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Adapting the CHEMTAX Method for Assessing Phytoplankton Taxonomic Composition in Southeastern U.S. Estuaries

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ABSTRACT: CHEMTAX is a matrix factorization program used to derive taxonomic structure of phytoplankton from photosynthetic pigment ratios. The program was originally developed from and applied to the analysis of oceanic phytoplankton assemblages. We found that application of the original CHEMTAX reference matrix to southeastern United States estuarine systems produced inaccurate results, as verified by microscopy. Modification of the matrix, based primarily on the pigment ratios of 33 estuarine isolates, improved the predictive capabilities of CHEMTAX for our samples. Limitations of the method included an overestimation of diatom biomass (due to the inability to differentiate diatoms from taxa with chloroplasts derived from diatom endosymbionts, notably some dinoflagellates) and a tendency to exclude some raphidophyte species. In complement with microscopic verification, the method was shown to improve assessment of phytoplankton taxonomic composition.

Introduction

Quantitative taxonomy of phytoplankton assemblages traditionally have been determined by microscopic examination of water samples. This process is time consuming, requires great skill and expertise, and is often subjective. Nanoplankton and picoplankton dominated assemblages are particularly difficult to identify and quantify, and require a combination of light, epifluorescent, and electron microscopy. In recent years, high performance liquid chromatography (HPLC) has been used to estimate phytoplankton composition by identifying photosynthetic pigments of chemotaxonomic relevance. New analytical techniques allow an increasing number of algal pigments to be resolved, and HPLC is used extensively (Wright et al. 1991; Van Heukelem et al. 1994; Jeffrey et al. 1997; Van Heukelem and Thomas 2001). Advantages to HPLC pigment analyses include rapid turnover and reproducible results (Millie et al. 1993; Wright et al. 1996).

HPLC does not provide the same taxonomic resolution as microscopy. Early HPLC analyses were qualitative and relied on the detection of diagnostic (marker) pigments present in only one or two groups; e.g., peridinin for Dinophyceae, alloxanthin for Cryptophyceae, prasinoxanthin for some Prasinophyceae (Gieskes and Kraay 1986; Klein and Sournia 1987). Quantitative methods were developed later to estimate the abundance of various phytoplankton classes from ratios of marker pigments to chlorophyll *a* (chl *a*; Gieskes et al. 1988; Everitt et al. 1990; Letelier et al. 1993). These methods gave unreliable results for taxa containing ambiguous pigment markers (Mackey et al. 1996).

Mackey et al. (1996) introduced CHEMTAX, a MATLAB (The MathWorks, Inc., Natick, Massachusetts) program for describing the relative abundances of taxonomic groups. Rather than using simple ratios of marker pigments, CHEMTAX uses a steepest-descent algorithm to fit a matrix of expected pigment ratios for several taxa, to one consisting of the actual pigment ratios from unknown

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samples. It has been used to describe oceanic phytoplankton assemblages, using pigment data obtained from literature (Wright et al. 1996; Mackey et al. 1998; Higgins and Mackey 2000; Riegman and Kraay 2001; Furuya et al. 2003).

Although it does not rely on marker pigments to form groups, greater resolution and accuracy is achieved when taxa with distinctive markers are present. CHEMTAX requires that the number of pigments be slightly greater than the number of taxonomic groupings (i.e., phytoplankton classes). The reference matrix must also include each major class likely to be present in the samples (Mackey et al. 1996). Because the predictive capabilities of CHEMTAX depend on the pigment ratios used for the reference matrix, it is critical to calibrate CHEMTAX to the assemblages from which samples will be taken (Mackey et al. 1996; DiTullio et al. 2003). These pigment ratios should be obtained from isolates representing taxa local to the area of investigation.

In our study, we applied CHEMTAX analyses to generate estimates of southeastern United States estuarine phytoplankton assemblages. We performed dual analyses, comparing results from Mackey et al.'s (1996) matrix (based on oceanic assemblages), to our matrix of pigment ratios primarily from estuarine phytoplankton isolates.

Materials and Methods

Cultures

A pigment matrix was developed that included 12 taxonomic groups (Table 1). Nine of these groups were based on pigment compositions of 33 estuarine isolates. Estuarine representatives of prasinoxanthin-containing prasinophytes, and flagellates with 19′-hexanoyloxyfucoxanthin or 19′-butanoyloxyfucoxanthin, were not available to the project. Groups 5 (Prasino-B), 9 (Hapto-B), and 10 (Chryso-B) were based on Mackey et al.'s (1996) Prasinophyceae Type 2, Haptophyceae Type 4, and Chrysophyceae Type 2, respectively. All cultures were routinely maintained at 24°C, on a 14:10 h light:dark cycle (60–80 $\mu E\ m^{-2}\ s^{-1}$). Culture media was f/2-enriched (nutrient, trace metal, and vitamin additions following Guillard 1975, but without silicate) filtered North Inlet, South Carolina, water with salinity adjusted to 16‰ or 30‰, depending on the species.

The effects of physiological status on pigment composition were examined in 10 isolates (designated by * in Table 1) by growing cultures under light-limiting or light-saturating conditions and harvesting cultures during exponential (i.e., nutrient-replete) and stationary (nutrient-deplete) growth phases. Isolates were grown in 250 ml batch cultures in f/2 media at 24°C (n = 3 per light treatment). A 14:10 h light:dark cycle was used under two light treatments. Low light (LL) was set at 25 $\mu E\ m^{-2}\ s^{-1}$, and high light (HL) was set at 250 $\mu E\ m^{-2}\ s^{-1}$. *Heterosigma akashiwo* did not grow under LL, so an intermediate irradiance of 65 $\mu E\ m^{-2}\ s^{-1}$ was used. Cells were kept in suspension with gentle shaking (100 rpm).

Cultures were first acclimated to ambient conditions by making two successive transfers into fresh media, during the exponential growth stage. Each transfer resulted in an abundance of 10^4 cell ml^{-1}. Following a third transfer, cell abundance in each culture was monitored daily, using a hemacytometer (Hauser Scientific, Horsham, Pennsylvania). Subsamples of each culture were withdrawn during mid exponential and stationary growth phases and filtered under gentle vacuum (−20 mm Hg) onto 25 mm glass fiber filters (GF/F; 0.7 μm nominal pore size). For *H. akashiwo* cultures grown at 65 $\mu E\ m^{-2}\ s^{-1}$, samples were taken during stationary growth phase only.

Field Samples

Water samples were collected from the North Inlet (Georgetown, South Carolina) and Murrells Inlet (Murrells Inlet, South Carolina) saltmarsh estuaries (Table 2). Sample bottles were placed in coolers at ambient water temperature and transported immediately to the Baruch Marine Laboratory (Georgetown, South Carolina). Aliquots were either preserved with Lugols iodine (3 ml in 100 ml sample), preserved with 50% glutaraldehyde solution (200 μl in 10 ml sample), or filtered onto GF/Fs for HPLC analyses. Diatoms and dinoflagellates were enumerated from Lugols-preserved samples, using Utermöhl chambers, and examined with an inverted microscope. All other cells were counted by filtering a known volume of glutaraldehyde-fixed water onto 0.4 μm black polycarbonate membrane filters (Osmonics, Inc.) and enumerated by epifluorescent microscopy.

Biovolumes were estimated using the geometric shapes and mathematical formulas presented in Hillebrand et al. (1999). A biovolume estimate of 1.3 μm^3 was used for *Synechococcus* spp., based on Kana and Glibert (1987). The effects of preservation on biovolume can be significant and vary with fixative, species, and analytical method (Booth 1987; Verity et al. 1992; Montagnes et al. 1994; Menden-Deuer et al. 2001). We did not attempt to apply correction factors to biovolume because of the great variability of effects on individual species, which can swell or shrink depending on fixative or physiological state. The overall correction factor of Montagnes et al. (1994) for analysis of Lugols-fixed samples by light microscopy (25% shrinkage) and

TABLE 1. Species used to derive pigment ratio for CHEMTAX matrix based on 12 taxonomic groups. Culture collections: CCMP = Provasoli-Guillard National Center for Culture of Marine Phytoplankton, HP = Horn Point, and SCAEL = South Carolina Algal Ecology Laboratory. MA = Massachusetts, MD = Maryland, and SC = South Carolina. allo = alloxanthin, but = 19′-butanoyloxyfucoxanthin, chl b = chlorophyll *b*, chl c1 = chlorophyll c_1, chl c2 = chlorophyll c_2, diadino = diadinoxanthin, diato = diatoxanthin, fuco = fucoxanthin, hex = 19′-hexanoyloxyfucoxanthin, lut = lutein, neo = neoxanthin, per = peridinin, prasino = prasinoxanthin, viola = violaxanthin, and zea = zeaxanthin. undes = undescribed. * = strains used in light-growth phase experiments (see Figs. 1 and 2).

Group	Class	Species	Source	Pigments
1 = Diatom/Dino-A	Bacillariophyceae	**Thalassiosira* cf. *miniscula* HP9101	Choptank River, MD	chl c1, chl c2
	Bacillariophyceae	*Cylindrotheca closterium* (undes strain)	North Inlet, SC	diadino, diato
	Bacillariophyceae	*Nitzschia* sp. HP9101	Choptank River, MD	fuco
	Bacillariophyceae	*Cylindrotheca closterium* (undes strain)	North Inlet, SC	
	Dinophyceae-A	*Kryptoperidinium foliaceum*	North Inlet, SC	
2 = Dino-B	Dinophyceae-B	**Amphidinium carterae* CCMP1314	Falmouth Great Pond, MA	chl c1, diadino
	Dinophyceae-B	*Prorocentrum minimum* SCAEL010403	Murrells Inlet, SC	diato, per
3 = Cyano	Cyanobacteria	filamentous species (undes strain)	North Inlet, SC	zea
	Cyanobacteria	*Limnothrix* sp. HP9101	Choptank River, MD	
	Cyanobacteria	*Anabaenopsis elekenii* SCAEL010524	Kiawah Island, SC	
	Cyanobacteria	**Synechococcus* sp. HP9101	Choptank River, MD	
4 = Prasino-A	Prasinophyceae-A	**Nephroselmis* sp. HP9001	Choptank River, MD	chl b, lut, neo
	Prasinophyceae-A	*Pyramimonas* sp. HP9001	Choptank River, MD	viola
5 = Prasino-B	Prasinophyceae-B	Mackey Prasinophyceae Type 2	Not estuarine	chl b, prasino, viola, zea
6 = Chloro	Chlorophyceae	unknown species (undes strain)	North Inlet, SC	chl b, lut, neo
	Chlorophyceae	unknown species (undes strain)	North Inlet, SC	viola, zea
	Conjugatophyceae	**Ankistrodesmus* sp. HP9101	Choptank River, MD	
	Chlorophyceae	unknown species (undes strain)	North Inlet, SC	
	Chlorophyceae	unknown species (undes strain)	North Inlet, SC	
	Chlorophyceae	unknown species (undes strain)	Choptank River, MD	
	Chlorophyceae	**Chlorella* sp. HP9101	Choptank River, MD	
7 = Crypto	Cryptophyceae	**Storeatula major* HP9001	Choptank River, MD	allo, chl c2
	Cryptophyceae	*Chroomonas* sp. HP9101	Choptank River, MD	
	Cryptophyceae	*Cryptomonas* sp. HP9002	Choptank River, MD	
	Cryptophyceae	*Cryptomonas* sp. HP9102	Choptank River, MD	
	Cryptophyceae	*Hemiselmis* sp. HP9001	Choptank River, MD	
	Cryptophyceae	*Cryptomonas* sp. HP9101	Choptank River, MD	
8 = Hapto-A/Chryso-A/Dino-C	Haptophyceae-A	unknown species (undes strain)	North Inlet, SC	chl c1, chl c2
	Haptophyceae-A	unknown species (undes strain)	North Inlet, SC	diadino, diato
	Haptophyceae-A	*Isochrysis* sp. CCMP1324	South Pacific	fuco
	Haptophyceae-A	**Pavlova* sp. HP9101	Choptank River, MD	
	Chrysophyceae-A	**Ochromonas* sp. SCAEL970626	North Inlet, SC	
9 = Hapto-B/Dino-D	Haptophyceae-B	Mackey Haptophyceae Type 4	Not estuarine	but, diadino, diato, hex
10 = Chryso-B	Chrysophyceae-B	Mackey Chrysophyceae Type 2	Not estuarine	but, chl c2, diadino, diato, fuco
11 = Raphido-A	Raphidophyceae-A	**Heterosigma akashiwo* HP9001	Choptank River, MD	chl c1, chl c2, diadino, fuco, viola, zea
12 = Eugleno	Euglenophyceae	*Euglena* sp. SCAEL010403	Murrells Inlet, SC	chl b, diadino, diato, lut, neo, zea
	Euglenophyceae	*Euglena* sp. SCAEL010408	Murrells Inlet, SC	

TABLE 2. Field sample locations and data.

Sample	Date	Estuary	Site	Coordinates	Salinity (‰)	Temperature (°C)	Chl *a* ($\mu g\ l^{-1}$)
1	August 5, 1998	North Inlet	NI3	33°22′10.18N 79°9′55.00W	36	28	6.9
2	October 2, 1998	Murrells Inlet	MI3B	33°33′23.43N 79°1′53.24W	38	26	8.4
3	January 28, 1999	North Inlet	NI2B	33°19′56.12N 79°11′34.83W	31	14	4
4	January 28, 1999	North Inlet	NI3C	33°22′10.18N 79°9′55.00W	26	14	3
5	January 28, 1999	Murrells Inlet	MI2B	33°34′48.43N 79°0′14.97W	30	13.5	2.9
6	January 28, 1999	Murrells Inlet	MI6A	33°31′25.68N 79°3′36.83W	35	14	2.1
7	May 11, 1999	North Inlet	NI6A	33°19′50.56N 79°10′12.32W	28	22	7.3
8	May 11, 1999	Murrells Inlet	MI8A	33°31′53.13N 79°2′17.15W	34	20.5	5.6
9	July 9, 1999	North Inlet	NI7A	33°18′30.77N 79°10′40.14W	36	30	10.8
10	August 24, 1999	Murrells Inlet	MI3B	33°33′23.43N 79°1′53.24W	36	29	6.6

Verity et al. (1992) for analysis of glutaraldehyde-fixed samples by epifluorescence microscopy (29% shrinkage) are reasonably close, suggesting that the methodological errors may not greatly affect estimates of the relative contributions of taxa to total community biomass. Menden-Deuer et al. (2001) found that estimates of cellular carbon (C) from fixed-cell volumes approximated those from live-cell volumes, when the preservation effects on several species were combined.

Carbon $cell^{-1}$ was estimated using biovolume-to-carbon conversion formulas taken from Menden-Deuer and Lessard (2000). Regression equations from the authors' Table 4 were applied to the corresponding taxonomic group. Equations based on the Protist plankton categories were used for Cryptophyceae, Euglenophyceae, Raphidophyceae, filamentous Cyanophyceae, and unidentified flagellates. For *Synechococcus* spp., 0.25 pg C $cell^{-1}$ was used, based on the measurements of Kana and Glibert (1987).

HPLC Analyses

Under subdued lighting, frozen filters were placed in a 10 ml glass tissue homogenizer. HPLC-grade acetone was added (2 ml, 100%), and the sample was pulverized with a teflon pestle over an ice bath. The resulting slurry was filtered through a 0.2 μm polytetrafluoroethylene (PTFE) syringe filter (Pall Gelman Laboratory, Ann Arbor, Michigan) into amber-glass vials, which were stored at −20°C until analysis.

Extracts were analyzed using a Beckman Gold HPLC system (Beckman Coulter, Fullerton, California) equipped with a Beckman 125 dual pump unit. Samples were injected manually, using a 200 μl sample loop, and separated on an Eclipse XDB-C8, reverse-phase column (150 × 4.6 mm, 3.5 μm particle size; Agilent Technologies, Palo Alto, California). Column temperature was maintained with an Eldex CH-150 column heater (Eldex Laboratories, Napa, California). Chromatograms were generated from the absorbance of individual pigments at 450 nm (20 nm bandwidth) using a Beckman 168 Diode Array Detector. Pigments were identified by comparing their retention times and absorption spectra to those of pure standards.

Pigments were separated using the method of Van Heukelem et al. (1994), modified for the Beckman instrument. All solvents were HPLC grade, and filtered through a 0.22 μm PTFE membrane. Solvent A was 30:70 (v:v) aqueous tetrabutyl ammonium acetate buffer (TBAA, 28 mM, pH 6.5):methanol. Solvent B was 100% methanol. Solvent flow rate was 1.1 ml min^{-1}, and initial conditions were 5% Solvent B. Column temperature was maintained at 60°C. Samples were mixed 50:50 (v:v) with TBAA, and allowed to equilibrate for 5 min. A 200 μl aliquot of the mixture was injected onto the column, initiating a linear solvent gradient from 5% to 95% methanol over 22 min. This was followed by an isocratic hold (95% methanol) for another 5 min. Initial conditions were restored over a 3-min gradient between injections. Statistical significance between means was analyzed by *t*-test using a probability level of 95%.

Results

Culture Experiment

The cellular chl *a* content in LL treatments was greater than that in HL in all species regardless of

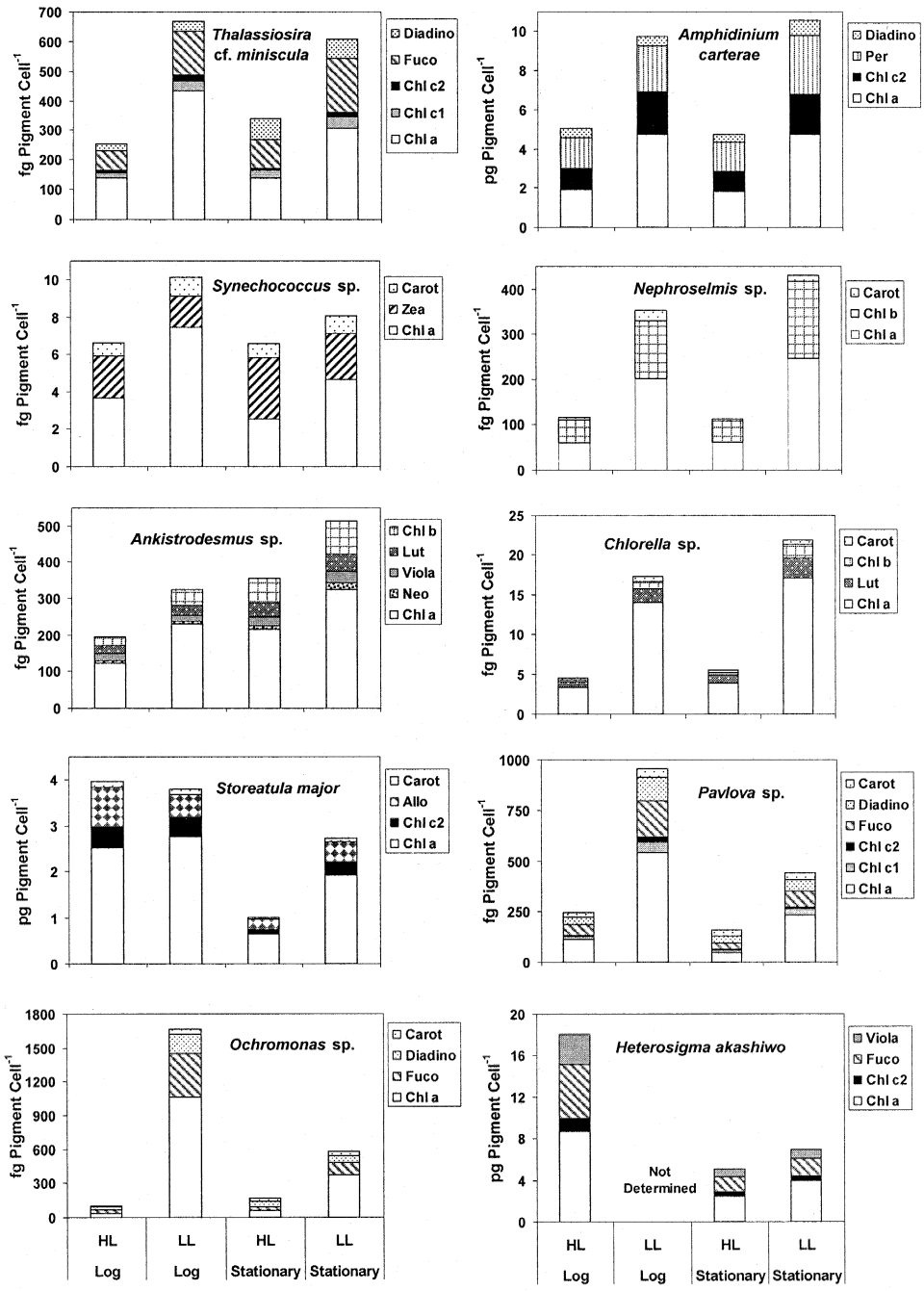

Fig. 1. The effects of different growth irradiances (high light [HL] versus low light [LL]) and growth phases (log versus stationary phase) on cellular pigment concentrations in *Thalassiosira* cf. *miniscula, Amphidinium carterae, Synechococcus* sp., *Nephroselmis* sp., *Ankistrodesmus* sp., *Chlorella* sp., *Storeatula major, Pavlova* sp., *Ochromonas* sp., and *Heterosigma akashiwo.* Pigment abbreviations: Allo = alloxanthin, Carot = carotenes, Chl b = chlorophyll *b,* Chl c1 = chlorophyll c_1, Chl c2 = chlorophyll c_2, Diadino = diadinoxanthin, Diato = diatoxanthin, Fuco = fucoxanthin, Lut = lutein, Neo = neoxanthin, Per = peridinin, Viola = violaxanthin, Zea = zeaxanthin.

growth stage (Fig. 1). A relatively greater pigment concentration cell^{-1} in LL also was found for most accessory pigments in all species with two notable exceptions. Zeaxanthin cell^{-1} was significantly higher in *Synechococcus* cultures grown at HL than at LL during exponential and stationary growth phase (Fig. 1) and alloxanthin cell^{-1} was higher in *Storeatula major* cultures during exponential, but not stationary, growth phase (Fig. 1). The difference between HL and LL pigmentation was excep-

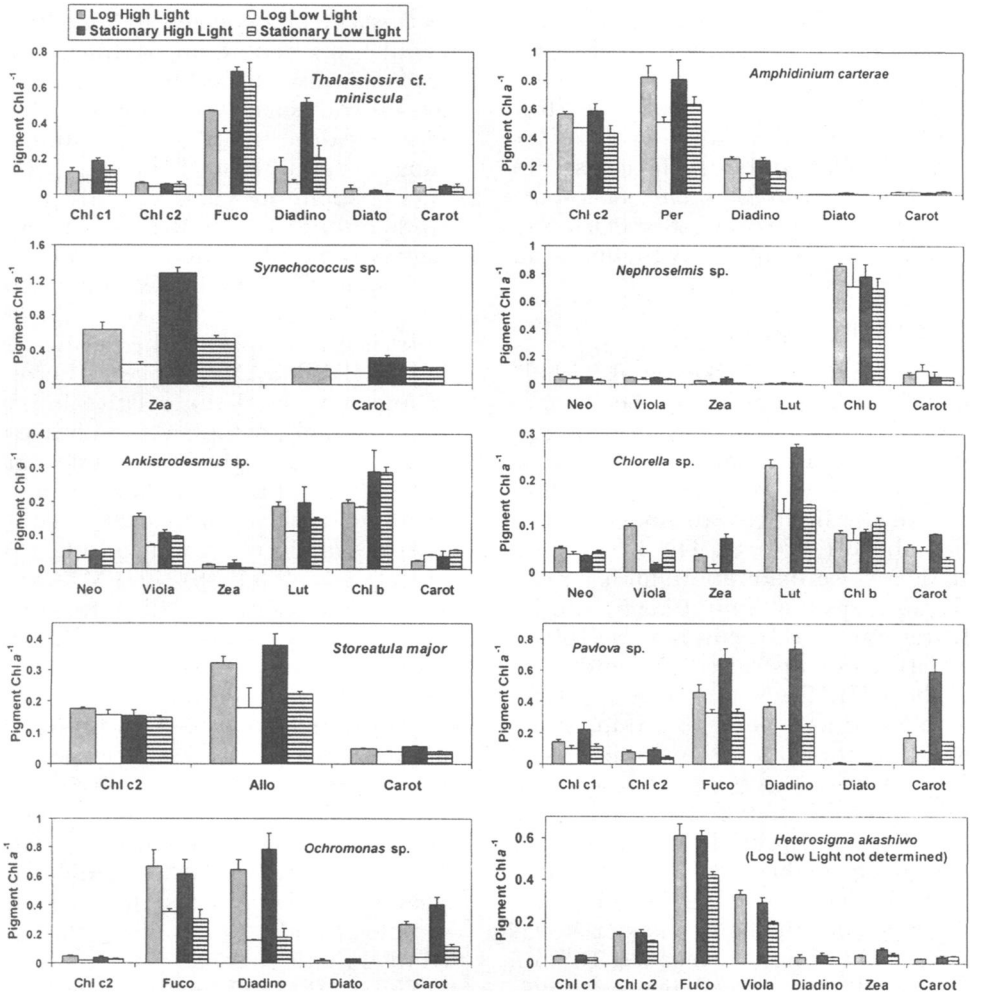

Fig. 2. The effects of different growth irradiances (high light versus low light) and growth phases (log versus stationary phase) on accessory pigment chlorophyll a^{-1} in *Thalassiosira* cf. *miniscula, Amphidinium carterae, Synechococcus* sp., *Nephroselmis* sp., *Ankistrodesmus* sp., *Chlorella* sp., *Storeatula major, Pavlova* sp., *Ochromonas* sp., and *Heterosigma akashiwo.* Pigment abbreviations: Allo = alloxanthin, Carot = carotenes, Chl b = chlorophyll *b,* Chl c1 = chlorophyll c_1, Chl c2 = chlorophyll c_2, Diadino = diadinoxanthin, Diato = diatoxanthin, Fuco = fucoxanthin, Lut = lutein, Neo = neoxanthin, Per = peridinin, Viola = violaxanthin, and Zea = zeaxanthin.

tional in *Ochromonas,* with pigment cell^{-1} in exponential phase LL cultures exceeding that in HL cultures by 27-fold (chl *a*), 11-fold (chl c_2), 14-fold (fucoxanthin), 7-fold (diadinoxanthin), or 4-fold (carotenes; Fig. 1, except chl c_2 not shown).

The effect of growth phase on pigment content varied with species, pigment, and growth irradiance. In 6 species, chl *a* cell^{-1} was significantly greater in exponential phase cultures than in stationary phase; e.g., *Thalassiosira* (Fig. 1, LL only), *Synechococcus* (Fig. 1), *Storeatula* (Fig. 1), *Pavlova* (Fig. 1), *Ochromonas* (Fig. 1, LL only), and *Heterosigma* (Fig. 1, data only available for HL). In the other species, chl *a* cell^{-1} did not vary significantly with growth phase, with the exception that a significantly greater value was observed in stationary phase *Ankistrodesmus* cultures than in exponential phase cultures (Fig. 1). The effects of growth stage on cellular content of the accessory pigments were the same as the effects on chl *a,* with three exceptions. Diadinoxanthin was significantly greater in *Thalassiosira* (HL and LL) and *Amphidinium* (LL) stationary phase cultures than in exponential phase cultures (Fig. 1), and zeaxanthin cell^{-1} was greater in stationary phase *Synechococcus* cultures (Fig. 1).

When normalized to chl *a,* treatment effects on accessory pigments showed a consistent trend with growth irradiance but not growth phase (Fig. 2). In 9 of 10 species, significantly greater accessory pigment:chl *a* ratios were observed in HL cultures than in LL cultures, regardless of growth phase. This ratio did not vary with irradiance treatment in *Nephroselmis* (Fig. 2). The effect of growth phase

on accessory pigment chl a^{-1} ratio varied with species, but only in rare cases did exponential phase cells contain greater ratios than stationary phase cells; e.g., violaxanthin in HL *Ankistrodesmus* (Fig. 2) and neoxanthin and violaxanthin in HL *Chlorella* (Fig. 2). Growth phase did not affect this ratio in 4 species (*Amphidinium, Nephroselmis, Storeatula, Heterosigma*), and in the remaining 6 species, stationary phase ratios were significantly higher in all or some of the accessory pigments.

CHEMTAX Analyses

Construction of the initial Mackey et al. (1996) CHEMTAX matrix was based on taxon-specific medians (Table 3). For the present study matrix, means of accessory pigment chl a^{-1} ratios were used, based on several culture trials, including those derived from the light-growth phase experiments described above (Table 1). The Prepro settings for CHEMTAX were iteration limit (500), weight bound (30), epsilon limit (0.005), initial step size (10), step ratio (1.3), cutoff step (1000), verbosity (normal), elements varied (5), and subiterations (5). The CHEMTAX output is the fraction of chl *a* contained in each algal group.

Using HPLC pigment profiles from 10 field samples, application of the Mackey et al. (1996) matrix (Fig. 3) and the present study's matrix (Fig. 3) yielded greatly different results. Differences were especially pronounced in the relative fractions of Haptophyceae and Cryptophyceae, which were consistently much greater in the Mackey et al. results, and Diatoms, which never exceeded 10% of chl *a* in Mackey et al., but ranged between 24% and 82% (mean = 53%) in the present study's matrix results.

Microscopic evaluations were used to determine whether CHEMTAX results approximated phytoplankton taxonomic composition. This method was limited by several uncertainties inherent in analyzing taxonomically diverse assemblages. The taxonomic classification of several unidentified flagellates was unclear, and these made up from 1% to 8% of total phytoplankton biovolume, 1% to 14% of phytoplankton C, 2% to 46% of total flagellate biovolume, and 2% to 38% of flagellate C (data not shown). Another factor confounding the ability to microscopically ground-truth the CHEMTAX results is the fact that dinoflagellates can contain one of four alternative pigment compositions, depending on species. Dinophyceae made up 10 ± 8% of phytoplankton biovolume and 19 ± 9% of phytoplankton C, and only a few of the species could be classified by pigment type. Another flaw in this method for testing the accuracy of the CHEMTAX results is the use of biovolume and cellular C as biomass indices because these would not necessarily be expected to correlate precisely with cellular pigmentation. Sample preservation often leads to distortions in biovolume that can vary greatly with species, fixative type (lugols versus glutaraldehyde), sample preparation protocol (settling versus filtering), microscopic method (light versus epifluorescence), and preserved sample storage time (Booth 1987; Verity et al. 1992; Montagnes et al. 1994; Menden-Deuer et al. 2001).

Despite the limitations, some gross patterns in taxonomic composition were revealed that served as basis for comparing the predictability of the different CHEMTAX matrices. Microscopic biomass estimates verified the relative importance of diatoms to total phytoplankton biomass (Fig. 4), as is consistent with previous studies in these waters (Kawaguchi et al. 1997; Lewitus et al. 1998, 2000). Diatoms made up 72 ± 20% and 53 ± 22% of phytoplankton biovolume and C, respectively. The Mackey et al. (1996) CHEMTAX matrix consistently underestimated diatom biomass, while the present study's matrix agreed in some cases. For example, estimates of diatom biomass based on cellular C were within 25% of these CHEMTAX fractions in samples 1–5 and 9–10 (Fig. 4). In two of the three samples where diatom C was more than 20% lower than our CHEMTAX estimates (samples 7 and 9), the sum of diatom and dinoflagellate C biomass brought the agreement to within 5% (sample 7) and 4% (sample 9; Fig. 4). One possible explanation is that the Dinophyceae in samples 7 and 9 were predominantly composed of Dino-A species.

The CHEMTAX matrices were compared for their ability to predict a subset of flagellate groups. For the Mackey et al. (1996) matrix, Prasinophyceae Types 1 and 3, Chlorophyceae, Haptophyceae Types 1 and 4, and Chrysophyceae Type 2 were combined and compared to the present study matrix combination of Prasino-A, Prasino-B, Chloro, Hapto-A, Hapto-B, Chryso-B, and Raphido-A. The Mackey et al. (1996) matrix consistently predicted higher contributions of these collective taxa to phytoplankton composition than the present study's matrix, and in 6 of 10 samples, both methods indicated substantially higher fractions than the microscopic estimates, based on biovolume or C (Fig. 4). Close approximation by the present study's CHEMTAX predictions was only found in samples 1 and 7, which were within 93% and 80%, respectively, of the C-based biomass estimates. When dinoflagellates were added to the microscopy-based biomass estimates, the agreement between C biomass and the present study's CHEMTAX results was improved in samples 2–5 and 10 (Fig. 4). The results again point to the possibility that dinoflagellates with pigment compositions

TABLE 3. Matrix ratios for CHEMTAX used in present study and Mackey et al. (1996). Pigment abbreviations: Allo = alloxanthin, Diadino = diadinoxanthin, Diato = diatoxanthin, Fuco = fucoxanthin, Lut = lutein, Neo = neoxanthin, Per = peridinin, Prasino = prasinoxanthin, Viola = violaxanthin, Zea = zeaxanthin, But = 19′-butanoyloxyfucoxanthin, Hex = 19′-hexanoyloxyfucoxanthin, Chl c = chlorophyll *c*, Chl b = chlorophyll *b*, Chl a = chlorophyll *a*. For the present study's matrix, chlorophyll *c* was derived from the sum of chlorophylls c_1 and c_2.

	Allo	Diadino	Diato	Fuco	Lut	Neo	Per	Prasino	Viola	Zea	But	Hex	Chl c	Chl b	Chl a
Present study															
Diatom/Dino-A	0	0.124	0.025	0.546	0	0	0	0	0	0	0	0	0.239	0	1
Dino-B	0	0.211	0.064	0	0	0	0.787	0	0	0	0	0	0.568	0	1
Cyano	0	0	0	0	0	0	0	0	0	0.368	0	0	0	0	1
Prasino-A	0	0	0	0	0.007	0.064	0	0	0.073	0.007	0	0	0	0.480	1
Prasino-B	0	0	0	0	0	0	0	0.024	0.008	0.003	0	0	0	0.131	1
Chloro	0	0	0	0	0.221	0.056	0	0	0.060	0.002	0	0	0	0.322	1
Crypto	0.389	0	0	0	0	0	0	0	0	0	0	0	0.292	0	1
Hapto-A/Chryso-A/Dino-C	0	0.238	0.034	0.356	0	0	0	0	0	0	0	0	0.127	0	1
Hapto-B/Dino-D	0	0.243	0.090	0.485	0	0	0	0	0	0	0.272	0.539	0.150	0	1
Chryso-B	0	0.438	0.051	0.625	0	0	0	0	0	0	0.933	0	0.127	0	1
Raphido-A	0	0.046	0	0.573	0	0	0	0	0.266	0.064	0	0	0.185	0	1
Eugleno	0	0.086	0.058	0	0.022	0.077	0	0	0	0.020	0	0	0	0.828	1
Mackey et al. (1996)															
Diatom	0	0.239	0.135	0.457	0	0.002	0	0	0	0	0	0	0.092	0	1
Dino	0	0.121	0.101	0	0	0	0.532	0	0	0	0	0.192	0	0	1
Cyano	0	0	0	0	0	0	0	0	0	0.223	0	0	0	0	1
Prasino Type 1	0	0	0	0	0.043	0.051	0	0	0.033	0.075	0	0	0	0.812	1
Prasino Type 2	0	0	0	0	0	0	0	0.024	0.008	0.003	0	0	0	0.131	1
Chloro	0	0	0	0	0.142	0.037	0	0	0.028	0.059	0	0	0	0.285	1
Crypto	0.136	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Hapto Type 1	0	0.181	0.007	0.654	0	0	0	0	0	0	0	0	0.193	0	1
Hapto Type 4	0	0.243	0.090	0.485	0	0	0	0	0	0	0.272	0.539	0.150	0	1
Chryso Type 2	0	0.438	0.051	0.625	0	0	0	0	0	0	0.933	0	0.268	0	1
Eugleno	0	0.230	0.027	0	0	0.015	0	0	0	0	0	0	0	0.406	1

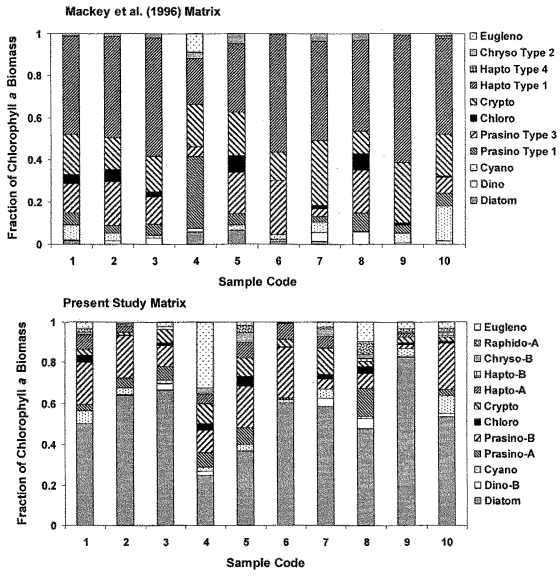

Fig. 3. Comparison of predicted phytoplankton composition from the Mackey et al. (1996) CHEMTAX matrix and the present study's CHEMTAX matrix based on HPLC-derived pigment concentrations obtained from 10 South Carolina estuarine samples (sample codes described in Table 2). Each phytoplankton taxon is shown as the fraction of total chlorophyll *a*.

that derive from 1 or more of these flagellate groups (e.g., Dino-C, Dino-D) may be important in these samples.

The capability of the present study's CHEMTAX matrix to predict the contribution of individual taxa to phytoplankton C biomass was generally poor (Fig. 5). The method may have been limited by the relatively low biomass of each taxon with respect to total phytoplankton composition. The combination of diatoms and dinoflagellates comprised > 50% of phytoplankton C in 9 of 10 samples and more than 90% in 4 samples (Figs. 4 and 5). C estimates from any other taxon exceeded 10% of phytoplankton biomass in only 6 of 70 samples (Fig. 5). The discrepancy shown in Fig. 5 between Dino-B estimates of fractional biomass (always < 5% of chl *a*) and microscopic biomass estimates (> 15% of phytoplankton C in 8 of 10 samples) is evidence that the majority of dinoflagellates in these samples did not contain peridinin, and supports the contention that dinoflagellates at times contributed highly to the Diatom and some flagellate CHEMTAX estimates (Fig. 4). CHEMTAX prediction of raphidophytes was also exceptionally poor (Fig. 5). This is not surprising, because this group was derived from one species (*H. akashiwo*) and pigment composition can vary within this class (Hymel unpublished data).

The relationship between the present study's CHEMTAX estimates and microscopically determined C estimates was examined for individual taxa, based on samples where the fraction of phytoplankton C for an individual taxon exceeded 2% (Fig. 6). Estimates from Dino-B and Raphido classes were not included. The correlation coefficient ($R^2 = 0.14$) was poor, but removal of two apparent outliers (Prasino in sample 5, Hapto/Chryso in sample 8) improved the correlation ($R^2 = 0.60$). It is possible that prasinophytes were not identified by microscopic analysis in sample 5 (this sample

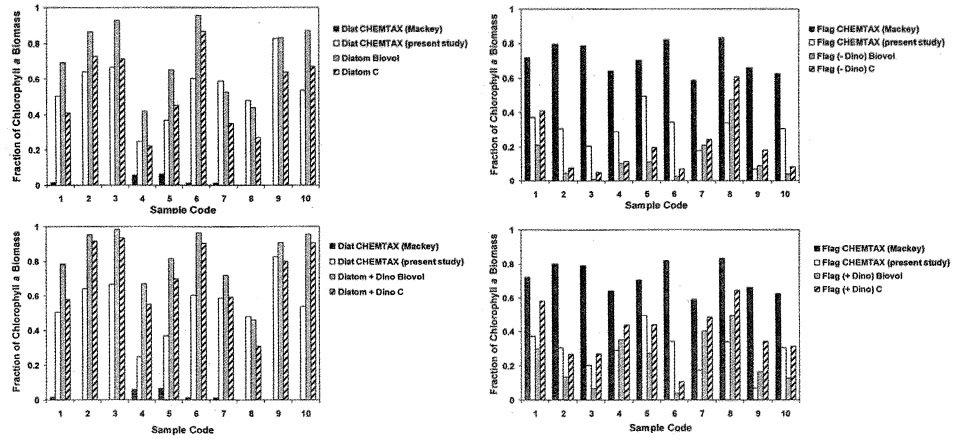

Fig. 4. The fraction of total chlorophyll *a* biomass predicted by the Mackey et al. (1996) CHEMTAX matrix (black), predicted by the present study's CHEMTAX matrix (white), estimated microscopically as biovolume (gray), and estimated microscopically as carbon (cross-hatched) for Diatoms, Diatoms using the sum of Diatom and Dinoflagellate biomass for biovolume and carbon estimates, Flagellates omitting Dinoflagellate biomass in biovolume and carbon estimates, and Flagellates including Dinoflagellate biomass in biovolume and carbon estimates. Flagellates for Mackey et al. application are the sum of Prasinophyceae Types 1 and 3, Chlorophyceae, Haptophyceae Types 1 and 4, and Chrysophyceae Type 2. Flagellates for the present study's application are the sum of Prasino-A, Prasino-B, Chloro, Hapto-A, Hapto-B, Chryso-B, and Raphido-A.

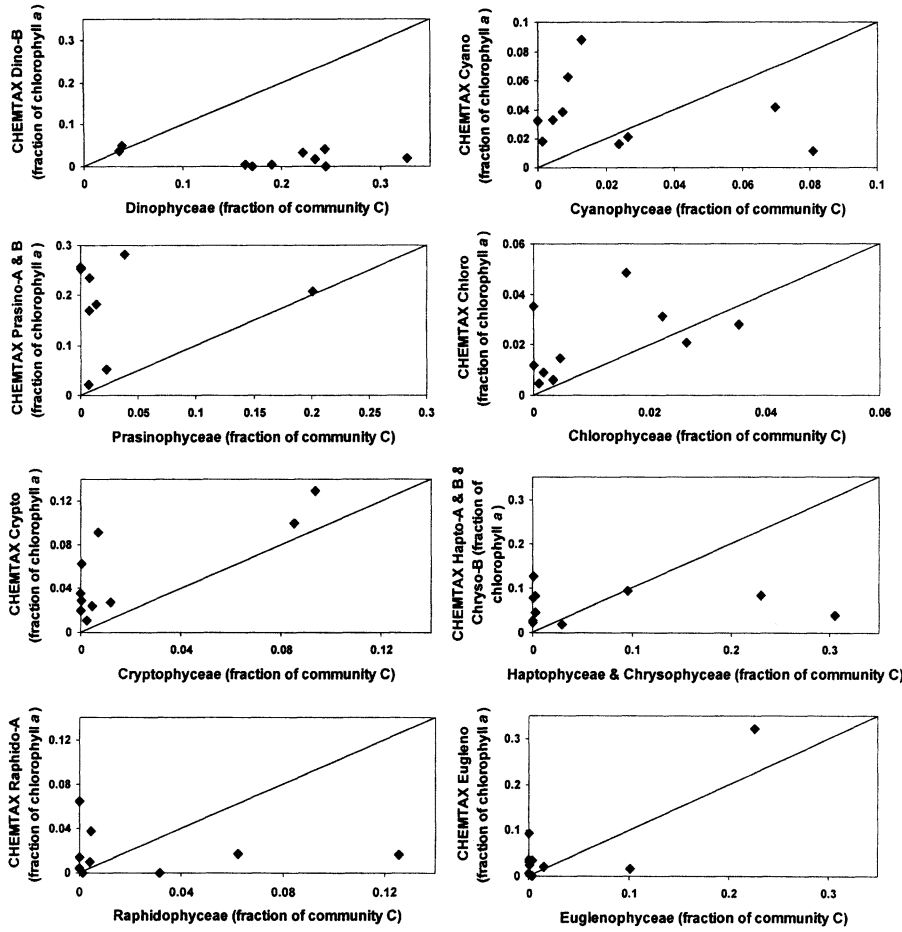

Fig. 5. Relationship between the present study's CHEMTAX prediction of fraction of chlorophyll *a* biomass and the microscopically estimated fraction of community carbon for Dino-B versus Dinophyceae, Cyano versus Cyanophyceae, sum of Prasino-A and Prasino-B versus Prasinophyceae, Chloro versus Chlrophyceae, Crypto versus Cryptophyceae, sum of Hapto-A, Hapto-B, and Chryso-B versus sum of Haptophyceae and Chrysophyceae, Raphido-A versus Raphidophyceae, and Eugleno versus Euglenophyceae.

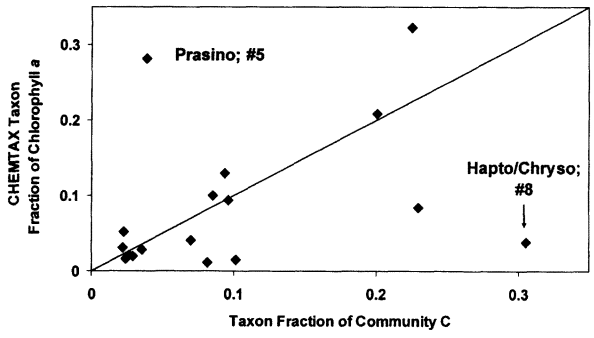

Fig. 6. Relationship between the present study's CHEMTAX prediction of fraction of chlorophyll *a* biomass and the microscopically estimated fraction of community carbon for individual taxa when the estimates of carbon fraction exceeded 2% of the total community biomass, and excluding groups Diatom, Dino-B, and Raphido-A. Apparent outliers are indicated as open circles; number indicates sample code.

had the highest proportion of unidentified flagellates at 14% of phytoplankton C, data not shown).

Discussion

Advances in remote sensing technology and expansion of monitoring effort have led to an amassment of environmental data from the world's estuaries. This increased research emphasis is driven largely by observed or expected impacts of coastal development on estuarine ecosystem properties. A critical element of the link between anthropogenic influences and ecosystem dynamics is the biomass and composition of estuarine primary producers. The rate of allochthonous nutrient turnover and efficiency of trophic transfer can vary greatly with phytoplankton taxonomic composition. Microscopic assessments of phytoplankton taxonomic composition can provide detailed resolution of assemblage structure, but requirements for taxonomic expertise and lengthy analysis restrict their

use as a complement to intensive environmental data sets. The application of chemotaxonomic pigments has emerged as an alternate method with improved throughput capability. Pigment-based phytoplankton assemblage assessments are inherently ambiguous, because pigments are rarely confined to one taxonomic class, and cellular pigment composition can vary widely with physiological state. As is the case with any chemical-based or molecular-based method for microbial community assessment, adequate microscopic ground-truthing is essential.

The CHEMTAX matrix factorization program, originally developed for oceanic phytoplankton evaluation, has the potential to be a valuable tool in estuarine systems. As Mackey et al. (1996) cautioned, effective application of the program requires calibration with isolates representative of the targeted aquatic system. In our study, the application of Mackey et al.'s (1996) matrix to estuarine samples from two South Carolina saltmarsh estuaries resulted in relatively poor predictive capabilities, compared to a matrix generated primarily from estuarine isolates. In the same way as Mackey et al.'s (1996) original matrix has required modifications for adapting to different oceanic regions (e.g., DiTullio et al. 2003), the application of CHEMTAX to estuaries needs to take a typological approach; that is, calibration with species typifying the sampled estuary. Microscopic verification should also be a prerequisite to application of the method. Our microscopic ground-truthing revealed some significant limitations in program application that will require further method development; e.g., integration of more local isolates into the calibration scheme and expansion of chemotaxonomic pigments.

In order to establish appropriate ranges of calibration pigment ratios that are representative of phytoplankton experiencing variable natural conditions, the effects of two growth variables were tested, irradiance and stage (i.e., reflecting nutrient status). As expected, pigment cell^{-1} was higher in LL than HL cultures. Exceptions were zeaxanthin in *Synechococcus* (both growth phases) and alloxanthin in *S. major* (exponential phase only). These species can be distinguished from the others in containing phycobiliproteins as their major light-harvesting pigments, and the lack of LL stimulation of these carotenoids may reflect a role in photoprotection rather than light harvesting (Kana et al. 1988; Bidigare et al. 1989; Schlüter et al. 2000; Henriksen et al. 2002). In general, the ratio of accessory pigments to chl *a* was higher under HL than LL conditions. This pattern is more easily explained in carotenoids involved with photoprotection than those functioning in light harvesting, but interspecific variability in the photoresponse of the ratio of light-harvesting carotenoids (e.g., peridinin, fucoxanthin) to chl *a* is not unusual (e.g., Schlüter et al. 2000; Henriksen et al. 2002). The response of pigment composition to irradiance and nutrient availability can vary greatly between species in the same class, underlining the need to calibrate CHEMTAX using species common to the targeted environment (Mackey et al. 1996; Schlüter et al. 2000).

One of the greatest deterrents to any pigment-based taxonomic scheme is the presence of fucoxanthin-containing dinoflagellates, because their pigment composition is undistinguishable from taxa from which their endosymbiont chloroplasts are derived. Our Groups 1 (Diatom/Dino-A), 8 (Hapto-A/Chryso-A/Dino-C), and 9 (Hapto-B/Dino-D) potentially include dinoflagellates in proportions that cannot be determined by pigment data alone. In the estuarine waters we sampled, dinoflagellates constituted 19 ± 9% of phytoplankton C, while peridinin concentrations never exceeded 5% of chl *a* concentrations (data not shown), and CHEMTAX predictions of Dino-B were always below 5% of chl *a* biomass (Fig. 3). These results indicate that a relatively large percentage of dinoflagellate biomass in these assemblages came from species without peridinin (i.e., Dino-A, -C, or -D). The contribution of the combined C biomasses of diatoms and dinoflagellates were within 5% of the contribution of pigment biomass of the CHEMTAX Diatom/Dino-A group in Samples 7 and 9 (Fig. 4), suggesting the importance of Dino-A dinoflagellates in these assemblages. Adding dinoflagellate C biomass to the sum of several flagellate groups (Fig. 4) improved predictability in Samples 2–5 and 10, suggesting the importance of Dino-C types in these samples. The contribution of dinoflagellates must be resolved by microscopy. An important step in improving CHEMTAX application is the establishment of a pigment library for local dinoflagellate species, to allow precise categorization by pigment type.

Despite the uncertainties from dinoflagellate contributions, unidentified flagellates, inadequate preservation of some species, and relating pigment biomass to C biomass, the predictability of the present CHEMTAX matrix holds promise for use with the targeted estuaries. The percent contribution of Diatom/Dino-A and total flagellates could be reasonably predicted in most cases when fucoxanthin-containing dinoflagellates are taken into account. The potential for accurate biomass estimates of several individual taxa was also suggested by comparisons between CHEMTAX- and microscopy-based biomass, when the latter exceeded 2% of phytoplankton C. Further tests of these relationships are

needed in assemblages less dominated by diatoms and dinoflagellates.

Adequate application of CHEMTAX to South Carolina estuarine communities will require continual modifications. For example, isolation of local clones for improving representation of the calibration matrix is ongoing. Local estuarine isolates representing Prasino-B, Hapto-B/Dino-D, and Chryso-B are being sought for replacement of Mackey et al.'s (1996) Prasinophyceae Type 2, Haptophyceae Type 4, and Haptophyceae Type 1, respectively. Additional groups not presently included in the matrix are raphidophytes with pigment composition differing from *H. akashiwo*. We have not been successful in applying the present study's CHEMTAX matrix to the characterization of brackish detention pond assemblages, in which raphidophytes such as *Chattonella subsalsa, C.* cf. *verruculosa,* and *Fibrocapsa japonica* are commonly abundant (Lewitus et al. 2003, 2004). This problem in application among nearby estuaries of different typology emphasizes the need to validate the method in any new environment, even similar estuarine types in different regions. It is not surprising that application of CHEMTAX calibrated with oceanic isolates to estuarine systems can lead to inaccurate predictions of phytoplankton taxonomic composition, as originally cautioned by Mackey et al. (1996).

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