

Nodularin concentrations in Baltic Sea zooplankton and fish during a cyanobacterial bloom

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Received: 8 August 2007 / Accepted: 19 August 2008 / Published online: 10 September 2008
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Abstract Toxic cyanobacterial blooms, dominated by *Nodularia spumigena*, are a recurrent phenomenon in the Baltic Sea during late summer. Nodularin, a potent hepatotoxin, has been previously observed to accumulate on different trophic levels, in zooplankton, mysid shrimps, fish as well as benthic organisms, even in waterfowl. While the largest concentrations of nodularin have been measured from the benthic organisms and the food web originating from them, the concentrations in the pelagic organisms are not negligible. The observations on concentrations in zooplankton and planktivorous fish are sporadic, however. A field study in the Gulf of Finland, northern Baltic Sea, was conducted during cyanobacterial bloom season where zooplankton (copepod *Eurytemora affinis*, cladoceran *Pleopsis polyphemoides*) and fish (herring, sprat, three-spined stickleback) samples for toxin analyses were collected from the same sampling areas, concurrently with phytoplankton community samples. *N. spumigena* was most abundant in the eastern Gulf of Finland. In this same sampling area,

cladoceran *P. polyphemoides* contained more nodularin than in the other areas, suggesting that this species has a low capacity to avoid cyanobacterial exposure when the abundance of cyanobacterial filaments is high. In copepod *E. affinis* nodularin concentrations were high in all of the sampling areas, irrespective of the *N. spumigena* cell numbers. Furthermore, nodularin concentrations in herring samples were highest in the eastern Gulf of Finland. Three-spined stickleback contained the highest concentrations of nodularin of all the three fish species included in this study, probably because it prefers upper water layers where also the risk of nodularin accumulation in zooplankton is the highest. No linear relationship was found between *N. spumigena* abundance and nodularin concentration in zooplankton and fish, but in the eastern area where the most dense surface-floating bloom was observed, the nodularin concentrations in zooplankton were high. The maximum concentrations in zooplankton and fish samples in this study were higher than measured before, suggesting that the temporal variation of nodularin concentrations in pelagic communities can be large, and vary from negligible to potentially harmful.

Communicated by M. Kühl.

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Introduction

Cyanobacteria dominate phytoplankton communities in the open Baltic Sea during late summer (Sivonen et al. 1989; Kononen et al. 1996). Their occurrence and frequency has, however, increased during the last decades, probably due to anthropogenic eutrophication (Kahru et al. 1994; Poutanen and Nikkilä 2001). In addition, the ratio of toxic to non-toxic cyanobacterial species seems to have increased since the early 1990s (HELCOM 2003). Dominating toxic species in the Baltic Sea cyanobacterial blooms is *Nodularia*

spumigena (Sivonen et al. 1989), which produces hepatotoxic nodularin in the Baltic Sea (Laamanen et al. 2001).

There are species-specific differences in the risk of nodularin accumulation in zooplankton, mainly due to their feeding preferences, and vertical distribution in the water column (Karjalainen et al. 2006). Species capable of grazing directly on *N. spumigena* (e.g., *Eurytemora affinis*), or the associated heterotrophic community, are more prone to accumulate nodularin than species that avoid feeding on toxic *N. spumigena* filaments (Engström et al. 2000; Koski et al. 2002; Kozłowski-Suzuki et al. 2003). Also, species performing vertical migration can avoid contact with toxic *N. spumigena* occurring mainly in the uppermost water column (Kononen et al. 1998).

The largest concentrations of nodularin in fish have been observed in species feeding on benthic organisms, such as in European flounder and roach (Sipiä et al. 2001b, 2002a, 2006), especially in their liver, whereas the average concentrations found in their flesh have been lower (Sipiä et al. 2001a, 2006). This is in accordance with experimental studies on organotropism of microcystins, a closely related group of hepatotoxins, which have been observed to accumulate mainly in the liver and digestive tract of exposed fish (Bury et al. 1998). In planktivorous fish like herring the concentrations have been generally lower, as have been the concentrations in predatory fish feeding on them, such as Atlantic salmon (Sipiä et al. 2002b). However, it is known that the accumulation of nodularin can be very variable between fish individuals (Kankaanpää et al. 2005), as well as the concentration of nodularin in zooplankton can rapidly vary (Karjalainen et al. 2006). Therefore, even low concentrations of nodularin in zooplankton can affect the condition of planktivorous fish, as has been demonstrated experimentally (Karjalainen et al. 2005; Pääkkönen et al. 2008).

The most important pathway for nodularin accumulation and transfer from cyanobacteria in planktivorous fish is probably via zooplankton grazing on cyanobacteria. It is known that planktivorous fish in the Baltic Sea do not consume cyanobacterial filaments directly, because in fish stomach analyses no traces of toxic cyanobacteria have been found in their guts (e.g., Raid and Lankov 1995). There is practically no evidence on transfer of dissolved nodularin directly from the water to fish, but the observed low concentration of dissolved nodularin in the open sea suggest that this pathway plays a minor role in nodularin transfer to fish (Kononen et al. 1993; Kankaanpää et al. 2001).

The risk of cyanobacterial toxin transfer in the open sea food web via zooplankton to planktivorous fish was studied in 2004 in order to evaluate simultaneously the concentrations of cyanobacteria cells found in the water, toxin concentrations in grazing zooplankton, as well as toxin

concentrations in the planktivorous fish species inhabiting the open sea areas during cyanobacterial blooms. The aim was to collect samples for toxin analyses from the most abundant zooplankton and pelagic fish species, and compare these results with cell numbers of *N. spumigena* in the water. In addition, the concentration of nodularin in *N. spumigena* biomass was compared with concentrations in zooplankton and fish biomasses to evaluate the accumulation of nodularin in studied species per surface area in the various parts Gulf of Finland.

Material and methods

Field sampling

The research cruise was conducted with two research vessels, R/V Aranda (Finnish Institute of Marine Research) and R/V Muikku (Finnish Environment Institute) during 20–29 July 2004 (sampling stations and trawling areas in Fig. 1). Plankton sampling was carried out onboard R/V Aranda during daytime, whereas fish trawling was conducted with R/V Muikku.

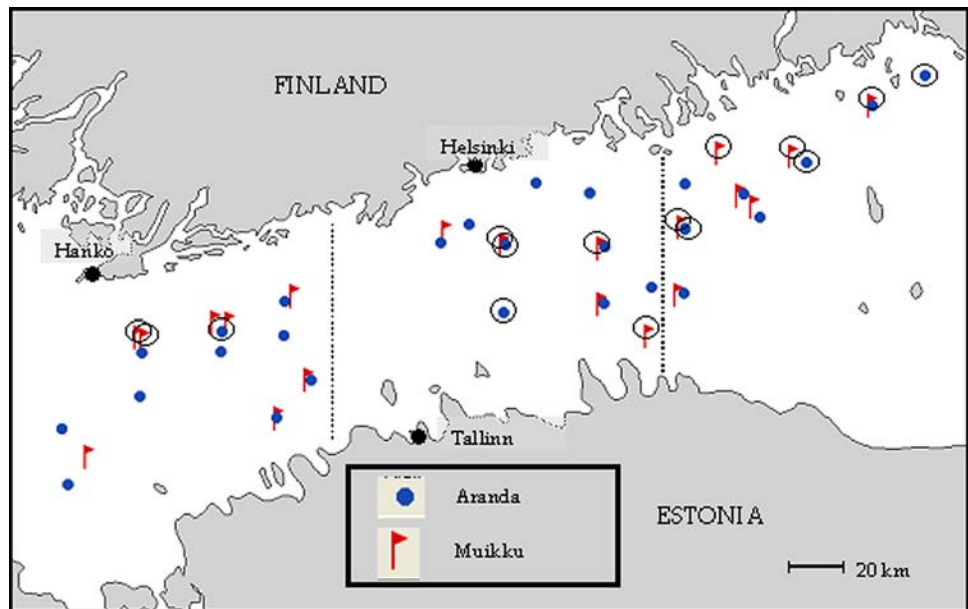
The phytoplankton community samples were taken with a Rosette-type water sampler onboard R/V Aranda. Hundred milliliter of water collected at 0, 7 and 18 m depths at each sampling station were preserved with acid Lugol's solution in glass bottles. Phytoplankton cell numbers, including cyanobacteria, were counted using the method presented by Utermöhl (1958). Zooplankton community samples were taken onboard R/V Aranda with a 100- μm WP-2 type plankton net with a closing mechanism from each sampling station from the surface to the bottom with 10-m net hauls (0–10, 10–20, 20–30, 30–40, 40–50, 50–bottom), preserved in 4% buffered formaldehyde solution, and counted under binocular microscope.

Based on cyanobacterial abundance in the water, the sampling area was divided into three sections, western, middle and eastern Gulf of Finland (Fig. 1). The results from toxin analyses are grouped in these three areas, since no simultaneous measurements could be made with two research vessels from the same sampling stations. The stations where toxin samples were collected are shown in Fig. 1.

Zooplankton toxin samples and their preparation

Zooplankton for toxin analyses was collected with 200- μm WP-2 type net from the bottom to 12 m depth to avoid contamination with surface-floating filaments of cyanobacteria. At each sampling station, zooplankton samples for toxin analyses were taken immediately after the zooplankton community samples were collected in order to get samples

Fig. 1 Sampling stations during the research cruise in 2004. Dots denote the plankton sampling stations visited by R/V Aranda, and flags denote the trawling areas sampled by R/V Muikku. The stations and trawling areas where toxin samples were collected are marked with circles. Broken lines separate the three sampling areas, the western, middle and eastern Gulf of Finland



from the same community to both analyses. Freshly-collected zooplankton for toxin analyses was placed in 30-l containers of cool water taken below the thermocline, and gentle aeration was added. At least 30 living individuals of the most abundant copepods species that were found at each sampling station (*Eurytemora affinis*), and 50 individuals of cladocerans (*Pleopsis polyphemoides*) were collected per sample ($n = 25$ and 7, respectively), under a binocular microscope. The animals were rinsed with filtered seawater three times before placing them with forceps into glass scintillation vials and freezing them in -30°C (see Karjalainen et al. 2006). Zooplankton samples were freeze-dried (Edwards 12K Super Modulyo Freeze Drier, West Sussex, England), extracted with 4 ml of 100% MeOH, and sonicated with a tip sonicator (Braun Labsonic-U, Melsungen, Germany). The samples were then filtered with a syringe-operated filtering unit with GF/F filters, and 3 ml of the filtrate was evaporated with nitrogen flow. The samples were resuspended with 40 ml of 50% MeOH, and gradually diluted with Milli-Q water to the final volume of 320 μl , corresponding to a methanol concentration of 6% (Metcalf et al. 2000).

Collection of fish samples and sample preparation

Fish samples were collected onboard R/V Muikku with a pelagic trawl. The most abundant planktivorous fish species, herring (*Clupea harengus membras*), sprat (*Sprattus sprattus*) and three-spined stickleback (*Gasterosteus aculeatus*) were collected for the samples.

At least ten individuals of each fish species from each trawl haul were measured, weighed and pooled for toxin samples ($n = 5$, $n = 8$, and $n = 3$ for herring, sprat and three-

spined stickleback, respectively). Fish samples were frozen at -30°C and freeze-dried (Edwards, 12K Super Modulyo Freeze Drier, West Sussex, England). The dry tissues were then weighed, homogenized using a planetary mill (Fritz, Planetary Mill Pulverisette 5, Idar-Oberstein, Germany) and stored at -30°C until extraction.

Pooled and homogenized fish samples were extracted with 30 ml of 100% MeOH (Williams et al. 1997) in a Branson 3200 (Dansbury, CT, USA) ultrasonic bath at $50\text{--}60^{\circ}\text{C}$ for 8 h with occasional shaking. Extracts were centrifuged at 10°C (10 min \times 1,300 g) for 15 min in a Megafuge 2.0 R (Heraeus Sepatech, Osterode, Germany). The supernatant was dried in vacuo (50°C) and the residue resuspended to 350 μl of 100% MeOH and centrifuged at 10°C (10 min \times 5,200 g) for 15 min. 100 μl of that sample volume was taken and diluted were diluted 1:10 to 1:200 with Milli-Q water (Millipore), and then filtered (0.45 μm , Millex-HV, Millipore). For final results, the nodularin concentrations in dried fish samples were converted to wet weight, assuming the dry weight of fish samples to be ca. 20% of their wet weight (Arrhenius and Hansson 1998).

Toxin analyses

Samples were analyzed by commercial ELISA plate kit (EnviroLogix, Portland, ME, USA). Commercial ELISA kit is primarily designed for analysis of microcystins, but can also be used for detection of nodularin. Absorbance was measured at 450 nm with a Benchmark Microplate reader (Bio-Rad, Hercules, CA, USA). For zooplankton samples, the standard solutions provided by the manufacturer were used following the kit instructions. The nodularin used as a standard for fish samples (analyzed in

triplicate) was purified from a bloom of *Nodularia spumigena* (Karlsson et al. 2005b). Nodularin solutions ranging from 0.25 to 1.5 $\mu\text{g l}^{-1}$ were used for calibration.

Phytoplankton and zooplankton data were tested for normality and homogeneity of variances, and statistically tested using one-way ANOVA for resolving whether there was a difference in the concentrations of *N. spumigena* cells, as well as in the nodularin concentrations in zooplankton samples between the three designated sampling areas. Regression analysis was used in determining if there was a relationship between the concentration of nodularin in zooplankton samples, and the *N. spumigena* cell counts from the same sampling area. No statistical tests were made to nodularin concentrations in the fish samples, due to the lack of replicate samples from each trawl haul and low number of overall samples.

Results

The numbers of *N. spumigena* cells in three sampling areas differed significantly from another (ANOVA $F_{2,18} = 5.825$, $P = 0.011$). The highest concentrations of *N. spumigena* cells were observed in the eastern Gulf of Finland, and the lowest in the western Gulf of Finland (Fig. 2). The abundances of the two dominant zooplankton species as well as

the studied planktivorous fish species in the whole study area can be seen in Fig. 3 and 4, respectively.

Nodularin was detected in both zooplankton species collected for the analyses (Fig. 5a). There was no difference in the nodularin concentrations of *E. affinis* between the areas (ANOVA $F_{2,19} = 0.386$, $P = 0.685$), but for *P. polyphemoides* the concentrations differed from another (ANOVA $F_{2,6} = 14.820$, $P = 0.014$). The concentrations in *P. polyphemoides* were significantly higher in the eastern Gulf of Finland than in the other parts of this sea area (Tukey's HSD $P = 0.020$). The highest concentrations in all zooplankton samples were observed in *P. polyphemoides* in the eastern Gulf of Finland, 2.36 $\mu\text{g g}^{-1}$ ww. There was not, however, any linear relationship between *N. spumigena* abundance in the water, and the nodularin concentrations in zooplankton ($r^2 = 0.036$, $P = 0.25$).

For herring the size range in fish collected for the toxin analyses was 7.6–12.2 cm and 2.61–11.94 g, for sprat 7.2–12.6 cm and 1.58–9.21 g, and for three-spined stickleback 3.4–6.6 cm and 0.35–1.92 g. Of fish samples, 14 out of 16 samples contained nodularin (Fig. 5b). Variation in the triplicate measurements from the same sample was 6.95% ($n = 14$, all positive samples included). Highest concentrations were measured from three-spined sticklebacks in the middle part of the Gulf of Finland, 0.80 $\mu\text{g g}^{-1}$ dw (0.16 $\mu\text{g g}^{-1}$ ww). For herring, the highest concentrations

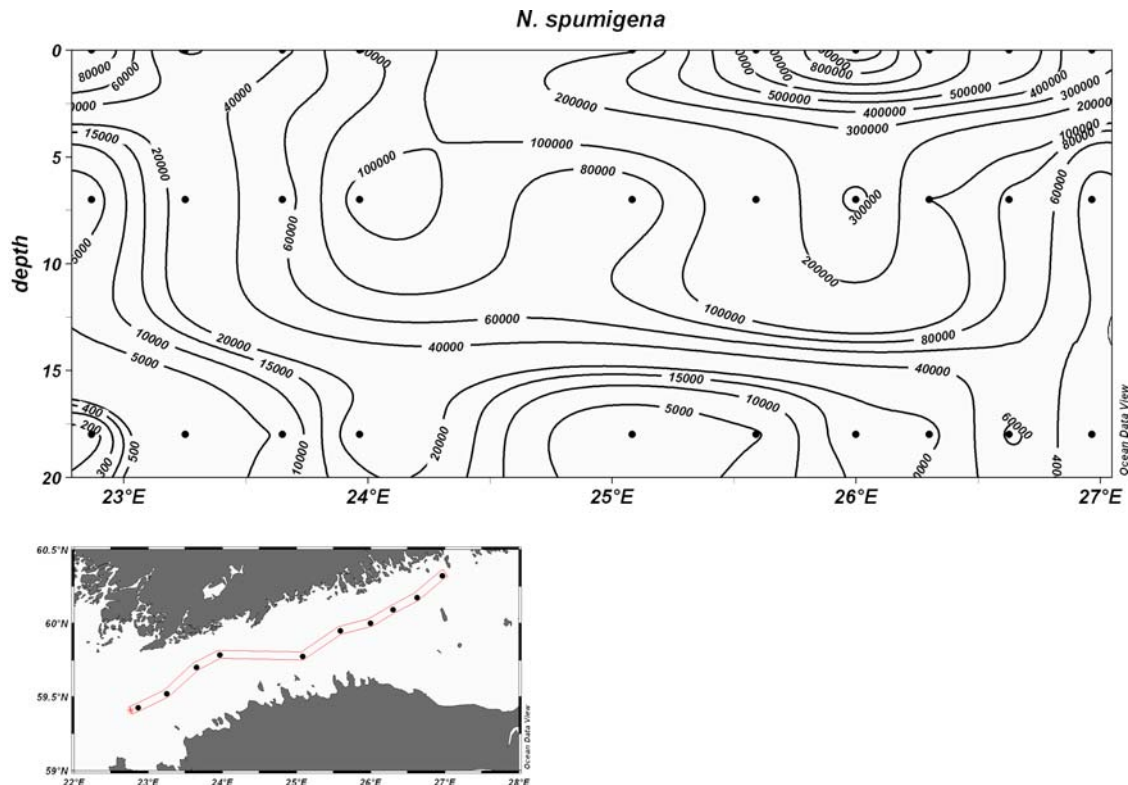


Fig. 2 The abundance of *Nodularia spumigena* (number of cells l^{-1}) along a gradient across the Gulf of Finland (from west to east, see the *small map* for the positioning of the sampling stations). Sampling was conducted at 0, 7 and 18 m depths

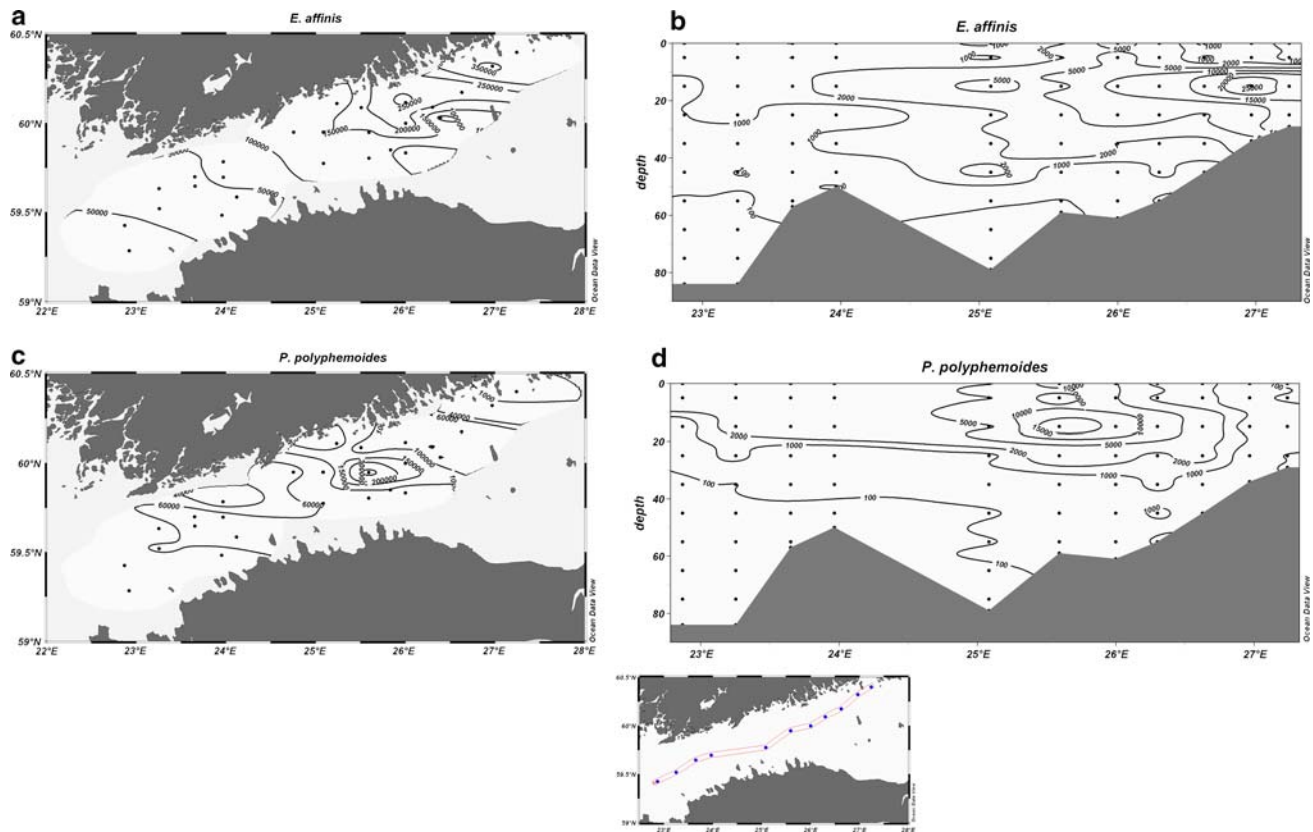


Fig. 3 Abundances of zooplankton (*Eurytemora affinis* and *Pleopsis polyphemoides*) in the Gulf of Finland. On the left (**a**, **c**) numbers of adult individuals in the whole water column, (ind m⁻²), on the right

(**b**, **d**) numbers of adult individuals in vertical net hauls collected at 10 m intervals (ind m⁻³) along a gradient from west to east (see the *small map* for sampling stations)

were measured from the eastern Gulf of Finland (0.22 $\mu\text{g g}^{-1}$ dw, corresponding 0.03 $\mu\text{g g}^{-1}$ ww), whereas for sprat, the highest values were recorded from the western Gulf of Finland (0.10 $\mu\text{g g}^{-1}$ dw, i.e., 0.02 $\mu\text{g g}^{-1}$ ww).

Discussion

The concentrations observed in zooplankton were higher than in field-collected zooplankton collected previously (Karjalainen et al. 2006). Measurements from zooplankton in Baltic Sea during 2001 and 2002 in cyanobacterial blooming season have ranged from 0 to 0.62 $\mu\text{g g}^{-1}$ ww, and during this study the measured concentrations were at their highest 2.36 $\mu\text{g g}^{-1}$ ww. It has been demonstrated that the tendency of *E. affinis* to accumulate nodularin in its tissues is high (Karjalainen et al. 2006), due to its preference to feed on the most available food item (Gasparini and Castel 1997), and to inhabit the near-surface water layers (Burris 1980) where majority of *N. spumigena* filaments occur. However, in this study the highest concentrations in zooplankton were found in the cladoceran *P. polyphemoides* in the eastern part of the Gulf of Finland, where there were dense *N. spumigena* aggregations in the water, and in

relatively shallow sampling stations, emphasizing the potential for nodularin accumulation in zooplankton in this area. No measurements from *P. polyphemoides*, or from any other zooplankton species from these shallower easternmost stations has been done previously, so no direct comparisons can be made, but the shallower water mass and dense *N. spumigena* bloom in 2004 in this area could explain the higher nodularin concentrations in zooplankton as well.

The highest concentrations of nodularin in fish were found from three-spined stickleback, the values being as high as 0.80 $\mu\text{g g}^{-1}$ dw. The concentrations in whole fish samples of this study cannot be directly compared with earlier measurements from the open Baltic Sea, where only the viscera of this species have been studied, but the results of this study are still higher than in the viscera samples in 1999 (0.17 $\mu\text{g g}^{-1}$ dw), or in 2003 (0.70 $\mu\text{g g}^{-1}$ dw), suggesting a slightly larger overall accumulation of nodularin in 2004 compared with earlier measurements (Kankaanpää et al. 2001; Sipiä et al. 2007). In littoral areas, measurements from three-spined stickleback juveniles (measured from the whole fish) have been recorded to contain nodularin 0.50 \pm 0.35 $\mu\text{g g}^{-1}$ dw (Pääkkönen et al. 2008). Generally, three-spined stickleback prefers the surface layer of the

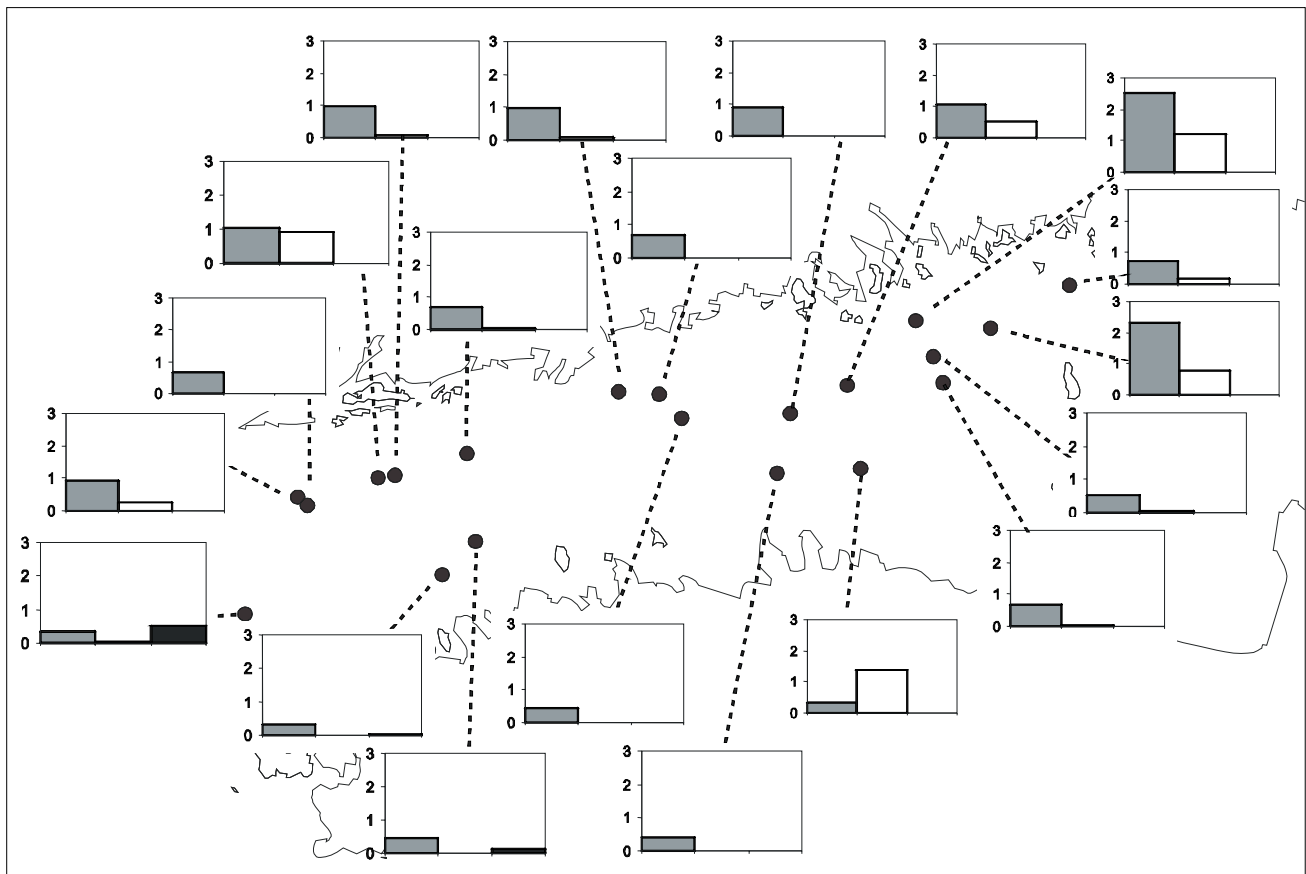


Fig. 4 Fish biomasses (g m^{-2}) in each trawling area. Grey bars denote sprat, white bars herring, and black bars three-spined stickleback. All three species were encountered in each trawling area

open sea water mass (Peltonen et al. 2004) where also the largest concentrations of *N. spumigena* can be found, and hence may be feeding more on nodularin-exposed zooplankton than species feeding in deeper waters.

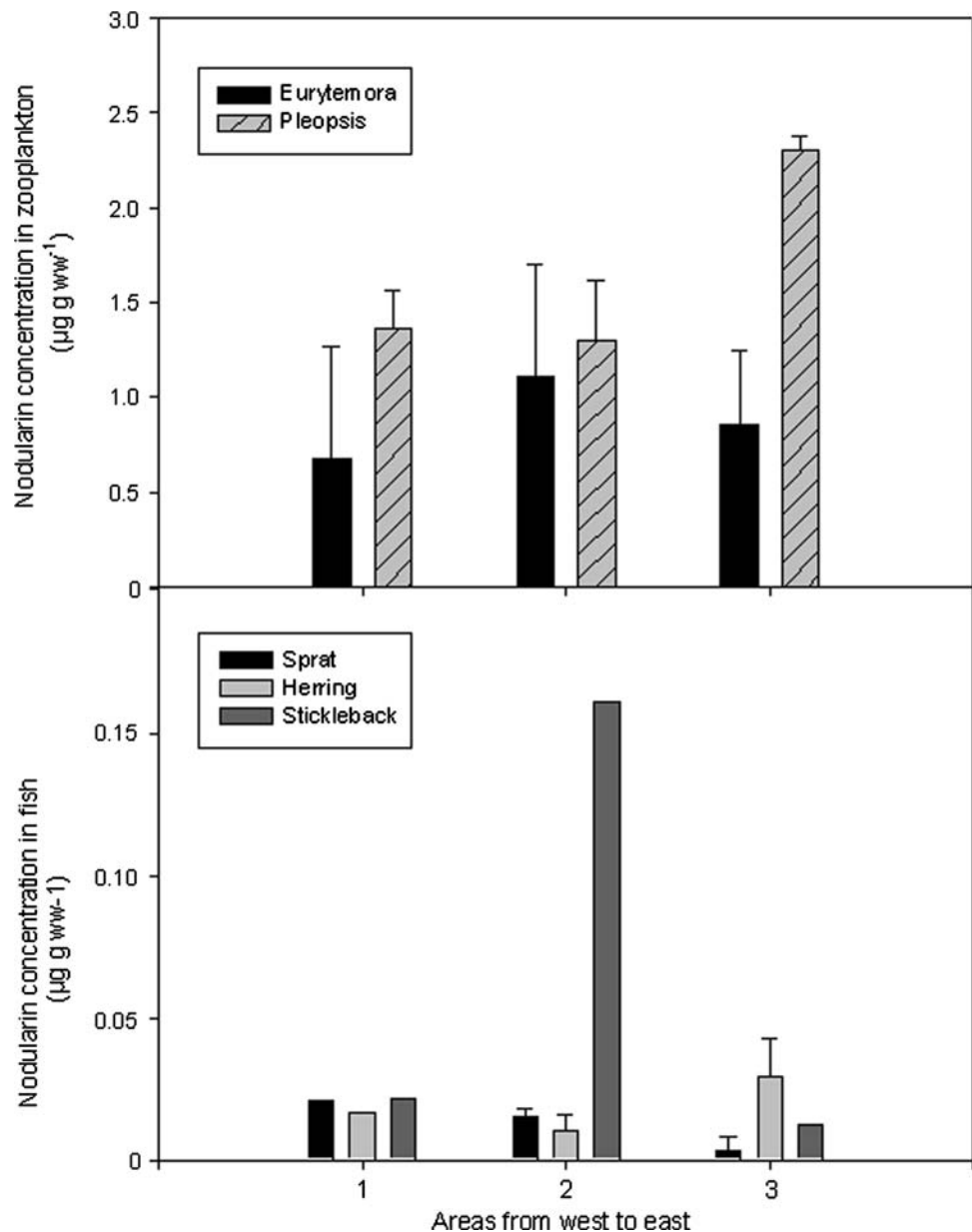
This study showed that the concentration of nodularin in herring samples can be as high as $0.22 \mu\text{g g}^{-1}$ dw. So far, the highest observed values in herring have been in 1999 in herring muscle $0.0065 \mu\text{g g}^{-1}$ dw (Sipiä et al. 2002b), and in 2002–2003 in the stomachs of herring $0.09 \mu\text{g g}^{-1}$ dw (Sipiä et al. 2007). Partly the earlier, lower observations can be explained by the fact that Baltic Sea herring muscle samples have been taken later during the cyanobacterial bloom season (in September in 1999), when there was probably less toxic *N. spumigena* in the water, and hence also the exposure via zooplankton was lower. Also, it is likely that the intestines and especially liver of herring contain most of the accumulated nodularin, as is the case with other fish species as well (Sipiä et al. 2001 a, b), and has not been included in the previous measurements. In addition, the sampling stations where the highest concentrations in herring were measured were situated in a relatively shallow sea area, where probability of zooplankton encountering *N. spumigena* is higher. Due to the shallowness of this area,

probably also the risk of herring to feed on exposed zooplankton is higher.

There are no previous records about concentrations of nodularin in sprat tissues, but they are in the same range with herring tissues in this study, suggesting that the exposure to nodularin via zooplankton to these two species is similar. This is much expected, since their feeding habits and preferences, at least for the smaller fish size classes, are much alike (Casini et al. 2004). However, herring was most abundant in the easternmost stations with fewer copepod species available (dominated by *E. affinis*) making them more prone to accumulate nodularin via zooplankton, whereas sprat was more numerous in the western Gulf of Finland (Peltonen et al. 2007) where more diverse copepod community prevails (Flinkman et al. 2007), and chance of more selective feeding on zooplankton species that do not accumulate nodularin so effectively (Karjalainen et al. 2006).

Since ELISA method does not discriminate between nodularin and microcystin variants, it is not known whether a fraction of the measured hepatotoxins in the samples could have been microcystins. However, according to phytoplankton counts, no *Microcystis* spp. were found at any of

Fig. 5 a Nodularin concentrations in zooplankton, *Eurytemora affinis* and *Pleopsis polyphemoides* and **b** in fish, three-spined stickleback, sprat and herring ($\mu\text{g g ww}^{-1}$, mean \pm S.E.) samples, from three sampling areas. Note the different scales on y-axes



the sampling stations during the cruise. Other cyanobacteria with potential for microcystin production, *Anabaena lemmermannii* (Rajaniemi et al. 2005) or unidentified *Anabaena* species were present (cell counts varying from $1,586$ to 2.8×10^6 and 0 to 0.8×10^6 cells l^{-1} , respectively), but it is probable that their contribution to the total hepatotoxin concentration in the open Baltic Sea is low, as observed by e.g., Karlsson et al. (2005a).

High accuracy of toxin analyses is required for reliable results. It is known that with zooplankton samples the ELISA method gives valid results of nodularin and its derivatives, or even exaggerates them (Karjalainen et al. 2006). For fish tissues, the matrix is more problematic, and mean recovery of 30.2% was obtained with spiked salmon liver samples with ELISA assay (Sipiä et al. 2002b), and

only 28% with stickleback viscera samples with LC-MS method (Sipiä et al. 2007). Therefore, the methodology for fish sample analyses should be improved, especially in connection with risk analyses.

Calculations for tolerable daily intake for human consumption should be corrected with the recovery factor. Probably a large fraction of nodularin is situated in the liver and digestive tract of planktivorous fish, but this certainly raises the question whether any monitoring of fish flesh should be carried out, in order to be on the safe side concerning human consumption of herring or sprat. Anyway, predatory fish and mammals feeding on planktivorous fish, and consuming them with their viscera, can be affected by nodularin.

Individual variation in fish samples, even collected from the same sampling area, appears to be large (Kankaanpää

Table 1 Calculation of nodularin concentrations in *Nodularia spumigena* (mg m^{-2}), zooplankton ($\mu\text{g m}^{-2}$) and fish (ng m^{-2}) per unit area in three sampling areas, western, middle and eastern part of the Gulf of Finland

	Western	Middle	Eastern
<i>Nodularia spumigena</i> (mg m^{-2})	0.46	0.63	11.57
<i>Eurytemora affinis</i> ($\mu\text{g m}^{-2}$)	0.90	2.61	2.13
<i>Pleopsis polyphemoides</i> ($\mu\text{g m}^{-2}$)	1.77	2.28	13.66
Sprat (ng m^{-2})	19.46	9.44	5.37
Herring (ng m^{-2})	3.96	0.01	3.60
Three-spined stickleback (ng m^{-2})	0.22	18.01	0.05

et al. 2005). More homogeneous toxin accumulation in fish would be expected, if nodularin accumulated directly from the water in their tissues. This appears to be unlikely, since closely related hepatotoxin microcystin-LR has a low $\log D_{ow}$ value (e.g., the value expressing the potential for bioconcentration by passive diffusion), in the pH range of Baltic Sea (De Maagd et al. 1999). This suggests that majority of nodularin in planktivorous fish tissues originates from their food. Since no *N. spumigena* filaments have been found in the stomachs of planktivorous fish (Raid and Lankov 1995), mesozooplankton seems to play a major role in nodularin transfer to planktivorous fish. At least the two dominant zooplankton species in 2004 regularly contained nodularin, and composed a major part of the diet of all the planktivorous fish species collected for toxin analyses in this study (M. Vinni, University of Helsinki, and H. Peltonen, Finnish Environment Institute; unpublished).

When nodularin concentrations are applied to the biomass estimations made during the research cruise, a rough calculation about nodularin accumulation on different trophic levels per surface area can be made. The average cell-bound nodularin concentration ($1.617 \text{ pg cell}^{-1}$ from *N. spumigena* samples taken on the same cruise; K. Sivonen, University of Helsinki, unpublished) was used in these calculations with *N. spumigena* mean abundance in the top-most 7 m (Table 1; Fig. 2). As expected, in the eastern part of the Gulf of Finland, where the number of *N. spumigena* cells was the highest, the total cell-bound concentration of nodularin appears to be highest as well, 11.56 mg m^{-2} . In zooplankton, especially high nodularin concentrations per square meter ($13.66 \mu\text{g m}^{-2}$) could be observed in the eastern Gulf of Finland in *P. polyphemoides*. This species had the highest nodularin concentrations in eastern Gulf of Finland, and was also abundant (Table 1; Fig. 3). In fish, as nodularin concentration expressed per fish biomass and surface area, the highest concentrations were found in sprat biomass in the western Gulf of Finland (Table 1; Fig. 4).

To conclude, the observations made in this study suggest that there is no linear relationship between the number of

N. spumigena cells in the water, nodularin concentrations in grazing zooplankton and in planktivorous fish. However, nodularin could be detected in all areas of Gulf of Finland, and in all dominating species in different trophic levels. Even though the spatial and temporal variations of nodularin concentrations in biota can be large, some of the concentrations can be potentially harmful to pelagic organisms.

Acknowledgments Laura Helenius, Eveliina Lindén, Satu Viitasalo, and the whole crew onboard R/V Aranda during TROFIA04 cruise are warmly acknowledged for their help in sample collection. Kaarina Sivonen and Mika Vinni are thanked for all their help and discussions during the manuscript preparation. Two anonymous referees gave valuable comments on the manuscript. This study was financed by the Academy of Finland (project numbers 202437 and 205048), Walter and Andrée de Nottbeck Foundation, and Maj and Tor Nessling Foundation.

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