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Potential impact of secondary wastewater treatment plant effluent on the concentration and antibiotic resistance of bacteria in river water

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Abstract

Effluent from wastewater treatment facilities can have a major impact on the bacterial populations in water downstream of the outfall point. We sought to assess the impact of wastewater effluent from the Northwest Langley Wastewater Treatment Plant on the concentration of bacteria and the occurrence of antibiotic-resistant bacteria in the Fraser River. We hypothesize that effluent from the plant will increase the amount of antibiotic-resistant bacteria downstream of the plant. In order to assess this, we took five samples of water downstream and five samples of water upstream from the treatment plant's outfall point and cultured the bacteria in these samples on Mueller Hinton agar, with half of the agar plates containing Ampicillin. We then counted the number of bacterial colonies that grew on each plate. Our results displayed that there were the same amount of bacteria downstream compared to upstream from the wastewater outfall point. This may be because secondary wastewater treatment is effective enough to remove antibiotics and other toxins from the wastewater effluent. We also observed that antibiotic resistance can be detected in the bacteria before they reach the wastewater effluent. This may be due to exposure to other compounds in the water or from changes in the river flow patterns that allow for the upstream bacteria to come in contact with the effluent. Our results suggest that substances present in wastewater effluent in the Fraser river do not reduce the quantity of bacteria in river water.

Keywords — Ampicillin, Sewage treatment, Aquatic bacteria, River contamination

1. Introduction

Treatment of sewage water is important in urban societies for preserving the quality of civic water. Current sewage treatment methods, especially in secondary and tertiary wastewater treatment plants (WWTPs), were found by Environment Canada [1] to be effective at treating sewage; secondary treatment removes approximately 85% of suspended solids and biochemical oxygen demand (BOD), while tertiary treatment removes as much as 99% of wastewater impurities. Suspended solids refers to large visible solid particles in the effluent. BOD accounts for the amount of oxygen dissolved in the river water used by aerobic organisms at 20 °C in five days. These

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conventional metrics do not account for other problematic pollutants. Many studies raise concerns about the occurrence of biologically active substances, specifically antibiotic substances, in river water downstream from WWTPs [2, 3, 4]. While it is unlikely that the treatment plants themselves are the sources of resistance, as they do not use antibiotics in their filtration process, it is possible that they do not remove all of the antibiotic substances that were in the water beforehand [1]. Antibiotics from wastewater effluent have been measured in rivers at distances of up to 500m downstream from WWTPs [3]. This may have impacts on the sensitivity of the local micro-organisms to these antibiotics, thus rendering them less effective. A study conducted by Drury et al. [5] has shown a significant decrease in the overall number of bacteria downstream of two secondary WWTPs in two rivers in Chicago, IL. They hypothesize that unfiltered antibiotic substances from WWTP effluent may play a role in this decline in bacterial concentration.

Conventional medical treatment practices that promote the overuse of antibiotic agents has contributed to an increase in antibiotic resistance, which may have deleterious consequences for the future of biomedicine [5, 6]. The increased exposure of aquatic bacteria to antibiotics due to WWTP effluent may increase the prevalence of antibiotic-resistant bacteria (ARB) in receiving bodies of water. Indeed, numerous studies have demonstrated an increase in the quantity of aquatic ARB downstream from a WWTP when compared to bacteria upstream from a WWTP [2, 3, 7, 8, 9, 10]. However, there is a lack of research investigating the effect of WWTP effluent on the bacteria at the North Langley WWTP. Indeed, this is the first study to investigate the impacts of a WWTP on bacteria or the implications for antibiotic resistance in British Columbia.

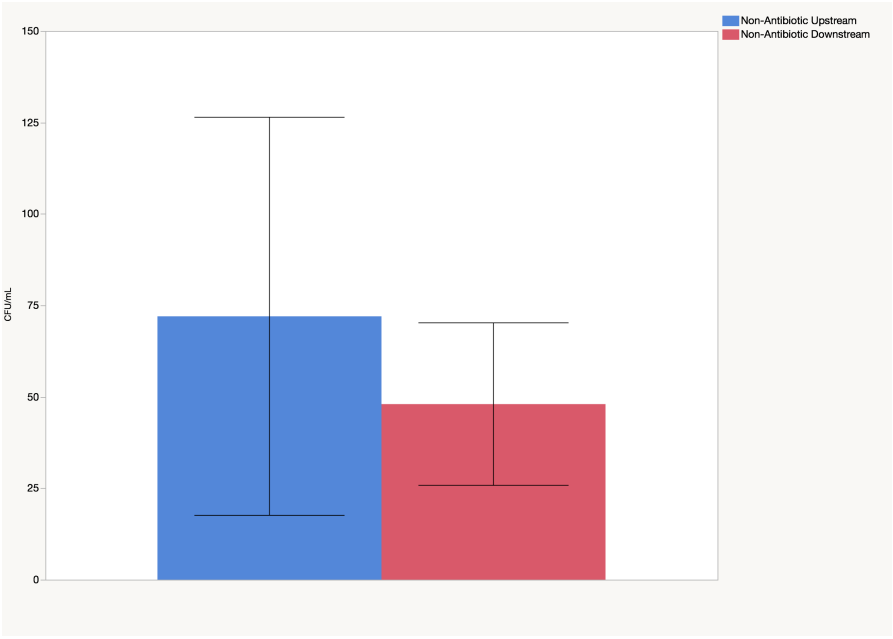

Figure 1: *Comparison of the number of bacterial colonies on non-antibiotic plates from the Downstream site and the Upstream site with error bars for 95% confidence intervals.*

This study compares (I) the concentration and (II) the antibiotic resistance of bacteria

2.8km upstream and 1.7km downstream of the Northwest Langley WWTP–an urban secondary WWTP on the Fraser River in British Columbia, Canada. We chose to test at these locations because they were the most accessible. To determine bacterial concentration and resistance to antibiotics, water samples were taken from these locations and plated on Mueller-Hinton agar, half of which contained ampicillin. We hypothesize that there will be (I) a decrease in overall concentration and (II) an increase in antibiotic resistance in the bacteria downstream from the WWTP.

2. Results

2.1. Comparison (I): Non-antibiotic plates

Figure 1 shows that there was no significant difference in the number of colony forming units (CFUs)/mL on non-antibiotic plates from the Upstream site than the Downstream site; $t(8) = 1.1339$, $p = 0.2897$.

2.2. Comparison (II): Antibiotic plates

We found that there was no significant difference in the number of ARB between the Upstream site and Downstream site (Figure 2); $t(8) = 0.5108$, $p = 0.6233$.

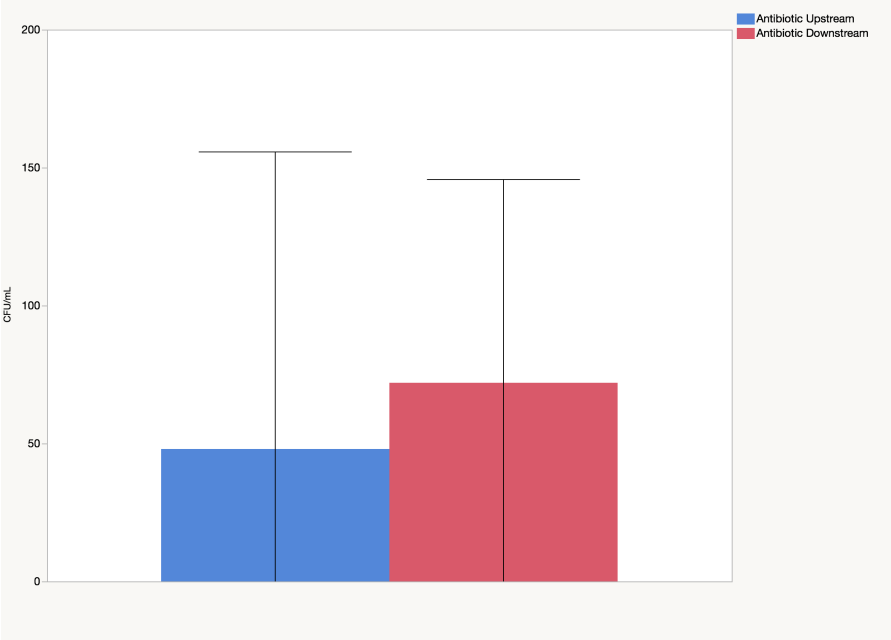

Figure 2: *Comparison of the number of antibiotic-resistant bacterial colonies on antibiotic plates from the Downstream site and the Upstream site with error bars for 95% confidence intervals.*

2.3. Comparison (III): Upstream plates

We found that there was no significant difference in the average number of culturable bacteria versus ARB at the Upstream site; $t(8) = 0.8660$, $p = 0.4117$.

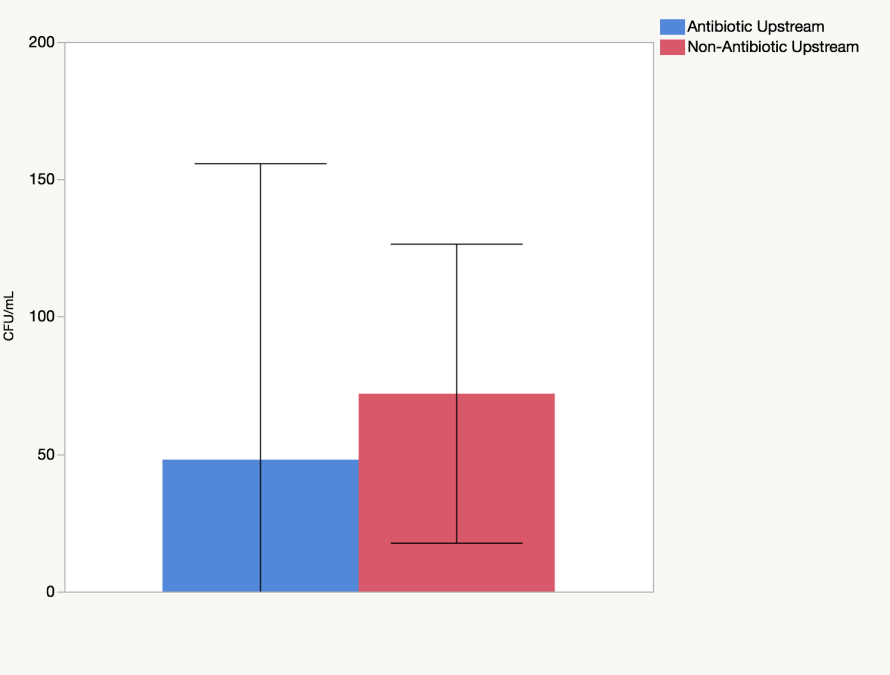

Figure 3: *Average number of culturable bacteria versus antibiotic-resistant culturable bacteria from the Upstream site with error bars for 95% confidence intervals.*

2.4. Comparison (IV): Downstream plates

We found that there was no significant difference between average culturable bacteria versus ARB at the Downstream site; $t(8) = 0.5523$, $p = 0.5958$.

3. Discussion

3.1. Comparison (I)

The results suggest that secondary treatment does not reduce the number of bacteria. This may mean that the WWTP does not release any substances that could be toxic to the bacteria in the Fraser River (Figure 1). This result may also suggest that secondary treatment is effective at removing antibiotics substances from wastewater. Another possible explanation could be that our testing site was far enough downstream for the WWTP that the effluent no longer had an effect on the CB. However, it is unknown whether this decrease in bacterial concentration is due to the WWTP or to other factors such as nutrient levels, interactions with other organisms, or sunlight exposure. Additionally, it is possible that the bacteria at both sites were already resistant to ampicillin prior to their exposure to the effluent.

Our results from comparison (I) (Figure 1) are contradicted by that of Drury et al. [5] who found a significantly lower quantity of CB downstream compared to upstream from two WWTPs. Their study collected river water and sediment at sites upstream and downstream from a major urban secondary WWTP, as well as a suburban secondary WWTP. They streaked these samples onto soy extract agar and assessed the quantity of

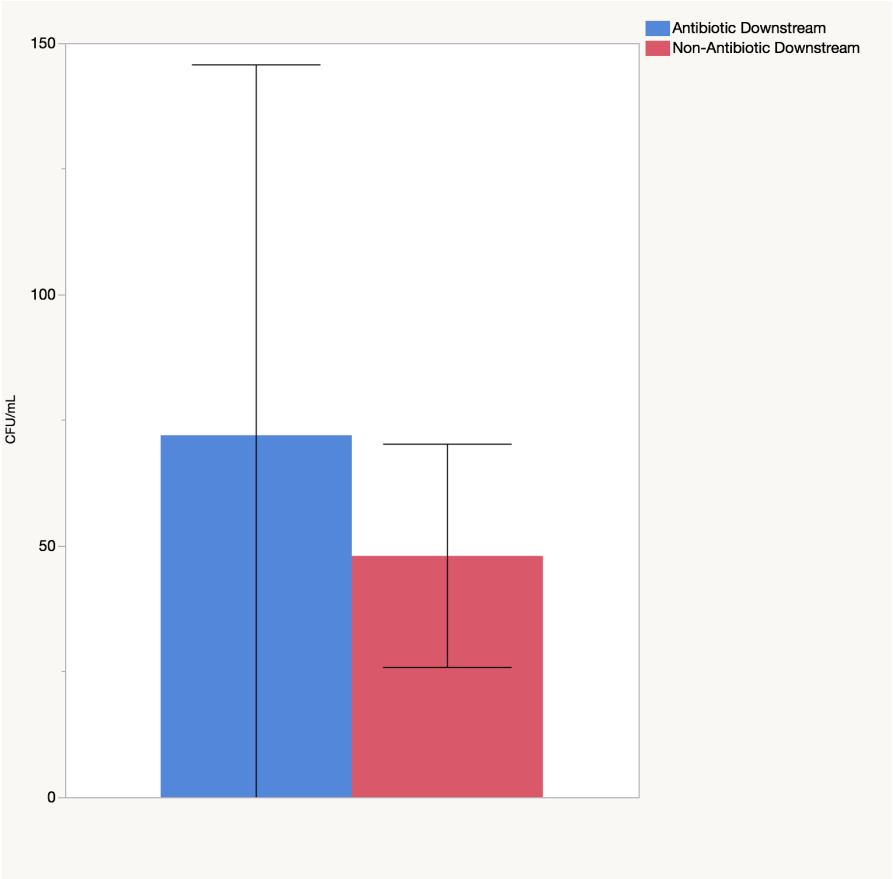

Figure 4: *Average number of culturable bacteria versus antibiotic-resistant culturable bacteria from the Downstream site with error bars for 95% confidence intervals.*

bacterial colonies that grew. Drury et al. [5] suggested that the decrease in bacterial populations in river water that contains wastewater effluent may be attributed to the increase in the inorganic substances in the river water. They found that the wastewater significantly increased the amount of nitrate, ammonium, and phosphate in the river water and sediment downstream from both WWTPs. It was suggested that some of these compounds may be toxic to the indigenous bacteria in the water. Although our study did not examine these compounds, this offers an alternative explanation to our antibiotic-based hypothesis. However, since our results suggest that there is no change in the quantity of CB, it may be the case that the Downstream site is too far from the WWTP for these inorganic compounds to be at a high enough concentration to have any deleterious effects on the bacterial populations. Alternatively, these contrary results may be attributed to the discrepancies in our methodology. For instance, we used Mueller-Hinton agar as opposed to soy agar because it accommodates a wide range of bacterial species. Future studies should be undertaken at the Northwest Langley WWTP to determine if the bacterial compositions between the upstream and downstream locations differ. Furthermore, since we tested different WWTPs than Drury et al., there may be variable levels of effluent output, which may have influenced the results.

Our findings are also contradicted by that of Wakeline et al. [7], who observed a higher quantity of CB downstream compared to upstream from a WWTP. Their study used the chloroform extraction technique [7] to quantify the amount of bacteria at each test site, compared to our agar-streaking technique, which may explain why their study observed a statistically significant increase in CB downstream compared to upstream from the WWTP. Additionally, their study tested the receiving waters at a maximum of 1.04km from the effluent outfall point, whereas our study collected water 1.7km downstream of the outfall point. Interestingly, they observed greater levels of bacteria at closer distances than our study did. This may indicate that the wastewater effluent does not affect bacterial concentration at greater distances from the outfall point. It is possible that this is because the effluent has become too diluted to have a significant impact. The discrepancies in our results may also be due to our different sampling methods. While we took samples of the river water mixed with some sediment, Wakelin et al. took samples of sediment that were dried before analysis exclusively. This could suggest that bacterial composition in the sediment is impacted more so than that of the surrounding waters. The results of this study also contradict that of Drury et al. [5], which may indicate that there are other factors influence bacterial concentration that have not been accounted for. Firstly, there is a substantial amount of variety between bacterial communities in general, which may explain why it is difficult to directly compare these results. Furthermore, the size of the WWTPs and other geographical factors may be influencing the results.

The quantity of bacteria in an aquatic environment may have important implications for the broader ecosystem, such as the exchange of organic material and nutrients among organisms [11] and the ability of the ecosystem to exchange CO_2 with the atmosphere [12]. Since some studies have found that primary WWTP effluent can significantly decrease the quantity of CB downstream from a WWTP [5], and our study suggests that secondary WWTP does not have this effect, it may be beneficial for all municipalities to upgrade their WWTPs to secondary treatment status. However, since one study on a secondary WWTP did show an increase in CB downstream [7], it is evident that further research needs be done to distinguish if this correlation is actually due to the presence of the WWTP effluent.

3.2. Comparisons (II, III, IV)

The results from Figure 3 indicate that there is no significant difference in the number of culturable antibiotic-resistant bacteria (CARB) compared to CB in the upstream site. This suggests that the bacteria in the river upstream from the wastewater treatment plant are already resistant to Ampicillin before they come in contact with the effluent. If the bacteria are indeed resistant to Ampicillin before they reach the wastewater effluent, then it is difficult to determine if the effluent is having any impact on the resistance of the bacteria to antibiotics. The presence of antibiotic resistant bacteria in river water has been well-documented in various rivers in the United States [13] and Tokyo [14], however, this is the first study to identify Ampicillin resistance in a west coast Canadian river. A study by Ash, Mauch, and Morgan [13] found that 98% of the bacteria from 22 sample sites were resistant to Ampicillin. Furthermore, they found that the resistance was plasmid-borne, which may suggest why it is observed with such ubiquity. While

we did not conduct any genome analyses on our sample bacteria, it is possible that they have developed and transferred antibiotic resistance through a similar mechanism.

Similarly, we did not observe a significant difference in the number of CARB compared to CB at the Downstream site (Figure 4). While a direct comparison between the two sites cannot be made due to potential differences in the environments at the two sites, Ash et al. [13] suggest that variations in temperature and pH does not correlate to Ampicillin resistance. This corroborates the hypothesis that the bacteria in the water are already antibiotic resistant. However, this does not definitively rule out any potential impacts of the wastewater effluent on the bacteria. If the bacteria are indeed resistant to Ampicillin prior to contact with the effluent, they may still be affected by other antibiotics that were not considered in this study. A study by Costanzo et al. [3] found that bacteria downstream from a WWTP developed resistance to six different antibiotics that were detected in the wastewater effluent, including ciprofloxacin, tetracycline, ampicillin, trimethoprim, erythromycin and trimethoprim/sulphamethoxazole. Further research should be conducted on the Fraser River to see if the bacteria near the Northwest Langley WWTP are resistant to other antibiotics in addition to Ampicillin and test to see if these antibiotics are present in the effluent.

Furthermore, it is possible that the observed resistance to Ampicillin is indeed due to exposure to antibiotics in the effluent of the river. The water may not flow in a direct downwards direction at all times, depending on the season, water volume, and wind [15]. If this is the case, then it is likely that the effluent can have impacts on the bacteria upstream from the WWTP as well as downstream. In addition to coming in contact with antibiotics in the WWTP, it is possible that the bacteria may come into contact with antibiotic-resistant strains in the downstream waters, giving them the opportunity to pass on the resistant genes via transmissible agents like plasmids. However, it is currently unknown whether the river water flow patterns vary drastically enough for any mixing to take place between sites that are large distances apart. We recommend that future studies conduct genome analyses on the bacteria at the upstream and downstream sites to identify if they are indeed resistant to Ampicillin and if this resistance is carried on the same plasmid.

However, there are numerous other factors that may be contributing to the apparent resistance of the bacteria to Ampicillin. Firstly, it is possible that the bacteria developed resistance through a naturally-occurring intrinsic pathway [16]. Secondly, we observed that both the Downstream and Upstream sites were regular sites of human activity–both recreation and industrial. These activities offer a multitude of sources of contaminants that may be affecting the bacteria. To further investigate this hypothesis, further studies should be conducting at more remote areas of the Fraser River to determine if Ampicillin-resistance is still present.

4. Materials and methods

We collected water samples upstream and downstream of the Northwest Langley WWTP and then plated these samples onto Mueller Hinton agar containing standard nutrients required for bacterial growth. The samples were monitored for nine days and the number of colonies on the plates were recorded.

4.1. Collection of water samples from the Fraser River

On June 29th, 2015, we collected water samples from two sites that are near the Northwest Langley WWTP outfall into the Fraser River, in Langley, British Columbia, Canada. The temperature outside was 28 °C. The Downstream Site was located at the end of 104th Avenue near a ferry parking lot in Surrey, British Columbia, Canada – approximately 1.7km downstream of the wastewater outfall. We chose this distance because these sites were readily accessible for testing. The Upstream Site was located at Derby Reach Regional Park, Langley, along a recreational beach and campground site – approximately 2.8km upstream of the wastewater outfall. The shoreline at the Upstream Site was rockier than the Downstream Site.

At each location, we collected five water samples at a distance of 2.5m apart parallel to the shoreline and a depth of approximately 10cm, in sterile 50mL Greiner Bio-one Cellstar® Tubes (Cat #210-261, USA). We chose to sample water at this depth as it is more likely to contain bacteria from both the surface water, sand, and sediment [17]. We also measured the temperature of the water at each site and found both to be 21 °C using a thermometer at a depth of 10cm. To ensure that we were gathering bacteria from both the surface and bottom of the river, we mixed the water and underlying sediment using a meter stick by jabbing the sediment with the stick for approximately five seconds before collection. Then the samples were collected approximately 5cm below the surface of the water. All samples were stored in a beaker that was on ice in a cooler, at an air temperature of approximately 4 °C, to ensure that the bacteria did not overheat during transportation [3].

4.2. Preparation of Mueller Hinton agar plates

To provide a medium that could accommodate a wide range of bacteria, we prepared 60 petri dishes (Fisher 08-757-1, Fisherbrand™, Canada) with 15mL each of Mueller Hinton II Agar (Ref #211438, Fisherbrand™, USA) with a concentration of 50.16g/L. To ensure that only bacterial colonies grew on the agar, we added 0.0132g/L of crystal violet dye (Aldrich Chemical Company Inc., USA) to act as an anti-fungal agent. We chose this concentration based on a recommendation by Atlas & Snyder [18]. To determine the concentration of culturable antibiotic-resistant bacteria (CARB), we also prepared 60 agar plates following the same procedure as above, with the addition of 0.051g/L of Ampicillin A9393-5G (Lot #103M4844V, Sigma-Aldrich, Canada) based on the recommendation from the article "Addgene" [19]. To ensure that the Ampicillin was not degraded by heat or light, we kept it in a refrigerator at 4 °C and covered it in aluminum foil (Alcan, USA) until we added it to the agar solution. We added the Ampicillin to the agar solution once the agar had cooled to 55 °C, to prevent heat-degradation [11]. Additionally, the plates containing Ampicillin were covered with aluminum foil for four hours after their preparation.

4.3. Agar plating methods to determine concentration of culturable bacteria and antibiotic-resistant culturable bacteria between sites

For comparison (I), we plated 25μL of each sample onto separate Mueller Hinton agar plates containing Ampicillin. For comparison (II), we repeated the above procedure for

the Mueller Hinton plates that did not contain Ampicillin. For each sample, there were 6 replicates which included 1 original concentrated sample + 5 dilutions with a 20X dilution factor. We prepared a total of 120 agar plates. Afterwards, we stored all of the plates under aluminium foil in a dark cupboard at room temperature (20-26 °C). We chose this temperature to mimic the conditions of the Fraser River (see subsection 4.1). We counted the number of colonies on the plates nine days after inoculation.

4.4. Statistical Analysis

To determine if there is a statistically significant difference in the quantity of culturable bacteria (CB) in the Downstream site versus the Upstream site, a *t*-test was performed. We graphed the average number of colony-forming units (CFUs)/mL on the non-antibiotic plates from each site. We also plotted and compared the average number of CFUs/mL on the ampicillin-treated plates from the Downstream site and the Upstream site to determine if there was a statistically significant difference in the quantity of antibiotic-resistant culturable bacteria between the sites. In each graph, we included 95% confidence intervals.

5. Conclusion

Our data suggest that the concentration of naturally-occurring bacteria in the Fraser River was not significantly affected by the presence of wastewater effluent in river water downstream from a secondary WWTP. Additionally, we found no significant difference in the quantity of CARB from the Downstream site compared to the Upstream site. Since the bacteria upstream of the WWTP seem to already show antibiotic resistance, it is not surprising that the downstream bacteria also display resistance. Our results may also be due to our testing at large distances from the WWTP or because we tested water rather than sediment communities. Further research should be conducted on the mechanism of antibiotic resistance in these bacteria as well as on the effect of tides on the spread of WWTP effluent in the river to see if it can explain the ubiquity of antibiotic resistance in the river.

6. Acknowledgements

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