

Final Report

Development of HPLC Technology
for Detection of *Gymnodinium breve*

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NOTE: *The following report will be submitted for publication in the Journal of Phycology. A copy of the manuscript has been submitted as the Final Report for this project. Within the past year, Gymnodinium breve has been renamed Karenia brevis and the manuscript below reflects the new name.*

Quantification of the toxic dinoflagellate, *Karenia brevis* using
photopigment biomarkers.¹

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ABSTRACT

Diagnostic photopigment analysis is a useful tool for determining the presence and relative abundance of algal groups in mixed species assemblages. This approach is especially useful when a genus has a unique photopigment composition. The toxic dinoflagellate *Karenia brevis* has a relatively unique photopigment composition, in that it shares the diagnostic pigment gyroxanthin-diester with only a few other dinoflagellates and it lacks peridinin, one of the major diagnostic pigments of most dinoflagellate species. Here measurements of gyroxanthin-diester and other diagnostic pigments of *K. brevis* were incorporated into the initial pigment ratio matrix of the Chemical Taxonomy program (CHEMTAX) to resolve the relative contribution of *K. brevis* biomass in mixed estuarine phytoplankton assemblages. Water samples containing *K. brevis* were obtained from Florida and Galveston Bay, Texas. The phytoplankton community composition of the bloom in Galveston Bay was estimated based on cell enumeration and biovolumetric measurements in addition to chlorophyll *a* specific photopigment estimates of biomass (HPLC and CHEMTAX). The CHEMTAX and biovolume estimates of phytoplankton community structure were not significantly different and suggest that the HPLC-CHEMTAX approaches provides reasonable estimates of *K. brevis* biomass in mixed algal assemblages. Incorporating gyroxanthin-diester into the CHEMTAX initial ratio matrix was essential for a true representation of the phytoplankton community in the presence of *K. brevis*. Without the proper initial matrix, *K. brevis* biomass (chl *a*) was contributed to diatoms and haptophytes. Our analysis of gyroxanthin-diester content per cell of *K. brevis* from Galveston Bay was significantly higher than in *K. brevis* collected from the West Coast of Florida. We conclude that the pigment-based approach (HPLC and CHEMTAX) provides a useful tool for resolving spatiotemporal distribution of phytoplankton in the presence of *K. brevis* blooms, when appropriate initial ratio matrix is applied.

INTRODUCTION

The toxic dinoflagellate, *Karenia brevis* (Davis) G. Hansen & Moestrup *comb. nov* (formerly known as *Gymnodinium breve* (Davis)) (Daugbjerg et al. 2000) is of ubiquitous distribution within Gulf of Mexico. The species occurs in low (background) concentrations of <10 to 100 cells per liter throughout the Gulf (Geesey and Tester 1993; Review by Kusek et al. 1999) but extensive blooms mainly occur in three regions; off the West coast of Florida, Texas, and the Yucatan peninsula (Tester and Steidinger, 1997). The regional blooms are possibly favored by the overlap of the depth of the thermocline with the continental shelf, localized upwelling of nutrient rich water and onshore advection of surface water (Rounsefell and Dragovich 1966; Tester and Steidinger 1997). Blooms off Western Florida are almost a yearly occurrence and 70% of the blooms occur in the autumn months (Tester and Steidinger 1997). The distribution of *K. brevis* is mainly determined by salinity and temperature (see reviews by Steidinger et al., 1998 and Kusek et al., 1999). *K. brevis* exhibits optimal growth at 22-28 °C and 31-34 psu, but has been detected across a range of temperature (9-33°C) and salinities (24-37 psu) (Kusek et al. 1999). The temperature and salinity of Gulf of Mexico water overlaps the physiological envelope of *K. brevis* through much of the year, thus it is not surprising that blooms have been detected in all seasons (Review by Kusek et al. 1999).

The frequency and times of *K. brevis* blooms in Texas waters is not as well documented. However, it has been proposed that bloom initiation off the coast of Texas is initiated by the same sequence of events as off the Western coast of Florida (Tester and Steidinger, 1997). Consequently the extent and breadth of knowledge on *K. brevis* physiology and bloom dynamics from Texas waters is very limited compared to our understanding of its bloom dynamics in Florida. Two cases of *K. brevis* associated fish kills have been reported for Texas coastal waters (Wilson and Ray 1956; Trebatoski 1988). However, phytoplankton induced fish kills are frequently observed/reported, but the causative species are uncertain (Harper and Guillen 1989; Zimmerman 1998). Although *K. brevis* blooms are infrequently observed in Texas, the economic consequences associated with fish kill and shellfish closures are substantial and of growing concern (ECO HAB 1995; Zimmerman 1998). Increased understanding of the bloom initiation, transport, and spatial coverage of *K. brevis* are an essential component in the development of an early warning and tracking system for the fisheries in coastal and estuarine ecosystems of Texas.

Diagnostic photopigment analysis is a useful tool for determining the presence and relative abundance of algal groups in mixed species assemblages. Photopigment-based approaches have proven useful and reliable for determining phytoplankton community composition (Mantoura and Llewellyn 1983; Wright et al. 1991; Jeffrey et al. 1999; Wright and van den Enden 2000) and community succession across broad spatial and temporal scales (Wilhelm et al. 1991; Letelier et al. 1993; Tester et al. 1995; Pinckney et al. 1998). One of the main advantages of the photopigment approach is the automated, rapid sample analysis and quantification of natural phytoplankton samples compared to tedious microscopic identification and enumeration of small volume subsamples of water (Millie et al. 1993). The pigment based approach also holds the potential for more consistent results among individual research teams than the more conventional microscopy methods (Schlüter 2000), because pigment based methods are well defined and the challenges associated with identification of small flagellates can be avoided.

The phytoplankton community composition can be resolved from the photopigment profile using CHEMTAX (Chemical Taxonomy) (Mackey et al. 1997). This algorithm partitions the total biomass (chl *a*) of phytoplankton groups in a sample by comparing the pigment composition of the sample with the diagnostic pigment composition of predetermined phytoplankton groups. The complexity of the community is constrained by the number of phytoplankton groups initially characterized by the researcher, (i.e. included in the initial pigment ratio matrix used for comparison in community composition analysis). Therefore, it is valuable to have insight into the phytoplankton community of the ecosystem for which the initial pigment ratio matrix is to be applied (Wright et al. 1996). CHEMTAX has been used to resolve phytoplankton community composition of oceanic and estuarine waters (Mackey et al. 1998; Pinckney et al. 1998; Higgins and Mackey 2000; Schlüter et al. 2000; Wright and van den Enden 2000), providing reliable information on large scales and long term distributions of phytoplankton groups.

A more specific determination of phytoplankton community structure is possible when a single species or few species share a unique diagnostic photopigment. This provides the opportunity to address species-specific questions based on the presence/absence as well as the relative concentration of the diagnostic pigment in environmental samples. Most autotrophic dinoflagellates contain the carotenoid peridinin as their primary light-harvesting pigment (Jeffrey

et al. 1975, Millie et al. 1993). *Karenia brevis*, however belongs to a group of dinoflagellates that do not have peridinin but instead have fucoxanthin and 19'-acylofucoxanthins as primary light harvesting pigments (Jeffrey et al. 1975, Bjørnland and Liaaen-Jensen 1989, Johnsen and Sakshaug 1993). A few gymnodinoid species, in addition to *K. brevis*, also have the carotenoid gyroxanthin-diester as an accessory photopigment (Johnsen and Sakshaug 1993, Hansen et al. 2000). Gyroxanthin-diester, which has been observed in only a few toxic dinoflagellates, is uncommon and provides a relatively unique biomarker for *K. brevis* (Millie et al. 1995). The chemical structure and biosynthesis of gyroxanthin-diester was recently described by Bjørnland et al. (2000). In the Gulf of Mexico, the only known gyroxanthin-containing dinoflagellates are *Karenia brevis* and *Karenia mikimotoi*. Although *K. brevis* and *K. mikimotoi* cannot be differentiated based on photopigment characteristics, both species are relatively easy to identify. When gyroxanthin is detected, samples should be examined using qualitative microscopy to determine the species present.

The usefulness of gyroxanthin-diester as a proxy for *K. brevis* abundance is defined by its relationship to chl *a*. Millie et al. (1995) demonstrated a consistent gyroxanthin-diester/chlorophyll *a* ratio in *K. brevis* cultures grown at various irradiances and inferred that gyroxanthin-diester could be used as a diagnostic pigment for *K. brevis* in Florida waters. Later Millie et al. (1997) demonstrated that gyroxanthin-diester concentrations corresponded to cell counts and chlorophyll *a* concentrations during a *K. brevis* bloom in Florida, allowing the quantification of *K. brevis* abundance based on gyroxanthin-diester pigment concentrations from the field. In this paper we expand the gyroxanthin-based methods to investigate their applicability to *K. brevis* in Texas waters.

The purpose of our work was to incorporate gyroxanthin-diester as a diagnostic photopigment in CHEMTAX to resolve the relative contribution of *K. brevis* biomass in mixed estuarine phytoplankton assemblages, from Galveston Bay, Texas and the West Coast of Florida.

MATERIAL AND METHODS

Culture material. Cultures of *Karenia brevis* (Wilson clone) at NOAA were grown in f/2 media made with Gulf Stream water adjusted to 30 psu at 23°C on a 14:10 hour light:dark cycle. Illumination was from a bank of Sylvania Paylight 20-watt fluorescent bulbs, providing a quantum scalar irradiance of 100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ as measured with a LiCor 4 pi sensor.

Cultures were shipped by overnight carrier to College Station. Immediately upon arrival the cultures were stored at room temperature for 24 hours before filtration under low-light conditions. Replicate (six) aliquots of the culture were filtered under low vacuum (80 mmHg) onto 25mm Whatman GF/F filters. The filters were folded and gently pressed between absorbent paper towels to remove excess water and were immediately frozen at -80°C until analyzed.

Natural samples. Water samples containing *K. brevis* were obtained from Florida and Galveston Bay, Texas. The samples from Florida were collected in September 1999 along the panhandle coast by S. Varnam for P. Tester. Samples (500 –1000 ml) were filtered onto 47 mm Whatman GF/F filters, placed in small cryo-vials and immediately frozen in liquid nitrogen. The samples were shipped to our facility at TAMU on dry ice and stored at -80°C until analyzed. In Galveston Bay, samples were collected from the uppermost meter of the water column using an integrated water sampler. Samples were gently poured into acid cleaned 10 liter carboys and transported in an insulated cooler (chilled with ice) to the lab at Texas A&M University at Galveston. Aliquots (75 to 150ml) were filtered onto 25mm GF/F filters under low vacuum, immediately frozen on dry ice, and stored at -80°C until analyzed.

Enumeration. Subsamples of water collected from Galveston Bay were preserved for phytoplankton identification and enumeration. Aliquots (48 ml) of each sample were preserved with 2 ml of 25 % glutaraldehyde (1% final concentration) (Booth 1993) and stored at 5°C until analyzed. Phytoplankton cells were concentrated by settling 5 ml of the sample in a settling chamber for 24 hours after which the overlying water was discarded (Utermöhl, 1958). Phytoplankton and cyanobacteria were enumerated in twenty fields of view at 3 magnifications; 200x, 400x and 1000x, counting at least 200 cells. Cells larger than 20 µm, 2-20 µm and smaller than 2 µm in diameter were counted at each magnification, respectively. Phytoplankton cells were identified to genus level when possible. Cell dimensions and species identifications were determined simultaneously for each organism and their biovolume estimated based on the assigned shape of each organism (Hillebrand et al. 1999).

HPLC methods. The phytoplankton containing GF/F filters were extracted in 100% acetone (0.75ml), and were sonicated and stored at -20°C for 15-20 hours. Filtered extracts were spiked with 1M ammonium acetate ion-pairing solution (final concentration 0.2M) and 375 µl of sample was injected into a Shimadzu HPLC equipped with a single monomeric (Rainin Microsorb-MV, 0.46x10 cm, 3µm) and one polymeric (Vydac 201TP, 0.46x25 cm , 5µm)

reverse-phase C18 column in series. A non-linear, binary gradient was used for pigment separation (for details, see Pinckney et al. 1996). Solvent A consisted of 80% methanol:20% ammonium acetate (0.5 M adjusted to pH 7.2) and solvent B was composed of 80% methanol:20% acetone. Absorption spectra and chromatograms (440 nm) were acquired using a Shimadzu SPD-M 10av photodiode array detector. Pigment peaks were identified by comparison of retention times and absorption spectra with pure crystalline standards of chlorophylls *a*, *b*, β -carotene (Sigma Chemical Company), fucoxanthin, lutein, canthaxanthin, and zeaxanthin (Hoffman-LaRoche and Company). Other pigments were identified by comparison to extracts from phytoplankton cultures (Wright et al. 1991) and our own standard of gyroxanthin-diester obtained from culture of *K. brevis*. Photopigment concentrations were quantified using chromatogram peak area and the appropriate extinction coefficients (Rowan 1989; Jeffrey et al. 1997).

CHEMTAX. CHEMTAX (CHEMical TAXonomy) is a matrix factorization routine for calculating algal class abundances based on the concentrations of diagnostic chlorophyll and carotenoid photopigments (Mackey et al. 1996; Wright et al. 1996; Pinckney et al. 1998). The program uses a steepest descent algorithm to determine the best fit based on an initial estimate of pigment ratios for algal classes. Input for the program consists of a raw data matrix of photopigment concentrations obtained by HPLC analyses and an initial pigment ratio file. The data matrix is subjected to a factor minimization algorithm that calculates a best fit pigment ratio matrix and a final phytoplankton class composition matrix. The class composition matrix can be expressed as relative or absolute values for specified photopigments. The absolute chlorophyll *a* (chl *a*) contribution of each class is particularly useful because it partitions the total chl *a* into major phytoplankton groups. The complexity of the estimated community structure depends upon the number of phytoplankton groups defined *a priori* by the researcher. We took a conservative approach in defining the initial ratio matrix, restricting our resolution to *K. brevis*, diatoms, chlorophytes, cyanobacteria, cryptophytes, dinoflagellates and two groups of haptophytes (hapto3s and hapto4s). Hapto3s and hapto4s are distinguished by the diagnostic pigments 19'hexanoyloxyfucoxanthin and 19'butanoyloxyfucoxanthin, respectively (Jeffrey and Wright 1994). These eight categories, which are commonly detected in estuarine waters (Roy et al. 1996; Tester et al. 1995), represent the major groups present in the estuarine samples used for our analysis.

The input ratio matrix was compiled from literature values and our own estimates of pigment composition of *K. brevis* culture. The eight functional groups of phytoplankton described above were defined in the initial ratio matrix based on ten diagnostic pigments (Table 1A). For *K. brevis* the diagnostic pigment concentrations were determined for two culture batches of *K. brevis* and the absolute concentrations were normalized to chl *a* and added to the input ratio matrix (Table 1A). Other functional groups were characterized by adding values obtained from the literature (Mackey et al. 1996) to our initial ratio matrix. We took a similar approach as Wright and van den Enden (2000) and applied the maximum ratio values reported from Mackey et al. (1996), except for cyanobacteria. Zeaxanthin, the diagnostic pigment of cyanobacteria, was characterized based on the maximum ratios of zeaxanthin reported for *Synechococcus* and *Trichodesmium* (Mackey et al. 1996), thus the zeaxanthin/chl *a* ratio is the average of the maximum value of the two groups.

Statistics. The relationship between cell abundance and gyroxanthin-diester concentration was analyzed using standard linear regression procedures. The null hypothesis of no significant difference between biovolume and CHEMTAX estimates of phytoplankton community composition was tested nonparametrically, using Wilcoxon's signed rank test (Sokal and Rohlf 1981).

RESULTS

K. brevis chromatograms. Diagnostic pigment profiles of *K. brevis* and the characteristic absorption spectra of gyroxanthin-diester were obtained from cultures of *K. brevis* (Fig. 1). The chromatograms demonstrated good separation between the diagnostic pigments including gyroxanthin-diester. The absorption spectra of the gyroxanthin-diester showed two pigment peaks, one at 444 and the other at 469 nm, which were slightly different from the maxima reported in Millie et al. (1995) (446 and 468 nm). The concentrations of gyroxanthin-diester showed a strong positive linear relationship with *K. brevis* cell abundance (Fig. 2) ($r^2 = 0.999$, $p < 0.01$). The absorption spectra and pigment retention time (25.6 min) of gyroxanthin-diester from the culture was used to resolve the presence of gyroxanthin-diester in field samples. Furthermore, the pigment composition of cultured *K. brevis* was used to determine the pigment to chl *a* ratio for the diagnostic pigments; gyroxanthin-diester, 19-butanoyloxyfucoxanthin,

fucoxanthin, 19-hexanoyloxyfucoxanthin and diadinoxanthin. These five pigments and their ratios in *K. brevis* cultures were used in the initial input ratio matrix to quantify the abundance of *K. brevis* in field samples using CHEMTAX (Table 1).

The linear relationship derived from the regression between gyroxanthin-diester concentration and cell abundance in *K. brevis* cultures (Fig. 2) was used to estimate a theoretical detection limit for *K. brevis*. Using our standard phytoplankton HPLC protocol (750 μ l acetone for extraction and 300 μ l injection volume) and a minimum peak area of 10,000 units (Shimadzu SPD-M10a PDA), we estimate that the lower limit of detection for our system is approximately 5,000 cells per extraction. In practical terms, this means that each filter used for the collection of phytoplankton (GF/F) must retain a minimum of 5,000 cells for reliable detection of *K. brevis* in natural phytoplankton samples. Using this relationship, we further estimated the amount of natural water that one would need to filter through a glass-fiber filter to detect *K. brevis* abundances at low cell concentrations (Fig. 3). The 5,000 cell per liter natural abundance is of particular interest because this is the critical value for oyster and other shellfish harvesting closures (National Shellfish Sanitation Program, US Food & Drug Administration).

CHEMTAX algorithm. Phytoplankton community composition estimated from CHEMTAX depends heavily on the starting values in the initial pigment ratio matrix formerly described. The initial ratio matrix used for our analysis was designed based on a conservative approach as phytoplankton groups containing the same diagnostic pigments were treated as a single group. Therefore, a few of the algal groups defined in Table 1 represent more than one taxonomic group of phytoplankton due to similarities in pigment composition. Chlorophytes were indistinguishable from euglenophytes and prasinophytes as all of the groups contain the diagnostic pigment chlorophyll *b*, separating them from other algal groups. Similarly, two fucoxanthin containing groups of haptophytes and a few dinoflagellates could not be separated from diatoms. Type 4 haptophytes (hapto4s) (Jeffrey and Wright, 1994) and chrysophytes were also indistinguishable in our analysis. Other groups (cyanobacteria, dinoflagellates, cryptophytes, hapto3s and *K. brevis*) defined in Table 1 represent phytoplankton of clearly defined taxonomic groups. In accordance with previous publications (Mackey et al. 1996; Wright and van den Enden 2000) and recommendations (Mackey et al. 1997) we constructed an initial ratio matrix where the number of diagnostic pigments exceeds the number of phytoplankton groups defined. Additionally our ratio matrix is consistent with previous work in representing cryptophytes,

cyanobacteria, and dinoflagellates by chl *a* and one additional pigment (Table 1), despite recommendations to characterize each phytoplankton group by two or more pigments other than chl *a* (Mackey et al. 1997).

The initial ratio matrix (Table 1) was tested using phytoplankton pigment data from the estuarine waters of Galveston Bay, Texas (Fig. 4) by comparing *K. brevis* biomass estimates to the initial gyroxanthin-diester concentration of each sample. HPLC pigment profiles obtained during a bi-weekly sampling effort from May 1999 through December 2000 at seven locations were subjected to the analysis. Some of the samples were known to contain gyroxanthin-diester while the majority of the samples were gyroxanthin-diester free. Phytoplankton community composition obtained from CHEMTAX using initial ratio matrix shown in Table 1, reflected the pigment composition of the samples showing the presence of high biomass of *K. brevis* in samples from September 4 and 18 (Table 2). In gyroxanthin-diester free samples either no or trace amounts of biomass (chl *a*) were contributed to *K. brevis* (represented in Table 2 by data from August 18 and October 14). The presence of *K. brevis* on Aug 18 demonstrates the sensitivity of the CHEMTAX algorithm to the pigment composition (ratios) of natural samples. However the fraction attributed to *K. brevis* is only 0.7% of the total biomass and does not alter the picture we obtain of the phytoplankton community structure of the sample (Wilcoxon signed rank test, $p > 0.05$). When the diagnostic pigment profile of *K. brevis* was not included in the initial ratio matrix, the chl *a* specific biomass was incorrectly attributed to diatoms, hapt3s and hapt4s (Table 2) based on commonalities in diagnostic pigment composition of these groups (Table 1A). The *K. brevis* bloom was not attributed to the dinoflagellate group because *K. brevis* lacks peridinin, a primary diagnostic pigment of most autotrophic dinoflagellates.

When running the algorithm, CHEMTAX was allowed to vary the initial pigment ratio matrix by as much as 500% to minimize the differences between the ratio matrix and the data set. Comparison of the initial ratio matrix and the final ratio matrix calculated by CHEMTAX (Table 1B) demonstrated that most pigment ratios remained the same or similar while others changed many fold. The greatest changes in the ratios were apparent in zeaxanthin of cyanobacteria, alloxanthin of cryptophytes, and lutein of chlorophytes (Table 1C). The pigment/chl *a* ratio increased two to almost fourfold for alloxanthin and zeaxanthin respectively whereas it decreased threefold for lutein.

Galveston Bay bloom. A *Karenia brevis* bloom was detected at the mouth of Galveston Bay in early September of 2000 during the bi-weekly sampling program carried out by the Estuarine Ecology Lab (TAMU). Two weeks later, the bloom was more dense and its intrusion was detected in the lower and center portion of the bay (Fig. 4). *K. brevis* cell abundance in mid-September ranged from 551 to 30,901 cells ml⁻¹ with the highest concentration observed in a sample taken from the surface in a patch of discolored water (Table 3). The high cell abundance was reflected in large peaks of gyroxanthin-diester and elevated phytoplankton biomass (chl *a*) detected by HPLC. The total phytoplankton biomass in the bloom ranged from 22-28 µg chl *a* l⁻¹ with an exceptionally high value of 426 µg chl *a* l⁻¹ in the sample of discolored water. In October the *K. brevis* bloom was not detected and the phytoplankton biomass in the lower bay ranged from 5-13 µg chl *a* l⁻¹, comparable with previously measured biomass in the area (data not shown).

CHEMTAX vs cell biovolume estimates. The phytoplankton community, determined by CHEMTAX, was dominated by diatoms (43%) and *K. brevis* (34%) on September 4 in Galveston Bay (Fig. 5). Two weeks later *K. brevis* (88%) was the dominant fraction of the biomass (chl *a*) and other algal groups formed a minor component (Fig. 5). Biovolumetric measurements of the same samples illustrated comparable trends in phytoplankton community structure. Diatoms (51%) and *K. brevis* (40%) accounted for the largest fraction of the biomass in early September (Fig. 5) but the community was dominated by *K. brevis* (83%) by mid-September (Fig. 5). The phytoplankton community composition estimates obtained from the two independent measures (CHEMTAX vs biovolume) were not significantly different (Wilcoxon signed ranks test, n=24, p>0.05).

Gyroxanthin-diester content of Texas vs. Florida blooms. Gyroxanthin-diester content per cell of *K. brevis* was significantly different among *K. brevis* from Galveston Bay and Florida (n=52, df=2, p<0.05). Post hoc analysis of variance (SPSS, Tamhane) showed that the gyroxanthin-diester content of *K. brevis* cells from Galveston Bay were significantly higher than gyroxanthin content of *K. brevis* detected from the West coast of Florida in 1994-95 (Millie et al. 1997) and 1999 (samples from Dr. Tester analysed by our lab) (Tamhane p<0.05) (Fig. 6). However, the gyroxanthin-diester content of the two populations of *K. brevis* from West Florida were not significantly different from one another (Tamhane, p>0.05).

DISCUSSION

This work demonstrates the applicability and advantages of HPLC and CHEMTAX approaches to assess phytoplankton community composition at ecosystem scales, and emphasizes the potential for screening large numbers of samples for presence of *K. brevis* or other phytoplankton species of unique pigment composition. Our HPLC protocol provides quantitative estimates of gyroxanthin-diester concentrations. This relationship is defined by a strong positive correlation between concentration of the detected pigment and the number of cells analyzed. In addition to the firm relationship established for the culture, our limited number of field observations also indicates the existence of a positive correlation between cell abundance and the gyroxanthin-diester concentration in natural samples (see Fig.6). Our findings are in agreement with the work of Millie et al. (1995,1997) and Kirkpatrick et al. (2000) demonstrating that gyroxanthin-diester concentrations are positively correlated with *K. brevis* cell abundance detected from the field. Because of the apparent linearity and the quantitative measure of *K. brevis* abundance, pigment based monitoring for *K. brevis* offers an additional tool for tracking spatio-temporal dynamics of the organism.

The absorption spectra obtained for gyroxanthin-diester (Fig. 1) is consistent with the chromatogram published by Millie et al. (1995, 1997). The minor shifts in the peak of maximum absorbance, 2 and 1 nm shift in wavelength respectively, probably reflects differences in the calibration of our photodiodearray used for the spectra rather than pigment associated properties. Therefore, we feel confident in applying the culture-obtained absorption spectra of gyroxanthin-diester as a reference for its identification in field samples.

Calculations of the potential limits of detection for *K. brevis* abundance reveals that the method outlined in this paper is capable of detecting natural cell abundances at densities less than 5 cells ml⁻¹, which is the critical value for oyster and other shellfish harvesting closures (National Shellfish Sanitation Program, US Food & Drug Administration). However, this level of sensitivity requires the filtration of nearly 1 liter of natural water using standard phytoplankton filtration and analysis protocols (see Materials and Methods). In estuarine waters, the filtration of >1 liter of water through the standard glass fiber filters used for phytoplankton analyses (Whatman GF/F) is impractical for most routine applications. Alternative filtration methods, which employ less-selective glass fiber filters or fine (ca. 10 µm mesh) Nitex mesh, may allow

the filtration of the large volumes of water necessary for detection of *K. brevis* in natural samples.

Karenia brevis cells from Galveston Bay contained higher concentrations of gyroxanthin-diester per cell than has been reported from samples from the West coast of Florida, on average the cells contained 3.09 (± 1.33) and 0.26 (± 0.10) picograms gyroxanthin-diester, respectively. The photopigment concentration of *K. brevis* has been observed to decrease with increasing irradiance (Millie et al. 1995). Furthermore, Millie et al. (1995) demonstrated that the relative proportion of chlorophylls remained constant. However, the relative contribution of fucoxanthin, gyroxanthin-diester and β -carotene to the carotenoid pool decreased with increased irradiance at the same time as diadinoxanthin contribution to the carotenoid pool increased. Therefore, the higher concentration of gyroxanthin-diester per cell in Texas *K. brevis* bloom may be a photoacclimation response to the more turbid estuarine waters of Galveston Bay, but further research is necessary.

In nature, *K. brevis* will be exposed to widely varying irradiance levels. Accumulations at the surface will expose them to full strength sunlight. This organism may have mechanisms for photoprotection but this has not been studied in detail. Vertical migrations will expose *K. brevis* to light of widely varying spectral quality and quantity. Millie et al. (1995) note that blooms of *K. brevis* vary in coloration from red to brown to yellow, and that differences in color are likely due to cell pigments in response to varying irradiance. The role of gyroxanthin-diester in photoacclimation has not been examined in detail. Because cell enumerations are dependent on the mathematical relationship between gyroxanthin concentrations and cell abundance, photoacclimation responses to different light environments needs to be more carefully explored. However, Millie et al. (1997), working with *K. brevis* cultures incubated under a range of light and nutrient conditions, found that gyroxanthin-diester concentrations are consistent, generally comprising 3 - 10% of the total carotenoid pigment pool in *K. brevis* (Millie et al. 1997). Therefore, the relationship between gyroxanthin concentration and *K. brevis* cell abundance should be determined for specific habitat types (i.e., estuary, shelf, deep-water, etc.).

Pigment detection is to some extent instrument dependent, but here we present our own analyses of the pigment composition of *K. brevis*, from Florida as well as from Galveston Bay. Our estimates of the gyroxanthin-diester content of *K. brevis* obtained from Florida waters (P. Tester, NOAA) are similar to the values reported for *K. brevis* of the West coast of Florida

(Millie et al. 1997). Furthermore, our estimates of the gyroxanthin-diester content of *K. brevis* from West Florida and Galveston Bay were significantly different from one another. An instrument or methodological explanation of the observed regional difference is unlikely, thus supporting regional differences in photoacclimation response and/or possibly strain differences between populations of *K. brevis* in Texas and Florida waters.

The phytoplankton community composition obtained from CHEMTAX was very similar to the biovolume estimates of phytoplankton community structure. The initial ratio matrix constructed from the pigment composition of *K. brevis*, literature values (Wright and van den Enden 2000) and insight into Galveston Bay phytoplankton community composition is consistent with direct microscopic examinations of water samples. However, previous comparisons of CHEMTAX and other measures of phytoplankton community structure show mixed results (Schlüter et al. 2000; Wright and van den Enden 2000). Wright and van den Enden (2000) found that in Antarctic waters the cell abundance of large diatoms was positively correlated with chl *a* specific biomass of diatoms as determined by CHEMTAX, whereas no correlation was apparent between the two parameters for other phytoplankton groups. Similarly, Schlüter et al. (2000) reported that the group specific chl *a* biomass estimate from CHEMTAX and carbon biomass estimates showed consistent results for coastal environmental samples dominated by diatoms and dinoflagellates whereas in mixed phytoplankton samples from the same region a correlation was only apparent for diatoms and dinoflagellates. The lack of agreement between the two methods was attributed to the inherent difficulty in enumerating and identifying small phytoplankton (nanoflagellates, pico-cyanobacteria etc.) by microscopy. In our case the phytoplankton community was dominated by diatoms and dinoflagellates (*K. brevis*) therefore the inherent challenges of microscopic identification of the phytoplankton community were minimal.

Mackey et al. (1996) recommend that the best CHEMTAX results are obtained when the initial ratio matrix incorporates phytoplankton group specific pigment ratios characterized from the ecosystem of concern. In the present study, the pigment composition of *K. brevis* was obtained from a culture and incorporated into the initial ratio matrix of CHEMTAX to facilitate a realistic measure of the phytoplankton community composition in Galveston Bay during a red-tide bloom. Incorporating gyroxanthin-diester into the ratio matrix was essential for a true representation of the phytoplankton community in the presence of *K. brevis*. Without the addition of gyroxanthin-diester to the matrix, the total chl *a* in the sample is partitioned among

the other groups, and results in an overestimation of the biomass contribution of individual groups. In our analysis, the exclusion of gyroxanthin-diester resulted in a large over-estimation of diatoms and haptophyte abundance because the chl *a* associated with *K. brevis* was not accounted for in the CHEMTAX analysis. This highlights an important potential weakness of the CHEMTAX approach. All phytoplankton in the sample must be represented by at least one unique pigment in addition to chl *a*. *Karenia brevis* presents a good example in which most of the accessory pigments are shared with diatoms and haptophytes. The inclusion of gyroxanthin-diester in the ratio matrix allows CHEMTAX to correctly partition the total chl *a* into the major taxa in the sample. Phytoplankton studies conducted in areas where *K. brevis* blooms are known to occur should include gyroxanthin-diester in all CHEMTAX analysis.

These results support the statement that ultimately the results of CHEMTAX are a product of the scientist's intuition (Wright and van den Enden 2000). In the present study, the biovolume and CHEMTAX estimates of the community structure were consistent in resolving the phytoplankton assemblage, thus confirming that HPLC and CHEMTAX approaches provide a useful tool for resolving spatiotemporal distribution of phytoplankton in the presence of *K. brevis* blooms.

The combined HPLC - CHEMTAX approach for quantifying the abundance of *K. brevis* in field samples uses analytical techniques that are rapid, automated, reliable, and inexpensive. The low cost and short analysis time of HPLC-based photopigment analyses makes it well suited for monitoring programs designed to assess microalgal community composition and biomass. The spatial distributions of algal groups, including near-background concentrations of *K. brevis*, can be mapped over large geographical areas (Pinckney et al. 1998). This approach offers an early warning system for potential blooms of nuisance or toxic species (HABs) and overall water quality conditions. In addition, the protocols established in this paper can be easily applied to other areas in the Gulf of Mexico where *K. brevis* monitoring is a high priority.

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Table 1. Pigment ratio matrices for CHEMTAX. A - The initial ratio matrix used for CHEMTAX. B - The final output pigment ratio matrix results from the best fit of CHEMTAX to Galveston Bay photopigment data set. Pigment ratios are normalized to a Chl α value of 1. C - Comparison of the changes in the final ratio matrix from the initial matrix. A value of 1 indicates no changes whereas values larger and smaller than 1 are indicative of increase and decrease of the pigment ratio in the final matrix, respectively.

A

Algal class	Perid	But-fuco	Fuco	Hex-fuco	Diadino	Allo	Lut	Zea	Chl <i>b</i>	Gyro	Chl α
<i>K. brevis</i>	-	0.0996	0.2905	0.1086	0.1856	-	-	-	-	0.1364	1.00
Diatoms	-	-	0.7554	-	0.1400	-	-	-	-	-	1.00
Cyanobacteria	-	-	-	-	-	-	-	0.3820	-	-	1.00
Chlorophytes	-	-	-	-	-	-	0.2030	0.0090	0.2632	-	1.00
Dimoflagellates	1.0630	-	-	-	-	-	-	-	-	-	1.00
Hapto3s	-	-	-	1.7000	0.1400	-	-	-	-	-	1.00
Hapto4s	-	0.2500	0.5800	0.5400	0.1200	-	-	-	-	-	1.00
Cryptophytes	-	-	-	-	-	0.2292	-	-	-	-	1.00

B

Algal class	Perid	But-fuco	Fuco	Hex-fuco	Diadino	Allo	Lut	Zea	Chl <i>b</i>	Gyro
<i>K. brevis</i>	-	0.1303	0.2905	0.1087	0.1855	-	-	-	-	0.1365
Diatoms	-	-	0.5406	-	0.1955	-	-	-	-	-
Cyanobacteria	-	-	-	-	-	-	-	1.4319	-	-
Chlorophytes	-	-	-	-	-	-	0.0625	0.0090	0.2895	-
Dinoflagellates	1.0631	-	-	-	-	-	-	-	-	-
Hapto3s	-	-	-	1.7001	0.1400	-	-	-	-	-
Hapto4s	-	0.2500	0.5799	0.5401	0.1200	-	-	-	-	-
Cryptophytes	-	-	-	-	-	0.5366	-	-	-	-

C

Algal class	Perid	But-fuco	Fuco	Hex-fuco	Diadino	Allo	Lut	Zea	Chl <i>b</i>	Gyro
<i>K. brevis</i>	-	1.31	1.00	1.00	1.00	-	-	-	-	1.00
Diatoms	-	-	0.72	-	1.40	-	-	-	-	-
Cyanobacteria	-	-	-	-	-	-	-	3.75	-	-
Chlorophytes	-	-	-	-	-	-	0.31	1.00	1.10	-
Dinoflagellates	1.00	-	-	-	-	-	-	-	-	-
Hapto3s	-	-	-	1.00	1.00	-	-	-	-	-
Hapto4s	-	1.00	1.00	1.00	1.00	-	-	-	-	-
Cryptophytes	-	-	-	-	-	2.34	-	-	-	-

Table 2. Phytoplankton biomass (Chl *a* $\mu\text{g l}^{-1}$) at station 6 in Galveston Bay before, during, and after the *Karenia brevis* bloom.

Phytoplankton community composition and abundance was estimated using two CHEMTAX analyses, which included and excluded the diagnostic pigment composition of *K. brevis* in the initial ratio matrix. Bold letters mark the groups showing major shifts in biomass between CHEMTAX analysis including and excluding the pigment composition of *K. brevis*.

Phytoplankton group	Biomass (Chl <i>a</i> $\mu\text{g l}^{-1}$) from CHEMTAX							
	Including <i>K. brevis</i>				Excluding <i>K. brevis</i>			
	18-Aug	4-Sep	18-Sep	14-Oct	18-Aug	4-Sep	18-Sep	14-Oct
<i>K. brevis</i>	0.03	5.17	25.11	0.00	0.00	0.00	0.00	0.00
Chlorophytes	0.90	1.22	0.62	0.69	0.90	1.46	1.35	0.70
Cryptophytes	0.47	0.91	0.08	1.01	0.35	0.86	0.70	0.76
Cyanobacteria	0.19	0.32	0.00	0.05	0.16	0.36	0.20	0.05
Dinoflagellates	0.08	0.13	0.23	0.14	0.08	0.15	0.37	0.14
Hapto3+4	0.04	0.18	1.06	0.03	0.06	1.81	16.26	0.03
Diatoms	2.49	4.98	1.21	2.35	2.67	8.29	9.42	2.60

Table 3. *Karenia brevis* cell abundance and photopigment concentrations observed in Galveston Bay, Texas on 18 August 2000 during a *K. brevis* bloom. Numbers of sampling sites refer to locations in Fig. 4.

Parameter measured	Sampling locations			
	ST 4	ST 5	ST 6	Bloom
Cells $\times 10^5 \text{ l}^{-1}$	6.29	5.51	13.75	309.01
Gyroxanthin ($\mu\text{g l}^{-1}$)	2.25	2.31	3.38	55.66
<i>K. brevis</i> biomass (Chl a $\mu\text{g l}^{-1}$)	17.2	19.9	25.1	375.0
Total biomass (Chl a $\mu\text{g l}^{-1}$)	23.7	21.6	28.3	425.7

FIGURE LEGENDS:

Fig. 1. HPLC chromatogram for the *Karenia brevis* culture. The retention time of Gyroxanthin-diester allows for good spatial separation and quantification. The pigments detected in *K. brevis* are; chlorophyll c_3 (Chl c_3), chlorophyll c_1c_2 (Chl c_1c_2), butanoyloxyfucoxanthin (But-fuco), fucoxanthin (Fuco) hexanoyloxyfucoxanthin (Hex-fuco), diadinoxanthin (Diadino), gyroxanthin-diester (Gyro), chlorophyll a (Chl a) and $\beta\psi$ -carotenoid ($\beta\psi$ -Car). Inserted in the graph is the absorption spectra of gyroxanthin-diester.

Fig. 2. Gyroxanthin-diester concentration ($\mu\text{g sample}^{-1}$) vs. *Karenia brevis* cell abundance (cells sample^{-1}). Each data point represents a subsample of *K. brevis* culture of known cell concentration ($7227 \text{ cells ml}^{-1}$) that was concentrated on a filter from seven volumetric measurements (1, 2, 3, 5, 7 10 and 15 ml), spanning the range of 7227 to 108,405 cells. The bars indicate ± 1 standard deviation (0.1-0.3 ng) and are based on the results of six replicates of each volumetric filtration (five replicates for the 15ml samples).

Fig. 3. Estimated detection limits for *Karenia brevis* in natural phytoplankton samples. This graph illustrates the volume of water that would need to be filtered to detect a given concentration of *K. brevis* using the methods outlined in the text. The dashed line shows that ca. 800 ml of water would have to be filtered to reliably detect a *K. brevis* abundance of 5,000 cells per liter in the sample water using the HPLC-based approach.

Fig. 4. Sampling locations within Galveston Bay, Texas. Sampling locations are characterized by filled squares and circles. *Karenia brevis* was detected at locations identified by filled circles (4, 5 and 6, and from a dense bloom (B)).

Fig. 5. Biovolume and CHEMTAX estimates of phytoplankton community composition (percent) on 4th and 18th of September 2000 at station 6 (see Fig. 4). Gray columns represent biovolume estimates whereas filled columns represent CHEMTAX results.

Phytoplankton groups present are: *Karenia brevis* (*K. brevis*), diatoms (Diato), chlorophytes (Chloro), cryptophytes (Crypto), cyanobacteria (Cyano), dinoflagellates (Dino) and haptophytes (Hapto).

Fig.6. Gyroxanthin-diester concentration of field samples of *Karenia brevis* from Texas and Florida (both scales are logarithmic). Diamonds represent samples taken from Galveston Bay, Texas September 2000. Stars represent samples taken off the West Coast of Florida near the panhandle in 1999. Filled circles represent samples taken in 1994 and 1995 of the West Coast of Florida and published by Millie et al. (1997). The environmental samples form two clusters, high and low gyroxanthin-diester content per cell, defined by the origin of the samples, Texas and Florida, respectively.

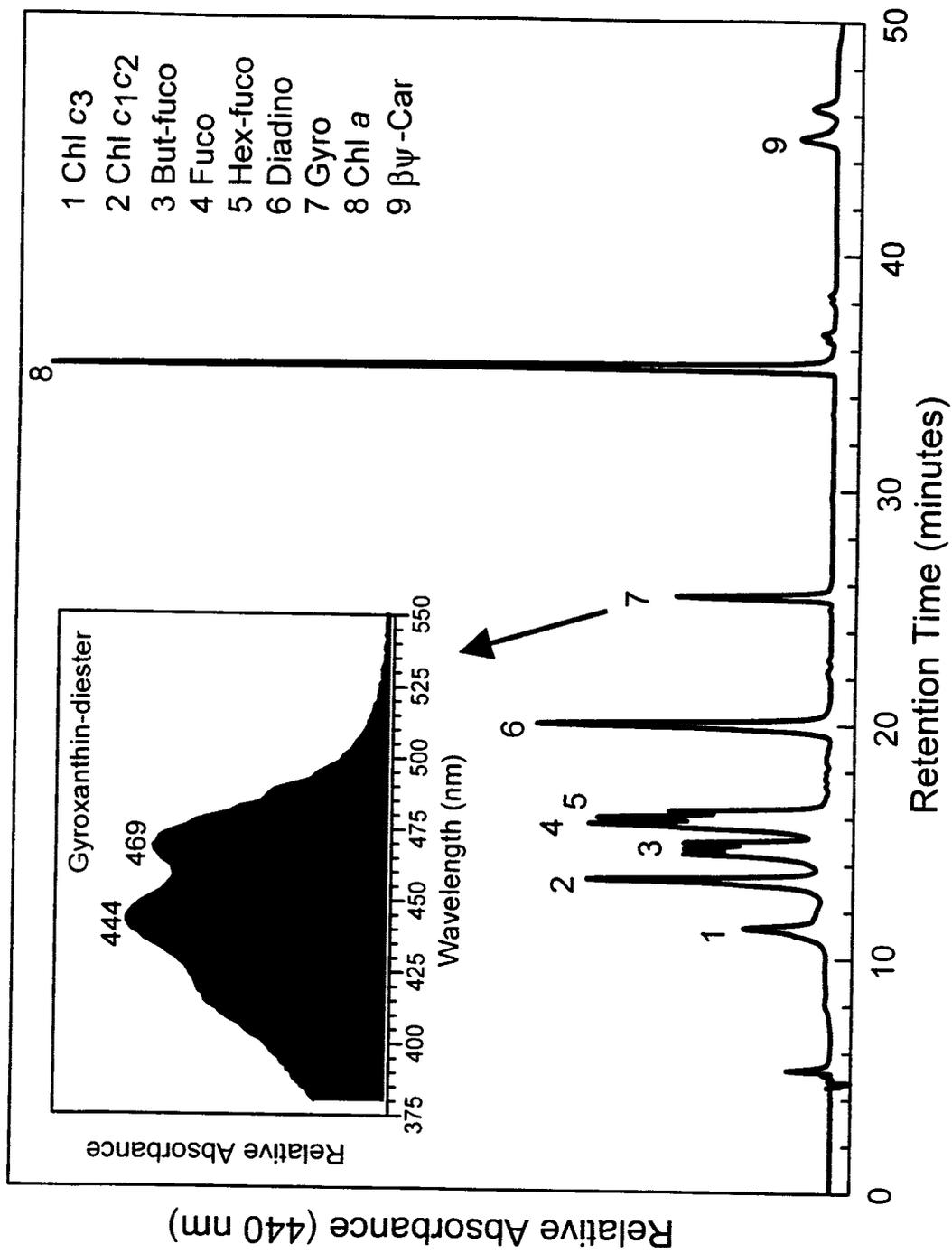


Figure 1

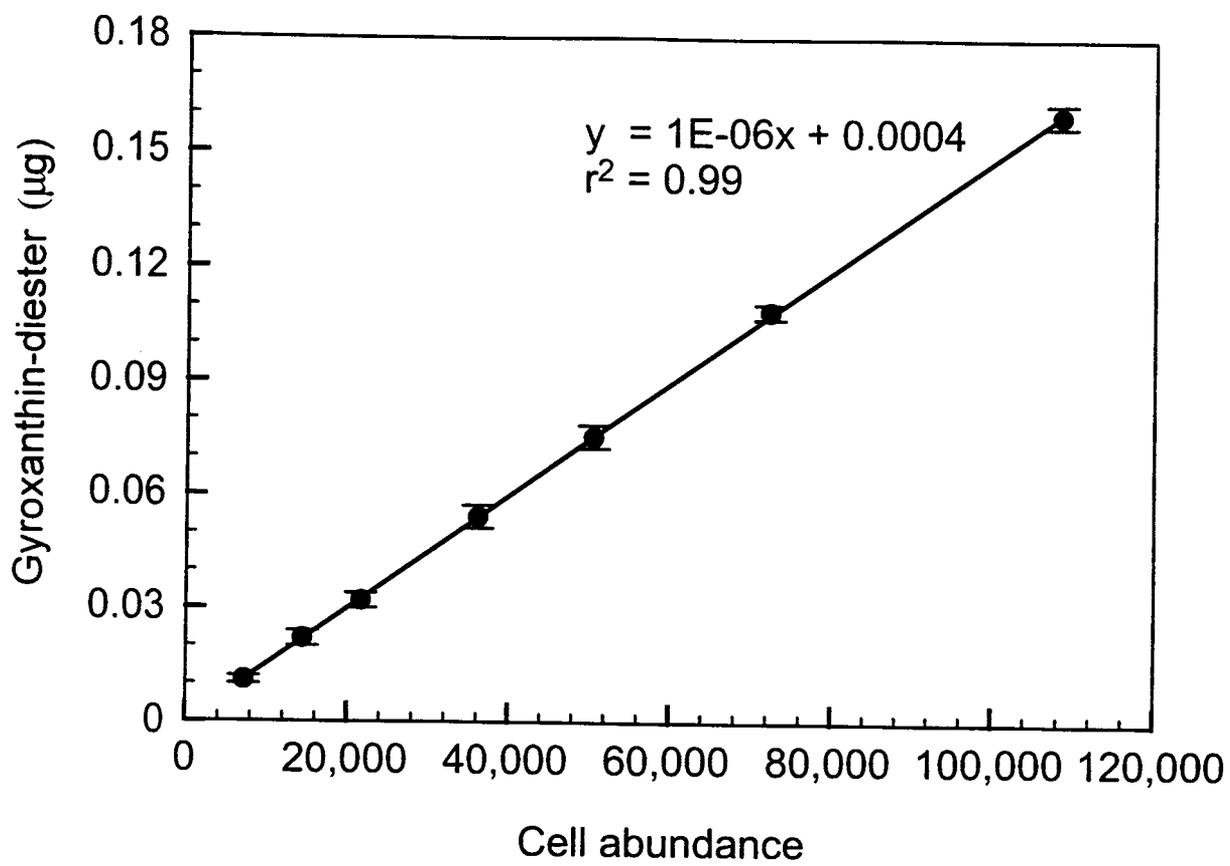


Figure 2

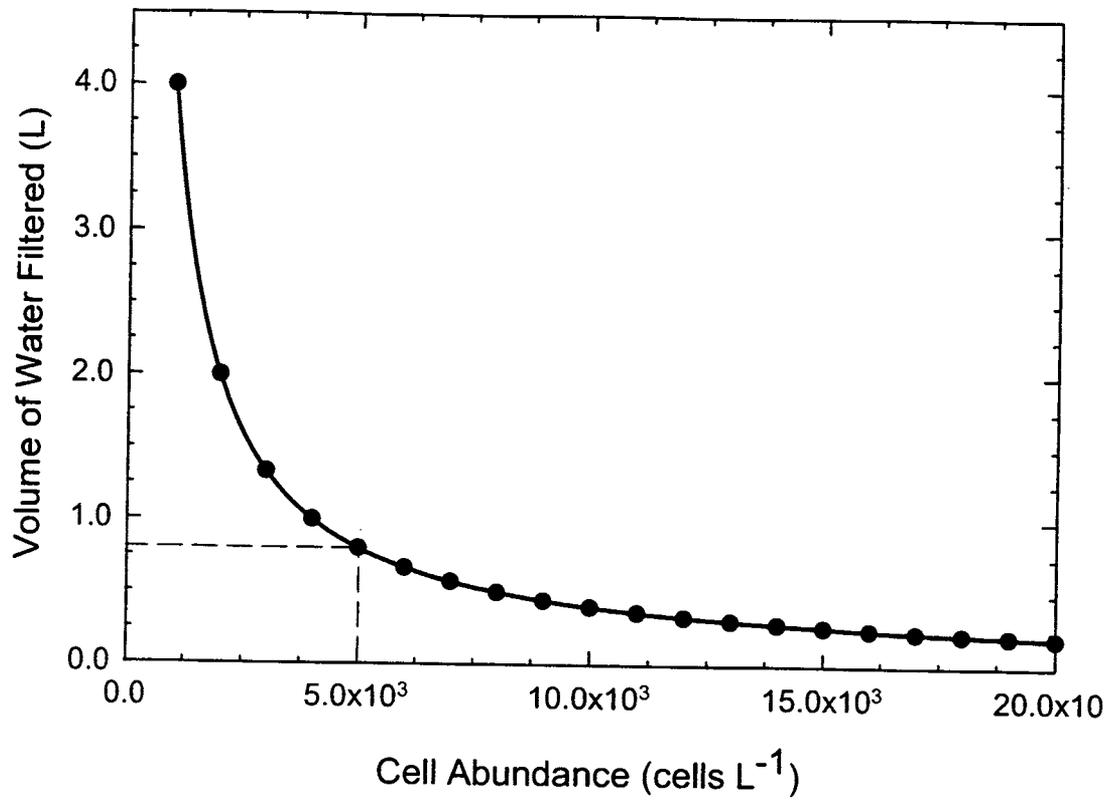


Figure 3



Figure 4

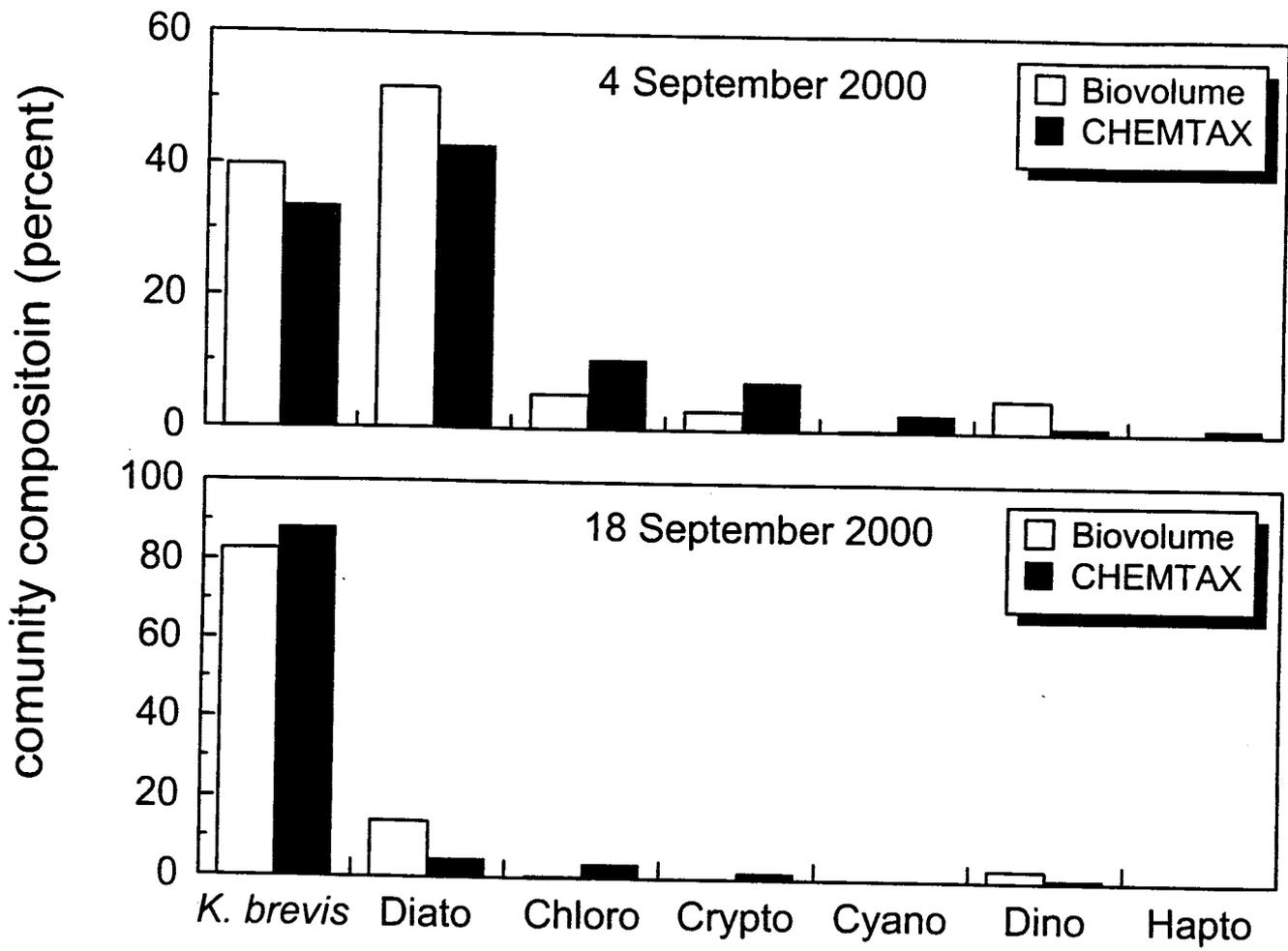


Figure 5

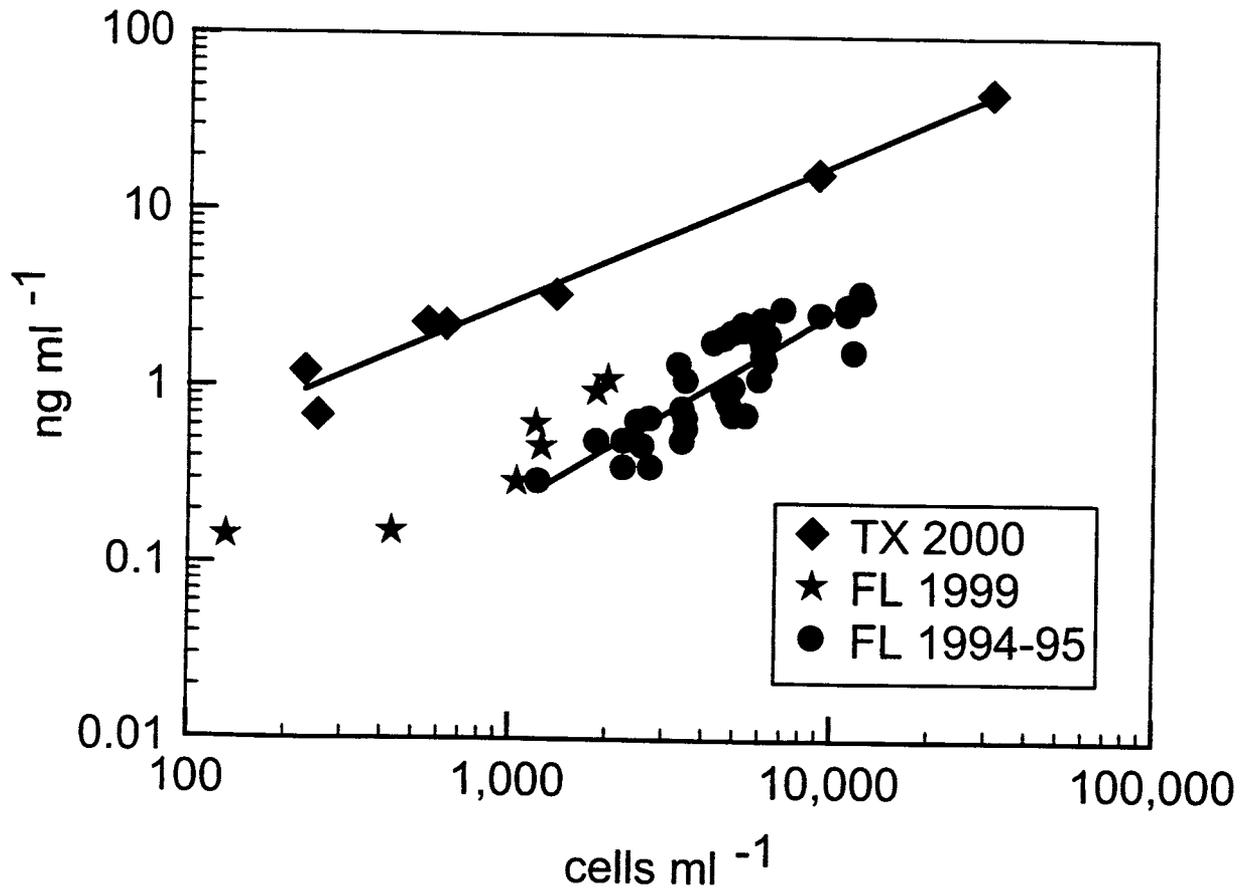


Figure 6