Physiological response of *Ostreococcus* to nutrient depletion

Mentors: Dr. Alex Worden and Dr. Amy Zimmerman

Summer 2014

Keywords: Biogeochemistry, Marine Biology, Microbiology, Phytoplankton and Stoichiometry

ABSTRACT

Phytoplankton are an important primary producer, which populate all of the oceans on Earth. One of the key environmental variables that control population growth of phytoplankton is nutrient availability. With rising levels of carbon dioxide in our atmosphere and increasing density stratification due to warming oceans, the nutrients available to phytoplankton in the euphotic zone will change. To further understand the nutrient limitation of phytoplankton, I investigated how the growth rate and elemental quotas of the pico-phytoplankton *Ostreococcus* respond to the depletion of nitrogen and phosphorus. I addressed this question by monitoring the growth of *Ostreococcus* cultures in multiple nutrient conditions (nutrient replete and deplete) using flow cytometry. In addition to calculating growth rates, I collected samples for cellular elemental quotas from the different treatments and across different phases of growth. These results will provide insight into the physiological plasticity of phytoplankton, which is a poorly characterized parameter in current biogeochemical models. Ultimately, the results of this study will give us a deeper understanding of the role of phytoplankton in marine biogeochemical cycles and how their populations may be affected in the future.

INTRODUCTION

Picoplankton are the smallest of the plankton with a diameter of 0.2-3μm, and are abundant throughout the world's oceans (Li 1994). Accounting for less than 1% of the planet's photosynthetic biomass, phytoplankton contribute almost 50% of the primary production on Earth (Field et al. 1998; Falkowski 2012). Therefore, a change in phytoplankton population will severely affect the biogeochemical cycles. Out of the diverse community of picoplankton, picoeukaryotic phytoplankton have been found to be significant players in terms of both biomass and primary production (Li 1994; Worden 2004).

The picoeukaryote *Ostreococcus* represents a diverse and widely distributed genus within the green algae (Le Bihan 2011). *Ostreococcus* is the smallest of the picoeukaryotes, with a very simple cell structure, including only one chloroplast and mitochondrion and no flagella (Cardol 2008). *Ostreococcus* strains have been isolated from the environment and grown successfully in laboratory cultures previously, allowing for controlled experiments (Worden 2004). In this study, *Ostreococcus lucimarinus* CCMP2972 will be used to study the influence of different nutrient conditions on cell physiology. Specifically, I will focus on the question: How do the growth rate and elemental quotas of the pico-phytoplankton *Ostreococcus* respond to the nutrient depletion of nitrogen and phosphorus?

Determining how environmental factors such as nutrient availability affect phytoplankton physiology is important to understanding potential effects on population stability as well as future changes in the biogeochemical cycles. The current rising carbon dioxide concentration in our atmosphere is causing Earth's oceans to grow warmer, making the stratification of ocean density increase, which will in turn limit the available nutrients at the surface layers (Sarmiento 1998). Many of the models used to understand and predict marine biogeochemical cycles are based on the Redfield ratio (Geider 2002), which specifies that the nutrients carbon (C), nitrogen (N), and phosphorus (P) are consumed by plankton at a ratio of 106:16:1 (Redfield 1958). As the levels of carbon dioxide in our atmosphere increase due to anthropogenic processes, the biogeochemical cycles of our oceans will change along with the ratio of nutrients available to all

phytoplankton (Falkowski 2000). These deviations from the current biogeochemical cycles and therefore Redfield's ratio will affect the phytoplankton uptake ratios, which will in turn affect higher trophic levels, the recycling of nutrients in the euphotic zone and the transportation of nutrients out of the euphotic zone. Having a better understanding of the plasticity of cellular quotas and the variability of the Redfield ratio is key to understanding and predicting how the biogeochemical cycles will change under future climates. Therefore, the ultimate goal of this study is to contribute to the deeper understanding of the role of phytoplankton in mediating Earth's biogeochemical cycles. In addition, my immediate goals are to:

1) Analyze *Ostreococcus* growth under gradients of nitrogen and phosphorus availability

2) Identify the deplete concentrations of nitrogen and phosphorus that impact *Ostreococcus* growth

3) Determine the degree of plasticity of carbon, nitrogen and phosphorus cellular quotas.

4) Describe potential implications for carbon, nitrogen and phosphorus cycling

When the Redfield ratio is used to model the relationship of plankton and the elements in their environment, there is little plasticity in the relationship between the C, N and P biomass compositions of plankton. Therefore, I hypothesize that picoeukaryotes will follow this theory and have constrained elemental ratios, but may have flexible quotas. In support of this hypothesis, I expect that:

P1) As the phosphorus in the media is depleted, *Ostreococcus* will adjust and decrease the cellular quota of phosphorus to match the low concentration of the media. The cellular quota of nitrogen and carbon will also decrease in accordance with the Redfield ratio. These physiological adjustments will in turn reduce growth rate.

P2) As the nitrogen in the media is depleted *Ostreococcus* will adjust and decrease the cellular quota of nitrogen to match the low concentration of the media. The

cellular quota of phosphorus and carbon will also decrease in accordance with Redfield's ratio. These physiological adjustments will cause a decrease in the growth rate.

An alternative hypothesis would be that both the cellular quotas of picoeukaryotes and their biomass nutrient ratios are flexible. Results that would support this alternative hypothesis would be:

$P1_A$) As the phosphorus in the media is depleted the cellular quota of phosphorus will decrease to match the low concentration of the media and the cellular quotas of carbon and nitrogen will not differ from the replete cultures.

$P2_A$) As the nitrogen in the media is depleted the cellular quota of nitrogen will decrease to match the low concentration of the media and the cellular quotas of carbon and phosphorus will not differ from the replete cultures.

MATERIALS AND METHODS

I am investigating how the growth rate and elemental quotas of the pico-phytoplankton *Ostreococcus* respond to the depletion of nitrogen and phosphorus in this experiment by monitoring the growth of *Ostreococcus* cultures in multiple nutrient conditions (nutrient replete and deplete). The strain that was used in this experiment is axenic *Ostreococcus* lucimarinus CCMP2972. Cell density was recorded daily with the use of an Accuri Flow Cytometer (BD Biosciences). From this data I calculated daily growth rates (GR) with Equation 1, where N_0 is the initial cell abundance, N_1 is the current cell abundance and Δt is the time between these two measurements.

$$GR = \frac{\ln\left(\frac{N_1}{N_0}\right)}{\Delta t} \quad (1)$$

Optimization of culture conditions

Before growing *Ostreococcus* in media with varying concentrations of nitrogen and phosphorus, I tested the optimal media type and cell density that these

picoeukaryotes prefer. Three different types of media were evaluated: L1, f/2 and f/4 (Guillard et al. 1993; Guilllard et al. 1975) using filtered natural sea water from CalCOFI (California Cooperative Ocean Fisheries Investigations) Line 67 station 135 (33.953°N, 128.048°W), an oligotrophic region, sampled during the 2011CANON cruise. Selenium (1mL L-1) was added to each media (Worden 2004). The cell density was recorded daily and used to calculate growth rate as described above. At each generation, when the entire population has doubled, the cultures were transferred to $5x10^6$ cells mL^{-1}. This transferring kept the cells in semi-continuous growth, which better represents the exponential phase of the growth curve and confirmed that the cultures were in a consistent physiological state. The optimal media that was chosen was determined by the highest and most stable growth rate (see Equation 1). After the media was chosen, I tested for the optimal cell density. The lowest cell abundance possible for *Ostreococcus* to grow must be used to guarantee the cultures will not be affected by the availability of dissolved carbon dioxide (i.e. carbon limitation). The chosen media was inoculated with four different cell densities: $1x10^5$, $5x10^5$, $1x10^6$ and $5x10^6$ cells mL^{-1}, with $5x10^6$ cells mL^{-1} being the highest because this concentration of cells has grown successfully in cultures in the past. These flasks were sampled and transferred every day for four generations and therefore kept in a stable, semi-continuous growth phase. After these four generations the cultures were left to grow out until stationary phase when the population reached its carrying capacity or the growth rate was close to zero more than one consecutive day. The cell density was recorded daily by flow cytometry. The lowest cell density that the picoeukaryotes kept a stable growth rate in was chosen as the inoculation density for the experimental flasks.

Determination of low phosphorus and nitrogen treatments

After the media and cell density had been chosen, nine treatments were created: one nutrient replete condition (36.2 μM P and 882 μM N) in quadruplicates, a gradient of four nitrogen concentrations (0.5X N or 441 μM, 0.15X N or 132.3 μM, 0.1X N or 88.2 μM and 0.05X N or 44.1 μM) each in duplicates, and a gradient of four phosphorus concentrations (0.5X P or 18.1 μM, 0.15X P or 5.43 μM, 0.1X P or 3.62 μM and 0.05X P

or 1.81 μM) each in duplicates. Each of the twenty cultures were inoculated with the chosen cell density. These cultures were transferred and grown semi-continuously for four generations. Transfers were used to keep the cultures in the exponential growth phase and samples were taken daily to measure cell density using flow cytometry. The cultures were then allowed to grow out until stationary phase and daily flow cytometry readings continued. From the daily cell density readings the growth rate (GR) was calculated using Equation 1. During the stationary phase of each culture, the pH was recorded and later referred to when analyzing the effects of the population density on carbonate chemistry. From the data collected the lowest phosphorus and nitrogen treatments that showed a stable growth curve were selected as the nutrient deplete treatments in the subsequent experiment.

Sample collection for elemental composition

The cultures chosen for the three treatments (i.e. deplete nitrogen, deplete phosphorus, and nutrient replete) were inoculated in a large volume (40 times the volume used in the preliminary experiments) at $2.5x10^5$ cells mL^{-1} in quadruplicate and allowed to grow out in until stationary phase. They were maintained in exponential growth through dilution with media to generate a large volume for inoculation. Flow cytometry measurements were taken daily over this time period to confirm that the picoeukaryotes had a stable growth rate. Over the course of the grow-out, the cell density was measured daily (as done previously) and additional samples were fixed with gluteraldehyde (0.25% final concentration) and flash frozen in liquid nitrogen for later analysis on the more sensitive Influx Flow Cytometer (BD Biosciences). In addition, the following samples were taken from the parent cultures and during mid-exponential and stationary (defined as two or more consecutive days of a growth rate close to zero) growth phases. To quantify the particulate organic carbon (POC), nitrogen (PON), and phosphorus (POP), which represent the cellular quotas of these elements, samples were taken on either 25 mm glass fiber (for POC/PON, 0.3 μm nominal pore size) or polycarbonate (for POP, 0.2 μm nominal pore size) filters and stored at -20°C. Filtrate was also collected to measure dissolved inorganic nutrients (NO^{3-} + NO^{2-}, PO_4^{3-}, NH^{4+}, H_4SiO_4) in the spent media.

Particulate and dissolved nutrient samples were sent to the SOEST Laboratory for Analytical Biogeochemistry (S-LAB) at the University of Hawai'i at Manoa for analysis (http://www.soest.hawaii.edu/S-LAB/index.htm). POC and PON were measured simultaneously on an Exeter Analytical model CE 440 elemental analyzer. Dissolved nutrients and POP (after high-temperature ashing) were both quantified on a Seal Analytical AA3 HR Nutrient Autoanalyzer. To analyze the affects of carbon limitation on the cultures, the pH was measured at each collection time point. Samples were also taken for staining with 4', 6- diamidino-2-phenylindole (DAPI), which was used to confirm that the cultures were axenic.

RESULTS

Optimal growth conditions

To better understand the optimal growth conditions of *Ostreococcus* the preferred medium and cell abundance for this organism was evaluated. After four generations it was clear that there was no apparent differentiation between the growth rates of the three media types and I concluded that *Ostreococcus* does not seem to prefer any one of these three types of media (Fig. 1). L1 was chosen because it has been shown to be preferred by multiple types of phytoplankton in previous experiences in the Worden Lab. From the cell density test (Fig 2) I found that at lower cell abundances, specifically $1x10^6$ and $5x10^5$ cells mL^{-1}, the cultures grew at similar, if not better growth rates than the cultures kept at $5x10^6$ cells mL^{-1}. $1x10^6$ cells mL^{-1} was chosen as the optimal cell abundance due to the accuracy of flow cytometry readings decreasing with low biomass.

Growth of nitrogen deplete treatments

Five different nitrogen conditions, including a replete condition that served as the control, were inoculated at $2.5x10^5$ cells mL^{-1} to test the growth response of *Ostreococcus*. During the mid-exponential phase, all cultures shared a common growth rate, but as the cultures entered stationary phase the carrying capacity decreased with the decreasing of nitrogen concentration (Fig 3 and 5). The lowest concentration of nitrogen that the cells were grown in (0.05X N or 44.10 μM) did go through all three stages of

growth but in a shorter amount of time, 7 days versus 12 days for the replete culture. Therefore, to allow a longer window for sampling at each stage of growth the culture grown in a gradient of 0.1 (88.20μM) was chosen to represent the deplete conditions of *Ostreococcus*.

Growth of phosphorus deplete treatments

One replete culture and four phosphorus deplete cultures were inoculated at $2.5x10^5$ cells mL^{-1} to determine the affects of phosphorus depletion on *Ostreococcus*. During the grow out period, it was clear that though they shared a similar growth rate during the mid-exponential phase, as the gradient of phosphorus decreased so did the carrying capacity of each culture (Fig 4 and 6). The concentration of 0.1X P (3.62μM) was chosen to represent the phosphorus deplete conditions of *Ostreococcus* because of the practicality of longer sampling periods during each phase of growth.

Growth under nutrient deplete conditions

Three treatments, with four biological replicates, were inoculated at $2.5x10^5$ cells mL^{-1} to test and compare the growth of replete, nitrogen deplete (0.1XN or 88.20μM) and phosphorus deplete (0.1XP or 3.62μM) cultures. It was clear after undisturbed growth over nine days there was no apparent difference in growth rates between the three different treatments (Fig 7).

Sample collection for elemental composition

Samples that represented the mid-exponential phase were taken on day 3 or 4 depending on the treatment (Fig 7). Mid-exponential samples for both phosphorus and nitrogen deplete cultures were taken on day 3 in the cell density range of $2.6x10^6$ to $4x10^6$ cells mL^{-1}. Samples for the replete cultures were taken on day 4 in the cell density range of $6x10^6$ to $8x10^6$ cells mL^{-1}. The mid-exponential range was determined from the growth curves plotted during the nitrogen and phosphorus deplete preliminary experiments (Fig 3 and 4) and was chosen in accordance to the highest period of growth

rate. Stationary samples for all three treatments were taken on day 9 because all cultures had a growth rate close to zero for more than one day (Fig 7). Results from the particulate organic phosphorus, carbon and nitrogen and dissolved inorganic nutrients have not been received yet from the SOEST Laboratory for Analytical Biogeochemistry. DAPI analysis was performed in the lab and all cultures were found to be axenic. pH data was collected at each growth phase (Fig 8), which showed an increase in pH from the exponential to stationary phase.

DISCUSSION

The results of this experiment give us an insight into the physiological plasticity of phytoplankton. From the phosphorus deplete, nitrogen deplete and nutrient deplete experiments it can be seen that during the mid-exponential phase all treatments had very similar growth rates (Fig 3, 4 and 7). This shows that even in low resource conditions *Ostreococcus* still managed to grow and adjust to their environment. The reason for *Ostreococcus* remarkable abilities to survive in low nutrient environments is due to the fact that *Osreococcus* is the smallest of the picoeukaryotes and therefore has a much high surface area to volume ratio making them more efficient at obtaining resources from the environment (Cardol 2008). These results give us a deeper understanding of the range of environmental conditions *Ostreococcus* can tolerate and therefore where they can be found in our oceans.

From both preliminary experiments it is apparent that as the availability of nitrogen or phosphorus decreases so does the carrying capacity (Fig 5 and 6). *Ostreococcus* is capable of surviving in low nutrient environments, but may not be very abundant. The cellular quota results that will be gathered from the samples collected at each growth phase will give us a better understanding of how exactly the cells were affected by nutrient deplete conditions and therefore the plasticity of Redfield's ratio.

The results of the nutrient deplete experiment differed greatly from the preliminary experiments. Though three different treatments were used (phosphorus deplete, nitrogen deplete and replete), the carrying capacity and growth rates were very similar (Fig 7). This similarity may be due to the fact that these cultures were grown in a

larger volume (almost 40 times larger than the preliminary experiments). The deeper water column in the flasks could have introduced carbon limitation and possibly light limitation. Indeed, between exponential and stationary phase the pH of these cultures increased (Fig 8), which supports the possibility that these cultures were limited by dissolved inorganic carbon.

From the cellular quota results collected from the samples we will have a better understanding of how *Ostreococcus* specifically copes with nutrient deplete conditions as well as the plasticity of the cellular quota. With these anticipated results we will better understand the role of nutrients in controlling cellular quotas, as well as how they relate to Redfield's ratio. In addition, the plasticity of *Ostreococcus* cellular quota and its limiting environmental conditions can be directly related to other picoeukaryotes. These results can be used to design new ecological models and to better predict the consequences of future climate change on biogeochemical cycles.

ACKNOWLEDGEMENTS

I would like to acknowledge and thank the Worden lab, including Alex Worden, Amy Zimmerman and Susanne Wilken, George Matsumoto, Corey Garza, Mary McCormick, Patrick Mulcahy, NSF-REU program, MBARI, the 2014 MBARI interns, CSUMB, and the David and Lucille Packard Foundation.

References:

Cardol, P. 2008. An Original Adaption of Photosynthesis in the Marine Green Alga Ostreococcus. *PNAS*, 105, No. 22: 7881-7886.

Falkowski, P. 2000. The global carbon cycle: A test of our knowledge of Earth as a system. *Science* 290: 291–296.

Falkowski, P. 2012. Ocean science: The power of plankton. *Nature, 483*, No. 7387: S17-S20.

Field, C, Michael Behrenfeld, James Randerson, and Paul Falkowski. 1998. Primary Production of the Biosphere: Integrating Terrestrial and Oceanic Components. *Science*. 281, no. 5374: 237-240.

Geider, R. 2002. Redfield Revisited: Variability of C:N:P in Marine Microalgae and Its Biochemical Basis. *European Journal of Phycology*. 37, no. 1: 1-17.

Guillard, R.R.L. 1975. Culture of phytoplankton for feeding marine invertebrates. In Smith W.L. and Chanley M.H (Eds.) Culture of Marine Invertebrate Animals. Plenum Press, New York, USA. 26-60.

Guillard, R. R. L., and P. E. Hargraves. 1993. Stichochrysis immobilis is a diatom, not a chrysophyte. Phycologia 32.3: 234-236.

Le Bihan, T. 2011. Shotgun Proteomic Analysis of the Unicellular Alga *Ostreococcus Tauri*. *Journal of Proteomics*. 74, no. 10: 2060-2070.

Li, W. K. W. 1994. Primary production of prochlorophytes, cyanobacteria, and eukaryotic ultraphytoplankton: Measurements from flow cytometric sorting. Limnol. Oceanogr. 39: 169–175.

Moore, C. 2013. Processes and Patterns of Oceanic Nutrient Limitation. *Nature Geoscience*. 6, no. 9: 701-710.

Redfield, A.C. 1958. The biological control of chemical factors in the environment. Am. Sci., 46: 205–221.

Sarmiento, J.L. 1998. Simulated response of the ocean carbon cycle to anthropogenic climate warming. *Nature*. 393: 245-249

Worden, A. 2004. Assessing the Dynamics and Ecology of Marine Picophytoplankton: The Importance of the Eukaryotic Component. *Limnology & Oceanography*. 49, no. 1: 168-179.

Figures:

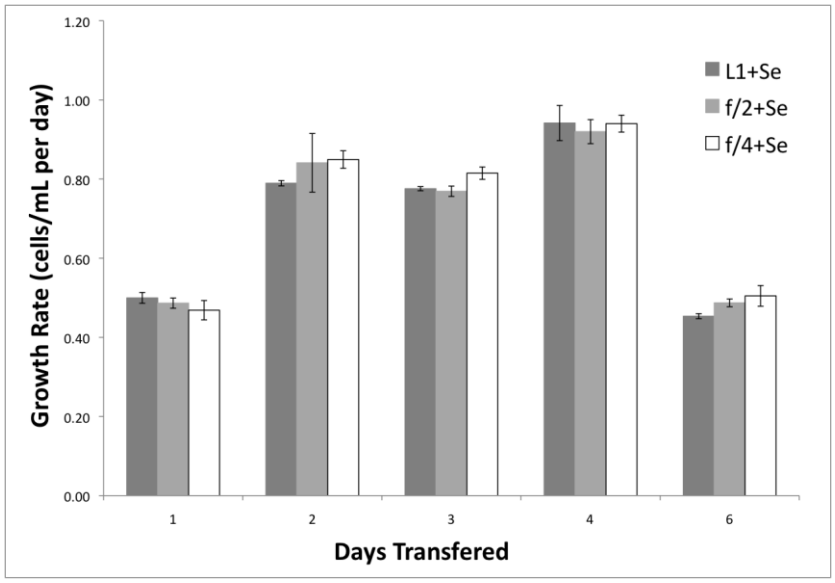

Figure 1: Media Optimization. Over six days, cultures grown in L1, f/2 and f/4 media were transferred to the same cell abundance. All three media showed supported similar growth rates.

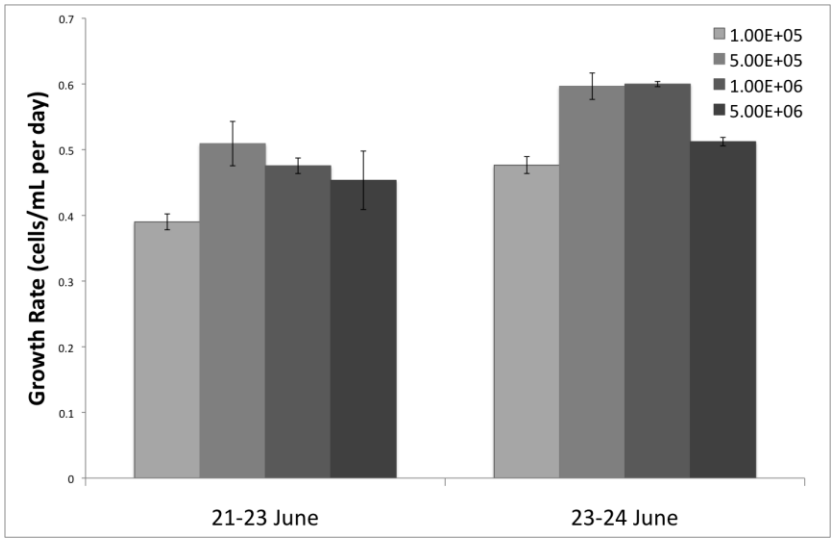

Figure 2: Cell Abundance Test. The cultures were transferred daily to the target abundance shown in the legend. At lower cell abundances ($5x10^5$ and $1x10^6$ cells mL^{-1}) the growth rate shows that the cultures grew the same if not better than the cells kept at $5x10^6$ cells mL

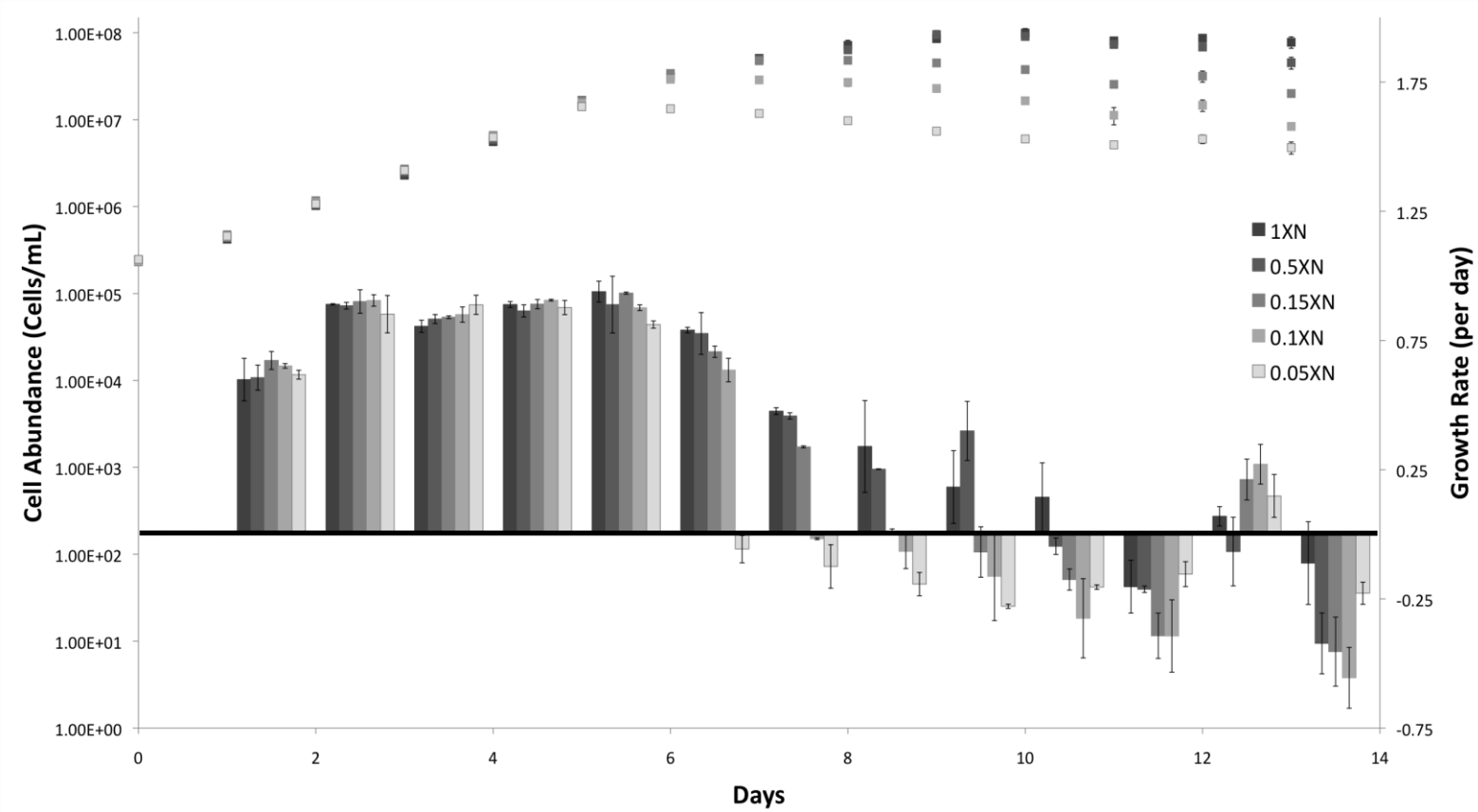

Figure 3: Nitrogen Depletion. Dots represent cell density (left axis) on a logarithmic scale, while bars represent the growth rate (right axis) of the cultures. As the concentration of nitrogen decreased so did the carrying capacity of each culture. Throughout the exponential phase all treatments had similar growth rates.

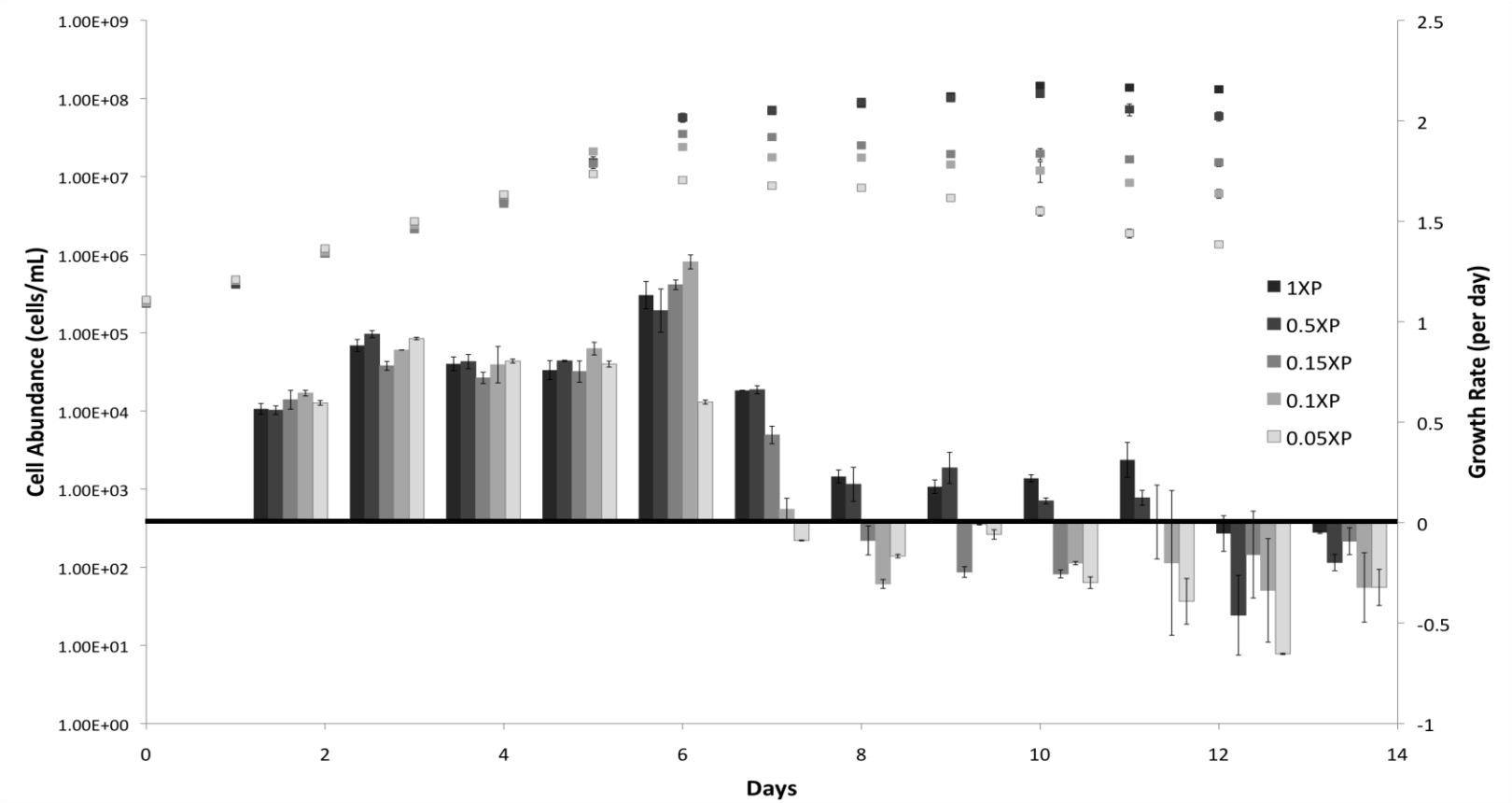

Figure 4: Phosphorus Depletion. Dots represent cell abundance (left axis) on a logarithmic scale, while bars represent the growth rate (right axis) of the cultures. As the concentration of phosphorus in the media decreased so did the carrying capacity of each culture. Throughout the exponential phase all treatments had similar growth rates.

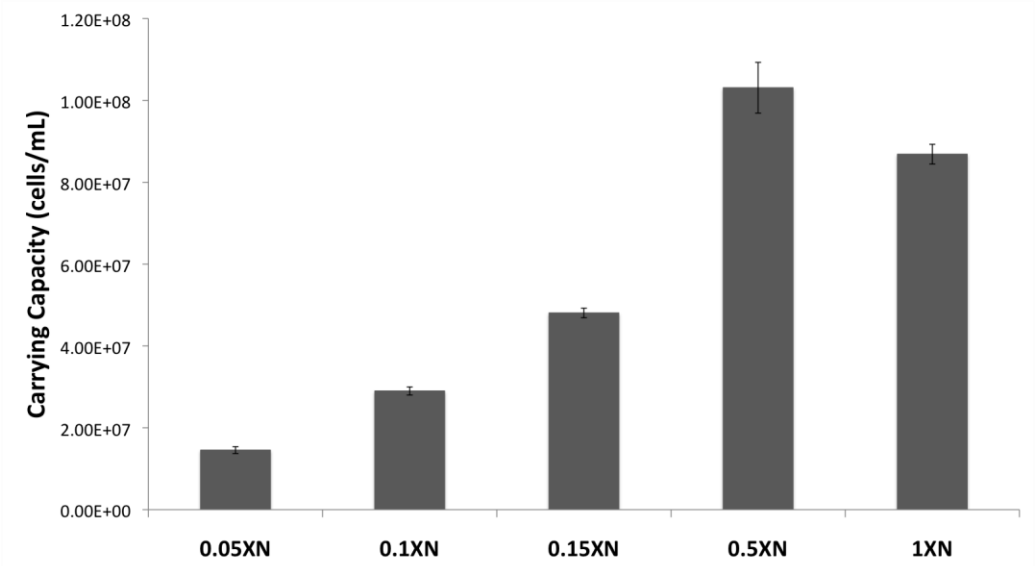

Figure 5: Carrying Capacity of Nitrogen Depletion Culture. The effect of nitrogen availability on the carrying capacity of *Ostreococcus*.

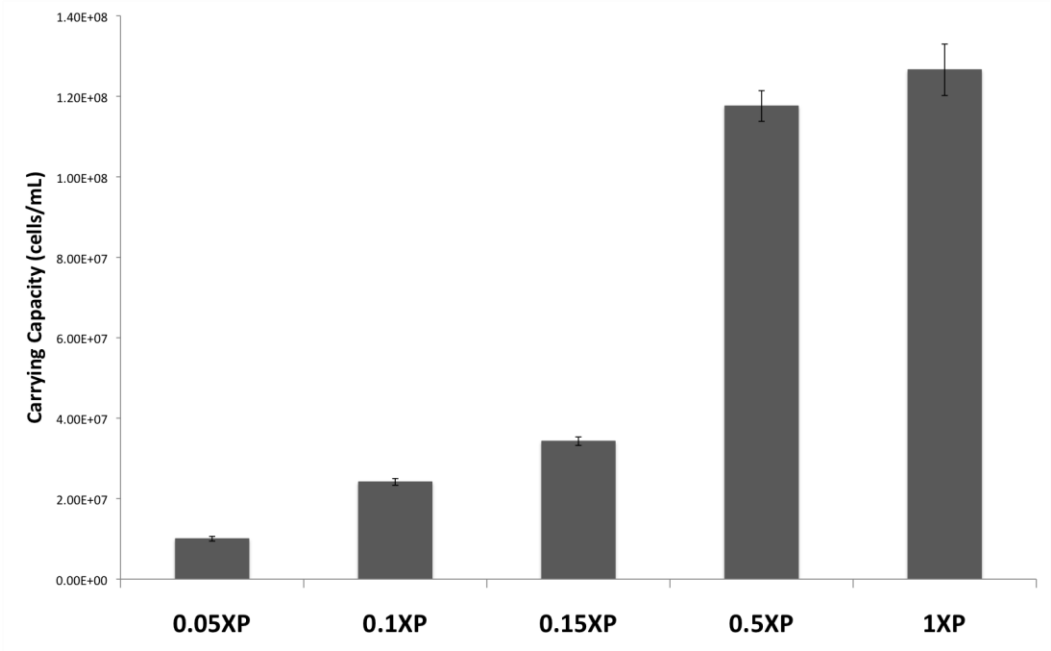

Figure 6: Carrying Capacity of Phosphorus Deplete Cultures. The effect of phosphorus availability on the carrying capacity of *Ostreococcus.*

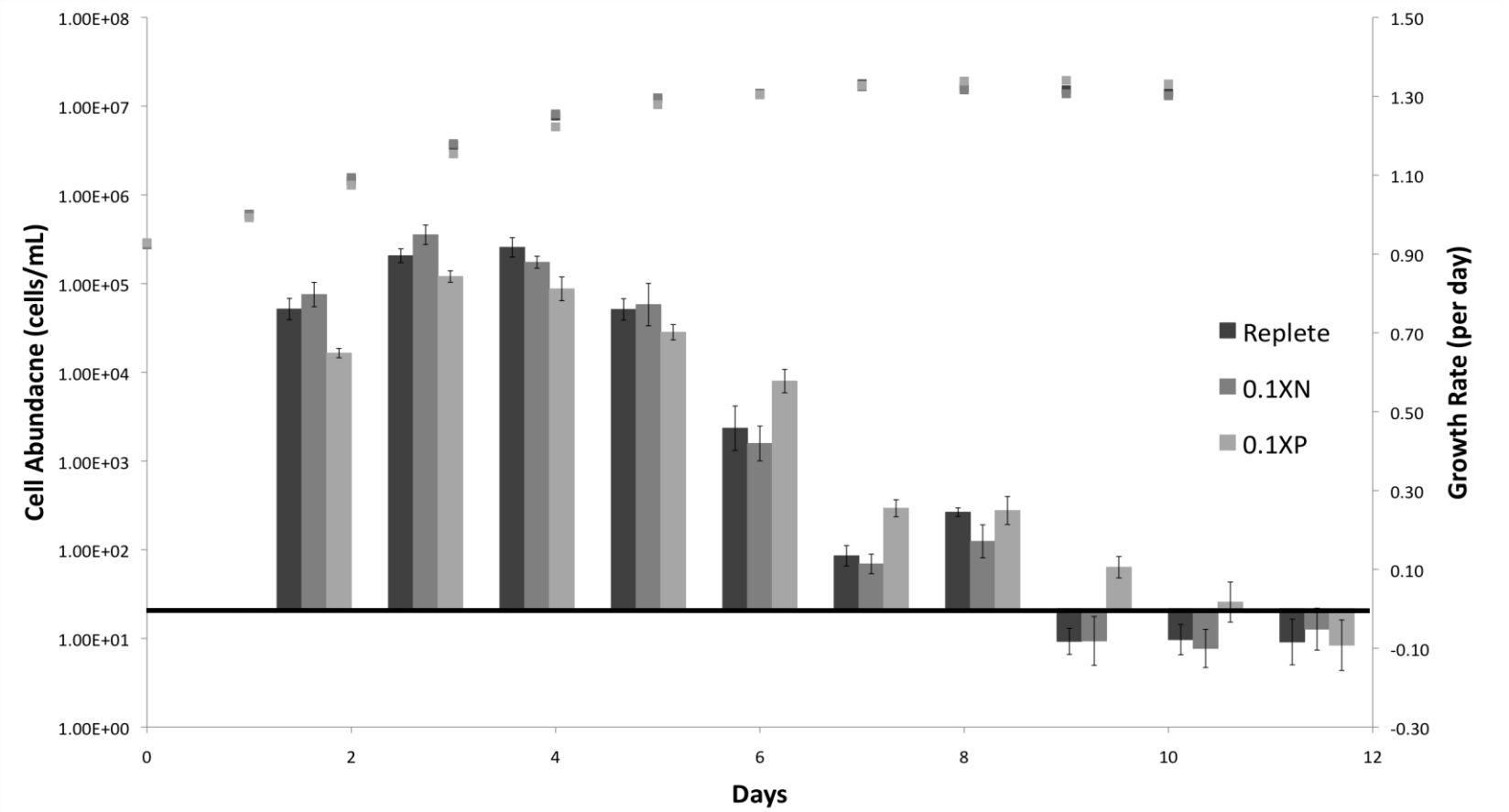

Figure 7: Nutrient Depletion. Dots represent cell abundance (right axis) on a logarithmic scale, while bars represent the growth rate (left axis) of the cultures. The depletion of nutrients did not appear to have an effect on the cultures. The carrying capacity and growth rate of all three treatments were very similar.

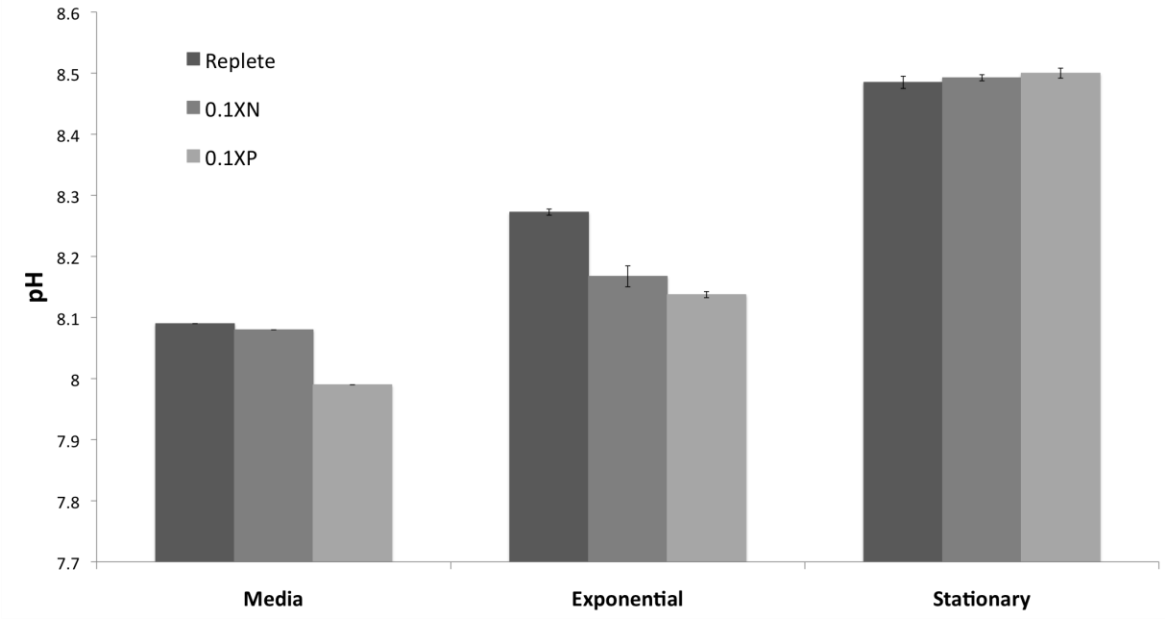

Figure 8: pH Changes in Nutrient Depletion Experiment. The change in pH over time in the nutrient deplete experiment shows that the pH of every treatment increased over the growth period. The higher pH values during stationary phase show that the cultures may have been subject to carbon limitation.