DEVELOPMENT OF A RAPID BIOASSESSMENT FOR WATER QUALITY MONITORING IN THE BELIZE RIVER WATERSHED

A Thesis
By
GRANT ALLAN BUCKNER

Submitted to the School of Graduate Studies
at Appalachian State University
in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE

August 2020,
Department of Biology

DEVELOPMENT OF A RAPID BIOASSESSMENT FOR WATER QUALITY MONITORING IN THE BELIZE RIVER WATERSHED

A Thesis
By
GRANT ALLAN BUCKNER
August 2020

APPROVED BY:

Dr. Shea R. Tuberty
Chairperson, Thesis Committee

Dr. Ed Boles
Member, Thesis Committee

Mr. Dave Penrose
Member, Thesis Committee

Dr. Robert Creed
Member, Thesis Committee

Dr. Zack E. Murrell
Chairperson, Department of Biology

Dr. Mike McKenzie
Dean, Cratis D. Williams School of Graduate Studies

Copyright by Grant Allan Buckner 2020
All Rights Reserved

Abstract

DEVELOPMENT OF A RAPID BIOASSESSMENT FOR WATER QUALITY MONITORING IN THE BELIZE RIVER WATERSHED

Grant Allan Buckner
B.S., University of North Carolina at Asheville
M.S., Appalachian State University

Chairperson: Dr. Shea R. Tuberty

Belize is a small country located on the Yucatan peninsula. Due to its large tracts of forest and its proximity to the Mesoamerican reef, Belize is home to some of the most biodiverse ecosystems in the world. Agriculture and development are becoming more frequent in Belize, which negatively impacts water quality, and could lead to losses in biodiversity. Belize has not established a rapid biological assessment method for monitoring river water quality based on aquatic macroinvertebrates. As such, the Belizean Rapid Bioassessment Protocols (BRBP) was created by collecting aquatic macroinvertebrates and water chemistry data from 31 sites during the dry season 2019-2020 within the Belize River Watershed (BRW). The BRW is the largest and one of the most impacted watersheds in Belize. A reference collection of over 5,000 aquatic macroinvertebrates from 150 different taxa including 29 new records was created for Belize. Also, standardized methods for collecting aquatic macroinvertebrates were created for the BRW. A Multimetric Index using box plots to detect metrics that were sensitive to impaired water quality was produced for the BRW. The Multimetric Index resulted in four valid metrics: Number of Ephemeroptera Collected, Total Taxa Richness, Biological Monitoring Working Party modified for Brazil (BMWP-Brazil), and % 3

Dominant Families. These metrics were used to create four categories of water quality for the BRW: “excellent”, “good”, fair”, and “poor”. Tolerance values (TV) for 29 families and 36 genera were calculated, starting with “excellent” and using cumulative percentiles to calculate how far into increasingly poor water quality a taxon was found. Watershed size, seasonality, and high elevation streams remain major areas that should be addressed in future studies. The Multimetric Index and TVs can be adjusted with more sampling, and eventually serve as a guideline for expansion outside of lowland streams in the BRW. Although this project represents an initial phase of biomonitoring in Belize, it is a vital step toward using aquatic macroinvertebrates as a critical component in detecting changes in water quality.

Acknowledgements

I would like to acknowledge my Thesis Advisor Dr. Shea Tuberty for the opportunity to join Appalachian State University and work on an exciting and unique project. I would like to acknowledge Dr. Ed Boles for his expertise and commitment to water quality for the country of Belize. I would like to thank Dave Penrose for sparking my interest in aquatic macroinvertebrates, providing input on my thesis, and helping with identifications in Belize. I would like to acknowledge Dr. Robert Creed for his input and advice on the ecology of streams and its implications for biomonitoring. I would also like to thank Dr. Jeff Colby who helped me with the GIS component of my work.

A special thank you to Jasper Yoke for his hard work and dedication that led to the completion of this project. I would also like to thank members of the Tuberty Lab, William McMahan and Cristina Sanders, for their support on this project and especially for welcoming me into the Tuberty Lab.

I would like to acknowledge the Department of Biology at Appalachian State University, the Office of Student Research, and the Wayne Richardson Memorial Fellowship for funding. Finally, I would like to acknowledge the Government of Belize, especially the Belize Fisheries Department and Forestry Department, for scientific research permits as well as everyone in Belize that helped support this effort, including: Monkey Bay Wildlife Sanctuary, Birds Eye View Lodge, and Friends of Conservation and Development.

Dedication

I dedicate this thesis to Dave Penrose, who sparked my interest in aquatic macroinvertebrates and whose commitment to water quality is an inspiration for all who love rivers and streams.

Table of Contents

Foreword

The research in this thesis has been formatted for and will be submitted to *Hydrobiologia* which is a peer-reviewed international journal focused on aquatic sciences owned by Springer Nature which is a part of Springer publishing company.

Introduction

Bioindicators are living organisms used to monitor an aquatic environment. Bioindicators are important because they are continually present in their environments and are therefore subject to environmental conditions, making them more useful than water grab samples to measure physical and chemical characteristics that represent a brief snapshot of conditions (Rosenberg & Resh, 1993). There is a consensus that there is no perfect stream bioindicator for all situations, but steps should be taken to try to select the best bioindicator possible given the goals of the effort (Bonada et al., 2006; Resh, 2008). For stream biomonitoring, there are several different candidates for bioindicators, including fish, algae, and aquatic macroinvertebrates each having an array of positive and negative attributes (Resh, 2008). Using a combination of different bioindicators is often the best choice (Barbour et al., 1999) but can be costly and not always possible due to time constraints or expertise of scientists.

Algae are easy to collect and good for indicating the presence of herbicides but has a short generation time and few indexes/metrics that can be easily expanded to new regions (Resh, 2008). Fish are long-lived, have important ecological roles, and are easy to identify; however, fish are extremely mobile, which is problematic for locating specific areas where pollution is occurring (Resh, 2008). Aquatic macroinvertebrates do not live as long as fish but are semi-long lived (months to a few years). They are easy to sample but are prone to issues of seasonality and are hard to identify without formal training (Resh, 2008). Aquatic macroinvertebrates are inexpensive to sample and can relate information about the long term water quality of a site (Resh, 2008). Aquatic macroinvertebrates have been used extensively around the world for monitoring water quality and are also used by many state and government agencies in North America (Chutter, 1972; Armitage et al., 1983; Lenat, 1993;

Barbour et al., 1996; Weigel et al., 2002; Baptista et al., 2007; NC Department of Environment Quality, 2016; Patang et al., 2018). Aquatic macroinvertebrates were selected to monitor water quality in Belize because they are inexpensive to sample and have been used extensively for monitoring water quality.

Aquatic macroinvertebrates have been used as bioindicators over 100 years to monitor aquatic ecosystem health and water quality (Bonanda et al., 2006). One of the first methods was the saprobic system developed in the early 1900s in Germany by Kolkwitz & Marsson (1908, 1909). The saprobic system related water quality at sites based on the type of organisms present and their reliance on dissolved oxygen. Other than the saprobic method, most of the early water quality monitoring programs focused on bacteria in sewage effluents (Hynes, 1960). This changed through the 1970s into the 1990s when there was an increase in the use of bioindicators for water quality, specifically using aquatic macroinvertebrates (Chutter, 1972; Hilsenhoff, 1987 & 1988; Barbour et al., 1996). Chutter (1972) developed a Biotic Index for South African streams by creating quality values for different invertebrates based on where they occurred in relation to water quality impairment. Likewise, Hilsenhoff's widely known Family Level Biotic Index worked similarly but chose to focus solely on arthropods (Hilsenhoff, 1987 & 1988). Hilsenhoff's Family Level Biotic Index was widely used and/or adapted for the expansion into new regions. North Carolina used a similar method to establish the North Carolina Biotic Index (Lenat, 1993; NC Department of Environment Quality, 2016) and focused on the genus and species level, while considering the different regions in North Carolina (Mountain, Piedmont and Coastal Plain). In Florida, a Multimetric method using aquatic macroinvertebrates was created based on methods for creating a fish Multimetric Index (Barbour et al., 1996; Karr et al., 1986). It also considered different regions and ecosystems of Florida. Eventually, the Environmental Protection Agency standardized a method utilizing benthic macroinvertebrates, periphyton, and fish to monitor water quality (Barbour et al., 1999). In Europe, similar scoring systems also arose. The Biological Monitoring Working Party (BMWP), developed in Europe, also used a scoring system at the family level with the score attributing to how sensitive macroinvertebrates are to pollution (Chesters, 1980). The Average Score Per Taxon was also developed and was shown to be a useful tool for monitoring water quality (Armitage et al., 1983).

There are many approaches to how aquatic macroinvertebrates can be used to monitor water quality. Multimetric, multivariate, biomarkers, and several other approaches have been proposed (Bonada et al., 2006). In North America, many are iterations and modifications to the Hilsenhoff Family Level Biotic Index, developed in Wisconsin in the late 1980s (Hilsenhoff, 1987 & 1988). As better keys and taxonomic resolution were formalized, there was a push toward genus and species level identification resulting in specific tolerance values that relate an individual aquatic macroinvertebrate species to its sensitivity to water quality pollution (Hilsenhoff, 1987, 1988; Barbour et al., 1996; NC Department of Environment Quality, 2016). As of 2006 all 50 states in the U.S. either had or were developing a water quality monitoring program using aquatic macroinvertebrates (Carter et al., 2006). Although it is difficult to generalize aspects of aquatic macroinvertebrate monitoring programs across continents, there are a few general principles that hold. Chang et al. (2014), found that after reviewing tolerance values from 29 different regions from all 6 continents, the basic assumptions about the tolerance of different orders and families of aquatic insects are true. An example of these assumptions was that Ephemeroptera, Plecoptera, and Trichoptera

(EPT) were always more sensitive than non-EPT insects (Chang et al., 2014). The basic assumptions that held true are useful for trying to implement a biomonitoring program in a new place like Belize. There have been some protocols developed for Central and South America that are useful, such as the many Multimetric indexes that have been developed in Brazil, Costa-Rica and Mexico (Fernández, 2002; Weigel et al., 2002; Baptista et al., 2007; Ferreira et al., 2011; Helson & Williams, 2013).

In Belize, water quality is important for the many organisms that make up its large biodiversity. Practices such as agriculture, human development, and deforestation occurring near streams and rivers are threats to water quality (Dudgeon, 2006), and are all currently taking place in the Belize River Watershed (BRW) (Young, 2008). Water withdrawals for agriculture and saltwater intrusion are also areas of concern in Belize. The more northernly distributed New River watershed experienced water quality pollution, causing a fish and crocodile kill in 2019, highlighting the need for increased biomonitoring of water quality. Belize has had previous work published regarding the use of aquatic macroinvertebrates for monitoring water quality (Carrie et al., 2015, 2017). Carrie et al. (2015) showed that there is a need to consider seasonality and the underlying geology of a stream when developing a rapid bioassessment tool. The work of Carrie et al. (2017) in Southern Belize also found that family-level classifications had limited capacity to detect moderate pollution. They concluded that while family level identification is useful for detecting heavily impacted streams, identification at the genus level may uncover impacts of moderate pollution (Carrie et al., 2017). Additionally, identifying macroinvertebrates to the lowest taxonomic level has been shown to provide more discriminatory power when detecting pollution (Lenat & Resh, 2001). This is problematic in Belize, where identification to the genus level with confidence was often not possible due to taxonomic keys not being compiled into one volume (Carrie et al. 2015). However, a new key published in 2018 for the Neotropics, (*Thorp and Covich's Freshwater Invertebrates. Volume III, Keys to Neotropical Hexapoda*) enabled mostly genus and some species level identification for this project. This, in turn, might increase the ability to detect mild to moderate water quality pollution (Lenat & Resh, 2001; Thorp, 2018). This project also focused on the larger, more centrally located BRW which has experienced increased development and deforestation (Karper & Boles, 2004). Due to the lack of knowledge of aquatic macroinvertebrates in Belize and the human impacts in the BRW, the project had several goals, which involved documenting aquatic macroinvertebrates and monitoring water quality. The objectives of this project were to: identify and document the diversity of the aquatic macroinvertebrates in the Belize River Watershed (BRW), provide a reference collection and standardized protocol for the collection of aquatic macroinvertebrates in the BRW (that can be expanded to other watersheds), develop a Multimetric Index for monitoring water quality for wadeable streams in the BRW, and propose initial tolerance values based on their distribution in water quality bioclassifications from the Multimetric Index.

Materials and Methods

Study Area

The focus area of this project was the Belize River Watershed (BRW), which is the largest watershed in Belize with approximately one-third of its watershed located in Guatemala. The BRW is heavily impacted by humans, and is subject to large amounts of pollution, making it a prime area for monitoring water quality (Karper & Boles unpublished, 2004; Young, 2008; Cherrington et al., 2010). Also, between 2010-2012 of all the deforestation in Belize, 36.7% of it occurred in the BRW, which was the highest of any Belizean watershed (Cherrington et al., 2010). This is especially concerning for the riparian forests, which are vital for stabilizing stream and riverbanks, providing shade that decreases water temperature, and providing leafy and woody debris inputs for aquatic macroinvertebrates (Quinn et al., 1997; Mark & Planner, 2003; Valente-Neto, et al., 2015).

Collection of Aquatic Macroinvertebrates

Aquatic macroinvertebrates, water chemistry, and GPS coordinates were collected at each site in the BRW during the dry season from December 29^{th,} 2019 to January 14^{th,} 2020 (Fig 1). The dry season was selected for sampling to increase the likelihood of wadeability, avoid the sampling of intermittent streams, and increase the prospect of discovering a difference in assemblages between reference and impaired streams. With lower flows in the dry season this could potentially increase stress on aquatic macroinvertebrates (Jacobsen et al., 2008; Damanik-Ambarita et al., 2016; Castellanos Romero et al., 2017; Patang et al., 2018). Additionally, flows during the dry season tend to be more stable and heaving flooding in the wet season can scour habitats of aquatic macroinvertebrates. Sites were selected based on three criteria: they were positioned in the BRW, they were publicly accessible, and easily wadeable. These criteria were chosen to make resampling efforts as easy and straightforward as possible. Collection of aquatic macroinvertebrates at each site involved the use of kick seines, dip nets, leaf packs, sand sieves, and visual searches adapted from the North Carolina Department of Environmental Quality, Virginia Department of Environmental Quality and the United States Environmental Protection Agency protocols (Barbour et al., 1999; VDEQ, 2008; NC Department of Environmental Quality, 2016).

At each site, a 500µm kick net (BioQuip Products, Rancho Dominguez, CA, USA) sample was collected by disturbing the substrate within a 3.0 m^2 area of riffle for one minute. All bugs were picked from the net for up to fifteen minutes or until nothing was left. Up to but no more than five jabs using a 500µm D-net (BioQuip Products, Rancho Dominguez, CA, USA) was used to collect macroinvertebrates from submerged vegetation or undercut banks. A jab was adapted from the Virginia protocols and defined as moving the D-net through the habitat for approximately five seconds or shaking the habitat in the net for approximately five seconds (VDEQ, 2008). Leaf packs, if present, were collected by gathering a handful of submerged leafy debris into a collecting pan. Sand samples were collected by filling an 8-inch #10 (2mm wire mesh) brass sieve (Cole Parmer, Vernon Hills IL, USA) with sand and washing away all silt/sand until only coarse sand or gravel was left. Sand samples were collected based on the amount of suitable sampling habitat available, but no more than 5 sand samples were taken at a site. Lastly, visual searches involved flipping over rocks and submerged woody debris or investigating any other unique habitats not already sampled. Visual searches lasted until all unique habitats were sampled or until fifteen minutes passed since visual searches first began. All macroinvertebrates collected were preserved in a labeled jar in the field in 80% Ethanol (produced from over-proof rum) and transported to a lab for identification. All aquatic macroinvertebrates collected were viewed under dissection microscopes and identified to the lowest taxonomic level possible, primarily using *Thorp and Covich's Freshwater Invertebrates, Volume III, Keys to Neotropical Hexapoda* but also with *A Natural History of the Bladen Nature Reserve and its Gastropods* (Doruson, 2009), and placed in labeled 1 or 6-dram archival grade vials (Discount Vials, Madison, WI, USA) with 80% ethanol. Additionally, head capsules of Chironomids were mounted and identified using the *Identification manual for the larval Chironomidae (Diptera) of North and South Carolina* (Epler, 2001).The specimens remain in Belize as vouchers of aquatic macroinvertebrate diversity and provide a training tool for local biologists to support continued monitoring of water quality.

The estimated discharge was also calculated at each site using the float method to estimate rough discharge (Dobriyal et al., 2017). Water chemistry data were recorded using a YSI Professional Plus multimeter probe (YSI Inc, Yellow Springs, OH, USA) to collect pH, temperature, conductivity, chloride, and dissolved oxygen at each site. Additionally, more than a liter of water was collected in the field in plastics bottles at each site. Bottles were rinsed with water from the collection site, then filled. These samples were transported to the lab and were then vacuum filtered using Whatman student grade filter paper (GE Healthcare Biosciences, Pittsburgh, PA, USA). Once a liter of the sample was filtered, it was then pushed by a 60 mL slip tip syringe (Becton, Dickson and Company, Franklin Lakes, NJ, USA) through an Oasis Prime HLB Plus Short Cartridge (Waters, Franklin, MA, USA), labeled and stored for transportation to the United States. Unfortunately, water samples were not processed by non-target screening (NTS) for the determination of pollutants by an innovative technique (UHPLC-Orbitrap MS/MS) due to complications with the Covid-19

Pandemic of 2020. Currently, they are stored on the cartridges in a secure freezer located at the Department of Biology at Appalachian State University.

Watershed Delineation, Underlying Geology, and Land Use

For every site sampled, the upstream watershed was delineated, as was the entire BRW. Land use and underlying geology were determined using ArcMap Version 10.6 (ESRI, Redlands, CA, USA) for all sites except 4, 6, and 19, due to the lack of available data for the Guatemalan portions of the watershed. For delineating the BRW and all sub-basins, ASTER Global Digital Elevation Model V003 (DEM) were downloaded from the National Aeronautics and Space Administration (NASA) earth explore website (https://search.earthdata.nasa.gov/search) and opened in ArcMap. The DEM were converted into a single mosaic and then the watershed was delineated using the Hydrology toolset in the spatial analyst tool pack. A flow accumulation threshold was set at 500 for the entire watershed, as this best represented the streams in the BRW based on aerial photography and sites sampled during field collections. Once every sub-basin upstream of each site were delineated, the size of the basin was calculated using the field calculator in the attributes table in ArcMap.

For streams in Belize, 2017 Land Use data were downloaded from the Belize Spatial Data Warehouse (Meerman & Clabaugh 2017). This data were created for the Central American Ecosystems Map, but has been continuously updated with new Landsat data. The land use data were downloaded into ArcMap, the land use upstream of sites were obtained by overlaying the sub-basin upstream for each site, and land use was extracted. Using the field calculator in ArcMap, the total area and area of each land use were obtained. These tables were exported into excel where all anthropogenic disturbance was combined into the category of “disturbed”. This included agriculture, urban area, mining, and fire-induced thickets. Lastly, for the determination of the geology upstream of each site located in Belize, geologic maps were downloaded from DATA BASIN (www.databasin.org). These geologic maps were uploaded by the Conservation Biology Institute and came from a 2004 planning project by the Selva Maya Consortium. The resulting map was reclassified in ArcMap into two categories for geology (limestone and non-limestone) and then underlying geology was extracted by overlaying each catchment upstream of each site.

Site Classification

Samples were collected from sites with a variety of conditions of water quality, ranging from pristine to heavily impacted. Reference sites were those that met at least four of the five following conditions: total percent area disturbed in the watershed less than 25%; dissolved oxygen greater than 7.0 mg/L; chloride less than 15 mg/L; and conductivity values less than 50 µS/cm or less than 500 µS/cm for watersheds with more than 25% limestone. Impacted sites were those that included at least two of the three following conditions: total percent disturbed in the watershed >75%; dissolved oxygen less than 5.5 mg/L; chloride greater than 15 mg/L. Impacted sites were also included if they had extremely high chloride and conductivity values but did not have any limestone within the watershed.

Developing and Selecting Metrics

Sites were split into two categories of high elevation (greater than 100m above sea level) and low elevation (less than 100m above sea level). This is due to the impact of elevation on aquatic macroinvertebrate assemblages and the fact that the high elevation sites occurred in the Caribbean Montane Pine Forest, which is a different bioregion from the low elevation tropical forest/savannah streams. The procedure for testing metrics was only applied to the low elevation sites (below 100m above sea level) and was adapted from Karr et al. 1986, Barbour et al. 1996, and Baptista et al. 2007. For an overview of this process, see the flow chart (Fig. 2). There were several tests that the metrics needed to pass. The first was a "no value" and range test, where metrics with a range of less than 5 and/or a value of 0 for more than ten sites (~ 30%) were excluded. This was done to avoid issues with rare taxa or small ranges that would make assigning scores for the sensitivity test too small for detecting impairment. An example of this is the order Plecoptera which has only one genus in Belize. Secondly, the remaining metrics were then put through a sensitivity test that involved comparing data via box and whisker plots of metrics at reference and impaired sites to detect metrics that can distinguish between impaired and nonimpaired sites (Karr et al 1986; Barbour et al., 1996; Baptista et al., 2007). If the metric had no overlap in the interquartile range (IQR) and no overlap in the medians, it was given a score of 3. Metrics that had IQRs overlapping but both medians occurring outside the IQR overlap resulted in a score of 2. A score of 1 was given if there was overlap in the IQR but only one of the medians overlapped with one of the other IQRs. A score of 0 was given to metrics that had either both medians occurring in the overlap range of the IQRs, or if one of the IQRs was inside the IQR of another (Fig. 3). A metric was considered sensitive if the metric sensitivity scored a 3 and results were confirmed with a two-sample t-test. Or when the data were not normally distributed, a Mann-Whitney U test was used.

After the sensitivity tests, Box and whisker plots for metrics at reference sites were used to normalize the metrics into a score of a 5, 3, or 1 (Fig. 4). For metrics expected to increase in value with increased water quality impairment, values at or below the 75th percentile were assigned a score of 5; above the 75th percentile but below the maximum received a score of 3; and above the maximum values received a score of 1. For metrics expected to decrease with increasing water quality degradation, values above the 25th percentile were assigned a score of 5. If the value fell below the 25th percentile and above the minimum, it received a score of 3. Lastly, a score of 1 was given to metrics if they occurred outside the minimum value. A score of 5 would indicate that a metric at the site is comparable with reference conditions. A score of 3 indicates moderate water quality impairment. A score of 1 indicates a severe water quality impairment.

All metrics were also checked for redundancy through a Spearman's correlation test to simplify the index and to avoid metrics that contribute the same information from influencing the final classification. If two metrics were in the same category and highly correlated, one was excluded. The final metrics were tested for correlations (Spearman correlations) with watershed size and percent limestone. If they were significantly correlated, the metrics were linearly regressed with that factor to investigate the strength of the relationship. This was done to prevent metrics from responding to watershed size and underlying geology instead of impairment, as both have been shown to influence aquatic macroinvertebrate assemblages (Paller et al., 2006; Carrie et al., 2015). Metrics determined to be responding to watershed size or underlying geology were excluded.

Generation of Genus Level Tolerance Values

Tolerance values (TVs) were created by adapting the method used by the North Carolina Department of Environmental Quality (Lenat, 1993). Using the Multimetric Index developed by this project, each site was assigned a bioclassification of water quality. The abundances for each specimen collected were converted into relative abundances based on Lenat (1993). The three categories were: rare, which was assigned a 1 if they were collected 1-2 times at each site; common, which was assigned a 3 if it was collected 3-9 times at a site; and abundant, which was assigned a 10 if it was collected 10 or more times at a site. Relative abundances were used instead of the actual number collected. This was done in order to prevent tolerance values from indicating where they had the highest number collected instead of the category of water quality they can tolerate. These relative abundances were averaged in the four classifications produced from the Multimetric Index: "excellent," "good," "fair," and "poor". These classifications were converted into the numeric values 1, 2, 3, and 4, respectively. The mean of the relative abundances in each bioclassification was converted into cumulative percentiles, starting at "excellent" (1) and extending to "poor" (4). Calculating the slope and y-intercept based on the cumulative percentiles for each of the four categories resulted in a linear equation that could be used to interpolate TVs based on cumulative percentiles. Taking the cumulative 75^{th} percent for taxa resulted in TVs that ranged usually between -0.5 to 4. The 75^{th} cumulative percentile was chosen as it has been shown to give the best spread between sensitive and tolerant TVs (Lenat, 1993). This resulted in the preliminary TVs that need to be converted to the desired range of 0-10 so it can be easily compared to other TVs. By graphing the minimum and maximum for the preliminary range and the desired range (0-10), preliminary TVs were converted to final proposed TVs. This was done for all genera that were collected in at least three different sites. The final TVs were split into three categories: sensitive (TV <4), intermediate (TV between 4 and 7), and tolerant (TV>7). TVs for families were calculated by averaging genera in that family with a known TV weighted by the number of individuals collected in each genus.

Results

Reference vs Impaired Sites

The average time spent per site was 49.8 ±1.8 minutes with either a team of 3 or 4 people. Overall, reference sites had a lower specific conductivity, chloride, and percent disturbed area than the impacted sites (Table 1). Impacted sites had a lower percent dissolved oxygen (DO) compared to reference sites (Table 1).

Aquatic Macroinvertebrate Diversity

A total of 5,532 individual aquatic macroinvertebrates were collected during the sampling from 59 different families and 120 different genera. In total, 150 different taxa were collected, including 29 new records for Belize, but not including the the family Chironomidae. The orders Ephemeroptera, Trichoptera, Odonata, and Diptera had the greatest number of different families collected (6, 8, 6 and 6, respectively). Odonatan diversity was high, especially the Libellulidae family with 13 different genera that were collected. 14 different species of Chironomidae were collected and identified, showing that Chironomidae was also a diverse group. The most diverse Ephemeroptera families were Baetidae, Leptohyphidae, and Leptophlebiidae with 8, 6, and 7 genera collected, respectively. The most diverse Trichopteran family was the Hydropsychidae family with 5 different genera collected. Other diverse groups included the family Elmidae in the order Coleoptera with 9 different genera collected, and the Phylum Mollusca with 7 families, 6 genera, and 6 species collected.

Developing the Belizean Multimetric Index

The collected data was organized into 68 different metrics and were tested for their integration in the index based on low elevation sites only (Appendix A). After the sensitivity test, 12 metrics remained, including Number of Ephemeroptera Collected, Number of EPT Collected, Number of Leptophlebiidae Collected, Total Number of Scrapers Collected, % Diptera, Total Taxa Richness, EPT Taxa Richness, BMWP-Brazil, Ephemeroptera Taxa Richness, % Dominant Family, % 2 Dominant Families, and % 3 Dominant Families (Fig 5a-l, Table 2). Of the 12-candidate metrics, Number of EPT Collected, EPT Richness, Ephemeroptera Richness, Number of Leptophlebiidae Collected, and % Diptera were all rejected due to small ranges in the scope for assigning scores of a 1, 3, or 5 (Table 3). The % 3 Dominant Family metric was highly correlated with % Dominant Family and % 2 Dominant Family (Table 4) but was kept because it had more separation in IQR when comparing reference and impaired sites (Fig 5d-e). Of the remaining metrics, none were significantly correlated with percent limestone (Table 4). However, only % 3 dominant families were not correlated with watershed size (Table 5). The Number of Ephemeroptera Collected and Total Number of Scrapers Collected were the only metrics that had a significant linear relationship with watershed size (Fig 6c-d). The coefficient of determination (r^2) was low for the Number of Ephemeroptera Collected (Fig 6c) and acceptable for Total Number of Scrapers Collected (Fig 6d). The three largest sites (4, 6 & 19), which were much larger than the other locations (Table 6) were excluded, and correlations were run again due to suspicion that those three sites may have been causing the significance and resulted in only Total Number of Scrapers Collected being correlated with watershed size (Table 7). No other metrics were rejected, although they were correlated with one another (Table 4), because they were contributing different information into the index. For example, both taxa richness and BMWP-Brazil were highly correlated (Table 4), but BMWP-Brazil is combining the sensitivity and presence of families while taxa richness is measuring the number of different taxa at a site. The final four metrics selected for the Multimetric Index were Number of Ephemeroptera Collected, Taxa Richness, BMWP-Brazil, and % 3 Dominant Families (Table 8).

Classification of Sites and Performance of Multimetric Index

The range score for the Multimetric Index was 4 to 20 and was divided into four classifications: "poor", "fair", "good", and "excellent" (Table 9). In total, 20 lowland streams were classified based on the Multimetric Index: six sites were classified as "excellent"; three were classified as "good"; four were classified as "fair", and seven were classified as "poor" (Table 10). All reference sites scored "excellent" with the exception of one site that scored "good". Additionally, impaired sites all scored "poor" except for one site that scored "fair". This indicates a close alignment with scores and expected classifications.

The Multimetric Index had a significant relationship with watershed size (Fig 7). However, it was a poor fit with low r^2 values (Fig. 7). When sites 4, 6, and 19 were excluded, the relationship with watershed size disappeared. This result suggests that the Multimetric Index should be used with caution with watersheds greater than 3,000 km^2. The Multimetric Index also largely agreed with the BMWP and the few instances it did not they were only separated by a few points (Table 11).

Tolerance Values

Proposed Tolerance Values (TVs) for 29 families (Table 12) and 36 genera (Table 13) were calculated for the taxa collected in the BRW. They ranged from 0-10 and were split into 3 categories of sensitive, intermediate, and tolerant. TVs are proposed based on collections from this project only and need more sampling to adjust further and to increase reliability.

Discussion

The Belizean Rapid Bioassessment Protocols (BRBP) were created as a proposed strategy to standardize collection efforts for aquatic macroinvertebrates in Belize. As of right now, there is still no standardize method established in Belize. The proposed method will need to be tested, adapted, or modified in the future for it to be implemented. The BRBP represents a clearly defined starting point rather than a complete finished product. The methods used were optimized to standardize effort at all sites while sampling the greatest diversity of habitats possible. The BRBP method is semiquantitative rather than quantitative. Other quantitative methods are good for studies concerned with density but do not obtain the diversity of semiqualitative collections (Stein et al., 2008). The BRBP was designed to be highly functional, as all collection sites were accessible via public roads and easy to approach. The effort was also designed to be rapid and conducted with a small team of 3-4 collectors in approximately one hour to increase the appeal of the BRBP for use by Belizean government agencies and trained community assessment volunteers.

All aquatic macroinvertebrates were identified to the lowest taxonomic level possible (mostly genus) which has not been the norm for Central America as it can be difficult to find proper keys. This resulted in identifying a large diversity of aquatic macroinvertebrates in Belize, including 29 new records. This increased the knowledge of genera and, in some cases, families of aquatic macroinvertebrates in Belize and Central America. The genus level identification is preferred over the family level in aquatic biomonitoring, as it gives the results a greater ability to detect impairment (Lenat & Resh, 2001). Species-level would have been the ideal level, as species that make up genera can have different sensitivities to pollution (Resh & Unzicker, 1975); however, current information and keys about species in the Neotropics are severely lacking (Springer, 2008; Carrie et al., 2017; Thorp, 2018). This lack of knowledge at the species level represents a key area of focus for future taxonomic studies in Belize.

This study used the new *Thorp and Covich's Freshwater Invertebrates, Volume III, Keys to Neotropical Hexapoda* which was recently published (Thorp, 2018). Not every identification was taken to the genus level, as there were cases when going past family was not possible. The key highlights several groups in each order that are problematic to identify or have been based on only a few specimens. A good example of this is the Philopotamidae family, in which the only reported genus in Belize, *Chimarra* spp, has difficult or no features for distinguishing between other genera in the neotropics (Thorp, 2018). Some keys did not go past the family level, for example, the family Scirtidae. It is the hope that the reference collection will serve as a tool that will enable Belizeans researchers and government agencies to train water quality researchers. The reference collection will eventually be stored in the Ministry of Forestry office to represent the diversity of aquatic macroinvertebrates in the BRW. Specimens from other watersheds could be added to the collection in the future.

In total four metrics were chosen for the Multimetric Index. Total Taxa Richness was one of the metrics that passed all the criteria. Richness measures can also act as good indicators of pollution, as cleaner, less disturbed sites will have higher richness (Resh et al., 1995; Barbour et al., 1996; Baptista et al., 2007). However, richness metrics can be problematic, given the potential of impacted sites retaining high richness of tolerant taxa. This is balanced by including the metric of % 3 Dominant Families. The metric % 3 Dominant Families is related to the lack of evenness of populations in an assemblage and should increase with impairment. For example, a similar metric, dominant taxa, have been shown to increase in response to nutrient enrichment (Camargo et al., 2004).

The BMWP adapted for Brazil was included in the final metrics (Alba-Tercedor & Ortega, 1988; Uherek & Pinto Gouveia, 2014). The BMWP originated in Europe and has been implemented and adapted for many parts of South America and Central America (Alba-Tercedor & Ortega, 1988, Junqueira et al., 2000, Uherek & Pinto Gouveia, 2014). The BMWP adopted for Costa Rica (Executive Decree No. 33903-S-MINAE Ministerio de Ambiente y Energía, Propuesta de Ley del Recurso Hídrico) in 2007 did not pass our metric sensitivity test. The BMWP is related to richness but focuses on the family level and associates families with their sensitivity to water pollution (Chestsers, 1980). Because the BMWP is calculated at the family level, it is prone to possible misclassifications of water quality (Hilsenhoff, 1988).

The Number of Ephemeroptera Collected is a metric that has not been commonly used. Given the nature of sampling with semiquantitative methods, measures of numbers collected are avoided as quantitative methods are better for representing the number of specimens collected (Lenat et al., 1988; Everall et al., 2017). However, it was incorporated as it passed the sensitivity test, and although initially correlated with watershed size, it no longer correlated once the three largest sites were excluded. Also, the order Ephemeroptera is one of the most sensitive aquatic insect orders (Chang et al., 2014), although there are tolerant families and genera of mayflies. It was incorporated given the nature of using multiple metrics, which have built in safeguards, because a site would need to score well in all metrics to receive a better classification of water quality. Metrics focused on the most sensitive groups of aquatic insects, Ephemeroptera, Plecoptera and Trichoptera (EPT) (Chang et al.,

2014) did pass the sensitivity test but lacked the ranges for use in our assessment tool despite their use elsewhere (Barbour et., 1996; Baptista et al. 2007; Helson & Williams, 2013; Macedo et al., 2016). However, EPT taxa are still contributing to the four adopted metrics.

Tolerance Values

There has been no effort to generate TVs for genera in Belize and little effort in Central America in general; therefore, this work represents an important first attempt to do so in Belize. Given the nature of our limited sample size, TVs developed in this study should be used with caution. TVs from this study are meant to be a starting point for TVs that can be adjusted with future sampling efforts. With more sampling, tolerance values should be pushed closer to their theoretically true values (Lenat, 1993). They were generated to aid in the creation of a Biotic Index for Belizean streams that could also be incorporated in the Multimetric Index. With more sampling, these TVs could even be applied to other regions of Central and South America. Additionally, a Biotic Index, is more quantitative. These TVs are meant to serve as a baseline that can be consistently reevaluated and improved with future research.

Watershed Size and Aquatic Macroinvertebrates

Stream size has been previously shown to influence the richness and abundance of aquatic macroinvertebrates (Paller et al., 2006), and the River Continuum Concept predicts increasing richness and abundance with increasing stream size (Vannote et al., 1980). Taxa richness and number of Ephemeroptera have been shown to increase with increasing stream width in South Carolina (Paller et al., 2006). While watershed size is different than stream size, watershed size was correlated with the Number of Ephemeroptera Collected, Total Number of Scrapers Collected, BMWP-Brazil, and Total Taxa Richness (Table 5). Likewise, Number of Ephemeroptera Collected and Total Number of Scrapers Collected showed significant linear relationships with watershed size (Fig 6c-d). However, except for Total Number of Scrapers Collected, the strength of these relationships fell apart when the three largest sites were excluded. The Multimetric Index was also shown to have a significant positive linear relationship with watershed size, although it does not explain a lot of the variation. However, when the three largest sites are removed the relationship is no longer significant. Based on this information, the Multimetric Index developed should be used with extreme caution on large watersheds (>3000 km^2) and further sampling is needed to account for watershed size. Although watershed size is a factor, the three largest sites were still classified with the Multimetric Index and used for calculating TVs. This is due to site scores for the BMWP and the Multimetric Index mostly agreeing at the the larger sites. Sites 4, 6, and 19 also had water quality values in the range of reference sites, giving confidence for their placement as having excellent water quality (Table 10).

Underlying geology

Underlying geology was a crucial factor that was considered when selecting metrics. Carrie et al., (2015) highlighted the impact that limestone or volcanic drainages had on aquatic macroinvertebrate assemblages. The most noticeable was an increase in the non-insect invertebrates, such as gastropods. Gastropods could impact three of the four metrics but would serve only as a component of Total Taxa Richness, BMWP-Brazil, and % 3 Dominant taxa. However, none of the metrics were correlated with percent limestone. Also, reference and impaired sites both included a varying amount of limestone upstream of sites and therefore metrics should have been less impacted by underlying geology when testing for sensitivity. The amount of limestone was also shown not to be a factor as sites with a high or low amount of limestone in their upstream catchments ranged in bioclassification from excellent to poor. If limestone had an impact on the Multimetric Index, it would be expected that sites would consistently classify as either lower or higher depending on the amount of limestone. This was not the case. For example, both the Macal River at Black Lodge (catchment 14% limestone) and Miguel creek (catchment 91% limestone) were classified as excellent. Billy White Creek (catchment 0% limestone) and Garbot (catchment 100% limestone) were both bioclassified as fair. Still, for future studies, it may be necessary for some areas to tease out underlying geology as a key factor especially if outside lowland streams in the BRW.

Elevation

High elevation sites (above 100m sea level) were not included in the analysis for the Multimetric Index. This was due to the differences in the water chemistry, such as higher levels of dissolved oxygen and lower conductivity. Also, in-stream habitat was different compared to low elevation streams, and high elevation streams were in a vastly different bioregion (Mountain Pine Forests vs Lowland Rainforests and Savannahs). High elevation streams had different Trichopteran taxa (Leptoceridea, Glossosomatidae, Calamoceratidae, *Leptonema spp*, *Macronema spp*) that did not occur in low elevation sites and few snails which were common in low elevation sites. Many high elevation sites had different families of Trichoptera that were only collected at one site. These trends would complicate input into the metrics selected. Taxa richness, % 3 Dominant families, and BMWP would be influenced by increases in Trichoptera diversity, potentially resulting in a higher classification. However, in low elevation sites, there is not the Trichoptera diversity that would help increase scores at sites. A taxa list including genera that were unique to high elevation sites can be found in Appendix B. Elevation is an important driver of aquatic macroinvertebrate richness and density in the Neotropics (Rezende et al., 2014). Due to impacts of elevation, indexes are usually adjusted to account for the differences (Lenat at el., 1988; NCDEQ, 2016); however, in this study there were not enough obviously impacted sites in the high elevation Mountain Pine bioregion to allow for any adjustments to the metrics. Ecosystem and bioregion are also accounted for in other Multimetric Indexes (Barbour et al., 1996) and scores for bioclassification. Lastly, high elevation sites were not included, as the lowland elevation streams are where the human impacts are most severe and frequent. All lowland elevation streams had some amount of human impact (19 out of 30) ranging up to 96% disturbed. Hardly any high elevation sites had a human impact. If human impact on high elevation sites was present, it was only a small percentage of the entire watershed (Max = 7.0%), although legacy effects of logging may be present. Further sampling and investigation in high elevation streams will be necessary to adjust or create a new Multimetric Index for high elevation streams in the BRW.

Seasonality

Seasonality was not assessed during this project and sampling currently would need to occur at the beginning or middle of the dry season. It has been shown in the neotropics and Belize that abundances of aquatic macroinvertebrates can vary widely when comparing wet and dry seasons (Righi-Cavallaro et al., 2010; Helson & Williams 2013; Carrie et al., 2015). Carrie et al., (2015) even found differences in assemblage when comparing the beginning and end of the dry season. The dry season could also be more informative, as water quality conditions tend to be worse during the dry season, partly due to lower flows and therefore less dilution of contaminants (Jacobsen et al., 2008; Damanik-Ambarita et al., 2016; Castellanos Romero et al., 2017; Patang et al., 2018). Although sampling could be more problematic in the wet season, it remains an important aspect, as degradation in water quality can occur during any season. For this reason, more sampling is needed at the beginning of the wet season and throughout the wet season to determine whether the selected metrics are seasonally stable.

Future Studies

Future efforts in Belize need to focus on four key issues: Sampling Efforts, Elevation, Watershed Size, and Seasonality. Primarily, the most important aspect of biomonitoring would be to increase sampling efforts. As of right now, work has been done in the BRW and on a few watersheds in southern Belize (Carrie et al., 2015, 2017). There are 16 watersheds in Belize (Cherrington et al., 2010) and the knowledge of aquatic macroinvertebrates in most of those watersheds is largely absent. Increased sampling will allow the ability to reassess the metrics selected for the Multimetric Index and the ranges of those metrics for determining ranges for scoring a 1, 3, or 5. It will also increase the accuracy of tolerance values proposed by this study which could be expanded to all of Belize and other parts of Central America with more sampling. A larger sample size would also increase the ability to address the other three issues, which are elevation, watershed size, and seasonality.

Elevation is a large issue that this study was not able to address. The Multimetric Index created was based solely on low-level sites (~100M above sea level or lower). Streams sampled in the high elevation were in a different bioregion than lowland streams and its impact on metrics and final bioclassification needs to be accounted for (Barbour et al., 1996). Also, elevation has been shown to impact the density and diversity of aquatic macroinvertebrates in neotropical streams (Rezende et al., 2014). Given the implications for elevation on aquatic macroinvertebrates, it would have been inaccurate to pair them with lowland elevation data.

Watershed size was also a factor that influenced metrics and the Multimetric Index. As a result, sampling rivers with watersheds larger than 3000 km^2 is not advised. Similarly, sampling methods were not meant to be and should not be applied to larger, unwadeable rivers with sandy bottoms, slow flows, or wetland areas. Therefore, bioclassification for water quality in larger rivers is not possible under the BRBP.

Seasonality remains an area that needs to be addressed. All the samples were collected at the beginning of the dry season between December 29th, 2019 through January 14th, 2020. This means that the Multimetric Index cannot be used for collections conducted in the wet season with confidence. Wet seasons have, in some cases, been shown to have lower abundances of aquatic macroinvertebrates (Righi-Cavallaro et al., 2010) and are thought to impact sampling so much that some studies suggest only sampling during the dry season (Helson & Williams, 2013). This could be due to heavy floods in the wet season scouring the bottoms of rivers where aquatic macroinvertebrates exist. Not only are there differences in the wet and dry season, but Carrie et al., (2015) even found some differences in assemblages between the end and beginning of the dry season. This warrants more studies involving seasonality and its impact of the Multimetric Index.

Conclusion

Water Quality impairment is an issue in Belize, especially in the Belize River Watershed (BRW). This project set out to create a water quality monitoring program using aquatic macroinvertebrates. The Belizean Rapid Bioassessment Protocols (BRBP) created standardized protocols for the collection of aquatic macroinvertebrates, and a functioning Multimetric Index was also created for lowland streams in the BRW. A reference collection for training Belizean scientists and or government agencies was created and is housed in Belize to aid in building local capacity. This was crucial, as there is a lack of infrastructure for biomonitoring using aquatic macroinvertebrates in Belize. Finally, tolerance values were derived and proposed for families and genera collected in the BRW. These TVs are proposed and will take more sampling efforts to adjust but could potentially be employed in other Central American regions. With continued sampling, the BRBP can improve or adjust the metrics and proposed TVs. Issues with seasonality, elevation, and watershed size are factors that still need to be addressed with more sampling due to their implications for the assemblages of aquatic macroinvertebrates. Studies on the taxonomy and identification of aquatic macroinvertebrates to the genus and eventually species level are a priority. The BRBP, Multimetric Index and TVs were created to increase biomonitoring in Belize to detect issues that negatively impact water quality.

References

Alba-Tercedor, J. & A. Sanchez Ortega, 1988. Un metodo rapido y simple para evaluar la calidade biologica de las aguas corrientes basado en el metodo de Hellawell. Limnetica 4:51–56.

Armitage, P. D., D. Moss, J. F. Wright, & M. T. Furse, 1983. The performance of a new biological water quality score system based on macroinvertebrates over a wide range of unpolluted running-water sites. Water Research 17: 333–347.

Baptista, D. F., D. F. Buss, M. Egler, A. Giovanelli, M. P. Silveira, & J. L. Nessimian, 2007. A multimetric index based on benthic macroinvertebrates for evaluation of Atlantic Forest streams at Rio de Janeiro State, Brazil. Hydrobiologia 575: 83–94.

Barbour, M. T., J. Gerritsen, G. E. Griffith, R. Frydenborg, E. Mccarron, J. S. White, & M. L. Bastian, 1996. A framework for biological criteria for Florida streams using benthic macroinvertebrates. Journal of the North American Benthological Society 15: 185–211.

Barbour, M.T., J. Gerritsen, B.D. Snyder, & J.B. Stribling, 1999. Rapid bioassessment protocols for use in streams and wadeable rivers: periphyton, benthic macroinvertebrates and fish, second edition. EPA 841-B-99-002. U.S. Environmental Protection Agency; Office of Water; Washington, D.C.

Bonada, N., N. Prat, V. H. Resh, & B. Statzner, 2006. Developments in aquatic insect biomonitoring: A comparative analysis of recent approaches. Annual Review of Entomology Annual Reviews 51: 495–523.

Camargo, J. A., A. Alonso, & M. De La Puente, 2004. Multimetric assessment of nutrient enrichment in impounded rivers based on benthic macroinvertebrates. Environmental Monitoring and Assessment 96: 233–249.

Carrie, R., M. Dobson, & J. Barlow, 2015. The influence of geology and season on macroinvertebrates in Belizean streams : implications for tropical bioassessment. Freshwater Sciences 34: 648–662.

Carrie, R., M. Dobson, & J. Barlow, 2017. Challenges using extrapolated family-level macroinvertebrate metrics in moderately disturbed tropical streams: a case-study from Belize. Hydrobiologia 794: 257–271.

Carter, J. L., Resh, V. H., Rosenberg, D. M., & T. B. Reynoldson, 2006. Biomonitoring in North American rivers: A comparison of methods used for benthic macroinvertebrates in Canada and the United States. In G. Ziglio, M. Siligardi, & G. Flaim (Eds.), Biological monitoring of rivers. Chicester: Wiley, p. 203– 228.

Castellanos Romero, K., J. Pizarro Del Río, K. Cuentas Villarreal, J. C. Costa Anillo, Z. Pino Zarate, L. C. Gutierrez, O. L. Franco, & J. W. Arboleda Valencia, 2017. Lentic water quality characterization using macroinvertebrates as bioindicators: An adapted BMWP index. Ecological Indicators. 72: 53–66.

Chang, F. H., J. E. Lawrence, B. Rios-Touma, & V. H. Resh, 2014. Tolerance values of benthic macroinvertebrates for stream biomonitoring: Assessment of assumptions underlying scoring systems worldwide. Environmental Monitoring and Assessment 186: 2135–2149.

Cherrington, E. A., E. Ek, P. Cho, B. F. Howell, B. E. Hernandez, E. R. Anderson, A. I. Flores, B. C. Garcia, A. Sempris & D. E. Irwin, 2010. Forest cover and deforestation in Belize: 1980–2010. Water Center for the Humid Tropics of Latin America and the Caribbean (Cathalac), Panama, Panama City.

Chesters, R. K., 1980. Biological Monitoring Working Party The 1978 national testing exercise Department of the Environment Water Data Unit Technical Memorandum 19, 1-37.

Chutter, F. M., 1972. An empirical biotic index of the quality of water in South African streams and rivers. Water Research Water Research Pergamon Press 6: 19–30.

Damanik-Ambarita, M. N., K. Lock, P. Boets, G. Everaert, T. H. T. Nguyen, M. A. E. Forio, P. L. S. Musonge, N. Suhareva, E. Bennetsen, D. Landuyt, L. Dominguez-Granda, & P. L. M. Goethals, 2016. Ecological water quality analysis of the Guayas river basin (Ecuador) based on macroinvertebrates indices. Limnologica 57: 27–59.

Dobriyal, P., R. Badola, C. Tuboi, & S. A. Hussain, 2017. A review of methods for monitoring streamflow for sustainable water resource management. Applied Water Science Springer Berlin Heidelberg 7: 2617–2628.

Doruson, D., 2009. A Natural History of the Bladen Nature Reserve and its Gastropods. Belize Foundation for Research and Environmental Education, p. 1-152.

Dudgeon, D., 2006. The impacts of human disturbance on stream benthic invertebrates and their drift in north Sulawesi, Indonesia. Freshwater Biology 51: 1710–1729.

Epler, J. H. 2001. Identification manual for the larval Chironomidae (Diptera) of North and South Carolina. Special Publication SJ2001-SP13. North Carolina Department of Environment and Natural Resources, Raleigh, North Carolina, USA.

Everall, N. C., M. F. Johnson, P. Wood, A. Farmer, R. L. Wilby, & N. Measham, 2017. Comparability of macroinvertebrate biomonitoring indices of river health derived from semi-quantitative and quantitative methodologies. Ecological Indicators 78: 437–448.

Fernández, L., 2002. Uso de insectos acuáticos como bioindicadores de la calidad de agua de ríos utilizados por beneficios de café en la provincia de Alajuela, Costa Rica. Licenciatura, Escuela de Biología, San José, Universidad de Costa Rica, p. 69.

Ferreira, W., L. Paiva, & M. Callisto, 2011. Development of a benthic multimetric index for biomonitoring of a neotropical watershed. Brazilian Journal of Biology Instituto Internacional de Ecologia 71: 15–25.

Helson, J. E., & D. D. Williams, 2013. Development of a macroinvertebrate multimetric index for the assessment of low-land streams in the neotropics. Ecological Indicators 29: 167–178.

Hilsenhoff, W. L., 1987. An improved biotic index of organic stream pollution. Great Lakes Entomologist 20: 31–40.

Hilsenhoff, W. L., 1988. Rapid Field Assessment of Organic Pollution with a Family-Level Biotic Index. Journal of the North American Benthological Society 7: 65–68.

Hynes, H.B., 1960. The Biology of Polluted Waters. Liverpool University Press, Liverpool.

Jacobsen, D., C. Cressa, J. M. Mathooko, & D. Dudgeon, 2008. Macroinvertebrates: composition, life histories and production. In Dudgeon, D. (ed), Tropical Stream Ecology. Elsevier Inc., London: 65–105.

Junqueira, MV., M.C., Amarante, C.F.S, Dias, & E.S., Franca, 2000. Biomonitoramento da qualidade das águas da Bacia do Rio das Velhas (MG/Brasil) através de macroinvertebrados. Acta Limnologica Brasiliensia, vol. 12, no. 1, p. 73-87.

Karper, J., & E. Boles, 2004. Human Impact Mapping of the Mopan and Chiquibul Rivers within Guatemala and Belize With Comments on Riparian Forest Ecology, Conservation, and Restoration. Unpublished Access.

Karr, J. R., K. D. Fausch, P. L. Angermeier, P. R. Yant, & I. J. Schlosser, 1986. Assessing biological integrity in running waters: A method and its rationale. Illinois Natural History Survey Special Publication 5 5: 28.

Kolkwitz, R., & M., Marsson, 1908. Okologie der pflanzlichen Saprobien. Ber. dr. bot. Ges. 26, 505-519.

Kolkwitz, R., & M., Marsson, 1909. Okologie der tierische Saprobien. Beitrige zur Lehre yon der biologische Gewserbeurteilung. Int. Rev. Hydrobiol. 2, 126-152.

Lenat, D. R., 1988. Water Quality Assessment of Streams Using a Qualitative Collection Method for Benthic Macroinvertebrates. Journal of the North American Benthological Society 7: 222–233.

Lenat, D. R., 1993. A biotic index for the southeastern United States: Derivation and list of tolerance values, with criteria for assigning water-quality ratings. Journal of the North American Benthological Society 12: 279–290.

Lenat, D. R., & V. H. Resh, 2001. Taxonomy and stream ecology. The benefits of genus- and species-level identifications. Journal of the North American Benthological Society 20: 287–298.

Mark, J., & P. Planner, 2003. County of Santa Clara riparian corridor study: a background document for the development of a riparian protection ordinance for the county of Santa Clara. Planning Office, Environmental Resources Agency, County of Santa Clara: 3–31.

Macedo, D. R., R. M. Hughes, W. R. Ferreira, K. R. Firmiano, D. R. O. Silva, R. Ligeiro, P. R. Kaufmann, & M. Callisto, 2016. Development of a benthic macroinvertebrate multimetric index (MMI) for Neotropical Savanna headwater streams. Ecological Indicators 64: 132–141.

Meerman, J., & Clabaugh, J. 2017. Biodiversity and Environmental Resource Data System of Belize. Online. http://www.biodiversity.bz.

North Carolina (NC) Department of Environmental Quality. 2016. Standard operating procedures for the collection and analysis of benthic macroinvertebrates. Division of Water Resources. Raleigh, North Carolina. February 2016.

Paller, M. H., W. L. Specht, & S. A. Dyer, 2006. Effects of stream size on taxa richness and other commonly used benthic bioassessment metrics. Hydrobiologia 568: 309–316.

Patang, F., A. Soegianto, & S. Hariyanto, 2018. Benthic Macroinvertebrates Diversity as Bioindicator of Water Quality of Some Rivers in East Kalimantan, Indonesia. International Journal of Ecology 2018: 1–11.

Quinn, J.M., A.B. Cooper, R.J. Davies-Colley, J.C. Rutherford, & R.B. Williamson, 1997. Land-use effects on habitat, periphyton, and benthic invertebrates in Waikato Hill Country streams. New Zealand Journal of Marine and Freshwater Research 31:579-597.

Resh, V. H., 2008. Which group is best? Attributes of different biological assemblages used in freshwater biomonitoring programs. Environmental Monitoring and Assessment 138: 131–138.

Resh, V. H., & J. D. Unzicker, 1975. Water quality monitoring and aquatic organisms: the importance of species identification. Journal of the Water Pollution Control

Federation 47: 9–19.

Resh, V. H., R. H. Norris, & M. T. Barbour, 1995. Design and implementation of rapid assessment approaches for water resource monitoring using benthic macroinvertebrates. Australian Journal of Ecology 20: 108–121.

Rezende, R. S., A. M. Santos, C. Henke-Oliveira, & J. F. Gonçalves, 2014. Effects of spatial and environmental factors on benthic a macroinvertebrate community. Zoologia 31: 426–434.

Righi-Cavallaro, K. O., K. F. Roche, O. Froehlich, & M. R. Cavallaro, 2010. Structure of macroinvertebrate communities in riffles of a Neotropical karst stream in the wet and dry seasons. Acta Limnologica Brasiliensia 22: 306–316.

Rosenberg, D.M., and Resh, V.H. (Eds.), 1993. Freshwater Biomonitoring and Benthic Macroinvertebrates. Chapman and Hall, New York.

Springer, M., 2008. Aquatic insect diversity of Costa Rica: State of knowledge. Rev. Biol. Trop 56: 273-295.

Stein, H., M. Springer, & B. Kohlmann, 2008. Comparison of two sampling methods for biomonitoring using aquatic macroinvertebrates in the Dos Novillos River, Costa Rica. Ecological Engineering 34: 267–275.

Thorp H, J., 2018. Thorp and Covich freshwater invertebrates volume III: Keys to neotropical Hexapoda. Elsevier, London.

Uherek, C. B., & F. B. Pinto Gouveia, 2014. Biological monitoring using macroinvertebrates as bioindicators of water quality of maroaga stream in the maroaga cave system, presidente figueiredo, Amazon, Brazil. International Journal of Ecology 2014: 1–7.

Valente-Neto, F., R. Koroiva, A. A. Fonseca-Gessner, & F. Roque, 2015. The effect of riparian deforestation on macroinvertebrates associated with submerged woody debris. Aquatic Ecology 49: 115–125.

Vannote, R. L., G. W. Minshale, K. W. Cummins, J. R. Sedell, & C. E. Cushing, 1980. The River Continuum Concept. Canadian Journal of Fisheries and Aquatic Sciences 37: 130–137.

Virginia Department of Environmental Quality(VDEQ) , 2008. Biological Monitoring Program Quality Assurance Project Plan for Wadeable Streams and Rivers. Richmond, VA.

Weigel, B. M., L. J. Henne, & L. M. Martínez-Rivera, 2002. Macroinvertebrate-based index of biotic integrity for protection of streams in west-central Mexico. Journal of the North American Benthological Society 21: 686–700

Young, C., 2008. Belize's ecosystems: Threats and challenges to conservation in Belize. Tropical Conservation Science 1:18-33.

Tables

Table 1 Water chemistry and % disturbed for both impacted and reference sites (Meant±SE).

Site	% Disturbed	Dissolved Oxygen (mg/L)	Specific Conductivity (µS/cm)	Chloride (mg/L)
Reference	18±6	7.82±0.21	328.06±52.42	8.18±0.58
Impaired	66±15	4.37±0.64	1468.50±233.54	156.24±48.36

Table 2 Candidate metrics after removing all the metrics with a range of less than 5 or more than 33% of the sample with a value of zero.

Metric	Sensitivity Score	T Stat or U Value	P-Value
Total Number Collected	2	-	-
**Number of Ephemeroptera Collected*	*3*	*23.00*	*0.022*
Number of Trichoptera Collected	1	-	-
**Number of EPT Collected*	*3*	*22.00*	*0.014*
Number of Odonata Collected	0	-	-
Number of Diptera Collected	2	-	-
Number of Coleoptera Collected	2	-	-
*Number of Leptophlebiidae Collected	3	23.00	0.017
Number of Leptohyphidae Collected	2	-	-
Number of Hydropsychidae Collected	0	-	-
Total Number of Collectors-Gatherers Collected	2	-	-
Total Number of Filterers Collected	1	-	-
Total Number of Predators Collected	2	-	-
Total Number of Scrapers Collected	*3*	*2.93*	*0.043*
% Ephemeroptera	2	-	-
% Trichoptera	0	-	-

Metric	Sensitivity Score	T Stat or U Value	P-Value
%EPT	1	-	-
% Odonata	0	-	-
**% Diptera*	*3*	*50.00*	*0.014*
% Coleoptera	0	-	-
Taxa Richness	*3*	*-3.27*	*0.011*
Family Richness	2	-	-
Ephemeroptera Taxa Richness	*3*	*-7.45*	*<0.001*
Trichoptera Taxa Richness	2	-	
Odonata Taxa Richness	1	-	-
EPT Taxa Richness	*3*	*-4.84*	*0.002*
% Collectors-Gatherers	0	-	-
% Filterers	0	-	-
% Predators	0	-	-
% Scrapers	0	-	-
% Leptophlebiidae	2	-	-
% Leptohyphidae	0	-	-
% Hydropsychidae	0	-	-
% Dominant Taxa	2	-	-
% Non-insect	0	-	-
% 2 Dominant Taxa	2	-	-
% Dominant Family	*3*	*3.11*	*0.027*
% 2 Dominant Families	*3*	*2.79*	*0.023*
% 3 Dominant Families	*3*	*3.50*	*0.010*
BMWP-CR Score	2	-	-
Average Score Per Taxon-Cr	2	-	-
BMWP-Brazil	*3*	*3.48*	*0.009*
Average Score per Taxon-Brazil	*2*	-	-

Metrics that had a sensitivity score of 3 and confirmed difference with 2-sample t-test or a Mann Whitney U Test are italicized (* indicates Mann Whitney U Test).

Table 3 Box plot statistics for candidate metrics for the Index (Q1 is 25% and Q3 is the 75%) and ranges for each metrics with their corresponding score. Metrics used for the index are shown in italics.

Metric	Statistics					Score		
	Min	Q1	Median	Q3	Max	5	3	1
Number of Ephemeroptera Collected	*22*	*49*	*58*	*63*	*150*	*>49*	*49-22*	*<22*
Number of Leptophlebiidae Collected	1	4	20	23	81	>4	3-1	<1
Number of EPT Collected	59	61	72	125	214	>61	61-59	<59
Total Number of Scrapers Collected	15	36	39	57	78	>36	36-15	<15
% Diptera	0.54	1.82	2.50	2.75	6.35	<2.75	6.35-2.75	> 6.35
Taxa Richness	*16*	*21*	*24*	*28*	*30*	*>21*	*21-16*	*< 16*
EPT Taxa Richness	6	7	8	9	12	> 7	6	< 6
BMWP-Brazil	*66*	*88*	*101*	*109*	*110*	*>88*	*88-66*	*< 66*
Ephemeroptera Taxa Richness	4	5	6	6	7	> 5	4	< 4
% Dominant Family	20	26	29	31	31	<31	31	>31
% 2 Dominant Families	36	40	46	46	59	<46	46-58	>58
% 3 Dominant Families	*51*	*53*	*55*	*58*	*67*	*<58*	*58-67*	*>67*

Table 4 Spearman correlations for metrics, and underlying geology.

Sample 1	Sample 2	N	Correlation	95% CI for ρ	P-Value
Taxa Richness	%Limestone	17	0.310	(-0.212, 0.695)	0.226
BMWP	%Limestone	17	0.385	(-0.136, 0.739)	0.127
Number of Ephemeroptera Collected	%Limestone	17	0.397	(-0.123, 0.746)	0.114
Total Number of Scrapers Collected (Sc)	%Limestone	17	0.289	(-0.233, 0.682)	0.260
% Dominant Family	%Limestone	17	-0.299	(-0.688, 0.224)	0.244
% 2 Dominant Families	%Limestone	17	-0.242	(-0.652, 0.277)	0.350
% 3 Dominant Families	%Limestone	17	-0.271	(-0.671, 0.250)	0.292

Sample 1	Sample 2	N	Correlation	95% CI for ρ	P-Value
BMWP	Taxa Richness	17	0.909	(0.716, 0.973)	0.000
Number of Ephemeroptera Collected	Taxa Richness	17	0.600	(0.123, 0.852)	0.011
Total Number of Scrapers Collected (Sc)	Taxa Richness	17	0.565	(0.076, 0.835)	0.018
% Dominant Family	Taxa Richness	17	-0.800	(-0.936, -0.460)	0.000
% 2 Dominant Families	Taxa Richness	17	-0.816	(-0.941, -0.493)	0.000
% 3 Dominant Families	Taxa Richness	17	-0.876	(-0.962, -0.632)	0.000
Number of Ephemeroptera Collected	BMWP	17	0.677	(0.238, 0.886)	0.003
Total Number of Scrapers Collected (Sc)	BMWP	17	0.421	(-0.097, 0.760)	0.092
% Dominant Family	BMWP	17	-0.874	(-0.962, -0.626)	0.000
% 2 Dominant Families	BMWP	17	-0.900	(-0.970, -0.692)	0.000
% 3 Dominant Families	BMWP	17	-0.950	(-0.986, -0.835)	0.000
Total Number of Scrapers Collected (Sc)	Number of Ephemeroptera Collected	17	0.478	(-0.032, 0.791)	0.052
% Dominant Family	Number of Ephemeroptera Collected	17	-0.554	(-0.829, -0.061)	0.021
% 2 Dominant Families	Number of Ephemeroptera Collected	17	-0.588	(-0.846, -0.107)	0.013
% 3 Dominant Families	Number of Ephemeroptera Collected	17	-0.651	(-0.875, -0.197)	0.005
% Dominant Family	Total Number of Scrapers Collected (Sc)	17	-0.389	(-0.742, 0.132)	0.123
% 2 Dominant Families	Total Number of Scrapers Collected (Sc)	17	-0.358	(-0.724, 0.164)	0.158
% 3 Dominant Families	Total Number of Scrapers Collected (Sc)	17	-0.407	(-0.752, 0.112)	0.105

Sample 1	Sample 2	N	Correlation	95% CI for ρ	P-Value
% 2 Dominant Families	% Dominant Family	17	0.983	(0.940, 0.995)	0.000
% 3 Dominant Families	% Dominant Family	17	0.958	(0.860, 0.988)	0.000
% 3 Dominant Families	% 2 Dominant Families	17	0.983	(0.940, 0.995)	0.000

Table 5 Spearman correlation for metrics and watershed size.

Sample 1	Sample 2	N	Correlation	95% CI for ρ	P-Value
Taxa Richness	Total Area (km^2)	20	0.504	(0.050, 0.785)	0.023
BMWP-Brazil	Total Area (km^2)	20	0.451	(-0.012, 0.755)	0.046
Number of Ephemeroptera Collected	Total Area (km^2)	20	0.567	(0.130, 0.819)	0.009
Total Number of Scrapers Collected (Sc)	Total Area (km^2)	20	0.722	(0.361, 0.895)	0.000
% 3 Dominant Families	Total Area (km^2)	20	-0.344	(-0.690, 0.129)	0.137

Table 6 Watershed size for low elevation sites in the Belize River Watershed.

Site #	Site Name	Watershed Size (km^2)
Site #1	Beaver Dam Cr	221.69
Site #2	Mount Pleasant	8.91
Site #3	Macal	1420.64
Site #4	Mopan	3743.04
Site #5	Billy White Creek	24.81
Site #6	Belize River	5540.72
Site #7	Jenny Cr	40.45
Site #17	Cristo Ray Bridge Crossing	1.43
Site #18	Macal (Black Lodge)	1293.46
Site #19	Mopan	3516.15
Site #20	Garbot Cr	1.60
Site #21	Barton Cr	122.53
Site #22	Yalbac	275.70
Site #23	N/A	8.12
Site #24	Iguana Cr (?)	71.72
Site #25	Roaring River	286.11
Site #26	Miguel Cr	3.76

Site #	Site Name	Watershed Size (km^2)
Site #28	Trib of Spanish Cr	0.96
Site #29	Mexico Cr	142.97
Site #30	Unnamed Trib of Mexico Cr, West @ bridge	1.31

Table 7 Spearman correlation for metrics and watershed size excluding 3 largest sites

Sample 1	Sample 2	N	Correlation	95% CI for ρ	P-Value
Taxa Richness	Total Area (km^2)	17	0.347	(-0.176, 0.717)	0.173
BMWP-Brazil	Total Area (km^2)	17	0.280	(-0.242, 0.676)	0.277
Number of Ephemeroptera Collected	Total Area (km^2)	17	0.327	(-0.196, 0.705)	0.201
Total Number of Scrapers Collected (Sc)	Total Area (km^2)	17	0.598	(0.120, 0.851)	0.011
% 3 Dominant Families	Total Area (km^2)	17	-0.267	(-0.668, 0.254)	0.300

Table 8 Final Metrics and their ranges for their respective scores.

Metric	Statistics					Score		
	Min	Q1	Median	Q3	Max	5	3	1
Number of Ephemeroptera Collected	22	49	58	63	150	>49	49-22	<22
Taxa Richness	16	21	24	28	30	>21	21-16	< 16
BMWP-Brazil	66	88	101	109	110	>88	88-66	< 66
% 3 Dominant Families	51	53	55	58	67	<58	58-67	>67

Table 9. Range scores and their corresponding water quality classification. Numbers in bold should be taken with caution until more robust sampling can occur.

Classifications	Poor	Fair	Good	Excellent
Scores	4,5,6,**7**	**8,**9,10,11,**12**	**13**,14,15,**16**	**17**,18,19,20

Table 10 List of low elevation sites with corresponding water chemistry data, scores for the MMBI, and water quality bioclassification. Streams that were considered reference streams in italics and streams that were considered impaired are

Site Name	Conductivity (µS/cm)	Dissolved Oxygen	pH	Chloride (mg/L)	Score	Bioclassification
Beaver Dam Creek	481	88%	7.49	9.57	14	Good
Mount Pleasant Creek	708	62%	7.52	19.43	12	Fair
Macal River	219	92%	7.41	7.6	18	Excellent
Mopan River	408	108%	8.4	8.38	16	Good
Billy White Creek	1670	65%	7.53	200	4	Poor
Belize River	359.6	113%	8.11	9.61	18	Excellent
Jenny Creek	1332	49%	7.83	89	6	Poor
Cristo Ray Bridge	510	45%	7.4	7.95	8	Fair
Macal River (Black Lodge)	203.3	100%	7.21	6.83	20	Excellent
Mopan River San Succotz	405	111%	8.05	6.31	20	Excellent
Garbot Creek	604	48%	7.26	12.4	12	Fair
Barton Creek	348	88%	7.51	9.56	14	Good
Yalbac Creek	643	68%	8.02	38	4	Poor
Saturday Creek	1258	60%	7.69	191	4	Poor
Iguana Creek	1400	15%	7.56	85	8	Fair
Roaring River	389	98%	7.62	7.35	18	Excellent
Miguel Creek	891	48%	6.92	23.92	18	Excellent
Tributary of Spanish Creek	2632	49%	7.26	12.8	6	Poor
Mexico Creek	3505	51%	6.87	514	4	Poor
Tributary of Mexico Creek	2443	64%	6.61	353	4	Poor

Table 11. Site scores and classifications for both the BMWP-Brazil (Uherek & Pinto Gouveia, 2014) and the Multi metric Index (MMI) from Belize.

Site	BMWP Score	BMWP Classification	MMI Score	MMI Classification
Site #1	66	Acceptable	14	Good
Site #2	88	Acceptable	12	Fair
Site #3	110	Good	18	Excellent
Site #4	94	Acceptable	16	Good
Site #5	23	Critical	4	Poor
Site #6	100	Acceptable	18	Excellent
Site #7	56	Questionable	6	Poor
Site #17	65	Acceptable	8	Fair
Site #18	109	Good	20	Excellent
Site #19	100	Acceptable	20	Excellent
Site #20	83	Acceptable	12	Fair
Site #21	88	Acceptable	14	Good
Site #22	43	Questionable	4	Poor
Site #23	57	Questionable	4	Poor
Site #24	52	Questionable	8	Fair
Site #25	101	Good	18	Excellent
Site #26	143	Good	18	Excellent
Site #28	48	Questionable	6	Poor
Site #29	41	Questionable	4	Poor
Site #30	47	Questionable	4	Poor

Table 12. Tolerance values (TV) for families collected in the BRW. Families TVs are weighted averages of genera that had a calculated TV.

Family	Proposed Tolerance Value
Sensitive	
Ephemeridae	0.00
Perlidae	0.00
Ampullaridae	3.06
Platystictoidae	3.61
Psephenidae	3.80
Intermediate	
Gomphidae	4.02
Baetidae	4.39
Corydalidae	4.67

Family	Proposed Tolerance Value
Elmidae	4.83
Leptohyphidae	4.84
Leptophlebiidae	5.15
Dytiscidae	5.56
Thiaridae	5.57
Cosmopterigidae	5.74
Philopotamidae	5.97
Gerridae	6.17
Unionidae	6.30
Caenidae	6.49
Libellulidae	6.49
Physidae	6.52
Simullidae	6.64
Calopterygidae	6.85
Tolerant	
Pachychilidae	7.07
Coenagrionidae	7.10
Cambaridae	7.47
Scirtidae	7.80
Momphidae	8.00
Culicidae	8.42
Hydrophilidae	8.72

Table 13. Tolerance values (TV) for genera collected in the BRW, their average relative abundance in each bioclassification, and the number of individuals collected.

	Water Quality Bioclassification and Scores					
	Excellent 1	Good 2	Fair 3	Poor 4	N	Proposed TV
Sensitive						
Anacroneuria spp	3.50	0.00	0.00	0.29	73	0.00
Hexagenia spp	0.50	0.00	0.00	0.00	3	0.00
Traverella spp	2.50	0.00	0.00	0.00	64	1.11
Camelobaetidus spp	2.17	0.33	0.25	0.00	46	2.64
Pomacea flagellata	1.17	0.33	0.00	0.00	12	3.06
Phyllogomphoides spp	4.00	1.33	0.00	0.14	45	3.51
Palaemnema spp	0.83	0.33	0.00	0.00	9	3.61
Psephenops spp	2.17	1.00	0.00	0.00	27	3.80
Baetodes spp	0.67	0.33	0.00	0.00	42	3.89

	Water Quality Bioclassification and Scores					
	Excellent 1	Good 2	Fair 3	Poor 4	N	Proposed TV
Corydalus spp	3.17	3.33	0.25	0.14	94	4.67
Cabecar spp	2.67	4.33	0.25	0.00	73	4.75
Askola spp	1.67	4.33	0.00	0.00	130	4.79
Macrelmis spp	1.00	3.33	0.00	0.00	30	4.83
Americabaetis spp	3.83	3.33	1.00	0.00	94	4.86
Baetis/Fallceon	6.5	4.33	0.00	1.86	117	4.88
Farrodes spp	5.00	3.33	1.00	0.57	157	4.95
Tricorythododes spp	2.17	1.00	0.75	0.00	29	5.05
Tarebia granifera	5.67	6.67	0.50	2.00	183	5.15
Hesperagrion spp	0.50	3.33	1.00	0.00	22	5.42
Hagenulopsis spp	0.00	1.33	0.00	0.43	26	5.54
Chimarra spp	10.00	7.67	5.75	1.57	501	5.97
Progomphus spp	0.17	0.33	0.25	0.00	11	6.11
Melanoides tuberculata	3.50	4.00	1.25	1.71	127	6.16
Metrobates spp	0.33	0.00	0.75	0.00	9	6.17
Macrothemis spp	0.00	1.00	1.00	0.00	10	6.67
Pachychilus spp	0.50	1.33	2.50	0.00	26	6.81
Hetarina spp	0.50	1.33	2.75	0.00	33	6.85
Pachychilus largillierti	0.00	3.33	2.50	0.57	23	6.86
Caenis spp	0.17	0.33	1.00	0.00	10	6.94
Tolerant						
Paltothemis spp	0.50	0.33	2.50	0.14	31	7.13
Pachychilus corvinus	3.67	0.33	2.5	1.43	53	7.29
Argia spp	3.00	3.00	5.75	2.57	163	7.39
Smicridae	4.83	1.33	0.25	3.29	210	8.36
Tropisternus spp	0.00	0.00	0.75	0.57	8	8.72
Dythemis spp	0.17	1.00	0.50	1.43	23	8.80

Figures.

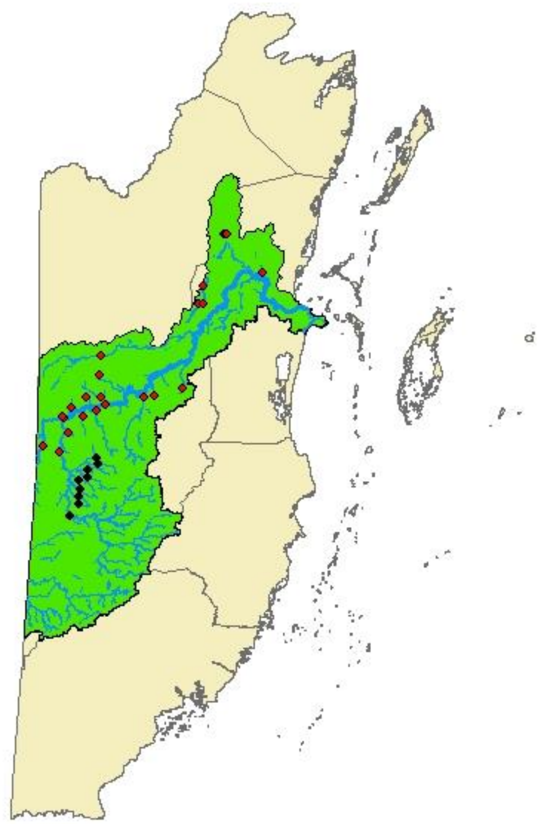


Fig 1. Map of sampling sites in the in the Belize portion of the Belize River Watershed. High elevation sites are black and low elevation sites are red.

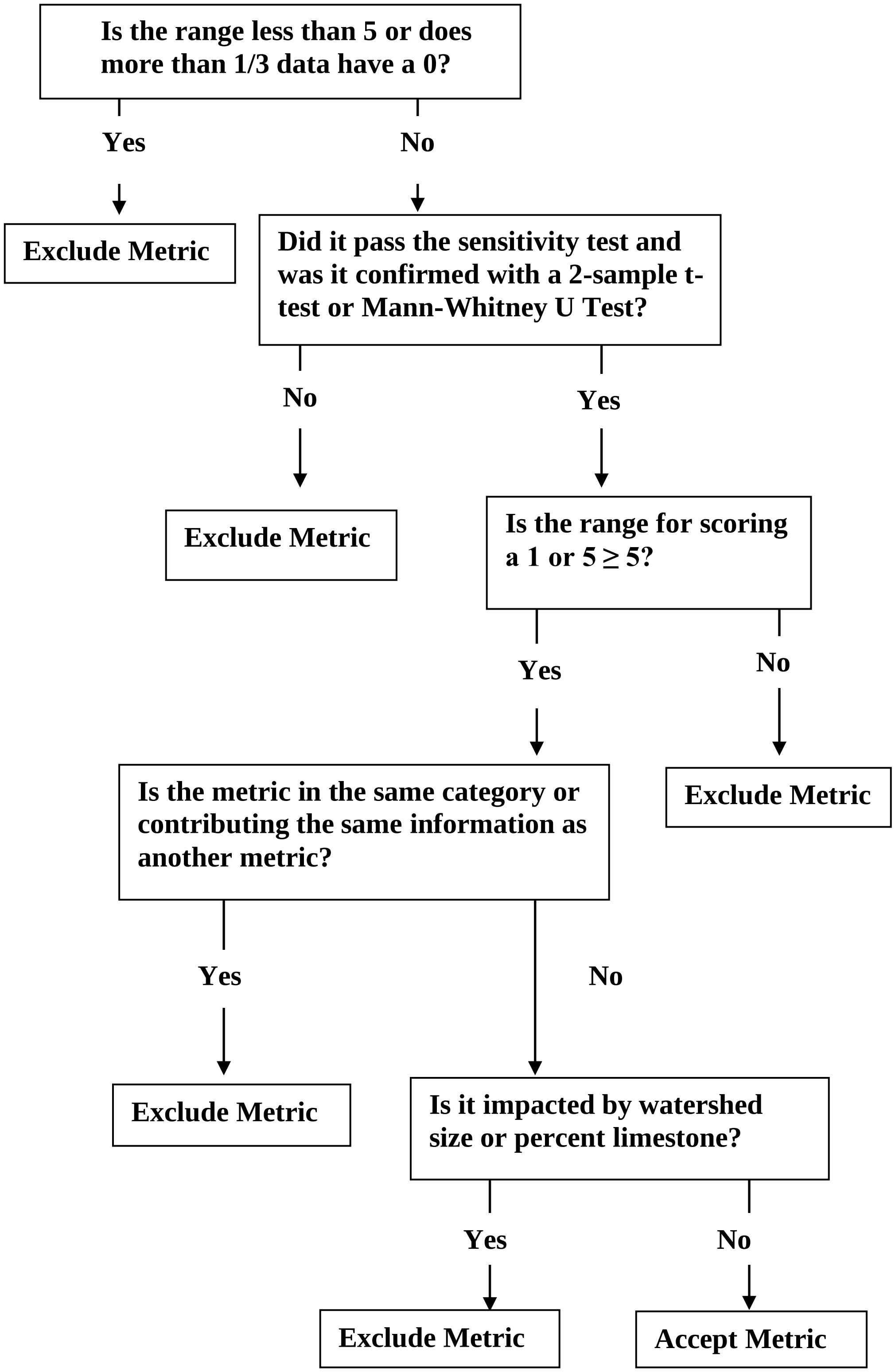


Fig 2. Flow chart on how metrics were either rejected or selected to be incorporated into the Multimetric index.

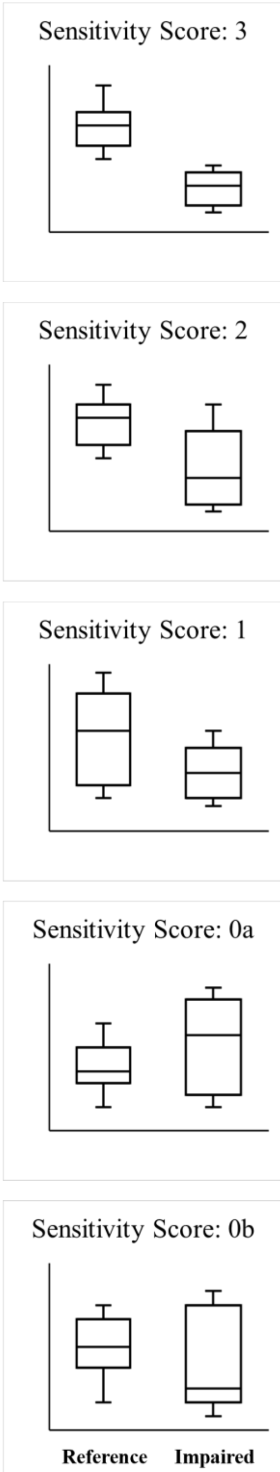


Fig 3. Box and Whisker-plots illustrating the point system for testing sensitivity of metrics at most and least impacted sites. Rectangles indicate interquartile ranges and horizontal lines are median values. Based on a figure from Barbour et al., 1996.

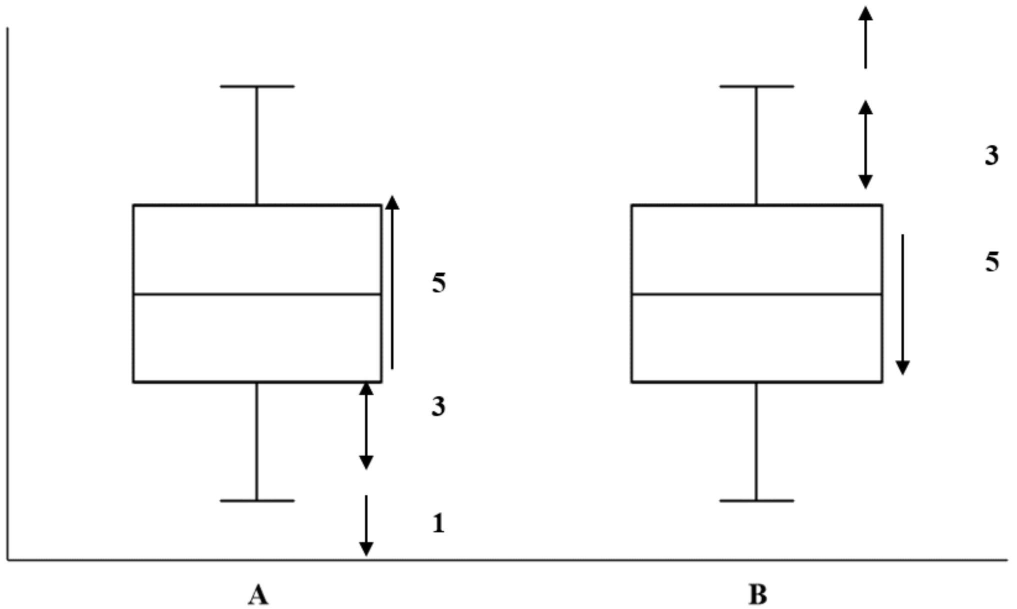
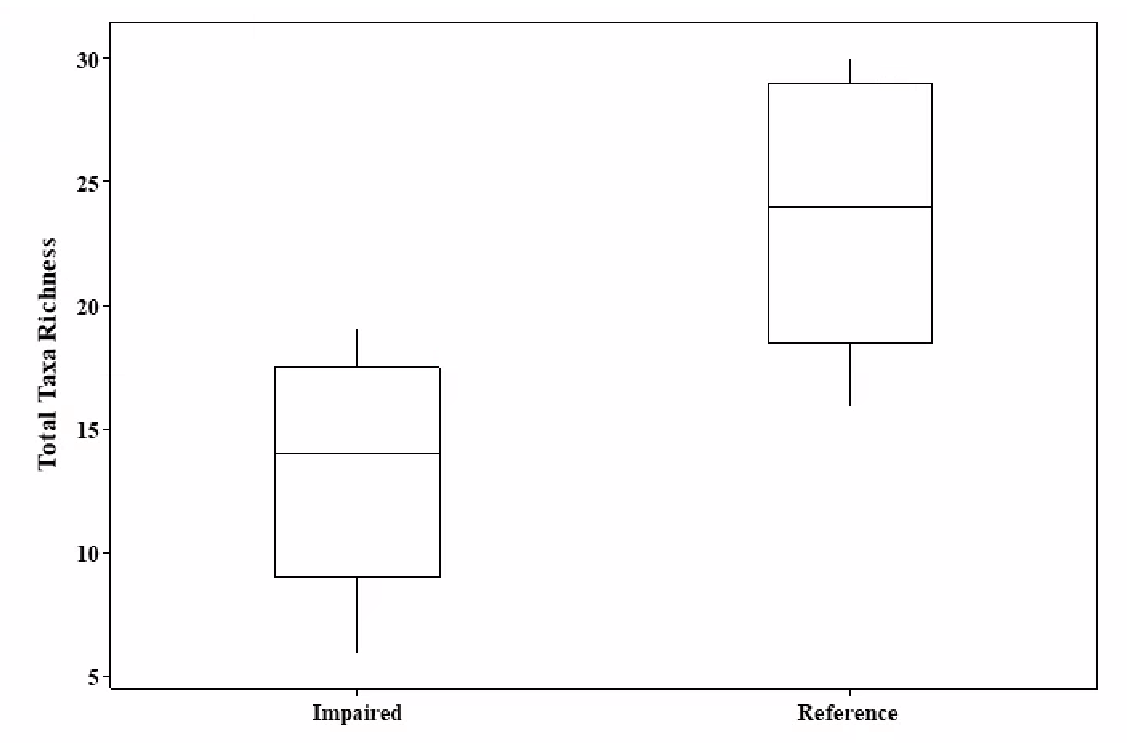
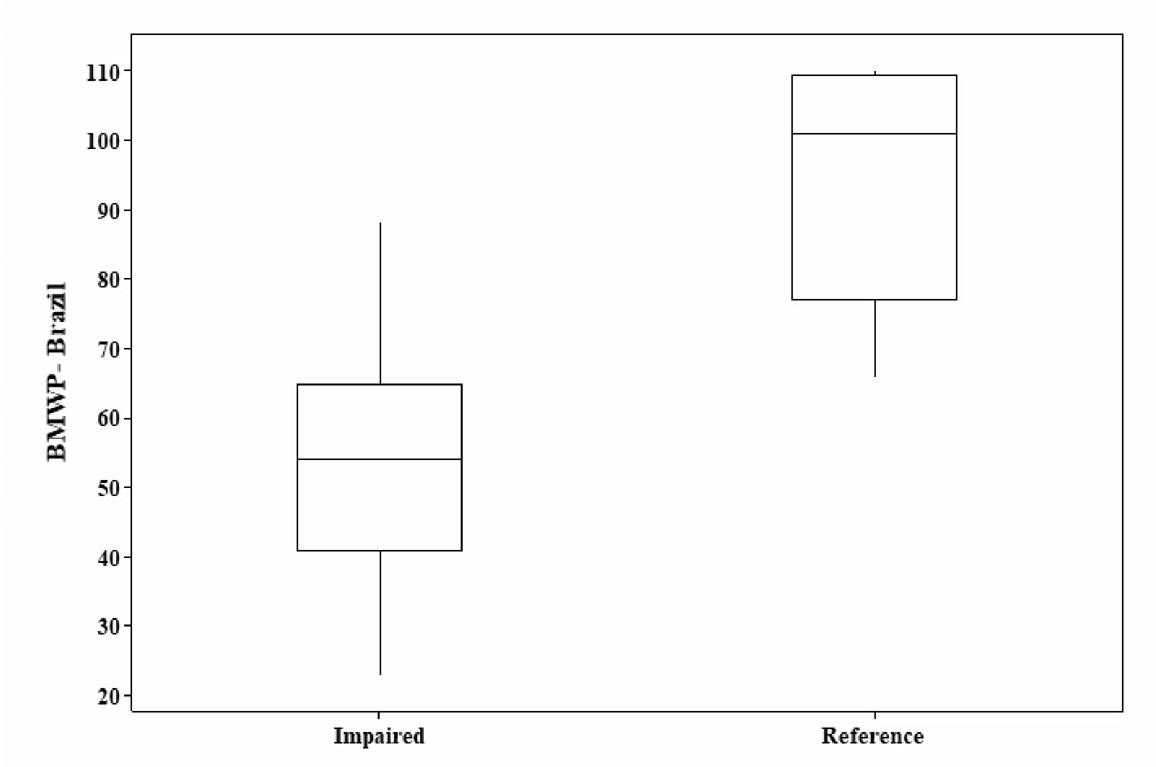


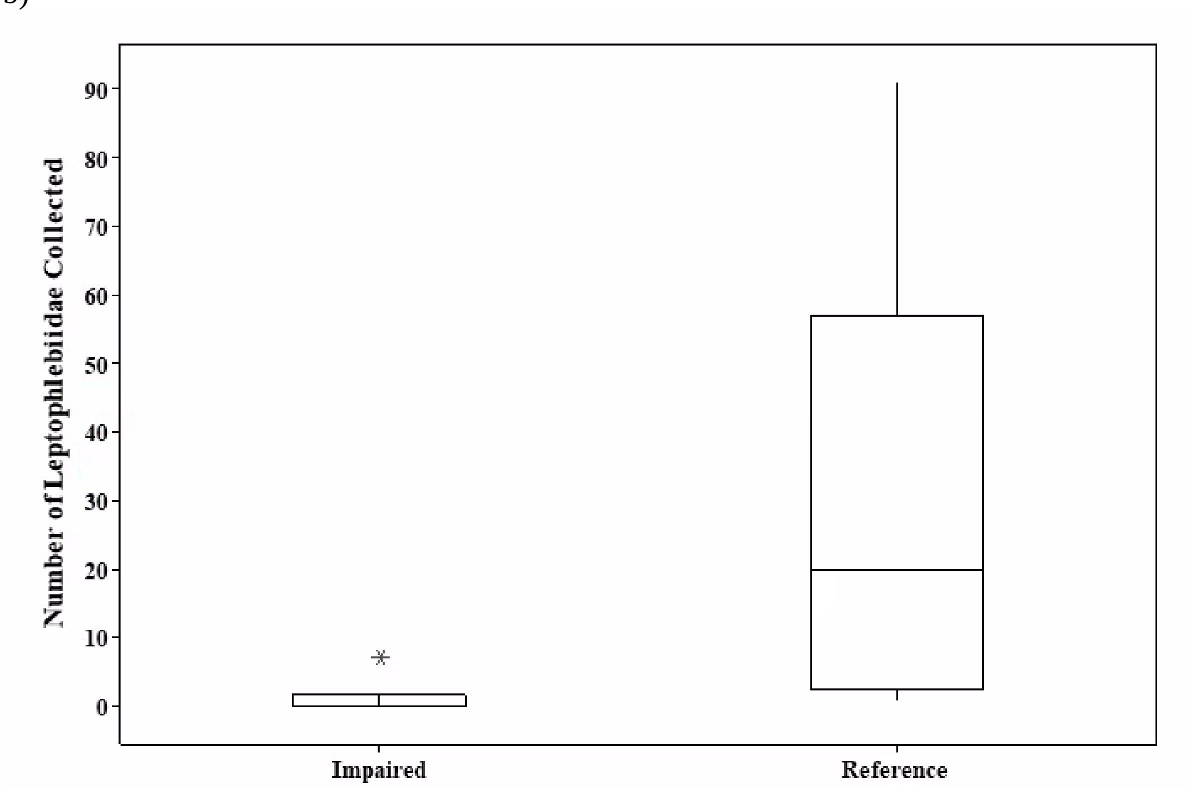
Fig 4. Box and Whisker-plots illustrating the assignment of scores of 5, 3, or 1 based on reference sites depending on if the metric is expected to decrease (A) or increase (B) with increasing impairment. Based on a figure from Baptista et al., 2007.



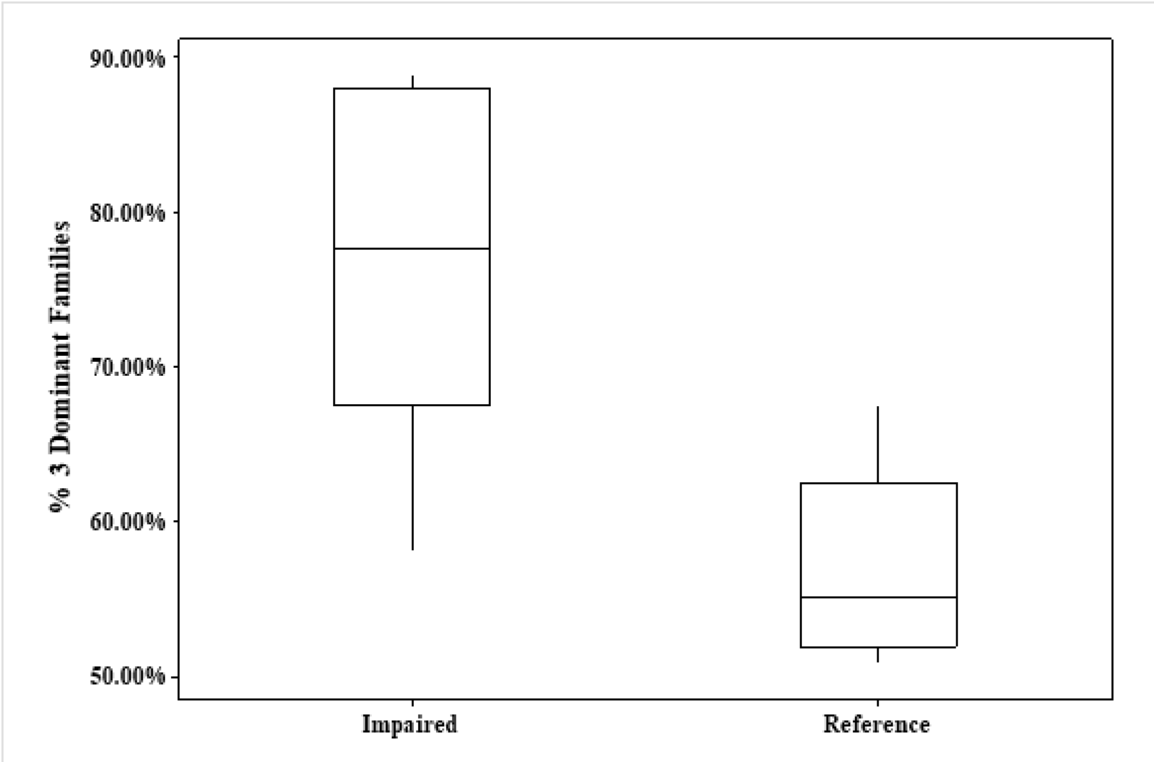
a)


BMWP- Brazil
110
100
90
80
70
60
50
40
30
20
Impaired
Reference

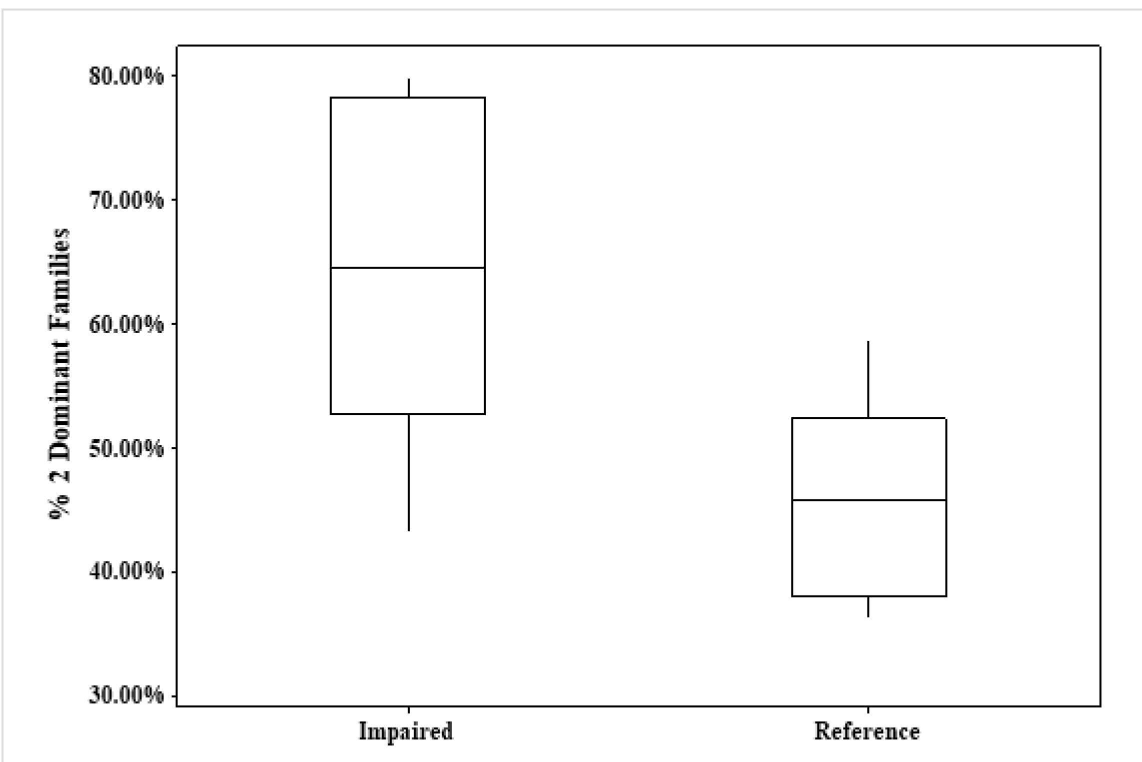
b)


Number of Leptophlebiidae Collected
90
80
70
60
50
40
30
20
10
0
Impaired
Reference

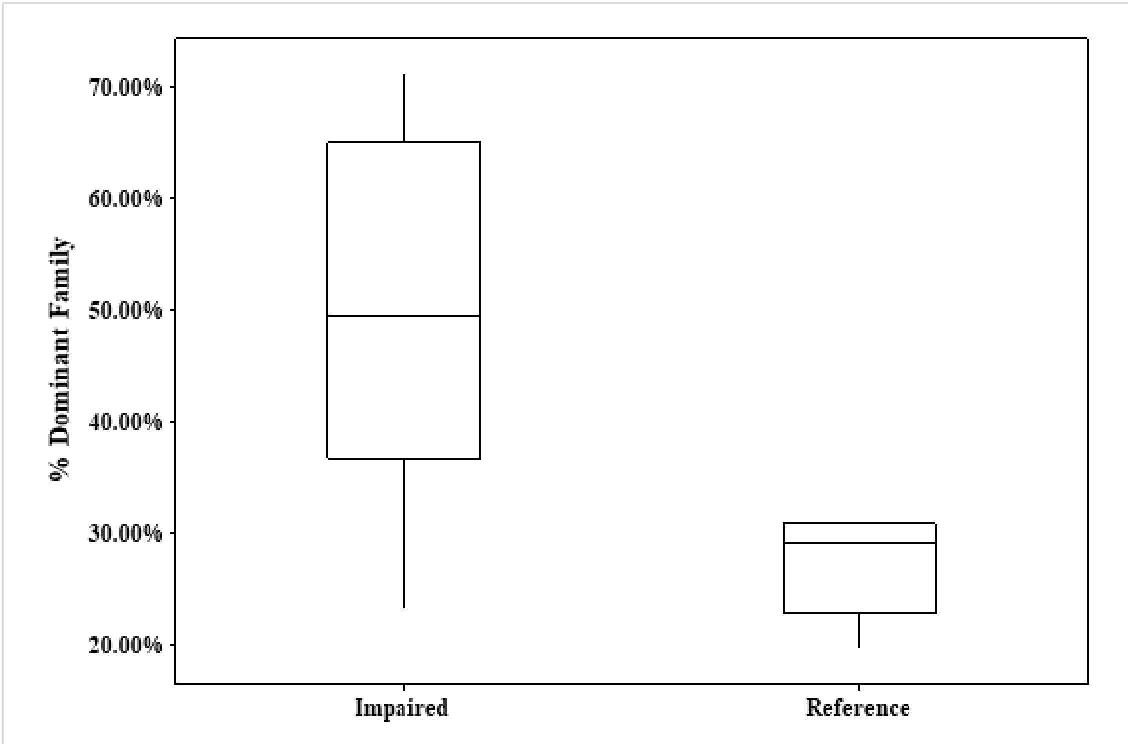
c)


90.00%
80.00%
70.00%
60.00%
50.00%
% 3 Dominant Families
Impaired
Reference

d)

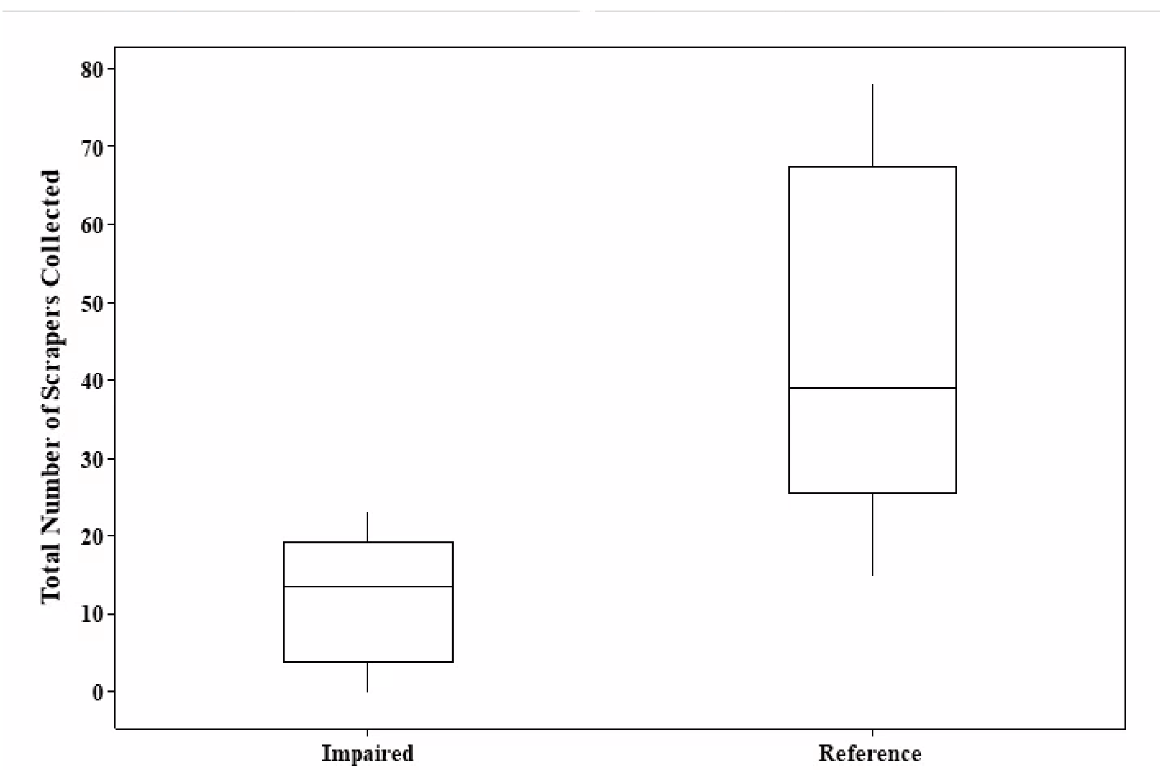

80.00%
70.00%
60.00%
50.00%
40.00%
30.00%
% 2 Dominant Families
Impaired
Reference

e)



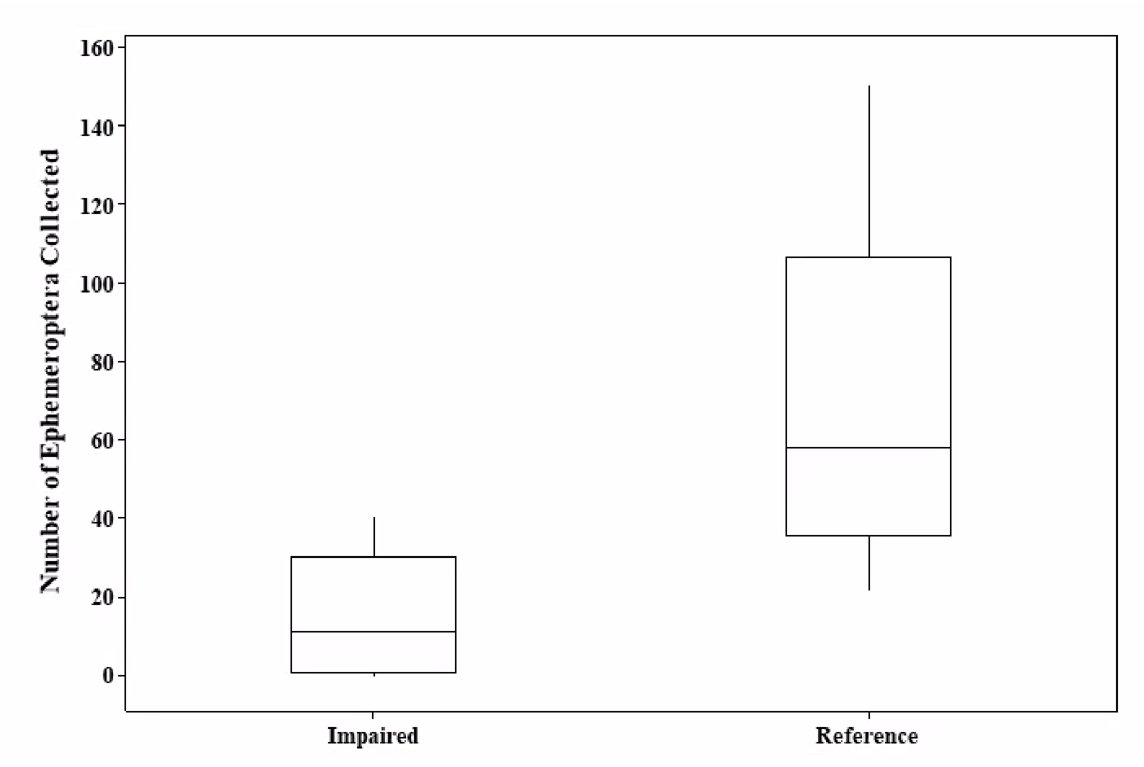
70.00%
60.00%
50.00%
40.00%
30.00%
20.00%
% Dominant Family
Impaired
Reference

f)



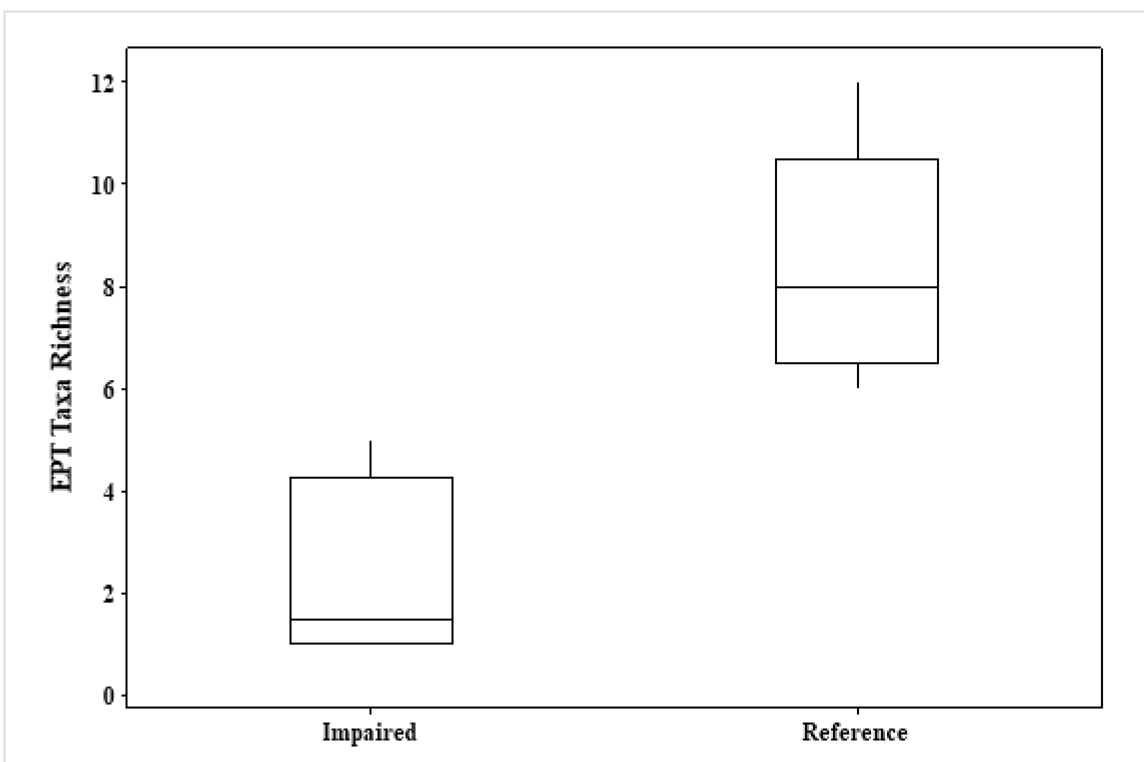
80
70
60
50
40
30
20
10
0
Total Number of Scrapers Collected
Impaired
Reference

g)



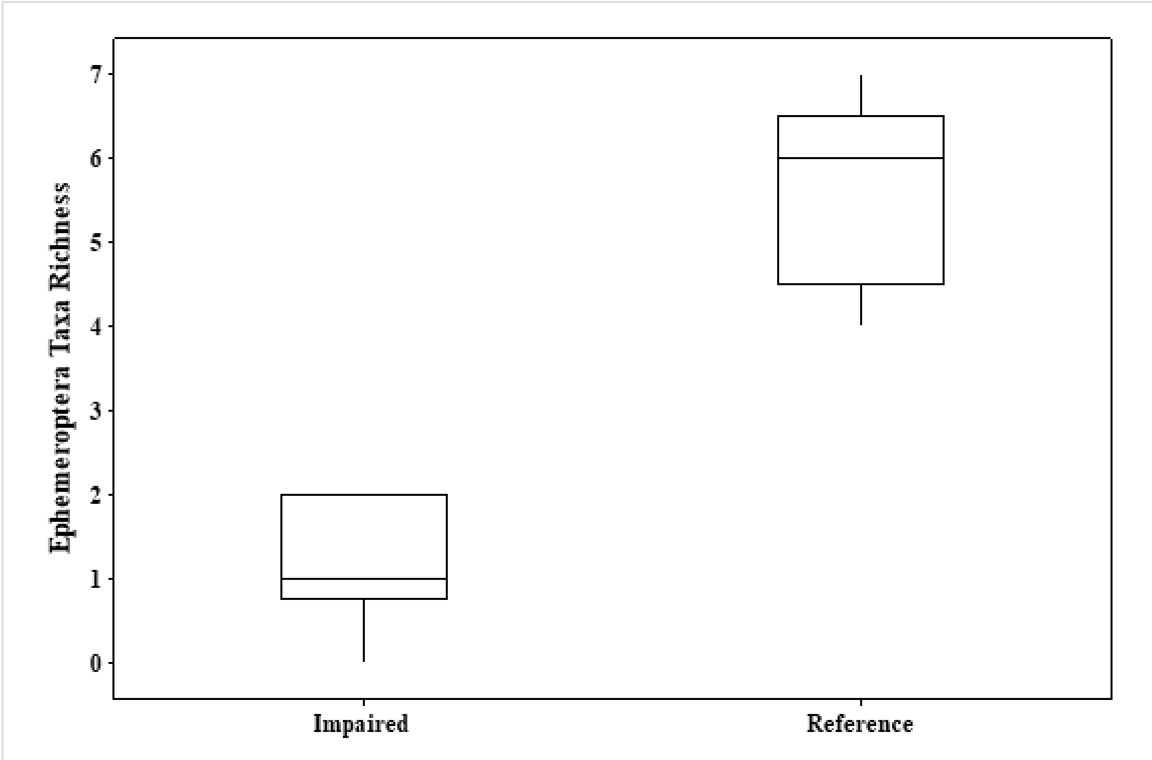
Number of Ephemeroptera Collected
160
140
120
100
80
60
40
20
0
Impaired
Reference

h)

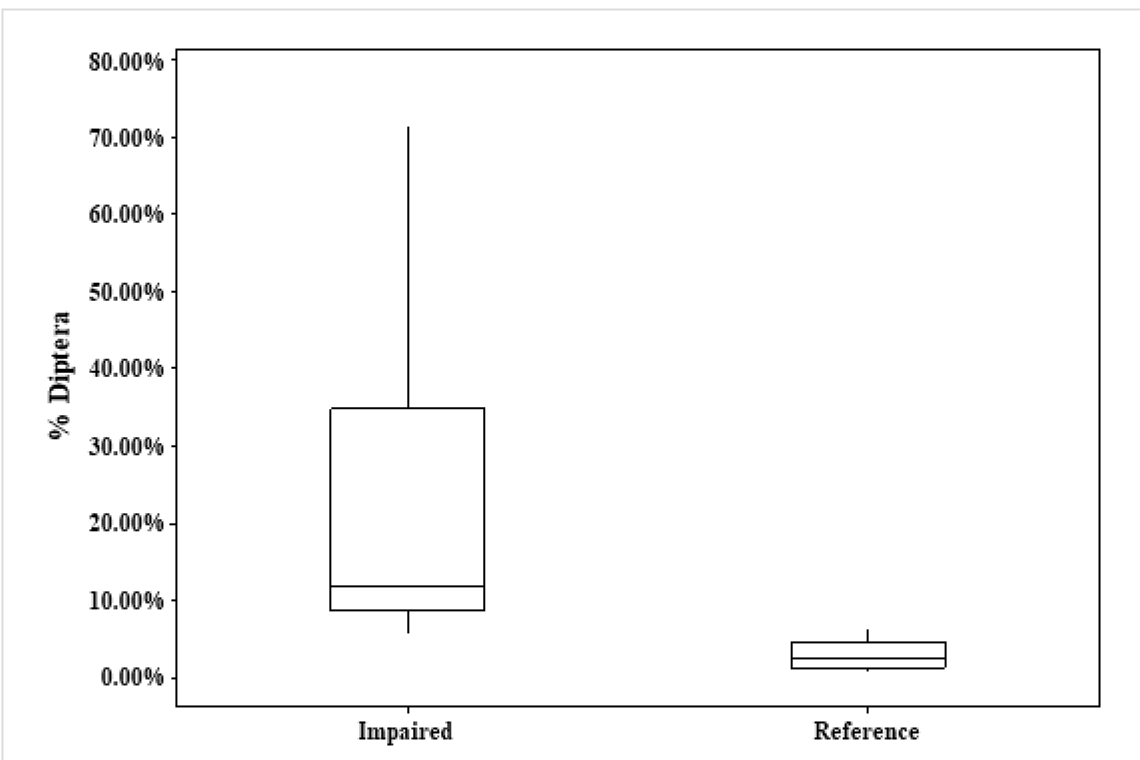


EPT Taxa Richness
12
10
8
6
4
2
0
Impaired
Reference

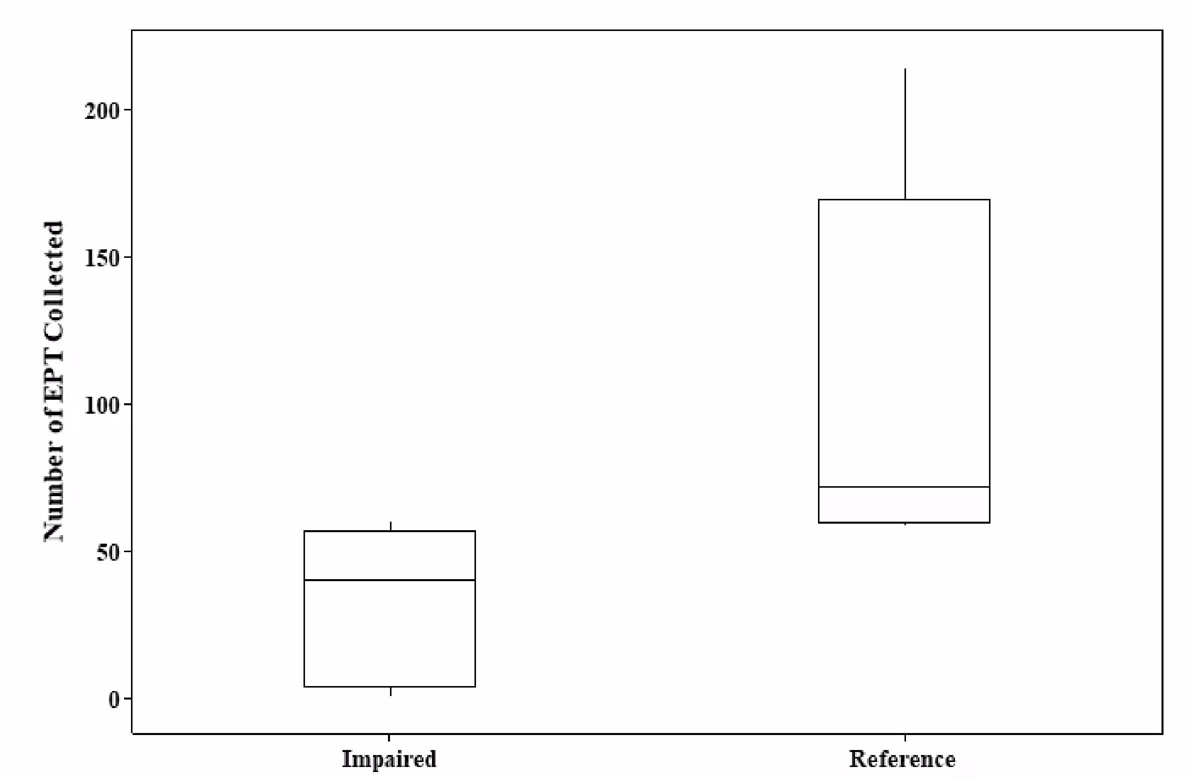
i)


Ephemeroptera Taxa Richness
7
6
5
4
3
2
1
0
Impaired
Reference

j)

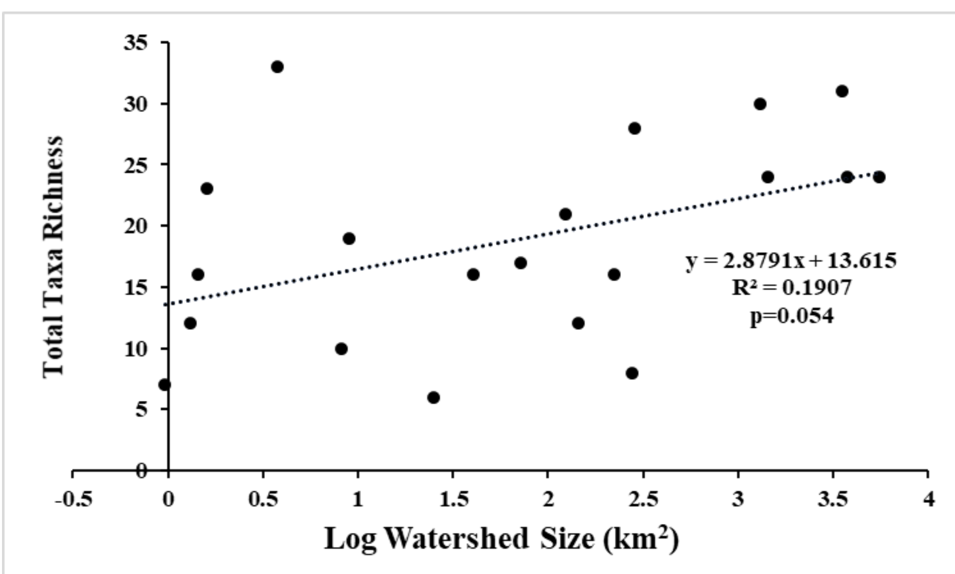

% Diptera
80.00%
70.00%
60.00%
50.00%
40.00%
30.00%
20.00%
10.00%
0.00%
Impaired
Reference

k)

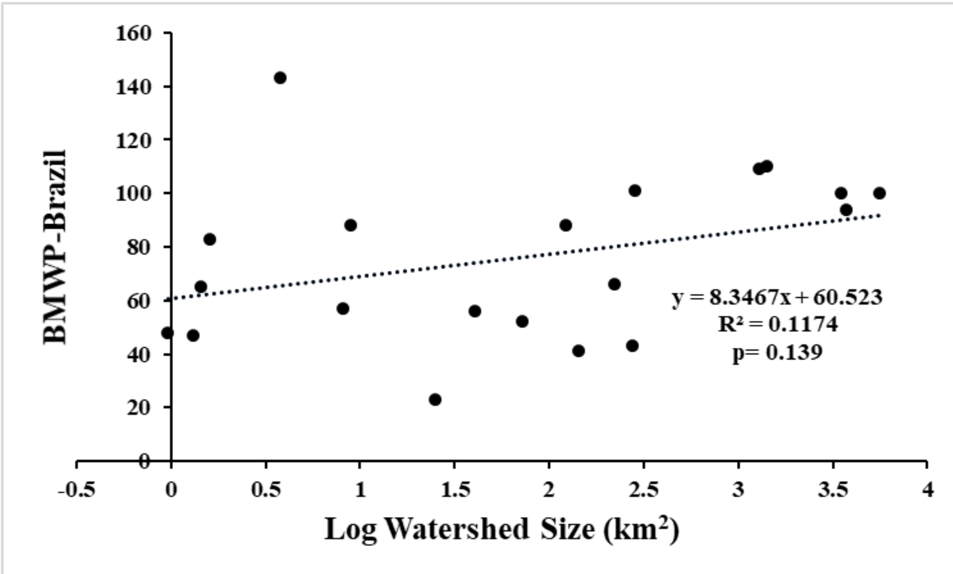


l)

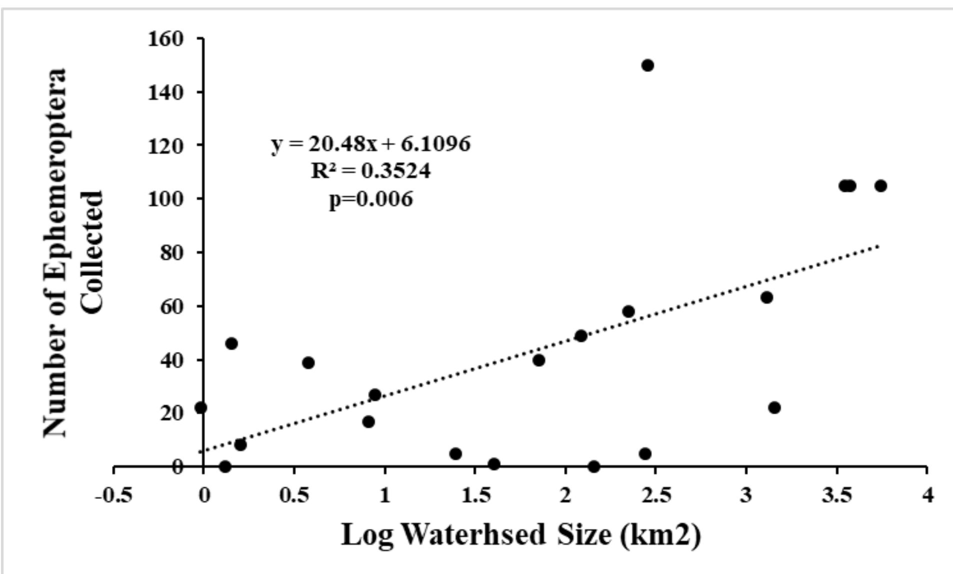
Fig 5a-l. Box and Whisker plots for metrics that passed the sensitivity



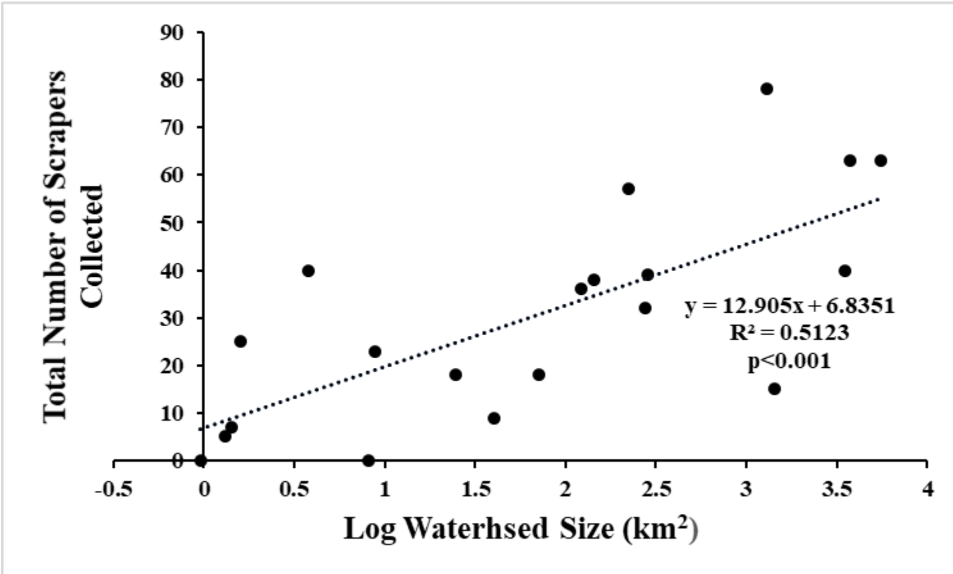
a)


160
140
120
100
80
60
40
20
0
BMWP-Brazil
-0.5
0
0.5
1
1.5
2
2.5
3
3.5
4
Log Watershed Size (km²)
y = 8.3467x + 60.523
R² = 0.1174
p= 0.139

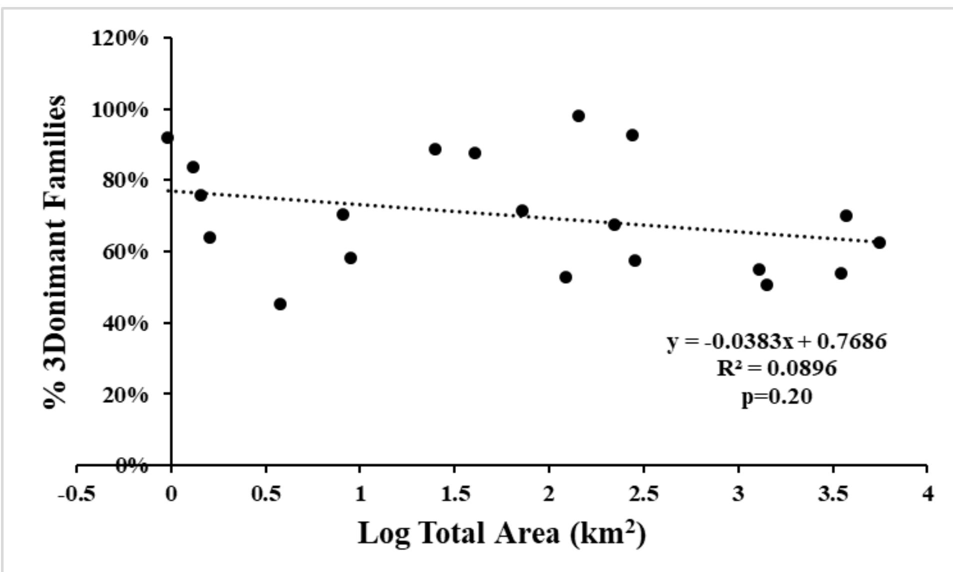
b)


160
140
120
100
80
60
40
20
0
Number of Ephemeroptera Collected
-0.5
0
0.5
1
1.5
2
2.5
3
3.5
4
Log Waterhsed Size (km2)
y = 20.48x + 6.1096
R² = 0.3524
p=0.006

c)

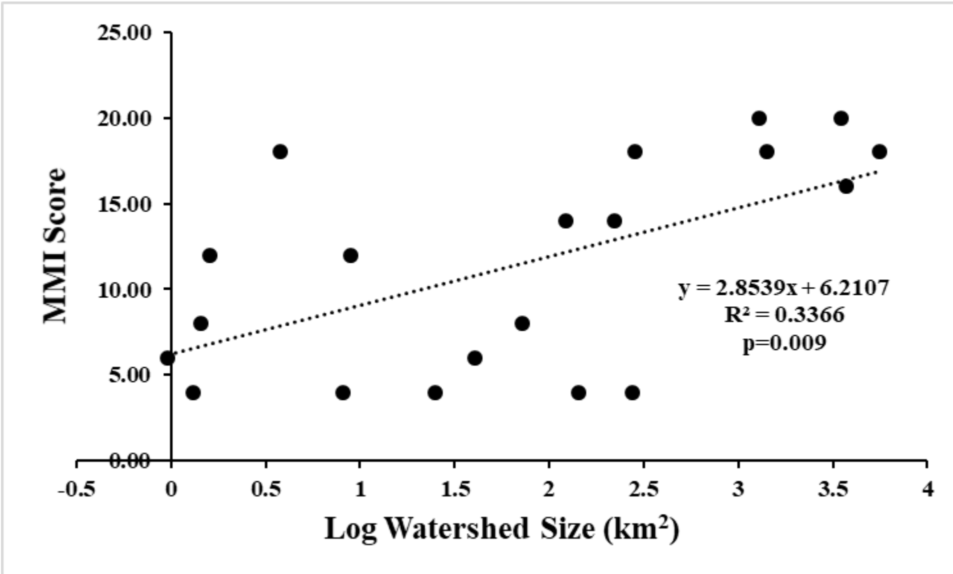


d)

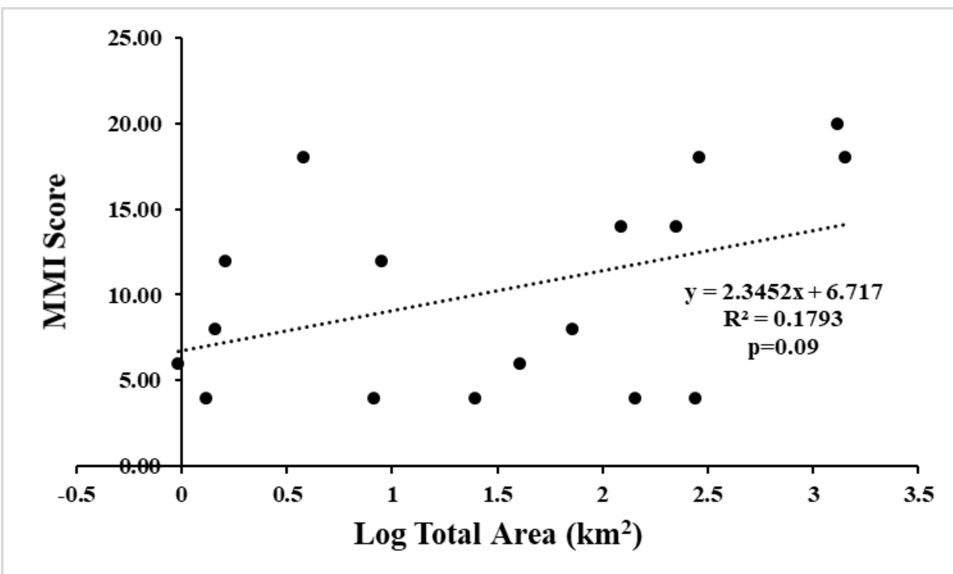


e)

Fig 6a-e. Linear regressions of the five-candidate metrics and their relationship with watershed size for low elevation sites in the Belize River Watershed.



a)



b)

Fig 7a-b. Linear regression of Multimetric Biotic Index scores and watershed size for low elevation sites in the Belize River Watershed with (a) and without (b) the three largest sites.

Appendix A. List of Metrics tested and their predicted response to impairment.

Metric	Predicted Response to Impairment	Description of Metric Categories
Abundance Measures		Number collected for each group. More sensitive groups should decrease with impairment and the opposite for less sensitive groups.
Total Number Collected	Decrease	
Number of Ephemeroptera Collected	Decrease	
Number of Trichoptera Collected	Decrease	
Number of Plecoptera Collected	Decrease	
Number of EPT Collected	Decrease	
Number of Odonata Collected	Variable	
Number of Diptera Collected	Increase	
Number of Megaloptera Collected	Decrease	
Number of Simuliidae Collected	Variable	
Number of Chironomidae Collected	Variable	
Number of Hemiptera Collected	Variable	
Number of Coleoptera Collected	Variable	
Number of Mollusca Collected	Variable	
Number of Annelida Collected	Increase	
Number of Leptophlebiidae Collected	Decrease	
Number of Leptohyphidae Collected	Decrease	
Number of Elmidae Collected	Decrease	
Number of Naucoridae Collected	Variable	
Number of Hydropsychidae Collected	Variable	

Metric	Predicted Response to Impairment	Description of Metric Categories
Composition Measures		Shows the relative contribution of each group to the total collection (Baptist et al., 2007)
% Ephemeroptera	Decrease	
% Trichoptera	Decrease	
% Plecoptera	Decrease	
%EPT	Decrease	
% Megaloptera	Decrease	
% Odonata	Decrease	
% Diptera	Increase	
% Simuliidae	Variable	
% Chironomidae	Increase	
% Coleoptera	Decrease	
%Hemiptera	Variable	
% Mollusca	Increase	
% Annelida	Increase	
% Leptophlebiidae	Decrease	
% Elmidae	Decrease	
% Leptohyphidae	Decrease	
% Naucoridae	Decrease	
% Hydropsychidae	Variable	
% Dominant Taxa	Increase	
% Dominant Family		
% 2 Dominant Families		
% 3 Dominant Families		
% Non-insect	Increase	
Richness Measures		Indicates the number of different taxa (genera) identified within the sample. Higher richness indicates better condition at sites (Baptista et al., 2007)
Total Richness	Decrease	
Ephemeroptera Taxa	Decrease	
Trichoptera Taxa Richness	Decrease	
Plecoptera Taxa	Decrease	
Diptera Taxa	Decrease	
Odonata Taxa	Decrease	
Coleoptera Taxa	Decrease	
Hemiptera Taxa	Decrease	
Mollusca Taxa	Decrease	
EPT Taxa	Decrease	
Family Richness	Decrease	
Trophic Measures		Measures the relative contribution of each functional feeding group to the entire sample (Baptista et al., 2007).
Total Number of Collectors-Gatherers Collected	Variable	

Metric	Predicted Response to Impairment	Description of Metric Categories
Total Number of Filterers Collected	Decrease	
Total Number of Predators Collected	Variable	
Total Number of Scrapers Collected	Decrease	
% Collectors-Gatherers	Variable	
% Filterers	Decrease	
% Predators	Variable	
% Shredders	Decrease	
% Scrapers	Decrease	
Tolerance Measures		Relates the tolerance of groups of aquatic macroinvertebrates to water quality impairment (Baptista et al., 2007).
BMWP-CR Score	Decrease	
Average Score Per Taxon-CR	Decrease	
BMWP-Brazil	Decrease	
Average Score per Taxon	Decrease	
EPT/Chironomids	Decrease	

Appendix B. List of Taxa. Gray highlight indicates taxa was only found in high elevation sites

TAXA LIST	Number of Individuals Collected
EPHEMEROPTERA	
Baetidae	
Americabaetis spp	94
Baetodes spp	42
Baetis/Fallceon	117
Callibaetiodes	42
Camelobaetidus spp	46
Callibaetis	6
Lugoiops	1
Caenidae	
Caenis spp	10
Ephemeridae	
Hexagenia spp	3
Heptageniidae	
Mccaffertium spp	2
Mccaffertium integrum	1
Leptohyphidae	
Amanahyphes spp	45
Cabecar spp	73
Haplohyphes spp	9
Macunahyphes spp	1
Tricorythodes spp	29
Vacupernius spp	13
Leptophlebiidae	
Askola spp	130
Demoulinellus spp	82
Hagenulopsis spp	26
Hylister spp	1
Farrodes spp	157
Traverella spp	64
Ulmeritoides spp	33
PLECOPTERA	
Perlidae	
Anacroneuria spp	73
TRICHOPTERA	3
Philopotamidae	
Chimarra spp	501
subgenus chimarra spp	480
subgenus chimarrita	21

TAXA LIST	Number of Individuals Collected
Calamoceratidae	
Phyloicus spp	10
Hydropsychidae	
Calesopsyche spp	27
Cheutomatapsyche spp	1
Leptonema spp	57
Macronema spp	4
Smicridae spp	210
Helicopsychidae	
Helicopsyche spp	35
Odontoceridae	
Marilia spp	3
Polycentropodidae	
Polyplectropus spp	4
Leptoceridae	
Mystacides alafimbiiata	1
Oecetis spp	1
Triplectides spp	6
Glossosomatidae	
Culoptila	9
COLEOPTERA	
Dytiscidae	
Hydroporus spp	2
Thermonectus	1
Desmopachria spp	1
Elmidae	
Austrelmis spp	10
Austrolimnius spp	10
Cylloepus spp	2
Macrelmis spp	30
Neoelmis spp	3
Heterelmis spp	4
Huleechius/Cylloepus spp	16
Phanocerus spp	1
Microcyllopeus spp	2
Hydraenidae	
Hydraena spp	2
Hydrophilidae	0
Tropisternus spp	8
Lilodactylidae	1
Lutrochidae	3
Psephenidae	

TAXA LIST	Number of Individuals Collected
Psephenops spp	27
Ptilodactylidae	
Anchytarsus spp	1
Scirtidae	27
DIPTERA	
Athericidae	1
Culicidae	5
Chironomidae	
Ablabesmyia rhamphe	7
Djalmabatista spp	1
Polypedilum aviceps	2
Polypedilum beckae	6
Polypedilum halterale	1
Polypedilum scalaenum	2
Polypedilum tritum	1
Parametriocnemus spp	1
Chironomus spp	51
Coelotanypus bicolor	4
Epoicocladius	89
Eukiefferiella devonica	2
Eukiefferiella gracei	2
Eukiefferiella tirolensis, another difficult/questionable ID	1
Goeldichironomus spp	3
Thienemannimyia spp	8
Larsia spp	6
Rheopelopia spp., difficult ID so I'm not 100% on this one	12
Fittkauimyia spp	3
Zavrelimyia spp	21
Dixidae	
Dixella spp.	3
Simullidae	439
Arauncepiodes spp	2
Stratiomyidae	1
Tipulidae	3
Hexatoma spp	2
HEMIPTERA	
Gerridae	
Metrobates spp	9
Naucoridae	
Ambrysus spp	45

TAXA LIST	Number of Individuals Collected
Limnocoris spp	12
Pelocoris spp	4
Procryphocricos spp	59
Notonectridae	
Enitharoides spp	1
Martarega spp	1
Veliidae	
Rhagovelia spp	1
LEPIDOPTERA	
Cosmopterigidae	15
Momphidae	1
MEGALOPTERA	
Corydalidae	
Corydalus spp	94
ODONATA	
Calopterygidae	
Hetarina spp	33
Coenagrionidae	
Acanthagrion spp	2
Argia spp	163
Enallagma spp	4
Hesperagrion spp	22
Neoneura spp	1
Gomphidae	
Aphylla spp	2
Phyllocycla spp	6
Progomphus spp	11
Erpetogomphus spp	20
Epigomphus spp	1
Phyllogomphoides spp	45
Libellulidae	
Brachymesia spp	4
Elasmothemis spp	1
Elga spp	3
Gynothemis spp	1
Macromia spp	1
Macrothemis spp	10
Tholymis spp	7
Dythemis spp	23
Pachydiplax spp	1
Paltothemis spp	31
Perithemis spp	1

TAXA LIST	Number of Individuals Collected
Orthemis spp	3
Libellula spp	1
Scapanea spp	8
Megapodagrionidae	
Philogenia spp	5
Heteragrion spp	4
Platystictidae	
Palaemnema spp	9
Sub Phylum Crustacean	
Pseudothalphusidae	3
O. Decapoda	
F. Cambaridae	5
F. Palaemonidae	3
P. MOLLUSCA	
C. Bivalvia	
Spaeridae	31
Fingernail Clam	8
Unionidae	6
C. Gastropoda	45
Pomacea flagellata	12
Tarebia granifera	183
Melanoides tuberculata	127
Pachychilus spp	26
Pachychilus corvinus	53
Pachychilus largillierti	23
Limpet	5
Physidae	26
Haitia spp	2
Hydrobiidae	3
Somatogyrus spp	2
C. Clitellata	23
Sub Class Hirudinea	6
Tubificidae	1
P. PLATYHELMINTHES	
C. Turbellaria	
Planaria spp	1
Amphipoda	605
Crangonyx	2
Hyalella	6
Isopoda	
Species 1	2
Species 2	55

Vita

Grant Allan Buckner was born in Burnsville, North Carolina, to Robert and Natalie Buckner. He graduated from the University of North Carolina at Asheville in 2018 with a Bachelor of Science in Biology. In the fall of 2018, he joined the master's program at Appalachian State University, where he received his Master of Science in Biology. He has plans to pursue a career in water quality or a related field. On weekends he enjoys his hobbies of fly fishing and birding.