Does DNA barcoding improve performance of traditional stream bioassessment metrics?

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Abstract

Benthic macroinvertebrate community composition is commonly used to assess the condition of streams and wetlands and help differentiate the effects of stressors between and among sites. DNA barcoding has been promoted as a way to increase taxonomic resolution, thereby increasing the sensitivity of existing bioassessment metrics. This hypothesis was tested by comparing the ability of several commonly used bioassessment metrics derived using traditional morphology and DNA barcoding to discern differences in stream condition. Six paired sites in southern California with relatively subtle impacts to habitat were assessed using standard bioassessment tools as well as DNA barcoding. At each site, two reaches were sampled: an upstream, (reference) reach and a downstream (impact) reach where the streambanks have been armored. For each sample, approximately 600 organisms were enumerated and identified based on morphological characteristics using a standardized taxonomic effort (generally to species, with midges to genus). mtDNA was then extracted from each individual and sequenced for the approximately 658 base pairs (bp) barcoding region of the cytochrome *c* oxidase subunit I (COI) gene. Although most (i.e., 91%) organisms yielded sequences >350 bp in length, high failure rates among all taxa collected from one stream required its exclusion from analysis. Results based on morphological identifications produced subtle differences in community composition, but no significant differences between armored and unarmored reaches using 16 commonly used metrics. In contrast, for 10 of the 16 metrics derived from DNA barcode identification, statistical power substantially increased; consequently, it was possible to discern differences between armored and unarmored reaches. These previously undetected differences were associated with the increased taxa richness for midges, mayflies, non-insects, caddisflies, and black flies that resulted from DNA barcoding. These results suggest that identifications based on DNA barcoding have the potential to improve power to detect minor changes in stream condition.

Introduction

Bioassessment is an attractive evaluation tool because resident organisms integrate the influences of environmental conditions over time and space and therefore can be more indicative of overall environmental health than measuring individual

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stressors or ecosystem attributes (Schoolmaster *et al.* 2012). Biotic indices provide information on the overall condition of a site based on the taxonomic composition and relative tolerances of resident taxa to pollution or other stressors (Karr and Chu 1999). However, component metrics of the overall biotic indices are often necessary in order to detect more subtle changes or effects of individual stressors (Hawkins 2006). Use of relatively coarse taxonomic resolution may obscure patterns in bioassessment metrics and can hinder detection of biological impacts. This makes fine scale taxonomic resolution desirable to maximize the diagnostic capability of assessment tools (Hawkins 2006, Jones 2008, Pfrender *et al.* 2010).

Obtaining detailed taxonomic data is challenging because identifications are typically done through observation of morphologic characteristics. Limited taxonomic resources, cryptic morphology, small size, damaged specimens, and polymorphism can make identification to the species level difficult or impossible in some instances. Incorporation of DNA barcoding into routine bioassessment has been promoted as alternative approach for taxonomic identification (Hebert *et al.* 2003, 2004; Stoeckle and Hebert 2008; Borisenko *et al.* 2009; Janzen *et al.* 2009). Barcoding involves identifying taxa based on a short DNA sequence from a standardized genetic locus, such as the mitochondrial gene cytochrome c oxidase I (COI) for most metazoans. Using standard molecular methods, DNA is extracted from specimen tissue and sequenced for the approximately 658-bp barcoding region of COI (Hebert *et al.* 2003). DNA from unknown specimens collected in benthic samples can be identified by comparing their barcode sequences to a reference library, such as the Barcode of Life Data Systems (BOLD; Ratnasingham and Hebert 2007).

Like any relatively new technique, DNA barcoding has potential advantages and disadvantages. The purported advantages include the potential to obtain taxonomic identifications in less time than traditional morphology-based methods, and the potential for increased metric sensitivity associated with improved taxonomic resolution (Waite *et al.* 2004, Chessman *et al.* 2007). Potential disadvantages include the need to develop and maintain capacity for genetic sequencing, the need to develop robust reference libraries, and increased bioinformatics and data management needs. In addition, some researchers have suggested that reliance on a single gene region (COI) may not be sufficient for the identification of all taxa in a community sample (Pfrender *et al.* 2010). For example, in an assessment of nematode diversity using genetic sequencing, Porazinska *et al.* (2009) found that analysis based on a single gene sequence underestimated the number of species. Others have expressed concern that reducing community composition to a list of operational taxonomic units (or putative species) has the ability to dilute the appreciation of actual community composition and mask the relationship between species behavior/adaptation and the requisite habitat characteristics (Cameron *et al.* 2006).

Previous studies have demonstrated that DNA barcoding has the ability to provide additional taxonomic information beyond that typically derived from traditional morphological identification, particularly for cryptic, small, or rare species (Pauls *et al.* 2010, Zhou *et al.* 2010, Sweeney *et al.* 2011, Jackson *et al.* In press). For example, Zhou *et al.* (2010) reported that DNA barcoding resulted in a greater than fivefold increase in the number of sensitive EPT taxa (Ephemeroptera, Plecoptera, and Trichoptera) widely used in bioassessment, and that improvements were most marked for rare species, which may be diagnostic of specific habitat or water quality conditions. Pilgrim *et al.* (2011) observed up to three times the number of EPT taxa using DNA barcoding than had been previously documented in Maryland. When averaged over all orders of aquatic insects, Sweeney *et al.* 2011 showed that only 51% of the specimens that were identified to species by barcoding could be identified to species by expert taxonomists given issues with specimen condition, size, level of maturity, or lack of taxonomic keys.

Increased taxonomic resolution has the potential to improve the ability of bioassessment metrics to discriminate effects of pollution or environmental degradation. Sweeney *et al.* (2011) compared bioassessment metrics derived from traditional and barcoding data at two stream reaches approximately 4 km apart, one surrounded by natural riparian forest and the other by orchards and farms. DNA barcoding allowed a 70% increase in the number of taxa that could be detected, which improved the sensitivity of taxonomic richness based bioassessment metrics to discern differences between the two sites.

In this study the work of Sweeney *et al.* (2011) was built upon by testing the ability of bioassessment metrics and indices derived from DNA barcodes to discern subtle environmental effects associated with

armoring of stream banks. This study took advantage of a companion study that evaluated the effects of bank armoring in five paired stream reaches; one armored and one unarmored in each location (Stein *et al.* 2013). Eroding stream banks are often reinforced (armored) with hard structures such as concrete walls, boulders, or gabions. These bank armoring structures are intended to stabilize channels and protect infrastructure, but they often result in increased stream power and decreased channel roughness, exacerbating stream channel responses to urbanization (Riley 1998, Jacobson *et al.* 2001). Traditional biological indicators showed subtle, mechanistic responses to physical changes. However, bioassessment metrics and traditional assessment indices (such as the Southern California Index of Biotic Integrity (IBI), Ode *et al.* 2005) did not differ between the armored and unarmored reaches (Stein *et al.* 2013).

As part of the companion study, DNA barcodes were obtained for nearly every organism collected at the study sites to answer the following questions: How does using DNA barcoding change the estimates of taxonomic richness and richness based bioassessment metrics? If DNA barcoding results in higher taxonomic richness, how does this affect statistical power of key metrics? Does DNA barcoding increase the ability to predict differences between impacted (armored) and unimpacted (unarmored) stream reaches?

Methods

Study Sites

Biological effects of stream channel armoring were investigated at five streams in the Los Angeles region, Southern California, USA (Table 1). Specimens were collected from six streams; however, sequence data was obtained for only five of the six sites. For the sixth site, Arroyo Simi (L1), barcode data were generated for only 11% percent of specimens. This low level of success was spread evenly among the different groups of benthic macroinvertebrates while these same taxonomic groups were barcoded successfully for the other five sites. This study attempted to re-extracting DNA from the Arroyo Simi samples at two labs (University of Guelph and EPA-Cincinnati), but DNA quality and quantity were too low for PCR amplification. Therefore, it was assumed that the low PCR amplification success for all specimens from this site resulted from some sort of sample contamination or mishandling of the DNA preservation protocol and are not representative of results from the rest of the study. Therefore, data from the Arroyo Simi site were omitted from subsequent analysis.

To ensure that the study sites represented the range of stream types where bank armoring projects are typically constructed in this region, sites were selected from three different watershed positions (mountain, transitional, lowland) with the following characteristics: watershed drainage areas of 50 to 300 km^2, channel slopes of 0.1 to 2%, pool-riffle morphologies, and discrete segments of bank armoring ranging from 100 to 200 m long (Table 1). The channel bottom at all sites consisted of natural substrates.

Each of the study reaches comprised a 150 m long, unarmored upstream control segment "A" and a 100 to 200 m long segment with armoring on at least one bank (the "impact" segment, "B"). Armored segments (B) were most often located along the outside of meander bends. In all cases, the bank armoring structures (which severely constrained the lateral movement of the study streams) enhanced the forcing of pool scour and meander development by constraining channel migration (Stein *et al.* 2013). This position is supported by observations of historical planform photos of the study sites, which show that all of the channels had been quite dynamic, both upstream of, and within, the bank segments that were eventually armored. Observation of historical photographs also indicated that bank armoring was at least ten years old at all sites. Thus, it was assumed that the present-day channel patterns strongly reflect the influence of the bank armoring structures, and that physical differences between stream segments can justifiably be interpreted as resulting, at least in part, from bank armoring.

Biological Assessment

Benthic macroinvertebrates (BMIs) were sampled using the multihabitat method described by Ode 2007. Each 150-m segment was divided into 11 equidistant transects, and a 500 µm mesh D-frame net was used to collect BMIs from a prescribed location along each transect (i.e., 25, 50, or 75% of the way across the stream), for a total of 0.9 m^2 of streambed sampled. The 11 subsamples were composited into one container and specimens were preserved immediately using 95% ethanol. Samples were drained and replenished with fresh ethanol within 24 hours of collection to maintain a minimum

Table 1. Summary of study sites.

Watershed Position Category	Site Code	Site Name	Upstream Drainage Area (km^2)	Upstream Effects on Hydrology	Landscape Setting	Slope (%)	Q10a (cfs)	Predominant Land Use	Nature and Bank Amoring Type
Mountainous/ upper watershed	M1	W. Fork San Gabriel River	215	Cogswell Dam; upstream fire in prior year	River gorge	1.68	8,667	Forested	Concrete structure on right bank of outside bend of active channel, ~45 degree angle and ~5 m tall
	M2	E. Fork San Gabriel River	205	none	Montane alluvial valley	1.59	8,218	Forested	Gabions filled with riprap on left bank of outside bend of active channel, 90 degree angle and ~5 m tall
Transition zone/ foothills	T1	Big Tujunga	298	Big Tujunga Dam; upstream fire in prior year	Proximal alluvial fan	1.39	5,119	Mixed scrub-shrub + urban	Concrete structure on left bank of outside bend of active channel, ~45 degree angle and ~5 m tall
	T2	Arroyo Seco	49	Upstream fire in prior year	River gorge	2.64	3,342	Mixed forested + urban	Concrete structure on right bank of inside bend of active channel, 90 degree angle and ~10 m tall
Lower watershed	L2	Conejo Creek	197	None	Alluvial valley	0.12	5,326	Agriculture	Concrete structure on right bank of outside bend of active channel, ~45 degree angle and ~5 m tall

Site L1 (Arroyo Simi) was excluded from the study because barcodes were obtained for only 11% of the specimens, despite the fact that the same species were successfully barcoded at other sites. This suggests a contamination issue which made extracts from this site unsuitable for this analysis.

90% ethanol concentration to prevent DNA degradation. A minimum of 600 BMIs were sorted and identified in the laboratory based on standard protocols and following the taxonomic standards of the Southwestern Association of Freshwater Invertebrate Taxonomists (i.e., level 2 in Richards and Rogers 2006). Species composition and abundance data were used to calculate a standard set of taxonomic richness (hereafter richness) and diversity metrics commonly used in the southern California Index of Biotic Integrity (Ode *et al.* 2005).

DNA Extraction, Amplification, and Sequencing

Following traditional taxonomic identification, a small piece of tissue was removed from each specimen and placed into 96-well plates. Plates were shipped to the Canadian Centre for DNA Barcoding (CCDB), where the standard COI DNA barcode was sequenced from each specimen using highly automated protocols established at the CCDB by Ivanova *et al.* (2006) and http://www.ccdb.ca/pa/ge/research/protocols. Once received by CCDB, well caps were removed to allow the ethanol to completely evaporate from the plates. Upon complete evaporation, lysis solution was added to plates, followed the next day by DNA extraction. DNA extracts were PCR amplified using standard barcoding forward and reverse primer-pairs (Table 2). If initial amplifications were unsuccessful, DNA extracts underwent additional PCR using primer-pair combinations developed for the taxa under analysis. For specimens that failed both first and second pass PCR, primers were deployed that target shorter barcode regions (~400 bp). PCR amplicons were bidirectionally sequenced using Sanger sequencing with BigDye v3.1 using an ABI 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA). Sequences and detailed information about all specimens were uploaded to the Barcode of Life Data Systems (Ratnasingham and Hebert 2007) and can be accessed there via the project codes CFWIA through CFWIJ. Nucleotide sequence data were exported from BOLD, and DNA sequences were aligned using the ClustalW with default parameters in MEGA 5.05 (Tamura *et al.* 2011).

Species Delimitation

Using barcode sequences greater than 350 bp in length, neighbor joining (NJ) trees were constructed for each taxonomic group at the order and class level according to the method of Saitou and Nei (1987) with the Kimura 2-parameter (K2P) distance nucleotide model (Kimura 1980). Individual specimens were assigned molecular operational taxonomic unit (MOTU) identifications based on the genetic distance thresholds determined in (Jackson *et al.* In press) so that all taxonomic groups were manually delimited using a 2% distance threshold applied to branch lengths, except *Simulium* and *Baetis* who were delimited at 3 and 1% distance thresholds, respectively. Specimens with a sequence length less than 350 bp were added to existing MOTUs using a K2P distance cutoff of 2%.

Data Analysis

Bioassessment metrics were calculated using taxonomic information derived from both morphologic identifications and DNA barcoding, and differences between armored and unarmored segments were tested using the Wilcoxon signed-rank

Table 2. Primers and primer sequences used in this study.

Name	Sequence (5' to 3')	Role	Reference
LCO1490	GGTCAACAAATCATAAAGATATTGG	External Forward	Folmer *et al.* 1994
HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	External Reverse	Folmer *et al.* 1994
LepF1	ATTCAACCAATCATAAAGATATTGG	External Forward	BOLD
LepR1	TAAACTTCTGGATGTCCAAAAAATCA	External Reverse	BOLD
GomphLCO	CAACAAATCATAAAGATATTGGAAC	External Forward	This Paper
MLepF1	GCTTTCCCACGAATAAATAATA	Internal Forward	BOLD
MLepR1	CCTGTTCCAGCTCCATTTTC	Internal Reverse	BOLD
TricorCOIF1	TYATTATRATTTTCTTTATAGT	Specific to *Tricorythodes*	This Paper
TricorCOIR1	AAGAARGARGTRTTTAAATAACG	Specific to *Tricorythodes*	This Paper

test. Unless otherwise stated, $\alpha = 0.05$ was used for all inferential analyses and significance was based on two-tailed tests. In order to obtain even sample sizes across all ten sites, samples from each site were randomly permuted without replacement and the average richness count of those permutations were used to calculate richness metrics. Power to detect observed differences was calculated for each metric for both morphology and barcode derived metrics.

Results

The five paired sites (i.e., ten sampling locations) produced 5,870 specimens that underwent DNA barcoding. DNA sequences were obtained from 5,478 (93.3%) of specimens (Figure 1). Of those sequences, 368 (6.7%) were between 125 and 500 base-pairs (so-called "partial-barcodes"), and 5,110 (87.1%) were between 500 and 658 base-pairs in length ("full barcodes").

Twelve of the 16 metrics evaluated showed higher median and ranges of richness values when calculated using barcoding data than when using morphology data (Figure 2). Differences were greatest for overall richness, as well as richness of Diptera, Baetidae, Chironomidae, and "GOLD" (Gastropoda, Oligochaeta and Diptera--a metric used in some Mediterranean streams (Morais *et al.* 2004). Increased richness associated with barcoding was observed across all sites and was particularly pronounced for common taxa such as Diptera and Chironomidae.

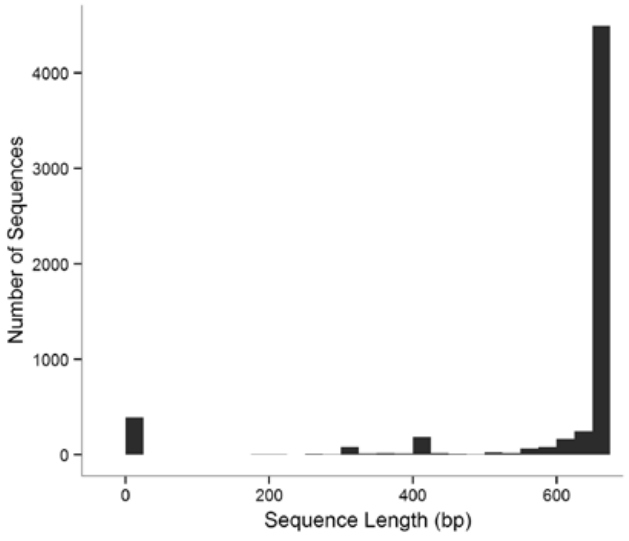

Figure 1. Relative abundance of base pair (bp) sequences as a function of sequence length used in this study.

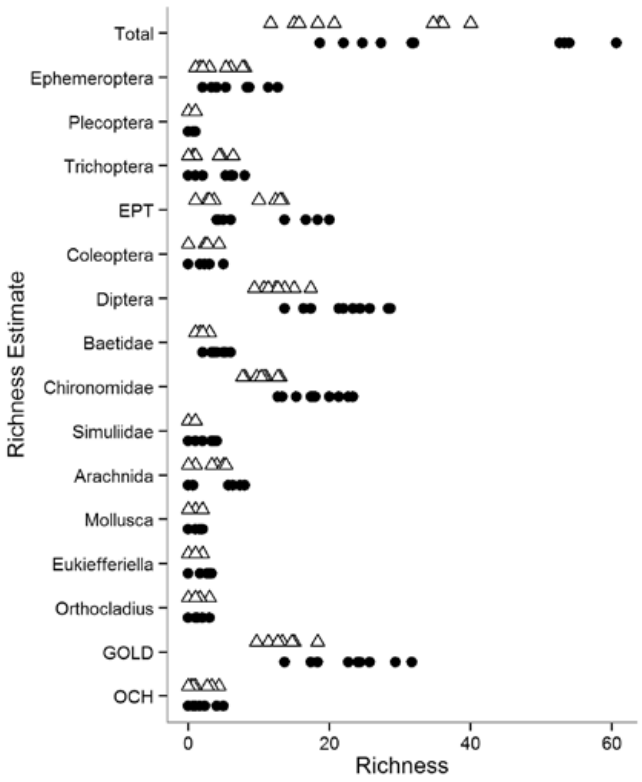

Figure 2. Taxonomic richness metrics for 16 different groupings of macroinvertebrates from five Southern California streams (2 reaches at each stream) based upon identifications made using DNA barcoding (solid circles) and morphology (open triangles).

Differences in metric values between unarmored and armored stream reaches reflect the increased taxonomic resolution associated with barcoding (Figure 3). In particular, the differences in Diptera, Chironomidae, and GOLD richness between unarmored and armored reaches are much larger when using barcoding vs. morphology based measures. Although barcoding-derived richness values were higher than morphology-based at all sites, the differences are most pronounced at higher quality sites (e.g., M1 and M2) where the channel armoring may be having a relatively larger effect on the instream biota (Figure 4). At lower quality sites (e.g., L2), richness values were still higher, but the difference in metric values between armored and unarmored reaches was not as great.

Statistical power increased for 10 of the 16 metrics evaluated when calculated using barcoding data, despite the generally low power values observed for all metrics (Figure 5). The largest increases (from 0 to 0.18 power) were observed for relatively common taxonomic groups where diversity is often underestimated due to the fact that organisms

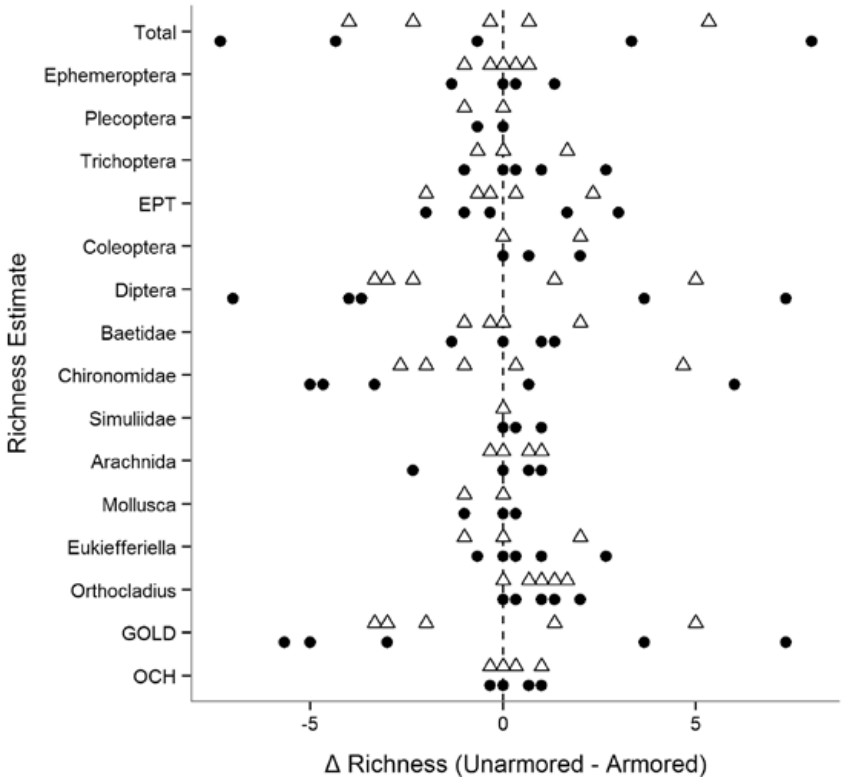

Figure 3. Differences in taxonomic richness between unarmored and armored reaches from five Southern California streams for 16 different groupings of macroinvertebrates as estimated by DNA barcoding (solid circles) and morphology (open triangles).

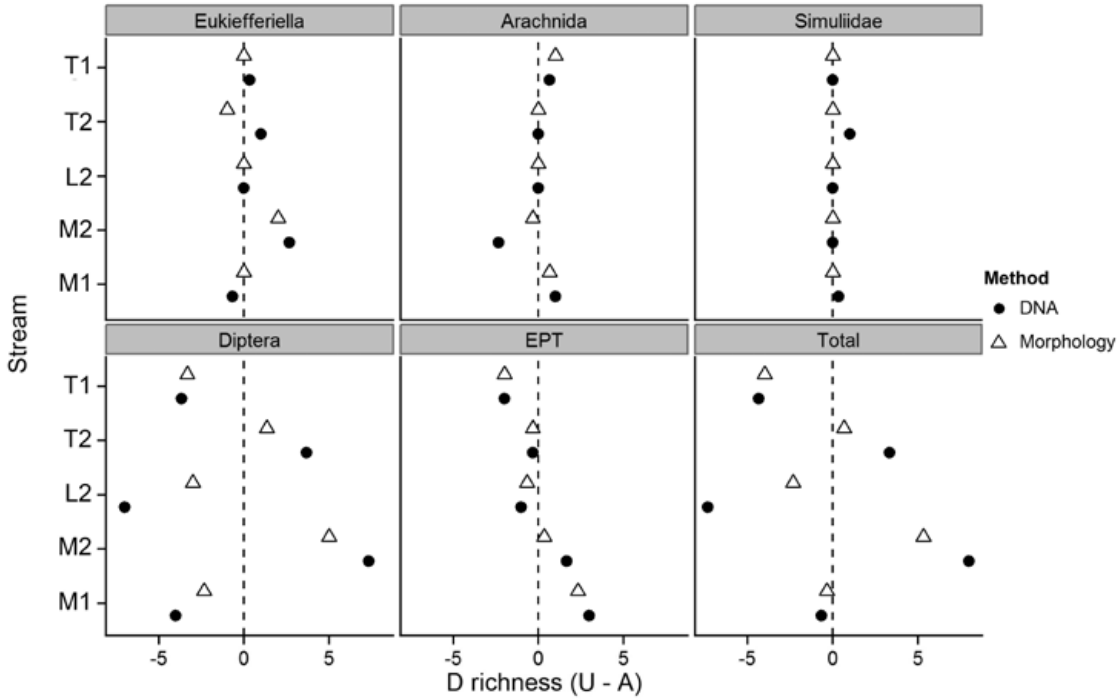

Figure 4. Differences in taxonomic richness between unarmored and armored reaches from five Southern California streams for six different groupings of macroinvertebrates as estimated by DNA barcoding (solid circles) and morphology (open triangles).

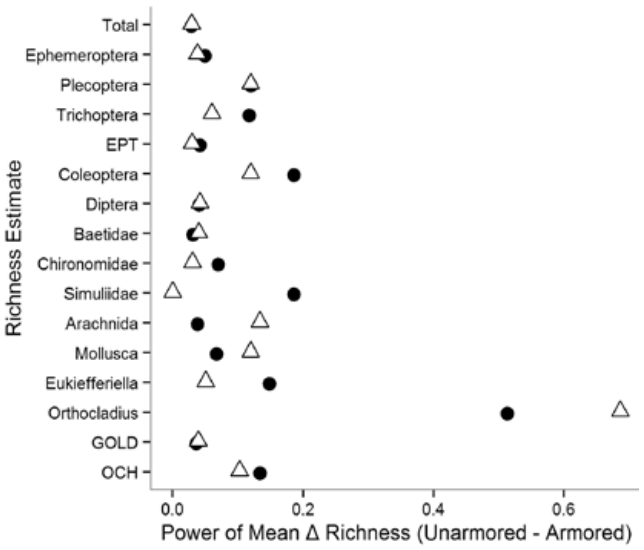

Figure 5. Relationship between taxonomic richness for 16 different groupings of macroinvertebrates as estimated by DNA barcoding (solid circles) and morphology (open triangles) and the average statistical power associated with them. Power was calculated as a paired, two-tailed t-test for the average difference between unarmored and armored reaches for the five Southern California streams.

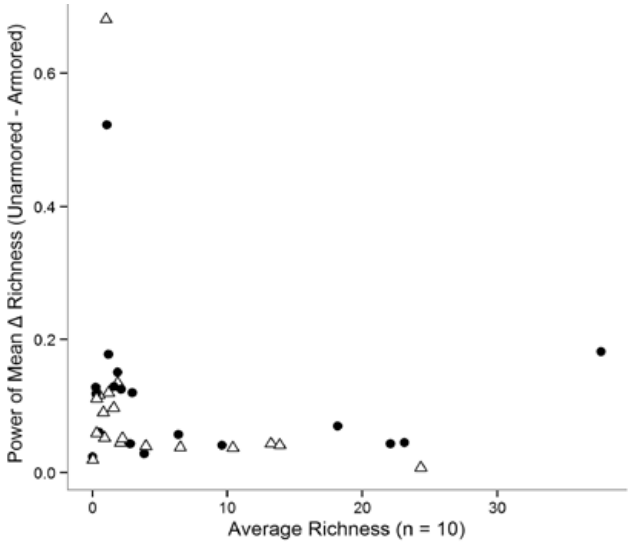

Figure 6. Relationship between the amount of statistical power (paired, two-tailed t-test for the average difference between unarmored and armored reaches) calculated for 16 different groupings of macroinvertebrates and the average taxonomic richness (averaged across 2 reaches and 5 streams) as estimated by DNA barcoding (solid circles) and morphology (open triangles).

are typically identified to genus level or higher. For example, the Simuliidae showed the greatest increase because larval simuliids are typically only identified to genus level even though they are known to be a speciose group. Other groups such as Trichoptera and Coleoptera also showed notable increases in statistical power. However, statistical power decreased for groups where site variance was high, such as Arachnida (Acari), Mollusca, and *Orthocladius* (a highly speciose genus of midge). Statistical power was independent of richness values and increases in power did not depend on the original power associated with the traditional taxonomy-derived metrics (Figure 6).

Discussion

The immense species richness and diversity of stress tolerances among benthic invertebrates make them one of the most commonly used organisms for bioassessment of streams (Bonada *et al.* 2006). However, patterns in species occurrences relative to stressors acting on a site are only interpretable to the taxonomic level at which individual species can be identified. Limitations in the ability to produce species level data either due to incomplete taxonomic knowledge, the condition of the specimen, poor taxonomic keys, shortage of trained taxonomists, or cost associated with getting high resolution taxonomy is one of the greatest challenges in implementing bioassessment programs.

DNA barcoding has potential to improve bioassessment by providing increased taxonomic resolution, improving data quality (improved accuracy and objectivity), and enhancing the diagnostic ability of existing assessment tools. Although existing tools adequately discriminate between reference and highly degraded streams, they are generally less sensitive to subtle changes along the mid-ranges of disturbance gradients and where there are multiple confounding stressors affecting a site (e.g., Stribling *et al.* 2008, Waite *et al.* 2004). Increased taxonomic resolution provided by barcoding improves the signal-to-noise ratio of bioassessment metrics and indices. For example, Sweeney *et al.* (2011) reported a 76% increase in metrics denoting changes in water quality between a natural and agricultural site in Pennsylvania. This study builds on this work by demonstrating similar success at five pairs of sites in a variety of environmental settings subject to minor localized effects associated with bank armoring. The fact that similar improvement was observed in responses relative to morphology based metrics,

despite differences in climate and stressors between the California and Pennsylvania studies, suggests broad applicability of the benefits associated with the improved taxonomic resolution.

Increased metric sensitivity associated with barcoding was most pronounced at high quality (i.e., relatively unimpacted) sites. These sites typically have higher species richness and are often inhabited by undescribed, cryptic, or regionally rare species. For example, 43% of the additional taxa identified through barcoding were one or two individuals and occurred at only one stream. The presence or absence of rare species may be diagnostic of specific environmental changes. The increased information provided by barcoding at taxa rich site allows for finer resolution of sources of stress and increases the ability to detect subtle changes in environmental quality.

The streams in this study were subjected to mild forms of degradation that resulted in subtle changes in the instream benthic community (Stein *et al.* 2013). Bank armoring causes indirect effects to the channel substrate in terms of increased fines and higher pool density; however, channel substrate remains largely natural. The effect of these differences on the biota are generally too subtle to be detected by richness-based metrics and can only be discerned when investigating functional traits of BMI that inhabit affected reaches (Stein *et al.* 2013). Unfortunately, with a few exceptions, information on functional traits, pollution tolerances, or niche preferences for many of the "new" species identified through barcoding is currently lacking. As basic research makes use of the species identifications from barcoding, relationships between changes in species or community composition and changes in environmental conditions begin to emerge. Incorporation of a functional traits approach holds promise for increasing the sensitivity and diagnostic power of bioassessment tools based on barcoding results to subtle environmental changes or impacts (Poff *et al.* 2006, Chessman *et al.* 2007).

The Simuliidae provide an example of the potential benefits associated with incorporation of life history information to newly identified species through barcoding to help discern subtle environmental effects. Barcoding results suggest that six species of *Simulium* Latreille were present at the study sites. Comparison to the public reference library in BOLD allowed us to identify five of the six species*, Simulium piperi, S. bracteatum, S. argus, S. paynei,* and *S. vittatum*. *S piperi* and *S. bracteatum* are typically associated with high quality, well-shaded streams with cool, flowing water (McCreadie and Colbo 1992, Adler *et al.* 2004). In contrast, *S. vittatum* is considered a more tolerant species that can be found in high temperature or low oxygen environments often associated with poor shading and lower velocity flows. The locations where these individuals were collected seem to generally correspond to respective documented habitat preferences, suggesting that species level identification could provide additional diagnostic power through development of new or modified metrics.

Previous researchers have also noted the potential improvement in diagnostic ability of bioassessment by focusing on groups where current taxonomy is poorly described or logistically difficult, such as Chironomidae (Pilgrim *et al.* 2011, Sweeney *et al.* 2011). The Chironomidae are a diverse group that exhibit a wide range of pollution tolerances (Lencioni *et al.* 2012), but are typically only identified to family or genus level. The difficulty in obtaining species level data deprives managers of information associated with the diversity, niche specialization, and relative disturbance tolerances of individual species (Pilgrim *et al.* 2011, Sweeney *et al.* 2011). Similar advantages for black flies were observed in the genus *Simulium* where higher resolution taxonomy using DNA barcoding to the species level could provide information on the specific stressors impacting the system, as well as improve the ability to detect impacts along a gradient of stress. This large genus contains about 40 subgenera worldwide, with 11 subgenera and 153 species recognized in North America (Adler *et al.* 2004). Distributions of black flies are often correlated with such factors as physiochemical gradients, substrate type, stream velocity and depth (Aldler and Kim 1984, Adler and McCreadie 1997). In addition, the impact of human stressors, such as the presence of impoundments, siltation, and various forms of pollution, is reflected in the tolerances of different black fly species (McCreadie and Colbo 1992, Adler *et al.* 2004).

In cases where positive identification of previously undetected species is not possible by comparing sequences to existing databases, barcoding results can be used to produce molecular operational taxonomic unit (MOTU) designations that can still be considered as distinct "species" for the purposes

of metric calculation. This can help provide additional insight into relative site conditions and effect of stressors. Gaps in the taxonomic knowledge of groups with diagnostic potential can be prioritized for additional work to refine morphologic keys that can ultimately lead to species identifications. This was case for the baetids in this study. Initial taxonomic analysis identified three species, *B. tricaudatus, B. adonis* and *Baetis sp. CA1*; however, barcoding analysis suggests that there may be up to six distinct species. Local taxonomists are currently using voucher specimens from this project that have been sorted into the six distinct haplotype groups in an attempt to refine taxonomic keys and resolve some of the cryptic nature within the Baetidae of California.

Application of DNA barcoding to routine bioassessment programs will necessitate additional efforts to advance the general science of freshwater benthic ecology. As new taxonomic units with potential diagnostic capability are identified, it will require basic alpha taxonomy to describe these species and update existing taxonomic keys and databases (Pfrender *et al.* 2010). Research on the life histories, niche preferences, and pollution tolerances of these newly identified species will be necessary to take full advantage of the enhanced information they provide (Poff *et al.* 2006, Van den Brink 2010).

As shown in this study and others, DNA barcoding can provide improvement to existing benthic macroinvertebrate based bioassessment programs by allowing development of new or improved metrics focusing on currently under described and underutilized taxonomic groups. Additional benefits include applications for quality control, taxonomic standardization, and improving keys (Pilgrim *et al.* 2011, Sweeney *et al.* 2011). It is likely that barcoding will be increasingly used to augment or support existing methods and to help improve taxonomic capacity at the local level in a relatively cost-effective manner.

Use of barcoding for bioassessment includes challenges that must be overcome before it can be fully integrated into routine bioassessment. First and foremost, a robust reference library must be developed and vouchered. Standard handling and quality control procedures must be developed to reduce risk of loss of samples due to contamination or DNA degradation (as happened in one of the sites in this study). Improved primers need to be developed for certain taxonomic groups to minimize bias due to differential amplification. Additional research needs to be conducted to fully understand the effect of short-sequence reads on conclusions about taxonomic resolution. For example, shorter sequences tend to increase genetic distances and may lead to erroneous conclusions about species richness (Kimura 1980). Given the small percentage of short sequences (i.e., <200 base pairs) used in the study, the likelihood that this issue affected the overall conclusions seems small. Finally, research involving the exploration of additional loci beyond COI is needed to provide more certainty in species delimitation (Pfrender *et al.* 2010). This needs to be accompanied by exploration and standardization of new delimitation methods, including model based approaches that predict species divergence using coalescent or other theories (Pons *et al.* 2006, Monaghan *et al.* 2009, Nuñez *et al.* 2012).

Looking to the future, the current single-specimen approach to DNA barcoding, using traditional Sanger sequencing, provides a critical stepping stone toward future applications of barcoding that have even greater potential to affect routine biomonitoring. Bulk sample sequencing using next generation methods show promise at allowing programs to process large volumes of composite samples in a mixed matrix, extract the DNA in bulk and produce a list of component species (Hajibabaei *et al.* 2011). This approach may increase the speed and reduce the effort associated with obtaining taxonomic information necessary for bioassessment. However, reference libraries produced using current single specimen Sanger methods will still be necessary to provide Linnaean taxonomic identities for the sequence data produced by next-generation methods.

Finally, DNA barcoding also has the potential to expand the breadth of the bioassessment arena by involving organisms not currently in widespread use, such as soft-bodied algae and hyporheic invertebrates. Despite the fact that these groups are known to be functionally important in freshwater ecosystems, they are rarely used for bioassessment because of limitations in existing taxonomic knowledge. Building on the momentum demonstrated here and by others on benthic macroinvertebrates, barcoding and other molecular methods seem to offer great promise in further advancing bioassessment using important but heretofore unavailable groups of organisms.

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