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Production and sedimentation of peptide toxins nodularin-R and microcystin-LR in the northern Baltic Sea

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Production of nodularin-R in pelagic Baltic Sea takes place in a scale of up to kilograms per square kilometre.

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ABSTRACT

This seven-year survey was primarily targeted to quantification of production of nodularin-R (NOD-R), a cyclic pentapeptide hepatotoxin, in Baltic Sea cyanobacteria waterblooms. Additionally, NOD-R and microcystin-LR (MC-LR; a cyclic heptapeptide toxin) sedimentation rates and NOD-R sediment storage were estimated. NOD-R production (70–2450 $\mu g\,m^{-3}$; ~1 $kg\,km^{-2}$ per season) and sedimentation rates (particles; 0.03–5.7 $\mu g\,m^{-2}\,d^{-1}$; ~0.3 $kg\,km^{-2}$ per season) were highly variable over space and time. Cell numbers of *Nodularia spumigena* did not correlate with NOD-R quantities. Dissolved NOD-R comprised 57–100% of total NOD-R in the predominantly senescent, low-intensity phytoplankton blooms and seston. Unprecedentedly intensive MC-LR sedimentation (0.56 $\mu g\,m^{-2}\,d^{-1}$) occurred in 2004. Hepatotoxin sedimentation rates highly exceeded those of anthropogenic xenobiotics. NOD-R storage in surficial sediments was 0.4–20 $\mu g\,kg^{-1}$ (~0.1 $kg\,km^{-2}$). Loss of NOD-R within the chain consisting of phytoplankton, seston and soft sediments seemed very effective.

1. Introduction

The Baltic Sea, the second largest brackish water body (415 000 km^2) in the world, exhibits probably the world's largest cyanobacteria blooms, covering areas of the order of 100 000 km^2 (e.g. Hansson, 2006). Cyanobacteria have been a nuisance in the area for millennia (Bianchi et al., 2000) although their historical toxicity is unknown. Seemingly, the frequency of cyanobacteria blooms has been increasing over the past decades (Kahru et al., 1994; Poutanen and Nikkilä, 2001; Suikkanen et al., 2007). Cyanobacterial blooms are supported by eutrophication especially due to phosphate emissions from municipal and waste waters plus diffuse agricultural sources.

Baltic Sea cyanobacteria blooms consist of the non-toxic *Aphanizomenon* and *Anabaena*, and the toxic *Nodularia spumigena*. *N. spumigena* produces non-ribosomically (Moffitt and Neilan, 2001) a hepatotoxic cyclic pentapeptide nodularin-R (NOD-R, Carmichael et al., 1988). Also the hepatotoxic heptapeptide microcystin-LR (MC-LR) has been observed in the pelagic (Karlsson et al., 2005). The high prevalence of these two toxins is one particular chemical characteristic of the Baltic Sea.

There are several studies describing NOD-R occurrence in different Baltic Sea compartments, but no comprehensive estimates on hepatotoxin production or sedimentation. For example, sporadic data describes NOD-R concentrations in northern Baltic Sea phytoplankton (<0.1–18.1 $g\,kg^{-1}$ dw, Kankaanpää et al., 2001; Kononen et al., 1993; Sivonen et al., 1989). Data on volumetric NOD-R production is scarce and based on analyses of either algal particles (filtered cells, in 1990 and 2001–2002, Henriksen, 2005; Kononen et al., 1993; Mazur-Marzec and Pliński, 2003) or water (in situ, Kankaanpää et al., 2001). NOD-R can also reach the benthic ecosystem (e.g. Sipiä et al., 2001) including soft sediments (Kankaanpää et al., 2001; Mazur-Marzec et al., 2007). Data on MC-LR is scarce (Karlsson et al., 2005).

The aims of the present study were quantitation of the volumetric production of NOD-R, and sedimentation and sediment storage (stratification) of NOD-R and MC-LR. Additionally, water-column high-resolution stratification of NOD-R, partitioning of NOD-R between the particulate and aqueous phases in the field samples plus stability of NOD-R in soft sediment were examined.

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2. Material and methods

2.1. Chemicals

HPLC-grade solvents (Rathburn; Walkerburn, Scotland; J.T. Baker, Deventer, Holland; E. Merck, Darmstadt, Germany) were used. Deionised water was from Millipore mQ plus system (Millipore S.A., Molsheim, France). All chemicals were of analytical grade. Diammoniumhydrogen phosphate was from Riedel-de Haën (Seelze, Germany). NOD-R was obtained either from Calbiochem (La Jolla, CA, USA) or as a gift from Prof. Wayne W. Carmichael (Wright State University, OH, USA). MC-LR was isolated and purified by Åbo Akademi University (Turku, Finland). All other chemicals were from Sigma–Aldrich Corporation (St. Louis, MO, USA).

2.2. Sampling and subsampling

Sampling was performed onboard the R/V Aranda (Fig. 1). One to ten water samples containing cyanobacteria [volumetric samples from 16 locations (Table 1); 24 samples] were collected at either 1 or 5 m, or from both depths, using a 30-l plastic sampler. First, aliquots for (in situ) analyses of dissolved hepatotoxins of these seawater samples were collected and frozen (−20 °C) immediately. Then the water samples collected at each station were pooled and concentrated (50 μm net) into 50–100 ml. Two aliquots from these concentrates were collected. To determine biomass weight, the first aliquots of the concentrates were filtered using (pre-weighed) 0.45 μm filters and stored frozen prior to lyophilisation. Phytoplankton for chemical analyses was separated from the second aliquots of the concentrates by gentle centrifuging (3000×*g*, 10 min) and frozen.

Furthermore, phytoplankton samples for non-volumetric determination of NOD-R in particles were collected using 50 μm net hauls (0–2 m; 18 locations; 31 net samples; Table 1). These samples were concentrated into approximately 50 ml, centrifuged (3000×*g*, 10 min) and frozen. Four parallel net samples for small-time-frame variability were collected from both LL15 (1–2 day intervals) and CYA06 (four consecutive 30-l samples). Five parallel samples, each consisting of 5–10 *N. spumigena* filaments, were collected by picking under a microscope (LL12, July 2003).

Hepatotoxin stratification was examined using an FIMR-designed device (organ), a 3-m-long rack equipped with 100-ml plastic syringes 0.2 m apart from each other (one sample per depth; 0–3 m section in Aug 2006 and 0–6 m section in 2007; Table 1). The samples were frozen upon retrieval. Seston (containing particles and aqueous phase) was collected at JML (deployments at 25, 20 and 61 m in 2000, 2002 and 2003, respectively) and LL3a (at 44 m depth in 2004) using a sediment trap (Technicap PPS 5/2, La Turbie, France; collection area 1.0 m^2), equipped with 250-ml polypropylene collection bottles (3% HCHO; 10 g l^{-1} NaCl; 3–6 day intervals). After collection, the bottles were frozen. Rates of sedimentation for the duration of collection were calculated as described earlier (Kankaanpää et al., 1997).

Soft sediments (Table 1) were collected using a Gemax gravity corer (ø 9 cm; http://www.kolumbus.fi/boris.winterhalter/GEMAX.pdf), sectioned into 1-cm subsamples and stored at −20 °C (dark). Pore water was collected as described by Tuominen et al. (1999). Briefly, a whole-core squeezer (FIMR design) with a hydraulic jack (2 ton) provided gentle pressure. Five to ten-millilitre aliquots were collected (resolution 1.7 mm) into plastic tubes and frozen. Benthic animals (*Macoma balthica*, *Monoporeia affinis*, *Saduria entomon* and unclassified worms; Table 1) were collected with a Van Veen sampler, washed with seawater and frozen.

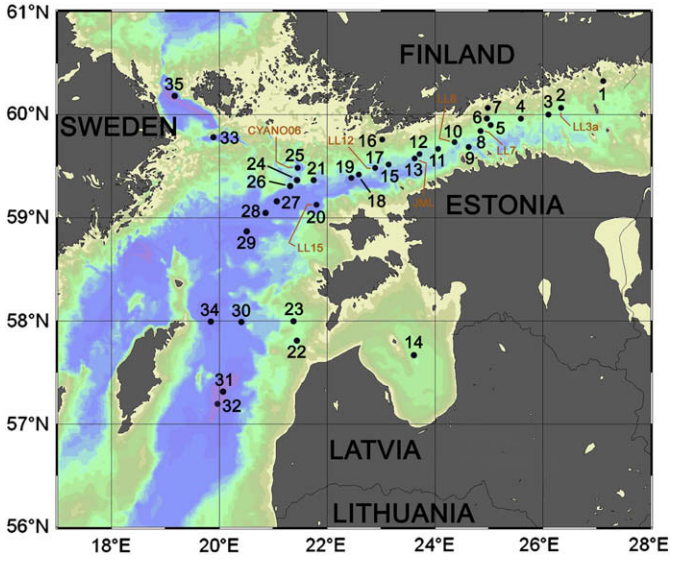

Fig. 1. Sampling locations during 2000–2007. See Table 1 for station list.

2.3. Cell counts

Cell counts were performed from 2001 phytoplankton (stations XV1, LL5, LL7, LL9, LL11, LL12 and BY15), 2002 seston samples (10 samples), and 30-l samples of 2006 (stations LL3a, JML, LL7, CB1 and X17) and 2007 (stations LL3a, LL4a, LL6a, 39a, LL7, GF1 and LL12), preserved using acidic Lugol solution. Cell counts (*N. spumigena*, *Anabaena* spp., *Aphanizomenon flos-aquae* and *Dinophysis* spp.) were performed by using an inverted microscope (Utermöhl, 1958). *N. spumigena* were counted as 100 μm units.

2.4. NOD-R degradation in sediment

In order to test NOD-R decay in sediments (mimicking natural decay and elucidating effect of storage), wet surficial sediment (LL7; hypoxic clayey mud; May 2003) was mixed (IKA T25 basic Ultra-Turrax; IKA-WERKE, Staufen, Germany) with a dried bloom sample (LL12, 0.8 g NOD-R kg^{-1} dw; Aug 2003; bloom material from LL7 not available). The theoretical NOD-R concentration in the spiked sediment (based on sediment weight and NOD-R concentration in phytoplankton) was 19.7 mg NOD-R kg^{-1} wet weight (ww). Aliquots of this sediment were stored in Falcon tubes at −78, −20, 4 and 23 °C. At 0, 2, 7, 14, 30, 60 and 120 days (each) four replicate samples per temperature were collected.

2.5. Sample pretreatment and hepatotoxin extraction

Anabaena lemmermannii filaments (170 ± 30; MC-LR analysis) from an LL3a seston sample (21–24 July 2004) were isolated by picking under the microscope and washed with filtered seawater. The aliquots of the aqueous phases from the 30-l sampler in 2006 and sediment trap (LL3a) were filtered (0.45 μm) and concentrated using Waters Sep-Pak cartridges (Milford, CA, USA; adsorbent weight 200 mg). Seawater samples obtained from organ sampling in 2006 (only) and 30-l sampling of 2007 were only filtered prior to analysis.

With the organ samples, particulate phase was obtained by evaporating 25 ml of the original sample (2006) or by filtration onto 0.45 μm filters, and weighing (2007). For the phytoplankton and seston from 2000–2002, pretreatment and hepatotoxin extraction were performed as previously described by Kankaanpää et al. (2001). Briefly, lyophilised and homogenised particulate materials (typically 0.05–0.2 g) were extracted with 20 ml of 75% MeOH in a 90-W, 47-kHz ultrasonic bath (Branson 3200, Danbury, CT, USA) in ambient temperature for 8 h. Extracts were further concentrated into 0.5–1 ml using gentle heating (<60 °C) and nitrogen flow.

To examine yield of NOD-R from phytoplankton and repeatability of NOD-R extraction, 30 parallel, homogenised subsamples (each 50 mg; station LL12, July 2003) were analysed. One set of 10 subsamples was first digested with chymotrypsin (1.0 mg ml^{-1}; 0.2 M TRIS, pH 7.6) at 55 °C (3 h) and incubated at ambient temperature for 19 h. Each of the next 10 subsamples were extracted with 3 ml of 75% MeOH using a 23-kHz probe sonicator (Sanyo Soniprep 150, Loughborough, UK) at 30 W power (10 μm amplitude) in ambient temperature for 5 min. The last 10 repeatability samples, plus phytoplankton and seston from 2003 onwards were extracted using three 15-min extraction cycles (each cycle using 3–5 ml of 75% MeOH; bath sonication). The particulate material from the organ sampler was treated similarly, except that 3 × 2 ml volumes were applied. All extracts were concentrated to 0.5–1 ml.

Spiked sediments (0.2 g) and filaments of *N. spumigena* and *Anabaena* were extracted with 3 × 2.0 ml of 75% MeOH (3 × 15 min bath ultrasonication, as above). Freeze-dried sediments (1.0 g) and benthic animals (0.01–0.1 g) were extracted with 10 and 5 ml of 75% MeOH, respectively, using 8-h bath ultrasonication. The extracts were concentrated to 0.15–0.5 ml.

2.6. Hepatotoxin analyses

Phytoplankton of 2000 and 2002, particulate material from 30-l sampling of 2007, particulate material from 30-l samples CYA06, RS1 and LF4 (2006), particulate material from organ samples (0.2–3.0 m) of 2007 and SPE concentrates of water samples from the 2004 sediment trap were analysed for NOD-R (not MC-LR) by HPLC/DAD. Either a Waters HPLC (M515 pumps; Milford, CA, USA) or an Agilent HPLC system (series 1100 pumps; Waldbronn, Germany) equipped with Waters Symmetry C18 and Xterra columns (3.9 × 150 mm; Milford, CA, USA) was employed. Mobile phase (isocratic, 1.2–1.5 ml min^{-1}) consisted of 0.01 M $(NH_4)_2HPO_4$ (pH 2.0; 280–300 ml) and MeCN (700–720 ml). Injection volume was 10–50 μl. Calibration was performed with NOD-R solutions (0.1–10 mg l^{-1}).

LC/MS analyses of benthos, pore water and 2004 seston (particles) were performed as described in Karlsson et al. (2005). Briefly, a triple quadrupole mass spectrometer (Quattro Micro, Micromass, Manchester, UK) was used, equipped with a Merck Purospher STAR RP-18e (3 μm, 30 × 4 or 55 × 4 mm; Darmstadt, Germany) column. Ultrapure water with 0.5% HCOOH was used as eluent (1 ml min^{-1}). NOD-R, demethyl NOD-R (dm-NOD-R) and MC-LR were monitored (*m*/*z* 825.5, 811.5 and 995.5; SIM) and further confirmed with the characteristic fragments of the (2*S*,3*S*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4(*E*),6(*E*)-dienoic acid moiety (Adda; *m*/*z* 135.2; MRM). Calibration was performed with NOD-R and MC-LR solutions (each 0.1–10 mg l^{-1}).