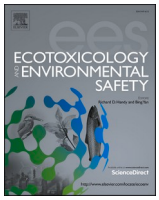
Ecotoxicology and Environmental Safety 245 (2022) 114119

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv



Abnormal neurobehavior in fish early life stages after exposure to cyanobacterial exudates

Wenwen Cai [a,b], Hugh J. MacIsaac [a,b], Runbing Xu [a], Jinlong Zhang [a], Xiaofu Pan [c], Yuanwei Zhang [c], Junxing Yang [c], Brian Dixon [d], Jiaojiao Li [a], Yuanyan Zi [a,b], Xuexiu Chang [b,e,*]

[a] School of Ecology and Environmental Science, Yunnan University, Kunming 650091, China
[b] Great Lakes Institute for Environmental Research, University of Windsor, Windsor, ON N9B 3P4, Canada
[c] Yunnan Key Laboratory of Plateau Fish Breeding, Yunnan Engineering Research Center for Plateau-Lake Health and Restoration, Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming 650223, China
[d] Department of Biology, University of Waterloo, Waterloo, ON N2L 3G1, Canada
[e] College of Agronomy and Life Sciences, Kunming University, Kunming 650214, China

ARTICLE INFO

Keywords:
Microcystis aeruginosa
Sinocyclocheilus grahami
Embryonic development
Neurotoxicity
Endangered species
Cyanobacterial blooms

ABSTRACT

Cyanobacterial harmful algal blooms (cHABs) pose a risk to exposed aquatic and terrestrial species. Numerous studies have addressed effects of single toxins while much less attention has been devoted to mixtures of cHAB metabolites that are continually released by living cyanobacteria. Neuro-impairment associated with cHABs has been reported in fish, though the mechanism remains unclear. Here we exposed embryos of *Sinocyclocheilus grahami*, an endangered fish, to *Microcystis aeruginosa* exudates (MaE) to evaluate neurotoxicity and the toxicity mechanism(s). We found that MaE affected embryonic development by increasing malformation and mortality rates and decreasing the fertilization rate. MaE also inhibited fish neurobehavior including touch response, social frequency, swimming distance, and aggravated light-stimulation response. Neurobehavior suppression resulted from a decrease in excitatory neurotransmitters acetylcholine and dopamine, even though receptors increased. MaE also affected gene and protein expression of neurotransmitters, synthetic and/or degrading enzymes, and receptors. Our findings shed light on specific mechanisms by which MaE induces neurotoxicity in early life stages in fish and contributes to improvement of the conservation strategy for this species.

1. Introduction

Freshwater ecosystems worldwide have been severely impacted by toxins produced by cyanobacterial harmful algal blooms (cHABs), with eutrophication and climate change contributing to this growing problem (Paerl et al., 2016; Huisman et al., 2018; Tang et al., 2018; Banerjee et al., 2021). cHABs harm a variety of plankton, fish, birds, and terrestrial species including cattle and elephants, and impair water sources for drinking, industry, and agriculture (Li et al., 2021a, 2021b; Hu and Rzymski, 2022; Breinlinger et al., 2021; Veerman et al., 2022; Spoof et al., 2020). Animals directly or indirectly exposed to cyanobacterial toxins may develop neurological illnesses, liver or renal damage, or tumour growth (Li et al., 2016; Hilborn and Beasley, 2015). cHABs may release hazardous chemicals either from lysed cells or as exudates from living cells. Hazardous cHAB metabolites from lysed cells include well-studied microcystin-LR, nodularin, and -N-methylamino-L-alanine (Banerjee et al., 2021). By contrast, cyanobacteria may also release mixtures of chemical exudates as they grow, which collectively may be toxic to other species (Zheng et al., 2013; Jiang et al., 2019).

Fish are typically abundant and widespread in lakes, and thus vulnerable to presence of cHABs. Available evidence has implicated cHABs in massive fish kills (Chellappa et al., 2008; Carmichael and Boyer, 2016; Drobac Backović et al., 2021). Neurotoxicity is one mode of toxicity by which fish may be impacted cHAB compounds (Jonasson et al., 2010; Jonas et al., 2015; Qian et al., 2018; Pípal et al., 2020) as the fish nervous system is particularly sensitive to toxins (Könemann et al., 2022). Identified mechanisms of neurotoxicity of microcystin and other cyanotoxins include ion-channel disorders and neuron cell damage (Florczyk et al., 2014). However, neurotoxicity associated with MaE mixtures remains unclear.

Lake Dianchi is the largest of the Plateau lakes in Yunnan Province, China, and has since the end of the last century suffered from recurrent,

* Corresponding author at: College of Agronomy and Life Sciences, Kunming University, Kunming 650214, China.
E-mail address: xchang@uwindsor.ca (X. Chang).

https://doi.org/10.1016/j.ecoenv.2022.114119
Received 13 July 2022; Received in revised form 16 September 2022; Accepted 23 September 2022
Available online 26 September 2022

massive cyanobacterial blooms dominated by *Microcystis aeruginosa* (Zhou et al., 2016). The golden-line barbel *Sinocyclocheilus grahami* was historically dominant the native fish community in the lake, but has become endangered in recent decades (Deng et al., 2014). Long-term reintroduction efforts have failed for unknown reasons (Zi et al., 2018). Exposure of fishes to *M. aeruginosa* exudates potentially hinder the species' recovery (Zi et al., 2018).

Early-life stages are highly sensitive period for fish, and, in fact, are used as a tool for evaluating toxicity of chemicals (eg., Fish Early Life Stage Toxicity Test; EPA, 2016; Coulter et al., 2019). In this paper, we hypothesized that MaE caused neurotoxicity to early-life stages of *S. grahami*. Specifically, we investigated whether MaE affects *S. grahami* embryonic development and neurobehavior. We then explored changes in neurotransmitter and receptor concentration, as well as their gene expression, to investigate possible toxicity mechanisms. Overall, our results revealed MaE-induced neurobehavioral effects in these early life stages, which may have consequences for management of this endangered fish species.

2. Materials and methods

2.1. MaE collection

M. aeruginosa (FACHB-905, toxic) was cultured with HGZ-145 nutrient solution at 25 ± 1 °C with 12 h/12 h light/dark with cell density which increasing from 2.0×10^6 cells/ml to 4.0×10^6 cells/ml after 72 h. We filtered the supernatant through a glass-fiber filter (0.22 μm, Xingya Purification Material Factory, Shanghai, China) to obtain our original MaE solution after centrifuging for 10 min at 15,314 RFC. Mineral water (Kunming Zhenming Food Co., Ltd. Yunnan, China) was employed as a control (*M. aeruginosa* cell density = 0 cells/ml). We diluted the stock MaE solution (cell density = 4.0×10^6 cells/ml) with mineral water to 30% (cell density 1.2×10^6 cells/ml), which was then employed as our MaE treatment incubation medium. Both control and MaE treatments had a pH of 7.0–7.2.

2.2. Embryonic harvesting

S. grahami eggs were provided by the Endangered Fish Conservation Center (EFCC) of the Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming, in March 2021. After artificial fertilization in the EFCC, eggs were brought to our laboratory (Yang et al., 2007). Transparent eggs before the 32-cell stage with uniform division and a normal yolk sac were used for experimentation. Eggs were sterilised for 3–5 min with 0.5 mg/L methylene blue, then rinsed three times with mineral water for 10 min each time. All procedures were conducted at 20 ± 1 °C.

2.3. Embryonic exposure test

We placed healthy *S. grahami* embryos that were ≤ 10 h post-fertilization (EPA, 2016) into a 24-well cell culture plate with mineral water (control treatment) or with the addition of 1.2×10^6 cells/ml MaE solution (MaE treatment). Embryos were incubated at 20 ± 1 °C. We calculated fertilization rate as 100% × N_1/N_0, malformation rate as 100% × N_2/N_1, and hatching rate as 100% × N_3/N_1, where N_0 was initial embryonic number (50 eggs), N_1 was the number of embryos living to gastrula, N_2 was number of malformed embryos, and N_3 was the number of hatched embryos. Heartbeat was calculated as number of beats per minute, and body length was measured by stereomicroscope (CellSens Standard, OLYMPUS-SZX16) every 24 h from 96 to 192 h after initial exposure (n = 15).

Embryonic chorion was removed by application of 0.1% protease E for no more than 10 min when embryos-initiated wiggling. De-chorion embryos were rinsed with incubation medium five times for 10 min each. After that, embryos were observed under stereomicroscope (OLYMPUS-SZX16) to record writhing-like-movements (embryonic whole body twisted as spine curved to left and/or right). At the same time, six embryos without malformation were employed for the touch-response test per treatment. Embryonic head was touched 20 times using one clean feather and responses were recorded when embryos violently twisted their tail. Every embryo was tested three times (interval = 1 h) to get an average response time (A). The touch response rate was calculated as A/20 × 100%.

We assessed behavioral changes using an observation box (Fig. S1). Dark-light response and light intensity response were conducted at 96 h after initial exposure to MaE with six embryos for each treatment. The process for the dark-light response test was five minutes of light followed by five minutes of dark. The light intensity response was tested by sequential exposure for five minutes to high light (15 μmol/m^2·s), darkness (0 μmol/m^2·s), intermediate light (7.5 μmol/m^2·s), darkness (0 μmol/m^2·s), low light (4 μmol/m^2·s), and then, finally, to darkness. After a 30 min recovery period, six embryos for each treatment were monitored to record writhe-movement times with a camera (Sony, FDR-AXP55) in the test. Light-dark and light intensity response tests were repeated three times.

The swimming activity test and social frequency test were performed with nine-day after initial fertilization larvae. Six larvae were monitored every 20 min for 60 min to record swimming activity in 12 well-plates. Records were analyzed by EthoVision XT to obtain swim distance, swimming speed and swimming trait (i.e. tracing the movement of S. grahami larvae). Six larvae were monitored for two hours to record social activity in six-well cell plates with two larvae per well (three replicates in total).

Embryo and larvae tests were performed in accordance with test guideline (TG) published by the organization for economic cooperation and development (OECD), including fish embryo acute toxicity TG236 and fish sexual development tests TG234. The ethical review number of this work is SMKX-SQ-20200408–066, National Natural Science Foundation of China.

2.4. Neurotransmitter metabolism test

We employed UHPLC-MS/MS to test neurotransmitter concentrations. We focused on 14 neurotransmitters: 5-hydroxyindoleacetic acid (5HIAA), 5-hydroxytryptophan (5HTP), acetylcholine (Ach), dopamine (DA), epinephrine (E), 4-aminobutyric acid (GABA), glutathione (GSH), 3,4-dihydroxyphenylalanine (DOPA), tryptamine (TrpA), melatonin (Mela), L-tyrosine (Tyr), norepinephrine (NE), serotonin (5HT), and L-phenylalanine (Phe). Fifty embryos at 96 h post-exposure for both control and MaE treatment were collected and sent to Biotree Biotech Co. Ltd. (Shanghai, China) for targeted neurotransmitters analysis (Li et al., 2021a, 2021b; Wu et al., 2021). Three replicates were used for both control and MaE treatments.

Briefly, an embryo sample (~100 mg) was ground with high-throughput tissue grinder (QILINBEIER QL866) for one minute under 60 Hz following addition of 10% methanol formate-ddH_2O (1:1) solution and one steel ball. This step was repeated two times and the samples were allowed to sit overnight at − 20 °C. Samples were then centrifuged at 16,128 RCF at 4 °C for 15 min. The supernatant (~100 μL) was mixed with 40 μL of Na_2CO_3 solution (100 mM) and 40 μL of 2% benzoyl chloride acetonitrile solution and incubated at room temperature for 30 min. After spiking 1.6 μL of stable-isotope-labeled standards (using ^{5}D-BzCl for labeling), the samples were centrifuged again at 16,128 RCF for 15 min at 4 °C. Supernatant (20 μL) was mixed with 10 μL of acetonitrile in 0.1% formic acid and transferred to an autosampler vial to measure the neurotransmitter concentrations on a UHPLC−MS/MS (QTrap 6500 plus, AB Sciex Co., Ltd., USA).

2.5. Gene expression analysis

Embryos at the hatching stage, 96 h after initial treatment, were harvested and preserved in 1.5 ml cryo tubes with 0.8–1 ml RNA-latter

solution at − 80 °C for RNA sequencing analysis. Total RNA was extracted using RNeasy Mini Kit (QIAGEN, Hilden, Germany). Three biological replicates of each treatment were conducted. RNA purity was assessed using a Nano Photometer spectrophotometer (IMPLEN, Westlake Village, CA, USA). RNA concentration was measured using a Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA) with the Qubit RNA Assay Kit. RNA integrity was assessed using a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA) with the RNA Nano 6000 Assay Kit (Agilent Technologies). Genes were annotated as published genomic information of *S. grahami* in National Center for Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov/genome/?term=Sinocyclocheilus+grahami).

Owing to significant differences in expressed neuron-related genes in RNA sequencing, we designed primers according to gene sequences published in NCBI. Target genes and their primer sequences are provided in Table S1. We selected Eef2 as a reference gene (Bustin et al., 2009; Zhang et al., 2017). This was followed by qPCR with thermal cycling program: enzyme activation (95 °C, 10 min, 1 cycles), denaturation (95 °C, 10 s, 40 cycles), annealing (60 °C, 20 s, 40 cycles), and extension (72 °C, 20 s, 40 cycles). Each target gene in each sample was normalized by subtracting the mean Ct value of the endogenous control gene (Eef2) from that of the target gene (Ct target gene - Ct endogenous control genes), thereafter the fold difference was calculated using the equation $2^{-\Delta\Delta Ct}$, (ΔΔ: two deltas). We sought to utilize additional endogenous control genes (beta-actin, G6PD, RPL17, B2M, tubulin, 18 S RNA, L13) for this project, but were unable to obtain useful, diagnostic information, thus only data for Eef2 are presented here. We applied a t-test to compare differences between the control and MaE treatment.

2.6. Determination of neuro components

All target receptors/enzymes in this study were tested by Fish Enzyme Linked Immunosorbent Assay Kit made against *Salmo salar* (Solarbio Life Science, Beyotime Biotechnology and Hengyuan Biotechnology). We measured 5-hydroxytryptamine receptor 3A-like (5HT3A), acetylcholine receptor (AchR), acetylcholinesterase (AchE), acetylcholine transferase (ChAT), sodium- and chloride-dependent GABA transporter 1-like (GAT1), aromatic-L-amino-acid decarboxylase (DDC), D (5)-like dopamine receptor (DRD5), beta-2 adrenergic receptor-like (ADRβ2), sodium-dependent noradrenaline transporter-like (SLC6A2), sodium/glucose cotransporter 1-like (SGLT1), phenylethanolamine N-methyltransferase (PNMT), dopamine beta-monooxygenase (DBMO), tyrosinase (TYR), and proto-oncogene (c-fos). All tests were performed according to kit instructions with three biological replicates. Because antibodies used in the kits were not designed for our fish, the golden line barbel, it may underestimate the concentration due to low cross reactivity. Potassium and sodium concentration were measured with atomic absorption spectroscopy.

All statistical analyses were performed using SPSS 23.0 (SPSS Inc., Chicago, IL, USA). The rejection level was set at $\alpha = 0.05$ and/or $\alpha = 0.01$. Unless stated otherwise, all values are reported as means and standard errors (SE). We use t-test for all statistical analysis when compared MaE treatment with the control. We employed different replicates based on different indices. Fertilization, malformation, mortality, and hatching rates were all performed with five replicates (n = 5). For heartbeat, the number of replicates were 15. Spontaneous twist, rate of touch response, and wriggling frequency of embryonic dark-light response, and swimming were all analyzed with six replicates. Social frequency, however, was performed with three replicates.

3. Results

3.1. MaE induced embryonic developmental toxicity of S. grahami

The most essential and immediate types of fish embryonic damage were based on our morphological indices (Fig. 1). When compared to the control (76.7%), fertilization rate in the MaE treatment was significantly lower (62.9%, $P < 0.01$). Conversely, embryonic malformation rates were higher in the MaE treatment (46.2%) than in the control (20.9%, $P < 0.01$). Mortality rates were also elevated with MaE exposure (40.4%), while the control value was only 26.7% ($P < 0.01$). Hatching was reduced from 79.3% in the control to 67.7% in the MaE treatment ($P <$

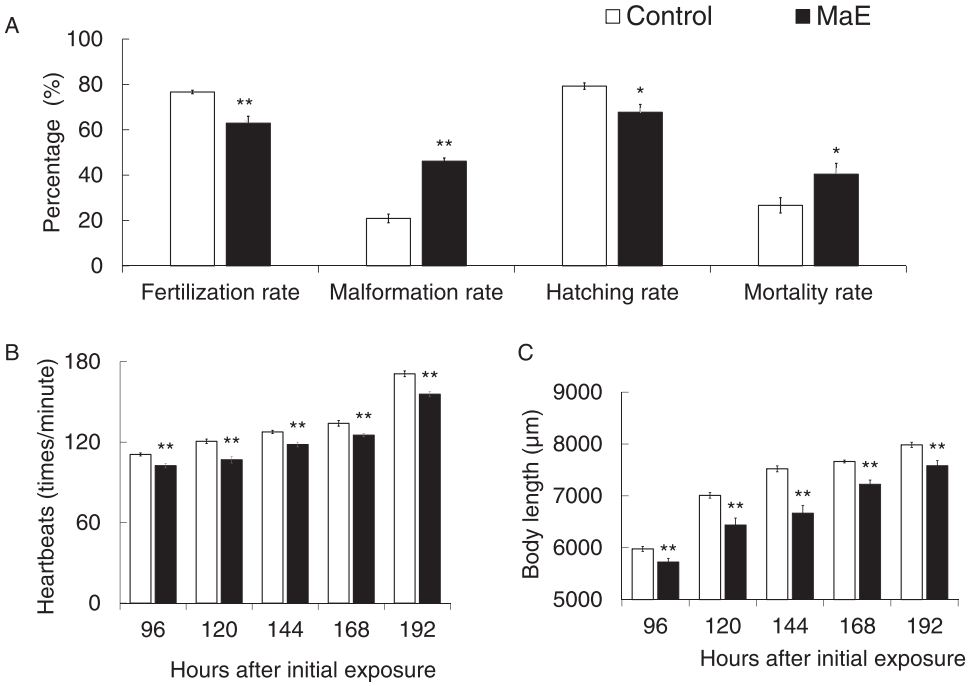


Fig. 1. Mean (± SE) effect of *M. aeruginosa* exudate (MaE) following 96-hour exposure on embryonic: A) fertilization, malformation, mortality and hatching rates; B) heartbeats; and C) body length (see Materials and methods) of *S. grahami*. *P < 0.05, * *P < 0.01 using t-test. Sample size is five for all assessments except heartbeats (n = 15).

0.05, Fig. 1A). MaE reduced *S. grahami* heartbeat from 76.8 (control) to 69.4 times/min at the 96th hour. This pattern continued to the 192nd hour as heartbeats decreased from 170.9 to 155.7 times/min, respectively ($P < 0.01$, Fig. 1B). Body length of *S. grahami* showed a similar pattern, decreasing from 5977 μm (control) to 5725 μm under MaE exposure at 96-hour, and from 7983 μm to 7581 μm at the end of the trial, respectively ($P < 0.01$, Fig. 1C).

3.2. MaE impaired neurobehavior

MaE had a negative effect on behavior of *S. grahami* embryos, with a significant reduction in twist time (10.0 versus 13.4 times/min, in controls; $P < 0.05$, Fig. 2A). Mechanical touch response of *S. grahami* embryos in the MaE treatment was also significantly lower (47.9%) than that of the control (73.3%, $P < 0.01$, Fig. 2B). Social frequency of larvae was also reduced by MaE exposure, to 2.8 versus 5.2 times/hour in the control ($P < 0.01$, Fig. 2C).

In both the control and MaE treatments, *S. grahami* embryos were more active in the dark than in the light. In the control treatment, embryos wriggled an average of 42.2 ± 4.5 times over five minutes in a dark environment versus 37.3 ± 3.0 times in a light environment. When exposed to MaE, values were 49.3 ± 3.9 and 43.9 ± 4.5 times in the dark and light, respectively. Overall, MaE-exposed embryos were always more active relative to the control ($P < 0.01$, Fig. 2D).

The light intensity response test revealed that embryos were very light sensitive in both control and MaE treatments (Fig. 2D). Control *S. grahami* embryos writhed 45.7 times over five minutes under high light exposure versus 50.2 times in the dark ($P < 0.01$, Fig. 2E). Similarly, under a medium light level, embryos writhed 46.6 times versus 49.9 times in the dark ($P < 0.01$, Fig. 2E). Under low light, embryos writhed 49.1 times versus 51.9 times in dark ($P < 0.01$, Fig. 2E). Embryonic twist times under high and medium light in the control did not differ, though both were significantly lower than the low light treatment ($P < 0.01$, Fig. 2E). For MaE treatment, twist times of *S. grahami* embryos was 53.9 times under high light exposure which was significantly lower than 58.9 times in the darkness. Under medium light it was 56.7 times, which was very similar to the 57.4 times in the followed darkness. Under low light, MaE induced a significant decrease to 60.6 times versus 62.4 times under the followed darkness ($P < 0.01$, Fig. 2E).

MaE also inhibited the swimming activity of embryos. Larvae in the control treatment moved equally over the circular walls' edges, but those in the MaE treatment were only active within a narrow range or even quiescent during the test period (Fig. 3A). MaE induced a decrease in swimming distance from 59.0 cm (control) to 21.4 cm ($P < 0.01$, Fig. 3B) and swimming speed from 0.049 cm/s (control) to 0.018 cm/s ($P < 0.01$, Fig. 3C) within 20 min.

3.3. Neurotransmitters disorder induced by MaE

Eleven of 14 studied neurotransmitters were significantly affected by MaE. Nine of these chemicals (5HIAA: 5-hydroxyindoleacetic acid, 5HTTP: 5-hydroxytryptophan, Ach: acetylcholine, DA: dopamine, E:

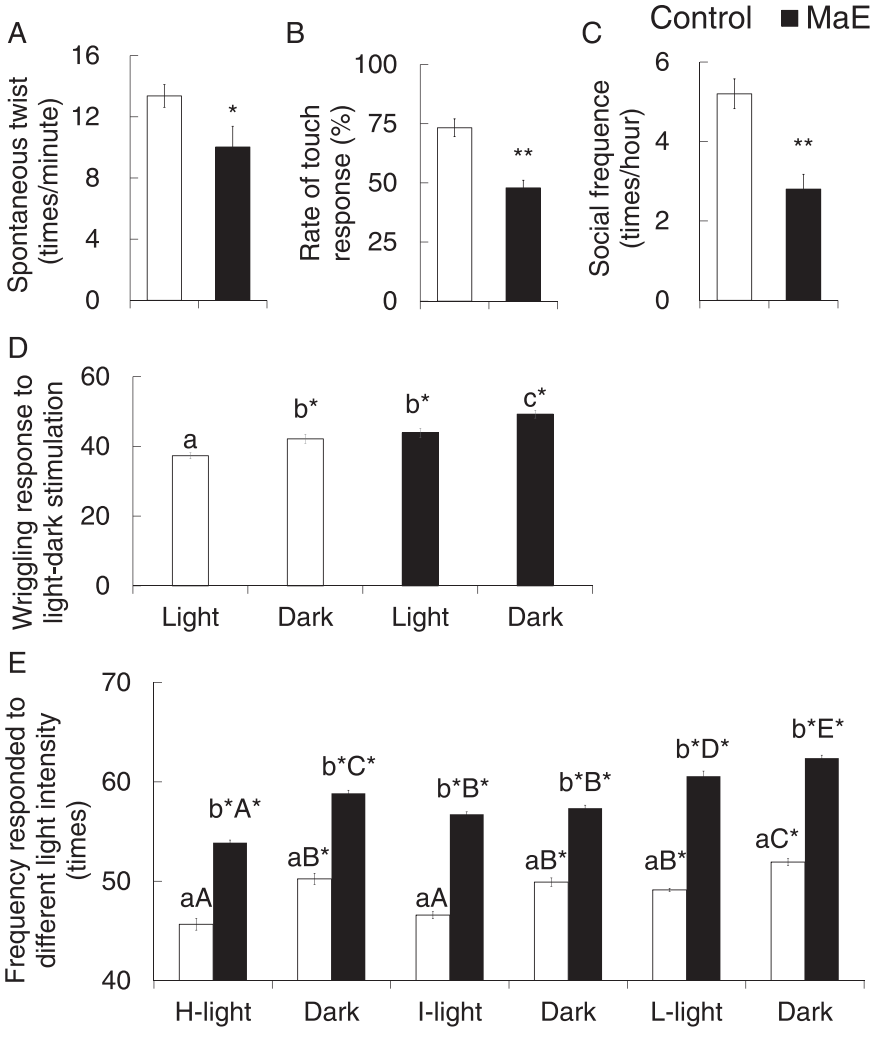


Fig. 2. Mean (± SE) effect of *M. aeruginosa* exudate (MaE) exposure on early-life *S. grahami*: A) spontaneous twist, B) rate of touch response, C) social frequency, D) wriggling frequency of embryonic dark-light response, and E) light intensity change response at 96 h after initial exposure (see Materials and methods). H-light: high light with 15 μmol/m^2·s; dark: 0 μmol/m^2·s; I-light: intermedium light with 7.5 μmol/m^2·s; L-light: low light with 4 μmol/m^2·s; lowercase letters represent differences between treatments, uppercase letters represent differences within treatments in E. *$P < 0.05$, * *$P < 0.01$ with t-test. n = 6.

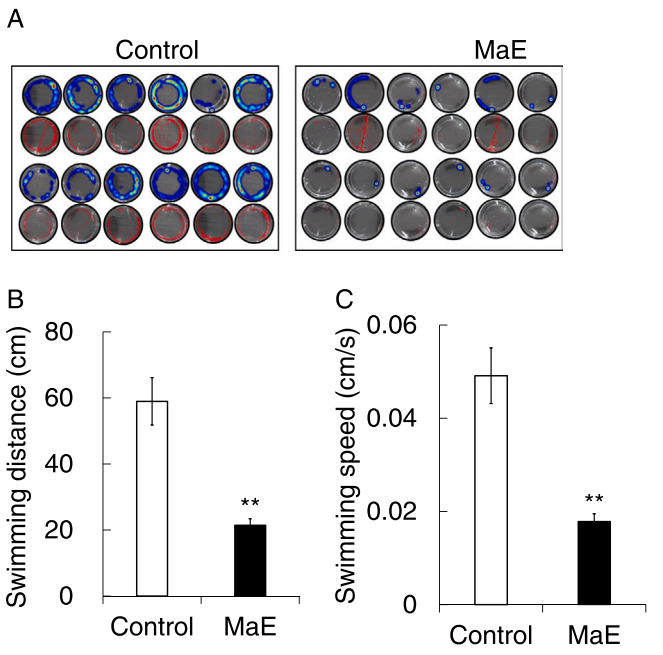


Fig. 3. Mean (± SE) effect of *M. aeruginosa* exudate (MaE) exposure on swimming of *S. grahami larvae*. Circle refers to well of 12-well cell culture plates. Hot map from blue to red indicates activity of *S. grahami*, the greater the activity, the darker the color. Red line in circle refers to movement track. * *P < 0.01 with t-test. n = 12.

epinephrine, GABA: 4-aminobutyric acid, GSH: glutathione, TrpA: tryptamine, and Tyr: L-tyrosine) were down-regulated by MaE exposure (P < 0.05, Table 1), while two others (DOPA: 3,4-dihydroxyphenylalanine and Mela: melatonin) were up-regulated with MaE exposure (P < 0.05, Table 1). The three remaining neurotransmitters (NE: norepinephrine, 5HT: serotonin, and Phe: L-Phenylalanine) were not affected by MaE.

3.4. MaE-inhibited connections among neuro-related compounds

MaE reduced sodium-dependent noradrenaline transporter-like (SLC6A2) gene transcription (Table 2), but had no effect on its gene expression or protein concentration (Fig. 4K,L). MaE treatment boosted expression of the sodium/glucose cotransporter 1-like (SGLT1) gene, which increased from 545.4 to 589.4 ng/L (P < 0.05, Table 2, Fig. 4M, N). MaE had no effect on proto-oncogene (c-fos) gene expression (Fig. 4O), but it did raise its protein content from 493.8 to 587.9 pg/ml in control and MaE treatment, respectively (P < 0.01, Fig. 4P).

Enzymes related to neuro-activity play important role in neurotransmitter-metabolism. Aromatic-L-amino-acid decarboxylase-like (DDC), responsible for synthesis 5HT and DA, increased from 216.6 to 253.7 IU/L in control and MaE exposure, respectively (P < 0.01, Fig. 4R). Likewise, acetylcholinesterase (AchE), which is responsible for decomposing acetylcholine, increased from 211.4 IU/L to 249.2 IU/L (P < 0.01, Fig. 4S). Acetylcholine transferase (ChAT), which is responsible for Ach synthesis, was not altered by MaE (Fig. 4T). Phenylethanolamine n-methyltransferase (PNMT) was not affected by MaE (Fig. 4V). Tyrosinase (TYR) concentration increased from 7.9 IU/ml in MaE versus 8.6 IU/ml in the control (Fig. 4U). Similarly, dopamine beta-monooxygenase (DBMO) increased from 180.6 to 209.6 IU/L (P < 0.01, Fig. 4W).

Increased AchE related to MaE resulted in a decreasing on Ach, while its receptor, acetylcholine receptor subunit beta-3-like (AchR) increasing with concentration from 336.6 pg/ml to 353.2 pg/ml in control and MaE treatments, respectively (P < 0.05, Table 2, Fig. 4C,D, Fig. 5). Similarly, elevated DBMO responded to a decline of DA, while D

Table 1
Mean (+/- SE) concentration of neurotransmitters affected by MaE of *S. grahami* (*P < 0.05; ** P < 0.01 with t-test. n = 3.).

	Control (nmol/g)	MaE (nmol/g)
5-Hydroxyindoleacetic acid (5HIAA)	0.0078 ± 0.0005	0.0058 ± 0.0003 *
5-Hydroxytryptophan (5HTP)	0.0107 ± 0.0001	0.0084 ± 0.0001 * *
Acetylcholine (Ach)	0.0270 ± 0.0008	0.0182 ± 0.0002 * *
Dopamine (DA)	0.0024 ± 0.0001	0.0015 ± 0.0001 *
Epinephrine (E)	0.1467 ± 0.0046	0.1049 ± 0.0073 *
4-Aminobutyric acid (GABA)	0.7710 ± 0.0362	0.5404 ± 0.0229 *
Glutathione (GSH)	1.5837 ± 0.0494	1.1147 ± 0.0224 *
3,4-Dihydroxyphenylalanine (DOPA)	0.0317 ± 0.0025	0.0931 ± 0.0083 *
Tryptamine (TrpA)	0.0072 ± 0.0001	0.0056 ± 0.0001 *
Melatonin (Mela)	0.0001 ± 0.0001	0.0003 ± 0.0001 *
L-Tyrosine (Tyr)	10.1880 ± 0.1454	7.7287 ± 0.2205 * *
Norepinephrine (NE)	0.0019 ± 0.0001	0.0017 ± 0.0001
Serotonin (5HT)	0.0016 ± 0.0001	0.0016 ± 0.0001
L-Phenylalanine (Phe)	14.2620 ± 0.7134	15.1924 ± 1.1160

Table 2
Differentially expressed gene by RNA-seq of *S. grahami* embryos exposed to MaE compared to control. log2FC is log2Fold Change in gene expression of the MaE treatment versus the control.

Gene name	Protein name	Gene description	log2FC	P value
LOC107548683	5HT3A	5-hydroxytryptamine receptor 3A-like	4.3896	0.0759
LOC107574616	AchR	Neuronal acetylcholine receptor subunit beta-3-like	-3.6478	0.0006
LOC107570615	GAT1	Sodium- and chloride-dependent GABA transporter 1-like	-4.4679	0.0077
LOC107601670	DDC	Aromatic-L-amino-acid decarboxylase-like	2.1103	0.0277
LOC107557345	DRD5	D (5)-like dopamine receptor	-3.4527	0.2093
LOC107603217	GABAB	Gamma-aminobutyric acid receptor subunit alpha-1-like	-4.6553	0.0252
LOC107554461	ADRB2	Beta-2 adrenergic receptor-like	-1.1745	0.0565
LOC107548932	SLC6A2	Sodium-dependent noradrenaline transporter-like	-2.3124	0.0191
LOC107562539	SGLT1	Sodium/glucose cotransporter 1-like	1.2483	0.0086
c-fos	c-fos	Proto-oncogene c-fos	-4.8006	0.0184

(5)-like dopamine receptor (DRD5) gene and protein expression were up-regulated, with concentrations rising from 9.0 ng/L in the control to 13.4 ng/L in the MaE treatment (P < 0.01, Table 2, Fig. 4E,F, Fig. 5). Beta-2 adrenergic receptor-like (ADRβ2) concentration increased from 176.5 ng/L in the control to 208.6 ng/L (MaE treatment, P < 0.01, Fig. 4H), when its ligand, E, decreased (Fig. 5). Similar to ADRβ2, MaE suppressed sodium- and chloride-dependent GABA transporter 1-like (GAT1) gene expression while increasing its concentration from 337.4 ng/L to 383.9 ng/L (P < 0.01, Table 2, Fig. 4I,J, Fig. 5). 5-hydroxytryptamine receptor 3A-like (5HT3A) increased from 31.7 pg/ml in the control to 37.3 pg/ml in the MaE treatment (P < 0.01, Table 2, Fig. 4A,B) without its ligand, 5HT, changing (Fig. 5).

4. Discussion

Many species are harmed by exposure to cHABs (Breinlinger et al., 2021). Cyanobacterial exudates released by living cells can be extremely hazardous to aquatic organisms (Xu et al., 2019). To determine these hazards, we exposed fish embryos to these exudates (Braunbeck et al., 2015). Our findings demonstrated that cyanobacterial exudates interfered with *S. grahami* embryonic development, resulting in decreased

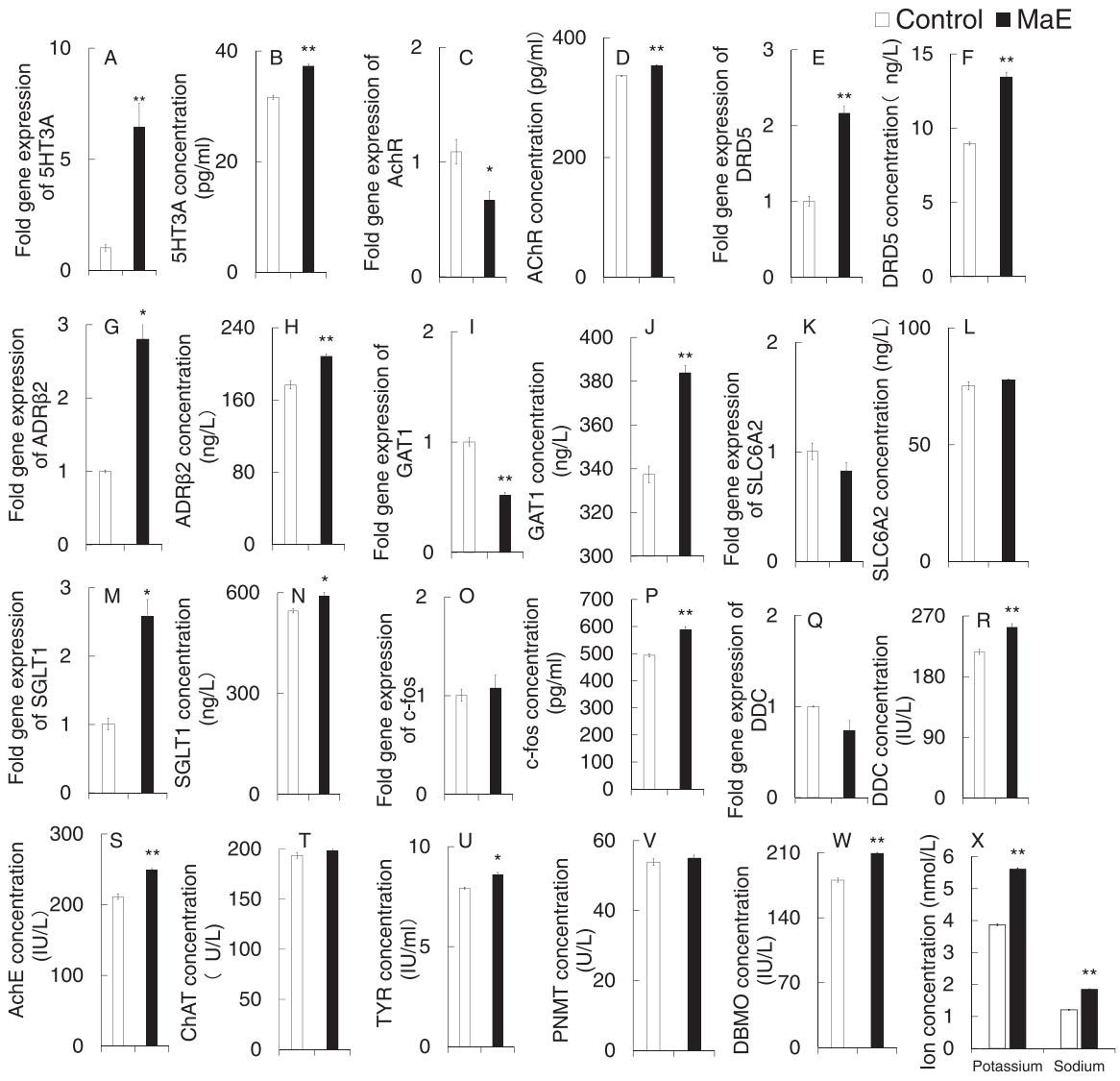


Fig. 4. Mean (± SE) effect of *M. aeruginosa* exudate (MaE) exposure on gene expression and concentration of neurobehavior-related components of *S. grahami*. * P < 0.05, * * P < 0.01 with t-test. Sample size is three for all assessments except ion concentration (X, n = 6).

fertilization, hatching rate, and heartbeat rate, as well as increased deformities and mortality rate (Fig. 1). Furthermore, MaE exhibited neurotoxicity to early-life stages of this fish species, mainly by disrupting neurotransmitters and their receptors (Table 1 and Fig. 4) which resulted in abnormal neurobehaviors (Figs. 2 and 3). Neurotransmitter receptors were over-expressed in response to ligand deprivation (Doğanlar et al., 2019). Similarly, MaE increased the expression of five targeted receptors while decreasing the levels of their respective neurotransmitters (Fig. 5). Our work illustrates that MaE toxicity caused changes to *S. grahami's* neurobehavior from genic expressions to neuro-related compound metabolites.

To explore MaE-induced photo response of *S. grahami*, healthy embryos had aberrant eye structure following MaE treatment, suggesting that eye damage might occur. MaE decreased the quantity of visual pigments on the photoreceptor cells of embryos at 120 h after initial exposure, and the various structural levels were not cleanly ordered (Fig. S2). Thus, photosensitivity and alert embryonic light sensitivity may be affected by abnormal eye development. A precondition for eye function is retinas with regular layers. Retinas are densely packed with a wide range of neurons. Fish retina consists of outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL), ganglion cell layer (GCL), and inner plexiform layer (IPL, Bejarano-Escobar et al., 2014). MaE caused retinal damage in *S. grahami* by failure of structured layers, which indicates disorder or lack of differentiation of different neurons. Retinal damage or degeneration may result in loss visual acuity (Angueyra and Kindt, 2018). However, additional work on effects of cyanobacterial exudates fish retinas is warranted.

Although we investigated a potential MaE-induced neurotoxicity in fish, mechanisms producing toxic effects are not entirely clear. Nerve impulses occur when the resting potential changes along with neurotransmitters release and bind to receptors. Potassium and sodium are the most necessary positive ions in changing resting potential (Bezine et al., 2018). In this work, we focused on potassium and sodium concentrations in the whole embryo. We found that they over-accumulated (P < 0.01, Fig. 4X), though we have no data for theoe ions in the nervous system. Hence, ions disturbed by MaE exposure also requires attention to increase understanding as well as the mechanism of MaE-induced neurotoxicity.

S. grahami was once the dominant fish species in Lake Dianchi, though it is now critically endangered. One of the roadblocks to *S. grahami* recovery could be the broad coverage of cyanobacterial blooms in the lake (Zi et al., 2018). Neurotoxicity of cyanobacterial exudates is a critical element that must be addressed, as it affects both the environment and recovery efforts for this and other native fish

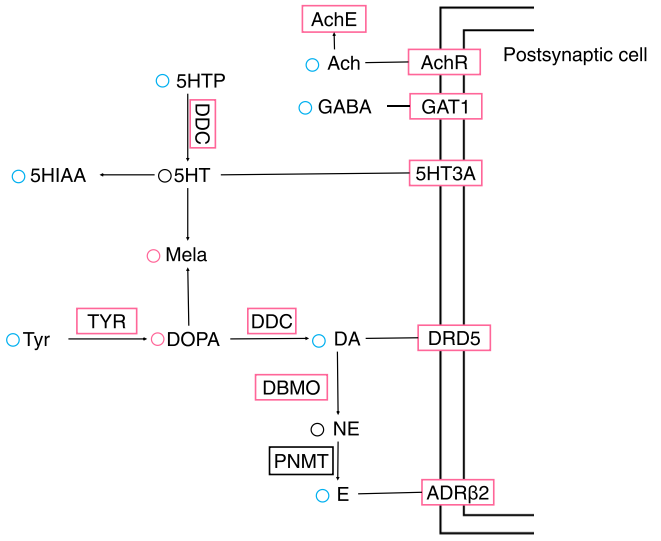


Fig. 5. Proposed MaE-induced neurobehavioral abnormalities in *S. grahami* by neurotransmitter disorders, as well as their metabolic enzymes and receptors. The double line indicates membrane; the lines between two components represent the relationship of receptors to ligands; the arrows point from upstream to downstream. The pink, blue, and dark borders indicate increasing, decreasing, and no change in *S. grahami* in MaE treatment compared to control, respectively. AchE: acetylcholinesterase; Ach: acetylcholine; AchR: acetylcholine receptor; GABA: 4-Aminobutyric acid; GAT1: sodium- and chloride-dependent GABA transporter 1-like; 5HTP: 5-Hydroxytryptophan; DDC: aromatic-L-amino-acid decarboxylase; 5HT: serotonin; 5HIAA: 5-Hydroxyindoleacetic acid; 5HT3A: 5-hydroxytryptamine receptor 3A-like; Mela: melatonin; Tyr: L-Tyrosine; TYR: tyrosinase; DOPA: 3,4-Dihydroxyphenylalanine; DA: dopamine; DRD5: D (5)-like dopamine receptor; DBMO: dopamine beta-monooxygenase; NE: norepinephrine; PNMT: phenylethanolamine N-methyltransferase; E: epinephrine; ADRβ2: beta-2 adrenergic receptor-like.

species.

CRediT authorship contribution statement

WC: conceptualization, methodology, data collection, formal analysis, data curation, writing – original draft; HJM: Conceptualization, writing - original draft and editing, funding, supervision; RX: methodology, data curation; JZ: data collection -morphological indices, collecting samples; XP, YZ, and JY: resources, methodology; BD: conceptualization, methodology; JL: methodology; YZ: collecting samples. XC: conceptualization, funding, data curation, writing- original draft and editing, supervision.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Xuexiu Chang reports financial support was provided by National Natural Science Foundation of China. Hugh J. MacIsaac and Xuexiu Chang reports financial support was provided by Great Lakes Fishery Commission. Xuexiu Chang reports financial support was provided by Yunnan Provincial Science and Technology Department grants. Junxing Yang reports financial support was provided by Yunnan Provincial Science and Technology Department grants. Hugh J. MacIsaac reports financial support was provided by NSERC Discovery grant and Canada Research Chair.

Data availability

Data will be made available on request.

Acknowledgements

This research was financially supported by National Natural Science Foundation of China (NSFC)-Yunnan Joint Key Grant (U1902202, to XC), Great Lakes Fishery Commission (to HJM and XC), Yunnan Provincial Science and Technology Department Grants (Nos: 2019FA043, 2018FY001-007, to XC; 202003AD150017, to JY), and an NSERC Discovery grant and Canada Research Chair (to HJM).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2022.114119.

References

Angueyra, J.M., Kindt, K.S., 2018. Leveraging zebrafish to study retinal degenerations. Front. Cell. Dev. Biol. 110, 1–19. https://doi.org/10.3389/fcell.2018.00110.

Banerjee, S., Maity, S., Guchhait, R., Chatterjee, A., Biswas, C., Adhikari, M., Pramanick, K., 2021. Toxic effects of cyanotoxins in teleost fish: a comprehensive review. Aquat. Toxicol. 240, 105971 https://doi.org/10.1016/j.aquatox.2021.105971.

Bejarano-Escobar, R., Blasco, M., Martín-Partido, G., Francisco-Morcillo, J., 2014. Molecular characterization of cell types in the developing, mature, and regenerating fish retina. Rev. Fish. Biol. Fish. 24, 127–158. https://doi.org/10.1007/s11160-013-9320-z.

Bezine, M., Namsi, A., Sghaier, R., Khalifa, R.B., Hamdouni, H., Brahmi, F., Badreddine, I., Mihoubi, W., Nury, T., Vejux, A., Zarrouk, A., 2018. The effect of oxysterols on nerve impulses. https://doi-org.ledproxy2.uwindsor.ca/ Biochimie 153, 46–51. https://doi.org/10.1016/j.biochi.2018.04.013.

Braunbeck, T., Kais, B., Lammer, E., Otte, J., Schneider, K., Stengel, D., Strecker, R., 2015. The fish embryo test (FET): origin, applications, and future. Environ. Sci. Pollut. Res. 22, 16247–16261. https://doi.org/10.1007/s11356-014-3814-7.

Breinlinger, S., Phillips, T.J., Haram, B.N., Mareš, J., Martínez Yerena, J.A., Hrouzek, P., Sobotka, R., Henderson, W.M., Schmieder, P., Williams, S.M., Lauderdale, J.D., 2021. Hunting the eagle killer: a cyanobacterial neurotoxin causes vacuolar myelinopathy. Science 371, eaax9050. https://doi.org/10.1126/science.aax9050.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., 2009. The MIQE guidelines: minimum information for publication of quantitative Real-Time PCR experiments. Clin. Chem. 55, 611–622. https://doi.org/10.1373/clinchem.2008.112797.

Carmichael, W.W., Boyer, G.L., 2016. Health impacts from cyanobacteria harmful algae blooms: Implications for the North American Great Lakes. Harmful Algae. 54, 194–212. https://doi.org/10.1016/j.hal.2016.02.002.

Chellappa, N.T., Chellappa, S.L., Chellappa, S., 2008. Harmful phytoplankton blooms and fish mortality in a eutrophicated reservoir of northeast Brazil. Braz. Arch. Biol. Technol. 51, 633–641. https://doi.org/10.1590/S1516-89132008000400022.

Coulter, D.P., Huff Hartz, K.E., Sepúlveda, M.S., Godfrey, A., Garvey, J.E., Lydy, M.J., 2019. Lifelong exposure to dioxin-like PCBs alters paternal offspring care behavior and reduces male fish reproductive success. Environ. Sci. Technol. 53, 11507–11514. https://doi.org/10.1021/acs.est.9b03460.

Deng, J., Zhang, X., Han, X., Tao, L., Bi, B., Kang, B., 2014. Dietary protein requirement of juvenile Dianchi golden-line barbell, *Sinocyclocheilus grahami*. J. World Aquacult. Soc. 45, 421–429. https://doi.org/10.1111/jwas.12137.

Doğanlar, Z.B., Güçlü, H., Öztopuz, Ö., Türkön, H., Dogan, A., Uzun, M., Doğanlar, O., 2019. The role of melatonin in oxidative stress, DNA damage, apoptosis and angiogenesis in fetal eye under preeclampsia and melatonin deficiency stress. Curr. Eye. Res. 44, 1157–1169. https://doi.org/10.1080/02713683.2019.1619778.

Drobac Backović, D., Tokodi, N., Marinović, Z., Lujić, J., Dulić, T., Simić, S.B., Đorđević, N.B., Kitanović, N., Šćekić, I., Urbányi, B., Meriluoto, J., 2021. Cyanobacteria, cyanotoxins, and their histopathological effects on fish tissues in Fehérvárcsurgó reservoir, Hungary. Environ. Monit. Assess. 193, 1–14. https://doi.org/10.1007/s10661-021-09324-3.

EPA (2016) Ecological effects test guidelines OCSPP 850.1400: Fish Early Life Stage Toxicity Test. ⟨https://nepis.epa.gov/Exe/ZyNET.exe/P100SH6O.TXT?ZyActionD=ZyDocument&Client=EPA&Index=2016+Thru+2020&Docs=&Query=&Time=&EndTime=&SearchMethod=1&TocRestrict=n&Toc=&TocEntry=&QFieldYear=&QFieldMonth=&QFieldDay=&IntQFieldOp=0&ExtQFieldOp=0&XmlQuery=&File=D%3A%5Czyfiles%5CIndex%20Dat%5C16thru20%5CTxt%5C00000000%5CP100SH6O.txt&User=ANONYMOUS&Password=anonymous&SortMethod=h%7C-&MaximumDocuments=1&FuzzyDegree=0&ImageQuality=r75g8/r75g8/x150y150g16/i425&Display=hpfr&DefSeekPage=x&SearchBack=ZyActionL&Back=ZyActionS&BackDesc=Results%20page&MaximumPages=1&ZyEntry=1&SeekPage=x&ZyPURL⟩.

Florczyk, M., Łakomiak, A., Woźny, M., Brzuzan, P., 2014. Neurotoxicity of cyanobacterial toxins. Environ. Biotechnol. 10, 26–43. https://doi.org/10.14799/ebms246.

Hilborn, E.D., Beasley, V.R., 2015. One health and cyanobacteria in freshwater systems: animal illnesses and deaths are sentinel events for human health risks. Toxins 7, 1374–1395. https://doi.org/10.3390/toxins7041374.

Hu, C., Rzymski, P., 2022. Non-photosynthetic melainabacteria (cyanobacteria) in human gut: characteristics and association with health. Life 12, 476. https://doi.org/10.3390/life12040476.

Huisman, J., Codd, G.A., Paerl, H.W., Ibelings, B.W., Verspagen, J.M., Visser, P.M., 2018. Cyanobacterial blooms. Nat. Rev. Microbiol. 16, 471–483. https://doi.org/10.1038/s41579-018-0040-1.

Jiang, M., Zhou, Y., Wang, N., Xu, L., Zheng, Z., Zhang, J., 2019. Allelopathic effects of harmful algal extracts and exudates on biofilms on leaves of *Vallisneria natans*. Sci. Total. Environ. 655, 823–830. https://doi.org/10.1016/j.scitotenv.2018.11.296.

Jonas, A., Scholz, S., Fetter, E., Sychrova, E., Novakova, K., Ortmann, J., Benisek, M., Adamovsky, O., Giesy, J.P., Hilscherova, K., 2015. Endocrine, teratogenic and neurotoxic effects of cyanobacteria detected by cellular in vitro and zebrafish embryos assays. Chemosphere 120, 321–327. https://doi.org/10.1016/j.chemosphere.2014.07.074.

Jonasson, S., Eriksson, J., Berntzon, L., Spáčil, Z., Ilag, L.L., Ronnevi, L.O., Rasmussen, U., Bergman, B., 2010. Transfer of a cyanobacterial neurotoxin within a temperate aquatic ecosystem suggests pathways for human exposure. Proc. Natl. Acad. Sci. 107, 9252–9257. https://doi.org/10.1073/pnas.0914417107.

Kõnemann, S., von Wyl, M., Vom Berg, C., 2022. Zebrafish larvae rapidly recover from locomotor effects and neuromuscular alterations induced by cholinergic insecticides. Environ. Sci. Technol. 56, 8449–8462. https://doi.org/10.1021/acs.est.2c00161.

Li, H., Ai, H., Kang, L., Sun, X., He, Q., 2016. Simultaneous *Microcystis* algicidal and microcystin degrading capability by a single *Acinetobacter* bacterial strain. Environ. Sci. Technol. 50, 11903–11911. https://doi.org/10.1021/acs.est.6b03986.

Li, W., Mao, F., Te, S.H., He, Y., Gin, K.Y., 2021a. Impacts of *Microcystis* on the dissemination of the antibiotic resistome in cyanobacterial blooms. Acs. Est. Water 1 (5), 1263–1273. https://doi.org/10.1021/acsestwater.1c00006.

Li, X., Liu, H., Li, D., Lei, H., Wei, X., Schlenk, D., Mu, J., Chen, H., Yan, B., Xie, L., 2021b. Dietary seleno-l-methionine causes alterations in neurotransmitters, ultrastructure of the brain, and behaviors in zebrafish (*Danio rerio*). Environ. Sci. Technol. 55, 11894–11905. https://doi.org/10.1021/acs.est.1c03457.

Paerl, H.W., Gardner, W.S., Havens, K.E., Joyner, A.R., McCarthy, M.J., Newell, S.E., Qin, B., Scott, J.T., 2016. Mitigating cyanobacterial harmful algal blooms in aquatic ecosystems impacted by climate change and anthropogenic nutrients. Harmful Algae 54, 213–222. https://doi.org/10.1016/j.hal.2015.09.009.

Pípal, M., Legradi, J., Smutná, M., Kočí, T., Priebojová, J., Bláhová, L., Krauss, M., Hilscherová, K., 2020. Neurobehavioral effects of cyanobacterial biomass field extracts on zebrafish embryos and potential role of retinoids. Aquat. Toxicol. 228, 105613 https://doi.org/10.1016/j.aquatox.2020.105613.

Qian, H., Liu, G., Lu, T., Sun, L., 2018. Developmental neurotoxicity of *Microcystis aeruginosa* in the early life stages of zebrafish. Ecotoxicol. Environ. Saf. 151, 35–41. https://doi.org/10.1016/j.ecoenv.2017.12.059.

Spoof, L., Jaakkola, S., Važić, T., Häggqvist, K., Kirkkala, T., Ventelä, A.M., Kirkkala, T., Svirčev, Z., Meriluoto, J., 2020. Elimination of cyanobacteria and microcystins in irrigation water-effects of hydrogen peroxide treatment. Environ. Sci. Pollut. Res. 27, 8638–8652. https://doi.org/10.1007/s11356-019-07476-x.

Tang, X., Krausfeldt, L.E., Shao, K., LeCleir, G.R., Stough, J.M., Gao, G., Boyer, G.L., Zhang, Y., Paerl, H.W., Qin, B., Wilhelm, S.W., 2018. Seasonal gene expression and the ecophysiological implications of toxic *Microcystis aeruginosa* blooms in Lake Taihu. Environ. Sci. Technol. 52, 11049–11059. https://doi.org/10.1021/acs.est.8b01066.

Veerman, J., Kumar, A., Mishra, D.R., 2022. Exceptional landscape-wide cyanobacteria bloom in Okavango Delta, Botswana in 2020 coincided with a mass elephant die-off event. Harmful Algae 111, 102145. https://doi.org/10.1016/j.hal.2021.102145.

Wu, J., Lang, H., Mu, X., Zhang, Z., Su, Q., Hu, X., Zheng, H., 2021. Honey bee genetics shape the strain-level structure of gut microbiota in social transmission. Microbiome 9, 1–19. https://doi.org/10.1186/s40168-021-01174-y.

Xu, R., Jiang, Y., MacIsaac, H.J., Chen, L., Li, J., Xu, J., Wang, T., Zi, Y., Chang, X., 2019. Blooming cyanobacteria alter water flea reproduction via exudates of estrogen analogues. Sci. Total. Environ. 696, 133909 https://doi.org/10.1016/j.scitotenv.2019.133909.

Yang, J.X., Pan, X., Li, Z., 2007. Preliminary report on the successful breeding of the endangered fish *Sinocyclocheilus grahami* endemic to Dianchi Lake. Zool. Res. 28, 329–331.

Zhang, Y.W., Pan, X.F., Wang, X.A., Jiang, W.S., Yang, K.F., Liu, Q., Yang, J.X., 2017. Selection of reliable reference genes for quantitative Real-Time PCR in golden-line barbell (*Sinocyclocheilus grahami*) during juvenile and adult stages. J. Fisheries Sci. Com. 11 https://doi.org/10.21767/1307-234X.1000122.

Zheng, G., Xu, R., Chang, X., Hilt, S., Wu, C., 2013. Cyanobacteria can allelopathically inhibit submerged macrophytes: effects of *Microcystis aeruginosa* extracts and exudates on *Potamogeton malaianus*. Aquat. Bot. 109, 1–7. https://doi.org/10.1016/j.aquabot.2013.02.004.

Zhou, Q., Zhang, Y., Lin, D., Shan, K., Luo, Y., Zhao, L., Tan, Z., Song, L., 2016. The relationships of meteorological factors and nutrient levels with phytoplankton biomass in a shallow eutrophic lake dominated by cyanobacteria, Lake Dianchi from 1991 to 2013. Environ. Sci. Pollut. Res. 23, 5616–15626. https://doi.org/10.1007/s11356-016-6748-4.

Zi, J., Pan, X., MacIsaac, H.J., Yang, J., Xu, R., Chen, S., Chang, X., 2018. Cyanobacteria blooms induce embryonic heart failure in an endangered fish species. Aquat. Toxicol. 194, 78–85. https://doi.org/10.1016/j.aquatox.2017.11.007.