Does DNA barcoding improve performance of traditional stream bioassessment metrics?

Eric D. Stein[1,5], Bryan P. White[1,6], Raphael D. Mazor[1,7], John K. Jackson[2,8], Juliann M. Battle[2,9], Peter E. Miller[3,10], Erik M. Pilgrim[4,11], and Bernard W. Sweeney[2,12]

[1] Southern California Coastal Water Research Project, Costa Mesa, California 92626 USA
[2] Stroud Water Research Center, Avondale, Pennsylvania 19311 USA
[3] Canadian Centre for DNA Barcoding, Biodiversity Institute of Ontario, University of Guelph, Guelph, Ontario, Canada N1G 2W1
[4] National Exposure Research Laboratory, US Environmental Protection Agency, Cincinnati, Ohio 45268 USA

Abstract: Benthic macroinvertebrate community composition is used to assess wetland and stream condition and to help differentiate the effects of stressors among sites. Deoxyribonucleic acid (DNA) barcoding has been promoted as a way to increase taxonomic resolution and, thereby, to increase the sensitivity of bioassessment metrics. We compared the ability of several commonly used bioassessment metrics calculated with data derived from morphology and from DNA barcoding to detect differences in stream condition of 6 paired sites in southern California with relatively subtle impacts to habitat. At each site, we sampled an upstream (reference) reach and a downstream (impact) reach with armored stream banks. We counted and identified ~600 organisms/sample based on morphology (generally to species, but to genus for midges). We then extracted mitochondrial (mt)DNA from each individual and sequenced the ~658-base pair (bp) barcoding region of the cytochrome *c* oxidase subunit I (COI) gene. Most (91%) organisms yielded sequences >350 bp in length, but high failure rates for all taxa collected from 1 stream required that we exclude it from analysis. Sixteen metrics calculated with morphological data showed subtle but not significant differences in community composition between armored and unarmored reaches. The statistical power of 10 of the 16 metrics was substantially higher when calculated with DNA than with morphological data, and we were able to discern differences between armored and unarmored reaches with the DNA data. These differences were associated with increased taxonomic richness detected for midges, mayflies, noninsects, caddisflies, and black flies when DNA data were used. Our results suggest that identifications based on DNA barcoding have the potential to improve power to detect small changes in stream condition.

Key words: DNA barcoding, bioassessment, stressor-response, metric evaluation, streambank armoring

Bioassessment is an attractive evaluation tool because resident organisms integrate the influences of environmental conditions over time and space and can be better indicators of overall environmental health than measurements of individual stressors or ecosystem attributes (Schoolmaster et al. 2012). Biotic indices provide information on the 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, the component metrics of the biotic indices often must be used to detect subtle changes or effects of individual stressors (Hawkins 2006). Use of coarse taxonomic resolution can obscure patterns in bioassessment metrics and hinder detection of biological impacts. Thus, fine-scale taxonomic resolution is 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 typically are done by observing morphologic characteristics. Limited taxonomic resources, cryptic species, small size, damaged specimens, and polymorphism can make identification to the species level difficult or impossible. Deoxyribonucleic acid (DNA) barcoding has been promoted as an alternative to taxonomic identification that could be used routinely in bioassessment (Hebert et al. 2003, 2004, Stoeckle and Hebert 2008,

E-mail addresses: [5]erics@sccwrp.org; [6]byranw@sccwrp.org; [7]raphaelm@sccwrp.org; [8]jkjackson@stroudcenter.org; [9]jbattle@stroudcenter.org
[10]pemiller@uoguelph.ca; [11]pilgrim.erik@epa.gov; [12]sweeney@stroudcenter.org

DOI: 10.1086/674782. Received 28 February 2013; Accepted 14 October 2013; Published online December 12, 2013.
Freshwater Science. 2014. 33(1):302–311.

Borisenko et al. 2009, Janzen et al. 2009). Barcoding is a method of identifying taxa based on a short DNA sequence from a standardized genetic locus, such as the mitochondrial gene cytochrome *c* oxidase subunit I (COI) for most metazoans. Standard molecular methods are used to extract DNA from specimen tissue and to sequence the ~658-base pair (bp) barcoding region of COI (Hebert et al. 2003). DNA from unknown specimens can be identified by comparing their barcode sequences to sequences in 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 be insufficient for identification of all taxa in a community sample (Pfrender et al. 2010). For example, in an assessment of nematode diversity based on genetic sequencing, use of a single gene sequence led to underestimation of the number of species (Porazinska et al. 2009). Others have expressed concern that reducing community composition to a list of operational taxonomic units (or putative species) might dilute appreciation of actual community composition and mask relationships between species behavior/adaptation and the requisite habitat characteristics (Cameron et al. 2006).

DNA barcoding has the ability to provide 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. 2014). For example, use of DNA barcoding resulted in a >5× increase in the number of sensitive Ephemeroptera, Plecoptera, and Trichoptera (EPT) taxa detected, and improvements were most marked for rare species, which may be diagnostic of specific habitat or water-quality conditions (Zhou et al. 2010). Pilgrim et al. (2011) used DNA barcoding and detected up to 3× more EPT taxa than had been previously documented in Maryland. When averaged over all aquatic insect orders, 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 (Sweeney et al. 2011).

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 2 stream reaches ~4 km apart, one surrounded by natural riparian forest and the other by orchards and farms. The number of taxa detected was 70% higher with barcode than with morphological data. The increased number of taxa improved the sensitivity of taxonomic-richness-based bioassessment metrics to discern differences between the 2 sites.

We built on the work of Sweeney et al. (2011) by testing the ability of bioassessment metrics and indices derived from barcode data to discern subtle environmental effects associated with armoring of stream banks. We took advantage of a companion study in which the effects of bank armoring were evaluated in 5 paired stream reaches, 1 armored and 1 unarmored reach 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, which exacerbate 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).

We obtained DNA barcodes for nearly every organism collected during the companion study to answer the following questions: 1) Does using DNA barcoding change estimates of taxonomic richness and richness-based bioassessment metrics? 2) If DNA barcoding results in higher taxonomic richness, how does this affect statistical power of key metrics? 3) Does DNA barcoding increase the ability to predict differences between impacted (armored) and unimpacted (unarmored) stream reaches?

METHODS

Study sites

We investigated biological effects of stream channel armoring at 5 streams in the Los Angeles region, southern California, USA (Table 1). Specimens were collected from 6 streams, but sequence data were obtained for only 5 of the 6 sites. For the 6th site, Arroyo Simi (L1), we generated barcode data for only 11% of specimens. This low level of success was spread evenly among different groups of benthic macroinvertebrates, and the same taxonomic groups were barcoded successfully for the other 5 sites. We tried reextracting DNA from the Arroyo Simi samples at 2 laboratories (University of Guelph and US Environmental Protection Agency [EPA]-Cincinnati), but DNA quality and quantity were too low for polymerase chain reaction (PCR) amplification. We think the low PCR amplification success for all specimens from this site resulted from some sort of

Table 1. Summary of study sites. Site L1 (Arroyo Simi) was excluded from the study because barcodes were obtained for only 11% of the specimens. Q_{10} = 10-y discharge, cfs = cubic feet/s, W. = west, E. = east, R. = river.

Watershed position	Site code	Site name	Watershed area (km^2)	Upstream effects on hydrology	Landscape setting	Slope (%)	Q_{10} ($cfs/m^3\ s^{-1}$)	Predominant land use	Nature and type of bank armoring
Mountainous/ upper watershed	M1	W. Fork San Gabriel R.	215	Cogswell Dam; upstream fire in prior year	River gorge	1.68	8667/245	Forest	Concrete structure on right bank of outside bend of active channel, ~45°, ~5 m tall
	M2	E. Fork San Gabriel R.	205	None	Montane alluvial valley	1.59	8218/233	Forest	Gabions filled with riprap on left bank of outside bend of active channel, 90°, ~5 m tall
Transition zone/foothills	T1	Big Tujunga	298	Big Tujunga Dam; upstream fire in prior year	Proximal alluvial fan	1.39	5119/145	Mixed scrub–shrub + urban	Concrete structure on left bank of outside bend active channel, ~45°, ~5 m tall
	T2	Arroyo Seco	49	Upstream fire in prior year	River gorge	2.64	3342/95	Mixed forest + urban	Concrete structure on right bank of inside bend of active channel, 90°, ~10 m tall
Lower watershed	L2	Conejo Creek	197	None	Alluvial valley	0.12	5326/151	Agriculture	Concrete structure on right bank of outside bend of active channel, ~45°, ~5 m tall

sample contamination or mishandling of the DNA preservation protocol and is 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 our results represented the range of stream types where bank armoring projects are typically constructed in our region, we selected study sites in 3 different watershed positions (mountain, transitional, lowland) with watershed areas of 49 to 298 km^2, channel slopes of 0.12 to 2.64%, pool–riffle morphology, and discrete segments of bank armoring ranging from 100 to 200 m long (Table 1). All channel bottoms consisted of natural substrates.

Each study reach consisted of a 150-m-long, unarmored upstream control segment A and a 100- to 200-m-long segment with armoring on at least 1 bank (impact segment B). Armored segments (B) were most often along the outside of meander bends. In all cases, 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 assertion is supported by observations of historical planform photographs of the 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 >10 y old at all sites. Thus, we think the present-day channel patterns strongly reflect the influence of the bank-armoring structures, and physical differences between stream segments can be interpreted as resulting, at least in part, from bank armoring.

Biological assessment

We sampled benthic macroinvertebrates (BMIs) using the multihabitat method described by Ode (2007). Each 150-m segment was divided into 11 equidistant transects, and we used a 500-µm-mesh D-frame net to collect BMIs from a prescribed location along each transect (i.e., 25, 50, or 75% of the distance across the stream). We sampled a total of 0.9 m^2 of stream bed, composited the 11 subsamples into one sample, and preserved specimens immediately with 95% ethanol. We drained samples and added fresh ethanol within 24 h of collection to maintain a minimum 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 (level 2; Richards and Rogers 2006). We used species composition and abundance data 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

After specimens were identified based on morphology, we removed a small piece of tissue from each specimen and placed it in a well in a 96-well plate. We capped the wells and shipped the plates 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; http://ccdb.ca/resources.php). Personnel at CCDB removed the well caps to allow the ethanol to evaporate completely from the plates. Upon complete evaporation, they added lysis solution to the plates and extracted DNA the next day. DNA extracts were amplified by PCR with 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 1^{st}- and 2^{nd}-pass PCR, primers that target shorter barcode regions (~400 bp) were used. PCR amplicons were sequenced bidirectionally by Sanger sequencing with BigDye v3.1 in an ABI 3730xl DNA Analyzer (Applied Biosystems, Foster City, California). Sequences and detailed information about all specimens were uploaded to the Barcode of Life Data Systems (Ratnasingham and Hebert 2007) and can be accessed via the project codes CFWIA through CFWIJ. Nucleotide sequence data were exported from BOLD, and DNA sequences were aligned with ClustalW with default parameters in MEGA 5.05 (Tamura et al. 2011).

Species delimitation

We used barcode sequences >350 bp to construct neighbor joining (NJ) trees for each taxonomic group at the order and class level (Saitou and Nei 1987) with the Kimura 2-parameter (K2P) distance nucleotide model (Kimura 1980). We assigned molecular operational taxonomic unit (MOTU) identifications to individual specimens based on the genetic distance thresholds identified by Jackson et al. (2014) so that all taxonomic groups were delimited manually with a 2% distance threshold applied to branch lengths, except *Simulium* and *Baetis*, which were delimited at 3 and 1% distance thresholds, respectively. We added specimens with a sequence length <350 bp to existing MOTUs based on a 2% K2P distance threshold.

Data analysis

We calculated bioassessment metrics with taxonomic information derived from morphologic identifications and from DNA barcoding. We tested for differences between armored and unarmored segments with Wilcoxon signed-rank tests. Unless otherwise stated, we used $\alpha = 0.05$ and 2-tailed tests for all inferential analyses. We obtained equal sample sizes across the 10 sampling locations by randomly permuting samples without replacement. We

Table 2. Primers and primer sequences used in our study. BOLD = Barcode of Life Database.

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

used the mean richness of the permutations to calculate richness metrics. We calculated the power of metrics to detect differences as a paired, 2-tailed *t*-test for the average difference between unarmored (U) and armored (A) reaches for the 5 southern California streams.

RESULTS

The 10 sampling locations yielded 5870 specimens that underwent DNA barcoding. DNA sequences were obtained from 5478 (93.3%) specimens (Fig. 1). Of those sequences, 368 (6.7% of the total) were between 125 and 500 bp (partial barcodes) and 5110 (87.1%) were between 500 and 658 bp (full barcodes).

Medians and ranges of richness values for 12 of the 16 metrics evaluated were higher when calculated with barcode data than with morphology data (Fig. 2). Differences were greatest for overall richness and richnesses 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 at all sites and was particularly pronounced for common taxa, such as Diptera and Chironomidae.

Differences in metric values between unarmored and armored stream reaches reflected the increased taxo-

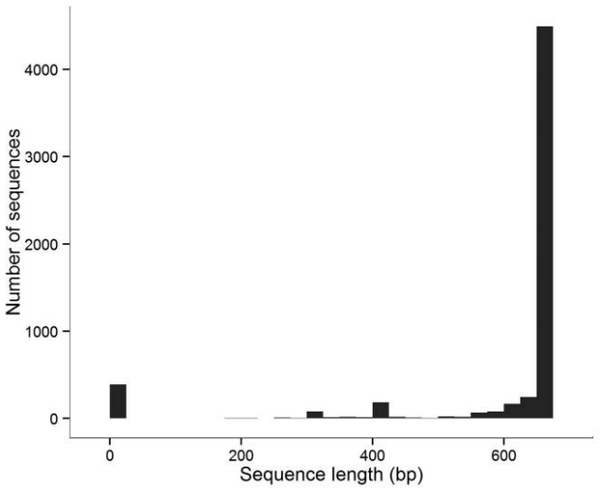

Figure 1. Relative abundance of base pair (bp) sequences as a function of sequence length.

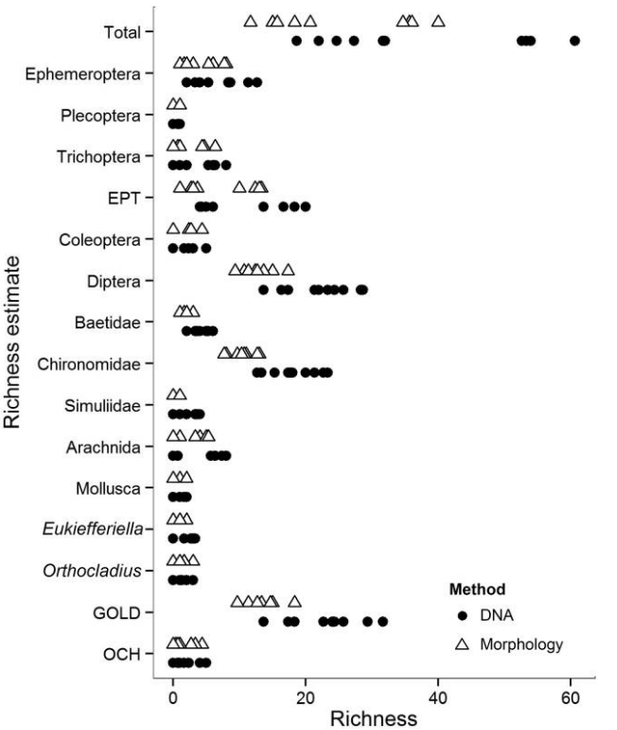

Figure 2. Taxonomic richness metrics for 16 groups of macroinvertebrates from 5 southern California streams (2 reaches at each stream) based on identifications made with deoxyribonucleic acid (DNA) barcoding and morphology. EPT = Ephemeroptera, Plecoptera, Trichoptera; GOLD = Gastropoda, Oligochaeta, Diptera; OCH = Odonata, Coleoptera, Hemiptera.

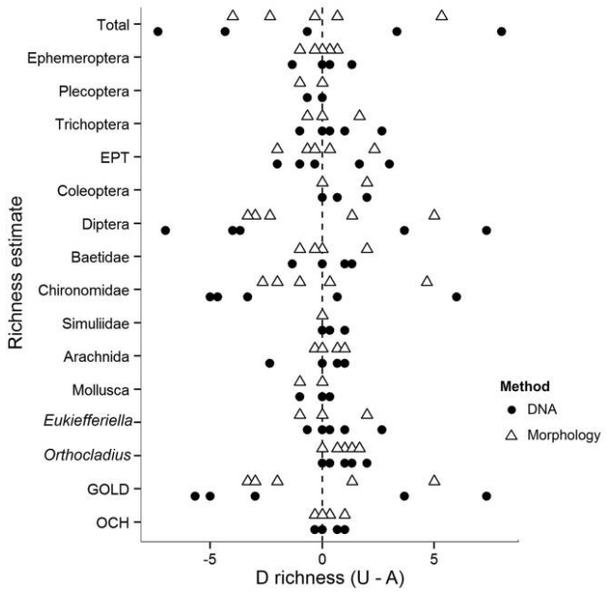

Figure 3. Differences in taxonomic richness between unarmored (U) and armored (A) reaches from 5 southern California streams for 16 groups of macroinvertebrates based on identifications made with DNA barcoding and morphology. See Fig. 2 for abbreviations.

nomic resolution associated with barcoding (Fig. 3). In particular, differences in Diptera, Chironomidae, and GOLD richness between unarmored and armored reaches were much larger for barcode-derived than for morphology-derived metrics. Barcode-derived richness values were higher than morphology-derived metrics at all sites, but the differences were most pronounced at higher-quality sites (M1 and M2), where channel armoring may have had a relatively larger effect on instream biota (Fig. 4). At lower-quality sites (e.g., L2), barcode-derived richness values were still higher at unarmored reaches, but the difference was smaller.

Power of all metrics was low, but it was higher for barcode- than for morphology-derived versions of 10 of the 16 metrics (Fig. 5). The largest differences in power (0–0.18 power) were observed for relatively common taxonomic groups for which diversity is often underestimated because specimens typically are identified to genus level or higher. For example, the increase in power was greatest for Simuliidae because larval simuliids typically are identified only to genus level even though they are a speciose group. Power also increased notably for Trichoptera and Coleoptera. However, power decreased for groups, such as Arachnida (Acari), Mollusca, and *Orthocladius* (a highly speciose genus of midge), because among-site variance was high. Power was independent of richness, and increases in power did not depend on the power associated with the taxonomy-derived metrics (Fig. 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 at a site can be interpreted only at the taxonomic level to which spe-

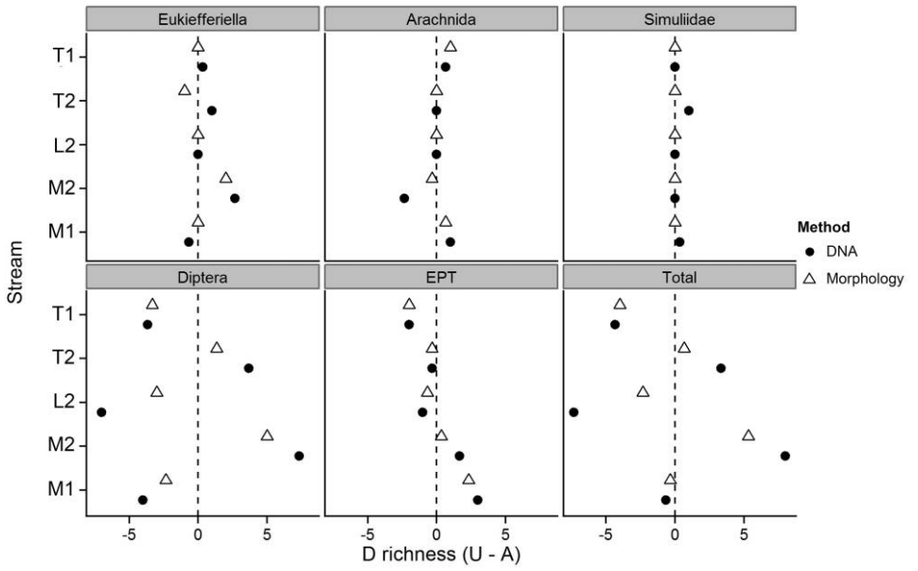

Figure 4. Differences in taxonomic richness between unarmored (U) and armored (A) reaches from 5 southern California streams for 6 groups of macroinvertebrates based on identifications made with DNA barcoding and morphology. See Fig. 2 for abbreviations.

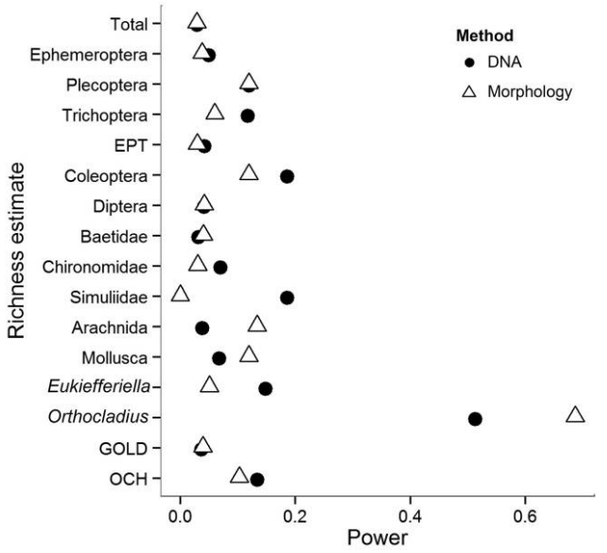

Figure 5. Relationship between estimates of taxonomic richness for 16 groups of macroinvertebrates and statistical power of metrics based on identifications made with DNA barcoding and morphology. See Fig. 2 for abbreviations.

cies can be identified. Limitation of our ability to produce species-level data because of incomplete taxonomic knowledge, the condition of the specimen, poor taxonomic keys, shortage of trained taxonomists, or cost 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. Existing tools discriminate adequately between reference and highly degraded streams, but they are less sensitive to subtle changes associated with the middle ranges of disturbance gradients or where multiple confounding stressors affect a site (Waite et al. 2004, Stribling et al. 2008). 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 the number of metrics denoting changes in water quality between a natural and agricultural site in Pennsylvania when barcode data were used instead of morphological data. We demonstrated similar success in a variety of environmental settings at 5 pairs of sites subjected to minor localized effects associated with bank armoring. Thus, the benefits associated with the improved taxonomic resolution of barcoded data are not specific to a particular geographic region, climate, or stressor.

Increased metric sensitivity associated with barcoding was most pronounced at high-quality (i.e., relatively unimpacted) sites, which often have higher species richness and are inhabited by undescribed, cryptic, or regionally rare species. For example, 43% of the additional taxa identified through barcoding consisted of 1 or 2 individuals and occurred at only 1 stream. The presence or absence of rare species may be diagnostic of specific environmental changes, so the increased information provided by barcoding at taxon-rich sites allows finer-scale resolution of sources of stress and increases our ability to detect subtle changes in environmental quality.

Our study streams were subjected to mild forms of degradation that resulted in subtle changes in the benthic community (Stein et al. 2013). Bank armoring affects channel substrate indirectly and leads to a higher percentage of fine sediments and higher pool density, but the substrate remains largely natural. The effect of these changes on the biota is generally too subtle to be detected with richness-based metrics and can be discerned only when investigating functional traits of BMIs in affected reaches (Stein et al. 2013). However, with a few exceptions, we currently lack information on functional traits, pollution tolerances, or niche preferences of many of the "new" species identified through barcoding. As basic researchers make use of the species identifications from barcoding, we can begin to relate changes in species or community composition to changes in environmental conditions. The functional-traits approach holds promise for increasing the sensitivity and diagnostic power of barcode-based bioassessment tools to subtle environmental changes or impacts (Poff et al. 2006, Chessman et al. 2007).

The Simuliidae provide an example of the potential benefits to bioassessment efforts of combining barcode-based taxonomy and the functional-traits approach to detect subtle environmental effects. Barcoding results suggest that 6 species of *Simulium* Latreille were present at

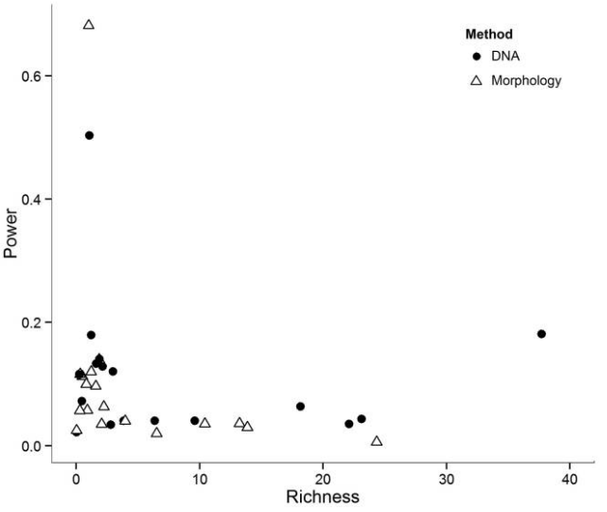

Figure 6. Relationship between statistical power of metrics and mean taxonomic richness (n = 10) calculated for 16 groups of macroinvertebrates based on identifications made with deoxyribonucleic acid (DNA) barcoding and morphology.

our study sites. We used the public reference library in BOLD to identify 5 of the 6 species as *Simulium piperi*, *Simulium bracteatum*, *Simulium argus*, *Simulium paynei*, and *Simulium vittatum*. *Simulium piperi* and *S. bracteatum* typically are associated with high-quality, well-shaded streams with cool, flowing water (McCreadie and Colbo 1992, Adler et al. 2004). In contrast, *S. vittatum* is a more tolerant species that can be found in high-temperature or low-O_2 environments often associated with poor shading and lower-velocity flows. The sites at which we collected these species generally corresponded to their documented habitat preferences, a result suggesting that species-level identification could provide additional diagnostic power through development of new or modified metrics.

Previous researchers have suggested that the diagnostic ability of bioassessment metrics might be improved if groups that are taxonomically difficult to identify could be used. Many of these potentially useful groups are not routinely identified to species because they are poorly described or technically difficult to identify (Pilgrim et al. 2011, Sweeney et al. 2011). For example, the Chironomidae are diverse and exhibit a wide range of pollution tolerances (Lencioni et al. 2012), but typically are identified only to family or genus level. The difficulty of 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). Similarly, the genus *Simulium* (black flies) contains ~40 subgenera worldwide, with 11 subgenera and 153 species recognized in North America (Adler et al. 2004). Distributions of black fly species often are correlated with environmental factors, such as physiochemical gradients, substrate type, stream velocity, and depth (Adler and Kim 1984, Adler and McCreadie 1997). In addition, black fly species have differing tolerances of human stressors, such as impoundments, siltation, and various forms of pollution (McCreadie and Colbo 1992, Adler et al. 2004). The higher taxonomic resolution provided by barcode data would enable use of such species-level information to understand specific stressors that might be affecting a site and would improve our ability to detect impacts along a gradient of stress.

In cases where positive identification of previously undetected species cannot be made by comparing sequences to existing databases, barcoding results can be used to produce MOTUs that can be used as fine-level taxa for the purpose of metric calculation. Use of MOTUs can provide additional insight into site conditions and effects of stressors. Gaps in the taxonomic knowledge of groups with diagnostic potential could be prioritized for additional research to develop morphologic keys that would yield species identifications. For example, in our study, 3 species (*Baetis tricaudatus*, *Baetis adonis*, and *Baetis* sp. CA1) were identified in the morphological taxonomic analysis, but barcode data suggest that as many as 6 species might exist at our study sites. Local taxonomists are using voucher specimens from our project that have been sorted into the 6 distinct haplotype groups in an attempt to develop taxonomic keys and resolve some of the cryptic taxa within the Baetidae of California.

Application of DNA barcoding in routine bioassessment programs will necessitate additional work to advance freshwater benthic ecology. As new taxonomic units with potential diagnostic capability are identified, taxonomists will have to describe these species and update existing taxonomic keys and databases (Pfrender et al. 2010). Research will be needed on the life histories, niche preferences, and pollution tolerances of these newly identified species to make full use of the enhanced information they provide (Poff et al. 2006, van den Brink et al. 2010).

The DNA barcoding approach can improve existing BMI-based bioassessment programs by enabling development of new or improved metrics based on taxonomic groups that currently are under-described and underused. Additional benefits include applications for quality control, taxonomic standardization, and improvement of taxonomic keys (Pilgrim et al. 2011, Sweeney et al. 2011). Barcoding probably will be used with increasing frequency to augment or support existing methods and to provide cost-effective improvement of taxonomic capacity.

However, full integration of barcode data in routine bioassessment will be challenging. First, a robust barcode reference library must be developed and vouchered. Standard handling and quality-control procedures must be developed to reduce risk of loss of samples because of contamination or DNA degradation (as happened for 1 of the sites in our study). Improved primers are needed for certain taxonomic groups to minimize bias caused by differential amplification. More research is needed on the effect of short-sequence reads on conclusions about taxonomic resolution. For example, inclusion of shorter sequences tends to increase genetic distances and may lead to erroneous conclusions about species richness (Kimura 1980). Given the small percentage of short sequences (<200 bp) in our study, the likelihood that this issue affected our overall conclusions seems small. Last, the use of loci in addition to COI should be explored to provide more certainty in species delimitation (Pfrender et al. 2010). This research should be accompanied by exploration and standardization of new methods of delimitation, 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).

The current single-specimen approach to DNA barcoding based on Sanger sequencing is a critical stepping stone toward future applications of barcoding that have even greater potential to affect routine biomonitoring. Bulk sample sequencing with next-generation methods are a promising way to process large volumes of composite samples in a mixed matrix, extract the DNA in bulk, and pro-

duce 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 with single-specimen Sanger methods will still be needed to provide Linnaean taxonomic identities for the sequence data produced by next-generation methods.

Barcoding and other molecular methods offer great promise in advancing bioassessment using important but, heretofore, unavailable groups of organisms, such as soft-bodied algae and hyporheic invertebrates. These groups are functionally important in freshwater ecosystems, but they are rarely used for bioassessment because of limitations in existing taxonomic knowledge.

ACKNOWLEDGEMENTS

We thank the teams from all institutions that cooperated to carry out this work. Kristin Smith of the Southern California Coastal Water Research Project (SCCWRP) was instrumental in site selection, reconnaissance, and field work. We thank Jeff Brown and Greg Lyon of SCCWRP for assistance with field work. Field assistance with BMI sampling was provided by California State University (CSU) Stanislaus students Veronica Menendez, Tamera Rogers, and Brittany Bjelde. Taxonomic expertise for benthic macroinvertebrates was provided by Daniel Pickard and the staff at the Department of Fish and Game Aquatic Bioassessment Laboratory at CSU Chico. Chris Solek provided assistance in interpreting the black fly data. Sequencing of the DNA barcodes was graciously provided by the Canadian Centre for DNA Barcoding with support from the Life Technologies Corporation. Funding for JKJ, JMB, and BWS was provided by the Pennswood Endowment Fund and the Stroud Water Research Center Endowment Funds.

LITERATURE CITED

Adler, P. H., D. C. Currie, and D. M. Wood. 2004. The black flies (Simuliidae) of North America. Cornell University Press, Ithaca, New York.

Adler, P. H., and K. C. Kim. 1984. Ecological characterization of two sibling species, IIL-1 and IS-7, in the *Simulium vittatum* complex (Diptera: Simuliidae). Canadian Journal of Zoology 62:1308–1315.

Adler, P. H., and J. W. McCreadie. 1997. The hidden ecology of black flies (Simuliidae): sibling species and ecological scale. American Entomologist 43:153–161.

Bonada, N., N. Prat, V. H. Resh, and B. Statzner. 2006. Developments in aquatic insect biomonitoring: a comparative analysis of recent approaches. Annual Review of Entomology 51:495–523.

Borisenko, A. V., J. E. Sones, and P. D. N. Hebert. 2009. The front-end logistics of DNA barcoding: challenges and prospects. Molecular Ecology Resources 9:27–34.

Cameron, S., D. Rubinoff, and K. Will. 2006. Who will actually use DNA barcoding and what will it cost? Systematic Biology 55:844–847.

Chessman, B., S. Williams, and C. Besley. 2007. Bioassessment of streams with macroinvertebrates: effect of sampled habitat and taxonomic resolution. Journal of the North American Benthological Society 26:546–565.

Folmer, O., M. Black, W. Hoeh, R. Lutz, and R. Vrijenhoek. 1994. DNA primers for amplification of mitochondrial cytochrome *c* oxidase subunit I from diverse metazoan invertebrates. Molecular Marine Biology and Biotechnology 3(5):294–299.

Hajibabaei, M., S. Shokralla, X. Zhou, G. A. C. Singer, and D. J. Baird. 2011. Environmental barcoding: a next-generation sequencing approach for biomonitoring applications using river benthos. PLoS ONE 6:e17497.

Hawkins, C. P. 2006. Quantifying biological integrity by taxonomic completeness: its utility in regional and global assessments. Ecological Applications 16:1277–1294.

Hebert, P. D. N., A. Cywinska, S. L. Ball, and J. R. deWaard. 2003. Biological identifications through DNA barcodes. Proceedings of the Royal Society London Series B: Biological Sciences 270:313–321.

Hebert, P. D. N., M. Y. Stoeckle, T. S. Zemlak, and C. M. Francis. 2004. Identification of birds through DNA barcodes. PLoS Biology 2:e312.

Ivanova, N. V., J. R. deWaard, and P. D. N. Hebert. 2006. An inexpensive, automation-friendly protocol for recovering high-quality DNA. Molecular Ecology Notes 6:998–1002.

Jackson, J. K., J. M. Battle, B. P. White, E. M. Pilgrim, E. D. Stein, P. E. Miller, and B. W. Sweeney. 2014. Cryptic biodiversity in streams—a comparison of macroinvertebrate communities based on morphological and DNA barcode identifications. Freshwater Science 33:312–324.

Jacobson, R. B., S. R. Femmer, and R. A. McKenney, 2001. Land-use changes and the physical habitat of streams: a review with emphasis on studies within the US Geological Survey Federal–State Cooperative Program. Circular 1175. US Geological Survey, Reston, Virginia. (Available from: http://pubs.usgs.gov/circ/circ1175/pdf/circ1175.pdf)

Janzen, D. H., W. Hallwachs, P. Blandin, J. M. Burns, J. M. Cadiou, I. Chacon, T. Dapkey, A. R. Deans, M. E. Epstein, B. Espinoza, J. G. Franclemont, W. A. Haber, M. Hajibabaei, J. P. W. Hall, P. D. N. Hebert, I. D. Gauld, D. J. Harvey, A. Hausmann, I. J. Kitching, D. LaFontaine, J. F. Landry, C. Lemaire, J. Y. Miller, J. S. Miller, L. Miller, S. E. Miller, J. Montero, E. Munroe, S. R. Green, S. Ratnasingham, J. E. Rawlins, R. K. Robbins, J. J. Rodriguez, R. Rougerie, M. J. Sharkey, M. A. Smith, M. A. Solis, J. B. Sullivan, P. Thiaucourt, D. B. Wahl, S. J. Weller, J. B. Whitfield, K. R. Willmott, D. M. Wood, N. E. Woodley, and J. J. Wilson. 2009. Integration of DNA barcoding into an ongoing inventory of complex tropical biodiversity. Molecular Ecology Resources 9:1–26.

Jones, F. C. 2008. Taxonomic sufficiency: the influence of taxonomic resolution on freshwater bioassessments using benthic macroinvertebrates. Environmental Reviews 16:45–69.

Karr, J. R., and E. W. Chu. 1999. Restoring life in running waters: better biological monitoring. Island Press, Washington, DC.

Kimura, M. 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. Journal of Molecular Evolution 16:111–120.

Lencioni, V., L. Marziali, and B. Rossaro. 2012. Chironomids as bioindicators of environmental quality in mountain springs. Freshwater Science 31:525–541.

McCreadie, J. W., and H. H. Colbo. 1992. Spatial distribution patterns of larval cytotypes of the *Simulium venustum/verecundum* complex (Diptera: Simuliidae) on the Avalon Peninsula, Newfoundland: factors associated with cytotype abun-

dance and composition. Canadian Journal of Zoology 70: 1389–1396.

Monaghan, M. T., R. Wild, M. Elliot, T. Fujisawa, M. Balke, D. J. Inward, D. C. Lees, R. Ranaivosolo, P. Eggleton, T. G. Barraclough, and A. P. Vogler. 2009. Accelerated species inventory on Madagascar using coalescent-based models of species delineation. Systematic Biology 58:298–311.

Morais, M., P. Pinto, P. Guilherme, J. Rosado, and I. Antunes. 2004. Assessment of temporary streams: the robustness of metric and multimetric indices under different hydrological conditions. Hydrobiologia 516:229–249.

Nuñez, J. J., A. Vejar-Pardo, B. E. Guzmán, E. H. Barriga, and C. S. Gallardo. 2012. Phylogenetic and mixed Yule-coalescent analyses reveal cryptic lineages within two South American marine snails of the genus *Crepipatella* (Gastropoda: Calyptraeidae). Invertebrate Biology 131:301–311.

Ode, P. R. 2007. Standard operating procedures for collecting macroinvertebrate samples and associated physical and chemical data for ambient bioassessments in California. California State Water Resources Control Board Surface Water Ambient Monitoring Program (SWAMP). California State Water Resources Control Board, Sacramento, Caliornia. (Available from: http://www.waterboards.ca.gov/water_issues/programs/swamp)

Ode, P. R., A. C. Rehn, and J. T. May. 2005. A quantitative tool for assessing the integrity of southern coastal California streams. Environmental Management 35:493–504.

Pauls, S. U., R. J. Blahnik, X. Zhou, C. T. Wardwell, and R. W. Holzenthal. 2010. DNA barcode data confirm new species and reveal cryptic biodiversity in Chilean *Smicridea* (Smicridea) (Trichoptera:Hydropsychidae). Journal of the North American Benthological Society 29:1058–1074.

Pfrender, M. E., C. P. Hawkins, M. Bagley, G. W. Courtney, B. R. Creutzburg, J. H. Epler, S. Fend, L. C. Ferrington, P. L. Hartzwell, S. Jackson, D. P. Larsen, C. A. Levesque, J. C. Morse, M. J. Peterson, D. Ruiter, D. Schindel, and M. Whiting. 2010. Assessing macroinvertebrate biodiversity in freshwater ecosystems: advances and challenges in DNA-based approaches. Quarterly Review of Biology 85:319–340.

Pilgrim, E. M., S. A. Jackson, S. Swenson, I. Turcsanyi, E. Friedman, L. Weigt, and M. Bagley. 2011. Incorporation of DNA barcoding into a large-scale biomonitoring program: opportunities and pitfalls. Journal of the North American Benthological Society 30:217–231.

Poff, N. L., J. D. Olden, N. K. M. Vieira, D. S. Finn, M. P. Simmons, and B. C. Kondratieff. 2006. Functional trait niches of North American lotic insects: traits-based ecological applications in light of phylogenetic relationships. Journal of the North American Benthological Society 25:730–755.

Pons, J., T. G. Barraclough, J. Gomez-Zurita, A. Cardoso, D. P. Duran, S. Hazell, and A. P. Vogler. 2006. Sequence-based species delimitation for the DNA taxonomy of undescribed insects. Systematic Biology 55:595–609.

Porazinska, D. L., R. M. Giblin-Davis, L. Faller, W. Farmerie, N. Kanzaki, K. Morris, T. O. Powers, A. E. Tucker, W. Sung, and K. Thomas. 2009. Evaluating high-throughput sequencing as a method for metagenomic analysis of nematode diversity. Molecular Ecology Resources 9:1439–1450.

Ratnasingham, S., and P. D. N. Hebert. 2007. BOLD: the barcode of life data system (www.barcodinglife.org). Molecular Ecology Notes 7:355–364.

Richards, A. B., and D. C. Rogers. 2006. List of the freshwater macroinvertebrate taxa from California and adjacent states including standard taxonomic effort levels. Southwest Association of Freshwater Invertebrate Taxonomists, Chico, California. (Available from: www.safit.org)

Riley, A. L. 1998. Restoring streams in cities: a guide for planners, policy makers, and citizens. Island Press, Washington, DC.

Saitou, N., and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4:406–425.

Schoolmaster, D. R., J. B. Grace, and E. W. Schweiger. 2012. A general theory of multimetric indices and their properties. Methods in Ecology and Evolution 3:773–781.

Stein, E. D., M. R. Cover, A. E. Fetscher, C. O'Reilly, R. Guardado, and C. W. Solek. 2013. Reach-scale geomorphic and biological effects of localized stream bank armoring. Journal of the American Water Resources Association 49:780–792.

Stoeckle, M. Y., and P. D. N. Hebert. 2008. Barcode of life. Scientific American 299:82–88.

Stribling, J. B., K. L. Pavlik, S. M. Holdsworth, and E. W. Leppo. 2008. Data quality, performance, and uncertainty in taxonomic identification for biological assessments. Journal of the North American Benthological Society 27:906–919.

Sweeney, B. W., J. M. Battle, J. K. Jackson, and T. Dapkey. 2011. Can DNA barcodes of stream macroinvertebrates improve descriptions of community structure and water quality? Journal of the North American Benthological Society 30:195–216.

Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei, and S. Kumar. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular Biology and Evolution 28:2731–2739.

van den Brink, P. J., A. C. Alexander, M. Desrosiers, W. Goedkoop, P. L. M. Goethals, M. Liess, and S. D. Dyer. 2010. Traits-based approaches in bioassessment and ecological risk assessment: strengths, weaknesses, opportunities and threats. Integrated Environmental Assessment and Management 7: 198–208.

Waite, I. R., A. T. Herlihy, D. P. Larsen, N. S. Urquhart, and D. J. Klemm. 2004. The effects of macroinvertebrate taxonomic resolution in large landscape bioassessments: an example from the Mid-Atlantic Highlands, U.S.A. Freshwater Biology 49:474–489.

Zhou, X., L. M. Jacobus, R. E. DeWalt, S. J. Adamowicz, and P. D. N. Hebert. 2010. Ephemeroptera, Plecoptera, and Trichoptera fauna of Churchill (Manitoba, Canada): insights into biodiversity patterns from DNA barcoding. Journal of the North American Benthological Society 29:814–837.