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Antibiotic-resistant bacteria in freshwater crayfish

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ANTIBIOTIC-RESISTANT BACTERIA IN FRESHWATER CRAYFISH

by

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ABSTRACT

Antibiotic-Resistant Bacteria in Freshwater Crayfish

(Under the direction of Colin Jackson, Ph.D)

The presence of antibiotic-resistant bacteria is increasing in natural aquatic environments. Alongside this, organisms that live in these ecosystems are increasingly harboring antibiotic-resistant bacteria. In this study, I analyzed the capacity for the crayfish species *Procambarus vioscai paynei* to harbor antibiotic-resistant bacteria. Crayfish, as well as water and sediment, were sampled from a pond at the University of Mississippi Field Station. The guts of crayfish were plated on TSA agar, as well as agar containing vancomycin, erythromycin, penicillin, tetracycline, or ciprofloxacin. Following incubation, counts of bacteria were determined. Selected bacterial isolates were tested for multiple antibiotic-resistance. Bacterial isolates were also treated with a Gram stain and the shape of bacterial cells were determined using a light microscope. Results of the study indicate that the pond water, sediment, and the crayfish samples all harbored antibiotic-resistant bacteria. All crayfish samples yielded bacterial growth on at least two of the five antibiotics used. Some of the bacterial isolates examined also displayed resistance to multiple antibiotics. The presence of antibiotic-resistant bacteria in aquatic environments and organisms outside of urban areas shows that they can become a more common and dangerous threat to people and organisms of all areas.

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Introduction:

One of the most studied topics in the area of human pathogens and disease is the concept of antibiotic-resistant bacteria. Antibiotic-resistant bacteria are strains of bacteria that carry genes that confer antibiotic resistance. This expression of these genes makes the bacterial strain less susceptible to the antibiotic that the genes specify resistance to (Marti et al., 2014). It is also possible for the gene to be passed along to other bacteria through the process of horizontal gene transfer (Marti et al., 2014). Antibiotic resistant bacteria have become increasingly important in recent years, largely because of the medical and health threats these bacteria pose to humanity. Since 2013, the Center for Disease Control and Prevention has considered antibiotic-resistant pathogens a "health crisis" for humanity, citing several types of infections as leading threats and causes of death in the United States, including Methicillin-Resistant *Staphylococcus aureus* (MRSA), Vancomycin-Resistant Enterococci (VRE), Drug-Resistant *Streptococcus pneumoniae*, and Drug-Resistant *Mycobacterium tuberculosis* (Ventola, 2015).

Selection for bacteria to become resistant to an antibiotic, requires exposure to the antibiotic. Therefore, as the usage of antibiotics has exponentially increased and as more new antibiotics have been developed, we have likewise seen a rapid and large increase in new strains of antibiotic-resistant bacteria. This increase in new antibiotic-resistant bacterial strains and the "health crisis" that accompanies them can be blamed on the gross overuse and misuse of antibiotics worldwide (Ventola, 2015).

There has been a large amount of research regarding antibiotic-resistant bacteria that focuses on human pathogens and treatment options, however, another field of study regarding this topic is the presence of antibiotic-resistant bacteria in natural environments and ecosystems. Freshwater lakes and rivers can easily be contaminated by antibiotics through a variety of ways including agricultural runoff and sewage discharge (Nnadozie & Odume, 2019). In northern Vietnam, a study examined the prevalence of antibiotics in natural water near agriculture sites as well as natural water near cities. The study found that the antibiotics used for agricultural purposes were found at higher rates in water near farm sites, and bacteria that were resistant to these drugs were found at higher rates near farms as well (Phuong Hoa et al., 2011). This study also found the antibiotics in freshwater closer to the city were not antibiotics used in agriculture, but instead the antibiotics found near the city were associated with human use (Phuong Hoa et al., 2011). Thus, there is evidence that agricultural pollution plays a role in the presence of antibiotics and antibiotic-resistant bacteria in freshwater. Another cause for the presence of antibiotics in freshwater is "clinical wastewater" (Voigt et al, 2020). A study conducted in Germany showed that there was a correlation of higher rates of antibiotic-resistant bacteria in freshwater near municipal areas where hospitals were present (Voigt et al, 2020), demonstrating that antibiotic waste from these clinical areas was polluting nearby water. Another study in India found that 33 out of 50 bacterial isolates obtained from the sediment of a freshwater lake were resistant to at least two antibiotics (Zothanpuia et al., 2016). From this research, it is known that through the aforementioned methods of contamination, antibiotics have polluted freshwater ecosystems and led to a rise in antibiotic-resistant bacteria within them.

Given that we know that water and sediment can contain antibiotic-resistant bacteria, how does this affect the macroorganisms that live in these environments? If the organisms living in these environments are exposed to antibiotics and/or antibiotic-resistant bacteria, can they become hosts to antibiotic-resistant bacteria? A study on fish in reservoirs in Spain found significant evidence of three different antibiotic-resistant genes in bacteria in the intestinal mucus of various fish species (Marti et al., 2018), showing that it is possible for aquatic organisms to harbor antibiotic resistant bacteria. Other studies support the idea that aquatic organisms can harbor antibiotic-resistant bacteria. Fish were caught from the Concepción Bay, Chile, near sites of urban sewage disposal, and they were examined for the presence of antibiotic-resistant bacteria (Miranda & Zemelman, 2001). There was a high occurrence of bacteria within these fish that were resistant to ampicillin, tetracycline, and streptomycin. While this study focuses on saltwater organisms instead of freshwater organisms, it does provide further evidence to support that organisms living in aquatic environments can harbor antibiotic-resistant bacteria.

A much less examined topic is the prevalence of antibiotic-resistant bacteria in freshwater macroinvertebrates, even though these organisms are common and often dominant in freshwater systems. Many freshwater macroinvertebrates are found in the waters of Mississippi, including a variety of crayfish species with 17 species endemic (https://www.srs.fs.usda.gov/crayfish/info.php). Crayfish live in a vast array of freshwater habitats including lakes, rivers, streams, and ponds. In these habitats crayfish tend to live in the water's sediment in self-made burrows (usda.gov). Crayfish are thus potentially exposed to not only antibiotics and antibiotic-resistant bacteria in the water, but also in the sediment. Crayfish also feed on multiple food sources, increasing their likelihood of ingesting various bacteria. The few studies on bacteria associated with crayfish have focused on crayfish bacterial infections in aquaculture farms. For example, a study in China found an antibiotic-resistant strain of *Enterobacter cloacae* in diseased crayfish from a crayfish farm (Dong et al., 2020). Another study, analyzing aquaculture in Iran, focused on the drug resistance of a specific bacterial pathogen (*Aeromonas*) in crayfish as well as other species. The study found that some of the bacterial isolates examined did show antibiotic-resistance, and that some of the isolates also showed resistance to multiple antibiotics (Ranjbar et al, 2019). However, there is no research on the presence of antibiotic resistant bacteria in wild crayfish.

The goal of this study was to determine if freshwater crayfish have the potential to harbor antibiotic-resistant bacteria. Crayfish were sampled from the University of Mississippi Field Station (UMFS), as were samples of water and sediment. Attempts were made to culture bacteria that were resistant to the antibiotics: vancomycin, penicillin, erythromycin, tetracycline, and ciprofloxacin. The first hypothesis I had was that crayfish would contain antibiotic-resistant bacteria, and I also hypothesized that larger crayfish would yield greater numbers of bacterial colonies.

Methods:

Crayfish (*Procambarus vioscai paynei*) were collected from UMFS pond 132 on 11/9/21. This field station, located outside Oxford Mississippi, is a 740 acre site consisting of a variety of ponds ranging in size, as well as several creeks and streams. This site is typically used as a research center for the University of Mississippi, but it is also open to the public. In the past, this site served as a fish hatchery.

Crayfish were collected from pond number 132 with nets. Eight captured individuals were placed in a large container containing water from the pond they were caught in, along with vegetation (*Myriophyllum aquaticum*) from their habitat. Sediment and water from the pond were also collected. Crayfish were taken to a laboratory on the University of Mississippi campus and those selected for sampling were sprayed with 70% ethanol, placed in individual plastic bags, and frozen overnight. Following freezing, the mass of each crayfish was recorded using a scale. Crayfish were then placed on a surface sterilized with 70% ethanol and their gut tracts were removed. The gut tracts were placed in 1 mL of sterile distilled water and vortexed to allow the gut contents to dissipate throughout the water. A sample of 0.1 mL from the sediment and water samples taken from the habitat were also placed in 1 mL of sterile water and received the same vortex treatment. A subsample of each suspension was serially diluted (100 fold) to yield dilutions of 10^{0}, 10^{-2}, and 10^{-4}.

For each sample, 0.1 mL from each dilution was pipetted and spread onto a regular trypticase soy agar (TSA) plate, as well TSA plates containing vancomycin, penicillin, erythromycin, tetracycline, and ciprofloxacin. The concentration of each antibiotic in the agar was chosen as 3x the minimum inhibitory concentration (Andrews, 2001): vancomycin (48mg antibiotic/1L agar), penicillin (48mg/1L), erythromycin (1.5mg/1L), tetracycline (48mg/1L), and ciprofloxacin (6mg/1L). Inoculated plates were incubated at room temperature for 48-72 hours, after which colonies were counted for all plates. Plates that contained the most colonies, without exceeding 300 colonies, were used for data collection. Colonies of antibiotic resistant bacteria were reported as colony forming units (CFUs) per mL of solution, adjusted for dilution as necessary. Using the mass of each crayfish, the amount of CFUs per gram for each crayfish was also calculated and recorded.

Selected bacterial colonies were transferred to new TSA plates and were further analyzed to determine if crayfish contained multi-antibiotic-resistant bacteria. Colonies showing resistance to one antibiotic were transferred onto TSA plates containing the other antibiotics (for example, if a colony was obtained from a plate containing vancomycin, it would be transferred onto four other plates, containing penicillin, erythromycin, tetracycline, and ciprofloxacin). Samples of isolates showing multi-drug resistance were then smeared onto microscope slides and treated with a Gram stain to determine cell morphology and whether the samples were Gram-positive or Gram-negative.

Results:

Bacterial growth on antibiotic plates:

Eight crayfish were sampled, along with samples of sediment and water taken from their environment. Crayfish were weighed with the mass measured in grams (Table 1). The amount of CFUs per mL were recorded (Table 2), as well as the amount of CFU/g for the eight crayfish (Table 3). All samples yielded multiple colonies on TSA media not containing any antibiotic. Bacterial growth on plates containing media with antibiotics varied greatly between individual crayfish. Erythromycin was the only antibiotic plate that yielded colonies from every sample, however, this varied greatly with some samples only producing 10 CFU/mL and some samples producing as many as 149,000 CFU/mL (Table 2). Penicillin was the next antibiotic that samples showed the most resistance towards. All samples except one crayfish (crayfish 1) yielded colonies that were resistant to penicillin (ranging from 10 CFU/mL to 123,000 CFU/mL). Six out of the eight crayfish yielded colonies that were resistant to vancomycin, as did the water and sediment samples. As with the other antibiotics, counts of vancomycin-resistant CFUs were highly variable between crayfish, with numbers ranging from 10 CFU/mL to 144,000 CFU/mL (Table 2). The antibiotics tetracycline and ciprofloxacin showed the most effectiveness against bacteria within the samples. Only three out of eight crayfish yielded colonies on plates containing tetracycline (ranging from 20 CFU/mL on two crayfish to 2000 CFU/mL on the other). The water and sediment samples also yielded colonies on tetracycline plates (70 and 5000 CFU/mL, respectively). Ciprofloxacin plates gave the smallest number of CFUs for the crayfish. Only three out of eight crayfish produced CFUs on ciprofloxacin plates, and the number of colonies was very low (10 to 120 CFU/mL). Water and sediment samples did yield ciprofloxacin-resistant CFUs (120 CFU/mL and 4000 CFU/mL, respectively; Table 2).

Comparing the number of bacteria in crayfish against the number of bacteria in their surrounding environment, water and sediment samples yielded CFUs that were resistant to each type of antibiotic tested, whereas none of the crayfish samples did. The sediment sample yielded much greater numbers of CFU/mL than the water sample for all antibiotics tested, and on unamended TSA. These numbers also exceeded those obtained from all but the largest crayfish (crayfish 4) which had similar numbers of CFU/mL. The water sample yielded fewer bacterial colonies than the sediment sample (70-510 CFU/mL).

The size of the crayfish (Table 1) seemed to also have an impact on the amount of antibiotic-resistant bacteria found within them (Table 3). The largest crayfish (crayfish 4) which weighed 4.25 g yielded CFUs on every plate except the plates containing ciprofloxacin with large values of CFU/g (470-33,882, Table 3). The next biggest crayfish samples (crayfish 6,7,and 8), whose weights varied from 1.13 to 1.27 g, yielded CFUs on three to four out of the five antibiotics. These numbers were lower and varied from 8-165 CFU/g. The smallest crayfish (crayfish 1), which weighed just 0.42 g, only yielded CFUs onto vancomycin and erythromycin plates with very small values for CFU/g (95 and 24 respectively).

Crayfish Sample Number	Crayfish Mass (g)	CFU/g on TSA Plates
1	.42	357
2	.50	1,620
3	.43	4,418
4	4.25	51,764
5	.88	1,159
6	1.20	500
7	1.13	53
8	1.27	976

Table 1: Crayfish Mass and CFU/g on TSA Plates. Crayfish were sampled 11/9/21 from a pond at the University of Mississippi Field Station outside Oxford, MS. The crayfish were labeled 1 through 8, and their corresponding mass is recorded in this table. The third column represents the total amount of CFU/g for each crayfish on regular TSA plates.

Crayfish Sample Number	CFU per mL solution				
& Water sample, Sediment Sample	Vancomycin	Penicillin	Erythromycin	Tetracycline	Ciprofloxacin
Cray 1	40	0	10	0	0
Cray 2	1,340	10	60	20	0
Cray 3	1,160	10	10	0	0
Cray 4	144,000	123,000	149,000	2,000	0
Cray 5	0	50	210	20	120
Cray 6	10	20	170	0	0
Cray 7	20	10	20	0	10
Cray 8	0	10	210	0	40
Water	510	320	420	70	120
Sediment	64,000	66,000	189,000	5,000	4,000

Table 2: Counts of antibiotic-resistant CFUs per mL on antibiotic plates. Colony forming units (CFUs) were counted for eight crayfish samples and a sample of water and sediment using plates containing vancomycin, penicillin, erythromycin, tetracycline, or ciprofloxacin.

Crayfish Sample Number	CFU/g				
	Vancomycin	Penicillin	Erythromycin	Tetracycline	Ciprofloxacin
1	95	0	24	0	0
2	2,680	20	120	40	0
3	2,697	23	23	0	0
4	33,882	28,941	3,505	470	0
5	0	57	239	23	136
6	8	17	142	0	0
7	18	9	18	0	9
8	0	8	165	0	31

Table 3: Counts of antibiotic-resistant CFUs per gram in each crayfish. Colony forming units (CFUs) were counted for eight crayfish using plates containing vancomycin, penicillin, erythromycin, tetracycline, or ciprofloxacin.

Further Analysis of Bacterial Colonies: Multi-resistance and Gram Stain Analysis

Seven colonies from five crayfish samples that showed resistance to an antibiotic, as well as one from water and one from sediment were tested for resistance to other antibiotics. These seven bacteria were chosen based on uniqueness in appearance of the colony compared to other bacteria.

All but one of the seven bacteria tested were able to grow on at least one other type of antibiotic-amended plate (Table 4). The most drug-resistant was a Gram positive rod shaped bacterium (Figure 1) obtained from a crayfish sample that had been plated onto a tetracycline plate. It also yielded growth on penicillin, vancomycin, and erythromycin, making it resistant to four of the five antibiotics tested (Table 4). A Gram negative coccus-shaped bacterium obtained from a crayfish sample on a vancomycin plate also yielded growth on penicillin and erythromycin plates, making it resistant to three out of five antibiotics. Similarly, a Gram negative rod-shaped bacterium taken from water also showed resistance to three antibiotics (vancomycin, erythromycin, and penicillin, from which it was originally obtained from). Another Gram negative rod-shaped bacterium obtained from a penicillin plate, in this case from a crayfish gut, also exhibited resistance to vancomycin and erythromycin. Two bacteria, a Gram negative coccus from a crayfish and a Gram negative rod-shaped bacterium from sediment, only showed resistance to erythromycin and penicillin. The only bacterium that did not yield growth on any other antibiotic-containing plate was a Gram positive coccus taken from a ciprofloxacin plate obtained from a crayfish. In contrast, none of the other bacterial isolates tested yielded growth on the plates amended with ciprofloxacin.

Bacterial Sample	Vancomycin	Penicillin	Erythromycin	Tetracycline	Ciprofloxacin	Bacteria Shape	Gram +/-
Crayfish Vancomycin-Resistant	X	X	X	O	O	Coccus	negative
Crayfish Penicillin-Resistant	X	X	X	O	O	Bacillus	negative
Crayfish Erythromycin-Resistant	O	X	X	O	O	Coccus	negative
Crayfish Tetracycline-Resistant	X	X	X	X	O	Bacillus	positive
Crayfish Ciprofloxacin-Resistant	O	O	O	O	X	Coccus	positive
Water Penicillin Resistant	X	X	X	O	O	Bacillus	negative
Sediment Erythromycin-Resistant	O	X	X	O	O	Bacillus	negative

Table 4: Multi antibiotic-resistance, cell shape, and Gram Stain results. Seven bacteria were tested for resistance to multiple antibiotics. 'X' signifies that the bacteria was able to grow on plates containing the corresponding antibiotic. 'O' signifies the bacteria was not able to grow on plates containing the antibiotic. The bacteria shape is also described (bacillus or coccus).

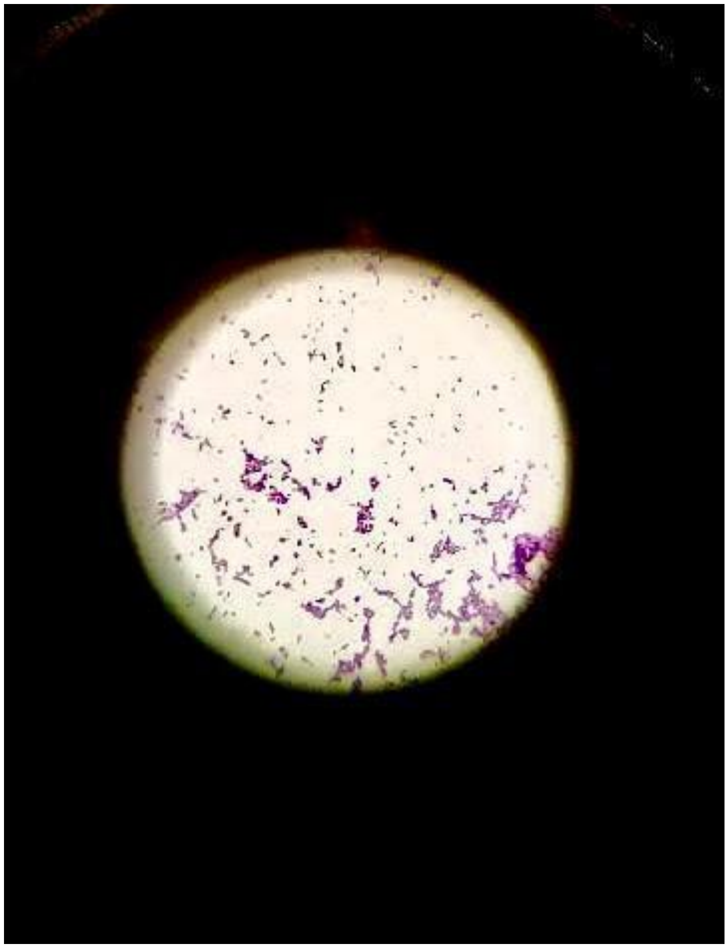

Figure 1: Multidrug-resistant bacterium. The bacterium in this image is a Gram positive rod shaped (bacillus) bacterium that was obtained from a crayfish. This bacterium showed resistance to multiple drugs: tetracycline, erythromycin, vancomycin, and penicillin.

Discussion

This study provides a glimpse into the potential for free-living crayfish to harbor antibiotic-resistant bacteria. The study sought to first determine if crayfish contained antibiotic-resistant bacteria in their guts, and what type of antibiotics the bacteria would be resistant against. It also sought to discover possible factors that influence the number of bacteria present in crayfish, such as size of the organism, as well as compare the numbers of bacteria in the crayfish compared to their habitat (water and sediment). Next, the study sought to determine if any of these bacteria found within crayfish had the potential to be resistant to multiple antibiotics.

The first and foremost observation is that crayfish did harbor bacteria in their guts that were able to grow and form colonies on antibiotic-amended media. Compared to the control group of regular TSA plates, the antibiotic-amended plates yielded a much lower number of bacterial growth, or no growth at all. Therefore, we can assume that the antibiotics were working as intended in inhibiting bacterial growth, otherwise the numbers for regular TSA plates and antibiotic plates would have been similar. So, the bacteria that grew on antibiotic amended plates shows that there was a presence of some bacteria that the antibiotics were not effective against.

Relating to how the size of the sampled crayfish affects bacterial growth and antibiotic resistance, my initial hypothesis was partially correct. Crayfish number 4, which weighed at least four times as much as any of the other crayfish, had much higher values for CFU/g than the other seven crayfish. On the unamended TSA plate, crayfish 4 yielded 51,764 CFU/g. The next highest recorded CFUs/g on unamended TSA was crayfish 3 with 4,418 CFUs/g (Table 3). The other seven crayfish were all within 1 gram of each other in terms of mass (0.42-1.27g), and the trend of a larger mass correlating with a higher number of CFUs/g was not observed in them. This would seem to suggest that larger crayfish do possess a higher presence of bacteria within them, but they must be at least multiple grams larger for this trend to be observed. Crayfish 4's gut yielded colonies on four out of five antibiotics, but crayfish 2 also yielded colonies on four out of five antibiotics despite being a fraction of the size as crayfish 4. This would suggest that the potential for a crayfish to harbor bacteria that is resistant to multiple antibiotics isn't influenced by size. Rather, size mainly determines the amount of bacteria and the density of the bacterial populations within crayfish.

Samples of the water and sediment that came from the same pond as the crayfish were also taken and tested. There are several points of observation that can be made from comparing these samples. The sample of sediment used yielded a large amount of CFUs, ranging from 4,000 CFU/mL on ciprofloxacin to 189,000 CFU/mL on erythromycin. This is greater than all of the crayfish samples. Crayfish 4, the largest crayfish, ranged from 0 CFU/mL on ciprofloxacin to 149,000 CFU/mL on erythromycin. The numbers for this crayfish were still less than the sediment sample, but they were the closest to it. The other seven crayfish yielded much lower colony counts than the sediment. In contrast, the sample of pond water yielded much lower numbers of bacterial growth. On none of the five antibiotic plates did the value of CFU/mL exceed 1,000 for the water sample. The water and sediment both produced some amount of colony growth on all five types of antibiotic, something that no crayfish samples achieved. We can likely conclude from this that the sediment in these ponds holds a higher prevalence of antibiotic-resistant bacteria than the water. We know that crayfish tend to live in and burrow into mud and sediment in ponds and streams (usda.gov). So, we can assume that the crayfish likely obtained these bacteria from the sediment in which they lived, but they could have also been exposed to some bacteria through their diet as well from the water.

However, if the crayfish were living in the sediment, then why did the sediment have such higher numbers and prevalence of antibiotic-resistant bacteria compared to the various crayfish? One possible answer is that crayfish possess natural physical defense barriers against bacteria and have a functional immune system that can destroy pathogens. The immune system of a crayfish is not as complex or effective as the human immune system, and it relies on innate immunity (Bouallegui, 2021). In other words, a crayfish lacks acquired immunity, and it is unable to form specific antibodies against viruses, bacteria, and other pathogens (Bouallegui, 2021). For this reason, crayfish are susceptible to various plagues because of their lack of immune system memory for specific pathogens. The antibiotic-resistant bacterial breakout in the agricultural crayfish mentioned earlier is an example of one of these plagues (Dong et al., 2020). Crayfish immunity is divided into two branches, cell mediated immunity (which includes phagocytosis and programmed killing of affected cells) and humoral mediated immunity (which includes enzymes and peptides that work against microbes and other pathogens) (Bouallegui, 2021). So, it is possible that the reason the crayfish yielded less colonies than the sediment is because their immune system removed a significant number of pathogens, however, the crayfish's lack of a highly advanced adaptive immune system means that they still contain a fairly large amount of bacteria as can be seen by the consistent prevalence of at least two types of antibiotic-resistant bacteria in each crayfish.

Another observation made is that erythromycin-amended plates yielded the greatest number of CFUs. Erythromycin is a macrolide that is effective against a majority of Gram positive bacteria, as well as some Gram negative bacteria. It acts against bacteria by inhibiting an important translocation reaction during the process of protein synthesis (Washington and Wilson, 1985). Erythromycin is typically prescribed today to treat a wide range of bacterial infections, including bronchitis, pneumonia, Legionnaires' Disease, whooping cough, diphtheria, syphilis and some other sexually transmitted diseases, ear infections, urinary tract infections, and skin infections (medlineplus.gov). Erythromycin is also an older antibiotic that was discovered in 1952, and has been prescribed to people since around that time (Washington and Wilson, 1985). That erythromycin has been in use for so long, and it is used to treat a variety of infections could explain why it was the antibiotic that most bacteria showed resistance to. The more an antibiotic is used, the higher the chance that bacterial strains can develop resistance to it.

Vancomycin yielded the next greatest amount of bacterial growth across the ten samples, and only two out of ten samples did not yield any growth on vancomycin plates. Vancomycin is reserved for use against very serious infections by Gram positive bacteria. It acts by inhibiting cell wall synthesis in the bacteria it targets (Patel et al., 2021). Some of the most common medical conditions that vancomycin is prescribed for are *Clostridium difficile* (C diff) and Staphylococcal infections (Patel et al., 2021). The observation that there were large numbers of some plates is puzzling since vancomycin is typically considered a stronger antibiotic and not used nearly as commonly as erythromycin. A possible explanation regarding growth on the vancomycin plates, as well as the other antibiotics, is that perhaps vancomycin simply is not effective against the bacteria in the pond due to it only targeting specific Gram positive bacteria. Often in freshwater environments, the dominant phylum of bacteria are *Proteobacteria*, which are Gram negative (Yannarell & Kent, 2009). *Proteobacteria* would not be inhibited by vancomycin, therefore, it is likely that they make up the majority of growth on vancomycin plates.

Nine of ten samples yielded bacterial growth on penicillin plates. This is less surprising than vancomycin. Penicillin is well known as the first antibiotic discovered almost a full century ago, and thus I expected to see higher numbers of CFUs on penicillin plates, since bacterial strains have had the longest amount of time to develop resistance against it. It acts by inhibiting the linking of peptidoglycan in the cell walls of bacteria, effectively destroying their cell wall and thus destroying the bacteria itself (Yip and Gerriets, 2021). Penicillin is effective against a wide range of bacteria, both Gram positive and Gram negative, and penicillin can be administered intravenously, intramuscularly, or orally (Yip and Gerriets, 2021). In all of the crayfish except crayfish 4, the number of CFUs/mL of penicillin-resistant bacteria were very low (ranging from 10-50), but yet seven out of the eight crayfish did yield penicillin-resistant growth. It seems that penicillin did kill the vast majority of bacteria, but some of the bacteria in the environment and crayfish were still resistant towards it.

Tetracycline and ciprofloxacin were both effective at inhibiting bacterial growth, Only half of the samples yielded bacterial growth on the plates of these two antibiotics, with a higher number of bacteria being tetracycline-resistant than ciprofloxacin-resistant across the samples. Tetracycline is effective against a wide range of microorganisms, including many types of Gram positive and Gram negative bacteria as well other microorganisms like protozoan parasites (Chopra and Roberts, 2001). Tetracycline acts by inhibiting protein synthesis, thus rendering cells unable to grow, function, or reproduce (Shutter and Akhondi, 2022). Tetracycline is used to treat a variety of medical conditions due to its wide range of targets including infections of the respiratory tract, skin, eye, urinary system, and intestine as well as infections spread by ticks, lice, mites, and any other small parasitic organisms (Tetracycline, National Library of Medicine). Tetracycline is usually prescribed as a capsule to be taken orally, similarly to the other antibiotics discussed so far. Tetracycline in this study proved to be extremely effective at inhibiting bacterial growth. It is known that it is effective against a diverse range of microorganisms, and it is sometimes used against more serious infections that may be resistant to other antibiotics such as MRSA and Vancomycin-Resistant Enterococcus (VRE) (Shutter and Akhondi, 2022). Likely, tetracycline yielded less growth than the other antibiotics so far because it is more versatile in its targets. Unlike vancomycin or erythromycin, tetracycline is able to target more than just a selective amount of Gram positive bacteria. Therefore, the lack of bacterial growth is due to it being successful at inhibiting both Gram negative bacteria, such as the aforementioned *Proteobacteria,* as well as Gram positive bacteria.

Ciprofloxacin yielded the lowest amount of CFUs across the ten samples. The three crayfish samples that did produce bacterial growth on the ciprofloxacin plates had very low numbers of CFU/mL, ranging from 10-120. Ciprofloxacin is a commonly prescribed antibiotic, and it was the fifth most commonly prescribed antibiotic in 2015 with 20.3 million prescriptions that year (cdc.gov). So why did it appear to be the most effective antibiotic against crayfish bacteria? Ciprofloxacin is most effective against Gram negative bacteria (especially Gram negative rods), but it is also effective against some Gram positive bacteria. It acts by inhibiting DNA synthesis in bacteria (Thai et al., 2021) and is primarily prescribed to treat urinary tract infections and STDs such as gonorrhea, but it is also prescribed for respiratory infections as well as infections of most other anatomical locations (Thai et al., 2021). It is mentioned that Gram negative *Proteobacteria* are the dominant type of bacteria found in aquatic ecosystems (Yannarell & Kent, 2009). Therefore, ciprofloxacin would be very effective at inhibiting these bacteria from growing on its plates. The bacterial colonies that did grow on ciprofloxacin plates could have been Gram positive bacteria. One of the next most common bacteria found in ponds after *Proteobacteria* are *Actinobacteria,* which are Gram positive (Newton et al, 2011).

Therefore the bacteria that grew on ciprofloxacin could have been an *Actinobacteria* or another Gram positive bacteria.

Some antibiotic-resistant bacteria are resistant to more than one antibiotic, and they are known as multidrug-resistant organisms (MRDOs) (portal.ct.gov). These multidrug-resistant bacteria can be generated by a couple of mechanisms. The first mechanism being that the bacteria accrues multiple genes with each gene individually coding resistance to a single antibiotic (Nikaido, 2009). The second mechanism is an increase in the expression of a gene that already codes for a multi-antibiotic efflux pump, and this then leads to more antibiotics being ineffective against the bacteria (Nikaido, 2009). The bacterial isolates tested in this study did show evidence of multidrug-resistance. Only one isolate did not yield growth on any of the other four antibiotics, a Gram positive coccus taken from a ciprofloxacin plate. This is likely because the other four antibiotics target Gram positive bacteria, and inhibited this bacterium's growth, while ciprofloxacin, which targets mainly Gram negative bacteria, was ineffective against it. Another Gram positive rod-shaped bacteria from a tetracycline plate (Figure 1) did show evidence of multidrug resistance. It yielded growth on all plates except for ciprofloxacin. Because ciprofloxacin targets Gram negative bacteria, and the bacteria grew on the other four antibiotics, this could indicate specific resistance to those, or there could have been inadequate incubation time for the isolate to grow, or perhaps an error made was made transferring the bacterium to the ciprofloxacin plate.

In conclusion, this study aimed to examine the capacity of freshwater ecosystems and organisms to harbor antibiotic-resistant bacteria. The study specifically focused on crayfish, a type of organism that has been seldom examined in the wild for this purpose. Crayfish clearly have the potential to harbor bacteria in their guts, and this study found evidence of antibiotic-resistant bacteria, including strains that were multi-resistant. However, the study was limited to a small sample size, with crayfish taken from just one environment, and it would be beneficial to continue this research with more crayfish taken from multiple environments. If such environments represented freshwater habitats with different levels of pollution of antibiotics, then the presence of antibiotics could be more easily related to the numbers of antibiotic-resistant bacteria. It is important to conduct studies like this on ecosystems because antibiotic-resistant bacteria are a danger to people and animals alike, and it is important to see how far reaching their presence can be.

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