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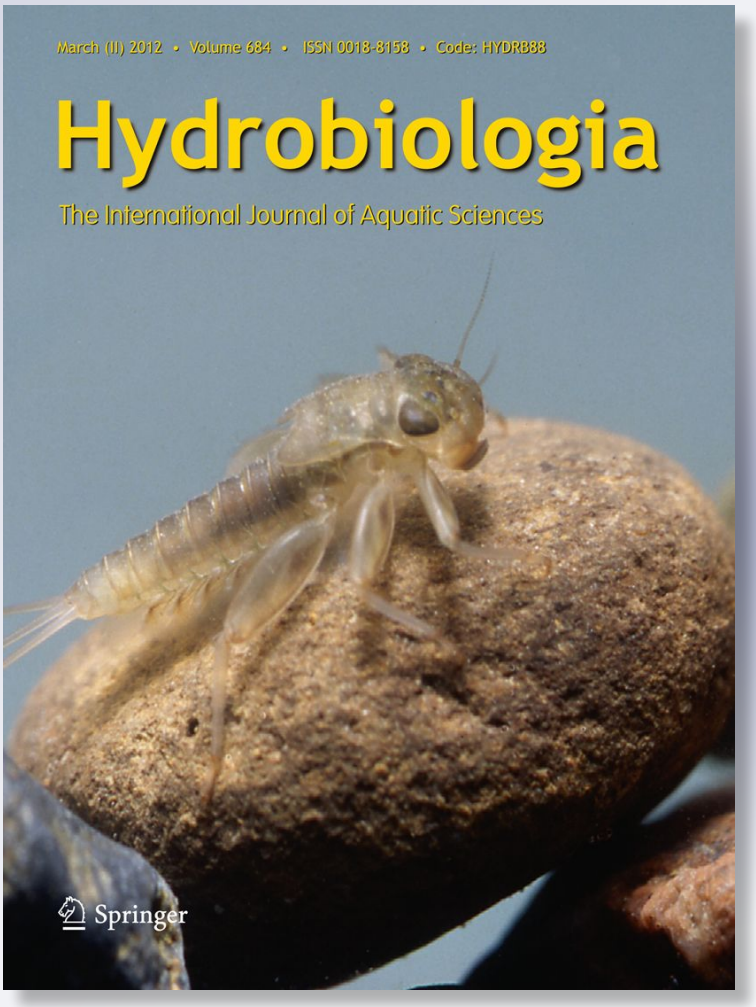

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PRIMARY RESEARCH PAPER

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 method for quantification of non-diatom algae that seeks to improve upon two important aspects of existing methods: separate processing of macroalgae and microalgae to avoid sample blending and consequent loss of macroalgal integrity, and 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 low 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 proportion of red algae 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.

Keywords Benthic algae · Quantification method · Bioassessment · Streams · California

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Introduction

Benthic algae (periphyton) serve as a primary source of energy in 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 & Wehr, 2003). There is insufficient information about benthic algal biodiversity, biomass, and community structure in arid areas, particularly 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 for 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 uninvestigated (Larned, 2010). However, numerous studies have proven their relationships with environmental variables (Griffith et al., 2002; Foerster et al., 2004; Hering et al., 2006; Porter et al., 2008; Schneider & Lindstrøm, 2009, 2011) suggesting benefits of inclusion of benthic non-diatom algae in biomonitoring efforts. Benthic algae are regarded as an important biological component of routine monitoring of the 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 and Assessment Program (EMAP; http://www.epa.gov/emap) and the United States Geological Survey's (USGS) National Water-Quality Assessment Program (NAWQA; http://water.usgs.gov/nawqa).

Whereas diatom sampling and laboratory protocols are well established (Stevenson & Bahls, 1999; Kelly et al., 2009), current methods for non-diatom algae assessment suffer from many shortcomings that limit their application in stream bioassessment. One possible reason non-diatom algal assemblages receive less attention than diatoms is that their quantitative assessment is challenging. General difficulties are related to:

1. morphological size differences;
2. life-stages and reproductive structures that must be observed for complete identification; and
3. the need for detailed and careful light microscope (LM) observations of cells or larger colonies or intact thalli for accurate identification to species.

Current approaches to assessing benthic algal species composition and biomass entail 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, whereas "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 the abundance of benthic algae in relative terms, on a five-score scale (Foerster et al., 2004; Schaumburg et al., 2004; Kelly et al., 2009), and Schneider & Lindstrøm (2009, 2011) used presence–absence data. Both semi-quantitative and qualitative approaches enable 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 procedures are quantitative, with the objective 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 different sizes (Stevenson & Bahls, 1999; Charles et al., 2002). These procedures require blending and breaking up of large filaments or colonies to suspend algae in 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 & Kilroy, 2007). Because disintegration of colonies of benthic algae can cause changes in color and distortion of sheaths or mucilage in cyanobacteria and other non-diatom algae, such potentially destructive manipulations of specimens are not recommended during taxonomic identification (Komárek, 2003). Furthermore, many macroalgae, for example *Vaucheria*, *Batrachospermum*, *Sirodotia*, and members of the Zygnemataceae, for which 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 because of 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 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 & 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 a 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).

This 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. To achieve that objective, we modified currently available procedures for benthic algae collection and enumeration. Our objectives were:

1. to present the novel method of quantification of benthic non-diatom algae used in this study 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 in respect of nutrients.

This information will improve our understanding of the efficacy of benthic non-diatom algae as a practical tool in stream bioassessment programs.

Materials and 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 (Fig. 1). A total of 104 stream sites were studied. Stream reaches included in the study were selected to represent a broad range of factors known or hypothesized to affect periphyton assemblage composition and biomass. These factors included different amounts 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 affect water chemistry downstream. In addition, on-site factors, for example dominant substratum type, amount of canopy cover, gradient, flow, and channel dimensions were taken into consideration.

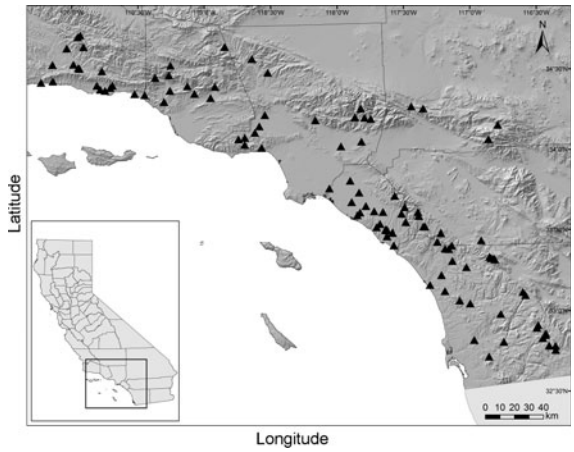

Fig. 1 Location map of the 104 stream study sites in southern California

Field sampling of benthic algae

Stream sites were sampled for benthic algae under dry-season (low-flow) conditions from May to July for two years (2007–2008). Samples were collected using a modification of the "multi-habitat" method of the USEPA (Peck et al., 2006), the purpose of which is 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 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 analysis 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 this paper.

The total surface area sampled for each reach, and the dilution of the sample, were recorded, to facilitate estimation of the biovolume of non-diatom algae, including cyanobacteria, per unit area sampled. For optimum preservation of cells, 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 objective 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, percentage 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 (Online Resource 1). Physical habitat data were recorded for 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, USA), and turbidity with a Hach 2100P portable turbidimeter (Hach, Loveland, CO, USA). For dissolved inorganic nutrients, for example nitrate/nitrite, ammonium, orthophosphate, total dissolved nitrogen, and total dissolved phosphorus, stream water samples were filtered through mixed cellulose ester membrane 0.45 μm pore-size syringe filters (Fisher Scientific, Pittsburg, PA, USA). Water chemistry analyses were carried out in accordance with APHA (2006). For landscape data analysis, watersheds were delineated for each site from 30-m digital elevation models using a geographic information system and the USGS National Elevation Database (http://ned.usgs.gov/).

Laboratory quantitative and qualitative analysis of 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 & 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 from the increase in volume (ml) in the tube. We used graduated centrifuge tubes (Fisher Scientific, Pittsburg, PA, USA) of different 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 LM. 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. After removal of

any macroalgae, 5 ml of well-mixed suspension from the remaining sample was concentrated to 1 ml with the aid of centrifugation (20 min at 3100 RPM, Medilite centrifuge; Thermo Scientific, Asheville, NC, USA), to prepare the sample for identification and enumeration of microalgae. A 0.05-ml subsample of this was pipetted on to a standard microscope slide and covered with a 22 mm × 30 mm coverslip. At a microscopic magnification of ×400, at least 300 natural algal counting entities (defined as each individual alga that is counted, whether it is a unicell, filament, coenocyte, tissue-like structure, colony, or crust, and irrespective of the number of cells) were identified and enumerated along a known number of optical transects across the microscope slide. Specimen observation and photomicrography were performed by use of an Olympus BX41 microscope and an Olympus SZ-40 stereo microscope with an attached Olympus MicroFire S99809 digital camera (Olympus Imaging America, Center Valley, PA, USA). A microscope slide at ×400 magnification consists of 44 horizontal optical transects with our optical conditions (Olympus eyepiece WHN ×10/22 and Olympus objective UPlanFL N ×40/0.75), 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, USA).

For the filamentous algae each piece of the filament regardless of its length was treated as separate algal counting entity. The biovolume of each microalgal taxon was calculated in accordance with Hillebrand et al. (1999) with individual microscopical 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, in accordance with Lowe & 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[1]

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

Biovolume calculations for microalgal fraction[2]

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

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

Non-diatom benthic algae processing: qualitative samples

In the laboratory, material from the unfixed qualitative samples was scanned under dissecting and compound LMs 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 further diluted with distilled water, 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

[1] Before incorporation 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.

[2] Before incorporation 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 glutaraldehyde was added, by multiplying by 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} is the a correction factor for sample dilution with fixative (assuming 5 ml fixative was added to the sample); V_t is the total initial volume in the sample vial (generally ~50 ml); V_m is the volume of macroalgal fraction in sample vial (which is 0 if no macroalgae are detected).

checked every 3 days and different stages of conjugation and development of reproductive cells were documented by use of photomicrographs.

Prescott (1951), Komárek & Anagnostidis (1999, 2005), John et al. (2002), and Wehr & Sheath (2003) were used as primary references for algal taxonomy, in addition to numerous specific ones, as needed.

Method uncertainty evaluation

To compare algal biovolume obtained by the novel laboratory quantification method with a quantitative field estimate of filamentous macroalgal cover, we regressed cubed-root-transformed algal biovolume on log-transformed macroalgal percentage cover by use of a quadratic fit. The transformations were used to improve normality of data distributions. The relationship between the two variables was assumed to be non-linear because the "percent" nature of the macroalgal cover data creates an upper bound to what is achievable for that variable (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 1,000 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 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 enabled us to begin assessing the importance of using quantitative, as opposed to solely 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 with heterocysts and thus capable of nitrogen fixation) and non-heterocystous taxa. Filamentous Zygnemataceae were separated from the other 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 for which distributions were not approximately normal were evaluated with the non-parametric Spearman's rank correlation coefficient. Holm–Bonferroni correction for multiple comparisons was applied at an α-level of 0.05, although some researchers, for example Gotelli & 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 in respect of nutrients

In accordance with established low and high-nutrient categories for diatoms in United States rivers (Potapova & 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 & 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, USA).

Species optima and tolerances were calculated by using a weighted averaging (WA) approach. Weighted averaging is a technique commonly used to estimate species indicator values or optima (ter Braak & Looman, 1986). Species preferences are calculated on the basis of 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, with their mean relative biovolume ("optima") and standard deviations ("tolerances"), were calculated by using the R language and environment for statistical computing (R Development Core Team, 2008). Because 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 & Charles, 2007).

Results

Algal species composition, biovolume, and taxonomic group proportions

A total of 260 non-diatom algal taxa were recorded in the streams studied; of these, 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 were 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 with the quantitative samples were frequently indispensable for arriving at correct species identifications, especially for the numerous macroalgal taxa listed in Online Resources 2 and 3. Photomicrographs in Online Resource 3 show some of the morphological features that were examined to aid identification of specimens to species level. For many genera, for example *Vaucheria*, *Sirodotia*, *Spirogyra*, *Zygnema*, and *Cylindrospermum*, species identification relied completely 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 $mm^3\ cm^{-2}$ (Fig. 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 six algal species in 16% of streams and 16 algal species in 11% of streams (Fig. 2b).

Method uncertainty

We compared algal biovolume obtained by the novel method with a quantitative field estimate of percentage macroalgal cover. The results from the two independent methods had a strong, positive relationship (Fig. 3; $r^2 = 0.52$, $P < 0.001$), corroborating the effectiveness of the novel laboratory method.

The counting error associated with estimating species number in the microalgal fraction was tested by counting at least 1,000 algal entities from each of three samples. This is in contrast with 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 the effort to 1,000 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

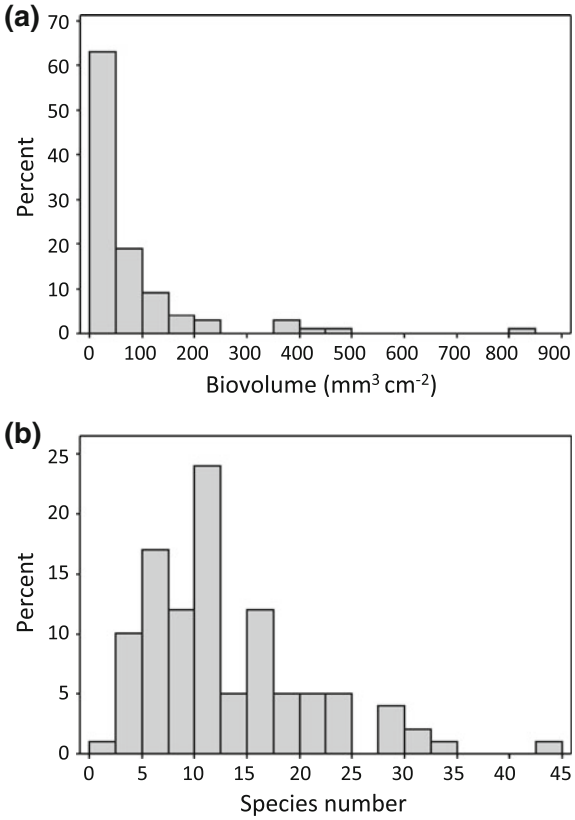

Fig. 2 Frequency histogram of total algal biovolume (**a**) and species number (**b**) of algal samples from 104 stream sites in southern California

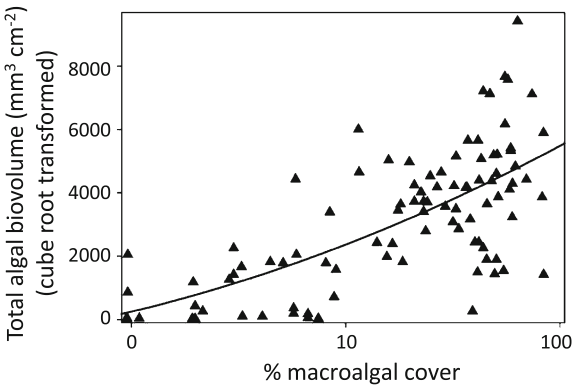

Fig. 3 Relationship between percentage macroalgal cover recorded in the field and laboratory-estimated total algal biovolume ($n = 104$). Regression equation: cubed-root total algal biovolume $= -759.7816 + 2926.7922 \times \log$ (% macroalgal cover + 1) + 750.06372 × (log (% macroalgal cover +1) − 1.16642)^2; $r^2 = 0.52$; $F = 54.92$; $P < 0.001$

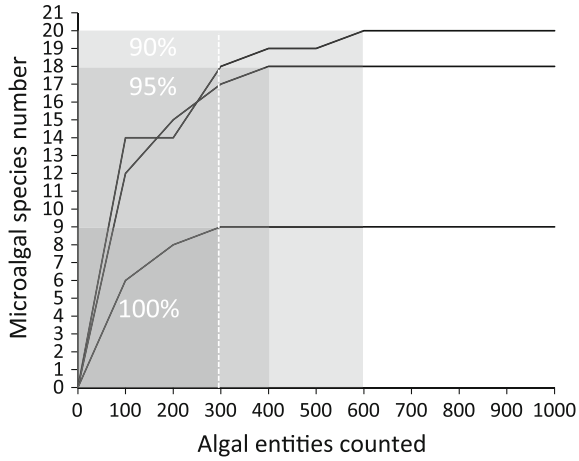

Fig. 4 Cumulative non-diatom microalgal species numbers recorded for increasing levels of effort (algal entity counts). Each line represents the "species-count" curve for a single sample. Samples were prepared on glass microscope slides

samples were new species encountered by counting beyond 600 entities (Fig. 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 revealed significant relationships between algal metrics and stream habitat conditions and anthropogenic stressors (Table 1). Both total algal biovolume and species number were significantly positively correlated with water temperature, and negatively with canopy cover, but not with any of the water chemistry data measured. In addition canopy cover was negatively correlated with green algae (without Zygnemataceae) proportions, and positively with non-heterocystous cyanobacteria and red algae. Percentage small-sized substrata (sand and silt) was negatively correlated with species number and heterocystous cyanobacteria proportions. Heterocystous cyanobacteria had negative correlations with all water chemistry data 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 had similar

Table 1 Spearman rank correlation coefficients between environmental variables for 104 stream sites in southern California and algal community metrics: total biovolume, species number, and taxonomic groups as proportions of species number and as proportions of species biovolume (values in parentheses)

Environmental variables	Total biovolume	Species number	Green algae	Zygnemataceae	Cyanobacteria heterocystous	Cyanobacteria	Non-heterocystous	Rhodophyta	Xanthophyceae
Temperature	**0.38****	**0.40****	**0.58**** (**0.37****)			−0.21		−0.20	
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****)	

Only significant correlations at the level $\alpha = 0.05$ are shown

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

correlation coefficients with water chemistry data, and green algae were positively correlated with TN, conductivity, and chlorides in terms of species number-based proportions, and with sulfate concentrations in terms of biovolume-based proportions.

Indicator species designations and species optima in respect of 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 yielded indicator values >10 ($P < 0.05$). These are listed in Online Resource 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, for the five most common species indicator values were >38. Of these, *N. verrucosum* and *Chamaesiphon polymorphus* were associated with low TN. *C. glomerata* was a good indicator of high TN, *R. hieroglyphicum* of both high TN and high TP, and the associated epiphyte *Leptolyngbya foveolara* of high TP.

In addition, the species abundance-weighted average approach determined 42 species to be associated with low or high nutrients (Online Resource 5). Most 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 Online Resource 6.

Discussion

Algal species composition and biovolume

Our novel method for quantification of non-diatom benthic algae sought to enhance the efficacy of the existing laboratory processing techniques for benthic samples used in stream water quality assessment programs over the last decade (Stevenson & Bahls, 1999; Charles et al., 2002). Our improvements consisted in separate processing of macroalgal and microalgal fractions 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 thorough characterization of algal taxonomic composition because of 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 estimate of counting error suggested that the errors are generally low, but ultimately depend on the size of the counts and on microalgal species number. For species-rich samples, the uncertainty using our method (of 300 entity counts) seems not to be high, although modest improvements were achieved by increasing the size of the counts. Alternatively, for samples with species numbers near the modal range found in our dataset (10–12 algal taxa) and below, representative species composition in samples is likely to be fully achievable by counting only the 300 algal entities recommended in our method. Determination of uncertainty associated with organism counts (for example diatoms or pollen grains) is important, but very rarely done. Our results agree with general knowledge that larger counts reduce uncertainty (Birks, 2010); however, we determined that the benefit of increasing counts above 300 was not high.

The algal species diversity observed in our stream dataset in southern California was comparable with results from semi-quantitative and qualitative methods known to ensure high taxonomic resolution (Stevenson & 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 & Lindstrøm (2011) identified 153 non-diatom algal benthic species as trophic state indicators in 387 rivers in Norway. Sheath & 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 estimate 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 conducted at genus or coarser level (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 by use of current procedures, for example uncertainty of identification, variability of taxonomic levels of identification, and inconsistencies in identification among laboratories. From the same dataset, Potapova & Charles (2005) analyzed the preferences of stream benthic algae for specific substratum types, and the only non-diatom indicator species recognized was *Calothrix parietina*; other non-diatom algae with indicator potential were identified to genus or coarser taxonomic level. Consequently, Porter (2008) stated that the "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 & Whitton, 1995), whereas others have insisted that the most precise taxonomic level achievable is required to minimize loss of valuable information (Schmidt-Kloiber & 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, have 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 objectives of algal identification at species level and precise estimation of biovolume. 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 estimates obtained by use of our method had a significant, positive correlation with other biomass measurements applied to the same stream sites, for example field macroalgal cover. Various estimates of periphyton biomass are typically highly correlated with one another (Vis et al., 1998), and our results provide support for the precision of the novel method for quantification of non-diatom benthic algae (Fig. 3).

Unfortunately, results from estimation of algal biovolume 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 procedures 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 & Charles, 2005; Porter et al., 2008). However, Potapova & 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 estimates 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 large macroalgae.

Algal metric relationships with environmental variables

Our results showed that total algal biovolume was correlated with physical habitat conditions (i.e., water temperature and canopy cover) but not with 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 significantly different 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 a strong relationship between algal biomass and nutrients in streams (Leland, 1995), because primary production depends on other factors, for example 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 many studies have reported no differences in species diversity of stream benthic-algal assemblages between sites with varying levels of urban pressure (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 the findings of Cattaneo et al. (1997) that substratum size affects periphyton biomass, taxonomic composition, and algal growth forms. The proportions of heterocystous cyanobacteria, represented to a large extent 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 data, particularly nutrients. We found strong negative correlations between heterocystous cyanobacteria and nitrate and TN concentrations, presumably because of 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 & Grimm, 1992). This is in contrast with Hill et al. (2000), who reported that "percentage of cyanobacteria" was not linked with water-quality constituents when considered as a entire group. These findings taken together suggest that identification to low taxonomic levels is an important first step in understanding the potential of algal taxa to serve as bioindicators for specific assessment objectives.

As expected, green algae (excluding Zygnemataceae) positive correlations with TN, conductivity, and chloride, in contrast with the Zygnemataceae, which were negatively correlated with TN and nitrates. Many studies have demonstrated that green algae dominate high-nutrient stream reaches (Vis et al., 1998; Leland & Porter, 2000; Lukavský et al., 2006), in contrast with our observations that Zygnemataceae are frequent and abundant in low-nutrient streams in southern California (see also Stancheva et al., 2012). Red algae proportions increased significantly with decreasing TP, which is in accordance with the previous finding that red algae are most abundant in environments with low concentrations of phosphates (Sheath, 2003).

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

Indicator species designations and species optima, in respect of nutrients

Algal species optima models are frequently used to characterize species responses to water-quality (Rott et al., 1999; Leland & Porter, 2000; Potapova et al., 2004) and used as foundations of periphyton indices (Schneider & 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 & Lindstrøm, 2011). Our results 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

also: *Calothrix parietina*, *Anabaena* sp., and *Mougeotia* sp. The species nutrient optima derived from our regional dataset were in agreement with autecological data known from algal floras and compilations of numerous literature sources (for example 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 effect of taxonomical resolution on our understanding of the 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 & Cummings, 1996). As a result, the most commonly published observations are, on the one hand, that excessive *Cladophora* biomass in freshwaters is stimulated by phosphorus addition, but, on the other hand, that *Cladophora* can also be abundant in habitats where nitrogen supply limits primary production (Dodds & Gudder, 1992).

Despite the potential for morphological overlap among *Cladophora* taxa, we were able to distinguish morphologically two *Cladophora* collections that had contrasting associations with nitrogen supply in southern California streams (Online Resource 4). *C. glomerata* emerged as a good indicator of high TN concentrations, in contrast with *C. fracta* which flourished under low nutrient conditions. Although freshwater *Cladophora* possibly comprises one ecologically and morphologically variable species (Marks & Cummings, 1996), proper distinguishing of morphotypes (or species) with different ecological preferences could increase 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 low 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, whereas heterocystous cyanobacteria, Zygnemataceae, and red algae proportions were significantly correlated with stream nutrient status and other water chemistry data. This indicates that non-diatom algae may be used for a variety of stream bioassessment objectives, 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 of 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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