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# Investigating the Impacts of Ammonium Phosphate-Based Fire Retardants on Cyanobacteria (Anabaena) Growth

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# Investigating the Impacts of Ammonium Phosphate-Based Fire Retardants on Cyanobacteria (Anabaena) Growth

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## ABSTRACT

In recent years the effects of climate change have taken a devastating toll on ecosystems around the world. With high temperatures and extreme droughts, wildfires have become increasingly common. In order to combat these natural disasters wildland firefighters, drop millions of gallons of fire retardant on public lands and forests. These fire retardants consist of between 80%-100% ammonium phosphate which are incredibly effective as fire suppressants yet is more commonly known for its use in fertilizer. Ammonium phosphate fertilizers can lead to stream eutrophication and undesirable environmental impacts. Our research aims to address the effects of fire retardant on growth in cyanobacteria, specifically *Anabaena* — a filamentous, nitrogen fixing genera common to North America and responsible for many of the large, toxic cyanobacteria blooms found during summer months. We hypothesized that fire retardant, which is made up of mostly ammonium phosphate, will act similarly to ammonium phosphate fertilizer and cause an increase in growth in *Anabaena* cultures grown in a lab environment. After a 11-day growth curve experiment, results showed no differences in growth between microcosms treated with ammonium phosphate or fire retardant, supporting the hypothesis that fire retardants can have similar effects to ammonium phosphate-based fertilizers when released in the environment.

## INTRODUCTION

In 2022, over 360,000 acres of land were burned by wildfires in the state of California and a 9 million gallons of fire retardant was dropped in firefighting efforts (1). These fire retardants often consist of between 80-100% ammonium-phosphate which is more commonly known as the active ingredient in agricultural fertilizers (2). Previous research has shown that there are "toxic" effects to aquatic ecosystems with the addition of fire retardants, but most of these studies have focused on the immediate effects rather than the possible longer-term consequences these fire retardants may play when introduced to the environment (3,4).

There have been many studies addressing agricultural runoff and stream eutrophication caused by ammonium phosphate, but less is known about fire retardants as a source of ammonium phosphate. Fertilizers are often composed of ammonium-phosphate salts to supplement both nitrogen and phosphorus to commercial crops. When these additional nutrients make their way into rivers during the months of summer, they create ideal conditions for cyanobacteria blooms (5). When cyanobacteria populations spike, a stream or lake can see large increases in the concentrations of cyanotoxins due to the high cell turnover "releasing" the intracellular toxins into the external environment. While most cyanobacteria are harmless, some produce cyanotoxins that in high concentrations can be harmful to both humans and other vertebrates (6,7). Not only do these bacteria produce toxins, but they can also use up dissolved oxygen, release harmful gasses, and block sunlight from entering the water (7). The negative consequences of excess cyanobacteria blooms have been clearly documented and understanding the sources of the nutrients responsible for them is critical in their prevention.

We examined the effects on growth of the cyanobacteria taxa *Anabaena* when treated with ammonium phosphate and fire retardant. *Anabaena*, is a filamentous, gram- negative, genera of cyanobacteria that is one of the more common cyanobacteria species in Humboldt County (8). The high abundance and widespread availability of *Anabaena* makes it a prime candidate to further understand how different eutrophication sources can affect its growth.

Previous growth experiments have shown that there is an overall increase in cellular growth when river water samples containing cyanobacteria were exposed to varying

levels of fire retardant (Sutter, Unpublished Data). The objective of this experiment is to generate growth curves of cyanobacteria (*Anabaena*) grown in a lab environment with varying concentrations of both ammonium-phosphate and fire retardant (2). By comparing the growth between ammonium phosphate and fire retardant, we will be able to see if there is a difference in growth when *Anabaena* cells have either ammonium phosphate or fire retardant to supplement the nitrogen and phosphorus necessary for their growth. Additionally, growth curve data during the exponential phase, will allow for comparisons of the growth rate between treatments which is an important factor when taking into consideration a cyanobacterium (or any microorganisms) ability trigger a bloom — large growth and die off event — which are known to cause negative effects on the surrounding environment. We hypothesized that there would be an overall increase in cyanobacteria growth in samples treated with ammonium phosphate fertilizer and fire retardant, and that increases in growth would be similar among the fire retardant and ammonium phosphate treatments.

## MATERIALS AND METHODS

### Culturing Anabaena

An *Anabaena* cyanobacteria isolate (50mLs) was acquired from Carolina Biological in March 2023 and stored at room temperature with access to window light (day-night cycle) for two weeks prior to use. In order to increase the amount of stock Anabaena culture, 1500mLs of AG-11 media (without nitrogen - *Anabaena* fix atmospheric nitrogen) was inoculated in a 2L Erlenmeyer flask with 15mLs of the original *Anabaena* sample. Aseptic technique and a laminar flow hood were used to reduce the risk of contamination during inoculation and media prep. The new sample was stored at room temperature with access to window light for 25 days leading up to the growth curve experiment.

### Determining Treatment Concentrations

A growth curve experiment was designed to look at the impacts on Anabaena growth with the addition of ammonium phosphate and fire retardant (Phos-Chek LC-95) in equal concentration. The concentration of fire retardant was calculated by the amount of fire retardant that would be expected in a cubic meter of water after a direct drop from an airtanker. Stream flow and dispersal rates were not considered in this study and would be an important area of future research. To calculate the average concentration, the ratio that Phos-Chek LC-95 is mixed with water for air tankers, 5.5 lbs. of Phos-Chek LC-95 to every gallon of water, was used to determine a standard concentration for firefighting use (9). This can be calculated as 658.2g/L of Phos-Chek LC-95. While the amount of fire retardant deposited per square meter during a drop is highly variable and dependent on the altitude released, an average amount of retardant dropped on a meter square area by air tankers was recently calculated at 1.3L m-2 (10). With the average quantity of fire retardant dropped per square meter and the concentration of fire retardant, the average concentration in a cubic meter of water hit in a direct drop would be 0.483g/L Phos-Chek LC-95. This concentration of fire retardant was used as the standard in growth curve experiments, and 50% and 150% (0.219g/L and 0.657g/L respectively) of this concentration was used to investigate variation in fire retardant concentrations.

**Table 1:**
AG-11 Liquid Media for culturing cyanobacteria cells. Creates 1L of media. NaNO3 was not added because Anabaena can fix its own nitrogen and the growth curve design was based on the addition of nitrogen in the form of fire retardant and ammonium phosphate.

**AG-11 Media - add to 999mL's H2O**

| Nutrients | Mass |
|---|---|
| $NaNo_3$ | 1.5g |
| $CaCl_2$-$H_2O$ | 0.001g |
| $FeNH_4$-Citrate | 0.04g |
| $Na_2$-EDTA | 0.075g |
| $K_2HPO_4$ | 0.04g |
| $MgSO_4$-$7H_2O$ | 0.075g |
| $Na_2CO_3$ | 0.20g |
| Haaglands Micro. Nutrients. | 1mL |

**Haaglands Micronutrients - add to 1L H2O**

| Nutrients | Mass |
|---|---|
| $H_3BO_3$ | 2.86g |
| $McCl_2$ | 1.81g |
| ZnSO4-$7H_2O$ | 0.222g |
| $Na_2MoO_4$-$2H_2O$ | 0.391g |
| $CuSO_4$-$5H_2O$ | 0.079g |
| $Co(NO_3)_2$ | 0.0494g |

Ammonium phosphate concentrations were calculated from the percent ammonium phosphate that is present in Phos-Chek LC-95 fire retardant. Perimeter solutions Phos-Chek LC-95 uses 91.80% ammonium phosphate (2). Ammonium phosphate concentrations were then calculated at 0.201g/L, 0.443g/L, and 0.603g/L (50%, 100%, and 150% respectively). Both fire retardant and ammonium phosphate treatments were prepared by making 10x concentrations in deionized water and autoclaved for sterility.

### Growth Curve Experiment

To observe the effects of adding fire retardant and ammonium phosphate to *Anabaena* samples, a growth curve experiment was designed to generate *Anabaena* growth curves with the fire retardant and ammonium phosphate treatments. Microcosms (50mL falcon tubes) were used as individual replicates and new AG-11 media, stock *Anabaena* culture, and 10x treatment stocks were used to create four replicates of: ammonium phosphate at 50% — (NP50), ammonium phosphate at 100% — (NP100), ammonium phosphate at 150% — (NP150), Phos-Chek LC-95 at 50% — (FR50), Phos-Chek LC-95 at 100% — (FR100), Phos-Chek LC-95 at 150% — (FR150), and control with only the addition of new media and *Anabaena* cells. Additionally, blanks were made from fire retardant and ammonium phosphate added to only new media to be used to calibrate fluorescence values when calculating cell concentration during the growth experiment.

Treatments were stored in Cal Poly Humboldt's C.O.R.E. facilities growth chambers for the duration of the growth experiment. Samples were kept at constant 20.0°C and exposed to a 16hr light 8hr dark cycle. Fluorescence values were gathered from each sample each day for 11-days. Manual cell counts were collected on day 5, 6, 7, 8, and 10 from a subset of the replicates in each treatment (see fluorescence and manual cell counts).

### Measuring Fluorescence and Manual Cell Counts

To track growth efficiently and accurately over time for the 42 samples, spectrophotometry was used to calculate the fluorescence of each sample every 24hrs for 11 days. Fluorescence values were collected by using Cal Poly Humboldt's C.O.R.E. facilities SpectraMaxi3 spectrophotometer. Samples were removed from the growth chamber between 1500hrs and 1800hrs each day, individually inverted three times to mix cells, and 200µL of sample was plated onto a 96 well plate (Costar Clear 96 Well Plate Flat Bottom - No Lid). The sample fluorescence was then measured in 2nm increments between 640nm and 700nm to capture the peak fluorescence of chlorophyll a — 667nm with an excitation wavelength of 440nm (11,12).

Manual cell counts were performed on an alternating treatment group for days 5, 6, 7, 8, and 10. Manual cell counts were performed by pipetting 10µL of sample onto a disposable Neubauer Improved hemocytometer (C-Chip DHC-N01) with a total chamber volume of 0.1mm3. Samples were examined under a Leica DM750 microscope with a Leica ICC50 HD digital viewfinder using 400x magnification. Originally, all cells were to be counted within the innermost grid of the slide, but due to the clumping nature of the cells, a cell count of one was recorded for every grid (smallest scale) that contained at least one cell. The cell counts from each grid were then multiplied by 100,000 to convert the units to cells/mL. Additionally, for filamentous chains of Anabaena, chain length, number of chains, and ratio of heterocyst to vegetative cells was recorded.

### Statistical Analysis

Fluorescence values were first "blanked" to remove interference from the media by subtracting the fluorescence value of the media/treatment blanks from their corresponding sample (i.e. the NP50 fluorescence values had the BNP50 subtracted from them). Fluorescence values were then added to a dataset that included the sample id, treatment, nutrient group, day sampled, fluorescence, manual cell counts, heterocyst ratio, chain count, chain length, and total chain cell count.

Manual cell counts were planned to be used to convert the fluorescence values to cells/mL via a standard curve and regression line. Despite best efforts, little to no correlation was found between the manually counted cell concentrations and the photometrically measured fluorescence values (Fig. 1). While this was inconvenient and created a proxy between our results and actual cell counts, fluorescence values clearly represented growth curves, and were used for the remainder of the analysis in the place of cell concentration.

All statistical analysis, data formatting, and plotting was performed in R version 4.2.1 using the RStudio IDE version 2022.7.1.554. Due to the repeated sampling of the same treatments each day, linear mixed models were used to prevent artificially inflating significance between treatments. The LME4 package was used to perform a LMM between treatments (NP50, FR150, control, etc.) and nutrient groups (fire retardant, ammonium phosphate, control) to compare the possible impacts of the fire retardant and ammonium phosphate on growth. Additionally, the maximum instantaneous growth rate during the exponential phase was calculated and a One-Way ANOVA with Tukey's Post Hoc test will be used to determine the difference in growth rate between treatments.

## RESULTS

### Data Analysis

To create standard curves from fluorescence data and manual cell counts, the raw fluorescence values were blanked — uninoculated media and corresponding concentration of nutrient were subtracted from the inoculated samples — resulting in a fluorescence value with less noise and more presentative of the fluorescence from *Anabaena* cells and not media or other reflective materials. Fluorescence data was then merged with manual cell counts of the corresponding day.

### Fluorescence to Cellular Concentration (cells/mL)

Standard curves were generated by correlating a sample's fluorescence value with its manual cell count from the same day (Fig. 1). Despite uniform cell counting strategies and breaking apart the data based on treatment and other factors, no strong association between cell number and fluorescence was found. While this was unfortunate and limits these results from being interpreted outside of this study, the rest of the analysis was performed with fluorescence values in place of cellular concentrations. Due to the known linear relationship between fluorescence and cell concentration, using fluorescence values in their place is only believed to impact the magnitude of the data, and have little impact on the relationship between samples. All statistical analyses and charts from this point forward will be calculated with fluorescence values in place of cell concentrations.

### Comparing Growth Curves between Treatments and Nutrient Groups

Despite the lack of cellular concentrations, growth curves were created by plotting treatment and nutrient group fluorescence values against the day that they were sampled (Fig. 2, Fig. 3). Treatments overall did have an impact on growth and a clear visual trend of increased initial growth rate is observable between both the ammonium phosphate and fire-retardant treatments when compared to the controls

**Figure 1.**
Standard curve generated from fluorescence values and manual cell counts taken on day 5, 6, 7, 8, and 10 (R2 = 0.011, y = 321027+-0.003x). There was no association between fluorescence and manual cell counts, resulting in the inability to convert fluorescence values from the entire growth period into their corresponding cellular concentrations (cells/mL).

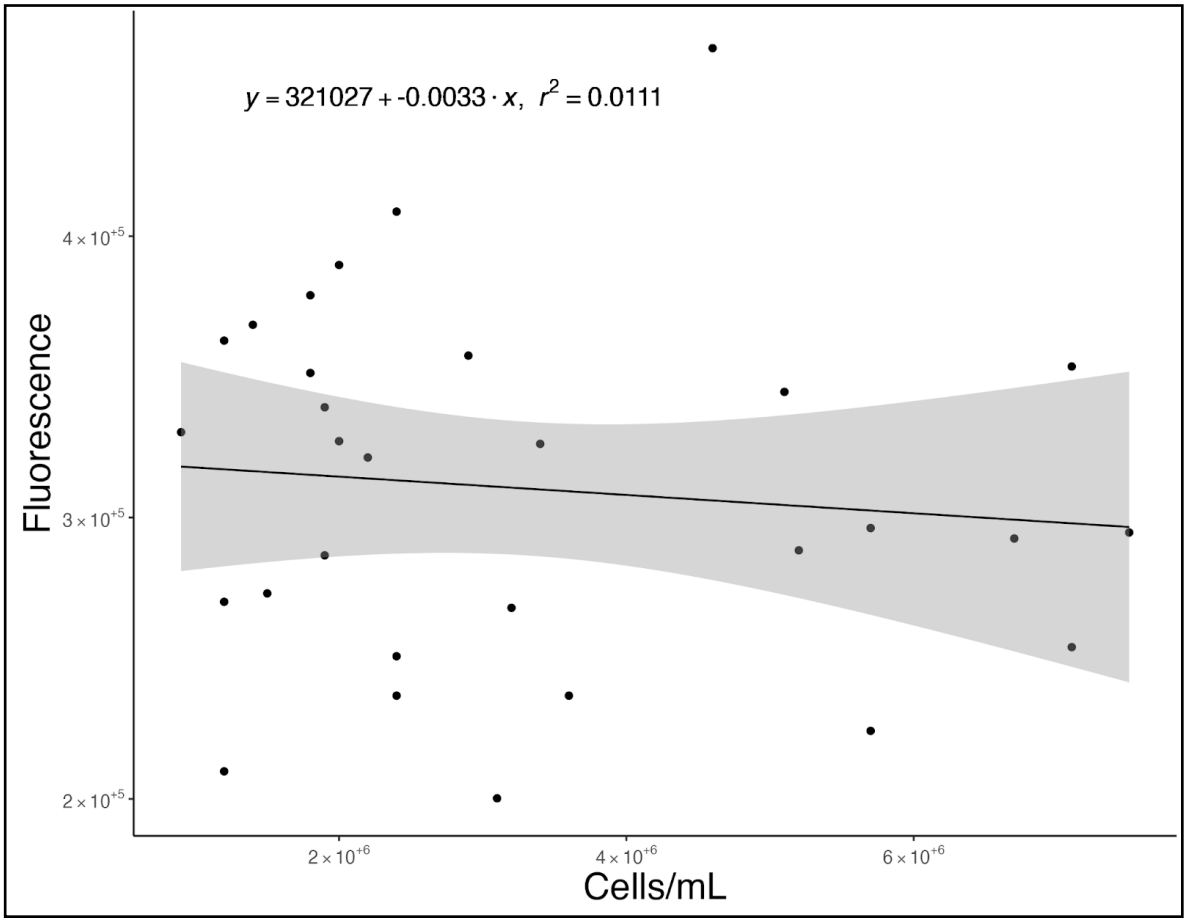


**Figure 2:**
Growth curves of all 6 treatments and control for the 11 day growth period. Overall, it was found that treatment did impact growth (P = 0.009634).

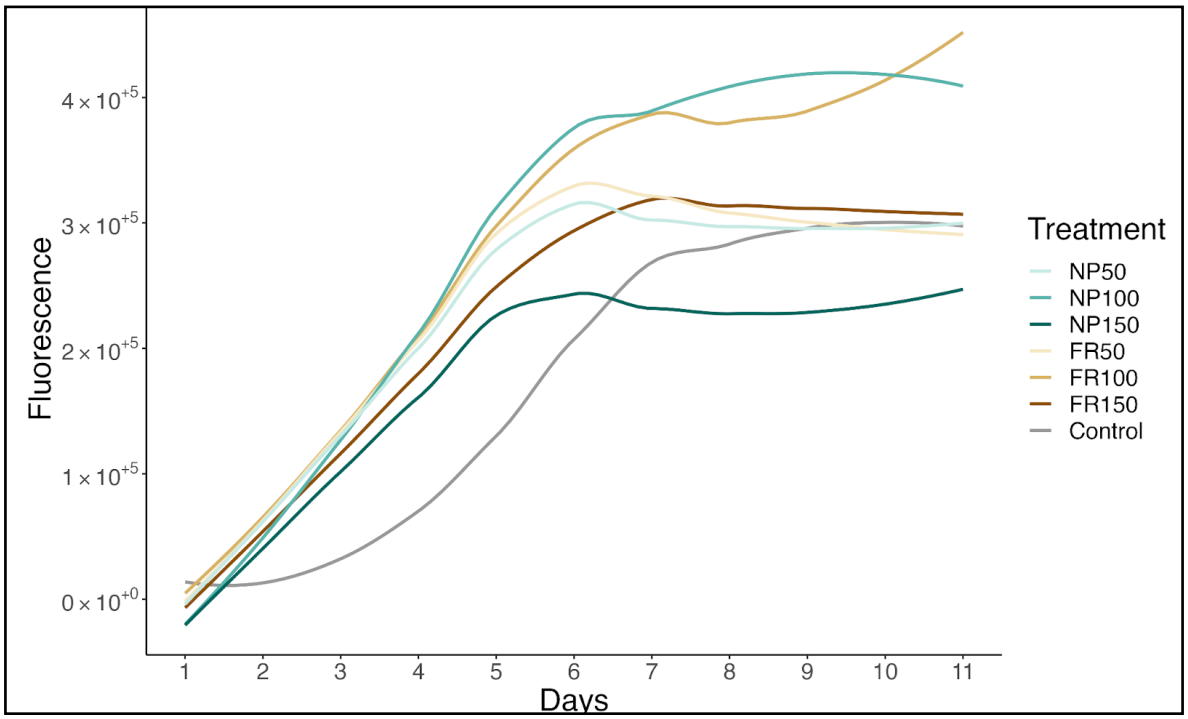



**Figure 3:**
Growth curves by the two nutrient groups and control for the 11-day growth period. Overall, there was no significant difference in growth between treatments (P = 0.12593). When nutrient groups are independently compared with the control, fire retardant is observed to be impacted by the nutrient group, but ammonium phosphate is not (P = 0.0001, P = 0.22; respectively).

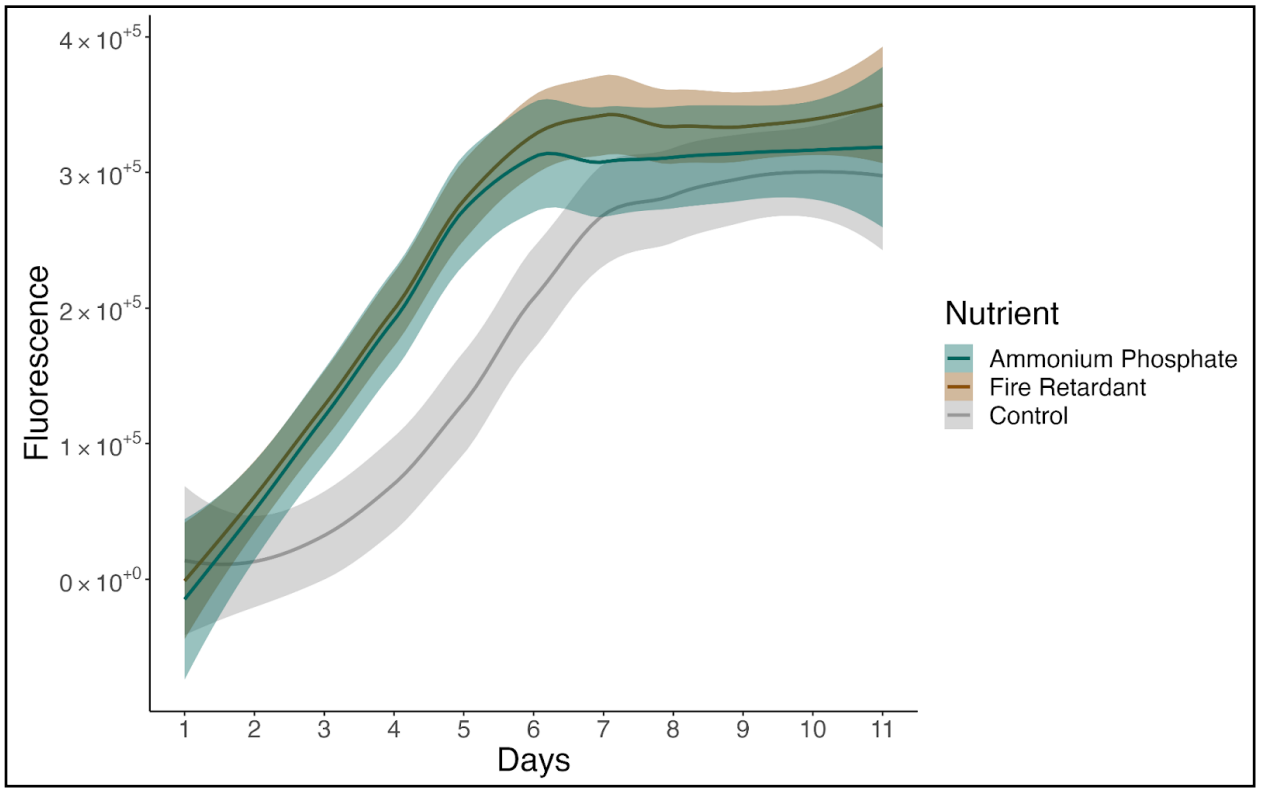
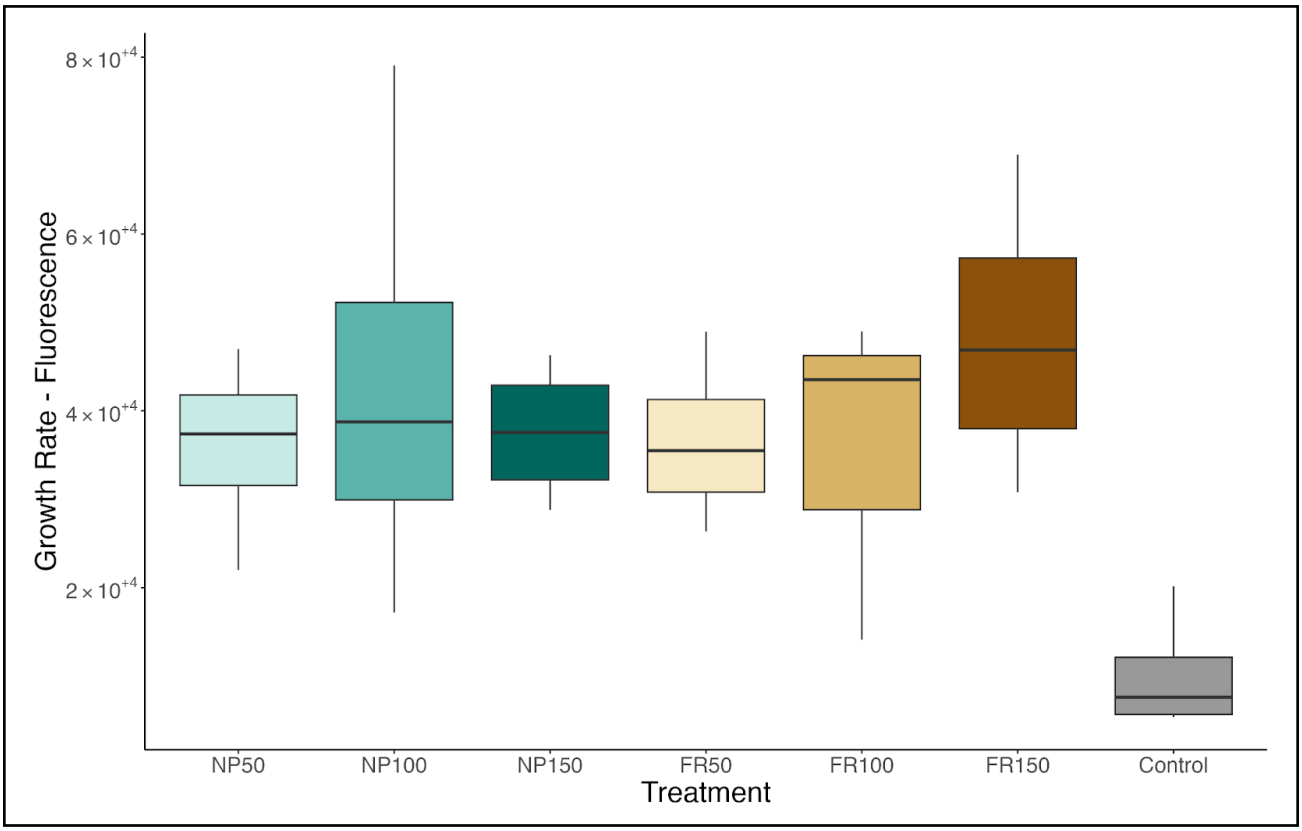


**Figure 4.**
Comparison of approximate growth rate on day 3 of all treatments by taking the average growth rate between days 2 and 4. Despite a clear visual trend between the treatments and control, no significant impact on growth rate was found between all the individual treatments (P = 0.051, F = 2.583, One-Way ANOVA). A Tukey's Post Hoc test was performed to compare individual treatments with each other. While none of the treatments were different from one another, there was a significantly less similarity when compared with the control, which is visibly apparent when comparing between the treatments and control.




(Fig. 2, P = 0.0096). When binned into nutrient groups, we did not see a difference when comparing all three groups — fire retardant, ammonium phosphate and controls (Fig. 3, P = 0.12593), yet we expect this to be caused by the overall variability and small sample sizes. Additionally, when comparing nutrient groups independently with the control, fire retardant did have a meaning difference in growth and the ammonium phosphate nutrient group did not (Fig. 3, P = 0.0001, P = 0.22; respectively).

### Comparing Growth Rate

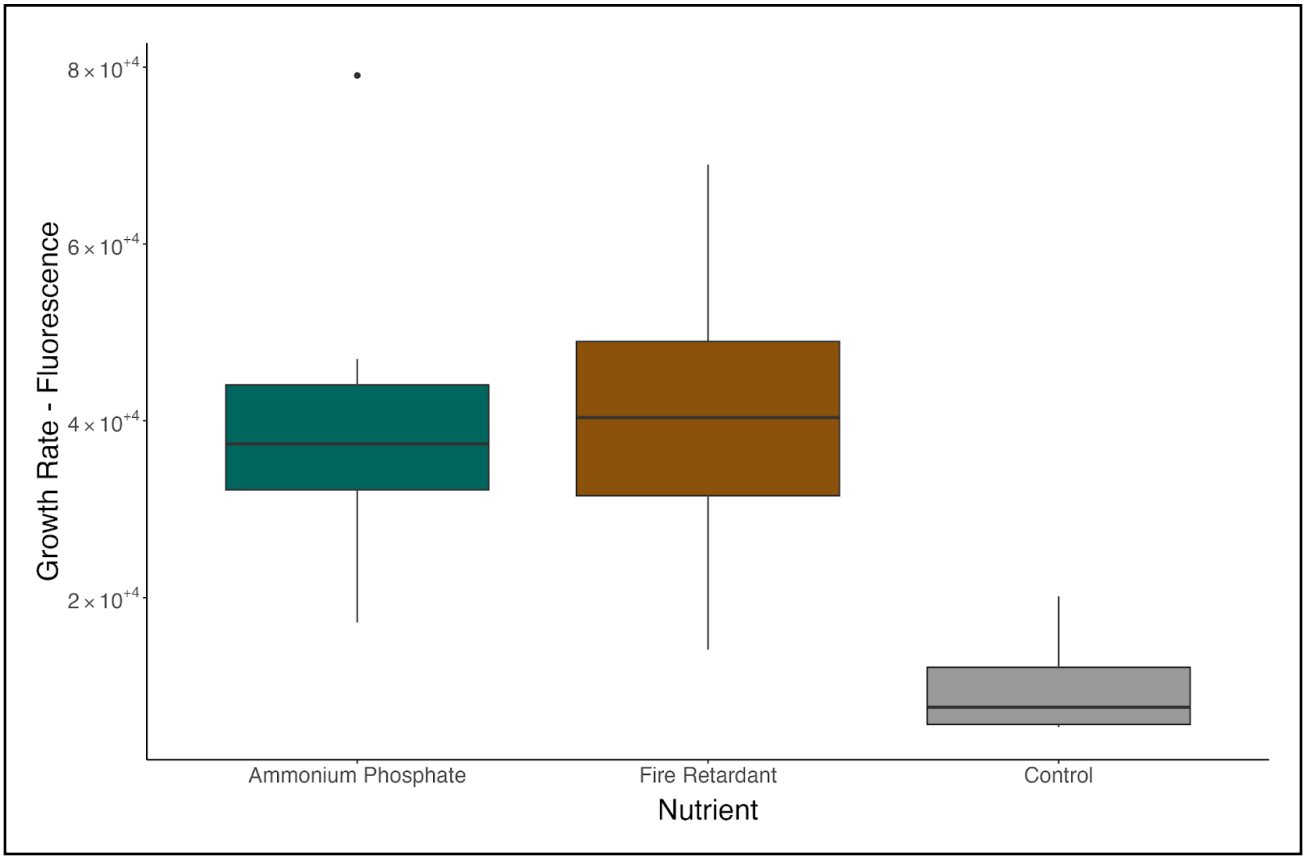
Growth rate was calculated for the exponential growth phase between day 2 and 4, and the mean maximum growth rate for day three was used to compare growth rate between treatments and nutrient groups. While there wasn't a difference between growth rate and treatment when compared with a One-Way ANOVA (P = 0.051, F = 2.583). A Tukey's Post Hoc Test did reveal that treatments did share more similar growth rates compared with one another and the control. Future work increasing sample sizes and cell counting methods may shine more light into whether the visible trends are representative of something meaningful, or insignificant as found in this small-scale experiment.

Like the growth curve analysis by nutrient group (Fig. 3), the average treatment growth rates were compared as nutrient groups due to the similarity in growth between all treatments of fire retardant and ammonium phosphate. When compared it was found that the nutrient group did impact growth rate (P = 0.00367, F = 7.146, One-Way ANOVA). The relationship between growth rate and nutrient group can be further explained by examining the results of a Tukey's Post Hoc test that showed that there was no significant difference between the ammonium phosphate and fire-retardant groups (P = 0.961), yet when compared with the controls both fire retardant and ammonium phosphate were found to significantly increase growth (P = 0.00545 and P = 0.00397, respectively).

## DISCUSSION

Despite the lack of direct cell concentrations, the observed increases in fluorescence among both fire retardant and

**Figure 5.**
Comparison of approximate growth rate on day 3 of all treatments by taking the average growth rate between days 2 and 4. Nutrient groups were found to increase growth rate compared with controls (P = 0.00367, F = 7.146, One-Way ANOVA). Additionally, Tukey's Post Hoc Test was performed — means that do not share a letter are significantly different.




ammonium phosphate treatments provide strong evidence that fire retardants are impacting growth similarly to ammonium phosphate fertilizers at the same concentration. While it was expected with the vast majority of fire retardant being composed of ammonium phosphate, this is the first time (to our knowledge) that this relationship has been tested and observed in a lab environment. The results show that there isn't a difference in growth between either fire retardant or ammonium phosphate when added to *Anabaena* cyanobacteria cultures, and both share a visibly increased growth rate compared to untreated *Anabaena* controls (fire retardant was found to be significantly increased, but due to the range of values in the ammonium phosphate nutrient group results could not be distinguished from the control). Further research is needed to confirm (or refute) many of the visible, yet insignificant trends found in this experiment. Due to small sample sizes (only four replicates in each treatment), lack of direct cell concentrations, and overall variability in some treatments, some results with strong visible trends were statistically no different than their controls. Moving forward, repeating these experiments with updated protocols that allowed for accurate cell counts and increased sample sizes would allow for more robust answers to the results that are provided in this preliminary analysis.

If indeed fire retardants are acting like concentrated fertilizers when in aquatic environments, it would become pertinent for land managers to weigh both short term and long-term effects of these fire retardants. Despite the overall dearth of research investigating the impacts of fire retardants on natural systems, what does exist has primarily focused on short term and toxic effects and often lacks insight into the possible long term and biotic repercussions of releasing large quantities of these chemicals into the environment.

Lastly, we'd like to acknowledge the inherent skepticism present in this report on the impacts of fire retardants on the environment. While we believe that this skepticism is warranted due to the lack of peer reviewed literature on the subject and the preliminary results shown in this experiment, we by no means refute the efficacy or usefulness of aerial fire retardants in forest fire suppression. Forest fires are going to continue to increase in size and severity with climate change and drought and finding innovative ways to manage them and protect human, ecological, and environmental resources will be critical. The

primary goal of researching the impacts of fire retardants is to provide land managers with the best data available to protect the environment, if that is using arial fire retardants to prevent severe burning or using other suppression techniques due to a fire's proximity to a stream, we want to support those who have made it their livelihoods to protect our natural environments.

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