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Detection of the toxic cyanobacteria *Nodularia spumigena* by means of a 4-keto-myxoxanthophyll-like pigment in the Baltic Sea

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ABSTRACT: The pigment composition of cyanobacterial species regularly forming blooms in the Baltic Sea was investigated. A 4-keto-myxoxanthophyll-like pigment was found in 2 strains of the toxic *Nodularia spumigena* isolated from the SE Baltic Sea. Strains of *Aphanizomenon* sp. and *Anabaena lemmermannii* did not contain this rarely observed pigment. The 4-keto-myxoxanthophyll-like pigment was also found in samples taken during intense blooms of *N. spumigena* and was found to be correlated with the concentration of the algal toxin nodularin (r = 0.97). *N. spumigena* could be detected by the 4-keto-myxoxanthophyll-like pigment at very low abundances by HPLC, i.e. down to 0.4 µg chlorophyll *a* (chl *a*) l^{-1}. As it is rapid, very sensitive and objective, this method can be used for early warning of toxic blooms of cyanobacteria in the Baltic Sea. CHEMTAX software was used to estimate the contribution of cyanobacteria species as well as other algal classes present to total chl *a*.

KEY WORDS: *Nodularia spumigena* · Phytoplankton pigments · Nodularin · Chemotaxonomy · CHEMTAX · HPLC

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INTRODUCTION

Blooms of cyanobacteria are a recurrent phenomenon during the late summer months in the brackish Baltic Sea, and 3 genera are commonly found in the blooms: *Nodularia*, *Aphanizomenon* and *Anabaena* (Kononen 1992, Kahru et al. 1994). In the genus *Nodularia*, the bloom-forming species is *N. spumigena*, and its blooms are problematic, since this species is toxic. It produces the algal toxin nodularin, a hepatotoxin which poses a health risk for humans and animals (Kononen 1992) by inhibiting protein phosphatases and being a potent tumor promotor (Kuiper-Goodman et al. 1999). It is therefore crucial to be able to identify and determine the presence, identity and abundance of this cyanobacterium.

Detection and determination of the abundance of harmful algae are usually carried out using microscopy. Microscopic enumerations are, however, time-consuming, imprecise, and require taxonomic skills (Schlüter et al. 2000), and particularly the quantification of filamentous species, such as the bloom-forming cyanobacteria in the Baltic Sea is difficult. There is a need for new methods that can identify the bloom-forming cyanobacteria precisely. Image analysis has recently been developed for the automatic determination of the biovolume of the 3 bloom forming cyanobacteria in the Baltic Sea (Congestri et al. 2000, 2003). Another chemotaxonomical method, that is being increasingly used, especially in the oligotrophic environment where algae cells are too small to be identified by normal microscopy methods, is HPLC analysis of the phytoplankton pigments to determine the species composition of the phytoplankton (e.g. Jeffrey et al. 1997). This method is fast and has been shown to have a high sensitivity and reproducibility in detecting and identifying the different phytoplankton groups (Schlüter et al. 2000). Furthermore, the biomass of the individual phytoplankton groups can be determined as chlorophyll *a* by using, e.g., the CHEMTAX program (M. D. Mackey et al. 1996, D. J. Mackey et al. 1998). As will be shown in this study, in some instances the

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HPLC method can be used to detect bloom-forming cyanobacteria to species level. The HPLC method has so far mostly been restricted to identification of phytoplankton to class level, although a few studies have identified species based on pigment analyses (e.g. Millie et al. 1995, Örnólfsdóttir et al. 2003).

To investigate the pigment content of the common bloom-forming cyanobacteria in the Baltic Sea, different strains of cyanobacteria isolated from the Baltic Sea were cultured under varying light climates and nutrient conditions. The results of the culture experiments were used for identifying and calculating the biomass of the bloom forming cyanobacteria as well as of other phytoplankton groups sampled in the summer months in the SE Baltic Sea.

MATERIALS AND METHODS

Culture experiments. Cultures of various cyanobacteria isolated from the southern and central parts of the Baltic Sea were obtained in 2001 from the Kalmar Algae Collection (KAC), University of Kalmar, Sweden: 2 strains of *Nodularia spumigena* (KAC 13 and KAC 66), 2 strains of *Aphanizomenon* sp. (KAC 15 and KAC 63), and 1 strain of *Anabaena lemmermannii* (KAC 16). The algae cultures were grown in a 16:8 h light:dark cycle at 17°C in Keller medium (Keller et al. 1987) adjusted to 7‰ in 1 l glass bottles. The algae cultures were aerated and stirred gently with a magnetic stirrer to keep them homogeneous, and were grown at 3 different light intensities using Pope fluorescent tubes (36W/33) — low light (LL), medium light (ML), and high light (HL) — as described by Schlüter et al. (2000). Incident irradiances were 23, 230, and 554 (light from 2 sides) µmol photons m^{-2} s^{-1}, for the LL, ML and HL treatments respectively, but the actual light intensity reaching the cells was lower due to self-shading.

Fig. 1. Sampling stations in the western part of the Baltic Sea. Stns 1, 2 and 3 were sampled in 2001; (•) sampling positions in 2002

In order to keep cell growth exponential, the cultures were maintained as semi-continuous batch cultures by adding fresh medium daily to maintain initial cell density, as described in Schlüter et al. (2000). The cultures were grown for 4 d to ensure that the cells were acclimated to the relevant light conditions. Cells were then harvested at noon of the fifth day by filtering replicate subsamples through GF/F filters, which were immediately frozen in liquid nitrogen, i.e. within 1 min after removal of the cultures from the light source. For the ML cultures, the experiment was continued without dilution until cell division terminated (i.e. stationary growth SG) usually within 7 d. Subsamples were then filtered as described above.

***In situ* samples.** Water samples were taken from 3 stations in the western part of the Baltic Sea from April to October 2001: Stn 1 near the coast in Køge Bay, Stn 2 in the middle of Køge Bay, and Stn 3 in Hjelm Bay (as part of the Danish monitoring program) (Fig. 1). At Stn 3, samples were taken weekly between 0 and 10 m by sampling at 1, 2.5, 5, 7.5, and 10 m. The subsamples were pooled and mixed gently in a purified jar, and samples for pigment and nodularin analyses were filtered onto GF/F filters and immediately frozen in liquid nitrogen on board. Samples for microscopic determination of phytoplankton were fixed in Lugol's solution. At Stns 1 and 2 samples were taken every 2 wk by sampling at the surface at 1 m depth. In addition, an integrated sample was taken at Stn 2 at 0 to 10 m as described above. The samples from Stn 1 and 2 were taken to a laboratory and filtered within 4 h of sampling.

In 2002, samples were taken specifically at locations where visible plankton-like particles were present in the water during the period from 30 July to 4 September. These samples were taken at the sea surface, mainly near the Danish shore and beaches with a mass occurrence of algae (Fig. 1). Samples were also collected at various stations offshore during the same period (Fig. 1). Subsamples for pigment analysis, nodularin analysis and microscopic determinations were prepared and treated as described above.

Pigment analysis. The filters were thawed, placed in 100% acetone, sonicated in an ice-cold sonication bath for 10 min, and extracted at 4°C for 24 h. The extracts were then filtered through Teflon syringe filters to remove filter and cell debris, and 1 ml was pipetted into HPLC vials and placed in the cooling rack of the HPLC. Prior to injection, 300 µl water (HPLC grade)

was injected into each vial using a pretreatment program that included careful mixing of the sample. The samples were then injected into a Shimadzu LC-10A HPLC system with Class-VP software using the method of Wright et al. (1991), with a slight modification to the gradient: 0 min 100% Solvent A, 0% Solvent B, 0% Solvent C; 1 min 10% A, 90% B, 0% C, 6 min 10% A, 75% B, 15% C; 10 min 10% A, 40% B, 50% C; 19 min 10% A, 35% B, 55% C; 25 min 0% A, 100% B, 0% C; 32 min 100% A, 0% B, 0% C. (Solvent A was 80:20 methanol:0.5 M ammonium acetate (pH: 7.2) (v/v), Solvent B was 90:10 acetonitrile: H_2O (v/v) and Solvent C was ethyl acetate.) The column was a Spherisorb ODS2, 25 cm × 4.6 mm inner diameter, 5 µm particle size. The HPLC system was calibrated with pigment standards from DHI Water & Environment, Denmark. Peak identities were routinely confirmed by on-line photo diode array (PDA).

The biomass of the phytoplankton groups detected by the pigments was calculated by CHEMTAX (Mackey et al. 1996) using the average pigment ratios from Schlüter et al. (2000) and the average pigment ratios of the cyanobacteria cultured in this study (see 'Results').

Microscopy of *in situ* samples. Prior to the CHEMTAX calculations, 25 to 50 ml of each Lugol-preserved sample was allowed to sediment for 24 h in Utermöhl settling chambers, and then screened in an inverted microscope to identify the major phytoplankton groups.

Nodularin analysis. The filters were thawed and frozen 3 times, placed in 75% methanol, sonicated in an ice-cold sonication bath for 10 min, and extracted for 30 min. Extracts were then filtered through Teflon syringe filters to remove filter and cell debris. The extract was purified on Waters C-18 SPE cartridges, evaporated, and redissolved in 1 ml 75% methanol and 20 to 50 µl was injected into a Shimadzu LC-10AD HPLC system with Class-VP software with a symmetry C18 column (150 mm, 3.9 mm) using a gradient of 10 mM ammonium acetate (A) and acetonitrile (B): 0 min 78% A, 22% B; 15 min 28% A, 72% B; 15.5 min 22% A, 78% B; 25 min 22% A, 78% B. The oven temperature was 40°C, and nodularin was detected at 239 nm using PDA. Calibration was carried out using nodularin standards from ICN Biomedicals.

RESULTS

Culture experiments

The pigments detected in the algae cultures are shown in Table 1 in the order they eluted from the HPLC as pigment/chlorophyll *a* (chl *a*) ratios. The qualitative and quantitative pigment content of the 2 strains of *Nodularia spumigena* (KAC 13 and KAC 66) were identical, and this was also the case for *Aphani-*

Table 1. Pigment/chlorophyll *a* ratios in cyanobacteria cultures for each pigment in different culture conditions. LL: low light, ML: medium light, HL: high light, Avg: average pigment/chlorophyll *a* ratios for LL, ML and HL treatments. SG: stationary growth in medium light. For *Nodularia spumigena*, average of 2 strains (KAC 13 and KAC 66: 2 replicates for each stain) is given. For *Aphanizomenon* sp. average of 2 strains (KAC 15 and KAC 63: 2 replicates for each stain) is given. For *Anabaena lemmermannii* only 1 strain (KAC 16 was tested in replicate). Pigments listed in order in which they eluted from HPLC

Species Treatment	Oscilla-xanthin	4-keto-myxo-xanthophyll-like pigment	Aphanizo-phyll	Myxoxan-thophyll	4-keto-myxo-xanthophyll-like derivative	Cantha-xanthin	Zea-xanthin	Echine-none	β-crypto-xanthin	β-carotene
Nodularia spumigena										
LL		0.131		0.014	0.006	0.042		0.116		0.012
ML		0.217		0.033	0.029	0.086		0.085		0.009
HL		0.314		0.048	0.045	0.132		0.078		0.010
Avg		0.221		0.032	0.027	0.087		0.093		0.010
SG		0.192		0.017	0.016	0.081		0.114		0.009
***Aphanizomenon* sp.**										
LL	0.010		0.138	0.072		0.045	0.006	0.097	0.007	0.008
ML	0.029		0.181	0.236		0.050	0.017	0.122	0.018	0.010
HL	0.049		0.196	0.317		0.057	0.017	0.103	0.026	0.007
Avg	0.029		0.172	0.208		0.051	0.014	0.107	0.017	0.009
SG	0.016		0.097	0.083		0.024	0.012	0.080	0.012	0.007
Anabaena lemmermannii										
LL	0.009		0.086	0.068		0.019	0.004	0.050	(trace)	0.006
ML	0.019		0.134	0.189		0.022	0.004	0.065	(trace)	0.009
HL	0.044		0.191	0.327		0.039	0.007	0.092	(trace)	0.008
Avg	0.024		0.137	0.195		0.027	0.005	0.069		0.008
SG	0.018		0.141	0.132		0.037	0.014	0.076	(trace)	0.006

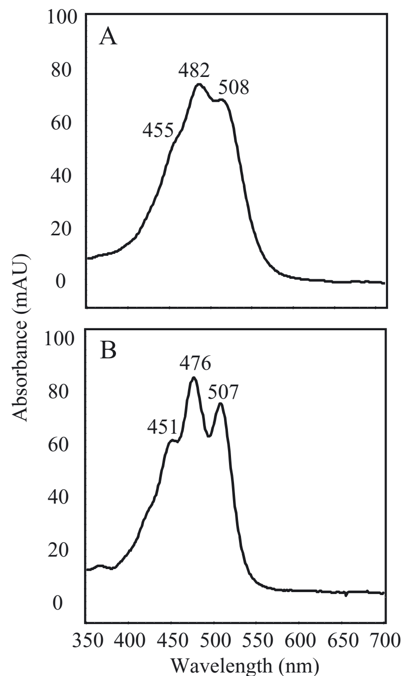

Fig. 2. Absorbance spectra in HPLC eluant of (A) 4-keto-myxoxanthophyll-like pigment (%III/II = 9, retention time 16.25 min) and (B) myxoxanthophyll (%III/II = 63, retention time 17.19 min)

zomenon sp. (KAC 15 and KAC 63) (Student's *t*-test, $p < 0.05$ for both species). The pigment/chl *a* ratios in Table 1 for *N. spumigena* and *Aphanizomenon sp.* are calculated as averages of the pigment concentrations of the 2 cultures, each with 2 replicates.

The pigment content of *Nodularia spumigena* was distinctly different from that of *Aphanizomenon* sp., and *Anabaena lemmermannii*, as it contained a pigment with an absorption spectrum similar to 4-keto-myxoxanthophyll that eluted before myxoxanthophyll (Fig. 2, Table 1). Furthermore, *N. spumigena* also contained a small amount of a derivative of this pigment, which absorbed similar to 4-keto-myxoxanthophyll, but eluted after myxoxanthophyll (Table 1). Beside these carotenoids, *N. spumigena* contained myxoxanthophyll, canthaxanthin, echinenone and β-carotene, which were also found in both *Aphanizomenon* sp. and *A. lemmermannii*. Furthermore, aphanizophyll and low amounts of oscillaxanthin and β-cryptoxanthin were found in *Aphanizomenon* sp. and *A. lemmermannii* (Table 1). The ratio of the 4-keto-myxoxanthophyll-like pigment to chl *a* was the highest pigment/chl *a* ratio observed, indicating that the 4-keto-myxoxanthophyll-like pigment is a significant pigment in *N. spumigena* (Table 1).

The pigment ratios were affected by the light intensity during culture, and most of the ratios increased at the higher light intensities from LL to HL, although echinenone/chl *a* ratios decreased in *Nodularia spumigena*. β-carotene/chl *a* ratios were relatively low and did not change much as a function of light intensity (Table 1). The pigment/chl *a*-ratios of cultures in the stationary growth phase were generally within the range of ratios in the light treatments (Table 1).

In situ samples

During 2001, pigment analyses of samples from the fixed stations revealed the presence of filamentous cyanobacteria since echinenone, myxoxanthophyll and canthaxanthin were detected in most samples. Furthermore, aphanizophyll and β-cryptoxanthin were detected in July 2001 at Stn 3, and on some occasions in lower concentrations at Stns 1 and 2. A 4-keto-myxoxanthophyll-like pigment was probably also present, since the HPLC chromatogram showed a minor peak, which eluted at the same time as the 4-keto-myxoxanthophyll-like pigment. However the peak was too small to be scanned and could not be properly identified in the *in situ* samples during 2001.

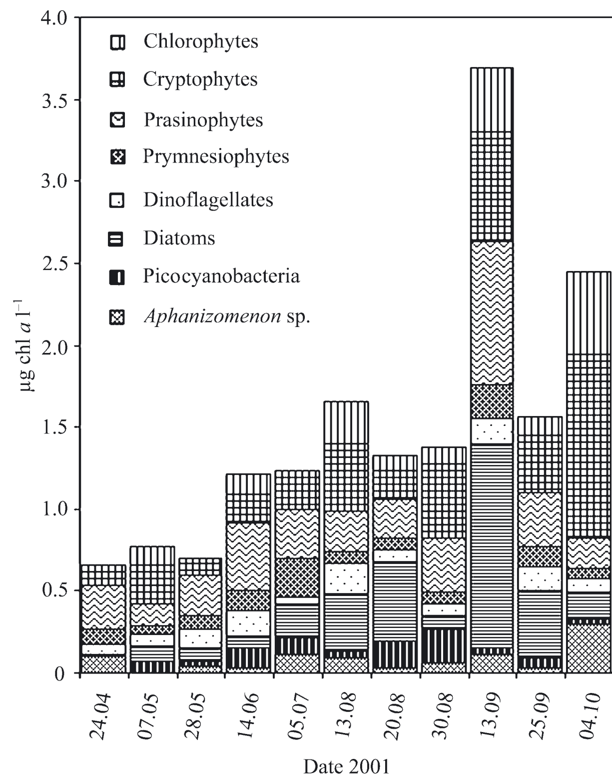

Fig. 3. CHEMTAX analyses of samples taken at surface at Stn 1. Here and in following figures, chlorophytes include euglenophytes; prasinophytes include both subtypes of prasinophytes (i.e. with and without prasinoxanthin)

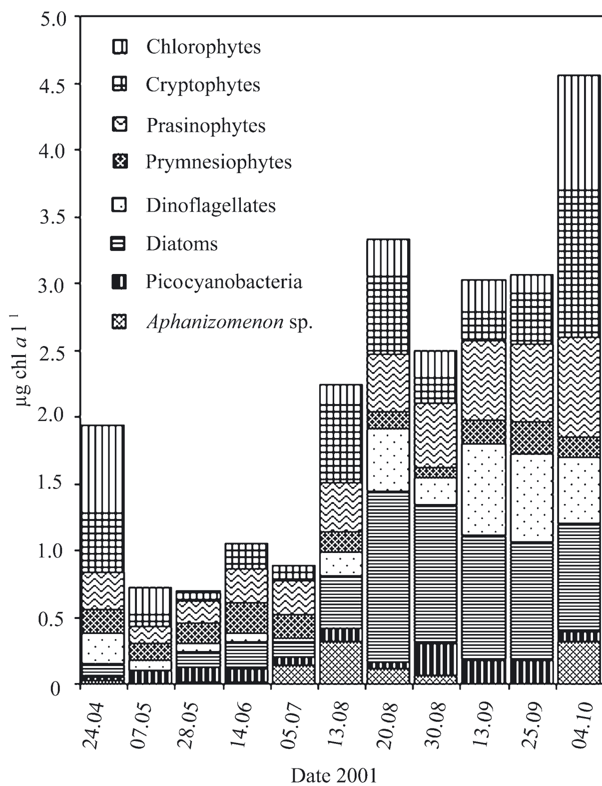

Fig. 4. CHEMTAX analyses of samples taken at surface at Stn 2

Besides these pigments, the major diagnostic pigments revealed by HPLC were: peridinin, 19′-butanoyloxyfucoxanthin, 19′-hexanoyloxyfucoxanthin, fucoxanthin, alloxanthin, zeaxanthin, prasinoxanthin and chlorophyll *b*, as well as other non-specific pigments. This indicated the presence of dinoflagellates, prymnesiophytes, diatoms, prasinophytes with prasinoxanthin, cryptophytes, and possibly also chlorophytes/euglenophytes, prasinophytes without prasinoxanthin, and picocyanobacteria. Pigment ratios were calculated as described by Schlüter & Møhlenberg (2003) for each data set to determine whether chlorophytes/euglenophytes, prasinophytes without prasinoxanthin, and picocyanobacteria were present, and revealed that these groups were generally also present.

The phytoplankton composition determined by HPLC analyses was generally in agreement with that determined under the microscope (data not shown). *Aphanizomenon* sp. was detected in most samples, especially in July and August 2001. A few colonies of *Nodularia spumigena* were found in the samples from Stns 1 and 2 during the summer months of 2001. Diatoms were generally the most abundant alga group present, but dinoflagellates were also quite common. Cryptophytes were also generally present, but in some instances the strict autotrophic ciliate *Mesodinium rubrum*, which contains a cryptophyte endosymbiont (Taylor & Blackbourn 1971) was present, and this ciliate was then included in the cryptophyte group by the HPLC analyses. Many of the smallest algae cells such as prymnesiophytes and prasinophytes could not be properly identified using the inverted microscope. Chlorophytes were not detected by the microscope, but euglenophytes were detected in most samples.

The CHEMTAX calculations revealed quite a diverse phytoplankton population on most occasions in 2001 (Figs. 3 to 6). The chl *a* biomass was relatively low at all stations, i.e. between 0.5 µg chl *a* l^{-1} in spring and 5.7 µg chl *a* l^{-1} in August. At Stn 2, the distribution and biomass of the phytoplankton groups at the surface and in the integrated samples were quite similar (Figs. 4 & 5), indicating that the water column was mixed. *Aphanizomenon* sp. was present on many occasions, especially during the summer, and particular at Stn 3 (Figs. 3 to 6). Usually diatoms dominated, as found when screening the samples in the microscope.

HPLC analyses of samples taken at locations with plankton-like particles in the water during 2002 revealed the presence of the 4-keto-myxoxanthophyll-like pigment in 68% of the samples, indicating that

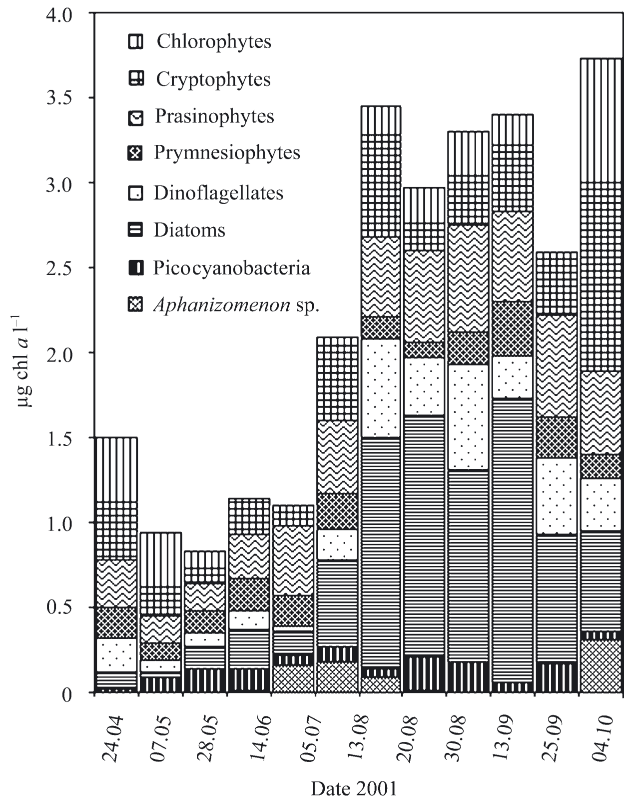

Fig. 5. CHEMTAX analyses of integrated samples taken at Stn 2

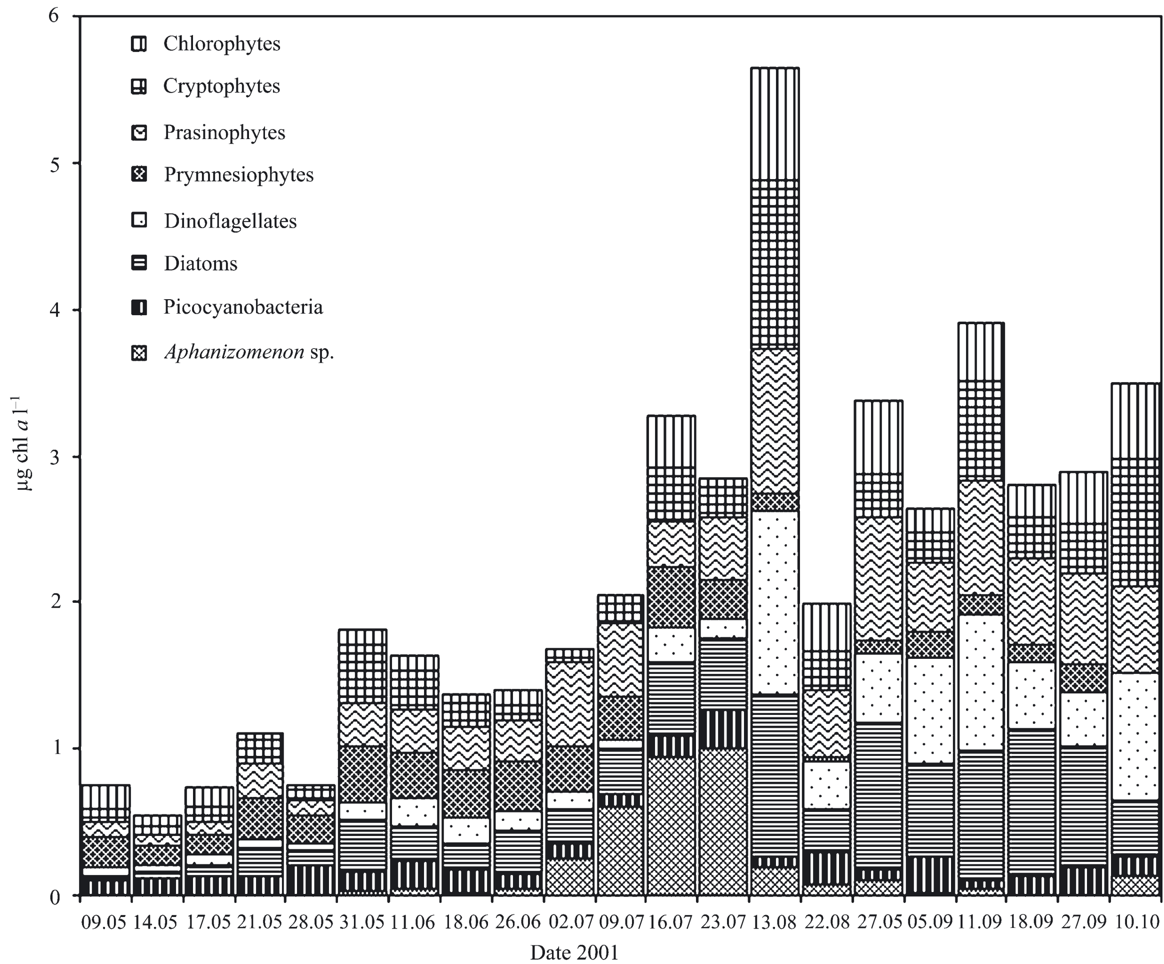

Fig. 6. CHEMTAX analyses of integrated samples taken at Stn 3

Nodularia spumigena was present on many occasions. Furthermore, echinenone, myxoxanthophyll, and canthaxanthin were detected in 77% of the samples, indicating that other filamentous cyanobacteria were also present.

Besides these pigments, the major diagnostic pigments found by HPLC were: peridinin, 19′-hexanoyloxyfucoxanthin, fucoxanthin, alloxanthin, zeaxanthin, prasinoxanthin and chlorophyll *b* (and other non-specific pigments), which indicated the presence of dinoflagellates, prymnesiophytes, diatoms, prasinophytes, cryptophytes, and possibly also chlorophytes/euglenophytes and picocyanobacteria. The calculation of pigment ratios according to Schlüter & Møhlenberg (2003) revealed that prasinophytes without prasinoxanthin, and picocyanobacteria were present, while chlorophytes/euglenophytes were most probably not present.

The phytoplankton composition determined by HPLC analyses of the samples in 2002 was in agreement with the microscopic screening (data not shown). *Nodularia spumigena* was present whenever the 4-keto-myxoxanthophyll-like pigment was found. At very high abundances of *N. spumigena* and *Aphanizomenon* sp., it was observed that besides these algae, *Anabaena* sp. was also sporadically present. Furthermore, dinoflagellates, diatoms, cryptophytes as well as other flagellates were detected by microscopy as well as by HPLC.

The biomass calculated by CHEMTAX was quite variable; in most of the samples the biomass was around 5 µg chl *a* l^{-1} or higher, *Nodularia spumigena* dominated, and *Aphanizomenon* sp. was also relatively abundant (Fig. 7). In 3 samples, the biomass of the phytoplankton was very high, i.e. between 360 and 550 µg chl *a* l^{-1} (right-hand graph in Fig. 7), and the bloom forming cyanobacteria constituted around ⅔ of the chl *a* biomass.

The algal toxin nodularin was not detected in the samples for 2001. In 2002 nodularin was present in the *in situ*

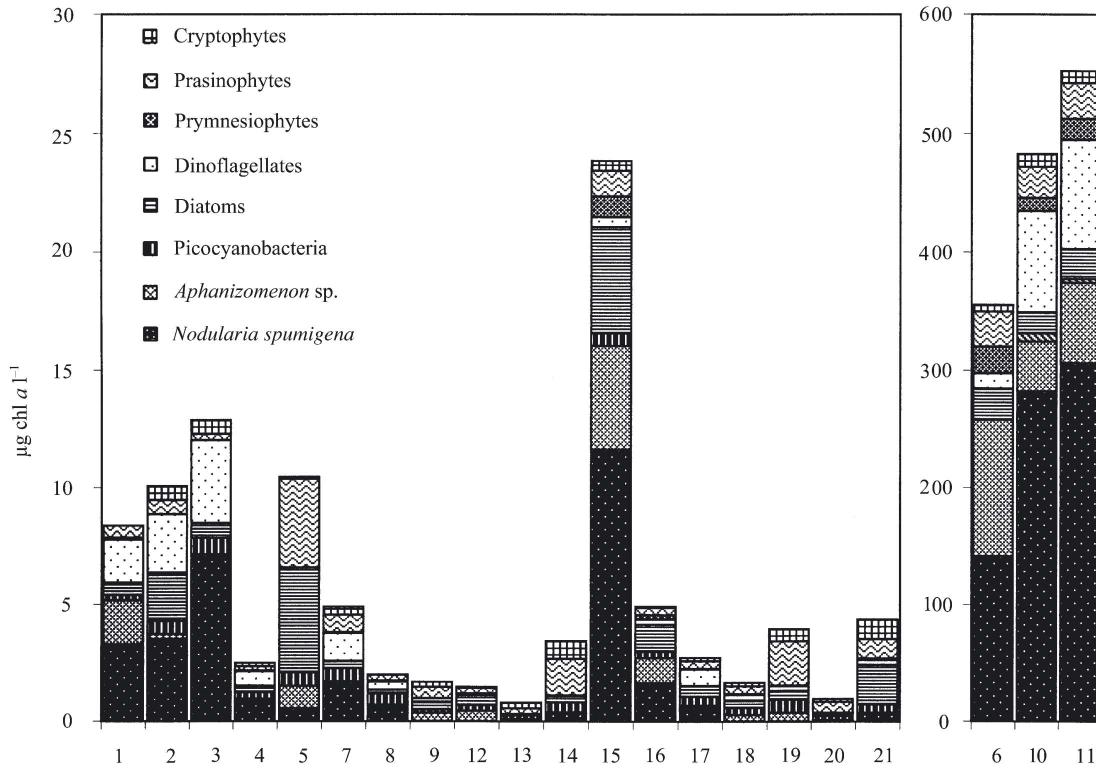

Fig. 7. CHEMTAX analyses of samples taken at different positions from 30 July to 4 September 2002. Numbers on the *x*-axis indicate order in which samples were taken. Graph on right shows data for 3 samples with exceptionally high biomass (note different scale of ordinate)

samples at intracellular concentrations of up to 251 µg l^{-1}, and even higher concentrations were found in the cultures, i.e. up to 338 µg nodularin l^{-1} in the SG cultures of *Nodularia spumigena*. Nodularin was not detected in the cultures of *Aphanizomenon* sp. or *Anabaena lemmermannii*. Nodularin was significantly correlated with the concentration of the 4-keto-myxoxanthophyll-like pigment ($r = 0.97$, $p < 0.05$, Fig. 8). Nodularin was also significantly correlated with the chl *a* biomass of *N. spumigena* derived from CHEMTAX, although the r-value was lower ($r = 0.89$, $p < 0.05$).

DISCUSSION

A 4-keto-myxoxanthophyll-like pigment was found in samples from the SE Baltic Sea during blooms of *Nodularia spumigena*, and was detected exclusively in 2 strains of *N. spumigena* (Table 1). Francis et al. (1970) first detected the glycoside 4-keto-myxoxanthophyll (4-keto-myxol-2′-methylpentoside) in *Oscillatoria limosa*. Hertzberg et al. (1971) listed the carotenoid composition of 28 different cyanobacteria: besides *O. limosa*, only 2 other species, *Anabaena flos-aqua* and *Phormidium faveolarum*, contained 4-keto-myxoxanthophyll, and then only in trace amounts (up to 8 % of the total carotenoids). Apparently, 4-keto-myxoxanthophyll has rarely been observed. Further studies should be conducted to reveal whether the 4-keto-

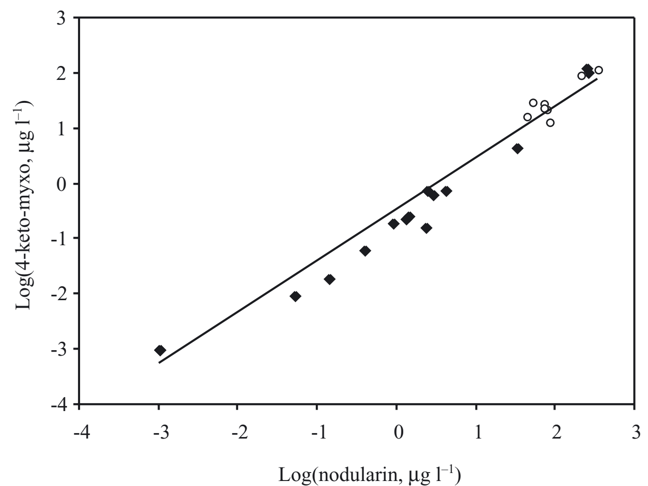

Fig. 8. Relation between nodularin and 4-keto-myxoxanthophyll-like pigment (4-keto-myxo). (○) Data from culture experiments (only *Nodularia spumigena*); (◆) *in situ* samples (2002). Note log scales of axes

myxoxanthophyll-like pigment found in this study is identical to 4-keto-myxoxanthophyll.

The pigment content of *Nodularia spumigena* isolated from the Baltic Sea was previously investigated by Piippola & Kononen (1995) who, as well as detecting chl *a*, detected echinenone, β-carotene, and 4 other 'unknown' pigments, which were most probably the 4-keto-myxoxanthophyll-like pigment, the derivative, myxoxanthophyll and canthaxanthin detected in this study (Table 1). All 4 unknown pigments were also detected in samples taken in the northern part of the Baltic Sea (Piippola & Kononen 1995).

There is only 1 planktonic *Nodularia* species in the Baltic Sea, *N. spumigena* (Barker et al. 1999, Laamanen et al. 2001), and it is always nodularin-producing (Kononen 1992, Laamanen et al. 2001). The correlation between nodularin and the 4-keto-myxoxanthophyll-like pigment found in this study (Fig. 8), the unique presence of this pigment in *N. spumigena*, and the occurrence of the pigment in natural samples during blooms of *N. spumigena*, suggests that the 4-keto-myxoxanthophyll-like pigment can be used as a diagnostic pigment for this toxic species in the Baltic Sea.

The pigment/chl *a* ratios in Table 1 generally increased from LL to HL. This was also found for light-protecting pigments by Schlüter et al. (2000), indicating that the carotenoids of the cyanobacteria investigated in this study are involved in photoprotection. An exception was the echinenone/chl *a* ratios, which decreased in *Nodularia spumigena*, increased in *Anabaena lemmermannii*, and were relatively stable for *Aphanizomenon* sp. at increasing light intensities (Table 1). Descy et al. (2000) detected echinenone in filamentous cyanobacteria (*Anabaena* spp. and *Oscillatoria* spp.) in freshwater. As for *A. lemmermannii* in the present study, the echinenone/chl *a* ratios and the ratios of other photoprotecting pigments in the study of Descy et al. (2000) increased with increasing light intensity from 0.200 to 0.269 when calculated by CHEMTAX.

Heresztyn & Nicholson 1997 have previously shown that the concentration of nodularin correlated well with *Nodularia spumigena* cell numbers, except toward the end of blooms, when the toxin content was lower than might be expected from the cell numbers. In this study, the correlation between the biomass as chl *a* (estimated from HPLC results) and nodularin was poorer (r = 0.89) than the correlation between the 4-keto-myxoxanthophyll-like pigment and nodularin (r = 0.97) (Fig. 8). This indicates that the concentration of the 4-keto-myxoxanthophyll-like pigment is a better indicator of the presence of the toxin than chl *a* biomass of *N. spumigena* calculated by CHEMTAX. Many factors, such as temperature, irradiance, nutrients, etc., have been shown to influence the nodularin production of *N. spumigena* (Lehtimäki et al. 1997, Hobson & Fallowfield 2003). The 4-keto-myxoxanthophyll-like pigment is apparently closely coupled to toxin production.

Although in the exceptionally warm and sunny summer of 2001, the coastal zone of the eastern part of Denmark was severely affected by blooms of cyanobacteria (Ærtebjerg et al. 2002), which had a great impact on water quality, especially for recreational use along the sea shores, the cyanobacteria were barely detected in our samples for 2001 (Figs. 3 to 6). When sampling at fixed positions, which is the procedure of the Danish aquatic monitoring program, predetermined positions and fixed depths and time intervals are used, and in 2001 our sampling program followed this procedure. As they are buoyant, cyanobacteria accumulate at the surface and are therefore susceptible to wind and currents. Even during the intense blooms of cyanobacteria that covered most of the coastline in the eastern part of Denmark in 2001, the cyanobacteria were almost undetectable between bloom-patches. During cyanobacteria blooms, it is possible that phytoplankton biomass estimated by monitoring programs using fixed positions and sampling intervals can fail to record the blooms. During 2002, samples were taken only at locations where visible plankton-like particles were present in the water, and the blooms of cyanobacteria were thus successfully sampled (Fig. 7). This sampling strategy is, however, seldom feasible. Remote-sensing, i.e. satellite images (Kahru et al. 1994), can be used during periods when blooms are intense to estimate the area covered and the bloom intensity, but *in situ* samples are still needed to determine whether the blooms are caused by the toxic *Nodularia spumigena*.

There are several advantages in using HPLC for detecting *Nodularia spumigena* in the Baltic Sea. The HPLC method is rapid, very sensitive, and objective. It was able to detect *N. spumigena* at very low abundance, i.e. down to 0.4 µg chl *a* l^{-1} in Sample 21 (Fig. 7). Hence, the method can be used for early warning of toxic blooms in the Baltic Sea if samples are taken specifically at locations with visible particles. Using HPLC, all algae groups present can be identified to group level, and increased levels of the toxic cyanobacteria *N. spumigena* can be revealed by the presence of the 4-keto-myxoxanthophyll-like pigment obviating the need for toxin analyses or microscopic counts. Microscope screening generally corroborated the results of the HPLC analyses in this study. However, it is impossible to determine the nature of the smallest algae cells under an inverted microscope when algal biomass is low. For correct identification of the smallest algae cells, more advanced methods such as epifluorescence microscopy are required.

The highest concentrations of phytoplankton in the blooms were encountered nearshore, where chl *a* concentrations were between 350 and 550 µg l^{-1} in surface waters (Fig. 7). Even under such extreme conditions as very high cyanobacteria biomass, the diversity and the density of the remaining phytoplankton was quite high, reaching 100–200 µg chl *a* l^{-1}. Microscopic determination of the biomass and composition of a 'background' phytoplankton population during a bloom is extremely time-consuming, since the water samples have to be divided into subsamples and large filter areas have to be screened under an epifluorescence microscope (Havskum et al. 2004). This is seldom feasible during routine monitoring. In contrast, the HPLC method is fast and reproducible and can characterize the diversity of a phytoplankton community at group level.

The relatively high biomass of the 'background' phytoplankton population found in this study during the *Nodularia spumigena* blooms (Fig. 7) indicated that the environmental conditions were also favorable for other phytoplankton groups despite the very high concentrations of nodularin present in *N. spumigena*. Hence, nodularin did not affect the growth of the phytoplankton.

CONCLUSIONS

A rare pigment, the 4-keto-myxoxanthophyll-like pigment, was detected in the toxic cyanobacteria *Nodularia spumigena*. Of the 3 bloom forming cyanobacteria in the Baltic Sea, only *N. spumigena* contained the hepatotoxin nodularin. The 4-keto-myxoxanthophyll-like pigment can apparently be used as a diagnostic pigment for toxic bloom forming cyanobacteria in the Baltic Sea. Further studies are required to reveal the structure and exact identity of this pigment and to determine whether it can be used as a specific marker of *N. spumigena* in other brackish environments also.

The CHEMTAX software can be used to estimate the contribution of cyanobacteria species as well as the other algae classes present to total chlorophyll *a*. This method can be used for early warning of, and for evaluating the intensity of blooms of potentially toxic cyanobacteria in the Baltic Sea.

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