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Miina Karjalainen

Fate and effects of *Nodularia spumigena* and its toxin,
nodularin, in Baltic Sea planktonic food webs

Om giftet dödar havet, vad blir havet av?
Det finns inom oss om det så försvinner
långt bortom synranden där månen brinner
och sträcker, som en blind, sin vita stav

ut över vågorna som aldrig hinner
till havsstranden inom oss. Vi är hav.
Ur havet steg vi och mot havet rinner
vårt liv, vår kärleks mönja, dödens krav.

För vi var hav med tusen måsar över,
ett hav vid hav som ingen stiltje söver
där vi skall tömmas, lämningar och drav.

I havet sjunker stunden vi har varit.
Bråddjupet famnar oss. Vi har befarit
ett hav som återtagit oss. Vi är ett hav.

Lars Forssell

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Fate and effects of *Nodularia spumigena* and its toxin, nodularin,
in Baltic Sea planktonic food webs

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LIST OF ORIGINAL ARTICLES

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:

- I** Karjalainen, M., Reinikainen, M., Lindvall, F., Spoof, L. & Meriluoto, J.A.O. 2003: Uptake and accumulation of radiolabeled nodularin in Baltic Sea zooplankton. *Environmental Toxicology* 18: 52-60.
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- III** Kozlowsky-Suzuki, B., Karjalainen, M., Lehtiniemi, M., Engström-Öst, J., Koski, M. & Carlsson, P. 2003: Feeding, reproduction and toxin accumulation by the copepods *Acartia bifilosa* and *Eurytemora affinis* in the presence of toxic *Nodularia spumigena*. *Marine Ecology Progress Series* 249: 237-249.
- IV** Karjalainen, M., Kozlowsky-Suzuki, B., Lehtiniemi, M., Engström-Öst, J., Kankaanpää, H. & Viitasalo, M.: Nodularin accumulation during cyanobacterial blooms and experimental depuration in zooplankton. *Marine Biology* (in press).
- V** Karjalainen, M., Reinikainen, M., Spoof, L., Meriluoto, J.A.O., Sivonen, K. & Viitasalo, M. 2005: Trophic transfer of cyanobacterial toxins from zooplankton to planktivores: consequences to pike larvae and mysid shrimps. *Environmental Toxicology* 20: 354-362.

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Fate and effects of *Nodularia spumigena* and its toxin, nodularin, in Baltic Sea planktonic food webs

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ABSTRACT

Cyanobacterial blooms occur every year in the Baltic Sea, and their frequency and intensity have increased during recent decades. Since one of the dominant species in these blooms, *Nodularia spumigena*, is toxic, the effect of these blooms in food webs was investigated. The aim of these studies was to increase the level of understanding of the effects of *N. spumigena* on zooplankton and planktivores feeding on zooplankton. In addition, the fate of nodularin in planktonic food webs was studied both experimentally and by collecting samples from the field.

In nodularin uptake experiments it was observed that zooplankton can take up dissolved nodularin directly from the water. In both ciliates and copepods the concentrations of nodularin attained higher values than in the surrounding water, indicating that bioconcentration of nodularin occurred in the zooplankton. Therefore, no direct contact with cyanobacterial filaments and zooplankton was needed for dissolved nodularin to accumulate in zooplankton. The nodularin accumulation was about 20 times larger when obtained via grazing, compared with its uptake directly from the water. There was a positive relationship between the ingestion rates for *N. spumigena* and the nodularin level found in the copepods.

Only a minor fraction of nodularin was detected in copepods, compared with the levels found in ingested cyanobacteria. This indicates that copepods can effectively metabolize or detoxify nodularin after ingestion or that filamentous cyanobacteria are broken down before ingestion and some of the toxin is released to the surrounding water. The concentrations of nodularin were 6-12 times higher in copepods than in their faecal pellets, suggesting that faecal pellets are unlikely to act as vectors for nodularin recycling in the pelagic zone.

The degradation of nodularin occurred rapidly in copepods. In depuration experiments the degradation rate, i.e. the half-life, of nodularin varied from 7.4 to 13.9 h, depending on the method used in the toxin analysis. Rapid decrease in the toxin concentrations indicates effective detoxification capacity in copepods. However, after 24 h the exposed copepods still contained 51-58% of the initial concentrations of nodularin.

Nodularin was detected in the field-collected zooplankton samples. Herbivorous species as well as species with a limited capacity to vertically migrate from the dense surface accumulations of toxic cyanobacteria were more vulnerable to nodularin uptake, whereas species capable of selective feeding had lower concentrations in their tissues. Larger, mainly carnivorous zooplankton species contained nodularin as well, suggesting that nodularin may be transported between trophic levels in pelagic food webs.

During the feeding experiments, copepods fed actively on toxic *N. spumigena*, as well as on the associated ciliate community. The egg production rates decreased with increasing *N. spumigena* concentrations in the food solution. These findings further clarify the view that copepods can utilize cyanobacteria as a food source, but the poor nutritional quality of cyanobacteria as well as their toxins can negatively affect their reproduction.

In artificial food chain experiments, trace amounts of nodularin were transferred to planktivorous fish and mysid shrimps via zooplankton. These trace amounts of nodularin did not affect the growth of mysid shrimps, but decreased the ingestion rates of pike larvae. Since detoxication is an energy-demanding process, it may affect the reproduction and gross growth efficiency of zooplankton and, along the food chain, also the condition of zooplanktivorous fish.

Even though the vectorial transfer of nodularin does not appear to be very effective and only relatively small concentrations of nodularin are transported to higher trophic levels, due to its toxicity, carcinogenicity and potential neurotoxicity, the effects of chronic exposure to organisms of the Baltic Sea food webs call for further investigation.

Key words: *Nodularia spumigena*, nodularin, copepods, mysid shrimps, fish larvae, accumulation, trophic transfer, depuration, Baltic Sea

1. INTRODUCTION

Ecotoxicology is defined as the science of the ecological effects of pollutants on all living organisms, especially on populations and communities within defined ecosystems (Duffus 1993). Pollutants are substances that “occur in the environment at least in part as a result of man’s activities, and which have a deleterious effect on living organisms” (Moriarty 1983). The effect of pollutants is related to the exposure and dose of these substances and, in some cases, also to their chemical form. For example, the chemical form of a metal such as copper will alter the severity with which organisms are affected (Moriarty 1983). Some of the toxic substances are artificial, the so-called xenobiotica, i.e. not produced in nature (Butler 1978). There are, however, some toxins that are of natural origin, such as those produced by phytoplankton (reviewed by Landsberg 2002).

Cyanotoxins produced by cyanobacteria are one group of harmful substances and are of special interest with respect to human-induced eutrophication, especially since the abundance of cyanobacteria in the water has increased due to cultural eutrophication (Mur & al. 1999). Recorded occurrences of cyanobacterial blooms and their deleterious effects date back for over 100 years (Francis 1878, cited by Sivonen & Jones 1999), and there is evidence that during the Pleistocene large mammals were killed by toxic cyanobacteria (Braun & Pfeiffer 2002). Currently, increasingly severe cyanobacterial blooms affect several aspects of human life, in particular the quality of drinking water for humans and livestock (Falconer 1993), but also the recreational use of water systems (Kuiper-Goodman & al. 1999). In arid regions where there may be a shortage of good-quality drinking water, the occurrence of cyanobacterial blooms in drinking water reservoirs may cause especially severe problems if no alternative water supply is available (Falconer 1999).

Cyanobacteria are one of the oldest groups of organisms on the earth, and considerable diversity as early as 3465 million years ago can be seen in the microfossil records of Archaean cyanobacteria (Schopf 1993). Hepatotoxin production can be found not only in several genera of aquatic cyanobacteria (Sivonen & Jones 1999), but also in cyanobacteria from soils as well as those associated with terrestrial lichens (Prinsep & al. 1992, Oksanen & al. 2004). Phylogenetic analyses suggest that microcystin synthetase genes were originally present in the last common ancestor of a large group of cyanobacteria (Rantala & al. 2004) and that the divergence between the three domains (Archaea, Bacteria and Eukaryota) occurred more than 1200 million years ago (Heckman & al. 2001).

Resistance against grazers has been suggested as the ultimate reason for toxin existence in cyanobac-

teria (Lampert 1981). However, since the ability to produce hepatotoxins is probably an older trait than would be expected if they were targeted towards grazers, other explanations have been evoked as well. These include allelopathic activity (Keating 1977), provision of essential secondary metabolites for cyanobacteria (Turner & Tester 1997), intracellular signal and/or regulatory compounds (Dittmann & al. 2001) or quorum-sensing compounds (Swift & al. 1994).

Cyanotoxins, like other toxic substances, affect populations via effects at the individual level, directly or indirectly, but the effects may still not be ecologically significant at the ecosystem level. In contrast, individuals may not be severely affected by toxic substances, while toxicants can nevertheless have considerable consequences on population and ecosystem scales (Moriarty 1983). Therefore, conclusions of the effects of a single toxicant on whole communities cannot be drawn by assessing their effect on individuals alone. The general aim of this thesis is to study the effects of the toxic cyanobacterium *Nodularia spumigena* Mertens, and the fate of its toxin, nodularin, in Baltic Sea planktonic food webs.

1.1 Cyanobacteria in the Baltic Sea – an ancient phenomenon

The Baltic Sea is a relatively young brackishwater basin that has undergone several marine and lacustrine phases since the latest glaciation (Winterhalter & al. 1981). Sediment analyses showed that nitrogen-fixing cyanobacteria have been present in the Baltic Sea for the last 7000 years (Bianchi & al. 2000), corresponding approximately to the latest brackishwater phase of the Baltic Sea (Winterhalter & al. 1981). However, it was postulated that cyanobacterial bloom intensity as well as frequency have increased during recent decades (Kahru & al. 1994, Finni & al. 2001, Poutanen & Nikkilä 2001), possibly due to human-induced eutrophication. There is also preliminary evidence for the increasing ratio of toxic/non-toxic cyanobacterial species in several Baltic Sea subregions (HELCOM 2003).

1.2 Why do cyanobacteria form blooms in the Baltic Sea?

Cyanobacterial dominance during late summer is a recurrent phenomenon in the Baltic Sea (Sivonen & al. 1989, Kononen & al. 1996). These blooms are dominated by *N. spumigena*, *Aphanizomenon flos-aquae* and *Anabaena* spp., all capable of nitrogen fixation. Even though these species possess different requirements regarding nutrients, salinity, temperature and light regime, they all share some common

features for cyanobacteria that enable them to dominate the pelagic communities during late summer, i.e. 1) the capability to fix molecular nitrogen, 2) good capacity for taking up and storing phosphorus and 3) ability to adjust their position in the stratified water column with gas vesicles (reviewed by Mur & al. 1999). They can also grow over a wide range of temperatures, further enhancing their occurrence during late summer (Robarts & Zohary 1987).

Diazotrophic cyanobacteria, i.e. cyanobacteria that fix gaseous nitrogen, usually have specialized cells called heterocytes (Stewart 1973), but there are genera capable of nitrogen fixation without heterocyte formation (Carpenter & al. 1992). High salinity can restrict nitrogen fixation (Fernandes & al. 1993), since iron (Michaels & al. 1996) and sulphate (Stal & al. 1999) appear to limit the activity of nitrogenase, the enzyme responsible for nitrogen fixation. The physiological salinity tolerance of cyanobacteria does not restrict their distribution in marine environments, but the inhibition of nitrogenase affects their ability to compete with other phytoplankton taxa in the oceans compared with estuaries and lakes.

Cyanobacteria have high affinities and a good storage capacity for phosphorus (reviewed by Mur & al. 1999). Therefore they can retain high growth rates and outcompete other phytoplankton species during low nitrogen and phosphorus concentrations. Cyanobacteria reach their maximum growth rates at higher temperatures compared with, for example, green algae and diatoms (Mur & al. 1999), thus further explaining their occurrence during late summer, when the surface temperatures in the Baltic Sea are highest (Kullenberg 1981). The tendency of cyanobacteria to accumulate at the water surface and form large surface scums results from their ability to regulate buoyancy with gas vacuoles (Walsby & al.

1995). As a sinking device cyanobacteria use the carbohydrates they have photosynthesized in the euphotic zone (Mur & al. 1999).

Low nitrogen to phosphorus ratios favour the occurrence of cyanobacteria in the Baltic Sea, excluding the northernmost part, the Bothnian Bay (Niemi 1979), where N:P ratios are generally higher due to the efficient precipitation of phosphorus with iron, which is transported from the land with humic substances (Alasaarela 1979). Salinity, nutrient concentrations and hydrographic conditions in the Baltic Sea favour the occurrence of cyanobacterial blooms, especially in late summer. The Baltic Sea is one of the largest brackishwater basins in the world where cyanobacteria regularly occur and where cyanobacterial blooms can cover thousands of square kilometres (Kahru & al. 1994).

1.3 Harmfulness of *Nodularia spumigena*

The genus *Nodularia* occurs rarely in freshwater reservoirs, but does occur mainly in slightly saline or brackish waters and in saline and coastal lakes (Komárek & Hauer 2004). In addition to the Baltic Sea area, the distribution of *N. spumigena* covers several brackish as well as saline water environments in Australia (Baker & Humpage 1994), New Zealand (Carmichael & al. 1988, Rinehart & al. 1988), Canada (Hammer 1981), and USA (Galat & al. 1990). The toxicity of cyanobacteria in the Baltic Sea has become widely known to the public after it was observed that one of the dominant species produces hepatotoxic compounds, and after some intoxication incidents (Table 1). It was concluded that in the Baltic Sea the symptoms were primarily caused by the hepatotoxic nodularin produced by *N. spumigena*.

Table 1. Observed effects of *Nodularia spumigena* on Baltic Sea organisms.

Observation	Reference
<i>Intoxications</i>	
400 ducks died in Jasmunder Bodden, Germany, in 1963	Kalbe & Tiess 1964
30 dogs sickened and 20 died in Aarhus, Denmark, in 1975.	Lindström & al. (1976), cited by Nehring, (1993)
1 dog and 3 puppies died in Porvoo, Finland, in 1984.	Persson & al. (1984)
9 dogs died in Gotland, Sweden, in 1982.	Edler & al. (1985)
16 young cattle died in Stelasund, Germany, in 1983.	Gußmann & al. (1985)
2 dogs died in Banter See, Germany, in 1990.	Nehring (1993)
2 dogs died in Gulf of Finland in 1997.	ICES (1998)
<i>Phytoplankton</i>	
<i>N. spumigena</i> was able to inhibit the growth of other phytoplankton species, especially during the exponential growth phase.	Suikkanen & al. (2004)
<i>N. spumigena</i> could stimulate the growth of some phytoplankton groups, and negatively affect others.	Suikkanen & al. (2005)

Observation	Reference
<i>Zooplankton & invertebrates</i>	
Cyanobacterial filaments patches of high bacterial productivity and are colonized by a rich heterotrophic community.	Hoppe (1981)
Copepod grazing decreased with increasing concentrations of cyanobacteria. <i>Bosmina</i> fed regularly on cyanobacteria.	Sellner & al. (1994)
Grazing on cyanobacteria and production rates were low in cyanobacterial blooms	Sellner & al. (1996)
<i>N. spumigena</i> increased copepod mortality, decreased egg production and hatching success.	Koski & al. (1999)
Copepods ingested and assimilated cyanobacteria during mass occurrences.	Meyer-Harms & al. (1999)
Some copepod species were capable of selective feeding and could avoid feeding on toxic <i>N. spumigena</i> .	Engström & al. (2000)
Nitrogen-fixing cyanobacteria may be an important food source for all plankton size classes during summer.	Rolff (2000)
In the presence of <i>Nodularia</i> the feeding rate of mysid shrimps decreased, but no increase in mortality occurred during 7-week trials.	Engström & al. (2001)
Decaying cyanobacteria accompanied a rich community of heterotrophic organisms, i.e. bacteria, flagellates and ciliates.	Engström-Öst & al. (2002)
Copepods fed on decaying cyanobacteria filaments and the community associated with them.	Koski & al. (2002)
Toxic cyanobacteria were consumed by copepods if no other food was available. Most of the toxin was not egested in faecal pellets.	Paper II
Copepod survival was marginally affected by high concentrations of dissolved nodularin, but no effect on egg hatching success.	Reinikainen & al. (2002)
Copepods, cladocerans and rotifers were able to survive and reproduce within a decaying cyanobacterial bloom.	Schmidt & al. (2002)
Zooplankton could take up dissolved nodularin directly from water, without feeding on cyanobacteria.	Paper I
Copepods actively fed on <i>N. spumigena</i> , both alone and with other food items. High <i>N. spumigena</i> ingestion rates decreased the gross growth efficiency.	Paper III
<i>N. spumigena</i> affected the acetylcholinesterase activity of the clam <i>Macoma balthica</i>	Lehtonen & al. (2003)
Male copepods were more sensitive to <i>N. spumigena</i> exposure than females.	Ojaveer & al. (2003)
Concentrations of <i>N. spumigena</i> in the field were unrelated to abundances of copepods, cladocerans and rotifers.	Repka & al. (2004)
When fed with <i>N. spumigena</i> , mussels produced pseudofaeces, but not when they were grazing on faeces.	Svensen & al. (2005)
Toxic <i>N. spumigena</i> caused a slight decrease in egg production and increased the abortion rate of embryos from the brood pouch, but did not affect survival.	Korpinen & al., in press
<i>Vertebrates</i>	
Three-spined sticklebacks were found floating on the surface during a dense bloom of <i>N. spumigena</i> .	Kankaanpää & al. (2001a)
<i>N. spumigena</i> caused severe, but reversible liver damage in sea trout.	Kankaanpää & al. (2002)
The embryonic and larval development of herring was negatively affected by <i>N. spumigena</i> .	Ojaveer & al. (2003)
Pike larvae had a lower ingestion rate of zooplankton that had been pre-exposed to cyanobacteria, possibly affecting their growth.	Paper V
In <i>N. spumigena</i> blooming conditions decrease in growth rate of juvenile three-spined sticklebacks was observed due to reduced protein synthesis (protein/RNA, RNA/DNA).	Pääkkönen & al., submitted
The feeding rate of pike larvae on zooplankton decreased with the presence of non-toxic <i>Nodularia</i> .	Engström-Öst & al., submitted

Nodularia spumigena always produces nodularin in the Baltic Sea (Laamanen & al. 2001). Nodularin production has been studied widely in the laboratory, and several factors apparently affect toxin production in *N. spumigena* (Lehtimäki & al. 2000). In general, the nodularin concentration is higher within the cells when they are actively growing (Lehtimäki & al. 1994, 1997). Recently *N. spumigena* (and several other cyanobacterial strains) was observed to produce BMAA (β -N-methylamino-L-alanine), a neurotoxic amino acid (Cox & al. 2005). Possible linkage between neurodegenerative diseases and BMAA was suggested (Cox & al. 2003, Murch & al. 2004).

In addition to hepato- and neurotoxins, cyanobacteria produce a wide variety of bioactive compounds such as peptides, alkaloids and lipopolysaccharides (Sivonen & Jones 1999). These compounds alone or in combination with hepatotoxins can be harmful to other biota (Lindholm & al. 1992, Reinikainen & al. 2001, Best & al. 2002, Rohrlack & al. 2004). For example, lipopolysaccharides potentially reduce the activity of glutathione-S-transferases (Best & al. 2002), which have been identified as the first step in detoxication of several aquatic organisms (Pflugmacher & al. 1998).

In freshwater environments, mass occurrences of cyanobacteria have been observed to alter the chemical properties of the water, e.g. by consuming oxygen during bloom decay (Jewel & al. 2003) or by depleting the CO₂ and raising the pH of the water due to photosynthetic activity prior to bloom senescence (Shapiro 1990). These indirect effects, in turn, can be deleterious for fish, e.g. by causing oxygen deficiency and gill damage (Gaete & al. 1994, Bury & al. 1996). Cyanobacterial filaments in the water can also cause disturbance to other aquatic biota, especially larger organisms dependent on visual predation (Engström-Öst & al. submitted). Direct contact with cyanobacterial filaments can clog the feeding apparatus of mysid shrimps (Engström & al. 2001), or interfere with the grazing of littoral amphipods on filamentous macroalgae (Korpinen & al., in press). Since many aquatic organisms, such as fish, rely on chemosensory signals in finding food, mating partners or perceiving information on predators (Hara 1993), the dampening of these chemically mediated signals by chemical overload of toxins can affect aquatic biota and their behaviour (Baganz & al. 1998, 2004).

From the point of view of human populations, cyanobacteria not only pose a threat to human health, but also hamper the recreational use of water (reviewed by Kuiper-Goodman & al. 1999). Swimming in the water with a dense biomass of cyanobacteria can cause skin and eye irritation symptoms (Torokne & al. 2001), and cyanobacterial pigments and other compounds can cause allergic reactions (Cohen & Reif 1953). If accidentally ingested, in-

toxication symptoms e.g. vomiting, diarrhoea, central abdominal pain, blistering of the lips or sore throats can occur (Turner & al. 1990). There are no reported fatal poisonings of humans caused by *N. spumigena* in the Baltic Sea area, but livestock, dogs and ducks have been killed (Table 1).

1.4 Properties of nodularin

Nodularin is a hepatotoxin that closely resembles the microcystins, a well-studied group of cyanotoxins in freshwater ecosystems (e.g. Sivonen & Jones 1999, Meriluoto 2000). It is a cyclic pentapeptide and, like the microcystins, nonselectively inhibits the eukaryotic serine-threonine protein phosphatases PP1 and PP2A (Yoshizawa & al. 1990), but unlike microcystins does not covalently bind to these protein phosphatases (Annala & al. 1996). The protein phosphatases are a principal component in the cellular response to external stimuli that control the degree of phosphorylation of proteins, hence defining the activity of protein molecules. The protein phosphatases regulate a wide variety of metabolic processes within the cell, e.g. glycogen metabolism, muscle contraction, membrane transport and cell differentiation (reviewed by Mehrotra & al. 1997). Nodularin also exposes the affected cells to oxidative stress, which can be a mechanism underlying the observed hepatotoxic and carcinogenic effects (Bouaïcha & Maatouk 2004, Jos & al. 2005).

Microcystins and nodularin are unable to diffusively pass the membranes of cells (Eriksson & al. 1990); therefore uptake occurs via membrane transporters, such organic anion-transporting polypeptides (Fischer & al. 2005). Microcystins and nodularin are potent hepatotoxins, accumulating in the liver (Carmichael 1992). They are tumour promoters (Nishiwaki-Matsushima & al. 1992), and nodularin is also carcinogenic (Ohta & al. 1994). As a guideline, the World Health Organization (WHO) defined the tolerable daily intake (TDI) of microcystins in drinking water as 1.0 $\mu\text{g l}^{-1}$ or 0.04 $\mu\text{g kg}^{-1}$ body weight per day (WHO 1998). No guidelines have been set for nodularin, but since its toxicity resembles that of the microcystins, the same TDI values can be used for both of these toxins (Kuiper-Goodman & al. 1999).

Nodularin is a predominantly intracellular toxin, and only a minor fraction of it (usually less than 10–20% of the total toxin amount) is released to the surrounding water in laboratory cultures (Sivonen & Jones 1999). In the field, nodularin is released to the surrounding water after cell lysis (Kankaanpää & al. 2001a). In the northern Baltic Sea, the highest dry weight (DW) concentrations of this toxin observed in the cell-bound biomass from the open sea were 18.1 mg (g DW)⁻¹ (Kononen & al. 1993). In the Gulf of Gdansk, southern Baltic Sea, the highest concentrations of nodularin in dissolved form were ob-

served in dense surface accumulations of up to 18.135 mg l⁻¹ (Mazur & Plinski 2003), but usually lower concentrations are encountered in the field, since strong turbulence dilutes the dissolved toxin (Henriksen 2005). Recently trace amounts of microcystin-LR were observed in pelagic cyanobacterial blooms in the northern Baltic Sea (Karlsson & al. 2005).

Nodularin, like the microcystins, is a relatively stable compound that is not very easily degraded e.g. by light, temperature or microwaves (Twist & Codd 1997, Metcalf & Codd 2000, Mazur & Plinski 2001). They are degraded by specialized microbial communities that thrive especially in bodies of water with earlier histories of cyanobacterial blooms (Mazur & Plinski 2001, Christoffersen & al. 2002, Hyenstrand & al. 2003). Microcystins can persist in lake water for several weeks after a bloom (Lahti & al. 1997), and relatively high concentrations of nodularin (14 µg l⁻¹) were detected in the water after a mass occurrence of cyanobacteria (Vuori & al. 2001). In experimental studies the degradation rate (i.e. the degradation half-life or the time required for 50 % of the substance to decompose) of dissolved microcystins was 0.5-1 d⁻¹ (Christoffersen & al. 2002). The degradation of dissolved organic carbon correlates with the degradation of microcystins (Christoffersen & al. 2002) and N₂O production in denitrifying sediments (Holst & al. 2003), suggesting that these processes are coupled.

1.5 Interactions of zooplankton and *N. spumigena* in the Baltic Sea – what is known so far?

In the Baltic Sea, zooplankton apparently do not avoid areas or water layers where cyanobacteria are abundant, since in a field survey conducted during the cyanobacterial blooming season rotifers, copepods and cladocerans were positively correlated with the abundance of *A. flos-aquae*, whereas no association was found between zooplankton abundance and nodularin concentration, or *N. spumigena* abundance (Repka & al. 2004). Since the seasonal maximum of zooplankton (Viitasalo 1992) often coincides with maximum cyanobacterial abundance (Kononen & al. 1993) and since cyanobacteria are being consumed by zooplankton in the field (Sellner & al. 1994, Meyer-Harms & al. 1999, Rolff 2000), zooplankton must cope with cyanobacteria in their diets, as well as with toxins in the surrounding water.

There are numerous published results on the effects of hepatotoxin-producing (i.e. producing microcystins and nodularin) cyanobacteria on zooplankton (reviewed by Landsberg 2002). In general, cyanobacteria are considered to be harmful for zooplankton (Lampert 1981), for several reasons. In addition to toxicity (DeMott & al. 1991, Reinikainen & al.

1994), difficult mechanical manageability (Infante & Abella 1985) can negatively affect zooplankton grazing on cyanobacteria. Furthermore, cyanobacteria are of low nutritional value to zooplankton, e.g. due to lack of essential fatty acids (Ahlgren & al. 1992).

Some of the results are controversial, however. For example, high uptake rates of cyanobacteria were observed, based on pigment analyses from copepods collected in the central Baltic Sea during cyanobacterial blooms (Meyer-Harms & al. 1999). Stable isotopes originating from nitrogen fixation can be found in all plankton size classes, indicating that cyanobacterial blooms may serve as a food source for the planktonic community during nutrient-poor summer conditions (Rolff 2000). Low feeding rates on cyanobacteria in the laboratory and high uptake rates in the field may derive from the fact that degradation and ageing of cyanobacterial filaments can enhance their usability by zooplankton (Repka & al. 1998, Meyer-Harms & al. 1999). Furthermore, cyanobacteria can be utilized by zooplankton when available in small quantities (Schmidt & Jónasdóttir 1997) or when offered in mixtures with good food algae (Koski & al. 1999). The responses of zooplankton to cyanobacteria are complex, since their preferences are affected by the ratio of food items, their starvation level and toxicity of the cyanobacteria in question (DeMott & Moxter 1991). Cyanobacterial blooms colonized by other organisms, such as heterotrophic bacteria, flagellates and ciliates, are more attractive food sources for copepods (Koski & al. 2002, Engström-Öst & al. 2002, Schmidt & al. 2002) than monocultures of cyanobacteria (Sellner & al. 1994, 1996, Koski & al. 1999, Engström & al. 2000). Despite the close coupling of zooplankton and cyanobacteria in the Baltic Sea, little is known about the quantity of toxin accumulation in zooplankton, the efficiency of different exposure routes or the time in which the toxin is retained within the individuals.

1.6 Transfer of nodularin and effects on zooplanktivores

The prerequisites for toxin accumulation in aquatic organisms include grazing on cyanobacteria, uptake via water or feeding on other organisms or particles containing toxins. The implication of toxin bioaccumulation in zooplankton is that zooplankton can act as packages of toxin to planktivorous organisms, such as fish larvae, mysid shrimps and carnivorous zooplankton, which otherwise would not be exposed to nodularin via their diet.

In artificial food-chain experiments it was demonstrated that Baltic Sea zooplankton can act as vectors for nodularin transfer from cyanobacteria to planktivorous mysid shrimps and juvenile fish (Eng-

ström-Öst & al. 2002). With microcystins, similar food-chain experiments proved to be lethal to zooplanktivores, since the mortality of *Chaoborus* larvae increased, even though no traces of microcystins could be observed in their tissues after consuming *Daphnia pulex* fed with *Microcystis aeruginosa* (Laurén-Määttä & al. 1995). These results indicated that zooplankton could mediate the negative effects of cyanobacteria along the food chain to zooplanktivores, even if the toxin concentrations in their tissues were below detection limits.

However, no information is available on the possible sublethal effects of such chronic exposure to zooplanktivores in the Baltic Sea or on about the magnitude of transfer of dissolved nodularin via zooplankton to planktivores. Especially sensitive life stages, such as larval or juvenile fish feeding on toxin-containing zooplankton, can be more sensitive to toxins and also less capable of escaping from areas with heavy cyanobacterial accumulations than adult fish. There appears to be very little transfer of dissolved cyanobacterial toxins to fish eggs before they hatch, and therefore the effect of toxins on egg hatching success or the development of the embryo at natural toxin concentrations is minimal (Fischer & Dietrich 2000, Ojaveer & al. 2003, Jacquet & al. 2004). However, the uptake of dissolved toxins increases when the fish larvae begin to feed (Fischer & Dietrich 2000). Estimates for the transfer of nodularin and its effect on higher trophic levels are important, since cyanobacterial toxins appear to affect vertebrates more strongly than invertebrates (Kiviranta & al. 1991).

2. OBJECTIVES OF THE STUDY

The present study aims at enhancing the present level of understanding of the fate and effects of *N. spumigena* and nodularin in the planktonic food webs of the Baltic Sea. Therefore, the following main objectives were set: a) to study the pathways taken and efficiencies in the transfer of nodularin in planktonic food webs, b) to measure toxin concentrations in the field from different zooplankton species and c) to investigate the effects of nodularin and *N. spumigena* on zooplankton and zooplanktivores.

These objectives were approached by both field studies and experimental work. The studies focused on the following topics:

1. Accumulation of dissolved nodularin directly from the water in zooplankton (Paper I)
2. Toxin accumulation, via grazing on toxic *N. spumigena*, in zooplankton and their faecal pellets (Paper II)
3. Grazing and reproduction of the two dominant copepod species in natural cyanobacterial

bloom (containing *N. spumigena*) and cultured *N. spumigena*, and comparing the effects with the tissue toxin concentration (Paper III)

4. Susceptibility of different zooplankton species to toxin accumulation in the field (Paper IV)
5. Toxin depuration from zooplankton, i.e. the disappearance from their tissues after cyanobacterial exposure (Paper IV)
6. The effects of cyanobacteria-exposed zooplankton on the growth of planktivores (Paper V).

3. METHODS

3.1 Dissolved toxin uptake experiments

Toxin uptake experiments (I) were done at Umeå Marine Sciences Centre, in which the accumulation of dissolved nodularin from water was studied with two calanoid copepods, *Eurytemora affinis* and *Acartia tonsa*, and an oligotrich ciliate, *Strombidium sulcatum*. The cultured copepods and ciliates were exposed to radiolabelled ³H-dihydronodularin at natural concentration (5 µg l⁻¹), and after certain time intervals (with copepods 1, 2, 4 and 6 d and with ciliates 0.25, 1, 4, 8 and 24 h) the amounts of radiolabel in the tissues of the zooplankton were measured with a scintillation counting technique (see Chapter 3.7). Based on their biovolume and the observed toxin concentrations, the bioconcentration factors of nodularin for these three zooplankton species were calculated.

3.2 Toxin fate experiments

To study the fate of nodularin (II) in the copepod *E. affinis*, experiments were performed aboard the R/V Aranda (Finnish Institute of Marine Research) in August 2000. The food suspensions during the experiments consisted of toxic *N. spumigena* (strain AV1, 876.5 µg C l⁻¹), a non-toxic flagellate *Brachionas submarina* (strain TV15, 1012.6 µg C l⁻¹) or a natural phytoplankton assemblage prefiltered through a 100 µm plankton net (predominantly *Aphanizomenon flos-aquae*, but also containing *N. spumigena*, 587.1 µg C l⁻¹). *Eurytemora affinis* females starved for 24 h were fed with the experimental food suspensions for 24 h at ambient temperature. After the incubations the females were collected for toxin analyses performed with the enzyme-linked immunosorbent assay (ELISA) and protein phosphatase inhibition assay PPI (see below). A total of 98 copepods from the *N. spumigena* treatment (divided into 5 samples), 104 copepods from the natural community treatment (divided into 5 samples) and 23 from the *B. submarina* treatment

(1 pooled sample) were analysed. The number and sizes of the faecal pellets produced with each diet were noted, and 200 pellets from each treatment were collected for the toxin analyses.

3.3 Feeding and reproduction experiments

To investigate the feeding, reproduction, survival and toxin accumulation in copepods of toxic *N. spumigena* alone and in mixtures with other food items (III) experiments were conducted aboard the R/V Aranda in August 2000. *Eurytemora affinis* and *Acartia* sp. were sampled from one station in the Gulf of Finland, and *E. affinis* from another in the Bothnian Bay. The copepods were starved overnight in filtered seawater and then fed with the experimental food suspensions for 72 h at ambient temperature. The food suspensions in the Gulf of Finland consisted of a natural phytoplankton community, prefiltered through a 100 µm plankton net (440 µg C l⁻¹), toxic *N. spumigena* culture (strain AV1, 1281 µg C l⁻¹), a combination of *N. spumigena* and non-toxic flagellate *B. submarina* (app. 1:1 carbon, 1330 µg C l⁻¹), and filtered seawater. In the Bothnian Bay the food suspensions consisted of a natural phytoplankton community pre-screened through a 100 µm net (363 µg C l⁻¹), a toxic *N. spumigena* culture (907 µg C l⁻¹), a combination of *N. spumigena* and the natural community (731 µg C l⁻¹) and filtered seawater. During the first 24 h the feeding was estimated with the method described by Frost (1972) and during the consecutive 48 h the egg production and hatching rates were measured. After the experiments the females were collected for toxin analyses with the ELISA and protein phosphatase inhibition assay (PP1), as described below.

3.4 Toxin disappearance experiments

The toxin depuration experiments (IV) were performed aboard the R/V Aranda in August 2000. The aim of the experiments was to determine how long the toxin accumulated in copepods would remain in their tissues and gut after the animals had been removed from the toxic food suspension. *Eurytemora affinis* females were starved overnight, fed with toxic *N. spumigena* food suspension (strain AV1, app. 500 µg C l⁻¹) for 24 h and transferred back to filtered seawater. The aim was to determine how long the toxin accumulated in copepods would remain in their tissues after removing the animals from the toxic food suspension. After 0, 0.5, 3, 6, 10 and 24 h, three replicate samples of the animals were

collected for toxin analyses with ELISA and PP1 (see below).

3.5 Zooplankton sampling in the field for toxin analyses – field measurements

Samples for toxin analyses (IV) were collected during the cyanobacterial bloom season from the Baltic Proper, Gulf of Finland and southern Bothnian Sea aboard the R/V Aranda in August 2001, July 2002 and August 2002. Zooplankton was sampled with a 200 µm WP-2 plankton net. Immediately after sampling about 30 adult female copepods and 50 cladocerans from the most numerous species were collected for toxin analyses with ELISA (see below). The animals were individually rinsed three times in GF/F filtered seawater to dispose of all the cyanobacterial filaments in the sample, and picked with forceps into glass vials. To calibrate the analysis method for zooplankton samples, a series of samples with cultured *Artemia salina* brine shrimp and known amounts of purified nodularin were prepared and measured, using methods similar to those used with the field-collected samples.

3.6 Trophic transfer experiments on planktivores

In the trophic transfer experiments (V) two planktivores, mysid shrimps (*Neomysis integer*) and pike larvae (*Esox lucius*), were fed with zooplankton that had been exposed either to purified nodularin (20 µg l⁻¹) or crude extract of toxic *N. spumigena* (nodularin concentration 18.5 µg l⁻¹). The growth of planktivores with these diets was monitored for two weeks. For mysid shrimps the length of the moulting cycle was measured, whereas the pike larvae were weighed three times during the experiments. Faecal pellet production and, after the experiments, the carbon:nitrogen (C:N) ratios were measured. To resolve whether pre-exposure with dissolved cyanotoxins affects the feeding rates of pike larvae, ingestion experiments were conducted.

In addition, the transfer of dissolved, radio-labelled toxin was measured using zooplankton as vectors. In these experiments a natural zooplankton community (dominated by *Acartia* sp. and *Synchaeta cf. baltica*) was exposed to dissolved, radio-labelled nodularin (100 µg l⁻¹) for 12 h and then fed to the fish and mysid shrimps. After feeding on radiolabelled zooplankton for 12 and 48 h, the fish larvae and mysid shrimps were collected for scintillation counting analyses (see below).

3.7 Toxin analyses

All zooplankton and faecal pellet samples for toxin analyses (II-V) were rinsed three times with filtered seawater and frozen until analysis. They were freeze-dried for 3 days, extracted in 100% methanol and sonicated until the tissues and pellets were broken down. The samples were filtered or centrifuged in order to dispose of the remnants of the tissues, whereafter the samples were dried with a nitrogen flow. The samples were then redissolved in 50% methanol and diluted with Milli-Q water to a final methanol concentration of 6% (IV, V) or 10% (II, III).

Enzyme-linked immunosorbent assay (ELISA) plate kit (EnviroGard; Strategic Diagnostics, Newark, DE, USA) assay was used for the zooplankton and pellet samples (II-V), using calibration standards provided by the manufacturer (0.1, 0.4 and 1.6 µg microcystin-LR l⁻¹); thus all the results are presented as microcystin-LR equivalents. Absorption was measured using an optical plate reader. Colorimetric (Ward & al. 1997, with modifications) and fluorimetric protein phosphatase (PP1) inhibition assays (Fontal & al. 1999) were used as second methods for toxin detection from zooplankton and pellets in II and III, respectively. In addition, high-performance liquid chromatography (HPLC; Lawton & al. 1994, with modifications) was used (II and III), to quantify the toxin content of the food suspensions.

³H-dihydronodularin method was utilized (I and V) when the quantitative toxin accumulation and transfer studies were performed. Tritium-labelled nodularin was prepared according to Spooft & al. (2003). The measurements were performed using a scintillation counting technique (reviewed by Sorokin 1999).

4. RESULTS AND DISCUSSION

4.1 Toxin uptake in zooplankton

Bioaccumulation is defined as “progressive increase in the amount of a substance in an organism or part of an organism which occurs because the rate of intake exceeds the organisms ability to remove the substance from the body” (Duffus 1993). Uptake of chemicals by aquatic organisms can occur from the abiotic environment (e.g. via gills, skin etc.), or via food uptake (Rand & al. 1995). Bioconcentration is the process leading to higher concentration of a substance in an organism than is found in the surrounding water (Duffus 1993). The term biomagnification is used when the concentration of a substance

is higher in the consumer than in its food, resulting in higher concentrations in the organisms at the higher trophic levels (Duffus 1993).

Direct uptake of nodularin from the water

After the exposure to dissolved radiolabelled nodularin, it could be detected in zooplankton, ciliates (*Strombidium sulcatum*) and copepods (*Acartia tonsa* and *Eurytemora affinis*) (I). Bioconcentration factors (i.e. the ratio of chemical concentration in the organism to that in the surrounding water, Rand & al. 1995) were determined for these 3 species, being 12 for *A. tonsa*, 18 for *E. affinis* and 22 for *S. sulcatum*. Bioconcentration factors larger than 1 indicate that there are larger concentrations of substances within the animal than in the surrounding medium. Ciliates took up nodularin from the water very rapidly (within 15 min) reaching steady-state in nodularin accumulation, while copepods (*Acartia tonsa* and *Eurytemora affinis*) progressively accumulated toxin within several days. Therefore, in addition to the dissolved nodularin bioconcentration in zooplankton, it also bioaccumulated in them as a function of time. No direct contact with cyanobacterial filaments containing nodularin and zooplankton was needed for nodularin bioconcentration, or bioaccumulation into zooplankton.

Nodularin was taken up by ciliates very rapidly; e.g. the equilibrium between intake and regulation of nodularin was already attained after 15 min exposure (I). Part of this nodularin transfer to ciliates may have occurred via feeding, since *S. sulcatum* fed on bacteria that could not be removed from the experimental units. Therefore, the bioconcentration factor of 22 noted for *S. sulcatum* may be an overestimation, since some of the nodularin within their tissues may have been transferred with bacteria. The feeding mode used by ciliates (phagocytosis) also enables some water to enter the food vacuoles (Fenchel 1987) and hence allows nodularin to be taken up more efficiently. The maximum ingestion volume of ciliates per hour is, however, no larger than the volume of the cell (Fenchel 1987); therefore direct uptake (active or passive) through the outer membrane must have occurred in order to reach such concentrations.

In copepods, one possible route for the uptake of dissolved nodularin may have been via the dermal glands, since copepods are capable of taking up dissolved organic material from the surrounding water (Chapman 1981). Therefore, earlier findings of the low mortality of calanoid copepods exposed to dissolved nodularin (Reinikainen & al. 2002) are probably not due to the lack of direct uptake via water. In fact, our results support the view presented by Gray (2000) that uptake from the surrounding water seems to be a common way for accumulation of organic compounds in aquatic organisms.

Uptake via feeding

Uptake of nodularin via grazing of cyanobacterial filaments was about 20 times more effective than the bioconcentration from the surrounding water. Copepods took up toxins after grazing on cyanobacteria under different experimental conditions, both when cultured toxic *N. spumigena* was offered alone and with other food algae, including natural communities with cyanobacteria (**II**, **III**, Table 2). After feeding on *N. spumigena* monoculture, the copepod *E. affinis* collected from the Åland Sea contained 0.031 ± 0.007 ng nodularin ind⁻¹ (mean \pm SD), corresponding to concentrations of $5.6 \mu\text{g g}^{-1}$ DW (**II**). This is comparable to exposures in which *E. affinis* from two separate locations, the Gulf of Finland and Bothnian Bay, were fed with *N. spumigena* monoculture, i.e. 0.011 and 0.006 ng ind⁻¹ (2.0

and $1.1 \mu\text{g g}^{-1}$ DW, respectively) (**III**). In similar experiments *Acartia bifilosa* in the Gulf of Finland contained 0.011 ng ind⁻¹ (i.e. $1.8 \mu\text{g g}^{-1}$ DW). No previous results are available on the concentrations of nodularin in Baltic Sea zooplankton, but compared with the maximum concentrations of microcystins in zooplankton collected during experimental studies (Thostrup & Christoffersen 1999, $24.5 \mu\text{g g}^{-1}$ DW in *Daphnia*) or from lakes with *Microcystis* sp. blooms (e.g. Watanabe & al. 1992, $1387 \mu\text{g g}^{-1}$ DW, Kotak & al. 1996, $67 \mu\text{g g}^{-1}$ DW, Ferrão-Filho & al. 2002, $16.4 \mu\text{g g}^{-1}$ DW), the concentrations in our experiments were distinctly lower. On the other hand, the nodularin concentrations in zooplankton were similar to those found in Baltic Sea blue mussels and mysid shrimps and clearly higher than in fish samples from the Baltic Sea (Table 2).

Table 2. Observed concentrations of nodularin in Baltic Sea biota, from field-collected samples and after experimental exposures in the laboratory.

Source		Nodularin concentration	Unit	Sampling time	Reference
Water	Field	<0.5 – 2.6	$\mu\text{g l}^{-1}$	Aug 1999	Kankaanpää & al. 2001a
Water	Field	14	$\mu\text{g l}^{-1}$	Aug 1999	Vuori & al. 2001
Water	Field	0.02 – 0.05	$\mu\text{g l}^{-1}$	Jun – Jul 1998, 1999	Repka & al. 2004
Water	Field	90 – 18135	$\mu\text{g l}^{-1}$	Jun – Sep 2001	Mazur & Plinski 2003
Phytoplankton	Field	100 – 2400	$\mu\text{g g}^{-1}$ DW	Aug 1986	Sivonen & al. 1989
Phytoplankton	Field	0 – 18100	$\mu\text{g g}^{-1}$ DW	Jul – Aug 1990	Kononen & al. 1993
Phytoplankton	Field	500 – 2300	$\mu\text{g g}^{-1}$ DW	Aug 1999	Kankaanpää & al. 2001a
Phytoplankton	Field	200 – 6000	$\mu\text{g g}^{-1}$ DW	Aug 2000	Laamanen & al. 2001
Phytoplankton	Field	3000 – 3520	$\mu\text{g g}^{-1}$ DW	Jun – Sep 2001	Mazur & Plinski 2003
Phytoplankton	Field	0.004 – 565	$\mu\text{g l}^{-1}$	Jun – Sep 2002	Henriksen 2005
Phytoplankton	Field	9800 ± 7000	$\mu\text{g g}^{-1}$ DW	Jun – Sep 2002	Henriksen 2005
Phytoplankton	Field	149 – 804	$\mu\text{g l}^{-1}$	Jul 2003	Luckas & al. 2005
Phytoplankton	Field	100 – 1000	$\mu\text{g g}^{-1}$ DW	Jul 2003	Karlsson & al. 2005
Zooplankton	Field	0 – 0.62	$\mu\text{g g}^{-1}$ DW	Aug 2001, 2002	Paper IV
Zooplankton	Exp.	0.8 – 1.3	$\mu\text{g g}^{-1}$ DW	exp., fed with natural phytoplankton community	Paper III
Zooplankton	Exp.	4.94	$\mu\text{g g}^{-1}$ DW	exp., fed with <i>Nodularia</i>	Paper II
Zooplankton	Exp.	1.3 – 4.5	$\mu\text{g g}^{-1}$ DW	exp., fed with <i>Nodularia</i>	Paper III
Zooplankton	Exp.	0.1 – 0.2	$\mu\text{g g}^{-1}$ WW	exp., via water	Paper I
Blue mussels	Field	2.15 ± 0.06	$\mu\text{g g}^{-1}$ DW	Jun – Sep 1999	Sipiä & al. 2001a
Blue mussels	Field	0.04 – 1.49	$\mu\text{g g}^{-1}$ DW	Aug 1999 – Sep 2000	Sipiä & al. 2002a
Blue mussels	Field	0.5 – 0.64	$\mu\text{g g}^{-1}$ DW	Jul – Aug 2001	Karlsson & al. 2003a
Blue mussels	Exp.	0.1	$\mu\text{g g}^{-1}$ DW	exp., seawater	Svensen & al. 2005
Blue mussels	Exp.	80.4	$\mu\text{g g}^{-1}$ DW	exp., fed with <i>Nodularia</i>	Svensen & al. 2005
Clams	Field	0.1 ± 0.13	$\mu\text{g g}^{-1}$ DW	Aug 2000	Sipiä & al. 2002a
Clams	Exp.	0.32	$\mu\text{g g}^{-1}$ DW	exp., via water	Kankaanpää & al. 2001b
Clams	Exp.	1.6 – 16.6	$\mu\text{g g}^{-1}$ DW	exp., fed with <i>Nodularia</i>	Lehtonen & al. 2003
Mysid shrimps	Field	0.21 – 0.56	$\mu\text{g g}^{-1}$ DW	Jul – Aug 2004	Karjalainen & al., unpubl.
Mysid shrimps	Exp.	0.7	$\mu\text{g g}^{-1}$ DW	exp., via zooplankton	Engström-Öst & al. 2002

Source		Nodularin concentration	Unit	Sampling time	Reference
Mysid shrimps	Exp.	0.59	$\mu\text{g g}^{-1}$ DW	exp., via zooplankton	Paper V
Herring muscle	Field	0.0025 – 0.0065	$\mu\text{g g}^{-1}$ DW	Aug 1997	Sipiä & al. 2002b
Pike larvae	Exp.	0.41	$\mu\text{g g}^{-1}$ DW	exp., via zooplankton	Paper V
Stickleback, viscera	Field	0.035 – 0.17	$\mu\text{g g}^{-1}$ DW	Aug 1999	Kankaanpää & al. 2001a
Stickleback	Exp.	0.14	$\mu\text{g g}^{-1}$ DW	exp., via zooplankton	Engström-Öst & al. 2002
Flounder liver	Field	0.082 – 0.637	$\mu\text{g g}^{-1}$ WW	Aug 1995	Karlsson & al. 2003b
Flounder liver	Field	0.137 – 0.399	$\mu\text{g g}^{-1}$ DW	Aug 1999	Sipiä & al. 2001b
Flounder liver	Field	max 0.41 \pm 0.012	$\mu\text{g g}^{-1}$ DW	Aug 1999 – Sep 2000	Sipiä & al. 2002a
Flounder liver	Field	0.24 – 0.35	$\mu\text{g g}^{-1}$ DW	Jul – Aug 2001	Karlsson & al. 2003a
Cod liver	Field	0.053 – 0.056	$\mu\text{g g}^{-1}$ DW	Aug 1998	Sipiä & al. 2001b
Salmon liver	Field	0.0049	$\mu\text{g g}^{-1}$ DW	Aug 1997	Sipiä & al. 2002b
Sea trout liver	Exp.	max 1.6	$\mu\text{g g}^{-1}$ DW	exp., oral exposure	Kankaanpää & al. 2002
Eider liver	Field	0.003 – 0.018	$\mu\text{g g}^{-1}$ DW	Aug – Sep 2002	Sipiä & al. 2003

There was a significant positive relationship between the ingestion rates for *N. spumigena* and nodularin found in the copepods (III), suggesting a dose-dependent uptake of nodularin to zooplankton. Since copepods ingested more cyanobacteria in monocultures, a larger toxin uptake was observed in the tissues in treatments with monocultures, compared with treatments in which natural communities containing cyanobacteria were used alone or in combination with *N. spumigena* culture. In addition, the toxin concentrations in zooplankton followed those observed in the food solution.

In *Daphnia galeata* the dose of microcystin needed for killing daphnids within 2 days varied from 10.2 to 18.3 $\mu\text{g g}^{-1}$ wet weight (WW) (Rohr-lack & al. 2005). After our grazing experiments, the highest concentrations of nodularin in the copepods after grazing solely on toxic *N. spumigena* were 0.4 $\mu\text{g g}^{-1}$ WW, a value more than one order of magnitude smaller. This concentration did not affect the survival of *E. affinis* females within 72 h, but did negatively affect their egg production (III). Therefore, exposure to *N. spumigena* may have affected copepod production negatively, even though the mortality rates would not have increased during the exposure.

Detoxication and depuration

Only a minor fraction (less than 0.1 %) of the calculated nodularin amount in ingested *N. spumigena* cells was detected in zooplankton (III). This may be explained by detoxication or metabolization of the toxin by zooplankton (Beattie & al. 2003), sloppy feeding (i.e. breaking the cells before ingestion causing leakage of organic material including the intracellular toxins; Roy & al. 1989), bacterial

degradation in the copepod guts (Hansen & Bech 1996) or faecal pellets (Thor & al. 2003) or low toxin recovery by the toxin analyses (Sipiä & al. 2001b). Toxin levels were 6-12 times higher in copepod tissues than in their faecal pellets (II); thus defecation did not explain the toxin disappearance, although some toxin may have leaked to the surrounding water due to sloppy feeding or from faeces (Møller & al. 2003). The spiked sample series from zooplankton showed that toxin recovery was also sufficient (IV).

Nodularin can be efficiently removed from the hepatopancreas of prawns fed with nodularin-containing feed (Kankaanpää & al. 2005), suggesting that enzymatic transformation and excretion of nodularin are efficient, as was also observed in copepods after feeding on toxic cyanobacteria (IV). The nodularin concentrations found in copepods were relatively high when the depuration experiment was initiated (e.g. compared with the concentrations measured in other Baltic Sea organisms, Table 2.), but rapidly (within 30 min – 3 h) decreased to app. 51-58% of the initial concentrations. This indicated that a large part of the nodularin was detoxified or excreted from their tissues, although after 24 h about half of the toxin could still be detected. The theoretical degradation rate, i.e. the half-life of nodularin in zooplankton, was 7.4 h when measured with ELISA and 13.9 h when measured with PP1 (IV). Even though during the experiments the copepods had no alternative food source available and hence probably grazed more on the toxic *N. spumigena* culture than would have occurred in the field, the capacity of nodularin detoxication appears to be high.

Effective detoxication and excretion of nodularin can probably occur in copepods, even though there appears to be no increase in the activities of two

detoxication enzymes, 7-ethoxyresorufin-*O*-deethylase (EROD) and glutathione (GSH) transferase after exposure to toxic cyanobacteria (Kozłowski-Suzuki 2004). However, detoxication of nodularin was observed in zooplankton, e.g. in *Artemia salina* brine shrimp (Beattie & al. 2003). The largest decreases between two sampling times differed when measured with two different methods, being 0-0.5 h with ELISA, and 0.5-3 h with PP1, suggesting that conjugation or transformation to less toxic compounds may occur in copepods, either within their own tissues, or in their intestines by bacterial flora (Hansen & Bech 1996). However, this remains speculative since no measurements were performed to detect detoxication products in the copepods.

4.2 Toxin concentrations in the field

Nodularin could be detected in field-collected zooplankton (IV). The variation between species and sampling times was large, but the highest concentrations in zooplankton peaked with the highest phytoplankton toxin concentrations per dry weight, not with the highest abundance of cyanobacterial filaments in the water. The amount of cyanobacterial filaments in the water probably affects the nodularin accumulation curvilinearly at lower cyanobacterial densities, but when the peak cyanobacterial biomass is attained in the surface water, the toxin concentrations in copepods remain at a relatively low level.

There are species-specific differences in nodularin accumulation of zooplankton during peak cyanobacterial abundance. No nodularin was detected in *Acartia* sp. in July 2002 when there were large surface-floating blooms in the Gulf of Finland, whereas nodularin was found in *E. affinis* from the same sampling stations (IV). This can probably be explained by differences in their feeding behaviour and tendency to migrate in the water column. *Acartia* sp. decreases its feeding rate when low-quality food is abundant, whereas *E. affinis* feeds more readily on the most abundant food source (Gasparini & Castel 1997, Engström & al. 2000). *Acartia* sp. also performs deeper vertical migration than *E. affinis* (Burris 1980), thus escaping the cyanobacterial filaments near the surface. The same was observed in cladocerans with different vertical migration patterns (Burris 1980) and feeding types (Kim & al. 1989), since significantly larger concentrations of nodularin were detected in the filter-feeding and non-migrating *Bosmina longispina maritima* than in the raptorially feeding and vertically migrating *Evadne nordmanni* (IV). Non-migrating suspension-feeding species thus appeared to be more prone to accumulate nodularin than vertically migrating raptorial feeders.

Species with mainly predatory diets, such as *Limnocalanus macrurus* and *Cercopagis pengoi* (Vanderploeg & al. 1998, Uitto & al. 1999), take up toxins at approximately the same level per dry weight compared with more herbivorous zooplankton species (IV). Being large species, they are also preferred food items for planktivorous fish (Flinkman & al. 1998) and may therefore form an important link for nodularin transfer from zooplankton to planktivorous fish. Interestingly, the glacial relict *L. macrurus* living in deeper water layers (Ackefors 1969) contained similar concentrations of nodularin as mainly near surface occurring *C. pengoi* (Uitto & al. 1999), suggesting that vertical transport of nodularin can take place even in the deeper areas of the Baltic Sea.

4.3 Nodularin transfer via zooplankton to planktivores

Since zooplankton are capable of taking up nodularin in their tissues, some of this toxin can be transferred to pike larvae and mysid shrimps as well (V). The concentrations after 12 h of feeding on nodularin-exposed zooplankton were 0.41 and 0.59 $\mu\text{g g}^{-1}$ DW in pike larvae and mysid shrimps, respectively. However, the nodularin concentrations in the tissues of pike larvae and mysid shrimps were, 0.12 % and 0.03 % of the calculated nodularin amount that would have been expected if all the toxin within the ingested zooplankton were found in the tissues of zooplanktivores. Similarly to copepods, pike larvae and mysid shrimps (III, V), low concentrations of nodularin compared with the ingested toxin were found in sea trout (Kankaanpää & al. 2002), prawns (Kankaanpää & al. 2005) and blue mussels (Svensen & al. 2005). These results suggest that the process of excretion or detoxication of nodularin were efficient in many aquatic organisms and that the nodularin concentrations retained in the tissues of target organisms were low in comparison to the exposure. This explains the biodilution of nodularin when it is transferred from one trophic level to another, a phenomenon observed also with microcystins (e.g. Kotak & al. 1996, Ibelings & al. 2005).

As with copepods, lower concentrations of nodularin were detected in the faeces than in the tissues of pike larvae. Similar results after *N. spumigena* exposure were obtained in prawns and blue mussels (Kankaanpää & al. 2005, Svensen & al. 2005). The highest concentrations of microcystin-LR in fish faeces after cyanobacterial exposure were found during the depuration period, but the concentrations were still one order of magnitude smaller than in the fish liver (Soares & al. 2004). These ob-

servations indicate that effective detoxication and transformation of nodularin into less toxic compounds occur at every trophic level, and that very little of the ingested toxin is excreted via faeces to the water.

4.4 Effects on zooplankton and planktivores

Nodularin is not the only compound causing the negative effects of *N. spumigena*; other toxic secondary metabolites also play a role, and these compounds may have additive or even synergistic effects on aquatic organisms (Lindholm & al. 1992, Reinikainen & al. 2001). Cell-bound toxins (DeMott & al. 1991) or crude extracts of cyanobacteria (Oberemm & al. 1999) are more deleterious to aquatic organisms than purified toxins alone.

After ingestion of toxic cells, damage to the midgut epithelial cells was observed in prawns (Lightner 1978), mosquito larvae (Saario & al. 1994) and daphnids (Rohrlack & al. 2005). These injuries were probably not caused solely by hepatotoxins, but also by some other factors connected with cyanobacteria, that enables microcystins or nodularin to enter the blood stream and cause intoxication (Rohrlack & al. 2005). It appears that microcystins and nodularin, to be effective against grazers, must be ingested with cyanobacterial cells along with other bioactive compounds that are present in the cyanobacterial cells. When organisms are exposed to hepatotoxins alone, the toxins must occur at very high concentrations compared with their naturally occurring concentrations to cause any adverse, acute effects.

Effects on zooplankton: reduced feeding and secondary production

Feeding experiments revealed that both *A. bifilosa* and *E. affinis* actively grazed on *N. spumigena* (III), contradicting some earlier experimental findings for Baltic Sea zooplankton (Sellner & al. 1996, Koski & al. 1999, Engström & al. 2000). The observed high ingestion rates of toxic cyanobacteria may indicate compensatory feeding, which aims to compensate for either the poor-quality food (Cruz-Rivera & Hay 2000), or the additional energy consumption caused by toxic substances in the diet (Dutz 1998, Frangópulos & al. 2000). The total ingestion rate of *E. affinis* increased in the Bothnian Bay with increasing food concentration (as well as with *N. spumigena* concentration), indicating food limitation in the environment (III). In addition, both species fed selectively on ciliates even when other food items were available, in agreement with the results of Stoecker & Egloff (1987) and Koski & al. (2002) and supporting the view that ciliates are a

nutritionally favourable food source for copepods (Stoecker & McDowell Capuzzo 1990).

The egg production rates in *A. bifilosa* decreased with increasing *N. spumigena* concentrations in their food suspension (III), while no difference was observed in the hatching success. Feeding on toxic *N. spumigena* apparently caused an additional energy expenditure in copepods, since there was a dose-dependent negative relationship between nodularin concentration in *A. bifilosa* and their gross growth efficiency (GGE) (III). Again, a lower GGE may also derive from poorer food quality when more cyanobacteria are consumed (Ahlgren & al. 1992). The nutritional status of the experimental animals before the initiation of the experiments, as well as the acclimation period before the experiments, can affect the feeding rates (Reinikainen & al. 1995, Gustafsson & Hansson 2004), and hence also the toxin uptake rates. Zooplankton that have been acclimated to feed on cyanobacterial filaments may have higher ingestion rates than non-acclimated zooplankton. Cyanobacteria experiments conducted outside the blooming season, or with laboratory-reared culture, may therefore be one reason for the variable responses between different experiments.

There are species-specific characteristics (DeMott & al. 1991, Reinikainen & al. 2002) and population-specific differences (Colin & Dam 2002) in the sensitivity of zooplankton to harmful algal bloom toxins. It can also be presumed that the various life stages react differently to cyanobacterial exposure (Reinikainen & al. 1994). A previous history of feeding on toxic cyanobacteria, or low food availability in general, can affect the egg viability of daphnids (Reinikainen & al. 1995), since starved and stressed animals are affected more severely (Reinikainen & al. 1994). On the other hand, it was shown experimentally that the tolerance of zooplankton to cyanobacterial toxins can be improved by pre-exposure to toxic cyanobacteria (Gustafsson & Hansson 2004) and that even lake-wide shifts in daphnid genotypic variance towards higher resistance against cyanobacteria can occur during increased eutrophication and consequent cyanobacterial blooms (Hairston & al. 1999, 2001).

Despite their geographically distinct distributions and differing histories with toxic cyanobacteria, there were no differences in the feeding rates and reproductive responses of the two populations of *E. affinis* (i.e. from the Gulf of Finland and Bothnian Bay) to *N. spumigena* (III). It was suggested that variable resistance of different copepod populations to toxic algae would result from their geographical distribution, enabling tolerance to toxin to develop when occurring in the same regions (Colin & Dam 2002). However, our results showed that *E. affinis* populations in the Bothnian Bay (an area with no recorded toxic blooms) are equally well adapted to cyanobacterial exposure as the populations in the

Gulf of Finland (an area with yearly blooms; Ranta-järvi 2003). In the Bothnian Bay, the close proximity of the Bothnian Sea, where *N. spumigena* regularly forms mass occurrences, may explain the tolerance of *E. affinis* to this cyanobacterial species.

Direct and indirect effects on planktivores

We observed a significant decrease in the predation rate and faeces production of pike larvae when fed zooplankton pretreated with cyanobacterial crude extract, but no such effect could be observed with mysid shrimps (V). The good tolerance of mysid shrimps to *N. spumigena* was observed previously in long-term experiments (Engström & al. 2001). There are some indications that invertebrates are generally more tolerant to cyanobacterial toxins than vertebrates (Kiviranta & al. 1991) and detoxify them efficiently (Kankaanpää & al. 2005). In addition, mysid shrimps in the Baltic Sea feed on phytoplankton and zooplankton (Arndt & Jansen 1986, Viherluoto & al. 2000), while pike larvae feed on zooplankton for only a few weeks before they switch to larger prey (Urho & al. 1989). This suggests that planktivorous mysid shrimps have good tolerance to cyanobacterial toxins, whereas pike larvae may not have adapted to cyanobacteria equally well.

The decreased predation rate by pike larvae may affect the growth of fish larvae negatively, even without direct contact between the fish and cyanobacteria. Lower ingestion rates will eventually lead to slower growth of the larvae, as suggested by the decreased condition of fish larvae after the experiment. Therefore the low C:N ratios observed in pike larvae could be interpreted as decreased growth condition (von Westerhagen & al. 1998). Decreased predation rates by pike larvae could also be a function of zooplankton behaviour, since the larvae are known to prefer motile zooplankton (Urho & al. 1989), while possible behavioural changes in zooplankton caused by nodularin are still unresolved. It is known, however, that ingestion of microcystin can affect the behaviour of daphnids (Rohrlack & al. 2005).

Other indirect effects of cyanobacterial exposure on fish larvae include the increased energy demand for detoxication of nodularin that may be especially important for fast-growing fish larvae (Blaxter & Hempel 1963, Peterson & Wroblewski 1984). For example, feeding directly on toxic *Microcystis* sp. can completely inhibit the growth of common carp during long-term exposures (Li & al. 2004) or even result in negative growth rates of juvenile roach (Kamjunke & al. 2002), either through energy cost to the fish due to detoxication or by low or incomplete digestion due to mucilage cover in *Microcystis* sp.

Furthermore, starved fish are more vulnerable to the negative effects of hepatotoxins, which themselves cause decreased feeding rates and therefore starvation. Microcystin-LR decreases hepatic protein phosphatase activity and glycogen reserves in fasted juvenile goldfish (Malbrouck & al. 2004) and also damages gill structures in fish, further increasing their sensitivity to dissolved toxins (Zambrano & Canelo 1996). In addition, exposure to cyanobacteria may increase the drinking rates of fish, accelerating the uptake of toxins from the water (Best & al. 2003). *N. spumigena* exposure via zooplankton could have affected the condition of pike larvae in our experiments in several ways, but the concentrations needed to cause observable chronic effects were very low compared with the short-term exposures resulting in adverse effects on fish. An additional chronic risk linked to nodularin exposure is its capacity to promote tumours (Ohta & al. 1994); e.g. liver tumours have been observed in Baltic Sea flounders (Wiklund & Bylund 1994), a species known to accumulate nodularin in its liver (Sipiä & al. 2001a).

Fish avoid areas with dense *N. spumigena* blooms in the Peel-Harvey Estuary system in Australia, based on fish catches in the same areas during different years with variable bloom conditions (Potter & al. 1983). To date, no such information is available for the Baltic Sea, but it can be assumed that fish respond to cyanobacterial abundance and adjust their behaviour accordingly. Dead sticklebacks were observed within a dense bloom of *N. spumigena* in the Baltic Sea, but it is not known whether the ultimate cause of death was nodularin in their tissues, even though relatively high concentrations of nodularin were found in their viscera (Kankaanpää & al. 2001a). Fish behaviour is complex and easily disrupted by exposure to environmental pollutants (Scott & Sloman 2004), and behavioural changes can reveal very low levels of stress caused by environmental pollutants in fish (Steinberg & al. 1995). Whether nodularin affects the behaviour of fish as microcystin-LR does, by affecting motility and swimming activity (Baganz & al. 1998, 2004), is a matter for future investigation.

4.5 Possible ecosystem consequences

Little information is available on the chronic effects of cyanobacteria on Baltic Sea organisms. Even though there has been a long history of cyanobacteria in the Baltic Sea (Bianchi & al. 2000) and possible tolerance may have evolved in some of the Baltic Sea organisms (Reinikainen & al. 2002), the increased frequency and intensity (Kahru & al. 1994) of these blooms may pose a threat to Baltic Sea organisms. Long-term effects e.g. on zooplank-

ton production, distribution and abundance are not known. In freshwater ecosystems decline in zooplankton abundance and decrease in body size was observed both experimentally (Ghadouani & al. 2003) as well as after large cyanobacterial blooms (Threlkeld 1979, Gliwicz & Lampert 1990). Some effects may be expected, since exposure to toxic cyanobacteria affects the reproductive success (Koski & al. 1999), and even behaviour of zooplankton (Rohrlack & al. 1999, 2001, 2005). Even though the vectorial transfer of nodularin does not appear to be very effective and only relatively small concentrations of nodularin are transported to higher trophic levels, due to its toxicity (reviewed by Sivonen & Jones 1999), carcinogenicity (Ohta & al. 1994) and potential neurotoxicity (Lehtonen & al. 2003), the effects of chronic exposure to organisms of the Baltic Sea food webs call for further investigation.

There are two mechanisms, in addition to morphological features, that can prevent cyanobacteria from being eaten: toxic substances used as feeding deterrents (Nizan & al. 1986) and post-ingestion physiological symptoms that decrease the feeding rate of consumers (DeMott & al. 1991, Rohrlack & al. 2005). According to Kurmayer & Jüttner (1999), it is advantageous for cyanobacteria to produce toxins against herbivorous zooplankton if there are species capable of selective feeding. If the grazing pressure is targeted towards other primary producers especially during nutrient limitation, selective feeding decreases competition from other algae (Guissande & al. 2002), hence favouring the occurrence of cyanobacteria. If zooplankton species capable of selective feeding and vertical migration accumulate less nodularin and hence are less affected by it, there could be some long-term effects of *N. spumigena* blooms on zooplankton species composition, depending on the sensitivity of the zooplankton species in question. Cyanobacterial blooms may even have some positive effects on zooplankton. A decaying bloom can be an especially favourable place for certain copepod species, since the heterotrophic community supported by cyanobacterial filaments provides good food for copepods (Engström-Öst & al. 2002, Koski & al. 2002, Schmidt & al. 2002) and since turbidity in the water is known to decrease feeding rates in visually preying fish (reviewed by Utne-Palm 2002), making dense cyanobacterial bloom areas potential refuges for zooplankton.

It was proposed that fish species originating from oligotrophic environments are more vulnerable to hepatotoxins than species native to eutrophic habitats (Xie & al. 2004), possibly indicating adaptation of fish to hepatotoxins in eutrophic environments. Since some of the fish species inhabiting the Baltic Sea originate from freshwater and some are of marine origin (Koli 1990), they have variable tolerances against cyanobacterial toxins as well.

The chronic effects of cyanotoxins at higher trophic levels are also largely unknown. Due to the organotropism of nodularin and its tendency to accumulate in inedible parts – the liver/hepatopancreas and viscera/digestive gland (Kankaanpää & al. 2002, 2005, Svensen & al. 2005) and in some cases the brain and heart (Kankaanpää & al. 2005), combined with the very low concentrations of nodularin found in the muscle tissues of fish (Table 2) – the risk of nodularin exposure via Baltic Sea organisms to human populations appears to be small (e.g. Sipiä 2001). However, Baltic Sea fish, birds and mammals usually consume their prey items entirely and may therefore be more at risk regarding the negative effects of nodularin. Nodularin was detected especially in blue mussels (Sipiä & al. 2001a,b, 2002a, Karlsson & al. 2003a) and organisms that prey on them, such as flounders (Sipiä & al. 2001a,b, Karlsson & al. 2003a,b) and eiders (Sipiä & al. 2003). This benthic food web connected with filter-feeding mussels appears to be more effective in transferring hepatotoxins, both nodularin and microcystins, to higher trophic levels than the pelagic food web, due to their larger hepatotoxin concentrations (Table 2) and relatively long periods of depuration from their tissues (Vasconcelos 1995, Amorim & Vasconcelos 1999, Sipiä & al. 2002a, Dionisio Pires & al. 2004, Svensen & al. 2005). Hence the intensified cyanobacterial blooms in the Baltic Sea can potentially have an effect on the structure of food webs if some species are favoured by their occurrence and others are negatively affected by them.

5. CONCLUSIONS

The results of this thesis suggest that Baltic Sea zooplankton can act as vectors for toxin transfer to higher trophic levels. In this way organisms that do not consume cyanobacteria themselves may also be affected by cyanobacteria via feeding on zooplankton containing nodularin. The more that zooplankton feed on *N. spumigena*, the more nodularin can be found in their tissues. The same applies for *N. spumigena* concentrations in the field, except for the extraordinarily high concentrations of cyanobacterial filaments in the surface layer during the densest blooms. The highest concentrations of nodularin in zooplankton are found immediately after feeding on *N. spumigena* filaments. The initial depuration of nodularin from zooplankton is a rapid process, but about half of the toxin is still retained in their tissues after 24 h. Zooplankton also take up nodularin directly from the water and bioconcentrate it, compared with the concentrations in the surrounding water.

The experimental studies of this thesis, as well as previous observations of nodularin concentrations in

Baltic Sea organisms (Table 2) suggest that at every trophic level nodularin is efficiently transformed and degraded to less harmful compounds. This degradation occurs rapidly compared with abiotic degradation (Twist & Codd 1997, Metcalf & Codd 2000). Part of the dissolved nodularin pool is also taken up by zooplankton and hence detoxified.

Detoxication, being an energy-demanding process, affects the reproduction and gross growth efficiency of zooplankton and the grazing rate of zooplanktivorous fish feeding on exposed zooplankton. The effects of nodularin at the cellular level interfere with several functions of cell metabolism, so that even very low concentrations of nodularin probably cause negative effects on fish larvae that already have considerable energy demands for sustaining sufficient growth. Invertebrates appear to be more tolerant to chronic, low exposures of nodularin.

Despite the low concentrations of nodularin that can be seen in transfer between trophic levels the energy expenditure demanded for detoxication of nodularin and possibly repairing the damage caused by it at the cellular level suggests that nodularin may have long-term effects on Baltic Sea biota. The magnitude of the negative effects is dependent on the toxicity and timing of the cyanobacterial mass occurrences, as well as on the exposed species in question. Ultimately, cyanobacterial exposure could affect the food web productivity if the most sensitive species and life stages are affected.

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