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Macroinvertebrate community diversity and habitat quality relationships along a large river from targeted eDNA metabarcode assays

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Abstract

Species compositions and diversity levels of aquatic macroinvertebrate communities provide important indicators of ecosystem health. However, such community analyses typically are limited by time and effort of sampling, sorting, and identifications, as well as morphological character uncertainty for some taxa, especially at early life stages. Our objective was to evaluate a suite of targeted metabarcode high-throughput sequencing assays to characterize the macroinvertebrate communities (specifically targeting Annelida, Bryozoa, Crustacea, Insecta, and Mollusca) from environmental (e)DNA water samples along 160 km of the Maumee River, OH (a major Lake Erie, Laurentian Great Lakes tributary). Multiple alpha (richness, Shannon Index, and Simpson's Index) and beta (Bray–Curtis and Sørensen dissimilarities) diversity metrics from the metabarcode assays were compared with an Invertebrate Community Index (ICI) metric calculated from traditional morphological sampling surveys conducted by the Ohio Environmental Protection Agency. The 15 Maumee River sites varied in their ICI scores (ranging from 4 to 46), with seven sites rated as "poor" or "very poor" and eight scoring "fair" or "good." Metabarcoding assays yielded greater gamma richness, delineating 181 Operational Taxonomic Units versus 172 taxa from morphology (the latter often limited to family or genus-level identifications). Both datasets supported similar river-wide trends, with comparable gamma, alpha, and beta diversity patterns and community compositions across habitat types and habitat quality scores. Metabarcode assays revealed similar detection of important bioindicator Insecta, but missed most Trichoptera (caddisflies). eDNA identified eight aquatic invasive species on the GLANSIS (Great Lakes Aquatic Nonindigenous Species Information System) list, including three missed by the morphological surveys (*Branchiura sowerbyi*, *Potamothrix bedoti*, and *Skistodiaptomus pallidus*; with *Lophopodella carteri*, *Faxonius rusticus*, *Corbicula fluminea*, *Dreissena polymorpha*, and *Valvata piscinalis* in both datasets). Findings illustrate the utility of eDNA sampling

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and targeted metabarcode assays to enhance and complement environmental assessments of aquatic ecosystems.

KEYWORDS

aquatic insects, biodiversity assessment, environmental DNA, Great Lakes, high-throughput sequencing, invasive species, Lake Erie, Maumee River

1 | INTRODUCTION

1.1 | eDNA and metabarcoding in environmental assessments

Biodiversity assessment and ecosystem monitoring require accurate measures of community composition to address environmental comparisons and identify negative anthropogenic impacts (Hillebrand et al., 2018; US Environmental Protection Agency, 2011). In aquatic systems, macroinvertebrates commonly comprise a focal group for biomonitoring, due to their high diversity and range of responses to environmental conditions (Menezes, Baird, & Soares, 2010; Resh, Norris, & Barbour, 1995). However, current biomonitoring methods often have coarse taxonomic resolution leading to false negatives for rare or cryptic macroinvertebrate taxa (Haase et al., 2006; Pfrender et al., 2010) and frequently miss important differences between closely related species (Macher et al., 2016). Conventional routine monitoring with morphological surveys relies on time-consuming collection and sample sorting steps, and often has inconsistencies in taxonomic accuracy and resolution (i.e., species-level; Hajibabaei, Shokralla, Zhou, Singer, & Baird, 2011; Jackson et al., 2014).

Over the past two decades, "barcoding" identification of individual morphological specimens with DNA sequences (see Hebert, Cywinska, Ball, & deWaard, 2003) has increased taxonomic diagnosis (Hajibabaei et al., 2011; Radulovici, Archambault, & Dufresne, 2010). When used with an accurate reference sequence database, DNA barcode identifications can significantly aid monitoring and management programs by identifying morphologically difficult, rare, invasive, and/or cryptic species (Pilgrim et al., 2011). This can be especially useful for early life stages that lack diagnostic morphological characters. For example, DNA barcode sequences can be employed to identify indicator species (including macroinvertebrates) for monitoring applications (Baird & Hajibabaei, 2012; Lejzerowicz et al., 2015; Sweeney, Battle, Jackson, & Dapkey, 2011). However, traditional DNA barcoding requires the sampling, sorting, and separation of each individual organism (Orlofske & Baird, 2014).

A newer alternative approach is DNA metabarcoding (here termed META) and accompanying bioinformatics, which employ amplicon-based high-throughput sequencing (HTS) to simultaneously identify and distinguish multiple individuals in samples (Taberlet, Coissac, Pompanon, Brochmann, & Willerslev, 2012). Notably, META of bulk invertebrate samples can yield cost-effective and quicker taxonomic identifications (Baird & Hajibabaei, 2012; Carew, Pettigrove, Metzeling, & Hoffmann, 2013; Emilson et al., 2017; Yu et al., 2012), along with increased taxonomic coverage and resolution (Carew, Kellar, Pettigrove, & Hoffmann, 2018; Elbrecht, Vamos, Meissner, Aroviita, & Leese, 2017; Pfrender et al., 2010; Soininen et al., 2015). META of environmental (e)DNA in water samples (i.e., genetic material from urine, waste, mucus, or sloughed cells) from aquatic habitats can be used to detect multiple taxa, elucidating much or most of an ecosystem's biodiversity (Cristescu, 2014; Fernández, Rodríguez-Martínez, Martínez, Garcia-Vazquez, & Ardura, 2019; Shokralla, Spall, Gibson, & Hajibabaei, 2012).

Although many studies have used eDNA water samples to assess the presence/absence of important bioindicator species (Czechowski, Stevens, Madden, & Weinstein, 2020; Deiner, Fronhofer, Mächler, Walser, & Altermatt, 2016; Macher et al., 2016; Mächler et al., 2019), water samples may discern less diversity than direct analyses of bulk samples of organisms, which contain their entire bodies or pieces, such as appendages (e.g., collected by kick-net sampling, benthic grabs, sieves, or plankton nets; Hajibabaei et al., 2019; Macher et al., 2018). Moreover, eDNA frequently is distributed ephemerally and unevenly in the water column (Macher & Leese, 2017), which can affect chances of detecting traces of larger organisms, such as macroinvertebrates and fishes. Water samples also contain entire microorganisms, whose DNA often dominates the eDNA pool, resulting in swamping of HTS reads when non-specific primers are used (Collins et al., 2019; Elbrecht & Leese, 2015).

Other challenges of META include uncertain quantification due to primer bias and lack of comprehensive sequence databases for taxon identifications (Cristescu & Hebert, 2018; Dowle, Pochon, Banks, Shearer, & Wood, 2016; Hering et al., 2018; Macher & Leese, 2017); the latter also is true of traditional barcoding (Ekrem, Willassen, & Stur, 2007; Moritz & Cicero, 2004). Despite such factors, eDNA META analyses indicate strong potential as a complementary monitoring tool. Yet, studies that compare results from eDNA analyses using META and traditional morphological surveys (here termed MORPH) are rare for macroinvertebrate communities.

1.2 | Comparing MORPH and META along a large river

The present investigation evaluates the use of multiple targeted META assays and accompanying bioinformatics on eDNA water samples to characterize the macroinvertebrate community across a large river, in comparison with MORPH surveys and analyses conducted by the Ohio Environmental Protection Agency (OEPA,

https://www.epa.state.oh.us/). The OEPA regularly evaluates in-state rivers to determine whether designation uses (e.g., dams and water plants) are meeting the goals of the U.S. Federal Clean Water Act (33 U.S.C. 1251–1376, Chapter 758, P.L. 845, June 30, 1948, 62 Stat. 1155). In 2012, the OEPA sampled 23 locations in the Maumee River, OH—a major tributary of Lake Erie in the Laurentian Great Lakes—for aquatic life and habitat quality (OEPA, 2014; Figure 1). They assessed the macroinvertebrate communities using the Invertebrate Community Index (ICI), which ranks habitat quality from 1–60 in classifications of Very Poor (1–11), Poor (12–24), Fair (25–41), Good (42–51), and Excellent (52–60; OEPA, 2015). In brief, the ICI employs a 10 metric system, based on the diversity and relative abundances of pollutant intolerant taxa (e.g., Ephemeroptera (mayflies), Plecoptera (stoneflies), and Trichoptera (caddisflies), and pollutant tolerant taxa (e.g., Chironomidae ("bloodworms"), and annelid worms; DeShon, 1995).

The OEPA (2014) classifies aquatic habitat regions of the Maumee River as either warm water habitat (WWH) or modified warm water habitat (MWH), and further as free-flowing riverine habitat, modified impoundment habitat, or low-flow lacustrine habitat (with the latter located in close proximity to Lake Erie). These habitat classification criteria are used by the OEPA to evaluate attainment status of a location or river system, by comparing the biological indices to a predetermined biocriteria value. According to the OEPA's 2012 Maumee River status report, 11 of the 12 free-flowing riverine locations were in full attainment (ICI > 34), while all five lacustrine and six impoundment locations had ICI scores <25, indicating poor quality habitat (OEPA, 2014). The Maumee River macroinvertebrate monitoring revealed significant decline in community condition, as the average ICI score from free-flowing riverine locations decreased from 52.2 in 1997 to 37.1 in 2012 (29% reduction in habitat quality over 15 years; OEPA, 2014). These management findings demonstrate the necessity for continued monitoring and assessment of human impacted environments, for which eDNA META assays could constitute a powerful evaluation tool in tandem with their MORPH assessments.

In the present study, water samples were collected by the OEPA from 15 sites throughout ~160 river kilometers (km) of the Maumee River, where the OEPA simultaneously conducted their traditional MORPH quantitative and qualitative macroinvertebrate survey (detailed above). We employed a suite of META assays to target taxonomic groups of the aquatic macroinvertebrate community and compared gamma, alpha, and beta diversity measures between our approach and the OEPA's survey. We hypothesized that eDNA META would (1) generate higher taxonomic richness by alleviating difficulties of MORPH identification, providing greater resolution, (2) reveal comparable diversity trends along the river, and (3) increase detection of aquatic invasive species (AIS), in comparison with MORPH. The aim is that our META methodology and results will be useful for future macroinvertebrate community and habitat assessments.

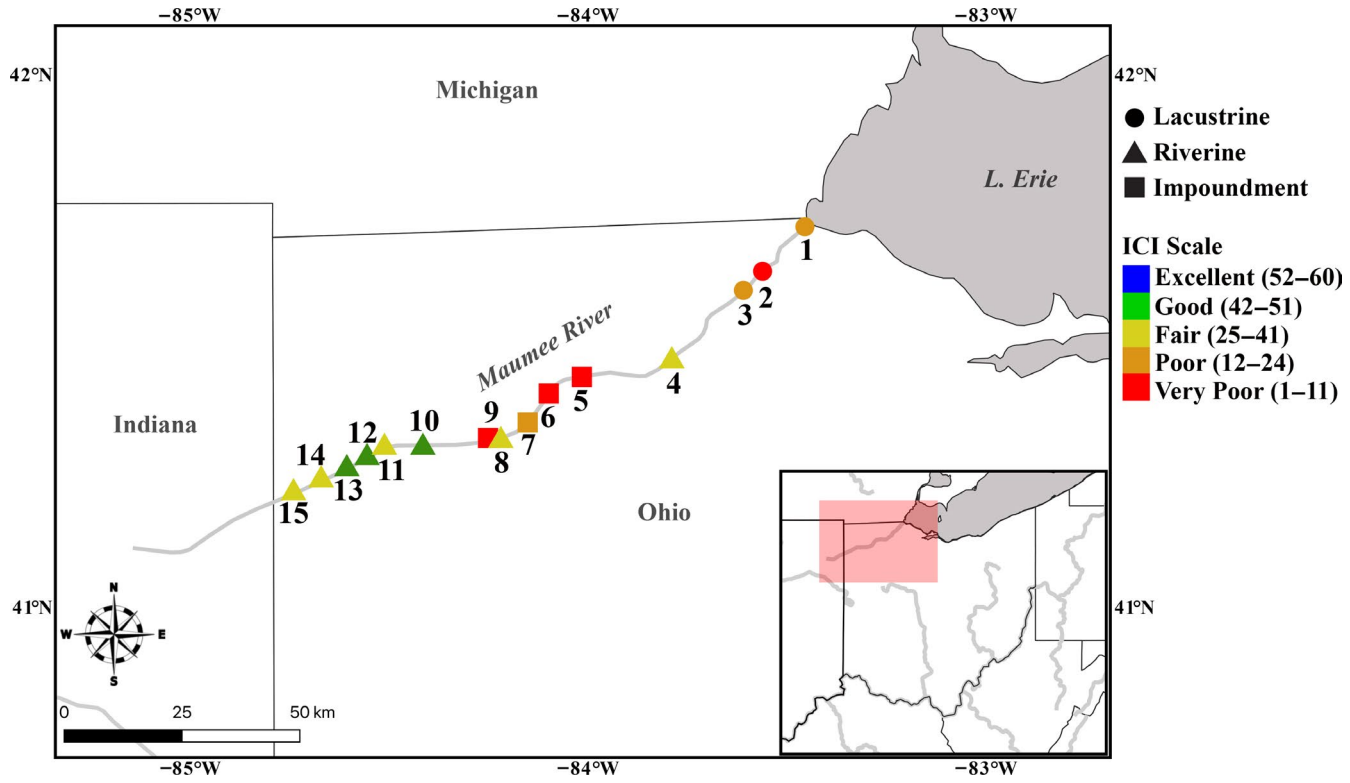

FIGURE 1 Sites 1–15 along the Maumee River, Ohio, where eDNA water samples were collected in correspondence to a quantitative and qualitative macroinvertebrate survey, conducted by the Ohio Environmental Protection Agency during August 2012. Colors correspond to habitat quality calculated by an Invertebrate Community Index (ICI) rating

2 | METHODS

2.1 | Sampling and DNA extraction

The OEPA sampled the macroinvertebrate assemblages at 23 sites along a ~160 river km stretch of the Maumee River, OH, from 0.80 river km (near the mouth of Lake Erie; 41.69N, −83.47W) to 158.40 river km (near the Indiana–Ohio border; 41.18N, −84.73W) using their standard quantitative and qualitative sampling methods in August 2012 (OEPA, 2014, 2015; Table 1; Figure 1). In short, standard EPA quantitative sampling consists of placing five eight-plated Hester-Dendy artificial substrate samples within each site for ≥6 weeks, accompanied by qualitative evaluations that entailed collections with a dip net and visual inspection of coarse substrate (OEPA, 2014, 2015). Macroinvertebrates sampled by the OEPA were identified by them following their standard methods, with some taxa limited to family or genus-level classifications (OEPA, 2014, 2015).

Just prior to their sampling, the OEPA collected 1 L water samples from 10 cm below the surface in sterilized bleach-washed Nalgene containers at 15 sites. The labeled containers were placed on ice in a designated clean cooler and returned within 1–3 hr to the Stepien laboratory, where they were stored at −80°C. In our laboratory, DNA from three of the 15 samples was extracted and processed during an earlier study (Klymus, Marshall, & Stepien, 2017), for which 500 ml was filtered through a polyethersulfone (PES) membrane using a vacuum pump, and DNA from the filter was extracted using a cetyl trimethyl ammonium bromide (CTAB) protocol. For the remaining 12 samples, 500 ml was pelleted in 10 sterile 50 ml falcon tubes at 7,500 *g* centrifugation for 30 min following Marshall and Stepien (2019). Resultant pellets from each single sample were combined in a 1.5-ml tube and extracted using the Qiagen DNeasy® Blood and Tissue Kit (Qiagen Inc.). The DNA was processed with a Zymo Research One Step PCR Inhibitor Removal kit (Zymo Research). A negative control of 250 ml deionized water was simultaneously extracted to test for possible laboratory contamination.

2.2 | MiSeq HTS library preparation

Three META markers targeting the mtDNA 16S gene, here designated as (a) Copepod (Clarke, Beard, Swadling, & Deagle, 2017), (b) Insect (Epp et al., 2012), and (c) Mollusk (Klymus et al., 2017), were used to analyze the macroinvertebrate communities from the 15 sample sites. Primers for each were attached to a spacer region, which increased library nucleotide (nt) diversity of sequence reads and enhanced cluster formation, improving sequencing quality (Klymus et al., 2017; Marshall & Stepien, 2019). The same two-step PCR library preparation was used for all markers: the 1st PCR included 1× PCR buffer, 0.3 mM dNTPs, 0.5 μM of each primer, an additional 1.5 mM $MgCl_2$, 5 U AllTaq® (Qiagen), 5 μl template DNA, and ddH_2O to total 50 μl. Conditions were 2 min initial denaturation at 95°C, followed by 40 cycles of 95°C for 5 s, T_A for 15 s, and 72°C for 10 s, with no final extension. First-step PCR products were processed with a 0.7× HighPrep™ bead clean-up (MagBio Genomics, kit/AC60050), yielding the template for the 2nd step. The 2nd PCR incorporated Nextera paired-end indices (Illumina, kit FC-121-1011), *p5*/*p7* adaptor sequences to allow the sample to bind onto the Illumina MiSeq flow cell, and contained eight bases that allowed samples to be identified in the raw read data. Each sample for each marker on the two MiSeq runs had a unique index pair. This final 25 μl reaction contained 1× PCR buffer, 0.2 mM dNTPs, 2.5 μl of each primer, 1.57 U NEB Hotstart Taq polymerase (New England

TABLE 1 Sites in the Maumee River, Ohio, where eDNA water samples were collected in August 2012, alongside a companion macroinvertebrate Invertebrate Community Index (ICI) survey by the Ohio Environmental Protection Agency, showing locations, habitat types, ICI scores, and habitat qualities

Site #	River km	Lat	Long	Habitat type	ICI score	Habitat quality
1	0.80	41.6942	−83.4667	Lacustrine	18	Poor
2	15.04	41.6089	−83.5794	Lacustrine	06	Very poor
3	21.28	41.5704	−83.6245	Lacustrine	12	Poor
4	42.72	41.4481	−83.7858	Riverine	30	Fair
5	65.92	41.4114	−84.0328	Impoundment	04	Very poor
6	75.36	41.3838	−84.1301	Impoundment	10	Very poor
7	83.36	41.3276	−84.1526	Impoundment	14	Poor
8	92.96	41.2906	−84.2444	Riverine	36	Fair
9	96.64	41.2914	−84.2819	Impoundment	10	Very poor
10	110.40	41.2842	−84.4344	Riverine	42	Good
11	121.76	41.2753	−84.5150	Riverine	40	Fair
12	128.16	41.2625	−84.5611	Riverine	42	Good
13	136.48	41.2378	−84.6022	Riverine	46	Good
14	146.40	41.2219	−84.6697	Riverine	38	Fair
15	158.40	41.1839	−84.7325	Riverine	38	Fair

Note: ICI ranges from 1–60 (Very Poor (1–11), Poor (12–24), Fair (25–41), Good (42–51), and Excellent (52–60)).

Biolabs® Inc.), 2.5 μl from the previous PCR cleanup, and ddH_2O to total 25 μl. Conditions were 30 s initial denaturation at 95°C, followed by eight cycles at 95°C for 30 s, 55°C for 30 s, and 68°C for 1 min, with a final 2 min 68°C extension.

PCR products were sized and quantified on a 2100 Bioanalyzer (Agilent Technologies) prior to Illumina® MiSeq analysis conducted at the Ohio State University's Molecular and Cellular Imaging Center in Wooster, OH (http://mcic.osu.edu/). To avoid sequencing dimer products, targeted fragments were size-selected with a 1.5% agarose gel cassette on Pippin Prep (Sage Science). Samples from each primer set were individually pooled based on equimolar concentrations. These primer set pools then were combined by the sequencing center, based on the expected fragment size and product concentrations from a Qubit fluorometer (Invitrogen), and the final pools were sequenced with 2× 300 nt V3 Illumina MiSeq chemistry. An additional 40%–50% PhiX DNA spike-in control was added to improve data quality of low nt diversity samples. A PCR no-template negative control was run for each library preparation step and checked for possible contamination using gel electrophoresis, with the final step checked on the 2100 Bioanalyzer to verify the absence of product. No contamination was found in any PCR during library preparation. As negative PCR and extraction controls are highly unlikely to yield no sequencing results using HTS, we did not run our negative controls on the MiSeq, since gel electrophoresis results showed no amplification and no contamination. Samples were sequenced on two MiSeq runs, with the Insect marker on run 1, and the Copepod and Mollusk markers on run 2.

2.3 | Bioinformatic analyses

MiSeq results were independently processed for each marker after demultiplexing each index combination. The primers and spacer regions were removed using a custom Perl script (https://github.com/ntmarshall406/HTS-Metabarcdoing-Primer-Trimming-Scripts). This step also removed any reads that had the wrong spacer region, which might result from index-hopping (MacConaill et al., 2018). Using the denoising *R* package DADA2 (Callahan et al., 2016), reads were filtered and trimmed using a "maxEE" of five for the reverse and forward reads, and trimmed to a "truncLen" for each marker (Table S1). Using DADA2, error rates were estimated, sequences were merged and dereplicated into Amplicon Sequence Variants (ASVs), and any erroneous or chimeric sequences were removed.

Unique ASVs then were clustered into Operational Taxonomic Units (OTUs) using QIIME's *pick_de_novo_otus* python workflow script, with the default 97% similarity threshold and UCLUST option (Caporaso et al., 2010; Edgar, 2010). OTUs were identified to broad taxonomic level using the Basic Local Alignment Search Tool (BLAST +, https://blast.ncbi.nlm.nih.gov/Blast.cgi, Camacho et al., 2009) against the NIH NCBI GenBank database (https://www.ncbi.nlm.nih.gov/GenBank). Due to the absence of some macroinvertebrate mt16S rDNA taxa reference sequences in published databases (see Trebitz et al., 2017), those OTUs often could not be resolved to species-level (e.g., just 26% of OTUs had >97% sequence identity). Thus, we retained OTUs having >80% identity and >90% query coverage, which matched sequences of Annelida, Bryozoa, Crustacea, Insecta, or Mollusca in the NCBI database, with Insecta OTUs identified to the order-level. We considered this broad phylum/subphylum classification a conservative approach, since other research found that >80% identity matches yielded accurate class-level identifications (Cawthorn, Steinman, & Witthuhn, 2012). Additionally, any OTU having >99% percent identity and >99% query coverage to an AIS on the NOAA Great Lakes Aquatic Nonndigenous Species Information System (GLANSIS; NOAA, 2019) was scored as detected.

2.4 | Statistical analyses

For both the MORPH and META datasets, diversity measures were calculated for the macroinvertebrate community of taxa from Annelida, Bryozoa, Crustacea, Insecta, and Mollusca. The MORPH taxa dataset is a combination of family, genus, and species-level classifications, based on OEPA macroinvertebrate identification guidelines (OEPA, 2015). MORPH data matrices were rarefied to the sampling site having the lowest abundance (Site 13—3,154 individuals), to equalize the number of individuals. For META data, Copepod, Insect, and Mollusk OTUs were independently rarefied to the sample having their respective lowest read count (Copepod: Site 11—36,773 reads; Insect: Site 8—18,003 reads; Mollusk: Site 8—41,696 reads). Next, community diversity analyses used the Crustacea OTUs from the Copepod marker, the Annelida, Bryozoa, and Mollusca OTUs from the Mollusk marker, and the Insecta OTUs from the Insect marker. Since each marker did not have similar sequencing depth across macroinvertebrate OTUs, read counts were not considered representative of abundances among different markers. Instead, for abundance-based community analyses, proportions of reads within each marker were used to normalize the data for abundance estimates. Linear regression analyses compared the numbers of taxa or OTUs within each major taxonomic grouping resolved by MORPH and/or META. For both datasets, diversity measures were compared for (a) habitat type (i.e., Lacustrine vs. Impoundment vs. Riverine) and (b) quality of habitat (i.e., grouped by OEPA-calculated ICI scores corresponding to Very Poor/Poor vs. Fair/Good).

Gamma diversity was calculated as MORPH taxa or META OTU richness across the entire Maumee River, as well as compared for habitat type and habitat quality. Diversity further was evaluated within-communities (alpha diversity) and between communities (beta diversity) for both datasets. Alpha metrics were conducted in *R* (R Core Team, 2017) with the "vegan" package (Oksanen et al., 2019) and included taxon/OTU richness, Shannon Index (Sh-I), and Simpson's Index (Si-I). Within each dataset, possible differences in diversity values among habitat types and habitat qualities were analyzed with *t* tests and ANOVA (analysis of variance), using Tukey's HSD post hoc tests. Regression analyses tested possible correlations of the alpha diversity matrices between the two datasets, and their correlations

with the OEPA-calculated ICI scores. Non-phylogenetic beta diversities were estimated using (a) a qualitative metric (based on presence/absence)—Sørensen dissimilarity (SD) and (b) a quantitative metric (based on rarified abundances for MORPH, and rarified proportion of reads for META)—Bray–Curtis dissimilarity (B-C) with the *R* package "betapart" (Baselga, Orme, Villeger, De Bortoli, & Leprieur, 2017). The latter partitioned beta diversity into a nestedness component (i.e., an abundance gradient) and a turnover component (i.e., balanced variation in species abundances; Baselga et al., 2017).

Mantel (1967) tests with 999 permutations assessed correlations between the MORPH and META datasets, using Non-metric Multidimensional Scaling (NMDS; Kruskal, 1964) to visualize beta diversity values, and PerMANOVAs (Anderson, 2014; vegan function *adonis2*) to test for possible differences between groups. In the latter, homogeneity of multivariate dispersions was calculated using the *betadisper* function and comparisons were statistically tested with ANOVA. Procrustes analyses (Gower, 1975) within vegan were used to determine correlations between ordinations of the datasets. Diversity measures in relation to river distances were examined using linear regression of the alpha diversity metrics versus river km, and with Mantel tests of 999 permutations comparing beta diversities versus Euclidean river km distances.

3 | RESULTS

3.1 | HTS raw reads and taxonomic comparisons

All 15 samples amplified successfully using all three markers (Table S2). These totaled 5,282,031 reads overall (mean per sample = 117,378.5 ± 14,075.0, Table S2), of which 3,947,989 (75%) contained the correct primers and spacer regions, and were longer than possible primer dimer lengths (mean correct per sample = 87,733.1 ± 8,610.6, Table S2). DADA2 merged a mean proportion of 0.83 ± 0.01 reads per sample, averaging 32.49 ± 3.17 OTUs. The Copepod marker revealed 92 macroinvertebrate OTUs (26 Crustacea OTUs), the Insect marker 93 (78 Insecta OTUs), and the Mollusk marker 97 (36 Annelida, 18 Bryozoa, and 23 Mollusca OTUs; Table S3).

Gamma diversity values across the Maumee River were based on 172 macroinvertebrate taxa from MORPH (ranging from family to species-level designations) compared to 181 OTUs from META (Table 2a). For both datasets, the riverine habitats possessed the greatest richness among all habitat types (Table 2b), and habitats ranked Fair/Good in quality contained greater richness than those designated as Very Poor/Poor (Table 2c). Broad taxonomic rank orders were similar between MORPH and META results (linear regression–R^2 = .88, p = .012*). The sole significant trend in broad taxonomic richness was for Insecta (linear regression—R^2 = .68, p = <.001***; Figure S1). MORPH discerned more taxa of Insecta and Mollusca, while META identified more Annelida, Bryozoa, and Crustacea (Figure 2a).

There was high within-order richness correlation along the Maumee River between the two datasets (linear regression—R^2 = .65, p = .002**; evident in the Insecta order heat map; Figure 2b).

MORPH and META results each identified eight AIS species listed by GLANSIS; however, the latter identified more occurrences (21 vs. 30, respectively). Of these, five species were discerned by both methods (i.e., the freshwater bryozoan *Lophopodella carteri* (Hyatt, 1865), rusty crayfish *Faxonius rusticus* (Girard, 1852), Asian clam *Corbicula fluminea* (Müller, 1774), zebra mussel *Dreissena polymorpha* (Pallas, 1771), and valve snail *Valvata piscinalis* (Müller, 1774)). Three species were uniquely identified with MORPH (the freshwater amphipod *Gammarus fasciatus* Say, 1818, faucet snail *Bithynia tentaculata* (Linnaeus, 1758), and mystery snail *Cipangopaludina japonica* (von Martens, 1861)), and three were unique to META (the tubificid worms *Branchiura sowerbyi* Beddard, 1892 and *Potamothrix bedoti* (Piguet, 1913), and the calanoid copepod *Skistodiaptomus pallidus* (Herrick, 1879), Figure 3). META found the same GLANSIS species in seven of the 21 MORPH occurrences (33.3%; Figure 3).

3.2 | Alpha diversity measures

For MORPH and META results, Lacustrine versus Riverine sites and Impoundment versus Riverine sites significantly differed in alpha diversity measures (except for MORPH Simpson's Index and META richness; Table 3a). There was not a difference between Lacustrine

TABLE 2 Gamma diversity, represented as species richness, from morphological surveys (MORPH) and eDNA metabarcoding (META) across the entire Maumee River (a), different habitat types (b), and varying quality of habitats (c)

(a) Entire river			
MORPH	172		
META	181		
(b) Habitat type	**Lacustrine**	**Impoundment**	**Riverine**
MORPH	46	70	126
META	45	43	158
(c) Habitat quality	**Very poor/poor**	**Fair/good**	
MORPH	95	126	
META	68	158	

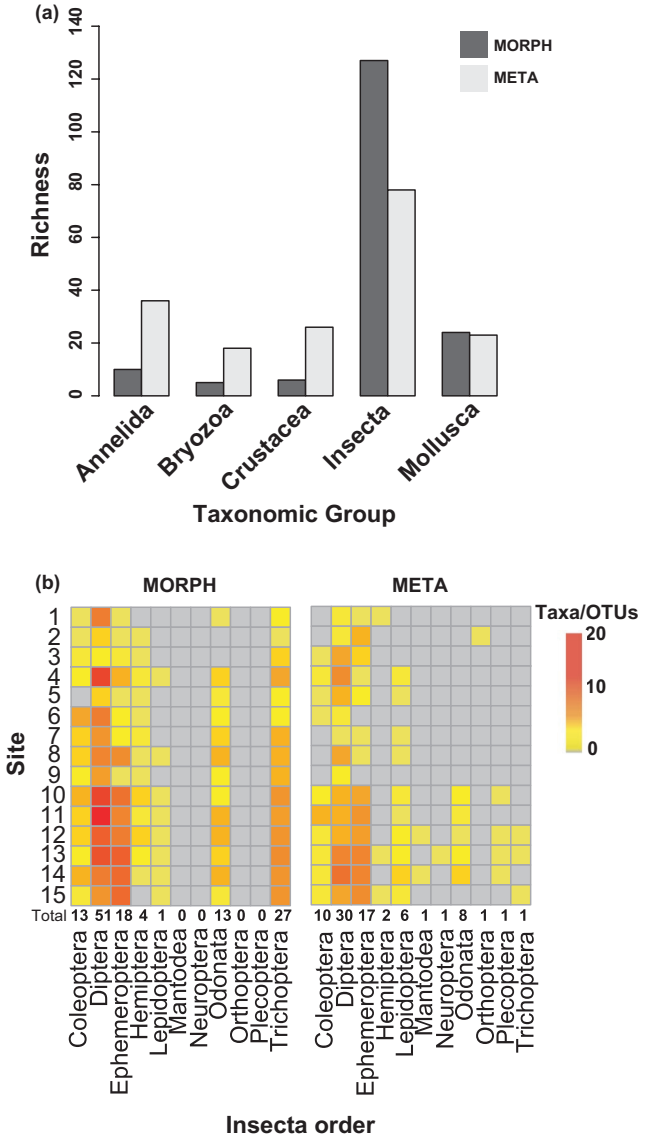

FIGURE 2 Taxon/OTU richness for the five target macroinvertebrate taxonomic groups identified by the quantitative and qualitative morphology survey (MORPH) or from eDNA metabarcoding (META; a). Comparative richness for Insecta orders across the Maumee River sites using MORPH or eDNA META analyses (colored according to legend; b). Gray cells indicate no detection (i.e., apparent taxon absence)

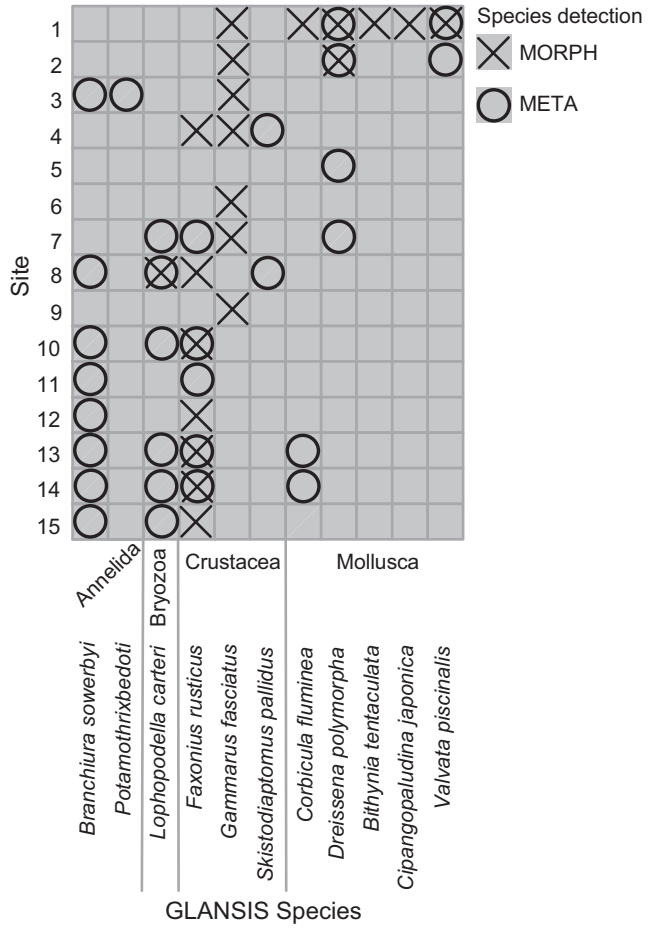

FIGURE 3 Aquatic Invasive Species (AIS; GLANSIS) detected in Maumee River sites by the quantitative and qualitative morphology survey (MORPH) and eDNA metabarcoding (META). Empty cells indicate no detection (i.e., apparent species absence) by either method

versus Impoundment sites in alpha diversity. Habitat quality (i.e., Very Poor/Poor vs. Fair/Good ICI scores) also significantly differed using all three alpha diversity metrics in both datasets (Table 3b). Regression analyses indicated significant relationships to ICI scores with both datasets (MORPH: Richness—R^2 = .84, p < .001***; Sh-I—R^2 = .54, p = .001**; Si-I—R^2 = .36, p = .01*; META: Richness—R^2 = .66, p < .001***; Sh-I—R^2 = .77, p < .001***; Si-I—R^2 = .65, p < .001***; Figure 4a,b). Taxonomic richness was the sole alpha diversity measure that displayed correlation between the two datasets (Richness—R^2 = .69, p < .001*** (Figure 4c); Sh-I–R^2 = .19, p = NS; Si-I—R^2 = .09, p = NS).

3.3 | Beta diversity measures and environmental factors

MORPH discerned significant community differences among the three types of habitats with SD (R^2 = .64, p < .001*** [Figure 5a]), and B-C dissimilarity (R^2 = .65, p < .001*** [Figure S2a]), and for those having different habitat quality rankings (SD: R^2 = .53, p = .002** [Figure 5c]; B-C: R^2 = .61, p < .001*** [Figure S2c]). META revealed congruent findings, including among habitat types (SD: R^2 = .34, p < .001*** [Figure 5b]; B-C: R^2 = .38, p = .002** [Figure S2b]) and their qualities (SD: R^2 = .24, p = .002** [Figure 5d]; B-C: R^2 = .21, p < .001*** [Figure S2d]). For SD and B-C in both methods, the turnover component had greater contribution to the dissimilarity than did a nestedness-related component, which was more pronounced in MORPH than META (Figures S3 and S4).

Distribution patterns of pairwise beta diversities were similar in both datasets for habitat type (Figure S5) and habitat quality (Figure S6). Furthermore, beta diversity values were highly correlated between MORPH and META, showing congruent trends

TABLE 3 Mean alpha diversity metrics (taxa/OTU richness, Shannon Index (Sh-I), and Simpson's Index (Si-I)), for the morphological (MORPH) and eDNA metabarcoding (META) datasets grouped by habitat type (i.e., Lacustrine vs. Impoundment vs. Riverine; a), and habitat quality (i.e., Very Poor/Poor vs. Fair/Good; b)

(a) Habitat type					*p*-value		
Diversity measure	**Dataset**	**Lacustrine**	**Impoundment**	**Riverine**	**L versus I**	**L versus R**	**I versus R**
Richness	MORPH	14.33	16.00	31.38	NS	.001**	.001**
	META	20.33	14.5	50.00	NS	NS	.01*
Sh-I	MORPH	0.69	1.22	1.88	NS	.001**	.03*
	META	1.72	1.55	2.61	NS	.005**	<.001***
Si-I	MORPH	0.30	0.55	0.71	NS	.002**	NS
	META	0.77	0.73	0.88	NS	.04*	.002**

(b) Habitat quality Diversity measure	Dataset	Very poor/poor	Fair/good	*p*-value
Richness	MORPH	15.29	31.38	<.001***
	META	17.00	50.00	.003**
Sh-I	MORPH	0.99	1.88	<.001***
	META	1.62	2.61	<.001***
Si-I	MORPH	0.45	0.71	.009**
	META	0.75	0.88	.003**

Note: Significance between groups calculated with ANOVA and Tukey's HSD post hoc tests (a), and Student's *t* tests (b). (NS = $p > .05$, * = .01, ** = .001, *** < .001).

for explaining differences among sites (SD: $R^2 = .72$, $p < .001$***; B-C: $R^2 = .38$, $p < .001$***). NMDS plots also revealed significant similarity of the two datasets with Procrustes tests and ordinations (SD Correlation = 0.81, $p < .001$***; B-C Correlation = 0.61, $p = .004$**).

3.4 | Diversity in relation to geographic distance

For both datasets, alpha diversities displayed a strong relationship to river km distances among sites (MORPH: Richness—$R^2 = .40$, $p = .007$**; Sh-I—$R^2 = .27$, $p = .03$*; Si-I—$R^2 = .22$, $p = .04$*; META: Richness—$R^2 = .43$, $p = .005$**; Sh-I—$R^2 = .36$, $p = .01$*; Si-I—$R^2 = .17$, p = NS). Likewise, beta diversities showed correlation to Euclidean river km (MORPH: SD—$R^2 = .61$, $p < .001$***; B-C—$R^2 = .50$, $p = .005$**; META: SD—$R^2 = .40$, $p = .004$**; B-C—$R^2 = .57$, $p < .001$***; Figures S3 and S4).

4 | DISCUSSION

Our findings demonstrate the use and application of targeted eDNA META analyses for discerning river-wide trends in aquatic macroinvertebrate community compositions. META results show significant relationships to habitat type and habitat quality, which are congruent with conventional sampling and MORPH. As META costs continue to decrease, this approach displays strong potential to complement and improve effort, time, and output from existing environmental monitoring programs. Our research furthers prior studies that used eDNA to evaluate differences in macro-eukaryotic community compositions among habitats (Deiner et al., 2016; Fernández et al., 2018) and to examine seasonal changes in communities (Bagley et al., 2019; Bista et al., 2017; Macher & Leese, 2017).

4.1 | Taxonomic resolution and diversity trends (Hypotheses 1 and 2)

In support of Hypothesis 1, we obtained higher gamma richness with META due to finer and increased taxonomic resolution. These results are similar to findings by Cowart et al. (2015) and Serrana, Miyake, Gamboa, and Watanabe (2019). Moreover, our eDNA META dataset discerned a wide realm of taxa, potentially including identifications of smaller organisms and planktonic invertebrates (e.g., *S. pallidus*), which would have been missed by the OEPA's benthic surveys. It is possible that META data might overestimate diversity of some taxa, such as Bryozoa, since the 18 OTUs that we recovered were more than half of the known freshwater bryozoan richness in all of North America (Massard & Geimer, 2008). However, many aquatic invertebrate taxa are considerably under-studied, possessing undetected cryptic species (see Witt, Threloff, & Hebert, 2006) and/or substantial intraspecific variation (Meier, Zhang, & Ali, 2008; Vivien, Wyler, Lafont, & Pawlowski, 2015). This may explain the high number of Bryzoa OTUs found here.

Supporting Hypothesis 2, the MORPH and META datasets both discerned significantly greater diversity in the riverine habitats versus the lacustrine and impoundment habitats, with no significant differences between the latter two. Additionally, higher quality ICI

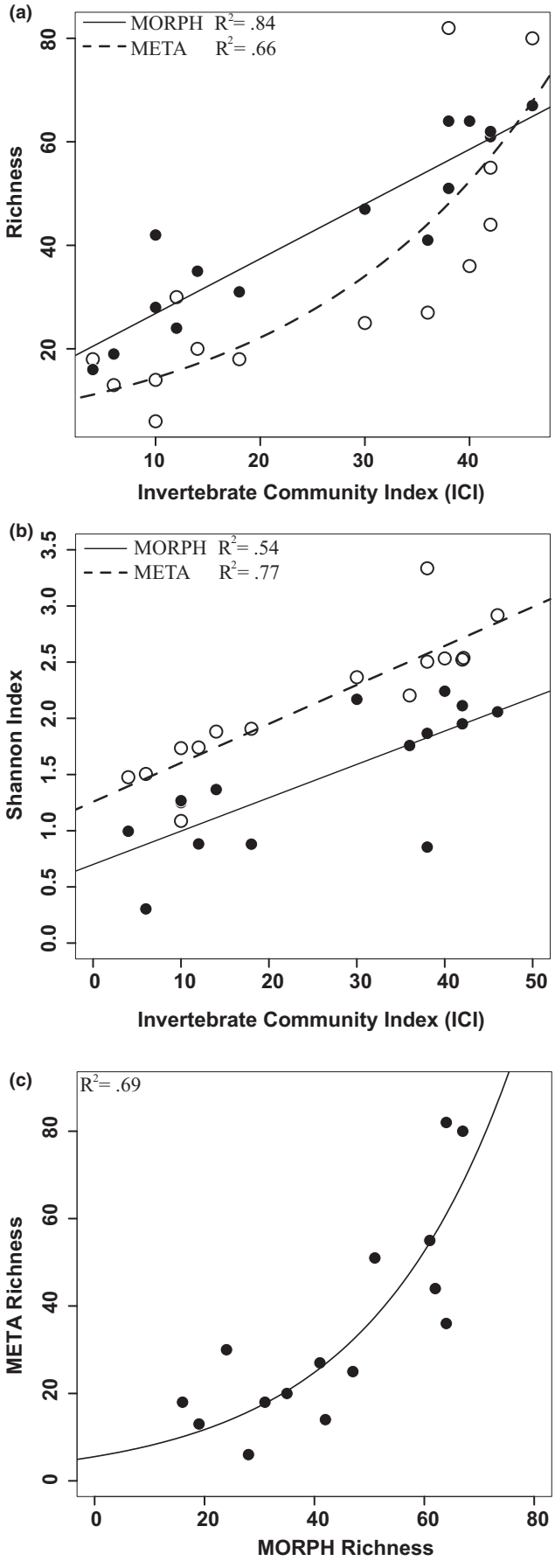

FIGURE 4 Diversity measures from morphology (MORPH) and eDNA metabarcoding (META) assessment for 15 sites along the Maumee River, displaying relationships between taxa/OTU richness versus OEPA-calculated Invertebrate Community Index (ICI) (a), Shannon Index versus ICI (b), and taxa/OTU richness (c)

habitats (Fair/Good ICI sites) supported significantly greater diversity than the lower quality habitats (Very Poor/Poor ICI sites). In the present investigation, it is difficult to separate the relative importance of habitat type versus habitat quality, since the lacustrine and impoundment sites contained all of the poor and very poor habitats. However, both MORPH and META successfully differentiated high-quality, free-flowing water sites from low-quality, stillwater sites.

Furthermore, significant correlations in both beta diversity estimates indicate that interpretations of community compositions were congruent between the MORPH and META approaches. Similarly, Gibson et al. (2015) accurately estimated site- and regional-level macroinvertebrate benthic biodiversity in the aquatic Canadian Peace-Athabasca river delta region, using a META approach on bulk samples. In the present study, we expanded upon previous META approaches by comparing traditional surveys with an eDNA targeted META approach. On a broad taxonomic scale, our eDNA META results were similar to the MORPH analyses at the phyla/subphyla levels, and yielded similar resolution for orders of Insecta. However, MORPH detected a much greater number of Trichoptera (caddisfly) taxa, suggesting need for a targeted tricopteran assay and further research that calibrates the amounts of eDNA that they shed. Our examination of the 16S rDNA region, including 160 Trichoptera unique sequences available on GenBank, found little to no differences in primer binding regions (including no differences in the final 10 nt), indicating no apparent primer inefficiencies (see Wilcox et al., 2013). Although Trichoptera DNA has been amplified from bulk macroinvertebrate benthic grab samples using META (Hajibabaei et al., 2019; Hajibabaei, Spall, Shokralla, & van Konynenburg, 2012), similar inefficiencies were found for their detection with META COI markers from eDNA water samples (Hajibabaei et al., 2019; Mächler, Deiner, Steinmann, & Altermatt, 2014). Many tripcopteran species live in cases constructed of various materials, which may reduce their eDNA shed into the water column (Hajibabaei et al., 2019). However, Mächler et al. (2019) used eDNA water and a META COI assay to detect most of the Trichoptera captured with a kicknet in Switzerland. Thus, there might be differences in DNA shedding among various trichopteran species, related to their different types of encasements. A quantitative (q)PCR analysis targeting Trichoptera in laboratory mesocosms could provide valuable information about their eDNA recovery from water.

Comparable to results of Deiner et al. (2016) and Fernández et al. (2018), we discerned eDNA from terrestrial taxa, such as the Insecta Orthoptera (e.g., grasshoppers, crickets, katykids) and Mantodea (mantises), and several livestock species. A DNA signal from livestock species was expected, since there are an estimated 775 hog, cattle, poultry, and dairy operations along the Maumee

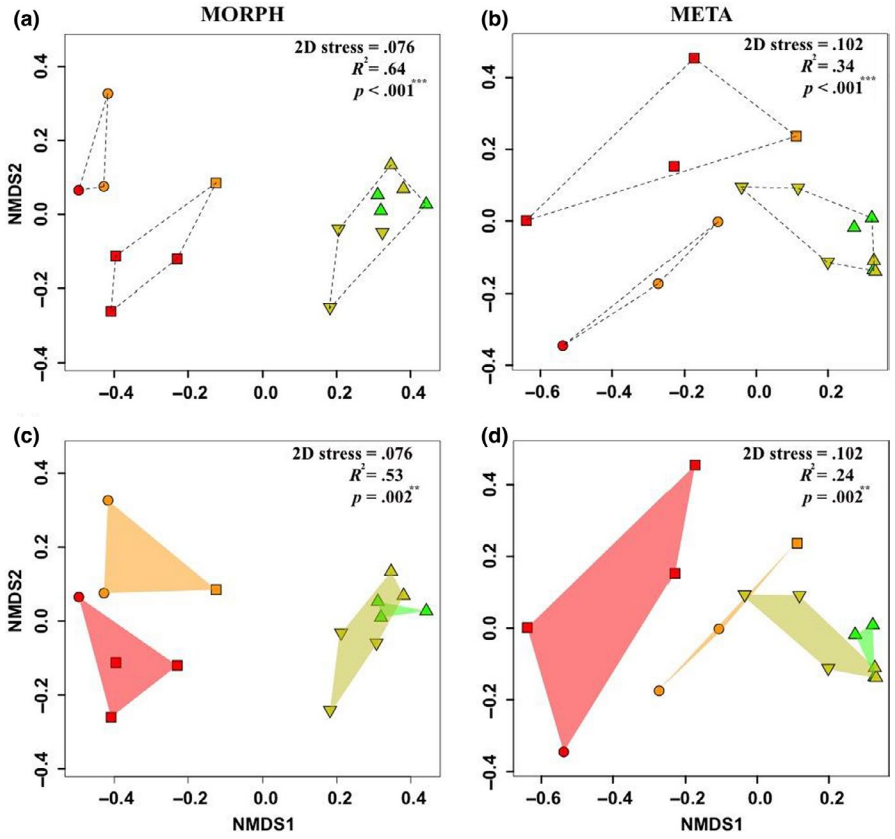

FIGURE 5 Non-Metric Multidimensional Scaling (NMDS) plots of Sørensen distance metric of the presence/absence data for: morphology (MORPH; a, c) and eDNA metabarcoding (META; b, d). Sites are clustered based on habitat type (a, b) and Ohio Environmental Protection Agency Invertebrate Community Index (ICI) habitat quality (c, d). Circles = Lacustrine, Squares = Impoundment, and Triangles = Riverine habitat. Red = Very Poor, Orange = Poor, Yellow = Fair, and Green = Good ICI habitat quality. Inverted triangles represent eDNA extraction from previous study by our laboratory (Klymus et al., 2017) [Colour figure can be viewed at wileyonlinelibrary.com]

River watershed (EWG, 2019). Therefore, a well-developed sequence reference database is necessary to accurately assess and eliminate eDNA of terrestrial origin, when those species are not the target for detection. Due to the limited taxonomic assignments obtained, largely due to lack of reference species' sequences on GenBank, we could not compute a comparable ICI index from META-identified taxa. However, our META alpha diversities displayed a strong correlation with ICI scores, suggesting that eDNA META can be used to produce a comparable biotic index. In accordance, other studies found high correspondence between biotic indices inferred from META and MORPH approaches (Fernández et al., 2019; Lejzerowicz et al., 2015; Pawlowski et al., 2018).

Targeted META has shown great promise in resolving fish diversity from eDNA water samples, often correlating well with traditional fish sampling counts (Civade et al., 2016; Gillet et al., 2018; Hänfling et al., 2016; Shaw et al., 2016; Thomsen et al., 2016). Further development of sequence reference libraries (e.g., NCBI GenBank and Barcode of Life Data System (BOLD)) is needed alongside META assay design, to propel this methodology into a practical biomonitoring tool for macroinvertebrates (Cordier et al., 2017; Ekrem et al., 2007; Zimmermann et al., 2014). Additionally, future work should explore the capability of META for quantitative biomonitoring assessments, since differences in PCR primer compatibility and varying biological traits (e.g., eDNA shedding, taxon, organism size and life stage) may affect correlations between the number of HTS reads and the abundance or biomass of a species (Clarke et al., 2017; Elbrecht & Leese, 2015; Zhou et al., 2013). Use of internal standards for quantification analysis of target sequences (see Pierce, Willey, Crawford, et al., 2013; Pierce, Willey, Palsule, et al., 2013) could lead to better abundance estimates, facilitating comparisons of HTS read counts among samples for community analyses (Hardwick et al., 2018; Ushio et al., 2018).

Other investigations have employed META on bulk benthic grab or sieved samples of macroinvertebrates, yielding stronger signal and greater resolution than from eDNA water samples (Carew et al., 2013, 2018; Gibson et al., 2015; Hajibabaei et al., 2011; Serrana et al., 2019). In water samples, swamping of HTS reads from non-target taxonomic groups may reduce signal if general or "universal" primers are used (Collins et al., 2019). For example, low target taxa amplifications affected studies by Deiner et al. (2016; only ~3% of reads were metazoans), Macher and Leese (2017; <20% of OTUs were metazoans), Macher et al. (2018; 21% of OTUs were metazoans), Bagley et al. (2019; ~8% of OTUs were metazoans), and Hajibabaei et al. (2019; ~9% of reads were arthropods). Most sequence reads from these studies matched stramenopiles (heterokonts; Protista), a non-target taxonomic group. Likewise, preliminary tests of a META COI primer (BF1/BR2; Elbrecht & Leese, 2015) on a subset of our samples, yielded few reads from macroinvertebrates (16% of OTUs and 15% of sequence reads), which instead similarly were dominated by stramenopiles (52% of OTUs and 67% of reads). This is an inherent problem with eDNA water samples and the use of general primers, since the proportion of reads from macroinvertebrates often is rare in comparison to numerous small micro-taxa found free-floating in the water column (Collins et al., 2019; Cristescu & Hebert, 2018; Macher & Leese, 2017). Although river-wide community composition trends may be comparable between micro- and macro-eukaryotic

organisms within an ecosystem (Bagley et al., 2019), inclusion of microfauna OTUs might lead to habitat assessment misinterpretation.

Our multi-assay META analyses for taxon-specific 16S regions yielded a much greater proportion of reads from target macroinvertebrate groups (73% of classified OTUs for Copepod (48% of reads), 60% of classified OTUs for Mollusk (43% of reads), and 93% of classified OTUs for Insect (95% of reads). Further development of taxon-specific META primers would greatly improve these results, as the markers used here were not specifically designed for the aquatic macroinvertebrate groups (i.e., the Copepod marker was not designed for all Crustacea, the Insect marker was designed for terrestrial Coleoptera (beetles), and the Mollusk marker amplifies a wide range of non-target taxa). Additionally, the use of blocking primers can significantly decrease non-target amplification and should be incorporated into META analyses when using degenerate or non-specific markers (Klymus et al., 2017). A further benefit of targeted META is the inclusion of population-genetic information for closely related species (Elbrecht, Vamos, Steinke, & Leese, 2018; Marshall & Stepien, 2019; Parsons, Everett, Dahlheim, & Park, 2018; Stepien, Snyder, & Elz, 2019).

4.2 | Application for AIS detection and monitoring (Hypothesis 3)

Our META and MORPH datasets both identified most of the AIS species found by Ram, Banno, Gala, Gizicki, and Kashian (2014) in their benthic sampling of the Maumee River and accompanying individual specimen barcoding. An exception was that our META missed the faucet snail *B. tentaculata* (here solely found at the mouth of the Maumee River by the OEPA), and neither MORPH nor META identified the chironomid *Lipiniella* sp. (which may or may not have been present at the time). Since GenBank lacked 16S reference sequences for *Lipiniella* spp., it was not identifiable with our META markers. Many Diptera spp. were detected by our META 16S data (30 OTUs), and a more complete reference sequence database is needed to accurately identify them to species-level. All other AIS GLANSIS species detected in our study have been reported from Lake Erie or nearby waters (USGS, 2019).

As anticipated in Hypothesis 3, META resulted in more detections of AIS across the Maumee River than the MORPH data, agreeing on only 33% of detections. Thus, both approaches display advantages and biases for detecting AIS taxa. For example, PCR interference and/or sequencing primer biases, plus the lack of reference sequences, may lead to false negatives for META (Clarke et al., 2017; Elbrecht & Leese, 2015; Zhou et al., 2013). Additionally, MORPH surveys often fail to sample and distinguish taxa due to low population numbers, crypsis, and/or capture method biases (Darling & Mahon, 2011). MORPH identifications also are limited by lack of distinctive morphological characters among related taxa, especially at egg and larval stages, and require taxonomic expertise. Since these biases can lead to differences in detection probability, a complete management approach for AIS early diagnosis and monitoring should incorporate both META and MORPH, dependent on the system and/or taxa of interest.

4.3 | Methodology uncertainties and suggestions

The range of sites along the Maumee River displayed relatively low community heterogeneity, with proximate sites containing similar communities. The extent of eDNA transport along lotic systems is uncertain (Barnes & Turner, 2016), with some studies reporting up to 20 km (Deiner & Altermatt, 2014; Pont et al., 2018), which is more than double the mean distance between our study sites. Thus, eDNA transport might limit beta diversity measures along a heterogeneous system, where large community changes occur over small distances. Here, eDNA movement might explain why community differences appeared more pronounced with the MORPH data, which could influence interpretations of META results.

Our analysis of some samples from a previous study that used a different extraction method could have led to varying quantities and differential false negatives (per Cristescu & Hebert, 2018; Deiner, Walser, Mächler, & Altermatt, 2015). However, this does not appear to be an issue here, as there was no difference in the riverine alpha or beta diversity measures between the two extraction protocols. As META studies become more prevalent, management and monitoring programs may alleviate such issues by implementing standardized sampling methodologies (e.g., sample replicates) and laboratory protocols (e.g., extraction and HTS library preparation; see Cristescu & Hebert, 2018).

5 | CONCLUSIONS

Our findings show that with proper marker design and implementation, eDNA META and accompanying bioinformatics uncover macroinvertebrate biodiversity and community trends across a large river, offering a complementary biomonitoring tool to traditional MORPH surveys. Since it is not practical for management agencies to conduct annual monitoring and habitat assessments for all streams and rivers, significant changes in habitat quality might go unnoticed for many years. For example, the average ICI score in the Maumee River displayed a 29% reduction in habitat quality over 15 years (mean ICI 52.2 in 1997 and 37.1 in 2012; OEPA, 2014). Incorporating eDNA META in biomonitoring programs can provide valuable biodiversity assessment and macroinvertebrate community specifics during non-sampling years and/or seasons. Our eDNA META methodology has the capability to monitor changes in biodiversity measures (alpha diversity) and elucidate shifts in community composition (beta diversity) across broad geographic and temporal scales. We agree with Bagley et al. (2019), who argued that eDNA META should supplement traditional MORPH monitoring programs, rather than replace them. The present multi-targeted taxa-specific META approach shows broad potential application to improve bioassessments by scientists and managers. In concert with MORPH,

META analyses will advance the ability to document community compositions and habitat relationships, in reference to environmental alterations.

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AUTHOR CONTRIBUTIONS

C.A.S. conceived the study, wrote the grants, and obtained the samples and the funding. N.T.M. conducted the laboratory analyses and developed the figures and tables. Data analysis, manuscript drafting, and manuscript revising and editing were done together by N.T.M. and C.A.S.

DATA AVAILABILITY STATEMENT

Raw sequence data are provided in the NCBI GenBank repository under BioProject PRJNA600479 with accession numbers SAMN13825690–SAMN13825734. Analysis scripts are available on https://github.com/ntmarshall406/HTS-Metabarcoding-Primer-Trimming-Scripts.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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