Determining the effects of Wastewater Treatment Plant effluent on sediment microbial communities and nitrogen-cycling in three study sites

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EXECUTIVE SUMMARY

Background

This study aims to determine whether point source water pollution from wastewater treatment plants (WWTP) changes the quantity and quality of specific nitrogen-cycling river sediment microbial communities in order to evaluate the ecosystem-level effects of WWTP outflow. Nitrogen, in the form of dissolved inorganic nitrogen (DIN - NO_3^-, NO_2^-, and NH_4^+), is a primary nutrient and present naturally in waterways at a concentration varying around 0.12 mg/L (2). Yet with more point and nonpoint source pollution and less forested areas to act as riparian buffers (using excess N for growth), dissolved inorganic nitrogen (DIN) can increase to 4.0 mg/L or more, creating benthic anoxia and dead zones in the waterways as a result of eutrophication (nutrient overloading) (2). In our own Chesapeake Bay watershed, eutrophication, dead zones, and fish kills have become a widespread problem. The Chesapeake Bay Total Maximum Daily Load assessment – an evaluation by the EPA of the maximum pollution amount that a water body can handle while meeting water quality standards – calls for a 25% reduction in nitrogen throughout the watershed for bay health restoration by 2025 (17). In order to achieve this, any nitrogen added to the watershed must be within the processing capabilities of the ecosystem. If so, the nitrogen cycle will naturally convert DIN to nitrogen gas, which is then released into the atmosphere (reducing problems of eutrophication). Problems occur, however, when the additive DIN becomes too great for the nitrogen cycle to naturally process out of the water. Because unique microorganisms (bacterial and archaeal communities) handle every step in the cycle, each of these steps can be correlated to DNA sequences specific to the functioning of these organisms in the cycle. Genetic analyses of these unique sequences can therefore be used to assess an ecosystem's N-cycling response to nitrogen contributors (21).

One such point source contributor is wastewater treatment plant (WWTP) effluent, typically containing high levels of NH_4^+(21). For this project, DNA was extracted from sediment samples collected around WWTP outflow pipes in three sites – Lexington, VA in the Maury River, Buena Vista, VA in the Maury River, and Glasgow, VA at the confluence of the Maury and James Rivers (where the WWTP effluent flows into the James just upstream of the confluence). Samples were collected approximately 500 m upstream, directly upstream, directly downstream, 500 m downstream, and 1 km downstream of the outflow pipe. Quantitative Polymerase Chain Reaction (qPCR) was used to identify the quantity of genes unique to certain stages of the nitrogen cycle in each sediment sample. The same procedure was used to identify the quantity of genes unique to human fecal coliform because of the recent impaired citing of the Maury River.

Results

The levels of total generalized bacteria and archaea (identified using genetic sequences universal to either group rather than those specific to stages of the nitrogen cycle) at the varied distances from the WWTP outflow had few statistically significant differences. However, the unique microorganisms responsible for an early stage in the nitrogen cycle (oxidation of ammonium, NH_4^+) were significantly less present about 1 km downstream from the outflow pipe than at the upstream sites for the sampling schemes in Lexington and Buena Vista. Disturbingly, human intestinal *Bacteroides* levels were noticeably present at all sites, with no statistically significant trend around the WWTP outflow. Ammonium, sodium, and total nitrogen showed a clear

increase downstream of the WWTP pipe. Most of the indicators of anthropogenic pollution correlated strongly.

Conclusions

Although the primary differences in N-cycling bacteria and archaea levels did not occur directly downstream of the WWTP effluent, the tailing off of ammonium oxidizers far downstream may indicate that the early stages of processing are being adequately handled by the river ecosystem. This downstream decrease in levels may be a result of the completion of this early step close to the outflow pipe, with no need for ammonium oxidizers further downstream. The ubiquitous presence of human intestinal *Bacteroides* in all three rivers is of great concern for both human and environmental health. Although added DIN is a problem for eutrophication, we should ensure that our wastewater plants are processing out all fecal bacteria before moving our priorities to nutrient pollution because of the greater threat to public health of the former. The lack of any great response in the microbial communities combined with the high ion and *Bacteroides* levels indicates an ecosystem that is heavily polluted (even before the WWTP outflow) and likely needs restoration to augment its natural ability to clear nitrogen.

Policy Implications

The results of this study indicate that the ecosystem is not significantly responding to the added nitrogen from the WWTP outflow – likely due to high background levels of DIN from nonpoint source polluters (mostly agricultural operations) upstream. For this reason, efforts should be focused on eliminating human fecal coliform from the waters first and foremost, then on reducing DIN inputs from WWTP and agriculture. Because of the ease of identifying and regulating point source polluters like wastewater treatment plants (as compared to nonpoint source polluters), efforts should be made to reduce the additive input of these facilities to background DIN. Recent developments in policy have shown promise for such incentive and regulation programs, including wetland and riparian buffer restoration, nutrient trading, and incorporating technologies such as biotic nitrogen removal to mimic the natural processing of nitrogen within the WWTP facility (4). With climate change, nitrogen loading is expected to increase, meaning more drastic regulatory and incentive measures will likely be needed in the near future (12).

Future work in this area should be done in both more restored ecosystems (with few anthropogenic DIN contributors) and more urban ecosystems (where WWTP DIN will make up a larger proportion of nitrogen loading) to better understand how background levels of DIN affect the response of the microbial communities to a point source pollutant. A major caveat of this study is that this data is correlational, not causational; future studies should be set up to understand the spatial, temporal, mechanistic, and causal details of the ecosystem response.

INTRODUCTION

Eutrophication and the Chesapeake Bay

Water quality is a growing environmental concern around the globe for reasons of human health and ecosystem integrity. Over one billion people do not have access to safe drinking water; pollution and habitat destruction tied to anthropogenic water use are causing a freshwater biodiversity crisis (2). Water quality can be degraded through point and nonpoint source (NPS) pollution from industrial, agricultural, municipal, and residential processes (2). Eutrophication (organic matter increase) is one critical anthropogenic effect on water quality with major ecological implications. When additional organic matter settles in a water body and decays, it requires increases in decomposer respiration, lowering dissolved oxygen (DO) levels. This occurs most drastically in the deep areas, where DO is not replenished as quickly through photosynthetic activity of organisms or mixing with surface water (5). This process is the causal link between anthropogenic nutrient loading, benthic anoxia, fish kills, decreased water clarity, and aquatic ecosystem change (5).

In our own Chesapeake Bay watershed, eutrophication has become a serious problem. The bay suffers from sediment, nitrogen, and phosphorus pollution causing “dead zones” without viable fish or shellfish and subsequent fishery and tourism collapse (17). The Chesapeake Bay Executive Council – consisting of EPA representatives, state governors, legislators, and other stakeholders – reached an agreement in 2000 to work towards removing the tidal waters from the impaired list under the Clean Water Act and reducing nutrient (nitrogen and phosphorous) loadings by 40% (5). Yet a major obstacle to reaching these goals is identifying not only major point and nonpoint source pollutants, but also the mechanisms for nutrient cycling and ecosystem

change from eutrophication so that restoration can be more effective by coordinating with natural stream ecology. By understanding contributing pollutant sources as well as the processes that can cause the ecosystem to be overwhelmed by nutrient levels, restoration efforts can be more spatially and temporally targeted.

Recent water restoration efforts – especially in the Chesapeake Bay – have been directed toward assessment at the entire ecosystem or watershed level. For example, the recent Bay Total Maximum Daily Load allocations for pollutants is based on models and water quality data from all possible inputs in the watershed; limits for nutrients are placed on tributaries in VA, MD, D.C., DE, WV, NY, and PA (17). Understanding and mediating the broad-scale impacts of certain pollutants requires research on the fine-scale dynamics of nutrient processing throughout the stream. Fine-scale mechanisms can then be applied to these watershed-level restoration efforts as government and citizen groups work to regulate inputs in a cost-effective manner.

Microbial Communities, Nutrient Cycling, and Water Quality

Many different techniques can be used to estimate water quality or classify a water body as impaired. Macroinvertebrate indicators, fecal coliform levels, dissolved oxygen, conductivity, total dissolved solids and direct pollutant (nitrogen, phosphorus, lead, etc.) measurements are all commonly used to evaluate the relative impairment of water bodies. With developments in identifying, quantifying, and qualifying unique functional gene sequences, however, microbial communities are increasingly being used to monitor ecosystem function and response to nutrient loading (13, 21). Nitrogen is a serious (if not the most crucial) contributor to eutrophication; because the aquatic nitrogen cycle is a biogeochemical cycle controlled by microbial

communities, genetic analyses of DNA in water and sediment samples provide unique insights to biological capacity for N-cycling (21).

Nitrogen, a primary nutrient largely limiting rates of primary production and heterotrophic microbial activity, is a natural part of aquatic chemistry – influenced by stream geology, soil and sediment composition, climate, vegetation, and anthropogenic influxes (2). Total nitrogen in an aquatic ecosystem includes dissolved inorganic nitrogen (NO_3^-, NO_2^-, and NH_4^+), dissolved organic nitrogen, and particulate organic nitrogen. Without anthropogenic contributors, world river averages for dissolved inorganic nitrogen (DIN) levels, which are typically 40-90% of total N, is about 0.12 mg/L (2). Yet, with more point and nonpoint source pollution and less forested areas to act as riparian buffers (using excess N for growth), DIN can increase to 4.0 mg/L or more, with adverse consequences for aquatic life (2). The Chesapeake Bay TMDL calls for a 25% reduction in nitrogen throughout the watershed for bay health restoration by 2025 (17).

As shown in Figure 1, the nitrogen cycle in aquatic ecosystems "begins" with atmospheric N_2 fixation by cyanobacteria, and NH_4^+ and NO_3^- assimilation by other microbes for structural synthesis or energy (2). Nitrification, driven by specialized bacteria and archaea under aerobic conditions, transforms NH_4^+ to NO_2^- and finally to NO_3^-, which is transported downstream (2). Denitrification, also driven by specialized archaea and bacteria (but under anaerobic conditions),

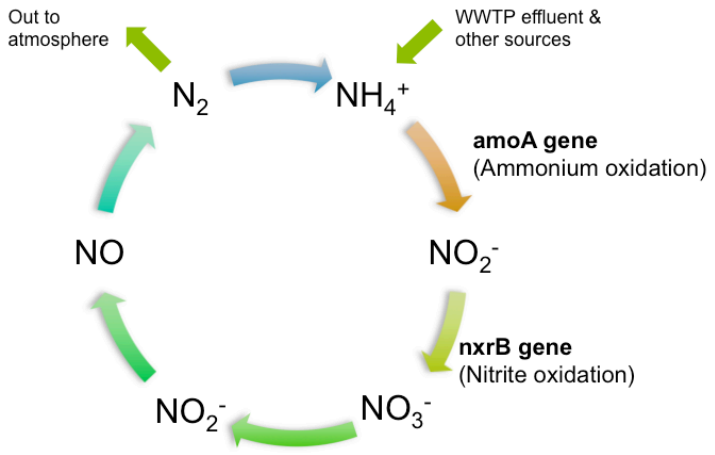


Figure 1. The aquatic nitrogen cycle. As noted, WWTP effluent contains high levels of ammonium (nitrate is also a primary anthropogenic pollutant). After being cycled to nitrogen gas (N_2), the nitrogen is no longer biologically available and is cleared from the ecosystem. Genes noted in bold are targeted by this study and control the marked steps of the cycle.

transforms NO_3^- to NO_2^- to N_2, which is released back into the atmosphere – dissipating excess N in the ecosystem (2).

One important point source contributor to total N is wastewater treatment plant (WWTP) effluent, which contains high levels of ammonium, sodium, fluoride, and chloride as a result of human inputs (21). Bacteria in the beta subgroup of *Proteobacteria* facilitate the oxidation of ammonium, which begins the processes of nitrification and subsequently denitrification (15). The *amoA* gene, which encodes the α-subunit of ammonia monooxygenase (a catalyst for the oxidation of ammonium to hydroxylamine), provides an effective molecular marker for these communities (11, 15). Recently, many *Crenarchaeota* have been found to also play a key role in ammonia oxidation, particularly because they have a higher abundance of the *amoA* gene than many ammonia-oxidizing bacteria (7). Similarly, nitrite-oxidizing bacteria, for which the nitrite oxidoreductase β subunit (*nxrB*) gene of *Nitrobacter* spp. can be a marker (encoding for an isomerase that aids in folding the enzyme for nitrite oxidation), carry out the process of nitrite oxidation to nitrate (10, 18). Real-time quantitative PCR (qPCR) using targeted primers can quantify the abundance of these functional genes in DNA extracted from environmental samples. Because bacterial and archaeal abundance (and therefore activity) tend to be much greater in sediments, benthic cores can provide better samples for DNA extraction and analyses than water samples (7). Quantification of gene abundances (particularly those related to N-cycling) in these sediment samples can then effectively indicate the biological capacity of the ecosystem to respond to nitrogen loading, as well as the ecosystem effects of nutrient stress (2).

Along similar lines, qPCR can also be used to assess the quantity of human intestinal flora in environmental DNA samples. By targeting human-associated *Bacteroides* 16S rRNA genes (HuBac), concentrations and sources of fecal pollution can be determined (13). *Bacteroides* are

intestinal flora that can cause or worsen infections if they become introduced to other areas of the body or are introduced into open wounds; they are good indicators of *E. coli* contamination in environmental samples (13). Contamination with any intestinal flora is both an environmental and public health risk. *HuBac* is targeted in this study because WWTP facilities handle human waste and because it is a clear indicator of anthropogenic pollution. High levels of nitrogen will also accompany intestinal flora because of the high nitrogen content in human waste (13).

Although gene abundances can be good indicators of ecosystem functioning, these data must be compared with physiochemical conditions to reveal mechanistic changes. Because the nitrogen cycle and other anthropogenic inputs play an important role in eutrophication, data on nitrite, nitrate, ammonium, chloride, sodium, and fluoride are most useful when correlating to the genetic data discussed above.

Current Wastewater Treatment Plant Regulatory Scheme

Wastewater treatment plants currently serve more than 75% of the United States population, with the remaining using septic systems and other alternative treatments (4). Unless the WWTP is situated on a coastline, the effluent is deposited into a nearby river after processing (4). WWTPs use some combination of chemical treatment and UV light to kill intestinal bacteria that present a public health hazard before emptying into the river. Primary treatment of waste entails the settling out of coarse solids, while secondary treatment uses biological processing of remaining organic matter (4). Over 70% of existing WWTP facilities use both primary and secondary treatment to handle waste, but a very small proportion are including tertiary measures to remove nitrogen and other problem pollutants from the effluent (4).

METHODS

Site Sampling

5-cm sediment cores were taken using PVC pipe 3 cm in diameter at various distances in relationship to the WWTP outflow pipe. Sediment cores were taken in the Maury around the Lexington WWTP outflow and around the Buena Vista outflow, and in the Maury and the James around the Glasgow WWTP outflow. The Glasgow WWTP discharges into the James at the confluence of the two rivers, so samples were taken upstream in both rivers and downstream of the confluence. Distances varied depending on the location of available access points to the river. Distances from outflow were estimated in the field and determined with specificity using Google Maps. See Table 1 for the distances of specific sites and Figure 2 for satellite images of the locations.

Table 1. Sediment core sampling distances from WWTP outflow at three sites. Upstream and downstream denote the spatial relationship of sampling sites to the WWTP outflow pipe.

Lexington (Maury)	Buena Vista (Maury)	Glasgow (James & Maury)
310 m upstream	950 m upstream	James – 1.09 km upstream
5 m upstream	2 m upstream	James – 2 m upstream
10 m downstream	2 m downstream	Maury – 370 m upstream
470 m downstream	300 m downstream	Maury – 70 m upstream
900 m downstream	720 m downstream	James – 3 m downstream
		After confluence – 300 m downstream
		After confluence – 700 m downstream

Samples were taken by pressing an angled PVC pipe into the sediment, sealing the top underwater (creating a vacuum seal), and removing the pipe from the river. The top layer of river water was removed using a baster, leaving 4-5 cm of water on top of the core. Upon collection,

sediment core and water samples were transferred to sterile 100-mL Whirl-Pak® bags and kept on ice until returned to the lab.

Sample Processing

The cores were incubated at 4°C for 25 days to allow the water to extract ions from the sediment. Samples were then centrifuged at 5000 rpm for 5 minutes to separate the water from the sediment core. The water was vacuum-filtered through 0.45 μm filter paper and the volume measured using a graduated cylinder. The filtered water and remaining core were then frozen at - 80°C until further analysis.

DNA Extraction and qPCR Analysis

DNA was extracted using an UltraClean Soil DNA Extraction Kit (MoBio Laboratories, CA) and following the associated protocol. Approximately 1 g of sediment was used per sample, with specific measurements recorded. Total dsDNA present in the samples was determined using the Tecan Infintite 200 PRO NanoQuant Absorbance Reader (Tecan Group Ltd., Switzerland).

Target functional genes, enzymes, and processes are listed in Table 2. All reactions were conducted on a BioRad iQ5 using 10 μL of iQ SYBR Green Supermix (BioRad USA, CA) with 1 μL of template DNA in a 20 μL total reaction. The quantification of each gene was based on the protocols cited in the reference papers.

Table 2. Primers used in qPCR analyses				
Target Gene	Protein	Relevance to nutrient cycling	Gene Sequence (5'-3', forward listed first, reverse after)	Ref.
amoA	α-subunit of ammonia monooxygenase	Oxidation of ammonium to hydroxylamine ($NH_3 + 0.5O_2 \rightarrow NH_2OH$)	GGGGHTTYTACTGGTGGT CCCCTCKGSAAAGCCTTCTTC	(15)
Archaeal *amoA*	Same as above (in archaea)	Same as above	STAATGGTCTGGCTTAGACG GCGGCCATCCATCTGTATGT	(9)
nxrB	β-subunit of nitrite oxidoreductase	Oxidation of nitrite to nitrate ($2NO_2^- + O_2 \rightarrow 2NO_3^-$)	CGTCGTGCGCAAGATGGAGA CCCGGCTTGGTCTCCACGT	(18)
rpoB	β-subunit of bacterial RNA polymerase	Total bacteria	CGAACATCGGTCTGATCAACTC GTTGCATGTTCGCACCCAT	(16)
Archaeal 16S rRNA gene	16S rRNA	Total archaea	GCYTAAAGSRICCGTAGC TTMGGGGCATRCIKACCT	(3)

The *amoA* gene for both bacteria and archaea was quantified using standards from serial dilutions of a purified 491 and 635 bp plasmid, respectively. Plasmids were obtained from Karen Adair per her paper protocol (1). Each *amoA* reaction used 0.4 μL of each primer pair and the following thermocycling conditions: 95°C for 2 minutes, followed by 40 cycles of 95°C for 30 s, 55°C for 1 min, 72°C for 45 s, and 83°C for 30 s, followed by 7 minutes at 72°C, and 81 cycles (20 s each) increasing by 5°C each cycle from 55°C to 95°C. The *nxrB* gene quantification used 0.125 μL of each primer and followed a similar thermocycling protocol of: 95°C for 5 min, followed by 40 cycles of 95°C for 1 min, 61°C for 2 min, and 76°C for 1.5 min, followed by 69 cycles of 20 s each increasing from 61°C to 95°C in increments of 5°C per cycle. Standards for this gene were serial diluted from DNA extracted from bacteria in the *Nitrobacter* genus. Quantification of the *rpoB* gene was standardized using purified extract of K12 E. coli DNA and used 0.5 μL of each primer and the following thermocycling protocol: 95°C for 15 s, followed by

40 cyles of 95°C for 5 s, 55°C for 20 s, 72°C for 30 s, then 81 cycles of 30 s each, increasing by 5°C each cycle from 55°C to 95°C. The universal archaea gene was quantified using 0.7 μL of each primer and the following protocol: 94°C for 2 mins, followed by 40 cycles of 94°C for 30 s, 60.5°C for 1 min, and 72°C for 1 min, then one cycle of 88°C for 30 s, another of 72°C for 10 min, and 81 cycles of 20 s each, increasing by 5°C each cycle from 55°C to 95°C. Standards for this gene were serial diluted from extracted DNA from *Halobacterium* species NRC-1. Quantification of the *HuBac* gene was standardized using purified extract of bacteria in the *Bacteroides* genus using 0.8 μL of each primer (20 pmol/μL concentration) and the following protocol: 5 minutes at 95°C, followed by 40 cycles of 30 s at 95°C, 45 s at 55°C, and 1.5 min at 72°C, then one cycle of 5 mins at 72°C, and 81 cycles of 20 s each, increasing by 0.5°C each cycle from 55°C to 95°C. All assays were performed in duplicate, with DNA melting curve analyses to prevent errors caused by primer-dimers or other amplification artifacts. Negative controls without any DNA were also be analyzed to minimize artifact error. Gene copy numbers were calculated from standard curves taken from the log transformation of known concentrations plotted against the threshold cycle data. Gene copy numbers were standardized according to the concentration of total bacteria (from the rpoB primer results) in order to approximate standardization according to total prokaryotic DNA. Concentration of ds-DNA found in the sample, which was quantified using spectrophotometric determination, was not found to be a useful standardization even though this is typical for qPCR methods. The relationship between log gene copy number of total bacteria (rpoB) and total archaea (UA) should be 1:1 with the DNA concentration in ng/μL. Yet, after graphing this relationship, there was only a very weak correlation – likely due to a high presence of eukaryotic DNA in the extractions (Figure 3). For

this reason, rpoB values were instead used to standardize. Gene copy numbers were then standardized against sediment extraction weight to report copy number per gram of sediment.

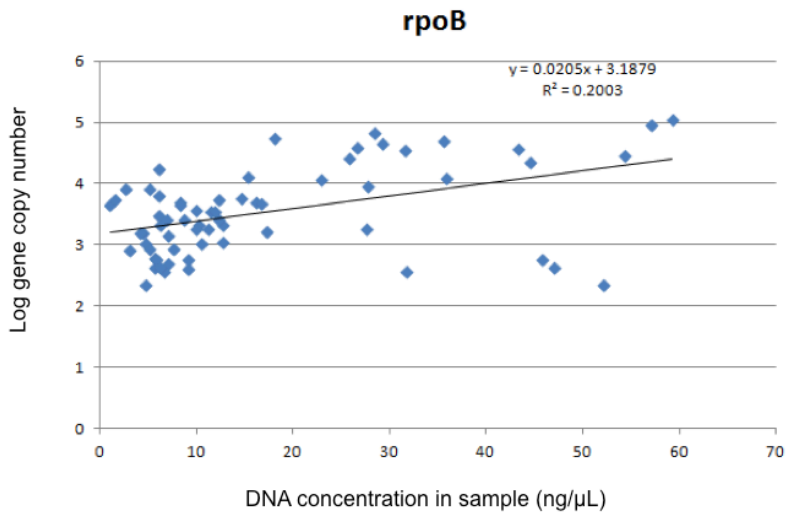


Figure 3. Graph of rpoB (total bacteria) log gene copy number versus DNA concentration in sample. All 70 sample extractions (from all sites) were used as points for the scatterplot. The weak (although statistically different than 0) relationship justifies standardizing according to total bacteria rather than DNA concentration. Total archaea (not pictured) followed a very similar trend.

Physiochemical Analyses

Ion chromatography was used to determine the concentrations of ions in the sediment samples using the filtered water frozen after sample processing. Anion and cation analyses were performed to determine levels of Cl^-, Fl^-, Na^+, NH_4^+, NO_3^-, and NO_2^-. Standard curves for ions of interest were created using serial dilutions of solutions with known concentrations of ions. Data were calculated as concentrations in mg/g sediment to be analogous to other sediment water quality analyses.

Statistical Analyses

Frequentist statistics were used to determine statistical significance of comparisons across sampling sites. A one-way ANOVA was used to identify differences among physiochemical

concentrations and microbial abundances at the different distances from the WWTP outflow. Correlation analyses were used to determine relationships between microbial community abundance and physiochemical properties of sediment samples.

RESULTS

Quantitative PCR Results

Quantitative PCR results are shown in Figure 4. Across most sites, the differences in total bacteria and archaea levels were not statistically significant ($p > 0.05$). The one exception to this pattern was seen in Glasgow, where total archaea increases far downstream ($p < 0.05$). Interestingly, total archaea is higher than total bacteria at all sites, which verifies the relatively new discovery that archaea are ubiquitous in natural ecosystems and not just in extreme environments. Nitrite oxidizing bacteria (*nxrB* gene) were also consistent across sites, with no statistically significant differences within sites. Ammonium-oxidizing bacteria (AOB) and ammonium-oxidizing archaea (AOA) both show statistically significant differences in Glasgow at the downstream sites, with AOA increasing and AOB decreasing ($p < 0.05$). This strange effect could be due to the impact of the confluence of the two rivers. In Lexington and Buena Vista, AOB decreased downstream of the confluence ($p < 0.05$). AOA followed a similar trend in Lexington ($p > 0.05$), but increased downstream in Buena Vista ($p > 0.05$). The fewer statistically significant differences in AOA might be due to the greater within-site variation for this variable.

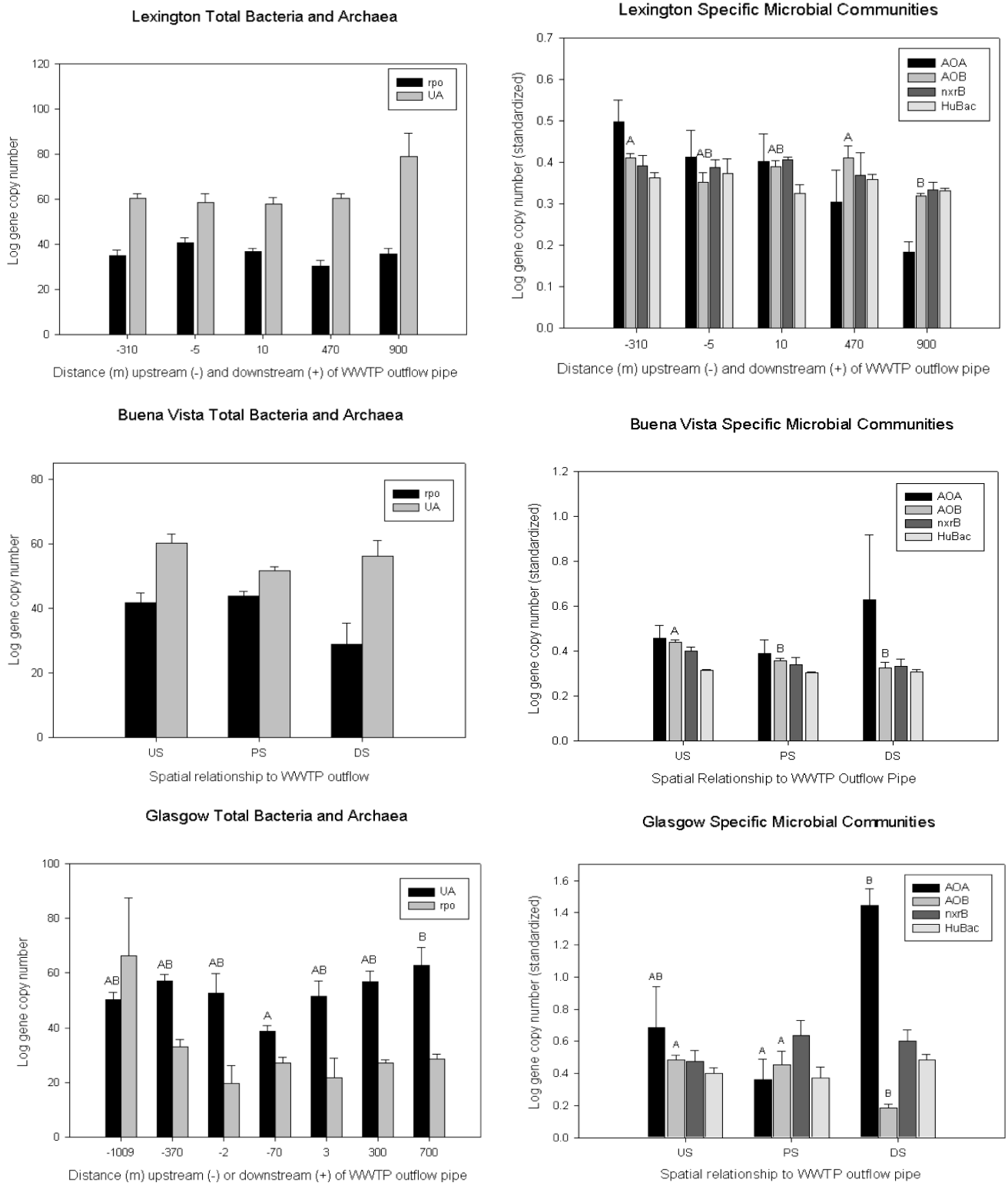


Figure 4. qPCR results for the three study sites. The Y-axis for all graphs shows log gene copy number standardized for sediment extract weights. AOA (ammonium-oxidizing archaea – *arch-amoA* primer) is further standardized according to total archaea levels and the other functional genes are standardized according to total bacteria levels. Numbered sites indicate meters from outflow pipe, while the grouped sites (US – upstream, PS – point source, DS – downstream) show data pooled for those locations. Glasgow sites alternate between the James and the Maury on the X-axis, with 300 and 700 being meters downstream of the confluence. Letters indicate differences significant at $p < 0.05$.

HuBac, which can be used as an indicator of human fecal coliform contamination, shows no statistically significant differences across sites. Alarmingly, however, the levels of human *Bacteroides* were very high at all sites, in concentrations comparable to those of the naturally-occurring nitrogen-cyclers.

Ion Chromatography Results

Ions of interest from these analyses included ammonium (NH_4^+), nitrate (NO_3^-), nitrite (NO_2^-), sodium (Na^+), fluoride (Fl^-), and chloride (Cl^-) because these are the primary anthropogenic ions in waterways. DIN (ammonium, nitrate, and nitrite) is of particular interest because WWTP effluent should contribute high levels of ammonium, which should then be processed and cleared to N_2 gas by the river ecosystem. See Figure 5 for results of interest. In Lexington, sodium and ammonium levels in sediment cores spiked drastically just around the point source and declined further downstream ($p < 0.05$). Sodium has a similar, yet not statistically significant, trend in Glasgow. Fluoride peaks far downstream in Glasgow ($p < 0.05$) but at the point source in Lexington ($p > 0.05$). In Buena Vista, chloride is much higher 300 m downstream than 2 m downstream from the outflow pipe ($p < 0.05$). In Glasgow, chloride is much higher at the 1009 m upstream site in the James than at the 70 m upstream site in the Maury ($p < 0.05$). Nitrite and nitrate have no statistically significant differences across sites. Total nitrogen (determined by converting DIN to total N equivalents and summing) shows a clear spike downstream in Lexington and Buena Vista ($p < 0.05$), but a decrease in Glasgow downstream ($p < 0.05$) – which may likely be due to the diluting effect of the confluence of the two rivers.

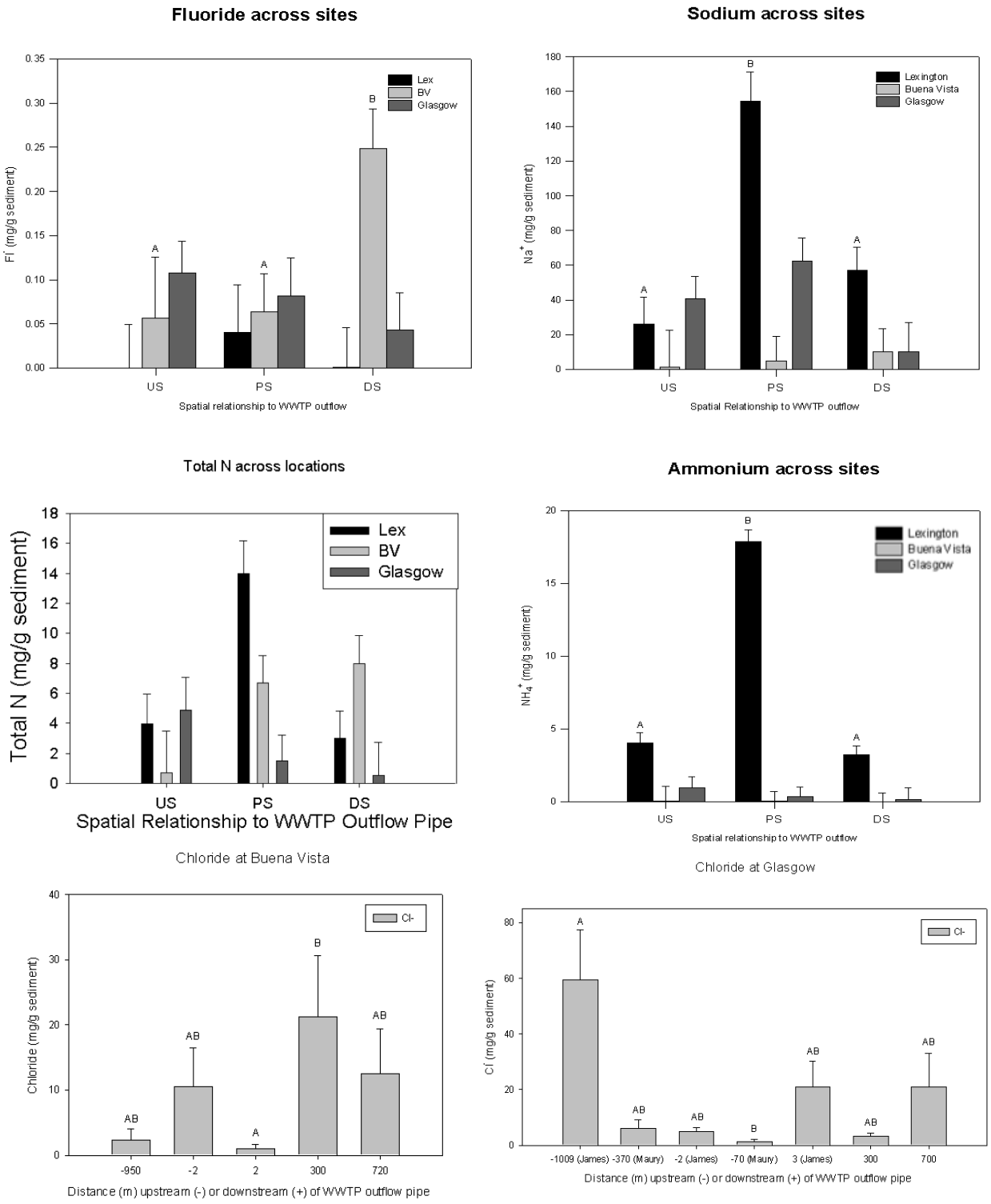
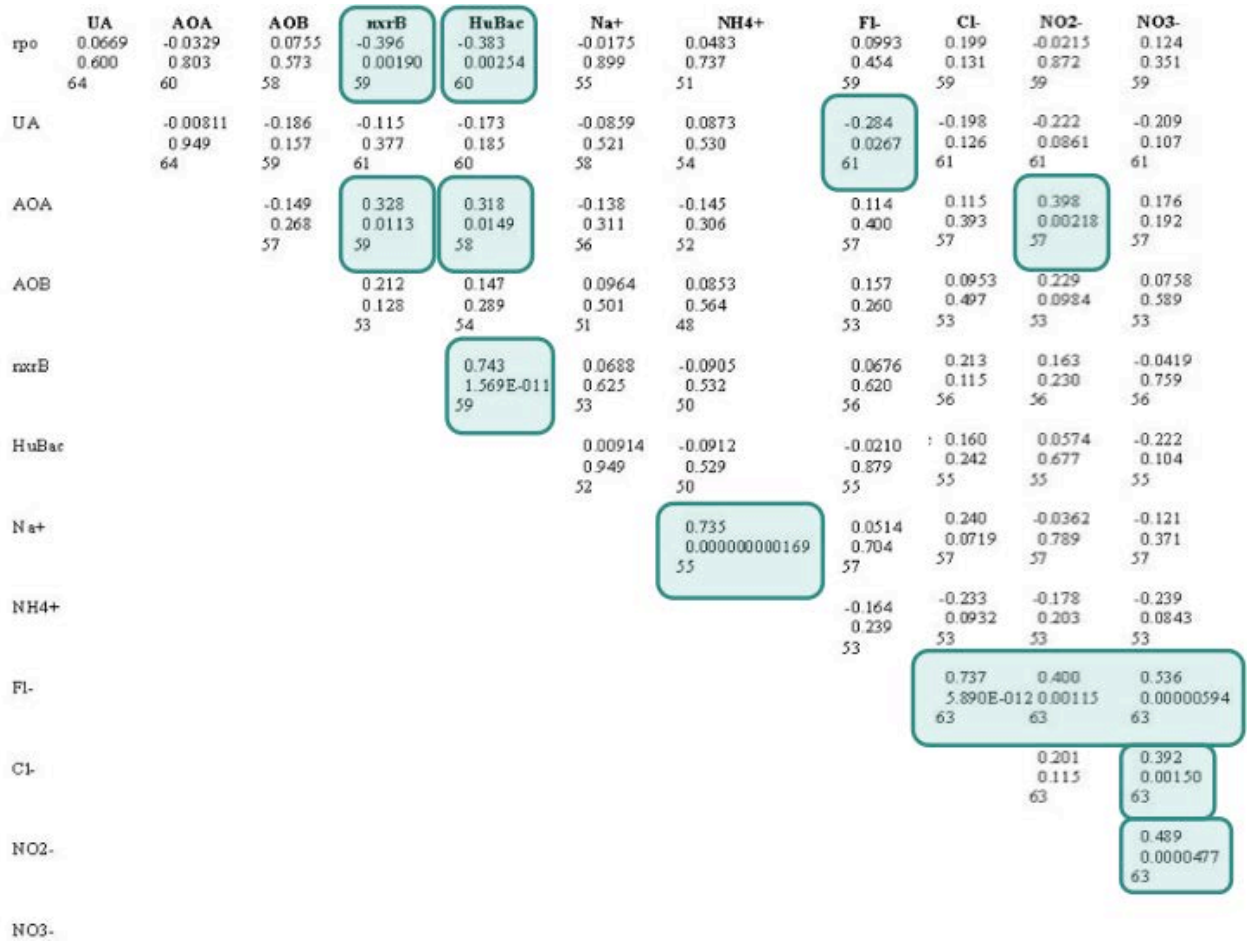


Figure 5. Ion chromatography results. Only ions with statistically significant results are shown. Sites are pooled according to upstream (US), point source (PS), and downstream (DS). All relationships are statistically significant for total N, and lettered relationships are statistically significant for fluoride, ammonium, and sodium. All differences are significant at $p < 0.05$.

Correlation Analysis

The results from a Pearson correlation analysis are shown in Table 3. Total bacteria are inversely correlated with nitrite-oxidizing bacteria ($p = 0.0019$) and human *Bacteroides* ($p = 0.0025$). Universal archaea is inversely correlated with fluoride ($p = 0.027$). All other relationships are positive correlations. Generally, indicators of anthropogenic contributions are correlated with one another. Ammonium-oxidizing archaea (AOA) are positively correlated with nitrite ($p = 0.0022$) and nitrite-oxidizing bacteria ($p = 0.0113$), both of which are to be expected because these three factors are closely linked in the nitrogen cycle. AOA and nxrB are also positively correlated with human *Bacteroides* (HuBac) at $p = 0.0149$ and $p = 1.57 \times 10^{-11}$, respectively. Sodium and ammonium, both components of wastewater treatment plant outflow, are strongly correlated ($p = 1.7 \times 10^{-10}$). Fluoride is correlated with all other anions. Chloride and nitrite are both correlated with nitrate ($p = 0.0015$ and $p = 0.00005$, respectively).



	UA	AOA	AOB	nxrB	HuBac	Na+	NH4+	Fl-	Cl-	NO2-	NO3-
rpo	0.0669 0.600 64	-0.0329 0.803 60	0.0755 0.573 58	-0.396 0.00190 59	-0.383 0.00254 60	-0.0175 0.899 55	0.0483 0.737 51	0.0993 0.454 59	0.199 0.131 59	-0.0215 0.872 59	0.124 0.351 59
UA		-0.00811 0.949 64	-0.186 0.157 59	-0.115 0.377 61	-0.173 0.185 60	-0.0859 0.521 58	0.0873 0.530 54	-0.284 0.0267 61	-0.198 0.126 61	-0.222 0.0861 61	-0.209 0.107 61
AOA			-0.149 0.268 57	0.328 0.0113 59	0.318 0.0149 58	-0.138 0.311 56	-0.145 0.306 52	0.114 0.400 57	0.115 0.393 57	0.398 0.00218 57	0.176 0.192 57
AOB				0.212 0.128 53	0.147 0.289 54	0.0964 0.501 51	0.0853 0.564 48	0.157 0.260 53	0.0953 0.497 53	0.229 0.0984 53	0.0758 0.589 53
nxrB					0.743 1.569E-011 59	0.0688 0.625 53	-0.0905 0.532 50	0.0676 0.620 56	0.213 0.115 56	0.163 0.230 56	-0.0419 0.759 56
HuBac						0.00914 0.949 52	-0.0912 0.529 50	-0.0210 0.879 55	0.160 0.242 55	0.0574 0.677 55	-0.222 0.104 55
Na+							0.735 0.000000000169 55	0.0514 0.704 57	0.240 0.0719 57	-0.0362 0.789 57	-0.121 0.371 57
NH4+								-0.164 0.239 53	-0.233 0.0932 53	-0.178 0.203 53	-0.239 0.0843 53
Fl-									0.737 5.890E-012 63	0.400 0.00115 63	0.536 0.00000594 63
Cl-										0.201 0.115 63	0.392 0.00150 63
NO2-											0.489 0.0000477 63
NO3-											

Table 3. Results of a Pearson correlation analysis between all of the parameters measured by this study. Statistically significant correlations are highlighted. The top number in each square indicates the correlation coefficient, the second is the p value, and the last is the number of samples in the comparison.

Conclusions and Recommendations for Future Work

qPCR and Ion Data

The relatively equal abundances of total bacteria and total archaea across sites indicates that, while WWTP outflow is not stimulating microbial community growth, it is also not adversely

impacting microorganisms important to the natural river ecosystem. The latter situation would likely occur when WWTPs use harsh chemicals to sanitize and process the water. The higher counts of archaea (than bacteria) at all sites could be a result of the pollution in the rivers, which tends to create anaerobic environments in the sediment – an extreme environment ideal for archaeal community growth. The ubiquitous presence of human *Bacteroides* – a good indicator of human fecal coliform – is a disturbing result, particularly because it is present in levels comparable to the nitrogen-cycling bacteria that occur naturally. Without anthropogenic contributions, these bacteria would not be in the river sediment; yet they have climbed to high levels throughout the study sites. Furthermore, these bacteria do not show a pattern in relationship to the WWTP effluent, which indicates that they are being contributed elsewhere in the river – possibly from leaking septic tanks or polluted tributaries. This verifies the recent classification of the Maury River as impaired with fecal coliform by the Virginia Department of Environmental Quality (20).

The nitrogen-cycling bacteria and archaea do not show drastic differences around the outflow pipe, but ammonium oxidizers (AOA and AOB) do show a general trend with numbers trailing off far downstream. This is likely due to the reduced need for ammonium oxidizers far downstream because most of the ammonium is processed closer to the outflow pipe. The actual ion levels, however, show a clearer influence of the WWTP effluent – particularly ammonium and sodium in Lexington, and total N in Lexington and Buena Vista. Fluoride and chloride – two other indicators of anthropogenic inputs – also show general trends of increase downstream in Buena Vista and Lexington. The different trends in Glasgow may be a result of the mixing and dilution of the two rivers downstream of the confluence. Further differences among locations may also be due to differences in WWTP functioning. Lexington, for example, has a continuous

flow and a comparatively large processing volume. Buena Vista and Glasgow handle much smaller volumes, and Buena Vista flows only intermittently.

Correlation Analysis

The primary indicators of anthropogenic pollution were correlated to one another. Of course, this analysis shows only correlation, not causation, but some possible explanations for the relationships shown can be inferred. The inverse relationships, for example, between total bacteria and human *Bacteroides* and between total archaea and fluoride might indicate that anthropogenic pollution is adversely affecting the sediment microbial communities. Both may, of course, be impacted by some third variable not included in this study, but considering the scope of pollution found, adverse impacts in microbial communities is not unreasonable.

The correlations between AOA and nxrB and nitrite are all logical because AOA is responsible for creating nitrite, which is then processed by nxrB (see Figure 1). The correlations between AOA and nxrB and HuBac might be explained by the presence of nitrogen in human waste, which would also have high levels of human *Bacteroides*. Sodium and ammonium have a strong correlation because they are both products of WWTP effluent and indicators of anthropogenic pollution. Most of the anions are also strongly correlated; again, likely due to the combined indicators of human influence.

Final Conclusions and Recommendations

One area for future work is the need for better standardization measures for qPCR data. Because of the relationship found between DNA concentration and total bacteria copy number (Figure 3), this project standardized according to total bacteria as an approximation of total prokaryotic DNA concentration extracted in the original samples. One potential future avenue would be to

run a total eukaryotic primer and standardize according to total DNA concentration after subtracting the amount of eukaryotic DNA present. Unfortunately, I was not able to troubleshoot this method before the completion of this project.

The correlation analysis, combined with the high anthropogenic ion levels and ubiquitous presence of human intestinal flora, point toward impaired rivers with a history of pollution. The *Bacteroides* result further indicates that we may be drastically underestimating the impact of anthropogenic pollution on the rivers; we have contributed enough pollution for human intestinal bacteria to be present at levels comparable to natural nitrogen-cyclers in the sediment. Despite these indicators, I found few to be impacted directly by the WWTP outflow – sodium and ammonium were really the only clear spikes in some sites. This is likely due to the high background nitrogen in Rockbridge County from nonpoint source polluters; the additive nitrogen from WWTP effluent may not be great enough to cause a response from microbial communities. Effluent can contribute anywhere from 20-80% of total nitrogen in a waterway, depending on the population concentration and land-use practices in the watershed – with large metropolitan areas in the northeast on the high end of that range (8). Because of the high levels of nonpoint source pollution in Rockbridge and the small populations handled by the WWTPs, we are most likely closer to the 20% side of that range. Similar projects are needed in very restored and very urban areas, then, to represent the full spectrum of relative WWTP nitrogen contributions. Only by encompassing this range of restored, unrestored with primarily nonpoint source pollution, and unrestored with primarily point source pollution can the interacting dynamics of various polluters be understood. Many of the problems associated with regulating pollution involve understanding and demonstrating the relative contributions of and interactions between multiple polluters in a waterway.

For these reasons, the lack of a drastic microbial community response in this project should not be interpreted as an indicator of a healthy ecosystem, particularly considering the high ion and *HuBac* levels in the sediment cores. Based on these other parameters, the rivers are more likely to have such high background nitrogen that the nitrogen cyclers cannot grow further to adapt to the WWTP effluent inputs. Future work in the area of local nitrogen pollution should – ideally – assess nitrogen levels and microbial communities (including *E. coli* with the use of bacterial plating) throughout the watershed in both sediment, water, and directly from outflow pipes. While this project is likely too involved to undertake, targeted sampling of water and sediment around tributaries, pipes, and agricultural operations (as well as far upstream in the watershed, before pollution has a large influence) can give a clearer picture of watershed health and where to best direct restoration efforts.

Policy Implications

General Recommendations

The most important finding of this study to target for restoration is the ubiquitous presence of human intestinal bacteria in the sediment, which presents a risk for both human and environmental health. These findings support the recent listings of the Maury and James as impaired with intestinal bacteria by the Virginia Department of Environmental Quality, and suggest that human inputs are a significant portion of this impairment (20). However, because there were no significant trends upstream and downstream of the WWTP outflow pipe, these bacteria seem to be contributed from other sources throughout the waterways. Some possibilities include leaking septic tanks, sewer pipes, and the contributions of polluted tributaries. A finer-

scale analysis aimed at these various sources would be a good first step to developing appropriately targeted policies. If septic systems are the main culprit (in tributaries as well as main channels), for instance, a state-wide tax credit for updating a septic system may be an effective policy. On the other hand, if leaking sewer pipes contribute contamination, than local governments should plan for reinforcement and remodeling of those structures, and individual homeowners would not be targeted by policy directly. The pollution may very easily be caused by all three and other factors, in which case a more holistic policy approach would be needed.

In order to reduce nitrogen loadings in the Chesapeake Bay by 40%, serious reduction is needed for all sources of nitrogen in all sub-watersheds (5). Because of this, point and nonpoint source pollution in Rockbridge need to be limited for both our own watershed health and the health of the Chesapeake Bay. The results of this project show that while WWTP effluent is not the most serious pollution problem in Rockbridge, it contributes high levels of ammonium and sodium to already polluted waterways – which may not have the microbial capacity for clearing the added nitrogen. Because of the comparative ease of targeting point source polluters like WWTPs, local governments may want to target their (albeit small) contributions of nitrogen before moving on to nonpoint source polluters. Ideally, research such as this would target the entirety of local waterways to assess the differential impacts of various polluters and target restoration accordingly. However, since many local rivers have been found impaired by the VA DEQ, and because the results of this study point to impaired waterways, restoration efforts should begin immediately and target as many sources as possible – particularly to achieve the drastic reduction in nitrogen needed for the Chesapeake Bay. With climate change, too, nitrogen loadings are projected to increase in many scenarios, meaning that even further reductions may be necessary than currently estimated (12).

Options for Management

One relatively new policy development for improving water quality is pollution trading, which typically involves trading nutrients (14). This option – essentially a "cap and trade" for watersheds – might provide a more economically efficient way of reducing pollution than strict regulatory policies. The legal framework for such a program has already been implemented in VA and a number of trades for both nitrogen and phosphorus took place starting in 2011 (19). Even so, this program is relatively new and has yet to be implemented on a large scale.

In general, removal of nitrogen from WWTP effluent before it exits the facility is costly and inefficient, but new methods of biotic nitrogen removal (BNR) mimic the natural sediment microbial communities within the facility to clear nitrogen before the effluent is expelled (8). These projects have been shown to reduce nitrogen by 67% from secondary WWTP treatment and by 87% beyond primary treatment (8). Of course, restoring natural river habitat in order to support the natural clearing of nitrogen can create similar effects. Common methods for river restoration include riparian buffer or wetland restoration, both of which increase retention time for runoff, allowing excess nitrogen to be used by those ecosystems before reaching the river (4). Constructed wetlands are becoming popular to pair with WWTP facilities for denitrification and uptake of biologically reactive nitrogen (4). An additional supplement to these programs is the pairing of WWTP effluent with aquaculture in artificial wetlands. Such a program in Los Angeles reduced inorganic nitrogen by 97% while producing 1000 kg of tilapia (4). Considering the unpredictable nature of nitrogen inputs from polluters, wetlands are consistent, economically viable methods for "sinking" nitrogen (6). While promising, one of the concerns with wetland restoration is incomplete denitrification, which occurs when wetlands are overloaded with nutrients (as is likely to occur in these locations). In this case, nitrogen is released as N_2O gas – a

potent greenhouse gas – instead of N_2, with potentially serious implications for climate change (4). In Rockbridge, riparian buffer restoration might be both more accessible and more effective, particularly for targeting both point and nonpoint source pollutants. An interesting option would be to implement some type of wetlands or riparian buffer trading programs according to nitrogen inputs from polluters in the area.

In general, there are many options for reducing nitrogen from polluters – targeting point sources from within the facility or targeting all pollution by supporting natural clearance in the receiving ecosystems. The most effective avenue is likely to be a combination of all of these methods and others, but fine-scale analyses of microbial communities and nutrient levels can assist restoration in cooperating with the natural clearing of pollutants. Based on the results of this study, anthropogenic impacts on our local rivers are widespread, and restoration efforts should be implemented as soon and as widely as possible – with adjustments later as pollution sources are further pinpointed – to reduce the pollution load on both our local streams and the Chesapeake Bay.

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