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Research Article

**CYANOBACTERIAL TOXINS IN FRESH AND BRACKISH WATERS
OF POMORSKIE PROVINCE (NORTHERN POLAND)**

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Abstract

Hepatotoxic cyclic peptides and neurotoxic alkaloids are the most common groups of cyanobacterial toxins. They pose a serious threat to human and animal health; each year they are responsible for several cases of animal fatalities (mammals, fish and birds). In Polish water bodies toxic blooms of cyanobacteria are also frequently observed. In this work, a procedure for anatoxin-a analysis has been modified to obtain a sensitive and selective method for routine studies of bloom samples. The presence of cyanobacterial neuro and hepatotoxins in fresh and brackish waters of Pomorskie Province was examined. Hepatotoxins, microcystins or nodularin, were detected in all phytoplankton samples dominated by the cyanobacteria of *Microcystis* and *Anabaena* genera or by *Nodularia spumigena*. HPLC-PDA analysis showed the presence of anatoxin-a only in one sample collected in coastal waters of the Gulf of Gdańsk and dominated by *Anabaena*.

INTRODUCTION

Cyanobacterial blooms occur in eutrophic fresh and brackish waters worldwide and are frequently dominated by toxin-producing species. Most of the species contain gas vesicles that enable them to optimise their vertical

position to water level suitable for their growth. Due to buoyancy, cyanobacteria cells may rise to the surface and at high solar radiation form characteristic scums with increased concentration of toxins. The surface scums, pushed by the leeward wind to the shore, have been responsible for several adverse health effects in humans and animals (Falconer 1996, Codd *et al.* 1999). Some cyanobacterial genera, such as *Microcystis*, *Anabaena*, *Oscillatoria*, *Nostoc* and *Nodularia*, produce hepatotoxic cyclic peptides, microcystins and nodularin, which are the most abundant cyanobacterial toxins. They are secondary metabolites released to water mostly during cyanobacterial cell senescence, death and lysis. In hepatocytes of eukaryotic organisms, they bind to protein phosphatases 1 and 2A and inhibit activity of the enzymes (Falconer and Yeung 1992). The LD₅₀ value for cyanobacterial hepatotoxins ranges from 25 to 150 µg kg⁻¹ body weight (b.w.). Neurotoxins are the other group of cyanobacterial toxins and include: anatoxin-a, anatoxin-a(s), homoanatoxin-a, saxitoxin and neo-saxitoxin. Anatoxin-a, has been found in *Anabaena flos-aqua*, *Anabaena* spp., *Anabaena planktonica*, *Aphanizomenon*, *Oscillatoria*, *Microcystis* and *Cylindrospermum* (Sivonen and Jones 1999). Anatoxin-a was the first cyanobacterial toxin to be chemically defined as the secondary amine, 2-acetyl-9-azabicyclo[4.2.1]non-ene (Devlin *et al.* 1977). The toxin is a potent postsynaptic, depolarizing, neuromuscular blocking agent with i.p. LD₅₀ of about 250 µg kg⁻¹ b.w. (Astrachan *et al.* 1980, Devlin *et al.* 1977). In animals, typical symptoms of poisoning include muscle fasciculation, gasping and convulsion; due to respiratory arrest death occurs within minutes. The number of reports on the occurrence of anatoxin-a is rather limited when compared with microcystins. It may seem to be rather surprising, especially in view of the fact that the toxin can be synthesised by common bloom-forming species of cyanobacteria.

It is known that one strain of cyanobacteria produces more than one toxin. In some cyanobacteria, simultaneous neurotoxin and hepatotoxin production can take place *i.e.* *Anabaena flos-aquae* strain NRC 525-17 produces anatoxin-a(S) and several microcystins (Matsunaga *et al.* 1989, Harada *et al.* 1991). The mass development of toxic cyanobacteria may have a negative impact on water quality for water supply. In Polish water bodies the presence of cyanobacterial toxins has been reported by some authors. Several microcystin analogues have been detected in lakes Sulejów, Jeziorsko and Włocławek (Kabziński *et al.* 2000). These lakes are used as sources of potable water *e.g.* for the city of Łódź, where the concentration of microcystin-LR exceeds the World Health Organization (WHO) guideline value of 1.0 µg l⁻¹. On the other hand, in summer months *Nodularia spumigena* blooms cause serious deterioration of sanitary state of bathing sites on beaches along the Gulf of Gdańsk (Pliński and

Józwiak 1996, Mazur and Pliński 2003). In all *Nodularia* bloom samples which have been collected for the last several years, nodularin, a cyclic pentapeptide hepatotoxins, was found. So far there have been no published and documented reports on anatoxin-a presence in any Polish water bodies.

The aim of this work was to study the presence of anatoxin-a and other cyanobacterial toxins in some Kashubian (northern Poland) lakes and the Gulf of Gdańsk. The analysis of anatoxin-a required a new procedure to be developed. It was mainly because after September 2001 a commercial standard of the neurotoxin has not been available. There are also some difficulties in HPLC analysis of anatoxin-a as the compound creates peak “smearing” and has no specific UV absorbance. All the analytical problems have been overcome in

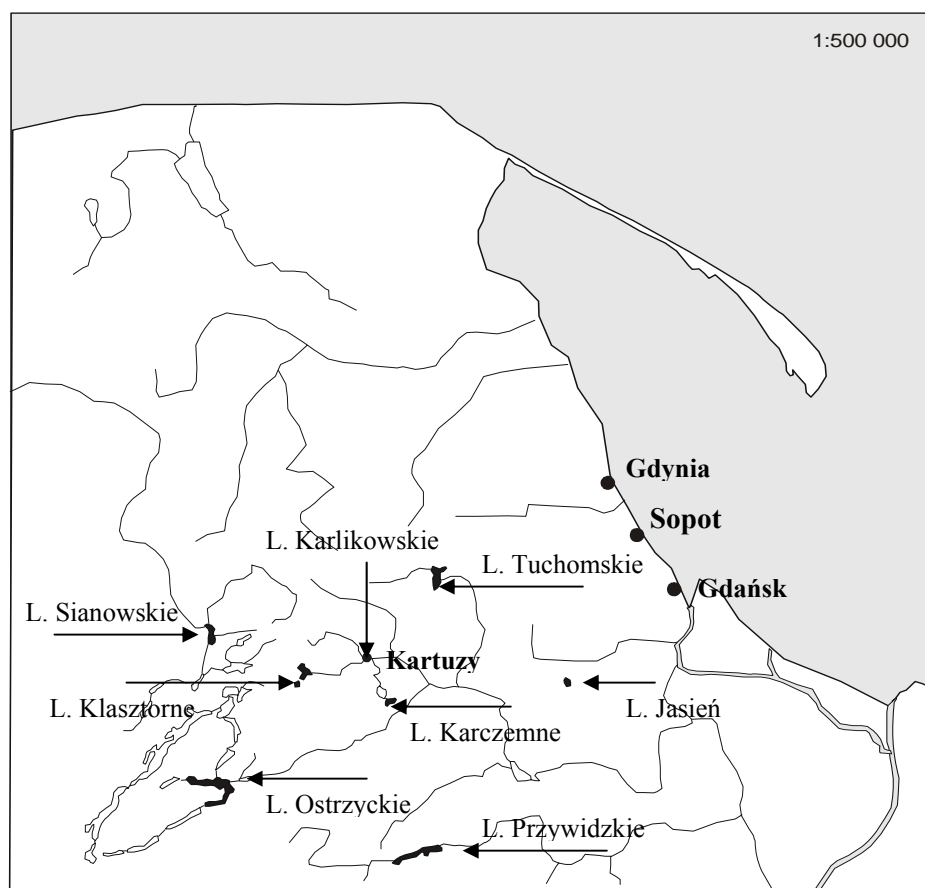


Fig. 1. Places of cyanobacterial bloom samples collection.

this work and a modified method for routine analysis of anatoxin-a in phytoplankton samples has been applied.

MATERIAL AND METHODS

Extraction of anatoxin-a from Anabaena flos-aquae

Anatoxin-a was isolated from *Anabaena flos-aquae* (Lyngbye) de BrEbisson strain NRC 44-1, purchased from the Culture Collection of the Göttingen University. The strain of cyanobacteria was grown in batch cultures in BG-11 medium at 21 °C and photon flux 30 $\mu\text{mol m}^{-2} \text{s}^{-1}$. The cells were harvested after 3 weeks by filtration through a glass microfibre filter (Whatman GF/C). After storage at -20 °C for a certain time, the material was added to MilliQ water from the ultra-pure water system and sonicated for 5 min with HD 2070 Sonopuls ultrasonic homogeniser (BANDELIN, Berlin, Germany). Then the sample was centrifuged with the Eppendorf miniSpin centrifuge (12000 g, 15 min.) and the supernatant the cartridge and was subjected to solid phase extraction (SPE) on 500-mg Sep-Pak tC₁₈ cartridges (Waters, Milford, MA, USA) activated with 10 cm³ of 100% methanol followed by 20 cm³ of MilliQ water. The centrifuged water extract was introduced onto passed at a flow rate of about 5 cm³ min⁻¹. Then the cartridge was washed with 10 cm³ of MilliQ water and anatoxin-a was eluted with 20 cm³ of 50% methanol. The solvent was evaporated to dryness *in vacuo* at 35 °C. The isolated anatoxin-a was analysed by HPLC and HPLC-MS.

Extraction and analyses of toxins from cyanobacterial bloom material

Field samples of cyanobacteria were collected in summer 2002 from blooms in Lakes Jasień, Karczemne, Karlikowskie, Klasztorne, Ostrzyckie, Przywidz, Sianowskie and Tuchom in the Pomorskie Province (northern Poland) and in the coastal waters of the Gulf of Gdańsk (Fig. 1). Concentrated cyanobacteria cells for the analyses of toxins were collected with a phytoplankton net (100 μm mesh size) towed horizontally in the surface layer. The samples were freeze-dried and kept at -20°C prior to toxin extraction and analysis. Live or preserved with Lugol's solution, sub-samples were examined to determine the contribution of different cyanobacteria species to the phytoplankton population (Fig. 2).

About 50 mg of lyophilised cyanobacteria was suspended in 90% methanol and sonicated for 5 min with the HD 2070 Sonopuls ultrasonic homogeniser

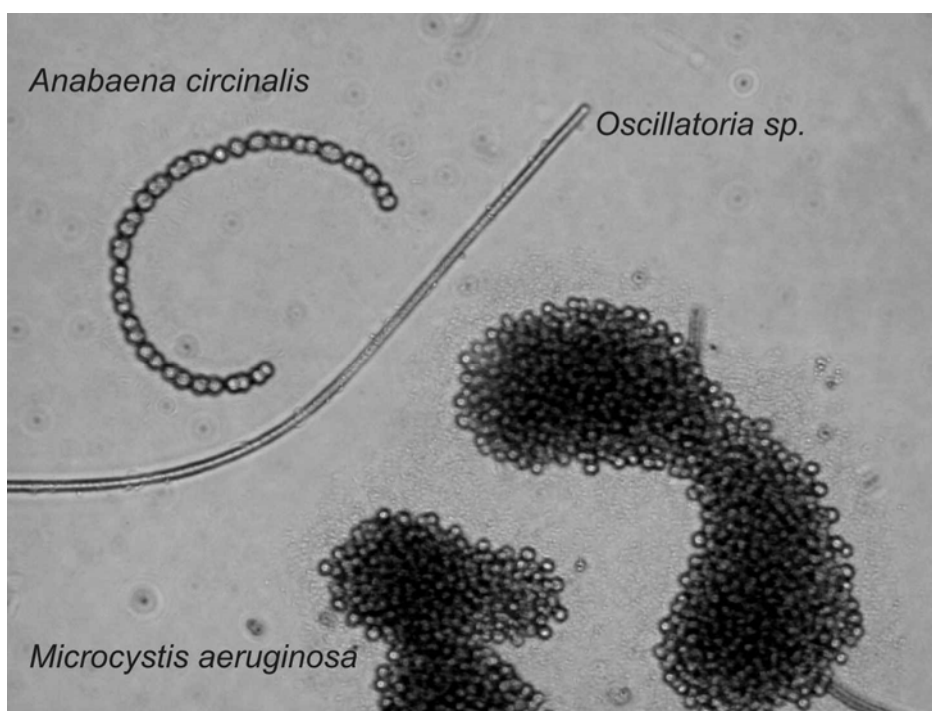


Fig. 2. Image of cyanobacterial bloom sample collected from Lake Karlikowskie.

(BANDELIN, Berlin, Germany). After the addition of 1 cm³ of 90% methanol the sample was vortex for 1 min and left for 2 hours at room temperature. It was clarified by centrifugation at 12000 g for 15 min and the supernatant was analysed by the HPLC system. The system consisted of a model 626 pump with model 600S controller, model 917plus auto-sampler, model 996 photodiode-array detector (PDA) operating in a range of 200-300 nm (hepatotoxins) or 200-550 (anatoxin-a, after a derivatization reaction with NBD-F) and a model 717plus fluorescence detector (FL) with the excitation and emission wavelengths set at 495 and 530 nm, respectively. Separations were performed on a LiChrospher 100 RP-18 column (25 cm x 0.4 cm I.D., 5 µm particle size) and a 100 RP-18e LiChroCart cartridge (Merck, Darmstadt, Germany). The mobile phase was 10% aqueous acetonitrile containing 0.05% TFA : acetonitrile

containing 0.05% TFA (60 : 40). The flow-rate was maintained at $1 \text{ cm}^3 \text{ min}^{-1}$ and the auto-injection volume was $20 \text{ }\mu\text{l}$. Microcystin-RR, Microcystin-LR and Nodularin standards were purchased from Calbiochem Novabiochem (La Jolla, CA, USA). Concentrated toxin solutions ($10 \text{ }\mu\text{g cm}^{-3}$) were prepared in methanol. The derivatisation reagent, 4-fluoro-7-nitrobenzofurazan (NBD-F) was purchased from Fluka All reagents used for analyses were of the HPLC grade. Methanol and acetonitrile were purchased from J.T. Baker (Deventer, The Netherlands). Deionized-distilled water was obtained using the Milli-Q ultra-pure water system (Millipore, Bedford, MA, USA). The microcystins (RR and LR) and nodularin were identified by their retention time and characteristic absorption spectrum with maximum at 238 nm. Anatoxin-a fluorescent derivative was analysed simultaneously with PDA and fluorescence detectors. All HPLC data were collected and processed using the Waters Millennium software. The quantitative analysis of hepatotoxins was carried out using a calibration curve based on peak area measurements for standard solution.

Derivatisation of anatoxin-a with 4-fluoro-7-nitro-2,1,3-benzoxadiazol (NBD-F)

The derivatisation reaction of anatoxin-a was carried out according to the procedure described by James *et al.* (1998) and Namera *et al.* (2002). Sodium borate solution (0.1 M, $300 \text{ }\mu\text{l}$) was added to an *Anabaena flos-aquae* extract containing anatoxin-a and the solution was mixed thoroughly. Then, NBD-F in acetonitrile (1 mg cm^{-3} , $50 \text{ }\mu\text{l}$) was added to the mixture and reacted for 10 min in the dark at room temperature. The reaction was stopped with hydrochloric acid (0.1 M, $50 \text{ }\mu\text{l}$). Before HPLC analyses the sample was diluted with the mobile phase ($300 \text{ }\mu\text{l}$), mixed thoroughly and filtered. The structure of the obtained derivative NBD-anatoxin-a was confirmed by HPLC-MS analysis.

HPLC-MS analysis of anatoxin-a and its fluorescent derivative NBD-anatoxin-a

Analyses of anatoxin-a and its fluorescent derivative were performed on the Agilent 1100 HPLC-MS system consisting of a gradient pump, diode array detector and mass spectrometer detector. The following chromatographic conditions were used: RP-Select B Lichrospher 60 ($250 \times 4 \text{ mm I.D.}$) column, isocratic elution at $1 \text{ cm}^3 \text{ min}^{-1}$ with the mobile phase including 35% A (A = water + 1% formic acid) and 65% B (B = 50% acetonitrile: 50% water + 1% formic acid). The detection of anatoxin-a was at 227 nm and NBD-anatoxin-a at 227 and 350 nm. The APCI ionisation mode was used at 70 eV. Full scan acquisitions were made over a mass range of 200 – 600. Additionally, selective ion monitoring (SIM) was performed at m/z 166 $[\text{M}+\text{H}]^+$ of anatoxin-a and 329 $[\text{M}+\text{H}]^+$ of NBD-anatoxin-a.

RESULTS AND DISCUSSION

Due to the difficulties with obtaining a commercial standard of anatoxin-a, the toxin had to be isolated from *Anabaena flos-aque* strain NRC 44-1, which is a well known producer of a large amount of the toxin, especially in old cultures (Harada *et al.* 1993). The presence of anatoxin-a in the extract and the adequacy of the extraction steps were confirmed by the combined HPLC-MS method. The absorption spectrum of the isolated compound, with maximum at 227 nm, was comparable to anatoxin-a. The base peak in the mass spectrum of the toxin was a protonated molecular ion at m/z 166 $[M+H]^+$ (Fig. 3). Since UV detection is not very effective for the HPLC analysis of anatoxin-a in cyanobacterial samples, we used the derivatisation reaction with NBD-F fluorescent reagent. The obtained derivative, NBD-anatoxin-a could be analysed with both UV and fluorescence detectors, which increases the sensitivity and selectivity of the method. To confirm the structure of the obtained derivative, HPLC-MS analysis was conducted. In the mass spectrum of NBD-anatoxin-a a protonated molecular ion at m/z 329 $[M+H]^+$ and a fragment ion at m/z 166 $[M]^+$ were observed (Fig. 4).

In 2002, phytoplankton samples were collected from 8 lakes (Pomorskie Province) which are used for recreation or fishing. In some of the lakes cyanobacteria often form a thick bloom with surface scums, which may pose a serious threat to water users. Microscopic analyses of collected samples showed the presence of many toxic or potentially toxic cyanobacterial species with the dominance of *Anabaena* and *Microcystis* genera (Tabl. 1, Fig. 2). On the other hand, production of hepatotoxins by cyanobacteria in the examined water bodies was proved by HPLC-PDA analyses. Microcystins were detected in 22 out of 28 samples of natural blooms. Two of the microcystins (McySt) were identified as McySt-LR and McySt-RR. Their concentrations ranged from just traces to $15.5 \mu\text{g g}^{-1}$ in Lake Karlikowskie. The measured level of microcystin concentration seems to be rather moderate when compared to other water bodies, *e.g.* in China, Japan, Australia and Finland, where it occasionally exceeds $1,000 \mu\text{g g}^{-1}$ (Sivonen and Jones 1999). No anatoxin-a was detected by SIM of m/z 166 in all field samples of the present study. It should be pointed out here that the samples were collected only twice in the summer of 2002. As the composition of the bloom forming species changes significantly over the whole summer season we can not exclude the presence of anatoxin-a producing cyanobacteria in the examined water bodies. More frequent sampling is required to draw any definite conclusion on the presence of neurotoxic cyanobacteria in the lakes.

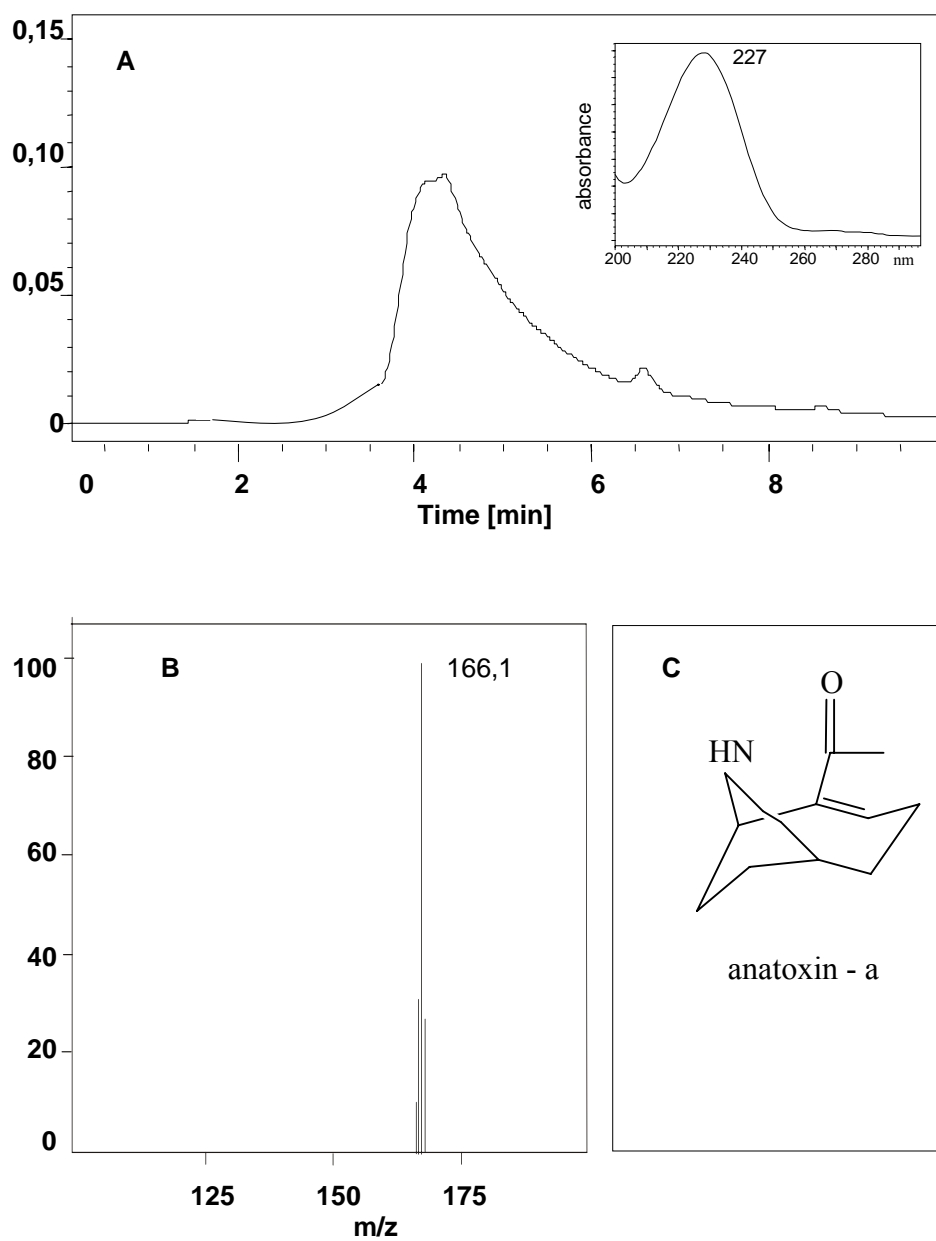


Fig. 3. HPLC-MS of *Anabena flos-aquae* extract. A: total ion current chromatogram and absorption spectrum of anatoxin-a; B: mass spectrum; C: chemical structure of anatoxin-a.

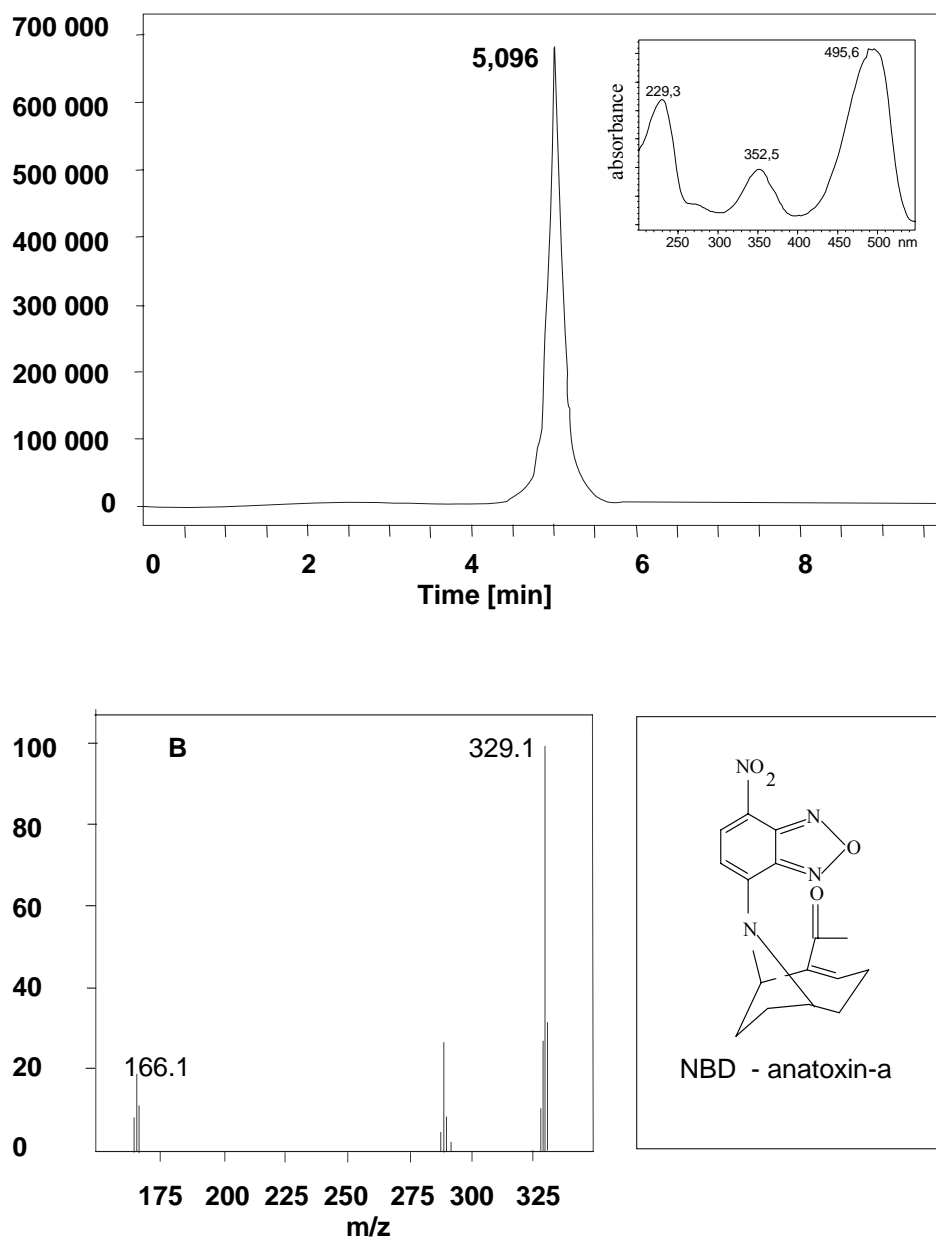


Fig. 4. HPLC-MS of NBD-anatoxin-a. A: total ion current chromatogram and absorption spectrum of NBD-anatoxin-a; B: mass spectrum; C: chemical structure of NBD-anatoxin-a.

Table 1

Dominating cyanobacterial species and toxins identified in the lakes of Pomorskie Province (northern Poland).

Lake	Dominating cyanobacterial species	Toxin identified	Concentration [$\mu\text{g g}^{-1}$]
Jasień	<i>Anabaena spiroides</i> , <i>A. flos-aquae</i> , <i>A. planctonica</i> , <i>A. circinalis</i>	Mcyst-LR Mcyst-RR	1.2 0.2
Karczemne	<i>A. spiroides</i> , <i>Microcystis flos-aquae</i> , <i>Oscillatoria</i> sp., <i>Phormidium</i> sp.	Mcyst-LR Mcyst-RR	0.1 – 1.7 0.9 – 3.1
Karlikowskie	<i>M. aeruginosa</i> , <i>M. flos-aquae</i> , <i>A. circinalis</i> ,	Mcyst-LR Mcyst-RR	2.4 – 15.5 0.3 – 0.7
Klasztorne	<i>M. aeruginosa</i> , <i>A. spiroides</i> , <i>Oscillatoria</i> sp.,	Mcyst-LR Mcyst-RR	Traces 1.3 – 11.9
Ostrzyckie	<i>Gloeotrichia echinulata</i>	n.d.	-
Przywidz	<i>M. aeruginosa</i> , <i>M. flos-aquae</i> , <i>M. wesenbergii</i>	Mcyst-RR	Traces
Sianowskie	<i>A. spiroides</i> , <i>A. circinalis</i> , <i>M. flos-aquae</i> , <i>M. aeruginosa</i> , <i>Aphanizomanon flos-aquae</i>	Mcyst-LR Mcyst-RR	Traces Traces
Tuchomskie	<i>M. aeruginosa</i> , <i>A. spiroides</i> ,	Mcyst-LR Mcyst-RR	0.7 – 8.4 0.4 – 10.2

In coastal waters of the Gulf of Gdańsk *Nodularia spumigena* and *Aphanizomenon flos-aquae* form a bloom throughout the whole summer. Nodularin was detected in all samples where *Nodularia spumigena* was present. In 2002 its concentration ranged from 5 – 919 $\mu\text{g g}^{-1}$. Apart from nodularin, in some samples where *Microcystis* made up about 10% of the phytoplankton population, microcystins were detected as well; two of them were identified as microcystin-LR (1.4 $\mu\text{g g}^{-1}$) and microcystin-RR (0.9 $\mu\text{g g}^{-1}$). The HPLC analyses of samples collected off the coast of Gdynia at the beginning of September revealed the presence of a peak with the retention time and spectrum characteristic of anatoxin-a. In this sample *Anabaena*, a potential producer of anatoxin-a, made up about 20% of the phytoplankton population. Unfortunately, a lack of sub-samples made it impossible to confirm the presence of anatoxin-a by HPLC-MS analysis.

On the basis of the published reports it can be concluded that, in general, the blooms of hepatotoxic cyanobacteria are formed more frequently than the neurotoxic ones. There are only few reports on the presence of anatoxin-a and

they come mainly from Canada, Finland, Japan and Ireland (Sivonen *et al.* 1989, Harada *et al.* 1993, James *et al.* 1997). The highest concentration of anatoxin-a 4,400 $\mu\text{g g}^{-1}$ was measured in bloom samples from Finland (Sivonen *et al.* 1989).

To our knowledge, there have been no published reports on the presence of anatoxin-a in Polish water bodies. In this work we also failed to supply convincing evidence of the presence of anatoxin-a in the examined samples. However, the analytical method modified and used in this work can contribute to routine water quality control and further studies into the occurrence of neurotoxin producing strains of cyanobacteria.

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