Effects of compounds in aquatic environment on early development of animals:

Retinoid-like effects of cyanobacteria on zebrafish embryos

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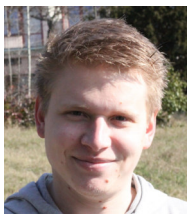

Introduction

- Cyanobacteria release various bioactive compounds
- For most of them there is only scarce information on their potential relevance for effects, especially *in vivo*
- Numerous hints of teratogenic metabolites and indications of potential presence of retinoid-like compounds were reported
- In this study field cyanobacterial biomass samples are investigated and characterized in terms of *in vitro* and *in vivo* bioactivity and production of retinoids

Objectives

Determine the role of retinoid-like compounds in teratogenicity of field cyanobacterial water blooms.

- Characterize *in vitro* retinoid-like potential
- Investigate its *in vivo* relevance using zebrafish embryo toxicity (zFET) test
- Conduct an effect-driven fractionation of complex environmental biomass extracts and characterization of effective fractions
- Measure retinoid compounds suspected from contribution to the observed effects

Conclusions

- Teratogenic effects caused by the cyanobacterial biomass extracts showed high similarity with effects of standard retinoid all-*trans* retinoic acid (ATRA; Fig. 3)
- The *in vitro* retinoid-like, teratogenic and behavioural effects were caused by the same fraction (Fig. 1 and 3)
- The fraction causing teratogenicity and behavioral effects contained most of the suspected retinoid compounds.
- Detected compounds do not completely explain the toxic effects suggesting there are more retinoid-like compounds or mixture effects contributing to the toxicity

Results & Discussion

- *In vitro* bioassays showed a significant total retinoid-like activity in both tested biomass samples Ma and Ak (Fig. 1 and 2).
- The samples were fractionated in 12 fractions and for both samples the retinoid-like activity *in vitro* was localized in fractions 4 and 5 and *in vivo* effects- malformations and behavioural in fraction 5 (Fig. 1 and 2).
- The other fractions tested at the same concentration did not show any bioactivity in used assays (Fig. 1 and 3).
- Malformations typical for retinoid signalling disruption were detected for both extracts (comparable to effects of ATRA; Fig. 3).
- The analysed retinoids were detected in ng g^{-1} of biomass dry weight range (Tab. 1). Their occurrence in two (out of 12) separated fractions 4 and 5 correspond with effects detected by bioassays.
- The effects of mixture cannot be sufficiently explained by the detected concentration of analysed compounds (Tab. 1)

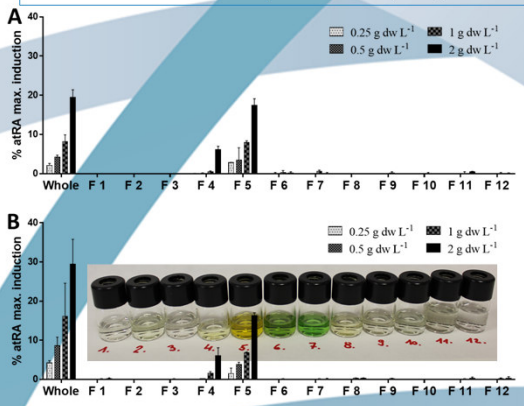

Fig. 1 Illustrative results of p19 *in vitro* assay screening of samples Ma (A) and Ak (B) fractions. Four concentrations were measured for each sample and activity is expressed as percents of calibration (ATRA).

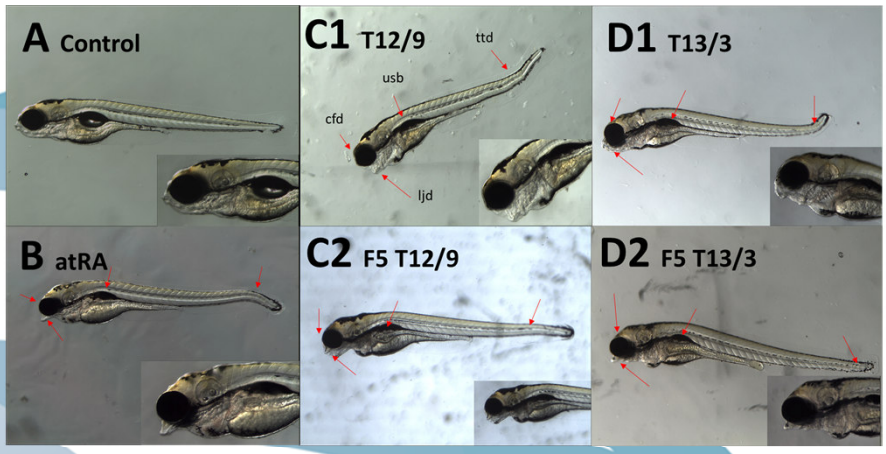

Fig. 2 Comparison of control embryos (**A**) to exposed embryos at 120 hpf. Individuals showing typical representative phenotype for each treatment are displayed: ATRA at concentration 2 000 ng/L (**B**); biomass extract Ma (T12/9) at concentrations 0.6 g dw/L: whole (**C1,** REQ 1 822 ng ATRA/g) and Fraction 5 (**C2,** REQ 1 099 ng ATRA/g); and biomass extract Ak (T13/3): whole at concentration 0.3 g dw/L (**D1**, REQ 896 ng ATRA/L) and Fraction 5 at 0.6 g dw/L (**D2**, REQ 1 099 ng ATRA/L). Magnification 30x (whole fish photos) and 90x (head detail). cfd - cranio-facial deformation; usb - uninflated gas bladder; ljd - lower jaw deformation, ttd - tail tip deformation

Table 1: Results of *in vitro* and chemical analyses for the selected biomass extract and fractions. *In vitro* results are presented as all-*trans* retinoic acid equivalent (REQ). 9-*cis* retinoic acid (RA) and 13-*cis* RA co-eluted and had the same parent and fragment ions, therefore they are reported as a sum. n.i. - no induction, n.d. - not detected, LOD - Limit of Detection, LOQ - Limit of Quantification

	REQ in vitro			Retinoids					
Extract Ma	[ng ATRA g dw^{-1}]			[ng g dw^{-1}]					
Microcystis aeruginosa (100 %)				ATRA	9cis/13cis-RA	retinal	4keto-ATRA	5,6epoxy-ATRA	4keto-retinal
			REP	1.00	0.33	0.02	0.36	0.73	0.11
Whole	2850 ± 505			296	n.d.	20-80	383	119	12-40
Fraction 4	697 ± 91			n.d.	n.d.	n.d.	329	n.d.	n.d.
Fraction 5	1640 ± 229			227	n.d.	459	n.d.	52	12-40
Fraction non-RA	n.i.			n.d.	n.d.	777	n.d.	n.d.	59
	LOD	16	LOD	12	20	20	20	12	12
			LOQ	120	40	80	40	40	40
Extract Ak									
Aphanizomenon klebahnii (92 %)									
				ATRA	9cis/13cis-RA	retinal	4-keto ATRA	5,6 epoxy atRA	4-keto retinal
Whole	2869 ± 467			848	216	142	2136	222	n.d.
Fraction 4	1027 ± 131			n.d.	n.d.	n.d.	1840	n.d.	116
Fraction 5	1601 ± 165			791	178	437	n.d.	83	129
Fraction non-RA	n.i.			n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	LOD	16	LOD	12	12	20	20	20	20
			LOQ	120	40	40	40	40	40

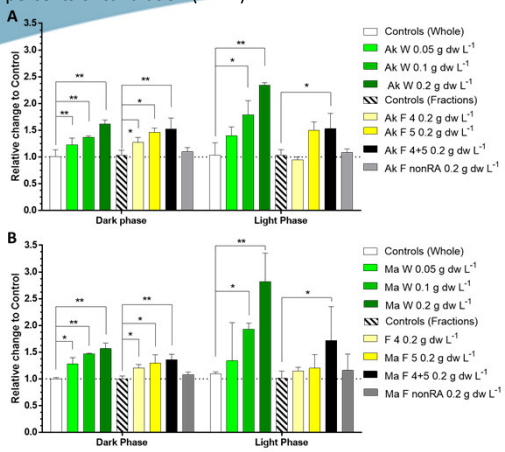

Fig. 3: Relative change in total activity (swimming time) of 120 hpf zebrafish embryos after 3 h exposure to cyanobacterial biomass extract Ak (A; MU strain) and Ma (B; VU strain) and their fractions. The displayed bars represent average combined total activity of all embryos in both dark/light phases normalized to their respective controls (N=3). The asterisks indicate statistical significance from control using Mann-Whitney U test (N=12, $p<0.05$; */** means there was statistically significant difference from control in two/three out of three independent experiments). W- whole extract, F x- Fraction x of the extract, F nonRA- Combination of fractions 1-3 and 6-12 that did not show any retinoid-like activity in vitro

Materials & Methods

Sample processing

The biomasses were collected by planktonic net from water bodies in the Czech Republic. Lyophilized biomasses were extracted by sonication with solvent for 2x2 min with 5 mL of methanol and re-extractions. Biomasses were centrifuged and supernatants were pooled from all extraction runs.

***In vitro* assay**

Retinoid-like activity was measured after 24 h exposure on transgenic cell line P19/A15 (Retinoic Acid Receptor, RAR). Effects are shown as percent of maximal induction of standard compound all-*trans* retinoic acid (ATRA). Total retinoid-like activity is shown as equivalent concentration of ATRA (**REQ**), calculated by comparison of sample EC_x and standard compound concentration that caused the same effect.

Zebrafish embryos exposures

Zebrafish embryo toxicity (zFET) test was used for the detection of bioactivity *in vivo*. Embryos at the stage of 4hpf were exposed to extracts at 0.05, 0.1, 0.2, 0.3, 0.6 and 1 g dw L^{-1}. Embryos were exposed without media renewal until 120 hpf. The malformations were observed daily. The behavioural assay was performed in 24-well plates in Zebrabox system (Viewpoint, France) after 3h exposure of 120 hpf embryos.

Fractionation and chemical analysis

Fractionation method was developed with Agilent LC/DAD on column Waters X-Bridge C18. The sample was separated in twelve fractions in increasing methanol gradient (from 25 % to 100% methanol). Seven compounds were analysed by LC-MS/MS. The separation was conducted on Waters Acquity UPLC (Waters, Milford, MA, USA). Detection was performed with mass spectrometer Waters Xevo TQ-S (Waters, Milford, MA, USA) in positive ESI mode.

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