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# Sample Collection and DNA Extraction Methods for Environmental DNA Metabarcoding in Headwater Streams

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# SAMPLE COLLECTION AND DNA EXTRACTION METHODS FOR ENVIRONMENTAL DNA METABARCODING IN HEADWATER STREAMS

By

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DEDICATION

To my parents, Mike and Kathy, for the encouragement to pursue my interests.

To my wife, Jennifer, and our children for inspiring me to go and see what is just around the river bend.

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First, I would not be who I am today without the support and encouragement from my parents, my wife, and our children. Thank you to my parents for encouraging me to play and learn in the "cricks" of the "hollers" we called home. My wife, Jennifer, and our children have inspired me to be a man of love and to always seek the adventures that are out there.

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ABSTRACT

DNA is found to be free and ubiquitous in the environment where it is no longer associated with the source organism, and is also known as environmental-DNA (eDNA). Methods optimized for specific environments may be able supplement insight to local taxa richness. With the advent of high throughput sequencing and the proliferation of sequence data in public repositories, insights to the biodiversity of communities at the molecular level have been possible. Thus, this study compared commonly used DNA capture (water precipitation and filtration) and extraction (MoBio's PowerWater, Qiagen's DNeasy Blood and Tissue Kit, and a CTAB protocol) methods in their ability to isolate eDNA for the purpose of metabarcoding a section of the ribosomal small subunit 18 S (18s) and the cytochrome oxidase I (COI) gene regions. The 18s sequence data is non-reportable due to lack of sequence quality, and MoBio's PowerWater did not yield DNA suitable concentrations. CTAB and DNeasy extractions yielded successful PCR reactions and high-throughput sequencing (HTS). When combined with their respective replicates, CTAB and DNeasy were determined to have genus richness ($\alpha$-diversity) of 25 and 24, respectively of benthic macroinvertebrates with 20 taxonomic determinations being shared between the two methods. After conducting Jaccard's dissimilarity index and constructing ordination plots using non-metric multidimensional scaling (NMDS), this study was not able to reveal differences in the amount of taxa richness between CTAB and DNeasy, which implied extraction methods may not be a limiting factor in detected taxa richness.

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# CHAPTER I

## INTRODUCTION

DNA is found to be free and ubiquitous in the environment (Bohmann et al., 2014), being described as environmental-DNA (eDNA) (Lodge et al., 2012; Bohmann et al., 2014; Barnes & Turner, 2015). Studying the ecology of eDNA and enhancing the methods for eDNA study is relevant to ecologists because "understanding the origin of eDNA… can inform our understanding about the taxa and environments for which eDNA represents and effective conservation and research tool" (Barnes & Turner, 2015). Sources of eDNA may include saliva, scent mark, sloughed/shed skin, exuviae, urine, feces, and other biological material (Beja-Pereira et al., 2009), though little is known about the physiological origins and decay rates of eDNA (Barnes & Turner, 2015). Both abiotic (i.e. conductivity, pH, DO, and light) and biotic (i.e. intra- and extra-cellular enzymes) factors influence the decay rates (Nielsen, et al. , 2007). eDNA is often degraded material (~80-250 bp) (Bohmann et al., 2014), and when samples are processed without lysis of cells, the DNA collected is considered extracellular DNA (Taberlet et al., 2012). eDNA may be able to give accurate biodiversity estimates of environments, which would be beneficial for invasive species detection, functional diversity, wildlife and conservation biology (Thomsen et al., 2011; Bohmann et al., 2014; Elbrecht & Leese, 2015). eDNA used as a biological survey tool causes less of a disruption to organisms and their habitat because of the non-invasive nature of sampling. Sample methods include but are not limited to filtering water from the water column (Goldberg et al., 2011; Jerde et

al., 2011; Thomsen et al., 2012), water precipitation (Ficetola et al., 2008), soil cores (Taberlet et al., 2012), and ice cores (Willerslev et al., 2007); all forms of eDNA sampling collect relatively small amounts of source material (<1gram) for laboratory processing ( Willerslev et al., 2007; Ficetola et al., 2008; Goldberg et al., 2011; Jerde et al., 2011; Taberlet et al., 2012; Thomsen et al., 2012).

eDNA has been used to survey for specific species (Goldberg et al., 2011; Jerde et al., 2011) or detect many species within a community ( Dejean et al., 2011; Kermarrec et al., 2014; Deiner et al., 2015a; Deiner et al., 2015b). eDNA amplified to detectable levels by means of polymerase chain reaction (PCR) has been demonstrated to denote the recent presence of the source species (Ficetola et al., 2008; Dejean et al., 2011; Civade et al., 2016; Souza et al., 2016), and eDNA concentration has also been positively correlated with biomass in the laboratory controlled settings (Elbrecht & Leese, 2015) and in the field (Jane et al., 2014). Detection of eDNA has been found up to 12 km away from the home range and habitat type of a source organism(aquatic systems; waterfleas and mussels) (Deiner & Altermatt, 2014). Thus, water flow velocity has been proposed to be an influential factor on the distance of detection (Civade et al., 2016). Even though eDNA signal detection has been found to be less than probable persistence of eDNA in the site, additional research needs to be conducted on factors influencing persistence and transport in lotic systems (Civade et al., 2016). Limiting the spatial scale of sample points by watershed size in lotic systems would be a prudent experimental design decision to prevent biased comparisons. In addition, PCR inhibiting compounds from decaying litter of leaf fall events have been shown to decrease amplification

and eDNA detection (Jane et al., 2014). PCR may also cause replication bias towards more abundant template DNA, so adequate PCR replication is needed to reduce false absences (Ficetola et al., 2014).

All of the initial processing of eDNA follows the same workflow as "capturing DNA from an environmental sample, followed by the extraction and purification of eDNA" (Deiner et al., 2015) with capture being defined as the concentration of cellular and extracellular DNA (Pilliod et al., 2013). Aquatic eDNA is commonly captured by either water filtration or water precipitation (Deiner & Altermatt, 2014), and eDNA extraction may be processed with a range of methods including but not limited to DNA lysis and precipitation using cetyl trimethyl ammonium bromide (CTAB) (Saghai-Maroof et al., 1984), a CTAB extraction protocol with ONESTEP PCR Inhibitor Removal Kit (Turner, Uy, & Everhart, 2015), Qiagen's DNeasy Blood and Tissue Kit (DNeasy) (Amberg et al., 2015), and MoBio's Powerwater DNA Isolation Kit (PowerWater) (Goldberg et al., 2011; Amberg et al., 2015). Studies have found different extraction protocols provide varying levels of biodiversity ( Goldberg et al., 2011; Amberg et al., 2015; Deiner et al., 2015), and the lack of experimental replication amongst extraction treatments in previous freshwater studies has been addressed (Deiner et al., 2015). The only study with appropriate replication of habitat of study was the outflow of a lentic system (Deiner et al., 2015). Few studies have tried to optimize eDNA metabarcoding in natural aquatic environments, especially headwater streams (Thomsen et al., 2012 Deiner, et al., 2015; Miya et al., 2015; Civade et al., 2016; Valentini et al., 2016).

With the advent of high throughput sequencing (also known as next generation sequencing), such as Illumina MiSeq (Bálint el al., 2014) and pyrosequencing (Chariton et al., 2010), and the proliferation of sequence data in public repositories, such as non-curated GenBank and GreenTrees and curated Barcode of Life and SILVA, insights to the biodiversity of communities at the molecular level have been possible ( Hajibabaei et al., 2007; Chariton et al., 2010; Dejean et al., 2011 Baird & Hajibabaei, 2012; Bohmann et al., 2014; Cristescu, 2014; Hugerth et al., 2014; Kermarrec et al., 2014; Mächler et al, 2014). DNA reference libraries are used to make taxonomic assignments from eDNA sequences, which allows for community level analysis, also known as metabarcoding (Barnes & Turner, 2015). Metabarcoding may make use of whole organisms, particularly microorganisms (Chariton et al., 2010; Zhan et al., 2014) or environmental DNA (Taberlet et al., 2012; Deiner et al., 2015a). In cases when sequences are analyzed before being assigned to taxonomies or when sequences may not be taxonomically identified because of insufficient DNA reference sequence, taxa may be reported as Molecular Operational Taxonomic Units (MOTUs) (Smith & Peay, 2014;Deiner, Walser, et al., 2015b). When working in new model systems or with new primers, newly discovered MOTUs may provide indication of species presence where it was not seen using physical detection (Yu et al., 2012; Kermarrec et al., 2014; Zhan et al., 2014). Metabarcording also has provided insights to community level comparisons with water quality (Chariton et al., 2010; Yu et al., 2012; Kermarrec et al., 2014; Mächler et al., 2014; Zimmermann et al., 2014).

PCR is currently a limiting factor for species detection when metabarcoding eDNA because of the need to anneal and amplify target DNA templates with DNA primers (Zhan et al., 2014). Aquatic macroinvertebrates have been found extensively associated with the commonly used gene region Cytochrome c oxidase subunit one (COI), a mitochondrial region of DNA (Folmer et al., 1994; Zhan et al., 2014; Deiner et al., 2015b). Nuclear regions of DNA, may also give the breadth of sequence diversity needed for community level analysis at the order and family taxonomic levels (Fonseca et al., 2010; Wu et al., 2015), but with less confidence in species level resolution (Hugerth et al., 2014; Zhan et al., 2014).

Metabarcoding has been used for understanding eukaryotic community diversity in relation to ecosystem health (Chariton et al., 2010), which may suggest metabarcoding of eDNA may be complimentary to water-quality of streams. If strong associations have been found between detected presence of species using metabarcoding of eDNA and those species found in traditional bioassessments (Chariton et al., 2010; Civade et al., 2016), then this method has the potential value to provide supplementary data to what monitoring agencies report on water quality. Community level surveys analyzing metabarcoded eDNA have been paired with ecoregions (Smith & Peay, 2014), biodiversity estimates (Kermarrec et al., 2014), bioindicators (Mächler et al., 2014), and impacted streams (Chariton et al., 2010). With eDNA methods exhibiting such promise as a survey tool, studies should focus on refining the implementation of methods for capture and extraction.

To date, relatively few studies have tried to compare and assess the performance of eDNA capture and extraction methods. This study has tried to

bridge this knowledge gap by assessing eDNA capture and extraction methods in a headwater stream. This study has two main objectives: (1) compare eDNA capture methods water precipitation (Ficetola et al., 2008) and water filtration ( Goldberg et al., 2011; Amberg et al., 2015), and (2) compare three eDNA extraction methods, which are MoBio's PowerWater DNA Isolation Kit, Qiagen's DNeasy Blood and Tissue Kit, and a CTAB based DNA precipitation extraction [1](Saghai-Maroof et al., 1984; Figure 1).

[1]All tables, figures, and supplementary material are presented in the appendices at the end of thesis

# CHAPTER II

## METHODS

### Site Selection

Filter and water eDNA capture samples were collected at the headwater stream, Bucket Branch in Morgan county, Kentucky (latitude 38.05474N, longitude -83.31615W) about 50 m above its confluence. Bucket Branch is an intermittent stream located within the Licking River basin with a 4.22 $km^2$ catchment area. The Bucket Branch site has been sampled for benthic macoinvertebrates (Supplementary Material A, and Roberts pers comm.), and used as a reference site by the Kentucky Division of Water (KDOW).

### Lab Quality Control for DNA Capture

Filters, gloves, forceps, and pipettes were UV sanitized for 15 minutes and then placed in polyethylene bags until use. Filters were placed in Whirl-Paks rather than polyethylene bags. Filter sampling apparatus components were soaked in 10% Sodium hypochlorite aqueous solution, and then rinsed with deionized water. Lab benches were all wiped down with 33% Sodium hypochlorite aqueous solution the night prior to all work done. The filter sampling apparatus was assembled (Figure 3), rinsed with deionized water, the filter head was disassembled, and a new glass fiber filter (47mm, 0.7μm) was placed on the filter head with flame-sanitized forceps (Goldberg et al., 2011). The filter was reassembled, and 2L of deionized water was filtered in the lab. The filter was then placed in 10mL of 95% ethanol, and

stored at -20° C (Goldberg et al., 2011). The filter sampling apparatus was disassembled and placed in new, UV sanitized polyethylene bags. For lab controls of water samples, new 50mL centrifuge tubes were filled with 15mL of deionized water, and preserved with 33 mL of 95% ethanol and 1.5mL of Sodium acetate 3M (Ficetola et al., 2008). Samples were immediately put into -20° C storage until future use. For PCR samples, the PCR box was UV sanitized for 35 minutes and filtered pipette tips were used.

Sample Collection, Preservation, and Storage

Sampling was performed on May 26, 2015 between 10:00 AM and 12:30 PM. Before entering the site, chest waders were soaked in 15% Sodium hypochlorite solution for 10 minutes. Once at the site, a new 55 gallon trash bag was inverted and placed on the ground to provide a work area. Filter apparatus was assembled, and then primed with 2L of stream water from site (Goldberg et al., 2011). Water used for pump priming was from upstream of the work area from the middle of the water column and the center point of the stream. Using flame-sanitized forceps, a new glass fiber filter was placed on the filter head, and 2L of sample was filtered upstream of the work area from the middle of the water column and the center point of the stream. The filter was rolled on the filter-head using forceps, and then placed in 15mL centrifuge tubes with 10mL of ethanol (Goldberg et al., 2011). Field samples were placed on ice until the samples could be placed in -20° C storage within 6 hours.

Measuring with a graduated conical vial, 15mL of stream water was collected from a point proximately the same as water filtration in new 50 mL centrifuge tubes, and preserved with 33 mL of 95% ethanol and 1.5mL of Sodium acetate 3M (Ficetola et al., 2008). All samples were placed on ice, and frozen within 6 hours.

DNA Extraction- Overnight Drying Step and Water Sample DNA Pelleted by Centrifugation

Per filter extraction method, three stream filter samples and one lab control filter were placed on UV sanitized aluminum pans using flame-sanitized forceps. A UV sanitized aluminum pan was placed askew overtop of the filter containing aluminum pan, and allowed to dry overnight (Goldberg et al., 2011). For water eDNA capture, three stream water samples and one lab control water sample were each centrifuged at 5500 x g, 6° C, 35 minutes (Ficetola et al., 2008). The supernatant was decanted, and the remaining pellet was allowed to dry overnight with the tubes inverted.

DNA Extraction- CTAB with OneStep PCR Inhibitor Removal Kit, DNeasy Blood and Tissue Kit, and PowerWater DNA Isolation Kit

One quad from three individual filters was assigned a DNA extraction with the remaining quad archived. Each filter quad was sliced into smaller pieces (about the size of a matchhead) and placed in the initial vessel for each extraction. The first DNA extraction chosen was PowerWater DNA Isolation Kit, in which the manufacture's protocol was followed except for the modifications made by Amberg

et al. (2015) and using 1.25mL of PW1 for the cell lysis step. The second extraction was Qiagen's DNeasy Blood and Tissue Kit (Qiagen GmbH, Hilden, Germany), in which the manufacture's protocol was followed except for the modifications made by Amberg et al. (2015). The elution step for DNeasy and PowerWater was conducted 25µl of nuclease-free water for 2 minutes, and centrifuged for 1 minute at ≥13,000 x rpm. The elution step was repeated with 25µl of nuclease-free water for 2 minutes and centrifuged at ≥13,000 x rpm for increased yield. The samples assigned to the CTAB protocol followed the instructions by Saghai-Maroof et al. (1984), with the exception the DNA was precipitated with 1mL of water and 0.2mL of each CTAB extraction replicate was cleaned with OneStep PCR Inhibitor Removal Kit (Zymo Research Irvine, CA, USA) (Turner et al., 2015). Samples were stored at -20° C until polymerase chain reaction.

DNA Amplification- PCR Optimization for Two 18s Primer Sets

Using eDNA positive controls (10 ng/µl), annealing temperatures for 18s primer sets 563f (5'-GCCAGCAVCYGCGGTAAY-3') and 1132r (5'-CCGTCAATTHCTTYAART-3') (Hugerth et al. 2014), 574*f (5'-CGGTAAYTCCAGCTCYV-3') and 1132r (5'-CCGTCAATTHCTTYAART-3') (Hugerth et al., 2014) were optimized for PCR by having a thermal-cycling regime of 98° C for 1 minute, followed by 30 cycles of 98° C for 1 second, and an annealing gradient of 45.0° C, 46.2° C, 48.2° C, 51.5° C, 55.4° C, 58.4° C, 60.6, or 62.0° C for 5 seconds, and 72° C for 15 seconds. The final extension was 72° C for 1 minute and then a hold at 4° C. A Nanodrop spectrophotometer was used to quantify the amount of DNA for

each extraction sample, which was all standardized to ≤10 ng/µl. Aliquots of 10 µl from filter samples were composited into 2 ml microcentrifuge tubes to make 40 µl of composited eDNA per filter.

18s PCR Amplification and Site Indexer Addition

For 18s metabarcodes, PCR was conducted in 20 µl reactions consisting of 10 µl of Thermo Scientific 2x Phusion Flash PCR Master Mix, 1 µl of UT_563f at 10 µM, 1 µl of UT_1132r at 10 µM, 6 µl of Qiagen nuclease-free $H_2O$, and 2 µl of template eDNA. The thermal-cycling regime was 98° C for 1 minute, followed by 30 cycles of 98° C for 1 second, 51.5° C for 5 seconds, and 72° C for 15 seconds. The final extension was 72° C for 1 minute and then a hold at 4° C. Each DNA capture sample of composited filter quad extractions and water extractions had eight PCR replicates (Ficetola et al. 2014). The PCR product confirmation with gel electrophoresis happened within 20 minutes of completion of PCR.

Gels were made with ~125 ml of 1% agarose with 2 µl of Ethidium bromide per 100 mL of agarose gel. All of the PCR reaction was loaded into gel wells with 4 µl of loading dye. Gel electrophoresis was conducted at 100 Volts and 400 mAmps max. Gel extraction was followed and modified from QIAquick Gel Extraction Kit (Qiagen 2001). PCR product (~550-650 bp) was excised using flame sanitized scalpel and forceps, and was placed in 2 ml microcentrifuge tubes. A 3:1 ratio of 300 µl of QG buffer per 100 mg of PCR product in agarose was added and incubated at 50° C for 20 minutes, vortexing briefly every 3 minutes. Dissolved agarose and QG buffer solution was loaded onto QIAquick spin column in a 2 ml collection tube in

650 µl increments, and centrifuged at ≥10,000 x g for 1 minute. The flow through was discarded, and the dissolved agarose and QG buffer solution was loaded and centrifuged until all of the solution was had been put through the QIAquick spin column. The QIAquick spin column was rinsed with 500 µl by centrifuging for 1 minute at ≥10,000 x g. The QIAquick spin column was rinsed with 750 µl of PE buffer by centrifuging for 1 minute at ≥10,000 x g. PE buffer flow through was discarded, and the QIAquick spin column was dried by centrifuging for 2 minutes at ≥10,000 x g. The spin filter basket was placed in a new 1.5 ml microcentrifuge tube, and DNA was eluted by adding 25 µl of nuclease-free water (Qiagen). The QIAquick spin column stood for 3 minutes, and was centrifuged for 1 minute at ≥10,000 x g. Elution step was repeated again to make 50 µl of DNA solution.

Each DNA capture sample of composited filter quad extractions and water extractions were dual-indexed for bioinformatic identification using universal tail primers (Carew et al., 2013). Each dual site-indexer was unique to DNA sample (Kozich et al., 2013). MiSeq adapters and dual site-indexer addition was conducted in 20 µl reactions consisting of 10 µl of Thermo Scientific 2x Phusion Flash PCR Master Mix, 1 µl of forward site-indexer primer at 10 µM, 1 µl of corresponding reverse site indexer primer at 10 µM, 6 µl of Qiagen nuclease-free $H_2O$, and 2 µl of template eDNA. The thermal-cycling regime was 98° C for 1 minute, followed by 30 cycles of 98° C for 1 second, 51.5° C for 5 seconds, and 72° C for 15 seconds. The final extension was 72° C for 1 minute and then a hold at 4° C. The PCR product confirmation with gel electrophoresis happened within 20 minutes of PCR completion.

Gels were made with ~125 ml of 1% agarose stained with 2 µl of Ethidium bromide. All of the PCR reaction was loaded into gel wells with 4 µl of loading dye. Gel electrophoresis was conducted at 100 Volts and 400 mAmps max. Gel extraction was followed and modified from QIAquick Gel Extraction Kit (Qiagen 2001). PCR product (~650-750 bp) was excised using flame sanitized scalpel and forceps, and was placed in 2 ml microcentrifuge tubes. A 3:1 ratio of 300 µl of QG buffer per 100 mg of PCR product in agarose was added and incubated at 50° C for 20 minutes, vortexing briefly every 3 minutes. Dissolved agarose and QG buffer solution was loaded onto QIAquick spin column in a 2 ml collection tube in 650 µl increments, and centrifuged at ≥10,000 x g for 1 minute. The flow through was discarded, and the dissolved agarose and QG buffer solution was continued to be loaded and centrifuged until all of the solution had been put through the QIAquick spin column. The QIAquick spin column was rinsed with 500 µl by centrifuging for 1 minute at ≥10,000 x g. The QIAquick spin column was rinsed with 750 µl of PE buffer by centrifuging for 1 minute at ≥10,000 x g. PE buffer flow through was discarded, and the QIAquick spin column was dried by centrifuging for 2 minutes at ≥10,000 x g. The spin filter basket was placed in a new 1.5 ml microcentrifuge tube, and DNA was eluted by adding 25 µl of nuclease-free water (Qiagen). The QIAquick spin column stood for 3 minutes, and was centrifuged for 1 minute at ≥10,000 x g. Elution step was repeated again to make 50 µl of DNA solution.

COI PCR Amplification and Nextera XT Kit

For COI metabarcodes, primers LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer et al., 1994) were used, and PCR was conducted in 20 µl reactions consisting of 10 µl of Thermo Scientific 2x Phusion Flash PCR Master Mix, 1 µl of LCO1490 at 10 µM, 1 µl of HCO2198 at 10 µM, 6 µl of Qiagen nuclease-free $H_2O$, and 2 µl of template eDNA. The thermal-cycling regime was 98° C for 1 minute, followed by 30 cycles of 98° C for 1 second, 45° C for 5 seconds, and 72° C for 15 seconds. The final extension was 72° C for 1 minute and then a hold at 4° C. Each eDNA capture and extraction replicate had eight PCR replicates (Ficetola et al., 2014). The PCR product confirmation with gel electrophoresis happened within 20 minutes of PCR completion.

Gels were made with ~125 ml of 1% agarose stained with 2 µl of Ethidium bromide. 5 µl of the PCR reaction was loaded into gel wells with 1 µl of loading dye. Gel electrophoresis was conducted at 100 Volts and 400 mAmps max. Samples with confirmed PCR amplification were composited with corresponding replicates. Composited samples underwent post-PCR cleanup with AMPure XP beads following recommended manufacturer's protocol except 0.6 x bead concentration was used for the COI 710 bp fragment size (p. 31, Nextera XT DNA 96 kit, Illumina, Inc., San Diego, CA, USA) and sequencing preparation with the Nextera XT DNA 96 Kit (Illumina, Inc., San Diego, CA, USA) (Deiner et al., 2015a).

Next-Gen Sequencing with Illimina MiSeq Platform

Amplicons of the 18s gene region (Hugerth et al., 2014) and the COI region (Folmer et al., 1994) were sequenced using the MiSeq Illumina platform (Illumina, San Diego, CA) at the UKy HealthCare Genomic Core Laboratory. DNA was quantified using the Qubit fluorometer (ThermoFisher Scientific) and qPCR to test for sample quality prior to high-throughput sequencing.

Bioinformatic Analysis

A data request was completed for all aquatic invertebrates sampled and morphologically identified by Kentucky Division of Water (KDOW) biologists for the Kentucky macroinvertebrate bioassessment index (MBI) completed for the bucket branch site (Supplementary Material A). A species search was performed in GenBank using the taxa lists KDOW, and the first ten sequences in the COI target region (Folmer et al., 1994) of each taxon were used to make a reference database (Supplementary Material B). Sequenced community data was processed using a bioinformatics pipeline in QIIME (Caporaso et al., 2010). Paired end sequence reads were joined with 50 bp overlaps and 90% similarity threshold using the join_paired_ends.py command (Aronesty, 2011). The resulting FASTQ files and validated mapping files (validate_mapping_file.py) were quality filtered with the split_libraries_fastq.py command using default parameters with a quality score threshold of 30 allowing for 0-5 low quality base calls and a singleton reads were removed. OTU picking and reference based taxonomic assignments were made performing the pick_closed_reference_otus.py command using default parameters

(Caporaso et al., 2010) and the reference database in FASTA format (Supplementary Material B).

Statistical Analyses

Taxa assignments from CTAB and DNeasy filter extractions were analyzed by calculating alpha-diversity using the alpha_diversity.py command (Caporaso et al., 2010). Additional statistical computations performed were Jaccard's dissimilarity index and by means of the package VEGAN in R (Oksanen et al., 2016; R Core Team, 2016). In order conduct the Jaccard dissimilarity index, each extraction replicate was recorded as having a taxa present or absent, then the dissimilarity was compared across extraction replicates individually. The data was visualized in ordinal plots using non-metric multidimensional scaling (NMDS) by means of the package VEGAN in R (Oksanen et al., 2016; R Core Team, 2016).

# CHAPTER III

## RESULTS

When using gel electrophoresis for confirmation of successful PCR for DNA capture (precipitation from water or filtration) combined with type of DNA extraction (MoBio Power Water, Qiagen DNeasy, or CTAB), banding in the 550-650 bp (18s) or ~710 bp (COI) ranges were deemed successful (Table 1). PowerWater samples of all types were deemed unsuccessful and were not carried forward with the study. With each DNA extraction (Qiagen DNeasy or CTAB) of filter samples eight PCR replicates for each primer combination were carried out, and reactions with gel banding were deemed acceptable to be ran on the Illumina MiSeq (Illumina, Inc., San Diego, CA, USA) after the AMPure XP bead cleanup (Illumina, Inc., San Diego, CA, USA)(Table 2). DNA concentrations were recorded for both pre-PCR and post-PCR [1](Table 3). Lab and negative controls did not yield bands with gel electrophoresis confirmation or after AMPure XP bead cleanup, and so were not ran on the Illumina MiSeq. Based upon low FASTQC sequence quality scores, the 18s dataset was deemed non-reportable, and so was not analyzed further.

The FASTQ files of the raw paired end reads for the COI data were received and after being subjected to the bioinformatics pipeline discussed above, CTAB and DNeasy extractions when combined with the respective replicates were found to yield a genus richness ($\alpha$-diversity) of n=25 and n=24, respectively (Table 3), with taxonomic assignments made at 97% similarity to corresponding reference sequences (Table 4). Twenty taxa were shared between CTAB and DNeasy

extractions, with five taxa only being found by CTAB and four taxa only being found by DNeasy. Jaccard's dissimilarity analysis was used to compare the relationship of species identified between all replicates without regard to extraction type (Table 5). NMDS plots did not reveal differences in the amount of taxa richness between the two methodologies (Non-metric fit, $R^2$ = 0.986, stress=0.1199294, two dimensions, Figure 4), which demonstrated a lack of dissimilarity between DNA of genera captured between CTAB and Qiagen DNeasy extractions ($\beta$-diversity).

# CHAPTER IV

## DISCUSSION

### Effect of methods on detection of invertebrate species

The data from this study supports the current literature in that sampling capture of eDNA is most effective using filtration methods over DNA precipitation from water samples (Barnes & Turner, 2015; Deiner et al., 2015b; Table 2). This is possibly due to filtration has 2L of the water column pass through the filter versus DNA precipitation from water, which only uses 15 mL of stream water. However, other research has predicted water filtration to be more advantageous than water precipitation in estimating eukaryotic diversity with capture COI eDNA when the two methods are estimated to have even capture volume (Deiner et al., 2015b).

This study's data also supports research demonstrating PowerWater is less effective in capturing eDNA than DNeasy and CTAB (Deiner et al., 2015b; Table 2), though my data does suggest that when comparing DNeasy and CTAB, the community diversity estimates are similar. The CTAB extraction did display an advantage over DNeasy in successful PCR attempts (Table 3), which may be due to individual extraction protocols handling of PCR inhibition removal or low eDNA concentrations.

This study did not try to make relative abundance or population size assumptions based upon number of sequencing reads or eDNA concentration, due to the variability and heterogeneity of eDNA in streams (Pilliod et al., 2013; Klymus, et al., 2015; Turner et al., 2015; Civade et al., 2016; Souza et al., 2016), but instead

focused on detecting the presence of species previously found at the sample site through taxonomic assignment of eDNA recovered at the site. OTU picking was made via a closed reference database with only species found to be recently present at the site (Supplementary Material A), which limited the likelihood of spurious identifications of species. By limiting reference database and lowering the chances of detecting spatially distance species with the sample site being located in a headwater stream, the likelihood of making false presence errors was limited (Deiner & Altermatt, 2014; Rees et al., 2014; Roussel et al., 2015; Franklin, 2016).

The downside to limiting the bioinformatics analyses to a close-reference library limited the species detected to only those species sampled and identified through traditional netting and morphological identification with sequences in Genbank. An example of this, would be that only one Dytiscidae sequence was used in the reference library, which gives only a family level resolution for "the largest family of water beetles in North America" with 50 genera, 503 species (Thorp & Covich, 2010). This leads to the possibility of missed eDNA detection of species with DNA sequences input for OTU picking. This study was only able to determine 29 species (Table 5) of 93 reference taxa (Supplementary Material B), which were derived from the 95 taxonomic determinations made by KDOW [1](Supplementary Material A).

More species with records in surrounding watersheds could have possibly been detected if the species had their COI sequences DNA barcoded and shared in a public DNA repository. More work needs to be completed in adding and curating

DNA sequences to public reference libraries in order to make using local reference libraries a powerful tool in eDNA studies and future monitoring programs.

Conclusions

This study was unable to determine differences in detected $\beta$-diversity of taxonomically assigned COI sequences found between CTAB and DNeasy extraction methods (Table 6; Figure 4). CTAB protocols are relatively cheap and may be modified to suit an investigator's needs (Turner et al., 2015, Mullins unpublished data), and so it is this study's recommendation CTAB be used over DNeasy when budgetary constraints are a factor in designing eDNA studies, though DNeasy may be more time efficient since it lacks overnight steps.

Environmental DNA practices are still being refined and will continue to do so as capture and molecular technologies advance. This study compared commonly used extraction protocols, and found relatively little difference between the effect of CTAB and DNeasy DNA extractions on species detection. Factors, which were not assessed in this study, that should be studied in the future are the sampling of streams between different sub-habitats in the reach scale and the different geomorphology and physiochemical processes associated with those sample points and their effects on extraction types. In order for eDNA to be considered a viable monitoring tool put into common use, method results must be validated and give insights that would otherwise be missed. Metabarcoding of eDNA from natural aquatic environments shows promise to aid in ecological management programs (Civade et al., 2016), but more research needs to completed to account for eDNA

performance in a variety of chemical, physical, and fluvial conditions to make the use of this tool a reality.

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# APPENDICES

APPENDIX A:

Tables

Table 1. Successful DNA capture and extraction methods confirmed by polymerase chain reaction (PCR) gel electrophoresis.

| | | | | DNA precipitation from water | | | DNA on glass fiber filter | | |
|---|---|---|---|---|---|---|---|---|---|
| Forward Primer | Reverse Primer | Reference | Locus | MoBio PowerWater | Qiagen Dneasy | CTAB | MoBio PowerWater | Qiagen Dneasy | CTAB |
| LCO1490 | HCO2198 | Folmer et al. 1994 | COI | - | - | - | - | + | + |
| 563f | 1132r | Hugerth etl al. 2014 | 18s | - | - | - | - | +* | +* |
| 574f* | 1132r | Hugerth etl al. 2014 | 18s | - | - | - | - | - | - |

Note(s): (+) indicates at least one successful reaction and (-) indicates a complete absence of positive reactions

*Sequences not bioinformatically analyzed due to low quality

Table 2. Gel confirmation of polymerase chain reactions (PCR)with primers LCO1490 and HCO2198 and 563f and 1132r successes of DNA extracted from glass fiber filters using Qiagen DNeasy or CTAB.

| DNA extraction Type | CTAB | | | Qiagen Dneasy | | |
|---|---|---|---|---|---|---|
| Extraction Replicate | CTAB 1 | CTAB 2 | CTAB 3 | DNeasy 1 | DNeasy 2 | DNeasy 3 |
| Number of Successful Reactions COI (n=8) | 8 | 8 | 8 | 8 | 2 | 7 |
| Number of Successful 18s Reactions (n=8) | 8 | 8 | 8 | 8 | 8 | 8 |

Table 3. Number of sequences left after each bioinformatic step and number of OTUs found in Qiagen Dneasy and CTAB extractions.

| DNA extraction Type | CTAB | | | Qiagen Dneasy | | |
|---|---|---|---|---|---|---|
| Extraction Replicate | CTAB 1 | CTAB 2 | CTAB 3 | DNeasy 1 | DNeasy 2 | DNeasy 3 |
| Pre-PCR DNA concentration (ng/µl) Nanodrop (±SD) | 21.9 (±3.86) | 19.7 (±0.306) | 17.4 (±0.551) | 2.77 (±1.68) | 6.77 (±5.30) | 2.73 (±1.93) |
| Pre-PCR 260/280 (nm) wavelength ratio Nanodrop | 1.30 | 1.17 | 1.24 | 3.77 | 1.26 | 3.41 |
| Post-PCR DNA concentration (ng/µl) Qubit | 6.6 | 3.3 | 14.2 | 10.3 | 8.3 | 5.3 |
| Mean Raw Read Sequence Length (±SD) | 441.59 (±77.84) | 441.77 (±77.74) | 448.67 (±74.00) | 444.38 (±75.82) | 446.54 (±76.03) | 438.6 (±79.81) |
| Raw reads | 1044409 | 1052501 | 1027892 | 780586 | 2282526 | 1062014 |
| Joined Paired End Reads | 1037885 | 1046887 | 1020970 | 775772 | 2264984 | 1053971 |
| Split Libraries FASTQ filtering | 929851 | 557470 | 1004725 | 695309 | 2073958 | 986111 |
| Reads assigned to MOTUs | 2461 | 1801 | 4803 | 3922 | 12612 | 4582 |
| Taxa Richness after MOTU taxa assignment | 6 | 10 | 23 | 15 | 21 | 9 |

Table 4. Genus level taxonomic assignments made from each filter sample replicate with corresponding DNA extraction technique.

| Genus | CTAB_1 | CTAB_2 | CTAB_3 | Dneasy_1 | Dneasy_2 | Dneasy_3 |
|---|---|---|---|---|---|---|
| *Cheumatopsyche* | | + | | | + | |
| Dytiscidae | | + | + | | + | |
| *Thienemanniella* | + | | + | + | + | + |
| *Thienemannimyia* | | | + | + | + | |
| *Trissopelopia* | | | + | | | |
| *Zavrelimyia* | | + | + | | + | |
| Tanypodinae | | | + | | | |
| *Prosimulium* | | | | | + | |
| *Simulium* | | | | + | + | |
| *Oulimnius* | | | | | + | |
| *Stenelmis* | | | | + | + | |
| *Pseudolimnophila* | | | + | | + | |
| *Habrophlebia* | + | | + | + | + | |
| *Leptophlebia* | | | + | | | |
| *Paraleptophlebia* | | | + | | | |
| *Pycnopsyche* | + | | + | | + | |
| *Microtendipes* | | | + | | | |
| *Polypedilum* | + | + | + | + | + | + |
| *Tanytarsus* | | | + | + | + | + |
| Leuctridae | | + | + | + | + | + |
| *Cinygmula* | | | + | + | + | + |
| *Epeorus* | | + | + | + | + | + |
| *Maccaffertium* | | + | + | | + | + |
| *Stenacron* | | | + | + | + | |
| Heptageniidae | | | + | | | + |
| *Physa* | | + | + | + | | + |
| *Amphinemura* | + | + | + | + | + | |
| *Alloperla* | | + | | + | + | |
| *Cambarus* | + | | + | + | | |

Note(s): (+) - indicates operational taxonimic unit assigned to genus reference sequence to 97% similarity

Table 5. Jaccard dissimilarity index between DNA extraction replicates.

| | CTAB 1 | CTAB 2 | CTAB 3 | DNeasy 1 | DNeasy 2 |
|---|---|---|---|---|---|
| CTAB 2 | 0.86 | | | | |
| CTAB 3 | 0.74 | 0.68 | | | |
| DNeasy 1 | 0.69 | 0.68 | 0.54 | | |
| DNeasy 2 | 0.77 | 0.59 | 0.48 | 0.43 | |
| DNeasy 3 | 0.85 | 0.64 | 0.61 | 0.59 | 0.70 |

APPENDIX B:

Figures

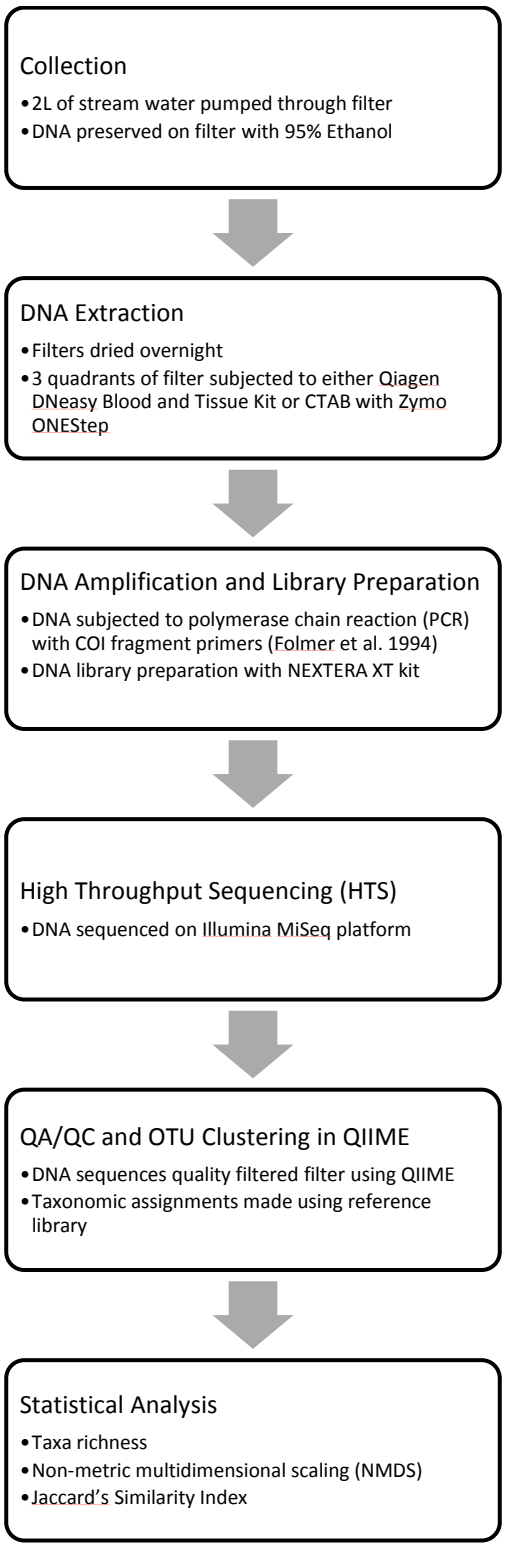


Figure 1. Workflow of processing environmental DNA samples from collection, extraction, amplification, and sequencing for targeting COI gene region.

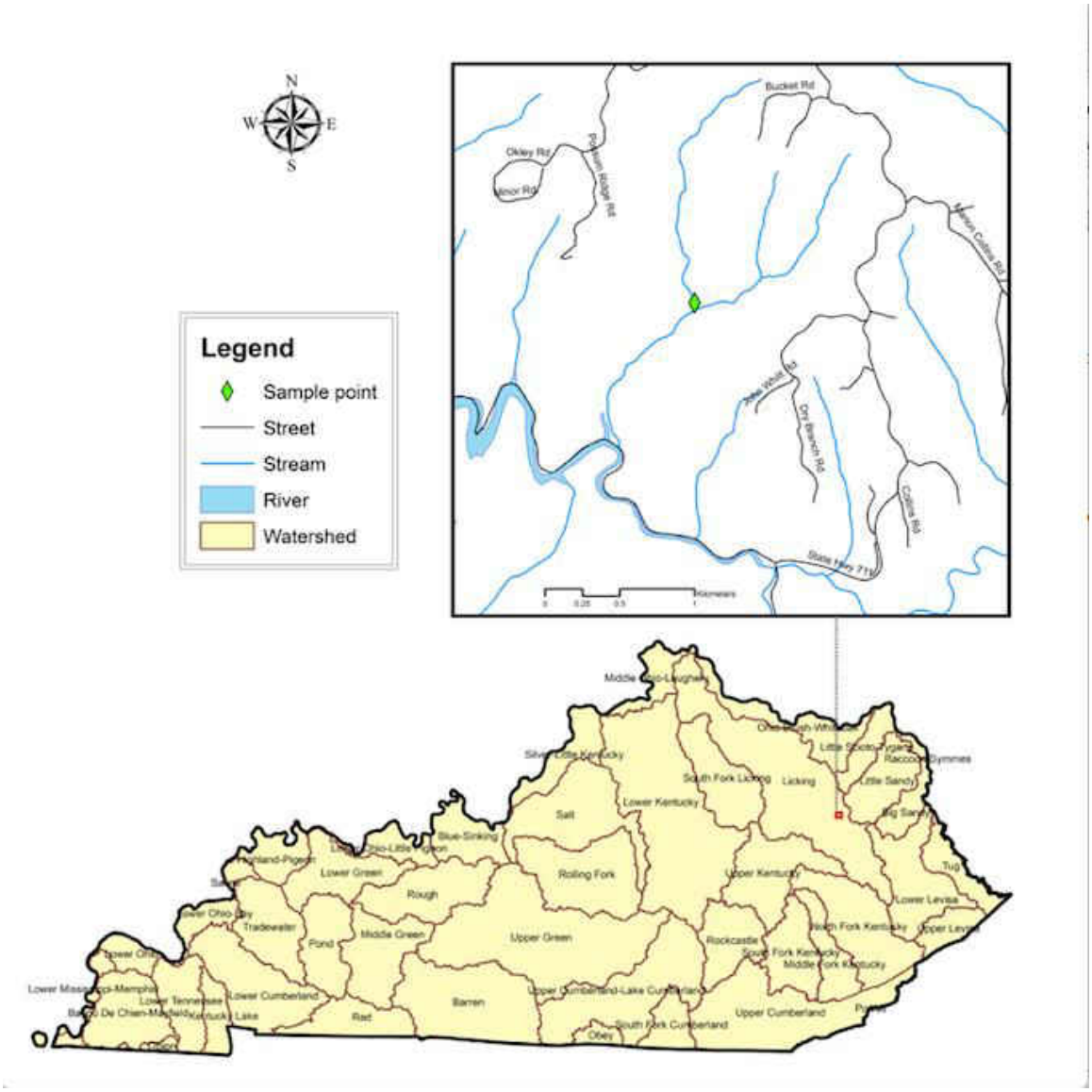


Figure 2. Location Kentucky Division of Water reference site in the watershed of the headwater stream Bucket Branch (38.05474N, longitude -83.31615W), located within the 8-HUC Licking River watershed.

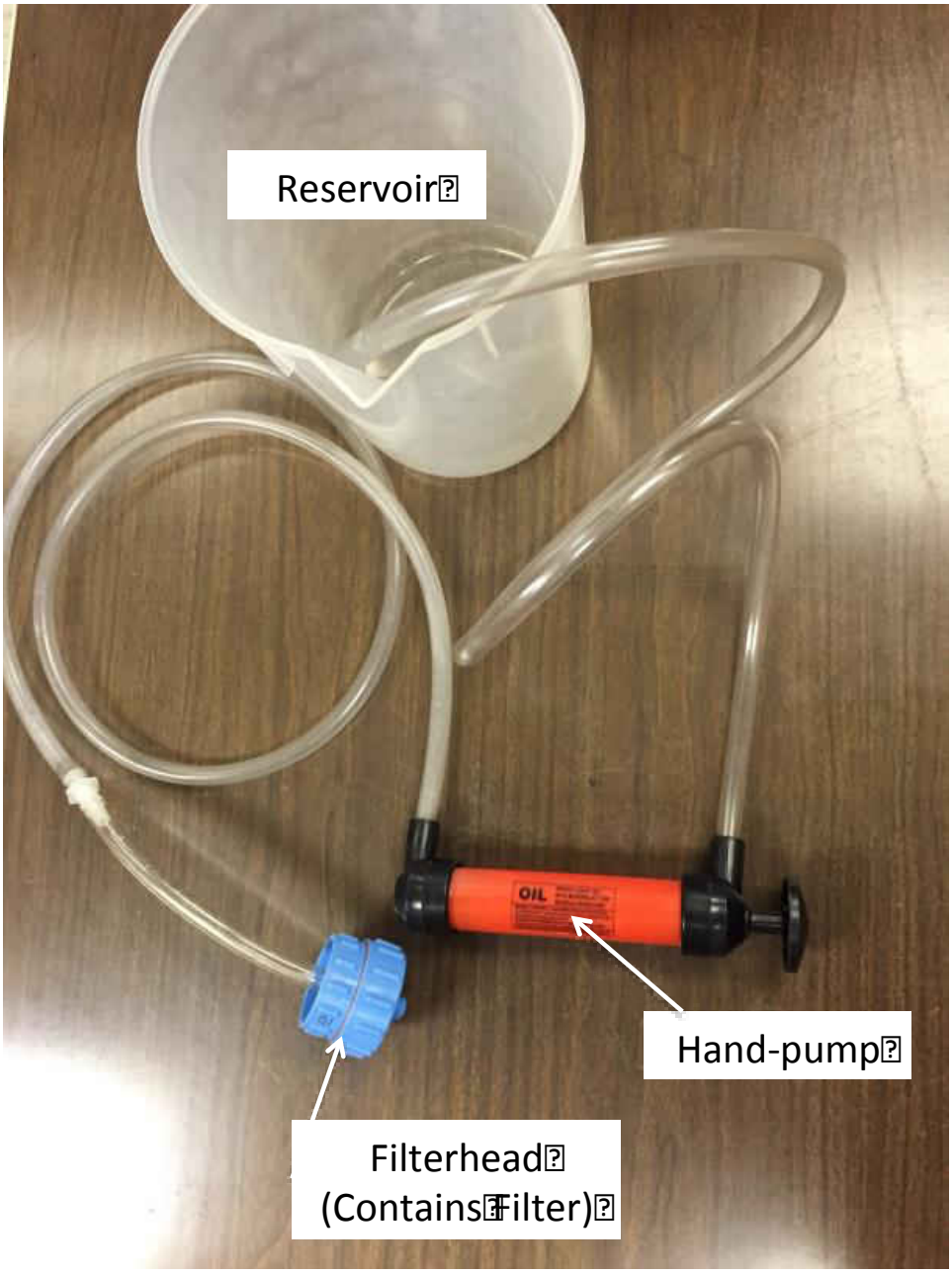


Figure 3. Pumping apparatus for filter capture of eDNA. Stream water is drawn through filterhead by pumping the hand-pump, which is measured in the reservoir at the end of the polyethylene tubing connecting the filterhead, hand-pump, and the reservoir.

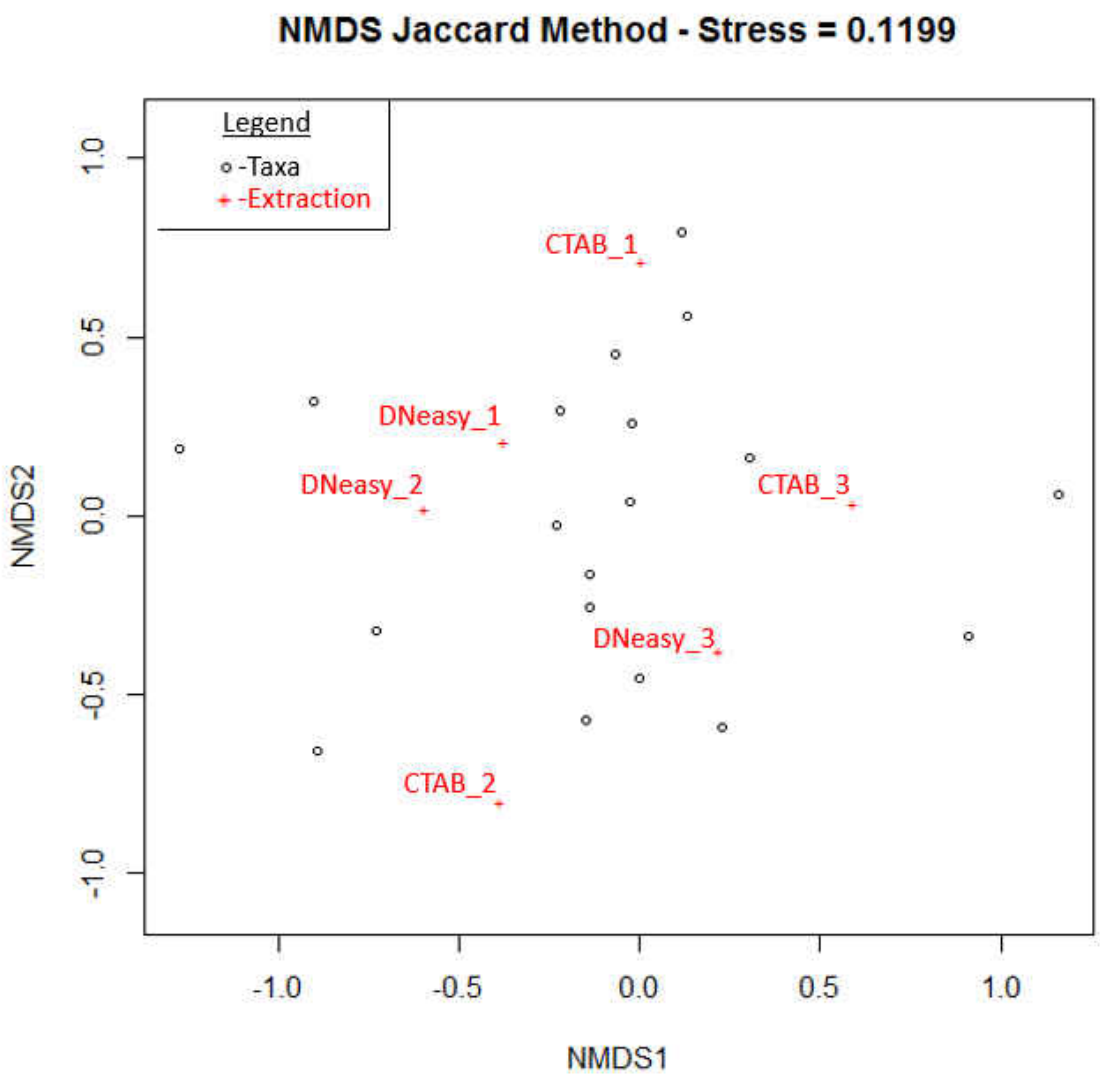


Figure 4. Non-metric multidimensional scaling (NMDS) using Jaccard method for presence data of taxonomic assignments made from Illumina MiSeq data made to 97% similarity to reference sequences derived from CTAB and DNeasy extractions captured by filtration of stream water at Bucket Branch, KY.

# APPENDIX C:

## Supplementary Material

Supplementary Material A. List of aquatic invertebrate species collected in 2014 and identified by Kentucky Division of Water (KDOW) biologists from Bucket Branch, KY.

| # | Species Identified |
|---|---|
| 1 | *Acentrella turbida* |
| 2 | *Acroneuria abnormis* |
| 3 | *Acroneuria carolinensis* |
| 4 | *Agapetus* sp. |
| 5 | *Alloperla* sp. |
| 6 | *Ameletus* sp. |
| 7 | *Amphinemura* sp. |
| 8 | *Antocha* sp. |
| 9 | *Baetis* sp. |
| 10 | *Bezzia/Palpomyia* gp |
| 11 | *Calopteryx maculata* |
| 12 | *Cambarus bartonii cavatus* |
| 13 | *Cambarus* sp. |
| 14 | *Cheumatopsyche* sp. |
| 15 | *Chimarra aterrima* |
| 16 | *Cinygmula subaequalis* |
| 17 | *Clinocera* sp. |
| 18 | *Cordulegaster erronea* |
| 19 | *Diamesa* sp. |
| 20 | *Diphetor hageni* |
| 21 | *Diplectrona modesta* |
| 22 | *Dixa* sp. |
| 23 | *Ectopria* sp. |
| 24 | *Epeorus* sp. |
| 25 | *Ephemera blanda* |
| 26 | *Ephemerella hispida* |
| 27 | *Ephemerella* sp. |
| 28 | *Eukiefferiella claripennis* gp |
| 29 | *Eurylophella funeralis* |
| 30 | *Glossosoma nigrior* |
| 31 | *Habrophlebia* sp. |
| 32 | *Haploperla brevis* |
| 33 | *Helichus basalis* |
| 34 | *Helochares* sp. |
| 35 | *Hexatoma* sp. |
| 36 | *Hydrobius melaenus* |
| 37 | *Ironoquia* sp. |
| 38 | *Isoperla* sp. |
| 39 | *Lanthus* sp. |

40 *Leptophlebia* sp.
41 *Leucrocuta* sp.
42 *Maccaffertium* sp.
43 *Maccaffertium vicarium*
44 *Microtendipes* sp.
45 *Natarsia baltimorea*
46 *Neophylax aniqua*
47 *Neophylax* sp.
48 *Neoporus* sp.
49 *Nigronia serricornis*
50 *Nyctiophylax moestus*
51 *Orconectes cristavarius*
52 *Oulimnius latiusculus*
53 *Paraleptophlebia* sp.
54 *Phylocentropus* sp.
55 *Physa* sp.
56 *Polycentropus* sp.
57 *Polypedilum aviceps*
58 *Polypedilum illinoense*
59 *Polypedilum* sp.
60 *Prosimulium* sp.
61 *Psephenus herricki*
62 *Pseudolimnophila* sp.
63 *Psilotreta* sp.
64 *Pycnopsyche* sp.
65 *Rheotanytarsus exiguus* gp
66 *Simulium* sp.
67 *Somatochlora* sp.
68 *Stempellinella* sp.
69 *Stenacron interpunctatum*
70 *Stenacron minnetonka*
71 *Stenacron pallidum*
72 *Stenelmis crenata*
73 *Stenelmis* sp.
74 *Stenonema femoratum*
75 *Stictochironomus* sp.
76 *Stylogomphus sigmastylus*
77 *Sweltsa* sp.
78 *Tanytarsus* sp.
79 *Thienemanniella* sp.
80 *Thienemannimyia* gp
81 *Trissopelopia ogemawi*
82 Unid. Chloroperlid sp.
83 Unid. Glossosomatidae sp.
84 Unid. Heptageniid sp.

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|---|---|
| 85 | Unid. Hydracarina (mite) sp. |
| 86 | Unid. Leptophlebiid sp. |
| 87 | Unid. Leuctrid sp. |
| 88 | Unid. Lumbriculid sp. |
| 89 | Unid. Naidid sp. |
| 90 | Unid. Orthoclad sp. |
| 91 | Unid. Perlodid sp. |
| 92 | Unid. Polycentropodid sp. |
| 93 | Unid. Tanypodinae sp. |
| 94 | Unid. Tanytarsini sp. |
| 95 | *Zavrelimyia* sp. |

Supplementary Material B. Reference library used for bioinformatic analysis arranged by accession numbers in alphabetical and numerical order

| # | GenBank accession number | Taxonomic Classification |
|---|---|---|
| 1 | AY165634.1 | *Stenonema femoratum* |
| 2 | AY165680.1 | *Psephenus herricki* |
| 3 | AY326800.1 | *Diphetor hageni* |
| 4 | AY326850.1 | *Stenacron interpunctatum* |
| 5 | AY326854.1 | *Maccaffertium vicarium* |
| 6 | AY326855.1 | *Stenonema femoratum* |
| 7 | AY326869.1 | *Stenacron interpunctatum* |
| 8 | AY326870.1 | *Stenacron interpunctatum* |
| 9 | AY326872.1 | *Stenacron interpunctatum* |
| 10 | AY326874.1 | *Stenacron interpunctatum* |
| 11 | AY326876.1 | *Stenacron interpunctatum* |
| 12 | AY326889.1 | *Stenonema femoratum* |
| 13 | AY326891.1 | *Stenonema femoratum* |
| 14 | AY326942.1 | *Stenacron interpunctatum* |
| 15 | DQ393853.1 | *Tanytarsus* sp. |
| 16 | DQ393864.1 | *Cladotanytarsus* sp. |
| 17 | DQ393871.1 | *Tanytarsus* sp. |
| 18 | DQ393876.1 | *Tanytarsus* sp. |
| 19 | DQ393877.1 | *Cladotanytarsus* sp. |
| 20 | DQ393878.1 | *Tanytarsus* sp. |
| 21 | DQ411780.1 | *Cambarus* sp. |
| 22 | DQ411781.1 | *Cambarus* sp. |
| 23 | DQ411782.1 | *Cambarus* sp. |
| 24 | EU038373.1 | *Physa gyrina* |
| 25 | EU038374.1 | *Physa gyrina* |
| 26 | EU038398.1 | *Physa gyrina* |
| 27 | FJ373016.1 | *Physa acuta* |
| 28 | FJ819855.1 | *Helochares* sp. |
| 29 | FJ819856.1 | *Helochares* sp. |
| 30 | FJ819857.1 | *Helochares* sp. |
| 31 | FJ819858.1 | *Helochares* sp. |
| 32 | FJ819859.1 | *Helochares* sp. |
| 33 | FJ819918.1 | *Helochares* sp. |
| 34 | FJ819919.1 | *Helochares* sp. |
| 35 | FJ819920.1 | *Helochares* sp. |
| 36 | GQ329628.1 | *Kalyptogaster erronea* |
| 37 | GQ415038.1 | *Physa acuta* |
| 38 | GQ415040.1 | *Physa* sp. |
| 39 | GQ415041.1 | *Physa* sp. |

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|---|---|---|
| 40 | GQ415042.1 | *Physa* sp. |
| 41 | GQ415043.1 | *Physa* sp. |
| 42 | GU066813.1 | Lumbriculidae sp. |
| 43 | GU115794.1 | *Isoperla* sp. |
| 44 | GU115795.1 | *Isoperla* sp. |
| 45 | GU115797.1 | *Isoperla* sp. |
| 46 | GU115799.1 | *Isoperla* sp. |
| 47 | GU247995.1 | *Physa acuta* |
| 48 | GU247996.1 | *Physa acuta* |
| 49 | GU667740.1 | *Agapetus* sp. |
| 50 | GU667742.1 | *Agapetus* sp. |
| 51 | GU667745.1 | *Agapetus* sp. |
| 52 | GU682377.1 | Heptageniidae sp. |
| 53 | GU711736.1 | *Neophylax* sp. |
| 54 | GU711791.1 | *Neophylax* sp. |
| 55 | GU711792.1 | *Neophylax* sp. |
| 56 | GU711793.1 | *Neophylax* sp. |
| 57 | GU711814.1 | *Neophylax* sp. |
| 58 | GU711817.1 | *Neophylax* sp. |
| 59 | HE651537.1 | *Baetis* sp. |
| 60 | HE651539.1 | *Baetis* sp. |
| 61 | HE651540.1 | *Baetis* sp. |
| 62 | HE651541.1 | *Baetis* sp. |
| 63 | HM102054.1 | *Phylocentropus carolinus* |
| 64 | HM102105.1 | *Psilotreta* sp. |
| 65 | HM102106.1 | *Psilotreta* sp. |
| 66 | HM102110.1 | *Psilotreta* sp. |
| 67 | HM102701.1 | *Diplectrona modesta* |
| 68 | HM102702.1 | *Diplectrona modesta* |
| 69 | HM102963.1 | *Neophylax aniqua* |
| 70 | HM103095.1 | *Phylocentropus carolinus* |
| 71 | HM103101.1 | *Phylocentropus lucidus* |
| 72 | HM900435.1 | *Eurylophella funeralis* |
| 73 | HQ105379.1 | *Trissopelopia longimana* |
| 74 | HQ105383.1 | *Zavrelimyia* sp. |
| 75 | HQ105384.1 | *Zavrelimyia* sp. |
| 76 | HQ150602.1 | *Ameletus* sp. |
| 77 | HQ150603.1 | *Ameletus* sp. |
| 78 | HQ150604.1 | *Ameletus* sp. |
| 79 | HQ150783.1 | *Leucrocuta* sp. |
| 80 | HQ151285.1 | *Ameletus* sp. |
| 81 | HQ151286.1 | *Ameletus* sp. |
| 82 | HQ151438.1 | *Ironoquia* sp. |
| 83 | HQ151486.1 | *Maccaffertium vicarium* |
| 84 | HQ151559.1 | *Diphetor hageni* |

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|---|---|---|
| 85 | HQ151584.1 | *Epeorus* sp. |
| 86 | HQ151668.1 | *Eurylophella funeralis* |
| 87 | HQ151670.1 | *Eurylophella funeralis* |
| 88 | HQ151671.1 | *Eurylophella funeralis* |
| 89 | HQ151672.1 | *Eurylophella funeralis* |
| 90 | HQ151673.1 | *Eurylophella funeralis* |
| 91 | HQ151810.1 | *Acroneuria carolinensis* |
| 92 | HQ151811.1 | *Acroneuria carolinensis* |
| 93 | HQ151820.1 | *Ameletus* sp. |
| 94 | HQ151821.1 | *Ameletus* sp. |
| 95 | HQ151822.1 | *Ameletus* sp. |
| 96 | HQ151899.1 | *Ephemerella* sp. |
| 97 | HQ151901.1 | *Ephemerella* sp. |
| 98 | HQ151902.1 | *Ephemerella* sp. |
| 99 | HQ151903.1 | *Ephemerella* sp. |
| 100 | HQ151904.1 | *Ephemerella* sp. |
| 101 | HQ151905.1 | *Ephemerella* sp. |
| 102 | HQ151906.1 | *Ephemerella* sp. |
| 103 | HQ151907.1 | *Ephemerella* sp. |
| 104 | HQ151908.1 | *Ephemerella* sp. |
| 105 | HQ151909.1 | *Ephemerella* sp. |
| 106 | HQ152066.1 | Perlodidae sp. |
| 107 | HQ152155.1 | *Diphetor hageni* |
| 108 | HQ152356.1 | *Cinygmula subaequalis* |
| 109 | HQ152357.1 | *Cinygmula subaequalis* |
| 110 | HQ152358.1 | *Cinygmula subaequalis* |
| 111 | HQ152359.1 | *Cinygmula subaequalis* |
| 112 | HQ152360.1 | *Diphetor hageni* |
| 113 | HQ152361.1 | *Diphetor hageni* |
| 114 | HQ152362.1 | *Diphetor hageni* |
| 115 | HQ152510.1 | *Acroneuria carolinensis* |
| 116 | HQ152522.1 | *Cinygmula subaequalis* |
| 117 | HQ152524.1 | *Diphetor hageni* |
| 118 | HQ152575.1 | *Ephemerella* sp. |
| 119 | HQ152697.1 | Perlodidae sp. |
| 120 | HQ152698.1 | Perlodidae sp. |
| 121 | HQ152699.1 | Perlodidae sp. |
| 122 | HQ152700.1 | Perlodidae sp. |
| 123 | HQ152701.1 | Perlodidae sp. |
| 124 | HQ152702.1 | Perlodidae sp. |
| 125 | HQ152703.1 | Perlodidae sp. |
| 126 | HQ152704.1 | Perlodidae sp. |
| 127 | HQ152705.1 | Perlodidae sp. |
| 128 | HQ152706.1 | Perlodidae sp. |
| 129 | HQ152728.1 | *Diphetor hageni* |

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|---|---|---|
| 130 | HQ152729.1 | *Diphetor hageni* |
| 131 | HQ152730.1 | *Diphetor hageni* |
| 132 | HQ152741.1 | *Eurylophella funeralis* |
| 133 | HQ261162.1 | *Stenacron* sp. |
| 134 | HQ261163.1 | *Stenacron* sp. |
| 135 | HQ261164.1 | *Stenacron* sp. |
| 136 | HQ571191.1 | *Leucrocuta* sp. |
| 137 | HQ571211.1 | *Ephemera blanda* |
| 138 | HQ571212.1 | *Ephemera blanda* |
| 139 | HQ571213.1 | *Ephemera blanda* |
| 140 | HQ571214.1 | *Ephemera blanda* |
| 141 | HQ571215.1 | *Ephemera blanda* |
| 142 | HQ571216.1 | *Ephemera blanda* |
| 143 | HQ660033.1 | *Physa gyrina* |
| 144 | HQ939489.1 | *Diphetor hageni* |
| 145 | HQ943407.1 | *Maccaffertium vicarium* |
| 146 | HQ943408.1 | *Eurylophella funeralis* |
| 147 | HQ943441.1 | *Maccaffertium vicarium* |
| 148 | HQ943471.1 | *Stenacron interpunctatum* |
| 149 | HQ943495.1 | *Stenacron interpunctatum* |
| 150 | HQ943502.1 | *Stenacron interpunctatum* |
| 151 | HQ979249.1 | Dytiscidae sp. |
| 152 | JF286647.1 | *Antocha* sp. |
| 153 | JF286648.1 | *Antocha* sp. |
| 154 | JF286649.1 | *Antocha* sp. |
| 155 | JF286650.1 | *Antocha* sp. |
| 156 | JF286651.1 | *Antocha* sp. |
| 157 | JF286652.1 | *Antocha* sp. |
| 158 | JF286654.1 | *Antocha* sp. |
| 159 | JF286655.1 | *Antocha* sp. |
| 160 | JF286960.1 | *Ephemerella* sp. |
| 161 | JF286961.1 | *Ephemerella* sp. |
| 162 | JF286964.1 | *Ephemerella* sp. |
| 163 | JF287254.1 | Heptageniidae sp. |
| 164 | JF287373.1 | Leuctridae sp. |
| 165 | JF287375.1 | Leuctridae sp. |
| 166 | JF287376.1 | Leuctridae sp. |
| 167 | JF287643.1 | *Oulimnius latiusculus* |
| 168 | JF287644.1 | *Oulimnius latiusculus* |
| 169 | JF287647.1 | *Oulimnius latiusculus* |
| 170 | JF287649.1 | *Oulimnius latiusculus* |
| 171 | JF287650.1 | *Oulimnius latiusculus* |
| 172 | JF287774.1 | *Prosimulium* sp. |
| 173 | JF287775.1 | *Prosimulium* sp. |
| 174 | JF287851.1 | *Psephenus herricki* |

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|---|---|---|
| 175 | JF287852.1 | *Psephenus herricki* |
| 176 | JF287853.1 | *Psephenus herricki* |
| 177 | JF288032.1 | *Stenelmis crenata* |
| 178 | JF288033.1 | *Stenelmis crenata* |
| 179 | JF288034.1 | *Stenelmis crenata* |
| 180 | JN197443.1 | *Amphinemura* sp. |
| 181 | JN197444.1 | *Amphinemura* sp. |
| 182 | JN197445.1 | *Amphinemura* sp. |
| 183 | JN197446.1 | *Amphinemura* sp. |
| 184 | JN197447.1 | *Amphinemura* sp. |
| 185 | JN197448.1 | *Amphinemura* sp. |
| 186 | JN197449.1 | *Amphinemura* sp. |
| 187 | JN197450.1 | *Amphinemura* sp. |
| 188 | JN197496.1 | *Epeorus* sp. |
| 189 | JN197497.1 | *Epeorus* sp. |
| 190 | JN197498.1 | *Epeorus* sp. |
| 191 | JN197499.1 | *Epeorus* sp. |
| 192 | JN197500.1 | *Epeorus* sp. |
| 193 | JN197501.1 | *Epeorus* sp. |
| 194 | JN197502.1 | *Epeorus* sp. |
| 195 | JN197549.1 | *Leucrocuta* sp. |
| 196 | JN197554.1 | *Neophylax* sp. |
| 197 | JN197559.1 | *Pycnopsyche* sp. |
| 198 | JN197560.1 | *Pycnopsyche* sp. |
| 199 | JN198316.1 | *Ironoquia* sp. |
| 200 | JN198318.1 | *Ironoquia* sp. |
| 201 | JN198320.1 | *Ironoquia* sp. |
| 202 | JN198324.1 | *Ironoquia* sp. |
| 203 | JN198326.1 | *Ironoquia* sp. |
| 204 | JN198329.1 | *Ironoquia* sp. |
| 205 | JN198349.1 | *Neophylax* sp. |
| 206 | JN198370.1 | *Pycnopsyche* sp. |
| 207 | JN198371.1 | *Pycnopsyche* sp. |
| 208 | JN198372.1 | *Pycnopsyche* sp. |
| 209 | JN198373.1 | *Pycnopsyche* sp. |
| 210 | JN198374.1 | *Pycnopsyche* sp. |
| 211 | JN200280.1 | *Amphinemura* sp. |
| 212 | JN200281.1 | *Amphinemura* sp. |
| 213 | JN200355.1 | Heptageniidae sp. |
| 214 | JN200356.1 | Heptageniidae sp. |
| 215 | JN200506.1 | *Cinygmula subaequalis* |
| 216 | JN200511.1 | *Diplectrona modesta* |
| 217 | JN200634.1 | *Maccaffertium vicarium* |
| 218 | JN200635.1 | *Maccaffertium vicarium* |
| 219 | JN200636.1 | *Maccaffertium vicarium* |

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|---|---|---|
| 220 | JN200637.1 | *Maccaffertium vicarium* |
| 221 | JN200638.1 | *Maccaffertium vicarium* |
| 222 | JN200639.1 | *Maccaffertium vicarium* |
| 223 | JN291792.1 | *Stenacron interpunctatum* |
| 224 | JN419463.1 | *Calopteryx maculata* |
| 225 | JN419948.1 | *Lanthus parvulus* |
| 226 | JN419949.1 | *Lanthus parvulus* |
| 227 | JN582236.1 | *Prosimulium* sp. |
| 228 | JN658996.1 | *Nyctiophylax moestus* |
| 229 | JN659016.1 | *Nyctiophylax moestus* |
| 230 | JQ662785.1 | *Eurylophella funeralis* |
| 231 | JQ663071.1 | *Acentrella turbida* |
| 232 | JQ663087.1 | *Acentrella turbida* |
| 233 | JQ663098.1 | *Acentrella turbida* |
| 234 | JQ663196.1 | *Acentrella turbida* |
| 235 | JQ663197.1 | *Acentrella turbida* |
| 236 | JX514462.1 | *Cambarus dubius* |
| 237 | JX514491.1 | *Cambarus robustus* |
| 238 | KC263060.1 | *Trissopelopia* cf. |
| 239 | KC502459.1 | *Hydroporus* sp. |
| 240 | KF000131.1 | *Zavrelimyia* sp. |
| 241 | KF000197.1 | *Zavrelimyia* sp. |
| 242 | KF000315.1 | Naididae |
| 243 | KF437320.1 | *Cambarus* sp. |
| 244 | KF437321.1 | *Cambarus* sp. |
| 245 | KF437323.1 | *Cambarus* sp. |
| 246 | KF489818.1 | *Thienemanniella* sp. |
| 247 | KF489841.1 | *Thienemanniella* sp. |
| 248 | KF489854.1 | *Thienemanniella* sp. |
| 249 | KF489864.1 | *Thienemanniella* sp. |
| 250 | KF489867.1 | *Thienemanniella* sp. |
| 251 | KF489873.1 | *Thienemanniella* sp. |
| 252 | KF489879.1 | *Thienemanniella* sp. |
| 253 | KF563009.1 | *Ameletus* sp. |
| 254 | KJ203823.1 | *Neoporus superioris* |
| 255 | KJ203895.1 | *Hydroporus* sp. |
| 256 | KJ449711.1 | *Ectopria nervosa* |
| 257 | KJ449712.1 | *Ectopria nervosa* |
| 258 | KJ449714.1 | *Ectopria nervosa* |
| 259 | KJ449715.1 | *Ectopria nervosa* |
| 260 | KJ449716.1 | *Ectopria nervosa* |
| 261 | KJ450824.1 | *Stenelmis crenata* |
| 262 | KJ450825.1 | *Stenelmis crenata* |
| 263 | KJ450826.1 | *Stenelmis crenata* |
| 264 | KJ450827.1 | *Stenelmis crenata* |

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|---|---|---|
| 265 | KJ450828.1 | *Stenelmis crenata* |
| 266 | KJ674900.1 | *Neophylax aniqua* |
| 267 | KJ674901.1 | *Maccaffertium vicarium* |
| 268 | KJ674933.1 | *Maccaffertium vicarium* |
| 269 | KJ674946.1 | *Eurylophella funeralis* |
| 270 | KJ674950.1 | *Eurylophella funeralis* |
| 271 | KJ674953.1 | *Amphinemura* sp. |
| 272 | KJ675056.1 | *Cinygmula subaequalis* |
| 273 | KJ675152.1 | *Stenonema femoratum* |
| 274 | KJ675153.1 | *Stenonema femoratum* |
| 275 | KJ675160.1 | *Cinygmula subaequalis* |
| 276 | KJ675187.1 | *Cinygmula subaequalis* |
| 277 | KJ675240.1 | *Cinygmula subaequalis* |
| 278 | KJ675372.1 | *Stenonema femoratum* |
| 279 | KJ961891.1 | *Dryops luridus* |
| 280 | KM206493.1 | Naididae sp. |
| 281 | KM206494.1 | Naididae sp. |
| 282 | KM206504.1 | Naididae sp. |
| 283 | KM206505.1 | Naididae sp. |
| 284 | KM206506.1 | Naididae sp. |
| 285 | KM206507.1 | Naididae sp. |
| 286 | KM206508.1 | Naididae sp. |
| 287 | KM206509.1 | Naididae sp. |
| 288 | KM206518.1 | Naididae sp. |
| 289 | KM445076.1 | *Dryops nitidulus* |
| 290 | KM445386.1 | *Pomatinus substriatus* |
| 291 | KM532299.1 | *Sweltsa* sp. |
| 292 | KM532433.1 | *Sweltsa* sp. |
| 293 | KM532633.1 | *Sweltsa* sp. |
| 294 | KM532974.1 | *Sweltsa* sp. |
| 295 | KM534699.1 | *Sweltsa* sp. |
| 296 | KM537031.1 | *Sweltsa* sp. |
| 297 | KM569819.1 | *Hexatoma* sp. |
| 298 | KM570489.1 | *Hexatoma* sp. |
| 299 | KM571243.1 | *Hexatoma* sp. |
| 300 | KM571592.1 | *Hexatoma* sp. |
| 301 | KM571629.1 | *Hexatoma* sp. |
| 302 | KM630869.1 | *Tanytarsus* sp. |
| 303 | KM979427.1 | *Cambarus robustus* |
| 304 | KM988740.1 | *Tanytarsus* sp. |
| 305 | KM990976.1 | *Tanytarsus* sp. |
| 306 | KP182981.1 | *Physa* sp. |
| 307 | KP182982.1 | *Physa* sp. |
| 308 | KR085274.1 | *Stictochironomus* sp. |
| 309 | KR085322.1 | *Stictochironomus* sp. |

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|---|---|---|
| 310 | KR134497.1 | *Pelonomus* sp. |
| 311 | KR140980.1 | *Chimarra aterrima* |
| 312 | KR141078.1 | *Somatochlora* sp. |
| 313 | KR141081.1 | *Stenacron* sp. |
| 314 | KR141684.1 | *Chimarra aterrima* |
| 315 | KR141716.1 | Heptageniidae sp. |
| 316 | KR141756.1 | *Acroneuria abnormis* |
| 317 | KR141818.1 | *Pycnopsyche* sp. |
| 318 | KR141825.1 | Heptageniidae sp. |
| 319 | KR142140.1 | *Leucrocuta* sp. |
| 320 | KR142144.1 | *Somatochlora* sp. |
| 321 | KR142202.1 | *Alloperla* sp. |
| 322 | KR142256.1 | Glossosomatidae sp. |
| 323 | KR142287.1 | Heptageniidae sp. |
| 324 | KR142407.1 | *Leucrocuta* sp. |
| 325 | KR142719.1 | *Somatochlora* sp. |
| 326 | KR142775.1 | *Somatochlora* sp. |
| 327 | KR142793.1 | *Leptophlebia* sp. |
| 328 | KR142894.1 | *Leptophlebia* sp. |
| 329 | KR142978.1 | *Leptophlebia* sp. |
| 330 | KR143163.1 | *Alloperla* sp. |
| 331 | KR143229.1 | *Leptophlebia* sp. |
| 332 | KR143344.1 | *Calopteryx maculata* |
| 333 | KR143496.1 | *Leucrocuta* sp. |
| 334 | KR143811.1 | *Leucrocuta* sp. |
| 335 | KR143915.1 | *Baetis* sp. |
| 336 | KR143976.1 | *Alloperla* sp. |
| 337 | KR144126.1 | *Baetis* sp. |
| 338 | KR144506.1 | *Alloperla* sp. |
| 339 | KR144641.1 | *Alloperla* sp. |
| 340 | KR144679.1 | *Baetis* sp. |
| 341 | KR145070.1 | Polycentropodidae sp. |
| 342 | KR145400.1 | Glossosomatidae sp. |
| 343 | KR145700.1 | *Leucrocuta* sp. |
| 344 | KR145830.1 | Heptageniidae sp. |
| 345 | KR145877.1 | *Isoperla* sp. |
| 346 | KR145964.1 | Heptageniidae sp. |
| 347 | KR145994.1 | *Stenacron* sp. |
| 348 | KR146139.1 | Glossosomatidae sp. |
| 349 | KR146151.1 | *Alloperla* sp. |
| 350 | KR146184.1 | *Leptophlebia* sp. |
| 351 | KR146206.1 | *Somatochlora* sp. |
| 352 | KR146219.1 | *Baetis* sp. |
| 353 | KR146477.1 | *Chimarra aterrima* |
| 354 | KR146506.1 | *Leptophlebia* sp. |

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| 355 | KR146558.1 | *Epeorus* sp. |
| 356 | KR146698.1 | *Leptophlebia* sp. |
| 357 | KR146704.1 | *Maccaffertium* sp. |
| 358 | KR146709.1 | *Chimarra aterrima* |
| 359 | KR146751.1 | *Maccaffertium* sp. |
| 360 | KR146791.1 | *Maccaffertium* sp. |
| 361 | KR146824.1 | *Habrophlebia* sp. |
| 362 | KR146837.1 | *Maccaffertium* sp. |
| 363 | KR146965.1 | *Leucrocuta* sp. |
| 364 | KR147018.1 | *Alloperla* sp. |
| 365 | KR147212.1 | *Leptophlebia* sp. |
| 366 | KR147223.1 | *Maccaffertium* sp. |
| 367 | KR147239.1 | *Somatochlora* sp. |
| 368 | KR147253.1 | *Habrophlebia* sp. |
| 369 | KR147304.1 | *Leucrocuta* sp. |
| 370 | KR147370.1 | *Paraleptophlebia* sp. |
| 371 | KR147439.1 | *Leptophlebia* sp. |
| 372 | KR147522.1 | *Paraleptophlebia* sp. |
| 373 | KR147551.1 | *Paraleptophlebia* sp. |
| 374 | KR147645.1 | *Baetis* sp. |
| 375 | KR147732.1 | *Paraleptophlebia* sp. |
| 376 | KR147737.1 | *Alloperla* sp. |
| 377 | KR147850.1 | *Paraleptophlebia* sp. |
| 378 | KR147857.1 | Leuctridae sp. |
| 379 | KR147872.1 | *Maccaffertium* sp. |
| 380 | KR147962.1 | *Haploperla brevis* |
| 381 | KR147978.1 | Leuctridae sp. |
| 382 | KR147986.1 | *Paraleptophlebia* sp. |
| 383 | KR148013.1 | Leuctridae sp. |
| 384 | KR148096.1 | Leuctridae sp. |
| 385 | KR148118.1 | Leuctridae sp. |
| 386 | KR148133.1 | *Isoperla* sp. |
| 387 | KR148149.1 | *Paraleptophlebia* sp. |
| 388 | KR148194.1 | Glossosomatidae sp. |
| 389 | KR148243.1 | *Cheumatopsyche* sp. |
| 390 | KR148265.1 | *Cheumatopsyche* sp. |
| 391 | KR148363.1 | *Cheumatopsyche* sp. |
| 392 | KR148396.1 | *Paraleptophlebia* sp. |
| 393 | KR148498.1 | Leuctridae sp. |
| 394 | KR148515.1 | *Habrophlebia* sp. |
| 395 | KR148556.1 | Leuctridae sp. |
| 396 | KR148557.1 | *Leptophlebia* sp. |
| 397 | KR148560.1 | *Habrophlebia* sp. |
| 398 | KR148593.1 | Leuctridae sp. |
| 399 | KR148620.1 | *Pycnopsyche* sp. |

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|---|---|---|
| 400 | KR148642.1 | *Cheumatopsyche* sp. |
| 401 | KR148661.1 | *Habrophlebia* sp. |
| 402 | KR148695.1 | *Amphinemura* sp. |
| 403 | KR148703.1 | Leuctridae sp. |
| 404 | KR148722.1 | *Epeorus* sp. |
| 405 | KR148744.1 | *Alloperla* sp. |
| 406 | KR148804.1 | *Habrophlebia* sp. |
| 407 | KR148849.1 | *Alloperla* sp. |
| 408 | KR148863.1 | *Habrophlebia* sp. |
| 409 | KR382538.1 | *Pseudolimnophila inornata* |
| 410 | KR383491.1 | *Pseudolimnophila inornata* |
| 411 | KR388602.1 | *Pseudolimnophila inornata* |
| 412 | KR388620.1 | *Pseudolimnophila inornata* |
| 413 | KR394099.1 | *Pseudolimnophila inornata* |
| 414 | KR394426.1 | *Pseudolimnophila inornata* |
| 415 | KR397763.1 | *Pseudolimnophila inornata* |
| 416 | KR398958.1 | *Pseudolimnophila inornata* |
| 417 | KR432840.1 | *Prosimulium* sp. |
| 418 | KR435636.1 | *Prosimulium* sp. |
| 419 | KR438615.1 | *Rheotanytarsus* sp. |
| 420 | KR445573.1 | *Prosimulium* sp. |
| 421 | KR468882.1 | *Stempellinella* sp. |
| 422 | KR470087.1 | *Thienemannimyia* sp. |
| 423 | KR480591.1 | *Stenelmis crenata* |
| 424 | KR480641.1 | Dytiscidae sp. |
| 425 | KR484340.1 | Dytiscidae sp. |
| 426 | KR484375.1 | *Psephenus herricki* |
| 427 | KR485770.1 | *Stenelmis* sp. |
| 428 | KR486476.1 | *Stenelmis* sp. |
| 429 | KR486700.1 | *Stenelmis* sp. |
| 430 | KR491184.1 | *Stenelmis crenata* |
| 431 | KR512256.1 | *Stempellinella* sp. |
| 432 | KR514078.1 | *Zavrelimyia* sp. |
| 433 | KR522705.1 | *Polypedilum aviceps* |
| 434 | KR523055.1 | *Rheotanytarsus* sp. |
| 435 | KR524726.1 | *Pseudolimnophila inornata* |
| 436 | KR525334.1 | *Thienemannimyia* sp. |
| 437 | KR589114.1 | *Natarsia punctata* |
| 438 | KR620728.1 | *Rheotanytarsus* sp. |
| 439 | KR622665.1 | *Rheotanytarsus* sp. |
| 440 | KR624529.1 | *Diamesa* sp. |
| 441 | KR635375.1 | *Thienemannimyia* sp. |
| 442 | KR635409.1 | *Polypedilum aviceps* |
| 443 | KR635862.1 | *Thienemannimyia* sp. |
| 444 | KR636146.1 | *Thienemannimyia* sp. |

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|---|---|---|
| 445 | KR640316.1 | *Thienemanniella* sp. |
| 446 | KR640496.1 | *Thienemanniella* sp. |
| 447 | KR641314.1 | *Thienemannimyia* sp. |
| 448 | KR642406.1 | *Thienemannimyia* sp. |
| 449 | KR644344.1 | *Simulium* sp. |
| 450 | KR644416.1 | *Simulium* sp. |
| 451 | KR644480.1 | *Simulium* sp. |
| 452 | KR644515.1 | *Simulium* sp. |
| 453 | KR644530.1 | *Polypedilum aviceps* |
| 454 | KR644545.1 | *Simulium* sp. |
| 455 | KR644848.1 | *Simulium* sp. |
| 456 | KR645039.1 | *Simulium* sp. |
| 457 | KR651776.1 | *Thienemanniella* sp. |
| 458 | KR657545.1 | *Thienemanniella* sp. |
| 459 | KR680511.1 | *Thienemannimyia* sp. |
| 460 | KR681360.1 | *Tanytarsus* sp. |
| 461 | KR681370.1 | *Tanytarsus* sp. |
| 462 | KR681757.1 | *Polypedilum* sp. |
| 463 | KR681890.1 | *Polypedilum* sp. |
| 464 | KR682013.1 | *Polypedilum* sp. |
| 465 | KR683112.1 | *Polypedilum* sp. |
| 466 | KR683332.1 | *Polypedilum* sp. |
| 467 | KR683635.1 | *Tanypodinae* sp. |
| 468 | KR683710.1 | *Tanypodinae* sp. |
| 469 | KR683905.1 | *Polypedilum* sp. |
| 470 | KR683972.1 | *Polypedilum* sp. |
| 471 | KR684069.1 | *Tanypodinae* sp. |
| 472 | KR684651.1 | *Zavrelimyia* sp. |
| 473 | KR687018.1 | *Zavrelimyia* sp. |
| 474 | KR689674.1 | *Zavrelimyia* sp. |
| 475 | KR692622.1 | *Eukiefferiella claripennis* |
| 476 | KR693595.1 | *Microtendipes pedellus* group sp. |
| 477 | KR694766.1 | *Microtendipes pedellus* group sp. |
| 478 | KR695130.1 | *Rheotanytarsus* sp. |
| 479 | KR697378.1 | *Tanytarsus* sp. |
| 480 | KR697452.1 | *Tanytarsus* sp. |
| 481 | KR714609.1 | *Stempellinella* sp. |
| 482 | KR714631.1 | *Thienemanniella* sp. |
| 483 | KR721133.1 | *Stempellinella* sp. |
| 484 | KR728001.1 | *Palpomyia* sp. |
| 485 | KR730033.1 | *Polypedilum aviceps* |
| 486 | KR731239.1 | *Polypedilum aviceps* |
| 487 | KR731965.1 | *Polypedilum aviceps* |
| 488 | KR741312.1 | *Diamesa* sp. |
| 489 | KR746080.1 | *Eukiefferiella claripennis* |

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|---|---|---|
| 490 | KR746623.1 | *Diamesa* sp. |
| 491 | KR747981.1 | *Eukiefferiella claripennis* |
| 492 | KR748435.1 | *Eukiefferiella claripennis* |
| 493 | KR753030.1 | *Eukiefferiella claripennis* |
| 494 | KR753383.1 | *Rheotanytarsus* sp. |
| 495 | KR754740.1 | *Diamesa* sp. |
| 496 | KR755785.1 | *Microtendipes pedellus* group sp. |
| 497 | KR755815.1 | *Microtendipes* sp. |
| 498 | KR756059.1 | *Tanytarsus* sp. |
| 499 | KR756118.1 | *Eukiefferiella claripennis* |
| 500 | KR756228.1 | *Eukiefferiella claripennis* |
| 501 | KR756375.1 | *Eukiefferiella claripennis* |
| 502 | KR756446.1 | *Tanypodinae* sp. |
| 503 | KR757131.1 | *Tanypodinae* sp. |
| 504 | KR757230.1 | *Microtendipes pedellus* group sp. |
| 505 | KR757336.1 | *Eukiefferiella claripennis* |
| 506 | KR757627.1 | *Eukiefferiella claripennis* |
| 507 | KR759106.1 | *Prosimulium* sp. |
| 508 | KR918929.1 | Glossosomatidae sp. |
| 509 | KR957882.1 | *Natarsia punctata* |
| 510 | KR958647.1 | *Eukiefferiella claripennis* |
| 511 | KR960127.1 | *Eukiefferiella claripennis* |
| 512 | KR960373.1 | *Eukiefferiella claripennis* |
| 513 | KR960828.1 | *Rheotanytarsus* sp. |
| 514 | KR961074.1 | *Rheotanytarsus* sp. |
| 515 | KR961487.1 | *Diamesa* sp. |
| 516 | KR962025.1 | *Diamesa* sp. |
| 517 | KR962209.1 | *Diamesa* sp. |
| 518 | KR964003.1 | *Zavrelimyia* sp. |
| 519 | KR966200.1 | *Stempellinella* sp. |
| 520 | KR966677.1 | *Stempellinella* sp. |
| 521 | KR967305.1 | *Stempellinella* sp. |
| 522 | KR971266.1 | *Pseudolimnophila inornata* |
| 523 | KR981444.1 | *Prosimulium* sp. |
| 524 | KT084036.1 | *Palpomyia* sp. |
| 525 | KT085519.1 | *Palpomyia* sp. |
| 526 | KT085628.1 | *Bezzia* sp. |
| 527 | KT086163.1 | *Palpomyia* sp. |
| 528 | KT087754.1 | *Bezzia* sp. |
| 529 | KT088829.1 | *Bezzia* sp. |
| 530 | KT088866.1 | *Bezzia* sp. |
| 531 | KT088938.1 | *Bezzia* sp. |
| 532 | KT089178.1 | *Bezzia* sp. |
| 533 | KT089430.1 | *Palpomyia* sp. |
| 534 | KT089889.1 | *Bezzia* sp. |

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| 535 | KT091618.1 | *Palpomyia* sp. |
| 536 | KT096662.1 | *Palpomyia* sp. |
| 537 | KT099059.1 | *Bezzia* sp. |
| 538 | KT115240.1 | *Rheotanytarsus* sp. |
| 539 | KT117770.1 | *Tanytarsus* sp. |
| 540 | KT117828.1 | *Tanytarsus* sp. |
| 541 | KT118027.1 | *Tanypodinae* sp. |
| 542 | KT118102.1 | *Tanytarsus* sp. |
| 543 | KT118775.1 | *Tanytarsus* sp. |
| 544 | KT118797.1 | *Tanytarsus* sp. |
| 545 | KT118907.1 | *Tanypodinae* sp. |
| 546 | KT119167.1 | *Tanypodinae* sp. |
| 547 | KT119216.1 | *Orthocladiinae* sp. |
| 548 | KT119307.1 | *Tanytarsus* sp. |
| 549 | KT119308.1 | *Orthocladiinae* sp. |
| 550 | KT282412.1 | *Orconectes cristavarius* |
| 551 | KT282414.1 | *Orconectes cristavarius* |
| 552 | KT759635.1 | *Cambarus bartonii* |
| 553 | KT759641.1 | *Cambarus bartonii* |
| 554 | KT759645.1 | *Cambarus bartonii* |
| 555 | KU980986.1 | Leptophlebiidae sp. |
| 556 | KU980993.1 | Leptophlebiidae sp. |
| 557 | KU981001.1 | Leptophlebiidae sp. |
| 558 | KX039562.1 | *Hexatoma* sp. |
| 559 | KX039573.1 | Leptophlebiidae sp. |
| 560 | KX039574.1 | Leptophlebiidae sp. |
| 561 | KX039575.1 | Leptophlebiidae sp. |
| 562 | KX039576.1 | Leptophlebiidae sp. |
| 563 | KX039628.1 | *Tanypodinae* sp. |
| 564 | KX039629.1 | *Tanypodinae* sp. |
| 565 | KX102703.1 | *Agapetus* sp. |
| 566 | KX102832.1 | *Neophylax* sp. |
| 567 | KX103370.1 | *Diplectrona modesta* |
| 568 | KX103387.1 | *Diplectrona modesta* |
| 569 | KX105155.1 | *Pycnopsyche* sp. |
| 570 | KX139049.1 | *Koenikea* sp. |
| 571 | KX139052.1 | *Limnesia* sp. |
| 572 | KX139053.1 | *Limnesia* sp. |
| 573 | KX139054.1 | *Limnesia* sp. |
| 574 | KX139055.1 | *Limnesia* sp. |
| 575 | KX139056.1 | *Krendowskia* sp. |
| 576 | KX139057.1 | *Krendowskia* sp. |
| 577 | KX139059.1 | *Koenikea* sp. |
| 578 | KX142783.1 | Glossosomatidae sp. |
| 579 | KX142887.1 | *Agapetus* sp. |

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| 580 | KX142936.1 | Polycentropodidae sp. |
| 581 | KX143815.1 | Polycentropodidae sp. |
| 582 | KX144371.1 | Polycentropodidae sp. |
| 583 | KX144433.1 | *Agapetus* sp. |
| 584 | KX144530.1 | Polycentropodidae sp. |
| 585 | KX271859.1 | *Zavrelimyia* sp. |
| 586 | KX291842.1 | *Neophylax* sp. |
| 587 | KX293446.1 | *Diplectrona modesta* |
| 588 | KX293883.1 | Polycentropodidae sp. |
| 589 | KX294140.1 | Polycentropodidae sp. |
| 590 | KX294335.1 | Polycentropodidae sp. |
| 591 | KX294832.1 | *Agapetus* sp. |
| 592 | KX295041.1 | Polycentropodidae sp. |
| 593 | KX295408.1 | *Psilotreta* sp. |
| 594 | KX295509.1 | *Neophylax* sp. |
| 595 | KX295568.1 | *Agapetus* sp. |
| 596 | KX296009.1 | Polycentropodidae sp. |
| 597 | KX296464.1 | *Neophylax* sp. |
| 598 | KX296624.1 | Glossosomatidae gen. sp. |
| 599 | KX453764.1 | *Dixa submaculata* |
| 600 | KX890920.1 | *Lanthus vernalis* |
| 601 | KX890945.1 | *Stylogomphus sigmastylus* |
| 602 | KX890996.1 | *Stylogomphus sigmastylus* |
| 603 | LC096195.1 | *Trissopelopia longimana* |
| 604 | LN810271.1 | Lumbriculidae sp. |
| 605 | LN810272.1 | Lumbriculidae sp. |
| 606 | LN897584.1 | *Diamesa cinerella/tonsa* group sp. |
| 607 | LN897587.1 | *Diamesa* sp. |
| 608 | LN897608.1 | *Diamesa* sp. |
| 609 | LN897619.1 | *Diamesa cinerella/tonsa* group sp. |
| 610 | LN897620.1 | *Diamesa cinerella/tonsa* group sp. |

Supplementary Material C. Plotted coordinates for species and extractions in ordinal space on Figure 4.

| | NMDS1 | NMDS2 |
|---|---|---|
| CTAB_1 | 0.004868538 | 0.71446701 |
| CTAB_2 | -0.389236263 | -0.80063627 |
| CTAB_3 | 0.592091461 | 0.03508303 |
| Dneasy_1 | -0.376132357 | 0.21013333 |
| Dneasy_2 | -0.593815356 | 0.02013655 |
| Dneasy_3 | 0.219897239 | -0.37581669 |
| *Cheumatopsyche* | -8.92E-01 | -0.65962517 |
| Dytiscidae sp. | -1.50E-01 | -0.56786729 |
| *Thienemanniella* | -2.14E-02 | 0.25958593 |
| *Thienemannimyia* | -2.17E-01 | 0.29265435 |
| *Trissopelopia* | 1.16E+00 | 0.06020264 |
| *Zavrelimyia* | -1.50E-01 | -0.56786729 |
| *Tanypodinae* | 1.16E+00 | 0.06020283 |
| *Prosimulium* | -1.27E+00 | 0.18623173 |
| *Simulium* | -9.03E-01 | 0.31991032 |
| *Oulimnius* | -1.27E+00 | 0.18623195 |
| *Stenelmis* | -9.03E-01 | 0.31991032 |
| *Pseudolimnophila* | 3.05E-01 | 0.15889753 |
| *Habrophlebia* | -6.74E-02 | 0.44928134 |
| *Leptophlebia* | 1.16E+00 | 0.06020226 |
| *Paraleptophlebia* | 1.16E+00 | 0.06020226 |
| *Pycnopsyche* | 1.24E-01 | 0.56137718 |
| *Microtendipes* | 1.16E+00 | 0.06020262 |
| *Polypedilum* | -1.36E-01 | -0.16552498 |
| *Tanytarsus* | -2.60E-02 | 0.03903263 |
| *Leuctridae* | -1.37E-01 | -0.2541858 |
| *Cinygmula* | -2.60E-02 | 0.03903263 |
| *Epeorus* | -1.37E-01 | -0.2541858 |
| *Maccaffertium* | 7.86E-05 | -0.45539669 |
| *Stenacron* | -2.17E-01 | 0.29265435 |
| Heptageniidae sp. | 9.10E-01 | -0.3347903 |
| *Physa* | 2.27E-01 | -0.59037475 |
| *Amphinemura* | -2.31E-01 | -0.02815366 |
| *Alloperla* | -7.26E-01 | -0.32342699 |
| *Cambarus* | 1.20E-01 | 0.79558582 |

# VITA

M. Triston Mullins was born in Ashland, Kentucky on December 15, 1988. He graduated from Russell Independent High School in June, 2007. The following fall semester, he attended Georgetown College (Georgetown, Ky.), and in 2011 received a degree of Bachelor of Science in Environmental Studies with a minor in Chemistry. After graduating Triston pursued work in the bourbon industry for Wild Turkey Distillery, fulfilling a variety of supervisory roles. In the fall of 2013, he started his graduate studies at Eastern Kentucky University, and received his Master of Science in Biology in the summer of 2017.

Triston is currently an ecologist for Eco-Tech Consultants in Louisville, Ky.