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Isolation and Morphology of Potentially Novel Actinomycetes in Centralia, Pennsylvania

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Abstract

Centralia, Pennsylvania is a town best known for the underground mine fire that started in 1962, which is expected to continue burning for another 100 years. The intense heat from this underground fire has caused a change in the ecosystem, specifically within the microbial communities that reside in the soil. The increased temperatures have provided a selective pressure for thermophilic actinomycetes, a bacteria species well known for its antibiotic production and resistance. Past metagenomic analyses have shown several novel genes of interest in the bacteria of these soil samples including two multi-drug transporter genes and an efflux pump gene used with arsenates. Soil samples taken in the Fall of 2019 have shown promise in being able to be cultured on actinomycete isolation agar at 50°C. In order to study these effects in greater detail, the proper growth conditions for these microbes must be determined in order to yield the most diverse novel species possible. A 1.5% phenol soil pre-treatment has shown great promise in eliminating common *Streptomyces* strains and yielding more diverse colonies based on their morphology. This pre-treatment showed morphologically different novel colonies similar to actinomycetes when compared to untreated soil samples. Using a soil pre-treatment in conjunction with defined media may provide an avenue for more novel species isolation which can lead to new antibiotic discovery in this age of antibiotic resistance.

Keywords: Actinomycetes, Centralia, Antibiotic Resistance

1. Introduction

The small town of Centralia, Pennsylvania appears abandoned and docile on the surface, but dig just a few feet below and you'll be met with a teeming world of everchanging microorganisms. Due to a devastating mine fire that started in 1962, and continues to burn to this day, Centralia has become a microbiological hotspot. The presence of fire has shown to disrupt usual microbial communities as well as the soil conditions they reside in.[6] Each microbial community is composed of complex processes, most of which fuel naturally occurring cycles: carbon, nitrogen, sulfur, etc. Changing environmental conditions pose a disruption to these systems and can impact the abundance and diversity of a microbial system.[7,9] Changing populations in soil microbes via fire are most evidently seen at surface levels and heat can cause an immediate change in bacterial populations.[10, 14] These changing populations can have an effect on competition in these communities and by proxy, the phenotypes these bacteria exhibit.[16]

In today's world, the looming threat of antibiotic resistance is becoming increasingly alarming. The CDC's most recent report shows that while infection rates have been declining since 2013, there are 2.8 million antibiotic resistant infections and over 35,000 related deaths each year.[11] Antibiotic resistance is a result of evolutionary adaptations, that are exacerbated by selective pressures.[12] "The extraordinary genetic capacities of microbes have benefitted from man's overuse of antibiotics to exploit every source of resistance genes and every means of horizontal gene transmission to develop multiple mechanisms of resistance for each and every antibiotic introduced into practice

clinically, agriculturally, or otherwise."[12] Many antibiotics that are still used in clinical practice today were derived from actinomycetes.[13]

Given Centralia's current soil conditions and the fact that actinomycetes are a common thermophilic soil bacteria,[14] Centralia may provide a unique avenue to culture novel actinomycetes that can lead to new antibiotics being produced, in an effort to combat the growing resistant infections. There also comes the idea that other cell management processes must be developed in order to continue living in an environment that continues to increase in toxicity.[5] Developing the proper growth conditions for these microbes is crucial in being able to maintain and study them. Typically, humic acid vitamin agar offers the best nutrient profile[4] while various pre-treatment to soil can restrict the growth of common bacteria strains.[15] Using either, or a combination, of these methods may allow more novel actinomycetes to be cultured in lab, opening the pathway for future metagenomic analyses.

2. Methods

3.1. Sample Collection

Soil samples were collected in the Fall of 2019 at various sites spread out across Centralia, PA. Samples were collected in 100mL tubes and immediately placed in an ice chest for transportation to the laboratory.

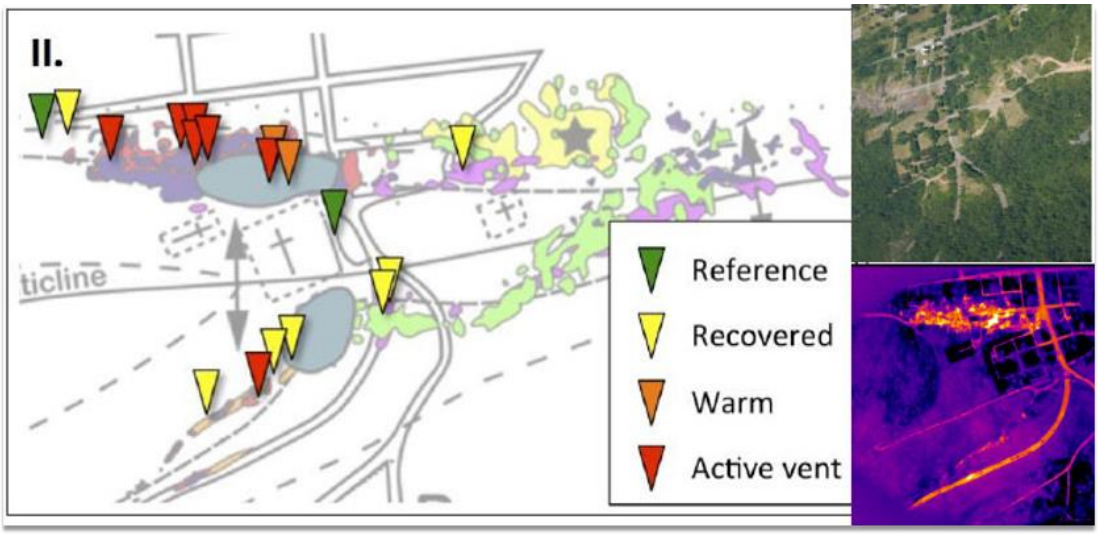

Figure 1. The above map shows the distribution of sample sites across Centralia with thermal imaging for heat reference. Reference locations were never affected by the fire and serve as a control. Recovered sites are sites that once were affected by the fire but are no longer. Warm and Active vent sites are both currently affected by the fire but at different temperatures, the latter being the hottest.

3.2. Plate Streaking

The plates used were made with commercial actinomycetes agar. Each plate contained approximately 30mL of agar. Soil supernatant was made by adding 1 gram of a soil sample into 10mL of sterile water; this was then vortexed for 5 seconds. 10uL of soil supernatant was pipetted onto the plates and the streak using aseptic technique with a sterile inoculating loop. The plates were then stored upside down in an incubator at 50°C. Each day the plates were checked for growth for up to 7 days. If after the seven days no growth was seen, the plates were discarded.

3.3. Phenol Pre-Treatment

A 1.5% phenol stock solution was prepared by diluting a 35% phenol solution with deionized water. 1 gram of each soil sample was placed into 10mL of the solution in a sterile 15mL tube, vortexed for 5 seconds, and incubated at 30°C

for 30 minutes. After the 30 minutes had passed, the samples were taken out of the incubator and 1mL of the supernatant was transferred to a clean 15mL tube along with 9mL of sterile water. This was vortexed for 5 seconds and then 10uL of the second supernatant was taken for streaking. This procedure was modeled after that used by M. Hayakawa.[15]

3.4. Colony Descriptions

Colonies' morphology was determined by describing pictures of them backlit by normal white light. The plates were photograph for reference. Each colony of interest is marked on the photographs with a corresponding number.

4. Results

Table 1. Tabulated colony descriptions of novel colonies selected from non-treated plates. Each colony was visually different per plate and has its morphology described below. Data is tabulated based on which soil sample was used to grow on the plate.

Soil Site:	Colony ID:	Colony Description:
Cen08	8.1	Rhizoid, filamentous, cream colored, slightly translucent
	8.2	Circular almost conjoined, endulate, cream colored
	8.3, 8.5	Circular, entire, cream colored
	8.4, 8.6	Irregular, endulate, Cream colored
	8.7	Filamentous, erose, white colored, slightly translucent
Cen10	10.1, 10.12	Filamentous, lobate, white colored
	10.2	Circular, undulate, white colored
	10.3	Irregular, entire, dark black center with transulucent body
	10.4, 10.10, 10.11	Irregular, undulate, cream colored
	10.5	Circular, entire, cream colored
	10.6, 10.7, 10.8, 10.9	Irregular, entire, dark ring around body, white colored
Cen13	13.1, 13.6	Filamentous, filiform, cream colored
	13.2	Rhizoid, lobate, white colored
	13.3, 13.4, 13.7	circular, entire, white colored
	13.5	Circular, entire, white colored, translucent with darker outer ring
	13.8	Irregular, filamentous, white colored, slihgtly translucent
	13.9	Irregular, undulate, cream colored
Cen14	14.1, 14.2	Irregular, undulate, white colored
	14.3	Circular, entire, cream colored
Cen15	15.1	Irregular, undulate, white colored, slightly darker spot
	15.2	Circular, entire, cream colored
	15.3	Irregular, undulate, cream colored with dark center
	15.4, 15.5, 15.6, 15.8	Circular, undulate, dark colored
	15.7	Irregular, undulate, cream colored with dark spots
Cen16	16.1	Irregular, entire, white colored
	16.2, 16.3	Circular, entire, cream colored
	16.4	Circular, filiform, cream colored
Cen17	17.1	Irregular, undulate, cream colored
	17.2	Circular, entire, white colored, slightly translucent
	17.3	Irregular, lobate, white colored, dark center
Cen19	19.1	Filamentous, filiform, cream colored, dark center
	19.2	Circular, enitre, dark colored, light center
	19.3, 19.6	Circular, enitre, dark colored, light center
	19.4	Irregular, undulate, dark colored, cream colored
	19.5	Circular, entire, cream colored, dark center
	19.7	Irregular, lombate, cream colored
Cen20	20.1, 20.2	Circular, entire, cream colored with dark outer ring
	20.3	Circular, entire, cream colored, aligned in a rod
Cen21	21.1, 21.2	Irregular, undulate, cream colored
	21.3	Circular, entire, cream colored with dark outer ring
	21.4	Circular, entire, white colored, translucent with darker outer ring
	21.5	Circular, entire, cream colored, dark center
	21.6	Circular, entire, dark outer ring, inside another colony
	21.7	Circular, entire, dark colored, inside another colony

Table 2. Tabulated colony descriptions of novel colonies selected from phenol-treated plates. Each colony was visually different per plate and has its morphology described below. Data is tabulated based on which soil sample was used to grow on the plate.

Soil Site:	Colony ID:	Colony Description:
Cen01	1.1	Circular, entire, dark colored
Cen03	3.1	Filamentous, cream colored, filiform, colony covers almost entire plate
Cen06	6.1, 6.2	Circular, undulate, white colored with dark center, clustered into group
Cen07	7.1	Irregular, undulate, white colored almost translucent with dark center
	7.2, 7.3	Circular, entire, dark colored
Cen08	8.1	Filamentous, cream colored, filiform, colony covers almost entire plate
Cen10	10.1	Circular, entire, dark colored with darker center
	10.2	Rhizoid, filiform, cream colored
	10.3, 10.4, 10.5	Irregular, entire, cream colored with dark center
	10.6, 10.7	Circular, entire, dark colored
Cen13	13.1	Filamentous, lobate, cream colored
	13.2	Irregular, undulate, cream colored with dark center
	13.3	Irregular, undulate, dark colored
	13.4	Rhizoid, cream colored, filiform
	13.5	Irregular, undulate, white colored with dark center
	13.6	Irregular, lobate, dark colored with white center
	13.7	Rhizoid, dark colored, filiform
	13.8, 13.9	Rhizoid, lobate, cream colored
	13.10, 13.12	Irregular, undulate, dark colored
	13.11	Circulaer, entire, white colored with dark ring

Soil Site:	Colony ID:	Colony Description:
Cen15	15.1	Rhizoid, filiform, cream colored
	15.2, 15.4	Irregular, undulate, dark colored with darker center
	15.3, 15.5,	Circular, entire, dark colored with white center
	15.7	Circular, entire, white colored with dark center
	15.8, 15.9	Circular, entire, cream colored
Cen16	16.1, 16.2, 16.5	Irregular, entire, cream colored with dark center
	16.3	Filamentous, filiform, cream colored
	16.4	Circular, entire, white colored tansparent
Cen19	19.1	Filamentous, filiform, cream colored
	19.3, 19.4, 19.5, 19.6,	Irregular, undulate, cream colored with dark center
Cen20	20.1, 20.3	Circular, entire, dark colored
	20.2	Filamentous, filiform, dark colored with slightly darker center
	20.4	Circular, entire, white colored, slightly transparent
Cen21	21.1, 21.7	Circular, entire, cream colored
	21.2	Rhizoid, filiform, white colored slightly transparent
	21.3, 21.6	Circular, undulate, white colored with a dark central ring
	21.4	Irregular, filiform, cream colored
	21.5	Irregular, undulate, dark colored
	21.8	Irregular, undulate, white colored with a dark central ring and outer edge
	21.9, 21.10	Irregular, undulate, cream colored with dark center
Cen22	22.1	Irregular, undulate, white colored with dark center
	22.2, 22.3, 22.4	Irregular, undulate, dark colored

Table 3. This table compiles the soil samples, their air temperature, and their fire classification. Soil temperatures were taken between 10/3 and 10/4 of 2018. Fire Classification references Figure 1 above.

Soil Site:	Soil Temperature (Celsius):	Fire Classification:
Cen01	20.7	Recovered
Cen02	20.03	Recovered
Cen03	16.9	Recovered
Cen04	17.8	Recovered
Cen05	20.7	Recovered
Cen06	20.02	Fire Affected
Cen07	16.4	Recovered
Cen08	20.5	Reference
Cen09	18.9	Fire Affected
Cen10	21.8	Fire Affected
Cen11	17.7	Fire Affected
Cen12	17.5	Fire Affected
Cen13	16.6	Fire Affected
Cen14	17.2	Fire Affected
Cen15	22.5	Fire Affected
Cen16	21.2	Fire Affected
Cen17	18.3	Reference
Cen18	19.7	Recovered
Cen19	24	Fire Affected
Cen20	17.1	Fire Affected
Cen21	23.1	Fire Affected
Cen22	17.3	Fire Affected
Cen23	19.9	Reference

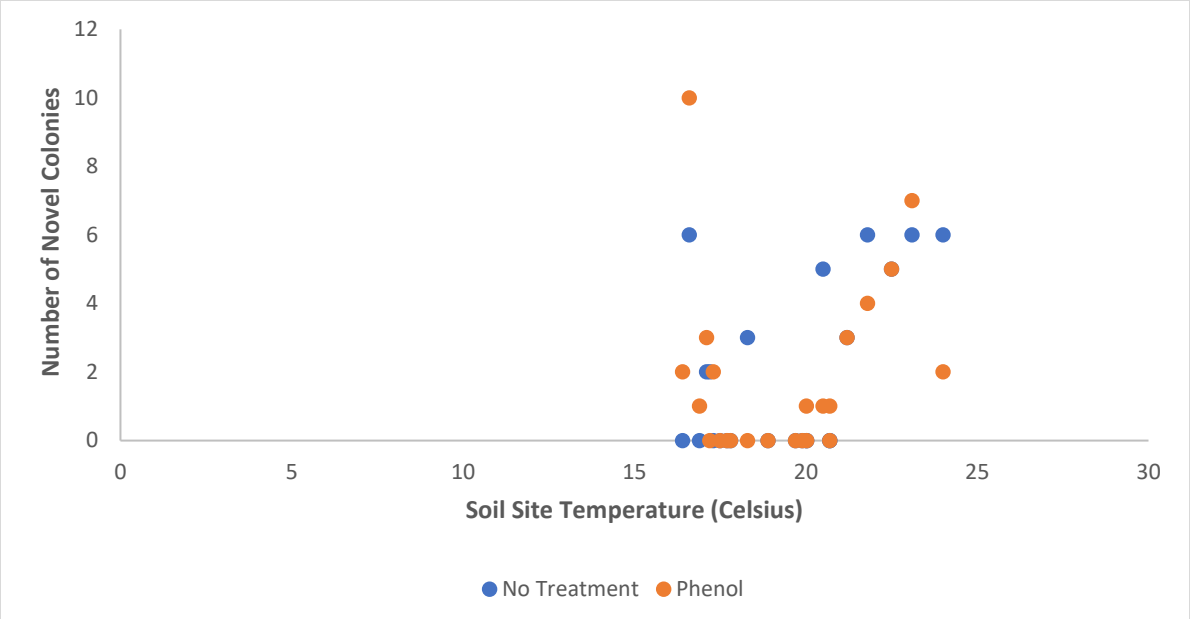

Figure 2. The figure above illustrates the relationship between soil temperature, treatment type, and the number of novel colonies visualized. Not all soil samples are shown here due to growth not being seen from each sample. If a sample did not yield any growth then the colony number is designated to be 0. R^2 value of "No Treatment" = 0.2652 R^2 value of "Phenol" = 0.0297.

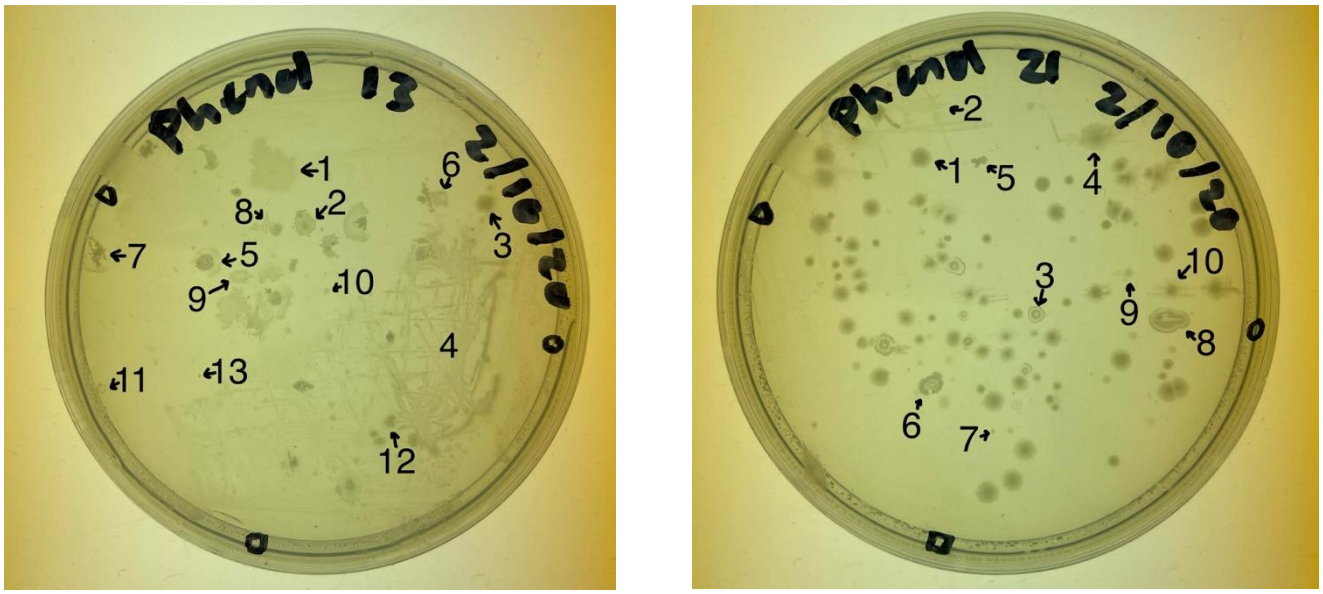

Figure 3. These photos show two of the phenol treated soil growth plates. Each type of colony morphology described in table 2 above can be seen on one of these plates. Each novel colony is marked with its corresponding number. The various shapes drawn on the plates are simply there to ensure correct plate orientation.

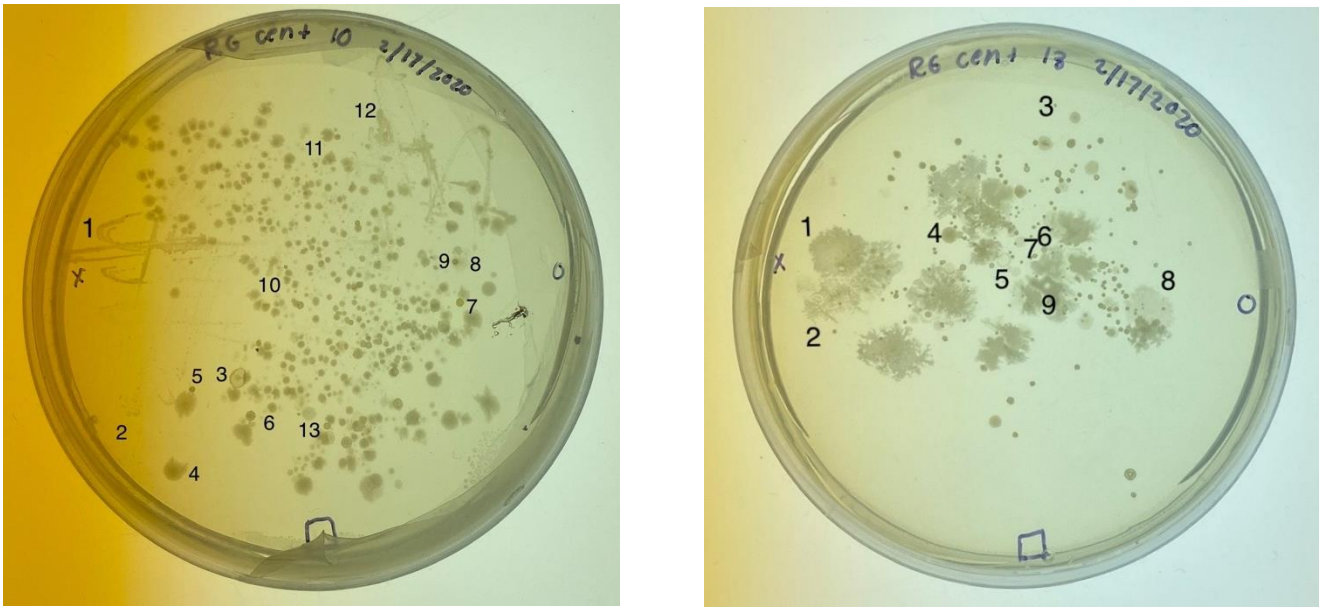

Figure 4. These photos show two of the untreated soil growth plates. Each type of colony morphology described in table 1 above can be seen on one of these plates. Each novel colony is marked with its corresponding number. The various shapes drawn on the plate, are simply there to ensure correct plate orientation.

5. Discussion

5.1. Morphology Analysis

Based on the morphology of the colonies that grew we can see several things. First, is that pre-treating the soil does have an impact on the growth of the microbes. It actually showed that pre-treating the soil resulted in three more soil samples being able to grow up on a culturing plate. Secondly, it shows that there is an effect on microbial diversity when talking about pre-treatments and media types. Looking at Tables 1 and 2 above, it appears as though the non-treated soil plates grew more diverse species because there are more different colony morphologies than in the ones pre-treated with phenol. However, looking at the morphology descriptions of the phenol plates, you will see that the descriptions closely match those associated with actinomycetes, while the descriptions from plate not treated with phenol don't as heavily.

This can mean one of several things; the phenol pre-treatment helps to mimic the soil conditions that actinomycetes are most respondent to or, the pre-treatment eliminates and kills off more commonly seen strains of bacteria which allows less competition for the actinomycetes to grow. We believe it to be a combination of the two, with more emphasis on the latter. What this means is that if soils are pre-treated with a 1.5% phenol solution, we can expect to see different strains than what's typically seen in the sample's plates which gives new avenues for metagenomic analysis. The phenol solution typically kills off most major *Streptomyces* species[15]—something we believe to be observed here.

5.2. Soil Temperature Effects

Figure 2 above looks at the relationship between soil temperature and the number of novel colonies cultured from each sample. The graph illustrates that there are more novel colonies at higher temperatures which becomes magnified with a soil pre-treatment. It's also apparent that between 20-25^{0}C is when we see the highest number of novel colonies in both the pre-treated and untreated soil samples. This can show that the higher temperatures are having an effect on

the microbe population by eliminating competition as well as providing more adequate environments for these thermophilic actinomycetes.

5.3. Limitations and Sources of Error

This study has a few limitations. First and foremost, the original aim of this project was to not only determine the best growth conditions for novel colonies but also to further identify those colonies using metagenomic analyses and determining whether these new growth conditions actually aided in novel colony development. Unfortunately, the second half of the project was unable to be completed due to the effects of COVID-19. As such, the project was not able to be finished and the conclusions drawn from this study were made using only available preliminary data.

Additionally, a second agar type was used, humic acid vitamin (HVA) agar, to see if this also offered another avenue to designing more favorable conditions for these microbes. Due to the constraints and time of the project, this path was not fully studied as no colonies grew on the media during testing before the project was suspended due to COVID-19. This is most likely due to the improper mixing of the agar as it was not bought commercially. The agar recipe used was referenced from M. Hayakawa.[4]

Lastly, the figures above depicting the bacterial plates are not showing fully isolated colonies but rather initial growth plates from the soil directly. While isolation plates were streaked and grown up in the incubator there was no access to them at this time to photograph and analyze them for more accurate novel colony determination. The photos above may show colonies that are growing together and may not necessarily represent one specific species by itself.

5.4. Future Directions

Expanding upon this research, there are several different avenues that could be explored. Fully isolating the novel colonies and having their DNA sequenced would offer insight into life inside these soil sites as well as show if there are any novel genes of interest being expressed that typically are not. This study would also benefit from a wider variety of pre-treatments and agar types. This study only looked at a 1.5% phenol soil pre-treatment before inoculating onto growth plates, however there are several other types of pre-treatments and media types that could yield similar of better results.[3, 2] This study could also be expanded to other areas with similar soil temperature conditions either organically or synthetically made. This would give insight as to whether the data shown here is specific to this area or something that is experienced on a much larger scale. There has also been successful Actinomycete isolations in marine environements,[1] so employing these techniques in various environments may present a more realistic avenue to being able to study these microbes around the world, and not just in one environment type.

6. Conclusion

Using a 1.5% phenol pretreatment yielded less morphologically different bacterial colonies but more novel colonies as compared to the untreated soil samples. Higher soil temperatures correlate with more novel colony development in Centralia.

7. Acknowledgements

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