

On the Ecophysiology of Baltic Cyanobacteria, Focusing on Bottom-up Factors



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Ph.D. thesis
Department of Marine Ecology
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Doctoral Thesis



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ABSTRACT

Cyanobacterial blooms in the Baltic Sea are dominated by diazotrophic cyanobacteria, i.e. *Aphanizomenon* sp. and *Nodularia spumigena*. The blooms coincide with a stable stratification and the organisms are concentrated to the surface water, exposed to high levels of both photosynthetically active radiation (PAR, 400–700 nm) and ultraviolet radiation (UVR, 280–400 nm), in combination with low ratios of dissolved inorganic nitrogen and inorganic phosphorus (DIN:DIP). The ability of nitrogen fixation, a high tolerance to phosphorus starvation and photo-protective strategies (production of mycosporine-like amino acids, MAAs) may explain their competitive advantage in the Baltic Sea. However, intraspecific variation in the response to environmental factors has been commonly overlooked.

The seasonal succession with peaks of *Aphanizomenon* sp. in early summer followed by peaks of *N. spumigena*, has been related to their interspecific preferences and response to abiotic conditions. *N. spumigena* dominates in late summer forming extensive toxic blooms, and its toxin nodularin, a hepatotoxin lethal to wild and domestic animals, may act as a tumour promotor. It has been suggested that the accumulation of nodularin within the *N. spumigena* cells and its release from the cells are affected by environmental factors. Hence, the seasonal succession may be explained by an allelopathic effect of nodularin on *Aphanizomenon* sp.

The aim of this thesis is to elucidate the factors controlling the cyanobacterial blooms, prevailing seasonal succession, intraspecific differences, toxin production and release. Moreover, to analyze the potential of future toxic blooms in a predicted climate change, e.g. increased UVR and stronger stratification due to increased precipitation and temperature. With a multi-factorial approach in the laboratory and in outdoor experiments, interactive effects of radiation (photosynthetic active radiation PAR and PAR + UV-A + UV-B), nutrients (nutrient replete, nitrogen limited, phosphorus limited) and species composition (monocultures of *N. spumigena* and *Aphanizomenon* sp. and mixed cultures with the respective species) were tested on these two species.

Although strain-specific differences in UV-B radiation tolerance were observed, *N. spumigena* is a species that is not generally negatively affected by UV-B radiation corresponding to ambient sea surface intensities/doses. *N. spumigena* tolerates high ambient UVR also under nutrient-limiting conditions and maintains positive growth rates even under severe phosphorus limitation. Interestingly, the specific growth rate of *N. spumigena* was stimulated by the presence of *Aphanizomenon* sp. and in contrast to our hypothesis, *Aphanizomenon* sp. was not negatively affected by the presence of *N. spumigena*. Nodularin accumulation and release were dependent on environmental conditions, but the released nodularin did not affect the co-existing species *Aphanizomenon* sp. The highest intra- and extracellular nodularin concentrations were observed under nitrogen limitation when shielded from UVR. In conclusion, I suggest that the seasonal succession, with peaks of *Aphanizomenon* sp. followed by peaks of *N. spumigena* is a result from species-specific preferences of environmental conditions and/or stimulation by *Aphanizomenon* sp., rather than an allelopathic effect of *N. spumigena*. Moreover, a possible increased toxin content of

the *N. spumigena* should be considered when planning sewage treatment, since nitrogen removal may cause problems on a recreational level and increased accumulation of nodularin higher up in the food web. The results from this thesis, together with a predicted stronger stratification and increased UVR due to effects of climate change in the Baltic Sea, reflect a scenario with a continuing future dominance of the toxic *N. spumigena*.

Keywords: Allelopathy; *Aphanizomenon* sp.; Baltic Sea; Cyanobacteria; Diazotrophic; Multifactorial; Nitrogen; Nodularin; *N. spumigena*; photosynthetic active radiation; Phosphorus; ultraviolet radiation; UV-A; UV-B.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Under sommarmånaderna de senaste tio åren har man kunnat läsa i dagstidningarna om ”giftiga mördaralger” som omringar Gotland eller om ”mördaralger” som rör sig mot skånska kusten. Ofta tillsammans med bilder där badande människor flyr i panik mot stranden eller flygfoton på skepp som plöjer genom en grågrön sörja.

Det är inga alger som mördar, det är cyanobakterier som blommar.

Forskarna är idag oeniga om anledningen till att dessa blomningar har ökat. En del anser att det beror på övergödningen som vi människor orsakat genom utsläpp av kväve och fosfor i Östersjön. Andra har undersökt Östersjöns botten sediment och hävdar att det är ett naturligt fenomen som har pågått i över 7000 år och att det snarare beror på klimatförändringar. En viktig anledning till att cyanobakterierna trivs på sommaren är bl.a tillgången och förhållandet mellan näringsämnen fosfor och kväve. Dessa näringsämnen finns i riklig mängd under vintern men på våren blommar ett annat växtplankton, kiselalger, som förbrukar det mesta av kvävet och lite av fosfor. Det finns dock tillräckligt med fosfor kvar i ytvattnet för att cyanobakterier, som kan fixera kvävgas, ska kunna blomma senare under sommaren. Solljus är en annan viktig anledning som blivit förbisedd i forskarvärlden. En ökad mängd ultraviolet (UV) ljus, orsakad av uttunning av ozonskiktet, är inte bara ett problem på södra halvklotet utan även här på nordliga breddgrader. Yt blommande växtplankton skadas av UV-ljus men har olika strategier för att undvika denna skadliga del av solens ljus. Många arter har utvecklat ett solskydd i form av UV-absorberande ämnen. Eftersom dessa ämnen innehåller mycket kväve, gynnas kvävefixerande cyanobakterier vid kvävebrist. Därför kan ljus, i samverkan med de näringsämnen som finns i ytvattnet, påverka vilka arter som blommar i Östersjön. Den art som har bäst strategier är den som kommer att dominera. Olika arter av kvävefixerande cyanobakterier blommar vid olika tidpunkter. I maj-juni brukar *Aphanizomenon* blomma, men i juli-augusti dominerar i allmänhet den giftiga arten *Nodularia spumigena* (även kallad katthårsalg). Den fortsätter att dominera så länge ytvattnet är varmt och stilla. Giftet den producerar heter nodularin och är ett hepatotoxin, dvs. ett gift som angriper levern. Boskapsdjur och hundar kring Östersjön har dött efter att ha fått i sig stora mängder av det giftiga vattnet under blomningarna. Anledningen till att den giftiga arten dominerar under sensommaren anses bero på att dessa två arter föredrar olika temperaturer och näringsförhållanden.

Syftet med min avhandling är att förstå hur ljus och näringsämnen kontrollerar och framförallt samverkar till att cyanobakterierna är så framgångsrika. Ett annat syfte med avhandlingen är att se om samverkan av ljus och näringsämnen har olika effekt på de dominerande arterna eller om det är så att den giftiga *N. spumigena* använder sig av giftet nodularin för att vinna över *Aphanizomenon*. Resultaten från mina studier kan sammanfattas med att *N. spumigena* har en fortsatt god tillväxt trots ökad intensitet av UV-ljus, även vid fosforbrist. *Aphanizomenon* växer överlag mycket sämre än *N. spumigena*. Det verkar inte vara så att *N. spumigena* har någon som helst påverkan på *Aphanizomenon*, varken med sitt gift eller med

sin närvaro. Vilken art som kommer att dominera styrs snarare av deras olika sätt att hantera situationer med ökad mängd UV-ljus och brist på näringsämnen, än av någon sorts kemisk krigföring. Något oväntat växer *N. spumigena* mycket bättre tillsammans med *Aphanizomenon* än ensam, vilket tyder på att *N. spumigena* gynnas av dess sällskap. *N. spumigena* producerar mest gift när det är lite kväve i vattnet men tillräckligt mycket fosfor. Man kan därför förvänta sig att *N. spumigena* innehåller mest gift i början av blomningen då dessa förhållanden råder. Om avloppsvatten renas från i huvudsak kväve, kan det få stora konsekvenser vad gäller mer gift i blomningarna. Om övergödning tillsammans med uttunning av ozonskiktet fortskrider, vilket forskarna förutspår, kommer cyanobakterierna och i synnerhet *N. spumigena* att gynnas.

There is a theory which states that if ever anybody discovers exactly what the Universe is for and why it is here, it will instantly disappear and be replaced by something even more bizarre and inexplicable. There is another theory which states that this has already happened.

Douglas Adams (1952 - 2001)
English humorist and science fiction novelist

Till Kent, Johannes och David

LIST OF PAPERS

This thesis is based on following publications/manuscripts. Publications will be referred to in the text by Roman numerals as follows:

- I. Wulff, A., **Mohlin, M.** & Sundbäck K. (2007) Intraspecific variation in the response of the cyanobacterium *Nodularia spumigena* to moderate UV-B radiation, *Harmful Algae*, 6:388-399
- II. **Mohlin, M.** & Wulff, A. (2009) Interaction effects of ambient UV-radiation and nutrient limitation on the toxic cyanobacterium *Nodularia spumigena*, *Microbial Ecology*, 57:675-686
- III. Pattanaik, B., Roleda, M.Y., Garde, K., Wulff, A. & **Mohlin, M.** (2010) Production of the cyanotoxin nodularin – a multifactorial approach, *Harmful Algae*, 10:30-38
- IV. **Mohlin, M.**, Pattanaik, B., Roleda, M.Y. & Wulff, A. (2010) Allelopathic and combined effects of radiation and nutrient limitation on Baltic cyanobacteria (Manuscript)

Related publications not included in the thesis:

Roleda, M.Y., **Mohlin, M.**, Pattanaik, B. & Wulff, A. (2008) Photosynthetic response of *Nodularia spumigena* to UV and photosynthetically active radiation depends on nutrient (N and P) availability, *FEMS Microbial Ecology*, 66: 230-242

Lindberg, V., **Mohlin, M.** & Wulff A. (2008) UV responses in three strains of the cyanobacterium *Nodularia spumigena*, Proceedings of the 12th International Conference on Harmful Algae. Ø. Moestrup et al. (Eds). ISSHA and IOC of UNESCO 2008, page 44

ABBREVIATIONS

AD	anno dato
APHA	<i>Aphanizomenon</i> sp.
BMAA	β -N-methylamino-L-alanine
BP	before present
BWF	biological weighting function
chl <i>a</i>	chlorophyll <i>a</i>
CIE	Commision Internationale de l'Eclairage
DIN	dissolved inorganic nitrogen
DIP	dissolved inorganic phosphorus
F_v/F_m	maximum quantum yield
HPLC	high-pressure liquid chromatography
MAAs	mycosporine-like aminoacids
MAPHA	<i>Aphanizomenon</i> sp. in mixed culture with <i>N. spumigena</i>
MNOD	<i>N. spumigena</i> in mixed culture with <i>Aphanizomenon</i> sp.
N	Nitrogen
-N	f/2 medium without nitrate
NO_2^-	Nitrite
NO_3^-	Nitrate
NH_4^+	Ammonium
NOD	<i>Nodularia spumigena</i>
NodApha	<i>N. spumigena</i> in mixed culture with <i>Aphanizomenon</i> sp.
NP	f/2 medium
P	phosphorus
-P	f/2 medium without phosphate
PAR	photosynthetic active radiation
PO_4^{3-}	phosphate
POC	particulate organic carbon
PON	particulate organic nitrogen
POP	particulate organic phosphorus
UV-A	ultraviolet-A radiation
UV-B	ultraviolet-B radiation
UVR	ultraviolet radiation

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1. INTRODUCTION

The Baltic Sea is situated between latitude 53°N to 66°N and between longitude 20°E to 26°E, and is one of the largest brackish-water bodies in the world. It consists of a number of basins, including the Gulf of Bothnia, Gulf of Finland and Gulf of Riga as well as the Baltic proper (Fig. 1). The anthropogenic stress to the Baltic Sea is high, as 16 million people live on the coast and a total of 85 million within the catchment area. The water exchange from the North Sea is restricted, due to the shallow sill and the narrow link situated between Denmark and Sweden. Together with a large river inflow those features give the Baltic Sea its brackish character.

1.1. *THE HISTORY OF THE BALTIC SEA AND CYANOBACTERIAL BLOOMS*

It has a very young history that have been influenced by the last glaciations of the Baltic basin, the Weichselian glaciations, and Baltic Sea as we know it today is a result of meltwater from the glaciations combined with saltwater from the North Sea when the straits between Sweden and Denmark opened. The postglacial history of the Baltic Sea is characterized by isostatic rebound, i.e. uplift of land masses that were depressed by the weight of ice together with eustatic changes in the volume of water in the world oceans caused by climate change (Winterhalter, 1992; reviewed by Björck, 1995). This resulted in a succession of freshwater and marine stages with either brackish condition with connections to the North Sea or isolated freshwater lake conditions (reviewed by Björck, 1995). Variations between oxic and hypoxic conditions in the deep water are recognizable in the sediment stratification. Under oxic conditions the sediments are homogenous because of the activity of the fauna in the sediments. Under hypoxic conditions the fauna that homogenize the sediments are eliminated and therefore sediments are laminated. By analyzing sediment cores and the stratigraphic succession including biogenic remains, researchers have been able to reconstruct the history of the Baltic Sea.



Fig. 1. Map made by John Malham in 1795. www.gracegalleries.com/images/S&B/S&B109.jpg

1.1.1. STRATIGRAPHIC STUDIES

The history of the Baltic Sea is traditionally divided into four main stages, i.e. the Baltic Ice Lake, the Yoldia Sea, the Ancylus Lake and the Litorina Sea (Fig. 2a- d). Deglaciation of the Baltic basin started around 17000–15000 before present (BP) and continued for at least 3000 years (Berglund, 1979; Andrén et al., 2000a and references therein). The ice-free freshwater stage, called the Baltic Ice Lake, began to form 16000 BP when large parts of the southern Baltic became ice-free relatively quickly (reviewed by Björck, 1995; Jensen, 1995). It existed until 10300 BP when the dam broke at Mount Billingen (arrow in Fig. 2b) and the melt water was flooding out in the North Sea (Andrén et al., 2002) resulting in a considerable fall of the Baltic lake-level (25 m), (Björck, 1995).



Fig. 2. Post-glacial stages of the Baltic basin. a) the Baltic Ice Lake, b) the Yoldia Sea, arrow indicate where the dam broke, c) the Ancylus Lake, arrow indicate where the connection to the North Sea closed d) the Litorina Sea, arrow indicate where the broadening of connection to the North Sea, Öresund (www.smf.se)

The Baltic Ice Lake was succeeded by the Yoldia Sea, 10300-9500 BP which consisted of two freshwater phases and a short brackish-water phase in between, caused by inflow of marine water from the North Sea (reviewed by Björck, 1995; Raukas, 1995). Sediment cores examined from this first freshwater phase do not contain siliceous microfossils or cyanobacteria, probably due to low primary production (Andrén et al., 2000a). The scenario would change when saline nutrient-rich water from the North Sea were brought into the Yoldia Sea, the upwelling of this nutrient-rich marine water caused high diatom abundances (Andrén et al., 2000a). The increase of diatom abundance has also been recorded in cores from the Bornholm Basin (Andrén et al. 2000b). According to a study by Andrén and Sohlenius (1995), the inflow of marine water during the brackish phase had probably the same periodicity as present in the Baltic Sea, approximately 15 years (Stigebrandt, 1987). The transition between the brackish and last freshwater phase in the Yoldia Sea is visible as a shift in the diatom composition to a freshwater flora dominated by a few taxa (Andrén et al., 2000a).

A new phase called the Ancylus Lake started around 9500 BP and ended around 8000 BP. The connections to the North Sea (at Mount Billingen, arrow in Fig. 2c) became shallow because of continuous land-uplift and forced the Baltic level to rise above sea level (Björck,

1995). This dammed-up freshwater lake is the most discussed of the many Baltic phases (summarized by Fredén, 1967). The land-uplift in the northern part of the basin was faster during this stage and this caused the Ancylus Lake to attain a link to the Kattegatt-Skagerack through the Great Belt region, 10 000 BP (Björck, 2008). This connection was broadened around 8500–7800 BP, and began to function as an important inlet of saltwater, the Sound (Öresund) (arrow in Fig. 2d), (Andrén et al., 2000a; Andrén et al., 2000b; Sohlenius et al., 2001). Until now there has been no particular sign of cyanobacterial blooms in the respective phases of the Baltic basin. The first indications were found during the transition phase between Ancylus Lake and the Litorina Sea. Sediment cores analysed from this transition phase showed a rapid increase in the organic carbon content (Sohlenius et al., 1996), thought to have been caused by cyanobacteria (Andrén et al., 2000b).

Finally we have reached the most interesting phase in the history of the Baltic Sea; the Litorina Sea 8000-4000 BP. I consider it most interesting, since it is in sediment layers from this phase that cyanobacteria have been recorded. The Litorina Sea was a marine stage (reviewed by Munthe, 1894) and the inflow of marine water created a halocline that most likely resulted in a decreased vertical mixing of the water column and may explain the extensive laminated sediments found from this stage (Fig. 3), (Bianchi et al., 2000 and references therein). It seems like anoxic conditions and dead bottoms existed in the Baltic basin already during the Litorina Sea.

In order to measure historical outbreaks of cyanobacterial bloom, researchers have measured the concentrations of the cyanobacteria-specific pigments echinenone, myxoxanthophyll (Poutanen and Nikkila, 2001) and zeaxanthin (Bianchi et al., 2000; Poutanen and Nikkila, 2001) in laminated sediments. Although common in cyanobacteria in general, it has been shown that *N. spumigena* contain no or very low concentrations of zeaxanthin (Paper I, II, IV; Henriksen, 2005; Schlüter et al., 2004), indicating that the zeaxanthin concentrations found in the laminated sediments originate from other cyanobacteria species or green algae (Jeffrey, 1997). Those findings have however been revised lately; Schlüter et al. (2008) found that zeaxanthin is a pigment found in *N.spumigena* from the Baltic Sea.

Bianchi et al. (2000) report the occurrence of cyanobacterial blooms already in the early stages of the Litorina Sea (7500-7000 BP), further supported by Poutanen and Nikkila (2001). By comparing sediments from early Litorina stage and modern sediments from the Gotland Basin, Struck et al. (2000), found that the productivity was nitrate based in spring and based on cyanobacterial nitrogen fixation in summer. Bianchi et al. (2000) suggest that the cyanobacterial blooms were initiated by increased availability of phosphorus, i.e. inflow of phosphorus-rich seawater and increased phosphorus-release from anoxic sediments. In addition, they detected the cyanobacterial pigment zeaxanthin in such high concentrations that

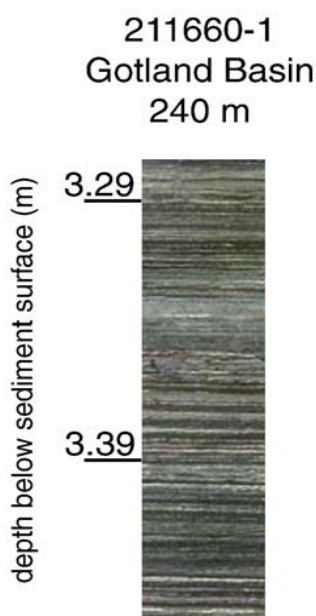


Fig. 3 Photo of laminated sediments dated to the Litorina Sea (from Zillén et al. 2008)

the blooms during this phase were similar in magnitude to the cyanobacterial blooms that we experience today. They further suggest, based on the results and on the stability of this pigment (Bianchi et al. 1993), that the absence of zeaxanthin in sediments from the Ancylus Lake is likely to be a result of low virtual abundances rather than a degradation of the pigment. During the Late Litorina Sea (4000 BP – AD 1800) there was a succession between homogeneous sediments and laminated sediments due to oxic or hypoxic conditions, temperature increase or decrease, intrusion of saline water from expanded opening in the Danish Strait, increased or decreased population in the coastal area (reviewed by Zillén et al., 2008).

The studies on stratigraphic succession mentioned above have been, among others, the bases in a confusing public debate during the last decades concerning to what extent the occurrence of the cyanobacterial blooms reflects natural variability rather than anthropogenic impacts. There is no doubt that the human impact has increased dramatically from the medieval time to present time. During that time there have been major changes in agriculture, cutting of trees, population increase and an industrial revolution resulting in an increased nutrient load to the Baltic Sea drainage area (reviewed by Zillén et al., 2008). Pigment data from stratigraphic studies by Poutanen and Nikkila (2001) clearly show an increased intensity of algal blooms in the Baltic Sea since the early 1960s. Furthermore, from dated sedimentary records of organic compounds, Struck et al. (2000) show a clear history of eutrophication with enhanced nutrient supply after the start of using fertilizers in agriculture, with a subsequent increased deposition of organic matter in the Baltic from 1920s to 1980s (Jonsson and Carman, 1994). There is no doubt that this increased occurrence and intensity of cyanobacterial blooms is a result of increased nutrient load and nutrient concentrations in the Baltic Sea (HELCOM, 1996; HELCOM, 2001).

1.1.2. *PLANKTON STUDIES*

The history of quantitative plankton research in the Baltic Sea goes back to the 19th century. Victor Hensen (1835-1924), a German planktologist from Kiel, developed sampling nets,



Plankton sampling at the beginning of the 20th century onboard R/V Nautilus. Photo: Finnish Institute of Marine Research (from Finni et al., 2001)

quantitative plankton analysis and conducted pioneer expeditions in the Baltic Sea in 1880 to 1890 (reviewed by Finni et al., 2001; Olenina et al., 2006). The first coordinated plankton research in the Baltic with a frequency of 4 times per year began after an inaugural meeting in Copenhagen 22 July, 1902, when The International Council for the Exploration of the Seas, ICES, was established (Finni et al., 2001).

It was mainly from the coastal area that most blooms of cyanobacteria were

recorded and *Aphanizomenon* (nitrogen-fixing cyanobacterium) was the dominant genus (reviewed by Finni et al. 2001), also in waters near the large cities (Välikangas, 1926). Many researchers thought that this was the freshwater species *Aphanizomenon flos-aquae* (reviewed by Finni et al., 2001) but this idea was argued by Aurivillius (1896) among others, claiming that this was a brackish-water species. They should, however, have to wait until 1994 when Janson et al. (1994) demonstrated the difference in ultrastructure of the vegetative cells of the freshwater and brackish-water species, however they suggested that the Baltic form should be referred to as *Aphanizomenon* sp.. Häyrén (1921) also found that *Aphanizomenon flos-aquae*, *Nodularia spumigena* and *Anabaena baltica* were dominating the blooms outside Tvärminne, Finland, 1913. In september 1921 in the coastal area between the Island Ven and south of Sweden, Sjöstedt (1922) observed a bloom of *Nodularia spumigena*.

Records of open-sea blooms rarely occurred but in July 1854 Lindström et al (1855) were among the first to observe an intensive bloom in the Baltic proper. Local fishermen informed him that the layer of the bloom sometimes was so thick that the boats had difficulties to pass through. Thirty years later (14 August to 15 September in 1884), the Prince of Monaco made an expedition in the Baltic Sea with his yacht l'Hirondelle. Professor Pouchet and Baron Jules de Guerne (1885) observed a bloom that covered the area between Gotland, the entrance to Gulf of Finland and Prussia. They noticed that the colour of the bloom was olive-green. However, in none of these two studies did the researchers determine species composition of the bloom. As far as I know, we would have to wait until summer of 1925 when Hessle and Vallin (1934) started a three year study along the Swedish coast and around Gotland. During their expeditions, *Aphanizomenon* was the most common genus, being more abundant than *Nodularia*. The dominance of *Aphanizomenon* in relation to *Nodularia* was also reported in other studies between 1924 and 1930 (reviewed by Finni et al., 2001). The earliest record of a *Nodularia* dominated open-sea bloom as we know them today, was from the expedition by Rothe in August 1938 (Rothe, 1941). From this period and to date, records of *Nodularia spumigena* as the dominating species in the open-sea blooms became common (reviewed by Finni et al. 2001)

In summary, after a closer look into the history of cyanobacterial blooms, I can only agree that the abundance of *Nodularia spumigena* as well as *Aphanizomenon* sp. in the Baltic Sea have indeed increased since the beginning of the 20th century.

1.2. *PRESENT SITUATION IN THE BALTIC SEA AND CONSPICUOUS BLOOMS OF CYANOBACTERIA*

1.2.1. *NUTRIENT SITUATION*

There is an increased supply of nutrients and organic matter to the Baltic Sea, and the main sources are the industrial and agricultural activities of the human population, aquaculture, municipal sewage water, river run-off and erosion, atmospheric deposition and nitrogen fixation (Elmgren and Larsson, 2001; HELCOM, 2002; HELCOM, 2003). The nutrient supply is estimated to a four-fold increase in nitrogen and eight-fold in phosphorus during the 20th century (Larsson et al., 1985). It has been debated over the relative importance of dissolved inorganic nitrogen (DIN) and dissolved inorganic phosphorus (DIP) for the phytoplankton productivity and species composition in the Baltic Sea. The most limiting nutrient may vary between area and season (Moisander et al., 2003; Rahm and Danielsson, 2007). For example, periods with a molar DIN:DIP of 50, well above the Redfield ratio of 16, suggests phosphorus limitation, but occasional phosphorus release from oxygen depleted (hypoxic) sediments reduce this ratio and leads to nitrogen limitation (Pitkänen and Tamminen, 1995; Elmgren and Larsson, 2001). The Baltic Sea surface water is separated from the deep water with a permanent halocline at 60–70 m depth. In summer, when the temperature starts to increase in the surface water, a seasonal pycnocline is built up in the upper mixed layer at 10–20 m depth from the underlying nutrient rich winter water. During spring, a yearly diatom bloom removes DIN from the surface water but leaves high concentrations of DIP, resulting in a low DIN:DIP ratio in May. This changed ratio between the major nutrients will favour nitrogen fixing, i.e. diazotrophic, cyanobacteria due to their ability to fix atmospheric nitrogen (N₂) (Granéli et al., 1990; Larsson et al., 2001; Rydin, 2002; Kangro et al., 2007; Rolff et al., 2007; Sivonen et al., 2007). It is also considered that the availability of DIP is the most important factor determining spatial and temporal distribution and induction of diazotrophic cyanobacterial blooms (Niemi, 1979; Niemi, 1981; Panosso and Granéli, 2000; Paper II, IV).

1.2.2. *HYPOXIA – CAUSES AND CONSEQUENCES*

During the last decades, increased frequency and intensity of cyanobacterial blooms have been linked to the ongoing eutrophication of the Baltic Sea (Niemi, 1981; Larsson et al., 1985). Eutrophication has been defined as an increased input of nutrients or organic matter into an aquatic ecosystem, resulting in an increased primary production (Nixon, 1995). Following increased sedimentation in deep waters of the Baltic Sea will support an increased consumption of the bottom water oxygen by heterotrophic organisms. Nutrients will initially accumulate in the deep water as DIN and DIP, but in basins with limited water exchange like the Baltic Sea, the increased respiration leads to temporary or sometimes permanently hypoxic conditions in the near bottom area. During the 20th century, large areas of formerly

oxic bottoms in the deep basins of the Baltic Sea have turned hypoxic (Fig. 4a) and dead zones have developed (Fig. 4b) (Karlson et al., 2002; Díaz and Rosenberg., 2008).

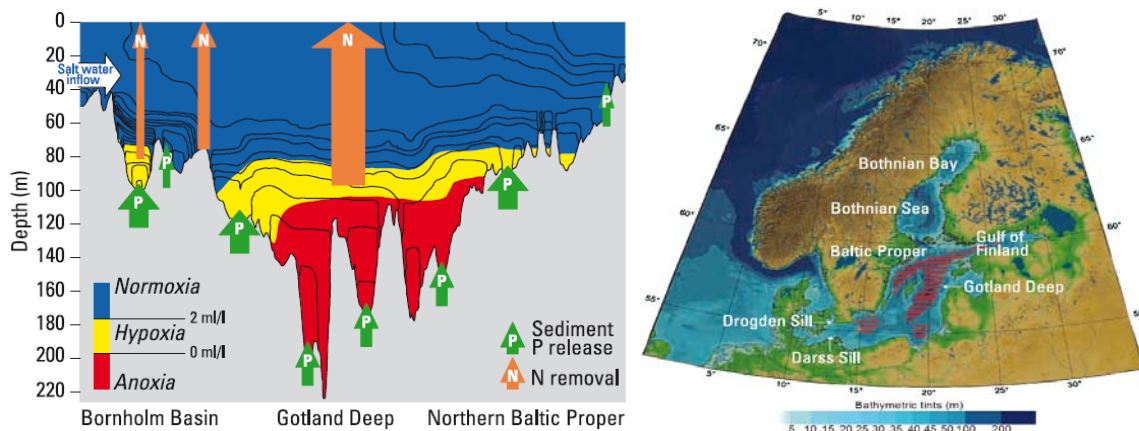


Fig. 4. a) Diagram of hypoxia in the Baltic Sea and the major basins. b) Map of the Baltic Sea identifying its major basins and sills governing the inflow of saltwater. The red lines designate the maximum hypoxic area that occurs in the Baltic Sea (from Conley et al. 2009)

When the deep water turns hypoxic, DIP increases in concentration due to phosphorus release from hypoxic sediments (HELCOM 1987; Vahtera et al. 2007a). Furthermore, a negative relationship between total amount of DIN in the Baltic Proper and hypoxic water volume indicate removal of nitrogen (Vahtera et al. 2007a). The processes behind this nitrogen removal are not yet understood but it seems like hypoxic conditions will alter the nutrient biogeochemical cycles (Conley et al., 2009). Of interest and very alarming is that the release of DIP from hypoxic sediments is approximately 1 order of magnitude greater than the anthropogenic total phosphorus loading to the Baltic Sea (Conley et al., 2002). The consequent decreasing DIN:DIP ratio will favour diazotrophic cyanobacterial blooms, and this will in turn lead to more sedimentation of organic matter and more hypoxia in the Baltic Sea. Vahtera et al. (2007a) have termed this internal acceleration of eutrophication the “vicious circle”.

In short, nutrient and nutrient ratios are important factors controlling and affecting cyanobacterial blooms. But when dealing with photosynthetic organisms, radiation (intensity and dose), and the spectral composition are all factors with the same magnitude of importance (Paper I, II, III, IV, Roleda et al., 2008).

1.2.3. RADIATION SITUATION

The Commission Internationale de l'Éclairage (CIE) has defined ultraviolet-B (UV-B) radiation as wavelengths of 280-315 nm. However, many aquatic scientists accept 320 nm as the upper limit and throughout this thesis I will use the definition of UV-B radiation as 280-320 nm.

An increased UV-B radiation caused by the ozone depletion is a well-known threat not only in the Antarctic region but also on Nordic latitudes (Aldhous, 2000; Shindell et al., 2001; Anonymous, 2002; Anonymous, 2006). It is difficult to assess the underwater UV radiation condition from space-based measurements (summarized by Vassilkov et al., 2001), but the long-term ultraviolet radiation (UV-B+UV-A, 280-400 nm) measurements on land with broadband radiometers show an overall increase towards late 1990s (Chubarova, 2005; Josefsson, 2006). At our latitudes, the UV-B radiation has increased by 6-14% during the last 20 years (Anonymous, 2002) and erythemal UV dose enhancements of 10–15% were observed in northern Norway during the in summer 2000 (Orsolini and Limpasuvan, 2001). This increase is due to the distribution and trends of atmospheric ozone in the Northern hemisphere, and is changing as a result of both natural and anthropogenic activities (Orsolini and Limpasuvan, 2001). Satellite ozone data have shown that since the late-1970, there has been a significant ozone decline over the northern mid-latitudes (Fig. 5) (Bojkov and Balis, 2001; Stahelin et al., 2001; Stahelin et al., 2002).

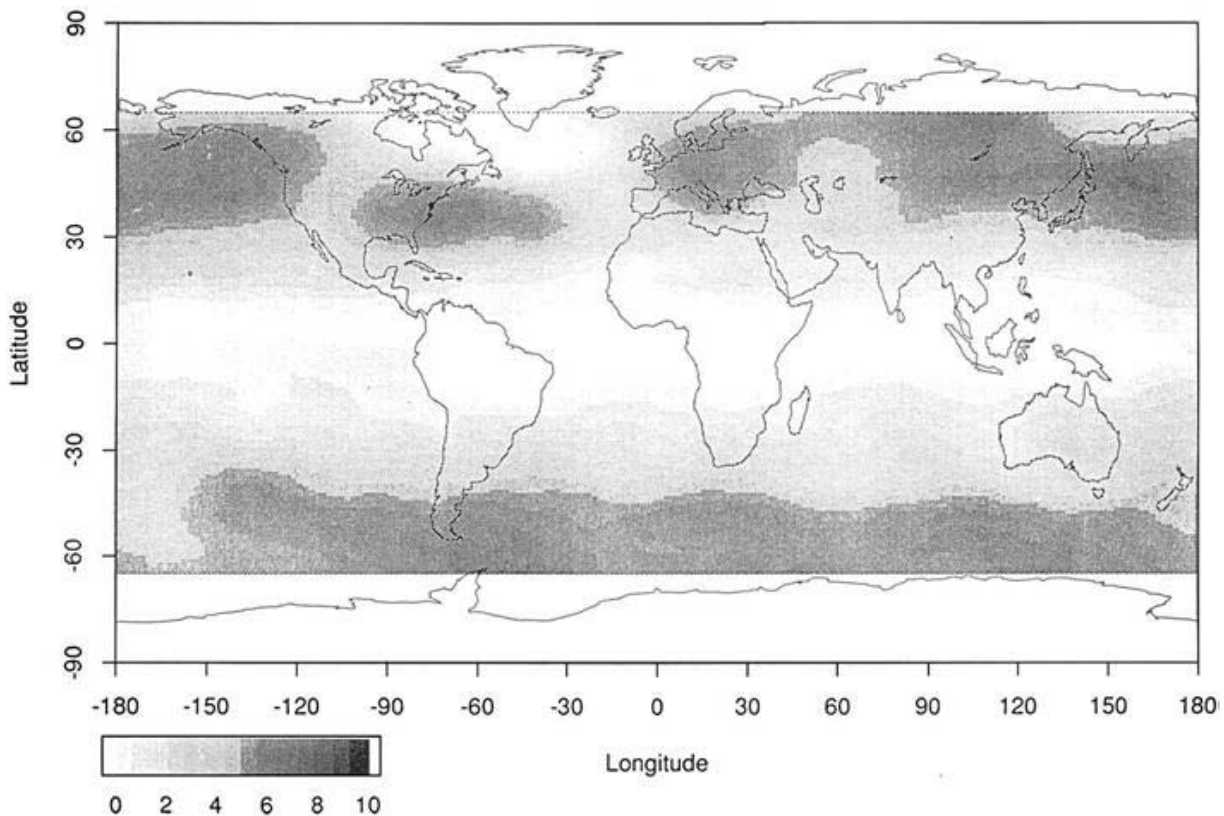


Fig. 5. Variation of ozone trends (percent change per decade) with longitude and latitude derived from Total Ozone Mapping Spectrometer (TOMS) satellite measurements for December–March, 1978–1991 (from Stahelin et al., 2001)

An additional threat to the ozone layer is the increasing level of greenhouse gases in the atmosphere (Shindell et al., 1998; Aldhous, 2000; Anonymous, 2002). The chemical reactions responsible for stratospheric ozone depletion are extremely sensitive to temperature. Greenhouse gases warm the Earth's surface but cool the stratosphere radiatively and therefore affect ozone depletion (Shindell et al., 1998 and references therein). Thus, climate change could prolong ozone depletion in the Arctic by many years despite the success of the Montreal Protocol of 1987 in reducing emissions of ozone-destroying chemicals (Shindell et al., 1998; Aldhous, 2000; Anonymous, 2006). The increase of longer wavelengths, i.e. UV-A radiation (320–400 nm), is a result of decreases in both aerosol optical thickness and effective cloud amount (Josefsson, 2006), and not related to the decreased ozone layer. Therefore, the role of clouds must be included in studies of long-term variations of UV, i.e. to be able to detect the recovery of the ozone layer in UV monitoring data, accurate measures of the effect of clouds is necessary (Josefsson, 2006).

High intensities and/or doses of both UV-B and UV-A could negatively affect phytoplankton and there are species-specific strategies to reduce UV-exposure and hence the amount of photo-damage (Karentz et al., 1991; Karentz, 1994, 2001). Cyanobacteria are among the oldest photosynthetic organisms (Summons et al., 1999) and they have evolved various strategies to limit the amount of photo-damage, including the production of mycosporine-like amino acids (MAAs) (Sinha et al., 2003; Sinha and Häder, 2008; Xue et al., 2005) and scytonemin (Garcia-Pichel and Castenholz, 1991). MAAs have absorbance peaks between 310 and 362 nm, while scytonemin absorbs the longer wavelengths in the UV range up to 382. Together they could effectively dissipate absorbed radiation without producing reactive oxygen species (Carreto et al., 1990; Conde et al., 2000). Although common in terrestrial and some benthic marine environments, scytonemin is not found in planktonic cyanobacteria. Due to their broad absorbance spectrum, MAAs can be an additional strategy to reduce negative effects of excessive photosynthetic active radiation (PAR, 400-700 nm). The exact location of MAAs in cyanobacteria is not known, but in *Nostoc commune*, MAAs are extracellular and bound to oligosaccharides in the sheath (Böhm et al., 1995).

Results from previous studies on plankton communities or cultures suggest that available inorganic nutrients are important to reduce negative effects of UVR (Neale et al., 1998; Wulff et al., 2000). Thus, since MAAs are nitrogen-containing compounds (Rozema et al. 2002), the prevailing nutrient situation during summer in the Baltic Sea might lead to a decreased production among the phytoplankton, giving N₂-fixing cyanobacteria an additional competitive advantage over other MAA-producing phytoplankton. Several laboratory studies have explored the effect of radiation on the ecology of Baltic cyanobacteria (Lehtimäki et al., 1994; Rapala et al., 1997), but the major weakness of these studies are the use of too low radiation intensities excluding UVR. Relevant radiation spectra and intensities are needed and are interesting from an ecological point of view (Roleda et al. 2008, Paper I, II, III, IV).

1.2.4. DOMINATING SPECIES

Cyanobacterial blooms in the open Baltic Sea consist of the N₂-fixing and filamentous genera *Nodularia*, *Aphanizomenon*, and *Anabaena* (Stal et al. 2003): all three belong to the *Nostocalean* taxa. Earlier morphological studies of phytoplankton samples from the Baltic Sea suggested three species of *Nodularia*: *N. spumigena*, *N. litorea*, and *N. baltica* (Komárek et al. 1993). However, detailed molecular studies of different strains from these species led to the conclusion that there was only one genetically distinct planktonic *Nodularia* species in the Baltic Sea, *N. spumigena* Mertens (Barker, 1999; Lehtimäki et al., 2000; Laamanen et al., 2001;). It has been suggested that genetic exchange may occur in the *Nodularia* populations

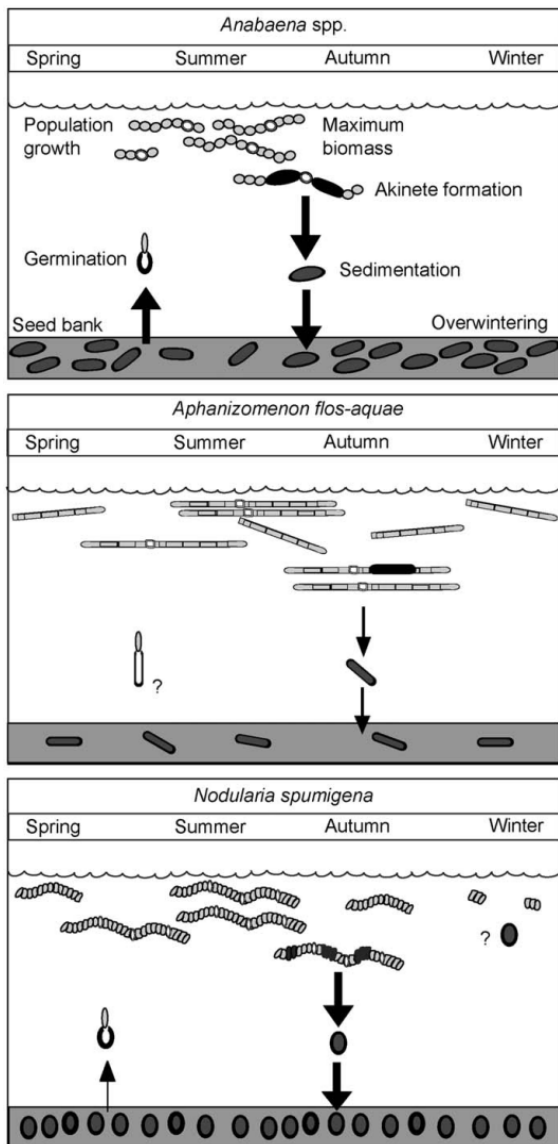


Fig. 6. The different lifecycles of the dominating cyanobacterial species in the Baltic Sea. Arrows denote the akinete flux to and germination from the sediment. Arrow size is proportional to the observed or assumed magnitude of respective fluxes (from Suikkanen et al. 2010).

(Barker et al. 2000). A large number of various *Nodularia*-infecting cyanophages have been found (Jenkins and Heyes, 2006) which putatively transfer genetic information between *Nodularia* strains and shape the structure of populations. The Baltic Sea *Aphanizomenon*, previously reported as *A. flos-aquae* (Linné) Ralfs, is suggested to be a genotype of the freshwater *A. flos-aquae* (Laamanen et al., 2002). Although Baltic Sea *Anabaena* show high morphological diversity (Hällfors, 2004), results from recent molecular studies have shown that there is only one genetically valid planktonic species (Barker et al. 2000; Halinen et al., 2008).

The previously secret annual life cycles of the dominating species in the Baltic Sea has finally been revealed in very interesting studies in 2005 and 2006 (Suikkanen et al. 2010) (Fig. 6). Using pelagial monitoring, sedimentation traps and germination of akinetes, they found that the three co-occurring species have different overwintering strategies in the Baltic Sea. *N. spumigena* overwinters with a smaller fraction of vegetative filaments in the water column and a larger fraction as sedimented resting stages, i.e. akinetes. *A. flos-aquae* has the opposite strategy with vegetative filaments in the water column year-round and are considered holoplanktonic despite the fact that they form akinetes. *Anabaena* spp. has a meroplanktonic lifecycle, with the planktonic stage during summer and formation of akinetes in fall, germinating the following spring. Suikkanen et

al. (2010) suggest that the akinetes formed by *N. spumigena* do not alone act as seeding population for cyanobacterial blooms, the contribution of vegetative filaments are more important. Nevertheless, the significance of the sediment akinetes in providing the inoculum for rapid onset of *Nodularia* blooms has been reported from Peel-Harvey Estuary (Western Australia) by Huber (1984). Huber (1985) also found that among several environmental conditions tested, light was shown to be the most important factor affecting germination.

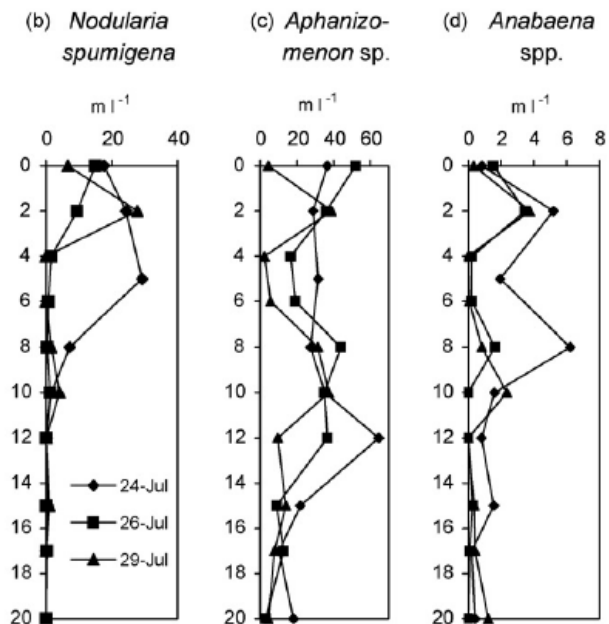


Fig. 7. Vertical distribution of dominating species in July 1994 in Hajdu et al. (2007)

In addition to different life cycle patterns, the three species have different vertical distribution pattern in the water column. One of the first studies in the open Baltic Sea was performed in the summer 1994 and 1997 (Hajdu et al. 2007). They reported that *N. spumigena* accumulated mainly in the top 5 m of the water mass, while *Aphanizomenon* sp. was found in the whole water column (0 - 20 m) and had bimodal vertical distributions (Fig. 7). *Anabaena* spp. was also found down to 20 m and had bimodal abundance depth distributions with the deeper peak somewhat shallower than *Aphanizomenon* sp..

1.3. APHANIZOMENON SP. VERSUS NODULARIA SPUMIGENA

1.3.1. SEPARATED IN TIME AND SPACE

In my thesis I have focused on *Aphanizomenon* sp. and *N. spumigena*. Over the year, the respective dominance of these two species is both temporally and spatially separated (Kononen et al., 1998, Sellner, 1997). It has been suggested that the temporal separation, e.g. seasonal succession, with peaks of *Aphanizomenon* sp. followed by peaks of *N. spumigena* are related to prevailing physical conditions (salinity, radiation and temperature) in the Baltic Sea during summer (Kononen, 1992; Lehtimäki et al., 1994, 1997; Mazur- Marzec et al., 2005, Paper III; Paper IV) and species-specific niches have been proposed for the two species (Niemistö et al., 1989; Kononen et al., 1996; Vahtera, 2007b).

In a field study, Andersson et al. (1996) observed that as DIP concentrations decreased during the summer, a peak of *Aphanizomenon* sp. was followed by a peak of *N. spumigena*.

Degerholm et al. (2006) reported that *Aphanizomenon* sp. was better adapted to environments with elevated concentrations of phosphorus or repeated intrusions of phosphorus-rich water. In contrast, *N. spumigena* seems to have an ecological advantage in stratified surface waters when phosphorus availability is low (Degerholm et al. 2006) due to a higher affinity for low phosphorus levels than *Aphanizomenon* sp. (Wallström et al. 1992). In addition, *N. spumigena* may utilize dissolved organic matter (DOM) as a nutrient source (Panosso and Granéli, 2000; Pöder et al., 2003).

As shown in Fig. 7, *Aphanizomenon* sp. has a deeper biomass maximum than *N. spumigena* (Niemistö et al. 1989, Kononen et al. 1998; Vahtera et al. 2005; Hajdu et al., 2007). The vertical distribution pattern of these two species reoccurs in areas with similar environmental conditions, suggesting species-specific niche separation (Hajdu et al., 2007). It has been related to the variation in prevalent phosphorus source with depth (Vahtera et al., 2005, 2007b). Furthermore, stratification and temperature are two important factors with implications on the vertical pattern. Although having a wide temperature window (Lehtimäki et al., 1994, 1997; Mazur-Marzec et al., 2005), *N. spumigena* thrives in high temperatures in the surface water (Wasmund, 1997). Thus, living near the surface is advantageous for *N. spumigena*. *Aphanizomenon* sp. prefers lower temperature (Wasmund, 1997), and seems better adapted to areas and periods of hydrodynamic activity with weaker stratification (Niemistö et al., 1989; Vahtera et al., 2005).

Moreover, the vertical separation potentially reflects their different sensitivity to high radiation and their differences in photoprotective strategies. During a summer bloom, cells are often concentrated to the upper water layers where they are exposed to high radiation of both PAR, UV-A, and UV-B. In order to change the vertical positioning, both species can change their buoyancy using cellular gas vesicles (Paerl, 1988; Staal et al., 2003.). The deeper biomass of *Aphanizomenon* sp. could be a photoprotective strategy, while *N. spumigena*, often found in the surface water, produce MAAs. Despite the fact that *N. spumigena* is considered one of the most important phytoplankton species in the Baltic Sea, except from our work (Paper I, II, III, IV; Lindberg et al., 2008; Roleda et al., 2008) only one study has considered the impact of UV-B on this surface-blooming species (Sinha et al., 2003), and only in terms of the presence of MAAs.

1.3.2. TOXIN PRODUCERS

The two genera *Aphanizomenon* and *Anabaena* from the Baltic Sea have not been as intensively studied as *N. spumigena*, perhaps because of their assumed non-toxicity. The first scientific report of *N. spumigena* causing animal poisoning came from Australia (Francis, 1878). It took another 100 years until scientists from New Zealand identified the chemical structure of the toxin (Rinehart et al. 1988). At about the same time Sivonen et al. (1989) found that *N. spumigena* from the Baltic Sea produced this pentapeptide hepatotoxin and also that there must be non-toxic strains of *N. spumigena*. However, lately researchers have agreed that *N. spumigena* in the Baltic Sea is always nodularin-producing (Kononen, 1992; Chorus and Bartram, 1999; Laamanen et al. 2001).

Nodularin inhibit the protein phosphatase of eukaryotic cells; these enzymes play a major role in regulating cell division and influence the structure and function of cytoskeletal fibres (Runnegar et al., 1995; Annila et al., 1996 and references therein). The liver is the target organ of nodularin. Liver cells are damaged under acute intoxication and the toxin may also acts a tumour promotion under long-term exposition of small toxin doses (Runnegar et al., 1988; Nehring, 1993; Ohta et al., 1994; Humpage and Falconer, 1999; Song et al., 1999). Nodularin may cause toxic effects (Sellner, 1997; Ibelings and Havens, 2008) and lethal effects on wild and domestic animals have been reported (Edler et al., 1985; Nehring, 1993). Fishkills in Gulf of Finland in 1999 were suggested to be linked to the *N. spumigena* bloom that occurred during the same period (Kankaanpaa et al., 2002). The transfer of nodularin in the food web has been reported with accumulation in zooplankton (Karjalainen et al., 2006, 2008), blue mussels (*Mytilus edulis*) (Sipiä et al., 2002), tissues and liver of fish (Kankaanpaa et al., 2002; Sipiä et al., 2002; Persson et al. 2009) and in eiders (*Somateria mollissima*) (Sipiä et al., 2008).

Changes in nodularin production as a response to various environmental factors including temperature, salinity, radiation, and nutrient concentrations have been studied in laboratory experiments (Lehtimäki et al., 1994; Granéli et al., 1998; Repka et al., 2001; Mazur-Marzec et al., 2005; Hobson et al., 1999; Paper III). The increased production with radiation could imply that nodularin is produced as a photoprotective strategy. Gorokhova and Engström-Öst (2009) hypothesized a positive relation between intracellular nodularin concentration and grazing, but the grazing of the copepod *Eurytemora affinis* did not increase the concentration, on the contrary, a significant decrease was observed.

It appears that toxin production is highest under conditions that also favour growth of *N. spumigena*; high temperature and high irradiance, which correspond to conditions in late summer water column of the Baltic Sea (Lehtimäki et al., 1997). However, the effects of phosphorus concentration on nodularin production do not seem to be significant (Repka et al. 2001). It has been suggested that cyanobacterial toxins accumulate within the cells and are only passively released into the surrounding water due to cell lyses (Heresztyn and Nicholson, 1997). On the other hand, Hobson and Fallowfield (2003) suggest that high temperature and high irradiances could increase an active exudation of nodularin during natural blooms. The above cited studies on the effect of radiation on the toxin production of cyanobacteria used

artificial radiation in the laboratory and the spectral composition differed from ambient solar radiation (e.g. Lehtimäki et al., 1994, 1997; Hobson et al., 1999). Once again I would like to stress the fact, that for ecologically relevant studies of radiation effects including UVR, it is crucial that the spectral composition should be realistic (Karentz, 1994; Neale et al., 1998).

To our knowledge, the interactive effect of nutrient limitation and radiation on nodularin production, is until now neglected in the literature.

1.3.3. ALLELOPATHY

The word allelopathy originates from the greek word ἀλλήλων (allelon) meaning mutually, and πάθος (pathos) meaning pathos. In a publication from 1937, Molisch coined the term allelopathy as the impact of a plant on another plant. According to Molisch (1937) allelo could mean either mutual impact, impact among each other, and pathos mean agony. The allelopathic inhibitory effects of secondary metabolites, e.g. nodularins, and its importance in phytoplankton competition have been reviewed by Legrand et al. (2003). Keating (1977) showed that allelopathy can affect the seasonal succession in a phytoplankton community. Furthermore, the release of cyanobacterial toxins have been suggested to play an ecological role in the interspecific competition via stimulating the abundance of the same or other cyanobacterial species in the community, rather than inhibiting the abundance of competitors to cyanobacteria (Suikkanen et al., 2004, 2005). Suikkanen et al. (2006) further suggest that nodularin is not the main allelopathic compound produced by *N. spumigena*. Interestingly, nodularin has been shown to inhibit growth of prokaryotic cells (Mazur-Marzec et al., 2009), but to my knowledge there are no studies made on the potential allelopathic effect of *N. spumigena* on the co-existing *Aphanizomenon* sp.. I find this surprising since evidence from field studies show clear seasonal succession between these two (Andersson et al., 1996), and that this pattern could be explained by allelopathy.

Hepatotoxin and neurotoxin are likely not expressed in the Baltic Sea bloom-forming *A. flos-aquae* (Sellner, 1997; Sivonen et al. 1989; Willén and Mattsson, 1997). However, several observations show that freshwater strains are producing compounds which may alter species composition and activity in at least some environments including the Baltic Sea (reviewed by Sellner, 1997). In a study by Suikkanen et al. (2006), cell-free filtrates of *A. flos-aquae* from the Baltic Sea significantly inhibited the growth and production of the cryptophyte *Rhodomonas* sp. In the same study, *A. flos-aquae* was observed to inhibit *Rhodomonas* sp. more strongly than *N. spumigena* was. These results imply that *A. flos-aquae* from the Baltic Sea may produce allelopathic compounds that affect the species composition. Even if earlier studies have shown that *Aphanizomenon* sp. from the Baltic Sea do not produce toxins, more recent studies on isolates of *A. flos-aquae* from the Baltic Sea have detected a neurotoxic amino acid, β -N-methylamino-L-alanine (BMAA) (Cox et al. 2005; reviewed by Jonasson et al., 2008). BMAA is a neurotoxic non-protein amino acid produced by most cyanobacteria, and has been proposed to cause neurodegenerative diseases (Cox et al. 2003; Murch et al. 2004). In a study from the Baltic Sea, Jonasson et al. (2010) suggested that BMAA is

transferred from cyanobacteria and bioaccumulated via zooplankton to organisms at higher trophic levels (e.g. fish) in both pelagic and benthic ecosystems.

1.4. *BOTTOM-UP VERSUS TOP-DOWN*

In this project we have focused on bottom-up factors, but we are aware of other factors controlling primary production. It has been a key question in ecology whether primary production is controlled by nutrient and/or light (bottom-up), or by grazers (top-down). According to Tilman's resource competition theory (Tilman et al., 1982), those species which have either the lowest requirement for the limited resource (light or nutrients) or the highest ability to utilize it, will succeed in competition. Thus, a change in the resource will potentially change the species composition of the phytoplankton community (Tilman et al., 1982). Worm et al. (2002) conclude that bottom-up and top-down factors are greatly dependent on each other, but a eutrophic system such as the Baltic Sea, is supposed to be more strongly controlled by bottom-up factors (Lotze et al., 2001).

2. AIMS OF THE THESIS

This thesis aims at increasing the knowledge about factors controlling the occurrence and distribution of toxic cyanobacterial blooms, a knowledge crucial for predicting toxic blooms. The approach was to investigate the interactive effect of radiation (PAR and UVR) and nutrients (N, P) on the performance of bloom-forming cyanobacteria from the Baltic Sea.

The specific aims of this thesis are:

1. To detect and describe strain-specific differences of *Nodularia spumigena* in the response to UV-B radiation. (Paper I)

In Paper I, the UV-B tolerance of four strains of *N. spumigena*, isolated from the Baltic Sea, was investigated in the laboratory. The working hypothesis was that there is an intra-specific variation in the response of *N. spumigena* to UV-B radiation. Parameters were chosen to elucidate UV-B treatment effects on photosynthesis and growth. Therefore, the variables measured included growth rate, photosynthetic capacity (fluorescence), photosynthetic pigments, and content of MAAs.

2. To improve our understanding of factors controlling bloom dynamics of *Nodularia spumigena*. (Paper II)

In Paper II, we investigated if ambient solar radiation and nutrient limitation interact in their effects on the performance of *N. spumigena*. We hypothesized an additive negative effect of radiation when nutrient conditions are limiting. A two-factor outdoor experiment was designed with specific growth rate, cell concentrations of MAAs, and photosynthetic pigments as response variables. We also recorded heterocyst frequency, cell size, and particulate carbon (POC), particulate nitrogen (PON), and particulate phosphorus (POP). Radiation treatments were exposure to PAR and PAR+UV-A+UV-B (PAB), and nutrient treatments were NP (f/2 medium), -N (f/2 medium without nitrate) and -P (f/2 medium without phosphate).

3. To improve our understanding of factors controlling production and release of the toxin nodularin. (Paper III)

In Paper III we investigated the interactive effects of radiation, nutrient limitation, and species composition on the accumulation and release of nodularin. We performed one laboratory experiment and two outdoor experiments. In addition, we investigated if the presence of *N. spumigena* would have an allelopathic effect on the specific growth rate of

the co-existing *Aphanizomenon* sp. Radiation treatments were exposure to PAR and PAR+UV-A+UV-B (PAB), and nutrient treatments were NP (f/2 medium), -N (f/2 medium without nitrate) and -P (f/2 medium without phosphate). Species treatments were Nod (*N. spumigena*), Apha (*Aphanizomenon* sp.) and NodApha (*N. spumigena* in presence of *Aphanizomenon* sp.). Variables measured were intracellular nodularin, extracellular nodularin and specific growth rate of *Aphanizomenon* sp.

4. To improve our understanding of factors controlling the succession during the diazotrophic cyanobacterial blooms in the Baltic Sea. (Paper IV).

In Paper IV we tested the interactive effects between radiation and nutrient limitation, and how the presence (e.g. competition) of *N. spumigena* will affect *Aphanizomenon* sp. and vice versa. Radiation treatments were exposure to PAR and PAR+UV-A+UV-B (PAB), and nutrient treatments were -N (f/2 medium without nitrate) and -P (f/2 medium without phosphate). Species composition treatments for *Nodularia spumigena* are NOD (monoculture of *N. spumigena*), MNOD (*N. spumigena* in mixed culture with *Aphanizomenon* sp.). Species composition treatments for *Aphanizomenon* sp. are APHA (monocultures of *Aphanizomenon* sp.) and MAPHA (*Aphanizomenon* sp. in mixed cultures with *N. spumigena*). Response variables measured were specific growth rate, cell concentrations of MAAs, and photosynthetic pigments. We also recorded heterocyst frequency, particulate carbon (POC), particulate nitrogen (PON), and particulate phosphorus (POP).

3. COMMENTS ON THE METHODOLOGY

The difficulties that you encounter when dealing with radiation in the laboratory, are to obtain ecologically relevant radiation spectra, ratios and intensities. The ambient radiation intensities of UV-B, UV-A, and PAR that phytoplankton encounter in surface water is decreasing with depth (Table 1). Ambient solar spectra are of course preferable, but not always possible.

It could be argued that the UV-B levels we used in the laboratory were too low when compared with the levels that can be expected to occur at the water surface in the Baltic Sea, or even too high if we consider a bloom around 5 m depth. Despite our efforts to create an ecologically relevant light regime, our laboratory experiment (like all experiments using UV lamps) will remain more or less mechanistic due to technical difficulties in mimicking the solar spectrum over a surface large enough to allow for replicate samples. We used realistic ratios between PAR and UV-B, which is relevant for studies of UV-B effects, since DNA repair mechanisms after UV-B damage are dependent on these ratios (Karentz et al., 1991).

Table 1. The radiation intensities of UV-B, UV-A, and PAR measured, with broadband sensors in mid-summer 2008 at Askö Laboratory, Sweden.

Depth	UV-B (Wm⁻²)	UV-A (Wm⁻²)	PAR ($\mu\text{mol photons m}^{-2} \text{s}^{-1}$)
In air	1.1	20	1470
Surface	0.7	12.4	720
0.5 m	0.06	5.2	
1 m	0.004	1.5	518

In Paper I we measured the chlorophyll fluorescence in order to assess the effect of radiation treatments on the photosynthetic capacity. Measurements of chlorophyll fluorescence give information about the efficiency of PSII and hence the photosynthetic capacity. Thus, any damage in PSII will result in a decrease of the maximum quantum yield of photosynthesis, F_v/F_m (Maxwell and Johnson, 2000). For the procedure and equipment see section 2.3 in Paper I.

In Paper III the results from the two outdoor studies were conflicting; the interactive effect on the nodularin production was significant in Experiment C but not in Experiment B. The major difference between the two outdoor experiments was the seasonal timing: Experiment B was conducted from 5 July with decreasing day-length (1 h) and radiation intensity during the experimental period. In Experiment C, on the contrary, the radiation intensity and day-length increased (1 h), during the experimental period. This was reflected in a higher daily average dose in PAR, UV-A and UV-B during the last 5 days of Experiment C compared to Experiment B.

Biological effects of UVR are generally a function of wavelength; therefore, they are best quantified with a spectral biological weighting function (BWFs). BWFs are sets of spectral weights that account for the wavelength dependency of the photoinhibition and properly scale the exposure spectra to the effective biological response and are usually obtained from polychromatic exposures (for reviews see Cullen and Neale, 1997; Neale, 2000). Photoinhibition is likely to be a function of both UVR and the ratio between UVR and PAR. A good model of spectral dependence is particularly important for studies of aquatic photosynthesis, because both UVR and the ratio UVR:PAR change with depth in the water column. To be able to understand to what extent the diminishing ozone layer on the Nordic hemisphere would have on *N. spumigena*, determination of BWFs were done in 2009 (Mohlin et al., unpublished). We studied the effects of P-limitation on carbon fixation in three strains of *N. spumigena* under two different radiation exposures; high PAR: 250 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ and low PAR: 50 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. Vulnerability of photosynthesis to UVR was estimated using BWFs for the inhibition of photosynthesis and a model that predicts primary productivity under PAR and UVR exposures. As appropriate for predicting the response to in situ irradiance, BWFs were derived from polychromatic experimental treatments in which UVR and PAR from a solar simulator (xenon) lamp was varied using long-pass cut-off filters. The results show a strain-specific difference in sensitivity in the UV-spectrum and the sensitivity increased in P-deplete growth conditions.

4. SUMMARY OF RESULTS

4.1. PAPER I: INTRASPECIFIC VARIATION

Intraspecific differences were observed both regardless of treatment and as a result of UV-B exposure. The only consistent UV-B effect on all strains was significantly higher MAAs content at the end of the experiment. Most negative effects of UV-B were found for the maximum quantum yield (F_v/F_m), mainly in the beginning of the experiment. One out of four strains showed a lower growth rate at UV-B exposure. For photosynthetic pigments, either no UV-B effects were found, or the concentration of pigments increased.

Table 2. Summary of significant treatment effects (PAR + UV-A vs. PAR + UV-A + UV-B) on measured variables during the experiment. Numbers denote days when significant ($p < 0.05$) treatment effects were observed (ANOVA, $p < 0.05$). (+) denotes a higher value and (-) a lower value in the UV-B treatment.

Strain	F_v/F_m	Cells	Growth	Chl <i>a</i>	Carotenoids	MAAs
KAC 11	2 (-)			8 (+)	8 (+)	8 (+)
KAC 64	4 (+)			8 (+)	8 (+)	8 (+)
KAC 7	2 (-), 4 (-), 8 (-)					8 (+)
KAC 66	2 (-)	4 (-), 8 (-)	0-8 (+), 4-8 (+)	8 (+)	8 (+)	8 (+)

4.2. PAPER II: INTERACTION OF RADIATION AND NUTRIENT LIMITATION

The specific growth rate of *N. spumigena* was surprisingly positive despite changes in solar radiation and nutrient conditions. Of interest, but not of surprise, was the lack of effect of nitrogen limitation on the specific growth rate, but there was an overall effect of phosphorus limitation on variables measured. Effects on variables measured are summarized in Table 2. The table does not show the individual concentrations of photosynthetic pigments, POC, PON, or POP. Those variables can be found in Paper II.

Table 3. Summary of most interesting results from Paper II. Radiation treatments are exposure to PAR and PAR+UV-A+UV-B (PAB), respectively, and nutrient treatments are NP (f/2 medium), -N (f/2 medium without nitrate) and -P (f/2 medium without phosphate). Particulate organic carbon (POC), particulate organic nitrogen (PON), particulate organic phosphorus (POP), chlorophyll *a* (chl *a*), mycosporine-like amino acids (MAAs).

Variable	General results
Specific growth rate	Although overall positive regardless of treatment, there was a nutrient effect; lowest in -P
Heterocyst cell ⁻¹	Nutrient effect; more heterocysts in -N
Cell size	Interactive effect ; under PAB cells are smallest in -N, under PAR cells are small in both -N and -P
POC:PON	Interactive effect; in -P, the ratio was significantly higher in PAR compared to PAB, but in -N and NP, no effect of radiation was found. Under PAR, the ratio was higher for -P compared to NP.
POC:POP	Nutrient effect; the ratio was higher in -P compared to -N and NP.
PON:POP	Interactive effect; the ratio was higher in -P compared to -N and NP, irrespective of radiation treatment. In PAB the ratio was significantly lower in -N compared to NP.
MAAs (abs units cell ⁻¹)	For total MAAs, no significant interaction effect between radiation and nutrient limitation was observed. Cells exposed to PAB had significantly higher MAAs concentrations in -P compared to -N (nutrient effect). Under -P conditions, significantly more MAAs were found in PAB compared to PAR (radiation effect).
Chl <i>a</i> (ng cell ⁻¹)	Under PAR, significantly less chl <i>a</i> was found in -P compared with -N, and a radiation effect was observed in the NP and -N treatments where PAB-treated cells showed lower concentrations compared to PAR-treated cells.
Photosynthetic pigments (ng cell ⁻¹)	Lowest concentrations were observed under -P conditions, higher concentrations in cells exposed to PAR compared to PAB-treated cells. Individual pigments showed some differences with respect to the different radiation and nutrient treatments.

4.3. *PAPER III: A MULTIFACTORIAL APPROACH ON THE PRODUCTION AND RELEASE OF NODULARIN*

Highest intra- and extracellular nodularin concentrations were observed in –N, when shielded from UVR. Conversely, the lowest intracellular nodularin concentrations were found under phosphorus limitation under UVR. The presence of *Aphanizomenon* sp. did neither increase the intracellular nodularin production, nor the release of nodularin. The presence of *N. spumigena* had no impact on the specific growth rate of *Aphanizomenon* sp., irrespective of radiation and nutrient treatments. A significant correlation between intracellular nodularin and extracellular nodularin ($r^2 = 0.4132$, Pearson correlation=0.643) indicated that accumulation and release of nodularin were showed similar response, increased intracellular nodularin resulted in increased extracellular nodularin.

Table 4. Summary of results from Paper III. Radiation treatments are exposure to PAR and PAR+UV-A+UV-B (PAB), respectively, and nutrient treatments are NP (f/2 medium), -N (f/2 medium without nitrate) and -P (f/2 medium without phosphate). Species treatments were Nod (*N. spumigena*), Apha (*Aphanizomenon* sp.) and NodApha (*N. spumigena* in presence of *Aphanizomenon* sp.).

Experiment	Treatment factors	Variable	General results
A Laboratory	Radiation Nutrients	Intracellular nodularin	Nutrient effect: highest intracellular nodularin concentrations in –N and NP compared to –P, (–N=NP > –P)
B Outdoor	Radiation Nutrients	Intracellular nodularin	Nutrient effect: highest intracellular nodularin concentrations in –N and NP compared to –P, (–N=NP > –P)
C Outdoor	Radiation Nutrients Species	Intracellular nodularin	Interactive effect, radiation and nutrient: Higher concentration in –NPAR compared to –NPAB, –P PAR and –PPAB (–NPAR > –N PAB > –P PAB=–PPAR) Interaction effect, radiation and presence of <i>Aphanizomenon</i> sp.: Higher concentration in NodPAR compared to NodPAB, NodAphaPAR and NodAphaPAB (NodPAR > NodPAB=NodAphaPAR=NodApha PAB)
		Extracellular nodularin	Interactive effect, nutrient and presence of <i>Aphanizomenon</i> sp. Highest concentration (e.g. release of nodularin) was found in Nod –N PAR compared to all other treatments.

Specific growth rate of <i>Aphanizomenon</i> sp.	There was no effect of treatment
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4.4. PAPER IV: FACTORS CONTROLLING THE SEASONAL SUCCESSION

Our results indicate an interspecific difference regarding nitrogen versus phosphorus limitation. *N. spumigena* had a lower specific growth rate in -P compared to -N, and for *Aphanizomenon* sp. the results were opposed. The presence of did not affect *Aphanizomenon* sp. negatively however, the growth rate of *N. spumigena* was stimulated by the presence of *Aphanizomenon* sp.. Furthermore, *N. spumigena* had a higher specific growth rate compared to *Aphanizomenon* sp., regardless of treatment. In the variables measured *N. spumigena* seemed better adapted to both -N and -P in interaction with UVR than *Aphanizomenon* sp.

Table 5. Summary of results from Paper IV. Radiation treatments were exposure to PAR and PAR+UV-A+UV-B (PAB), and nutrient treatments were -N (f/2 medium without nitrate) and -P (f/2 medium without phosphate). Species composition treatments for *Nodularia spumigena* are NOD (monoculture of *N. spumigena*), MNOD (*N. spumigena* in mixed culture with *Aphanizomenon* sp.). Species composition treatments for *Aphanizomenon* sp. are APHA (monocultures of *Aphanizomenon* sp.) and MAPHA (*Aphanizomenon* sp. in mixed cultures with *N. spumigena*). Particulate organic carbon (POC), particulate organic nitrogen (PON), particulate organic phosphorus (POP), chlorophyll *a* (chl *a*), and mycosporine-like amino acids (MAAs).

Variable	General results <i>Aphanizomenon</i> sp.	<i>Nodularia spumigena</i>
Specific growth rate	Interaction effect; radiation and nutrient. Highest in -P, regardless of radiation treatment (PAB-P=PAR-P). In -N, lower in PAB compared to PAR (PAR-N>PAB-N). In PAB, lower in -N compared to -P (PAB-N<PAB-P).	Interaction effect; nutrient and competition. Higher in MNOD-N compared to MNOD -P. In -N, higher in MNOD compared to NOD. In -P, no difference between MNOD and NOD.
Heterocyst cell ⁻¹	Nutrients effect Higher frequency in -N compared to -P	Nutrient effect Higher frequency in -N compared to -P Species composition effect Lower in MNOD compared to NOD.
POC:PON	Nutrient effect Higher ratio in -P compared to -N	Nutrient effect Higher ratio in -P compared to -N Radiation effect Higher ratio in PAB compared to PAR
POC:POP	Interactive effect; radiation and nutrient In -N, irrespective radiation treatment In -P, there was an additional effect of radiation; higher in PAB -P compared to PAR-P.	Nutrient effect Higher ratio in -P

PON:POP	Nutrient effect Higher ratio in -N compared to -P	Nutrient effect Higher ratio in -P compared to -N
MAAs (abs units cell ⁻¹)	No treatment effects	Interactive effect; radiation and nutrient In PAR, higher in -P compared to -N In PAB, no difference between -N and -P. Furthermore, In -P, higher in PAR compared to PAB In -N, no difference between PAR and PAB
Chl <i>a</i> (ng cell ⁻¹)	No treatment effects	Radiation effect Higher in PAR compared to PAB Nutrient effect Higher in -N compared to -P
Photosynthetic pigments (ng cell ⁻¹)	No treatment effects	Nutrient effect Higher 4-ketomyxoxanthophyll concentration -N compared to -P The same pattern for carotenoids
Phycocyanin (pg cell ⁻¹),	No treatment effects	No treatment effects

5. DISCUSSION

5.1. *EFFECTS OF UV-B RADIATION AND INTRASPECIFIC VARIATIONS*

The first paper (Paper I) revealed the strain specific differences in the response of *N. spumigena* to UV-B radiation.

We hypothesised:

No negative effect of UV-B on the specific growth rate of *N. spumigena*.

There would be species-specific differences in response to UV-B radiation.

5.1.1. *UV-B, THEY DON'T CARE!*

The results in Paper I show that *N. spumigena* is a species that is not generally negatively affected by moderate levels of UV-B radiation. Despite the fact that *N. spumigena* is considered one of the most important phytoplankton species in the Baltic Sea, only one study has considered the impact of UV-B on this surface-blooming species (Sinha et al., 2003), and only in terms of the presence of mycosporine-like amino acids (MAAs).

The maximum quantum yield, (F_v/F_m) has been shown to be a sensitive non-specific marker of UV-B exposure (Cordi et al., 1997). For all strains, this value was low in the beginning of the experiment; however, the photosynthetic activity was high enough to sustain a positive growth rate among the strains. A decrease in the F_v/F_m ratio could reflect either dynamic or chronic photoinhibition (Hanelt et al., 2003). Dynamic photoinhibition is a regulative mechanism for protection against excessive radiation (Hanelt et al., 2003, and references therein), a mechanism that most certainly caused the lowering of F_v/F_m in our study, since no negative impact on growth was observed. For the genus *Anabaena*, Kumar et al. (2003), observed that although growth was inhibited at UV-B intensities as low as 0.2 W m^{-2} , in the presence of white light, a positive growth was found up to 2.4 W m^{-2} . The positive role of photoreactivation by PAR and UV-A in UV-B-damaged cyanobacterial cells has also been shown by Blakefield and Harris (1994) and Ivanov et al. (2000). Although we never tested the impact of photoreactivation, the PAR and UV-A levels used in our study were high enough to repair or reduce the damage of the UV-B-damaged cells (cf. Kumar et al., 2003).

UV-B-treated cells, in all strains, showed a significant accumulation of total MAAs, indicating that UV-B had an important role in the induction or accumulation of MAAs. Sinha et al. (2001, 2003) found that UV-B played a vital role in the accumulation of MAAs in cyanobacteria; UV-B was shown to be most efficient in the induction of shinorine (Portwich and Garcia-Pichel, 2000). Based on spectra and retention time and other studies (Sinha et al.,

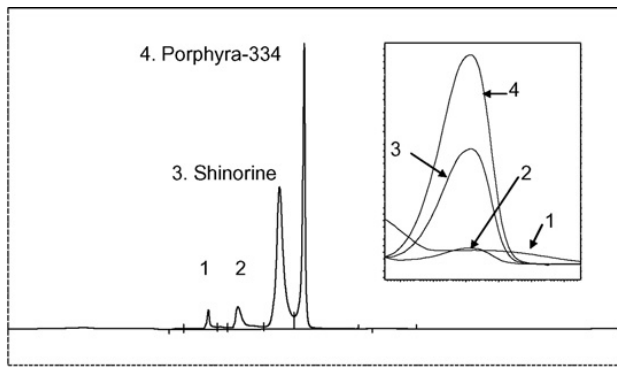


Fig. 8. High-pressure liquid chromatography (HPLC) chromatogram showing the different mycosporine-like amino acids found in *Nodularia spumigena*, together with their normalized absorbance spectra.

2003) we concluded that the different MAAs present in our *Nodularia* strains were shinorine and porphyra-334 (Fig. 8).

On a cell basis, the MAAs concentrations provide a sunscreen factor of 0.11–0.26 at 320 nm (Garcia-Pichel and Castenholz, 1993), implying that at least one out of ten photons are prevented from hitting their target. However, as the authors point out, a colonial growth form (like in our case) will provide a substantially higher sunscreen effect. In our study we cannot conclude that the accumulation of MAAs was the sole factor enabling the cells to retain (or

increase) their photosynthetic activity and growth in the presence of UV-B. The significantly lower F_v/F_m in KAC7 does not support the theory that MAAs provide a protection against UV-B. On the other hand, support is found in the F_v/F_m data for the other three strains.

Although MAAs have been described as giving protection against UV-B damage through their sunscreen properties (Garcia-Pichel and Castenholz, 1993; Shick and Dunlap, 2002), there have been suggestions that this is not their only role. Dunlap and Yamamoto (1995) proposed that some MAAs may act as antioxidants, thereby preventing UV-induced cellular damage from active oxygen species. UV-B has been shown to increase the production of reactive oxygen species in *Anabaena* sp. (He and Häder, 2002). In addition, MAAs have been proposed to have a role as osmolytes (Shick and Dunlap, 2002 and references therein). In *Chlorogloeopsis* the steady-state concentration of MAAs was positively related to environmental salinity: the osmotically induced mycosporine-glycine was enhanced by UV-B, and shinorine accumulated due to UV-B only (Portwich and Garcia-Pichel, 1999). Although we did not test any variables other than light on the induction and accumulation of MAAs, our study also showed that shinorine accumulated due to UV-B.

5.1.2. *STRAIN SPECIFIC DIFFERENCES, SHOULD WE CARE?*

The results from Paper I suggest that *N. spumigena* is a species that is not negatively affected by moderate UV-B intensities, since no lasting overall negative effects of the UV-B treatment were evident. However, assuming this study was performed on one of the strains, for example KAC66, the conclusion would have been different. For this strain, significant negative UV-B effects were observed for cell numbers and growth rate and although not statistically significant, also the F_v/F_m was lower by Day 8. For photosynthetic pigments the conclusion would have been that the pigments myxoxanthophyll and canthaxanthin are not affected by UV-B. On the other hand, conclusions drawn from KAC64 points in a different direction. No or positive UV-B effects were found for cell numbers, growth rate and F_v/F_m . In this strain, the pigments myxoxanthophyll and canthaxanthin increased in the UV-B treatment but 4-keto-myxoxanthophyll-like pigment, now defined as 4-keto-myxoxanthophyll (Schlüter et al., 2008), and b-cryptoxanthin-like remained unaffected. Interestingly, 4-keto-myxoxanthophyll showed the largest variation among strains. Considering the fact that this pigment has been suggested as a biomarker for both *N. spumigena* and nodularin in the Baltic Sea (Schlüter et al., 2004, 2008), further studies are needed to elucidate how its content varies between strains and with environmental conditions. An intraspecific variation in the requirement of nutrients for optimal growth was shown for the dinoflagellate *Gymnodinium catenatum* (Bolch et al., 1999). It is possible that also our variation in growth rate was a consequence of strain-specific requirements for nutrients.

To answer the question I would say, yes we should care!

Thus, we conclude that studying one strain is not enough to draw conclusions about a species response to environmental stress. Burkholder and Glibert (2004) found that “different and often opposite interpretations at the species level, would have resulted from consideration of individual strains”. In a 5-year literature survey of the harmful algal literature they conclude that intraspecific variation is commonly overlooked in characterizations of harmful algal species and they stress the importance of including multiple strains in research studies.

5.2. NUTRIENT LIMITATION + UVR, THEY DON'T CARE!

In Paper II, we investigated if ambient solar radiation and nutrient limitation have interactive effects on the performance of *N. spumigena*

We hypothesized:

Ambient UVR would have an additive negative effect when nutrients are limiting

N. spumigena had surprisingly a positive growth rate, despite changes in solar radiation and nutrient conditions (Paper II). We expected that ambient UVR used in this outdoor experiment, compared to artificial UVR used by Wulff et al. (Paper I), would have a significant impact on the growth rate of *N. spumigena*. In addition, we expected a additive effect of radiation when the nutrient conditions were limiting (Wulff et al. 2000). Although strain-specific differences in UV-B tolerance have been observed, we conclude that *N. spumigena* is a species that is not negatively affected by moderate levels of UV-B (Paper I). Another explanation might be that they are already stressed by the high PAR. In other studies (Lehtimäki et al., 1994), where growth rate was stimulated by increased radiation, the highest radiation intensities used were less than 20% of the radiation intensities in Paper II. However, despite an overall positive growth rate regardless treatment, there were interactive effects of radiation and nutrient limitation on important functions for fitness of *N. spumigena*.

P-limitation had a negative impact on growth rate in this study. According to Goldman et al. (1979), phytoplankton deprived of phosphorus during growth have PON/POP ratios greater than 30:1, whereas phytoplankton deprived of nitrogen during growth have PON/POP ratios less than 10:1. In Paper II, the POP/PON ratios found in -P treatment indicate severe phosphorus limitation (Healy, 1975; Paasche and Erga, 1988). The low PON/POP (-N and NP treatments) rather indicated internal storage of P than an indication of nitrogen limitation since nitrogen limitation is not expected in the diazotrophic *N. spumigena*. This is supported by the fact that the POC/PON ratio did not indicate nitrogen limitation.

When light energy is the most limiting factor, cells adjust chemically and physically to capture more light, but when nutrient deficiency is the growth-limiting factor, pigment concentrations depend upon precursors for pigment synthesis (Rosen and Lowe, 1984). In Paper II, pigment concentrations were generally lower under phosphorus limited conditions. Latasa and Berdalet (1994) studied phosphorus starvation on the dinoflagellate, *Heterocapsa* sp. The authors hypothesize that when phosphorus is depleted the cells can no longer synthesize RNA, and thus, pigment synthesis stops (no transcription of DNA into RNA). It has been shown that concentrations of nitrogen-containing pigments like chl *a* decrease more than carotenoids under low nitrogen availability (e.g., Latasa and Berdalet, 1994; Staehr et al.

2002). No such decrease was observed in Paper II, that is, no difference between the NP and –N treatments was found. This suggests that *N. spumigena* was not N-limited due to their ability to fix atmospheric nitrogen.

Photosynthesizing diazotrophic cyanobacteria encounter ecophysiological problems because oxygen (O₂) evolved during photosynthesis inhibits nitrogenase, the enzyme responsible for N₂-fixation (Bothe, 1982). To solve the problem some genera form O₂ deplete heterocysts where nitrogenase is located (Paerl, 2000). The frequency of these specialized cells is usually indicative of the N₂-fixation activity level in cyanobacterial filaments. N₂-fixation activity was not measured in our studies, but it has previously been shown that changes in nitrate concentrations do not affect nitrogenase activity in *Nodularia* spp. (Sanz-Alferez and del Campo, 1994, and references therein). Further, Vintila and El-Shehawey (2007) showed that the genes involved in heterocyst differentiation and N₂-fixation in *N. spumigena* strain AV1 are continuously expressed and not affected by changed concentrations of the nitrogen source. Others have reported that the formation of these specialized cells is enhanced under nitrogen limited conditions (Paerl, 2000), explaining the higher heterocyst frequency in–N compared to –P (Paper II). Additionally, it has been shown that low phosphorus availability may suppress heterocyst formation, with a subsequent reduction in N₂-fixation activity and hence, cellular nitrogen content (Healey, 1973; Layzell et al., 1985). In our study, there was a lower heterocyst frequency and lower PON inP PAR comp ared to–N PAR. The results from Paper II show that heterocyst frequency is not a good indicator of nitrogen limitation in the surrounding water since the frequency vary depending on environmental conditions.

Despite the negative effect of –P, the growth rate was still positive (Paper II); the reason might be a capability of storing phosphorus for future phosphorus scarcity. Similar results was found by Degerholm et al. (2006) and are further supported by a study by Vahtera et al. (2007b), where a phosphorus limitation (PO₄³⁻, <0.5 μM; NO₃⁻ + NO₂⁻, 0.5–2.0 μM) showed the most distinct increase in PON/POP caused by decrease in cellular phosphorus stores (TEM images). The authors concluded that PON/ POP of *N. spumigena* has high plasticity, and efficient growth can occur at DIN/DIP, far exceeding the Redfield ratio of 16:1 (up to 80:1). The high POC/POP value in –P treatment compared to –N and NP treatments (paper II) indicate that internal phosphorus storages are used to maintain a low and positive growth rate (Walve and Larsson, 2007). DIP was quickly vanished within 1 day after addition of new medium in the nutrient treatments. Also Vathera et al. (2007b) and Roleda et al. (2008) noticed a rapid decline in DIP concentrations in phosphorus limited treatments.

As we observed in Paper I, UV-B is responsible for the induction and/or accumulation of MAAs in *N. spumigena*. In Paper II the exposure to UVR did not increase MAAs except in –P. Thus, apparently ambient PAR intensities in our study were so high that additional UVR did not have any further impact on the MAAs concentrations. Compared to radiation conditions in our laboratory study (Paper I) and others (Portwich and Garcia-Pichel 2000; Sinha et al., 2003), the ambient PAR intensity was 10 to 20 times higher. Our results stress the fact that ecologically relevant UVR studies should be performed under ambient solar radiation. We expected lower MAAs concentrations in –N conditions because those

compounds contain nitrogen (Karentz, 2001). This was not the case, obviously the cells fix enough atmospheric nitrogen (or use a different nitrogen source) to account for the MAAs production. Despite the cost of N₂-fixation and MAAs production, the overall fitness reflected as specific growth rate was not lower in the –N treatment compared with the NP treatment. Interestingly and unexpectedly, significantly higher MAAs concentrations were found in UV-exposed cells under phosphorus limitation, although the cell density was higher compared with the PAR treatment. Possibly, the higher MAAs concentration was due to the larger cell volume (25% larger cells in PAB compared to PAR).

5.3. COMBINED EFFECTS ON ACCUMULATION AND RELEASE OF NODULARIN, SHOULD ANYONE CARE?

In Paper III we investigated if ambient radiation, nutrient limitation and the presence of *Aphanizomenon* would have interactive effects on the production and/or release of the toxin nodularin. We investigated if the presence of *N. spumigena* would have an allelopathic effect on the specific growth rate of the co-existing *Aphanizomenon* sp.

We hypothesized:

Nodularin production would be negatively affected in –N and UVR would have additive negative effects.

Extracellular nodularin would increase in presence of *Aphanizomenon* sp.

N. spumigena would have a negative effect on the specific growth rate of the co-existing *Aphanizomenon* sp.

We expected lower intracellular nodularin concentration in –N, because nodularin is a nitrogen-rich compound, 8 mol N (mol nodularin)⁻¹ (Stolte et al., 2002). Surprisingly, no such correlation was found: highest concentrations were observed in –N. Stolte et al.(2002) did not find any significant differences in nodularin production rate due to different nitrogen source (NO₃⁻, N₂ and NH₄⁺), implying that *N. spumigena* is able to fix enough atmospheric N₂ to account for the nodularin production. This is further supported by Paper II where POC:PON did not indicate that *N. spumigena* was nitrogen limited even under severe N-limited growth conditions.

Earlier results from single-factor experiments are contradictory regarding intracellular nodularin with respect to phosphorus conditions. There are observations of increased nodularin in phosphorus limited conditions (Granéli et al. 1998) while others report increased

nodularin in phosphorus replete conditions (Lehtimäki et al. 1997) and finally no impact of phosphorus on nodularin accumulation (Repka et al. 2001; Stolte et al. 2002; Vuorio et al., 2005; Lagus et al., 2007). Our study show that the intracellular nodularin concentration was lowest in -P and that UVR had an additional reducing effect. The results suggest that ambient radiation and nutrient conditions do have significant interactive effects on the accumulation of nodularin and stress the importance of multi-factorial approaches. Further support for multi-factorial approaches were provided by Hobson et al. (1999) and Hobson and Fallowfield (2003); whereby combined effects of radiation and salinity (Hobson et al., 1999) and of temperature and radiation (Hobson and Fallowfield, 2003) on the nodularin production were suggested.

It has been suggested that most of the nodularin produced remain within the cells (Repka et al. 2004) and is only released upon cell lyses (Park et al., 1998; Sivonen and Jones, 1999; Suikkanen et al., 2004). Others have reported that environmental conditions seem to affect the release of nodularin and other cyanobacterial toxins. In single-factor experiments the release has been reported to increase with increased phosphate concentrations (Lehtimäki et al. 1997), increased radiation (Sivonen, 1990; Lehtimäki et al. 1997) and increased temperature (Sivonen, 1990). Only a few studies have investigated the effect of combined environmental conditions. Hobson and Fallowfield (2003) showed that interaction of temperature and high radiation increased the release of nodularin into the surrounding water and they also observed that the release originated from intact cells, not from degraded cells. Our results suggest that ambient radiation and nutrient conditions have significant interactive effects on the release of nodularin. To our knowledge, few have studied bloom dynamics and nodularin concentrations of *N. spumigena* in the field. In filtered water samples collected three days after the peak of the bloom, a dramatic decrease of nodularin due to water mixing or degradation by the natural microbial community was observed (Mazur-Marzec et al. 2006a). The maximum nodularin concentration where found in *N. spumigena* blooms of moderate intensity.

The correlation between intracellular nodularin and extracellular nodularin (Paper III) indicated that accumulation and release of nodularin were dependent on each other. Jonasson (2006) suggested that when intracellular nodularin concentration reaches a certain threshold, the excess nodularin will either be released or degraded within the cell. We could speculate that those threshold intracellular concentrations vary with respect to environmental conditions. Thus the release of extracellular nodularin is only related to those threshold values and not to environmental conditions.

The presence of *Aphanizomenon* sp. did not increase the release of nodularin and the growth rate of *Aphanizomenon* sp. was not affected by the presence of *N. spumigena*. That was unexpected since we hypothesized that the presence of *N. spumigena* (e.g. nodularin) would have an allelopathic effect on *Aphanizomenon* sp.. On the contrary, highest extracellular nodularin concentration found was in absence of *Aphanizomenon* sp. Thus, we suggest that the allelopathic effect of nodularin on *Aphanizomenon* sp. could be neglected. This is further supported in a mesocosm study by Engström-Öst et al. (2002). They created an artificial *N. spumigena* bloom in mesocosms and investigated the development of a natural plankton

community; the results did not reveal harmful effects of nodularin on the organisms studied. Our results support the suggestion that *N. spumigena* and *Aphanizomenon* sp. occupy different niches (Kononen et al., 1996; Lehtimäki et al., 1997; Vahtera et al., 2007b) rather than perform a chemical warfare. In contrast and further discussed in Paper IV and in Section 5.4., cyanobacterial allelochemicals have been proposed to have an ecological role in maintaining Baltic cyanobacterial dominance by stimulating the abundance of the same or other species of cyanobacteria, rather than inhibiting the abundance of competitors to cyanobacteria (Suikkanen et al., 2004, 2005, and 2006).

We believe that the photoprotective role of nodularin is negligible; we observed lower nodularin concentrations under UVR compared to PAR. The absorbance spectrum of nodularin peaking at 238 nm (Harada, 1995), could imply a possible photoprotective role of this compound. This is relevant from an evolutionary perspective when UVC still reached the Earth's surface; at present day radiation condition this is no longer of ecological relevance. The lower concentration under UVR could be explained by a degradation of nodularin. It has been shown that both intracellular and extracellular nodularin are degraded by UVR (Twist and Codd, 1997; Mazur-Marzec et al., 2006b).

To answer the question, two answers are needed:

No, *Aphanizomenon* sp. should not care since its presence did not increase the release of nodularin. Thus the potential target species of chemical defence or attack of *N. spumigena* is probably found somewhere else.

Yes, we should definitely care since the nodularin concentration was highly variable depending on treatment. Discussions concerning further increase of nitrogen removal in sewage treatment, should take into account that this may lead to increased toxicity during the *N. spumigena* blooms. The results further suggest that *N. spumigena* may be expected to produce less nodularin in the end of the bloom when phosphate concentrations are very low in the surface water.

5.4. SEASONAL SUCCESSION; PREFERENCES OR ALLELOPATHY?

To improve our understanding of factors controlling the succession during the diazotrophic cyanobacterial blooms in the Baltic Sea.

We hypothesized:

Nutrient limitation in interaction with UVR would negatively affect *Aphanizomenon* sp.

The presence of *N. spumigena* would have additive negative effects on the performance of *Aphanizomenon* sp.

The presence of *Aphanizomenon* sp. would not have additive negative effects on the performance of *N. spumigena*.

Even if *N. spumigena* had an overall higher specific growth rate compared to *Aphanizomenon* sp., our results indicate an interspecific difference regarding nitrogen versus phosphorus limitation. *N. spumigena* had a lower specific growth rate in –P compared to –N and for *Aphanizomenon* sp. the results were opposite. This is consistent with a study by Lehtimäki et al. (1997), who suggested that *A. flos-aquae* has lower phosphorus demand compared to *N. spumigena*. Kononen et al. (1998) suggested that this species difference in utilizing surplus phosphorus could be explained by different nutrient uptake kinetics. It has been shown that *N. spumigena* has higher affinity for phosphorus than *A. flos-aquae* ($K_s = 0.016$ and 0.048 pM, respectively) (Wallström et al. 1992), but of explicable reasons this was not obvious in –P treatment in our study. We observed that *N. spumigena* efficiently absorbed all phosphorus after addition of new –N media while minor amounts were absorbed by *Aphanizomenon* sp., the observations indicate their different affinity for phosphorus. Despite the fact that *N. spumigena* absorbed phosphorus from the media, POC:POP remained high, most probably because of the high growth rate and the consumption of the internal storage. The opposite situation was found in the slow growing *Aphanizomenon* sp.; excess phosphorus in –N treatment was not absorbed from the media and a low POC:POP indicated that the internal storage of phosphorus was not consumed. The POC:POP ratio further indicates that both species are under severe phosphorus limitation, however, *Aphanizomenon* sp. have a lower ratio and more phosphorus stored per cell than *N. spumigena*. We suggest that these results, together with the lower specific growth rate in –N compared to –P for *Aphanizomenon* sp., indicate a lower efficiency in N_2 fixation of *Aphanizomenon* sp. rather than having a lower affinity for phosphorus compared to *N. spumigena*. This is consistent with the higher growth rate in –P with surplus of N.

We did not expect *N. spumigena* to be nitrogen limited based on previous results (Paper II) and this was the case (POC:PON <7). The opposite was found for *Aphanizomenon* sp. (POC:PON ratio >7) and their contrasting results could be explained by a previous study where Stal et al. (2003) reported a higher efficiency of N₂ fixation of *N. spumigena* than *Aphanizomenon* sp. The PON:POP found in the -P treatment (our study), indicated severe phosphorus limitation (Healey, 1975; Paasche and Erga, 1988): 40 for *N. spumigena* and 20 for *Aphanizomenon* sp.. The negative impact of -P was reflected in the specific growth rate of *N. spumigena* and our results corroborate with previous studies (Lehtimäki et al., 1997; Paper II). Vahtera et al. (2007b) found that an increase in PON:POP is caused by decrease in cellular phosphorus stores (TEM images). Furthermore, Walve and Larsson, (2007) found that a high POC:POP indicated that internal phosphorus storages were used for growth. According to the POC:POP ratio in our study *N. spumigena* was phosphorus limited even in -N where DIP was added in surplus (10 µM), but not reflected in the specific growth rate. We noticed that DIP declined from ~5µM to 0.3-1 µM within one day after addition of new medium in the nutrient treatments. Also, Vahtera et al. (2007b) and Roleda et al. (2008) noticed a rapid decline in phosphate concentrations in DIP-replete treatments. The rapid decline must be due to the high specific growth rate in -N treatment and a high consumption of DIP. Vahtera et al. (2007b) concluded that PON:POP of *N. spumigena* has high plasticity and efficient growth can occur at DIN:DIP far exceeding the Redfield ratio of 16:1 (up to 80:1).

As in Paper II, the heterocyst frequency was high in -N, but this time for both *N. spumigena* and *Aphanizomenon* sp. Furthermore, *N. spumigena* and *Aphanizomenon* sp. had lowest heterocyst frequency in -P conditions with excess of DIN. It is known that DIN tends to suppress heterocyst formation (Adams and Duggan, 1999). In a field study, Laamanen et al. (2005) observed that the annual pattern of development of *N. spumigena* had a rather invariable frequency with an abrupt decrease in autumn when DIN increased in the surface layer, these findings corroborate our results. Laamanen et al. (2005) also found that *A. flos-aquae* displayed a more variable annual development pattern that was associated with changes in temperature, increasing biomass and nutrients. They observed that *A. flos-aquae* had lower average heterocyst frequency than *N. spumigena* and corroborate our results with an overall threefold higher heterocyst frequency for *N. spumigena*. Surprisingly, we found that *N. spumigena* had lower heterocyst frequency in presence of *Aphanizomenon* sp. compared to monocultures.

The additional negative effect of UVR under nutrient limitation was obvious in the specific growth rate of *Aphanizomenon* sp. The specific growth rate was significantly lower in -N; however the effect of UVR was not reflected in the MAAs concentration. Previous observations *in situ* have revealed that *Aphanizomenon* filaments are usually evenly distributed down to 10 m, but *N. spumigena* is concentrated near the surface (Niemistö et al., 1989; Kononen et al., 1998). In our study *Aphanizomenon* sp. was not able to avoid radiation by vertical migration in the water column, and the overall high concentration of MAAs per cell indicates that the radiation exposure was high in the PAR treatment. This was reflected in the lack of treatment effect and an increased concentration per cell from initial conditions. Our results, together with previous studies (Niemistö et al., 1989; Kononen et al., 1998)

indicate that the vertical positioning in the water column can be a photoprotective strategy of *Aphanizomenon* sp. We observed that there was a difference of vertical positioning in the aquaria when bubbling was interrupted, *N. spumigena* had positive and *Aphanizomenon* sp. had negative buoyancy. The buoyancy of cyanobacteria in general is affected by environmental factors and has markedly different behavior in different species, explaining the occurrence of blooms under various conditions (reviewed by Oliver, 1994). *A. flos-aquae* seem to lose buoyancy when radiation intensities increase (Kromkamp et al. 1986; Oliver and Walsby, 1984).

N. spumigena was not negatively affected by UVR and this is consistent with our previous studies (Paper II). In laboratory studies, the importance of UV-B in the induction or accumulation of MAAs in cyanobacteria has been observed (Portwich and Garcia-Pichel, 2000, Sinha et al. 2003, Paper I, Paper II). In the present study the highest MAAs concentration in *N. spumigena*, was found under PAR and –P in contrast to Paper II, where the highest concentration was found under PAB and phosphorus limitation. Results from both studies confirm our hypothesis that under high ambient PAR additional UVR do not have any further impact on the MAAs concentrations. The lowest MAAs concentration was not found in –N, despite the fact that MAAs is a nitrogen containing compound (Karentz 2001). In accordance with previous studies (Paper I and Paper II), *N. spumigena* fix enough N₂ (or use a different N-source) to account for the MAAs production.

For *N. spumigena* cells in this study, radiation and nutrient effects, respectively, were found for both chl *a* and carotenoids. The higher concentrations in PAR compared to PAB is probably due to a down regulation of the rate of light absorption through a decrease in the concentration of chl *a* and light harvesting pigments (cf. Miskiewicz et al 2000). However, myxoxanthophyll has been suggested to have a photoprotective role (Miskiewicz et al 2000) but in our study, myxoxanthophyll did not increase in the PAB treatment. Although not statistically significant, the phycocyanin concentrations in *N. spumigena* followed the same pattern as chl *a* and carotenoids. Consistent with our results, Collier and Grossman (1992) showed decreased phycobilisome concentrations under phosphorus depleted conditions. Due to fixation of N₂, it is of no surprise that the pigment composition was not affected by the –N treatment. Due to severe phosphorus limitation in the –P treatment, the low pigment concentrations could be explained by a disrupted pigment synthesis (Latasa and Berdalet, 1994). The pigment 4-keto-myxoxanthophyll has been suggested as a biomarker for both *N. spumigena* and nodularin in the Baltic Sea (Schlüter et al., 2004, 2008). In Paper I, this pigment showed the largest variation between different strains of *N. spumigena*. In the present study, 4-keto-myxoxanthophyll was the only pigment being affected by the presence of *Aphanizomenon* sp. why its variation with environmental conditions as well as with co-existing species should be further investigated.

The growth rate of *Aphanizomenon* sp. was unaffected by the presence of *N. spumigena*. The results are consistent with previous observations in a parallel study (Paper III); no correlation between increased nodularin concentration and the specific growth rate of *Aphanizomenon* sp. was found. The temporal succession seen in the Baltic Sea is better explained by the species-

specific niches that have been proposed for the two species (Niemistö et al., 1989; Kononen et al., 1996; Vahtera et al., 2007b). *N. spumigena* had higher specific growth rate compared to *Aphanizomenon* sp., regardless of treatment, and most surprisingly the growth rate of *N. spumigena* was stimulated in presence of *Aphanizomenon* sp. As I discussed earlier, it has been suggested that the release of cyanobacterial toxins may play an ecological role in the interspecific competition via stimulating the abundance of the same or other cyanobacterial species in the community, rather than inhibiting the abundance of competitors to cyanobacteria (Suikkanen et al., 2004, 2005). Even if earlier studies have shown that *Aphanizomenon* sp. from the Baltic Sea do not produce toxins (Willén and Mattsson, 1997) more recent studies on isolates of *A. flos-aquae* from the Baltic Sea have detected a neurotoxic amino acid, β -N-methylamino-L-alanine (BMAA) (Cox et al. 2005); Jonasson et al (2008). BMAA is a neurotoxic non-protein amino acid produced by most cyanobacteria, has been proposed to cause neurodegenerative diseases on the island of Guam in the Pacific Ocean. BMAA has been related to neurodegenerative diseases among Chamorroan and Canadian patients (Cox et al. 2003; Murch et al. 2004). It may not be this compound that is responsible for the stimulated specific growth rate of *N. spumigena*, but it shows that *Aphanizomenon* spp. produce compounds that can affect other organisms.

To answer the question:

The seasonal succession, with peaks of *Aphanizomenon* sp. followed by peaks of *N. spumigena* is a result from species-specific preferences and/or stimulation by *Aphanizomenon* sp., rather than the allelopathic effect of *N. spumigena*.

CONCLUSIONS AND FUTURE PROSPECTS

How will the future appear with respect to environmental conditions, cyanobacterial blooms, dominating species and toxin production?

Global models, scenarios, and data sets are tools that we have at hand to make predictions of future climate changes. Although difficult to predict with certainty, it has been projected that the surface water temperature will increase about 3°C to 5°C during the 21st century in the Baltic area. This scenario will result in a decreasing ice cover period and a consequent prolongation of the growing season (1 to 3 months) (HELCOM 2007; Neumann, 2010). The predicted increase in temperature together with a general increase in annual precipitation will promote a stronger stratification, beneficial conditions for bloom forming cyanobacteria. The climate change is further predicted to increase the UV-B radiation, due to prolonged reductions of the ozone layer (Shindell et al., 1998; Aldhous, 2000; Anonymous 2002; Anonymous, 2006), despite the success of the Montreal Protocol of 1987 in reducing emissions of ozone-destroying chemicals.

Sweden and other countries around the Baltic Sea, through the Helsinki Commission (HELCOM), have agreed to reduce the emissions of nutrients to sustainable levels (HELCOM, 2007). From 1990 to 2000, inputs of phosphorus and nitrogen decreased by 68% and 60%, respectively (HELCOM, 2010). Despite the success in reducing the inputs of nitrogen and especially phosphorus to the Baltic Sea, almost all open basins and coastal waters are still classified as areas affected by eutrophication. The only coastal areas not affected by eutrophication are found in the Gulf of Bothnia (HELCOM, 2010). If the eutrophicated nutrient conditions will continue, where diatoms are promoted to form spring blooms and remove DIN, and the subsequent hypoxic bottom-water will increase DIP, the situation will most certainly continue to supply an advantageous situation for N₂-fixing cyanobacteria during late summer.

After studying the historical and predicted environmental conditions in the Baltic Sea together with our results, I get more and more convinced that the ratio between the major nutrients will decide whether there will be extensive cyanobacterial blooms or a more diverse phytoplankton composition. As long as the N/P ratio can be reduced by a diatom spring bloom to a threshold that counteracts a diverse phytoplankton composition, the N₂-fixing cyanobacteria will remain the “Lords of the Baltic” in the future.

I am also convinced that the ultimate “Lord of Summer blooms”, either *Aphanizomenon* sp. or *N. spumigena*, without hesitation is a question of their respective response to the future scenario of UVR in combination with nutrient conditions. Our results together with previous research stress the fact that under predicted environmental conditions with stronger stratification, low N:P ratios and increased UV-B radiation, *N. spumigena* is the most

qualified to win this title. We have shown that this domination over *Aphanizomenon* sp. is not a question of chemical arms (e.g. allelopathy), just a question of a successful adaptation to UV-B and nutrient limitations.

The environmental (e.g. radiation and phosphorus concentrations) conditions on nodularin production of *N. spumigena* should nevertheless be taken into account. Discussions concerning further increase of nitrogen removal in sewage treatment, should take into account that this may lead to increased toxicity during the *N. spumigena* blooms. Our results suggest that the highest nodularin concentrations during a *N. spumigena* bloom may occur during the initiation of the bloom when phosphorus is still present in excess while nitrogen is limited and when filaments are found deeper in the water column not exposed to UVR.

For the future

From our study (Paper I) it is obvious that culture experiments that aim at testing the response of a phytoplankton species to external stressors should be implemented using several strains.

Nodularin production and release from *N. spumigena* is not yet clearly understood. Interactive effects of several different environmental factors on more strains should be included in future outdoor studies to answer these questions. In situ monitoring of intra- and extracellular nodularin during *N. spumigena* bloom relative to ambient nutrient concentration and other environmental factors is needed to support the result of our study.

Furthermore, future studies should include the effect of increased CO₂ and the predicted acidification on the performance and nodularin production of *N. spumigena*. The response of increased CO₂ on the phytoplankton composition in the Baltic Sea should also be emphasized in future studies. The overriding impression of cyanobacteria is their diverse ways of adapting to a wide spectrum of environmental variability (Reynolds, 1987), but will their reputation as a prominent and successful group of organisms remain even in an acidified Baltic Sea?

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