

The effects of road salt on mushroom-forming saprotrophic fungi



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Abstract

Saprotrophic Fungi are valuable members of ecosystems for their role in nutrient cycling and ability to degrade and/or sequester environmental pollutants. This study explores the effect road salt may have on the growth and ecological functionality of mushroom-forming primary decomposers of lignin in oak trees through the increase of substrate salinity and thus lowered water potential. A significant negative response to increased levels of substrate salinity was seen through measures of radial mycelial growth and extracellular enzyme activity. These findings call for research into other means of road deicing that do not hinder the role of fungi.

Introduction

Saprotrophic fungi recycle nutrients bound in detritus through the excretion of extracellular enzymes from their mycelium. The mycelium is a vast network of microscopic filaments called hyphae and is the main body of the fungus (as opposed to the carpophore or mushroom, which is a temporary reproductive structure). Hyphae are fine, far-stretching strands of single, exposed eukaryotic cells (Stamets 2005). From the surfaces of these strands saprotrophic fungi excrete specialized enzymes to break down complex molecules in its environment. Plants, for example, through photosynthesis can sequester carbon in the form of the complex organic polymers cellulose and lignin, providing structure and rigidity to secondary cell walls and creating wood (Vishal & Nerud 2002). This process adds a great amount of biomass to the ecosystem but it isn't in the form of nutrients and a carbon source in their complex forms. Through the activity of specialized extracellular enzymes on such complex molecules minerals, hydrogen, nitrogen and carbon are released from complex forms in dead organisms into simpler forms usable by the fungus, plants and other microorganisms (Stamets 2000). Several recent studies show that these fungi and the enzymes they secrete are also ecologically valuable in filtering out and biodegrading environmental toxins including industrial wastes, harmful bacteria and phenolic resins (Gusee et al 2006, Vishal & Nerud 2002, Minussi et al 2001, Stamets 2001 & 2006).

Paul Stamets identifies three types of fungi: mycorrhizal, parasitic and saprophytic

(synonymous with saprotrophic) (2000). Mycorrhizal fungi grow in forest soils in direct and necessary symbiosis with plant roots to the benefit of both the fungus and the host plant. Mycorrhizal species are quickly becoming an interest of foresters and forest ecologists as their benefits to plant health and nutrient cycling are realized. Parasitic mushrooms take living trees and insects or whole plant or insect communities as hosts, such as the 2,200 acre honey mushroom (*Armillaria ostoyae*) culture in Washington State (Stamets 2000).

Carthage College is located in Wisconsin which, due to cold winters and snow accumulation, applies road salt in the form of sodium chloride (NaCl) as a deicing agent. The amount of road salt purchased for application to roads by the state and local government rapidly rose from 405,000 tons of sodium chloride during the 2006-2007 winter to 700,000 tons in the following 2007-2008 winter (State Legislatures 2008). This trend has continued with the 2008-2009 winter with road salt purchases totaling 1,300,000 tons per winter (Williams 2009). Deicing has come to be expected by motorists and some method is essential for road safety in dangerous conditions. Before such large applications of road salt are applied and the trend of increased road salt usage continues its effects on roadside and downstream ecosystems should be explored.

Of particular interest to this study is the effect of different sodium chloride concentrations on the ability of saprotrophic fungi to perform their ecological functions. The vast network of hyphae that make up the fungus' mycelium create a large surface area which, while allowing the fungus area to excrete enzymes, exposes the entire organism to potentially harmful chemicals and conditions outside its cells.

The negative effects of roads on ecosystems are many and well described. In their construction roads displace any above-ground organisms in their path and physically alter the area. Roads along with traffic cause far-reaching habitat fragmentation and mortality through car collisions (Trombulak & Frissell 2000). Roads are also a vector for biotic pollution in the form of exotic and potentially

invasive species. Cars not only provide a vehicle for foreign seeds to enter an ecosystem but also deicing chemicals applied to roads and road sides can alter soil chemistry in favor of invasive plant species (Trombulak & Frissell 2000).

Sodium chloride is one of the chemicals responsible for altering soil chemistry. Sodium chloride is the most commonly used deicing agent in the United States and is being used in increasing amounts each year, totaling 20.3 million tons in 2008 (MSNBC 2008). Although this chemical is being released in copious amounts along our roadways its effects on the environment are yet to be fully explored.

Fungi would not be the only organisms potentially harmed by increased concentrations of road salt in the environment. Previous plant research shows that relatively low amounts of sodium chloride applied in the field to barley plants inhibits their ability to transport calcium from the roots to the shoot portions by interrupting the active transport of calcium into the root xylem (Lynch & Lauchli 1985). Romero and Maranon (1996) demonstrated the significant changes in morphology and mineral content and location in plant species *Melilotus segetalis* across a soil salt gradient. Their study found that increasing soil sodium chloride concentrations yielded plants with lower biomass and also higher leaf to root and shoot biomass ratios. Sodium chloride's presence altered both the amount and location of storage for several essential minerals. Sodium chlorides increasing presence increased sodium content of the roots of *M. segetalis* and decreased their potassium content as compared to controls. The effects of road salt on fungi, though, are less understood.

Various types of fungi have previously been demonstrated to be sensitive to other environmental pollutants. Wilkinson and Lucas (1969) observed the effects of various herbicides popular at the time on various species soil fungi. Growing the fungi across a gradient of each herbicide showed the sensitivity of fungi to a wide range of common agricultural pollutants in the form of decreased radial mycelium growth. They demonstrated that different species of soil fungi had differing thresholds for

sensitivity, but that sensitivity was maintained across different growth mediums. These results were replicated by Smith and Lyon (1976) looking at the effects of the herbicide Paraquat on soil fungi. They not only found decreased radial growth but also the more reliable measure of dry fungal mass.

Experiments with the aquatic fungi *Dendryphiella salina* showed a negative correlation between the presence of sodium ions and glucose uptake along with dry fungal weight (Allaway and Jennings 1970). Although not directly analogous to terrestrial fungi the mycelium is similarly exposed to a high-solute environment as a fungus in a salt-polluted substrate might. From their findings Allaway and Jennings go on to claim that, though the point of inhibition varies between species, the vegetative growth of all fungi is inhibited by sodium.

Allaway and Jennings' demonstration of the negative effect of solutes, specifically sodium ions, on fungal growth raises the question of water potential and its effects on fungi health. Water potential is a measure of the potential energy of water movement compared to pure water and decreases with increasing solutes. Water potential reflects how freely water can flow. Water with lower potential than that within the fungal cells should disrupt transport between the cells and their environment. Griffin (1977) applies this fundamental idea to wood-decaying fungi, stating that lower water potentials should inhibit the ability of fungi to move extracellular enzymes and take up the simple sugars they help form.

This idea was demonstrated to be reality by Griffith and Boddy (1991) concerning ash and oak twig decomposing fungi. Various species of primary decomposers isolated from wild twigs and branches were grown in two dimensional substrates across a water potential gradient. As expected, lower levels of water potential significantly or drastically changed the radial growth but also morphology and pigmentation of the fungi. A decrease in growth over time in relation to decreasing water potential was common to all species, though the point and degree of inhibition was dependent on species. Species isolated from decaying oak samples were the most sensitive to water potential with complete growth inhibition and sometimes mortality at water potentials less than -4.0 MPa, a relatively

low figure compared to the rest of the fungi observed. Heavily pigmented species decreased in pigmentation with decreasing water potential and one reverted to a more primitive morphology. Griffith and Boddy call for future observation of extracellular enzyme activity, citing the change in pigmentation as a likely marker for changes in enzyme production.

This study seeks to demonstrate the effects of road salt in the form of sodium chloride on four species of saprotrophic mushroom-forming fungi native to Kenosha County, Wisconsin with a common ecological niche: oak forests. In particular, the effect of road salt on lignin-degrading saprotrophs will be examined. Lignin is the second most abundant organic polymer on earth and is constructed by woody plants to provide structure for vertical growth and vascular tissues (Boerjan & Baucher 2003, Vishal & Nerud 2002). Lignin-degrading saprotrophic fungi, also known as white-rot fungi, are the most efficient organisms at breaking down lignin, demonstrating their ecological importance (Vishal & Nerud 2002). These fungi are also growing in interest for use breaking down industrial pollutants and the remediation of polluted environments (Vishal & Nerud 2002, Stamets 2005, Minussi et al 2001). The expectation of this study is that increasing concentrations of road salt in the substrate of a saprotrophic fungus will lower its ability to fulfill its ecological role excreting enzymes. The first hypothesis is that there will be a negative morphological response in the form of decreased radial growth with increased substrate sodium chloride concentration. The second hypothesis is that activity of the extracellular enzymes used to degrade lignin will also decrease with increasing amounts of road salt in the fungus' immediate environment.

Methods and Materials

Species Descriptions

Four species of fungi were chosen for their similar ecological roles in southeastern Wisconsin. The species *Laetiporus sulphureus* (Bull. ex Fr.), *Ganoderma lucidum* (Curt. ex Fr.), *Trametes*

versicolor (L. ex Fr.) and *Pleurotus ostreatus* (Jacq. ex Fr.) are mushroom-forming fungi native to the area (Murphy 1996). These species were selected for research because they are ecologically redundant, all being a primary decomposer of hardwoods, specifically oak trees (Stamets 2005). The species *L. sulphureus* is unique among the four because it is a 'brown rot' fungus, breaking down cellulose as opposed to the other three lignin-decomposing 'white rot' fungi (Stamets 2005). This research measures the activity of lignin-degrading enzymes and *L. sulphureus* was selected as an out group for comparison. Since *L. sulphureus* does not use phenol oxidases there should be no reaction for its extracellular enzyme analysis, providing comparison to the other three species which should show strong activity.

Culture Collection and Propagation

Commercial varieties bred and selected for mushroom farming were purchased from Mushroom Harvest in Athens, Ohio. These came in the form of culture storage slants and were stored at 2 degrees Celcius until used, less than six months later. During the summer of 2008 wild fruit bodies of *L. sulphureus*, *P. ostreatus* and *T. versicolor* were also collected in forests in or adjacent to Kenosha County, Wisconsin. A living specimen of *G. lucidum* was never found so the ecologically and morphologically similar *Ganoderma applanatum* was collected (Stamets 2005). Whole mushrooms were collected in paper bags and let sit overnight to eject spores. Species were identified by observing morphological features of the carpophores, mycelium appearance and spore print color.

The collected carpophores for the wild fungi were transferred to and grown on nutrient agar. This was done in a laminar flow hood to decrease chances of airborne contamination of the growing medium. Fruit bodies were rubbed with iodine tincture to reduce the possibility of their own spores germinating and contaminating the culture. This was a concern as genetic differences between supposedly identical cultures could account for variation in growth and enzyme activity. Inside the flow hood the carpophores were torn open, exposing clean flesh. Using a scalpel, small sections of condensed hyphae within or near the base of the stem were transferred to potato dextrose agar. The inoculated petri dishes were observed daily and the leading edges of clean mycelia was transferred away from contaminants until clean and healthy cultures of each species were isolated. Growth rates, visual appearance and hyphal density of wild cultures were compared with dishes of the commercial types to verify isolation.

Deep-welled dishes of autoclave-sterilized potato dextrose agar (PDA) were used to propagate

the mycelium isolates. In each plate was poured 10ccs of PDA using a syringe to standardize the nutrients available to each culture. Small sections from the commercial culture slants or cultured wild clones were transferred to agar plates and incubated for 9 days. On the leading edges of the growing fungus was cut a grid creating 2mm by 2mm sections for inoculation plugs of a standardized size and dimension with the purpose of obtaining vigorously growing mycelium with a common amount of hyphae per area for each culture.

Salt Treatments

After propagation of the isolated cultures, inoculation plugs were transferred to plates containing one of the three different levels of added sodium chloride: 0ppm, 461ppm and 890ppm. The 0ppm level gave comparison for the other two salt levels observed in the environment. An Analytical Chemistry class at Carthage College taught by Dr. Blaine in the Spring of 2008 measured 461ppm of sodium chloride in the Pike River in Kenosha, Wisconsin after the snow melt. 890ppm of sodium chloride has been observed in areas exposed to long term road salt usage 5 meters from the road side (Hofstra & Smith 1984).

Treatments were applied to the fungi by preparing petri dishes with 10.0ml of PDA prepared with either 0ppm, 461ppm or 890ppm of sodium chloride. The NaCl was added while mixing the agar before autoclaving. To these plates were transferred the 2mm by 2mm sections of the appropriate culture.

Morphological and Functional Analysis

The effects of road salt on the different species of fungi was observed morphologically and functionally through measuring radial growth over time and extracellular enzyme analysis.

Culture diameter was measured using a ruler and averaging the most narrow and widest dimensions of each culture for the commercial varieties of each species after incubating in petri dishes after 6 days of growth. Each plate was kept in the same environment to limit error from variables such as temperature that could affect the growth rate of the fungi.

The primary mechanism of white-rot fungi in breaking down lignin has been identified as extracellular phenol oxidase enzymes, particularly laccase (Vishal & Nerud 2002, Minussi et al 2001, Sinsabaugh et al. 1991). Extracellular enzyme analysis focused on the activity phenol oxidases. The

extracellular enzyme analysis was done on a 96 well microplate as described by Sinsabaugh, 1991. The sample, in this case colonized nutrient agar, is homogenized and suspended in an acetate buffer to which L-DOPA is applied. The L-DOPA gives a colored response which can then be read by a spectrophotometer. Microplates treated with L-DOPA were incubated for 19 hours before analysis. A BioTek x800 reader was used to measure the absorbance created in the microplates by the colored L-DOPA and phenol oxidase reaction.

Data Analysis

Variance between the radial mycelial growth over time of the commercial types of the four species was quantified using a full factorial ANOVA in SPSS 17.0 with culture width as the dependent variable and species and sodium chloride concentration as fixed nominal and ordinal factors, respectively. This demonstrates the relationship between culture width and species, culture width and sodium chloride concentration and the interaction between species and salinity.

Spectrophotometer readings were converted to umol per hour per gram of substrate per the Sinsabaugh, 1991, enzyme analysis protocol. The results of these calculations were analyzed in the same way as radial growth but using phenol oxidase activity as the dependent variable.

Results

Radial Growth

There was a statistically significant relationship of $P < 0.05$ between radial growth over time and sodium chloride concentration for the commercial types of each species (Table 1). *T. versicolor* was the most rapid growing species with *P. ostreatus* showing the least radial growth per time. *L. sulphureus* showed the most sensitive species to sodium chloride as can be measured by radial growth over time between the control and 461ppm treatment while *G. lucidum* reacted most negatively in the transition to 890ppm. There was not shown to be a significant interaction between fungi species and sodium chloride concentration tolerance. Figure 1 shows the growth differences visually with 95%

confidence intervals provided as error bars.

In addition to ruler measurements, *P. ostreatus* cultures in higher salinity treatments appeared in the petri dishes to be less dense in mycelium due to their translucence. Another observation on growth patterns was that *L. sulphureus* cultures were visibly less opaquely orange as the substrate sodium chloride level increased. It was unclear if this visible change was a decrease hyphal density or only pigmentation.

Figure 1, Growth data for commercial cultures of each species after six (6) days of growth demonstrating the response to sodium chloride concentration in the substrate as measured by radial growth.

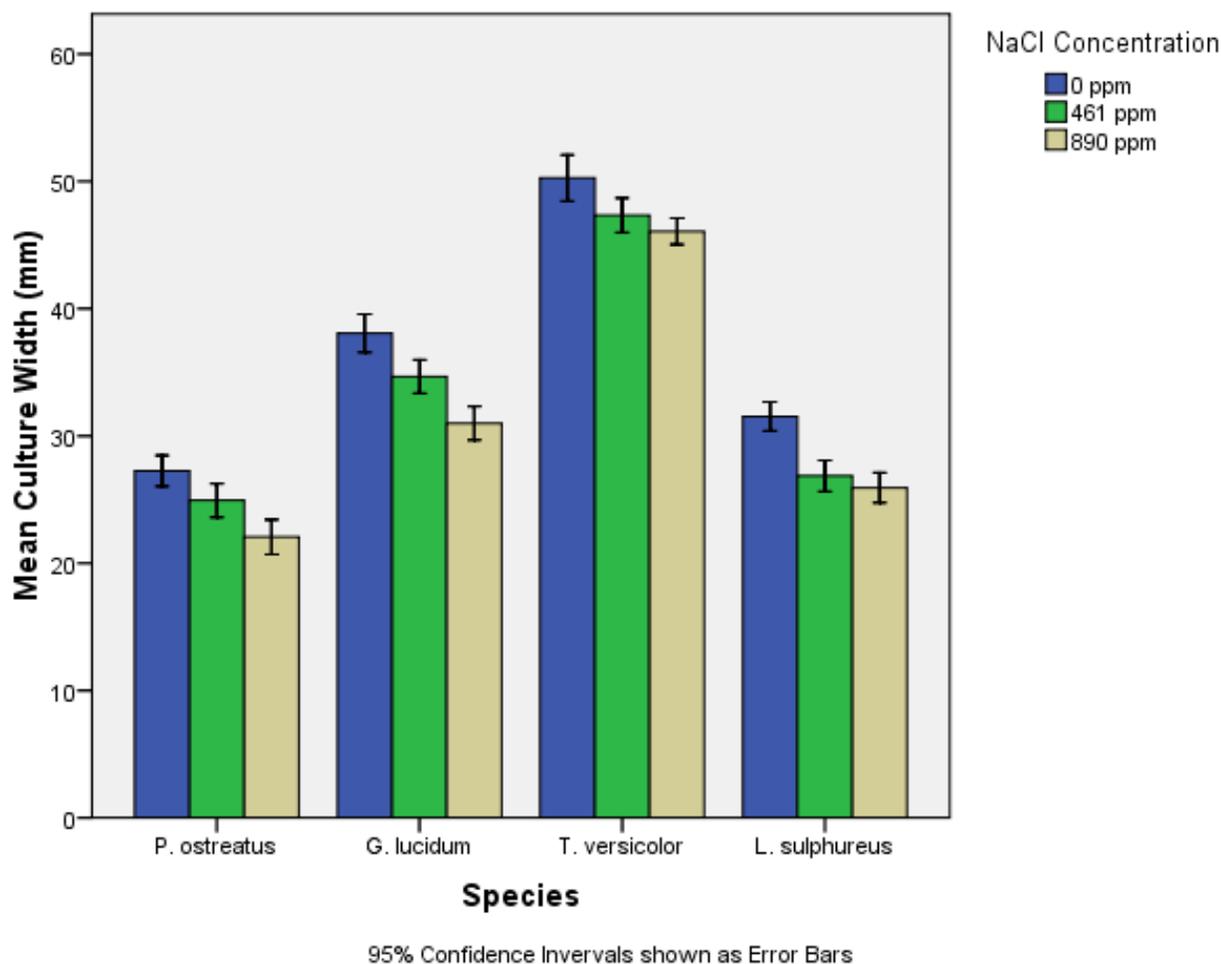


Table 1, Factorial ANOVA results of growth data of commercial types of each species (*G. lucidum*, *P. ostreatus*, *L. sulphureus* and *T. versicolor*).

Tests of Between-Subjects Effects

Dependent Variable: Culture Width (mm)

| Source | Type III Sum of Squares | df | Mean Square | F | Sig. |
|-------------------|-------------------------|-----|-------------|-----------|------|
| Corrected Model | 15084.333 ^a | 11 | 1371.303 | 237.831 | .000 |
| Intercept | 206045.000 | 1 | 206045.000 | 35735.265 | .000 |
| Species | 14096.822 | 3 | 4698.941 | 814.957 | .000 |
| ppmNaCl | 926.233 | 2 | 463.117 | 80.320 | .000 |
| Species * ppmNaCl | 61.278 | 6 | 10.213 | 1.771 | .108 |
| Error | 968.667 | 168 | 5.766 | | |
| Total | 222098.000 | 180 | | | |
| Corrected Total | 16053.000 | 179 | | | |

a. R Squared = .940 (Adjusted R Squared = .936)

Extracellular Enzyme Activity

There was little to no phenol oxidase activity measured for *L. sulphureus* (Figure 3). Figure 3 shows the mean values of activity measured through absorbance by the microplate reader for the commercial *L. sulphureus* samples. Each 151ppm sodium chloride treatments' activity after conversion from absorbance was less than one, which is unfeasible as there can not be less than no activity of any certain enzyme. Negative values were thus set to zero for the purpose of analysis and Figure 3.

Samples for *G. lucidum* and *G. applanatum* both exhibited a large amount of variance relative to

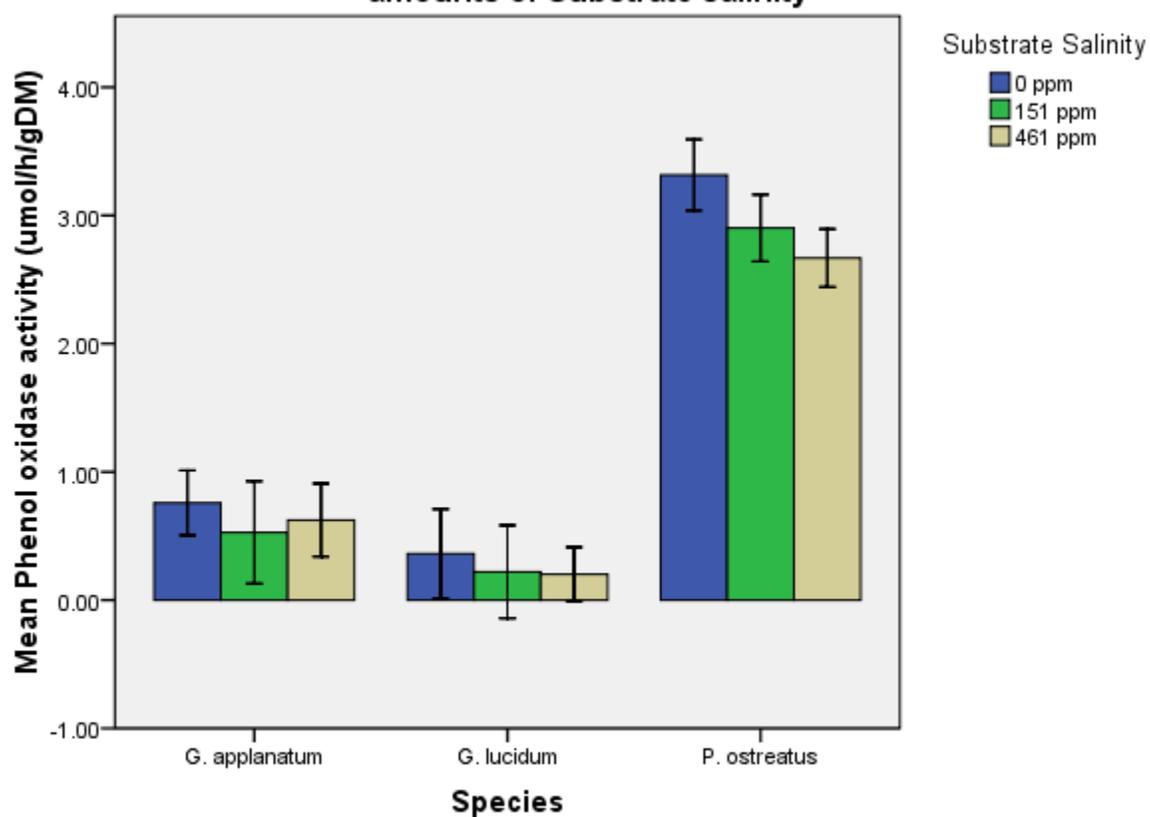
the mean phenol oxidase activity with their 95% confidence intervals' range sometimes exceeding that of the mean value (Figure 2). An ANOVA was performed on *G. applanatum* to explore the strength of the relationship between parts per million of sodium chloride in the substrate and measured extracellular enzyme activity. With $P > 0.05$ ($P = 0.390$ for the ppm NaCl factor) a negative response to sodium chloride concentration through extracellular enzyme activity was not strong enough to come to the conclusion that the two are correlated. A factorial ANOVA was performed with both species (*G. lucidum* and *G. applanatum*) and P decreased to $P = 0.224$, still not strong enough of a trend to establish a relationship between substrate salinity and phenol oxidase activity (Table 4). There was no trend seen in the interaction between species and parts per million of sodium chloride (Table 4). Although statistically a consistent negative trend between sodium chloride concentration and extracellular enzyme activity wasn't demonstrated visually there was a distinct and consistent pattern viewable by eye, with increased levels of salinity showing lighter color reaction, suggesting decreased extracellular enzyme activity.

In contrast with the *Ganoderma spp.* results, *P. ostreatus* showed a distinct negative reaction in measured phenol oxidase activity to increased concentrations of sodium chloride in the substrate (Figure 2). The variance was much smaller in relation to the mean than with both *Ganoderma spp.* also, with the 151ppm and 461ppm treatments both being outside the 95% confidence interval of the 0ppm control samples.

The results of *G. lucidum*, *G. applanatum* and *P. ostreatus*' extracellular enzyme activity were used as the dependent variable for another factorial ANOVA with sodium chloride concentration and species. This analysis showed a statistically significant response to substrate salinity with $P < 0.05$ (Table 2). The interaction between species and response to parts per million of sodium chloride was not significant ($P = 0.132$) but was much lower and thus stronger than that between only the *Ganoderma spp.* (Table 2, Table 4).

Figure 2, Mean extracellular phenol oxidase activity of species *G. lucidum*, *G. applanatum* (wild) and *P. ostreatus*. Error bars show the 95% confidence interval for each treatment.

Phenol Oxidase activity of *G. applanatum*, *G. lucidum* and *P. ostreatus* in varying amounts of Substrate salinity



Error Bars Show 95% Confidence Interval

Table 2, ANOVA between extracellular phenol oxidase activity, substrate salinity and species for *G. lucidum*, *G. applanatum* (wild) and *P. ostreatus*

Tests of Between- Subjects Effects

Dependent Variable: Phenol oxidase activity (umol/h/gDM)

| Source | Type III Sum of Squares | df | Mean Square | F | Sig. |
|-----------------|-------------------------|----|-------------|----------|-------------|
| Corrected Model | 65.491 ^a | 8 | 8.186 | 142.216 | .000 |
| Intercept | 74.508 | 1 | 74.508 | 1294.378 | .000 |
| ppm | .851 | 2 | .426 | 7.394 | .002 |
| species | 64.203 | 2 | 32.102 | 557.676 | .000 |
| ppm * species | .437 | 4 | .109 | 1.897 | .132 |
| Error | 2.072 | 36 | .058 | | |
| Total | 142.072 | 45 | | | |
| Corrected Total | 67.563 | 44 | | | |

a. R Squared = .969 (Adjusted R Squared = .963)

Table 3, The relationship between substrate salinity and extracellular enzyme (phenol oxidase) activity for *G. applanatum* (wild).

Tests of Between-Subjects Effects

Dependent Variable: Phenol oxidase activity (umol/h/gDM)

| Source | Type III Sum of Squares | df | Mean Square | F | Sig. |
|-----------------|-------------------------|----|-------------|--------|-------------|
| Corrected Model | .134 ^a | 2 | .067 | 1.019 | .390 |
| Intercept | 6.085 | 1 | 6.085 | 92.291 | .000 |
| ppm | .134 | 2 | .067 | 1.019 | .390 |
| Error | .791 | 12 | .066 | | |
| Total | 7.011 | 15 | | | |
| Corrected Total | .926 | 14 | | | |

Figure 3, Extracellular phenol oxidase activity of *L. sulphureus*. The 151ppm treatment is zero because all values were less than one.

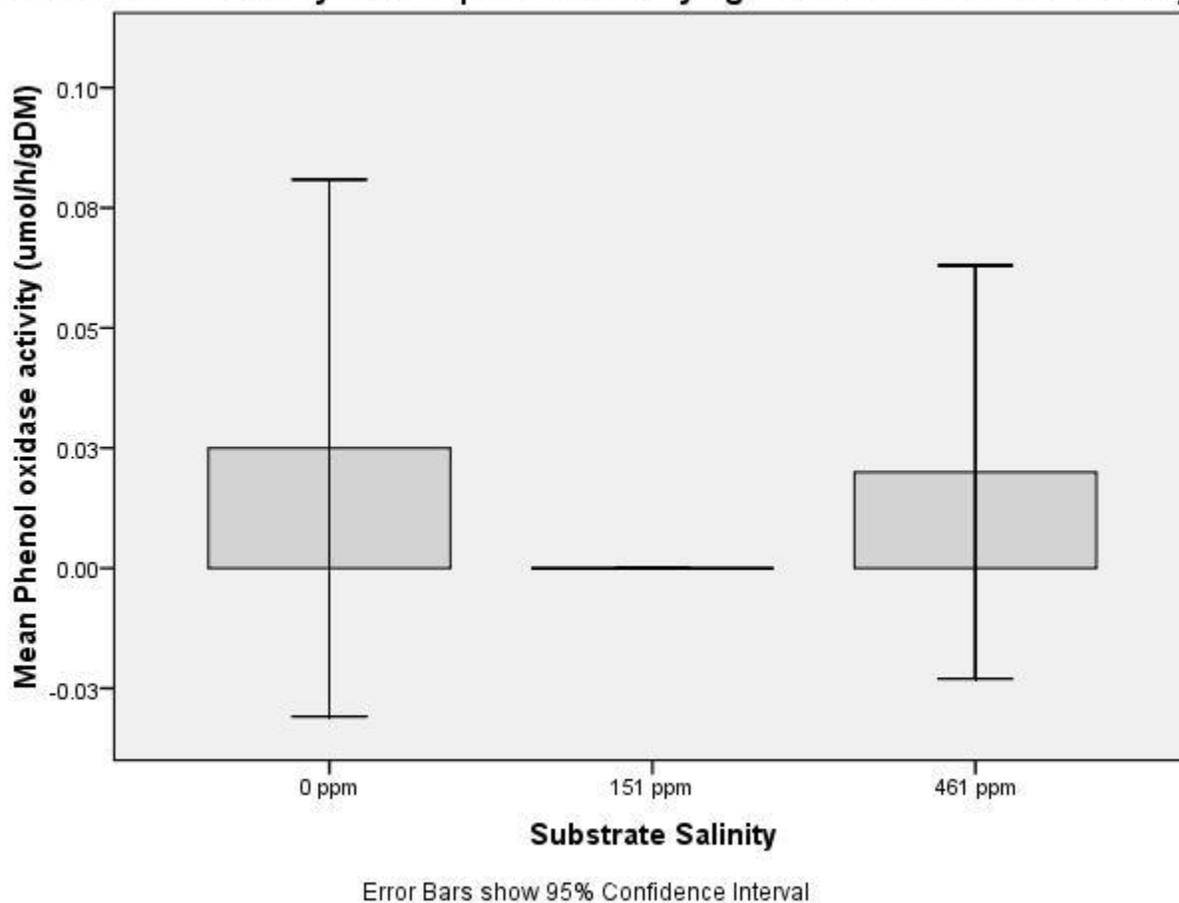
Phenol oxidase activity of *L. sulphureus* in varying amounts of Substrate salinity

Table 4, Showing the relationship substrate salinity and extracellular enzyme activity for *G. lucidum* and *G. applanatum* (wild), exploring the differences in response to salinity between the commercial *G.*

lucidum culture and the related *G. applanatum* culture cloned from the field.

Tests of Between- Subjects Effects

Dependent Variable: Phenol oxidase activity (umol/h/gDM)

| Source | Type III Sum of Squares | df | Mean Square | F | Sig. |
|-----------------|-------------------------|----|-------------|--------|------|
| Corrected Model | 1.342 ^a | 5 | .268 | 3.934 | .010 |
| Intercept | 5.918 | 1 | 5.918 | 86.759 | .000 |
| ppm | .217 | 2 | .109 | 1.593 | .224 |
| species | 1.115 | 1 | 1.115 | 16.349 | .000 |
| ppm * species | .009 | 2 | .005 | .068 | .934 |
| Error | 1.637 | 24 | .068 | | |
| Total | 8.896 | 30 | | | |
| Corrected Total | 2.979 | 29 | | | |

a. R Squared = .450 (Adjusted R Squared = .336)

Discussion

Radial Growth

The first hypothesis was statistically supported in that there was a strong and consistent negative morphological reaction to increased concentrations of sodium chloride in the substrate for each of the four species (Commercial varieties of *G. lucidum*, *L. sulphureus*, *T. versicolor* and *P. ostreatus*) examined as measured by radial growth rate .

In addition, morphological appearance changed with increasing levels of sodium chloride in the agar growth medium for species *L. sulphureus* and *P. ostreatus* with decreases in opacity. This suggests decreased hyphal density with increased substrate salinity. Considering *L. sulphureus* it is unclear whether it was a decrease in hyphal density or simply a loss of orange pigment. Griffith and Body (1991) observed decreased pigmentation in some primary decomposing fungi as water potential lowered (as caused by an increase of substrate solutes) and suggest pigmentation may be an indicator for enzyme activity in some fungi. If the change was instead a decrease in hyphal density then for both *P. ostreatus* and *L. sulphureus* the amount of hyphal surface area anecdotally decreased with increased parts per million of substrate salinity. Less surface area of hyphae translates to less cells exposed to

their environment from which the fungi secrete its enzymes.

For both radial growth and enzyme activity a significant interaction between NaCl concentration response and species was not strongly established, not supporting Wilkinson and Lucas' observation in 1969 that different species of fungi exhibit varying tolerances to salinity.

Extracellular Enzyme Activity

In agreement with the second hypothesis, increased concentrations of sodium chloride in the substrate yielded decreased activity in lignin-degrading extracellular enzymes when analyzing *G. lucidum*, *G. applanatum* and *P. ostreatus* together. Individually and when analyzed together *G. applanatum* and *G. lucidum* exhibited too much variance to suggest a trend. Only preliminary data was taken for *T. versicolor* but all three lignin degrading species showed a consistent visual decrease in L-DOPA reaction (which indicates phenol oxidase activity) after incubation, though it could not be quantified as consistent. Phenol oxidase was verified as the enzyme of interest because little to no activity was detected in the cellulose-degrading *L. sulphureus*.

There was a varying amount of systematic error in the measuring of extracellular enzyme activity. The homogenizing of *G. lucidum*, *G. applanatum* and *T. versicolor* in the acetate buffer was only partial because of the blending unit used. These three species formed dense mats of mycelium, as opposed to *P. ostreatus* and *L. sulphureus* which formed rhizomorphic and wispy aggregated filaments of hyphae, respectively. The partial homogenization of *G. lucidum*, *G. applanatum* and *T. versicolor* samples left visible pieces of interwoven hyphae in the solution pipetted into the microplate wells. Some of the absorbance measured by the spectrophotometer would have been due to the difficulty of light passing through the dense mycelium. The results for *L. sulphureus* make some systematic error clear also due to negative and positive values. Positive values could be due to contamination from airborne microbes that produce phenol oxidases in the samples and should occur from the mycelium in the wells. Note though that the mycelium for *L. sulphureus* was wispy with very low hyphal density in

relation to the other species. The source for negative values could be airborne contamination to the reference wells, whose values were subtracted from the absorbance from the samples.

Although a negative relationship was supported between substrate salinity and extracellular enzyme activity, it's important to note that there was also a negative relationship between radial growth and sodium chloride concentration and anecdotal evidence of a decrease in hyphal mass for some species. If the spectrophotometer is reading falsely inflated absorbance values due to mycelium in the microplate wells then the decreased amount of mycelium in high salinity samples would yield lower enzyme activity measurements. Without a way to separate the mycelium and/or calculate the difference in hyphal mass between treatments the extent of the effect is unknown.

Ecological Implications

Griffin (1997) stated that decreased water potentials should inhibit the fungus in excreting enzymes. Increased levels of solutes such as sodium chloride (road salt) do lower water potential and thus Griffin's assumptions would agree with my results, systematic error notwithstanding. Decreased amounts of excreted lignin-degrading enzymes by these fungi would slow down their decomposition of freshly fallen hardwoods and the cycling of the nutrients in those hardwoods back into bioavailable forms for other plants.

The affected areas would be along salted roadways. These are ecosystems already subject to large amounts of mechanical and chemical disturbance from road use while at the same time their aesthetic is appreciated by a large amount of people. The quicker and more complete biomass litter is decomposed the quicker eyesores will reenter soils and the healthier the roadside should appear.

Due to the chemical pollution from asphalt and traffic roadsides are areas that are particularly in need of these fungi both for human health and that of the local and downstream ecosystems. *P. ostreatus* showed a strong negative response in enzyme activity to increasing substrate salinity. *P.*

ostreatus is particularly relevant to controlling roadside pollution because it readily breaks down used automotive oils for food (Stamets 2005). It's also shown in Stamets' research to be more effective than experimental chemical and biological (through bacteria) remediation processes at breaking down diesel fuel in contaminated soils. Lignin-degrading fungi are being found to break down various phenolic pollutants such as resins and industrial dyes, breaking them down into less complex and easier to degrade forms (Gusse et al 2006, Shah & Nerud 2002).

Fungi can also hyperaccumulate heavy metals from their substrate into their fruitbodies, which can then be picked, transported and disposed of in safer locations (Stamets 2005). The mycelium of saprotrophic fungi are also effective environmental filters due to their mesh-like webs of hyphae (Stamets 2005). The particularly dense mats formed by *T. versicolor*, *G. applanatum* and *G. lucidum* should be effective at slowing and sequestering pollutants along the roadways that could otherwise be carried by storm water to surface waters or infiltrate soils and percolate into drinking water.

Continued Research

The sources of systematic error in this study question whether the trends seen in extracellular enzyme activity are due directly to road salt concentrations in the substrate. It may be that the road salt is decreasing extracellular enzyme activity indirectly through the decrease in radial growth instead. It is plausible though that the decrease in radial growth is due to a hindering of their digestion of the substrate.

To answer this question the extracellular enzyme analysis protocol must be refined. Mycelium need to first be removed from the homogenized solution to read the absorbance of the L-DOPA reaction without undue absorbance from the hyphae. If this was done there should be far less variation in the enzyme analysis data from the solution not being perfectly homogenized. A centrifuge should be able to separate both the agar and the mycelium from the solution while still suspending the enzymes. The pellet formed in the centrifuge could then be washed over a filter to separate the hyphae from the agar.

That hyphae could then be measured and hyphal mass could be used instead of radial growth as a more accurate measure of how the growth rate is affected by substrate salinity. Observing the pellet along with the hyphal mass data could also make clearer the answer to the question of whether it was a pigmentation indication of decreased enzyme activity as suggested by Griffin (2007) or if the perceived pigmentation loss was simply due to decreased hyphal density.

It would also be worthwhile to further explore possible differences in responses to road salt concentrations in the substrate between wild and commercial varieties as was originally intended. The assumption would be that wild fungi found in salt-polluted habitats would have grown somewhat salt tolerant over time through natural selection. These cultures should then be more salt tolerant than commercial species, who are bred to grow on engineered substrates that have no threat of road salt exposure.

Although a sensitivity to road salt has been demonstrated, these findings do not suggest that any alternative to road deicing is more safe for local ecosystems. To explore the potential harm of other possible solutions to the hazard of frozen roads similar testing must be done with those chemicals.

Conclusion

There were statistically significant negative responses by both measures, radial growth over time and extracellular enzyme activity, to increased concentrations of road salt in the substrate, supporting the two original hypotheses. Saprotrophic fungi fulfill a vital ecosystem role and also serve to reduce the amount of chemical pollution introduced by traffic on roadways. There was a notable amount of systematic error in the analysis of extracellular enzyme activity and so further research should be done to make clear the cause for and true results of the analysis of lignin-degrading extracellular enzymes.

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