

Baltic Cyanobacteria - a source of biologically active compounds

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Abstract:

Cyanobacteria are effective producers of bioactive metabolites, including both acute toxins and potential pharmaceuticals. In the current work, the biological activity of 27 strains of Baltic cyanobacteria representing different taxonomic groups and chemotypes were tested in a wide variety of assays. The Cyanobacteria showed strain-specific differences in the induced effects. The extracts from *Nodularia spumigena* CCNP1401 were active in the highest number of tests, including protease and phosphatase inhibition assays. Four strains from Nostocales and four strains from Oscillatoriales increased proliferation of mitogen-stimulated human T cells. In antimicrobial assays, *Phormidium* sp. CCNP1317 (Oscillatoriales) strongly inhibited the growth of six fouling Gammaproteobacteria. The growth of monocotyl *Sorghum saccharatum* was inhibited by both toxin-producing and “non-toxic” strains. The Baltic cyanobacteria were also found to be a rich source of commercially important enzymes. Among the 19 enzymatic activities tested, alkaline phosphatase, acid phosphatase, esterase (C4 and C8), and naphthyl-AS-BI-phosphohydrolase were particularly common. In the cyanobacterial extracts, different peptide structures which may have been responsible for the observed effects were classified to microcystins, nodularins, anabaenopeptins, cyanopeptolins, aeruginosins, spumigins and nostocyclopeptides were identified using LC-MS/MS.

Keywords: antimicrobial compounds, Baltic cyanobacteria, enzyme activity, enzyme inhibitors, immunological activity, natural products, nonribosomal peptides, plant growth regulators

INTRODUCTION

Cyanobacteria are Gram-negative bacteria which are widely distributed in many water bodies worldwide. In the Baltic Sea, the cyanobacterial community is composed of the single-celled *Synechococcus* sp. and other members of the Chroococcales (e.g. *Microcystis*, *Aphanocapsa*, *Aphanothece*), the surface bloom-forming members of Nostocales (e.g. *Aphanizomenon*, *Nodularia*, *Dolichospermum/Anabaena*), and the less frequently observed in the Baltic representatives of Oscillatoriales (e.g. *Phormidium*, *Lynngbya*, *Planktothrix*) (Hällfors, 2004). Cyanobacteria differ significantly with respect to the metabolites produced, their structures and biological activity. Due to the harmful effects on humans and animals, the toxic compounds produced by *Nodularia spumigena* and other Baltic Cyanobacteria are of the highest scientific interest and ecological concern (Karjalainen *et al.*, 2007). These include the cyclic hepatotoxic cyanopeptides nodularin (NOD) and the microcystins (MCs), which are strong inhibitors of eukaryotic-type protein phosphatases 1 and 2A (PP) (McKintosh *et al.*, 1990; Ohta *et al.*, 1994). In eukaryotic cells, protein phosphatases PP1 and PP2A are involved in fundamental cellular processes such as cell growth and proliferation, protein synthesis, DNA replication and repair, neuronal signaling, apoptosis and carbohydrate metabolism. Deregulation of PP activity can lead to the changes in signal transduction and disturbed balance in cellular homeostasis. In Baltic cyanobacteria, two neurotoxic metabolites have also been detected: anatoxin-a (Rantala-Ylinen *et al.*, 2011) and β -N-methylamino-L-alanine (BMAA) (Cox *et al.*, 2005; Jonasson *et al.*, 2010). Anatoxin-a irreversibly binds to the nicotinic acetylcholine receptor. In extreme cases this so-called very fast death factor (VFDF) may cause severe overstimulation of respiratory muscles, finally leading to respiratory arrest and rapid death (Stevens & Krieger, 1991). In contrast, BMAA is classified as a “slow toxin”, possibly linked to the development of atypical neurodegenerative diseases known as the amyotrophic lateral sclerosis/parkinsonism-dementia complex (ALS/PDC) (Murch *et al.*, 2004).

In addition to toxins, cyanobacteria produce a wide variety of other metabolites, including alkaloids, lactones, amino acids, peptides, lipopeptides, polyketides and lipids. It has been suggested that at least some of these compounds constitute an element of the adaptive strategies of cyanobacteria, enabling them to survive in a wide range of physical and chemical conditions. The metabolites can also be involved in interactions with co-occurring organisms, from bacteria and viruses to aquatic animals (Rohrlack *et al.*, 2004; Sedmak *et al.*, 2008; Sønstebø & Rohrlack, 2011).

As many cyanobacterial products are characterized by a unique biological activity, they have become a focus of high interest as chemicals of potential biotechnological and industrial application (Rastogi & Sinha, 2007; Tan, 2007; Abed *et al.*, 2013). In agriculture, cyanobacteria have long been

used as biofertilizers, soil texture improving or growth stimulating agents (Upasana & Pabbi, 2004). This type of activity has been detected in, amongst others, *Calothrix* sp., *Hapalosiphon intricatus*, *Nostoc* sp. and *Arthronema africanum* (Stirk *et al.*, 2002; Karthikeyan *et al.*, 2007). Cyanobacteria of the genera *Fischerella*, *Scytonema*, *Nostoc*, *Hapalosiphon* and *Oscillatoria* can also produce compounds with algicidal activity directed against photosynthesis (Abdel-Raouf *et al.*, 2012). As the active metabolites degrade easily, their application as agrochemicals in place of many currently used fertilizers, herbicides or insecticides, can be of benefit for the health of the environment.

Cyanobacterial metabolites, or their synthetic analogues, are also considered to be commercially appealing as immunosuppressants and antimicrobial, anticancer and protease inhibiting agents (Burja *et al.*, 2001; Rastogi & Sinha, 2007; Tan, 2007, Imhoff *et al.*, 2011; Costa *et al.*, 2012; Nagarajan *et al.*, 2012; Abed *et al.*, 2013). It was estimated that out of the 660 new compounds identified in marine bacteria in the years 1997–2008, 33% were derived from cyanobacteria (Imhoff *et al.*, 2011). The most promising compounds of pharmaceutical application were isolated from two cyanobacterial genera, *Lyngbya* and *Symploca*. *Lyngbya majuscula* from tropical waters produces curacin A, a potent inhibitor of microtubule assembly (Gerwick *et al.*, 1994), and apratoxin D, a compound which is cytotoxic to human lung cancer cells (Gutierrez *et al.*, 2008). Dolastatin 10, originally isolated from the sea hare *Dolabella auricularia*, but produced by endosymbiotic *Symploca* sp., acts as an anticancer agent (Mooberry *et al.*, 2003) and synthetic analogues of dolastatin 10 entered phase III of clinical trials (Schöffski *et al.*, 2004). Largazole, another metabolite from *Symploca* sp., is a signal transduction inhibitor – cytotoxic to human lung cancer cells (Taori *et al.*, 2008). Various cyanobacterial taxa produce linear tetrapeptides classified as aeruginosins. Currently, several synthetic analogues of the peptides are in clinical trials as blood anti-clogging agents (Ersmark *et al.*, 2008).

The majority of studies testing the activity of cyanobacterial compounds have been focused on Cyanobacteria in tropical waters. There has been little work on Baltic Cyanobacteria as a potential source of novel drugs. However, among the bioactive agents identified from Baltic cyanobacteria have been inhibitors of the serine proteases: spumigins, aeruginosins and anabaenopeptins (Fewer *et al.*, 2009; Mazur-Marzec *et al.*, 2013); nostocyclopeptides which inhibit MCs and NOD uptake by hepatocytes (Herfindal *et al.*, 2011); the cytolytic lipopeptides (Jokela *et al.*, 2012) and unknown compounds which induced apoptosis in acute myeloid leukemia cells (Ofstedal *et al.*, 2010).

We expected that the brackish water Baltic Sea, with its unusual physical conditions, would harbour organisms producing metabolites with unique features. Therefore, we assessed the biological activity of the extracts from 27 Baltic cyanobacteria isolates using enzymatic, immunological,

antimicrobial and plant growth tests. The overall aim of the experiments was to identify strains showing the most promising biological activity for potential biotechnological application.

MATERIALS AND METHODS

Isolation, culture and extraction of Cyanobacteria

Cyanobacteria were isolated from the littoral and pelagic zones of the Baltic Sea (Table 1). In the laboratory, single trichomes or colonies were picked up and purified by multiple transfers to a liquid or solid (1% bacterial agar) media (Table 1) (Kotai, 1972). The growth of eukaryotes was inhibited by the addition of cycloheximide at 50 $\mu\text{g ml}^{-1}$ (Rippka, 1988). Once monospecies cultures were obtained, the isolated strains were grown for biomass in 5000 ml bottles at room temperature ($22 \text{ }^{\circ}\text{C} \pm 1 \text{ }^{\circ}\text{C}$). Continuous light of 10–30 $\mu\text{M photons m}^{-2} \text{ s}^{-1}$ was provided by standard cool white fluorescent lamps (36 W). Cultures were aerated by bubbling air which was sterilized by passing through 0.2 μm filters (Millex[®]-FG, Millipore, USA). Each culture was monitored for bacterial contamination by spreading the cell suspension (100 μl) on agar Z8 medium. If visible colonies of bacteria occurred during two-week incubation, the culture was discarded. The biomass of cyanobacteria was harvested in the mid-exponential growth phase by centrifugation at $2000\times g$ for 20 min (Eppendorff Centrifuge 5810R, Germany) or by passing through a 10 μm phytoplankton net. The collected cyanobacterial material was frozen and lyophilized.

Freeze-dried samples of cyanobacterial material (20 mg) were extracted (1:10 w/v) with different solvents: 80% ethanol (gradient grade for liquid chromatography, Merck KGaA, Darmstadt, Germany) and Milli-Q water (Merck Millipore, Billerica, MA, USA). For immunological tests the extracts were prepared in 50% methanol (HPLC Gradient Grade, Mellinckordt Baker B.V., Denver, The Netherlands). Samples were subjected to 30 min probe sonication with an ultrasonic disrupter (HD 2070 Sonopuls, Bandeline, Berlin, Germany) followed by 15 min bath sonication (Sonorex, Bandeline, Berlin, Germany). After centrifugation (10,000g for 15 min) the supernatants were collected and the residue was extracted again with 1:10 w/v of the same solvent overnight at $15 \text{ }^{\circ}\text{C}$ on an orbital shaker (200 rpm, Shaker DOS-10L, neoLab, Heidelberg, Germany). The supernatants were combined and evaporated to dry residue. All assays were performed in triplicate.

Cyanobacteria

The isolated cyanobacterial strains were deposited in the Culture Collection of Northern Poland (CCNP), University of Gdańsk (Poland). For molecular characterization, 5 ml of cyanobacterial culture was centrifuged at $13,000\times g$ for 5 min. The remaining pellet was suspended in 0.1 ml of TRIS buffer (A&A Biotechnology, Gdynia, Poland) and 0.05 ml of lysozyme in water (15 mg ml^{-1} ,

Sigma-Aldrich, St. Louis, USA) was added. The sample was mixed thoroughly by vortex and incubated for 10 min at 37°C. Afterwards, genomic DNA was extracted using Genomic Mini Kit (A&A Biotechnology), according to the manufacturer's instructions. DNA was amplified with cyanobacteria-specific 16S rRNA gene primers: CYA359F (Nübel *et al.*, 1997) and 23S30R (Lepère *et al.*, 2000). Each PCR reaction had a final volume of 25 µl and contained 0.5 µl of target DNA, 250 mM dNTP (A&A Biotechnology), 1 mg ml⁻¹ albumine (Sigma-Aldrich), 500 mM MgCl₂ (Blirt S.A., Gdańsk, Poland), 1x *TaqNova* reaction buffer with 0.4U *TaqNova* polymerase (Blirt S.A.) and 0.5 µM of each primer. The PCR reaction was performed according to Koskenniemi *et al.* (2007) with minor changes (annealing temperature changed to 57°C). The amplified PCR products were purified using Clean-up Kit (A&A Biotechnology). The nucleotide sequences of PCR products were obtained from Genomed (Warsaw, Poland) edited with Chromas Lite 2.1 and compared to the sequences in the NCBI GenBank (<http://www.ncbi.nlm.nih.gov>) using the nucleotide BLAST. Nucleotide sequences were deposited in GenBank under the accession numbers listed in Table 1.

Chemical analysis of cyanobacterial extracts

For each individual strain, a profile of metabolites extracted from dry biomass in 5% aqueous acetic acid (2 mg ml⁻¹) was determined using a nano-LC (nanoAquity, Waters, Massachusetts, USA) coupled to a QStar Elite hybrid quadrupole-time-of-flight tandem mass spectrometry system (Q-TOF MS/MS, Applied Biosystems MDS Sciex, Ontario, Canada). The analyses were conducted using a NanoSpray II source and Heated Interface (150 °C). Chromatography was performed with 5% acetonitrile in MilliQ water plus 0.1% formic acid (solvent A) and 0.1% formic acid in acetonitrile (solvent B). Samples (0.5 µl) were injected on a nanoAquity UPLC Symmetry C18 trapping column (180 µm × 20 mm, 5 µm, Waters, USA) and washed with solvent A at a flow rate of 15 µl min⁻¹ for 2 min. Compounds were separated on a nanoAquity UPLC BEH130 C18 column (75 µm × 100 mm, 1.7 µm, Waters, USA). The gradient started at 5% B and went to 30% B within 30 min at a flow rate of 0.4 µl min⁻¹. The content of phase B was then increased to 99% within the next 15 min and kept at that level for 10 min before returning to the starting conditions. MS spectra were acquired over the range 350–2000 Da. The instrument was operated in the positive mode. Ion spray voltage was 2.4 kV, with the nebulizing gas nitrogen pressure and curtain gas nitrogen pressures set at 25 p.s.i and 20 p.s.i., respectively (1 p.s.i. = 6894.76 Pa). Data acquisition and processing were accomplished with the Analyst[®] QS 2.0 software.

For the quantitative analysis of microcystins and nodularin, the HPLC system (Agilent 1200, Agilent Technologies, Germany) equipped with a photodiode array detector (PDA) was used; the absorbance at 238 was monitored. The separation was performed on a Luna RP-18 column (3.0 mm × 150 mm;

3 μm) kept at temperature of 40°C. Gradient elution with the mobile phase A (5% acetonitrile in MilliQ water with 0.05% trifluoroacetic acid TFA) and B (100% acetonitrile with 0.05% TFA) was used. The mobile phase was delivered at a flow rate of 0.5 ml min⁻¹. Phase B was linearly increased from 30% to 70% in 7 min and then to 100% in 3 min. The column was washed with 100% phase B for 10 min then the mobile phase composition was brought back to the initial conditions (70% B) in 1 min. The total concentration of MCs and NOD in the analysed samples was calculated as MC-LR or NOD equivalents using a calibration curve prepared with standards.

Screening for enzyme inhibitors

For all enzyme inhibition assays, serial dilutions of cyanobacterial extract (20mg; 1:1 – 1:10,000) and the solutions of inhibitors were prepared in MilliQ water.

Protein phosphatases inhibition assays. Protein phosphatase inhibition assay with PP1 (New England Biolabs, Ipswich, UK) was performed according to the procedure described by Rapala *et al.* (2002). PP1 and the substrate, *p*-nitrophenyl phosphate disodium salt hexahydrate (*p*-NPP), were purchased from Sigma-Aldrich (Irvine, UK). Nodularin standard (Alexis Biochemicals, Lausen, Switzerland) (0.125–4.0 ng ml⁻¹) was used as a protein phosphatase inhibitor. The enzyme was dissolved in buffer solution A (50 mM Tris at pH 7.4, 1 mg ml⁻¹ bovine serum albumin (BSA), 1 mM MnCl₂, 2 mM DTT; Sigma-Aldrich, St. Louis, USA). The substrate *p*-NPP (5.5 mg ml⁻¹) was dissolved in buffer solution B (50 mM Tris, pH 8.1, 0.5 mg ml⁻¹ BSA, 20 mM MgCl₂ × 6H₂O, 200 mM MnCl₂ × 4H₂O). The assay was performed in 96 multiwell plate at 37°C. Each well contained: 10 μl of sample or standard and 10 μl of enzyme solution (1.7 U ml⁻¹). The reaction was started with the addition of substrate *p*-nitrophenyl phosphate (*p*-NPP, 200 μl). After 120 min, the absorption measurements were conducted at 405 nm using microplate reader (Molecular Devices, Sunny Vale, USA).

The protein phosphatase 2A (Millipore, USA) inhibition assay was performed according to Rivasseau & Hennion (1999). Sample or standard solutions (125 μl) were placed in wells containing 25 μl of buffer (250 mM Tris at pH 8.4). Then, 50 μl of enzyme (0.05 U ml⁻¹) dissolved in buffer solution A (20 mM Tris, pH 7.4, 1 mg ml⁻¹ BSA, 0.1 mM MgCl₂) was added. After incubation for 8 min, 50 μl of *p*-NPP (20 mg ml⁻¹) in buffer solution B (50 mM Tris, pH 8.4) was added to start the reaction. After 60 min, the absorbance was measured at 405 nm.

Trypsin inhibition assay. The trypsin inhibition assay was performed according to the method described by Pluotno & Carmeli (2005). Trypsin from porcine pancreas (T0303) and the inhibitor aprotinine (A6103) were purchased from Sigma-Aldrich (St. Louis, USA) while the substrate *N* _{α} -

benzoyl-L-arginine 4-nitroanilide hydrochloride (BAPNA) was from Acros Organics (227740050, Geel, Belgium). Trypsin (1 mg ml^{-1}) and BAPNA (2 mM) were dissolved in buffer solution (50 mM Tris-HCl, 100 mM NaCl, 1 mM CaCl₂, pH 7.5). Five solutions of inhibitor ($10\text{--}60\text{ }\mu\text{g ml}^{-1}$) were used. The mixtures containing the sample ($10\text{ }\mu\text{l}$), inhibitor ($10\text{ }\mu\text{l}$), enzyme ($10\text{ }\mu\text{l}$) and buffer ($100\text{ }\mu\text{l}$) were preincubated for 15 min. Then, the substrate solution ($100\text{ }\mu\text{l}$) was added and the mixture was incubated for another 15 min at $25\text{ }^{\circ}\text{C}$. The absorbance was measured at 405 nm .

Elastase inhibition assay. The elastase inhibition assay was based on the procedure described by Kwan *et al.* (2009). Elastase from porcine pancreas (E0258, Sigma-Aldrich, St. Louis, USA) was dissolved in buffer (0.2 M Tris-HCl, pH 8.0) to prepare $75\text{ }\mu\text{g ml}^{-1}$ solution. Elastatinal (Sigma-Aldrich, Tokyo, Japan) was applied as an inhibitor. The substrate solution contained 2 mM of *N*-succinyl-Ala-Ala-Ala-p-nitroanilide (S4760, Sigma-Aldrich) in 0.2 M Tris-HCl, pH 8.0 buffer. To each microplate well the solutions of the cyanobacterial extract ($10\text{ }\mu\text{l}$), inhibitor ($10\text{ }\mu\text{l}$, $5\text{--}125\text{ }\mu\text{g ml}^{-1}$), enzyme ($10\text{ }\mu\text{l}$) and buffer ($150\text{ }\mu\text{l}$) were added and preincubated at $25\text{ }^{\circ}\text{C}$ for 15 min. Then, $30\text{ }\mu\text{l}$ of substrate solution () was added to start the reaction. After 10 min, absorbance was measured at 405 nm .

Thrombin inhibition assay. Thrombin inhibition assay was performed according to Ocampo Bennet (2007). Thrombin from bovine plasma (T4648, Sigma-Aldrich, St. Louis, USA), inhibitor 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride-AEBSF (A8456) and the substrate, *N*-p-tosyl-Gly-Pro-Lys-p-nitroanilide acetate salt (T6140) (Sigma-Aldrich, Buchs, Switzerland) were used. Thrombin and the substrate were dissolved separately in buffer (0.2 M Tris-HCl, pH 8.0) so that the final concentration of both solutions was 0.5 mg ml^{-1} . To each microplate well the solutions of cyanobacterial extract ($10\text{ }\mu\text{l}$), inhibitor ($10\text{ }\mu\text{l}$, $24\text{--}600\text{ }\mu\text{g ml}^{-1}$), thrombin ($10\text{ }\mu\text{l}$) and buffer ($170\text{ }\mu\text{l}$) were added and preincubated at $37\text{ }^{\circ}\text{C}$ for 10 min after which the substrate solution ($20\text{ }\mu\text{l}$) was added. The absorbance was measured at 405 nm .

Carboxypeptidase-A inhibition assay. Carboxypeptidase-A inhibition assay was performed with the use of the chromogenic substrate *N*-(4-methoxyphenylazoformyl)-Phe-OH (M-2245.0100, Bachem, Bubendorf, Germany). Carboxypeptidase-A ($1.6\text{ }\mu\text{M}$) (C9268, Sigma-Aldrich, St. Louis, USA) was dissolved in Tris-HCl buffer (50 mM , pH 7.5). The carboxypeptidase inhibitor (CPI) was purchased from Sigma-Aldrich (C0297). Substrate was dissolved in 1% DMSO to final concentration 0.6 mM . To each microplate well, the solutions of cyanobacterial extract ($10\text{ }\mu\text{l}$), CPI ($10\text{ }\mu\text{l}$, $0.1\text{--}5.0\text{ }\mu\text{g ml}^{-1}$), buffer ($150\text{ }\mu\text{l}$) and enzyme ($10\text{ }\mu\text{l}$) were added and preincubated for 5 min at $37\text{ }^{\circ}\text{C}$. Finally, the substrate ($20\text{ }\mu\text{l}$) was added to each well. Absorbance was measured at 350 nm after 15 min of incubation.

Screening for enzyme activities

The activities of 19 enzymes in the cyanobacterial aqueous extracts were tested by employing the API ZYM test (BioMerieux, Lyon, France). Manufacturer's instructions were strictly followed. The extracts were diluted in MilliQ water to the concentration of 1 mg ml^{-1} and $65 \text{ }\mu\text{l}$ of the solutions were dispensed on each cupule of the API ZYM strip. Demineralized water was used as a control. Strips were placed in the incubation box ($37 \text{ }^{\circ}\text{C}$) for 4 hours. After incubation, 1 drop of ZYM A reagent and 1 drop of ZYM B reagent were added to each cupule. Strips were incubated at room temperature for 5 minutes. Then, the intensity of color in the cupules was read and compared to the reading table (attached to the API ZYM test)..

Immunological assay

A cohort of 10 pre-screened, healthy volunteers of both sexes (6 women, 4 men) was recruited. None of the healthy volunteers were taking medication that would influence the immune system. All the individuals involved in the study were informed about the purpose of the experiment and gave their written consent. The project was accepted by the Local Independent Committee for Ethics in Scientific Research at the Medical University of Gdańsk (permission number NKEBN/269/2011, 2011.08.03). Peripheral blood mononuclear cells were obtained from fasting venous blood samples. Blood was collected into the EDTA-containing Vacutainer[®] tubes (BD Pharmingen, San Diego, USA) and then peripheral blood mononuclear cells (PBMC) were isolated by Histopaque[™] (Sigma-Aldrich, St. Louis, USA) flotation. Isolated PBMC were stained supravivally with fluorescent CFSE (Carboxyfluorescein succinimidyl ester) dye according to the method described by Witkowski (2008), re-suspended in culture medium RPMI 1640 supplemented with 10% foetal bovine serum, 2 mM-glutamine, penicillin and streptomycin (Sigma-Aldrich) at $2 \times 10^6 \text{ ml}^{-1}$ and stimulated with immobilized anti-CD3 (BD Pharmingen). Then, the cells were exposed to cyanobacterial extracts at $10 \text{ }\mu\text{g ml}^{-1}$. The samples and the control variants without cyanobacterial extracts were incubated for 72 hours at 37°C in 5% CO_2 and 95% air. After 72 hours the cells were harvested, washed, counterstained with fluorochrome-coupled antibodies (RPE-Cy5-anti-CD4 (DAKO, Glostrup, Denmark) and PE-anti-CD25 (BD Pharmingen) and analysed by flow cytometry. For this purpose, the fluorescence-activated cell sorting FACScan system was used (BD Biosciences, San Jose, USA).

Antimicrobial activities

Broth microdilution assays and agar disc diffusion assays (pathogenic microorganism strains 3, 5, 7, 9, 11, 13–23 only, Table 5) were used to determine the ability of cyanobacterial extracts to inhibit the growth of bacteria and fungi. Pathogenic microorganisms were obtained from the Goa Medical

College, Dept. Microbiology (strains 3, 9, 11, 14, 22–23, Table 5) and Microbiologics Inc. (strains 2, 4, 10, 24, Table 5). Strains 1 and 12 were isolated at the Medical University Hospital in Gdańsk, and identified with the use of VITEK Legacy (BioMerieux, Lyon, France). Strain 6, isolated from the human throat (Furmanek *et al.*, 1999) and strain 8 were kindly provided by Prof. T. Kaczorowski, Dept. Molecular Biology, University of Gdańsk. The identification of strain 6 was confirmed by Elżbieta Mączak. The fouling bacteria (5, 7, 13, 15–21, Table 5) were isolated from coastal waters off Goa, India. For the purpose of the antimicrobial screening, lyophilized cyanobacterial extracts were dissolved in water, or in the case of strains 2, 4, 10 and 24 (Table 5) in DMSO. Due to a limited amount of cyanobacterial material, the microorganisms used in the tests were not exposed to all cyanobacterial extracts (Table 5).

Agar disc diffusion assay. Sterilized Whatman[®] filter paper discs (6 mm diameter) (Sigma-Aldrich, St. Louis, USA) were saturated with the cyanobacterial extract (50 µg/disc) and placed on Mueller Hinton II Agar (Becton Dickinson, Loveton Circle, USA) plates seeded with a lawn of the tested microorganism. Positive controls (streptomycin, trimethoprim, chloramphenicol, methicillin, amphotericin B at 5 mg ml⁻¹) and negative control (water) were run simultaneously. After 24 h (bacteria) or 48 h (fungi) incubation at 37°C, the diameter of the inhibition zone was measured. When inhibition zone larger than 10 mm was observed, a microdilution assay was performed to determine the MIC value (minimum inhibitory concentration).

Broth microdilution assay. MIC values for microbial cultures treated with the crude cyanobacterial extracts were determined according to Balows (1991). Reference antibiotics were used as positive controls on every assay plate. Bacterial strains 1, 6, 8 and 12 (Table 5) were precultured in LB medium (Sigma-Aldrich, St. Louis, USA) at 37°C for 16 h at 150 rpm. The initial number of colony-forming units in the experimental cultures was 2×10⁵ CFU ml⁻¹. The cultures were incubated for 16 h in the presence of cyanobacterial extracts at concentrations from 0.01 mg ml⁻¹ to 1.0 mg ml⁻¹ (n = 3). Then, the optical density was measured at 570 nm.

In the case of the strains 2, 4, 10 and 24 (Table 5), antimicrobial assays were performed following the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (2012) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) (<http://www.eucast.org>). The bacterial strains were precultured in Mueller Hinton II Broth (Becton Dickinson) at 37°C for 16–20 h at 100 rpm. At the beginning of the experiment the density of the culture was 5 × 10⁵ CFU ml⁻¹. The fungal strains were precultured on Sabouraud Dextrose Agar (Becton Dickinson) plates at 28°C for 18–24 h. Then the strains were transferred to sterile 0.9% saline solution and the suspension was diluted into RPMI-1640 medium (with l-glutamine, w/o NaHCO₃ and supplemented with 2%

glucose and 0.165 M MOPS, buffered to pH 7; Lonza, Basel, Switzerland) to reach 2.5×10^3 CFU ml⁻¹ in the assay. Microbial cultures were incubated in the presence of cyanobacterial extracts at concentrations of 0.1 mg ml⁻¹ for 24 h (n=3). Assay plates were incubated for 24 h at 37°C (bacteria) or 48 h at 28°C (fungi), then optical density was measured at 620 nm.

For all microbial strains used in the broth microdilution assay, growth stimulation effects were considered to be significant if the absorbance of the treated culture was at least 50% higher than in the control (untreated) culture. Growth inhibition effect was considered as significant if absorbance of the treated culture was at least 30% lower than that of control. Otherwise, effects were considered to be non-significant.

Plant growth test

Effects of cyanobacterial extracts on the growth of young roots and shoots of monocotyl sorghum (*Sorghum saccharatum*) were tested according to the procedure provided by the producer of the Microbiotest Phytotestkit (Microbiotests Inc., Gent, Belgium). The extracts (20 mg) were dissolved in 20 ml MiliQ water. Each test plate contained 10 sorghum seeds. The test was carried out in incubator ST 1B50 (POL-EKO, Wodzislav Slaski, Poland) for 3 days in darkness, at 25°C. At the end of the exposure period, length of roots and shoots was measured using ImageJ Software (Bethesda, Maryland, USA).

Statistical analysis

Data from the plant growth tests were analysed with the non-parametric Mann-Whitney U test ($p < 0.01$) using STATISTICA v.9 software (StatSoft, Tulsa, OK, USA).

RESULTS AND DISCUSSION

Extraction, molecular characterization & chemical analysis of extracts

All tested strains belonging to the Chroococcales (7), Oscillatoriales (10) and Nostocales (10) orders (Table 1) were genetically and chemically characterized. A phylogenetic tree of cyanobacterial 16S rRNA gene sequences, illustrating diversity of the microorganisms is presented in Figure 1S.

Those strains for which any compound was detected as a peak in the LC-MS chromatogram (Figure 2S) are listed in Table 2. Among the detected structures (Table 2, Figure 3S–5S) there were known inhibitors of protein phosphatase (microcystins, nodularins and anabaenopeptins) (McKintosh *et al.* 1990; Ohta *et al.* 1994; Sano *et al.*, 2001), serine proteases (aeruginosins, spumigins, anabaenopeptins, cyanopeptolins) (Sano & Kaya, 1995; Ishida *et al.*, 1999; Itou *et al.*, 1999;

Murakami *et al.*, 2000; Ploutno *et al.*, 2002; Radau *et al.*, 2003; Elert *et al.*, 2005; Fewer *et al.*, 2009, 2013) and compounds cytotoxic against human cancer cell lines (nostocyclopeptides) (Golakoti *et al.*, 2001) (Figure 3S). Some of these compounds might be responsible for the observed activities, however, we cannot exclude the possibility that the extraction procedure or/and the conditions of the LC-MS/MS analyses were not optimal for the detection of the active agent, which would therefore not appear in the LC-MS chromatogram .

Inhibition of enzyme activity

Protein phosphatases. Cyanobacterial toxins, microcystins and nodularin, are known inhibitors of PP1 and PP2A (McKintosh *et al.*, 1990; Ohta *et al.*, 1994). In our tests, all of the six extracts which contained these peptides, inhibited the activity of the enzymes (Table 3).

Compounds that modify the activity of PP have great potential in the treatment of diseases such as Parkinson's disease, cancer, myeloid leukemia or cardiac hypertrophy (McConnell & Wadzinski, 2009; Janssen & Rebollo, 2012). Due to the inhibitory effect on PP, the acute toxins, microcystins and nodularins, are also considered to be potential anticancer drug candidates (Monks *et al.*, 2007; Sainis *et al.*, 2010). In mammalian organisms exposed to microcystins, the toxins cross cell membranes of hepatocytes through the organic anion-transporting polypeptides (OATP). It was recently found that in cancer tissue, certain OATPs are over-expressed compared with normal tissues. As a consequence, the cancer cells are more sensitive to MCs and experience cytotoxic effects at lower concentrations than normal cells. Work on MC variants with high selectivity against cancer-specific OATPs is in progress (Monks *et al.*, 2007; Sainis *et al.*, 2010).

Another group of PP inhibitors detected in cyanobacterial extracts analysed in this work belong to the cyclic hexapeptides called anabaenopeptins (APs) (Sano *et al.*, 2001). The LC-MS/MS analyses revealed the presence of these compounds in all *Nodularia spumigena* strains (Table 2). Therefore, the observed inhibition of PP in samples with *N. spumigena* extracts probably results from the additive effects of nodularin and anabaenopeptins. As five extracts that did not contain any of these peptides also showed the activity against PP (CCNP1104, 1108, 1310, 1406 and 1411) (Table 3), we presume that the tested cyanobacteria produce other, as yet unknown, inhibitors of the enzymes.

Proteases: trypsin, elastase, thrombin & carboxypeptidase-A. In our study, 9 of the 27 analysed cyanobacteria (33%) were shown to produce metabolites active against at least one of the three proteases: trypsin, thrombin or carboxypeptidase-A, but none of the extracts was active against elastase (Table 3). Serine proteases are an important group of hydrolytic enzymes which take part in

such processes as digestion, blood coagulation, fibrinolysis, immune response and apoptosis (Di Cera, 2009). Naturally produced inhibitors of serine proteases are considered to be potential therapeutic agents in cardiovascular disorders, thrombosis, hypertension, viral infections and other health problems. Cyanobacterial extracts have been frequently reported to inhibit the activity of proteolytic enzymes of medicinal relevance such as chymotrypsin, trypsin, elastase, plasmin and thrombin (Kodani *et al.*, 1998; Ishida *et al.*, 1999; Itou *et al.*, 1999; Taori *et al.*, 2007, Lifshits *et al.*, 2011). Peptidic structures with protease inhibition activity (microginins, aeruginosins, cyanopeptolins, micropeptins, anabaenopeptins and microviridins) have been identified from *Microcystis*, *Planktothrix*, *Anabaena*, *Nostoc*, *Lyngbya*, and *Symploca* (Grach-Pogrebinsky *et al.*, 2003; Bubik *et al.*, 2008; Nagarajan *et al.*, 2012).

In this study extracts from the three *N. spumigena* strains and from *Spirulina subsalsa* CCNP1310 showed activity in a widest array of enzymatic assays, including protease inhibition assays (Table 3). In *N. spumigena*, (in addition to nodularin and APs), other linear and cyclic peptides classified to spumigins and aeruginosins were detected (Table 2). APs have been reported to be active towards trypsin (AP MM85 and Schizopeptin 791), chymotrypsin (AP MM823, AP MM850 and Oscillamide Y), elastase (AP MM823 and AP MM850) and carboxypeptidase-A (AP G, H, T, I, J and AP 915) (Sano & Kaya, 1995; Itou *et al.*, 1999; Murakami *et al.*, 2000). In *in vitro* assays, spumigins and aeruginosins, especially those with argininal at the C-terminus, inhibited the activity of thrombin and trypsin, even at nanomolar level (Ishida *et al.*, 1999; Fewer *et al.* 2009; Anas *et al.*, 2012). Inhibitory activity against thrombin and trypsin was also assigned to the presence of such peptides as micropeptins, cyanopeptolins, microviridins, banyasin, banyasides, and dehydroradiosumin (Namikoshi & Rinehart, 1996; Jaspar & Lawton, 1998; Kodani *et al.*, 1998; Ploutno *et al.*, 2002; Pluotno & Carmeli, 2005; Lifshits *et al.*, 2011).

In assays performed in this work, thrombin was selectively inhibited by the extracts from two strains of *Anabaena* (CCNP1405 and CCNP1406), while *Nostoc cf. edaphicum* (CCNP1411) inhibited only the activity of trypsin (Table 3). Among numerous peptides identified in CCNP1411, cyclic depsipeptides, cyanopeptolins, are the most probable metabolites responsible for the observed inhibition of trypsin (Namikoshi & Rinehart, 1996).

The activity of carboxypeptidase-A was selectively inhibited by the extracts from *Leptolyngbya* sp. CCNP1301 and *Lyngbya cf. aestuarii* CCNP1314. So far, APs are the only cyanobacterial metabolites whose activity against carboxypeptidase-A has been reported (Itou *et al.*, 1999; Murakami *et al.*, 2000), however, APs were not found in these two extracts.

Enzyme activities in cyanobacterial extracts

The API ZYM test indicated that the Baltic Cyanobacterial extracts constituted a rich source of various enzymes. Among the 19 enzymatic activities tested, alkaline phosphatase, acid phosphatase, esterase (C4 and C8), and naphthyl-AS-BI-phosphohydrolase were particularly common and were seen in 21–27 extracts (Table 4). However, the activities of cystine arylamidase, lipase (C14), α -galactosidase, β -galactosidase, and β -glucuronidase were detected in relatively few extracts (1–3). Significant differences were also observed in the number of enzyme activities detected in individual strains. The extracts from *Microcystis aeruginosa* CCNP1102, *Synechocystis salina* CCNP1104, *N. spumigena* CCNP1401 and CCNP1402, and *Anabaena* sp. CCNP1406 were the richest (11–15) in enzymatic activities, but only 2–4 were detected in *S. subsalsa* CCNP1310, *L. cf. aestuarii* CCNP1314, CCNP1316, *N. spumigena* CCNP1403, and *Anabaena* sp. CCNP1416 (Table 4). What is interesting, similar differences in the occurrence of enzymatic activities were observed within one cyanobacterial order when phenotypic and genotypic variabilities among *Microcystis* strains were studied (Yoshida *et al.*, 2008). These results indicate that production of specific classes of enzyme is a strain specific feature.

It is difficult to compare the level of occurrence of various enzymatic activities in Cyanobacteria tested in this work with Cyanobacteria from other geographic regions, as analogous reports are very rare in the literature. Usually, when considering cyanobacterial extracts, either specific enzymatic activities were analysed (see, for example: Haystead *et al.*, 1970; Schönherr & Keir, 1972; Papen *et al.*, 1983, 1986; Hai *et al.*, 2001; Chiba *et al.*, 2013) or researchers looked for enzyme inhibitors or activators rather than active proteins (Cannell *et al.*, 1987; Lau *et al.*, 1993; Tang *et al.*, 2007; Taori *et al.*, 2008; Zelik *et al.*, 2009; Bláha *et al.*, 2010; Anas *et al.*, 2012; Malloy *et al.*, 2012; Makhoulouf Brahmī *et al.*, 2013).

Due to their higher stability and activity, the enzymes produced by marine microorganisms are attracting increasing attention and are already in use as pharmaceuticals, food additives, cleansing additives, cosmetics components and industrial biocatalysts (Zhang & Kim, 2010; Trincone, 2011).

Immunological assay

In our work, 8 of the 27 tested cyanobacterial extracts caused an increase in the proliferation of mitogen-stimulated human T cells (Table 3). The stimulatory effects were observed in strains from the Oscillatoriales and Nostocales orders. This includes *Spirulina* strains whose positive effects on several immunological functions in mammals have been previously documented (Løbner *et al.*,

2008; Rasool & Sabina, 2009; Nielsen *et al.*, 2010). Cyanobacteria have been found to modulate the functioning of the immune system in different ways. Observed effects include, changes in phagocytic activity of macrophages, proliferation of T and B lymphocytes, activity of natural killer (NK) cells and production of antibodies and cytokines (Shen *et al.*, 2003; Løbner *et al.*, 2008; Nielsen *et al.*, 2010; Palikova *et al.*, 2013).

The role of cyanotoxins in the observed immunological activities has been considered by numerous authors. In some studies, the inhibition of immune functions by the toxin-producing cyanobacteria or cyanotoxins was observed (Yea *et al.* 2001; Shen *et al.*, 2003; Lankoff *et al.*, 2004; Palikova *et al.*, 2013). Shen *et al.* (2003) reported no changes in the proliferation of the concanavalin A-induced T cells, but there was reduced phagocytosis in mice exposed to the toxic *M. aeruginosa*. Stimulation of $\gamma\delta$ + T lymphocyte proliferation in the peripheral blood and spleen of rats with the externally added MCs ($5,000 \mu\text{g kg}^{-1}$) was revealed by Palikova *et al.* (2013). Other authors did not find any effects of the most toxic microcystin variant MC-LR on T cells (Teneva *et al.*, 2005), or decrease in their proliferation was only at the highest dose of the toxin ($25 \mu\text{g ml}^{-1}$) (Lankoff *et al.*, 2004). The tests performed in our experiments showed an increase in the proliferation of T cells on exposure to two of the three extracts from nodularin-producing *N. spumigena* and a lack of effect with the microcystin-producing *M. aeruginosa* (Table 3). It was postulated that different effects of cyanobacterial extracts and cyanotoxins on T cells might result from the differences in the experimental conditions or from the activity of unknown components of cyanobacterial cells (Palikova *et al.*, 2013). Due to the relatively small number of individuals tested, our results should be treated as preliminary. Still, they show potentially interesting effects of extracts from the Baltic cyanobacteria on the proliferation of human T lymphocytes.

Antimicrobial activity

Of the 27 cyanobacteria screened for antimicrobial activity, nine strains (33%) were shown to produce metabolites which inhibited the growth of at least one of the microorganisms (Table 5). This includes six cyanobacteria which also showed stimulatory effects on bacteria. For the most active extracts, the MIC₅₀ values were determined. The ethanol extract from *Phormidium* sp. CCNP1317 was characterized by having the widest spectrum of activities. It strongly inhibited the growth of six fouling Gammaproteobacteria: *Pseudomonas mendocina* Fo-6 (MIC₅₀ $1 \mu\text{g ml}^{-1}$), *Vibrio furnissii* Fo-10 (MIC₅₀ $1 \mu\text{g ml}^{-1}$), *Aliivibrio salmonicida* Fo-8 (MIC₅₀ $2 \mu\text{g ml}^{-1}$), *Alcanivorax borkumensis* Fo-7 (MIC₅₀ $1 \mu\text{g ml}^{-1}$), *Aeromonas hydrophila* subsp. *hydrophila* Fo-3 ATCC7966 (MIC₅₀ $4 \mu\text{g ml}^{-1}$) and *Pseudoalteromonas* sp. Fo-9 (MIC₅₀ $0.6 \mu\text{g ml}^{-1}$). In the agar disc diffusion assay, the inhibition zone

for these bacteria reached 10–15 mm. Sensitivity of the fouling Gram-positive *Planococcus donghaensis* Fo-2 (5 mm inhibition zone, MIC₅₀ 4 µg ml⁻¹) to *Phormidium* extract was also observed. The crude extract of *Cyanobacterium* sp. CCNP1105 showed mild antifouling activity against *A. hydrophila* subsp. *hydrophila* ATCC7966 and against *Pseudoalteromonas* sp. Fo-9 (inhibition zone 10 mm). On the other hand, the same extract significantly stimulated the growth of five other bacterial strains. A similar effect was seen with *Leptolyngbya* sp. CCNP1301 which showed mild inhibitory activity against *Pseudoalteromonas* sp. Fo-9, but stimulated the growth of five other strains (Table 5). Both the water and ethanol extracts from *M. aeruginosa* CCNP1102 were highly active against four strains representing different bacterial phyla: *Staphylococcus aureus* DMB-pR-1-20 (MIC₅₀ 42 µg ml⁻¹), *Micrococcus luteus* DMB-pR1-1 (MIC₅₀ 42 µg ml⁻¹), *Serratia mercerscens* DMBpR-1-2 (MIC₅₀ 74 µg ml⁻¹) and *Pseudomonas aeruginosa* DMB-pR-1-23 (MIC₅₀ 168 µg ml⁻¹). The same strains of bacteria were sensitive to extracts from *Anabaena* sp. CCNP1406 (MIC₅₀ 250-300 µg ml⁻¹), and (with the exception of *P. aeruginosa*), to the extracts from *S. salina* CCNP1104 (MIC₅₀ 250-300 µg ml⁻¹), *M. aeruginosa* CCNP1103 (MIC₅₀ 165 µg ml⁻¹) and *N. spumigena* CCNP1401 (weak activity) (Table 5). Only the extract from *Leptolyngbya* sp. CCNP1301 inhibited the growth of fungus, *Candida albicans* ATCC90028. Unfortunately, none of the Baltic cyanobacteria had influence on the methicillin-resistant *S. aureus* D2 used in our study.

As growing number of clinically important pathogens have developed multiresistance to the currently used antibiotics, there is a strong need for a new generation of antimicrobial agents. The potential of cyanobacteria to produce antimicrobial compounds has been explored by numerous research groups. The published works referred to such effects as inhibition/stimulation of Gram-positive, Gram-negative bacteria, fungi, algae and protozoa. The proportion of strains producing antimicrobial agents varied from 7% (Patterson *et al.*, 1994) to almost 50% (Jaki *et al.*, 1999). Significant activity was found mainly among the representatives of the Nostocales, Oscillatoriales and Chroococcales. Similarly to our work, Martins *et al.*(2008) tested the effects of aqueous and organic solvent extracts from 14 strains of *Synechococcus* and *Synechocystis* (Chroococcales). Nine of the extracts inhibited the growth of Gram-positive bacteria (*Clavibacter michiganensis* subsp. *insidiosum* and *Cellulomonas uda*), while no effects on Gram-negative bacteria and the fungus *Candida albicans* were observed. High resistance of Gram-negative bacteria was attributed to the protective role of the lipopolysaccharide layer of the outer membrane. In the work by Silva-Stenico *et al.* (2010), most of the metabolites with antimicrobial activity were produced by cyanobacteria from the Chroococcales. Of the 100 ethyl acetate and methanol extracts tested, 24 showed antimicrobial activity, especially against *Bacillus subtilis* and *Salmonella typhimurium*. Generally,

cyanobacterial extracts were more frequently active against bacteria than fungi (Silva-Stenico *et al.*, 2010). Such tendency was also observed in our study.

Although many tests have been performed, and strong antimicrobial activities were revealed in numerous cyanobacterial strains, few structures responsible for these activities have been identified. The compounds have mainly been classified to alkaloids, fatty acids, peptides, lipopeptides and terpenoids (Asthana *et al.*, 2006; Silva-Stenico *et al.*, 2010; Chlipala *et al.* 2011; Guedes *et al.*, 2011; Nagarajan *et al.*, 2012). In our studies, more than half of the pathogenic strains were sensitive to the extracts from the Baltic cyanobacteria. In further work, the metabolites responsible for the strongest inhibitory effects, (e.g. from CCNP1102, CCNP1317 and CCNP1406), should be identified and tested as potential antimicrobial agents.

Plant growth test

The most significant inhibition was observed in young sorghum plants exposed to the extracts from the nodularin-producing *N. spumigena* CCNP1403 (2.9 $\mu\text{g NOD ml}^{-1}$) (Table 6). In both water and ethanol extracts from this strain, the lengths of roots and shoots were reduced to 35.1– 42.7% of the control plants. Inhibitory effects were also seen in shoots of sorghum exposed to aqueous extract from *N. spumigena* CCNP1401 (2.6 $\mu\text{g NOD ml}^{-1}$), characterized by the same set of detected metabolites as CCNP1403 (Table 2). In the case of the third *N. spumigena* strain (CCNP1402) (1.5 $\mu\text{g NOD ml}^{-1}$), with a different pattern of metabolites (Table 2), no statistically significant effects were observed ($p < 0.01$) (Table 1S). Sorghum was also inhibited by the extracts from two of the three microcystin-producing *M. aeruginosa* strains: CCNP1101 (roots and shoots) and CCNP1103 (roots) (Table 6). The total concentrations of microcystins in the media were 4.5 $\mu\text{g ml}^{-1}$ (CCNP1101), 4.9 $\mu\text{g ml}^{-1}$ (CCNP1102) and 1.2 $\mu\text{g ml}^{-1}$ (CCNP1103), so they were in the range of concentrations used in other studies (0.1– 50 $\mu\text{g ml}^{-1}$). Some other extracts from the Baltic cyanobacteria, both with and without cyanotoxins, also caused slower growth of sorghum, compared to the control, but only for four of them the effects were statistically significant (CCNP1104, CCNP1105C, CNP1108, CCNP1312) (Table 1S). In general, it was the shoots of sorghum that were more frequently affected (Table 6).

The activity of cyanobacterial extracts against higher plants has mainly been tested using microcystin or nodularin-producing strains (e.g. McElhiney *et al.*, 2001; Gehringer *et al.*, 2003; Pflugmacher, 2007; Chen *et al.*, 2010; Lehtimäki *et al.*, 2011; El Khalloufi *et al.*, 2012). The activity of pure toxins on the growth and physiological functions in higher plants was also examined (McElhiney *et al.*, 2001; Gehringer *et al.*, 2003; Pflugmacher, 2007). Among the adverse effects, a

decrease in the rate of seed germination, inhibition of growth of roots and shoots and necrotic changes on leaves and shoots were most commonly observed. In addition, the toxin-containing extracts caused a decrease in photosynthetic activity, and changes in the activity of enzymes involved in antioxidative response (Pflugmacher, 2007; Chen *et al.*, 2010; El Khalloufi *et al.*, 2012; Shunmugam *et al.*, 2013). Lehtimäki *et al.* (2011) did not observe significant changes in the photosynthetic performance in spinach leaves exposed to the extract from *N. spumigena* AV1. However, the authors revealed an increased amount of cytochrome oxidase and a high respiratory activity in the treated leaves. Further studies (El Khalloufi *et al.*, 2012) confirmed that oxidative stress in spinach, regularly watered with the solution of AV1 extract, was related to the upregulation of mitochondrial respiratory electron transfer complexes in the plant. The adverse effects of cyanobacterial toxins on higher plants were also assigned to the inhibition of protein phosphatases 1 and 2A (McKintosh *et al.*, 1990).

In some of the published studies, more pronounced effects were seen in plants treated with the crude cyanobacterial extracts rather than with pure cyanotoxins. In our work, not all extracts from toxin-producing strains were active against sorghum. On the other hand, significant inhibitory activity was observed with non-toxic cyanobacteria (e.g. *S. salina* CCNP1104, *Synechocystis* sp. CCNP1108, *Cyanobacterium* sp. CNP1105 and *Pseudanabaena* cf. *galeata* CCNP1312) (Table 6). In the view of these findings we support the hypothesis that microcystins and nodularin are not the only cyanobacterial metabolites that show herbicidal activity (Gehring *et al.*, 2003; Pflugmacher, 2007).

CONCLUSIONS

On the basis of the screening experiments performed with the application of a wide array of assays, the strains most interesting as producers of active agents were selected. *N. spumigena* CCNP1401 turned out to be active in the highest number of tests. Together with other *N. spumigena* strains and *Nostoc* cf. *edaphicum*, it also produces the highest number of non-ribosomal peptides. As activity of these peptides was previously documented, part of the effects observed in our work was attributed to their presence in the extracts. *Phormidium* sp. CCNP1317 also deserves further attention as it showed strong antimicrobial activity against the highest number of pathogenic bacteria.

However, a broad spectrum of activities can be a disqualifying feature of an extract, if the observed activities are attributed to the same metabolite. The natural products which are highly active in screening experiments can have little or no application as they often appear to be too toxic and/or cause different side effects. Therefore, the second criterion that we used to select the strains

for further studies was the selectivity in the induced effects. In this respect, *Anabaena* CCNP1405 and CCNP1406 which strongly inhibited activity of thrombin, and had no effect on trypsin, seem to be of some interest. In addition, *Anabaena* sp. CCNP1406 was shown to produce the highest number of commercially important enzymes.

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AUTHOR CONTRIBUTIONS

H. Mazur-Marzec: LC-MS/MS analyses, writing the manuscript, G. Węgrzyn: text revision and manuscript editing, A. Felczykowska (contribution to writing text on enzymatic activity), S. Bloch, B. Nejman-Faleńczyk: enzymatic activity and antimicrobial activity tests, A. Błaszczuk: enzyme inhibition assays, contribution to writing text on enzyme inhibition assays, N. Hohlfeld: enzyme inhibition assays, plant growth assay, Justyna Kobos: isolation and culturing of cyanobacteria, Anna Toruńska-Sitarz: genetic analyses, antimicrobial tests, contribution to text on antimicrobial activity, P. Devi, S. Montalvão, L. D'Souza, P. Tammela: antimicrobial tests, Anna Mikosik: immunological tests, contribution to writing text on immunological activity.

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Table 1. Baltic cyanobacterial strains used in this study, time and place of isolation, culture media and accession numbers.

No.	Strain of Cyanobacteria	Strain Code	Isolation		Culture Medium	Accession Number (GenBank)
			Year	Area		
Chroococcales						
1	<i>Microcystis aeruginosa</i>	CCNP1101	2005	Vistula Lagoon	Z8	KJ161446
2	<i>Microcystis aeruginosa</i>	CCNP1102	2005	Vistula Lagoon	Z8	FN678904
3	<i>Microcystis aeruginosa</i>	CCNP1103	2005	Vistula Lagoon	Z8	FN678905
4	<i>Microcystis viridis</i>	CCNP1106	2011	Southern Baltic Sea	Z8	KJ161449
5	<i>Synechocystis salina</i>	CCNP1104	2006	Gulf of Gdańsk	Z8S	KJ161448
6	<i>Synechocystis</i> sp.	CCNP1108	2012	Gulf of Gdańsk	Z8S	KJ161447
7	<i>Cyanobacterium</i> sp.	CCNP1105	2010	Gulf of Gdańsk	Z8S	KJ161433
Oscillatoriales						
8	<i>Leptolyngbya</i> sp.	CCNP1301	2004	Gulf of Gdańsk	Z8S	KJ161434
9	<i>Leptolyngbya</i> sp.	CCNP1302	2005	Puck Bay	Z8S	KJ161435
10	<i>Leptolyngbya</i> sp.	CCNP1308	2009	Gulf of Gdańsk	Z8S	KJ161436
11	<i>Spirulina subsalsa</i>	CCNP1310	2009	Puck Bay	Z8S	KJ161437
12	<i>Pseudanabaena</i> sp.	CCNP1311	2009	Gulf of Gdańsk	Z8	KJ161438
13	<i>Pseudanabaena</i> cf. <i>galeata</i>	CCNP1312	2010	Gulf of Gdańsk	Z8	KJ161439
14	<i>Pseudanabaena</i> cf. <i>galeata</i>	CCNP1313	2010	Gulf of Gdańsk	Z8	KJ161440
15	<i>Lynngbya</i> cf. <i>aestuarii</i>	CCNP1314	2010	Gulf of Gdańsk	Z8S	KJ161441
16	<i>Lynngbya</i> cf. <i>aestuarii</i>	CCNP1316	2011	Puck Bay	F/2	KJ161442
17	<i>Phormidium</i> sp.	CCNP1317	2011	Gulf of Gdańsk	Z8S	KJ161443
Nostocales						
18	<i>Nodularia spumigena</i>	CCNP1401	1997	Gulf of Gdańsk	Z8S	KJ161425
19	<i>Nodularia spumigena</i>	CCNP1402	2005	Gulf of Gdańsk	Z8S	KJ161426
20	<i>Nodularia spumigena</i>	CCNP1403	2009	Gulf of Gdańsk	Z8S	KJ161427
21	<i>Trichormus variabilis</i>	CCNP1404	2005	Gulf of Gdańsk	Z8S	KJ161428
22	<i>Anabaena cylindrica</i>	CCNP1405	2005	Gulf of Gdańsk	Z8Sx	KJ161429
23	<i>Anabaena</i> sp.	CCNP1406	2005	Gulf of Gdańsk	Z8x	KJ161430
24	<i>Anabaena</i> sp.	CCNP1407	2005	Gulf of Gdańsk	Z8Sx	KJ161431
25	<i>Anabaena</i> sp.	CCNP1416	2011	Gulf of Gdańsk	Z8Sx	KJ161432
26	<i>Anabaena</i> sp.	CCNP1417	2010	Gulf of Gdańsk	Z8Sx	KJ161444
27	<i>Nostoc</i> cf. <i>edaphicum</i>	CCNP1411	2010	Gulf of Gdańsk	Z8x	KJ161445

Table 2. Metabolites detected in the Baltic cyanobacteria: **MC** – microcystin, **NOD** – nodularin, **AP** – anabaenopeptin, **AER** – aeruginosin, **SPU** – spumigin, **CPL** – cyanopeptolin, **NCP** – nostocyclopeptide.

CCNP Strain Code	Detected Metabolites and <i>m/z</i> of their [M+H] ⁺ ion	Total Concentration of MCs or NOD [$\mu\text{g g d.w.}^{-1}$]
1101	Unknown: 1044 MC : 1009 (MC-HilR), 995 (MC-LR), 981 ([Asp ³ ,Dhb ⁷]MC-LR) AER 102: 653	4.5
1102	Unknown: 1044, 1004 MC : 1009 (MC-HilR), 995 (MC-LR), 981 ([Asp ³ ,Dhb ⁷]MC-LR) AER 102: 653	4.9
1103	Unknown: 1044, 1022 MC : 1009 (MC-HilR), 995 (MC-LR), 981 ([Asp ³ ,Dhb ⁷]MC-LR) AER 102: 653	1.2
1106	CPL : 1049, 1038, 1021, 993	
1310	Unknown: 613, 617, 622	
1314	Unknown: 640, 638, 622, 618	
1316	Unknown: 708, 694	
1401	AP : 842, 808 NOD : 825, 811 ([Asp ³]NOD) AER : 603, 589, 587, 559 SPU : 655, 653, 641, 639, 627, 613, 611, 599, 597, 583, 575, 457	2.6
1402	AP : 932, 916, 914, 900, 898, 884, 882, 880, 872, 856 NOD : 825, 811 ([Asp ³]NOD) AER : 603, 589, 587, 559 SPU : 627, 613, 611, 599, 595, 597, 457	1.5
1403	AP : 842, 808 NOD : 825, 811 ([Asp ³]NOD) AER : 603, 589, 587, 559 SPU : 655, 653, 641, 639, 627, 613, 611, 599, 597, 583, 575, 457	2.9
1417	Unknown: 1153	
1411	CPL : 1021, 1017, 1012, 1010, 1007, 996, 994, 985, 982, 981, 979, 972, 968, 960, 952, 902 NCP : 809, 794, 776, 757 Unknown: 1007, 994 Unknown: 628	

Table 3. Inhibition of enzymes by cyanobacterial extracts (CPA – Carboxypeptidase-A; PP 1 – Protein phosphatase 1; PP 2A - Protein phosphatase 2A; W – water extracts; E – ethanol extracts) and effect of the extract on the proliferation of the mitogen-stimulated human T cells (T). I – inhibition observed up to 100×dilution; I* - inhibition observed up to 1,000×dilution, I** - inhibition observed up to 10,000×dilution; S – increased proliferation of T cells; S* - the highest proliferation of T cells (200–230% of control); ● - no effect.

No.	Strain of Cyanobacteria	Strain Code	PP 1		PP 2A		Elastase		Trypsin		Thrombin		CPA		T
			W	E	W	E	W	E	W	E	W	E	W	E	
1	<i>Microcystis aeruginosa</i>	CCNP1101	I**	I**	I**	I**	●	●	●	●	●	●	●	●	●
2	<i>Microcystis aeruginosa</i>	CCNP1102	I**	I**	I**	I**	●	●	●	●	●	●	●	●	●
3	<i>Microcystis aeruginosa</i>	CCNP1103	I**	I**	I**	I**	●	●	●	●	●	●	●	●	●
4	<i>Microcystis viridis</i>	CCNP1106	●	●	●	●	●	●	●	●	●	●	●	●	●
5	<i>Synechocystis salina</i>	CCNP1104	I	I	●	●	●	●	●	●	●	●	●	●	●
6	<i>Synechocystis</i> sp.	CCNP1108	●	●	●	I	●	●	●	●	●	●	●	●	●
7	<i>Cyanobacterium</i> sp.	CCNP1105	●	●	●	●	●	●	●	●	●	●	●	●	●
8	<i>Leptolyngbya</i> sp.	CCNP1301	●	●	●	●	●	●	●	●	●	I	●	●	●
9	<i>Leptolyngbya</i> sp.	CCNP1302	●	●	●	●	●	●	●	●	●	●	●	●	●
10	<i>Leptolyngbya</i> sp.	CCNP1308	●	●	●	●	●	●	●	●	●	●	●	●	●
11	<i>Spirulina subsalsa</i>	CCNP1310	I*	I*	I*	I*	●	●	I*	●	I*	●	I*	●	S*
12	<i>Pseudanabaena</i> sp.	CCNP1311	●	●	●	●	●	●	●	●	●	●	●	●	S
13	<i>Pseudanabaena</i> cf. <i>galeata</i>	CCNP1312	●	●	●	●	●	●	●	●	●	●	●	●	●
14	<i>Pseudanabaena</i> cf. <i>galeata</i>	CCNP1313	●	●	●	●	●	●	●	●	●	●	●	●	●
15	<i>Lyngbya</i> cf. <i>aestuarii</i>	CCNP1314	●	●	●	●	●	●	●	●	●	●	●	I	S*
16	<i>Lyngbya</i> cf. <i>aestuarii</i>	CCNP1316	●	●	●	●	●	●	●	●	●	●	●	●	S*
17	<i>Phormidium</i> sp.	CCNP1317	●	●	●	●	●	●	●	●	●	●	●	●	●
18	<i>Nodularia spumigena</i>	CCNP1401	I**	I**	I**	I**	●	●	I*	I*	I*	I*	I*	I*	S
19	<i>Nodularia spumigena</i>	CCNP1402	I**	I**	I**	I**	●	●	I*	I*	I*	I*	I*	I*	●
20	<i>Nodularia spumigena</i>	CCNP1403	I**	I**	I**	I**	●	●	I*	I*	I*	I*	I*	I*	S
21	<i>Trichormus variabilis</i>	CCNP1404	●	●	●	●	●	●	●	●	●	●	●	●	S
22	<i>Anabaena cylindrica</i>	CCNP1405	●	●	●	●	●	●	●	●	I	I	●	●	S
23	<i>Anabaena</i> sp.	CCNP1406	I	I	●	●	●	●	●	●	I	I	●	●	●
24	<i>Anabaena</i> sp.	CCNP1407	●	●	●	●	●	●	●	●	●	●	●	●	●
25	<i>Anabaena</i> sp.	CCNP1416	●	●	●	●	●	●	●	●	●	●	●	●	●
26	<i>Anabaena</i> sp.	CCNP1417	●	●	●	●	●	●	●	●	●	●	●	●	●
27	<i>Nostoc</i> cf. <i>edaphicum</i>	CCNP1411	I*	I*	●	●	●	●	●	I	●	●	●	●	●

Table 4. Enzymatic activities in cyanobacterial extracts; A – enzymatic activity; • – lack of enzymatic activity.

CCNP Strain Code	Enzyme																		
	Alkaline phosphatase	Esterase (C4)	Esterase lipase (C8)	Lipase (C14)	Leucine arylamidase	Valine arylamidase	Cystine arylamidase	Trypsin	α -chymotrypsin	Acid phosphatase	Naphtyl-AS-BI-phosphohydrolase	α -galactosidase	β -galactosidase	β -glucuronidase	α -glucosidase	β -glucosidase	N-acetyl- β -glukozaminidase	α -mannosidase	α -fukosidase
1101	A	A	A	A	A	A	•	•	•	A	A	•	•	•	•	A	•	•	•
1102	A	A	A	•	A	A	•	•	•	A	A	•	A	A	A	•	A	•	•
1103	A	A	A	•	A	A	•	•	•	A	A	•	•	•	•	A	•	•	•
1106	•	A	A	•	A	•	•	•	•	A	A	•	A	•	A	•	•	•	•
1104	A	A	A	•	A	A	•	A	•	A	A	A	•	•	A	A	A	A	A
1108	A	A	A	•	A	A	•	A	•	A	A	•	•	•	•	•	•	•	•
1105	A	A	A	•	A	A	•	A	•	A	A	•	•	•	•	•	•	•	•
1301	A	A	A	•	•	•	•	•	A	A	A	•	•	•	•	•	•	•	•
1302	A	A	A	•	A	•	•	•	•	A	A	•	•	•	•	•	•	•	•
1308	A	A	A	•	A	•	•	•	•	A	A	•	•	•	•	•	•	•	•
1310	A	•	•	•	•	•	•	A	•	•	A	•	•	•	•	•	•	•	•
1311	•	•	•	•	A	A	•	A	•	•	A	•	•	•	•	A	•	•	•
1312	A	A	A	•	A	A	•	A	•	A	A	A	•	•	•	•	A	•	•
1313	A	A	A	•	A	A	•	A	A	•	A	•	•	•	A	A	•	•	•
1314	A	•	•	•	•	•	•	•	•	•	A	•	•	•	•	•	•	•	•
1316	A	•	•	•	•	•	•	•	•	A	A	•	•	•	•	•	•	•	•
1317	A	A	A	•	•	•	•	•	A	A	A	•	•	•	•	•	•	•	•
1401	A	A	A	•	•	•	•	A	•	A	A	•	•	•	A	A	A	A	A
1402	A	A	A	•	A	A	•	A	•	A	A	•	•	•	A	•	A	•	A
1403	•	•	•	•	•	•	•	•	•	A	A	•	•	•	•	•	•	•	•
1404	A	A	A	•	A	•	•	A	•	A	A	•	•	•	•	•	A	•	•
1405	A	A	A	•	•	•	•	•	•	A	A	•	•	•	•	•	•	•	•
1406	A	A	A	A	A	A	A	A	A	A	A	•	•	•	•	A	A	A	A
1407	A	A	A	•	•	•	•	•	•	A	A	•	•	•	•	•	•	•	•
1416	•	•	•	•	•	A	•	•	•	A	A	A	•	•	•	•	•	•	•
1417	A	A	A	•	•	•	•	•	•	A	A	•	•	•	•	•	•	•	•
1411	A	A	A	•	A	•	•	•	A	A	A	•	•	•	•	•	•	•	•

Table 5. Effect of crude cyanobacterial extracts on the growth of bacteria and fungi; W – water extracts; E – ethanol extracts; I – growth inhibition; S – growth stimulation; ● – no effect; “-“ – no data.

CCNP Strain Code	BACTERIA Gram+										BACTERIA Gram-												FUNGI					
	1. <i>Staphylococcus aureus</i> DMB-pR1-20 2. <i>Staphylococcus aureus</i> ATCC25923 3. MRSA D2 4. <i>Enterococcus faecalis</i> ATCC29212 5. <i>Planococcus donghaensis</i> Fo-2 6. <i>Micrococcus luteus</i> DMB-pR1-1 7. <i>Erythrobacter litoralis</i> Fo-5										8. <i>Serratia mercrescens</i> DMB-pR-1-2 9. <i>Klebsiella pneumoniae</i> B7 10. <i>Escherichia coli</i> ATCC25922 11. <i>Shigella flexneri</i> B4 12. <i>Pseudomonas aeruginosa</i> DMB-pR1-23 13. <i>Pseudomonas mendocina</i> Fo-6 14. <i>Vibrio cholerae</i> B6 15. <i>Vibrio furnissii</i> Fo-10 16. <i>Aliivibrio salmonicida</i> Fo-8 17. <i>Alcanivorax borkumensis</i> Fo-7 18. <i>Alcanivorax</i> sp Fo-1 19. <i>Aeromonas hydrophila</i> Fo-3 ATCC7966 20. <i>Aeromonas salmonicida</i> Fo-4 21. <i>Pseudalteromonas</i> sp. Fo-9												22. <i>Aspergillus fumigatus</i> F1 23. <i>Cryptococcus neoformans</i> F5 24. <i>Candida albicans</i> ATCC 90028					
	W	E	E	E	E	E	W	E	E	E	W	E	E	E	E	W	E	E	E	E	E	E	E	E	E	E	E	E
1101	S	•	•	•	•	S	•	•	•	S	•	•	•	•	S	•	•	•	•	•	•	•	•	•	•	•	•	•
1102	I	I	•	•	•	I	I	•	•	I	I	•	•	•	I	•	•	•	•	•	•	•	•	•	•	•	•	•
1103	•	I	•	-	•	•	I	I	-	•	I	-	•	•	•	-	-	-	-	-	-	-	-	-	-	-	-	•
1106	•	•	•	•	-	•	S	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
1104	I	•	•	•	•	I	•	•	•	I	I	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
1108	•	•	-	-	-	S	•	-	-	•	•	•	-	-	•	-	-	-	-	-	-	-	-	-	-	-	-	•
1105	S	S	S	-	-	•	S	S	•	S	•	•	•	S	•	I	•	•	•	•	I	•	•	I	•	•	•	•
1301	•	S	S	•	-	•	S	•	•	S	S	•	•	•	S	•	•	•	•	•	•	•	I	•	•	•	I	•
1302	S	S	-	-	-	S	S	-	-	S	•	•	-	-	S	•	-	-	-	-	-	-	-	-	-	-	-	-
1308	S	S	-	-	-	S	S	-	-	S	S	-	-	-	S	S	-	-	-	-	-	-	-	-	-	-	-	-
1310	S	•	•	•	•	•	S	S	•	•	•	•	•	•	S	S	•	•	•	•	•	•	•	•	•	•	•	S
1311	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
1312	S	S	•	•	•	•	•	•	•	S	•	•	•	•	S	S	•	•	•	•	•	•	•	•	•	•	•	•
1313	•	•	S	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
1314	•	•	•	•	•	•	I	•	•	•	•	•	•	•	S	•	-	-	-	-	-	-	-	-	-	-	-	•
1316	S	•	-	-	-	S	S	-	-	•	•	•	•	•	S	S	-	-	-	-	-	-	-	-	-	-	-	•
1317	•	•	•	•	•	I	S	•	•	•	•	•	•	•	S	I	•	I	I	I	I	•	I	•	I	•	•	•
1401	•	I	•	•	•	•	I	•	•	•	I	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	S
1402	S	•	•	-	•	-	S	S	-	S	•	-	•	-	S	S	-	-	-	-	-	-	-	-	-	-	-	•
1403	•	•	•	•	•	•	S	•	•	•	•	•	•	•	S	S	•	•	•	•	•	•	•	•	•	•	•	•
1404	S	•	•	•	•	•	S	•	•	•	•	•	•	•	S	•	•	•	•	•	•	•	•	•	•	•	•	•
1405	S	•	S	•	•	•	S	•	•	•	•	•	•	•	S	•	•	•	•	•	•	•	•	•	•	•	•	•
1406	I	I	•	•	•	•	I	I	•	I	I	•	•	•	I	I	•	•	•	•	•	•	•	•	•	•	•	S
1407	S	S	•	-	•	-	S	-	-	S	S	-	•	•	-	-	-	-	-	-	-	-	-	-	-	-	-	•
1416	•	•	•	-	•	-	•	•	-	•	•	-	•	•	•	-	-	-	-	-	-	-	-	-	-	-	-	•
1417	•	•	•	-	•	-	•	•	-	S	•	-	•	•	•	-	-	-	-	-	-	-	-	-	-	-	-	•
1411	S	S	•	•	•	•	S	•	•	S	S	-	•	•	S	•	•	•	•	•	•	•	•	•	•	•	•	-

Table 6. Effect of cyanobacterial extracts on the growth of young roots and shoots of monocotyl *Sorghum saccharatum*; W – water extracts; E – ethanol extracts; I – inhibition 50-70% Control; I* - inhibition 28-49% Control; • – no effect.

No.	Strain Cyanobacteria	of Strain Code	<i>Sorghum saccharatum</i>			
			Shoot (W)	Root (W)	Shoot (E)	Root (E)
1	<i>Microcystis aeruginosa</i>	CCNP1101	•	•	I*	I*
2	<i>Microcystis aeruginosa</i>	CCNP1102	•	•	•	•
3	<i>Microcystis aeruginosa</i>	CCNP1103	•	I	•	•
4	<i>Microcystis viridis</i>	CCNP1106	•	•	•	•
5	<i>Synechocystis salina</i>	CCNP1104	I*	•	•	•
6	<i>Synechocystis</i> sp.	CCNP1108	•	•	I*	•
7	<i>Cyanobacterium</i> sp.	CCNP1105	I	•	I*	•
8	<i>Leptolyngbya</i> sp.	CCNP1301	•	•	•	•
9	<i>Leptolyngbya</i> sp.	CCNP1302	•	•	•	•
10	<i>Leptolyngbya</i> sp.	CCNP1308	•	•	•	•
11	<i>Spirulina subsalsa</i>	CCNP1310	•	•	•	•
12	<i>Pseudanabaena</i> sp.	CCNP1311	•	•	•	•
13	<i>Pseudanabaena</i> cf. <i>galeata</i>	CCNP1312	I*	•	•	•
14	<i>Pseudanabaena</i> cf. <i>galeata</i>	CCNP1313	•	•	•	•
15	<i>Lyngbya</i> cf. <i>aestuarii</i>	CCNP1314	•	•	•	•
16	<i>Lyngbya</i> cf. <i>aestuarii</i>	CCNP1316	•	•	•	•
17	<i>Phormidium</i> sp.	CCNP1317	•	•	•	•
18	<i>Nodularia spumigena</i>	CCNP1401	I	•	•	•
19	<i>Nodularia spumigena</i>	CCNP1402	•	•	•	•
20	<i>Nodularia spumigena</i>	CCNP1403	I*	I*	I*	I*
21	<i>Trichormus variabilis</i>	CCNP1404	•	•	•	•
22	<i>Anabaena cylindrica</i>	CCNP1405	•	•	•	•
23	<i>Anabaena</i> sp.	CCNP1406	•	•	•	•
24	<i>Anabaena</i> sp.	CCNP1407	•	•	•	•
25	<i>Anabaena</i> sp.	CCNP1416	•	•	•	•
26	<i>Anabaena</i> sp.	CCNP1417	•	•	•	•
27	<i>Nostoc</i> cf. <i>edaphicum</i>	CCNP1411	•	•	•	•

Supplementary Information

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Figure 2S. Total ion current nLC-MS chromatograms illustrating the metabolite diversity among the cyanobacteria: *Nostoc cf. edaphicum* CCNP1411 (A), *Nodularia spumigena* CCNP1401 (B), *Spirulina subsalsa* 1310 (C), *Phormidium* sp. CCNP1317 (D).

Figure 3S. Ion product mass spectra of peptides identified in *Nostoc cf. edaphicum* CCNP1411 from the Baltic Sea: cyanopeptolin with m/z 982 (A), cryptophycin with m/z 628 (B), nostocyclopeptide with m/z 809 (C).

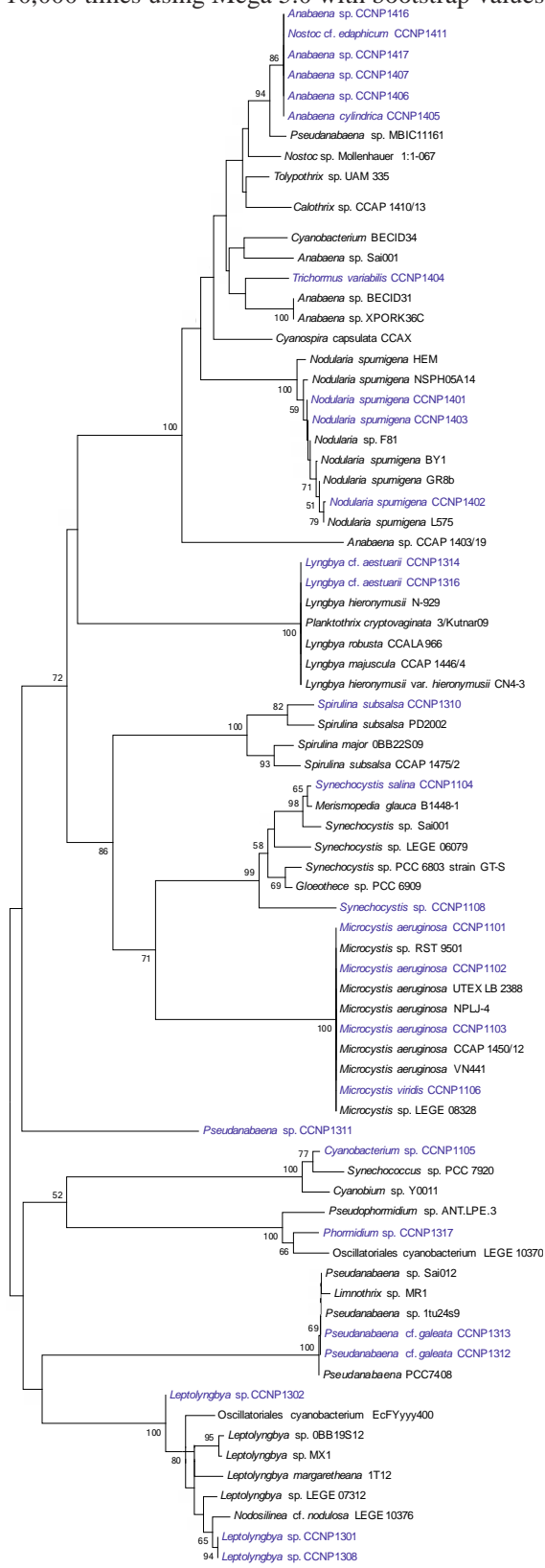
Figure 4S. Ion product mass spectra of peptides identified in the Baltic isolates of *Microcystis aeruginosa*: aeruginosin 102 with m/z 653 (CCNP1101) (A), microcystin [Asp³, Dhb⁷]MC-LR with m/z 981 (CCNP1103) (B).

Figure 5S. Ion product mass spectra of peptides identified in *Nodularia spumigena* CCNP1402 from the Baltic Sea: spumigin with m/z 611 (A), anabaenopeptin with m/z 900 (B).

Table 1S Effect of cyanobacterial extracts on the growth of young roots and shoots of monocotyl *Sorghum saccharatum*; results analysis carried out with the non-parametric Mann-Whitney U test ($p < 0.01$) using STATISTICA.

No.	Strain of Cyanobacteria	Strain Code	<i>Sorghum saccharatum</i>			
			Level of Significance $p < 0,01$			
			Shoot (W)	Root (W)	Shoot (E)	Root (E)
1	<i>Microcystis aeruginosa</i>	CCNP1101	0.499	0.505	0.0002	0.0001
2	<i>Microcystis aeruginosa</i>	CCNP1102	0.087	0.0920	0.754	0.770
3	<i>Microcystis aeruginosa</i>	CCNP1103	0.016	0.009	0.065	0.067
4	<i>Microcystis viridis</i>	CCNP1106	0.274	0.286	0.011	0.019
5	<i>Synechocystis salina</i>	CCNP1104	0.00004	0.06	0.039	0.039
6	<i>Synechocystis</i> sp.	CCNP1108	0.02	0.06	0.0003	0.017
7	<i>Cyanobacterium</i> sp.	CCNP1105	0.003	0.02	0.0005	0.02
8	<i>Leptolyngbya</i> sp.	CCNP1301	0.111	0.115	0.416	0.431
9	<i>Leptolyngbya</i> sp.	CCNP1302	0.754	0.770	0.472	0.488
10	<i>Leptolyngbya</i> sp.	CCNP1308	0.416	0.431	0.348	0.362
11	<i>Spirulina subsalsa</i>	CCNP1310	0.045	0.046	0.067	0.070
12	<i>Pseudanabaena</i> sp.	CCNP1311	0.851	0.865	0.014	0.04
13	<i>Pseudanabaena</i> cf. <i>galeata</i>	CCNP1312	0.0005	0.020	0.075	0.069
14	<i>Pseudanabaena</i> cf. <i>galeata</i>	CCNP1313	0.253	0.356	0.654	0.598
15	<i>Lyngbya</i> cf. <i>aestuarii</i>	CCNP1314	0.154	0.167	0.024	0.038
16	<i>Lyngbya</i> cf. <i>aestuarii</i>	CCNP1316	0.288	0.300	0.049	0.049
17	<i>Phormidium</i> sp.	CCNP1317	0.110	0.115	0.875	0.890
18	<i>Nodularia spumigena</i>	CCNP1401	0.011	0.055	0.014	0.025
19	<i>Nodularia spumigena</i>	CCNP1402	0.022	0.095	0.085	0.098
20	<i>Nodularia spumigena</i>	CCNP1403	0.00002	0.00008	0.00001	0.00007
21	<i>Trichormus variabilis</i>	CCNP1404	0.014	0.019	0.075	0.085
22	<i>Anabaena cylindrica</i>	CCNP1405	0.111	0.115	0.021	0.025
23	<i>Anabaena</i> sp.	CCNP1406	0.070	0.072	0.012	0.110
24	<i>Anabaena</i> sp.	CCNP1407	0.005	0.004	0.011	0.012
25	<i>Anabaena</i> sp.	CCNP1416	0.654	0.633	0.094	0.096
26	<i>Anabaena</i> sp.	CCNP1417	0.453	0.469	0.033	0.029
27	<i>Nostoc</i> cf. <i>edaphicum</i>	CCNP1411	0.339	0.413	0.052	0.043

Figure 1S. Neighbor-joining phylogenetic tree illustrating the diversity of cyanobacterial strains from CCNP collection, based on partial 16S rRNA gene sequences (547 bp). Phylogenetic relationships were bootstrapped 10,000 times using Mega 5.0 with bootstrap values >50% shown.



A 16S rRNA gene phylogeny was constructed to gain insight into the cyanobacterial diversity among the tested strains. The sequences obtained from CCNP isolates were queried using BLASTn, and the corresponding top-scoring sequences were used in the phylogenetic analysis.

Figure 2S. Total ion current nLC-MS chromatograms illustrating the metabolite diversity among the cyanobacteria: *Nostoc cf. edaphicum* CCNP1411 (A), *Nodularia spumigena* CCNP1401 (B), *Spirulina subsalsa* 1310 (C), *Phormidium* sp. CCNP1317 (D).

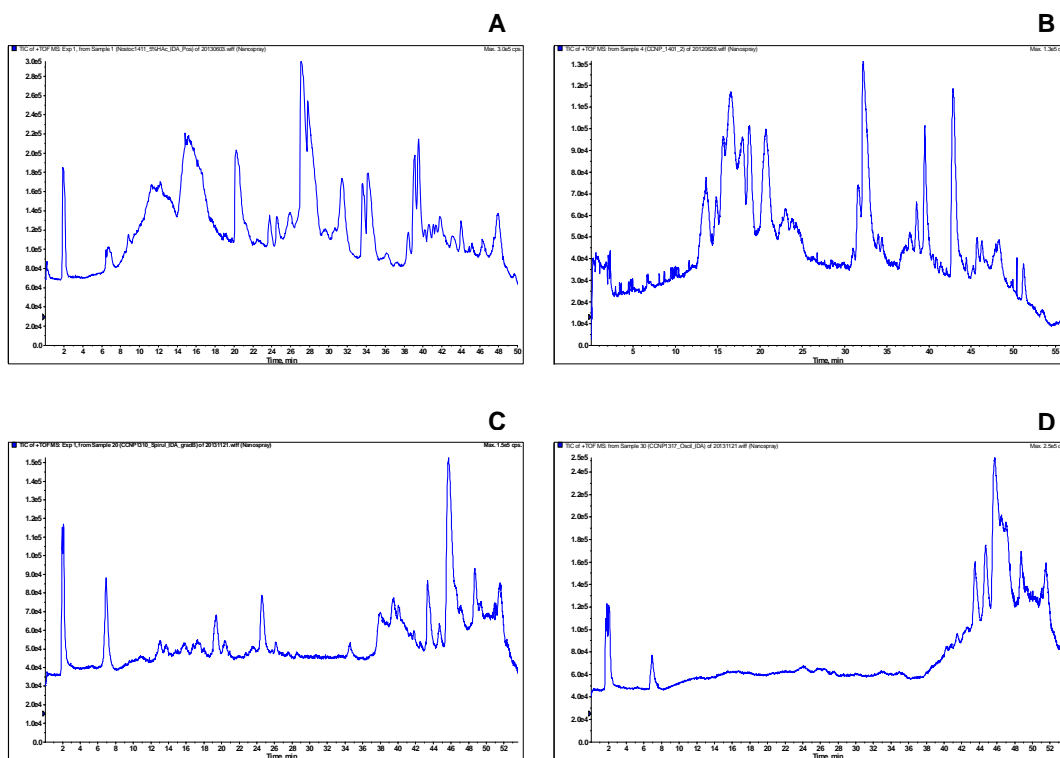


Figure 3S. Ion product mass spectra of peptides identified in *Nostoc cf. edaphicum* CCNP1411 from the Baltic Sea: cyanopeptolin with m/z 968 (A), nostocyclopeptide with m/z 809 (B).

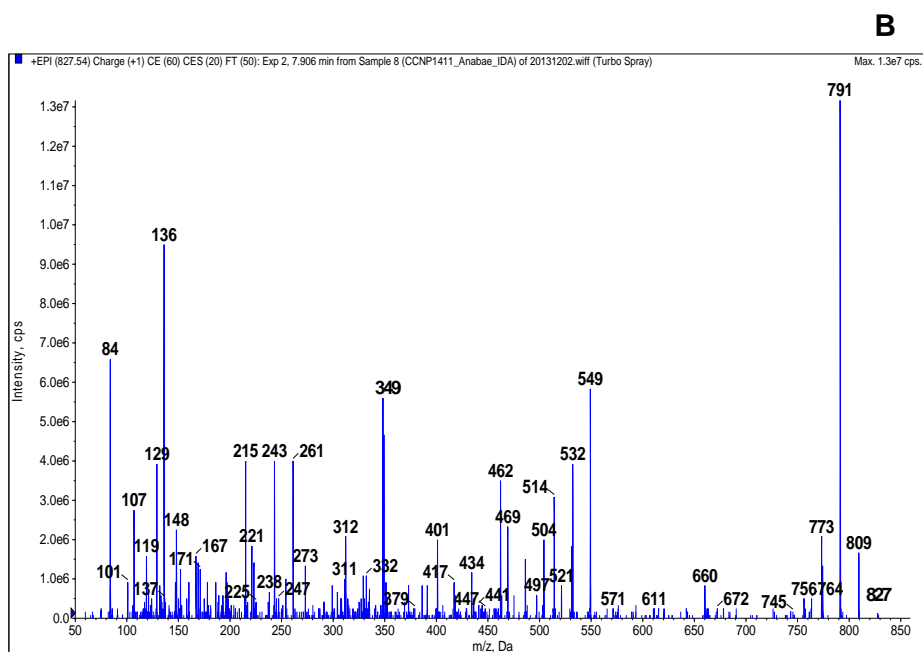
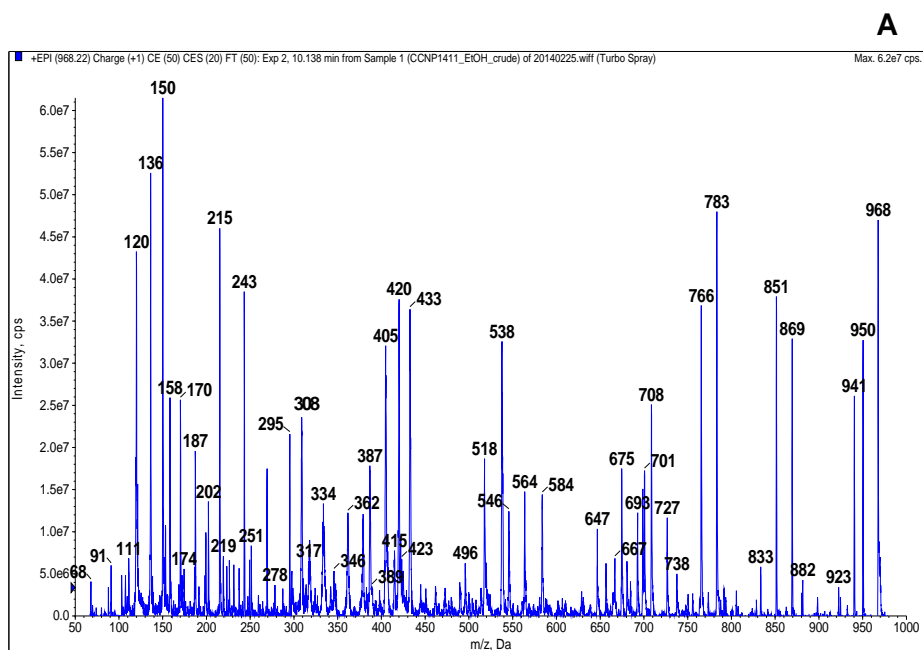


Figure 4S. Ion product mass spectra of peptides identified in the Baltic isolates of *Microcystis aeruginosa*: aeruginosin 102 with m/z 653 (CCNP1101) (A), microcystin [Asp³, Dhb⁷]MC-LR with m/z 981 (CCNP1103) (B).

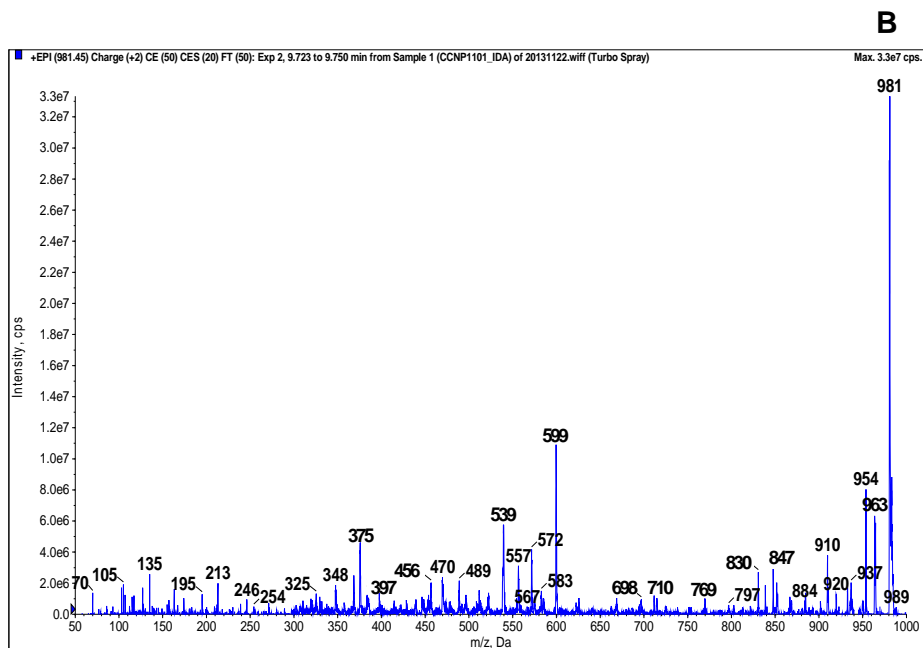
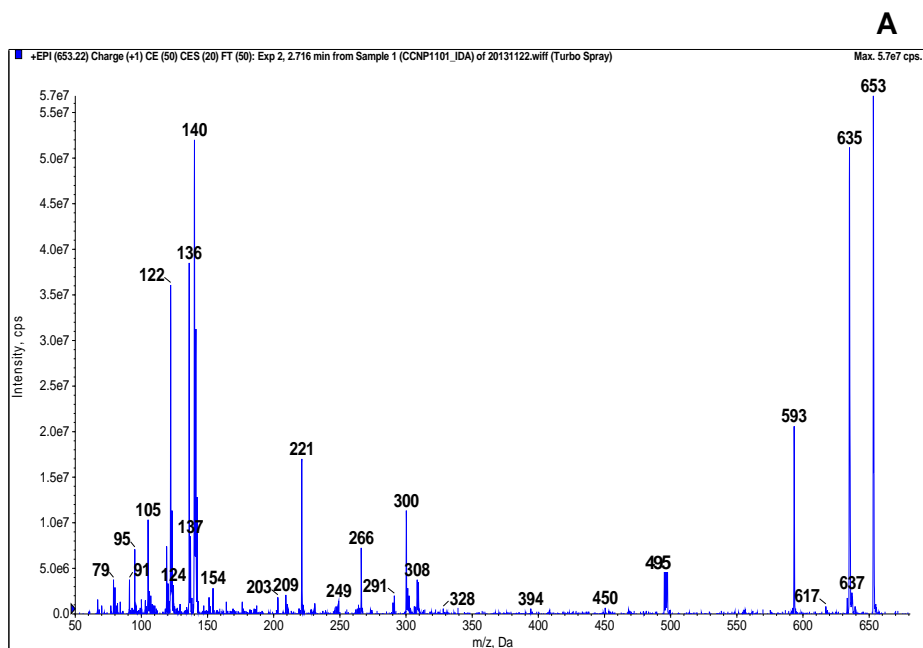


Figure 5S. Ion product mass spectra of peptides identified in *Nodularia spumigena* CCNP1402 from the Baltic Sea: spumigin with m/z 611 (A), anabaenopeptin with m/z 900 (B).

