Sinister Superbugs Skulking in Streams?

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**Abstract**

Urban pollutants, such as heavy metals and antibiotics, affect most of the waterways in the United States. While the effect of these pollutants are well understood for many aquatic organisms, their effect on stream microbial communities is not well studied. In Upstate New York there is a history of industry that dumped its heavy metal waste into streams; this practice has since stopped, but the effects may still be there. It has been shown that active heavy metal pollution can lead to antibiotic resistance, but it is unknown if legacy heavy metal pollution can have the same effect. By growing stream bacteria on four different treatments (unamended agar, agar with copper, agar with ciprofloxacin, and agar with copper plus ciprofloxacin), we aimed to determine the effects of legacy heavy metals and other urban pollutants on antibiotic resistance in stream bacteria, and identify any microbial community shifts.

**Introduction:**

Microbial communities are integral to the structure and functioning of stream ecosystems, and while urban and industrial pollutants affect nearly every stream in the United States, we don’t know the intricacies of how the microbial communities are impacted by those inputs. Streams are heterotrophic environments, and therefore essentially all of the energy (in the form of carbon) that cycles throughout a stream, passes through microbial communities to be decomposed and incorporated into material usable for higher trophic level organisms (Kaplan and Bott et al., 2006). Because of the significance of this role, it is very difficult to predict any affects a change in the microbial population will have on stream health. All kinds of human sourced pollution have deleterious effects on our environment. Industrial waste, wastewater effluent, urban runoff, landfills, plastic substances, agricultural runoff and many other pollutants have caused serious detrimental changes to the environment by affecting everything from bacteria to mammals (Huddleston et al., 2006). As the types of pollutants that make their way into streams and waterways continues to change, new biogeochemical interactions will occur and potentially have harmful and unpredictable effects on the environment. Current research into the effects of novel substances like microplastics, polychlorinated biphenyls (PCBs), caffeine, antibiotics, hormones and other chemicals continues to show the disruption these pollutants can cause to aquatic ecosystems (Rosi-Marshall et al., 2013). Many of these pollutants are found in streams at only trace levels, but persistent and long-term exposure, even at low concentrations, may have significant consequences, particularly on small organisms such as bacteria (Ellis, 2006).

In this study we examined a new environmental issue caused by an old pollutant: heavy metals. In many parts of the United States, including upstate New York, towns were built in the late 19th century around industries no longer active today, including tanneries and paper mills (Starr, 2010). These factories discharged wastewater rich in heavy metals into small headwater streams. Due to economic changes these industries were abandoned many decades ago, however the metals released into the environment are still present in the sediment bed of the streams (Starr, 2010). This study endeavoured to determine if these legacy metals are still impacting modern microbial communities and thus altering stream ecosystems as a whole.

Recent research has found that exposure to heavy metals can result in antibiotic resistant bacteria. This has been demonstrated in the environment in areas with current heavy metal point sources, like ash settling basins in coal fired power plants (Calomiris et al., 1984; Gadd et al., 1978). However, the effect of historical heavy metal pollution on antibiotic resistance in bacteria is still unknown.  It is well understood that improper usage of antibiotics can lead to antibiotic resistance (Martinez et al., 2009), and as the use of antibiotics has increased the development of antibiotic resistant strains of pathogenic bacteria has become a serious health concern (da Silva et al., 2007). New research is showing that heavy metal pollution may be a significant source of antibiotic resistance in the environment (Matyar et al., 2008). The way in which each of these pollutants affect bacterial communities in the environment is different and understanding their interactions and mechanisms of action is essential for determining appropriate strategies to mitigate the effects caused by these pollutants (Silver and Misra 1984).

*Heavy Metals*

Heavy metals are naturally occurring in the environment and are introduced into aquatic systems through weathering of rocks and soil, as many heavy metals are a component of the earths crust. The term heavy metals describes any metallic element that has an atomic density greater than 4 g/cm3 and some common heavy metals are lead, cadmium, zinc, mercury, arsenic, copper and iron (Duruibe et al., 2007). Heavy metals are persistent in the environment because they cannot be degraded, and high concentrations of heavy metals have detrimental implications for both the ecosystem and human health.

In addition to natural occurrences, a major source of heavy metals in the environment is anthropogenic causes, namely pollution. The main source of heavy metal pollution is mining and other industrial activities (Duruibe et al., 2007). Abandoned mines continue to contaminate streams and groundwater long after mining activity stops due to ongoing seepage and runoff, and metals released from factories remain in the environment due to the persistent properties of heavy metals (Schaider et al., 2014).

*Antibiotic Resistance*

An organism is antibiotic resistant when it is able to combat the effect of antibiotics, thus rendering the antibiotic ineffective. The result is that antibiotic products, which are our predominant tool in combating bacterial infections, are made useless. Many bacteria possess natural antibiotic resistant qualities and produce antibiotics themselves, though there are anthropogenic sources that increase the frequency of antibiotic resistance in bacteria (Huddleston et al., 2006). The primary way that bacteria develop a resistance is through exposure to antibiotics. Because some bacterium naturally possess resistant qualities, when there is large scale exposure to antibiotics, many bacteria will die, but individual bacterium or certain species with higher resistance will survive. This allows the resistant bacteria to proliferate, while the affected bacteria will perish, thus increasing the frequency of resistant genes in the population. Bacteria are often exposed to antibiotics unintentionally through pollution. Most antibiotics introduced into waterways originate from human sources such as hospitals and farms as a result of medications and sterilization procedures (Baquero et al., 2008). As much as 30-90 % of a dose of antibiotics administered to humans or animals is excreted through urine as active substances (Rang et al., 1999). It has been demonstrated that since the onset of widespread antibiotic usage, the frequency of resistant bacteria has increased drastically, (Huddleston et al., 2006) particularly in areas with high concentrations of human waste (Tomoney et al., 1978; Ternes et al., 2002). Kolpin et al (2002) found more than 15 different types of antibiotics in streams receiving urban and agricultural runoff, out of 139 US streams tested, 22% contained detectable concentrations of antibiotics. This suggests that human antibiotic use is directly affecting the way in which stream bacteria are developing and evolving, however most streams do not have detectable levels of antibiotics.

Once in the population, the antibiotic resistant genes from one bacterium can be inserted into genetically mobile platforms such as transposons, integrons or plasmids. The traits can be shared with other stream and soil bacteria, including potentially pathogenic species (Baquero et al., 2008). This can have widespread effects on the environment, such as decreased biodiversity (Matinez, 2009; Kong et al., 2006), altered biomass and alterations to algal structuring, as well as changes in nutrient processing and natural food web functioning (Halling-Sorenson et al., 1998). Instances of antibiotic resistant bacteria have been found in raw sewage, untreated stream water, and drinking water (Calomiris et al., 1984). The ability for bacteria to perform horizontal gene transfer means that if any pollutant has selected for certain resistant gene, that resistance can spread.

*Heavy Metals and Antibiotic Resistance*

Many studies have shown that heavy metals can cause antibiotic resistance in bacteria. Specifically, heavy metal pollution in streams can lead to increased antibiotic resistance in stream bacterial communities (McArthur et al., 2000; Tuckfield et al, 2008). However, the areas around Saratoga Springs, NY are no longer releasing heavy metals into streams and instead have trace amounts of the metals, which have been present for many decades (Starr, 2010) and the potential effects of these legacy metals is unknown. While the antibiotic levels in many U.S. waterways are very low, metal contamination is often high at industrial sites and heavy metals persist for long periods of time in stream sediments, (Stepanauskas et al., 2006) which may indicate that metal contamination, and not antibiotic pollution, is leading to antibiotic resistance.

Both environmental and laboratory studies have confirmed the relationship between current heavy metal exposure and antibiotic resistance. Wright et al. (2006) analyzed ash settling basins in coal-fired power plants as sites heavily contaminated with metals and further analyzed reference sites free of contamination. They found that antibiotic tolerance levels increased with pollution levels, which suggests that metal pollution is acting as a selective pressure for antibiotic resistance (Wright et al., 2006). Other studies have examined this phenomenon in the lab using freshwater microcosms and have found that metal amendments lead to increased resistance not only to metals, but antibiotics as well. The inverse relationship was also observed; bacteria exposed to antibiotics were tolerant of higher metal concentrations (Stepanauskas et al., 2006). While past research has confirmed the relationship between metal exposure and antibiotic resistance, the effect of long term, low level exposure has not been examined.

Determining the mechanisms that cause metal exposure to increase resistance to antibiotics is another aspect to this emerging research. Many genes that encode for metal and antibiotic resistance are commonly found on the same plasmids or transposons (Stepanauskas et al., 2005). This is described as co-resistance, which occurs when the genes that code for the resistant phenotypes are on the same genetic element (Baker-Austin et . al., 2006). It has been demonstrated that metal resistance and antibiotic resistance are often found on the same plasmid. One study showed this by removing a plasmids from an antibiotic resistance bacterial strain, which then  became sensitive to certain metals and antibiotics. Another mechanism for the co-selection of metal and antibiotic resistance is cross-resistance. This occurs when different antagonistic agents have the same method or pathway to cause cell death. Therefore, by developing a method to combat one type of antimicrobial agent, the organisms also develop a way to combat unrelated agents that have the same method of causing cell death. An example of this type of resistance is an efflux pump developed to combat heavy metal exposure that is also effective against antibiotics (Baker-Austin et al., 2006).

While the effects of ongoing heavy metal pollution have been well studied in larger freshwater systems (Duruibe et al. 2007; Merroun, 2007; Schaider et al 2014), few studies have examined the effects of legacy heavy metals on small stream ecosystems, such as those in the Saratoga Springs area. None of the sites examined in this study contain sources of significant modern heavy metal inputs. The effect of legacy metals on microbial communities may follow the trends found by studies examining areas of modern metal pollution, but they may also be significantly different. If metals deposited historically have been sequestered by sediments or severely diluted, they may have no effect on the microbial communities. However, persistent exposure, even at low levels, may have surprising consequences for the microbial environment.

*Saratoga Springs*

Historically the Saratoga county area of upstate New York was heavily industrialized with factories and industrial sites commonly located on, or in close proximity to streams and rivers. As a result, there are high levels of toxins and heavy metals that continue to persist within different aquatic communities throughout the area (Starr, 2010). In addition to a history of metal contamination, streams such as Spring Run, in Saratoga Springs, flow through urban centers, picking up contaminants and pollutants from human waste, including, but not limited to antibiotics (Yang and Carlson, 2003). The inputs in these waterways make the streams in Saratoga prime candidates for increased antibiotic resistant populations.

The intersection of industrial pollution and waste water discharge make Saratoga an excellent location to study the effects of legacy metals and urban runoff on antibiotic resistance in stream bacteria. In this study we will look at the effects of legacy metals and urban runoff on the community structure and functioning of stream bacteria, in order to understand if antibiotic resistance in the stream is a function of legacy metals, antibiotic pollution, or some combination. We will also attempt to induce antibiotic resistance in bacteria in a controlled, laboratory environment by exposing stream bacteria from an uncontaminated site to heavy metals. The legacy heavy metal pollution in Saratoga Springs may be leading to the accumulation of antibiotic resistant bacteria in the local environment, which could pose a threat to the entire stream ecosystem.

It is the intention of this study to determine the effect that different pollutants in Saratoga area streams are having on the antibiotic resistance and community structure of stream bacteria. Three streams, a reference site, urban site, and site of past industry were examined. We expect that the reference site will show the greatest responses to exposure to copper and the antibiotic ciproflaxin, in the form of decreased respiration, while the urban and legacy metal sites will be less sensitive to these contaminants. We also expect that microbial diversity will be greatest at the reference site while the other streams will show higher tolerances to metal and ciproflaxin exposure, but have less community diversity. This study provides a new perspective on the issue of heavy metal pollution with implications for future environmental management and policy.

**Methods**

*Study Sites*

Site 1: Hans Creek (Tributary of Lake Desolation), Middle Grove NY (43.177, -73.992)

A stream located in a sparsely developed forested area near the Adirondack Mountains. It is currently listed as not impaired by the New York Department of Environmental Conservation (NYDEC).

Site 2: Spring Run, Saratoga Springs NY (43.089, -73.770)

An urban stream located in close proximity to downtown Saratoga Springs (pop. 27,000) that receives urban runoff from the surrounding area((McCarty, 2012). Spring Run is currently listed on the EPA’s 303(d) list of highly impaired waters(EPA).

Site 3: Cayadutta Creek, Gloversville NY (43.010, -74.376)

A stream that has been historically impacted by pollutants from nearby tanneries (Starr, 2010). Pollution from tanneries includes a variety of heavy metals including; cadmium, zinc, iron, lead, nickel, chromium, copper, and others (Deepali and Gangwar 2010). Cayadutta Creek is currently listed as slightly impaired by the NYDEC.

In order to test the growth of stream bacteria in the presence of different pollutants, we deployed CuSO4, ciprofloxacin, and combined CuSO4/cipro diffusing substrata into three streams in the Saratoga Springs area. The creeks chosen were Spring Run, an urban stream, Cayadutta creek, and industrial stream, and Hans Creek, a forested site.

To construct the substrata we followed the procedure detailed in Rosi-Marshal et al. (2013). We used 0.36 g/l of CuSO4, 0.013 M ciprofloxacin, and a combination of CuSO4 and ciprofloxacin at the same concentrations (agar of 3 % by weight was used for the combined substrata), and we also prepared control cups of plain agar. We capped each substrate with a cellulose sponge to promote colonization by bacteria, and ten replications of each condition were prepared for each stream location. Cellulose sponges were used to record biofilm heterotroph response (Rosi-Marshal et al. 2013). The cups were deployed and secured in the stream in the same manner, and for the same duration as stated by Rosi-Marshal et al. (2013). The arrays were left in the streams for either 18 or 19 days starting in the beginning of November and were removed near the end of November.

When transporting the substrata from the stream back to the laboratory, they were kept in a cooler and wrapped in cellophane.

*Bacterial Respiration*

The dissolved oxygen was measured for each of the treatments according to the methods described in Rosi-Marshall et al. (2013).

The respiration was measured for each site on the same day the arrays were removed from the stream. The substrates (cellulose sponges) were removed from the arrays and placed in 50 mL centrifuge tubes filled with filtered stream water. The stream water was filtered using a Millipore filter (Bedford, MA) with a pore size of 0.45μm.The tubes were capped underwater to minimize the amount of air trapped in the container and the initial DO was measured while the tubes were submerged. The subsequent DO measurements were taken twice more after 2-3 hours and 13-15 hours. DO was measured using a handheld optical meter (ProODO meter; YSI, Yellow Springs, Ohio, USA).

The centrifuge tubes were wrapped in aluminum foil to prevent light from affecting the samples and they were stored in a dark room with minimal light. The DO was also measured in five blank tubes for each site containing only filtered stream water to account for background changes in DO.

*Community structure: 16s rRNA Analysis*

In order to assess any changes in community structure, such as diversity and genus composition, 16s rRNA analysis was utilized to build a community profile for each site and treatment type. One sponge from each of the four treatments from the three sites was suspended in 20 ml of sterile water and vortexed thoroughly. 100 microliters of this solution was pipetted onto half strength R2A plates and 10-1 and 10-2 dilutions were prepared and 100 microliters of each solutions was also plated onto half strength R2A plates. The solution was spread using a flame sterilized glass hockey stick. The spread plates were incubated at room temperature for 24 hours. The colonies on each plate were isolated using standard streak plate techniques. The streak plates were incubated at room temp for 48 hours and then refrigerated. Standard PCR was carried out on the isolated colonies to amplify the 16S rRNA gene. The DNA was purified and sent for analysis at Yale University. The sequenced data was run using BLAST analysis and the genus of each sample was determined.

*Statistical Analysis*

An Anova was run on the respiration data to determine if the the communities varied between the treatments within each site, and the communities between the sites responded differently.

We examined the effects of sites, treatments, and the interaction between treatment and sites on biofilm respiration rates using a two-way ANOVA. The respiration levels at each site were normalized by creating a ratio between the respirations of each treatment in relation to the respiration of the control treatment; this ratio was called the Response Ratio.

**Results**

At the forested reference site, additions of copper, ciprofloxacin, and copper plus ciprofloxacin led to a decline in biofilm respiration rates (One way ANOVA, F<0.0001, p<0.05). The average respiration rate in the control treatment (0.12 ± 0.0075~~)~~ was three times higher than in the Cu and Cipro treatments (0.036 ± 0.0068 and 0.052 ± 0.012, respectively) (Tukey Post-Hoc, P<0.001). The copper treatment and ciproflaxin treatment had similar respiration rates and were not statistically different from each other. However, the average respiration rate for the CipCu treatment was significantly lower than other three treatments (0.004 ± 0.009, p<0.001)(Figure 1).

At the urban stormwater site, biofilm respiration rates did not vary with the addition of copper, cipro, or copper plus cipro (One-way ANOVA, F=0.4859, p>0.05)(Tukey Post-Hoc, P<0.001) (Figure 2). The plain and copper treatments had the same average respiration (0.27 ± 0.039, 0.032 respectively). The CipCu treatment had a slightly, though not significantly lower average respiration rate (0.24 ± 0.022), and the Cip had the lowest respiration at Spring Run with an average rate (0.21 ± 0.029).

Similarly, at the legacy industrial waste site, biofilm respiration rates were statistically equal across a four treatments (One way ANOVA, F=0.0554, p<0.05)(Tukey Post-Hoc P<0.001)(Figure 3). The plain treatment had the highest average respiration (0.23 ± 0.009), followed by the copper (0.22 ± 0.026). The average respiration for the Cip treatment was slightly lower than the control and copper (0.21 ± 0.028) while the CipCu had the lowest respiration rate (0.15 ± 0.018).

Respiration response ratios varied significantly across sites (F<0.0001, p<0.01) and treatments (F<0.0001, p<-0.05). In addition the interaction between site and treatment was significant indicating that the site has a significant effect on biofilm response to treatment (F<0.0001, p<0.05). At forested, urban and industrial sites the copper treatment had the highest response ratios (0.42 ± 0.094, 1.01 ± 0.12 and 0.96 ± 0.12 respectively). The forested site had the lowest response ratio with the copper treatment, less than one half of either of the other two sites; in the urban and industrial sites the respiration ratios were not significantly different from 1, indicating virtually no change in respiration. The urban site had its lowest response ratio in the cipro treatment (0.78 ± 0.11). In the forested site the ratio in the cipro treatment was lower than that in the urban site by almost three times (0.29 ± 0.053); the industrial site had a slightly higher ratio than the urban site in the cipro treatment (0.93 ± 0.11) The ratio in the forested and industrial sites was the lowest when exposed to the copper plus cipro treatment (-0.03 ± .069 and 0.66 ± 0.082 respectively), in the urban creek there was a slightly increase between the cipro treatment to the copper plus cipro (0.89 ± 0.084). The forested site had the lowest ratios in every treatment (Figure 4).

In the forested site the number of genre present at each treatment increased from only one genre (Pseudomonas) on the control treatment, to two on the copper, three on the cipro, and four on the copper plus cipro. The genus present on the control treatment was not present on any of the other treatments (Figure 5).

A similar trend was seen in the urban site. The control treatment had a greater number of genera present than the copper site, but from copper to cipro, and cipro to copper plus cipro, the number of genre present increased (Figure 6).

The pattern present at the industrial site was inconsistent with the other two sites. The copper treatment had the most genera, and then the control and cipro treatments, and the fewest genera were present in the copper plus cipro treatment. However, the two most abundant genera in the copper and cipro treatments are the only two genera present in the copper plus cipro treatment (Figure 7). While these abundance data indicate trends of changes in microbial community compositions, we were unable to run statistical tests on these data due to a lack of replication.

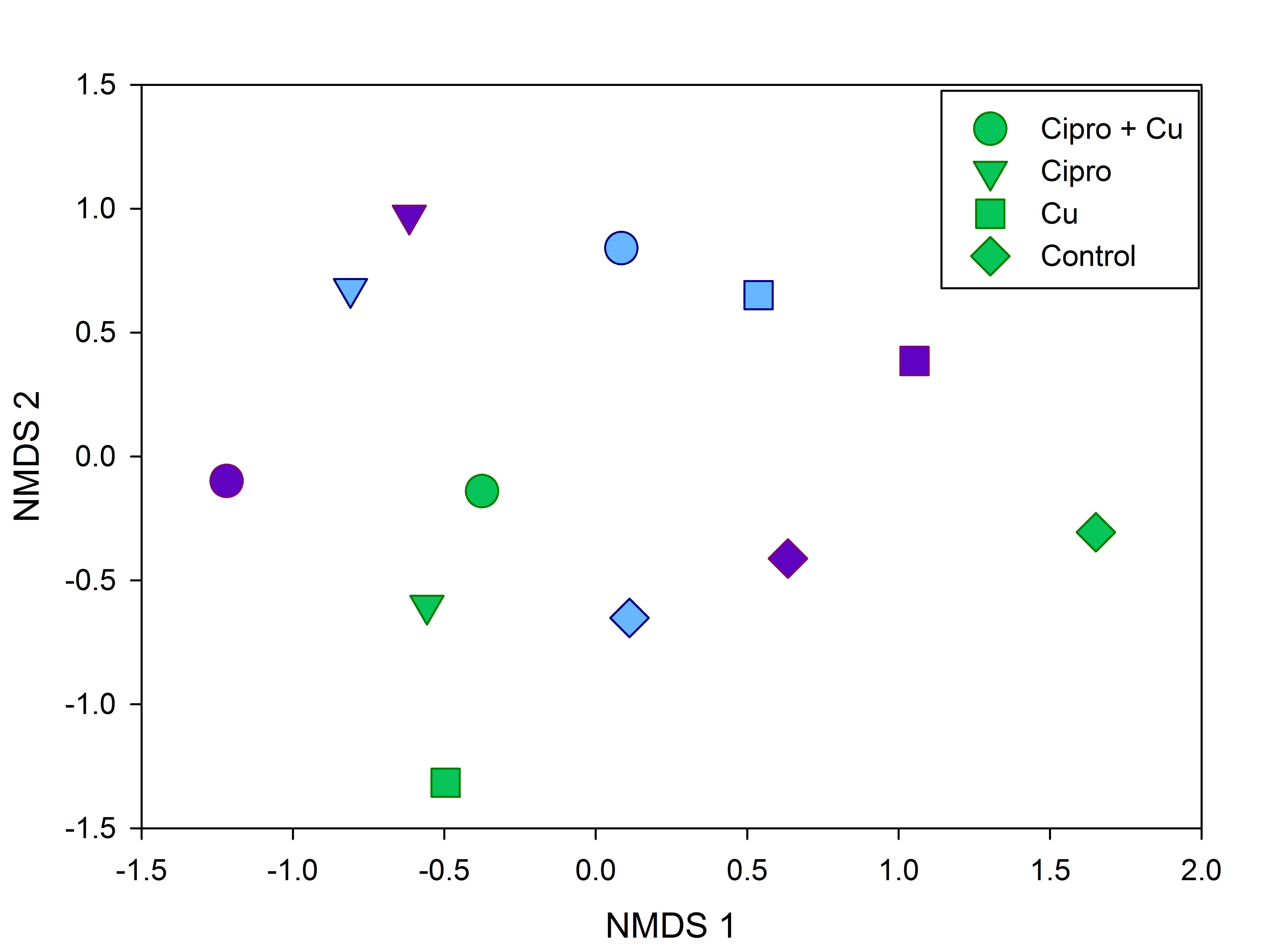


Figure 7. Similarity metric of genus-level microbial community composition of forested (green), urban (purple), and industrial (blue) streams when grown on unamended agar (diamond), copper (square), cipro (triangle), or copper plus cipro (circle).

Our NMDS indicated shifts in the communities both between sites and after the addition of treatments. The urban and industrial control communities were much more similar to each other than to the forested control community. Once the treatments were added to the forested site, the communities shifted to become similar to those in the urban and industrial controls. When the treatments were added to the urban and industrial sites, their communities (except the urban cipro plus copper) shifted to be similar in community composition to each other, and dissimilar to any of the control treatments. Again, we were unable to perform statistical tests on our NMDS due to a lack of experimental replication.

**Discussion**

Our data indicate that copper, ciprofloxacin, and the combination of the two pollutants affected the respiration rates in streams differently. This demonstrates that each substance may be having a different effect on the microbial communities in the streams. The differences in respiration changes elicited by each treatment suggests increased resistance to certain treatments. Each treatment decreased the respiration in the streams, but the copper treatment had the highest response ratio, indicating that it, overall, affected stream respiration the least. This indicates that the stream bacteria communities in the Saratoga area may have a higher resistance to copper than to ciprofloxacin. This may be a result of natural resistance to copper, or induced resistance as a result of exposure to copper from natural sources (Duruibe et al., 2007). However, the increased resistance to copper may be a result of chronic exposure to the pollution from years of industry in the Saratoga area (Schaider et al., 2014; Starr, 2010). The ciprofloxacin treatment affected the respiration on average more than the copper treatment. The combination treatment of CipCu caused the greatest decrease in stream respiration, greater than copper or ciprofloxacin alone in the industrial and forested creeks. This may indicate that there is not a complete overlap in the substances to which the bacterial communities are resistant, suggesting that copper resistance is not always indicative of antibiotic resistance.

In addition to the treatments affecting respiration differently, our data indicate that each site responded differently to the treatments. The respiration rate at the forested site was lowered by the treatments much more than the respiration rates at the other two sites. Because the forested site was our unpolluted, pristine site, this pattern may indicate that the bacteria in our other sites are acquiring resistance to the treatments. This creek is far from sources of copper or ciprofloxacin pollution, meaning that the bacterial communities have not been exposed to those substances. The urban site has many pollutants in it, particularly antibiotic and other personal items (Yang and Carlson, 2003). The industrial site has a history of heavy metal pollution (Schaider et al., 2014; Starr, 2010), exposure to these heavy metals may have allowed the bacterial communities to develop resistance to the copper treatment, which may have also resulted in antibiotic resistance; the history of heavy metal exposure may have reduced the impact of all of the treatments by co-selecting for both heavy metal and antibiotic resistance (McArthur et al., 2000; Tuckfield et al, 2008).

Each treatment affected the forested site the most, and each site was most heavily affected by the CipCu treatment. The urban site was the least affected by the copper and CipCu treatments. The industrial site was the least affected by the ciprofloxacin treatment. This further suggests that the history of heavy metal pollution in the river has lead to increased resistance to ciprofloxacin a well as copper.

At the forested site the respiration rates of the four treatments varied significantly. The three treatments were significantly different from the control, indicating that within this site the presence of heavy metals and antibiotics lowers the respiration rates and affects the survival of the microbes. The Copper and Ciprofloxacin treatments had significantly lower respiration rates than the control, and the combined Ciprofloxacin plus Copper treatment had the lowest respiration rate.  Because when bacteria were exposed to only one substance, either copper or ciprofloxacin, they maintained activity more than when they were exposed to the combination, it suggests that some bacteria are resistant to either copper or cipro alone, but few are resistant to both.  Since the forested site is not directly affected by human related inputs of heavy metals or antibiotics, it was expected that resistance would be low at this site. This site is located in a remote area that is relatively devoid of human related pollution and the NYDEC assessed the water quality of the creek as non-impacted, with diverse macroinvertebrate communities.The differences in respiration between the control and the treatment groups demonstrates that the bacterial communities at this site were not resistant to heavy metals and antibiotics.

At the urban and industrial sites the bacterial respiration rates were the same for all four treatment groups, including the control. Each treatment had effectively the same respiration rate, indicating that the bacterial communities in these streams were not affected by the pollutants. Past studies have shown that exposure to heavy metals can lead to a resistance to both heavy metals and antibiotics (McArthur et al., 2000; Tuckfield et al, 2008). The urban site is classified as impaired by the NYDEC and is on the 303(d) list of impaired water bodies as a result of inputs of runoff containing sewage and pollutants. Antibiotics often enter streams through wastewater, urban runoff, agriculture, and other sources and it would be expected that streams located in urban areas would have higher anthropogenic inputs of antibiotics that would lead to antibiotic resistant bacteria. Thus it is not surprising that the bacteria treated with the cipro within the urban site had the same respiration rate as the control. The bacteria at this site have most likely been previously exposed to antibiotics, and are thus resistant to antibiotics. The bacteria in the heavy metal treatment also had the same respiration as the other three groups, even though there was not a point source of heavy metal pollution in the urban site. Research has shown that heavy metal exposure can result in co-resistance in which the same plasmid encodes for heavy metal and antibiotic resistance. Since antibiotic and heavy metal resistance are often related through co-resistance, it would be expected that exposure to just one of these factors would result in resistance in both. The industrial creek is classified as slightly impacted (DEC), and the sample site has been historically polluted with inputs of heavy metals from a nearby tannery (Starr, 2010). Within the industrial site the bacterial respiration rates were all the same, which shows that treatment with heavy metals and antibiotics did not negatively impact the survival of the microbial communities. All the treatments at both the urban and industrial sites did not impact the respiration of the bacterial communities, which indicates that the bacteria at both sites had mechanisms to deal with the presence of heavy metals and antibiotics.

The composition of microbes at each site shifted when the bacteria were exposed to our treatments. In the forestd site the control community was composed completely of *Pseudomonas* and once treatments were added this genus was lost and others appeared. There was the greatest diversity of genera in the combined cooper-cipro treatment. The urban site had the greatest number of genera overall and the same trend of highest diversity in the combined treatment was also seen. The treatments could be killing off the most abundant bacteria present in the stream, allowing other less common genera to colonize this sponge. This would explain the higher diversity in the combined copper-cipro treatment. In the industrial site this trend is not present, however there is an interesting pattern in the structure of the communities of each treatment. In the copper treatment one third of the community is *Flavobacterium* and in the cipro treatment over half the community belongs to *Methylobacterium*. The combined treatment at the industrial site is composed entirely of these two genera and this suggests that these genera are the most resistant to copper and cipro.

When the composition of the control treatments are compared there is a clear pattern. *Pseudomonas* is the only genus in the forested control and is present in both the urban and industrial controls. When copper is added to the forested site *Duganella* appears, which is also found in the industrial control. In the cipro and combined treatments at the forested site we see *Janthinobacterium*, which is also found in the urban control. Finally, in the combined treatment at the forested site 20% of the community belongs to *Aeromonas*, which is found in both the industrial and urban controls. This pattern supports our NMDS findings which shows that when  the treatments were added to the forested site the communities shifted to become more like the urban and industrial controls. This suggests that the bacterial communities in our urban and industrial streams have already been altered do to exposure to heavy metals and antibiotics.

Many of the genera found at our sites have been associated with heavy metal contamination in soil, including *pseudomonas, janthinobacterium, duganella, bacillus, flavobacterium* and *serratia* (Karelova et al. 2011, Kuffner et al. 2008).The genus *aeromonas*, found at all three sites, is a known human pathogen and causes a variety of human diseases (Janda and Abbott 1998). All of the bacteria found at our sites belong to 3 phylum proteobacteria, alpha, beta, and gamma, as well as bacteroides and actinobacteria all of which have been found in soils contaminated with heavy metals (Navarro-Noyo et al. 2010).

Our respiration data shows that the bacterial communities at Cayadutta Creek and Spring Run are more resistant to heavy metals and antibiotics then the control site, Hans Creek. The only way this resistance can develop is through exposure to these pollutants. While the waste water an urban pollution at Spring Run may contain antibiotics as well as heavy metals, it is less likely that Cayadutta Creek has a major source of antibiotic pollution. Therefore, any antibiotic resistance found at that site may be a result of heavy metal exposure. The composition of the bacterial communities at each of our sites shifted when exposed to copper and ciprofloxacin and the forested site became more like the controls of the urban and industrial sites when exposed to these pollutants. Stream ecosystems rely on a specific composition of bacteria to perform essential functions such as decomposition and nutrient cycling. Therefore,any change in the structure of stream bacterial communities due to urban pollution could have significant detrimental affects on stream health. We also found genre that are potential human pathogens, which raises the concern over “superbugs” and the proliferation of antibiotic resistant bacteria in the environment.

In future studies a minimal inhibitory experiment could be conducted to determine the amount of ciprofloxacin and copper that is required to inhibit bacterial growth. This would provide the baseline levels to use for another experiment to induce antibiotic resistance through exposure to the heavy metal copper. These studies would further support previous research and be a useful reference when investigating persistent heavy metals in the environment.

Using replicates when analyzing the microbial community structure would significantly improve this study. One sponge from each treatment and site was used to culture bacteria and compare the microbial communities. It would be beneficial to use more than one sponge in order to get a more accurate picture of the community structure and allow for more advanced statistical analyses.

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