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Cytotoxicity and Reproductive Impairment in Rainbow Trout Cell Lines Exposed to Microcystis aeruginosa Extracellular Metabolites

Keira Harshaw
University of Windsor

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Keira Harshaw

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by

Keira Harshaw

APPROVED BY:

B. Dixon
University of Waterloo

C. Chandrasekera
Department of Chemistry and Biochemistry

X. Chang
Great Lakes Institute for Environmental Research

H. MacIsaac, Advisor
Great Lakes Institute for Environmental Research

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ABSTRACT

Current trends in cyanobacterial harmful algal blooms (cHABs) demonstrate increasing risks to human health and the health of aquatic ecosystems around the globe. Expansion of algal blooms, both geographically and temporally, serve to place increasing numbers of freshwater species, including fish, in peril. *Microcystis aeruginosa*, one of the most common species of bloom-causing cyanobacteria, is capable of producing a vast diversity of biologically active compounds, however *Microcystis* studies are often dominated by microcystins. How non-microcystin metabolites contribute to *Microcystis* toxicity, particularly in freshwater fish, has been the subject of a limited, but growing, body of research. To contribute to the bridging of this knowledge gap, my thesis examined the effects of extracellular metabolites produced by *M. aeruginosa* on *in vitro* fish cell lines derived from multiple tissues of the freshwater salmonid rainbow trout (*Oncorhynchus mykiss*), incorporating not only changing metabolite production over the lifespan of a bloom, but also toxicity in the absence of microcystins. I found that non-microcystin-producing strains of *M. aeruginosa*, commonly referred to as 'non-toxic,' were capable of producing and releasing metabolites that significantly reduced the viability of cell lines derived from the brain, gills, and milt of fish. Impairment of reproduction can seriously impact the sustainability of a population. In examining the effects of 'non-toxic' extracellular metabolites on gene expression, I found that these cyanobacterial mixtures were able to dysregulate genes associated with reproduction and steroidogenesis in brain and gonadal tissue-derived cell lines. While the correlation between *in vitro* cytotoxic and sub-lethal effects and *in vivo* ramifications requires further investigation, overall, this thesis highlights the need to integrate non-microcystin metabolites into risk assessments for freshwater systems and fish species impacted by *Microcystis* algal blooms.

DEDICATION

To my mother, who instilled in me an endless love of nature and who is always willing to accompany me into whatever wilderness I can find.

And to Willow, Murphy, Lily, and Finn, who are terrible study buddies and wonderful friends.

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CHAPTER 1 - General Introduction

1.1 Harmful Algal Blooms (HABs)

Cyanobacterial algal blooms, the dense accumulation cyanobacterial biomass, pose a serious threat to important and vulnerable aquatic ecosystems across the globe. Expanding research into harmful algal blooms (HABs) and their distribution has revealed an increasing trend in the intensity of cyanobacterial blooms in freshwater ecosystems (Ho et al., 2019) – including expansion within Canadian lakes and rivers (Pick, 2016) – and have linked this increased abundance with the intensification of agriculture and its role in the shifting of aquatic trophic states toward eutrophication (Taranu et al., 2015). The addition of excess nutrients, especially phosphorus and nitrogen, from urban and agricultural sources is a major component to the development and persistence of cyanobacterial blooms (Giani et al., 2005; Paerl et al., 2014) and it is compounded by a variety of environmental and climatic conditions. Rising water temperatures, persistent and stronger vertical stratification of the water column, longer residence time, increasing nutrient inputs through greater run-off and increasing salinity of freshwater systems all provide a competitive advantage for cyanobacteria over their eukaryotic counterparts (Paerl & Huisman, 2008; Paerl & Paul, 2012). Even the introduction of invasive species may provide more favourable conditions for cyanobacteria, as appears to be the case with dreissenid mussels in Lake Huron and Lake Erie (Vanderploeg et al., 2001).

The presence of cyanobacterial blooms in aquatic systems can have significant negative effects on the health of the waterbodies they occupy as well as the communities therein. As cyanobacterial biomass increases during bloom conditions, nuisance surface scum can form, fouling terrestrial areas when swept to shore (Carmichael & Boyer, 2016) and sufficient densities of cyanobacteria can contaminate local drinking water (Pick, 2016). Increased turbidity

associated with blooms can shade other photosynthetic phytoplankton and aquatic macrophytes and may also negatively affect benthic and pelagic organisms, such as fish, that rely on visual cues for hunting and foraging, conspecific interactions such as mate selection and schooling, and other vision-reliant behaviours (Engström-Öst & Mattila, 2008; Sundin et al., 2010; Sukenik et al., 2015). The senescence of these dense blooms can also drive the depletion of oxygen in hypolimnetic zones, generating localized anoxia that can result in fish kills (Sukenik et al., 2015) and, combined with loss of aquatic plants, can lead to the degradation of critical benthic habitats (Havens, 2008; Ludsin & Hook, 2013).

Another pressing concern regarding HABs is the ability of many cyanobacterial genera to produce a variety of secondary metabolites, including hepatotoxins, such as nodularin and microcystins, and neurotoxins, such as anatoxin-a and saxitoxin (Smith et al., 2008). Consolidation of prior research into accessible databases, such as CyanoMetDB, has identified more than two thousand secondary metabolites produced by species of freshwater and marine cyanobacteria (Jones et al., 2021). These include species of *Anabaena, Cylindrospermopsis,* and *Microcystis*, all of which have been identified in North American freshwater systems within recent years (O'Neil et al., 2012; Steffen et al., 2014; Pick, 2016).

1.2 The freshwater cyanobacteria *Microcystis*

The *Microcystis* genus are unicellular, colony-forming cyanobacteria that are broadly distributed across more than 100 countries and are best known for their production of the hepatotoxin microcystin (MC), a cyclic heptapeptide (**Figure 1.1**) that inhibits protein phosphatases and disrupts the normal regulation of protein phosphorylation in cells, resulting in significant damage to cell structures and oxidative stress, particularly in the liver (Smith et al., 2008; Harke et al., 2016). High structural variation within MCs has led to the identification of

more than 300 congeners (Jones et al., 2021), although toxicological information for many of these forms is lacking (Chorus & Welker, 2021). Studies using microcystin-LR (MC-LR), considered one of the most common and most toxic variants (Chorus & Welker, 2021), form the basis of the World Health Organization's (WHO) provisional guidelines for MCs of 24 µg/L for recreational water and 1 µg/L for drinking water (WHO, 2020). These cyanotoxins have been implicated in multiple mass mortality events involving contaminated water sources – resulting in the deaths of fish, herbivorous and piscivorous birds, and terrestrial animals – including critically endangered mammals (Lopez-Rodas et al., 2008; Bengis et al., 2016). In one case in Caruaru, Brazil, the use of *Microcystis*-containing water for haemodialysis treatment was implicated in the deaths of 60 people (Pouria et al., 1998), however, more common sources of human exposure to MCs include physical contact with blooms, the ingestion of contaminated water or tissues of aquatic animals in which MCs can accumulate (Papadimitriou et al., 2012; Carmichael & Boyer, 2016) and through inhalation of spray aerosol from bloom-affected lakes (Plaas & Paerl, 2021).

Similarly, exposure to MCs in fish and other aquatic organisms can occur through the consumption of cyanobacterial cells, purposefully or otherwise, and immersion in a bloom-contaminated environment where MCs may be absorbed through the skin and gills (Sukenik et al., 2015). Alongside their well-known hepatotoxicity, MC exposure can also inflict neurotoxicity, reproductive toxicity, immunotoxicity and significantly impact the development of early life stages in fish, including embryos and larvae (see reviews by Malbrouck & Kestemont, 2006; Banerjee et al., 2021). For example, exposure to MCs has been linked to significant histopathological changes in the liver and the dysregulation of proteins associated with numerous critical biological functions including metabolic processes, homeostasis and detoxification (Le Manach et al., 2016). In the early life stages of fish, MCs significantly impair proper embryonic

and larval development, increased malformation rates and mortalities, altered the timing of hatching, depressed heart rates and decreased growth rates (Malbrouck & Kestemont, 2006; Qi et al., 2016) as well as altering swimming behaviour and decreasing neurotransmitter levels (Wu et al., 2016). Additionally, larvae exposed to MCs suffered significant dysregulation of genes associated with detoxification, metabolism, development, and nervous system functioning (Rogers et al., 2011; Wu et al., 2016).

1.3 Metabolites beyond microcystins

Alongside MCs, the *Microcystis* genus produces numerous other secondary metabolites which have been the subject of similar efforts to catalog and characterize their structure, bioactivity, and toxicity. These include aeruginosins and microviridins – inhibitors of serine proteases such as trypsin and plasmin (Murakami et al., 1995; Namikoshi & Rinehart, 1996; Huang & Zimba, 2019) as well as cyanopeptolins and aerucyclamides, which induce toxic effects in freshwater zooplankton, crustaceans, and fish (Portmann et al., 2008; Faltermann et al., 2014; Kohler et al., 2014). Analysis of metabolites produced by different *Microcystis* strains has highlighted the impressive chemodiversity of the genus (see for example Welker et al., 2004; Sotton et al., 2017) however many compounds and their variants remain poorly characterized and research in this area has been hampered by the limitations of current analytical methods to identify, quantify, and isolate individual metabolites for toxicological assessment (Janssen, 2019). On the other hand, complex cyanobacterial mixtures serve as an important avenue for studying ecological impacts of HABs in environmentally-relevant contexts.

Current ecotoxicological research has utilized live algal cultures and lyophilized cells to examine the effects of compounds produced by the *Microcystis* genus (e.g. Rogers et al., 2011; Qian et al., 2018) as well as crude extracts from lysed cells and exudates – the cell-free media of

algal cultures (e.g. Zheng et al., 2013). Depending on the health of the culture, exudates allow researchers to study primarily extracellular metabolites with limited inclusion of intracellularly produced compounds, including MCs, which are released during cell lysis (Chorus & Welker, 2021). Recent studies with extracts and exudates support the idea that at least some of these cyanobacterial metabolites are potential allelochemicals, providing *Microcystis* spp. with a competitive advantage against other phytoplankton and aquatic macrophytes. Under both co-culturing conditions and exposure to exudates, *M. aeruginosa* is capable of inhibiting the growth of green algae and diatoms (Wang et al., 2017) and these inhibitory effects increased as water temperature increased in green algae co-cultures (Ma et al., 2015). Similarly, in submerged macrophytes, multiple studies have found that *M. aeruginosa* extracts and exudates inhibit photosynthesis as well as negatively impact seed germination and fresh weight of seedlings (Zheng et al., 2013), decrease the percentage of healthy seedlings, inhibit root and shoot development (Xu et al., 2015), increase oxidative stress and alter the structure and abundance of microbial communities in leaf biofilms (Jiang et al., 2019). When comparing the effects of extracts and exudates the impacts on photosynthesis, seedling health and development of roots and leaves were stronger in the latter group (Zheng et al., 2013; Xu et al., 2015).

In the same vein, while previous research has identified *Microcystis* spp. as a poor or actively harmful food source for a number of zooplankton species – either through poor nutritional properties or the inhibition of growth by potential toxins (Lampert, 1987; Zhou et al., 2020) – Xu et al. (2019) found that *M. aeruginosa* exudates increased the net reproductive rate of exposed water flea *Daphnia magna* individuals and significantly elevated levels of important reproductive hormones, including ecdysone, juvenile hormone, and vitellogenin. An egg yolk protein precursor produced in the liver, changes in vitellogenin production have been an

important indicator for aquatic contamination by estrogenic compounds, particularly in male fish (Sumpter & Jobling, 1995; Wheeler et al., 2005). This potential estrogen-like activity has also been previously identified in *in vitro* luciferase reporter gene assays using human breast carcinoma cell lines, although as with the previous comparisons, *M. aeruginosa* exudates appeared to be more strongly estrogenic than their extracts (Sychrová et al., 2012; Jonas et al., 2015).

Studying complex cyanobacterial mixtures, such as extracts and exudates, can allow researchers to examine the effects of HABs under conditions closer to natural or field contexts, highlighting interactive toxicity that may be lacking in the study of isolated constituent compounds. Researchers have found that the exposure of fish embryos to live *M. aeruginosa* cultures, extracts, and exudates has been associated with declines in hatching rates, earlier hatching, increased mortalities, and increased malformations (Jonas et al., 2014; Jonas et al., 2015; Saraf et al., 2018), similar to the effects found in pure toxin studies (Malbrouck & Kestemont, 2006). However, while MC-LR increases malformation rates in embryos (Qi et al., 2016) treatments using *M. aeruginosa* extracts and live cultures induce significant developmental deformations at much lower equivalent concentrations of MC-LR (Ghazali et al., 2009; Li et al., 2021). In addition, embryos exposed to high concentrations of algal cultures during development showed decreased expression of both growth- and oxidative stress-related genes (Li et al., 2021) as well as decreased locomotor behaviour and dysregulation in genes associated with both normal nervous system function and neuronal development (Qian et al., 2018).

Microcystis aeruginosa exudates can dysregulate genes related to heart function and development in fish larvae, leading to impairment of developing heart structures and potentially heart failure (Zi et al., 2018). Decreased heart rates have been detected in experiments using

algal cultures (Li et al., 2021), however Saraf et al. (2018) found that in comparisons between live cultures and purified MC-LR the depression of heart rates in fish embryos could not be explained by MCs alone. Another study comparing *M. aeruginosa* lyophilized cells and MC-LR exposure on zebrafish embryos found 126 genes that were differentially expressed only in cell-treated groups, including genes involved in cell signalling and development, neurological function, visual perception, and endocrine activity (Rogers et al., 2011).

Adult fish exposed to complex mixtures of *Microcystis* spp. metabolites appear to face a similarly broad variety of sublethal effects. Exposure to live cultures of *M. aeruginosa* in adult zebrafish (*Danio rerio*) induced damage to intestinal tissues, increased abundance of pathogenic bacteria in intestinal microbiota and potentially inhibited immune responses by down-regulating inflammation-related genes (Qian et al., 2019) as well as significantly altering expression of proteins in the brain involved in pathways critical to normal physiological functioning, including synaptic vesicle cycles, insulin signalling and oocyte meiosis (Yu et al., 2021). Liu et al. (2018) found significant histopathological damage was also evident in the liver and gonads of female zebrafish exposed to *M. aeruginosa* cultures along with decreased plasma levels of the sex steroid hormones 17β-estradiol and testosterone. Significant dysregulation was detected in genes associated with reproductive pathways, steroidogenesis, and endocrine activity in the brain, liver and ovaries of treated fish and following exposure, female fish produced eggs that had significantly lower fertilization rates and hatchability, indicators of potential transgenerational effects of *M. aeruginosa* exposure (Liu et al., 2018).

As with research conducted with the early life stages of fish, comparisons between complex cyanobacterial mixtures and purified MCs in adult fish also highlight the potential for enhanced toxicity in mixtures, possibly through synergistic or additive interactions between MCs

and other metabolites. When comparing the effects of lyophilized *M. aeruginosa* cells to MC-LR, Chen et al. (2017) found that both treatments increased sex steroid hormone levels in adult male Nile tilapia (*Oreochromis niloticus*) – in contrast to the live culture study by Liu et al. (2018) – and dysregulated genes associated with growth and steroidogenesis; however, treatments with *M. aeruginosa* cells affected a greater number of the tested genes or produced more intense dysregulation (Chen et al., 2017). In the same vein, research by Qiao et al. (2016) found that chronic exposure to *M. aeruginosa* extracts and MC-LR treatments in medaka fish dysregulated genes associated with metabolic processes and signalling pathways; however, under extract treatments female fish experienced a significantly greater number of dysregulated genes, including those involved in nuclear receptor signalling, despite similar concentrations of MCs in each treatment.

1.4 "Non-Toxic" *Microcystis* strains

The potential interactions of cyanobacterial metabolites are not the only confounding factors of note in current cHABs research. Variation in compound production by different strains, and in particular the presence of so-called "non-toxic" strains in algal blooms, are also critical aspects to consider when studying the impact of cyanobacteria on freshwater ecosystems. Non-microcystin-producing (Non-Myc) variants are naturally occurring and have the potential to make up a significant proportion of a bloom's *Microcystis* population (Wilson et al., 2005; Rinta-Kanto et al., 2009). In field samples, these "non-toxic" strains are often categorized by the absence of genes within a *mcy* gene cluster (Davis et al., 2009; Rinta-Kanto et al., 2009) which has been linked to microcystin production (Tillet et al., 2000), however the differences between these strains extend beyond these specific hepatotoxins. In comparisons between the metabolites present in isolated laboratory cultures, Non-Myc strains can produce an array of compounds with

very little overlap with their "toxic" counterparts, either through differences in the variants produced, as previously seen with aeruginosins and microginins (Le Manach et al., 2016; Le Manach et al., 2018) or with potentially uniquely produced metabolites, such as cryptophycins (Sotton et al., 2017). Shared metabolites can also vary in relative concentrations produced across strains as seen by Zhou et al. (2023) in their broad-scale examination of *M. aeruginosa* metabolomes of both MC-producing and non-producing strains, wherein the latter strain produced elevated levels of metabolites such as 7-ketocholesterol and sinapyl alcohol.

Exposure to Non-Myc cultures in adult medaka fish produced distinct patterns of protein dysregulation in the liver – with proteins associated with heme transport, metabolic processes, oogenesis, redox homeostasis and other biological processes uniquely dysregulated only in "non-toxic" treatments (Sotton et a., 2017). Chronic exposure to non-MC-producing strains induced significant histopathological changes in the livers of both male and female medaka in addition to producing significant dysregulation of proteins involved in the cytoskeleton and cellular processes, metabolic processes, stress response, and reproduction (Le Manach et al., 2018). Similarly, when comparing the long-term effects of MC-LR and the extracts of different *M. aeruginosa*/*Microcystis*-dominated cultures on adult medaka, although natural bloom-derived extracts generated the greatest cellular changes and protein dysregulation, extracts of the non-MC-producing strain induced significant changes in the regulation of proteins involved in metabolic, reproductive, and homeostatic processes (Le Manach et al., 2016). While the protein level modulations of these "non-toxic" strains present a significant sublethal molecular effect on exposed fish, studies of this kind are still limited both in number and anatomical scope. *In vitro* studies on non-Myc *Microcystis* spp. are similarly limited. Sorichetti et al. (2014) found that

extracts from a *M. aeruginosa* strain previously described as ‘non-toxic’ (CPCC, 2013) were capable of significantly decreasing the viability of the fish cell line RTgill-W1.

1.5 Study System

Fish have long been critical components of ecotoxicological research. Their extensive diversity in both number of species and characteristics such as habitat and body size have made them excellent model systems in not only ecotoxicology, but also neurobiology and embryology (Powers, 1989). Studying the impact of ecological contaminants on fish has been important not only in elucidating the mechanisms of toxicity, both in the study species themselves and in predicting the risk to other fish species and their predators (including humans), but also as a means of monitoring aquatic ecosystem health, particularly in areas subject to prolific infiltration of contaminants through agricultural or industrial runoff or sewage effluent (Bols et al., 2005).

Rainbow trout (*Oncorhynchus mykiss*) is a member of the family Salmonidae and native to the eastern Pacific Ocean and freshwater systems of western North America. Valued both as a sport fish and for consumption, rainbow trout have been broadly and successfully introduced across Canada and around the world (Scott & Crossman, 1973), lending them a similarly cosmopolitan distribution as *Microcystis* cyanobacteria (Crawford & Muir, 2008; Harke et al., 2016). As a study species, rainbow trout are readily available – both in distribution and life stage, amenable to culturing and husbandry – and can act as representatives for salmonid species that are more difficult to acquire or maintain. Although the general sensitivity of rainbow trout compared to other species is still being debated (Besser et al., 2020), they have been effectively utilized as surrogate species in toxicity research in place of other imperiled salmonids and cold-water species (Sappington et al., 2001; Raimondo et al., 2008). These characteristics as well as their economic and recreational importance has led them to being well-studied across many

disciplines including immunology, virology, and physiology (Wolf & Rumsey, 1985). Rainbow trout are also prolific experimental animals in ecotoxicology research, particularly with human-sourced pollutants, such as pesticides and sewage effluent (Sumpter & Jobling, 1995; Topal et al., 2015), but also biological contaminants including cyanotoxins (Shahmohamadloo et al., 2021). Their widespread use, extensive knowledge base, and favourable research characteristics have also placed them among the recommended test species for the Organisation for Economic Co-operation and Development's (OECD) Guidelines for testing the toxicity of chemicals to both early-life stage and adult fish (OECD, 2013; OECD, 2019), cementing their place as a key species in ecotoxicological research.

Rainbow trout's well-earned popularity as a research model has extended into *in vitro* work as well. The first piscine cell line was described in 1962 by researchers Wolf & Quimby – RTG-2, derived from the gonadal tissue of juvenile rainbow trout. Since then, the rainbow trout catalogue of cell lines has expanded to include more than 50 immortal lines from a variety of tissues, including the brain, liver, heart, and spleen, contained in repositories or stored and shared amongst scientists (Bols et al., 2017).

Cell cultures offer an alternative to *in vivo* live animal assays, mitigating some of the limitations of whole animal use while also aligning with a growing desire in research to pursue more ethical testing methodologies — a pursuit that seeks to embody the Three Rs Principle to replace, reduce, and refine the use of animals in science (Canadian Council on Animal Care, n.d.). Cell cultures can provide faster results than their live animal counterparts while reducing the costs of raising and maintaining experimental stocks (Bols et al., 2005). Continuous cell lines also provide much greater reproducibility, even more than their initiating primary cultures (Bols et al., 2005). In comparison to mammalian cultures, while fish-derived cell lines grow much

slower, they will also tolerate a greater range of temperatures, aligning them closer to the exposure conditions of their progenitors' habitats (Bols et al., 2005). Fish cell lines, including RT lines, have been used extensively in fish virology since their inception (Bols et al., 2017), however increasing emphasis has been placed on their value in toxicological research. Rainbow trout lines have been used to evaluate the toxicity of many common aquatic pollutants including pesticides and herbicides (Wang et al., 2015; Weeks Santos et al., 2019), pharmaceuticals (Bain & Kumar, 2014), and polycyclic aromatic hydrocarbons (PAHs) (Araújo et al., 2000) as well as mixtures of contaminants such as those found in sediments or effluent sources (Schirmer, 2006; Wu et al., 2016).

When comparing *in vitro* and *in vivo* toxicity tests, the relative sensitivity of fish cell lines were strongly correlated with the results of whole fish assays, although the absolute sensitivity of cell lines is much lower than *in vivo* findings (Schirmer, 2006). Fish cell lines do have unique advantages compared to whole animal assays by allowing more focused evaluations of cellular responses and mechanisms of toxicity and by extension cell-type specific sensitivities, whether they be organ-, tissue-, or species-specific assessments (Fent, 2001; Lakra et al., 2011). To this end more than 880 fish cell lines have been produced since RTG-2 was first passaged across a large variety of species and tissue types, including the RT cell lines previously mentioned (Goswami et al., 2022). Fish cell lines' growing roles in ecotoxicology are also reflected in the OECD's recent RTgill-W1 acute toxicity assay, which highlights the cell line's potential as a predictive or preliminary test in conjunction with *in vivo* testing to reduce the number of animals needed (OECD, 2021). When it comes to HABs, fish cell lines have been previously used to evaluate not only the *in vitro* toxicity of isolated microcystins (Pichardo et al.,

2006) and *M. aeruginosa* metabolites (Sorichetti et al., 2014), but also the extracts and exudates of other cyanobacteria or bloom-causing algae (Teneva et al., 2013; Franco et al., 2019).

1.6 Thesis Objectives

My thesis aims to compare the toxicity of extracellular metabolites produced by microcystin-producing and non-microcystin-producing strains of the prolific cHABs contributor *M. aeruginosa*, specifically looking at their potential impacts – both lethal and sublethal – on freshwater fish. Fish kills are a well-understood consequence of algal blooms, whether it is through toxins or hypoxic conditions, however, detrimental impacts on fish populations can also occur through sublethal effects, such as waterborne contaminants inhibiting reproduction or the development of subsequent generations (Kidd et al., 2007). Additionally, while MC-focused research is extensive, that regarding the potential of other extracellular metabolites – particularly those produced by non-MC strains – to contribute to these toxic outcomes has received little attention. To this end, my thesis is divided into two data chapters.

The main aim of Chapter 2 explores cytotoxicity of *M. aeruginosa* extracellular metabolites, with particular interest paid to how the toxicity of "non-toxic" strains may differ from or overlap with their "toxic" counterparts. To this end, this chapter utilized dye-based cell viability assays alongside an array of rainbow trout (*O. mykiss*) cell lines to potentially elucidate differences in toxic mechanisms or tissue targets. With current methodological difficulties in isolating and testing non-microcystin *Microcystis* spp. metabolites (Janssen, 2019), research into their toxic modes of action has been limited. Target tissues of these metabolites and the complex mixtures of non-microcystin-producing strains are also poorly explored, often focusing on key organs such as the liver (e.g., Sotton et al., 2017). The methodologies of this chapter aim to potentially expand upon these unknown variables through assessments of viability focusing on

different aspects of cellular function as well as cell lines derived from a variety of both internal and external tissues, such as the brain, gonads, gills, and skin. Additionally, previous studies have highlighted variability in the inhibitory effects of *M. aeruginosa* complex metabolite mixtures depending on the growth phase of the originating culture (Xu et al., 2016; Wang et al., 2017), however this research was largely focused on allelopathy between toxin-producing cyanobacteria and co-occurring aquatic macrophytes or other phytoplankton. To better understand this aspect of cHABs both in regards to non-microcystin-producing strains and effects on fish species, I incorporated exudates from both exponential and stable growth phases of cyanobacterial cultures into cytotoxicity testing.

The main aim of the third chapter in this thesis was to explore the potential sublethal impacts of *M. aeruginosa* extracellular metabolites on reproduction-related pathways. Real-time quantitative polymerase chain reaction (RT-qPCR) assays were used to monitor changes in the transcription of genes in rainbow trout cell lines treated with the exudates of the two strains of *M. aeruginosa.* As with the cytotoxicity experiments, these studies expand upon the potential target tissues of non-microcystin-producing *M. aeruginosa* strains, focusing specifically on how extracellular metabolites of both MC-productive and non-productive strains alter expression of genes related to reproduction. By monitoring changes in key reproduction-related genes, I hope to provide a better understanding of how cyanobacterial blooms can impact freshwater fish populations through impairment of reproductive processes.

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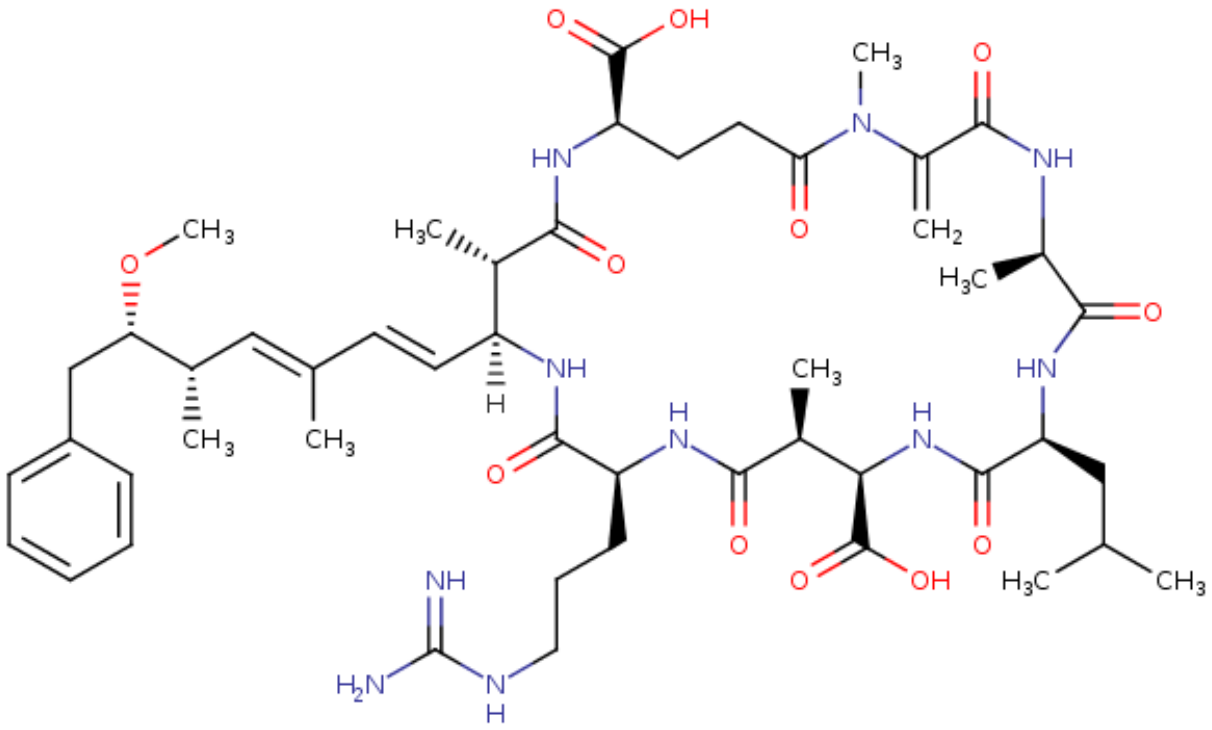

Figure 1.1: Chemical structure of microcystin-LR. Image sourced from Chemical Entities of Biological Interest (ChEBI) database (Hastings et al., 2016).

CHAPTER 2: Cytotoxicity in rainbow trout cell lines: extracellular metabolites of microcystin-producing versus non-producing *Microcystis* strains

2.1 Introduction

The *Microcystis* species of cyanobacteria are prolific contributors to freshwater harmful algal blooms (HABs). These planktonic, unicellular cyanobacteria are colony-forming and capable of producing dense surface scums (Chorus & Welker, 2021) and *Microcystis*-dominated blooms and their toxins have been recorded in more than 100 countries around the world (Harke et al., 2016). Trends of increasing intensity and abundance have been broadly identified in freshwater HABs (Ho et al., 2019; Huisman et al., 2018) and the distribution and density of *Microcystis* blooms are likely to follow this pattern. Often through anthropogenic inputs, the eutrophication of freshwater systems shifts phytoplankton assemblages toward cyanobacterial dominance, while changing climatic conditions such as rising water temperatures and salinity and alterations in hydrological cycles that favour longer residence times and increased stratification further tip the scales toward bloom development and reoccurrence (Paerl & Paul, 2012; Taranu et al., 2015). During peak bloom periods, HABs can have significant impacts on freshwater environments including increasing local turbidity, shading aquatic macrophytes and disrupting visual cues of other aquatic organisms (Sukenik et al., 2015). As blooms senesce, decomposition of cyanobacterial biomass can deplete dissolved oxygen, creating fatal hypoxic zones and negatively impacting benthic habitats (Havens, 2008; Sukenik et al., 2015).

In addition to the visible blue-green film and biomass buildup during bloom accumulation, the *Microcystis* genus is most often associated with their most well-known toxin, the hepatotoxin microcystin (MC). Originally called Fast-Death Factor, MCs are cyclic

heptapeptides that bind to protein phosphatases (PP1, PP2A, and PP5) in cells and inhibit their activity (Harke et al., 2016; Chorus & Welker, 2021). These phosphatases are critical to many aspects of normal cell function including cytoskeletal structure, cell replication, and DNA repair and their inhibition disrupts normal patterns of phosphorylation in cells, leading to cellular degradation, apoptosis, and tumour promotion (WHO, 2020; Banerjee et al., 2021; Chorus & Welker, 2021). The liver is a major target of MC accumulation and the site of significant tissue damage, however numerous other organs, including the kidneys, intestines, and respiratory tissues can be the site of absorption and adverse affects (Malbrouck & Kestemont, 2006; Banerjee et al., 2021). Histopathological evidence of MC intoxication can present as swelling or haemorrhaging of these affected tissues as well (Lopez-Rodas et al., 2008; Bengis et al., 2016). Microcystins pose a risk to humans and other terrestrial animals most often through contaminated drinking water and contact with bloom biomass or potentially through the inhalation of spray aerosol from affected water bodies (Carmichael & Boyer, 2016; Plaas & Paerl, 2021). MCs may also accumulate in the tissues of edible fish and crustaceans – at times reaching levels possibly unsafe for human consumption (Poste et al., 2011; Papadimitriou et al., 2012). Likewise, fish and other aquatic organisms are exposed to MCs through the consumption of cyanobacterial cells and contaminated food sources or contact with bloom biomass to gill structures during respiration (Sukenik et al., 2015).

In ecotoxicological research, the toxicity of MCs to fish has been evaluated through a variety of experimental exposure routes including intraperitoneal injection, oral gavaging and immersion in both adults and early life stages (Malbrouck & Kestemont, 2006). These experiments have linked MC exposure to tissue damage, particularly in the liver, kidneys and gills, reproductive toxicity – disrupting proper development and egg production in adults as well

as increasing malformations and mortality of early life stages – and dysregulation of genes leading to potential immuno- and neurotoxicity (Malbrouck & Kestemont, 2006; Banerjee et al., 2021). Much of the current research on microcystins focuses on MC-LR – considered the most potent of MC variants; however, microcystins display significant structural variation and little toxicological information has been recorded for the majority of the more than 300 MC congeners identified so far (Jones et al., 2021; Chorus & Welker, 2021). This impressive chemodiversity extends beyond microcystins as well. While the potent toxicity of MCs places them at the forefront of cHABs research, *Microcystis* spp. produce a variety of other metabolites, including microviridins, aeruginosins, and cyanopeptolins (Huang & Zimba, 2019). These metabolites have been identified as potent inhibitors of a variety of proteases such as trypsin, elastase, and thrombin (Huang & Zimba, 2019). Microviridins, cyanopeptolins and aerucyclamides – another *Microcystis*-produced compound – negatively affect zooplankton, crustaceans, and fish (Portmann et al., 2008; Faltermann et al., 2014; Kohler et al., 2014; Amaral et al., 2021). In a similar vein to the diversity of MC congeners detected, amongst *Microcystis* strains isolated for research there is significant variability in metabolite production with different strains varying both in families of metabolites and specific congeners produced (e.g., see Welker et al., 2004; Sotton et al., 2017). However, many metabolite families are poorly characterized with toxicological research hampered by the limitations in current methodologies to identify and isolate individual compounds (Janssen, 2019).

Variability in metabolite production, especially MCs, between different *Microcystis* strains can also include strains that fail to produce MCs altogether. *Microcystis* strains that do not produce their titular primary toxin are often dubbed “non-toxic” in research papers where they are used, delineated during cultivation by the absence of detectable MC congeners or an

incomplete *mcy* gene cluster (Rinta-Kanto et al., 2009; CPCC, 2013), the latter of which has been linked to their production (Tillet et al., 2000). However, this nomenclature can be misleading. In studies using the freshwater fish species medaka (*Oryzias latipes*) non-microcystin-producing strains of *M. aeruginosa* induced distinct histopathological damage to the livers of exposed fish (Le Manach et al., 2018), which resulted in significant dysregulation of proteins associated with stress responses, reproductive processes, metabolism, and homeostasis and other important biological processes (Le Manach et al., 2016; Sotton et al., 2017, Le Manach et al., 2018). While studies of this kind are limited, these non-microcystin-producing strains are naturally occurring in freshwater systems (Wilson et al., 2005), coexisting with their toxic counterparts. Indeed, these non-toxic strains may constitute anywhere between 65 and 99.9% of *Microcystis* blooms (Davis et al., 2009; Rinta-Kanto et al., 2009). How these strains contribute to HABs toxicity dynamics is an emerging research subject (Bittencourt-Oliveira et al., 2014).

Microcystin uptake appears to be facilitated by organic anion transporting polypeptides (OATPs), critical transport proteins of organic compounds such as bilirubin, prostaglandins, and thyroid hormones, as well as xenobiotics and toxins (Hagenbuch & Stieger, 2013). In humans, these proteins are widely expressed in a variety of tissue types, including tissue-specific expression in the liver and brain (Hagenbuch & Stieger, 2013). Likewise, expression of OATPs in zebrafish were highest in brain, liver, and kidney tissues and high expression of an OATP was identified in rainbow trout liver tissue, highlighting that these tissues are particularly vulnerable to deleterious impacts of MCs (Steiner et al., 2014; Steiner et al., 2016). Indeed, sensitivity of the brain and liver of fish to MCs, as well as the liver's role in xenobiotic metabolism, has made them well-studied tissues in cHABs research (see review, Malbrouck & Kestemont, 2006); however, little is known about the toxicokinetics of other *Microcystis* metabolites – including

their potential reliance on specific transport proteins – making identifying potential target tissues more difficult. In this vein, current studies of non-microcystin-producing strains have been largely focused on hepatic damages of these cultures, neglecting possible novel metabolite and tissue interactions.

Metabolite production in cyanobacteria is likely to also vary within a strain as a culture enters different phases of growth and changing metabolic processes in turn shift compound abundance and diversity. For *Microcystis* spp., blooms adhere to a recognizable pattern of initiation and recruitment followed by a period of exponential (or log) growth. Once these blooms reach a point of maximal density, cyanobacterial populations stabilize before a precipitous decline in which a majority of cells die or return to the sediment for overwintering (Reynolds et al., 1981). The timing and duration of these bloom stages are driven by local physical and climatological factors including increasing light levels and temperature, changing nutrient ratios and oxygen levels, leading to seasonal and location-based variation in peaks and declines (Reynolds et al., 1981) – for example in large lacustrine systems, the timing and density of bloom peaks can vary across multiple sampling sites depending on site-specific conditions (e.g., Wang et al., 2009).

For microcystins, the senescent stage of an algal bloom can be particularly dangerous due to high numbers of lysing cells releasing MCs – which are primarily intracellular – into the water column (Chorus & Welker, 2021). However, extracellular compounds produced during largely non-lytic stages may also have variable toxicity. This has been observed in the strength of allelopathy within stationary and exponential growth phase filtrates of *Nodularia*, *Oscillatoria* and *Cylindrospermopsis* species (Śliwińska-Wilczewska et al., 2021). Distinct differences in allelopathy have also been found in the extracellular metabolites of different growth phases of *M.*

aeruginosa. Log phase cell-free medium was found to increase growth in the cyanobacteria and green algae species, while decline phase exudates produced an inhibitory effect (El-Sheekh et al., 2010). Conversely, a similar study on green algae and diatom species exposed to *M. aeruginosa* exudates found that log phase exudates were strongly inhibitory alongside those of the stationary phase, while decline phase exudates promoted growth when concentrations were low (Wang et al., 2017). These findings also align with previous research with aquatic macrophyte seedlings in which exudates collected from the exponential stage of growth were primarily inhibitory in most tested growth parameters, including seedling weight, leaf number, and photosynthetic activity, whereas weaker or even stimulatory effects were observed in decline phase treatments (Xu et al., 2016). Within *M. aeruginosa* strains, differences in growth phase significantly alter metabolite production – both in concentration and type – when assessed using liquid chromatography-mass spectrometry (LC-MS), likely contributing to the differences in toxicity observed in these aforementioned studies (Zhou et al., 2022).

While this is an expanding aspect of research on allelopathic interactions between cyanobacteria and other phytoplankton and aquatic macrophytes, how these differences in metabolite composition may impact *Microcysits'* effect on vertebrates, including fish, is an important aspect of future cHABs research. Sorichetti et al. (2014) compared the toxicity of log and stable phase extracts and exudates of *Microcystis aeruginosa* cultures on rainbow trout gill cells and found that stable phase metabolites were much more strongly cytotoxic than their log phase equivalents. This appears to be one of the only comparisons of this kind on fish in current *Microcystis* algal bloom research.

Considering the need for further exploration into all of these previously discussed areas of cHABs research, this study compares the toxicity of extracellular metabolites produced by

MC-producing strains of *M. aeruginosa* – denoted "Toxic" strains – from those of their "Non-Toxic" non-microcystin-producing counterparts, with particular focus on their effects on freshwater fish. I used fluorescent dye-based cytotoxicity assays to assess differences in the cell viability of rainbow trout (*Oncorhynchus mykiss*) cell lines exposed to the cell-free exudates of each *Microcystis* strain. By using a broad array of tissue types, I hoped to capture a more thorough catalogue of potential target sites for the non-MC metabolites of both strains. When examining the effects of MCs and algal cultures on the heart rates of fish embryos, previous research found that MCs could not fully explain the strength of heart rate depression in culture exposures, lending evidence to the possibility of additive interactions between MCs and other metabolites present (Saraf et al., 2018). Based on previous work with non-MC-producing strains and these potentially additive interactions, I hypothesize that the "Non-Toxic" strains will induce significant changes in the viability of the fish cell lines and there will be distinct overlap in the patterns of toxicity between strains. The second goal of this study was to compare *M. aeruginosa* exudates from different growth phases and their toxicity in fish cell lines. I predict that, as previously observed with allelopathic interactions between cyanobacteria and other algae and macrophytes (El-Sheekh et al., 2010; Wang et al., 2016), there will be distinct differences in the strength of inhibitory effects between metabolites of different growth phases. This study will not only provide further information on non-microcystin-producing *M. aeruginosa* strains and their effects on fish, but also highlight considerations necessary for future research on the impacts of expanding cHABs in freshwater ecosystems.

2.2 Materials and Methods

2.2.1 *Microcystis* Cultures

Two strains of *M. aeruginosa* were acquired from the University of Waterloo's Canadian Phycological Culture Centre – CPCC 300, a microcystin-producing toxic strain isolated in Alberta, CA and CPCC 633, a 'non-toxic' non-microcystin-producing strain originating from Ontario, CA. Both strains of algae were maintained in BG-11 growth medium (See Appendix 1.1) and incubated at 23±1.5°C in a 12:12 light-dark cycle with fluorescent light of 19.80 ± 1.42 µmol/m^2/s. Light levels in the incubator were reduced using aluminum foil covers to the intensity recommended by the CPCC. Culture density was measured by manual counting using a compound microscope and haemocytometer.

2.2.2 Rainbow Trout Cell Lines

Rainbow trout cell lines were generously provided by Dr. B. Dixon and Dr. N.T.K. Vo from the University of Waterloo (**Table 2.1**). All cell lines were routinely grown in 175 cm^2 plug-seal tissue culture flasks at 19±1.5°C in Leibovitz's L-15 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). Flasks were considered confluent when cell monolayers covered 80-90% of the flask surface, at which point cells were passaged using TrypLE (Gibco) to new flasks or plated for use in experimental treatments. Flasks were routinely passaged at ratios between 1:3 and 1:5.

2.2.3 Experimental Cultures and Exudate Preparation

Experimental cultures of each *M. aeruginosa* strain were inoculated at cell densities of $3.01 \pm 0.14 \times 10^7$ cells/mL in 1000 mL Erlenmeyer flasks from healthy stock cultures that had been growing for at least five weeks and were consolidated prior to being divided into

experimental flasks. Flasks were manually shaken every two to three days by gentle swirling and randomly redistributed within the incubator to account for minor differences in fluorescence between shelves. Every six days, three flasks of each strain were randomly selected for exudate collection. Samples were collected for cell density monitoring and the total volume of each flask was centrifuged to remove intact cells and debris – such as fragments of dead cells and loose cell contents. Cultures were initially centrifuged for 25 minutes at 4400 rpm and 15°C, but equipment malfunctions necessitated a shift to 10 minutes at 4500 rpm and 22°C in later samples. The supernatant was then collected and filtered using 0.2 µm polyethersulfone (PES) membrane syringe filters to remove any remaining debris. The greater presence of debris in CPCC 633 cultures necessitated a second centrifugation of the supernatant before exudates were filtered. Exudates were then aliquoted into 15 mL falcon tubes and stored frozen at -80 °C until lyophilization.

For cell line treatments, exudates were selected from within the exponential and stable growth phases – six days and thirty days in culture respectively – and lyophilized using a benchtop freeze dryer (Labconco, Missouri, USA). Samples, including a BG-11 control, were re-aliquoted into 5 mL volumes and lyophilized just until all liquid was removed and they had reached a dry and powder-like consistency – between 18 to 24 hours. Dried samples were then resuspended in phenol-free L-15 medium up to a 20X concentration and triplicate flasks for each strain/phase were combined to generate the final treatments, referred to hereafter as Toxic-Log (TS), Toxic-Stable (TL), Non-Toxic-Log (NTL), and Non-Toxic-Stable (NTS). These treatments were aliquoted out to volumes ≤ 1000 µL and stored frozen at -80 °C. Immediately prior to each cell line treatment, concentrated *M. aeruginosa* exudate (MaE) and BG-11 samples were thawed in a 37°C warm water bath and dilutions of 2.5, 5, and 10% (v/v) were prepared in phenol-free

L-15 exposure medium (L-15/ex, United States Biological, Massachusetts, USA), a simplified medium developed by Schirmer et al. (1997) for final concentrations equivalent to 0.5X, 1X, and 2X (**Table 2.2**).

Unconcentrated samples were also set aside for microcystin content quantification via enzyme-linked immunosorbent assay (ELISA) using an Abnova Microcystin-LR ELISA Kit (Abnova, Taiwan) per the manufacturer's recommended protocol. The resulting concentrations were expressed in MC-LR equivalents (μg/L).

2.2.4 Cytotoxicity assays

Cell lines were plated in 96-well Poly-D-Lysine-coated (Gibco) black plates at densities of 2.5 x 10^4 cells per well in 50 - 100 μL of complete L-15 media. Seeded plates were sealed with Parafilm and incubated for 24h at 19°C to allow cells to attach. Cell culture media was removed via vacuum pipette and 100 μL of each treatment was added to each well in triplicate for exudate treatments and duplicate for BG-11 controls. For untreated wells, complete media was replaced with L-15/ex of equal volume.

Changes in cell viability were assessed using two fluorescent dye-based assays – AlamarBlue (AB) and 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM) based on the protocols previously described by Dayeh et al. (2013). AlamarBlue monitors cellular metabolic activity by measuring the ability of living cells to convert the non-fluorescent resazurin compound to its fluorescent form, resorufin via oxidoreductases. Similarly, CFDA-AM measures plasma membrane integrity through the conversion – via non-specific esterases – of the CFDA-AM dye to the fluorescent form, 5-carboxyfluorescein. Since their final fluorescent products are detected at different emission wavelengths, these dyes were used concurrently during cell viability assays with only minor modifications from the protocols described by Dayeh

et al. (2013). Briefly, mixed dye working solutions were prepared in the dark immediately before applying to plates. The AlamarBlue (purchased ready-to-use, Invitrogen) and CFDA-AM (dissolved in DMSO for a final concentration of 4nM, Invitrogen) were diluted 1:10 and 1:1000 respectively in L-15/ex media. After a 24h exposure period at 19°C, treatment solutions were decanted and plates briefly blotted on paper towels. Using a multi-channel pipette, 100 µL of the AB/CFDA-AM dye solution was quickly added to each well, including an additional three cell-free wells to remove background fluorescence during readings. Plates were re-sealed with Parafilm and incubated in the dark for 2h. Fluorescence readings were taken every hour at excitation and emission wavelengths of 530 and 590 nm for AB and 485 and 530 nm for CFDA-AM respectively, using a Varioskan LUX microplate reader (Thermo Fisher Scientific).

2.2.5 Lysotracker dye assays

For evaluating lysosomal activity, cells were seeded into 96-well Poly-D-Lysine-coated clear plates at densities of 2.5 x 10^4 cells per well in the same manner as the cytotoxicity assays and allowed to attach overnight at 19°C. Following the attachment period, cell culture media was removed via vacuum pipette and replaced with 100 µL of either the 5 or 10% concentrations of each treatment or phenol-free L-15/ex for the untreated wells. After a 24h 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 minutes, cells were visualized using a EVOS m5000 inverted microscope (Invitrogen) (**Appendix 1.2**). Lysotracker Red selectively localizes to acidic organelles, including lysosomes, and is used as method of visualizing lysosomal activity and accumulation. NucBlue binds to DNA, localizing to cell nuclei, and was used quantify cells

during visualisation. The mean fluorescence of Lysotracker Red was then normalized to the number of counted cells.

2.2.6 Data analysis and statistics

As discussed previously, individual cyanobacterial compounds are poorly characterized largely due to the difficulties current methods encounter in isolating and identifying these metabolites (Janssen, 2019). Quantification of these components is likewise difficult and even in well-studied metabolites such as microcystins, proxy measurements are required where specific compounds cannot be individually measured or suitable standards have not been produced, i.e. 'MC-LR equivalents' as a representative measurement for all microcystin congeners present in a sample (for example: El-Sheekh et al., 2010). Nevertheless, utilizing MC-LR measurements as a stand-in for other cyanobacterial metabolites can lead to significant errors in quantification (Natumi & Janssen, 2020). Culture density may also be a useful tool in proxy measurements as the production of microcystins as well as other metabolites are strongly correlated with cell density (Natumi & Janssen, 2020; Wang et al., 2021). However, when it comes to extracellular metabolites, previous studies with *Microcystis* species found a poor to no correlation between extracellular MC concentrations and cell abundance (Wood et al., 2012; Wu et al., 2014) and it remains to be seen whether this is also true for other cyanobacterial compounds. With these considerations in mind, the effects of the exudate treatments on cell viability were compared using both the percent volume (% v/v) concentration of the solution applied to cells and their 'equivalent' cell densities, a proxy measurement calculated from the cell density of the progenitor flasks immediately prior to cell removal (**Table 2**).

All data were expressed as mean ± standard error of the mean (SEM). Data was analyzed using GraphPad Prism 8.0.2 software. The normality of data distribution and homogeneity of

variances were assessed using the Shapiro-Wilks test and Brown-Forsythe test, respectively. Potential interactions between the effects of cyanobacterial strain, growth phase, and concentration on cell viability were analyzed via 3-way ANOVA followed by Tukey's multiple comparisons post hoc test. Statistical differences between treatments (TL, TS, NTL, or NTS) within each concentration were evaluated by one of following tests: 1) Ordinary one-way ANOVA followed by Tukey's post hoc test; 2) Welch's ANOVA with Games-Howell's post hoc test; or 3) the non-parametric Kruskal-Wallis test with Dunn's post hoc test. The latter two test sets were utilized when the data was found to violate the assumptions of equal standard deviations or normal distribution needed for the ordinary one-way ANOVA respectively. Student's t-tests were used to assess the statistical differences between exudate treatments and controls. P-values of < 0.05 were considered statistically significant.

For the lysotracker dye assays, some cell lines were suitable for analysis with either two-way or three-way ANOVAs based on the heteroscedasticity and distribution of the data as assessed using the same Brown-Forsythe and Shapiro-Wilks tests respectively. Where applicable, these results will be noted as well, otherwise, analysis was conducted with One-Way ANOVAs and post hoc tests as previously described.

2.3 Results

2.3.1 Microcystin concentration

Microcystin concentrations were assessed in each of the three unconcentrated biological replicates of the log and stable phases of my "Toxic" and "Non-Toxic" strains. The upper detection limit of the ELISA was 2.5 μg/L, necessitating an initial dilution of my "Toxic" exudates. The MC concentrations of TL and TS exudates were 1.25 ± 0.35 μg/L and 1.02 ± 0.09 μg/L respectively. While higher than WHO's lifetime drinking-water provisional guideline of 1.0

µg/L, these values are in line with previously detected extracellular MC concentrations in *Microcystis* spp. dominated blooms (WHO, 2020; Pham et al., 2021). Microcystin concentrations for "Non-Toxic" exudates were also evaluated, however, unsurprisingly these fell under the limit of detection for this particular method of MC quantification (<0.1 µg/L).

2.3.1 BG-11 Media as a vehicle control

Since each exudate treatment was a solution containing both the putative bioactive metabolites and cyanobacterial growth media, it was necessary to examine the effects of this media alone on treated cells. During initial analysis of cytotoxicity results, I found that the cyanobacterial growth media BG-11 induced significant changes in the viability of cells from some of the tested cell lines. For example, in the AB assays BG-11 treatments significantly increased fluorescence in RTBrain cells (**Figure 2.1)** while RTHDF cells experienced high cell death in BG-11-treated wells (**Figure 2.2**). Overall, compared to untreated cells, a majority of tested cell lines experienced significant changes in cell viability following treatment with at least one BG-11 concentration during either cytotoxicity assay (**Figure 2.3**). Considering these results and since BG-11 could not be feasibly removed from my treatments at this time, the decision was made to treat BG-11 as the "control" value in all subsequent statistical analyses in an effort to remove the potential influence of media components from further toxicity assessments.

2.3.2 Cytotoxicity responses

For the cytotoxicity assays, significant changes in cell viability or cellular activity were assessed using the fluorescent indicator dyes AlamarBlue (AB) and CFDA-AM, which monitor cellular metabolic activity and membrane integrity/esterase activity respectively.

The AB assay (see Appendix 1.4 **Table A1.4.1** and **Table A1.4.3**) was more sensitive to potential negative effects of Non-Toxic exudates treatments compared to CFDA-AM (**Table**

A1.4.2 and **Table A1.4.4**). The cell lines RTBrain, RTgill-W1, and RT-milt5 all experienced significantly reduced AB fluorescence following treatment with NT solutions (**Figure 2.4)**, impairments which were not reflected in their CFDA-AM assessments (**Figure 2.8**). Additionally, these significant effects were apparent in the majority of treatments – both Toxic (T) and Non-Toxic (NT) – at the lowest tested concentrations for all three cell lines. When comparing the T and NT treatments (**Table A1.4.5**), differences in the responses for the RTBrain (**Figure 2.5.B**) and RT-milt5 (**Figure 2.5.G**) cell lines were only statistically significant at the 2X concentration, however; for RT-milt5 this difference was dependent on the growth phase of the NT exudates – with no difference between the stable phase NT treatment and either T treatment. This was also the case for the RTgill-W1 cell line, although in this cell line, statistical differences between the response of the log phase NT treatment and the others became evident at a lower treatment dosage (**Figure 2.5.D**).

When comparing the responses of these cell lines based on their relative culture densities similar patterns were evident in their responses (**Figure 2.6**), however; the growth phase of the T treatments appeared more critical in determining comparative toxicity (**Table A.1.4.6**). While there were no significant differences in the responses between treatments for RT-milt5 at either density (**Figure 2.7.G**) or RTBrain at the medium relative density (**Figure 2.7.B**), TL treatments produced significantly greater reductions in AB fluorescence compared to NT exudates at the high density for RTBrain and both densities for RTgill-W1 (**Figure 2.7.D**).

The RTP-2 cell line also experienced significant reductions in cell viability in response to both T and NT treatments, although this was only evident at the 1X concentration (**Figure 2.5.H**). In density- based comparisons, log phase exudates produced significant decreases in AB fluorescence at the medium relative density, but not at the high density and vice versa was true of

the stable phase exudates (**Figure 2.7.H**). Lack of significant results, particularly at the 2X concentration, may be a consequence of RTP-2's weak adherence while undergoing treatment and assay methodologies – an issue during lysotracker assays as well, as I will discuss – potentially leading to loss of cells and increasingly disparate results.

In addition to these previous four cell lines, the RBT4BA and RTG-2 cell lines were also sensitive to the negative effects of T exudates on cell viability (**Figure 2.5.A** and **C**). At the highest tested concentration (2X), both T treatments significantly reduced the AB fluorescence of RBT4BA cells compared to controls (**Figure 2.5.A**). For RTG-2, cell viability was only significantly reduced by the stable phase T treatment (**Figure 2.5.C**), although there were no statistical differences between T treatments in either cell line (**Table A1.4.5**). When compared via relative density, only the high-density treatment of TL produced a significant reduction in RBT4BA cell viability (**Figure 2.7.A**), while RTG-2 was not significantly affected by any treatment in this comparison method (**Figure 2.7.C**).

Viability of the RTL-W1 cell line was also significantly reduced by T exudate treatments at the 2X concentration (**Figure 2.5.F**), similar to RBT4BA and RTG-2. However, at the lowest tested concentration (0.5X) the stable phase exudates of both T and NT treatments produced significant *increases* in AB fluorescence compared to controls and at the 2X concentration, while T treatments reduced viability, the log phase NT treatments significantly increased fluorescence. This pattern of responses was reflected in density-based comparisons as well, although only the log phase T treatments produced a significant reduction in viability at the high density (**Figure 2.7.F**). Finally, the AB fluorescence of RTHDF cells was significantly increased by TL treatments at the highest concentration used (**Figure 2.5.E**) and – when compared via density – the highest density (**Figure 2.7.F**).

While AB assays (**Figure 2.4** and **Figure 2.6)** were largely dominated by reductions in fluorescence and therefore impairments of viability and focal enzyme activity (i.e. oxidoreductases), increases in fluorescence like those observed in RTL-W1 and RTHDF were much more common among cell responses in the CFDA-AM assays (**Figure 2.8** and **Figure 2.10**). RTHDF, as well as RT-milt5, experienced significant increases in CFDA-AM fluorescence following treatments with both T and NT exudates (**Figure 2.9.E** and **G**). For the RTHDF cell line, fluorescence was significantly increased at the 0.5X concentration with the stable phase T treatments, while at 2X, log phase T treatments produced a significant positive response alongside stable phase NT treatments (**Figure 2.9.E**). Similarly, in density-based comparisons, TS and TL treatments significantly increased fluorescence at the medium and high densities respectively (**Figure 2.11.E**).

For RT-milt5, stable phase NT treatments increased CFDA-AM fluorescence at every tested concentration (**Figure 2.9.G**), while increases from stable phase T treatments were only statistically significant at the 0.5X and 2X concentrations. For log phase treatments, TL produced significant increases only at 0.5X, while NTL treatments increased fluorescence only at 2X. For density-based comparisons, NTS treatments significantly increased fluorescence at both densities while TS treatment increases were only significant at the medium density (**Figure 2.11.G**). Similar to concentration-based comparisons, NTL treatments only increased fluorescence significantly compared to controls at high densities.

For the cell lines RTG-2 and RTgill-W1, exudate treatments produced a mixture of inhibitory and stimulatory responses for CFDA-AM assays (**Figure 2.9.C** and **D**). In the RTG-2 cell line, stable phase treatments produced significant increases in CFDA-AM fluorescence at 1X for T exudate treatments and 2X for NT treatments (**Figure 2.9.C**). However, for the log phase T

treatments, exposure to 2X concentrations significantly decreased cell viability. Comparisons via density follow a similar pattern, although only TS treatments produced a significant increase in fluorescence at the high density, while at the same density, TL treatments significantly reduced viability (**Figure 2.11.C**). For the RTgill-W1 cell line, CFDA-AM fluorescence was significantly increased only at the 0.5X concentration by TS treatments, while at 2X these treatments significantly decreased viability (**Figure 2.9.D**). In addition, TL treatments also significantly reduced viability of RTgill-W1 cells at both 1X and 2X concentrations. Again, density-based comparisons were similar with significant increases in fluorescence by TS treatments at medium density and significant decreases by TL treatments at both densities (**Figure 2.11.D**).

In contrast to these previous cell lines, exudate treatments of RBT4BA, RTBrain, and RTL-W1 cell lines produced only significantly negative responses in CFDA-AM fluorescence, in particular following exposure to the 2X concentration (**Figure 2.9.A, B,** and **F**) and high density (**Figure 2.11.A, B,** and **F**) log phase T treatments. Finally, the CFDA-AM assays for the RTP-2 cell line did not result in any significant changes in viability following exudate treatments compared to controls (**Figure 2.9.H** and **Figure 2.11.H**).

2.3.2.1 Three-way ANOVA analyses

In addition to examining the effects of each treatment individually, I also examined how different treatment factors and their interactions contributed to cell line responses using three-way ANOVAs. The factors I focused on were the exudates' strain type, growth phase, and either concentration or density depending on the type of comparison conducted. For concentration-based comparisons, treatment concentration, strain type, and the interaction between these two factors were the dominant source of variation in cellular responses for the majority of tested cell lines for both AB and CFDA-AM assays (**Table 2.3**). These three factors combined accounted

for between 15.94 and 81.92% of total variation in each three-way ANOVA conducted for AB and between 19.11 and 79.59% of total variation for CFDA-AM assays. For AB assays, concentration contributed the highest percentage of variation in six out of the eight tested cell lines, with the two exceptions being RTBrain and RTP-2 in which strain was the major contributing factor. Similarly, for CFDA-AM the interaction between concentration and strain made up the highest percentage of response variation in half of the tested cell lines, with the remaining lines split between concentration (RBT4BA, RTHDF, and RTP-2) and strain type (RTBrain). The remaining key attribute – growth phase – and its interactions with other factors were only significant contributors to response variation in three cell lines for AB assays (RTgill-W1, RTHDF, and RT-milt5) and four cell lines for CFDA-AM assays (RTBrain, RTG-2, RTL-W1, and RT-milt5).

For density-based comparisons, there was greater variety in the major factors contributing to response variation in the AB and CFDA-AM assays (**Table 2.4**). In the AB assays, three-way ANOVA analysis found that strain type contributed the highest percentage of variation in three out of eight of the tested cell lines (RBT4BA, RTBrain, and RTG-2), concentration was the major contributing factor in two cell lines (RTgill-W1 and RTL-W1), and growth phase and the interaction of growth phase and strain type were the highest percentage contributors in one cell line each (RTHDF and RT-milt5, respectively). No factors produced significant variation in response for RTP-2. These four factors were responsible for between 43.47 and 96.96% of total variation in cellular responses for AB assays. For CFDA-AM assays, the major contributing factors in cell responses were very similar – strain type contributed the highest percentage of variation in the same three cell lines as the AB assays, however; the interaction between density and strain type was the major contributing factor for two cell lines (Rtgill-W1 and RT-milt5) as

well as the interaction between density and growth phase (RTL-W1 and RTP-2). Growth phase alone was also the highest percentage contributor for the RTHDF cell line, in line with the findings of the AB assays. For the CFDA-AM assays, the contributions of each factor to the total variation of responses were much more varied than the AB assays and these four major contributing factors were responsible for between 22.61 and 60.65% of total variation in responses across cell lines.

2.3.2.2 Growth-phase dependent toxicity

According to the results of the three-way ANOVA analyses – in concentration-based comparisons (**Table 2.3**) – growth phase and its interactions with other treatment factors were significant sources of variation in the AB cell viability responses of the RTgill-W1, RTHDF, and RT-milt5 cell lines and in the CFDA-AM responses of the RTBrain, RTG-2, RTL-W1, and RT-milt5 cell lines. When comparisons were made via relative density (**Table 2.4**), growth phase and its interactions were significant sources of variation in nearly every cell line for both cell viability assays, the only exception being the AB assay for RTP-2. While these analyses indicate that growth phase can be a significant contributor to effects on cell viability, delineations between the growth phases of each strain type and the strength of their effects was a much more limited occurrence among my cytotoxicity results and depended both on the assay and comparison type being assessed. In the concentration-based comparisons, there were significant differences between the AB viability responses of RTgill-W1 cells to log and stable phase NT treatments at both 1X and 2X concentrations with the NTS treatments producing significantly stronger negative effects (**Figure 2.5.D**). Similar results were evident for the RT-milt5 cell line at the 2X concentration in the AB assays as well (**Figure 2.5.G**). For the CFDA-AM assays,

significant differences were observed for the RTL-W1 cell line between log and stable phase T treatments at the 2X concentration (**Figure 2.9.F**).

Differences between growth phases were much more common within density-based comparisons. In the AB assays, there were significant differences between growth phases of T treatments in the RTgill-W1 cell line at both medium and high relative densities (**Figure 2.7.D**). At high treatment densities, log and stable phase T treatments also produced significantly different responses in RBT4BA (**Figure 2.7.A**) and RTL-W1 cells (**Figure 2.7.F**). Similarly, for CFDA-AM assays, log and stable phase T treatments produced significantly different responses at high density treatments for RBT4BA (**Figure 2.11.A**), RTG-2 (**Figure 2.11.C**), and RTL-W1 cell lines (**Figure 2.11.F**). In these assays, when differences between Toxic exudates were evident, log phase treatments consistently produced greater reductions in cell viability compared to stable phase.

2.3.2.3 Cell line sensitivity

When comparing the responses of each of the tested cell lines, the NT-sensitive cell lines RTBrain, RTgill-W1, and RT-milt5 were also frequently the most sensitive cell lines for all treatment types in regards to the lowest tested concentrations. However, from a statistical standpoint, there were limited differences between the responses of each cell line (**Table A1.4.7** and **Table A1.5.8**). At the 0.5X concentration in AB assays, TL treatments significantly reduced viability in all three cell lines (**Figure 2.12.A**), although only the response of RTBrain was significantly different from the responses of other cell lines – specifically RBT4BA, RTHDF, and RTL-W1 (**Table A1.5.7**). For the stable phase T treatments, at 0.5X concentrations only RTBrain and RT-milt5 experienced significant reductions in cell viability (**Figure 2.13.A)** and both responses were only statistically distinct from the responses of RBT4BA and RTL-W1

(**Table A1.5.7**). On the other hand, the AB fluorescence in RTL-W1 cells was significantly increased at the lowest tested concentration for TS treatments. For my NT treatments, at 0.5X concentrations the log phase exudate treatments significantly reduced AB fluorescence in both RTBrain and RTgill-W1 cell lines (**Figure 2.14.A**), although only the response of RTBrain cells were significantly different from other cell lines – specifically RBT4BA and RTL-W1 (**Table A1.5.7**). Finally, for the NTS treatments, 0.5X concentrations produced significant decreases in the viability of the RTBrain, RTgill-W1, and RT-milt5 cell lines (**Figure 2.15.A**) and these responses were statistically distinct from those of RBT4BA and RTL-W1 (**Table A1.5.7**). The response of RTBrain and RT-milt5 cell lines were also significantly different from the RTG-2 cell line. At this concentration, RTL-W1 cells also experienced a significant increase in fluorescence following NTS treatments.

As indicated in the prior sections, the CFDA-AM assay appeared to be less sensitive to the negative toxic effects of my treatments than AB assays, potentially due to the differences in the focal enzymes employed by the reactions of each fluorescent dye. At the lowest tested concentration (0.5X), only significant increases in fluorescence were detected in cell lines for all treatments – when significant results were detected at all (**Figure 2.12.B, Figure 2.13.B, Figure 2.14.B,** and **Figure 2.15.B**). There were also no significant differences between treatment responses at this concentration (**Table A.1.5.8**). For the TL treatments, at the 0.5X concentration, CFDA-AM fluorescence was significantly increased in RT-milt5 cells (**Figure 2.12.B**). At the 1X concentration, RTgill-W1 cells had significantly reduced viability following treatment (**Figure 2.12.D**), however; this was not significantly different from the responses of the other cell lines. For the stable phase T treatments, the 0.5X concentration significantly increased fluorescence in RTgill-W1, RTHDF, and RT-milt5 cells (**Figure 2.13.B**). Significant reductions

in viability were only detected in these treatments at the 2X concentration in the RBT4BA and RTgill-W1 cell lines (**Figure 2.13.F**), although no significant differences were detected. Similarly, in the NTL treatments, a significant response in either direction was only detected at the 2X concentration with a significant increase in CFDA-AM fluorescence of RT-milt5 cells (**Figure 2.14.F**) and once again this response was not significantly different from those of the other cell lines. For the NTS treatments, the 0.5X concentration induced a significant increase in the fluorescence of RT-milt5 cells (**Figure 2.15.B**). Unlike the other treatments, stable phase NT exudates did not produce a significantly negative response in any of the tested cell lines even at the highest tested concentration (**Figure 2.15.F**).

2.3.3 Lysosomal activity

To assess lysosomal accumulation and activity, a Lysotracker-NucBlue dye solution was used to quantify fluorescent dye localized in lysosomes and related organelles to the number of cells present. Unlike in the cytotoxicity assays, significant responses in my lysosomal activity assays were exclusively increases in Lysotracker fluorescence compared to the controls. Additionally, NT treatments induced significant effects in nearly every tested cell line with the exceptions of RBT4BA (**Figure 2.16.A** and **Figure 2.17.A**) and RTBrain (**Figure 2.16.B** and **Figure 2.17.B**), which were not significantly affect by any treatment.

In the RTG-2 cell line, along with the RTHDF cell line in density-based comparisons, there were no significant differences in the responses of cells to T or NT treatments (**Table A.1.4.10**). For RTG-2, NTL treatments significantly increased Lysotracker fluorescence at both tested concentrations (**Figure 2.16.C**) and at the 1X concentration, TS treatments also increased lysosomal activity. These results were reflected at the high density in density-based comparisons as well (**Figure 2.17.C**). For RTHDF, when comparing treatments via relative density,

significantly increased fluorescence by NTS treatments (**Figure 2.17.E**) was not statistically distinct from other treatments at high relative densities (**Table A1.4.10**). However, when assessed via concentrations, this increase was significantly different from RTHDF cells' response to TL treatments (**Figure 2.16.E**).

Delineations between the effects of treatments by strain type were more common among the remaining cell lines. In the RTgill-W1 cell line, TS, NTL, and NTS treatments all significantly increased lysosomal activity at both tested concentrations (**Figure 2.16.D**) and at high relative densities (**Figure 2.17.D**). The responses of all three treatments were significantly different from the response of cells to TL treatments at both the 2X and high-density treatments, however; only the TS treatment was significantly different from TL at the 1X concentration (**Table A.1.4.10**). For the RTL-W1 cell line, stable phase NT treatments significantly increased Lysotracker fluorescence at both 1X and 2X concentrations, while all other treatments only increased fluorescence at the higher concentration (**Figure 2.16.F**). In terms of significant differences across treatments, at the 2X concentration, TL treatment responses were distinct from those of the log phase NT treatments (**Table A1.4.10**). When compared at the high relative density, all treatments except TS significantly increased lysosomal activity (**Figure 2.17.F**). In density-based comparisons, TL treatments produced significantly different responses from both growth phase of NT exudate treatments (**Table A1.4.10**). In the RT-milt5 cell line, all treatments produced significant increases in lysosomal activity regardless of concentration or density (**Figure 2.16.G** and **Figure 2.17.G**). Within this cell line, responses to the TS treatments were statistically distinct from all other treatments in both concentration- and density-based comparisons (**Table A1.4.10**).

Finally, in RTP-2 cell lines, significant increases in Lysotracker fluorescence were only evident in 2X concentrations of NTS treatments (**Figure 2.16.H** and **Figure 2.17.H**), the response of which was significantly different from those of cells treated with NTL (**Table A1.4.10**). However, as mentioned previously, adherence of RTP-2 cells appeared to be particularly susceptible to disruption by exudate treatments. For all TL treatments with the Lysotracker assay, cells were absent from treated wells during imaging, potentially due to dead or loosely adherent cells being physically removed during the replacement of treatment media with the dye solution. Therefore, the effects of log phase T exudates on the lysosomal activity of RTP-2 cells are still unknown.

2.3.3.1 ANOVA analyses and growth phase effects

Multi-factor analysis of Lysotracker assay data via ANOVAs was limited to only datasets that obeyed the necessary assumptions – most importantly those of normal distribution of data and of equal variations as detailed previously. These included the lysosomal responses of the RTG-2, RTgill-W1, and RT-milt5 cell lines (**Table 2.5**), which could be assessed using three-way ANOVAs similar to the cytotoxicity assays, and the RTHDF cell line (**Table 2.6**), which was assessed using two separate two-way ANOVAs to examine the effects of strain type and growth phase and their interactions on lysosomal activity. For both RTgill-W1 and RT-milt5, the growth phase of treatments contributed the largest proportion of response variation among significant factors, followed by the interaction between strain type and growth phase for RTgill-W1 and strain type alone for RT-milt5. In addition to the effect of growth phase on lysosomal activity detected in the three-way ANOVA analyses, there were also significant differences observed in the lysosomal responses to TL and TS treatments in both RTgill-W1 and RT-milt5 cells at both tested concentrations (**Table A1.4.10**) with greater increases in fluorescence with

TS treatments (**Table A1.4.9**). Growth phase was also the main and only significant factor contributing to response variation in RTHDF cells treated with 1X treatment concentrations. However, no significant effects on lysosomal activity were detected for any factors in either the 2X treatments or in the three-way ANOVA for RTG-2. Finally, in addition to the differences within T treatments in RTgill-W1 and Rt-milt5 cell lines, significant differences were also observed between growth phases of NT treatments at the 2X concentration in RTP-2 cells (**Table A1.4.10**).

2.3.3.2 Cell line sensitivity

When comparing the lysosomal responses of cell lines at the lowest tested concentration (1X), RT-milt5 appeared to be the most sensitive to the induction of lysosomal accumulation by exudate treatments, with significant increases in Lysotracker fluorescence in all treatments (**Figure 2.18**). However, similar to cell line comparisons in the cytotoxicity assays, statistically relevant differences between the responses of cell lines were rare (**Table A1.4.11**). At the 1X concentration, significant increases in lysosomal activity were only observed in RT-milt5 cells (**Figure 2.18.A**), however, there were no statistical differences among cell line responses to this treatment. For both TS (**Figure 2.18.C**) and NTL (**Figure 2.18.E**) treatments at the 1X concentration, the RTG-2, RTgill-W1, and RT-milt5 cell lines produced significantly greater Lysotracker fluorescence compared to controls, however; the only significant differences between responses were in the NTL treatment between the RTG-2 and RTL-W1 responses (**Table A1.4.11**). Finally, in the NTS treatments at 1X, lysosomal activity was significantly increased in cells from the RTgill-W1, RTHDF, RTL-W1, and RT-milt5 cell lines (**Figure 2.18.G**), although there were no significant differences between responses. Even at 2X concentrations, cell line responses were largely similar except for the response of RTL-W1 cells

treated with TL exudates (**Figure 2.18.B**), which was both significantly different from every other cell line besides RTG-2 (**Table A1.4.11**) and the greatest increase in fluorescence across all treatments – more than 4 times the Lysotracker fluorescence observed in the controls (**Table A1.4.9**).

2.4 Discussion

2.4.1 Cytotoxicity in *O. mykiss* cell lines

Non-toxigenic strains can constitute a significant fraction of *M. aeruginosa*-dominated algal blooms (Rinta-Kanto et al., 2009), however little research has been conducted on how they contribute to bloom toxicity. These strains have the capacity to produce numerous bioactive metabolites (Le Manach et al., 2016; Sotton et al., 2017) yet their use in toxicology testing for fish has been largely limited to liver-focused studies only (for example, Le Manach et al., 2016; Sotton et al., 2017; Le Manach et al., 2018). Expanding on this gap in *Microcystis* research, I compared the cytotoxicity of extracellular metabolites derived from microcystin-producing and non-producing *M. aeruginosa* strains on a breadth of rainbow trout tissue-derived cell lines. My results indicate that the exudates of these "Non-Toxic" strains have the potential to induce significant cellular damage or disruption, particularly in sensitive cell lines derived from tissues of the brain, gills, and milt of rainbow trout. I found that in the cell lines RTBrain, RTgill-W1, and RT-milt5 (and to a lesser extent, RTP-2), fluorescence of the AlamarBlue viability dye was significantly reduced in both "Toxic" and "Non-Toxic" treatments, indicating disruption of normal cellular metabolic functioning, and the cytotoxicity of these treatments overlapped with each other to varying degrees (**Figure 2.4** and **Figure 2.6**).

The potential neurotoxicity of *M. aeruginosa* cultures has been highlighted in previous studies with live zebrafish (*Danio rerio*), particularly during vulnerable early life stages (Qian et

al., 2018) in which cyanobacterial cultures or their compounds altered locomotive behaviour and disrupted genetic and proteomic biomarkers of neurological development and function (Qian et al., 2018; Yu et al., 2021). Using embryos of the endangered freshwater fish species *Sinocyclocheilus grahami,* Cai et al., (2022) found that the exudates of a toxic *M. aeruginosa* strain also induced significant neurotoxicity – impairing development, disrupting neurotransmitter levels, and significantly altering normal neurobehaviour. In my study, sensitivity of RTBrain cells to exudates of both microcystin- producing and non-producing strains (**Figure 2.5.B**) could indicate the presence of other neurotoxic compounds among *M. aeruginosa* metabolites in the absence of microcystins – which negatively affect neurological function in zebrafish (Wu et al., 2016). Cyanopeptolins, for example, are putatively neurotoxic (Faltermann et al., 2014), and have been produced previously in lab cultures of "non-toxic" *M. aeruginosa* strains (Sotton et al., 2017; Le Manach et al., 2018). More work is needed to identify the specific compounds involved and how these cellular disruptions may translate into more concerning neurological impairments; however, this represents one of the first studies to identify the cytotoxic effects of *M. aeruginosa* exudates in fish using brain tissue-derived cells and adds to the limited number of studies exploring the potential neurotoxicity of these exudates, alongside the *S. grahami* study of Cai et al., (2022) and one other study using the nematode, *Caernorhabditis elegans* (Ju et al., 2014).

As with my RTBrain cell line, I found that exposure to exudates of both "Toxic" and "Non-Toxic" strains of *M. aeruingosa* significantly impacted the cellular metabolism of RTgill-W1 cells (**Figure 2.5.D**). The relative sensitivity of RTgill-W1 cells in this study aligns with previous comparisons between RTgill-W1, RTL-W1, and RTG-2 cell lines, in which gill cells appeared to be the most sensitive of tested lines to the cellular organic matter (primarily

intracellular metabolites) of stable *M. aeruginosa* cultures (Šrédlová et al., 2021). However, my results strongly diverge from those of Sorichetti et al. (2014) who did not find any significant cytotoxic effects in RTgill-W1 cells exposed to log phase *M. aeruginosa* exudates, instead finding strong toxicity of metabolites from the stable phase. While the differences between the results of this study and my own may be a consequence of the choice in the *M. aeruginosa* isolates used in each (CPCC 300 and CPCC 633 versus CPCC 124 and CPCC 299), it is possible this lack of significant response may be due to lower relative densities used in the 2014 study, although the differing methodologies used for measuring culture density between the two stymie such comparisons.

Numerous studies in freshwater tilapia (*Oreochromis spp.*) and their gill tissues have found significant cellular injury or impairment when exposed to *M. aeruginosa* and their metabolites. Dietary exposure to *M. aeruginosa* cells has been linked to histopathological damage to gill structures and increased oxidative stress (Preeti et al., 2016) and these findings are similarly reflected in experiments with immersion in cyanobacterial extracts (Abdel-Latif & Abou Khashaba, 2017). Studies using excised gill tissues found *M. aeruginosa* extracts disrupted of ion transport in the gills through the inhibition of Na^+/K^+-ATPase activity and ATP-driven Ca^{2+} transport following treatment – a result that was not reflected in exposure to purified microcystins (Bury et al., 1996; Bury et al., 1998). Here, I can draw a similar conclusion as my "Non-Toxic" exudates still inflicted a significant effect on gill cells in the absence of MC-production. As such, impairment of ion-dependent physiological processes, damage to critical tissue architecture, and the potential subsequent loss of normal respiratory function places fish encountering freshwater blooms of *Microcystis* at significant risk and my results reinforce the need for assessments of this risk to consider metabolites beyond microcystins.

The RT-milt5 cell line used in this study was cultivated specifically from somatic cells found in rainbow trout milt, with the majority of sperm having been removed in the culture process (Vo et al., 2015). In addition to sperm cells, fish milt contains seminal fluid which appears to play a protective role both in external fertilization (Billard, 1983) and internal storage of spermatozoa (Nynca et al., 2014) as well as containing proteins associated with initiation of motility, maintenance of sperm quality, and metabolic support (Dietrich et al., 2014; Nynca et al., 2014). The sensitivity of the RT-milt5 line to *M. aeruginosa* exudates (**Figure 2.5.G**) suggests the potential for milt quality to be degraded during external fertilization in a bloom-contaminated environment, possibly leading to decreased fertilization success (Billard, 1983). Although, it remains unclear what the origin was for the source somatic cells of RT-milt5 (Vo et al., 2015) and with very little prior characterization work conducted so far, it is difficult to determine how closely it aligns with whole milt and the intersection between its functionalities and those of its *in vivo* counterparts.

Critically, however, *M. aeruginosa* exudates significantly decrease both sperm motility and lifespans in the bloom-affected freshwater fish *S. grahami* (Zi et al., 2018). With these injurious effects on sperm, along with the negative effects of MCs on gonadal tissues and sperm maturation (Trinchet et al., 2011; Liu et al., 2016) and the potential degradation of seminal fluid highlighted here, the quality of gametes and the supporting reproductive fluids of male fish could be significantly impacted by the presence of *Microcystis* blooms. Bloom peaks tend to occur in the summer and early fall (Michalak et al., 2013; Zhu et al., 2014; Pham et al., 2021), placing fall-spawning fish at greater risk of overlapping reproductive events with bloom toxins, especially with climate change expected to increase bloom duration (Pick, 2016; Huisman et al., 2018). With the use of cell lines derived from rainbow trout, my results are most relevant to

closely related salmonids with further research needed to confirm general toxicity across more distantly related species. However, fall-spawners in the Laurentian Great Lakes include multiple members of the Salmonidae family, such as Atlantic salmon, brown and lake trout, and multiple species of whitefish and cisco (Lane et al., 1996). Additionally, the overlap in cytotoxicity between the exudates of microcystin-producing and non-producing strains could indicate that cellular impairment is due to a non-MC compound to which milt cells are particularly sensitive.

In these gill and milt cell line assays, and several of my other cell lines, I also found that changes in cell viability appeared to be not only dependent on the strain of *M. aeruginosa* from which my exudate treatments were derived from, but the growth phase at which they were collected, particularly at higher tested concentrations/densities. In the RTgill-W1 cell line, for example, exudates from the stable phase of either strain had comparable cytotoxicity when measured with the AlamarBlue dye. For log phase exudates, this overlap varied with the method of comparison – in concentration-based analyses, TL treatments reduced viability to a degree not significantly different from either TS or NTS treatments (**Figure 2.5.D**), however when comparing based on relative density there appeared to be no difference in the negative effects of either NT treatment and the TS exudates, while the toxicity of TL exudates was significantly greater than all other treatments (**Figure 2.7.D**).

For RT-milt5 AlamarBlue assays, another NT-sensitive line, statistical differences between treatments were only evident at the highest concentrations tested, in which, similar to RTgill-W1, the effects of NTL exudates were significantly weaker than those of all other treatments (**Figure 2.5.G**) – a pattern that was repeated, albeit without significant differences, for both lower concentrations and for high relative density comparisons. In fact, there were no differences in the toxicity of treatments at either tested culture densities (**Figure 2.7.G**).

For all of the tested cell lines, I found that log phase exudates of the "Toxic" *M. aeruginosa* strain strongly impacted cell viability in both AlamarBlue and CFDA-AM assays to a degree equal to or greater than their stable phase counterparts. On the other hand, the opposite was true of the "Non-Toxic" treatments in which, in the few cases where log and stable phase treatments diverged (**Figure 2.5.D** and **G**), the stable phase exudates appeared to be more strongly cytotoxic. Wang et al. (2017) compared the toxicity of log, stable, and decline phase exudates and found that inhibitory effects of *M. aeruginosa* metabolites on green algae and diatoms were largely similar across the former two phases, consistent with my findings in the majority of my assays. However, these results appear to be at odds with the findings of Sorichetti et al. (2014), who found significant toxicity to the rainbow trout gill cell line from exudates of stable phase *M. aeruginosa* cultures but no detectable toxicity in those of the exponential phase. Differences in the methodologies used to harvest exudates, application of test solutions, or *M. aeruginosa* strains used may provide possible explanations for the divergences between the conclusions of this study and mine, however it is clear that further research is needed to better integrate growth phase-dependent toxicity into *in vitro* cyanobacterial risk assessments, particularly in regards to differences in toxicity between MC-producing and non-producing strains and cell line-specific sensitivity.

A general trend in my cytotoxicity assays was the apparent greater sensitivity of the AlamarBlue assay compared to CFDA-AM. In the latter assays, significant cytotoxicity was mostly confined to the highest tested concentrations of my "Toxic" strain exudates. As noted by Dayeh et al. (2013), differences between these assays may be a consequence of the nature of their fluorescent indicator dyes, which rely on oxidoreductases in the case of AB and an intact cell membrane (and non-specific esterases) in the case of CFDA-AM. It is possible that

impairment of cellular metabolism is a more sensitive endpoint, especially for testing with "Non-Toxic" exudates which appeared to have no *negative* effects on membrane integrity. Conversely, for some of my tested cell lines, exudate treatments resulted in increased fluorescence readings compared to the controls, potentially suggesting a positive or stimulatory effect on the assays' focal enzymes. For example, the RT-milt5, RTG-2, RTgill-W1, and RTHDF experienced increased CFDA-AM fluorescence compared to the controls for one or more exudate treatments (**Figure 2.9.C, D, E,** and **G**) and the same was true for RTHDF and RTL-W1 in my AlamarBlue assays (**Figure 2.5.E** and **F**).

2.4.2 Lysosomal activity in *O. mykiss* cell lines

Lysosomes are critical to maintaining cell health, for both normal cell functioning and cellular responses to injury, stress, and disease, by recycling cellular components and sequestering and degrading damaged or impaired organelles and proteins through a process called autophagy (Moore et al., 2008). The Neutral Red dye uptake assay is a common methodology for assessing the impact of toxicants on lysosomal function, and by extension cell viability, by evaluating the integrity of lysosomal membranes (Moore et al., 2008; Dayeh et al., 2013) and is the last in a battery of three tests recommended by Dayeh et al. (2013) for use in fish cell lines and the basis of my thesis methodology. However, during early optimization of my assays, I found that some of my cell lines became weakly adherent following treatment, particularly RTP-2, resulting in cells lifting free of the tissue culture surface and being lost during the addition and removal of multiple reagents during the NR process. Since this compromised the assay's ability to evaluate lysosomal effects the Neutral Red assay was substituted with my Lysotracker/NucBlue dye combination as previously described. Lysotracker dyes localize to acidic organelles, which include not only lysosomes, but also endosomes,

autophagosomes, and autolysosomes (Rodriguez-Enriquez et al., 2006), and all of which are associated with the cellular digestion of macromolecules – either intracellular cell components or internalized extracellular materials (Lenz et al., 2018).

In my assays, I found that exudates from my "Non-Toxic" strain produced a significant increase in lysosomal activity in the majority of tested cell lines, with their stimulatory effect on par with one or both "Toxic" exudates in each assay. Differences in the effects of the growth phases appears to be line-specific, with significantly greater lysosomal accumulation in the stable phase of "Toxic" exudates compared to those of the log phase for RTgill-W1 and RT-milt5 (**Figure 2.16.D** and **G**). For the "Non-Toxic" exudates, there was a significant difference between log and stable phase exudates in RTP-2 treated cells, however this difference disappeared when comparing treatments by density rather than concentration (**Figure 2.16.H** and **Figure 2.17.H**). These increases in Lysotracker fluorescence could indicate two potential cellular responses to *M. aeruginosa* exudate treatments: 1) an increase in autophagic vacuoles removing damaged or impaired organelles and cell components or 2) an increase in lysosomal activity as exudate components are internalized and degraded (Moore et al., 2008; Lenz et al., 2018). Autophagy may also degrade organelles to recycle their contents during stress, such as nutrient deprivation (Rodriguez-Enriquez et al., 2006). Previous studies have found that *M. aeruginosa* exudates can contain metabolites capable of stimulating autophagic responses (Zhou et al., 2023) or impairing autophagy and related organelle-recycling pathways (Li et al., 2022).

Based on the findings of my cytotoxicity assays, one interpretation of these results is that, while both strains of exudates induced a lysosomal response in tested cells, the combined cytotoxic effects of the compounds of the "Toxic" strain were sufficient to overwhelm autophagic processes and impair cellular function, leading to an 'adverse' accumulation of

lysosomes, as evidenced by decreased viability (Lenz et al., 2018). For example, the highest observed increase in lysosomal accumulation in my study occurred in my RTL-W1 cells treated with TL exudates at the highest tested concentrations and coincided with a significant decrease in cell viability in both cytotoxicity assays. At the next lowest concentration, neither lysosomal activity nor cell viability were significantly different from the controls.

2.4.3 Study limitations and conclusions

While my study offers significant findings regarding the comparative toxicity of *M. aeruginosa* exudates of different strains, more work is needed to elucidate the risks posed by these metabolites in the wild. RTBrain, RTgill-W1 and RT-milt5 cell lines all displayed pronounced sensitivity compared to the majority of tested cell lines, however, assays utilizing the latter two lines likely represent a closer approximation of *in vivo* exposure. Externally-oriented tissues, such as the gills, skin, and – to a certain extent – digestive tracts of fish, or cells released during reproduction, such as gametes and milt, are in more direct contact with the aquatic environment and thus any dissolved toxicants than their internal organ counterparts. However, in live fish these barrier tissues are sheathed in a mucosal layer that serves to protect them from injury or infection among other roles (Reverter et al., 2018) and, while there is some evidence that RTgill-W1 can be prompted to develop mucus-secreting goblet cells (Lee et al., 2009), I did not find any evidence of mucus production in my cultured cells, leaving these cell lines potentially at greater risk of the harmful effects of *Microcystis* metabolites than corresponding *in vivo* tissues.

Comparing my treatments to field conditions, the lower treatment concentrations (0.5X and 1X) overlap with previously recorded high-density *Microcystis* spp.-dominated blooms – between 1.92 and 7.64 x 10^7 cells/mL in freshwater reservoirs and estuaries (Lehman et al.,

2010; Javůrek et al., 2015; Pham et al., 2021). These measurements often consolidate *Microcystis* spp., although when bloom contents are defined *M. aeruginosa* can reach densities of 1.23 x 10^7 cells/mL and 3.82 x 10^7 cells alone or in blooms with other *Microcystis* spp. in large freshwater reservoirs (Javůrek et al., 2015). Furthermore, *M. aeruginosa* densities as high as 1.3 x 10^8 cells/mL have been recorded in brackish estuaries where conditions of bloom consolidation are ideal (Atkins et al., 2001). However, while strong correlations have been identified between whole fish and cell line testing in isolated pollutants and moderate predictive capabilities in mixed effluent solutions, fish cell lines are often less sensitive than their live animal counterparts, necessitating in the inclusion of higher density treatments as well to better encapsulate cell responses (Schirmer, 2006). Studies of this kind would greatly benefit from further analysis of the contents of *M. aeruginosa* exudates as isolation of these compounds would facilitate both examination of individual toxicity and their toxicokinetics as well as directing improvements to the veracity of *in vitro* fish cell line assays with cyanobacterial materials. Schirmer (2006) recommended in their proposal to improve vertebrate cell cultures as a substitute for *in vivo* testing, that sensitivity can be improved by aligning cell line selection with the known or potential mechanisms of toxicity of the tested compounds – for example, lack of OATPs in the RTL-W1 line renders it insensitive to microcystins (Boaru et al., 2006). Thus, the results of my liver cell line assays may be the result of non-microcystin metabolites produced by my "Toxic" strain. My cell lines were chosen to encompass as many potential targets of adverse effects as possible, however, advancements in both my understanding of the toxic mechanisms of cyanobacterial metabolites as well as further characterization of known and novel cell lines will improve this field of research immensely.

Overall, my results indicate that in the absence of microcystin production, non-toxigenic strains of *M. aeruginosa* are capable of producing extracellular metabolites that are toxic to sensitive fish cell lines, and call into question the validity of the 'non-toxic' label. I also found that both strains can express varying levels of growth phase-dependent toxicity, with greater negative effects in the log phase for "Toxic" strains and in the stable phase for "Non-Toxic." Both of these factors should be considered in future risk assessments of *Microcystis*-dominated blooms to avoid underestimating the impact they may have on affected aquatic organisms, including fish.

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Table 2.1: Cell lines provided by the University of Waterloo and if applicable, the associated study in which they were cultivated. FBS % refers to the percentage of fetal bovine serum supplemented in the growth media needed to maintain healthy cultures.

Cell line	Tissue Origin	Morphology	FBS %	Reference
RBT4BA	Heart (bulbus arteriosus)	Epithelial/Fibroblastic	15	Provided by B. Dixon & N.T.K. Vo
RTBrain	Brain	Astroglial-like	15	Vo et al., 2014
RTG-2	Gonads	Fibroblastic	10	Wolf & Quimby, 1962
RTgill-W1	Gill	Epithelial	10	Bols et al., 1994
RTHDF	Skin	Fibroblastic	10	Ossum et al., 2004
RTL-W1	Liver	Epithelial	10	Lee et al., 1993
RT-milt5	Milt	Fibroblastic	10	Vo et al., 2015
RTP-2	Pituitary	Epithelial	10	Bols et al., 1995

Table 2.2: Equivalent cell densities in cells per mL for each exudate treatment of Toxic and Non-Toxic *M. aeruginosa.* Equivalent densities are based on cell counts taken immediately prior to the removal of cells via centrifugation and filtration.

Treatment	Concentration	Equivalent density (cells/mL)	Relative density category
TL	**0.5X** *(2.5% v/v)*	2.3×10^7	Low
	1X (*5% v/v)*	4.6×10^7	Medium
	2X *(10% v/v)*	9.2×10^7	High
TS	**0.5X** *(2.5% v/v)*	4.9×10^7	Medium
	1X (*5% v/v)*	9.8×10^7	High
	2X *(10% v/v)*	19.6×10^7	Very High
NTL	**0.5X** *(2.5% v/v)*	2.3×10^7	Low
	1X (*5% v/v)*	4.5×10^7	Medium
	2X *(10% v/v)*	9.0×10^7	High
NTS	**0.5X** *(2.5% v/v)*	4.3×10^7	Medium
	1X (*5% v/v)*	8.5×10^7	High
	2X *(10% v/v)*	17.1×10^7	Very High

Table 2.3: Summary of three-way ANOVA examining the effects of exudates' strain type, growth phase stage, and concentration (0.5X, 1X, and 2X) on the cell viability of rainbow trout cell lines as measured by AlamarBlue and CFDA-AM.

Cell Line	Source of Variation	AlamarBlue		CFDA-AM	
		% Total variation	*P value*	*% Total variation*	*P value*
RBT4BA					
	Concentration	**34.48**	**<0.0001**	**25.13**	**<0.0001**
	Strain	**34.14**	**<0.0001**	**24.32**	**<0.0001**
	Growth Phase	0.08619	0.6643	0.5231	0.3794
	Concentration x Strain	**13.30**	**<0.0001**	**23.60**	**<0.0001**
	Concentration x Growth Phase	0.8033	0.4186	1.194	0.4140
	Strain x Growth Phase	0.7271	0.2119	0.5761	0.3565
	Concentration x Strain x Growth Phase	0.2452	0.7632	0.8876	0.5170
RTBrain					
	Concentration	**29.48**	**0.0001**	1.447	0.6719
	Strain	**34.26**	**<0.0001**	**21.86**	**0.0019**
	Growth Phase	0.06263	0.8150	**9.907**	**0.0275**
	Concentration x Strain	5.398	0.1111	**12.87**	**0.0437**
	Concentration x Growth Phase	1.385	0.5469	6.497	0.1852
	Strain x Growth Phase	1.822	0.2142	1.632	0.3494
	Concentration x Strain x Growth Phase	0.7276	0.7256	2.727	0.4780
RTG-2					
	Concentration	**47.51**	**<0.0001**	**2.868**	**0.0056**
	Strain	**17.16**	**0.0007**	**29.40**	**<0.0001**
	Growth Phase	1.148	0.3049	**12.21**	**<0.0001**
	Concentration x Strain	**15.58**	**0.0041**	**47.32**	**<0.0001**
	Concentration x Growth Phase	0.04945	0.9763	**5.419**	**0.0003**
	Strain x Growth Phase	1.187	0.2970	**3.310**	**0.0008**
	Concentration x Strain x Growth Phase	2.934	0.2664	**5.780**	**0.0002**
RTgill-W1					
	Concentration	**70.12**	**<0.0001**	**9.472**	**0.0005**
	Strain	**3.016**	**<0.0001**	**11.16**	**<0.0001**
	Growth Phase	**3.848**	**<0.0001**	0.2840	0.4709
	Concentration x Strain	**3.356**	**<0.0001**	**42.97**	**<0.0001**
	Concentration x Growth Phase	**5.498**	**<0.0001**	2.231	0.1356
	Strain x Growth Phase	**2.611**	**<0.0001**	0.8430	0.2162
	Concentration x Strain x Growth Phase	0.1539	0.4650	1.054	0.3824
RTHDF					
	Concentration	**47.91**	**<0.0001**	**36.81**	**<0.0001**
	Strain	3.828	0.0731	**12.17**	**0.0011**
	Growth Phase	**6.198**	**0.0249**	0.1045	0.7375
	Concentration x Strain	4.651	0.1406	**16.08**	**0.0011**
	Concentration x Growth Phase	5.314	0.1085	0.05201	0.9719
	Strain x Growth Phase	0.8400	0.3909	2.804	0.0907
	Concentration x Strain x Growth Phase	0.4413	0.8204	3.772	0.1457

Cell Line	Source of Variation	AlamarBlue		CFDA-AM	
		% Total variation	*P value*	*% Total variation*	*P value*
RTL-W1					
	Concentration	**37.70**	**<0.0001**	**9.928**	**0.0162**
	Strain	**13.71**	**<0.0001**	0.2257	0.6526
	Growth Phase	0.1470	0.6271	3.660	0.0745
	Concentration x Strain	**13.04**	**0.0002**	**12.04**	**0.0073**
	Concentration x Growth Phase	2.555	0.1364	**8.442**	**0.0287**
	Strain x Growth Phase	2.152	0.0675	3.636	0.0755
	Concentration x Strain x Growth Phase	1.171	0.3932	**8.358**	**0.0297**
RT-milt5					
	Concentration	**32.89**	**<0.0001**	**11.34**	**0.0063**
	Strain	2.408	0.1024	**12.68**	**0.0009**
	Growth Phase	**17.84**	**<0.0001**	**9.375**	**0.0036**
	Concentration x Strain	4.075	0.1073	**27.31**	**<0.0001**
	Concentration x Growth Phase	4.856	0.0721	1.456	0.4790
	Strain x Growth Phase	**5.999**	**0.0120**	0.4707	0.4903
	Concentration x Strain x Growth Phase	1.066	0.5426	2.490	0.2890
RTP-2					
	Concentration	3.395	0.4601	**19.11**	**0.0196**
	Strain	**15.94**	**0.0102**	2.717	0.2655
	Growth Phase	4.112	0.1748	2.007	0.3371
	Concentration x Strain	5.715	0.2766	1.093	0.7731
	Concentration x Growth Phase	2.836	0.5214	11.18	0.0878
	Strain x Growth Phase	4.152	0.1728	0.3559	0.6840
	Concentration x Strain x Growth Phase	0.9028	0.8104	4.781	0.3354

Table 2.4: Summary of three-way ANOVA examining the effects of exudates' strain type, growth phase stage, and relative culture density on the cell viability of rainbow trout cell lines as measured by AlamarBlue and CFDA-AM.

Cell Line	Source of Variation	AlamarBlue		CFDA-AM	
		% Total variation	*P value*	*% Total variation*	*P value*
RBT4BA					
	Density	**17.30**	**<0.0001**	**16.00**	**0.0005**
	Strain	**35.74**	**<0.0001**	**22.16**	**<0.0001**
	Growth Phase	**10.61**	**0.0010**	**11.39**	**0.0024**
	Density x Strain	**7.917**	**0.0036**	**13.04**	**0.0014**
	Density x Growth Phase	0.8166	0.3111	0.1197	0.7316
	Growth Phase x Strain	**9.013**	**0.0022**	**10.78**	**0.0031**
	Density x Phase x Strain	0.2999	0.5365	2.645	0.1159
RTBrain					
	Density	**18.59**	**0.0011**	**9.390**	**0.0336**
	Strain	**40.51**	**<0.0001**	**23.07**	**0.0023**
	Growth Phase	**8.809**	**0.0146**	**10.42**	**0.0263**
	Density x Strain	**5.408**	**0.0476**	**14.88**	**0.0100**
	Density x Growth Phase	0.1740	0.7055	0.1166	0.7979
	Growth Phase x Strain	**7.051**	**0.0262**	**8.212**	**0.0449**
	Density x Phase x Strain	0.6532	0.4668	3.934	0.1508
RTG-2					
	Density	**22.41**	**0.0032**	0.9127	0.0927
	Strain	**27.01**	**0.0017**	**28.77**	**<0.0001**
	Growth Phase	**11.68**	**0.0220**	**10.11**	**<0.0001**
	Density x Strain	**9.915**	**0.0324**	**21.77**	**<0.0001**
	Density x Growth Phase	1.356	0.3919	**8.646**	**0.0001**
	Growth Phase x Strain	**12.89**	**0.0171**	**25.53**	**<0.0001**
	Density x Phase x Strain	4.569	0.1280	**18.01**	**<0.0001**
RTgill-W1					
	Density	**54.81**	**<0.0001**	0.9694	0.4021
	Strain	**8.990**	**<0.0001**	**8.925**	**0.0142**
	Growth Phase	**18.39**	**<0.0001**	5.040	0.0608
	Density x Strain	**1.617**	**0.0326**	**24.32**	**0.0001**
	Density x Growth Phase	0.07517	0.6351	0.0001439	0.9918
	Growth Phase x Strain	**14.77**	**<0.0001**	**17.18**	**0.0010**
	Density x Phase x Strain	0.2974	0.3474	0.7934	0.4481
RTHDF					
	Density	**19.49**	**0.0021**	**17.38**	**0.0135**
	Strain	4.461	0.1045	4.850	0.1654
	Growth Phase	**26.05**	**0.0006**	**22.61**	**0.0059**
	Density x Strain	1.494	0.3362	4.341	0.1882
	Density x Growth Phase	**19.54**	**0.0021**	9.242	0.0613
	Growth Phase x Strain	3.623	0.1409	1.416	0.4448
	Density x Phase x Strain	0.04538	0.8653	0.06302	0.8709

Cell Line	Source of Variation	AlamarBlue		CFDA-AM	
		% Total variation	*P value*	*% Total variation*	*P value*
RTL-W1					
	Density	**20.39**	**<0.0001**	0.02621	0.8774
	Strain	**14.09**	**0.0003**	1.371	0.2694
	Growth Phase	**10.06**	**0.0019**	**5.055**	**0.0386**
	Density x Strain	**9.989**	**0.0020**	**10.62**	**0.0038**
	Density x Growth Phase	1.491	0.2016	**23.76**	**<0.0001**
	Growth Phase x Strain	**11.27**	**0.0011**	**8.533**	**0.0086**
	Density x Phase x Strain	**4.641**	**0.0281**	**14.62**	**0.0009**
RT-milt5					
	Density	**13.61**	**0.0146**	1.115	0.4108
	Strain	**9.486**	**0.0378**	**19.40**	**0.0019**
	Growth Phase	0.4735	0.6277	2.415	0.2299
	Density x Strain	2.539	0.2665	**20.76**	**0.0014**
	Density x Growth Phase	6.414	0.0831	3.831	0.1338
	Growth Phase x Strain	**20.37**	**0.0036**	**13.45**	**0.0077**
	Density x Phase x Strain	0.02881	0.9046	0.8428	0.4737
RTP-2					
	Density	0.2457	0.7779	7.798	0.1279
	Strain	12.38	0.0549	0.02288	0.9321
	Growth Phase	3.070	0.3238	**16.05**	**0.0343**
	Density x Strain	5.195	0.2027	0.05736	0.8926
	Density x Growth Phase	7.811	0.1217	**19.51**	**0.0212**
	Growth Phase x Strain	8.738	0.1027	0.4058	0.7200
	Density x Phase x Strain	0.7927	0.6131	2.663	0.3633

Table 2.5: Summary of three-way ANOVA examining the effects of exudates' strain type, growth phase stage, and concentration (1X and 2X) on the lysosomal activity of rainbow trout cell lines RTG-2, RTgill-W1, and RT-milt5.

Cell Line	**Source of Variation**	***% Total variation***	***P value***
RTG-2	Concentration	14.78	0.0995
	Strain	0.4430	0.7653
	Growth Phase	6.918	0.2483
	Concentration x Strain	0.05717	0.9145
	Concentration x Growth Phase	2.933	0.4463
	Strain x Growth Phase	0.04013	0.9283
	Concentration x Strain x Growth Phase	2.003	0.5278
RTgill-W1	Concentration	1.471	0.3060
	Strain	**5.981**	**0.0452**
	Growth Phase	**41.35**	**<0.0001**
	Concentration x Strain	**10.29**	**0.0105**
	Concentration x Growth Phase	1.410	0.3159
	Strain x Growth Phase	**17.27**	**0.0014**
	Concentration x Strain x Growth Phase	1.716	0.2697
RT-milt5	Concentration	0.7234	0.3593
	Strain	**24.56**	**<0.0001**
	Growth Phase	**30.11**	**<0.0001**
	Concentration x Strain	0.04280	0.8227
	Concentration x Growth Phase	0.9771	0.2876
	Strain x Growth Phase	**8.523**	**0.0028**
	Concentration x Strain x Growth Phase	1.421	0.2011

Table 2.6: Summary of two-way ANOVA examining the effects of exudates' strain type and growth phase stage on lysosomal activity in rainbow trout cell line RTHDF at tested concentrations of 1X and 2X.

Cell Line	Source of Variation	*1X*		*2X*	
		% Total variation	***P value***	***% Total variation***	***P value***
RTHDF	Strain	0.3150	0.7856	0.1533	0.8867
	Growth Phase	**66.03**	**0.0036**	30.77	0.0706
	Strain x Growth Phase	1.801	0.5201	12.42	0.2220

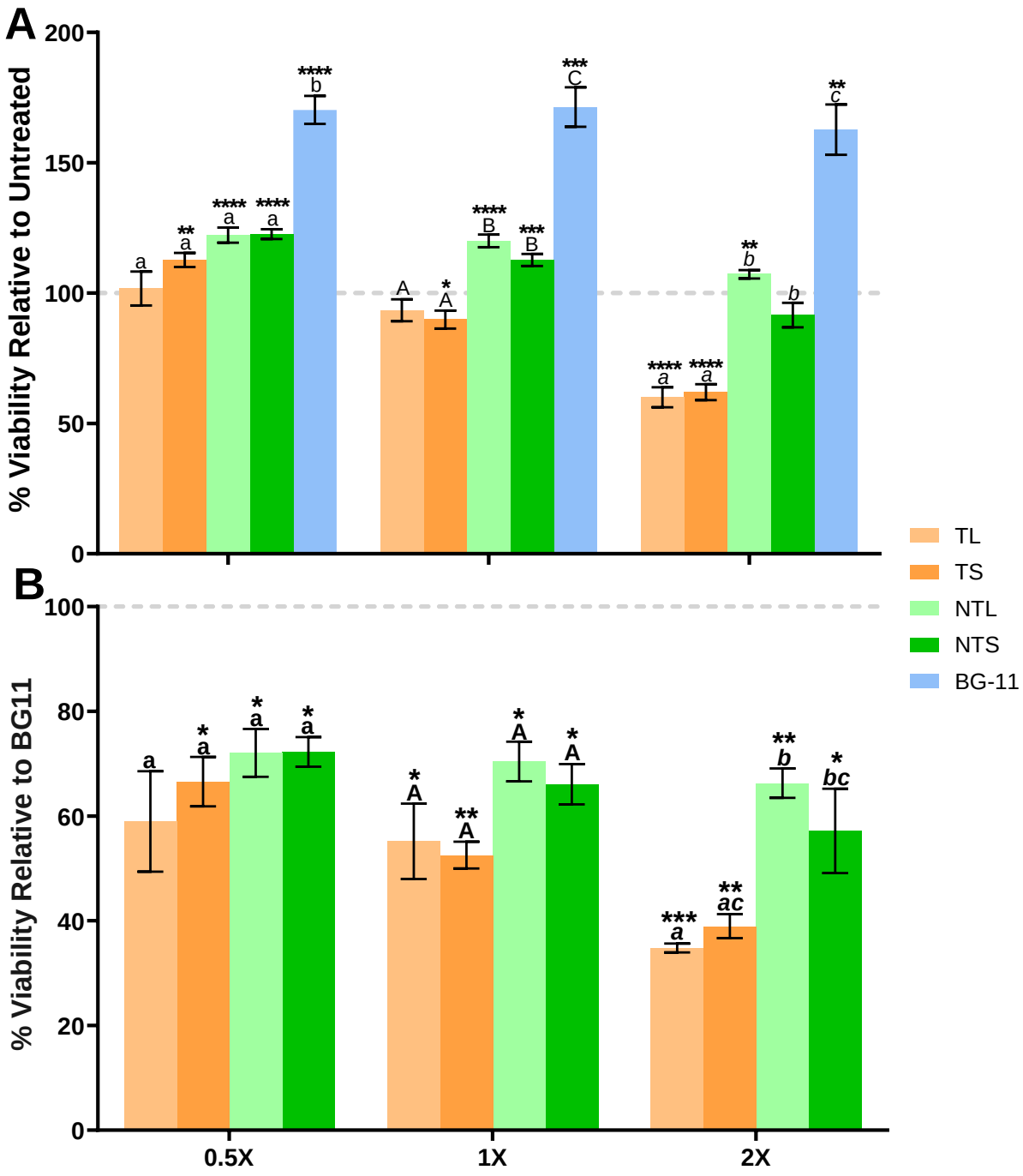

Figure 2.1: Mean (± standard error of the mean (SEM)) cell viability values for RTBrain cells compared as percent viability of Untreated cells (A) and BG-11 cells (B). Significant differences between treatments and respective controls are indicated with asterisks (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001) and significant differences between treatments within each concentration are indicated by different letters.

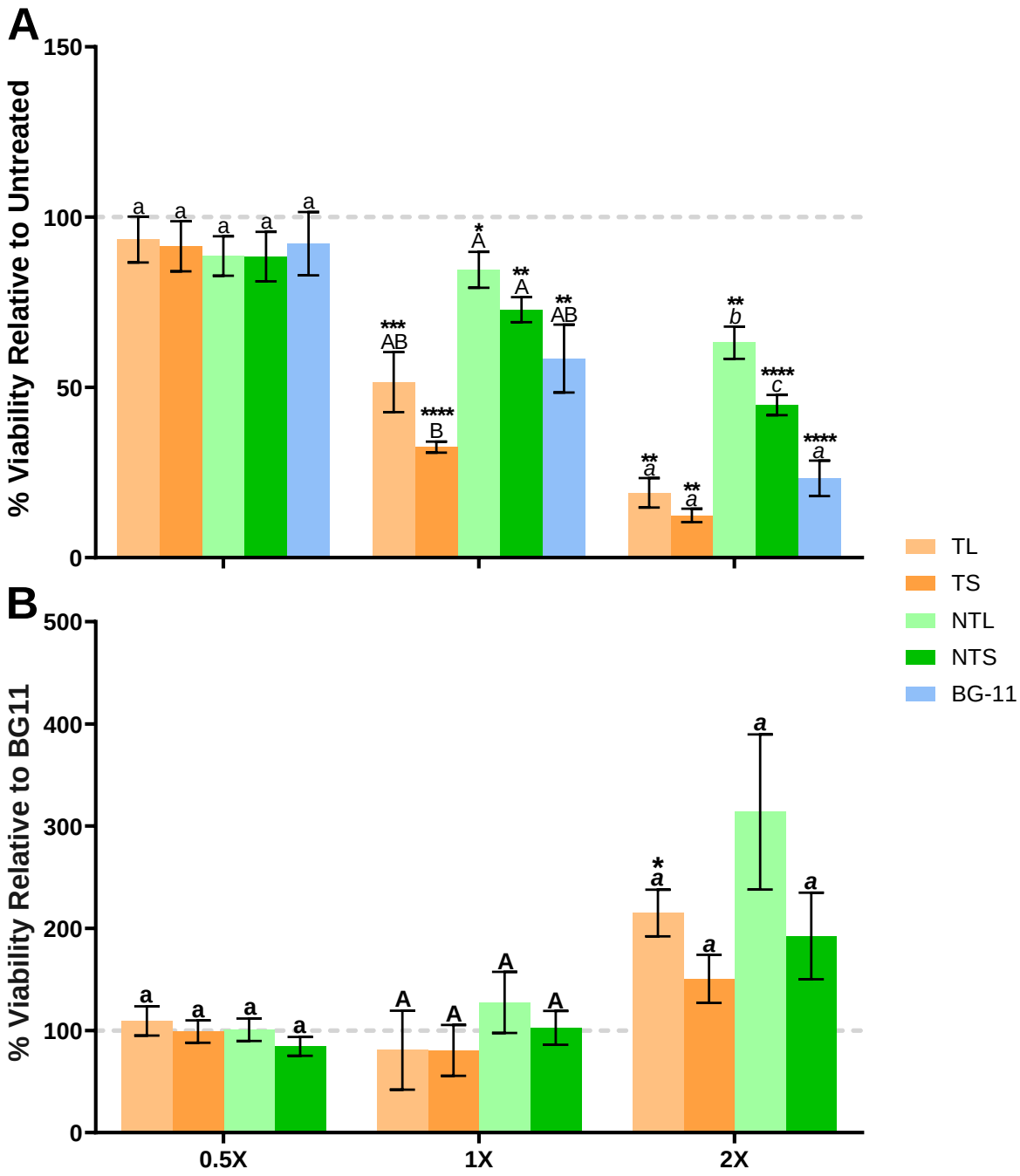

Figure 2.2: Mean (± SEM) cell viability values for RTHDF cells compared as percent viability of Untreated cells (**A**) and BG-11 cells (**B**). Significant differences between treatments and respective controls are indicated with asterisks (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001) and significant differences between treatments within each concentration are indicated by different letters.

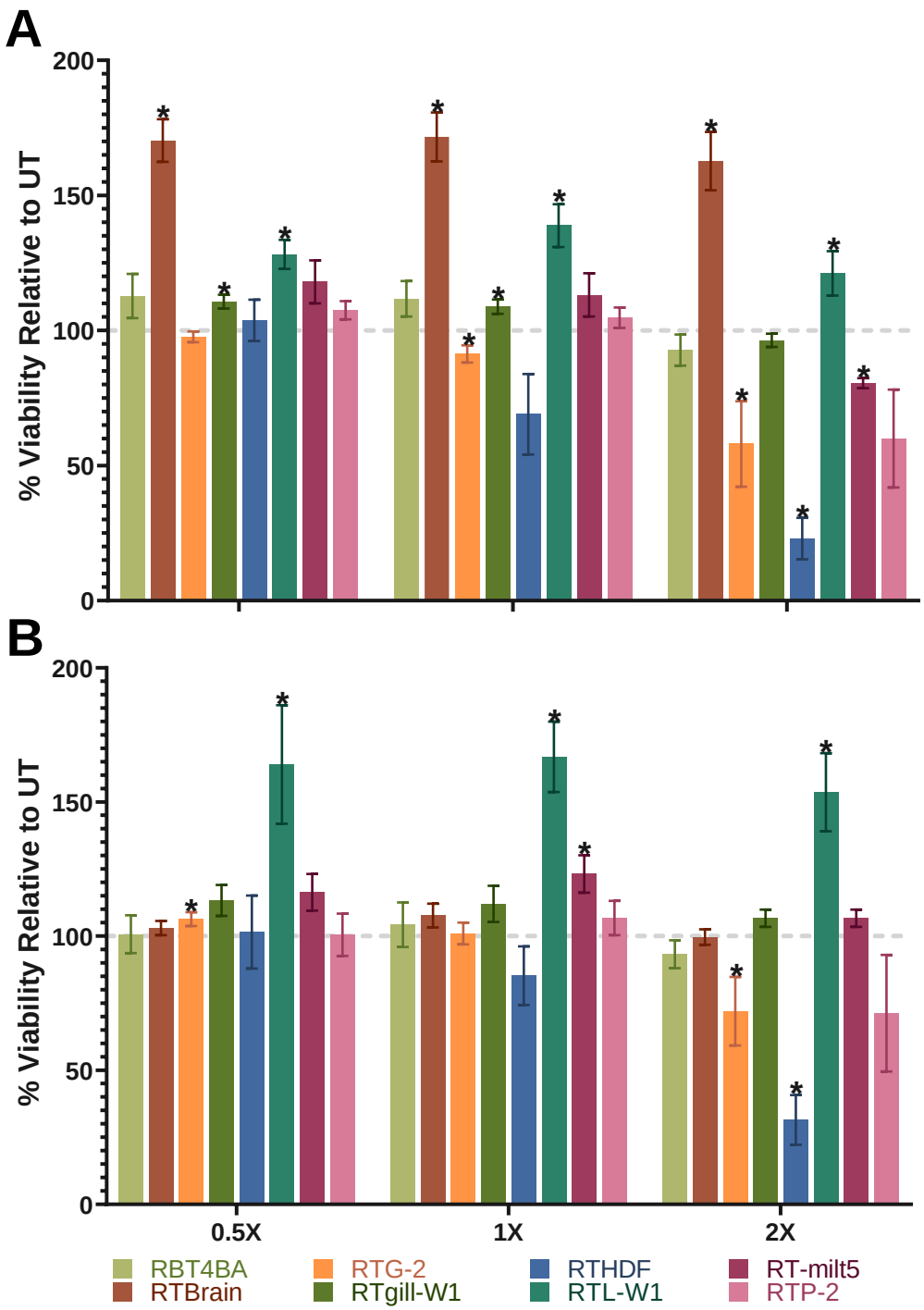

Figure 2.3: Comparisons between BG-11 treatments and untreated cells for each cell line (n = 3 to 6) for the cytotoxicity assays AlamarBlue (**A**) and CFDA-AM (**B**). Each experiment was conducted in triplicate and repeated on 3 to 6 96-well plates. Exposure periods were 24h. Values are presented as mean (± SEM) and significant differences ($P < 0.05$) from untreated are indicated by asterisks (*****).

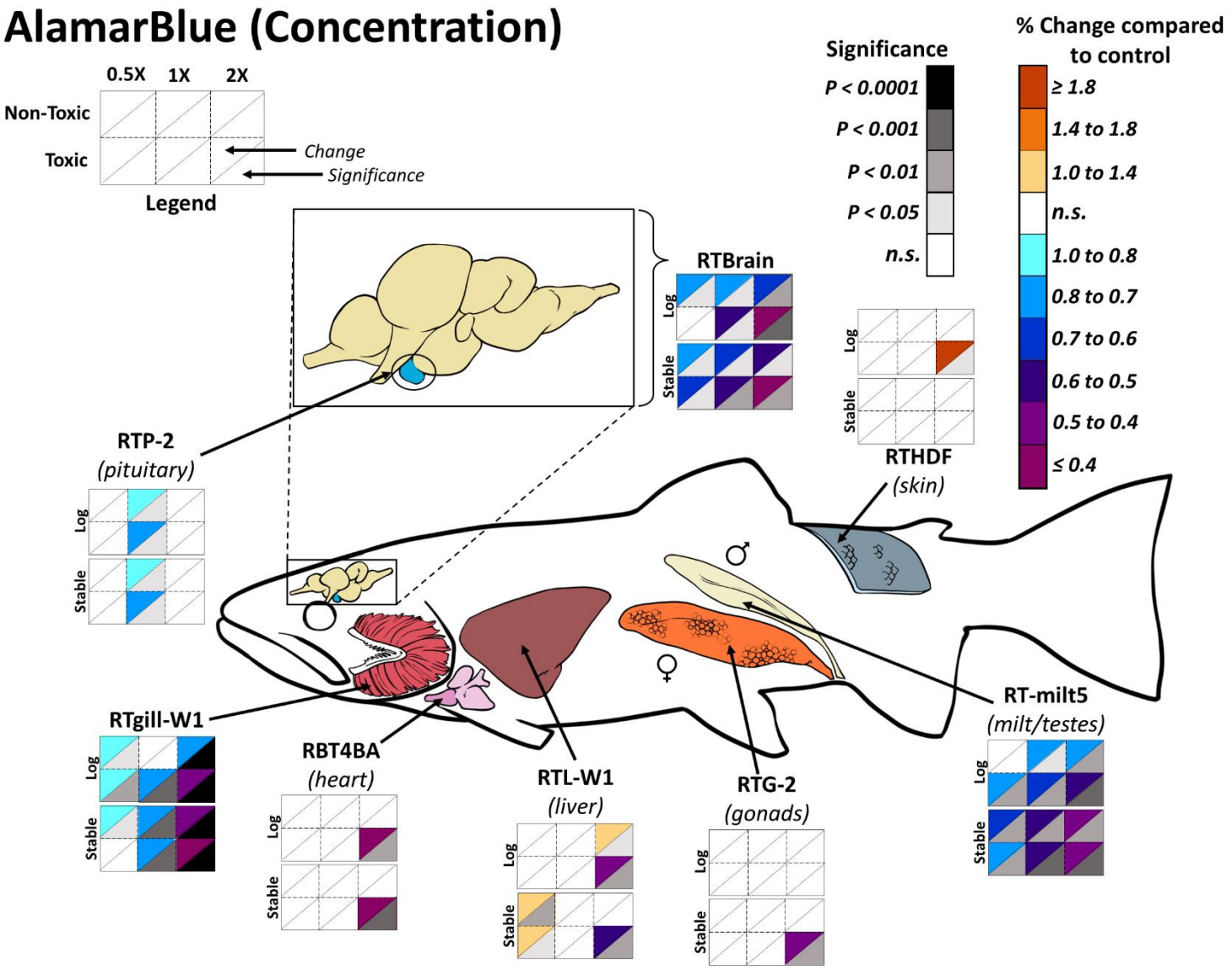

Figure 2.4: Summary of cell viability effects of exudate treatments on rainbow trout cell lines as measured by AlamarBlue and compared via exudate concentrations of 0.5X, 1X, and 2X.

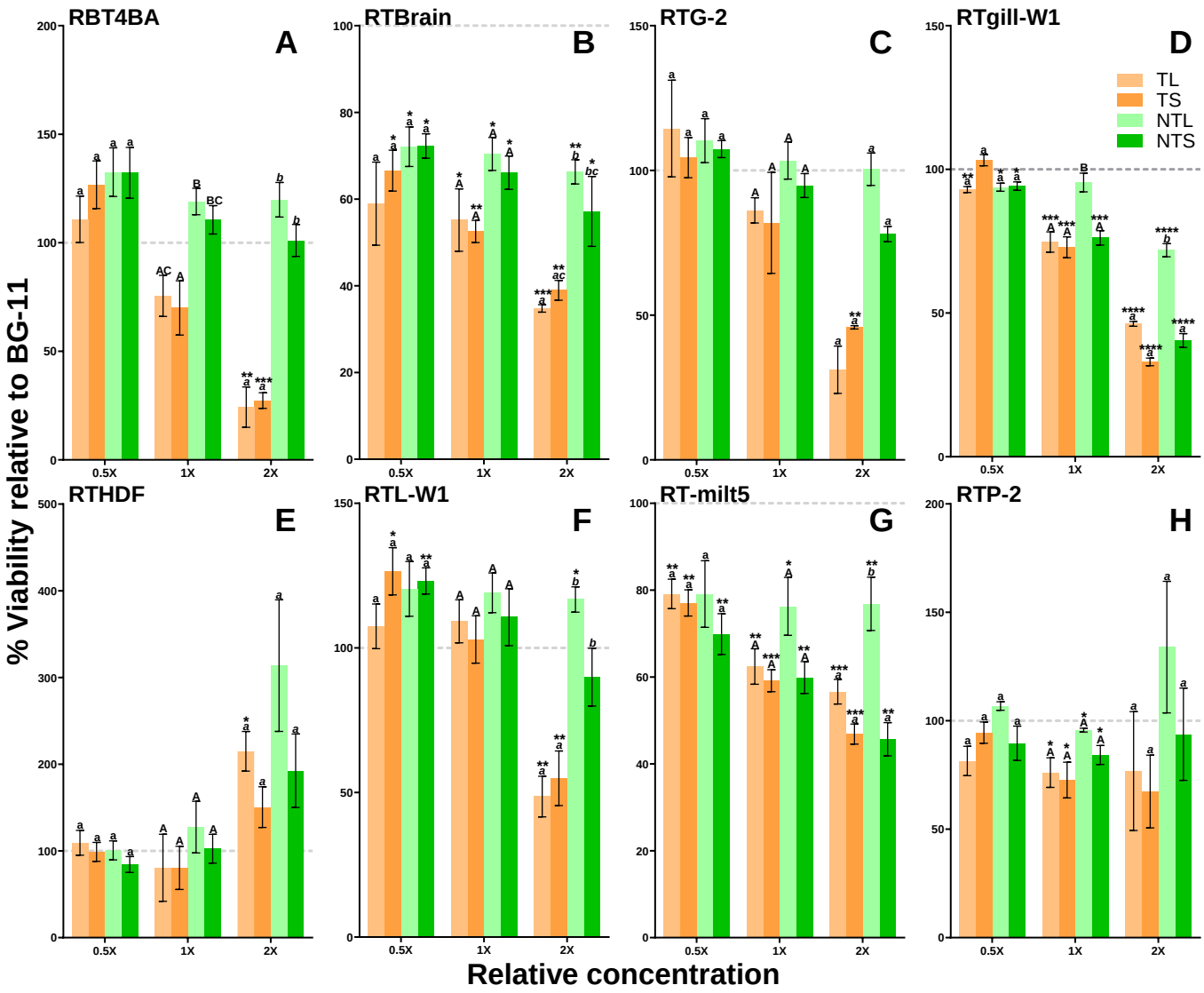

Figure 2.5: Mean (± SEM) differences in AlamarBlue cell viability between cell lines treated with TL, TS, NTL, and NTS exudate treatments at concentrations of 0.5X, 1X, and 2X. Significant differences between treatments and the BG-11 vehicle control are indicated with asterisks (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001) and significant differences between cell lines are indicated by different letters.

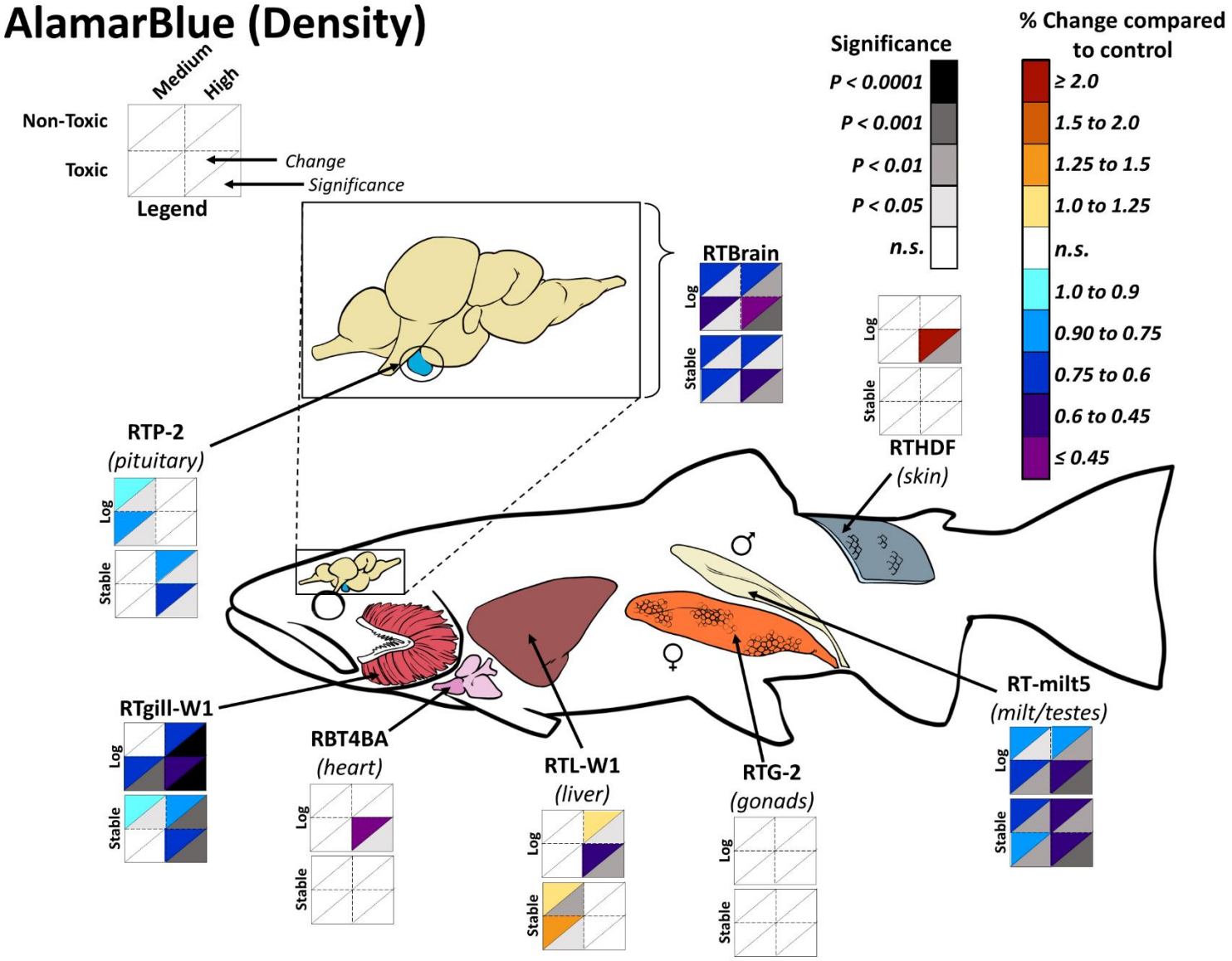

Figure 2.6: Summary of cell viability effects of exudate treatments on rainbow trout cell lines as measured by AlamarBlue and compared at medium and high relative culture densities.

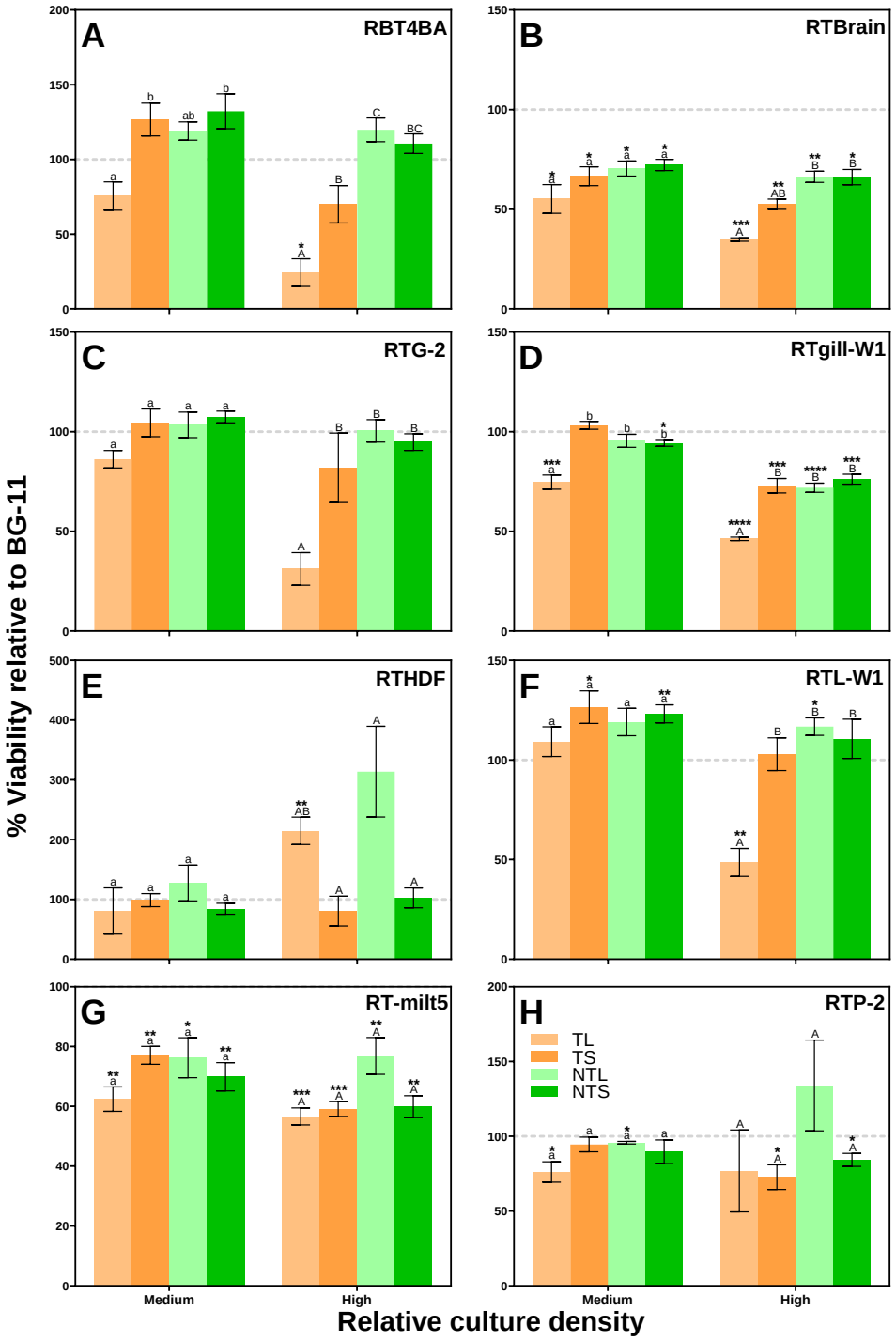

Figure 2.7: Mean (± SEM) differences in AlamarBlue cell viability between cell lines treated with TL, TS, NTL, and NTS exudate treatments at medium and high relative culture densities. Significant differences between treatments and the BG-11 vehicle control are indicated with asterisks (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001) and significant differences between cell lines are indicated by different letters.

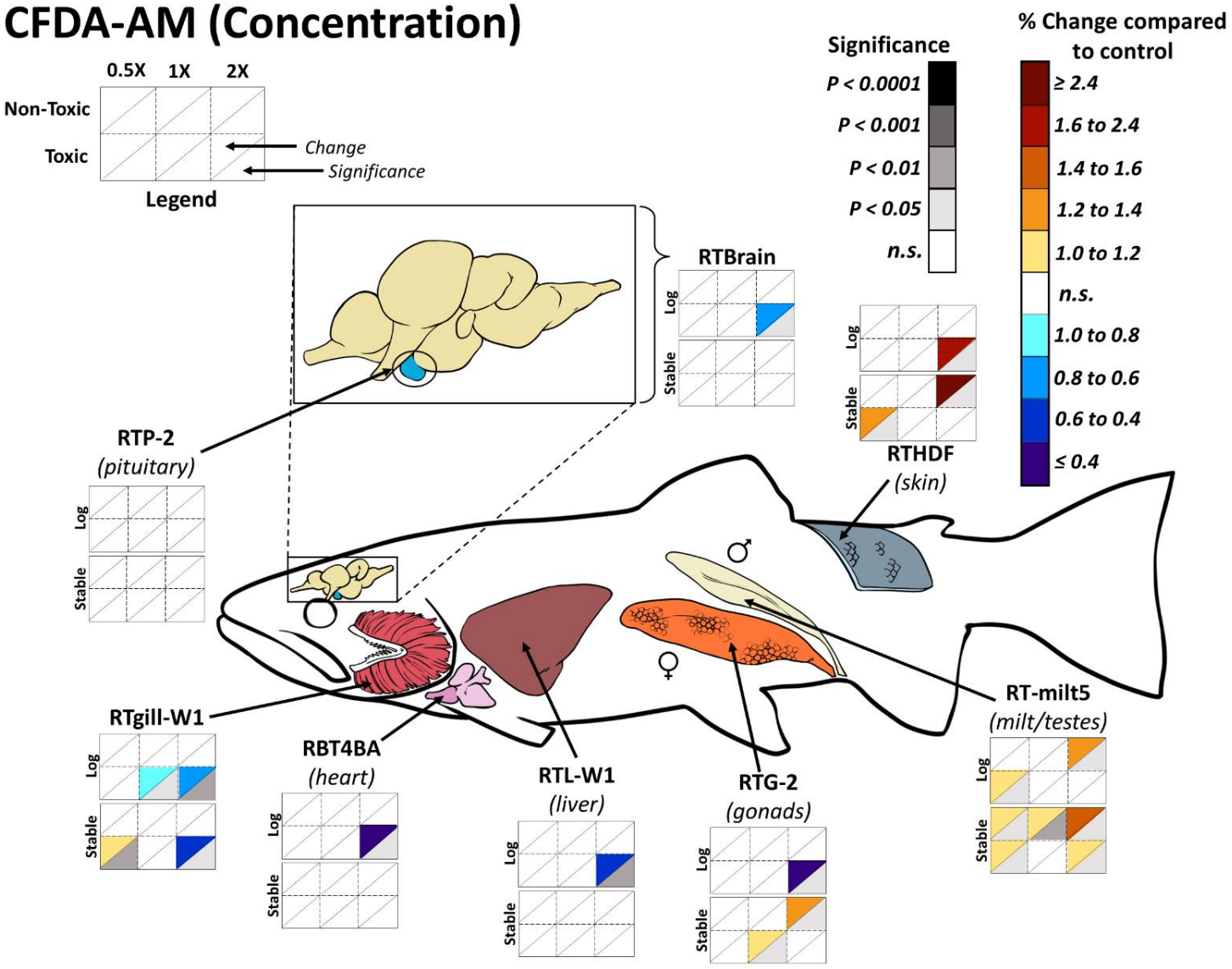

Figure 2.8: Summary of cell viability effects of exudate treatments on rainbow trout cell lines as measured by CFDA-AM and compared via exudate concentrations of 0.5X, 1X, and 2X.

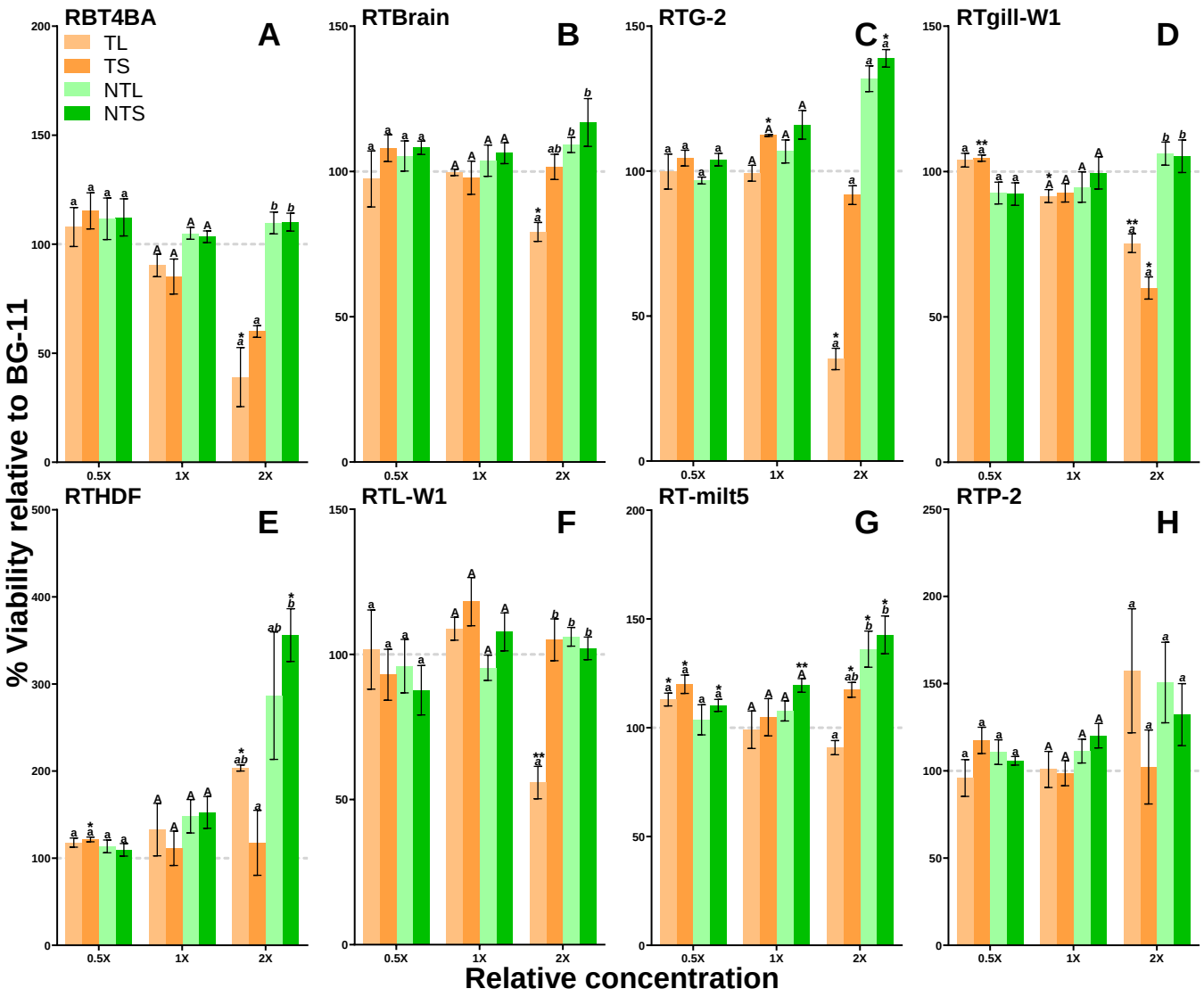

Figure 2.9: Mean (± SEM) differences in CFDA-AM cell viability between cell lines treated with TL, TS, NTL, and NTS exudate treatments at concentrations of 0.5X, 1X, and 2X. Significant differences between treatments and the BG-11 vehicle control are indicated with asterisks (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001) and significant differences between cell lines are indicated by different letters.

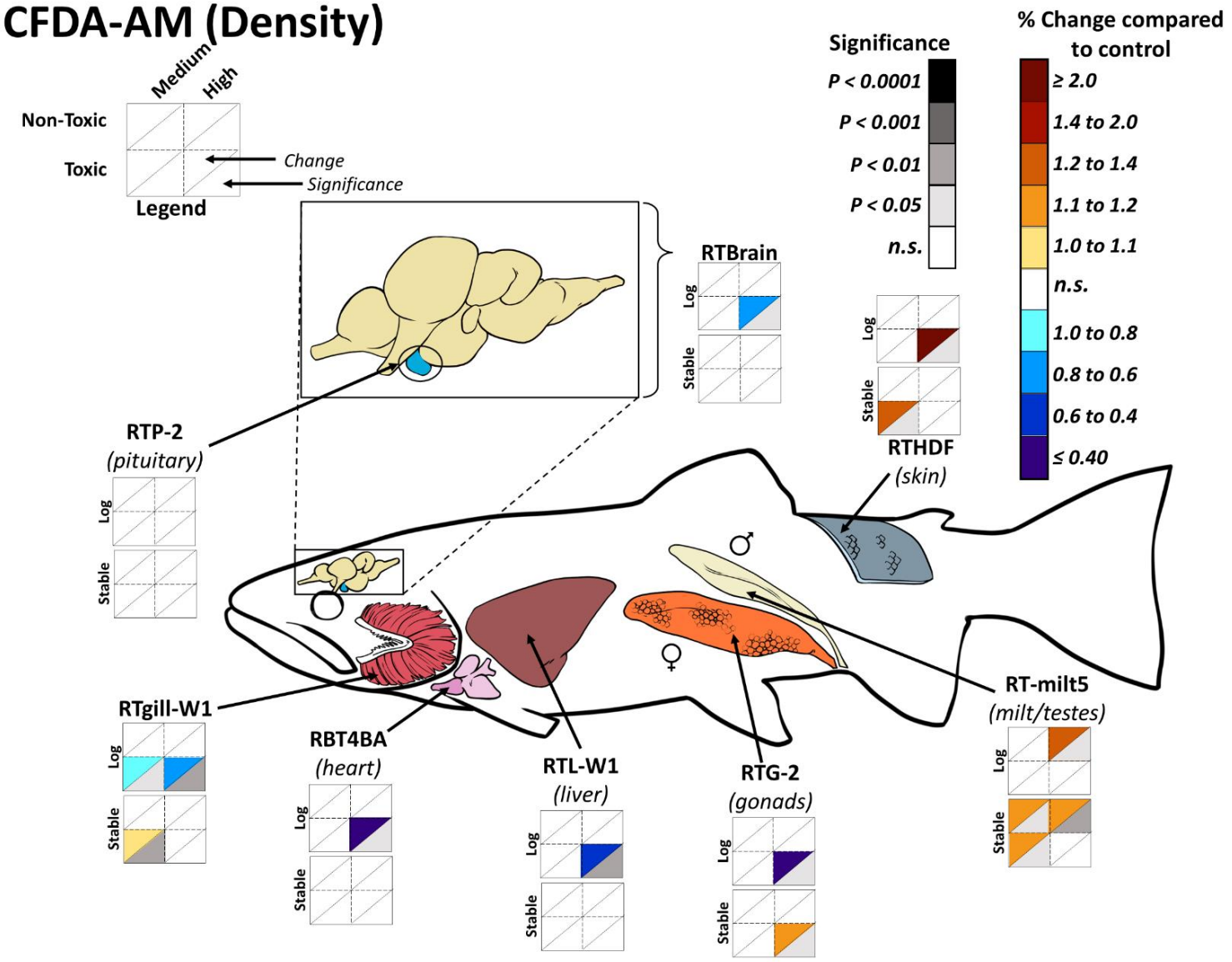

Figure 2.10: Summary of cell viability effects of exudate treatments on rainbow trout cell lines as measured by CFDA-AM and compared at medium and high relative culture densities.

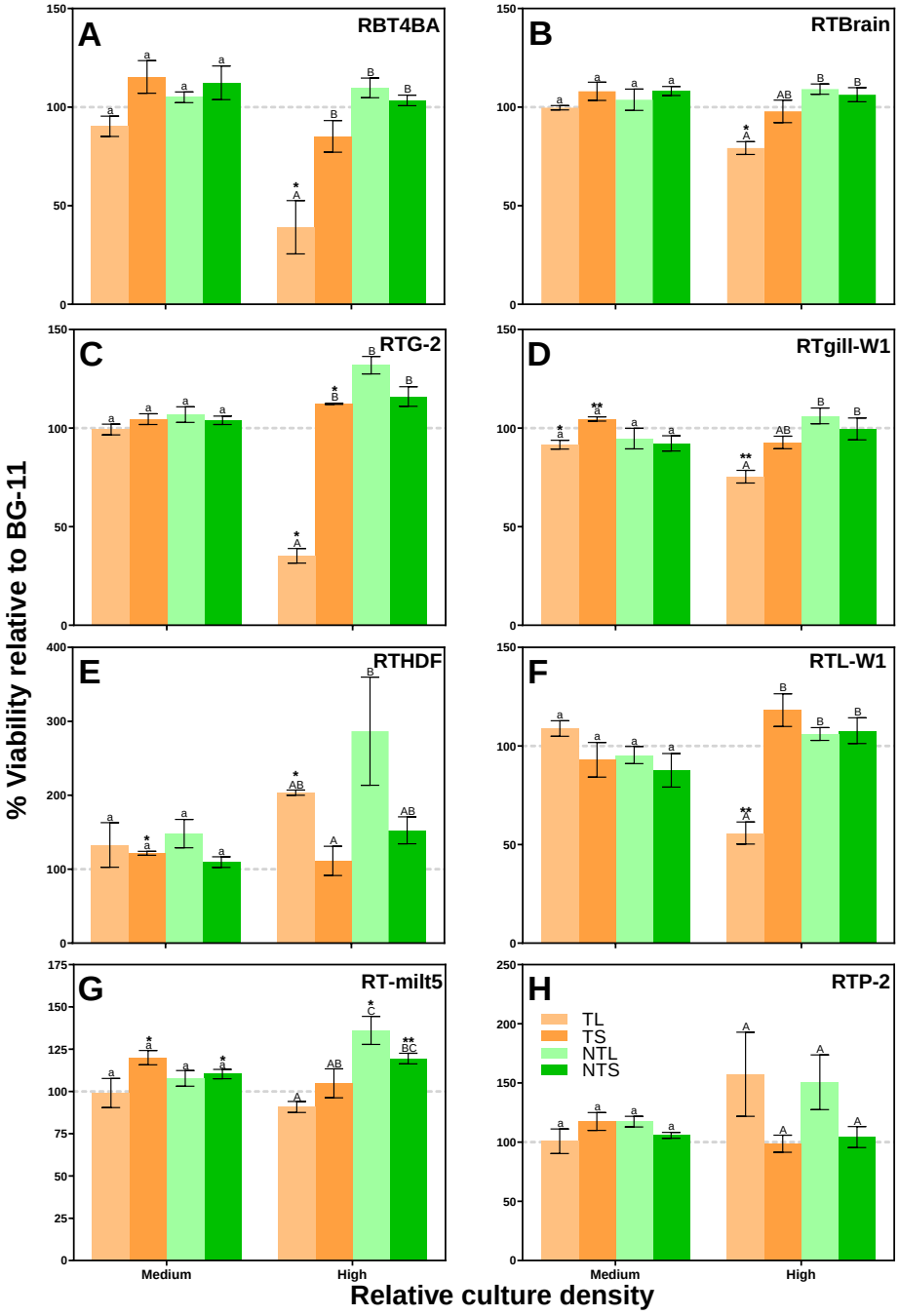

Figure 2.11: Mean (± SEM) differences in CFDA-AM cell viability between cell lines treated with TL, TS, NTL, and NTS exudate treatments at medium and high relative culture densities. Significant differences between treatments and the BG-11 vehicle control are indicated with asterisks (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001) and significant differences between cell lines are indicated by different letters.

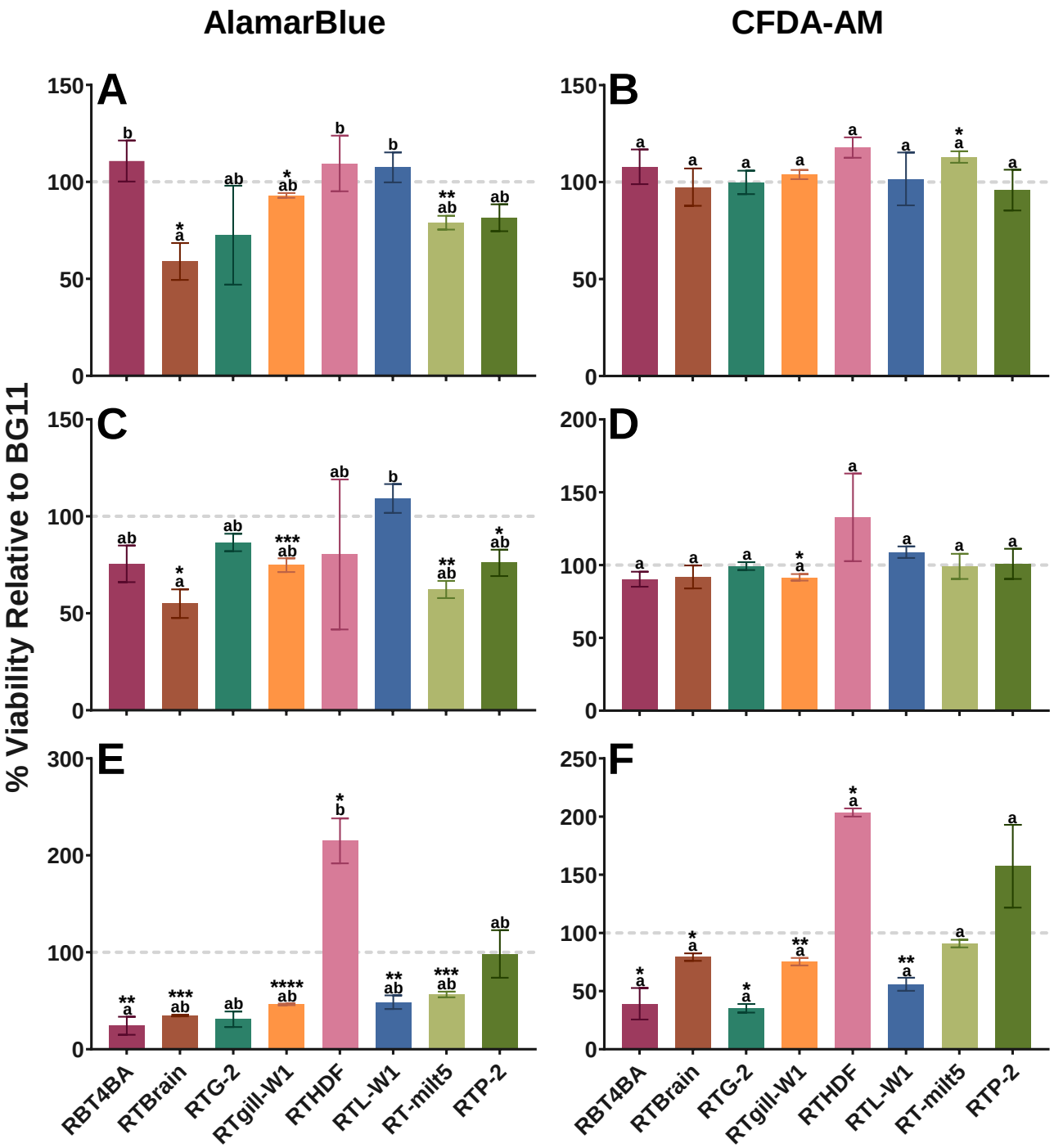

Figure 2.12: Mean (± SEM) differences in cell viability between cell lines treated with Toxic Log (**TL**) exudates at concentrations of 0.5X (**A, B**), 1X (**C, D**), and 2X (**E, F**) and measured using AlamarBlue and CFDA-AM. Significant differences between treatments and the BG-11 vehicle control are indicated with asterisks (*P < 0.05, **P < 0.01, ***P < 0.001) and significant differences between cell lines are indicated by different letters.

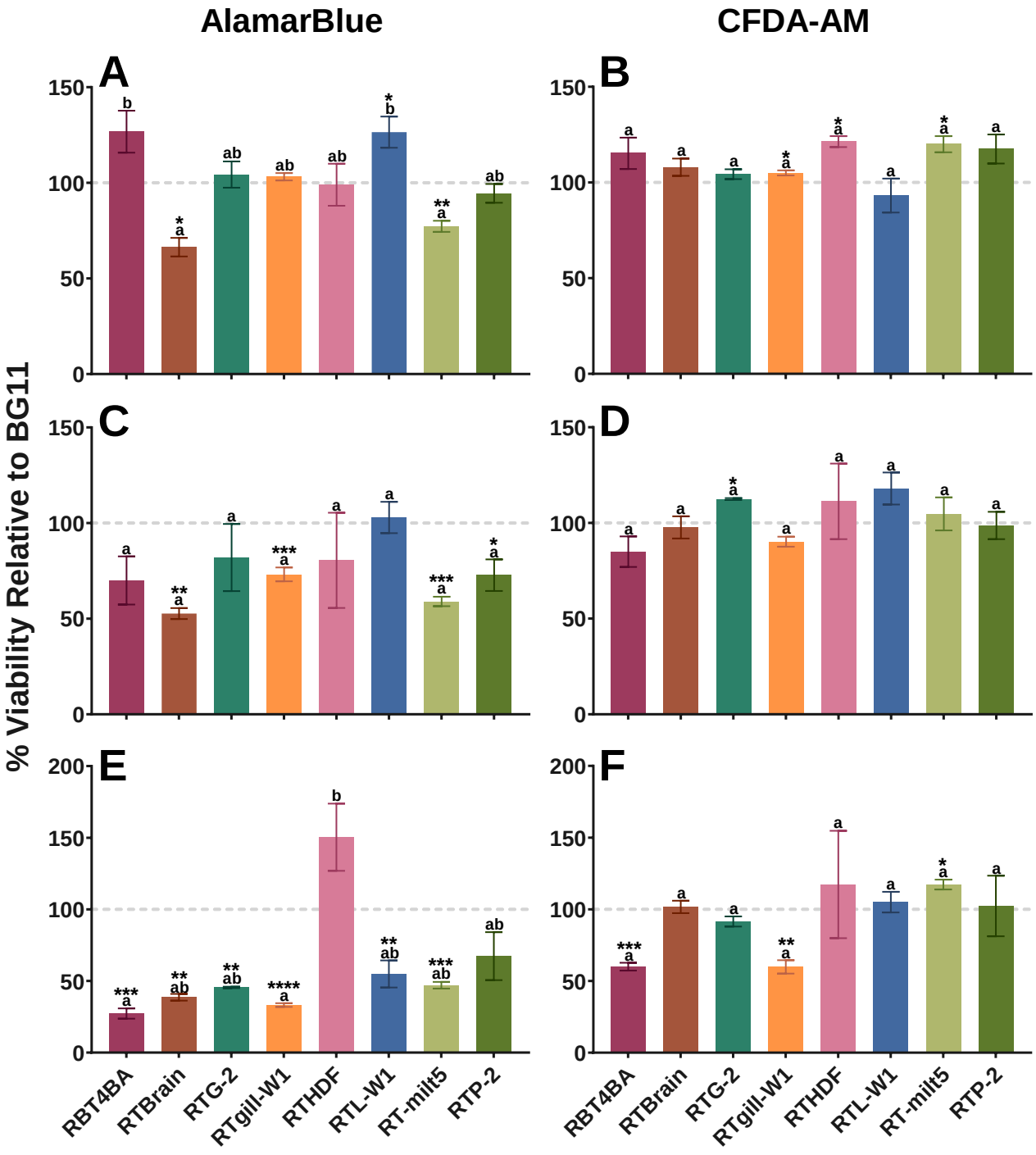

Figure 2.13: Mean (± SEM) differences in cell viability between cell lines treated with Toxic Stable (**TS**) exudates at concentrations of 0.5X (**A, B**), 1X (**C, D**), and 2X (**E, F**) and measured using AlamarBlue and CFDA-AM. Significant differences between treatments and the BG-11 vehicle control are indicated with asterisks (*P < 0.05, **P < 0.01, ***P < 0.001) and significant differences between cell lines are indicated by different letters.

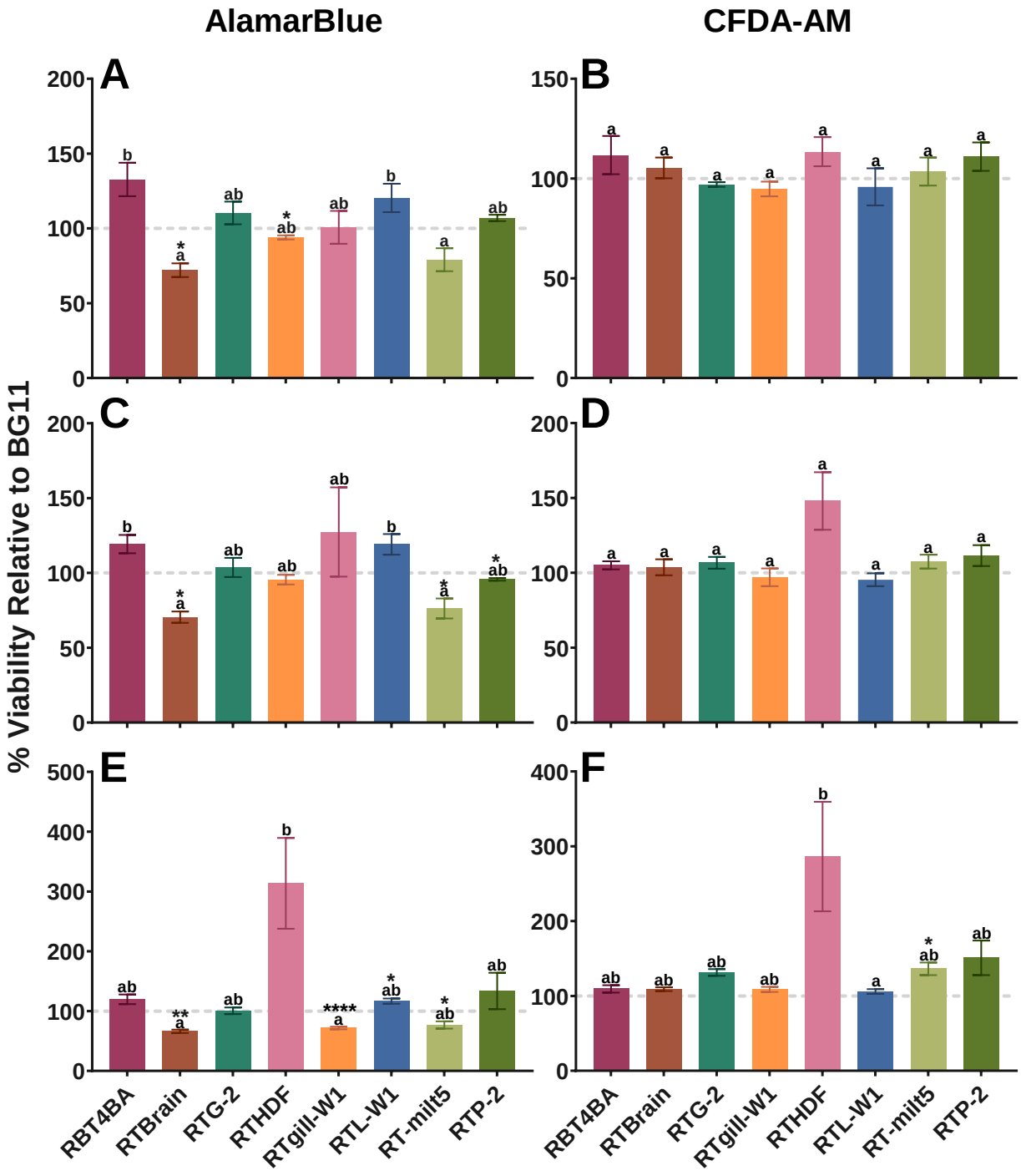

Figure 2.14: Mean (± SEM) differences in cell viability between cell lines treated with Non-Toxic Log (**NTL**) exudates at concentrations of 0.5X (**A, B**), 1X (**C, D**), and 2X (**E, F**) and measured using AlamarBlue and CFDA-AM. Significant differences between treatments and the BG-11 vehicle control are indicated with asterisks ($P < 0.05$) and significant differences between cell lines are indicated by different letters.

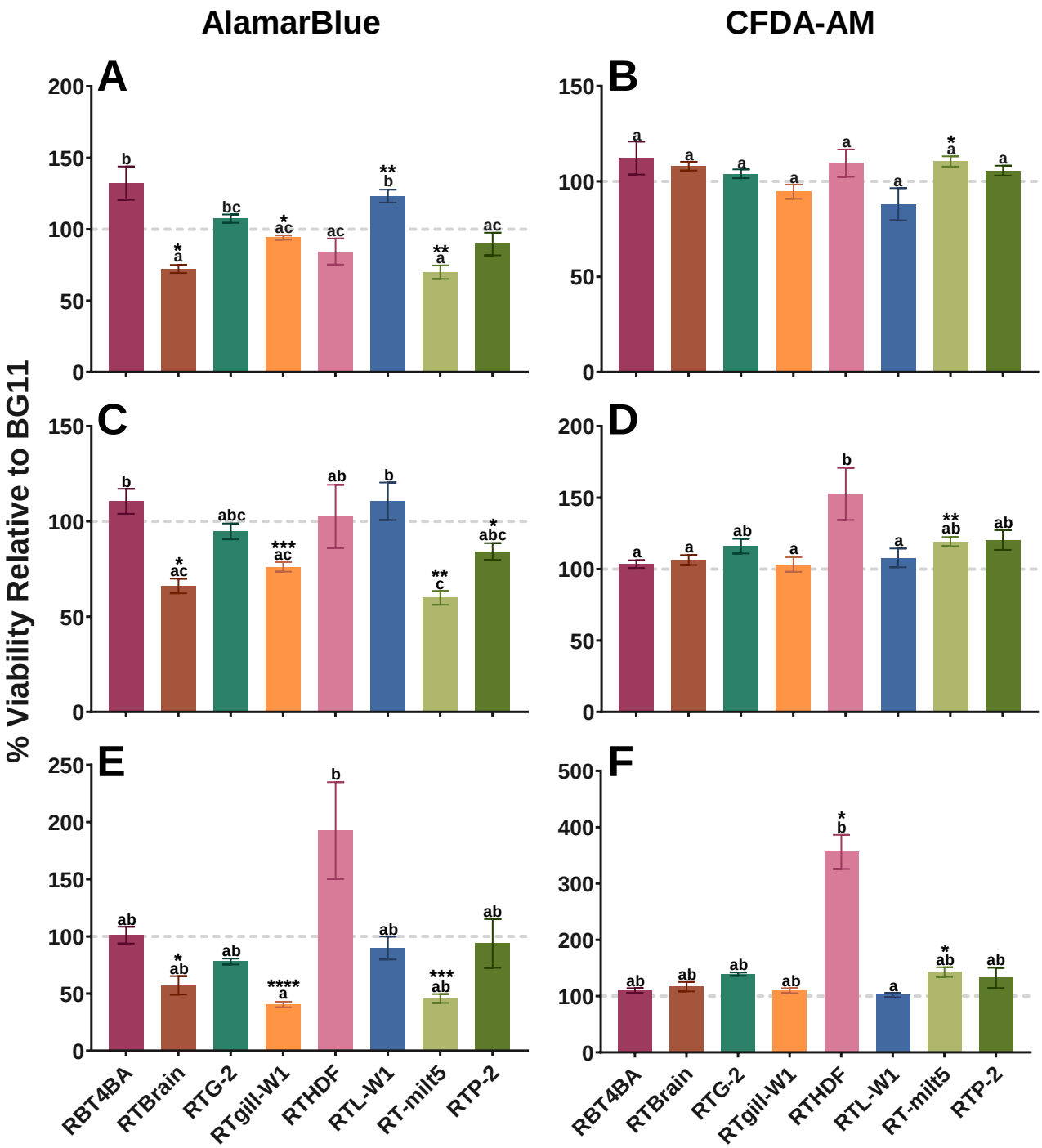

Figure 2.15: Mean (± SEM) differences in cell viability between cell lines treated with Non-Toxic Stable (**NTS**) exudates at concentrations of 0.5X (**A, B**), 1X (**C, D**), and 2X (**E, F**) and measured using AlamarBlue and CFDA-AM. Significant differences between treatments and the BG-11 vehicle control are indicated with asterisks (*P < 0.05, **P < 0.01, ***P < 0.001) and significant differences between cell lines are indicated by different letters.

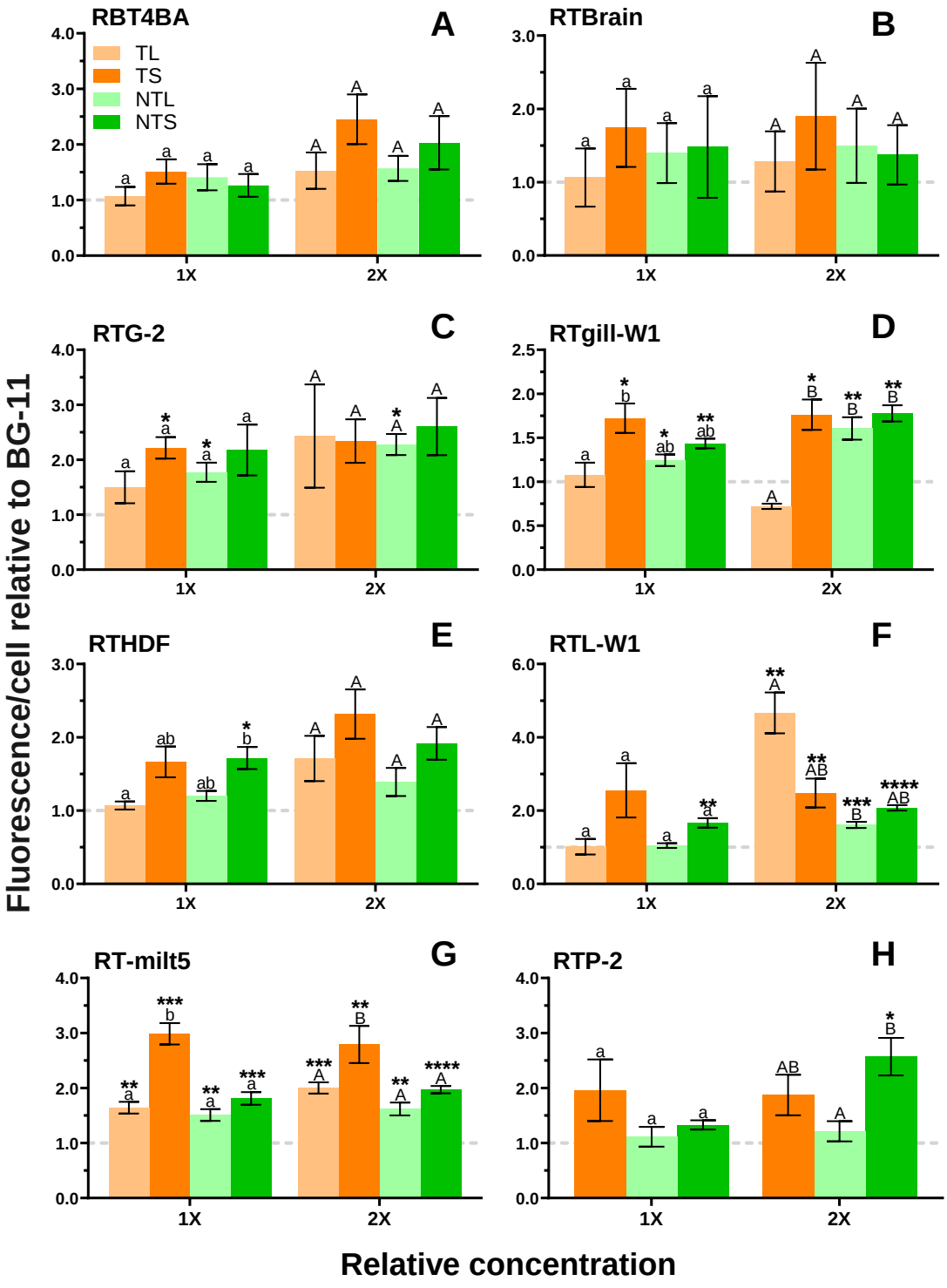

Figure 2.16: Mean (± SEM) differences in lysosomal activity between cell lines treated with exudate solutions at concentrations of 1X and 2X. Significant differences between treatments and the BG-11 vehicle control are indicated with asterisks (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001) and significant differences between cell lines are indicated by different letters.

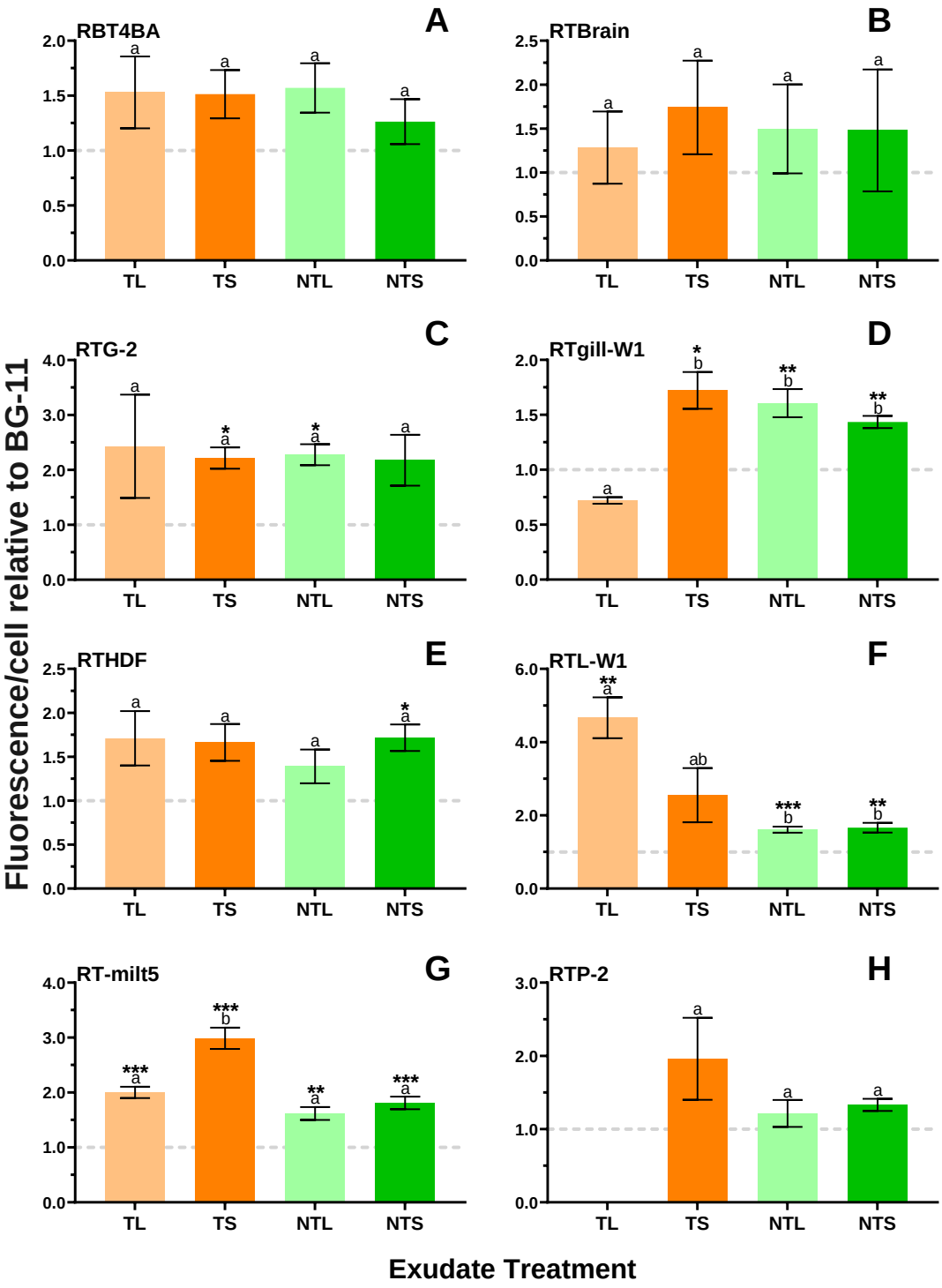

Figure 2.17: Mean (± SEM) differences in lysosomal activity between cell lines treated with exudate solutions at high relative density concentrations. Significant differences between treatments and the BG-11 vehicle control are indicated with asterisks (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001) and significant differences between cell lines are indicated by different letters.

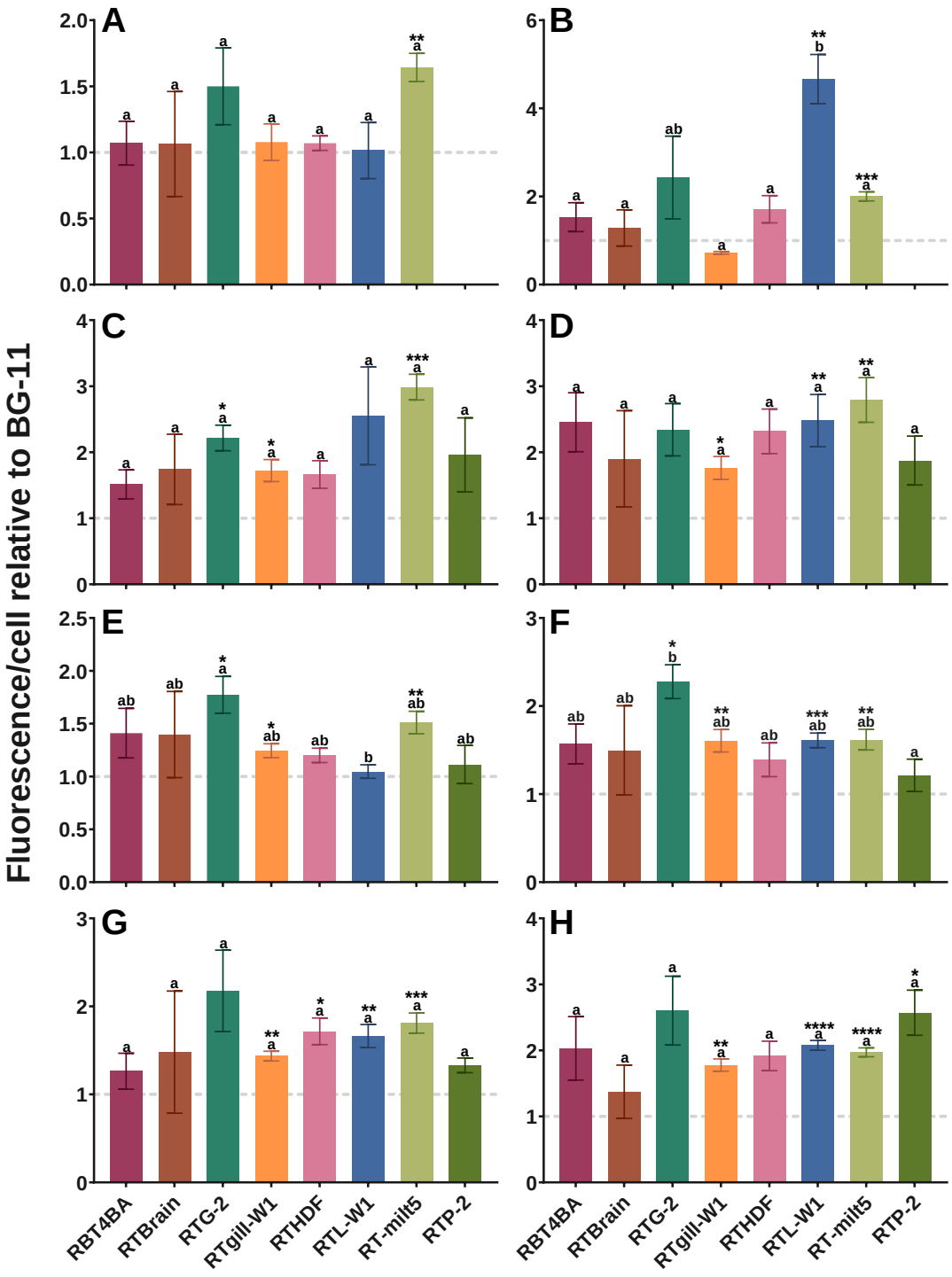

Figure 2.18: Mean (± SEM) differences in lysosomal accumulation between cell lines treated with TL (**A, B**), TS (**C, D**), NTL (**E, F**), and NTS (**G, H**) exudates at concentrations of 1X (**A, C, E, G**) and 2X (**B, D, F, H**). Significant differences between treatments and the BG-11 vehicle control are indicated with asterisks (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001) and significant differences between cell lines are indicated by different letters.

CHAPTER 3: Reproductive impairment by *Microcystis aeruginosa* extracellular metabolites – a study in rainbow trout cell lines

3.1. Introduction

Freshwater cyanobacterial algal blooms (cHABs) have gained notoriety for their lethal toxin components (Carmichael & Boyer, 2016), often with particular attention paid to their impact on terrestrial animals or humans encountering blooms at the height of their toxicity – including their scum washing ashore and contaminating areas of drinking water access (Bengis et al., 2016). The *Microcystis* genus of cyanobacteria – a major contributor of freshwater blooms – is often discussed solely in relation to their major toxin, microcystins (MCs), the risk they pose to humans, and their lethal effects on local wildlife (Harke et al., 2016). During bloom senescence, the concentration of MCs in the water column can spike as cyanobacterial cells die and lyse, releasing the intracellular compound into the environment and leading to lethal levels of the toxins, in some cases high enough to overcome local water treatment efforts (Steffen et al., 2017). Before these cyanobacterial crashes, healthy blooms can accumulate and persist in freshwater environments for long periods of time (Havens, 2008) and this duration is predicted to only increase without intervention (Huisman et al., 2018). During this time, local aquatic flora and fauna must contend with the sublethal impacts of not only MCs, but also of the many other compounds produced by the *Microcystis* cyanobacteria, which have been gaining attention in cHABs research for their contributions to cyanobacterial toxicity (Huang & Zimba, 2019; Janssen, 2019).

Mortality events are obviously a major concern regarding the health of freshwater fish populations, however, sublethal levels of contaminants, including cyanobacteria, can also impose population level consequences through the disruption of reproductive processes (Kidd et al.,

2007). In many well known endocrine-disrupting compounds as well as other toxic contaminants, lethal concentrations are much higher than the concentrations at which impairment of reproduction, for example through disruption of gonadal development, normal spawning behaviours, and gamete and sex steroid hormone production, becomes evident (Mills & Chichester, 2005; Scheuhammer et al., 2007). In *Microcystis* spp., reproductive toxicity also appears at sublethal concentrations of MCs (Malbrouck & Kestemont, 2006; Chen et al., 2016), highlighting the need for monitoring cyanobacterial blooms and their impacts outside of bloom declines to capture potential consequences for at-risk populations of freshwater fish, particularly when it comes to reproductive success.

In line with other vertebrates, major developmental and reproductive processes (**Figure 3.1)** in teleost fish are regulated by the Hypothalamic-Pituitary-Gonadal (HPG) axis, a multi-organ endocrine system that governs sexual development, differentiation, and maturation as well as gamete production and release (Arcand-Hoy & Benson, 1998; Yaron & Levavi-Sivan, 2011). Production of gonadotropin-releasing hormones (GnRH) in the hypothalamus stimulates the release of gonadotropins GtH I and II from the pituitary. These gonadotropins - often interchangeably referred to as follicle-stimulating hormone (FSH) and luteinizing hormone (LH) respectively due to their analogous nature with mammalian hormones of the same names – play critical roles in directing major reproductive events primarily through the stimulation of sex steroid hormone synthesis (Arcand-Hoy & Benson, 1998). FSH is strongly associated with gonadal development, early gametogenesis, and steroidogenesis, while LH is involved in gamete maturation and the timing of spawning, including ovulation and spermiation, with elevated expression of each shifting with progress toward reproductive culmination (Arcand-Hoy &

Benson, 1998; Tenugu et al., 2021) or in the case of iteroparous species such as rainbow trout, along seasonal trends in annual reproductive cycles (Chen et al., 2021).

Gonadal steroidogenesis in teleosts, once initiated by the binding of gonadotropins, is a multi-step process in which the steroid precursor cholesterol is converted to the major biologically active sex steroids – estrogens, progestogens, and androgens – via multiple steroidogenic enzymes (Tenugu et al., 2021). The key sex steroid hormones in fish, 17β-estradiol (E2), 11-ketotestosterone (11-KT), and 17α,20β dihydroxy-4-pregnen-3-one (DHP) are responsible for directing various stages of gametogenesis and reproductive development, including oocyte proliferation and growth (estrogens), spermatogenesis (androgens), and the final stages in the maturation of follicles and spermatozoa up to and including spermiation and ovulation (progestogens) (Yaron & Levavi-Sivan, 2011). Levels of these sex steroid hormones vary with reproductive and developmental events and expression of enzymes within this pathway are similarly tied to normal biological functioning, for example the cytochrome P450 side chain cleavage enzyme (*P450scc* or *cyp11a1*) is highly expressed during late stages of follicular maturation or in mature testis during spawning (Schulz et al., 2010; Tenugu et al., 2021), while transcription of 3β-hydroxysteroid dehydrogenase (*3β-hsd*), which catalyzes multiple steps in progestogen synthesis, peaks during mid-spermatogenesis (Kusakabe et al., 2006). Impairment of steroidogenesis can have significant impacts across multiple life stages, for example, another member of the cytochrome family, aromatase (*cyp19a1*) controls the conversion of androgens to estrogens and plays a key role in gonadal differentiation in fish, making dysregulation of this enzyme particularly disruptive to both sexual development in juvenile fish and sex hormone balance in adults (Tenugu et al., 2021).

Vitellogenesis, the primary process of oocyte growth, has been of particular interest in studies of xenobiotics and reproductive toxicity. When E2 binds to estrogen receptors in the liver, it stimulates the production of the yolk precursor protein vitellogenin. Vitellogenin and other critical egg proteins such as choriogenins are then incorporated into the growing oocytes, a process which is also stimulated by the binding of FSH (Arukwe & Goksøyr, 2003; Yaron & Levavi-Sivan, 2011). In female fish, elevated levels of vitellogenin and the *vtg* gene are expected in normal development and reproductive cycles; however, detectable levels of vitellogenin in male fish can be indicative of exposure to estrogenic compounds, leading to disrupted gonadal development and sperm production and eventually population level consequences (Sumpter & Jobling, 1995; Kidd et al., 2007); thus, vitellogenin and its associated genes may be useful for monitoring the estrogenicity of contaminant inputs into aquatic systems, including sewage treatment system effluent (Folmar et al., 2001).

The reproductive toxicity of isolated microcystins, particularly MC-LR, has been well-studied in fish. MC-LR exposure in fish as well as mammals and birds has been established to damage gonadal tissues, alter sex hormone levels, and decrease gamete production and quality (see reviews Chen et al., 2016 and Zhang et al., 2021). In fish specifically, MC exposure induces histopathological changes in both testes and ovaries as well as the liver (Trinchet et al., 2011). When exposed to MC-LR, female fish produce fewer eggs, with less of those eggs going on to be successfully fertilized and hatch (Zhao et al., 2015). These effects may be potential consequences of disruptions to normal oocyte maturation and yolk protein production (Qiao et al., 2013; Zhao et al., 2015). In both male and female fish, MC exposure has also been linked to the imbalance of hormones such as 17β-estradiol and testosterone, critical to reproduction and development and likewise, the perturbation of gene expression in steroidogenic and HPG axis pathway (Liu et al.,

2016; Hou et al., 2016). Disruptions to reproductive processes and the passage of MC from parent to offspring can also have trans-generational consequences (Zhang et al., 2021), including decreased embryonic survival and increased developmental malformations (Zhan et al., 2020).

While these studies are critical to our understanding of the potential damage cHABs can impart on fish populations, more research is necessary to understand the consequences of bloom exposure in the field and how non-MC metabolites contribute to possible population level effects. Reproduction-related studies utilizing complex *Microcystis* mixtures, including extracts, exudates, lyophilized cells, and live cultures, are a particularly critical area of study in this respect. Studies using live cultures of microcystin-producing *M. aeruginosa* strains have found similar patterns of negative effects as MC studies in female zebrafish, with exposure leading to decreased egg quality, altered sex hormone levels, and dysregulation of reproductive critical genes (Liu et al., 2018). Likewise, in a study comparing the effects of MC-LR and lyophilized *M. aeruginosa* cells on male Nile Tilapia (*Oreochromis niloticus*), both treatments significantly increased serum levels of the sex hormones testosterone and 17β-estradiol and dysregulated genes associated with steroidogenesis and growth hormone pathways; however, the strength and direction of this dysregulation was treatment-dependent (Chen et al., 2017).

Other *Microcystis* studies have focused on the hepatic consequences of exposure, including histopathological, proteinaceous, or genetic alterations and their downstream effects on fecundity. *Microcystis aeruginosa* extracts, when compared to equivalent concentrations of MC-LR, produced significantly greater dysregulation of hepatic genes in female medaka fish (*Oryzias latipes*), including genes that could negatively impair reproductive success, such as those involved in estrogen receptor signaling pathways or lipid and amino acid metabolism (Qiao et al., 2016). In a similar vein, female medaka fish exposed to living cultures of MC-producing

M. aeruginosa experienced greater dysregulation of hepatic proteins than their male counterparts, including those related to metabolic processes and stress response, and this dysregulation was more pronounced than exposure to extracts from the same culture (Le Manach et al., 2018). Additionally, in this same study exposure to a culture of non-MC-producing *M. aeruginosa* also generated significant histopathological damage and distinct proteomic effects in both male and female fish, including uniquely dysregulated proteins (Le Manach et al., 2018). How these non-MC metabolites contribute to algal bloom hepatoxicity and the downstream impact they play on oogenesis and egg quality is an important subject of future research. Furthermore, how non-MC-producing strains affect other tissues within critical reproductive pathways remains largely unexplored in current cyanobacterial research.

In this study, I investigate the potential reproductive toxicity of extracellular metabolites produced by the cyanobacteria *M. aeruginosa*, through the dysregulation of genes associated with critical reproductive processes. As detailed above, a more comprehensive understanding of the reproductive impacts of *Microcystis* blooms on freshwater fish has been hampered by limitations in the scope of current research, both in regards to the focus on MCs and toxin-producing varieties of *Microcystis* as well as anatomical scope. To this end, I monitored changes in gene expression following exposure to *M. aeruginosa* exudates, using cell lines from a diversity of tissue sources and using genes selected from previous studies using rainbow trout to capture potential disruption along the breadth of the reproductive pathways, including the HPG axis and the steroidogenic and vitellogenic pathways. These genes included follicle-stimulating hormone beta (*fshb*), FSH receptor (*fshr*), luteinizing hormone receptor (*lhr*), estrogen receptor alpha 1 (*era1*), vitellogenin (*vtg*), side chain cleavage enzyme (*P450scc*), and 3β-hydroxysteroid dehydrogenase (*3β-hsd*). Previous studies have indicated that non-MC-producing strains of *M.*

aeruginosa and their metabolites can alter the expression of genes within the liver tissue of exposed freshwater fish, including those related to oogenesis (Le Manach et al., 2016; Sotton et al., 2017). My study aims to expand upon these initial findings by utilizing cell lines derived from other tissues such as the brain and gonads, to better understand the reproductive influence these cyanobacteria may have on fish populations. I predict that the extracellular metabolites of both the MC-producing and non-productive *M. aeruginosa* strains will produce significant dysregulation in genes within the reproductive pathways in the rainbow trout cell lines, despite the exudates used in these experiments being harvested outside of the decline phase of cyanobacterial growth – when peak MC toxicity would be expected – and/or from a variety of cyanobacteria classically referred to as "Non-Toxic." From this study I hope to gain a better understanding of the potential sublethal impacts of *M. aeruginosa* cyanobacterial blooms on freshwater fish over the entire bloom duration and highlight the need for greater research into the effects of natural bloom conditions on fish populations, including the influence of strain diversity.

3.2 Materials and Methods

3.2.1 *Microcystis* Cultures and Exudate Preparation

Two strains of *M. aeruginosa* – CPCC 300, a MC-producing strain and CPCC 633, a non-MC-producing strain – were acquired from the University of Waterloo's Canadian Phycological Culture. Both strains of cyanobacteria were maintained in BG-11 growth medium (See Appendix A) and incubated at 23±1.5°C in a 12:12 light-dark cycle with fluorescent light of 19.80 ± 1.42 µmol/m^2/s. Culture density was measured by manual counting using a compound microscope and haemocytometer.

Experimental cultures of each *M. aeruginosa* strain were inoculated at cell densities of $3.01 \pm 0.14 \times 10^7$ cells/mL in 1000 mL Erlenmeyer flasks from healthy stock cultures that had been growing for at least five weeks. Flasks were randomly selected after six and 30 days of growth to capture cultures during log and stable growth phases respectively (See **Appendix 1.2**). Cultures from each flask were then centrifuged and filtered using 0.2 µm polyethersulfone (PES) membrane filters to remove cells and other debris from the exudates. Exudate samples and BG-11 media controls were lyophilized to dryness and resuspended in phenol-free Leibovitz's L-15 medium to a 20X concentration stock solution for each growth phase of each strain, referred to hereafter as Toxic-Log (TS), Toxic-Stable (TL), Non-Toxic-Log (NTL), and Non-Toxic-Stable (NTS). Working solutions of each treatment and control were prepared immediately prior to dosing applications with 5% (v/v) dilutions of the concentrated exudates in phenol-free L-15/ex medium for a working concentration of 1X (**Table 3.1**). (For more details on *M. aeruginosa* culturing and exudate preparation see Chapter 2).

3.2.2 Rainbow Trout Cell Lines

Rainbow trout cell lines were generously provided by Dr. B. Dixon and Dr. N.T.K. Vo from the University of Waterloo (**Table 2.1**). All cell lines were routinely grown in 175 cm^2 plug-seal tissue culture flasks at 19±1.5°C in Leibovitz's L-15 medium (Cytiva) supplemented with 10-15% (v/v) fetal bovine serum (FBS, Gibco), 1% (v/v) penicillin/streptomycin (Gibco) and 1% (v/v) L-glutamine (GlutaMAX, Gibco). For both routine culturing and plating for experiments, flasks were passaged when they reached between 80 and 90% confluency, generally at 1:3 and 1:5 splitting ratios, using TrypLE (Gibco).

3.2.3 Gene expression assays

Cell lines were plated in 96-well Poly-D-Lysine-coated black plates at densities of 2.5 x 10^4 cells per well in 50 - 100 µL of complete L-15 media. To achieve sufficient RNA, each treatment was allotted 16 wells for RNA collection. In more sensitive cell lines (RTG-2, RTP-2, RTHDF), an additional 48 wells were seeded for each of the TL and TS treatments after preliminary attempts failed to yield enough RNA. After allowing cells to attach overnight at 19°C, culture media was removed via vacuum pipette and replaced with 100 µL of 5% treatment solutions. Media from untreated wells was replaced with phenol-free L-15/ex media. Following a 24h exposure period, treatment solutions were decanted, wells were rinsed with DPBS, and RNA was collected and processed 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.

3.2.4 Quantitative real-time PCR

Once three biological replicates for each cell line were collected, 0.5 µg of RNA for each sample was reverse transcribed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) according to manufacturer's protocol. Target genes were selected from rainbow trout reproductive processes and related biological systems including the HPG axis (*fshb, fshr, lhr,* and *erα1*), steroidogenic enzymes (*P450scc* and *3β-HSD*), and oocyte production (*vtg*). Taqman primer and probe sequences were obtained from previously published studies on rainbow trout (**Table 3.2**).

Real-time PCR reactions were performed in 384-well plates with 10 µL reaction volumes containing 5 µL of TaqMan™ Fast Advanced Master Mix (2X), 0.5 µL of each primer (20X), 3.5 µL Nuclease-free water, and 1 µL of cDNA diluted 1:10. Each reaction was performed in

duplicate and amplification was carried out using a QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems) according to the manufacturer's recommendations with the following run protocol: an initial stage of 95°C for 20s then 40 cycles of 95°C for 1s and 60°C for 20s. The relative expression of each of the target genes was calculated using the comparative CT ($2^{-\Delta\Delta CT}$) method with the reference gene *18s rRNA* acting as an internal control.

3.2.5 Data analysis and statistics

All data were expressed as mean ± standard error of the mean and analyzed using GraphPad Prism 8.0.2 software. The normality of data distribution and homogeneity of variances were assessed using the Shapiro-Wilks test and Brown-Forsythe test, respectively. Differences between exudate treatments were evaluated by either: 1) ordinary one-way ANOVA followed by Tukey's multiple comparisons post hoc test for normally distributed data; 2) the nonparametric Kruskal-Wallis test with Dunn's post hoc test when data had a non-normal distribution; or 3) Welch's ANOVA with Games-Howell's post hoc test when standard deviations in the data were unequal. Differences between the treatments and the BG-11 controls were evaluated using the appropriate Student's *t*-test. P-values of < 0.05 were considered statistically significant.

3.3 Results

3.3.1 Selecting primers within cell lines

Before running RT-qPCR analysis for all treatments on each cell line, untreated samples were run with all available primers first to determine whether each gene had basal expression within each specific cell line. Since the comparative CT method relies on analysis through relative expression compared to both control samples and housekeeping genes, genes that were undetectable in untreated samples were removed from future analysis with those lines.

3.3.2 Reproduction-relevant cell lines

Changes in gene expression were assessed in cell lines derived from tissues involved in reproduction and related processes (See **Appendix 1.6, Table A1.6.1**). These included the RTBrain and RTP-2 cell lines derived from brain and pituitary tissues, RTG-2 derived from the gonads, RTL-W1 derived from the liver, and RT-milt5 derived from the seminal fluid, milt.

For RTBrain, mRNA levels of *fshb, p450scc,* and *3β-hsd* were significantly decreased when cells were treated with TL exudates (**Figure 3.2.B**). Additionally, *p450scc* transcription was also significantly reduced by TS, NTL and NTS treatments. These treatments also appeared to reduce expression of the *fshb* and *3β-hsd* genes as well, however these changes were not statistically significant. Likewise, there were no significant differences between strains or growth phases of each treatment.

For RTG-2, expression of *fshr* and *3β-hsd* were significantly reduced by all treatments (**Figure 3.2.C**). The transcription of *p450scc* was also reduced by all treatments; however, only TL and TS exudates produced significant changes in gene expression. mRNA levels of *lhr* also appeared to be influenced by exudate treatments, although these changes were not considered significantly different from control expression. There were no significant differences between each of the different treatments.

For the liver cell line, there were no significant changes from control levels in the expression of any tested genes for any of the treatments (**Figure 3.3.B**). There were also no significant differences between treatments in respect to either growth phase or strain type.

For RT-milt5, TS treatments significantly decreased the expression of *fshb* and *3β-hsd*, while significantly increasing mRNA levels of *era1* (**Figure 3.3.C**). TL treatments also appeared to alter the expression of *era1* and *3β-hsd* in the same manner as TS, however these changes

were not statistically significant. Between the treatments, the only significant difference observed was between the effects of TS and NTS treatments on *3β-hsd* (**Table A1.6.3**)

Finally, for the pituitary cell line RTP-2, TL treatments significantly reduced the expression levels of *fshb*, *p450scc*, and *3β-hsd* (**Figure 3.3.D**). For *p450scc*, TS and NTL treatments also appeared to decrease relative mRNA levels, however these changes were not significant. All treatments appeared to increase the expression of *lhr*, although only NTL exudates produced a significant response. When comparing between treatments, there were significant differences observed between the effects of TL and NTS on both the expression of *fshb* and *3β-hsd*. There were no other significant differences between treatment types.

3.3.3 Non-reproduction-related cell lines

In addition to the cell lines directly related to reproduction, gene expression changes were also monitored in the remaining three cell lines used in the cytotoxicity assays – RBT4BA, RTgill-W1, and RTHDF (**Table A1.6.2**). For the heart cell line RBT4BA, none of the treatments produced changes in mRNA levels that differed significantly from controls (**Figure 3.2.A**). In addition, there were no significant differences between the treatments themselves or their growth phases. For RTgill-W1, mRNA levels of *fshr* were significantly reduced by NTS exudates (**Figure 3.2.D**). For the *era1* gene, expression was significantly decreased in TS treatments. There was also an apparent, but non-significant increase in *3β- hsd* expression following treatment with TS exudates. As with the previous cell lines, there were no significant differences between the strain types or growth phases of treatments.

Finally, for RTHDF, transcription of *fshb* and *3β-hsd* was significantly decreased by both TL and TS treatments (**Figure 3.3.A**). Expression of *fshb* was also significantly reduced by NTL treatment, although this down-regulation was not as severe as the TL and TS treatments (fold-

change of 0.732 ± 0.031 versus 0.270 ± 0.032 and 0.335 ± 0.013 respectively). The mRNA levels of *p450scc* appeared reduced after all treatments, however only the reduction following TS treatment was statistically significant. Between the growth phases of each strain, there were no significant differences between the effects of TL and TS treatments for any of the genes in question, however for the NT treatments there was a significant difference in the effects of log and stable exudates on the expression of *fshb* (**Table A1.6.4**). There were also significant differences in the effects of T versus NT treatments on the mRNA levels of *fshb* and *3β-hsd*. There were no differences, however, between the effects of treatments on *p450scc* and *era1* expression levels.

3.4 Discussion

Understanding the sub-lethal effects of environmental toxicants is critical to accurate risk assessments for the sustainability of aquatic populations, particularly those that affect reproductive efforts – a consequence that could lead to the eventual extirpation of sensitive species from affected areas (Kidd et al., 2007). For *Microcystis* algal blooms, little information exists for the reproductive toxicity of bloom components beyond microcystins, a knowledge gap that also neglects to account for the toxicity of non-microcystin-producing strains. My study compared how gene expression of reproduction-related genes was altered by exposure to exudates of both "Toxic" and "Non-Toxic" strains of *M. aeruginosa* in rainbow trout cell lines. I found that the expression of these genes was significantly dysregulated in four out of five cell lines derived from tissues directly or indirectly involved in reproduction, including RTBrain, RTG-2, RT-milt5 and RTP-2 (**Figure 3.4**).

The HPG axis functions as the core of the fish reproductive system, from which critical processes are directed. Dysregulation of genes within this system could lead to disruption of

these downstream processes and eventually significant reproductive impairment (Mills & Chichester, 2005). In both of the brain tissue-derived cell lines, I found that *fshβ* was significantly downregulated when cells were treated with exudates from the log phase of the "Toxic" strain (**Figure 3.2.B** and **Figure 3.3.D**). FSH is associated with the early stages of gametogenesis in fish, including vitellogenesis and spermatogenesis (Yaron & Levavi-Sivan, 2011) and along with LH, functions by binding to its respective receptors, stimulating steroid hormone production (Saha et al., 2022). While my two cell lines shared similar responses to the TL exudates, they diverged significantly with respect to the others. In the RTBrain cell line, although only TL treatments produced a significant decrease in expression, there was no statistical difference between the exudate treatments, with slight (but not significant) decreases across the board (**Figure 3.2.B**). However, in the RTP-2 cell line, TL treatments produced significantly greater down-regulation of *fshβ* compared to treatments with stable phase exudates of the "Non-Toxic" strain, which appeared to slightly increase expression, although there was no difference statistically between these results and the other treatments (**Figure 3.3.D**). Interestingly, RTP-2 cells also experienced increased expression of the luteinizing hormone receptor (LHR) gene, with significant increases following NTL exposure, basal expression of which was lacking in the RTBrain cell line. *Lhr* expression has been detected in African catfish (*Clarias gariepinus*) brain tissue as well (Vischer & Bogerd, 2003), however this, along with their relative sensitivity to treatments, highlights differences between these two cell lines with respect to *in vivo* functionalities. While RTP-2 can be traced back to pituitary tissues specifically (Bols et al., 1995), RTBrain has a less clear origin, a consequence of the limited amount of characterization conducted with the line thus far.

LHR, along with the FSH receptor (FHR), were also expressed in my gonadal cell line RTG-2 (**Figure 3.2.C**). Although there appeared to be a stimulatory effect on the expression of *lhr* in all treatments, these increases were not statistically significant. However, *fshr* on the other hand was strongly and significantly downregulated following all treatments. Gonadotropin hormone receptors are localized in the gonadal tissues, including the Sertoli and Leydig cells in the testes of males and the theca layer and granulosa cells of the ovaries in females (Yaron & Levavi-Sivan, 2011), and upon the binding of their respective gonadotropins, initiate the synthesis of sex steroid hormones (Tenugu et al., 2021).

Sex steroid synthesis occurs primarily in the gonads, although steroidogenesis also takes place in the interrenal tissues and brain as well, and relies on a number of enzymes to catalyze the process (Tenugu et al., 2021). The very first step of which is the conversion of cholesterol to pregnenolone by the cytochrome P450 side-chain cleavage enzyme (Tenugu et al., 2021). I found that *p450scc* was significantly downregulated by all exudate treatments in the RTBrain cell line (**Figure 3.2.B**). In contrast, in the RTP-2 cell lines, only TL exudates significantly decreased *p450scc* expression, although this response was not significantly different from those of the other exudates (**Figure 3.3.D**). In my RTG-2 cells, *p450scc* was downregulated in all treatments, however only exudates from my "Toxic" strain produced significant decreases in expression (**Figure 3.2.C**).

The other steroidogenic enzyme examined in my study was 3β-hydroxysteroid dehydrogenase, which is involved in multiple stages of steroidogenesis but, primarily converts pregnenolone to progesterone (Tenugu et al., 2021). Between the RTBrain and RTP-2 cell lines, the pattern of expression for *3β-hsd* was very similar to that of *fshβ* – that is, only significant decrease in expression followed treatment with TL exudates in both cell lines, however there was

no statistical difference in treatments for RTBrain while only significant difference between TL and NTS exudates in RTP-2 (**Figure 3.2.B** and **Figure 3.3.D**). For the RTG-2 cell line, *3β-hsd* was significantly downregulated across all treatments and there were no significant differences among them (**Figure 3.2.C**).

In previous studies, *Microcystis* and MCs appear to be able to dysregulate the expression of gonadotropins and their receptors, as well as significant steroidogenic genes, with potentially significant effects on sex steroid concentrations and gamete quality (Zhao et al., 2015; Hou et al., 2016; Liu et al., 2016; Liu et al., 2018). Short-term exposure to live *M. aeruginosa* cultures in female zebrafish resulted in significantly increased expression of *fshβ* and *lhβ* in the brain and expression of *fshr* and *lhr* in the ovaries (Liu et al., 2018). This strongly contrasted my findings, with the exception of upregulation of *lhr* (and a non-significant increase in expression of *fshβ*) in my RTP-2 cell line as well as my RTG-2 line, although this was not significant (**Figure 3.2.C** and **Figure 3.3.D**). Similarly, the downregulation of steroidogenic genes *p450scc* and *3β-hsd* in my study was not mirrored in live culture exposure, in which there was no significant change in expression (Liu et al., 2018). However, both 17β-estradiol and testosterone levels were significantly decreased in this study as well as the expression of other steroidogenic enzymes, indicating *M. aeruginosa* and/or MCs may inhibit steroidogenesis further downstream than my focal enzymes. These findings were also paired with histopathological damage in the ovaries and decreased fertilization and hatching success in the eggs of treated fish (Liu et al., 2018).

Chronic exposure to MCs in female zebrafish increased the expression of *fshr*, particularly at lower doses, and decrease the expression of *lhr* with increasing concentrations (Liu et al., 2016); however, both upregulation (Kawan et al., 2019; Hou et al., 2016) and downregulation of *fshβ* has been observed following long-term MC exposure (Zhao et al., 2015).

With the exception of the latter study, the findings of this prior body of work contrasts strongly with the results of my study, however they do highlight another critical factor in *Microcystis*/MC effects – sex-dependent sensitivity. Liu et al. (2016) also compared effects of MC-LR chronic exposure on male zebrafish, with treated fish experiencing significant decreases in *fshr* expression as well as that of *lhβ* and *lhr*. In terms of my focal steroidogenic enzymes, *3β-hsd* was significantly upregulated at low treatment doses in female zebrafish but reversed to downregulation at higher concentrations (Kawan et al., 2019). In male zebrafish *3β-hsd* was significantly downregulated at all treatment concentrations (Liu et al., 2016), while in male Nile Tilapia it was downregulated following chronic exposure to MC-LR or to lyophilized *M. aeruginosa* cells (Chen et al., 2017). Similarly, *p450scc* expression was upregulated in females and downregulated in male zebrafish (Liu et al., 2016; Kawan et al., 2019).

With the complexity of reproductive pathways, especially in whole organisms, and the differences of chronic versus acute exposure it is perhaps unsurprising that the results of my cell line studies only partially overlap with the trends of *in vivo* research. As evident in these previous studies, the length of the exposure period is a major factor in determining gene expression responses in fish exposed to *Microcystis* metabolites – MCs or otherwise – and a highlights a limitation of my study, which utilized a single 24-hour exposure period. Expanding the methodologies used here to capture multiple time points across an exposure period can better capture trends in gene expression changes, including potential effects of *M. aeruginosa* exudates missed during my single time-point approach. Nevertheless, common consequences of *M. aeruginosa*/MC exposure, whether they be acute or chronic, in the form of delayed gamete maturation (Zhao et al., 2015; Liu et al., 2018), decreased fertilization and hatching success (Zhao et al., 2015; Liu et al., 2018; Kawan et al., 2019), and dysregulated sex hormone levels (all

studies previously mentioned studies, including Hou et al., 2016) indicate potentially serious effects on regulation of reproduction, of which these gene expression changes could be symptom (i.e. response of a feedback system to tissue damage) or cause (upstream dysregulation of gonadotropin-driven pathways including steroidogenesis, resulting in hampered gamete development and degraded quality) (Yaron & Levavi-Sivan, 2011).

Differences in sensitivity of the sexes to effects of *Microcystis* and MCs should also be considered as both a potential explanatory influence and a limitation. My RTP-2 cell line was derived from the pituitary of a female rainbow trout (Bols et al., 1995), however there was very little overlap between the results of my study (**Figure 3.3.D**) and the findings of other authors who used female fish – although species-specific variation may also play a role (Liu et al., 2018). Likewise, my RTG-2 cell line originated from pooled immature gonads, the majority of which were observed to be developing ovaries (Wolf & Quimby, 1962), and the changes in gene expression observed in these cells (**Figure 3.2.C**) also diverged from the findings of whole female fish (Liu et al., 2018). Whether these discrepancies were the result of *in vitro* versus *in vivo* differences, differences in the exposure lengths, or whether they represent true differences between the effects of MCs and *Microcystis* extracellular compounds remains to be seen, ideally with additional research on these subjects in the future.

Either way, this highlights another factor that needs to be considered during cell line selection and would benefit from both increased attention on *Microcystis* exudate contents and cell line characterization as previously discussed. On the one hand, RTBrain, was referred to in the cell line database Cellosaurus as originating from an unspecified sex (available at https://www.cellosaurus.org/, Bairoch, 2018), rendering difficult comparisons to *in vivo* work. On the other hand, RTL-W1 was derived from the tissue of the liver of a male rainbow trout (Lee

et al., 1993), and which was not significantly affected by any of my treatments (**Figure 3.3.B**). Initial analyses of changes in gene expression included monitoring changes in the expression of the vitellogenin gene *vtg*, however, this gene is not expressed at detectable levels in male fish (Sumpter & Jobling, 1995), rendering it unsuitable for my use of the comparative CT method of gene expression analysis. In addition, the lack of response from these cells in tandem with their apparent insensitivity to MCs (Boaru et al., 2006) may indicate that RTL-W1 was not negatively impacted by non-MC metabolites.

In addition to my other reproduction-related cell lines, I also observed significant changes in gene expression in my RT-milt5 cell line. Cultivated from the somatic cells harvested from the milt of male rainbow trout, the nature of this cell line in unclear, muddying potential interpretations of gene expression changes. Its originating paper by Vo et al. (2015) suggested that, among other possibilities, these cells may have been shed from the reproductive tract, i.e. the spermatogenic tubules where germ cells mature (Schulz et al., 2010). Gonadal origins, particularly from the Sertoli cells would reasonably explain the basal gene expression detected in RT-milt5, which included *fshr*, *p450scc,* and *3β-hsd*, whose expression in the gonads has already been described, as well as *era1* (**Figure 3.3.C**). Estrogen receptors are expressed in the male gonads and are associated with a number of estrogen-dependent genes (Schulz et al., 2010). Whether gonadal in origin or from some other somatic cell present in rainbow trout milt, my results found that RT-milt5 was particularly sensitive to the effects of the stable phase "Toxic" exudates, however without further characterization of the cell line *in vivo* potentialities were not the focus of my study.

Delineating the effects of *Microcystis* metabolites on reproductive gene expression from those of MC-LR remains a challenge in reproductive toxicity research. However, the results of

my study and from the RTG-2 cell line in particular suggest that the metabolites found in the exudates of *both* “Toxic” and “Non-Toxic” strains of *M. aeruginosa* are capable of dysregulating genes within both the signalling pathways of the HPG axis and the workhorse of the reproductive system, the steroidogenic pathway. Impairment of the processes driven by these systems could adversely affect the reproductive success of freshwater fish subject to *Microcystis* algal blooms, as these dysregulated genes are intrinsically tied to the regulation of gametogenesis, the maintenance of sex hormone levels, and the timing of spawning (Yaron & Levavi-Sivan, 2011; Chen et al., 2021). However, further research is needed to better explore the relationship between these *in vitro* results and *in vivo* outcomes, as my study represents potentially the only instance of reproductive toxicity assessments using *M. aeruginosa* extracellular metabolites.

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Table 3.1: Equivalent cell densities in cells per mL for each exudate treatment of Toxic and Non-Toxic *M. aeruginosa* used in gene expression assays. Equivalent densities are based on cell counts taken immediately prior to the removal of cells via centrifugation and filtration.

Strain	Growth Phase	Equivalent density (cells/mL)
CPCC 300 (Toxic)	Log (TL)	4.6×10^7
	Stable (TS)	9.8×10^7
CPCC 633 (Non-Toxic)	Log (NTL)	4.5×10^7
	Stable (NTS)	8.5×10^7

Table 3.2: 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	

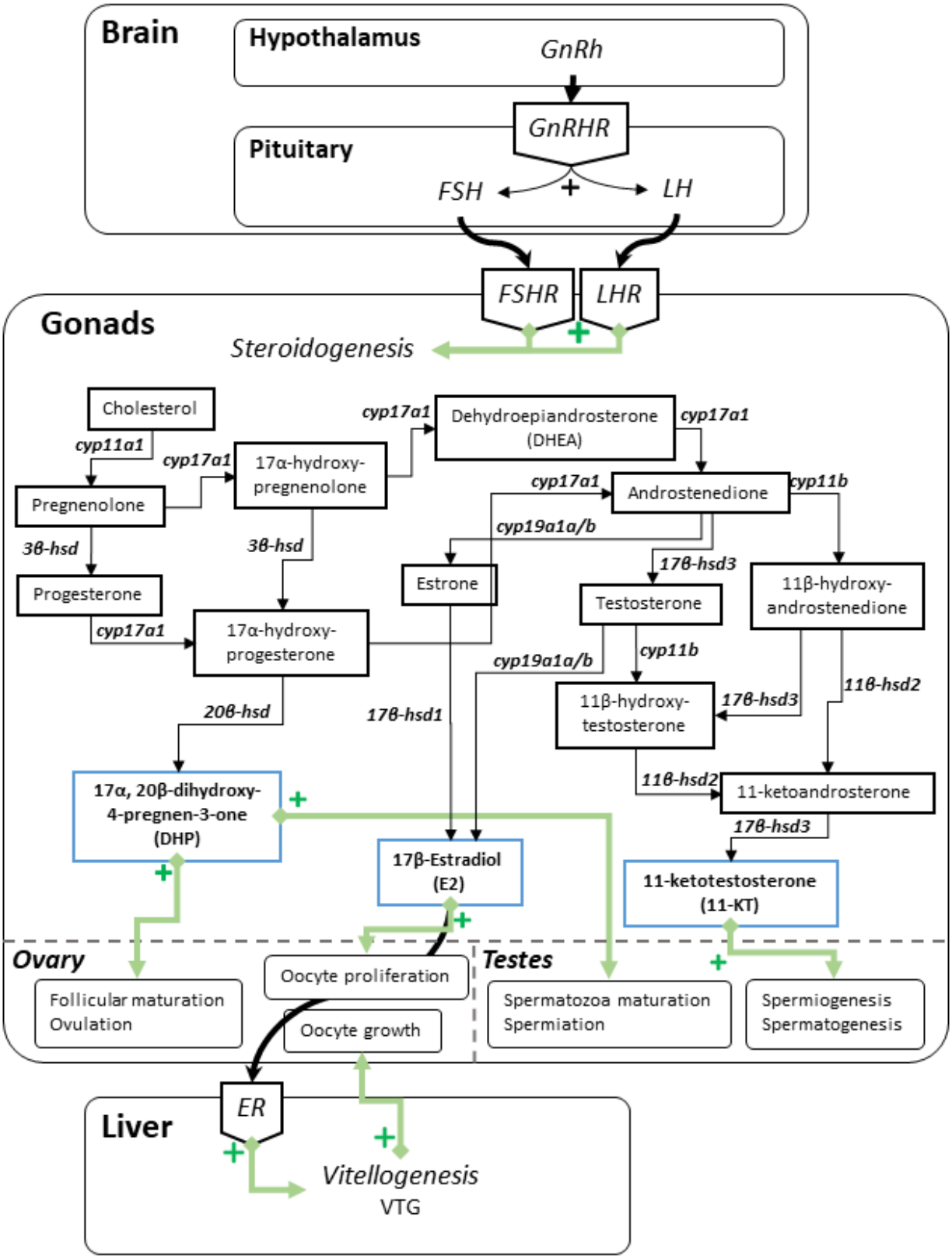

Figure 3.1: An overview of the processes driving the major reproductive and developmental systems in teleost fish. Adapted from work of Arcand- Hoy & Benson, 1998; Yaron & Levavi-Sivan, 2011; and Tenugu et al., 2021.

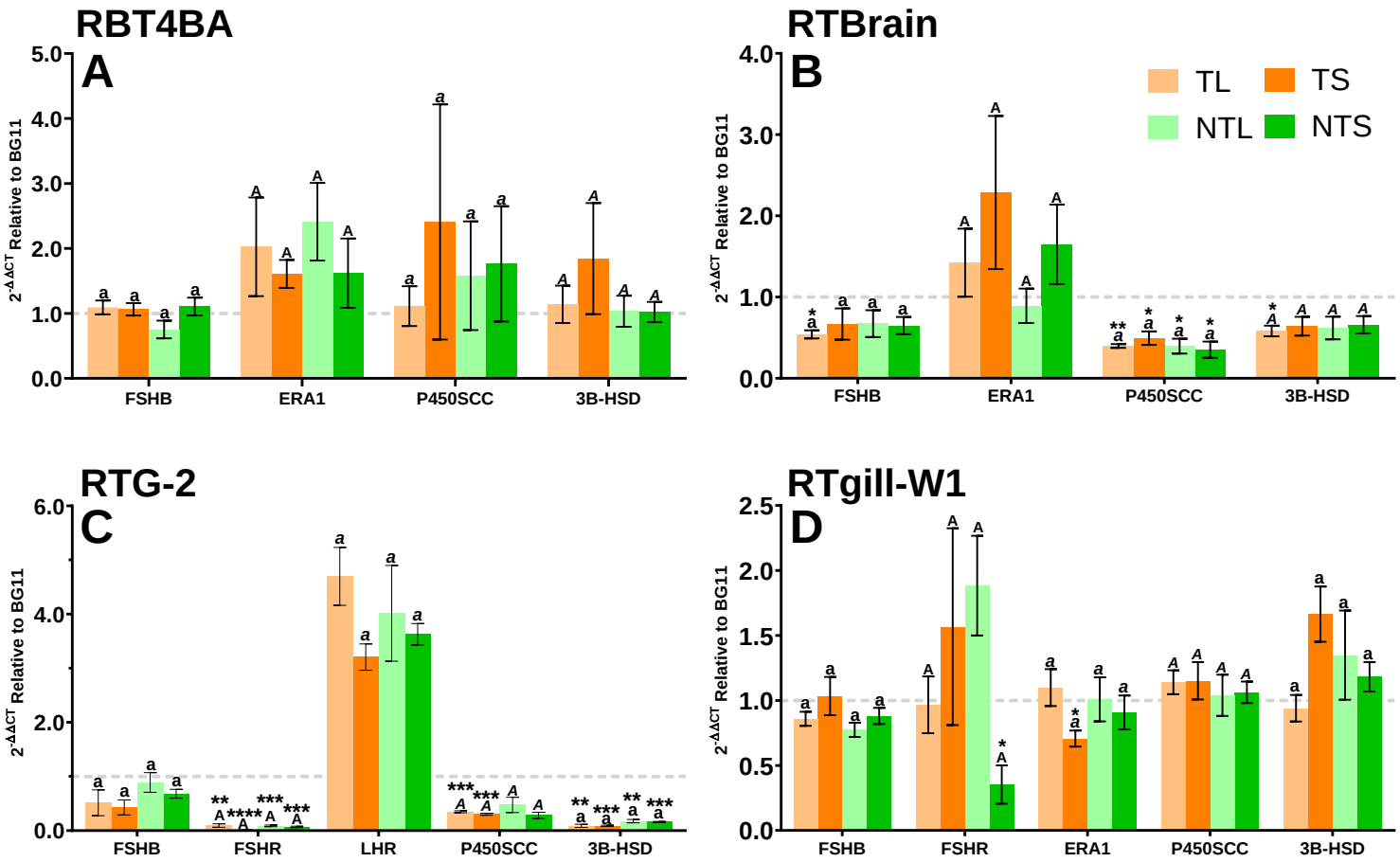

Figure 3.2: Mean (± standard error of the mean (SEM)) changes in gene expression in rainbow trout cell lines RBT4BA (**A**), RTBrain (**B**), RTG-2 (**C**), and RTgill-W1 (**D**) following 24h exposure to TL, TS, NTL, and NTS exudates. Significant differences between treatments and the BG-11 vehicle control are indicated with asterisks (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001).

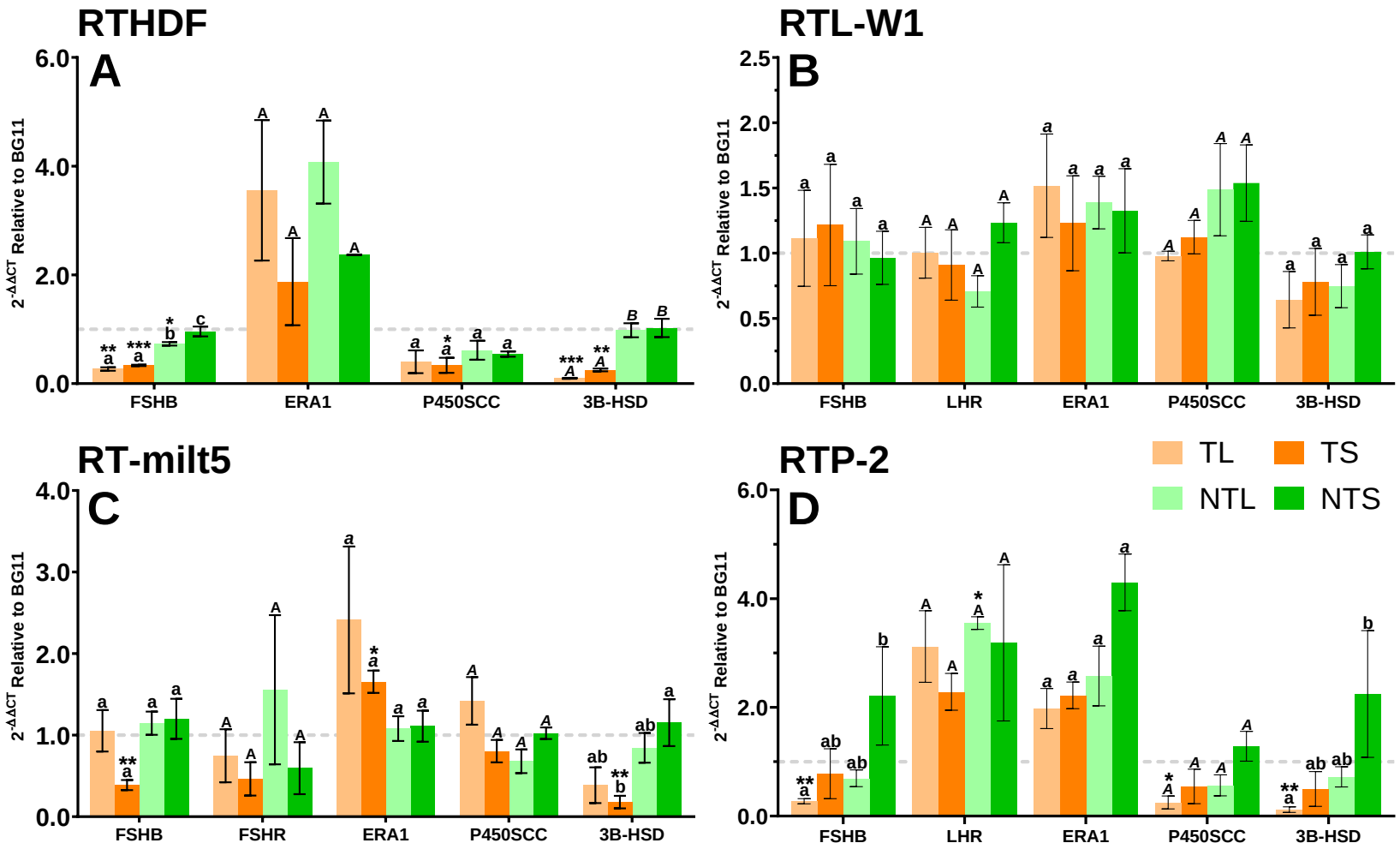

Figure 3.3: Mean (± SEM) changes in gene expression in rainbow trout cell lines RTHDF (**A**), RTL-W1 (**B**), RT-milt5 (**C**), and RTP-2 (**D**) following 24h exposure to TL, TS, NTL, and NTS exudates. Significant differences between treatments and the BG-11 vehicle control are indicated with asterisks (*P < 0.05, **P < 0.01, ***P < 0.001).

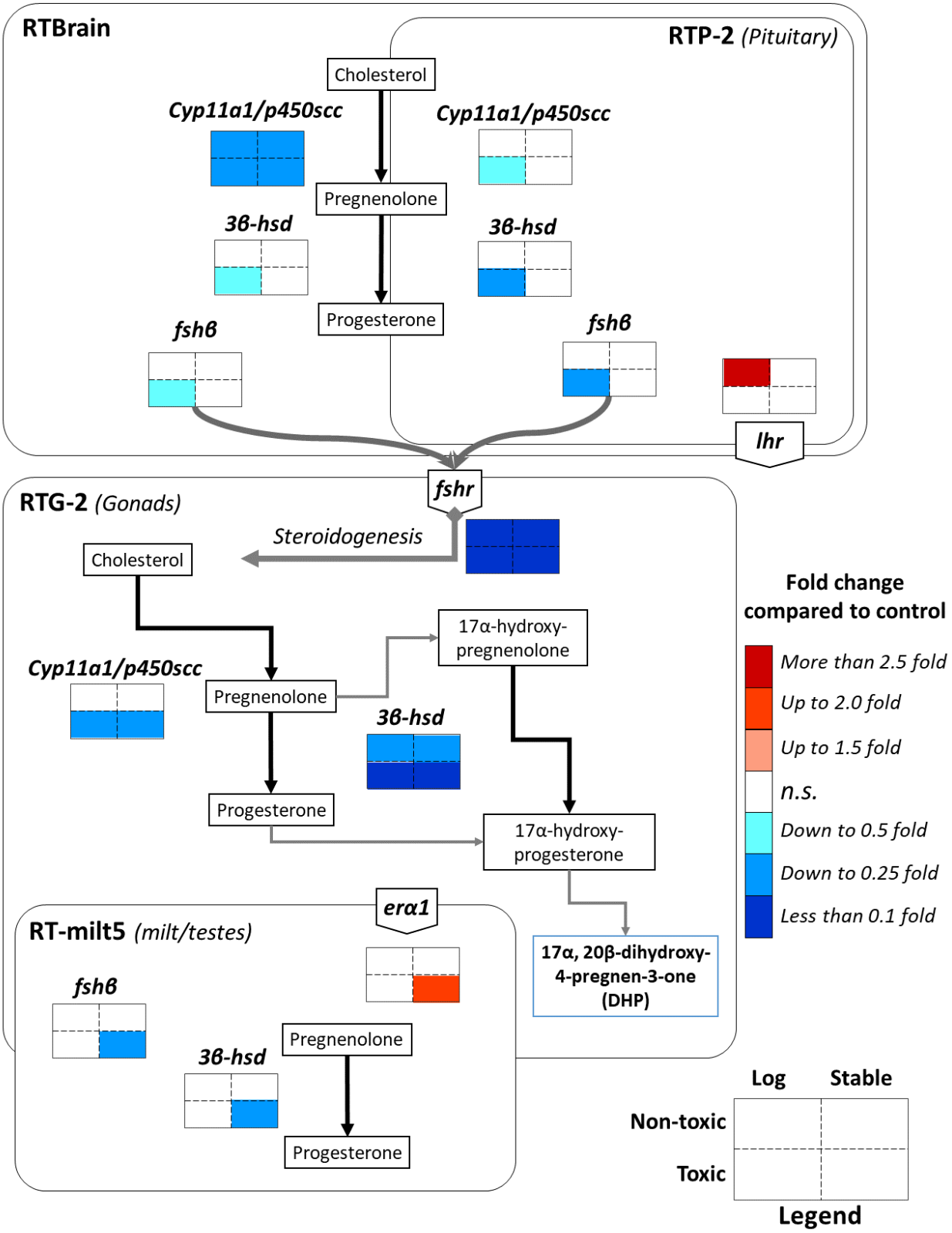

Figure 3.4: Significant effects of *M. aeruginosa* exudates on gene expression in cell lines derived from the HPG axis of rainbow trout.

CHAPTER 4 – General Discussion

4.1 Summary

Harmful algal blooms represent a significant and expanding concern in freshwater ecosystems (Ho et al., 2019), endangering not only clean drinking water but also the health and habitats of aquatic organisms including fish (Havens, 2008; Steffen et al., 2017). The *Microcystis* genus of cyanobacteria is a common constituent of freshwater harmful algal blooms globally and is capable of producing a diversity of biologically-active metabolites (Harke et al., 2016; Janssen, 2019). However, *Microcystis* toxicological research has been dominated by the study of microcystins, their most infamous and lethal toxin. To better encapsulate the effects of bloom exposure, contemporary efforts to study *Microcystis* toxicity have incorporated the use of complex cyanobacterial mixtures, such as living cultures, extracts, and exudates (Abdel-Latif & Abou Khashaba, 2017; Zi et al., 2018; Li et al., 2021), highlighting not only potentially synergistic interactions between MCs and other metabolites (Saraf et al., 2018) but the deleterious effects of non-MC compounds as well (Le Manach et al., 2018).

In this thesis, I examined the cytotoxic and reprotoxic effects of *Microcystis aeruginosa* extracellular metabolites derived from both microcystin-producing ("Toxic") and non-microcystin-producing ("Non-Toxic") *M. aeruginosa* strains on freshwater fish using *O. mykiss* immortalized cell lines as an *in vitro* alternative to whole fish testing. My objectives were two-fold: first, to evaluate the toxicity of *M. aeruginosa* exudates and how it varies between culture strains, over the lifespan of a bloom, and in potential target tissues, and secondly, to examine how these extracellular metabolites may contribute to sub-lethal effects of bloom exposure, specifically in regards to reproductive toxicity. Improving our knowledge of potential toxicants is the first step in improving the accuracy of risk assessments for cyanobacterial algal blooms,

especially as the expansion of blooms serves to imperil increasing numbers of freshwater ecosystems and species (Pick, 2016; Ho et al., 2019).

In Chapter 2, rainbow trout cell lines were treated with the exudates of *M. aeruginosa* cultures from both toxigenic and non-toxigenic strains as well as from periods of exponential and stable culture growth and monitored for changes in cell viability and normal cell function. Fish cell lines, particularly those of the well-studied rainbow trout, facilitate a broad examination of cytotoxicity across a number of potential tissue types in the absence of whole animal testing, which is both ethically and practically advantageous (Bols et al., 2005). With so little known regarding the mechanisms of toxicity and target tissues of *Microcystis* metabolites, it was imperative that I include as many tissue types as possible rather than taking cues solely from MC and its hepatotoxicity. Indeed, my liver-derived cell line was largely unaffected by my treatments, save at the highest densities of my 'toxic' strain and even this effect may be disconnected from MC toxicity as RTL-W1 lacks the OATP transport proteins that facilitate MC uptake (Boaru et al., 2006).

RTBrain, RTgill-W1, and RT-milt5 were the most sensitive cell lines identified in my cytotoxicity assays, with significant effects observed in the AlamarBlue assays even at my lowest tested concentration (**Figure 2.5.B, D,** and **G**). Additionally, these cell lines, along with RTP-2 (**Figure 2.5.H**), were negatively affected by my "Non-Toxic" exudates as well as those from my "Toxic" strain (**Figure 2.4**). Injurious effects have been observed in the brain and gill tissues of fish exposed to *M. aeruginosa* materials (Šrédlová et al., 2021; Yu et al., 2021); however, decreased cell viability following treatments with exudates of "Non-Toxic" *M. aeruginosa* cultures, particularly in RTgill-W1 where the toxicity of exudates overlapped across strains (**Figure 2.5.D**), implicates the contribution of non-microcystin metabolites in the adverse

outcomes of my treatments. RTgill-W1 has earned a reputation as a sensitive and robust cell line assay (Tanneberger et al., 2013) and its strong predictive capacity likely contributed to its selection for the OECD guideline for fish cell line acute toxicity testing (OECD, 2021). Fish gills are in direct contact with their external environment at all times during respiration as water, and any dissolved toxicants, passes through the gill tissues, an exposure method that shares some similarities with my presentation of exudates to my test cells. In my study, the response of RTgill-W1 suggests that both toxigenic and non-toxigenic strains of *M. aeruginosa* produce metabolites capable of impairing cellular functions in gill cells, findings that could lead to serious health consequences for fish encountering *Microcystis* algal blooms. However, further research is needed to determine the true predictive capabilities of RTgill-W1 when it comes to *Microcystis* metabolites and larger scale consequences for gill tissues and respiratory functions. As extracellular metabolites are isolated and characterized, knowledge of their toxic modes of action can be used to improve the sensitivity and relevance of this assay.

My third sensitive cell line, RT-milt5, may represent a novel tissue for studying the effects of *M. aeruginosa* and its metabolites. *Microcystis aeruginosa* exudates can negatively impact the motility and lifespan of fish sperm (Zi et al., 2018), however, how somatic cells found in milt contribute to reproductive success is still unknown. Fish spawning under bloom conditions could be at risk of degraded seminal fluid, depriving gametes of the potential protective elements (Billard, 1983), although this intersection is more likely in fall spawning fish such as lake trout (Lane et al., 1996). However, with limited characterization of the cell line at this time, more work is needed to draw definitive conclusions on potential whole animal consequences and subsequent reproductive effects.

Cell injury and impairment was measured using three dye-based assays: AlamarBlue, CFDA-AM, and Lysotracker Red + NucBlue. Fluorescent dye assays, such as AB and CFDA-AM, rely on the conversion of cell permeable dyes from their non-fluorescent form to their non-permeable fluorescent form via critical cell enzymes, in this case, oxidoreductases associated with cell metabolism and non-specific esterases associated with cell membrane integrity respectively (Dayeh et al., 2013). My results found that AB-based assays were more sensitive to impairment by *M. aeruginosa* metabolites across all of my tested cell lines (**Figure 2.12, 2.13, 2.14,** and **2.15**). This may indicate that these compounds compromise the reducing environment of cells or inhibit other aspects of cellular metabolism with effects on membrane integrity or esterase inhibition largely absent (Dayeh et al., 2013).

Lysosomal activity in cells can either be indicative of normal cell functioning as cells degrade and recycle redundant or aging cell components or it can be a sign of increased cell stress as cells remove damaged or malfunctioning organelles and foreign materials (Moore et al., 2008; Lenz et al., 2018). In my final viability assay, only significant increases in lysosomal activity were observed across my treated cell lines (**Figure 2.16**), indicating that cells were increasing autophagic processes (Lenz et al., 2018). When these increases coincide with cytotoxicity observed in my other assays, this may suggest that the cell's ability to mitigate cellular damages has been overwhelmed, furthering the toxic effects of compounds present in my treatments (Lenz et al., 2018).

Growth phase-dependent toxicity was evident in some of my cell lines, with greater toxicity observed in the log phase exudates of my toxigenic strain. In my "Non-Toxic" strain, however the deleterious effects of exudates were generally equal or greater following exposure to exudates of the stable growth phase (**Figure 2.5.D** and **G**). These results strongly contrast the

results of Sorichetti et al., (2014), in which log phase extracellular metabolites of either toxigenic or non-toxigenic *M. aeruginosa* had no effect on cell viability while stable phase exudates were strongly cytotoxic. While variability in metabolite production across strains could contribute to the differences between these studies, to the best of my knowledge these two studies are the extent of current research on *Microcystis* spp. growth phase-dependent toxicity in fish, highlighting a significant gap in current cyanobacterial knowledge.

In Chapter 3, I examined how *M. aeruginosa* extracellular metabolites altered the expression of genes critical to reproductive processes. In imperiled populations, sub-lethal consequences of toxicant exposure that impair reproductive success can greatly impact the sustainability of that population (Kidd et al., 2007) on top of the loss of individuals through direct fatalities. *M. aeruginosa* cultures and microcystins have negatively impacted reproduction through the disruption of signalling pathways, steroidogenesis, and gamete quality (Zhao et al., 2015; Liu et al., 2018). Limited research has been conducted; however, on the contributions of non-toxigenic strains and non-MC metabolites to this reproductive toxicity.

RT-qPCR analysis revealed that both "Toxic" and "Non-Toxic" strains of *M. aeruginosa* produced extracellular metabolites capable of dysregulating genes within the reproductive pathway in both brain- and gonadal tissue-derived fish cell lines (**Figure 3.4**). The HPG axis is responsible for the regulation and timing of critical reproductive processes, including the generation, maturation, and release of gametes (Yaron & Levavi-Sivan, 2011), and which are conducted primarily through the production and circulation of the major sex steroid hormones: estrogens, progestogens, and androgens (Tenugu et al., 2021). I found that the gonadotropin FSH and its receptor were downregulated following exudate treatments (**Figure 3.2.C** and **Figure 3.3.C** and **D**). FSH is associated with the early stage of gametogenesis and its binding to the

gonads not only initiates steroidogenesis, but also increases the uptake of vitellogenins by growing oocytes (Lubzens et al., 2010; Yaron & Levavi-Sivan, 2011), implicating the disruption of these genes in potential degradation of gamete quality and delayed germ cell development. On the other hand, upregulation of the LH receptor may indicate a disturbance in gamete maturation that could lead to early ovulation/spermiation (Yaron & Levavi-Sivan, 2011).

In terms of steroidogenesis, downregulation of the genes *p450scc* and *3β-hsd* could result in impaired sex steroid synthesis in the brain and gonadal tissues, particularly progestogen production for the latter and steroid hormone synthesis in general for the former, as P450scc catalyzes the rate-limiting step of steroid synthesis upstream of all sex steroid hormones (Tenugu et al., 2021). However, much more work is needed to determine how well my *in vitro* assays align with whole fish effects and their capacity to predict consequences of exposure *in vivo*. From previous studies, dysregulation of these genes by *M. aeruginosa* complex mixtures and MCs co-occurs with decreases in hatching and fertilization success, increases in immature gametes, and alterations to sex steroid hormone levels (Zhao et al., 2015; Liu et al., 2016; Liu et al., 2018).

Overall, it is important to keep in mind the limitations of *in vitro* fish cell lines to effectively utilize them in further research with *Microcystis* metabolites and other compounds. Knowledge of a cell line's functionalities is critical to cell line selection and this knowledge base is constantly expanding through *in vitro* research utilizing these lines, for example, my brain tissue-derived cell lines RTBrain and RTP-2 had different patterns of basal expression for my focal genes, i.e. differences in the presence of *lhr* gene expression (**Figure 3.2.B** and **Figure 3.3.D**). With this in mind, further reproductive toxicity testing would greatly benefit from the use of a broader complement of relevant genes to better capture putatively altered gene expression in

minimally studied cell lines. Lack of cultivation or characterization information for many cell lines may limit the information that can be gathered from those lines. In this case, sex-dependent differences in gene expression were observed in previous MC-based studies of reproductive toxicity (Liu et al., 2016); however, only some of the cell lines used here have recorded sexes for their donor animals – for example, RTL-W1 was cultured from tissue of a male trout (Lee et al., 1993), while RTBrain is of unspecified origin, hampering more thorough comparisons between my study and those previously conducted. As current lines are utilized in more studies and new cell lines are generated, good record-keeping will also greatly benefit future *in vitro* research using these cell lines.

Nevertheless, my study represents a novel examination of the effects of *M. aeruginosa* extracellular metabolites on gene expression in fish. The ability of exudates from a non-toxigenic *M. aeruginosa* strain to significantly downregulate the expression of genes within the HPG axis in my gonadal cell line RTG-2 as well as steroidogenic genes in both RTG-2 and RTBrain, as strongly as exudates of a microcystin-producing strain, indicates the potential for 'non-toxic' *M. aeruginosa* strains and non-MC metabolites to significantly contribute to the disruption of reproductive processes in fish exposed to cyanobacterial algal blooms.

4.2 Conclusions and future research

Non-microcystin-producing *M. aeruginosa* strains are common contributors to *Microcystis*-dominated algal blooms (Wilson et al., 2005; Davis et al., 2009), sometimes overshadowing the contributions of their toxigenic counterparts (Rinta-Kanto et al., 2009). However, expanding cyanobacterial research has made their title of 'non-toxic' increasingly erroneous. Several studies have highlighted the impact of 'non-toxic' *M. aeruginosa* cultures and extracts on hepatic histopathological integrity and protein dysregulation (Le Manach et al., 2016;

Sotton et al., 2017b; Le Manach et al., 2018). In this study I present an examination of cytotoxic and sub-lethal reproductive effects on an anatomical scope much wider than ever previously studied. Through the use of rainbow trout cell lines, my research revealed that "Non-Toxic" strains of *M. aeruginosa* produced metabolites that can severely impair cell viability in cells derived from the tissues of the brain, gills, and milt of fish as well as significantly dysregulate reproduction-critical genes in brain and gonadal cells.

These results highlight the need to integrate these non-toxigenic strains and non-MC metabolites into cHABs risk assessments to more accurately encapsulate the true potential harm *Microcystis* blooms can inflict on aquatic populations, both in terms of their own toxicities and their synergistic interactions with MCs. *Microcystis* cyanobacteria can produce a multitude of biologically active compounds and the experimental use of complex cyanobacterial mixtures offers a closer approximation of field conditions than MCs alone (Sotton et al., 2017b; Saraf et al., 2018). However, identification and isolation of cyanobacterial metabolites remains a limited area of research. In conjunction with my study, mass spectrometry (MS) was performed using exudates of our *M. aeruginosa* CPCC 300 and 633 strains in the hopes of determining the compounds produced and released by *Microcystis*, information that will ultimately be critical in identifying toxic metabolites (unpublished data). This pilot study revealed over 100 potential compounds using the METLIN metabolome database (METLIN, >22,000 metabolites). The power of MS and related techniques to ascertain potential Microcystis-produced compounds has been instrumental in furthering cyanobacterial research (Racine et al., 2019), however further validation must be performed to concretely identify these metabolites.

Isolation of these metabolites would be invaluable in advancing knowledge of algal bloom toxicity, not only by facilitating study of toxic mechanisms but, in studies such as this, aid

in the separation of the adverse effects of cyanobacterial exudates from those of the culture's growth media. In this regard, individual or mixed solutions of cyanotoxins would be incredibly advantageous in further *in vitro* testing, particularly in the cell lines such as RTHDF, which displayed significantly pronounced sensitivity to my own cyanobacterial growth media, BG-11. Expanding upon the methodologies of my study, in addition to the media control used here, the incorporation of other controls for testing may also be useful in further elucidation of cyanobacterial toxic effects. For example, with MC concentrations derived from the ELISA assays, equal concentrations of dissolved MC-LR could be utilized to compare mixed solution and purified toxin effects as used in previous studies (e.g. Saraf et al, 2018) to examine potential synergistic effects from the metabolites produced by the CPCC 300 *M. aeruginosa* strain. Another potential option for further testing would be to harvest exudates from benign species of green algae grown in BG-11, such as *Scenedesmus* spp. or *Chlorella* spp., which have also been previously used in prior *M. aeruginosa* studies (Sotton et al., 2017a), to better approximate the more depleted media in exudate solutions.

Microcystis-dominated cyanobacterial blooms can pose a significant risk to freshwater fish species and the potential effects of these blooms can not be accurately measured by assessments that rely on MC concentrations alone, as highlighted in both my study and previous research (Saraf et al., 2018; Natumi & Janssen, 2020). While further testing is needed to confirm *in vivo* consequences for my *in vitro* results, non-MC metabolites are a significant factor that should be considered in future risk assessments in freshwater environments in the future. In addition, with blooms expected to continue expanding into novel ecosystems (Ho et al., 2019), future conservation efforts must take into account the potential for *M. aeruginosa* blooms to

impair reproductive processes in co-habiting fish when making decisions regarding imperiled species in both current and future bloom-affected ecosystems.

Finally, characterization of not only these non-MC compounds, but also of fish cell lines that are currently minimally described, would greatly aid in cell line selection in future assays and beyond that, improve the sensitivity and accuracy of future *in vitro* research with not only cyanobacterial compounds, but other ecotoxicants that pose a risk to fish populations.

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APPENDICES

APPENDIX 1.1: BG-11 CYANOBACTERIAL GROWTH MEDIA

BG-11 growth medium recipe was provided by CPCC and modified from medium recipe by Rippka et al. (1979).

	Media Component	*Stock Solution*	*mL/Litre*
1	$NaNO_3$	150 g/L	10 mL
2	K_2HPO_4	30 g/L	1 mL
3	$MgSO_4 \cdot 7H_2O$	75 g/L	1 mL
4	$CaCl_2 \cdot 2H_2O$	36 g/L	1 mL
5	Citric Acid combined with Ammonium iron (III) citrate	6 g/L 6 g/L	1 mL
6	$Na_2EDTA \cdot 2H_2O$	1 g/L	1 mL
7	Na_2CO_3	20 g/L	1 mL
8	Trace Metal Solution	*See below	1 mL
9	F/2 Vitamin Solution	*See below	1 mL

Add components #1 through 8 to 900 mL of dH_2O. Bring total volume up to 1000 mL with additional dH_2O and stir on a magnetic stirrer for at least 30 to 40 minutes. Adjust pH to 7.5 and dispense media into two 1000 mL Erlenmeyer flasks. Autoclave media for 60 minutes at 121°C. Once cooled, add the F/2 Vitamin Solution aseptically in a biological safety cabinet.

Trace Metal Solution

Solution Component	*g/Litre*
H_3BO_3	2.86 g
$MnCl_2 \cdot 4H_2O$	1.81 g
$ZnSO_4 \cdot 7H_2O$	0.222 g
$Na_2MoO_4 \cdot 2H_2O$	0.390 g
$CuSO_4 \cdot 5H_2O$	0.079 g
$Co(NO_3)_2 \cdot 6H_2O$	0.0494 g

In the order listed, add components to 900 mL of dH_2O and stir until fully dissolved. Add additional dH_2O up to 1000 mL total volume.

F/2 Vitamin Solution

Solution Component	Stock Solution	mL/100 mL or mg/100 mL
Vitamin B12	1 mg/mL	0.1 mL
Biotin	0.1 mg/mL	1.0 mL
Thiamine HCl	--	20 mg

For a working solution of the F/2 Vitamins, add the listed amounts of each stock solution/reagent to 100 mL of dH2O. This solution cannot be autoclaved and must be filter sterilized with a 0.22 µm filter. Once sterilized, aliquot solution into 1 mL cryovials and store at -20 °C. Remaining working solution should be stored in containers sealed with Parafilm.

APPENDIX 1.2: MICROCYSTIS AERUGINOSA GROWTH CURVE

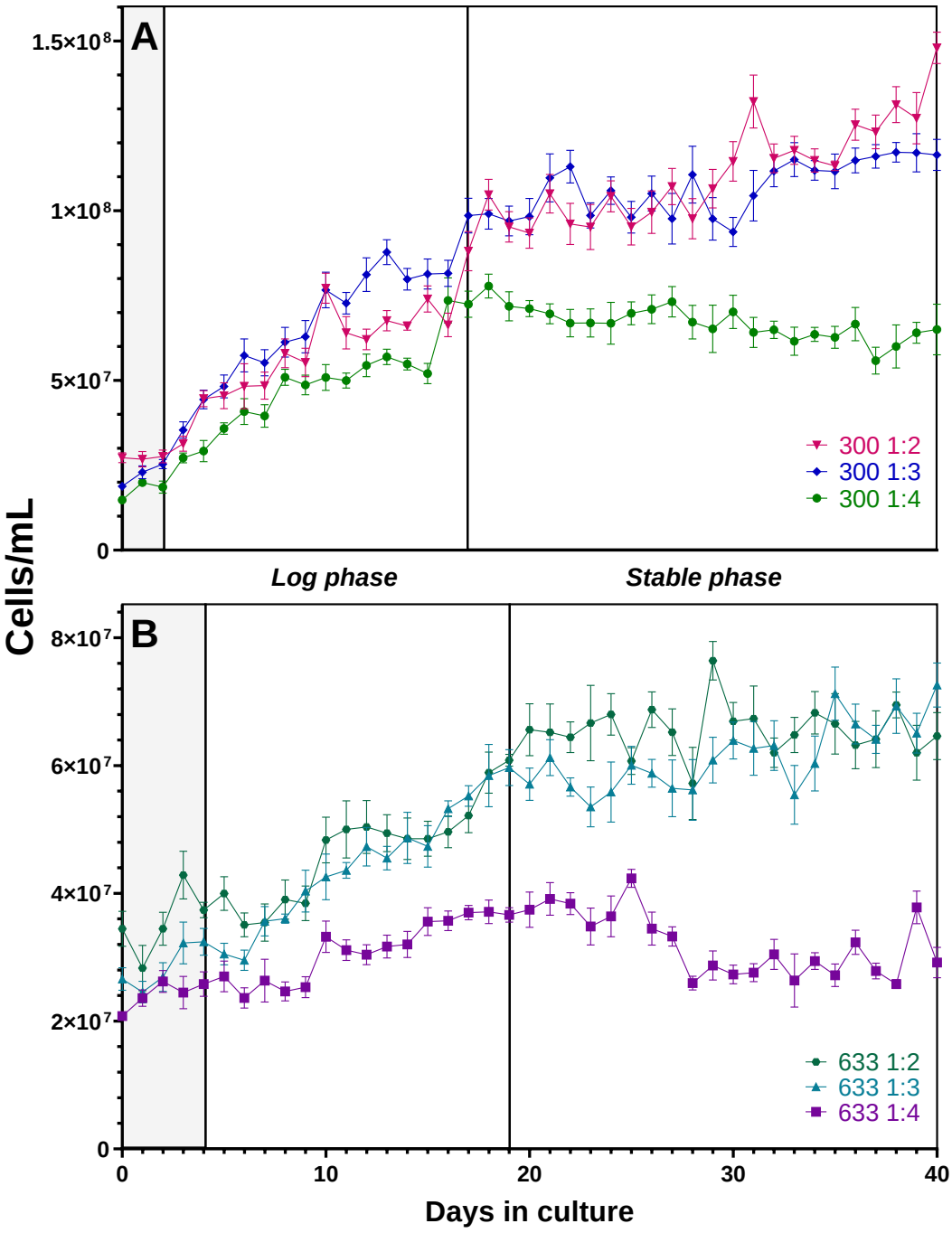

Figure A1.2.1: Growth curves of *M. aeruginosa* strains CPCC 300 (**A**) and 633 (**B**), grown from three starting dilutions of lab cultures and counted daily using a compound microscope and haemocytometer. Values are presented as mean ± SEM. Following an initial lag phase, approximate delineations for log and stable growth phases are labelled as such.

APPENDIX 1.3: LYSOTRACKER IMAGES

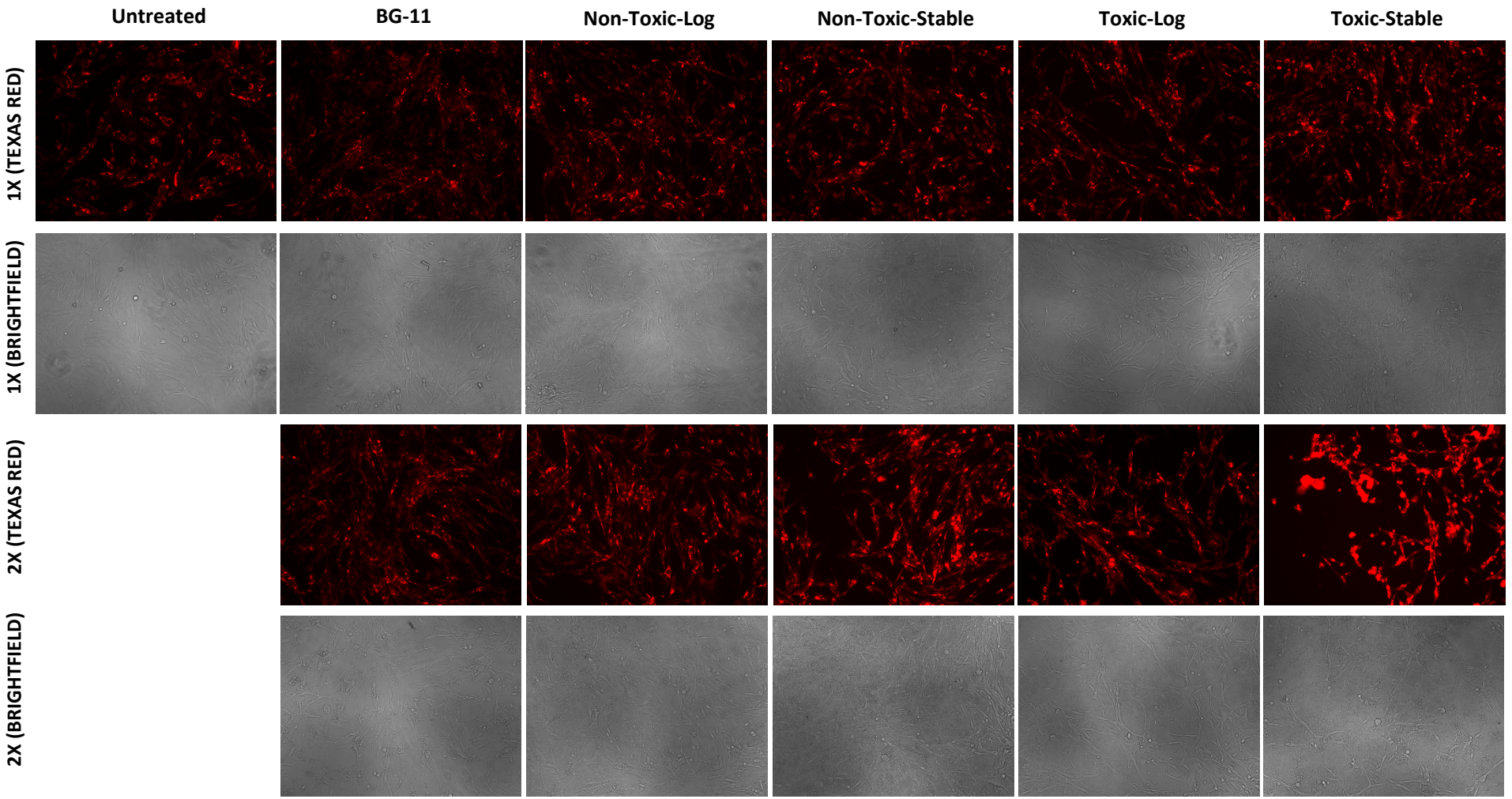

Figure A1.3.1: Lysotracker Red DND-99 staining of lysosomes in the heart cell line RBT4BA alongside brightfield images of cell morphology following exposure to TL, TS, NTL, and NTS exudates as well as controls. Images were taken on an EVOS m5000 inverted microscope (Invitrogen) at 10X magnification.

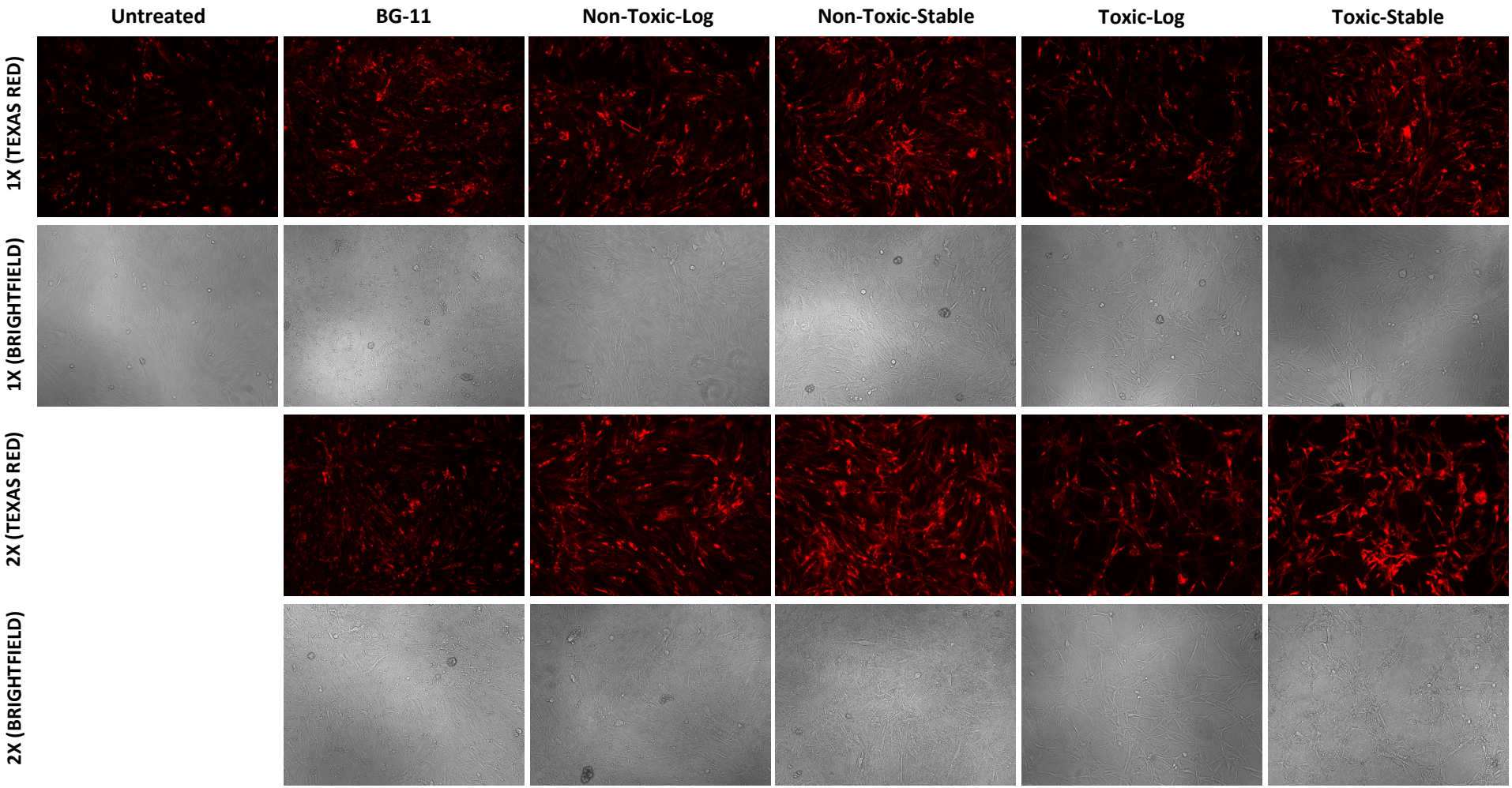

Figure A1.3.2: Lysotracker Red DND-99 staining of lysosomes in the heart cell line RTBrain alongside brightfield images of cell morphology following exposure to TL, TS, NTL, and NTS exudates as well as controls. Images were taken on an EVOS m5000 inverted microscope (Invitrogen) at 10X magnification.

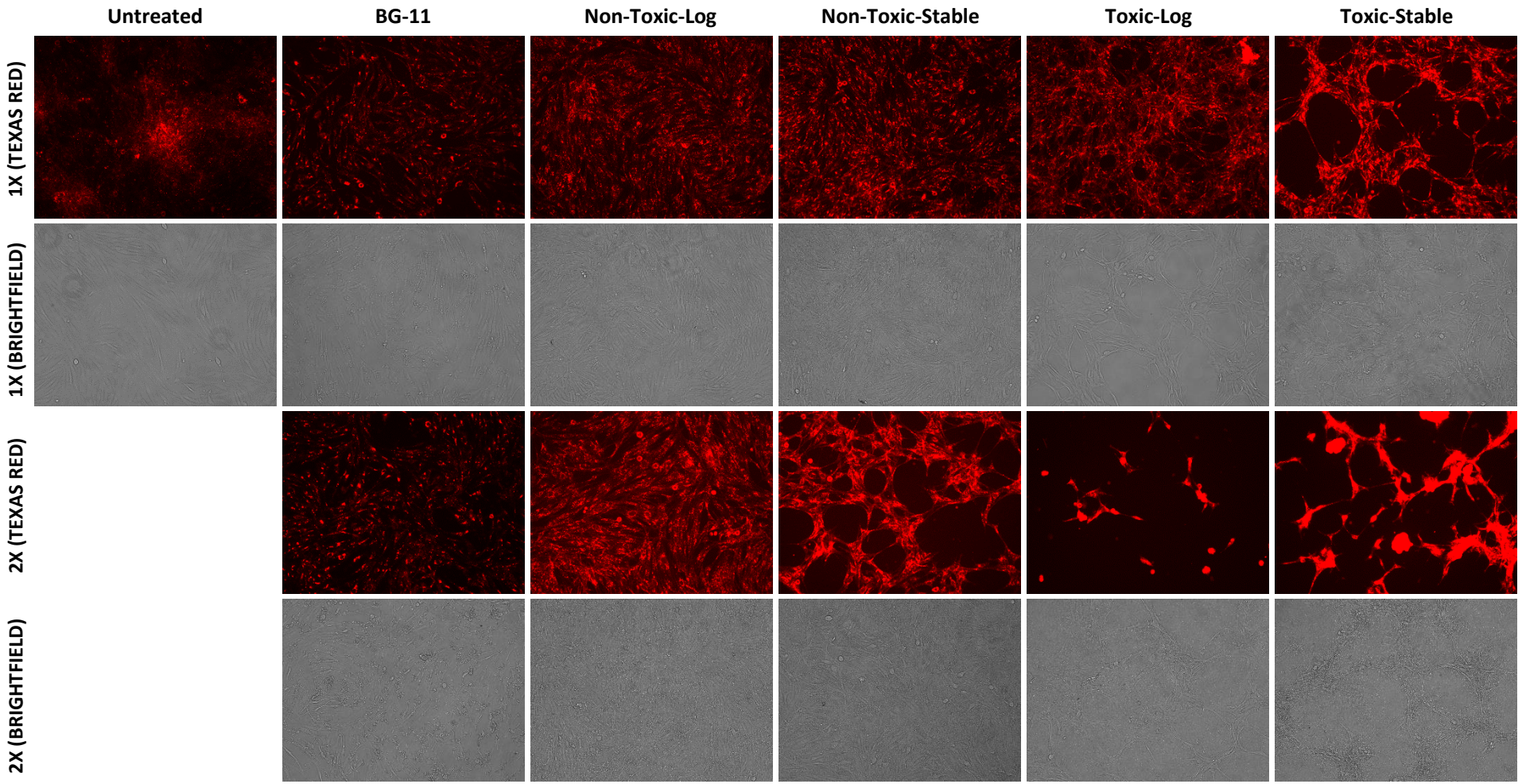

Figure A1.3.3: Lysotracker Red DND-99 staining of lysosomes in the heart cell line RTG-2 alongside brightfield images of cell morphology following exposure to TL, TS, NTL, and NTS exudates as well as controls. Images were taken on an EVOS m5000 inverted microscope (Invitrogen) at 10X magnification.

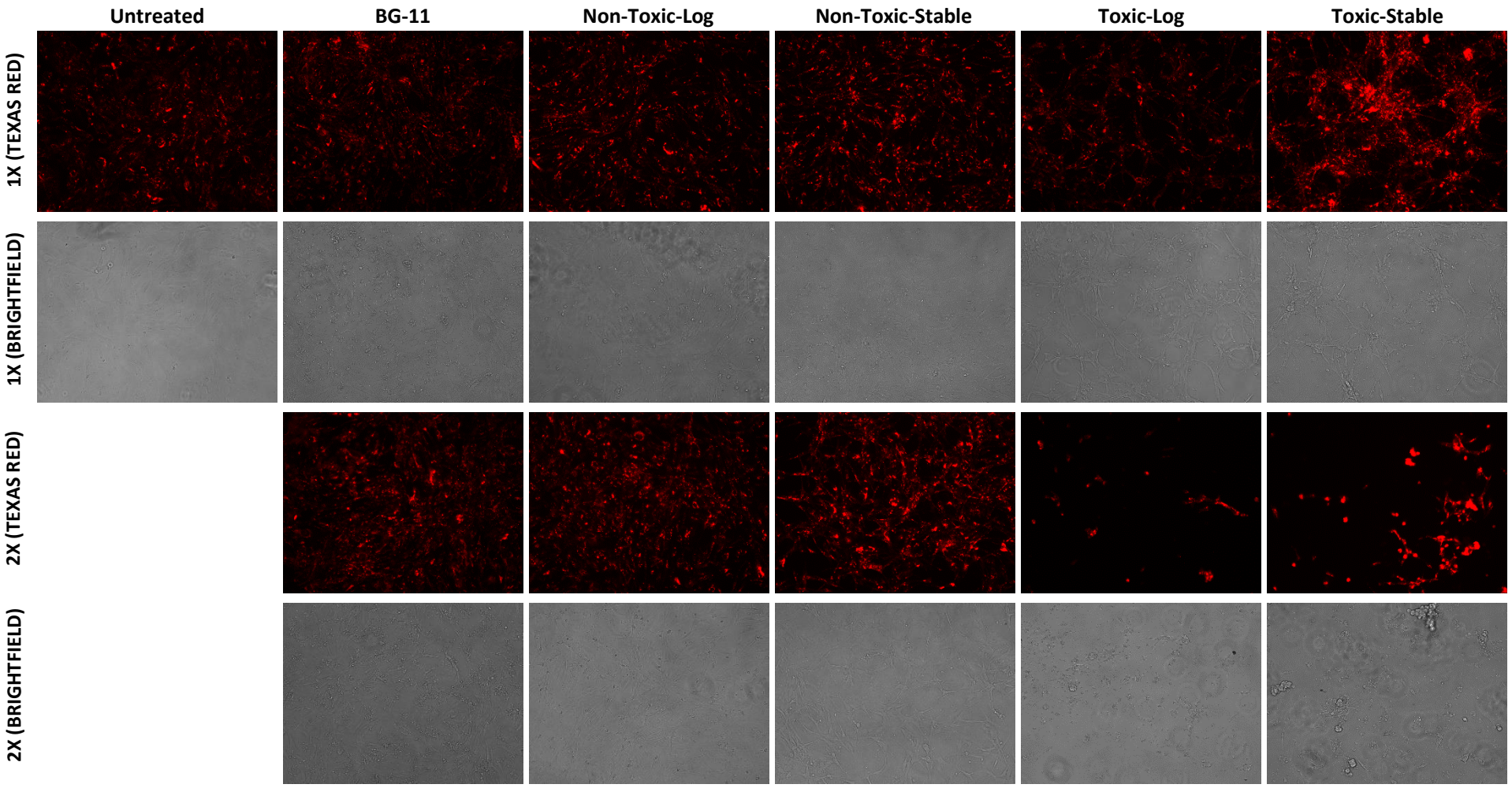

Figure A1.3.4: Lysotracker Red DND-99 staining of lysosomes in the heart cell line RTgill-W1 alongside brightfield images of cell morphology following exposure to TL, TS, NTL, and NTS exudates as well as controls. Images were taken on an EVOS m5000 inverted microscope (Invitrogen) at 10X magnification.

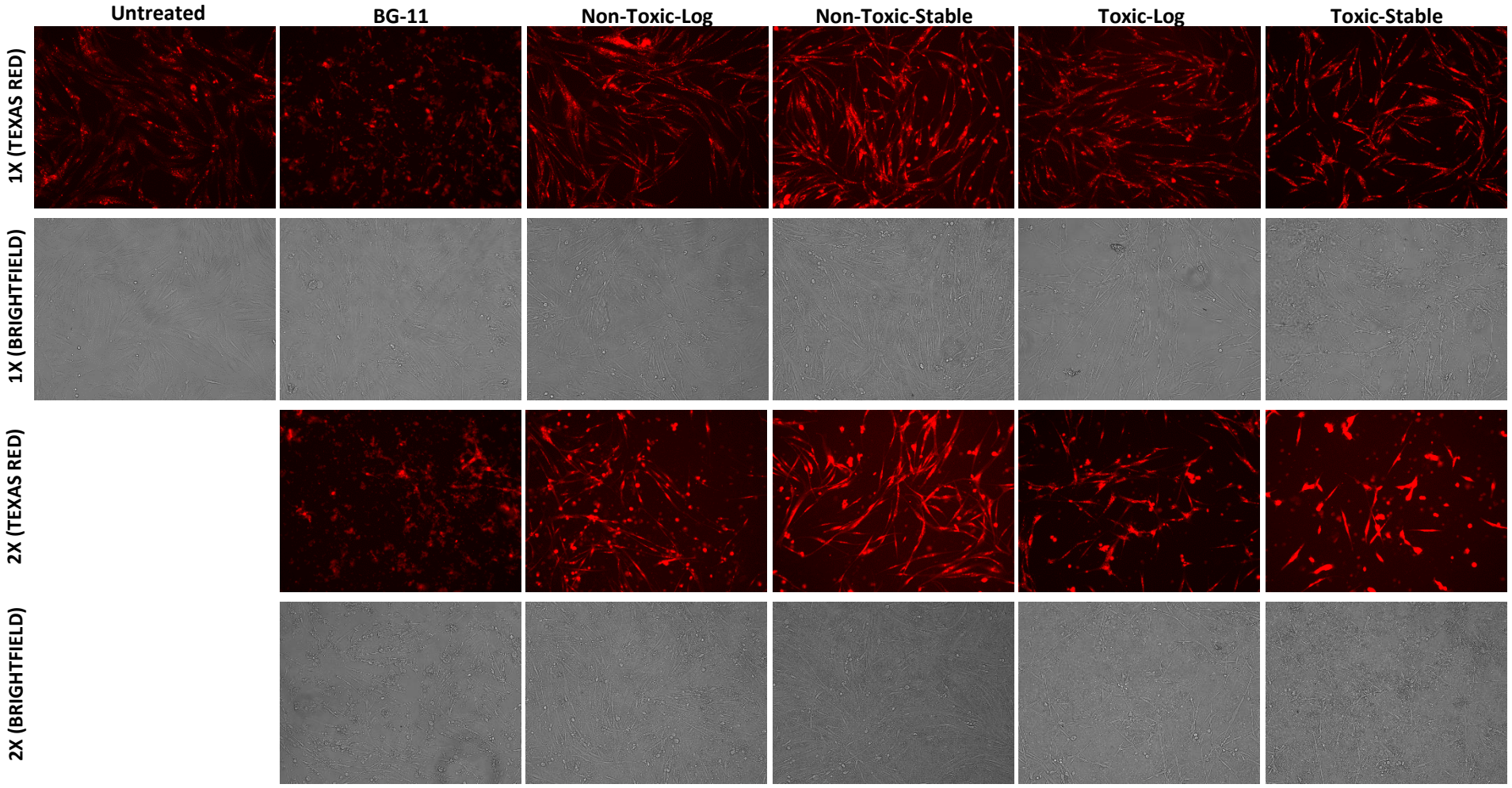

Figure A1.3.5: Lysotracker Red DND-99 staining of lysosomes in the heart cell line RTHDF alongside brightfield images of cell morphology following exposure to TL, TS, NTL, and NTS exudates as well as controls. Images were taken on an EVOS m5000 inverted microscope (Invitrogen) at 10X magnification.

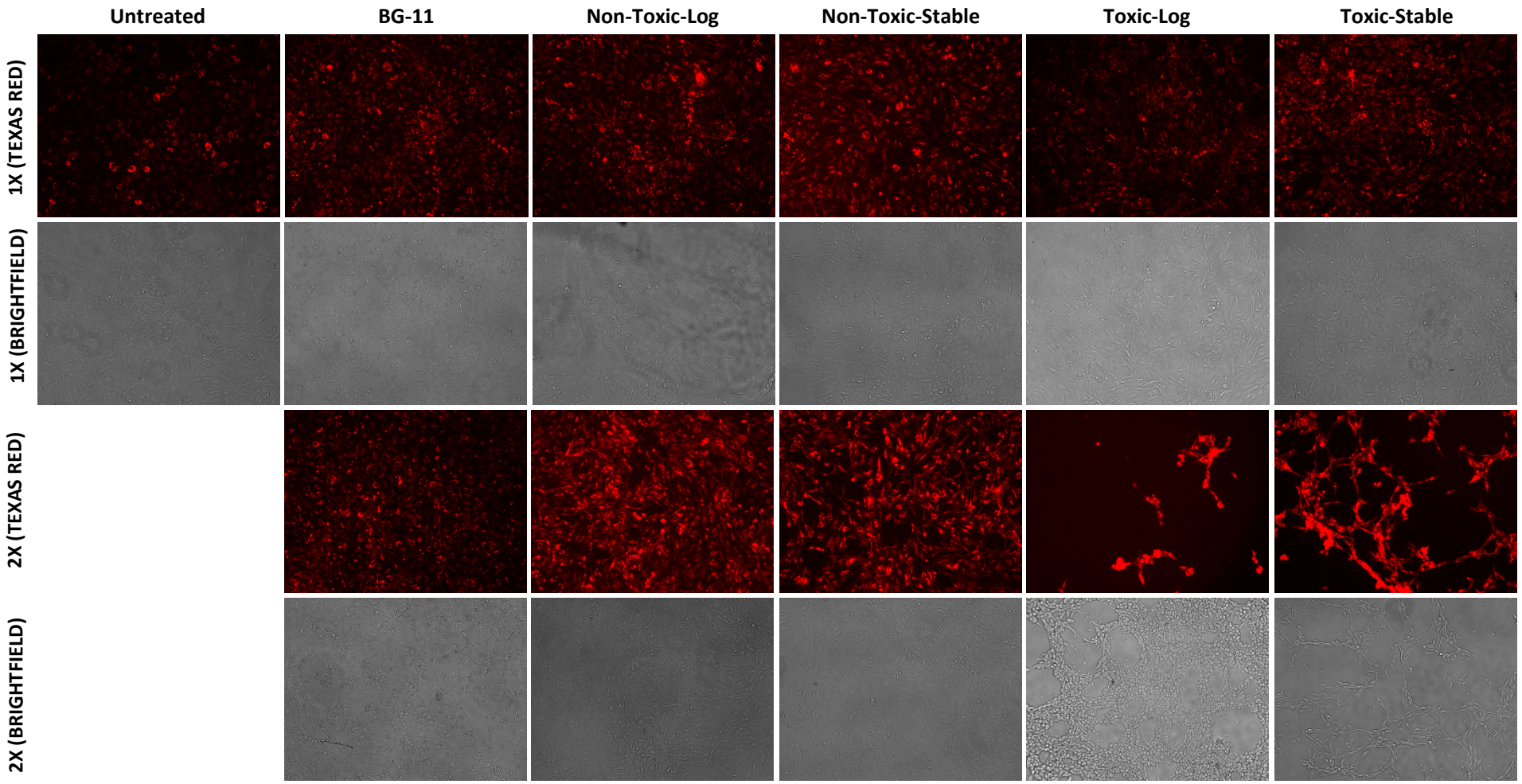

Figure A1.3.6: Lysotracker Red DND-99 staining of lysosomes in the heart cell line RTL-W1 alongside brightfield images of cell morphology following exposure to TL, TS, NTL, and NTS exudates as well as controls. Images were taken on an EVOS m5000 inverted microscope (Invitrogen) at 10X magnification.

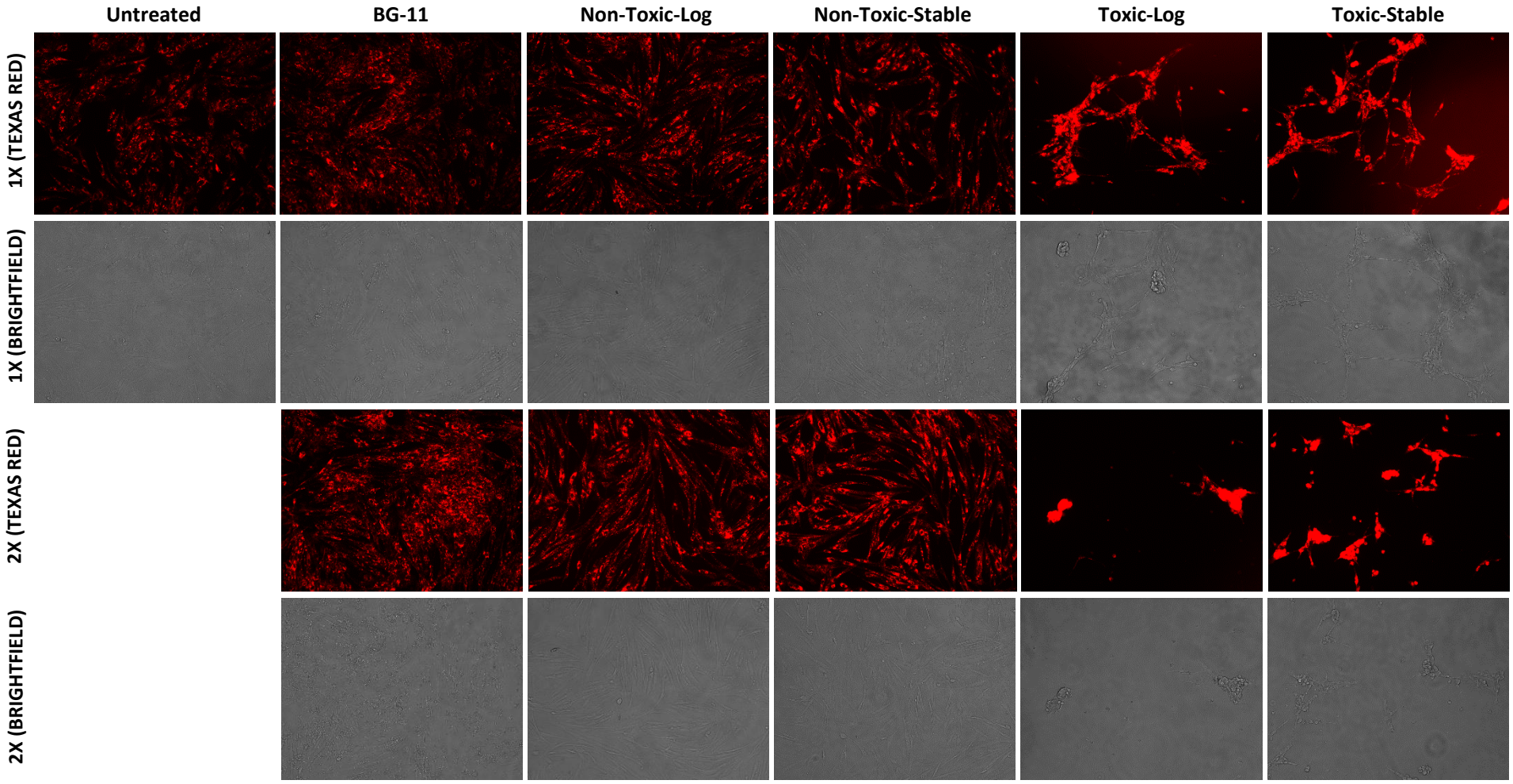

Figure A1.3.7: Lysotracker Red DND-99 staining of lysosomes in the heart cell line RT-milt5 alongside brightfield images of cell morphology following exposure to TL, TS, NTL, and NTS exudates as well as controls. Images were taken on an EVOS m5000 inverted microscope (Invitrogen) at 10X magnification.

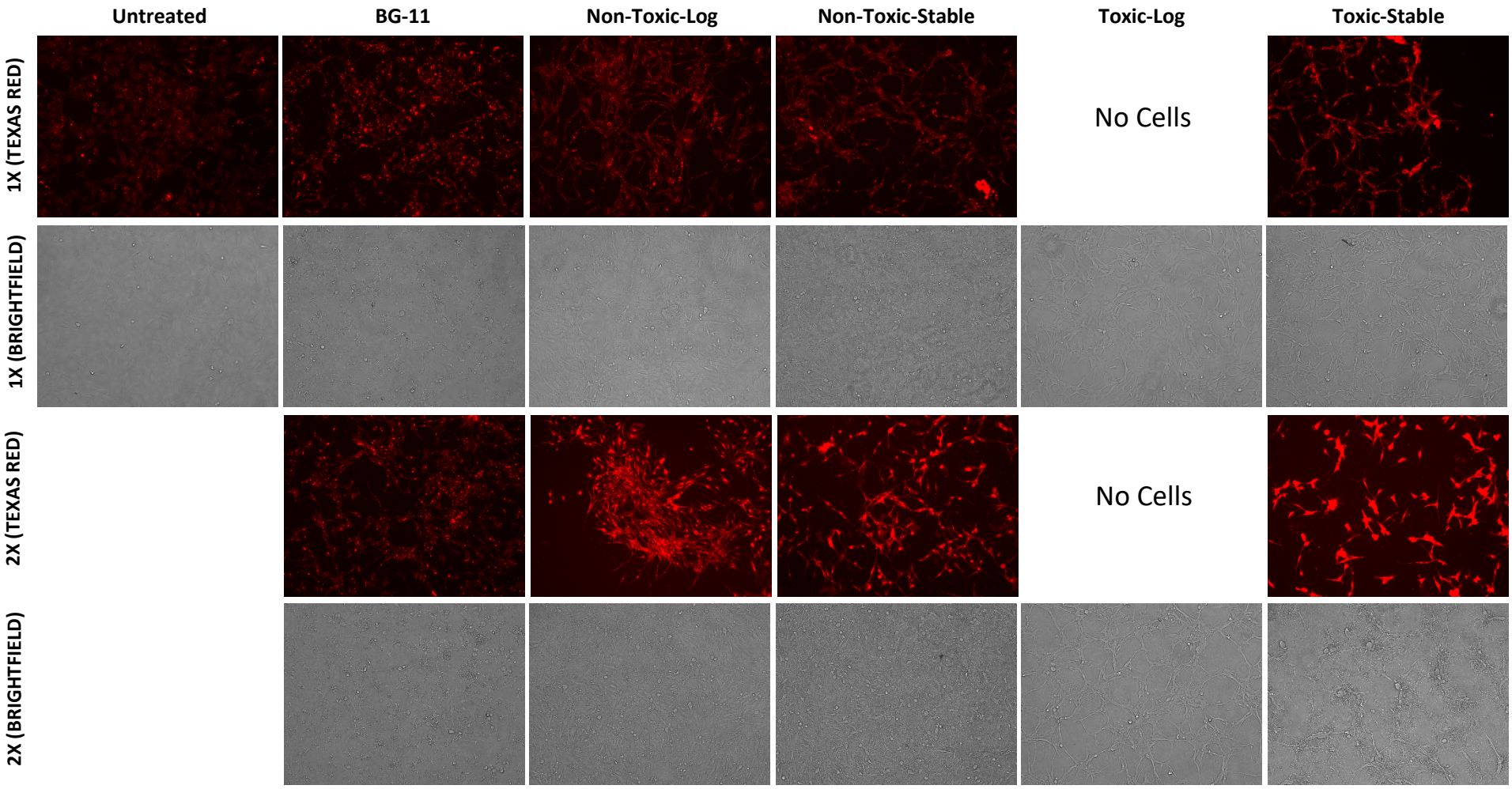

Figure A1.3.8: Lysotracker Red DND-99 staining of lysosomes in the heart cell line RTP-2 alongside brightfield images of cell morphology following exposure to TL, TS, NTL, and NTS exudates as well as controls. Images were taken on an EVOS m5000 inverted microscope (Invitrogen) at 10X magnification.

APPENDIX 1.4 CHAPTER 2 SUPPLEMENTAL TABLES

Table A1.4.1: Mean (± SEM) **AlamarBlue** cell viability values and significance compared to vehicle controls of rainbow trout cell lines treated with TL, TS, NTL, and NTS exudate solutions at concentrations of 0.5X, 1X, and 2X. **Bolded** values indicate differences from controls with *p* values < 0.05.

Cell Line	Concentration	Toxic-Log		Toxic-Stable		Non-Toxic-Log		Non-Toxic-Stable	
		Mean Viability	*P* value	Mean Viability	*P* value	Mean Viability	*P* value	Mean Viability	*P* value
RBT4BA	0.5X	110.8225 ± 10.697	0.3862	126.713 ± 10.971	0.0929	132.535 ± 11.188	0.0621	132.210 ± 11.684	0.0703
	1X	75.4975 ± 9.444	0.0808	69.970 ± 12.497	0.0956	119.000 ± 6.079	0.0523	110.555 ± 6.552	0.2056
	2X	**24.2675 ± 9.283**	**0.0039**	**27.330 ± 3.596**	**0.0003**	119.818 ± 7.949	0.0882	101.065 ± 7.353	0.8940
RTBrain	0.5X	58.957 ± 9.582	0.2500	**66.56 ± 4.723**	**0.0194**	**72.080 ± 4.569**	**0.0257**	**72.240 ± 2.819**	**0.0102**
	1X	**55.163 ± 7.196**	**0.0248**	**52.537 ± 2.562**	**0.0029**	**70.417 ± 3.792**	**0.0160**	**66.080 ± 3.840**	**0.0126**
	2X	**34.790 ± 0.861**	**0.0002**	**38.953 ± 2.292**	**0.0014**	**66.283 ± 2.789**	**0.0068**	**57.160 ± 8.066**	**0.0337**
RTG-2	0.5X	114.475 ± 16.705	0.5455	104.430 ± 6.940	0.5886	110.290 ± 7.597	0.3083	107.36 ± 2.922	0.1281
	1X	86.190 ± 4.360	0.1947	81.883 ± 17.444	0.4081	103.423 ± 6.411	0.6467	94.767 ± 4.143	0.3338
	2X	31.140 ± 8.180	0.0753	**45.775 ± 0.575**	**0.0068**	100.410 ± 5.610	0.9536	77.995 ± 2.625	0.0756
RTgill-W1	0.5X	**92.980 ± 1.039**	**0.0066**	103.178 ± 1.952	0.1644	**93.778 ± 1.418**	**0.0219**	**94.160 ± 1.462**	**0.0104**
	1X	**74.707 ± 3.529**	**0.0008**	**72.867 ± 3.628**	**0.0007**	95.427 ± 3.269	0.2207	**76.160 ± 2.497**	**0.0002**
	2X	**46.233 ± 0.840**	**<0.0001**	**33.008 ± 1.326**	**<0.0001**	**71.873 ± 2.277**	**<0.0001**	**40.433 ± 2.408**	**<0.0001**
RTHDF	0.5X	109.373 ± 14.336	0.5599	98.900 ± 10.929	0.9262	100.685 ± 10.957	0.9541	84.373 ± 9.168	0.1868
	1X	80.640 ± 38.661	0.6662	80.485 ± 24.893	0.4903	127.443 ± 29.814	>0.9999	102.577 ± 16.604	0.8909
	2X	**214.867 ± 22.890**	**0.0375**	150.490 ± 23.466	0.1643	313.747 ± 75.880	0.1063	192.520 ± 42.348	0.1605
RTL-W1	0.5X	107.464 ± 7.728	0.3888	**126.488 ± 8.161**	**0.0315**	120.382 ± 9.468	0.0977	**123.148 ± 4.513**	**0.0068**
	1X	109.198 ± 7.437	0.2838	102.906 ± 8.191	0.7407	119.042 ± 6.879	0.0504	110.600 ± 9.839	0.3420
	2X	**48.602 ± 7.019**	**0.0019**	**54.920 ± 9.437**	**0.0088**	**116.728 ± 4.374**	**0.0187**	89.872 ± 10.008	0.6250
RT-milt5	0.5X	**79.123 ± 3.395**	**0.0086**	**77.060 ± 3.005**	**0.0047**	79.105 ± 7.668	0.0722	**69.893 ± 4.708**	**0.0077**
	1X	**62.423 ± 4.086**	**0.0027**	**59.155 ± 2.526**	**0.0005**	**76.258 ± 6.668**	**0.0378**	**59.895 ± 3.662**	**0.0016**
	2X	**56.638 ± 2.832**	**0.0003**	**46.873 ± 2.304**	**0.0003**	**76.858 ± 6.139**	**0.0011**	**45.665 ± 3.831**	**0.0014**
RTP-2	0.5X	81.475 ± 6.776	0.0761	94.518 ± 4.903	0.3440	106.797 ± 1.978	0.0782	89.623 ± 7.926	0.2830
	1X	**76.100 ± 6.831**	**0.0384**	**72.665 ± 8.234**	**0.0456**	**95.683 ± 0.882**	**0.0390**	**84.230 ± 4.368**	**0.0390**
	2X	76.815 ± 27.402	0.4644	67.373 ± 16.768	0.1906	133.960 ± 30.349	0.3833	93.790 ± 21.271	0.7940

Table A1.4.2: Mean (± SEM) **CFDA-AM** cell viability values and significance compared to vehicle controls of rainbow trout cell lines treated with TL, TS, NTL, and NTS exudate solutions at concentrations of 0.5X, 1X, and 2X. **Bolded** values indicate differences from controls with p values < 0.05.

Cell Line	Concentration	Toxic-Log		Toxic-Stable		Non-Toxic-Log		Non-Toxic-Stable	
		Mean Viability	*P* value	Mean Viability	*P* value	Mean Viability	*P* value	Mean Viability	*P* value
RBT4BA	0.5X	107.885 ± 8.945	0.4429	115.308 ± 8.254	0.1607	111.668 ± 9.516	0.3076	112.313 ± 8.525	0.2444
	1X	90.268 ± 5.150	0.1552	85.178 ± 8.019	0.1617	104.980 ± 2.712	0.1636	103.398 ± 2.674	0.2935
	2X	**39.060 ± 13.532**	**0.0204**	59.958 ± 2.680	0.1250	109.748 ± 4.986	0.1456	110.125 ± 4.115	0.0908
RTBrain	0.5X	97.423 ± 9.637	0.8142	108.023 ± 4.605	0.2236	105.320 ± 5.202	0.4140	108.163 ± 2.300	0.0710
	1X	99.680 ± 1.100	0.8198	97.847 ± 5.710	0.7424	103.727 ± 5.385	0.5605	106.283 ± 3.585	0.2217
	2X	**79.210 ± 3.257**	**0.0237**	101.553 ± 4.331	0.7542	109.117 ± 2.598	0.0725	116.863 ± 8.240	0.1773
RTG-2	0.5X	99.855 ± 6.055	0.9848	104.503 ± 2.720	0.2396	96.773 ± 1.181	0.1120	103.950 ± 2.139	0.2061
	1X	99.260 ± 2.740	0.8321	**112.320 ± 0.320**	**0.0165**	106.800 ± 3.963	0.2283	115.990 ± 4.937	0.0836
	2X	**35.215 ± 3.695**	**0.0363**	91.705 ± 3.225	0.2361	131.875 ± 4.435	0.0880	**138.900 ± 3.030**	**0.0495**
RTgill-W1	0.5X	103.910 ± 2.352	0.1574	**104.615 ± 1.069**	**0.0076**	92.605 ± 3.738	0.1048	92.245 ± 3.881	0.1022
	1X	**91.558 ± 2.238**	**0.0130**	92.685 ± 3.171	0.0692	94.675 ± 5.231	0.3554	99.547 ± 5.549	0.9381
	2X	**75.333 ± 3.219**	**0.0046**	**59.940 ± 3.832**	**0.0313**	106.158 ± 3.974	0.1819	105.247 ± 5.590	0.3910
RTHDF	0.5X	117.800 ± 5.262	0.0774	**121.503 ± 2.704**	**0.0154**	113.485 ± 7.299	0.1618	109.603 ± 7.201	0.3139
	1X	132.765 ± 30.095	0.3559	111.290 ± 19.795	0.6084	148.115 ± 19.178	0.0870	152.567 ± 18.213	0.1020
	2X	**203.490 ± 3.480**	**0.0214**	117.590 ± 37.301	0.6837	286.587 ± 73.145	0.1254	**356.137 ± 30.309**	**0.0137**
RTL-W1	0.5X	101.646 ± 13.626	0.9097	92.980 ± 8.774	0.4685	95.937 ± 9.223	0.6779	87.648 ± 8.511	0.2426
	1X	108.834 ± 3.955	0.0892	118.146 ± 8.305	0.0942	95.396 ± 4.372	0.3517	107.747 ± 6.534	0.2891
	2X	**55.830 ± 5.585**	**0.0014**	105.010 ± 7.212	0.5372	106.045 ± 3.222	0.1573	102.068 ± 3.896	0.8750
RT-milt5	0.5X	**112.958 ± 2.944**	**0.0218**	**119.975 ± 4.203**	**0.0177**	103.613 ± 6.913	0.6374	**110.280 ± 2.762**	**0.0338**
	1X	99.055 ± 8.629	0.9197	104.805 ± 8.565	0.6140	107.735 ± 4.631	0.1934	**119.425 ± 3.131**	**0.0084**
	2X	90.830 ± 3.262	0.0672	**117.423 ± 3.414**	**0.0146**	**136.095 ± 8.317**	**0.0226**	**142.688 ± 8.666**	**0.0160**
RTP-2	0.5X	95.893 ± 10.542	0.7228	117.473 ± 7.530	0.1250	110.737 ± 7.073	0.2683	105.767 ± 2.455	0.1433
	1X	100.770 ± 10.354	0.9454	98.667 ± 7.220	0.8705	111.315 ± 6.828	0.1961	120.153 ± 6.998	0.1024
	2X	157.370 ± 35.569	0.2481	102.210 ± 21.204	0.9265	150.620 ± 23.093	0.1597	132.217 ± 17.787	0.2118

Table A1.4.3: Mean (± SEM) **AlamarBlue** cell viability values and significance compared to vehicle controls of rainbow trout cell lines treated with TL, TS, NTL, and NTS exudate solutions at medium and high relative culture densities. **Bolded** values indicate differences from controls with p values < 0.05.

Cell Line	Relative Density	Toxic-Log		Toxic-Stable		Non-Toxic-Log		Non-Toxic-Stable	
		Mean Viability	*P* value	Mean Viability	*P* value	Mean Viability	*P* value	Mean Viability	*P* value
RBT4BA	Medium	75.498 ± 9.444	0.0808	126.713 ± 10.971	0.0929	119.000 ± 6.079	0.0523	132.210 ± 11.684	0.0703
	High	**24.268 ± 9.283**	**0.0039**	69.970 ± 12.497	0.0956	119.818 ± 7.949	0.0882	110.555 ± 6.552	0.2056
RTBrain	Medium	**55.163 ± 7.196**	**0.0248**	**66.560 ± 4.723**	**0.0194**	**70.417 ± 3.792**	**0.0160**	**72.240 ± 2.819**	**0.0102**
	High	**34.790 ± 0.861**	**0.0002**	**52.537 ± 2.562**	**0.0029**	**66.283 ± 2.789**	**0.0068**	**66.080 ± 3.840**	**0.0126**
RTG-2	Medium	86.190 ± 4.360	0.1947	104.430 ± 6.940	0.5886	103.123 ± 6.411	0.6467	107.360 ± 2.922	0.1281
	High	31.140 ± 8.180	0.0753	81.883 ± 17.444	0.4081	100.410 ± 5.610	0.9536	94.767 ± 4.143	0.3338
RTgill-W1	Medium	**74.707 ± 3.529**	**0.0008**	103.178 ± 1.952	0.1644	95.427 ± 3.269	0.2207	**94.160 ± 1.462**	**0.0104**
	High	**46.233 ± 0.840**	**<0.0001**	**72.867 ± 3.628**	**0.0007**	**71.873 ± 2.277**	**<0.0001**	**76.160 ± 2.497**	**0.0002**
RTHDF	Medium	80.640 ± 38.661	0.6662	98.900 ± 10.929	0.9262	127.443 ± 29.814	>0.9999	84.373 ± 9.168	0.1868
	High	**214.867 ± 22.890**	**0.0375**	80.485 ± 24.893	0.4903	313.747 ± 75.880	0.1063	102.577 ± 16.604	0.8909
RTL-W1	Medium	109.198 ± 7.437	0.2838	**126.488 ± 8.161**	**0.0315**	119.042 ± 6.879	0.0504	**123.148 ± 4.513**	**0.0068**
	High	**48.602 ± 7.019**	**0.0019**	102.906 ± 8.191	0.7407	**116.728 ± 4.374**	**0.0187**	110.600 ± 9.839	0.3420
RT-milt5	Medium	**62.423 ± 4.086**	**0.0027**	**77.060 ± 3.005**	**0.0047**	**76.258 ± 6.668**	**0.0378**	**69.893 ± 4.708**	**0.0077**
	High	**56.638 ± 2.832**	**0.0006**	**59.155 ± 2.526**	**0.0005**	**76.858 ± 6.139**	**0.0327**	**59.895 ± 3.662**	**0.0016**
RTP-2	Medium	**76.100 ± 6.831**	**0.0395**	94.518 ± 4.903	0.3449	**95.683 ± 0.882**	**0.0393**	89.623 ± 7.926	0.2817
	High	76.815 ± 27.402	0.4596	**72.662 ± 8.234**	**0.0451**	133.960 ± 30.349	0.3795	**84.230 ± 4.368**	**0.0365**

Table A1.4.4: Mean (± SEM) **CFDA-AM** cell viability values and significance compared to vehicle controls of rainbow trout cell lines treated with TL, TS, NTL, and NTS exudate solutions at medium and high relative culture densities. **Bolded** values indicate differences from controls with p values < 0.05.

Cell Line	Relative Density	Toxic-Log		Toxic-Stable		Non-Toxic-Log		Non-Toxic-Stable	
		Mean Viability	*P value*	*Mean Viability*	*P value*	*Mean Viability*	*P value*	*Mean Viability*	*P value*
RBT4BA	Medium	90.268 ± 5.150	0.1552	115.308 ± 8.254	0.1607	104.980 ± 2.712	0.1636	112.313 ± 8.525	0.2444
	High	**39.060 ± 13.532**	**0.0204**	85.178 ± 8.019	0.1617	109.748 ± 4.986	0.1456	103.398 ± 2.674	0.2935
RTBrain	Medium	99.680 ± 1.100	0.8198	108.023 ± 4.605	0.2236	103.727 ± 5.385	0.5605	108.163 ± 2.300	0.0710
	High	**79.210 ± 3.257**	**0.0237**	97.847 ± 5.710	0.7424	109.117 ± 2.598	0.0725	106.283 ± 3.585	0.2217
RTG-2	Medium	99.260 ± 2.740	0.8321	104.503 ± 2.720	0.2396	106.800 ± 3.963	0.2283	103.950 ± 2.139	0.2061
	High	**35.215 ± 3.695**	**0.0363**	**112.320 ± 0.320**	**0.0165**	131.875 ± 4.435	0.0880	115.990 ± 4.937	0.0836
RTgill-W1	Medium	**91.558 ± 2.238**	**0.0130**	**104.615 ± 1.069**	**0.0076**	94.675 ± 5.231	0.3554	92.245 ± 3.881	0.1022
	High	**75.333 ± 3.219**	**0.0046**	92.685 ± 3.171	0.0692	106.158 ± 3.974	0.1819	99.547 ± 5.549	0.9381
RTHDF	Medium	132.765 ± 30.095	0.3559	**121.503 ± 2.704**	**0.0154**	148.115 ± 19.178	0.0870	109.603 ± 7.201	0.3139
	High	**203.490 ± 3.480**	**0.0214**	111.290 ± 19.795	0.6084	286.587 ± 73.145	0.1254	152.567 ± 18.213	0.1020
RTL-W1	Medium	108.834 ± 3.955	0.0892	92.980 ± 8.774	0.4685	95.396 ± 4.372	0.3517	87.648 ± 8.511	0.2426
	High	**55.830 ± 5.585**	**0.0014**	118.146 ± 8.305	0.0942	106.045 ± 3.222	0.1573	107.747 ± 6.534	0.2891
RT-milt5	Medium	99.055 ± 8.629	0.9197	**119.975 ± 4.203**	**0.0177**	107.735 ± 4.631	0.1934	**110.280 ± 2.762**	**0.0338**
	High	90.830 ± 3.262	0.0672	104.805 ± 8.565	0.6140	**136.095 ± 8.317**	**0.0226**	**119.425 ± 3.131**	**0.0084**
RTP-2	Medium	100.770 ± 10.354	0.9454	117.473 ± 7.530	0.1250	117.347 ± 4.526	0.0618	105.767 ± 2.455	0.1433
	High	157.370 ± 35.569	0.2481	98.667 ± 7.220	0.8705	150.620 ± 23.093	0.1597	104.317 ± 8.856	0.6741

Table A1.4.5: Calculated *p* values for comparisons of rainbow trout cell line responses to exudate treatments at concentrations of 0.5X, 1X, and 2X and measured using both AlamarBlue and CFDA-AM. **Bolded** values indicate significant differences between treatments.

		AlamarBlue			**CFDA-AM**		
Cell Line	**Comparison**	*0.5X*	*1X*	*2X*	*0.5X*	*1X*	*2X*
RBT4BA	TL vs. TS	0.7476	0.9715	0.9907	>0.9999†	0.8946	0.2631
	TL vs. NTL	0.5351	**0.0231**	**<0.0001**	>0.9999†	0.2317	**0.0001**
	TL vs. NTS	0.5469	0.0729	**<0.0001**	>0.9999†	0.3158	**0.0001**
	TS vs. NTL	0.9819	**0.0108**	**<0.0001**	>0.9999†	0.0754	**0.0028**
	TS vs. NTS	0.9847	**0.0345**	**<0.0001**	>0.9999†	0.1085	**0.0027**
	NTL vs. NTS	>0.9999	0.9092	0.3185	>0.9999†	0.9961	>0.9999
RTBrain	TL vs. TS	0.8057	0.9773	0.9080	0.6219	0.9940	0.0579
	TL vs. NTL	0.4535	0.1756	**0.0045**	0.7939	0.9427	**0.0137**
	TL vs. NTS	0.4440	0.4058	**0.0302**	0.6127	0.8040	**0.0035**
	TS vs. NTL	0.9116	0.1011	**0.0104**	0.9883	0.8060	0.7276
	TS vs. NTS	0.9048	0.2481	0.0766	>0.9999	0.5964	0.2247
	NTL vs. NTS	>0.9999	0.9106	0.5045	0.9864	0.9782	0.7137
RTG-2	TL vs. TS	0.8411	0.9929	0.5568††	0.7077	0.2848	>0.9999†
	TL vs. NTL	0.9849	0.7216	0.0635††	0.8858	0.6136	0.3972†
	TL vs. NTS	0.9336	0.9494	0.1674††	0.7764	0.1084	0.1485†
	TS vs. NTL	0.9468	0.4936	0.1151††	0.2661	0.7929	>0.9999†
	TS vs. NTS	0.9926	0.8117	0.0807††	0.9988	0.9234	0.9183†
	NTL vs. NTS	0.9926	0.9300	0.2303††	0.3174	0.3890	>0.9999†
RTgill-W1	TL vs. TS	**0.0028**	0.9779	**0.0018**	0.9923††	0.9909††	0.1411
	TL vs. NTL	0.9895	**0.0012**	**<0.0001**	0.1202††	0.9442††	**0.0013**
	TL vs. NTS	0.9583	0.9888	0.2515	0.1199††	0.5735††	**0.0017**
	TS vs. NTL	**0.0055**	**0.0005**	**<0.0001**	0.0798††	0.9872††	**<0.0001**
	TS vs. NTS	**0.0030**	0.8903	0.0569	0.0821††	0.7140††	**<0.0001**
	NTL vs. NTS	0.9984	**0.0024**	**<0.0001**	0.9999††	0.9170††	0.9987
RTHDF	TL vs. TS	0.9157	>0.9999	0.7643	0.9771	0.9027	0.6673
	TL vs. NTL	0.9490	0.6847	0.4771	0.9576	0.9607	0.6882
	TL vs. NTS	0.4473	0.9504	0.9855	0.8132	0.9360	0.2527
	TS vs. NTL	0.9995	0.6385	0.1371	0.7929	0.6594	0.1345
	TS vs. NTS	0.8085	0.9393	0.9161	0.5945	0.6340	**0.0343**
	NTL vs. NTS	0.7507	0.9303	0.3210	0.9684	0.9992	0.7274

† : Analyzed via Kruskal-Wallis and Dunn's post hoc test, ††: Analyzed via Welch's ANOVA with Games-Howell's post hoc test

Cell Line	Comparison	AlamarBlue			CFDA-AM		
		0.5X	*1X*	*2X*	*0.5X*	*1X*	*2X*
RTL-W1	TL vs. TS	0.3318	0.9465	0.9434	0.9341	0.7244	**<0.0001**
	TL vs. NTL	0.6425	0.8286	**<0.0001**	0.9768	0.4521	**<0.0001**
	TL vs. NTS	0.4925	0.9993	**0.0107**	0.8069	0.9992	**0.0001**
	TS vs. NTL	0.9419	0.5185	**0.0003**	0.9966	0.0857	0.9991
	TS vs. NTS	0.9895	0.9081	**0.0327**	0.9858	0.6217	0.9804
	NTL vs. NTS	0.9940	0.8831	0.1244	0.9448	0.4863	0.9542
RT-milt5	TL vs. TS	0.9911	0.9542	0.3635	0.6976	0.9277	0.0550
	TL vs. NTL	>0.9999	0.1855	**0.0189**	0.4882	0.7962	**0.0016**
	TL vs. NTS	0.5826	0.9778	0.2728	0.9742	0.1919	**0.0005**
	TS vs. NTL	0.9913	0.0807	**0.0010**	0.1002	0.9892	0.2253
	TS vs. NTS	0.7490	0.9994	0.9965	0.4583	0.4430	0.0705
	NTL vs. NTS	0.5841	0.0981	**0.0007**	0.7286	0.6177	0.8863
RTP-2	TL vs. TS	0.4741	0.9803	0.9922	0.2538	0.9979	0.4594
	TL vs. NTL	0.0821	0.2029	0.4181	0.6016	0.7784	0.9974
	TL vs. NTS	0.7960	0.7555	0.9628	0.8329	0.4106	0.8933
	TS vs. NTL	0.5655	0.1181	0.3473	0.9373	0.7214	0.5591
	TS vs. NTS	0.9379	0.5386	0.8939	0.7531	0.3818	0.8353
	NTL vs. NTS	0.2989	0.6336	0.7175	0.9776	0.8792	0.9534

Table A1.4.6: Calculated *p* values for comparisons of rainbow trout cell line responses to exudate treatments at medium and high relative culture densities and measured using both AlamarBlue and CFDA-AM. **Bolded** values indicate significant differences between treatments.

		AlamarBlue		**CFDA-AM**	
Cell Line	**Comparison**	*Medium density*	*High density*	*Medium density*	*High density*
RBT4BA	TL vs. TS	**0.0172**	**0.0432**	0.3089	**0.0048**
	TL vs. NTL	0.0613	**<0.0001**	0.8570	**<0.0001**
	TL vs. NTS	**0.0066**	**<0.0001**	0.4607	**<0.0001**
	TS vs. NTL	0.9990	**0.0217**	0.9746	0.3306
	TS vs. NTS	0.9999	0.0957	>0.9999	0.6812
	NTL vs. NTS	0.9735	0.9967	0.9966	0.9986
RTBrain	TL vs. TS	0.4942	0.0873	0.8752	0.0693
	TL vs. NTL	0.1872	**0.0008**	0.9974	**0.0017**
	TL vs. NTS	0.1079	**0.0009**	0.8661	**0.0043**
	TS vs. NTL	0.9962	0.2840	0.9927	0.5106
	TS vs. NTS	0.9659	0.2994	>0.9999	0.7967
	NTL vs. NTS	>0.9999	>0.9999	0.9912	0.9995
RTG-2	TL vs. TS	0.8538	**0.0325**	0.9653	**<0.0001**
	TL vs. NTL	0.8844	**0.0064**	0.8227	**<0.0001**
	TL vs. NTS	0.7462	**0.0061**	0.9808	**<0.0001**
	TS vs. NTL	>0.9999	0.8444	0.9995	0.0701
	TS vs. NTS	>0.9999	0.9493	>0.9999	0.9953
	NTL vs. NTS	>0.9999	0.9998	0.9980	0.1273
RTgill-W1	TL vs. TS	**<0.0001**	**<0.0001**	0.2549	0.1025
	TL vs. NTL	**<0.0001**	**<0.0001**	0.9989	**0.0002**
	TL vs. NTS	**0.0002**	**<0.0001**	>0.9999	**0.0056**
	TS vs. NTL	0.4593	>0.9999	0.5898	0.2216
	TS vs. NTS	0.2736	0.9867	0.3169	0.9009
	NTL vs. NTS	>0.9999	0.9440	0.9998	0.9169
RTHDF	TL vs. TS	0.9999	0.0982	>0.9999	0.5462
	TL vs. NTL	0.9702	0.4519	0.9999	0.7136
	TL vs. NTS	>0.9999	0.3053	0.9991	0.9661
	TS vs. NTL	0.9975	**0.0009**	0.9978	**0.0107**
	TS vs. NTS	>0.9999	0.9995	>0.9999	0.9713
	NTL vs. NTS	0.9727	**0.0052**	0.9803	0.1124

Cell Line	Comparison	AlamarBlue		CFDA-AM	
		Medium density	*High density*	*Medium density*	*High density*
RTL-W1	TL vs. TS	0.6972	**0.0002**	0.6690	**<0.0001**
	TL vs. NTL	0.9771	**<0.0001**	0.8190	**0.0003**
	TL vs. NTS	0.8688	**<0.0001**	0.3909	**<0.0001**
	TS vs. NTL	0.9955	0.8740	>0.9999	0.9117
	TS vs. NTS	>0.9999	0.9945	0.9992	0.9303
	NTL vs. NTS	>0.9999	0.9987	0.9921	>0.9999
RT-milt5	TL vs. TS	0.3186	0.9999	0.2498	0.7123
	TL vs. NTL	0.3848	0.0610	0.9649	**0.0004**
	TL vs. NTS	0.9274	0.9994	0.8777	**0.0420**
	TS vs. NTL	>0.9999	0.1370	0.8240	**0.0205**
	TS vs. NTS	0.9407	>0.9999	0.9382	0.6663
	NTL vs. NTS	0.9678	0.1707	>0.9999	0.5157
RTP-2	TL vs. TS	0.9763	>0.9999	0.9866	0.2161
	TL vs. NTL	0.9810	0.1820	0.9918	>0.9999
	TL vs. NTS	0.9966	>0.9999	>0.9999	0.3185
	TS vs. NTL	>0.9999	0.1275	>0.9999	0.3417
	TS vs. NTS	>0.9999	0.9987	0.9990	>0.9999
	NTL vs. NTS	>0.9999	0.3221	0.9994	0.4756

Table A1.4.7: Calculated *p* values for comparisons between the **AlamarBlue** responses of rainbow trout cell lines to exudate treatments at concentrations of 0.5X, 1X, and 2X. **Bolded** values indicate significant differences between treatments.

Cell line comparisons	TL			TS			NTL			NTS		
	0.5X	*1X*	*2X*	*0.5X*	*1X*	*2X*	*0.5X*	*1X*	*2X*	*0.5X*	*1X*	*2X*
RTBrain vs. RBT4BA	**0.0258**	>0.9999†	>0.9999†	**0.0243**†	0.9945††	>0.9999†	**0.0017**	**0.0334**†	0.2880†	**<0.0001**	**0.0085**	>0.9999†
RTBrain vs. RTP-2	0.7535	>0.9999†	0.6281†	>0.9999†	0.8575††	>0.9999†	0.2080	>0.9999†	0.4443†	0.6578	0.7066	>0.9999†
RTBrain vs. RTG-2	0.9916	>0.9999†	>0.9999†	>0.9999†	0.9919††	>0.9999†	0.1331	0.9741†	>0.9999†	**0.0496**	0.2568	>0.9999†
RTBrain vs. RTL-W1	**0.0303**	**0.0317**†	>0.9999†	**0.0083**†	0.0633††	>0.9999†	**0.0111**	**0.0287**†	0.2746†	**0.0003**	**0.0054**	>0.9999†
RTBrain vs. RTgill-W1	0.2909	>0.9999†	>0.9999†	0.6126†	0.0861††	>0.9999†	0.6602	>0.9999†	>0.9999†	0.2927	0.9699	>0.9999†
RTBrain vs. RTHDF	**0.0312**	>0.9999†	0.0813†	>0.9999†	0.9983††	0.6848†	0.3456	0.4027†	**0.0141**†	0.9182	0.0725	0.5044†
RTBrain vs. RTMilt	0.8435	>0.9999†	>0.9999†	>0.9999†	0.9704††	>0.9999†	0.9991	>0.9999†	>0.9999†	>0.9999	0.9990	>0.9999†
RBT4BA vs. RTP-2	0.3745	>0.9999†	0.2325†	>0.9999†	>0.9999††	0.2316†	0.4740	>0.9999†	>0.9999†	**0.0021**	0.1997	>0.9999†
RBT4BA vs. RTG-2	0.2995	>0.9999†	>0.9999†	>0.9999†	>0.9999††	>0.9999†	0.6361	>0.9999†	>0.9999†	0.2411	0.8248	>0.9999†
RBT4BA vs. RTL-W1	>0.9999	>0.9999†	>0.9999†	>0.9999†	0.8243††	0.3492†	0.9451	>0.9999†	>0.9999†	0.9637	>0.9999	>0.9999†
RBT4BA vs. RTgill-W1	0.8690	>0.9999†	>0.9999†	>0.9999†	>0.9999††	>0.9999†	0.0507	>0.9999†	0.4005†	**0.0027**	**0.0193**	0.0939†
RBT4BA vs. RTHDF	>0.9999	>0.9999†	**0.0201**†	>0.9999†	>0.9999††	**0.0065**†	0.1592	>0.9999†	>0.9999†	**0.0005**	0.9950	>0.9999†
RBT4BA vs. RT-milt5	0.2815	>0.9999†	0.4001†	**0.0463**†	0.9995††	0.4461†	**0.0028**	**0.0399**†	>0.9999†	**<0.0001**	**0.0009**	0.6175†
RTP-2 vs. RTG-2	0.9991	>0.9999†	0.8294†	>0.9999†	>0.9999††	>0.9999†	>0.9999	>0.9999†	>0.9999†	0.6358	0.9748	>0.9999†
RTP-2 vs. RTL-W1	0.4544	>0.9999†	>0.9999†	0.6880†	0.5743††	>0.9999†	0.9468	>0.9999†	>0.9999†	**0.0146**	0.1532	>0.9999†
RTP-2 vs. RTgill-W1	0.9854	>0.9999†	>0.9999†	>0.9999†	>0.9999††	0.5745†	0.9635	>0.9999†	0.6854†	0.9993	0.9855	0.2630†
RTP-2 vs. RTHDF	0.4268	>0.9999†	>0.9999†	>0.9999†	>0.9999††	>0.9999†	0.9996	>0.9999†	>0.9999†	0.9990	0.6958	>0.9999†
RTP-2 vs. RT-milt5	>0.9999	>0.9999†	>0.9999†	>0.9999†	0.9837††	>0.9999†	0.3777	>0.9999†	>0.9999†	0.4165	0.2788	>0.9999†
RTG-2 vs. RTL-W1	0.3625	>0.9999†	>0.9999†	>0.9999†	0.9983††	>0.9999†	0.9888	>0.9999†	>0.9999†	0.7108	0.7889	>0.9999†
RTG-2 vs. RTgill-W1	0.8989	>0.9999†	>0.9999†	>0.9999†	>0.9999††	>0.9999†	0.8893	>0.9999†	>0.9999†	0.8331	0.5971	>0.9999†
RTG-2 vs. RTHDF	0.3369	>0.9999†	0.1446†	>0.9999†	>0.9999††	>0.9999†	0.9935	>0.9999†	>0.9999†	0.3261	0.9971	>0.9999†
RTG-2 vs. RT-milt5	0.9999	>0.9999†	>0.9999†	>0.9999†	0.9979††	>0.9999†	0.2520	>0.9999†	>0.9999†	**0.0173**	0.0630	>0.9999†
RTL-W1 vs. RTgill-W1	0.9348	0.5459†	>0.9999†	>0.9999†	0.3528††	0.9005†	0.2932	>0.9999†	0.3584†	**0.0214**	**0.0107**	0.2202†
RTL-W1 vs. RTHDF	>0.9999	0.5238†	0.9272†	>0.9999†	0.9995††	>0.9999†	0.6392	>0.9999†	>0.9999†	**0.0034**	0.9932	>0.9999†
RTL-W1 vs. RT-milt5	0.3448	0.0729†	>0.9999†	**0.0150**†	0.1070††	>0.9999†	**0.0209**	**0.0327**†	>0.9999†	**<0.0001**	**0.0004**	>0.9999†
RTgill-W1 vs. RTHDF	0.9053	>0.9999†	0.6530†	>0.9999†	>0.9999††	**0.0162**†	0.9988	>0.9999†	**0.0140**†	0.9346	0.1966	**0.0062**†
RTgill-W1 vs. RT-milt5	0.9575	>0.9999†	>0.9999†	>0.9999†	0.2682††	>0.9999†	0.8965	>0.9999†	>0.9999†	0.1180	0.6457	>0.9999†
RTHDF vs. RT-milt5	0.3260	>0.9999†	>0.9999†	>0.9999†	0.9995††	>0.9999†	0.5888	0.5567†	0.0925†	0.7612	**0.0126**	0.0616†

† : Analyzed via Kruskal-Wallis and Dunn's post hoc test, ††: Analyzed via Welch's ANOVA with Games-Howell's post hoc test

Table A1.4.8: Calculated *p* values for comparisons between the **CFDA-AM** responses of rainbow trout cell lines to exudate treatments at concentrations of 0.5X, 1X, and 2X. **Bolded** values indicate significant differences between treatments.

	TL			**TS**			**NTL**			**NTS**		
Cell line comparisons	*0.5X*	*1X*	*2X*	*0.5X*	*1X*	*2X*	*0.5X*	*1X*	*2X*	*0.5X*	*1X*	*2X*
RTBrain vs. RBT4BA	0.9926	>0.9999†	>0.9999†	>0.9999†	0.9877	>0.9999†	0.9992	>0.9999††	>0.9999†	0.9995	>0.9999	>0.9999†
RTBrain vs. RTP-2	>0.9999	>0.9999†	>0.9999†	>0.9999†	>0.9999	>0.9999†	0.9998	0.9736††	>0.9999†	>0.9999	0.9178	>0.9999†
RTBrain vs. RTG-2	>0.9999	>0.9999†	>0.9999†	>0.9999†	0.9890	>0.9999†	0.9972	0.9993††	>0.9999†	0.9998	0.9884	>0.9999†
RTBrain vs. RTL-W1	>0.9999	>0.9999†	>0.9999†	>0.9999†	0.8352	>0.9999†	0.9856	0.8978††	>0.9999†	0.2981	>0.9999	>0.9999†
RTBrain vs. RTgill-W1	0.9994	>0.9999†	>0.9999†	>0.9999†	0.9994	>0.9999†	0.9785	0.9826††	>0.9999†	0.7046	>0.9999	>0.9999†
RTBrain vs. RTHDF	0.8376	>0.9999†	>0.9999†	>0.9999†	0.9817	>0.9999†	0.9963	0.4904††	0.2378†	>0.9999	**0.0104**	0.9730†
RTBrain vs. RTMilt	0.9354	>0.9999†	>0.9999†	>0.9999†	0.9997	>0.9999†	>0.9999	0.9982††	>0.9999†	>0.9999	0.9229	>0.9999†
RBT4BA vs. RTP-2	0.9748	>0.9999†	0.1042†	>0.9999†	0.9810	>0.9999†	>0.9999	0.9736††	>0.9999†	0.9922	0.7638	>0.9999†
RBT4BA vs. RTG-2	0.9993	>0.9999†	>0.9999†	>0.9999†	0.7297	>0.9999†	0.9050	0.9999††	>0.9999†	0.9720	0.9342	>0.9999†
RBT4BA vs. RTL-W1	0.9993	0.9302†	>0.9999†	>0.9999†	0.2375	0.7084†	0.7415	0.5920††	>0.9999†	0.0799	0.9997	>0.9999†
RBT4BA vs. RTgill-W1	>0.9999	>0.9999†	>0.9999†	>0.9999†	>0.9999	>0.9999†	0.7186	0.8951††	>0.9999†	0.2930	>0.9999	>0.9999†
RBT4BA vs. RTHDF	0.9946	>0.9999†	0.0900†	>0.9999†	0.5630	>0.9999†	>0.9999	0.5020††	0.1650†	>0.9999	**0.0029**	0.1396†
RBT4BA vs. RT-milt5	0.9999	>0.9999†	0.2105†	>0.9999†	0.8331	0.0839†	0.9937	0.9993††	>0.9999†	>0.9999	0.7545	>0.9999†
RTP-2 vs. RTG-2	>0.9999	>0.9999†	0.1401†	>0.9999†	0.9927	>0.9999†	0.9464	0.9966††	>0.9999†	>0.9999	>0.9999	>0.9999†
RTP-2 vs. RTL-W1	0.9996	>0.9999†	0.2793†	0.7729†	0.8665	>0.9999†	0.8493	0.5632††	0.4819†	0.4423	0.9052	>0.9999†
RTP-2 vs. RTgill-W1	0.9959	>0.9999†	>0.9999†	>0.9999†	0.9986	>0.9999†	0.8269	0.7460††	0.8386†	0.8563	0.7060	>0.9999†
RTP-2 vs. RTHDF	0.7253	>0.9999†	>0.9999†	>0.9999†	0.9881	>0.9999†	>0.9999	0.6595††	>0.9999†	0.9998	0.1416	>0.9999†
RTP-2 vs. RT-milt5	0.8587	>0.9999†	>0.9999†	>0.9999†	0.9999	>0.9999†	0.9978	0.9993††	>0.9999†	0.9988	>0.9999	>0.9999†
RTG-2 vs. RTL-W1	>0.9999	>0.9999†	>0.9999†	>0.9999†	>0.9999	>0.9999†	>0.9999	0.5778††	>0.9999†	0.5608	0.9900	0.9400†
RTG-2 vs. RTgill-W1	>0.9999	>0.9999†	>0.9999†	>0.9999†	0.8626	>0.9999†	>0.9999	0.8488††	>0.9999†	0.9313	0.9089	>0.9999†
RTG-2 vs. RTHDF	0.9454	>0.9999†	0.1051†	>0.9999†	>0.9999	>0.9999†	0.8447	0.5373††	>0.9999†	0.9981	0.0670	>0.9999†
RTG-2 vs. RT-milt5	0.9867	>0.9999†	0.2745†	>0.9999†	0.9997	>0.9999†	0.9991	>0.9999††	>0.9999†	0.9927	>0.9999	>0.9999†
RTL-W1 vs. RTgill-W1	>0.9999	0.5131†	>0.9999†	>0.9999†	0.3605	0.7194†	>0.9999	>0.9999††	>0.9999†	0.9832	0.9992	>0.9999†
RTL-W1 vs. RTHDF	0.9038	>0.9999†	0.2249†	0.1506†	0.9994	>0.9999†	0.6357	0.3582††	**0.0454**†	0.2192	**0.0034**	**0.0285**†
RTL-W1 vs. RT-milt5	0.9756	>0.9999†	0.5698†	0.1740†	0.9675	>0.9999†	0.9930	0.5810††	0.5046†	0.1235	0.9050	0.3316†
RTgill-W1 vs. RTHDF	0.9456	>0.9999†	>0.9999†	0.8500†	0.7406	>0.9999†	0.6152	0.3853††	0.0818†	0.5834	**0.0016**	0.0800†
RTgill-W1 vs. RT-milt5	0.9915	>0.9999†	>0.9999†	>0.9999†	0.9473	0.0752†	0.9882	0.8358††	0.8967†	0.4131	0.6863	0.8647†
RTHDF vs. RT-milt5	>0.9999	>0.9999†	>0.9999†	>0.9999†	0.9997	>0.9999†	0.9808	0.5571††	>0.9999†	>0.9999	0.0795	>0.9999†

† : Analyzed via Kruskal-Wallis and Dunn's post hoc test, ††: Analyzed via Welch's ANOVA with Games-Howell's post hoc test

Table A1.4.9: Mean (± SEM) **Lysotracker** lysosomal activity values and significance compared to vehicle controls of rainbow trout cell lines treated with TL, TS, NTL, and NTS exudate solutions at concentrations of 1X and 2X., as well as high relative densities. **Bolded** values indicate differences from controls with *p* values < 0.05.

		Toxic-Log		**Toxic-Stable**		**Non-Toxic-Log**		**Non-Toxic-Stable**	
Cell line	**Concentration/ *Density***	Mean Viability	*P* value	Mean Viability	*P* value	Mean Viability	*P* value	Mean Viability	*P* value
RBT4BA	1X	1.070 ± 0.165	0.7130	1.513 ± 0.220	0.1447	1.410 ± 0.234	0.2223	1.263 ± 0.204	0.3261
	2X	1.530 ± 0.327	0.2467	2.453 ± 0.447	0.0829	1.570 ± 0.225	0.1273	2.030 ± 0.481	0.1655
	High density	1.530 ± 0.327	0.2467	1.513 ± 0.220	0.1447	1.570 ± 0.225	0.1273	1.263 ± 0.204	0.3261
RTBrain	1X	1.063 ± 0.397	0.8880	1.740 ± 0.533	0.2991	1.397 ± 0.408	0.4336	1.480 ± 0.693	0.5604
	2X	1.283 ± 0.411	0.5615	1.900 ± 0.729	0.3421	1.497 ± 0.507	0.4303	1.373 ± 0.404	0.4533
	High density	1.283 ± 0.411	0.5615	1.740 ± 0.533	0.2991	1.497 ± 0.507	0.4303	1.480 ± 0.693	0.5604
RTG-2	1X	1.500 ± 0.291	0.2274	**2.217 ± 0.194**	**0.0245**	**1.773 ± 0.175**	**0.0474**	2.177 ± 0.462	0.1258
	2X	2.430 ± 0.940	0.3702	2.340 ± 0.396	0.0772	**2.277 ± 0.192**	**0.0219**	2.603 ± 0.521	0.0913
	High density	2.430 ± 0.940	0.3702	**2.217 ± 0.194**	**0.0245**	**2.277 ± 0.192**	**0.0219**	2.177 ± 0.462	0.1258
RTgill-W1	1X	1.078 ± 0.138	0.6020	**1.723 ± 0.167**	**0.0229**	**1.244 ± 0.066**	**0.0205**	**1.435 ± 0.055**	**0.0042**
	2X	0.720 ± 0.030	0.0680	**1.763 ± 0.173**	**0.0216**	**1.606 ± 0.128**	**0.0090**	**1.778 ± 0.093**	**0.0035**
	High density	0.720 ± 0.030	0.0680	**1.723 ± 0.167**	**0.0229**	**1.606 ± 0.128**	**0.0090**	**1.435 ± 0.055**	**0.0042**
RTHDF	1X	1.070 ± 0.055	0.3316	1.663 ± 0.210	0.0870	1.200 ± 0.068	0.0989	**1.717 ± 0.151**	**0.0415**
	2X	1.710 ± 0.309	0.1483	2.317 ± 0.337	0.0597	1.390 ± 0.192	0.1787	1.917 ± 0.223	0.0545
	High density	1.710 ± 0.309	0.1483	1.663 ± 0.210	0.0870	1.390 ± 0.192	0.1787	**1.717 ± 0.151**	**0.0415**
RTL-W1	1X	1.014 ± 0.212	0.9506	2.552 ± 0.740	0.1039	1.047 ± 0.064	0.2438	**1.663 ± 0.131**	**0.0015**
	2X	**4.665 ± 0.558**	**0.0072**	**2.479 ± 0.394**	**0.0072**	**1.610 ± 0.083**	**0.0002**	**2.076 ± 0.074**	**<0.0001**
	High density	**4.665 ± 0.558**	**0.0072**	2.552 ± 0.740	0.1039	**1.610 ± 0.083**	**0.0002**	**1.663 ± 0.131**	**0.0015**
RT-milt5	1X	**1.643 ± 0.106**	**0.0018**	**2.987 ± 0.195**	**0.0002**	**1.510 ± 0.107**	**0.0050**	**1.810 ± 0.114**	**0.0009**
	2X	**2.002 ± 0.103**	**0.0002**	**2.792 ± 0.338**	**0.0032**	**1.618 ± 0.117**	**0.0033**	**1.970 ± 0.067**	**<0.0001**
	High density	**2.002 ± 0.103**	**0.0002**	**2.987 ± 0.195**	**0.0002**	**1.618 ± 0.117**	**0.0033**	**1.810 ± 0.114**	**0.0009**
RTP-2	1X	—	—	1.960 ± 0.560	0.3362	1.113 ± 0.180	0.5937	1.330 ± 0.083	0.0582
	2X	—	—	1.873 ± 0.370	0.1422	1.213 ± 0.184	0.3663	**2.570 ± 0.342**	**0.0442**
	High density	—	—	1.960 ± 0.560	0.3362	1.213 ± 0.184	0.3663	1.330 ± 0.083	0.0582

Table A1.4.10: Calculated *p* values for comparisons of rainbow trout cell line lysosomal responses to exudate treatments at concentrations of 1X and 2X, as well as at high relative culture densities. **Bolded** values indicate significant differences between treatments.

Cell line	Concentration /*Density*	TL vs. TS	TL vs. NTL	TL vs. NTS	TS vs. NTL	TS vs. NTS	NTL vs. NTS
RBT4BA	1X	0.6775†	>0.9999†	>0.9999†	>0.9999†	>0.9999†	>0.9999†
	2X	0.3821	0.9998	0.7945	0.4163	0.8613	0.8306
	High Density	>0.9999	0.9994	0.8711	0.9984	0.8904	0.8196
RTBrain	1X	0.9374†	>0.9999†	>0.9999†	>0.9999†	>0.9999†	>0.9999†
	2X	>0.9999†	>0.9999†	>0.9999†	>0.9999†	>0.9999†	>0.9999†
	High Density	>0.9999†	>0.9999†	>0.9999†	>0.9999†	>0.9999†	>0.9999†
RTG-2	1X	0.3944	0.9165	0.4389	0.7344	0.9997	0.7838
	2X	0.9993	0.9965	0.9950	0.9996	0.9767	0.9575
	High Density	0.9865	0.9948	0.9779	0.9996	0.9999	0.9980
RTgill-W1	1X	**0.0074**	0.7114	0.1785	**0.0494**	0.3791	0.6587
	2X	**0.0043**	**0.0103**	**0.0039**	0.8195	0.9998	0.7769
	High Density	**0.0037**	**0.0069**	**0.0322**	0.8994	0.4115	0.7469
RTHDF	1X	0.0595	0.9039	**0.0403**	0.1535	0.9920	0.1044
	2X	0.4408	0.8379	0.9473	0.1522	0.7322	0.5493
	High Density	0.9987	0.7458	>0.9999	0.8215	0.9981	0.7344
RTL-W1	1X	0.1366†	>0.9999†	0.0965†	0.1451†	>0.9999†	0.0934†
	2X	0.3762†	**0.0005**†	0.1631†	0.0698†	>0.9999†	0.2158†
	High Density	0.1934††	**0.0334**††	**0.0322**††	0.6246††	0.6648††	0.9861††
RT-milt5	1X	**<0.0001**	0.8979	0.8210	**<0.0001**	**<0.0001**	0.4211
	2X	**0.0362**	0.4939	0.9994	**0.0015**	**0.0282**	0.5642
	High Density	**0.0003**	0.2308	0.7584	**<0.0001**	**<0.0001**	0.7584
RTP-2	1X	—	—	—	0.1579†	0.8902†	0.9519†
	2X	—	—	—	0.3523	0.3192	**0.0481**
	High Density	—	—	—	0.2175	0.3092	0.9383

† : Analyzed via Kruskal-Wallis and Dunn's post hoc test, ††: Analyzed via Welch's ANOVA with Games-Howell's post hoc test

Table A1.4.11: Calculated *p* values for comparisons between the lysosomal responses of rainbow trout cell lines to exudate treatments at concentrations of 1X, and 2X. **Bolded** values indicate significant differences between treatments.

	TL		TS		NTL		NTS	
Cell line comparisons	*1X*	*2X*	*1X*	*2X*	*1X*	*2X*	*1X*	*2X*
RTBrain vs. RBT4BA	>0.9999	0.9994	>0.9999	0.9932	>0.9999	>0.9999	0.9992	0.6819
RTBrain vs. RTP-2	0.8169	—	>0.9999	>0.9999	0.9448	0.9800	>0.9999	0.0745
RTBrain vs. RTG-2	>0.9999	0.5836	0.9967	0.9984	0.8027	0.2151	0.6606	0.0620
RTBrain vs. RTL-W1	>0.9999	**0.0002**	0.8916	0.9743	0.7436	0.9998	0.9992	0.3816
RTBrain vs. RTgill-W1	>0.9999	0.9722	>0.9999	>0.9999	0.9969	0.9999	>0.9999	0.9451
RTBrain vs. RTHDF	0.3971	0.9879	>0.9999	0.9988	0.9926	>0.9999	0.9987	0.8425
RTBrain vs. RTMilt	0.8270	0.7778	0.4750	0.8327	0.9994	0.9997	0.9769	0.6302
RBT4BA vs. RTP-2	>0.9999	—	0.9989	0.9910	0.9308	0.9331	>0.9999	0.8465
RBT4BA vs. RTG-2	>0.9999	0.7995	0.9691	>0.9999	0.8290	0.3206	0.3392	0.8045
RBT4BA vs. RTL-W1	>0.9999	**0.0004**	0.7146	>0.9999	0.7081	>0.9999	0.9229	>0.9999
RBT4BA vs. RTgill-W1	0.4102	0.8639	>0.9999	0.9652	0.9948	>0.9999	0.9997	0.9961
RBT4BA vs. RTHDF	0.6295	>0.9999	>0.9999	>0.9999	0.9892	0.9987	0.9425	>0.9999
RBT4BA vs. RT-milt5	0.7599	0.9598	0.2810	0.9992	0.9997	>0.9999	0.7573	>0.9999
RTP-2 vs. RTG-2	0.8270	—	>0.9999	0.9976	0.1973	**0.0317**	0.4304	>0.9999
RTP-2 vs. RTL-W1	0.9983	—	0.9897	0.9673	>0.9999	0.7625	0.9696	0.7740
RTP-2 vs. RTgill-W1	>0.9999	—	>0.9999	>0.9999	0.9988	0.8298	>0.9999	0.3823
RTP-2 vs. RTHDF	>0.9999	—	>0.9999	0.9983	>0.9999	0.9989	0.9750	0.6872
RTP-2 vs. RT-milt5	0.1641	—	0.8182	0.8117	0.6149	0.7818	0.8545	0.6240
RTG-2 vs. RTL-W1	>0.9999	**0.0290**	0.9993	>0.9999	**0.0481**	0.1868	0.7726	0.7146
RTG-2 vs. RTgill-W1	0.2588	0.2559	0.9939	0.9871	0.3096	0.2550	0.5109	0.3335
RTG-2 vs. RTHDF	0.4102	0.9154	0.9920	>0.9999	0.3461	0.1111	0.9382	0.6340
RTG-2 vs. RT-milt5	>0.9999	0.9876	0.9013	0.9953	0.9221	0.2408	0.9594	0.5614
RTL-W1 vs. RTgill-W1	0.8169	**0.0001**	0.8299	0.8784	0.9600	>0.9999	0.9938	0.9639
RTL-W1 vs. RTHDF	>0.9999	**0.0008**	0.8406	>0.9999	0.9961	0.9862	>0.9999	0.9996
RTL-W1 vs. RT-milt5	>0.9999	**0.0004**	0.9888	0.9974	0.2040	>0.9999	0.9991	0.9999
RTgill-W1 vs. RTHDF	>0.9999	0.7252	>0.9999	0.9898	>0.9999	0.9920	0.9941	>0.9999
RTgill-W1 vs. RT-milt5	0.3971	0.3414	0.3499	0.6169	0.8377	>0.9999	0.9282	0.9980
RTHDF vs. RT-milt5	0.8270	0.9965	0.4033	0.9936	0.8390	0.9867	>0.9999	>0.9999

APPENDIX 1.5 CHAPTER 2 SUPPLEMENTAL GRAPHS

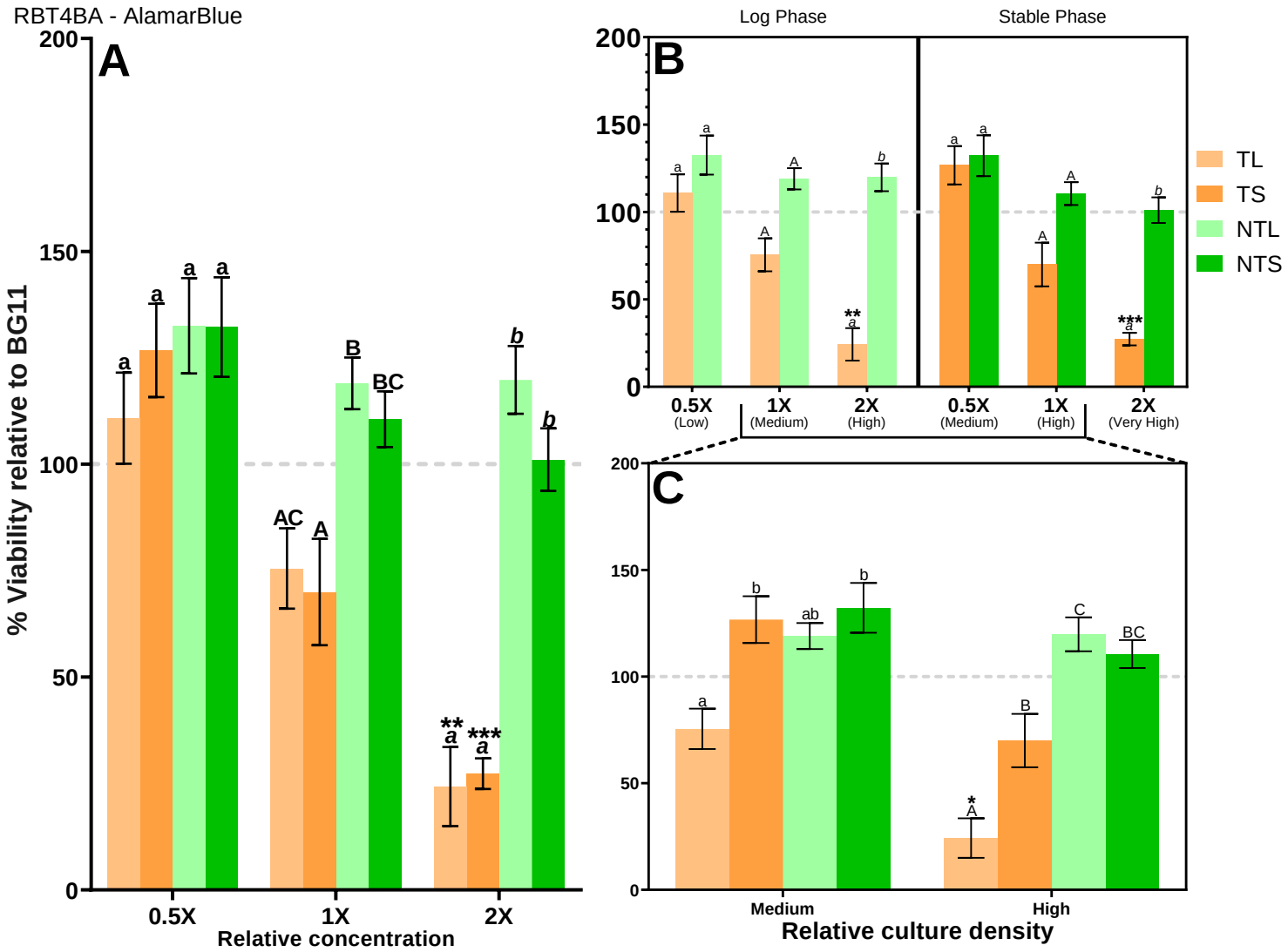

Figure A1.5.1: Mean (± SEM) cell viability values of RBT4BA cells from AlamarBlue cytotoxicity assays and compared via concentration (**A**) and relative culture density (**B, C**). Each experiment was conducted in triplicate and repeated on four separate 96-well plates. Significant differences between treatments and the BG-11 vehicle control are indicated with asterisks (*P < 0.05, **P < 0.01, ***P < 0.001) and significant differences between treatments within each concentration are indicated by different letters.

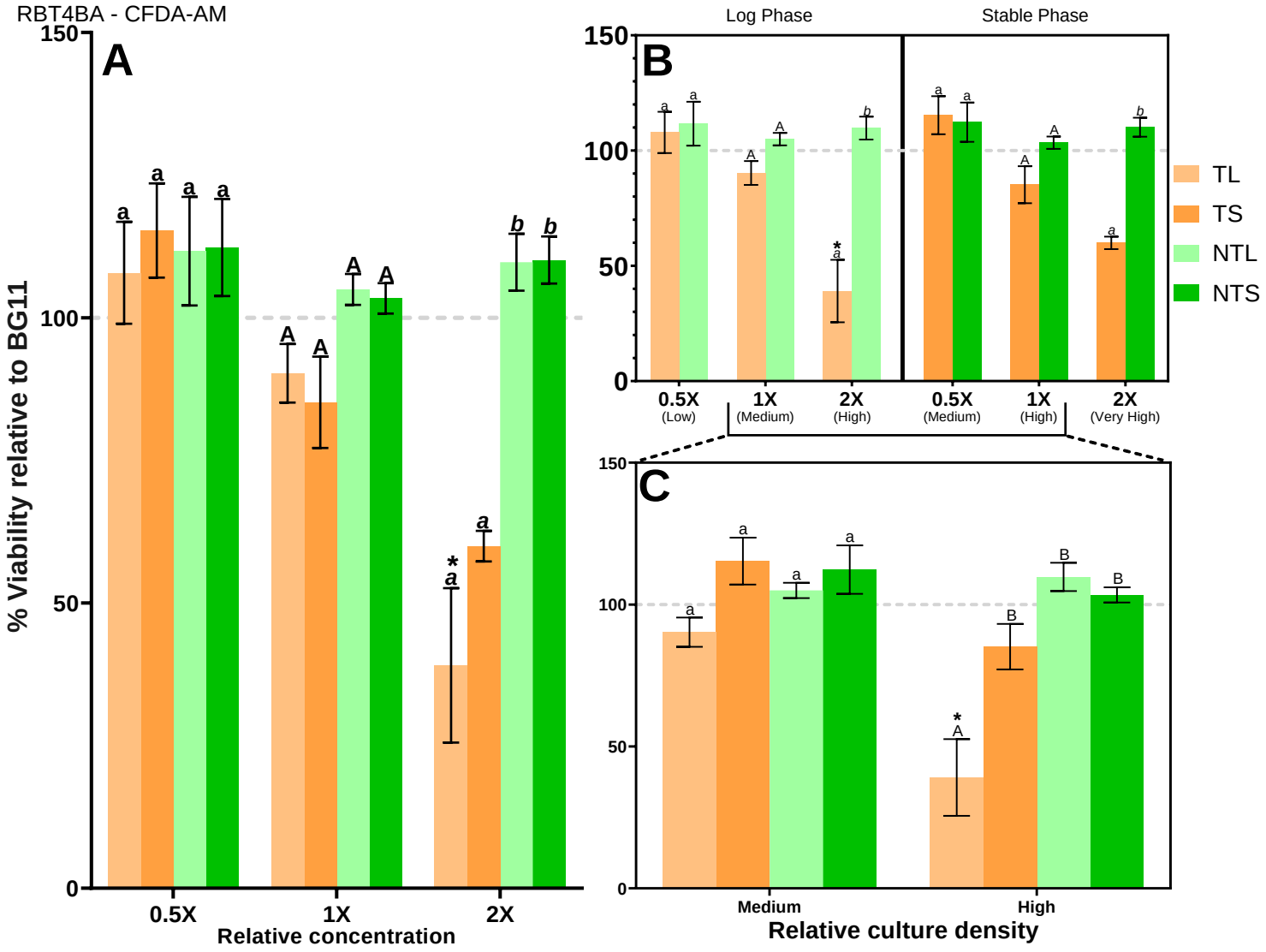

Figure A1.5.2: Mean (± SEM) cell viability values of RBT4BA cells from CFDA-AM cytotoxicity assays and compared via concentration (**A**) and relative culture density (**B, C**). Each experiment was conducted in triplicate and repeated on four separate 96-well plates. Significant differences between treatments and the BG-11 vehicle control are indicated with asterisks (*P < 0.05, **P < 0.01, ***P < 0.001) and significant differences between treatments within each concentration are indicated by different letters.

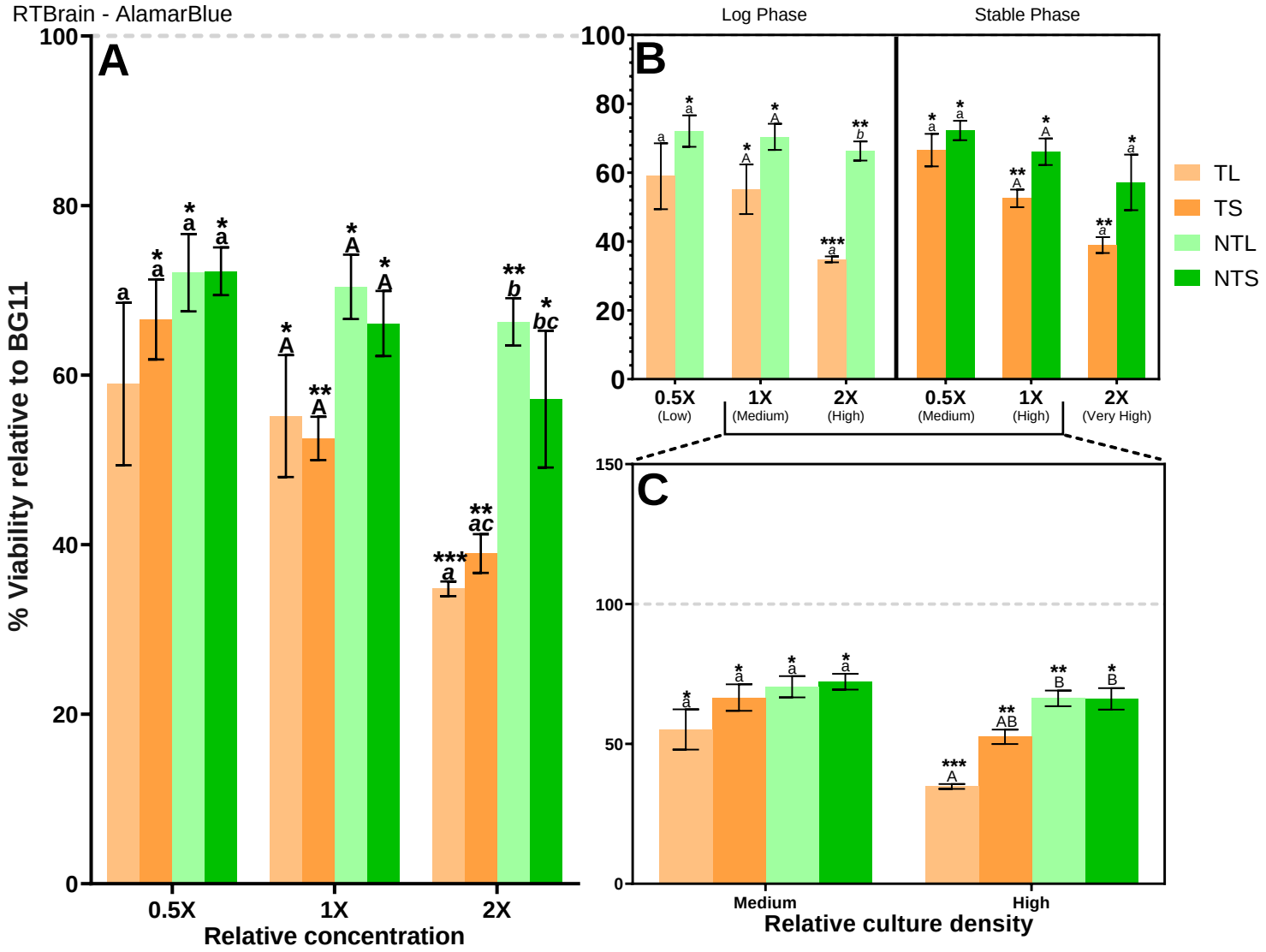

Figure A1.5.3: Mean (± SEM) cell viability values of RTBrain cells from AlamarBlue cytotoxicity assays and compared via concentration (**A**) and relative culture density (**B, C**). Each experiment was conducted in triplicate and repeated on three separate 96-well plates. Significant differences between treatments and the BG-11 vehicle control are indicated with asterisks (*P < 0.05, **P < 0.01, ***P < 0.001) and significant differences between treatments within each concentration are indicated by different letters.

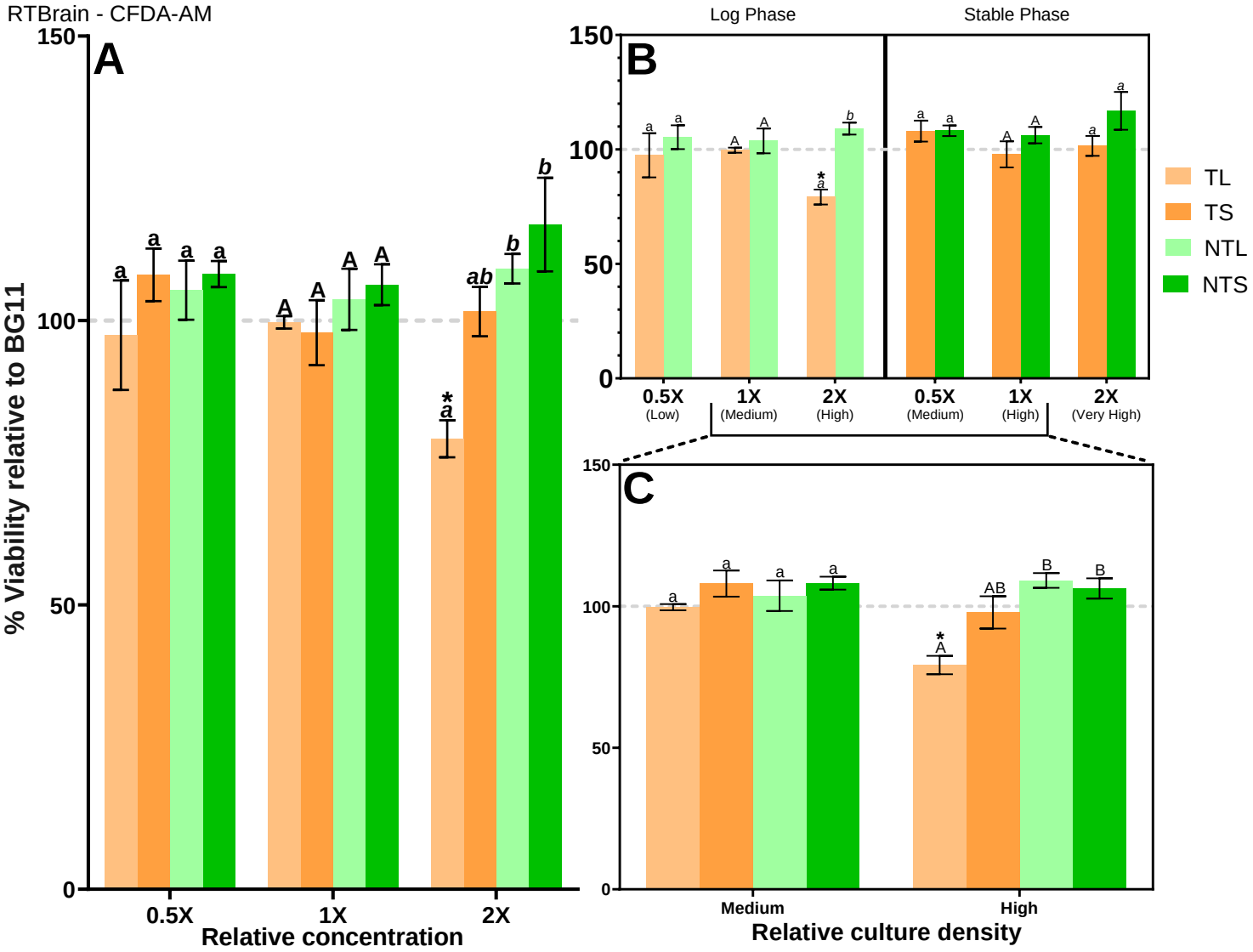

Figure A1.5.4: Mean (± SEM) cell viability values of RTBrain cells from CFDA-AM cytotoxicity assays and compared via concentration (**A**) and relative culture density (**B, C**). Each experiment was conducted in triplicate and repeated on three separate 96-well plates. Significant differences between treatments and the BG-11 vehicle control are indicated with asterisks (*P < 0.05, **P < 0.01, ***P < 0.001) and significant differences between treatments within each concentration are indicated by different letters.

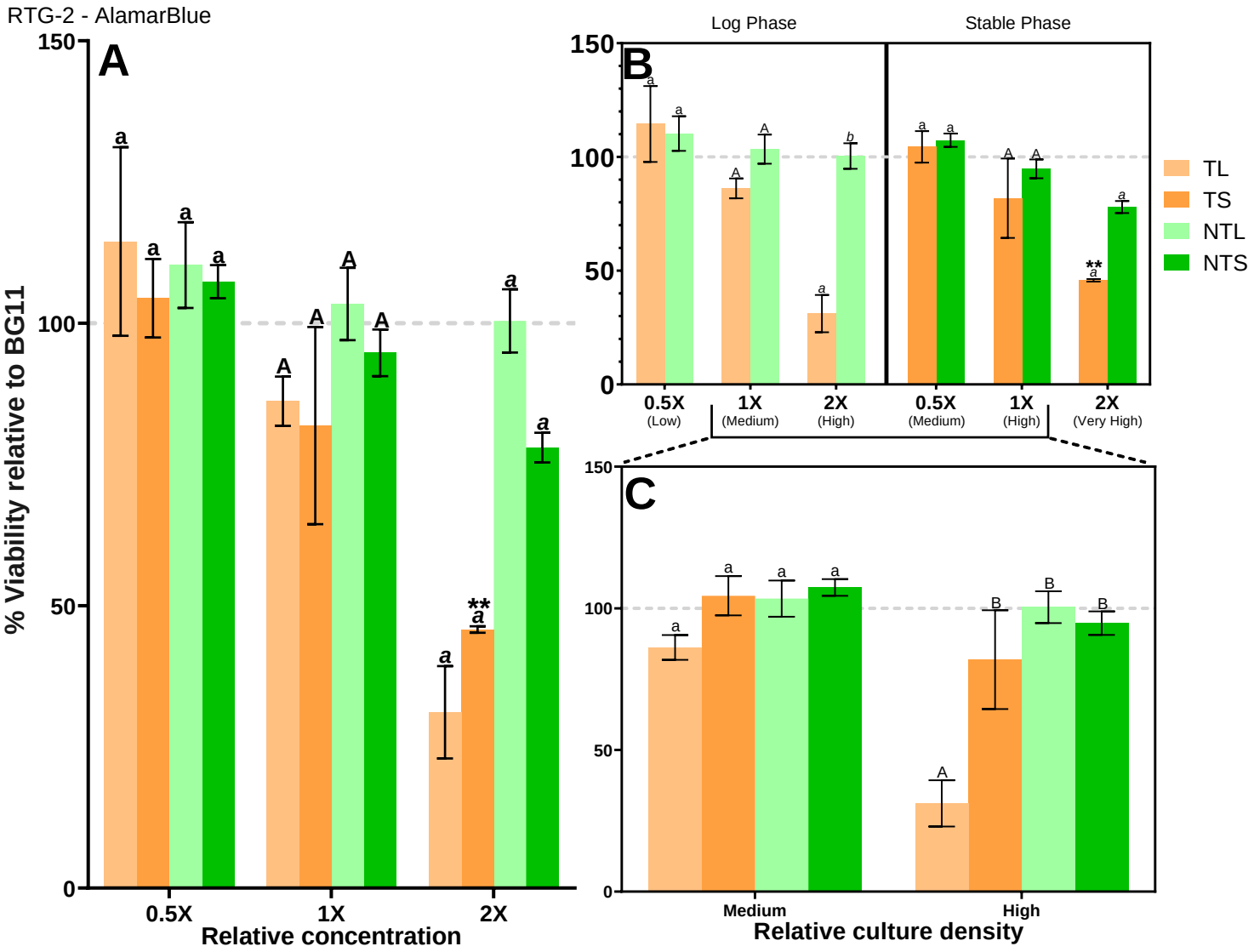

Figure A1.5.5: Mean (± SEM) cell viability values of RTG-2 cells from AlamarBlue cytotoxicity assays and compared via concentration (**A**) and relative culture density (**B, C**). Each experiment was conducted in triplicate and repeated on three separate 96-well plates. Significant differences between treatments and the BG-11 vehicle control are indicated with asterisks (*P < 0.05, **P < 0.01) and significant differences between treatments within each concentration are indicated by different letters.

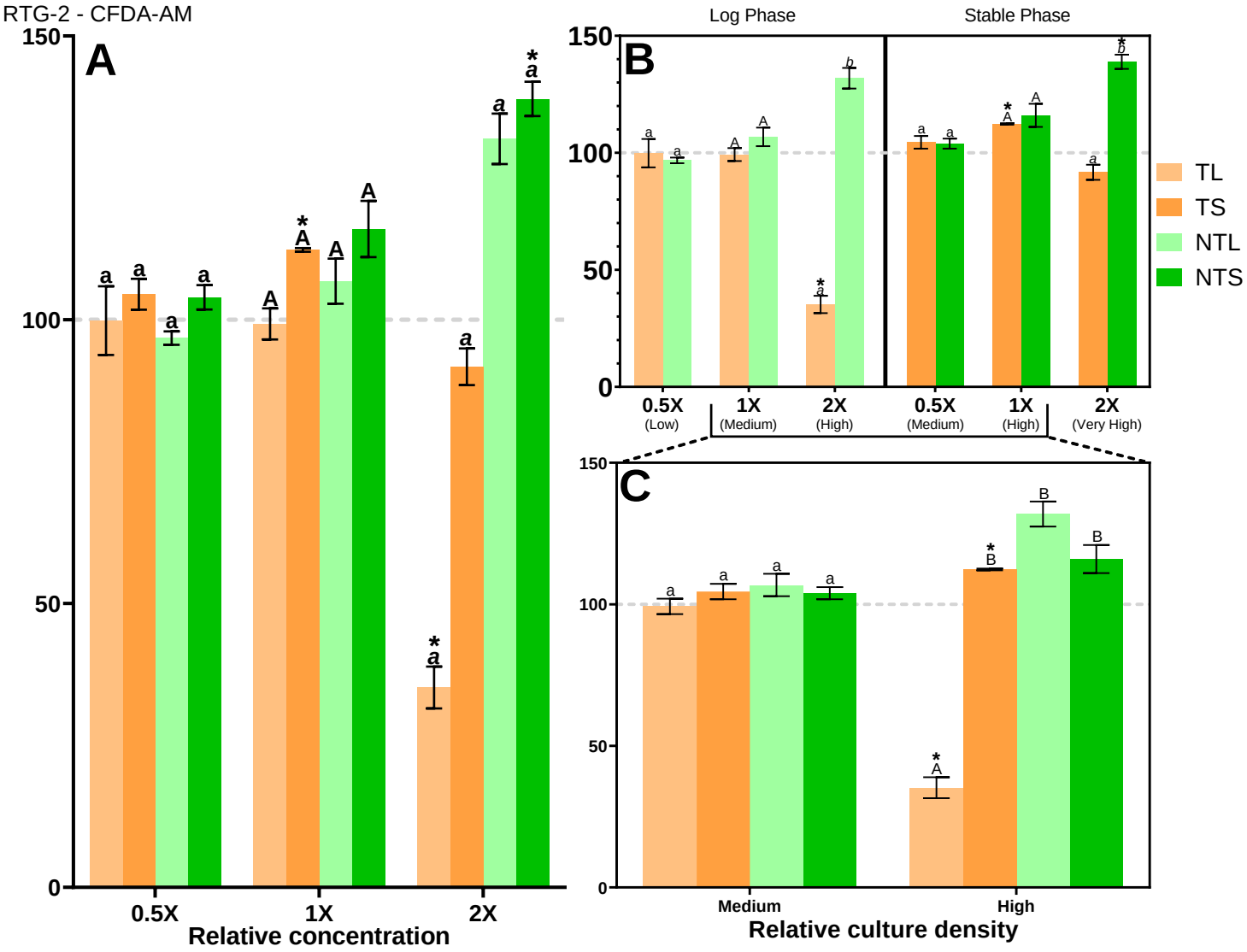

Figure A1.5.6: Mean (± SEM) cell viability values of RTG-2 cells from CFDA-AM cytotoxicity assays and compared via concentration (**A**) and relative culture density (**B, C**). Each experiment was conducted in triplicate and repeated on three separate 96-well plates. Significant differences between treatments and the BG-11 vehicle control are indicated with asterisks (*P < 0.05, **P < 0.01) and significant differences between treatments within each concentration are indicated by different letters.

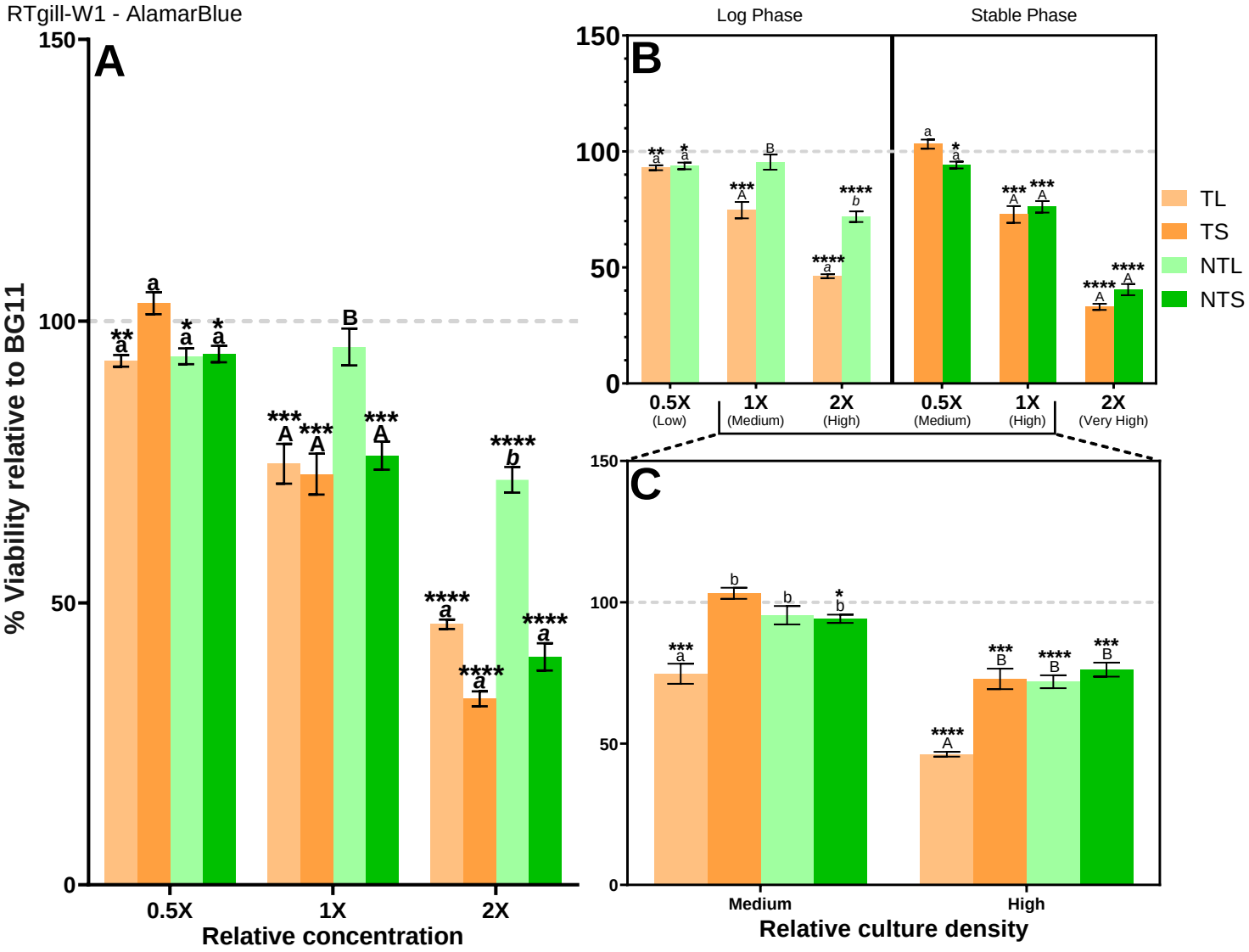

Figure A1.5.7: Mean (± SEM) cell viability values of RTgill-W1 cells from AlamarBlue cytotoxicity assays and compared via concentration (**A**) and relative culture density (**B, C**). Each experiment was conducted in triplicate and repeated on six separate 96-well plates. Significant differences between treatments and the BG-11 vehicle control are indicated with asterisks (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001) and significant differences between treatments within each concentration are indicated by different letters.

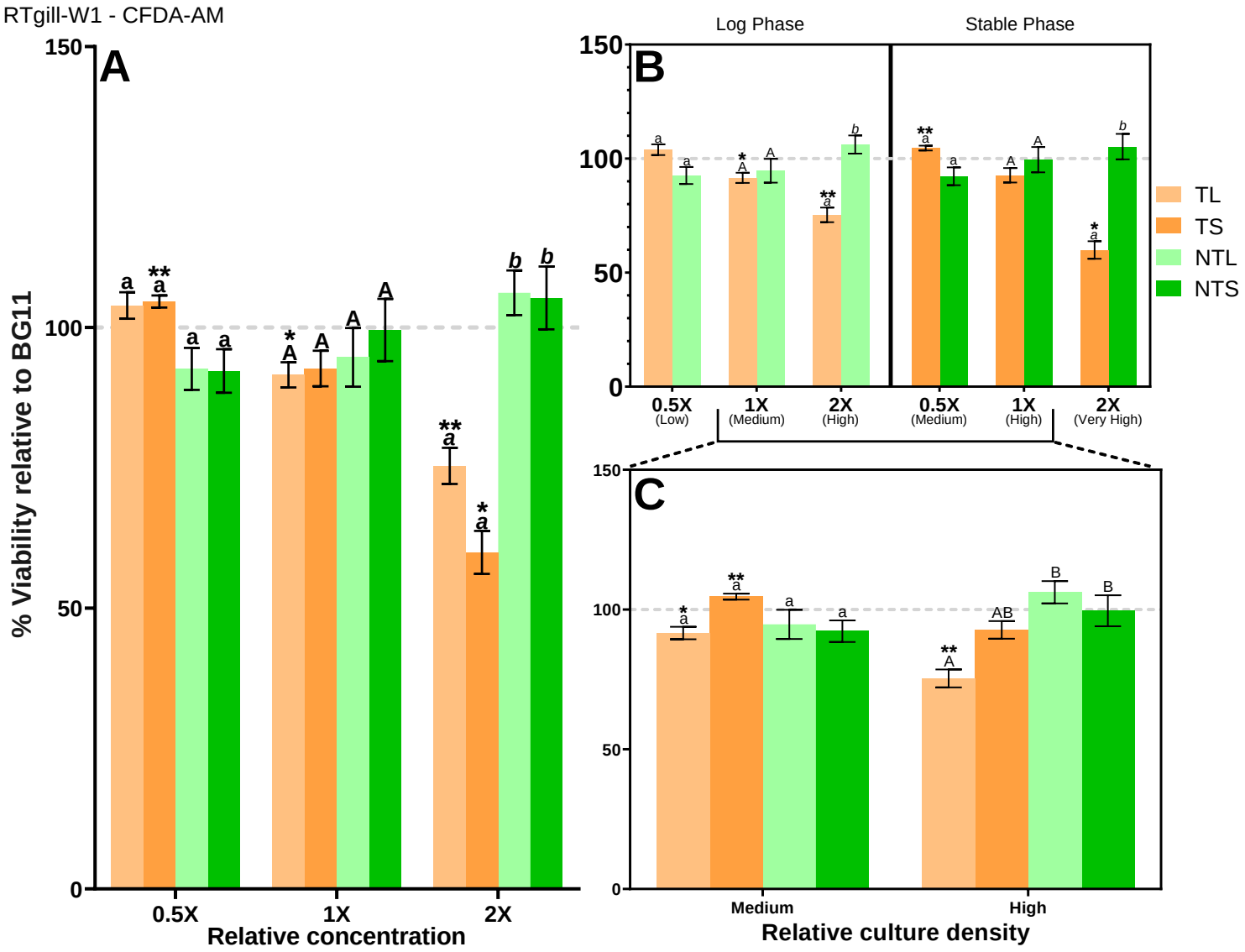

Figure A1.5.8: Mean (± SEM) cell viability values of RTgill-W1 cells from CFDA-AM cytotoxicity assays and compared via concentration (**A**) and relative culture density (**B, C**). Each experiment was conducted in triplicate and repeated on six separate 96-well plates. Significant differences between treatments and the BG-11 vehicle control are indicated with asterisks (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001) and significant differences between treatments within each concentration are indicated by different letters.

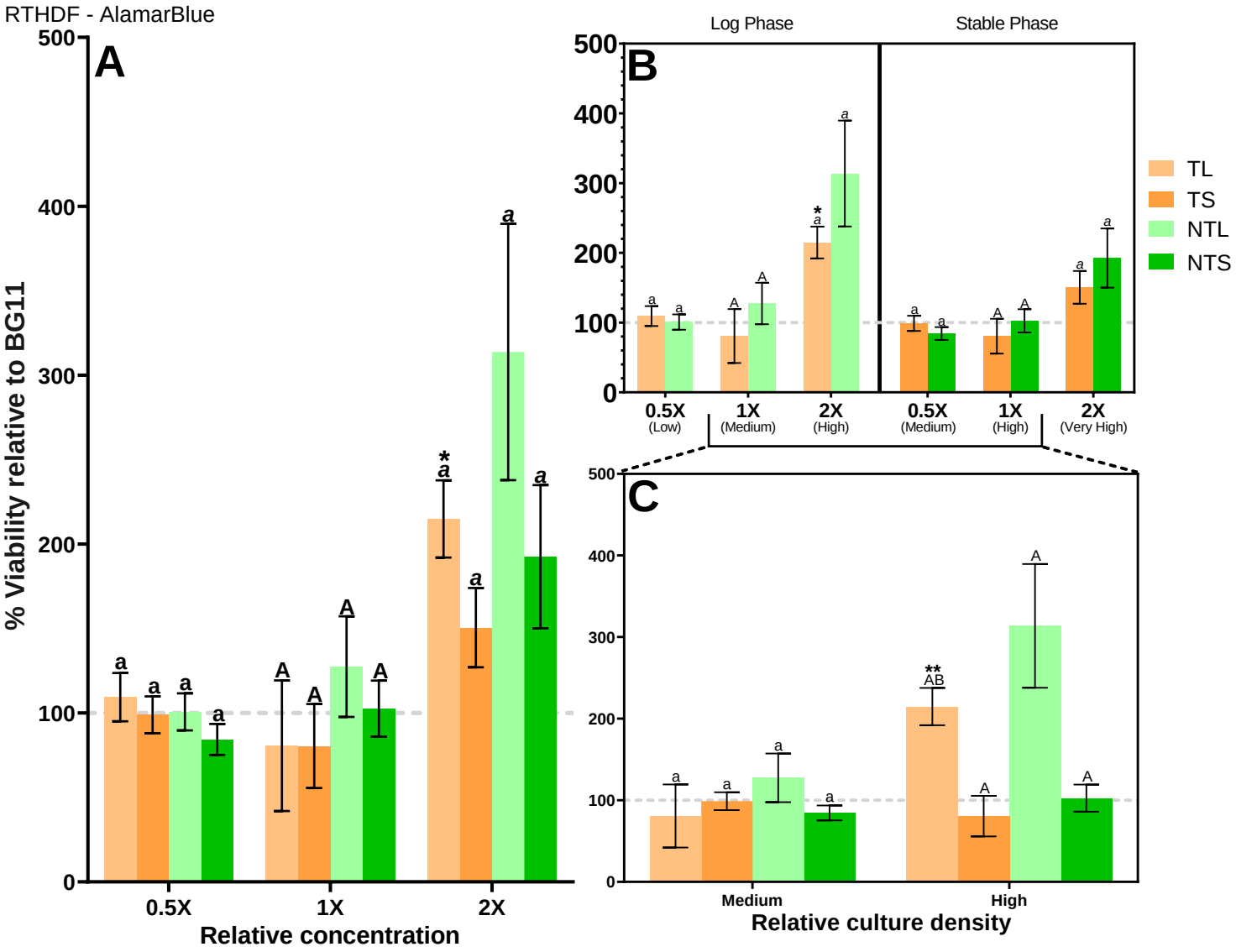

Figure A1.5.9: Mean (± SEM) cell viability values of RTHDF cells from AlamarBlue cytotoxicity assays and compared via concentration (**A**) and relative culture density (**B, C**). Each experiment was conducted in triplicate and repeated on four separate 96-well plates. Significant differences between treatments and the BG-11 vehicle control are indicated with asterisks ($P < 0.05$) and significant differences between treatments within each concentration are indicated by different letters.

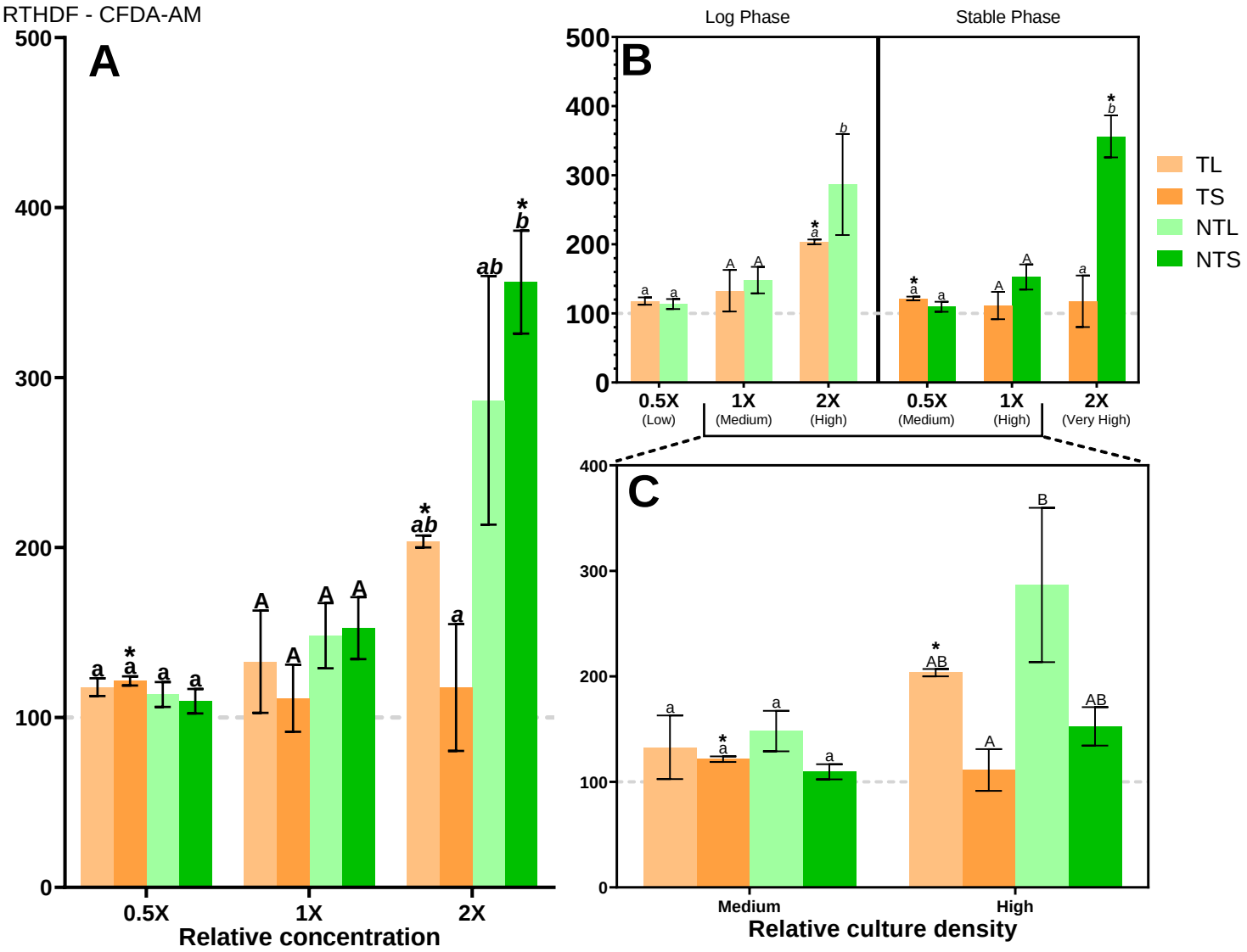

Figure A1.5.10: Mean (± SEM) cell viability values of RTHDF cells from CFDA-AM cytotoxicity assays and compared via concentration (**A**) and relative culture density (**B, C**). Each experiment was conducted in triplicate and repeated on four separate 96-well plates. Significant differences between treatments and the BG-11 vehicle control are indicated with asterisks ($P < 0.05$) and significant differences between treatments within each concentration are indicated by different letters.

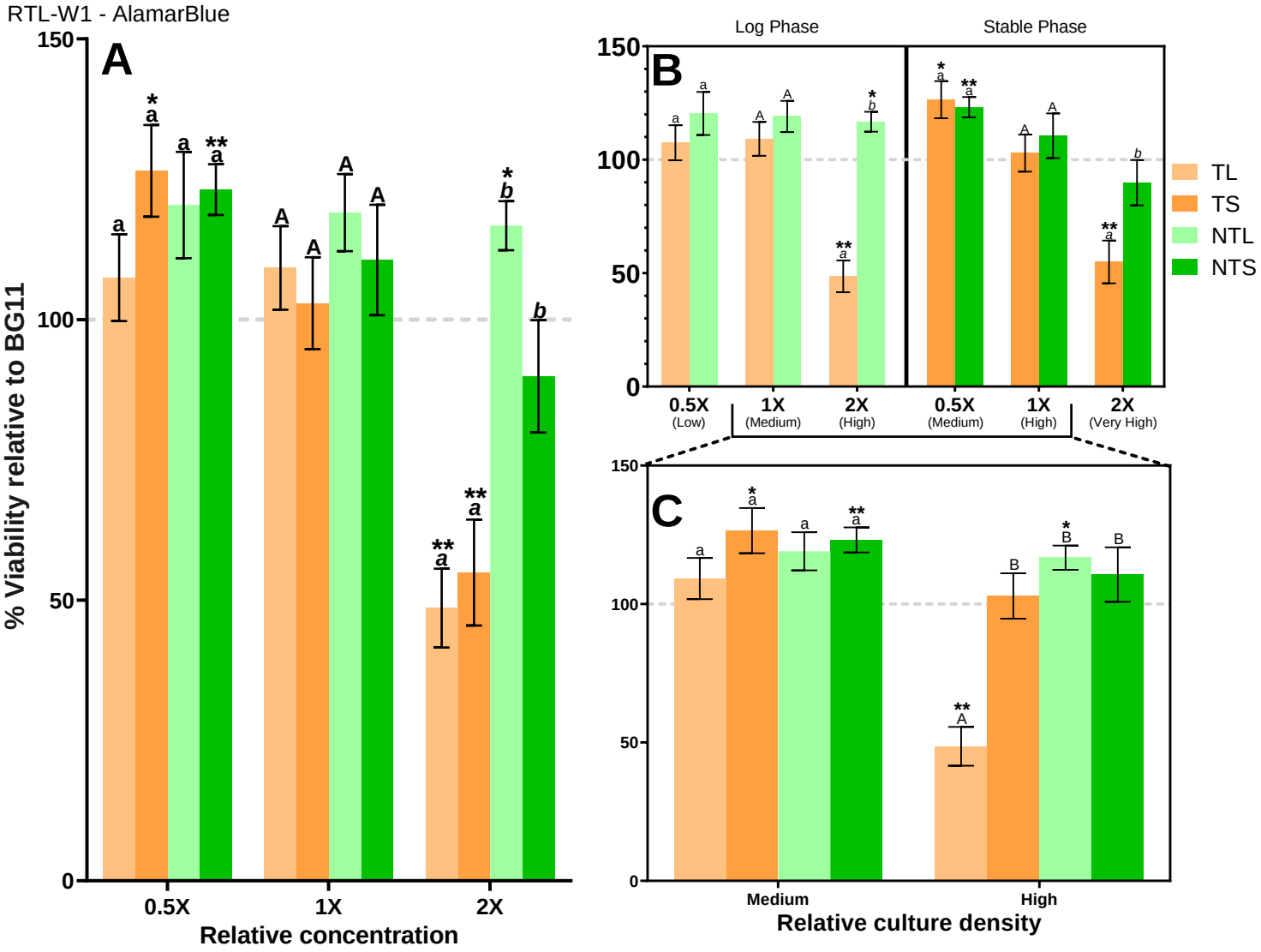

Figure A1.5.11: Mean (± SEM) cell viability values of RTL-W1 cells from AlamarBlue cytotoxicity assays and compared via concentration (**A**) and relative culture density (**B, C**). Each experiment was conducted in triplicate and repeated on six separate 96-well plates. Significant differences between treatments and the BG-11 vehicle control are indicated with asterisks (*P < 0.05, **P < 0.01) and significant differences between treatments within each concentration are indicated by different letters.

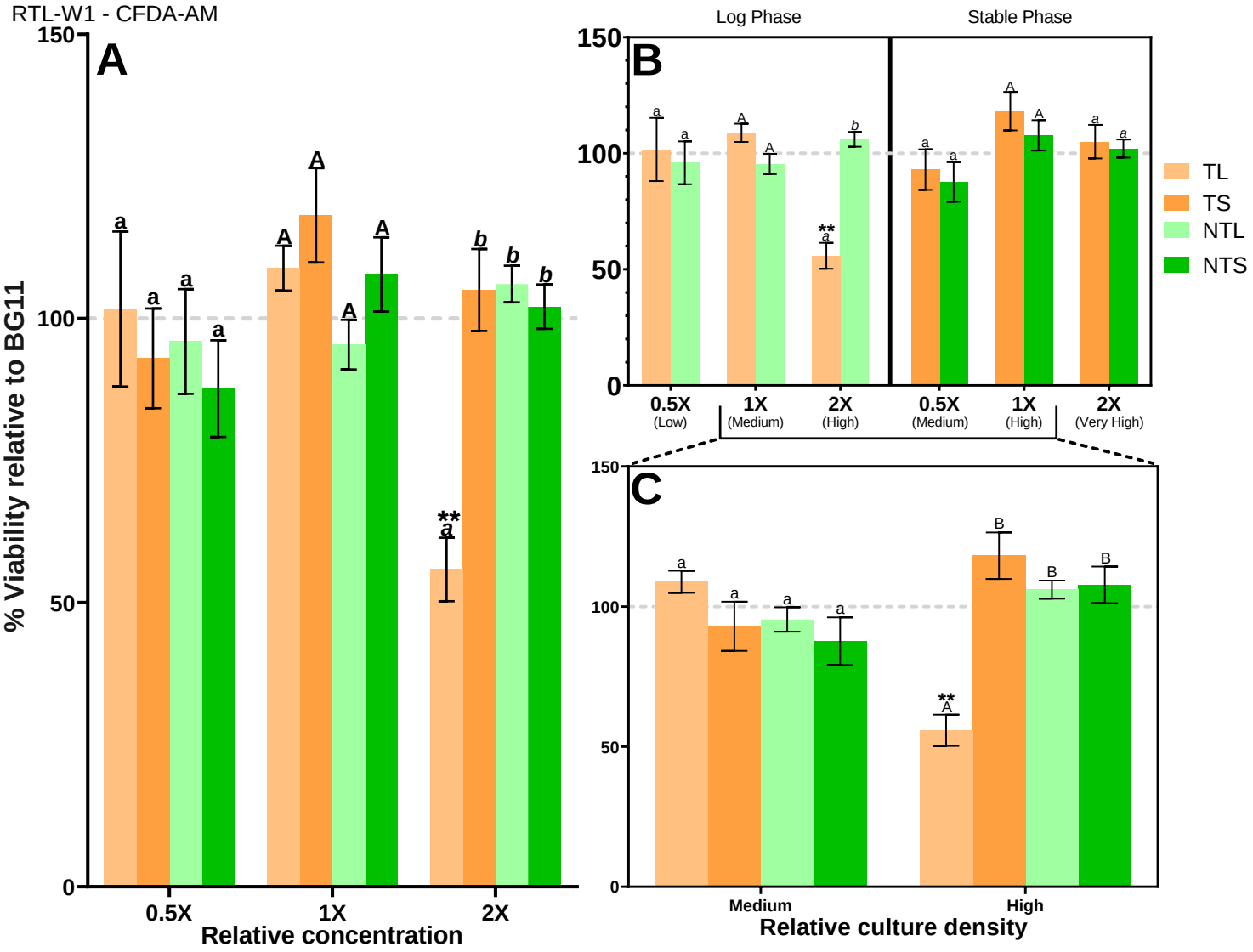

Figure A1.5.12: Mean (± SEM) cell viability values of RTL-W1 cells from CFDA-AM cytotoxicity assays and compared via concentration (**A**) and relative culture density (**B, C**). Each experiment was conducted in triplicate and repeated on 6 separate 96-well plates. Significant differences between treatments and the BG-11 vehicle control are indicated with asterisks (*P < 0.05, **P < 0.01) and significant differences between treatments within each concentration are indicated by different letters.

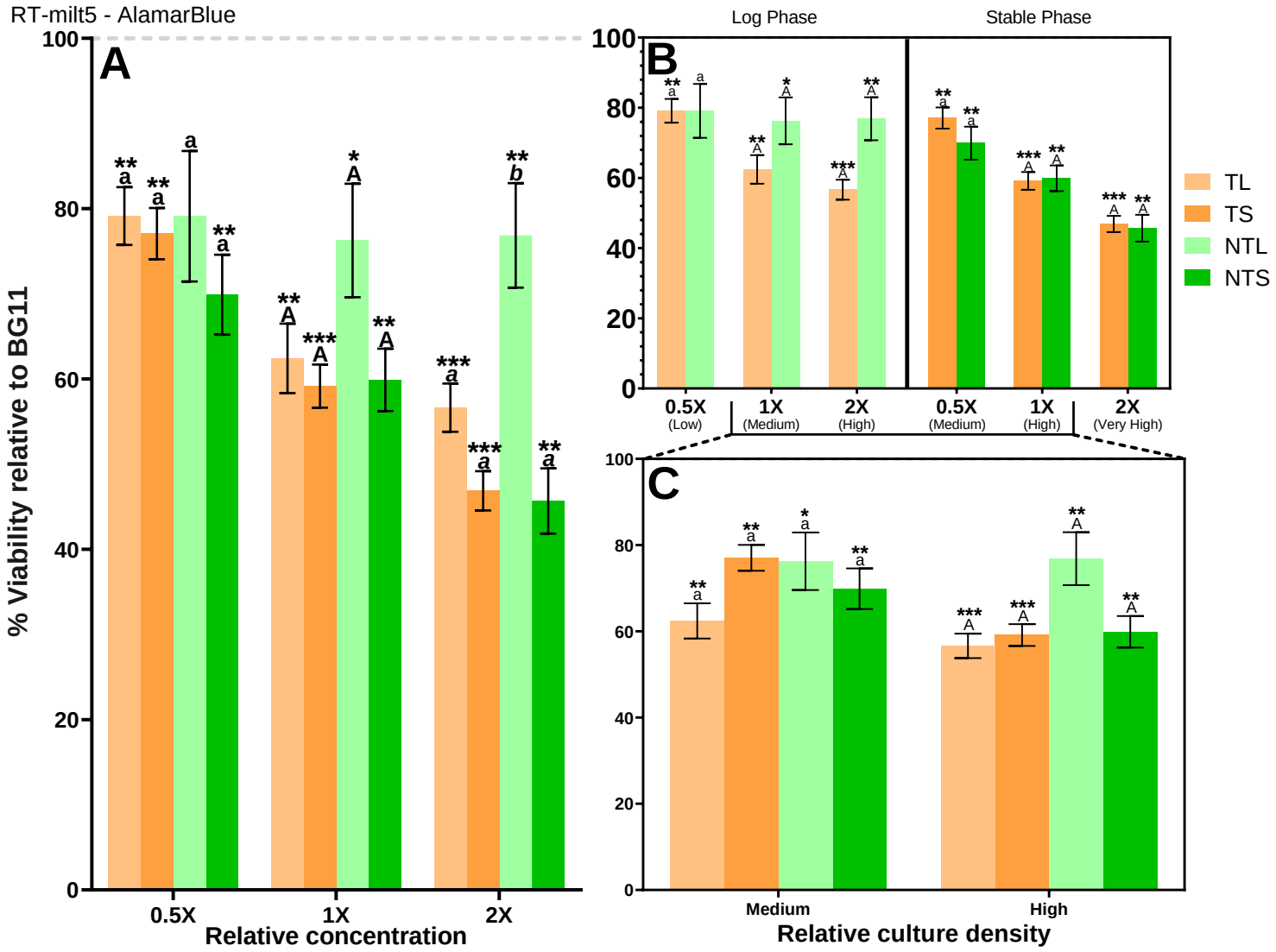

Figure A1.5.13: Mean (± SEM) cell viability values of RT-milt5 cells from AlamarBlue cytotoxicity assays and compared via concentration (**A**) and relative culture density (**B, C**). Each experiment was conducted in triplicate and repeated on six separate 96-well plates. Significant differences between treatments and the BG-11 vehicle control are indicated with asterisks (*P < 0.05, **P < 0.01, ***P < 0.001) and significant differences between treatments within each concentration are indicated by different letters.

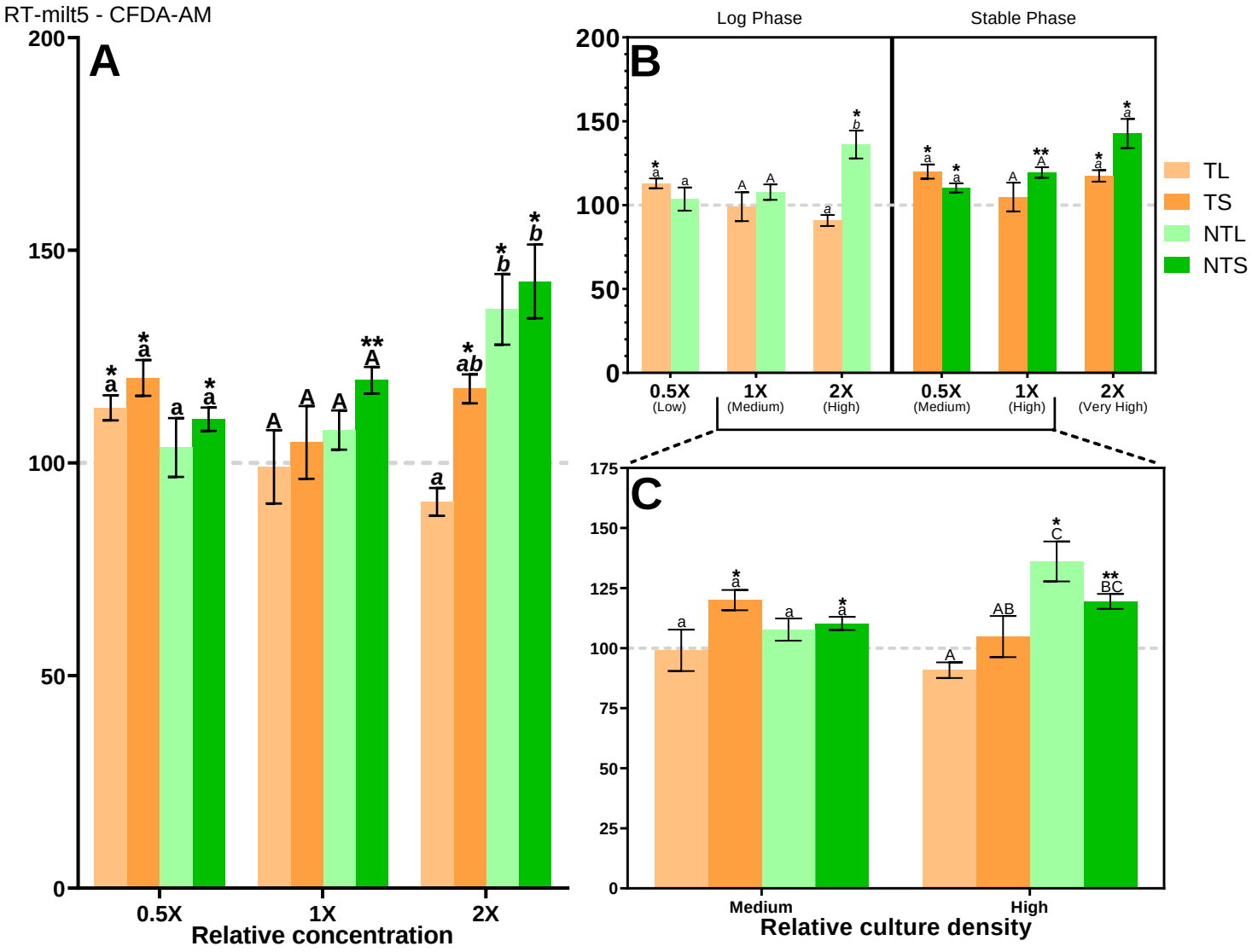

Figure A1.5.14: Mean (± SEM) cell viability values of RT-milt5 cells from CFDA-AM cytotoxicity assays and compared via concentration (**A**) and relative culture density (**B, C**). Each experiment was conducted in triplicate and repeated on six separate 96-well plates. Significant differences between treatments and the BG-11 vehicle control are indicated with asterisks (*P < 0.05, **P < 0.01, ***P < 0.001) and significant differences between treatments within each concentration are indicated by different letters.

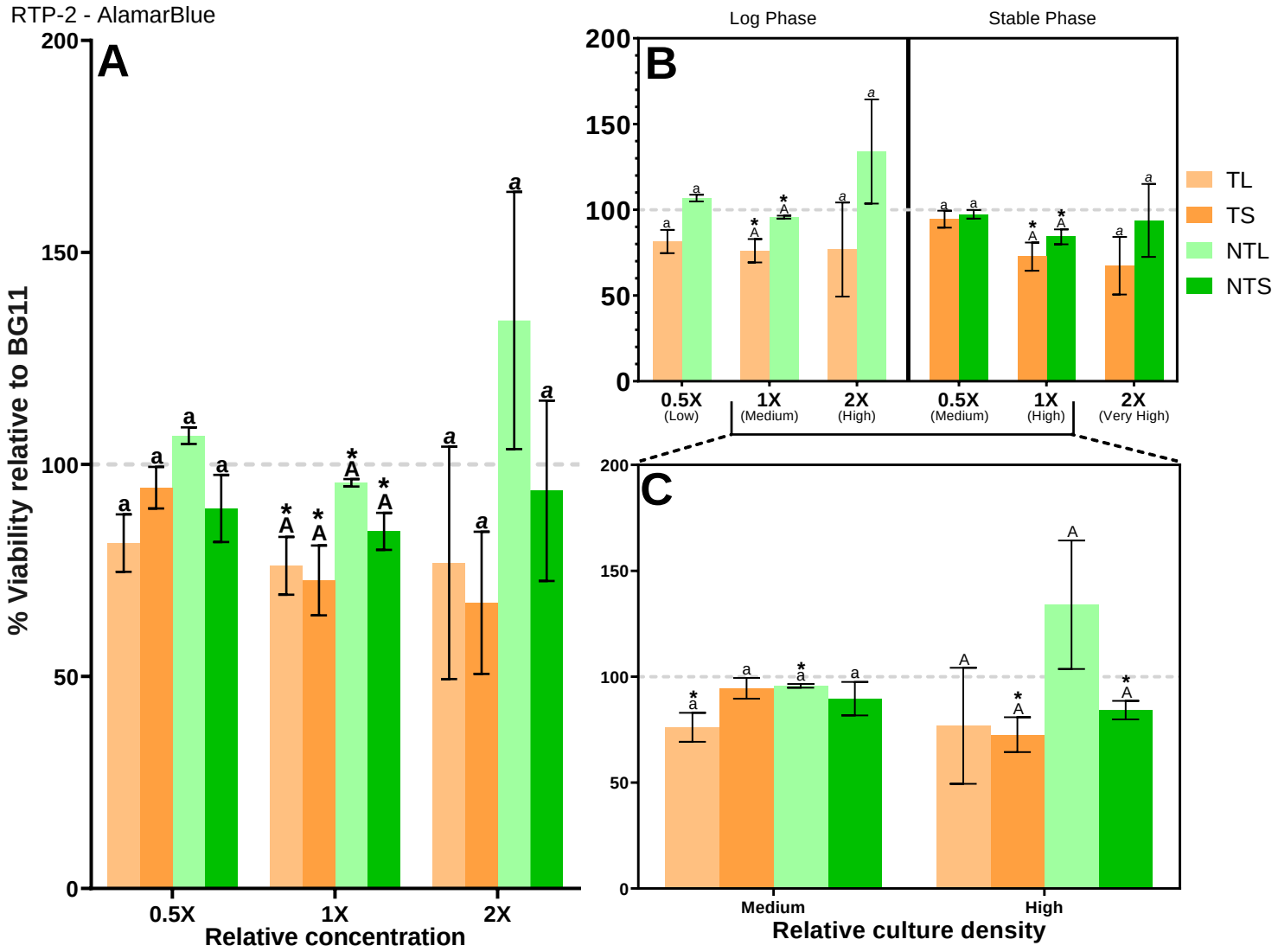

Figure A1.5.15: Mean (± SEM) cell viability values of RTP-2 cells from AlamarBlue cytotoxicity assays and compared via concentration (**A**) and relative culture density (**B, C**). Each experiment was conducted in triplicate and repeated on four separate 96-well plates. Significant differences between treatments and the BG-11 vehicle control are indicated with asterisks ($P < 0.05$) and significant differences between treatments within each concentration are indicated by different letters.

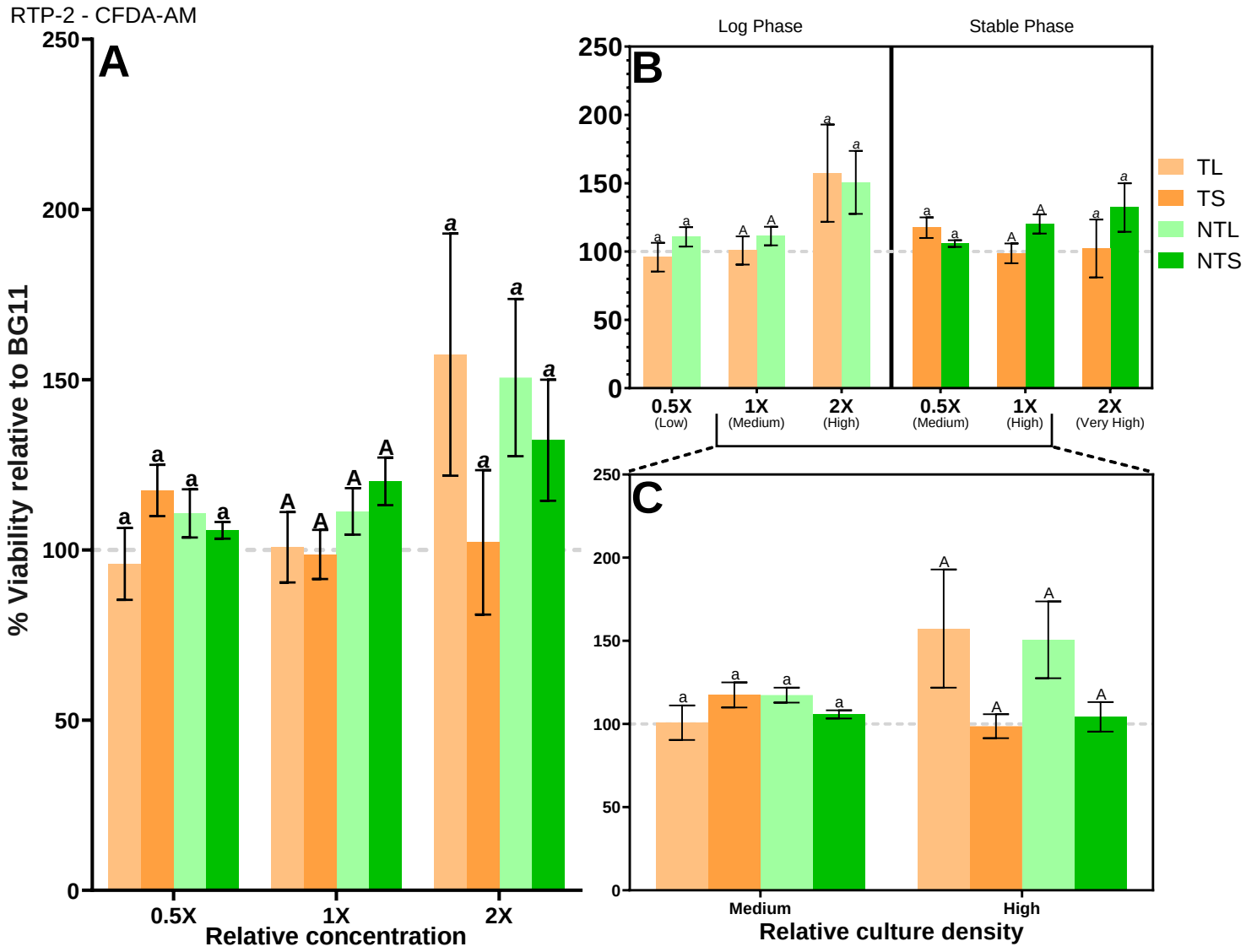

Figure A1.5.16: Mean (± SEM) cell viability values of RTP-2 cells from CFDA-AM cytotoxicity assays and compared via concentration (**A**) and relative culture density (**B, C**). Each experiment was conducted in triplicate and repeated on four separate 96-well plates. Significant differences between treatments and the BG-11 vehicle control are indicated with asterisks ($P < 0.05$) and significant differences between treatments within each concentration are indicated by different letters.

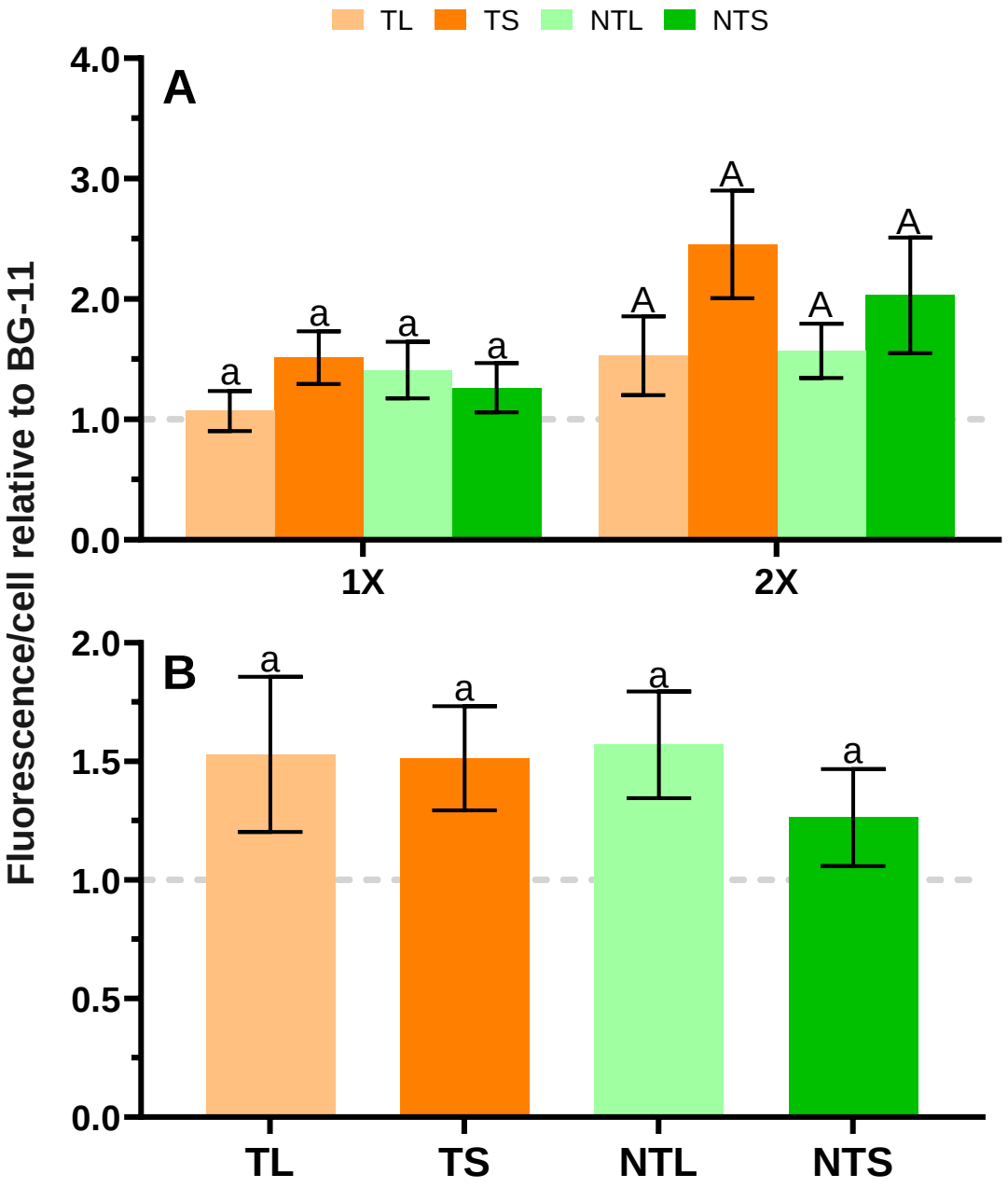

Figure A1.5.17: Differences in lysosomal accumulation in RBT4BA cells treated with TL, TS, NTL, and NTS exudate treatments at concentrations of 1X and 2X (**A**) and high relative culture density (**B**). Values are presented as mean ± SEM (n = 3) relative to BG-11 vehicle control values. Significant differences between treatments within each concentration are indicated by different letters.

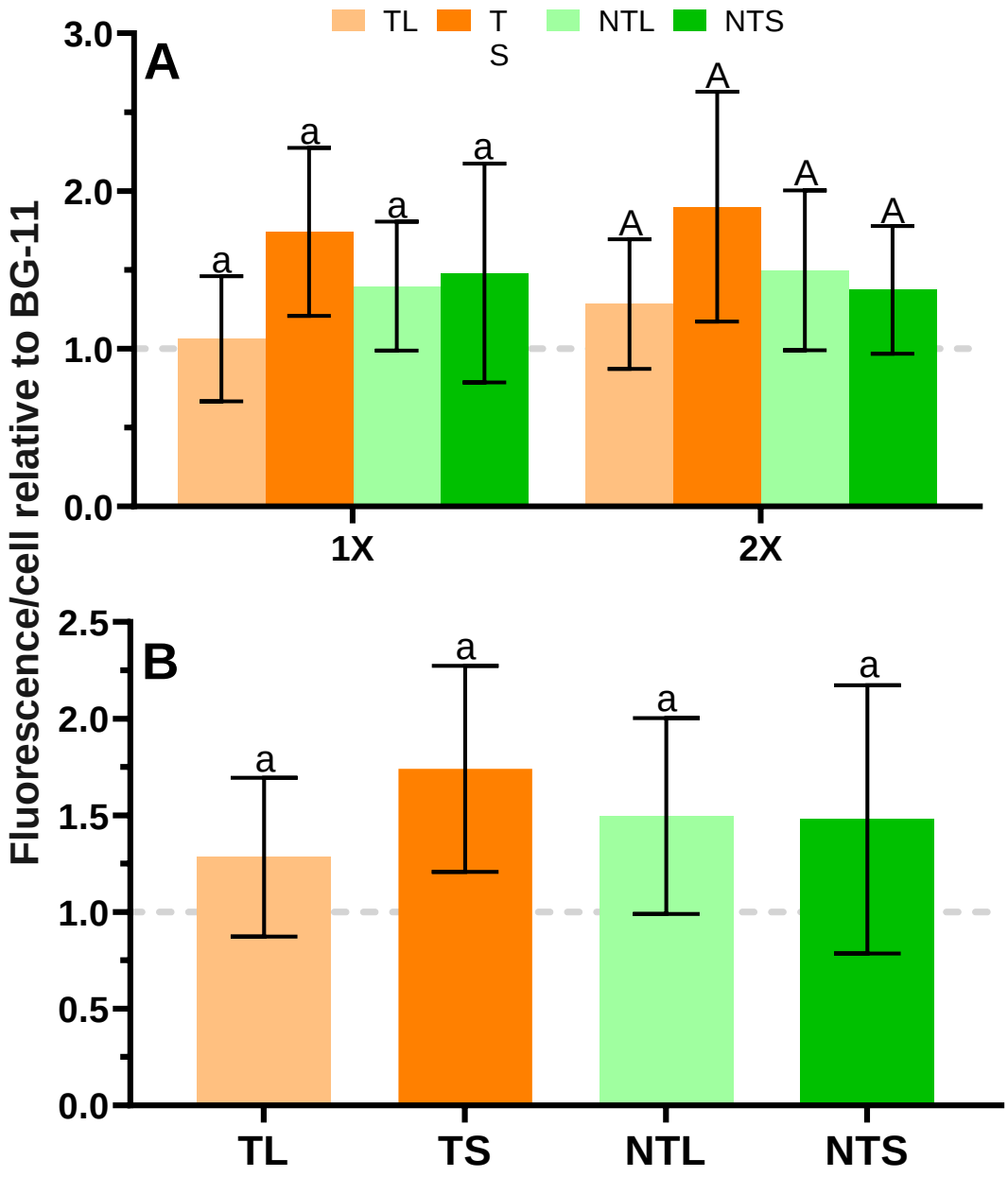

Figure A1.5.18: Differences in lysosomal accumulation in RTBrain cells treated with TL, TS, NTL, and NTS exudate treatments at concentrations of 1X and 2X (**A**) and high relative culture density (**B**). Values are presented as mean ± SEM (n = 3) relative to BG-11 vehicle control values. Significant differences between treatments within each concentration are indicated by different letters.

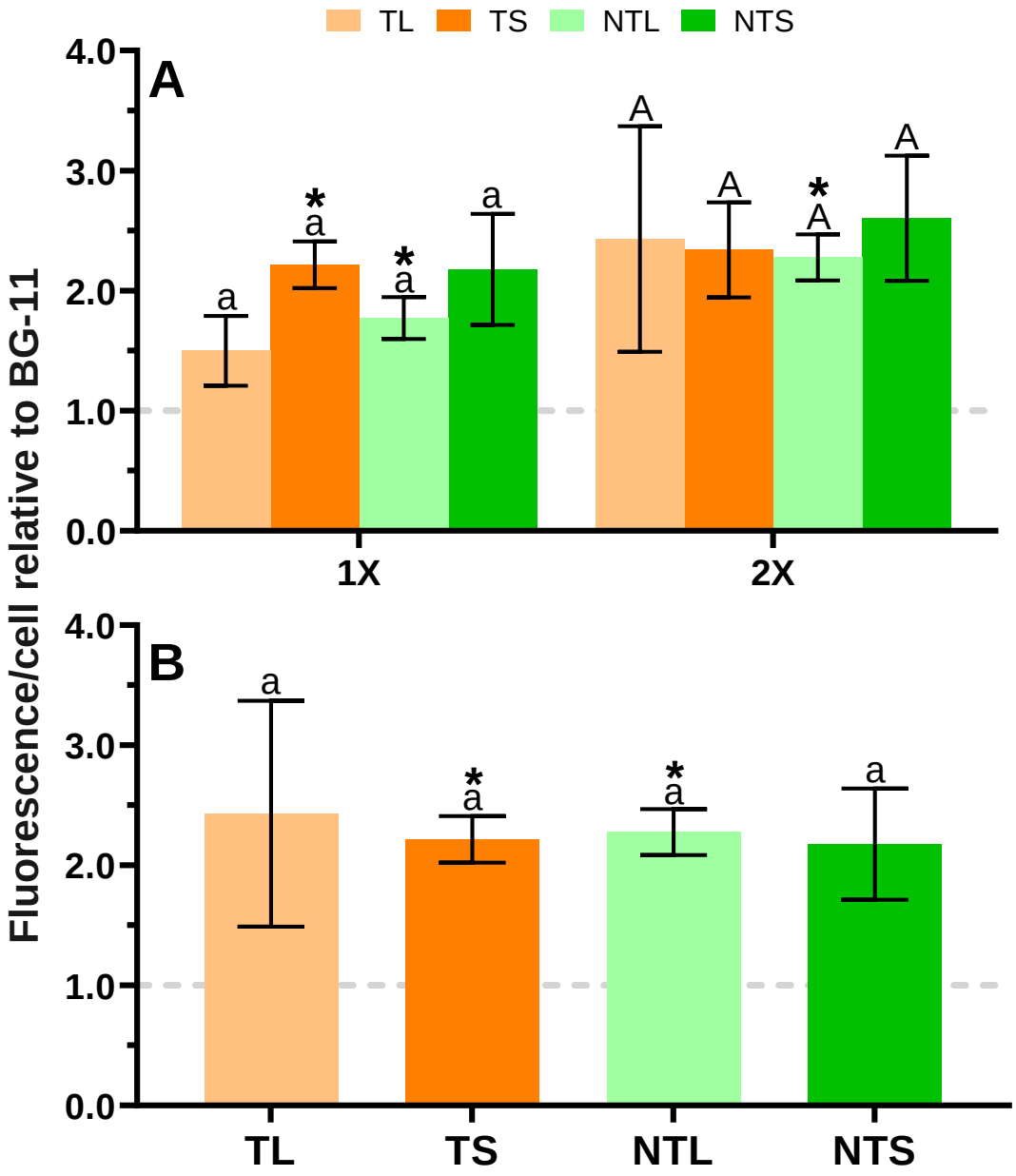

Figure A1.5.19: Differences in lysosomal accumulation in RTG-2 cells treated with TL, TS, NTL, and NTS exudate treatments at concentrations of 1X and 2X (**A**) and high relative culture density (**B**). Values are presented as mean ± SEM (n = 3) relative to BG-11 vehicle control values. Significant differences between treatments and the BG-11 vehicle control are indicated with asterisks (*P < 0.05) and significant differences between treatments within each concentration are indicated by different letters.

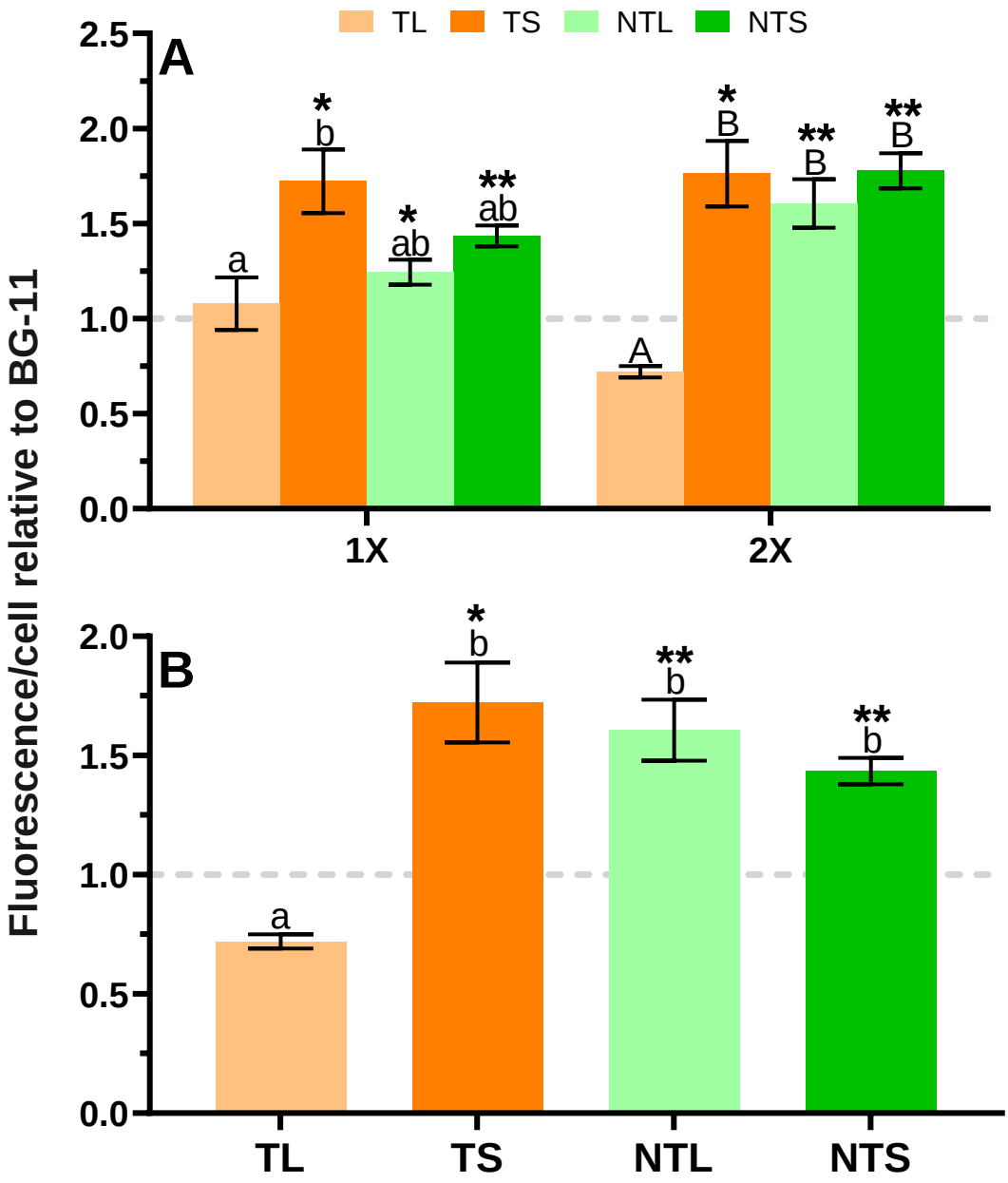

Figure A1.5.20: Differences in lysosomal accumulation in RTgill-W1 cells treated with TL, TS, NTL, and NTS exudate treatments at concentrations of 1X and 2X (**A**) and high relative culture density (**B**). Values are presented as mean ± SEM (n = 5) relative to BG-11 vehicle control values. Significant differences between treatments within each concentration are indicated by different letters.

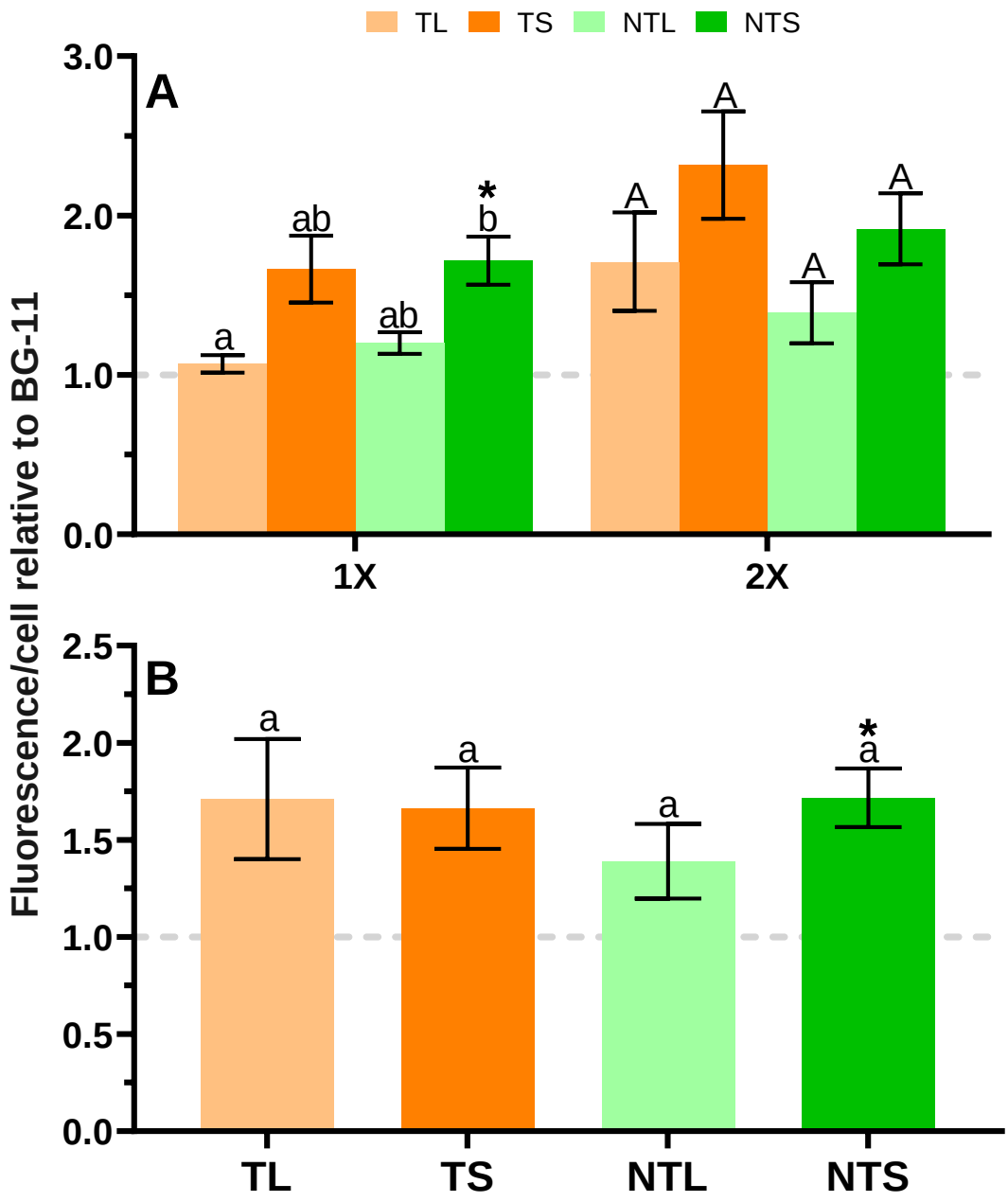

Figure A1.5.21: Differences in lysosomal accumulation in RTHDF cells treated with TL, TS, NTL, and NTS exudate treatments at concentrations of 1X and 2X (**A**) and high relative culture density (**B**). Values are presented as mean ± SEM (n = 3) relative to BG-11 vehicle control values. Significant differences between treatments and the BG-11 vehicle control are indicated with asterisks (*P < 0.05) and significant differences between treatments within each concentration are indicated by different letters.

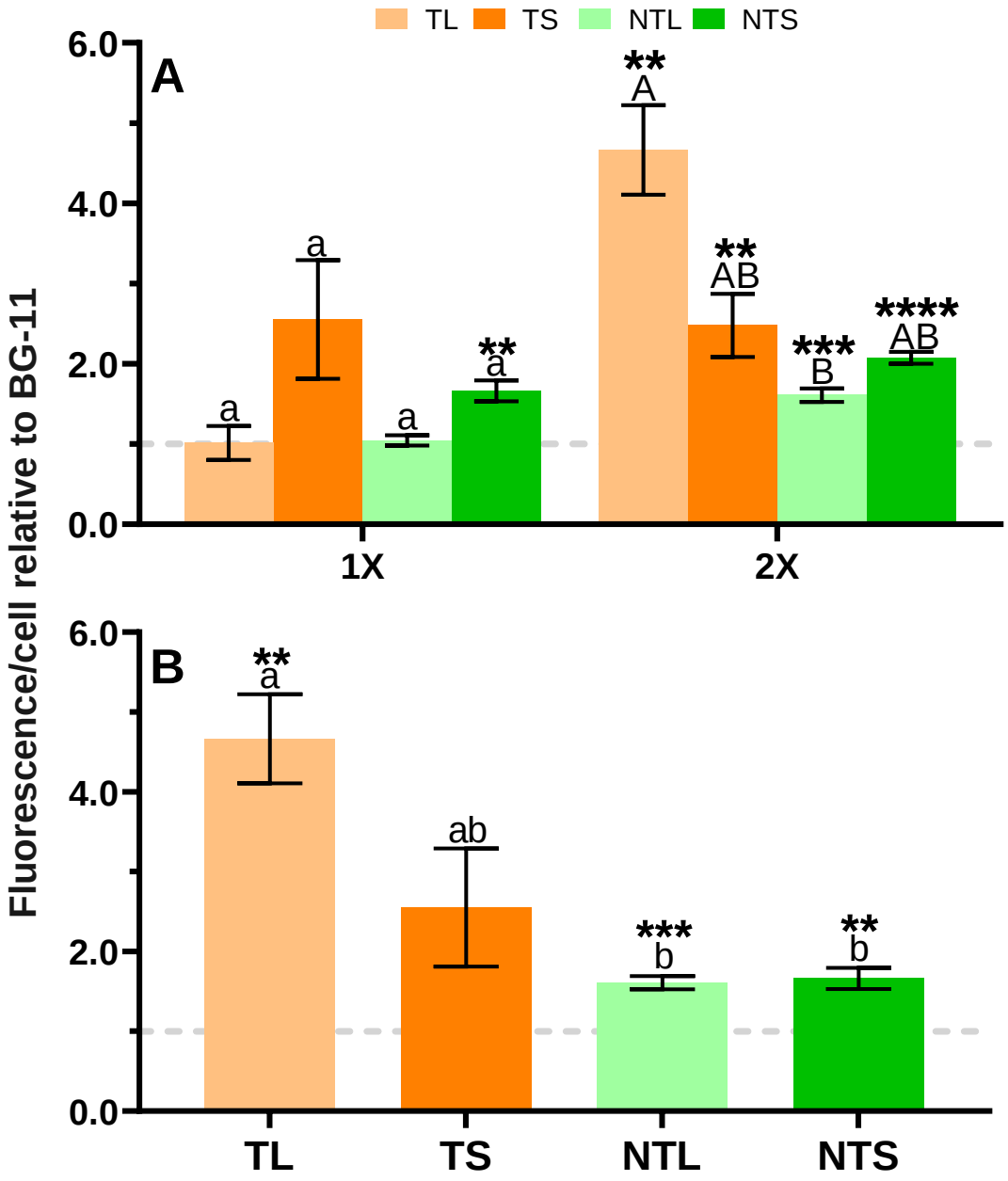

Figure A1.5.22: Differences in lysosomal accumulation in RTL-W1 cells treated with TL, TS, NTL, and NTS exudate treatments at concentrations of 1X and 2X (**A**) and high relative culture density (**B**). Values are presented as mean ± SEM (n = 8) relative to BG-11 vehicle control values. Significant differences between treatments and the BG-11 vehicle control are indicated with asterisks (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001) and significant differences between treatments within each concentration are indicated by different letters.

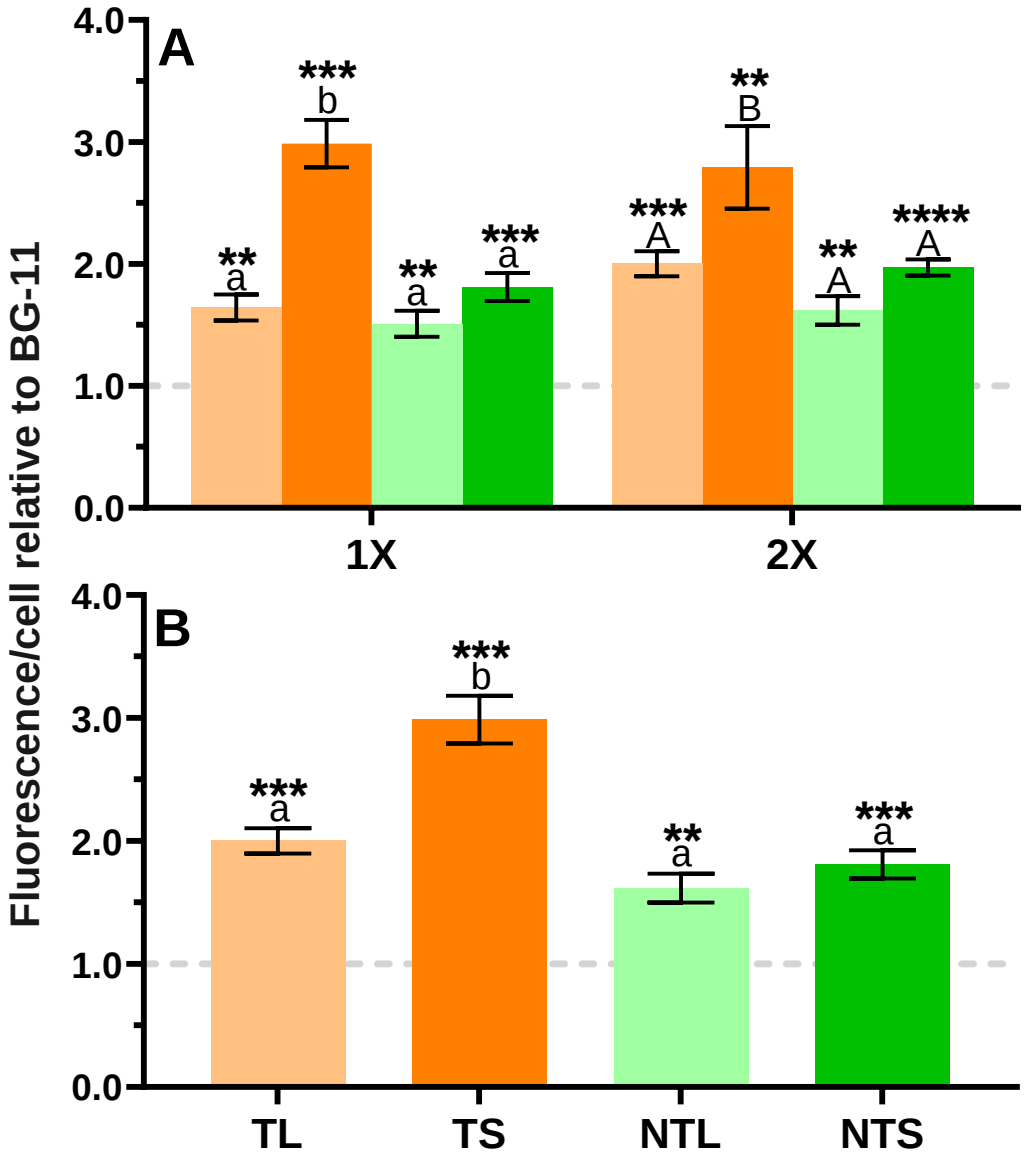

Figure A1.5.23: Differences in lysosomal accumulation in RT-milt5 cells treated with TL, TS, NTL, and NTS exudate treatments at concentrations of 1X and 2X (**A**) and high relative culture density (**B**). Values are presented as mean ± SEM (n = 8) relative to BG-11 vehicle control values. Significant differences between treatments and the BG-11 vehicle control are indicated with asterisks (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001) and significant differences between treatments within each concentration are indicated by different letters.

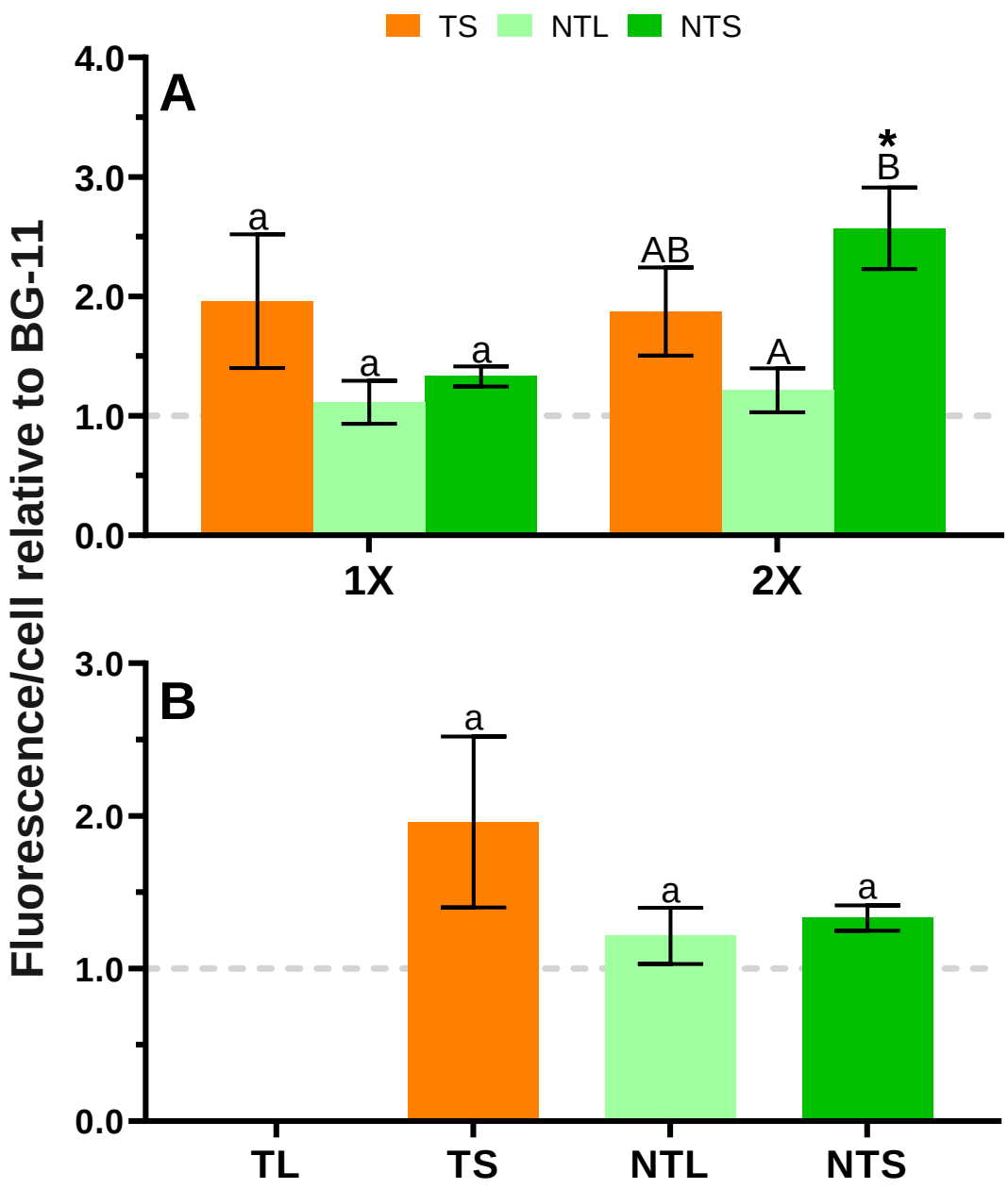

Figure A1.5.24: Differences in lysosomal accumulation in RTP-2 cells treated with TL, TS, NTL, and NTS exudate treatments at concentrations of 1X and 2X (**A**) and high relative culture density (**B**). Values are presented as mean ± SEM (n = 3) relative to BG-11 vehicle control values. Significant differences between treatments and the BG-11 vehicle control are indicated with asterisks (*P < 0.05) and significant differences between treatments within each concentration are indicated by different letters.

APPENDIX 1.6 CHAPTER 3 SUPPLEMENTAL TABLES

Table A1.6.1: Fold-change and significance compared to controls for genes expressed in reproduction-related rainbow trout cell lines treated with *M. aeruginosa* exudate treatments. **Bolded** values are significantly different from controls.

Cell Line		*fshβ*		*fshr*		*lhr*		*erα1*		*p450scc*		*3β-hsd*	
		Fold-change	***p***	**Fold-change**	***p***	**Fold-change**	***p***	**Fold-change**	***p***	**Fold-change**	***p***	**Fold-change**	***p***
RTBrain	TL	**0.541 ± 0.049**	**0.0112**	—	—	—	—	1.424 ± 0.419	0.4180	**0.397 ± 0.023**	**0.0015**	**0.582 ± 0.065**	**0.0235**
	TS	0.669 ± 0.192	0.2266	—	—	—	—	2.288 ± 0.943	0.3054	**0.494 ± 0.083**	**0.0260**	0.641 ± 0.115	0.0893
	NTL	0.673 ± 0.165	0.1866	—	—	—	—	0.893 ± 0.212	0.6639	**0.395 ± 0.091**	**0.0221**	0.619 ± 0.140	0.1123
	NTS	0.648 ± 0.106	0.0802	—	—	—	—	1.649 ± 0.490	0.3163	**0.352 ± 0.099**	**0.0227**	0.660 ± 0.106	0.0849
RTG-2	TL	0.514 ± 0.238	0.1786	**0.095 ± 0.031**	**0.0012**	4.698 ± 0.535	0.1266	—	—	**0.350 ± 0.018**	**0.0007**	**0.089 ± 0.029**	**0.0010**
	TS	0.428 ± 0.139	0.2968	**0.012 ± 0.002**	**<0.0001**	3.208 ± 0.243	0.0715	—	—	**0.302 ± 0.019**	**0.0008**	**0.088 ± 0.013**	**0.0002**
	NTL	0.890 ± 0.181	0.4912	**0.088 ± 0.016**	**0.0003**	4.016 ± 0.885	0.1272	—	—	0.475 ± 0.142	0.0657	**0.177 ± 0.030**	**0.0013**
	NTS	0.682 ± 0.082	0.5654	**0.070 ± 0.013**	**0.0002**	3.629 ± 0.200	0.2135	—	—	0.284 ± 0.058	0.2296	**0.160 ± 0.012**	**0.0002**
RTL-W1	TL	1.114 ± 0.368	0.7765	—	—	1.003 ± 0.194	0.9891	1.517 ± 0.397	0.3224	0.979 ± 0.037	0.6036	0.643 ± 0.216	0.1969
	TS	1.216 ± 0.465	0.6738	—	—	0.909 ± 0.270	0.7690	1.230 ± 0.364	0.5926	1.123 ± 0.128	0.4089	0.780 ± 0.256	0.4537
	NTL	1.091 ± 0.251	0.7404	—	—	0.707 ± 0.120	0.1340	1.388 ± 0.202	0.5000	1.487 ± 0.353	0.2620	0.748 ± 0.165	0.2242
	NTS	0.965 ± 0.203	0.8733	—	—	1.233 ± 0.153	0.2670	1.325 ± 0.323	0.4197	1.537 ± 0.293	0.1642	1.010 ± 0.130	0.9434
RT-milt5	TL	1.054 ± 0.255	0.8528	0.748 ± 0.325	0.5198	—	—	2.413 ± 0.900	0.2567	1.421 ± 0.293	0.2872	0.387 ± 0.219	0.1077
	TS	**0.388 ± 0.061**	**0.099**	0.464 ± 0.204	0.1197	—	—	**1.655 ± 0.137**	**0.0408**	0.804 ± 0.138	0.2913	**0.179 ± 0.077**	**0.0087**
	NTL	1.146 ± 0.142	0.4118	1.557 ± 0.914	0.6042	—	—	1.081 ± 0.152	0.6469	0.681 ± 0.146	0.1595	0.845 ± 0.182	0.4828
	NTS	1.200 ± 0.246	0.5009	0.596 ± 0.319	0.3326	—	—	1.110 ± 0.190	0.6202	1.024 ± 0.071	0.7705	1.154 ± 0.287	0.6463
RTP-2	TL	**0.273 ± 0.048**	**0.0043**	—	—	3.118 ± 0.658	0.0845	1.978 ± 0.368	0.1173	**0.250 ± 0.119**	**0.0244**	**0.119 ± 0.051**	**0.0033**
	TS	0.779 ± 0.460	0.7140	—	—	2.286 ± 0.339	0.1639	2.222 ± 0.246	0.1263	0.547 ± 0.317	0.3879	0.499 ± 0.320	0.3619
	NTL	0.695 ± 0.154	0.1860	—	—	**3.551 ± 0.116**	**0.0288**	2.578 ± 0.549	0.1028	0.566 ± 0.193	0.2500	0.722 ± 0.186	0.2738
	NTS	2.212 ± 0.905	0.2253	—	—	3.187 ± 1.435	0.3697	4.299 ± 0.522	0.0998	1.286 ± 0.275	0.4875	2.246 ± 1.165	0.2909

Table A1.6.2: Fold-change and significance compared to controls for genes expressed in non-reproduction-related rainbow trout cell lines treated with *M. aeruginosa* exudate treatments. **Bolded** values are significantly different from controls.

Cell Line		***fshβ***		***fshr***		***erα1***		***p450scc***		***3β-hsd***	
		Fold-change	***p***	**Fold-change**	***p***	**Fold-change**	***p***	**Fold-change**	***p***	**Fold-change**	***p***
RBT4BA	TL	1.092 ± 0.107	0.4808	—	—	2.025 ± 0.760	3.096	1.111 ± 0.307	0.7519	1.139 ± 0.287	0.6754
	TS	1.062 ± 0.096	0.5824	—	—	1.608 ± 0.215	0.1057	2.408 ± 1.809	0.5179	1.843 ± 0.855	0.4284
	NTL	0.752 ± 0.137	0.2109	—	—	2.412 ± 0.597	0.1419	1.579 ± 0.836	0.5601	1.034 ± 0.240	0.9001
	NTS	1.105 ± 0.137	0.5234	—	—	1.619 ± 0.534	0.3660	1.762 ± 0.887	0.4806	1.022 ± 0.156	0.9026
RTgill-W1	TL	0.859 ± 0.054	0.0800	0.967 ± 0.218	0.8876	1.100 ± 0.142	0.5345	1.140 ± 0.092	0.2231	0.940 ± 0.102	0.6192
	TS	1.034 ± 0.147	0.8312	1.568 ± 0.757	0.5316	**0.707 ± 0.062**	**0.0178**	1.152 ± 0.144	0.3686	1.664 ± 0.213	0.0523
	NTL	0.774 ± 0.055	0.0546	1.884 ± 0.383	0.1475	1.009 ± 0.169	0.9638	1.040 ± 0.159	0.8259	1.349 ± 0.343	0.3842
	NTS	0.881 ± 0.062	0.1521	**0.354 ± 0.147**	**0.0480**	0.908 ± 0.130	0.5302	1.063 ± 0.083	0.5038	1.183 ± 0.113	0.2477
RTHDF	TL	**0.270 ± 0.032**	**0.0019**	—	—	3.556 ± 1.293	0.1867	0.402 ± 0.209	0.1037	**0.099 ± 0.009**	**0.0001**
	TS	**0.335 ± 0.013**	**0.0004**	—	—	1.876 ± 0.801	0.3881	0.338 ± 0.139	0.0412	**0.252 ± 0.026**	**0.0012**
	NTL	**0.732 ± 0.031**	**0.0131**	—	—	4.076 ± 0.764	0.0566	**0.614 ± 0.176**	**0.1594**	0.981 ± 0.129	0.8943
	NTS	0.959 ± 0.091	0.7305	—	—	2.370 ± 0.004	0.2500	0.543 ± 0.049	0.0672	1.025 ± 0.170	0.8977

Table A1.6.3: Calculated *p* values for comparisons of gene expression changes in reproduction-related rainbow trout cell lines treated with *M. aeruginosa* exudates. **Bolded** values indicate significant differences between treatments.

Cell Line		***fshβ***	***fshr***	***lhr***	***erα1***	***p450scc***	***3β-hsd***
RTBrain	TL vs. TS	0.9132	—	—	0.7259	0.8254	0.9800
	TL vs. NTL	0.9053	—	—	0.9140	>0.9999	0.9949
	TL vs. NTS	0.9457	—	—	0.9922	0.9783	0.9573
	TS vs. NTL	>0.9999	—	—	0.3840	0.8197	0.9989
	TS vs. NTS	0.9996	—	—	0.8625	0.6155	0.9994
	NTL vs. NTS	0.9992	—	—	0.7952	0.9801	0.9934
RTG-2	TL vs. TS	0.9984	0.0578	0.9916	—	0.9957	>0.9999
	TL vs. NTL	0.6449	0.9940	0.9892	—	0.9341	0.0935
	TL vs. NTS	0.5912	0.7846	0.8776	—	0.7954	0.1958
	TS vs. NTL	0.7331	0.0824	0.9325	—	0.8486	0.0908
	TS vs. NTS	0.6801	0.2144	0.7409	—	0.6745	0.1903
	NTL vs. NTS	0.9997	0.8980	0.9708	—	0.9860	0.9485
RTL-W1	TL vs. TS	0.9964	—	0.9850	>0.9999†	0.7206††	0.9595
	TL vs. NTL	>0.9999	—	0.7064	>0.9999†	0.5600††	0.9812
	TL vs. NTS	0.9888	—	0.8319	>0.9999†	0.3834††	0.5724
	TS vs. NTL	0.9934	—	0.8768	>0.9999†	0.7743††	0.9994
	TS vs. NTS	0.9510	—	0.6498	>0.9999†	0.6093††	0.8432
	NTL vs. NTS	0.9931	—	0.2878	>0.9999†	0.9995††	0.7852
RT-milt5	TL vs. TS	0.1472	0.9791	—	0.6779	0.1522	0.8893
	TL vs. NTL	0.9856	0.7004	—	0.2638	0.0774	0.4435
	TL vs. NTS	0.9476	0.9966	—	0.2792	0.4537	0.1116
	TS vs. NTL	0.0916	0.4882	—	0.8239	0.9610	0.1807
	TS vs. NTS	0.0693	0.9978	—	0.8446	0.8255	**0.0409**
	NTL vs. NTS	0.9970	0.5847	—	>0.9999	0.5652	0.7210
RTP-2	TL vs. TS	>0.9999†	—	0.8639	0.9826	0.7663	>0.9999†
	TL vs. NTL	>0.9999†	—	0.9760	0.7597	0.6674	0.6573†
	TL vs. NTS	**0.0278**†	—	0.9999	0.0517	0.0524	**0.0278**†
	TS vs. NTL	>0.9999†	—	0.7201	0.9494	0.9999	>0.9999†
	TS vs. NTS	0.8232†	—	0.8677	0.1086	0.2174	0.4157†
	NTL vs. NTS	0.8379†	—	0.9887	0.1485	0.1818	>0.9999†

† : Analyzed via Kruskal-Wallis and Dunn's post hoc test, ††: Analyzed via Welch's ANOVA with Games-Howell's post hoc test

Table A1.6.4: Calculated *p* values for comparisons of gene expression changes in non-reproduction-related rainbow trout cell lines treated with *M. aeruginosa* exudates. **Bolded** values indicate significant differences between treatments.

Cell Line		***fshβ***	***fshr***	***lhr***	***erα1***	***p450scc***	***3β-hsd***
RBT4BA	TL vs. TS	0.9980	—	—	0.9507	0.8378	0.7264
	TL vs. NTL	0.2656	—	—	0.9601	0.9898	0.9985
	TL vs. NTS	0.9998	—	—	0.9541	0.9738	0.9979
	TS vs. NTL	0.3309	—	—	0.7482	0.9488	0.6390
	TS vs. NTS	0.9940	—	—	>0.9999	0.9744	0.6287
	NTL vs. NTS	0.2404	—	—	0.7554	0.9994	>0.9999
RTgill-W1	TL vs. TS	0.5409	0.7240	—	0.1661	0.9999	0.1114††
	TL vs. NTL	0.9258	0.4211	—	0.9609	0.9382	0.6909††
	TL vs. NTS	0.9980	0.7127	—	0.6955	0.9623	0.4750††
	TS vs. NTL	0.2896	0.9526	—	0.4118	0.9174	0.8598††
	TS vs. NTS	0.6421	0.2598	—	0.6616	0.9449	0.3125††
	NTL vs. NTS	0.8638	0.1249	—	0.9484	0.9992	0.9639††
RTHDF	TL vs. TS	0.6291	—	—	0.6766	0.9921	0.7473
	TL vs. NTL	**0.0002**	—	—	0.9838	0.7995	**0.0018**
	TL vs. NTS	**<0.0001**	—	—	>0.9999	0.9460	**0.0013**
	TS vs. NTL	**0.0006**	—	—	0.4829	0.6529	**0.0059**
	TS vs. NTS	**<0.0001**	—	—	0.6722	0.8581	**0.0042**
	NTL vs. NTS	**0.0256**	—	—	0.9848	0.9921	0.9909

††: Analyzed via Welch's ANOVA with Games-Howell's post hoc test

VITA AUCTORIS

NAME: Keira Aileen Harshaw

PLACE OF BIRTH: New Westminster, BC

YEAR OF BIRTH: 1995

EDUCATION: Vincent Massey Secondary School, Windsor, ON, 2012

University of Windsor, B.Sc., Windsor, ON, 2018

University of Windsor, M.Sc., Windsor, ON, 2023