The Effect of *Hordeum vulgare*, Rice Hull, and Oak Leaf Litter on the Prevalence of *Microcystis*

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Cyanobacterial harmful algal blooms are becoming more prevalent issues throughout the world, threatening ecosystems, posing risks to human health, and resulting in millions of dollars in economic damage. Conventionally, treatment approaches have been chemical or physical, but these are harmful to aquatic life or inefficient, respectively, which presents the biological approach as a favorable alternative. To assess the effects of low-effort and cost-effective treatments, *Hordeum vulgare*, rice hull, and oak leaf litter were individually applied to *Microcystis* cultures and compared to two negative controls (*Microcystis* alone and cotton on *Microcystis*) and a positive control ($CuSO_4$). This research was aimed at comparing the capabilities of these treatments on minimizing the size of cyanobacterial blooms, through optical density. It was hypothesized that *Hordeum vulgare* would be the most effective treatment since barley straw releases phenolic compounds. Separately, observations of the effects of these treatments on *Daphnia magna* populations were noted in separate cultures without *Microcystis*. Optical density was taken of the groups with cyanobacteria at 730 nm prior to adding treatments and repeated twice on weekly intervals. After calculating the decrease in OD, an ANOVA at alpha equals 0.05 was used to determine whether there were any significant differences between the means. The ANOVA determined that the values were not significant, as p>alpha ($F(5, 37) = 1.31$, $p=0.283$), showing that none of the treatments had significantly different effects. Thus, the hypothesis that *Hordeum vulgare* would be the most effective in decreasing *Microcystis* prevalence was not supported.

Introduction

As the global population continues to climb exponentially, the availability of natural resources are quickly becoming an issue to satisfy the needs of an ever-increasing human population. However, throughout the world, cyanobacterial harmful algal blooms are becoming more prevalent, which prevents the attainment of required high-quality water sources to sustain the global populace. According to the United States Geological Survey, the presence of these blooms have caused an increase in drinking-water treatment costs, a decrease in recreational revenues, and lowered property values across the board.[1] In recent years, these damages have totaled to losses of over $82 million annually in the seafood, tourism, and restaurant industries.[2] Not only do they mean vast negative implications for the economy, but cyanobacterial HABs are also a threat to flora, fauna, and the aquatic ecosystem. Many species produce cyanotoxins, which can have disastrous effects on microorganisms.[3] Furthermore, these toxins can cause illness or death in human populations due to the passage of these toxins through the food web.[4] In addition to being a threat to the environment, changes in the environment can also contribute to excessive cyano-HAB presence. Recent studies have shown that climate change can lead to the dominance of particularly toxic species of cyanobacteria by speeding growth rates and encouraging colonization in various geographic locations, in addition to numerous other factors.[5] In terms of health, HABs have caused illness and death in humans and animals in approximately 43 states across the United States.[4] As of 2016, the US declared public health advisories in around 19 states due to the presence of cyanobacterial HABs.[6] Various EPA-approved methods exist to treat HABs, but the usage of these methods is paradoxical. They often contain compounds detrimental to the survival of microorganisms, so even though they effectively kill the algal blooms, they worsen ecological habitats, defeating the initial purpose of the treatment.

Cyanobacteria are photosynthetic microorganisms that are similar to algae, and they naturally occur in ponds, lakes, and other bodies of water.[6] However, when they multiply, they are referred to as blooms. A specific and relatively common type of bloom-forming cyanobacteria is *Microcystis aeruginosa*, which is a unicellular, freshwater cyanobacteria that utilizes the process of photosynthesis to produce energy. This bacterium produces microcystin toxins, and drinking or swimming in contaminated water can directly cause liver and kidney disease in humans.[7] *Microcystis* is a non-toxin-producing cyanobacteria that includes the common HAB *Microcystis aeruginosa* strain, which makes it applicable for use in this experiment, rather than creating risks associated with toxin production.

Hordeum vulgare, more commonly known as barley straw, is an annual grass grown in temperate regions around the world and one of the most widely grown cereal crops. This straw is a common treatment for cyanobacterial blooms due to its low cost, high effectiveness, and easy obtainability.[8] Rice hull, otherwise known as rice husk, refers to the protective covering of certain grains. In the past, it was often discarded due to the lack of use, but nowadays, researchers and eco-friendly companies have begun to look at more ways to utilize this byproduct.[9] Oak leaf litter, a type of deciduous leaf litter, presents a favorable aspect for use as a treatment as it is a common byproduct of a range of trees and can easily be obtained.[10]

Optical density quantifies the amount of of light that is absorbed by bacterial cells through the use of a spectrophotometer. OD 730nm, the wavelength typically used for cyanobacterial cultures, was used in this experiment to quantify the amount of *Microcystis* in each trial. Optical density was measured prior to the application of the treatment and at regular intervals throughout experimentation to determine how effectively each method affected the size of the *Microcystis* cultures by calculating the decrease in OD from the initial measurement to the final measurement. *Daphnia magna* are crustaceans that are frequently used in lab settings to assess water quality due to their relatively short life cycle, size, and general adaptability to laboratory experiments.[11] Their transparency allows for easy heartbeat measurements, and as they are also freshwater organisms, it makes them applicable to use to study *Microcystis*, a freshwater cyanobacteria.[11] As another component to this experiment, the effect of the treatments on local organisms was studied by observing the survival of *Daphnia* populations when placed in treated environments without cyanobacteria. Every few days, *Daphnia* populations were counted, in order to understand the extent of how these treatments can alter aquatic ecosystems where these treatments could potentially be employed.

There are three main methods of short-term treatment for cyanobacterial blooms: chemical approaches, physical approaches, and biological approaches, each of which have positive and negative aspects.[12] Shao et al. states that overall, the chemical approach is effective and fast, but a particular downside to its implementation is that some chemical methods can create secondary pollution and kill non-target organisms.[12] Types of chemical treatments include copper, chlorine, aluminum, and various herbicides.[12-13] On the other hand, while physical approaches use great amounts of energy while being relatively inefficient and can cause harm to non-target organisms, they create less secondary pollution.[12] Methods such as using an air compressor to mix bodies of water and using pressure devices to deter growth of the cyano-HAB are common in the physical

approach. As neither the chemical nor the physical approach seems desirable, scientists are turning to the eco-friendly biological approach, although its efficiency is variable due to biotic and abiotic factors in the environment. *Hordeum vulgare* is a natural treatment method that is often used to treat the occurrence of these blooms. Other natural treatment methods are deciduous leaf litter (such as oak leaves), rice straw, and rice hull, all of which are agricultural byproducts or byproducts of the natural environment.[12] Although not as heavily researched as barley straw, these treatment methods have been shown to have great potential. The exact mechanisms employed by barley straw to effectively decrease the size of *Microcystis aeruginosa* blooms is still relatively unknown. Scientists theorize that exposing barley straw to air allows for certain reactions to occur, promoting the release of phenolic compounds and further combating excessive growth of cyanobacteria.[8] Similarly, rice hull extract and deciduous leaf litter have been shown to release inhibitory phenolic compounds that have been effective in combating *M. aeruginosa* blooms, although not much else is known about the processes.[10-13] In addition, as demonstrated in research conducted by Park, Kim, Chung, & Hwang, rice hull extract has a low toxicity to *Daphnia magna* populations.[13]

Cyano-HABs are occurring more often, and as they do, they continue to harm the local environment and cause millions of dollars in damage to the economy. In addition, signs of a cyanobacterial infestation are not always readily apparent. This an issue for those who are unable to recognize the signs of HAB presence, since illness and death can result when the proper precautions are not taken. Although extensive research has resulted in several EPA-approved and efficient methods, they are not perfect. A consistent problem with these methods is that they involve toxic chemicals, which often harm the microorganisms that inhabit these bloom-affected bodies of water. This counteracts the reason for removing the cyanobacterial blooms because the naturally occurring organisms perish anyway. The purpose of this experiment was to more thoroughly research the biological approach and natural methods of treatment to determine which ones are effective in killing cyanobacterial blooms, while remaining safe to the local environment to prevent this paradoxical situation from occurring.

It was hypothesized that if *Hordeum vulgare*, rice hull, and oak leaf litter were used to treat *Microcystis*, *Hordeum vulgare* would be most effective in treating the cyanobacteria since barley straw releases phenolic compounds that have been shown to inhibit cyanobacterial growth.

In the experiment, various treatments (*Hordeum vulgare*, rice hull, oak leaf litter, no treatment, cotton balls, and copper (II) sulfate) were applied to the *Microcystis* in separate groups to study the effects of the treatments on the prevalence of the cyanobacteria. To measure the prevalence of the cyanobacteria, initial optical density measurements were taken at 730 nm prior to adding in treatments. Every week, these measurements were retaken until the end of experimentation. Using this data, the decrease in optical density was calculated and used to understand the effects of the treatments. In another component, the same treatments were applied to populations of *Daphnia magna* to observe the effects of the treatments on *Daphnia magna* survival by counting population sizes every three days. After initial inoculation of the cyanobacteria and allowing a period of growth, the experiment was run for a period of 21 days to gather data. *Daphnia* responses to the treatments were observed during a simultaneous 15 day segment.

Methods

Microcystis cultures, Alga-Gro Freshwater Medium, and *Daphnia magna* were obtained from Carolina Biological. *Hordeum vulgare* was purchased from Summit Chemical, rice hull was purchased from Home Brew Ohio, and the oak leaf litter was purchased from theBioDude. Copper (II) sulfate pentahydrate was obtained from Flinn Scientific. Sixty separate containers were set up to simulate freshwater ecosystems for the *Hordeum vulgare*, rice hull, oak leaf litter, two negative controls (no treatment and cotton), and positive ($CuSO_4$) control treatments.

Nine liters of the Alga-Gro Freshwater Medium were prepared by using a ratio of 20 mL of concentrate to 980 mL of spring water. In each of the 60 containers, 150 mL of the prepared medium was added. One container represented 1 trial.

Using a hemocytometer, it was calculated that the cell density of the *Microcystis* cultures was approximately 3857.5 cells/µL (Figure 1). From the culture tubes, 1.2 mL (1200 µL) containing approximately 4629 *Microcystis* cells was measured using a micropipette and deposited into each of the 60 containers.

These cultures were placed under a 24-hour operating light table emitting 650 lux for 7 days to encourage growth and allow the cultures to acclimate to the environment. Initial optical density measurements were taken after these 7 days passed (prior to adding treatments) using a spectrophotometer.

Afterwards, each treatment was applied in their optimal quantities to 10 containers each. Barley straw was applied in a 7.2 g/L concentration, rice hull was applied in a 0.0006 g/L concentration, and oak leaf litter was applied in a 5.5 g/L concentration. In the first negative control group, no treatment was added to the *Microcystis* cultures to understand how an untreated environment would react in the same conditions experienced by all 60 simulated ecosystems. In the second negative control group, the cotton ball treatment group, two cotton balls were applied to each container to study how a material with no supposed effect on *Microcystis* cultures would affect cyanobacterial growth. In the positive control group, in which copper (II) sulfate was being used as a treatment, a 1.5 g/L concentration of $CuSO_4$ was introduced to study the effects of a common chemical method that is employed today. To be constant, each treatment was placed at the surface of the medium, even though only the agricultural products require it to allow necessary chemicals to be released. In this way, the application of the treatment was controlled to eliminate other variables. To prevent evaporation of the medium, clear plastic cling film was used to loosely cover the containers, while still allowing for aeration. The light table continued to emit 650 lux for 24 hours every day during the experimentation period. Cultures were stirred every few days to prevent them from growing directly on the container.

As a separate component to observe how organisms were impacted by the treatments, 90 *Daphnia magna* were equally separated into another set of 60 containers each (10 trials per treatment where one container represented 1 trial) with 150 mL of spring water. These containers did not have any cyanobacteria present, so only the effects of the prescribed treatment would be observed. A yeast suspension was prepared by adding enough Brewer's yeast to 0.5 L of spring water until the suspension looked milky. *Daphnia* cultures were fed every day using a few drops of the prepared yeast suspension.

The experiment was run for a total of 28 days, including the initial inoculation period of the cyanobacteria for 7 days, the following testing period of 21 days where optical density data was collected, and a simultaneous period of 15 days where *Daphnia* cultures were observed. Every week for two additional weeks, optical density measurements were taken at 730 nm using a spectrophotometer. Prior to collecting OD readings, the medium was homogenized using a stirring rod to ensure that a generalized measurement was taken. In addition, *Daphnia* population counts were taken every three days in order to establish trends and observe which treatments significantly impacted *Daphnia* populations.

To understand the data, the decrease in the optical density was calculated for each trial to understand how well each treatment effectively decreased cyanobacterial prevalence (Figure 2). In addition, *Daphnia* population counts were observed to understand which treatment groups had the most toxic effects on the organisms.

After the testing period ended, the *Microcystis* cultures were disposed of with bleach. Analysis of the results was aimed at unveiling any effects the various treatments may have had on the overall prevalence of the cyanobacteria. Data was analyzed at alpha equal to 0.05 with an ANOVA.

Results

In order to study the effectiveness of each treatment, optical density measurements were recorded at 730 nm every week for three weeks. Table 1 shows an analysis of the data concerning the decrease in optical density from the first reading to the last reading for the various treatments. For the *Hordeum vulgare* treatment, the mean optical density was -0.1401, with a standard deviation of 0.1751. The data for this treatment had a range of 0.4136. The mean for the rice hull treatment was slightly lower at -0.268, with a greater standard deviation and range of 0.355 and 1.118, respectively. The oak leaf litter treatment yielded a mean of 0.0577, a standard deviation of 0.2329 mL, and a range of 0.8130. Next, data pertaining to the first negative control, the singular *Microcystis* treatment, exhibited a mean of -0.393, a standard deviation of 0.561, and a range of 1.419. In the second negative control, the *Microcystis* and cotton treatment, the mean was -0.149, the standard deviation was 0.508, and the range was 1.870. The data for the last treatment, the $CuSO_4$ positive control, displayed a mean of 0.0037, a standard deviation of 0.0883, and a range of 0.2670. Boxplots of this data are presented in Figure 4.

The data were normal, so an ANOVA test was conducted to determine significance. The F value was 1.31, and the p value was 0.283, which is greater than the alpha value of 0.05 (Table 2). As a result, the null hypothesis, which states the means are equal, was not supported. This indicates none of the treatments were significantly different in their function from the other treatments.

Discussion

The purpose of this experiment was to more thoroughly research natural, low-effort methods of cyano-HAB treatment to determine which ones are effective in killing cyanobacterial blooms. While being effective, these treatments must also not negatively impact non-target aquatic organisms. Although there are other options such as chemical and physical approaches (the prior method being more efficient), each have downsides associated with them. Both of these approaches have been found to be either toxic or in some way harmful to organisms that inhabit the areas where these eradicative methods are employed, while also creating secondary pollution. It was hypothesized that if *Hordeum vulgare*, rice hull, and oak leaf litter were used to treat *Microcystis*, *Hordeum vulgare* would be most effective in treating the cyanobacteria since barley straw releases phenolic compounds that have been shown to inhibit cyanobacterial growth. The hypothesis was not supported because the data evaluating the decrease in optical density of the cultures was found to be insignificant.

It was found that the oak leaf litter treatment had the highest mean (M = 0.0577) for the decrease in optical density measurements (Table 1). The highest positive value was most relevant to this experiment because the objective was to find the treatment with the greatest decrease from the initial OD reading to the final OD reading, thus indicating which treatment was most effective in eradicating *Microcystis*. In addition to the oak leaf litter, a positive mean was present in the positive control ($CuSO_4$) group, as expected because copper (II) sulfate is a common cyano-HAB treatment. Negative values, on the other hand, indicated an increase in *Microcystis* growth, which was observed for the following treatments: *Hordeum vulgare*, rice hull, *Microcystis* (negative control #1), and cotton (negative control #2). Interestingly, the lowest mean was present in the *Microcystis* group (M = -0.393). Although it was expected to see an increase in optical density in the two negative control groups since no treatments were present, it was interesting to note that in this experiment, the *Hordeum vulgare* and rice hull were shown to be ineffective in targeting cyanobacterial cells. The ANOVA test yielded an F value of 1.31 and a p value of 0.283, which is greater than the alpha value of 0.05 (Table 2). As a result, the null hypothesis was not supported, indicating that there were no significant differences between the means, ultimately showing that none of the treatments caused significantly varied effects to the *Microcystis* blooms. Although the data suggests that the treatments did not cause significant declines in *Microcystis* blooms (or in some cases, they permitted increases), valuable insight can be gained when strictly observing the means (Table 1). Table 1 shows that the oak leaf litter, to a small extent, had a negative effect on the persistence of the cyanobacteria (represented by its positive mean), whereas the other two treatments, *Hordeum vulgare* and rice hull, did nothing to target the growth of the cyanobacteria, letting it prosper (M = -0.1401 and M= -0.268, respectively).

In terms of the response of *Daphnia magna* to the various treatments, a general trend that was observed was that all of the populations significantly declined in the first few days, which can likely be attributed to trauma. Of the populations, the most dramatic declines were immediately noted in the *Hordeum vulgare*, oak leaf litter, and copper (II) sulfate (positive control) treatment groups. In the remaining groups (rice hull, *Microcystis* negative control, and cotton negative control), small populations persisted for up to two weeks before staggering off. It was expected to see a general decline in populations only in the $CuSO_4$ positive control group and stable populations in the remaining five groups as the treatments are natural and supposedly non-toxic. However, it is interesting to note that of the *Daphnia* cultures that did survive, the *Microcystis* negative control group was most successful and supported stable and increasing populations until the end of the 15 day testing period. Additionally, the cotton negative control group and the rice hull group were also similarly relatively successful in sustaining *Daphnia* populations.

In a study conducted by Shao, Li, Lepo, and Gu, barley straw was found to be the most effective and practical natural treatment method when compared to other biologically derived substances.[12] In this experiment, however, oak leaf litter showed the most promising results, which may have been due to differences in barley straw application, making it less effective. In the same study by Shao et al. and another study conducted by Park, Kim, Chung, & Hwang, rice hull was found to be minimally harmful to the survival of *Daphnia magna*, allowing between 50%-90% survival rates during a 7 day period, depending on the concentration.[13] Similarly, in this experiment, rice hull was found to be one of the least harmful treatments to *Daphnia magna*, and during a 7 day period, this treatment achieved similar survival rates. Sellner et al. also determined that barley straw was highly effective in eradicating *Microcystis aeruginosa* blooms and lowered the severity of successive *M. aeruginosa* infestations as years passed.[8] However, in this experiment, likely due to differences in the deployment of the treatment, a decline in cyanobacterial prevalence was not noted.

Certain adjustments to the methods could be made to improve this research. In terms of methodology, barley straw is most effective when placed in bodies of water a few months prior to the appearance of *Microcystis*, but this was unable to be replicated in this experiment due to time constraints. As a result, the necessary phenolic compounds that allow barley straw to be successful in eradicating blooms may not have been released. This could be improved by starting experimentation early enough to allot enough time for proper *Hordeum vulgare* introduction. In addition, due to the

placement of the cultures underneath the light table, which emitted 24 hours of continuous light every day, the medium in a few of the containers evaporated, disqualifying the container as a trial and preventing optical density readings from being taken. This issue was more pronounced in some groups than others (particularly the *Microcystis* negative control, $CuSO_4$ positive control, and the *Hordeum vulgare* groups), and may have skewed data, resulting in an ill-represented statistic communicating the efficiency of the treatments. The issue was noticed during experimentation, and cling film was used to prevent further evaporation, but to avoid this in future experiments, cling film must be used from the very beginning of experimentation, and more trials should be run. One last issue that came up during experimentation was a noticeable early decline in *Daphnia* populations across all treatment groups. This may have been due to the trauma of being transferred from stable cultures to new environments. In order to solve this issue, more *Daphnia* could be placed in a single container, and more trials could be run.

One idea for future research involves testing other novel treatment methods, specifically using extracts of known cyanobacterial inhibitors, such as the *Ephedra equisetina* root and rice hulls, rather than the agricultural products themselves. Other interesting treatment options include testing certain acid derivatives from various materials, such as salicylic acids from rice hull and tannic acid from oak extract. Another venue of cyanobacterial research could be to increase favorable conditions for *Microcystis* growth by introducing an abundance of phosphorus and increasing temperatures to evaluate the rate at which the treatments can properly eradicate the cyanobacterial cells despite unfavorable conditions.

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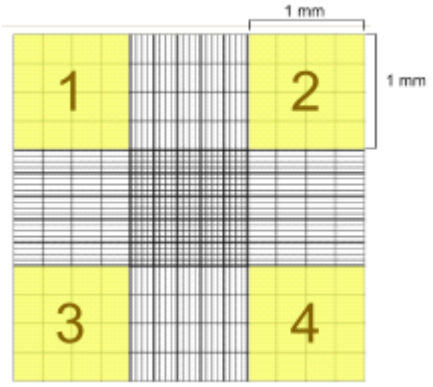
Figure 1. Calculating cell density using a hemocytometer

Quadrant	# of cells
1	291
2	373
3	470
4	409
Total	1543
Average	385.75



# of cells per cubic millimeter = # of cells counted per square millimeter * dilution * 10
# of cells per cubic millimeter = (385.75) * (1) * (10)
# of cells per cubic millimeter = 3857.5 cells/mm^3
# of cells per Microliter = 3857.5 cells/µl

*Each highlighted square on the hemocytometer grid represents an area of 1 square mm.
*No dilution was employed, so a dilution factor of 1 was used in the formula.

Figure 2. Decrease in optical density calculations and relevance

Decrease = Initial - Final

*Positive values indicate a decrease in cyanobacterial presence.
*Negative values indicate an increase in cyanobacterial presence.

Figure 3. Experimental design diagram

Title: The effect of *Hordeum vulgare*, rice hull, and oak leaf litter on the prevalence of *Microcystis*

Hypothesis: If *Hordeum vulgare*, rice hull, and oak leaf litter were used to treat *Microcystis*, *Hordeum vulgare* would be most effective in treating the cyanobacterial bloom growth since barley straw releases phenolic compounds that have been shown to inhibit cyanobacterial growth.

Independent Variable: cyanobacteria treatment

Levels of IV	*Microcystis* treated with *Hordeum vulgare*	*Microcystis* treated with rice hull	*Microcystis* treated with oak leaf litter	*Microcystis* (negative control #1)	*Microcystis* and cotton (negative control #2)	*M*
# of trials	10	10	10	10	10	

Dependent Variable: decline in cyanobacterial presence measured via decrease in optical density at 730 nm

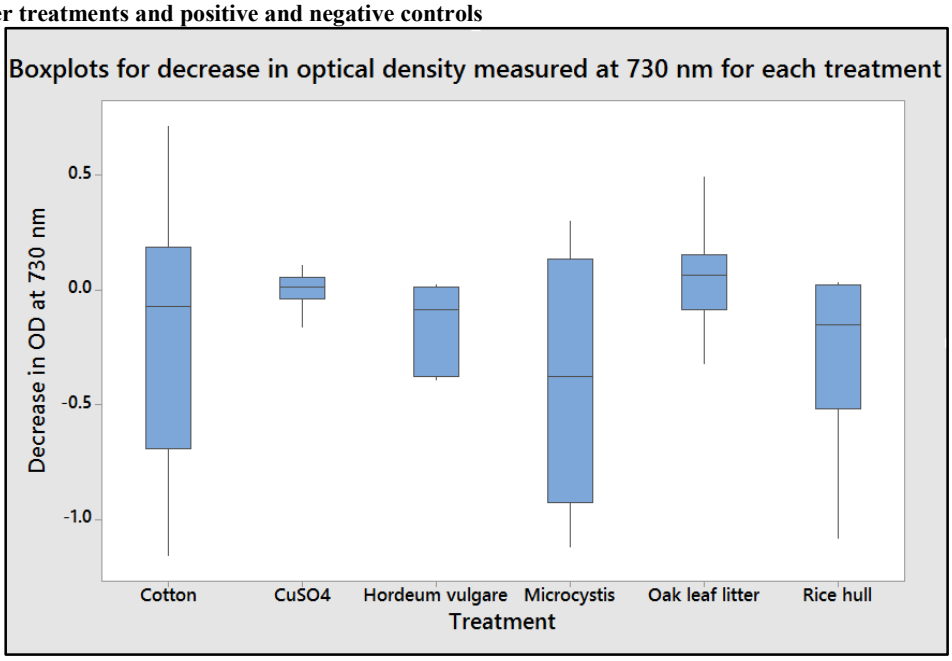
Constants: amount of sunlight, amount of *Microcystis*, temperature, humidity, space of simulated environment, quantity of medium

Table 1. Mean, range, and standard deviation for the decrease in optical density of *Hordeum vulgare*, rice hull, and oak leaf litter treatments and positive and negative controls

Treatment	Mean	Range	Standard Deviation
Microcystis treated with *Hordeum vulgare*	-0.1401	0.4136	0.1751
Microcystis treated with rice hull	-0.268	1.118	0.355
Microcystis treated with oak leaf litter	0.0577	0.8130	0.2329
Microcystis (negative control #1)	-0.393	1.419	0.561
Microcystis and cotton (negative control #2)	-0.149	1.870	0.608
Microcystis and $CuSO_4$ (positive control)	0.0037	0.2670	0.0883

The oak leaf litter treatment had the greatest mean of 0.0577, followed by the $CuSO_4$ treatment with a mean of 0.0037, and the *Hordeum vulgare* treatment with a mean of -0.1401. Following the *Hordeum vulgare*, the cotton treatment had a mean of -0.149, the rice hull had a mean of -0.268, and finally, the lowest mean of -0.393 was present in the *Microcystis* treatment. The cotton treatment (1.870) had the greatest range (0.2670), followed by the *Microcystis* treatment (1.419), and the rice hull treatment (1.118). The three lowest ranges, in descending order, were present in the oak leaf litter (0.8130), the *Hordeum vulgare* (0.4136), and the $CuSO_4$ treatment (0.2670). The cotton treatment had the greatest standard deviation at 0.608, followed by the *Microcystis* at 0.561, and the rice hull treatment at 0.355. Next, the oak leaf litter treatment followed with a standard deviation of 0.2329, the *Hordeum vulgare* at 0.1751, and the $CuSO_4$ at 0.0883.

Figure 4. Boxplots for the decrease in optical density of *Hordeum vulgare*, rice hull, and oak leaf litter treatments and positive and negative controls



Asterisks represent outliers in the data set. The *Microcystis* treatment had the largest IQR, followed by the cotton treatment and the rice hull treatment. The three smallest IQRs were present in the *Hordeum vulgare*, oak leaf litter, and $CuSO_4$ treatments, in descending order. The mean was the highest for the oak leaf litter treatment and the lowest for the *Microcystis* treatment.

Table 2. ANOVA summary table for decrease in optical density of *Hordeum vulgare*, rice hull, and oak leaf litter treatments and positive and negative controls

	dF	SS	MS	F	P
Factor	5	0.9205	0.1841	1.31	0.283
Error	37	5.2190	0.1411		
Total	42	6.1395			

An ANOVA test was conducted because the data were normal, and data were analyzed at alpha equal to 0.05. The null hypothesis stated that all means were equal, and it was not supported because p>alpha ($F(5, 37) = 1.31$, $p=0.283$). This means there are no significant differences in the data to indicate that at least one mean is different.