Characterizing the presence of *P. cinnamomi* on reclaimed mines in Eastern Kentucky

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INTRODUCTION

Coal Mining & Reclamation Efforts in Appalachia

Surface coal mining is the dominant form of land cover change in the central Appalachian region of the United States (Bernhardt et al. 2012). Mountaintop removal, a form of surface mining widespread throughout eastern Kentucky, has been used in the United States since the 1970s (Palmar et al. 2010, Fox 1999). The process involves clearing montane forests, removing topsoil, and using both dynamite and draglines to expose underlying coal seams (Fox 1999). The displaced soil and rock from the stripped mountaintop are then deposited into adjacent valleys, known commonly as valley fills. While the intensive nature of surface mining can cause notable environmental damage, reclamation practices are able to convert these barren landscapes back to productive and diverse forested ecosystems (Palmar et al. 2010, Zipper et al. 2011).

Traditionally, reclamation practices have focused on ensuring substrate stability through excessive soil compaction in order to prevent heavy erosion and landslides (Zipper et al. 2011). Since native trees are unable to establish in overly compacted soils, grass-dominated landscapes prevail on reclaimed coal mine sites instead of forested ecosystems. Restored forest ecosystems are preferable to grasslands because they recover natural biodiversity, provide marketable goods such as timber, and act as carbon sinks (Zipper et al. 2011). In recent years, concerned groups have come together to rewrite traditional practices and shift the focus of reclamation to restoring forested lands. The Appalachian Regional Reforestation Initiative (ARRI) is a partnership between citizen groups, the coal industry, and governmental agencies that advocates for the restoring of forests on mined lands (About ARRI n.d.). The partnership developed the Forestry Reclamation Approach (FRA), a five step reclamation technique that involves creating a loosely graded rooting medium suitable for tree growth and planting both early successional species and commercially valuable crop trees (About ARRI n.d.). ARRI is dedicated to promoting the FRA across Appalachia in order to increase the survival of planted trees, expedite natural succession, restore wildlife habitat, and improve soil stability (About ARRI n.d.). As a result of efforts by ARRI, the FRA has been implemented for the reclamation of thousands of hectares of mined land (Zipper et al. 2011).

A secondary goal of ARRI is to aid organizations such as the American Chestnut Foundation in restoring American Chestnut populations in Appalachia. The American Chestnut remains functionally extinct after the chestnut blight fungus devastated populations in the early 20th century (American Chestnut Foundation, n.d.). FRA reclaimed mine sites were proposed for

planting this species due to the low competition and fast potential growth possible on these newly planted sites, as well as the perceived lack of soil pathogens due to high soil infiltration (About ARRI n.d., French et al. 2007). American Chestnuts have since been incorporated in FRA plantings.

An Emerging Threat

Research has shown that the FRA is effective in reestablishing native tree species on post-mining sites, and produces higher survival and growth rates than traditional reclamation practices (Zipper et al. 2011, Fields-Johnson et al. 2010). In spite of these strides, current reforestation efforts may be thwarted by the emergence of a soil-borne pathogen, *Phytophthora cinnamomi* (Pc), infecting trees in Appalachia. A highly invasive and deadly pathogen, Pc may pose a threat to newly-established trees on reclaimed mine lands (Bergot et al. 2004) because its hosts are mainly woody trees, including red oak, white oak, and American chestnut (Bergot et al. 2004). These tree species are native to Appalachia and are commonly used by ARRI in coal mine reforestation efforts (Sena et al. in prep). Symptoms of infection by Pc include root rot and stem/collar necrosis, usually resulting in tree death (Bergot et al. 2004). Therefore, it is important to inform reforestation efforts by characterizing the distribution of Pc across reclaimed mines in Appalachia, evaluating colonization of mine spoils by Pc over time, and determining any predictors of infection such as specific soil conditions.

Distribution and Dispersion

Pc likely originated in Taiwan or Papua New Guinea and has since spread globally, reaching the United States in the late 19th century (Corsa 1896). This long distance dispersal between continents was likely due to the exportation of ornamental plants that may have been infected with Pc (Sena et al. in prep). Pc has now reached Appalachia, creating a regional source from which the pathogen may spread to reforested mine sites. Pc reproduces asexually using motile zoospores which travel through bodies of water, as well as water on plant surfaces and water in soils, allowing them to travel effectively through most ecosystems (Ridge et al. 2014, Sena et al. in prep). They are also able to use chemical cues to find nearby potential host species (O'Gara et al. 2015). This combination of characteristics allows Pc to efficiently disperse between host populations. Pc has been isolated using soil baiting methods from forested sites across Appalachia such as in Southern Ohio, Western North Carolina, Western South Carolina, and sites in Eastern Kentucky, including Robinson Forest and Berea Forest (Adank et al. 2008, Balci et al. 2010, Hwang et al. 2009, Meadows et al. 2011). These studies establish that Pc is present in this heavily mined region, but very few studies have tested reclaimed mine sites themselves. In Ohio and Eastern Kentucky, reclaimed mine sites tested negative for Pc presence suggesting that these soils may be unfavorable for the pathogen (Adank et al. 2008, Hiremath et al. 2013). Further sampling of reclaimed mine sites for the presence of Pc is necessary to determine if these sites truly are unfavorable or if Pc is already established in these areas. Due to Pc's demonstrated ability to disperse, there is cause for concern that mined sites in Eastern Kentucky targeted for

reforestation could become infected posing a threat to these newly established forests. To understand the susceptibility of these sites to Pc invasion, the prefered environmental conditions of this pathogen must be examined.

Environmental Variables

Exotic species can be spread via media diversity of mechanisms, but in order for an alien species to establish a viable population it must first colonize a region where the environmental conditions are favorable for its growth, survival, and reproduction. Pc survives under a wide range of environmental conditions and can even persist under unfavorable conditions for extended periods of time by forming chlamydospores. Therefore, Pc is a serious pathogen threat (Sena et al. in prep). However, there are certain soil environmental conditions that facilitate its spread including soil moisture, pH, conductivity, and compaction. Although many studies have investigated the soil conditions that are suitable for Pc in natural and agricultural systems, none have looked at the presence of Pc in soils of reclaimed mine sites. By investigating how these variables correlate with the presence of Pc, we can begin to discern if reclaimed coal mine sites are viable for Pc establishment and propagation. Moreover, quantifying these relationships within reclaimed coal mine sites can help determine if Pc responds differently to these environmental conditions in highly disturbed soils compared to natural and agricultural systems.

Soil Moisture: Soil moisture is an important variable to consider, as the zoospores use the water in soil as a means of dispersal (The Threatened Species Network, 2008). One study focused on the decline of white oaks in Ohio found a positive, exponential correlation between soil moisture and Pc population on a seasonal scale in 2008. (Nagle et al. 2010). Another study suggests that high soil moisture also creates an anaerobic environment, which is favorable for water molds such as Pc. (Nesbitt et al. 1979). Therefore, we hypothesize that soil moisture and the percent occurrence of Pc will be positively related.

Soil Water pH: Soil water pH is another environmental factor that may affect the establishment of the pathogen. Pc flourishes best in the acidic soil conditions also fit for Chestnut Trees (slightly acidic, between a pH of 4.5 and 6.5) (Barton et al. 2010). Although no previous studies have linked pH directly to Pc, it would be a measurable variable of interest when determining correlations between the presence/absence of ink disease caused by Pc and favorable tree pH soil.

Soil Conductivity: Conductivity must also be considered. Although its effect on Pc has not been studied, conductivity is one of the most commonly altered soil environmental conditions during mitigation. One study investigated how salinity affects the presence of a similar pathogen (*Phytophthora ramorum*) around the perimeter of agricultural nurseries. This research provides a window of salinity suitability for the pathogen: from 0–45 g l^-1, or 0-0.045sg. (Preuett et al. 2016). This preexisting study gives us some reference to compare our *P. cinnamomi* findings

with, and our study will be novel in that it may be the first to provide basic information about the soil conductivity conditions tht are necessary for Pc presence on reclaimed coal mines.

Soil Compaction: Soil compaction can affect a variety of physical, chemical, and biological soil variables. (Whalley et al. 1995). Studies have shown that probability of the presence of Chestnut Ink Disease, which is caused by Pc, increases with increasing soil compaction (Fonseca et al. 2004). Additionally, advancement and dispersal of the disease has been shown to occur more rapidly within soils that are both moist and more compact, indicating that denser, finer soils (clay-based) result in higher seedling mortality when Pc is present (Rhoades et al. 2003).

Understanding the ideal growth conditions of Pc is important for knowing what areas may be susceptible to invasion. The environmental conditions of our study sites were measured in order to relate them to the presence of Pc and determine how susceptible the sites may be to this pathogen. One must also consider that mountaintop removal mining alters these environmental conditions, which may impact the likelihood of Pc establishing. By looking at sites that have been affected by the mining process in various stages of recovery, as well as forest sites that have not been directly influenced by mining, we hope to determine whether or not these areas are viable for the establishment and propagation of Pc.

Host Species & Symptoms

Objectives

Reclaimed mountaintop mines in Appalachia potentially provide an opportunity for extensive reforestation, specifically of American Chestnuts (Sena et al. in prep). The prevalence of Pc on these sites is not well understood and may dictate the success of current and future reforestation efforts. In one study, sites reclaimed under the guidelines of ARRI all tested negative for presence of Pc (Hiremath et al. 2013). This suggests that these sites may be a safe environment, at least temporarily, for trees susceptible to Pc. If reclaimed mine sites truly are unfavorable for Pc, it may be due to the upturning of previously buried soils in the FRA reclamation process which Pc has not yet reached. Another possible factor is the loose grading of soil in the FRA which results in high infiltration, keeping soil moisture low and therefore limiting Pc dispersion. By this logic, recently reclaimed mine sites should not have Pc present, but over time Pc may still colonize these sites. By testing a chronosequence of reforested coal mines, we hope to determine if and when Pc colonizes these sites after reclamation. We also examine the environmental variables of soil moisture, compaction, pH and conductivity in order to compare to the ideal growth conditions of Pc and relate to its prevalence.

METHODS

Study Sites

Our study site was in Clayhole, Kentucky in and around Robinson (Figure 1). The climate of this area during the early spring varies from 0 to 10 degrees celsius with slim chances of occasional snowfall and rain. Geographically, Robinson Forest is a mountainous region with several valleys. Three, approximately 1 acre plots, were sampled at each treatment level. Sites reclaimed 10 years ago and undisturbed sites (our control) were located within Robinson Forest. Sites reclaimed 20 years ago and 0-1 year ago were located on Starfire Mine. Starfire Mine is a mountain top removal mine that has been in operation since the 1980s (Cotton et al. 2012). All experimental plots were on valley fills produced by mountaintop removal coal mining that were reclaimed using the FRA. Treatment plots were all relatively flat, while undisturbed plots were all on steep heavily forested hillsides. Plots reclaimed 20 and 10 years ago contained young trees, while plots reclaimed 0-1 year ago contained saplings. Tree density was highest in undisturbed plots, then 20 year plots, and lowest in 10 year plots. The tree density of 0-1 year plots was difficult to compare since they only contained saplings.

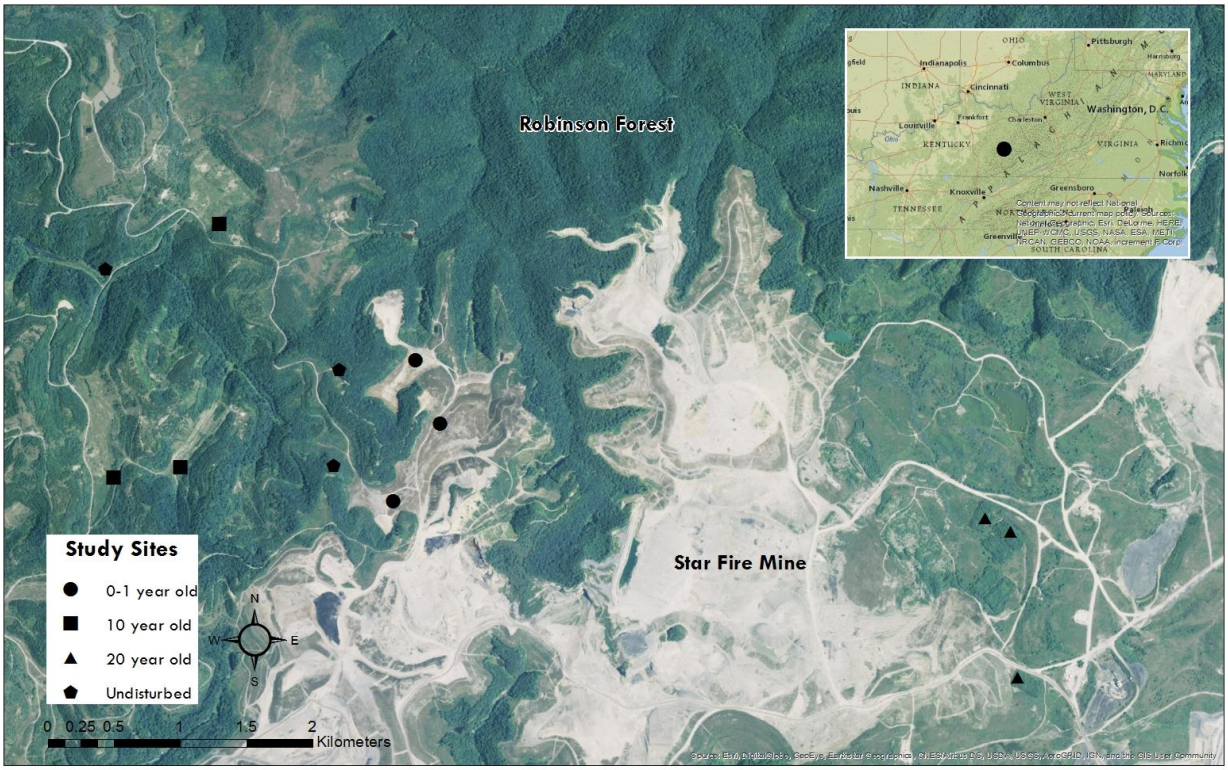

Figure 1. Spatial distribution of study sites across Robinson Forest and Starfire Mine. Three sites were sampled for pathogen presence and soil variables at each treatment level (0-1 year, 10 year, 20 year, undisturbed).

Field Sampling Methods

Six subsamples were selected at random within each reclaimed mine site. Each subsample was a composite of six smaller soil samples in order to account for small scale variation to reduce the chance of false negatives in pathogen detection (Pryce et al. 2002).

Soil Samples: The composite subsample was made from six full trowels, with each trowel being filled with approximately 80g of soil, for a sample total of approximately 480g. The six trowel-fulls of soil were collected within 2 square meters of each other. The soil was extracted from the top 10-15 cm of the soil surface, with the manual exclusion of rocks and gravel. The equipment used for sampling was sterilized with ethanol between each of the subsamples and also between each of the sites to eliminate cross-contamination.

Soil Moisture: We collected soil moisture measurements for our sites by placing two WaterScout SMEC 300 Soil Moisture/EC/Temperature Sensor soil probes about half a meter apart at the first subsample area in each of the six sample sites. The first subsample in each area was determined randomly. We completely covered the collection surface of the probes with soil (about 10 cm deep) and left the probes for at least 24 for hours to equilibrate. The probes are connected to lead wires that extend to the soil surface.We then attached the probes to a portable handheld meter and recorded values for both probes in each site. The two measurements were then averaged to attain a single value for each site.

Soil Compaction: To measure soil compaction, we used a DICKEY-john soil compaction tester. At each site, we took six readings per subsample to account for microenvironmental variance, averaged these values across the subsample sites, and averaged them again to get a single replicate value for the site. To effectively use the soil compaction tester, we applied slow, constant pressure until the tester reached the compacted layer of soil. The compacted layer can be determined by the gauge indicator increasing upward into the red range and then moving back down into the yellow or green after passing through the compacted layer. The compaction measurements were taken at the beginning of the compacted layer. This made for a total of 18 compaction measurements per study site.

Lab Analysis of Soil Characteristics

Sample Preparation: The composite soil subsamples were prepared by kneading the sample bag for approximately two minutes to attain a homogenous sample. From this, 50 g of homogenized soil were mixed with 50 mL of distilled water in a labeled container. The mixture was homogenized by vigorous shaking for one minute.

pH: The pH of the soil sample was measured with a PHH-37 Handheld pH/mV Meter. The measurement was recorded after submerging the pH probe into the soil mixture for one minute. The probe was rinsed with distilled water between uses to avoid cross-contamination between different soil subsamples. The six pH measurements for each site were averaged to attain a single replicate value for that site.

Conductivity: To measure soil conductivity, an additional 20 mL was added to each soil mixture. The new mixtures were homogenized by vigorous shaking the container for one minute. The conductivity of each soil sample was then measured with a YSI Model 30 digital, handheld conductivity meter. The measurements were recorded in millisiemens per centimeter after submerging the probe into the soil mixture. The probe was rinsed with distilled water between each measurement. The six conductivity measurements for each site were averaged to attain a single replicate value for that site.

***Phytophthora Cinnamomi* Detection**

Composite soil samples were analyzed for presence of Pc using molecular methods at University of Kentucky. Detection and identification of Pc was done by a nested PCR approach from DNA extracted from the soil samples, according to the method developed by Sena et al, in prep, for evaluation of Pc presence directly from soil samples rather than from a soil culture. The PCR method is advantageous over the conventional soil-baiting methods because of its improved sensitivity of detection from soil as well as its ability to complete the process in a more expedited manner (Sena et al, in prep). Once complete, the number of samples positive for Pc was divided by the total number of samples at each site (6) to achieve a percent presence value for each replicate.

DNA Extraction: Soil DNA was extracted from 0.25g aliquots, using the MoBio PowerSoil DNA Extraction Kit, according to manufacturer instructions.

Molecular Methods: The nested PCR method involves an initial round of amplification by conventional PCR, followed by another set of conventional PCR, adapted from Engelbrecht et al. First-round conventional PCR reactions were carried out with a PCR mix of 2.5 µL of PCR reaction buffer, 2.5 mM MgCl2, 200 µM each dNTP, 0.2 µM of each primer, 1 U of Fast Start Taq DNA Polymerase and 20 ng of template DNA. Amplification conditions were as follows: 95°C for 5 min, 15 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, with a final step at 72°C for 10 min.

Second-round conventional PCR was then performed using a PCR mix of 0.25 µM forward and reverse primer, 2 µl of template, (2 µl of the first-round PCR product), and 3 µl of PCR-grade water. Amplification conditions were as follows: preincubation at 95°C for 10 min, followed by 40 cycles at 95°C for 5 s, 60°C for 5 s, 72°C for 5 s. Negative controls contained water as a template.

Gel Electrophoresis: PCR products were separated by gel electrophoresis using 2% agarose gel. DNA from *P. cinnamomi* RF5, at a concentration of 10^4 ng/µl served as the positive control. Distilled water served as the non-template control.

Data Analysis: An analysis of covariance (ANCOVA) was completed in R to assess whether pathogen presence/absence was affected by the categorical time since reclamation variable and the continuous environmental variables. The ANCOVA model found no statistically significant interactive effects between time since reclamation and the environmental variables so main and covariate effect p-values could be interpreted. To determine significant differences in average pathogen presence between individual treatment levels, a tukey's comparison of multiple means test was performed with a 95% family-wise confidence level.

RESULTS

Pc presence vs. Time since reclamation:

In sites between 0 and 1 year since reclamation, the percentage of samples from each site that tested positive for Pc ranged between 50% and 67%, with an average presence of 61%. In 10 year-old sites, percent presence was between 50% and 83%, with an average presence of 61%. In 20 year-old sites, percent presence was between 50% and 83%, with an average presence of 67%. Percent presence in undisturbed sites ranged between 0% and 67%, with an average of 33%. A 20 year-old site had the highest percent presence of 83% while an undisturbed site had the lowest percent presence of 0% (Figure 2). ANCOVA found no significant relationship between presence and time since reclamation with a p value of 0.295 (Appendix Table 1). Additionally, Tukey's test confirmed no significance difference among any paired combination of treatment levels (Appendix Table 2)

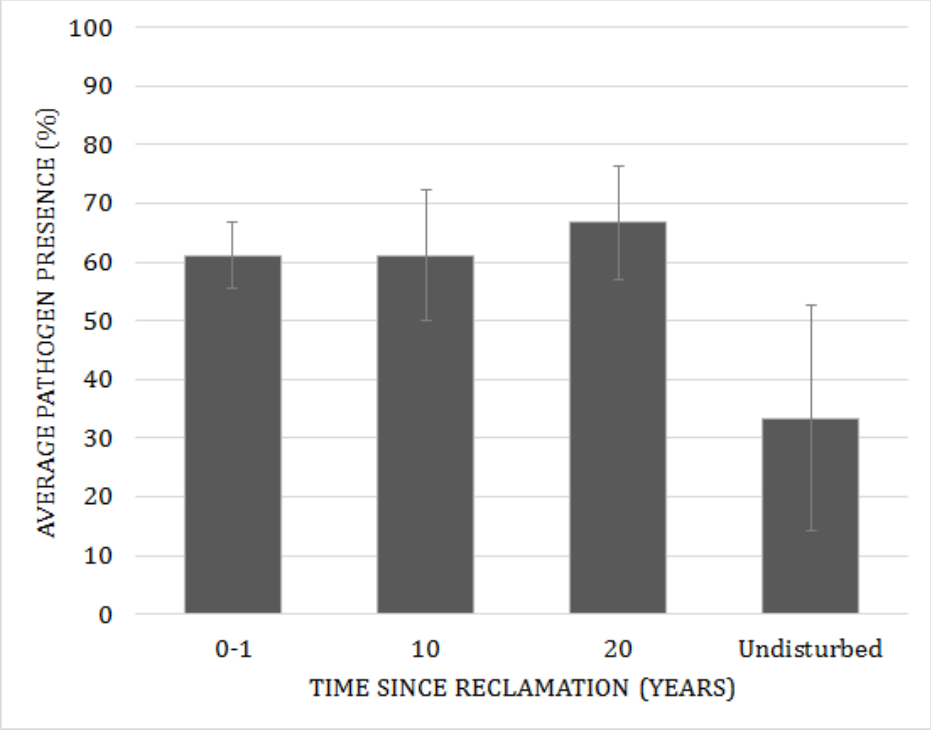

Figure 2. Average percent pathogen presence across the three different treatment levels (time since reclamation) and control sites (undisturbed).

Pc presence vs Soil Variables:

Average soil moisture levels ranged between 5.25% and 20.5% but there was no detectable pattern between soil moisture and P. cinnamommi presence. The R-squared value for the relationship was 0.0049 and the p-value was 0.823 (Figure 3). (Appendix Table 3).

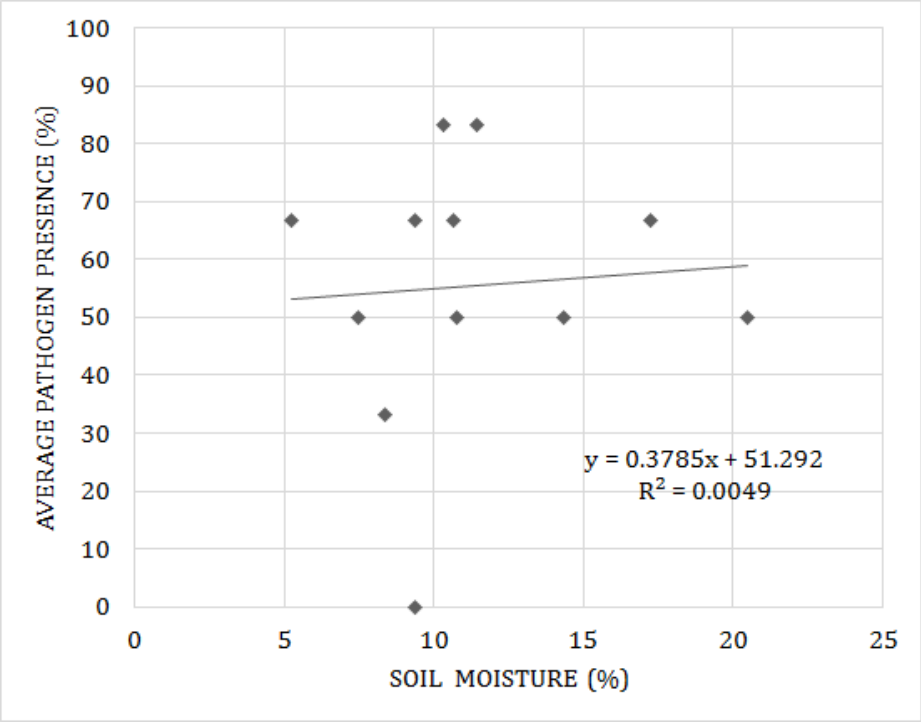

Figure 3. The linear relationship between average percent pathogen presence and soil moisture percentage across all test sites. R-squared value is 0.0049.

Soil compaction ranged from 86.875 psi to 149.1 psi and averaged 150.7 across all sites. Similar to soil moisture, soil compaction had no detectable effect on the presence of *P. cinnamomi* with anR-squared of 0.1692 (Figure 4) and p-value of 0.0898 (Appendix Table 4).

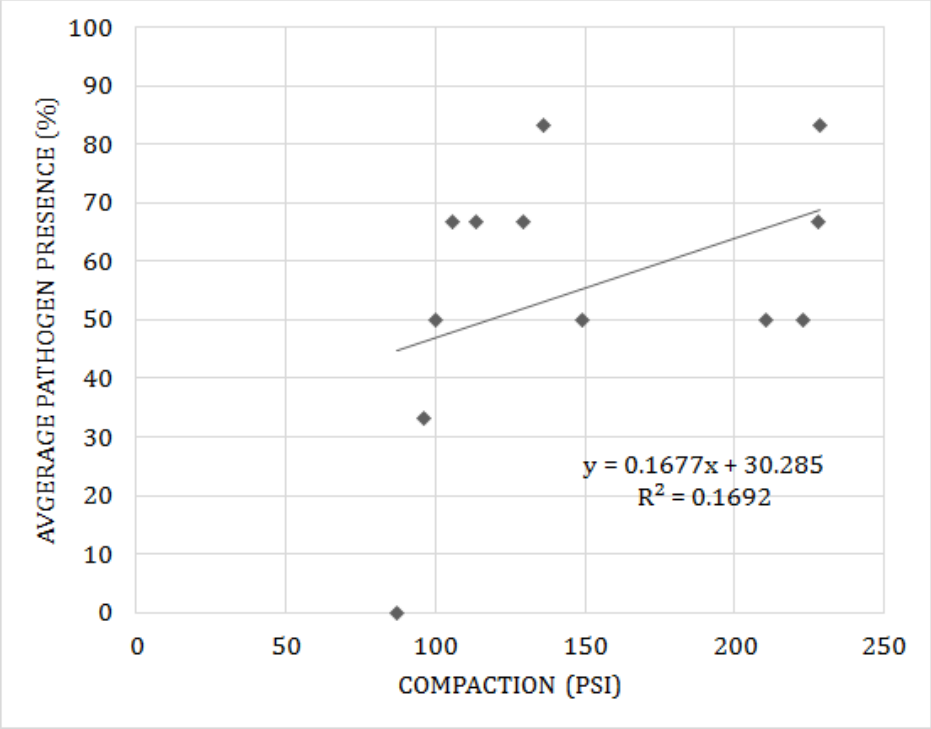

Figure 4. The linear relationship between compaction (psi) and average percent pathogen presence across all test sites. R-squared value is 0.1692.

Soil water pH ranged from 4.4 to 6.7 and averaged 5.8 across all sites. Just like the other two soil variables, soil pH had no effect on P. cinnamomi presence with an R-squared 0.0613 (Figure 5) and p-value of 0.512 (Appendix Table 5).

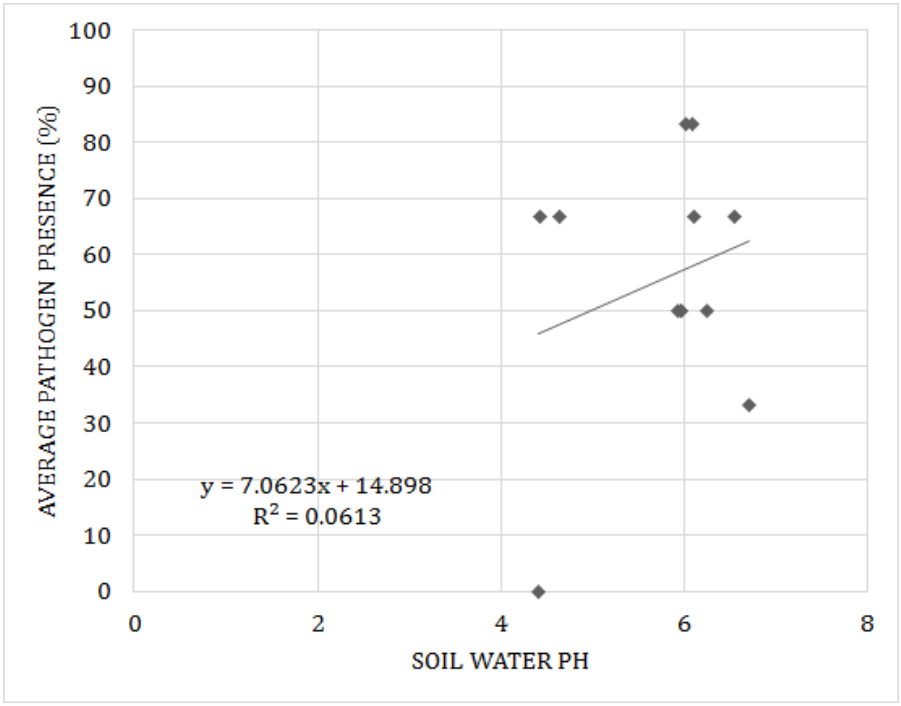

Figure 5. The linear relationship between average percent pathogen presence and soil water pH is shown across all test sites. R-squared value is 0.0613.

Conductivity ranged between 50.25 mS/cm 687.9 mS/cm and averaged 194.8 mS/cm across sites. The functional relationship between conductivity and *P. cinnamomi* was weak (Fig. 6; R^2 = 0.0251) and statistically not significant with a p-value of 0.65 (Appendix Table 6).

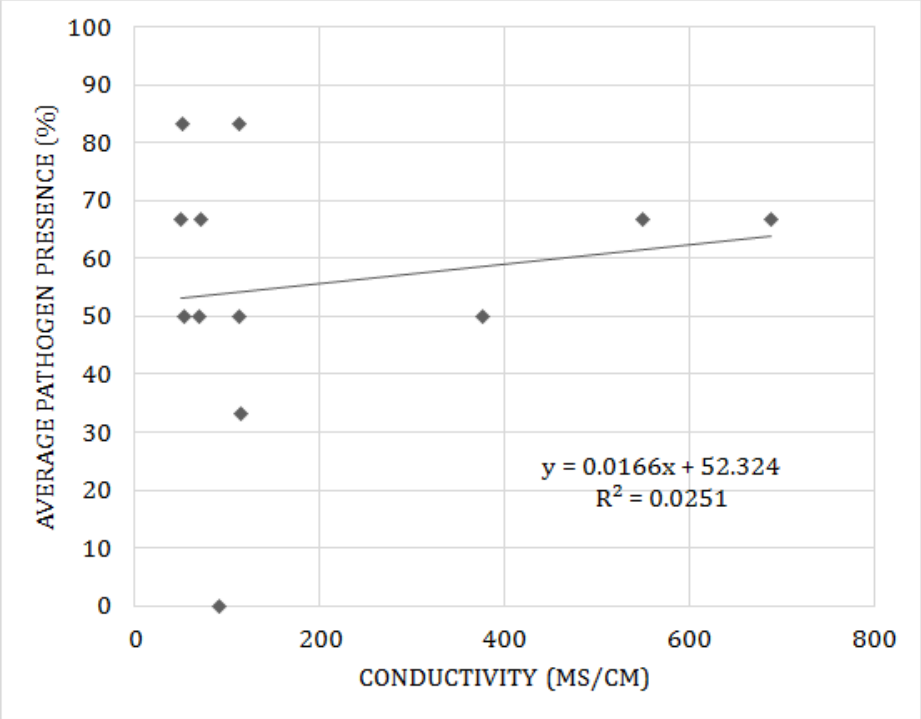

Figure 6. The linear relationship between average percent pathogen presence and conductivity (ms/cm) across all test sites. R-squared value is 0.0251.

DISCUSSION

The present findings indicate that *Phytophthora cinnamomi* is widespread throughout the reclaimed mine sites included in this study. These mine spoils, situated in Eastern Kentucky and reclaimed by the FRA Approach, may provide a suitable habitat for colonization by Pc. The present findings indicate that variations in soil variables do not readily predict the percent pathogen presence. The percent pathogen presence was not appreciably related to soil moisture, compaction, pH, or conductivity. Likewise, there was no significant relationship between time since FRA reclamation and pathogen presence on the reclaimed mine sites included in our study.

While Pc has been isolated from forest soils across Appalachia, the presence and distribution of the pathogen on reclaimed mine sites is not well-characterized (Adank et. al 2008). Our findings indicate presence of Pc in undisturbed forested sites in Robinson Forest, since Pc was isolated from two of the three undisturbed sites. In addition, our findings indicate ubiquitous presence of Pc across the reclaimed mine sites included in this study. Previous studies have been unable to isolate Pc from reclaimed mine sites in Ohio and Eastern Kentucky (Adank et al. 2008, Hiremath

et al. 2013). However, Pc was isolated from each reclaimed mine site in this study, and the average percent pathogen presence ranged from 50% to 83%. Why this pathogen is present at our reclaimed mine sites but not in Ohio is unknown.

Ideal soil conditions for Pc are not well-characterized on reclaimed coal mines. Since Pc was isolated from each mine site, our results indicate that reclaimed mine spoils in Eastern Kentucky may have environmental conditions favorable for colonization by Pc. Average soil conditions are described in Appendix Figure 1. Average soil conductivity on these reclaimed mine sites ranged from 50.25 mS/cm to 548.62 mS/cm, non-saline to moderately saline conditions (USDA n.d.), which may be favorable for growth of Pc. Soil water pH ranged from 4.43 to 6.55, acidic conditions that may support the growth of Chestnut trees (Barton et al. 2010), but may also be ideal for invasion by Pc. On those undisturbed sites from which Pc was isolated, average soil conductivity varied between 71.90 mS/cm and 114 mS/cm, considered to be non-saline (USDA n.d.), and average soil water pH varied between 4.64 and 6.71, acidic conditions which may be appropriate for Pc.

Based on the present data, we could not identify any of the soil variables as predictors of percent pathogen presence. Percent pathogen presence was not attributable to soil moisture, compaction, pH, or conductivity when the linear model was analyzed with ANCOVA. At this time, soil conditions may not inform reclamation practices with consideration to distribution and presence of Pc. However, these results may be due to the limited sample size of this study and the limited variation in soil parameters studied.

Similarly, the present research was unable to indicate susceptibility of reclaimed mine sites to Pc colonization over time. It was expected that mine sites most recently reclaimed by the FRA would be unfavorable for Pc, since the FRA creates loose graded soils with high infiltration. Over time, Pc may be expected to colonize reclaimed mine sites based on the invasive nature of the pathogen (Adank et al. 2008). That is to say, the percent presence of the pathogen is expected to increase with increasing time since reclamation. However, our results indicate ubiquitous presence of Pc across mine sites included in this study, regardless of time since reclamation. Percent pathogen presence was not significantly different between sites reclaimed recently, reclaimed 10 years ago, or those reclaimed 20 years ago based on ANCOVA analysis among means. Based on these results, reclamation efforts may be wary of colonization of Pc at any stage of FRA reclamation, and may investigate other factors that may influence colonization by Pc.

The results from this study may be influenced by anthropogenic activity in this region. Within this study, there was a single site where Pc was not isolated. The percent presence of Pc was 0% at a single undisturbed site, which had been unaltered by anthropogenic activity. This site was further removed from neighboring mine sites than the other two control sites, suggesting that removal from nearby anthropogenic activity may be protective against invasion by Pc. The site

with the greatest percent presence of Pc (83%) was a retired mine site reclaimed 20 years ago. This site on Starfire Mine hosts many research projects and has been subject to heavy anthropogenic activity that may perhaps explain the notable presence of Pc at this site. Thus, future studies should note land-use and neighboring anthropogenic activity while surveying for Pc. Coupled with the use of GIS, this information may further highlight the relationship between anthropogenic disturbances and the fate and transport of the invasive Pc.

This study, and thus the implications of these results, are limited by the sample size of the current study. While we were able to access nine reclaimed mine sites and three control sites, it is possible that these sites are not entirely representative of the presence and distribution of Pc along all reclaimed mine sites in Eastern Kentucky. The time since reclamation in this study refers only to the time since the site was reclaimed with the FRA approach. Some sites had been reclaimed with traditional methods years prior, and then re-tilled for FRA reclamation. Thus, "time since reclamation" is not entirely representative of the history of each site. Perhaps further investigation into the soil alterations over time would reveal a relationship with percent presence of Pc. A longitudinal study may better reveal the colonization of Pc over time at a single site.

The accuracy of the percent presence of Pc at each site may be limited due to the notable small-scale random distribution of Pc (Pryce et al. 2002). The risk of false negatives was reduced by taking multiple subsamples at each site, each a composite sample of the sampling point. In addition, the presence of *P. cinnamomi* was indicated by the detection of DNA specific to the pathogen. Presence in this sense does not indicate viable organisms of *P. cinnamomi*, nor does it indicate successful colonization by the pathogen at these sites. However, the results from this study do exemplify the applications for the use of PCR methods to detect Pc in environmental samples.

Restoration programs may consider surveying sites for presence of Pc using this novel molecular approach in order to inform restoration efforts, specifically as it relates to the reforestation of native trees and American chestnuts. Pc causes chestnut ink disease in American chestnuts and caused steep declines in populations even before the chestnut blight fungus arrived in the United States (Anagnostakis 2001). Chestnut ink disease has recently resurfaced as a significant concern as it causes high mortality in American chestnut restoration efforts (Brosi 2001). If Pc is present on reclaimed mine sites, the species used for reforestation may need to be evaluated since it may not be cost-effective to plant expensive blight-resistant American chestnuts.

Restoration programs may benefit from identifying and investing in trees that are resistant to Pc. Some host species of Pc have shown certain amounts of resistance to the pathogen (Mbaka, 2013). For instance, mature shortleaf pines on one Australian plantation were not killed by Pc despite being infected (Bumbieris, 1981). Species that show resistance to Pc are often still infected by the pathogen but are able to contain the infection to one area, allowing the host to

survive (Phillips et al, 1984). On mine sites where Pc has been isolated, reclamation efforts may plant trees that have noted resistance to Pc in order to promote survival of new forest growth.

CONCLUSION

An emerging threat to restoration efforts, *Phytophthora cinnamomi* has been isolated from nine reclaimed mine sites in Eastern Kentucky. Present findings did not establish a relationship between the percent presence of Pc at reclaimed mine sites and various soil variables, nor do our results establish a relationship between present presence of Pc based on the time since reclamation of a retired mine site. However, our results do suggest that reclaimed mine sites may be susceptible to invasion by Pc. This study justifies further investigation into the distribution and presence of Pc among FRA reclaimed mine sites in Appalachia, especially those sites identified for restoration of the American Chestnut. To ensure tree survival and growth on reclaimed mine land, restoration programs may identify and invest in trees resistant to the emerging Pc pathogen.

Citations

About ARRI. (n.d.). Retrieved April 10, 2017, from https://arri.osmre.gov/About/AboutARRI.shtm

Adank, K.M., Barton, C.D., French, M.E., & De Sá, P.B. (2008). Occurrence of Phytophthora on reforested loose-graded spoils in Eastern Kentucky. *Proceedings of the American Society of Mining Reclamation*, 1-13.

American Chestnut Foundation. (2017). Saving the American Chestnut. Retrieved from https://www.acf.org/.

Anagnostakis, S. (2001). The effect of multiple importations of pests and pathogens on a native tree. *Biol Invasions, 3,* 245-254.

Barton, C.D., M.E. French, S. Fei, K.A. Ward, R. Paris, and P. Angel. 2010. Appalachian surface mines provide a unique setting for the return of the American Chestnut. Solutions. Published Online: July 20, 2010.

Balci, Y., Long, R., Mansfield, M., Balser, D., & Macdonald, W. (2010). Involvement of *Phytophthora* species in white oak (*Quercus alba*) decline in southern Ohio. *Forest Pathology, 40,* 430-442.

Bergot, M., Cloppet, E., Pérarnaud, V., Déqué, M., Marçais, B. & Desprez-Loustau, M.-L. (2004). Simulation of potential range expansion of oak disease caused by *Phytophthora cinnamomi* under climate change. *Global Change Biology, 10,* 1539–1552.

Bernhardt, E.S., Lutz B.D., King, R.S., Fay, J.P., Carter, C.E., Helton, A.M, Campagna D. & Amos J. (2012) How many mountains can we mine? Assessing the regional degradation of Central Appalachian rivers by surface coal mining. *Environmental Science Technology, 46,* 8115-22.

Brosi, S.L. (2001). *American chestnut seedling establishment in the Knobs and Eastern Coalfields regions of Kentucky*.

Bumbieris, M. (1981). Association of Phytophthora cinnamomi with Pinus radiata in South Australia. *Australian Journal of Botany, 29,* 11-15.

Corsa, W.P. (1896). Nut culture in the United States: embracing native and introduced species. *USDA Division of Pomology*.

Cotton, C., Barton, C., Lhotka, J., Angel, P.N., Graves, D. (2012) Evaluating Reforestation Success on a Surface Mine in Eastern Kentucky. *USDA Forest Proceedings,* 68, 16-23.

EPA. Basic Information about Surface Coal Mining in Appalachia. Environmental Protection Agency, 06 Oct. 2016. Retrieved April 10, 2017, from https://www.epa.gov/sc-mining/basic-information-about-surface-coal-mining-appalachia#what.

Engelbrecht, J. (2013) Development of a Nested Quantitative Real-Time PCR for Detecting Phytophthora cinnamomi in Persea americana Rootstocks. *Plant Disease, 97* 1012 - 1017.

Fields-Johnson C., Zipper, C.E., Burger, J.A., Evans D.M. (2010). Second Year Response of Appalachian Mixed Hardwoods on Soil Surface Grading and Herbaceous Ground Cover on Reclaimed Mine Land. *National Meeting of the American Society of Mining and Reclamation.*

Fonseca, T.F., Abreu, C.G., Parresol, B.R. (2004). Soil Compaction and Chestnut Ink Disease. *Forest Pathology, 34,* 273-283.

Fox, J. (1999). Mountaintop Removal in West Virginia: An Environmental Sacrifice Zone. *Sage Journals, 12,* 163-183.

French, M. E., Barton, C. D., Graves, D., Angel, P. N., & Hebard, F. V. (2007, June). Evaluation of mine spoil suitability for the introduction of American chestnut hybrids in the Cumberland Plateau. In *Proceedings of the 2007 national meeting of the American Society of Mining and Reclamation. ASMR, Lexington, KY* (pp. 249-258).

Hiremath, S., Lehtoma, K., & Bauman, J.M. (2013). Survey for the presence of Phytophthora cinnamomi on reclaimed mine lands in Ohio chosen for restoration of the American Chestnut. *Journal of the American Society of Mining and Reclamation, 2,* 68.

Hwang, J., Oak, S.W., Jeffers, S.N. (2009). Monitoring occurrence and distribution of *Phytophthora* species in forest streams in North Carolina using baiting and filtration methods. *Proceedings of the Proceedings of the fourth meeting of the International Union of Forest Research Organizations (IUFRO),* 91-95.

Kuhlman, G.E. (1964). Survival and pathogenicity of Phytophthora cinnamomi in several western Oregon soils. *Forest Science, 10,* 151-158.

Mbaka, J.N. (2013). *The ecology, distribution and population structure of Phytophthora cinnamomi associated with root rots and trunk cankers of macadamia in Kenya.* Retrieved from *JKUAT.*

Meadows, I.M., Jeffers, S.N. (2011). Distribution and recovery of *Phytophthora cinnamomi* in soils of mixed hardwood-pine forests of the south-eastern USA. *New Zealand Journal of Forestry Science, 41.*

Nagle, A.M., Long, R.P., Madden, L.V., and Bonello, P. (2010) Association of *Phytophthora cinnamomi* with white oak decline in southern Ohio. Plant Dis. 94:1026-1034.

Nesbitt H. J., Malajczuk, N., Glenn, A. R., Effect of Soil Moisture and Temperature on the Survival of *Phytophthora cinnamomi rands* in Soil. *Soil Biol. Biochem., 11,* 137-140.

O'gara, E. Howard, K., Mccomb, J., Colquhoun, I., & Hardy, G.S.J. (2015). Penetration of suberized periderm of a woody host by Phytophthora cinnamomi. *Plant Pathology, 64,* 207-215.

Palmer, M.A., Bernhardt, E. S., Schlesinger, W. H., Eshleman, K. N., Foufoula-Georgiou, E., Hendryx, M. S., Lemly A. D., Likens G. E., Loucks, O. L., Power M. E., White, P. S. & Wilcock, P. R. (2010) Mountaintop Mining Consequences. *Science, 327.*

Phillips, D., & Weste, G. (1984). Field resistance in three native monocotyledon species that colonize indigenous sclerophyll forest after invasion by Phytophthora cinnamomi. *Australian Journal of Botany, 32,* 339-352.

Preuett J.A., Collins, D.J.,Luster D.J., Widmer, T.L. (2016) The effect of salinity on the survival, growth, sporulation and infection of *Phytophthora ramorum. Fungal Ecology, 23,* 123-130.

Pryce, J., Edwards, W., & Gadek, P.A. (2002). Distribution of Phytophthora cinnamomi at different spatial scales: When can a negative result be considered positively? *Austral Ecology, 27,* 459-62.

Rhoades, C.C., Brosi, S.L., Dattilo, A.J., & Vincelli, P. (2003). Effect of soil compaction and moisture on incidence of phytophthora root rot on American chestnut (Castanea dentata) seedlings. *Forest Ecology and Management, 184,* 47-54.

Ridge, G., Jeffers, S., Bridges, Jr.W., & White, S. (2014). In situ production of zoospores by five species of Phytophthora in aqueous environments for use as inocula. *Plant Disease, 98,* 551-558.

Sena, K., Crocker, E., Vincelli, P., & Barton, C. (in prep). Phytophthora cinnamomi: A review. *Unpublished review.*

The Threatened Species Network (2008). Managing Phytophthora Dieback in Bushland. http://www.bpginc.info/informationsheets/Managing%20Dieback%20Edition%204%202008%20DWG20Handbook.pdf

United States Department of Agriculture (USDA). Soil Electrical Conductivity: Soil Quality Kit - Guides for Educators (n.d.) Retrieved May 7, 2017 from https://www.nrcs.usda.gov/Internet/FSE_DOCUMENTS/nrcs142p2_053280.pdf

Whalley, W.R., Dimitru, E., Dexter, A.R. (1995). Biological Effects of Soil Compaction. *Soil and Tillage Research, 35,* 53-68.

Zipper, C.E., Burger, J.A., Skousen, J.G., Angel, P.N., Barton, C.D., Davis, V., & Franklin, J.A. (2011). Restoring forests and associated ecosystem services on appalachian coal surface mines. *Environ Manage, 47,* 751-765.

Appendix

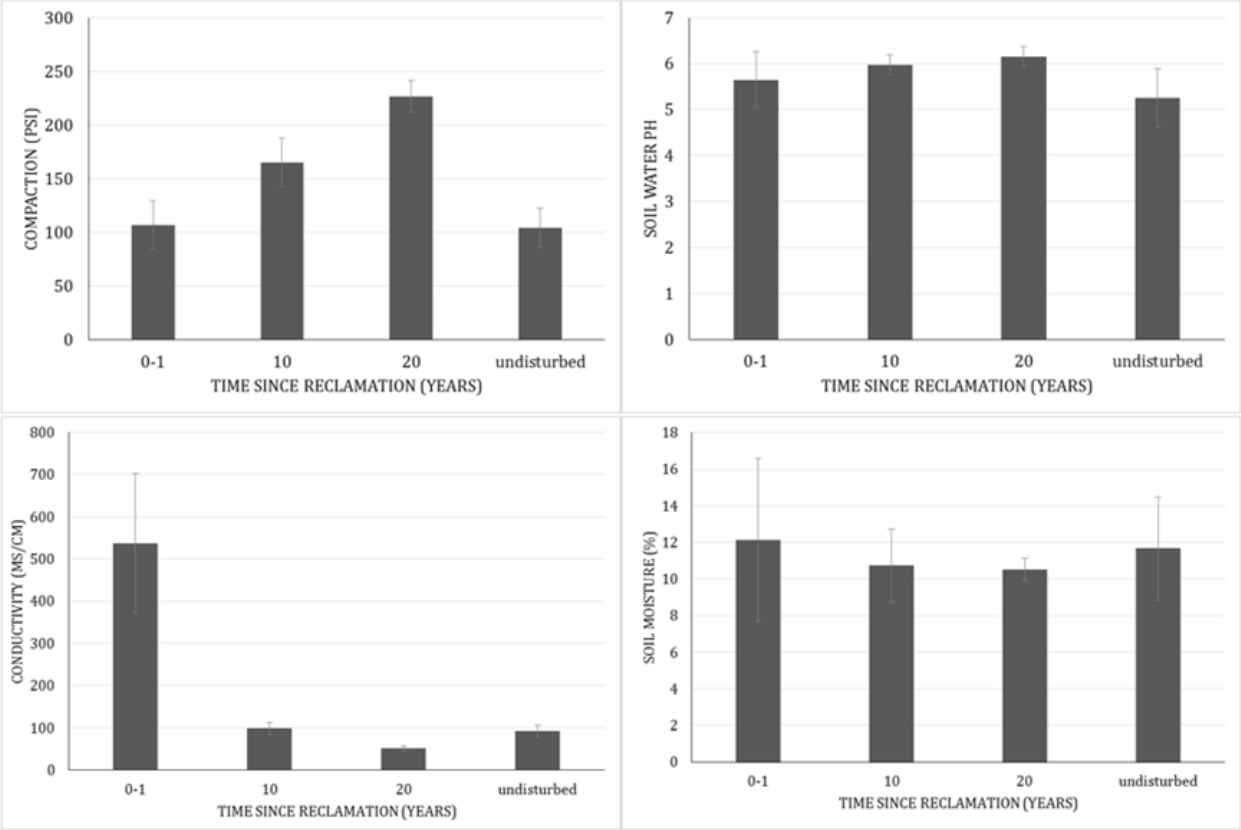

Figure 1. For each of the soil variables, average soil variable values are displayed for sites at each treatment level (time since reclamation) and control (undisturbed).

```
treatment_presence <- aov(pathogen$Percent.Presence~pathogen$Treatment)
summary(treatment_presence)
```

	DF	Sum sq	Mean sq	F value	Pr(>F)
Treatment	3	0.2041	0.06803	1.468	0.295
Residuals	8	0.3708	0.04635		

Table 1. ANCOVA results performed in R for differences in pathogen presence across all treatment levels.

```
treatment_presence <- aov(pathogen$Percent.Presence~pathogen$Treatment)
tukeytest <- TukeyHSD(treatment_presence)
tukeytest
```

	diff	lwr	upr	p adj
10 yr - 0-1 yr	-0.00333333	-0.5662554	0.5595888	0.9999973
20 yr - 0-1 yr	0.05333333	-0.5095888	0.6162554	0.9895630
Undisturbed - 0-1 yr	-0.28000000	-0.8429221	0.2829221	0.4334074
20 yr - 10 yr	0.05666667	-0.5062554	0.6195888	0.9875559
Undisturbed - 10 yr	-0.17666667	-0.8395888	0.2862554	0.4427191
Undisturbed - 20 yr	-0.33333333	-0.8962554	0.2295888	0.3014341

Table 2. Tukey's multiple comparisons of means test computed in R with a 95% family-wise confidence level.

```
moist_anc <- aov(pathogen$Percent.Presence~pathogen$Soil.Moisture.Percent +
pathogen$Soil.Moisture.Percent*pathogen$Treatment)
summary(moist_anc)
```

	DF	Sum sq	Mean sq	F value	Pr(>F)
Soil Moisture	1	0.00287	0.00287	0.057	0.823
Treatment	3	0.20878	0.06959	1.392	0.367
Soil Moisture* Treatment	3	0.16332	0.05444	1.089	0.450
Residuals	4	0.19993	0.04998		

Table 3. ANCOVA results performed in R for soil moisture across all treatment levels.

```
comp_anc <- aov(pathogen$Percent.Presence~pathogen$Soil.Compaction +
pathogen$Soil.Compaction*pathogen$Treatment)
summary(comp_anc)
```

	DF	Sum sq	Mean sq	F value	Pr(>F)
Compaction	1	0.09677	0.09677	4.965	0.0898
Treatment	3	0.11812	0.03937	2.020	0.2536
Compaction* Treatment	3	0.28202	0.09401	4.823	0.0813
Residuals	4	0.07797	0.01949		

Table 4. ANCOVA results performed in R for compaction across all treatment levels.

```
pH_anc <- aov(pathogen$Percent.Presence~pathogen$pH + pathogen$pH*pathogen$Treatment)
summary(pH_anc)
```

	DF	Sum sq	Mean sq	F value	Pr(>F)
pH	1	0.03387	0.03387	0.518	0.512
Treatment	3	0.17022	0.05674	0.867	0.528
pH*Treatment	3	0.10904	0.03635	0.555	0.678
Residuals	4	0.26175	0.06544		

Table 5. ANCOVA results performed in R for pH across treatment levels

```
cond_anc <- aov(pathogen$Percent.Presence~pathogen$Conductivity +
pathogen$Conductivity*pathogen$Treatment)
summary(cond_anc)
```

	DF	Sum sq	Mean sq	F value	Pr(>F)
Conductivity	1	0.01541	0.01541	0.240	0.650
Treatment	3	0.20152	0.06717	1.046	0.464
Conductivity* Treatment	3	0.10105	0.03368	0.524	0.689
Residuals	4	0.25690	0.06423		

Table 6. ANCOVA results performed in R for compaction across all treatment levels.