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Analysis on the Impact of Human-Induced Pollutants on River Microbiology

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ABSTRACT

In recent decades, water quality and contaminant concentrations have been tightly regulated by relevant laws and monitoring. However, detailed microbial composition in different environments and their interactions with human activities has yet to be fully characterized. This paper shows how different environments, including city environments and highways, can affect the properties of water bodies closely associated with them geographically. Two pairs of locations along Schuylkill and Wissahickon river were sampled. Through 16s rRNA metagenomic diversity sequencing and a functional gene prediction approach, the taxonomic and predicted gene profiles of samples from various locations were elucidated. Through comparative study of these samples, the effect of human activity on the river between the locations were evaluated. In the Wissahickon river, metagenome analysis indicates that human-induced pollutants potentially fostered the growth of bacteria that are able to utilize them, but suggests no increment of genes' abundance that resist their damaging effects, such as heavy metals exporting ATPase, and various antibiotic resistance genes. In the Schuylkill river, the analysis indicates that the growth of the aforementioned bacteria is insignificant, and the resistance genes were predicted to decrease in the urban area where it was anticipated to receive more influence from human activities, rendering the result inconclusive. We anticipate that this study will become the starting point for future research on microbial populations in water bodies so that the dynamics of how human activities influence river microbiology can be determined more clearly.

Introduction

The impact of human-induced pollutants on macro-organisms, such as aquatic animals and humans, had been long elucidated and acknowledged. Government regulations that limit the discharge of harmful substances such as heavy metals and industrial pollutants in water bodies, such as the Clean Water Act, have been in place for years. In recent decades, how bacterial, fungal, and other microbial species in aquatic communities respond to and interact with human activities has also emerged as another focus of study (Ekwanzala et al., 2020; Mehrshad et al., 2016; Ng et al., 2017).

In order to conduct analysis on such a micro scale, many researchers utilize metagenomic analysis. Metagenomic analysis is arguably one of the most important technologies in genetic studies that advented in the past decade. Rather than focusing on only one gene, metagenomics can provide access to the description of a much broader scope, including the functional gene composition of microbial communities as a whole (Thomas et al., 2012). Until a few years back, scientists could only study those microbes that are able to be successfully cultivated in the lab, which is only a fraction of all environmental microbes that had previously been known. By allowing the sequencing of microbial genomes directly taken from the environment, metagenomic studies opened a gate towards a plethora of research directions focusing on analyzing the composition, variety, and abundance of environmental microbes. A subset technique in metagenomic analysis, 16s rRNA sequencing, has reduced costs yet focuses mainly on the sequencing of the titular rRNA, while shotgun sequencing examines all genes and thus provides a more exhaustive and comprehensive view of the genes.

Overall, this study is similar to previous studies on micro-biosystems. It employs 16s rRNA metagenomic analysis and focuses on the microbial composition of several sites along the Schuylkill River and Wissahickon River in Philadelphia, Pennsylvania. The primary objective of this study is to determine the human impact such as highways and human communities to the dynamics of microbial composition in natural aquatic resources. The process of 16s rRNA metagenomic analysis, which is employed in this study, can be illustrated by the graph below:

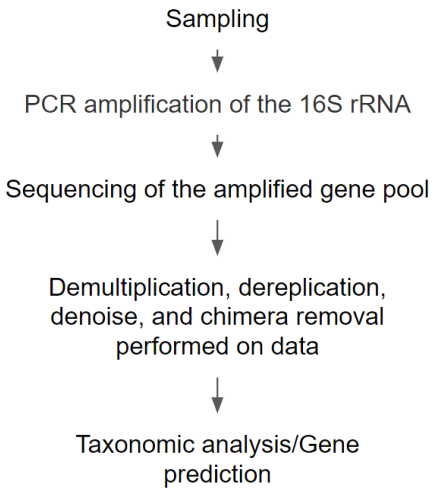

Figure 1. A flow chart illustration of the main processes of 16s rRNA sequencing that was employed in this study

Using the taxonomic and gene profile generated by the metagenomic analysis and prediction, the features of a sample from a certain river location, such as the presence of certain bacteria or genes for temperature resistance, salt/certain metal ion resistance, antibiotic/pharmaceutical resistance, can be analyzed. By doing so comparatively in pairs of locations, the effect that the human activities had on the river segment between them can be illustrated effectively.

Methods

The samples were collected in pairs using falcon tubes and gloves at each of the sites recorded in the table below. Each pair of samples was collected in 20-minute intervals. Each falcon tube was filled with around 35ml of sediments and 15ml of water. The table and figure below shows the details of the locations from which the samples were taken.

Table 1. A detailed description of the samples taken, including the coordinates at which they are taken, the surrounding environment, river speed, sediment properties, and the numbers of samples taken (* indicates auxiliary locations that are not involved in subsequent analyses).

Name	Coordinate	Environment	River speed	Sediment Property	Pairs of Sample Collected
Schu 1	(40.1104476, -	In a state park with well maintained	Flowing fast	Greater	3

	75.4286632)	forest on its shores. The river is relatively undisturbed and has visible aquatic organism populations.		Gravels	
Schu 2	(40.0701437,-75.3101562)	In a relatively urban area, bordered by a strip of spaced trees and buildings, with a visible population of tadpoles present.	Moderately flowing	Gravel and Mud	3
Schu 3*	(40.0101955, -75.2000303)	Relatively urban area, with shores bordered by a relatively busy road and scarce vegetation.	Visually Still	Very fine	1
Wiss 1*	(40.1843798, -75.2545911)	Relatively undisturbed river, with its two shores surrounded by trees.	Very fast	Mostly big rocks	1
Wiss 2	(40.1275943, -75.2191981)	Under a bridge of highway, with shores covered by short bush and the river covered by large amounts of leaf litter.	Visually Still	Fine, mud-like	3
Wiss 3	(40.1271803, -75.2189795)	Same as Wiss 2.	Visually Still	Fine, mud-like	3

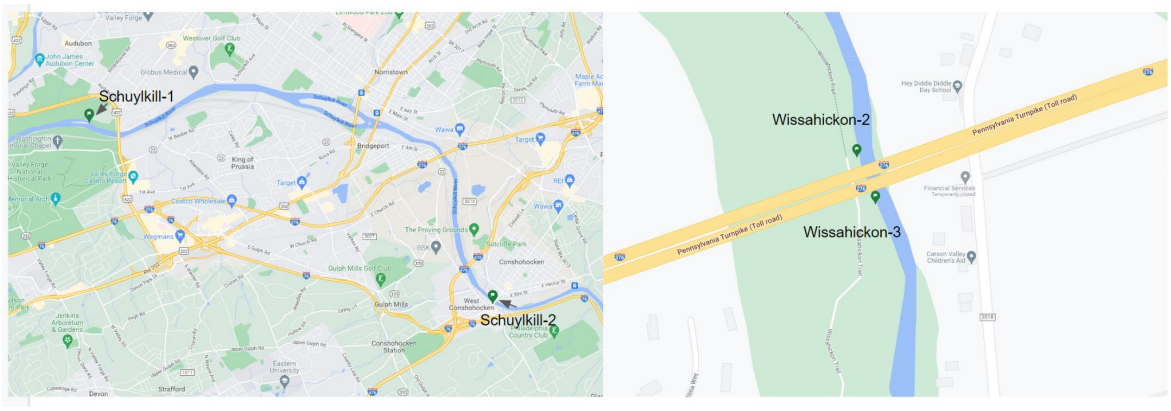

Figure 2. The locations of samples graphed on Google Maps.

The samples were sealed and shipped refrigerated to Mr. DNA laboratory overnight, where downstream processing steps including DNA extraction, DNA purification and 16s rRNA metagenomic sequencing and taxonomic analysis were conducted.

16s rRNA Sequencing

The 16S rRNA gene V4 variable region PCR primers 515/806 were used in a 30 cycle PCR using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) under the following conditions: 95°C for 5 minutes, followed by 30-35 cycles of 95°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute, after which a final elongation step at 72°C for 10 minutes was performed. Sample data are then multiplexed using unique dual indices and

pooled together in equal proportions based on their molecular weight and DNA concentrations. Pooled samples are purified using calibrated Ampure XP beads. Then the pooled and purified PCR product is used to prepare an Illumina DNA library. Sequencing was performed at MR DNA (www.mrdnalab.com, Shallowater, TX, USA) on a MiSeq following the manufacturer's guidelines. The data were demultiplexed and exported as Casava 1.8 paired-end demultiplexed fastq before subsequent analyses.

Taxonomic Analysis

The 16S metagenome sequence data were processed using MR DNA analysis pipeline (MR DNA, Shallowater, TX, USA), in which sequences are joined, sequences <150bp removed, and sequences with ambiguous base calls removed. Sequences were quality filtered using a maximum expected error threshold of 1.0 and dereplicated. The dereplicated or unique sequences are denoised; unique sequences identified with sequencing and/or PCR point errors and removed, followed by chimera removal, thereby providing a denoised sequence or zOTU (zero-radius Operational Taxonomic Unit). Final zOTUs were taxonomically classified and labeled using BLASTn against a curated database derived from NCBI (www.ncbi.nlm.nih.gov).

Functional Gene Prediction

The 16S metagenome sequence data were processed using the DADA2 pipeline of QIIME2 (Bolyen et al., 2019; Callahan et al., 2016), in which sequences were joined, those less than 150bp removed, and finally quality filtered using a maximum expected error threshold of 1.0. They were then replicated and removed of chimeras, resulting in a denoised ASV (Amplicon Sequence Variant) sequence and ASV abundance table. These were exported into fasta and biom formats, respectively, and analyzed using the PICRUSt2 pipeline (Douglas et al., 2020; Barbera et al., 2018; Czech et al., 2020; Louca et al., 2017), which uses these data to predict the abundance of various gene families. They were finally classified and described using data from Enzyme Commission Numbers and Kyoto Encyclopedia of Genes and Genomes.

Results

Figures 3-6 are pie charts detailing the bacterial composition at Schuylkill locations 1 and 2 and Wissahickon locations 2 and 3, with specific taxonomic abundance displayed for each prominent genus of bacteria. The most amount of relative abundance was observed to be *Pedosphaera* with an abundance of around 4% in locations 1 and 2 of Schuylkill River and location 2 of Wissahickon River, while for location 3 of Wissahickon, *Geobacter* became the dominant species with a percentage of 4.5%.

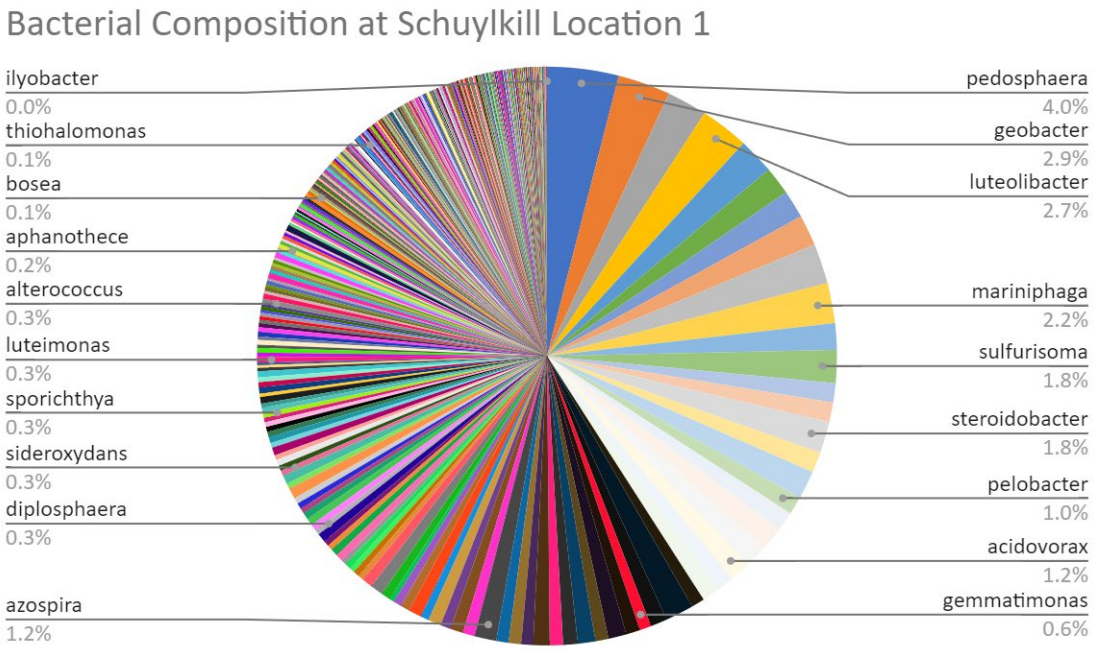

Figure 3. A pie chart detailing the bacterial composition at Schuylkill location 1, with specific taxonomic abundance displayed for each prominent genus of bacteria.

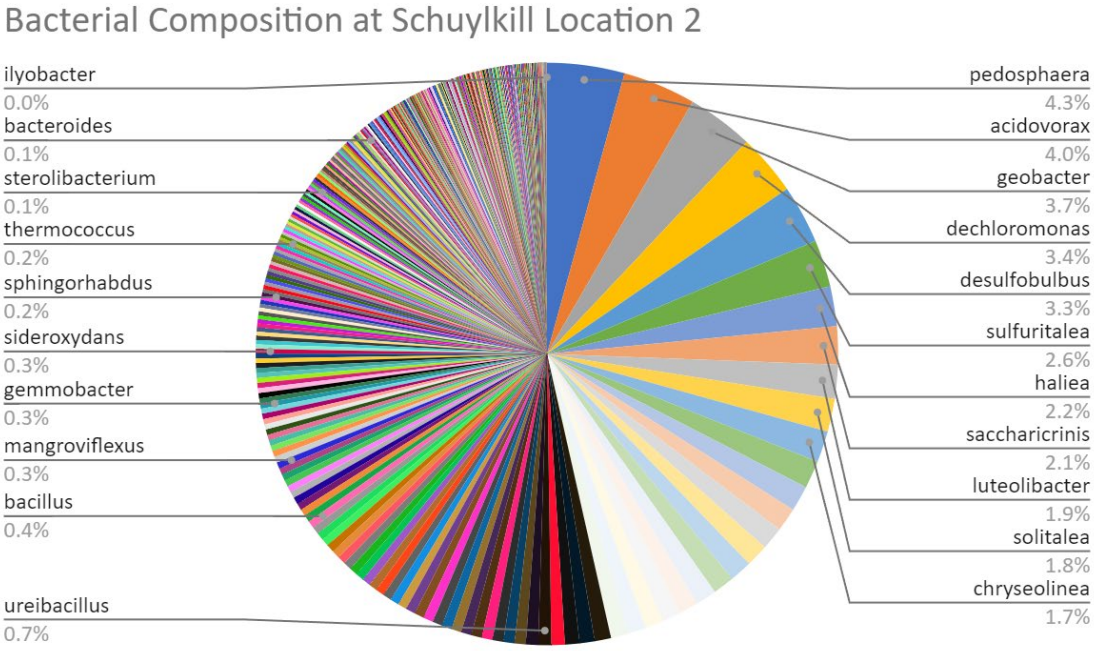

Figure 4. A pie chart detailing the bacterial composition at Schuylkill location 2, with specific taxonomic abundance displayed for each prominent genus of bacteria.

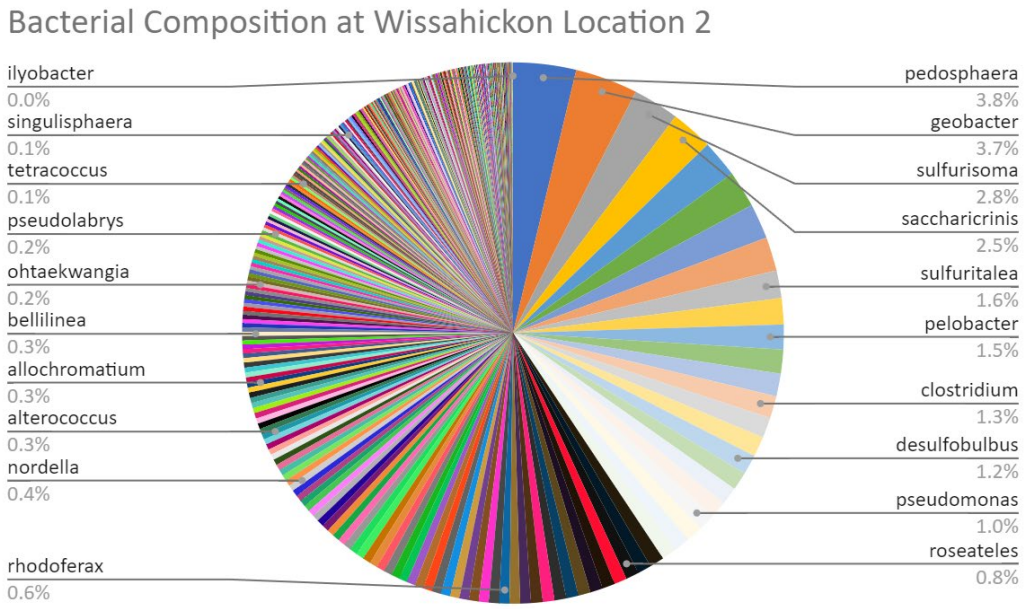

Figure 5. A pie chart detailing the bacterial composition at Wissahickon location 2, with specific taxonomic abundance displayed for each prominent genus of bacteria.

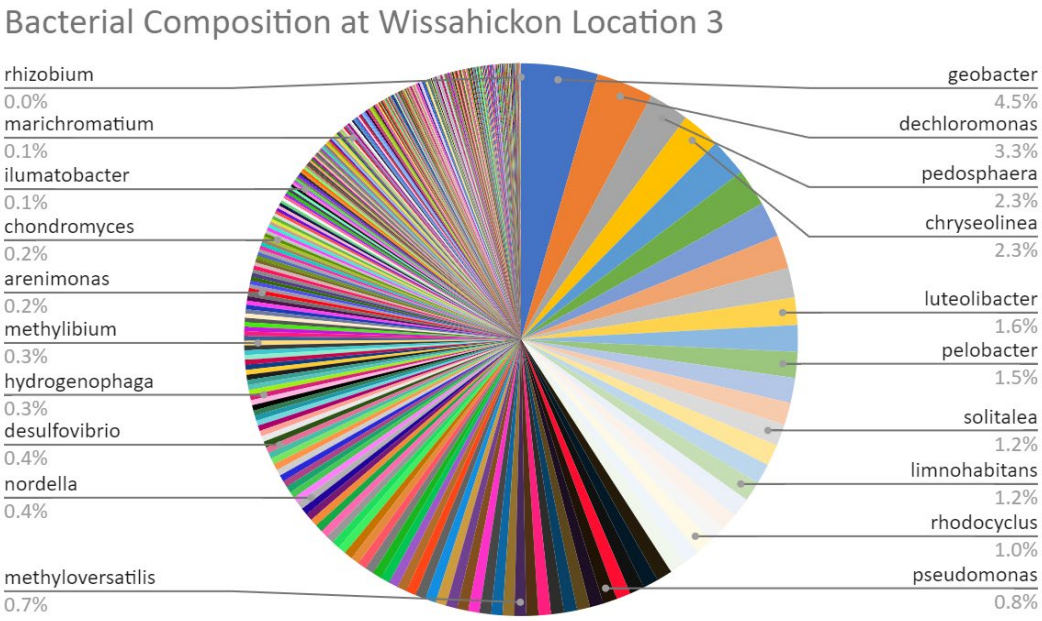

Figure 6. A pie chart detailing the bacterial composition at Wissahickon location 3, with specific taxonomic abundance displayed for each prominent genus of bacteria.

Discussion

The human-induced pollutants can influence the microbiology of the river from two means: the pollutants that *is* beneficial to certain bacteria can cause them to thrive; the pollutants that *is not* beneficial to bacteria can act as stress factors, promoting increment of features that prevents the pollutants from damaging them. For the former way, we used analysis of these certain bacteria's percentage in the whole micro-population; for the latter way, we used functional gene prediction to analyze the genes that result in resistance towards these detrimental pollutants.

Bacterial Analysis

A Foci Genus is defined in our study as a certain genus which (1) is a significant part of the microbial community and (2) can utilize a certain human-induced pollutant to benefit itself. By comparing the abundance of foci groups in pairs of locations, the effect of human activities between these locations on the river can be elucidated. We chose four genuses as Foci Groups for this study: *Geobacter*, *Dechloromonas*, *Desulfobulbus*, and *Pelobacter.*

Geobacter is a genus of bacteria with a fair presence across all samples. During its metabolism cycle, *Geobacter* uses heavy metal compounds like Fe(III) oxide, Mn(IV) oxide, and even uranyl ions as electron acceptors to oxidize organic compounds such as pollutants derived from petroleum into carbon dioxide (Childers, 2002; Anderson et al., 2003). Given that both heavy metals and petroleum are major human-induced pollutants, the abundance of *Geobacter* serves as a great indicator for human influence on water quality; the greater the abundance, the greater the concentration of pollutants.

Table 2. This table shows the percentage of Geobacter present in each sample taken in four different sites, the average of the three samples taken, their standard deviation, and p-values (t-test, one-tailed).

Name\Sample #	1st sample	2nd sample	3rd sample	AVERAGE	STDEV	p-Value
Schu-1	3.620847303	1.899131527	3.326710479	2.948896436	0.9209418504	0.2955790821
Schu-2	3.531017229	3.725457291	2.91714646	3.391206994	0.4219025673	
Wiss-2	2.168269965	2.009859689	2.108777788	2.095635814	0.08001866956	0.008098352223
Wiss-3	3.507775332	2.882802972	3.422744691	3.271107665	0.338958631	

For the Schuylkill river pair, location 2, which was closer to the urban area, had less percentage of *Geobacter* present than location 1, which was in a forested area. Given that the p-value was relatively big (0.296), this result was insignificant and will not be further analyzed. For the Wissahickon river pair, location 3, located after the highway bridge, had significantly more presence of *Geobacter* than location 2, and this difference was statistically significant with a p-value of 0.008.

The second foci genus is *Dechloromonas*, a genus of bacteria reliant on anaerobic hydrocarbon metabolic processes. They are able to metabolize linear and branched alkanes from C6 to C20 along with cycloalkanes, BTEX, and various aromatic compounds, and oxidize Fe(II), using nitrate as electron acceptors (Ward et al., 2009; White et al., 2016). Similar to *Geobacter*, *Dechloromonas*' metabolism is also closely related to human activities and pollution, thereby qualifying it as a suitable foci genus.

Table 3. This table shows the percentage of Dechloromonas present in each sample taken in four different sites, the average of the three samples taken, their standard deviation, and p-values (t-test, one-tailed).

Name\Sample #	1	2	3	AVERAGE	STDEV	p-Value

Schu-1	2.188357208	2.615883645	1.905344134	2.236528329	0.3577106961	
Schu-2	3.002217547	4.581305588	3.459998903	3.681174013	0.8124460488	0.02516436346
Wiss-2	4.00061078	3.420553659	3.760653721	3.72727272	0.2914657514	
Wiss-3	4.388269545	4.77880031	4.403455219	4.523508358	0.2212196577	0.05565907468

For the Schuylkill river pair, the urban location 2 had significantly more percentage of *Dechloromonas* present than forested location 1, and as the p-value is only 0.025, the difference was statistically significant. This hints that location 2 may had more presence of petroleum products and Fe than location 1. The *Dechloromonas* percentage of the Wissahickon river pair displayed a similar pattern as *Geobacter*: the downstream location has more *Dechloromonas* than the upstream. Although the p-value (0.055) did not qualify the difference between the locations as statistically significant, it was still notable and was taken into consideration.

The third foci genus is *Desulfobulbus*, a genus of bacteria predominantly reliant on sulfur in its metabolism. It reduces sulfate to sulfite and sulfide and uses hydrogen and simple carbon compounds as electron donors (Lens, 2009; Fauque et al., 2012). Given that sulfur compounds in human-induced pollution, such as sulfur dioxide and sulfur trioxide, are readily converted to sulfuric acid and thus sulfate in atmospheric oxidation and hydrolyzation, the relative abundance of *Desulfobulbus* is a great indicator of the presence of sulfur compounds.

Table 4. This table shows the percentage of Desulfobulbus present in each sample taken in four different sites, the average of the three samples taken, their standard deviation, and p-values (t-test, one-tailed).

Name\Sample #	1	2	3	AVERAGE	STDEV	p-Value
Schu-1	1.322767449	2.082243382	1.706658515	1.703889782	0.3797455366	
Schu-2	2.700858589	2.76332718	2.352360586	2.605515452	0.2214522992	0.03168391834
Wiss-2	1.725454268	1.706484642	1.484931025	1.638956645	0.1337268869	
Wiss-3	2.080522616	1.918172746	2.50698188	2.168559081	0.3041165289	0.08398605583

For the Schuylkill river pair, the urban location 2 had significantly more percentage of *Desulfobulbus* present than forested location 1, and as the p-value is only 0.032, the difference was statistically significant. This suggested that location 2 may have more presence of sulfur pollution and sulfate than location 1. The *Desulfobulbus* percentage of the Wissahickon river pair again displayed the same pattern as previous bacteria. Given the slightly big p-value of 0.0839, the difference cannot be considered statistically significant, but it was still sizable and had been taken into consideration.

The fourth foci genus is *Pelobacter,* a genus of bacteria both taxonomically and functionally similar to *Geobacter*. Its metabolism involves indirect Fe(III) reduction through the usage of sulfur and simple organic compounds as reductants (Lovley et al. 2011). Therefore, because of its relationship with iron and sulfur, *Pelobacter* is also an effective indicator of human activities.

Table 5. This table shows the percentage of Pelobacter present in each sample taken in four different sites, the average of the three samples taken, their standard deviation, and p-values (t-test, one-tailed).

Name\Sample #	1	2	3	AVERAGE	STDEV	p-Value

Schu-1	1.280097531	0.5702626347	1.207397218	1.019252461	0.3905319862	
Schu-2	1.68874737	0.8614420764	0.5154356528	1.021875033	0.6028840243	0.4973428208
Wiss-2	0.5267979844	0.4778156997	0.4129689834	0.4725275558	0.05709845635	
Wiss-3	1.015408649	0.7428761503	1.06514256	0.9411424531	0.1734949872	0.02641085663

The Schuylkill river pair of locations had a very close percentage of *Pelobacter* present. As the p-value is very big (0.497), their difference can be considered very statistically insignificant. Conversely, the *Geobacter* percentage of the Wissahickon river pair displayed a strong difference: the p-value was only 0.026 and qualified the difference between the locations as statistically significant.

For the Schuylkill river pair, two of the four genuses of bacteria displayed a strong, statistically significant difference in percentage, but the other two genuses barely displayed any. Besides, within the four genuses, *Geobacter, Pelobacter,* and *Dechloromonas* all utilize iron in metabolism, but their percentage pattern was very different; only *Dechloromonas* displayed a considerable difference in percentage. Both of these undermined the strength of the data to prove the presence of influence from human-induced pollutants, and thus no definitive conclusion could be drawn.

For the Wissahickon river pair, all four genuses of bacteria displayed the same tendency of having more percentage immediately downstream to the highway than its upstream counterpart. Two genuses' differences were statistically significant, and the other two had p-value only slightly above the threshold of 0.05 (0.055 and 0.084). There were also no inconsistencies in data like the one displayed in Schuylkill river pair's data. Thus, it could be concluded that in the Wissahickon river pair, it was very likely that the highway's presence had an influence on the microbiology of the river.

Functional Gene Analysis

As previously mentioned, while bacterial analysis focuses on human-induced pollutants that *do* play an important role in metabolism, such as sulfur, petroleum-derived simple hydrocarbons, and iron, the functional gene analysis focuses on those that *do not* benefit the bacteria, such as antibiotics and trace heavy metals like zinc and copper.

To this end, we centered our analysis mainly on genes that result in heavy-metal resistance and antibiotic resistance. For the former, three specific genes that code for Zn2+/Cd2+ exporting ATPase, Cu2+ exporting ATPase, and Cu+ exporting ATPase were chosen as the subject. These enzymes play crucial roles in the efflux of their respective heavy metal ions, as suggested by their names (Nies, 2003). For the latter, we chose genes that code Beta-lactamase, Sialate O-acetylesterase, Protein-glutamate methylesterase, 11 Acetyltransferases, 13 Lyases, 4 Monooxygenases, and 6 nucleotidyltransferase genes and phosphotransferase. These genes were relatively abundant in the predicted gene profile of the samples, and the enzymes they code are known to play an important role in modifying and resisting antibacterial drugs, which makes them great subjects for our study (Egorov et al., 2018).

Heavy Metal Exporting ATPases

Table 6. This table shows the abundance of the Cd2+/Zn2+ Exporting ATPase gene present in each sample taken in four different sites, the average of the three samples taken, their standard deviation, and p-values (t-test, one-tailed).

Cd2+/Zn2+-Export	1	2	3	AVERAGE	STDEV	p-Value

Schu-1	107919.72	115873.31	97625.84	107139.6233	9148.713213	0.06124100538
Schu-2	57098.42	91838.6	84860.59	77932.53667	18377.11808	
Wiss-2	123119.97	140672.19	148977.83	137589.9967	13201.59717	0.3577188863
Wiss-3	125512.19	169950.23	80649.95	125370.79	44650.30792	

Table 7. This table shows the abundance of the Cu2+ Exporting ATPase gene present in each sample taken in four different sites, the average of the three samples taken, their standard deviation, and p-values (t-test, one-tailed).

Cu2+ export	1	2	3	AVERAGE	STDEV	p-Value
Schu-1	125568.69	124871.23	113554.96	121331.6267	6743.813531	0.09489286289
Schu-2	67587.6	107746.81	102382.66	92572.35667	21803.02905	
Wiss-2	138631.25	158881.45	163008.63	153507.11	13047.09974	0.3468241598
Wiss-3	140826.93	185409.99	96050.76	140762.56	44679.64978	

Table 8. This table shows the abundance of the Cu+ Exporting ATPase gene present in each sample taken in four different sites, the average of the three samples taken, their standard deviation, and p-values (t-test, one-tailed).

Cu+ export	1	2	3	AVERAGE	STDEV	p-Value
Schu-1	215995.56	202128.65	207796.53	208640.2467	6971.849941	0.045365389 07
Schu-2	113340.17	171431.49	147582.53	144118.0633	29200.20996	
Wiss-2	194869.93	219695.57	218431.24	210998.9133	13982.40716	0.2755074248
Wiss-3	198639.48	231850.67	143074.43	191188.1933	44854.72608	

The abundance of all three genes in these locations were predicted to display a similar pattern. For the Schuylkill river pairs, the urban location 2 had much less abundance of all three samples than forested location 1. The difference of Cu+ exporting ATPase was statistically significant (p=0.045) and the other two also have a relatively great difference given their small p-value. For the Wissahickon river pair, the two locations had barely any difference. The average abundance of them was very close, and the p-value was relatively big.

Taking both pairs into account, it could be concluded that either due to the amount of these heavy metals released or that their concentration barely affected the ecology, human-induced pollution did not have a definitively significant influence on the river microbes, and no conclusive statement can be drawn.

Antibiotic Resistance Genes

Figures 7 through 11 show the predicted abundance of several groups of genes that code for enzymes that increase bacteria's antibiotic resistance in four different locations along the Schuylkill and Wissahickon rivers.

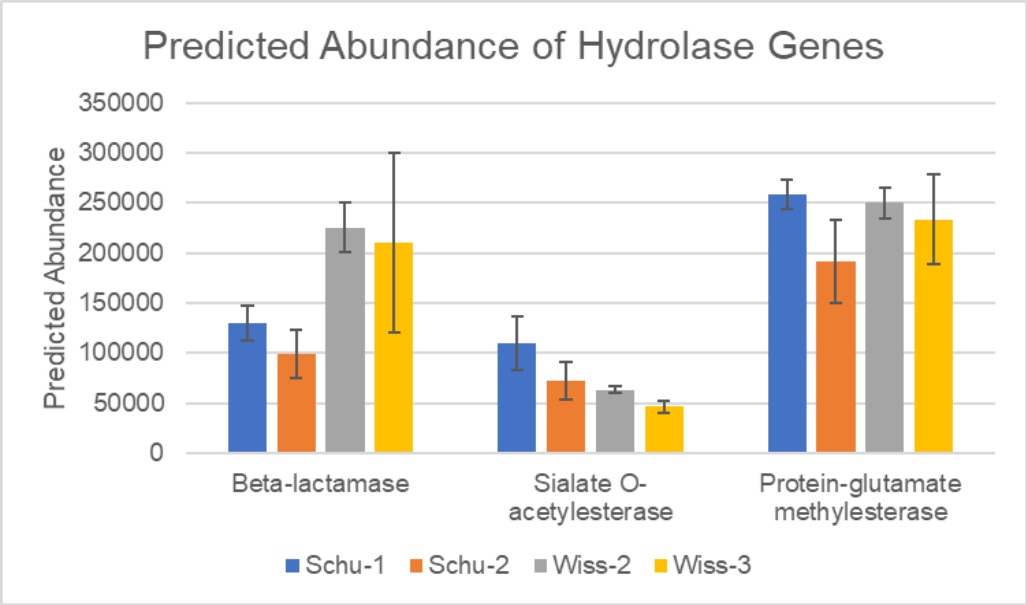

Figure 7. A bar graph showing the predicted abundance of several hydrolase genes in four locations. Error bars indicate standard deviation.

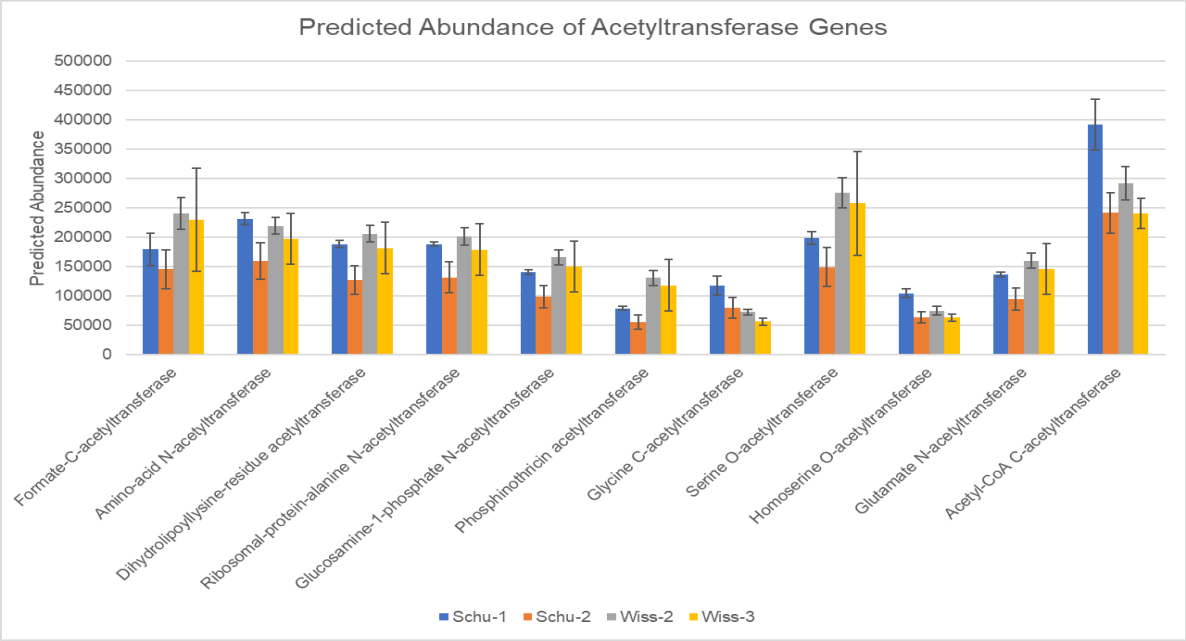

Figure 8. A bar graph showing the predicted abundance of several acetyltransferase genes in four locations. Error bars indicate standard deviation.

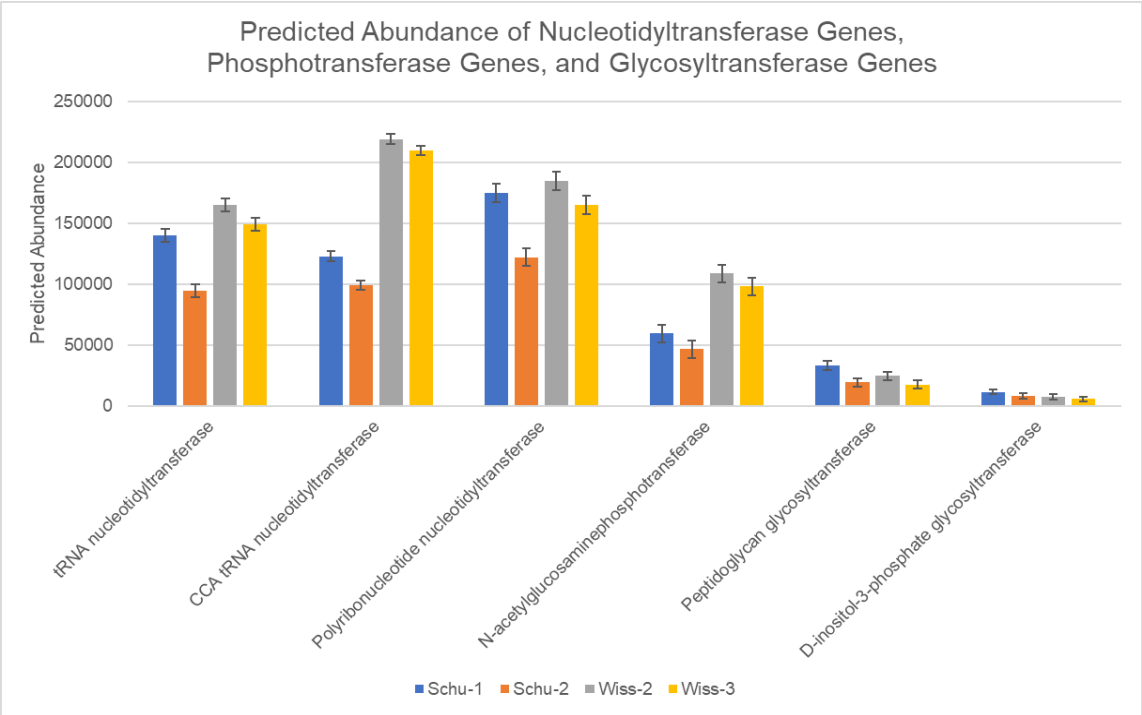

Figure 9. A bar graph showing the predicted abundance of several nucleotidyltransferase genes, phosphotransferase genes, and glycosyltransferase genes in four locations. Error bars indicate standard deviation.

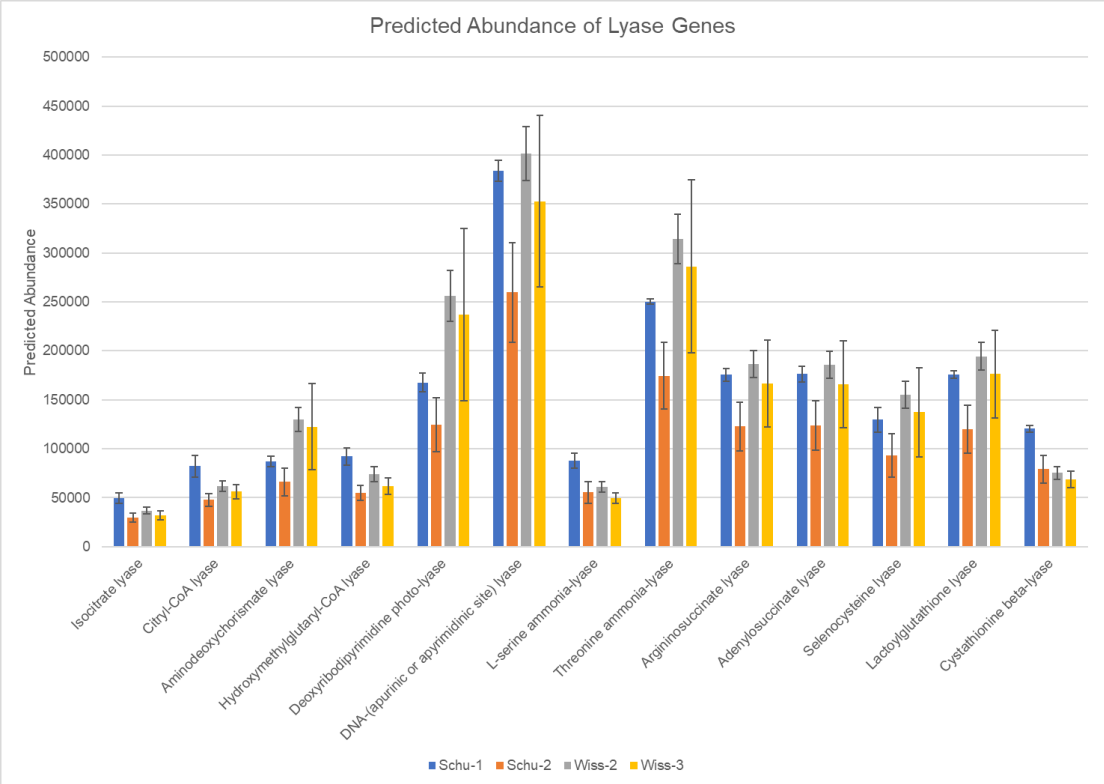

Figure 10. A bar graph showing the predicted abundance of several lyase genes in four locations. Error bars indicate standard deviation.

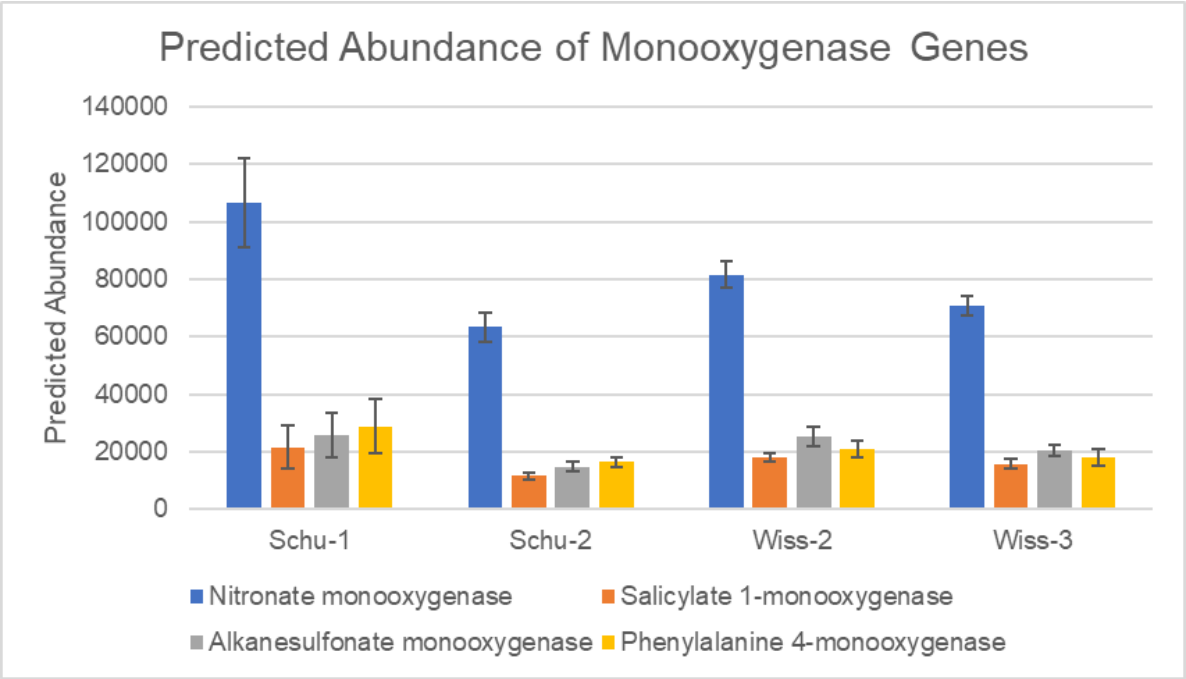

Figure 11. A bar graph showing the predicted abundance of several monooxygenase genes in four locations. Error bars indicate standard deviation.

The antibiotic resistance genes were again predicted to display a very similar pattern to the heavy metal resistance genes. The Schuylkill river location 2 samples had significantly less in abundance of almost all antibiotic resistance genes. Having p-values barely exceeding 0.05, most of these differences were very statistically significant. The Wissahickon river pairs again displayed little difference in abundance of these genes. For all genes location 3 is less abundant than location 2, and with a few exceptions, the differences are statistically insignificant ($p>0.05$).

Considering the results for heavy metal metabolizing genes and antibiotic resistance genes altogether, it could be seen that through means of human-induced pollutants that might had damaging effects, the human activity did not have any significance on the river's biology.

Limitations

Due to economic constraints, we were unable to conduct analysis on more samples or more accurate shotgun metagenomic analysis. It would be instrumental to include more samples and process them with shotgun sequencing to definitively observe the abundance of specific genes for a more robust analysis. Besides, the prediction conducted by PICRUSt2 only reflects the tendency and pattern of the gene abundances, and cannot represent the exact number of the abundance. This is again a problem that can only be solved using the shotgun metagenomic analysis for a more robust analysis. Finally, for the Schuylkill river pair, the environment between the two locations is treated as an aggregate blackbox, within which the conditions might be so various and unpredictable that the environment cannot be treated as a whole. More pairs of locations should be investigated in the future.

Conclusion

The impact of human-induced pollutants on river microbiology were studied in this paper. Through 16s rRNA Metagenomic Analysis and gene prediction, the taxonomic and gene profile of samples from various locations were elucidated. Through comparatively studying these samples, the effect of human activity on the river between the locations were found. In the Wissahickon river, human-induced pollutants fostered the growth in population of bacteria that are able to utilize them, such as *Geobacter*, but according to metagenomic predictions, the pollutants did not promote the increment of genes that resist their damaging effects, such as heavy metals exporting ATPase, and various antibiotic resistance genes. In the Schuylkill river, the change in the relative abundance of the aforementioned bacteria was much more insignificant, and the genes' abundance were predicted to decrease in the urban area where it is hypothesized to receive more influence from human activities. Even though human-induced pollutants are within the acceptable boundaries of law such as the clean water act, the results taken from the Wissahickon and Schuylkill rivers in this study indicated a certain level of change in microbial abundance from human influence. This result showed that although the concentrations of human-induced pollutants is low in the macroenvironment, human activities may still impact microbial populations in microenvironments. This study serves as a stepping stone for future research on microbial compositions in water bodies in order to more comprehensively determine the dynamics that human activities influence river microbiology and other conditions that might affect aquatic microbial communities.

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