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The Effects of Growth Conditions on Bacterial Inhibition of Water Mold

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Abstract

Amphibian populations have been declining over the past 40 years in part due to water mold infection. Interestingly, various bacterial species can inhibit water mold growth *in vitro*. Understanding the mechanisms underlying bacterial inhibition of water mold can possibly provide applications in nature to stop amphibian population declines. The Peterson laboratory studies the mechanisms underlying bacterial inhibition of water mold. Previous researchers hypothesized that pH changes could be important for this inhibition. The research presented here focused on the effects that growth conditions (pH, energy sources, and glucose concentrations) have on the ability of bacteria to inhibit water mold growth. It was found that water mold by itself is inhibited at basic pH and that bacteria create alkaline and acidic environments when given protein and carbohydrate media, respectively. Additionally, bacteria can inhibit water mold when given protein but not when given carbohydrate as the primary energy source. Bacteria have very weak growth at high concentrations of glucose and this seemed to affect their ability to inhibit water mold. Bacteria's ability grow substantially should be further studied to determine its importance for inhibition of water mold.

Keywords: Water mold, Bacteria, Inhibition

1. Introduction

Amphibian populations have been declining since as early as 1970. For a long time, the causes of these amphibian population declines were unknown. Many frog species were among the amphibians most affected. Because the causes of these population declines were unknown, no conservation remedies or intervention methods were created. With a lack of conservation remedies and intervention methods, many of these amphibian species now face extinction[13]. More recently, it was found that infectious diseases play an important role in these population declines. Such infectious diseases reduce biodiversity, cause mortality, and ultimately result in extinction for some amphibian species[8]. *Saprolegnia* species are known to be pathogenic for vertebrates such as fish[1] and invertebrates such as crayfish[9]. Specifically, infection by *Saprolegnia* species kills amphibian larvae and egg masses. *Saprolegnia* species are one type of water mold[10,11]. The term "water mold" is somewhat of a misnomer. Although it has fungus-like characteristics, this pathogen is actually a protist[4]; it is eukaryotic, not prokaryotic. This factor adds to the complexity and difficulty of treating and preventing water mold infection. This protist can be found in many soil and aquatic environments[4]. Interestingly, it has been shown that certain bacterial species are able to inhibit *Saprolegnia* growth in the laboratory[6]. Stable bacterial communities can live on egg masses. Egg masses can cooperate with their bacterial communities to form mutually beneficial symbiotic relationships[12]. Therefore, it is conceivable that this bacterial inhibition of water mold seen in the laboratory also happens on egg masses *in vivo*. This is important because if the mechanisms underlying this bacterial inhibition of water mold in the laboratory can be understood, it is possible that this mechanism can have applications in nature for stopping amphibian population declines.

The Peterson laboratory studies the mechanisms underlying this bacterial inhibition of water mold using a water mold species obtained from a pond on the North Central College campus. Although this species has not yet been sequenced, it is believed to be of the genus *Saprolegnia* because this water mold is commonly found in this body of water. Previously, researchers in the Peterson laboratory found that diverse species of bacteria were able to inhibit water mold growth on the protein-rich Luria-Bertani (LB) medium. They found that these bacterial species inhibit water mold by secreting an unidentified molecule; it was hypothesized that this unidentified molecule causes the inhibition by changing the pH and creating an alkaline environment (unpublished data). It is known that bacteria secrete metabolites that can alter their environment[2]. Therefore, the metabolites produced by bacterial protein or carbohydrate metabolism should alter the environment. Specifically, given the well-known products of carbohydrate metabolism (carbon dioxide) and protein metabolism (ammonia), it makes sense that the medium or energy source provided for bacteria should determine the pH of the environment. Accordingly, the research presented here focused on the effects that growth conditions have on the ability of bacteria to inhibit water mold growth. Specifically, the researchers studied the effects that pH changes have on the ability of water mold to grow and the effects that alerting energy sources and glucose concentrations have on bacterial inhibition of water mold.

2. Methods and Materials

2.1 Media:

A variety of media was utilized in conducting this research. Bacteria were grown on Difco™ LB Broth, Miller (Luria-Bertani) medium for general maintenance of cultures. Water mold was grown on either Difco™ R2A Agar, a nutrient poor medium, or cornmeal medium for general maintenance of cultures. To make cornmeal medium, 20 grams of cornmeal was steeped for an hour in one liter of deionized water. Cornmeal was filtered out and 15 grams of agar was added per one liter of water. Plugs from these water mold cultures were removed using a metal spatula, a 0.6 cm diameter glass tube, and sterile technique. All plugs were placed on new agar plates with the water mold growth side facing down. The R2A medium was used both by itself and with various percentages of glucose and tryptone. For pH analysis, appropriate buffer was added to the LB medium.

2.2 Protocol For Typical Inhibition Assays:

Using a sterile cotton swab, a ring of bacteria (either *Bacillus anthracis*, *Aeromonas jandaei*, or *Escherichia coli*) from an overnight LB broth culture was spread around the outer edge of an 8 cm diameter agar plate containing the desired media. The bacteria were spread in a ring only one time around the plate to prevent overgrowth. This plate containing the bacteria was incubated for 12-16 hours at 37° C. A 0.6 cm water mold plug was added to the center of the plate on the following day. This plate containing bacteria and water mold was left at room temperature for three days. The diameter of the water mold growth was measured daily. To measure water mold growth in a consistent manner, the diameter was measured in one spot, the plate was rotated 180°, and the diameter was measured in another spot. These two diameter measurements were averaged. Both spots were marked so that measurement could be consistent from day to day. Two or three replicates were done for each assay.

2.3 Determining Water Mold Inhibition at Varying pH Levels:

A 0.6 cm water mold plug was plated without bacteria on LB agar made with 0.1 M buffer and with the pH adjusted to 5, 6, 7, 8, or 9. Homo-PIPES buffer (pKa 4.6) was used for pH 5, PIPES (pKa 6.8) buffer was used for pH 6, HEPES (pKa 7.5) buffer was used for pH 7 and pH 8, and AMPSO (pKa 9.0) buffer was used for pH 9. Plates were left at room temperature for three days and water mold growth diameter was measured daily. Water mold growth diameter was measured as described in section 2.2. Five replicates were done for each pH level.

2.4 Testing The pH of Agar Plates:

Any time the pH of an agar plate was measured, a small strip of pH paper was placed on a clear zone of the plate (a spot with no bacteria or water mold growth). After it was added to the plate, the pH paper was mositened with distilled water from a spray bottle. The distilled water was verified to have a neutral pH. When the pH paper became

wet, it changed color according to the pH of the plate. The color of the pH paper was compared to the key to determine the pH of the plate.

3. Results

To study the effects of growth conditions on bacterial inhibition of water mold, the effect of pH on water mold inhibition was studied. To determine if changes in pH lead to inhibition of water mold, water mold was grown without bacteria on LB plates buffered at pH 5, 6, 7, 8, and 9 at room temperature for three days. LB media by itself (without HEPES, water mold, or bacterial growth) was found to have a neutral pH, so it was expected that water mold would experience no inhibition at pH 7. The diameter of the water mold growth was measured daily (Fig. 1).

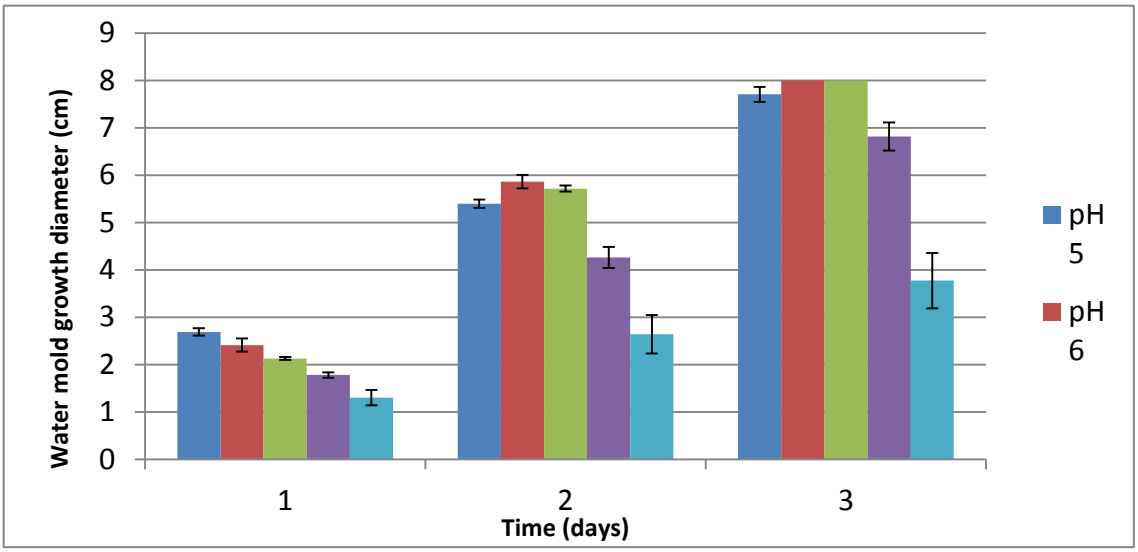

Figure 1: Water mold is inhibited at various pH levels

Figure 1: Water mold was grown without bacteria on LB buffered at pH 5, 6, 7, 8, and 9 (n=5 at each pH). Plates were left at room temperature for three days. Water mold growth diameter was measured daily. Water mold growth was significantly inhibited at basic pH levels (two-tailed t-test, unequal variances, $p<0.01$). Error bars represent standard deviation.

The Y-axis displays the diameter of the water mold growth measured in centimeters and the X-axis axis displays the number of days that water mold was allowed to grow. After the first day, the water mold growth diameter was similar at each of the different pH levels. Only one day of growth was too early for any trends to emerge. By the second day, however, growth patterns began to appear. On day two water mold growth at pH 5, 6, and 7 was greater than 5 cm in diameter while growth at pH 8 and 9 was almost 4 cm and and less than 3 cm, respectively. By the third day, water mold at pH 6 and 7 grew to 8 cm, which was the full diameter of the plate. This indicated that water mold growth was uninhibted at these pH levels. At pH 5, water mold growth was slightly less than 8 cm. At pH 8 and 9, water mold growth was significantly ($p<0.01$) less than at pH 6 and 7; growth reached less than 7 cm and less than 4 cm at pH 8 and pH 9, respectively. Five trials were performed at each pH level; error bars are notably small. Overall, these results indicate that water mold growth is inhibited by extreme changes in pH; specifically, water mold growth is most inhibited at basic pH.

After determining that water mold is inhibited by changes in pH, the ability of bacteria to alter the pH of the environment was studied. Specifically, the researchers wanted to know if altering the primary energy source in the media would change bacterial secretions and ultimately change the pH of the environment. It was hypothesized that bacteria would produce carbon dioxide and create an acidic environment when given carbohydrate as the primary energy source and that bacteria would produce ammonia and create a basic environment when given protein as the primary energy source. To test this, *B. anthracis*, *E. coli*, and *A. jandaei* were grown on a variety of media. These three species were chosen because they are fairly different from one another: *B. anthracis* is Gram-positive and

spore-forming, *E. coli* is Gram-negative, and *A. jandaei* was isolated from a frog egg mass. All can be found in ponds where frogs breed. Determining if an effect is observed over a variety of diverse species was desired. These bacterial species were grown on six different types of media: LB, R2A alone, and R2A with 1% glucose, 2% glucose, 1% tryptone, or 2% tryptone. Media with added glucose ensured that bacteria received carbohydrate as the primary energy source. LB medium and media with added tryptone ensured that bacteria received protein as the primary energy source. As a nutrient poor medium, R2A ensured that bacteria received neither carbohydrate nor protein as the primary energy source; this media acted as somewhat of a control and was expected to result in a neutral environment. After three days of bacterial growth, the pH of the plate was determined (Fig. 2).

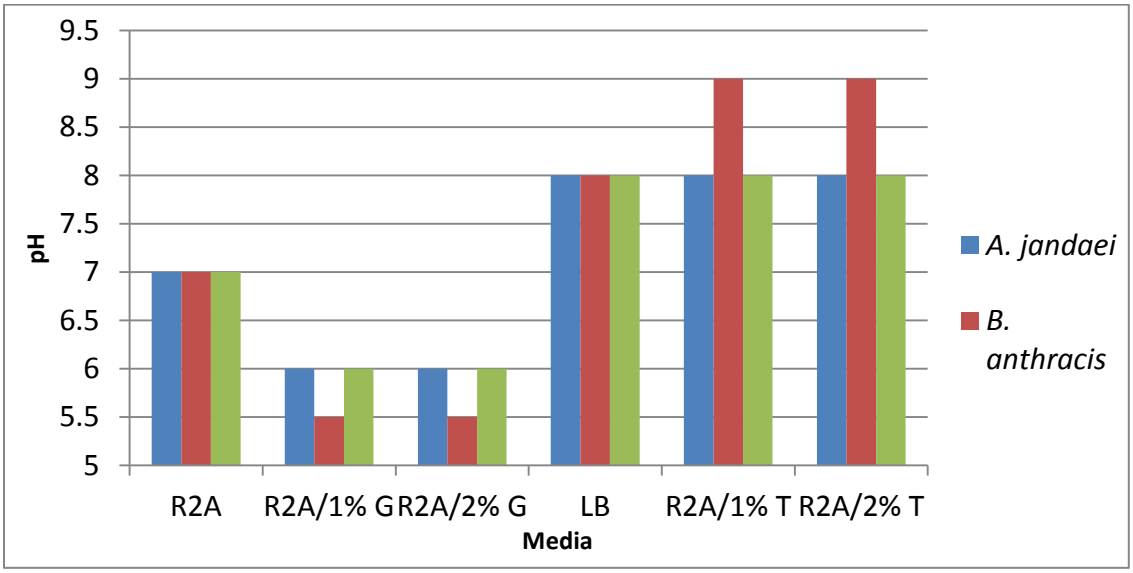

Figure 2: Altering media changes the pH of the environment

Figure 2: Bacteria were grown alone on R2A, 1% glucose, 2% glucose, LB,1% tryptone, and 2% tryptone media (n=3 for each). Plates were left at 37° C for three days. pH of the environment was tested after three days. Bacteria created basic and acidic environments on protein and carbohydrate media, respectively. The standard deviation was zero; no statistical analysis could be performed. The standard deviation was always zero because pH paper was used as the testing method. pH paper involves qualitative analysis (observing color changes) and is not very sensitive.

The Y-axis displays the pH of the plate after three days of bacterial growth and the X-axis axis displays the different types of media bacteria was grown on. All three bacterial species created a neutral environment (pH 7) on the nutrient poor R2A medium. When given glucose as the primary energy source (1% and 2% glucose), *B. anthracis* created an acidic environment of pH 5.5 while *E. coli* and *A. jandaei* created acidic environments of pH 6. When given protein as the primary energy source (LB and 1% and 2% tryptone), *B. anthracis* created a basic environment of pH 9 while *E. coli* and *A. jandaei* created basic environments of pH 8. Notably, *B. anthracis* seemed to grow more substantially than either *E. coli* or *A. jandaei* on all types of media. Three trials were done for each medium for each bacterial species. Overall, these results indicate that altering the primary energy source in the media changes bacterial secretions and ultimately changes the pH of the environment. Specifically, when bacteria are given carbohydrate as the primary energy source they presumably produce carbon dioxide and create an acidic environment. When bacteria are given protein as the primary energy source they presumably produce ammonia and create a basic environment.

After determining that bacteria create a basic environment when given protein as the primary energy source and an acidic environment when given carbohydrate as the primary energy source, the effect of primary energy source on bacterial inhibition of water mold was studied. Specifically, the difference in bacterial inhibition of water mold on glucose media versus protein media was of interest. To test this, typical inhibition assays using *B. anthracis* and water mold were staged on a variety of media. Only *B. anthracis* was used for these inhibition assays because it created the most acidic and the most basic environments; therefore, it was expected that this bacterial species would

result in the greatest water mold inhibition. These inhibition assays were performed on five different types of media: LB and R2A with 1% glucose, 2% glucose, 1% tryptone, or 2% tryptone. As negative controls, water mold was grown without bacteria on each of the different types of media described to compare growth on different media without bacterial inhibition. The diameter of the water mold growth was measured daily (Fig. 3)

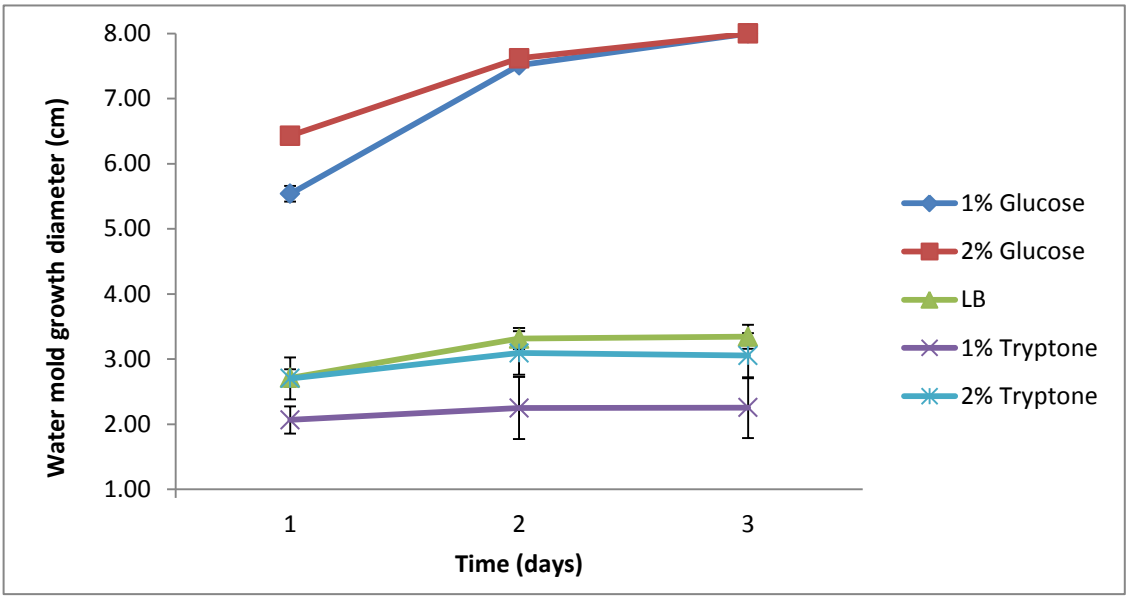

Figure 3: Altering media changes bacterial inhibition of water mold

Figure 3: Bacteria and water mold were grown together in typical inhibition assays on LB and R2A with 1% glucose, 2% glucose, 1% tryptone, or 2% tryptone (n=3 for each). Bacteria and water mold were grown together at room temperature for three days. Water mold growth diameter was measured daily. Bacteria inhibited water mold on protein-rich media (two-tailed t-test, unequal variances, $P<0.01$). Error bars represent standard deviation.

The Y-axis displays the diameter of the water mold growth measured in centimeters and the X-axis axis displays the number of days that water mold was allowed to grow. When grown without bacteria on 1% added glucose, 2% added glucose, LB, 1% added tryptone, and 2% added tryptone plates, water mold growth reached 8 cm in diameter (the full diameter of the plate) by day three. This indicated that water mold was uninhibited on these different types of media when bacteria were absent. It is important to note that water mold grew well on all different types of media, but it really thrived on media with added glucose. Water mold grew quickly and showed white, fluffy overgrowth when given carbohydrate as the primary energy source (data not shown). In contrast, with *B. anthracis* present, there was a clear difference between water mold growth on protein inhibition assays and water mold growth on glucose inhibition assays. Water mold growth diameters on 1% and 2% glucose inhibition assays were greater than 5 cm and 6 cm, respectively. Water mold growth diameters on LB and 1% and 2% tryptone inhibition assays were all between 2 cm and 3 cm. By day two, water mold growth diameters on 1% and 2% glucose inhibition assays were nearly 8 cm; at this point, the water mold grew up to the place where bacteria were streaked. Water mold growth diameters on LB and 2% tryptone inhibition assays were around 3 cm while the diameter on 1% tryptone inhibition assay was less than 2 cm. Finally, by day three water mold growth diameters on 1% and 2% glucose inhibition assays reached 8cm, the full diameter of the plate, growing over the bacteria. This indicated that bacteria were unable to inhibit water mold when given carbohydrate as the primary energy source. On day three, water mold growth on LB and 1% and 2% tryptone inhibition assays was almost the same as it was day two, indicating that water mold had been nearly completely inhibited. Water mold growth was significantly ($p<0.01$) less on these three protein inhibition assays than on the uninhibited controls. It is important to note that bacterial growth looked rather weak (spotty, sparse) on glucose media and very substantial (thick, dense) on protein media. Three trials were performed for each of the different types of media; error bars represent standard deviation and are notably small. Overall, these results indicate that bacteria can inhibit water mold when given protein as the primary energy source but bacteria cannot inhibit water mold growth when given carbohydrate as the primary energy source.

It was determined that bacteria inhibit water mold when given protein as the primary energy source but not when given carbohydrate as the primary energy source. At the same time, it was noticed that bacteria have very weak growth when given carbohydrate as the primary energy source and very substantial growth when given protein as the primary energy source. Glucose seemed to inhibit bacterial growth. Therefore, it was hypothesized that bacteria's ability to inhibit water mold growth depends on their ability to produce substantial growth. In other words, it was hypothesized that bacteria would inhibit water mold best at low concentrations of glucose because this is when bacteria should grow best. To test this, typical inhibition assays using *B. anthracis* and water mold were staged on media with varying concentrations of glucose. Only *B. anthracis* was used for these inhibition assays because it was the only species used for inhibition assays on varying energy sources (Fig. 3). These inhibition assays were performed on media that had 1% tryptone plus one of four concentrations of glucose: 0.125%, 0.25%, 0.5%, or 1%. These plates are referred to by their concentration of glucose only because they all had 1% tryptone as well. As controls, water mold was grown without bacteria on each of the four different types of media described where it was expected that water mold growth would be uninhibited. The diameter of water mold growth was measured daily (Fig. 4).

The Y-axis displays the diameter of the water mold growth measured in centimeters and the X-axis axis displays the number of days that water mold was allowed to grow. When grown without bacteria on 0.125%, 0.25%, 0.5%, and 1% glucose plates, water mold growth reached 8 cm in diameter, which was the full diameter of the plate. This indicated that water mold was completely uninhibited on these different types of media when bacteria were absent (data not shown). For the inhibition assays, the water mold growth diameter was almost the same at each concentration of glucose on the first day. However, water mold growth diameters on 1% and 0.5% glucose inhibition assays were slightly greater than diameters on 0.25% and 0.125% glucose inhibition assays. On day two, water mold growth diameters on 1% and 0.5% glucose inhibition assays were less than 8 cm while diameters on 0.25% and 0.125% glucose inhibition assays were about 6 cm and 5 cm, respectively.

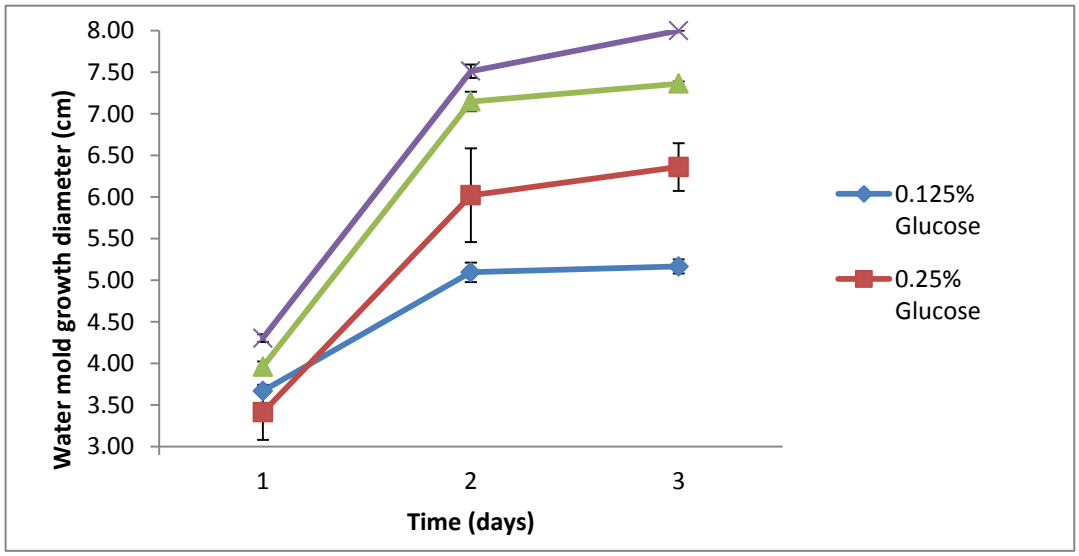

Figure 4: Altering glucose concentration changes bacterial inbhition of water mold

Figure 4: Bacteria and water mold were grown together in typical inhibition assays on media with 1% tryptone and 0.125% glucose (n=3), 0.25% glucose (n=3), 0.5% glucose (n=2), and 1% glucose (n=2). Bacteria and water mold were grown together at room temperature for three days. Water mold growth diameter was measured daily. Bacteria inhibited water mold growth best at the lowest concentration of glucose (two-tailed t-test, unequal variances, $P<0.01$). Error bars represent standard deviation.

By day three the water mold growth diameter on the 1% glucose inhibition assay reached 8cm, the full diameter of the plate; at this point, the water mold was covering the bacteria. The diameter on the 0.5% glucose inhibition assay was 7.3 cm, significantly ($p<0.05$) less than the uninhibited control. The diameters on the 0.25% and 0.125% glucose inhibition assays were roughly the same as they were on day two, significantly less than the uninhibited

controls ($p<0.05$ and $p<0.01$, respectively). Three trials were performed for inhibition assays on 1% and 0.5% glucose. Only two trials were performed for inhibition assays on 0.25% glucose and 0.125% glucose because one trial for each became containinated. Error bars represent standard deviation and are notably small. It is important to note that, as expected, bacterial growth was the most substantial at 0.125% glucose and weakest at 1% glucose. Overall, these results indicate that increasing the concentration of glucose in the media impacts the bacteria's ability to inhibit water mold growth. This likely occurs because bacteria does not grow substantially at high concentrations of glucose.

4. Discussion

Water molds such as *Saprolegnia* species infect amphibian egg masses and are partially responsible for the recent amphibian population declines. Interestingly, this pathogen can be inhibited by various species of bacteria *in vitro*. Bacteria act to inhibit water mold at a distance, so they must inhibit by secretion. The current study attempted to analyze the mechanisms underlying bacterial inhibition of water mold. Firstly, it was found in this study that water mold is in fact inhibited at basic pH levels; specifically, water mold is significantly inhibited at pH 8 and pH 9. We did not notice any macro-morphological changes in the water mold but it is possible that there are microscopic differences such as changes in zoospore release or formation. Admittedly, water mold growth is slightly inhibited at pH 5; however, this inhibition is not nearly as great as the inhibition at basic pH levels. This supports the hypothesis that pH changes can contribute to bacterial inhibition of water mold. In other words, because water mold growth is inhibited at increased pH levels, it is possible that this is one mechanism underlying bacterial inhibition of water mold. More specifically, bacteria's ability to create a basic environment could contribute to their ability to inhibit water mold growth. Because water mold growth was not greatly inhibited at acidic pH levels, bacteria's ability to create an acidic environment does not seem likely to contribute to their ability to inhibit water mold growth. It was also found that when bacteria are given carbohydrate or protein as the primary energy source, they create an acidic or basic environment, respectively. We hypothesize this is due to carbon dioxide (an acidic molecule) and ammonia (a basic molecule); these are metabolic waste products of carbohydrate and protein metabolism, respectively[5]. Notably, *B. anthracis* produced more basic and more acidic environments than either *E. coli* or *A. jandaei*. This likely occurred because *B. anthracis* grew more substantially than *E. coli* and *A. jandaei* on all different types of media and, therefore, produced more secretions.

Because bacteria change the pH of the environment based on the primary energy source consumed, it is possible that energy source contributes to bacterial inhibition of water mold. More specifically, because water mold is inhibited at basic pH, the availability of protein in the environment should allow bacteria to inhibit water mold. Indeed, this is what was found. When given protein as the primary energy source, bacteria were able to inhibit water mold growth; when given carbohydrate as the primary energy source, they were unable to do so. This makes sense in terms of what happens *in vivo*. When bacterial inhibition of water mold happens in nature, it likely happens directly on amphibian egg masses. Amphibian egg masses are largely protein[3], so bacteria receive protein as the primary energy source and, therefore, should be able to inhibit water mold. Moreover, because bacteria can only inhibit water mold growth on protein-rich media and not on carbohydrate-rich media, it seems likely that one of the products of protein metabolism such as ammonia or urea could be at least partially responsible for this inhibition. Additionally, it was noticed that bacteria had weak, spotty growth on carbohydrate-rich media and thick, substantial growth on protein-rich media. Glucose seemed to inhibit bacterial growth and water mold inhibition was weakest on this media. Glucose likely inhibited bacterial growth on this media by creating a hypertonic environment. When grown on media with 1% tryptone and either 0.125%, 0.25%, 0.5%, or 1% glucose, bacteria inhibited water mold best at 0.125% glucose and worst at 1% glucose. Bacterial growth was weakest at 1% glucose and most substantial at 0.125% glucose. This suggests that bacteria have to grow substantially in order to inhibit water mold and the effect is titratable. If bacteria are unable to grow substantially, they are probably unable to produce a significant amount of inhibitory secretions.

Overall, from this study the researchers found that, although pH of the environment seems to be an important aspect of bacterial inhibition of water mold, the bacteria's ability to grow seems to be an important factor as well. Interestingly, the primary energy source in the media seems to affect both pH of the environment and the bacteria's ability to grow. Possibly, a combination of energy source, pH, and substantial bacterial growth set the stage for water mold inhibition. Therefore, water mold inhibition should be further studied with respect to bacterial protein and carbohydrate metabolism. GC-MS and/or LC-MS would be useful tools for analyzing such metabolites. In addition to studying how bacteria's ability to grow affects bacterial inhibition of water mold, it would also be

beneficial to study how water mold's ability to grow affects this phenomenon. It was found that water mold grew extremely well when given carbohydrate as the primary energy source; bacteria did not grow well when given glucose. Accordingly, bacteria were unable to inhibit water mold on this media. Therefore, it is possible that bacterial inhibition of water mold does not occur on carbohydrate media due to a combination of water mold overgrowth and bacterial growth inhibition. In other words, the ability of water mold to grow may be just as important as the ability of bacteria to grow. Further studies should be conducted to look into this possibility. Finally, although *B. anthracis* was used for all inhibition assays performed in this study, the efficacy of *Aeromonas* species to inhibit water mold should be further addressed as well. The Peterson laboratory has a frozen stock of *Aeromonas jandaei* that was isolated from a frog. The inhibition of water mold by this species should be addressed because one study[7] found that an *Aeromonas* strain had antagonistic activity of *Saprolegnia* and helped reverse saprolegniosis in infected fish. Inhibition assays where energy sources and glucose concentrations are varied should be conducted with *A. jandaei*. Although the Peterson laboratory has some preliminary results using this species, more needs to be done in order to address its ability to inhibit water mold growth compared to *B. anthracis*. It is important to determine any differences because *B. anthracis* is a laboratory strain while *A. jandaei* was isolated from a frog. Studies with the latter would more closely resemble the bacterial inhibition of water mold that would actually be observed *in vivo*.

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