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Acknowledgements

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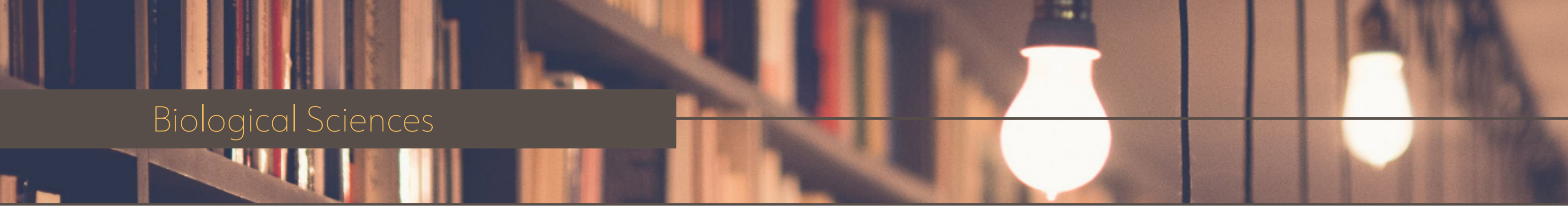

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Keywords: Sequoia semperviren, Picea stichensis, Alnus rubra, redwood chemistry, leaf leachate, bark leachate, Flavoparmelia sp., Hypogymnia sp., Parmelia sp., Parmotrema sp., Ramalina sp., Sphaerophorus sp., Usnea sp., lichen, lichens

Introduction

The redwood-dominated coastal forest of Northern California is a unique environment that is home to several endemic, and many closely associated, tree and lichen species. Associated tree species such as the red alder, *Alnus rubra,* Bong., and Sitka spruce, *Picea sitchensis* (Bong.) Carriere, can often be found within the same forest habitat as the coast redwood, *Sequoia sempervirens* (Lamb. ex D. Don) Endl. Although these associated trees share the same space and common epiphytes, the lichens that are present on the branches and in the canopy of these associated trees are much less abundant on the redwoods, if they appear at all.

Lichens exist as epiphytes with an ecologically obligate mutualistic symbiosis that is shared between a fungal partner, the mycobiont, and an algal partner, the photobiont. Their photobiont can be either green algae, known as a chlorobiont, and/or cyanobacteria, a cyanobiont. These partners provide the lichen with sugars, or, in the case of the chlorobiont, both sugars and fixed nitrogen. The structure of lichens, as dictated by their mycobiont, can vary in form from crustose to foliose, fruticose, or pendulous, and often take on various intermediate forms, allowing for their survival in almost any environment. These organisms are poikilohydric which means they experience varying concentrations of water in their body, the thallus, as water is absorbed passively through fluctuations in concentration gradients on their surfaces. As poikilohydric organisms, moisture is required for the transport of nutrients and water into the lichen and, accordingly, most lichens are found in areas that can meet their hydration as well as their climatic and nutritional needs. This poikilohydric nature renders lichens vulnerable to water-soluble environmental toxins, as they are not able to filter out or discern between harmful and beneficial molecules.

About thirty-four percent of the water that is annually available in the coastal redwood forest comes directly from the fog drip collected by the foliage of the redwoods, and contributes to up to sixty-six percent of its water during the hottest parts of the year (Dawson, 1998). The rainfall in redwood forests is less than 1 meter per year, so the redwoods use the limited rainfall in conjunction with the trapped coastal fog to drip for hydration. It would make sense, then, for lichens to take advantage of the redwood canopies, as there is plenty of moisture and sunlight; however, even when an associated tree harboring a diverse community of lichens extends to touch the redwood's branches, most of the lichen communities will not transfer over and establish on the redwoods as they would on other trees. Based on the observations of lichen species within the Douglas fir-dominated forests, the limited presence of lichens on non-redwoods has been hypothesized to have been due to dispersal limitations (Sillett et at., 2000). However, later observations in the redwood-dominated forest showed that even when a lichen was given ample redwood substrate, lichens (especially cyanolichens) were still resistant to habitation on the available redwood's surface (Williams et al., 2007). Another hypothesis for the cause of the reduced presence of lichens on redwood substrate might be related to the low-light tolerance of redwoods, which could inhibit the growth of other epiphytic species. However, lichens are adapted to become light-saturated at very low light levels, conditions that would inhibit other species, affording lichens the advantage.

If redwoods exhibit ample open substrate space for lichen colonization, and the lichens are not limited by dispersal methods or low-light conditions, then it stands that there must be another explanation as to why lichens do not colonize on redwoods. Redwood trees are well known to have a unique chemical composition in their foliage as well as their bark, and it is thought that these compounds may be the limiting factor to many other genera of fungi, plants, and animals taking up residence within the redwood forest. The bark of redwood trees exhibit response mechanisms against herbivory such as the swelling of polyphenolic parenchyma, an increase in cell wall lignification, and traumatic resin duct development within the stems of the redwood (Hudgins, 2004). Polyphenolic compounds have been identified to retard the growth of pathogenic fungi that might try to inhabit the tree (Hall, 1985). This method of protection works to deter herbivores as well, but redwoods also exhibit other forms of protection. The inhibition of endophytic fungi growth is not limited to the bark; different terpenes found within the foliage of redwoods have also been identified as inhibitors of the growth of some of their endophytic fungal partners (Espinosa-Garcia, 1991). The foliage of redwood trees contains many known and identified terpenes, some of which have varying concentrations depending on the stage of maturation of the tree, suggesting yet another form of herbivory defense (Okamoto et al., 1981).

Given that lichens have ample available substrate, water, and light within the redwood forest, it seems most likely that chemical toxicity is the true limiting factor for lichen presence in the lower strata of redwoods. Lichens are more likely to colonize the bark of their hosts, rather than the foliage, and taking into consideration that lichens absorb nutrients as well as toxins through their hydrated thallus, we hypothesize that it is the chemical composition of the redwood bark that is the main deterrent to lichen colonization on redwood trees, and that these chemicals are leached out of the bark through rainwater passing over the outer surface of the tree. Therefore, if the chemical composition of either the bark or the foliage prove to be detrimental to lichen growth, the observable decrease in lichen coverage when exposed to leachates from different parts of the redwood tree will not be zero. In other words, if there are chemicals within these parts of the tree that are inhibiting the growth of lichens, then there will be a significant negative change in the percent coverage of lichens. This study investigated the effect of redwood leaf leachate and redwood bark leachate on the growth of lichens from redwood forest associated tree species. We hypothesized that the chemical compounds in *S. sempervirens* will negatively affect the health of the lichen populations found on the neighboring associated tree species.

Materials and Methods

To perform this experiment, we watered the specimens of collected sticks, which were covered in representative lichens from the different associated tree species of the redwood forest, with leachates prepared from different parts of the redwood tree: the bark and leaves. The prepared leachates are meant to mimic the natural chemistry that might be found in the water that passes down the trunk of a redwood or cascades down through the needles during rainfall. To set up an experimental area, a metal kitchen rack with four metal wire shelves, measuring 4 feet x 2 feet x 5 feet, was placed in a protected area behind the greenhouse of Humboldt State University. The area was shaded by tall surrounding buildings, protected from excessive wind, and the rack was placed close to a wall, with no direct canopy cover. The rack was adorned with twenty-four metal hooks that were placed at the front and the back of each shelf in three sets of two on each level, forming three columns of four sticks each, with the capability of holding a total of twelve sticks on the rack (see Figure 1). The twelve experimental sticks were then gathered from the floor of Arcata Community Forest the day following a significant windstorm, to ensure the freshness of fallen material. Through casual observation, the main associated tree species within this portion of the redwood forest were determined to be red alder (*Alnus rubra*) and Sitka spruce (*Picea sitchensis*).

Healthy sticks were gathered at random and kept for the experiment based on three main criteria. First it was determined by visual identification if the stick belonged to either a red alder or a Sitka spruce tree. The second criterion was the overall good health of the lichens present on the stick, as assessed by observational analysis. Finally, the third criterion was that the stick be about 1 to 2 inches in diameter and at least 24 inches long, or be able to be cut to that length without damaging the lichen communities on the stick. Macrolichen species were identified using *Macrolichens of the Pacific Northwest* (McCune and Geiser, 1997, Table 1). The first six sticks of each associated tree species that met these criteria were transported to the greenhouse in a 5-gallon bucket and hung from the metal hooks. The different stick species were separated such that each of the three columns on the rack had two Sitka spruce branches and two red alder branches to ensure replication between the three treatment groups. The sticks were labeled with a number 1 through 12 that was written with a permanent marker on plant tape, which was then tied around one end of each stick starting with 1 on the top left and then numbering down.

The initial percent-coverage of lichens on each stick was determined by dividing the stick into four equal quadrants on the front side of the stick and four equal quadrants on the back. Each side of the stick was observed individually as its own whole and treated as a two-dimensional surface, where each of the four equal quadrants represented 25% coverage. Calculated results of both sides were then added together and divided by two to get the total percent coverage of the stick. This is also how final percent coverage was calculated, and the difference between the final and initial percent coverage yielded our observed decrease in lichens. After initial coverage was determined, the sticks received their first treatment.

Each of the three columns received a different treatment and thus were numbered vertically. The far-left column of sticks, numbered 1-4, were the control group. In the middle column, the sticks were numbered 5-8 and given the bark leachate experimental treatment, and in the far-right column, the sticks were numbered 9-12 and received the leaf leachate experimental treatment. The treatments were given in vertical groups to avoid cross-contamination of treatment types due to inevitable dripping.

The three treatments were prepared as follows; the control group was treated with untreated rainwater, which was collected in a 5-gallon bucket in the backyard of HSU's greenhouse. The second treatment, bark leachate, was prepared with approximately 700 grams of dry redwood outer-bark, weighed with a balance, then macerated with gardening shears and saturated with 8 liters of rainwater in its own 5-gallon bucket. The third treatment was composed of approximately 700 grams of redwood foliage, which was cut from the attached lateral woody branches, weighed with a balance, and covered with 8 liters of rainwater in a third 5-gallon bucket. These leachates were prepared once a week, two days prior to the first treatment day of the week. The mass of redwood bark and foliage used was determined by the availability of the sample that could be collected without damaging the redwood tree and the amount of rain water used was determined by how much it would take to completely submerge the redwood bark and leaf samples inside the 5-gallon buckets without depleting our limited rainwater reserves. The treatments were administered every Tuesday, Thursday, and Saturday morning before the hottest part of the day to ensure maximum retention of the treatment solutions given. The pH of each leachate solution was measured after each treatment with the Thermo Scientific Orion Star A-111(c) pH meter, which was calibrated with a pH 3 and pH 7 buffers prior to each use. Each treatment group was given one full liter of leachate per treatment by means of a hand-held spray bottle, which was split amongst the four specimens of each group. To administer the treatments, each stick was carefully removed from the hooks and sprayed until saturated, mimicking heavy rainfall, then placed back on the hooks in its column to drip dry as it would in nature. The treatments were repeated for five weeks.

During the treatment duration, observational data was recorded by means of bi-weekly photos. Twice a week, on days that alternate treatment days, photos were taken of each stick in each treatment group with an Apple iPhone and then uploaded to a google doc. To do this, each stick was carefully removed from its hooks and placed in a flat black-colored tray alongside a 24-inch ruler. Pictures were then taken of the front and the back sides of each of the twelve sticks, paying particularly close attention to areas that may be exhibiting signs of impact from the treatment being administered. The photos at the end were then compared to the photos taken before treatment began, and % coverage decrease was recorded using a quadrat and the method described above.

During this experiment, the pH values of each of the treatments were recorded, and although the pH values that were recorded seemed as if they may provide some valuable information in this experiment, we decided not to use this data. The averages that were recorded were based on the treatments that were collected only in the first three weeks of experimentation. After the first three weeks, data collection was discontinued due to campus closures relating to the COVID-19 outbreak, which began during the latter half of our experiment and caused the loss of access to the Thermo Scientific Orion Star A-111(c) pH meter.

When analyzing the data, a one-way ANOVA test was used to compare the mean decrease in lichen coverage of the three treatment groups, pooling the data from all host sticks regardless of species. A one-way ANOVA test was also used to compare the difference in decrease of lichen coverage between host-stick species that were given the same treatment, and the test was run for each treatment group. We then used the p-values to determine statistical significance.

Results

The bark leachate treatment had no significant effect on the percent decrease of the lichen population on either stick species (p-value= 0.55). The leaf leachate did have a significant effect on the percent decrease of the lichen population on both host stick species (p-value= 0.001). The pH of the bark leachate averaged 3.5, the leaf leachate averaged 5.73, and the control averaged 6.8 (See Table 2, Figures 2 and 3).

Discussion

Our first analysis yielded a graph that showed the percent decrease in lichen coverage on the host sticks in each treatment and compared the three results (Figure 2). The difference in the lichen coverage decrease between the lichens on the Sitka spruce host sticks and the red alder host sticks was not statistically significant in any of the treatment groups, so we pooled the data to make a more concise graph that better summarized the data and helped recognize the total difference between treatments (Figure 3). Although the bark leachate did have a slightly higher percent decrease in lichen coverage than the control, the magnitude of the necrosis was not statistically significant. This may have been due to the use of outer bark, versus the use of inner heartwood, which is known to have a higher concentration of polyphenolic compounds and thus may have rendered different results if we used this wood instead. The use of the inner heartwood could more accurately illustrate the negative impact of the polyphenolic compounds on the lichen's overall health through closer proximity to these compounds.

Redwood outer bark, contrarily, is very fibrous and sloughs off easily, which could deter the establishment of macrolichens and may also suggest a different chemical composition than what is found in the inner heartwood. The treatment of leaf leachate, however, had a drastically more significant effect (Figures 2 and 3). Based on the data collected, it can be inferred that lichen growth on redwoods is not significantly impacted by the polyphenolic compounds in the outer bark but is more likely impacted by the compounds in the foliage leaching out and down the bark's surface through fog accumulation and rain. It is true that the wood has its own set of inhibitory, aromatic compounds, but they are found deep within the heartwood of the tree. One hypothesis we propose, based on this fact, and our findings with this experiment, is that the toxic nature of the foliage is attributed to the polyphenolic compounds of the heartwood being transferred into the sapwood and conducted through the vascular tissue and into the leaves. However, further experimentation on this would be needed to confirm these inferences.

Our results demonstrated that the percent decrease in lichen coverage was virtually the same for both the bark leachate treatment and the control. Therefore, the loss of these lichens could be attributed to initially being moved to a new environment, stress from the constant disturbance of being removed from the rack to be treated and photographed, or other natural and experimental variables. It is also possible that the bark generally has less of these polyphenolic compounds on the outer surface, which is where we obtained our bark samples. Although the bark leachate did not render statistically significant results, one observation was made but not quantified within this treatment group: the foliose *Parmotrema* spp. lichens showed an abundant increase in the production of marginal soredia when photographically compared to its initial photos and the control sticks. Likewise, the fruticose *Usnea* spp. demonstrated an observable increase in apothecia production. When certain lichens experience environmental stresses, it is not uncommon to see an overproduction of reproductive structures as an attempt to reproduce when experiencing conditions that may lead to the death of the lichen. This behavior suggests that the lichens were, in fact, experiencing stress, and over a longer duration it is likely we would have observed an even greater production of these structures and possibly eventual death of the lichen.

Continuing this experiment for a longer duration in the future, as well as using heartwood over bark, may provide better insight into how these lichen communities interact with the chemistry of the redwood trees. Additionally, future research on the chemical composition of water extractions from the leaves and the heartwood may prove to be useful in determining which compounds are present and in what concentration these compounds may become toxic to certain lichen communities. Ultimately, the expansion of this information, when taken into consideration with our experimental findings, could provide better insight into why some lichens are better suited to live in the canopies of redwoods and on neighboring associated trees rather than in the lower strata of redwoods.

Table 1. Identified macrolichen genera found on each stick.

	Flavoparmelia	*Hypogymnia*	*Parmelia*	*Parmotrema*	*Ramalina*	*Sphaerophorus*	*Usnea*
Stick 1 (alder)	x		x	x			x
Stick 2 (alder)		x	x	x	x		x
Stick 3 (Sitka)		x	x	x			x
Stick 4 (Sitka)		x		x	x	x	x
Stick 5 (alder)	x	x	x	x		x	x
Stick 6 (alder)	x			x	x		x
Stick 7 (Sitka)		x		x			x
Stick 8 (Sitka)			x	x	x		x
Stick 9 (alder)			x	x	x		x
Stick 10 (alder)			x	x	x	x	x
Stick 11 (Sitka)			x	x			x
Stick 12 (Sitka)			x	x			x

Table 2. Mean decrease in percent coverage of lichens on red alder and Sitka spruce sticks after treatment with control (rain water), redwood bark leachate, or redwood leaf leachate experimentaltreatments.

	Control Alder	**Control Sitka**	**Bark leachate Alder**	**Bark leachate Sitka**	**Leaf leachate Alder**	**Leaf leachate Sitka**
Coverage Decrease (%) Stick 1	5.0	4.0	5.0	3.0	14.0	10.0
Coverage Decrease (%) Stick 2	4.0	3.0	4.0	7.0	12.0	12.0
Average Coverage Decrease (%)	4.5	3.5	4.5	5.0	13.0	11.0

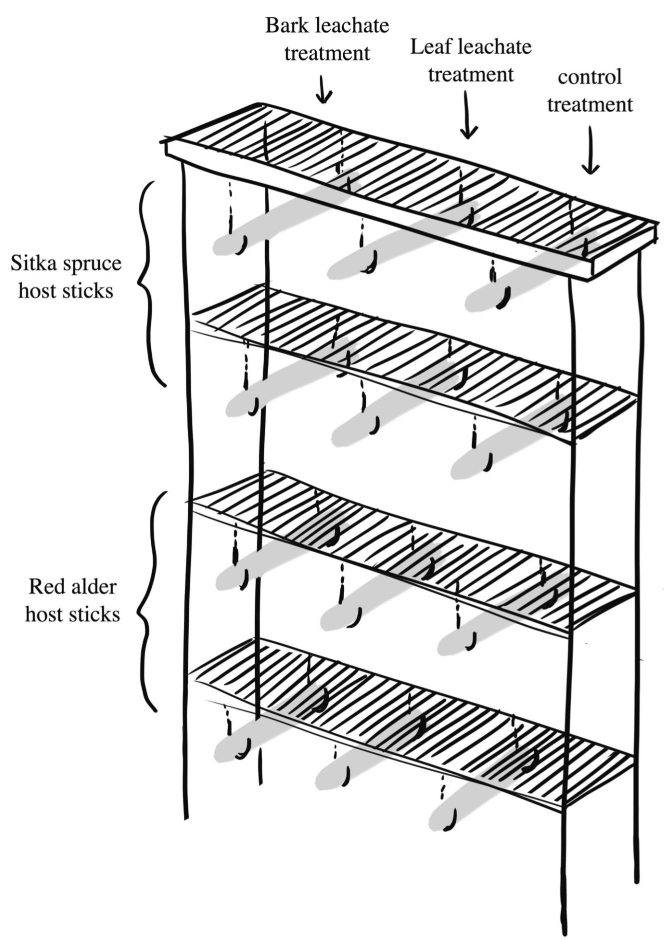

Figure 1. Setup of the kitchen rack, hooks, experimental sticks, and treatment groups.

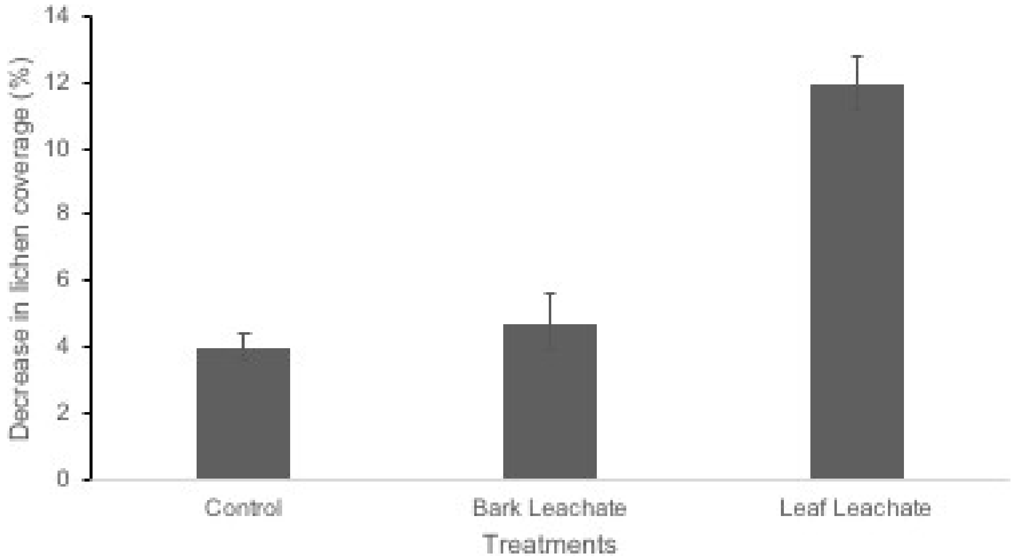

Figure 2. Total percent decrease in lichen coverage between species of host sticks (red alder, Sitka spruce) per treatment group (control rain water, bark leachate, and leaf leachate). Standard error for eachtreatment is shown. The p-value between the control and the bark leachate is >0.05, which isn't significant. The p-value between both control and leaf leachate, and bark and leaf leachate is <0.05, and is therefore statistically significant.

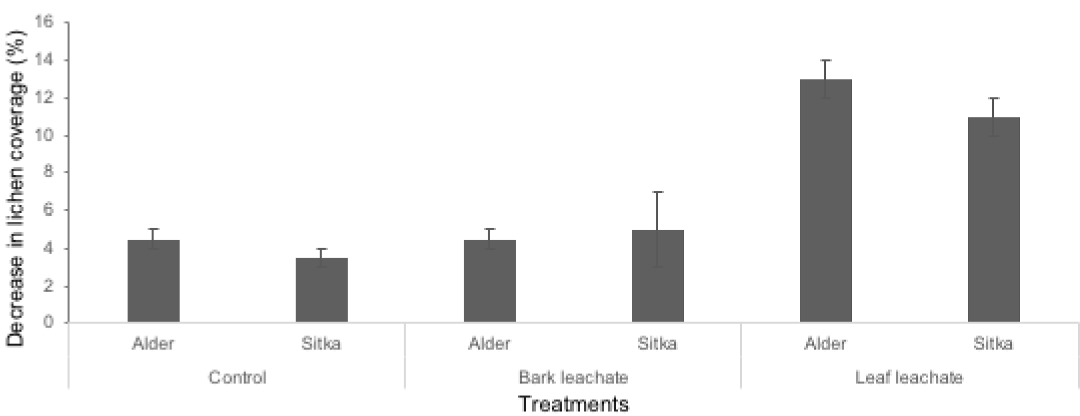

Figure 3. Mean percent decrease in lichen coverage between treatment groups (control rain water, redwood bark leachate, and redwood leaf leachate). The p-values between the host sticks within each ofthe treatment groups was >0.05 and therefore not significant.

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Citations

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