UNIVERSITY OF CALIFORNIA SAN DIEGO

Physiological responses of *Thalassiosira pseudonana* in over 70 days in continuous co-culture with *Ruegeria pomeroyi* under nitrate and B12 starvation

A thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Marine Biology

by

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Committee in charge:

Professor Andrew Allen, Chair
Professor Eric Allen
Professor Douglas Bartlett

2026

The Thesis of Kaitlyn Michelle Broersma is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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LIST OF ABBREVIATIONS

PBR	Photo-bio reactor
Tp	*Thalassiosira pseudonana*
Rpom	*Ruegeria pomeroyi*
B12	Vitamin B12 (cobalamin)
FSC	Forward Scatter
SSC	Side scatter

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This thesis is coauthored with George, Emma and Hamilton, Maria. The thesis author: Broersma, Kaitlyn was the primary author of this chapter.

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ABSTRACT OF THE THESIS

Physiological responses of *Thalassiosira pseudonana* in over 70 days in continuous co-culture with *Ruegeria pomeroyi* under nitrate and B12 starvation

by

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Master of Science in Marine Biology

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Nitrogen and cobalamin (B12) are essential for diatom growth, specifically for the species *Thalassiosira psuedonana* (Tp) which is a B12 auxotroph. Here, continuous culture systems called photobioreactors (PBRs) were utilized to assess the physiological response of Tp under chronic nitrate and B12 limitation with and without bacteria present. Continuous culture systems allow diatom cultures to reach steady state conditions during nutrient limitation, unlike

batch cultures where the diatom cells quickly run into limitation conditions. In this experiment, the bacterium *Ruegeria pomeroyi* (Rpom) was added to cultures of Tp experiencing chronic nitrate stress and then was left in the continuous culture system for the remainder of the experiment, which included shifts into replete nitrate medium and later a B12-free medium. The addition of Rpom was associated with an increase in Tp fluorescence and cell size when under nitrate limitation, however it did not affect the photosynthetic efficiency of Tp, an indicator for cellular health. When B12 was removed, Rpom allowed for the continuous survival of Tp and was also associated with larger Tp biovolume and forward scatter in continuous culture conditions. These results suggest a beneficial relationship between the two species. All treatment types had varying effects on Tp, providing information into how different types of limitation can have varying effects on diatom physiology. The insights from this experiment can help identify locations where similar diatom-bacteria interactions may occur naturally, enabling lab-based recreation, and further study.

INTRODUCTION

Phytoplankton are known for their role as primary producers, fixing inorganic carbon and providing an estimated 50% of oxygen globally (Petsch, 2003). While total phytoplankton biomass is miniscule compared to the biomass of primary producers in terrestrial systems, phytoplankton support large marine food webs and contribute significantly to the biological carbon pump. Heterotrophic marine bacteria are also essential for oceanic processes. There are more than a million microbial cells in just one drop of seawater (Raina, 2018). These small but mighty bacteria recycle essential nutrients in the water column and also can synthesize various vitamins (Azam et al., 1983). Phytoplankton are also diverse, with diatoms alone having over 100,000 species (Wang et al., 2022). Diatoms specifically are of interest because of their extremely fast growth rates, making them extremely pervasive and photosynthetically productive (Inomura et al., 2023).

Diatom growth is limited by the availability of various nutrients, along with reliance on a balance of carbon fixation and cellular redox states. Nitrogen, specifically nitrate and ammonium, is one of the main limiting nutrients for diatoms in open ocean regions (Browning & Moore, 2023). Nitrate limitation in diatoms can cause them to reprogram many metabolic pathways including how carbon and lipid contents are stored (Giri et al., 2022). Other physiological effects can also occur like a decrease in diatom cell size and chlorophyll fluorescence (Hennon et al., 2014). Since diatoms are specific about the form of nitrogen they consume, bacterial recycling of essential nutrients is extremely common, this recycling supports around 80% of primary productivity in euphotic zones (Bristow et al., 2017). This recycling of specifically nitrogen may also buffer nitrogen limitation for diatoms.

Another limiting nutrient for many diatoms is cobalamin or vitamin B12. This vitamin is a large molecule only produced by some bacteria and archaea due to the extensive metabolic pathway involved in its formation (Fang et al., 2017). B12 is an important cofactor involved in the biosynthesis of methionine, an essential amino acid (Banerjee & Matthews, 1990). Without B12-producing bacteria or archaea present, many auxotrophic diatoms are unable to survive. Therefore, vitamin B12 may help drive interactions between bacteria and diatoms. Not all diatoms require B12 because some encode a B12-independent methionine synthase protein (MetE)(Bertrand et al., 2013). However, *Thalassiosira pseudonana* (Tp), our model marine diatom, only has MetH, a B12-dependent methionine synthase, thus it has an obligate demand for B12 (Bertrand et al., 2013).

For this experiment Tp was chosen due to its well characterized genome of 34 million base pairs, making it a model diatom that is widely used in diatom studies (Armbrust et al., 2004). Due to its high usage in diatom research it also has many comparable published studies involving its physiology and molecular mechanisms. Similarly, *Ruegeria pomeroyi* (Rpom) was also studied due to its known interactions with diatoms and for its ability to produce vitamins for phytoplankton species (Kaur et al., 2018) (Moran et al., 2004). Previous experiments have shown that in cases of B12 limitation, Rpom provides Tp with B12, while the Tp diatom provides Rpom with fixed carbon (Croft et al., 2005). This proposed interaction could be mutualistic, however it is important to note that Rpom does not release B12 in culture alone, and only releases B12 when in culture with a diatom species (Durham et al., 2015) (Mars Brisbin et al., 2023). Thus, in coculture in the absence of B12 in the media, diatoms can survive off of Rpom produced B12, while Tp provides Rpom with organic forms of nitrogen and carbon for its consumption.

When assessing diatom growth it is common to use batch culturing methods, however these methods are unable to achieve steady state conditions needed for exploring environmental perturbations. Continuous culturing systems allow us to simulate various oceanic conditions and probe perturbations involving pH, light, temperature, and nutrient concentrations. Because these systems have a continuous flow of medium in and out of the system, longer-term acclimation to chronic stress conditions can be explored. While using PBRs, cells can be easily monitored through a series of metrics that include: fluorescence, cell counts, pH, forward scatter, Fv/Fm, biovolume, and growth rate.

Although interactions between Tp and Rpom have been previously studied, most studies have focussed on batch culture systems and have not addressed the role of chronic nutrient limitation in these interactions. To address this gap in knowledge this study investigated physiological responses of Tp while in co-culture with Rpom under vitamin and nutrient limitation, within a dynamic continuous culture system. By utilizing a controlled dynamic environment (PBRs), this study allowed for the examination of Tp responses under sustained limitation.

This study hypothesized that vitamin or nutrient limitation conditions would result in physiological changes in Tp, including changes in fluorescence, biovolume, growth rate, and photosynthetic efficiency (Fv/Fm). Additionally, under zero B12 conditions, B12 produced by Rpom will facilitate Tp growth and survival. Together these hypotheses aim to test how interactions between diatoms and bacteria can change diatom physiology, while also testing the sustained usage of a PBR.

METHODS

Preparation of Media:

F/2 was made by using an artificial seawater medium (Harrison et al., 1980) along with sodium phosphate ($3.62E^{-05}$ M), sodium silicate ($1.06E^{-04}$ M), and sodium nitrate ($8.82E^{-04}$ M). During manipulation periods, nitrate and B12 were changed in the F/2 media. For the 50 μM nitrate media only 56.68 microliters per liter nitrate stock at a concentration of $8.82E^{-1}$ M was added to the F/2 media. The vitamin solution was mixed beforehand at its normal concentrations for biotin and thiamine, however no B12 was added to the vitamin stock solution.

Setting up PBRs:

In a sterilized laminar flow hood, the culturing vessels were prepared one at a time. Once PBR was placed into the hood, a peristaltic pump was added with its sterilized tubing and culture vessel. The autoclaved vessel caps were placed onto the ports of the PBR, along with the outflow ports. Once the outflow ports were connected, sterile tubing was attached to the vessel and also to the media bottle. Once the media bottles with one way valves were attached, the valves were closed. At the top of the vessel, the screws were removed in order to replace the screw with pH and temperature probes. Before probes were placed into the vessel they were sterilized with ethanol. Outflow tubing was then attached to the outflow port at the top of the vessel, once attached, the outflow port was closed, then attached to the outflow bottle. Lastly the glass window was carefully placed on top of the vessel, then the PBR was removed from the hood. Once the PBR is secure within its stand and temperature jacket, the heating ring and light fixture were carefully placed on top of the culture vessel. This process was repeated for the remaining PBRs. See Figure 1 for detail on the PBR set up. Once all PBR were set up, 400ml of media was added to each of the culture vessels. Once the PBR was running and all parameters were turned

on Tp could be inoculated into the vessels. These parameters include: light, temperature, pH, flow rate, and mixing. Finally, a timepoint zero was taken to monitor initial Tp concentration in the chamber. During the first few days of the pregrowth stage, Tp was monitored closely to assure the culture started off healthy and acclimated to a dynamic environment. After Tp is inoculated, cover sampling ports with ethanoled aluminum foil in order to prevent contamination of the sampling port.

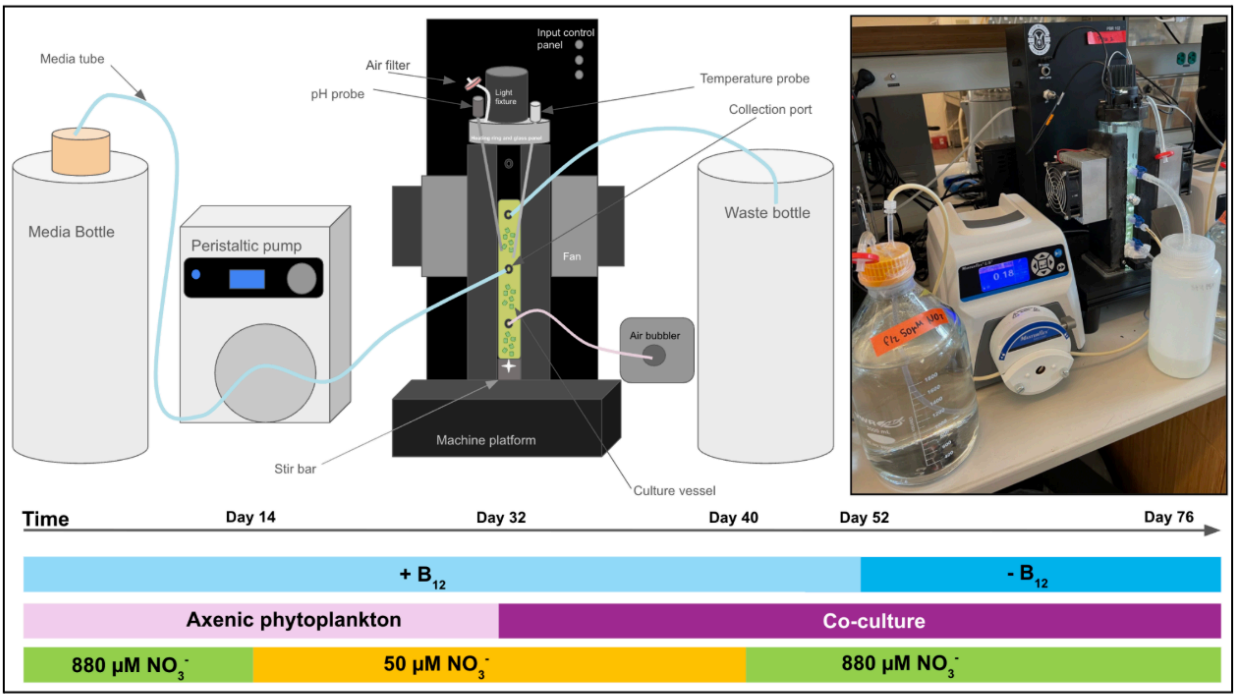

Figure 1: Diagram of PBR machinery and image of physical set up. The left panel is a diagram with labels of PBR machinery that is essential for our experiment. The panel on the right is a physical image of what our set up looked like during the experiment. All four PBRs were set up in the same manner along one single lab bench. The bottom panel describes the experimental treatment types as time goes on.

Management of diatom species:

Thalassiosira pseudonana (CCMP 1335) (Armbrust et al., 2004) (Robert Guillard 1958) was grown up in F/2 medium, once at a healthy and dense culture cells were treated with a series of antibiotic treatments in order to assure the cultures were axenic. Cells were treated with a series of antibiotics to ensure cleanliness. The first was Provasoli's antibiotic (Sigma P8029)

concentrated solution added at final concentrations of: 480 units/mL penicillin G, 2 µg/mL chloramphenicol, 12 units/mL polymyxin B, and 2.4 µg/mL neomycin. 750-1000 microliters of this cocktail was added to 25ml of culture medium. Between each treatment, 1 mL of treated culture was transferred into a new flask with 24mL of F/2 media. The second treatment was 6.25mg of Cefotaxime in 25 ml of culture. Lastly, 12.5mg of Carbopenicillin was added to 25 ml of media. After cells were treated and checked through microscopy and streaking for cleanliness, cells were counted with an end goal concentration of around 10^6 cells per milliliter. Once this was assured, cells were added to each culture vessel at 10^6 cells per milliliter using a sterile syringe into the sampling port.

Bacterial Addition:

The bacterial species *Ruegeria pomeroyi* (DSS-3) (Moran et al., 2004) was grown on ½ YTSS (in 500 ml, 2 g yeast extract (BD Biosciences), 1.25 g tryptone (BD Biosciences), 10 g sea salts (Sigma-Aldrich) (Gao et al., 2020). Then, a single Rpom colony was inoculated into liquid ½ YTSS media and grew up for another 24 hours at 30 degrees celcius. On the day of experimental additions to the PBR environment, the cells were washed. Before adding into the PBRs, bacterial cultures were spun down at 4,000 G for five minutes and the supernatant was removed. The cells were resuspended in F/2 medium and respun down. In total cells were washed five times in order to prevent mixing of mediums within the culture vessel. After final the wash cells were counted using FCM and microscopy. Once Rpom was counted it was added to each PBR using a syringe needle through the sampling port with a final concentration of 10^6 cells/ml.

Sampling methods:

Samples were taken everyday at the same time in 24 hour increments, 1:00 pm was chosen for this experiment's sampling time. Flow cytometry data was taken everyday, each PBR had three replicates. Once the 1pm timepoint occurred, media bottles were weighed in order to calculate the amount of volume that flowed out of the media bottle between timepoints. This value was for the weight based media dilution calculation Then one milliliter from each of the PBRs was removed using a 3ml syringe. The syringe was gently inserted into the sample port and slowly pulled culture out of the vessel. Then an ethanoled foil was placed over the sampling port to prevent contamination. This 1ml aliquot was placed into separate epi tubes. This was repeated for the remaining PBRs. The aliquots were then divided up into separate sampling methods that included: flow cytometry, Fv/Fm, microscopy, and a sample to freeze for secondary lab analysis.

Flow cytometry:

Flow cytometry samples were acquired on a BD Accuri C6 Sampler using the C-Flow software (v 1.0.227.4). In a 96 well plate, 180 ul of F/2 was added and 20 ul of the allocated PBR sample per well. A dilute sample was used in order to prevent clogging of the flow cytometer. Between each PBR (set of three wells) a blank well was placed. Having a blank in between each triplicate decreased the amount of Tp cells transferring between PBR triplicates. The cytometer was run on medium speed (35 ul per minute) for one minute.

Multiple methods for calculating Tp cell densities were utilized. First, after every time point cells were individually gated per time point using the BD Accuri C-Flow software (v. 1.0.227.4). This was a root of variability and inconsistency within the data set because each time point cells were gated differently per day, thus the gating was not uniform. Due to this variable

gating system, this method was not used. A second method used involved gating the cells three times. This gating is different from our final method applied because it includes a gating for single cells only. This method was voted out because it was removing almost ten fold cells from our final cell count. This was most likely due to cells being stuck together during flow cytometry or possibly gating out large cells. The final method tested was chosen to count cells due to its robustness and consistency with the original cell counts found per time point using the first method.

Cell Gating Methods:

All flow cytometry files were removed from the desktop computer and converted into ".fcs" files that were analyzed using FlowJo V10 software. All replicates were analyzed using the same gate in order to remain unbiased between conditions. First, Tp cells were gated by alive or dead cells by choosing the most dense population. The first gating had an x-axis of FSCA (forward scatter) and a y-axis of SSCA (side scatter). After the initial gating, a second gate was added using a FL3A histogram that blocked out all cells not fluorescing at a high enough level (y-axis FL3A and x-axis histogram). The second gate assured that all cells captured in the first gate were alive and fluorescing. Once all samples were gated the following metrics were analyzed: fluorescence, cell counts, forward scatter, side scatter, and the volume of the well analyzed. Individual cell counts per milliliter were calculated using the number of cells counted in cells per microliter divided volume analyzed in microlitters then multiplied by 1000 to get cells per milliliter. Finally, per day all values were averaged by PBR, for example each sample day each PBR had three replicates tested using flow cytometry with a total of 12 wells, thus every day 4 values per each parameter measured.

$$cells = \left(\frac{events}{vol\ sampled} \right) \cdot 1000$$

Photosynthetic efficiency:

To measure photosynthetic efficiency, samples were incubated in the dark for 15 minutes and then 200 ul of each sample was added to 1,800 ul of F/2. The diluted samples were analyzed using a Walz WATER-PAM fluorometer, and the Fv/Fm, the ratio of variable fluorescence over maximum fluorescence after dark adaptation, was recorded (Hao et al., 2021). Fv/Fm was measured daily.

Biovolume:

Microscopy was conducted on a Zeiss Axio Imager M1 using a Zeiss Axiocam 820 mono camera and the Zeiss Zen software (v. 3.10). Cell length and width was measured with ImageJ.JS online browser and only cells oriented with the length and width visible were used to calculate biovolume. Once two measurements were taken per cell, all Tp cells were assumed to be cylindrical (Hillebrand & Durselen, 1999; Sun & Lu, 2003). Cell biovolume was calculated at least four times per replicate per treatment type using the below formula for the volume of a cylinder:

$$V = \frac{\pi}{4} \left(\frac{\text{width}^2}{\text{length}} \right)$$

Biovolume trends were confirmed using forward scatter since both are proxies for cell size. FSC is the result of diffractions collected along the same axis as the laser beam that is pulsed through the cell when it is counted in the cytometer. This metric can be proportional to cell surface area or detecting larger sized particles (Adan et al., 2017). FSC was compared to

biovolume because of its robustness, biovolume most likely had human error from by hand measurements.

Flow cytometry gating methods:

All flow cytometry files were removed from the desktop computer and converted into ".fcs" files that were analyzed using FlowJo V10 software. All replicates were analyzed using the same gating in order to remain unbiased between conditions. First, Tp cells were gated by alive or dead cells by choosing the most dense population. The first gating had an x-axis of FSCA (forward scatter) and a y-axis of SSCA (side scatter). After the initial gating, a second gate was added using a FL3A histogram that blocked out all cells not fluorescing at a high enough level (y-axis FL3A and x-axis histogram). Finally, per day all values were averaged by PBR, for example each sample day each PBR had three replicates tested using flow cytometry with a total of 12 wells, thus every day 4 values per each parameter measured.

Smoothing growth rates:

In order to smooth out growth rates and assign steady state periods, growth rates were calculated using a moving average. The moving average was calculated using a moving three day average. The cell counts and dilution rates were both averaged before calculating the growth rates. Comparable growth rates had dilution by weight added, while when calculating steady state, dilution was not added. Each method for smoothing out growth rates was tested to assure that it was not removing a large variability. The following tests were conducted: repeated ANOVA and linear mixed-effects model.

$$GR = dilution + \left(\ln \frac{\left(\frac{cells_2}{cells_1} \right)}{\left(day_2 - day_1 \right)} \right) \qquad Steady\ State\ GR = \left(\ln \frac{\left(\frac{cells_2}{cells_1} \right)}{\left(day_2 - day_1 \right)} \right)$$

Choosing steady state periods:

Parameters were analyzed only using steady state periods. Steady state periods were chosen by reviewing physiological responses along with growth rate calculations. Steady state periods were chosen in order to differentiate most between treatment types during analysis. Choosing steady state periods per treatment type was difficult, since in this experiment it did not follow ideal conditions where dilution rate equals growth rate. In these light periods of time where FSC, cell counts, and fluorescence seemed stable for at least three days were concluded to be in steady state. While variations between treatment types with transitional periods still had variation, the variation between steady states is greater. These perturbation events could have been due to bottle changes, flow rate changes, or full sampling time points. It is important to note that included in this experiment larger time points also occurred that would remove 200 ml from each culture vessel, these samples were taken for transcriptomics and metabolomics. This large removal of culture from the vessel is a large cause of perturbation events within this experiment. Due to these events, some periods of time could not be considered steady state. Steady state periods were chosen to be two days after a perturbation event to confirm the culture had acclimated to the event. Steady state is considered to be achieved when net growth rate is equal to the dilution rate, this is also when physiological changes are considered to be at a minimum (Lucker et al., 2014). Thus if you were to increase the dilution rate past the growth rate, you would wash out cells from the system (Okay et al., 2001).

$$Steady\ state = dilution\ rate = growth\ rate \qquad dilution = \frac{\left(weight_2 - weight_1\right)}{400}$$

In order to confirm steady state periods, the individual variation (standard deviation of the replicate mean) in Fv/Fm, forward scatter (FSC), and relative fluorescence (FL3) within a

proposed steady state period was compared to the variation for each replicate across the entire experiment. Steady state was defined when the variation for all three parameters during that period was less than the overall variation, with the exception of the B12-limitation period due to high variation in Fv/Fm throughout the treatment.

Statistical Analyses:

All statistical analyses and data visualization were performed in R version 2023.12.1+402 using the packages dplyr, tidyr, stringr, ggplot2, ggh4x, patchwork, ggnewscale, car, multcompView, lme4, lmerTest, modelsummary, and emmeans. For all statistical analyses used to compare treatment types, ANOVA was used. In order to check for normality and variance, a Shapiro and Levene test were conducted. If tests for normality and variance failed, a non parametric Kruskal-Wallis test was conducted (Table 1).

Determining cellular proxies:

Parameters were considered in pairs in order to compare cellular effects. The following pairs were used to explain these measurements: cell size (biovolume and FSC), cell physiology(fluorescence and Fv/Fm), cell population (Cell counts and pH), and lastly growth rate was a considered as factor on its own due to its variability and its dependence on other cellular factors. pH was considered a proxy for cell population due to the ability of diatoms to change pH dependent on carbon drawdown. Low pH is associated with decreased cell counts.

RESULTS

PBR machinery:

PBRs were successfully run for a total of 76 days including the pre-growth period. Four PBRs were used in order to assure biological replication. All four PBRs successfully followed similar trends during the 76 day period. Throughout the experiment there were five different treatments for Tp using a baseline of F/2 medium with differing nitrate and B12 concentrations: 880μM nitrate axenic culture (14 days) [880axenic], 50μM nitrate axenic (18 days) [50axenic], 50μM nitrate plus Rpom (8 days)[50Rpom], 880μM nitrate plus Rpom (12 days)[880Rpom], and a 880μM nitrate plus zero B12 plus Rpom condition (24 days)[0B12] (Figure 1). All treatment types successfully produced data for analysis.

Treatment variability:

When comparing treatment effects on measured parameters an ANOVA was used in order to assure the parameter had confirmed variance and normality. For biovolume and growth rate, ANOVA was used to assure the null hypothesis with p values of $5.13e^{-11}$ and $9.02e^{-05}$. The kruskal-wallis test was used for Fv/Fm ($5.88e^{-07}$) and fluorescence ($1.52e^{-13}$) since they failed both assumptions for normality and variance. For the last three parameters at least one test for normality or variance failed so a kruskal-wallis test was utilized for cell counts ($3.59e^{-05}$), forward scatter ($1.32e^{-12}$), and pH ($6.10e^{-06}$). Even though all tests passed ANOVA, further statistical analysis was used to assure the assumptions. All parameters passed the Kruskal-Wallis test. Overall, each parameter was confirmed to be statistically different between treatments to a significant extent ($p<0.001$). After confirming that treatments were statistically different, a Tukey HSD test was utilized in order to assess which treatment groups were different from each other (Table 1).

Tp cell size and structure: Biovolume and FSC:

Tp cell size and structure varied significantly between treatments. Biovolume (cell size) differed over time with increases in cell size under nutrient enriched and zero B12 amendment media conditions when Rpom was present (Figure 2). Mean biovolume (um^3) was highest in zero B12 conditions (61.955) and again when Rpom was present in 880µM nitrate conditions (50.404), with the lowest biovolume being in 880µM nitrate axenic conditions (28.852) (Table 2). B12 starvation conditions were significantly greater than 50 µM nitrate axenic, 50 µM nitrate Rpom, and 880µM nitrate axenic ($p<0.001$), with 880 µM nitrate Rpom also significant ($p<0.05$) (Table 3). Biovolume increased throughout the treatments over the 76 day period (Figure 2). Forward scatter (cell size proxy) also followed a similar pattern, but had a major decline during the 50µM nitrate axenic period (Figure 2). Forward scatter (FSC) is another metric found using a flow cytometer, this metric can be proportional to cell surface area or detecting larger sized particles (Adan et al., 2017). One way to confirm FSC is to visually measure cells, or get their biovolume. These metrics can then be input into a formula that best suits their geometry, in Tp's case it follows a cylindrical (Sun & Lu, 2003). Notably, zero B12 condition still had the highest FSC and biovolume. This was confirmed to be statistically significant from all other treatments (Table 3). Unexpectedly, 880µM nitrate axenic, 50µM nitrate axenic, and 50µM nitrate Rpom were found to not be statistically different even though the presence of nitrate is drastically different between the treatments (Figure 3).

Tp productivity: Fluorescence and Fv/Fm:

Similarly to the trends seen in biovolume and FSC, fluorescence and Fv/Fm also displayed trends that seem to mimic each other. Fv/Fm or the photosynthetic efficiency is the ratio of variable over maximum fluorescence after dark adaptation also known as Fv/Fm (Hao et

al., 2021). Secondly, fluorescence (photosynthetic activity) can be measured using flow cytometry, when a cell is fluorescing more they are likely to be more healthy and perform more photosynthesis.Over time, fluorescence exhibited a dramatic decrease during the 50µM nitrate axenic conditions then increase again once in coculture with Rpom, then increasing when nitrate is back up to 880µM nitrate, and finally following a small dip toward the end of the experiment where there is no B12 within the culture vessel (Figure 2). Notably, fluorescence had distinct mean values in the box plots shown (Figure 3, Table 2). However, in the Fv/Fm plot, the values are much more grouped together with only four comparisons having distinct significance levels, out of which 880µM nitrate Rpom showed to have the most distinctions (Table 3). One thing of note is that during the 50 µM nitrate Rpom condition, Tp fluorescence and Fv/Fm did not follow the same pattern. Tp fluorescence decreased during the 50 µM nitrate axenic condition, while Fv/Fm slightly increased (Figure 2 and 3). Secondly it appears the fluorescence decreased more during the zero B12 period, denoted by a new grouping, while in Fv/Fm, 880µM nitrate Rpom, and zero B12 plus Rpom are denoted with the same grouping (Figure 3).

Cell population: Cell counts and pH:

Cellular population proxies followed similar trends for cell counts and pH, however pH had more statistical differences between groupings. Figure 2 displays the replicates over time have similar spikes in pH and cell counts, however cell counts are much more variable. Mean cell counts in the same grouping for treatments: 880µM nitrate axenic (2383333.333), 50µM nitrate axenic (1,887,125), 50µM nitrate Rpom (16,49,166.667), and zero B12 (1,213,208.333), however 880µM nitrate Rpom (4,094,000) (Table 2) was considered to be in its own grouping having extreme significance ($p<0.001$) (Table 3). More notably pH had more significant groupings for its Tukey HSD results with 50µM nitrate Rpom and zero B12 forming a new

statistical group ($p<0.01$) (Table 3). In both cases for cell counts and pH, 880µM nitrate Rpom had the highest distinction and highest values (Figure 3). Notably, this increase in cell counts likely contributed to the increase in pH due to the drawdown of carbon dioxide (CO_2) in the system.

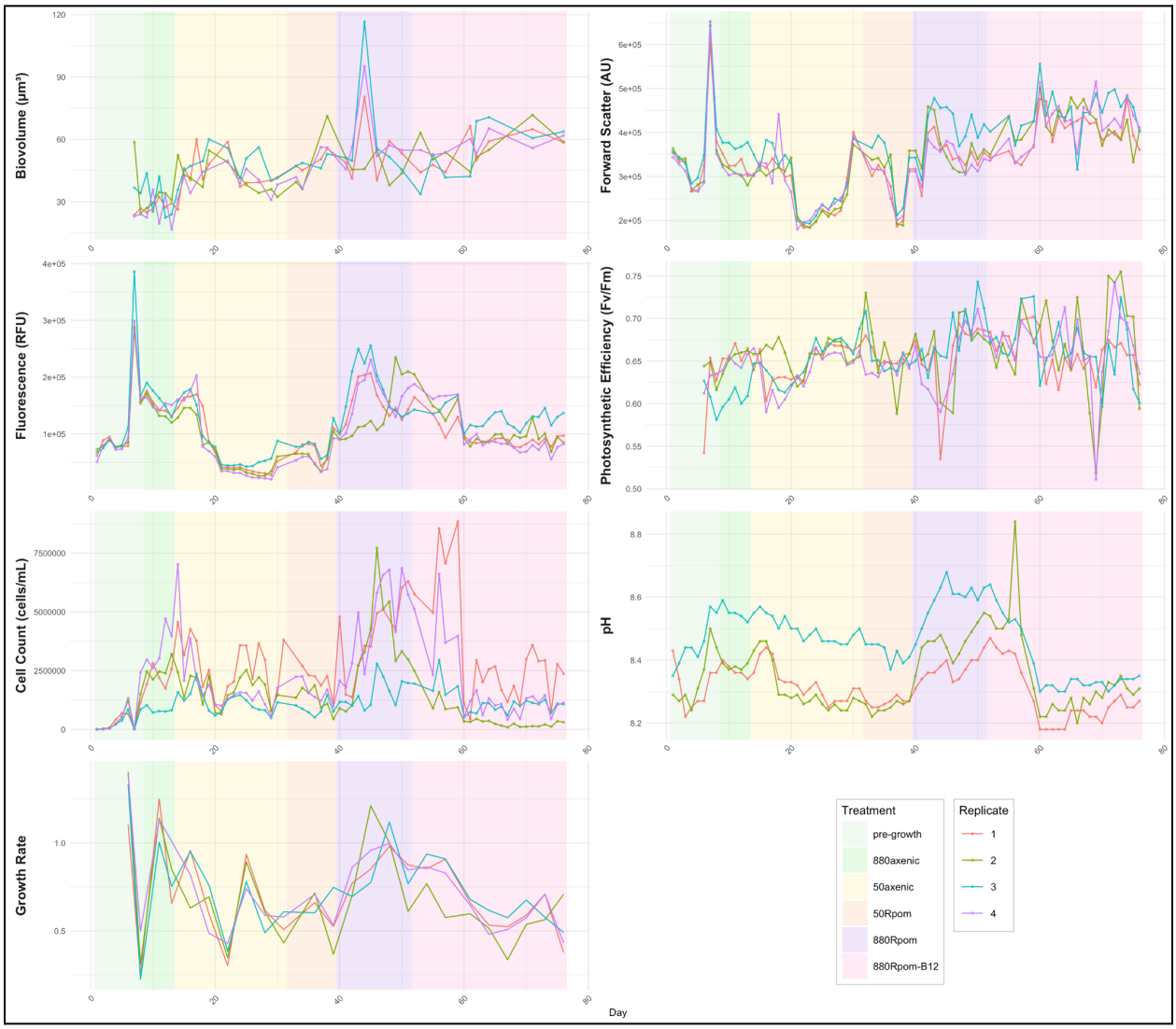

Figure 2: Time series of biological and physiological responses under varying treatment conditions. Panels show changes in biovolume (µm³), cell concentration (cells mL^{-1}), fluorescence (RFU), forward scatter (arbitrary units), photosynthetic efficiency (Fv/Fm), growth rate (d^{-1}), and pH. Each line represents a replicate PBR1 (red), PBR2 (green), PBR3 (blue), and PBR (purple). Shaded regions behind the lines denote the different treatment types.

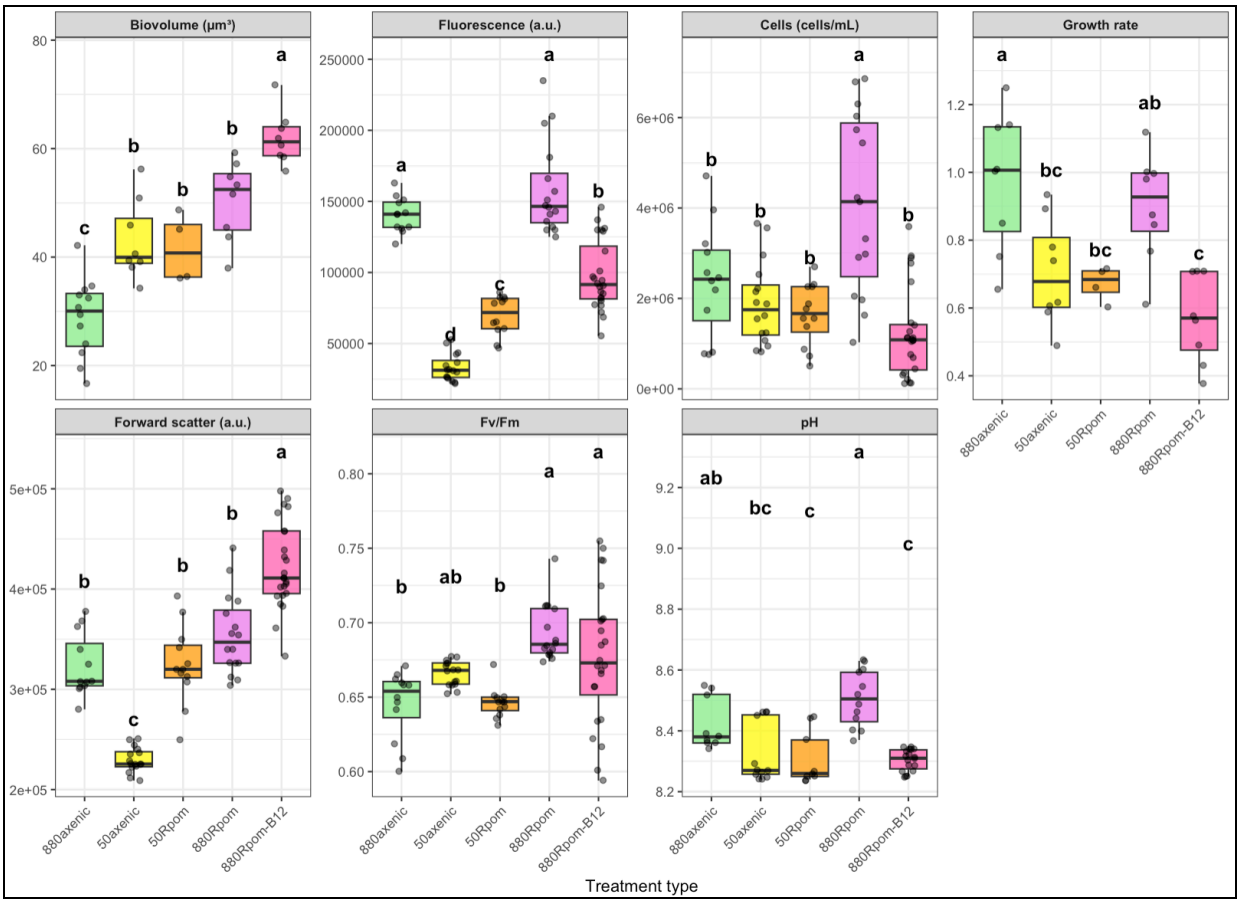

Figure 3: Steady-state biological responses across treatment conditions. Box plots show the distribution of various parameters including: biovolume (µm³), cell concentration (cells mL^{-1}), fluorescence (a.u.), forward scatter (a.u.), photosynthetic efficiency (Fv/Fm), growth rate, and pH across five treatment types (880 axenic, 50 axenic, 50 Rpom, 880 Rpom, and 880 Rpom + B12). Points represent individual replicate measurements, while boxes represent the interquartile range of each parameter. Letters above each box represent the results from Tukey's significant difference (HSD) post hoc tests, following one way ANOVA. Treatments that do not share a common letter are significantly different ($p<0.05$).

Cellular growth rate:

Growth rate was analyzed separately from other parameters due to its variability and overall effect on cell population. Specifically a moving average was used instead of a daily timepoint. Specific growth rates (µ) were variable (Figure 2), however when referring to Table 3, there is a statistical difference between growth rates. Growth rate was determined by taking a three day moving average. By referring to Figure 4 it is noted that there is extreme variance between methods to measure growth rate, the every day growth rate seeming to fluctuate

inconsistently, the every other day removing some variance, and finally measuring growth rate every three days smoothing out the line. In comparison with Figure 5, that portrays box plots of each of the methods during each treatment, again showing measuring growth rate every three days helped remove some variance. When comparing methods to measure growth rates a repeated anova test was used to confirm that the method in which growth rate was measured did not affect overall trends, this was found to have weak to no effect ($p = 0.095$) (Table 4). One more method used to assure there was no effect on treatment was a linear mixed effects model, this result had no interaction on treatment ($p = 0.957$) (Table 4).

After confirming the method of tracking growthrate, steady state periods were tested and added to the steady state box plot Table (Table 2). The mean averages of each growth rate from smallest to largest are as follows: zero B12 plus Rpom (0.571), 50µM nitrate Rpom (0.672), 50µM nitrate axenic (0.706), 880µM nitrate Rpom (0.899), and lastly 880µM nitrate axenic (0.974) (Table 2). The grouping showing largest statistical differences were zero B12 plus Rpom having the lowest growth rate and 880µM nitrate axenic having the highest growth rate ($p<0.001$) (Table 3). Secondly with 50µM nitrate conditions both axenic and Rpom to both be grouped similarly with no significance between them. It is important to note that it looks like Rpom is changing growth rate because of the large differences during 0B12 (figure 2), however this is most likely due to nutrient limitation and not the addition of the bacterial species.

Overall, all of the results provided indication that an increase in nitrate and bacterial additions influence Tp physiological parameters. Some changes occur when Rpom is added, and the strongest changes occur when B12 is completely eliminated from the media and Rpom is present. Refer to figure six for an artistic representation of Tp change in biovolume and fluorescence over time.

DISCUSSION

In this study, Tp was successfully co-cultured with Rpom in a continuous culture system under nitrate limitation and B12 starvation for 76 days. This could not have been achieved within batch culture experiments due to the expenditure of nutrients and die off within batch culture, not allowing for a continuous nutrient limited state. While Tp has been previously cultured in batch cultures with Rpom, their interactions under continuous culture systems has not been explored, especially the physiological response of Tp cells in these conditions. Previously Rpom cultured axenically will not release traceable amounts of B12, this experiment expresses that when in the presence of a diatom Rpom will release B12, and support the survival of the auxotrophic diatom (Mars Brisbin et al., 2023).

The ability to sustain the co-culture under nutrient limitation demonstrates that the interactions between species can remain stable under continuous culture conditions. This was of concern due to the ability of the bacterial cells to "wash out" of the PBR outflow system. In this experiment the lowest bacterial cell counts were 1.8 $E10^6$, if cell counts were to decrease any further, there would be a decrease in interactions between species.

It is important to note that this experiment was successful in achieving steady states within a 76 day experimental period within the PBR culturing system, while also observing several physiological changes dependent on nutrient limitation. Specifically how biovolume was largest under zero B12 amendment media, and lowest in 880 nitrate replete media. Secondly, that fluorescence was highest under 880 nitrate replete media conditions. Observing these large physiological changes infers that the addition of Rpom does affect Tp physiology while also providing Tp necessary B12 to survive in zero B12 amendment media.

Decreased fluorescence and FSC induced by nitrate limitation:

Under nitrate limitation, Tp experienced an overall negative effect regardless of the presence of Rpom compared to the nitrate replete conditions. During axenic, nitrate limited conditions, there was a decline in Tp fluorescence, FSC, and growth rate, similar to findings from previous studies on nitrate limitation in Tp (Hennon et al., 2014) (Lin et al., 2017). However, a smaller inhibition of Fv/Fm was measured in our study than previously observed (Lin et al., 2017). Nitrogen limitation decreases the abundance of structural proteins in Tp leading to reduced efficiency in photosystem I and II, and therefore lower Fv/Fm (Lin et al., 2017). However, the continuous culture system may provide enough nitrogen even under nitrate limitation to alleviate this effect.

When Rpom was added to the 50μM nitrate condition, Rpom seemed to alleviate some stress that was shown in some of Tp's physiology. However Tp did not receive benefits in terms of cellular performance which would have been indicative in an increased Fv/Fm and growth rate. Instead, when Tp was in coculture with Rpom, it seemed to have increased FSC, biovolume, and fluorescence; these attributes are more indicative of physiological changes. When in limitation it is possible that fluorescence is still present however if photosystems are impaired due to the decreased flow of electrons, Fv/Fm would not increase (Chokshi et al., 2017). However, without transcriptional data it is uncertain if a specific process is occurring. We hypothesized that the presence of Rpom may help alleviate the effects of nitrate limitation on Tp by remineralizing the organic forms of nitrogen released by Tp and then releasing ammonium that could be taken up by the diatom. However, Tp growth rate was still low during nitrate depletion, matching results from a previous experiment with Tp (Parslow et al., 1984). When under nitrate limitation Tp is also accumulating several lipids (Bromke et al., 2013), these lipids

if released extracellularly would be of use to Rpom in the form of organic carbons. This study hypothesizes that Tp is releasing lipids extracellularly for Rpom to utilize as a carbon source.

Overall, the addition of Rpom in nitrate limited conditions did not improve cellular performance, but it did alter the physiology of Tp by increasing the forward scatter and chlorophyll fluorescence. It is important to note that this study is one of the first to add bacteria in a continents culture system while a diatom is under nutrient limitation, thus comparing it to other studies can be relatively challenging. However, this study aims to produce results that show how nitrate limitation affects cellular physiology and elucidate how nutrients affect diatom growth.

Increased cell size under zero B12 amendment media:

In this experiment, Rpom was the B12 producer and sole supplier for Tp under zero B12 amendment media. The survival of Tp during the zero B12 amendment media period indicates a beneficial interaction between Rpom and Tp, and supports previous studies that found Rpom provides Tp with B12, while the Tp provides Rpom with fixed carbon (Croft et al., 2005). Under zero B12 amendment media with Rpom, Tp cell size increased both in terms of biovolume and FSC compared to normal B12 conditions with Rpom. This was unexpected because nutrient limitation generally causes diatom cells to decrease in size. This increase in cell size could possibly be due to an increase in cellular storage of carbon, nitrogen, or phosphorus. Another hypothesis proposed that Tp could also possibly be remobilizing carbon stores (Hockin et al., 2012). The remobilization of carbon stores could be due to cascading effects on changing cellular physiology. Due to decreased growth rates under zero B12 amendment media, there would be a decreased demand for fixed carbon and biosynthetic precursors that would be used for reproduction (Bertrand et al., 2013; Geider et al., 1997). The accumulation of carbon

intracellularly leads to an increased risk of over-reduction in photosynthetic electron transport chains and the formation of reactive oxygen species (Asada, 2006; Niyogi, 1999). This redox imbalance could be causing Tp to redirect its carbon storage in the form of triacylglycerols and chrysolaminarin, which act as metabolic electron sinks (Bailleul et al., 2015; Hu et al., 2008). This cascading effect can be described as "overflow metabolism" since the excess intracellular carbon is stored (causing increased biovolume) instead of used for cellular growth in the form of multiplication. Overall, the increased biovolume and FSC under zero B12 amendment media conditions suggest that Tp is most likely storing carbon internally rather than utilizing it for reproduction.

This experiment also showed that under zero B12 amendment media Tp experiences reduced chlorophyll fluorescence and cell density. In a similar study involving how Tp grows under zero B12 amendment media, they also established that the cell density and relative fluorescence decreased under reduced B12 concentrations (Sultana et al., 2023). The Sultana study also established that when a provider bacteria strain is not present, Tp will not grow, however the species of bacteria present can influence how much the diatoms will grow. This gives us some insight into what an 880μM nitrate and zero B12 condition could have looked like in our PBR experiment. This provides insight into how the bacterial community can influence vitamins available in times of limitation for phytoplankton species like in blooming conditions. In a similar semi-continuous culture studies, they were also able to coculture phytoplankton with bacteria (*Chlamydomonas nivalis* and *Mesorhizobium*) (*Ostreococcus tauri* and *Dinoroseobacter shibae*) exhibiting a relationship forced by zero B12 amendment media (Kazamia et al., 2012) (Cooper et al., 2019). They found that there is a series of complex relationships revolving around the sharing of B vitamins due to the complexity of molecular shape and complex formation

(Kazamia et al., 2012) (Cooper et al., 2019). These other relationships indicate that the sharing of B12 is common, thus more research on bacterial interactions could increase the understanding of how this vitamin may be a main point of interaction for diatoms and bacteria.

Lastly, one observation of note is that even when Rpom is present under zero B12 amendment media, there are still observed physiological changes, even though B12 is provided by the bacterial species. The physiological changes under zero B12 amendment media include: a decrease in growth rate, fluorescence, and amount of cells present, but notably Fv/Fm was not significantly affected. The insignificant change in Fv/Fm suggests that Tp could be buffering, reducing power, and preventing the photoinhibition, despite nutrient stress (Maxwell & Johnson, 2000). The overall minimal changes to Fv/Fm highlight that nutrient limitation in diatoms is not only a growth constraint, but also a complex reorganization of metabolic processes in order to remain redox homeostasis and cellular integrity.

Overall, zero B12 amendment media had a strong effect on Tp cell physiology, but these changes were different from nitrate limitation effects. Tp is a model diatom, its physiological changes can provide insight into how oceanic limitation can affect other species of diatoms leading to possible future study.

pH and hypothesized carbon drawdown:

CO_2 draw down is of specific interest in this study especially when comparing to oceanic conditions. Currently the ocean is acidifying rapidly due to anthropogenic impact, understanding how acidification can harm diatoms is of specific interest. Within this study there were a series of pH changes within the system. For example, when nutrients were limited cell density and pH decreased, while in replete conditions cell density and pH increased. Since ambient air from the lab was bubbled into the system and not pure CO_2, it is possible that CO_2 was a limiting factor

for Tp growth under high cell densities. Since CO_2 concentrations were not measured, pH was indicative of CO_2 drawdown. Within our experiment pH rose up to about 8.4-8.5, this is strikingly different to oceanic conditions which usually sit around 8.07 (Jiang et al., 2019). A similar study resulted in a significant increase in carbon fixation rates, causing a decrease in pH for Tp (Shi et al., 2019). In oceanic conditions that are limited by nutrients one could suspect that the carbon fixation rates would decrease, while in areas with replete nutrients and lots of diatom activity, carbon fixation rates would increase. The relationship increased cell populations have with the increased amounts of photosynthesis they can undergo could give us insight into how larger populations can fix more carbon. However, the role that marine bacteria play in these regions should be studied further in order to assess how they could possibly alleviate pH fluctuations by producing viable products for diatoms, or instead also decreasing pH due to cellular respiration and increasing the amount of CO_2.

Challenges of continuous culture systems:

Continuous culture systems are not without their caveats. It is important to understand how the state of a continuous culture system can be perturbed or negatively impacted in minor ways. For example, in this experiment the perturbation effects were observed when the media bottles were changed. This can cause changes in cellular physiology depending on treatment type. In order to consider a steady state period it must be considered if the system was perturbed during that period. A point of large perturbation events in this experiment was due to periodic RNA and metabolite sampling where 200ml of culture was removed from the 400 mL chamber . This sampling was only performed once during each steady state and transitional state in order to decrease the perturbation effect, and this had minimal impact on Tp physiology. As time progressed throughout the experiment, the sampling port seals developed small leaks, causing the

PBRs to lose an unmeasurable amount of culture. One benefit of PBRs is that they are closed off to the outside environment which decreases the risk for contamination, but PBR culture vessels have a small surface area to volume ratio for lighting due to its cylindrical shape which may cause some shading during dense culture conditions (Chanquia et al., 2022). Finally, PBRs also have many probes used to monitor the culture, some however can fail. For example, this was the case with the pH probe in PBR 4, which was found to not measure pH properly. Overall, while PBRs are extremely useful in keeping cultures at exponential states and in constant limitation they do have some limitations and important factors to consider before using.

Future directions

Within this study, the physiological responses of Tp have been explored under multiple types of limitation, however it is clear what mechanisms underlie these changes, since only physiology was analyzed. Based on other papers it can be inferred what other mechanisms are being regulated. When B12 is limited it has been previously observed that the cobalmin acquisition protein synthesis, specifically the CB1A protein, was upregulated as a response to try and uptake more B12 (Bertrand et al., 2012). When Tp is in zero B12 amendment media, thus only receiving B12 from Rpom it is important to assess if similar mechanisms are being upregulated due to the increase in B12 demand. Another important point of future study is to assess what is initiating the relationship between a diatom and bacteria interaction. In a similar study from Durham in 2015, with the coculture of Tp and Rpom, it was found that Rpom was upregulating its metabolism for 2,3-dihydroxypropane-1 sulfonate (DHPS). Possibly meaning that Tp is releasing DHPS and cuing the relationship between the two species (Durham et al., 2015). They raise the question on the importance of this metabolite between the two species and how sulfur metabolism is involved with limitation. These inferences from other experiments will

be studied in depth for future directions of this research paper and will be studied by analyzing metabolomics and transcriptomics data taken throughout the experiment during steady state periods.

Diatoms are constantly being manipulated by their environment, understanding how these limiting nutrients and vitamins affect diatoms allows us to better predict how oceanic processes can affect phytoplankton productivity. Thus, understanding how Tp and Rpom are able to coexist in a dynamic setting like the open ocean, has important implications for understanding diatom interactions with other species of bacteria. The upkeep of a continuous culture system under nutrient limitation could have potential applications in algal biotechnology, where the microbial interactions could possibly produce metabolites that are viable for industry.

CONCLUSION

This study is important for providing insight into how dynamic conditions can influence diatom growth under nutrient and vitamin stress. The bacterial community present during times of limitation can play an important role in B12 and nitrogen available to phytoplankton species. It is clear that B12 and nitrate limitation have a large impact on cells, but more investigation into metabolomics and transcriptomics is needed in order to assess its impact on cellular processes when bacteria is present. However, it is important to note that B12 had differing effects compared to nitrate. B12 starvation appears to affect Tp physiology more than any other limitation parameter.

In terms of oceanic context it is important to note that this study is set in a dynamic continuous culture system, which is different to many other studies already conducted that do studies in batch culture systems. The coculture of both species in an environment that mimics oceanic conditions allows us to provide insight into what types of mechanisms are being upregulated in environmental conditions.

This thesis is coauthored with George, Emma and Hamilton, Maria. The thesis author: Broersma, Kaitlyn was the primary author of this chapter.

SUPPLEMENTAL FIGURES

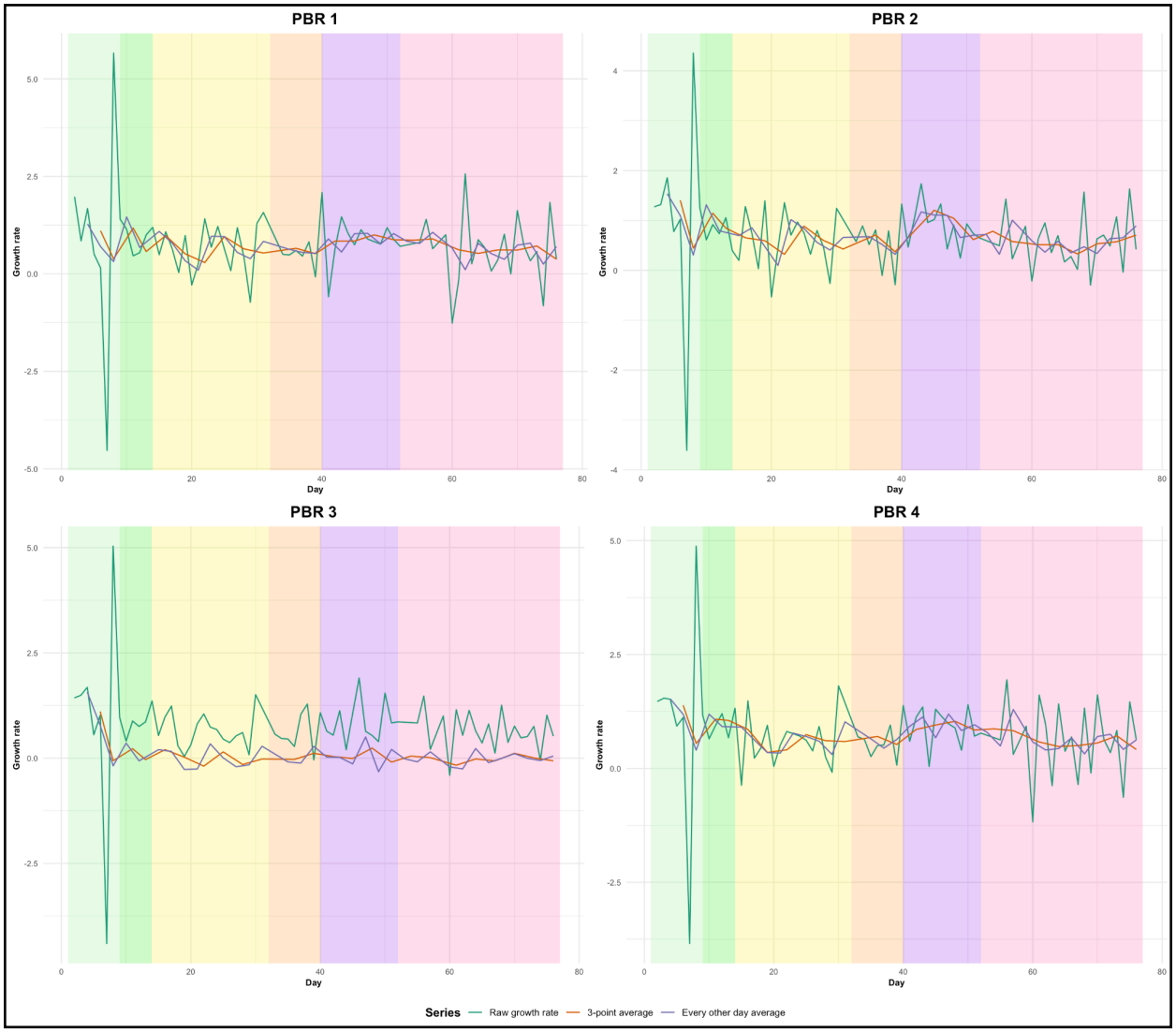

Figure 4: Comparisons of methods for calculating growth rate. The green line denoting growth rate taken every day, the blue line every other day, and finally the red line denoting the moving average over a three day period. Highlighted behind is treatment type over time.

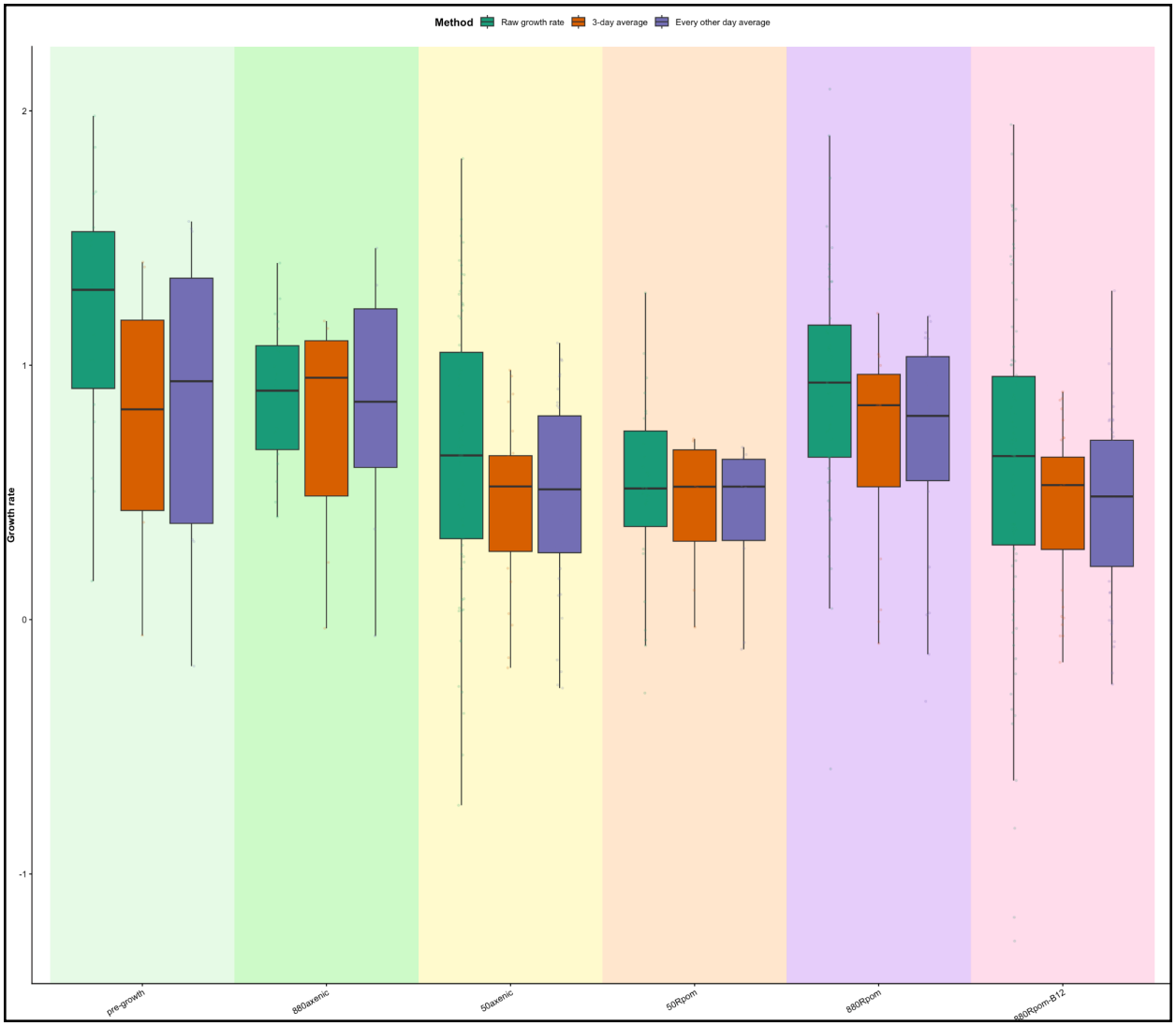

Figure 5: Growth rate by treatment and calculation method. Three different methods of calculating growth rate were tested in order to smooth out data. A raw growth rate calculated every day (green), a three day average growth rate (orange), and finally a growth rate average every other day (indigo). Behind denotes treatment type. Linear mixed-effects models and ANOVA were conducted in order to determine if the way growth rates were averaged had an effect on the significance of the trends per treatment type; this was found insignificant for method type ($p=0.685$), and for method type on treatment ($p=0.38$), and ANOVA ($p=0.095$) having a weak interaction.

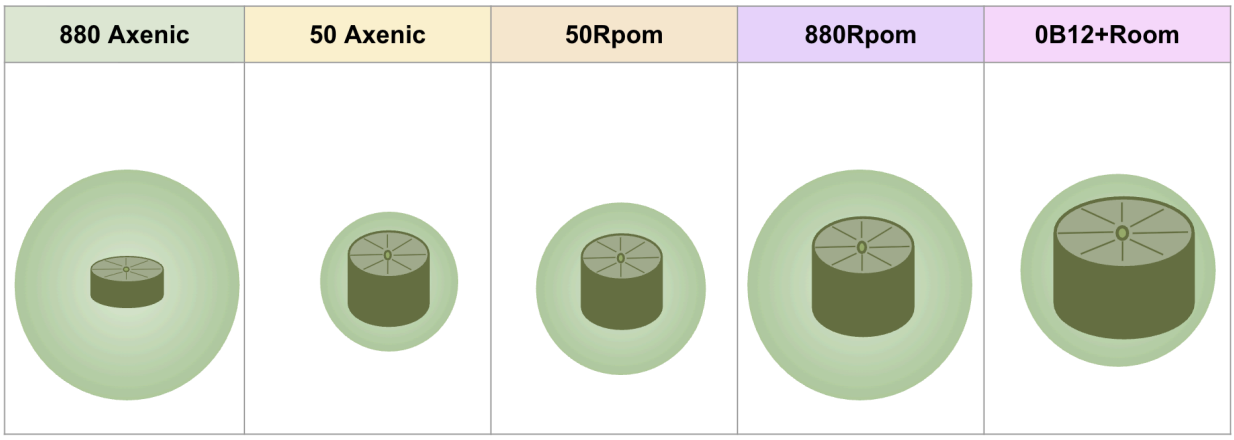

Figure 6: Artistic representation of parameters over time. This simplified figure shows how cell physiology changed over time in correspondence with treatment type. Showing changes in biovolume (cell size) and fluorescence (portrayed in light green circles).

SUPPLEMENTAL TABLES

Table 1: Statistical analysis of treatments to determine statistical difference. Each parameter was tested in order to determine if each parameter was statistically different between treatments. ANOVA was first used and reported. Then to check for normality and variance a shapiro and Levene testes were conducted and reported. If the assumptions in ANOVA were not met, a Kruskal-Wallis test was conducted. Red highlighting denotes where each assumption was not met.

Parameter	ANOVA (p, F)	Shapiro (p)	Levene (pr(>F)	Kruskal-Wallis (p)	Test used
Cells	p= 2.95e-08 F=13.39	0.1612	0.0001728	3.59E-05	Kruskal-Wallis (p)
Biovolume	p= 5.13E-11 F=30.75	0.7426	0.8446	2.65E-06	ANOVA (p, F)
Growth rate	p=9.03e-05 F= 8.55	0.8364	0.2875	09.22 e-04	ANOVA (p, F)
Fv/Fm	p=4.61e-05 F=7.372	0.0005278	7.76E-06	5.88E-07	Kruskal-Wallis (p)
Forward Scatter	p= <2e-16 F=72.27	0.09273	0.04203	1.32E-12	Kruskal-Wallis (p)
pH	p=1.69e-08 F=15.37	0.0004382	0.1665	6.10E-06	Kruskal-Wallis (p)
Fluorescence	p=<2e-16 F=84.15	0.001277	0.02402	1.52E-13	Kruskal-Wallis (p)

Table 2: Descriptive statistics from box plots analysis varying over treatment type and parameter. Columns represent the variable, treatment type, how many samples in that treatment (n), mean, standard deviation (SD), Median, Quartile 1, quartile 3, and finally interquartile range (IQR). Each treatment type has a corresponding treatment type given the parameter.

Variable	Treatment	n	Mean	SD	Median	Q1	Q3	IQR
Forward scatter (AU)	880axenic	12	323666.67	31303.02	308000.00	303500.00	345750.00	42250.00
Forward scatter (AU)	50axenic	16	229375.00	12500.00	225500.00	222750.00	237750.00	15000.00
Forward scatter (AU)	50Rpom	12	324333.33	38929.62	320000.00	311500.00	344000.00	32500.00
Forward scatter (AU)	880Rpom	16	354437.50	39871.41	347000.00	326000.00	379000.00	53000.00
Forward scatter (AU)	880Rpom-B12	24	422791.67	42877.97	411000.00	395500.00	458000.00	62500.00
Biovolume (µm³)	880axenic	12	28.85	7.23	30.05	23.56	33.29	9.73
Biovolume (µm³)	50axenic	8	43.05	7.36	39.97	38.86	47.14	8.28
Biovolume (µm³)	50Rpom	4	41.58	6.34	40.76	36.32	46.01	9.69
Biovolume (µm³)	880Rpom	8	50.40	7.36	52.47	44.99	55.39	10.40
Biovolume (µm³)	880Rpom-B12	8	62.00	4.91	61.25	58.68	64.03	5.35
Fluorescence (AU)	880axenic	12	140416.67	12287.90	141000.00	131750.00	149500.00	17750.00
Fluorescence (AU)	50axenic	16	33143.75	9620.81	31200.00	26150.00	38075.00	11925.00
Fluorescence (AU)	50Rpom	12	69662.83	13784.11	71815.01	60405.97	81725.00	21319.03
Fluorescence (AU)	880Rpom	16	158437.50	32739.31	146500.00	135000.00	169750.00	34750.00
Fluorescence (AU)	880Rpom-B12	24	97716.67	24439.02	91500.00	81400.00	118500.00	37100.00
Fv/Fm	880axenic	12	0.65	0.02	0.65	0.64	0.66	0.02
Fv/Fm	50axenic	16	0.67	0.01	0.67	0.66	0.67	0.01
Fv/Fm	50Rpom	12	0.65	0.01	0.65	0.64	0.65	0.01
Fv/Fm	880Rpom	16	0.69	0.02	0.69	0.68	0.71	0.03
Fv/Fm	880Rpom-B12	24	0.68	0.05	0.67	0.65	0.70	0.05
pH	880axenic	9	8.42	0.09	8.38	8.36	8.52	0.16
pH	50axenic	12	8.33	0.10	8.27	8.26	8.45	0.20
pH	50Rpom	9	8.31	0.09	8.26	8.25	8.37	0.12
pH	880Rpom	12	8.51	0.09	8.51	8.43	8.59	0.16
pH	880Rpom-B12	18	8.30	0.04	8.31	8.28	8.34	0.06
Cells (cells/mL)	880axenic	12	2383333.33	1249045.48	2425000.00	1507750.00	3067500.00	1559750.00
Cells (cells/mL)	50axenic	16	1887125.00	912661.74	1750000.00	1190000.00	2297500.00	1107500.00
Cells (cells/mL)	50Rpom	12	1649166.67	687316.63	1665000.00	1254250.00	2260000.00	1005750.00
Cells (cells/mL)	880Rpom	15	4094000.00	1989852.11	4140000.00	2480000.00	5880000.00	3400000.00
Cells (cells/mL)	880Rpom-B12	24	1231208.33	992081.08	1085000.00	419000.00	1422500.00	1003500.00
Growth rate	880axenic	8	0.97	0.21	1.01	0.83	1.13	0.31
Growth rate	50axenic	8	0.71	0.16	0.68	0.60	0.81	0.21
Growth rate	50Rpom	4	0.67	0.05	0.68	0.65	0.71	0.06
Growth rate	880Rpom	8	0.90	0.16	0.93	0.83	1.00	0.17
Growth rate	880Rpom-B12	8	0.57	0.13	0.57	0.48	0.71	0.23

Table 3: Inferential statistics from Tukey post hoc (HSD) test. Asterix denotes level of significance between each grouping. Shown are the measures parameters: biovolume (μm³), cell concentration (cells mL^{-1}), fluorescence (a.u.), forward scatter (a.u.), photosynthetic efficiency (Fv/Fm), growth rate, and pH. Each treatment type (880 axenic, 50 axenic, 50 Rpom, 880 Rpom, and 880 Rpom + B12) was compared to each other for a total of ten different results to confirm if each treatment was statistically different from one another. Columns represent the parameter, comparison of which two treatments, the difference in mean values, p value, and finally their significance grouping.

Variable	Comparison	Difference	p value	Significance group
Biovolume	50Rpom-50axenic	-1.47	1.00	Not significant
Biovolume	880axenic-50axenic	-14.19	0.00	***
Biovolume	880Rpom-50axenic	7.36	0.22	Not significant
Biovolume	880Rpom-B12-50axenic	18.95	0.00	***
Biovolume	880axenic-50Rpom	-12.72	0.02	*
Biovolume	880Rpom-50Rpom	8.83	0.24	Not significant
Biovolume	880Rpom-B12-50Rpom	20.42	0.00	***
Biovolume	880Rpom-880axenic	21.55	0.00	***
Biovolume	880Rpom-B12-880axenic	33.14	0.00	***
Biovolume	880Rpom-B12-880Rpom	11.59	0.01	*

Variable	Comparison	Difference	p value	Significance group
Forward scatter	50Rpom-50axenic	94958.33	0.00	***
Forward scatter	880axenic-50axenic	94291.67	0.00	***
Forward scatter	880Rpom-50axenic	125062.50	0.00	***
Forward scatter	880Rpom-B12-50axenic	193416.67	0.00	***
Forward scatter	880axenic-50Rpom	-666.67	1.00	Not significant
Forward scatter	880Rpom-50Rpom	30104.17	0.19	Not significant
Forward scatter	880Rpom-B12-50Rpom	98458.33	0.00	***
Forward scatter	880Rpom-880axenic	30770.83	0.17	Not significant
Forward scatter	880Rpom-B12-880axenic	99125.00	0.00	***
Forward scatter	880Rpom-B12-880Rpom	68354.17	0.00	***

Variable	Comparison	Difference	p value	Significance group
Fv/Fm	50Rpom-50axenic	-0.02	0.40	Not significant
Fv/Fm	880axenic-50axenic	-0.02	0.34	Not significant
Fv/Fm	880Rpom-50axenic	0.03	0.06	Not significant
Fv/Fm	880Rpom-B12-50axenic	0.01	0.72	Not significant
Fv/Fm	880axenic-50Rpom	0.00	1.00	Not significant
Fv/Fm	880Rpom-50Rpom	0.05	0.00	***
Fv/Fm	880Rpom-B12-50Rpom	0.03	0.03	*
Fv/Fm	880Rpom-880axenic	0.05	0.00	***
Fv/Fm	880Rpom-B12-880axenic	0.03	0.02	*
Fv/Fm	880Rpom-B12-880Rpom	-0.02	0.42	Not significant

Variable	Comparison	Difference	p value	Significance group
Fluorescence	50Rpom-50axenic	36519.08	0.00	***
Fluorescence	880axenic-50axenic	107272.92	0.00	***
Fluorescence	880Rpom-50axenic	125293.75	0.00	***
Fluorescence	880Rpom-B12-50axenic	64572.92	0.00	***
Fluorescence	880axenic-50Rpom	70753.84	0.00	***
Fluorescence	880Rpom-50Rpom	88774.67	0.00	***
Fluorescence	880Rpom-B12-50Rpom	28053.84	0.00	**
Fluorescence	880Rpom-880axenic	18020.83	0.20	Not significant
Fluorescence	880Rpom-B12-880axenic	-42700.00	0.00	***
Fluorescence	880Rpom-B12-880Rpom	-60720.83	0.00	***

Variable	Comparison	Difference	p value	Significance group
Cells	50Rpom-50axenic	-237958.33	0.99	Not significant
Cells	880axenic-50axenic	496208.33	0.83	Not significant
Cells	880Rpom-50axenic	2206875.00	0.00	***
Cells	880Rpom-B12-50axenic	-655916.67	0.47	Not significant
Cells	880axenic-50Rpom	734166.67	0.59	Not significant
Cells	880Rpom-50Rpom	2444833.33	0.00	***
Cells	880Rpom-B12-50Rpom	-417958.33	0.87	Not significant
Cells	880Rpom-880axenic	1710666.67	0.01	**
Cells	880Rpom-B12-880axenic	-1152125.00	0.07	Not significant
Cells	880Rpom-B12-880Rpom	-2862791.67	0.00	***

Variable	Comparison	Difference	p value	Significance group
pH	50Rpom-50axenic	-0.02	0.99	Not significant
pH	880axenic-50axenic	0.10	0.05	Not significant
pH	880Rpom-50axenic	0.18	0.00	***
pH	880Rpom-B12-50axenic	-0.02	0.95	Not significant
pH	880axenic-50Rpom	0.12	0.02	*
pH	880Rpom-50Rpom	0.20	0.00	***
pH	880Rpom-B12-50Rpom	0.00	1.00	Not significant
pH	880Rpom-880axenic	0.08	0.13	Not significant
pH	880Rpom-B12-880axenic	-0.12	0.00	**
pH	880Rpom-B12-880Rpom	-0.20	0.00	***

Variable	Comparison	Difference	p value	Significance group
Growth rate	50Rpom-50axenic	-0.03	1.00	Not significant
Growth rate	880axenic-50axenic	0.27	0.02	*
Growth rate	880Rpom-50axenic	0.19	0.13	Not significant
Growth rate	880Rpom-B12-50axenic	-0.14	0.44	Not significant
Growth rate	880axenic-50Rpom	0.30	0.03	*
Growth rate	880Rpom-50Rpom	0.23	0.16	Not significant
Growth rate	880Rpom-B12-50Rpom	-0.10	0.83	Not significant
Growth rate	880Rpom-880axenic	-0.07	0.88	Not significant
Growth rate	880Rpom-B12-880axenic	-0.40	0.00	***
Growth rate	880Rpom-B12-880Rpom	-0.33	0.00	**

Significance:
***** = p<0.001**
**** = p<0.01**
*** = p<0.05**
Not significant = **p>0.05**

Table 4: Determining significance between growth rate counting method. Growth rate was counted using three different methods involving the days between each count. The method and method of treatment was considered when determining statistical significance. Tests proved that growth rate method did not affect significance on method or treatment type ($p>0.05$).

Tested parameter	Test	F-value	p-value	Interpretation
Method (GR)	Repeated ANOVA	2.37	0.095	Weak
Method (GR)	Linear mixed-effects model	0.39	0.685	Not significant
Method on treatment	Linear mixed-effects model	0.38	0.957	No interaction

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