A novel quantification method for stream-inhabiting, non-diatom benthic algae, and its application in bioassessment

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Abstract

Non-diatom benthic algae from 104 streams in southern California were studied. We present a novel quantification method for non-diatom algae that seeks to improve upon existing methods in terms of the following key elements: 1) processing macroalgae separately from microalgae in order to avoid sample blending and consequent loss of macroalgal integrity, and 2) for better viewing, counting a well-mixed microalgal subsample on a standard microscope slide instead of using a counting chamber. Our method provided high quality taxonomic and quantitative data with a low degree of uncertainty. A total of 260 algal taxa were recorded, 180 of which were identified to species level. The median total algal biovolume per site was 22.7 mm^3 cm^{-2} (range: <0.001 - 836.9 mm^3 cm^{-2}), the median species number was 11 (range: 2 - 43). Total algal biovolume and species number correlated with canopy cover (negative) and water temperature (positive), but not with measured water chemistry constituents. The proportion of heterocystous cyanobacteria and Zygnemataceae were strongly negatively correlated with nitrate concentrations and TN. The red algae proportion was negatively correlated with TP. Species optima calculations combined with indicator species analysis identified >40 algal species as potential indicators of nutrient conditions. Proposed here is a practical tool for non-diatom algal quantification that enhances its application to stream bioassessment.

Introduction

Benthic algae (periphyton) serve as a primary source of energy to aquatic food webs in many streams and rivers (Stevenson 1996). They belong to several classes, representing a wide variety of evolutionary traits, life-forms, and strategies (Sheath and Wehr 2003). There is insufficient information about benthic algal biodiversity, biomass and community structure in more arid areas, and in particular, those of the southern California Mediterranean climatic region (Busse *et al.* 2006). Many studies have shown the value of using diatoms as a component of periphyton to ascertain the ecological conditions and general impairment of streams (Stevenson *et al.* 2010) and consequently the most commonly used periphyton indices are based on diatom metrics. In contrast, non-diatom benthic algae are less studied and therefore many topics in periphyton ecology remain unexplored (Larned 2010). However, numerous studies have proven their relationships with environmental variables (Griffith *et al.* 2002; Foester *et al.* 2004; Hering *et al.* 2006; Porter *et al.* 2008; Schneider and Lindstrøm 2009, 2011) suggesting benefits to inclusion of benthic non-diatom algae in biomonitoring efforts. Benthic algae are considered an important biological component of routine monitoring of ecological status of rivers in European Water Management (European Communities (EC) 2000) and ongoing national biomonitoring programs of the United States Environmental Protection Agency's (USEPA) Environmental Monitoring

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and Assessment Program (EMAP; http://www.epa.gov/emap) and United States Geological Survey's (USGS) National Water-Quality Assessment Program (NAWQA; http://water.usgs.gov/nawqa).

While diatom sampling and laboratory protocols are well established (Kelly *et al.* 2009, Stevenson and Bahls 1999), current methods for non-diatom algae assessment suffer from many shortcomings that limit their application in stream bioassessment. One possible reason that non-diatom algal assemblages receive less attention than diatoms is their challenging quantitative assessment. General difficulties are related to: 1) morphological size differences, 2) life-stages and reproductive structures that need to be observed for complete identification, and 3) the necessity for detailed and careful light microscope (LM) observations of cells or larger colonies or intact thalli for accurate identification to species.

The current approaches to assessing benthic algal species composition and biomass consist of identifying and counting cells microscopically. Ratios of species-specific cell densities or biovolumes to total density or biovolume are used with cell counts to define the proportions of communities (relative abundances) composed by different taxa. According to Stevenson (1996), this method accurately assesses algal biomass and taxonomic shifts, but is time-consuming and may have high error variances. However, this general method is applied with variations and different levels of accuracy in routine laboratory procedures of various programs. For instance, the European Project for the Standardisation of River Classifications protocol (STAR, http://www.eu-star.at/frameset.htm) estimates "macroscopic benthic algae cover" based on the percentage of the stream bottom occupied, while "microscopic benthic algae" are collected and counted separately in a counting cell. As part of the same European Project, the Bavarian Water Management Agency utilizes another method that estimates benthic algae abundance in relative terms, on a 5-score scale (Foerster *et al.* 2004, Schaumburg *et al.* 2004, Kelly *et al.* 2009), while Schneider and Lindstrøm (2009, 2011) applied presence-absence data. Both semi-quantitative and qualitative approaches allow accurate taxonomic identification of benthic algae to species level, but do not yield estimates of their absolute biovolume within the stream reach.

In contrast, USEPA and USGS laboratory protocols are quantitative, with a goal of estimating precisely the density or biovolume of benthic algae (including diatoms and non-diatom algae) from a composite sample which may contain diverse taxa of various sizes (Stevenson and Bahls 1999, Charles *et al.* 2002).

These protocols require blending and breaking up of large filaments or colonies in order to suspend algae into a counting chamber. Biggs (1987) proposed mechanical blending of periphyton samples for dispersing algal cells and clumps in order to reduce subsampling variation by nearly 90% and to improve the accuracy of periphyton analysis. He also states that the blending process generally does not greatly damage cells, although large colonies, long narrow cells, and filamentous algae can be fragmented (Biggs and Kilroy 2007). Because disintegration of colonies of benthic algae can cause changes in color and distortions in sheaths or mucilage in cyanobacteria and other non-diatom algae, such potentially destructive manipulations of specimens are not recommended during the taxonomic identification (Komárek 2003). Furthermore, many macroalgae, such as *Vaucheria*, *Batrachospermum, Sirodotia*, and members of the Zygnemataceae, for which the identification is especially problematic, require examination of their reproductive structures, and destruction of specimen integrity can compromise the ability to do this (Sheath 2003).

Finally, some of the key diagnostic characters of benthic taxa could be lost in the standard 0.4 mm deep counting chamber due to overlapping by larger clumps, or insufficient sections of large filamentous algae for observation. For instance, identification of *Cladophora* species requires viewing of a large portion of the thallus in order to assess branching pattern and apical cell features (van den Hoek 1963). The Palmer-Maloney counting chamber was designed originally for processing planktonic samples (Palmer and Maloney 1954), and therefore may not be well designed for microscopic observation of benthic algae of different sizes and estimation of their biovolume. However, this issue can be mitigated by placing enough microalgal material on glass microscope slide to observe many entities, well mixed and non-overlapping, and to capture high quality LM pictures for taxonomic and documentation purposes (John *et al.* 2002).

The shortcomings of existing quantification methods for benthic non-diatom algae have probably contributed to the frequency with which they are excluded from periphyton bioassessment (STAR; Yagow *et al.* 2006). As a result, important aspects

of the primary producer community may be missed and our knowledge about ecosystem structure and functioning may be affected (Bortolus 2008). The current study was based on extensive sampling of coastal perennial and non-perennial streams in southern California, as part of a project to develop an Algal Index of Biotic Integrity (IBI) for that area. In order to achieve that goal, we modified current available protocols for benthic algae collection and enumeration. Our aim is to 1) present the novel method for benthic non-diatom algae quantification that was applied in this study in order to overcome difficulties with previous methods, and 2) to evaluate the potential of the benthic non-diatom algae to serve as indicators of conditions in southern Californian streams, particularly with respect to nutrients. This information will improve our understanding about the efficacy of benthic non-diatom algae as a practical tool in stream bioassessment programs.

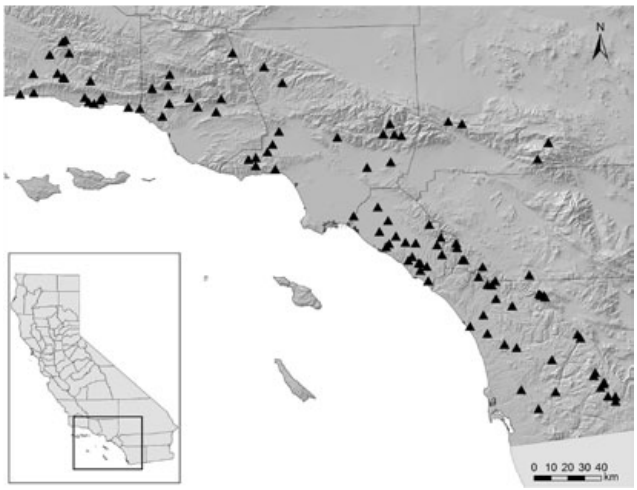

Figure 1. Location map of the 104 stream study sites.

Methods

Selection and Classification of Study Sites

The study region consisted of streams draining coastal watersheds along southern California, from Point Conception in Santa Barbara County to the Mexican border (Figure 1). A total of 104 stream sites were studied. Stream reaches included in the study were selected in order to represent a broad range of factors known or hypothesized to influence periphyton assemblage composition and biomass. These factors included varying degrees of human activity in the contributing watershed in terms of the amount and nature of development (i.e., residential/commercial, industrial, agricultural, and grazing, in addition to pristine or essentially undeveloped open space), which, in conjunction with basin geology, could influence water chemistry parameters downstream. In addition, on-site factors, such as dominant substratum type, amount of canopy cover, gradient, flow, and channel dimensions were taken into consideration.

Field Sampling of Benthic Algae

Stream sites were sampled for benthic algae under dry-season (low-flow) conditions from May through July for two years (2007-2008). Samples were collected using a modification of the "multi-habitat" method of the US Environmental Protection Agency (Peck *et al.* 2006), which is geared toward cost-effective sampling for ambient regional stream surveys (Fetscher *et al.* 2009). It entails collection of material at 11 objectively selected locations spaced evenly across a 150 m or 250 m long stream reach (depending upon whether the average wetted width of the stream is less or greater than 10 m). Within each reach, samples were obtained from whatever substrata (e.g., cobble, silt/sand, gravel, bedrock, wood, concrete) were present at each of the 11 locations. These subsamples were combined into a single, well-mixed composite sample from which aliquots were drawn for: 1) quantitative analyses of the non-diatom algal assemblages, 2) diatom enumeration, 3) chlorophyll *a* (chl *a*) quantification, and 4) ash-free dry mass quantification. Diatom and ash-free dry mass data are not discussed in the present paper.

The total surface area sampled for each reach, as well as the dilution of the sample, were recorded, in order to facilitate estimation of the biovolume of non-diatom algae, including cyanobacteria, per unit area sampled. For optimal cellular preservation, all samples were fixed immediately upon collection in 2.5% histological grade glutaraldehyde and kept cold and in the dark until laboratory analysis. Additional "qualitative" samples of fresh, unfixed macroalgae were also collected from each stream reach. The goal was to be as exhaustive as possible in order to capture the diversity of macroalgae at each study site and to observe as many morphologies and diagnostic features as possible. All types of benthic algae visible within the stream reach were removed by hand and stored in Whirlpak® bags. The qualitative samples were kept cool and in the dark, and were delivered to the laboratory for examination as quickly as possible (Fetscher *et al.* 2009).

In addition, percent macroalgal cover was estimated in the field by a point-intercept approach that entails collecting information about the presence of attached and floating benthic macroalgae filaments and mats at each of the points along the transects (Fetscher *et al.* 2009).

Environmental Variables

Several environmental variables were measured in conjunction with the sampling of benthic algae (Supplemental Information (SI) Table SI-1; Supplemental Information is available at ftp://ftp.sccwrp.org/pub/download/DOCUMENTS/AnnualReports/2012AnnualReport/ar12_14SI.pdf). Physical habitat data were recorded at each stream reach as described by Fetscher *et al.* (2009). Conductivity, pH, and water temperature were measured with a field meter (Waterproof pH/CON 10 Meter, OAKTON Instruments, Vernon Hills, IL), and turbidity with a Hach 2100P portable turbidimeter (Hach Company, Loveland, CO). For dissolved inorganic nutrients, such as nitrate/nitrite, ammonium, orthophosphate, total dissolved nitrogen, and total dissolved phosphorus, stream water samples were filtered using mixed cellulose ester membrane 0.45 µm pore-size syringe filters (Fisher Scientific, Pittsburg, PA). Water chemistry analyses were carried out after APHA (2006). For the landscape data analysis, watersheds were delineated for each site from 30-m digital elevation models using a geographic information system and USGS National Elevation Database (http://ned.usgs.gov/).

Laboratory Analysis of Quantitative and Qualitative Benthic Algal Samples

Non-diatom Benthic Algae Processing: Quantitative Sample

For proper species identification and quantitative enumeration of non-diatom algal taxa, we processed macroalgae separately from the microscopic algal fraction of each sample. We adopted the concept of macroalgae as defined by Sheath and Cole (1992). Macroalgae were removed gently with forceps from the original sample, squeezed to remove as much liquid as possible, and then placed into a graduated centrifuge tube with a known volume of distilled water. The total volume of macroalgae was determined by the increase in volume (ml) in the tube. We used graduated centrifuge tubes (Fisher Scientific, Pittsburg, PA) with varying capacity (10, 15, or 50 ml) depending upon the volume of macroalgae. When the water displacement was not detectable because of the very low volume of algae, their biovolumes were calculated as dimensions measured under the light microscope. The macroalgal fraction with known total volume was spread out in a gridded Petri dish, and using a dissecting microscope, the number of macroalgal species and the proportion of each were determined. When mosses, higher plant stems, or roots were encountered in the sample, their biovolumes (also determined as described above) were subtracted from the total volume of the macroalgal fraction originally quantified. Identification of each macroalgal taxon encountered was carried out by microscopic examination, and the biovolume of each was calculated as the proportion of total volume that the fraction represented in a gridded Petri dish. Following removal of any macroalgae, a 5 ml aliquot of well mixed suspension from the remainder of the sample was concentrated down to 1 ml with the aid of centrifugation (20 minutes at 3100 RPM, Medilite Centrifuge, Thermo Scientific, Asheville, NC), in order to prepare the sample for microalgal identification and enumeration. A 0.05 ml subsample of this was pipetted onto a standard microscope slide and covered with a 22 x 30 mm coverslip. At a microscopic magnification of 400x, at least 300 natural algal counting entities were identified and enumerated along a known number of optical transects across the microscope slide. Specimen observation and photomicrography were performed using an Olympus microscope BX41 and Olympus stereo microscope SZ-40 with an attached Olympus MicroFire S99809 digital camera (Olympus Imaging America Inc., Center Valley, PA). A microscope slide at 400x magnification consists of 44 horizontal optical transects with our optical conditions (Olympus eyepiece WHN 10x/22 and Olympus objective UPlanFL N 40x/0.75), each representing a known volume of counted sample, thus aiding calculation of absolute biovolumes for all taxa identified. The size measurements were taken by Rincon image analysis software (Imaging Planet, Goleta, CA).

The natural algal counting entity was defined as each individual alga that is counted, whether it is a unicell, filament, coenocyte, tissue-like structure, colony, or crust, and regardless of the cell number. For the filamentous algae each piece of the filament regardless of its length was treated as separate entity. The biovolume of each microalgal taxon was calculated according to Hillebrand *et al.* (1999) with individual microscopic measurements of each

algal entity to the cell level at which it could be determined to the lowest taxonomic level possible. The biovolume of each macroalgal and microalgal taxon encountered was calculated as individual biovolume (μm^3) per cm^2 of area sampled after Lowe and Laliberte (1996) and converted to mm^3 cm^{-2}. The resulting absolute biovolume of each algal taxon could also be reported as relative biovolume, calculated as a percentage of total algal biovolume represented by that individual taxon.

*Biovolume calculations for macroalgal fraction**

$$V_i = V_a A^{-1}$$

*Biovolume calculations for microalgal fraction***

$$V_i = V_a V_s V_c^{-1} A^{-1}$$

Where: V_i = biovolume of *i*-species (μm^3) per 1 cm^2 stream bottom area sampled; V_a (macroalgae) = biovolume of *i*-species (μm^3) per sample counted (50 ml sample tube; known number of optical transects in which at least 300 algal counting entities were enumerated); V_s = composite sample volume (ml); V_c = volume of sample counted (ml) [this is the number of transects counted multiplied by the sample volume per transect]; A = stream bottom area of substratum sampled (cm^2).

*Prior to incorporating into the formula, the biovolume of *i*-species (V_a) was converted from (ml) to (μm^3), and then multiplied by 4 because we analyzed one fourth of the total macroalgae collected from a total stream bottom area of substratum sampled.

** Prior to incorporating into the formula, the biovolume of i-species (V_a) was corrected for the dilution factor, caused by variable sample volumes to which the 5 ml of glutaraldehyde was added, by multiplying with the correction factor (V_{cr}) calculated as follows:

$$V_{cr} = (V_t - V_m)\,(V_t - V_m - 5 \text{ ml fixative})^{-1}$$

Where: V_{cr} = a correction factor for sample dilution with fixative (assuming 5 ml of fixative was added to the sample); V_t = total initial volume in the sample vial (generally ~50 ml); V_m = volume of macroalgal fraction in sample vial (which is 0, if no macroalgae detected).

Non-diatom Benthic Algae Processing: Qualitative Samples

In the laboratory, material from the unfixed qualitative samples was scanned under dissecting and compound light microscopes in order to identify each non-diatom macroalgal and cyanobacterial taxon to the lowest possible taxonomic level. In many cases, the material contained different life stages of macroalgae. Live samples containing reproducing filaments of Zygnemataceae were incubated initially in water from the site where they were collected, and eventually diluted with distilled water for further intervals, to facilitate completion of sexual or asexual reproduction resulting in mature zygospores, akinetes, or aplanospores. The samples were placed on the north-facing window of the laboratory at room temperature (held constantly at 20°C). The reproductive filaments were checked every three days and different stages of conjugation and development of reproductive cells were documented by photomicrographs. Prescott (1951), Komárek and Anagnostidis (1999, 2005), John *et al.* (2002), and Wehr and Sheath (2003) were used as primary references for algal taxonomy, in addition to numerous specific ones, as needed.

Method Uncertainty Evaluation

In order to compare algal biovolume obtained by the novel laboratory quantification method with a quantitative field estimation of filamentous macroalgal cover, we regressed the cube-root transformed algal biovolume on log-transformed macroalgal percent cover using a quadratic fit. The transformations were used to improve normality of data distributions. The relationship between the two variables was presumed to be non-linear because the "percent" nature of the macroalgal cover data creates an upper bound to what is achievable for that parameter (100%), but the same type of constraint is not directly applicable to the biovolume data. The fit applied was intended to accommodate the potential for an asymptotic relationship between the two. The potential counting error for microalgal fraction species number estimation was tested by counting at least 1000 algal counting entities from three samples with different microalgal species numbers (9, 18 and 20 algal taxa).

Analysis of Algal Assemblages

A number of algal metrics were designed in order to compare characteristics of algal communities

along the environmental gradient, to begin evaluating their utility for assessing the ecological (biological and stressor) conditions of a habitat. Algal metrics were expressed in terms of species numbers, total algal biovolume estimated, and proportions of total species number and total algal biovolume associated with different taxonomic groups. Looking at both presence-absence and biovolume data types allowed us to begin assessing the importance of using quantitative, as opposed to just qualitative, estimates of algal community composition in bioassessment applications.

We hypothesized that the two main algal groups, i.e., green algae (Chlorophyta and Charophyta) and cyanobacteria, each consist of two subgroups with contrasting ecological preferences. Cyanobacteria were divided into heterocystous taxa (i.e., those possessing heterocysts and thus capable of nitrogen fixation) and non-heterocystous taxa. Filamentous Zygnemataceae were separated from the rest of the green algae and treated as a subgroup for separate data interpretation. Other taxonomic groupings included Rhodophyta and Xanthophyceae. Strength of associations between algal metrics and environmental variables that did not exhibit approximately normal distributions were evaluated with the non-parametric Spearman's rank correlation coefficient. Holm–Bonferroni correction for multiple comparisons was applied at α-level of 0.05, although some researchers (e.g., Gotelli and Ellison 2004) have suggested letting "the raw p-values stand and interpret them with some common sense, rather than constantly downgrading the data using Bonferroni adjustments." Therefore, we present results both with and without the Holm–Bonferroni correction, and focus our discussion of results on corrected values.

Indicator Species Analysis and Species Optima with Respect to Nutrients

In accordance with established low- and high-nutrient categories for diatoms in United States rivers (Potapova and Charles 2007), all benthic samples collected from streams with total dissolved phosphorus (TP) $\leq$0.01 mg L^{-1} were designated as "low-TP" samples, and those with TP $\geq$0.1 mg L^{-1} as "high-TP" sites. Likewise, those with total dissolved nitrogen (TN) $\leq$0.2 mg L^{-1} were designated as "low-TN", and those with TN $\geq$3 mg L^{-1} as "high-TN" samples.

An indicator species analysis (Dufrêne and Legendre, 1997) was carried out to identify which species were associated with the most nutrient-poor and the most nutrient-rich sites. Statistical significance of each species indicator value was tested using a Monte-Carlo method (999 permutations, $p < 0.05$). Indicator values can vary from 0 for a taxon that has the same occurrence and abundance in all groups of samples to 100 for a taxon that is confined to one group of samples and present in each. This analysis reveals species that not only have the highest specificity (mean relative abundance), but also the highest fidelity (frequency of occurrence), to a certain group of samples. Indicator species analysis was carried out with PC-ORD (version 6, MjM Software, Gleneden Beach, OR).

Species optima and tolerances were calculated using a weighted averaging (WA) approach. Weighted averaging is a technique commonly used to estimate species indicator values or optima (ter Braak and Looman 1986). Species preferences are calculated based on the values of specific environmental variables at sites where a species occurs weighted by the species' abundance at those sites. This approach was used in addition to indicator species analysis because it can identify less common species that might also be good indicators. TP and TN abundance-weighted means where each species occurs, along with their mean relative biovolume ("optima") and standard deviations ("tolerances"), were calculated using the R language and environment for statistical computing (R Development Core Team 2008). Since no strict rules or guidelines about the number of occurrences that is sufficient to obtain a reliable WA estimate are available, the following criteria were used to include species in the indicator list: species occurrence in at least five samples, WA optima either in the lowest (for low-nutrient indicators) or highest (for high-nutrient indicators) quartile of the species list, and tolerance-to-optimum ratio below 3 (Potapova and Charles 2007).

Results

Algal Species Composition, Biovolume and Taxonomic Group Proportions

A total of 260 non-diatom algal taxa were recorded in the studied streams, of which 180 were identified to species level. The taxonomic groups represented were the green algae (151 taxa total), including Zygnemataceae (31 taxa), cyanobacteria (83 taxa total), of which 63 taxa non-heterocystous cyanobacteria, and 20 taxa heterocystous cyanobacteria, Xanthophyceae (13 taxa), Rhodophyta

(7 taxa), Euglenozoa (4 taxa), and Cryptophyta (2 taxa). The most common taxa were *Cladophora glomerata* (48% of the sites), *Nostoc verrucosum* (31% of the sites), *Rhizoclonium hieroglyphicum* (30% of the sites), and unidentified *chantransia* stage of Rhodophyta and *Vaucheria* sp. 1 (29% of the sites, each). The most abundant taxa in the study often dominated benthic algal communities, sometimes reaching up to ~ 99% relative biovolume. These included (with median relative biovolume, among the sites where they were recorded, provided in parentheses) *C. glomerata* (51%), *N. verrucosum* (16%), *R. hieroglyphicum* (10%) and *Vaucheria* sp. 1 (8%).

Intact qualitative samples collected along with the quantitative samples were frequently indispensable for arriving at correct species identifications, especially in the case of the numerous macroalgal taxa listed in Table SI-2. Photomicrographs in Figure SI-3 show some of the morphological features that were examined in order to aid identification of specimens to species level. For many genera, such as *Vaucheria, Sirodotia, Spirogyra*, *Zygnema,* and *Cylindrospermum*, species identification completely relied on qualitative samples in which their reproductive structures were observed.

The median algal biovolume per site, estimated by the novel quantification method, was 22.7 mm^3 cm^{-2} (range: <0.001 - 836.9 mm^3 cm^{-2}), and in 60% of studied streams the total algal biovolume was less than 50 mm^3 cm^{-2} (Figure 2a). The number of algal species per site, obtained from quantitative samples ranged from 2 to 43, with median number of species (11) recorded in 23% of the streams, followed by 6 algal species in 16% of streams, 16 algal species in 11% of streams with (Figure 2b).

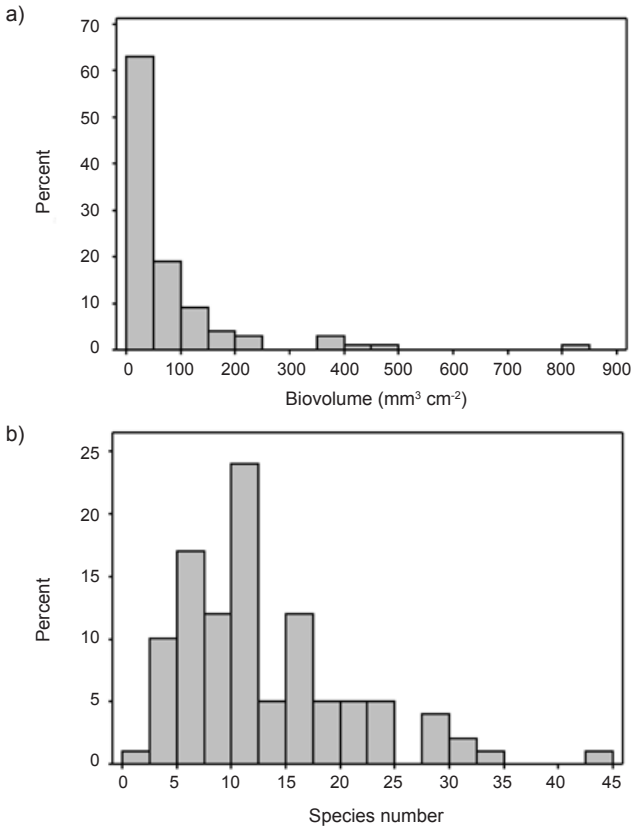

Figure 2. Frequency histogram of total algal biovolume (a) and species number (b) of algal samples from 104 study sites.

Method Uncertainty

We compared algal biovolume obtained by the novel method with a quantitative field estimation of percent macroalgal cover using the regression equation below. The results from the two independent methods exhibited a strong, positive relationship (Figure 3; $r^2 = 0.52$, $p < 0.001$), corroborating the effectiveness of the novel laboratory method.

Regression Equation

cube-root total algal biovolume = -759.7816 + 2926.7922 x log (% macroalgal cover +1) + 750.06372 x (log (% macroalgal cover +1) - 1.16642)^2

The counting error associated with estimating species number in the microalgal fraction was tested by counting at least 1000 algal entities from each of three samples. This is in contrast to the 300 entities prescribed by our method. For the sample with the lowest microalgal species number (9), all species were recorded within the first 300 entities counted, and increasing effort to 1000 entities added no new species. For the sample with 18 algal taxa, only 5% of them were encountered after the first 300 counts, and for the sample with 20 algal taxa, only 10% of them were added after the routine 300 counts. In none of the samples were new species encountered by counting beyond 600 entities (Figure 4). Hence, for the 82% of samples in this study that contained up to 18 macroalgal and microalgal taxa, the uncertainty associated with counting only 300 entities was presumably very low.

Algal Metric Relationships with Environmental Variables

Spearman rank correlation showed significant relationships between algal metrics and stream habitat conditions and anthropogenic stressors (Table 1). Both total algal biovolume and species number

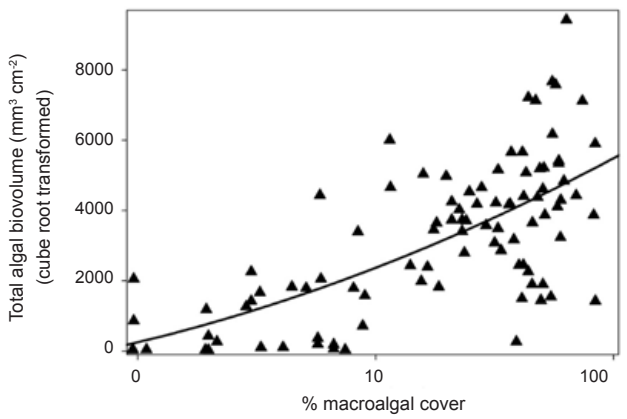

Figure 3. Relationship between percent macroalgal cover recorded in the field and laboratory-estimated total algal biovolume (n=104). $r^2 = 0.52$; $p < 0.001$.

were significantly positively correlated with water temperature, and negatively with canopy cover, but not with any of the water chemistry parameters measured. In addition canopy cover was negatively correlated with green algae (without Zygnemataceae) proportions, and positively with non-heterocystous cyanobacteria and red algae. Percent small-sized substrata (sand and silt) was negatively correlated with species number and heterocystous cyanobacteria proportions. Heterocystous cyanobacteria showed negative correlations with all water chemistry parameters in Table 1. The proportions of heterocystous cyanobacteria and Zygnemataceae were strongly negatively correlated with nitrate concentrations and TN. The red algae were significantly negatively correlated with TP. Both biovolume-based and species number-based proportions of heterocystous cyanobacteria, Zygnemataceae and red algae showed similar correlation coefficients with water chemistry parameters, while green algae were positively correlated with TN, conductivity, chlorides in terms of species number-based proportions, and with sulfate concentrations in terms of biovolume-based proportions.

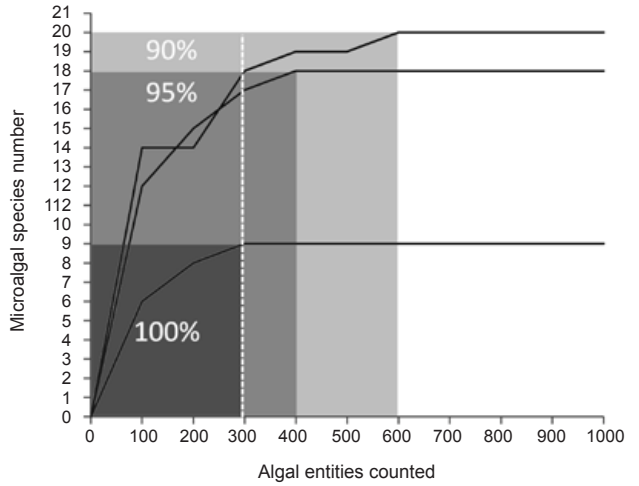

Figure 4. Cumulative non-diatom microalgal species numbers recorded across increasing levels of effort (algal entity counts). Each line represents the "species-count" curve for a single sample. Samples were prepared on glass microscope slides.

Indicator Species Designations and Species Optima with Respect to Nutrients

Indicator species analysis showed that most of the taxa recorded in our study either have broad nutrient tolerances, or were not common enough to yield large and significant indicator values. Only 15 of our taxa expressed indicator values >10 ($p < 0.05$). These are listed in Table SI-4 as possible indicators of low or high nutrient concentrations. Indicator values can vary from 0, for a taxon that has the same occurrence and abundance in all groups of samples, to 100, for a taxon that is confined to, and always present in, one group of samples. In our analysis, indicator values rarely exceeded 50; however, the five most common species demonstrated indicator values >38. Of them, *N. verrucosum* and *Chamaesiphon polymorphus* were associated with low TN. *C. glomerata* was a good indicator for high TN, *R. hieroglyphicum* for both high TN and TP, and the associated epiphyte *Leptolyngbya foveolara* for high TP.

In addition, the species abundance-weighted average approach determined 42 species to be associated with low or high nutrients (Table SI-4). The majority of these species were potential indicators of low-nutrient concentrations. The results from indicator species analysis and WA analyses were in concordance. For instance, they revealed that the two *Cladophora* species had contrasting ecological preferences. *C. glomerata* was identified as an indicator of high TN with a TN optimum of 3.14 mg L^{-1}, (maximum 23.2 mg L^{-1}, n = 50 sites), in contrast to *C. fracta*, with a TN optimum of 0.15 mg L^{-1} (maximum 0.63 mg L^{-1}, n = 13 sites). The distribution of the two *Cladophora* species along the TN gradient is presented in Figure SI-2.

Discussion

Algal Species Composition and Biovolume

Our novel method for identification and quantification of non-diatom benthic algae sought

Table 1. Spearman rank correlation coefficients between environmental variables for 104 southern California study sites and algal community metrics: total biovolume, species number, and taxonomic groups as proportion of species number, and as proportion of species biovolume (values in parentheses). Only significant correlations at level $\alpha = 0.05$ are shown.

Environmental Variables	Total Biovolume	Species Number	Green Algae	Zygnemataceae	Cyanobacteria		Rhodophyta	Xanthophyceae
					Heterocystous	Non-Heterocystous		
Temperature	**0.38****	**0.40****	**0.58**** **(0.37**)**			-0.21	-0.2	
pH	0.33*	0.25*	0.23 **(0.35*)**			(-0.27*)		-0.22
Canopy	**-0.36****	**-0.52****	**-0.52**** **(-0.40**)**			**0.34***	**0.34*** (0.31*)	
Sand+Silt	-0.24	**-0.33***		-0.31* (-0.30*)	**-0.36**** **(-0.35**)**	0.29* (0.24)	-0.22 (-0.22)	
Sulfate			0.32* **(0.37**)**	-0.22 (-0.22)	**-0.36**** **(-0.38**)**			
Chloride			**0.37**** (0.25)	-0.33* (-0.32*)	**-0.49**** **(-0.52**)**			
Conductivity			**0.34*** **(0.34*)**	-0.30* (-0.29*)	**-0.44**** **(-0.49**)**			
DOC			0.29* (0.28*)	-0.21	**-0.37**** **(-0.38**)**			
Nitrate		-0.29*	0.28* (0.33*)	**-0.36**** **(-0.41**)**	**-0.64**** **(-0.67**)**	0.24		-0.23 (0.23)
TN			**0.42**** (0.33*)	**-0.34*** **(-0.36**)**	**-0.59**** **(-0.59**)**			
OrthoPhosphate		-0.24		-0.32* (-0.32*)	**-0.34*** **(-0.36*)**		-0.25* (-0.30*)	
TP			0.30*	-0.25* (-0.27*)	**-0.35*** **(-0.37*)**		**-0.35*** **(-0.40**)**	

*p <0.01, **p <0.001; bold text indicates values that are still significant after Holm–Bonferroni correction; total dissolved nitrogen (TN) and total dissolved phosphorous (TP) were used in the analysis.

to enhance the efficacy of the existing laboratory processing techniques for benthic samples employed in stream water quality assessment programs over the last decade (Stevenson and Bahls 1999, Charles *et al.* 2002). Our improvements consisted of separate processing of macroalgal and microalgal fractions in order to avoid blending of the composite sample, and for better viewing, counting microalgae on glass microscope slides instead of using counting chambers. These modifications facilitated a thorough characterization of algal taxonomic composition due to the high-quality preservation of macroalgal vegetative and reproductive structures during laboratory processing. Subsampling of a well-mixed microalgal fraction for viewing on a standard microscope slide was essential for correct estimation of species composition and algal biovolumes (Lund *et al.* 1958).

Our counting error estimation suggested that the errors are generally low, but ultimately depend on the size of the counts and on the microalgal species number. For species-rich samples, the degree of uncertainty using our method (of 300 entity counts) appears not to be high, although modest improvements were achieved by increasing the size of the counts up to a point. Alternatively, for samples with species numbers near the modal range found in our dataset (10 to 12 algal taxa) and below, representative species composition in samples is likely fully achievable by counting only the 300 algal entities recommended in our method. Determination of uncertainty associated with organism counts (such as diatoms or pollen grains) is important, but very rarely done. Our results agree with general knowledge that larger counts decrease uncertainty (Birks 2010); however, we determined that the benefit of increasing counts above 300 was not high.

The algal species diversity obtained in our stream data set in southern California was comparable to the results from semi-quantitative and qualitative methods known to ensure high taxonomic resolution (Stevenson and Smol 2003). For instance, Schaumburg *et al.* (2004), in a study of 143 rivers of different types in Germany and Austria, recorded 196 non-diatom algal benthic taxa, and Schneider and Lindstrøm (2011) identified 153 non-diatom algal benthic species as trophic state indicators from 387 Norway rivers. Sheath and Cole (1992) reported 221 non-diatom macroalgal infrageneric taxa from a large stream survey in North America. The novel method presented here achieved a comparable estimation of algal species diversity, with 260 algal taxa identified, of which 70% were to species level. Non-diatom

algal taxonomic data from ongoing US national stream water quality assessment programs are not available for comparison with our study, because identification in these programs has been carried at genus or coarser level (e.g., Pan *et al.* 1999, Hill *et al.* 2000, Griffith *et al.* 2002).

Potapova (2005) reported environmental optima for 245 non-diatom taxa identified to the "lowest practical level" (species and genus) using a quantitative counting method (Charles *et al.* 2002) from more than 6,000 stream benthic samples collected across the US during a ten-year period. However, Potapova (2005) concluded that the NAWQA algal dataset is associated with many unresolved problems that could not be avoided under current protocols, such as uncertainty of identifications, variability of taxonomic levels of identification, and inconsistencies in identifications among laboratories. From the same data set, Potapova and Charles (2005) analyzed the preferences of stream benthic algae for specific substratum types, and the only non-diatom indicator species recognized was *Calothrix parietina,* while the rest of the non-diatom algae with indicator potential were identified to genus or coarser taxonomic level. Consequently, Porter (2008) stated that "autecology of many algal species (particularly soft algae) is unknown or poorly understood" and concluded that analysis of algal assemblage structure requires taxonomic resolution to species level in order to maximize the ecological signal. Depending on the purpose of the study and the algal group being studied, some authors have suggested that reducing taxonomic resolution does not affect the ability to derive ecological information from algal assemblage composition (Kelly and Whitton 1995), while others have insisted that the most precise taxonomic level achievable is required to minimize the loss of valuable information (Schmidt-Kloiber and Nijboer 2004). This latter view was exemplified by our finding that *C. glomerata* and *C. fracta*, green algal congeners that are common in our region, exhibit very different ecological tolerances, a phenomenon that would be important to take into account within bioassessment applications of algal assemblage data.

The most important advantage of the novel counting method is its reasonable balance between the goals of algal identification at species level and precise biovolume estimation. Nearly all methods for measuring algal community attributes are prone to statistical errors (Alverson *et al.* 2003, Stevenson *et al.* 2010), and the measurement accuracy of the presented method is associated with some of the same general problems encountered in all counting methods. This includes difficulty measuring the third cell dimension, and overestimation of the cellular volume of larger cells with a higher relative vacuole volume (Stevenson 1996, Hillebrand *et al.* 1999). For this study, we took the dimensions of each algal entity counted, and in this way we avoided using median cell sizes based on examination of a subset of randomly selected cells per species (usually 15 or more representative cells; Hillebrand *et al.* 1999, Porter *et al.* 2008). Algal biovolume estimation obtained by presented method showed a significant, positive correlation to other biomass measurements applied to the same stream sites, such as field macroalgal cover. Various estimates of periphyton biomass are typically highly correlated to one another (Vis *et al.* 1998), and our results provide support for the precision of the novel method for non-diatom benthic algae quantification (Figure 3).

Unfortunately, data for algal biovolume estimations are scarce, especially for the streams in southern California. For comparison purposes, we chose a NAWQA large-scale study of rivers throughout the US, despite the fact that NAWQA sampling protocols were designed for separate sampling of microalgae and macroalgae (http://water.usgs.gov/nawqa/protocols/OFR-93-409/algp13.html) and total algal biovolumes reported considered diatoms in addition to non-diatom microalgae (Potapova and Charles 2005, Porter *et al.* 2008). However, Potapova and Charles (2005) specified 22 $mm^3\ dm^{-2}$ (0.22 $mm^3\ cm^{-2}$) median non-diatom algae biovolume on hard substrata, and 24 $mm^3\ dm^{-2}$ (0.24 $mm^3\ cm^{-2}$) on soft substrata. In general, these algal biovolumes fall within the range of total algal biovolume estimations from our study area. As could be expected, the median algal biovolume estimated in this study was higher, because all non-diatom algal types present in our samples were quantified, including the large-sized macroalgae.

Algal Metric Relationships with Environmental Variables

Our results showed that total algal biovolume was correlated to physical habitat conditions (i.e., water temperature and canopy cover), but not to measured water chemistry constituents. Canopy cover was an important factor negatively associated with total algal biovolume, in agreement with the acknowledged effect of light availability on the

net biomass production of autotrophic organisms (Hill 1996). Similarly, Porter *et al.* (2008) did not find significant differences in total algal biovolume among major river catchments or land-use classifications at the United States national scale, and reported only a weak correlation with nitrate (positive) and suspended sediment concentrations (negative). Algal-nutrient interactions in streams are complex, and many studies often fail to show strong relationship between algal biomass and nutrients in streams (Leland 1995), because primary production depends on other factors such as frequency and intensity of floods (Power *et al.* 2008), grazers community structure (Power *et al.* 2009), and substratum type and size (Cattaneo *et al.* 1997).

The pattern typically expected as a result of human impact is a decrease in species diversity, but there are many studies that have reported no differences in species diversity of stream benthic-algal assemblages between sites with varying levels of urban pressure (e.g., Vis *et al.* 1998, Lukavský *et al.* 2006). Our study did not reveal significant correlations between algal species number and measured water chemistry constituents. In addition to water temperature and canopy cover, species number was correlated significantly to fine substrata (negatively), in concordance with Cattaneo *et al.* (1997) findings that substratum size affects periphyton biomass, taxonomic composition and algal growth forms. The proportions of heterocystous cyanobacteria, represented to large extend by *Nostoc verrucosum*, a colonial cyanobacterium attached to stones and rocks, decreased with increasing of fine substrata, which supported more loosely attached periphyton than rocks (Cattaneo *et al.* 1997).

The taxonomic composition and structure of benthic algal communities in southern California reflected differences in water-quality parameters, particularly nutrients. We found strong negative correlation between heterocystous cyanobacteria and nitrate and TN concentrations presumably due to their ability to fix atmospheric N_2 as an alternative nitrogen source. Our results are in agreement with observed relationships between nitrogen-fixing algae and nitrate in Californian streams (Porter *et al.* 2008), and with studies that showed nitrogen as limiting nutrient in non-urban streams in the southwestern United States (Peterson and Grimm 1992). This is in contrast to Hill *et al.* (2000), who had reported that "percentage of cyanobacteria" was not linked to water-quality constituents when considered as an entire group. These findings taken together suggest that identification to low taxonomic levels is an important first step to understanding the potential of algal taxa to serve as bioindicators for specific assessment goals.

As expected, green algae had positive correlations with TN, conductivity, and chloride, in contrast to the Zygnemataceae, which were negatively correlated with TN and nitrates. Many studies have demonstrated that green algae dominate high-nutrient stream reaches (e.g., Leland and Porter 2000, Lukavsky *et al.* 2006, Vis *et al.* 2008) in contrast to our observations that Zygnemataceae are frequent and abundant in low-nutrient streams in southern California (see also Stancheva *et al.* In Press). Red algae proportions increased significantly with the decrease of TP, which is in accordance with the previous finding that red algae are most abundant in environments with low concentrations of phosphates (Sheath 2003).

The algal groups that best respond to nutrient changes and other water quality parameters (i.e., heterocystous cyanobacteria, Zygnemataceae and red algae), showed similar trends based on biovolume and presence-absence data, supporting the concept that changes in species composition provide strong signals of aquatic organisms, and particularly of benthic algae, to environmental alterations (Schindler 1990, Schneider and Lindstrøm 2011).

Indicator Species Designations and Species Optima with Respect to Nutrients

Algal species optima models are frequently used to characterize species responses to water-quality parameters (e.g., Rott *et al.* 1999, Leland and Porter 2000, Potapova *et al.* 2004) and applied as foundation of periphyton indices (Schneider and Lindstrøm 2009, 2011). Therefore, studies at small regional scales are perhaps necessary to develop sufficiently sensitive algal indicators of river health (Porter *et al.* 2008, Schneider and Lindstrøm 2011). Our data showed that >40 algal species could be potential indicators of nutrient conditions in southern California streams. On the United States national scale, Potapova (2005) defined three indicator non-diatom algal species for low TN (<0.9 mg L^{-1}), which were captured by our analysis as well: *Calothrix parietina*, *Anabaena* sp. and *Mougeotia* sp. The species nutrient optima derived from our regional data set were in agreement with autecological data known from algal floras and compilations of numerous

literature sources (such as Rott et. al. 1999), which testifies to the taxonomic consistency and adequate quantification of algal biodiversity achieved by our novel method.

An interesting example of the influence of taxonomical resolution on our understanding of autecology of some common and ecologically important freshwater species was illustrated by our observations of *Cladophora* distribution along a nutrient gradient. Taxonomic identification of *Cladophora* species has challenged phycologists for decades because many morphological features vary with plant age and environment (van den Hoek 1963), and as a consequence, phycologists often refrain from keying *Cladophora* to species and either assume their samples are *C. glomerata* or report them as *Cladophora* sp. (Marks and Cumming 1996). As a result, the most commonly published observations are that, on one hand, excessive *Cladophora* biomass in freshwaters is stimulated by phosphorous additions, but on other hand, *Cladophora* also can be abundant in habitats where nitrogen supply limits primary production (Dodds and Gudder 1992).

Regardless of the potential for morphological overlap among *Cladophora* taxa, we were able to distinguish morphologically two *Cladophora* species that had contrasting associations with nitrogen supply in southern California streams (Table SI-3). *C. glomerata* emerged as a good indicator of high TN concentrations, in contrast to *C. fracta* which flourished under low nutrient conditions. Though freshwater *Cladophora* comprises possible one ecologically and morphologically variable species (Marks and Cumming 1996), proper distinguishing of morphotypes (or species) with different ecological preferences could magnify the power of algal community analysis.

In conclusion, our study demonstrated that the novel quantification method for stream-inhabiting, non-diatom benthic algae provides high quality taxonomic and quantitative data with a low degree of uncertainty. The algal metrics discussed here represent various characteristics of algal communities that could be used to assess stream ecological (biological and stressor) conditions. Total algal biovolume and species number corresponded to physical habitat conditions, while heterocystous cyanobacteria, Zygnemataceae and red algae proportions were significantly correlated with stream nutrient status and other water chemistry parameters. This indicates that non-diatom algae may be applied to various stream bioassessment objectives, from the standpoints of both community composition assessment (e.g., via an IBI) and algal primary productivity quantification (e.g., to evaluate impairment in terms of algal nuisance). Indicator species designations and species optima revealed more than 40 species as potential indicators of nutrient status, and highlighted the importance of species-level taxonomic resolution in order to maximize the ecological signal from stream benthic algae analysis. This work was conducted in parallel with similar diatom community studies from the same streams. Taken together, these methods will produce an integrated approach that enhances the breadth and quality of information achievable in stream bioassessment programs.

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Supplemental Information

Supplemental Information is available at ftp://ftp.sccwrp.org/pub/download/DOCUMENTS/AnnualReports/2012AnnualReport/ar12_14SI.pdf