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ARTICLE

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Zeta diversity patterns in metabarcoded lotic algal assemblages as a tool for bioassessment

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Abstract

Assessments of the ecological health of algal assemblages in streams typically focus on measures of their local diversity and classify individuals by morphotaxonomy. Such assemblages are often connected through various ecological processes, such as dispersal, and may be more accurately assessed as components of regional-, rather than local-scale assemblages. With recent declines in the costs of sequencing and computation, it has also become increasingly feasible to use metabarcoding to more accurately classify algal species and perform regional-scale bioassessments. Recently, zeta diversity has been explored as a novel method of constructing regional bioassessments for groups of streams. Here, we model the use of zeta diversity to investigate whether stream health can be determined by the landscape diversity of algal assemblages. We also compare the use of DNA metabarcoding and morphotaxonomy classifications in these zeta diversity-based bioassessments of regional stream health. From 96 stream samples in California, we used various orders of zeta diversity to construct models of biotic integrity for multiple assemblages of diatoms, as well as hybrid assemblages of diatoms in combination with soft-bodied algae, using taxonomy data generated with both DNA sequencing as well as traditional morphotaxonomic approaches. We compared our ability to evaluate the ecological health of streams with the performance of multiple algal indices of biological condition. Our zeta diversity-based models of regional biotic integrity were more strongly correlated with existing indices for algal assemblages classified using metabarcoding compared to morphotaxonomy. Metabarcoding for diatoms and hybrid algal assemblages involved rbcL and 18S V9 primers, respectively. Importantly, we also found that these algal assemblages, independent of the classification method, are more likely to be assembled under a process of niche differentiation rather than stochastically. Taken together, these results suggest the potential for zeta diversity patterns of algal assemblages classified using metabarcoding to inform stream bioassessments.

KEYWORDS
algae, biotic integrity, diatoms, diversity indices, eDNA, environmental DNA, landscape diversity, watershed health, zeta diversity

INTRODUCTION

Biological assemblages in streams provide a variety of ecosystem services such as breaking down organic matter in runoff (Schäfer et al., 2012), sustaining the health of fisheries (Colvin et al., 2019), and regulating the formation of algal blooms (Castilla et al., 2015). Quantification of the condition of these streams has often utilized comparisons of the compositions of biological assemblages at locations of interest against their expected composition without the effects of human activities (Abbasi & Abbasi, 2011; Danielson et al., 2011). These bioassessments have often utilized measures of their alpha diversity, such as species and functional group richness, in order to quantify the ecological impacts of human activities (Hitt & Angermeir, 2011; Mazor et al., 2016; Xu et al., 2021). One such set of assemblages used in the creation of bioassessments of stream conditions is benthic algae.

A particular region of interest in assessing the impacts of human activities is the State of California, USA which covers a wide variety of ecological and anthropogenic gradients (Ode et al., 2016; Mazor et al., 2016), across a near-continental scale landscape. In California, stream biotic integrity is assessed using algal assemblages to calculate the Algal Stream Condition Index (ASCI; Theroux et al., 2020), a multimetric index that scores sites based on their deviation from reference (minimally impacted) expectation. This index can be used to assess biological integrity via diatoms communities in the phylum Bacillariophyta (D_ASCI), as well as hybrid assemblages of diatoms and soft-bodied algae including cyanobacteria and other eukaryote species in the phyla Charophyta, Chlorophyta, Rhodophyta, Ochrophyta, Miozoa, Euglenozoa, Heterokontophyta, and Cryptophyta (H_ASCI). The ASCI indices allow for the assessment of algal communities on a site-by-site basis. However, algal assemblages are connected through mechanisms such as dispersal, and may be more accurately assessed as components of regional-scale assemblages (Grönroos et al., 2013; Socolar et al., 2016; Vanschoenwinkel et al., 2007). As a result, it may therefore be more accurate to pair both local and regional measures of diversity in assessing the biotic integrity of algal assemblages in streams as means of performing bioassessments (Heino, 2013; Socolar et al., 2016).

To assess potential relationships between environmental gradients and multiassemblage diversity, several studies have used beta or "landscape" diversity (i.e., shared species diversity between sites) (Gál et al., 2019; Ruhí et al., 2017). Although beta diversity is a well established ecological concept, it is somewhat limited in describing large numbers of assemblages. First, as beta diversity involves only a pairwise comparison of interassemblage diversity it can only fully quantify diversity patterns concurrently across two assemblages (Chao et al., 2008). Second, although various measures of average beta diversity have been developed to account for regional trends, they are potentially biased toward the presence of rare organisms found only in a few sampled assemblages (Beck et al., 2013; Latombe et al., 2017). These limitations necessitated the recent development of a new ecological measure, called zeta diversity, to expand upon and complement alpha and beta diversity (Figure 1).

Zeta diversity, a generalized extension of alpha and beta diversity, was developed to describe the number of unique organisms held in common between two or more assemblages (Hui & McGeoch, 2014). In general, zeta diversity, denoted as ζ_N, quantifies the average number of unique organisms held in common between N assemblages. By simultaneously comparing the compositions of arbitrarily large groups of assemblages, zeta diversity can capture regional trends in the gain or loss of both widespread and rare taxa, whereas beta diversity tends to be biased toward changes in the presence of rare taxa (Latombe et al., 2017). There is the well established practice of using changes in populations of widely distributed organisms to capture the effects of environmental degradation at individual locations (Baker et al., 2019; Gaston & Fuller, 2008; Pond, 2012), and the role of environmental gradients and spatial separation in influencing variations in the diversity of stream assemblages across a landscape (Heino, 2013; Patrick & Swan, 2011). Investigations of the roles of environmental gradients in shaping spatial patterns of biodiversity have been carried out using zeta diversity (Latombe et al., 2017; McGeoch et al., 2019). Further investigations using zeta diversity have been applied to assessing the ecological condition of streams on a regional basis, where changes in patterns of both local and landscape diversity were strongly associated with regional ecological degradation (Simons et al., 2019).

The use of zeta diversity can also help to investigate other questions relevant to the use of biological assemblages to track the effects of environmental changes, such as determining the relative likelihoods for different

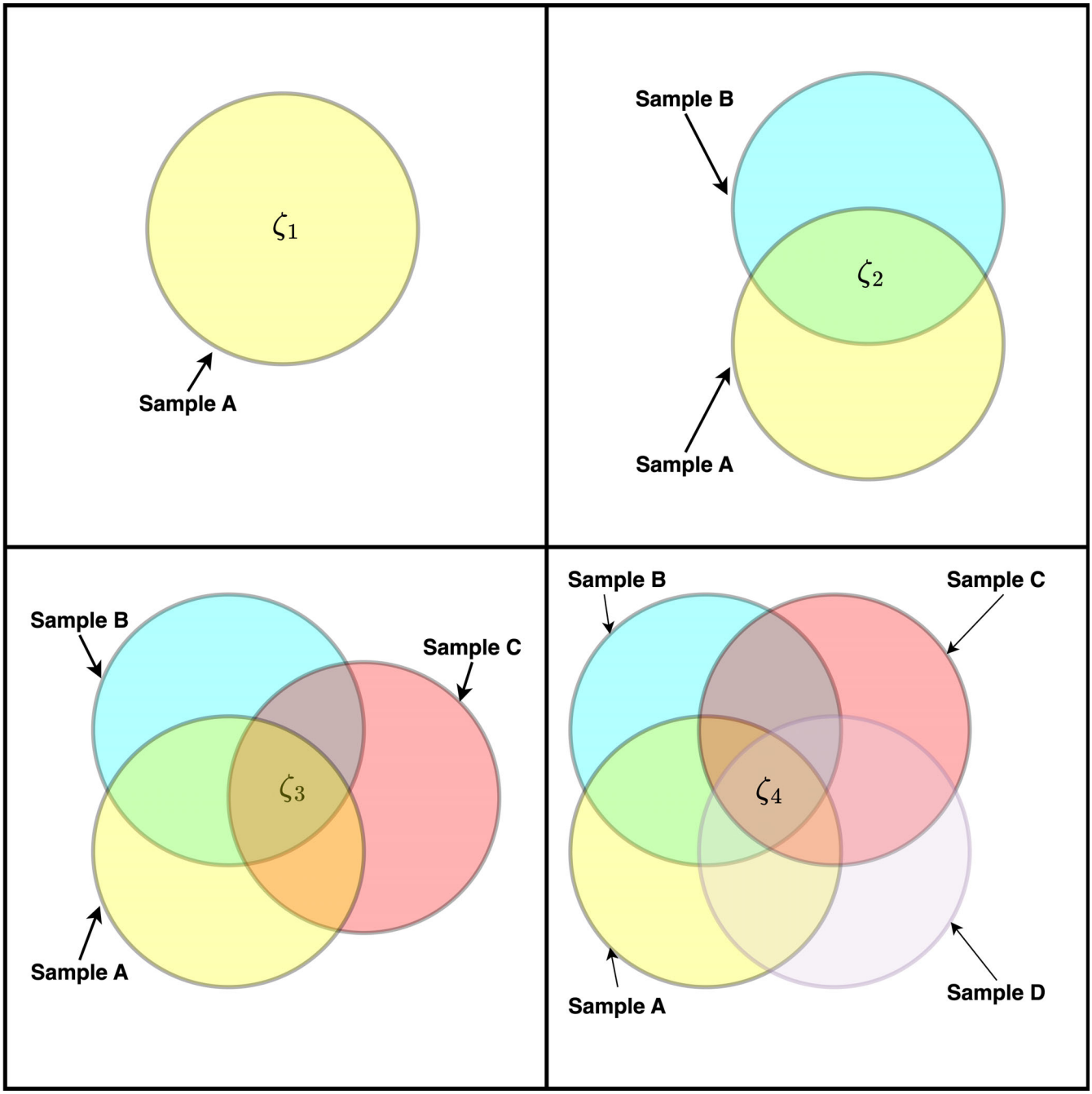

FIGURE 1 An illustration of the first four orders of zeta diversity. The mean number of unique categories of organisms per sample, a measure of alpha diversity, is represented by the value ζ_1. In comparing two or more samples the average number of unique categories of organism held in common between any two samples is represented by the value ζ_2. This process can be extended to N samples, allowing for a determination of the values ζ_1 through ζ_N.

models of community assembly. Prior studies of groups of organisms used as biological indicators of anthropogenic activities have illustrated the importance of selecting assemblages that contain taxa with a diverse array of ecological niches and environmental sensitivities (Hilty & Merenlender, 2000; Resh, 2008; Simonin et al., 2019; Smol & Stoermer, 2010), with evidence also suggesting a process of niche differentiation being more likely than a stochastic one in such assemblages (Gillett et al., 2021; Passy & Legendre, 2006; Simons et al., 2019). The relative likelihood of these two models of community assembly, niche differentiation, and a stochastic process, can then be respectively calculated from the relative likelihoods of a power law and exponential decay models of how ζ_N decays with N (Hui & McGeoch, 2014; McGeoch et al., 2019). This is of particular relevance to the

selection of assemblages as indicators of anthropogenic environmental changes, with correlations having been observed between degrees of ecological disturbance and an increase in the relative likelihood of a stochastic over niche-differentiated process of community assembly (Britton et al., 2017).

In addition to the application of zeta diversity to assessing stream health, this study also compared the use of DNA metabarcoding to traditional morphotaxonomy in determining the composition of biological assemblages. Stream bioassessments have relied on morphologically classified algae (Fetscher et al., 2014; Theroux et al., 2020), however this is a time-consuming and error-prone process (Brown et al., 2015; Hebert et al., 2004; Lanzén et al., 2016; Pauls et al., 2010; Ristau et al., 2013). With the recent development of metabarcoding it is now possible to rapidly and reliably classify organisms using DNA fragments from environmental samples (Banerji et al., 2018; Elbrecht & Leese, 2017; Keck et al., 2017), and with it the potential for developing bioassessments which are unencumbered by the limitations of morphologically based methods (Hajibabaei et al., 2011; Ji et al., 2013; Pawlowski et al., 2018; Ratnasingham & Hebert, 2007; Taberlet et al., 2012). With this technique, the composition of several algal assemblages used in the bioassessment of freshwater streams has been evaluated, including both soft-bodied algae (Banerji et al., 2018; Minerovic et al., 2020; Wolf & Vis, 2020), and diatoms (Bailet et al., 2019; Valentin et al., 2019; Vasselon et al., 2017). The use of metabarcoding-based methods has demonstrated its potential for both improved efficiency for the detection of rare organisms, as well as finer taxonomic resolution (Baird & Sweeney, 2011; Gibson et al., 2015; Stein, Martinez, et al., 2014; Stein, White, et al., 2014; Sweeney et al., 2011; Zhan et al., 2013). This indicates its potential for far more sensitive and nuanced bioassessments than those derived from traditional morphology-based methods (Elbrecht & Leese, 2017; Mortágua et al., 2019; Stein, White, et al., 2014).

The application of zeta diversity in bioassessments of multisite assemblages has been demonstrated in several environments including islands (Leihy et al., 2018), protected areas in aquatic environments (Britton et al., 2017), and forests (Lazarina et al., 2019). Furthermore, evidence of relationships between environmental gradients and measures of landscape diversity has been found in streams for soft-bodied algae (Passy & Blanchet, 2007; Wu et al., 2018) and diatoms (Jyrkänkallio-Mikkola et al., 2016; Soininen et al., 2004). Using the zeta diversity framework, we were also able to demonstrate that the effects of ecological degradation of streams in a region could be assessed by the change in patterns of local and landscape diversity alone (Simons et al., 2019). Building on previous work, we used zeta diversity patterns of algal assemblages, classified via metabarcoding and by morphology, to construct stream bioassessments on a regional scale. This study compares these zeta diversity-based regional-scale bioassessments, constructed using algal assemblages classified using either morphotaxonomy or metabarcoding, to existing algal indices. The goal of this study was as follows:

1. Demonstrate that patterns in zeta diversity, across multisite algal assemblages found in streams, can be used as indicators of their biotic integrity on a regional scale.
2. Compare the performance of morphotaxonomic versus DNA metabarcode sequencing for assessing the performance of these zeta diversity-based indices of regional biotic integrity.
3. Use zeta diversity to assess the relative likelihoods for models of community assembly for these multisite algal assemblages, classified using both morphotaxonomy and metabarcoding, and test if algal assemblages in streams are more likely to be assembled via a process of niche differentiation rather than stochastically.

With this work, we aimed to evaluate the potential of zeta diversity as an additional tool for assessing the ecological health of streams across a region, and to investigate the impact of morphology versus DNA-based taxonomy identification on this metric.

MATERIALS AND METHODS

Sample collection and scope

The samples used in this study were collected at wadable stream reaches located throughout California as part of four regional monitoring programs, the Stormwater Monitoring Coalition (SMC), the Perennial Stream Assessment (PSA), the Reference Condition Monitoring Program (RCMP), and the Reference Monitoring Program (RMP). The data used in this study were taken from 96 samples across 92 stream reaches (Figure 2) during the period 2016–2018. These stream reaches represented a spectrum of ecological conditions, ranging from coastal to alpine environments, as well as a wide scope of human impacts, ranging from relatively undisturbed "reference" sites with low to no modifications of the immediate landscape (as defined in Ode, Rehn, et al., 2016) to sites under high levels of anthropogenic stress from human landscape modifications.

Briefly, this protocol involves the collection of benthic algae subsamples along 11 transects within a 150 m stream reach, which are used to create a composite algal

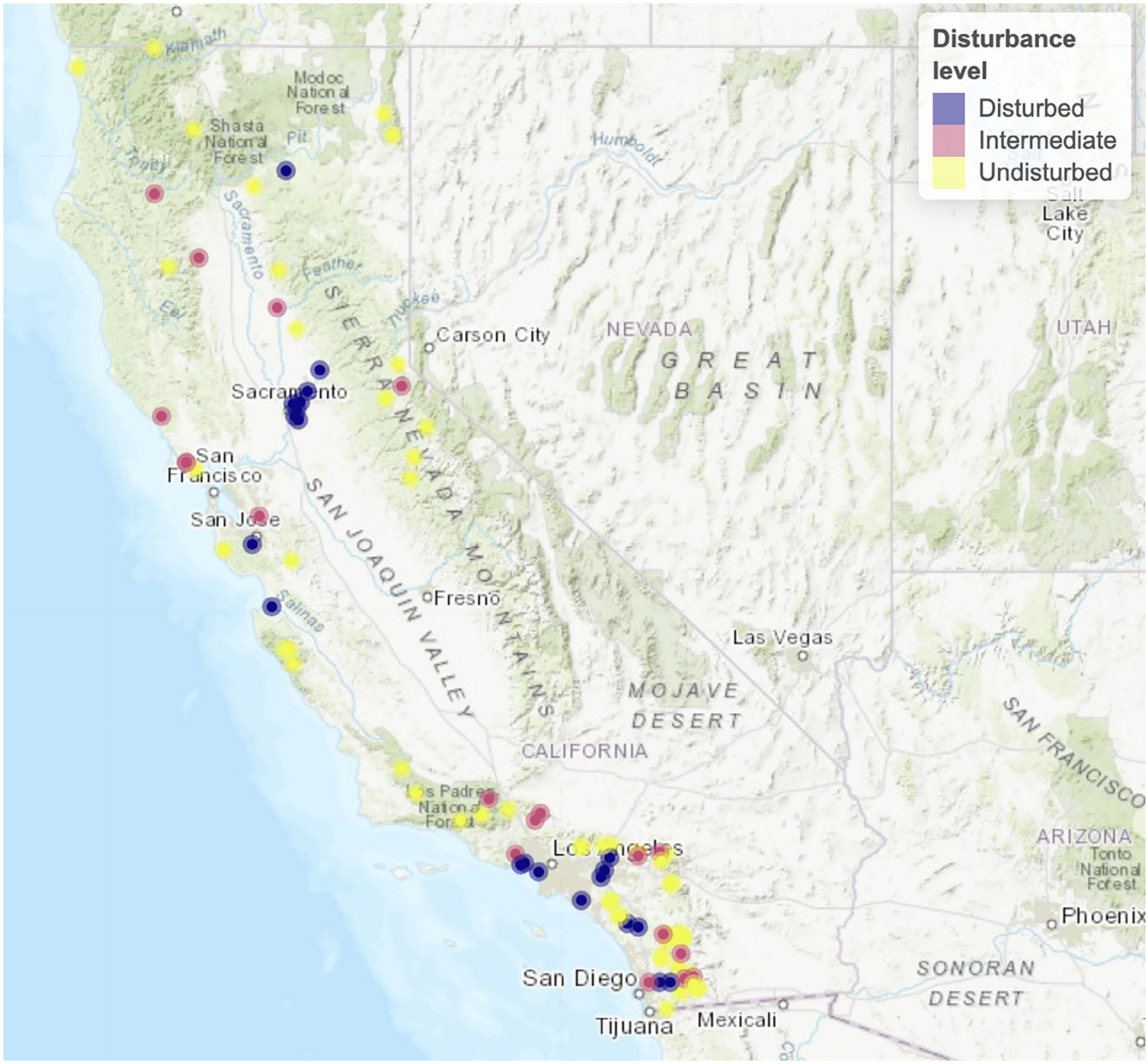

FIGURE 2 Sample sites colored by their level of disturbance based on upstream land use.

sample representative of multiple stream substrata (e.g., cobbles, sand, large wood, etc.) in proportion to their abundance across the reach. This composite sample is subsampled for diatom and soft-bodied algae taxonomic analyses as well as DNA analyses (detailed below). Laboratory procedures for morphological classifications and enumerations of both the soft-bodied algae and diatom communities followed Stancheva et al., 2015. The abundance of diatoms was recorded as a valve count, and those of soft-bodied algae were recorded as a biovolume, although both of these sets of values were converted to a simple presence/absence format for ASCI index calculations (Theroux et al., 2020).

DNA extraction, metabarcoding, and bioinformatics

We collected a fraction of the composite algal samples described above for DNA extraction and analysis. For each sample we filtered between 10 and 50 ml of composite material, composed of a homogenate of diatoms and soft-bodied algae, onto 0.2-μm Whatman Nucleopore polycarbonate filters (GE Healthcare Life Sciences, Buckinghamshire, UK). We first preserved each filter in bead solution storage buffer (Mo Bio Laboratories, Inc., Carlsbad, CA) at −80°C. Following a thaw at 4°C, we performed DNA extractions on the filters using the

Mini-Beadbeater-96 homogenizer (BioSpec, Bartlesville, OK) and PowerSoil DNA kit and protocols (Mo Bio Laboratories, Inc., Carlsbad, CA). Following extractions, we quantified DNA yield using a NanoDrop 8000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

Because of the multiphyletic nature of algae, we chose a universal 18S V9 primer (Amaral-Zettler et al., 2009) to target combined populations of soft-bodied algae and diatoms. To specifically target diatoms alone we used an rbcL primer (Vasselon et al., 2017). Paired-end sequencing of all of the resulting barcoded amplicons was performed on an Illumina MiSeq platform by Laragen, Inc. (Culver City, CA, USA) using either 2 × 150 reads (18S V9) or 2 × 250 reads (rbcL). The resulting DNA amplicons were processed and demultiplexed using the Illumina MiSeq Recorder, which enabled the removal of barcodes, primers, and adapter sequences. We stored the processed amplicons on the Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra) as BioProject no. PRJNA545290.

We processed DNA sequences using QIIME2 2019.07 (Bolyen et al., 2019, http://qiime2.org). In our workflow, forward and reverse reads were first merged into contigs. These contigs were then filtered by first removing chimeric sequences, then removing reads which occurred less than two times in any individual sample. We then generated Amplicon Sequence Variants (ASVs) within QIIME2 using DADA2 (Callahan et al., 2016). Taxonomic assignments for algae were then performed for 18S ASVs using BLAST within QIIME2 against the SILVA v132 database (Quast et al., 2012). As with our morphotaxonomic classifications, we classified taxa within the phylum Bacillariophyta as diatoms, with taxa within the phyla Charophyta, Chlorophyta, Rhodophyta, Ochrophyta, Miozoa, Euglenozoa, Heterokontophyta, and Cryptophyta as members of the soft-bodied algal community. Hybrid algal communities then contain taxa within either diatoms or soft-bodied algae. This filtering step yielded 2632 unique 18S ASVs covering 2,356,473 reads from an initial set of 11,793 ASVs covering 4,663,161 reads. For ASVs classified using the rbcL primer we used BLAST taxonomic assignment as implemented in QIIME2 against a custom reference database comprised of the R-Syst database (Rimet et al., 2016) and diatom rbcL sequences from GenBank for diatom-specific taxonomic assignments. This filtering step yielded 5513 unique rbcL ASVs covering 712,791 reads from an initial set of 7049 ASVs covering 815,672 reads. ASV counts were then converted into a percentage of total reads per sample as a way to normalize differential read counts. Rarefaction was not used on read counts as it has been found to increase the likelihood of false positives (McMurdie & Holmes, 2014). Code documenting the workflow to produce ASV tables is available online (10.5281/zenodo.7402027).

Calculating the D_ASCI and H_ASCI

For each sample, algal multimetric index scores were calculated based on diatom (D_ASCI) or diatom and soft-bodied algae (hybrid, H_ASCI) taxonomy data after Theroux et al. (2020). In brief, morphotaxonomic counts were converted to presence/absence data and a series of individual metrics are calculated (proportion planktonic species, proportion low nitrogen taxa, etc.). These metrics were scored based on the expected value predicted from a site's local environment, such as geology, climate, and watershed area (Theroux et al., 2020); the larger the deviation from reference expectation, the lower the metric score. The metric scores were aggregated and the final D_ASCI and H_ASCI scores were calculated, ranging from ~0 (highly impacted) to ~1.5 (reference). Code documenting the workflow to calculate the D_ASCI and H_ASCI are available online (10.5281/zenodo.7465435).

Land use

To determine the geographic distribution of land use upstream of each sampling site we used National Land Cover Data (NLCD) acquired in the year 2011 (Homer et al., 2015). We define land use as the total percentage of land cover in a designated area dedicated to human activity, such as agricultural land, parks, and impervious urban surfaces. These designated areas are defined by a watershed-clipped buffer running 5 km upstream from the sampling site as determined with ArcGIS tools (version 10.3; Environmental Systems Research Institute). We selected this geographic scale for defining land use as it has been commonly used in assessments by SCCWRP to capture local variations in anthropogenic activity (Mazor et al., 2016; Simons et al., 2019; Theroux et al., 2020). Sites were then classified into one of three categories of disturbance based on the percentage land use dedicated to human activity: undisturbed (0%–3%), intermediate (3%–15%), and disturbed (15%–100%). Of the 96 sample sites in this study, 56 were categorized as undisturbed, 19 as intermediate, and 21 as disturbed.

Sample data structure

For each sample the structure of our data was as follows: morphologically classified soft-bodied algae and diatoms, soft-bodied algae and diatoms classified by an 18S V9 primer, diatoms classified using an rbcL primer, site altitude in meters, the percentage of total developed land use (agricultural, urban, and managed landscapes) within a 5 km clipped buffer of the watershed upstream of the

sampling site, and scores of the biotic integrity of hybrid algal and diatom assemblages as measured by H_ASCI and D_ASCI respectively. For algae classified both by morphology and metabarcoding we converted count and abundance data to a presence/absence sample by taxa matrix for use in calculating zeta diversity.

Sample group selection

To analyze patterns between measures of zeta diversity and environmental conditions we randomly subsampled 15 sites, 100 times, from each of the three disturbance categories described above (undisturbed, intermediate, and disturbed) (Figure 3), producing a set of 300 sample groups. Each sample group was kept at a uniform size of 15 samples, a size sufficiently larger to calculate the decay of zeta diversity beyond zeta10, the highest zeta diversity order used in this study. In total, 100 subsamples were chosen, as this was sufficient for capturing a representative and balanced set of sample groups for each disturbance category, while avoiding oversampling within both the intermediate and disturbed sample groups given the number of samples within each sample group. For each subsample group the following environmental values were calculated: the mean percent developed upstream land use ("land use"), mean sample site altitude ("altitude"), and mean geographic separation distance between samples ("distance"). Distance between sample sites was calculated, in meters, using the function

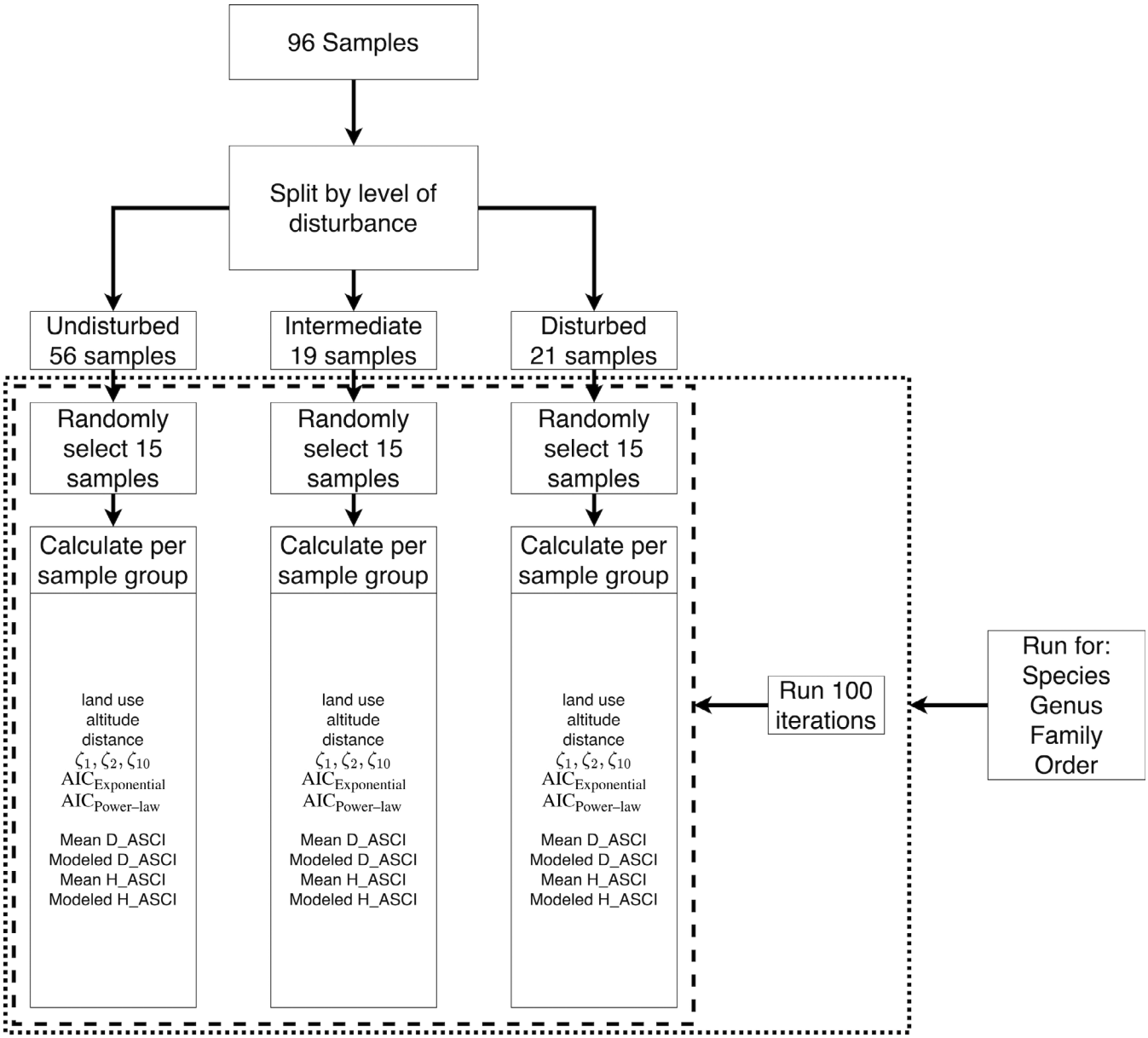

FIGURE 3 Analysis workflow diagram.

"distm" within the R package *geosphere* (Hijmans et al., 2017). For each group of 15 samples, using assemblages classified both by morphology and metabarcoding, we also calculated the values of ζ_1, ζ_2, and ζ_{10}, the mean values of the D_ASCI and H_ASCI, linear models of the D_ASCI and H_ASCI built using our three zeta diversity measures, as well as Akaike Information Criterion (AIC) scores for the relative likelihoods of two models of how zeta diversity decays with sample order (Figure 3).

Calculations of zeta diversity

For this study we focused on three orders of zeta diversity, ζ_1, ζ_2, and ζ_{10}, to describe changes in both local and landscape diversity for our sampled communities (Simons et al., 2019). All three of these zeta diversity orders were calculated using the function "Zeta.decline.ex" within the R package *zetadiv* (Latombe et al., 2018). The value of ζ_1, our measure of local diversity, was calculated as the average taxonomic richness per sample in a group of samples. For measures of zeta diversity of order N, the function "Zeta.decline.ex" calculated the average fraction of unique taxa held in common between any N samples selected from a group of samples. For our measures of landscape diversity, ζ_2, and ζ_{10}, we therefore calculated the average fraction of unique taxa held in common between any 2 or 10 samples selected from our group of 15 samples.

We then investigated how the number of unique taxa shared between N sampled communities within a sample group decayed with N. The two models we tested, a power law of the form $\zeta_N = \zeta_1 N^{-b}$ and an exponential of the form $\zeta_N = \zeta_1 e^{b(N-1)}$, were chosen as they had been found to account for the majority of cases for how zeta diversity distributions most likely decayed with N (Hui & McGeoch, 2014). The value of the exponent for our zeta diversity decay models (b) is a measure of intercommunity dissimilarity within our sample groups, with a greater value indicating a more rapid decay in similarities between communities. Both models were fitted using the first 10 orders of zeta diversity, ζ_1 through ζ_{10}, for each group of 15 samples. Only the first 10 orders of zeta diversity were chosen, as it was found to be sufficient to demonstrate an asymptotic level of similarity in the average number of unique taxonomic groups held in common across our sample groups. The relative likelihoods of both models of zeta diversity decaying with sample order, the first a power law and the second an exponential decay model, were then assessed using an AIC score generated using the function "Zeta.decline.ex."

Modeled index scores

We constructed linear models, based on zeta diversity, of the mean regional value of algal biotic integrity. Zeta diversity-based models of the mean biotic integrity (D_ASCI and H_ASCI) per sample group were constructed using the "lm" function within the R package *stats* (v3.5.1 R Core Team, 2018), with ζ_1, ζ_2, and ζ_{10} as predictors. We then compared the behavior of these mean and modeled indices to changes in land use, altitude, and distance. Two sets of linear models were then constructed using land use, altitude, and distance as predictors: the first predicting the mean biotic integrity and the second predicting the zeta diversity-based model of biotic integrity. To calculate the relative importance of variables in our models of biotic integrity we then used the function "calc.relimp" within the R package *relaimpo* (Grömping, 2006). The function "calc.relimp" was used to calculate the percentage variation in our zeta diversity-based models of biotic integrity attributed to zeta diversity orders. The function "calc.relimp" was similarly used to calculate the percentage variation attributed to land use, altitude, and distance in predicting the mean or modeled indices of biotic integrity. To summarize how well our linear models predicted regional algal biotic integrity, we performed a 10-fold cross-validation, with 100 repeats, between the mean and modeled algal biotic integrity scores per sample group. These cross-validations were performed using Pearson correlation coefficients and the "train" function within the R package *caret* (Kuhn, 2008).

RESULTS

We found that our zeta diversity-based models of the biotic integrity of groups of algal assemblages, both for diatoms alone as well as in combination with soft-bodied algae, tended to be strongly and significantly correlated with the mean values of their biotic integrity (Figures 3 and 4). The strength of these correlations varied by method of classification, community type, and the taxonomic level at which community members were aggregated. We found that the mean biotic integrity of groups of algal assemblages could be more reliably predicted with zeta diversity-based models for combined assemblages of diatoms and soft-bodied algae classified via metabarcoding rather than by morphotaxonomy, with the strength of correlations generally increasing when using more finely resolved taxonomic levels (Table 1). In comparing the relative likelihoods for two models of how zeta diversity for algal assemblages decay with sample order we consistently found a power-law model to be

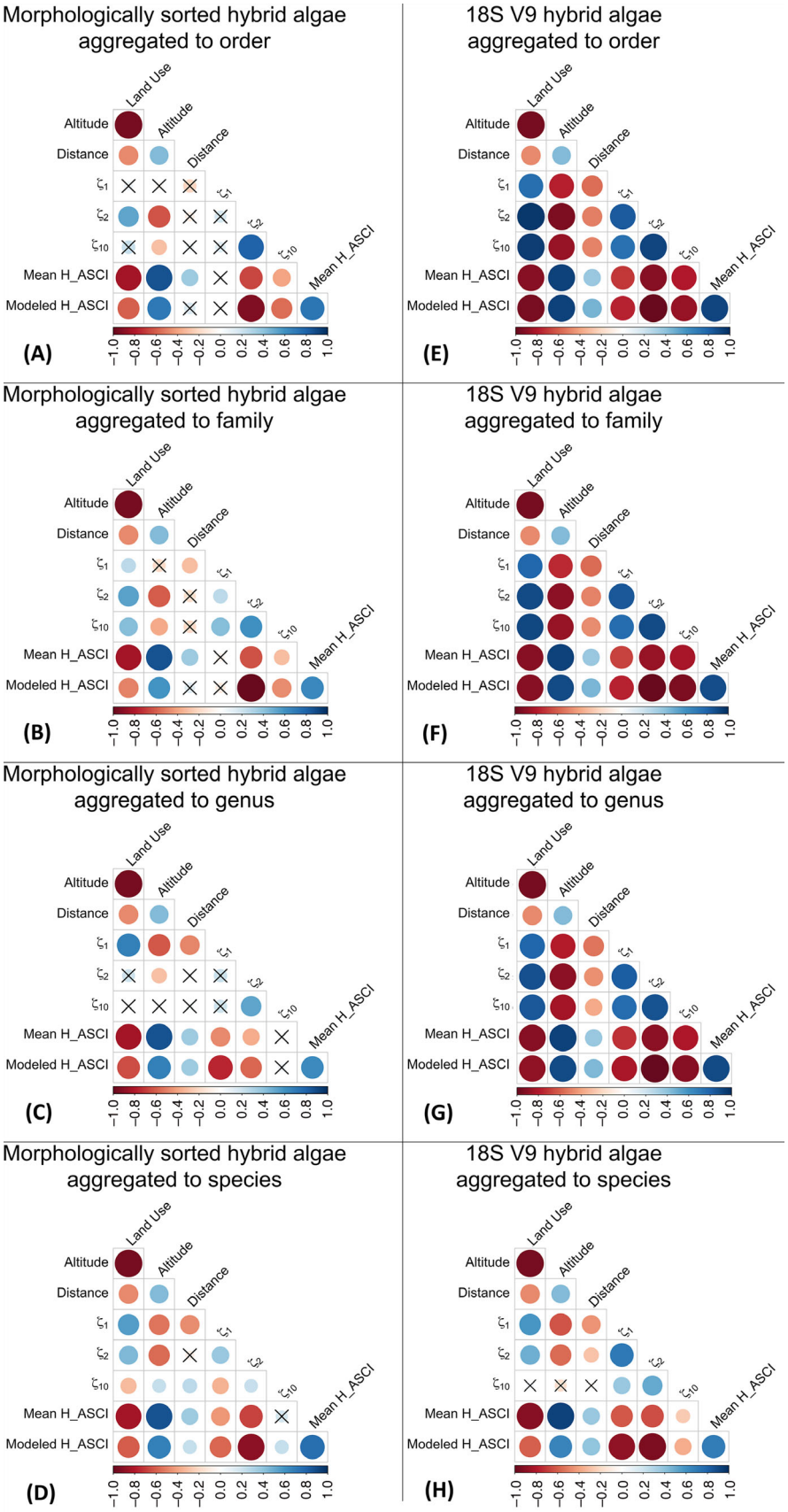

FIGURE 4 Pearson correlations between environmental variables (A–H), measures of zeta diversity, mean and modeled indices for algal communities ($p < 10^{-4}$). × indicates lack of statistical significance.

more likely than an exponential one (Table 2), providing evidence that all of the algal assemblages in this study were more likely to be assembled via a process of niche differentiation rather than stochastically.

Linear models of diversity and biotic integrity

Using three parameters, ζ_1, ζ_2, and ζ_{10} linear models were constructed to predict the mean value of biological index scores for various communities aggregated at taxonomic levels ranging from species to order. The strength of the Pearson correlation coefficients between the mean value of various index scores for assemblages within a set of samples ranged from 38%, for diatoms classified using an rbcL primer and aggregated to order, to 87% when these same sequences were aggregated to family order (Table 1). We observed that linear models which could most strongly predict the mean index of biotic integrity, across all levels of taxonomic aggregation were constructed using hybrid algal communities classified using an 18S V9 primer (Table 1). We found the performance of these linear models tended to be improved by the use of assemblages classified via metabarcoding rather than morphotaxonomy, with the exception of diatoms aggregated to the level of species (Table 1).

The behavior of zeta diversity-based models of the D_ASCI or H_ASCI on a regional basis was found to be similar to their mean values, across the taxonomic levels considered, when varying land use, altitude, and distance (Figures 4 and 5). The behavior of both the mean and modeled algal stream condition indices was similar whether the algal assemblages were classified by morphotaxonomy or metabarcoding (Figures 4 and 5).

TABLE 1 Ten-fold cross-validated Pearson correlations between mean and modeled stream condition indices, D_ASCI for regional groups of assemblages of diatoms and H_ASCI, using regional groups of assemblages aggregated to various taxonomic levels ($p < 10^{-4}$).

Taxonomic level	Morphological algae	18S V9 algae	Morphological diatoms	rbcL diatoms
Species	0.75	0.80	0.65	0.38
Genus	0.63	0.79	0.77	0.81
Family	0.64	0.78	0.53	0.87
Order	0.72	0.71	0.49	0.71

Zeta diversity patterns and the environment

Across our study area we found that both the average taxonomic richness of hybrid algal assemblages (ζ_1), as well as the number of unique taxa shared between any two or ten samples (ζ_2 and ζ_{10} respectively), tended to increase with land use (Figure 4). However, the opposite of these trends was observed when only the diatom component of these assemblages was considered (Figure 5). For hybrid algal assemblages both measure local (ζ_1) and landscape diversity (ζ_2 and ζ_{10}) were found to be negatively correlated with both altitude and distance (Figure 4), while the trends were reversed for diatoms (Figure 5). In both cases these trends were independent of whether the algal assemblages were classified by morphotaxonomy or metabarcoding, although the correlations were stronger when metabarcoding was used to classify algal assemblages (Figures 4 and 5).

The relative importance of local and landscape diversity table

We found a series of distinct trends regarding the relative importance of measures of local (ζ_1) and landscape (ζ_2 or ζ_{10}) diversity in our zeta diversity-based models of algal biotic integrity. For assemblages of diatoms classified through metabarcoding or by morphology, we found variations in landscape diversity to be of greater relative importance to our models of the D_ASCI than changes in local diversity (Table 3). However, for hybrid assemblages of diatoms and soft-bodied algae we found measures of landscape diversity to be of relatively lower importance in our models of the H_ASCI (Table 3). For hybrid algal assemblages classified by morphotaxonomy local diversity

TABLE 2 Akaike Information Criterion scores of exponential (power-law) models of zeta diversity decay with sampling order ($p < 10^{-4}$).

Taxonomic level	Morphological algae	18S V9 algae	Morphological diatoms	rbcL diatoms
Species	−7.4 (−30.7)	−7.4 (−44.4)	−11.6 (−19.7)	4.3 (16.3)
Genus	−12.6 (−48.2)	−11.6 (−54.1)	−16.4 (−44.4)	−4.8 (−25.1)
Family	−19.8 (−49.2)	−16.0 (−62.8)	−26.7 (−43.0)	−16.3 (−48.4)
Order	−26.9 (−60.6)	−22.9 (−65.4)	−33.4 (−64.1)	−24.1 (−52.4)

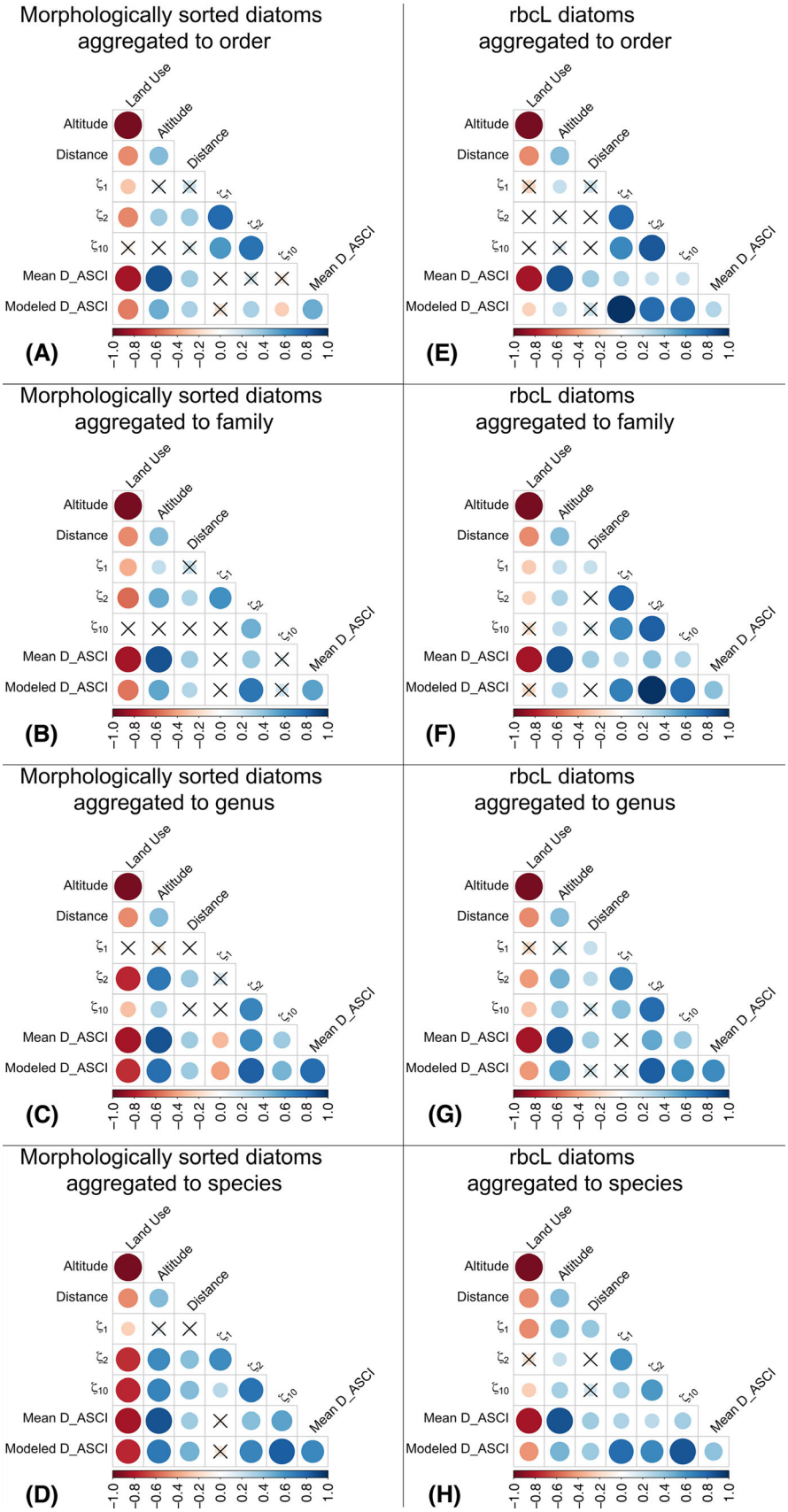

FIGURE 5 Pearson correlations between environmental variables (A–H), measures of zeta diversity, mean and modeled indices for diatom communities ($p < 10^{-4}$). × indicates lack of statistical significance.

tended to be of greater importance than measures of landscape diversity in models of the H_ASCI, however all three measures of zeta diversity were of similar importance in our models of the H_ASCI when algal assemblages were classified using metabarcoding (Table 3).

The relative importance of environmental measures in our modeled indices

We observed similar patterns in the relative importance (altitude > land use > distance) of our measures of the physical environment in both the mean and modeled H_ASCI scores for hybrid algal assemblages (Table 4). However, the relative importance of these variables was far higher for models of the H_ASCI constructed using hybrid algal assemblages classified using metabarcoding rather than morphotaxonomy. For diatoms we generally found a different order (land use > altitude > distance) of variable importance in our models of the D_ASCI. As with hybrid algal assemblages, the relative importance of these variables was also higher in models of the D_ASCI constructed using metabarcoding rather than morphologically classified assemblages.

Of the environmental measures considered, we tended to observe altitude and land use as having the greatest relative importance for our modeled stream-health indices for hybrid algal assemblages or diatoms, respectively (Table 4). We also tended to observe opposite trends comparing modeled indices to either altitude or distance as those compared to land use (Figures 4 and 5). While we used calculations of relative importance to help account for collinearity in our environmental variables, we do note broadly negative trends between land use and altitude ($r = -0.95$, $p < 10^{-4}$) as well as land use and distance ($r = -0.47$, $p < 10^{-4}$) across our sample groups.

Statistics for assemblages classified by morphology and metabarcoding

For our assemblages classified via metabarcoding we obtained more than three times as many reads using an 18S V9 primer to classify hybrid algal assemblages than using an rbcL primer to classify diatoms (Appendix S1: Table S1). We did find a greater proportion of DNA sequence reads could be assigned a taxonomy for hybrid algal assemblages classified using an 18S V9 primer as compared to diatom assemblages classified using an rbcL primer (Appendix S1: Figure S1A). Although this reflects the greater numbers of taxonomic groups could be resolved for our hybrid algal assemblages as compared to diatoms (Appendix S1: Figure S1B). For algae that could be resolved to species, we did find the most common ones were detected in more samples using morphotaxonomy rather than metabarcoding (Appendix S1: Table S2). For both diatoms and hybrid algal assemblages we found metabarcoding identified unique taxonomic groups, including a greater number of total phyla as well as total species (Appendix S1: Figure S1B).

TABLE 3 The percentage relative importance of zeta diversity orders in models of the mean value of biotic integrity, D_ASCI for assemblages of diatoms and H_ASCI for assemblages of diatoms and soft-bodied algae, using regional groups of assemblages aggregated to various taxonomic levels.

Taxonomic level and ζ order		Morphological algae	18S V9 algae	Morphological diatoms	rbcL diatoms
Species	ζ_1	9.7	**36.8**	8.9	2.6
	ζ_2	**41.4**	25.6	14.6	**11.4**
	ζ_{10}	5.4	2.3	**18.2**	0.1
Genus	ζ_1	**20.8**	25.9	15.0	11.9
	ζ_2	13.9	**30.9**	**36.7**	**43.5**
	ζ_{10}	5.0	5.9	7.0	10.0
Family	ζ_1	0.5	**23.8**	5.5	11.0
	ζ_2	**36.0**	17.5	**20.4**	**54.6**
	ζ_{10}	4.9	19.4	2.3	9.8
Order	ζ_1	1.2	**17.9**	5.1	5.0
	ζ_2	**39.9**	15.8	**12.7**	**22.9**
	ζ_{10}	10.9	17.0	6.5	21.8

Note: The zeta diversity order with the greatest relative importance per model is marked in bold.

TABLE 4 The relative importance of land use, altitude, and distance for both modeled and (mean) indices of biotic integrity for regional groups of assemblages of diatoms, assessed using the D_ASCI, and regional groups of assemblages of diatoms and soft-bodied algae, assessed using the H_ASCI.

Taxonomic level and variable		Morphological algae	18S V9 algae	Morphological diatoms	rbcL diatoms
Species	Land use	18.1 (38.8)	31.9 (38.8)	**26.9 (32.5)**	**20.4 (32.5)**
	Altitude	**27.3 (42.4)**	**32.0 (42.4)**	22.2 (38.3)	11.9 (38.3)
	Distance	2.0 (4.8)	7.5 (4.8)	8.9 (4.3)	1.9 (4.3)
Genus	Land use	19.0 (38.8)	31.2 (38.8)	25.1 (32.5)	**35.2 (32.5)**
	Altitude	**22.9 (42.4)**	**33.0 (42.4)**	**27.5 (38.3)**	34.2 (38.3)
	Distance	4.4 (4.8)	7.0 (4.8)	4.6 (4.3)	3.1 (4.3)
Family	Land use	14.3 (38.8)	27.9 (38.8)	**13.5 (32.5)**	35.1 (32.5)
	Altitude	**24.9 (42.4)**	**31.9 (42.4)**	12.3 (38.3)	**37.9 (38.3)**
	Distance	1.4 (4.8)	5.2 (4.8)	3.0 (4.3)	4.8 (4.3)
Order	Land use	20.0 (38.8)	20.5 (38.8)	**12.3 (32.5)**	**29.3 (32.5)**
	Altitude	**33.6 (42.4)**	**29.2 (42.4)**	10.7 (38.3)	25.4 (38.3)
	Distance	2.1 (4.8)	3.9 (4.8)	4.1 (4.3)	5.0 (4.3)

Note: The variable with the greatest relative importance per model is marked in bold.

Zeta diversity and models of community assembly

We found evidence supporting a greater likelihood that the structure of algal assemblages in streams tend to be shaped by a process of niche differentiation rather than a stochastic one. This was found in hybrid algal assemblages across all taxonomic levels, classified by morphology and an 18S V9 primer, where a power-law model was likelier than an exponential one in describing the decay of zeta diversity with sample number (Table 2). A similar pattern was found with diatoms, classified using an rbcL primer as well as by morphology, with the only exception being with metabarcoded diatoms classified to species (Table 2).

DISCUSSION

For both diatom and hybrid algal assemblages, we found that zeta diversity indices were able to reliably predict the biotic integrity across a region and, in particular, these indices performed best when algal assemblages were identified using metabarcoding rather than morphology. Unlike current indices of algal biotic integrity, these zeta diversity-based modeled indices do not rely on the use of undisturbed reference communities or the presence of particular indicator species, but simply patterns of algal diversity across a landscape. For these reasons, we believe they may be used to develop tools to assess environmental conditions in streams in regions beyond the original geographic scope of this study.

Metabarcoding and limitations with taxonomic assignments

Between hybrid algal and diatom assemblages, and between assemblages classified using metabarcoding or morphology, we found large differences in the number of unique groups which could be resolved at each taxonomic level (Appendix S1: Figure S1B). Many of these differences could be attributed to the limited ability to generate species-level taxonomic assignments for DNA metabarcode data, a common limitation in sequence-based taxonomy (Clarke et al., 2014; Debroas et al., 2017; Elbrecht & Leese, 2017; Meyer et al., 2021). Likewise, many of the California algae species currently lack DNA reference sequences and therefore taxonomic assignment is constrained to genus or higher levels of taxonomy (Somervuo et al., 2017; von Ammon et al., 2018). Prior work in developing indices of algal biotic integrity has shown improvements in reliability using assemblages resolved to species (Fetscher et al., 2014; Stancheva & Sheath, 2016), although certain indices can attain adequate sensitivity with taxonomic resolution limited to higher levels such as genus or family (Mueller et al., 2013). With this in mind, there is the potential to use zeta diversity-based regional bioassessments of algal assemblages in streams with relaxed levels of taxonomic

sufficiency, greatly reducing the amount of time needed to perform taxonomic assignments as part of bioassessments.

The importance of local and landscape diversity in predicting regional measures of stream community health

We observed a significant role for both our measures of local (ζ_1) and landscape diversity (ζ_2, and ζ_{10}) in our models of regional biotic integrity for both diatoms as well as hybrid algal assemblages, potentially reflecting a number of ecological processes. As zeta diversity order increases, for example from ζ_2 to ζ_{10}, there is an increased sensitivity to changes in the presence of common categories of organisms across a landscape (Latombe et al., 2017). Consequently, we propose that assessments of the impacts of environmental degradation cannot be described solely from the loss of relatively rare or common categories of organisms, but that such changes must be accounted for together.

We did find some key distinctions between patterns in the behavior of local and landscape diversity and regional biotic integrity for hybrid algal assemblages or diatoms. For example, the regional biotic integrity of hybrid algal assemblages tends to decline with increases in both local and landscape diversity (Figure 4), while the opposite trend is observed with just diatoms (Figure 5). This may indicate, at least using our two primers, that the less similar the taxonomic compositions of a group of hybrid algal assemblages the greater their average biotic integrity, with the opposite pattern found when focusing on diatoms alone.

Greater environmental stress has been found to act as an environmental filter with diatoms, whereby a reduced set of resilient species persist in degraded environments (Huttunen et al., 2020; Pound et al., 2019). Across California we do find that assemblages of diatoms in degraded environments tend to be more similar to one another, which is reflected in the positive correlations we observed between regional biotic integrity and landscape diversity (Figure 5). However, this taxonomic convergence is absent, as seen with negative correlations between landscape diversity and biotic integrity (Figure 4), when considering soft-bodied algae in combination with diatoms. This difference may in part reflect differences in the rates of dispersal between diatoms, which are free-floating, and soft-bodied algae, and which are often sessile (Schneider et al., 2012). With hybrid algal assemblages, environmental degradation may lead to a convergence in the compositions of their more mobile diatom components, but locally unique declines in more sessile populations of soft-bodied algae. These trends have been observed elsewhere in soft-bodied algae, where degraded assemblages undergo both a decline in local taxonomic richness (Schneider et al., 2012, 2013; Stancheva & Sheath, 2016) as well as interassemblage similarity (Dunck et al., 2021).

Measures of regional stream health and environmental variables

The behavior of the modeled H_ASCI, whereby the compositions of hybrid algal assemblages converge while their biotic integrity is degraded as a result of greater land use, reflects prior observations of a decline in the beta diversity of soft-bodied algal assemblages under elevated anthropogenic stresses (Passy & Blanchet, 2007). That is, elevated land use degrades regional biotic integrity for hybrid algal assemblages, with degraded assemblages tending to look more similar in composition (Figure 4). This in turn indicates that the modeled H_ASCI is capturing the effects of strong environmental filtering (Dunck et al., 2019) on regional groupings of hybrid algal assemblages.

As altitude is negatively correlated with land use, the tendency of hybrid algal assemblages to have greater biotic integrity at higher elevations, and with it the divergence in the compositions of hybrid algal assemblages with altitude (Figure 4), may indirectly reflect the correlation observed between measures of landscape diversity and land use. Elevated sites in our data tended to be found on mountain slopes scattered across California (Figure 2), which is reflected in the negative correlation between distance and altitude (Figure 4). With elevated sites tending to be more isolated from one another geographically, dispersal limitation in soft-bodied algae may underly the negative relationship between measures of landscape diversity and altitude (Branco et al., 2020). All three orders of zeta diversity for our hybrid algal assemblages are negatively correlated with distance, and given that our zeta diversity-based model of regional biotic integrity for hybrid algal assemblages is positively correlated with all three of these diversity measures, may then at least partially underlie the tendency of elevated sites to have greater biotic integrity.

With assemblages of diatoms, as with hybrid algal assemblages of diatoms and soft-bodied algae, we find that increases in land use, or declines in altitude, are associated with a decline in interassemblage similarity (Figure 5). These patterns suggest that increases in land use effectively fragment habitats across a landscape and interfere with dispersal (Matthiessen et al., 2010; Vander Laan & Hawkins, 2014) and therefore reduce regional biotic integrity for diatoms. In many cases, there is a lack

of uniformity in the local loss of free-floating species such as diatoms and invertebrates, leading to the decline in the compositional similarity of assemblages scattered across networks of streams subjected to environmental stresses (Fugère et al., 2016; Hawkins et al., 2010; Libório & Tanaka, 2016; Mykrä et al., 2017; Pound et al., 2019). While the ecological response of diatoms to anthropogenic stress differs from that of hybrid algal assemblages, our zeta diversity-based model of D_ASCI appears to capture reliable patterns connecting environmental gradients and regional biotic integrity of diatom assemblages.

Zeta diversity decay and models of community assembly

Using models of how zeta diversity decays with sample order we found evidence of freshwater algal assemblages, both diatom and hybrid, tending to be assembled via a process of niche differentiation, which is in line with earlier studies (Dunck et al., 2021; Narwani et al., 2013; Pan et al., 1999; Passy & Legendre, 2006; Soininen, 2007), These results supported the use of zeta diversity in analyzing the ecology of freshwater algae, particularly with identifying likely processes of assembly. This is important in identifying the type of niche-differentiated assemblages useful in constructing bioassessments, ranging from marine invertebrates (Gillett et al., 2021) to lichens (Nascimbene et al., 2013).

Not only was the process of assembly for algal assemblages found to be dominated by a process of niche differentiation, but it was found to be increasingly likely as assemblages were aggregated from the level of species up toward order (Table 2). The only exceptions to this pattern appear to be metabarcoded diatoms aggregated at the level of species (Table 2), but this may be an artifact of the relatively few numbers of reads with species-level taxonomic assignments disrupting any diversity-based analyses. Given broad correspondences between functional and taxonomic diversity at higher taxonomic levels (Anacker & Strauss, 2016; Keck et al., 2016; Lu et al., 2016), our results are in line with observations of stronger relationships between measures of functional, rather than taxonomic, diversity and the biotic filters driving assembly via a process of niche differentiation (Khalil et al., 2018; Siefert et al., 2013). Both diatoms (He et al., 2020; Rimet & Bouchez, 2012) and soft-bodied algal (Branco et al., 2020) species tend to be highly diverse and endemic, but more cosmopolitan at higher taxonomic levels, which may also underlie part of the shift in likelihood that we observed from a stochastic model of community assembly as we aggregated assemblage data at coarser taxonomic levels. That we identify the same general pattern in the relative likelihood of two community assembly processes across multiple taxonomic levels, and that assemblages containing diatoms (Minerovic et al., 2020; Rimet & Bouchez, 2012) can be reliably used in bioassessments when aggregated to taxonomic levels higher than species, implies zeta diversity may provide useful information regarding key ecological processes and assessments when using algal assemblages aggregate to genus or even higher levels of taxonomy.

CONCLUSION

With this study we demonstrate the potential for zeta diversity to be used in analyzing ecological processes in freshwater algal assemblages, as well as developing regional-scale assessments of freshwater stream ecosystems. We found that biotic integrity on a regional scale, for hybrid algal assemblages, as well as diatoms alone, could be reliably predicted using zeta diversity alone. This suggests that much of the information needed in assessing the regional biotic integrity of algal assemblages is encoded in diversity patterns, potentially enabling the development of new bioassessment metrics which can be more rapidly determined than methods dependent on the presence of particular taxa. However, it should be noted that these results were dependent on how these assemblages were classified, with our best results derived from hybrid algal assemblages classified using an 18S V9 primer.

We have also demonstrated the utility of applying analysis of zeta diversity patterns of various types of algal assemblages, as classified using metabarcoding, as a means of assessing their ecological health. However, the accuracy of this approach appears to be strongly dependent on the completeness of the reconstructed assemblages, whether from morphotaxonomic or DNA-based taxonomy data, in addition to the assemblage type, primer, and the completeness of the reference library used to assign taxonomies (Baker et al., 2010; Christensen & Olsen, 2018; Lan et al., 2012; Mande et al., 2012; Marcy et al., 2007; Rinke et al., 2013). To overcome these limitations, future work on the development of biological assessments of streams using zeta diversity and sequence data should evaluate a shift toward reference-free based approaches of classifying sequences and organizing them into assemblages (Dubinkina et al., 2016; Linard et al., 2019; Ren et al., 2018; Tang et al., 2018; Zielezinski et al., 2019). Such approaches have shown a significant improvement over standard taxonomic approaches in the completeness with which sequences can be incorporated into profiles of assemblage composition (Apothéloz-Perret-Gentil et al., 2017), and have begun to demonstrate promise in building tools for biological assessment (Pawlowski et al., 2018).

Future work on the development of biological assessments based on patterns of zeta diversity is therefore likely to improve with the incorporation of assemblage data constructed using reference-free approaches.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Code to analyze biodiversity patterns (Simons, 2022) is available in Zenodo at https://doi.org/10.5281/zenodo.7402027 and code documenting the workflow to calculate the D_ASCI and H_ASCI (Theroux, 2022) is available in Zenodo at https://doi.org/10.5281/zenodo.7465436. The 2011 National Land Cover Database is available in the USGS ScienceBase-Catalog at https://www.sciencebase.gov/catalog/item/53715219e4b07ccdd78bad4a. Sequence data for samples are available in the NCBI SRA repository under PRJNA545290 at https://www.ncbi.nlm.nih.gov/bioproject/PRJNA545290.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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