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Research Article

**EFFECT OF TWO DIFFERENT *NODULARIA SPUMIGENA*
EXTRACTS ON *DANIO RERIO* EGG MORTALITY AND
HATCHING TIME**

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Abstract

Every summer the coastal area of the Gulf of Gdansk is affected by intensive blooms of *Nodularia spumigena*. This cyanobacterium produces nodularin (NOD), hepatotoxic pentapeptide containing a unique amino acid residue – Adda. There is strong evidence that nodularin can cause liver damage in mammals and fish and promote liver cancer. Some incidents of dog, duck, cattle and fish death attributed to *N. spumigena* bloom have been reported. In our work, bloom samples of toxic *Nodularia* were collected and extracts were prepared with two solvents of different polarity. In laboratory experiment the effect of the extracts on a small tropical cyprinid *Danio*

rerio, was investigated. The results showed that methanol extract had more pronounced effect on eggs and larvae development. Increased egg mortality, earlier hatching and malformations of larvae were observed. The different activity of the two extracts, containing equal amounts of nodularin, seemed to support the hypothesis that it is not nodularin, but probably other *Nodularia* cell components, that are responsible for the observed changes in *Danio* development.

INTRODUCTION

Many cyanobacterial species are able to produce toxic substances, which have been classified as: hepatotoxins (microcystins, nodularins, cylindrospermopsin), neurotoxins (anatoxin-a, anatoxin-a(s), saxitoxins, neosaxitoxins), dermatotoxins (lyngbyatoxin) and endotoxins, e.g. lipopolisaccharides (LPSs). The latter are characteristic for all gram-negative prokaryotes (Carmichael 1992). Hepatotoxins inhibit activity of the serine-threonine protein phosphatases (PP 1 and PP 2A type), the enzymes which influence the structure and function of cytoskeletal fibers and play an important role in many processes, including cell division, apoptosis and signal transduction, carbohydrate and lipid metabolism, (Runnegar *et al.* 1994, Annila *et al.* 1996). Acute poisoning may lead to liver cell disruption and liver haemorrhage, while long-term exposure to small doses of the toxins promotes tumours of the organ (Ohta *et al.* 1994). There is strong evidence that cyanobacterial hepatotoxins such as microcystins and structurally and functionally similar nodularin have a negative effect on fish and other aquatic animals. In studies carried out by Fladmark *et al.* (1994) it was proved that salmon hepatocytes were more sensitive to algal toxins than rat hepatocytes.

In the Gulf of Gdansk the intensive blooms of cyanobacteria, mainly *Aphanizomenon flos-aquae* and *Nodularia spumigena*, are among the most spectacular consequences of the increased level of nutrients (Pliński *et al.* 1998, Witek and Pliński 1998). The blooms can seriously affect water quality and result in decreased transparency of water, increased pH, dramatic oxygen depletion and increased sulphide concentration. *N. spumigena* is a well known producer of nodularin (NOD), cyclic pentapeptide hepatotoxin containing a unique amino acid Adda. In mice, when administrated intraperitoneally the LD₅₀ of NOD is approximately 50 µg kg⁻¹ b.w. (Carmichael *et al.* 1988). In July 2003, the concentration of nodularin in coastal areas of the Gulf of Gdańsk amounted to over 25 mg dm⁻³ (Mazur and Pliński 2003). The coastal zone is a spawning area and habitat for several fishes. If the bloom is a local event, the fish can try to avoid it. It happens quite often, however, that the bloom occurs in the whole Baltic proper and covers an area of over 60,000 km². Under such circumstances nodularin accumulation in different organisms, including fish is probable. Sipia *et al.* (2001) and Kankaanpää *et al.* (2002) detected nodularin in

flounder (40 - 400 ng g⁻¹ d.w.), cod (50 - 60 ng g⁻¹ d.w.), threespine stickleback (35 - 170 ng g⁻¹ d.w.), herring (0.7-6.5 ng g⁻¹ d.w.) and salmon (1.1 - 4.9 ng g⁻¹ d.w.). Fish eggs and offspring are even more threatened by toxic *Nodularia*, as these organisms cannot escape from the area of the bloom.

In this study the effect of *N. spumigena* on the breeding success of *Danio rerio* was investigated. *D. rerio* is a small (up to 4-5 cm) tropical cyprinid often kept in home tanks. Due to its high fertility, well known life cycle and genetics, simple care and low costs of maintenance, *D. rerio* is one of the most popular model aquatic organisms used for toxicity testing, genetic experiments or embryology (Sprague *et al.* 2001).

MATERIAL AND METHODS

Bloom samples of *N. spumigena* were collected on 17 July 2003 from the Gulf of Gdansk using a plankton net with mesh size 100 µm. The samples were freeze-dried and kept in the freezer until the experiment.

The phytoplankton extracts were prepared with two solvents differing in polarity: methanol and water. Cells were disrupted in one of the solvents by sonication (1 min) and then centrifuged for 15 min at 10,000 rpm. The solvents were removed by rotary evaporation and the residue was re-dissolved in 30 cm³ of MilliQ water. The samples were subjected to solid phase extraction on Sep-Pak Vac C18 cartridges (200 mg, Waters, Massachusetts, USA), which had been previously conditioned with 15 cm³ of methanol and washed with 15 cm³ of water. The fraction containing nodularin was eluted using 10 cm³ of 100% methanol, and then the solvent was removed by rotary evaporation at 35 °C. The residue was re-dissolved in egg medium and used in the experiment. Nodularin concentration in the extracts were analysed with the Waters HPLC system equipped with photodiode-array detector (Waters, Milford, MA, USA). NOD was separated and quantified by isocratic elution performed on Symmetry PR-18 column (5 µm; 150 x 3.9mm, Waters) with acetonitrile: water (33:67) both containing 0.05% TFA. The flow rate was maintained at 1 cm³ min⁻¹ and absorbance at 238 nm was monitored. The HPLC data were analyzed by Millennium 32 computer software (Waters).

The test medium was prepared with salts at concentrations: 120 mg dm⁻³ NaCl, 6 mg dm⁻³ KCl, 5.37 mg dm⁻³ Na₂HPO₄, 9 mg dm⁻³ KH₂PO₄, 21.6 mg dm⁻³ CaCl₂, 18.03 mg dm⁻³ MgSO₄, 52.5 mg dm⁻³ NaHCO₃. Extracts were added to give final nodularin concentrations 0, 5, 50, 500, 5000 and 50000 µg dm⁻³. Fig. 1 shows the flow chart of the experimental procedure.

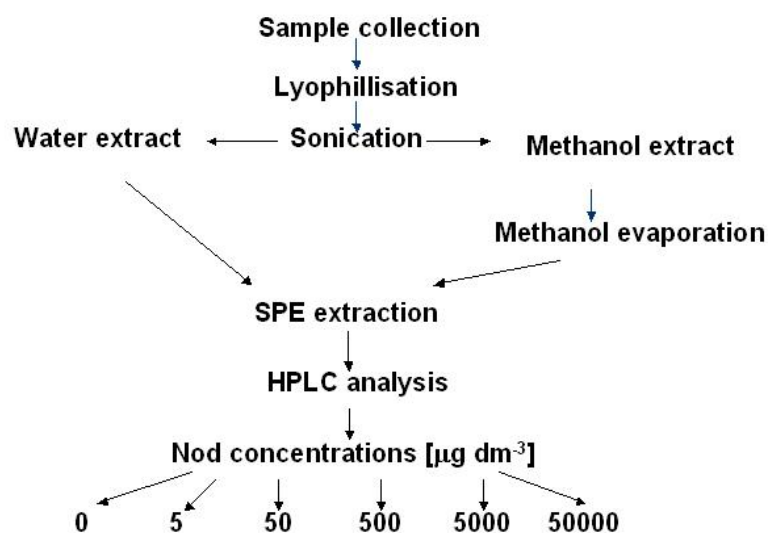


Fig. 1. Flow chart of experimental procedure

Adult fish, 8 females and 6 males, were kept in a 60dm³ tank filled with dechlorinated tap water, which was constantly filtered and aerated. Water temperature was set at 25°C and the photoperiod 13h light/11h dark was established. Fish were fed with frozen glass worms and black mosquito larvae 2 times a day. The tank was cleaned and 1/3 of the tank water was changed every two weeks. Spawning was induced by the onset of light. The eggs were laid on the artificial plant placed on the gridded tray. 1 hour after laying, the eggs were collected from the tank, checked for fertilization, rinsed with water and an appropriate test medium, then placed in a number of 10 per Petri dish in 5 cm³ of medium and incubated for 8 days at 25°C. Every 24 h the eggs were checked for mortality and hatching rate. The experiments were run in triplicate.

RESULTS AND DISCUSSION

There have been some incidents of dog, duck, cattle and fish death attributed to toxic *Nodularia* blooms (Edler *et al.* 1985, Nehring 1993). Bioaccumulation of cyanobacterial toxins has been proved in the tissues of such aquatic organisms as plants (Wiegand & Pflugmacher 2001), zooplankton (Ferrão-Filho *et al.* 2002b), snails (Kotak *et al.* 1996, Ozawa *et al.* 2003), mussels and clams (Yokoyama & Park 2003, Eriksson *et al.* 1989, Prepas *et al.*

1997, Williams *et al.* 1997), as well as in higher animals such as crayfish (Vasconcelos 1995, Lirás *et al.* 1998) and fish (Xie *et al.* 2004, Magalhães *et al.* 2001, Mohamed 2001). Intoxication of aquatic animals can be caused by direct ingestion of cyanobacterial cells (planktivorans), consumption of toxic food *e.g.* zooplankton, small fish, faeces, or uptake of toxin dissolved in water via the dermal surface (worms, snails, crayfish, fish) or drinking (fish) (White *et al.* 2005).

The results obtained in our studies showed that compounds included in the extracts of the *N. spumigena* bloom sample had a significant effect on *D. rerio* egg development. The mortality of eggs exposed to the *Nodularia* extract was observed only in the first 24 hours of incubation, and mainly in the solutions with the highest concentration of NOD. The observed changes were more pronounced in organisms exposed to the medium with organic solvent-extractable *Nodularia* cell components. These effects cannot be attributed to the activity of the solvent (methanol) as it was thoroughly evaporated before exposure. In test solutions where nodularin concentration was 5,000 and 50,000 $\mu\text{g dm}^{-3}$, egg mortality reached 96% and 100%, respectively. In the solution with methanol extract added and at NOD concentration up to 500 $\mu\text{g dm}^{-3}$, egg mortality did not differ significantly from the control (Fig. 2). Water extract turned out to be less toxic and such high egg mortality (100%) was observed only in the samples with nodularin concentration of 50,000 $\mu\text{g dm}^{-3}$. No significant changes in egg mortality were recorded at NOD concentration up to 5,000 $\mu\text{g dm}^{-3}$ (Fig. 2).

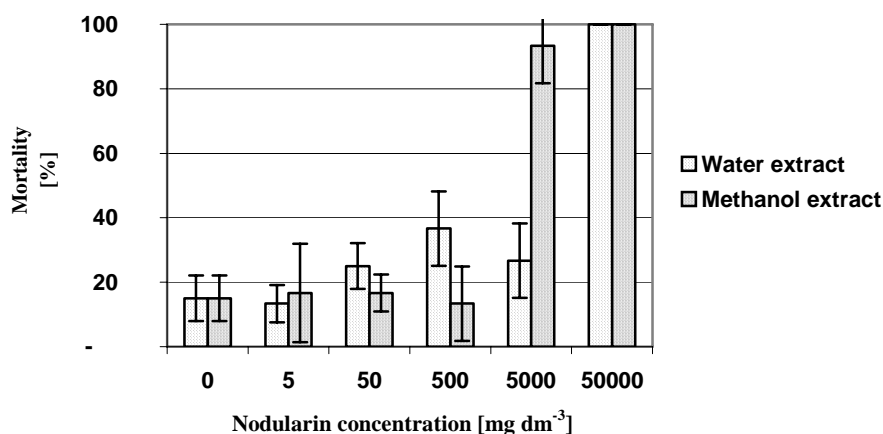


Fig. 2. Egg mortality after exposure to solutions containing methanol or water extract of *Nodularia spumigena*. \pm SE.

Presumably some detoxication mechanism is functioning in *Danio*, which collapses when the concentration of nodularin, or possibly other active compounds present in the extracts, is very high. Biochemical processes triggered by cyanobacterial toxins in *D. rerio* were studied by Wiegand *et al.* (1999). They observed a significant increase in glutathione transferases (GST) and glutathione oxidase activity in *Danio rerio* embryos treated with 500 $\mu\text{g dm}^{-3}$ of microcystin LR. These enzymes are well known elements of the chain of reactions neutralising the effects of toxic substances in many plant and animal organisms. What is more, the role of GST in conjugating microcystin LR and nodularin to glutathione was also proved by Pflugmacher *et al.* (1998) and Kankaanpää *et al.* (2001).

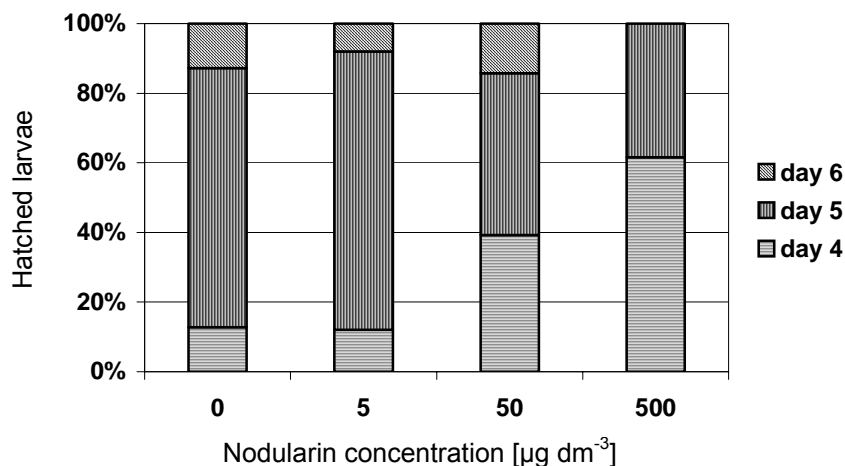


Fig. 3. Hatching time of eggs exposed to solutions containing methanol extract of *Nodularia spumigena*. In the test solution containing 5,000 $\mu\text{g dm}^{-3}$ only one egg was hatched, on day 5.

In our study the exposure of *D. rerio* eggs to *Nodularia* extract caused changes in hatching time. Generally, the eggs treated with methanol extract, at higher concentrations of nodularin, hatched faster. An increase in the number of eggs hatched on day 4 with the increase in NOD concentration up to 500 $\mu\text{g dm}^{-3}$ was recorded. In comparison to the control, there were no differences in hatching time at the lowest concentration of NOD (5 $\mu\text{g dm}^{-3}$) and on day 4 only 13% of eggs were hatched. On that day, 40% of eggs exposed to extract containing 50 $\mu\text{g dm}^{-3}$ of NOD and 62% of eggs exposed to extract containing 500 $\mu\text{g dm}^{-3}$ of NOD were hatched (Fig. 3). In the solution with 5,000 $\mu\text{g dm}^{-3}$ NOD, the only egg which survived hatched on day 5. The opposite tendency

was observed in the medium with water extract. The higher the NOD concentration, the fewer eggs were hatched on day 5: from 61% of eggs at $5 \mu\text{g dm}^{-3}$ NOD to 42% of eggs at $500 \mu\text{g dm}^{-3}$ NOD (Fig. 4). The influence of cyanobacterial toxins on egg hatching time was recorded by Oberemm *et al.* (1998). According to the authors, rainbow trout eggs hatched earlier when treated with microcystin. The hatching time was delayed when the effect of neurotoxic saxitoxin was investigated.

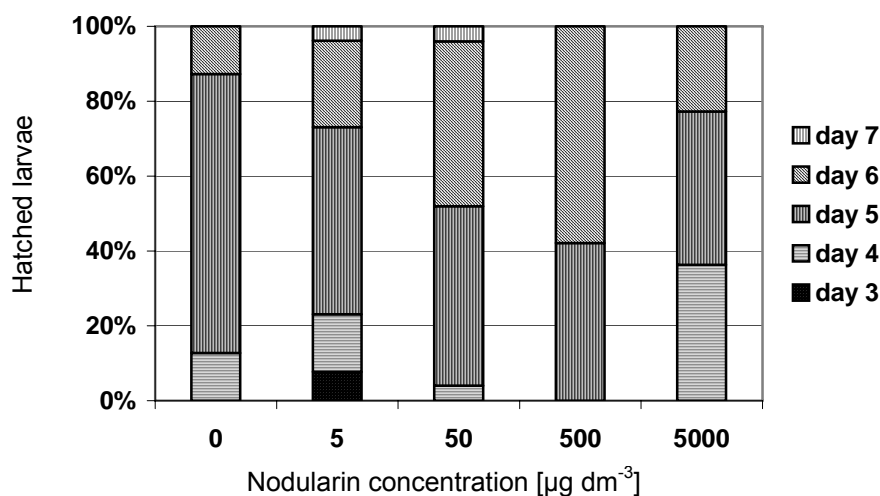


Fig. 4. Hatching time of eggs exposed to solutions containing water extract of *Nodularia spumigena*

Apart from egg mortality and changes in hatching time, the *Nodularia* extract caused some malformations in *D. rerio* larvae (Fig. 5a). These effects were observed only in the organisms exposed to the solution containing methanol extract of *Nodularia* cells. In the test variant where nodularin concentration was $500 \mu\text{g dm}^{-3}$, curved tail and oedema were recorded in 25% of individuals and in the only one that hatched in the $5000 \mu\text{g dm}^{-3}$ variant. Such effects were not observed in the other variants.

Since solvents of different polarity were used to prepare the extracts, they varied in composition and in biological activity (as was proved in the experiments). All polar compounds included in the extract were dissolved in water, while fewer polar ones became components of methanol extract. There are some *Nodularia* cell components, including nodularin, which are of intermediate polarity, and soluble in both solvents. We suppose that the content of both extracts was rather complex, and therefore it is not clear if the observed

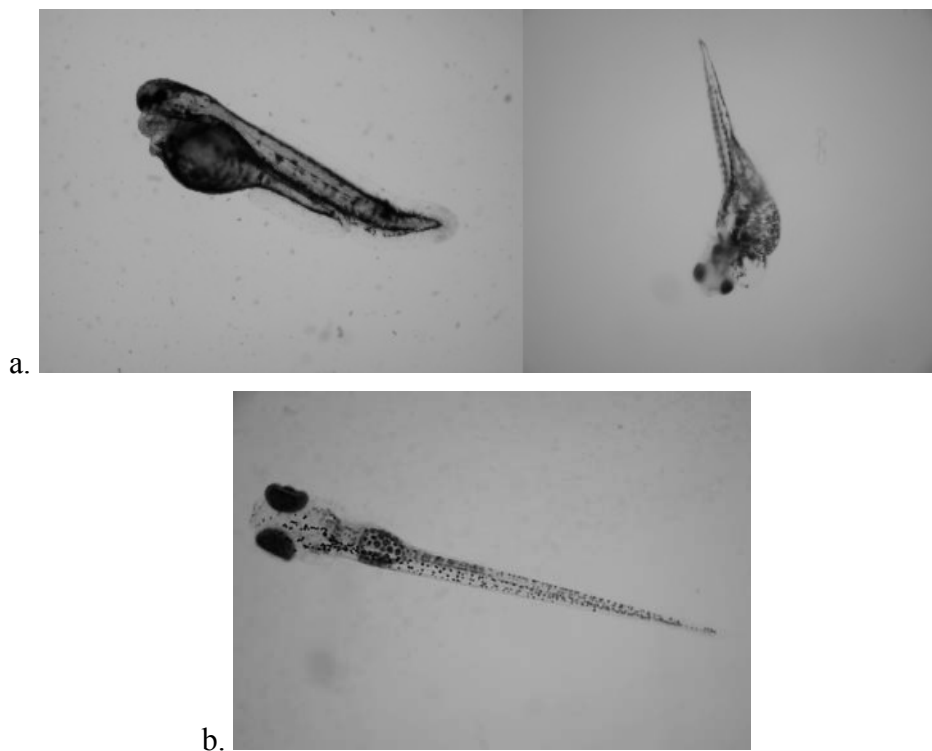


Fig. 5. **a** - Body malformations of larvae hatched in the solution containing methanol extract with $500 \mu\text{g dm}^{-3}$, **b** - well developed larva body.

activities could be attributed to the presence of individual compounds or to synergistic or antagonistic interactions of some *Nodularia* cell components. On the other hand, the more pronounced effects observed in *Danio* eggs and larvae treated with solvent-extractable *Nodularia* cell components rather than with the water-extractable ones can be explained by their chemical nature. The cell membrane, due to its structure, is more permeable to lipophilic *Nodularia* cell components. Polar substances from water *Nodularia* extract require some mechanism of active transport to cross the membrane and get to the cell. Additionally, polar substances are more readily excreted by organisms and in this way the exposure time to their toxic effects is shorter. In this work, nodularin concentration was used as a parameter characterizing the test solutions. However, it is not clear whether the effects observed in *Danio* could be attributed to the presence of the toxin in a test medium. Oberemm *et al.* (1999) showed that crude extract of cyanobacteria cells had much more pronounced effects on fish development than pure toxin alone. They also hypothesised that other substances produced by these microorganisms might be

responsible for the adverse effects on fish. The different activity of methanol and water *Nodularia* extracts used in our experiments, both containing equal concentration of nodularin, seemed to support the hypothesis.

In the natural environment, during bloom senescence and cell lysis, all cyanobacterial cell components can be present in the water. As was proved in our studies, at least some of them have a harmful effect on fish embryos. However, the exact mechanism of action on different stages of fish development has not been fully recognized yet.

REFERENCES

- Annala A., Lehtimäki J., Mattila K., Eriksson J.E., Sivonen K., Rantala T.T., Drakenberg T., 1996 *Solution structure of nodularin an inhibitor of serine/threonine-specific protein phosphatases*. J. Biol. Chem., 271, 16695-16702.
- Carmichael W. W., 1992, *Cyanobacterial secondary metabolites – the cyanotoxins*. J. Appl. Bacteriol., 72, 445-459.
- Carmichael, W. W., 1988, *Toxins of freshwater algae*. [in:] *I: Handbook of natural toxins. Vol. 3. Marine toxins and venoms*. Ed. A.T. Tu. Marcel Dekker Inc., New York.
- Eidler L., Fernö S., Lind M. G., Lundberg R., Nillsson P. O., 1985, *Mortality of dogs associated with bloom of the cyanobacterium Nodularia spumigena in the Baltic Sea*, Ophelia, 24, 103-109.
- Eriksson J. E., Meriluoto J. & Lindholm T., 1989, *Accumulation of a peptide toxin from the cyanobacterium Oscillatoria agardhii in the freshwater mussel Anodonta cygnea*. Hydrobiologia, 183, 211 – 216.
- Ferrão-Filho A., Kozłowsky-Suzuki B. & Azevedo S. M. F. O., 2002b, *Accumulation of microcystins by a tropical zooplankton community*, Aquat. Toxicol. 59, 201 – 208.
- Fladmark K. E., Serres M. H., Larsen N. L., Yasumoto T., Aune T. & Doskeland S. O., 1998, *Sensitive detection of apoptogenic toxins in suspension cultures of rat and salmon hepatocytes*, Toxicon, 36, 1101-1114
- Kankaanpää H. T., Sipilä V. O., Kuparinen J. S., Ott J. L., Carmichael W. W., 2001 *Nodularin analyses and toxicity of a Nodularia spumigena (Nostocales, Cyanobacteria) water-bloom in the western Gulf of Finland, Baltic Sea, in August 1999*, Phycologia, 40 (93), 268-274
- Kotak B. G., Zurawell R., Prepas E. & Holmes C. F., 1996, *Microcystin-LR concentration in aquatic food web compartments from lakes of varying trophic status*, Can. J. Fish Aquat. Sci, 53, 1974 – 1985

- Lirås V., Lindberg M., Nystrom P., Annadotter H., Lawton L. & Graf B., 1998, *Can ingested cyanobacteria be harmful to the signal crayfish (Pacifastacus leniusculus)?*, Freshwat. Biol., 39, 233 – 242
- Magalhães V. F., Soares R. & Azevedo S., 2001, *Microcystin contamination in fish from the Jacarepaguá Lagoon (Rio de Janeiro, Brazil): Ecological implication and human health risk*, Toxicon, 39, 1077 – 1085
- Mazur H., Pliński M., 2003, *Nodularia spumigena blooms and occurrence of hepatotoxin in the Gulf of Gdańsk*, Oceanologia, 45 (2), 305-316
- Mohamed Z. A., 2001, *Accumulation of cyanobacterial hepatotoxins by Daphnia in some Egyptian irrigation channels*, Ecotoxicol. Environ. Safety, 50, 4 – 8.
- Nehring S., 1993, *Mortality of dogs associated with mass development of Nodularia spumigena (Cyanophyceae) in a brakish lake at the German North Sea coast.*, J. Plankton Res., Vol. 15 no 7, 867-872
- Oberemm A., Becker J., Codd G. A., Steinberg C. E. W., 1999 *Effects of cyanobacterial toxins and aqueous crude extracts of cyanobacteria on the development of fish and amphibians*, Environ. Toxicol., 14, 77-88
- Ohta T., Sueoka E., Iida N., Komori A., Suganuma M., Nishiwaki R., Tatematsu M., Kim M., Carmichael W. W., Fujiki H., 1994, *Nodularin, a potent inhibitor of protein phosphatases 1 and 1A, is a new cacarcinogen in male F344 rat liver*, Cancer Res., 54, 6402-6406
- Ozawa K., Yokoyama A., Ishikawa K., Kumagi M., Watanabe M. & Park H.-D., 2003, *Accumulation and depuration of microcystin produced by the cyanobacterium Microcystis in a freshwater snail*, Limnology, 4, 131–138
- Pflugmacher S., Wiegand C., Oberemm A., Beattie K. A., Krause E., Codd G. A., Steinberg C. E. W., 1998, *Identification of an enzymatically-formed glutathione conjugate of the cyanobacterial hepatotoxin microcystin-LR. The first step of detoxication*, Biochim. Biophys. Acta, 1425, 527-533
- Pliński M., Codd G. A., 1997, *Zakwity sinic – zagrożenie dla zdrowia zwiażat.* Medycyna Wet., 53, 8-10
- Pliński M., Musiał A., Ostrowski B., 1998, *Blue-green algae blooms in the Gulf of Gdańsk and surrounding area*, Oceanol. Stud., 1, 55-60
- Prepas E. E., Kotak B. G., Campbell L. M., Evans J. C., Hruday S. E. & Holmes C. F., 1997, *Accumulation and elimination of cyanobacterial hepatotoxins by the freshwater clam, Anodonta grandis simpsonia*, Can. J. Fish Aquat. Sci., 54, 41-46
- Runnegar M. T. C., Jackson A. R. B., Falconer I. R., 1988, *Toxicity of the cyanobacterium Nodularia spumigena Mertens*, Toxicon, 26, 143-151
- Sipia V., Kankaanpää H., Lahti K., Carmichael W. W., Meriluoto J., 2001, *Detection of nodularin in flounders and cod from the Baltic Sea*, Eviron.

- Toxicol., 16, 121-126
- Sivonen K., 1996, *Cyanobacterial toxins and toxin production*, Phycologia, 35 (Suppl), 12-24
- Sprague J., Doerry E., Douglas S., Westerfield M., 2001, *The Zebrafish Information Network (ZFIN): a resource for genetic, genomic and developmental research*, Nucleic Acids Res., 29, 87-90
- Vasconcelos V. M., 1995, *Uptake and depuration of the heptapeptide toxin microcystin-LR in Mytilus galloprovincialis*, Aquat. Toxicol., 32, 227 – 237.
- White S. H., Duivenvoorden L. J., Fabbro L. D., 2005, *A decision-making framework for ecological impacts associated with the accumulation of cyanotoxins (cylindrospermopsin and microcystin)*, Lakes & Reservoirs: Research and Management, 10, 25-37.
- Wiegand C. & Pflugmacher S., 2001, *Uptake of Microcystin-LR in aquatic organisms*. [in:] *Cyanotoxins Occurrence, Causes, Consequences* (ed. I. Chorus) pp.249 – 252. Springer-Verlag, Berlin
- Wiegand C., Pflugmacher S., Oberemm A., Meems N., Beattie K., Steinberg C. E. W., Codd G. A., 1999, *Uptake and effects of microcystin LR on detoxication enzymes of early life stages of the zebrafish (Danio rerio)*, Environ. Toxicol., 14, 89-95
- Williams D. E., Dawe S. C., Kent M. L., Andersen R. J., Craig M. & Holmes C. F., 1997, *Bioaccumulation and clearance of microcystins from salt water mussels, Mytilus edulis, and in vivo evidence for covalently bound microcystins in mussel tissues*, Toxicon, 35, 1617-1625
- Witek B., Pliński M., 1998, *Occurrence of blue-green algae in phytoplankton of the Gulf of Gdańsk in the years 1994-1997*, Oceanol. Stud., 3, 77-82
- Xie L., Xie P., Ozawa K., Honma T., Yokoyama A. & Park H.-D., 2004, *Dynamics of microcystins-LR and -RR in the phytoplanktivorous silver carp in a sub-chronic toxicity experiment*, Environ. Pollut., 127, 431-9
- Yokoyama A. & Park H., 2003, *Depuration kinetics and persistence of the cyanobacterial toxin microcystin-LR in the freshwater bivalves Unio douglasiae*, Environ. Toxicol., 18, 61-7
- Yoshizawa S., Matsushima R., Watanabe M., Harada K., Ichihara A., Carmichael W. W., Fujiki H., 1990, *Inhibition of protein phosphatases by microcystin and nodularin associated with hepatotoxicity*, J. Cancer Res. Clin. Oncol., 116, 609-614