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Non-microcystin extracellular metabolites of *Microcystis aeruginosa* impair viability and reproductive gene expression in rainbow trout cell lines

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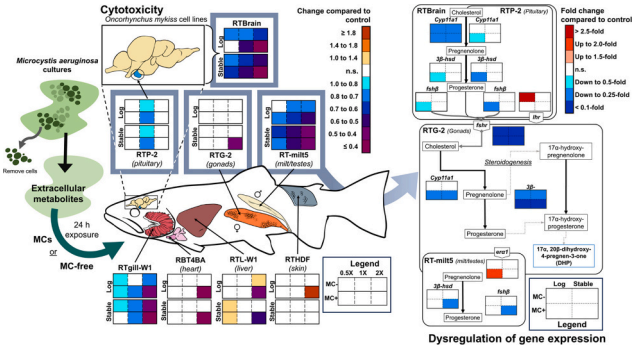
HIGHLIGHTS

- "MC-free" MaE impaired viability of RTBrain, RTgill-W1, and RT-milt5 cell lines.
- Cytotoxicity of MC+ and MC- strains overlapped, especially in stable growth phase.
- Exudates from both strains altered expression of key reproductive genes.

GRAPHICAL ABSTRACT



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ABSTRACT

Microcystis aeruginosa is a ubiquitous freshwater cyanobacterium best known for producing hepatotoxic microcystins; however, this common bloom-forming species also produces myriad biologically active and potentially deleterious other metabolites. Our understanding of the effects of these non-microcystin metabolites on fish is limited. In this study, we evaluated cytotoxicity of extracellular metabolites harvested from both microcystin-producing (MC+) and non-producing (MC-) strains of *M. aeruginosa* on rainbow trout (*Oncorhynchus mykiss*) cell lines derived from tissues of the brain, pituitary, heart, gonads, gills, skin, liver, and milt. We also examined the influence of *M. aeruginosa* exudates (MaE) on the expression of critical reproduction-related genes using the same cell lines. We found that exudates of the MC- *M. aeruginosa* strain significantly reduced viability in RTBrain, RTgill-W1, and RT-milt5 cell lines and induced significant cellular stress and/or injury in six of the eight cell lines—highlighting potential target tissues of cyanobacterial cytotoxic effects. Observed sublethal consequences of *Microcystis* bloom exposure occurred with both MC+ and MC- strains' exudates and significantly altered expression of developmental and sex steroidogenic genes. Collectively, our results emphasize the contributions of

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non-MC metabolites to toxicity of *Microcystis*-dominated algal blooms and the need to integrate the full diversity of *M. aeruginosa* compounds—beyond microcystins—into ecotoxicological risk assessments.

1. Introduction

The prolific, bloom-forming cyanobacterium *Microcystis aeruginosa* is well-studied for its production of hepatotoxic microcystins (MCs), cyclic heptapeptides and potent inhibitors of protein phosphatases that impair cellular function and cause significant damage to tissue integrity (e.g., Chorus and Welker, 2021). However, harmful algal blooms (HABs) dominated by *M. aeruginosa* and other cyanobacteria contain a cocktail of bio-active and potentially harmful metabolites and the identification, isolation, and toxicological assessment of these compounds is the subject of ongoing research (Zhou et al., 2023). On their own, some deleterious metabolites have the potential to disturb cellular processes by inhibiting critical serine proteases (Huang and Zimba, 2019), produce broad systemic effects (e.g., neurotoxicity; Faltermann et al., 2014), or induce DNA damage and inflammatory responses (Moosová et al., 2019; Ujvárosi et al., 2020). Individual compound toxicity is only one component of current HABs research, as growing interest has focused on effects of complex cyanobacterial mixtures (CCMs) – including studies of algal cultures, cells, extracts, and exudates – on aquatic fauna (e.g., Le Manach et al., 2016; Huang et al., 2020; Zhang et al., 2023).

Within HABs-affected waterways, the health of freshwater fish populations is of critical economic and ecological importance. Fish may be exposed to *Microcystis* metabolites by ingesting algal cells or by contact with exuded compounds through their skin and gills throughout the duration of a bloom (Sukenik et al., 2015). During vulnerable early life stages (ELS), exposure to *M. aeruginosa* cultures or extracts in fish impaired embryonic development and viability, leading to increased malformations, decreased heart rates, and mortality (Ghazali et al., 2009; Li et al., 2021). For adult fish, exposure to *M. aeruginosa* CCMs can produce histopathological damage in affected tissues as well as dysregulating expression of genes related to growth and metabolism, reproduction, and other biological processes (Le Manach et al., 2016; Le Manach et al., 2018). Comparisons of the effects of CCMs to purified MCs has revealed that compound mixtures may produce distinct effects such as dysregulation in a greater number of genes and pathways than MC exposure alone (Saraf et al., 2018; Chen et al., 2017; Qiao et al., 2016).

To better delineate the effects of non-MC metabolites from those of MCs, expanding HABs research has utilized non-microcystin-producing (MC-) *M. aeruginosa* strains, which are naturally occurring components of many *Microcystis* blooms (Rinta-Kanto et al., 2009). Similar to their "toxic" counterparts, extracts of MC- *M. aeruginosa* strains may alter development and mortality in ELS (De Almeida Torres et al., 2023), and histopathological liver damage in adults (Le Manach et al., 2018). These mixtures also cause dysregulation of proteins associated with metabolic, reproductive, and other biological and cellular processes in adult fish (Le Manach et al., 2016; Sotton et al., 2017). MC- strains also alter zooplankton and bivalve feeding behaviour and reproductive success (Huang et al., 2020; Zhang et al., 2023).

Growing research on MC- strains has only scratched the surface regarding their overall contribution to HABs toxicity to fish and their capacity to affect freshwater ecosystems. Sublethal alterations to physiological processes - especially reproduction (Le Manach et al., 2016; Sotton et al., 2017) - hint at potentially larger-scale impacts on fish health. Pollutants in aquatic systems that disturb critical endocrine signalling and regulatory systems have the potential to negatively impact reproduction (Marlatt et al., 2022). *Microcystis aeruginosa* metabolites have been implicated in the failure of recovery efforts for an endangered fish species (Zi et al., 2018).

Fish-derived cell lines are now commonly used in toxicological research, as they offer rapid, reproducible results and reduced costs relative to whole animal assays (Bols et al., 2005). Cell lines from the common model species rainbow trout (RT, *Oncorhynchus mykiss*) have been used in toxicity testing of many common pollutants and cyanotoxins (e.g., Pichardo et al., 2006; Wang et al., 2015). For *Microcystis* CCMs, both extracts and exudates of a non-MC-producing *M. aeruginosa* strain negatively affected viability of gill-derived fish cell line RTgill-W1 (Sorichetti et al., 2014). This cell line is just one of a growing number of RT cell lines currently in use (Bols et al., 2017), including those derived from the tissues of the brain, skin, heart, liver, and gonads utilized in the current study. In turn, increased use of cell lines contributes to a growing knowledgebase, expanding their applicability and functionality in in vitro research.

Prior proteomic analyses revealed possible disruption of physiological and reproductive processes within liver tissues by *M. aeruginosa* metabolites, including those from MC- strains (Le Manach et al., 2016; Sotton et al., 2017). However, teleost reproduction relies on the coordination of multiple pathways within the Hypothalamus-Pituitary-Gonadal (HPG) axis (Fig. 1) and adjacent systems, comprising a multi-organ network to function (Arcand-Hoy and Benson, 1998). Critical signalling and feedback systems within the brain and gonads of teleost fish and other vertebrates governs the timing and maintenance of key reproductive/developmental events, primarily through the regulation of sex steroid hormones – i.e., estrogens, androgens, and progestogens (Arcand-Hoy and Benson, 1998; Yaron and Levavi-Sivan, 2011). Compounds capable of disrupting these systems may affect reproductive fitness of fish and other species (Marlatt et al., 2022).

The aim of our study was to utilize a broad complement of RT cell lines to investigate the potential of extracellular metabolites produced by both MC-producing (MC+) and non-producing (MC-) strains of *M. aeruginosa* to negatively affect fish cells, to explore tissue-specific sensitivities absent in previous studies, and to determine the potential of these exudates to alter expression of reproductive genes in cells of relevant tissues. We hypothesized that both MC+ and MC- exudates of *M. aeruginosa* would impair the viability of RT cells derived from other tissues in addition to liver and gill cells, as well as disturbing transcription of genes associated with reproductive pathways. To assess the effects of a bloom's changing extracellular metabolome across its lifespan, we examined cytotoxicity and reproductive impairment of extracellular metabolites derived from both exponential and stable growth phase cultures.

2. Materials and methods

2.1. M. aeruginosa cultures and exudate preparation

The MC-producing *M. aeruginosa* CPCC 300 and non-MC-producing CPCC 633 were obtained from the Canadian Phycological Culture Centre (CPCC) at the University of Waterloo (Ontario, Canada). Stock cultures of both strains were maintained in BG-11 growth medium and incubated at 23 ± 1.5 °C in a 12:12 light-dark cycle with fluorescent light of 19.8 ± 1.4 μmol m^{-2} s^{-1}. For experimental cultures of each strain, we inoculated 1000 mL Erlenmeyer flasks with 300 mL of consolidated stock cultures at cell densities of 3.0 ± 0.1 × 10^7 cells/mL. Cultures were manually shaken every two to three days by gentle swirling but were otherwise undisturbed.

Three flasks were harvested from each strain at six days and 30 days in culture for log and stable phase exudates, respectively, and their total contents were centrifuged at 4700 xg for 10 min. We then carefully collected the supernatant and filtered it using 0.2 μm polyethersulfone (PES) filters to remove cell debris. For the CPCC 633 strain, after the pellet was discarded from the initial centrifugation, the cell-free media was centrifuged again to further pelletize debris and prevent clogging of

filters.

To confirm 'MC+' versus 'MC-' designations for the *M. aeruginosa* strains, microcystin concentrations for exudate samples of each strain and growth phase were determined using a 96-well microcystin enzyme-linked immunosorbent assay (ELISA, Beacon Analytical Systems Inc., Maine, USA) according to the manufacturer's protocol (Table 1). This ELISA kit provides broad detection of MC congeners, primarily the highly toxic MC-LR as well as (in descending order of relative cross-reactivity) MC-RR, MC-YR, MC-LW, MC-LF, and MC-LA, and the closely related cyanotoxin, nodularin. The detection limit of this kit is between 0.1 and 2.0 ppb or μg/L. *Microcystis aeruginosa* exudates (MaE) and BG-11 controls were lyophilized in a benchtop freeze dryer (Labconco, Missouri, USA) and resuspended in phenol-free Leibovitz's L-15 medium (Cytiva) to generate a 20× concentrated stock solution. We then consolidated triplicate flasks for final treatment solutions for each *M. aeruginosa* strain and growth phase. These solutions were aliquoted and stored frozen at −80 °C until use.

Table 1
Equivalent cell densities in cells per mL and microcystin (MC) concentration for each exudate treatment of the microcystin-producing (MC+) and non-producing (MC-) *Microcystis aeruginosa* strains. Equivalent densities are based on cell counts taken immediately prior to the removal of cells via centrifugation and filtration and MC concentrations were determined by enzyme-linked immunosorbent assays (ELISA).

Strain	Growth phase	Treatment	Equivalent density (x 10^7 cells/mL)	MC concentration (μg/L)
CPCC 300 (MC+)	Log	LMC+	4.60 ± 0.10	1.042 ± 0.312
	Stable	SMC+	9.78 ± 0.01	0.841 ± 0.082
CPCC 633 (MC-)	Log	LMC-	4.52 ± 0.07	*Below limit of detection
	Stable	SMC-	8.53 ± 0.06	*Below limit of detection

2.2. *O. mykiss* cell cultures

Rainbow trout cell lines were provided by Dr. B. Dixon and Dr. N.T.K. Vo (Table 2). All cell lines were routinely grown in 175 cm^2 plug-seal tissue culture flasks at 19 ± 1.5 °C in L-15 growth medium (Cytiva) supplemented with 10–15 % (*v*/v) fetal bovine serum (FBS, Gibco), 1 % (v/v) penicillin/streptomycin (Gibco) and 1 % L-glutamine (GlutaMAX, Gibco). When cell monolayers covered 80–90 % of the flask surface flasks were considered confluent, at which point cells were passaged using TrypLE (Gibco) to new flasks. We plated cell cultures for use in experimental treatments at passages 3 to 8.

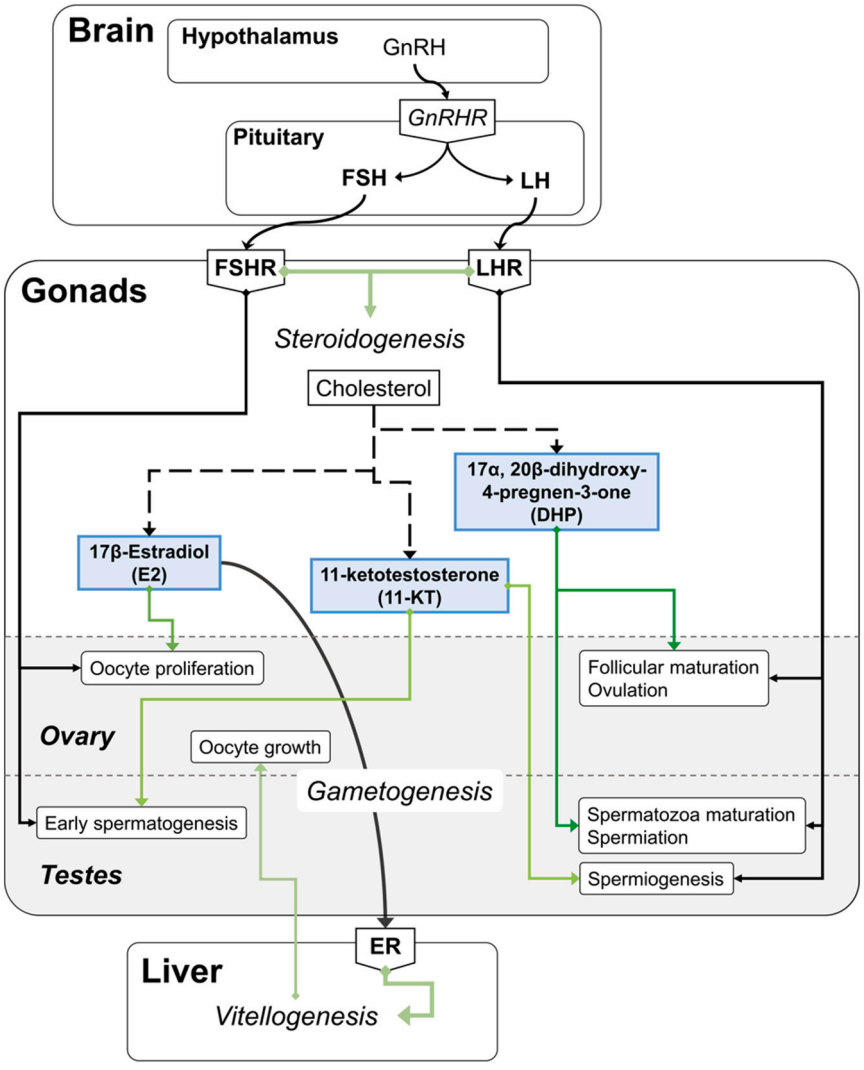

Fig. 1. A simplified view of the hypothalamus-pituitary-gonadal (HPG) axis of the teleost fish and the reproductive and developmental processes driven by sex steroid hormones and gonadotropins.

Table 2
Summary of rainbow trout (*O. mykiss*) cell line information. FBS % refers to the percentage of fetal bovine serum supplemented in the growth media required to maintain healthy cultures.

Cell line	Tissue origin	Morphology	FBS %
RBT4BA	Heart (bulbus arteriosus)	Epithelial/Fibroblastic	15
RTBrain	Brain	Astroglial-like	15
RTG-2	Gonads	Fibroblastic	10
RTgill-W1	Gill	Epithelial	10
RTHDF	Skin	Fibroblastic	10
RTL-W1	Liver	Epithelial	10
RT-milt5	Milt	Fibroblastic	10
RTP-2	Pituitary	Epithelial	10

2.3. Exposure conditions

Briefly, cell lines were seeded in 96-well plates at densities of 2.5 × 10^4 cells/well in complete L-15 media and incubated them overnight at 19 ± 1.5 °C. Treatment solutions were prepared immediately prior to exposure and applied to cells at concentrations of 0.5×, 1×, and 2× for cell viability assays, 1× and 2× for lysosomal assays, and 1× only for gene expression analysis by diluting concentrated MaE and BG-11 stock solutions with phenol-free L-15 exposure medium (L-15/ex, United States Biological, Massachusetts, USA). Initial growth medium was removed using a vacuum pipette and replaced it with 100 μL of each treatment, in triplicate or duplicate for cytotoxicity assays. For sufficient RNA collection, between 16 and 64 wells were exposed to each treatment solution. Complete media was replaced with L-15/ex of equal volume for untreated controls. After 24 h, we removed the treatment solutions and replaced them with the respective assay solutions as detailed below. Each assay was repeated in at least three separate plates for each cell line with cells from different passages.

2.4. Cytotoxicity assays

Cell viability was determined using a combination of two fluorescent dye-based assays – AlamarBlue (AB, purchased ready-to-use, Invitrogen) which measures cellular metabolic activity and CFDA-AM (dissolved in DMSO for a final concentration of 4 nM, Invitrogen), which monitors membrane integrity – utilized the protocol described by Dayeh et al. (2013) with minor changes. Briefly, we prepared a mixed dye solution in L-15/ex with dilutions of 1:10 and 1:1000 of AB and CFDA-AM stock solutions, respectively. Following a 24 h exposure period, treatment solutions were decanted and 100 μL of the dye solution was added to each well and plates were incubated in the dark for 2 h. Fluorescence readings were taken at excitation/emission wavelengths of 530/590 nm for AB and 485/530 nm for CFDA-AM, respectively, using a Varioskan LUX microplate reader (Thermo Fisher Scientific). Results were expressed as percentage of the mean fluorescence of BG-11 media controls.

As noted by Dayeh et al. (2013) in their protocol, the use of multiple assays can be useful in evaluating potential mechanisms of toxicity. However, with the diversity of cell lines utilized, a multi-assay approach also allowed us to explore the suitability of assay-cell line combinations for future studies. Indeed, initial pilot studies found that the lysosome-based Neutral Red uptake assay – the third assay suggested by Dayeh et al. (2013) – was unsuitable for weakly-adherent cells such as RTP-2. In its stead, we evaluated lysosomal accumulation following MaE treatments, using Lysotracker Red DND-99 stain (Invitrogen) in conjunction with NucBlue Live Cell Stain (Invitrogen) to quantify lysosomal accumulation in treated cells. After a 24 h exposure, treatment solutions were removed and replaced with 100 μL of a dye solution made up of 10 μL of 15 nM Lysotracker Red DND-99 (Invitrogen) in L-15/ex and 2 drops of NucBlue Live Cell Stain (Invitrogen) per 10 mL of L-15/ex. After 40 min of incubation in the dark, two images were taken of each well using a EVOS m5000 inverted microscope (Invitrogen). The mean fluorescence of Lysotracker Red DND-99, which selectively localizes to acidic lysosomes, in each well was then normalized to the number of cells in each well as quantified by the EVOS M5000 Imaging System's automated cell counts using the NucBlue-stained cell nuclei. Results were presented as lysosomal accumulation compared to controls.

2.5. RNA collection and quantitative real-time PCR

After 24 h, we decanted the treatment solutions from the wells, rinsed the cells with DPBS, and collected and processed RNA using PureLink RNA Mini Kits (Invitrogen). The concentration and purity of isolated RNA were measured using a Varioskan Lux microplate reader before samples were stored at −80 °C for future analysis. For qPCR, each sample was reverse transcribed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) according to manufacturer's protocol. Primers were selected from previously published *O. mykiss* studies and are shown in Table 3. We selected genes from major reproductive regulatory pathways (*fshβ, fshr, lhr, erα1*), as well as those involved in sex steroid hormone production (*cyp11a1, 3β-hsd)* and egg development (*vtg*). Real-time PCR reactions were performed in 384-well plates with 10 μL reaction volumes of 5 μL of TaqManTM Fast Advanced Master Mix (2×), 0.5 μL of each primer (20×), 3.5 μL Nuclease-free water, and 1 μL of cDNA diluted 1:10. Amplification reactions were carried out in duplicate using a QuantStudioTM 6 Flex Real-Time PCR System (Applied Biosystems) with the following run protocol: an initial stage of 95 °C for 20 s then 40 cycles of 95 °C for 1 s and 60 °C for 20 s. Using the reference gene *18 s rRNA* as an endogenous control, the relative expression of each of the target genes was calculated using the comparative CT (2-ΔΔCT) method.

2.6. Data analysis and statistics

All results were expressed as mean ± standard error of the mean (SEM) and analyzed using GraphPad Prism 8.0.2 software. For both cell viability and gene expression assays, the normality of data distribution and homogeneity of variances were assessed using the Shapiro-Wilks test and Brown-Forsythe test, respectively. Depending on whether assumptions of parametric tests were met, differences between treatments were evaluated by one of following tests: 1) One-way analysis of variance (ANOVA) followed by Tukey's post hoc test; 2) Welch's ANOVA with Games-Howell's post hoc test; or, in cases where assumptions were violated 3) the non-parametric Kruskal-Wallis test with Dunn's post hoc test. Student's *t*-tests were used to assess the differences between exudate treatments and controls. Main and interactive effects of cyanobacterial strain and concentration on cell viability were analyzed via 2-way ANOVA followed by Tukey's multiple comparisons post hoc test. *P*-values of ≤0.05 were considered statistically significant.

3. Results

3.1. Microcystin production

Microcystin concentrations for exudates of the MC+ *M. aeruginosa* strain were 1.0 ± 0.3 μg/L and 0.84 ± 0.1 μg/L for the log and stable growth phases, respectively. For the MC- strain, MCs could not be detected given the detection limit of ELISA kits used for MC production determination (<0.1 μg/L).

3.2. Cytotoxicity assays

In the AB assays, while MC-containing exudates of the MC+ *M. aeruginosa* strain induced cytotoxic effects in every tested cell line (Fig. 2, see also Supplemental Fig. S1), at the lowest tested concentrations (0.5×, approximately 0.5 and 0.4 μg/L for log and stable phases, respectively) only RTBrain, RTgill-W1, RT-milt5, and RTL-W1 cells were

Table 3
Nucleotide sequences of primers used in RT-qPCR and their originating studies.

Target		Sequence (5′-3′	Reference
fshb	Forward primer:	AGGACTGTCACGGAAGCATCA	Middleton et al., 2019
	Reverse primer:	GTTCAGGTCCGTTGTTTCGC	
	Probe:	TCACCACCTGCGCCGGCC	
fshr	Forward primer:	CAGTCACCTGACGATCTGCAA	Kusakabe et al., 2006
	Reverse primer:	TGCAGGTCCAGCAGAAACG	
	Probe:	ACTGGACTGAGGGTTCTACCTAACTTCTCCCG	
lhr	Forward primer:	CAACTGAATATACTGCAATGAACCTGT	Kusakabe et al., 2006
	Reverse primer:	CGGTTATTCTTCAAAACCAATTTATTT	
	Probe:	TCTTGGTCCCATTAAAGGCATAGTCTTGTATTTCTCTA	
vtg	Forward primer:	GAGCTAAGGTCCGCACAATTG	Celius et al., 2000
	Reverse primer:	GGGAAACAGGGAAAGCTTCAA	
	Probe:	CCTGCAAAATTTGCAGCACAGCTTGAC	
erα1	Forward primer:	CCCCCCAAGCCACCAT	Casanova-Nakayama et al., 2018
	Reverse primer:	TGATTGGTTACCACACTCGACCTATAT	
	Probe:	CATACTACCTGGAGACCTCGTCCACACCC	
P450scc	Forward primer:	ACATGCTACAGATGCTGAAGATGAT	Kusakabe et al., 2006
	Reverse primer:	TGGATGAAGCCTCAGCGTTT	
	Probe:	TCAGCGCTCCTTTGACCAGCGG	
3β-hsd	Forward primer:	TCCACACTGCGTCCCTCAT	Kusakabe et al., 2006
	Reverse primer:	CTGGGTTCCTTTGACGTTGAC	
	Probe:	TGAAGCTCACTGTATAACACCTTCCCGGTG	
18S rRNA (Reference)	Forward primer:	CACGCGAGATGGAGCAATAA	Salaberria et al., 2009
	Reverse primer:	CGCAGAGTAGACACACGCTGAT	
	Probe:	TGCCCTTAGATGTCC	

significantly affected by treatments. At the 0.5× concentration, RTBrain appeared to be the most sensitive – especially to the stable MC+ treatments – with viability decreased to 66.6 ± 4.7 % (Fig. 2B), followed by RT-milt5 (down to 79.1 ± 3.4 and 77.1 ± 3.0 % for log and stable treatments, respectively, Fig. 2G). RTgill-W1 viability was also significantly reduced by log MC+ treatments (down to 93.0 ± 1.0 %), an effect that was strengthened with increasing concentration – down to 74.7 ± 3.5 % at the 1× concentration, alongside a significant decrease to 72.9 ± 3.6 % by the 1× stable phase (Fig. 2D).

Alongside RTP-2, the RTBrain, RTgill-W1, and RT-milt5 cell lines were also strongly affected by MC- strain exudates. RTBrain and RT-milt5 cells were most sensitive to the negative effects of stable phase MC- exudates at the lowest concentration used – with viability reduced to 72.2 ± 2.8 % and 69.9 ± 4.7 % of control, respectively – however, decreases in fluorescence values in log phase treatments were only significant for RTBrain (down to 72.1 ± 4.6 % of control values, Fig. 2B, G). Similar to the MC+ treatments, RTgill-W1 displayed a weak, but significant response to MC- exudates at the 0.5× concentration, with viability reduced to 93.8 ± 1.4 % and 94.2 ± 1.5 % of control in log and stable phase treatments, respectively, and decreasing to 76.2 ± 2.5 % for the 1× stable phase (Fig. 2D).

Interestingly, at the lowest tested concentration, both MC+ and MC- stable phase exudates significantly increased the metabolic activity of RTL-W1 cells, up to 126.5 ± 8.2 % control values for the MC+ strain and up to 123.2 ± 4.5 % following MC- treatments (Fig. 2F). Comparing MC- and MC+ strain treatments in the five MC- affected cell lines (i.e., RTBrain, RTgill-W1, RTL-W1, RT-milt5, and RTP-2), statistical differences between log phase treatments were evident only in the RTgill-W1 cell line (at the 1× concentration) and the RTBrain and RTL-W1 cell lines (at 2×). No differences were detected between stable phase treatments of each *M. aeruginosa* strain at any tested concentration.

For the CFDA-AM assays, cytotoxic effects were observed in five (RTgill-W1, RBT4BA, RTBrain, RTG-2, and RTL-W1) cell lines, though apart from RTgill-W1, these effects were limited to log phase treatments of MC+ exudates at the highest tested concentration (Fig. 3, see also Supplemental Fig. S2). RTgill-W1 appeared to be the most sensitive cell line to cytotoxic effects, with a significant reduction in membrane integrity of 91.6 ± 2.2 % of control following exposure to 1× log phase MC+ exudates. As with the AB assays, stimulatory effects were also observed following both MC+ and MC- exudate treatments, specifically in the RTG-2, RTgill-W1, RT-milt5, and RTHDF cell lines. At the lowest tested dosage, stable phase MC+ exudates produced the greatest increase in apparent cell viability in RTHDF cells – up to 121.3 ± 2.9 % compared to controls (Fig. 3E), while the strongest effect by MC- exudates at the 0.5× concentration was observed in the RT-milt5 cell line with stable phase treatments – up to 113.0 ± 2.9 % (Fig. 3G). Among these cell lines, differences in the effects of each strain only became significant at the highest tested concentration.

RTBrain, RT-milt5, and RTgill-W1 appeared to be the most sensitive (in descending order) to cytotoxic effects of the exudates of both strains of *M. aeruginosa* according to our AB assay. Exudate concentration was a significant factor in both the log and stable phase for all three of these cell lines, except for log phase treatments of RT-milt5 (two-way ANOVAs; see Supplemental Table S1, S2). On the other hand, strain type was a significant source of response variation in all log phase treatments of RTBrain, RTgill-W1, and RT-milt5, however; in the stable phase treatments, strain type was only significant in the RTBrain cell line. The interaction between strain and concentration of treatments produced a significant effect on viability in both growth phases of RTgill-W1 only, indicating that the effect of strain type is dependent on the dose applied.

3.3. Lysosomal accumulation

MaE treatments induced increased lysosomal accumulation of Lysotracker Red in 6 of 8 tested cell lines relative to the BG-11 solvent control (Fig. 4) – with no significant effects observed in the RBT4BA and RTBrain cell lines – and with a greater number of cell lines significantly affected by MC- exudates. However, when comparing the effects of each strain, significant differences between MC- and MC+ treatments were only evident in RTgill-W1, RTL-W1, and RT-milt5. For RT-milt5, stable phase MC+ exudates consistently produced a stronger increase in lysosomal activity (Fig. 4E). Conversely, for RTgill-W1 and RTL-W1, significant differences between the strains were only apparent at the 2× concentration and only for log phase exudates. For RTgill-W1, MC- exudates produced a greater increase in lysosomal accumulation than MC+ exudates (Fig. 4B), while the inverse was true of RTL-W1 cells (Fig. 4D). In fact, when applied at the 2× concentration, log-phase MC+ strain exudates produced the greatest increase in lysosomal accumulation across all treatments and cell lines in the RTL-W1 cell line (4.7 ± 0.6 times control values, $p = 0.0072$).

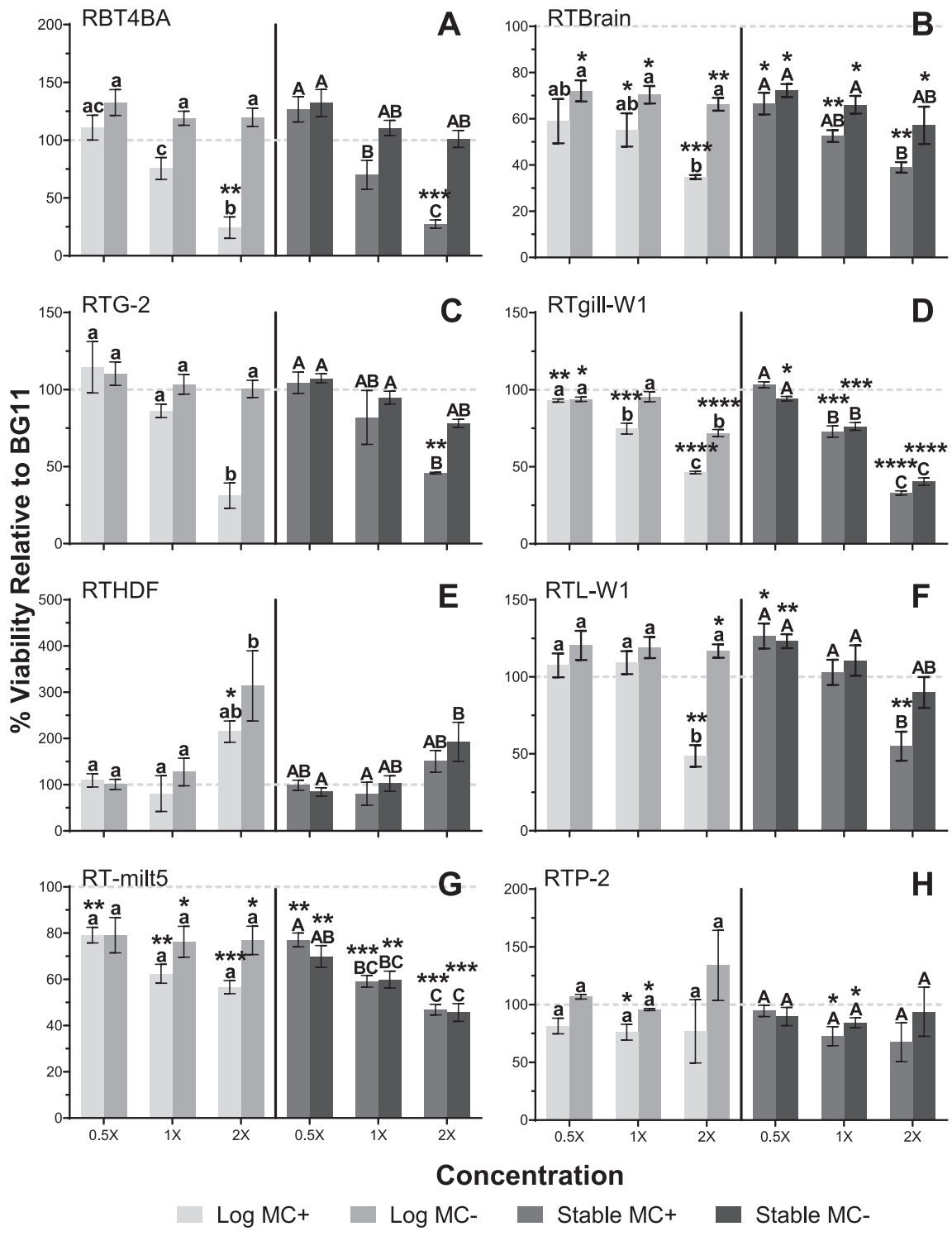

Fig. 2. Effects of MC+ and MC- *M. aeruginosa* exudates from log (LMC+, LMC-) and stable (SMC+, SMC-) growth phases on the AlamarBlue fluorescence compared to control. RBT4BA (A). RTBrain (B), RTG-2 (C), RTgill-W1 (D), RTHDF (E), RTL-W1 (F), RT-milt5 (G) and RTP-2 (H). Values are expressed as the mean ± SEM. $N = 3$ to 6. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ vs. control; different letters denote significant differences between treatments.

3.4. Gene expression

Initial qPCR analysis utilized all selected primers and only untreated samples for each cell line. When basal expression was detected, those primers were used for subsequent gene expression analysis. Compared to our media control, exudates from both MC+ and MC- strains produced significant changes in genes associated with reproduction across multiple cell lines (Fig. 5).

In the RTBrain cell line, *p450scc* transcript levels declined significantly following all treatments. For log and stable MC+ treatments, *p450scc* expression was downregulated to 39.7 % and 49.4 % of control levels, respectively; while for log and stable MC- treatments, transcript levels were reduced to 39.5 % and 35.2 %, respectively. Additionally, downregulation of both *fshβ* and *3β-hsd* expression was observed in log MC+ treatment groups. Transcript levels of *fshβ* were reduced to 54.1 %, while expression of *3β-hsd* declined to 58.2 % of control levels (Fig. 6A).

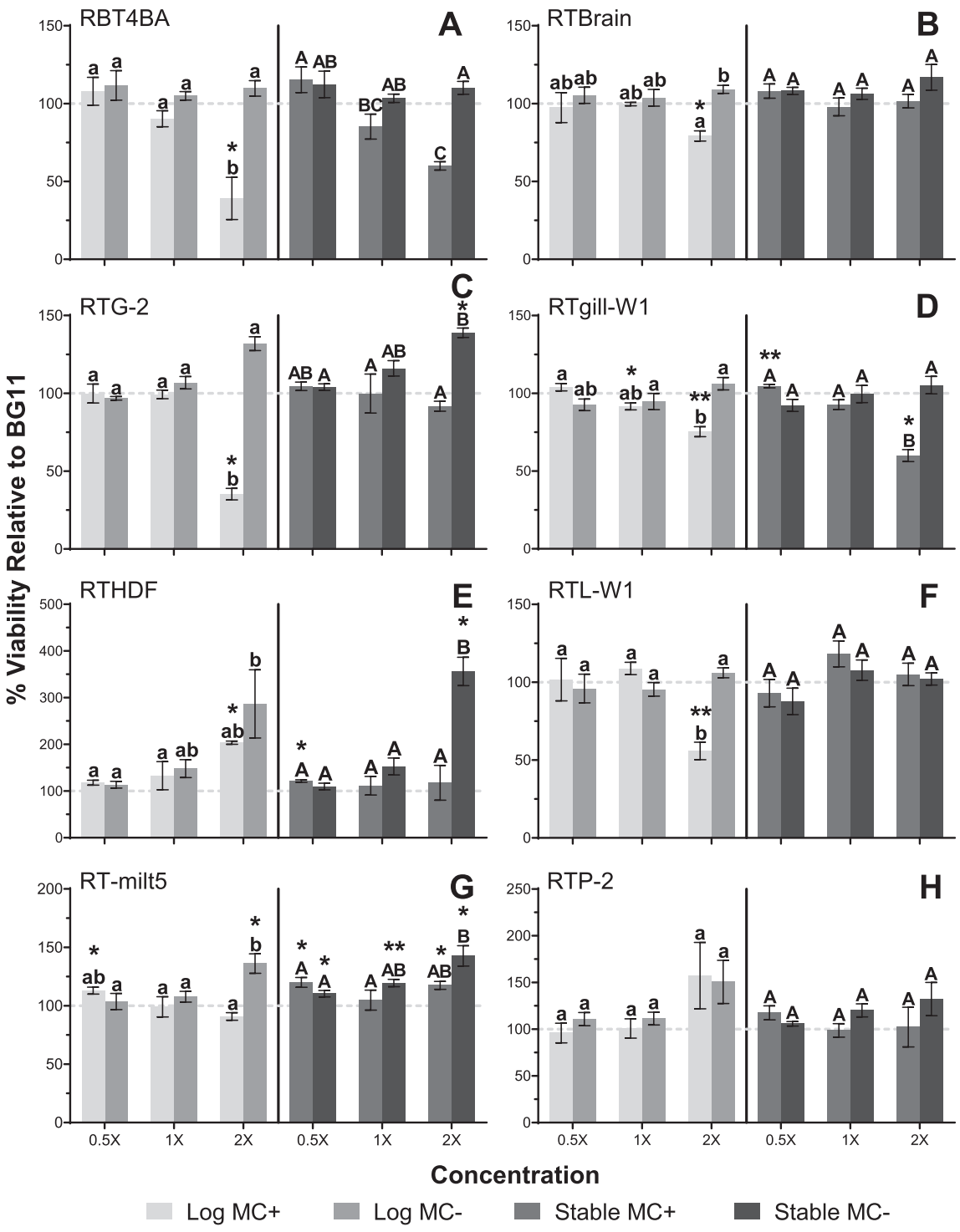

Fig. 3. Effects of MC+ and MC- *M. aeruginosa* exudates from log (LMC+, LMC-) and stable (SMC+, SMC-) growth phases on the CFDA-AM fluorescence compared to control. RBT4BA (A). RTBrain (B), RTG-2 (C), RTgill-W1 (D), RTHDF (E), RTL-W1 (F), RT-milt5 (G) and RTP-2 (H). Values are expressed as the mean ± SEM. N = 3 to 6. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ vs. control; different letters denote significant differences between treatments.

In the pituitary cell line RTP-2, log MC+ treatments also reduced the expression of *fshβ, p450scc,* and *3β-hsd* genes to 27.3 %, 25.0 %, and 11.9 % of control levels. However, unlike the RTBrain cell line, *lhr* expression following log MC- treatment was significantly increased to 3.55-fold higher than that in the control group (Fig. 6E).

In the gonadal cell line RTG-2, expression of both *fshr* and *3β-hsd* were downregulated in all treatments. In the log and stable MC+ treatments, *fshr* expression declined to 9.5 % and 1.2 % of control levels, respectively, while following log and stable MC- treatments, transcript levels were reduced to 8.8 % and 7.0 % of controls, respectively (Fig. 6B). Similarly, *3β-hsd* expression was downregulated to 8.9 %, 8.8 %, 17.7 %, and 16.0 % of control levels for the LMC+, SMC+, LMC-, and SMC- treatments, respectively. In addition, transcript levels of *p450scc* were reduced in both log and stable MC+ treatments, to 35.0 % and 30.2 % of controls, respectively.

In the RT-milt5 cell line, expression of *fshβ, erα1,* and *3β-hsd* were

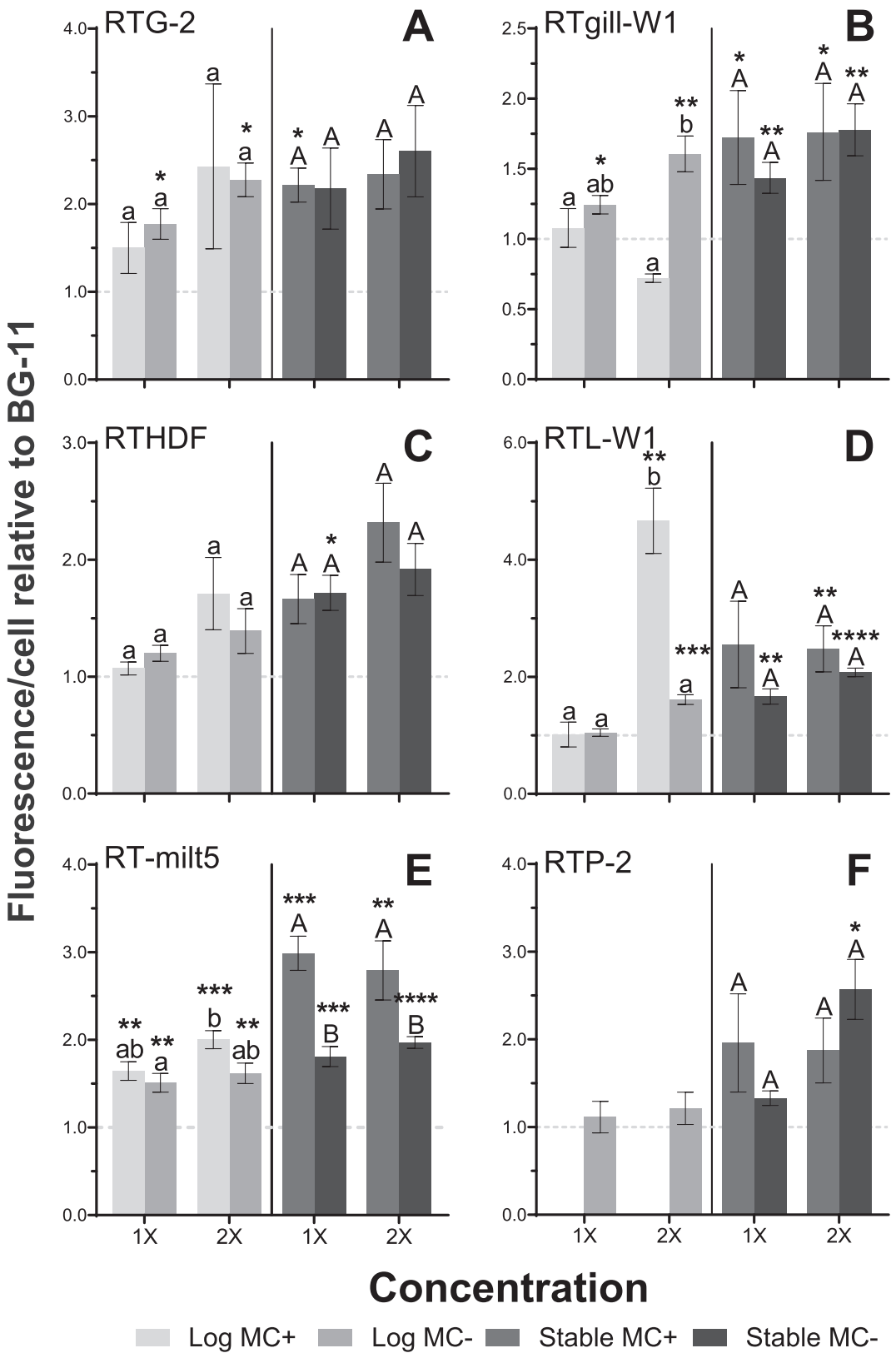

Fig. 4. Effects of MC+ and MC- *M. aeruginosa* exudates from log (LMC+, LMC-) and stable (SMC+, SMC-) growth phases on the Lysotracker fluorescence per cell compared to the control. RTG-2 (A), RTgill-W1 (B), RTHDF (C), RTL-W1 (D), RT-milt5 (E) and RTP-2 (F). Values are expressed as the mean ± SEM. N = 3 to 8. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ vs. control; different letters denote significant differences between treatments.

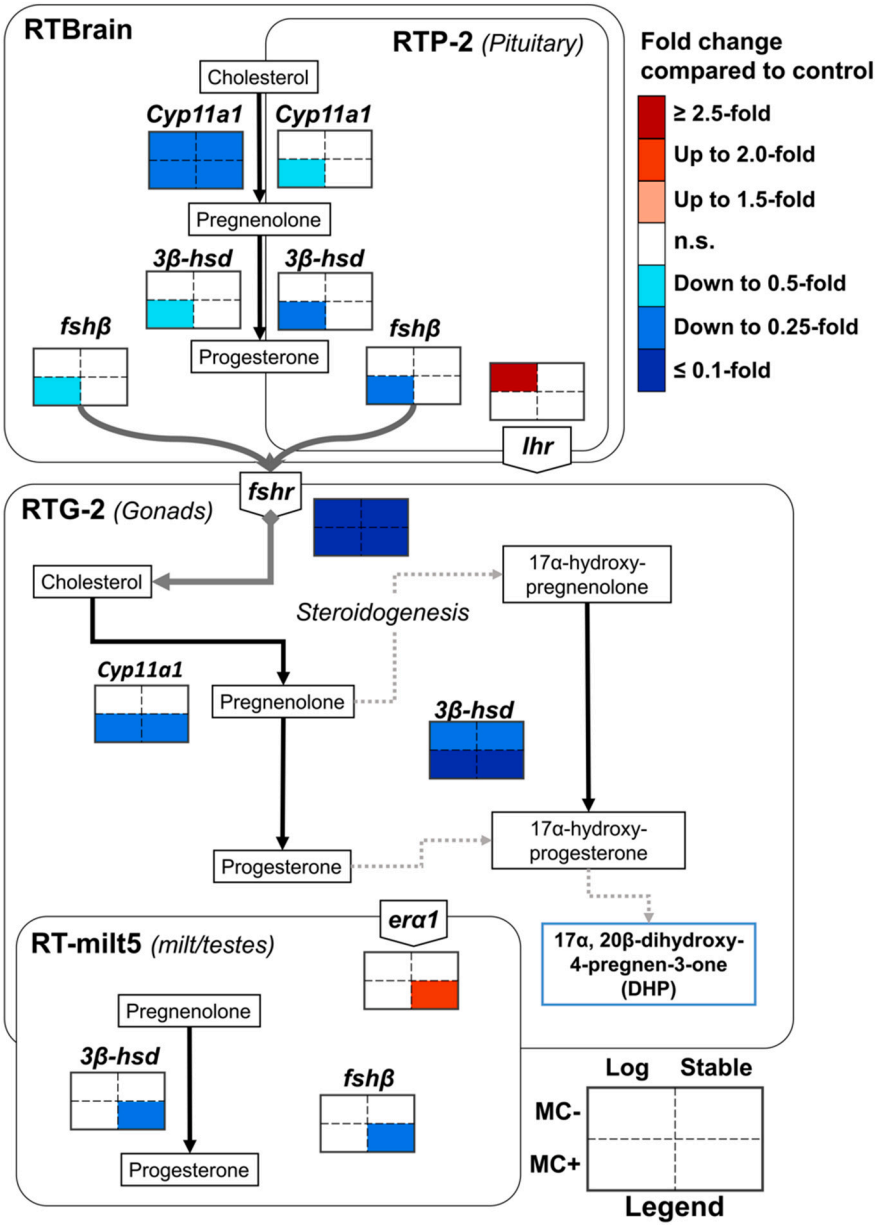

Fig. 5. Significant effects of *M. aeruginosa* exudates on gene expression in cell lines derived from the HPG axis of rainbow trout.

dysregulated in MC+ treatments for stable-phase cultures. We observed that transcript levels of *fshβ* and *3β-hsd* were significantly reduced to 38.8 % and 17.9 % of control levels, respectively. We observed a 1.7-fold increase for *erα1* in the stable MC+ treatment (Fig. 6D). No significant changes in gene expression were observed in the RTL-W1 liver cell line (Fig. 6C).

4. Discussion

Toxicity of *M. aeruginosa*-dominated blooms encompasses both microcystins and non-MC metabolites. Previous studies have found that non-MC metabolites negatively impacted both histopathological condition and critical functions in liver and gill tissues and cells (Bury et al., 1996; Le Manach et al., 2016; Sotton et al., 2017; Sorichetti et al., 2014). Our study expands upon this work and represents a novel examination of the effects of extracellular metabolites produced by MC- *M. aeruginosa* strains for many of our tested cell lines. Exudates produced by this strain significantly impaired normal cell functions in sensitive rainbow trout cell lines derived from brain, gill, and milt, with severity of impairment overlapping - in some cases - that of MC+ strains (Fig. 2). These findings highlight that these tissues are potential targets for bioactive non-MC metabolites. Additionally, both strains of *M. aeruginosa* produced significant dysregulation in transcription of genes associated with reproduction and related physiological pathways, further highlighting possible sublethal consequences of exposure to *Microcystis* blooms.

4.1. Effects of MaE on cell viability and lysosomal accumulation

We found that MC- exudates significantly decreased the viability of RTgill-W1 cells, with cytotoxicity largely overlapping that of the MC+ strain, particularly for stable growth phase cultures (Fig. 2D). Loss of integrity and functioning of gill tissues, as the site of respiration in teleost fish, can be catastrophic. While immersion exposure to CCMs containing MCs induced histopathological damage in gills of treated fish (Abdel-Latif and Abou Khashaba, 2017; Malbrouck and Kestemont, 2006), decline of RTgill-W1 cells in our study by extracellular metabolites in the absence of MCs further highlights the deleterious effects of these “non-toxic” strains. Šrédlová et al. (2021) also observed high

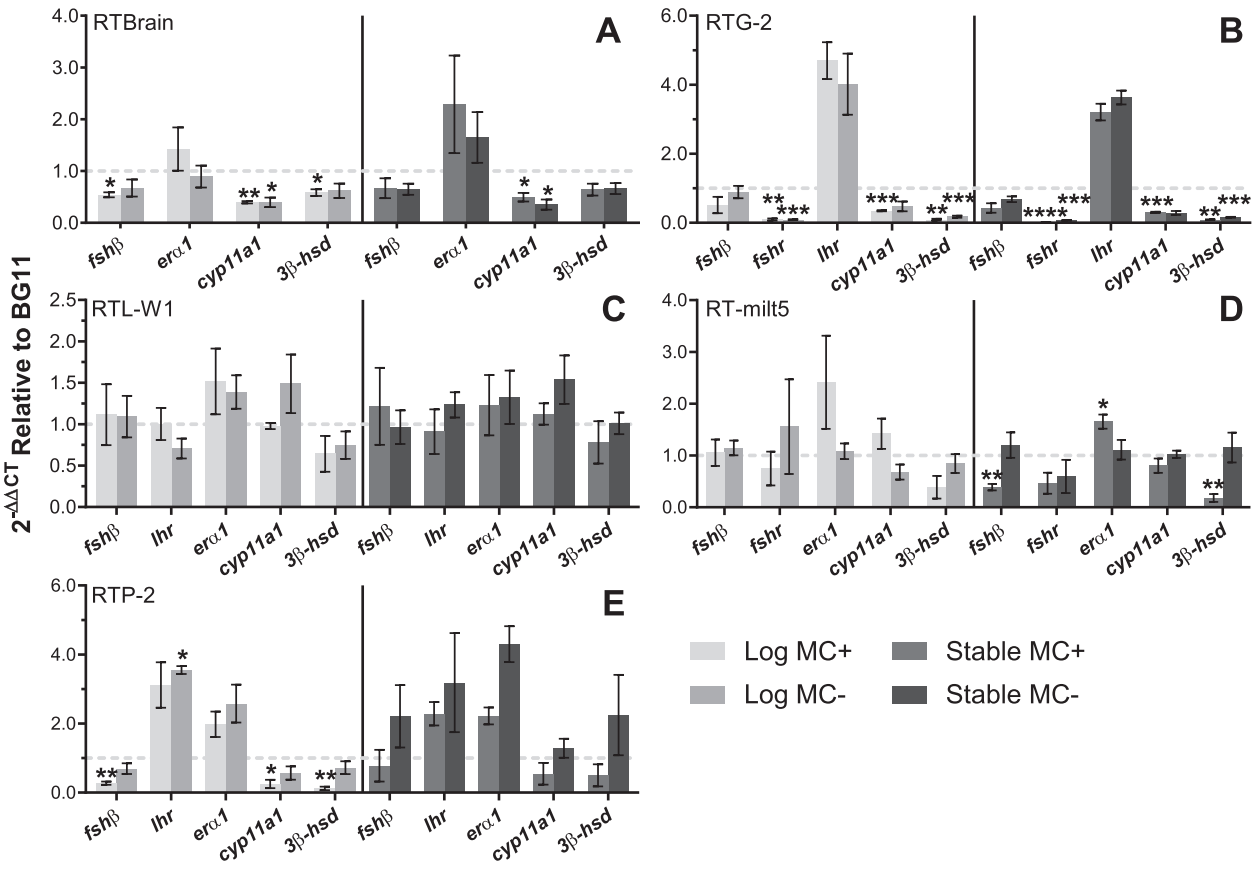

Fig. 6. Changes in gene expression in rainbow trout cell lines RTBrain (A), RTG-2 (B), RTL-W1 (C), RT-milt5 (D) and RTP-2 (E) following 24 h exposure to MC+ and MC- *M. aeruginosa* exudates from the log (TL, NTL) and stable growth phases (TS, NTS). Values are expressed as the mean ± SEM. N = 3 to 4. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ vs. control.

sensitivity in RTgill-W1 cells to *M. aeruginosa* intracellular metabolites, however; cytotoxicity was greatly diminished or absent when cells were exposed to MCs on their own or in artificial mixtures.

As an interface between the aquatic environment and internal systems, gill tissues are particularly vulnerable to any toxicants present. The overlapping cytotoxicity observed between MC+ and MC- stable phase exudates in our own study implicate the presence of non-MC metabolites within blooms that are capable of significant damage to gill tissues. In a similar study, Sorichetti et al. (2014) also observed that stable phase exudates of both MC+ and MC- strains impaired viability, however; log phase exudates failed to produce significant cytotoxic effects. The metabolome of *M. aeruginosa* can vary significantly within strains as well as across growth phases (Racine et al., 2019; Zhou et al., 2023). For the strains used in our study, comparisons between the major metabolites produced by each strain found distinct metabolomes with, for example, microviridin, anabaenopeptin, and microginin production detected only in CPCC 633 while microcystins, cyanobactins, and aeruginosins were only produced by CPCC 300 (McDonald et al., 2023).

In contrast, metabolomic analysis of the chemical composition of exudates from exponential and stationary growth phases of CPCC 300 and CPCC 633 found high similarity across growth phases with greater relative concentrations in the exponential phase (Zi et al., 2023, Preprint). Allelopathic interactions between exudates of *M. aeruginosa* and other phytoplankton and macrophytes also show growth-phase-dependent variation within strains (Zheng et al., 2013; Wang et al., 2017), however; little is known on how growth phase may alter the effects on vertebrates. Thus, further testing is needed to elucidate the cause of the differences in toxicity observed in our study.

The sensitivity to exudates in the MC+ treatment of our RTBrain cell line, and to a lesser extent the RTP-2 pituitary line, align with these trends of neurotoxicity in MC+ strains. In comparisons of CCM and MC-LR exposure on ELS of fish, changes in behaviour and neurotransmitters levels were induced at lower MC concentrations in CCM exposures versus MCs alone (Qian et al., 2018; Wu et al., 2016), indicating a putative enhancement of neurotoxicity by other metabolites present in *Microcystis* blooms. While isolated MCs have profoundly negative effects on nervous system of fish (Wu et al., 2016), exudates of our MC- strain also produced significant reductions in cell viability following exposure and these changes were only significantly distinct from those of the MC+ strain at the highest tested concentrations (Fig. 2B). McDonald et al. (2023) also found that both *M. aeruginosa* strains produced cyanopeptolins, metabolic compounds found to have potentially neurotoxic effects (Faltermann et al., 2014). Recent studies on neurotoxicity in *Microcystis* CCMs and MCs have highlighted their ability to alter neurobehaviour, disturb neurotransmitter levels and their associated receptors, and dysregulate expression of genes associated with nervous system function and development in embryos and larvae (Cai et al., 2022; Qian et al., 2018) and adult fish (Yu et al., 2021). Our results represent a novel example of potential neurotoxicity in non-MC-producing *M. aeruginosa* CCMs, adding to growing evidence of detrimental effects of non-MC metabolites present in blooms on the brain and nervous system-related tissues.

Our third sensitive cell line, RT-milt5 was cultivated from sperm-free, somatic cells derived from the milt of male rainbow trout (Vo et al., 2015). In our study, this cell line was significantly affected by both MC+ and MC- exudates at even the lowest tested concentrations and particularly by those of the stable growth phase, wherein no differences were detected between strains (Fig. 2G). The seminal fluid of fish milt maintains a protective environment during spermatozoa storage (Nynca et al., 2014) and may also play an important role in maintaining sperm

fertility during external fertilization (Billard, 1983). In bloom-affected systems, impairment of these functions by *M. aeruginosa* metabolites could impact the ability of male fish to fertilize eggs. Indeed, exudates of a MC-producing strain significantly decreased sperm motility and lifespan following direct exposure of sperm of an endangered fish (Zi et al., 2018).

In contrast to our AlamarBlue assays, our CFDA-AM assays were marked by primarily increased viability in treated groups compared to controls, including in our NT-sensitive cell lines (Fig. 3B, D, G). The disparity between these assays could indicate differences in the interaction of MaE with each assays' focal enzyme groups – oxidoreductases in the case of AB and non-specific esterases for CFDA-AM. In our study, the greater sensitivity of the AB assay to the negative effects of tested exudates could indicate disturbance of cellular metabolic processes by *M. aeruginosa* metabolites through the inhibition of relevant enzymes. These findings are consistent with Šrédlová et al. (2021), who found that the AB assay was more sensitive to toxicity of *M. aeruginosa* cyanobacterial organic matter – primarily intracellular compounds – than the CFDA-AM assay.

Increased lysosomal activity can be indicative of cell injury, as autophagic processes remove and recycle damaged organelles (Moore et al., 2008). However, lysosomal activity can also increase as foreign materials are internalized and degraded, a process which ultimately may have little to no impact on cell survival (Lenz et al., 2018). In our study, both MC+ and MC- exudates significantly increased lysosomal activity in the majority of tested cell lines, though only some of these increases were associated with loss of viability (Fig. 4). We observed these potentially adverse accumulations of lysosomes in the RT-milt5 and RTgill-W1 cell lines (Fig. 4D, G) as well as RTL-W1 cells, although only following treatment with MC+ exudates (Fig. 4F). Possible lysosomal dysfunction induced by both MC+ and MC- exudates suggests a non-MC origin for this mechanism of toxicity, consistent with previous studies that identified non-MC metabolites capable of impacting autophagic and organelle-recycling pathways (Li et al., 2022). The examination of how non-MC metabolites interact with these critical cellular processes is only a preliminary stage to determining their modes of toxicity.

4.2. Changes in reproductive gene expression following MaE exposure

Prior research has revealed that both MC-LR and *M. aeruginosa* cultures can dysregulate genes within the HPG axis and downstream pathways, with potential ramifications for both sex steroid levels and gamete quality (Zhao et al., 2015; Liu et al., 2016; Chen et al., 2017; Liu et al., 2018). Gonadotropins – commonly referred to as follicle-stimulating hormone (FSH) and luteinizing hormone (LH) – are critical drivers in this system through their stimulation of sex steroid hormone synthesis. In our study, expression of the follicle-stimulating hormone gene (*fshβ*) – which is associated with the early stages of gamete production in adult fish (Arcand-Hoy and Benson, 1998) - was down-regulated in RTBrain and RTP-2 cell lines by MC+ treatments (Fig. 6A, D, E), while both MC+ and MC- exudates decreased expression of the FSH receptor gene (*fshr*) in the RTG-2 gonadal cell line (Fig. 6B). With its association with early oogenesis, disruption of the FSH pathway could have significant impacts on the number of eggs produced (Lister et al., 2009), leading to reduced clutch sizes. On the other hand, expression of *lhr* – the receptor for LH, which is associated with gamete maturation (Arcand-Hoy and Benson, 1998) – was upregulated following MC- MaE treatments in our RTP-2 pituitary line (Fig. 6E). Exposure to *M. aeruginosa* cultures in female zebrafish increased the expression of *lhr, fshr,* and other reproduction-critical genes; however, this upregulation coincided with histopathological tissue changes and decreased fertilization and hatching rates in the eggs of affected fish (Liu et al., 2018), emphasizing a need for caution when attempting to discern whole animal effects from gene expression alone.

Similarly, these findings highlight one of the major hurdles of comparing in vitro results to whole animal experimentation, which is that cell-based studies lack the interactions of the feedback systems within reproductive pathways, specifically between different tissues. As noted by Liu et al. (2018) and Hou et al. (2016) in their *M. aeruginosa* culture and MC-LR exposure studies, respectively, significant changes in sex steroid hormone levels can spur compensatory mechanisms in upstream regulatory pathways, including gonadotropin-related gene expression. Critically, in our study changes in the *fshr*, as well as *lhr*, by MC- exudates suggests a non-MC driven interaction between *M. aeruginosa* metabolites and this component of reproduction regulation, while alterations in the expression of steroidogenic genes may indicate a complementary ability by non-MC metabolites to alter sex steroid hormone levels as well.

Steroidogenesis is a multi-step process in which steroidogenic enzymes convert cholesterol into the major sex steroid hormones, which then drive reproductive events, including the development and maturation of both male and female gametes and the timing of spawning (Yaron and Levavi-Sivan, 2011). The *cyp11a1* and *3β-hsd* genes, associated with the initial stages of steroidogenesis i.e., the conversion of cholesterol to pregnenolone and then pregnenolone to progesterone, respectively, were significantly down-regulated in the majority of our cell lines (all but RT-milt5 for *cyp11a1* and RTL-W1 in general). In particular, MC- treatments decreased expression of *cyp11a1* and *3β-hsd* in the RTBrain and RTG-2 cell lines, respectively (Fig. 6A, B). Not only does the dysregulation of these early steroidogenic genes implicate a potential disturbance of sex steroid production and their associated signalling pathways, *cyp11a1* is also crucial to the production of all other steroid hormones as well, including mineralocorticoids and glucocorticoids (Tenugu et al., 2021).

Dysregulation of steroidogenic genes appears to be a commonly observed effect of *M. aeruginosa* CCM and MC exposures, however; specific patterns of disturbance, especially with *cyp11a1* and *3β-hsd* expression, are varied. For example, *M. aeruginosa* cultures appear to have no significant effect on the expression of these genes in female zebrafish (Liu et al., 2018) while exposure to lyophilized *M. aeruginosa* cells in male Nile tilapia decreased *3β-hsd* expression (Chen et al., 2017). Similarly, potentially sex- and dose-dependent patterns of dysregulation of *cyp11a1*, *3β-hsd*, and other steroidogenic genes have been reported in MC-based treatments as well (Liu et al., 2016; Hou et al., 2018). Similarly, in our RT-milt5 cell line the estrogen receptor (*erα1*) was upregulated following exposure to MC+ MaE (Fig. 6D). While in female zebrafish, gonadal expression of estrogen receptors was upregulated following both MC-LR (Hou et al., 2016) and *Microcystis* culture exposures (Liu et al., 2018), MC-LR exposure resulted in decreased expression of estrogen receptors in the liver and brain of male fish (Hou et al., 2018). Although upregulation of these receptors could be an avenue for estrogenic effects, the effects of *Microcystis* metabolites on this signalling pathway in male gonads and their downstream consequences are largely unexplored.

The influence of both MC+ and MC- MaE on gene transcription across the reproductive system highlights a need for further investigation into both the breadth of influence of *M. aeruginosa* metabolites on critical gene expression, and how transcription disruptions translate to greater system-wide effects. The capacity to influence reproductive gene expression as identified in our study, complements current evidence that *M. aeruginosa* blooms can inflict serious sublethal effects on freshwater fish, altering signalling pathways and critical hormone levels throughout the system. The effects observed in our MC- treatments reveal that this influence extends beyond MCs to other metabolites present in *M. aeruginosa* blooms.

High-density cyanobacterial HABs are major ecosystem-altering forces and *Microcystis* species-dominated blooms can reach concentrations between 10^7 and 10^9 cells/mL (Zohary and Madeira, 1990; Javůrek et al., 2015; Wood et al., 2021), while previous studies have found that exudates produced by cultures at densities as low as 10^6 cells/mL are sufficient to impair hatching and survival rates (Zi et al., 2018; Cai et al., 2022). The concentrations used in our study (between 2.3 and 19.6 ×

10^7 cells/mL) aim to balance observed real-world bloom densities with one of the major limitations of in vitro cell line research – their lower absolute sensitivity compared to their in vivo counterparts, a characteristic that necessitates the use of higher concentrations of test compounds (Schirmer, 2006). Extracellular metabolites represent an understudied component of HABs toxicity. The sensitivity of the brain-, gill-, and milt-derived cell lines identified in this study highlight avenues for further study both in terms of potential target tissues and mechanisms of toxicity in future in vitro or in vivo investigations.

5. Conclusions

Non-MC-producing strains of *M. aeruginosa* produce metabolites that significantly impaired viability of rainbow trout cells derived from the brain, gill, and milt tissues, with cytotoxicity (in some cases) on par with that of an MC-producing strain. Further study is needed to extrapolate in vitro findings to histopathological consequences for whole fish, though the RTgill-W1 and RT-milt5 cell lines represent external tissues potentially directly exposed to *M. aeruginosa* exudates in the field. Furthermore, exudates from both strains contained compounds capable of disrupting the expression of reproduction-related genes, suggesting additional sublethal effects beyond cytotoxicity. With *Microcystis aeruginosa*-dominated blooms expected to continue to increase in occurrence and severity, risk assessments for bloom-affected ecosystems must consider both MC and non-MC metabolites. Further characterization of non-MC metabolites is imperative to improve our understanding of overall impacts of *Microcystis aeruginosa*-dominated blooms.

CRediT authorship contribution statement

Keira Harshaw: Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Ambreen Fahim:** Investigation, Methodology, Resources. **Jinmei Zi:** Methodology, Resources. **P. Charukeshi Chandrasekera:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing. **Xuexiu Chang:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing. **Brian Dixon:** Conceptualization, Funding acquisition, Writing – review & editing. **Hugh J. MacIsaac:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2024.170747.

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