x10^{-6}$, and $132.3\ x10^{-6}$ M. The replete Nitrogen concentration for L1 media is $882\ x10^{-6}$ M.

RCC299 was transferred everyday for a minimum of four days in a culture flask with a total volume of 15mL, and a cell volume of $1x10^6$ cells/mL prior to analyzing and growing out the cultures to get a full cell growth-curve. Before growing out the cells for a full growth curve, the cells were diluted down to 150,000 cells/mL and grew until the cells reached stationary phase and did not increase in cell abundance anymore. It was determined that the cells have reached maximum abundance when the growth rate dropped to or below zero. The diluted concentration gradients that were chosen were based on which cells gave the most meaningful and controlled growth curve, as well as which group of cells did not run into carbon limitation. For example, the lowest concentration gradient that was used for both Nitrogen and Phosphorus caused the cells to die relatively quickly with regards to the other samples, so analysis of this growth curve wouldn't yield as much information about the cells response to the elemental environment. After analyzing the growth curves (see figures 1a, b in results), it was determined that the second concentration for both Phosphorus and Nitrogen would be used for the actual experimentation of the nutrient depleted RCC299.

Nutrient Analysis

In order to analyze samples for dissolved inorganic nutrients and for particulate organic phosphorus, carbon, and nitrogen, a vacuum filtration device was set up. Samples for dissolved inorganic nutrients, Particulate organic carbon, nitrogen, and phosphorus were all collected at different time points throughout the duration of the grow-out for RCC299. These time points were the lag phage (beginning of the inoculation), mid exponential phase (middle of the exponential growth), and the second day after the cells reached stationary phase. It was determined that these cells reached stationary phase when the growth rate of the cells reached either zero or below zero. All dissolved organic and inorganic nutrient samples were collected within the same hour using a filtration device consisting of an erlen meyer-flask, vacuum hose and pump, pre-combusted glass fiber filter, filter collection glassware and stopper, clamp, and a glass vial to pour the sample through. 50 mL of the culture were poured through the glass vial, and the vacuum

pump was turned on to draw the filtrate through. A piece of pre-combusted aluminum foil was used to carefully wrap the folded filter. The sample was then stored in the freezer at -20 °C. For the dissolved inorganic nutrients, 30 mL of the filtrate was sampled from the bottom of the erylyn meyer-flask after the filter was collected and stored. The dissolved inorganic nutrient samples were then collected -and placed in a 50 mL conical tube which was also labeled appropriately and stored in the freezer at -20°C. When the cell density became too large for the culture to properly be filtered, like during stationary phase, a lower amount of volume was then used. For the stationary phase for the replete cells, 30 mL was used to collect the PON/C samples, and the same amount of filtrate for the DIN was collected as well.

POP (particulate organic phosphorus) samples were collected by using a microbiology grade polycarbonate filter and the same filtration set-up that was used for the particular organic nitrogen and carbon was used for the collection of the particulate organic phosphorus. 200 µL of 1:10 diluted HCl was used to rinse the filter and to get rid of any trace of other organic components. 30 mL of the sample were then placed in the vial, the vacuum pump was turned on, and the sample was then drawn through just as in the previous step. All other techniques were identical as described in the filtration of particulate organic carbon and nitrogen.

Flow Cytometry

Cell density and fluorescence was determined on a daily basis by using the BD Accuri C6 flow cytometer. 1mL total in volume was used per sample, and this included 100 µL of the culture sample, 20 µL 3rd dilution of red beads, 20 µL 3rd dilution of yellow/green beads, and 860 µL of artificial sea water (ASW). A control was used as well with each sampling using the Accuri. The control consisted of 960 µL, 20 µL 3rd dilution of red beads, and 20 µL 3rd dilution of yellow/green beads. In addition to cell density, the flow cytometer was also used to analyze the pigments within the chlorophyll a content of the cells. The flow cytometer has multiple plots that can be analyzed, and in order to analyze the pigments within the cells, the fluorescence vs. forward scatter data was used to quantify the amount of pigments within the cell. This was done by taking the fluorescent measurement of the red beads that I put in with my sample, and having the fluorescence of the cells normalized to that of the red beads. The excitation of the red beads as well as the chlorophyll a content within the cell was done with the blue laser within the flow cytometer.

Samples were also taken at each major time point along the growth curve for flow cytometry analysis using a different flow cytometer. Technical dublicates were taken and 5 µL of 25% glutaraldehyde were used to fix 0.5 mL of each sample. Once these samples were fixed, they were inverted and incubated in the dark for twenty minutes. After twenty minutes, these samples were taken out and flash frozen in liquid nitrogen. They were then transferred to the -80 °C for later analysis. The calculation of the growth rate was also taken on a daily basis. The growth rate equation that was used for this experiment was $\mu=(N/N_o)/\Delta t$.

pH testing

After the cells had completed their growth curve and the maximum cell abundance was reached according to the growth curve, the pH of these cells were measured quantitatively using a standard lab pH probe. The pH was recorded and was compared to that of the starting media pH. The pH of the media used to inoculate these cells was around 8.2, so we wanted the pH of the cultures to be around 8.2 as well, or at least not high enough to be concerned that carbon was starting to become a limiting factor. The main purpose for taking the pH measurement at the end of the cultures grow-out period was to be sure that inorganic carbon was not a limiting factor. Inorganic carbon is in excess in the environment, and this should not be a factor for experimental conditions since we wanted to the media to be either Nitrogen or Phosphorus limiting, depending on the media.

Epifluorescence microscopy, DAPI-staining (4, 6-diamidino-2-phenylindole)

DAPI staining and microscopy were used to determine that the RCC299 cultures were axenic, meaning that there were no bacteria growing and utilizing the nutrients within these cultures. DAPI samples were collected at each time of protein and nutrient sampling (lag, mid-exponential, and stationary phase). DAPI analysis was done at the end, and we started analyzing the stationary phase samples first so that way we knew that if these samples were axenic, then so were the other time points for these cultures. In order to fix and store the DAPI samples, 100 µL of 37% formaldehyde was pipetted into 1 mL of each of the samples, inverted once, and stored in the lab refrigerator.

pre-acid washed plastic filters were used to hold the filters in place and were attached to the filtration devices. The samples used for DAPI were collected during each of the three time points for the growth curve, and were fixed within the same time period as the rest of the allocation and nutrient analysis samples (at least within four hours). a glass fiber filter, as well as a black polycarbonate filter, were used to draw the sample through. It's important to not introduce new bacteria during the process, so it is important to be as clean as possible at all times.

A glass fiber filter was first placed on each of the filtering devices and a black 0.2 μM polycarbonate filter was placed on top. DAPI was used to dye the sample once the filtrate was drawn through. This was timed for 60 seconds while keeping aluminum foil around the samples, and the sample was drawn through. The filter was then placed on a microslide and covered with cover slide. These samples were stored in the freezer for later analysis.

Protein quantification for cultures

In order to determine the average amount of protein content per cell, a QuantiT kit and Qubit fluorometer were used to record a raw reading that was later interpreted and manipulated by a linear regression to determine μg of average protein content per cell. Cellular allocation samples were collected at each time point along the growth curve for the lag, mid-exponential, and stationary phase. In order to collect the samples for protein analysis, the same filtration set-up was used as the POC/N samples. Firstly, the filtration device was set-up and a glass fiber filter was placed on top of the glass filter collection device. A clamp and a vial were then placed on the filtration set up, and 50 mL of sample was drawn through the filter using the vacuum pump. Again, if the cell density were too high, then it was appropriate to use a smaller volume such as 30 mL. Once the cells were collected on the filter, ethanol cleaned tweezers were used to carefully fold the filter in to fourths, and placed in a twist microtube with glass beads. This microtube was then flash frozen using liquid nitrogen, and then placed in the -80 °C.

The samples for the protein allocation needed to be sampled within two weeks. The samples were thawed for several minutes and 1 mL of protein buffer were added to the thawed samples. Once the buffer was added, the samples were bead beaten twice for one minute each and put on ice for five minutes in between. Three standard samples were used to create a linear regression, and the concentration of average protein content per cell was calculated using this linear regression.

RESULTS

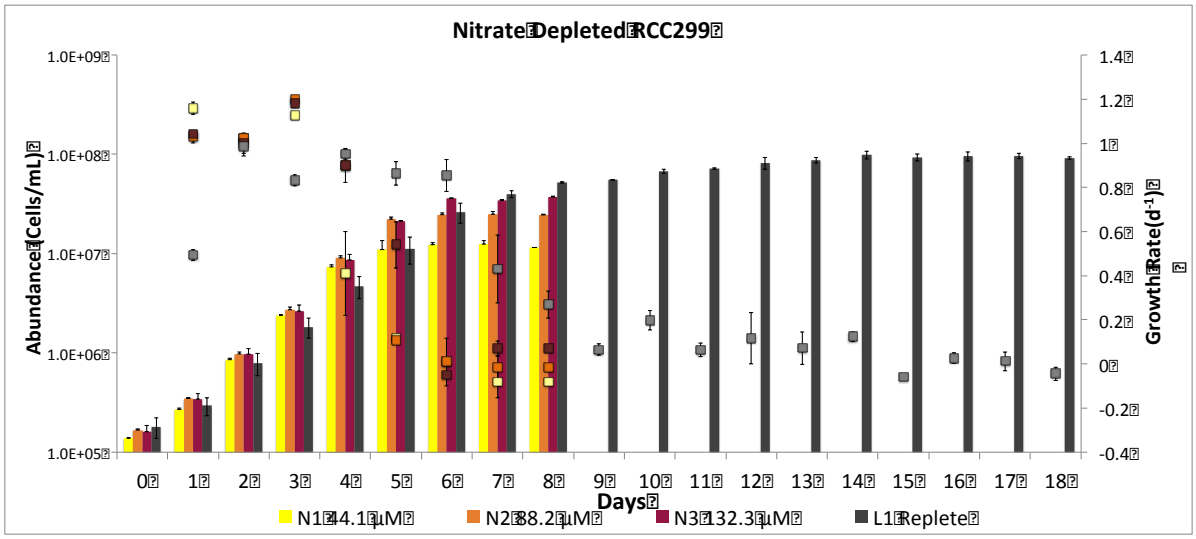
For the preliminary experiment both the second concentration for both Nitrate and Phosphate depleted medias were chosen to be the best medias to use to study Nitrogen and Phosphorus limitation in *Micromonas* strain RCC299. The media chosen was determined by analyzing which media gave the cells the most meaningful growth curve, as well as which cells would not run into carbon limitation. It was determined that the second concentration was the best to use because it gave a full S-shaped growth curve. The second concentration gradient was also determined to be the best because once the cells reached stationary phase, the pH measurement did not seem to run into carbon limitation since the pH of the culture was similar to that of the media for the beginning of the experiment.

In order to determine the best Phosphate limiting media to use to study the effect of Phosphorus depletion, the second concentration gradient for Phosphate limiting media

was also used. This was determined in a similar manner to that of the Nitrate limiting media, meaning that the second concentration gradient for Phosphate limiting media gave a useful S-shaped growth curve as well as not being able to run into carbon limitation by measuring the pH once the maximum cell abundance was reached at the beginning of stationary phase.

Due to time constraints, the cellular allocation only included average protein quantification per cell, as well as the quantification of the pigments within the chlorophyll a content within the cell. For the average protein quantification, the results weren't as promising for the exponential phase as they were for the stationary phase sampling time point. For the exponential phase, there is a large error bar for the quantity of the average amount of protein content within the Nitrogen depleted cells. However, the replete samplings seem to be the same for the exponential time point compared to the stationary time point. The low phosphate concentrated media did not seem to have an effect on the average protein content of the cell between the exponential and stationary time points.

The analysis of the fluorescence and forward scatter data indicated that with the depletion of phosphorus from the media, the cells grew brighter and brighter. The brightness of the fluorescence for the replete (L1 media) cells stayed relatively the same throughout the duration of the experiment. The cells that were cultured within the Nitrate-depleted media seemed to fluoresce with less of an intensity compared to the Phosphate-depleted and replete-L1 media cell cultures. For the large volume grow-out experiment, the samples were shipped and sent to the University of Hawaii to have the particulate organic carbon and nitrogen matter analyzed and to also have the elemental ratios of the cells analyzed as well to get measurements for the cellular quotas, we will have those samples back in September, and that data will be available then.



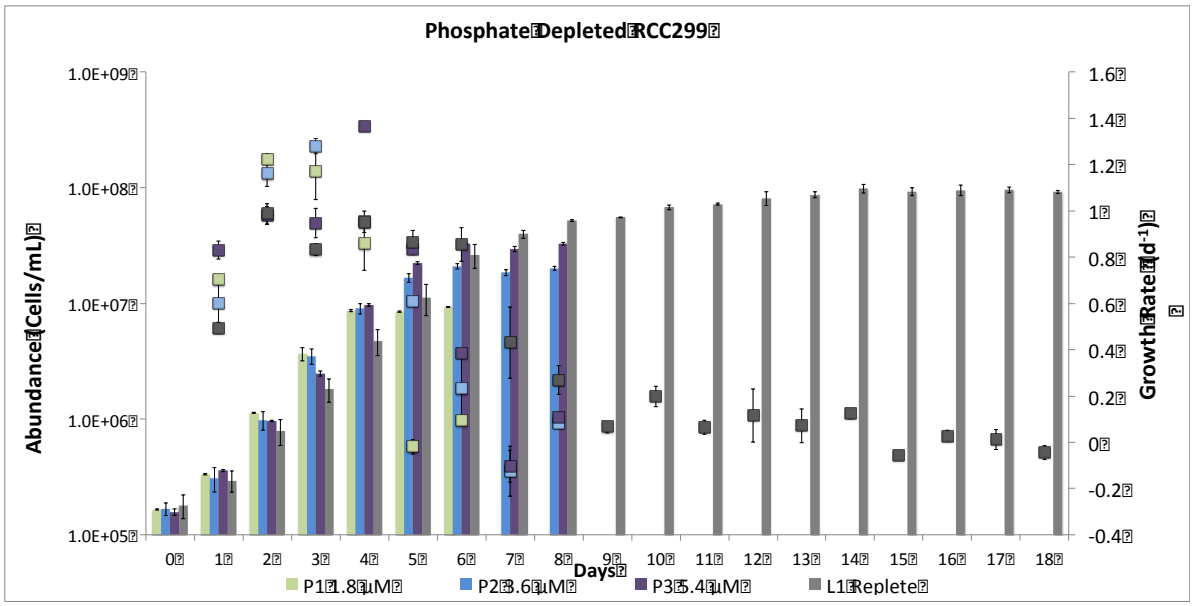
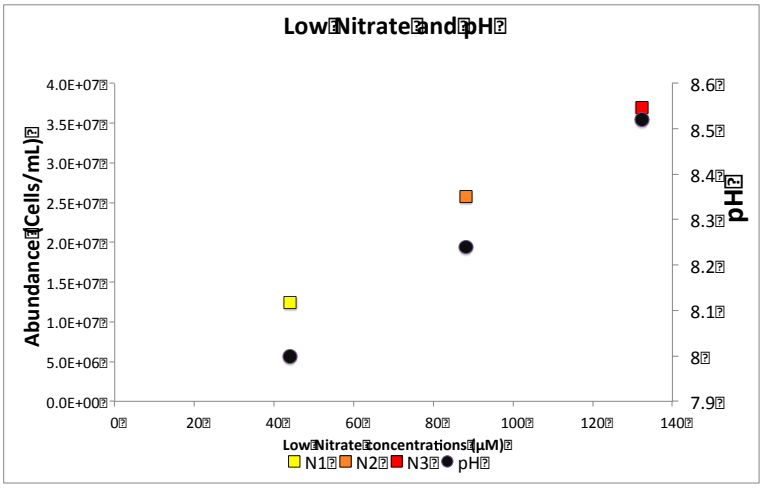


Figure 1a (top): The cell abundance vs. day of RCC299 under Nitrogen depleted conditions.

Figure 1b (bottom): The cell abundance vs. day of RCC299 under phosphorus depleted conditions.



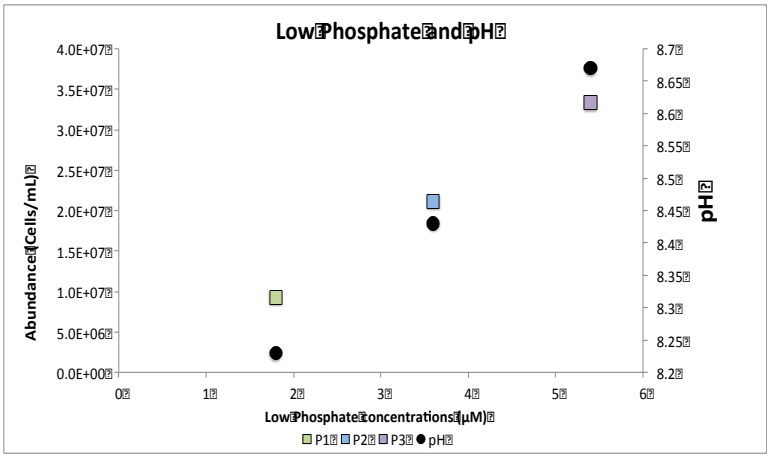
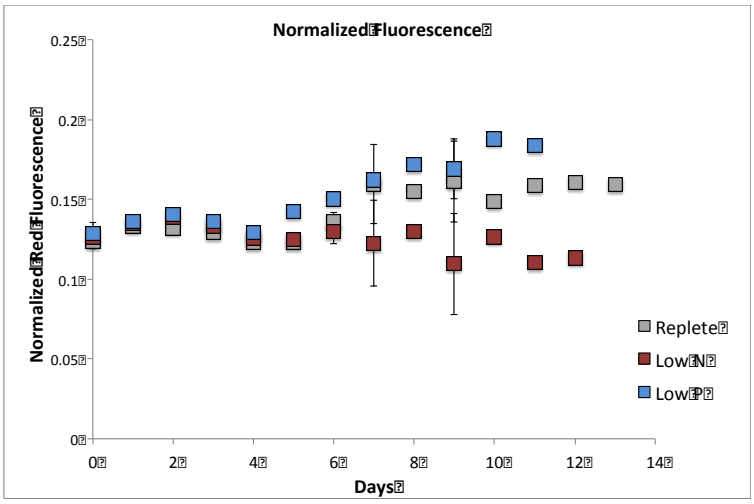


Figure 2a (top): This graph represents the maximum cell abundance once the cell reached stationary phase for each of the Nitrate depleted cells. The pH is represented by black dots and corresponds to the far right y-axis. The pH for each intial media was around 8.2, and the pH for the cultures using the media once it reached stationary phase was slightly more than 8.2

Figure 2b (bottom): The pH for each of the medias for the Phosphate-depleted media started at about 8.2 for each media.



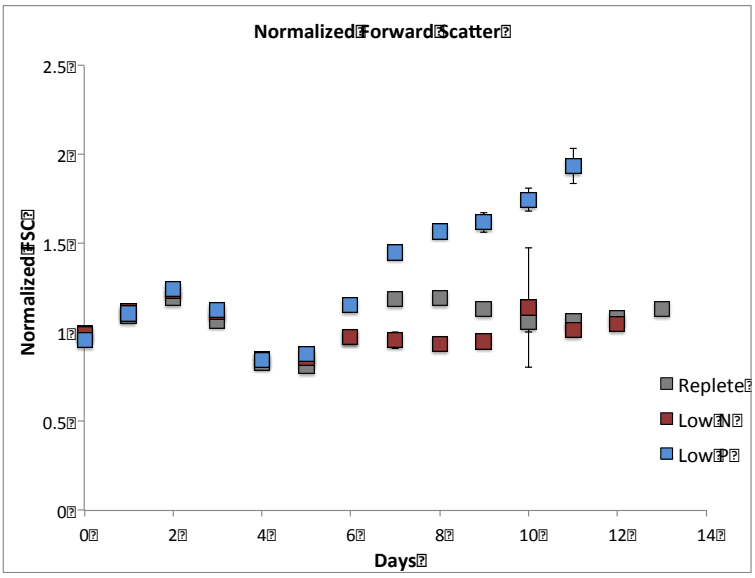


Figure 3a (top): This graph is a representation of how bright the cells are getting over the course of 13 days which is the duration of the large volume grow-out experiment.

Figure 3b (bottom): This graph is showing how large the cells are getting over the thirteen day-long experiment.

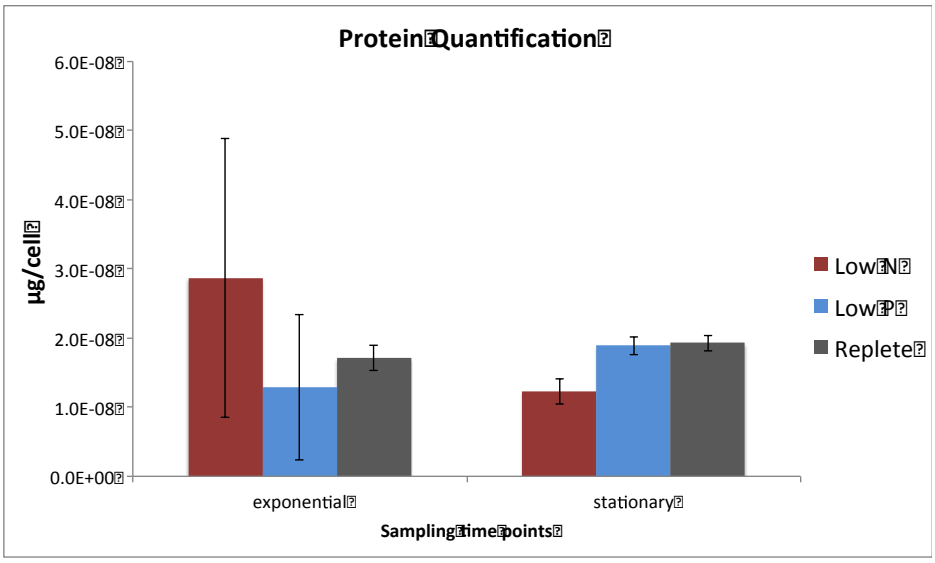


Figure 4: This graph shows the average protein content per cell for both the exponential and stationary time points of the S-shaped growth curve for all three of the different sets of cultures.

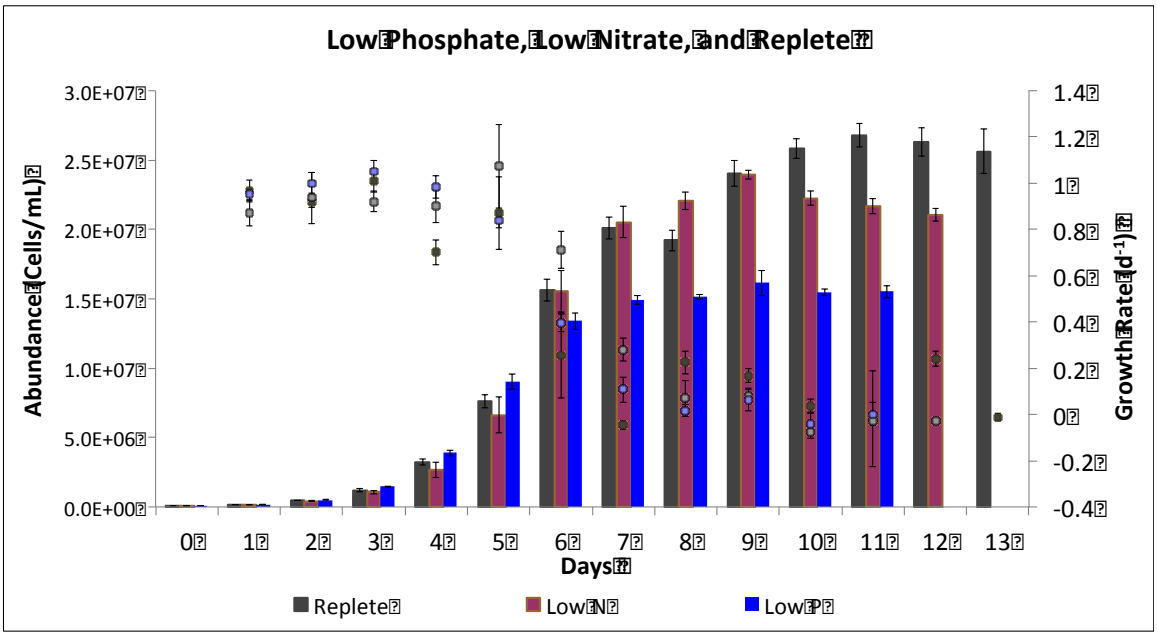


Figure 5: This graph shows the cell abundance indicated by the dark colored bars and addressed on the left y-axis, as well as the growth rate represented by small boxes and represented on the far right y-axis.

DISCUSSION

From the interpretation of the results, it seems that *Micromonas* strain RCC299 invest less in protein content when Nitrogen is limiting compared to the replete and Phosphate-deplete cultures. By analyzing the protein quantification data, it seems that there is no plasticity within the cells between the exponential and stationary time points. Therefore, I would not expect them to be bloomers during the exponential phase and survivalists during the stationary phase, hence no plasticity. Because the cells did not

have as much Nitrogen to invest within their proteins and pigment content within the chlorophyll, it makes sense that the cells had a lower protein content. Most of the data will be analyzed at the end of September 2014 when the results are returned from the University of Hawaii so we can analyze the results of the nutrient analyses as well as have the data for the elemental ratios.

CONCLUSIONS/RECOMMENDATIONS

For the future directions of this work, it would be recommended that this experiment be repeated with multiple strains as well as with multiple species of different types of phytoplankton. This would create a baseline and a better interpretation for what could be considered to be bloomers and survivalists within the natural environment. This study does not incorporate Phosphorus and Nitrogen limitation within the same culture, which could be also be a future study.

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