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Stephan Pflugmacher, Miikka Olin, Harri Kankaanpää. Nodularin induces oxidative stress in the Baltic Sea brown alga (Phaeophyceae). Marine Environmental Research, 2007, 64 (2), pp.149. 10.1016/j.marenvres.2006.12.011 . hal-00501900

# HAL Id: hal-00501900 https://hal.science/hal-00501900

Submitted on 13 Jul 2010

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## Accepted Manuscript

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PII:	S0141-1136(07)00007-4
DOI:	10.1016/j.marenvres.2006.12.01
Reference:	MERE 3092
To appear in:	Marine Environmental Research
Received Date:	18 May 2006
Revised Date:	19 December 2006
Accepted Date:	20 December 2006



Please cite this article as: Pflugmacher, S., Olin, M., Kankaanpä ä, H., Nodularin induces oxidative stress in the Baltic Sea brown alga *Fucus vesiculosus* (Phaeophyceae), *Marine Environmental Research* (2007), doi: 10.1016/j.marenvres.2006.12.011

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## Nodularin induces oxidative stress in the Baltic Sea brown alga

Fucus vesiculosus (Phaeophyceae)

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#### Abstract

In the Baltic Sea regular, intensive cyanobacterial blooms rich in the cyanobacterium *Nodularia spumigena* occur during the summer season. *Nodularia spumigena* is known to produce the cyclic pentapeptide nodularin (NOD) in high concentrations. Marine macroalgae, together with sea-grass meadows, are an extremely important habitat for life in the sea. In addition to this, the decaying macroalgae substantially contribute to the substrate for the microbial loop in coastal food webs. Uptake of nodularin into the brown macroalga *Fucus vesiculosus* was assessed using an ELISA technique resulting in an uptake of up to 45.1  $\mu$ g kg<sup>-1</sup> fresh weight (fw). Nodularin was also detected in the reproductive part of the algae (receptacle) at 14.1  $\mu$ g kg<sup>-1</sup> fw. The induction of oxidative stress in *F. vesiculosus*, after exposure to NOD, was also shown by monitoring cellular damage as changes in lipid peroxidation and the activation of antioxidative defence systems (antioxidative capacity, superoxide dismutase and soluble glutathione S-transferase).

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#### Introduction

During the past one hundred years the phosphorous load into the Baltic Sea has increased by about eight-fold and the nitrogen load about four-fold (Larsson et al., 1985). As a consequence of this, the pelagic primary production has increased by an estimated 50% (Elmgren, 1989) and sedimentation of organic carbon five to ten-fold (Jonsson & Carman 1994). In the northern Baltic Sea regular, intensive cyanobacterial blooms rich in the cyanobacterium *Nodularia spumigena* occur during the summer season (Sivonen et al., 1989). *Nodularia spumigena* is known to produce the cyclic pentapeptide hepatotoxin nodularin (NOD) in high concentrations (Sivonen et al., 1989). The most common NOD is NOD-R, or cyclo(-D-*erythro*- $\beta$ -methylAsp(iso)-L-Arg-Adda-D-Glu(iso)-2-(methylamino)-2-(Z)-dehydrobutyric acid), where Adda stands for 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4(E),6(E)-dienoic acid (Carmichael et al., 1988). The concentration of NOD in the Baltic Sea has been shown to range from 0.5 µg L<sup>-1</sup> to 25.8 µg L<sup>-1</sup> with a maximum cellbound concentration of 18.1 mg kg<sup>-1</sup> (Kononen et al., 1993; Kankaanpää et al., 2001; Mazur and Plinski, 2003).

Several cases of animal poisonings in the southern Baltic Sea have been reported (Edler et al., 1985; Nehring 1993). Changes in liver histopathology have also been observed in trout exposed *in vivo* with *Nodularia* cells containing NOD (Kankaanpää et al. 2002). Other recent studies have indicated that NOD can accumulate into blue mussels (*Mytilus edulis;* Sipiä *et al.*, 2001a) and some Baltic fish species, e.g. flounder (*Platichthys flesus;* Kankaanpää et al. 2002), Atlantic cod (*Gadus morhua*; Sipiä et al., 2001b) and the three-spined stickleback (*Gasterosteus aculeatus*; Kankaanpää et al., 2001). One feature of rocky seashores in the Baltic Sea is the growth of macroalgae belonging to three different phyla: Rhodophyta (red algae), Phaeophyta (brown algae) and Chlorophyta (green algae). These algae, together with sea-grass meadows, are extremely important for life in the sea. Macroalgae are not to a great extent utilised by primary consumers as a food source, but for numerous smaller animals,

such as isopods (especially *Idotea baltica*), benthic isopods (e.g. *Saduria entomon*), blue mussels, crustaceans, scallops, starfish and lobsters they do provide shelter. Several of the economically most important fish species, herring or pollock, are entirely dependent on the macroalgae zones in their early life stages. Macroalgae also increase the available substrate for benthic microalgae, which in turn are the main food for small herbivores. In addition to this, the decaying macroalgae are substantially contributing to the substrate for the microbial loop in coastal food webs (Mann 1982, Lüning 1990). It has been shown that cyanobacterial toxins can promote oxidative stress in freshwater macrophytes (Pflugmacher, 2004). To prevent cellular damage caused by reactive oxygen species (ROS), cells have an elaborate system involving antioxidative enzymes which include superoxide dismutase (SOD), glutathione peroxidase (GPx), ascorbate peroxidase (APx), catalase (CAT) and glutathione reductase (GR). Superoxide Dismutase (SOD) catalyzes the dismutation (reduction) of superoxide anions into oxygen and hydrogen peroxide. Hydrogen peroxide is reduced by CAT, GPx or APx to water, the latter using ascorbate as an electron donor. Glutathione reductase is part of an oxido-reduction cycle involving glutathione and ascorbate (Foyer & Halliwell, 1976; Smith et al., 1990). Glutathione reductase catalyses the reduction of glutathione disulfide (GSSG) in an NADPH-dependent reaction and therefore plays a pivotal role in protection against oxidative damage and the adjustment processes of metabolic pathways.

Marine macroalgae, many of which play an important role in the marine coastal ecosystem, have so far been overlooked in relation to cyanobacterial toxins. The aim of this study was to examine any effects of a NOD-containing crude cell extract of *N. spumigena* on the most abundant Baltic Sea brown algae, the bladder wrack *Fucus vesiculosus* and in particular the promotion of oxidative stress and the distribution of NOD throughout the plant. Reactive oxygen metabolism plays a major role in seaweed with regard to stress tolerance (Collen & Davison, 1997, 1999a, b) and stress factors such as cyanobacterial toxins may be able to

promote oxidative stress which in turn may have a long-term ecological significance on the ecosystem of the Baltic Sea as a whole.

#### **Material and Methods**

#### **Algal material**

*Fucus vesiculosus* was collected from a rocky bottom (5-10 m depth) by SCUBA south of the Helsinki University Zoological station in Tvärminne (Hanko Peninsula, SW Finland) in May 2005. The algae, attached to its original rock material, were cultivated and acclimatised in circulating natural Baltic Sea water (pumped from the vicinity of the station through filters) under laboratory conditions (12h/12h diurnal cycle,  $+10^{\circ}$ C, salinity of 6, ambient dim light) for two months prior to the experiments. Prior to the experiments the algae were detached from the rock material by carefully cutting the holdfast from the rock.

#### Preparation of a nodularin-containing crude cell extract

In order to mimic the natural dissolution of the chemical constituents of *Nodularia spumigena* into Baltic Sea water, a crude cell extract was prepared. One gram of freeze-dried *N. spumigena*, cells collected in July 2003, was extracted with 10.0 ml of filtered Baltic Sea water (salinity 6). The extraction was performed over 15 minutes using an ultrasonic bath (Branson 3200). After ultrasonication, the mixture was centrifuged at 10000 x g for 10 min, and the supernatant collected. The extract obtained contained 150 mg NOD L<sup>-1</sup> and was stored at + 4 °C until required.

#### Exposure of *F. vesiculosus* to the crude extract

*Fucus vesiculosus,* maintained in Baltic Sea water were exposed to a nodularin-containing crude cell extract by producing a solution (diluted with seawater of salinity 6) containing 1  $\mu$ g L<sup>-1</sup> NOD in a 15 L tank. Control plants were kept in a 40 L tank. During the 48-h

experimental exposure water circulation was stopped (static exposure). Triplicate algal samples were taken (whole thallus) for analyses of oxidative stress (lipid peroxidation, ROS) and GST induction from both the exposed and control tank at 0, 0.5, 1, 4, 6, 12, 24 and 48 hours from the start of exposure. The concentration of NOD in the exposure medium was measured by ELISA (EP 022; EnviroLogix Inc, Portland, USA) according to the manufacturers' instructions, prior to exposure and at the end of the exposure period to control possible toxin degradation. Material for distribution analysis of total extractable NOD in the holdfast, stripes, blades and the gametangial part (receptacle) were collected at 48 h only.

The sectioned algal material collected at the end of the experiment was first gently washed with 75% (v/v) methanol to remove any extracellular NOD from the surface of the plant. The sections were wrapped in aluminium foil, snap frozen in liquid nitrogen, placed on dry ice and stored at -78°C prior to extraction.

#### Extraction of F. vesiculosus for toxin analysis

Exactly 1.0 g of fresh weight (fw) of whole algae was frozen in liquid nitrogen, ground to a fine powder and NOD extracted according to Pflugmacher (2004) using 10 ml of 70% (v/v) methanol for the first extraction. These samples were shaken for 4 h at room temperature, centrifuged at 5000 x g for 10 min and the supernatant removed and stored at 4°C. The algal material (pellet) was further extracted with 100% methanol as previous. The supernatants were combined and evaporated to dryness under a constant flow of nitrogen and gentle heating at 30°C and finally redissolved in 1.0 ml of 70% (v/v) methanol prior to analysis.

### Total extractable hepatotoxin (TEH) analysis using ELISA

The extracts in 70% methanol were further diluted with Milli-Q water to produce dilutions of 1:10, 1:100 and 1:1000. The dilutions were analysed in triplicate using EnviroLogix EP 022 (Portland, Maine, USA) ELISA plates according to the manufacturer's instructions. Results

are based on those dilutions falling within the range of calibration range (0.25 – 2.0  $\mu$ g NOD /L) used.

#### Lipid peroxidation

Exactly 1.0 g of algae was suspended in 2 mL 0.1 M phosphate buffer (pH 7.4), centrifuged at 13.500 x g for 5 min and the pellet was homogenised 1 mL 20 mM phosphate buffer (pH 7.4). 10 µL of butylated hydroxytoluene (BHT) (Merck, Darmstadt, Germany) was added to prevent sample oxidation. The precipitate was removed by centrifugation 3000 x g at 4 °C for 10 min. The supernatant was used for the lipid peroxidation assay and for protein determination by Bradford (1976) method. Lipid peroxidation (LPO) in F. vesiculosus was measured according to Botsoglou et al. (1994) as amount of malondialdehyde (MDA) and 4hydroxynonenal (4-HNE) (Merck, Darmstadt, Germany), which are end products of unstable and decomposing lipid peroxides. The reaction of the chromogenic reagent N-methyl-2phenylindole with MDA gives a stable chromophore with maximum absorbance at 586 nm. A calibration curve was made using MDA. For the analysis of MDA, 200 µL of the extract was used and 600 µL of N-5 methyl-2-phenylindole in acetonitrile was added and gently mixed. A volume of 150 µL of 12 M HCl was added, mixed again and incubated at 45 °C for 1h. Samples were centrifuged at 15.000 x g for 10 min and the clear supernatant was transferred into a plastic cuvette for measurement. For the analyses of MDA and 4-HNE 200 µL of the sample was mixed with 150 µL of methane sulfonic acid (MSA) and incubated at 45 °C for 1h. Samples were cooled down and centrifuged at 15.000 x g for 10 min and the clear supernatant was transferred into a plastic cuvette for measurement at 586 nm.

#### Total antioxidant capacity

This assay was performed according to the method of Miller et al. (1993) and relies on the ability of antioxidants in the sample to inhibit the oxidation of 2,2'-azino-di-[3-ethylbenz-thiazoline sulphonate] (ABTS) by metmyoglobin. The amount of oxidized ABTS is monitored at 600 nm with the absorbance of ABTS at 600 nm proportional to the concentration of antioxidants in the sample. The assay is linear for antioxidant levels less than 2.5 mM.

#### Algal preparation for enzyme assays

An algal extract was prepared according to Pflugmacher et al. (1999) with minor modifications, listed below. A total of 5 g wet weight of algae was used in three replicates for enzyme isolation. The algae were ground in liquid nitrogen to a fine powder, transferred into 0.1 M sodium phosphate buffer (pH 6.5) containing 14 mM -dithioerythritol (SIGMA) and 5 mM EDTA (SIGMA) and homogenized on ice using a glass homogenizer on ice. The resulting slurry was shaken for 30 min and centrifuged at 5 000 x g for 5 min. Soluble proteins in the supernatant were concentrated by ammonium sulphate precipitation using 35-80% saturation and centrifugation at 25 000 x g for 30 min. The pellet (soluble fraction) was resuspended in 20 mM sodium phosphate buffer (pH 7.0) and desalted by gel filtration on NAP-10 columns (Amersham Pharmacia, Sweden). Protein determination was performed using the method of Bradford (1976).

#### Measurement of SOD, CAT and GST activities

Superoxide dismutase (SOD) (E.C. 1.15.1.1) activity was measured in the algal preparation according to Sun et al. (1988) using the xanthine/xanthine oxidase assay. Activity of catalase (CAT) was measured according to Aebi et al (1984). Activity of soluble (cytosolic)

glutathione S-transferases (sGST) (E.C. 2.5.1.18) was determined using the standard model substrate 1-chloro-2,4-dinitrobenzene (CDNB) according to Habig et al. (1974) and 4-hydroxynonenal according to Alin et al. (1985). Enzyme activities are given as nkat mg<sup>-1</sup> protein (a katal is equivalent to the conversion rate of one mole of substrate per second).

### Statistical analysis

One-way analysis of variances (ANOVA) and the Tukey's test at p = 0.05 were used to test differences between exposures and control.

### Results

After being exposed for 48 h to water containing an extract of *N. spumigena*, at 1 µg  $1^{-1}$  NOD, a total toxin uptake of 131 µg kg<sup>-1</sup> fw was detected in the thallus of *F. vesiculosus*. The highest uptake was measured in the holdfast with 45.1 ± 4.2 µg kg<sup>-1</sup> (fresh weight) fw, followed by the lower part of the stipe with 39.5 ± 2.9 µg kg<sup>-1</sup> fw. The stipe within the blades exhibited a NOD concentration of 20.8 ± 4.5 µg kg<sup>-1</sup> fw and the blades itself 13.9 ± 1.1 µg kg<sup>-1</sup> fw. Nodularin was also detected in the gametangial, which is the reproductive part of these algae, at a concentration of 14.1 ± 3.0 µg kg<sup>-1</sup> fw.

One of the possible indicators of oxidative stress is lipid peroxidation (LPO) (Fig. 1), which was measured using the two metabolites malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE). 4-Hydroxynonenal was detectable after 1 h of exposure and MDA after 4 h. The concentration of MDA was always 10-fold lower than the concentration of 4-HNE. The concentration of both metabolites was seen to increase until the end of the experiment at 48 h. The measurement of the total antioxidative status of *F. vesiculosus* revealed that, with time, the elevated concentration induced an 11-fold increase in the exposed algae when compared

to controls. A significant increase of the total antioxidative status was observed after 6 h exposure to NOD (Fig. 2).

Activity of superoxide dismutase (SOD) (Fig. 3) was significantly elevated after 30 min of exposure of the algae to NOD and leveled-off over a further 4 h. After 6 h of exposure, a second elevation occurred with a slight increase until the end of the experiment at 48 h. As a whole, the SOD activity increased 3-fold during the course of the experiment.

Catalase (CAT) activity (Fig. 4) was significantly elevated after 1 h of exposure and continued to increase until 24 h attaining the highest activity of 28  $\mu$ kat mg<sup>-1</sup> protein. Between 24 and 48 h exposure the activity of CAT decreased 2-fold when compared to the 24 h value. Glutathione S-transferase (GST) activity (Fig. 5A), when measured with CDNB, increased almost immediately on exposure with NOD with a constant elevation and a total 4.2-fold increase by 48 h.

Based on the use of 4-HNE (one of the metabolites of lipid peroxidation) as substrate, the GST activity significantly elevated after 4 h of exposure reaching a maximum activity of 140 nkat mg<sup>-1</sup> protein after 12 h (Fig. 5B). Apparently, the activity diminished towards the end of the experiment at 48 h, although the decrease was statistically not significant.

#### Discussion

The use of a crude cell free extract made from a natural phytoplankton bloom sample was selected for its resemblance to naturally decaying bloom material. The concentration of NOD used in this study was environmentally relevant ( $1.0 \ \mu g \ l^{-1}$ ), when compared to concentrations detected in the pelagic Baltic Sea during cyanobacterial blooms (Kankaanpää et al., 2001). However, the cyanobacteria in the material will contain a complex mixture of several bloactive peptides which includes NOD (Sivonen et al., 1989). Thus, any responses observed in *F. vesiculosus* can not solely be attributed to NOD.

Based on the exposure of *F. vesiculosus* to a NOD-containing cell free extract from *N. spumigena* bloom material, the uptake of NOD was evident. Nodularin concentration was determined using an ELISA, which, strictly speaking, is providing the total concentration of molecules able to bind to the antibodies of the ELISA plate (i.e. other hepatotoxins detected by the assay). It is however assumed that most of the total extractable hepatotoxins detected will consist of NOD as it is the dominant hepatotoxin found in the Baltic Sea.

Detectable concentrations of NOD were observed in different parts of *F. vesiculosus*, especially in the stem and basal parts. The total uptake of toxin by *F. vesiculosus* was 131  $\mu$ g kg<sup>-1</sup> fw, which is in between the concentrations observed in other marine organisms such as herring (2.5-6.5  $\mu$ g kg<sup>-1</sup> dw), stickleback (35-170  $\mu$ g kg<sup>-1</sup> dw) and flounder liver (240-2500  $\mu$ g kg<sup>-1</sup> dw) (Kankaanpää et al., 2001; 2005, Karlsson et al., 2003).

An ecologically important effect potentially initiated by NOD in marine macroalgae can be the formation of reactive oxygen species (ROS) ie  $O_2$ ,  $O_2$ ,  $H_2O_2$ , and HO<sup>•</sup>. The promotion of oxidative stress by cyanobacterial toxins in the limnic macrophyte *Ceratophyllum demersum* has previously been demonstrated (Pflugmacher 2004). Cellular damage can include the breakdown of membrane integrity through peroxidation of lipids. Lipid peroxidation takes part in two distinct sequential reactions namely the initiation and the propagation of oxidation. In the first reaction, the formation of lipid hydroperoxides occurs, yielding the production of MDA. Secondly, propagation is an iron-catalysed breakdown of these hydroperoxides generating reactive intermediates, rapid formation of MDA and additional lipid hydroperoxides. The oxidation of polyunsaturated fatty acids in the membrane, particularly arachidonic acid and linoleic acid, leads to the formation of the major unsaturated aldehyde, 4-Hydroxynonenal (4-HNE). 4-HNE is a highly reactive compound known to have numerous cellular effects (Esterbauer et al., 1991). The concentration of 4-HNE in the cells is regulated by  $\alpha$  class glutathione S-transferases (GST A4-4 and hGST5.8) which catalyse the formation of glutathione-HNE. This conjugate is further transported by a 76 kDa ral-binding, ATP-

dependent GTPase-activating protein (RLIP76) (Yang et al., 2003). As shown by the present data, GST activity using 4-HNE as a substrate was elevated significantly after 4 h of exposure of *F. vesiculosus* to NOD. This corresponds to the formation of 4-HNE via lipid peroxidation. Using CDNB as a substrate to measure GST activity, a constant elevation was detected which differs from the response detected with 4-HNE (the latter showing a decline of GST activity after 24 h of exposure). Taking into account that NOD, as shown by Beattie et al. (2003), can be conjugated to glutathione via a GST-catalysed reaction, this difference may be explained by the biotransformation activity of the GST system.

The increase in total antioxidative capacity is a further indication of NOD-promoted oxidative stress in *F. vesiculosus*. Significant elevation was observed after 6 h of exposure and resulted in an 11-fold activity increase when compared to control algae.

The activities of enzymes (SOD and CAT) involved in the detoxification of reactive oxygen species were significantly elevated after 4 h of exposure. Other studies have demonstrated that antioxidative enzymes in fucoids are a major factor in these brown algae to determine the resistance against oxidative stress (Collen & Davison, 1999b). The observed activities of these enzymes in the present study are in the range observed in studies describing the development of oxidative stress during seasonal and thermal acclimation of *F. vesiculosus* (Collen & Davison, 2001).

Comparing the reactions of plants and macroalgae to cyanobacterial toxins, the promotion of oxidative stress seems to be a common occurrence and possibly how cyanobacteria may be able to create their own ecological niche. Similar reactions were seen in the freshwater plant *Ceratophyllum demersum* after exposure to purified cyanobacterial toxins and a cell-free cyanobacterial crude extract (Pflugmacher, 2004).

The survival of plants under stress conditions is possible only if several antioxidants cooperate together with antioxidant enzymes. This work shows that exposure of *F. vesiculosus* to a NOD-containing solution induced the formation of reactive oxygen species as well as the

antioxidative defence. We intend to carry out further studies to elaborate the ecological consequences of toxin uptake by marine macroalgae in the Baltic Sea environment.

#### Acknowledgements

We wish to thank the staff of the Tvärminne Zoological Station (University of Helsinki) for excellent assistance and the use of laboratory premises. This project has received funding through DAAD and the Academy of Finland (HK; grant numbers 208778 and 211645), which is gratefully acknowledged. Thanks also to K. A. Beattie for help with the manuscript.

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### Legends

#### Figure 1

Lipid peroxidation activity (pmol mg<sup>-1</sup> protein) measured as the formation of malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) in *Fucus vesiculosus* after exposure to  $1 \mu g l^{-1}$  nodularin in seawater.

### Figure 2

The total antioxidative capacity (TAOC) ( $\mu$ mol mg<sup>-1</sup> protein) in *Fucus vesiculosus* exposed to 1  $\mu$ g<sup>-1</sup> nodularin in seawater and compared to non-exposed control algae.

## Figure 3

The activity of superoxide dismutase (SOD) (nkat  $mg^{-1}$  protein) in *Fucus vesiculosus* exposed to 1  $\mu$ g l<sup>-1</sup> nodularin in seawater and compared to non-exposed control algae.

#### Figure 4

The activity of catalase (CAT) ( $\mu$ kat mg<sup>-1</sup> protein) in *Fucus vesiculosus* exposed to 1  $\mu$ g l<sup>-1</sup> nodularin in seawater and compared to non-exposed control algae.

### Figure 5

The activity of soluble glutathione S-transferase determined using the substrates 1-chloro-2,4dinitrobenzene (CDNB) (A) and 4-hydroxynonenal (4-HNE) (B) in *Fucus vesiculosus* after exposure to  $1 \mu g l^{-1}$  nodularin in seawater and compared to non-exposed control algae.



















