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Toxins and other bioactive compounds produced by cyanobacteria in Lake Ladoga

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Abstract. Biologically active compounds were detected in water blooms on Lake Ladoga for the first time. Screening for cytotoxicity and for trypsin and acetylcholinesterase inhibitors revealed a degree of inhibitory activity by the water bloom biomass, which indicates the presence of cyanobacterial toxins in Lake Ladoga. High performance liquid chromatography (HPLC) of extracts of selected samples from the lake demonstrated a rather high diversity of bioactive compounds including hepatotoxic cyclic peptides (microcystins), enzyme inhibitors (cytotoxins), and a series of unidentified substances. Thirteen toxins and protease inhibitors were identified by the HPLC. The toxins were most diverse in the most eutrophic southern part of the lake (up to 12 bioactive compounds). Chromatograms showed that planktopeptin BL dominated in most of the samples investigated, except those from Svir Bay, where mcyst-LR prevailed. The results obtained indicate anthropogenic eutrophication of Lake Ladoga and a decrease in water quality. This lake is used as the only source of drinking water for the city of St. Petersburg.

Key words: Lake Ladoga, cyanobacteria, toxin diversity.

INTRODUCTION

Many cyanobacteria produce compounds with potent biological activities (Sivonen & Jones, 1999). These compounds are generally considered to be secondary metabolites, that is, compounds that are not essential for general metabolism or growth of the organism and are present in restricted taxonomic groups.

Many secondary metabolites are potent toxins, causing health problems for animals and humans when the producer organisms occur in masses in water bodies. The toxins produced by cyanobacteria (cyanotoxins) are grouped into two categories on the basis of the bioassay methods used to screen them: cytotoxins and bio-

toxins (Carmichael, 1997). Cytotoxins are studied with cultured cell lines; there are still no data on cytotoxins from natural sources that are lethal to animals. Biotoxins are lethal to whole organisms.

Cyanotoxins are of three chemical types: peptides (cyclic and linear), alkaloids, and lipopolysaccharides (Sivonen & Jones, 1999). The mechanisms of cyanobacterial toxicity are diverse, ranging from hepatotoxic and neurotoxic effects to the inhibition of protein synthesis. Microcystins, cyclic heptapeptide hepatotoxins, are by far the most prevalent of the cyanotoxins. Seventy-one varieties of microcystins have been reported in the literature (Codd et al., 2005). Microcystins inhibit eukaryotic serine/threonine protein phosphatases 1 and 2A. They cause acute poisoning of animals and humans and act as tumour promoters (Kuiper-Goodman et al., 1999). For example, cyanobacterial hepatotoxic microcystins were responsible for the deaths of 60 patients subjected to renal dialysis with toxin-contaminated water in Brazil (Codd et al., 2005). Chronic low-level exposure to microcystins causes human hepatocellular carcinoma in China (Kuiper-Goodman et al., 1999). Apart from their toxicity, cyanobacterial water blooms make water bodies taste and smell unpleasant. Toxic water blooms have become common in North European lakes (Sivonen et al., 1990, 1995; Skulberg, 1996; Willen & Mattsson, 1997; Lepistö et al., 2005). A survey in North-West Russia in 1990–1992 based on mouse bioassays revealed that toxic blooms occurred on 40% of the lakes studied (Gromov et al., 1996).

Lake Ladoga is the largest lake in Europe and ranks among the top 15 freshwater bodies in the world. The ecological condition of Lake Ladoga is of concern to several million inhabitants of St. Petersburg and surrounding territories, for whom the lake is the only source of domestic and industrial water (Drabkova et al., 1996). Under natural conditions, the lake ecosystem was characterized by high water quality. As a result of human impact, conditions have changed towards saprobity and toxicity of the water, and the phytoplankton biomass has increased. Cyanobacterial blooms have been observed on the lake surface since the 1990s.

The purpose of this paper is to evaluate the variety of cyanobacterial toxins in selected bloom samples from Lake Ladoga using modern methods.

MATERIAL AND METHODS

Sampling

Cyanobacterial blooms were monitored in the northern and southern bays and in the deep-water part of Lake Ladoga in July–August 2004–2005. Bloom samples were obtained at 0–5 m depth from five sampling sites (Fig. 1) using a plankton net (25 μm mesh). The concentrated biomass was air-dried on paper filters and stored at 4 °C. For laboratory analyses, the biomass was resuspended in sterile water and lyophilized.

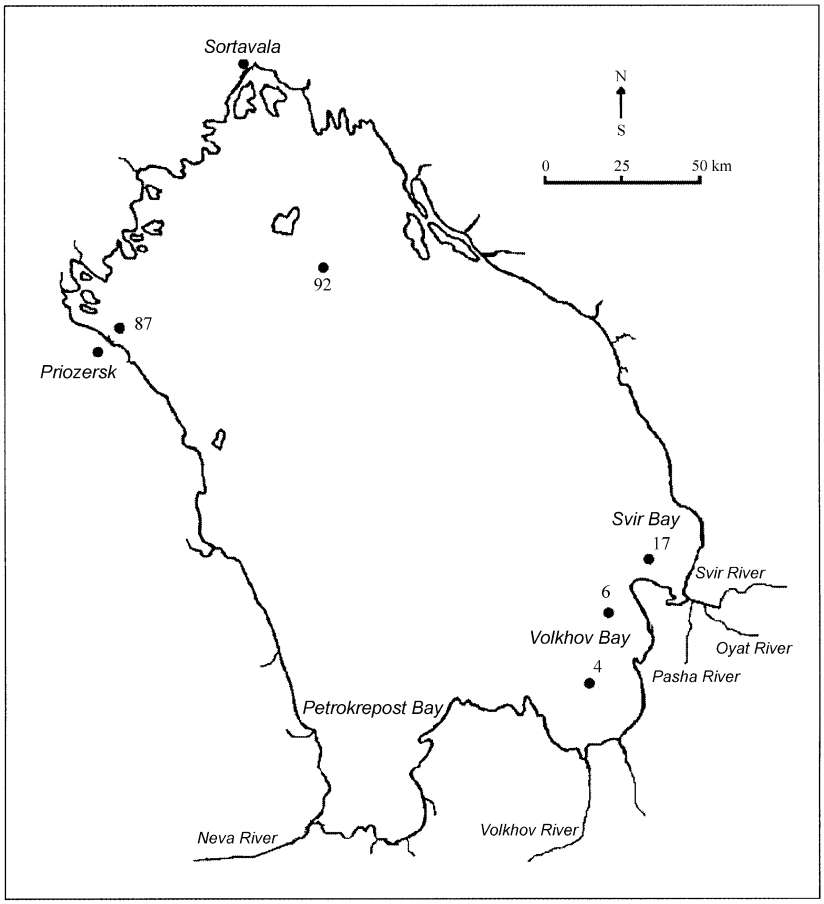

Fig. 1. Lake Ladoga with the sampling sites denoted with numbers.

Extraction

Lyophilized bloom biomass (200 mg) was extracted with 6 mL of 70% aqueous methanol for 1 h at room temperature. The extract was centrifuged at 4000 rpm for 15 min to obtain a pale green extract. This crude extract was used directly for HPLC analysis and biological activity assays (cytotoxicity, trypsin, and acetyl-cholinesterase).

Cytotoxicity assay

Cytotoxicity in vitro (MTT test) is an effective method for testing cell viability. It was optimized for the assessment of cyanobacterial cytotoxicity using the murine cell line SP2 (Mosmann, 1983). The colorimetric method is based on the

measurement of dehydrogenase activity (yellow tetrazolium salt is oxidized to blue formazan). Optical density was quantified using a microplate reader.

Crude extract of bloom biomass (10 µL) was inoculated (3 × 3 µL) into microtiter wells in a 96-cell plate. Triplicate wells without extracts served as controls. The plate was pre-incubated in a thermostat at 37 °C for 1 h. Suspensions of the murine cells (200 µL) were added to wells with or without the extract and kept in the thermostat at 37 °C for 12 h. Thiazol blue tetrazolium was added (10 µL) and the plate was returned to thermostat for 5 h. The cytotoxic effect as revealed by optical density was estimated at 590–640 nm using a spectrophotometer (Sunrise-Tecan). Cytotoxicity decreased the dehydrogenase activity, i.e. the ability of cells to oxidize the tetrazolium salt to formazan. If < 20% of the cells were dead, this was evaluated as negligible cytotoxicity, 20–50% indicated low toxicity, 50–80% indicated moderate, and > 80% high toxicity.

Trypsin inhibition assay

Trypsin inhibition was determined by a modification of the method of Cannell et al. (1988). Trypsin (Sigma Chemical Co) was dissolved in 50 mM Tris-HCl (pH 7.6) to a concentration of 150 units mL^{-1}. *N*-benzoyl-D,L-arginine-*p*-nitroanilide (BAPNA, 4.6 mg) was dissolved in 100 µL DMSO and used as substrate. Thirty microlitres of 0.4 M Tris-HCl (pH 7.6), 50 µL of trypsin solution, and 10 µL of test solution were added to each microtiter plate well and pre-incubated at 37°C for 5 min, then 115 µL of substrate solution was added to start the reaction. The absorbance of the well was immediately read at 405 nm. The developed colour was measured after incubation at 37°C for 30 min or 60 min using a Sunrise-Tecan spectrophotometer. The extract was considered active if the decrease of absorbance (compared with control) was >50%.

Acetylcholinesterase assay

Inhibition of acetylcholinesterase (AChE) was determined by a colorimetric procedure based on the Ellman reaction (Ellman et al., 1961; Mahmood & Carmichael, 1987). For each assay, 125 µL of phosphate buffer, pH 8.0, 50 µL of 0.4 units mL^{-1} AChE solution, 25 µL of 7.6 mM 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), and 20 µL of the extract were placed in a microwell of a 96-well polystyrene plate. The reaction was started by adding 30 µL of 6.2 mM acetylthiocholine iodide (ATCI). The absorbance was measured spectrophotometrically at 412 nm in 10 cycles at 12 s intervals at 30°C. Three replicates were measured for each extract. The inhibition of the enzyme was calculated from the slope of the linear part of the reaction (absorption vs time) in relation to control (no inhibition, 100% activity). The concentration of the inhibitor that reduced the enzyme activity by 50% was determined by interpolation of the data.

HPLC analysis

HPLC analysis was performed using an Agilent 1100 series liquid chromatograph equipped with a MSD SL ion trap mass-spectrometer (Meriluoto & Eriksson, 1988). Samples were separated on a Zorbax XDBC8 analytical column (4.6 × 150 mm). The mobile phase was methanol–water (linear gradient from 30% to 100% methanol over 30 min) at a flow rate of 0.6 mL min^{-1} at 30 °C. The volume of the injected extract was 20 µL. An ion trap mass spectrometer and PDA detector were used to monitor the eluted compounds. Toxins were identified by absorbance at 230 nm over 10–25 min (total analysis time 35 min). Particular toxin structures were putatively identified and evaluated by comparing the molecular masses (*m/z* values) of the eluted compounds with literature data.

RESULTS AND DISCUSSION

Dominant cyanobacterial species in the water blooms collected were *Aphanizomenon flos-aquae* (L.) Ralfs and *Woronichinia naegeliana* (Ung.) Elenk. in association with *Anabaena* (*A. flos-aquae* (Lyngb.) Bréb., *A. spiroides* Kleb., *A. lemmermannii* P. Richt., and *A. circinalis* (Kütz.) Hansg.) and *Microcystis* species (*M. wesenbergii* Komárek, *Microcystis aeruginosa* Kütz. em. Elenk., *M. viridis* (A.Br.) Lemm., and *M. grevillei* (Hass.) Elenk.). About 40 species of toxigenic cyanobacteria are currently known (Skulberg, 1993). However, it has been established that toxin production is strain- and not species-specific (Sivonen, 1996). Field studies and laboratory work with cultures have revealed that a cyanobacterial species may include toxigenic strains as well as strains that produce no toxin (Skulberg, 1993; Gromov et al., 1996). The species composition shows that 50% of the cyanobacteria in Lake Ladoga may include toxin-producing strains (Skulberg, 1993). The mean biomass of cyanobacteria varied between 3 and 5 g m^{-3} with a maximum value up to 8–10 g m^{-3}.

Assays to assess cyanobacterial cytotoxicity in vitro using the murine cell line SP2 revealed moderate inhibitory activity (50%) in extracts of plankton samples from all stations investigated (Table 1). Screening for acetylcholinesterase inhibitors in cyanobacterial biomass by the enzyme inhibition method (Mahmood & Carmichael, 1987) demonstrated 26–31% inhibitory activity, indicating the possible presence of neurotoxic alkaloids in the southern bays of Lake Ladoga. Bloom-forming species of *Aphanizomenon*, *Woronichinia*, *Anabaena*, and *Planktothrix* include neurotoxic strains (Table 1). An organophosphate inhibitor of acetylcholinesterase, anatoxin-*a*(*s*), is produced by *Anabaena flos-aque* and *A. lemmermannii* (Sivonen & Jones, 1999). Both species of *Anabaena* dominated in the water blooms examined. The LD_{50} of anatoxin-*a*(*s*) in mouse is 20 µg kg^{-1}. Anatoxins are known to overstimulate muscles, and if respiratory muscles are affected, the animal may suffer convulsions and die of suffocation. Anatoxin-*a*(*s*) has been shown to be a potent inhibitor of acetylcholinesterase that makes vertebrates salivate excessively (Carmichael, 1994). The method used is very sensitive, but the

Table 1. Inhibitory effects (%) of the extracts of lyophilized biomass taken from Lake Ladoga water blooms on cell viability (cytotoxic activity) and on trypsine and acetylcholinesterase activities (–, no data)

Assay	Stations in Lake Ladoga				
	87	92	4	6	17
Cytotoxic activity	–	–	50	50	50
Trypsine inhibition activity	93	68	93	96	99
Acetylcholinesterase inhibition activity	–	–	27	26	31

presence of other acetylcholinesterase inhibitors, e.g. organophosphate pesticides, can affect the bioassay results. Isolation and purification of the toxin for structural analysis will help to prove the presence of anatoxin-*a*(*s*) in Lake Ladoga.

Extracts of the lyophilized bloom biomass showed strong serine protease inhibition of trypsin (93–99%) in bays of Lake Ladoga and a lower level of inhibition (68%) in the deepwater zone (Table 2). These data indicate the presence of peptides (linear and cyclic) in the water body. However, cyanobacteria sometimes contain significant amounts of phosphorylase, which can inhibit protein phosphatases and mask (or add to) the inhibitory activities of microcystins (Carmichael, 1997).

Today HPLC is the most frequently used analytical method for identifying peptide microcystins (Bláha & Maršálek, 2000). HPLC of extracts of selected samples from Lake Ladoga revealed a rather high diversity of toxins, including hepatotoxic cyclic peptides (microcystins), enzyme inhibitors (cytoxins), and numerous unidentified substances (Table 2; Fig. 2). Microcystins, being cyclic

Table 2. Toxins and other bioactive compounds produced by cyanobacteria in Lake Ladoga (T, toxic effect; PI, protease inhibitor; ChTI, chymotrypsin inhibitor)

m/*z* [M + H]	Toxin	Effect	References	Stations in Lake Ladoga				
				87	92	4	6	17
609	Microcin SF608	PI	Banker & Carmeli (1999)	–	–	–	–	+
851	Anabaenopeptin F	PI	Banker & Carmeli (1999)	–	–	–	+	+
858	Oscillamide Y	PI	Sano & Kaya (1995)	–	–	–	+	–
915	Nodulapeptin B	PI	Fujii et al. (1997)	+	+	–	–	+
972	Mcyst-VF	T	Bateman et al. (1995)	–	–	–	+	+
995	Mcyst-LR	T	Botes et al. (1985)	–	–	–	–	+
992	Mcyst-N15dMeLR	T	Bateman et al. (1995)	–	–	–	+	–
1011	Micropeptin T-20	ChTI	Okano et al. (1999)	–	+	–	–	–
1014	Oscillapeptilide 97-B	PI	Fujii et al. (2000)	+	+	–	+	+
1028	Oscillapeptilide 97-A	PI	Fujii et al. (2000)	–	–	–	–	+
1037	[ADMAdda5]mcyst-LHar	T	Namikoshi et al. (1990)	–	–	–	–	+
1062	Planktopeptin BL 1061	PI	Grach-Pogrebinsky et al. (2003)	–	+	+	+	+
1073	Aeruginopeptin 917S-A	PI	Harada et al. (2001)	+	–	–	–	+
	Total			3	4	1	6	10

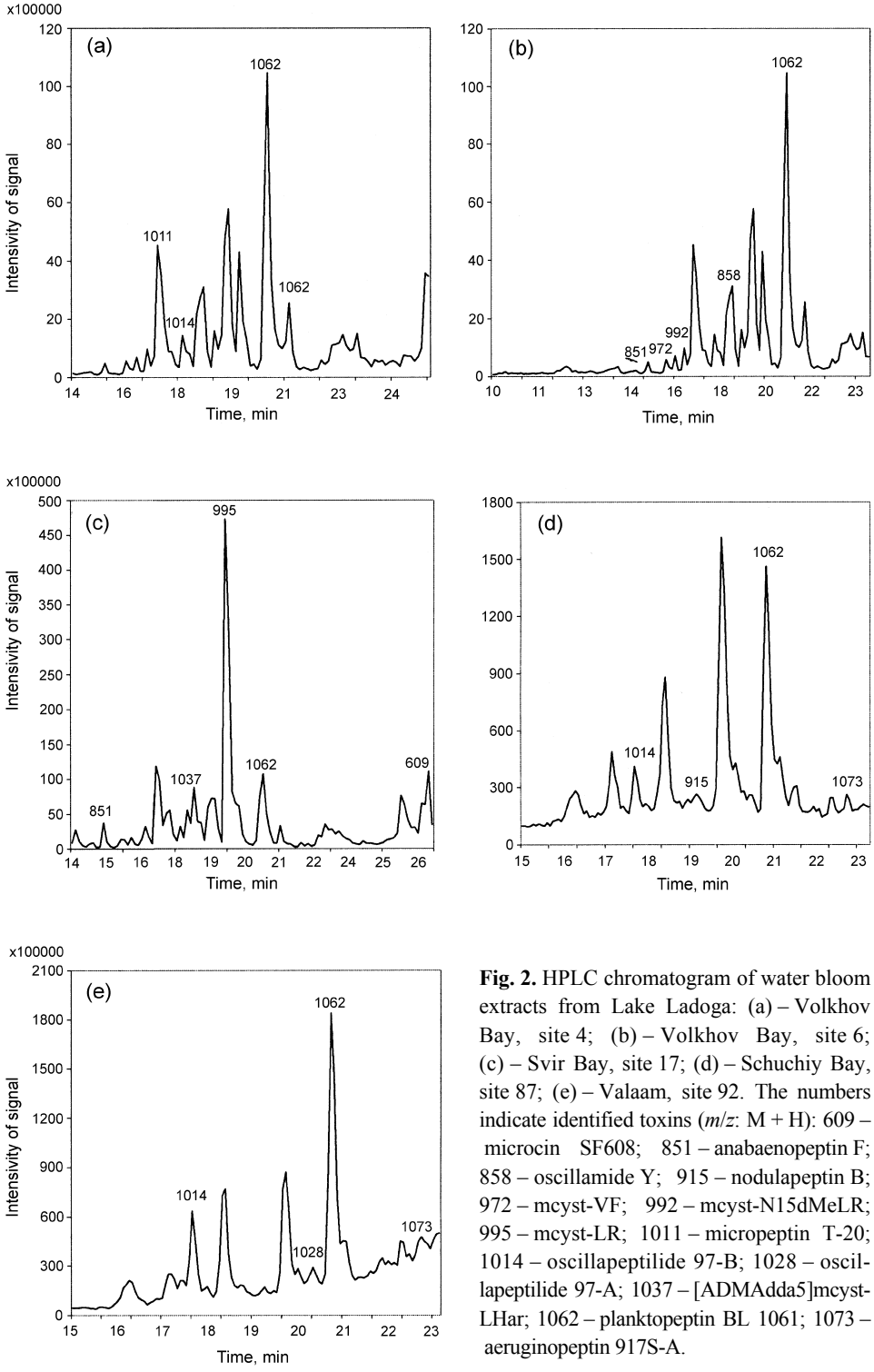

Fig. 2. HPLC chromatogram of water bloom extracts from Lake Ladoga: (a) – Volkhov Bay, site 4; (b) – Volkhov Bay, site 6; (c) – Svir Bay, site 17; (d) – Schuchiy Bay, site 87; (e) – Valaam, site 92. The numbers indicate identified toxins (*m/z*: M + H): 609 – microcin SF608; 851 – anabaenopeptin F; 858 – oscillamide Y; 915 – nodulapeptin B; 972 – mcyst-VF; 992 – mcyst-N15dMeLR; 995 – mcyst-LR; 1011 – micropeptin T-20; 1014 – oscillapeptilide 97-B; 1028 – oscillapeptilide 97-A; 1037 – [ADMAdda5]mcyst-LHar; 1062 – planktopeptin BL 1061; 1073 – aeruginopeptin 917S-A.

peptides, are extremely stable. In natural waters (especially in the dark) they may persist for months or years (Sivonen & Jones, 1999). Microcystins are produced by strains of the distantly-related cyanobacterial genera *Microcystis*, *Anabaena*, *Planktothrix*, and others. In Lake Ladoga, the microcystins (mcyst) identified with high toxicity (LD_{50} = 50 μg kg^{-1}) were mcyst-LR and [ADMAdda^5]mcyst-LHar. In addition, microcystins with undetermined LD_{50} were found: mcyst-VF and mcyst-N^{15}dMeLR. The variant mcyst-LR is considered the most widespread and is regarded as one of the most toxic microcystins. The World Health Organization declared a provisional limiting dose of 1.0 μg L^{-1} for this toxin, as the most dangerous compound in nature (WHO, 1998). All microcystins were found in the most eutrophicated shallow southern part of the lake (Table 2; Fig. 2). It is well known that hepatotoxic strains produce more toxins when the phosphorus concentration is high (Sivonen, 1996). The phosphorus load fed by rivers into the southern bays of Lake Ladoga is three times higher than that from any other incoming rivers (Sorokin et al., 1995).

Cyanobacteria can produce a wide variety of linear (e.g. aerugenosins and microginins) and cyclic (e.g. anabaenopeptins, anabaenopeptilides, microviridins, nostopeptilides) peptides, which may not be acutely toxic but have other bioactivities such as serine protease inhibition (Rantala et al., 2004). They are responsible for oral and gastrointestinal inflammation, allergic reactions, and skin irritation resulting from the ingestion of cyanobacterial cells or after contact with water blooms. From the HPLC analysis results we identified 9 different protease and chymotrypsin inhibitors in the natural samples, such as aeruginopeptin 917S-A, anabaenopeptin F, microcin SF608, micropeptin T-20, nodulapeptin B, planktopeptin BL 1061, oscillamide Y, oscillapeptilide 97-A, and oscillapeptilide 97-B (Table 2; Fig. 2).

From one to ten biologically active compounds were found in each bloom sample. The greatest diversity of toxins and other compounds was found in Volkhov and Svir bays (6 and 10 compounds, respectively). Peak values on the chromatograms indicate that planktopeptin BL dominated in most of the stations investigated, the exception being station 17 (Svir Bay), where mcyst-LR prevailed (Fig. 2).

CONCLUSIONS

Cyanobacterial blooms are diagnostic of accelerating eutrophication of freshwater and estuarine ecosystems. Both acute and chronic exposure to cyanobacterial hepatotoxins may be significant human health risks. Biologically active compounds were detected in water blooms in Lake Ladoga for the first time. Screening for cytotoxic activity and for trypsin and acetylcholinesterase inhibitors revealed that the biomass had inhibitory activity, indicating the presence of cyanobacterial peptides and neurotoxic alkaloids in Lake Ladoga. HPLC of the extracts of selected samples from the lake revealed a rather high diversity of bioactive compounds including hepatotoxic cyclic peptides (microcystins), inhibitors of enzymes

(cytoxins), and numerous unidentified substances. Four different microcystins were identified, including two structural variants of microcystin-LR, which is considered the most toxic chemical compound in nature. The toxins were most diverse in the most eutrophicated shallow southern part of the lake, Volkhov and Svir bays. The results indicate anthropogenic eutrophication of Lake Ladoga and a decrease in its water quality. This lake is the only drinking water source of the city of St. Petersburg. To minimize problems from harmful water blooms, effective water management is necessary to control the factors causing the eutrophication of Lake Ladoga.

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Tsüanobakterite poolt produtseeritavate toksiinide ja muude bioloogiliselt aktiivsete ühendite mitmekesisus Laadoga järves

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On selgitatud esmakordselt mitmesuguste bioloogiliselt aktiivsete ühendite, eelkõige tsüanobakterite toksiinide esinemist Laadoga järves veeõitsengute ajal. Tsüanobakterite toksiinide olemasolu vees näitavad tsütotoksilisuse ja trüpsiini ning atsetüülkoliini esteraasi inhibiitorite aktiivsuse testid. Kõrgsurve vedelikkromatograafia (HPLC) abil on selgitatud, et järves on üsna palju bioloogiliselt aktiivseid ühendeid, nagu hepatotoksilised tsüklilised peptiidid (mikrotsüstiinid), ensüümide inhibiitorid (tsütotoksiinid) ja mitmed ained, mida pole suudetud identifitseerida. Kokku on HPLC abil identifitseeritud 13 toksiini ja proteaasi inhibiitorit. Toksiinide mitmekesisus on suurim järve eutroofsemas lõunaosas, sealt on leitud 12 ühendit. Kromatogrammid näitavad, et planktopeptiin BL domineerib enamikus uuritud proovidest, vaid Sviri lahes on ülekaalus mcyst-LR. Tulemustest nähtub, et Laadoga järv on eutrofeerumas ja vee kvaliteet halveneb. Toksiinide esinemine on alarmeeriv, sest Laadoga vett kasutab joogiks Peterburi linn.