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SEVENTH FRAMEWORK PROGRAMME

WISER

Water bodies in Europe: Integrative Systems to assess Ecological status and Recovery

DELIVERABLE

Deliverable D4.1-2: Assessment of pigment data potential for multi-species and assemblage indices

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Content

Non-technical summary

The European Water Framework Directive (WFD) requires the Member States to assess the ecological status of the marine coastal and estuarine waters. In this assessment, several aspects of the phytoplankton communities, such as composition, abundance and biomass, must be included.

In the first round of WFD intercalibration only the sub element chlorophyll a (Chl a) of the biological quality element phytoplankton was intercalibrated. The present report provides results from analysis of phytoplankton composition described from pigment content. The phytoplankton communities analysed were sampled as part of the WISER field work during the summer of 2009.

While the total concentration of Chl a was significantly correlated with TN across the geographically different WISER sampling localities, the distribution patterns of pigment samples and communities showed the major correlation with salinity and temperature and only minor correlation with TN as a measure of eutrophication. The variation in phytoplankton composition at a Danish station included in the analyses showed inter-annual variations over a three year sampling period in the same range as variations found among the WISER stations.

The concentration of individual pigments increased with increasing TN whereas no clear relationships were found for relative contributions of the different phytoplankton groups.

A prerequisite for the use of pigment based community composition as a WFD indicator is the establishment of reference conditions. The major influence from salinity and temperature on the distribution pattern of the WISER samples hindered the use of any of these sampling stations as reference sites for a pigment based phytoplankton indicator. Different and commonly unknown accumulation and preservation rates of the different pigments in sediments reduce the possibility of describing quantitative reference phytoplankton communities from the fossil record.

The most specific pigments may have a potential as indicators. However, this requires that the phytoplankton group they characterise is also a useful indicator. At present very few such single species/group indicators have been identified

Introduction

Phytoplankton is one of the biological quality elements to be used for assessing the ecological status of coastal waters according to the European Water Framework Directive (WFD). One of the main aims of the WFD is that by 2015 the biological quality elements used for assessment show less than "moderate" deviations from undisturbed conditions. If this is not the case, actions to improve the ecological status are required. Prerequisites for specifying such actions will be 1) knowledge on the coupling between anthropogenic pressures and the biological responses of the marine ecosystem and 2) definition of reference conditions (i.e. conditions representing no anthropogenic disturbance) and acceptable deviations from the reference conditions for the given quality element.

For the biological quality element phytoplankton, the biomass, composition and bloom frequency should be addressed in the assessment of ecological status (European Commission, 2000). During the initial European intercalibration of assessments only the concentration of chlorophyll a and a few area-specific indicator species (e.g. *Phaeocystis* sp. which forms nuisance blooms in the southern North Sea) were included as metrics (Carletti and Heiskanen, 2009). However, the WFD requires that future assessments also include indicators (metrics) that reflect phytoplankton composition.

In addition to affecting the total phytoplankton production and biomass, changes in the physical-chemical environment may lead to shifts in the phytoplankton community structure. Margalef (1978) and Margalef et al. (1979) provided a frame (Margalef's Mandala) for species succession under varied physical (turbulence) and chemical (nutrients) conditions in marine waters. The initial ideas in this Mandala have subsequently been developed further to characterize marine dinoflagellate assemblages in relation to nutrient accessibility, water column mixing and irradiance (Smayda and Reynolds, 2003). While this conceptual understanding of phytoplankton community structure and species/life-form composition relates to concurrent changes in the physical and the chemical environment either seasonally or across ecosystems (near-shore coastal to open ocean) it will be of greater importance to the WFD and assessments in general to focus on within-ecosystem patterns and indicator species related to changes in e.g. only nutrients. However, a common problem related to defining indicator species in specific systems (e.g. fjords) is the lack of a sufficient within-system pressure gradient to establish empirical relationships between the pressure and the indicator.

Currently the global climatic changes act as an additional external pressure that impose further complexity on the driver/response relationships, the non-linearity of these interactions and the

biogeographical differences are difficult to predict (Edwards and Richardson 2004, Wilby et al. 2006, Abigail et al. 2008)

Over recent years, phytoplankton composition in relation to anthropogenic nutrient inputs has been investigated worldwide. For example, possible eutrophication indicators have been proposed in the Baltic Sea (Sagert et al. 2005; Jaanus et al. 2009), in the Mediterranean coast (Spatharis et al. 2007), on the Florida shelf (Heil et al. 2007) and in the mesohaline Neuse River Estuary (Rothenberger et al. 2009). Despite many efforts to understand how eutrophication affects phytoplankton composition in marine waters, some authors have noted that a direct correlation cannot be established between species abundance and nutrients, which is a prerequisite of the WFD indicators (Nielsen et al. 2003, Cloern et al. 2005 Yunev et al. 2007, Domingues et al. 2008). As a consequence, for marine waters there are very few methodologies in accordance with the WFD that include composition (e.g. Devlin et al. 2007; 2009).

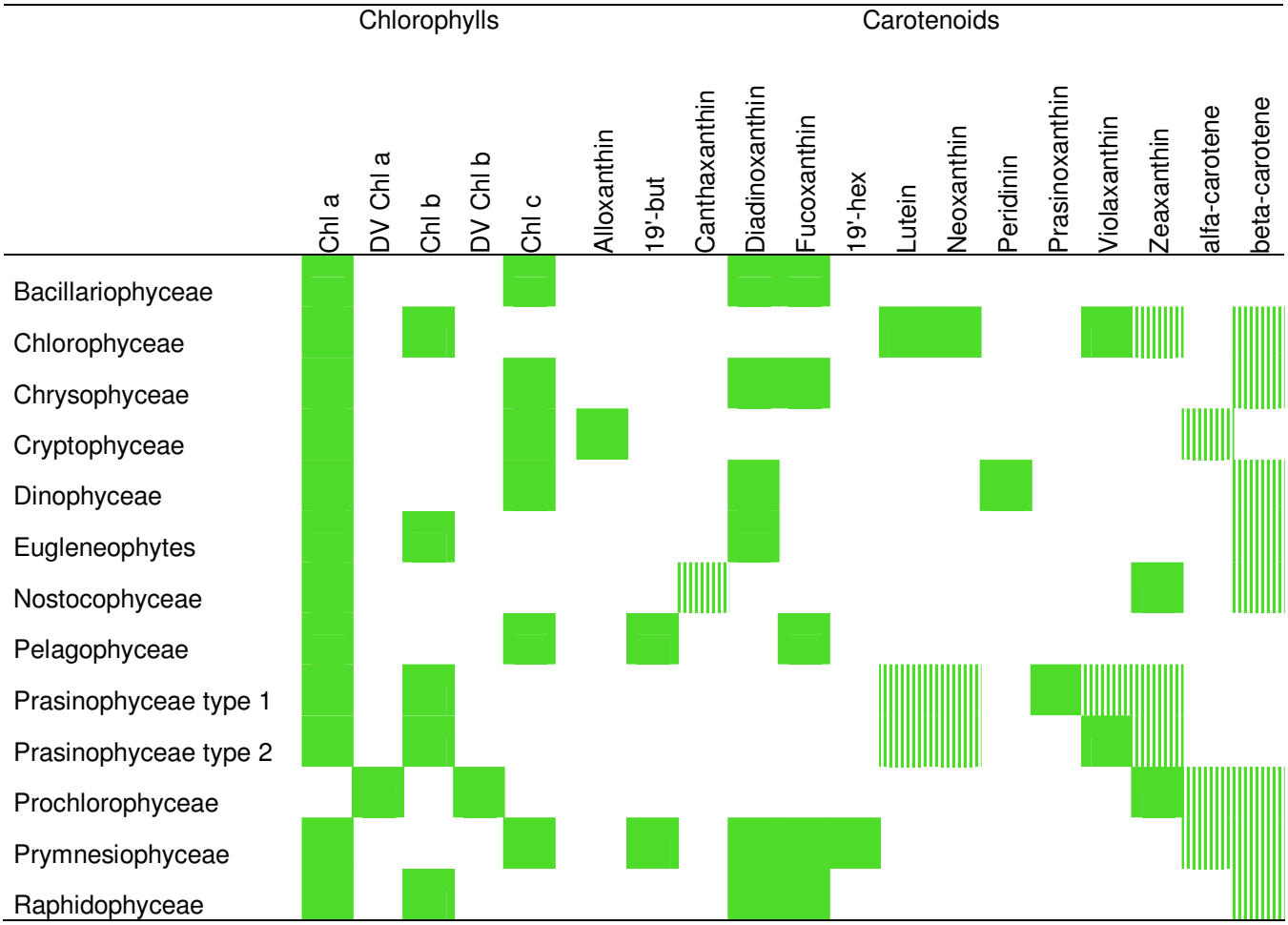
The changes in the phytoplankton assemblages are usually addressed by light microscopy techniques, such as Utermöhl (Uthermöhl 1958), since these techniques are species-specific. However, due to the taxonomic complexity of the phytoplankton communities and the multiple bottom-up and top-down interactions that they are subject to, confident relationships are difficult to establish between the species and the environment (not only nutrients, but also other physico-chemical and biological elements). One way to overcome this problem is simplifying the communities through partition of species into guilds, functional groups or functional types. For example, macroalgal-dominated communities, when examined at the functional group level, appear to be more temporally stable and predictable than when examined at the species level (Mouillot et al. 2006). In this sense, for phytoplankton, methodologies based on pigments as a surrogate for functional groups can be promising (Paerl et al. 2003).

Pigments in phytoplankton and application to description of community structure

Phytoplankton contains numerous different pigments of which chlorophyll a (Chl a) is found in all phytoplankton species. In addition, phytoplankton organisms contain accessory pigments that are more or less unique (’marker pigments’) to specific taxonomic groups (e.g. prasinoxanthin in some prasinophytes and peridinin in most dinoflagellates) and others that are found mainly in one or few groups (e.g. 19’-hexanoyloxyfucoxanthin in prymnesiophytes and some dinoflagellates, and fucoxanthin in diatoms, chrysophytes, prymnesiophytes, and raphidophytes). An overview of pigments commonly found in different groups of phytoplankton is provided in Table 1. This overview is not fully comprehensive since continued studies on further species reveal more details in pigment composition and more exceptions to these general patterns. Thus, just within the prymnesiophytes Jeffrey and Wright (1994) characterised four different types

based on pigment content while subsequent studies of more laboratory strains of prymnesiophytes led to distinction of eight different 'pigment types' (Zapata et al. 2004).

Table 1. Major (filled marker) and minor (hatched marker) pigments in different phytoplankton groups. Based on Jeffrey et al (1997). 19'-but = 19'-butanoyloxyfucoxanthin, 19'-hex = 19'-hexanoyloxyfucoxanthin.



While Table 1 provides a very rough overview of the general pigment content of different phytoplankton groups it should be emphasised that there are numerous exceptions to this overall pattern. Several common, important and sometimes dominant species contain pigments normally associated with other groups of organisms. These include e.g. chloroplast-containing species of *Dinophysis* and the ciliate *Myrionecta rubra* that have alloxanthin as a major pigment similar to cryptophytes and several dinoflagellates and 'chrysophytes' like the dictyocophyceaen *Dictyocha speculum* containing pigments similar to prymnesiophytes (Meyer-Harms & Pollehne 1998, Hansen et al. 2000, Daugbjerg & Henriksen 2001).

Chl a and the accessory pigments can be simultaneously quantified using high-performance liquid chromatography (HPLC) and the associated chemotaxonomical classification of phytoplankton communities based on the pigment contents has increased.

The quantified amounts of the individual pigments in environmental samples provide the basis for calculating the contribution of individual phytoplankton groups to the total amount of Chl a given sufficient knowledge of the relationship between cellular content of marker pigments and chlorophyll a in different taxa. Thus, seasonal or vertical light-induced variations in the ratio of carbon or biovolume to Chl a will also be reflected in estimates of the biomass of different groups using microscopy and pigment analysis, respectively. Pigment analysis may provide information about the physiological state of the phytoplankton and about zooplankton grazing. For instance, Jeffrey (1974) used chlorophyllide a as an *in situ* marker of scenescent cells, and pheophytins, pheophorbides and pyropheophorbide a have been used as indicators of protozoan or copepod grazing (see Jeffrey et al. 1997).

Characterization of phytoplankton communities using pigment analysis is cost-efficient and much less time consuming than traditional analysis in the microscope. Consequently, it can be more useful than microscopy techniques in large-scale monitoring programmes, such as those required by the WFD. However, it should be emphasized that the results are not directly comparable to those obtained by the traditional microscopic method. The chemotaxonomical approach provides information at only the class or group level while microscopy provides information about individual species. therefore chemotaxonomical characterization should be treated with caution and supplemented with screenings of dominant species in the microscope. A major strength of the pigment approach is, however, that groups of small or fragile to fixatives organisms, impossible to identify in the microscope but containing specific pigments, may be quantified by pigment analysis. Other advantages of the HPLC-based techniques are their high analytical reproducibility which avoids large variations in the results within and between laboratories (Sherrard et al., 2006) and it allows for a rapid processing of large numbers of samples from numerous locations and depths, thereby providing better temporal and spatial resolution matching the scale of phytoplankton variability (Sarmento and Descy, 2010).

Algorithms for deriving contributions from different phytoplankton groups to Chl a have been obtained by multiple regressions or by inverse methods based on individual marker pigments (Gieskes and Kraay, 1983; Letelier et al., 1993; Tester et al., 1995; Kohata et al., 1997). Another, and by now commonly used, approach has been application of a matrix factorization program, 'CHEMTAX' (Mackey et al., 1996), using input matrixes of, in principle, all identified and quantified pigments in samples and the corresponding pigment ratios of phytoplankton taxa potentially present. The output from the calculations provides the best fit of contributions from the predefined taxa to the true measured Chl a. While the analysis of pigment content is very reproducible between laboratories the interpretation of the phytoplankton community composition is, however, much more subjective and dependent on the input to calculation when applying CHEMTAX analysis to samples from natural phytoplankton communities (Irigoien et al. 2004).

The present report represents an analysis of pigment data collected from seven European localities during the WISER 2009 field campaign and from five Danish stations during 1997-2000. Pigment data as well as phytoplankton community structure calculated from pigments were analysed for relationships with trophic status of the different regions and, in addition, salinity and temperature.

Materials and methods

The WISER field work campaign was undertaken during summer 2009. Samples for pigment analysis were collected from seven different geographical areas covering the range from brackish waters of the Baltic Sea to full oceanic salinity off the Basque coast and around the Ballerian Island Mallorca. Sampling locations also included a transitional water, the Mondego Estuary in Portugal (Table 2) For the analysis of pigment profiles from different geographical regions additional samples collected during seasonal studies at five Danish locations in 1997-1998 were incorporated. Only samples collected during the summer season (May-September) at the Danish stations were included.

Environmental parameters accompanying the samples for pigment analysis included salinity, temperature and nutrients as shown in Table 2.

*Table 2. Environmental parameters measured at the sampling sites. Values represent averages of all stations and samples within the given area/year. *: data from Lesina were collected during sampling in 2007 and thus not synoptic with the WISER field campaign. Data from Lesina represents three of the five WISER stations.*

	TN (µM)	TP (µM)	DIN (µM)	DIP (µM)	Si (µM)	Salinity	Temperature
Basque Coast (ES)	10.5	0.45	6.82	0.28	1.24	35.2	20.8
Helsinki Sea area (FI)	26.3	0.59	< 0.20	0.13	0.33	5.0	18.0
Lesina (I)	47.5 *	0.92 *	4.54	0.01	15.67	17.0	24.7
Mallorca (ES)	2.8	0.32		0.22	2.26	38.1	28.0
Mondego (P) – Oligohaline part			73.9	0.75	55.2	6.5	22.3
Mondego (P) – Mesohaline part			50.0	0.75	38.9	17.7	20.4
Mondego (P) – Polyhaline part			23.5	0.31	10.8	34	16.4
Oslo Fjord (NO)							
Varna (BG)		0.92	10.66	0.84	5.97	15.7	24.1
Århus Bugt 1997 (DK)	18.7	0.40	0.75	0.07		17.7	16.6
Århus Bugt 1998 (DK)	16.1	0.79	2.01	0.36		22.8	13.5
Århus Bugt 2000 (DK)	17.1	1.04	3.20	0.24		23.2	12.8
Nivå Bugt (DK)	16.4	0.53	0.93	0.17		13.2	14.2
Gilleleje (DK)	13.6	0.49	0.97	0.17		17.9	13.9
Roskilde Fjord (DK)	34.1	2.55	3.05	1.48		17.2	15.5
Horsens Fjord (DK)	33.4	1.33	2.90	0.37	9.49	22.2	15.1

Samples for pigment analysis were collected at three to four stations within each water body. In the Mondego Estuary three stations were sampled in each of three salinity regions of the estuary. At each station two replicate water samples were collected. From each of these water samples

one to two sub samples were taken and subsequently divided into two sub-sub samples analysed for pigment content by HPLC. In addition, replicate samples for pigment analysis were collected at four "satellite" stations located in close vicinity of the main stations in each water body.

One hundred to 3000 ml of sea water were gently filtered onto 25 or 47 mm glass fibre filters and immediately frozen. Filters were shipped on dry ice to AU, Denmark, where they were kept at -80 ºC until analysis. The frozen filters were transferred to 2.5 or 5.0 ml methanol, sonicated for 30 sec, and left to extract for 24 h at -20 ºC prior to filtering (0.2 µm) 1 ml extract into HPLC-vials and mixing with 250 µl water. HPLC analyses were performed on a Shimazu LC 10A system with a Supelcosil C18 column (250 x 4.6 mm, 5 µm) using a slight modification of the Wright et al. (1991) method as described in Schlüter and Havskum (1997). Pigments were identified by retention times and absorption spectra identical to those of authentic standards, and quantified against standards purchased from the International Agency for 14C Determination, Hørsholm, Denmark.

Pigment filters (47 mm) from the Helsinki sea area (FI) and the Basque coast (ES) were unfortunately extracted in too small a volume of MeOH (2.5 ml) resulting in large variations in extracted amounts of pigment among replicates. Due to this variation in absolute amount of pigments these samples were included in analyses of relative contributions of pigments/phytoplankton groups only.

Phytoplankton composition was calculated by the use of the matrix-factorisation program, 'CHEMTAX' (Mackey et al. 1996) for estimating algal class abundances from concentrations of different pigments. CHEMTAX uses an input matrix of measured pigment concentrations and a matrix of pigment ratios for the different algal groups to provide a final matrix of the phytoplankton composition, expressed as group-specific contributions to total Chl a, in all samples. As input matrixes we used pigment concentrations of natural samples and pigment ratios obtained from Henriksen et al. (2002) (Table 3). To account for 19'-butanoyloxyfucoxanthin detected in several samples data on pigment ratios of pelagophytes from Mackey et al. (1996) were included. Prochlorophytes that are present in the Mediterranean contain divinyl chlorophyll a (DV Chl a) which could not be separated from Chl a by the HPLC method used. For calculation of the possible contribution of prochlorophytes to the total Chl a concentrations the pigment ratio of *Prochlorococcus* (Mackey et al. 1996) was included where DV Chl a was substituted by Chl a with which it will co-elute.

Table 3. Ratios of individual pigments to chlorophyll a in phytoplankton groups included in the CHEMTAX analyses. Prasinophycea_1 and _2 represent prasinophytes with and without prasinoxanthin, respectively. Ratios from Henriksen et al. (2002) with addition of pigment ratios from Prochlorococcus (Mackey et al. 1996) representing prochlorophytes.

Class	Chl c1+c2	Perid	But-fuco	Fuco	Hex-fuco	Neo	Pras	Viola	Allo	Lut	Zea	Chl b
Dinophyceae	0.707	0.731										
Pelagophyceae	0.127		0.782	0.487								
Prymnesiophyceae	0.136			0.089	0.735							
Bacillariophyceae	0.287			0.478								
Cryptophyceae	0.180								0.442			
Prasinophyceae_1						0.080	0.346	0.080		0.011	0.054	0.663
Chlorophyceae						0.045		0.065		0.139		0.334
Prasinophyceae_2						0.097		0.197		0.011	0.010	0.601
Nostocophyceae											0.627	
Prochlorophycea											0.321	1.074

Separation of samples and locations based on pigment profiles or calculated (CHEMTAX) phytoplankton community composition was done using the PRIMER software (PRIMER 6 version 6.1.12, PRIMER-E Ltd.). Multidimensional scaling (MDS) was used to illustrate separation of sampling regions based on measured concentrations of pigments in individual samples or based on calculated community compositions (contributions of individual chemo-taxonomical phytoplankton groups to total chlorophyll a). Significance of similarity between sampling regions/stations was analysed by ANOSIM with a 0.1% significance limit. A BIOENV analysis (PRIMER) was undertaken to find the best match between the multivariate among-sample patterns of the pigment/phytoplankton assemblages and that from environmental variables associated with those. For this analysis it was necessary to restrict the localities to those with accompanying data on TN, TP, salinity and temperature. These were Mallorca, Helsinki sea area, the Basque coast, Århus Bugt, Gilleleje, Nivå Bugt, Horsens Fjord and Roskilde Fjord (Table 2).

A ratio of phytoplankton pigments has been suggested as a measure of trophic status of oceanic waters (Claustre, 1994). This Fp ratio was calculated as

$$Fp = (fucoxanthin + peridinin) / (fucoxanthin + peridinin + 19'\text{-}butanoyloxyfucoxanthin + 19'\text{-}hexanoyloxyfucoxanthin + alloxanthin + zeaxanthin + chlorophyll\ b)$$

and tested for relationship with TN and TP.

Results

Total Chl a

A very strong correlation ($R^2 = 0.844$) between TN and total chlorophyll a was found across the different geographical sampling stations (Fig. 1). Chlorophyll a also increased with increasing TP up to a TP concentration of approximately 1 µM.

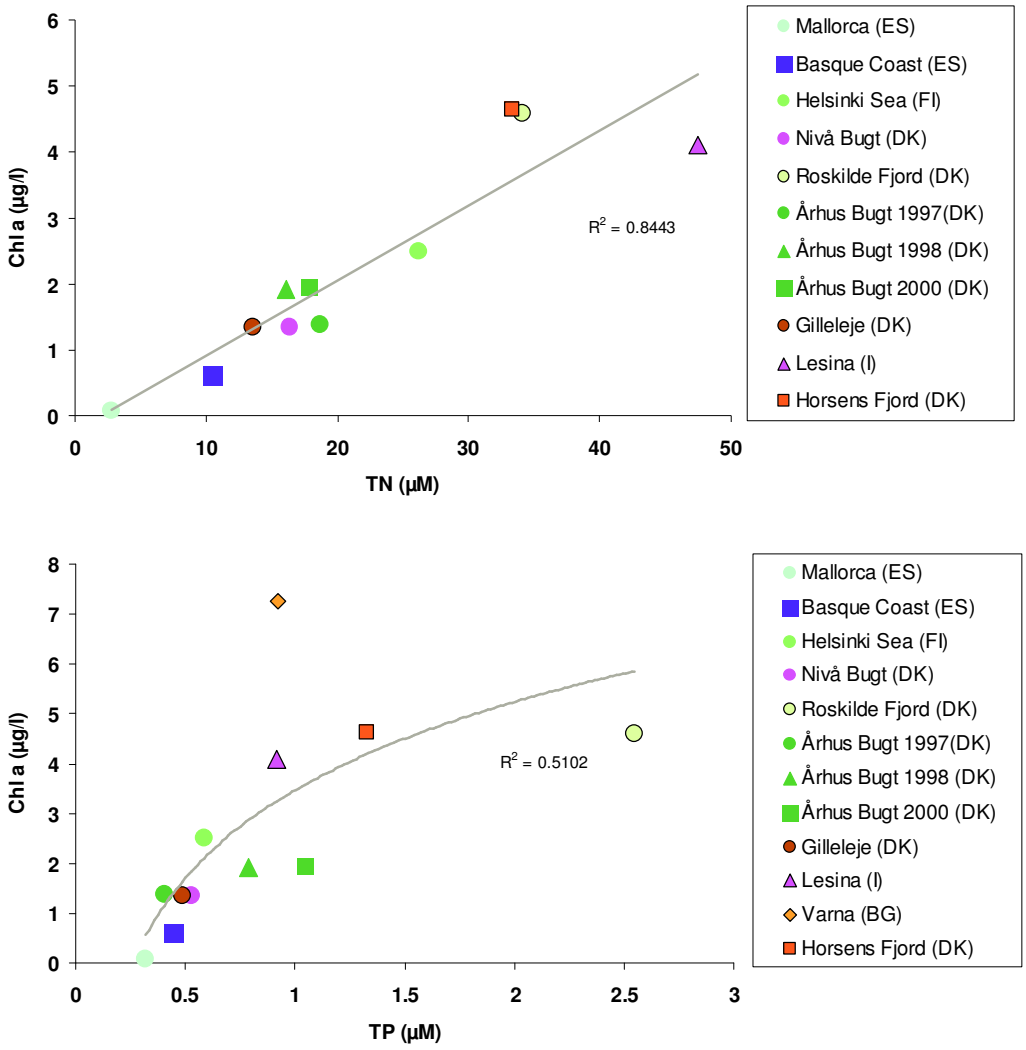


Fig. 1. Relationships between chlorophyll a total N (TN) and total P (TP), respectively. Markers represent average values of all samples from the given sites. Due to problems during extraction of pigments from samples from the Helsinki sea area and the Basque coast markers from these areas represent chlorophyll a concentrations determined by photo spectrometry. TN and TP values from Lesina represent measurements of annual values during 2007.

Phytoplankton pigment content and community composition

The relative contribution from individual pigments to total pigment content (chlorophylls plus carotenoids) differed between sampling sites and also between sampling years in Århus Bugt where samples were collected during three years (1997, 1998 and 2000) (Figs. 2 and 3). Phytoplankton community structure calculated using CHEMTAX showed different compositions in different areas. The most dominant phytoplankton groups were diatoms and dinoflagellates. Several minor groups, most notably cryptophytes, chlorophytes and cyanobacteria, contributed up to 40% of the total chlorophyll a (Fig. 2).

The phytoplankton community in Århus Bugt differed between the years 1997, 1998 and 2000 with a pronounced dominance by diatoms in 1997 and approximately equal contributions from diatoms and dinoflagellates in 1998 and 2000. The seasonal variation in the community composition in Århus Bugt was as pronounced as the variation between all sampling locations (Figs. 2 and 3). Based on the input pigment ratio for CHEMTAX a minor contribution (ca. 5%) from prochlorophytes was calculated for the samples from the Balearian Island Mallorca. This site also had the highest contribution from cyanobacteria.

MDS analysis showed separation of sampling locations based on pigment content as well as based on calculated phytoplankton groups (Fig. 3). When using phytoplankton groups calculated by CHEMTAX all sampling locations were significantly different except samples from the three Danish stations Gilleleje, Nivå Bugt and Århus Bugt 1997. Samples from Århus Bugt 1997 and Århus Bugt 1998 were different at a significance level of < 1% while those from Århus Bugt in 1997 and 2000 were not significantly different at the 5% significance level. Using relative pigment content all sampling locations were different at the < 1% significance level except the Danish stations Gilleleje and Århus Bugt 1997 and Nivå Bugt and Århus Bugt 1997 that were not different at the 5% significance level.

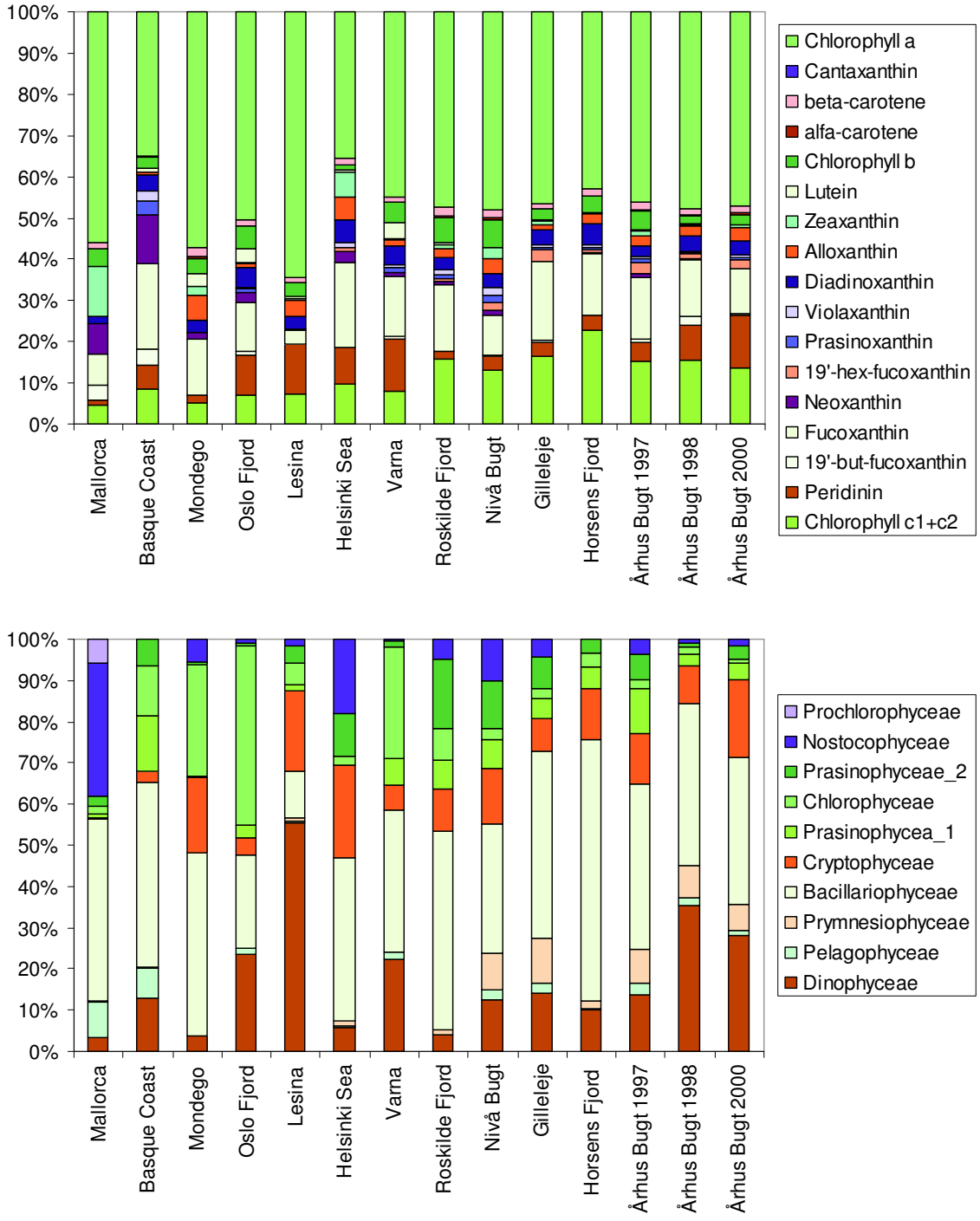


Fig. 2. Pigment and phytoplankton community composition at different sampling sites. Top panel shows average relative contributions to total pigment content and bottom panel average relative contribution from individual phytoplankton groups to total chlorophyll a calculated using CHEMTAX. Prasinophytes with and without prasinoxanthin are represented as prasinophytes_1 and _2, respectively.

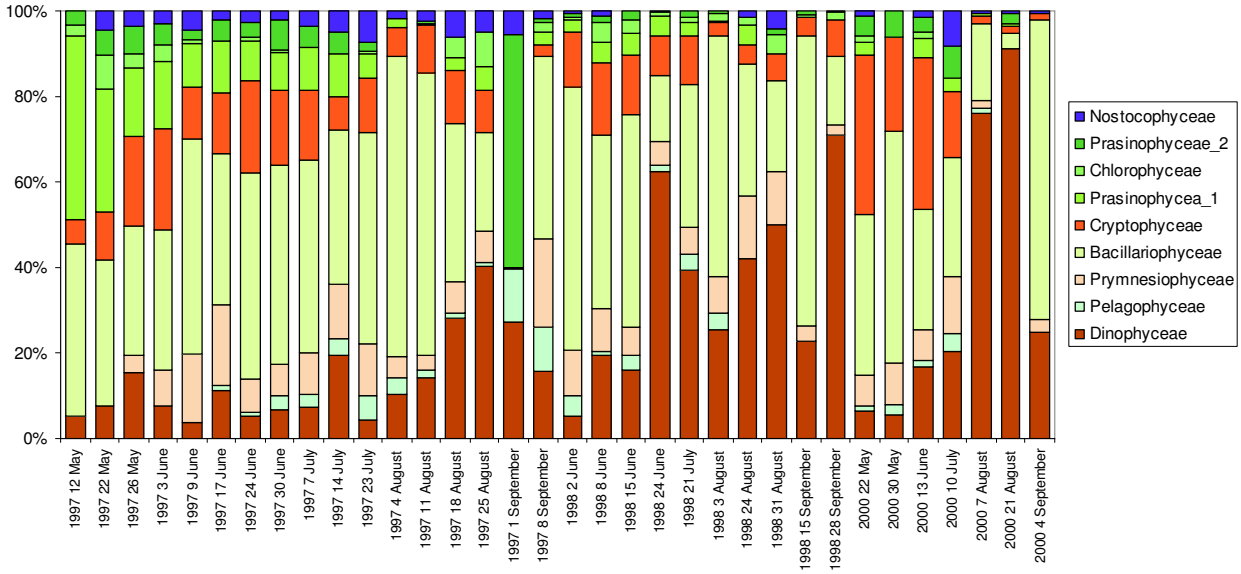


Fig. 3. Phytoplankton community composition in Århus Bugt (DK) at different sampling dates during the summers 1997, 1998 and 2000. Composition is show as relative contribution from individual phytoplankton groups to total chlorophyll a calculated using CHEMTAX. Prasinophytes with and without prasinoxanthin are represented as prasinophytes_1 and _2, respectively.

When the MDS pattern of samples based on pigment content was analysed, BIOENV revealed the best match (correlation = 0.564) between the sample pattern and a combination of the TN, salinity and temperature as environmental parameters. An almost similar correlation (0.559) was obtained for a combination of salinity and temperature only while TN alone, TN and TP, or TP alone provided much lower correlations (0.313, 0.186 and 0.005, respectively) (Table 4).

BIOENV analysis of samples distribution pattern of samples based on CHEMTAX calculated phytoplankton communities showed the best match (correlation = 0.490) with a combination of the environmental parameters TN, salinity and temperature. Excluding salinity and temperature from the analysis reduced the correlation to 0.299 when matched to TN only and 0.186 when matched to both TN and TP (Table 4).

Thus, the BIOENV analyses showed a major influence from salinity and temperature on the among-sample patterns. The main explanatory "eutrophication" parameter was TN but with only a limited importance relative to salinity and temperature.

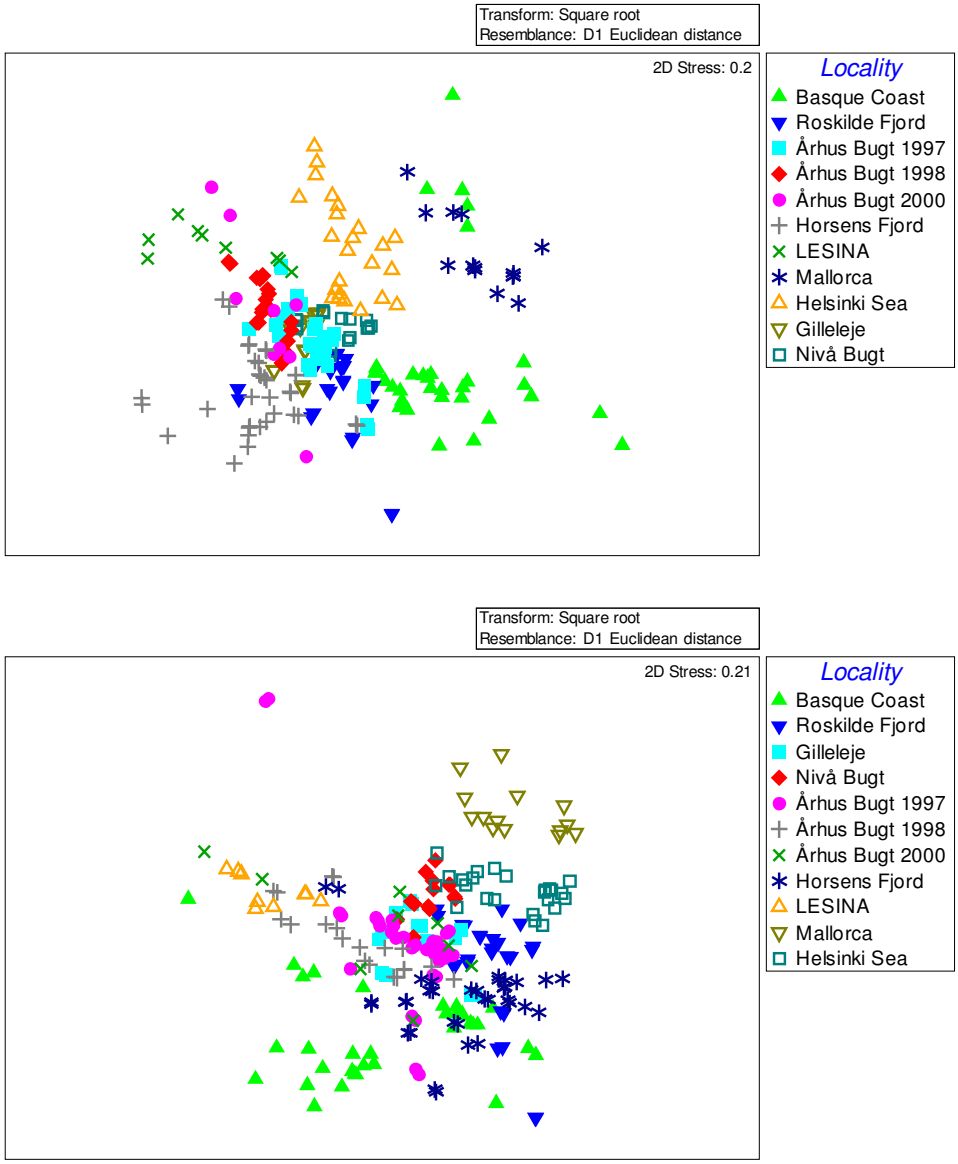


Fig. 3. Multidimensional scaling of individual samples from the different localities. Top panel shows samples distributed according to relative content of pigments, bottom panel shows distribution of samples based on phytoplankton community composition as calculated using CHEMTAX. Only localities with associated environmental data on TN, TP, salinity and temperature are included. TN and TP data from Lesina originated from a previous study in 2007covering only three of the five WISER stations.

Table 4. Spearman rank correlation between sample resemblance and combinations of the environmental variables TN, TP, salinity and temperature (BIOENV analysis, PRIMER software).

	Pigment concentrations		Phytoplankton groups	
Environmental variables	Correlation	Environmental variables included	Correlation	Environmental variables included
1 TN	0.564	1,3,4	0.490	1,3,4
2 TP	0.559	3,4	0.460	3,4
3 Salinity	0.511	1,3	0.429	1,3
4 Temperature	0.497	3	0.395	1,4
	0.427	1,4	0.374	All
	0.421	All	0.359	3
	0.397	4	0.343	4
	0.396	2-4	0.334	2-4
	0.341	1-3	0.302	1-3
	0.313	1	0.299	1
	0.186	1,2	0.186	1,2
	0.005	2	0.010	2

Individual phytoplankton pigments

Concentrations of individual pigments correlated better with TN than with the other environmental parameters as exemplified for fucoxanthin in Figure 4. However, when including all samples the scatter around the regression line was very large and correlations generally poor. Scatter was reduced and correlations improved when analyses were performed on averaged values for the individual stations (Fig. 5).

In general the concentrations of individual pigments were significantly correlated with TN (Figs 5, 6). The ratio Fp was neither correlated to TN nor TP (Fig. 7).

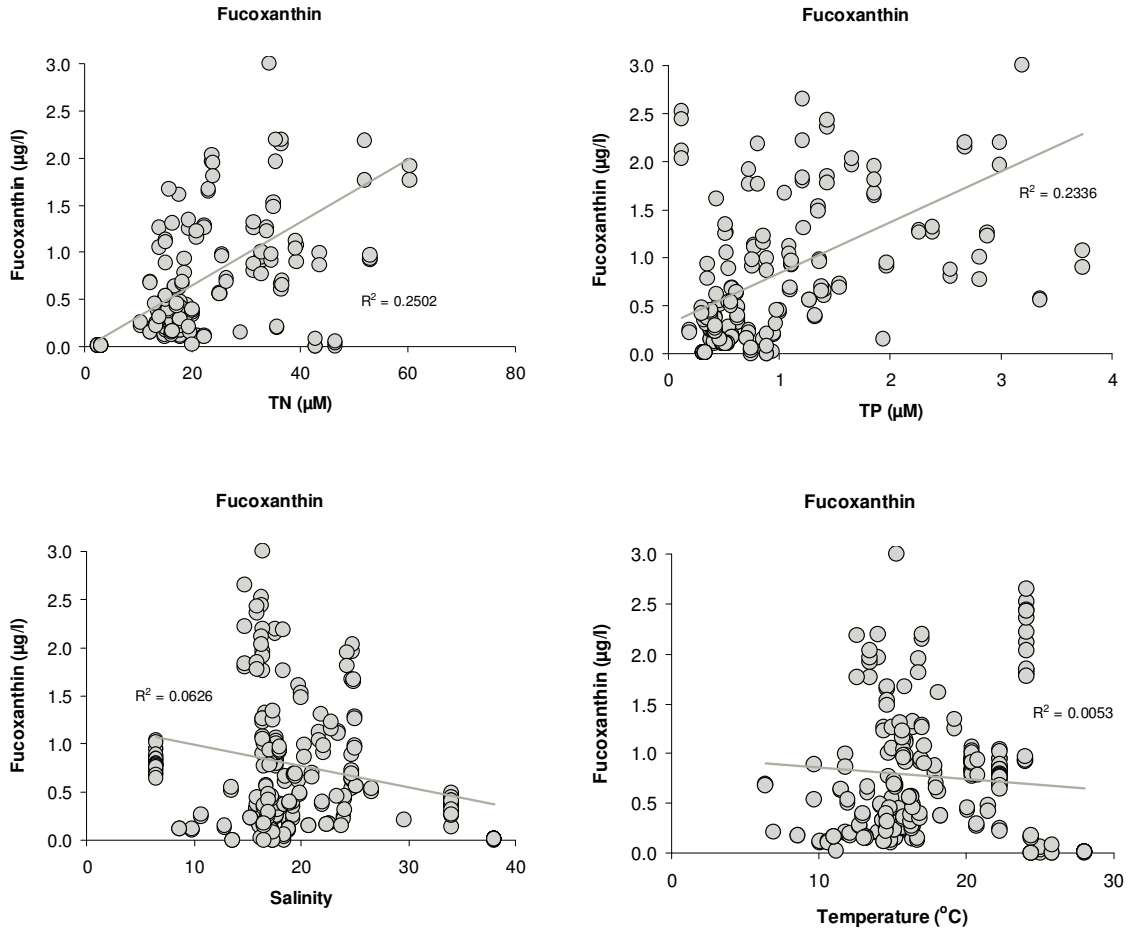


Fig. 4. Relationships between concentrations of fucoxanthin and environmental parameters. All samples except those from the Basque coast and Helsinki sea area included.

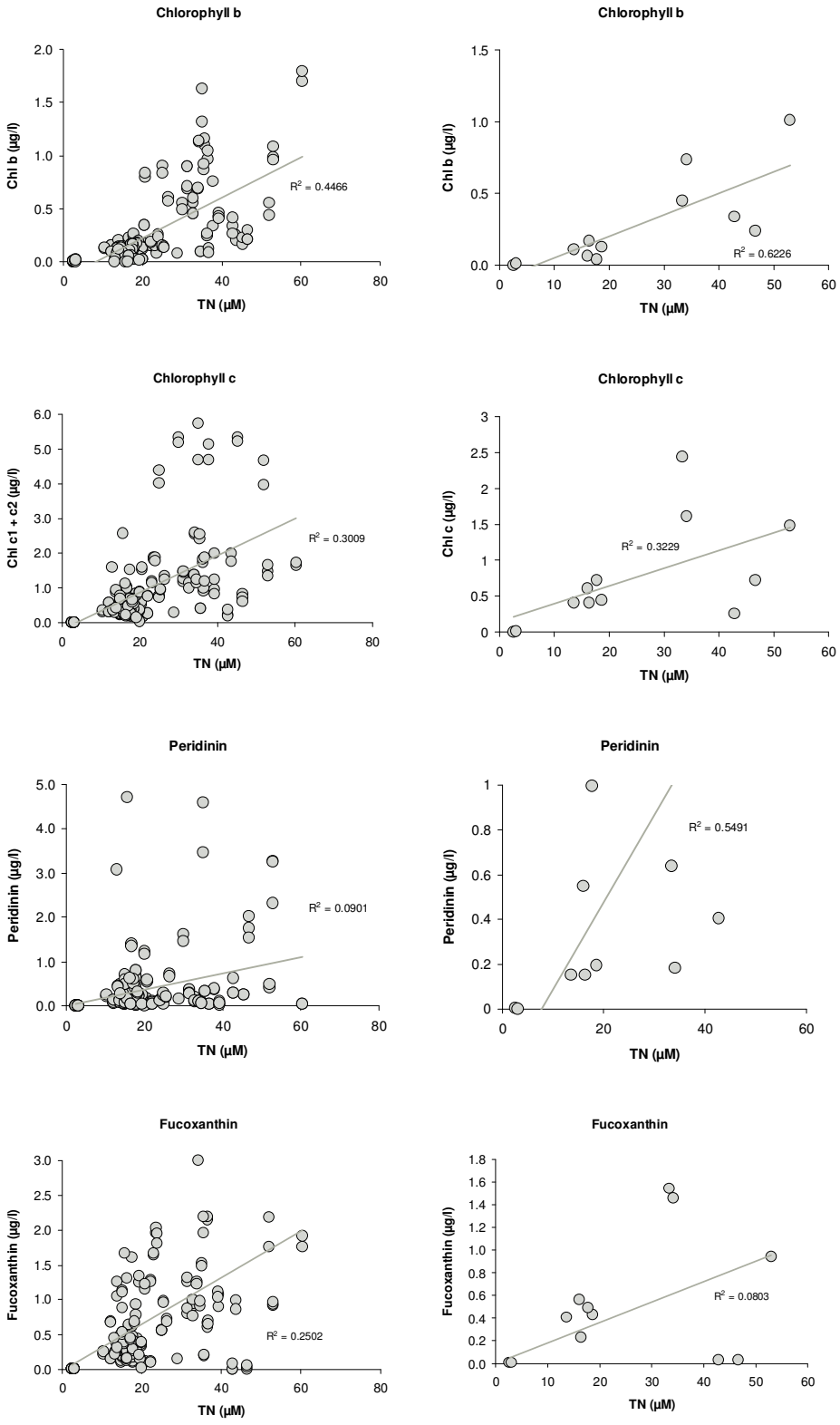


Fig. 5. Relationships between TN and chlorophylls and carotenoids when including all samples (left panels) or averages for individual stations only (right panels).

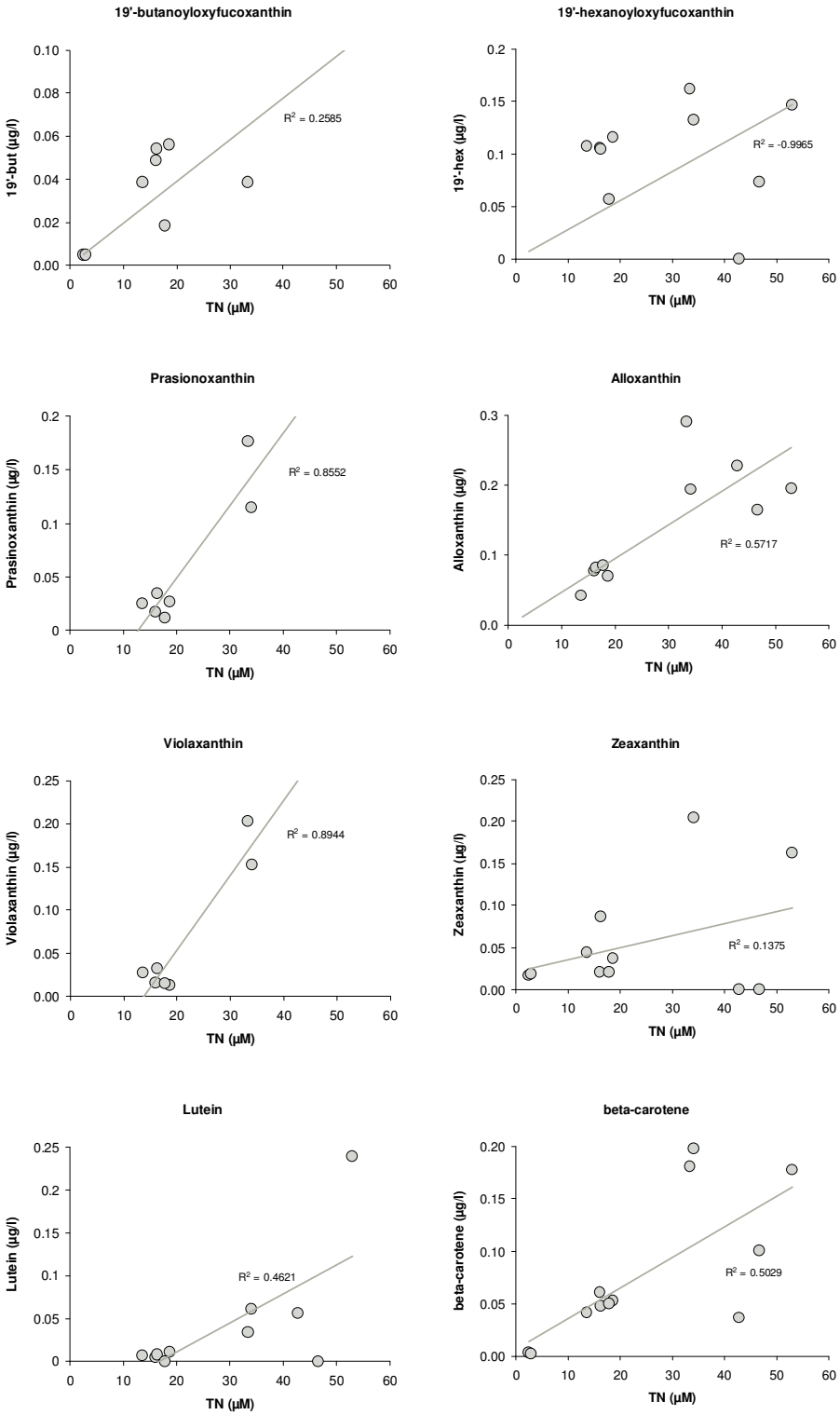


Fig. 6. Relationships between TN and carotenoids at stations with concurrent data on pigments and environmental parameters. Markers represent station averages.

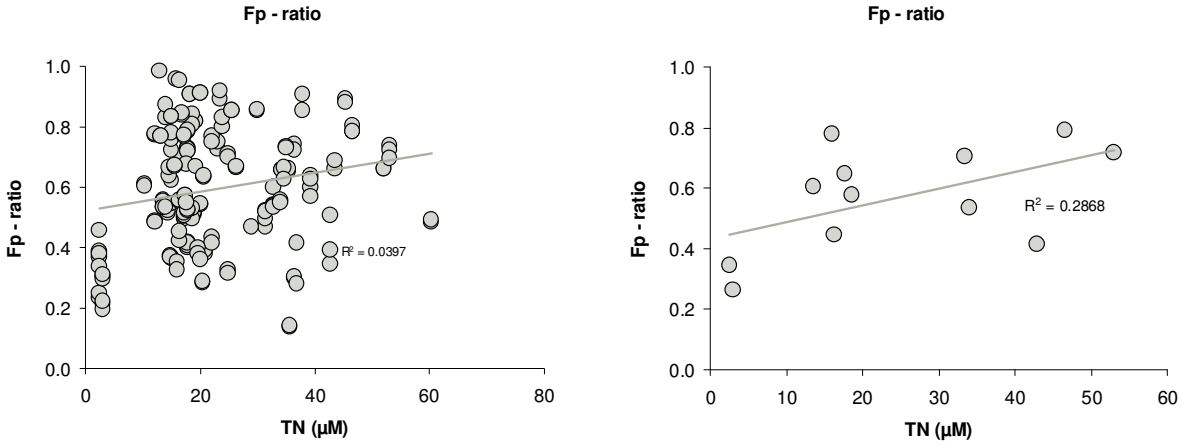


Fig. 7. Relationships between TN and the Fp ratio when including all data (left panel) and average values for each station (right panel).

Phytoplankton chemotaxonomical groups

BIOENV analyses on distribution patterns of samples characterised by either relative pigment content or calculated phytoplankton groups showed correlation with TN. Thus, for the analyses of relationship between individual phytoplankton groups and pigments TN was chosen as the environmental parameter representing tropic status of the different sampling localities.

With the chosen input ratio file for CHEMTAX the contribution from each of eight phytoplankton groups to the total Chl a was calculated. No distinct correlations with TN were found (Fig. 8). For some groups (dinoflagellates and prasinophytes type 2) the highest contributions were found at stations with the highest TN concentrations. In contrast, the highest contributions from pelagophytes, cyanobacteria, prochlorophytes and prasinophytes type 1 were found at stations with low TN concentrations (Fig. 8). These station averages do, however, cover very large within-station variations in contributions from the given groups to the individual samples as exemplified for dinoflagellates in Fig. 9.

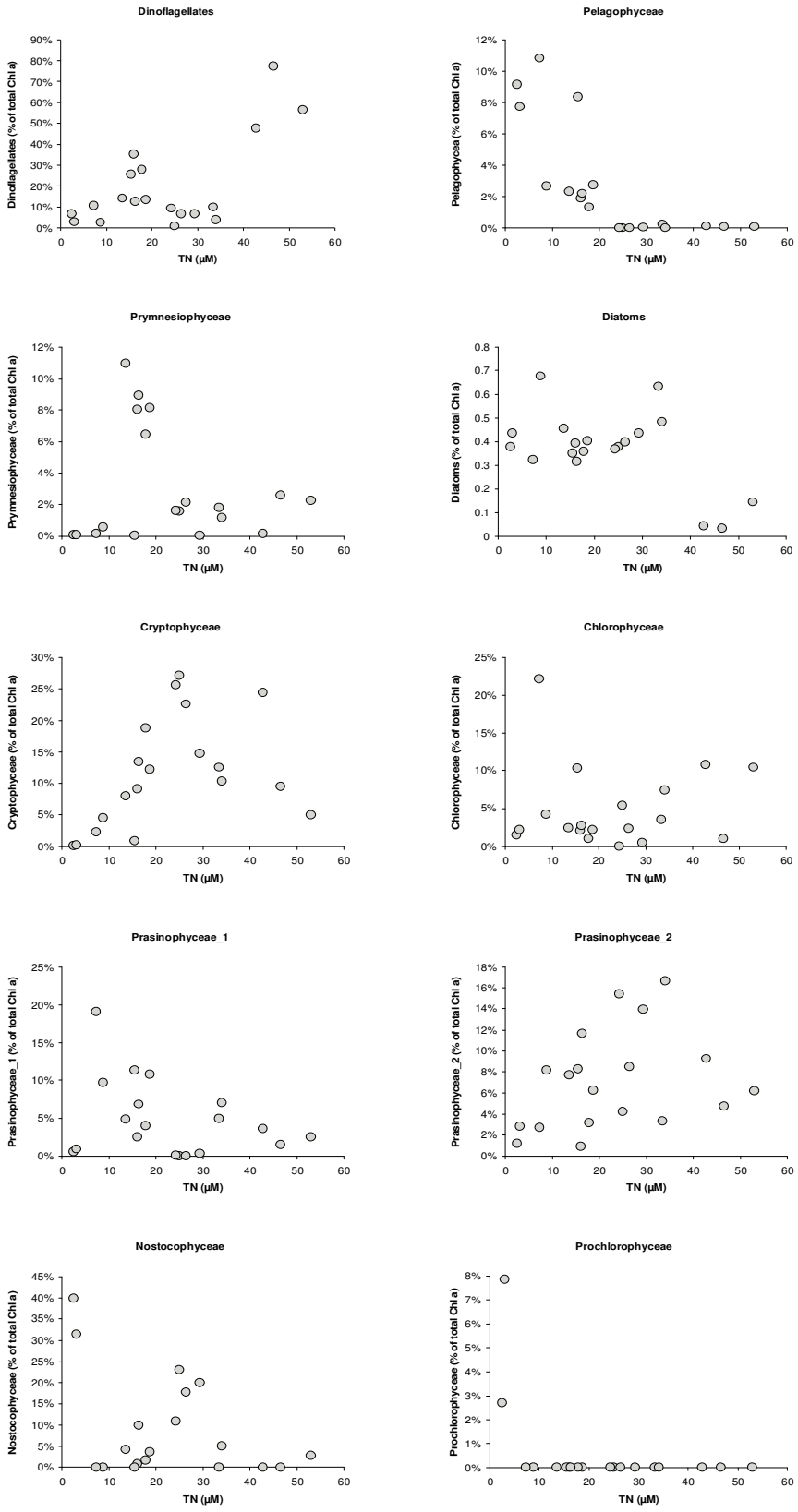


Fig. 8. Relationships between TN and contribution from different chemotaxonomical phytoplankton groups to total Chl a. Markers represent average values at stations

with concurrent data on pigments and TN.

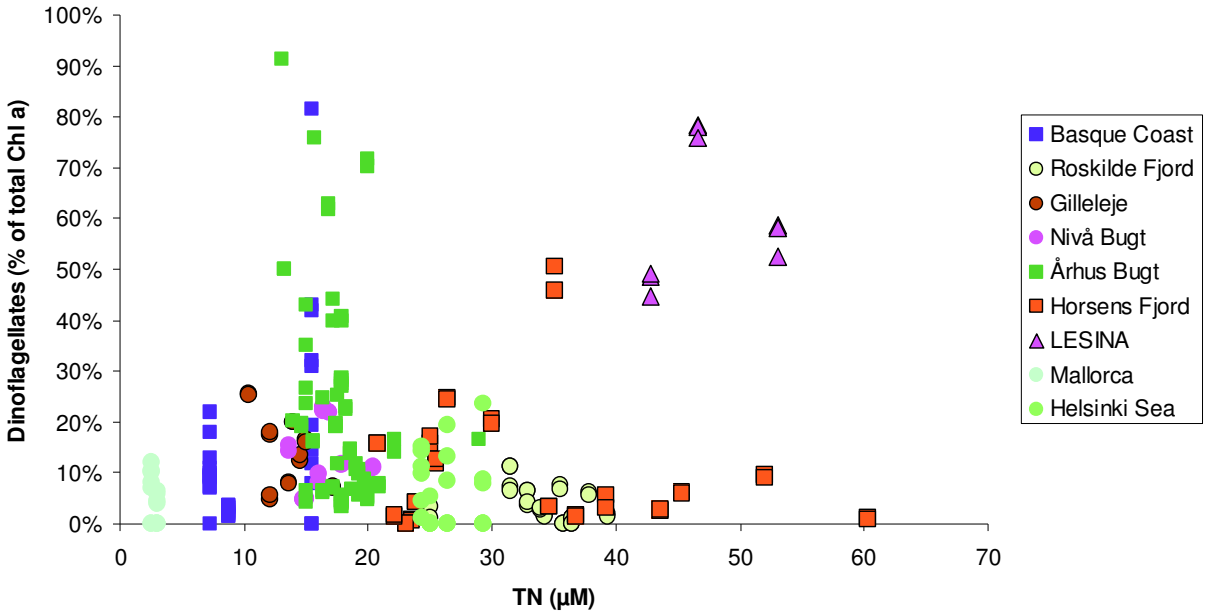


Fig. 7. Relationships between TN and the contribution from dinoflagellates to total Chl a in individual samples from all stations with measurements of TN.

Discussion

Classification of ecological status according to the WFD requires 1) indicators ('metrics') for the biological quality elements that respond to specific pressures, 2) establishment of dose-response relationships between the pressure and the indicator and 3) definition of reference conditions and acceptable deviations from reference conditions.

Chl a has a long history as one of the most important biological parameters in oceanography (Jeffrey et al. 1997), and Chl a - which is the only sub element of the BQE phytoplankton that was intercalibrated in the first round of WFD intercalibration – was significantly correlated with TN across the geographically different WISER sampling localities. The Chl a measurement is a very rapid, cost-effective and easily reproducible technique. However, it only estimates the

biomass, without giving any information about the community composition that the WFD requires (Domingues et al., 2008). Therefore, data on the whole suite of phytoplankton pigments found in the WISER samples were used for analysis of phytoplankton communities at the different localities.

Whether characterised by pigment composition or by composition calculated using CHEMTAX, communities differed between all but a few Danish sampling locations. The difference between years at the Danish station Århus Bugt was as significant as differences between stations. This illustrates major inter-annual as well as seasonal variations in phytoplankton communities at single stations. Thus, the inter-annual variation needs to be addressed prior to attempts to establish assessment systems based on community structure. A WFD-compliant tool taking into account seasonal succession of taxonomic groups was described by Devlin et al. (2007). This tool is based on characteristic seasonal variations in abundance of four different phytoplankton groups/species (diatoms, dinoflagellates, flagellates and *Phaeocystis*). Seasonal abundances of organisms belonging to one of these groups are compared to the seasonal 'growth envelope' at reference sites. This approach requires very good knowledge of the seasonal and the inter-annual variability of different phytoplankton groups and therefore several years of data collected at high frequency. Most EU Member States suffer from a lack of sufficient data sets or reference sites – or both – necessary to develop and apply this type of tool.

In addition to Chl a, the distribution pattern of the WISER phytoplankton pigment samples and calculated communities correlated to TN as a measure of eutrophication. However, this correlation was in it self low and the major environmental parameters affecting the distribution of samples were salinity and temperature. Even within a much more narrow salinity range, as that found in the Baltic Sea region, the salinity has proved a major structuring factor the phytoplankton community composition (Gasiunaite et al. 2005). In Helsinki Sea area, for example, the changes in phytoplankton composition in the outer archipelago were linked to variations in nutrient concentrations, salinity and temperature during 1969-2003 (Pellikka et al. 2007). In the case of the phytoplankton, classification systems for assessment of environmental status should address eutrophication only. Similarly neither of the calculated contributions of single phytoplankton groups nor the Fp-ratio showed distinct significant responses to eutrophication. The reason for this could be a general lack of response of phytoplankton community composition to eutrophication or an underlying change in dominant species/groups that does not appear in the very broad-brushed characterisation of phytoplankton communities using pigments. Quoting Tett et al. (2007): "*In the case of freshwater phytoplankton, shifts from desmids, chrysophytes or diatoms to cyanobacteria are known to be associated with nutrient enrichment (Hutchinson, 1969; Talling and Heaney, 1988). In contrast, and except for the Baltic Sea, where blue–green bacteria have increased with nutrient enrichment (Finni et al., 2001), the marine situation is less clear. Increases in the ratio of N to Si may cause increases in the proportion of non-silicified algae (Gillbricht, 1988; Tett et al., 2003b), and this has led to*

proposals for indicators based on the ratio of diatoms to dinoflagellates. Care must be taken in the use of simple, growth-season-averaged, ratios of this sort, since they can underestimate the effect of nutrient pressure on well-stirred waters where diatoms, including resuspended benthic diatoms, are natural dominants. Setting EQSs from such ratios tends to reflect the view that 'diatoms' are 'good' and 'flagellates' or 'dinoflagellates' are 'bad', which misunderstands the multiple roles that each group plays in marine ecosystems. For example, dinoflagellate lipids can make important contributions to the diet of crustacean zooplankton. More generally, it is apparent that the phytoplankton encompasses a wide range of biochemical, taxonomic and functional diversity (Delwiche et al., 2004; Jeffrey and Vesk, 1997; Tett et al., 2003b), and it seems unwise to ignore this diversity in assessing the health of the plankton. It is also desirable to take into account the natural, especially seasonal, variability that is an essential part of phytoplankton ecology".

MDS patterns of the WISER samples correlated with TN but more importantly with the environmental parameters salinity and temperature. WFD classification requires definition of reference conditions. Reference conditions can be established from data collected at reference sites or through modelling of conditions from established dose-response relationships between the pressure and the given indicator. The major influence from salinity and temperature on the distribution pattern of the WISER samples hinders the use of any of these sampling stations as reference sites for a pigment based phytoplankton indicator. Thus, reference conditions need to be defined within the WFD typologies which will in some regions, like the Baltic Sea, not be possible due to the generally impaired conditions.

Reference conditions for pelagic pigment concentrations at the examined localities are not available. In a limited number of other areas records of fossil pigments may be available. At best, and given a continued sampling of pelagic pigments for comparison over a longer period, these fossil records may provide reference conditions for individual pigments. However, due to different and commonly unknown accumulation and preservation rates of the different pigments in sediments (Leavitt & Findlay 1994) it will not be possible to establish a quantitative reference phytoplankton community from the fossil record. Some pigments are very labile and therefore rarely found in the sediments (e.g. the 'marker pigment' peridinin found in most dinoflagellates) and an attempt to correlate pelagic monitoring data with sediment pigment profiles in four northern European estuaries was unsuccessful (Reuss et al. 2005).

Very few pigments are unique to specific phytoplankton groups. Most pigments are shared by several groups leaving the individual pigments as poor indicators. Several authors have noted the ambiguous character of some pigments and their potential to erroneous class assignation (Irigoien et al., 2004; Llewellyn et al., 2005; Laza-Martínez et al., 2007; Eker-Develi et al., 2008). The inclusion of not only major, but also minor non-conventional pigment markers (e.g.

Zapata et al., 2004) in CHEMTAX matrices is essential to attain a real picture of the community. In addition, a rapid screening at the light microscope has been recommended to assign some pigments to the correct phytoplankton class (Seoane et al., in revision). This may improve the description of phytoplankton communities by the use of pigments.

The most specific pigments may have a potential as indicators. However, this requires that the phytoplankton group they characterise is also a useful indicator. At present very few single species/group indicators have been identified (*Phaeocystis* (Gypens et al. (2007), possibly filamentous cyanobacteria in the Baltic (Finni et al. 2001)). Based upon field data, in the Baltic Sea, Sagert et al. (2005) suggested the autotrophic ciliate *Myrionecta rubra* as a possible indicator for eutrophication, whereas Jaanus et al. (2009) proposed oscillatorialean cyanobacteria and two diatoms species *Cyclotella choctawhatcheeana* and *Cylindrotheca closterium*. In Mediterranean coastal waters Spatharis et al. (2007) related the harmful diatom *Pseudo-nitzschia calliantha* with extreme nutrient enrichment. Also, experiments have showed that diatoms have higher growth efficiency in favourable conditions comparing to other groups (Cermeño et al., 2005). The occurrence of these individual potential indicator species will, however, be masked when analysing communities using pigments only. The ciliate *Myrionecta rubra* will 'appear' as cryptophytes while the mentioned diatoms will be included in the generally large pool of diatoms. Another difficulty when developing a composition-based assessment tool is that a particular taxon can behave similarly in response to nutrient enrichment due to anthropogenic factors (such as disposals) or to natural factors (such as upwelling, rain events or salinity-gradients).

At the group level, the increases in the biomasses of chrysophytes and cyanobacteria during 1969-2003 in Helsinki Sea area correlated with the decrease in salinity, whereas the decrease in the biomasses of cryptophytes correlated with the decrease in the concentrations of inorganic and total nitrogen (Pellikka et al. 2007). At species level, for instance, the decrease in the biomass of *Achnanthes taeniata*, an abundantly occurring diatom in the outer archipelago, correlates with an increase in the concentrations of inorganic phosphorus. The total biomasses (as biovolume and Chl a) and the biomasses of the cyanobacterial species, *Aphanizomenon* and *Nodularia spumigena*, were high in the Gulf of Finland in the early 2000s (Raateoja et al. 2005, Pellikka et al. 2007, Suikkanen et al. 2007), which can be explained by the release of inorganic phosphorus from the sediment due to internal loading (Pitkänen et al. 2001, Vahtera et al. 2007). In case of *Nodularia spumigena* the characteristic pigments of this species may be used as a measure of the abundance of this species (Henriksen 2005) which combined with reference conditions and dose-response relationships with eutrophication may serve as a useful pigment based indicator.

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