EVALUATING INVERTEBRATE COMMUNITY DIVERSITY USING EDNA FOR BIOMONITORING IN FRESHWATER ECOSYSTEMS

by

Rachael Mae Rowe

A thesis submitted to the faculty of
The University of North Carolina at Charlotte
in partial fulfillment of the requirements
for the degree of Master of Science in
Earth Sciences

Charlotte

2023

Approved by:

_____
Dr. Sandra Clinton

_____
Dr. Sara Gagné

_____
Dr. Adam Reitzel

ABSTRACT

RACHAEL MAE ROWE. Evaluating invertebrate community diversity using eDNA for biomonitoring in freshwater ecosystems. (Under the direction of Dr. SANDRA CLINTON).

Traditional invertebrate biomonitoring has low taxonomic resolution, low accuracy, and is plagued with human error and biases. Molecular monitoring, using environmental DNA (eDNA), is a practical alternative due to the high accuracy, lack of human error influencing results, and the potential for standardizing techniques across systems. This study evaluates molecular monitoring by using accumulation curves from temporally distributed samples across an impervious cover gradient, comparing traditional samples processed by Charlotte-Mecklenburg Storm Water Services to molecular samples, and eDNA sampling to quantify invertebrate communities across uncommonly monitored freshwater systems including beaver ponds, beaver wetlands, restored streams, and tribally protected Catawba Nation Reservation streams. Molecular monitoring of sites resulted in a total of 14 phyla compared to 3 phyla from traditional monitoring. Traditional versus molecular communities were significantly different (PERMANOVA, df = 1, p = 0.001). Molecular samples had greater dissimilarity in community structure compared to traditional samples. Distribution of samples based on percent impervious cover were similar across traditional and molecular samples, implying differences between sampling types does not misconstrue community structure. Lastly, multi-site type analysis revealed invertebrate community structure differences among beaver ponds, restored streams, and Catawba Nation streams at the order (PERMANOVA, df =3, p = 0.042) and family (PERMANOVA, df = 3, p = 0.043) levels. The results of this study are an indicator that molecular biomonitoring using eDNA for invertebrate communities is a successful, and more efficient, alternative to traditional biomonitoring.

ACKNOWLEDGEMENTS

I would like to extend the following thank you notices to all the individuals and organizations that supported this work. Thank you to my committee for support in developing and completing this study: Dr. Sandra Clinton, Dr. Sara Gagné, and Dr. Adam Reitzel; the Reitzel lab for running gel electrophoresis; Bill Taylor and UNC Charlotte Department of Bioinformatics for sequencing; the Catawba Nation Environmental Services (Scott Hansen, Tiffany Witherington, and Marvin Bouknight) for access to sites and sampling assistance; Charlotte Mecklenburg Storm Water Services (Tony Roux, Matthew Phillips, and Olivia Edwards) for site access, environmental data, and traditional sample processing; Jamil Millner, Emma Dyson, Jordan Landis, and Stephen Rowe for sample collection assistance; University of North Carolina at Charlotte Department of Geography & Earth Sciences for supporting this research and providing laboratory space; the McCreary family for financial support through the McCreary Memorial Award.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

SECTION 1: INTRODUCTION

1.1 Molecular Ecology

Molecular ecology is the unification of field work and lab work to provide a detailed understanding of natural processes on a microscopic scale. Molecular ecology tends to focus on DNA and RNA analysis, with the prominent community tier technique using environmental DNA. Environmental DNA, known as eDNA, is the heterogenous mixture of DNA not attached to an organism in an environmental matrix (Barnes et al. 2014). eDNA is more spatially integrated than individual organisms, implying that eDNA is present throughout a habitat (Blackman et al. 2019). Most eDNA studies have focused on microbial work, but since 2010 there has been an increase in quantifying community level dynamics (Thompson et al. 2017; Mathieu et al. 2020). eDNA can provide accuracy and precision where visual analysis fails, such as with degraded or damaged specimens (Nørgaard et al. 2021). eDNA is a viable solution to rapidly evaluate distribution changes among populations, which is increasingly important with climate change (Tsyrlin et al. 2022). eDNA is effective even in low densities of target taxa (Tsyrlin et al. 2022).

A common concern within eDNA analysis is how long DNA will last in an environment (Barnes et al. 2014). Based on lab-based analysis, lentic eDNA does not become detectable until approximately seven days, but lotic eDNA is detectable within the first 48 hours (Trimbos et al. 2021). This represents the initial detection, while post removal of organisms followed a trend of minimal detection within 14 days (Trimbos et al. 2021). Lab based work has more control than natural systems, but the DNA presence is much more likely to be shorter in situ since eDNA is degraded by solar radiation, extracellular enzymes, and pH deviations (Barnes et al. 2014). Sediments can preserve DNA molecules through a binding process allowing it to last longer than

in water; however, sediment sampling provides low yields of non-microbial DNA(Barnes et al. 2014; Pawlowski et al. 2022).

1.2 Biomonitoring

Multiple studies support eDNA as a macroinvertebrate biomonitoring technique (Lim et al. 2016; Hering et al. 2018; Carew et al. 2021; Reinholdt Jensen et al. 2021; Aunins et al. 2023). Traditional sampling is disruptive to ecosystems while eDNA is minimally invasive (Hering et al. 2018). eDNA has higher taxonomic resolution and has a higher accuracy than traditional sampling (Lim et al. 2016; Blackman et al. 2019; Nørgaard et al. 2021; Reinholdt Jensen et al. 2021). Having high accuracy and precision allows for eDNA to provide a detailed community structure. Identification during the traditional biomonitoring process is also highly affected by human error (Haase et al. 2010). Traditional biomonitoring requirements for aquatic invertebrates lack taxonomic resolution or require differing levels, which complicates analyses and does not provide the full picture in terms of community structure, and samples are limited by organism size in collection (Birk et al. 2012; Reinholdt Jensen et al. 2021; Keck et al. 2022). Freshwater ecosystems are highly variable in size, structure, and flow, which leads to multiple traditional sampling techniques creating a lack of standardization across methods (Bonada et al. 2006; Birk et al. 2012). Consequently, most metrics, such as diversity and richness, are reliant on sampling technique (Barton and Metcalfe-Smith 1992).

Despite the dominance of traditional biomonitoring, these methods fall short when looking at cryptic, invasive, and undescribed species. In contrast, eDNA is capable of detecting cryptic species and invasive species (Hering et al. 2018; Reinholdt Jensen et al. 2021). Early detection of invasive species through molecular monitoring is vital for eradication and control

(Dejean et al. 2012; Goldberg et al. 2013; Keskin 2014). Traditional sampling of important taxa, such as chironomids or other indicator groups, presents issues with identification, the taxa traits may differ only slightly, along with the issue of cryptids being difficult to identify within these already diverse groups (Czechowski et al. 2020; Campbell et al. 2022). There are limitations on studies done in most regions of the world, meaning that morphological identification would be starting with minimal background information, which would be difficult when encountering undescribed taxa (Czechowski et al. 2020). Traditional identification usually involves a dichotomous key which is generally focused to region or taxa. When encountering an invasive species or undescribed species, this allows for the misrepresentation of taxonomic composition of a community. Due to anthropogenic influences, multiple species have been assumed to have gone extinct or extirpated from their range as well, but eDNA can provide evidence of the opposite, allowing for future protections to be implemented where failure was seen previously (Lim et al. 2016).

Molecular techniques also come with analytical challenges. First eDNA methods have multiple biases. A single DNA molecule can provide a positive hit for an organism in an environment, despite being non-representative of what is in the system, called a false positive (Hering et al. 2018). The non-detection of a present species is a false negative. Traditional sampling has a higher rate of false negatives compared to the rate of false positives in eDNA analysis, and data processing can eliminate false positives by using quality control methods such as requiring a certain pairwise identity to be approved (Burian et al. 2021). Invertebrate eDNA has been shown to be limited in the range of the species, with a vast number of invertebrates not sequenced or officially classified, lowering the chances of false positives (Tsyrlin et al. 2022).

Second, the use of eDNA to calculate abundance has been an ongoing focus of research and discussion. Multiple studies claim eDNA cannot provide relative abundance due to issues like primer bias (Piñol et al. 2014). While earlier eDNA work was not suitable for abundance measurements, recent development of primers allows for abundance to be considered (Yates et al. 2019). Primer bias is low with the BF2/BR2 combination (planned for use in this study) due to the development targeting broad ranges of arthropods and equal amplification (Elbrecht and Leese 2017; Reinholdt Jensen et al. 2021). Finally, standardizing any biomonitoring technique is not possible due to the wide variety of existing taxa and ecosystem conditions. A one-size-fits-all method is not applicable on a planet thriving in diversity. Despite the lack of an overall standardization, classifying eDNA methods based on target taxa and sample type allows for repeatable methods (Thalinger et al. 2021). Traditional sampling of macroinvertebrates varies based on environment, which can account for the difference between results of diversity whereas eDNA sampling can provide comparable data between ecosystem types (Hering et al. 2018).

One of the reasons for current biomonitoring to move away from biologic analysis is cost and time. Despite using newer technology and requiring more materials, eDNA is significantly cheaper compared to traditional monitoring (Hering et al. 2018; Burian et al. 2021; Carew et al. 2021). Traditional identification to family level is 1 to 3 times the cost of using metabarcoding to species level (Carew et al. 2021). To obtain species level, the cost can range from 6 to 18 times the cost per sample compared to metabarcoding (Carew et al. 2021). Molecular analysis is faster than traditional sampling due to the incorporation of technology, which is becoming available at rapid rates, and the time of training (Hering et al. 2018). The caveat of this method is the existence of databases to compare sequences, but these are adding new sequences every day. Molecular techniques are similar in process, and the protocols generally do not require advanced

knowledge and training compared to traditional sampling and identification procedures (Hering et al. 2018).

1.3 Transition to eDNA Biomonitoring

The transition to using eDNA for biomonitoring began in microbiology. In 2008, the first study of metazoan eDNA detection was published, using an in-lab control and known populations of bullfrogs, assessing the capability of DNA technologies to detect DNA in natural samples (Ficetola et al. 2008). After this, eDNA usage increased dramatically in freshwater.

Amphibians are sensitive to environmental disturbances, including distress from sampling. Using non-invasive eDNA sampling, amphibian biomonitoring is successful in detecting key species indicating stream health (Goldberg et al. 2011). Studies in fisheries management have used eDNA to successfully monitor rare species and protect habitats (Jerde et al. 2011). Damage from invasive species, including the Asian Carp in the Great Lakes and American Bullfrog in France, can be limited by early detection from eDNA (Jerde et al. 2011; Dejean et al. 2012; Goldberg et al. 2013; Keskin 2014).

Macroinvertebrates have been monitored via traditional sampling for a long period of time and are able to be sampled routinely. Due to the difficulties of monitoring vertebrates, the initial eDNA jump did not focus on already established monitoring of invertebrates, despite the lack of studies looking at meiofauna as well. As of 2014, there has been a shift to focusing on macroinvertebrates. Rare and bioindicator species have been detected via eDNA and confirmed with traditional sampling (Mächler et al. 2014). Diversity estimates of macroinvertebrate communities using eDNA have been successful, which provides an even greater understanding of freshwater ecosystem health (Sweeney et al. 2011; Stein et al. 2014; Deiner et al. 2016).

Molecular monitoring is a staple of freshwater biomonitoring in the age of information and big data, contributing to the goals across the world of collecting as much information as possible (Keck et al. 2017). This study will provide a basis of using molecular methods for biological monitoring in freshwater ecosystems using invertebrates as a model group. Biologic monitoring complements chemical and geomorphological monitoring by connecting the structure and function of an ecosystem.

1.4 Invertebrates

Aquatic invertebrates can be defined as animals without a backbone and live at least one life cycle stage in an aquatic environment. Macroinvertebrates are visible to the naked eye, usually consisting of arthropods, mollusks, and worms in freshwater environments. Most macroinvertebrates are insects, in which 12 orders have larval or adult stages in freshwater environments (Cardé and Resh 2009). Macroinvertebrates are the primary target of traditional sampling due to their size and visibility, but molecular monitoring can simultaneously monitor meiofauna, such as rotifers and tardigrades, as well. Invertebrates are vital to ecosystem structure by providing a base of the food web, contributing to multiple ecosystem processes and are contributors to stream function and stability (Wallace and Webster 1996).

Invertebrates have been used as a biological indicator for over 100 years in freshwater ecosystems (Cairns and Pratt 1993). Considering the ubiquity and abundance of invertebrates, they are one of the easier groups to use as a model for stream function (Wallace and Webster 1996; Birk et al. 2012). This is true for molecular methods as well, as invertebrates provide a substantial number of potential specimens, without the complex gene transfer and diversity present in microbial work (Birk et al. 2012; Thompson et al. 2017; Blackman et al. 2019).

Macroinvertebrates are the best representative for traditional biomonitoring techniques across the world due to longer lifespans compared to microorganisms, low mobility, and variety of tolerance to environmental factors (Buffagni et al. 2001). Their range of tolerance indicates that when a taxon is no longer present, stressors such as pollution are present in the stream (Gerhardt 2009; Deborde et al. 2016). Multiple macroinvertebrates are used as bioindicators for stream health, with increasing importance on EPT taxa (Ephemeroptera, Plecoptera, and Trichoptera). EPT taxa are an invaluable bioindicator due to their intolerance to any pollution (Ab Hamid and Md Rawi 2017). While not commonly monitored, multiple groups of meiofauna are sensitive to specific stressors, implying they would be an excellent bioindicator as well (Balsamo 1980; Dahms et al. 2011).

SECTION 2: RESEARCH QUESTIONS AND HYPOTHESES

My overall objective was to quantify and evaluate eDNA as a biomonitoring method in freshwater ecosystems.

1. *How does invertebrate community structure differ with successive eDNA sampling?*

H_{a1}: I hypothesize that increasing temporal sampling of eDNA will increase invertebrate richness since streams have a high number of rare species that are not detected frequently (Sgarbi and Melo 2017).

H_{01}: Increasing temporal sampling of eDNA will not increase invertebrate richness.

2. *How do invertebrate communities from eDNA samples compare to traditional sampling in regularly monitored streams in Mecklenburg County, North Carolina?*

H_{a2}: I hypothesize that invertebrate communities from eDNA sampling will have higher richness than those from traditional sampling since eDNA can detect all invertebrates while traditional sampling can only identify macroinvertebrates.

H_{02}: eDNA developed invertebrate communities will be the same as those from traditional sampling.

3. *How do invertebrate communities vary across uncommonly monitored freshwater ecosystem types?*

H_{a3}: I hypothesize that Catawba Nation streams will have higher diversity metrics compared to beaver ponds and wetlands due to the consistent mixing of water in lotic ecosystems and Catawba Nation streams will have higher diversity metrics compared to restored streams due to limits on macroinvertebrate dispersal in urban environments (Brederveld et al. 2011; Blackman et al. 2019).

H_{03}: There will be no difference between diversity metrics in eDNA samples across sites.

SECTION 3: METHODS

3.1 Site Selection

3.1.1 Temporal Sampling

Ten sites were chosen from stream sites monitored for macroinvertebrates by Charlotte-Mecklenburg Storm Water Services in Mecklenburg County, NC (Figure 1). These ten sites were chosen since they span a range of impervious surface cover while maintaining similar drainage size (Table 1). Impervious cover data provided by Charlotte-Mecklenburg Storm Water Services. During the sampling period, all ten sites were sampled 5 times each at randomized intervals between 20 June and 9 August 2022. Environmental data for these samples were retrieved via the CMANN network (https://cmann.mecknc.gov/).

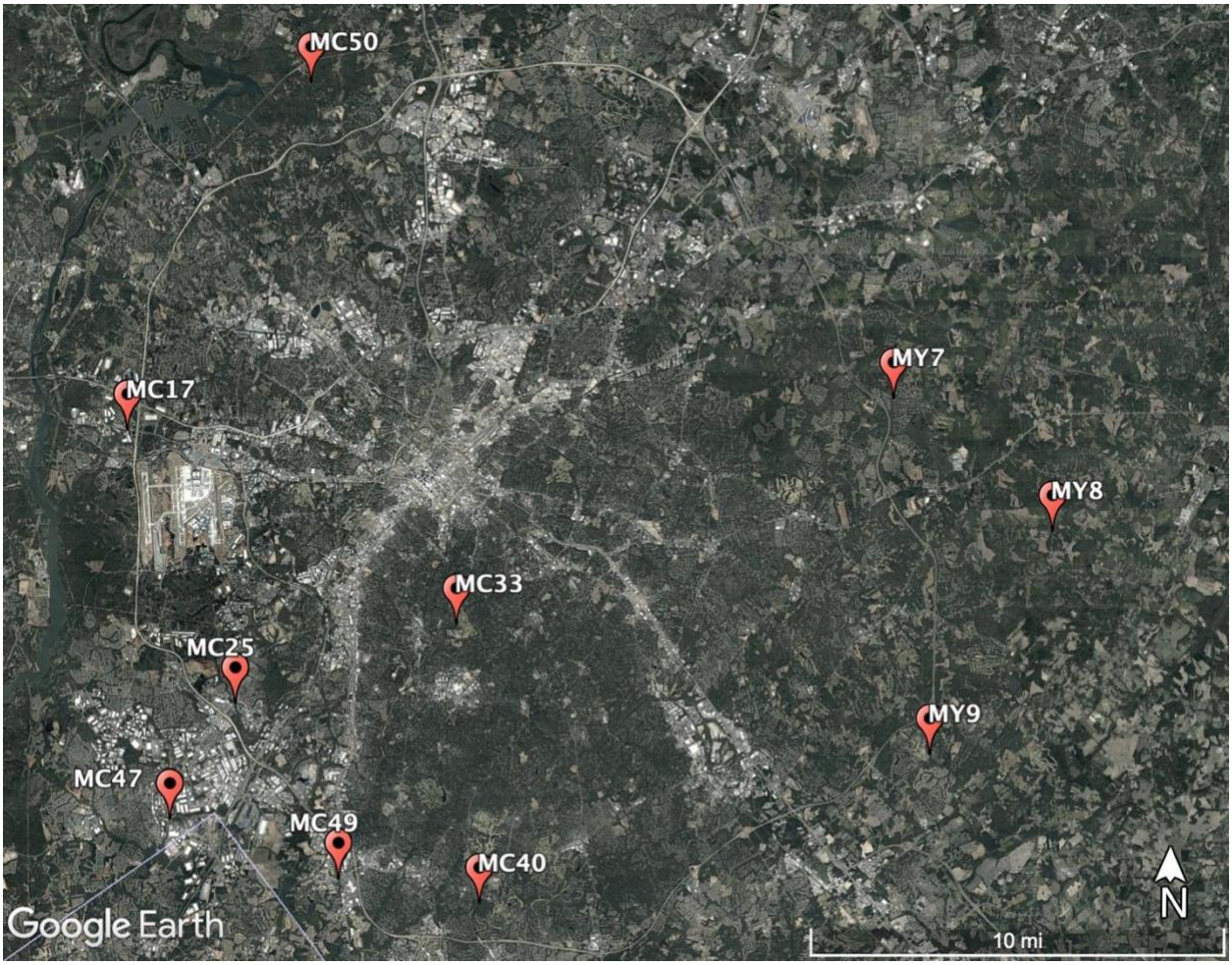

Figure 1. Location of sites in Mecklenburg County sampled for temporal sampling using molecular methods and compared to traditional sampling.

Table 1. List of County Sites sampled with molecular methods and their corresponding percent impervious cover. Adjusted ID represents a simplified version of County ID for this study only used to reference sites. Sampling year is the year the traditional taxonomic data was collected. Impervious cover data provided by Charlotte-Mecklenburg Storm Water Services.

Site Name	County ID	Adjusted ID	% Impervious Cover	Sampling Year
McKee Cr	MY7B	MY7	45.82	2021
Clear Cr	MY8	MY8	26.60	2022
Goose Cr	MY9	MY9	38.78	2021
Paw Cr	MC17	MC17	78.19	2021
Coffey Cr	MC25	MC25	59.75	2022
Briar Cr	MC33	MC33	96.18	2021
Fourmile Cr	MC40A	MC40	78.86	2021
Steele Cr	MC47A	MC47	77.65	2022
Little Sugar Cr	MC49A	MC49	96.50	2020
Gar Cr	MC50	MC50	13.70	2022

3.1.2 Traditional versus Molecular

The same samples used for temporal sampling were also used to compare the molecular method to traditional biomonitoring. These samples were treated as both individuals & summative per site. Macroinvertebrate samples were collected, processed, and identified to the lowest taxonomic rank possible by Charlotte Mecklenburg Storm Water Services on various days throughout the sampling period. One traditional sample was taken per site. Due to county schedules and time restraints, some traditional sample data was used from the previous year (Table 1).

3.1.3 BioBlitz

Uncommon types of sites that are typically not monitored for invertebrates were sampled once per site across the sampling period, between 20 June and 8 August 2022, for analysis using molecular methods. Site types included beaver ponds, beaver wetlands, restored streams, and tribal-protected streams under the Catawba Nation Reservation (Figure 2). These sites collectively are termed “BioBlitz”.

3.1.3.1 Beaver Ponds (n = 3)

Samples in this study were collected from an accessible edge of Castor canadensis (North American Beaver) ponds. These sites were highly established with multiple ponds and dams.

3.1.3.2 Beaver Wetlands (n = 3)

Samples in this study were collected from a central point of intermittent Castor canadensis (North American Beaver) wetlands. These sites were highly established with multiple ponds and dams.

3.1.3.3 Restored Streams (n = 7)

Streams were restored between 2018 and 2023 across Charlotte, NC.

3.1.3.4 Catawba Nation Streams (n = 6)

Streams are under ownership of the Catawba Nation Indian Reservation, York County, SC. All streams are low-flow streams in the Catawba river watershed. Streams are considered natural rather than rural or urban due to the maintenance and protection established by the Reservation.

Figure 2. Distribution of sites for each site type across their relative areas. (a) Catawba Nation streams, (b) restored streams, (c) beaver ponds & wetlands.

3.2 Molecular Sample Collection

3.2.1 Sterilization

All bottles, filter funnels, and forceps used in sampling & processing were sterilized following the protocol by Genidaqs (Blankenship and Schumer 2017). Items were sterilized using a 20% bleach soak for a minimum of 2 minutes. Bleach was fully rinsed using DI water. Finally, items were sprayed with 70% ethanol and allowed to dry in a sterile environment.

3.2.2 Collection

Water sample collection was based partially on Laramie et al. (2015). Sampling was conducted during dawn hours to avoid excess UV exposure to samples and the sampler. Water was collected from an accessible point in a portion of sites to fully submerge the bottles. Bottles were capped underwater and immediately placed in a dark cooler on ice. Samples were stored in the dark at 4°C for a maximum of 5 days (Djurhuus et al. 2017).

3.2.3 Filtration

Samples were filtered using a Pall filter funnel attached to a vacuum pump on 0.45 μM, 47 mm sterile cellulose nitrate filters. Two filters were used per sample, with 500 mL of water per filter to reach targeted volumes (Mächler et al. 2016).

3.3.4 Extraction

After filtration, DNA was extracted using either Qiagen DNeasy PowerWater Kit or NucleoSpin water eDNA kit. Extraction quality and DNA concentration was measured using a Nanodrop. Differences between kit quality were assessed for influence on quality and were negligible via t-test of the quality control variable A260/A280 ($p > 0.05$).

3.3.5 Amplification

Amplification was completed by polymerase-chain reaction using the BF2/BR2 primer (GCHCCHGAYATRGCHTTYCC/ TCDGGRTGNCCRAARAAYCA) and methods described by Elbrecht and Leese (2017). All samples received 125 ng of sample DNA and 1 ng of marine snail, the Common Periwinkle (*Littorina littorea*), DNA as a quality control during sequencing. Target amplicon size (400 – 500 bp) was confirmed using gel electrophoresis.

3.3.6 Purification & End Prep

Purification of samples was completed using magnetic beads and end-prep as prescribed by Oxford Nanopore Technologies.

3.3.7 Sequencing

Sequencing was conducted on an Oxford Nanopore Technologies PromethION 24. Sequences were demultiplexed & paired end reads were merged by the sequencing technician.

3.3.8 Processing sequences

Sequence processing was completed in Geneious Prime 2022.2.2 (https://www.geneious.com). Sequences were corrected and normalized using BBNorm, primers were trimmed, and low-quality reads (PHRED < 40) were removed. Remaining sequences were compared to the NCBI (https://www.ncbi.nlm.nih.gov/) Nucleotide database using MegaBLAST to obtain organism name and accession ID with a maximum e-score of 1e-6. Alignments were filtered to remove bit scores lower than 40, and query coverages less than 80%. Organism names were normalized using the Species matching tool by Global Biodiversity Information Facility (https://www.gbif.org/) to obtain taxonomic information. Names representing no match or multiple matches had taxonomic information verified by accession ID. To ensure up to date and valid taxonomy, all names were compared to the Integrated Taxonomic Information System

(https://www.itis.gov/). Reads matching to kingdom Metazoa (Animalia) were isolated. For taxa that were missing level classifications (i.e., not having an order classification but having a family level classification) I used the taxonomic level above the missing grouping to ensure the organism was included in metrics. Reads matching to phylum Chordata were removed. Reads matching to *Littorina littorea* were counted and removed. Reads of *L. littorea* were treated as presence/absence indicator of successful sequencing. Unsuccessful sequencing runs were not used in taxonomic analysis.

3.4 Data Analysis

Relative abundance was calculated as organism reads per total sample invertebrate reads. Relative abundance tables were created for the order, family, and species taxonomic levels. All data analysis were conducted in R version 4.2.2 (R Core Team 2022). Richness (R), Shannon's Diversity Index (H'), and Pielou's Evenness Index (J') were calculated using R package vegan (Oksanen et al., 2022). Shapiro's test of normality was applied to all numeric data to determine normality (normal if $p > 0.05$). Normal data will undergo parametric statistics while non-normal will use non-parametric statistics.

Environmental data from the CMANN network (https://cmann.mecknc.gov/) were averaged over the 24-hour period prior to the sampling date & time for all temporal samples. Metrics included specific conductivity, dissolved oxygen, turbidity, water temperature, discharge, and pH. Results exclude one sample of MY8 due to damage caused to the sensor.

3.4.1 Statistical Analysis and Multivariate Analysis

Results of the Shapiro's test indicate that the data is not normally distributed. All significance tests used were non-parametric.

3.4.1.1 Correlation

R^2 and p-values were calculated using linear regression modeling using Spearman's correlation between diversity metrics, environmental data, and quality control metrics to determine any predictor relationships between sample characteristics and post-sequencing metrics.

3.4.1.2 Assessment of temporal sampling

Temporal samples were fit to a logarithmic function of sample count versus richness to develop curves. Diversity metrics were also plotted on a box plot by site using R package ggplot2 (Wickham 2016).

3.4.1.3 Assessing traditional versus molecular methods

Diversity metrics of individual molecular samples were plotted on a box plot and differences were determined via Kruskal-Wallis tests and post-hoc Dunn's test. Non-metric dimensional scaling (NMDS) plots were developed for (1) traditional sample similarity and (2) summative molecular sample similarity and assessed individually via PERMANOVA. Select significant taxa ($p < 0.05$) were plotted as well. Taxa selection was based on commonly known or recognized groups. NMDS plots were also developed comparing traditional sampled communities to (1) all temporal samples and (2) summative county samples. Significance was tested using PERMANOVA.

3.4.1.4 Assessment of BioBlitz sites

Diversity metrics of site types were plotted on a box plot and differences were determined via Kruskal-Wallis tests and post-hoc Dunn's test using R package dunn.test (Dinno 2017). NMDS plots were developed based on community data and assessed using a PERMANOVA.

SECTION 4: RESULTS

The count of quality sequences, which are sequences that passed the quality filters described in methods before being filtered for target taxa, totaled at 47,738 reads. Target taxa sequences (excluding *L. littorea*) composed of 1,723 reads. Targeted taxa reads included 14 phyla, 35 classes, 106 orders, 383 families, 579 genera, and 687 species. Fifty-three percent 53% of reads were in phylum Arthropoda, 13% in Mollusca, and 11% in Annelida. Twenty-seven percent of reads were in class Insecta, 9% in Arachnida, 8% in Hydrozoa, and 8% in Gastropoda. Thirteen percent of reads were in order Diptera, 6% in Ploima (Rotifer), 6% in Cyclopoida (Copepod), and 6% in Tubificada. The most abundant family was Chironomidae at 7% of reads, followed by Naididae at 6% of reads. The most abundant species was *Corbicula fluminalis* with 3.25% of all reads.

Not all reads were capable of identification to the species level, likely due to the condition of the eDNA and the availability of species-level sequence data. 98.96% of reads were classified to the class level, 96.92% to the order level, 93.27% to the family level, 79.92% to the genus level, and 54.50% to the species level. Reads identified to genus but not species were listed as *Genus sp.* to allow comparisons. The relationships between level of identification skew comparing richness and diversity metrics across taxonomic level.

Phyla detected molecularly were Annelida, Arthropoda, Byozoa, Cnidaria, Gastrotricha, Hemichordata, Mollusca, Nematoda, Nemertea, Onychophora, Platyhelminthes, Porifera, Rotifera, and Tardigrada. Phyla detected traditionally were Annelida, Arthropoda, and Mollusca. Molecular monitoring recovered 4.7x more phyla than traditional monitoring.

4.1 Correlation

Shapiro's test of normality applied to all numeric metrics supported using non-parametric tests ($p < 0.05$). Counts of quality sequences (whether invertebrate or not) were deemed normal ($p = 0.19$) but were treated as non-normal due to being tested against non-normal variables. All correlations were done using Spearman's Rho.

Correlation analysis, completed to identify predictor relationships, of non-diversity variables supported the counts of quality sequences to be significantly correlated with sample quality measured as A260/A280 ($R^2 = -0.20$, $p = 0.02$). Counts of quality sequences were also significantly correlated with percentage impervious cover in temporal sites ($R^2 = -0.44$, $p = 0.002$). In terms of environmental factors, counts of quality sequences were correlated with specific conductivity in μS ($R^2 = -0.35$, $p = 0.013$), dissolved oxygen in mg/L ($R^2 = 0.46$, $p = 0.000803$), and water temperature in °C ($R^2 = -0.46$, $p = 0.000994$). Specific conductivity and water temperature were positively correlated with A260/A280 ($R^2 = 0.31$, $p = 0.0294$; $R^2 = 0.3$, $p = 0.0394$ respectively).

Diversity metrics across levels had multiple correlated factors. Diversity metrics and environmental factor significant correlations are presented in Table 2. Sequence count was correlated with all diversity metrics at all levels (Figure 3). Similarly, percent impervious cover was correlated with order richness, family evenness, and diversity at all levels (Figure 3).

Table 2. Significant Spearman's Rho correlations between diversity metrics and environmental factors.

Metric	Factor	R2	p-value
Order Richness (S)	Water Temperature (°C)	-0.4	0.00489
Family Richness (S)	Water Temperature (°C)	-0.36	0.0104
Order Diversity (H)	Specific Conductivity (µS)	-0.53	7.53E-05
	Dissolved Oxygen (mg/L)	0.56	2.41E-05
Family Diversity (H)	Specific Conductivity (µS)	-0.52	0.000126
	Dissolved Oxygen (mg/L)	0.56	3.31E-05
Species Diversity (H)	Specific Conductivity (µS)	-0.51	0.00021
	Dissolved Oxygen (mg/L)	0.54	5.76E-05
Order Evenness (J)	Specific Conductivity (µS)	-0.58	1.10E-05
	Dissolved Oxygen (mg/L)	0.61	2.70E-06
Family Evenness (J)	Specific Conductivity (µS)	-0.58	1.45E-05
	Dissolved Oxygen (mg/L)	0.61	3.34E-06
Species Evenness (J)	Specific Conductivity (µS)	-0.54	5.22E-05
	Dissolved Oxygen (mg/L)	0.6	6.02E-06

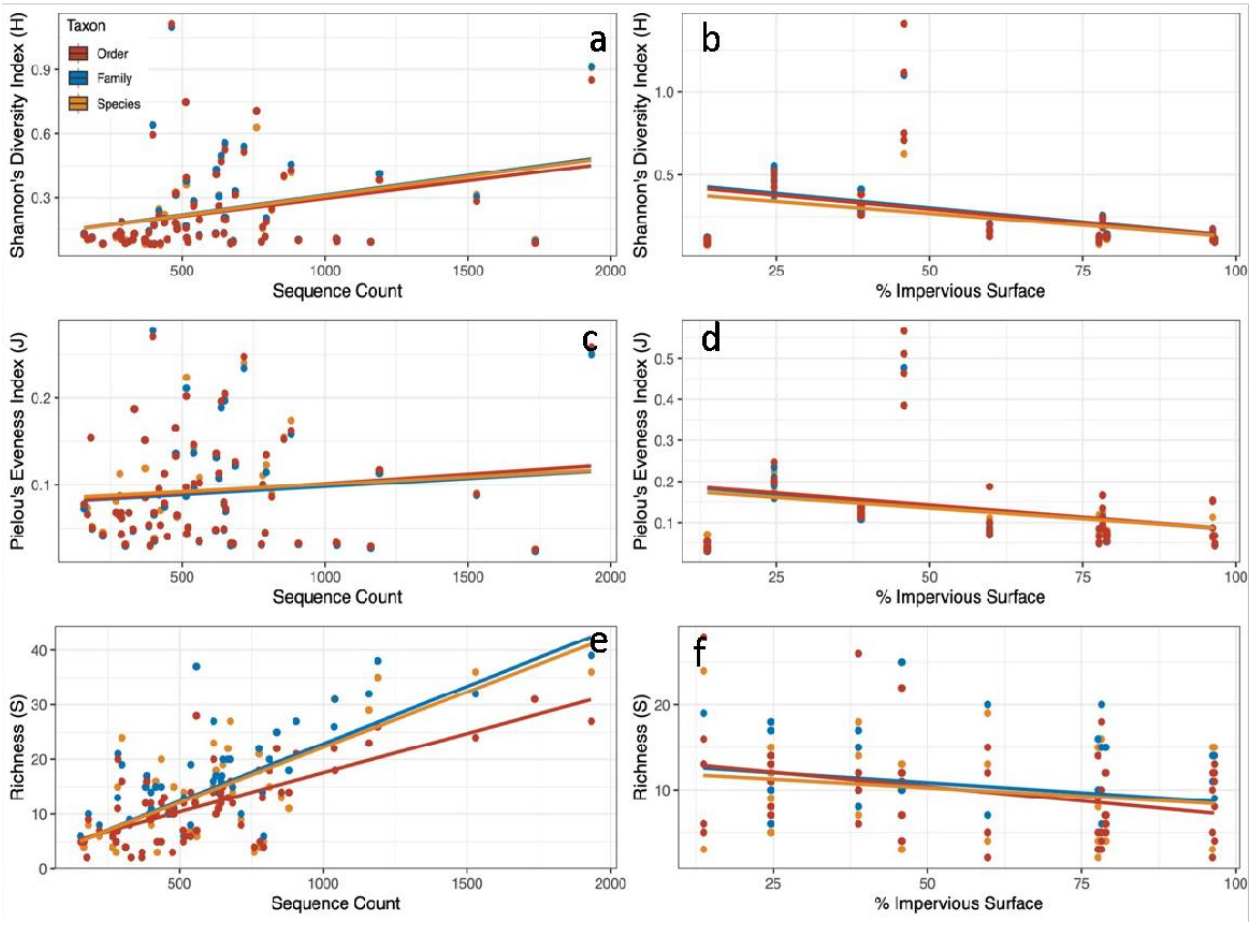

Figure 3.. Linear regression models of relationships between diversity metrics and sequences count or percent impervious surface. (a) Shannon's Diversity Index vs. Sequence Count, (b) Shannon's Diversity Index vs % Impervious Surface, (c) Pielou's Evenness Index vs. Sequence Count, (d) Pielou's Evenness Index vs. % Impervious Surface, (e) Richness vs. Sequence Count. (f) Richness vs% Impervious Surface.

4.2 Temporal samples

4.2.1 Accumulation curves

As shown in Figure 4, there is a relationship between the curve plateau, representing theoretical maximum richness based on number of samples and percent impervious surface (R2 = -0.39, p = 0.03144). Maximum projected richness is aligned with the first quartile of impervious surface (Site MY9, S = 157, %IS = 0.3878) while minimum projected richness is aligned with the third quartile of impervious surface (Site MC40, S = 35, %IS = 0.7886).

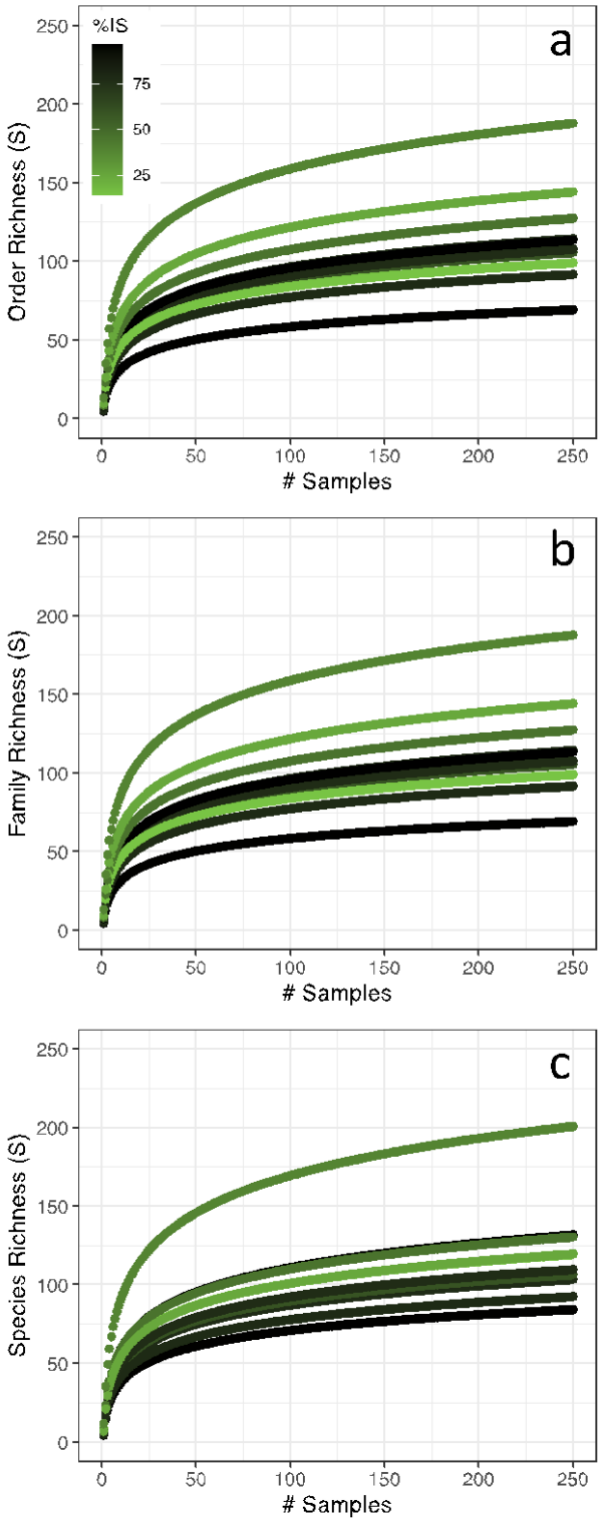

Figure 4. Projected accumulation curves for county sites based on molecular sampling. Color represents percentage of impervious surface in each watershed. Curves fitted via log model (sites = 10, n = 50). (a) order level, (b) family level, (c) species level.

4.2.2 Community Structure

Diversity metrics for individual samples were compared among sites based on taxonomic level (Figure 5). At all taxonomic levels, there were significant differences among Shannon's Diversity Index and Pielou's Eveness Index as determined by a Kruskal-Wallis's test. Primarily, MY7 differed from MC40, MC47, MC49 on all levels in both indices, and MY8 differed from MC49 and MC50 (Dunn's Test, $p < 0.05$).

Summative samples were plotted on NMDS for determining similarity to traditional sites, while individual communities were tested via PERMANOVA for significant differences (Figure 6b,d,f). On the order level ($df=9$, $p = 0.044$), MY8 was different from MC17 and MC33, and MC17 also different from MC47. Orders driving this differentiation are Araneae, Diptera, Podocopida, Sarcoptiformes, Venerida, Ploima, Stylommatophora, and Tubificida. On the family level ($df= 9$, $p=0.006$), MC50 was different from all other sites, MC47 differed from MY7, MC17, and MC33, and MY7 differed from MC33. Families driving these differences include Cypridídae, Cyrenidae, Chironomidae, Darwinulidae, Chondrinidae, Brachionidae, Raspailiidae, Aeolosomatidae, Synchaetidae, Cecidomyiidae, Dendronotidae, Pteronyssidae, and Agalmatidae. Finally, on the species level ($df=9$, $p=0.068$) differences were marginally significant, but a further pairwise adonis test showed MC50 differing from MC17, MC25, MY7, and MY8 ($p < 0.05$). Species driving these differences include *Polyarthra dolichoptera*, *Michthyops parva*, *Tanytarsus sp.*, and *Stenostomum sp.*

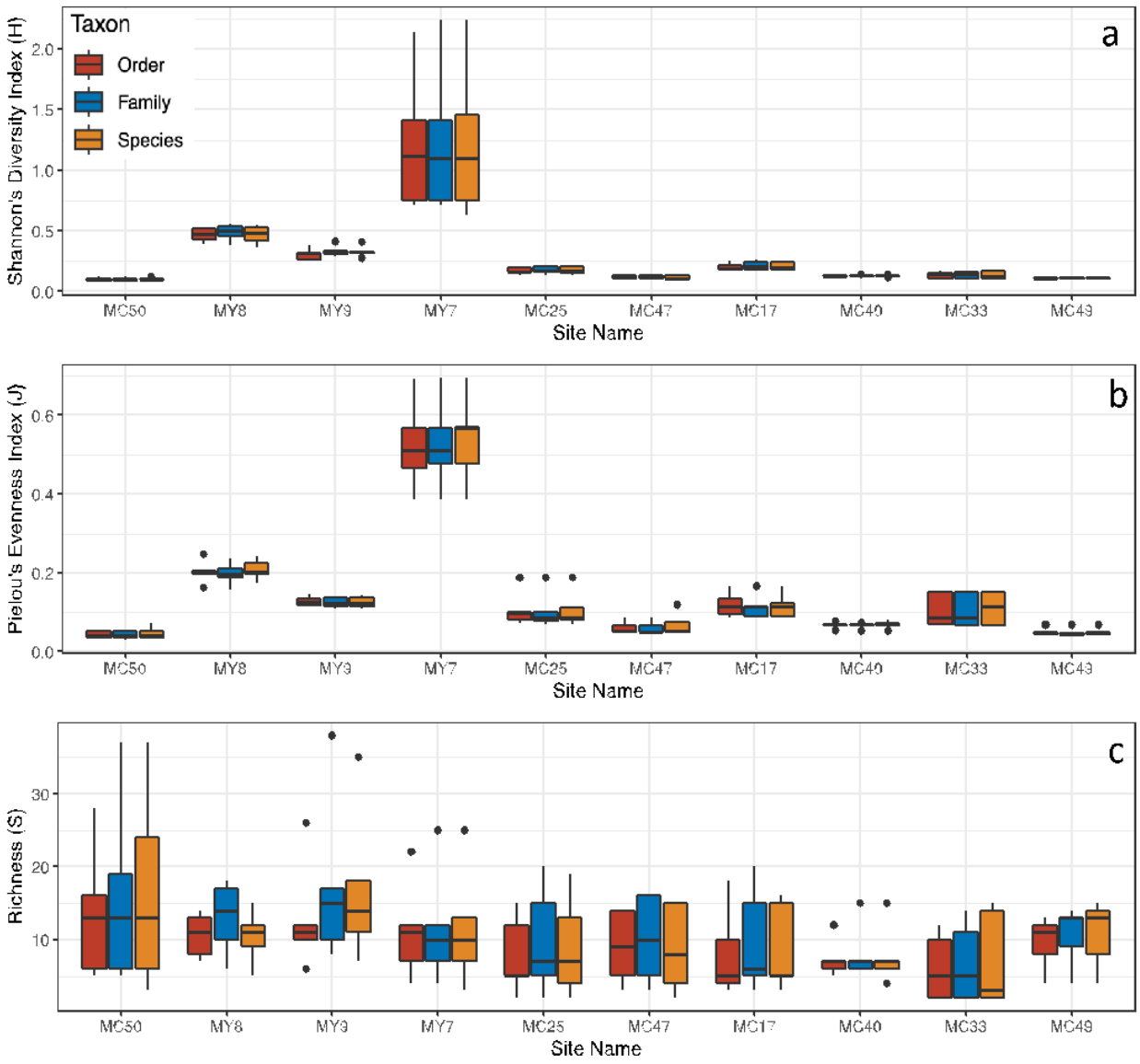

Figure 5. Boxplots comparing diversity metrics between county molecular sites. Kruskal-Wallis results indicate significant differences between sites for Shannon's Diversity Index on the order (df = 9, p =1.042e-06), family (df = 9, p =1.352e-06) and species (df = 9, p =1.769e-06) level. Kruskal-Wallis results indicate significant differences between sites for Pielou's Evenness Index on the order (df = 9, p = 2.54e-06), family (df = 9, p = 3.137e-06) and species (df = 9, p = 8.36e-06) level. (a) Shannon's Diversity Index, (b) Pielou's Evenness Index, (c) Richness.

4.3 Traditional vs Molecular

4.3.1 Community Structure

Traditional communities were compared to each other using NMDS (Figure 6a, c, e). Taxa contributing to differences are plotted, but due lack of replicates for traditional samples, no significant differences were detected between groups, but significant orders include Diptera, Ephemeroptera, and Trichoptera ($p < 0.05$). Significant families include Chironomidae, Heprtageniidae, and Hydrospychidae ($p < 0.05$). Significant species include *Labiobaetis propinquus, Tribelos jucundum, Polypedilum scalaenum, Tanytarsus sp., Cheumatopsyche sp., Stenochironomus sp., Ablabesmyia mallochi, Chironomus sp., Hydropsyche betteni, Cricotopus sp., Zavrelimyia sp., Paracloeodes minutus, Cricotopus bicinctus, Paratendipes sp.,* and *Stenelmis sp.* Visual expression of differences in site similarity based on form are plotted in Figure 6.

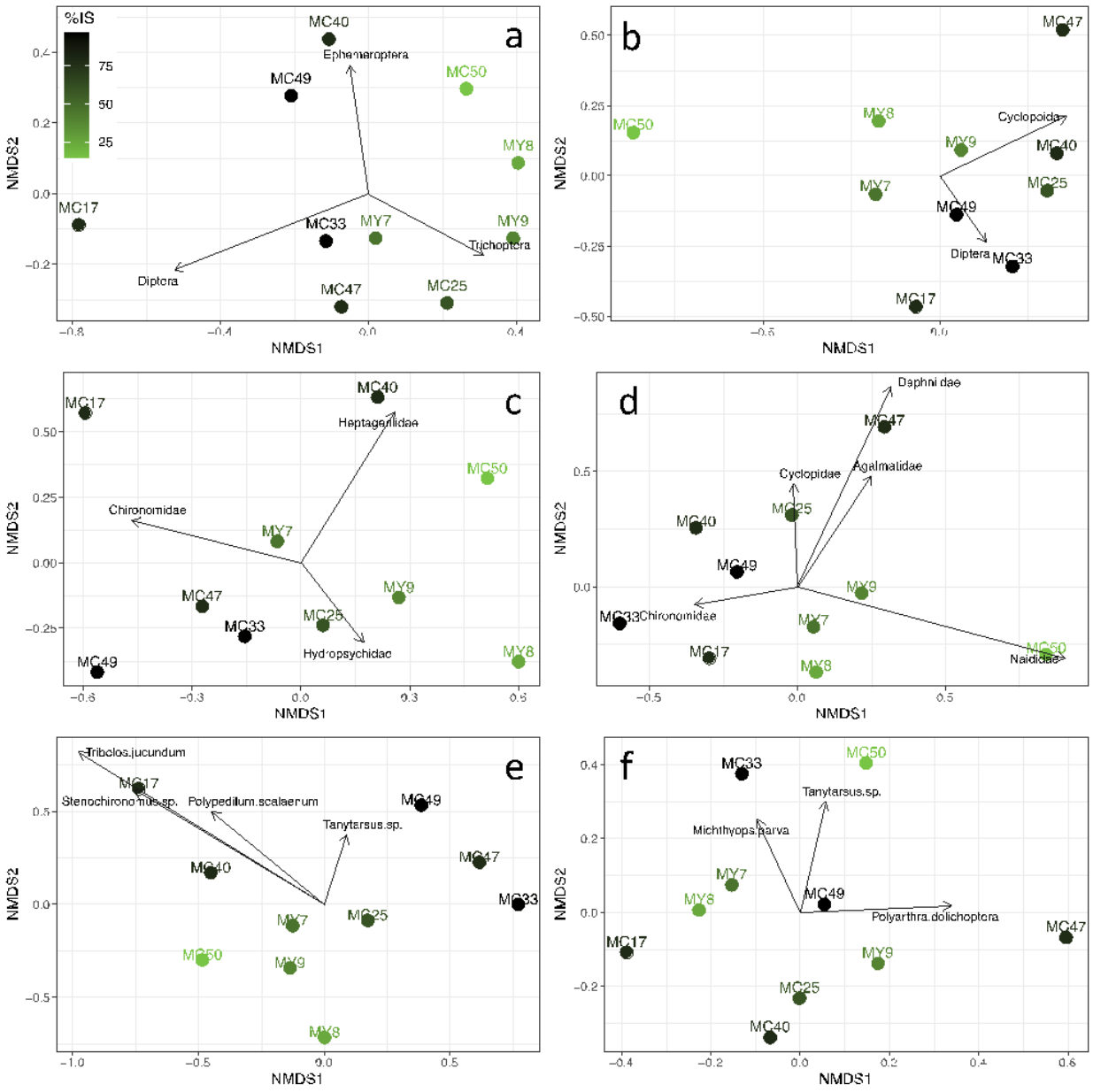

Figure 6. NMDS representing order, family, and species level of molecular samples and traditional samples based on community structure. Arrows represent significant taxa. (a) traditional order level (stress= 0.09), (b) molecular summative order level (stress=0.19), (c) traditional family level (stress=0.08), (d) molecular summative family level (stress=0.17), (e) traditional species level (stress=0.10), (f) molecular summative species level (stress=0.10).

4.3.2 Sample Form

Traditional community structure was compared to both summative county structure and individual communities from molecular samples (Figure 7). PERMANOVA results indicate that communities resolved by traditional sampling are more alike to each other than to molecular samples, which are spread out across the NMDS plot (df = 1, p = 0.001).

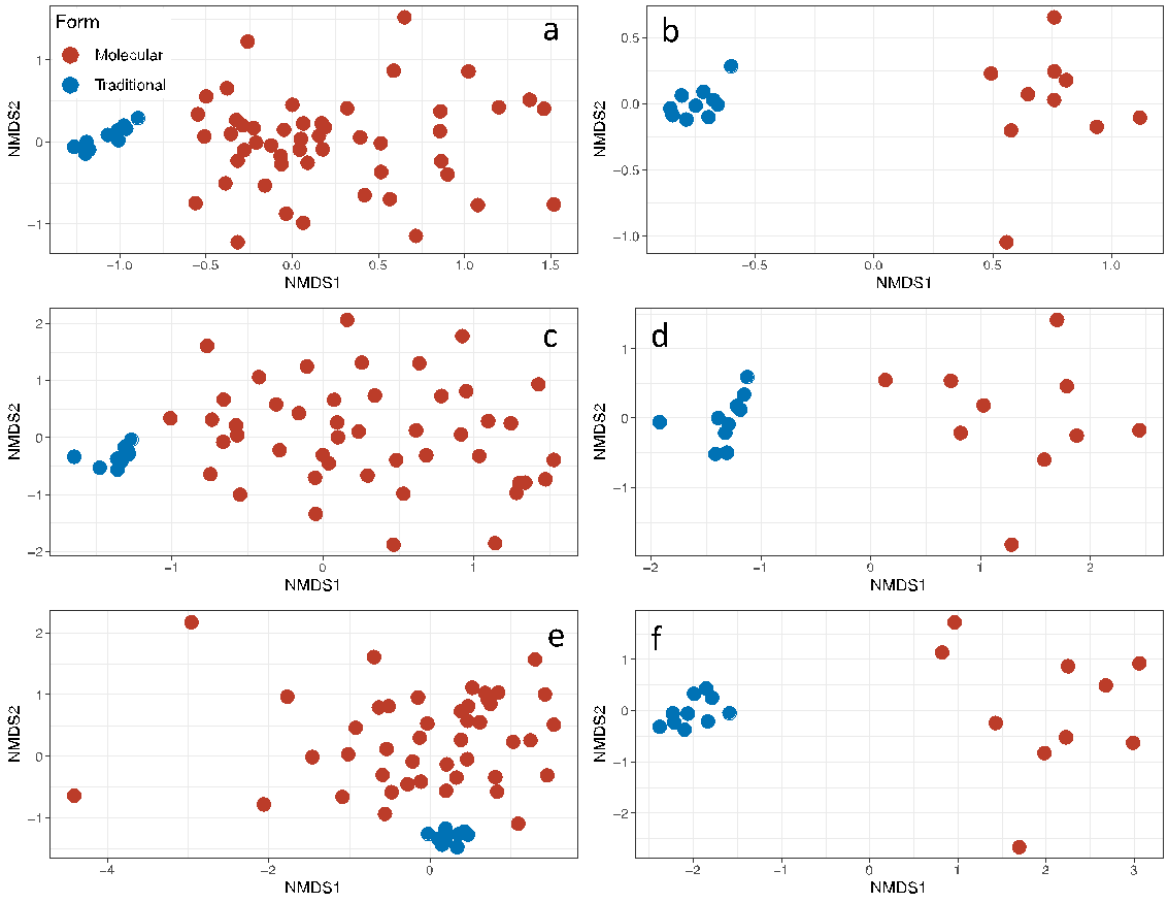

Figure 7. NMDS representing order, family, and species level of molecular samples versus traditional samples based on community structure. PERMANOVA indicates significance on all taxonomic levels for comparisons with individual samples (df = 1, p = 0.001) and summative samples (df = 1, p = 0.001), (a) traditional and all county samples order level (stress= 0.17), (b) traditional and summative county samples order level (stress=0.07), (c) traditional and all county samples family level (stress=0.16), (d) traditional and summative county samples family level (stress=0.10), (e) traditional and all county samples species level (stress=0.09), (f) traditional and summative county samples species level (stress=0.10).

4.4 BioBlitz

4.4.1 Diversity Metrics

Kruskal-Wallis's test supports evenness being significantly different across site types at all levels (df = 3, p_{order}=0.04988, p_{family}=0.03295, $p_{species}$=0.04791). Richness and Diversity were not significantly different, as indicated in Figure 6.

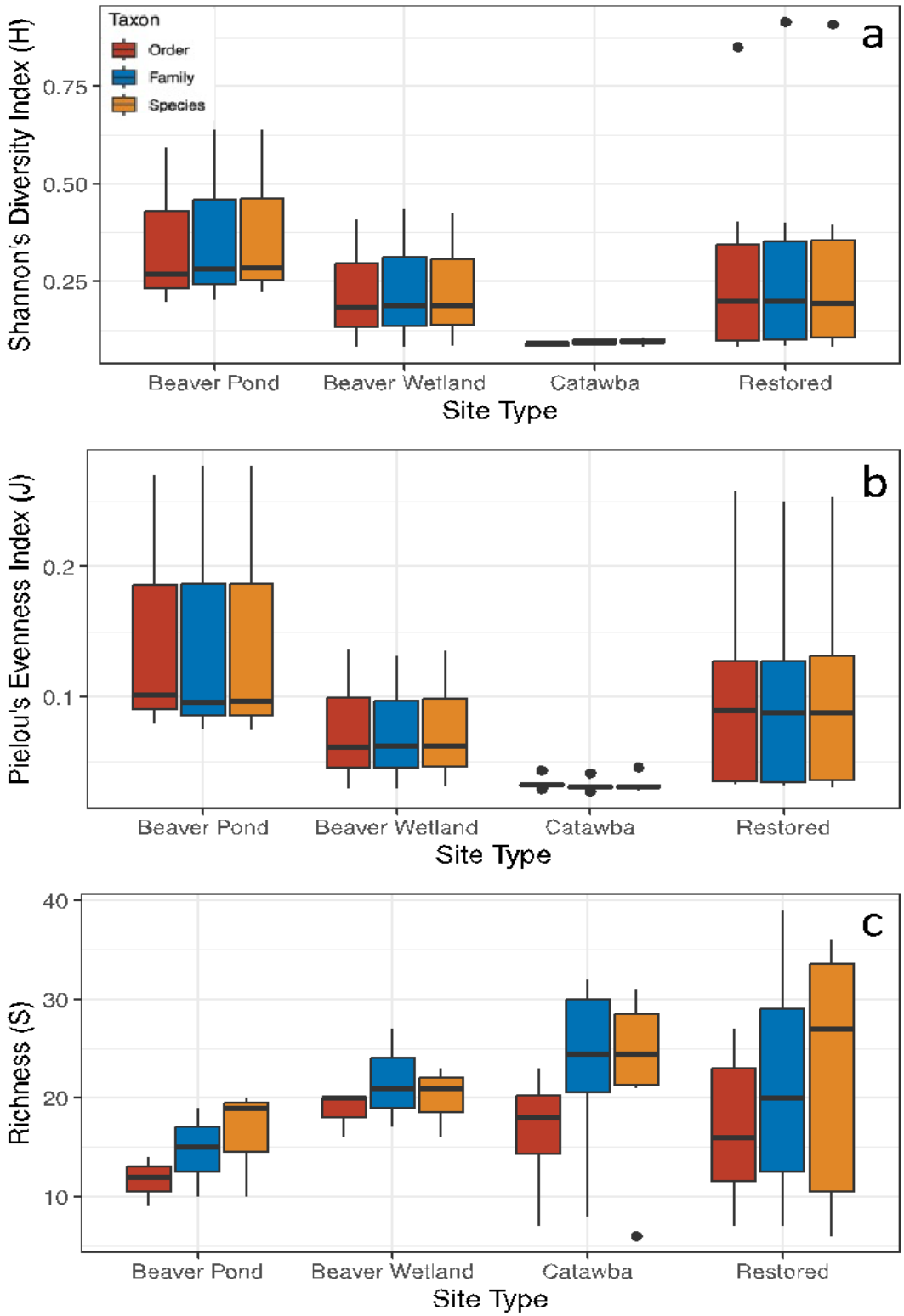

Figure 8. Boxplots comparing diversity metrics between different site types. Kruskal-Wallis results indicate significant differences between site types for Pielou's Evenness Index on the order (df = 3, p = 0.04988) family (df = 3,p = 0.03295) and species (df = 3, p = 0.04791) level. (a) Shannon's Diversity Index, (b) Pielou's Evenness Index, (c) Richness.

4.4.2 Community Structure

While diversity metrics may be similar across site type, community structure was not for the order and family levels and was marginally different on the species level (PERMANOVA, df=3, p_{order}=0.042, p_{family}=0.043, $p_{species}$=0.064). On the order level, structure differed between beaver ponds, restored streams, and Catawba Nation streams (Figure 9). Driving orders include Littorinimorpha, Ploima, Cyclopoida, Podocopida, Ephemeroptera, Lepidoptera, and Sarcoptiformes. On the family level, beaver ponds were significantly different from restored streams and the Catawba Nation streams. Driving families include Chaetonotidae, Macrothricidae, Chironomidae, Cyclopidae, Neritidae, Cecidomyiidae, Camaenidae, Cypridiidae, Gnaphosidae, and Agelenidae. While the species level indicates marginally significant differences, a pairwise adonis test indicated that restored streams were different from beaver ponds and Catawba Nation streams. Driving species include *Corbicula fluminalis, Mesocyclops pehpeiensis, Acanthocyclops brevispinosus,* and *Macrothrix sp.*

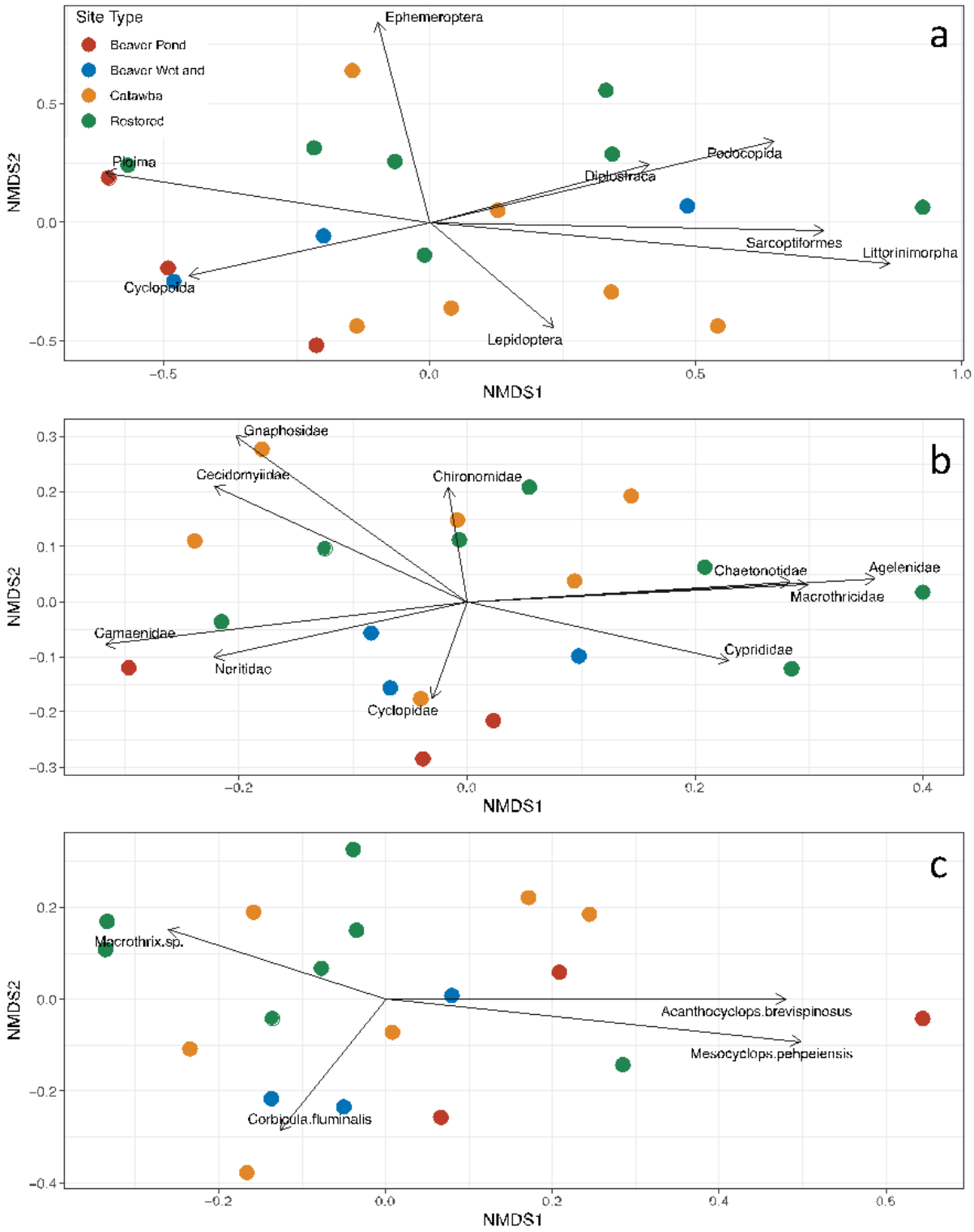

Figure 9.NMDS representing order, family, and species level of BioBlitz sites based on community structure. PERMANOVA indicates significance on order (df = 3, p = 0.042), and family (df = 3, p = 0.043) level. Arrows represent significantly contributing taxa. (a) order level (stress=0.21), (b) family level(stress=0.22), (c) species level (stress = 0.13).

SECTION 5: DISCUSSION

5.1 Temporal Sampling

Like any kind of sampling or monitoring, richness is expected to increase with sampling effort till reaching a plateau. This is no different for the temporal samples in this study. Initial curves did not support a plateau due to having limited samples, but using projected logarithmic models it is possible to determine the richness ceiling of a site. This theoretical ceiling is the assumed maximum richness of a site. Species accumulation curves are technically limitless in terms of increasing richness, so maximum richness was calculated to be the point where the slope of the model (i.e., derivative) reaches less than 1, meaning less than 1 unit of richness would be gained per sampling period after this point. Interestingly, the maximum projected richness for all metrics was from MY9 which has an impervious cover of 39%. This result is unexpected, but moderate impervious surface is not detrimental to invertebrate diversity (Maher et al. 2022). The minimum projected richness, from MC40 (impervious surface = 79%), supports claims made in other studies that high levels of percent impervious cover are negatively associated with richness (Moore and Palmer 2005). Landscapes with high levels of percent impervious cover are still capable of providing successful habitats for invertebrate groups when paired with high canopy cover (Moore and Palmer 2005; Maher et al. 2022).

Supporting my hypothesis, richness does increase with successive sampling. Additionally, it is evident from the projection curves and Figure 5c that multiple samples ($n > 5$) are needed to see differences in richness in these sites. When looking at diversity and evenness in Figure 5, MY7 stands out. The statistical results separate the streams in the Yadkin- Pee Dee River basin and the Catawba River basin. Potential differences could come from the comparatively lower percent impervious cover in the MY sites compared to MC sites, except for MC50 being an

outlier in this. A reasonable explanation is that MC50 is extremely low flow, with discharge averaging at 0.15 f^3/s during the sampling period. Lack of flow means no DNA movement and low flow conditions can drastically decrease eDNA recovery (Stoeckle et al. 2017). Additionally, according to the Environmental Working Group 2023 maps, the Catawba River basin has a higher density of polluters compared to Yadkin-Pee Dee River basin (EWG 2023). This would be influential when looking at sensitive groups, including certain dipterans which were important in driving differences among sites.

5.2 Traditional versus Molecular Sampling

Traditional sampling, which typically uses net mesh sizes of 500 μm, target larger taxa (i.e., insect groups) (Buss and Borges 2008). Meiofauna net sampling typically uses 44 μm mesh sizes (Palmer 1992). Molecular sampling uncovers macroinvertebrate and meiofauna diversity. When focusing on the similarities indicated in Figure 6, one of the standout taxa are the dipterans, specifically the chironomids. The family Chironomidae trends towards streams with higher percent impervious cover, representing higher urbanization, than more natural sites in both molecular and traditional sampling, likely due to chironomids having high tolerance scores (NC Department of Environmental Quality 2016). High tolerance values are a life-history trait of Chironomidae that supports being found in degraded streams in other studies (Maher et al. 2022). Interestingly, the genus *Tanytarsus*, a member of Chironomidae, trends towards more urban sites in traditional sampling, but towards the most natural site in molecular sampling. This can be explained by the fact that *Tanytarsus* is extremely diverse, with 44 different species, which may prefer different habitats (USGS 2023). In general, communities tend to be more similar depending on percent impervious cover rather than based on sampling method. These results

support the use of eDNA as a biomonitoring technique due to the lack of site distribution differences between molecular and traditional samples.

When classifying communities as traditionally sampled versus molecularly sampled, there is a clear dichotomy as seen in Figure 7. Traditional samples at all taxonomic levels are grouped together tightly and isolated from molecular samples. Molecular samples are spread out, showing differentiation across sites, but are still grouped in a way to show similarity across taxa. This difference is most likely due to the fact eDNA can detect invertebrates that are usually not seen such as rotifers, tardigrades, and multiple species (unclassified) of worms. While these data strongly support molecular monitoring, and my hypothesis, it is important to consider the importance of traditional monitoring as well (Aunins et al. 2023). Having available sequences for invertebrates relies on previous identification and taxonomic classification. Molecular monitoring is mostly able to provide quantitative information about communities, and traditional monitoring can collect qualitative information such as morphological anomalies.

5.3 BioBlitz

Across site types, diversity and richness were not significantly different, but evenness significantly varied across site types but was consistent among the taxonomic levels of each site type. The Catawba Nation Reservation streams stand out as the lowest. The Catawba Nation sites are low flow and in a region with low human impacts. The location of these streams is likely to limit dispersal of invertebrates from other regions, and these streams likely have strongly established populations (Bohonak and Jenkins 2003). Similarly, to the case of MC50, low flow is likely a key driver in eDNA detection, meaning that these sites are potentially misrepresented due to the lack of mixing of water. This evidence appears to support traditional monitoring, but

the disturbance and destruction needed to collect invertebrate samples from a low-mixed area could lead to a more negative impact compared to the increased diversity metrics. While not significantly different, there were differences between the evenness and richness of beaver ponds and beaver wetlands. This may be due to the fact the wetlands sampled are intermittent, and therefore taxa that require longer times for nymph or larval growth would not be capable of surviving. Additionally, wetlands are shallower than ponds and have more emergent vegetation, creating different micro-habitats between the two site types. Richness in restored streams is highly variable, which is understandable due to the difference in ages of sites and limitations for dispersal (Brederveld et al. 2011).

Community structure differences are less evident among sites. At the order level, the group Lepidoptera (moths and butterflies) separates the Catawba Nation streams from other sites. While most lepidopterans are terrestrial, one of the semi-aquatic groups are native to the southeastern USA and have a high dependency on nitrogen (Taylor 1984). The Catawba Nation has a large area of surrounding farmland, meaning it is likely to have high nitrogen input. At the family level, it is evident that there is a cluster of restored streams that are driven by the presence of Chaetonotidae (gastrotrichs), Agelenidae (grass spiders), Cyprididae (ostracods), and Macrothricidae (water fleas). These families, except Agelenidae, are all early successionists as they have short life spans, ease of dispersal, or are ubiquitous (Ferguson 1944; Dole-Oliver et al. 2000; Strayer et al. 2010). These early successionists are typical for early restored streams before a committed population establishes (Bohonak and Jenkins 2003). Spiders are typically terrestrial but will enter water systems to hunt insects (Graham et al. 2003; Kelly et al. 2019). Additionally, there is a significant difference between beaver pond and Catawba Nation stream communities. This is likely due to the established communities of these sites having preference

of lotic or lentic habitats (Ribera 2008). My hypothesis was not supported, most likely due to limited water flow, limited mixing, and potentially limited invertebrate dispersal in Catawba Nation streams.

5.4 Limitations

Due to time and novelty of this study in the Carolina Piedmont, there were limitations and learning curves that will be addressed in future work. Initial work involved multiple adaptations to the methodology, such as determining pore size of filters, before reaching an effective protocol. Two different extraction kits were used because of limited shipping availability. While the BF2/BR2 primer combination was supported by the present literature, the primer became outdated very quickly, with rapid successions of new primers being announced and validated multiple times. The primer combination did detect a wide range of invertebrates, but multiple non-target taxa, including bacteria and fungi, were detected as well. Newer primers reduce noise from non-target taxa (Leese et al. 2021). Lastly, the novelty of nanopore sequencing likely created errors or missed reads in sequencing which can be improved through more studies with eDNA using nanopore sequencing.

5.5 Conclusions

Molecular monitoring is comparable, and in multiple facets, exceeds traditional monitoring. Molecular monitoring is less influenced by human bias but is highly influenced by extraneous factors such as water flow, primer choice, and environmental factors. Implications of this study would be useful in developing a standardized molecular biomonitoring program in both currently monitored and unmonitored sites. Additionally, site type comparisons are capable

due to the standardization of methods, meaning that monitoring is possible in sites rarely surveyed for invertebrates such as ephemeral wetlands and restored streams. Improvements and future work to be done would focus on primer evaluation and the influence of further sampling. Primer development for specific groups has skyrocketed, and therefore there is likely a better alternative for detecting invertebrates or even entire system studies. Successive samples would provide greater stability for accumulation curve projections, allow for greater understanding of diversity in established sites, and provide a method of tracking invertebrate succession patterns in restored streams.

Biomonitoring is essential to fully understanding any ecosystem, including freshwater environments. Due to the difficulties with traditional biomonitoring, molecular biomonitoring provides a realistic, functional alternative for evaluating invertebrate communities in freshwater systems.

REFERENCES

Ab Hamid S, Md Rawi CS. 2017. Application of Aquatic Insects (Ephemeroptera, Plecoptera and Trichoptera) in Water Quality Assessment of Malaysian Headwater. TLSR. 28(2):143–162. doi:10.21315/tlsr2017.28.2.11.

Aunins AA, Mueller SJ, Fike JA, Cornman RS. 2023. Assessing arthropod diversity metrics derived from stream environmental DNA: spatiotemporal variation and paired comparisons with manual sampling. PeerJ. 11:e15163. doi:10.7717/peerj.15163.

Balsamo M. 1980. Spectral sensitivity in a fresh-water Gastrotrich (Lepidodermella squamatum Dujardin). Experientia. 36(7):830–831. doi:10.1007/BF01978596.

Barnes MA, Turner CR, Jerde CL, Renshaw MA, Chadderton WL, Lodge DM. 2014. Environmental Conditions Influence eDNA Persistence in Aquatic Systems. Environ Sci Technol. 48(3):1819–1827. doi:10.1021/es404734p.

Barton DR, Metcalfe-Smith JL. 1992. A COMPARISON OF SAMPLING TECHNIQUES AND SUMMARY INDICES FOR ASSESSMENT OF WATER QUALITY IN THE YAMASKA RIVER, QUI~BEC, BASED ON BENTHIC MACROINVERTEBRATES. Environmental Monitoring and Assessment.:225–244.

Birk S, Bonne W, Borja A, Brucet S, Courrat A, Poikane S, Solimini A, van de Bund W, Zampoukas N, Hering D. 2012. Three hundred ways to assess Europe's surface waters: An almost complete overview of biological methods to implement the Water Framework Directive. Ecological Indicators. 18:31–41. doi:10.1016/j.ecolind.2011.10.009.

Blackman R, Mächler E, Altermatt F, Arnold A, Beja P, Boets P, Egeter B, Elbrecht V, Filipe AF, Jones J, et al. 2019. Advancing the use of molecular methods for routine freshwater macroinvertebrate biomonitoring – the need for calibration experiments. MBMG. 3:e34735. doi:10.3897/mbmg.3.34735.

Blankenship S, Schumer G. 2017. Field Collection Procedure for Aquatic Environmental DNA sample collection and analysis.

Bohonak AJ, Jenkins DG. 2003. Ecological and evolutionary significance of dispersal by freshwater invertebrates. Ecol Letters. 6(8):783–796. doi:10.1046/j.1461-0248.2003.00486.x.

Bonada N, Prat N, Resh VH, Statzner B. 2006. DEVELOPMENTS IN AQUATIC INSECT BIOMONITORING: A Comparative Analysis of Recent Approaches. Annu Rev Entomol. 51(1):495–523. doi:10.1146/annurev.ento.51.110104.151124.

Brederveld RJ, Jähnig SC, Lorenz AW, Brunzel S, Soons MB. 2011. Dispersal as a limiting factor in the colonization of restored mountain streams by plants and macroinvertebrates: Dispersal restricts stream colonization. Journal of Applied Ecology. 48(5):1241–1250. doi:10.1111/j.1365-2664.2011.02026.x.

Buffagni A, Kemp JL, Erba S, Belfiore C, Hering D, Moog O. 2001. A Europe-wide system for assessing the quality of rivers using macroinvertebrates: the AQEM Project*) and its importance for southern Europe (with special emphasis on Italy). J Limnol. 60(1s):39. doi:10.4081/jlimnol.2001.s1.39.

Burian A, Mauvisseau Q, Bulling M, Domisch S, Qian S, Sweet M. 2021. Improving the reliability of eDNA data interpretation. Mol Ecol Resour. 21(5):1422–1433. doi:10.1111/1755-0998.13367.

Buss DF, Borges EL. 2008. Application of rapid bioassessment protocols (RBP) for benthic macroinvertebrates in Brazil: comparison between sampling techniques and mesh sizes. Neotrop entomol. 37(3):288–295. doi:10.1590/S1519-566X2008000300007.

Cairns J, Pratt JR. 1993. A History of Biological Monitoring Using Benthic Macroinvertebrates. In: Rosenberg M, Resh V, editors. Freshwater biomonitoring and benthic macroinvertebrates. London: Chapman and Hall. p. 10–27.

Campbell CD, Gleeson DM, Furlan EM, Muirhead KA, Caron V. 2022. Detection of a cryptic terrestrial insect using novel eDNA collection techniques. Environmental DNA. 4(4):820–829. doi:10.1002/edn3.295.

Cardé RT, Resh VH. 2009. Encyclopedia of insects. 2nd ed. Amsterdam London: Elsevier/Academic Press.

Carew ME, Coleman RA, Robinson KL, Hoffmann AA. 2021. Using unsorted sweep-net samples to rapidly assess macroinvertebrate biodiversity. Freshwater Science. 40(3):551–565. doi:10.1086/716215.

Czechowski P, Stevens MI, Madden C, Weinstein P. 2020. Steps towards a more efficient use of chironomids as bioindicators for freshwater bioassessment: Exploiting eDNA and other genetic tools. Ecological Indicators. 110:105868. doi:10.1016/j.ecolind.2019.105868.

Dahms H-U, Hagiwara A, Lee J-S. 2011. Ecotoxicology, ecophysiology, and mechanistic studies with rotifers. Aquatic Toxicology. 101(1):1–12. doi:10.1016/j.aquatox.2010.09.006.

Deborde DDD, Hernandez MBM, Magbanua FS. 2016. Benthic Macroinvertebrate Community as an Indicator of Stream Health: The Effects of Land Use on Stream Benthic Macroinvertebrates. Science Diliman. 28(2):5–26.

Deiner K, Fronhofer EA, Mächler E, Walser J-C, Altermatt F. 2016. Environmental DNA reveals that rivers are conveyer belts of biodiversity information. Nat Commun. 7(1):12544. doi:10.1038/ncomms12544.

Dejean T, Valentini A, Miquel C, Taberlet P, Bellemain E, Miaud C. 2012. Improved detection of an alien invasive species through environmental DNA barcoding: the example of the American bullfrog *Lithobates catesbeianus*: *Alien invasive species detection using eDNA*. Journal of Applied Ecology. 49(4):953–959. doi:10.1111/j.1365-2664.2012.02171.x.

Dinno A. 2017. _dunn.test: Dunn's Test of Multiple Comparisons Using Rank Sums_. R package version 1.3.5, <https://CRAN.R-project.org package=dunn.test>.

Djurhuus A, Port J, Closek CJ, Yamahara KM, Romero-Maraccini O, Walz KR, Goldsmith DB, Michisaki R, Breitbart M, Boehm AB, et al. 2017. Evaluation of Filtration and DNA Extraction Methods for Environmental DNA Biodiversity Assessments across Multiple Trophic Levels. Front Mar Sci. 4:314. doi:10.3389/fmars.2017.00314.

Dole-Olivier M, Galassi DMP, Marmonier P, Creuzé Des Châtelliers M. 2000. The biology and ecology of lotic microcrustaceans. Freshwater Biology. 44(1):63–91. doi:10.1046/j.1365-2427.2000.00590.x.

Elbrecht V, Leese F. 2017. Validation and Development of COI Metabarcoding Primers for Freshwater Macroinvertebrate Bioassessment. Front Environ Sci. 5. doi:10.3389/fenvs.2017.00011. [accessed 2022 Apr 2]. http://journal.frontiersin.org/article/10.3389/fenvs.2017.00011/full.

EWG. 2023. Suspected industrial discharges of PFAS. https://www.ewg.org/interactive-maps/2021_suspected_industrial_discharges_of_pfas/map/.

Ficetola GF, Miaud C, Pompanon F, Taberlet P. 2008. Species detection using environmental DNA from water samples. Biol Lett. 4(4):423–425. doi:10.1098/rsbl.2008.0118.

Ferguson E. 1944. Studies on the Seasonal Life History of Three Species of Freshwater Ostracoda. American Midland Naturalist. 32(3):713. doi:10.2307/2421245.

Gerhardt A. 2009. Bioindicator Species and Their Use in Biomonitoring. In: Daniels JL, Inyang HI, editors. ENVIRONMENTAL MONITORING. Vol. 1. Oxford: EOLSS Publications. p. 77–123.

Goldberg CS, Pilliod DS, Arkle RS, Waits LP. 2011. Molecular Detection of Vertebrates in Stream Water: A Demonstration Using Rocky Mountain Tailed Frogs and Idaho Giant Salamanders. Gratwicke B, editor. PLoS ONE. 6(7):e22746. doi:10.1371/journal.pone.0022746.

Goldberg CS, Sepulveda A, Ray A, Baumgardt J, Waits LP. 2013. Environmental DNA as a new method for early detection of New Zealand mudsnails (*Potamopyrgus antipodarum*). Freshwater Science. 32(3):792–800. doi:10.1899/13-046.1.

Graham AK, Buddle CM, Spence JR. 2003. HABITAT AFFINITIES OF SPIDERS LIVING NEAR A FRESHWATER POND. Journal of Arachnology. 31(1):78–89. doi:10.1636/0161-8202(2003)031[0078:HAOSLN]2.0.CO;2.

Haase P, Pauls SU, Schindehütte K, Sundermann A. 2010. First audit of macroinvertebrate samples from an EU Water Framework Directive monitoring program: human error greatly lowers precision of assessment results. Journal of the North American Benthological Society. 29(4):1279–1291. doi:10.1899/09-183.1.

Hering D, Borja A, Jones JI, Pont D, Boets P, Bouchez A, Bruce K, Drakare S, Hänfling B, Kahlert M, et al. 2018. Implementation options for DNA-based identification into ecological status assessment under the European Water Framework Directive. Water Research. 138:192–205. doi:10.1016/j.watres.2018.03.003.

Jerde CL, Mahon AR, Chadderton WL, Lodge DM. 2011. “Sight-unseen” detection of rare aquatic species using environmental DNA: eDNA surveillance of rare aquatic species. Conservation Letters. 4(2):150–157. doi:10.1111/j.1755-263X.2010.00158.x.

Keck F, Blackman RC, Bossart R, Brantschen J, Couton M, Hürlemann S, Kirschner D, Locher N, Zhang H, Altermatt F. 2022. Meta-analysis shows both congruence and complementarity of DNA and eDNA metabarcoding to traditional methods for biological community assessment. Molecular Ecology. 31(6):1820–1835. doi:10.1111/mec.16364.

Keck F, Vasselon V, Tapolczai K, Rimet F, Bouchez A. 2017. Freshwater biomonitoring in the Information Age. Front Ecol Environ. 15(5):266–274. doi:10.1002/fee.1490.

Kelly SP, Cuevas E, Ramírez A. 2019. Urbanization increases the proportion of aquatic insects in the diets of riparian spiders. Freshwater Science. 38(2):379–390. doi:10.1086/703442.

Keskin E. 2014. Detection of invasive freshwater fish species using environmental DNA survey. Biochemical Systematics and Ecology. 56:68–74. doi:10.1016/j.bse.2014.05.003.

Laramie M, Pilliod D, Goldberg CS, Strickler KM. 2015. Environmental DNA sampling protocol - filtering water to capture DNA from aquatic organisms. Techniques and Methods.

Leese F, Sander M, Buchner D, Elbrecht V, Haase P, Zizka VMA. 2021. Improved freshwater macroinvertebrate detection from environmental DNA through minimized nontarget amplification. Environmental DNA. 3(1):261–276. doi:10.1002/edn3.177.

Lim NKM, Tay YC, Srivathsan A, Tan JWT, Kwik JTB, Baloğlu B, Meier R, Yeo DCJ. 2016. Next-generation freshwater bioassessment: eDNA metabarcoding with a conserved metazoan primer reveals species-rich and reservoir-specific communities. R Soc open sci. 3(11):160635. doi:10.1098/rsos.160635.

Mächler E, Deiner K, Spahn F, Altermatt F. 2016. Fishing in the Water: Effect of Sampled Water Volume on Environmental DNA-Based Detection of Macroinvertebrates. Environ Sci Technol. 50(1):305–312. doi:10.1021/acs.est.5b04188.

Mächler E, Deiner K, Steinmann P, Altermatt F. 2014. Utility of environmental DNA for monitoring rare and indicator macroinvertebrate species. Freshwater Science. 33(4):1174–1183. doi:10.1086/678128.

Maher GM, Johnson GA, Burdine JD. 2022. Impervious surface and local abiotic conditions influence arthropod communities within urban greenspaces. PeerJ. 10:e12818. doi:10.7717/peerj.12818.

Mathieu C, Hermans SM, Lear G, Buckley TR, Lee KC, Buckley HL. 2020. A Systematic Review of Sources of Variability and Uncertainty in eDNA Data for Environmental Monitoring. Front Ecol Evol. 8:135. doi:10.3389/fevo.2020.00135.

Moore AA, Palmer MA. 2005. INVERTEBRATE BIODIVERSITY IN AGRICULTURAL AND URBAN HEADWATER STREAMS: IMPLICATIONS FOR CONSERVATION AND MANAGEMENT. Ecological Applications. 15(4):1169–1177. doi:10.1890/04-1484.

NC Department of Environmental Quality. 2016. Standard Operating Procedures for the Collection and Analysis of Benthic Macroinvertebrates.

Nørgaard L, Olesen CR, Trøjelsgaard K, Pertoldi C, Nielsen JL, Taberlet P, Ruiz-González A, De Barba M, Iacolina L. 2021. eDNA metabarcoding for biodiversity assessment, generalist predators as sampling assistants. Sci Rep. 11(1):6820. doi:10.1038/s41598-021-85488-9.

Oksanen J, Simpson G, Blanchet F, Kindt R, Legendre P, Minchin P, O'Hara R, Solymos P, Stevens M, Szoecs E, Wagner H, Barbour M, Bedward M, Bolker B, Borcard D, Carvalho G, Chirico M, De Caceres M, Durand S, Evangelista H, FitzJohn R, Friendly M, Furneaux B, Hannigan G, Hill M, Lahti L, McGlinn D, Ouellette M, Ribeiro Cunha E, Smith T, Stier A, Ter Braak C, & Weedon J. 2022. _vegan: Community Ecology Package_. R package version 2.6-4, <https://CRAN.R-project.org/package=vegan>.

Palmer MA. 1992. Incorporating lotic meiofauna into our understanding of faunal transport processes. Limnol Oceanogr. 37(2):329–341. doi:10.4319/lo.1992.37.2.0329.

Pawlowski J, Bruce K, Panksep K, Aguirre FI, Amalfitano S, Apothéloz-Perret-Gentil L, Baussant T, Bouchez A, Carugati L, Cermakova K, et al. 2022. Environmental DNA metabarcoding for benthic monitoring: A review of sediment sampling and DNA extraction methods. Science of The Total Environment. 818:151783. doi:10.1016/j.scitotenv.2021.151783.

Piñol J, San Andrés V, Clare EL, Mir G, Symondson WOC. 2014. A pragmatic approach to the analysis of diets of generalist predators: the use of next-generation sequencing with no blocking probes. Mol Ecol Resour. 14(1):18–26. doi:10.1111/1755-0998.12156.

R Core Team. 2022. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Reinholdt Jensen M, Egelyng Sigsgaard E, Agersnap S, Jessen Rasmussen J, Baattrup-Pedersen A, Wiberg-Larsen P, Francis Thomsen P. 2021. Seasonal turnover in community composition of stream-associated macroinvertebrates inferred from freshwater environmental DNA metabarcoding. Environmental DNA. 3(4):861–876. doi:10.1002/edn3.193.

Ribera I. 2008. Habitat constraints and the generation of diversity in freshwater macroinvertebrates. In: Lancaster J, Briers RA, editors. Aquatic insects: challenges to populations. 1st ed. UK: CABI. p. 289–311. [accessed 2023 Apr 17]. http://www.cabidigitallibrary.org/doi/10.1079/9781845933968.0289.

Sgarbi LF, Melo AS. 2017. You don’t belong here: explaining the excess of rare species in terms of habitat, space, and time. Oikos. 127(4):497–506. doi:10.1111/oik.04855.

Stein ED, White BP, Mazor RD, Jackson JK, Battle JM, Miller PE, Pilgrim EM, Sweeney BW. 2014. Does DNA barcoding improve performance of traditional stream bioassessment metrics? Freshwater Science. 33(1):302–311. doi:10.1086/674782.

Stoeckle BC, Beggel S, Cerwenka AF, Motivans E, Kuehn R, Geist J. 2017. A systematic approach to evaluate the influence of environmental conditions on eDNA detection success in aquatic ecosystems. Doi H, editor. PLoS ONE. 12(12):e0189119. doi:10.1371/journal.pone.0189119.

Strayer DL, Hummon WD, Hochberg R. 2010. Gastrotricha. In: Ecology and Classification of North American Freshwater Invertebrates. Elsevier. p. 163–172. https://linkinghub.elsevier.com/retrieve/pii/B9780123748553000078.

Sweeney BW, Battle JM, Jackson JK, Dapkey T. 2011. Can DNA barcodes of stream macroinvertebrates improve descriptions of community structure and water quality? Journal of the North American Benthological Society. 30(1):195–216. doi:10.1899/10-016.1.

Taylor MFJ. 1984. The dependence of development and fecundity of Samea multiplicalis on early larval nitrogen intake. Journal of Insect Physiology. 30(10):779–785. doi:10.1016/0022-1910(84)90014-3.

Thalinger B, Deiner K, Harper LR, Rees HC, Blackman RC, Sint D, Traugott M, Goldberg CS, Bruce K. 2021. A validation scale to determine the readiness of environmental DNA assays for routine species monitoring. Environmental DNA. 3(4):823–836. doi:10.1002/edn3.189.

Thompson LR, Sanders JG, McDonald D, Amir A, Ladau J, Locey KJ, Prill RJ, Tripathi A, Gibbons SM, Ackermann G, et al. 2017. A communal catalogue reveals Earth’s multiscale microbial diversity. Nature. 551(7681):457–463. doi:10.1038/nature24621.

Trimbos KB, Cieraad E, Schrama M, Saarloos AI, Musters KeesJM, Bertola LD, Bodegom PM. 2021. Stirring up the relationship between quantified environmental DNA concentrations and exoskeleton-shedding invertebrate densities. Environmental DNA. 3(3):605–618. doi:10.1002/edn3.157.

Tsyrlin E, Robinson K, Hoffmann A, Coleman RA. 2022. Climate warming threatens critically endangered wingless stonefly Riekoperla darlingtoni (Illies, 1968) (Plecoptera: Gripopterygidae). J Insect Conserv. 26(1):59–68. doi:10.1007/s10841-021-00361-4.

USGS LBT. 2023. Integrated Taxonomic Information System (ITIS). doi:10.5066/F7KH0KBK. [accessed 2023 Apr 17]. http://www.itis.gov.

Wickham H. 2016. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York.

Wallace JB, Webster JR. 1996. The Role of Macroinvertebrates in Stream Ecosystem Function. Annu Rev Entomol. 41(1):115–139. doi:10.1146/annurev.en.41.010196.000555.

Yates MC, Fraser DJ, Derry AM. 2019. Meta-analysis supports further refinement of eDNA for monitoring aquatic species-specific abundance in nature. Environmental DNA. 1(1):5–13. doi:10.1002/edn3.7.

APPENDIX A: P-VALUES

Spearman's Rho correlations p values for Figure 3.

Metric	Level	Sequence Count p value	Impervious Surface p value
Diversity (H)	Order	0.01208193	0.01244
	Family	0.00907565	0.01218
	Species	0.01181386	0.01144
Evenness (J)	Order	0.02959057	0.04842
	Family	0.03610548	0.04341
	Species	0.03523049	0.03784
Richness (S)	Order	0.00026861	0.04287
	Family	7.23E-05	0.03925
	Species	0.00180805	0.165

APPENDIX B: LOG MODELS

Log model accumulation curve equations

Site	Order	Family	Species
MC17	y = 5.92 + 11.74 * log(x)	y = 6.31 + 19.58 * log(x)	y = 5.52 + 17.71 * log(x)
MC25	y=6.09 + 10.02 * log(x)	y = 6.85 + 17.89 * log(x)	y = 5.72 + 18.23 * log(x)
MC33	y = 4.02 + 10.28 * log(x)	y = 4.42 + 11.74 * log(x)	y = 4.28 + 14.48 * log(x)
MC40	y = 5.77 + 9.65 * log(x)	y = 5.32 + 15.64 * log(x)	y = 4.8 + 15.87 * log(x)
MC47	y = 7.29 + 10.49 * log(x)	y = 6.85 + 18.27 * log(x)	y = 5.23 + 18.91 * log(x)
MC49	y = 8.03 + 10.79 * log(x)	y = 7.52 + 19.19 * log(x)	y = 6.97 + 22.58 * log(x)
MC50	y = 11.5 + 17.18 * log(x)	y = 8.53 + 16.39 * log(x)	y = 8.97 + 19.97 * log(x)
MY7	y = 8.98 + 15.39 * log(x)	y = 7.98 + 21.63 * log(x)	y = 7.83 + 22.2 * log(x)
MY8	y = 9.06 + 10.76 * log(x)	y = 9.32 + 24.42 * log(x)	y = 6.94 + 20.41 * log(x)
MY9	y = 11.37 + 13.11 * log(x)	y = 13.27 + 31.61 * log(x)	y = 11.88 + 34.21 * log(x)