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The profound effect of harmful cyanobacterial blooms: From food-web and management perspectives

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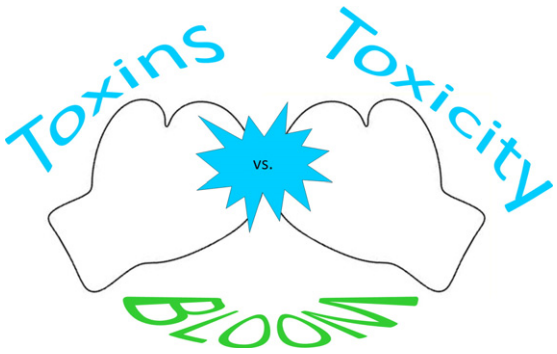
HIGHLIGHTS

- The 'bathing water profile' does not reflect the toxicological potential of HABs.
- The growth-inhibiting potential of the HAB varies between different HABs.
- The growth-inhibiting potential of the HAB varies between different species and strains of organism.
- Bioassays should become standard routine procedure in aquatic monitoring programs.

GRAPHICAL ABSTRACT



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ABSTRACT

Sustainable and effective water management plans must have a reliable risk assessment strategies for harmful cyanobacterial blooms (HABs) that would enable timely decisions to be made, thus avoiding the trespassing of ecological thresholds, leading to the collapse of ecosystem structure and function. Such strategies are usually based on cyanobacterial biomass and/or on the monitoring of known toxins, which may, however, in many cases, under- or over-represent the actual toxicity of the HAB. Therefore, in this study, by the application of growth-inhibition assays using different bacteria, algae, zooplankton and fish species, we assessed the toxicological potential of two cyanobacterial blooms that differed in total cyanobacterial biomass, species composition and cyanopeptide profiles. We demonstrated that neither cyanobacterial community composition nor its relative abundance, nor indeed concentrations of known toxins reflected the potential risk of HAB based on growth-inhibition assays. We discuss our findings in the context of food-web dynamics and ecosystem management, and suggest that toxicological tests should constitute a key element in the routine monitoring of water bodies so as to prevent under-/over-estimation of potential HAB risk for both ecosystem and public health.

1. Introduction

Harmful cyanobacterial blooms (hereafter referred to as HABs) are a worldwide phenomenon, characterized by a massive proliferation of one or several species that have an adverse effect on ecosystems and

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public health (De Figueiredo et al., 2004; Ibelings and Chorus, 2007; Nováková et al., 2013; Chen et al., 2016), and are accompanied by economic losses to various business sectors, including, tourism, fisheries, agriculture, recreation and real estate value in waterfront areas (Engle et al., 1995; Dodds et al., 2009; Ahlvik and Hyytiäinen, 2015). Consequently, these blooms raise serious environmental management challenges, requiring a thorough understanding of the principles and integration of aquatic food-web dynamics and community ecology, along with the demands made by society, economic welfare, governmental policy and regulation, as well as practical considerations (Paerl and Otten, 2013; Qin et al., 2015; Sun et al., 2015; Brooks et al., 2016). From the perspective of ecosystem functioning and recreational activities, it is essential that the effective management of water bodies is based on reliable surveillance and dependable alert and action plans (Ibelings et al., 2015), which, in turn, take into account the risk assessment strategies that have been adopted by many countries and are based on WHO Guidelines (World Health Organization, 1993; World Health Organization, 1996) and, in Europe, are also based on several different Water Directives (Council Directive 91/271/EEC, 1991; Council Directive 91/676/EEC, 1991; Directive 2000/60/EC, 2000; Directive 2006/7/EC, 2006), and, thus involve the establishment of so called "bathing water profiles" (Ibelings et al., 2015). The "bathing water profile" framework usually encompasses physical, chemical, hydrological and biological parameters, and, in most cases, includes microscopical identification and biomass assessment of cyanobacteria and analysis of the toxins that they synthesize. However, setting tolerance levels for the assessment of HAB risk remains a major problem in the development of effective and sustainable environmental management plans (D'Anglada, 2015; Zingone and Oksfeldt Enevoldsen, 2000). Inappropriate management and action plans for mitigating HABs and reducing their harmful effects can lead to the extensive damage of aquatic ecosystems and substantial socio-economic losses (Ahlvik and Hyytiäinen, 2015), that can potentially even exceed the losses sustained if no actions are implemented at all. Therefore, there is an urgent need to improve our understanding of the toxicological potential of HABs (Brooks et al., 2016; Ibelings et al., 2015), especially for coastal ecosystems that experience ever growing demand for environmental goods and services and are highly vulnerable to human activities (Halpern et al., 2008).

Toxicological studies of HABs can provide data on acute and chronic toxicity, tolerance and dose-response relationships for HAB material and test organisms, and, therefore, may provide a foundation for the knowledge-based management of aquatic ecosystems. Generally, toxicological assessments of cyanobacteria can be subdivided into those involving only one or several specific cyanobacterial compounds, usually purified cyanotoxins (e.g. microcystin and its variants), and those that assess HAB extracts containing a mixture of a wide range of unidentified compounds. It would seem that aqueous extracts from complex natural HABs containing several different species of cyanobacteria exhibit considerably greater growth-inhibition and toxicity effects compared with pure cyanobacterial toxins, probably because of additive or synergistic interactions between many different compounds (Burýšková et al., 2006; Palíková et al., 2007; Frazão et al., 2010). From an ecological perspective, species of cyanobacteria differ significantly with respect to the metabolites they produce, including their chemical structures and biological activity (e.g. (Dittmann et al., 2013; Wang et al., 2014)), that challenge co-existing species (e.g. (Christoffersen, 1996; Smith et al., 2008)). Conversely, these species possess various protection strategies enabling them to either tolerate or counteract the harmful effect of cyanobacterial compounds (Jones et al., 1994; Pietsch et al., 2001; Saqrane et al., 2007). Therefore, different blooms that either involve different species or produce metabolites of different chemical structure or at different rates and concentrations (Palíková et al., 2007), may have different effects on the pelagic food-web, thereby having an adverse effect on ecosystem productivity (Persson et al., 2013). Finally, environmental factors are able to modulate the effect or interactions of biologically active compounds (Lehman et al., 2008; White et al., 2011; De Senerpont Domis et al., 2013), making it difficult to generalize the impact of HAB on aquatic biota in a specific habitat or environment based solely on species biomass and composition. Therefore, the application of an eco-toxicological approach in the context of trophic and community interactions allows one to evaluate how organisms respond to HAB products, the variability of these responses as a function of the composition, concentration and structure of HAB extracts, and may provide insights into how the ecotrophic efficiency of the food-web changes in response to different HABs.

The aim of this study was, therefore, to examine the toxicological potential of two cyanobacterial blooms collected from the Curonian Lagoon (SI Fig. 1) and that differed in total cyanobacterial biomass, species composition and cyanopeptide profiles (refers to "bathing water profile"). We assessed the growth-inhibiting effect of aqueous HAB extracts between and within the trophic levels, including bacteria, algae, zooplankton and juvenile fish. Further, we demonstrated the relationship between HAB biomass and the response of different test organisms. We discuss our findings in the context of food-web dynamics and risk assessment procedures for the management of aquatic ecosystems. Our data indicate that the presence of HAB redirects carbon and energy flow within the pelagic food-web toward heterotrophic bacteria-dominated processes, primarily through the inhibition of algal growth and enhancement of bacterial proliferation. We also suggest that more attention should be paid to the risk assessment of mixtures of components that are present in the complex cyanobacterial biomass, especially when defining targets for the effective management of harmful cyanobacterial blooms.

2. Experimental

2.1. Collection of HAB samples

Two different natural cyanobacterial water blooms were collected from the Curonian lagoon (SI Fig. 1) in September 2014 (hereafter referred to as HAB-14) and 2015 (hereafter referred to as HAB-15). Samples for phytoplankton and cyanobacterial community analysis were taken from the water surface using 50 mL plastic containers, fixed with acid Lugol's solution and kept in the dark at +4 °C prior to microscopical analysis. Samples for the preparation of HAB extracts were collected just beneath (~0.05–0.1 m) the surface using a plankton mesh of pore size 50 μm, placed in 200 mL plastic containers, frozen at −20 °C and kept in the dark prior to further processing.

2.2. Microscopical analysis of HAB samples

Cyanobacterial and algal species composition and biomass were determined using an inverted microscope at 400× magnification in accordance with the Utermöhl counting technique (Utermöhl, 1958).

2.3. Preparation of HAB extracts for inhibition assays and LC-MS/MS analysis

The phytoplankton biomass was harvested by centrifugation (10 min, 4000 rpm), frozen and lyophilized. For the inhibition assays, freeze-dried samples of bloom material were homogenized with a mortar and pestle and crude cell extracts were obtained by re-suspending the material in Milli-Q water. After resuspension, samples were subjected to probe sonication (10 min, cycle 0.9, 30 kHz) with an ultrasonic disrupter (Labsonic M, Sartorius, Germany) and left overnight at ~4 °C in the dark. Samples were then centrifuged (15 min, 4000 rpm), and the supernatant collected and used in preparation for inhibition assays, except for zooplankton and fish assays, for which crude (non-centrifuged) extracts were used.

For the LC-MS/MS analysis, the lyophilized phytoplankton biomass (50 μg) was extracted in 70% methanol solution for the analysis of cyanopeptides; in 20% methanol solution for the analysis of anatoxin-a

and cylindrospermopsin; and in a mixture of 4 mM ammonium formate (pH 3.5) (A) and 5% phase (A) in acetonitrile (pH 3.5) (40:60; v:v) for the analysis of saxitoxins (Halme et al., 2012). Samples were extracted by vortexing (15 min) and bath sonication (15 min) (Sonorex, Germany). After centrifugation (10 min, 10,000 ×*g*), the supernatants were transferred to chromatographic vials and analyzed by LC-MS/MS.

2.4. LC-MS/MS analysis

Chromatographic separation of cyanopeptides, anatoxin-a and cylindrospermopsin was performed using Agilent 1200 (Agilent Technologies, Germany) with a Zorbax Eclipse XDB-C18 column (4.6 × 150 mm, 5 μm; Agilent Technologies, USA). Compounds were eluted with a mobile phase composed of 5% acetonitrile in Milli-Q water (A) and acetonitrile (B), both containing 0.1% formic acid. For the separation of saxitoxin, a TSK-gel Amide-80 hydrophilic column (2.0 × 250 mm, 5 μm; Tosoh Bioscience, Japan) and a mixture of phase A (composed of 4 mM ammonium formate (pH 3.5)) and phase B (composed of 5% phase A in acetonitrile (pH 3.5) (40:60; v:v)) was used.

The mass spectrometer (5500 QTRAP, AB Sciex) was operated in positive mode, with a turbo ion spray (550 °C) at a voltage of 5.5 kV. For the analysis of cyanobacterial metabolites, multiple reaction monitoring mode (MRM) was used (Table 1). In the case of cyanopeptides, the information dependent acquisition method (IDA) was also used. For ions with *m/z* in a range 500–1000 and signal above the threshold of 500,000 cps, fragmentation spectra were collected. The relative amounts of peptides in the extract were estimated based on the intensity of the signal in the extracted ion chromatogram.

2.5. Growth-inhibition assays

For inhibition assays, five different treatments (except for green algae - please see below) were applied (differing in their concentration of dry cyanobacterial biomass: 200 mg, 100 mg, 50 mg, 25 mg and 12.5 mg L^{-1}). All incubation assays were run in triplicate for each treatment. Test organisms and culture isolates grown in their corresponding media and conditions without the addition of cyanobacterial extracts were used as controls.

2.5.1. Bacterial assays

Bacterial isolates of *Pseudomonas xanthomarina* Pxan-D3-151, *Stenotrophomonas rhizophila* Srhi-D3-151 and *Bacillus aryabhattai* Bary-D3-151, used in this study were isolated from *Aphanizomenon flos-aquae* filaments (for details see Šulčius et al., 2017) and characterized based on the partial sequence of the 16S rRNA gene (SI Fig. 2). Prior to analysis, bacteria were grown overnight in TSB medium and then diluted to OD of ~0.3. Inhibition assays were performed in 2 mL Eppendorf tubes containing 900 μL of TSB media with the appropriate concentration of cyanobacterial biomass and 100 μL of bacterial inoculum. Incubations were performed for 24 h at 25 °C in the dark. The number of colony-forming units (CFU) was determined by dilution plating onto TSA medium. In addition, in order to ensure that no bacterial contamination could arise from the cyanobacterial biomass extracts, 100 μL of extract was spread onto a Petri dish containing TSA medium and incubated for 48 h (data not shown).

Table 1
Peptides detected in the extracts obtained from the cyanobacterial biomass collected during the natural bloom in the Curonian Lagoon for 2014 (HAB-14) and 2015 (HAB-15). Peptides for which quantitative data were obtained in MRM mode (SI Table 1) are marked with an asterisk.

m/z	Peptide	t_r	Peak area	
			HAB-14	HAB-15
639	Aeruginosin Hpla-?*-Choi-Argal	3.3	$0.5e^{8}$	–
618	Aeruginosin Hpla-Lys-Choi-Argal	2.5–3.9	$56.3e^{8}$	$42.7e^{8}$
607	Aeruginosin Hpla-Val-Choi-Argal	3.6–4.1	$38.5e^{8}$	$40.1e^{8}$
603	Aeruginosin ?-Tyr-Choi-Argal	5. 5	$0.5e^{8}$	$2.1e^{8}$
568	Aeruginosin ?-Lys-Choi-Argal	3.0	$28.3e^{8}$	$11.5e^{8}$
Sum of aeruginosins			**$124.1e^{8}$**	**$96.4e^{8}$**
561	Aeruginosamide MeOTzl-Pro-Val-Ile-(Pren)$_2$	10.5	$7.3e^{7}$	$2.2e^{8}$
995	MC-LR	8.6	$1.4e^{4*}$	traces
519	MC-RR	7.6	$1.8e^{6*}$	$6.1e^{4*}$
512	dmMC-RR	7.4	traces	traces
Sum of MCs			**$182.4e^{4*}$**	**$6.1e^{4*}$**

2.5.2. Algal assays

The green algae (*Chlorophyceae*) isolates, *Scenedesmus quadricauda* isolate 2012/KM1/G2 and *Selenastrum capricornutum* isolate 2013/DUO/B5, were obtained from the Collection of pure cultures of algae and cyanobacteria of the Nature Research Centre (Vilnius, Lithuania) (Koreivienė et al., 2016). Culture isolates were propagated as unialgal (but non-axenic) cultures in a modified MWC medium with the addition of selenium (MWC^{+Se}) under continuous light at approximately 100 μmol photons m^{-2} s^{-1} using cool white fluorescent illumination at 21 °C. Exponential growth phase cultures were used for bioassays following OECD recommendations for growth-inhibition testing of freshwater alga and cyanobacteria (OECD, 2011). Bioassays were performed in 100 mL Erlenmeyer flasks (Schott Duran, Mainz, Germany) containing 50 mL MWC^{+Se} medium with a green algal inoculum of ~10^4 cells mL^{-1}. All assays were run in triplicate for 96 h. Cell numbers were scored through a light microscope (Nikon T_i) using a Fuchs-Rosenthal chamber at ×400 magnification. In order to calculate the median effective EC_{50} concentrations for algal cultures, additional treatments were performed using concentrations of HAB biomass of 6.25 mg L^{-1}, 3.15 mg L^{-1}, 1.56 mg L^{-1}, following OECD recommendations (OECD, 2011).

2.5.3. Daphnia assays

Daphnia magna neonates, taken from laboratory culture, were used for inhibition assays performed according to ISO 6341:2012 standard (ISO 6341:2012, 2012) and OECD recommendations for acute immobilization test of *Daphnia* sp. (OECD, 2004). Prior to analysis, *D. magna* were cultivated in Elendt M4 medium at 20 °C under a 16/8 h light-dark cycle. All assays were run in 50 mL Erlenmeyer flasks (Schott Duran, Mainz, Germany) containing five neonates (<24 h) for 48 h. The observed changes were expressed as percentages after correction for control treatments.

2.5.4. Fish assays

Early ontogenesis (juvenile) stage rainbow trout (*Oncorhynchus mykiss*), derived from eyed eggs and obtained from the Simnas experimental hatchery (Alytus, Lithuania) were used in the assay in accordance with descriptions given in ISO 7346-1:1996 (1996). Prior to analysis, juvenile rainbow trout were cultivated under static conditions in the dark in a constant climate cabinet (Bronson PGC-660, Germany). All assays were run for 96 h in 1000 mL flasks containing 10 6-week-old juveniles. The observed changes were expressed as percentages after correction for control treatments. Experimental procedures involving fish were carried out according to the requirements of the Directive 2010/63/EU on the protection of animals used for scientific purposes (Directive 2010/63/EU, 2010).

2.6. Calculations and statistical analysis

The EC_{50} and LC_{50} values were calculated using the trimmed Spearman-Karber method according to USEPA guidelines (USEPA, 2002), and further expressed in Toxic Units (TU) by the following equation: $TU = 100 / x$, where x is the LC_{50} or EC_{50}. Then, the observed effects for each test organism were classified in one of the five ecotoxicity classes: class – $TU < 0.4$, non-toxic; II class – $0.4 \le TU < 1.0$, low toxicity; III class – $1.0 \le TU < 10$, toxic; IV class – $10 \le TU \le 100$, high toxicity; V class – $TU > 100$, extremely toxic (Persoone et al., 1993). The concentration of concern (COC) was determined according recommendations

presented in P2 Framework Manual 2012 EPA-748-B12-001 (USEPA, 2012). For setting acute COCs the following assessment factors was used: acute COC for *O. mykiss* = LC_{50} / 5; acute COC for *D. magna* = LC_{50} / 5; acute COC for algal isolates = EC_{50} / 4.

Prior to statistical analyses, data were log_{10}-transformed to improve homogeneity of variance and normality. We used analysis of covariance (ANCOVA) to reveal differences in the response of bacterial and algal isolates (dependent variables) to treatment of the two HAB samples (factor) with concentration of cyanobacterial biomass as covariate. We also used ANCOVA to analyze differences in growth (dependent variable) among different bacteria and algae isolates (factor) treated with the same HAB sample using concentration of cyanobacterial biomass as covariate. Significant differences between HAB biomass concentrations for each isolate were confirmed by one-way analysis of variance (ANOVA) followed by post hoc comparison of the means using the *Bonferroni* test. Linear regression analysis was performed in order to assess the relationship between HAB biomass concentration and the tested isolates. All statistical analyses were performed with Statistica 10 software (StatSoft Inc., USA), a p value of <0.05 being considered as significant.

3. Results

3.1. Characterization of bloom samples

A detailed list of species of algae and cyanobacteria that comprise the HAB samples, including their biomass and relative contribution to the phytoplankton community is given in SI Table 2. In the HAB-14 sample, the total cyanobacterial biomass was 204.4 mg L^{-1}, with the members of *Microcystis* contributing to ~80% of the community (Fig. 1A). The second largest contributor, which comprised ~19% of the total cyanobacterial biomass, was the filamentous cyanobacterium *Aphanizomenon flos-aquae* (Fig. 1A, SI Table 2) which occurs embedded in typical fascicle-like colonies. In the HAB-15 sample, members of genus *Aphanizomenon* contributed ~95% of the total of 321.1 mg L^{-1} of cyanobacterial biomass (Fig. 1B, SI Table 2). The contribution of other algal and cyanobacterial species to the total biomass of HAB-14 and HAB-15 samples was negligible (rarely exceeding 3%) (Fig. 1, SI Table 2).

LC-MS/MS analysis of HAB samples did not reveal the presence of anatoxin-a, saxitoxin or cylindrospermopsin in either of the HAB samples. Of the identified compounds, five were aeruginosins, one was an aeruginosamide and three were microcystins: MC-LR, MC-RR and dmMC-RR (Table 1). The concentrations of total and different variants of microcystins (MC) were different for HAB-14 and HAB-15 samples (Table 1). The overall concentration of MCs in HAB-14 sample was 0.95 μg g^{-1} of DW, and was ~32-fold greater than that of the HAB-15 sample (0.03 μg g^{-1} of DW). The MC-RR variant was dominant in both HAB samples, constituting 89% and 100% of the total MC concentration in HAB-14 and HAB-15 samples, respectively (Table 1).

3.2. Effect of HAB extracts on the bacterial isolates

The analysis of covariance revealed significant differences between the two HAB samples for all tested bacterial isolates (Table 2). Generally, treatment with HAB-14 biomass had a strong positive effect on bacterial density compared to HAB-15 extracts, in which the effect was either negative or relatively marginal (Fig. 2). Comparison between isolates treated with the same HAB biomass revealed significant differences between *P. xanthomarina* and *B. aryabhattai* (Bary-D3-151) in the HAB-14 treatments (*Bonferroni* $p < 0.01$), whereas in the HAB-15 treatments, *P. xanthomarina* differed significantly from both other isolates used in this study (*Bonferroni* $p < 0.01$). In the HAB-14 treatments, the increase in CFU (mL^{-1}) was somewhat stepwise for all isolates. However, some differences were observed in the patterns of CFU increase. For *P. xanthomarina* isolate, significant differences in CFU (mL^{-1}) were observed between the HAB biomass concentration range of 0 (control treatment, C) – 25 mg dw L^{-1} and 50–200 mg dw L^{-1} ($F = 11.7$, df = 5, $p < 0.01$). For *S. rhizophila*, significant differences were found between the control (C), HAB biomass concentration of 12.5 mg dw L^{-1}, 25–100 mg dw L^{-1} and 200 mg dw L^{-1} ($F = 96.4$, df = 5; $p < 0.01$). For *B. aryabhattai* isolate significant differences were found between the control (C), HAB biomass concentration of 12.5–25 mg dw L^{-1}, 50–100 mg dw L^{-1} and 200 mg dw L^{-1} ($F = 52.8$, df = 5; $p < 0.01$). No significant effect of the HAB-15 biomass concentration on bacteria growth was observed (Table 2), except for *B. aryabhattai* treated with 200 mg dw L^{-1} of HAB-15 biomass, which showed significant differences (*Bonferroni* $p < 0.05$) compared to the control (Fig. 2).

3.3. Effect of HAB extracts on the algal isolates

The overall effect of HAB biomass on the green algal isolates was negative (Fig. 3, Table 3). The analysis of covariance revealed significant differences between both HAB samples for each isolate and between two isolates for each HAB sample (Table 2). In HAB-14 samples, the growth inhibition of *S. quadricauda* was directly proportional to the concentration of HAB biomass ($r^2 = 0.97$, $b = -0.98$, $p < 0.01$, $F = 1300$, df = 8, $p < 0.01$ for all pairwise comparisons), whereas the effect on *S. capricornutum* was somewhat stepwise, with significant differences ($F = 99.5$, df = 8, $p < 0.01$) found between concentration ranges of 0–6.25 mg dw L^{-1}, 12.5–100 mg dw L^{-1} and 200 mg dw L^{-1} (data not shown). Similar results were found for HAB-15 treatments, but the negative effect was greater compared to that for HAB-14 (Fig. 3, Table 3). *Scenedesmus quadricauda* was more sensitive to exposure to HAB extracts than was *S capricornutum*, with 50% growth inhibition (EC_{50}) occurring at concentrations of 9.2 mg dw L^{-1} (range 8.9–10.7 mg dw L^{-1}) and 4.7 mg dw L^{-1} (range 4.4–8.9 mg dw L^{-1}) for HAB-14 and HAB-15 samples, respectively (Table 3). Alternatively, *S. capricornutum* was found to be less sensitive to HAB extracts addition, with effective concentration (EC_{50}) values of 154.2 mg dw L^{-1} (range 112.8–

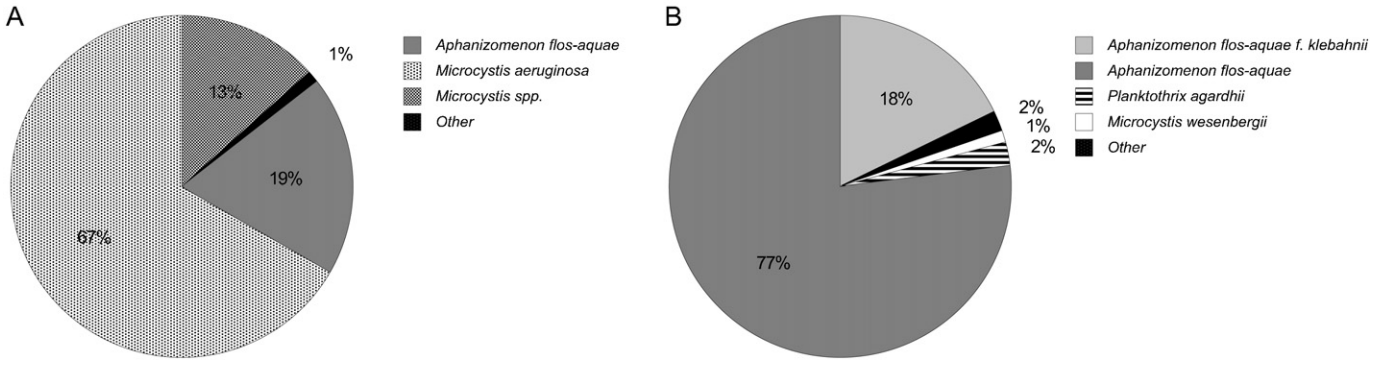

Fig. 1. Cyanobacterial community composition in the HAB samples for 2014 (A) and 2015 (B). For detailed species list see SI Table 2.

Table 2
Results of analysis of covariance (ANCOVA) representing differences between different HAB samples among tested bacterial and algal isolates, and differences between bacterial and algal isolates within the same HAB sample.

	Treatment	df	MS	F	*p*
Between HAB effect					
Bacterial isolates					
P. xanthomarina	HAB	1	0.81	27.16	0.00
	Concentration	1	0.17	5.69	0.04
	Error	9	0.03		
S. rhizophila	HAB	1	0.45	11.89	0.01
	Concentration	1	0.23	6.12	0.04
	Error	9	0.04		
B. aryabhattai	HAB	1	0.98	22.47	0.00
	Concentration	1	0.41	9.33	0.01
	Error	9	0.04		
Algal isolates					
S. quadricauda	HAB	1	0.27	4.75	0.04
	Concentration	1	4.40	76.65	0.00
	Error	15	0.06		
S. capricornutum	HAB	1	0.20	58.69	0.00
	Concentration	1	0.39	113.14	0.00
	Error	15	0.00		
Within HAB effect					
HAB-14					
Bacteria	Isolate	2	0.09	6.11	0.01
	Concentration	1	1.46	103.96	0.00
	Error	14	0.01		
Algae	Isolate	1	0.18	9.98	0.01
	Concentration	1	0.80	43.62	0.00
	Error	15	0.02		
HAB-15					
Bacteria	Isolate	2	0.08	7.21	0.01
	Concentration	1	0.00	0.18	0.68
	Error	14	0.01		
Algae	Isolate	1	0.25	2.93	0.11
	Concentration	1	3.32	38.54	0.00
	Error	15	0.09		

210.8 mg dw L^{-1}) and 17.6 mg dw L^{-1} (range 12.6–24.7 mg dw L^{-1}) for HAB-14 and HAB-15, respectively (Table 3).

3.4. Effect of HAB extracts on Daphnia mortality

The effect of HAB biomass on the mortality of *Daphnia magna* was different for HAB-14 and HAB-15 treatments (Fig. 4A, Table 3). No effect of HAB-14 treatments on the mortality of *D. magna* was observed, except at the highest (200 mg dw L^{-1}) HAB-14 concentration, in which 10% mortality rate occurred (Fig. 4A). *Daphnia magna* mortality increased significantly in HAB-15 compared to HAB-14 treatment (Fig. 4A, Table 3). However, within HAB-15 treatment, the only significant ($F = 13.8$, $df = 5$, $p < 0.01$) effect of HAB-15 biomass was found at the highest HAB concentration, in which nearly all *D. magna* did not survived the treatment (Fig. 4A).

3.5. Effect of HAB extracts on fish mortality

The effect of HAB biomass on fish mortality was rather ambiguous, with no clear pattern or consistency (Fig. 4B). However, as with other assays, the effect of HAB-15 treatments (at least in some cases) appeared to be more severe compared to HAB-14 treatments (Fig. 4B, Table 3).

4. Discussion

The results of this study demonstrated the following: 1) the effect of HAB on the growth of the same organism differed significantly between two HABs, without there being a clear relationship with the concentration of known toxins or sample biomass, and 2) different organisms are affected differently by the same HAB extracts.

From the perspective of food-web dynamics, the impact of HAB at both community and population levels can be either direct or indirect (Carpenter et al., 2011; Ger et al., 2016), and may vary from being neutral (no changes in species density) to positive (increase in species density) or negative (decrease in species density) (Filstrup et al., 2014). In addition, the relationship between the HAB and species density and growth of organisms can be either linear to threshold concentration-dependent. Together, these effects lead to a complex interplay between the HAB and aquatic biota. Generally, bacteria and algae were directly and to a greater extent (Figs. 2 and 3) affected by HAB addition compared to zooplankton and fish (Fig. 4, Table 3), yet the direct effect was either positive (e.g. HAB-14, Fig. 2) or neutral (e.g. HAB-15, Fig. 2) for bacteria and always negative for algal species (Fig. 3). This strongly implies that during bloom conditions, and, in particular, upon bloom decay, a significant amount of energy that had accumulated in the phytoplankton biomass is redirected toward bacteria, increasing the carbon flow through the pelagic microbial loop (Engström-Öst et al., 2013; Engström-Öst et al., 2002). Significant inhibition of algal growth also suggests taxonomical and functional reduction in this group of primary producers (Huisman et al., 1999). This corresponds well with our observations relating to species richness and evenness (number of species and their relative contribution to total biomass, SI Table 2) in the HAB-15 samples, in which community diversity was significantly lower compared to the HAB-14 sample, and which also had a significantly greater harmful effect on all tested organisms used in this study (Fig. 3). *Daphnia magna* was either not affected (neutral direct impact, HAB-14 treatments) or affected negatively (HAB-15 treatments) by HAB addition, yet to a much lesser extent (Table 3; the average mortality of *D. magna*,

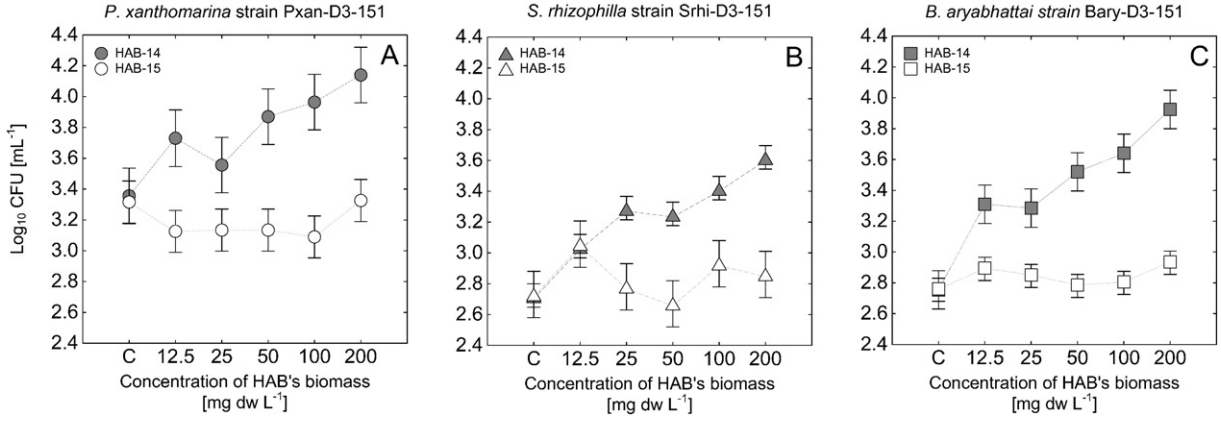

Fig. 2. The effect of HAB biomass extracts on the growth of different bacterial isolates: (A) - *Pseudomonas xanthomarina* isolate Pxan-D3-151, (B) - *Stenotrophomonas rhizophila* isolate Srhi-D3-151and (C) - *Bacillus aryabhattai* isolate Bary-D3-151. Error bars represent 95% confidence limits.

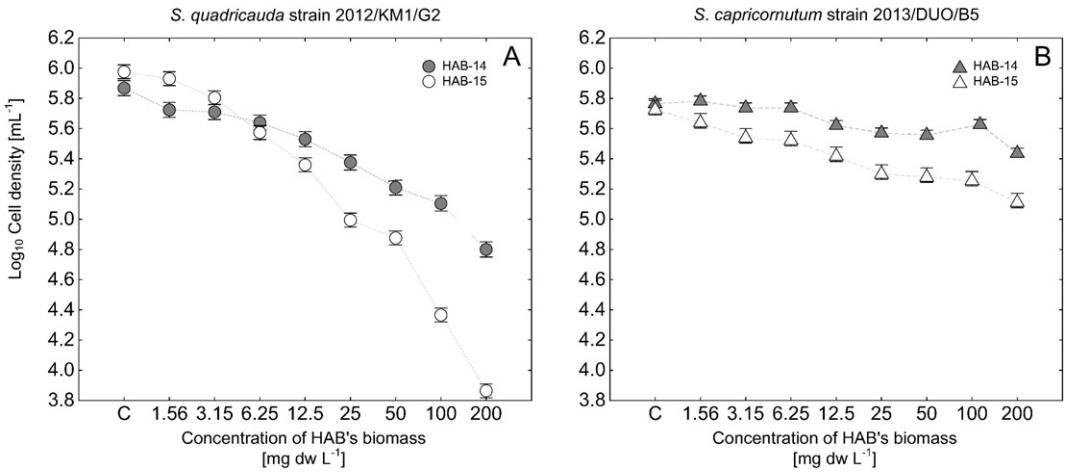

Fig. 3. The effect of HAB biomass extracts on the growth of different green algal isolates: (A) – *Scenedesmus quadricauda* isolate 2012/KM1/G2 and (B) – *Selenastrum capricornutum* isolate 2013/DUO/B5. Error bars represent 95% confidence limits.

except at the highest HAB-14 concentration (Fig. 4), was ~25%), suggesting that these organisms are either more resistant or have more effective protection mechanisms (e.g. detoxification, temporal feeding inhibition etc.) that lowers their vulnerability to toxic HAB compounds, and, in turn, increases their survival rate during HAB. However, it is important to notice, that relatively marginal effect of HAB extracts on *D. magna* and *O. mykiss* compared to bacteria and algae isolates might be due to differences in measured responses. In case of *D. magna* and *O. mykiss* mortality instead of changes in body mass (proxy of growth rate) has been assessed, which is a more drastic endpoint then measuring organism's sensitivity. Therefore, a more subtle effect of HAB extracts on zooplankton and fish may probably be underestimated by the assays used in this study.

The direct effects of HAB that are mostly pronounced at the base of the food-web, may generate a wide spectrum of indirect effects that can cascade throughout the ecosystem. For example, both HAB and dead algal cells release compounds that can promote bacterial growth that may also lead to an increase in the abundance of heterotrophic nano-flagellates and a shift in the zooplankton community toward smaller species (e.g. protists, rotifers and small cladocerans) (Ger et al., 2016; Gilbert, 1990; Jeppesen et al., 2000). Dead algal cells also reduce competition for cyanobacteria and availability of high quality food for grazers (e.g. *Scenedesmus*) that in turn may promote bloom development and negatively affect zooplankton reproduction, growth and mortality (Huisman et al., 1999). It has also been shown that cyanobacteria can have an indirect effect through changes in zooplankton and fish feeding behaviour (DeMott, 1999), or zooplankton and fish may migrate to areas where the concentration of cyanobacteria is lower (Karjalainen et al., 2005; Reichwaldt et al., 2013). Furthermore, this would also reduce top-down control of cyanobacteria having positive feedback for the extension of HAB.

Table 3
Values of the 48 h (for *D. magna*) and 96 h (for algae isolates and *O. mykiss*) EC_{50} and LC_{50} (mg dw L^{-1}) of tested organisms, and assessment of their sensitivity to different HAB extracts (COC (concentration of concern) and TU (toxic units)).

Isolates	Sample	EC^1_{50} (LC^2_{50})	TU	Ecotoxicity class	COC
S. quadricauda	HAB-14	9.2[1]	10.9	IV	2.3
S. quadricauda	HAB-15	4.7[1]	21.5	IV	1.2
S. capricornutum	HAB-14	154.0[1]	0.93	II	38.5
S. capricornutum	HAB-15	17.6[1]	5.70	III	4.4
D. magna	HAB-14	–	–	–	–
D. magna	HAB-15	114.0[2]	0.88	II	22.8
O. mykiss	HAB-14	670.0[a2]	0.15	I	134.0
O. mykiss	HAB-15	115.0[2]	0.87	II	23.0

[a] Value determined from dose-response curve.

The use of different isolates belonging to the same trophic level enables insights into differences between individual species of the community in their use of, or tolerance to compounds present in the HAB extracts. In this study, we found that HAB extracts can differentially support the growth of various bacterial species, as it has been found in field observations (Wilhelm et al., 2011; Eiler and Bertilsson, 2004). For example, although all bacterial isolates were dependent on the concentration of HAB-14 extracts, the concentration levels that induced significant changes in bacterial growth for *S. rhizophila* and *B. aryabhattai* isolates (as revealed by significant differences in abundance) were lower compared to that of *P. xanthomarina*, suggesting that these species would benefit faster as the bloom develops, leading to changes in bacterial community structure. However, some counteracting effects of the bloom on bacteria growth may also occur as can be seen in HAB-15 treatments (Fig. 2). Intriguing, the preventive effect of HAB-15 on the tested isolates showed different species specificity and was independent of the extracts concentration (Table 2), in opposite to growth-stimulating effect of HAB-14 (Fig. 2). Similar results were also observed for algal isolates, indicating that *S. capricornutum* is able to tolerate higher concentrations of HAB extracts than is *S. quadricauda*. Their sensitivity to HAB concentration was also different, suggesting that proliferation and distribution of these species depends not only on the cyanobacterial bloom itself, but is also proportional to bloom intensity.

From the perspective of HAB management, the selection of appropriate measurements for evaluation of HAB risk is of critical importance if one is to avoid interfering with or disrupting ecosystem processes and

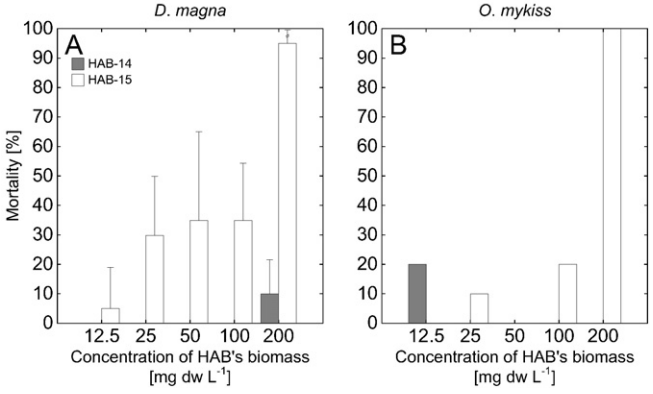

Fig. 4. The effect of HAB biomass extracts on the mortality of *Daphnia magna* (A) and rainbow trout (*Oncorhynchus mykiss*) (B). Values are corrected for the control treatments. Error bars represent standard deviation of the mean.

food-web dynamics, while providing maximum benefits for society in its use of ecosystem goods and services, including fishing and bathing activities. The decisions to take actions recommended in the action plans of the environmental protection agencies or respective authorities usually rely on the available data, obtained from either biological monitoring (that includes measurements of changes in phytoplankton composition and biomass, including enumeration of cyanobacterial cells) or from the monitoring of cyanotoxins, that are mainly based on concentration measurements of microcystin variant MC-LR (Qin et al., 2015; Ibelings et al., 2015; Stroom and Kardinaal, 2016). In this study, measurements of phytoplankton structure, its biomass distribution and cyanotoxin profiles in the HAB samples, supplemented by the toxicological study of HAB extracts, demonstrate that neither the composition nor the relative abundance or toxin concentration of the cyanobacterial community reflected the true potential risk of HAB as demonstrated by growth-inhibition assays (Table 3). This suggests that, for example, the harmfulness of cyanobacterial bloom for 2014 can be overestimated if evaluation is based on cyanobacterial composition, biomass and toxin concentration measurements alone (in HAB-14 microcystin concentration was ~32-fold greater than in HAB-15 sample). The converse could be said for the cyanobacterial bloom that appeared in 2015. These data point to the importance of growth-inhibition assays, which, as our and other studies indicate, must now be included in the current framework of "bathing water profile" proposed by EU Directives (Directive 2006/7/EC, 2006). Toxicological studies are also useful from a practical viewpoint. They can be standardised easily, do not require expensive and sophisticated analysis tools or expertise in the field, they are relatively time-efficient (from 24 to 96 h) and data can be compared between different organisms and sites (Burýšková et al., 2006; Palíková et al., 2007; OECD, 2011; ISO 6341:2012, 2012). Thus the use of growth-inhibition assays may provide a valuable tool in the monitoring and evaluation of HAB risk, and significantly improve the accuracy of the decision making process.

5. Conclusions

In this study we assessed the ecological impacts of harmful cyanobacterial blooms, taking into consideration typically occurring levels of toxins, biomass and species composition, and demonstrated the potential for both direct and indirect effects of HABs that eventually lead to changes in food-web structure and subsequent ecosystem malfunction. Although, many aspects of HAB - species interactions have not been covered here (e.g. adaptation, temporal feeding inhibition, depuration, excretion and the detoxification capabilities of different species), our findings strongly suggest that neither biomass, nor toxin-monitoring-based programs are sufficient for adequate evaluation of HAB risk. We thus strongly argue that toxicological tests have to become a standard routine procedure in aquatic monitoring programs, in particular for those water bodies that are highly dynamic on spatial and temporal scales (e.g. coastal ecosystems), or are vulnerable to rapid environmental changes and experience increased anthropogenic pressure. Ecotoxicological tests that reflect changes to human health should also be considered for water bodies that are exploited for recreational purposes.

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