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Problems and pitfalls in using HPLC pigment analysis to distinguish Lake Michigan phytoplankton taxa

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ABSTRACT

High pressure liquid chromatography (HPLC) has become a standard for analysis of phytoplankton pigment in marine and freshwaters. In marine systems, such data have been combined with optimization algorithms (e.g., CHEMTAX) to quantitatively predict the taxa present in a given sample; such studies are less common in freshwaters, particularly in oligo- and mesotrophic lakes. HPLC/CHEMTAX methods were compared with taxonomic identification and quantification using traditional microscopy and volume to chlorophyll *a* conversions in Lake Michigan phytoplankton communities collected on six cruises during summer (June to August) 2008. Chlorophyll *a* reached maxima (approximately 1.5 μg L^{-1} nearshore and 0.5 μg L^{-1} offshore) in late June/early July, with the exception of the offshore metalimnion where chlorophyll *a* peaked (3.0 μg L^{-1}) in early July. Taxonomic groups were consistently misidentified by HPLC/CHEMTAX, relative to microscope methods; of 18 points of comparisons, only 5 were significantly related (judged by regression of relative proportion of chlorophyll *a* estimated by HPLC/CHEMTAX versus cell counts/volume). Confusion between diatoms and chrysophytes was particularly serious. For example, in late July when counts indicated that biovolume at epilimnetic stations was dominated by diatoms, HPLC/CHEMTAX indicated dominance by chrysophytes. Grouping diatoms and chrysophytes as a single taxon improved comparisons, so that there was significant agreement in 7 out of 18 cases. While some specific improvement to the HPLC method (e.g., selecting a smaller subset of pigments relevant to the specific phytoplankton assemblages found) might help, our work emphasizes the point made repeatedly in the literature, that HPLC methods cannot replace microscopy.

Introduction

Understanding changes in phytoplankton communities is essential to interpreting ecosystems dynamics, as well as biogeochemical cycles (Jeffrey et al., 1997; Li et al., 2002; Reynolds, 2006). Although changes in cell size and shape distribution can occur, in most cases, phytoplankton communities respond to environmental changes (e.g., irradiance, nutrients, grazing) with changes in species composition (e.g., Berquist and Carpenter, 1986; Carpenter and Kitchell, 1993).

Unfortunately, monitoring species composition in phytoplankton is difficult. One of the most common measurements, chlorophyll *a* (chl *a*), can provide biomass estimates, but offers no taxonomic resolution (see Kruskopf and Flynn, 2005). Thus chl *a* is usually paired with identification and enumeration of preserved samples (e.g., Havskum et al., 2004; Li et al., 2002). Even with modified cell counting techniques to correct for non-random settling of cells (Sandgren and Robinson, 1984), accuracy is largely dependent upon the skills of the observer. Recently, molecular-based methods have been introduced, for example, using 18 s rRNA genes to determine the groups present (Beszteri et al., 2005; Countway et al., 2005), but such methods are expensive and currently limited by the availability of reference sequences in databases.

One alternative is to examine taxon-specific phytoplankton pigments, quantified using high pressure liquid chromatography (HPLC, reviewed by Jeffrey et al., 1997) and allocated quantitatively to taxa using approaches such as regression (e.g., Descy et al., 2000) or optimization techniques, including CHEMTAX (Mackey et al., 1996). HPLC analyses are rapid relative to cell counting, and can potentially resolve taxa that are otherwise difficult to distinguish microscopically (Jeffrey et al., 1999). While HPLC methods have been used extensively in marine waters (see Roy et al., 2011), they have only more recently been used extensively in freshwaters and are still relatively rarely applied to large meso- or oligotrophic systems such as the Laurentian Great Lakes (e.g., Descy et al., 2009; Lauridsen et al., 2011; Millie et al., 2003).

Pigment analysis is not straightforward because pigments will vary with the taxa present, but they also change with environmental conditions such as irradiance and the degree of nutrient limitation (see Descy et al., 2009). Thus, there are two critical issues: 1) correct diagnostic pigment ratios must be found that are appropriate to the ecosystem being considered, and 2) samples must be analyzed together in groups that are similar in terms of environmental conditions (typically termed

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'bins'). The first issue is relatively clear and much effort has been expended in finding such ratios (e.g., Lauridsen et al., 2011; Schlüter et al., 2006). The second point is more subtle. CHEMTAX is an optimization method which is based on an input matrix of different pigment to chl *a* ratios. Importantly, the CHEMTAX procedure actively changes these ratios, through a series of iterations. This means that the pigment ratios are adjusted with each run and change with the specific samples that are being analyzed. Stated another way, analyzing the same samples in different groupings will give different results. Unfortunately, phytoplankton ecologists often need to make comparisons across such groupings, which means that results derived with different matrices must be compared.

In order to explore these issues in practice, applying HPLC pigment analyses in freshwater communities, we chose paired nearshore–offshore stations in Lake Michigan, which provided a taxonomically rich phytoplankton community. By sampling seasonally, we were able to observe relatively large changes in taxa. Our overall objectives were to determine how accurately HPLC/CHEMTAX analysis of algal pigments could predict the broad taxonomic composition of the phytoplankton communities and to assess the use of the method in practice.

Methodology

Sampling sites

Field sampling was performed twice per month (June, July, August) during the summer of 2008 using the R/V Neeskay at two sites on Lake Michigan: Linnwood (43° 04.58′ N, 87° 50.29′ W, 20 m depth, nearshore) and Fox Point (43° 11.67′ N, 87° 47.26′ W, 100 m depth, offshore). These stations have served as reference locations for many studies conducted at the Great Lakes Water Institute (See http://www.waterbase.glwi.uwm.edu/monthly.php. Note that the Linnwood station is called "North Milwaukee" in these online datasets).

At each site, CTD casts (Sea-Bird Electronics Model 25 SeaLogger CTD) were taken for conductivity, dissolved oxygen, chl *a* fluorescence, quantum irradiance, and temperature. Appropriate sampling depths were identified from temperature–depth, irradiance–depth, and fluorescence–depth profiles. Epilimnetic samples were collected from a depth with 30% surface irradiance, and metalimnetic samples from the depth at which deep chlorophyll maxima were identified.

Water for biomass determination was collected with a 30-L Niskin bottle, and stored in brown polypropylene bottles on ice after onboard filtration (see below). Whole water samples for phytoplankton cell counts were preserved using acid Lugol's solution. Replicate samples for chl *a* were filtered onto Pall Supor filters (47 mm, 0.2 μm pore size), which were wrapped in aluminum foil, stored on ice, and frozen (−20 °C) on return to the laboratory. Samples for HPLC (≥2000 mL, pre-screened with 153-μm Nitex mesh) were filtered onto Whatman GF/F filters (47 mm, 0.7 μm nominal pore size), which were flash-frozen in liquid nitrogen and stored (−70 °C), for up to 12 months until analyzed.

Chlorophyll analyses

In the laboratory, pigments were extracted from chlorophyll filters and analyzed using a modification of EPA Method 445 (EPA, 1997), where pigments were extracted in 90% (buffered with 0.1% magnesium carbonate) acetone and measured using a Turner Designs TD 700 fluorimeter (configured with a daylight lamp, excitation filter 10-050R, emission filter 10-051R, and red-sensitive PMT tube), and an acidification correction for phaeopigments (Parsons et al., 1984).

Phytoplankton

Phytoplankton samples were counted, dimensions measured, biovolumes calculated and converted to chl *a* for comparison with HPLC/CHEMTAX estimates, within two years of collection. Phytoplankton were settled overnight using a 50-mL Utermöhl chamber and counted using an Olympus IX70 inverted microscope at magnification levels of 100–400×, using appropriate taxonomic keys (Dillard, 2007; Nygaard, 1976; Prescott, 1951). Samples were counted using random fields, multiple transects, or half-chamber scans, depending on the abundance of the taxa. Dimensions of the first five individuals of a given taxon were measured in order to calculate cell biovolume (using simple geometric approximations; Hillebrand et al., 1999). Biovolume was converted to chl *a* using the chl *a*:volume equation found in Montagnes et al. (1994). Although the equations used in this paper are based on marine species, they compare favorably with available relationships for freshwater species. The equations have critical advantages in that they account for volume shrinkage in Lugol's iodine and consider a comparable range of cell sizes.

HPLC methods

The HPLC method selected to detect taxon-specific pigments in Lake Michigan was slightly modified from Pinckney et al. (1996) by extending the time of the run and adjusting the flow rate to compensate for a slightly different column diameter (Electonic Supplementaary Material (ESM) Table S1), using a Shimadzu Prominence series HPLC system, outfitted with a Shimadzu Premier C-18 column, a Vydac 201TP C-18 column, and a Shimadzu diode array detector (Model SPD-M20A) set to produce an output with detection at 435 nm and 440 nm. An 82-min binary gradient was selected (A: 80:20 methanol:ammonium acetate 0.5 M, B: 80:20 methanol:acetone) with a 1.25-mL min^{-1} flow rate and 500-μL injection volume (ESM Table S1). The column oven temperature was kept at 40 °C and the autosampler at 4 °C. The HPLC was calibrated (following Roy et al., 2011) using pigment standards from DHI Lab Products (Denmark).

Cultures of representative algal taxa (ESM Table S2) were used to derive initial pigment:chl *a* input ratios. All cultures were grown at 18 °C in liquid DY-V growth medium (Lehman, 1976), with the exception of the cyanobacteria which were grown in liquid BG-11 growth medium (Stanier et al., 1971). Cultures were harvested after at least 1 week, with a minimum of 50 mL of algal culture filtered onto Whatman GF/F filters (47 mm, 0.7-μm nominal pore size). Filters were flash-frozen in liquid nitrogen and stored (−70 °C) until analysis (<3 months). Filters were removed from storage and extracted in 3 mL of 100% acetone overnight at −20 °C. Extracts were sonicated on ice for 1 min (Fisher Scientific Sonic Dismembranator Model 100, 15 W) and filtered through a 0.2-μm syringe filter. A final volume of 800 μL was placed into an autosampler vial. Immediately prior to injection, 200 μL of 0.5 M ammonium acetate was added to aid in the separation of the pigments (Pinckney et al., 1996). Taxon-specific pigments were identified by HPLC column retention time and absorbance spectra using previously published data (Jeffrey et al., 1997) and during runs, identity of pigments was routinely checked by examining the photo diode array spectra (cf. Descy et al., 2009). Each filter extract was analyzed with analytical replicates (i.e., two separate runs were done for every filter collected).

CHEMTAX analyses

HPLC pigment data (expressed as ratios to chl *a*, hereafter "pigment ratios") were analyzed using the CHEMTAX (V 1.95) program (Mackey et al., 1996), obtained from S. Wright (CSIRO, Australia). This program uses the steepest-descent algorithm to fit a matrix of pigment ratios that define key taxa (determined from reference cultures) to the pigment ratios in an unknown sample. With assistance from D. Millie (Great Lakes Research Center, Michigan Tech, USA), a ratio matrix for Lake Michigan samples was created using a custom-designed spreadsheet. An input table of pigment ratios was used that included: fucoxanthin (diatoms and chrysophytes), neoxanthin (chlorophytes),

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