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Short-Term Effects of Critical Metal Concentrations on Usnic Acid Production in *Cladonia rangiferina*

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ABSTRACT

Cladonia rangiferina is a lichen that produces usnic acid, a selectively toxic biomolecule, allowing the lichen to protect itself from predators and regulate populations of microorganisms, other fungi, and animals in its vicinity. Unfortunately, metal pollution associated with human industrial activity has hindered *C. rangiferina's* metabolism, as indicated by its decreasing population in areas with high metal concentrations. It was unclear whether *C. rangiferina's* ability to produce usnic acid was also hindered in polluted environments and whether the type of metal pollutant led to different effects. A short-term experimental study was used to isolate the effects of concentrated copper, nickel, and zinc ions on this metabolic pathway. It was found that nickel (+2) does not significantly affect usnic acid production, whereas even small concentrations of copper (+2) dramatically reduce it. Meanwhile, the concentration of zinc (+2) seems to be negatively correlated with usnic acid production in the short term. This means that priority regions for *C. rangiferina* population restoration projects include areas with critically high concentrations of copper and zinc, but not nickel. Further research is needed to a) explain how usnic acid synthesis changed rapidly under varying copper conditions compared to zinc and b) identify changes to this molecule's synthesis under a combination of metal stresses. This study could clarify the metabolic pathway of usnic acid production and refine plans for artificial dispersal (a lichen conservation method).

Introduction

As evidenced by their presence on every continent, lichens are a diverse and hardy class of biological structures (Grube et al., 2015). A lichen is not a single organism, but rather the result of a symbiotic relationship between a fungus and an algae. The algal component tends to be a single species while fungal components vary greatly. Lichens' properties and environmental roles are starkly different from those of individual algae or fungi, so each pair is given its own species name and taxonomic classification (Salgado et al., 2017). In some lichen varieties, the fungus is paired with a cyanobacteria – a bacteria that conducts photosynthesis – instead of an algae (Nash, 2008).

With the combined photosynthetic and decomposing abilities of the algae and fungus respectively, lichens can conduct a wide variety of chemical reactions. This gives them flexibility in how they process energy, obtain nutrition, and reproduce. As a result, a single lichen species is often able to tolerate a large range of moisture, nutrient, sunlight, and temperature conditions, allowing each species to spread over wide geographic regions (Salgado et al., 2017). The union of algae and fungi also results in the formation of entirely new metabolic pathways that are not observed in the isolated components. A metabolism is the sum of transfers of substances into and out of an organism's body as well as the chemical reactions occurring within. A metabolic pathway is a series of interconnected chemical reactions, and organic substances which are produced by these

lichen-only metabolic pathways are referred to as secondary metabolites (Nybakken, 2010).

Usnic acid is a secondary metabolite produced by the *lecanorales* taxonomic group of lichens. This compound is yellow, mildly acidic, and toxic to some plants and animals (Araujo et al., 2017). *Cladonia rangiferina*, informally called reindeer moss, is a species within the *lecanorales* taxonomic group that produces usnic acid. Even compared to other lichens, *C. rangiferina* has a remarkably large geographic range that spans Canada, Europe, and the northern United States (Grube et al., 2015).This study focused on *C. rangiferina* because changes in this lichen have the potential to affect many ecosystems worldwide.

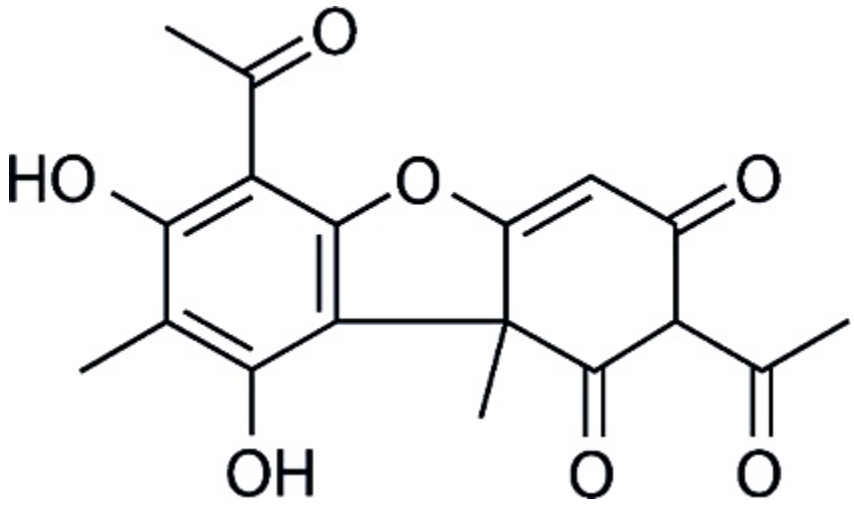

Figure 1. Chemical structure of usnic acid – PubChem, National Institute of Health.

Literature Review

Properties of Usnic Acid

Previous studies on usnic acid synthesis in lichens have identified several of the chemical reactions needed to make this molecule, but certain steps in the metabolic pathway remain unknown (Nunes et al., 2015). As a result, the majority of usnic acid's properties cannot be predicted, and they are generally discovered through sampling or experimental designs instead. One such finding is that usnic acid is toxic to species that commonly threaten *lecanorales* lichens (Nybakken et al., 2010). Lichen metabolisms are much slower than plant and animal metabolisms. As a result, lichen growth is slow, and risks being outpaced by consumption from herbivores. Usnic acid often acts as a defense against these predators. For example, in 2017, the Federal University of Brazil used an experimental method to indicate that usnic acid kills snails. Their methods involved the use of positive and negative controls as well as measurement techniques supported by the World Health Organization. Because snails regularly eat lichens, this research implies that usnic acid acts as a defense from predators in order to maintain stable lichen populations over time (Araujo et al., 2017). This is supported by the results of a 2010 experimental study which found that voles (a type of herbivorous rodent) are reluctant to eat usnic acid-rich lichens. This pattern was observed by offering the voles samples of *C. rangiferina* and several other species inraw and acid-free forms. The voles' preference for acid-free lichens indicated that usnic acid is a significant deterrent to herbivores (Nybakken et al., 2010).

Second, usnic acid is antibacterial, as shown by a 2012 study that tested the efficacy of usnic acid in preventing reproduction of 10 species of bacteria. It was found that the minimum concentration of usnic acid

needed to prevent bacterial spread was often a fraction of the concentration needed from commonly used antibiotics, demonstrating how powerful this property is. Additionally, the species in the 2012 study were from diverse taxonomic groups, soit is reasonable to infer that usnic acid affects most bacteria species (Rankovic et al., 2012). When this is combined with the idea that *lecanorales* lichens secrete usnic acid into their surroundings, it becomes clear that usnic acid plays a role in regulating the populations of nearbymicroorganisms and predators in *lecanorales* lichens' environments.

Usnic acid has also been proposed as an anticancer and anti-inflammatory agent, butstudies on these properties have produced mixed or even contradictory results at times (Cocchietto et al., 2002; Tang, 2020). A 2008 review by the Food and Drug Administration states that usnic acid is antiviral to some degree. This was determined based on sped up recoverytimes in infected cell samples receiving usnic acid-based treatments when compared cells that received a placebo, but only 3 viral diseases were included in this study (FDA staff, 2005). The applications of usnic acid in human health continue to be investigated and explored (Tang, 2020).

Since usnic acid production is closely intertwined with lichen populations, lichen consumption, and the populations of many other organisms, it is a key factor in defining the ecological role of *lecanorales* lichens. It is crucial to understand the factors influencing usnic acid production in lichens because variation in environmental usnic acid concentrations can havediverse and widespread effects.

Factors Influencing Usnic Acid Production

Nutrient availability in a lichen's environment impacts its usnic acid production. In 2009,the Agharkar Research Institute demonstrated this by growing a single lichen species (*Usnea ghattensis*) in artificial environments with excessively low, moderate, and excessively high amounts of glucose and nitrogen. It was observed that usnic acid production steadily increased as the nutrient concentrations increased, and this pattern held true even in extreme excesses of glucose and nitrogen. In a publication summarizing this research, the authors explain that this positive correlation indicates both nutrients are involved in the chemical reactions needed to create usnic acid molecules (Verma & Sonone, 2009). As of 2020, there are not many sources which indicate that the metabolic pathway for usnic acid production differs among different lichen species. Therefore, it was assumed for this project that the factors causing usnic acid variation in any lichen species have the potential to cause usnic acid variation in *C. rangiferina* (Gao et al.,2020).

Light radiation has been found to influence usnic acid production in several *lecanorales* species over the last 20 years. According to a publication by the Norwegian University of Life Sciences, increases in some light energy can lead to increased usnic acid production in *C. rangiferina*. Unlike most other sources mentioned so far, this study employed a sampling method. Begora and Fahselt collected lichens from a range of locations in Canada and measuredeach location for two types of light radiation: UV-A and UV-B. The amount of usnic acid withinthe lichen bodies were recorded, and these amounts were graphed in relation to each sample's UV conditions. A strong positive correlation was observed between increasing UV-A radiation and usnic acid, while increasing UV-B radiation was associated with a moderate decrease in usnic acid (Begora & Fahselt, 2001). These results are supported by two experimental designs conducted on other lichen species in 2005 and 2009 (Bjerke et al., 2005; Larsson, 2009).

Variation in usnic acid production by lichens often mimics how the lichens' overall metabolism has changed. For example, increased UV-A radiation and increased glucose are both associated with faster growth in *C. rangiferina* lichens, which implies overall metabolic productivity (Begora & Fahselt, 2001; Muggia et al., 2017). This matches the increases in usnic acid synthesis as described in previous paragraphs (Verma & Sonone, 2009). Similarly, unusually high UV-B radiation is associated with reductions in both lichen growth and usnic acidproduction (Begora & Fahselt, 2001). However, some studies have indicated that effects on general metabolism cannot be used to predict effects on individual pathways in unusual environments. For example, harsh cold conditions tend to slow lichen growth but encourage usnic acid production (Bjerke et al.,

2005). As mentioned previously, environments with unusually high nitrogen concentrations encourage usnic acid production, but they have also beenshown to cause significant decreases in lichen growth rates and even death in long-term studies (Hutchinson et al., 2016; Verma & Sonone, 2009). Therefore, there remains a debate on how to predict usnic acid variation caused by unusual environmental conditions, even if the trend for general metabolism under those conditions is known.

Cladonia rangiferina Metal Sensitivity

Cladonia rangiferina populations tend to sharply decline in regions with high concentrations of metallic elements (Hutchinson et al., 2016). A 2010 study by Moscow State University revealed that these sensitivities are likely caused by a characteristic of lichen cells which allows rapid intake of metal ions (Meychek et al., 2010). To express this sensitivity in quantitative terms, Folkeson and Bringmark used a sampling method and recorded *C. rangiferina* population density, along with various metal concentrations, at several distances from an industrial facility. The researchers gathered over 100samples before calculating the correlations between lichen population density and metal concentrations. It was found that copper, zinc, and nickel have correlations of -0.95, -0.92, and
-0.76 with *C. rangiferina* populations, respectively (Folkeson & Bringmark, 2011). A correlationof -1 would indicate a perfectly linear, negative relationship; therefore, it is evident that these three metals pose a significant risk to *C. rangiferina* (Grube et al., 2015).

Furthermore, Folkeson and Bringmark used these mathematical models to identify *C. rangiferina*'s critical concentrations of copper and zinc as 350 and 600 ppm, respectively. A critical concentration is the maximum concentration of a substance that a species can survive inlong-term (Folkeson & Bringmark, 2011). These values were confirmed in a 2019 study by theRussian Academy of Sciences. In the 2019 study, lichen samples were taken from an environment with drastically different light, temperature, and moisture conditions, implying thatthere were not any unintended variables impacting the results of the first study (Popova, 2019). Critical concentration estimates for nickel varied slightly between two studies in 2019 and 2020(17.36 and 18.66 ppm, respectively). Lichen death implies that a significant change in overall metabolism occurred, so it can be inferred that copper, zinc, and nickel impair or hinder *C. rangiferina*'s metabolic processes in some way (Gao et al., 2020).

Figure 2. Cladonia rangiferina lichens in Vermont. US Forest Service

Artificial Dispersal

Artificial dispersal is a technique used to restore damaged lichen populations. In this procedure, lichens are collected from their native environment, stimulated to rapidly reproduce ina controlled setting such as a lab, and then released with their offspring back into the environment (Zabrabska et al., 2015). According to the Swedish University of Agricultural Sciences, this has been carried out successfully for two *lecanorales* lichen species. The University reported in 2003 that over 80% of *Evernia divaricata* and *Ramalina dilacerata* lichens redistributed into their environments remained alive for at least 1 year after artificial dispersal, indicating that artificial dispersal could be a long-term solution to reduced lichen populations in harsh environments (Liden et al., 2004). This is important because *C. rangiferina*is also a threatened species, but there are many potential locations to conduct artificial dispersalat (Gao et al., 2020). If this technique were used for *C. rangiferina*, it would be beneficial to understand where it is most urgently needed.

Research Gap, Research Question, and Thesis

There is little available information regarding the effects of critically high copper, zinc, and nickel concentrations on usnic acid production in *Cladonia rangiferina* lichens.This gap has occurred despite usnic acid being intensely studied for about twenty years, possibly because recent focus has shifted toward the intersection of usnic acid and human health (FDA staff, 2005). However, it was necessary to understand this topic because *Cladonia rangiferina* is a widespread lichen species, and usnic acid affects many organisms other than its producer (Grube et al., 2015; Nybakken et al., 2010). Therefore, variation in this species' usnic acid production has serious implications for many environments worldwide. This research was significant because the conditions that were found to cause dramatic decreases in usnic acid production indicated priority regions for artificial dispersal of *C. rangiferina* (Gao et al., 2020).

In sum, the research question was "How do environments with critically high copper (+2), zinc (+2), and nickel (+2) ion concentrations affect usnic acid production in *Cladonia rangiferina* lichens?" It was hypothesized that all three metals would reduce usnic acid production with copper causing the greatest change, zinc slightly less, and nickel the least. In other words, the trend for usnic acid production was expected to mimic general metabolic trends (Folkeson & Bringmark, 2011).

Method

Only an experimental method could demonstrate the causality that this project aimed to identify (Salgado et al., 2017). Long-term studies for lichens require about two months per trial, and it would be unreasonable to employ such a method without receiving an indication that there were significant results in a short-term study first (Nash, 2008). It was necessary to ensure that the metals, their concentrations, and the usnic acidproduction were the only variables in this design.

Lichen Preparation

Six lichen bodies were purchased – three per trial for two trials. It was confirmed by the supplier that the bodies were collected from a singleforest in North Carolina and that all six were of the species *C. rangiferina*. Once the lichens were received, they were cleared of debris with sterilized metal tweezers and washed thoroughly with deionized water. This was to ensure that the lichens did not have any contaminants before being placed in petri dishes (Ekmekyapar et al., 2006; Meychek et al., 2010). To make sure that each sample was equal, the lichen bodies were air dried for 24 hours before being divided into portions of equal mass (0.25 grams + - 0.001 grams), which is a common practice in lichen experiments (Begora & Fahselt, 2001; Liden et al., 2010; Meychek etal., 2010). Since younger sections tend to produce more usnic acid than older ones, only youngerparts of the lichen bodies were used in the trials. These sections were identified by the width of the lichen thalli, which are branch-like structures. All samples had a thallus width of 1 mm or smaller (Meycheck et al., 2010).

Artificial Environment

Media is an artificial watery environment that an organism is suspended in during a trial. Media includes essential nutrients for life, such as sugar and calcium, but it can also be altered based on the environmental conditions that are being imitated. The base formula of media used in this method was a pre-made mixture called Nutrient Broth (brand HiMedia). This is a commonmedia type, and it was chosen because it contained large amounts of glucose. As described in theliterature review, having excess glucose can enhance usnic acid production, and greater usnic acid production overall would allow for easier identification of differences among the treatment groups (Verma & Sonone, 2009). The treatment groups will be explained further in subsequent sections. An antibiotic compound – chloramphenicol – was added to all medias before they were poured into plastic, sterile petri dishes, and lichen samples were then placed inside these petri dishes (Salgado et al., 2017).

Another option would have been to use agar media instead of nutrient broth. Agar media contains all of the components of nutrient broth plus agar, a jelly-like substance. This has been preferred by some researchers because it contains even more glucose than plain nutrient broth, further increasing usnic acid production. However, agar media must be treated with several otherchemicals to alter its consistency before it can be analyzed for usnic acid concentrations (Verma & Sonone, 2009). It was unknown whether the addition of metals to the agar media could react with these chemical treatments and impact the usnic acid readings, so plain nutrient broth was chosen instead.

Other environmental factors which could have affected usnic acid production were kept constant, as

well. For example, the temperature was kept at 20 degrees Celsius. De-ionized waterwas occasionally added to the petri dishes to keep the amount of fluid in each sample at 20 mL because the design of petri dishes allows for evaporation. The lichens were kept equidistant froma light source, and they all remained in their respective treatment groups for 15 days (Grube et al., 2015).

Treatment Groups

	No Lichen	Metal-Free	High Cu	Low Cu	High Zn	Low Zn	High Ni	Low Ni
Trial 1 Replicates	A B C	A B C	A B C	A B C	A B C	A B C	A B C	A B C
Trial 2 Replicates	D E F	D E F	D E F	D E F	D E F	D E F	D E F	D E F

Figure 3. Setup of treatments and replicates in both trials

Figure 3 (above) displays the treatments given to lichen samples in this method. “No lichen” refers to a negative control. These were petri dishes with plain, metal-free media and no lichen, expected to show zero usnic acid production in order to confirm that there was no contamination (Grube et al., 2015). The “metal-free” group refers to the positive control, expected to show the most usnic acid production because there was not any copper, zinc, or nickel in the media (Salgado et al., 2017). The “High” groups had critically high concentrations of copper, zinc, or nickel (350, 600, or 18 ppm) while the “Low” groups had the US average soilconcentration of each metal (120, 200, or 6 ppm). The low metal treatments were included to help determine whether any observed changes in usnic acid were caused by the critically high amounts of metal or just the presence of the metal in general (Hutchinson et al., 2016; Popova 2019). Curiously, the critical concentrations for all metals in this project were roughly triple the US average soil concentration. All metals were added to the media by dissolving metal nitrates inthe pre-made Nutrient Broth because metal nitrates easily split into metal ions and nitrate ions.

Nitrate ions are not known to affect usnic acid production (Dahlman et al., 2004), so the only significant difference between treatment groups was the addition of metals. It should be noted that none of the treatment groups included combinations of metals. This is a significant limitation because in real environments, industrial metals are always present in combinations, and sometimes a single location can reach critical concentrations of multiple metals (Folkeson & Bringmark, 2011). The implications of this will be explained further in the Discussion section.

Measuring Usnic Acid Production

There are two common ways of measuring usnic acid production by lichens. The first is HPLC, or high-performance liquid chromatography, which uses chemical properties such as density to separate substances in a solution. The main benefit of HPLC is its remarkably high precision (Bishnu et al., 2017). Another option is spectrophotometry, which uses a liquid's colorintensity to measure how much of a colored compound is in the solution. Usnic acid is a bright yellow substance, and *C. rangiferina* does not excrete significant amounts of any other colored compound, so the intensity of yellow color in the lichens' media can be used to measure the amount of usnic acid produced by each lichen (Kutney & Sanchez, 2011). A machine called a spectrophotometer calculates color intensity of the media and represents this quantity as an absorbance value. According to the Beer-Lambert equation, these absorbance values are proportional to the concentration of usnic acid excreted by the lichens into the media (Nunes et al., 2015). This means that a 50% decrease in the absorbance reading would indicate a 50% decrease in usnic acid production. Benefits of the spectrophotometry method include the abilityto analyze small amounts of lichen media, as little as 1 mL, and high precision (Kutney & Sanchez, 2011).

This project used spectrophotometry to analyze the amount of usnic acid in the lichen samples' media because it had less risk for contamination compared to HPLC (Kutney & Sanchez, 2011). Although spectrophotometry is less precise than HPLC, the creation of a standard curve indicated that it was undoubtedly precise enough for this project. A standard curve is formed by taking absorbance readings of solutions with known concentrations of a colored compound, then calculating the linear correlation between the concentrations and theirrespective color intensities (Nash, 2008). The results of the standard curve are provided in subsequent sections.

A micropipette and test tubes were used to take small, exact samples of lichen media. Micropipettes are specialized tools for drawing liquid that limit variation between volumes to less than 0.1% (Salgado et al., 2017). Samples were measured for usnic acid production at 2, 5, 8, 11, and 15 days after the start of the experimental period. Readings were limited to these fivetimes because each additional measurement increased the risk of contamination, but it was also necessary to understand how usnic acid production changed over time.

Lichen Normalization

Even though the lichens were collected from a small region and measured to have approximately equal size and weight, there was still a possibility that each lichen body would have different usnic acid production rates. In order to have a valid experimental design with a small number of replicates, it was necessary to limit variation in usnic acid production among thesamples. Therefore, before the true experiments began, 15 samples from each lichen body were all placed in metal-free media for 3 days. On the 4th day, this was exchanged for more metal-free media. This process can be thought of as washing the insides of the lichens (Verma & Sonone, 2009). To determine whether it was effective, all 45 lichen samples (15 each from 3 lichen bodies per trial) were measured for usnic acid production on the 6th day. Samples which varied more than 5% from the mean usnic acid production were not used in the trials. If more than 7 samples fit the criteria for use in the trials, a random number generator was used to determine which ones would be discarded. The remaining samples were given experimental treatments withmetals one day later, which marked the start of the true trial period. This step is common among experiments with a small number of replicates, and it ensured that all variation in usnic acid was caused by the metal treatments only (Bacci et al., 1986; Nash, 2008; Verma & Sonone, 2009).

Safety, Cleaning, and Equipment

All procedures were completed under the supervision of one or more adults on the school campus. Gloves were worn throughout the experiments, and metal tools, glass materials, and labsurfaces were sterilized with alcohol. The lichens were killed and disposed of according to supplier recommendations once the trials were complete.

Graphing and Statistics

Absorbance readings were plotted against time in days to show the production of usnic acid over time (Hauck et al., 2009). However, only the final, 15-day absorbance readings were used for statistical analysis. The ANOVA test with Tukey Test Intervals was used to determine where there were significant changes in usnic acid production between treatment groups. This procedure was chosen because the ANOVA test has been used in several other published studiesregarding usnic acid production (Bjerke et al., 2005; Larsson, 2009; Nunes et al., 2015). Also, the accuracy of an ANOVA procedure does not depend on the number of replicates in an experiment, making it ideal for use in a method with only 6 lichens (Nash, 2008). This test also provides an estimate of the percent decrease in the response variable (usnic acid concentration) between two treatment groups, expressed as a range of possible values. The test software (StatCrunch) returned estimates that were spread over a large numerical range, so they were deemed unfit to use for analysis (Larsson, 2009). This means only the ANOVA values were usedin this project, and the exact percent decreases in usnic acid production were not calculated. However, this method did fulfill the project goal because an ANOVA test alone was able toindicate which metal conditions caused a significant decrease in usnic acid production.

Results

Standard Curve

The standard curve for potassium ferricyanide, an intensely yellow substance, yielded alinear correlation value of $r^2 = 0.9927$. The closer this value is to 1, the more precise the spectrophotometer is, so this instrument was certainly appropriate to use for the project (Nash 2008). The raw data for this standard curve is displayed in Figure 4 on the next page.

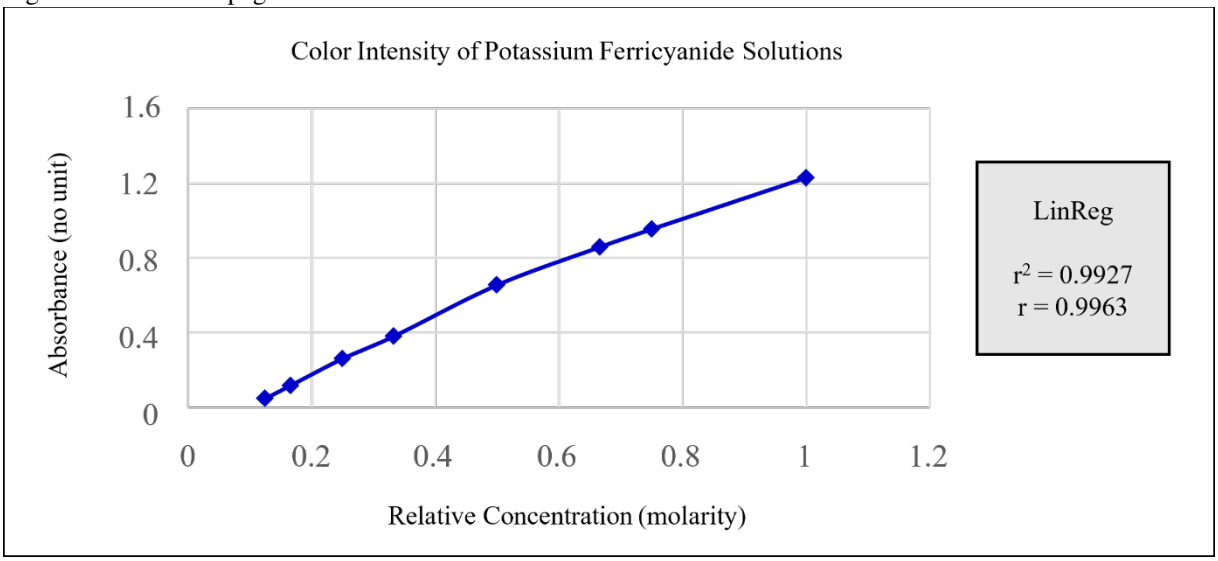

Figure 4. Standard curve to test accuracy of spectrophotometer

Lichen Health

All lichens remained alive throughout both trial periods, as evidenced by continuous increases in absorbance

readings (and, therefore, usnic acid levels) for every sample (Bjerke etal., 2005). Additionally, no visible contamination occurred in any lichen sample. Absorbance readings remained equal to zero for the negative controls throughout both trials, meaning that petri dishes without lichens did not show any usnic acid production, as expected.

Nickel

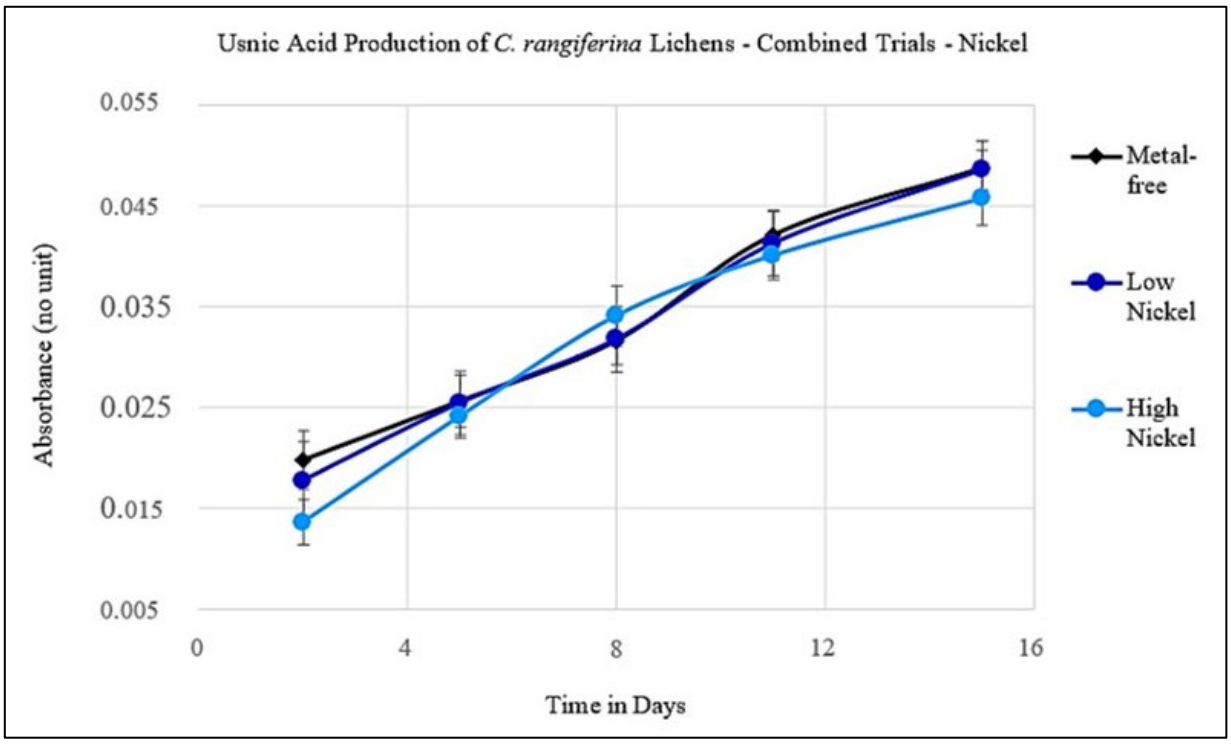

Figure 5. Graph of usnic acid production over time, nickel treatment groups and positive control

ANOVA Results - Nickel	
Treatment	P-value
Metal-free vs. low nickel	1.000
Metal-free vs. high nickel	0.3560
High nickel vs. low nickel	0.4281

Figure 6. ANOVA test results for nickel treatment groups

As indicated by Figure 5, the data do not imply any association between nickel concentration and usnic acid

production. Any p-value less than 0.05 in ANOVA testing shows asignificant difference between two treatments (Nunes et al., 2015). The large p-values for all three combinations in Figure 6 indicate that there were not any notable changes in usnic acid production within the metal-free, low nickel, and high nickel treatment groups. Real *C. rangiferina* populations most likely have the same level of usnic acid production in environmentswithout nickel, with the US average soil concentration of nickel, and with the critical concentration of nickel (Francolini et al., 2004). These treatment groups contradicted the hypothesis, which predicted a notable change between the positive control and high nickel group.

Zinc

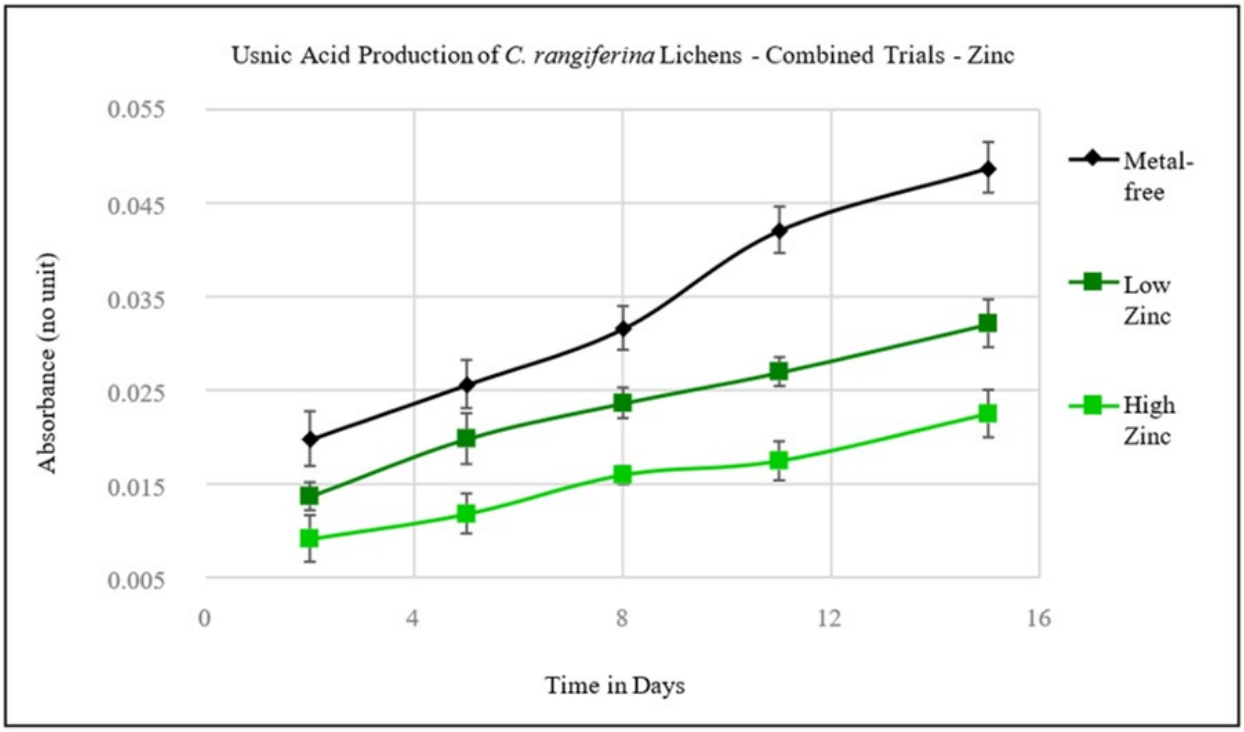

Figure 7. Graph of usnic acid production over time, zinc treatment groups and positive control

ANOVA Results – Zinc	
Treatment	P-value
Metal-free vs. low zinc	<0.0001
Metal-free vs. high zinc	<0.0001
High zinc vs. low zinc	<0.0001

Figure 8. ANOVA test results for zinc treatment groups

Figure 7 shows that lichens exposed to zinc behaved as predicted bythe hypothesis: the more zinc added to the lichens' environments, the less usnic acid was produced. ANOVA and Tukey statistics were in agreement with this. The p-values for all three combinations were less than 0.05, so there were significant reductions in usnic acid between the positive control and low zinc groups as well as the low and high zinc groups (Nunes et al., 2015).

Copper

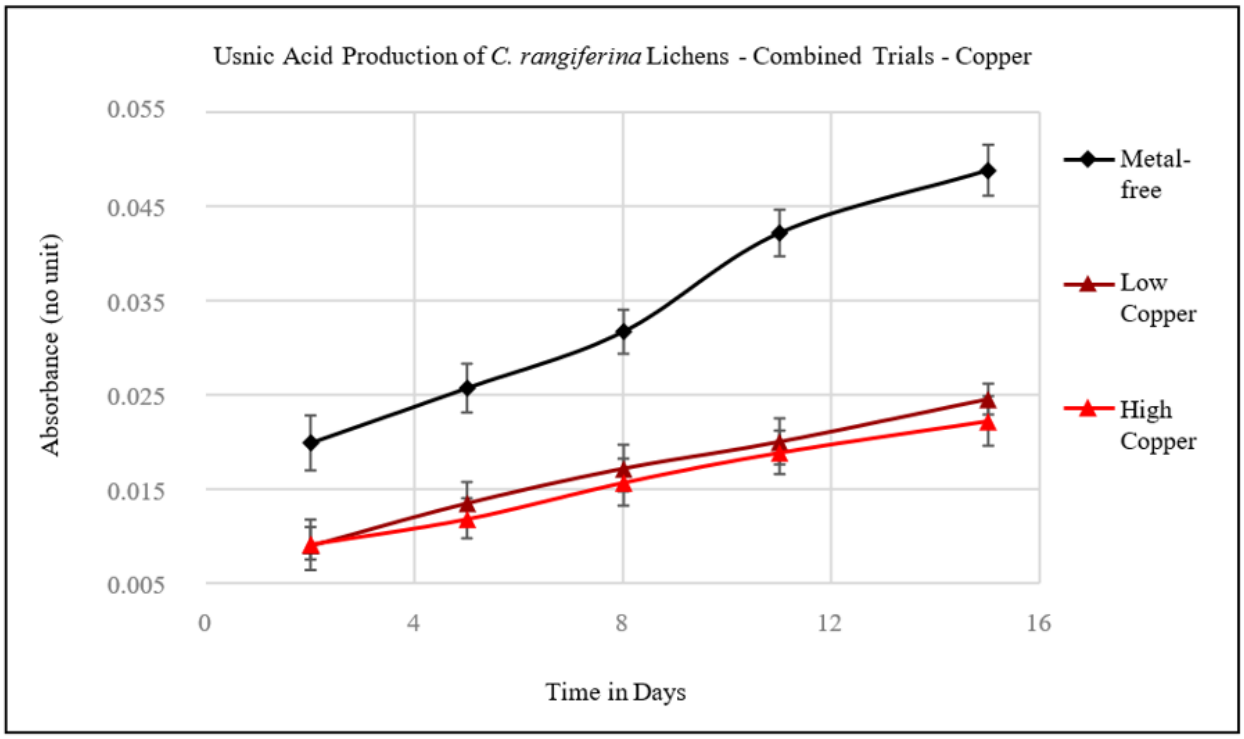

Figure 9. Graph of usnic acid production over time, copper treatment groups and positive control

ANOVA Results - Copper	
Treatment	P-value
Metal-free vs. low copper	<0.0001
Metal-free vs. high copper	<0.0001

High copper vs. low copper	0.6651

Figure 10. ANOVA test results for copper treatment groups

The copper treatment groups yielded curious results. ANOVA p-values for 1) positive control vs. low copper, 2) positive control vs. high copper, and 3) high copper vs. low copper were <0.0001, <0.0001, and 0.6651, respectively. This means that the presence of copper significantly reduced usnic acid production in *C. rangiferina* lichens when compared to a complete absence of metal, but there was no difference in usnic acid production depending on the amount (Nunes et al., 2015). This treatment group did still behave as predicted by the hypothesis because lichens exposed to critical concentrations of copper immediately slowed their usnic acid production.

Overall

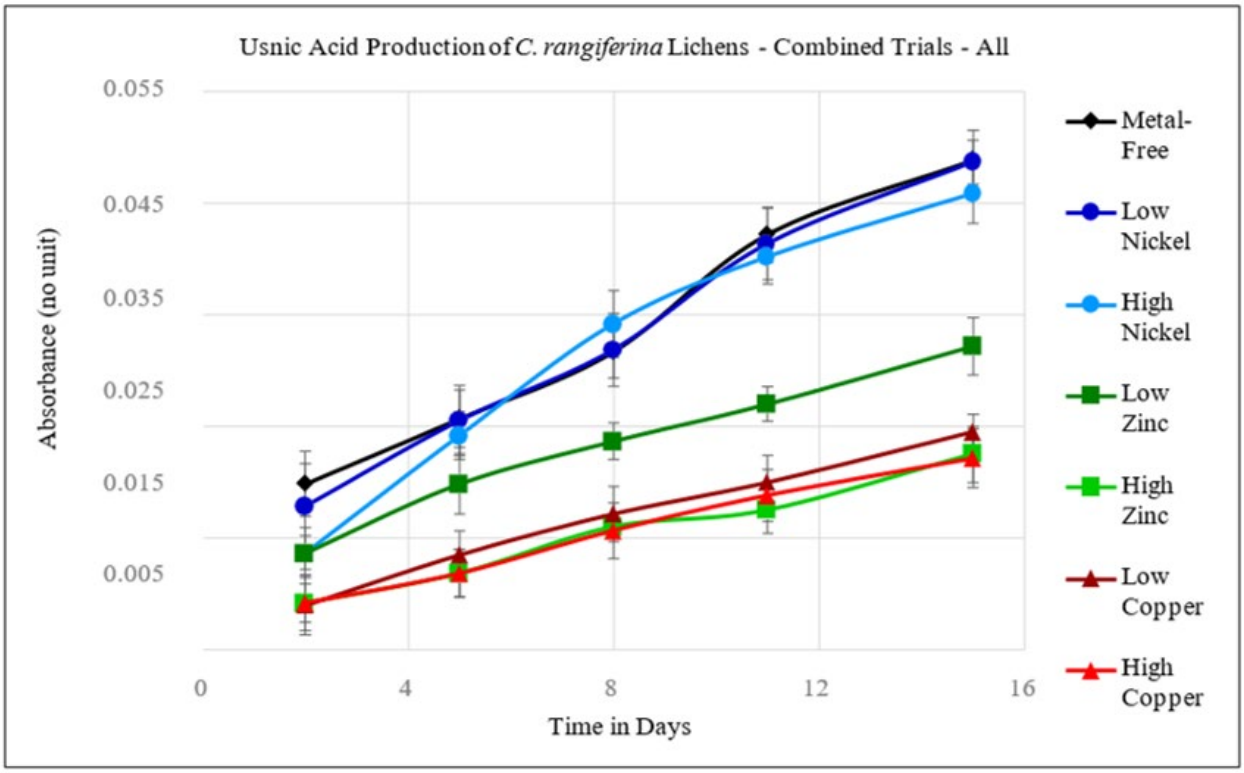

Figure 11. Graph of usnic acid production over time, all treatment groups

ANOVA Results – Comparing Metals	
Treatment	P-value

High copper vs. high zinc	1.000
High copper vs. high nickel	<0.0001
High zinc vs. high nickel	<0.0001

Figure 12. ANOVA test results for all critical concentrations

Figures 11 and 12 give a visual and statistical depiction of how different metal groups compared to each other. The ANOVA test gave a p-value of 1.000 for high copper vs. high zinc,meaning that there was no variation between the usnic acid production in these two treatment groups. This is significant because it negates the hypothesis, which predicted that critical concentrations of copper would cause lower usnic acid production than zinc. However, criticallyhigh nickel samples had the least dramatic change in usnic acid production, as expected. This is evidenced by the p-values for high copper vs. high nickel and high zinc vs. high nickel – both less than 0.05 – indicating notable differences (Nunes et al., 2015; Salgado et al., 2017). In sum,the hypothesis was correct in that critically high metal concentrations did hinder usnic acid production in *C. rangiferina* lichens, but it was incorrect in the ordering of usnic acid production within various treatment groups.

Discussion

Implications

Based on the lack of change in usnic acid production between the positive control and nickel treatments, it can be inferred that nickel is not heavily involved in the usnic acid synthesis pathway for *C. rangiferina* lichens. If nickel was involved in any way, then usnic acid productionwould progressively increase or decrease as the amount of nickel in the lichens' media changed; this did not occur. On the other hand, there was notable variation in usnic acid production undercritical zinc and copper concentrations when compared to the positive control. Therefore, both zinc and copper are most likely involved in usnic acid synthesis (Verma & Sonone, 2009). Because increased copper and zinc were associated with decreased usnic acid production, both metals most likely play a limiting or inhibiting role in the process of usnic acid synthesis (Francolini et al., 2004). Unexpectedly, usnic acid production in the low and high copper treatment groups were statistically indistinguishable, whereas the low and high zinc groups demonstrated a progressive slowing of usnic acid synthesis. This difference between the copperand zinc trends implies that the two metals are heavily involved in usnic acid production, but they do not play the same role.

Another explanation for the unanticipated pattern of the copper treatment groups is basedon what was labelled as "low" and "high" copper. Although the US average soil concentration ofcopper appears to be much lower than the critically high concentration, it could still be reflectiveof background pollution buildup over many decades (Hutchinson et al., 2016). This means that the current US average soil concentration of copper might be much higher than the conditions that *C. rangiferina* evolved in. If the experiment were to be completed with the "low" copper concentration being much lower, a trend similar to that of zinc might be observed.

In addition, this experiment indicated that usnic acid production change in *C. rangiferina*lichens is not reflective of general metabolic changes. While the difference in usnic acid production under critical copper and zinc conditions matched the pattern for general metabolic change (strong decrease), this was not the case for nickel. Therefore, in future research for this field, general metabolic trends should not be used as a predictor for usnic acid production.

Applications

The main application for this research is its relation to the field of artificial dispersal. Critical concentrations of copper and zinc dramatically reduced usnic acid production by *C. rangiferina,* and this substance is inextricably linked to the lichen's growth and survival (Araujoet al., 2017). Therefore, this study indicated a dire need for artificial dispersal of *C. rangiferina* in environments with zinc concentrations greater than 600 ppm and copper concentrations greater than 350 ppm. Artificial dispersal in areas with less than 350 ppm copper could also be beneficial because even the average US soil concentration of copper was enough to significantlyreduce usnic acid production (Zabrabska et al., 2015). Nonetheless, areas with critical zinc and copper concentrations should be prioritized. Artificial dispersal is not needed in areas with critical levels of nickel, at least when the goal of artificial dispersal is to maintain constant usnicacid levels in an ecosystem.

Limitations, Scope, and Further Research

The most significant limitation of this research is that the lichens did not reach an equilibrium by the end of the trial period. Equilibrium is a state in which a process has stopped significantly changing (Nash, 2008). In the context of this project, an equilibrium would have been indicated by nearly horizontal lines in the absorbance graphs toward the end of the trial period. However, the true data revealed that *C. rangiferina* lichens were still rapidly producing usnic acid after 15 days. This impacts the interpretation of this data because theoretically, new significant differences between the treatment groups could still emerge. It is unlikely for existing significant differences observed in short-term designs to become insignificant in long-term designs, meaning that the implications for copper and zinc are relatively clear (Nash, 2008). Only the results for critically high nickel environments remain ambiguous, and this could be resolved through another experimental design which extends the timing to two months per trial.

The small number of replicates (6 total) was chosen because *C. rangiferina* is a threatened species, and using many lichen bodies in the trials would reduce their wild populations. However, it seems as though this method choice resulted in much larger, unusable Tukey Test Intervals regarding percentage decrease in usnic acid (Nunes et al., 2015). Therefore,further research which mimics this project's design and uses a larger number of replicates wouldbe highly beneficial because it would allow the researcher to identify the exact percentage decreases.

Although the data in this project might be used to estimate the behavior of other taxonomically related lichen species, it should not be used to estimate changes in usnic acid production by *C. rangiferina* under different metal conditions. Similar studies on other metabolites such as chlorophyll and ethylene have indicated that some metals increase metabolicproductivity rather than diminishing it, so lichens' responses to unusual conditions can vary widely depending on the identity of the metal (Garty, 2002; Larsson, 2009). Applying the findings of this project to better understand lichens' sensitivity to nickel, zinc, and copper only would maintain alignment with the scope of this method. It should be noted that in this project, *C. rangiferina* was only exposed to positively charged metal ions. Trends in usnic acid production may differ when this species is placed in environments with excesses of neutral metalatoms, but such conditions are uncommon (Hutchinson et al., 2016). If further research were conducted to investigate new metals, it would be best to start with lead, which also has a moderately negative correlation (-0.62) with *C. rangiferina's* overall metabolic productivity. In addition, lead shares several chemical properties with nickel, zinc, and copper such as reactivity patterns (Folkeson and Bringmark, 2011).

Further research is certainly warranted in terms of understanding how *C. rangiferina's* usnic acid production responds to combinations of unusual metal conditions since combinations were completely excluded from this project. A buildup of multiple harsh environmental factors has the potential to reveal new,

unpredicted patterns in an organism's metabolism, so sampling or experimental designs to fill this new gap are urgently needed (Hutchinson et al., 2016). Most importantly, this research is among very few to indicate a need for the mapping of usnic acid's full synthesis pathway. Gaining a better understanding of the exact chemical reactions within thismetabolic process would help to explain and confirm why copper and zinc impacted usnic acid production while nickel did not (Garty, 2002). This type of research also has the potential to identify whether usnic acid production has its own critical concentrations – amounts of copper, zinc, or nickel that would cause usnic acid synthesis to halt completely in *C. rangiferina*. Finally,discovering the missing steps in this chemical process could allow researchers to effectively predict how other unusual environmental conditions might impact usnic acid production in *lecanorales* group lichens without the use of experiments (Grube et al., 2015).

Conclusion

By employing a short-term experimental design, it was demonstrated that usnic acid production in *Cladonia rangiferina* lichens tends to decrease in environments with critically highconcentrations of copper and zinc ions, but not nickel. This is significant because it provides insight into the chemical reactions involved in usnic acid synthesis. Although this project indicated which ecosystems would benefit the most from artificial dispersal of *C. rangferina* lichens, it also highlighted numerous research gaps that are yet to be explored.

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