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Detection of *Karenia mikimotoi* by spectral absorption signatures

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This study investigated the performance of a spectral similarity index and a multivariate partial least-squares regression technique for detecting the presence of the gyroxanthin-diester-carrying toxic dinoflagellate Karenia mikimotoi from measurements of spectral light absorption. The methods were applied to fourth-derivative absorption spectra of K. mikimotoi mixed with the toxic dinoflagellate Prorocentrum minimum, which does not contain gyroxanthin-diester. Acclimating the cultures to different light and nutrient conditions allowed us to evaluate the sensitivity of the methods to changes in pigmentation and intracellular light absorption (pigment packaging) of the algae. Both methods were able to determine the fraction of K. mikimotoi and the gyroxanthin-diester concentration. However, whereas the partial least-squares predictions were almost insensitive to the induced variability in optical properties of the algae, predictions based on the similarity index differed significantly depending on the acclimation of the algae. Furthermore, discriminating K. mikimotoi from natural phytoplankton assemblages indicated a significant influence of cell-size composition, through pigment packaging, on the accuracy of the similarity index. Our results suggest that optical discrimination of phytoplankton species from spectral absorption signatures will improve significantly by applying the partial least-squares regression technique.

INTRODUCTION

Frequent blooms of toxic algae in shallow coastal waters with deleterious effects on fish, benthic fauna and human health, stress the need for effective, quick and inexpensive means to monitor the development of such events (GEOHAB, 2003). Harmful algal blooms are often dominated by single species and can reach extremely high biomass for an extended period of time. Given that the algal species may have distinct bio-optical signatures, such blooms have the potential to be detected and monitored by a multi-platform optical approach, using remote sensing and *in situ*-moored technologies (Schofield *et al.*, 1999). In view of this context, Millie *et al.* introduced an interesting method by which the red tide dinoflagellate *Karenia brevis* (formerly *Gymnodinium brevis*) was detected and quantified on the basis of a spectral similarity index (SI) calculated from measurements of spectral light absorption (Millie *et al.*, 1997). The method required the collection of algae on filters, but a subsequent application yielded good results in an analysis of absorption measured using a liquid waveguide capillary cell (Kirkpatrick *et al.*, 2000). It is thus conceivable that spectral discrimination could be used in autonomous systems for detection of harmful algae. However, the promising results of preliminary studies require further examination. For example, absorption signatures vary significantly as a result of changes in environmental growth conditions (Bricaud *et al.*, 1988; Stæhr *et al.*, 2002) and previous light and nutrient history should therefore be assessed in the evaluation of optically based methods for monitoring phytoplankton blooms. The impact of nutrient depletion on the optical signatures of *K. brevis* was however not considered in the work by Millie *et al.* (Millie *et al.*, 1997). Since we know that differences in growth conditions affect both intracellular pigment concentration and pigment composition, our attempts to discriminate target species using absorption spectra should be able to take account of such variations. Acknowledging that the spectral SI only considers the shape of the absorption spectra, it seems apparent that

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differences in growth conditions will introduce uncertainty and error to the prediction of relative cell abundance from the SI. Also, it remains uncertain whether the SI can be applied to other bloom-forming phytoplankton species containing the gyroxanthin-diester marker pigment.

The objectives of this study were to examine the robustness of the spectral SI suggested by Millie *et al.* (Millie *et al.*, 1997) to differentiate between gyroxanthin-diester-containing species and other bloom-forming species from their spectral light absorption signatures. For this purpose we performed experiments with a gyroxanthin-diester-carrying dinoflagellate, *Karenia mikimotoi* (formerly *Gymnodinium mikimotoi*), and *Prorocentrum minimum*, a dinoflagellate without gyroxanthin-diester. The species have a rather similar pigment composition (Millie *et al.*, 1997); they are both bloom-forming, potentially toxic and may co-occur in coastal waters (Bjergskov *et al.*, 1990; Ærtebjerg *et al.*, 1998). *Karenia mikimotoi* was reported for the first time in European waters in the Oslofjord (Norway) in 1966 (Braarud and Heimdal, 1970) and has since formed large blooms ($>10^3$–10^6 cells l^{-1}) and caused serious mortality of fish and benthos in Danish waters in 1968, 1981, 1985, 1988, 1990 and 1997 (Bjergskov *et al.*, 1990; Ærtebjerg *et al.*, 1998). *Prorocentrum minimum* has also been observed frequently in Scandinavian coastal waters and the North Sea (Bjergskov *et al.*, 1990). In Danish coastal waters high concentrations of *P. minimum* ($>10^3$–10^7 cells l^{-1}) have been observed almost every year since the middle of the 1970s (Bjergskov *et al.*, 1990; Ærtebjerg *et al.*, 1998).

The ability of the spectral SI to differentiate between gyroxanthin-diester-containing species and other bloom-forming species was further compared with a multivariate regression technique. Multivariate regression has proven a strong tool in determining pigment composition and concentration from both spectral *in vitro* data (Moberg *et al.*, 2000) and spectral *in vivo* absorption data (Stæhr, 2003).

METHOD

Phytoplankton cultures and growth conditions

Two toxic dinoflagellates, *K. mikimotoi* (Miyake & Kominami ex Oda) G. Hansen & Moestrup (K-0260), transferred from *G. mikimotoi* by Daugbjerg *et al.* (Daugbjerg *et al.*, 2000) and *P. minimum* (Pavillard) Schiller (K-0336), were used in this study. The species were isolated from Scandinavian waters and obtained from the Scandinavian Culture Centre for Algae & Protozoa, University of Copenhagen. The algae were grown in L1-enriched, 0.45-μm-filtered Bedford Basin sea water (Guillard and Hargraves, 1993) growth medium at 18–20°C for several weeks prior to the experiment. Cool-white fluorescent lamps illuminated the culture flasks with 300 μmol quanta m^{-2} s^{-1} of photosynthetically active radiation (PAR) in a 14 h light:10 h dark cycle (lights on at 08:00 h). Duplicate cultures were grown under potentially limiting and non-limiting levels of dissolved inorganic nitrogen and under shade- and light-adapted conditions, thereby resulting in $2 \times 2 \times 2 = 8$ flasks (see Table I). Aseptic techniques were used in the maintenance and growth of all cultures. Semi-continuous culture techniques (Ukeles, 1973) kept cultures in exponential growth for >3 weeks. As described below, growth rates were low for both low-nitrogen and nitrate-replete cultures of *K. mikimotoi*. Consequently, the low-nitrogen cultures did not experience significant nitrogen stress during the 25 days of growth. Dilution did, however, maintain all cultures in exponential phase growth and concentrations of chlorophyll never exceeded 100 mg m^{-3} for any of the cultures, so neither carbon limitation nor significant shading of the cultures was encountered.

Growth rate and biomass

Samples were taken on a daily basis between 9:00 and 10:00 h (1–2 h after lights went on) to measure cell density and chlorophyll *a* (Chl *a*) and thus determine changes in growth rate and cellular Chl *a* content.

Table I: Experimental design with notations of nitrate (N) and irradiance (E) combinations (n = 2)

NO_3^-	Irradiance	Notation of N and E conditions
850	400	N^+ E^+
850	140	N^+ E^-
50	400	N^- E^+
50	140	N^- E^-

Units of measurement were as follows: NO_3^-, μmol l^{-1}; irradiance, μmol photons m^{-2} s^{-1}.

Growth rate (μ; day^{-1}) was calculated according to equation (1).

$$\mu = (\ln B_{T1} - \ln B_{T0})/\mathcal{T} \quad (1)$$

where B_{T1} represents the cell concentration (cells ml^{-1}) on the day of sampling, B_{T0} is the cell concentration at the previous sampling, separated by $\mathcal{T}$ days. Cell counts were measured on 20 ml aliquots using a Coulter counter. The Coulter counter was also used to determine cell size by the end of each growth experiment.

Concentrations of Chl *a* were determined fluorometrically in duplicate from samples concentrated on Poretics GF75 filters and then extracted in the dark for at least 24 h in 10 ml of ice-cold 90% acetone (Holm-Hansen *et al.*, 1965). Samples were allowed to stand in the dark for 30 min before fluorescence measurements were carried out on a Turner Designs 10-005-R fluorometer. Ten per cent HCl was then added and measurements were repeated to determine pheophytin (Holm-Hansen *et al.*, 1965). The carbon biomass of phytoplankton monocultures was determined at the end of the experiment as particulate organic carbon (POC) by filtering 30–100 ml aliquots onto precombusted Advantec GF 75 glass-fibre filters and analysed on a Perkin-Elmer 240C elemental analyser (Perkin-Elmer, Norwalk, CT, USA).

Bio-optical analyses

Spectral absorption (300–800 nm recorded with 0.5 nm resolution; slit width = 2 nm) of particulate material was determined using the quantitative filtering technique described previously (Kishino *et al.*, 1985) on a Shimadzu UV-2401PC UV-Vis recording spectrophotometer equipped with an integrating sphere. Samples were preserved in liquid nitrogen until analysis within 2 months. Optical density spectra were smoothed using a 10 nm running average. Smoothing did not, however, affect the ability to discriminate between phytoplankton species from spectral absorption spectra, as no significant differences were found on the calculated spectral SI or the partial least-squares (PLS) regression technique when smoothing over a 2, 5, 10 or 15 nm interval. Optical densities were corrected for differential scattering by subtracting the average optical density measured between 750 and 800 nm. The corrected optical densities were converted into particulate absorption coefficients (a_p) using the β-correction factor given elsewhere (Bricaud and Stramski, 1990):

$$a_p(\lambda) = 2.3 \times OD(\lambda)/V \times [1.63 \times OD(\lambda)^{-0.22}] \quad (2)$$

where V is the filter volume, AF is the effective filter area, OD is the optical density of the particulate material and λ is the measured wavelength. The importance of the β-correction was assessed by applying the β-correction method described by Arbones *et al.* (Arbones *et al.*, 1996). This, however, had no significant effect on the predictive abilities of the SI or the PLS method.

Absorption by phytoplankton pigments (a_{ph}) was determined as:

$$a_{ph}(\lambda) = a_p(\lambda) - a_{det}(\lambda) \quad (3)$$

where $a_{det}(\lambda)$—the spectral absorption by detritus and other non-photosynthetic particulate matter—was corrected for by measuring the absorption before and after methanol extraction according to a procedure described previously (Kishino *et al.*, 1985). Chl *a*-specific absorption, $a^*_{ph}(\lambda)$ (m^2 mg^{-1} Chl *a*), was calculated by normalizing $a_{ph}(\lambda)$ by the fluorometrically determined Chl *a* + pheophytin *a* concentration. The spectral mean Chl *a*-specific absorption coefficient ($\hat{a}^*_{ph}$) over the 400–700 nm region was finally calculated. This measure has previously proved to be more valuable than absorption at a given wavelength to characterize the overall phytoplankton absorption (Markager and Vincent, 2001; Stæhr *et al.*, 2002).

HPLC analysis provided estimates of the concentration of chlorophylls, carotenoids and other accessory pigments for *K. mikimotoi* samples at the end of the experiment. Samples were gently filtered onto 25 mm Advantec GF 75 glass-fibre filters and these were immediately stored in liquid nitrogen until analysis within 2 months. Filters were subsequently transferred to 2.5 ml of methanol, sonicated on ice for 30 s, and filtered (0.2 μm). One millilitre of filtrate was then transferred into HPLC vials containing 250 ml water. HPLC analyses were performed on a Shimadzu LC 10A system with a Supercosil C18 column (250 × 4.6 mm, 5 μm) using a slight modification of the Wright *et al.* method as described in Schlüter and Havskum (Wright *et al.*, 1991; Schlüter and Havskum, 1997). This method does not provide full baseline separation of lutein and zeaxanthin, and therefore we separated these pigments manually based on their absorption spectra recorded by a photodiode array detector. Pigments were identified and quantified by comparing their retention times and absorption spectra to those of authentic standards, purchased from the International Agency for ^{14}C Determination, Hørsholm, Denmark.

Mixing of phytoplankton species

To evaluate the ability of the spectral SI proposed by Millie *et al.* (Millie *et al.*, 1997) to differentiate the absorption spectra of *K. mikimotoi* from the spectra of *P. minimum*, mixed assemblages were established by physically mixing the species in known proportions and also by making

hypothetical combinations. Prior to the physical mixing, cultures were kept in exponential growth for 3 weeks at ~300 μmol photons m^{-2} s^{-1} in nutrient-replete medium. Cultures were mixed to obtain proportions of *K. mikimotoi* of 0, 20, 40, 60, 80 and 100%. Samples were retrieved and spectral absorption and pigment concentration were measured. To investigate the effect of optical acclimation on the predictive ability of the SI we also made hypothetical combinations of absorption spectra of *K. mikimotoi* and *P. minimum*, obtained in the acclimation experiment. The hypothetical combinations were calculated according to the descriptions of Millie *et al.* (Millie *et al.*, 1997). As with the physical mixing experiment, the hypothetical combinations were generated so that the contribution of *K. mikimotoi* ranged from 0 to 100% in increments of 20%.

Statistical analyses

To determine whether the different growth conditions (irradiance and nitrogen availability) significantly affected the optical properties of the algae, we applied a two-way ANOVA on the effect of two irradiance levels (400 and 150 μmol photons m^2 s^{-1}) and two nitrogen levels in the culture medium (850 and 50 μmol l^{-1}) on cellular Chl *a* content (pg Chl *a* $cell^{-1}$) and the mean Chl *a*-specific absorption coefficient ($\hat{a}^*_{ph}$) on samples collected on the last day of the experiment. Furthermore, this test enabled us to determine possible interactions between the different irradiance and nitrogen levels on cellular Chl *a* content and $\hat{a}^*_{ph}$.

The SI was computed from fourth-derivative spectra (Butler and Hopkins, 1970) calculated from phytoplankton absorption spectra. SI was computed for each of the four growth conditions and the physical mixing experiment. For each of these five data sets, SI was determined by computing the angle between the vectors comprising the fourth-derivative spectra of the sample with 100% *K. mikimotoi* (= standard) and the unknown mixed sample as:

$$SI = 1 - \left(\frac{2 \times \arccos\left(\frac{A_{std} \bullet A_{unk}}{|A_{std}| \times |A_{unk}|} \right)}{\pi} \right) \qquad (4)$$

where A_{std} and A_{unk} are the fourth derivative of absorption spectra (a_{ph}) of the standard and unknown samples respectively (Millie *et al.*, 1997; Kirkpatrick *et al.*, 2000). The arc-cosine transformation and division by $\pi/2$ were chosen in agreement with the previous studies (Millie *et al.*, 1997; Kirkpatrick *et al.*, 2000). The transformation converts the non-linear relationship between SI and % biomass of the gyroxanthin-diester-holding species into a linear relationship between zero and one.

As an alternative to the spectral SI proposed by Millie *et al.* (Millie *et al.*, 1997), a multivariate regression technique was applied to predict the fraction of *K. mikimotoi* as well as the concentration of gyroxanthin-diester from spectral absorption data. The multivariate analysis consisted of a PLS regression model which can be used for predicting one or several dependent variables on the basis of several independent variables (Martens and Næs, 1989; Esbensen *et al.*, 1994). The model was computed from the fourth-derivative spectra (400–700 nm by 0.5 nm) of phytoplankton absorption spectra (smoothed using a 10 nm running average), used as independent *X*-variables, and the fraction of *K. mikimotoi* and absolute concentration of gyroxanthin-diester as dependent *Y*-variables. PLS is a multivariate calibration method for relating two data matrices, *X* and *Y*, by regression. *X* is a matrix of predictor variables and *Y* is a matrix of response variables, both with one variable in each column and one observation in each row. The multivariate model for *X* and *Y* is simply a regression relationship between linear combinations $t = Xw$ of *X* and $u = Yc$ of *Y* where *w* and *c* are eigenvectors (with $w'w = c'c = 1$) which maximize the covariance between *u* and *t*. PLS allows simultaneous determination of several *Y*-variables and has the advantage over other regression techniques that it uses the *Y*-data structure as a guiding hand in the decomposition of the *X*-matrix, which optimizes the regression outcome. In addition, PLS is well suited for modelling absorption spectra since it can handle co-varying *Y*-variables and co-linearity of *X*-variables. Each PLS model was established using the full cross-validation technique, which develops the PLS models on parts of the data and tests the models on other parts (Esbensen *et al.*, 1994). A more thorough description of PLS is available elsewhere (Martens and Næs, 1989; Esbensen *et al.*, 1994).

For the prediction of the fraction (%) of the samples consisting of *K. mikimotoi* by SI and PLS, absorption spectra were normalized to the mean spectral value prior to calculation of the fourth derivative (Figure 1). Prediction of absolute concentrations of gyroxanthin-diester by the SI and the PLS methods, was performed on non-normalized absorption spectra. Processing of absorption spectra to the fourth-derivative spectra was performed in the SAS/STAT software package (SAS Institute Inc., 1994), while multivariate regression (PLS) was carried out using the Unscrambler software, Version 6.11, CAMO ASA.

RESULTS

Acclimation to irradiance and nitrogen limitation

The low-nitrogen treatments had little differential effect on *K. mikimotoi* because of low growth rates (< 0.2 day^{-1}) even at high irradiances and dilutions sufficient to maintain adequate concentrations of nitrate in the

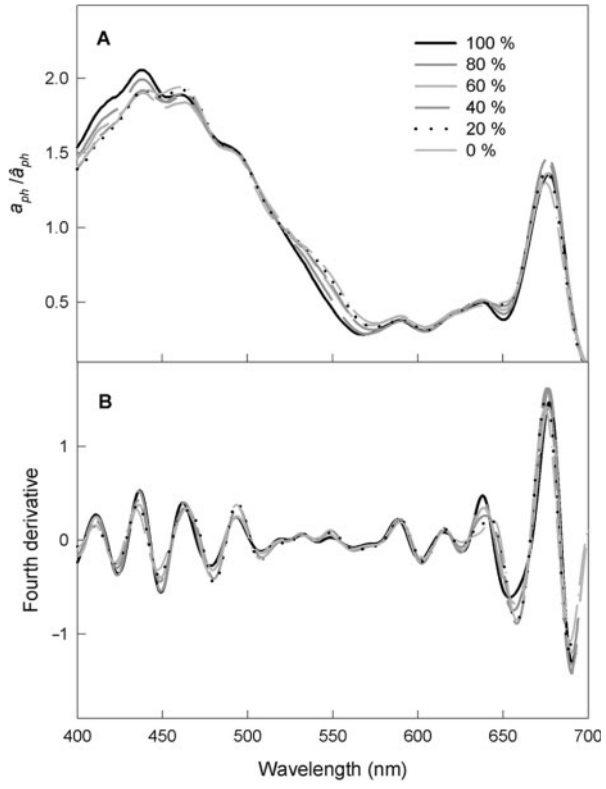

Fig. 1. Representative mean normalized absorption spectra (**A**) and corresponding fourth-derivative spectra (**B**) of mixed assemblages of *P. minimum* and *K. mikimotoi*. Spectra ranged from 0 to 100% contribution by *K. mikimotoi* to the total Chl *a* biomass in increments of 20%. Each spectrum is the average of two replicates.

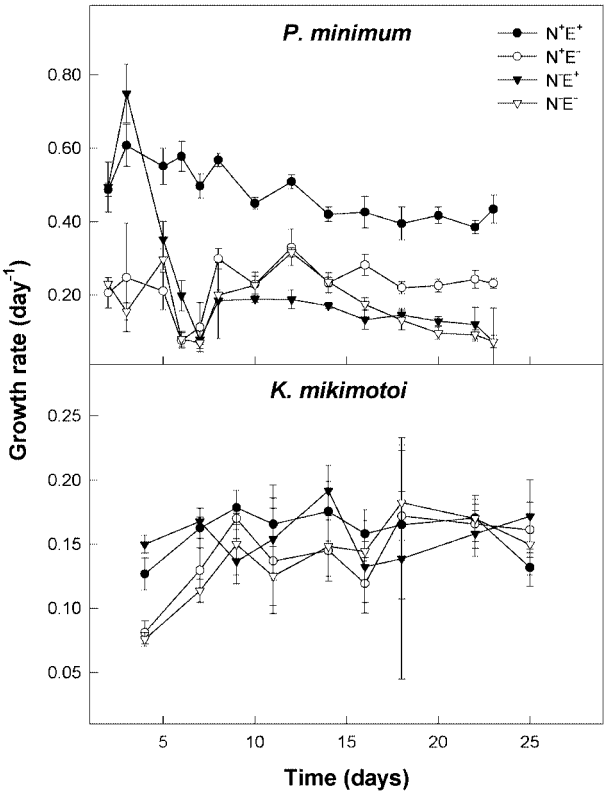

Fig. 2. Acclimation of the specific growth rate of *P. minimum* and *Karenia mikimotoi* to different levels of nitrate (N) and irradiance (E) in a semi-continuous growth experiment. All data are average ± 1 SD ($n = 2$).

medium >10 μM. Consequently, four physiological conditions were imposed on *Prorocentrum minimum* and two on *K. mikimotoi*.

Specific growth rate showed large variations for both species, but tended to be higher for *P. minimum* than *K. mikimotoi* throughout the growth period (Figure 2). This was especially evident for *P. minimum* cultures growing at high nitrate and irradiance levels. However, *P. minimum* cultures in depleted nitrate acclimated asymptotically towards a lower growth rate of 0.07 ± 0.04 day^{-1} (mean ± 1 SD) compared with cultures replete with nitrate, which had a growth rate of 0.33 ± 0.11 day^{-1} (mean ± 1 SD). *Karenia mikimotoi* maintained an overall low growth rate around 0.15 ± 0.04 day^{-1} (mean ± 1 SD) for all growth conditions (Figure 2).

Cellular Chl *a* content of *P. minimum* was highest in the nitrate-replete cultures at low irradiance, and lowest in nitrate-depleted cultures at high irradiance (Figure 3). Two-way ANOVA for the cellular Chl *a* content of *P. minimum* on the last experimental day showed significant effects of both irradiance and nitrate levels (Table II). Furthermore, a significant interaction between these factors was found, indicating that the resulting cellular Chl *a* content of *P. minimum* was caused by a combination of the experienced irradiance and nitrate levels. *Karenia mikimotoi* only adjusted its cellular Chl *a* content to the different levels of growth irradiance (Figure 3), while no significant effect of nitrate depletion was found on the final day of the experiment (Table II).

Normalizing the phytoplankton absorption spectra (a_{ph}) to the Chl *a* + pheophytin *a* concentration produced the Chl *a*-specific absorption spectra in Figure 4. Differences in shape and level of the spectra suggest that *P. minimum* acclimated to both nitrate and irradiance levels, whereas *K. mikimotoi* acclimated almost only to irradiance. This was further documented by performing a two-way ANOVA (Table II) on the spectrally averaged (400–700 nm) Chl *a*-specific absorption coefficient ($\hat{a}^*_{ph}$). *Prorocentrum minimum* had the highest $\hat{a}^*_{ph}$ value in cultures grown at high irradiance and in depleted nitrate (Figure 5), corresponding to cultures with the lowest cellular Chl *a* content (Figure 3). The lowest $\hat{a}^*_{ph}$ value of *P. minimum* was found in cultures grown at low irradiance replete in nitrate, corresponding to cultures with the highest cellular Chl *a*

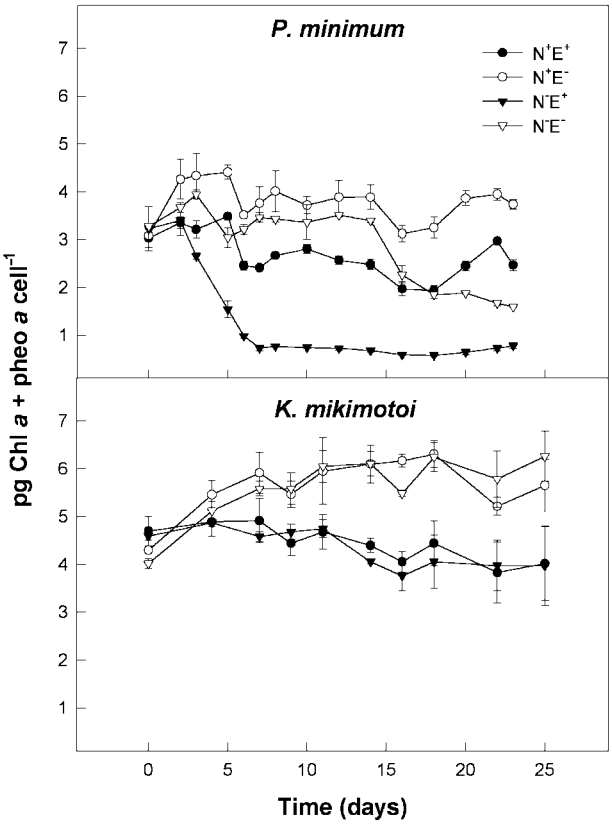

Fig. 3. Acclimation of the cellular Chl *a* content of *P. minimum* and *K. mikimotoi* exposed to different levels of nitrate (N) and irradiance (E) in a semi-continuous growth experiment. All data are average ± 1 SD ($n = 2$).

content. Significant differences in $\hat{a}^*_{ph}$ of *K. mikimotoi* were only found between cultures grown at high and low irradiance (Table II; Figure 5), which were acclimated to low and high cellular Chl *a* content respectively (Figure 3).

Cellular content of Chl *a*, as well as all the different accessory pigments of *K. mikimotoi*, was more than twice as high in the low-irradiance cultures as in the high-irradiance conditions (Figure 6). The similar response of all pigments indicates an almost constant relationship between the different pigments and Chl *a*. This was particularly the case for gyroxanthin-diester, which had an almost constant ratio to Chl *a* for all growth conditions (Table III). However, significantly different ($P < 0.001$, Student's *t*-test) relationships between gyroxanthin-diester and carbon biomass (mg C l^{-1}) and cell abundance (cells ml^{-1}) were found for *K. mikimotoi* cultures acclimated to low and high irradiance (Table III). Cultures grown at low irradiance had a two- to three-fold higher concentration of gyroxanthin-diester at the same carbon biomass and cell abundance as cultures grown at high irradiance.

Species differentiation by a spectral SI

The spectral SI comparing the fourth-derivative absorption spectra of a known with an unknown sample, was calculated to determine the unknown fraction (% Chl *a*) of *K. mikimotoi* (Figure 7A) and the concentration of gyroxanthin-diester (Figure 7C). SI was linearly related to the fraction of *K. mikimotoi* and the index differed little between growth conditions. However, using the SI from non-normalized spectra to model the gyroxanthin-diester concentration resulted in significantly different predictions depending on the choice of growth conditions

Table II: Two-way ANOVA on the effect of different levels of nitrate (N) and growth irradiance (E) on log transformed cellular Chl a and mean spectral (400–700 nm) Chl a-specific absorption ($\hat{a}^*_{ph}$) ($n = 2$)

Dependent variable	Species	Source of variation	DF	*F*	*P*
Cellular Chl *a*	*K. mikimotoi*	N	1	1	0.43
		E	1	33	< 0.0004
		N × E	1	1	0.36
	P. minimum	N	1	2289	< 0.0001
		E	1	677	< 0.0001
		N × E	1	33	< 0.0001
$\hat{a}^*_{ph}$	*K. mikimotoi*	N	1	0.01	0.91
		E	1	6.19	0.03
		N × E	1	0.61	0.45
	P. minimum	N	1	8.9	0.01
		E	1	5.4	0.04
		N × E	1	2.7	0.13

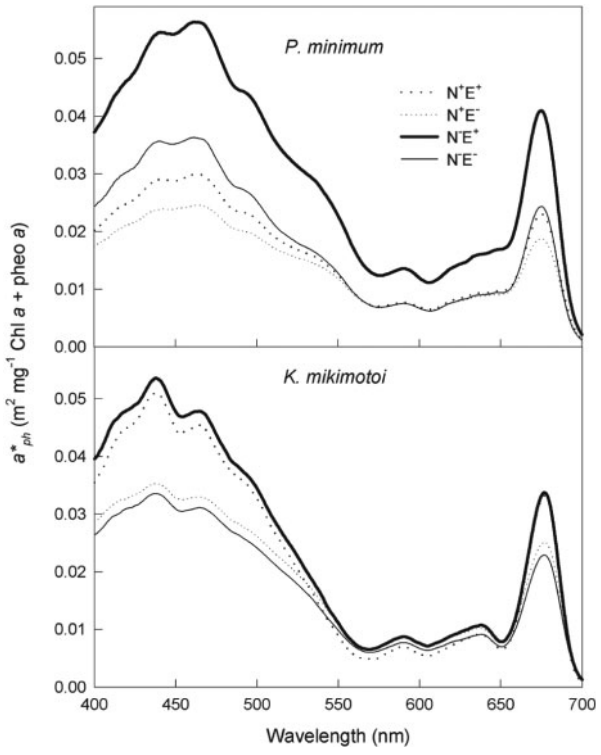

Fig. 4. Acclimation of spectral Chl *a*-specific light absorption of *P. minimum* and *K. mikimotoi* exposed to different levels of nitrate (N) and irradiance (E) in a semi-continuous growth experiment. Each spectrum is the average of two replicates.

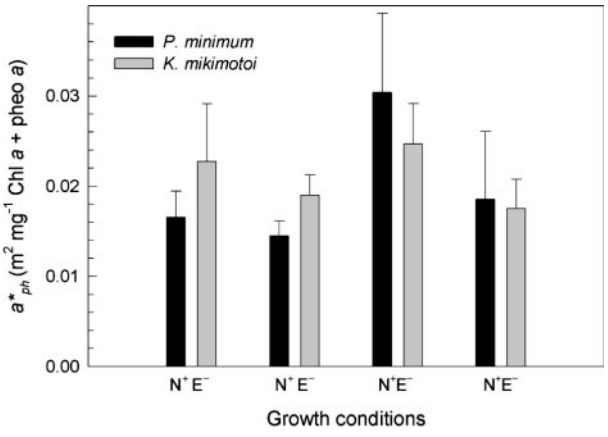

Fig. 5. Spectrally averaged (400–700 nm) Chl *a*-specific absorption coefficient of *P. minimum* and *K. mikimotoi* exposed to different levels of nitrate (N) and irradiance (E) in a semi-continuous growth experiment. All data are average ± 1 SD ($n = 2$).

(Figure 7C). Assemblages composed of high-irradiance cultures generally had a higher SI value than low-irradiance cultures and samples from the physical mixing experiment most resembled the low-irradiance cultures. Calculation of the 95% confidence of the regression line in Figure 7A and C, showed that the variation of the dependent variables (% Chl *a* and gyroxanthin-diester concentration) could not be accurately explained by applying only one regression model for all assemblages.

Species differentiation by multivariate regression

PLS regression models of fourth-derivative absorption spectra as a function of the fraction of *K. mikimotoi* (Figure 7B) and the concentration of gyroxanthin-diester (Figure 7D), were established. Three PLS components (approximate latent dimensions of the model) were optimal for modelling both the fraction of *K. mikimotoi* and the concentration of gyroxanthin-diester. PLS1 models explained >96% of the variance in both % *K. mikimotoi* and the concentration of gyroxanthin-diester. No outliers were detected from score-plots of PLS component 1 versus 2, and all samples were therefore included in the analysis. The PLS technique applied to non-normalized spectra was very accurate in predicting both the fraction of *K. mikimotoi* ($r^2 = 0.99$) and the concentration of gyroxanthin-diester ($r^2 = 0.94$). No systematic variation between phytoplankton assemblages was found for either of the PLS models. Calculation of the 95% confident limit of the regression line in Figure 7B and D, showed that the slopes of both models did not differ significantly from 1, and that the offsets were not significantly different from 0.

DISCUSSION

Acclimation to nitrate and irradiance

The semi-continuous culturing technique made it possible to maintain algae in exponential growth throughout the experiment. It should therefore be possible to explain differences in specific growth rate, pigmentation and light absorption using differences in nitrate and irradiance availability.

Differences in growth rates of *P. minimum* were primarily determined by nitrate availability, as nitrate-depleted cultures had significantly lower growth rates than the nitrate-replete cultures. Growth rates of *P. minimum* acclimated to low (35 µmol photons m^{-2} day^{-1}) and high (500 µmol photons m^{-2} day^{-1}) light under nitrate-replete conditions have previously been reported to range between 0.24 and 0.39 day^{-1}, although with considerable variability among culture strains (Johnsen and Sakshaug, 1993). However, by examining the combined effect of nitrate and irradiance limitation, we recorded a much larger range (0.07–0.45 day^{-1}) in the specific growth rates of *P. minimum* on the final day of sampling, further indicating the importance of nitrate

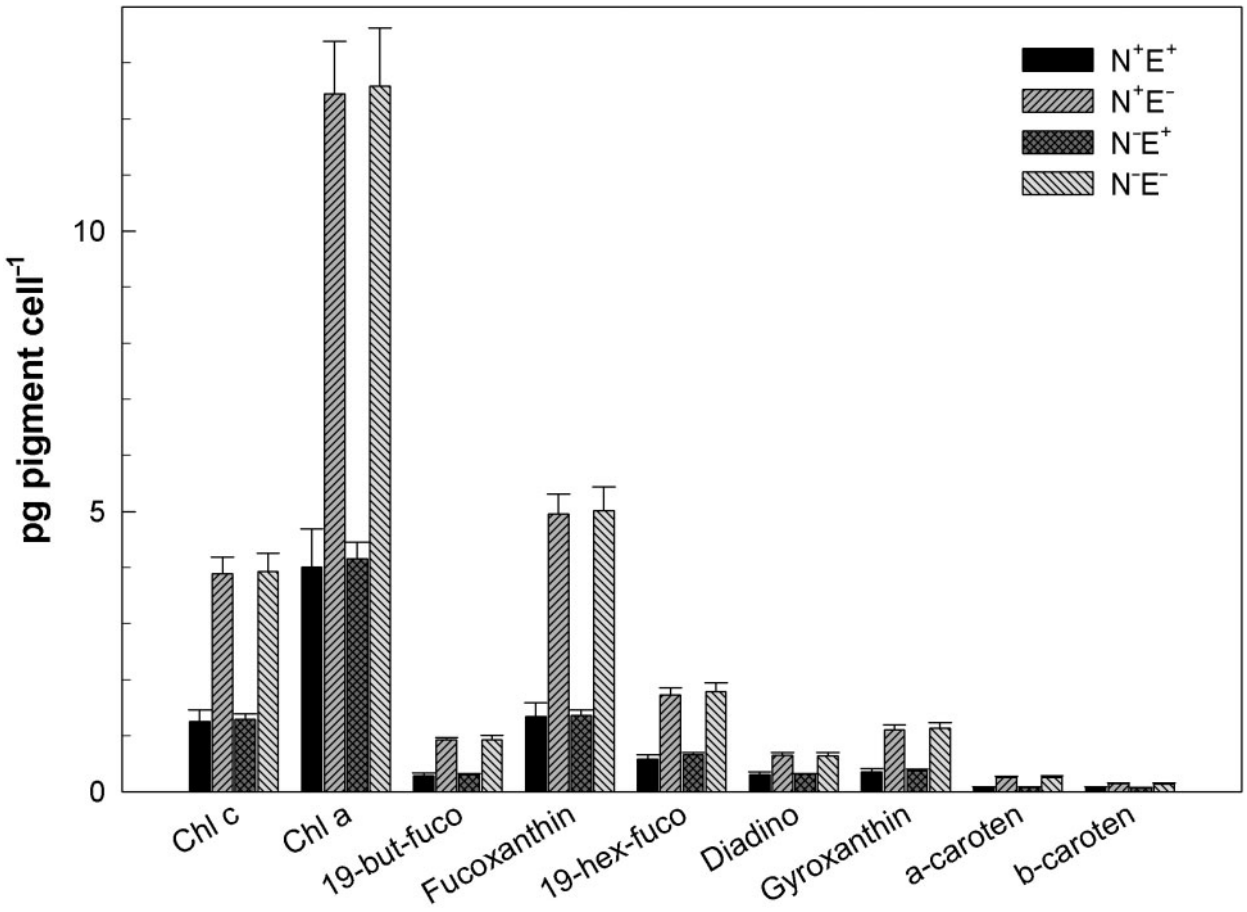

Fig. 6. Acclimation of the cellular pigment content of *K. mikimotoi* exposed to different levels of nitrate (N) and irradiance (E) in a semi-continuous growth experiment. All data are average ± 1 SD ($n = 2$).

availability for the performance of the algae. In comparison the growth rate of *K. mikimotoi* varied little (0.12–0.18 day^{-1}) and showed no significant differences between growth conditions. However, the measured growth rates were similar to the rates of *Gyrodinium aureolum* (original name of *K. mikimotoi*; 0.09–0.33 day^{-1}) reported by Johnsen and Sakshaug, although they grew their culture at lower irradiance [30 and 170 µmol photons m^{-2} day^{-1}; (Johnsen and Sakshaug, 1993)]. The lack of effect of irradiance on the growth rate of *K. mikimotoi* in this study probably resulted from the choices of growth irradiance (140 and 400 µmol photons m^{-2} day^{-1}), both of which, according to Nielsen (Nielsen, 1992), are high compared to the optimum irradiance for cell division of *G. aureolum* at ~170 µmol photons m^{-2} day^{-1}. Clearly, however, irradiance influenced the chemical composition of *K. mikimotoi* (Table III).

Table III: Ratio of gyroxanthin-diester (Gyro, g m^{-3}) to Chl a (mg m^{-3}), particulate carbon (g m^{-3}) and cell density (ml^{-1})

Growth condition	Gyro:Chl *a*	Gyro:carbon	Gyro:cells	Cells:gyro
N^+E^+	89.9 ± 1.5	668 ± 115	0.36 ± 0.06	2.8 ± 0.5
N^+E^-	89.4 ± 1.7	1518 ± 71	1.11 ± 0.08	0.9 ± 0.1
N^-E^+	91.5 ± 1.6	744 ± 79	0.38 ± 0.02	2.6 ± 0.2
N^-E^-	90.2 ± 0.5	1580 ± 128	1.13 ± 0.10	0.9 ± 0.1

The cell to gyro ratio has units of 10^6 cells l^{-1} µg gyro l^{-1}. All data are average ± SD of replicate cultures. Growth conditions are explained in Table I.

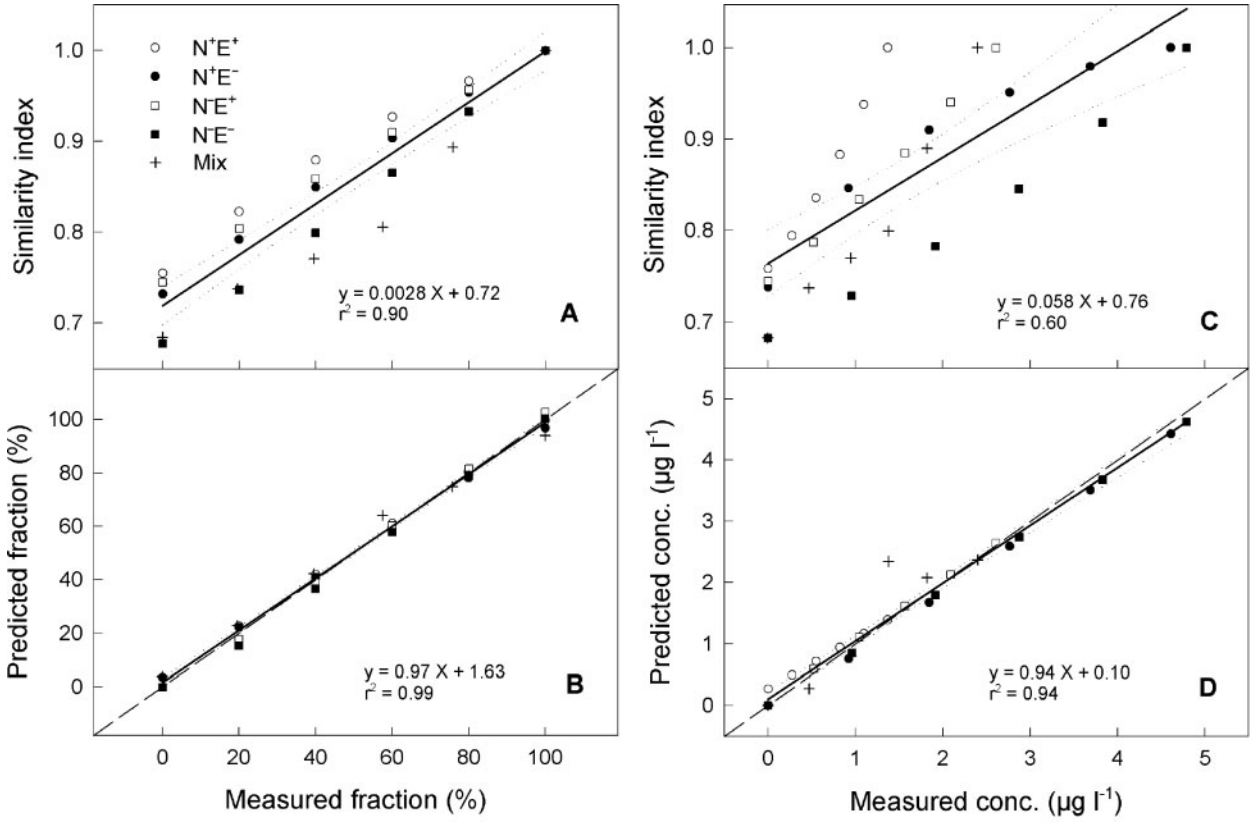

Fig. 7. Determination of the Chl *a* fraction of *K. mikimotoi* by (**A**) using a spectral similarity index and (**B**) by multivariate regression. Both methods were based on fourth-derivative spectra of mean-normalized absorption spectra. In comparison the ability to predict the concentration of gyroxanthin-diester using (**C**) the similarity index and (**D**) multivariate regression is shown. In this case both methods were based on fourth-derivative spectra of non-normalized absorption spectra. Hypothetical assemblages based on combinations of *K. mikimotoi* and *P. minimum* acclimated to different nitrate and irradiance conditions are compared with the results of a physical mixing experiment, shown as a cross. Calculations of SI for the different assemblages and concentration levels were determined using equation (4), where A_{std} = samples in which *K. mikimotoi* contributed 100%. The solid line of (B) and (D) is the linear regression; the dotted line represents the 95% confidence limit and the dashed lines indicate the identity (1:1) line.

The cellular Chl *a* content of *P. minimum* changed as a combined response to both nitrate and irradiance availability. Although Johnsen and Sakshaug (1993) exposed *P. minimum* to larger differences in irradiance, we observed a larger range in cellular Chl *a* content (0.7–3.9 pg Chl *a* cell^{-1}) [(Johnsen and Sakshaug, 1993); 1–2.6 pg Chl *a* cell^{-1}]. In both experiments, the highest cellular Chl *a* content was found at low irradiance. Our low-nitrate cultures did however have significantly lower cellular Chl *a* content than nitrate-replete cultures, indicating that differences were primarily determined by the availability of nitrate. The cellular Chl *a* content of *K. mikimotoi* cells exposed to low irradiance (6 ± 0.5 pg Chl *a* cell^{-1}, mean ± SD) was ~50% higher than cells grown at high irradiance (4 ± 0.4 pg Chl *a* cell^{-1}). Similarly, Johnsen and Sakshaug found an ~50% higher cellular Chl *a* content of shade-adapted cells of *Gyrodinium aureolum* (Johnsen and Sakshaug, 1993). No significant differences in cellular Chl *a* content were found between nitrate-replete and nitrate-depleted cultures, suggesting that *K. mikimotoi* did not experience nitrate limitation. This could be expected because of low growth rates throughout the experiment (~0.10–0.15 day^{-1}) combined with the duration of the experiment (25 days), resulting in only a few (~2–4) cell divisions, which would be insufficient to deplete nitrate and induce nitrogen limitation.

Differences in Chl *a*-specific light absorption spectra of phytoplankton are primarily determined by differences in the packing of pigments and to less extent pigment composition (Morel and Bricaud, 1981; Bricaud *et al.*, 1983; Kirk, 1994). Pigment packaging is strongly related to the product of the intracellular concentration of Chl *a* (c_i; mg Chl *a* m^{-3} of cellular material) and the cell diameter (d; µm). Using the product $c_i \times d$ as a measure of pigment packaging, 79% of the variation in the mean Chl *a*-specific light absorption ($\hat{a}^*_{ph}$) was

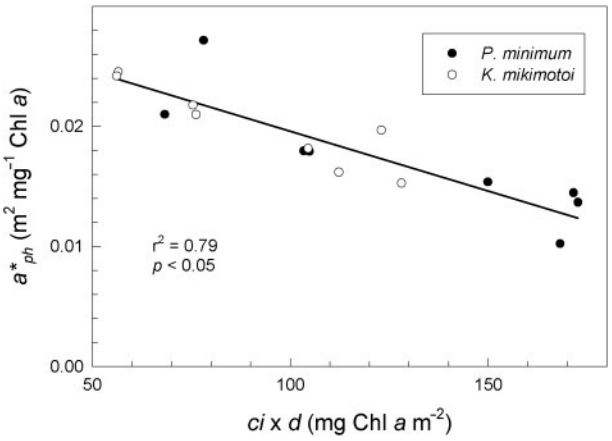

Fig. 8. Variability in the mean Chl *a*-specific absorption coefficient ($\hat{a}^*_{ph}$) as a function of the product of intracellular Chl *a* concentration (c_i) and cell diameter (d).

accounted for (Figure 8). *Prorocentrum minimum* clearly experienced a larger variability in $\hat{a}^*_{ph}$ and $c_i \times d$ than seen for *K. mikimotoi*, which only responded to differences in irradiance (Table II).

The concentration of the different accessory pigments of *K. mikimotoi* relative to Chl *a* were similar for all growth conditions. Thus, gyroxanthin-diester was clearly correlated with Chl *a*, suggesting that gyroxanthin-diester is as good as chlorophyll as an indicator of the abundance of *K. mikimotoi*. Unfortunately, variability in chlorophyll per cell and the ratio of chlorophyll to cellular carbon limits the usefulness of either pigment as a precise measure of biomass (Cullen, 1982). Gyroxanthin-diester has previously been suggested as a diagnostic pigment for *Karenia brevis* because of its stability to light acclimatization and also its distinct chromatographic characteristics, which allow an easy recognition and quantification (Millie *et al.*, 1995). This study, however, clearly demonstrated that changes in the physiological state of *K. mikimotoi*, caused by different irradiances, greatly affect the relationship between the concentration of gyroxanthin-diester and the abundance of *K. mikimotoi* measured as POC or cell abundance (Table III). It is therefore questionable whether the linear relationship between the concentration of gyroxanthin-diester and the cell abundance of *K. brevis* found in fieldwork (Millie *et al.*, 1997; Kirkpatrick *et al.*, 2000) can be applied under significantly different environmental conditions. This argument is supported by comparing the slope of the linear relationship shown by Millie *et al.* (Millie *et al.*, 1997) ([gyro] $= 3.8 \times 10^6$ cells l^{-1} $\times$ μg gyroxanthin-diester l^{-1}) with that of Kirkpatrick *et al.* (Kirkpatrick *et al.*, 2000) ([gyro] $= 3.0 \times 10^6$ cells l^{-1} $\times$ μg gyroxanthin-diester l^{-1}). Thus, the ability to predict cell abundance of *K. breve* from concentrations of gyroxanthin-diester seems to depend on the environmental setting.

Species differentiation

Application of fourth-derivative analysis to spectral light absorption spectra of phytoplankton enhances differences in the shape of the spectra, thereby indicating qualitative alterations of the light-harvesting pigments (Millie *et al.*, 1995). The calculated fourth-derivative spectra, therefore, carry useful information on the pigment composition. However, the extent to which this information can be applied for rapid assessment of the development of algal blooms depends not only on the technical means for acquiring highly resolved absorption spectra, but also on the sensitivity and robustness of the technique used to interpret the spectral information.

In this study we applied a spectral SI and a PLS multivariate regression technique to differentiate the abundance of *K. mikimotoi* from samples mixed with *P. minimum*. Both methods were able to determine the fraction of *K. mikimotoi* from fourth-derivative spectra normalized to their mean absorption value. The PLS predictions were, however, much less sensitive than the SI predictions to the induced variability in optical properties of the algae caused by mixing absorption spectra derived from different growth conditions. But why is the SI approach sensitive to photoacclimation? The *K. mikimotoi* cells grown at high irradiance were significantly less packed than cells growing at low irradiance (Figure 5). This resulted in significantly different shapes of the mean normalized absorption spectra, with those exposed to low irradiance being significantly flatter (not shown). Considering the fact that SI is only an index of the shape of the absorption spectra, it is thus not surprising that SI predictions were sensitive to differences in growth irradiances. Since *K. mikimotoi* did not experience nitrogen limitation, it is not possible to draw specific conclusions on the importance of nutrient conditions on the accuracy of species differentiation. However, differences in pigment packaging because of nitrogen limitation, such as those experienced by *P. minimum* (see Table II; Figure 5), would further exacerbate differences in the spectral flattening. If *K. mikimotoi* had experienced nitrogen limitation, this would most probably have resulted in an even larger uncertainty of the SI-predicted estimates.

The SI and PLS techniques were also applied to determine the concentration of gyroxanthin-diester, a potential indicator of the concentration of *K. mikimotoi*. Estimations of gyroxanthin-diester by the SI and PLS method were both derived from non-mean-normalized fourth-derivative spectra, since normalizing to the mean gave a larger scatter and a lower correlation of the relationships (not shown). Therefore, whereas normalization

of absorption spectra removes information about the absolute concentration level, which has no direct relationship to relative abundance of *K. mikimotoi*, estimations of absolute concentrations must be made on unnormalized spectra. Photoacclimation of the algae strongly affected the predictions of the gyroxanthin-diester concentrations from SI, and predictions were significantly different for the different growth conditions. The inability of the spectral SI to predict absolute concentrations of gyroxanthin-diester is not surprising since the index only compares similarities in the shape of the fourth-derivative spectra and does not account for differences in the overall absorption level.

Successful monitoring of toxic phytoplankton species based on light absorption spectra requires a method which can provide reliable estimates of cell abundance, during the development as well as decay of a bloom. Therefore the method should be robust to significant changes in both the photoacclimative state of the algae and species composition. To compare the performance of the presented methods under such conditions, hypothetical assemblages of absorption spectra of *K. mikimotoi* were made with natural mixed samples collected in the Greenland Sea ($n = 52$), Danish coastal waters ($n = 162$) and Danish estuaries ($n = 61$). These samples differ greatly in cell size composition and pigment packaging (Stæhr *et al.*, 2003), causing the mean-normalized absorption spectra of the estuarine samples to be much flatter than those of the coast and ocean. An average spectrum from each water type (oceanic, coastal and estuarine) was mixed with absorption spectra of *K. mikimotoi* acclimated to high and low irradiances, according to the previously described procedure. The SI varied significantly depending on whether the *K. mikimotoi* spectra were mixed with an oceanic, coastal or estuarine spectrum, but varied less as a result of light conditions. In comparison, the PLS-predicted fraction only showed a minor dependency on the origin of the natural sample. Predictions of the fraction of *K. mikimotoi* from the SI were consequently less accurate ($20 \pm 15\%$, mean $\pm$ SD) than predictions based on the PLS regression ($8 \pm 4\%$), both methods having largest uncertainties at low fractions of *K. mikimotoi* (Figure 9).

In summary, the results presented here showed that predictions of relative cell abundance by the SI approach may be seriously affected by the photoacclimative state of the algae and by phytoplankton assemblage structure. Futhermore, the SI approach cannot provide reliable estimates of absolute abundance in terms of cell numbers or pigment concentration. To the credit of the SI approach it must, however, be said that if irradiance conditions are constant over the sampling period, the SI method does provide reasonable

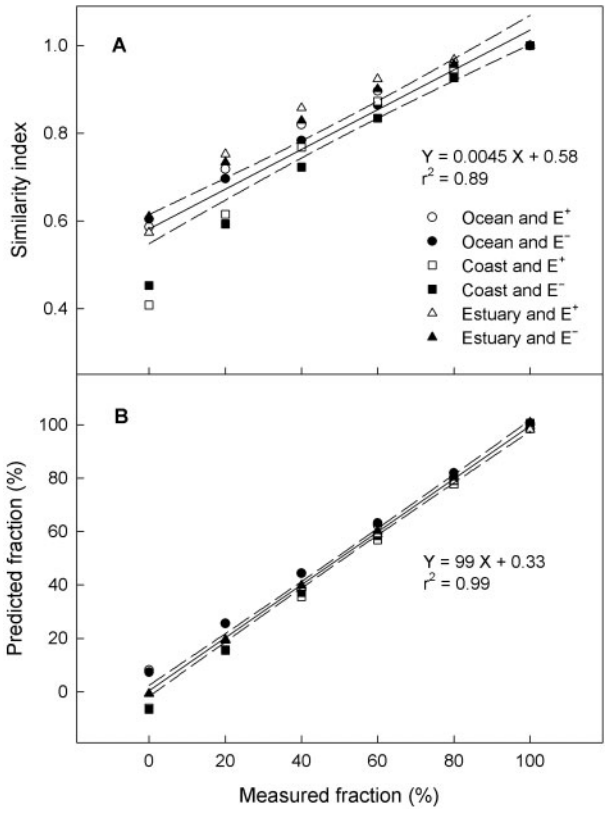

Fig. 9. Determination of the Chl *a* fraction of *Karenia* in natural phytoplankton assemblages (**A**) using a spectral similarity index and (**B**) by multivariate regression. Both methods were based on fourth-derivative spectra of mean-normalized absorption spectra. Phytoplankton assemblages derived from hypothetical combinations of absorption spectra of *K. mikimotoi* acclimated to high (E^+) and low (E^-) irradiances mixed with absorption spectra of oceanic, coastal and estuarine origin. The solid line is the linear regression; the dotted line is the 95% confidence limit.

measures of relative abundance, as shown for field samples by Kirkpatrick *et al.* and for laboratory cultures by Millie *et al.* (Kirkpatrick *et al.*, 2000; Millie *et al.*, 2002). Application of the SI in regular monitoring of microalgal phylogenetic groups and selected species, as suggested by Millie *et al.* (Millie *et al.*, 2002) seems, however, to require some level of continuous validation as a result of sensitivity to growth conditions and to differences in phytoplankton assemblage structure which may significantly affect the flattening of mean-normalized absorption spectra.

In comparison, the PLS predictions were almost insensitive to the photoacclimative state of the algae and the phytoplankton assemblage structure, and provided accurate estimates of both relative and absolute abundances of *K. mikimotoi*. The power of multivariate regression in predicting absolute concentration levels from spectral data is well documented for near-infrared spectra (Dardenne *et al.*, 2000) and has recently been successfully applied to determine the concentration of

photopigments from *in vitro* spectra (Moberg *et al.*, 2000) and fourth-derivative *in vivo* spectra of phytoplankton (Stæhr, 2003). The PLS method does not require more detailed data than the SI to calculate the relative or absolute cell abundance. Once the algorithm for natural samples has been established, the method simply requires measurements of spectral light absorption, similar to the SI method. One drawback of multivariate regression models is, however, that they initially require a large number of samples to be calibrated and validated with a high degree of confidence.

We recommend that future work aiming at detecting specific phytoplankton species on the basis of their absorption signatures should apply a PLS regression technique. Initial work should focus on establishing a large number of field samples to calibrate and rigorously validate the regression models to a high degree of confidence. The described PLS regression model, however, requires highly resolved information on the spectral absorption signatures, as does the SI. Therefore, to provide better determination of phytoplankton bloom history, models (PLS or SI) must be coupled with instruments collecting *in situ* multi-wavelength absorption measurements, such as the liquid wave-guide capillary cell.

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