Student Status	Number	Disciplines
Undergraduate	4	Biology
M.S.	2	Biology
Ph.D.	0	
Post Doc	0	
Total	6	

Technical Report

Title: Impact of Wastewater Treatment Plant Effluent on Nitrogen Cycling by Stream Bacteria

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Publications: None at the time this report was prepared. We anticipate that at least two manuscripts will be prepared for submission. The OWRRI will be notified of any future publications that result from this work.

Problem and Research Objectives:

Treated wastewater contains nutrients, such as ammonium (NH_4^+), and organic compounds, such as antibiotics. Nitrogen is both an essential nutrient and a potential pollutant that can lead to degradation of water quality when levels in the environment are high. Antibiotics at high concentrations can kill or inhibit the growth of bacteria. However, it has also been suggested that low concentrations of antibiotics play a role in bacterial communication in natural ecosystems and can cause changes in bacterial gene expression. The effects of contaminants, including nitrogen and antibiotics, in wastewater treatment plant effluent on nitrogen cycling in downstream bacterial populations are not well understood.

This study had four objectives.

1. To characterize bacterial diversity in a stream that receives wastewater treatment plant effluent, and compare upstream and downstream populations
2. To determine the abundance of bacterial genes encoding enzymes involved in nitrogen cycling in bacterial populations upstream and downstream of a wastewater treatment plant
3. To examine the expression of genes involved in nitrogen cycling in bacteria downstream of a wastewater treatment plant and compare to the upstream population
    a. In addition, in-stream uptake of ammonium (NH_4^+) upstream and downstream of the Tahlequah WWTP will be measured and compared.
4. To examine ammonium (NH_4^+) uptake by sediment bacteria and the effect(s) of antibiotics on bacterial nitrogen metabolism

Methodology:

Extraction of nucleic acids from stream sediments

Tahlequah Creek sediments, 200m upstream and 100m downstream of the Tahlequah wastewater treatment plant, (WWTP) were sampled twelve times over the course of this study. Sediment samples were stored on ice until processed. All samples were processed within 6 hrs of sampling. A MoBio Power Max® Soil DNA isolation kit was used to purify high quality DNA from 5g of sediment. DNA samples were concentrated by ethanol precipitation and stored at -80˚C in 10mM Tris pH 7.4 buffer. A MoBio PowerSoil® Total RNA Isolation kit was used to purify RNA from 2g of sediment. Although yields were high using the MoBio kit the RNA was contaminated with RNases, which resulted in degradation of the RNA when we attempted to purify mRNA from total RNA. mRNA is preferred over total RNA in gene expression studies as signal intensities are increased. Several attempts were made to obtain RNase-free total RNA including additional phenol extraction of the RNA samples, use of two RNA Capture columns during purification with the MoBio kit, and use of an RNA-binding column (MEGAclear™ Kit, Invitrogen) for removal of contaminants from RNA. However, none of the treatments was successful. Therefore, we decided to use total RNA rather than mRNA for preparation of fluorescently labeled cDNA. The RNA pellets obtained from the MoBio PowerSoil® Total RNA Isolation kit were resuspended in RNA Storage Solution (Ambion®) and stored at -80˚C. Nucleic acids were quantitated using a Qubit® 2.0 fluorometer and dsDNA Broad Range Assay or RNA Assay kits (Invitrogen). Nucleic acids were purified from triplicate biological replicates (sediment samples) taken at the upstream and downstream locations.

GeoChip hybridization and scanning

Fluorescently labeled genomic DNA (gDNA) was prepared and hybridized to GeoChip 4.0 (NimbleGen) arrays as described previously (Wu et al. 2006; Lu et al. 2011). In brief, 1 µg of gDNA was labeled with Cy-3 by random priming. Labeled gDNA was suspended in hybridization buffer containing a universal standard DNA labeled with the fluorescent dye Cy-5 (Liang et al. 2010). Fluorescently labeled cDNA was prepared from total RNA as described by He et al. (He et al. 2005). In brief, 500ng of total RNA was amplified using a MessageAmp™ II-Bacteria Kit (Invitrogen). Ten micrograms of amplified RNA (aRNA) was then used to prepare Cy-5 labeled cDNA using random primers and reverse transcriptase. Labeled cDNA was suspended in hybridization buffer containing a universal standard DNA labeled with the fluorescent dye Cy-3. Fluorescently labeled nucleic acids were denatured and loaded onto GeoChip 4.0 microarrays. Twelve arrays were used in these experiments: 3 arrays were hybridized with Cy-5 labeled gDNA from replicate upstream sediment samples, 3 arrays were hybridized with Cy-5 labeled gDNA from replicate downstream sediment samples, 3 arrays were hybridized with Cy-3 labeled cDNA derived from replicate upstream sediment RNA samples, and 3 arrays were hybridized with Cy-3 labeled cDNA derived from replicate downstream sediment RNA samples. Hybridization was performed overnight using a Hybridization Station (Maui; Roche). Arrays were washed and scanned using an MS 200 Microarray Scanner (NimbleGen).

GeoChip 4.0 data analyses

Raw data were analyzed using the Institute for Environmental Genomics data analysis pipeline (ieg.ou.edu) as described previously (He et al. 2010). Signal intensities of GeoChip hybridization spots were normalized across samples using the universal standards included in the hybridization

buffers. Positive spots were selected based on signal-to-noise ratios [(signal intensity-background intensity)/background standard deviation]. Cy-5 labeled gDNA positive spots had signal-to-noise ratios (SNRs) ≥ 2.0. Cy-3 labeled cDNA (from RNA) positive spots had SNRs ≥ 2.0 and SNRs ≥ 1.0 for the corresponding gDNA probes. All signal intensities were log-normal transformed. A minimum of two valid values was required for each gene included in the analyses.

Statistical analyses included calculation of diversity indices (Shannon and Simpson), cluster analysis, dissimilarity analysis, and analysis of gene categories and genes using a T-test.

Bacterial diversity based on pyrosequencing of 16S rRNA genes

Artificial substrates (Fluval Biomax bio rings, Hagen) were buried in Tahlequah Creek sediments upstream and downstream of the Tahlequah WWTP (200 m and 100 m, respectively) 30d prior to sampling to allow for colonization by resident bacteria. The bio rings are porous ceramic media designed for biological filtration in aquarium systems. A MoBio Power Max® Soil DNA isolation kit was used to purify high quality DNA from 10-12 g of bio rings and from 5g of sediment. Artificial substrate and sediment samples were harvested simultaneously from the locations described above. All samples were placed on ice and processed within 6 hours of sampling or stored at -20˚C until processed. DNA samples were concentrated by ethanol precipitation and stored at -80˚C in 10mM Tris pH 7.4 buffer.

Bacterial FLX-Titanium amplicon pyrosequencing (TEFAP) and data processing were performed at the Research and Testing Laboratory (Lubbock, TX) as described previously (Handl et al. 2011). In brief, small subunit rRNA gene primers, forward28F (GAGTTTGATCNTGGCTCAG) and reverse519R (GTNTTACNGCGGCKGCTG), were used to generate sequence data that included the V1–V3 hypervariable regions. Analysis of sequence data was performed in two stages. Stage I, sequence reads were checked for quality and denoised. Chimeras and poor quality sequences were removed. Stage II, sequencing data was used to examine bacterial diversity in the samples. Sequences were queried against a database of high quality sequences derived from NCBI (KrakenBLAST www.krakenblast.com) and classified at the appropriate taxonomic levels using the following criteria. Sequences with > 97% identity were resolved at the species level, between 95% and 97% at the genus level, between 90% and 95% at the family level, between 85% and 90% at the order level, between 80 and 85% at the class level, and between 77% to 80% at the phyla level. Files containing taxonomic information for each read and the number and percentage of each taxonomic group within each sample were generated.

Artificial substrate and stream sediment communities were compared to determine if the bacterial populations were similar or different using principal components analysis (CANOCO for Windows 4.53).

In-stream nitrogen uptake

Ammonium chloride was introduced to the stream at a constant rate 100m upstream from each sample site (upstream and downstream of the Tahlequah WWTP). Specific conductivity was used as a conservative tracer in-stream and to correct for dilution in samples analyzed in the laboratory. Once in-stream conductivity reached a stable maximum water samples were taken every 10m downstream of the injection point for 100m. These samples were placed on ice and transported back to the laboratory for analysis of total ammonia, nitrite, nitrate, total nitrogen,

and conductivity. These experiments were replicated three times at each site during the summer and fall of 2012.

Summary statistics, linear regressions, and graphical representations of data were accomplished in MS Excel.

Nitrogen cycling by bacteria on colonized artificial substrates with and without antibiotics

Artificial substrates (Fluval Biomax bio rings, Hagen) buried in Tahlequah Creek sediments for 60d were harvested and taken to the laboratory in sterile containers. Individual rings were placed in sterilized 300ml BOD bottles containing Tahlequah Creek water from the relevant study site (upstream or downstream of the Tahlequah WWTP). Ammonium chloride was added to achieve a concentration of 1mg/liter total ammonia as nitrogen, with and without an antibiotic. Dissolved oxygen was measured in each bottle. Bottles were then incubated at 20°C in the dark for 5d after which dissolved oxygen, total ammonia, nitrite, nitrate, and total nitrogen were measured in each replicate. Antibiotic concentrations were chosen to represent 0x, 1x, and 10x concentrations where x represents a previously measured environmental concentration for that antibiotic. The antibiotics used were azithromycin, ciprofloxacin, erythromycin, sulfamethoxazole, triclosan, trimethoprim, and tylosin (Table 1). Four replicates of each antibiotic concentration were analyzed for each site.

Table 1. Nominal concentrations and literature source for each of the antibiotics used in the experiments described above. Concentrations represent the 1x or environmental concentrations.

Antibiotic	**Nominal Concentration**	**Units**	**Source**
Triclosan	0.250	ug/L	(Haggard et al. 2006)
Erythromycin	0.175	ug/L	(Haggard et al. 2006)
Trimethoprim	0.190	ug/L	(Haggard et al. 2006)
Tylosin	0.012	ug/L	(Haggard et al. 2006)
Ciprofloxacin	0.039	ug/L	(Haggard et al. 2006)
Sulfamethoxazole	0.500	ug/L	(Haggard et al. 2006)
Azithromycin	0.042	ug/L	(Cisar *et al.*, manuscript submitted)

One-way ANOVA's with Fisher's LSD post-hoc comparisons to test a-priori comparisons of antibiotic screening experiments were done using IBM SPSS Statistics 19.

Principal Findings and Significance:

Bacterial diversity in sediments upstream and downstream of the Tahlequah wastewater treatment plant based on 16S rRNA gene pyrosequencing (Objective 1)

Bacterial operational taxonomic units (OTUs) identified in sediment samples from upstream and downstream of the Tahlequah wastewater treatment plant (WWTP) were not the same. At the phylum level, bacteria from 22 phyla were present in Tahlequah Creek sediments (Table 2). However, only 20 of the 22 phyla were present at each location. Chrysiognetes and Aquificae were present only in sediments downstream of the WWTP while Deferribacteres and Fusobacteria were present only in sediments upstream of the WWTP. The abundance of some phyla could be quite different between upstream and downstream sediments. For example, the percentage of Acidobacteria downstream of the WWTP was almost two times the percentage upstream of the WWTP. Upstream of the WWTP eight phyla were abundant (present at levels > 1%) making up > 96% of bacteria identified. Downstream of the WWTP nine phyla were abundant (present at levels > 1%) making up > 97% of bacteria identified. Most of these phyla were abundant (defined as 1% or greater of the total bacteria) in both upstream and downstream sediments. However, Cyanobacteria and Actinobacteria were present at < 1% in upstream sediment and Firmicutes were present at < 1% in downstream sediment. Two of the phyla, Nitrospirae and Cyanobacteria, were more abundant in downstream sediment than in upstream sediment and are particularly interesting as they play important roles in cycling of nitrogen in streams. Species of Nitrospirae convert nitrite (NO_2^-) to nitrate (NO_3^-). Cyanobacteria are more abundant in streams polluted with nitrogen and high numbers of cyanobacteria can be indicative of poor water quality.

Table 2. Bacteria Present in Tahlequah Creek Sediments by Phylum

Phylum	**Upstream of WWTP (%)**	**Downstream of WWTP (%)**
Proteobacteria	39.311	37.765
Bacteroidetes	20.368	12.804
Nitrospirae	17.963	24.333
Acidobacteria	6.888	13.569
Verrucomicrobia	5.879	1.902
Chloroflexi	3.593	2.745
Firmicutes	1.455	0.725
Planctomycetes	1.099	1.647
Cyanobacteria	0.831	1.784
Actinobacteria	0.445	1.000
Fibrobacteres	0.445	0.431
Lentisphaerae	0.386	0.510
Chlorobi	0.386	0.176
Gemmatimonadetes	0.297	0.078
Deinococcus-Thermus	0.267	0.157
Dictyoglomi	0.148	0.196
Spirochaetes	0.119	0.020
TM7	0.059	0.118
Deferribacteres	0.030	0
Fusobacteria	0.030	0
Chrysiogenetes	0	0.020
Aquificae	0	0.020

16S rRNA gene sequences obtained by pyrosequencing were used to compare bacterial diversity in sediments upstream and downstream of the Tahlequah WWTP at the phylum, genus, and species levels (Table 3). The data indicates that the bacterial populations at the two locations (sediments upstream and downstream of the WWTP) were similar, but not identical, at the phylum level. However, the populations appeared more different at the genus and species levels.

Table 3. Shannon diversity index and number of OTU's observed at the phylum level (20% sequence dissimilarity), genus level (5% sequence dissimilarity), and species level (3% sequence dissimilarity).

	Upstream sediment	Downstream sediment
Phylum level (20% dissimilarity)		
Shannon diversity index (H)	1.74	1.77
OTUs	20	20
Genus level (5% dissimilarity)		
Shannon diversity index (H)	4.12	5.17
OTUs	261	281
Species level (3% dissimilarity)		
Shannon diversity index (H)	4.25	3.92
OTUs	322	339

Pyrosequencing of 16S rRNA genes from bacteria in sediment samples taken upstream and downstream of a wastewater treatment plant showed that bacterial diversity was high at both sites (Table 3). Although the bacterial populations share similarities they also differ at all three levels with regard to their specific makeup. For example, Cyanobacteria is a highly diverse phylum. Current estimates of described species of Cyanobacteria range from 3,234 to 2,664 different species (http://www.environment.gov.au/biodiversity/abrs/publications/other/species-numbers/2009/pubs/08-nlsaw-others.pdf). Seventeen cyanobacterial species were identified in upstream sediment and sixteen cyanobacterial species were identified in downstream sediment (Table 4). Only nine of these species were present at both locations although the sites are only 300m apart. Preliminary analysis of data indicates that the wastewater treatment plant effluent, which is essentially the only environmental factor that is different between the two locations, does have an impact on downstream bacterial populations.

Table 4. Cyanobacterial species present in sediments

Species	Upstream sediment (%)	Downstream sediment (%)
Cyanothece sp[1]	0.089073634	0.294117647
Gloeocapsa sp	0.059382423	0.019607843
Gloeothece sp	0	0.039215686
Microcystis aeruginosa	0.059382423	0
Synechococcus sp	0	0.156862745
Chamaesiphon subglobosus	0	0.019607843
Tolypothrix sp	0.089073634	0
Anabaena sp	0.089073634	0.098039216
Cylindrospermum sp	0.029691211	0
Dolichospermum lemmermanii	0.029691211	0
Nodularia spumigena	0	0.019607843
Nostoc sp	0.029691211	0
Scytonema sp	0.029691211	0
Geitlerinema sp	0.029691211	0.078431373
Leptolyngbya frigida	0	0.019607843
Leptolyngbya sp	0.059382423	0.058823529
Lyngbya sp	0	0.019607843
Pleurocapsa sp	0.089073634	0.450980392
Dermocarpella sp	0.029691211	0.098039216
Myxosarcina sp	0.029691211	0.039215686
Solentia sp	0.029691211	0
Xenococcus sp	0	0.019607843
Gloeobacter violaceus	0.029691211	0
Chroococcidiopsis sp	0.029691211	0.352941176

[1]Due to incomplete taxonomic data for some organisms some entries have no specific species name. These are abbreviated <Genus> sp.

Analysis of microbial communities from sediments upstream and downstream of a WWTP using a functional gene array probed with genomic DNA (Objective 2)

The GeoChip 4.0 array contains 83,992 50-mer oligonucleotide probes targeting 152,414 genes in 410 gene categories for different microbial functional and biogeochemical processes. Functional gene categories include antibiotic resistance, bacteriophage, bioleaching, carbon cycling, energy processes, fungal genes, metal resistance, nitrogen cycling, organic remediation, phosphorous cycling, stress proteins, sulphur cycling, beneficial in soil, soil borne pathogens, and virulence. Arrays were hybridized with fluorescently labeled genomic DNA (gDNA) extracted from sediments upstream and downstream of the Tahlequah WWTP. Similar numbers of genes were detected on each array (Table 5). In addition, the gene overlap between arrays was high: 73-92% overlap among upstream samples, 85-89% overlap among downstream samples, 70-80% overlap between upstream and downstream samples.

Table 5. Total number of genes detected per GeoChip array and gene overlap between arrays[1]

	U/S I	**U/S J**	**U/S K**	**D/S I**	**D/S J**	**D/S K**
U/S I		24333(74.10%)	30049(91.50%)	26282(77.75%)	25414(75.47%)	25558(75.93%)
U/S J			24007(73.11%)	22589(71.81%)	22603(74.24%)	22211(71.73%)
U/S K				26612(80.28%)	25830(78.43%)	26011(79.11%)
D/S I					26213(88.11%)	26339(88.53%)
D/S J						25342(85.18%)
D/S K						
Total	31607	25565	31281	28480	27483	27609

[1]Triplicate biological replicates (I, J, K) were used in these experiments. U/S = upstream sediment, D/S = downstream sediment.

Microbial diversity and species evenness in upstream and downstream sediments was also measured using GeoChip array data (Table 6). Results indicate high levels of microbial diversity in both locations, upstream and downstream of the WWTP, and very high levels of species evenness.

Table 6. Diversity indices and species evenness for microorganisms in sediments upstream and downstream of the Tahlequah WWTP

	Shannon Index (H)	Simpson Index (D)	Pielou evenness (J)	Simpson evenness (Si)
Upstream	10.28 ±0.12	29235.7 ±3313.7	0.9996 ±0.00	0.9917 ±0.00
Downstream	10.23 ±0.02	27649.6 ±534.8	0.9996 ±0.00	0.9925 ±0.00

Cluster analysis of the GeoChip array data revealed that the microbial populations in upstream and downstream sediments are different (Figure 1). However, one of the upstream sediment samples (T1S J) is more similar to the downstream sediment samples than the other two upstream samples (T1S I and K).

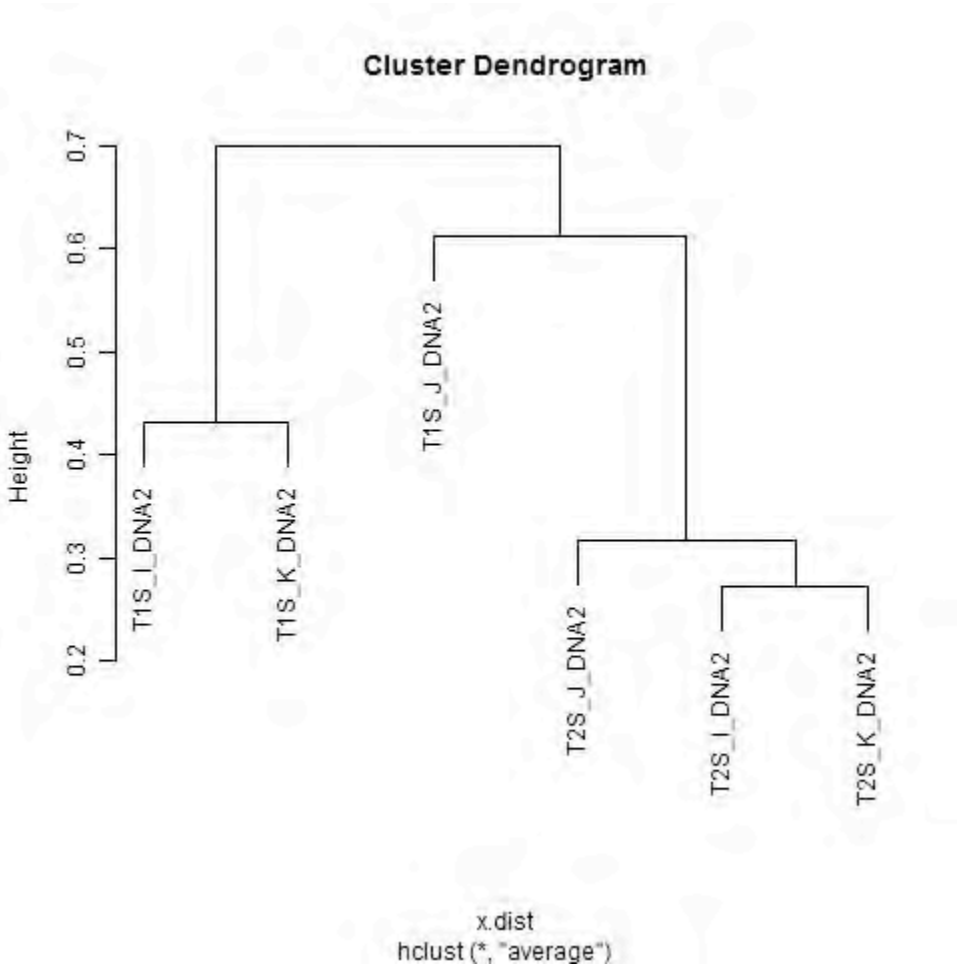

Figure 1. Hierarchical cluster analysis of GeoChip array data. T1S = upstream sediment (I, J and K are biological replicates). T2S = downstream sediment (I, J and K are biological replicates).

Figure 2 shows the relative abundance of the different categories of functional genes in sediments upstream and downstream of the Tahlequah WWTP. Very similar percentages of gene groups in upstream and downstream samples were observed. However, statistically significant differences were observed for 'other category' genes (T-test; p=0.03), which includes *gyrB* and genes for chlorophyllide reductase. Further examination of these results is planned.

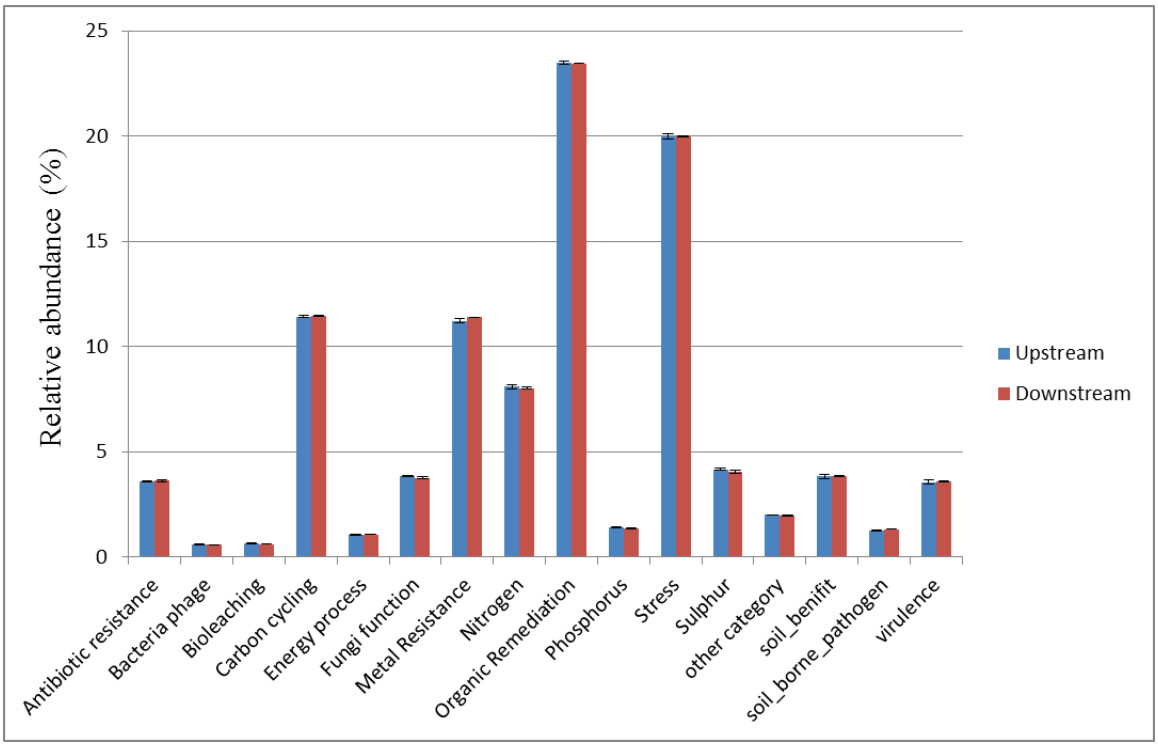

Figure 2. Relative abundances of functional genes by category on GeoChip 4.0 arrays hybridized with genomic DNA from sediment samples from upstream and downstream of a WWTP. Error bars are SD.

The analyses of the GeoChip 4.0 array data described above suggest that the microbial communities upstream and downstream of the WWTP are similar, *i.e.* in terms of diversity (Table 6), functional ability (Figure 2) and even community members (Table 5). However, when examining the structure of the communities (presence/absence and abundance of individual sequences) via clustering and dissimilarity analysis, differences between the upstream and downstream communities are apparent (Table 7).

Table 7. Dissimilarity analysis of upstream and downstream microbial communities at the level of gene category

adonis	euclidean		manhattan		bray curtis		binomial	
	Delta	p-value	Delta	p-value	Delta	p-value	Delta	p-value
Organic Remediation	0.36	0.038	0.393	0.001	0.398	0.042	0.434	0.001
Soil_benefit	0.372	0.046	0.429	0.033	0.433	0.056	0.483	0.07
Antibiotic resistance	0.366	0.001	0.405	0.063	0.406	0.011	0.449	0.068
Carbon cycling	0.376	0.001	0.428	0.049	0.432	0.059	0.475	0.056
Stress	0.363	0.001	0.405	0.001	0.407	0.065	0.445	0.04
Sulfur cycling	0.388	0.001	0.442	0.001	0.45	0.053	0.486	0.001
Virulence	0.388	0.001	0.442	0.001	0.445	0.001	0.499	0.001
Phosphorus cycling	0.367	0.001	0.428	0.016	0.433	0.055	0.464	0.057
Fungi function	0.378	0.001	0.446	0.035	0.45	0.033	0.497	0.001
Nitrogen cycling	0.389	0.001	0.445	0.001	0.448	0.061	0.49	0.075
Metal Resistance	0.369	0.001	0.419	0.043	0.421	0.001	0.467	0.036
Other category	0.384	0.017	0.445	0.001	0.452	0.059	0.491	0.001
Soil_borne_pathogen	0.382	0.001	0.456	0.072	0.454	0.054	0.509	0.001
Bioleaching	0.338	0.049	0.346	0.127	0.347	0.162	0.377	0.137
Energy process	0.289	0.001	0.299	0.183	0.308	0.088	0.326	0.049
Bacteriophage	0.366	0.001	0.407	0.05	0.416	0.001	0.44	0.046

Examination of expression of nitrogen cycling genes in sediments upstream and downstream of the Tahlequah WWTP using a functional gene array, GeoChip 4.0 (Objective 3)

The experimental results above describe microbial community functional structure, or more specifically, the similarities and differences between the upstream and downstream sediment communities based on the abundance of certain genes. The presence/abundance of genes in a habitat indicates the *potential* for that population to perform a particular function. However, knowledge of the *amount of messenger RNA* (a measure of gene expression) produced by these bacteria is a *better indicator of their actual capacity* for nitrogen cycling. Therefore, GeoChip arrays were hybridized with fluorescently-labeled cDNA prepared from RNA extracted from the sediments described above. As expected, far fewer genes were expressed than were present in these communities (compare Tables 5 and 8).

Table 8. Total number of genes expressed per GeoChip array and gene overlap between arrays[1]

	U/S I	**U/S J**	**U/S K**	**D/S I**	**D/S J**	**D/S K**
U/S I		6588(97.72%)	6596(97.81%)	6631(98.22%)	6131(90.87%)	6522(96.55%)
U/S J			6613(98.22%)	6634(98.22%)	6146(91.21%)	6529(96.67%)
U/S K				6649(98.52%)	6150(91.19%)	6540(96.85%)
D/S I					6185(91.62%)	6576(97.29%)
D/S J						6130(91.48%)
D/S K						
Total	6662	6668	6678	6720	6216	6615

[1]Triplicate biological replicates (I, J, K) were used in these experiments. U/S = upstream sediment, D/S = downstream sediment.

GeoChip 4.0 contains probes for 16 gene families coding for enzymes involved in functional processes such as N fixation, nitrification, denitrification, ammonification, dissimilatory N reduction, assimilatory N reduction, and anaerobic ammonium oxidation (annamox). In our experiments, six genes (Table 9) were expressed at significantly lower levels in microbial communities in sediments downstream of the Tahlequah WWTP than in sediments upstream of the WWTP.

Table 9. N cycling genes expressed at lower levels in microbial communities downstream of the Tahlequah WWTP

Process	**Gene Name**	**Enzyme**	**P**
Nitrification	*amoA*	ammonia monooxygenase	0.0006
Denitrification	*narG*	nitrate reductase	0.0001
Denitrification	*nirK*	nitrite reductase	0.0345
Ammonification	*ureC*	urease	0.0003
Assimilatory N reduction	*nasA*	nitrate reductase	0.0387
Assimilatory N reduction	*nir*	nitrite reductase	0.0108

The nitrogen cycle with GeoChip 4.0 functional gene family probes indicated is shown in Figure 3. The observed lower levels of expression in microorganisms downstream of the WWTP are intriguing and our analysis is ongoing.

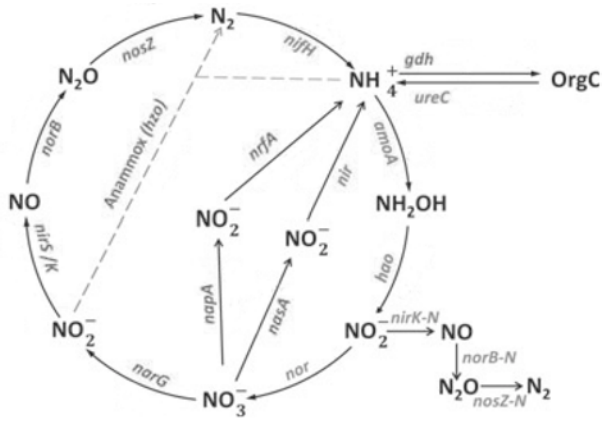

Figure 3. Nitrogen cycle. Diagram indicates N cycle gene probes present on GeoChip 4.0.

In-stream Nitrogen Uptake Experiments (Objective 3a)

Though stream discharge was always similar between sites on the same day, there was variability in discharge between sampling days. Results from the nitrogen uptake experiments were converted to percent of ambient concentrations for each experiment to help correct for this variability. A significant relationship ($P < 0.05$) for total ammonia and none of the other measured nutrients uptake was developed (Figure 4). This relationship was similar for both sites (Upstream = -0.0105 and Downstream = -0.0112) with the slope of the simple linear regression approximating the rate of nitrogen uptake or conversion by resident nutrient cycling communities. These uptake regressions also explained the majority of variability (Upstream = 59.39% and Downstream = 83.69%) in measured total ammonia concentrations.

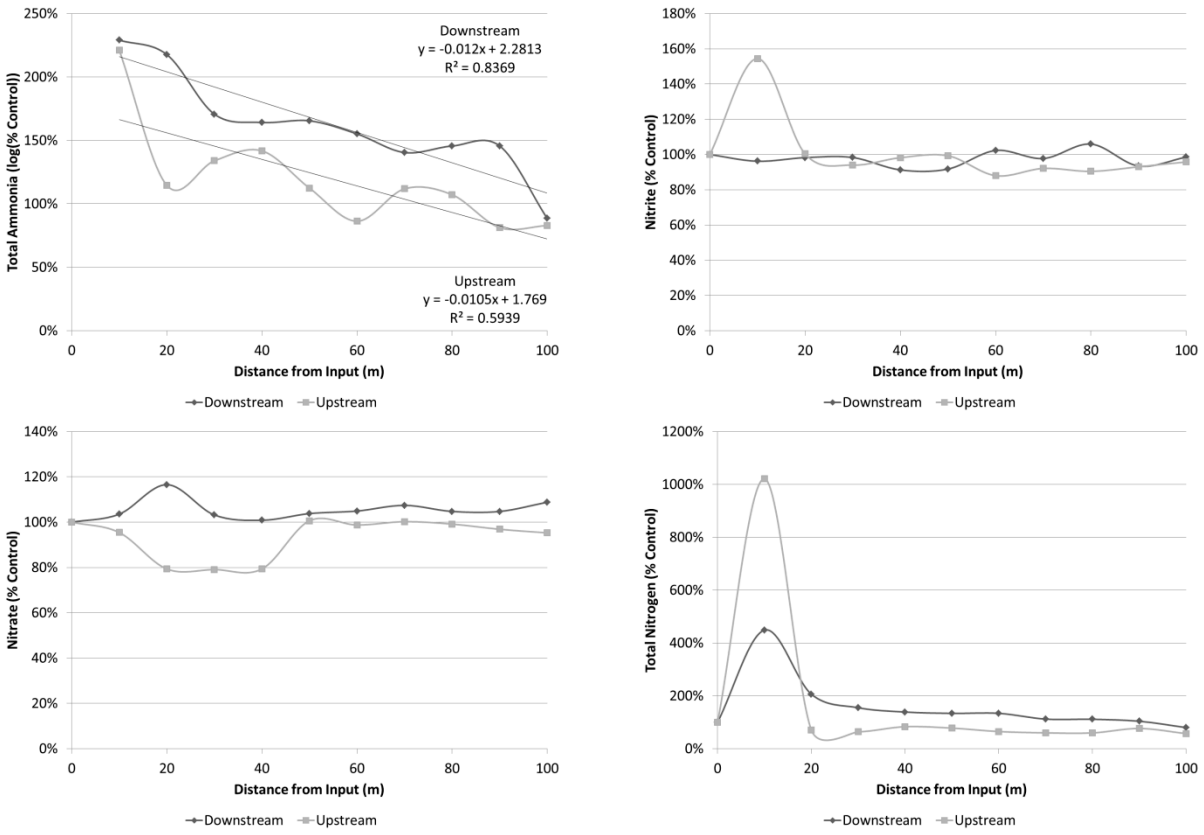

Figure 4. Results for both sites when Ammonium chloride was added to the stream. Uptake rates (linear slopes) could only be derived for ammonia concentrations (top left). Uptake rates for nitrite (top right), nitrate (bottom left) and total nitrogen (bottom right) did not differ from zero.

Comparison of bacterial communities in stream sediments and colonized artificial substrates (Objective 4)

16S rRNA gene sequences were used to identify bacteria present in stream sediments and on artificial substrates buried in stream sediments upstream and downstream of the WWTP for 30d. Bacterial phyla data were analyzed for correlations using a principal components analysis (PCA) (Figure 5). The first two axes of the PCA accounted for 73.71% of the variability in the observed data. The first axis represented the differences between the sediment samples and artificial substrates. The artificial substrates were positively correlated with Planctomycetes, whereas the sediment samples were positively correlated with all other phyla, especially Chloroflexi, Cyanobacteria, Verrucomicrobia, and Actinobacteria. The second axis plots the differences between the phyla present at the two different sample sites, but only for the sediment samples. Firmicutes and Verrucomicrobia were positively correlated with the upstream site, while Actinobacteria, Acidobacteria, Planctomycetes, Proteobacteria, and Nitrospirae were all strongly correlated with the downstream sediment. Preliminary analysis of the data indicates that based on bacterial phyla the artificial substrates were more similar to each other than to stream sediments. However, this analysis is ongoing.

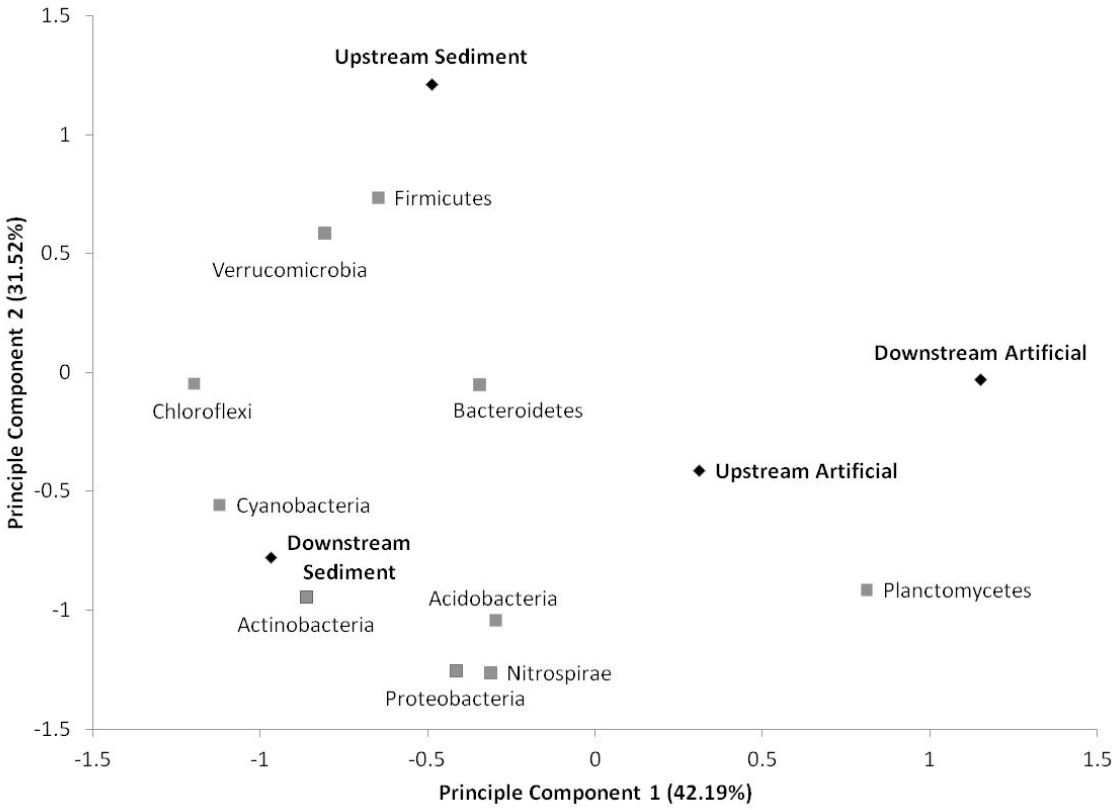

Figure 5. Symmetric biplot of a principal components analysis of bacterial phyla counts from sediment and artificial substrate samples from both study locations (upstream and downstream of the WWTP). Percentages on each axis label represent the percent of variability in the observed data explained by that axis. Sample types and locations are plotted as black diamonds. Bacterial phyla representing at least 1% of the sequences in one sample are plotted as gray squares.

Nitrogen cycling by bacteria on colonized artificial substrates with and without antibiotics (Objective 4)

Although preliminary analysis of the data on bacterial taxa present on artificial substrates colonized by stream bacteria and bacterial taxa present in stream sediments indicated that these populations may be different, experiments examining the effects of antibiotics on bacterial processes using colonized artificial substrates were still deemed valuable and were performed.

Experiments were performed to measure the effect of antibiotics on biochemical oxygen demand (BOD) using colonized artificial substrates from upstream and downstream of the WWTP. Five out of the seven antibiotics applied to laboratory microcosms containing stream water and artificial substrates significantly altered BOD (Figure 6). Sulfamethoxazole, triclosan, and tylosin at environmental (1x) concentrations significantly increased BOD in experiments with upstream colonized artificial substrates. Environmental concentrations (1x) of tylosin also affected BOD in experiments with artificial substrates harvested from the downstream site, but the effect was to reduce BOD. Erythromycin, sulfamethoxazole, triclosan, and trimethoprim increased BOD significantly at ten times (10x) environmental concentrations in experiments with artificial substrates harvested from both sites.

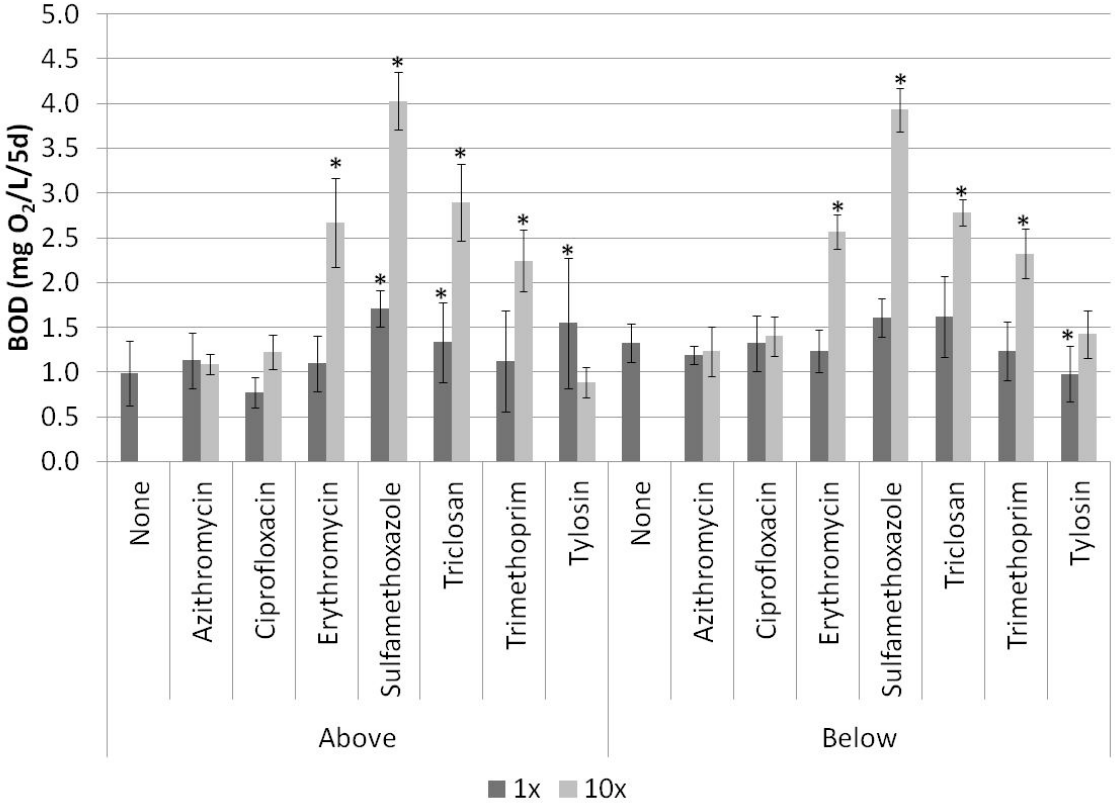

Figure 6. Bar chart representing the mean BOD (Biochemical Oxygen Demand) with colonized artificial substrates in the presence of ammonium chloride and the indicated antibiotics. Each grouping of bars indicates one antibiotic treatment within a site treatment (Above = upstream of WWTP and Below = downstream of WWTP). Dark gray bars represent 1x previously reported environmental antibiotic concentrations and light gray bars represent 10 times those reported concentrations. Asterisks indicate means that significantly differ from the treatment containing no antibiotics within each sampling location. Error bars are standard deviation for each treatment.

Four out of the seven antibiotics significantly affected nitrite production in the laboratory microcosms (Figure 7). At environmental concentrations, only experiments including the antibiotic tylosin showed a significant change in nitrite concentrations. In experiments with colonized artificial substrates from upstream of the WWTP nitrite levels were significantly higher in the presence of 1x tylosin. In experiments with colonized artificial substrates from downstream of the WWTP nitrite levels were significantly lower in the presence of 1x tylosin. Azithromycin, sulfamethoxazole, and triclosan, all at 10x environmental concentrations, significantly decreased nitrite concentrations in downstream treatments.

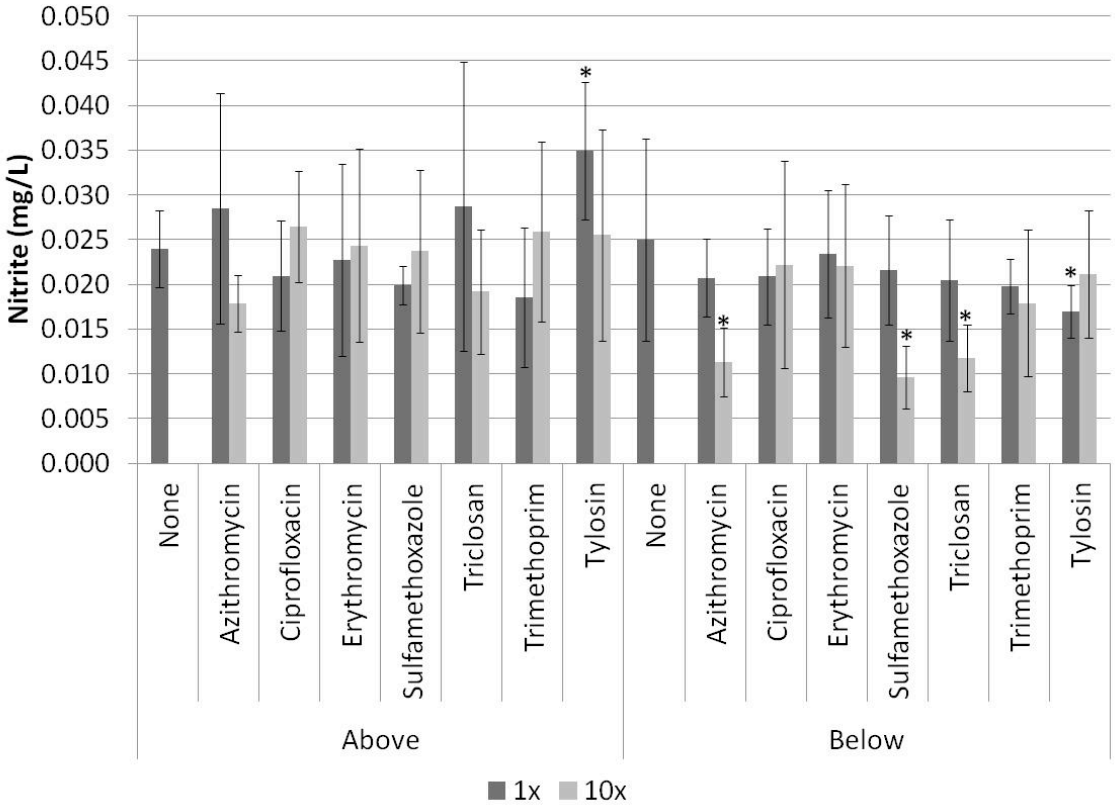

Figure 7. Bar chart representing the mean nitrite concentrations generated with colonized artificial substrates in the presence of ammonium chloride and the indicated antibiotics. Each grouping of bars indicates one antibiotic treatment within a site treatment (Above = upstream of WWTP and Below = downstream of WWTP). Dark gray bars represent 1x previously reported environmental antibiotic concentrations and light gray bars represent 10 times those reported concentrations. Asterisks indicate means that significantly differ from the treatment containing no antibiotics within each sampling location. Error bars are standard deviation for each treatment.

All seven antibiotics affected nitrate concentrations in our experiments, though most of the effects were limited to the downstream treatments (Figure 8). Sulfamethoxazole (10x concentration, upstream treatment) and azithromycin (10x concentration, downstream treatment) significantly reduced nitrate concentrations. In the downstream experimental units environmental concentrations of azithromycin, ciprofloxacin, triclosan, and trimethoprim significantly increased nitrate concentrations. Also in the downstream microcosms 10x concentrations of erythromycin, sulfamethoxazole, trimethoprim, and tylosin resulted in increased nitrate concentrations.

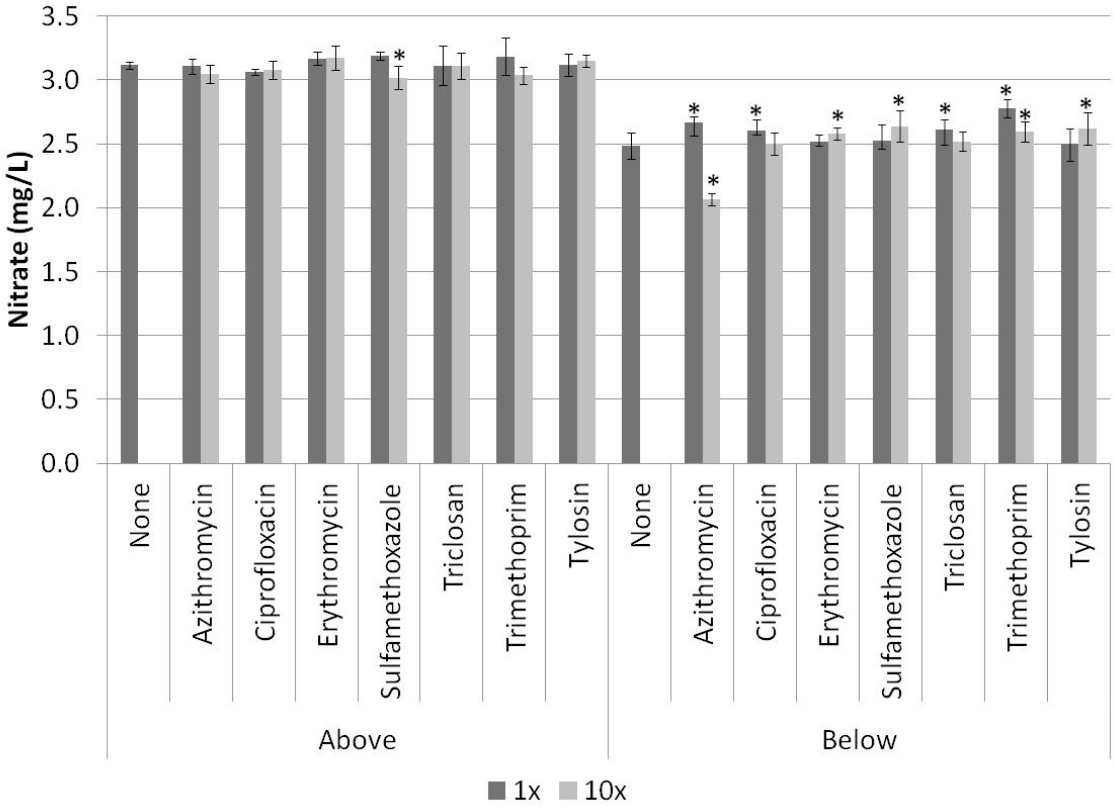

Figure 8. Bar chart representing the mean nitrate concentrations generated with colonized artificial substrates in the presence of ammonium chloride and the indicated antibiotics. Each grouping of bars indicates one antibiotic treatment within a site treatment (Above = upstream of WWTP and Below = downstream of WWTP). Dark gray bars represent 1x previously reported environmental antibiotic concentrations and light gray bars represent 10 times those reported concentrations. Asterisks indicate means that significantly differ from the treatment containing no antibiotics within each sampling location. Error bars are standard deviation for each treatment.

Summary

No differences were observed in ammonium uptake rates at the upstream and downstream study sites, indicating that the wastewater treatment plant has no effect on the ecological function of stream communities responsible for nitrogen processing. However, other data indicate that some aspects of nitrogen processing may be affected in downstream sediments, though more research

is needed, and that wastewater treatment plant effluent does have an impact on downstream microorganisms. For example, the upstream and downstream bacterial communities are different based on 16S rRNA gene pyrosequencing data. Dissimilarity analysis of the data from GeoChip 4.0 arrays probed with genomic DNA from sediment also indicates differences between the upstream and downstream sediment microbiota. Furthermore, several genes encoding enzymes involved in nitrogen cycling have been found to be expressed at lower levels in microbial communities downstream of the wastewater treatment plant. Analysis of abundance and expression of nutrient cycling genes in these populations is ongoing.

The data presented here indicate that environmentally relevant concentrations of antibiotics can significantly affect nutrient cycling in microbial stream communities. Several antibiotics increased the oxygen demand of these communities. The current data set does not indicate whether the increase in oxygen use is from stressing or stimulating the communities. Decreased nitrite concentrations in the presence of antibiotics could indicate that less nitrite was being produced or that the conversion of nitrite to other products was stimulated. Similarly, increased nitrate concentrations in the presence of antibiotics could indicate the stimulation of cellular processes that stimulate nitrate production, or that nitrate uptake/processing was inhibited. The experiments presented here are preliminary studies on the effects of environmentally relevant antibiotics on nutrient cycling. Additional experiments will be required to more precisely describe the impact of antibiotics on nitrogen cycling by microbial communities and to correlate laboratory results with ecological data.

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