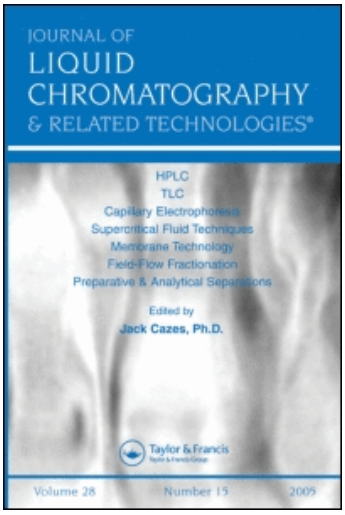


Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

HPLC-Based Chemotaxonomy of Florida Bay Phytoplankton: Difficulties in Coastal Environments

J. William Louda[a]
[a] Organic Geochemistry Group, Department of Chemistry and Biochemistry, Florida Atlantic University, Boca Raton, Florida, U.S.A

To cite this Article Louda, J. William(2008) 'HPLC-Based Chemotaxonomy of Florida Bay Phytoplankton: Difficulties in Coastal Environments', Journal of Liquid Chromatography & Related Technologies, 31: 3, 295 — 323
To link to this Article: DOI: 10.1080/10826070701780599
URL: http://dx.doi.org/10.1080/10826070701780599

Journal of Liquid Chromatography & Related Technologies®, 31: 295–323, 2008

ISSN 1082-6076 print/1520-572X online
DOI: 10.1080/10826070701780599

HPLC-Based Chemotaxonomy of Florida Bay Phytoplankton: Difficulties in Coastal Environments

J. William Louda

Organic Geochemistry Group, Department of Chemistry and Biochemistry, Florida Atlantic University, Boca Raton, Florida, U.S.A

Abstract: This report covers high performance liquid chromatography (HPLC) derived pigment based chemotaxonomy for phytoplankton studies in a turbid coastal environment. Sediment resuspension brings detritus with pheopigments and other chlorophyll (CHL) breakdown products into the water column and hampers data interpretation. Two hundred sixty six water samples from Florida Bay were analyzed from monthly collections (Sept. 2000–Aug. 2002). Comparing biomarker estimated CHL*a* to measured CHL*a*, we found a mean of 105.4% (Std. Dev. = 24.7; $R^2 = 0.9547$) and when data was restricted to those samples with $<10\%$ pheopigments, estimations improved to $102 \pm 16\%$. It is concluded that all HPLC studies on phytoplankton communities must report the percentage pheopigments.

Keywords: HPLC, Pigments, Chlorophyll, Pheopigments, Chemotaxonomy

INTRODUCTION

High performance liquid chromatography (HPLC) derived pigment based chemotaxonomy has been reviewed by Millie and co-workers[1] and expanded by Paerl and others.[2] Only through chromatographic advances, such as the ion pairing techniques popularized by Mantoura and Llewellyn[3] and many others, has dissection of the full range of polar through non-polar lipid

Correspondence: J. William Louda, Organic Geochemistry Group, Department of Chemistry and Biochemistry, Florida Atlantic University, Boca Raton, FL 33431, U.S.A. E-mail: blouda@fau.edu

soluble pigments been possible in a single analytical procedure. The popularity of pigment based chemotaxonomic studies on microalgae is reflected by studies on: (1) the separation of the chlorophylls-*c* to better define the chromophytes;[4] (2) community structure in lakes,[5–8] estuaries,[9–12] or the sea;[13–18] and (3) detailing anoxygenic photosynthesis by purple and green/brown sulfur bacteria in both lakes and the sea.[6,13,19–21]

The importance of studies of phytoplankton community structures is easily noted from The Scientific Committee on Oceanic Research (SCOR) of the United Nations Educational, Scientific and Cultural Organization (UNESCO) directing and publishing an in depth study and review of guidelines and recommendations for pigment based chemotaxonomy.[22]

At the heart of pigment based chemotaxonomic studies is the assertion that, once the overall pigment array has been analytically dissected, the distribution of the primary (chlorophylls) and secondary/accessory (carotenoids, phycobiliproteins) photosynthetic pigments can be mathematically deconvoluted in ways that reflect the taxonomic makeup of the sampled community. That is, specific biomarker pigments (examples in Appendix A) should be present in known ratios to a biomass commonality, such as Chlorophyll-*a* (CHL*a*) in the oxygenic photoautotrophs. As an example, a number of diatom species have been studied by the author and many more are reported in the literature[7,9,15,17] and these, except those reported from the Southern Ocean,[23,24] yield average chlorophyll-a -to- fucoxanthin molar ratios of 1.2 ± 0.3:1 (1.6:1 by wt.). Thus, the moles of diatom (or chrysophyte) contributed CHL*a* should be about 1.2 times the moles, or about 1.6 times the mass, of fucoxanthin found in a natural phytoplankton sample, if diatoms are the only fucoxanthin contributor in the community. Next, comparison of the 'diatom CHL*a*' to the CHL*a* similarly calculated for other taxa (e.g., from chlorophyll-*b* for chlorophytes, peridinin for dinoflagellates, 19′-hexanoyloxyfuxoxanthin and/or 19′-butanoyloxy-fucoxanthin for prymnesiophytes/nanoflagellate chrysophytes, alloxanthin for cryptophytes, prasinoxanthin for prasinophytes, and zeaxanthin, echinenone and/or myxoxanthophyll for cyanobacteria), or directly from divinyl-chlorophyll-*a* for the prochlorophytes (cf. Paerl et al.[2]), should then allow the relative percentage of each taxon (=taxon-specific CHL*a*) within the source community to be back calculated. The overall method employs (regression) formulae based upon the study of unialgal cultures, field samples, and/or literature values. Alternatively, CHEMTAX, a matrix factorisation program using a "steepest descent algorithm"[23–25] may be utilized. A comparison of multiple linear regressions (MLR) and the CHEMTAX algorithm has been published[26] and problems with CHEMTAX in certain coastal environments have been noted.[25,27] "Methods that rely more strongly on empirically determined pigment ratios"[27] are stressed herein, in order to "reflect the dominant phytoplankton species present".[25]

We have chosen a modified MLR approach for our studies in Florida Bay, the Everglades and Lake Okeechobee as well defined major species are known

and interest here is in rapidly assessing plankton functional groups (PFGs)[2] as a measure of bulk community dynamics for monitoring purposes.

Given that the Everglades and Florida Bay are linked within the Comprehensive Everglades Restoration plan and that methods for the rapid temporal and spatial monitoring of microalgal communities are needed (CERP-MAP),[28] an evaluation of HPLC derived pigment based chemotaxonomy in the high light turbid interface of these systems and within the bay proper was undertaken.

EXPERIMENTAL

Study Area and Field Samples

This study sampled sites within north-central and western Florida Bay (Figure 1).

Monthly sampling at thirteen main stations (1–12 plus OS5) occurred between September 2000 and August 2002. Water samples were collected at about 0.3 m into triple rinsed brown 2 L Nalgene bottles, occlusively sealed, and transported to shore in the shade. Water was filtered through Whatman GF/F filters, which were then folded, blotted, refolded (quarters), blotted and sealed in aluminum foil, and flash frozen by immersion in liquid nitrogen. Filtration and all subsequent operations were conducted in dim, usually yellow light. Samples were transported from Flamingo to the FAU lab on dry ice and stored frozen until analyzed within 1–2 weeks.

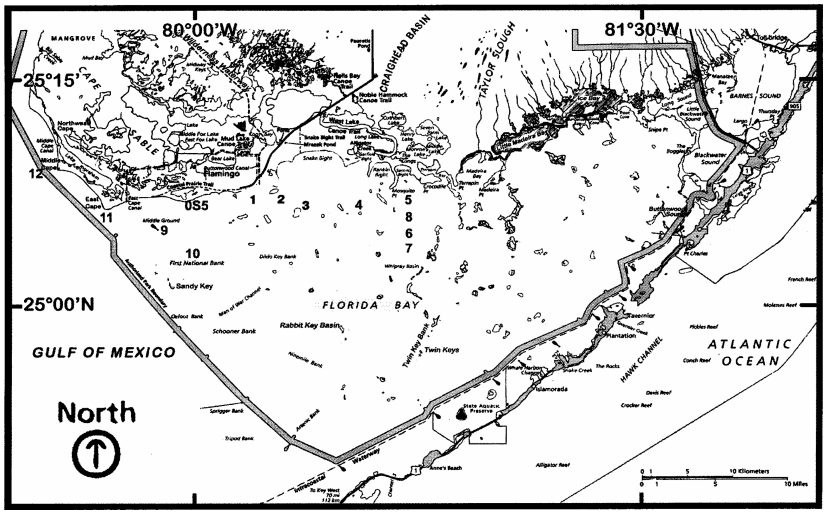


Figure 1. Map of Florida Bay with sampling sites labeled.

Unialgal Cultures

Numerous unialgal cultures were analyzed for biomarker pigment ratios. These cultures included the following groupings by source: (a) *Synechococcus elongatus*, *Cyclotella choctawatcheeana*, and a "2 micron prokaryotic picosphere", all isolated clones from Florida Bay and provided by Carmelo Tomas, then of the Florida Marine Research Institute in St. Petersburg. (b) *Isochrysis galbana* from John Scarpa at Harbor Branch Oceanographic Institute in Ft. Pierce, Florida. (c) 8 cyanobacteria, 10 chlorophytes, 1 euglenophyte, 4 non-diatom chrysophytes, 7 diatoms, and 4 dinoflagellates purchased from the Living Materials Division of Carolina Biological Supply or the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP). Several of these were ordered two or more times over the period of several years; and, (d) the dinoflagellates *Amphidinium carterae* (CCMP#1314), *Prorocentrum hoffmanianum* (CCMP#683), *P. micans* (CCMP#1591), *Heterocapsa triquetra* (CCMP#448), and 3 isolates of *Karenia brevis* (CCMP# 718, 2228, 2229) were gifts from K. S. Rein of the Advanced Research Cooperation In Environmental Health (ARCH) program at Florida International University.

Pigment Extraction and Analysis

Pigment extractions were performed at ice bath temperatures using pre-chilled solvent, containing a known amount of a procedural internal standard ("IS"; Cu-mesoporphyrin-IX-DME, aka Cu-MESO-IX-DME), by grinding (350–500 rpm) at ice bath temperature ($\sim$2–3°C) in a Teflon/glass homogenizer (Kontes "Duall"^{TM}, 15 mL) that had been previously stored in a freezer. The extractant/glass fiber mixture, in the mortar or the homogenizer, was sonicated in an ice bath 5–10 times in 3–5 second spurts and then allowed to steep at 2–3°C for 2–3 hours. Steeping for prolonged periods (6–72 h) of time was found to release only minor amounts ($<$5%) of additional pigment,[29,30] and likely leads to unwanted isomerizations and other artifacts (e.g., phytol/Mg^{2+} losses etc., cf. Chapters in Jeffrey et al.).[22] Throughout much of the present study, the extraction solvent was 90% aqueous acetone. We have also explored alternate extraction cocktails and found that both acetone/methanol water (45:45:10, v/v/v) and methanol/acetone/dimethylformamide/water (aka MADW; 30:30:30:10, v/v/v/v) are also excellent extractants, and lead to much better separation and peak shapes of the early eluting (polar) pigments.[29,30] The later extractant mixture was used during the last several months of the present study. Extracts were recovered by centrifugation and subsequently filtered through a 0.45 μm syringe filter. The overall UV/Vis spectrum (330–800 nm) of the filtered extract was recorded on a Perkin-Elmer Lambda-2 instrument, calibrated against holmium oxide for wavelength and absorbance. The

injectate was prepared using 1.00 mL of filtered extract and 0.125 mL of an ion pairing solution ("IP": ammonium acetate plus tetrabutylammonium acetate) made according to Mantoura and Llewellyn.[3]

Separation and identification of chlorophylls, chlorophyll derivatives, and carotenoids was by the 2D analytical technique of reversed-phase high performance liquid chromatography (RP-HPLC), coupled with full spectral (190–800 nm) photodiode array detection (PDA, also called diode array detection = DAD). RP-HPLC was performed using 3.9×150 mm (or 300 mm) Waters NovaPak 4-micron C_{18} columns, developed with a ternary gradient (Table 1) close to that we previously reported.[8,20–21,29] Prepared extracts were loaded and injected with a Rheodyne 7125 injector and solvents were delivered with a Thermo-Separations Products Model 4100 quaternary HPLC pump at 1.00 mL min^{-1}. The present HPLC method was developed using over 85 known carotenoids, chlorophylls, and chlorophyll derivatives. the known amount of Cu-MESO-IX DME (IS) in the injectate was divided by the detected amount and the response correction factor ($=IS_{added}/IS_{detected}$)which averaged 1.2 ± 0.1 was then applied to all peaks. The exact times of elution of the internal standardfucoxanthinzeaxanthinchlorophyll-*a*and β-carotene were utilized to adjust for slight run-to-run retention time drift due to operatorinjectionand solvent differencesas well as column aging. HPLC-PDA responses were standardized versus known pigments[21,31] Overall response (QA/QC) of the system was monitored with the procedural internal standard (IS) added to the extracting solvent mixture. Here the known amount of Cu-MESO-IX DME (IS) in the injectate was divided by the detected amount and the response correction factor ($=IS_{added}/IS_{detected}$) which averaged 1.2 ± 0.1 was then applied to all peaks. The exact times of elution of the internal standard, fucoxanthin, zeaxanthin, Chlorophyll-*a* and *b*-carotene were utilized to adjust for slight

Table 1. Solvent profile used with RP-HPLC

Time (minutes)	Solvent "A"	Solvent "B"	Solvent "C"
0	60	40	0
5	60	40	0
10	0	100	0
40	0	30	70
45	0	30	70
46	0	0	100
47	0	100	0
48	60	40	0

Solvents: "A" = 0.5 M ammonium acetate in methanol/water (85:15, v/v); "B" = Acetonitrile/water (90:10, v/v); "C" = ethyl acetate.

All gradients were linear. Column stored in methanol/water (85:15, v/v).

Profile as modified from Kraay et al.[4] (Mantoura and Llewellen[3]).

run-to-run reterntion time drift due to operator, injection, and solvent differences as well as column aging. HPLC-PDA responses were standardized versus known pigments[31] that were isolated from unialgal cultures, formed by in vitro derivatizations, purchased (Sigma, VKI-Denmark) or received as gifts (Hoffman-LaRoche). Periodic co-injections, notably with alloxanthin, were made as part of our routine QA/QC protocol.

Quantitation relied on the Beer-Lambert relation wherein peak areas at an appropriate wavelength (440 nm = chlorophylls, carotenoids; 410 nm = pheopigments, 394 nm = internal standard, 360 nm = bacteriochlorophyll/bacteriopheophytin-*a*, etc.) were divided by an extinction coefficient from the literature.[22,32] Extinction coefficients that were used but did not correspond to the exact maximum for which it was reported were adjusted by the ratio of the HPLC monitoring/integrating wavelength to the wavelength of the reported coefficient. This quantitation protocol was verified using solutions of authentic known pigments that were prepared using standard spectrophotometric techniques (Chapters in Jeffrey et al.).[22] Multiplication by the effective volume adjusted for dilutions, such as the addition of an ion pairing solution to the injectate, gave the overall yield of individual pigments.

Microscopic Examinations

Aliquots (~125 mL) from several of the water samples analyzed for pigments were sent to two separate commercial phytoplankton laboratories for cell counts and biovolume estimates to the species level and were totaled into Divisional values by the author. These aliquots were added to 125 mL brown Nalgene bottles, made up to a final concentration of 0.25–0.50% glutaraldehyde for preservation, occlusively sealed, placed on ice, and mailed to the microscopy laboratories (Labs "A" and "B") for identification to division and provision of cell numbers and estimated biomass.

RESULTS AND DISCUSSION

Chemotaxonomic Equation(s) Development

In order to convert HPLC pigment data into (chemo-) taxonomic groupings, one must relate the individual biomarker relationships to a commonality, such as chlorophyll-*a*. To that end, literature surveys, analyses of unialgal cultures, and interpretations of field data were performed.

The present compilation (Table 2) is not meant to be an all encompassing survey of pigment ratios. In each case, these data are given here both as the weight and molar ratios. In our studies, we prefer to use molar ratios as those data may eventually reveal additional insight concerning underlying pigment relationships in taxon-specific photosynthetic reaction centers.

Table 2. Biomarker pigment-to-chlorophyll-*a* (weight ratios)

CYANOS	GREENS		CHRYS-NANO			DIATS	DINOS	CRYPTO	PRASIN	
ZEA	LUT	CHL*b*	HEX	BUT	Hex + But	FUCO	PERI	ALLO	PRAS	Ref.
0.83		1.24			1.05	1.84	2.25	3.34		[81]
		0.90	1.14			1.34	2.79	3.60		[82]
0.69		2.95	0.73			1.72				[34]
1.32		0.70			0.68	1.07	2.50	3.34		[79]
		2.40	0.90	1.20		1.90	1.40	4.20	2.50	[17]
5.50	4.29					0.89	0.77	1.58		[83]
								2.50		[84]
0.63	5.56					0.87	2.22	4.00		[85]
		1.13	0.67			1.24	2.02			[86]
		2.5–3.0	1.23	1.12		1.3–1.9		2.1–2.7		[63]
		1.3–2.3	0.7–3.2	0.9–3.9		1.3–2.3	1.4–2.8	1.8–4.2	2.3–4.3	[17]
2.8	4.9	3.8	0.61			1.0	0.9	4.4	4.4	[87]
0.7		3.26				0.89	1.05	2.39		[88]

(*continued*)

Table 2. Continued (molar ratios)

CYANOS	GREENS		CHRYS-NANO			DIATS	DINOS	CRYPTO	PRASIN	
ZEA	LUT	CHL*b*	HEX	BUT	Hex + But	FUCO	PERI	ALLO	PRAS	Ref.
1.30		1.22			1.24	2.50	3.20	5.28		[81]
		0.88	1.32			1.82	3.96	5.69		[82]
1.08		2.89	0.85			2.34				[34]
2.07		0.69			0.80	1.46	3.55	5.28		[79]
		2.35	1.04	1.44		2.58	1.99	6.64	3.73	[17]
8.64	6.74					1.21	1.09	2.50		[83]
								3.95		[84]
0.99	8.73					1.18	3.15	6.32		[85]
		1.11	0.78			1.69	2.87			[86]
		2.4–2.9	1.43	1.30		1.8–2.6		3.3–4.3		[63]
		1.5–2.6	0.8–3.7	1.1–4.7		1.8–3.1	2.0.–4.0	2.8–6.6	3.4–6.4	[17]
4.4	7.7	3.9	0.7			1.4	1.3	7.0	6.6	[87]
1.10		3.20				1.20	1.50	3.80		[88]

Abbreviations, see Appendix B.

Most of the data in Table 2 are 'CHL*a*/taxonomic biomarker' values derived by the taking the reciprocal of published 'biomarker-to-CHL*a*' weight ratios with the exceptions of data reported as pM xanthophyll to nM CHL*a* (ref. [6] in Table 2) and values obtained by the author (ref. [13] in Table 2) directly as molar ratios.

Gieskes and Kraay[33] published one of the first mathematical chemotaxonomic descriptions of phytoplankton communities using multiple regression formulae to back calculate taxon-specific chlorophyll-*a* contributions. Gieskes et al.,[34] then published seasonal regression formulae for phytoplankton in the Banda Sea of Indonesia.

Ideally, a formula should predict total CHLa without a non-zero intercept or correction value that would indicate an unknown source (+) or sink (−) of CHL*a*. Additionally, multipliers for photosynthetic accessory pigments should remain relatively constant, or change only in ways that are known, such that the formulae can be corrected. We choose to develop and adjust a formula for Florida Bay samples that did not contain a value for 'unaccounted' CHL*a*. Therefore, we were aiming at a formula that would give close to 100% estimated CHL*a* when compared to the sum of CHL*a* actually measured. Ideally, this should result regardless of the dominance or co-dominance of the contributory taxa (PFGs) present.

Photoprotectorant pigments (PPs: Hex and Zea) in the Gieskes et al.,[34] report revealed a large decrease, relative to CHL*a*, in February of 1985, relative to August of 1984. Lowered photic flux, requiring less 'photoprotection', may be an explanation for the increased CHLa/PP ratios they reported. This point is especially noteworthy for the present Florida Bay study, as it is a high light ($2{,}000 + \mu$mol phota $m^{-2}\,sec^{-1}$ surface PAR irradiation) environment.

Various researchers have stressed the need to have pigment ratios reflect the known dominant species in any community and/or ecosystem under study.[25–26,35] This point is easily extended to include the use of pigment ratios characteristic of what Paerl and others[2] define as phytoplankton functional groups or PFGs.

The cyanobacterium *Synechococcus elongatus*, the diatom *Cyclotella choctawatcheeana*, and "a eukaryotic picosphere" have been the main species implicated in the "blue-green" or "blue-green/diatom/picosphere" blooms within Florida Bay.[36] During a 1996 Florida Bay chlorophyll intercalibration study (US-EPA and Interagency Florida Bay Program, W. L. Kruczynski facilitator), we[37,38] had the opportunity to analyze these 3 species as unialgal cultures provided by C. Tomas. Various *Synechococcus elongatus* samples gave CHL*a*/ZEA molar ratios of about 5:1. The 2 micron picosphere, originally reported as an eukaryote,[36] was found to have CHLa/MYXO (myxoxanthophyll) of about 7.5:1 and ZEA equal to about 1.56 [MYXO], again all in molar ratios. The presence of myxoxanthophyll, found only in certain cyanobacterial lines, then identified the 2 micron 'picosphere' as a prokaryote. Subsequent electron microscopy and other criteria

reconfirmed this pigment based identity (W. Richardson, pers. commun. 1999). The diatom, *Cyclotella choctawatcheeana*, was found to have a CHLa to FUCO (fucoxanthin) molar ratio of 1.0:1.

Using our HPLC data on unialgal cultures, including the three species given above plus some 36 species covering 8 divisions, we formulated a preliminary chemotaxonomic Equation (1) for Florida Bay.[39] The preliminary {molar} chemotaxonomic equation for the estimation of cyanobacteria, chlorophytes, diatoms, and dinoflagellates, respectively, took the form:

$$\Sigma\text{CHL}a = (7.5[\text{MYXO}] + 5.0[\text{ZEA}]) + 3.6[\text{CHLb}] + 1.1[\text{FUCO}] + 2.3[\text{PERI}] \quad (1)$$

This Equation (1), when applied to phytoplankton (seston) field data, was found to overestimate cyanobacteria (ZEA) and to not account for the cryptophyte contribution of alloxanthin (ALLO). The overestimation of cyanobacteria stemmed from the fact that zeaxanthin is a photoprotectorant pigment (PP), rather than a photosynthetic accessory pigment (PAP) stoichiometrically related to the amount of CHL*a*. Thus, using the values from lab cultures, grown under limited photic flux conditions, led to higher CHL*a*/ZEA values ($\sim$5:1; lower amounts of ZEA) than we found in the bay. Natural *Synechococcus* sp. blooms ([CHL*a*] $> 10\ \mu g\ L^{-1}$) from Whitewater Bay or the Snake-Rankin-North Whipray areas of Florida Bay were found to give CHL*a*/ZEA values of 2.5:1 or 1:1, respectively. The higher DOM loading (humics/brown water) in Whitewater Bay appears to have cut the 'felt' photic flux (e.g. $\sim 100\ \mu$mol phota $m^{-2}\ sec^{-1}$ at 1.0 m), relative to Florida Bay (e.g., $\sim 100\ \mu$mol phota $m^{-2}\ sec^{-1}$ at 3.0 m), and thereby lessened the requirement for the production of larger amounts of zeaxanthin. The values for photosynthetically active radiation (PAR; 400–700 nm) flux given are for periods with surface irradiation at about 2,000 to 2,500 μmol phota $m^{-2}\ sec^{-1}$. Again, attention to the photic field/history and the major species in the cyanobacterial 'functional group'[2] is concluded as being quite important in the high light environment of southern Florida and similar sub-tropical/tropical settings.

Given the above and other input, we adjusted our molar based equation to the following (2):

$$\Sigma\text{CHL}a = (7.5[\text{MYXO}] + 1.1[\text{ZEA}^*]) + 2.4[\text{CHLb}] + 1.2[\text{FUCO}] + 1.5[\text{PERI}] + 3.8[\text{ALLO}] \quad (2)$$

or, on a weight basis (3):

$$\Sigma\text{CHL}a = (9.2[\text{MYXO}] + 1.72[\text{ZEA}^*]) + 2.45[\text{CHLb}] + 1.63[\text{FUCO}] + 2.13[\text{PERI}] + 6.0[\text{ALLO}] \quad (3)$$

In the above Equations (2, 3), the amount of ZEA is adjusted in order to account for ZEA also contributed by the MYXO containing population, if

present. Thus, ZEA* = ZEA – 1.56[MYXO], as derived from data on the 2 micron picosphere. It must be noted that, even though MYXO, and most likely the 2 micron picosphere, has been found[40] in Florida Bay waters during 1994–1996, we did not find MYXO in any (seston) phytoplankton samples during 2000–2002.

Above, we described deriving an equation that included 2 main groups of cyanobacteria, one having only zeaxanthin as a biomarker and the second with myxoxanthophyll plus minor amounts of accompanying zeaxanthin. A zeaxanthin correction was therefore required. A third group of cyanobacteria to be considered contains many benthic forms that biosynthesize keto-carotenoids, echinenone and, to a lesser extent, canthaxanthin. A forth group may also be required in certain environments. That is, inclusion of aphanizophyll as an indicator of nitrogen fixing cyanobacteria should be considered when this pigment is detected.

Obviously, phytoplankton species containing highly specific biomarkers such as oscillaxanthin in *Oscillatoria rubescens*[41] or gyroxanthin in *Karenia brevis*[42] would require formula adjustment if and when present. The same holds for other taxa with specific biomarkers (19′-hexanoyl-or butanoyl-oxyfucoxanthins, prasinoxanthin, divinylchlorophylls, etc.). The reader is directed to the literature for extended discussion.[22–26]

Chemotaxonomic Equation (#2) Testing

The HPLC pigment analyses of 266 seston samples from north-central and western Florida Bay were performed and chemotaxonomic estimates were made using Equation (2).

Figure 2 is a plot of CHL*a* estimated from biomarker pigments using Equation (2) versus the sum of CHLa species measured by HPLC. That

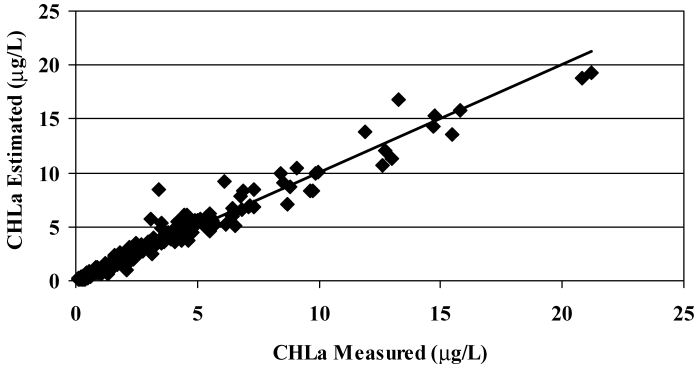


Figure 2. Plot of chlorophyll-*a* (CHL*a*) estimated from biomarker data using Equation (2) versus CHL*a* measured by HPLC. ($y = 1.0056x$, $R^2 = 0.9547$, mean = 105, Std. Dev. = 25).

is, measured CHL*a* (ΣCHL*a*) equals the sum of chlorophyll-*a*, chlorophyll-*a*-epimer, chlorophyll-*a*-allomer, the epimer of CHL*a*-allo, chlorophyllide-*a* and pyrochlorophyllide-*a*. These pigments all have the chromophore and spectrum of CHL*a* per se. This also accounts for isomerizations and the action of chlorophyllase, especially active in diatoms.[43] Good linearity of fit between estimated and measured CHL*a* is reflected by the regression of these data (Estimated = 1.0056 × Measured) and a correlation coefficient (R^2) of 0.9547. The mean and standard deviation for estimated CHL*a* from biomarker data are 105 ± 25%. The confidence of the mean at the 95% interval is 2.968 and the data set yielded a coefficient of variation of 0.9784. Given a wide spread in the consistency and agreement between the microscopic exams, as covered in the next section, the 105 ± 25% chemotaxonomic estimations are concluded here as quite workable for rapid spatial and temporal community surveys/monitoring. Additionally, given in the next section, lessening the deviation is quite possible. Our goal of developing a protocol to come close to 100% estimated versus found CHL*a* without an adjustment for 'unaccounted for CHL*a*' was achieved.

Pheopigments

An examination of the pigment data for any underlying causal relationship that could contribute to the large standard deviation in the chemotaxonomic estimation of total CHL*a* was undertaken. Immediately obvious is the fact that large amounts of pheopigments (viz. pheophytin-a, pyropheophytin-a, pheophorbide-a, pyropheophorbide-a inter alia) were often present. Figure 3 (upper) is a plot of the percent estimated CHL*a* (=[estimated/measured] × 100) versus the molar percentage of pheopigments (where {[CHLs-*a* + pheopigments]/CHLs-a} × 100 = % Pheopigments) and reveals a striking increase in the amount of estimated CHLa with increasing pheopigment content. Given that the overall data set yielded 105 ± 25% (Standard error of the mean {SEM} = 1.58) for estimated CHL*a* from biomarker data, we next constrained the data to include only those samples with <10% pheopigments. In this case (Figure 3 lower), the mean was 102 and the standard deviation dropped to ± 16% (SEM = 1.81). If the percent pheopigments were further constrained to below 7.5% (not shown), the mean becomes 102 with a standard deviation of 13% (SEM = 1.76). A relationship between increased CHLa breakdown products and lowered chemotaxonomic efficiency has been reported for fresh water lentic microalgae.[44] However, that study emphasized chlorophyllide-*a* as an indicator of "necromass". Chlorophyllide-*a* is quite often produced solely by induced decompartmentalization during extraction of living diatoms and is related to highly active chlorophyllase systems.[43] Therefore, the presence of chlorophyllides-*a* does not necessarily indicate dead or even senescent microalgae, notably diatoms.[35,41]

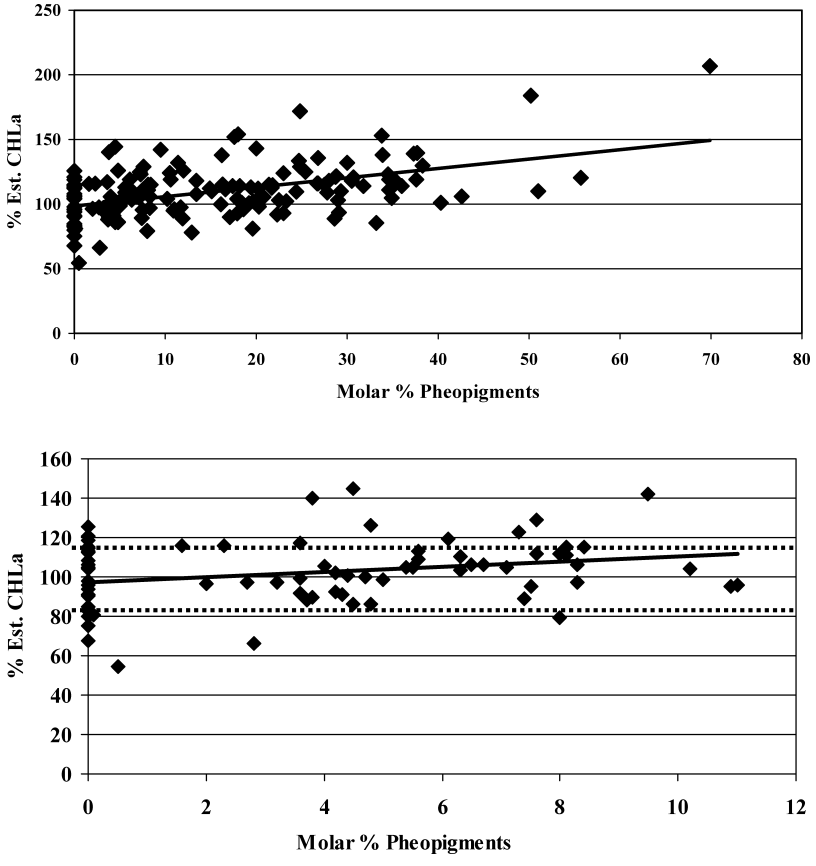


Figure 3. Percent estimated chlorophyll-*a* ([estimated CHLa/HPLC measured CHL*a*] × 100) versus molar percent pheopigments in the sample. (upper) all data (see Fig. 2), (lower) only samples with less than 10% pheopigments (mean = 102%, dotted line = Std. Dev. ± 16%).

Herein, all CHLa chromophoric species are added into the 'total CHL*a*' pool and CHL*a* derivatives without chelated Mg are termed pheopigments (aka phaeopigments) by convention. As senescent and dead phytoplankton convert CHL*a* to a variety of pheopigments[31,45] and both the flocs and surficial marl sediments of Florida Bay contain large amounts of pheopigments,[21,39] sediment resuspension and resultant turbidity was suspected as a prime source for the pheopigment load.

A plot of turbidity versus percent pheopigments is given as Figure 4. The relationship between turbidity and pheopigments in the water column (seston) exists but is certainly not linear. A trend line plotted as a linear, logarithmic, or

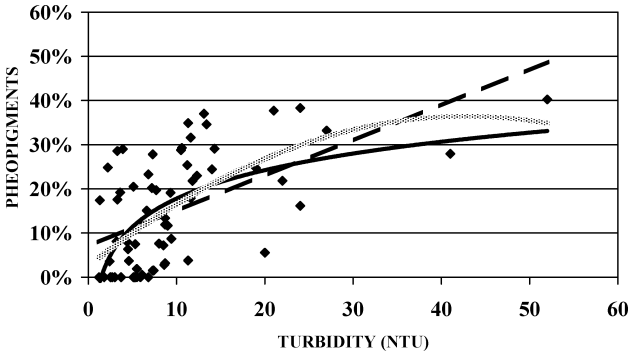


Figure 4. Plot of percent pheopigments versus turbidity (NTU, nephlometric turbidity units). Regression lines are: Linear (dashed, $R^2 = 0.3146$), Logarithmic (solid black, $R^2 = 0.3218$), and 2nd order polynomial (solid gray, $R^2 = 0.3606$).

even as a second order polynomial function, all indicate that turbidity only explains for about 31–36% of the pheopigment presence.

Only very small amounts of pheopigments are reported in the upper layers of open oceans but these breakdown products do routinely increase with depth.[46–49] In coastal, estuarine and lagoonal settings, the mixed layer extends to the benthos and, when enough wind/tidal energy is present, bottom sediments, detritus, and microphytobenthos are easily swept into the water column. The surficial floc and carbonate muds of Florida Bay are easily and routinely mixed into the water column increasing turbidity[39] and, as shown herein, pheopigments. Pheopigment loading into the water column of estuaries and coastal areas has been reported previously,[50–52] but accounting for linkages to lowered HPLC derived chemotaxonomy has been slow to emerge in these studies.

When pheopigments and other CHL*a* derivatives that are strongly linked to senescent and dead microalgae[31,45,53,54] are present, then one must assume that preferential destruction/retention of the chemotaxonomic biomarkers has occurred.[8,21,31,55] This would include a faster rate of chlorophyll-*b* (chlorophyte), fucoxanthin (diatom), and peridinin (dinoflagellate) destruction and relatively good retention of the carotenoid diols zeaxanthin (cyanobacteria) and alloxanthin (cryptophytes).[21,31,56]

HPLC Pigment Based Chemotaxonomy and Microscopic Analyses

Given the need to 'ground truth' HPLC based chemotaxonomy versus conventional microscopic taxonomy, several samples were preserved in glutaraldehyde (0.25–0.50% final concentration) and refrigerated immediately

following collection. Samples were then sent on ice (2–4°C) to 2 individual microscopic service companies (Labs "A" and "B") for taxonomic evaluation. Ideally, it would be advantageous to have an expert algal microscopist familiar with each specific ecosystem in every lab but this is rarely possible.

It was decided to compare the results from different microscopy services prior to comparing microscopy based with pigment based taxonomy. Given that this is very expensive (~$180.00 or $275.00/sample for genus or species level identifications with biovolume estimations) and time consuming (~1–2 months turn around time), only a limited number (13) of samples could be contracted. Of these, four samples were sent to each of two separate labs in order to compare interlaboratory data. Table 2 contains the data obtained from these independent laboratories. The biovolume data on samples from sites 6 and 11 from March 2001 exhibited some differences but did agree on the divisional dominance order (Cyanobacteria > Diatoms). However, conflicting results were obtained for samples from sites 8 and 11 collected in June 2001. Here, lab "A" reported strong cyanobacterial dominance while lab "B" reported nearly 100% diatom presence. Following up on reasons behind this, it was found that lab "A" employed epifluorescence plus light methodologies, while lab "B" used only light microscopy. Lab "A" was then utilized for all samples to which HPLC comparisons were made. Exactly the same results, disagreement between labs, and a general non-correlation of pigment data to biomass, have been reported using the CHEMTAX algorithm.[57]

Comparing HPLC derived chemotaxonomic estimations with microscopic taxonomy (Table 3) we found the following R^2 values for individual CHLa/biomarker relations: cyanobacteria (ZEA: 0.49 {0.80}), diatoms (FUCO: 0.39 {0.56}), chlorophytes (CHL*b*: -0.44 {0.91}), dinoflagellates (PERI: 0.63{0.92}), and cryptophytes (ALLO: −0.94). The higher R^2 values within the inner brackets indicate data after discarding 1–3 major outliers. However, it must be noted that, without *a priori* knowledge of the full data set, an 'outlier' would not be identifiable as such. Additionally, negative correlations between CHL*b*/chlorophytes and alloxanthin/cryptophytes makes the microscopic data suspect, since there is no other explanation for these biomarker pigments other than the presence of their source organisms.

Adequate relationships between HPLC and microscopy for cyanobacteria, diatoms, and dinoflagellates were concluded. Chlorophytes and cryptophytes were shown to definitely be present as their corresponding taxon specific biomarkers, chlorophyll-*b* and alloxanthin, were conclusively identified. As given below, chlorophytes and cryptophytes appear also to be adequately estimated, but solely on the basis of overall community estimated versus measured CHL*a*. The lack of more direct relationships between HPLC and pigment data for the chlorophytes and cryptophytes is an enigma

Table 3. Comparison of microscopic cell counts and biovolume estimates with HPLC data

SITE #	mo/yr	TYPE[a]	CHL*a*, ug/L	%Pheo[b]	TAXON PERCENTAGE[c] CYANO	CHLRO	DIAT	DINO	CRYPT
1	03/01	No.	6.9	12	80.7	0.1	19.2	0	0.1
		Vol			0.4	0.1	99.4	0	0.1
		HPLC			0	0	87.4	0	12.6
6	03/01	No.	0.5	29	80.5	0.1	18.7	0.1	0.4
		Vol			0.4	0.1	98.5	0.1	0.2
		HPLC			0.0	0.0	100.0	0	0
11	03/01	No.	3.6	1.5	93.8	0.1	4.2	0.1	1.6
		Vol			0.4	0.1	99.0	0.1	0.4
		HPLC			0.0	19.0	64.0	0.0	17.0
11	04/01	No.	8.3	14	78.5	0.1	20.6	0.1	0.7
		Vol			0.2	0.1	99.4	0.1	0.1
		HPLC			1.5	7.7	77.0	0.0	13.8
12	06/01	No.	0.7	11	97.8	0.1	0.2	0.2	0.1
		Vol			53.0	3.2	5.6	9.3	0.7
		HPLC			33.2	12.5	36.5	7.9	9.9
8	06/01	No.	0.8	14	96.2	0.1	3.4	0.2	0.1
		Vol			2.5	1.8	93.2	0.5	0.1
		HPLC			7.3	0.0	42.3	20.0	29.9
1	07/01	No.	4.0	7.5	0.0	2.8	89.8	0.0	2.5
		Vol			0.0	0.4	98.9	0.0	0.1
		HPLC			11.7	3.0	66.0	1.7	17.3
5	07/01	No.	0.7	3.6	11.8	0.0	23.5	13.6	50.9
		Vol			23.4	0.0	51.1	19.8	5.7
		HPLC			6.5	0.0	27.9	34.4	31.2
10	07/01	No.	6.0	9.8	0.7	2.4	89.5	2.3	2.7
		Vol			0.1	6.5	93.1	0.2	0.1
		HPLC			2.5	4.0	80.3	4.3	9.2

[a]No. = cell number, Vol = Biovolume. Data from Laboratory "A", as given in text.
[b]% PHEO = percent pheopigments ((PHEOs/[PHEOs + CHL*a*])100).
[c]Percent taxon of 100% community. CYANO = cyanobacteria, CHLRO = chlorophytes DIAT = diatoms {~chrysopytes}, DINO = dinoflagellates, CRYPT = cryptophytyes.

at present. Fortunately, the major phytoplankton functional groups (PFGs) in this ecosystem[58–60] are easily estimated. Additionally, it is reiterated here that the overall estimated (y) versus measured (x) CHL*a* for all samples studied had a mean of 105 $\pm$ 25%, a regression of y = 1.0056x, and an R^2 of 0.9547 (102 $\pm$ 16% at ΣPheos $<$10%). It therefore appears that, in spite

of perceived inadequacies to regress biomarker data to certain individual microscopic taxon estimates, chemotaxonomy is describing their presence within each community quite adequately.

A recent publication[61] had the title "Routine quantification of phytoplankton groups-microscopy or pigment analyses?" Their conclusion was to use pigment analyses for monitoring purposes and to augment that with microscopy for screening of dominant species and flow cytometry for quantification of picocyanobacteria.[61] As these and other authors [2,8,62,63] have concluded, there are indeed problems with all techniques.

It has been stated that microscopy is "a time consuming and costly procedure that requires a high level of expertise".[2] Additionally, such variables as preservation[64] and the "careful choice of appropriate volumetric shapes and taxa categories",[65] in addition to the microscopist's expertise, can inflict bias into the results. One study questioned the marine ecology dogma of assumed or claimed 100% accuracy in microscopy and concluded by stating: "Trained personnel can be expected to achieve 67–83% self-consistency and 43% consensus between people in an expert taxonomic labeling task. Experts who are routinely engaged in particular discriminations can return accuracies in the range of 84–95%".[66] Failure of pigment based (chemo-) taxonomy to agree with microscopic analysis has also been attributed to 'subjectivity' during microscopic exams, especially "when small phytoplankton cells dominate".[57] Biomass (~biovolume) estimations are reported to be prone to adverse effects due to preservation.[64] As stated earlier, the present study utilized a final concentration of 0.25–0.50% glutaraldehyde for preservation. This routine method has been reported to decrease the biovolume of diatoms and athecate dinoflagellates, relative to living cells.[64] An additional problem in the estimation of cell number and biovolume in the present case may derive from our need to ship samples. That is, turbulence during shipping, even though cells were preserved and shipped on ice, could have led to the disruption of certain fragile cells, such as the cryptophytes.[67]

Advantages-Disadvantages of HPLC Derived Pigment Based Chemotaxonomy

The methodology of pigment based chemotaxonomy has several advantages as well as certain pitfalls. First, pigment data are derived from a much larger integrated sample than is microscopy. That is, all of the phytoplankton in a sample of from 50 to 10,000 mL, depending upon seston concentrations, is analyzed. Secondly, the technique itself, except for extraction differences[29,30] that can be accounted for, is relatively unbiased (~objective) on the part of the operator. Data interpretation (formulae) forms the major part

of uncertainty in these studies. All in all, algal biomass[55] and average cell volume[68] are generally related to CHL*a* contents. Yet, the literature consensus is that much more is needed in these aspects as well. Again, it is the relation of biomarker pigments to taxon specific CHLa that presently forms the basis of chemotaxonomy.

Comparisons of microscopic and pigment based estimations of major taxa (aka PFGs[2]) are well known.[8,18,23,24,61–63] One of these studies found that CHLc_3, peridinin, alloxanthin, and fucoxanthin gave good quantitative results for the biomass of Phaeocystis sp., dinoflagellates, cryptophytes, and the sum of brown algae (diatoms etc.), respectively. However, they also reported that CHL*b* had no relationship to chlorophytes and attributed this to uncertainties in the microscopic counts of small (<5 μm) cells and/or cells with low visibility membranes.[62]

Effects of light on the C ('biomass') to CHL*a* ratios are known.[69] Specifically, for diatoms and dinoflagellates in "high" (>100 μmol phota m^{-2} sec^{-1}) or "low" (<100 μmol phota m^{-2} sec^{-1}) light, it was reported that CHL*a*/C gave R^2 of 0.24 or 0.75 and 0.65 and 'not reported', respectively. Overall, for total phytoplankton, they obtained R^2 values of 0.48 and 0.8, respectively. This they correlated to low CHL*a*/C and high CHL*a*/C values, respectively.[69] The effect of light on CHL*a* to non-stoichiometric biomarker (viz. PPs) ratios is also well known.[70–72] As described earlier, light (low to high) was found to significantly change the CHL*a* to ZEA ratio (5–1 to 1–1, respectively) in *Synechococcus elongatus*. As these studies continue in Florida Bay, corrections for seasonal photic flux, at least summer versus winter for example, will likely evolve.

The microscopic estimation of biovolume is also reported to be exceedingly complex,[73,74] especially when cells of less than 5 microns diameter are prevalent.[75] It is given that phytoplankton cell volume ranges over 9 orders of magnitude.[74] Additionally, it has been suggested that diatom cell volumes really need to be adjusted downward by the factor 0.54x in order to remove the volume of the siliceous frustules and intracellular vacuoles.[76]

Potential and known problems with chlorophyte and cryptophyte estimations follow here. Chlorophytes, in this and other studies, are estimated from the CHL*a*/CHL*b* ratio. Lutein is also a 'marker' for green algae but recent evidences describe it as a photoprotectorant.[72,77] We choose to use lutein as only a confirming or co-marker for chlorophytes, much as high levels of α-carotene are for cryptophytes. Therefore, CHL*b* is used and if present, then it must be concluded that chlorophytes are present.

The potential presence of prochlorophytes,[78,79] having divinyl chlorophyll-*b*, in the present samples, was not overlooked. For all of the samples from Florida Bay studied, the position of the Soret (violet) band of CHL*b* exhibited no bathochromic (red) shift relative to monovinyl

('regular') CHL*b* ($\lambda_{Soret} = 458$ nm, band I = 642 nm). DV-CHL*b* (DHI, Denmark) eluting from our C_8 system (Nova Pak-octyl, 3 μm, 3.9 × 150 mm) exhibits Soret and band-I absorptions at 464 and 648 nm, respectively. C_8 RP-HPLC, easily separating the mono-and divinyl-chlorophylls-*a*/-*b*, was therefore not required here. Additionally, chlorophytes (e.g., *Chlamydomonas*, several non-motile chlorococcales, *Oocystis*, and other chlorophytes) were identified (Lab "A") and are numerically included in the divisional microscopic data (Table 3). A lack of hard and fast correlation between CHL*b* and microscopically determined chlorophyte abundance has also been reported for the eastern English Channel.[62] Chlorophytes in the nanoplankton (5–20 μm) size class have previously apparently led to deviations between CHL*b*/biomass-biovolume correlations.[62]

Cryptophytes, in this and other studies,[23,27,62] are estimated from the CHL*a*/alloxanthin (ALLO) ratio. As given above, we found and used a molar CHL*a*/ALLO ratio of 3.8:1 (6.0:1 by wt.) for the estimation of cryptophyte contributed CHL*a* and obtained a negative correlation of $R^2 = -0.94$ when related to the microscopic data. Given that ratios in the literature[10,27] bracket (Table 2: 2.8–7.0) the value used here and since we are using a ratio obtained in our lab from the only genus (viz. *Rhodomonas*) reported by lab "A" from these samples, we can only conclude that HPLC is detecting more of the cryptophyte portion of these communities than is microscopy. This may derive from the fact that the cryptophytes (cryptomonads) are notoriously fragile,[67] hence the Latin names equating to 'hidden plant' ('hidden single cells'), and that the requirement to ship these samples for microscopy may have exacerbated such destruction. A strong effect of light has also been reported for marine cryptophytes. That is, the ALLO/CHL*a* weight ratio was found to increase 1.3- to 9- fold going from the exponential to N-limited stationary phases of growth.[80] Thus, as discussed for zeaxanthin, photic flux and history may need to be factored into cryptophyte estimation by alloxanthin abundance, especially in the high light (1,000–2,500 + μmol phota $m^{-2}\,sec^{-1}$) conditions of Florida Bay and other subtropical/tropical settings.

CONCLUSIONS

HPLC derived pigment based chemotaxonomy has been developed for use in the study and monitoring of phytoplankton community studies in Florida Bay. Florida Bay is a coastal ecosystem closely receiving input of fresh water and nutrients from the Everglades system and, as such, is intimately linked to the ongoing restoration efforts of the Comprehensive Everglades Restoration Plan (CERP).

Analyses of 266 seston samples were collected monthly from north-central and western Florida Bay between September 2000 and August 2002. Manipulation of a pigment based formula using empirically derived data gave excellent correlation ($R^2 = 0.9547$) and slope (estimated = 1.0056 × measured) with a mean of 105.4% (Std. Dev. = 24.7) for total chlorophyll-*a* (CHL*a*) estimated from individual divisional contributions when regressed against HPLC measured total CHL*a*. These data yielded a coefficient of variation of 0.9784 and a confidence on the estimated CHL*a* mean of 2.968 at the 95% interval.

It was found, that as the relative amounts of pheopigments increased so did the overestimation of CHL*a* from biomarker data. About 33% of the pheopigment load was found to be explainable by turbidity. The mean and standard deviation for estimated CHL*a* improved to 102 ± 16% when the data set was restricted to only those samples with less than 10% pheopigments (where $\Sigma\text{CHL}a + \Sigma\text{PHEOs} = 100\%$). Phytoplankton communities varied from nearly total cyanobacteria to nearly total diatoms, with numerous samples also containing moderate amounts of chlorophytes, cryptophytes, and/or dinoflagellates.

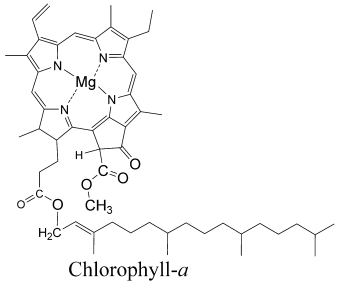
While there was excellent correlation between estimated and total chlorophyll-*a*, estimation of individual taxa yielded correlations (R^2) between −0.94 (cryptophytes) and 0.63. Significant increases in the correlation coefficients were found after the removal of 1–3 'outliers'. For example, R^2 for chlorophytes went from −0.44 to 0.91 and for dinoflagellates it improved from 0.63 to 0.92. However, without coincident microscopic data, outliers would not be identifiable.

A strong effect of light was found for the biomarker of *Synechococcus* in the bay. That is, the CHL*a* to zeaxanthin ratio changes from 5 to 2.5 to 1:1 for samples from lab cultures, Whitewater Bay (dark humic stained waters) to the open bay, respectively. This directly paralleled the amount of photic (PAR) flux received by the cells. The effect of high photic flux (e.g., surface >1,500 μmol phota m^{-2} sec^{-1}) on other pigment ratios requires additional study. In the future, chemotaxonomic formulae may require seasonal (viz. light level) adjustments.

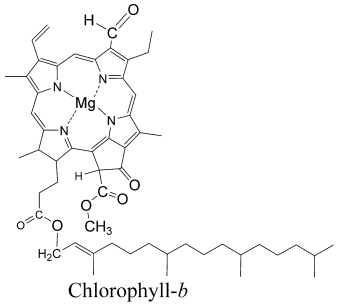
In conclusion, pigment based chemotaxonomy has been adequately developed for the study of Florida Bay phytoplankton and will become more robust in the future as light and pheopigment perturbations of the data are factored into chemotaxonomic formulae.

It may also be concluded that all studies using HPLC derived pigment based chemotaxonomy must not only consider the implications of pheopigments in their samples, but also list the percent pheopigments so that the reader may better understand each report. That is, pheopigments typically indicate senescent/dead/grazed/resuspended phytoplankton and, accompanying chlorophyll alteration, differential losses of biomarker pigments are known.

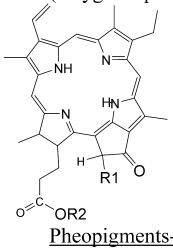
APPENDIX A: SELECTED PIGMENT BIOMARKER STRUCTURES



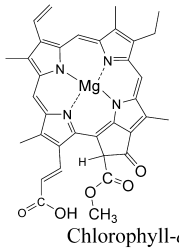
Chlorophyll-*a*
(Oxygenic photoautotrophs)



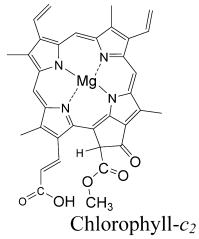
Chlorophyll-*b*
(Green algae and plants)



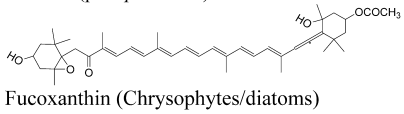
Pheopigments-*a*
R1 = -$COOCH_3$ ('native')
R1= -H (pyro derivative)
R2= Phytyl (pheo*phytin*)
R2= -H (pheo*phorbide*)



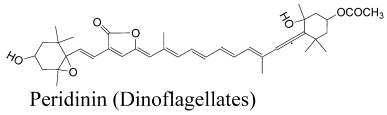
Chlorophyll-c_1



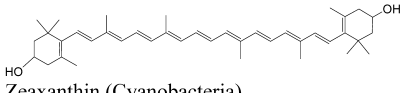
Chlorophyll-c_2



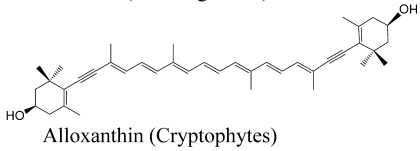
Fucoxanthin (Chrysophytes/diatoms)



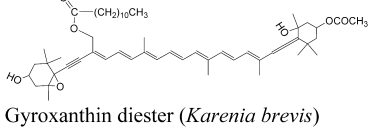
Peridinin (Dinoflagellates)



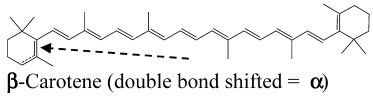
Zeaxanthin (Cyanobacteria)



Alloxanthin (Cryptophytes)



Gyroxanthin diester (*Karenia brevis*)



β-Carotene (double bond shifted = α)

APPENDIX B: ABBREVIATIONS

Abbreviation	Identity
CHL-*a*	chlorophyll-*a*
CHL-*a*′	chlorophyll-*a*-epimer

CHL-*a*-allo	chlorophyll-*a*-allomer
PHEOs	pheopigments (=pheophorbide-*a*, pyropheophorbide-*a*, pheophytin-*a* etc.
CHL-*b*	chlorophyll-*b*
CHLs-*c*	chlorophylls-c_1/-c_2
MYXO	myxoxanthphyll
ZEA	zeaxanthin
LUT	lutein
HEX	19′-hexanoyloxyfucoxanthin
BUT	19′-butanoyloxyfucoxanthin
FUCO	fucoxanthin
PERI	peridinin
ALLO	alloxanthin
PRAS	prasinoxanthin
PAP	photosynthetic Accessory Pigment
PP	photoprotectorant Pigment
CYANOS	cyanobacteria (aka 'cyanophytes')
GREENS	chlorophytes
CHRYS	chrysophytes
NANO	nonoflagellates
DIATS	diatoms
DINOS	dinoflagellates
CRYPTO	cryptophytes
PRASIN	prasinophytes
DOM	dissolved organic matter (∼organic carbon)
HPLC	high-performance liquid-chromatography
RP	reversed phase
PDA	photodiode array (detection)
λ	wavelength (nm, nanometers)
IP	ion pairing (aka Ion Supression)
IS	internal standard
QA/QC	quality assurance/quality control
CERP-MAP	comprehensive everglades rrstoration plan–monitoring and assessment program

ACKNOWLEDGMENTS

These studies were funded by the National Oceanographic and Atmospheric Administration (NOAA), South Florida Ecosystem Restoration and Modeling Program (SFERMP) through the National Marine Fisheries Service (NMFS). The financial support from NOAA/NMFS and the moral support of Dr. Larry Pugh are both greatly appreciated.

I thank the National Park Service, Everglades National Park, especially Ms. Lucy Given and Mr. Robert Zepp, for allowing access to facilities and provision of sampling permits (Permits # 2000088 and EVER-2001-SCI-0078).

Dr. Kelly S. Rein of the Advanced Research Cooperation in Environmental Health (ARCH) program at Florida International University is thanked for several samples of dinoflagellates, including 3 clones of *Karenia brevis*, the Florida Red Tide organism.

The provision of cultures of *Synechococcus elongatus*, *Cyclotella choctawatcheeana*, and the prokaryotic "2 micron picosphere", all isolated clones from Florida Bay, by Dr. Carmelo Tomas is gratefully acknowledged.

Dr. John Scarpa of Harbor Branch oceanographic Institute in Ft. Pierce, Florida is thanked for recurrent donations of *Isochrysis galbana*.

F. Hoffman-LaRoche in Basel Switzerland is thanked for gifts of isozeaxanthin, echinenone, and canthaxanthin.

I would like to acknowledge the expert laboratory assistance of Ms. Panne Monghkonsri for performing extractions and HPLC analyses. Likewise, I thank P. Monghkonsri, A. Singh, D. W. Louda, W. Gurney, D. Snedden, and others for assistance during sampling events.

I thank the anonymous review process and editorial expertise of JLC & RT for recommendations and improvements to this manuscript.

REFERENCES

1. Millie, D.F.; Paerl, H.W.; Hurley, J.P. Microalgal pigment assessments using high performance liquid chromatography: a Synopsis of organismal and ecological applications. Can. J. Fish. Aquat. Sci. **1993**, *50*, 2513–2527.
2. Paerl, H.W.; Valdes, L.M.; Pinckney, J.L.; Piehler, M.F.; Dyble, J.; Moisander, P.H. Phytoplankton photopigments as indicators of estuarine and coastal eutrophication. Bioscience **2003**, *52*, 953–964.
3. Mantoura, R.F.C.; Llewellyn, C.A. The rapid determination of algal chlorophyll and carotenoid pigments and their breakdown products in natural waters by reverse-phase high performance liquid chromatography. Analyt. Chim. Acta **1983**, *151*, 297–314.
4. Kraay, G.W.; Zapata, M.; Veldhuis, M.J.W. Separation of chlorophylls c1, c2 and c3 of marine phytoplankton by reversed-phase-C18-high-performance liquid chromatography. J. Phycol. **1992**, *28*, 708–712.
5. Wilhelm, C.; Rudolph, I.; Renner, W.A. Quantitative method based on HPLC–aided pigment analysis to monitor structure and dynamics of the phytoplankton assemblage- A Study from Lake Meerfelder Maar (eifel, Germany). Arch. Hydrobiol. **1991**, *123*, 21–35.
6. Villanueva, J.; Grimalt, J.; Dewit, R.; Keely, B.J.; Maxwell, J.R. Chlorophyll and carotenoid- pigments in solar saltern microbial mats. Geochim. Cosmochim. Acta **1994**, *58*, 4703–4715.
7. Winfree, N.M.; Louda, J.W.; Baker, E.W.; Steinman, A.D.; Havens, K.E. Application of chlorophyll and carotenoid pigments for the chemotaxonomic assessment of seston, periphyton, and cyanobacterial mats of Lake Okeechobee,

Florida. In *Molecular Markers in Environmental Chemistry*; Eganhouse, R.P., Ed.; ACS Symp. Series No. 671 American Chemical Society: Washington, D.C., 1997, 77–91.
8. Havens, K.E.; Steinman, A.D.; Louda, J.W.; Winfree, N.M.; Baker, E.W. Comparative analysis of lake periphyton communities using high performance liquid chromatography (HPLC) and light microscope counts. Aquat. Sci. **1999**, *61*, 307–322.
9. Bianchi, T.S.; Findlay, S.; Dawson, R. Organic matter sources in the water column and sediments of the Hudson River estuary: The use of plant pigments as tracers. Estuarine Coastal Shelf Sci. **1993**, *36*, 359–376.
10. Ansotegui, A.; Trigueros, J.M.; Orive, E. The use of pigment signatures to assess phytoplankton assemblage structures in estuarine waters. Estuarine, Coastal Shelf Sci. **2001**, *52*, 689–703.
11. Ansotegui, A.; Sarobe, A.; Trigueros, J.M.; Urrutxurtu, I.; Orive, E. Size distribution of algal pigments and phytoplankton assemblages in a coast–estuarine environment: contribution of small eukaryotic algae. J. Plankton Res. **2003**, *25*, 341–355.
12. Pinckney, J.L.; Richardson, T.L.; Millie, D.F.; Paerl, H.F. Application of photopigment biomarkers for quantifying microalgal community composition and in situ growth rates. Org. Geochem. **2001**, *32*, 585–595.
13. Repeta, D.J.; Simpson, D.J.; Jorgensen, B.B.; Jannasch, H.W. Evidence for anoxygenic photosynthesis from the distribution of bacteriochlorophylls in the Black Sea. Nature **1989**, *342*, 69–72.
14. Guillard, R.R.L.; Keller, M.D.; O'Kelly, C.J.; Floyd, G.L. *Pycnococcus provasolii* gen. et sp. nov, a coccoid prasinoxanthin-containing phytoplankter from the western North Atlantic and Gulf of Mexico. J. Phycol. **1991**, *27*, 39–47.
15. Barlow, R.G.; Mantoura, R.F.C.; Gough, M.A.; Fileman, T.W. Pigment signatures of the phytoplankton composition in the northeastern Atlantic during the 1990 spring bloom. Deep-Sea Res. II. **1993**, *40*, 459–477.
16. Wright, S.W.; Thomas, D.P.; Marchant, H.J.; Higgins, H.W.; Mackey, M.D.; Mackey, D.J. Analysis of phytoplankton of the Australian sector of the Southern Ocean: comparisons of microscopy and size frequency data with the interpretations of pigment HPLC data using the 'CHEMTAX' matrix factorisation program. Mar. Ecol. Prog. Ser. **1996**, *144*, 285–298.
17. Peeken, I. Photosynthetic pigment fingerprints as indicators of phytoplankton biomass and development in different water masses of the Southern Ocean during austral spring. Deep-Sea Res II. **1997**, *44*, 261–282.
18. Mackey, M.D.; Higgins, H.W.; Mackey, D.J.; Holdsworth, D. Algal class abundances in the western Pacific: Estimation from HPLC measurements of chloroplast pigments using CHEMTAX. Deep-Sea Res. I **1998**, *45*, 1441–1468.
19. Hurley, J.P.; Watras, C.J. Identification of bacteriochlorophylls in lakes via reverse-phase HPLC. Limnol. Oceanogr. **19**, *36*, 307–315.
20. Steinman, A.D.; Havens, K.E.; Louda, J.W.; Winfree, M.N.; Baker, E.W. Characterization of the photoautotrophic algal and bacterial communities in a large shallow subtropical lake using HPLC-PDA based pigment analysis. Can. J. Fish. Aquatic Sci. **1998**, *55*, 206–219.
21. Louda, J.W.; Loitz, J.W.; Rudnick, D.T.; Baker, E.W. Early diagenetic alteration of chlorophyll-a and bacteriochlorophyll-a in a contemporaneous marl ecosystem. Org. Geochem. **2000**, *31*, 1561–1580.
22. Jeffrey, S.W., Mantoura, R.F.C., Wright, S.W., Eds.; *Phytoplankton Pigments in Oceanography: Guidelines to Modern Methods*; UNESCO: Paris, 1997; 661 pp.

23. Mackey, M.D.; Mackey, D.J.; Higgins, H.W.; Wright, S.W. CHEMTAX- A program for estimating class abundances from chemical marker: Application to HPLC measurement of phytoplankton pigments. Mar. Ecol. Prog. Ser. **1996**, *144*, 265–283.
24. Mackey, M.D.; Higgins, H.W.; Mackey, D.J.; Wright, S.W. CHEMTAX user's manual: a program for estimating class abundances from chemical markers-application to HPLC measurements of phytoplankton pigments. CSIRO Marine Laboratries Report 229, Hobart Australia, **1997**, ISBN 0–643–06040–5, 47 pp.
25. Schluter, L.; Mohlenberg, F.; Havskum, H.; Larsen, S. The use of phytoplankton pigments for identifying and quantifying phytoplankton groups in coastal areas: testing the influence of light and nutrients on pigment/chlorophyll a ratios. Mar. Ecol. Prog. Ser. **2000**, *192*, 49–63.
26. Gibb, S.W.; Cummings, D.G.; Irigoien, X.; Barlow, R.G.; Fauzi, R.; Mantoura, R.F.C. Phytoplankton pigment chemotaxonomy of the northeastern Atlantic. Deep-Sea Res. **2001**, *48*, 795–823.
27. Goericke, R.; Montoya, J.P. Estimating the contribution of microalgal taxa to chlorophyll a in the field–variations of pigment ratios under nutrient- and light-limited growth. Mar. Ecol. Prog. Ser. **1998**, *169*, 97–112.
28. *Comprehensive Everglades Restoration Plan - Monitoring and Assessment Plan* (CERP-MAP); United States Army Corps of Engineers and South Florida Water Management District: Jacksonville, Florida, 2003.
29. Hagerthey, S.E.; Louda, J.W.; Mongkronsri, P. Evaluation of pigment extraction methods and a recommended protocol for periphyton chlorophyll *a* determination and chemotaxonomic assessment. J. Phycol. **2006**, *42*, 1125–1136.
30. Wright, S.W.; Jeffrey, S.W.; Mantoura, R.F.C. Evaluation of methods and solvents for pigment extraction. In *Phytoplankton Pigments in Oceanography: Guidelines to Modern Methods*; Jeffrey, S.W., Mantoura, R.F.C and Wright, S.W. Eds.; UNESCO: Paris, 1997, 261–282.
31. Louda, J.W.; Liu, L.; Baker, E.W. Senescence- and death-related alteration of chlorophylls and carotenoids in marine phytoplankton. Org. Geochem. **2002**, *33*, 1635–1653.
32. Britton, G. UV/Visible Spectroscopy. In *Carotenoids*, Volume 1B, Spectroscopy; Briton, G., Liaaen-Jensen, S., and Pfander, H. Eds.; Birkhauser, Basel, 1995, 13–62.
33. Gieskes, W.W.; Kraay, G.W. Unknown chlorophyll-a derivatives in the North Sea and the tropic Atlantic Ocean revealed by HPLC analysis. Limnol. Oceanogr. **1983**, *28*, 757–766.
34. Gieskes, W.W.; Kraay, G.W.; Nontji, A.; Setiapermana, D.; Sutomo. Monsoonal alteration of a mixed and a layered structure in the phytoplankton of the euphotic zone of the Banda Sea (Indonesia): A Mathematical analysis of algal fingerprints. Netherlands J. Sea Res. **1988**, *22*, 123–137.
35. Carreto, J.I.; Montoya, N.G.; Benavides, H.R.; Guerrero, R.; Carignan, M.O. Characterization of spring phytoplankton communities in the Rio de la Plata maritime front using pigment signatures and cell microscopy. Mar. Biol. **2003**, *143*, 1013–1027.
36. Steidinger, K.A.; Phlips, E.J. *Florida Bay Microalgal Blooms: Composition, Abundance, and Distribution*; 2nd Ann. Florida Bay Sci. Conf., Sea Grant, Key Largo, Florida, 1996, Abs. pp. 77–79.
37. Louda, J.W. *NOAA-SFERPM: Question #3 Algal Blooms, Chemotaxonomic Assessment of Phytoplankton and Epiphyte Succession in the Rankin Bight-*

Whipray Basin Areas of North Central Florida Bay. Final Report (September, 2002). <http.www.aoml.noaa.gov/ocd/sferpm/louda/louda_algal_blooms.html>.
38. Louda, J.W.; Monghkonsri, P. Comparison of spectrophotometric estimates of chlorophylls-a,b,c and 'pheopigments' in Florida Bay seston with that obtained by high performance liquid chromatography-photodiode array analyses. Fla. Scient. **2004**, *67*, 281–292.
39. Louda, J.W.; Loitz, J.W.; Melisiotis, A.; Orem, W.H. Hydrogel stabilization of Florida Bay marl sediments and implications for the preservation of organic matter. J. Coastal Res. **2004**, *20*, 448–463.
40. Richardson, L.L.; Ambrosia, V.G. *Hyperspectral Imaging Sensors and Assessment of Oceanic Littoral Zone Phytoplankton Dynamics* Oceans 96, MTS/IEEE. 1996, Sept. 23–26.
41. Zullig, H. Role of carotenoids in lake sediments for reconstructing trophic history during the late. Quaternary. J. Paleolimnol. **1989**, *2*, 23–40.
42. Millie, D.F.; Schofield, O.M.; Kirkpatrick, G.J.; Johnsen, G.; Tester, P.A.; Vinyard, B.T. Detection of harmful algal blooms using photopigments and absorption signatures: A case study of the Florida red tide dinoflagellate, *Gymnodinium breve*. Limnol. Oceanogr. **1997**, *42*, 1240–1251.
43. Jeffrey, S.W.; Hallegraeff, G.M. Chlorophyllase distribution in 10 classes of phytoplankton – a problem for chlorophyll analysis. Mar. Ecol. Prog. Ser. **1987**, *35*, 293–304.
44. Deydier-Stephan, L.; Berru, G.; LeRouzic, B. A chemotaxonomic method to quantify phytoplankton groups in freshwater lentic mesocosms: an approach including chlorophyll *a* breakdown products. C. R. Biologies **2003**, *326*, 95–105.
45. Louda, J.W.; Li, J.; Liu, L.; Winfree, M.N.; Baker, E.W. Chlorophyll degradation during senescence and death. Org. Geochem. **1998**, *29*, 1233–1251.
46. Llewellyn, C.A.; Mantoura, R.F.C. Pigment biomarkers and particulate carbon in the upper water column compared to the ocean interior of the northeast Atlantic. Deep-Sea Res.-I. **1996**, *43*, 1165–1184.
47. Thibault, D.; Roy, S.; Wong, C.S.; Bishop, J.K. The downward flux of biogenic material in the NE subarctic Pacific: Importance of algal sinking and mesozooplankton herbivory. Deep-Sea Res. II. **1999**, *46*, 2669–2697.
48. Fuchs, E.; Zimmerman, R.C.; Jaffe, J.S. The effect of elevated levels of phaeophytin in natural water on variable flouresence measured from phytoplankton. J. Plankton Res. **2002**, *24*, 1221–1229.
49. Walker, J.S.; Keely, B.J. Distribution and significance of chlorophyll derivatives and oxidation products during the spring phytoplankton bloom in the Celtic Sea April 2002. Org. Geochem. **2004**, *35*, 1289–1298.
50. Roy, S.; Chanut, J.P.; Gosselin, M.; Simengando, T. Characterization of phytoplankton communities in the lower St. Lawrence estuary using HPLC-detected pigments and cell microscopy. Mar. Ecol. Prog. Ser. **1996**, *142*, 55–73.
51. Brotas, V.; Plante-Cuny, M.R. Spatial and temporal patterns of microphytobenthic taxa of estuarine tidal flats in the Tagus Estuary (Portugal) using pigment analysis by HPLC. Mar. Ecol. Prog. Ser. **1998**, *171*, 43–57.
52. Metaxatos, A.; Ignatiades, L. Seasonality of Algal Pigments in the Sea Water and Interstitial Water/Sediment System of an Eastern Mediterranean Coastal Area. Estuarine, Coastal Shelf Sci. **2002**, *55*, 415–426.

53. Baker, E.W.; Louda, J.W. Porphyrins in the geologic record. In *Biological Markers in the Geological Record*; Johns, R.B., Ed.; Elsevier: Amsterdam, 1986, 125–225.
54. Baker, E.W.; Louda, J.W. The legacy of the Treibs' samples. In *Alfred Treibs Memorial Volume*; Prashnowsky, A., Ed.; University of Wurzburg: Wurzburg, 2002, 3–128.
55. Descy, J.-P.; Metens, A. Biomass – pigment relationships in potamoplankton. J. Plankton Res. **1996**, *18*, 1557–1566.
56. Neto, R.R.; Mead, R.N.; Louda, J.W.; Jaffe, R. Organic biogeochemistry of detrital flocculent material (floc) in a subtropical, coastal, wetland. Biogeochem. **2006**, *77*, 283–304.
57. Schluter, L.; Mohlenberg, F.; Havskum, H.; Larsen, S. The use of phytoplankton pigments for identifying and quantifying phytoplankton groups in coastal areas: testing the influence of light and nutrients on pigment/chlorophyll a ratios. Mar. Ecol. Prog. Ser. **2000**, *192*, 49–63.
58. Phlips, E.J.; Badylak, S. Spatial variability in phytoplankton standing crop and composition in a shallow inner-shelf lagoon, Florida Bay. Florida. Bull. Mar. Sci. **1996**, *58*, 203–216.
59. Phlips, E.J.; Badylak, S.; Lynch, T.C. Blooms of the picoplanktonic cyanobacterium *Synechococcus* in Florida Bay, a subtropical inner shelf lagoon. Limnol. Oceanogr. **1999**, *44*, 1166–1175.
60. Richardson, L.L.; Buisson, D.; Ambrosia, V.G. *Use of Remote Sensing Coupled with Algal Accessory Pigment Data to Study Phytoplankton Bloom Dynamics in Florida Bay*; Third Thematic Conf. on Remote Sensing for Marine and Coastal Environments. Seattle, Wash., Sept., 18–20, 1995, Proc. v. II 125–133.
61. Havskum, H.; Schluter, L.; Scharek, R.; Berdalet, E.; Jacquet, S. Routine quantification of phytoplankton groups – microscopy or pigment analyses? Mar. Ecol. Prog. Ser. **2004**, *273*, 31–42.
62. Breton, E.; Brunet, C.; Sautour, B.; Brylinski, J.-M. Annual variations of phytoplankton biomass in the Eastern English Channel: comparison by pigment signatures and microscopic counts. J. Plankton Res. **2000**, *22*, 1423–1440.
63. Garibotti, I.A.; Vernet, M.; Kozlowski, W.A.; Ferrario, M. Composition and biomass of phytoplankton assemblages in coastal Antarctic waters: a comparison of chemotaxonomic and microscopic analyses. Mar. Ecol. Prog. Ser. **2003**, *247*, 27–42.
64. Menden-Deuer, S.; Lessard, E.J.; Satterberg, J. Effect of preservation on dinoflagellate and diatom cell volume and consequences for carbon biomass predictions. Mar. Ecol. Prog. Ser. **2001**, *222*, 41–50.
65. Gosselain, V.; Hamilton, P.B.; Descy, J.-P. Estimating phytoplankton carbon from microscopic counts: an application for riverine systems. Hydrobiologia **2000**, *438*, 75–90.
66. Culverhouse, P.F.; Williams, R.; Reguera, B.; Herry, V.; Gonzales–Gil, S. Do experts make mistakes? A comparison of human and machine identification of dinoflagellates. Mar. Ecol. Prog. Ser. **2003**, *247*, 17–25.
67. Graham, L.E.; Wilcox, L.W. *Algae*; Prentice Hall: Upper Saddle River, New Jersey, 2000; 640.
68. Voros, L.; Padisak, J. Phytoplankton biomass and chlorophyll–a in some shallow lakes in central Europe. Hydrobiologia **1991**, *215*, 111–119.
69. Llewellyn, C.A.; Fishwick, J.R.; Blackford, J.C. Phytoplankton community assemblage in the English Channel: a comparison using chlorophyll a derived from

HPLC-CHEMTAX and carbon derived from microscopy cell counts. J. Plankton Res. **2004**, *27*, 103–119.
70. Higgins, H.W.; Mackey, D.J. Algal class abundances, estimated from chlorophyll and carotenoid pigments, in the western equatorial Pacific under El Nino and non-El Nino conditions. Deep-Sea Res. I. **2000**, *47*, 1461–1483.
71. Skoog, K.; Louda, J.W. Chemotaxonomic assessment of Lake Okeechobee phytoplankton: natural samples and in vitro experimentation into the effects of light levels. 67th *Annual Meeting of the Florida Academy of Sciences*; Orlando, Fl. 21–22, 2003, Abstract # SS-6 March.
72. Grant, C. *Photosynthetic Pigment Ratios in Relation to Photic Flux*; M.S. Thesis, Florida Atlantic University (J.W. Louda, supervisor), 2006; 129 pp.
73. Hillebrand, H.; Durselen, C.-D.; Kirschtel, D.; Pollingher, U.; Zohary, T. Biovolume calculation for pelagic and benthic microalgae. J. Phycol. **1999**, *35*, 403–424.
74. Sun, J.; Liu, D. Geometric models for calculating cell biovolume and surface area for phytoplankton. J. Plankton Res. **2003**, *25*, 1331–1346.
75. Buchaca, T.; Felip, M.; Catalan, J.A. Comparison of HPLC pigment analyses and biovolume estimates of phytoplankton groups in an oligotrophic lake. J. Plankton Res. **2005**, *27*, 91–101.
76. Landry, M.R.; Ondrusek, M.E.; Tanner, S.J.; Brown, S.L.; Constantinou, J.; Bidigare, R.R.; Coale, K.H.; Fitzwater, S. Biological response to iron fertilization in the eastern equatorial Pacific (IronEx II). I. Microplankton community abundances and biomass. Mar. Ecol. Prog. Ser. **2000**, *201*, 27–42.
77. DellaPenna, D. Carotenoid synthesis and function in plants: Insights from mutant studies in *Arabidopsis*. Pure Applied Chem. **1999**, *71*, 2205–2212.
78. Jeffrey, S.W.; Vesk, M. Introduction to marine phytoplankton and their pigment signatures. In *Phytoplankton Pigments in Oceanography: Guidelines to Modern Methods*; Jeffrey, S.W., Mantoura, R.F.C. and Wright, S.W. Eds.; UNESCO: Paris, 1997, 37–84.
79. Vidussi, F.; Marty, J.-C.; Chiaverini, J. Phytoplankton pigment variations during the transition from spring bloom to oligotrophy in the northwestern Mediterranean sea. Deep–Sea Res. I. **2000**, *47*, 423–445.
80. Henriksen, P.; Riemann, B.; Kaas, H.; Sorensen, H.M.; Sorensen, H.L. Effects of nutrient-limitation and irradiance on marine phytoplankton pigments. J. Plankton. Res. **2002**, *24*, 835–858.
81. Bustillos-Guzman, J.; Claustre, H.; Marty, J.C. Specific phytoplankton signatures and their relationship to hydrographic conditions in the coastal northwestern Mediterranean Sea. Mar. Ecol. Prog. Ser. **1995**, *124*, 247–258.
82. Barlow, R.G.; Mantoura, R.F.C.; Peinert, R.D.; Miller, A.E.J.; Fileman, T.W. Distribution, sedimentation and fate of pigment biomarkers following thermal stratification in the western Alboran Sea. Mar. Ecol. Prog. Ser. **1995**, *125*, 279–291.
83. Wilhelm, C.; Rudolph, I.; Renner, W. A Quantitative method based on HPLC–aided pigment analysis to monitor structure and dynamics of the phytoplankton assemblage- A Study from Lake Meerfelder Maar (eifel, Germany). Arch. Hydrobiol. **1991**, *123*, 21–35.
84. Bianchi, T.S.; Findlay, S.; Dawson, R. Organic matter sources in the water column and sediments of the Hudson River estuary: The use of plant pigments as tracers. Estuarine Coastal Shelf Sci. **1993**, *36*, 359–376.

85. Descy, J.-P.; Higgins, H.W.; Mackey, D.J.; Hurley, J.P.; Frost, T.M. Pigment ratios and phytoplankton assessment in northern Wisconsin lakes. J. Phycol. **2000**, *36*, 274–286.
86. Barlow, R.G.; Mantoura, R.F.C.; Gough, M.A.; Fileman, T.W. Pigment signatures of the phytoplankton composition in the northeastern Atlantic during the 1990 spring bloom. Deep-Sea Res.-II. **1993**, *40*, 459–477.
87. Jeffrey, S.W.; Wright, S.W. Qualitative and quantitative HPLC analysis of SCOR reference algal cultures. In *Phytoplankton Pigments in Oceanography: Guidelines to Modern Methods*; Jeffrey, S.W., Mantoura, R.F.C. and Wright, S.W. Eds. UNESCO: Paris, 1997; 343–360.
88. Louda, J.W. unpublished data (2002–2007).

Received August 24, 2007
Accepted September 10, 2007
Manuscript 6174