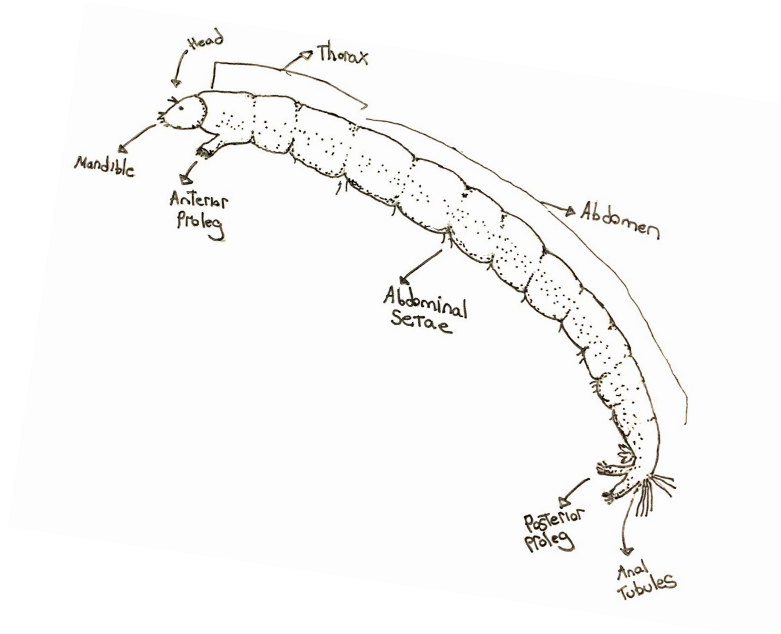
Entry to the Stockholm Junior Water Prize 2019

A Novel Method of Monitoring the Health of our Global Fresh Water Supply using DNA Barcoding of Chironomidae (Diptera)



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I. Abstract: It is forecast that 66% of our population will experience water scarcity within a decade, leaving us more dependent on surface water for drinking.[17] This requires more filtration infrastructure, and monitoring of surface water sources. Current methods rely on expensive and technically challenging manual identification of biological samples. Macroinvertebrates spend their larval lives within a small area of water, showing cumulative effects of habitat alteration and pollutants that chemical testing and field sensors do not.[12] Molecular methods enhance biomonitoring programs. This project explores deoxyribonucleic acid (DNA) barcoding, to measure waterway health with larval Chironomidae (order Diptera), the most widespread macroinvertebrate family.[5] Their complex taxonomy makes manual morphological identification difficult. A statistical sampling plan was designed that represents variation in geological, ecological, and land use factors. Four methods of isolation and amplification were compared. Statistical analysis shows DNA Barcoding of Chironomidae results in more accurate and precise waterway health data, adding significant value for monitoring scarce water resources. The learnings from these data are being applied building microbiology capability at a nonprofit scientific water study institute.

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III. Key Words: Public Water, Chironomidae, Water Scarcity, DNA Barcoding, Surface Water, Water Monitoring, Bioassessment, Nonpoint Source Pollution, Macroinvertebrate, COI (cytochrome c oxidase subunit 1)

IV. Abbreviations and Acronyms:

COI: Cytochrome c oxidase subunit 1
DNA: deoxyribonucleic acid
NJDEP: New Jersey Department of Environmental Protection
PCR: Polymerase chain reaction

V. Acknowledgements: This effort was designed and conducted by the author. Appreciation to Karen Lucci of Hopewell Valley Central High School for guidance on phylogenetic tree analysis. Based on previous training and experience, Cold Spring Harbor Laboratory graciously allowed open access to microbiology labs and equipment to conduct independent experimentation. Special appreciation to Dr. Cristina Fernandez for DNA Barcoding principles. Sample preparation, DNA extraction, macroinvertebrate identification and chemical analysis was performed in home laboratory underwritten by funding awarded for prior research. Additional thanks to Erin Stretz, Dr. Steve Tuorto, and Jim Waltman from The Watershed Institute for internships and opportunities to learn aquatic entomology and chemical environmental monitoring; Dr. Patricia Shanley for guidance on policy and advocacy; Lawrenceville Summer Scholars for robotics and programming.

VI. Biography:

Freshwater biology and bioassessment is an area I have been working in for nearly a decade. My independent research focuses on how environmental data can be gathered and used to inform decision making in terms of how and when we develop our natural and water resources. I have been a member of the Society for Freshwater Science since 2014, and I have presented data at their 2015 Mid-Atlantic Chapter meeting at the Academy of Natural Sciences in Philadelphia, USA, and the 2017 Annual Conference in Raleigh, North Carolina, USA. Additionally, I have presented at the United States Environmental Protection Agency (EPA), for receiving a United States Presidential Award, National Geographic Society in Washington, D.C., USA, as well as New York Academy of Sciences Bicentennial celebration, New York, NY, USA (2017). Massachusetts Institute of Technology (MIT) named a minor planet in my honor. Through chemical and biological stream assessment (certified for 8 years), I have been monitoring the health of our local waterways as an active member of a StreamWatch volunteer program since 2011. Encyclopedia Britannica published my definition of “macroinvertebrate.” I was the featured speaker and Watershed Hero at The Alliance for Watershed Education River Days 2017 & the East Coast Greenway River Days Kick-Off at Fairmount Water Works, Philadelphia, USA. I have developed and taught curriculua at a multitude of venues, consulted, and have been called upon to start monitoring programs.

My research, data collection, and advocacy have led to environmental improvements: data submission to the New Jersey Department of Environmental Protection (NJDEP) and modifications to a national pipeline construction project that minimized disturbance to streams, preserving ecologically critical forest and wetlands such as a critical wildlife habitat corridor, and locating and documenting the southernmost population of a threatened amphibian species to support a current proposal for

categorization of a special wetland habitat as a NJDEP C1 Stream (area of exceptional ecological significance protected from measurable degradation).

Through these experiences I became aware of the limitations of current bioassessment methods, such as reliance on expert taxonomists, accuracy of reference material, and treatment of damaged samples. I began making improvements, from developing monitoring devices, to adapting precise genetic techniques. I observed that Chironomidae were a common denominator across my sample sites, as well as across the globe.

1. Introduction: Utilizing DNA Barcoding of the larval Chironomid to monitor the health of freshwater adds significant value for understanding an increasingly scarce water resource. This method captures the cumulative effects of all stressors, from nonpoint source nutrient and heavy metal pollution, to temperature and dissolved oxygen, to flow alteration. Currently there are hundreds of bioassessment protocol in use globally, however expert error rates as high as 65% have been observed at the genus and species level.[1][5] The method developed here increases the accuracy, precision and statistical power of stream health assessment data. This is critical from an ecosystem perspective, and also from a human health perspective. Our population is relying more on surface water for drinking. Current bioassessment methods have limitations; we will need better ways to monitor our surface water.

Parts of the world are abundant with fresh water, but 2.7 billion people (about 40% of our population) experience water scarcity at least one month per year.[17] This is expected to grow to two-thirds of the world's population within a decade (Falkenmark Water Stress Indicator) as population and water usage increase.[17] Less than 1% of the world's water is accessible as a public water source.[4] Water scarcity affects every continent and was listed in 2015 by the World Economic Forum as the largest global risk in terms of potential impact over the next decade.[16][7]

As water scarcity increases, we become more and more dependant on surface water for drinking, therefore requiring more filtration infrastructure, and more monitoring of surface water sources. Currently 63% of public water (serving a population of 169 million) in the USA is from surface water.[13] New York City provides an example of wetlands as a natural water filtration resource for their public water. Over one million acres of protected land in the Catskill/Delaware watersheds provide natural filtration for 90% of New York City's population of 8.5 million.[14] New York is one of only five cities that can rely on simpler natural filtration for public water.[14] The New York City Land Acquisition Program purchased or protected over 130,000 acres since 1997 and restricts development.[10] A dedicated police force of more than 200 members guards the health of the wetlands and prevents illegal dumping.[15] Wetlands provide surface water filtration, however more than half the world's wetlands have disappeared.[17]

Bioassessment measures of taxa richness and relative abundance provide valuable information on trends in ecosystem health. Macroinvertebrates provide a logical choice since they can be seen with the naked eye and spend their larval lives in a small area of water, therefore showing the cumulative effects of habitat alteration, contaminants, and pollutants that chemical testing and field sensors do not. Additionally, macroinvertebrates play a significant part of the food web, preyed upon by fish, birds, reptiles, and amphibians. Many current waterway assessment methods are based on a procedure defined and popularized by Hilsenhoff[9] in 1977: a 100 organism sub-sample is obtained from a Stratified Random Sample taken in the field. Organisms are identified to the lowest practical taxonomic level with a microscope and taxonomic keys.[9][12] Macroinvertebrates are relatively easy to identify to family level manually by morphology, however genus and species level identification is exponentially more expensive and technically challenging. Highly detailed genus and species level data is more accurate and precise but difficult to obtain due to cost, specimen condition, incomplete taxonomic knowledge, poor taxonomic keys, and lack of trained taxonomists. Error rates of genus and species in samples identified manually by professional taxonomists have been found to be as high as 65%.[5] Taxonomic identification to family level by volunteers is widely used for citizen science programs and broad data gathering.

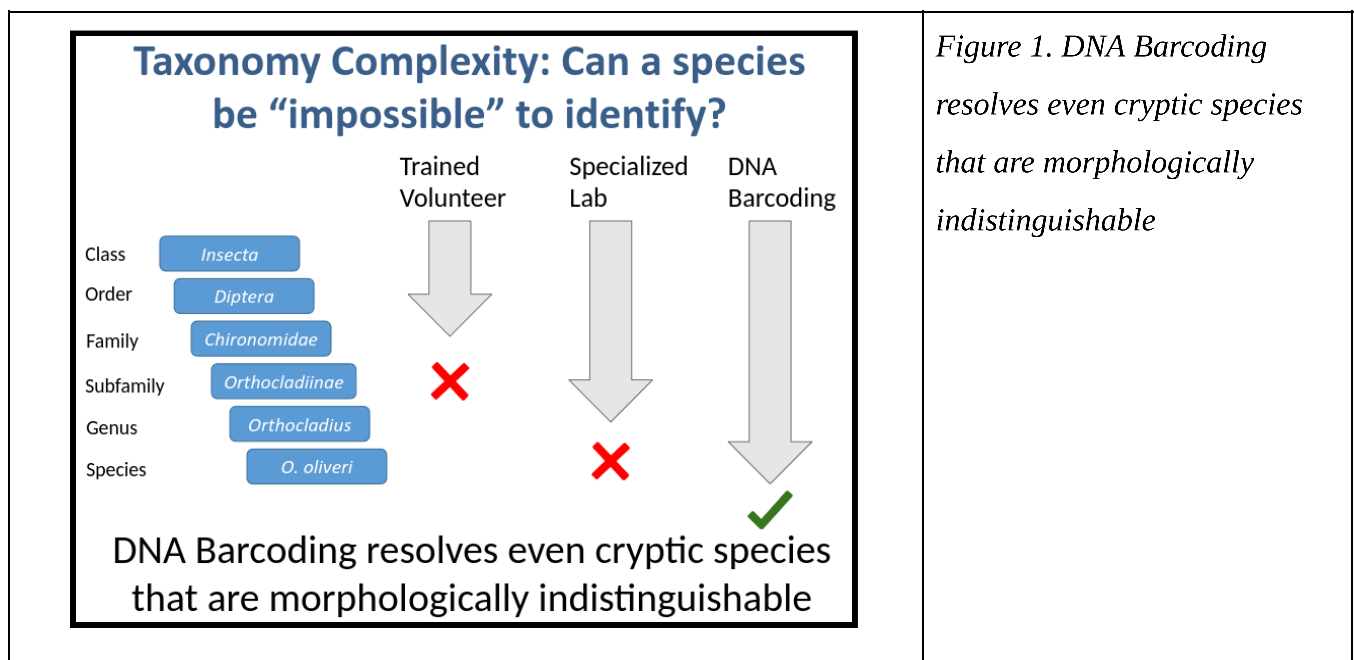

Figure 1. DNA Barcoding resolves even cryptic species that are morphologically indistinguishable

Molecular methods, such as DNA Barcoding from a region of the mitochondrial gene COI (cytochrome c oxidase subunit 1), are now available to enhance bioassessment programs. In 2003, Hebert et. al. started working on improving bioassessment with DNA Barcoding, and currently there are many open investigations in this area, including an EU COST Action.[8][11] DNA Barcoding overcomes many limitations of manual taxonomic identification and offers the promise of a more rapid, accurate (less human error), and precise (species level) identification of macroinvertebrate taxa, and therefore more accurate and precise environmental assessments. Importantly, DNA Barcoding also significantly

improves the statistical power of bioassessment tools.[18] However, there is currently no standard bioassessment method that leverages the power of DNA Barcoding.[1]

	Continuous / Cumulative Assessment	Includes All Stressors	Detects Subtle Changes	Scalable	Standard Methods Available
Chemical Monitoring	✗	✗	✓	✗	✓
Remote Sensors	✓	✗	✓	✓	✓
Bioassessment, Manual Taxonomy	✓	✓	✗	✗	✓
Bioassessment, DNA Barcoding	✓	✓	✓	✓	✗

Figure 2. DNA Barcoding offers a powerful, comprehensive, and scalable bioassessment. Currently no standard method exists.

This effort creates a methodology that allows DNA Barcoding to be integrated into existing water monitoring programs through sampling the larval non-biting midge Chironomidae (order Diptera), in order to improve accuracy, precision, and statistical power of results. Chironomidae are versatile macroinvertebrates and a common denominator among most aquatic sites.[5] They occupy many important parts of food webs, and includes all functional feeding groups: collector/gatherers, shredders, scrapers, filter-feeders, and predators.[5] They have a holometabolous, or complete metamorphosis, life cycle with; egg, larva, pupa, and adult. The Chironomidae are the only free-living (non-parasitic) holometabolous insect extant on every continent, including Antarctica, and in a great range of altitudes.[5] They have been found 5600 meters above sea level on glaciers in Nepal, and 1360 meters below the surface of freshwater Lake Baikal in Russia. This project is concerned with the larval form, which in some species occurs in water films a millimeter thick, and in other species dwells in arid regions and can tolerate drought (one Chironomid species even survived 18 months in the vacuum of space). Some species' larvae are found in glacial meltwater just above freezing, and another species' larvae are extant in hot springs over 40°C. There are fully marine species, and some have even been found in algae on sea turtle shells. Some Chironomid larvae have hemoglobin which allow them to absorb oxygen from and tolerate low-oxygen waters that other macroinvertebrates cannot survive. Due to their reddish color these are commonly called bloodworms. Unfortunately for taxonomists and citizen scientists alike, Chironomidae have complex taxonomy that makes manual morphological identification to genus and species level extremely difficult. The Hilsenhoff Family Tolerance Value for Chironomidae is 6. However, this average masks a great variability in genera tolerance values which have been shown to range greatly (e.g. from 2 to 10 for the genera sampled here). Since morphological identification is difficult, DNA Barcoding adds great value. Additionally, unlike some other macroinvertebrates, they lack inhibitors that impede amplification using the silica resin isolation method and polymerase chain reaction (PCR) primer beads.

This research hypothesized that a novel DNA Barcoding process utilizing Chironomidae (Diptera) would provide a more accurate and precise waterway health measures than manual taxonomic identification by morphology. The purpose was to contribute an improved method of bioassessment to aid in preservation of our freshwater resources. In Phase I, method development was explored. The independent variables were DNA extraction methods and primers used. The dependent variable was the percent amplification of samples. The control was the DNA ladder. In Phase II, The Chironomidae were explored as an index of waterway health. The independent variables were the sample sites, varying freshwater bodies with a statistically planned variety of geological, ecological, and anthropogenic factors. The dependent variables were the genera and species present. The positive control is a known healthy location (per statistical data) and manual identification. The negative control is a known unhealthy location.

The research questions explored here support creation of a microbiology lab at a nonprofit water study institute that supplements their existing citizen science water monitoring programs. 1) Can DNA Barcoding be used as a means of monitoring surface water sources? 2) How do Chironomidae genera and species vary in response to variation in geological, ecological, and land use factors? 3) How do Chironomidae genera and species vary in response to nutrient pollution? 4) Will this project add new species to the Chironomidae data sets in genetic sequence databases used by the scientific community? 5) What is the effect of different methods of PCR on the amplification of Chironomidae DNA?

2. Materials and Methods:

2.1 Risk and Safety: These procedures involve use of ethanol, a Lamotte Water Quality test kit, and microliter amounts of DNA isolation, PCR amplification, and gel electrophoresis reagents. Material Safety Data Sheets (MSDS) sheets were reviewed. Personal protective equipment was used to protect against risk of chemical exposure. Waste liquid was collected and given to Clean Harbors, a company specializing in hazardous waste disposal. Training was completed and up to date for equipment, chemicals, and taxonomic identification.

2.2 Procedures: The following methods of DNA isolation were selected (Rapid DNA Isolation, PowerSoil Isolation Method (Metabarcoding), Silica Resin Isolation). Research showed these to be more likely to work for macroinvertebrates and they are easy and economical for real world use. Sample sites were chosen according to a statistical sampling plan to capture a variety of geological, ecological, and anthropogenic factors: high gradient vs coastal plain, stream vs. pond, healthy ecosystem vs. unhealthy ecosystem.

2.3 Water Quality Chemical Analysis: Water quality chemical analysis certifications relevant to this project were up to date. Chemical sampling was performed with LaMotte water test kit and procedure. Nitrates, orthophosphates, dissolved oxygen, pH, and turbidity were monitored over 9 months at 13 sites.

2.4 Benthic Macroinvertebrate Sampling: Sampling was performed per NJDEP procedure. Freshwater macroinvertebrate samples were collected with D-frame net. The percentage of net jabs taken in each habitat type corresponded to the percentage of each habitat type's presence in the stream reach. The sample was stored in ethanol. Macroinvertebrates were identified, and those from the Chironomidae family (order Diptera) were identified under a microscope and removed for DNA Barcode analysis. Stream health was monitored over 9 months at 13 sites.

2.5 DNA Isolation Procedure: The membrane-bound organelles such as the nucleus and mitochondria were dissolved with lysis solution. A sterile plastic pestle was used to liquify the macroinvertebrate sample in a 1.5ml tube. Silica resin was used to bind DNA. The nucleic acids were eluted from the silica resin with laboratory grade distilled water. Samples were stored at -20 C prior to PCR amplification.

2.6 Polymerase Chain Reaction (PCR) Amplification: Primers were selected based on sample type. Different methods of PCR amplification were tested: Ready-To-Go PCR Beads were activated by adding a mix of loading dye and COI primers LCO1490 and HC02198. After bead dissolves, the DNA sample was added with micropipette. The PCR tubes were then mixed by lightly flicking, and centrifuged for 30 seconds at 13,400 RPM to spin the liquid to the bottom of the tube. Samples were thermal cycled with the appropriate temperature profile programmed. NEB Taq 2X Master Mix: 10μL of loading dye per sample was mixed with 12.5μL of NEB Taq 2X Master Mix per sample, combined in a 1.5ml tube, and shaken gently for mixing. 2μL of sample DNA was then added with micropipette to the correspondingly labeled PCR tubes. 23μL of the LCO1490 and HC02198 primer mix was added to each PCR tube. The PCR tubes were then mixed by lightly flicking, and centrifuged for 30 seconds at 13,400 RPM to spin the liquid to the bottom of the tube. Samples were then thermal cycled with the appropriate temperature profile programmed.

2.7 Gel Electrophoresis: Agarose gel was poured, and when it was solid it was placed into the electrophoresis chamber. Tris/Borate/EDTA (TBE) buffer was added. PCR samples were loaded, the gel was run at 130V and the images were captured. Images for samples prepared with PCR Beads and with Master Mix were used to verify DNA amplification.

2.8 Sequencing: Samples were then sent for DNA Sequencing. Bioinformatic analysis was completed by trimming and analyzing the Chironomidae genetic sequences. The final sequences were submitted and compared to multiple genetic sequence databases to determine the genus and species of each sample.

Software tools were programmed and developed to easily calculate biological health scores. The appropriate index was selected (High Gradient or Coastal Plain Macroinvertebrate Index).

3. Results: DNA Barcoding overcomes limitations of manual taxonomic identification and significantly improves the statistical power of bioassessment tools.[18] Hilsenhoff tolerance scores of the Chironomidae genera sampled and identified using the DNA Barcoding method developed here were used with GIS software to provide an overview of water quality.

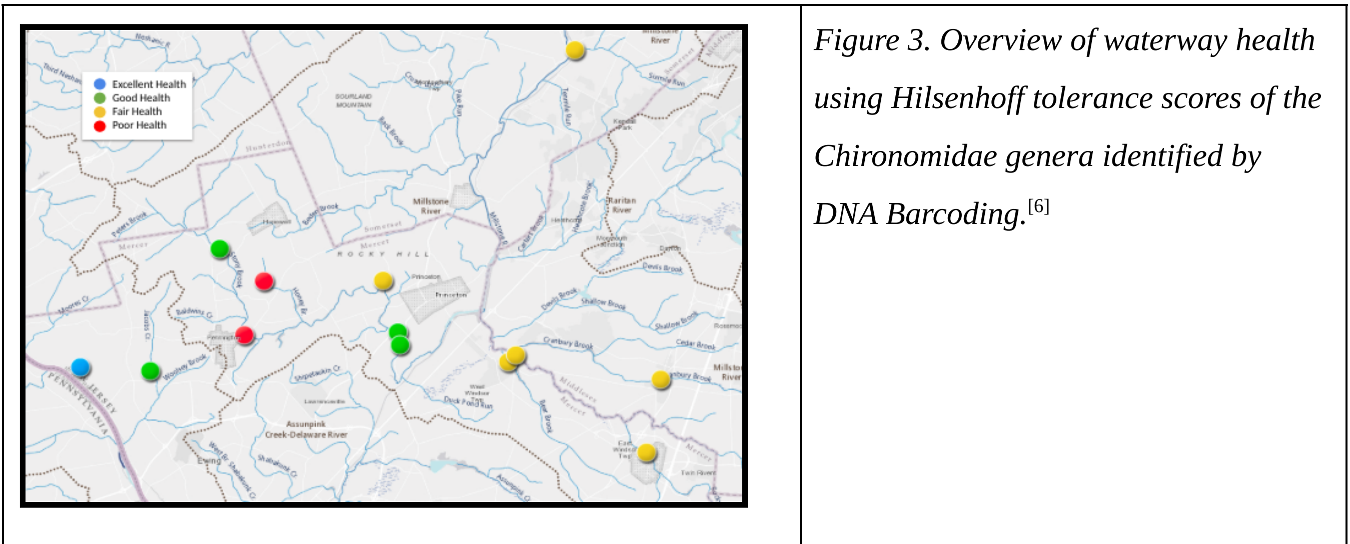

Figure 3. Overview of waterway health using Hilsenhoff tolerance scores of the Chironomidae genera identified by DNA Barcoding.[6]

Highly detailed genus and species level data provides a more accurate and precise bioassessment metric, but is difficult to obtain manually due to cost, specimen condition, incomplete taxonomic knowledge, poor taxonomic keys, and lack of trained taxonomists.

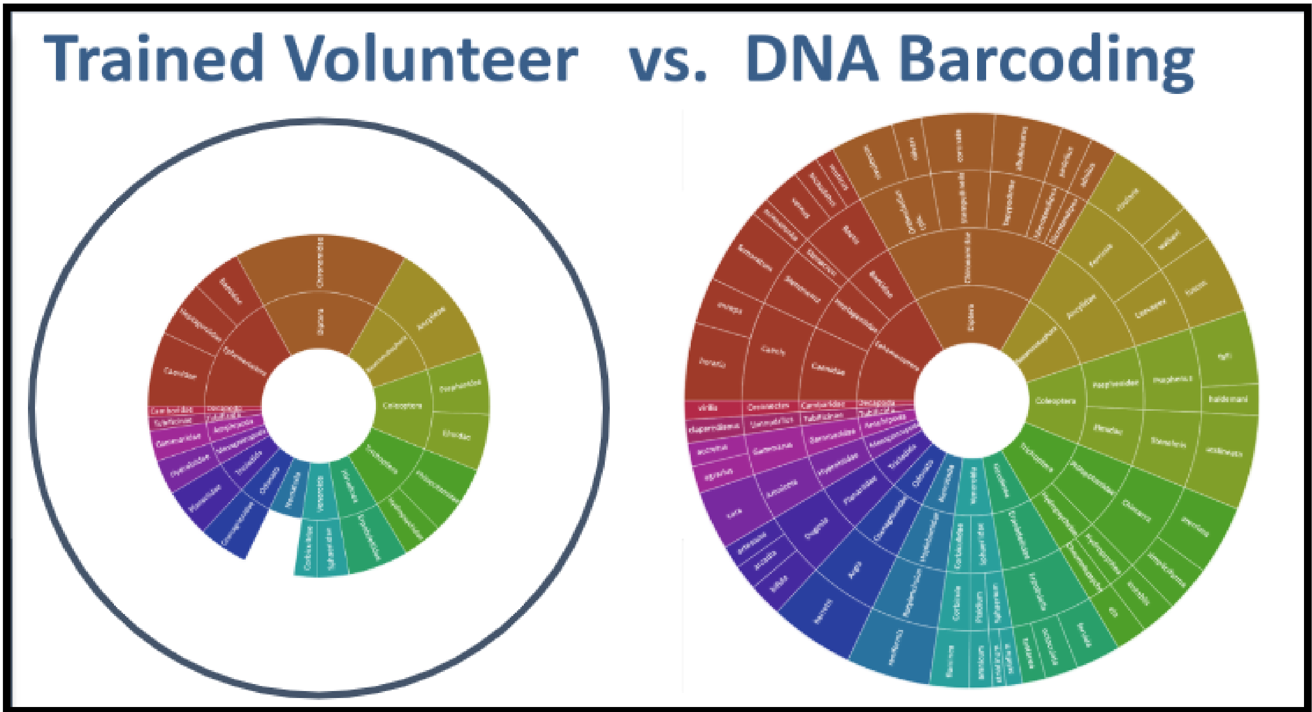

Figure 4. The left diagram shows a taxonomic macroinvertebrate sample identified to class and family level by a trained volunteer. The right diagram shows the sample identified to species level by DNA Barcoding, and reveals the additional resolution provided by DNA Barcoding.

An important step to developing a methodology for use of Chironomidae in bioassessment was comparing and evaluating molecular analysis methods. Silica resin and PCR bead successfully amplified 100% of the samples. Four approaches were evaluated, and had very different results in terms of the percent of samples that successfully amplified.

	eDNA Extraction & eDNA Primer	Rapid Method (chromatography paper) Extraction & PCR Bead	Silica Resin Extraction & PCR Bead	Silica Resin Extraction & MM Primer
% Successful amplification	< 1% ✗	40% ✗	100% ✓	28% ✗
No primer bias	✗	✓	✓	✓
Precise sample time/location	✗	✓	✓	✓

Figure 5. Summary of the molecular analysis methods evaluated. Silica resin and PCR bead successfully amplified 100% of the samples.

The Chironomidae samples showed the least undetermined nucleotides, best peak quality, and best *Phred* sequence quality. The Gammaridae also responded very well to barcoding, However, the Gammaridae do not have the range of geography and biotic indices that the Chironomidae do.

DNA Barcoding of Macroinvertebrate Examples: Chironomid Excellent

Electropherogram	Undetermined Nucleotides	Peak Quality	*Phred* Quality	Gel	Taxon
	57%	Undefined	Poor 64% high quality sequences		*haliplid*
	27%	Inconsistently Defined	Medium 73% high quality sequences		*physid*
	3%	Well Defined	Good 98% high quality sequences		*Gammarid*
	3%	Well Defined	Very Good 99% high quality sequences		*Chironomid*

images: Gammarus Encyclopedia of Life, halipid bugguide.net

Figure 6. Summary of various taxa samples identified by DNA Barcoding with silica resin and PCR beads. Taxa samples compared by their response to DNA Barcoding.

Phred scores were compared using two-sample t-tests (0.01 significance level). This test was selected since *n* (the number of samples) was less than 30.

Chironomidae vs. Physidae $p = 1.01 \times 10^{-6}$ indicating a statistically significant difference.

Chironomidae vs. Haliplidae p = 7.37 x 10^{-8} indicating a statistically significant difference. Chironomidae vs. Gammaridae p = .053 indicating a difference that is not significant, however Gammaridae were not chosen due to their more limited number of species, geographic range, and biotic index range.

The Chironomidae sampled here aligned by genera with either high gradient streams in piedmont geology with bedrock, cobble, pebble bottom composition, or coastal plain geology bottom composition of sand and silt. Only 13% of the genera sampled were found evenly in both geologies.

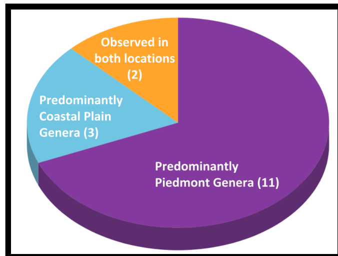

Figure 7. Percent of Chironomidae genera based on the surface geology they predominantly occur in.

Nutrient pollution was compared with the weighted average Hilsenhoff tolerance scores of the Chironomidae genera sampled at each site. The value for nutrient pollution was calculated from the average ppm of nitrate and orthophosphate sampled at each site, which was normalized to a value between zero and ten. This shows a statistically significant relationship with $p<0.05$. When nutrient pollution data for sites are graphed with the weighted average Hilsenhoff tolerance scores of the Chironomidae genera sampled, a moderate positive linear association is noted. There is a statistically significant relationship with $p<0.05$. In a linear regression, R^2=.67 indicating that 67% of the variation in the Hilsenhoff tolerance scores of the Chironomidae genera sampled were accounted for by overall nutrient pollution data. This means that 33% of the variation in tolerance score is influenced by factors other than nutrient pollution.

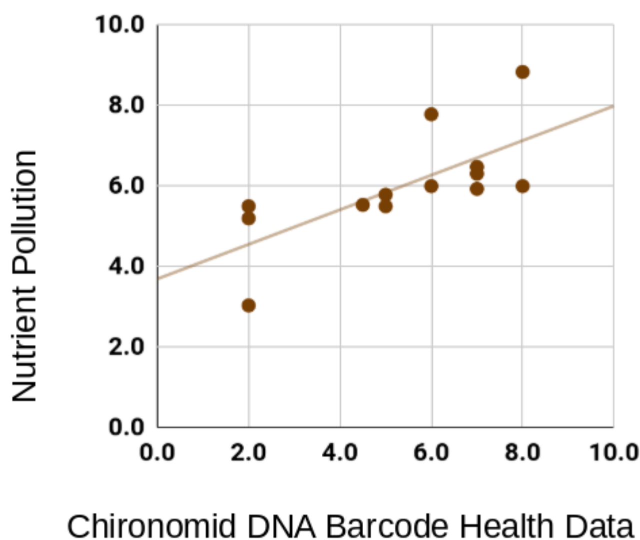

Figure 8. Relationship between the weighted average Hilsenhoff tolerance scores of the Chironomidae genera barcoded at each site and nutrient pollution.

When historical health data for sites are graphed with the weighted average Hilsenhoff tolerance scores of the Chironomidae genera sampled, a strong positive linear relationship is noted. There is a statistically significant relationship with $p<0.05$. In a linear regression, $R^2=.79$ indicating that 79% of the variation in the Hilsenhoff tolerance scores of the Chironomidae genera sampled were accounted for by overall historical waterway health data. This means that 21% of the variation in tolerance score is influenced by factors other than overall waterway health.

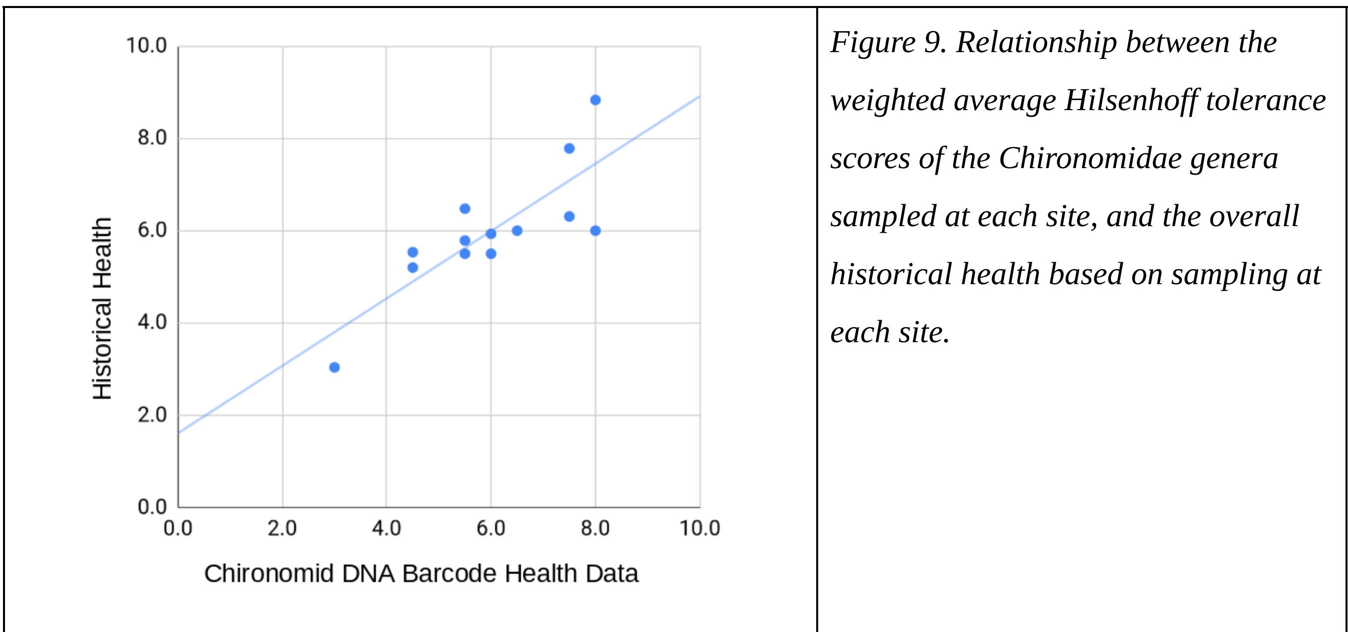

Figure 9. Relationship between the weighted average Hilsenhoff tolerance scores of the Chironomidae genera sampled at each site, and the overall historical health based on sampling at each site.

A Bland-Altman analysis showed limits of agreement of -0.853 and 0.868 between the weighted average tolerance values of Chironomidae genera barcoded and the current manual method that uses manual taxonomic identification by morphology. This indicates that the new method proposed here of DNA barcoding Chironomidae is in agreement with the current method to within 1.72 on a zero to ten health scale.

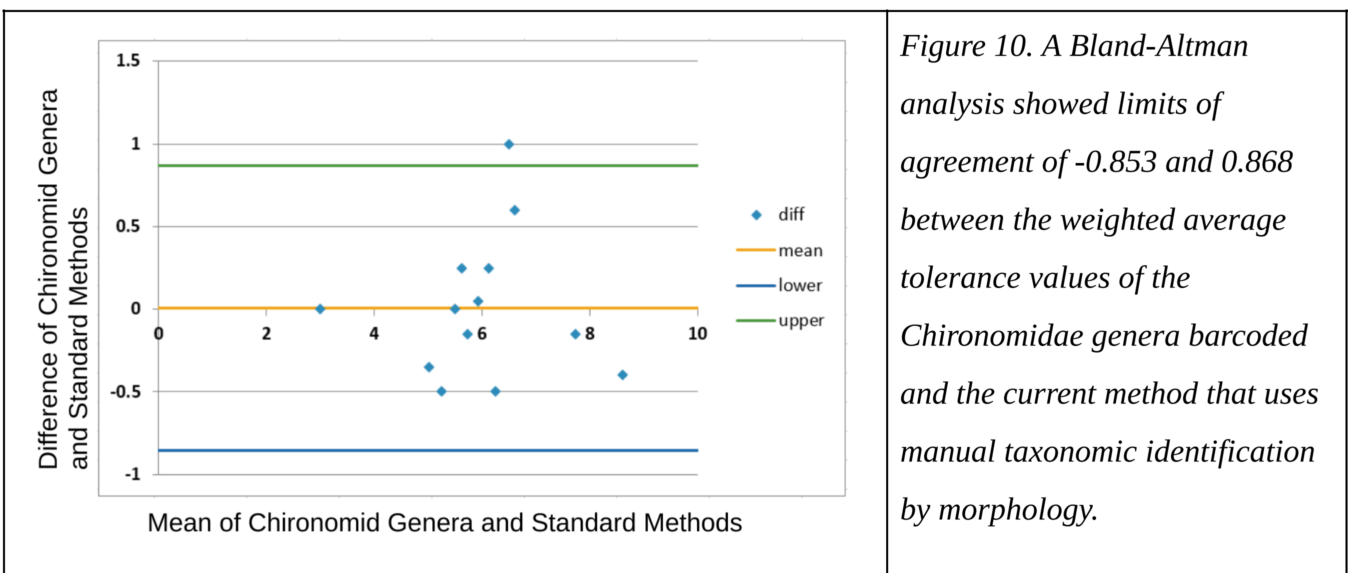

Figure 10. A Bland-Altman analysis showed limits of agreement of -0.853 and 0.868 between the weighted average tolerance values of the Chironomidae genera barcoded and the current method that uses manual taxonomic identification by morphology.

The following phylogenetic trees were used to analyze the genetic relationships between selections of the Chironomidae sampled with respect to site, taxa level identified, and biotic index.

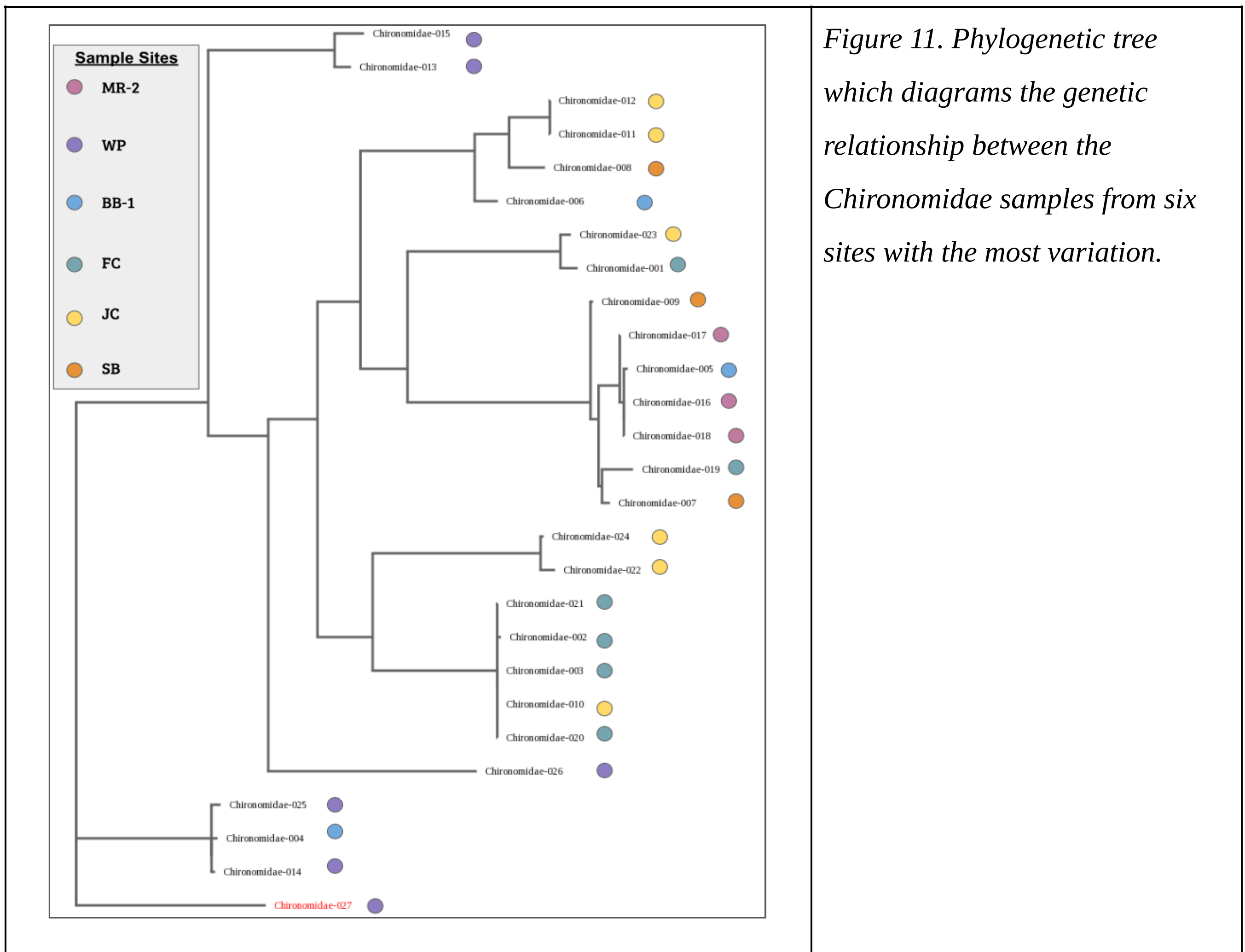

Figure 11. Phylogenetic tree which diagrams the genetic relationship between the Chironomidae samples from six sites with the most variation.

The following phylogenetic tree was used to analyze the genetic relationships between selections of the Chironomidae sampled with respect taxa level identified (e.g. subfamily, genus, or species). Identification down to species level indicates a match in the sequence databases. Identification to genus or subfamily indicates gaps in the sequence database that can be filled with a widespread barcoding initiative. The gaps could also allude to potential novel species.

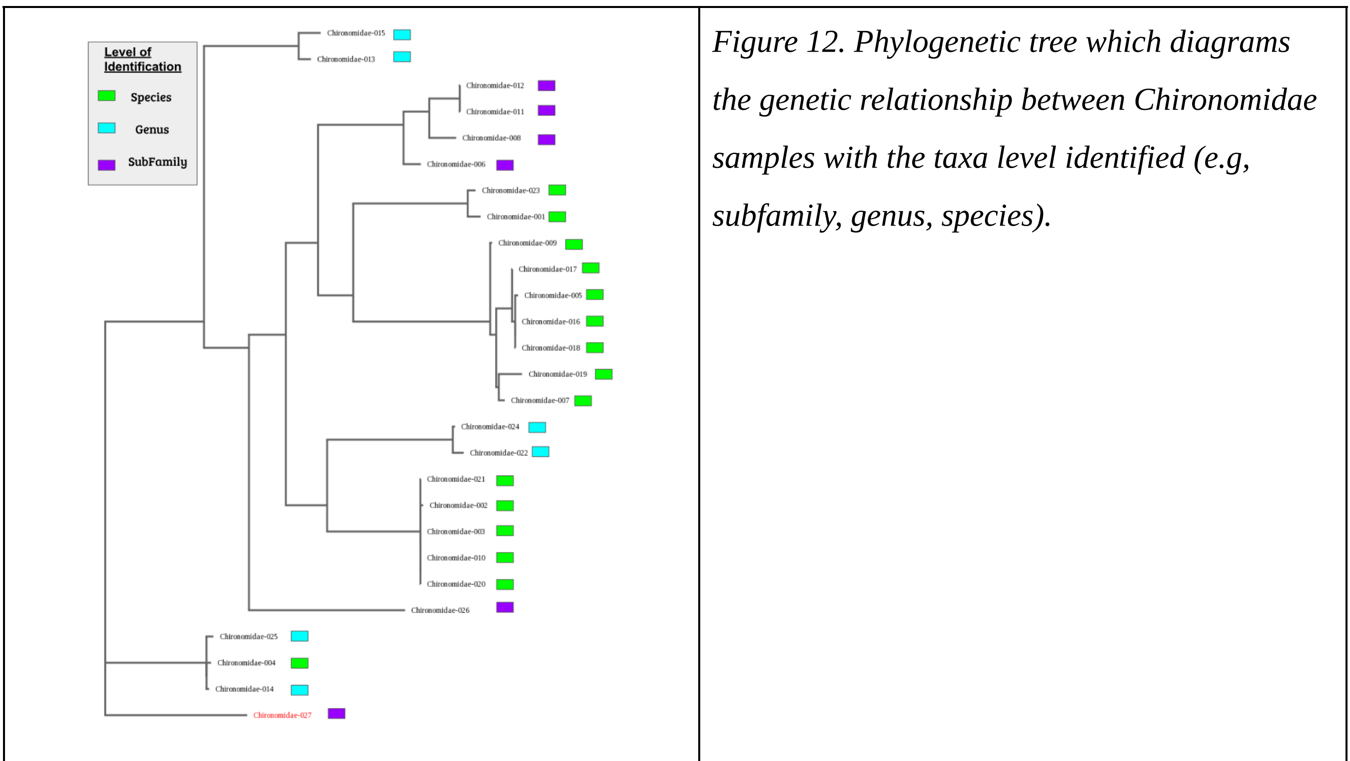

Figure 12. Phylogenetic tree which diagrams the genetic relationship between Chironomidae samples with the taxa level identified (e.g, subfamily, genus, species).

The family biotic index for Chironomidae is 6. This masks an underlying variability as the genera sampled for this study range in biotic index from 2 to 10 on a scale of 0 to 10 health scale.

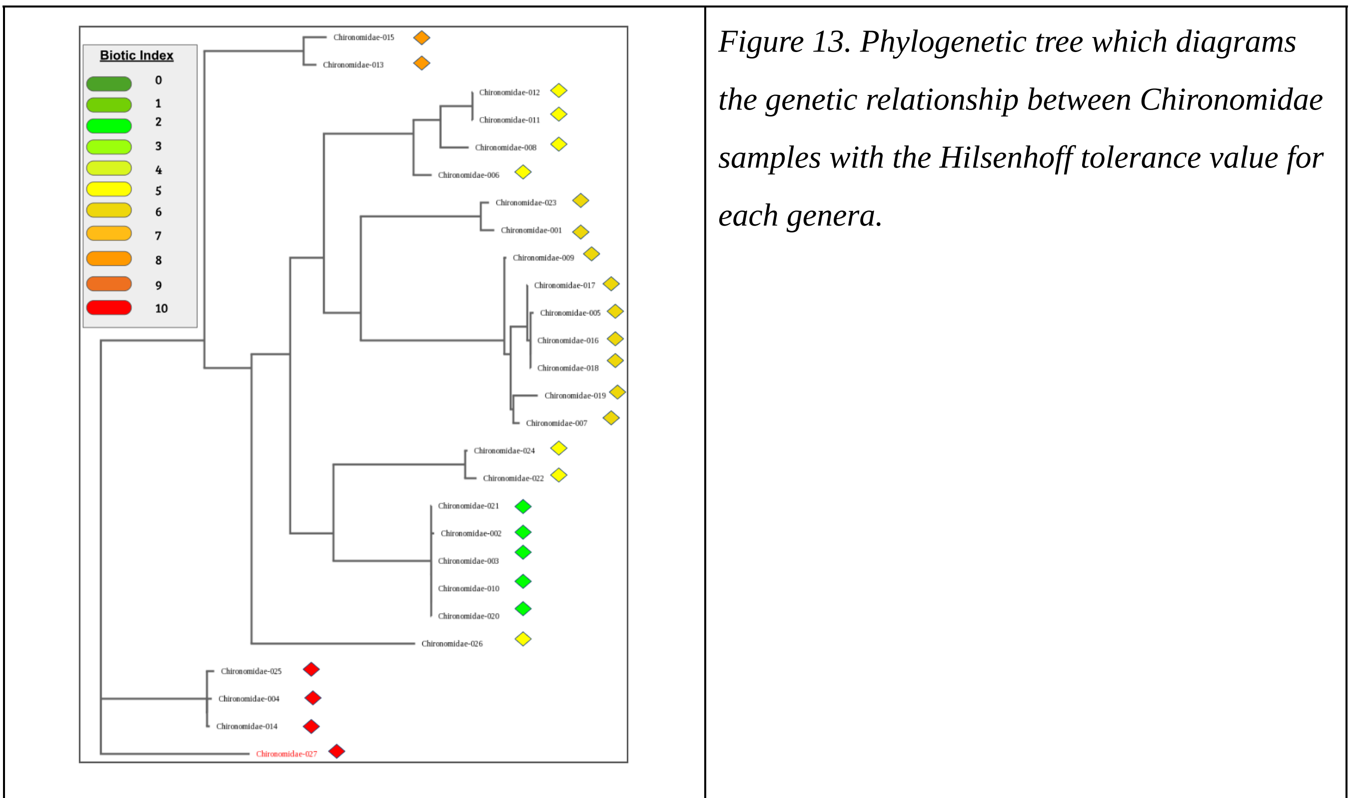

Figure 13. Phylogenetic tree which diagrams the genetic relationship between Chironomidae samples with the Hilsenhoff tolerance value for each genera.

4. Discussion

4.1 DNA Barcoding for Bioassessment: Highly detailed genus and species level data is more accurate and precise but difficult to obtain manually due to cost, specimen condition, incomplete taxonomic knowledge, poor taxonomic keys, lack of trained taxonomists. Error rates of genus and species in samples identified manually by experts have been found to be as high as 65%.[5] This demonstrated the value of DNA Barcoding, especially for identifying such versatile and phenotypically similar specimens as Chironomidae. Hilsenhoff tolerance scores of the Chironomidae genera sampled and identified using the DNA Barcoding method developed here were used with GIS software to provide a water quality overview map. Visualizations from this project's data were used in community land use decision making. In addition to the value of making data readily available to communities, it is important to note that DNA Barcoding enables an increase in the amount and accuracy of data available for community and land use decision making.

4.2 Comparison of Molecular Analysis Methods: An important step to developing a methodology for use of Chironomidae in bioassessment was comparing and evaluating molecular analysis methods. Four approaches were evaluated: eDNA Metabarcoding Extraction and eDNA Metabarcoding Primer, Rapid Method (chromatography paper) Extraction and PCR Bead, Silica Resin Extraction and PCR Bead, Silica Resin Extraction and MM Primer. Silica resin and PCR bead successfully amplified 100% of the samples. This result also verified that the appropriate laboratory and field practices and techniques had been used, and that the techniques and methods were not excessively cumbersome.

4.3 Selection of Chironomidae as a Global Common Denominator: Various macroinvertebrate families were identified by DNA Barcoding with silica resin and PCR beads. Selecting one family to focus on provided a natural limit that allowed effects of differences in extraction and amplification of DNA to be minimized, for example macroinvertebrates with tough exoskeletons or shells can be more difficult to extract DNA from, and many mollusks contain PCR inhibitors. The response of various macroinvertebrate families to DNA Barcoding and success at amplification was compared using measures of DNA sequence quality: visual analysis of electropherograms, *Phred* score, undetermined nucleotides, peak quality, sequence quality. The Chironomidae were identified as the best option with the best sequence quality, as they had the best *Phred* score, least undetermined nucleotides, and best peak quality. The Gammaridae also responded very well to barcoding, with a *Phred* score of 98% vs 99% for Chironomidae, however the Gammaridae do not have the range of geography and biotic indices that the Chironomidae do.

4.4 Chironomidae and Surface Geology Variation: The Chironomidae sampled here aligned by genera with either high gradient streams in piedmont geology, or sandy soils and coastal plain geology. Only 13% of the genera sampled were found evenly in both geologies.

4.5 Comparison of Genera Tolerance Values and Nutrient Pollution: This analysis compared the relationship between the weighted average Hilsenhoff tolerance scores of the Chironomidae genera sampled at each site, with the nutrient pollution. The value for nutrient pollution was calculated from the average ppm of nitrate and orthophosphate sampled at each site, which was normalized to a value between 0 and 10. When nutrient pollution data for sites are graphed with the weighted average Hilsenhoff tolerance scores of the Chironomidae genera sampled, a moderate positive linear association is noted. There is a statistically significant relationship with $p<0.05$. In a linear regression ran, $R^2 =.67$ indicating that 67% of the variation in the Hilsenhoff tolerance scores of the Chironomidae genera sampled were accounted for by overall nutrient pollution data. This means that 33% of the variation in tolerance score is influenced by factors other than nutrient pollution. Obviously not all variation in health as measured by Genera Tolerance Values can be explained by nutrient pollution as there are many other factors that contribute to a healthy waterway.

4.6 Comparison of Genera Tolerance Values and Overall Historical Health Values: The Chironomidae health data correlates to historical health measurements. When historical health data for sites are graphed with the weighted average Hilsenhoff tolerance scores of the Chironomidae genera sampled, a strong positive linear relationship is noted. There is a statistically significant relationship with $p<0.05$. In a linear regression, $R^2=.79$ indicating that 79% of the variation in the Hilsenhoff tolerance scores of the Chironomidae genera sampled were accounted for by overall historical waterway health data. This means that 21% of the variation in tolerance score is influenced by factors other than overall waterway health. Bottom composition is likely a part of that 21% as there are some Chironomidae genera that prefer a healthy, pebble-bottomed stream over a healthier mud-bottomed stream. Future plans for this study include finding out more about how bottom composition affects the residential Chironomid. The overall historical health score explains more of the variation in Hilsenhoff tolerance scores of the Chironomidae genera sampled.

4.7 Phylogenetic Tree Analysis: Phylogenetic trees were used to analyze the genetic relationships between selections of the Chironomidae sampled with respect to site, taxa level identified, and biotic index. The phylogenetic tree in Figure 12 was used to analyze the genetic relationships between selections of the Chironomidae sampled with respect to taxa level identified (e.g. subfamily, genus, or species). Identification down to species level indicates a match in the sequence databases. Identification to genus or subfamily indicates gaps in the sequence database that can be filled with a widespread

barcoding initiative. The gaps could also allude to potential novel species. The phylogenetic tree in Figure 13 diagrams the genetic relationship between Chironomidae samples with the Hilsenhoff tolerance value for each genus. The Hilsenhoff family biotic index for Chironomidae is 6. The genera sampled range in Hilsenhoff Biotic index from 2 to 10 on a scale of 0 to 10 health scale.

4.8 Statistical Tools and Analysis:

In Phase I, a two-sample t-test was used to compare *Phred* sequence quality scores between Chironomidae and other macroinvertebrates sampled to a 0.01 significance level. The two-sample t-test was selected since the sample quantity *n* was less than 30. The significance of 0.01 was chosen to emphasize the very low p value obtained for the Physidae and Haliplidae.

When the Chironomidae were compared with Physidae, Haliplidae, and Gammaridae, the null hypothesis was that the mean proportion of ideal *Phred* scores for Chironomidae would be equal to that of Physidae. The alternative hypothesis was that the mean proportion of ideal *Phred* scores would be greater for Chironomidae than, for example Physidae. Since $p= 1.01 \times 10^{-6}$ and is lower than the significance level of 0.01, the null was rejected, indicating that the Chironomidae DNA sequence quality was significantly better than the Physidae sequence quality. For Chironomidae vs. Haliplidae $p = 7.37 \times 10^{-8} < 0.01$ indicating a statistically significant sequence quality improvement. For Chironomidae vs. Gammaridae p = .053 indicating a difference that is not significant, however Gammaridae were not chosen due to their more limited number of species, geographic range, and biotic index range.

In Phase II, bioassessment measurement systems were compared. In order to compare waterway ecosystem bioassessment by weighted average tolerance values of the Chironomidae genera barcoded, and the current method that uses manual taxonomic identification by morphology, a Bland-Altman analysis was used.[2] The Bland-Altman test was selected as this is a common statistical tool used to compare two different methods of measurement when a true value or calibration standard is not available, and measurements must be made indirectly.[2]

Comparing two measurement systems by running a regression and calculating a correlation coefficient R value is not sufficient to compare measurement systems, as two methods of measuring the same value are nearly guaranteed to be correlated. Additionally, they can be correlated without being in agreement, such as a measurement of length in inches, and in centimeters.[2] Bland-Altman analysis determines the level of agreement between two measurement systems. This comparison showed limits of agreement of -0.853 and 0.868 between the weighted average tolerance values of the Chironomidae genera barcoded and the current method that uses manual taxonomic identification by morphology. This indicates that the new method proposed here of DNA barcoding Chironomidae is in agreement with the current method to within 1.72 out of 10. This finding is significant, especially considering that waterway

wellhealth data is often reported as good / fair / poor, and leads to the conclusion that the measurement method is sensitive enough, and waterway ecosystem bioassessment by DNA Barcoding of Chironomidae is a viable option for bioassessment globally.

Statistical power is the sensitivity of a test, or the ability of a test to find an effect if there is one to be found, or in other words the probability that the test will correctly reject a false null hypothesis. Statistical power = 1 – β, where β is the probability of making a Type II error and alpha α is the probability of making a Type I error. Statistical power is also a function of the sample size, alpha, and effect size. Increasing the sample size increases statistical power, but there is typically a cost or challenge to obtaining more samples. Increasing alpha also increases statistical power, however this merely exchanges this risk of a Type II error (β-risk) for the risk of a Type I error (α-risk). Where statistical significance determines if there is a difference between the two groups, effect size quantifies the difference between the two groups. Bigger effects are easier to detect than smaller effects. If the data being sampled has a large amount of variance, both from the value being measured and the noise in the data, this will decrease the statistical power.[3] Measurement error is also a source of noise. The goal of using DNA Barcoding to resolve taxa in more detail to the genus and species level, is to reduce variability and therefore increase statistical power. Increasing the precision of the measurement increases the statistical power and/or decreases sample size. A statistical power of 0.80 is typical, and indicates a 4:1 trade off between β-risk and α-risk. Highly consistent systems in engineering and physical sciences, as well as medical tests where the risk of a false negative (not detecting a disease) can have higher statistical power such as 0.90.

DNA Barcoding increases resolution from family level, to genus and species, as well as reducing errors from manual taxonomic identification by morphology. In the case of Chironomidae this means that genus level tolerance values ranging from 0 to 10 can be used instead of the family level tolerance value of 6. This increases the statistical power of the bioassessment method.

5. Conclusions:

5.1 Based on Bland-Altman and other analyses, waterway ecosystem bioassessment by DNA Barcoding of Chironomidae is a significantly improved option for bioassessment globally, providing more accuracy, more precision, and higher statistical power than manual taxonomic identification by morphology. Stream health data from Chironomidae genera also correlated with historical health data. (Statistically significant $p<0.05$)

5.2 The learnings from these data are being applied to fund and build a microbiology capability at a nonprofit scientific water study institute that supplements their existing citizen science and water

monitoring programs. This program has been approved by the organization's leadership. Laboratory space and ongoing support resources have been allocated. Partial capital funding has been received from multiple sources.

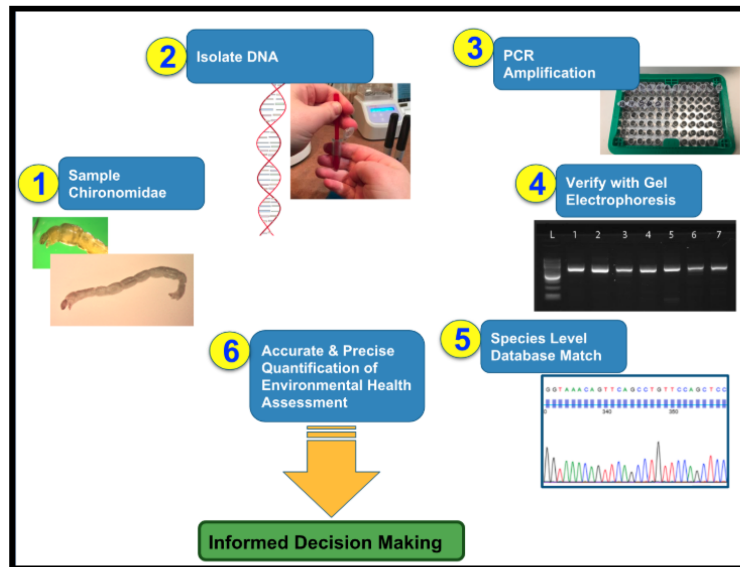

Figure 14. Methodology developed for bioassessment with DNA Barcoding with Chironomidae

5.3 All samples barcoded using the optimized method of silica resin isolation and PCR beads as was also observed by percent amplification in gel electrophoresis.

5.4 The family tolerance value for Chironomidae is 6, however identification to the genus level revealed Hilsenhoff tolerance values ranging from 0 to 10 on a scale of 0 to 10 health scale. The phylogenetic tree shows potential novel species where closely related samples correspond to gaps in the sequence database. New sequences were added to databases used by the scientific community. Phylogenetic tree groupings match geography and historical health data. Samples from the healthiest sites are nearly genetically identical. The most sensitive genus of Chironomid was only found in the healthiest sites.

5.5 DNA Barcoding of Chironomidae can be faster and lower cost than the current method. This method is robust, reproducible, and suitable for augmenting citizen science initiatives.

5.6 In analyzing the distribution of Chironomidae genera between streams with urban vs. open space catchment areas, there was not a statistical correlation. This may require further study with more detailed land use data. (Not statistically significant $p>.05$)

5.7 Finally, the investigation into the Chironomidae family shows that DNA Barcode analysis can result in waterway health data that is both more accurate and more precise, and therefore increase statistical power and significant value for monitoring an increasingly scarce water resource.

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