Using eDNA methods to extend biological sampling and identify candidate restorations for species reintroductions

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Executive Summary

We assessed the efficacy of using environmental DNA (eDNA) for stream restoration monitoring and for using stream sediment microbial communities to predict ecological condition. eDNA and stream sediment samples were collected at 27 stream restorations, paired with unrestored upstream controls for each. After DNA library preparation, sequencing, and bioinformatics, the resulting fish and benthic macroinvertebrate occurrences were combined with and compared against traditional monitoring data to: 1) assess whether restored sites showed improvements over their upstream unrestored sections; 2) identify if differences in assessments differed between fish and benthic macroinvertebrates; 3) identify any taxonomic or functional group bias with eDNA sampling; and 4) compare eDNA against traditional monitoring methods. We also assessed the ability of microbial communities in stream sediments to predict stream condition for use as a screening tool for future fish and benthic macroinvertebrate reintroductions.

Including eDNA in restoration monitoring increased the sensitivity of the monitoring results and was able to identify positive responses in restored sections that were not evident with traditional monitoring methods. This was especially evident for the benthic macroinvertebrates where more sensitive taxa, more EPT taxa, and greater overall taxa richness occurred in the restored sections. Restored sections also had significantly more fish species and sensitive fishes, but eDNA was not as useful because the electrofishing data were adequate to find this pattern. Both the fish and benthic macroinvertebrate communities described by eDNA were significantly different between the restored and control sections, and were different from the communities described by traditional monitoring. This in part is due to the increased taxa richness captured with eDNA.

Compared to traditional monitoring, eDNA identified significantly more taxa and sensitive taxa for both fish and benthic macrionvertebrates. The only attribute explored where eDNA was not equal to or greater for measuring biodiversity were the fish families Cyprinidae (minnows) and Percidae (perch), where electrofishing better sampled the diversity.

Despite substantial efforts and multiple approaches, we were unable to generate reliable predictions of stream condition using the stream sediment communities.

While not a complete replacement for traditional sampling methods, including eDNA in stream restoration monitoring has distinct advantages. It is cost effective, easy to collect samples, and tends to better sample the fish and benthic macroinvertebrate biodiversity. We believe it can effectively complement traditional sampling to give a broader and more detailed perspective on aquatic biodiversity.

Stream restorations are a major mitigation activity to combat the degradation of running waters in the mid-Atlantic, and Maryland in particular. Restoration activities have achieved substantial improvements in the physical structure and stability of stream banks and channels (Hilderbrand et al. 2019). In contrast, evidence for improvements in ecological recovery measures are largely lacking. However, a lack of evidence does not necessarily mean lack of success. At least two scenarios could show no ecological improvements despite a successful stream restoration because 1) our survey abilities are limited or 2) taxa targeted through restoration exhibit poor dispersal or establishment capabilities.

A restoration may be successful in providing all of the necessary conditions for desired taxa, but none are present because recolonization is limited by proximity to nearby donor sites (Sundermann et al. 2011). Alternatively, a stream restoration may be successful, but desired taxa have returned at low and patchy abundances which are near or below the limits of detection using current sampling methods. Under these conditions, many years may be required before desired taxa attain sufficient abundances as to allow detection. Finding rare and establishing taxa may be particularly problematic for benthic macroinvertebrates where the amount of physical sampling is necessarily limited, and the collections are typically subsampled due to the large numbers of individuals collected. Under these circumstances, taxa attempting to establish could easily be missed. Similar issues are encountered when characterizing fish communities, where the standard 75m sampling reach for fish can easily miss species in low numbers or with patchy distributions. Using eDNA may substantially advance our abilities to sample the more rare taxa in streams.

eDNA approaches are revolutionizing stream monitoring because they can detect rare, cryptic, common, and invasive species (Thomsen et al. 2012; Keller et al. 2017). eDNA can be more sensitive (Ficetola et al. 2008), detect taxa not found with traditional survey methods (Jerde et al. 2011; Pilliod et al. 2013), and do so over distances of up to several kilometers for invertebrate DNA (Deiner and Altermatt 2014). Primers exist that target fish and benthic macroinvertebrates (Leese et al. 2020; Zhang et al. 2020), and results can now replicate those found in known surveys of each group as well as detect cryptic and rarely observed taxa (Bessey et al. 2020). The advantage of eDNA for use within stream restorations is the ability to substantially extend detection of rare or patchily distributed taxa that may be in the process of establishing post-restoration.

A related, but very different application of DNA is to use microbes and 16S rRNA sequencing data to predict/classify ecological condition. Microbial communities from stream sediments can mirror a stream's BIBI (Benthic Index of Biotic Integrity) score with 80% accuracy (Hilderbrand et al. 2020) and presents a potential way to pre-screen restorations to determine if the conditions appear suitable to reintroduce desired fish and benthic macroinvertebrate taxa. Microbes are ubiquitous in the environment and not as subject to colonization/dispersal limitations as are fish and benthic macroinvertebrates. The proportions of different microbial groups can be associated with stream condition in similar fashion to the IBI. Just as with blood markers of human health, microbes may serve as biomarkers of stream conditions and may be broadly useful in aiding restorations where recovery and recolonization dynamics are in question. Streams classified as higher condition would be good candidates for reintroductions. While research into the effectiveness of reintroductions is valuable, it assumes that the restorations are suitable. Microbial communities may provide an *a priori* screen for potential recipient streams.

OBJECTIVES

Our major objective was to determine the utility of eDNA in complementing or even replacing traditional methods for stream restoration monitoring. Important components of this objective were to: 1) assess whether restored sites showed improvements over their upstream unrestored sections; 2) identify if differences in assessments differed between fish and benthic macroinvertebrates; 3) identify any taxonomic or functional group bias with eDNA sampling; and 4) compare eDNA against traditional monitoring methods. A second major objective was to assess the ability of microbial communities in stream sediments to predict stream condition for use as a screening tool for future fish and benthic macroinvertebrate reintroductions.

METHODS

Sample pairs were collected at 27 restorations during the spring 2022 (Table 1). Each pair consisted of a water and stream sediment sample collected 50-100m above the restoration and a water and stream sediment sample collected near the bottom of the stream restoration. This paired approach was used in order to determine if taxa found in restorations were also present in the unrestored upstream area as well as to compare the stream sediment microbial communities for suitability for species recovery. Samples were collected near the bottom of each restoration in order to maximize the restoration effect as well as to maximize distance from the control section sample to minimize detections of control section taxa that might not occur in the restoration.

Table 1. Restorations sampled for the project.

Restoration	County	Region	Date sampled	Latitude	Longitude	Age
Bachelors Run	Montgomery	Piedmont	3/29/2022	39.12102	-77.03378	2011
Bacon Ridge Lower	Anne Arundel	Coastal	3/28/2022	38.99429	-76.61335	2020
Bacon Ridge Upper	Anne Arundel	Coastal	3/28/2022	39.0369433	-76.6273815	2019
Bramhope (Red Hill Run)	Howard	Piedmont	3/11/2022	39.2385685	-76.813565	2012
Cat Branch	Anne Arundel	Coastal	3/28/2022	39.0317383	-76.4465417	2019
Cowhide Branch	Anne Arundel	Coastal	3/28/2022	38.990786	-76.528975	2018
Fosters Branch	Harford	Coastal	3/16/2022	39.41256	-76.33949	2017
Greenbriar Branch	Montgomery	Piedmont	3/29/2022	39.0544133	-77.2507692	2020
Howard's Branch	Anne Arundel	Coastal	5/24/2022	39.021164	-76.548155	2006
Kelly Branch	Baltimore	Piedmont	3/16/2022	39.4412188	-76.5911717	2021
Little Catoctin Creek	Frederick	Highland	3/17/2022	39.3378124	-77.6279083	2019
Little Patuxent River	Howard	Piedmont	3/11/2022	39.270915	-76.8518717	2015
Little Tuscarora	Frederick	Highland	3/17/2022	39.471274	-77.4141883	2015
Long Draught Branch	Montgomery	Piedmont	3/10/2022	39.1439583	-77.2301176	2019
Maple Dell	Howard	Piedmont	3/16/2022	39.3161533	-77.0616013	2018
Mill Creek	Anne Arundel	Coastal	3/28/2022	39.055835	-76.5087867	2021
Minebank I695	Baltimore	Piedmont	3/11/2022	39.404465	-76.5675333	2021
Muncaster Mill	Montgomery	Piedmont	3/29/2022	39.1359514	-77.1289402	2016
Plumtree Creek	Harford	Piedmont	3/16/2022	39.5031732	-76.3404117	2011
Red Hill Branch Meadowbrook Park	Howard	Piedmont	3/11/2022	39.2468183	-76.8234967	2012
Red Hill Branch upper	Howard	Piedmont	3/11/2022	39.2388878	-76.8130085	2012
Sandy Branch	Montgomery	Piedmont	3/29/2022	39.05456	-77.2510942	2020
Shephard's Cove	Anne Arundel	Coastal	3/28/2022	38.9718417	-76.595245	2021
UT Little Gunpowder	Baltimore	Piedmont	3/10/2022	39.5162604	-76.4829633	2019
UT Seneca Creek, Watkins Mill	Montgomery	Piedmont	3/1/2022	39.15897	-77.231202	2019
Valley Mill Creek	Montgomery	Piedmont	3/29/2022	39.0660833	-76.9792283	
Wheel Creek	Harford	Piedmont	3/16/2022	39.482255	-76.339685	2017

Water samples for fish and benthic macroinvertebrate eDNA were collected using a Smith-Root backpack eDNA sampler, which pulled stream water through 1.2um self-preserving PES filters. Stream water was drawn through each filter until the filter clogged and the volume recorded. Filters were kept in their housings, placed and sealed into their original packaging, labeled to identify each collection, and placed on ice until being brought back to the Appalachian Laboratory. The filters/packaging were placed into a -20C freezer until DNA extractions.

Each stream sediment sample was collected in triplicate and pooled to comprise an upstream unrestored sample and a restored sample for each site. A sterile 10cc syringe tube was inserted wide-end down into the stream sediment in a depositional area to collect the top 1 cm of sediment and its microbial communities. The syringe plunger was inserted to retain the sediments, water evacuated, the syringe capped, and then placed into a sterile, labeled collection bag and stored on ice until arrival at the Appalachian Laboratory. Sediment samples were placed into a -20C freezer until DNA extractions.

DNA Extraction

DNA Extraction from Water Filters

DNA was extracted from the water filters using the E.Z.N.A. Tissue DNA Kit from Omega Bio-Tek (Omega Bio-Tek, Inc., Norcross, Georgia) with the following modifications: One-half of a water filter was cut into smaller pieces (cleaning scissors and forceps between samples) and placed in a 2.0ml microcentrifuge tube. 567ul of buffer TL and 63ul of Proteinase-K were then added, the sample vortexed, and placed in a 65C water bath for 1 hour, during which the samples were vortexed about every 15 minutes. The samples were removed from the water bath after the 1 hour incubation and 630ul of buffer BL was added to each, vortexed, and incubated at 70C for 10 minutes. After this incubation 630ul of 100% ethanol was added to each sample and vortexed. 650ul of sample was added to a HiBind DNA Mini Column, centrifuged at maximum speed for 30 seconds, the filtrate discarded, and this step repeated until all of the sample had been passed through the column. The rest of the procedure was followed per kit instructions. In the final step, 65ul of elution buffer was added to the column and the column incubated at 70C for 5 minutes to increase DNA concentration. The DNA was then stored at -20C until ready to proceed with library amplification.

DNA Extraction from Sediment Cores

DNA was extracted from the three sediment cores from each site using the DNeasy PowerSoil Pro Kit (Qiagen, Germantown, MD) with the following modification for microbe lysis: after the addition of buffer CD1 and vortexing, the samples were placed in a Geno/Grinder (Cole-Palmer, Metuchen, NJ) and subjected to lysis at 1500 RPM, in 2.5 minute increments for a total time of 7.5 minutes. 20ul of Proteinase-K (stock concentration of 20mg/ml, Qiagen, Germantown, MD) was then added to each sample and incubated at 56C for 30 minutes. The rest of the protocol was followed per kit instructions with the DNA being eluted in a final volume of 50ul and stored at -20C until ready to proceed with library amplification.

Library Preparation

Library Preparation from Water Sample DNA

Three primer pairs were used to construct libraries from the extracted water sample DNA. The primer pairs used for PCR1 were: 1) fwhF2/EPTDr2n (Leese et al. 2020; hereafter referred to as Leese primers) which amplifies an approximately 150bp region and has been found to work well for the detection of aquatic insects. The Leese primer sequences are: fwhF2_F 5’ GGDACWGGWTGAACWGTWTAYCCHCC3’ and EPTDr2n_R 5’CAAACAAATARDGGTATTCGDTY3’; 2) the general primer set mlCOIintF/jgHCO2198

(Leray et al. 2013; Hereafter referred to as Leray Primers) which amplifies an approximately 313bp region and has been found to capture invertebrates as well as fish and substantially supplemented the biodiversity not detected by more taxa-specific primers. The Leray primer sequences are: mlCOIintF 5'GGWACWGGWTGAACWGTWTAYCCYCC3' and jgHCO2198 5'TAIACYTCIGGRTGICCRAARAAYCA3'. 3)

The fish-specific MiFish primers (Miya et al. 2015) which amplifies an approximately 150bp region. The MiFish primer sequences are: MiFish-U_F 5'GTCGGTAAAACTCGTGCCAGC3' and MiFish-U_R 5'CATAGTGGGGTATCTAATCCCAGTTTG3'. All PCR reactions were conducted using the Thermo Scientific Phire Tissue Direct PCR master mix (Thermo Fisher Scientific, Waltham, MA). PCR reactions consisted of 10.0ul of master mix; 1.6ul of BSA (stock concentration of 10mg/ml); 1.0ul of forward and reverse primer (stock concentration of 10uM); 5-10ng of DNA brought up to 20ul volume with nuclease free water. Positive controls used were Atlantic Salmon (Salmo salar) for the MiFish primers; European Honey Bee (Apis mellifera) for Leray primers; and a pollinator community containing several insect pollinators with no aquatic life history stage for the Leese primers. Negative controls were also included. PCR1 Parameters for both the Leese and Leray primer pairs were as follows: 98C for 5 min followed by 40 cycles of 98C for 5 seconds; 45.3C for 5 seconds; and 72C for 20 seconds; with a final extension at 72C for 1 minute and a hold of 4C until removed from the thermalcycler. PCR1 parameters for the MiFish primers were as follows: 98C for 5 min followed by 35 cycles of 98C for 5 seconds; 64.5C for 5 seconds; and 72C for 20 seconds; with a final extension at 72C for 1 minute and a hold of 4C until removed from the thermalcycler.

PCR2 primers were constructed with the addition of the following sequences to each of the PCR1 primers in order to be compatible with the Illumina sequencing platform: forward sequence 5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG3'; reverse sequence 5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG3'. PCR reaction mixes were set up as for PCR1 with 1ul of each primer at a stock concentration of 2uM being used along with 1ul of the PCR1 product. Cycling conditions were as for PCR1 for each primer set with the exception that 15 cycles instead of 35 or 40 were used for PCR2.
PCR3 was conducted using 1ul of PCR2 and primers containing sample specific dual indexing tags. The reaction mix was constructed as above, using 1 ul of each indexing primer at stock concentration of 10uM. The PCR cycling conditions were as follows: 98C for 5 min followed by 20 cycles of 98C for 5 seconds; 66C for 5 seconds; and 72C for 20 seconds; with a final extension at 72C for 1 minute and a hold of 4C until removed from the thermalcycler. Success of PCR was checked by running products on a 1.2% agarose gel.

The DNA of samples which did not amplify were then subjected to a clean-up using the Qiagen DNeasy PowerClean Pro Cleanup kit (Qiagen, Germantown, MD). The cleaned DNA was then used to repeat the PCR process and success visualized on a 1.2% agarose gel.
PCR products from the index reactions were cleaned and normalized using the SequalPrep Normalization Plate Kit (Applied Biosystems,Thermo Fisher Scientific) per manufacturer instructions. Indexed samples were then pooled per primer set, quantified using the Invitrogen Qubit HS dsDNA assay (Thermo Fisher Scientific), and sequenced on an Illumina MiSeq DNA Sequencer at the USGS Eastern Ecological Science Center, Kearneysville, WV. The MiFish and Leese libraries were each sequenced using the MiSeq Reagent v2 300 cycle kit (final amplicon size approximately 350bp) and the Leray library was sequenced using the MiSeq Reagent v3 600 cycle kit (final amplicon size approximately 500bp) (Illumina, San Diego, CA).

Stream Sediment Samples Library Preparation

PCR amplification was conducted as for the DNA water samples but with the following modifications: The primers used were U515F 5'GTGCCAGCMGCCGCGGTAA3' Parada et al. 2016) and 806R 5'GGACTACHVGGGTWTCTAAT3' (Apprill 2015), which amplify an approximately 250bp of the V4 region of the bacterial and archaeal 16S subunit ribosomal gene. Cycling conditions for PCR1 were 98C for 5min; then 35 cycles of 98C for 5 seconds, 65C for 5 seconds, 72C for 20 seconds; a final extension at 72C for 1 min; and a hold of 4C until removed from the thermalcycler. A microbial mock community (Microbial Mock Community B, catalog number HM-276D, BEI Resources) was used as the positive control along with negative controls. PCR1 was conducted on the DNA from each core sample from each site. The success of PCR1 was then visualized on a 1.2% agarose gel after which the PCR1 products for the three core samples from a site were combined. PCR2 primers were synthesized as for the PCR2 primers used on the DNA from the water samples. The rest of the amplification (including indexing), cleaning/normalization, and sequencing steps were as described for the water samples; a MiSeq Reagent v2 500 cycle kit (Illumina) was used for sequencing.

Bioinformatics

Stream Sediment Microbial Communities

The stream sediment microbial community sequencing data were analyzed using the mothur software package (v.1.48.0). Sequences were trimmed according to base quality scores using a 50 bp sliding window with an average quality score cutoff of 35. Reads with primer mismatches, ambiguous bases, homopolymers greater than 8 bp, and/or sequences less than 100 bp and greater than 250 bp were removed. In order to maintain backward compatibility with the original model for predicting stream condition using microbial communities (Hilderbrand et al. 2020), sequences were aligned to a reference alignment (SILVA v. 119), and sequences that did not align were removed. All remaining sequences were trimmed to the same start and end position, and any unnecessary gaps generated during alignment were removed. Prior to chimera removal, sequences were preclustered allowing a difference of up to 2 bp (1.5%) between sequences. Chimeras were detected using the UCHIME algorithm [41] and removed from the dataset. Sequences were classified using a Bayesian classifier with an 80% pseudobootstrap confidence score against the GreenGenes database (v. 13.8.99). All sequences that classified as unknown, chloroplasts, mitochondria, or Eukaryota were removed.

Fish and Benthic Macroinvertebrates

We used a using a customized pipeline developed by Louis Plough (Formerly at UMCES Horn Point Laboratory) that uses existing routines from USEARCH (Edgar 2010), VSEARCH (Rognes et al. 2016) and the NCBI BLAST package. The code for this routine will be provided in an Appendix at submission of the final report for others to use or adapt at their convenience. We removed any taxa from a sample that produced fewer than 75 reads in the sequencing data in order to avoid any false positives associated with contamination.

Statistical Analyses

Stream Sediment Microbe Communities

The microbial communities were analyzed using Discriminant Analysis of Principal Components (DAPC) in the adegenet (Jombart 2008; Jombart et al. 2011) package for R 4.4.2 to classify and predict the ecological condition of sampled streams. Analyses were based on the archaea and bacteria OTU identities within the collections at each site. The OTUs and their abundances were fed into the model developed in Hilderbrand et al. (2020) to predict stream condition based on the BIBI in increments of 0.5.

Fish and Benthic Macroinvertebrates

Multiple analyses were used to assess whether 1) including eDNA altered interpretations of restoration success; 2) the effectiveness of eDNA in capturing aquatic biodiversity when compared to traditional monitoring data; and 3) differences occurred in detection across taxonomic or functional groups when using eDNA. Fish and benthic macroinvertebrates were analyzed separately, but the analyses were essentially the same for each group.

The general analysis framework was similar across the comparisons listed above. We subtracted off the number of taxa found in the upstream unrestored control section from the number found in the restored section for each sample pair. Thus, we analyzed the difference for each pair to control for differences across watersheds. This is essentially a blocked design without the need for an additional factor in the model. We used a linear models (ANOVA, ANCOVA) framework to examine the responses in which significant positive values resulted in greater biodiversity in restored sections compared to controls. After determining if differences existed between restored and control sections, more complicated statistical models were constructed to examine any influences of landscape, watershed, and restoration attributes. The predictor attributes examined were: geographic region; basin area; channel slope; % Forest in upstream watershed; %Urban land uses in upstream watershed; % Impervious Surfaces in watershed; length of restored stream; and time since restoration. In order to better understand the role of including eDNA in restoration monitoring, analyses were run for three scenarios: 1) eDNA + traditional monitoring data; 2) eDNA only; and 3) traditional monitoring data only. While these scenarios make for substantially more comparisons and complicate interpretations, they also allowed us to better compare and contrast how eDNA may contribute to future restoration monitoring.

In addition to the linear models, we assessed community-wide differences for fish and benthic macroinvertebrates between the restored and control sections and between eDNA and traditional monitoring methods using PERMANOVA as implemented in adonis in the vegan package (Oksanen et al. 2024) in R. Each stream was treated as a blocking factor in order to control for stream-to-stream differences in community structure. Thus, the community comparisons were on a restoration-specific basis akin to using a paired design in linear models. Because the comparisons were paired, an overall ordination for visualizing community structure is not informative since the communities for each pair will be more similar than across restorations.

A final analysis was to compare the calculated BIBI scores for the D-Net traditional monitoring data against a modified version using the eDNA data in order to determine if eDNA made substantive changes to the overall BIBI. The eDNA BIBI version had to be slightly modified because eDNA does not create reliable abundance data, and it typically identifies vastly more taxa than does D-Net sampling, which would substantially alter the scoring for that metric. To deal with these differences, we implemented two changes to the BIBI calculations. The overall structure used a bootstrapping approach where the eDNA data were subsampled 1,000 times for each sample and the average value used as the BIBI score. The second change accounted for differences in taxa richness and proportion-based metrics by randomly generating the total number of individuals in a subsample from a uniform distribution ranging 100-120 to match the range of individuals in the BIBI subsampling protocols and then randomly drawing these individuals from the eDNA data.

All comparisons were considered statistically significant at $P < 0.05$

RESULTS

Including benthic macroinvertebrate eDNA sampling alters measured restoration outcomes

We found significant differences between the benthic macroinvertebrate communities in restored and control sections when using only the eDNA data (PERMANOVA, P=0.023). The strength of the differences increased when combining eDNA with the D-Net sampling data (PERMANOVA, P=0.001). In contrast, no community-wide differences were found when using only the D-Net data (PERMANOVA, P>0.05).

Adding eDNA sampling to supplement or even replace traditional D-Net sampling demonstrated positive benthic macroinvertebrate biodiversity responses in restored sections compared to each stream's unrestored upstream control (Table 2; Figure 1). The combined eDNA + traditional sampling data showed a slightly stronger positive restoration response than using exclusively eDNA data. In contrast, there were no differences in benthic macroinvertebrate responses between restored and unrestored sections when using only the traditional D-Net sampling data. Similar results were found in the previous paired-sections research in Hilderbrand and Acord (2019) when using traditional sampling.

Analyses using eDNA combined with traditional sampling resulted in restored sections having significantly greater taxonomic richness (ANOVA, P=0.002), more urban intolerant taxa (ANOVA, P=0.031), more Dipteran genera (ANOVA, P=0.04), and more EPT taxa (ANOVA, P=0.014) compared to each stream's upstream unrestored control section (Table 2, Figure 2). Similar results were obtained when using exclusively eDNA data without the traditional sampling. However, the eDNA-exclusive analyses found no differences between restored and control for urban intolerant taxa nor EPT. The eDNA-exclusive data tended to find more Dipterans, with an emphasis on the Chironomidae in restored compared to unrestored, but the signal was not as strong as when these data were used in conjunction with the physical D-Net collections (Table 2).

Table 2. Analysis results comparing attributes of benthic macroinvertebrate biodiversity in restored sections versus their upstream unrestored controls. Results are presented for three data scenarios: 1) combined eDNA and traditional D-Net sampling; 2) exclusively eDNA data; and 3) exclusively traditional D-Net (Physical sampling data. The 'Greater' column indicates which attribute produced a greater response. All results are considered statistically significant at P<0.05 and are highlighted.

	eDNA + Physical			eDNA only			Physical only		
	F	P	Greater	F	P	Greater	F	P	Greater
Total Richness	11.4	0.002	Restored	5.5	0.028	Restored	0.4	0.529	Restored
Intolerant < 3	5.2	0.031	Restored	2.0	0.165	Restored	2.0	0.176	Restored
Intolerant < 5	2.4	0.135	Restored	0.8	0.380	Restored	0.3	0.592	Restored
Diptera	4.7	0.040	Restored	6.4	0.018	Restored	0.1	0.728	Control
Ephemeroptera	2.9	0.102	Restored	0.6	0.446	Restored	1.1	0.300	Restored
EPT	7.1	0.014	Restored	0.1	0.767	Control	3.2	0.091	Restored
Chironomidae	2.4	0.137	Restored	7.2	0.013	Restored	1.1	0.315	Control
Shredder	0.5	0.477	Control	3.5	0.074	Restored	2.5	0.131	Control
Collector	3.7	0.066	Restored	7.4	0.012	Restored	3.2	0.090	Control
Predator	6.8	0.015	Restored	0.2	0.622	Restored	8.9	0.007	Restored
Scraper	1.9	0.183	Restored	0.1	0.774	Restored	0.9	0.365	Restored
Filterer	13.2	0.001	Restored	5.4	0.029	Restored	3.7	0.069	Restored

Despite significant differences in restoration responses between analyses using eDNA and exclusively physical data, there were no appreciable differences in the overall benthic IBI scores when comparing traditional collections with those augmented with eDNA data (ANOVA $P>0.05$). Any differences found in the biodiversity measures were not large enough to be captured in the IBI metrics or overall score. This is in part because overall taxonomic richness in eDNA cannot be correctly compared to physical collections because of the way the IBI is calculated. Therefore the overall richness score was dropped from the IBI calculations and the overall BIBI formula adjusted (the denominator was changed) to account for the lack of taxon richness score. Because the IBI is calculated mostly from counts of individuals and percentages, the bootstrapping approach described in the methods was used.

From a trophic functional perspective, including eDNA in the analyses resulted in restored sections containing significantly greater taxonomic richness of predators (ANOVA, $P=0.015$) and filterers (ANOVA, $P=0.001$) for the combined eDNA + physical samples (Figure 3). The eDNA exclusive analyses contained significantly greater richness of collectors (ANOVA, $P=0.012$) and filterers ($P=0.029$). In contrast, only the predators were higher in restored sections when using traditional sampling data (ANOVA, $P=0.007$; Table 2).

No landscape, watershed, or instream attributes were related to the differences identified between restored sections and their upstream unrestored controls (ANOVA $P>0.05$).

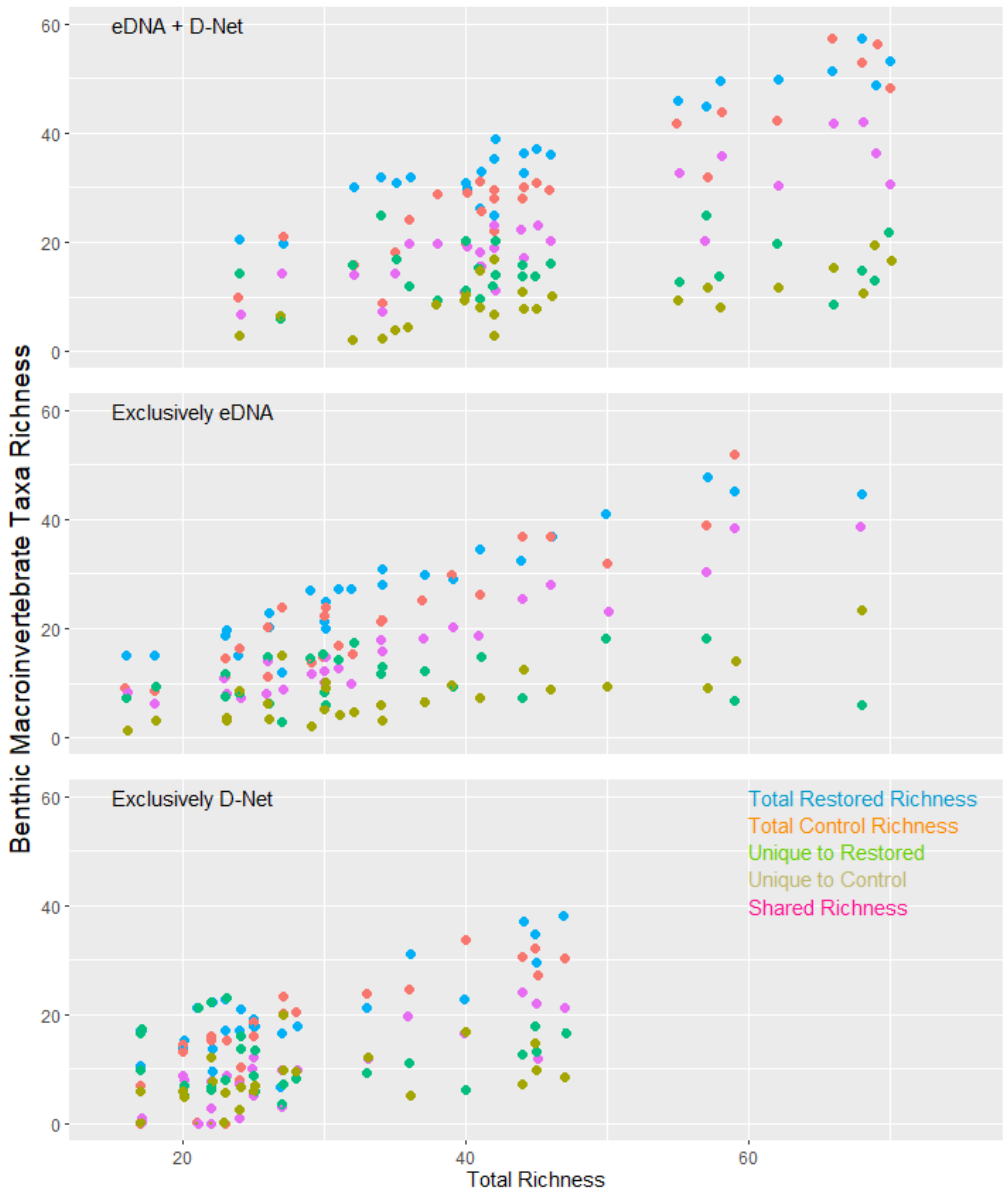

Figure 1. Plots of benthic macroinvertebrate taxa richness for combined eDNA + D-Net, eDNA-only, and D-Net only data that are arranged along the total richness gradient. Each column of points represents a restored+control section pair partitioned into the taxa richness for the restored (blue) and control (orange), taxa richness unique to restored (green) and control (brown), taxa shared among the sections (pink).

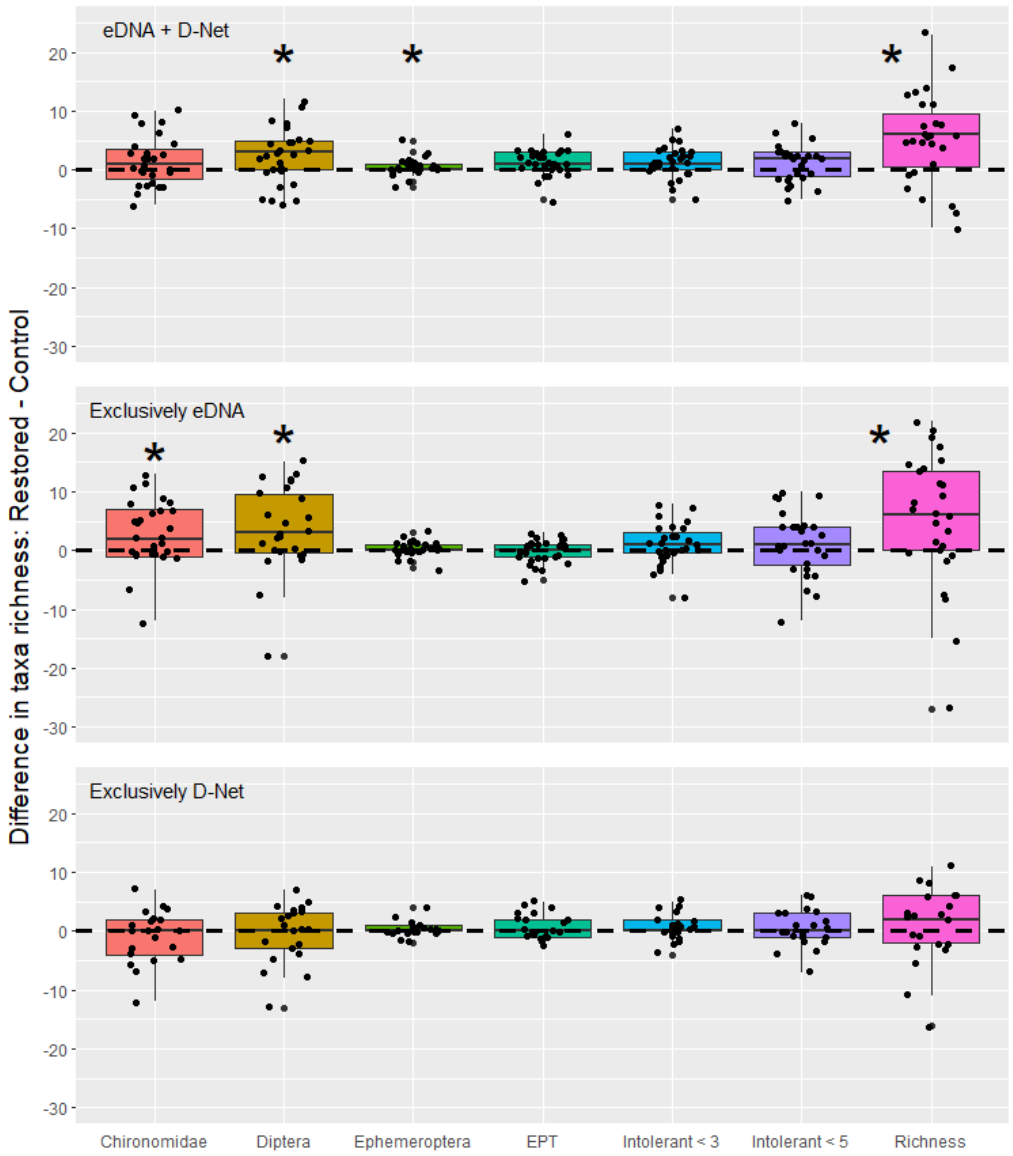

Figure 2. Differences in taxa richness for various benthic macroinvertebrate attributes for combined eDNA + D-Net, eDNA-only, and D-Net only data. Points above the horizontal line at zero indicate more taxa in the restored section. Attributes with an asterisk indicate a statistically significant difference between restored and control.

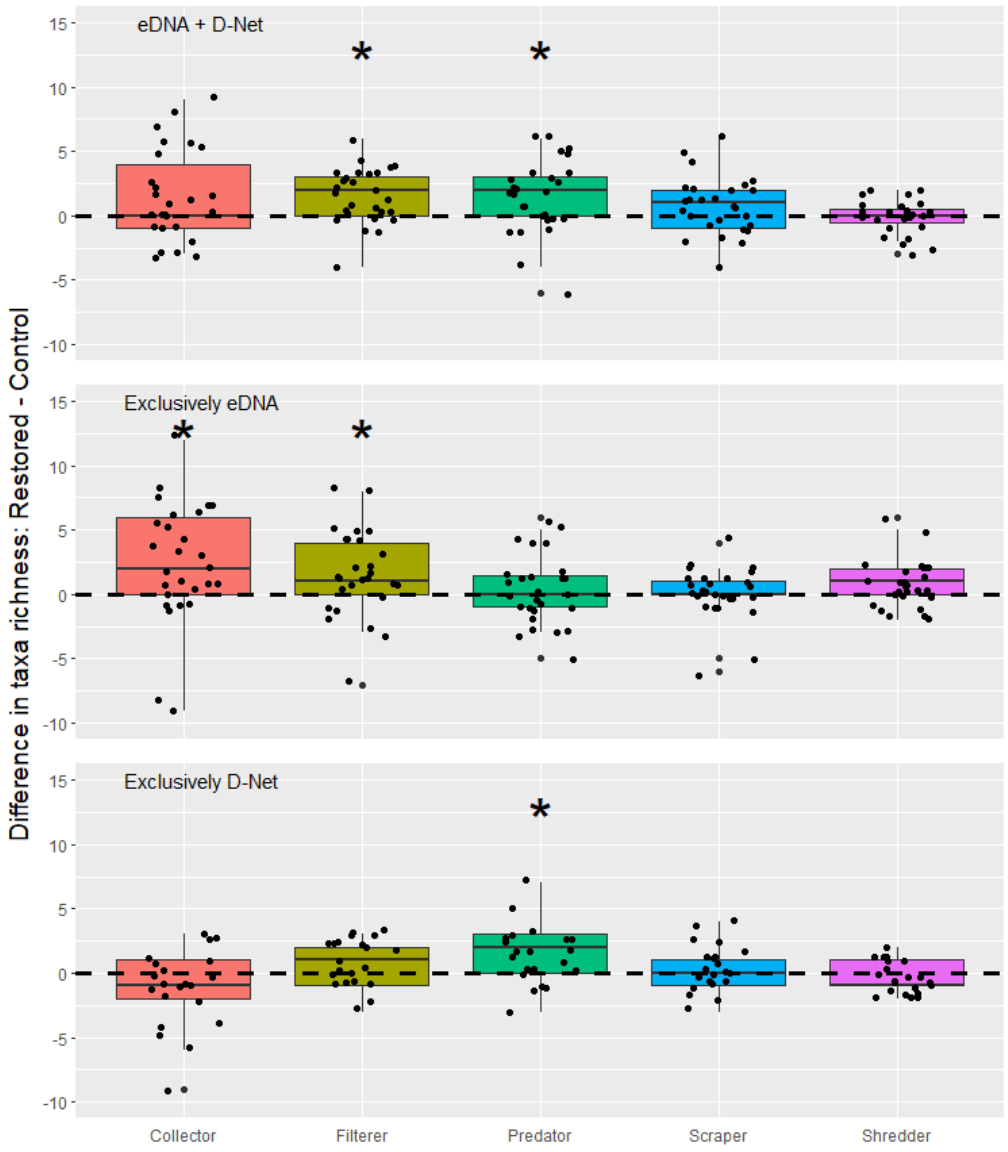

Figure 3. Differences in taxa richness of benthic macroinvertebrate trophic groups for combined eDNA + D-Net, eDNA-only, and D-Net only data. Points above the horizontal line at zero indicate more taxa in the restored section. Attributes with an asterisk indicate a statistically significant difference between restored and control.

Including fish eDNA sampling does not appreciably change measured restoration outcomes

Fish biodiversity was typically greater in restored sections compared to their upstream unrestored counterparts for most biodiversity and trophic attributes examined (Figures 4 and 5). In contrast to the benthos, significant differences were found regardless of whether eDNA or traditional electrofishing data were analyzed. More differences between restored sections and their unrestored control pair were identified when eDNA and electrofishing data were incorporated into analyses compared to analyzing exclusively electrofishing or exclusively eDNA data (Table 3). The greatest number of significant differences occurred when using the combined eDNA and electrofishing data and suggests the

additional biodiversity sampled with eDNA substantively contributes to the overall picture. The combined eDNA and electrofishing data identified restored sections to contain significantly greater fish species richness (ANOVA, P=0.001), intolerant species (ANOVA, P=0.019), silt intolerant species (ANOVA, P=0.001), silt tolerant species (ANOVA, P=0.003), and more native species (ANOVA, P=0.001; Table 3; Figure 5). From a phylogenetic perspective, restored sections contained significantly more species in Centrarchidae (sunfishes; ANOVA, P=0.005), Cyprinidae (minnows; ANOVA, P=0.008), Ictaluridae (catfishes; ANOVA, P=0.004), and Poecillidae (live bearers; ANOVA, P=0.032; Figure 6). Functionally, restored sections contained significantly more species of generalists (ANOVA, P=0.025), invertivores (ANOVA, P=0.016), omnivores (ANOVA, P=0.001), and top predators (ANOVA, P=0.009; Figure 7). Similar patterns existed when using exclusively electrofishing data, and more weakly, when using exclusively eDNA data (Table 3).

As with the benthic macroinvertebrates, we found no land use or restoration-specific attributes to explain the differences found between the restored and control sections (ANOVA, P>0.05)

While the electrofishing data alone was adequate to identify differences among many of the attributes measured, including eDNA resulted in differences in the structure of restored and control fish communities. The fish communities identified using only electrofishing were not different between the restored and control sections (PERMANOVA, P>0.05. However, significant differences existed when using exclusively eDNA (PERMANOVA, P=0.025) or when combining eDNA with the electrofishing data (PERMANOVA, P=0.011).

Table 3. Analysis results comparing attributes of fish biodiversity in restored sections versus their upstream unrestored controls. Results are presented for three data scenarios: 1) combined eDNA and traditional electrofishing; 2) exclusively eDNA data; and 3) exclusively traditional electrofishing data. The 'Greater' column indicates which attribute produced a greater response. All results are considered statistically significant at P<0.05 and are highlighted.

	eDNA + Physical			eDNA only			Physical only		
	F	P	Greater	F	P	Greater	F	P	Greater
Total Richness	15.8	0.001	Restored	5.8	0.024	Restored	12.7	0.003	Restored
Intolerant	6.3	0.019	Restored	2.0	0.170	Restored	5.4	0.037	Restored
Silt Intolerant	13.9	0.001	Restored	4.8	0.038	Restored	10.2	0.007	Restored
Silt Tolerant	10.5	0.003	Restored	3.7	0.065	Restored	7.4	0.018	Restored
Non Native	13.2	0.001	Restored	5.9	0.022	Restored	14.4	0.002	Restored
Anguillidae	0.0	1.000	Control	0.8	0.376	Restored	N/A	N/A	N/A
Catostomidae	2.9	0.103	Restored	4.3	0.048	Restored	1.0	0.336	Restored
Centrarchidae	9.3	0.005	Restored	2.7	0.115	Restored	4.4	0.057	Restored
Cyprinidae	8.3	0.008	Restored	1.0	0.327	Restored	9.0	0.010	Restored
Ictaluridae	9.9	0.004	Restored	8.2	0.008	Restored	3.1	0.104	Restored
Percidae	0.0	1.000	Control	0.1	0.713	Restored	0.0	1.000	Control
Poeciliidae	5.2	0.032	Restored	0.4	0.538	Restored	17.3	0.001	Restored
Salmonidae	1.0	0.327	Restored	N/A	N/A	N/A	N/A	N/A	N/A
Algivore	1.0	0.327	Restored	1.0	0.327	Restored	1.0	0.336	Restored
Filter Feeder	0.0	1.000	Control	1.0	0.327	Restored	0.0	1.000	Control
Generalist	5.7	0.025	Restored	2.3	0.143	Restored	0.7	0.435	Restored
Invertivore	6.7	0.016	Restored	4.3	0.049	Restored	6.3	0.026	Restored
Insectivore	0.0	1.000	Control	2.1	0.161	Restored	0.0	1.000	Control
Omnivore	15.7	0.001	Restored	7.1	0.013	Restored	19.2	0.001	Restored
Top Predator	7.9	0.009	Restored	0.8	0.376	Restored	1.7	0.218	Restored

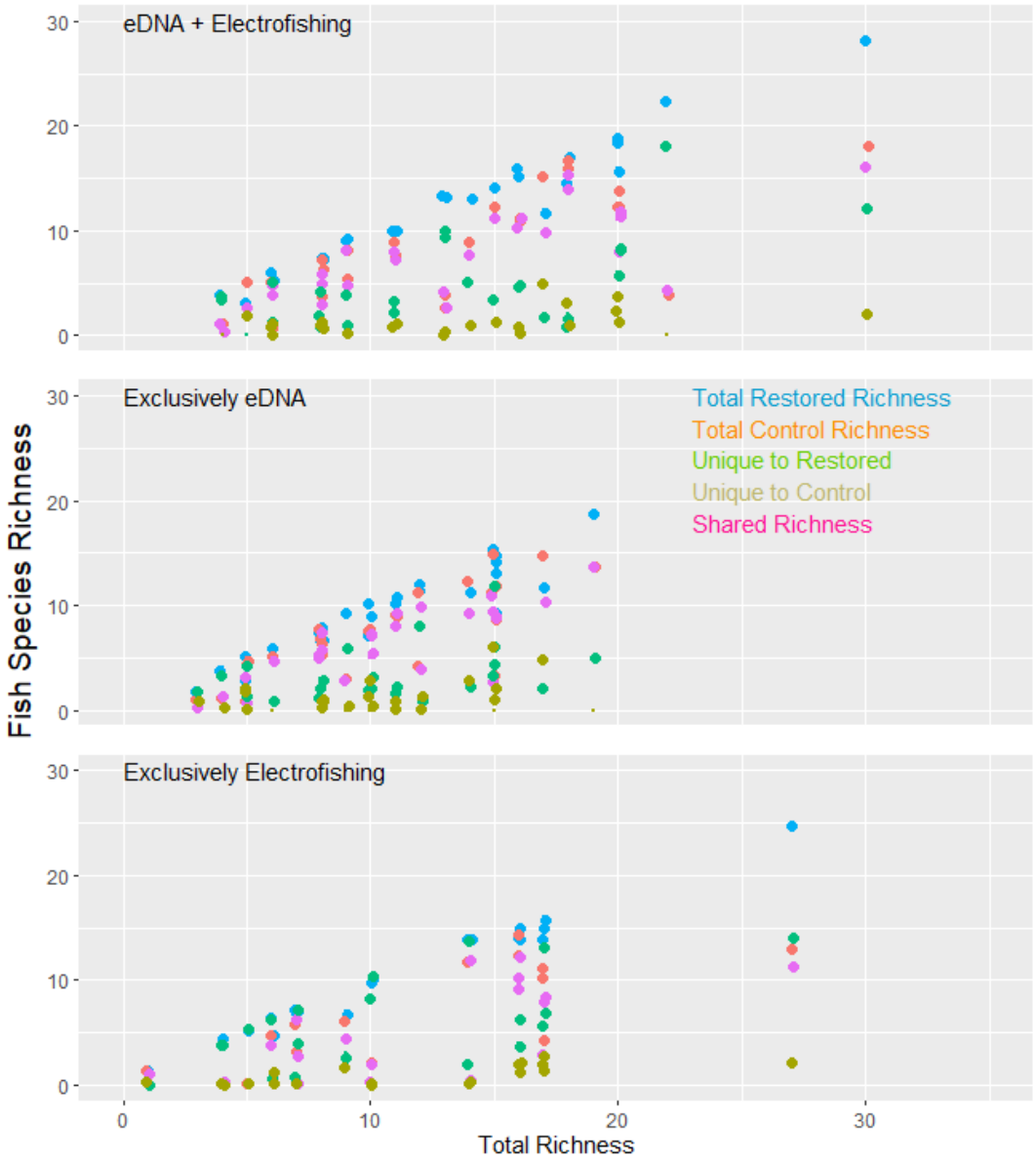

Figure 4. Plots of fish species richness for combined eDNA + electrofishing, eDNA-only, and electrofishing only data that are arranged along the total richness gradient. Each column of points represents a restored+control section pair partitioned into the taxa richness for the restored (blue) and control (orange), taxa richness unique to restored (green) and control (brown), taxa shared among the sections (pink).

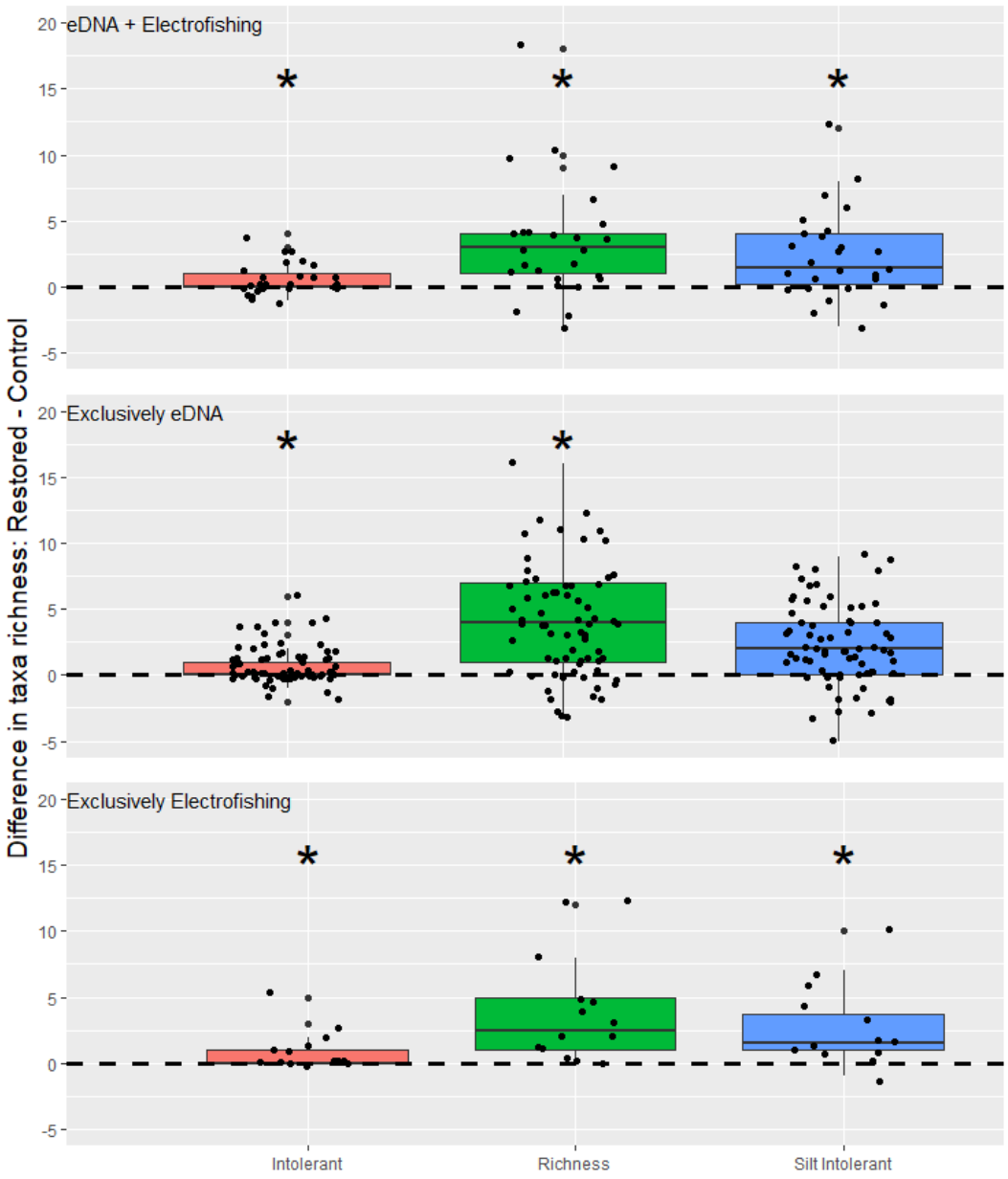

Figure 5. Differences in fish species richness for various attributes for combined eDNA + electrofishing, eDNA-only, and electrofishing only data. Points above the horizontal line at zero indicate more taxa in the restored section. Attributes with an asterisk indicate a statistically significant difference between restored and control.

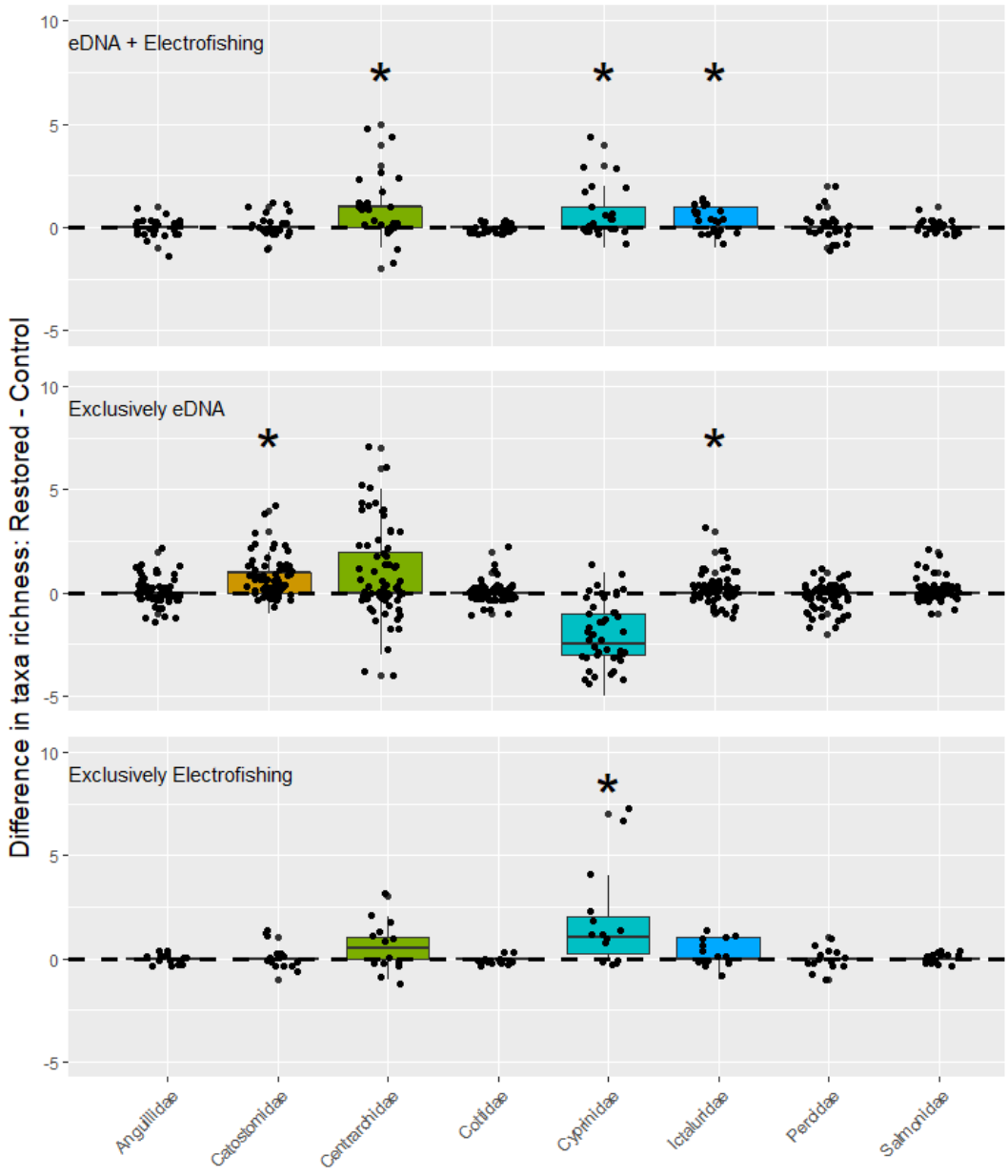

Figure 6. Differences in fish species richness for the different taxonomic Families for combined eDNA + electrofishing, eDNA-only, and electrofishing only data. Points above the horizontal line at zero indicate more taxa in the restored section. Attributes with an asterisk indicate a statistically significant difference between restored and control.

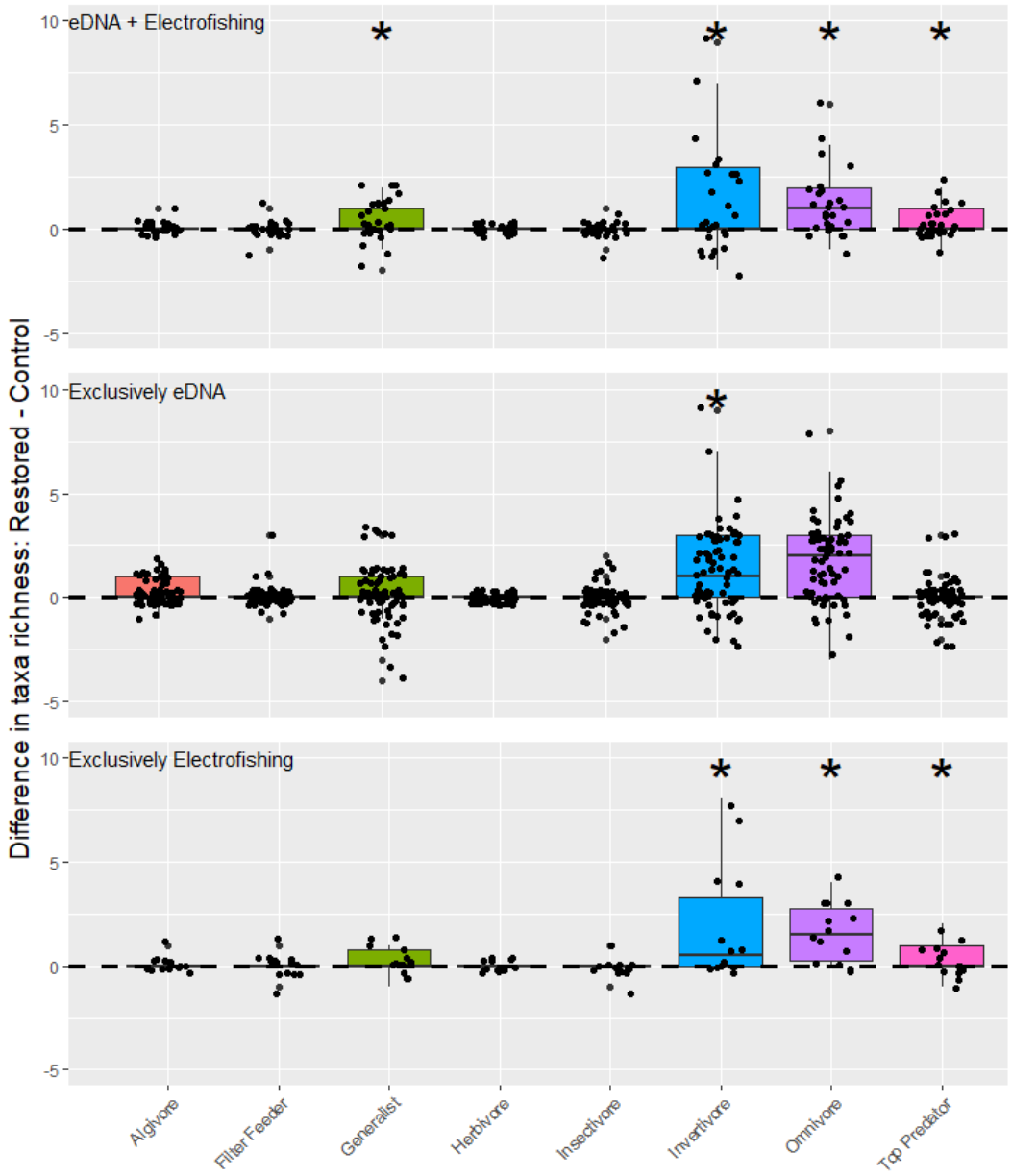

Figure 7. Differences in fish species richness for different trophic groups for combined eDNA + D-Net, eDNA-only, and D-Net only data. Points above the horizontal line at zero indicate more taxa in the restored section. Attributes with an asterisk indicate a statistically significant difference between restored and control.

eDNA identifies more biodiversity than traditional sampling for both benthic macroinvertebrates and fishes

Compared to traditional D-Net sampling, eDNA identified significantly more benthic macroinvertebrate taxa (ANOVA, $P<0.001$) and more pollution intolerant taxa at two different sensitivity cutoffs (Intolerant < 3; ANOVA, $P<0.001$, and Intolerant < 5; ANOVA, $P<0.001$) (Table 4; Figure 8) as well as greater biodiversity in the Diptera (ANOVA, $P<0.001$) and Chironomidae within the Diptera (ANOVA, $P<0.001$).

eDNA also identified significantly more taxa within the shredder (ANOVA, P<0.001) and collector (ANOVA, P<0.001) functional feeding groups. There were no community attributes where traditional D-Net sampling identified more biodiversity. The overall benthic macroinvertebrate communities described by eDNA was also significantly different from those described by D-Net sampling (PERMANOVA, P=0.001)

Table 4. Analysis results comparing eDNA against traditional D-Net sampling for various biodiversity and functional trophic attributes of benthic macroinvertebrate communities. The 'Greater' column indicates which attribute produced a greater response. All results are considered statistically significant at P<0.05 and are highlighted.

Metric	F	P	Greater
Total Richness	49.5	<0.001	eDNA
Intolerant < 3	27.3	<0.001	eDNA
Intolerant < 5	55.6	<0.001	eDNA
Diptera	47.9	<0.001	eDNA
Ephemeroptera	0.0	0.949	D-Net
EPT	0.3	0.56	eDNA
Chironomidae	39.3	<0.001	eDNA
Shredder	67.1	<0.001	eDNA
Collector	76.6	<0.001	eDNA
Predator	0.7	0.398	D-Net
Scraper	1.7	0.199	eDNA
Filterer	0.4	0.512	eDNA

Compared to electrofishing, eDNA identified significantly greater fish species richness (ANOVA, P<0.001), intolerant species (ANOVA, P<0.001), silt intolerant species (ANOVA, P<0.001), silt tolerant species (P<0.001), and native species (ANOVA, P<0.001; Table 5; Figure 9). eDNA also identified more species in the Catostomidae (suckers; ANOVA, P<0.001) and Centrarchidae (sunfishes; ANOVA, P=0.001). However, electrofishing identified significantly more species in the Cyprinidae (minnows; ANOVA, P<0.001) and Percidae (perch; ANOVA, P=0.033). From a trophic, functional perspective, eDNA identified significantly more invertivores (ANOVA, P<0.001) and omnivores (ANOVA, P<0.001) than traditional electrofishing. The overall fish communities described by eDNA was also significantly different than communities described by electrofishing (PERMANOVA, P=0.002).

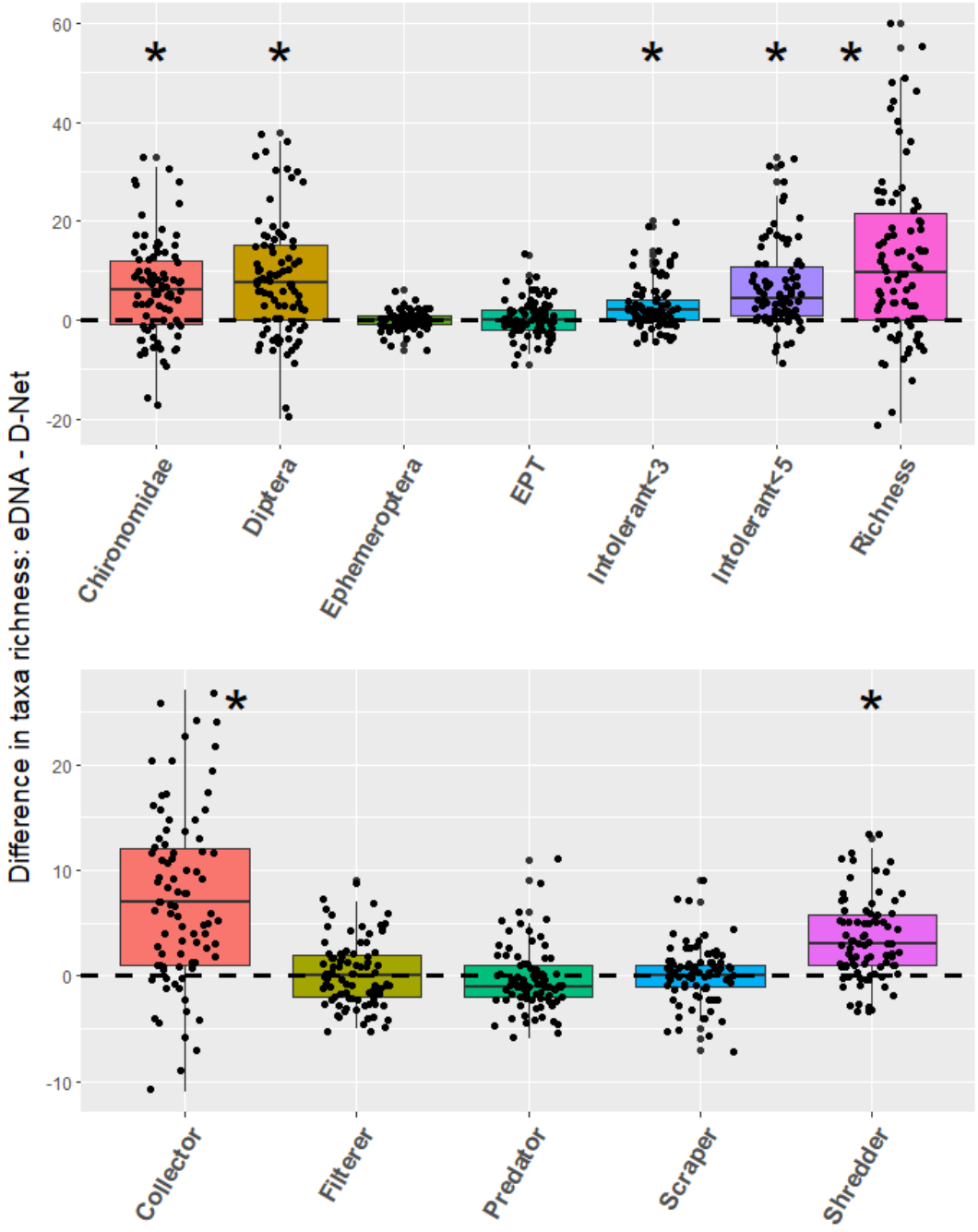

Figure 8. Differences in taxa richness comparing eDNA against D-Net sampling of benthic macroinvertebrate sensitivity, taxonomic, and trophic groups. Points above the horizontal line at zero indicate more taxa in the restored section. Attributes with an asterisk indicate a statistically significant difference between restored and control.

Table 5. Analysis results comparing eDNA against traditional electrofishing for various biodiversity and functional trophic attributes of fish communities. The 'Greater' column indicates which attribute produced a greater response. All results are considered statistically significant at P<0.05.

Metric	F	P	Greater
Total Richness	51.0	0.000	eDNA
Intolerant	19.9	0.000	eDNA
Silt Intolerant	37.0	0.000	eDNA
Silt Tolerant	26.4	0.000	eDNA
Non Native	57.5	0.000	eDNA
Anguillidae	2.7	0.103	
Catostomidae	45.5	0.000	eDNA
Centrarchidae	11.5	0.001	eDNA
Cottidae	0.1	0.784	
Cyprinidae	113.8	0.000	Electrofishing
Ictaluridae	3.7	0.057	
Percidae	4.7	0.033	Electrofishing
Poeciliidae	3.0	0.090	
Salmonidae	3.4	0.070	
Algivore	17.7	0.000	eDNA
Filter Feeder	0.7	0.409	
Generalist	1.6	0.215	
Invertivore	28.5	0.000	eDNA
Insectivore	0.2	0.658	
Omnivore	65.4	0.000	eDNA
Top Predator	0.1	0.704	

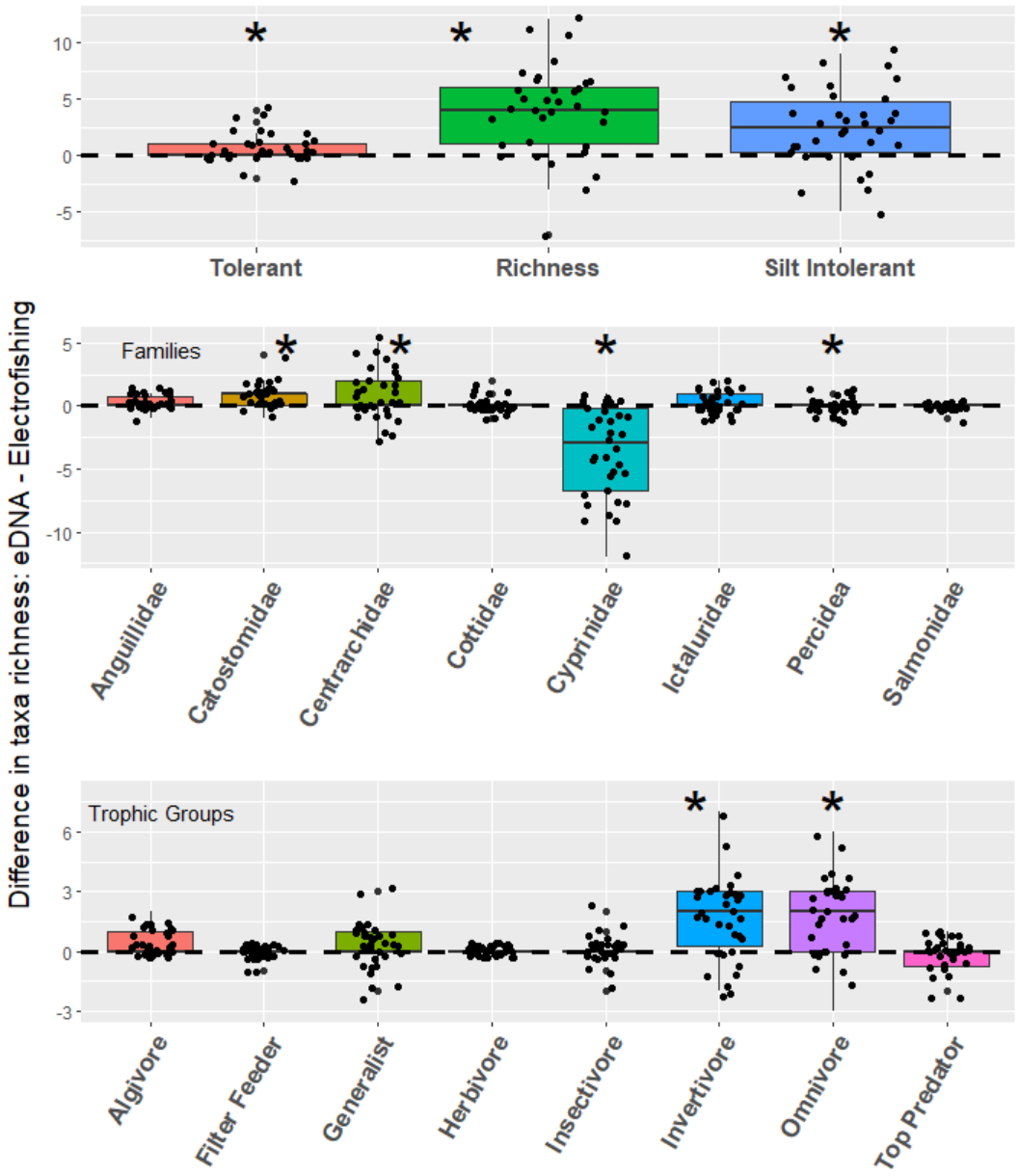

Figure 9. Differences in fish species richness comparing eDNA against electrofishing for various sensitivity, taxonomic, and trophic attributes. Points above the horizontal line at zero indicate more taxa in the restored section. Attributes with an asterisk indicate a statistically significant difference between restored and control.

Changes in the Fish Index of Biotic Integrity by including eDNA data could not be performed because too many metrics rely on biomass or numbers, and eDNA cannot be used for these purposes.

Stream sediment microbial communities could not differentiate levels of stream condition

Despite substantial efforts and multiple approaches, we were unable to generate reliable predictions of stream condition using the stream sediment communities (Table 6). Our best model using stream

sediment microbes predicted the BIBI correctly in only 30% of comparisons when allowing for a +/- 0.5 difference of Observed - Predicted. Relaxing the difference between observed and predicted to 0.75 increased the success rate to 60%, but at the expense of making the allowable interval too wide to be of use. The model had no observable bias in either over- or under-estimating stream condition (P=0.62). While some variation in the accuracy of predictions is expected, we believe the results are not particularly useful.

Table 6. Results of predicting stream condition by using the stream sediment microbial communities. Predicted and observed ratings, BIBI scores, and their differences are shown for each site. Sites without accompanying benthic macroinvertebrate samples were not included.

SITE	Predicted BIBI	Observed BIBI	Difference		SITE	Predicted BIBI	Observed BIBI	Difference
BachelorsRunControl	3	2.7	-0.3		MapleDellControl	3	3.7	0.7
BachelorsRunRestored	3	3.0	0.0		MapleDellRestored	3	2.3	-0.7
BaconRidgeLowerControl	1.5	3.9	2.4		MillCreekControl	3	3.0	0.0
BaconRidgeLowerRestored	3	3.0	0.0		MillCreekRestored	3	2.1	-0.9
BaconRidgeUpperControl	3	3.0	0.0		MinebankControl	4	2.0	-2.0
BaconRidgeUpperRestored	3	1.3	-1.7		MinebankLowerRestored	4	2.3	-1.7
BramhopeRestored	3	2.0	-1.0		MinebankRestored	1.5	3.0	1.5
CatBranchControl	3	2.4	-0.6		MuncasterMillControl	3	4.3	1.3
CatBranchRestored	1	3.0	2.0		MuncasterMillRestored	1	4.0	3.0
CowhideBranchControl	3	2.4	-0.6		PlumtreeCreekControl	3	2.3	-0.7
CowhideBranchRestored	3	2.4	-0.6		PlumtreeCreekRestored	3	2.3	-0.7
FostersBranchRestored	3	3.3	0.3		RedHillLowerRestored	3	2.3	-0.7
GreenbriarBranchControl	3	3.0	0.0		RedHillUpperRestored	3	3.0	0.0
GreenbriarBranchRestored	1	3.0	2.0		SandyBranchControl	1.5	2.0	0.5
HowardBranchControl	3	2.1	-0.9		SandyBranchRestored	3	3.3	0.3
HowardBranchRestored	3	1.6	-1.4		ShephardsCoveControl	3	1.6	-1.4
KellyBranchRestored	3	3.3	0.3		ShephardsCoveRestored	1	2.7	1.7
LittleCatoctinControl	3	2.0	-1.0		UTLGControl	3	5.0	2.0
LittleCatoctinRestored	3	1.8	-1.3		UTLGRestored	3	4.3	1.3
LittlePatuxentControl	3	2.3	-0.7		ValleyMillCreekRestored	3	1.7	-1.3
LittlePatuxentRestored	3	3.0	0.0		WatkinsControl	3	2.3	-0.7
LittleTuscaroraControl	3	2.5	-0.5		WatkinsRestored	3	2.7	-0.3
LittleTuscaroraRestored	3	2.3	-0.8		WheelCreekControl	4	2.3	-1.7
LongDraughtBranchControl	3	2.3	-0.7		WheelCreekRestored	1	2.3	1.3
LongDraughtBranchRestored	4	3.7	-0.3					

Discussion and Interpretations with Relevance to Restoration

While not a complete replacement for traditional sampling methods, including eDNA in stream restoration monitoring has distinct advantages. eDNA sampled a greater portion of the overall biodiversity of streams as well as better sampled habitats and taxa that are more difficult to sample with traditional methods. These included the more sensitive taxa. The eDNA sampling was able to differentiate differences between restored and the upstream unrestored controls when traditional monitoring methods found no differences for the benthic macroinvertebrates. Pairing eDNA with traditional monitoring data made for an even more effective description of the aquatic communities. Sampling with eDNA is also economically effective: the entire research project cost was less than the normal cost of traditional sampling for the same number of sites. We expand on each of these points below.

Including eDNA in restoration monitoring increased the sensitivity of the monitoring results and was able to identify differences between restored and control sections that were not evident with traditional monitoring methods. This was especially evident for the benthic macroinvertebrates. For example, no differences between restored and control sections were found when analyzing the D-Net sampling data for benthic macroinvertebrates. The lack of differences using traditional monitoring data is common in stream restoration studies (Stranko et al. 2012, Violin et al. 2011, Hilderbrand and Acord 2019) and has led to the conclusion that restorations are not successful. However, including eDNA suggests these conclusions require more nuance. It is clear that there are rarely large positive effects in urban stream restorations, but there appear to be smaller, positive restoration responses identified only by increasing the monitoring sensitivity provided by eDNA. These responses included greater overall biodiversity as well as greater taxonomic richness of Diptera, sensitive taxa, and the EPT. The increased sensitivity provided with eDNA also identified community-wide differences between restored and control sections for both fish and benthic macroinvertebrates.

The complementarity achieved in combining eDNA and traditional monitoring data sources may extend our understanding of restoration responses and points to new research questions. eDNA may provide insights into the degradation / recovery spectrum not captured with traditional monitoring with benthic macroinvertebrates. For example, the combined eDNA and D-Net data identified significantly more intolerant taxa and EPT taxa in restored sections compared to control sections, whereas neither data source in isolation identified any differences. The increased sensitivity of the pooled data suggests these desired, sensitive groups may be establishing in restorations, but are difficult to detect. If so, research into how to best jump start colonization or increase population numbers may be profitable.

eDNA effectively increases the sample size by providing an additional sample into the analysis. If the species-area curve has not been saturated, then we should expect additional taxa present merely by adding the additional sample regardless of it being eDNA or traditional. The more pronounced effects of eDNA seen in the macroinvertebrates is consistent with a sampling effect. While electrofishing conducts depletion sampling over the entire 75m segment, benthic macroinvertebrate sampling subsamples only a small part of the stream segment, and this subsample is further subsampled when using the MBSS laboratory protocols (Resource Assessment Service 2022). Increasing the numbers of benthic macroinvertebrates identified with D-Net sampling should increase the sensitivity. However, eDNA clearly outperformed D-Net sampling for biodiversity survey on a sample-to-sample comparison and suggests that a single eDNA sample provides substantially more information than a single D-Net sample.

In contrast to the benthic macroinvertebrates, eDNA did not appreciably alter or complement findings for fish attributes. Electrofishing data indicated restoration success for a number of attributes compared to the upstream control sections. The results suggest that eDNA is more likely equivalent to, rather than, superior to electrofishing. While including eDNA with the electrofishing data strengthened some of the relationships, eDNA data in isolation was not as effective in capturing restoration responses as the electrofishing data. This is somewhat confusing and paradoxical because eDNA outperformed electrofishing in detecting significantly more taxa at each site for most of the metrics analyzed. Despite this better on-site performance, eDNA did not adequately capture the overall fish community response. Nonetheless, if eDNA is being collected to monitor benthic macroinvertebrates or other groups, we still recommend including the primers for fish since there was no noticeable negative effects of adding the information to the electrofishing data, and additional fish species will likely be detected.

eDNA has many benefits and efficiencies compared to traditional monitoring. Sample collection for eDNA is substantially easier: a 1-2L water sample takes substantially less time to collect than a D-Net sample for benthic macroinvertebrates or the hours to electrofish a reach. eDNA samples can also be collected from bridges of other structures spanning a waterway, which could allow sampling of areas where access is limited for any number of reasons. Substantially more eDNA samples can be collected in a day by a single person than using traditional methods. An eDNA sample also has the possibility of describing the aquatic community outside of the sampling reach to provide a more integrated picture of the community. eDNA samples are also cost effective. The entire project cost (56 eDNA samples + research costs ~ $195,000) works out to roughly $3,500 per site, which is substantially lower than the cost of biological sampling using traditional methods.

Monitoring with eDNA has many demonstrated benefits, but there are several caveats relevant to our current study. Because the restored sites are downstream of the control sites, they had additional opportunity to identify organisms in the control section via DNA drifting into the restoration. In effect, restored sections were more densely sampled than the controls and should therefore have higher eDNA biodiversity. We cannot completely discount the increased sampling effect. However, eDNA samples were collected at or near the bottom of restored sections, and many of the restorations were sufficiently long to reduce the sampling effect. Another issue is the family Cyprinidae (minnows) were much better sampled with electrofishing than with eDNA. We suspect this has something to do with DNA amplification issues rather than habitat not adequately sampled. Regardless, minnows are a major constituent of most fish communities, so under-sampling with eDNA may lead to incorrect conclusions. Similarly, some taxa have genomes that don't amplify well because their higher GC (Guanine and Cytosine) content makes it more difficult for the DNA to melt at temperatures normally used for the PCR cycles (McDowell et al. 1998), and so their DNA is under-represented during sequencing. Because eDNA metabarcoding as done in this study cannot provide abundance information on the taxa present, any assessment metrics using numbers of individuals cannot be used. Thus, the fish IBI could not be reliably calculated using the eDNA data. Some claim the number of eDNA reads returned can be a proxy for relative abundance, but it is too unreliable to use for an actual assessment. All of these potential issues highlight that eDNA is not a perfect replacement for traditional monitoring methods, particularly when an assessment such as the IBI is used as legal biocriteria.

Despite our expectations of using stream sediment microbial communities as a proxy to assess stream condition, we were unsuccessful. The results were disappointing given its prior successes (Hilderbrand et al. 2020). We found no directional bias in the predictions as there were both over- and under-

estimates of condition with only about 30% accuracy. Although we spent substantial time and effort, the method did not work. Several reasons may explain the poor performance. The Hilderbrand et al. (2020) results may be an anomaly or an example of over-fitting despite the precautions employed. The results could also be time-specific where the environmental conditions during a given year or season resulted in the specific microbial communities reflecting ecological conditions. If so, then multiple communities could adequately predict ecological condition, but each year would be a special case. The more likely explanation stems from the disparity in the DNA sequencing since the original model was developed. The original model was built on 150 base pair, single end, forward read sequencing data available with the technologies in 2015. We could not replicate the same data in our current efforts despite repeated attempts at the bioinformatics, using the same databases for the original model, and working with the author of the mothur bioinformatics software package. We still believe the microbial communities can reflect a stream's overall ecological condition, but our approach was inadequate.

Summary and Next Steps

Including eDNA monitoring data can improve the overall sensitivity of the monitoring results, but it should complement rather than replace traditional methods. eDNA identified improvements in restored sections compared to their upstream controls in ways not previously identified when using traditional D-Net monitoring data for benthic macroinvertebrates. In contrast, improvements in restored sections were evident in the electrofishing data, and eDNA did little to improve the resolution. For both fish and benthic macroinvertebrates, including eDNA data resulted significant differences in the overall community structure of restored versus control communities compared to no overall differences present when using traditional monitoring methods. For both groups, eDNA identified more overall biodiversity than traditional monitoring methods and was equal to or superior for most attributes. Nonetheless, eDNA is not a replacement for traditional monitoring since it under-samples the minnows and cannot provide abundance information.

As with most research, our findings have generated additional questions to answer in order to advance the state of the science. Some of these include the following questions. Why are the minnows under-sampled with eDNA? Can eDNA information be incorporated into existing IBI approaches or developed into an entirely new assessment tool? How far upstream from a sample can eDNA be used to survey biodiversity, and do the distances change with taxonomic groups? How long will eDNA reside in a stream before degrading into something not useful for monitoring? How many traditional samples are equivalent to a single eDNA sample, and does it differ between fish and benthic macroinvertebrates?

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