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Aeromonas Phage Research and the Public Health Impact of Antibiotics in Aquaculture Workers

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AEROMONAS PHAGE RESEARCH AND THE PUBLIC HEALTH IMPACT OF ANTIBIOTICS IN AQUACULTURE WORKERS

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HONORS PROJECT

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Introduction:

History has shown to repeat itself in public health with workers being the guinea pigs for diseases. One early example includes scrotal cancer among young British chimney sweeps first noted in 1775. During this time boys were sent up naked and the soot from the chimney would lodge in the scrotum. There were preventative measures taken; however, “it took more than 200 years before other aspects of the health of chimney sweeps were systematically studied and reported” (Hogstedt, et al). It took hundreds of years before anyone did anything about this public health issue. Another prime example of this includes radium poisoning from watch workers who painted watch faces with radium. At the time, radium was seen as a type of magic medicine that could cure anything. There were warning signs as seen by the scientists losing fingers and getting terrible burns. The effects of radium were not studied and kept from the public. Many young women who worked in the factory lost teeth, developed cancer, had amputations and developed osteoporosis. It took several decades before any major changes were made and factories were shut down. A more recent example of this is popcorn lung where it was shown that artificial butter flavorings that are added to microwave popcorn were responsible for “severe respiratory impairment and obliterative bronchiolitis, a serious lung disease that is irreversible”(“CDC flavorings-related lung disease”). There was an alert made about the hazards of these chemicals, but there is still a lot unknown about the effects of these chemicals on human health. All of these examples provide us insight into how workers are usually the first to be exposed to harmful diseases. This might be the case for aquaculture workers.

There have already been signs of aquaculture workers getting infectious diseases from the working conditions. When workers come into contact with waste water from aquaculture ponds they are at an “increased risk of acquiring diarrheal diseases (DO eat al., 2007), skin diseases (van der Hoek et al., 2005) and liver fluke infections (Scripa et al., 2007)(Klase et al.). It has been shown that antibiotic resistant bacteria can make these infections more likely to happen. My project involves finding an alternative to antibiotic use such as bacteriophage. Bacteriophage live in the human gut microbiome and are harmless to humans. Recently, there have been new bacteriophage products used to control pathogenic bacteria in aquaculture that are “safe, simple and effective commercial phage products to target and destroy pathogenic bacteria (for instance, CUSTUS®YRS for *Yersinia ruckeri* control, LUXON for *Vibrio parahaemolyticus* control”) (Liu et al.). As seen there are products on the market that have shown to treat bacterial infections in fish using bacteriophage that are great alternatives to using antibiotics. Hopefully, if we catch this issue early on we can avoid the aquaculture workers getting sick in the first place.

Aquaculture is the controlled breeding and harvesting of marine animals such as fish, shellfish, and algae. There has been an increase in the human population which in turn creates an increase in the demand for food protein. Over the years there has been a reduction in wild-caught fish, so there has been an increase in aquaculture. “Aquaculture is the fastest-growing food-production sector worldwide” (Akmal et al.). This expanding sector of aquaculture has created many environmental problems as well such as water pollution, chemical run-off, and various fish diseases. The most common treatment for bacterial infections is antibiotics; however, in aquaculture, these antibiotics can run off into main water sources and cause the death of water ecosystems. Additionally, the bacteria in the fish can become antibiotic-resistant and perpetuate the disease. A huge limitation to the growth of the aquaculture industry is the presence of disease in fish. This allows for a huge financial loss. It has been estimated that aquaculture loses $6 billion per year due to various diseases in fish.

The most common bacteria to cause these infections in fish is *Aeromonas* which is a gram-negative, rod-shaped bacterium that can infect humans and fish alike. There are 24 species of *Aeromonas* in nomenclature today. Some of the most useful species of Aeromonas to be used in the laboratory include *A. hydrophila*, *A. salmonicida*, *A. media*, *A. caviae* and *A. veronii.* The strain I ended up using in my research was *A. salmonicola* that infects salmon and other species of fish. Infection caused by *A. salmonicola* can cause “hemorrhaging in the fin bases, mouth, abdominal walls, reproductive organs, viscera, liver, pyloric caeca, and heart; enlarged spleen; soft kidneys; erratic swimming; lack of feeding; and congested intestines” (Buschmann et al.). As shown above, infection from *Aeromonas salmonicola* is detrimental to the health of the fish and causes a huge economic loss for the aquaculture industry. *Aeromonas* can be “isolated from rivers, lakes, ponds, seawater (estuaries), drinking water, groundwater, wastewater, and sewage in various stages of treatment” (Janda et.al). The concentrations of aeromonads vary from type of water source with concentrations in drinking water being relatively low in comparison to the concentrations in sewage waste. In my work with *Aeromonas* I used sewage water samples to increase my chance of finding bacteriophage.

Bacteriophage are a type of virus that infects bacteria and has little to no environmental impact. There is a recent increase in phage research addressing the potential of phage therapy rather than antibiotics to treat bacterial infections. Several studies have looked at the impacts of phage therapy on human, animal, and aquaculture populations. In one study, three *Aeromonas* phage were isolated from sewage samples and one showed antibacterial properties. No antibiotic resistance genes were identified which is important for the health of humans and the fish. Another study used *Aeromonas* bacteriophage to treat *Aeromonas* infection in rainbow trout. The bacteriophage was shown to reduce the bacterial infection on the fish as well as provide some protection from further bacterial infections. Overall, there are various studies showing a correlation between *Aeromonas* bacteriophage treating bacterial infections in fish.

The issue of antibiotics in aquaculture is not only a biological problem, but a public health problem. Antibiotics can remain in commercialized fish products and be consumed by humans which creates a public health issue. “The major public health significances of antimicrobial residues include the development of antimicrobial drug resistance, hypersensitivity reaction, carcinogenicity, mutagenicity, teratogenicity, bone marrow depression, and disruption of normal intestinal flora” (Okocha et al.). Antibiotics in aquaculture “increases the risk of harmful bacteria becoming resistant to specific antibiotics, undermining the effectiveness of those antibiotics in treating human illness”(“Human Health Risk”). Most people in today’s society do not even know antibiotics are in their food and could become sick from consuming them. The main research question I am addressing is: How can the public health issue of antibiotics used in aquaculture be addressed using biological methods? With this honors project, I hope to expand my knowledge of *Aeromonas* bacteriophage and how my findings can help future research endeavors into this topic.

Literature Review:

Fish farmers use aquaculture to produce about “114.5 million metric tons[of fish], with an estimated first-sale value of US$160.2 billion” in 2018 for the purpose of consumption by humans (”Global Aquaculture”). *Aeromonas* infection is common in farmed fish. Fish farmers use antibiotics to cure *Aeromonas* bacterial infections. These antibiotics can run off into the main water supply and cause death of ecosystems. In addition, humans can consume these antibiotics in their food. Luckily, there have been new findings that bacteriophage can cure these infections as well. Bacteriophages do not harm the environment and human health like antibiotics do.

A study written in Sciencedirect characterized a new *Aeromonas* bacteriophage named MJG to treat *Aeromonas hydrophila* infection in rainbow trout. This new bacteriophage “meets the main prerequisites for a candidate therapeutic phage based on genomic characterization” (Cao et al.). The phage MJG was found to completely protect the fish from death due to *A.hydrophila* infection as well as restore liver tissue damage. One implication of MJG is that it only infects 4 out of 20 *A. hydrophila* different strains. Another journal article discusses the problems with the overuse of antibiotics in aquaculture and how it causes multidrug resistance to the *A. hydrophila* strains. One solution discussed is the use of bacteriophages that do not affect other flora and create no chemical residues. Scientists discovered five phages that have the potential to treat *Aeromonas* infection. Two of the phages named “G65 and Y81 showed considerable bacterial killing effect and potential in preventing formation of *A. hydrophila* biofilm”(Liu et al.). The other three samples had the potential to eradicate the *A. hydrophila* biofilm on the surface. Although *Aeromonas hydrophila* is not the strain I ended up discovering my bacteriophage with this information is still relevant because various strains of *Aeromonas* can infect different types of fish. It’s important to know that there are bacteriophage that can eradicate Aeromonas infection.

From these two findings I have discovered that phage therapy is a viable option for treating *Aeromonas* infection. Additionally, these sources confirm that the overuse of antibiotics is a huge problem for human health. Bacteriophage is a viable way to solve this problem and I hope to contribute to helping solve this problem with my research. These sources have further solidified my interest and passion for this topic.

In terms of the public health aspect of aquaculture workers they are more likely to get injured or sick on the job compared to other occupations. The hazards from aquaculture include chemical, biological and physical hazards. The workers are exposed to many chemicals while working such as “formalin, methanol, hypochlorite, oxygen-acetylene systems, fuels and solvents”(Fry et al.). There has not been much investigation into the specific occupational hazards of aquaculture workers, but there have been several case studies. One such example is an exposure to MS-222 that caused temporary blindness to a salmon aquaculture worker. Additionally there are pathogens in the water and fish that can cause disease in workers. Contact with the water can cause dermatitis, warts, and allergic reactions for the workers. Furthermore, antibiotics approved by the U.S food and drug administration for use in aquaculture such as Florfenicol and Oxytetracycline dihydrate have potential health hazards. These hazards include irritation to the upper respiratory tract, potential eye and skin irritation, and chronic exposure could damage fertility. All of these health hazards further indicate that there needs to be reform in the aquaculture industry for the safety of workers.

The Occupational Safety and Health Administration (OHSA) located within the U.S. Department of Labor helps to regulate worker and workplace safety. This administration helps to decrease the risk of aquaculture workers by putting rules and regulations in place. However, there still remains problems with regulating the aquaculture industry such as “employers with 10 or fewer employees are partially exempt from keeping workplace injury or illness records” (Fry et al.). There are many aquaculture practices that have 10 or fewer employees and are not covered under OHSA. Additionally, minor aquaculture farm workers are excused from any and all OHSA regulations. Work still needs to be done in the aquaculture industry to protect aquaculture workers and minors.

Methods:

Biological research methods: On February 16th, I made three plates with three different *Aeromonas salmonicola* strains and spotted three different sewage samples named AS1, AS2 and AS3. The sewage samples were treated with chloroform and put in a .2 centrifuge to eradicate all enveloped viruses such as COVID-19 and leave all bacteriophage viruses. I combined 100 µl of each bacteria strain of *Aeromonas* with 3 ml of agar and poured it on the respective t-plate. I then took 5 µl of each phage sample and spotted it on each plate. I left the plates out at room temperature because *Aeromonas* grows best at room temperature. In 24-48 hours, analyze which sewage samples create a zone of inhibition for *Aeromonas*.

On March 17th, I made one plate with the bacterial strain named ATT to see if any of the sewage samples with phage contained any phage that would kill the *Aeromonas* bacteria. The plate had nine phage samples plated including 2C, SB2, US2, AR, AR2, BG1oct, SBoct, BG3oct, and BG2 oct. The sewage samples were treated with chloroform and put in a .2 centrifuge to eradicate all enveloped viruses such as COVID-19 and leave all bacteriophage viruses. I combined 100 µl of the bacteria strain of *Aeromonas* with 3 ml of agar and poured it on the respective t-plate. I then took 5 µl of each phage sample and spotted it on each plate. I put the plate in the incubator for 24 hours. In 24-48 hours, analyze which sewage samples create a zone of inhibition for *Aeromonas*.

On March 21st, I made three plates with bacterial strains named AS1, AS2 and AS3 and 18 phage samples numbered from 1-18. The sewage samples were treated with chloroform and put in a .2 centrifuge to eradicate all enveloped viruses such as COVID-19 and leave all bacteriophage viruses. I combined 100 µl of each bacteria strain of *Aeromonas* with 3 ml of agar and poured it on the respective t-plate. I then took 5 µl of each phage sample and spotted it on each plate. I left the plates out at room temperature because the *Aeromonas* strain grows best at this temperature. In 24-48 hours, analyze which sewage samples create a zone of inhibition for *Aeromonas*.

On March 23rd, I checked my results from March 21st and did not find any conclusive data. My plan was to let the plates sit longer and see if anything grows. The cultures I used for the March 21st experiment were grown overnight; but for this new experiment I am going to use cultures that have been in the incubator. I made three plates with bacterial strains named AS1, AS2 and AS3 and 18 phage samples numbered from 1-18. The sewage samples were treated with chloroform and put in a .2 centrifuge to eradicate all enveloped viruses such as COVID-19 and leave all bacteriophage viruses. I combined 100 μl of each bacteria strain of *Aeromonas* with 3 ml of agar and poured it on the respective t-plate. I then took 5 μl of each phage sample and spotted it on each plate. I left the plates out at room temperature because the *Aeromonas* strain grows best at this temperature. In 24-48 hours, analyze which sewage samples create a zone of inhibition for *Aeromonas*.

On March 28th, I checked my plates from the March 23rd experiment and I got some cloudy plaques from bacterial strains AS1 and AS3. Today I made cultures using AS1 by putting 1ml of media in 10 tubes with two different controls. One control just had media and the other had media and bacterial cells of AS1. I put 100 μl of AS1 bacteria in each tube along with 10 μl of each phage sample from the AS1 plate that showed a plaque. I put the samples on the shaker at room temperature for 48 hours.

On March 30th, I came into the lab to check my samples and did not find that the phage killed the bacteria. I let the samples sit for another few days to see if anything killed the bacteria.

On March 31st, Dr. Larsen plated each sample of the media on a t-plate. Dr. Larsen made two plates with bacterial strains named AS1 and AS2 and 10 phage samples numbered from 1-10. Dr. Larsen spotted 5 μl of each media sample and spotted it on each plate. He left the plates out at room temperature because the *Aeromonas* strain grows best at this temperature. In 24-48 hours, analyze which sewage samples create a zone of inhibition for *Aeromonas*.

On April 4th, I analyzed the results from March 31st and saw that both plates with AS1 and AS2 strains had plaques. I took pictures of the plates under the light.

Public health research: I analyzed credible online sources for more information about various public risks associated with antibiotic use in aquaculture. Also, I discussed my project with my secondary faculty advisor, Dr. Welch about further implications for antibiotic use in aquaculture.

Results:

I tested 10 phage samples against three strains of *A. salmonicola* (I,II & III). The third strain was shown to be negative. The results are shown below with the positive indicating a plaque or a clear area on a lawn of bacteria that shows the dissolution of bacterial cells by a bacteriophage. A negative or minus sign indicates no plaque shown and no bacteria inhibited. The pool is a set of 10 more samples that were negative on both tests. The phage samples are from sewage from places such as Archibald, Perrysburg, Upper Sandusky, Port Clinton, Put in Bay and Bowling Green. I have discovered 6 phage samples on AS1 and 9 phage samples on AS3 that have the ability to be used as bacteriophage to kill *Aeromonas salmonicola* infection. According to my lab work there are bacteriophage found in sewage samples that infect *Aeromonas salmonicola* and could potentially be used in treating *Aeromonas salmonicola* infection in fish. For public health, this is a promising finding because it can be a possible solution for the issue with antibiotics found in food and infections in aquaculture workers. History has shown us to look at worker exposure to potentially harmful chemicals and industries as a warning sign. Aquaculture workers are in a potentially dangerous career that has higher rates of illness and injuries compared to other industries. To decrease the risk of illness and injury in the aquaculture industry OHSA has applied practices and standards to decrease this overall risk. However, there remains work to be done to improve aquaculture workers health and safety.

	I.	II
AR 2/27	+	+
PB 2/27	+	+
PB 2/22	+	+
AR 2/22	+	-
US 2/22	+	-
PC 2/22	-	+
PIB 2/22	+	+
BG 2/22	-	+
BG 2/13	-	-
PIB 2/13	-	-
Pool	NT	-

Figure 1: Table of results for Aeromonas salmonicola I&II

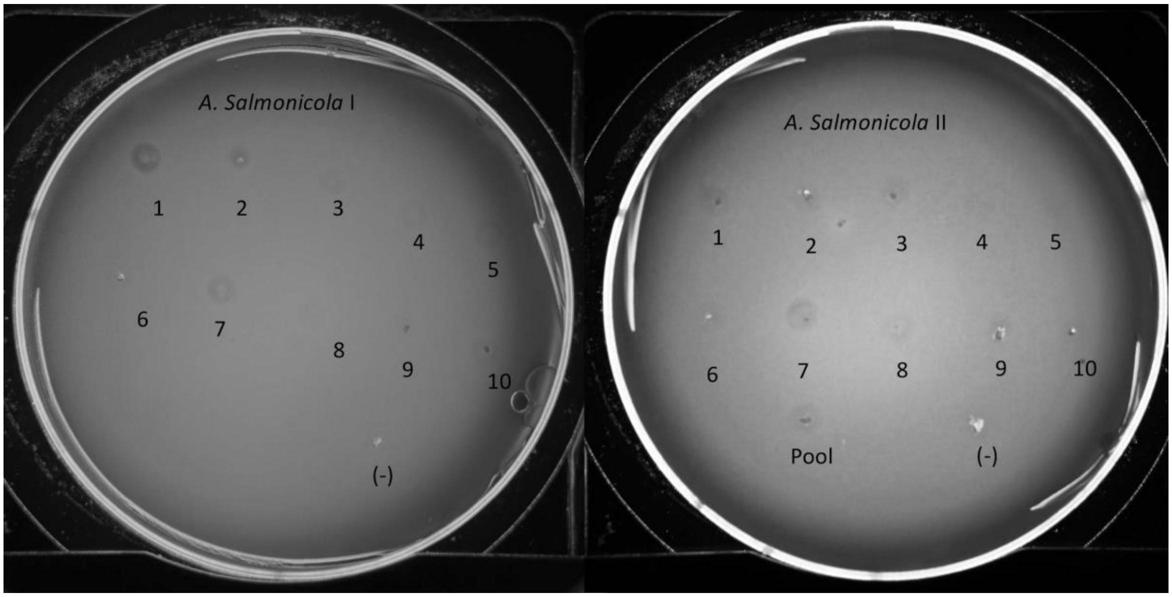

Figure 2: Aeromonas salmonicola 1&II plates with plaques shown

Implications for future research:

My project with Aeromonas can continue at Bowling Green State University and I intend to continue this project next semester. I can continue working in the laboratory to discover more bacteriophage that infect *Aeromonas salmonicola*. These strains can be sequenced and characterized for further use in place of antibiotics used in aquaculture. Once a bacteriophage is discovered it can be tested on fish to see if it reverses *Aeromonas salmoicola* infection. Bacteriophage therapy is a relatively new area of research, so there are a lot of questions to be answered including the safety and efficacy of phage therapy. Some issues that still need to be answered include: the host's immune response to the bacteriophage, route of administration and safety of phage therapy. However, before these questions can be answered there needs to be a bacteriophage to study its properties and effects. This is why it's so important for me to discover a bacteriophage that infects *Aeromonas* first.

Appendix:

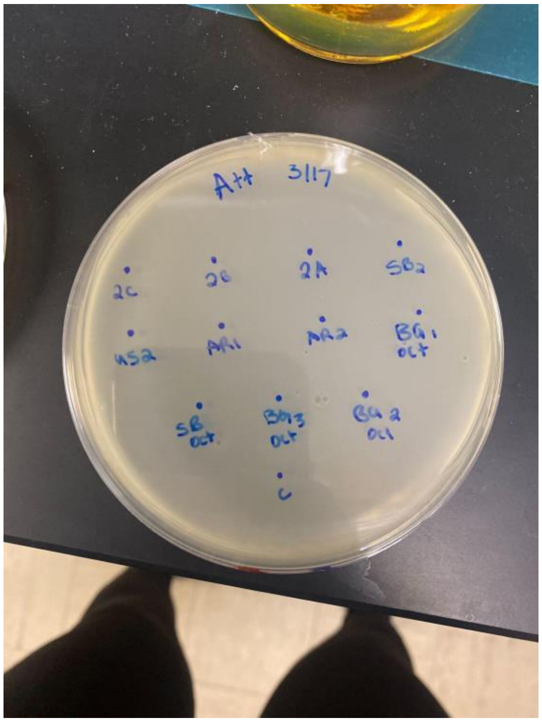

Figure 3: Results from 3/17 experiment: no plaques shown

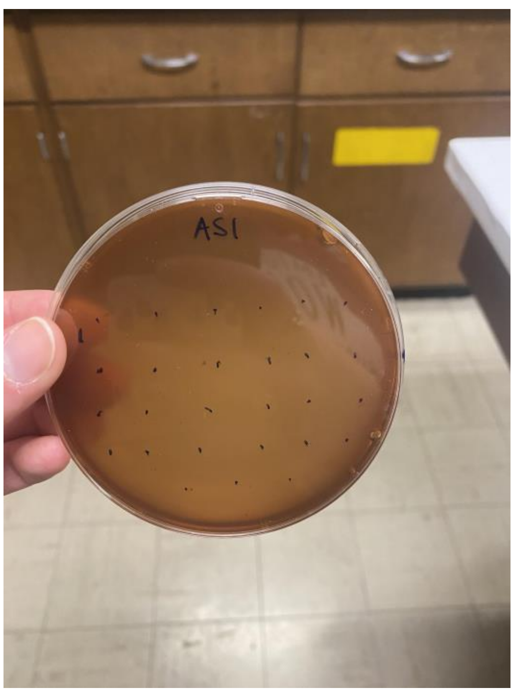

Figure 4: Results from 3/23 experiment with Aeromonas salmonicola I

Figure 5: Results from 3/23 experiment with Aeromonas salmonicola II

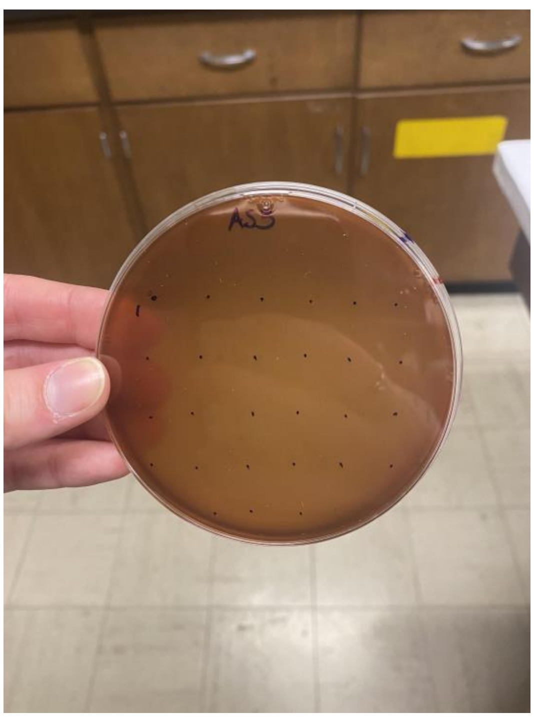

Figure 6: Results from 3/23 experiment with Aeromonas salmonicola III

Strengths and Limitations:

The strengths of my project include the widespread issue of antibiotics in aquaculture and the huge financial loss that comes from aquaculture. This issue affects not only the companies who produce fish from aquaculture, but the workers and consumers of the product as well. Bacteriophage are not a threat to human health because they only target specific pathogenic bacteria and not the human gut flora. There have already been seven companies who manufacture phage products for pathogenic bacteria in aquaculture. They have already created multiple safe and effective phage products to destroy pathogenic bacteria. Other methods have been used to replace antibiotics, but none have proved to effective. The limitations of my project include that I was not able to find anyone to interview about the public health aspects of aquaculture. This area of research is still relatively new and it was difficult to find anyone to interview. Additionally, I was not able to test the bacteriophage on fish to see if it could eradicate the Aeromonas infection.

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