WASTEWATER TREATMENT PLANT INFLUENCES ON MACROINVERTEBRATE ASSEMBLAGE, FUNCTIONAL FEEDING GROUPS, AND PRODUCTIVITY

A Thesis
by
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Abstract

WASTEWATER TREATMENT PLANT INFLUENCES ON BENTHIC MACROINVERTEBRATE ASSEMBLAGE, FUNCTIONAL FEEDING GROUPS, AND PRODUCTIVITY

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Organic waste from municipal wastewater treatment plants can alter stream benthic communities by contributing allochthonous energy sources as well as altering stream physicochemical habitat parameters. Nutrients may have a large impact on the primary and secondary production of receiving streams. The purpose of this study is to evaluate the impacts of wastewater treatment plant effluents on headwater systems in the Southern Appalachian Mountains. To do this the biological, physical, and chemical aspects along multiple stream continuums were analyzed. Benthic macroinvertebrates and stream physicochemical parameters were measured in 5 reference and 3 effluent-impacted streams in western North Carolina. Aquatic invertebrates were collected using a modified NCDEQ macroinvertebrate sampling protocol and keyed to the lowest possible taxonomic level. Benthic invertebrate data were used to compute community BI scores, diversity indices and the percentage of trophic feeding guilds. Water chemistry and habitat integrity were assessed at all sites. Benthic communities in impacted streams did not follow the RCC predicted feeding group distributions. In contrast benthic communities in reference streams

generally followed the predictions of the RCC. However, predator abundance was generally higher than predicted in reference streams, while lower than predicted in impacted streams. Overall trends among impacted sites were variable and community structure was likely affected by increased ion concentrations, increased nutrients, and increased levels of primary production at effluent-impacted sites. Increased abundance and richness of collector-gatherers, decreased abundance of Ephemeroptera, Plecoptera and Trichoptera, an increase in Diptera taxa, and lower stream BI scores downstream from effluent outfalls were found when compared to upstream control sites. Multiple regression analyses found that temperature, discharge, conductivity, pH, and elevation variables best predicted several macroinvertebrate metrics taxa in reference streams verses impacted streams. Principal component analyses using invertebrate metrics and water chemistry parameters revealed distinctions between reference and impacted streams, and non-metric dimensional scaling plots showed that temperature, conductivity, and multiple dissolved ions significantly differentiate these sites. Implications of this study are vast and applicable to water use stakeholders, planners, and management agencies. Understanding the degree to which these types of effluent sources impact headwater streams, managers can make better decisions about permitting and placement of new facilities to protect downstream ecosystems from adverse anthropogenic impacts.

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Dedication

I would like to dedicate this thesis to my parents (Debbie and Tom Park), my significant other; Spencer, and my friends. Without all of your love and support my time here would not have been the same, and none of my success would be possible.

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Foreword

This thesis manuscript will be submitted to the journal *Freshwater Science*. The thesis has been formatted according to the style guide for this journal for minimal revisions before publication.

Introduction

Water quality is important for the environment and biodiversity, but also human uses such as socio-economic and recreational purposes (Kowalik et al. 2015). There are multiple ecosystem services provided by headwater streams. For example, streams have a large impact on available water supply, nutrient and mineral fluxes, provide water for drinking and agriculture, and habitat for human food sources (Allan and Flecker 1993). Streams are also influential in creating transportation routes, waste removal, and renewable energy sources (Allan and Flecker 1993). Furthermore, high water quality may result in a sense of spiritual uplift for some people (Allan and Flecker 1993).

Aquatic macroinvertebrates play a large role in stream ecosystem services. They help process organic matter, take up nitrogen, support fish populations and aid in point source and non-point source pollution processing (Sweeney et al. 2004). Benthic invertebrate community structure can be used to monitor stream water quality, nutrient enrichment, and guide the design as well as placement of sewage treatment facilities in headwaters to ensure that the services provided by intact stream ecosystems and macroinvertebrate communities are maintained (Kowalik et al. 2015).

Physical and chemical attributes of headwaters may significantly influence downstream water quality and macroinvertebrate populations. Dodds and Oakes (2008) found that headwater stream buffers are critical in reducing diffuse pollution in downstream areas. Low order streams comprise the majority of stream mileage within drainages (Horton 1945, Leopold et al. 1964). Dodds and

Oakes (2008) found that >60% of the streams in prairie watersheds were first order channels and that more headwater riparian land cover was correlated with positive water quality parameters, such as nutrients and reduced pollutants, in downstream reaches.

The River Continuum Concept

The River Continuum Concept (RCC, Vannote et al. 1980) is a widely accepted model that proposes that communities change along a stream's course and that predictable gradients of physical, chemical, and biological attributes explain this shift. Thus, profound differences in community structure are observed within and between headwater (i.e., low order) and lowland (i.e., high order) streams. Community shifts are driven by differences in stream productivity and nutrient availability (allochthonous vs autochthonous), as well as changes in habitat conditions within a watershed (Vannote et al. 1980, Greathouse and Pringle 2006). Physical and chemical parameters of a stream directly influence stream biota. The RCC can be used to assess benthic macroinvertebrate communities in natural systems and can contribute to the evaluation of the overall health of headwaters with regard to their productivity and the dominant trophic assemblages. Stream benthic invertebrate communities are generally comprised of four trophic levels or functional feeding groups (FFGs): shredders, scrapers (also known as grazers), collectors, and predators. Each of these groups feed on the nutrients derived from upstream habitats. As described in the RCC, benthic macroinvertebrate community structure is determined by the productivity and the inefficiencies of upstream community functions (Vannote et al. 1980).

According to the RCC, invertebrate communities in forested low-order streams in eastern North America should be co-dominated by shredders and collectors with relatively few grazers. Communities in mid-order reaches are dominated by scrapers as well as a large population of collectors, but few shredders. Higher-order stream invertebrate communities are usually dominated by collectors with relatively fewer scrapers and no shredders. Predators are predicted to occur at a relatively constant abundance in both headwater and mid- to high-order streams.

Macroinvertebrate responses to their environment

Streams differ from each other with regard to biotic, chemical, and physical aspects. This means that macroinvertebrate assemblages in particular will differ amongst streams as they react to these variables differently. How macroinvertebrates respond and survive in their environments following exposure to anthropogenic changes is important to consider when using them for research.

To be mobile in the water, macroinvertebrates mostly rely on drifting to move any significant distance. By utilizing the water column and drifting they can move anywhere from a few centimeters to tens of meters (Brittain and Eikeland 1988, Giller and Malmqvist 1998). But, as is the case with most ecological patterns, there are multiple variables that impact the rate and distance at which macroinvertebrates drift. These variables include, but are not limited to, the time of day, seasonality, river discharge, river substrate, the type of macroinvertebrate, predation, life cycles, and random disturbances (Brittain and

Eikeland 1988). Important reasons for macroinvertebrates to drift are to avoid large physical or chemical alterations to the habitat, move to areas with less macroinvertebrate density (i.e. less competition), feeding activities, avoiding predators, or avoiding other forms of disturbance (Brittain and Eikeland 1988, Giller and Malmqvist 1998). Macroinvertebrates that drift more often are more resilient to disturbances, as they can move away from the impact (Poff et al. 2006).

While macroinvertebrate larvae drift downstream, their adult forms fly back upstream to lay their eggs. There are two accepted hypotheses for this. One hypothesis is that the adults are compensating for the downstream drift (Pearson and Kramer 1971, Madsen et al. 1973). The second hypothesis is that adults fly upstream to lay their eggs in a habitat that is more suitable than the area they have just emerged from (Pearson and Kramer 1971, Williams and Williams 1993, Winterbourn and Crowe 2001).

Anthropogenic development and water quality

Human development and impervious surface area have been increasing drastically over the past few decades (Hasse and Lathrop 2003). It is estimated that from 1982-1997 12 x 10^6 Ha of natural land in the USA was developed (NRCS 1999). Expanded infrastructure has led to increased runoff as well as surface and groundwater pollution. Both surface and groundwater pollution may impact local water quality and contribute to issues farther downstream (Allan and Flecker 1993). Moreover, increased ex-urban human populations means that more sanitation infrastructure is needed. Small-scale waste water treatment plants (WWTPs) are frequently constructed adjacent to ex-urban developments and may substantially impact water quality of receiving streams. Many ex-urban WWTPs discharge into headwater streams and therefore may be significant point-sources of pollution in these often oligotrophic watersheds (Nitschke and Schussler 1998, Kowalik et al. 2015).

Most of North America's mid-order and larger streams have been highly modified by human activities, and more recently development of headwater catchments is becoming more common (Statzner and Higler 1985). The rate, as well as the scale, of these changes may overwhelm the ability of riparian buffers and natural nutrient processing ability of these environments, resulting in impairment to both water quality and quantity (Kowalik et al. 2015).

With increased human development comes the need for more wastewater treatment plants (WWTP), and more discharge of sewage into surface water. Organic waste from municipal WWTPs can alter aquatic communities by altering both the origins of stream food web energy sources (i.e., shift from authochonous to allochthonous production) as well as stream physicochemical parameters (Schwartz and Gruendling 1985, Spieles and Mitsch 2000). The excess nutrients in particular can have a large impact on the primary and secondary production in the receiving streams. Increased nutrient inputs may dramatically alter both primary producer (e.g., shifting autotrophic production from diatoms to filamentous algae) and invertebrate communities (McMahan et al. 1972, Kadlec and Bevis 1990, Kurashov et al. 1996).

Appalachian headwater streams are cold-water, oligotrophic ecosystems. Wastewater discharges may rapidly increase temperature and primary productivity due to inputs of excessive nutrients. This may lead to increased DO saturation during times of peak productivity (Schwegler 1978). Spieles and Mitsch (2000) found that chlorophyll *a* was greatly increased by WWTP effluent. Further, chlorophyll *a* concentrations were associated with increased abundance of collector, predator, and scraper feeding guilds. Diversity scores in the impacted sites were reduced compared to the nonimpacted sites, however productivity was higher than nonimpacted sites at the two closest sites to the effluent discharge. The study also found that the Biotic Integrity (BI) scores, dissolved oxygen, pH, and nitrate/nitrite for the impacted sample sites were lower than the reference locations while the conductivity and soluble phosphate were higher in impacted sites.

Birge et al. (1989) found that macroinvertebrate communities negatively changed and water chemistry was altered due to WWTP impacts in higher order streams. Downstream from the effluent outfall collectors and grazers were dominant, species richness and BI scores were low, and there was a high coefficient of species dominance. Further downstream from the outflow, diversity levels recovered and populations of nutrient-sensitive feeding guilds increased as well. Some impacts to community structure were still apparent 37.5 km downstream from the effluent and recovered to reference levels >54 km downstream (Birge et al. 1989).

Objectives

The purpose of this study is to evaluate the impacts of WWTPs on headwater stream benthic community structure. Reference sites were compared to WWTP impacted sites to provide a context for understanding how the proliferation of ex-urban WWTPs may lead to changes in headwater stream productivity, community structure and ecosystem function. It is predicted that recent human population growth trends in high elevation North Carolina, including development of headwaters catchments, may lead to predicable changes to these communities. Specifically, it is hypothesized that in these nutrient poor watersheds WWTP effluent will be associated with increased productivity, reduced diversity, and a shift towards trophic guilds (e.g., collectors and filter-feeders) that are more characteristic of downstream reaches.

Materials and Methods

Study sites

In total 41 sites in 8 headwater stream catchments during summer 2016 and 2017 were sampled. Impacted streams had small watersheds and were affected by one wastewater treatment facility. Study streams impacted by wastewater treatment facilities were the North Toe River (NTR) (Figs 1 and 2) in Newland, North Carolina, the Middle Fork of the New River (MFNR) (Fig. 3) near Blowing Rock, North Carolina and Valley Creek (VC) (Fig. 4) in Seven Devils, North Carolina.

Reference streams were located in forested catchments with low levels of human disturbance. Reference streams included in this study are Wilson Creek (WC) (Fig. 5) in the Wilson Creek Wilderness Area in the Pisgah National Forest, North Carolina, Deep Creek (DC) (Fig. 6) and the Oconaluftee River (OR) (Fig. 7) in the Great Smoky Mountains National Park, North Harper Creek (NHC) (Fig. 8) a tributary to Wilson Creek in the Pisgah National Forest, and Flannery Fork (FF) (Fig. 9) a tributary of the New River and originates on the Blue Ridge Parkway.

Macroinvertebrate collection and identification

Macroinvertebrates were collected in June and July of 2016 and 2017 from 42 sites in 8 streams. Each site was sampled using a modified methodology (NCDEQ 2016 Qual 4). Three riffle kicks were done at each site in order to maximize the potential of collecting a more complete taxa list for each site. To further this, leaf packs, stream banks, and woody debris were also sampled for 10 minutes each in order to sample all stream niches. Macroinvertebrates were preserved in 80% ethanol and returned to the lab for identification.

Macroinvertebrates were identified to the lowest taxonomic level possible using keys in Morse et al. (2017) and Merritt et al. (2008). Most Ephemeroptera, Plecoptera, and Trichoptera (EPT) taxa were identified to the species or genus level. Non-EPT taxa were identified to genus or family. A total of 16,681 organisms and 261 different taxa were collected.

Macroinvertebrate data were used to calculate Simpsons Index (D’), Shannon Index (H), evenness, abundance, and richness. Simpsons Index was calculated using the equation $D= 1-(\Sigma n(n-1))/(N(N-1))$ where n is the number of organisms of a specific species and N is the total number of organisms of all species. Shannon Index was calculated using the equation $H=\Sigma p_i \ln(p_i)$ where p_i is the proportion if S (richness) made up by the *i*th species. Evenness was calculated using H/ln(S). Abundance was simply the total number of organisms at each site, while richness was the number of different taxa groups at each site.

Macroinvertebrates were also assigned functional feeding groups (Merritt et al. 2008) and tolerance values to calculate North Carolina Biotic Integrity (BI) (NCDEQ 2016). Ephemeroptera, Plecoptera, Trichoptera and other taxa percentages were calculated once the macroinvertebrates were identified as well. The other taxa category consisted primarily of Diptera and Coleoptera, but also included any other macroinvertebrates collected such as Odonata and Megaloptera.

Water chemistry sampling and analysis

A YSI Quattro™ multimeter probe was used to measure pH, temperature, and specific conductivity. Water samples were collected and preserved with 1% v/v nitric acid and stored at 4°C until analyzed for elements using Inductively Coupled Plasma – Optical Emission Spectroscopy (ICP-OES, Varian 710-ES with a Cetac ASX 520 autosampler). Elements analyzed included Al, B, Ba, Cd, Cr, Cu, Fe, K, Li, Na, Ni, Rb, S, Se, Sn, Sr, and Zn. Water samples for analysis of anion and nutrient concentrations (Cl, F, NO_3, PO_4, SO_4) were filtered and frozen without acidification and analyzed using ion chromatography (IC). For IC a

Dionex ICS-3000 with a Dionex IonPac AS11-HC column and a flow rate set at 1.5 mL/min along with an eluent of 30 mM NaOH, suppressor current of 112 mA, and injection volume of 20 μL.

Habitat evaluation

Site-specific habitat characteristics were evaluated using the US Environmental Protection Agency's Rapid Bioassessment protocols to create overall habitat scores for each site (USEPA 1999). ArcMap 10.3.1 was also used in order to determine land cover within a 1 km radius of the sample site using the USGS National Land Cover Data with 30m resolution (MRLC 2008). The types of land cover were compared for statistical significance ($p < 0.05$) between reference and impacted streams using a Dunn's test. For WWTP-impacted streams, percent effluent in the stream was also computed.

Statistical analyses

Kruskal- Wallis tests were performed to determine significant differences between the functional feeding group counts as well as taxa distribution in reference and impacted streams. Boxplots were constructed to compare EPT and FFG counts between impacted and reference streams using MiniTab (version 18.1, PA State University, State College, PA). Regressions made on Microsoft Excel (version 15.24, Microsoft, Redmond, WA), as well as t- tests and ANOVAs preformed in SigmaPlot (version 12.5, Microsoft, Redmond, WA) were used to test for significant relationships between the water chemistry, environmental variables, and the calculated biological values.

Multiple regressions were performed between water quality variables and calculated macroinvertebrate values to determine their relationships with taxa that were collected in reference and impacted streams. Regressions were tested for significance by calculating t values. When a calculated t value is greater than the critical t value then we reject the null hypothesis and conclude that there is a statistically significant difference between the variables being compared. Critical t values were obtained from tables in Glover and Mitchell (2002).

Ordination plots were created to compare multiple variables to each other. Principal Components Analysis (PCA) was performed on a dataset comprising physical (water chemistry, discharge, elevation, percent effluent and land cover) parameters using Minitab. ICP-OES and IC data were not included in PCAs because data were not available for three 2016 rivers. OR site 3 was excluded from the physical data PCA due to a missing data point for discharge. Habitat scores were not included in the physical PCA as well due to only having these values for the 2017 sites.

Four Non- Metric Dimensional Scaling (NMDS) plots were also created using RStudio (version 1.1.419, R Core Team, Auckland, New Zealand) using taxa data, FFG counts, EPT counts, calculated values, and water chemistry data from both the YSI Multimeter as well as the IC and ICP-OES data. These comparisons were split into four different graphs due to illegibility if combined in any way. The Vegan package (Oksanen et al. 2018) was used along with the Bray Curtis Dissimilarity Diversity index to determine the location of the centroid for each site using the taxa data. Only 2017 data was used in the NMDS plots due to the missing IC and ICP-OES data for 2016 sites. This was done to facilitate the comparison of the four plots.

Results

Reference vs effluent impacted streams

Overall the Shannon and Simpson diversity scores did not change when comparing the sites upstream and downstream of the wastewater effluent. Macroinvertebrate richness drastically decreased downstream from WWTP effluent in the NTR both years while richness in the MFNR and VC increased downstream from the effluent. Benthic invertebrate abundance decreased downstream of the NTR effluent both years, but increased downstream of the effluent in MFNR and VC. Evenness increased downstream of the effluent on the NTR and MFNR, but stayed about the same on VC. NCBI scores increased downstream of the effluent on both the NTR and VC, but stayed about the same for the MFNR. These measurements can all be seen in Table 1.

The average tolerance values were calculated with the individual tolerance scores of the macroinvertebrates seen upstream verses downstream of the wastewater effluent. Tolerance value averages increased downstream of the effluent in NTR and VC, but slightly decreased in MFNR. In the NTR in 2016 the average tolerance value increased from 3.60 to 4.41, and in 2017 increased from 3.32 to 3.90. For VC the average tolerance value upstream of the effluent was 3.02 but increased to 3.20 downstream of the effluent. The MFNR had the highest tolerance value averages at 5.50 upstream of the effluent and 5.68 downstream of it.

Macroinvertebrate functional feeding groups

Reference streams were dominated primarily by shredders (11%-89%). Collectors were typically co-dominant with shredders (2%-61%). Scrapers comprised a small amount of the sampled macroinvertebrates (1%-25%). These can be seen in Appendix Fig. 1.

Impacted stream sites, even upstream of the WWTPs, are all dominated by collectors (42%-99.5%). Shredders are in very low abundance at these sites, even though many sites were in well-forested catchments (0.25%-23%). Scrapers comprised varying portions of the sample (0.28%- 37%). All functional feeding group fluctuations between sites can be seen in Appendix Fig. 2.

Predator abundance was variable among sites in both reference (0%-43%) (Appendix Fig. 1) and impacted streams (0%-21%) (Appendix Fig. 2). Predators in reference streams were more abundant than expected in DC site 4 (43%), WC site 3 (38% in 2016) and North Harper Creek sites 1 (28%) and 2 (38%) (Appendix Fig. 1).

Comparisons of community structure between impacted and reference streams showed a significant difference in the abundance of shredders ($p < 0.001$), collector-filterers ($p < 0.001$), collector-gatherers ($p = 0.003$) and predators ($p < 0.001$) between reference and impacted streams (Fig. 10). Reference streams had more shredders and predators than impacted streams, whereas impacted streams had more collector-filterers and collector-gatherers. However, scraper abundance was not significantly different between reference and impacted streams ($p = 0.946$) (Fig. 10). The proportion of collectors increased downstream of the effluent in all three impacted streams. This was the one consistent trend in functional feeding group alterations downstream from the wastewater effluent. Other trends in functional feeding groups upstream and downstream of the WWTP were inconsistent among rivers.

Macroinvertebrate abundance

Macroinvertebrate communities in most reference streams were dominated by Plecoptera species and other taxa (Appendix Fig. 3). In contrast, WWTP-impacted streams had very low percentages of Plecoptera and communities were split evenly among Ephemeroptera, Trichoptera and other taxa (Appendix Fig. 4). Although Ephemeroptera abundance was similar between reference and impacted streams, the abundance of Trichoptera and other taxa was higher in impacted streams.

Dunn's tests revealed a significant difference in Plecoptera ($p < 0.001$), and Trichoptera ($p = 0.006$) abundance between reference and impacted streams (Fig. 11). The abundance of Ephemeroptera ($p = 0.072$) and other taxa ($p = 0.12$) were not significantly different between reference and impacted streams (Fig. 11).

Comparisons of EPT assemblages up and downstream from wastewater outfalls revealed decreased abundance in downstream reaches whereas the abundance of other taxa increased (Tables 2-9). The abundance of EPT taxa increased and the abundance of other taxa decreased considerably at sites farther downstream. Recovery of invertebrate community metrics to levels comparable to those observed at sites upstream of WWTPs was observed at a mean of 1.4 km (1.54 km for the NTR, 2.03 km for VC, and 0.68 km for the MFNR) downstream. Plecoptera abundance either slightly increased or decreased drastically downstream from the effluent.

Regressions illustrated that temperature, discharge, and conductivity were significant factors influencing the macroinvertebrate assemblages in the streams (Table 10). There were also individual differences as well between the reference and impacted streams regarding what variables had significant impacts. An interesting one to note is that elevation was significant in reference streams, but not impacted streams (Table 2).

Water chemistry

Temperature, pH, and specific conductivity measurements were made during macroinvertebrate sampling. Few differences were observed in stream physicochemical parameters between reference (Table 11) and impacted streams (Table 12). However specific conductance was significantly higher ($p<0.001$) in impacted stream sites (all orders) compared to reference streams and increased dramatically downstream from all WWTP outfalls.

The concentrations of elements and anions for the NTR in 2016 from the ICP-OES and IC can be seen in Appendix Table 1 as it was the only water chemistry analyzed that year. Element concentrations and anions for all reference streams sampled in 2017 can be seen in Appendix Table 2 and sampled impacted streams sampled in 2017 in Appendix Table 3. Overall, increased ion concentrations were observed in impacted streams compared to reference streams. Chloride concentration was the most dramatic and biologically relevant difference observed between reference and impacted streams. However, Ba ($p<0.001$), Na ($p<0.001$), Al ($p=0.012$), Fe ($p= 0.004$), K ($p=0.014$), Sr ($p<0.001$), Ca ($p<0.001$), Cl ($p<0.001$), and Mn ($p<0.001$), and PO_4 ($p=0.004$) were all statistically higher in WWTP-impacted relative to reference streams.

Within stream comparisons revealed increases of Na downstream from effluent outfalls in all three WWTP-impacted streams. Concentrations of Fe and N increased downstream from the NTR effluent during both years and in 2016 Al and S were also elevated at downstream sites. Sulfate concentrations in the NTR were elevated at downstream sites in 2017. Concentrations of F, B, and Cl were elevated downstream from both the VC and NTR outfalls. In the MFNR and VC concentrations of S and P were elevated downstream from both effluent outfalls.

Physical habitat parameters

Reference streams had slightly higher EPA habitat suitability index (HSI) scores compared to impacted streams, however even impacted streams had relatively high scores (Tables 11 and 12). The MFNR had the lowest HSI scores and North

Harper Creek had the highest scores. Habitat scores decreased downstream from the effluent outfall at both NTR and MFNR sites but slightly increased in VC.

Overall, 11 land cover categories were present at study sites and deciduous forest was the dominant coverage class (Appendix Table 4). Developed open space (i.e., farmland) was the second most common land cover occurring near sample sites. The proportion of impervious surface was low at most sites, but was highest in the NTR (3%-41%) and the MFNR (20%-43%) watersheds. Statistically significant differences ($p<0.001$) were observed in the relative abundance of deciduous forest, impervious surface, open field, hay/pasture, herbaceous, mixed forest, and shrub land types between impacted and reference streams. Barren land was also significantly different between reference and impacted streams ($p = 0.009$).

The discharge of sewage effluents at the streams were determined. The NTR in 2016 had a discharge of 0.07 m^3/s. The NTR in 2017 and the MFNR both had a discharge rate of 0.04 m^3/s in 2017. VC discharged at 0.0003 m^3/s. The proportion of effluent ranged from 0.04-17.2%. Directly downstream of the effluent outfall the MFNR had the highest percent effluent (17.2% of stream discharge downstream of the effluent), whereas at VC effluent was a very low proportion of stream flow (0.25%). The percent of effluent in the stream water was calculated for all downstream sites (Table 12).

Ordination plots

Principal Components Analysis (PCA) revealed that 2 components explained ~50% of the variability among sites (Fig. 12). Principal Component (PC) 1 explains 29.7% of the variation in the data whereas PC2 explains 17.7%. PC1 was most influenced most strongly by deciduous forest and hay/pasture land cover whereas PC2 was most influenced by stream order and discharge.

Non- Metric Dimensional Scaling (NMDS) analysis revealed a significant difference in macroinvertebrate community structure between reference and impacted streams ($p = 0.019$, Fig. 13). Examination of the composition of taxa revealed that the abundance of collector-filterers ($p= 0.004$), predators ($p= 0.009$), Plecoptera ($p= 0.031$), Trichoptera ($p= 0.003$), other taxa ($p= 0.009$), Simpson diversity ($p= 0.002$) and genus-level BI score ($p< 0.001$) as well as temperature and specific conductance ($p<0.01$) all contributed significantly to the ordination of sites in NMDS plots (Figs 14 and 15). Examination of the influence of different cations and anions on water chemistry revealed that Zn ($p=0.003$), F ($p=0.019$), Cl ($p<0.001$), NO_3 ($p=0.002$), Ba ($p<0.001$), Ca ($p=0.004$), Cr ($p<0.001$), Fe ($p=0.011$), K ($p<0.001$), Mn ($p<0.001$), Na ($p<0.001$), S ($p=0.027$), Sr ($p<0.001$), W ($p=0.035$) contributed significantly to the ordination of study sites in NMDS plots (Figs 16 and 17).

Discussion

Large-volume WWTP outfalls are widely known to alter the biological, chemical, and physical aspects of receiving rivers (McColl 1974, Schwartz and Gruendling

1985, Spieles and Mitsch 2001) Introduced toxins and nutrients may have large impacts on primary productivity and macroinvertebrate assemblages (McMahan et al. 1972, Kadlec and Belvis 1990, Kurashov et al. 1996). However, the impacts of small-scale WWTP outfalls on headwater streams are less well studied. Using macroinvertebrate, water chemistry and constituent concentration, and physical habitat analyses, significant impacts of sewage effluent in high elevation western North Carolina headwater stream systems were determined.

The River Continuum Concept and macroinvertebrates

The natural baseline condition of streams in relatively undisturbed watersheds from 1st to 3rd order streams in the Southern Appalachian Mountains of Western North Carolina were evaluated to compare to impacted streams.

One of the most widely-observed deviations from the River Continuum Concept (RCC) was that the abundance of predators was relatively inconsistent among sites and sometimes comprised the second largest feeding group in some reference streams (Appendix Figs 1 and 2). Perlidae, Perlodidae (both in the O. Plecoptera - stoneflies), and Rhyacophilidae (O. Trichoptera – caddisflies) were the most abundant families of predators collected. Grubaugh et al. (1997) studied low- and higher-order streams in the Appalachian Mountains and found that the RCC's predictions were generally met for feeding groups other than predators, which decreased with increasing stream order. Although the relative abundance of predators in this study did not increase with stream order, results of both studies suggest that predator abundance does not always follow the RCC. Data from this study supports that predators flourish in pristine water quality, as the predators commonly found in abundance were pollution sensitive species. This further explains why predators were found in higher abundance in reference stream conditions, verses impacted streams.

The main objective of this study was to determine how benthic macroinvertebrate functional feeding groups (FFGs) respond to WWTP effluent outfalls in headwater streams. The data shows that WWTP impacted streams do not follow the RCC's predictions; shredders were uncommon overall and collectors were numerically dominant at all sites (Fig. 10 and Appendix Table 2). *Hydropsyche* caddisflies, Simuliidae (blackflies), and some Baetidae (mayflies) were common tolerant collectors found downstream of the WWTPs. Reduced shredder abundance was surprising given that all sites were in largely forested watersheds and while riparian land cover was variable, most had abundant CPOM inputs. Previous studies have shown elevated collector-gatherer abundance downstream from WWTPs, likely due to increased concentrations of fine particulate organic matter originating in effluent. Nutrient enrichment from WWTP effluent also likely alters stream nutrient budgets and leads to shifts in primary producer and macroinvertebrate assemblages (Birge et al. 1989).

Shredders were rare and collectors were the dominant FFG at all sites in impacted streams and this pattern remained consistent in sites upstream from WWTP effluent sources. This suggests that one or several process could be at work. First, it is possible that conditions throughout the watershed are conducive to the formation of benthic communities dominated by collectors. However, most of these streams drain largely forested, montane catchments. Impoundments are rare and most streams have naturally low levels of suspended organic matter at base flows. Second it may be that WWTP effluent impacted reaches are large enough to act as an environmental barrier capable of not only altering downstream macroinvertebrate assemblages but also by limiting the potential for re-colonization of headwaters via intra-basin (e.g., adult dispersal to upstream reaches or among tributaries). Finally, because many shredders are intolerant of pollution, WWTP-induced mortality of drifting larvae may reduce the pool of adults dispersing along stream corridors (Madsen et al. 1973, Pearson and Kramer 1971 Williams and Williams 1993; Winterbourn and Crowe 2001, NCDEQ 2016). Moreover, macroinvertebrates that are tolerant of conditions near effluent outfalls may form large aggregations in downstream reaches (Brittain and Eikeland 1988). Because adults generally fly upstream to oviposit, large aggregations of collectors near WWTPs may serve as a source of colonists for upstream benthic communities (Madsen et al. 1973; Pearson and Kramer 1971, Williams and Williams 1993, Winterbourn and Crowe 2001). This may explain why sites upstream of WWTPs were dominated by collectors and had shedder numbers that were much lower than those observed in reference streams.

Approximately 1.5km downstream of the NTR WWTP and about 0.7km downstream of the MFNR WWTP macroinvertebrate abundance and BI scores decrease and diversity increases as tributaries bring clean water into these systems. Birge et al. (1989) found that recovery occurred ~8 km downstream of

WWTPs, however that study was in higher-order streams with a larger amount of effluent discharge.

VC and the MFNR demonstrated increases in macroinvertebrate richness, diversity (D' and H), and abundance downstream of the effluent (Table 1). Species richness increased downstream of the effluent in VC and MFNR as habitat improved from the upstream sites, in conjunction with the increased food availability. These results were similar to the Birge et al. study, as they found increased abundance directly downstream of the WWTP effluent. They also reported that abundance decreased downstream as the percent effluent in the stream decreased, which was also seen in VC and MFNR. The initial increase in abundance was supported by increased food availability from FPOM in the effluent (Birge et al. 1989). The NTR, however, saw decreased macroinvertebrate richness, diversity, and abundance downstream of the effluent (Table 1). The Birge et al. study similarly found low species richness downstream of the effluent. NTR may be different from VC and the MFNR due to farmland on the NTR next to the effluent input causing further adverse impacts on habitat quality.

Water physiochemical influences

Macroinvertebrate larvae undergo passive downstream dispersal (i.e., drift) to help locate optimal habitats (Brittain and Eikeland 1988). WWTP effluent restricts the extent of optimal habitat by altering specific conductance and FPOM concentrations, as well as contributing numerous known pollutants into surface waters (Loos et al. 2013), in downstream reaches. Elevated conductivity may trigger drift events. Brittain and Eikeland (1988) reported that roadway de-iceing agents increase conductivity and promote drift. Although drifting macroinvertebrate larvae may locate better conditions in downstream sites the distance adults must fly upstream to maintain populations is increased.

Chlorine (Cl) concentrations were significantly higher in impacted compared to reference streams and Cl concentrations were substantially elevated downstream from WWTP outfalls. This is not surprising, as most WWTPs have a final dechlorinating step to kill microbes before discharging effluent into streams. Cl concentrations are variable among WWTPs but the overall impacts to stream health as most macroinvertebrates are very sensitive to Cl (Brungs 1973). Cl levels were close to (VC) or exceeded (NTR and MFNR) the EPA aquatic standard for aquatic life (230mg/L) in this study (Appendix Table 3) (USEPA 2017).

Concentrations of several other ions increased downstream from WWTPs (Appendix Table 3). Birge et al. (1989) found that Na, P, Zn, and polar organics all increased downstream of WWTPs. This study found increased concentrations of Na at all sites downstream of WWTPs. Only very tolerant taxa can live in impacted streams with high specific conductivity levels resulting from increased metal and nutrient inputs downstream from wastewater discharges (Birge et al. 1989).

Physical parameters

Allan and Flecker (1993) found that land use alteration and impacts to stream habitats are highly correlated. A higher diversity of land use types occurred near impacted verses reference streams (Appendix Table 5). Land use may have contributed to altered water chemistry and macroinvertebrate assemblage structure in impacted streams. Although this study did not specifically address the influence of land use in invertebrate communities, local land cover likely influenced the results (Spieles and Mitsch 2000).

Elevation also played a significant role in predicting community structure in reference but not impacted streams (Table 10). This suggests that effluent is likely altering the baseline physiochemical parameters in streams that are regulated by elevation changes (DO, conductivity, temperature, pH). Increased FPOM from effluent alters natural patterns in food availability, resulting in an early shift to collector-dominated as opposed to shredder-dominated invertebrate communities (Vannote et al. 1980).

Study limitations

Other limitations to this project include low levels of statistical power originating from the fact that relatively few WWTPs discharge into headwater streams. Moreover, the WWTPs studied were not located on streams of the same stream order. Despite this limitation, this study provides some important insights into the impacts of WWTP effluent on headwater stream water chemistry, macroinvertebrate productivity, and assemblage structure. The extent of WWTP impacts is likely dependent on upstream land use and the volume and nature of the effluent outfall. VC, for example, received much lower volumes of effluent compared to other impacted streams and, as a result, impacts to stream health (as measured by invertebrate community composition) were much lower than in NTR and MFNR. Additionally, VC is relatively undisturbed and flows through a densely-forested catchment which may mitigate WWTP impacts. Effluent concentrations in the MFNR and the NTR were similar but had different results, likely because communities at the MFNR site are already impacted by a dam located several km upstream whereas the NTR is free-flowing upstream of the study reach.

Conclusions

This study suggests that WWTPs can substantially affect headwater stream water quality and macroinvertebrate community structure. Comparisons between reference and impacted streams showed clear differences in abundance, taxa richness and the relative abundance of different functional feeding groups. Specifically, WWTPs resulted in increased benthic productivity in impacted compared to reference streams as well as increased abundance of collectors and fewer shredders compared to reference streams. The factors that likely drove the observed differences between reference and impacted streams include land use and land cover as well as baseline ionic and nutrient concentrations in focal streams. Water quality managers and other stakeholders need to consider these potential impacts when designing and locating WWTPs in headwater catchments. As the ex-urban development increases, new regulations may be needed to help protect downstream ecosystems from adverse anthropogenic impacts of headwater WWTPs.

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Table 1. Calculated biological values for macroinvertebrate richness, Shannon Diversity (H’), Simpson Diversity (S’), abundance (Total N invertebrates), evenness, and the North Carolina Biotic Integrity (NC BI) for impacted streams.

Stream	Richness	H’	S’	Abundance	Evenness	NC BI
North Toe R 1, 16	11	2.02	0.85	62	0.84	3.87
North Toe R 2, 16	26	2.23	0.82	219	0.68	3.40
North Toe R 3, 16	18	2.45	0.89	121	0.85	4.35
North Toe R 4, 16	24	2.21	0.85	400	0.70	4.21
North Toe R 5, 16	37	2.64	0.91	434	0.73	3.70
North Toe R 6, 16	26	2.24	0.91	138	0.69	3.31
North Toe R 7, 16	26	2.55	0.88	155	0.78	4.33
Middle Fork N R 1	22	2.44	0.87	330	0.79	3.73
Middle Fork N R 2	12	0.96	0.49	1062	0.39	5.73
Middle Fork N R 3	18	1.47	0.70	1294	0.51	5.70
Middle Fork N R 4	17	1.27	0.54	780	0.45	6.21
Middle Fork N R 5	26	2.16	0.84	557	0.66	4.78
North Toe R 1,17	43	1.72	0.97	430	0.46	3.16
North Toe R 2,17	32	2.40	0.80	399	0.69	3.85
North Toe R 3, 17	35	2.40	0.84	524	0.68	3.64
North Toe R 4,17	18	2.42	0.89	81	0.84	5.31
North Toe R 5, 17	37	2.08	0.75	629	0.58	3.47
Valley Cr 1	25	2.55	0.90	328	0.79	2.95
Valley Cr 2	31	2.59	0.90	672	0.75	3.11
Valley Cr 3	26	2.39	0.85	417	0.73	2.69
Valley Cr 4	43	2.50	0.85	707	0.67	2.64

Table 2. Macroinvertebrate taxa, count (number of individuals), family pollution tolerance (FT), genus pollution tolerance (GT), and functional feeding group (FFG) in the North Toe River (2016) found upstream of the wastewater effluent outfall that were not found downstream of the outfall.

Order	Family	Genus/Species	Count	FT	GT	FFG
Diptera	Dolichopodidae	-	1	7	7	Predator
Gastropoda	Lymnaeidae	-	2	8	7	Scraper
Diptera	Tipulidae	-	2	5.34	5.34	Collector-gatherer
Ephemeroptera	Heptageniidae	*Epeorus vitreus*	2	3	1.33	Scraper
Ephemeroptera	Ephemerellidae	*Ephemerella dorothea*	15	2	2.1	Scraper
Trichoptera	Glossosomatidae	*Glossosoma nigrior*	3	1.2	1.4	Scraper
Odonata	Gomphidae	*Gomphus*	1	1.65	1.3	Predator
Trichoptera	Hydropschidae	*Hydropsyche bronta*	19	4.1	4.31	Collector-filterer
Trichoptera	Hydropschidae	*Hydropsyche sparna*	1	4.1	4.31	Collector-filterer
Ephemeroptera	Isonychidae	*Isonychia sicca*	1	3.6	3.6	Collector-filterer
Trichoptera	Thremmatidae	*Neophylax atlanta*	1		1.6	Scraper
Plecoptera	Perlidae	*Paragnetina immarginata*	1	2	1.7	Predator
Plecoptera	Perlidae	*Perlinella drymo*	1	2	1.3	Predator
Plecoptera	Pteronarcyidae	*Pteronarcys proteus*	1	1.8	1.8	Shredder
Plecoptera	Chloroperlidae	*Suwallia marginata*	6	1.3	2.6	Predator

Table 3. Macroinvertebrate taxa, individual count, family pollution tolerance, genus pollution tolerance, and functional feeding group (FFG) in the North Toe River (2016) found downstream of the wastewater effluent outfall but not upstream.

Order	**Family**	**Genus/Species**	**Count**	**Family Tol.**	**Gen Tol.**	**FFG**
Odonata	Calopterygidae	-	2	6	6	Predator
Trichoptera	Limnephilidae	*Hydatophylax argus*	1	3.86	2.4	Shredder
Trichoptera	Leptoceridae	*Oecetis*	1	3.15	5.1	Predator
Ephemeroptera	Leptophlebiidea	*Tricorythodes*	1	2.5	5	Collector-gatherer

Table 4. Macroinvertebrate taxa, individual count, family pollution tolerance, genus pollution tolerance, and functional feeding group (FFG) in the North Toe River (2017) found upstream of the wastewater effluent outfall.

Order	**Family**	**Taxon**	**Count**	**FT**	**GT**	**FFG**
Diptera	Simulidae	-	44	5.88	5.88	Collector-Filterer
Diptera	Tipulidae	-	2	5.34	5.34	Shredder
Plecoptera	Perlidae	*Acroneuria abnormis*	1	2	1.9	Predator
Plecoptera	Perlidae	*Agnetina annulipes*	2	2	1.1	Predator
Plecoptera	Nemouridae	*Amphinemura*	1	4.5	3.8	Shredder
Odonata	Calopterygidae	*Calopteryx*	1	6.2	7.5	Predator
Trichoptera	Philopotamidae	*Dolophilodes distincta*	105	2.23	1	Collector-Filterer
Ephemeroptera	Heptageniidae	*Epeorus subpallidus*	3	3	1.33	Scraper
Ephemeroptera	Heptageniidae	*Epeorus vitreus*	8	3	1.33	Scraper
Ephemeroptera	Ephemerellidae	*Ephemerella catawba*	1	2	2.1	Collector-Filterer
Ephemeroptera	Heptageniidae	*Heptagenia marginalis*	1	3	1.9	Shredder
Trichoptera	Hydropsychidae	*Hydropsyche alhedra*	27	4.1	4.31	Collector-Filterer
Trichoptera	Hydropsychidae	*Hydropsyche ventura*	5	4.1	4.31	Collector-Filterer
Plecoptera	Perlodidae	*Isoperla holochlora*	2	2.2	3.2	Predator
Plecoptera	Perlodidae	*Isoperla similis*	1	2.2	3.2	Predator
Plecoptera	Leuctridae	*Leuctra ferruginea*	3	1.5	1.5	Shredder
Plecoptera	Perlidae	*Paragnetina immarginata*	10	2	1.7	Predator
Plecoptera	Perlidae	*Paragnetina kansensis*	1	2	1.7	Predator
Trichoptera	Polycentropodidae	*Polycentropus*	2	6	3.1	Predator
Coleoptera	Elmidae	*Promoresia*	1	4.41	1	Scraper
Plecoptera	Pteronarcyidae	*Pteronarcys scotti*	1	1.15	1.15	Shredder
Trichoptera	Rhyacophilidae	*Rhyacophila fuscula*	2	1.025	1.03	Predator
Coleoptera	Elmidae	*Stenelmis*	3	4.41	5.6	Scraper

Table 5. Macroinvertebrate taxa, individual count, family pollution tolerance, genus pollution tolerance, and functional feeding group (FFG) in the North Toe River (2017) found downstream of the wastewater effluent outfall but not upstream.

Order	Family	Genus/Species	Count	Family Tol.	Gen Tol.	FFG
Annelida	-	-	13	8	8	Collector gatherer
Ephemeroptera	Baetiscidae	*Baetisca carolina*	3	4.2	3.2	Collector gatherer
Trichoptera	Hydropsychidae	*Cheumatopsyche*	2	4.1	6.6	Collector-filterer
Coleoptera	Elimidae	*Cylloepus*	2	4.41	3.3	Collector- gather
Ephemeroptera	Ephemerellidae	*Dannella simplex*	1	2	2	Collector-gather
Trichoptera	Thremmatidae	*Neophylax concinnus*	2	1.6	0.93	Scraper
Ephemeroptera	Baetidae	*Plauditus dubius*	3	6	4	Collector- gather

Table 6. Macroinvertebrate taxa, individual count, family pollution tolerance, genus pollution tolerance, and functional feeding group (FFG) found upstream of the wastewater treatment effluent outfall for Valley Creek that were not found downstream of the outfall.

Order	**Family**	**Genus/Species**	**Count**	**Family Tol.**	**Gen Tol.**	**FFG**
Gastropoda	-	-	11	7	7	Scraper
Plecoptera	Perlidae	*Agnetina flavescens*	1	2	1.1	Predator
Ephemeroptera	Ephemerellidae	*Drunella longicornis*	7	2	0.1	Scraper
Plecoptera	Perlidae	*Eccoptura xanthenes*	1	1.89	4.7	Predator
Ephemeroptera	Heptageniidae	*Epeorus vitreus*	30	3	1.33	Scraper
Trichoptera	Glossosomatidae	*Glossosoma nigrior*	7	1.2	1.4	Scraper
Trichoptera	Limnephilidae	*Pseudostenophylax*	1	3.86	0	Shredder
Trichoptera	Rhyacophilidae	*Rhyacophila fuscula*	2	1.025	1.03	Predator

Table 7. Macroinvertebrate taxa, individual count, family pollution tolerance, genus pollution tolerance, and functional feeding group (FFG) in Valley Creek found downstream of the effluent outfall but not upstream of it.

Order	**Family**	**Genus/Species**	**Count**	**Family Tol.**	**Gen Tol.**	**FFG**
Oligochaeta	-	-	8	8	8	Collector-gather
Coleoptera	Elmidae	-	2	4.41	4.41	Collector-gather
Diptera	Ephydridae	-	3	6	6	Shredder
Plecoptera	Perlidae	*Acroneuria abnorimis*	12	2	1.9	Predator
Plecoptera	Chloroperlidae	*Alloperla atlantica*	1	1.3	1	Predator
Diptera	Blephariceridae	*Blepharicera*	5	0	0	Scraper
Odonata	Gomphidae	*Gomphus*	3	4.41	4.9	Predator
Trichoptera	Limnephilidae	*Hydatophylax argus*	1	3.86	2.4	Shredder
Ephemeroptera	Heptageniidae	*Maccaffertium*	3	3	3.1	Scraper
Ephemeroptera	Leptophilebiidae	*Paraleptophlebia assimillis*	20	2.5	1.2	Collector-gather
Plecoptera	Perlidae	*Perlinella ephyre*	2	1.89	1.3	Predator
Trichoptera	Limnephilidae	*Pycnopsyche gentilis*	1	3.86	2.58	Shredder
Trichoptera	Rhyacophilidae	*Rhyacophila carolina*	5	1.025	1.03	Predator
Ephemeroptera	Ephemerellidae	*Teloganopsis deficiens*	4	2	2.6	Collector-gather

Table 8. Macroinvertebrate taxa, individual count, family pollution tolerance, genus pollution tolerance, and functional feeding group (FFG) found upstream but not downstream of the effluent outfall in the Middle Fork of the New River.

Order	**Family**	**Genus/Species**	**Count**	**Family Tol.**	**Gen Tol.**	**FFG**
Trichoptera	Hydropsychidae	*Hydropsyche morosa*	1	4.1	4.31	Collector-filterer
Trichoptera	Rhyacophilidae	*Rhyacophila fuscula*	1	1.025	1.03	Predator

Table 9. Macroinvertebrate taxa, individual count, family pollution tolerance, genus pollution tolerance, and functional feeding group (FFG) found downstream but not upstream of the effluent outfall in the Middle Fork of the New River.

Order	Family	Genus/Species	Count	Family Tol.	Gen Tol.	FFG
Leeches	-	-	3	10	10	Predator
Diptera	Ephydridae	-	1	6	6	Shredder
Ephemeroptera	Baetidae	*Baetis pluto*	10	6	6	Collector-gather
Trichoptera	Hydropsychidae	*Hydropsyche bronta*	2	4.1	4.31	Collector-filterer
Odonata	Lestidae	*Lestes*	1	9.4	9.4	Predator
Coleoptera	Psephenidae	*Psephenus*	2	3.3	2.3	Collector-gather
Plecoptera	Chloroperlidae	*Suwallia marginata*	2	1.3	2.6	Predator

Table 10. The calculated and critical t values for each significant (*T*calc > *T*crit) regression for reference and impacted streams as well as the direction of the relationship. Physical and chemical variables were compared to biological variables to determine which factors influenced the taxa seen. *T*crit for impacted streams was 2.11 and 2.10 for reference streams.

Dependent Variable	Impacted/ Reference	Conductivity	Discharge	Elevation	pH	°C
Abundance	I		2.50, -		2.42, +	
Collector-F	I	3.18, +	2.53, -			
Collector-G	I	2.28, -	2.79, +			2.42, +
Ephemeroptera	I	2.80, -	2.20, +			2.91, -
NCBI Score	I	4.25, +				4.57, +
Other Taxa	I	2.27, +				5.74, +
Plecoptera	I					3.41, -
Predator	I					4.62, -
Richness	I	4.02, -			2.15, +	2.99, -
Scraper	I		2.83, +			
Shannon	I		2.20, +			2.96, -
Shredder	I					
Simpson	I	2.58, -				3.25, -
Trichoptera	I		2.14, -			
Abundance	R					
Collector-F	R	2.32, +				
Collector-G	R		2.18, +	2.23, -		
Ephemeroptera	R	3.03, +				
NCBI Score	R		2.88, -			
Other Taxa	R		5.27, +	6.34, -		
Plecoptera	R		3.66, -	4.84, +		
Predator	R		3.56, +	3.77, -		
Richness	R	3.59, +				
Scraper	R	4.55, +				
Shannon	R		3.52, +	2.52, -		
Shredder	R	2.44, -	2.76, -	4.83, +		
Simpson	R		2.75, +	2.70, -		
Trichoptera	R		3.27, +			

Table 11. Temperature (T), pH, DO (mg/l) and specific conductivity (SC, (µS/cm), discharge (Q, m^3/second), elevation (Elev, m), and EPA Rapid Bioassessment habitat score (HS) measured at reference sites on the day of macroinvertebrate sampling.

River	Year	T	pH	SC	Q	Elev	HS
Deep Cr 1	2016	14.7	7.35	13.9	0.02	1196	-
Deep Cr 2	2016	16.4	7.39	14.2	0.60	963	-
Deep Cr 3	2016	18.6	7.39	12.5	0.55	804	-
Deep Cr 4	2016	20.3	7.04	13.1	-	638	-
Oconaluftee R 2	2016	15.4	5.79	35.0	0.11	1263	-
Oconaluftee R 3	2016	16.7	5.80	17.1	0.40	1261	-
Oconaluftee R 4	2016	17.9	5.93	14.1	1.05	822	-
Wilson Cr 1	2016	13.9	6.90	20.3	0.01	1332	-
Wilson Cr 2	2016	17.9	5.94	37.0	0.03	860	-
Wilson Cr 3	2016	21.6	6.60	22.4	0.66	494	-
Flannery Fk 1	2017	17.1	6.45	26.8	0.07	1060	151
Flannery Fk 2	2017	17	6.71	28.1	0.09	994	151
Flannery Fk 3	2017	16.7	6.43	30	0.14	993	140
North Harper Cr 1	2017	15.9	6.10	13.3	0.01	966	178
North Harper Cr 2	2017	16.4	7.20	20.3	0.07	960	169
North Harper Cr 3	2017	22.8	6.28	17.3	1.24	884	181
Wilson Cr 1	2017	11.3	7.12	15.3	0.01	1329	167
Wilson Cr 2	2017	14.6	6.73	25.7	0.26	860	167
Wilson Cr 3	2017	19.1	6.57	20.7	0.97	491	142

Table 12. Water chemistry data for temperature, pH, and specific conductivity (SC, (µS/cm) taken with a YSI probe the day of macroinvertebrate sampling as well as habitat parameters for stream discharge (Q, m^3/second), elevation (Elev, m), EPA Rapid Bioassessment habitat scoring, and percent effluent for impacted stream sites.

River	Year	T	pH	SC	Q	Elev	HS	%E
North Toe R 1	2016	21.1	5.88	72.2	0.47	1091	-	0.00
North Toe R 2	2016	17.3	6.21	80.2	0.55	1088	-	0.00
North Toe R 3	2016	20.8	5.25	174.7	0.55	1082	-	7.22
North Toe R 4	2016	12.3	6.99	71	0.55	1070	-	7.22
North Toe R 5	2016	19.4	5.98	86.4	0.91	1030	-	6.34
North Toe R 6	2016	18.4	6.00	89.1	0.64	1018	-	6.20
North Toe R 7	2016	18.5	7.40	90.7	0.57	1033	-	6.19
Middle Fork 1	2016	7.46	7.11	102.6	0.02	1091	154	0.00
Middle Fork 2	2016	21.8	6.77	107.4	0.21	1054	118	0.00
Middle Fork 3	2016	21.5	6.83	118.3	0.23	1054	100	17.22
Middle Fork 4	2017	23	6.36	126.5	0.24	1051	137	16.15
Middle Fork 5	2017	21.8	6.54	112.1	0.31	1049	113	12.77
North Toe R 1	2017	12.9	6.72	38.1	0.02	1125	127	0.00
North Toe R 2	2017	17.1	6.60	61.6	0.48	1093	139	0.00
North Toe R 3	2017	17.3	6.63	64.3	0.54	1093	131	0.00
North Toe R 4	2017	17.2	6.60	65.5	0.83	1091	72	7.88
North Toe R 5	2017	15.4	7.27	69.1	1.08	1082	155	6.01
Valley Cr 1	2017	15.4	7.08	53.1	0.20	1210	143	0.00
Valley Cr 2	2017	14.7	6.50	70	0.11	1102	145	0.25
Valley Cr 3	2017	15.4	7.22	73.3	0.16	1030	133	0.16
Valley Cr 4	2017	16.4	7.08	69.2	0.63	972	134	0.04

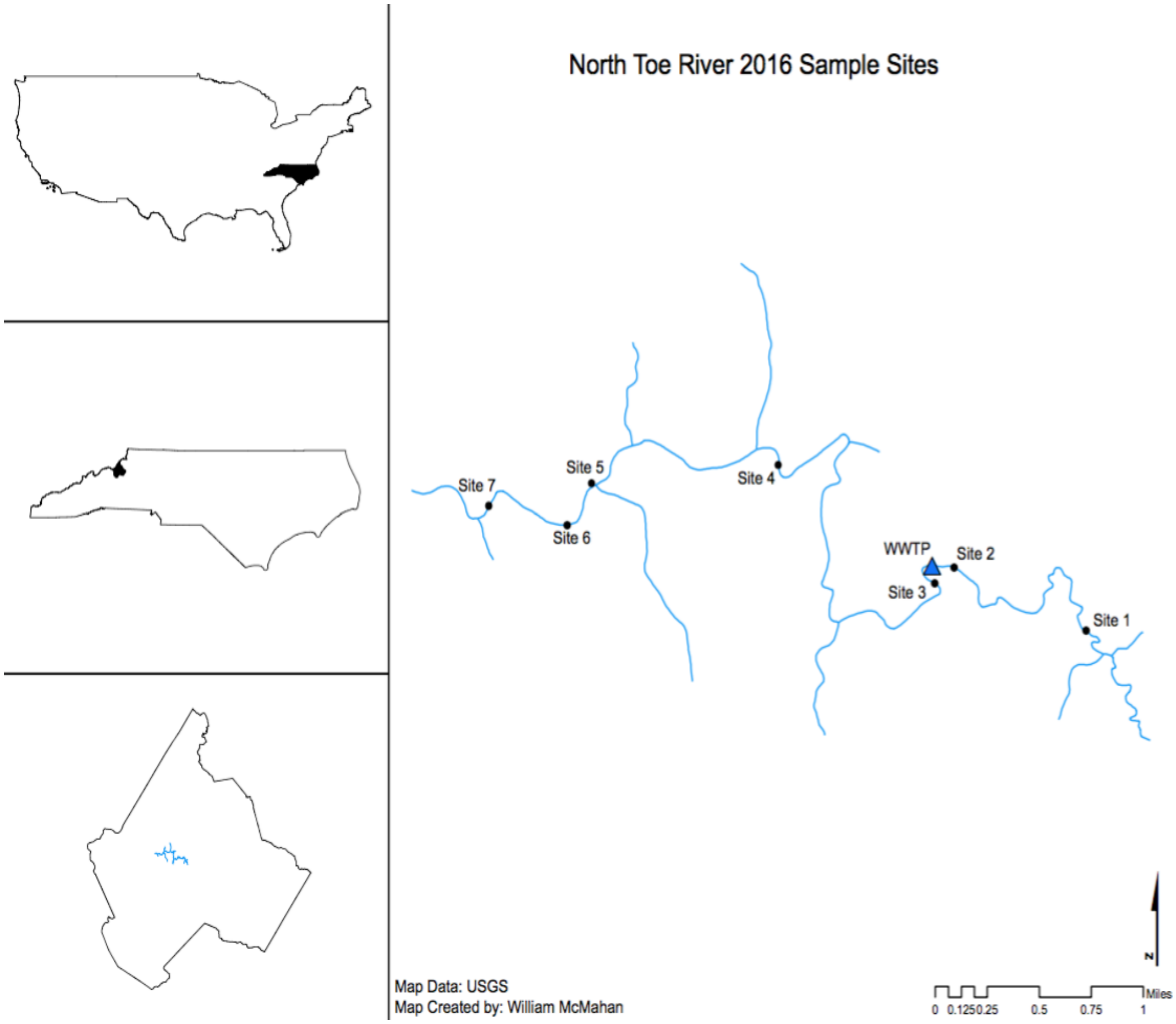


Figure 1. Map showing the location of North Carolina in the United States of America (top left), location of the county the river is in within North Carolina (middle left), the river within the county (bottom left), and finally North Toe River as a whole, as well as the location of each site sampled in 2016.

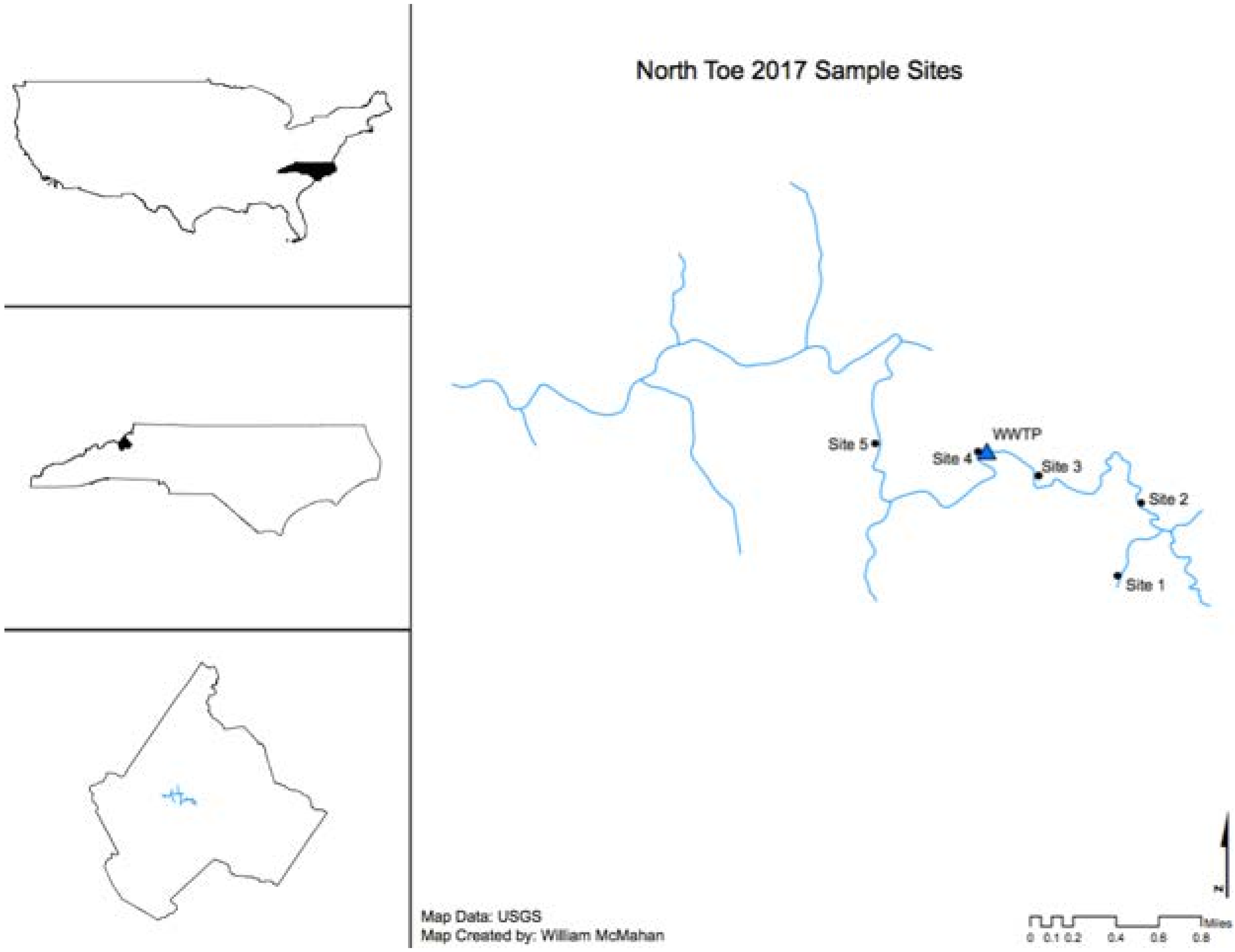


Figure 2. Map showing the location of North Carolina in the United States of America (top left), location of the county the river is in within North Carolina (middle left), the river within the county (bottom left), and finally North Toe River as a whole, as well as the location of each site sampled in 2017.

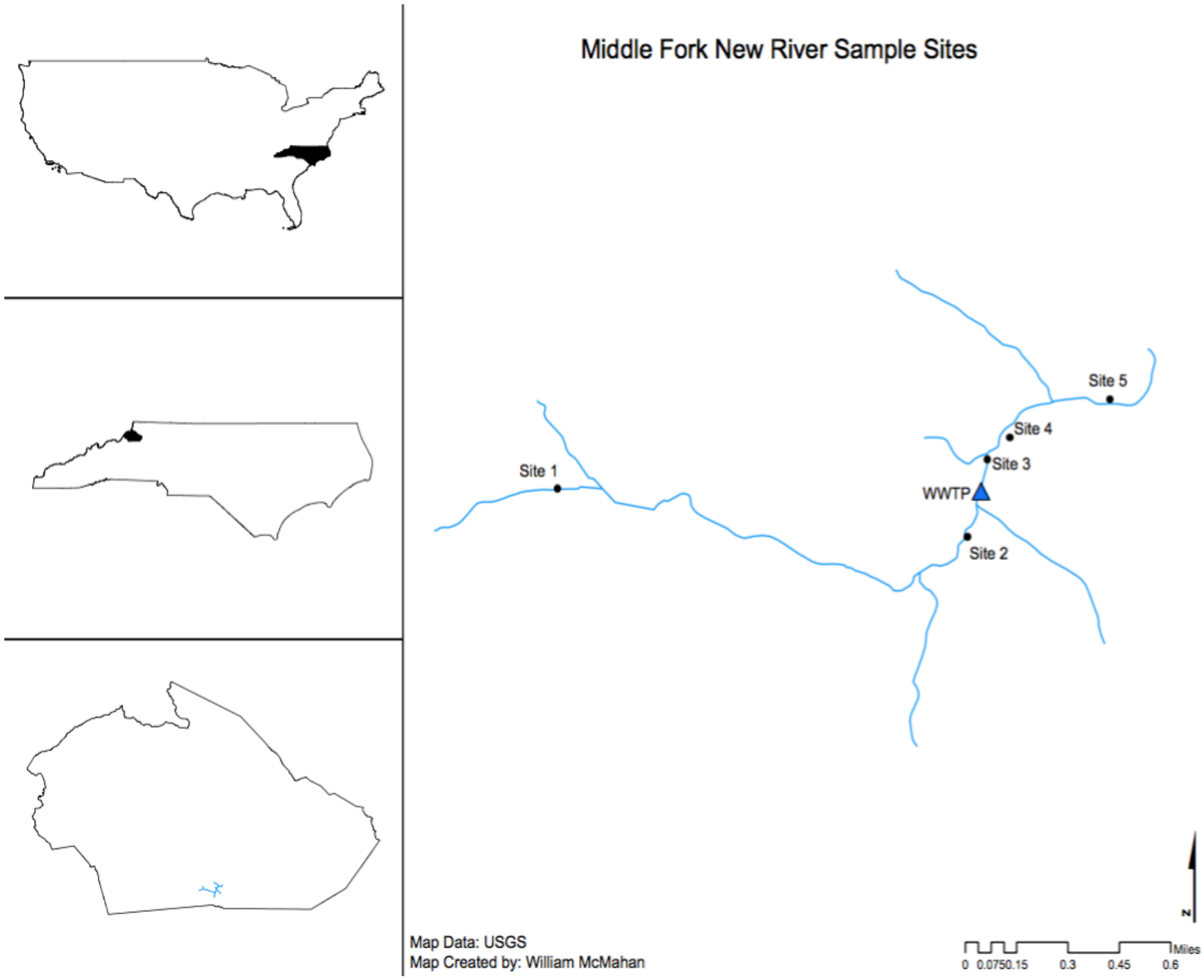


Figure 3. Map showing the location of North Carolina in the United States of America (top left), location of the county the river is in within North Carolina (middle left), the river within the county (bottom left), and finally Middle Fork of the New River as a whole, as well as the location of each site sampled.

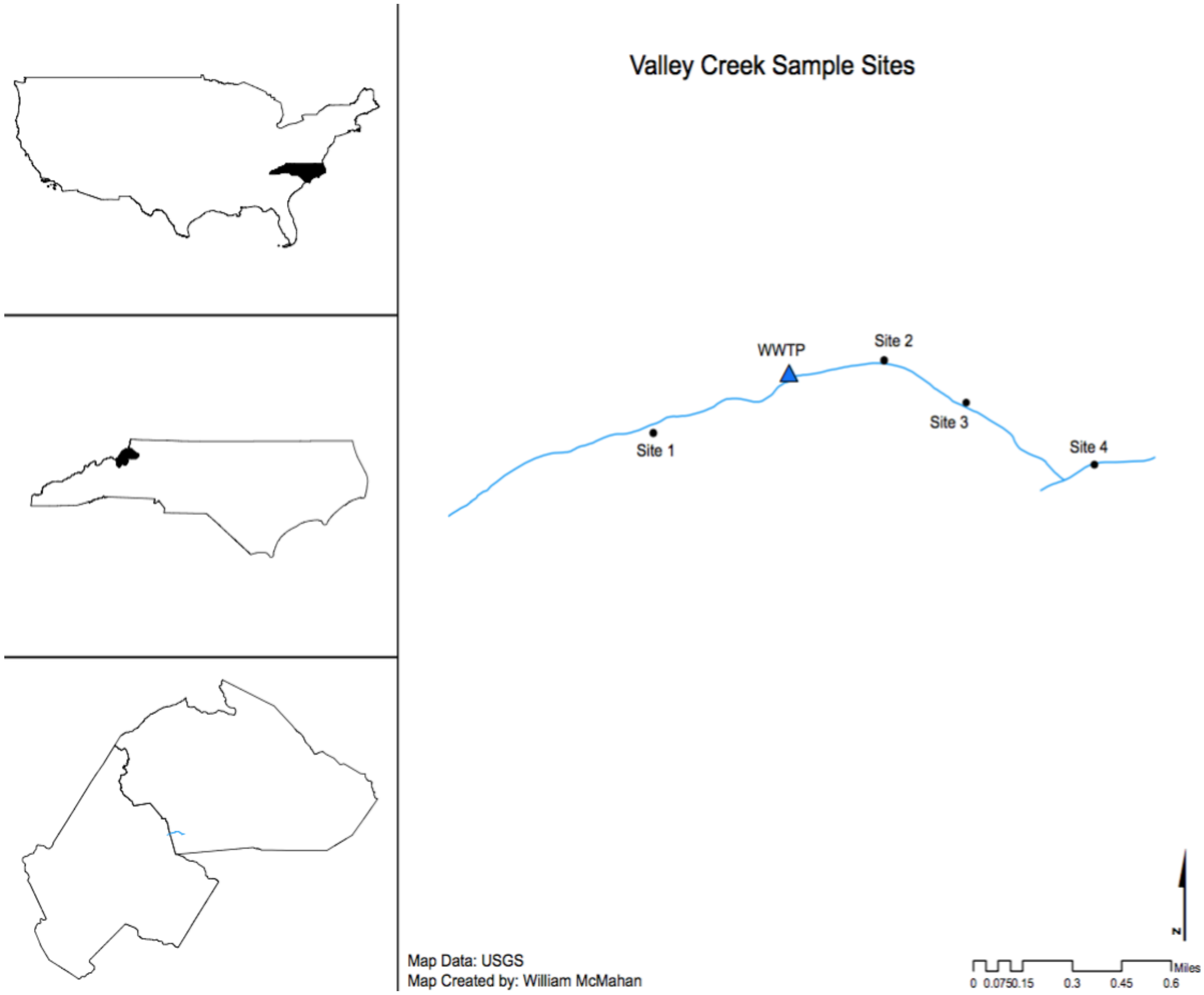


Figure 4. Map showing the location of North Carolina in the United States of America (top left), location of the county the river is in within North Carolina (middle left), the river within the county (bottom left), and finally Valley Creek as a whole, as well as the location of each site sampled.

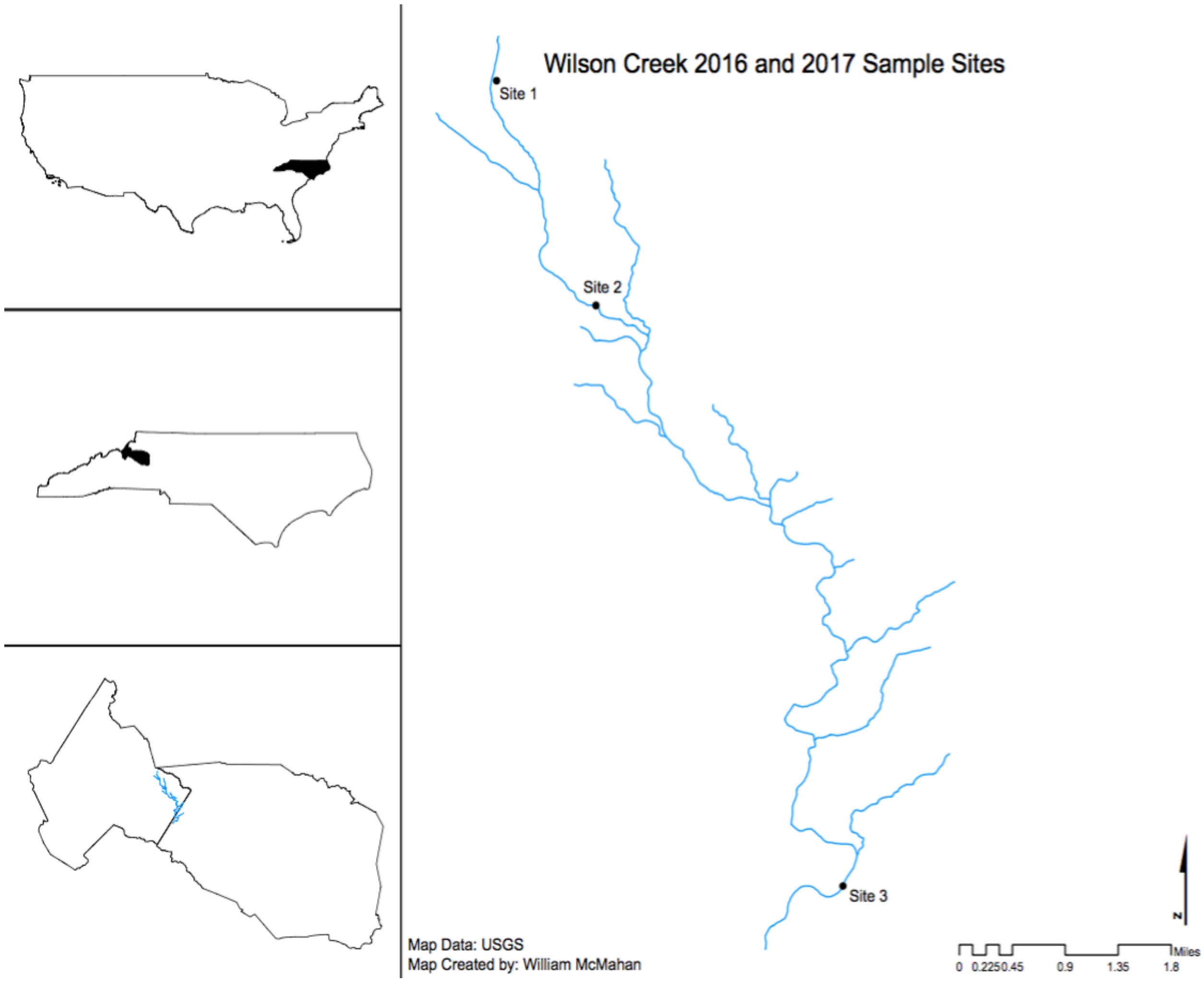


Figure 5. Map showing the location of North Carolina in the United States of America (top left), location of the county the river is in within North Carolina (middle left), the river within the county (bottom left), and finally Wilson Creek as a whole, as well as the location of each site sampled.

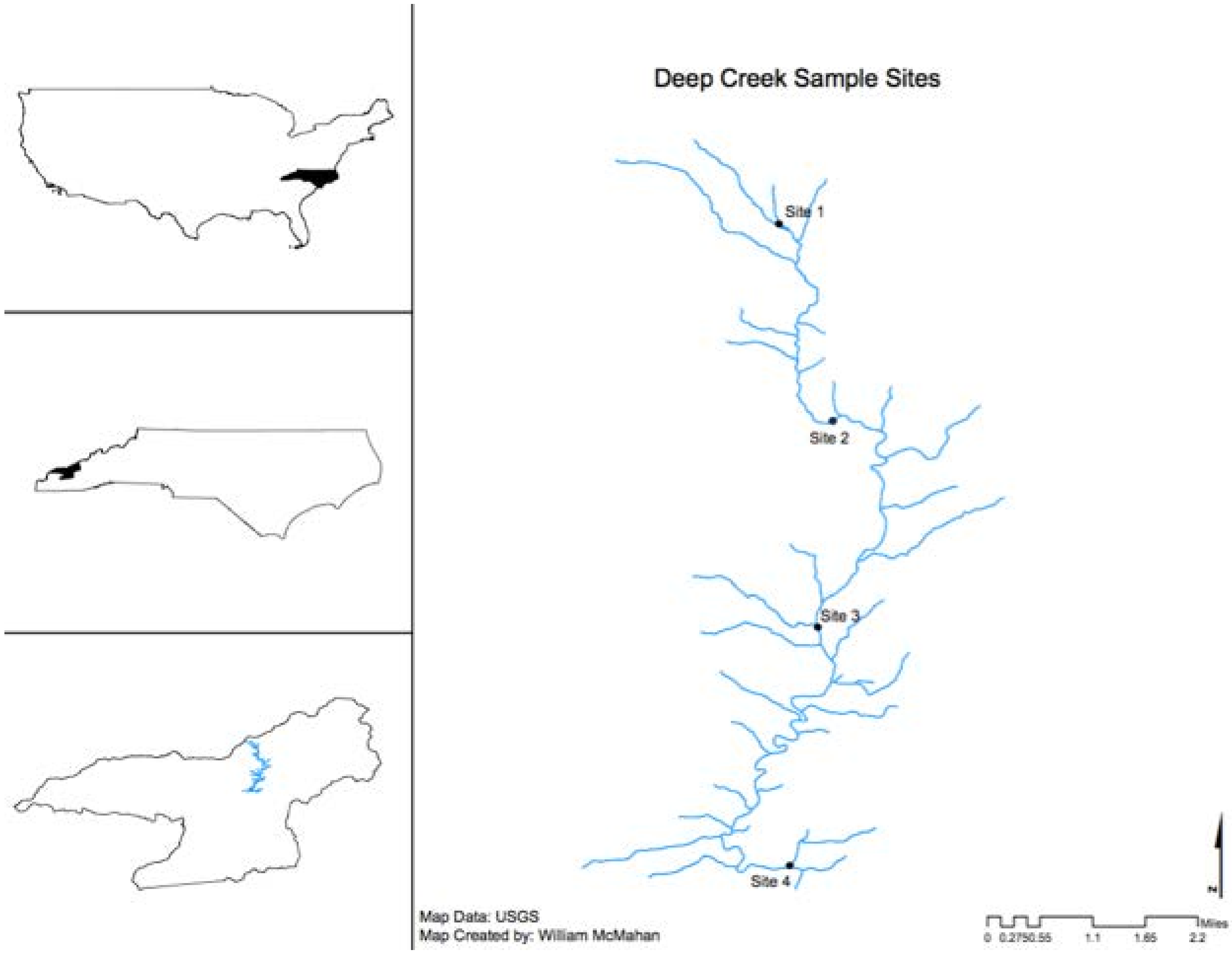


Figure 6. Map showing the location of North Carolina in the United States of America (top left), location of the county the river is in within North Carolina (middle left), the river within the county (bottom left), and finally Deep Creek as a whole, as well as the location of each site sampled.

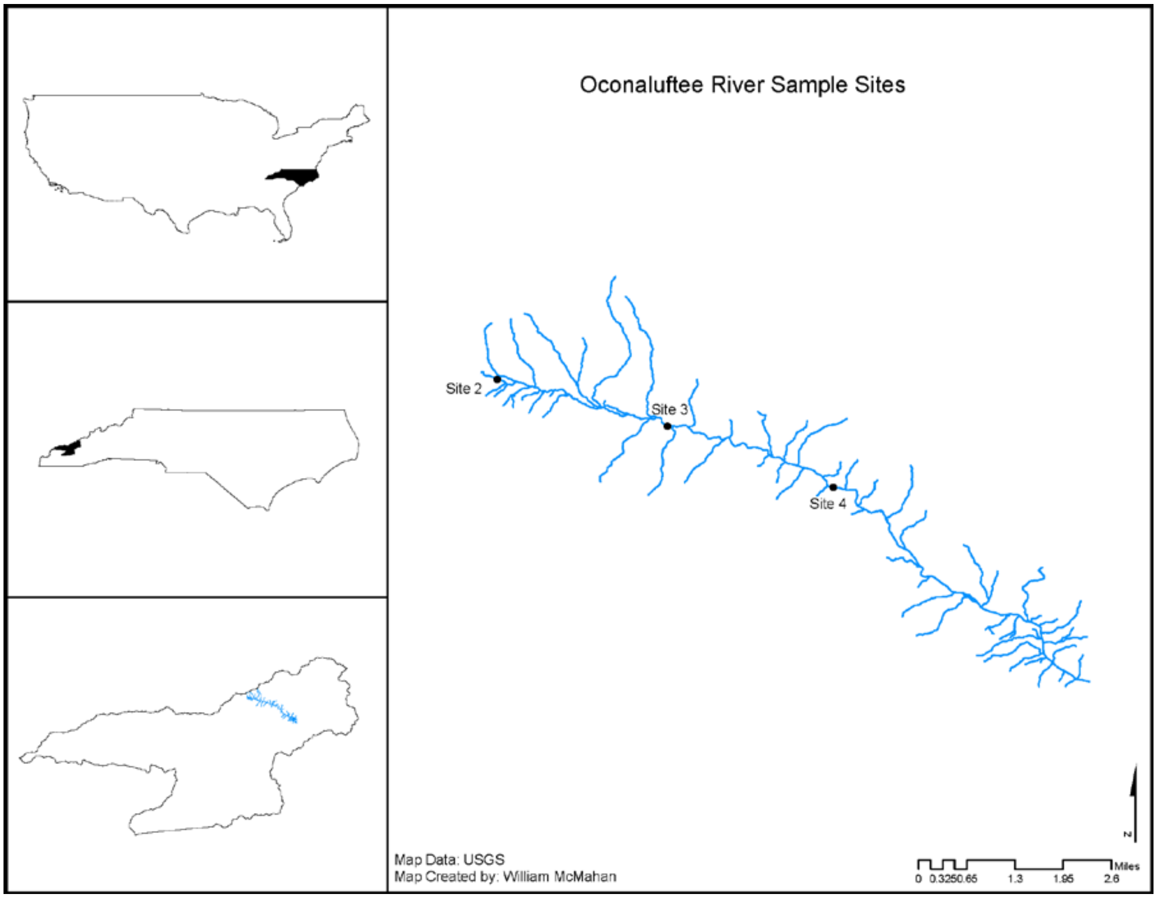


Figure 7. Map showing the location of North Carolina in the United States of America (top left), location of the county the river is in within North Carolina (middle left), the river within the county (bottom left), and finally Oconaluftee River as a whole, as well as the location of each site sampled. Site 1 coordinates were not recorded.

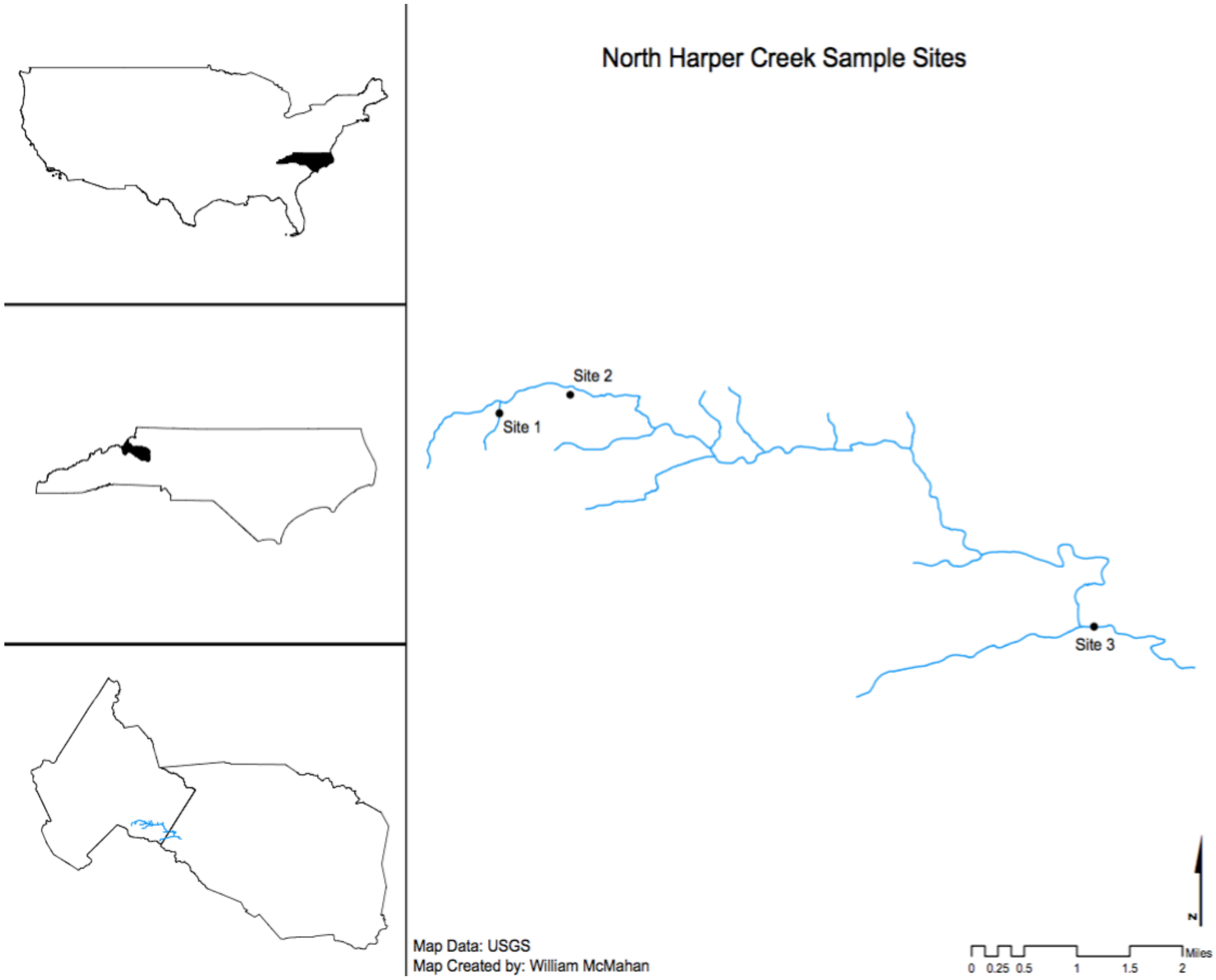


Figure 8. Map showing the location of North Carolina in the United States of America (top left), location of the county the river is in within North Carolina (middle left), the river within the county (bottom left), and finally North Harper Creek as a whole, as well as the location of each site sampled.

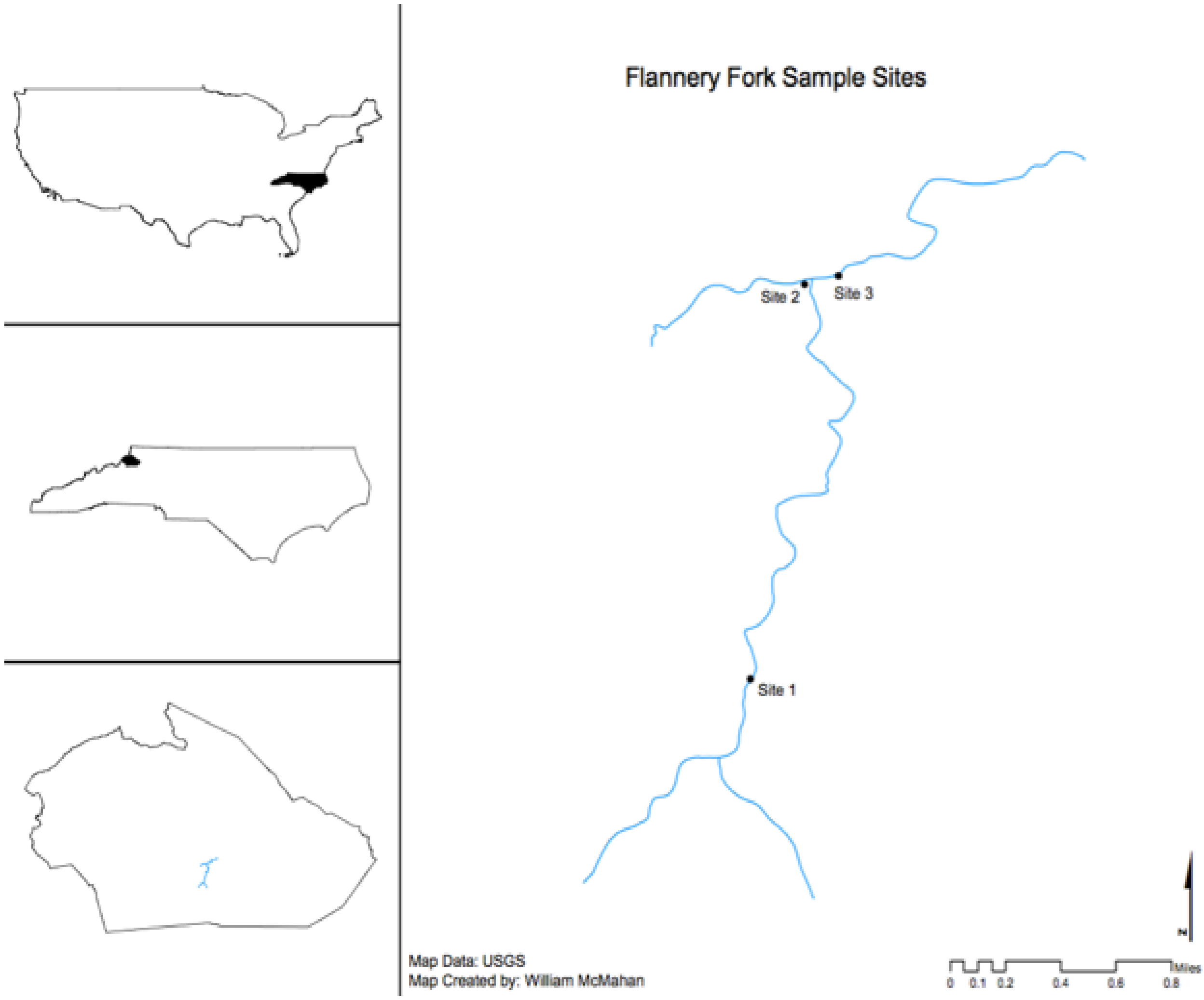


Figure 9. Map showing the location of North Carolina in the United States of America (top left), location of the county the river is in within North Carolina (middle left), the river within the county (bottom left), and finally of Flannery Fork as a whole, as well as the location of each site sampled.

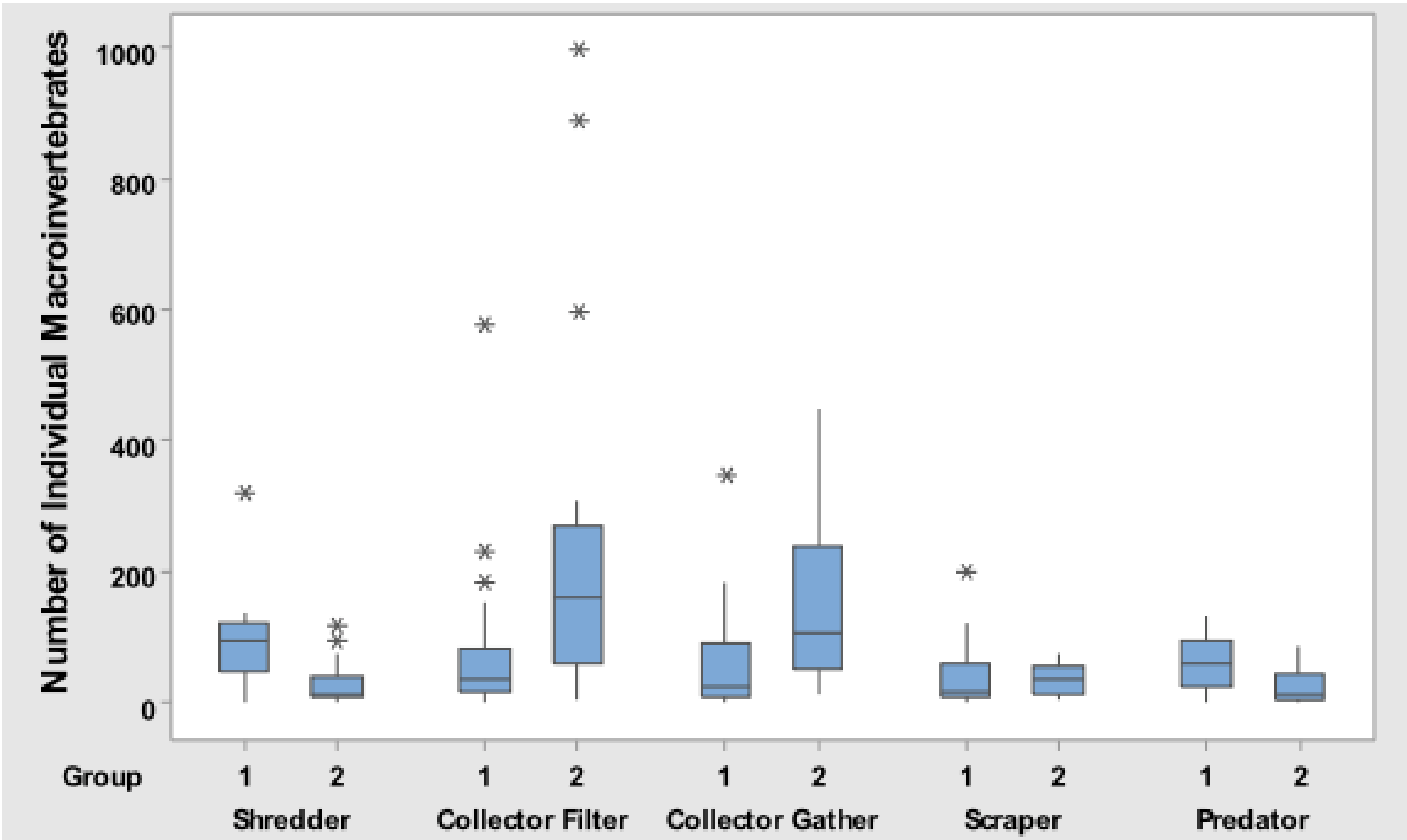


Figure 10. These boxplots show the distribution of numbers of each functional feeding group in reference streams (1) and impacted streams (2). The middle lines show the median, the boxes show the middle 50%, the lower line shows the lower 25%, the upper line shows the upper 25%, and the * symbol is an indication of outliers.

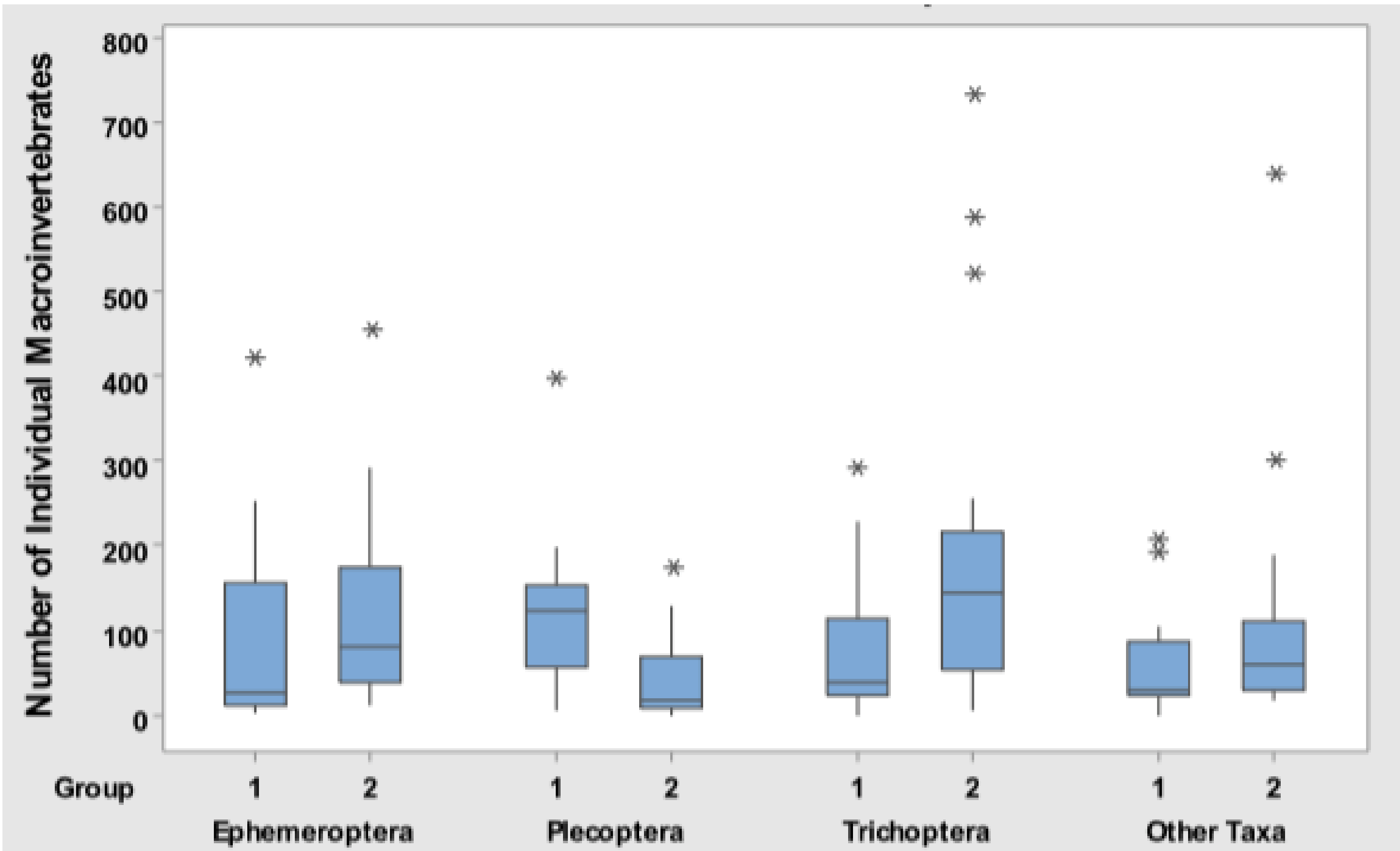


Figure 11. These boxplots show the distribution of numbers of each taxa group in reference streams (1) and impacted streams (2). The middle lines show the median, the boxes show the middle 50%, the lower line shows the lower 25%, the upper line shows the upper 25%, and the * symbol is an indication of outliers.

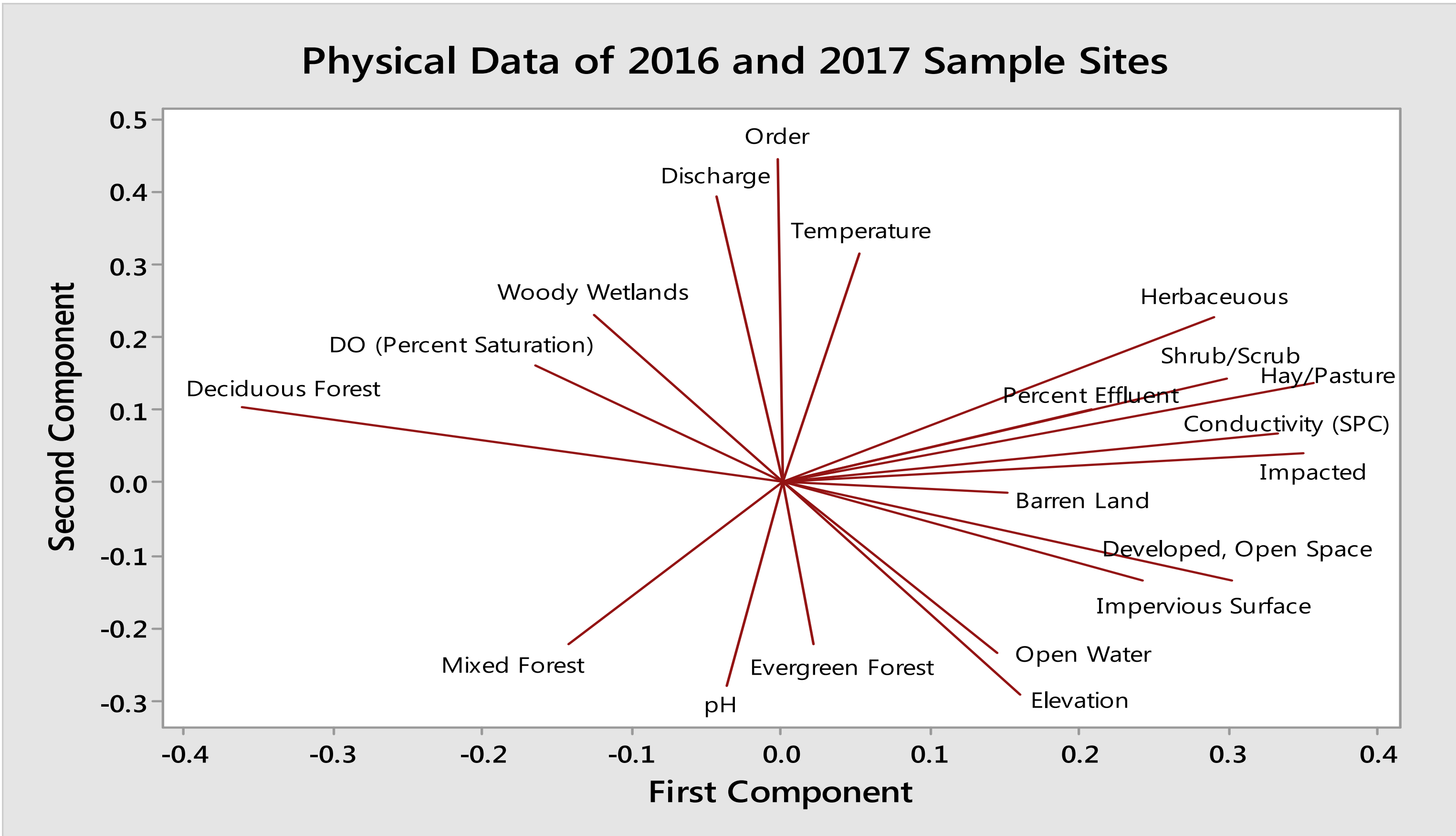


Figure 12. Principle Components Analysis (PCA) of physical data for all streams sampled, excluding Oconaluftee River site 1. The left side of the x axis shows characteristics more prevalent in reference streams, while the right side shows characteristics of impacted streams.

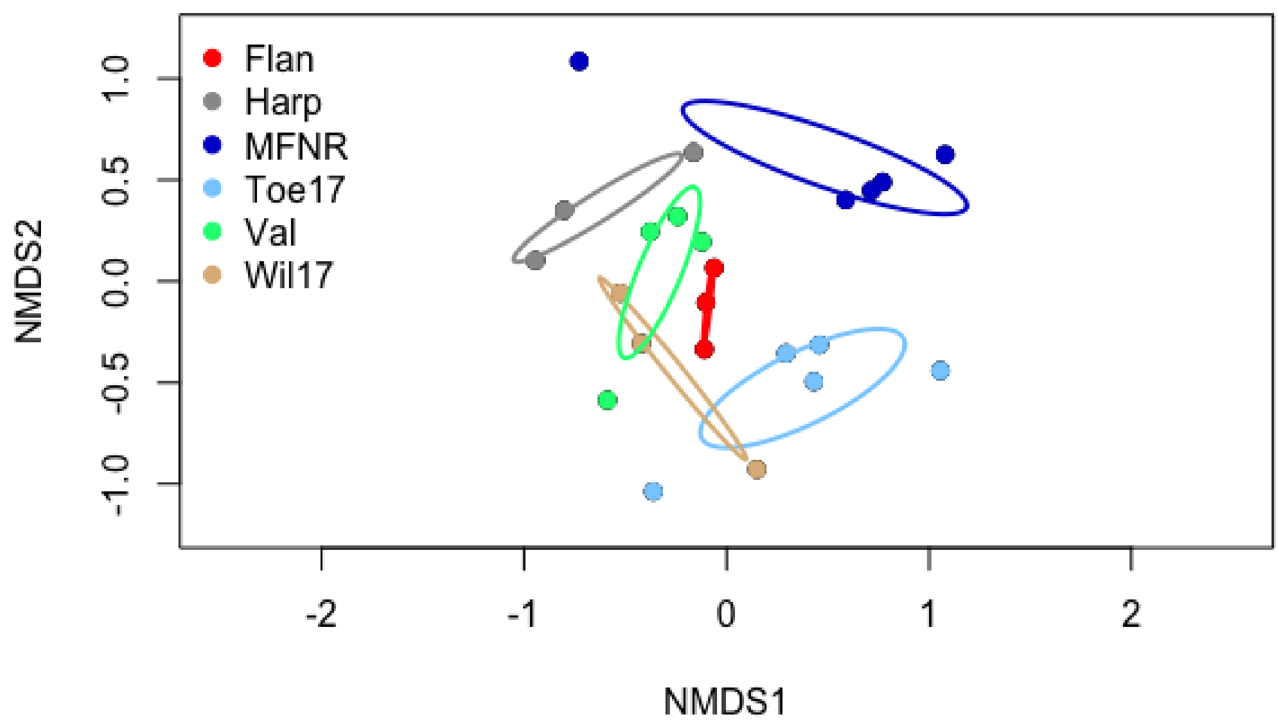


Figure 13. NMDS plot showing the similarity and dissimilarity of the macroinvertebrate taxa sampled in each river in 2017. The left side of the x axis shows taxa characteristics more prevalent in reference streams, while the right side shows taxa characteristics of impacted streams.

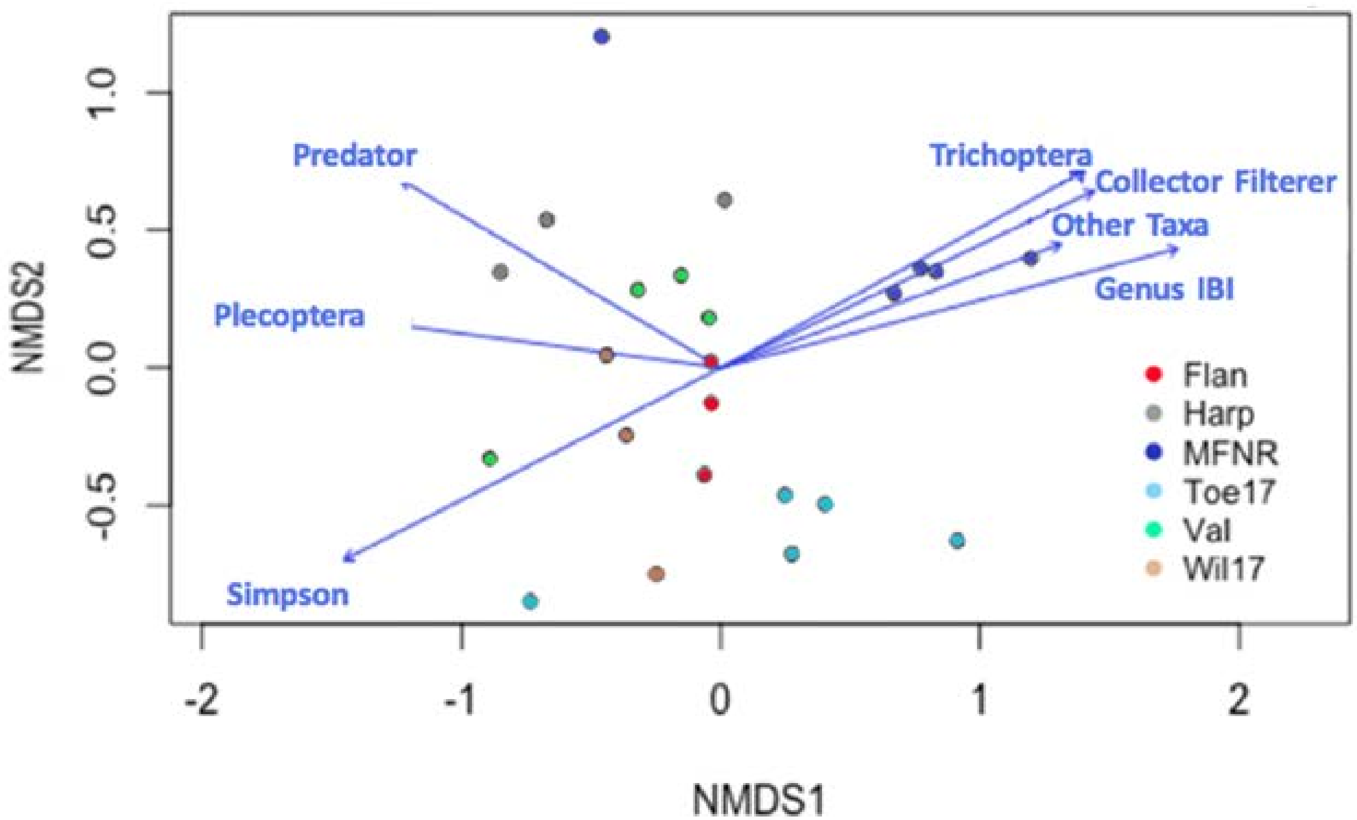


Figure 14. NMDS plot showing the significant calculated biological variables that drove taxa differences when looking at the differences between the impacted and reference streams. The left side of the x axis shows characteristics more prevalent in reference streams, while the right side shows characteristics of impacted streams.

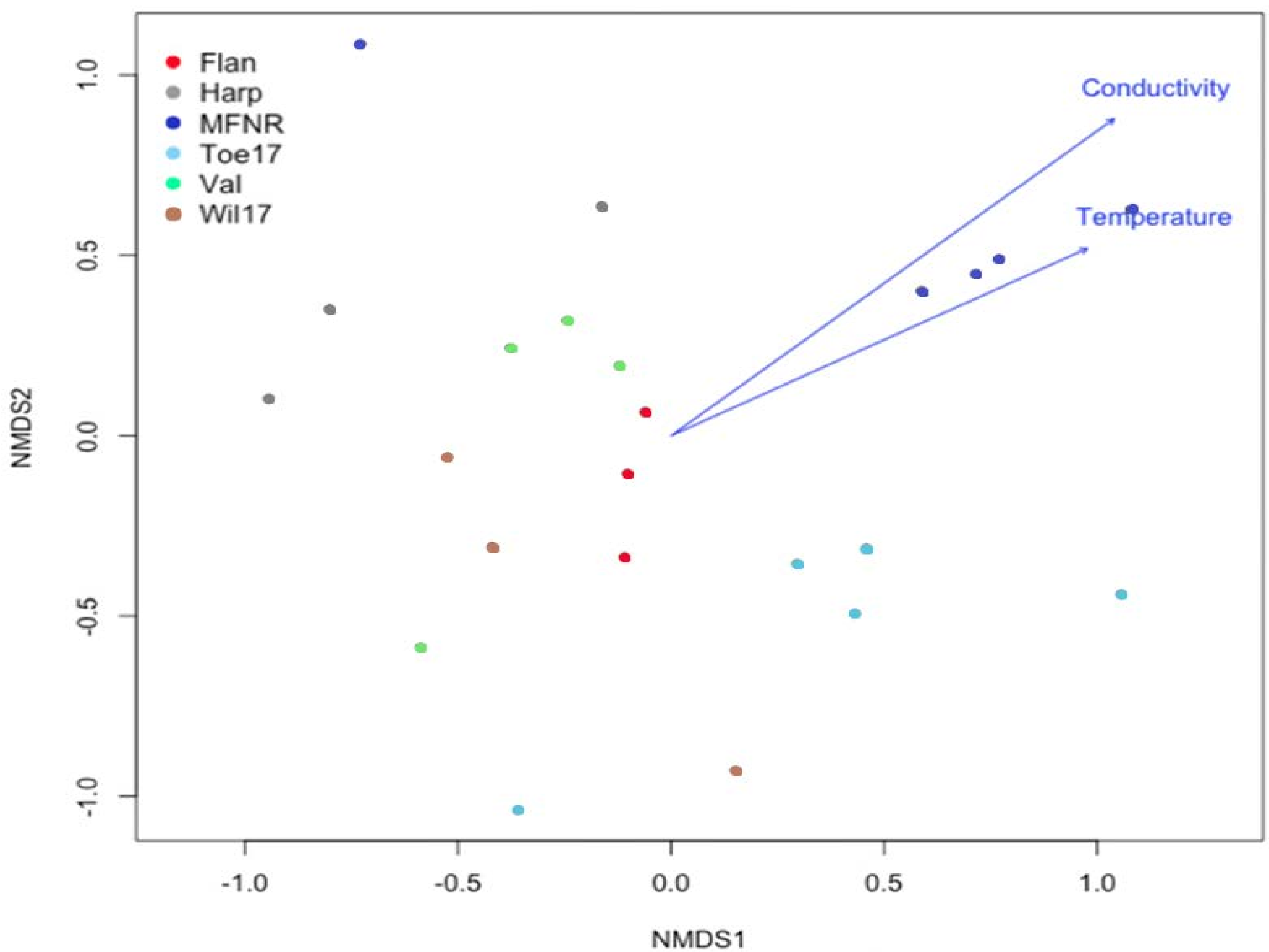


Figure 15. NMDS plot showing the significant water chemistry variables recorded the day of macroinvertebrate sampling that influence taxa species and abundance differences between the impacted and reference streams. The left side of the x axis shows characteristics more prevalent in reference streams, while the right side shows characteristics of impacted streams.

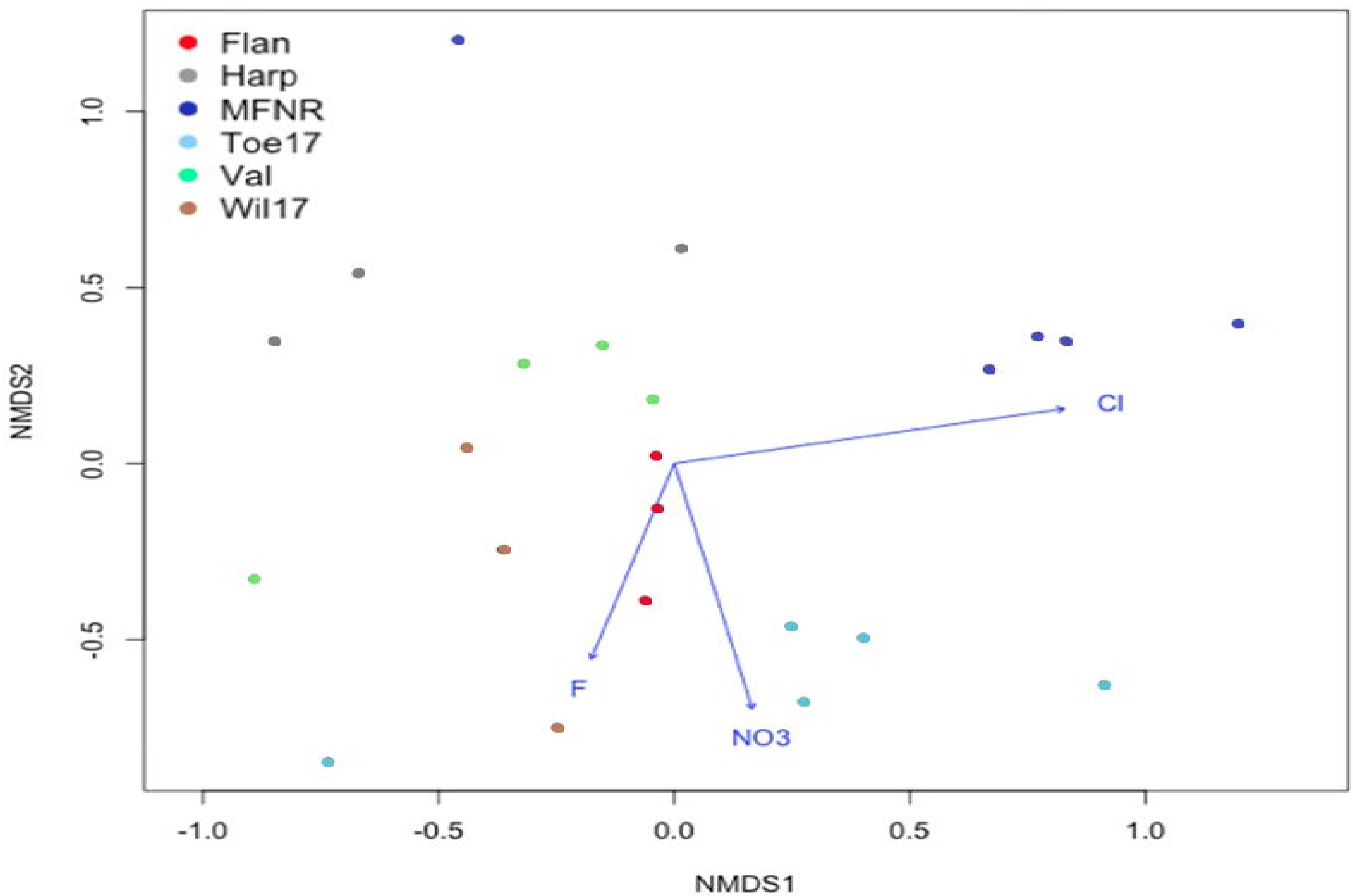


Figure 16. NMDS plot of the significant cations and anions (using IC) from water samples that influence differences of taxa species and abundance at the reference and impacted sample sites. The left side of the x axis shows characteristics more prevalent in reference streams, while the right side shows characteristics of impacted streams.

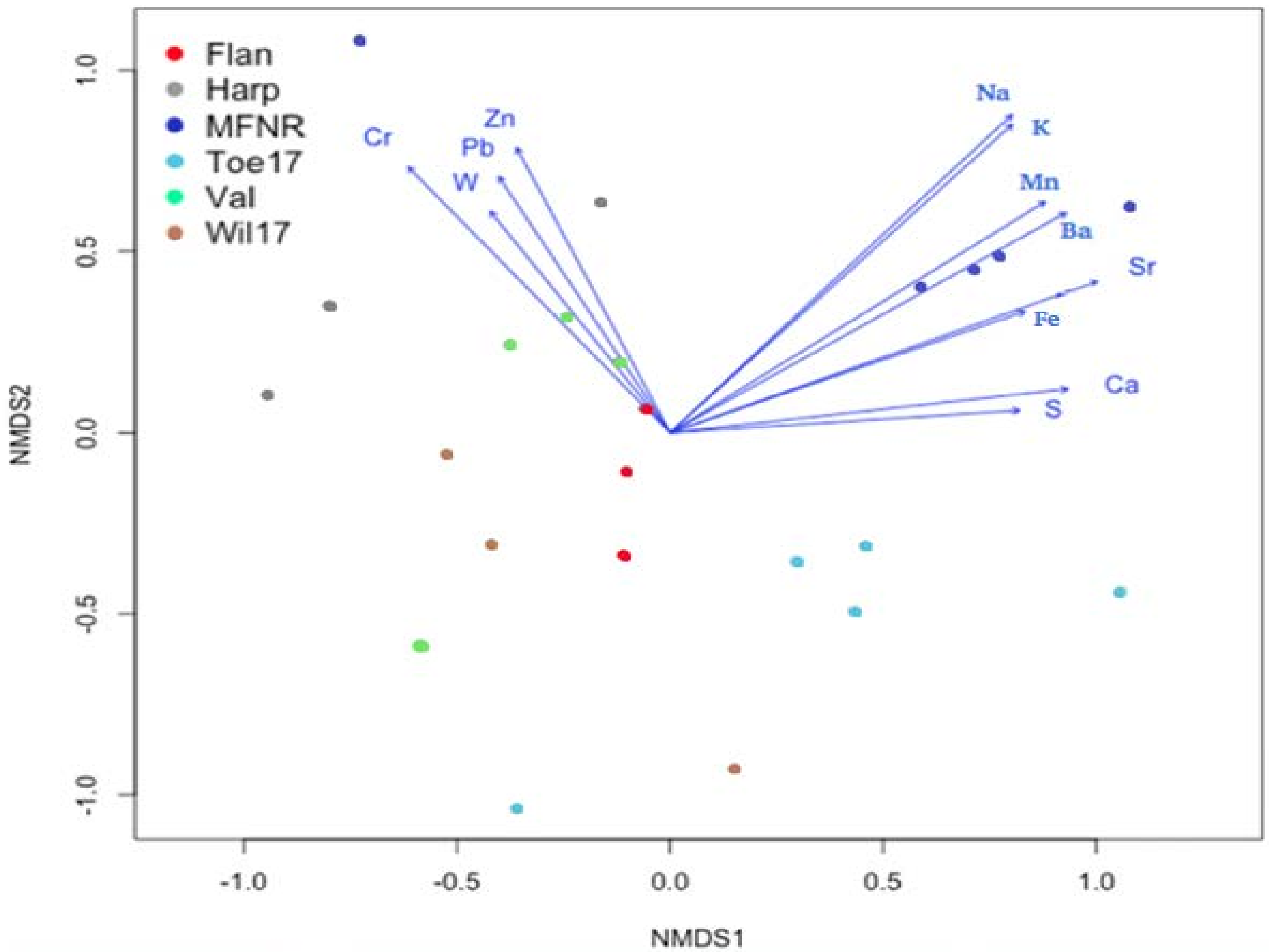


Figure 17. NMDS plot of the significant elements (using ICP-OES) from water samples that influence taxa species and abundance differences recorded from the reference and impacted sample sites. The left side of the x axis shows characteristics more prevalent in reference streams, while the right side shows characteristics of impacted streams.

Appendix Table 1. Inductively Coupled Plasma- Optical Emission Spectroscopy (ICP-OES) and Ion Chromatography (IC) elemental and nutrient data for North Toe River in 2016. Each concentration is in parts per million. Any cell with BDL means that the concentration was below the detection limit of the instrument. EPA indicates EPA standard for aquatic life.

Element	EPA	Site 1	Site 2	Site 3	Site 4	Site 5	Site 6	Site 7
Al		0.456	0.193	0.551	0.207	0.216	0.172	0.176
B		0.338	0.177	0.088	0.068	0.054	0.064	0.044
Ba		0.280	0.264	0.298	0.258	0.263	0.276	0.266
Cr	50	0.007	0.004	0.001	0.002	0.002	0.003	0.003
Cu	0.007	0.004	0.007	0.002	0.002	0.002	0.082	0.003
Fe	1.0	0.853	0.585	1.116	0.396	0.449	0.362	0.301
Li		0.000	0.002	0.000	0.001	0.001	0.001	0.001
Mn		0.071	0.042	0.083	0.018	0.019	0.016	0.011
Ni	0.088	0.003	0.003	0.001	0.003	0.006	0.001	0.002
Rb		0.004	BDL	0.004	0.002	0.004	0.005	0.001
S		1.203	1.251	1.400	1.564	1.679	1.750	1.806
Sn		0.004	0.004	0.002	0.002	0.004	0.001	0.001
Sr		0.061	0.065	0.064	0.069	0.069	0.073	0.070
W		0.058	0.034	0.016	0.014	0.011	0.009	0.009
Zn	0.050	0.044	0.058	0.027	0.057	0.137	0.061	0.031
F	1.8	BDL	0.361	BDL	BDL	BDL	BDL	BDL
Cl	17.0	10.862	10.384	11.201	9.956	10.527	10.187	6.672
SO_4		0.986	0.965	0.967	1.038	1.187	1.231	0.894
Br		0.096	0.091	0.095	BDL	BDL	BDL	BDL
NO_3		3.014	1.555	2.083	3.428	3.825	6.158	1.512
PO_4		BDL	BDL	BDL	0.094	0.121	0.113	0.068

Appendix Table 2. Inductively Coupled Plasma- Optical Emission Spectroscopy (ICP-OES) and Ion Chromatography (IC) elemental and nutrient data for reference rivers sampled in 2017. Each element concentration is in parts per million (mg/L). Any cell with BDL means that the concentration was below the detection limit of the instrument. EPA column indicates EPA standard for aquatic life.

Element	EPA	FF 1	FF 2	FF 3	NH 1	NH 2	NH 3	WC 1	WC 2	WC 3
Al	0.087	0.223	0.228	0.194	0.162	0.125	0.803	0.169	0.113	0.602
B		0.162	0.131	0.206	0.159	0.242	0.364	0.031	0.036	0.002
Ba		1.077	1.006	1.045	1.236	1.310	1.051	1.315	1.247	1.049
Cr	50	0.001	0.001	BDL	0.005	0.002	0.000	BDL	0.001	BDL
Cu	0.007	BDL	0.001	0.002	0.016	0.001	BDL	BDL	BDL	0.000
Fe	1.0	0.689	0.348	0.307	0.151	0.084	0.078	0.068	0.068	0.055
K		0.544	0.551	0.512	0.713	0.456	0.576	0.310	0.367	0.382
Li		0.001	0.000	0.001	0.004	0.002	0.001	0.001	0.000	0.000
Mn		0.020	0.009	0.005	0.014	0.006	0.006	0.006	0.004	0.004
Na		1.054	1.050	1.107	0.757	0.878	1.091	0.396	1.071	0.769
Ni	0.088	0.006	0.006	0.005	0.011	0.009	0.005	0.006	0.005	0.005
Rb		BDL	BDL	0.002	0.006	BDL	0.003	BDL	BDL	0.001
S		1.660	1.876	1.703	0.972	1.203	1.516	3.844	2.087	1.889
Se		0.007	BDL	BDL	0.005	0.011	BDL	0.010	0.006	0.012
Sn		0.003	0.004	BDL	0.008	0.002	BDL	0.001	0.000	0.003
Sr		0.030	0.030	0.029	0.022	0.019	0.019	0.019	0.022	0.022
Zn	0.050	0.011	0.005	0.014	0.025	0.015	0.013	0.014	0.002	0.007
F	1.8	0.210	0.525	0.427	0.174	0.466	0.269	0.474	0.419	0.719
Cl	17.0	3.439	5.042	4.036	0.772	3.075	2.228	3.273	5.592	4.664
SO_4		0.654	5.547	2.471	0.600	BDL	1.276	1.972	0.812	4.637
NO_3		0.028	BDL	BDL	0.334	6.144	0.856	BDL	0.083	0.155
PO_4		0.195	BDL	BDL	0.386	1.018	1.051	0.417	0.236	BDL

Appendix Table 3. Inductively Coupled Plasma- Optical Emission Spectroscopy (ICP-OES) and Ion Chromatography (IC) elemental and nutrient data for impacted rivers sampled in 2017. Each element (E) concentration is in parts per million. Any cell with BDL means that the concentration was below the detection limit of the instrument. EPA column indicates EPA standard for aquatic life.

E	EPA	NT 1	NT 2	NT 3	NT 4	NT 5	VC 1	VC 2	VC 3	VC 4	MF 1	MF 2	MF 3	MF 4	MF 5
Al	0.087	0.579	0.602	0.754	0.361	0.559	0.322	0.406	0.303	0.753	0.717	0.690	0.402	0.720	0.480
B		0.039	0.026	0.010	0.036	0.023	0.018	0.055	0.024	0.028	0.095	0.028	0.228	0.192	0.061
Ba		2.243	2.342	2.584	2.485	2.440	1.520	1.516	1.341	1.366	3.548	4.064	4.335	3.694	3.329
Cr	50	0.001	BDL	0.000	0.000	BDL	BDL	0.000	BDL	0.001	0.013	0.001	0.001	0.000	BDL
Cu	0.007	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	0.002	BDL
Fe	1.0	0.802	0.460	0.451	0.362	0.337	0.431	0.422	0.314	0.125	0.283	0.788	1.625	0.780	0.572
K		0.394	0.448	0.561	0.579	0.541	0.590	0.683	0.667	0.695	0.667	1.546	1.326	1.730	1.586
Li		0.001	0.000	0.000	0.001	0.000	0.001	0.001	0.001	0.000	0.000	0.001	0.001	0.001	0.001
Mn		0.028	0.032	0.027	0.025	0.021	0.059	0.023	0.014	0.007	0.016	0.228	0.357	0.173	0.111
Na		0.623	1.797	2.059	2.084	2.018	1.451	2.227	2.255	2.277	3.862	4.486	4.679	5.462	4.882
Ni	0.088	0.005	0.006	0.005	0.006	0.005	0.007	0.007	0.010	0.006	0.002	0.006	0.007	0.007	0.007
Rb		0.006	0.002	0.001	0.002	0.002	0.001	0.003	BDL	0.007	BDL	BDL	BDL	0.004	0.008
S		1.496	2.108	2.271	2.265	2.319	2.651	3.204	2.988	2.971	1.435	2.849	3.208	4.699	3.931
Sn		0.003	0.004	0.002	0.002	BDL	0.002	0.004	BDL	0.003	0.003	0.002	0.001	0.001	0.007
Sr		0.040	0.040	0.044	0.043	0.045	0.036	0.042	0.042	0.045	0.041	0.063	0.060	0.061	0.058
Zn		0.050	0.006	0.002	0.008	0.012	0.008	0.011	0.029	0.025	0.003	0.214	0.013	0.012	0.013
F	1.8	0.450	0.426	0.368	0.601	0.404	0.426	0.665	0.462	0.412	BDL	0.281	0.093	0.448	0.061
Cl	17.0	3.270	14.52	14.59	18.63	13.270	9.616	14.935	12.634	13.93	15.81	29.45	18.503	20.90	26.087
NO_3		0.050	BDL	0.552	0.544	0.572	0.715	0.829	0.909	0.826	0.199	0.530	0.586	0.511	0.613
PO_4		0.144	0.410	0.254	0.364	0.400	BDL	BDL	BDL	0.287	BDL	BDL	0.017	BDL	BDL
SO_4		5.942	3.846	3.468	9.188	4.530	0.654	3.629	3.928	3.361	0.530	BDL	1.367	3.399	1.319

Appendix Table 4. Land cover within a 1 km radius of each reference stream sample site. Reference streams were primarily forested.

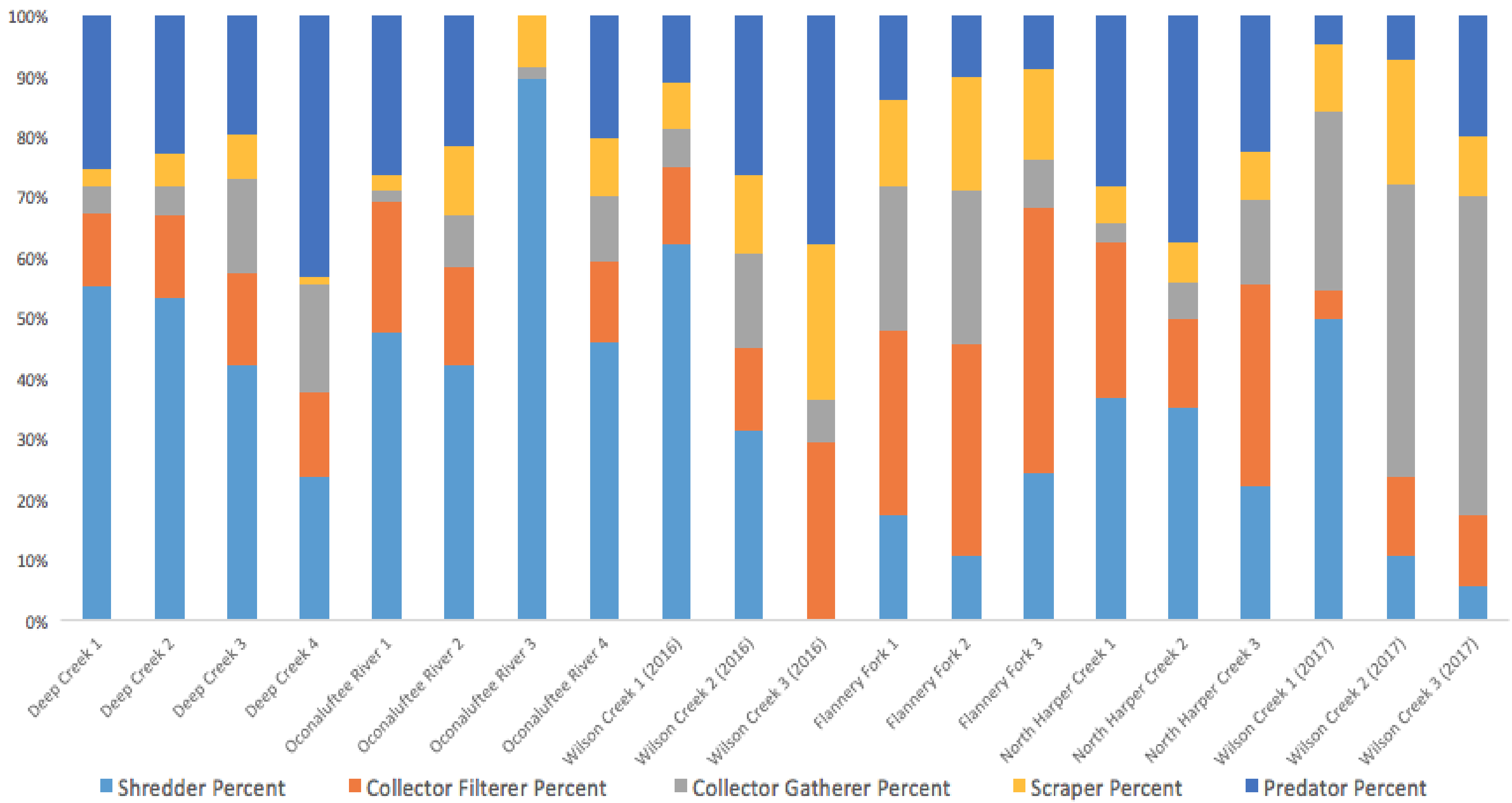
	Barren Land	Deciduous Forest	Impervi-ous	Open Space	Evergreen Forest	Hay/ Pasture	Herbace-uous	Mixed Forest	Open Water	Shrub/ Scrub	Woody Wetlands
DC 1		86.02	0.00		5.79			8.11		0.09	
DC 2		87.08	0.00		8.59			4.33			
DC 3		66.69	0.00		28.27			5.04			
DC 4		85.56	0.52	0.52	7.88			6.04			
FF 1	1.18	80.81	7.57	7.28	3.30	2.70	0.95	1.81	1.06	0.49	0.14
FF 2		68.28	19.50	11.93	2.47	3.13		4.82		0.49	
FF 3		64.15	23.17	12.76	2.35	2.27		4.42		0.49	
NH 1		96.96	2.35	2.35						0.69	
NH 2		96.82	2.49	2.49						0.69	
NH 3		85.83	0.00		8.83			5.33			
OR 1		77.89	11.66	10.94	8.62			1.80		0.03	
OR 2		93.76	5.12	5.10	0.14			0.97			
OR 3		93.55	5.82	5.82				0.63			
OR 4		84.24	10.75	10.75	0.37	1.29	0.14	2.98		0.23	
WC 1,16	0.14	74.85	6.02	5.42	4.16		0.14	12.82		1.86	
WC 2,16		91.45	6.31	6.31	0.66			1.23		0.34	
WC 3,16		86.32	3.27	3.24	2.93	0.49	1.06	5.08		0.17	0.69
WC 1,17	0.14	70.55	5.62	5.10	8.66		0.03	13.48		1.52	
WC 2,17		91.45	6.31	6.31	0.66			1.23		0.34	
WC 3,17		86.43	3.36	3.33	2.67	0.49	1.06	5.16		0.14	0.69

Appendix Table 5. Land use within a 1 km radius of each impacted stream sample site. Impacted streams had multiple land use types surrounding the sample site.

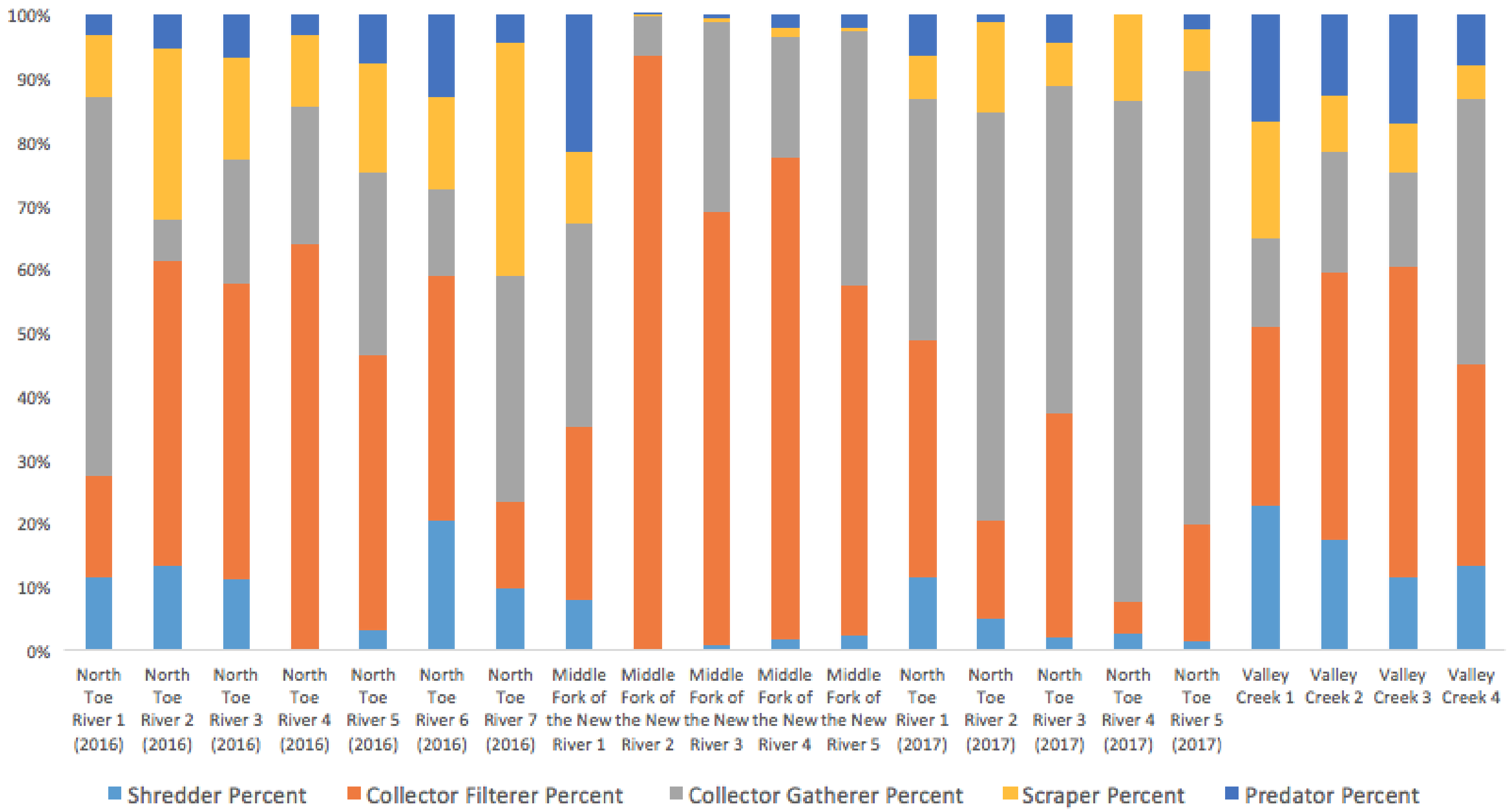
Site	Barren Land	Deciduous Forest	Impervi-ous	Open Space	Evergreen Forest	Hay/ Pasture	Herbace-uous	Mixed Forest	Open Water	Shrub/ Scrub	Woody Wetlands
MFNR1		53.31	20.13	14.65	17.64	3.47	0.77	1.92	2.18	0.54	
MFNR 2	0.26	34.13	42.50	26.21	8.92	6.83	1.15	2.95	1.03	1.06	
MFNR 3	0.20	43.91	30.77	21.54	9.75	8.35	1.00	3.38	1.03	1.06	
MFNR 4	0.20	46.00	28.62	20.25	8.86	9.41	1.06	3.30	1.03	1.00	
MFNR 5	0.17	53.59	23.67	16.49	5.13	11.27	0.89	3.36	0.69	0.83	
NT 1,16	0.03	35.70	40.52	22.77	3.84	7.66	2.93	0.37		6.62	
NT 2,16	0.34	46.14	14.83	11.90	3.18	18.01	7.08	0.92		9.44	
NT 3,16	0.34	48.01	11.53	10.04	3.30	18.18	7.71	0.92		10.01	
NT 4,16		73.07	6.31	5.85	0.49	12.62	3.99			3.53	
NT 5,16		92.95	2.61	2.58		1.84	0.98			1.29	0.34
NT 6,16		90.08	2.87	2.84	0.20	2.67	2.29			1.55	0.34
NT 7,16		92.66	3.04	3.01		1.66	1.35			0.95	0.34
NT 1,17		46.37	31.32	15.57	2.21	7.08	3.13	0.52		7.08	
NT 2,17	0.09	35.36	40.01	22.31	3.81	8.23	3.10	0.32		6.77	
NT 3,17	0.34	45.34	18.30	11.84	3.44	13.85	6.60	0.92		9.69	
NT 4,17	0.34	48.84	8.35	7.63	3.15	19.99	8.37	0.66		10.30	
NT 5,17	0.17	63.44	7.40	6.83	2.18	16.35	5.22			5.25	
VC 1	0.14	76.54	14.54	12.82		6.74	0.17			1.86	
VC 2		74.79	18.87	16.32		4.59	0.17			1.58	
VC 3		69.72	20.62	16.46	0.34	7.00	0.17	0.43		1.69	
VC 4		70.52	17.03	13.11	0.69	10.04	0.29	0.57		0.83	

Appendix Table 6. Calculated biological values for macroinvertebrate richness, Shannon Diversity (H'), Simpson Diversity (S'), abundance (Total N invertebrates), evenness, and the North Carolina Biotic Integrity (NC BI) for reference streams.

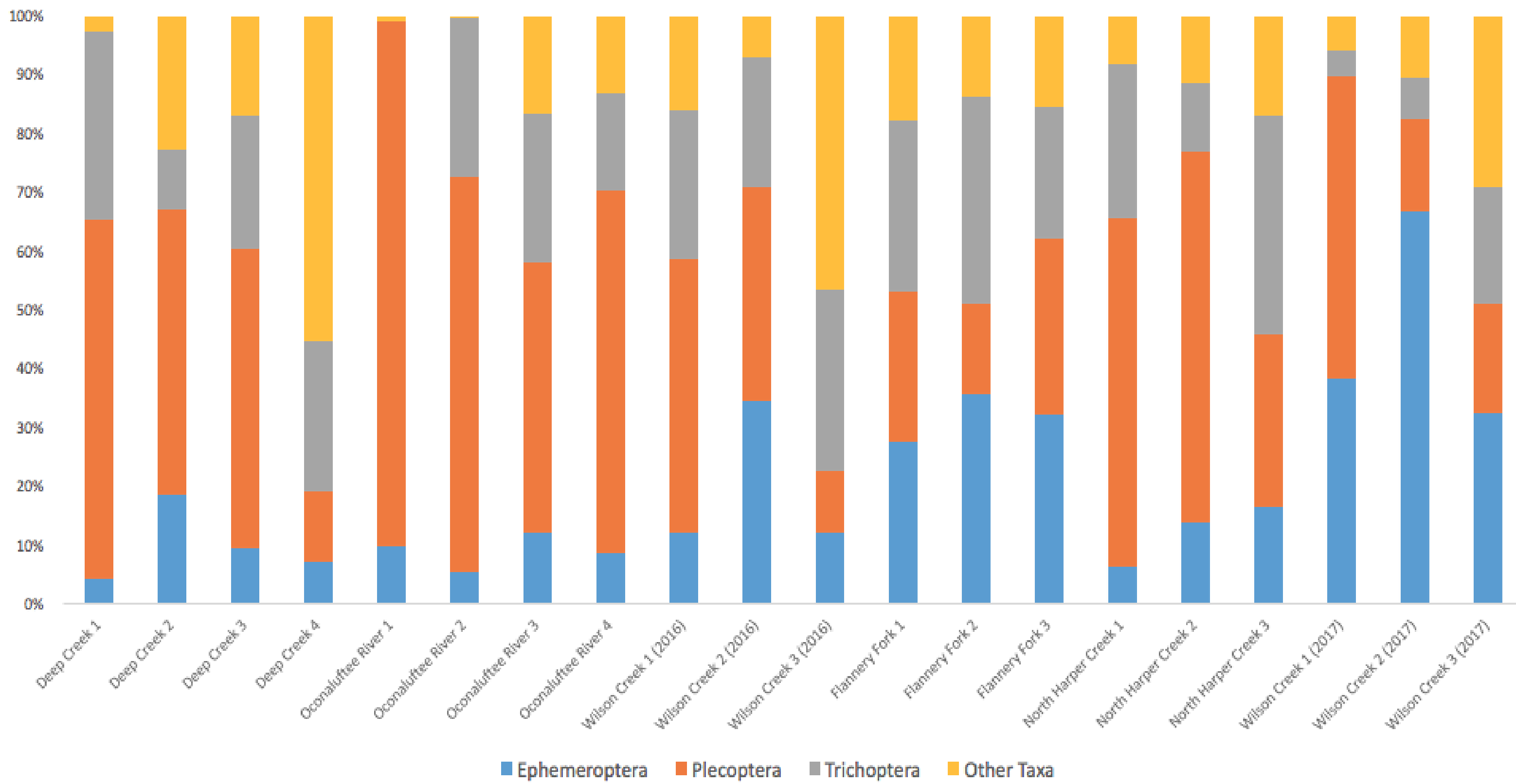
Stream	Richness	H'	S'	Abundance	Evenness	NC BI
Deep Cr 1	18	2.41	0.88	72	0.83	1.69
Deep Cr 2	26	2.59	0.89	109	0.79	1.90
Deep Cr 3	29	2.61	0.87	136	0.77	2.51
Deep Cr 4	26	2.78	0.92	154	0.85	3.72
Oconaluftee R 1	6	0.47	0.20	104	0.26	1.25
Oconaluftee R 2	19	1.97	0.78	229	0.67	2.33
Oconaluftee R 3	34	2.58	0.87	283	0.73	1.91
Oconaluftee R 4	30	2.40	0.79	198	0.71	2.01
Wilson Cr 1 (16)	22	2.28	0.82	222	0.74	3.42
Wilson Cr 2 (16)	62	3.38	0.95	434	0.82	2.61
Wilson Cr 3 (16)	18	2.37	0.88	58	0.82	3.16
Flannery Fk 1	41	2.95	0.92	596	0.79	2.98
Flannery Fk 2	38	2.86	0.91	652	0.79	3.10
Flannery Fk 3	60	2.79	0.90	1315	0.68	2.77
North Harper Cr 1	27	2.20	0.81	335	0.67	3.55
North Harper Cr 2	36	2.59	0.87	256	0.72	3.16
North Harper Cr 3	50	3.14	0.93	452	0.80	3.00
Wilson Cr 1 (17)	22	2.25	0.85	251	0.73	3.42
Wilson Cr 2 (17)	33	2.62	0.88	379	0.75	2.61
Wilson Cr 3 (17)	31	2.91	0.96	656	0.85	3.16



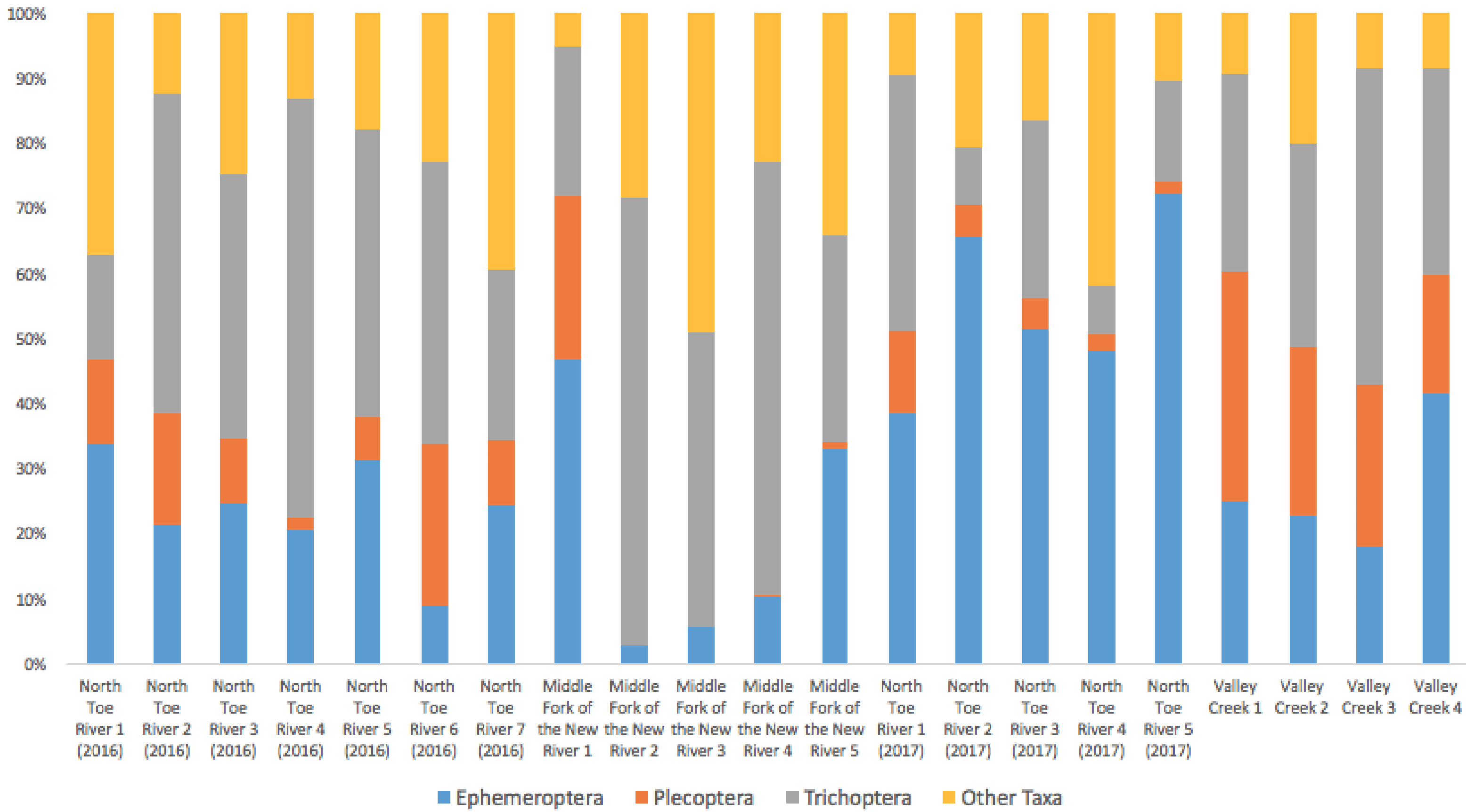
Appendix Figure 1. These 100% bar plots show the percentage and the progression downstream of functional feeding groups in each of the reference streams. Reference streams had high percentages of shredders, moderate percentages of collectors, small percentage of scrapers, and fluctuating percentages of predators.



Appendix Figure 2. These 100% bar plots show the percentage and the progression downstream of functional feeding groups in each impacted site. Impacted streams had low percentages of shredders, extreme percentages of collectors, moderate percentages of scrapers, and fluctuating percentages of predators.



Appendix Figure 3. The 100% bar graphs show the percentage and progression of Ephemeroptera, Plecoptera, Trichoptera, and other taxa in each reference site. Plecoptera primarily dominated reference streams. Ephemeroptera, Trichoptera, and Other Taxa were found in moderate amounts at most reference sites, but dominated a few sites.



Appendix Figure 4. The 100% bar graphs show the percentage and progression of Ephemeroptera, Plecoptera, Trichoptera, and other taxa in each impacted site. Ephemeroptera, Trichoptera, and Other Taxa dominated most impacted sites, while Plecoptera were typically found in low abundance.

Vita

Kelli Alexandra Park was born in Warrenton, Virginia, to Debbie and Tom Park. She graduated from Fauquier High School in June of 2012 and enrolled at James Madison University in the fall. In the summer of 2014, she interned in a water chemistry lab at a wastewater treatment facility in Woodbridge, Virginia. She also interned at the Virginia Department of Environmental Quality over the summers of 2015 and 2016 in Harrisonburg, Virginia. Kelli earned a Bachelor of Science in Biology concentrating in Ecology and Environmental Science as well as earning a minor in Environmental Management upon graduating in May of 2016. After graduation and her internship, she moved to Boone, North Carolina, to attend Appalachian State University and work in an Ecotoxicology lab. Kelli earned her Master of Science degree in Biology upon graduation in May 2018.

In her free time, Kelli is an avid equestrian. Her horse, Lily, has been by her side since becoming part of the family in 2006 and was boarded nearby to both James Madison University and Appalachian State University. Kelli also enjoys hiking, music, shopping, and good craft beer.