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Mitochondria Mediated Outcomes of Developmental Exposure to Low-level Chemical Mixtures in Zebrafish Danio rerio

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MITOCHONDRIA MEDIATED OUTCOMES OF DEVELOPMENTAL EXPOSURE TO LOW-LEVEL CHEMICAL MIXTURES IN ZEBRAFISH *DANIO RERIO*

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An Abstract of the Thesis Presented
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Exposure to drinking water contaminants has been linked to developmental outcomes in both epidemiological and model organism studies. However, low level mixture effects, on early development has yet to be explored. It is hypothesized that early chemical exposures may increase disease susceptibility later in life. This work aimed to investigate impacts of a variety of chemicals and concentrated on metals arsenic (As), cadmium (Cd), vanadium (V), and lead (Pb) due to their presence in drinking water and known developmental toxicity. To determine the effects of a metal and organic contaminant co-exposure, the ubiquitously used herbicide glyphosate was also explored.

The zebrafish (*Danio rerio*) model was used to elucidate developmental impacts of low-level chemical mixture exposure with a focus on mitochondrial function. An in-depth analysis exploring embryonic oxygen consumption rate (eOCR) in response to all iterations of a 5-part chemical mixture of glyphosate, As, Cd, V, and Pb showed that mitochondria are highly sensitive to mixture toxicity, and that pre-exposure to a metal mixture leave the mitochondria more susceptible to acute chemical stress through depleted reserve capacity. Altered mitochondrial function, along with changes in gene expression and histology suggested that early

mixture exposure may contribute to the endemic of chronic kidney disease of unknown etiology (CKDu).

To investigate underlying molecular mechanisms that may contribute to CKDu susceptibility, RNA seq data from zebrafish embryos exposed to mixtures of As, Cd, V, and Pb, (+/- glyphosate) and glyphosate alone, suggest that exposure to metal and organic mixtures may be altering the extracellular matrix of kidney tissue. This combined with impaired mitochondrial function, could leave individuals more susceptible to kidney injury CKDu progression.

To determine phenotypes associated with mixture exposure, changes in behavior after exposure to a large collection of water samples were explored. A cluster analysis of metals found in drinking water samples were coupled to changes in behavior and revealed that concentrations of Pb, Cd, As, Uranium (U) and Nickel (Ni), should be taken into special consideration when determining drinking water standards.

These data suggest that impaired mitochondria, as a result of low-level mixture exposure, may function in the early onset of disease, such as CKDu, and further impair organism development.

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CHAPTER 1

INTRODUCTION

Chemical exposure is of increasing concern in a world in which new chemicals are synthesized, mass produced, and used, often times without regulation, on a daily basis. Intensive studies have been done on some chemicals, such as metals, pesticides, and other prominent organic compounds, and have found these chemicals to be individually toxic at specific concentrations. Such toxicity assays include those that are in-vitro and measure cell viability, ATP depletion, and lipid peroxidation, or those that are *in vivo* such as changes in behavior, reproductive consequences, and cancer progression (Q. Zhang et al., 2018). Metals have been associated with neurological dysfunction, kidney and respiratory diseases, and cancer (Balali-Mood et al., 2021). Pesticides have been correlated to development of neurological disease, respiratory illness, reproductive toxicity, and cancer (Mostafalou & Abdollahi, 2017).

Developmental toxicity of chemicals is extremely important, as an early chemical exposure could alter overall health later in life, and perhaps contribute to the progression of disease. This hypothesis, commonly known as the Developmental Origin of Health and Disease (DOHAD), originated in the early 2000s when gestational diet was considered to be an integral part of fetal development, and that a poor diet could leave an individual more susceptible to disease. Since then, DOHAD considers additional factors beyond diet, such as stress and chemical exposures (Heindel, 2019; Hoffman et al., 2017). An exploration of epidemiological and model organism studies has identified potential molecular mechanisms that contribute to DOHAD, such as changes in gene expression, epigenetics, and alterations in mtDNA copy number (Aluru, 2017; Fukunaga, 2021; McMullen & Mostyn, 2009).

At present, with an ever-growing exposome, understanding developmental exposure toxicology is complex. This is because individuals are exposed to multiple chemicals at once, and chemical mixtures rather than individual chemicals, are likely driving biological changes. Depending upon the mixture, additions of chemicals may have synergistic or antagonistic effects on toxicity, making it crucial to understand mixture specific endpoints (Singh et al., 2017). Additionally, there is a critical need to understand how low dose exposure to chemicals, that at individual levels are considered safe, may be acting together to have biological impacts. This is especially important during early development, in which there is a critical window where the fate of tissue and organs may be more susceptible to low levels of chemicals acting in tandem (Heindel, 2019).

The biological impacts of chemical mixture exposures needs to be addressed on a global scale, as constant exposure to a wide range of chemicals and concentrations, may be occurring through drinking water alone. Drinking water has been shown to contain both organic contaminants and inorganic contaminants (Babich, Ulrich, et al., 2020; Han & Price, 2011), with the United States Environmental Protection Agency (EPA) regulating levels of 53 and 12 chemicals in the aforementioned categories respectively (*National Primary Drinking Water Regulations*, 2015). Further, water quality is considered to be a "wicked problem", in which the solution requires multiple players to act in agreement (i.e., consensus on lowering chemical limits in drinking water, involvement of multiple stakeholders, community-specific intervention strategies) (Kreuter et al., 2004). Water quality also has large ecological impacts, as poor water quality can be harmful to plants and animals, and ultimately impact our food supply. By understanding how chemicals commonly found in drinking water drive molecular changes in early development, we are contributing to the body of knowledge that ultimately advocates for;

the right to have access to a clean drinking water supply, increased wellbeing of our future generations, and protection of our ecosystems.

To better understand how chemical mixture exposure through drinking water may contribute to disease, water quality in Sri Lanka where there is an endemic of chronic kidney disease of unknown etiology (CKDu), was extensively explored. CKDu research in Sri Lanka emphasizes a key role for chemical exposure (e.g., heavy metals, herbicides, etc.) through drinking water due to contamination from agricultural practices (Ananda Jayalal et al., 2019; Cooray et al., 2019; Gunatilake et al., 2019; Jayasumana et al., 2014; Kulathunga et al., 2019; Wimalawansa, 2016). Metal composition data led to the identification of 4 chemicals as potential drivers of CKDu, both individually and in mixture. These chemicals include metals arsenic (As), cadmium (Cd), vanadium (V) and lead (Pb). Endemic regions were often located in agricultural communities, therefore, the herbicide glyphosate (Gly) was also considered (Gunarathna et al., 2018; Gunatilake et al., 2019). For this reason, As, Cd, V, Pb, and Gly, both alone and in mixture were the primary chemicals explored in this work.

In the studies that follow the zebrafish, *Danio rerio*, was used to shed light on developmental toxicity of multiple chemicals and chemical mixtures. The zebrafish provide an excellent *in vivo* model to understand the genetic consequences to early exposures to chemicals and chemical mixtures (Shankar et al., 2019; M. Zheng et al., 2018). They allow for high-throughput exposure studies, their genome is sequenced and annotated, and biological changes in response to chemical exposures in the zebrafish model have been related across species (Nishimura et al., 2015). Additionally, due to their ex vivo fertilization and rapid development time the impact of embryonic exposures can be easily explored (J.-H. He et al., 2014; Nishimura et al., 2015). The zebrafish also shares genetic similarities to humans that allows for molecular

consequences to exposures to be compared across species. In fact, humans have a zebrafish gene orthologue for 70% of genes and 82% of genes that are associated with disease (Bradford et al., 2017). This, along with similar organ system structure and function, has made the zebrafish an accepted toxicological disease model (Santoriello & Zon, 2012).

Here, the embryonic zebrafish was exposed to a series of anthropogenic chemicals, drinking water samples, and multiple concentrations and mixture variations of As, Cd, V, Pb and Gly. The impact of exposure on mitochondrial function, gene expression, histopathology, and behavior were explored in order to elucidate molecular mechanisms that may be contributing to low level chemical developmental toxicity. Mitochondrial electron transport chain dysfunction via whole organism embryonic oxygen consumption rate (eOCR) was broadly explored as a potential key player in organism susceptibility to environmental stressors.

CHAPTER 2
MITOCHONDRIA RESPONSE AND RESILIENCE TO ANTHROPOGENIC CHEMICALS

Chapter 2.1 Introduction

Mitochondria, which are essential for eukaryotic cellular development and function, are primarily recognized for maintaining energy homeostasis through the production of ATP. However, they are involved in a number of other processes including oxidative stress response, reactive oxygen species (ROS) signaling, apoptotic signaling, Ca^{2+} signaling and regulation, and biosynthesis of macromolecules (Bhatti et al., 2017; Pathak & Trebak, 2018; Pfanner et al., 2019; Spinelli & Haigis, 2018; Vakifahmetoglu-Norberg et al., 2017).

Mitochondria are membrane bound organelles, range in cellular density depending on tissue type, and uniquely, contain their own mitochondrial DNA (mtDNA). mtDNA codes for 13 ETC complex subunit proteins, 22 transfer RNAs and 2 ribosomal RNAs (El-Hattab et al., 2017), while the rest of the mitochondrial proteome (~ 1500 proteins) are nuclear encoded and transported into the mitochondria via inner and outer membrane translocase channels (Chacinska et al., 2009; Melber & Haynes, 2018). Successful transcription and translation of ETC proteins is necessary for oxidative phosphorylation and ATP production. Dysfunctional ETC complexes will also contribute to the accumulation of ROS, which at low-levels can interfere with signal transduction and at higher levels cause DNA damage and cell death (Sies & Jones, 2020).

Importantly, mitochondria play a critical role in viability and survival of developing embryos (Mishra & Chan, 2014). Throughout embryogenesis, aside from ATP synthesis, mitochondria facilitate signaling cascades and are involved in intracellular communication (Chandel, 2014; Nagaraj et al., 2017). Mitochondrial synthesis of molecules such as acetyl CoA

from glucose and NAD+ is important in acetylation and potential epigenetic modifications of the developing embryo (Moussaieff et al., 2015). Consequently, developmental perturbations to mitochondria may have significant later-life health impacts (Leung et al., 2013; Xia et al., 2014).

Mitochondria are highly sensitive to environmental toxin exposure and are often responsible for facilitating an adaptive response to stressors by meeting the energy demands of the cell through increased oxidative phosphorylation (OXPHOS), alterations in fusion or fission, and mitophagy (Eisner et al., 2018). Mitochondrial function (e.g., oxidative phosphorylation) and mitochondrial structures (e.g., lipid membranes and mitochondrial DNA) are key targets of anthropogenic compounds such as agrochemicals, industrial chemicals, and pharmaceuticals (Meyer et al., 2018). Furthermore, xenobiotic chemicals may affect mitochondrial regulation of nuclear-cytoplasmic processes (e.g., hormonal activity) and signaling (e.g., ROS, apoptotic), as well as alter mitochondrial ATP synthesis. Although mitochondria are multi-faceted, their primary job is to provide energy through OXPHOS to meet the cells increased energy demand. Therefore, oxygen consumption rate is a useful metric to determine chemical toxicity to mitochondria.

Despite growing interest on mitochondrial toxins and their potential role in the etiology of chronic diseases, research is just beginning to emerge on mitochondrial effects of chemical exposure during embryonic development. Furthermore, current studies are typically conducted using cell culture assays and primarily focus on acute mitochondrial effects (Nadanaciva et al., 2013). Significant limitations in these studies include lack of insight into putative effects of chemical metabolites derived *in vivo* from parent compounds, and persistent long-term effects.

Continuous discovery of persistent contaminants in the environment, such as PFOS (Perfluorooctanesulfonic acid; a perfluoroalkyl substance) in the drinking water, indicate the

limitations in determining health effects of environmental exposures solely through chemical composition analyses (Hu et al., 2016). Therefore, *in vivo* analyses of environmental samples can provide insights into long-term health consequences of environmental exposures. To this end, mitochondrial toxicity can be a useful end-point and provide broader insights into potential health outcomes of environmental exposures. Thus, high throughput approaches assessing mitochondrial effects in a whole organismal exposure context, particularly during vulnerable life-history stages (e.g., embryogenesis) are highly valuable when screening for persistent cellular effects of individual chemical contaminants.

2.2 Methods

2.2.1 Exposure protocol

Wildtype AB strain zebrafish embryos were collected and incubated at 28.5°C in egg water (1 embryo/1 mL) until 1 hpf at which time embryos were screened for viability. Embryos were moved to treatment solutions, within their chorions, (10 embryos / 10 mL) for 24 hr at 28.5°C. Embryos were treated with egg water supplemented with a given chemical as summarized in Table 1. These concentrations were determined via a literature search to determine environmentally relevant concentrations and low dose ranges that were previously determined to be non-lethal in aquatic organisms (See Table A1 for literature review). Each chemical treatment was conducted in triplicate and none of the treatments showed significant effects on survival or embryonic development.

Table 1: Mitochondrial toxicity of anthropogenic chemicals. Chemicals, manufacturer, primary source of entry into the environment and mechanisms of mitochondrial toxicity (if known), and concentrations used for treatment of 1 hpf zebrafish embryos in egg water.

Chemical	Primary source / mechanisms of toxicity in mitochondria	Chemical concentrations in egg water (μM). *Initial chemical stock solutions were diluted in 100% DMSO. (DMSO concentrations did not exceed 0.1% in egg water.)			
Amiodarone Hydrochloride (Sigma, 19774824)	Pharmaceutical / uncoupler	(0.001)	(0.01)	(0.1)	(1.0)
Arsenic (Sigma, S7400)	Naturally occurring, used in industrial applications / Disrupts MMP	2ug/L (0.026)	10 ug/ L (0.133)	50 ug/L (0.667)	500 ug/L (6.67)
Azoxystrobin (Sigma, 131860338)	Fungicide / inhibits complex III	0.5 ug/L (1.24)	2.0 ug/L (4.96)	3.5 ug/L (8.67)	5.0 ug/L (12.39)
BDE-47 (Sigma, 5436431)	Flame retardant, used in manufacturing	0.01 mg/L (0.0205)	0.1 mg/L (0.205)	1.0 mg/L (2.058)	10 mg/L (20.6)
Benzo(A)pyrene (Sigma, 50328)	Combustion of organic compounds	0.02 ug/L (0.000079)	0.2 ug/L (0.00079)	2.0 ug/L (0.0079)	20 ug/L (0.079)
Ezetimibe (Sigma, 163222331)	Pharmaceutical	(0.05)	(0.5)	(5.0)	(50.0)
FCCP (Sigma, 370865)	Mitochondrial uncoupler	(0.0125)	(0.025)	(0.05)	(0.15)
Fenpyroximate (Sigma, 134098616)	Pesticide / inhibits complex I	(0.05)	(0.1)	(0.2)	(0.4)
Fenazaquin (Sigma, 120928098)	Pesticide / inhibits complex I	0.05 ug/L (0.163)	0.5 ug/L (1.63)	5.0 ug/L (16.32)	20 ug/L (65.27)
Fluoranthene (Sigma, 206440)	Combustion of organic compounds	0.01 ug/L (0.005)	0.1 ug/L (0.029)	1.0 ug/L (0.148)	10 ug/L (0.741)
Naproxen Sodium (Sigma, 26159342)	Pharmaceutical	1.0 ug/L (0.004)	10 ug/L (0.043)	100 ug/L (0.434)	1000 ug/L (4.34)
Oligomycin (Sigma, 75351)	Mitochondrial ATP synthase inhibitor	(0.125)	(0.25)	(0.5)	(1)

Table 1 Continued

Oxybenzone (Sigma, 131577)	Key compound in Sunscreen	0.004 mg/L (0.0175)	0.04 mg/L (0.1753)	0.4 mg/L (1.753)	4 mg/L (17.53)
PFOA (Sigma, 335671)	Industry	0.0035 ug/L (0.000008)	0.007 ug/L (0.000017)	0.07 ug/L (0.00017)	0.7 ug/L (0.00169)
PFOS (Sigma, 77283)	Industry / decreases MMP	0.0035 ug/L (0.000007)	0.007 ug/L (0.000014)	0.07 ug/L (0.00014)	0.7 ug/L (0.00139)
Pyraclostrobin (Sigma, 175013180)	Fungicide / inhibits complex III	0.16 ug/L (0.0004)	0.8 ug/L (0.002)	4 ug/L (0.010)	20 ug/L (0.052)
Pyridaben (Sigma, 96489713)	Insecticide / inhibits complex I	0.006 ug/L (0.00003)	0.012 ug/L (0.0002)	0.06 ug/L (0.0008)	0.3 ug/L (0.004)
Rosiglitazone (Sigma, 122320734)	Pharmaceutical	(0.01)	(0.1)	(1)	(10)
Rotenone (Sigma, 557369)	Pesticide / inhibits complex I	0.005 ug/L (0.00001)	0.05 ug/L (0.00013)	0.5 ug/L (0.0013)	5.0 ug/L (0.0126)
Sodium Azide (Sigma, 26628228)	Industry / inhibits complex IV	(13.25)	(62.5)	(625)	(6250)
TDCPP (Sigma, 13674878)	Industry and pesticide	(0.001)	(0.01)	(0.1)	(1)
Toxaphene (Supelco, N13586)	Insecticide	1 mg/L (2.428)	5 mg/L (12.14)	10 mg/L (24.28)	20 mg/L (48.57)
Tributyltin (Sigma, 7486353)	Industry / Moussaieff	(0.0001)	(0.001)	(0.01)	(0.1)
Trifloxystrobin (Sigma, 141517217)	Pesticide	1 ug/L (0.00003)	6 ug/L (0.0003)	30 ug/L (0.0025)	150 ug/L (0.0245)

2.2.2 Mitochondrial toxicity analysis

Embryonic oxygen consumption rate (eOCR) was analyzed, per embryo, using XF96^{e} Extracellular Flux Analyzer (Agilent Technologies, CA) and was optimized following previous

studies (Souders et al., 2018; Stackley et al., 2011). Following a 24 hour incubation in treatment solutions, embryos were rinsed with egg water 3 times (10 mLs each). Subsequently, single embryos were transferred to an individual well, containing 150 uL of egg water, in a spheroid microplate (Agilent Technologies, CA). Each embryo was centered in the spheroid chamber in the bottom of the well and air bubbles were removed.

Two types of assays were conducted. First, an embryo mitochondrial toxicity profile was generated following 24 hours of exposure to amiodarone, BDE-47, ezetimibe, fluoranthene, naproxen sodium, TDCPP, and toxaphene. eOCR was measured under basal conditions, followed by eOCR measurements after injecting mitochondrial inhibitors to each well. To measure maximum respiratory capacity 6 μM FCCP (carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone, Sigma-Aldrich, CAS370-86-5) was used, and 65.8 μM oligomycin (Sigma-Aldrich, 75351) was used as an ATP synthase inhibitor. A given well was only injected with either FCCP or oligomycin. Subsequently 6.25 mM sodium azide (NaAz) (Sigma-Aldrich, CAS 26628-22-8) was injected into each well to completely inhibit mitochondria. These concentrations were determined based on previous studies (Souders et al., 2018; Stackley et al., 2011). The protocol consisted of basal (15 cycles), post injection FCCP or oligomycin (15 cycles), and post injection NaAz (20 cycles) with a 2:00 minute mix, 2:00 min wait, and 3:00 min measure period per cycle.

Secondly, the list of tested chemicals was expanded, and eOCR was assessed for ~510 minutes to determine the initial mitochondrial response as well as the recovery response. The time length of 510 minutes was chosen to avoid any potential hatching of embryos mid-run thus avoiding confounding results. This assay consisted of an initial 12 eOCR measurements followed by a 15-minute wait period and five eOCR measurement cycle. This cycle was repeated seven

times. Each measurement corresponded with a 2:00 minute mix, 2:00 minute wait, and 3:00 minute measure time.

Upon completion of each run, eOCR per embryo data were averaged per treatment group. Mitochondria toxicity profile data yielded seven different mitochondrial parameters; basal respiration, reserve capacity (RC = FCCP induced eOCR – basal eOCR), ATP-independent respiration (AI = basal eOCR – oligomycin induced eOCR), maximal respiration (FCCP-induced eOCR), ATP-linked respiration (AL-oligomycin induced OCR), mitochondrial respiration (basal eOCR – NaAz induced eOCR), and non-mitochondrial respiration (NaAz induced eOCR). Data are presented as a percent of control. The 510 min long basal eOCR data are visualized as mean eOCR per treatment group (i.e., chemical concentration) over time.

2.2.3 Statistical analysis

eOCR per treatment were reorganized in R (RStudio) and imported to GraphPad Prism 8.0 (GraphPad Software, San Diego California USA) for further analysis. To determine statistical significance between control and treatment groups for mean eOCR values for a given parameter (basal, reserve capacity, ATP-independent respiration, maximal respiration, ATP-linked respiration, and mitochondrial respiration) an ANOVA was conducted followed by a Tukey post-hoc test. Significance was determined by a $p\text{-}value < 0.05$. Time-series data for each chemical for a given concentration were analyzed using a non-liner regression to determine the slope (rate of change in eOCR over time) and the area under the curve (total eOCR). Statistical significance between control and treatment groups for the slope and area under the curve were determined based on two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli, with Q = 1%, without assuming a consistent standard deviation (GraphPad Prism 8.0, GraphPad Software, San Diego California USA). Significance was determined by a $p\ value < 0.001$. To

determine eOCR immediately following removal from the exposure solution, the first five data points were extracted separately.

2.3 Results

2.3.1 Chemical treatment specific mitochondrial toxicity profiles

Mitochondrial toxicity profiles for a given group of embryos were generated by injecting known mitochondrial inhibitors (NaAz and Oligomycin) and an uncoupler (FCCP) during the eOCR measurements to the wells of exposed and control embryos. Data showed that every chemical that was tested, with the exception of naproxen sodium, had at least some statistically significant effect on embryonic mitochondrial function. TDCPP (0.1 μM) was found to have the greatest effect on mitochondrial eOCR by significantly increasing basal eOCR, max respiration, and mitochondrial respiration (Figure 2.1). Toxaphene (24.28 μM) significantly decreased reserve capacity and increased basal respiration (Figure 2.1). BDE-47 (2.06 μM) significantly reduced non-mitochondrial respiration, whereas fluoranthene (0.148 μM) led to an increase in overall eOCR leading to higher ATP linked respiration (Figure 1). Amiodarone (0.1 μM) and ezetimibe (5.0 μM) treatment resulted in an increase in reserve capacity and mitochondrial respiration respectively.

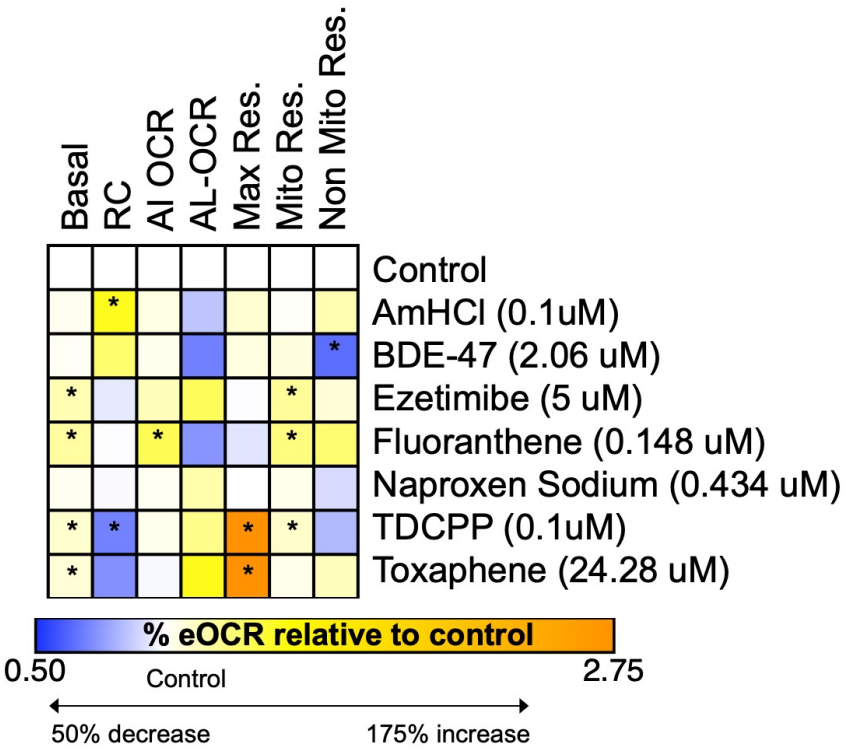


Figure 2.1: Mitochondrial toxicity profile of anthropogenic chemicals. Heat map representing embryonic oxygen consumption rates (eOCR) in zebrafish treated with AmHCl, BDE-47, ezetmibe, fluoranthene, naproxen sodium, TDCPP, and toxaphene. Embryos were in treatment solution from 1 to 25 hpf. Each square represents percent of control; blue indicates a per- cent decrease relative to control and yellow indicates a percent increase relative to control. ATP independent respiration (AI OCR) was calculated by subtracting ATP-linked respiration (AL-OCR) from basal values. Mitochondrial reserve capacity (RC) was calculated by subtracting basal value from maximal respiration (Max Res.). Mitochondrial respiration (Mito Res.) was calculated by subtracting non mitochondrial respiration (Non Mito Res.) from basal values. An independent T-Test was used to test for significance between treatment and control. Significance is represented by (*), p-value < .05, N = 9.

2.3.2 Recovery trajectory following chemical exposure

A unique mitochondrial response was detected per given chemical treatment when considering the recovery trajectory. Eleven chemicals had a significantly altered initial response (average of first 5 measurements, 24 minutes) relative to controls. Among these, treatments with sodium azide (0.01325, 0.0625, 0.625, 6.25 mM), pyridaben (0.00003, 0.0003, 0.0008, 0.004 µM) and fenpyroximate (0.05, 0.1, 0.2, 0.4 µM) were most toxic to mitochondria, with all 4

concentrations producing a significant increase or decrease in eOCR relative to controls (Figure 2.2). This was followed closely by ezetimibe (0.5, 5.0, 50 μM), in which three concentrations significantly increased OCR (Figure 2.2).

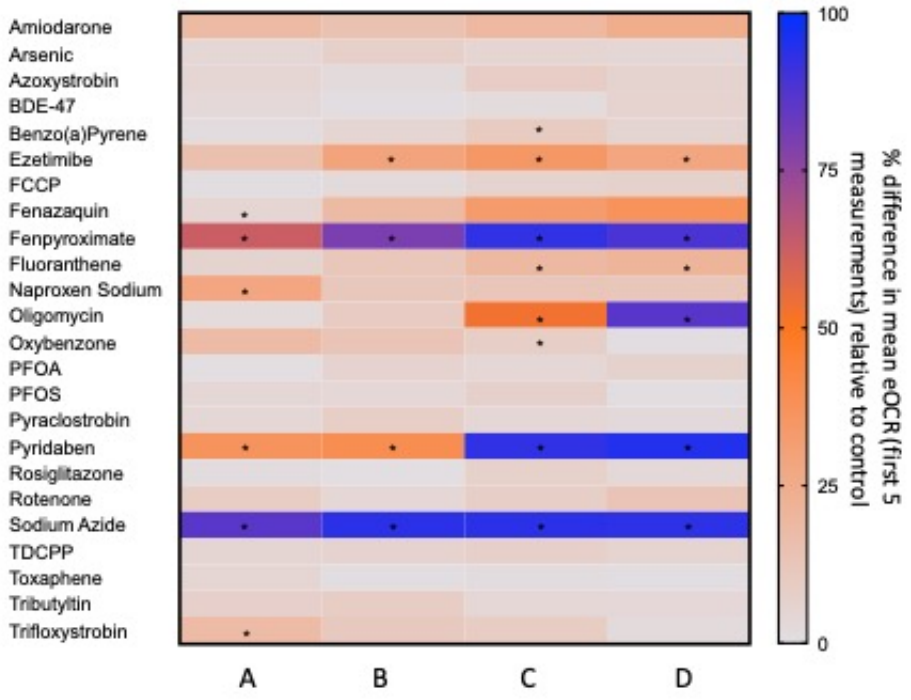


Figure 2.2: Changes in initial eOCR in zebrafish treated with a chemical for 24 hr. Heat map representing an initial response (average of first 5 measurements over 24 min) in embryonic oxygen consumption rates (eOCR) in zebrafish treated with a given chemical for 24 hr. Embryos were treated at 1 hpf. Each square re- presents percent difference from control, with white representing 0% change and blue represents maximum percent change. A, B, C, D represents the concentrations used for treatment with each chemical as listed in Table 1. An independent T-Test was used to test for significance between treatment and control. Significance is represented by (*), p- value < .05, N = 9 per treatment.

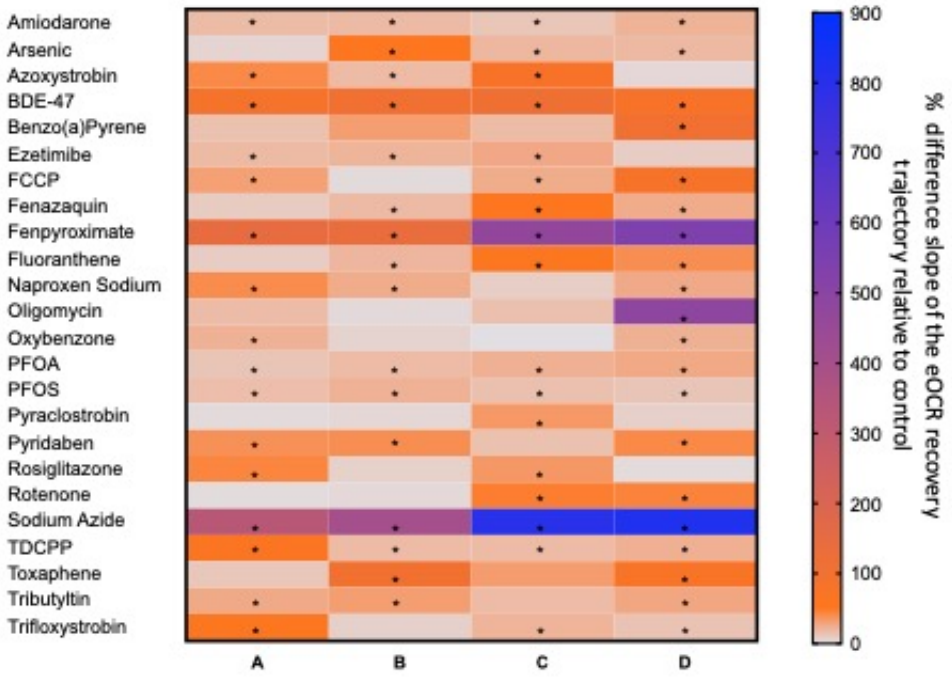


Figure 2.3: Changes in eOCR recovery trajectory in zebrafish treated with a chemical for 24 hr. Heat map representing slope of the recovery trajectory based on a non-linear regression analysis conducted on embryonic oxygen consumption rates (eOCR) over 510 min after zebrafish embryos were removed from a 24 h chemical treatment. Embryos were treated at 1 hpf. Each square represents percent difference from control, with white representing 0% change, and blue represents maximum percent change. A, B, C, D represents the concentrations used for treatment with each chemical as listed in Table 1. An independent T-Test was used to test for sig- nificance between slope of the recovery trajectory of treatment and control. Significance is represented by (*), p-value < 0.05, N = 9 per treatment. (For in- terpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Basal eOCR was significantly altered at the highest concentration of chemical treatments over 510 minutes except for azoxystrobin, ezetimibe, pyraclostrobin, and rosiglitazone (Figure 2.3). Treatments with amiodarone, BDE-47, fenpyroximate, PFOA, PFOS, sodium azide, and TDCPP significantly increased or decreased eOCR relative to control under all concentrations

(Figure 2.3). 16 of the 24 chemicals resulted in statistically significant difference in recovery trajectory after chemical treatment at the lowest concentration.

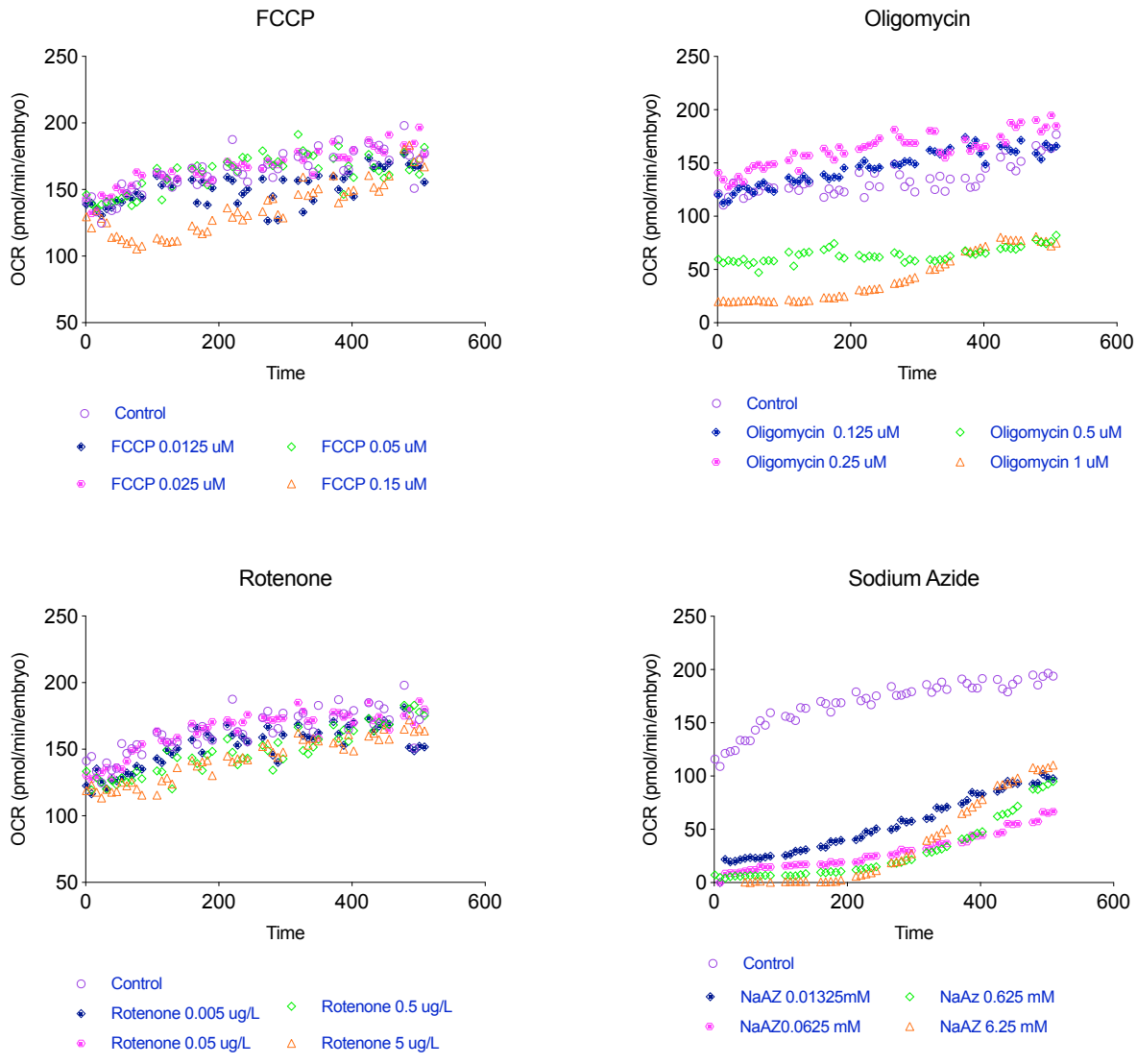


Figure 2.4: eOCR time series data. Several examples of time series data representing basal embryonic oxygen consumption rate (eOCR) over ~ 510 min of 25 hpf zebrafish after 24 h of treatment. Zebrafish were exposed to a gradient of concentrations of a given chemical for 24 h, removed from the exposure solution and eOCR rates were measured for 8 in hours to chemicals. Treatment with mitochondrial inhibition (FCCP, oligomycin, rotenone, sodium azide), chemical treatments depicting non- monotonic dose responses (fenazaquin and pyridaben), and chemicals resulting in mitochondrial hormesis (amidorone, and arsenic), N = 9 per treatment. Statistical significances of the slope and area under the curve for a given treatment is depicted in Fig. 3 and Supplemental Fig. S1

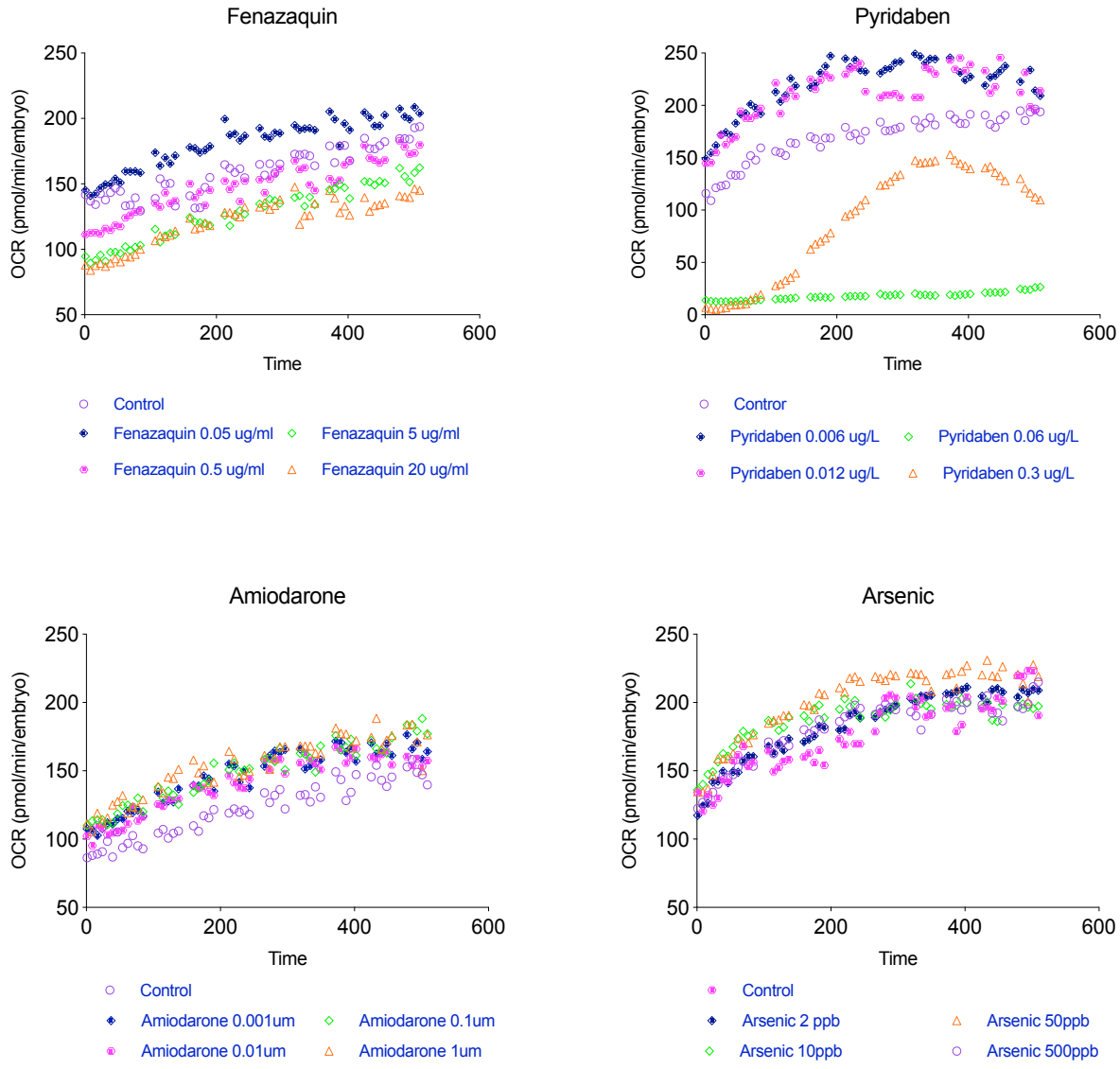


Figure 2.4 Continued: eOCR time series data. Several examples of time series data representing basal embryonic oxygen consumption rate (eOCR) over ~ 510 min of 25 hpf zebrafish after 24 h of treatment. Zebrafish were exposed to a gradient of concentrations of a given chemical for 24 h, removed from the exposure solution and eOCR rates were measured for 8 in hours to chemicals. Treatment with mitochondrial inhibition (FCCP, oligomycin, rotenone, sodium azide), chemical treatments depicting non- monotonic dose responses (fenazaquin and pyridaben), and chemicals resulting in mitochondrial hormesis (amidorone, and arsenic), N = 9 per treatment. Statistical significances of the slope and area under the curve for a given treatment is depicted in Fig. 3 and Supplemental Fig. S1

Exposures to known potent mitochondrial toxins (e.g., FCCP, oligomycin, rotenone, sodium azide) resulted in significantly decreased basal eOCR at the highest concentrations (Figure 2.4). Basal OCR was most impacted by sodium azide and oligomycin treatments with decreased levels at all concentrations for sodium azide and at 0.5 μM, and 1.0 μM oligomycin (Figure 2.4). Embryos treated with FCCP, oligomycin, rotenone, and sodium azide show that the recovery response is significantly different compared to the control. However, rotenone at $1e^{-5}$ (or 1.0×10^{-5}) μM and $1.3e^{-4}$ μM and FCCP at 0.025 μM were similar to the controls. Furthermore, oligomycin recovery trajectory (eOCR change over time) was parallel to that of controls, despite at much lower rates (i.e., the slope of the line was the same for three of the four concentrations tested) (Figure 2.4).

All other chemicals tested here showed an overall significant effect on eOCR recovery over time (Figure 2.3, Figure A1). This was independent of the effects measured immediately following removal from the 24 hour exposure (i.e., basal eOCR during the first 5 measurements as shown in Figure 1). 17 of the 24 chemical treatments altered eOCR recovery trajectory in at least 3 of the 4 concentrations tested. Notably, effects of PFOS, PFAS, amiadarone, and fenpyroximate on eOCR trajectory was statistically significant at all four concentrations tested. Benzo-a-pyrene and pyraclostrobin only affected eOCR trajectory at higher concentrations. Conversely, significant lower treatment concentration effects were not evident at high concentrations for azoxystrobin, ezetimibe, rosiglitazone, treatments. This indicates a non-monotonic dose response.

Fenazaquin and pyridaben treatments resulted in the most prominent non-monotonic dose responses. Fenazquin had significantly decreased basal eOCR at 65.27 and 16.32 μM and

increased eOCR at 0.163 μM (Figure 2.14). Pyridaben showed decreased eOCR at 0.004 μM and 0.0008 μM and an increase at 0.0002 μM and 0.00003 μM (Figure 2.4).

Amidarone (a pharmaceutical; 0.001, 0.01, 0.1, 1.0 μM) and arsenic (heavy metalloid; 0.133, 0.667, 6.67 μM) both show a potentially beneficial to mitochondria function. In this unique response, all concentrations of amidarone and the three highest of arsenic resulted in an increase in basal eOCR relative to control (Figure 2.4). Ezetimibe (a pharmaceutical; 0.5, 5.0, 50 μM) also showed a similar response at the three highest concentrations (Figure A2).

2.4 Discussion

The goal of this study was to elucidate vertebrate developmental mitochondrial effects of exposure to environmental contaminants, some of which are found in drinking water. Given that cellular oxygen is primarily utilized by the mitochondrial electron transport chain during ATP synthesis, changes in eOCR signified chemical impacts on early mitochondrial function. Further, by using Xfe96 Eflux technology embryos were subjected to specific ETC uncouplers and inhibitors, providing a more in-depth analysis on mitochondrial specific OCR chemical response. Results show a highly compound and concentration specific mitochondria toxicity profile for a given chemical.

Results also showed statistically significant long-term persistent effects of exposure to environmental contaminants, even at low-levels of exposure, but further investigations are warranted to determine biological mechanisms. Importantly, the analyses of changes in eOCR over time showed a remarkable capacity for mitochondria to recover. For example, direct exposure to potent mitochondrial toxins (e.g., oligomycin and NaAz) reduced cellular eOCR by more than 80%; however, when removed from this exposure solution, eOCR increased with time (Figure 2.4A). An additional consideration is the relationship between eOCR and oxygen utilized

by mitochondrial vs non-mitochondrial processes. As described previously, 80% of the total oxygen consumed during 24 hpf – 48 hpf is utilized by mitochondria. Potential changes in this percent utilization following chemical exposure was not captured in the recovery trajectory assay, but remains an important area of future studies. Further studies focusing on larval and tissue specific mitochondrial function are also likely to reveal persistent mitochondrial effects (Jayasundara et al., 2015; Raftery et al., 2017). Nonetheless, the significant differences detected in eOCR recovery trajectory for the majority of the treatment groups highlights an important consideration in defining mitochondrial toxicity of a given chemical.

eOCR effects following treatment with potent mitochondrial toxins suggest a concentration dependent, non-monotonic dose response effect. For example, exposure to FCCP, a mitochondrial uncoupler, at concentrations between 0.0125 μM – 0.05 μM induced a very different response compared to 0.5 μM. Similarly, eOCR profile following exposure to oligomycin, a mitochondrial ATP synthesis inhibitor, were significantly different between lower (0.125 μM and 0.25 μM) and higher concentrations (0.5 μM and 1 μM). Interestingly, despite being highly toxic to mitochondria, FCCP, oligomycin, rotenone and NaAZ, eOCR profiles indicated signs of complete or partial recovery. FCCP at low concentrations (30 – 100 nM) has been seen to increase mitochondrial function in mouse hearts, potentially through activation of ROS to initiate pathways that support mitochondrial recovery. Similarly, as to what was seen here, FCCP induced uncoupling at higher concentrations (300 nm) depleted mitochondrial recovery (Brennan et al., 2006). The increase in eOCR after treatment of oligomycin and return to control values, is likely due to the buildup of the proton gradient, created by the inhibition of ATP synthase. Eventually the proton gradient will exceed the energy created by electron transfer from NADH to O^2, and eOCR will decline. It is particularly remarkable that eOCR can increase

following complete mitochondrial inactivation by NaAz. In fact, partial or complete recovery after 8 hours following removal from the exposure solutions were detected with all the chemicals, except for pyridaben and fenpyroximate. It is likely that this recovery is temporary. For example, previous studies have shown zebrafish treated with 1 μM oligomycin starting at 5 hpf resulted in increased cardiac edema by 3 dpf (days post fertilization) and decreased survivability by 5 dpf (Byrnes et al., 2018). Nonetheless, these data indicate resilience of mitochondria to environmental perturbations during development and the potential capacity to regain homeostasis, at least to some extent. Further studies (e.g., mitochondrial DNA copy number and mitochondrial morphology analyses) are necessary to increase understanding of mitochondrial recovery following a chemical insult.

Several tested chemicals such as the pesticides fenazaquin and pyridaben, produced non-montotonic eOCR responses in a concentration dependent manner. Treatment with pyridaben at 0.0008 μM resulted in a significant decrease (~14 pmol/min) in eOCR (Figure 2.4). In contrast, pyridaben at 0.004μM leads to an initial eOCR response similar to 0.0008 μM exposure, but then increases peaking around 6.5 hours (just below control values) followed by a decrease. Pyridaben is known to be a mitochondrial complex I inhibitor and has been found to be toxic to rat N27 dopaminergic neuronal cells. At concentrations from 0.5 – 6 μM pyridaben significantly decreased OCR after 3 hours of exposure (Charli et al., 2016). Here we provide further support that pyridaben affects cellular OCR at very low concentrations in a significant concentration dependent manner.

Treatment with some chemicals led to an increase in eOCR relative to controls, which can potentially be characterized as mitochondrial hormesis. This pattern was seen with arsenic at 0.133 μM and especially at 0.667 μM. Amiodarone also showed a similar response at all the

concentrations tested. Data from the mitochondrial toxicity profile analyses (Figure 1) indicate that this increase in eOCR is coupled to an increase in mitochondrial reserve capacity. Increases in mitochondrial activity have been reported under low arsenic exposures (Schmeisser et al., 2013). It is speculated that mitohormesis may be playing a protective role potentially via signal transduction to the nucleus to ultimately regain homeostasis (Merry & Ristow, 2016; Meyer et al., 2018). When a mitohormesis response is consistently seen after exposure, it would be compelling to explore if there are any later-life fitness consequences, including those that are potentially beneficial.

Given that successful early development of healthy mitochondria is critical, a recovery in eOCR or an apparent insignificant response may still result in later life defects. Previous studies show that exposure to chemicals at the levels examined in our study resulted in long-term physiological effects that may be explained by mitochondrial changes. For example, a 96-hour embryonic exposure to low levels of BaP manifested an increase in cellular apoptosis and neurodegeneration in adult stages (Gao et al., 2017). Treatment with pyraclostrobin from 4 hpf to 5 dpf led to reduced body length, decreased OCR, and an increase in ROS related transcripts (Kumar et al., 2020). Studies also show that early chronic exposures to NaAz result in developmental delays and pyridaben perturbs larval endothelium development (Byrnes et al., 2018; McCollum et al., 2017). Therefore, it is possible that an initial change or significant difference in OCR over time is a key indicator that a chemical is impacting mitochondrial function which may lead to greater developmental stress and later life consequences.

While the eOCR recovery trajectory analyses clearly demonstrated persistent effects on cellular bioenergetics, mitochondrial profile analyses provided further insights into potential mechanisms of action for a given chemical. For example, the increase in OCR with TDCPP at

0.1 μM following 24 hours of exposure was coupled to an overall increase in maximum respiratory capacity, inferring an overall increase in total mitochondrial activity. However, given that reserve capacity was significantly reduced, it appears this increase in maximal respiration is a potential partial compensatory response to the increase in basal eOCR. Toxaphene also followed a similar mitochondrial profile following 24 hours of exposure. In contrast, compared to TDCPP, BDE-47 shows a complete opposite effect on mitochondria. Previous studies show that both these chemicals are linked to mitochondrial dysfunction (Byun et al., 2015; Chen et al., 2017, 2018).

Collectively, this study presents a tool to assess mitochondrial effects of chemical exposure during development using the zebrafish model. As shown, it can be used to determine mitochondrial toxicity of pharmaceuticals, industrial byproducts, and agrochemicals *in ovo*. It is possible that positive or negative selection of mitochondria during embryonic development may contribute to altered mitochondrial phenotype following chemical exposure (Otten et al., 2018). This is based on the hypothesis that mitochondria bottleneck during development, where the total mitochondrial copy number reduces from ~1.9 x 10^6 to ~150 (Otten et al., 2016), before rapidly increasing, is a sensitive window for selection. Additionally, our eOCR recovery analyses indicate the importance of considering long-term effects of exposure to chemical contaminants, even at low-levels. Future studies may be directed at exploring mechanisms (e.g., examining mitochondrial morphology) and later-developmental consequences of exposure to chemical contaminants toxic to mitochondria. Given that exposure to multiple chemicals at once is more likely on a day to day basis, identifying the impact of chemical mixtures on mitochondrial eOCR and how sensitive the mitochondria are to changes in mixture composition is a necessary avenue to explore, and is investigated in the following subchapter.

CHAPTER 3
MIXTURE SPECIFIC ALTERATIONS OF MITOCHONDRIAL eOCR

Chapter 3.1 Introduction

Interpreting the impacts of a chemical mixture on mitochondrial function, rather than a single agrochemical or pharmaceutical (as done in the previous subchapter), would provide further insight into how real-world chemical exposures may alter early development. Chemical and metal mixtures in drinking water supplies, and their potential impacts on human health, are beginning to be explored in literature. Previous studies, have found metal mixtures in drinking water, many of which include As and Pb, to have health implications such as altered birth outcomes (Hoover et al., 2018; Signes-Pastor et al., 2019). Model organism studies have also suggested developmental effects of chemical mixture exposure (F. Zhou et al., 2019). However, many times *in vivo* studies only assess mixtures of 2 or 3 chemicals. Here mixture toxicity on mitochondrial function is explored in depth, where a 5-part chemical mixture of As, Cd, V, Pb and Gly is heavily investigated and mitochondrial toxicity profiles for each iteration of the mixture are determined and compared.

3.2 Methods

3.2.1 Exposure protocol

Wildtype AB strain zebrafish embryos were collected and incubated at 28.5°C in egg water (1 embryo/1 mL) until 1 hpf at which time embryos were screened for viability. Embryos, remaining in their chorion, were moved to treatment solutions (10 embryos / 10 mL) for 25 h at 28.5°C. Embryos were treated with egg water supplemented with metals (2 ppb Cd, Sigma Aldrich Cat# 202908, 8 ppb As, Sigma Aldrich Cat# S7400, 8 ppb V, Sigma Aldrich Cat# 262935, and 10 ppb Pb, Sigma Aldrich Cat# 268690) and 8 ppb glyphosate (Sigma Aldrich Cat#

45521, fresh stock made bi-weekly), individually or in mixtures. These concentrations are environmentally relevant, are at levels often found in drinking water supplies and are all at levels considered safe for human consumption (Babich, Ulrich, et al., 2020; Babich et al., 2021). A total of 31 treatment solutions were synthesized and encompassed every possible mixture combination of the 5 chemicals (Table 2). Each chemical treatment was conducted in triplicate and none of the treatments showed significant effects on survival or embryonic development.

A subset of 1 hpf zebrafish embryos were exposed to the metal mixture of As, Cd, V, and Pb for 25 hours and were placed into a new solution that contained an acute high dose of either 150 ppm As or 10 ppm glyphosate. This experiment was conducted to explore if a low dose exposure to a chemical mixture impacted mitochondrial capacity to respond do an acute chemical stress.

Table 2: Chemical mixtures used for mitochondrial toxicity profiles. One through five-part chemical mixtures of As (8 ppb), Cd (2 ppb), Pb (10 ppb), V (8 ppb), and glyphosate (8 ppb) that were used to determine mitochondrial toxicity profiles after a 25 hour embryonic exposure. These mixtures include every iteration of the 5-part mixture.

Individual chemicals	2 chemical mixtures	3 chemical mixtures	4 chemical mixtures	5 chemical mixture
As	As Cd	As Cd Pb	As Cd Pb V	As Cd Pb V Gly
Cd	As Pb	As Cd V	As Cd Pb Gly	
Pb	As V	As Cd Gly	As Cd V Gly	
V	As Gly	As Pb V	As Pb V Gly	
Gly	Cd Pb	As Pb Gly	Cd Pb V Gly	
	Cd V	As V Gly		
	Cd Gly	Cd Pb V		
	Pb V	Cd Pb Gly		
	Pb Gly	Cd V Gly		
	V Gly	Pb V Gly		

3.2.2. Mitochondrial toxicity analysis

Embryonic oxygen consumption rate (eOCR) was analyzed, per embryo, using XF96^{e} Extracellular Flux Analyzer (Agilent Technologies, CA) and was optimized following previous studies (Stackley et al. 2011; Sounders et al. 2018). After a 25 hr incubation in chemical and mixture treatment solutions (Table 2), embryos were rinsed with egg water 3 times (10 mLs each). Subsequently, single embryos were transferred to an individual well, containing 150 uL of egg water, in a spheroid microplate (Agilent Technologies, CA). Each embryo was centered in the spheroid chamber in the bottom of the well and air bubbles were removed.

Embryo mitochondrial function was determined for each treatment by measuring whole embryo oxygen consumption rate. eOCR was measured under basal conditions, followed by eOCR measurements after injecting mitochondrial inhibitors to each well. To measure maximum respiratory capacity 6 μM FCCP (carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone, Sigma-Aldrich, CAS370-86-5) was used. Subsequently 6.25 mM sodium azide (NaAz) (Sigma-Aldrich, CAS 26628-22-8) was injected into each well to completely inhibit mitochondria. These concentrations were determined based on previous studies (Babich, Hamlin, et al., 2020; Souders et al., 2018; Stackley et al., 2011). The protocol consisted of basal (12 cycles), post injection FCCP (6 cycles), and post injection NaAz (20 cycles) with a 2:00 minute mix, 2:00 min wait, and 3:00 min measure period per cycle.

Upon completion of each run, eOCR per embryo data were averaged per treatment group. Mitochondria toxicity profile data yielded seven different mitochondrial parameters; basal respiration, reserve capacity (RC = FCCP induced eOCR – basal eOCR), maximal respiration (FCCP-induced eOCR), mitochondrial respiration (basal eOCR – NaAz induced eOCR), and non-mitochondrial respiration (NaAz induced eOCR). Data are presented as a percent of control.

3.3 Statistical analysis

eOCR was checked for normalization using Shapiro Wilke and boxplots were used to identify outliers. Extreme outliers, by mitochondrial eOCR parameter, were removed from the datasets (extreme outliers are identified in SPSS as those that lie outside of the [3rd quartile range + 3*interquartile range] or [1st quartile range + 3*interquartile range]. Whole embryo data for each parameter, were removed when ≥ 3 of the 5 parameters for a given embryo were identified as outliers (outliers are identified in SPSS as those that lie outside of the [3rd quartile range + 1.5*interquartile range] or [1st quartile range + 1.5*interquartile range]. To determine statistical significance between control and treatment groups for mean eOCR value for a given parameter (basal, reserve capacity, maximal respiration, mitochondrial respiration, and non-mitochondrial respiration) an ANOVA was conducted followed by a Tukey post-hoc test. Significance was determined by a *p-value* < 0.05.

3.3 Results

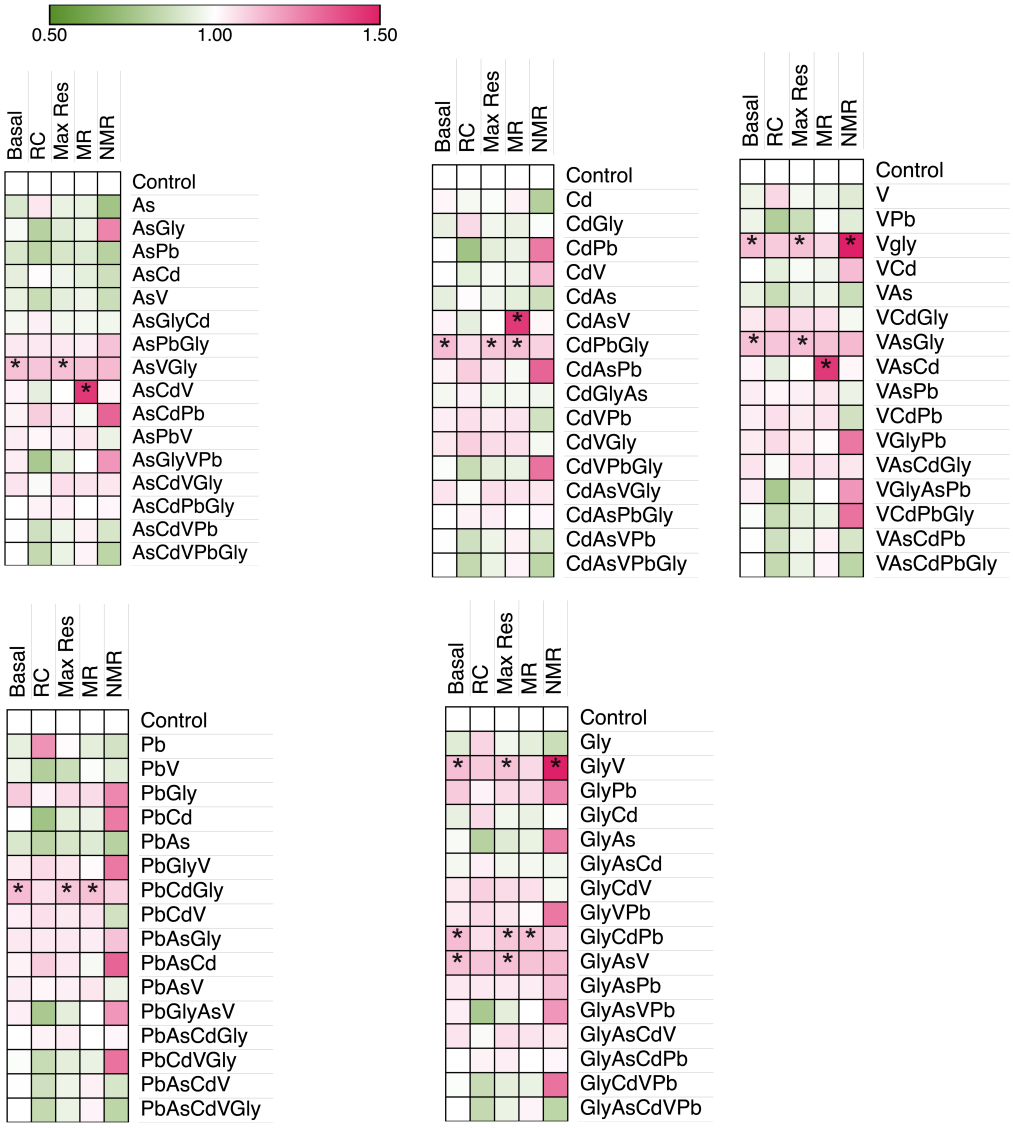


Figure 3.1: eOCR mitochondrial toxicity profiles for chemical mixtures. Heat maps representing embryonic oxygen consumption rates (eOCR) in zebrafish treated with 8 ppb As, 2 ppb Cd, 8 ppb V, 10 ppb Pb, and 8 ppb Gly both individually and in mixture. Panels are separated by chemical, in which each mixture following the individual exposure includes either As, Cd, V, Pb, or glyphosate. Embryos were in treatment solution from 1 to 25 hpf. Each square represents percent of control; green indicates a percent decrease relative to control and pink indicates a percent increase relative to control. Mitochondrial reserve capacity (RC) was calculated by subtracting basal value from maximal respiration (MR) Mitochondrial respiration (Mito Res.) was calculated by subtracting non mitochondrial respiration (NMR) from basal values. An ANOVA was used to test for significance between treatment and control. Significance is represented by (*), p-value < .05, N = 21.

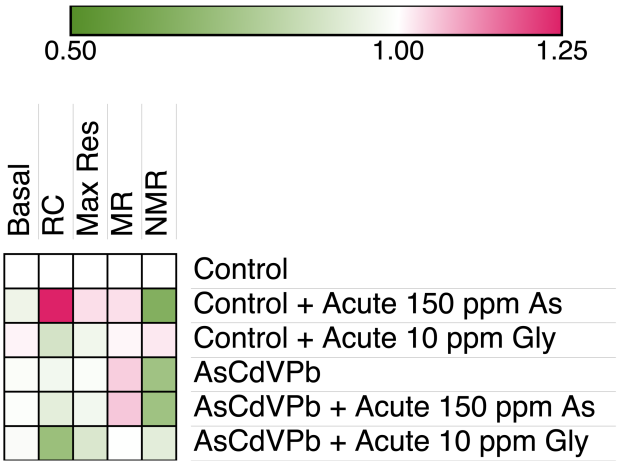


Figure 3.2: eOCR mitochondrial toxicity profile including pre-exposure and acute stressor. Heat map representing embryonic oxygen consumption rates (eOCR) in zebrafish treated pre-exposed to a mixture of 8 ppb As, 2 ppb Cd, 8 ppb V, 10 ppb Pb or egg water (control) from 1 – 25 hpf ± 150 ppm or 10 ppm As or glyphosate exposure. Each square represents percent of control; green indicates a percent decrease relative to control and pink indicates a percent increase relative to control. Mitochondrial reserve capacity (RC) was calculated by subtracting basal value from maximal respiration (Max Res.). Mitochondrial respiration (MR) was calculated by subtracting non mitochondrial respiration (NMR) from basal values. An ANOVA was used to test for significance between treatment and control. Significance is represented by (*), p-value < .05, N = 21.

3.3.1 Mixtures with As - Alteration in mitochondrial toxicity profiles

Zebrafish embryos exposed to low levels of chemicals from 1 to 26 hpf showed chemical and mixture specific alterations in eOCR. Specific trends were identified based upon mixture components. Arsenic alone and in small mixture (2 chemical mixtures) appeared to have an overall dampening effect on eOCR for all parameters relative to control (Figure 3.1). AsPb mixture exposure showed the greatest decrease in basal eOCR (0.90) relative to control. More complex mixtures (3+ chemical mixtures) with arsenic tended to increase eOCR in most parameters. Of note, AsVGly mixture resulted in an increase in all eOCR parameters, with basal (1.12) and max res (1.12) significantly increasing relative to controls. The increase in max res under AsVGly exposure was the highest eOCR for the max res parameter among all treatments. AsCdV treatment resulted in the highest eOCR for MR (1.44), and was statistically significant

relative to controls. Mixtures including Cd followed similar trends to those seen under As treatment conditions (Figure 3.1).

3.3.2 Mixtures with gly - Alteration in mitochondrial toxicity profiles

In contrast to As, most gly mixtures resulted in an increase in eOCR parameters. GlyV and GlyPb mixture resulted in an increase in all parameters, with NMR under GlyV treatment having the highest percent increase compared to all treatments (1.64). Along with NMR, GlyV treatment resulted in statistically significant increases in basal (1.13) and max res (1.12) parameters (Figure 3.1). Many triplicate mixtures containing glyphosate resulted in increases across parameters, including GlyCdPb mixture exposure, which resulted in significant increases in basal (1.14), max res (1.11) and MR (1.13) relative to controls. The increase in basal under GlyCdPb treatment was highest among all mixture exposures.

3.3.3 Mixtures with Pb and V have similar eOCR profiles

Pb and V toxicity profiles showed similar trends in their individual treatments (decrease in all parameters except for RC which was increased), as well as their mixtures with Gly, Cd, and As. PbGly and VGly treatments showed increases in all parameters, while PbAs and VAs resulted in decreases in all parameters. PbCd and VCd had minimal impacts on basal eOCR, mild decreases in RC, max res, and MR, and an increase in NMR. The mixtures PbAsCdGly and VAsCdGly showed minimal increases in all parameters relative to control (Figure 3.1).

3.3.4 Mixture impact on reserve capacity

Individual chemical treatments resulted in an increase in reserve capacity, Pb exposure was responsible for the greatest increase in reserve capacity relative to controls (1.23). This was often times reversed for mixtures of 2 metals, where reserve capacity tended to decrease. However, mixtures of Cd, V, and Pb with Gly also resulted in an increase in RC. Mixtures of 3

chemicals trended towards increases in RC, and more complex mixtures of 4+ chemicals typically resulted in a decrease in RC (Figure 3.1).

3.3.4 Pre-exposure to a mixture leaves mitochondria susceptible to an acute stress

To explore the impact of early chemical exposures on the mitochondria's ability to respond to an acute chemical stressor, an exposure to the metal mixture As, Cd, V, and Pb from one to 25 hours followed by a one-hour acute exposure to 150 ppm As or 10 ppm glyphosate was conducted. Results show that, compared to controls, a preexposure to a low dose metal mixture resulted in a diminished RC following 150 ppm As and 10 ppm Gly exposures (Figure 3.2). This was most apparent in those exposed to 150 ppm As, as the response to As in control embryos resulted in a 1.25 increase in RC compared to controls that were not subjected to the 1 hour acute As exposure. The most dramatic decline in RC (0.72) was seen in embryos pre-exposed to the metal mixture and then subjected to the one hour acute exposure to 10 ppm glyphosate. Both preexposure to the metal mixture as well as acute As exposure resulted in an increase in MR.

3.4 Discussion

This is the first time a mitochondrial toxicity profile has been established for every iteration of a five-part chemical mixture. Here, the data shows that responses in mitochondrial respiration are highly mixture specific, and trends in mitochondrial parameter alterations exist depending upon mixture components.

Arsenic is a widely discussed chemical as it is often present in drinking water supplies, especially those with wells where As can leach from the bedrock into the groundwater. Arsenic has also been thoroughly explored in scientific literature in the context of mitochondrial function. Developing rats exposed to As via gestation and lactation (maternal exposure 2 or 4 mg/kg daily), showed an increase in ROS and decrease in ETC complex I, II, and IV activity in brain tissue (Chandravanshi et al., 2018). Mammalian cells treated with 1 ug/mL As for 60 days

resulted in decreased OCR, decreased cytochrome c oxidase activity, and elongated mitochondria morphology relative to controls (Partridge et al., 2007). Additionally, As treatment of 2.5 mg/kg daily for 4 weeks in adult male rats has been shown to decrease activity of ETC complexes I, II, and IV in brain cells (Dwivedi et al., 2011). Here, As alone and in small mixture has a dampening effect on mitochondrial function via a decrease in eOCR, which may be occurring through alterations in ETC complex activity and generation of ROS (Garza-Lombó et al., 2019). Other individual metal exposures of Cd, Pb, and V, also resulted in a dampening effect of most mitochondrial parameters and may have similarly been interfering with ETC complex function and ROS generation.

Interestingly, Pb and V showed very similar mitochondrial toxicity profiles in small mixture (Figure 3.1). Similar to excess calcium, Pb has been shown to decrease mitochondrial membrane potential and activate apoptosis via caspace-c release (L. He et al., 2000). Pb exposure in adult *Clarius batrachus* at 37.8 and 75.6 mg/L for 40 and 60 days showed decreased ETC complex IV activity, as well as a decrease in ATP production and increase in lipid peroxidation (Maiti et al., 2010). In the rat brain, Pb was found to cause lipid peroxidation, and decrease activities of ETC complexes II, III, and IV after 15 days of exposure at 100 and 400 ppm through drinking water. This function was rescued upon co-exposure with MitoQ, a known peroxynitrite scavenger, suggesting that Pb-induced mitochondrial toxicity may stem from generation of reactive nitrogen species (Maiti et al., 2017). Little is known about vanadium regarding potential mitochondrial toxicity, however exposure in isolated mitochondria from rat liver also resulted in opening the mitochondrial transition pore and an increase in ROS production (Zhao et al., 2010). In an additional study exposing rat liver mitochondria to vanadate, it was determined that ROS generation may be a result of interaction with ETC complex III (Hosseini et al., 2013). Here,

given that Pb and V produced similar alterations in eOCR both individually and when in mixtures, at low levels they may be acting similarly on mitochondria possibly through ROS or RNS generation or interference with complex III. The finding that the toxicity profiles are similar across mixtures is especially enlightening and suggests that they may contribute comparably to mixture specific toxicity and may be drivers of mechanistic changes that resulted in altered eOCR.

Beyond metals, the herbicide glyphosate was also explored alone and in mixture. Previous studies have found environmentally relevant exposures to herbicides containing glyphosate to have an impact on mitochondrial function. For example, TouchDown, which contains the primary ingredient glyphosate, resulted in decreased OCR in C.elegans, along with depleted ATP production (D. C. Bailey et al., 2018). Adult zebrafish exposed to a glyphosate-based herbicide for 7 days at 0.065 and 1.0 ppm showed decreased complex I and IV activity in brain cells and increased ROS production (Pereira et al., 2018). Here, glyphosate alone acts similarly on mitochondrial function to metals, as well as the studies aforementioned, and resulted in a dampening effect among most mitochondrial parameters. Interestingly, mixtures that contain glyphosate tended to have a positive impact on mitochondrial function, increasing many of the eOCR parameters (a mitohormetic effect). This suggests that the mitochondria respond differently to low level mixtures of metals and organics than metal mixtures alone, and are sensitive targets to mixture effects. Although the impacts of complex glyphosate mixtures on mitochondrial function remain elusive, one study suggested potential synergistic effects of a glyphosate-based mixture exposure. In this study, a 12-week exposure in adult male rats to a glyphosate and hard water mixture resulted in increased mitochondrial fission in kidney tissue as well as increased renal injury score compared to controls. These results were more pronounced

than those seen in the counter treatments of glyphosate and hardwater individually (L. Zhang et al., 2021).

Upon exploration of a post exposure chemical stressor, it was found that a 24 hour early exposure to a low level metal mixture alters the response to an acute chemical stress. This was most apparent in the difference between RC in control and pre-treated embryos that were exposed to 150 ppm As for 1 hour. Here, the control embryos show an increase in RC after exposure, indicating a potential mitochondrial driven response to the additional arsenic treatment. The pre-treated embryos did not show an increase in RC, suggesting that the early exposure could be delaying or dampening the mitochondrial response. It has been suggested that early exposure to chemicals may leave an organism more susceptible to environmental stressors and alter development. For example, zebrafish embryonic exposure to the insecticide diazinon showed altered behavior throughout later life stages as well as altered mitochondrial function in brain and testes. Adult fish acutely exposed did not show changes in neurobehavioral and mitochondrial outcomes (Boyda et al., 2021). Further, compromised mitochondria, as investigated by using the Sod2 knockdown mouse model, showed that as mice aged there was increased ROS and ETC dysfunction in Sod2 KDs compared to controls (Kokoszka et al., 2001). There is potential, as shown here, that an embryonic exposure to chemicals is subtly altering mitochondrial function so that the response to additional stressors is compromised. This has long term implications for individuals, in that early exposure to mixtures may increase the likelihood of disease or alter reproductive capabilities.

This study has limitations, for example, the concentrations explored here, although environmentally relevant, remained the same. Additional insight on drivers of changes in mitochondrial function may be elucidated by increasing or decreasing chemical concentrations

within the mixture. Additionally, this data was analyzed by an ANOVA, a more robust clustering analysis to determine if specific chemicals and chemical combinations had clear impacts on specific mitochondrial parameters is necessary. This work would also benefit from a later stage assay, such as a larval behavioral study, to explore what mixtures may drive changes in phenotype and how these compare to early alterations in mitochondrial function. Nonetheless, this study was a crucial first step in exploring early low level exposure to mixtures on mitochondrial respiration and provided a large and complete dataset for future investigation.

The next chapter explores how metal and glyphosate mixture-induced mitochondrial toxicity, and other endpoints such as gene expression and histopathology, may be connected to the endemic of chronic kidney disease of unknown etiology (CKDu).

CHAPTER 4

CONNECTING WATER QUALITY TO DISEASE PREVALENCE: A CASE STUDY IN CHRONIC KIDNEY DISEASE OF UNKNOWN ETIOLOGY

4.1 Introduction

Chronic kidney disease (CKD) affects ~15% of the global population, with the highest percent prevalence found in Europe, Australia, USA, and Canada and is typically associated with systemic disorders (e.g., diabetes, age, and hypertension) (Hill et al., 2016; James et al., 2010). CKD is defined and staged by a decreased glomerular filtration rate for at least 3 months (G2 – G5 ranging from 60 mL/min – kidney failure at < 15 mL/min) or presence of albuminuria, and can be debilitating (Webster et al., 2017). If not treated, CKD will eventually lead to kidney failure and increased likeliness of developing cardiovascular disease or acute kidney injury (Levey & Coresh, 2012).

Most of the scientific literature to date focuses on identifying biomarkers for CKD, many of which are becoming more specific and allow for longitudinal studies to more appropriately stage CKD. CKD273, a protein biomarker identified in end stage CKD patients, is being used to detect stage III CKD, as it is more highly associated with eGFR than urinary albumin excretion rate (UAE) when treatment options are still applicable (Pontillo et al., 2017). Kidney transcriptomics were used to identify both transcript and protein expression of epidermal growth factor (involved in cellular regeneration and differentiation) as being correlated with estimated glomuler filtration rate (eGFR) (Ju et al., 2015). Renal fibrosis, which may be the most important metric in early identification of CKD as well as long-term morphological damage, which is not always reflected in typical GFR and proteinuria assays, has become measurable in real time by utilizing ESMA (an elastin-specific magnetic resonance imaging agent). It was

found that cortical/medulla elastin presence is significantly increased in CKD associated models when compared to healthy individuals, and could be repetitively used in order to monitor fibrosis in response to treatments (Sun et al., 2019). Other biomarkers include soluble tumor necrosis factors 1 & 2 (sTNFs), fibroblast growth factor (FGF) and neutrophil gelatinase-associated lipocalin (NGAL) (Siwy et al., 2019). Although the advancements in CKD biomarkers is extremely valuable to the disease community, those suffering in developing countries or without sufficient income may not be able to utilize these advancements, leaving them with eGFR as an identifier until end stage is reached. Therefore, it is becoming critical to not only expand and identify biomarkers but, of equal or greater importance, to remediate underlying causes of kidney disease and dysfunction. This is particularly applicable when considering chronic kidney disease of unknown etiology (CKDu), in which patients display none of the systematic disorders mentioned above.

In recent years, this un-traceable kidney disease (Gifford et al., 2017; Obrador & Levin, 2019) has emerged across farming communities in Central America and South Asia, with some prevalence data emerging in the United States (Aguilar & Madero, 2019; Kulathunga et al., 2019; Ramirez-Rubio et al., 2013). Genetic predisposition, dehydration, nutrition, and exposure to anthropogenic chemicals are thought to be players in CKDu onset (Friedman & Luyckx, 2019; Kulathunga et al., 2019; Levine et al., 2016). Sri Lanka is one of the most affected countries by CKDu, where disease prevalence has exceeded 20% in some communities (Rajapakse et al., 2016). Notably, 8.7% of children (ages 5 – 11) tested positive for kidney injury markers in CKDu endemic regions in Sri Lanka, suggesting a potential early life onset of this disease (Agampodi et al., 2018; Friedman & Luyckx, 2019). Next generation sequencing also confirms that ~ 20% of early-onset kidney disease in children and adolescents is due to genetics (de Haan

et al., 2019). Thus, the development of CKD may be hereditary further necessitating the need to understand risk factors associated with CKDu.

CKDu research in Sri Lanka highlights drinking water contaminated from agricultural practices (e.g., heavy metals, herbicides, etc.) as a main source of chemical exposure (Ananda Jayalal et al., 2019; Cooray et al., 2019; Gunatilake et al., 2019; Jayasumana et al., 2014; Kulathunga et al., 2019; Wimalawansa, 2016). Water and soils in CKDu endemic regions in Sri Lanka are contaminated with heavy metals and glyphosate, which are likely introduced via fertilizer and herbicide use, respectively (Atafar et al., 2010; Jayasumana et al., 2014). Importantly, anthropogenic contaminants (e.g., Cd, As, Pb, glyphosate) in the environment in endemic regions are found at concentrations below current maximum allowable limits in the drinking water (Gunarathna et al., 2018; Kulathunga et al., 2019; Levine et al., 2016). However, recent research using rodents has shown that even at levels considered "safe", the synergistic effect of chemical mixtures plays a role in biological outcomes (Wasana et al., 2017). Given that early onset of kidney disease in children may contribute to the overall prevalence of CKDu, effects of contaminants and their mixtures on kidney development may play a critical role in initiation and progression of CKDu (Agampodi et al., 2018; Friedman & Luyckx, 2019; Jayasekara et al., 2013).

Assessing mixture effects on kidney development has been difficult due to the lack of a higher throughput animal model. The zebrafish can be used to evaluate effects of individual compounds and their mixtures on kidney development. At 24 hpf, the zebrafish embryonic kidney, or pronephros, is formed, and are both structurally and functionally similar to the adult mammalian kidney (Elmonem et al., 2018). The pronephros retain their structure until 10 days post fertilization (dpf) until developing into the mesonephros and fully maturing (Drummond et al., 1998). Given

these attributes, the embryonic and larval zebrafish were used to assess effects of low level chemical contaminants and their mixtures on kidney development.

First, metal concentrations in environmental samples collected from reservoirs, wells, and rice-fields from CKDu endemic and non-endemic regions of Sri Lanka were determined. Based on the metal composition data, laboratory-derived mixtures (LMs) were designed. LMs contained arsenic (As), cadmium (Cd), lead (Pb), and vanadium (V) at levels below maximum allowable limits in the drinking water. To better understand interactive effects of metals and herbicides, metal mixtures that contain glyphosate at concentrations considered safe in drinking water, were also synthesized. Glyphosate is a ubiquitously used herbicide that is prevalent in the endemic region and has been postulated to play a role in CKDu (Gunarathna et al., 2018; Gunatilake et al., 2019). The overall objective of testing multiple lab mixtures was to uncover the potential role of synergistic effects and emphasize the need to consider mixtures when assessing risk of CKDu.

To examine the exposure effects of LMs, as well as environmentally derived mixtures on kidney development, gene expression analysis, histopathology, and mitochondrial function were explored. For the gene expression analysis, preliminary studies were conducted with gentamicin, a known nephrotoxin, to examine changes in expression of several genes involved in kidney development and injury. Based on results, transcript levels of *pax2a* and *kim1* were further investigated. *Pax2* is critical for precise kidney organ development and *kim1* is a key marker of kidney injury and is associated with kidney disease progression (Humphreys et al., 2013; Orisio et al., 1993; Patel & Dressler, 2013). Kidney histopathological changes following developmental exposure of zebrafish embryos to metal mixtures with and without the addition of glyphosate (LMs), as well as glyphosate alone, was assessed.

To uncover potential contribution of mitochondrial dysfunction on early development and the progression of CKDu, mixture induced mitochondrial toxicity profiles were generated. Renal damage as well as reduced kidney function has been associated with mitochondrial dysfunction and increased reactive oxygen species (ROS) (Dounousi et al., 2006; Gamboa et al., 2016; Granata et al., 2009). Notably, mitochondrial function was highlighted as one of the significantly altered biochemical processes in CKDu patients in Sri Lanka (Sayanthooran et al., 2018).

4.2 Methods

4.2.1 Environmental sample characterization

Environmental samples were collected from CKDu endemic and non-endemic regions in Sri Lanka (Figure 1A). Sediment samples were collected for metal analysis and toxicity studies. Endemic samples were collected from Medirigiriya, Pollonnaruwa District, and Padaviya, Anuradhapura District in Sri Lanka. Rice field samples were also collected from Monaragala District – an emerging CKDu area. Information was gathered from local health officials and patient and non-patient wells were determined – none of the researchers herein had access to patient data. Non-endemic (control-region) drinking well and rice field water samples were collected from Matara and Galle districts. A total of 9 reservoirs, 35 endemic region drinking water samples (18 patient affiliated and 17 non-patient affiliated), 18 endemic region rice field samples, and 8 (5 drinking water, 3 rice field) non-endemic region samples were used in this study.

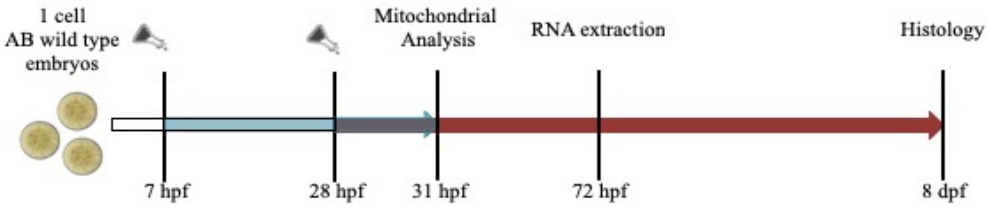
For the metal analysis and toxicity studies, sediment from reservoirs, drinking wells, and rice fields were collected by hand directly using an acid washed glass jar. Three random locations of a given reservoir were sampled and a composite sediment sample was created. Composite sediment extracts for each site were created as previously described (Clark et al.,

2013). Sediments were processed into extracts within one week of collection and stored at 4˚C during transportation. After being thoroughly mixed, 25 mL of composite sediment sample and 25 mL of deionized water were added to a 50 mL centrifuge tube. All tubes were placed (in darkness) horizontally on a rotary shaker for 24 hrs at 20°C. Tubes were then centrifuged at 1000 relative centrifugal force (RCF) for 25 min. The supernatant was decanted from each tube to a single vial. Samples were transported to University of Maine packed with ice and stored at -80˚C until further analyses.

4.2.2 Metal Analysis

The sediment extracts were acidified (1% v/v) with optima nitric acid prior to analysis. Samples were analyzed by inductively coupled plasma mass spectrometry (ICP-MS) using a triple quadrupole Agilent 8900 (Santa Clara, CA) in helium and oxygen modes. The ICP-MS was calibrated using NIST-traceable standards and calibration was verified using second source standards after the calibration standard and every ten samples. The laboratory control solutions used were NIST 1640a and a USGS proficiency test reference sample. Analytical duplicate and spikes were analyzed at a frequency of one each per 20 samples.

Table 3: Dosing regimen for CKDu case study. Schematic for dosing regimen and lab mixture (1-7) components, including glyphosate, cadmium chloride, sodium arsenite, vanadium, and lead (II) chloride used for treatments. 1-cell zebrafish embryos were dosed with a chemical, lab mixture, or environmental sample from 8 to 32 hpf for mitochondrial analysis (blue arrow). Zebrafish were maintained in dosing solution from 28 hpf to 72 hpf for RNA extractions and 28 hpf to 8 dpf (glyphosate, LM5, LM6 only) for histology (red arrow).



Lab mixtures in egg water (LM)					
Lab mix 1	10 ppb glyphosate	2 ppb cadmium chloride	-	-	-
Lab mix 2	10 ppb glyphosate	4 ppb sodium arsenite	-	-	-
Lab mix 3	10 ppb glyphosate	2 ppb cadmium chloride	4 ppb sodium arsenite	-	-
Lab mix 4	10 ppb glyphosate	2 ppb cadmium chloride	4 ppb sodium arsenite	15 ppb vanadium	-
Lab mix 5	10 ppb glyphosate	2 ppb cadmium chloride	4 ppb sodium arsenite	15 ppb vanadium	5 ppb lead (II) chloride
Lab Mix 6	2 ppb cadmium chloride	4 ppb sodium arsenite	15 ppb vanadium	5 ppb lead (II) chloride	-
Lab mix 7	2 ppb cadmium chloride	15 ppb vanadium	5 ppb lead	-	-

Environmental samples: Mixture of 20% sample + 80% egg water

RSV# = Reservoir (1-9)
#P = Patient associated wells from endemic regions (1-18)
#N = Non-patient wells from endemic regions (1-17)
Gal# = Non endemic region wells from Galle (1-5)
RFE# = Endemic region rice fields (1-18)
RFNE# = Non-endemic region rice fields (1-3)

4.2.3 Fish care and exposure studies

One cell AB strain zebrafish were incubated at 28.5°C in egg water (1 embryo/1 mL) until they were transferred to treatment solutions (at 7 hpf or 28 hpf depending upon the assay). Zebrafish embryos were treated with agrochemical and metal mixtures, as well as reservoir, well, and rice field samples from endemic and non-endemic regions as summarized in Table 3. Metals (Cd, Sigma Aldrich Cat# 202908, As, Sigma Aldrich Cat# S7400, V, Sigma Aldrich Cat# 262935, and Pb, Sigma Aldrich Cat# 268690) and glyphosate (Sigma Aldrich Cat# 45521, fresh stock made bi-weekly) were added to egg water to create a treatment solution. Complex mixtures were designed to understand how metal mixtures alone (LM6, LM7), and in the presence of glyphosate (LM3, LM4, LM5) may be perturbing kidney development. Metal concentrations in LMs were chosen based on concentrations found in CKDu endemic reservoirs and wells in the current study as described earlier.

Glyphosate was chosen since its ubiquitous use and the known chelation properties of this compound with metals. The environmental levels of glyphosate in the endemic regions ranges between 1ppb-1000 ppb (Gunarathna et al., 2018). Mixtures of glyphosate and cadmium (LM1), and glyphosate and arsenic (LM2) were used to specifically explore the interactive effects of a chelating compound and a known nephrotoxic metal (Gao et al., 2013; Jayasumana et al., 2014) (Table 3). Complementing LM exposure studies, we also exposed zebrafish to mixtures derived directly from reservoirs (RSVs), wells, and rice fields. Well samples are derived from a non-endemic region as well as from patient and non-patient wells in CKDu endemic regions. These samples were collected as described earlier. Each embryo treatment contained 20% of sample in 80% egg water. None of the treatments had significant effects on

mortality or embryonic development. This protocol was approved by the University of Maine IACUC, protocol number A2017-05-04.

4.2.4 RT-qPCR

Dechorinated embryos were incubated in 750 μL of treatment solution (LMs or environmental samples) from 28 to 72 hpf for RNA extraction. Treatments for RNA extraction occurred from 28 – 72 hpf because the kidney is beginning to form and *pax2a* expression levels are tightly regulated in these early time points (Drummond et al., 1998). RNA was extracted from whole larvae (10 larvae per extraction) at 72 hpf using Qiashredder and RNAeasy minikit (Qiagen, Valencia, CA) following manufacturers protocol. A total of 5-8 RNA extractions were completed per treatment (50-80 individuals). 500 ng RNA was then synthesized into cDNA using BioRad iScript cDNA synthesis kit (BioRad, Hercules, CA) for RT-qPCR. Target genes, *kim1* and *pax2a*, were amplified using iTaq SYBR green reagents (BioRad, Hercules, CA) with efficiencies optimized at 102%. Amplification procedures were completed using a CFX96 real time thermocycler (Biorad, Hercules, CA). See appendix for thermocycler parameters and primer sequences (Table A2, A3). Primers were synthesized by Integrated DNA Technologies (Skokie, IL). Given the difficulties in maintaining stable house-keeping genes during embryonic development in zebrafish under chemical treatment conditions, an exact amount of RNA (500 ng) was used along with the delta CT method to analyze differences in fold change relative to the control.

4.2.5 Histopathology

28 hpf embryos were dechorinated and incubated in control, 10 ppb glyphosate, LM5, and LM6 treatments until 8 dpf. These treatment groups and longer exposure durations were chosen to obtain a comprehensive analysis of the mixture effects of heavy metals on the

pronephros, and determine the potential synergistic effects of glyphosate and metal mixtures. Fish were euthanized with 0.025% MS-222, fixed in 4% PFA in 1X PBS overnight and subsequently dehydrated to 100% acetone. Four larvae from each treatment and control groups were infiltrated with Embed 812 (Epon 812) – Araldite resin mixture, embedded, and cured. Blocks were trimmed and sectioned at ~ 0.5 μm with microtome using glass knives. Sections were stained with toludine blue and imaged under a Nikon eclipse E200 using a Nikon DS-Fi2 digital camera. Sections were analyzed for kidney tubule malformations (such as tubule dilation and the presence of vacuoles).

4.2.6 Mitochondrial toxicity analysis

One cell AB strain zebrafish were incubated at 28.5°C in egg water (1 embryo/mL) until 7 hpf at which time they were transferred, within their chorions, to 25 mL glass plates containing treatment regimen and were plated at 10 embryos / plate with 10 mL of treatment solution. Treatment start time and duration for this assay differed from the gene expression and histology studies. The 7 – 31 hpf window of exposure was used, because this is a crucial interval in early embryogenesis in which mtDNA copy number decreases offering a potential to accumulate mtDNA mutations and was demonstrated to be a sensitive time period for mitochondrial measurements using the flux analyzer assay described below (Otten et al., 2016; Stackley et al., 2011).

Embryos were treated with LMs (Table 3) as well as samples derived from reservoirs and wells. Embryos were incubated in treatment solutions for 24 h, rinsed with egg water, and then introduced into spheroid microplates (Agilent Technologies, CA). Embryonic oxygen consumption rate (eOCR) was analyzed using XF96e Extracellular Flux Analyzer (Agilent Technologies, CA). We assessed basal mitochondrial rate per embryo followed by a 6 μM FCCP

(carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone, Sigma-Aldrich, CAS370-86-5) injection and 6.25 mM sodium azide (NaAz) injection (Sigma-Aldrich, CAS 26628-22-8). FCCP was used as an uncoupler (maximizing mitochondrial eOCR) and NaAz as a complete mitochondrial inhibitor (decreasing mitochondrial eOCR). The protocol consisted of 3 measurement types, basal (15 cycles), post injection FCCP (6 cycles), and post injection NaAz (20 cycles) with a 2:00 minute mix, 2:00 min wait, and 3:00 min measure period per cycle. Data were downloaded and eOCR per embryo was averaged per treatment group. These data yielded five different mitochondrial parameters; basal respiration, reserve capacity (FCCP induced OCR – basal OCR), maximal respiration (FCCP-induced OCR), mitochondrial respiration (basal OCR – NaAz induced OCR), and non-mitochondrial respiration (NaAz induced OCR). Data are presented as a percent of control.

4.2.7 Statistical Analysis

Shapiro and Wilke test was used to confirm normality and Levene's test was used to confirm homogeneity among variances for gene expression and mitochondrial function data. ANOVA was run for all gene expression data with a Tukey post-hoc. For mitochondrial studies, an ANOVA with Tukey post-hoc was used to analyze significance between treatment and control groups per given mitochondrial parameter. Significance was determined by a $p\text{-}value < 0.05$ for both gene expression and mitochondrial data. For metal analysis data, principal component analysis was used to generate loading plots to visualize relationships between environmental samples based upon chemical composition. Statistical tests were conducted using SPSS software (IBM, Armonk, NY).

4.3 Results

4.3.1 Metal levels in environmental mixtures

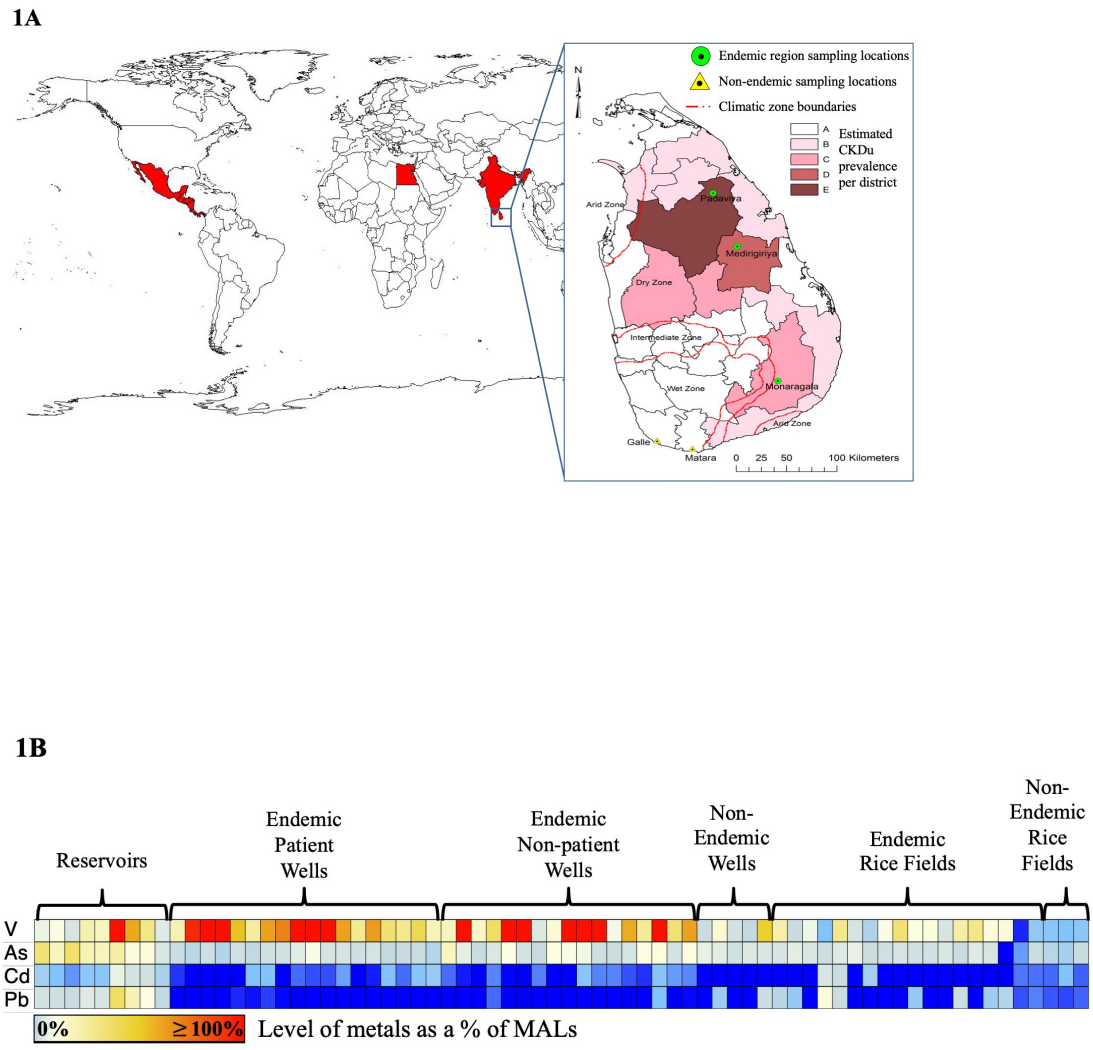


Figure 4.1: Exploration of CKDu region water samples. Sampling locations and results from heavy metal analysis (measured in ppb) of environmental samples from reservoirs (RSV), CKDu patient associated wells (#P), non-patient wells (#N), non-endemic wells (Gal#), endemic rice fields (RFE#), and non-endemic rice fields (RFNE#) in Sri Lanka. (A) World map, highlighting countries affected by CKDu including Sri Lanka where green circles depict endemic region sampling locations and yellow triangles depict non-endemic sampling locations. (B) Heat map, representing concentration of a given metal as a percent of its maximum allowable limit (MAL) in the drinking water; MALs for vanadium (V) - ~15 ppb, arsenic (As) -10 ppb, cadmium (Cd) -5 ppb, and lead (Pb)15 ppb. Blue colors indicate a lower percentage and reds indicate a higher percentage.

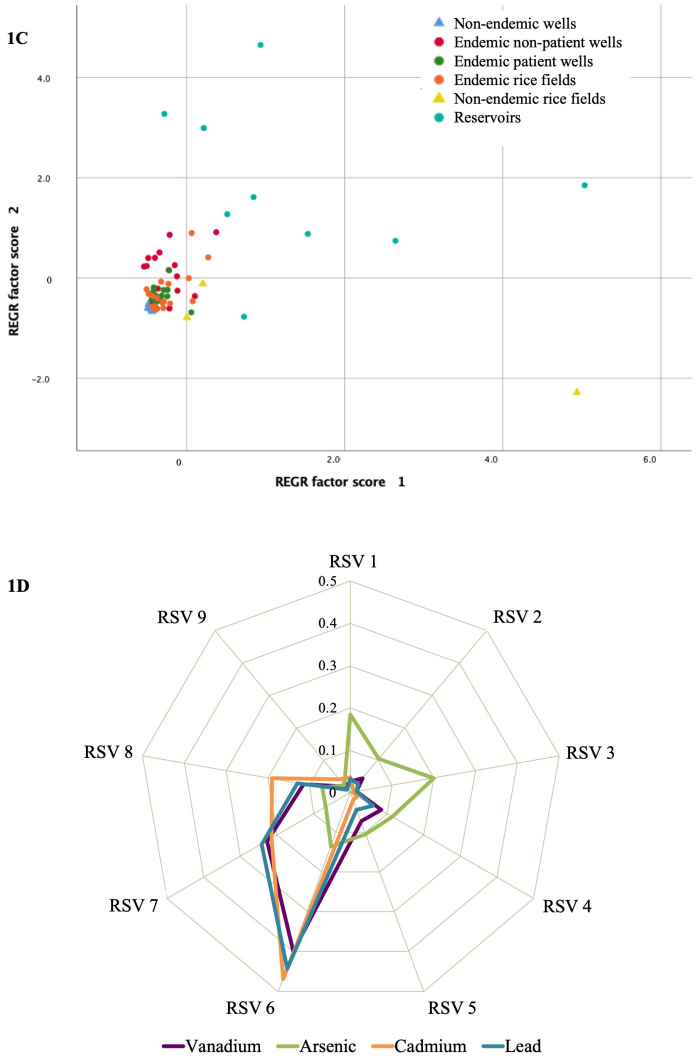


Figure 4.1 Continued: (C) PCA plot derived from linear regression factor score 1 and 2 (REGR) representing relationships among all environmental samples based upon heavy metal composition, designed by SPSS software. (D) Sediment chemistry profile plot for Cd, As, V, and Pb concentrations in reservoir samples (RSV) 1 –9 (RSV 1-5 collected from Medirigiriya, and 6-9 from Padaviya). Values 0-0.5 in the spider plot depict percent concentrations calculated by dividing the metal concentration of a given reservoir (e.g., Cd levels in RSV 1) by the sum of that metal from all nine reservoir samples. This was repeated for each of the four metals.

Irrespective of the region of origin or the source, all metals in the environmental samples, with the exception of vanadium, were below current regulatory thresholds (Figure 1B, Table S3). Notably, each environmental sample had a unique metal profile (Figure 4.1B, Table A8). When considering the four metals we focused on for deriving lab mixtures, V was found in the greatest

abundance, especially in well samples in the endemic region, followed by As, with trace amounts of Cd and Pb (Figure 4.1B). Principal component analysis illustrated broad relationships between metal profiles from different sources. Specifically, endemic well and rice field samples cluster together without any specific patterning (Figure 4.1C). In contrast, the non-endemic region wells had lower metal levels compared to the endemic regions, and also clustered together relative to wells from endemic regions (Figure 4.1C, Figure A3). Also, non-endemic samples showed less heterogeneity in overall metal levels among the wells analyzed compared to endemic region wells. Rice field samples did not show any differential clustering between endemic and non-endemic regions (Figure A4).

Reservoir-derived samples did not cluster with well and rice field samples, indicating a different metal profile (Figure 4.1C). Reservoir sample 6 (RSV6) had the highest concentration of metals, including V (15.6 ppb), Pb (5.5 ppb), and Cd (0.35 ppb), followed closely by RSV7 and RSV8 (Figure 4.1D). RSV3 had the highest concentration of As (3.55 ppb). Notably, RSV9 revealed low concentrations for all 4 metals (V – 0.73 ppb; Pb – 0.14 ppb; Cd – 0.03 ppb; As – 0.4 ppb) (Figure 4.1D).

4.3.2 Gene expression changes following laboratory-derived mixture treatments

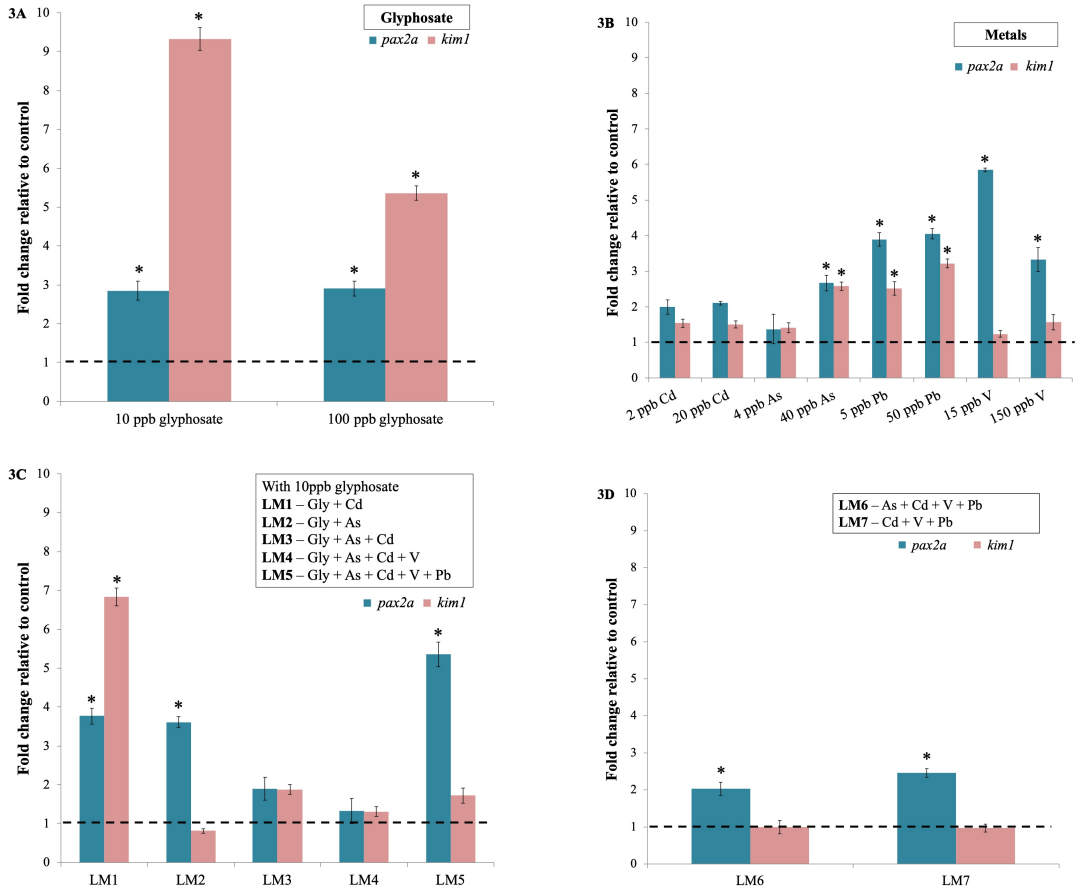


Figure 4.2: *Pax2a* and *Kim1* gene expression after exposure to lab-derived mixtures. Gene expression changes associated with kidney development (*pax2a*) and injury (*kim1*), following (A) herbicide glyphosate treatment (B) individual metal treatments (C) lab mixtures (LM) containing 10 ppb glyphosate, and (D) LM with no glyphosate. Embryos were treated from 28 hpf until the time of RNA extraction at 72 hpf. Bars represent fold change relative to control (dotted line, egg-water) +/- SEM. ANOVA was used to test for significance between treatment and control. Significance is represented by (*), *p-value* < *0.05* (See Supplemental Material Table S5 for exact p-values), N = 8.

Figure 4.2 indicates RT-qPCR analysis data for genes associated with kidney development (*pax2a*) and kidney injury (*kim1*) at 72 hpf. Data showed treatment specific changes in expression patterns following exposure to laboratory-derived mixtures (Table 3) and individual compounds. There was an increase in *pax2a* (>2-fold) and *kim1* (>5-fold) relative to control at both 10 and 100 ppb concentrations of glyphosate, with a more prominent increase

seen in *kim1* (Figure 4.2A). The effect of glyphosate in *kim1* increase was statistically significant when compared to all other individual metal treatments (Table A4). With metals, Pb and V treatments had a significant effect on *pax2a* levels (>2-fold increase) at both concentrations tested compared to the control. Cd and As treatment at 4 ppb had no effect on either of the genes. However, at 40 ppb, As exposure increased *pax2a* levels by ~2-fold. V and Cd showed no effect on *kim1*, however Pb exposure and As at 40 ppb led to an increase in this gene by ~2-fold compared to the control (Figure 4.2B).

Gene expression analyses with mixture treatments demonstrated synergistic effects. In mixtures containing glyphosate, LM1 treatment resulted in an elevated expression in both *kim1* (~ 7-fold) and *pax2a* (~ 3.5-fold) relative to the control (Figure 4.2C). In contrast, none of the other mixtures altered *kim1* expression. *pax2a* was increased with LM 2 (3-fold) and LM5 (5-fold) treatments compared to the control (Figure 3C). Metal mixtures without glyphosate showed a 2-fold increase in *pax2a,* but had no effect on *kim1* compared to the control (Figure 4.2D).

Overall, the effects of glyphosate on *kim1* were completely altered when in a mixture with metals, except in the case of LM1 (Glyphosate + Cd). However, changes in *pax2a* were still persistent with Pb, V, and some metal mixtures, but not with others. V effects on *pax2a* were also diminished when in mixture with other metals and/or glyphosate, with LM5 being the only exception. Collectively, these data show highly mixture specific effects on kidney development.

4.3.3 Gene expression changes following environmental sample treatments

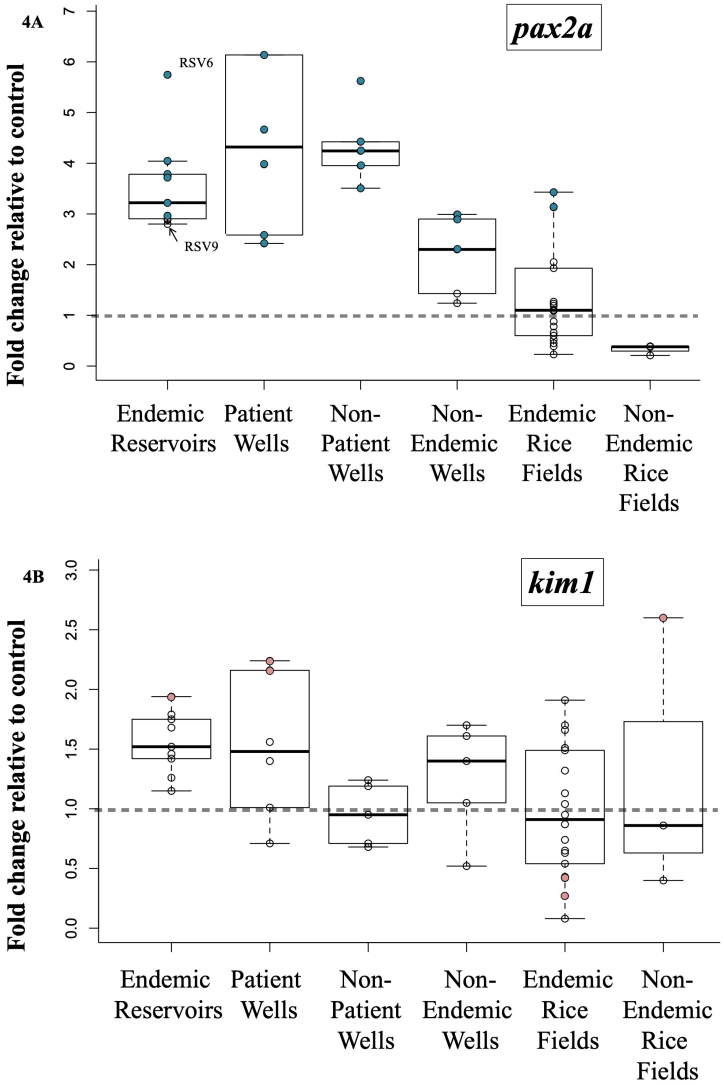


Figure 4.3: *Pax2a* and *Kim1* gene expression after exposure to water samples. Gene expression changes associated with kidney development (A) *pax2a* and injury (B) *kim1*, following reservoir sample, endemic region patient and non-patient well sample, non-endemic well sample, endemic rice field, emerging endemic rice field, and non-endemic rice field sample treatments from 28 hpf until the time of RNA extraction at 72 hpf. Boxplots were used here to represent the distribution gene expression fold change within a treatment sample group relative to control (depicted by dashed line). Circles represent individual fold change values. ANOVA was used to test for significance between treatment and control. Significance is represented by a solid circle, *p-value* < *0.05* (See Supplemental Material Table S5 for exact p-values), N = 8 (reservoir samples), N = 6 (wells), N= 5 (rice fields).

Pax2a and *kim1* transcript levels were also assessed following exposure to environmental samples derived from reservoirs, wells, and rice fields in CKDu endemic and non-endemic regions in Sri Lanka (Figure 4.3A, B). Reservoir samples 1-8 led to an increase in *pax2a* expression with the largest fold change (~ 5.7-fold) observed following reservoir 6 (RSV6) treatment—RSV6 had the highest levels of metals (Figure 4.3A). Reservoir sample 9, which borders an endemic region, treatment did not show any effect on *pax2a* expression (Figure 4.3A). Compared to the control, *pax2a* expression also increased (~2-6-fold) following well sample treatments from the endemic regions (Figure 4.3A). Effect on *pax2a* expression did not differ between samples derived from wells with a family member diagnosed with CKDu and non-patient wells. Treatment with some samples derived from the non-endemic regions did show an effect on *pax2a* expression, although the fold change ranged between 1.4- and 3-fold compared to the control and was significantly lower than endemic region samples (Figure 4.3A). Effects of rice-field derived samples from endemic and non-endemic regions were minimal on *pax2a* expression in which only two samples showed a 2-fold increase in expression compared to the control (Figure 4.3A).

Compared to results on *pax2a* expression, *kim1* was less affected by exposure to environmental samples. Six of the sample exposures, (1 reservoir, 2 patient wells, 2 endemic rice fields, 1 non-endemic rice field) led to an increase in *kim1* by ~2-2.5-fold compared to the control (Figure 4.3B).

4.3.4 Histopathological changes following laboratory-derived mixture treatments

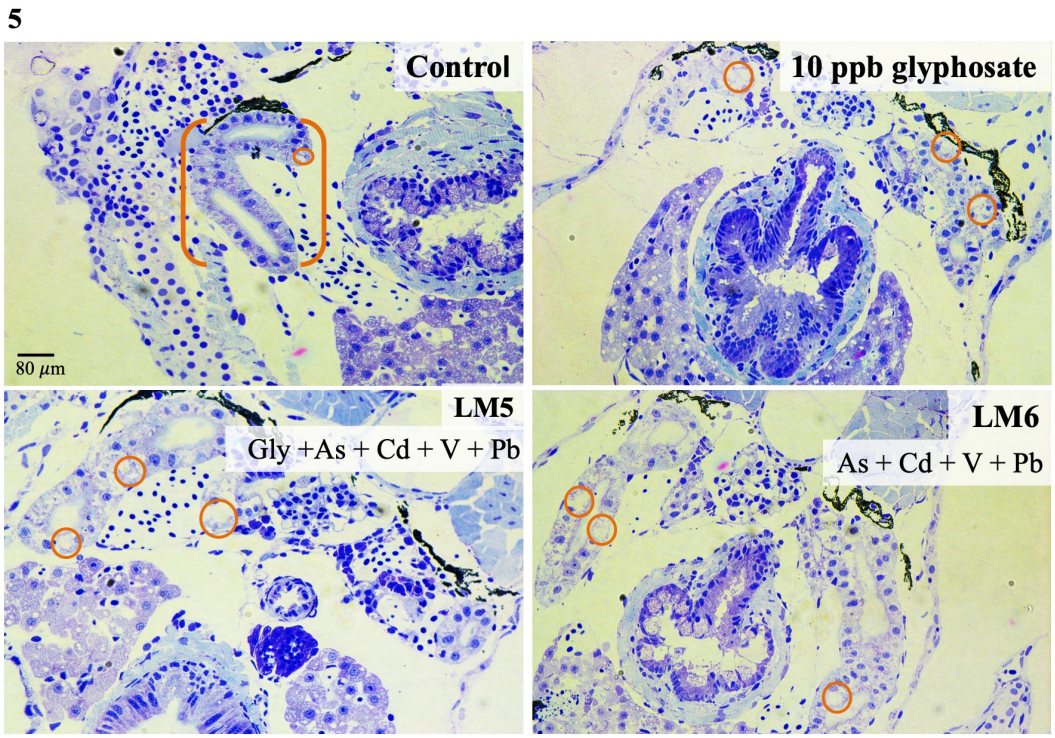


Figure 4.4: Histopathology of zebrafish pronephros after exposure to mixtures. 0.5 μm resin sections of zebrafish pronephric kidney at 8 dpf under control, 10 ppb glyphosate, LM5 (lab mix 5), and LM6 (lab mix 6) treatment conditions. Sections were stained with toluidine blue, the orange bracket is denoting a kidney tubules. Orange circles are referring to formation of vacuoles within kidney tubules (not all vacuoles are circled), N=4.

Histopathological analysis of zebrafish kidney tissue following exposure to a metal mixture (V + Pb + As + Cd) with and without glyphosate and glyphosate alone until 8 dpf showed that all treatments affected the kidney. These effects were most exacerbated following glyphosate + metal treatment, where images indicate an increased presence of vacuoles and improper formation of tubules when compared to the controls (Figure 4.4, Figure A5)

4.3.5 Mitochondrial analysis

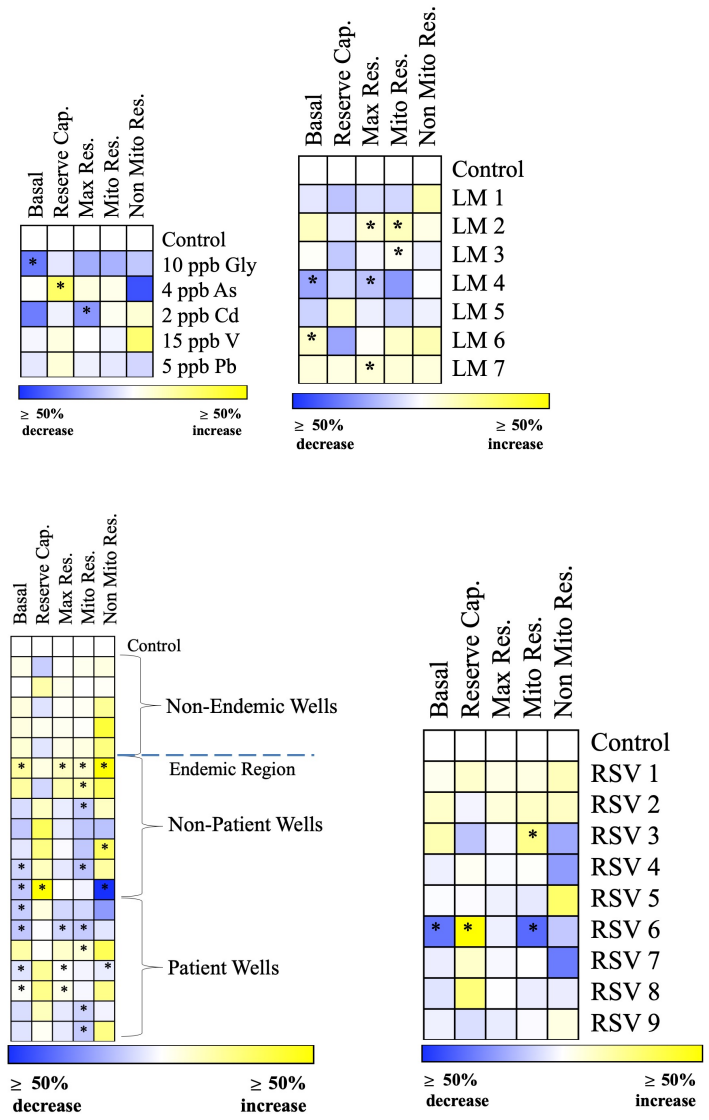


Figure 4.5: eOCR mitochondrial toxicity profiles after exposure to mixtures and CKDu region water samples. of Heat map representing oxygen consumption rates (OCR) in zebrafish treated with (A) individual chemicals; glyphosate (gly), arsenic (As), cadmium (Cd), vanadium (V), and lead (Pb), (B) lab mixtures (LM) 1 – 5, (C) well samples from non-endemic wells, non-patient wells, and CKDu patient wells and (D) reservoir samples Embryos were in treatment solution from 7 hpf to 31 hpf. Heat map squares represent percent of control with blue indicating a percent decrease relative to control and yellow indicating a percent increase relative to control. Mitochondrial reserve capacity (Reserve Cap.) was calculated by subtracting basal value from maximal respiration (Max Res.). Mitochondrial respiration (Mito Res.) was calculated by subtracting non mitochondrial respiration (Non Mito Res.) from basal values. An independent T-Test was used to test for significance between treatment and control. Significance is represented by (*), *p-value < 0.05,* (See Supplemental Material Table S6 for exact p-values), N = 21.

Mitochondrial function was significantly altered following exposure to certain chemical treatments. Notably, compared to the impacts of individual chemicals and lab-derived mixtures, environmental samples from the endemic region showed a greater effect on mitochondrial function relative to the control. Following zebrafish embryo exposure to individual chemicals, only glyphosate (10 ppb), Cd (2 ppb), and As (4 ppb) significantly altered mitochondrial bioenergetics. Mixtures, in particular LM4 (Glyphosate + As + Cd + V) had a greater impact on mitochondrial respiration (e.g., basal respiration and maximal mitochondrial respiration and mitochondrial reserve capacity) compared to individual chemical treatments (Figure 4.5A, B).

The greatest effect on mitochondrial bioenergetics occurred with well sample treatments, in which most of the well samples from endemic regions indicated altered basal, maximum respiration, and mitochondrial respiration measurements, when compared to controls and non-endemic region samples. Particularly, treatment with samples derived from endemic region patient and non-patient wells led to a decrease in overall mitochondrial parameters compared to the control (Figure 4.5C). In contrast, reservoir samples did not appear to significantly alter mitochondrial respiration parameters. The exception to this being treatment with reservoir 6, in which basal and mitochondrial respiration were significantly decreased and reserve capacity increased relative to controls (Figure 4.5D)

4.4 Discussion

Our toxicity studies and environmental analyses indicate that the chemical makeup of the reservoir and well water in CKDu endemic regions in Sri Lanka has the potential to alter kidney development and alter mitochondrial function, which might contribute to initiation and propagation of kidney dysfunction.

4.4.1 Chemical composition of environmental samples in the endemic region

Our metal and non-targeted analyses show a highly heterogeneous chemical composition in CKDu affected regions. Metal data show that metals appear variegated in distribution across endemic region samples compared to non-endemic samples which cluster together in a PCA analysis (Figure 4.1C). Further there is an increase in metals of interest (As, Cd, V, Pb) in endemic region samples, regardless of a patient or non-patient affiliation, compared to non-endemic region well samples (Figure 4.1B). Metal exposure to As and Cd have been linked to renal cancer and disease (Chowdhury et al., 2016). Further, Pb accumulation in blood has been correlated to developmental delays and en utero exposure to both As and Pb have influences on birth outcomes Hsueh et al., 2017; Signes-Pastor et al., 2019). This suggests that a consistent exposure to metals in drinking water, even at low levels, may be a concern to the development of children as well as those more susceptible to CKDu.

4.4.2 Presence of previously unidentified nephrotoxins in the water

Considering the likely scenario of individuals in endemic regions being vulnerable to CKDu later in life, we further characterized the organic contaminants in the drinking water (Babich, Ulrich, et al., 2020). These data show that ~2600 potential organic compounds are present within well water samples in the endemic zone of Sri Lanka, which demonstrates a highly diverse chemical burden in this water system. This indicates that there is a potential for human exposure to thousands of chemicals at trace levels.

Multiple classes of chemical compounds were present in the environmental samples, including agrochemicals and pharmaceuticals, some of which have been identified as nephrotoxic compounds. This may mean that an agrochemical, such as glyphosate, may only be one organic contaminant driver behind kidney toxicity in Sri Lanka. Also, it is conceivable that

the complex organic mixtures present in these drinking waters can enhance or even diminish the toxicity of organic contaminants and warrants further research to evaluate mixtures with respect to kidney toxicity (Cleuvers, 2004). This may explain the high heterogeneity of the disease even within regions that are most affected by CKDu. The presence of nephrotoxic chemicals could further be exacerbating kidney disease, especially for individuals who may be more susceptible given early-life exposure to heavy metals and warrants further research. It should also be noted that glyphosate was not identified in this analysis (Babich, Ulrich, et al., 2020). Since glyphosate is ubiquitously used in affected regions and was previously quantified by Gunarathna et. al 2018, it is highly likely that there are other chemicals that were not identified. However, this analysis provides the highest level of confidence that can be achieved in non-targeted mass spectrometry without authentic standards.

Overall, metal and non-targeted analyses demonstrate the presence of complex chemical mixtures in CKDu affected areas, especially in the drinking water. This highlights the critical need to identify drinking water sources that are potentially nephrotoxic, and to develop high throughput assays to determine at risk populations. To this end, here we utilize zebrafish as a model to examine chemical mixture effects on kidney development.

4.4.3 Developmental nephrotoxicity of laboratory-derived mixtures

We conducted a preliminary exposure study using gentamicin, a commonly used nephrotoxin to experimentally induce kidney injury, and also cadmium at 220 ppb to identify appropriate markers of altered kidney development and damage (Gao et al., 2013; Martínez et al., 2018). This study showed that compared to other genes tested (such as *Deltac*, *Lhx1a*, and *Ngal*), *pax2a* and *kim1* are highly sensitive markers of kidney toxicity. We then focused on

pax2a and *kim1* to further quantify effects of specific chemicals and their mixtures on kidney development.

Pax2a is a gene critical for the development of the kidney, and is involved in patterning in early developmental differentiation of the neck, podocyte, and proximal tubule boundaries (Drummond & Davidson, 2010). Its expression during embryonic kidney development is tightly regulated, first increasing in expression at 24 hpf in the pronephric ducts (Drummond et al. 1998) and refining to the pronephric tubules by 72 hpf (Drummond et al., 1998; Serluca & Fishman, 2001). Loss of *pax2* expression inhibits nephron tubule formation in both zebrafish and mice (Dressler & Woolf, 1999; Majumdar et al., 2000). In contrast, increased expression of *pax2* has been linked to renal cell carcinoma in endothelial cells and persistent expression leads to abnormal kidney development in mice (Fonsato et al., 2006; Patel & Dressler, 2013). While *pax2a* is also involved in development of other organ systems (e.g., the central nervous system), it is clear that tight regulation of *pax2a* expression during zebrafish embryogenesis is critical for proper kidney development. Therefore, any deviation in expression from controls, whether increased or decreased, is likely to have an impact on the developing kidney. Our data show that specific combinations of metals and herbicides may have different effects on kidney development (Figure 3.12). Notably, exposure to LMs containing glyphosate, *pax2a* expression returns to control levels with the introduction of As, Cd, and V, and does not increase until the addition of Pb (Figure 4.2). Furthermore, V significantly affects *pax2a* expression, but this effect is not apparent when in mixture with other metals.

KIM1 is a type 1 transmembrane protein. *Kim1* expression increases following kidney injury and is a known marker for both acute kidney injury (AKI) and chronic kidney disease (CKD). Specifically, embryonic overexpression in mice can reduce nephron quantity and may be

contributing to development of CKD (Humphreys et al., 2013). Induced expression of *kim1* in zebrafish also led to tubular damage and reduced glomerular filtration rate (GFR) (Yin et al., 2016). Our data show that glyphosate induces *kim1* expression by ~10-fold, but this effect is completely reversed when in mixture with other metals with the exception of Cd. (Figure 4.2).

Overall, *pax2a* and *kim1* results following exposure to LMs confirm the significance of considering differential developmental renal effects of metal-herbicide mixtures. This is further supported by the histopathological analyses, which showed that exposure to a metal mixture with or without glyphosate resulted in larval kidney tissue abnormalities, primarily in the form of increased vacuolization (Figure 4.4). Formation of vacuoles within kidney tubules has been observed in CKDu patients (Wijkström et al., 2013). Vacuolization of the kidney was also detected in mice exposed to cadmium, as well as in fish exposed to heavy metal toxicity (Zn, Cd, Pb, Cu) (Galindo-Riaño et al., 2015; Thijssen et al., 2007). In this study, the increased presence of vacuoles under treatment conditions could be an early sign of kidney perturbation, and if not remediated, could lead to a more detrimental tissue injury such as cellular apoptosis (Padanilam, 2003).

4.4.4 Developmental nephrotoxicity of environmentally-derived mixtures

The most notable finding in gene expression studies following exposure to environmental samples (reservoirs and endemic wells) was the significant increase in *pax2a* expression (Figure 4.3). When considered in comparison with lab-mixture data, this increase in *pax2a* expression with environmental sample treatments is likely a result of metal mixtures in the reservoirs and wells. For example, *pax2a* had the greatest increase under RSV6 treatment which also corresponds to the highest levels of metals detected among all reservoir samples. In contrast, RSV9, which is the outermost reservoir belonging to the cascade of reservoirs running through

the endemic region, has the lowest concentration of metals and does not show any significant changes in *pax2a* expression. A similar pattern is detected for non-endemic wells, where metal levels and *pax2a* expression changes are minimal (Figure 4.3). Notably, V levels are higher in endemic region well samples compared to reservoirs and non-endemic well samples, and *pax2a* expression was generally higher in endemic well samples relative to RSVs. This may be causally linked, given the prominent effect of V on *pax2a* as discussed earlier.

Compared to all the treatment groups, rice field sample treatments, irrespective of the region of origin, showed muted gene expression changes (Figure 4.3). This suggests that rice field sediment is comprised of very low levels of chemical constituents. Indeed, As, Cd, and V levels are noticeably lower in rice field samples compared to others (Figure 4.1). The induction of *kim1* detected following some of the rice field samples suggests that there are potentially other nephrotoxins present in these samples, but levels are likely to depend on seasonal weather patterns and agrochemical usage (Figure 4.3). Given the constant irrigation of these fields, compared to more stagnant water bodies such as wells and reservoirs, it is possible that the chemicals applied to rice fields may not persist.

Overall, environmental sample exposure data, especially from drinking wells, indicated the presence of chemical constituents that can alter kidney development. The heterogeneity of *pax2a* and *kim1* toxicity with each environmental sample also suggests that community members in the endemic region may be exposed to slightly different chemical profiles.

4.4.5 A multifaceted role for agrochemicals in CKDu

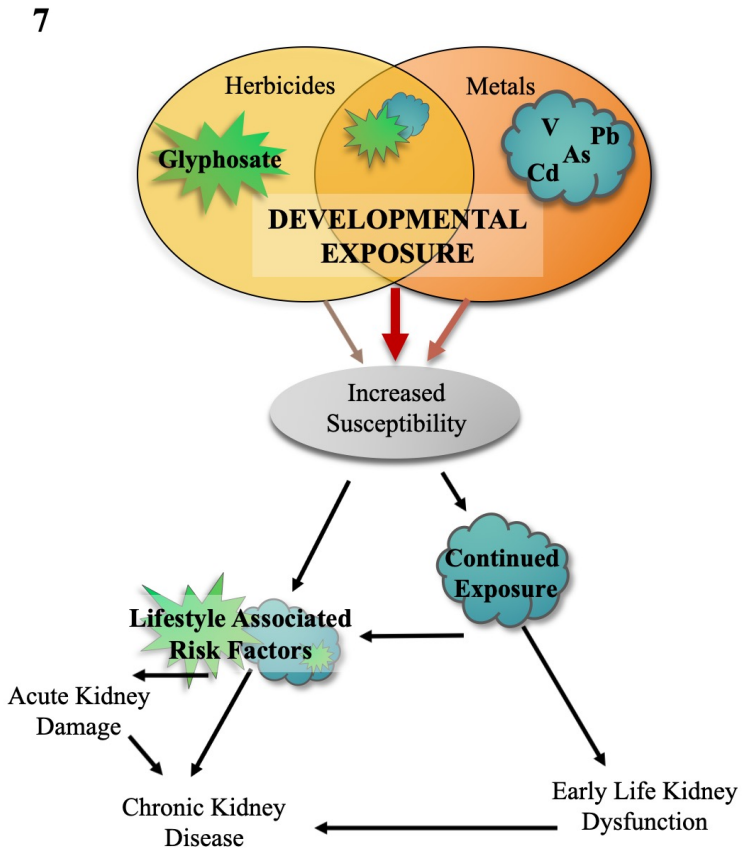


Figure 4.6: Conceptual map illustrating the potential progression of impaired kidney development to CKDu, beginning *in utero* through adulthood. Children are exposed to chemicals through development, in which it is most likely that chemical and metal mixtures impact the immature kidney leading to an increased susceptibility to CKDu. Continued exposure to mixtures may lead to early kidney dysfunction, whereas the introduction to nephrotoxins or other stressors (e.g., dehydration and heat stress) later in life may induce further kidney damage. Both of these pathways may then contribute to CKDu.

Collectively, gene expression data coupled with histology analyses indicate that metal mixtures are likely to have the greatest impact on early kidney development through manipulation of *pax2a* expression. This effect on *pax2a* is detected with mixtures containing metals at levels that are considered safe for human consumption. However, an important distinction is V—it is present at levels as high as ~150 ppb in some environmental samples, a

level that exceeds California state regulatory limits by 10-fold. Rat studies suggest that V is nephrotoxic but the environmentally safe levels are ambiguous for this metal. Therefore, the role of V warrants further examination in the context of CKDu (Tr, 2002). We speculate that metals in the endemic region, even at levels considered safe for drinking, can contribute to abnormal kidney development in children (Friedman & Luyckx, 2019). This may later increase the susceptibility of individuals to CKDu.

Using glyphosate as an example, we show that interactive nephrotoxic effects of organic agrochemicals and heavy metals are an important consideration. For example, glyphosate at 10 ppb can induce *kim1*, implying that exposure to this chemical even at very low-levels can contribute to kidney injury. Glyphosate has been found to enter mammalian cells via Na+ phosphate transporters, as well as amino acid transporters LAT1 and LAT2 (Xu et al., 2016). LAT2 is expressed in the mammalian renal cells (del Amo et al., 2008). We postulate that exposure to glyphosate coupled with individuals with impaired kidney development is likely to increase the initiation and progression of CKDu. (Figure 7). However, gene expression data also suggest, when in a mixture with metals such as arsenic, glyphosate may have alternate effects on kidneys, including effects that may not be detrimental.

4.4.6 A role for mitochondrial dysfunction

One potential mechanism by which these metal and organic chemical mixtures may contribute to the progression of kidney dysfunction is via mitochondrial toxicity. Studies show damaged mitochondria and mitochondrial dysfunction as a key factor underlying chronic kidney disease (Gamboa et al., 2016; Granata et al., 2009).

Our mitochondrial analyses, following exposure to laboratory- and environmentally-derived mixtures, showed that the most significant perturbation of OCR occurred from treatment

of endemic region well samples and lab mixtures compared to individual chemicals and reservoir samples (Figure 4.5). Notably, LM4 which includes the addition of vanadium, as well as endemic region well samples (which show higher concentrations of vanadium compared to non-endemic well samples and reservoirs (Figure 4.1) had the most significant differences when compared to controls.

Although each well sample treatment corresponds to a unique mitochondrial toxicity profile, treatments from endemic regions produce a trend in which mitochondrial parameters (specifically basal, maximum respiration, mitochondrial respiration, and non-mitochondrial respiration) are decreased (Figure 4.5). As demonstrated by the non-targeted chemical analysis, patient and non-patient well samples also have specific chemical constituent profiles (Babich, Ulrich, et al., 2020). However, further data are needed to make a direct correlation between the chemical profile of a given well and mitochondrial toxicity of that well water.

Reservoir samples showed little significant difference compared to controls in terms of mitochondrial toxicity. However, a similar dampening effect on mitochondrial respiration parameters that was seen in the endemic region well samples is also seen in treatments with reservoir samples. RSV6 had the greatest effect on eOCR with significant decreases in both basal and mitochondrial respiration and an increase in reserve capacity. Notably, these data also correspond to gene expression effects detected by RSV6 and high metal concentrations found in RSV6, suggesting that chemical mixtures in the reservoirs can not only induce developmental defects, but also have the potential to contribute to progression of kidney failure via mitochondrial perturbation. It is unlikely that community members drink the reservoir water at sufficient quantities for this to be a health hazard, unless they accumulate in certain plants and fish that are common food sources for community members. Nonetheless, these data strongly

support the presence of environmental contaminants that can contribute to the progression of CKDu in these regions. Furthermore, our assay may provide rapid screening system to identify drinking water sources that may contribute to CKDu. Collectively, mitochondrial data from individual chemicals and lab mixtures as well as environmental samples illustrate a mixture specific mitochondrial toxicity. Mitochondria are a common target of many metals and organic pollutants including herbicides (Meyer et al., 2018; Yamano & Morita, 1993). Kidney tissues are also known to accumulate or aggregate chemical compounds, and continuous perturbation of kidney mitochondria through exposure to chemical mixtures in the drinking water may have deleterious effects on renal structures and function (Jin et al., 1999; Shen et al., 2011). It is important to note that a recent transcriptomic analysis also showed significant changes in genes associated with mitochondrial function in CKDu patients from Sri Lanka, suggesting a potential mitochondrial etiology for CKDu (Sayanthooran et al., 2018).

4.4.7 Implications for CKDu

Our data highlight the need to evaluate developmental nephrotoxicity of chemical mixtures and provide a possible mechanistic explanation for the increasing number of younger individuals with kidney dysfunction. Given the differences in toxicity detected between rice field samples and reservoir and well samples, poor drinking water quality is likely a significant source contributing to the initiation and/or progression of CKDu. It is possible that children may be exposed to mixtures of metals *in utero* and throughout their youth, that are impacting kidney development (Wai et al., 2017). Contact with specific chemicals, such as the herbicide glyphosate or other nephrotoxic contaminants as found in their drinking wells, could increase their risk for kidney failure. Given that glyphosate has the ability to chelate and form complexes with metals, there is a potential that it is acting as a transporter, and after environmental cues,

may be releasing metals to specific organs (Gunatilake et al., 2019; Mertens et al., 2018). Further investigation of the biological effects of glyphosate and metals are needed to interpret these possible molecular interactions. Notably, glyphosate may be one of many herbicides or other chemicals in the endemic regions that may contribute to CKDu.

Coupled with childhood onset, differences in exposure to agrochemicals or other causative factors (e.g., heat stress), may explain the heterogeneity of disease prevalence within endemic zones (Figure 4.6). Mitochondrial toxicity of the drinking water from the endemic regions, despite low-abundance of metal and organic compounds, also emphasizes the importance of assessing not just the chemical composition of water, but also their biological effects. Assessing mitochondrial toxicity of well water and other water sources, in combination with mitochondrial integrity of individuals in these communities may serve as important indices in determining at risk patient populations. Conducting a more thorough investigation of changes in gene expression, such as RNA sequencing, will also provide information on biological functional pathways that are being altered in response to chemical mixture exposures. Finally, based on these data we emphasize the critical need to provide a clean water supply to affected communities to mitigate further exposure to chemical mixtures.

CHAPTER 5

MIXTURE INDUCED CHANGES IN GENE EXPRESSION AS A PROPAGATOR OF DISEASE

5.1 Introduction

RNA sequencing is a widely used tool to better understand the molecular impacts of chemical exposures. However, it is under-utilized when studying safe low levels of chemicals, as well as chemical mixtures. Here, we used an RNAseq dataset in response to exposures of mixtures of As, Cd, Pb, V (+ / - glyphosate) and glyphosate alone to further identify underlying mechanisms, that along with mitochondrial impediment, that may be contributing to disease progression and stressor susceptibility. This is relevant to understanding mechanisms behind chemical mixture-induced developmental nephrotoxicity, which was discussed in the previous subchapter.

Additionally, the zebrafish provide an excellent *in vivo* model to understand the genetic consequences to early exposures to chemicals and chemical mixtures and have been used in RNA sequencing based analyses (Barta et al., 2018; Qiu et al., 2019; Shankar et al., 2019; M. Zheng et al., 2018). The availability of other zebrafish RNAseq datasets, such as the zebrafish developmental atlas that was used here, are also beneficial to gain deeper insight into genotoxicity through cross comparison analysis techniques (Dong et al., 2021; Farnsworth et al., 2020).

5.2 Methods

5.2.1 Zebrafish care and exposure

One cell AB strain zebrafish were incubated at 28.5 °C in egg water (1 embryo/1 mL) until 28 hpf at which time they were screened and transferred to treatment solutions. Zebrafish embryos were manually dechorinated and treated with glyphosate (10 ppb), a metal mixture; As (4 ppb),

Cd (2 ppb), V (15 ppb), and Pb (5 ppb), and a glyphosate + metal mixture (glyphosate & metals). A subset of embryos were transferred to clean egg water and acted as controls. Metals (Cd, Sigma Aldrich Cat# 202908, As, Sigma Aldrich Cat# S7400, V, Sigma Aldrich Cat# 262935, and Pb, Sigma Aldrich Cat# 268690) and glyphosate (Sigma Aldrich Cat# 45521, fresh stock made bi-weekly) were added to egg water to create a treatment solution. Complex mixtures were designed to understand how metal mixtures alone, and in the presence of glyphosate may be altering gene expression profiles. Metal concentrations used were chosen as they are representative of concentrations found in drinking water samples (Babich, Ulrich, et al., 2020) Embryos remained undisturbed in treatment solutions until 72 hpf.

5.2.2 RNA extraction and sequencing

RNA was extracted from whole larvae at 72 hpf using Qiashredder and RNAeasy minikit (Qiagen, Valencia, CA) following manufacturers protocol. Each RNA extraction contained a total of 10 larvae. A total of 5–8 RNA extractions were completed per treatment (50–80 individuals). High quality RNA (as determined by nanodrop), was sent to the Duke sequencing facility. Five samples of control, glyphosate, metal mixture, and glyphosate & metals mixture treatments underwent quality control and were sequenced on the Illumina NovaSeq 6000 with strand specific Poly A+ libraries on an S-prime 50bp PE full flow cell.

5.2.3 Bioinformatics and analyses

Quality of raw reads was checked using FastQC and it was determined that reads had a quality score above Reads were processed, which included Truseq adaptor trimming and mapping to the zebrafish reference genome, by following the protocol by Pertea et al. 2016. Annotated gene counts were imported into R studio (Vs 4.1.2) where edgeR was used to normalize zebrafish gene counts. Gene counts were normalized by trimmed mean of M-values

(TMM) method. Following normalization, common dispersion was estimated, and tagwise dispersion was applied. Final normalized log2 counts per million (CPM) were used to determine fold change relative to control using topTags for pairwise comparisons. Genes were considered differentially expressed with an FDR adjusted p-value < 0.01 and absolute log2FC > 1. To determine biological function of differentially expressed genes, genes were annotated using online software gprofiler.

A principal component analysis was used to determine clustering of samples and was conducted in SPSS software (IBM, Armonk, NY). Normalized log2 CPM gene expression data were inserted into the PCA for individual samples. Principle components 1 and 2 were extracted per sample, plotted, and overlayed with treatment conditions. Inputs for the PCA included expression data for all genes sequenced as well as genes that were determined to be differentially expressed.

5.2.4 Exploration of developmental gene sets

RNA seq data were further analyzed based upon genes responsible for early zebrafish development. Zebrafish developmental gene sets, as determined by Farnsworth et al. 2020, were used. Normalized RNA seq expression data for treatments and control were identified for 31 tissue specific gene sets and used in the competitive gene set test Camera (R studio vs 4.1.2). A developmental gene set was considered differentially expressed between treatment and controls with an FDR adjusted p-value < 0.01. An excel file containing tissue specific gene sets is included in the appendix Table A8.

5.3 Results

5.3.1 Differentially expressed genes (DEGs)

Gene expression was analyzed and differentially expressed genes were identified between treatments and control. Genes were considered to be differentially expressed with an false discovery rate (FDR) adjusted P-value < 0.01 and an absolute log2FC > 1. A total of 105, 196, and 339 genes were considered differentially expressed relative to control for glyphosate, glyphosate & metals, and metals treatments respectively. Among these, 29 genes were determined to be specific to glyphosate, 82 for glyphosate & metal, and 215 for metal treatments (Figure 5.1). Volcano plots were used to visualize scattering of differentially expressed transcripts among treatments relative to control (Figure 5.2).

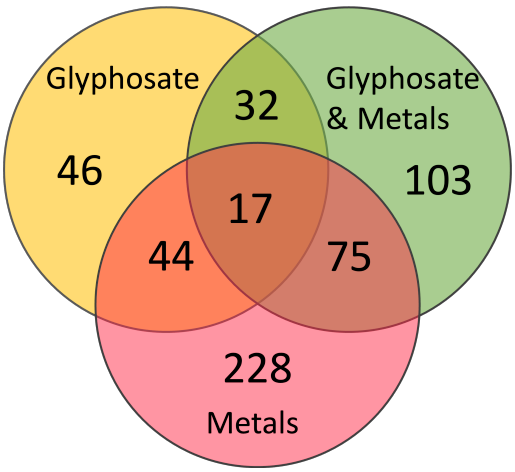


Figure 5.1: Differentially expressed genes from RNA seq analysis. Venn diagram showing the number DEGs that are unique to metals (red), glyphosate (yellow), and glyphosate & metals (green) as well as those that are shared among treatments.

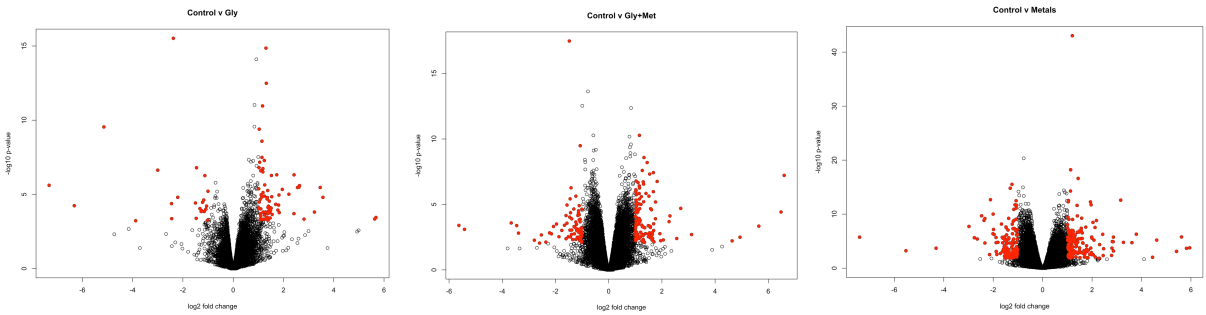
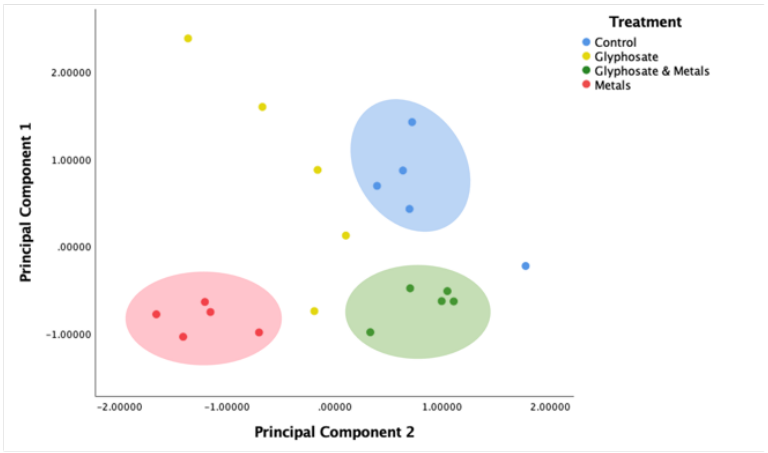


Figure 5.2: RNA seq volcano plots. Volcano plot displaying DEGs (red) compared to all genes between glyphosate and controls (A), glyphosate & metals and controls (B), and metals and controls (C). Genes are plotted by log2 Fold change (x-axis) and -log10 p-value (y-axis).

5.3.2 DEG clustering

Principal component plots were used to explore clustering of sample treatments based upon gene expression. First, normalized expression counts for all 23,437 transcripts sequenced were used as inputs for the principal component analysis (PCA). Two principal components (PC) were extracted, PC1 explained 36.215% of variance and PC2 explained 9.503% of variance, explaining a cumulative 45.719% of variance. Extracted regression factors PC1 and PC2 were used to build a scatter plot, which showed clear clusters of metals and glyphosate & metals treatments (Figure 5.3A) Clustering was further explored by inputting the 509 shared and treatment specific DEGs into the PCA. Here, PC1 explained 32.139% of variance and PC2 explained 18.136% of variance, explaining a cumulative 50.275% of variance. Extracted regression factors PC1 and PC2 were plotted and showed clear clustering of control, glyphosate & metals, and metals treatments (Figure 5.3B). In order to further understand what may be driving treatment specific gene expression clusters, a PCA was conducted on the 17 genes that were shared among all treatments. Extracted regression factors PC1 (51.079%) and PC2 (11.822%) were plotted. Clustering occurred among control and glyphosate & metal treatments. However, there was a loss of clustering among metals and glyphosate treatments (Figure A6).



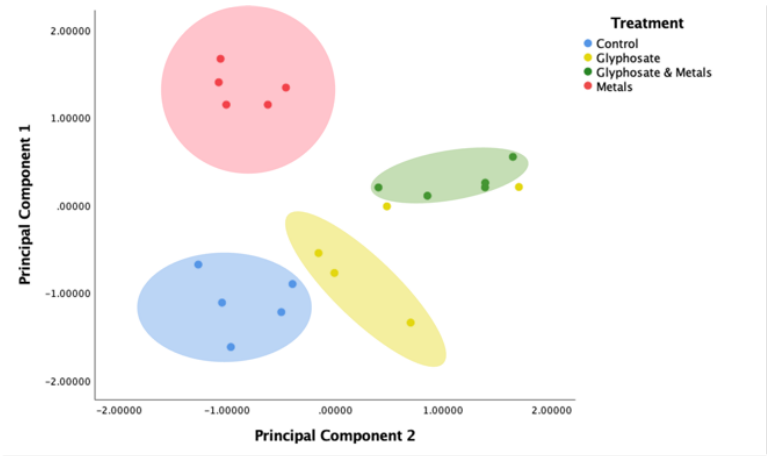


Figure 5.3: RNA seq clustering of genes by treatment condition. Principal Component plots representing clustering of gene expression, where gene expression values inserted into the PCA include all genes (A) and DEG for all treatments (B), and DEGs that are shared by all treatments (C). Clusters are labeled by treatment condition for control (blue), glyphosate (yellow), glyphosate & metals (green), metals (red). A principal component plot representing clustering of gene expression where gene expression values inserted into the PCA include only the DEGs shared by all treatments is included in the appendix (Figure A6).

5.3.3 Shared and treatment-specific gene ontology

Gene ontology for DEGs among treatments were explored. This resulted in 7 functional terms unique to glyphosate, 17 terms unique to glyphosate & metals, and 24 unique terms for metals. Among these, 10 /17 terms specific to glyphosate & metal treatment were involved in protein recycling, structural homeostasis, and cellular degradation. 16 / 24 terms specific to

metals treatment were involved in DNA maintenance and organization, of these 16 terms five are directly related to regulation of transcription and epigenetic modifications. Collectively, all three treatments shared terms DNA geometric change, DNA duplex unwinding, and DNA conformation change. When comparing two treatments at a time, each pair (glyphosate & metals / metals, glyphosate / glyphosate & metals, glyphosate / metals) shared four functional terms. Most shared terms include those that are specific to DNA processing and maintenance (i.e. DNA packaging complex and DNA helicase activity). Uniquely, GO terms shared by glyphosate & metals / metals included endopeptidase activity, extracellular space, extracellular region, and degradation of extracellular matrix (Table 4).

Table 4: RNA seq gene ontology functional terms. Biological function terms associated with treatments are shown below. The table is divided into sections including terms that are shared by 2 or more treatments, and terms that are treatment specific.

Glyphosate	Gly & Metals	Metals
Shared		
DNA geometric change	DNA geometric change	DNA geometric change
DNA duplex unwinding	DNA duplex unwinding	DNA duplex unwinding
DNA conformation change	DNA conformation change	DNA conformation change
DNA recombination	DNA recombination	-
DNA helicase activity	DNA helicase activity	-
Telomere organization	Telomere organization	-
Telomere maintenance	Telomere maintenance	-
Nucleosome	-	Nucleosome
DNA packaging complex	-	DNA packaging complex
Protein-DNA complex	-	Protein-DNA complex
Chromatin	-	Chromatin

Table 4 Continued

-	Endopeptidase activity	Endopeptidase activity
-	Extracellular space Extracellular region	Extracellular space Extracellular region
-	Degradation of the extracellular matrix	Degradation of the extracellular matrix
Treatment Specific		
DNA binding	cysteine-type peptidase activity	protein heterodimerization activity
Termination of O-glycan biosynthesis	cysteine-type endopeptidase activity	protein dimerization activity
Factor: p53; motif: NGRCWTGYCY	peptidase activity	neuropeptide receptor activity
Elevated bronchoalveolar lavage fluid neutrophil proportion	helicase activity	serine-type endopeptidase activity
Usual interstitial pneumonia	ATPase, acting on DNA	nucleosome assembly
Abnormal cellular composition of bronchoalveolar fluid	anatomical structure homeostasis	chromosome organization
	lysosome	chromatin assembly
	lytic vacuole	nucleosome organization
	vacuole	chromatin assembly or disassembly
	Phagosome	protein-DNA complex assembly
	RUNX1 regulates transcription of genes involved in differentiation of keratinocytes	DNA packaging
	Trafficking and processing of endosomal TLR	protein-DNA complex subunit organization
	Collagen degradation	chromatin silencing
	Assembly of collagen fibrils and other multimeric structures	negative regulation of gene expression, epigenetic
	Toll-like Receptor Cascades	chromatin organization
	Collagen formation	chromatin organization involved in negative regulation of transcription
	Cerebellar vermis atrophy	regulation of gene expression, epigenetic
		chromatin organization involved in regulation of transcription
		membrane disruption in other organism
		chromosome
		Calcium signaling pathway
		Cobalamin (Cbl, vitamin B12) transport and metabolism
		Digestion of dietary carbohydrate
-		DNA Damage/Telomere Unique Stress Induced Senescence

5.3.4 Tissue gene set expression is treatment specific

Competitive gene set tests for tissue-specific developmental gene sets were conducted and gene sets were biologically ranked among treatments. A total of 31 tissue specific gene sets were analyzed (Table A8). Eleven out of 31 gene sets were statistically differentially expressed in glyphosate treatment relative to control. There were 27 gene sets identified as differentially expressed for glyphosate & metals, as well as metals treatments, relative to control. The top five differentially expressed gene sets, ranked in order as most biologically relevant to least, for the treatments are as follows; glyphosate (FDR adjusted p-value < 4.28e-4) - pharyngeal arch, neuron, tailbud, pharyngeal endoderm, and central nervous system, glyphosate & metals (FDR adjusted p-value < 1.64 e-410) – thymus, spleen, intestine, tailbud, integument, metals (FDR adjusted p-value < 1.63e-12) – tailbud, thymus, pectoral fin bud, paraxial mesoderm, lens placode. Additionally, both glyphosate & metals, and metals treatments included 4 gene sets that were not considered to be differentially expressed compared to controls, these include the pineal gland, pancreas, central nervous system, and neuron (Figure 5.4).

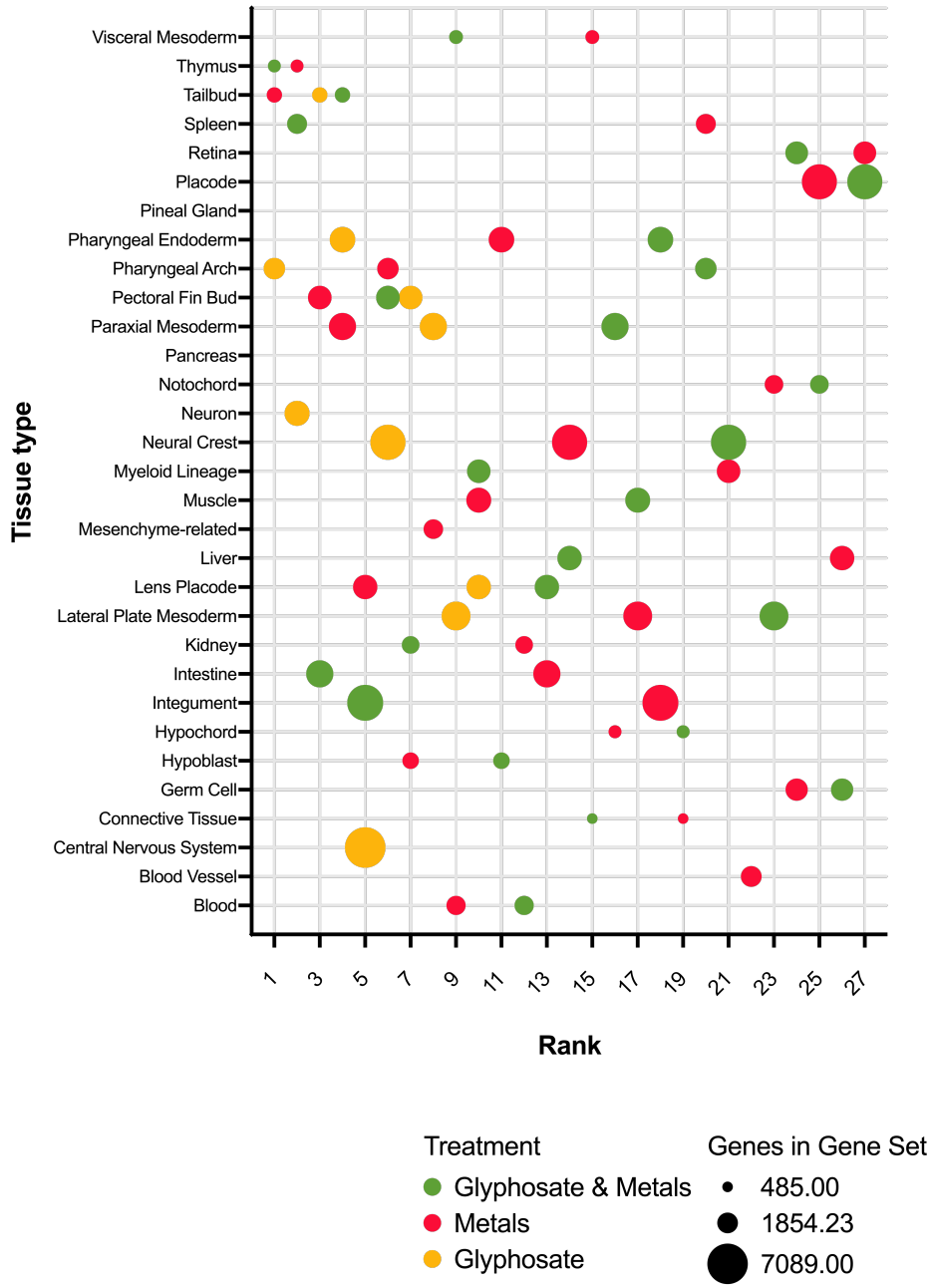


Figure 5.4: RNAseq comparison to a zebrafish developmental atlas. Multivariable dot plot representing the biological rank (x-axis) of a tissue type (y-axis) for a treatment condition as determined by the CAMERA test. The size of the dot represents the number of genes that were in a given tissue set, with a larger dot indicating a larger number of genes comprise a gene tissue set. The color of the dots represents treatment of either glyphosate (yellow), glyphosate & metals (green), or metals (red). If a tissue gene set was not determined to be significantly different from controls for a given treatment, a dot was not included for that tissue type.

5.4 Discussion

Exposure to low levels of chemical mixtures is highly relevant to humans and animals, and yet this area of study has only been minimally investigated. Although some studies are beginning to explore the biological consequences of mixture exposure compared to individual chemicals (such as multiple vs singular metals), few studies take into consideration mixtures of metals and commonly used organic compounds, such as herbicides or antibiotics (Heffern et al., 2018; Jia et al., 2022; Jijie et al., 2020). Early exposure to such mixtures may not manifest in a direct phenotype, however, alteration of molecular pathways may still leave an individual at risk for future stressors. Here, we have used RNA seq to explore the molecular impacts of chemical mixture exposures, and report that alterations in gene expression are mixture dependent and occur at concentrations considered safe for human consumption. By using predetermined zebrafish developmental gene sets, to our knowledge, we are the first to identify specific tissues that may be more at risk for mixture induced developmental toxicity. Additionally, an RNA-seq dataset exploring the developmental impacts of environmentally relevant glyphosate concentrations, both alone and in mixture, is a necessity given it's global ubiquitous use as a herbicide.

Insecticide organophosphates (OPs), such as malathion, have been shown to induce multiple organ toxicity, however the central nervous system is documented as being the most effected due to their inhibition of acetylcholinesterase activity (Badr, 2020). Prenatal OP pesticide exposure in humans has been linked to decreased cognitive performance at 2 months of age and decreased fine motor skills at 6 months of age (Suwannakul et al., 2021). Similarly, zebrafish embryonic exposure to the insecticide diazinon showed altered behavior throughout later life stages as well as altered mitochondrial function in brain and testes. Adult fish acutely

exposed did not show changes in neurobehavioral and mitochondrial outcomes (Boyda et al., 2021). OP pesticide exposure in zebrafish has also been shown to have transgenerational effects such as hyperactivity and increased acetylcholine concentrations in F2 generations (Schmitt et al., 2020).

OP pesticides have also been associated with DNA damage in both human and model organisms (D'Costa et al., 2018; Grover et al., 2003), and have been speculated to cause DNA damage through increased levels of ROS, which when left un-neutralized, can oxidize the DNA sugar-phosphate backbone or bases (Kaur & Kaur, 2018). Increases in ROS may stem from pesticide interaction with important antioxidants, such as glutathione peroxidase, which was confirmed after an exposure to organophosphate dimethoate to adult male rats showed increased lipid peroxidation and decreased glutathione peroxidase activity in serum after 48 hours (Yahia & Ali, 2018).

Glyphosate, although is considered an OP in chemical structure, acts differently by targeting a plant-specific enzyme 5-enolpyruvyl shikimate-3-phosphate synthase that is responsible for aromatic amino acid synthesis (Steinrücken & Amrhein, 1980). Vertebrates do not contain this enzyme, therefore were considered safe from potential harmful consequences of glyphosate exposure and glyphosate was ubiquitously used. Currently, scientific literature is lacking on the impacts of glyphosate on organismal development and was not previously thought to be associated with neurotoxicity. However, more recent literature points to neurological impacts from developmental glyphosate exposure in vertebrate models. Zebrafish exposed from 1.5 – 120 hpf to 1 ppm glyphosate showed decreased motor activity as well as altered genetic pathways related to neuronal physiology (Forner-Piquer et al., 2021). Additionally, gestational

exposure to glyphosate (500 uM) in rats resulted in altered reflex and motor activity in pups (Coullery et al., 2020).

We show that glyphosate exposure resulted in the least number of DEGs (105) and along with mixtures, induced changes in gene expression for a number of biological functions associated with DNA (such as DNA recombination, DNA conformation change, DNA helicase activity, and DNA duplex unwinding), as well as uniquely alters gene expression associated with DNA binding. When exploring developmental gene sets, tissues associated with the central nervous system, specifically those for the neuron, central nervous system, neural crest, and lens placode were deferentially expressed. We speculate that early exposure to a pesticide such as glyphosate changes expression of transcripts necessary for maintenance of DNA which may cause tissue specific alterations in the CNS. DEGs that were also identified in gene sets associated with the CNS include fosab, ier2b, and socs3a. fosab, has been shown to increase upon early exposure to the OP Diisopropyl fluorophosphate (DFP) as an indication of CNS hyperexcitation (Brenet et al., 2020). Similarly, fosab was significantly upregulated after glyphosate exposure relative to controls, further confirming the CNS as a target tissue. Given that the number of DEGs identified for glyphosate exposure was less than both metal mixtures, as well as the number of differentially expressed tissue gene sets, it is likely that developmental exposure to low levels of metals may have more biological consequences compared to glyphosate alone.

Exposure to a metal mixture of As, Cd, V, and Pb resulted in the greatest abundance of 339 differentially expressed genes relative to controls. Metals have been known to bind directly to DNA altering it's structure, interact with transcription factors to either activate or inhibit function, and interfere with DNA methylation, all of which will alter gene expression and induce

specific biological pathways associated with transcription regulation as seen here (Blanchard & Manderville, 2016; Kanellis & dos Remedios, 2018; Ryu et al., 2015). The impact of metals has also been shown to act differently on gene expression when in mixture. In a study comparing Pb, Mercury (Hg), As, and Cd exposure, alone and in mixture on zebrafish gene expression, it was found that individual metals stimulated changes in gene expression related to the immune response whereas mixtures altered those associated with the antioxidant pathway, further confirming that metal induced changes in gene expression are highly mixture specific (Cobbina et al., 2015).

With the addition of glyphosate to the metal mixture, the number of DEGs decreased from 339 to 196. Co-exposures can alter individual chemical toxicity by potentially facilitating or blocking transport into the cell, changing target tissues, and by activating or inhibiting antioxidant enzymes in response to oxidative stress (Wallace & Buha Djordjevic, 2020).For example, co-exposure of cadmium and the OP pesticide chlorpyrifos (CPS) on human hepatocytes resulted in hepatic lipid accumulation, which is thought to be facilitated by intracellular transport of the Cd and CPS complex (W. He et al., 2015). Currently there is not much published literature on the interactive effects of metals and glyphosate and the regulation of gene expression. However, this body of work has shown that expression of zebrafish kidney development genes *pax2a* and *kim1* have altered expression levels after exposure to metal mixtures with and without glyphosate (Babich, Ulrich, et al., 2020).

Interestingly, metal mixtures both alone and with glyphosate, did not target the CNS, as was seen with glyphosate exposure. Rather, glyphosate & metal mixture exposure was significantly associated with gene sets representing, in biological order, thymus, spleen, intestine, tailbud, and integument. While metal mixture exposure was significantly associated with genes

representing tailbud, thymus, pectoral fin bid, paraxial mesoderm, and lens placode. Although the first 5 biologically ranked gene sets targeted by the two mixtures are similar, spleen is ranked much higher for glyphosate & metals (2) compared to metal mixture (20) alone. Additionally, the thymus and kidney are ranked higher at number 1 and 7 compared to the metal mixture at 2 and 12. In teleost fish, these three organs comprise important players in the immune response, as they are production sites for T and B cells as well as macrophages (Bjørgen & Koppang, 2021). Here, development of tissues important to the adaptive immune response were impacted by low-level metal mixtures and may be targeted when glyphosate is introduced as can be seen by the higher tissue rank under glyphosate & metal exposure.

What was more striking, were the similarities and differences in biological function terms of DEGS between metals and glyphosate & metals mixtures. Both mixtures significantly altered genes associated with endopeptidase activity, extracellular space, extracellular region, and degradation of the extracellular matrix. The ECM plays a role in a number of critical functions, including cellular proliferation, migration, differentiation, and cell death (Karamanos et al., 2021). By changing the expression of genes related to the ECM, responses to environmental changes may be impeded and more pathological responses, such as tissue fibrosis or carcinomas may develop. Due to the feedback between the ECM and adjacent cells, cellular organelles such as lysosomes, vacuoles, and endopeptidases play an important role in recycling ECM protein constituents (Lu et al., 2011).

Metals have been shown to alter ECM dynamics. Specifically, As has been positively associated with DNA methylation of ECM remodeling genes (such as matrix metalloproteinases) in children that were exposed in utero (Gonzalez-Cortes et al., 2017). Mice treated with 100 ppb arsenic in their drinking water showed a maladaptive mitochondrial phenotype in connective

tissue fibroblasts as well as dysfunctional ECM (Anguiano et al., 2020). Additionally, fibroid cells exposed to CdCl2 showed a downregulation of genes associated with ECM components (such as collagen) and an upregulation in genes associated with ECM degradation (Yan et al., 2021).

Although both metal mixtures shared functional terms for the ECM and endopeptidases, glyphosate & metal mixture exposure uniquely altered genes associated with biological functions such as lysosome, lytic vacuole, and phagosome all which are important in the recycling of cellular organelles and proteins, as well as apoptosis. Additionally, collagen formation and degradation were also identified as a GO term associated with glyphosate & metals mixture exposure. Collagen is a main protein component of the ECM, as it provides structure and acts as a receptor for cell signaling (Karamanos et al., 2021). Changes in collagen deposition and degradation could lead to diseases in which stromal collagen is disorganized (such as fibrosis) (Ricard-Blum et al., 2018). The pathways associated with the ECM, collagen, and cell component recycling altered by glyphosate & metal mixture indicates a pathological response that may be facilitated by the presence of glyphosate. Given that glyphosate is a derivative of glycine, and glycine is necessary for proliferating cells and is a primary component of collagen, glyphosate may be interfering with normal ECM deposition and exacerbating metal mediated ECM toxicity (Kay et al., 2021; Martínez et al., 2018).

While both metal mixtures also shared biological functions associated with DNA structure and maintenance, metal mixtures alone had biological functions further associated with chromatin organization, DNA packaging, and epigenetic regulation of gene expression. Exposure to As and Cd are known to cause changes in global DNA methylation (Arita & Costa, 2009). Arsenic has also been shown modify histones (via acetylation or methylation) as well as alter

miRNA expression levels, both of which may have epigenetic consequences (Q. Zhou & Xi, 2018). For example, chronic As exposure to zebrafish F0 generation resulted in histone hypermethylation and altered motor activity in F2 generation individuals (Valles et al., 2020). Zebrafish embryonic Pb exposure at 10 and 17 ppb resulted in decreased expression of DNA methylation related gene Dnmt1 (Park et al., 2020). Although the concentrations of metals used in this study are lower than those that are typically associated with genotoxicity in literature, together low levels of metals may be acting in tandem to alter the transcriptome.

Limitations of this study include the lack of individual chemical exposure assessment on zebrafish gene expression. However, given limited resources, we find the mixtures that were explored to be highly relevant to real-world environmental exposures. Given glyphosates ubiquitous use, as well as direct exposure in farming communities, exploring the individual impacts of glyphosate on gene expression is also relevant. Future studies should aim to explore individual chemicals and sub mixtures to determine what chemicals may be contributing to the clear shifts in biological function pathways that were identified here.

We have identified clear mixture-specific alterations in gene expression in response to exposure to low level metal mixtures with and without glyphosate. Specifically, a mixture of glyphosate & metals may be disrupting ECM organization and additionally targeting tissues that are important players in the zebrafish immune response (including the kidney). This ties into the previous subchapter, in which kidney development was most exacerbated by complex mixtures compared to individual chemicals. Here, the addition of glyphosate to the metal mixture could be exceeding the organisms capacity for an adaptive response. Alternatively, we found that a metal mixture without glyphosate alters gene expression related to epigenetic modifications and transcription regulation, confirming that low-level metal mixtures can have genetic

consequences. These findings highlight the importance of studying how safe levels of chemicals, when in mixture, impact early development and alter the transcriptome.

In the final study included in this body of work, the phenotypic consequences of embryonic exposure to a large set of drinking water samples collected from the Maine and New Hampshire region, were determined. This work made connections between changes in larval zebrafish behavior and the metals present in the water samples, and is discussed in detail in the following chapter.

CHAPTER 6
IDENTIFYING DRINKING WATER METAL CONTAMINANT MIXTURE RISK BY COUPLING ZEBRAFISH BEHAVIORAL ANALYSIS WITH CITIZEN SCIENCE

6.1 Introduction

Clean drinking water is at risk due to chemical contamination from both natural and anthropogenic sources. This is a pressing issue in Maine and New Hampshire. Approximately 40% of New Hampshire residents and 40-45% of Maine residents depend on private wells for their drinking water (Andy et al., 2017; Ayotte et al., 2017; Smith et al., 2016). Over one million people across both states are at risk for drinking well water containing more than 10 ppb of Arsenic (As), the maximum contaminant level set for public water systems by the U.S. Environmental Protection Agency (USEPA). Of particular concern, is a high risk of metal contamination in wells in these two states, due to the bedrock geology (Andy et al., 2017; Yang et al., 2009). Neighboring states such as Massachusetts, Vermont, and New Jersey also report high utilization of well water and are at increased risk of chemical contamination (Baris et al., 2016; Möller et al., 2009).

Arsenic contamination of drinking water is an important public health concern in New England as well as around the world. Arsenic is present within bedrock in As-sulfide complexes, and can be easily released into the groundwater supply under alkaline and reducing conditions (Bondu et al., 2017). Exposure to As has been linked to many diseases including lung, bladder, liver, and skin cancers as well as vascular and neurological disorders (Abdul et al., 2015; Hong et al., 2014; Steinmaus et al., 2014; Tyler & Allan, 2014). Studies have also linked prenatal As exposure to increased risk of stillbirth as well as increased As exposure to deficits in intellectual function in adolescents (Shih et al., 2017; Wasserman et al., 2018). Considering the adverse

outcomes of As exposure, the US EPA reestablished the maximum contaminant level (MCL) of As in drinking water from 50 ppb to 10 ppb in 2001, giving municipalities with public water systems until 2006 to adhere to the new standards.

In Maine and New Hampshire, As remains a critical concern (Stanton et al., 2015). According to a USGS survey of As in private wells in Maine from 2005 to 2009, the majority of towns contained wells with maximum As concentrations between 10 and 50 μg/L and some exceeding 500 μg/L (Nielson et al., 2010). A 252 household study in Maine showed that As levels in 1/3 of wells tested exceeded 10 μg/L . Notably, children in households that exceeded 5 μg/L of As were found to have significantly lower IQ scores (Wasserman et al., 2018).

In addition to As, individuals are exposed to other metal contaminants such as lead (Pb), uranium (U), and cadmium (Cd) in the drinking water (Chowdhury et al., 2016). Early Pb and Cd exposures have been associated with lower IQs and behavioral disorders in children (Hou et al., 2013; Kippler et al., 2012). Gestational exposure to U has shown modifications in locomotor activity and memory in rodent models (Dinocourt et al., 2015). The additive or interactive effects of exposure to multiple metal contaminants through the drinking water is beginning to be studied. Importantly, an increasing number of studies show that exposure to chemical mixtures with concentrations below MCLs have biological consequences (Beaver et al., 2017; Cobbina et al., 2015; Ramsey et al., 2013). While integrating mixture impacts in drinking water quality assessment is just beginning to emerge, this is not a consideration at the regulatory level.

Private well water is not mandated by the law to be tested and homeowners are responsible for their own tests. In Maine, many homeowners who have had their wells tested for contaminants in the past have not done so again (even though the recommended testing frequency is 3-5 years) and are often optimistic that their water is clean compared to their

neighbors (Flanagan, Marvinney, & Zheng, 2015). Even for individuals who are taking action, mitigation methods, such as using a portable water filter or installing a reverse osmosis system, are not always effective, resulting in a false sense of security that can lead to inadvertent metal contaminant exposure (Flanagan, Marvinney, Johnston, et al., 2015; Y. Zheng & Flanagan, 2017).

In vivo studies based on model organisms offer a relatively quick and controlled effect-based approach to screening for chemical mixture toxicity of drinking water contaminants, while providing a real-world example for citizen engagement. In particular, the zebrafish (*Danio rerio*), is a prominent high throughput toxicological model with a rapid embryonic development time (Nishimura et al., 2015; Ogungbemi et al., 2020; Selderslaghs et al., 2013). Although the zebrafish has been used to assess toxicity of chemicals and chemical mixtures, zebrafish embryos are just beginning to be utilized in deriving biological outcomes from actual drinking water samples (Babich, Ulrich, et al., 2020).

There are two limitations that are currently impeding the effectiveness of determining drinking water quality. The first that is particularly relevant to Maine and New Hampshire, is a lack of homeowner participation in both water testing and mitigation processes. The second, and a more globally prevalent issue is the lack of information related to metal mixture effects and the difficulty in communicating mixture effects to the broader public.

Here, given the association of lower-IQ levels in children with arsenic-contaminated drinking water in Maine and other demonstrated effects of arsenic on neurobiology, we sought to explore neurobehavioral effects of exposure to drinking water collected from wells in Maine and New Hampshire (Wasserman et al., 2018). To improve homeowner participation in well water testing and increase awareness of physiological effects of metal mixtures, we implemented a

citizen science – scientific partner approach, facilitated by teachers in Maine and New Hampshire, who educated and guided students to collect local well water samples for heavy metal analysis. We used these student-collected water samples in blind studies to test behavioral toxicity using the zebrafish model organism to provide a more comprehensive analysis of water quality and potential underlying low-level and mixture effects.

6.2 Methods

6.2.1 Outreach and Sample collection

Well water samples were collected as part of an NIGMS Science Education Partnership Award (SEPA) project called "Data to Action: A Secondary School-Based Citizen Science Project to Address Arsenic Contamination of Well Water. This collaborative project engages teachers and students from rural schools in Maine and New Hampshire as citizen scientists in collecting well water from their homes for arsenic analysis (Figure A7). Teachers are recruited through several partnerships and receive training alongside scientist partners.

Well water samples were collected by students from their homes or from neighboring homes or summer camps between spring 2019 and winter 2020. The sampling protocol is as follows: The cold water tap is run for five minutes, after which 50 mL of water is collected in a plastic conical 50 mL tube. The caps to the tubes are wrapped in parafilm in order to prevent leakage when being shipped out for analysis. A second 100 mL water sample is collected from the running tap in a 100 mL sample plastic jar for fish behavior analyses. These samples are frozen for 24 hours at the student's home to kill microorganisms that could set up biogeochemical cycles within the jar and change the speciation of the arsenic (X. Wang et al., 2012). Otherwise, the samples are brought into the classroom and frozen for 24 hours by the teacher, who makes note of the time between sampling and freezing. Students collect metadata,

often on a paper datasheet, which is later entered into the "All About Arsenic" project on the citizen science data portal Anecdata.org. The metadata include the collector's name, the student name, the location of the well, the type of well, whether the water was filtered and whether the filter was for the whole home or located at the tap. Information about previous testing is also collected. The name and address of the sampler and exact well location are not available in the publicly available dataset on Anecdata. However, disclosure forms are provided to families so that they can give permission for data to be shared with the Maine Center for Disease Control, New Hampshire Department of Environmental Services, and with scientists conducting research on the well water samples.

6.2.2 Water analysis

The Trace Element Analysis (TEA) laboratory at Dartmouth College conducts low-level trace metal analysis on student-collected well water samples collected by students using inductively coupled plasma mass spectrometry (ICP-MS) with a triple quadrupole Agilent 8900 (Santa Clara, CA) in helium and oxygen modes. The ICP-MS was calibrated using NIST-traceable standards and calibration was verified using second source standards after the calibration standard and every ten samples. The laboratory control solutions used were NIST 1640a and a USGS proficiency test reference sample. Analytical duplicate and spikes were analyzed at a frequency of one each per 20 samples. The samples were tested for the following metals: Arsenic (As), Antimony (Sb), Barium (Ba), Beryllium (Be), Cadmium (Cd), Chromium (Cr), Copper (Cu), Iron (Fe), Lead (Pb), Manganese (Mn), Nickel (Ni), Selenium (Se), Thallium (Tl), Uranium (U).

6.2.3 Zebrafish Exposure

Zebrafish embryos (AB Wildtype) were collected and incubated at 28.5°C in egg water (60 µg/mL Instant Ocean sea salt in deionized water) at 1 embryo/1 mL until 24 hours post fertilization (hpf) and screened for viability. 15 embryos, remaining in their chorion, were then moved to treatment solutions containing 7.5 mL egg water and 7.5 mL of a given well water sample (or 15 mL egg water for control exposure) at 28.5°C until 5 days post fertilization (dpf) in a 30 mL (diameter 6.5 cm, depth 2.5 cm) glass petri dish. 15 embryos were dosed in triplicate, collected from 3 different batches of eggs, per treatment solution. The 50% dilution was used because it was non-lethal but still produced alterations in behavior and allowed the conservation of limited water samples. Of the total 382 samples collected from Maine and New Hampshire, 92 samples were chosen randomly for the behavioral analysis.

6.2.4 Behavioral studies

Zebrafish larvae were analyzed at 5 dpf for mortality, chorion presence, and any obvious deformities. For a given sample treatment, 8 larvae were randomly selected and placed individually into wells containing 2 mL of egg water each in a 24 well plate. This process was repeated 3 times, yielding behavior data for 24 larvae. There were certain instances where exposure to well water resulted in significant mortality or hatching inhibition. Larvae that remained in their chorion were manually dechorinated and allowed to equilibrate before being plated in 24 well plates, larvae that exhibited minimal movement post-dechorination were not used. In groups with high mortality, extra embryos from the same exposure group were used to obtain a sufficient number of larvae for the behavior analysis. The mortality and hatching data are indicated in Figure 4.1 and Appendix Table A7.

For the behavior analysis, plates containing larvae were kept in a water bath inside Danio Vision (Noldus, Leesburg VA) to maintain temperature at 28.5°C throughout the run. The Ethovision (Noldus, Leesburg, VA), a software used in tandem with Danio Vision, was used for a light/dark test, which included a 5:00 min dark habituation period, 5:00 min light period, 5:00 min dark period, 5:00 min light period, and 5:00 min dark period (for a total of 25 minutes). After the run was completed, larvae were removed and euthanized with MS-222. All zebrafish research were carried out in accordance with ARRIVE guidelines and were approved by the University of Maine IACUC committee, proposal number A2017-05-04.

6.2.5 Statistical analysis

The distance traveled by each larvae was extrapolated from Ethovision software at 30 frames/second as mm moved per minute and summed over 25 minutes to determine the total distance (TD) traveled by a given larvae during the experiment. Averages of TD over 25 minutes from each larvae were calculated for each treatment group. Prior to statistical analysis, TD data were checked for normal distribution (Shapiro-Wilk), homogeneity of variance (Levenes test), and outliers in SPSS software (IBM, Armonk, NY). To test for statistical significance between TD across sample treatment groups, a one-way analysis of variance (ANOVA) and Tukey post-hoc was conducted using GraphPad Software (Prism, San Diego, CA). This test designated control-like, hyperactive, and hypoactive behavioral groups. Subsequently, ANOVA and Tukey post-hoc was used to test for significance in mortality and chorion presence of samples associated with hypo/hyper/control-like activity relative to egg water controls. Here hypoactive is defined as a sample that results in a significant decrease in TD relative to egg water controls, hyperactive is defined as a sample that results in a significant increase in TD relative to egg

water controls, and control-like is defined as a sample that resulted in no significant change in TD relative to egg water controls.

To determine the potential for one chemical contaminant to drive the behavioral effects detected following exposure to a given well water sample, a simple correlation analysis was conducted where total distance traveled was plotted against the concentration of a given chemical of interest (Figure 6.2, Figure A8). Given previous behavior toxicity studies, we focused on examining a correlation between As, Cd, Pb, and U concentration and larval behavior (Figure A9).

To evaluate metal mixture effects, we conducted a principal component analysis (PCA) using SPSS software (IBM, Armonk, NY). The PCA generates 2 principal components (PC1, PC2) for each of the 92 well water samples based on their chemical composition, which was inserted into the PCA as raw concentration values in μg/L. The PCA can be performed based on all 12 chemical composition data or user picked combination of chemicals (e.g., PCA can be conducted based only on As, Cd, and Pb concentration data to determine principal components for all 92 wells). Given there are 12 metals, numerous combinations of metal concentration data, can be integrated into the PCA. To determine the number of combinations of metals (i.e. different permutations of As, Sb, Ba, Cd, Cr, Cu, Fe, Pb, Mn, Ni, Se, and U combinations, from a mixture containing all 12 down to that of three metals remaining in each combination) $\Sigma\, n! / r! (n-r)!$ [Eq 1] equation was used. Here, n = 12 and r is all integers between and including 11 and 3, resulting in a total of 4,016 combinations without repetition. (Although Be and Tl levels were determined in the metal analysis, they were excluded in subsequent mixture analyses, since they were undetected in the majority of well samples tested). PC1 and PC2 for all possible 4,016 combinations were generated.

PC1 and PC2 for each of the 92 samples were then plotted for each of the 4,016 combinations. Each of the 92 samples within a PCA plot was then overlayed with behavioral data (i.e. designated as hyperactive, hypoactive, or control-like behavior). This allowed for visualizing clusters of behavior among the 92 sample treatments, based on the metals and their corresponding concentrations for a given metal input combination. Subsequently, we focused on metal combinations that resulted in distinct clustering between groups of control-like and hypoactive associated samples or control-like and hyperactive associated samples.

To reduce ambiguity in determining different clusters, we utilized an approach as demonstrated by Goodpaster & Kennedy, 2011 to identify statistically significant clusters between different behavior response groups for a given metal combination and repeated it for all 4,016 possible metal combinations. Briefly, PC1 and PC2 were used to generate the Mahalanobis distance (MAH) between groups based upon activity level, e.g., MAH between control-like cluster and hypoactive cluster. Using Excel (Microsoft, Redmond, WA) the MAH was derived from $\sqrt{d'C_w^{-1}d}$, where $d'_{average} = PC1_{(control\text{-}like)} - PC1_{(hypoactive)}$ and $d_{average} = PC2_{(control\text{-}like)} - PC2_{(hypoactive)}$. C_w^{-1} is the inverse of the pooled variance and covariance matrices between control-like and hypoactive groups. The MAH was then used to determine the Hotteling's two-sample T^2 statistic using $T^2 = (n_1n_2)/(n_1+n_2)(MAH)$. Upon calculation of the T^2 statistic, an F-value = $(n_1+n_2-p-1)/p(n_1+n_2-2)T^2$, could be calculated and used in the F-test. Here, n_1 = # of well samples associated with control-like activity, and n_2 = # of well samples associated with hypoactivity, and $p = 2$ (comparison between 2 clusters). The F-test was then used to determine significance between 2 activity clusters generated via PCA derived principal components. The clusters were considered significantly different if the calculated F-value was greater than the critical F-value as determined by a web-based software

(https://www.danielsoper.com/statcalc/calculator.aspx?id=4). The F-critical value for control vs hypo and hyperactive clusters was calculated to be 5.81 and 5.79 respectively, with an alpha level of 0.005.

Statistically significant clusters of control-like and hypoactive or control-like and hyperactive groups were determined for all 4,016 metal combinations. From this, we generated two subsets of combinations, those combinations that resulted in significantly different clusters between control-like and hypoactive behavior and control-like and hyperactive behavior. For each subset, the number of times an individual metal appeared within a combination was calculated. This was conducted to determine the prevalence of metals that resulted in distinct hypoactive and hyperactive clusters compared to control-like clusters.

Further, to identify if 2 or 3 metals consistently appeared together within metal combinations of the 2 subsets, similar to earlier, the number of permutations to be tested were first calculated using $\Sigma\ (n! / r!\ (n-r)!)$. Here, n = 12 and r is 2 or 3. This resulted in 286 tri and bipartite combinations, which were subsequently identified within combinations comprising each subset.

6.3 Results

6.3.1 Metal analysis

Metal analysis revealed a heterogenous metal composition across the well samples based on the 14 chemical panel that was tested . Corresponding concentrations per each metal from each sample can be found in the appendix (Table A7 and Table A8).

Given previous data on zebrafish behavioral effects of exposure to As, Pb, Cd, and U (Figure A9), first, we focused on levels of these metals in our well water samples (Figure 6.1). The minimum and maximum As concentrations found among the 92 samples were 0 and 717.9

µg/L respectively with a median of 0.52 µg/L. Eight samples (487, 490, 501, 514, 515, 895, 950, 1238) exceeded the MCL of 10 µg/L (Table SI). The maximum level of Cd was 0.41 µg/L from sample 508, with a minimum of 0 and median of 0.007 µg/L. The maximum level of Pb was 20.1 µg/L from sample 907, and was the only sample to exceed the lead MCL of 15 µg/L. The minimum concentration was 0.01 µg/L and the median was 0.36 µg/L (Table A7). Uranium concentrations ranged from 0 to 3274.37 µg/L with a median of 1.0 µg/L. Twelve of the 92 samples exceeded the MCL for uranium of 30 µg/L (Table A7), while 21 out of the 92 samples contained either As, Pb, or U exceeding the MCLs. The remaining 77% of samples contained levels of As, U, Pb, and Cd considered safe in drinking water (Figure 6.1).

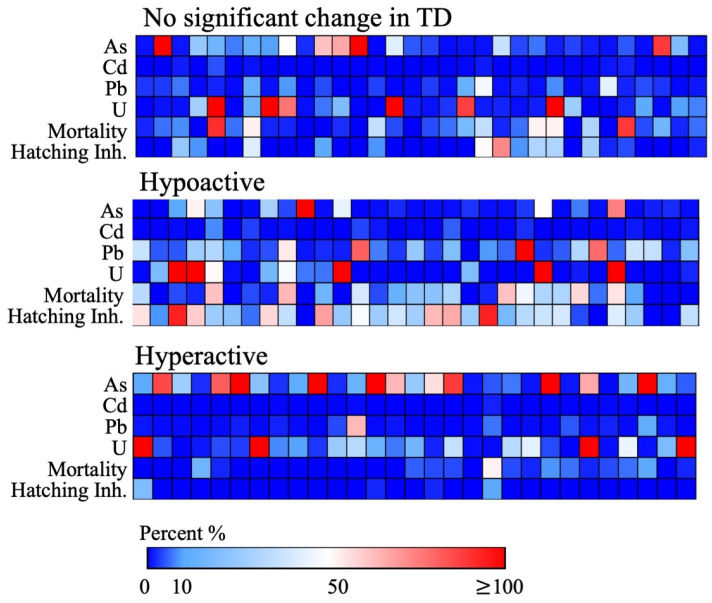


Figure 6.1: Metal concentrations in water samples collected from ME and NH. Heat map, representing concentration of a given metal as a percent of its maximum contaminant level (MCL) in a drinking water sample; MCLs for arsenic (As) – 10 ppb, cadmium (Cd) – 5 ppb, lead (Pb) -15 ppb, and uranium (U) - 30 ppb. Percent mortality and hatching inhibition at 5 dpf are also included. Samples are further categorized into exposures that resulted in no significant change in larval total distance (TD) traveled, significant hypoactivity, and significant hyperactivity relative to egg water controls. Blue colors indicate a lower percentage and reds indicate a higher percentage.

4.5. Behavioral toxicity – individual contaminant effects

Zebrafish exposed to a 1:1 well water sample to egg water solution resulted in altered TD traveled over a 25 minute light dark test period in 60 of the 92 wells tested. Light dark tests have been used extensively in toxicity studies to estimate behavioral effects of contaminant exposure (J. Bailey et al., 2013; Basnet et al., 2019; Pitt et al., 2018). 31 samples induced a significant decrease in activity and 29 induced a significant increase in activity relative to TD traveled of embryos reared in egg water. 32 of the 92 samples showed no significant change in TD compared to egg water controls (Table A7).

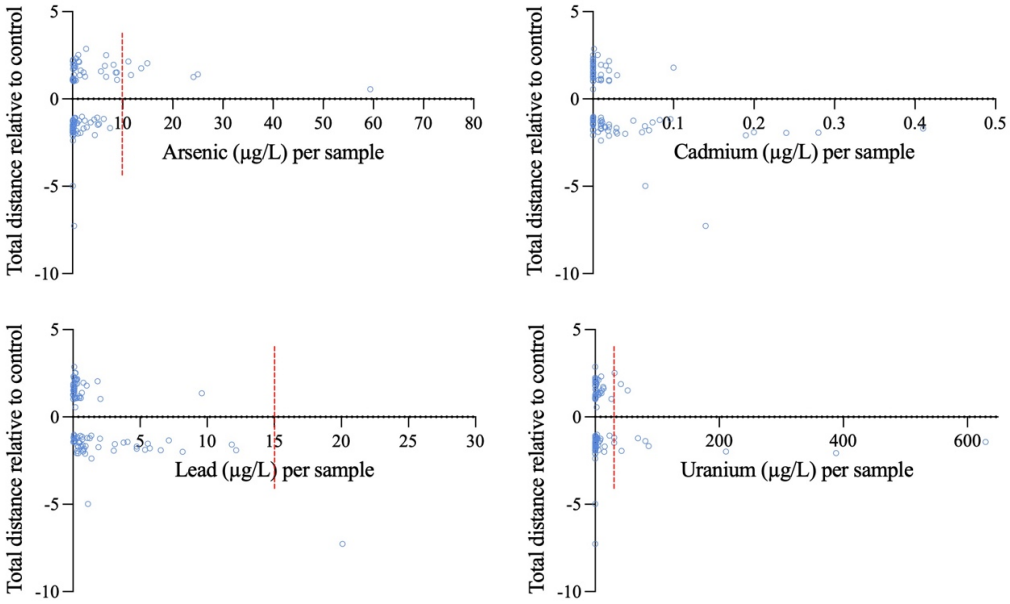


Figure 6.2: Comparison of changes in zebrafish behavior and metal concentrations. Dot plot representing total distance after a 1:1 sample / egg water exposure. Data is shown in the form of fold change difference between treatment relative to 100% egg water control against the amount of arsenic (µg/L), cadmium (µg/L), lead (µg/L), and uranium (µg/L) present in a given sample. Dashed red line represents current EPA maximum contaminant levels in drinking water. Samples that induced significant hyper or hypoactivity can be found in Supplemental Table SI, *p-value* < 0.05, ANOVA, n=24.

To examine potential correlations between known behavior toxicants, individual chemical concentrations of As, Pb, Cd, and U were plotted against TD as a percent of egg water controls (Figure 6.2). Eight out of the 92 samples tested contained As above the MCL of 10

µg/L, five of which (11.2, 11.7, 13.7, 14.9 and 25.0 µg/L,) were associated with samples that induced hyperactivity (Figure 6.2). Sample 187, with the highest concentration of As, at 717.9 µg/L did not significantly change TD traveled compared to controls. Additionally, from 10 of the samples containing arsenic levels between 5 -10 µg/L, four were associated with hyperactivity. Low-levels of As in the remaining samples associated with hyperactivity ranged from 0.05 – 2.7 µg/L, the highest concentration of As in all 33 samples associated with hypoactivity was 59.4 µg/L. All other samples associated with hypoactivity contained a range of As from 0 – 7.4 µg/L (Figure 6.2, Table A7).

Cadmium was low among all samples regardless of the associated activity level. The maximum concentration of Cd detected was 0.41 µg/L and was associated with a sample that produced hypoactivity. Well water samples with Cd ranging from 0.14 – 0.28 µg/L, aside from one sample that produced control-like behavior at 0.24 µg/L, were associated with hypoactivity, suggesting a potential connection between Cd exposure and decreased behavior (Figure 6.2).

One well sample contained Pb over the MCL (20.1 µg/L). Including this sample, a total of ten samples contained Pb over 5 µg/L, and seven were associated with hypoactivity (Figure 6.2). The remaining 24 samples associated with hypoactivity had a range of 0.03 – 4.75 µg/L Pb. The largest concentration of Pb found in those samples producing hyperactivity was 9.6 µg/L with a range of 0.01 – 1.8 µg/L for the remaining (Table A7).

Presence of U in the drinking water was also associated with behavioral effects (Figure 6.2). Thirteen (30.42 µg/L – 3274.4 µg/L) of the samples exceeded the MCL for U of 30 µg/L, and five (30.42 – 629.4 µg/L) were associated with hypoactivity in zebrafish. The highest concentration of U was found in a sample associated with hyperactivity at 3274.4 µg/L along with 4 other samples containing concentrations over the MCL (31.1, 41.5, 52.5, and 3274.3

µg/L). Control-like behavior also contained 4 samples exceeding the MCL (30.4, 42.6, 69.1, and 81.0 µg/L). 16 samples contained U over 5 µg/L, these included 4 producing hypoactivity (5.6 – 15.4 µg/L), 7 producing hyperactivity (5.5 – 13.6 µg/L), and 5 producing control-like behavior (5.6 – 26.3 µg/L). The remaining concentrations among all 3 activity levels collectively ranged from 0.01 – 4.9 µg/L (Table A7).

Overall, individual As, Pb, Cd, and U metal levels did not explain behavioral effects detected following exposure to a given well water sample, suggesting a potential role for interactive effects of multiple metals.

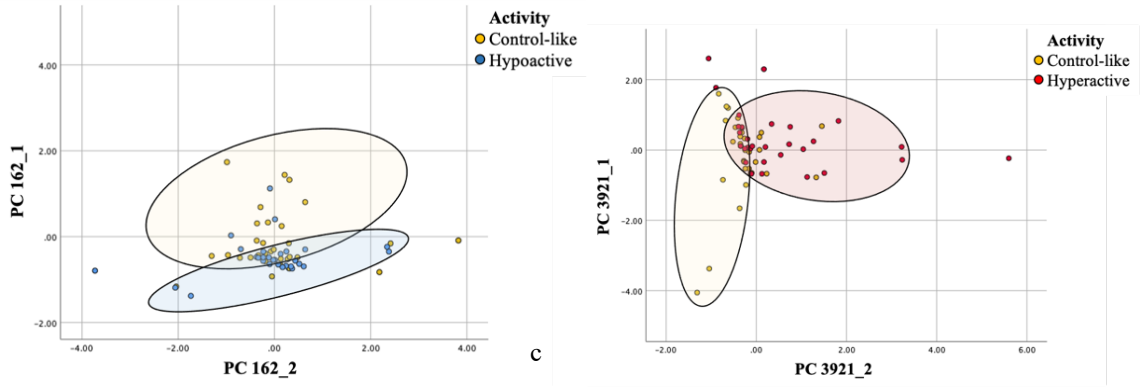


Figure 6.3: Impacts of exposure to mixtures in water samples on zebrafish behavior. Examples of principal component analysis (PCA) derived scatter plots that show significant clustering between control-like and hypoactive and control-like and hyperactive behaviors from metal combination inputs of Cr, Mn, Fe, Se, Cd, Sb, Ba, Pb, & U (combination 162) and Fe, Ni, Cu, Se, Pb, & U (combination 3921) respectively.

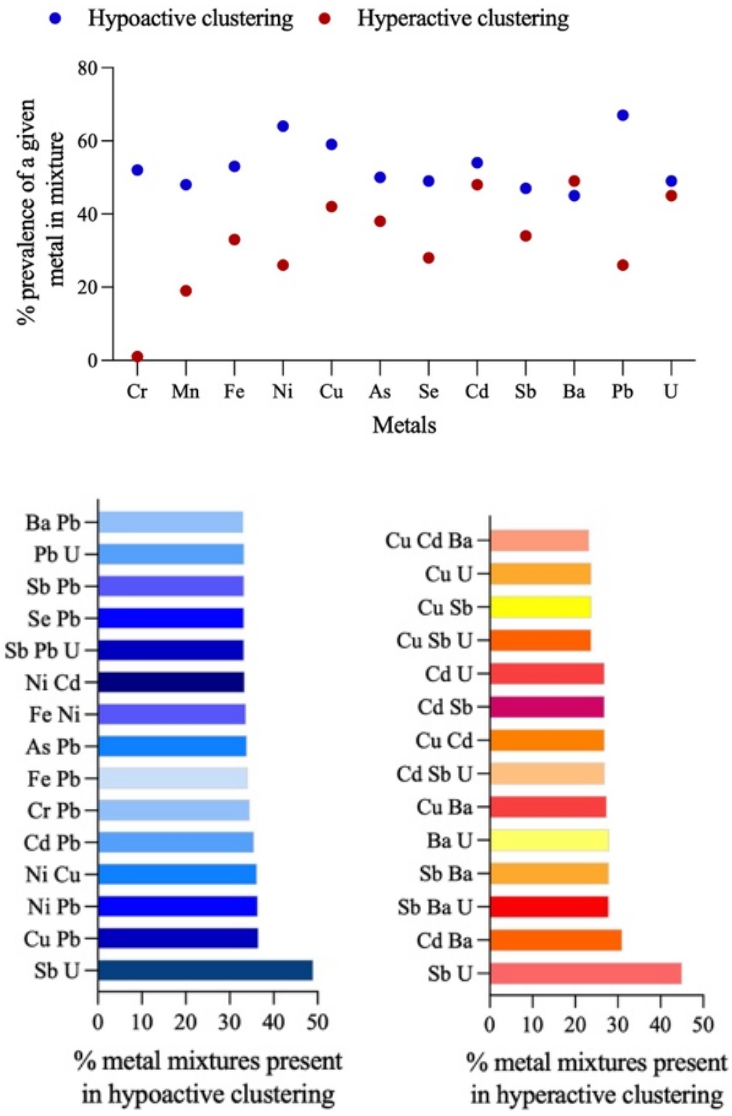


Figure 6.3 Continued: Dot plot representing the percent prevalence of a given metal in mixtures that resulted in significant clustering between control-like and hyperactive (red) as well as control-like and hypoactive (blue) behavior. Bar graphs representing the top 15 combinations of two or three metals within mixture subsets that resulted in significant clustering between control-like and hypoactive and control-like and hyperactive behavior. Clusters were created via PCA and an F-statistic was calculated to determine significance, p-value < 0.005.

6.3.3 Behavioral toxicity – mixture effects

Principal component analysis was used to extrapolate a total of 3,982 principal component pairs derived from 4,016 chemical combinations as inputs (the statistical software was unable to calculate PC pairs from 34 combinations). Behavioral activity was overlayed on top of principal components to determine if activity level clustered based upon what chemicals and their corresponding concentrations were present in the water samples. Examples of PCA derived scatter plots from a combination of Cr, Mn, Fe, Se, Cd, Sb, Ba, Pb, & U and Fe, Ni, Cu, Se, Pb, & U, that resulted in significant clustering between control-like and hypoactive and control-like and hyperactive behavior respectively, are provided in Figure 6.3. A subset of 2,777, out of the 3,982 tested, metal mixtures resulted in distinct clusters of samples that produced control-like behavior and hypoactivity. Metals that appeared the most within the 2,777 combinations included Pb (67%), Ni (64%), Cu (59%), and Cd (54%) (Figure 6.3). A second subset of 193 metal combinations, resulted in distinct clusters of samples that produced control-like behavior and hyperactivity. Ba, Cd, U, and Cu were present in 49%, 48%, 45%, and 42% of the 193 combinations, respectively (Figure 6.3).

Bipartite and tripartite combinations, containing either 2 or 3 metals, (286 total combinations) were also checked for frequency among the subsets of metal combinations that resulted in significant clustering between control-like samples and those that produced hypo or hyperactivity. The top bipartite combination for both activity trends was Sb and U which was present in 49% of hypoactive and 45% for hyperactive associated subsets (Figure 6.3). Six of the top 15 bipartite and tripartite combinations associated with hyperactivity contained Cd, and 10 of the 15 associated with hypoactivity contained Pb (Figure 6.3).

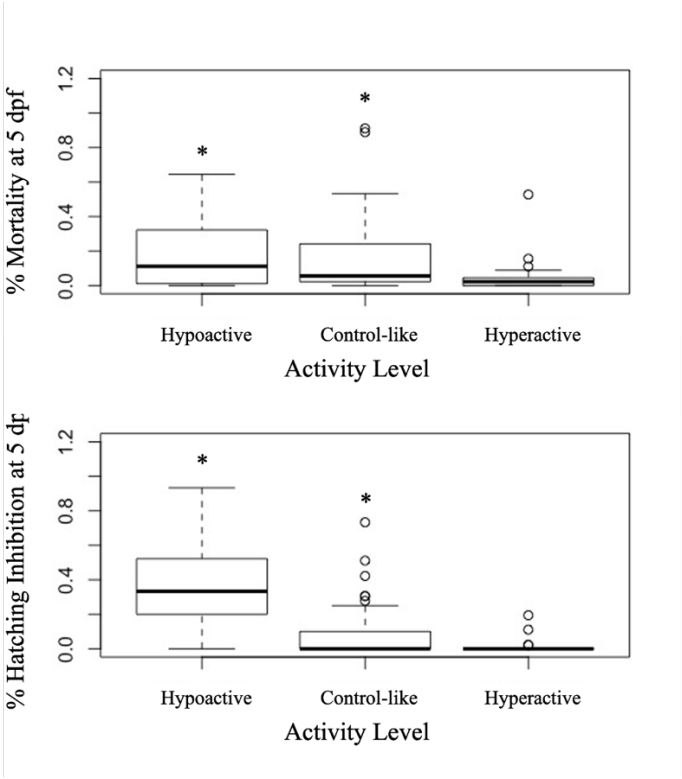


Figure 6.4: Association of zebrafish activity level, morphology, and mortality. Box plot representing % mortality and % hatching inhibition at 5dpf after a 1:1 sample / egg water exposure relative to 100% egg water control. Data is plotted based upon samples that significantly induced hypoactivity, hyperactivity, or no significance from egg water controls. Additional information regarding specific samples that induced significant hyper or hypoactivity can be found in Table S1, *p-value* < 0.05, ANOVA, n=24.

Samples associated with hypoactivity and control-like behaviors had a significant increase in % mortality (20%, 17%) and hatching inhibition (35%, 10%) at 5 dpf relative to egg water controls (1.7%, 0%, Figure 4.4). There was no significant difference in mortality and hatching inhibition in those samples associated with hyperactivity (4.6%, 1.2%) compared to egg water controls (Figure 6.1, Figure 6.4).

6.4 Discussion

In this study, we present a zebrafish-based toxicity analysis in determining behavioral effects of exposure to metal mixtures in the drinking water. The study was significantly enhanced by utilizing a citizen science approach that enabled collection of a large set of well

water samples from families in Maine and New Hampshire. Teachers and students involved in this study gained knowledge in the following areas: health risks due to metal exposure, methods for sampling well water, submitting metadata associated with the well water sample, analyzing the data using a data literacy software called Tuva, and public outreach about the well water results. In this way, we are sharing knowledge and preparing future citizens so that they may generate community awareness around water quality and demand better water quality monitoring standards.

This approach increased our likelihood of obtaining an increased number of well water samples across Maine and New Hampshire, while engaging local teachers and students to discuss the importance of clean drinking water. With our set of 92 samples, we show that although samples may be considered safe with most chemicals at concentrations below the MCLs, there are still potential adverse behavioral impacts. These results were shared with teachers in an annual training workshop. We developed an analogy-based video on PCA analysis for teachers and engaged them in an interactive discussion of our research results.

The results of our study demonstrate that adverse biological outcomes of environmental exposures, such as the behavioral effects detected in the current study, can result from exposure to multiple chemicals, even when individual chemicals are at low to moderate levels. Additionally, a subset of samples do not have an impact on behavior, as seen in our control-like group, which may be due opposing modes of action of metal contaminants and further highlights the role of mixture and concentration specific effects (Ogungbemi et al., 2021). Our results are consistent with other emerging studies, including in zebrafish, which show chemical mixture effects on development as well as behavior (Jijie et al., 2020; Valeri et al., 2017).

Our cluster analysis supports the hypothesis that a single chemical is not driving the changes in zebrafish activity levels, but rather a result of a mixture effect (Figure 6.3). By utilizing PCA we demonstrate that distinct clusters of samples that are associated with hypo or hyperactivity still occur, regardless if the metals within the mixtures exceed the MCL. Interestingly, Cd is in the top four metals that are present within both hypo and hyperactive clusters when compared to control-like behavior. In these instances, Cd concentrations remain well below the MCL of 5 ppb (Figure 4.3). Cd has been shown to be toxic when in mixture; for example, in *C. elegans* toxicity increased when exposed to Cd and Cu in tandem (Moyson et al., 2018). Although concentrations of Cd are lower than the MCL in our well water samples, Cd is in 7 / 15 of the top bipartite and tripartite combinations present in the subset of metal combinations associated with distinct hyperactive clustering, one of which is Cd and Cu (Figure 6.3).

Along with Cd, Pb was found to be present in 67% of the subset of mixtures that resulted in significantly different clusters between hypoactive and control-like behavior (Figure 6.3). Lead was also present in 10 / 15 most prevalent bipartite and tripartite combinations associated with distinct hypoactive clustering (Figure 6.3). However, both epidemiological and rodent studies suggest that Pb is more likely to cause hyperactivity at levels of 14.3 μg/L in cord blood and 50 mg/L in drinking water (Mansouri et al., 2012; Sioen et al., 2013). Interestingly, a study subjecting maternal zebrafish to 20 μg/L Pb found that larval offspring in the next generation also showed hyperactivity relative to controls. However, when the maternal generation was exposed to a mixture of 20 μg/L Pb and 5 μg/L crude oil, TD traveled by larval offspring significantly decreased, suggesting that transgenerational behavioral effects of Pb are mixture specific (Y. Wang et al., 2016). The concentrations examined in these studies are higher than

what was detected in Maine and New Hampshire well water, suggesting that possible interactions with other metals and potential chemicals in the water sample can alter zebrafish behavior.

Uranium was also present in the top four metals that appear in 45% of the subset of mixtures that resulted in significantly different clusters between hyperactive samples verses control-like (Figure 6.3). Uranium, in combination with antimony, is the most prevalent bipartite combination among both hyperactive and hypoactive associated mixture subsets. It is also present in 7 / 15 top bipartite and tripartite combinations present in the subset of mixtures responsible for hyperactive clustering (Figure 6.3). Specifically, in the zebrafish model, U at concentrations of 20 and 250 μg/L has been found to significantly delay hatching as well as decrease larval body length compared to controls (Bourrachot et al., 2008). In addition, U exposure at 100 μg/L has been linked to muscle tissue disruption in adult zebrafish in the form of degenerated myofibrils and abnormal mitochondrial localization (Barillet et al., 2010). A 10 day U exposure at 250 μg/L has also been shown to damage adult male zebrafish sensory organs, specifically the lateral line and olfactory systems(Faucher et al., 2012). Although more studies are needed on the impacts of U on development along with behavioral consequences, these data from previous studies may explain the changes in activity associated with U seen in this study.

Arsenic, which is a relevant metal of concern in New England, did not show any obvious trends regarding concentration or presence in samples associated with hyperactivity, hypoactivity, or control-like behavior (Figure 6.2). However, when determining prevalence in mixtures accounting for distinct clusters, As was present in 50% and 38% of mixtures associated with hypo and hyperactivity respectively. It was also higher in prevalence over 5 (Mn, Se, Sb, Ba, U) and 7 (Mn, Fe, Ni, Se, Sb, Pb) other metals in the panel for hypo and hyperactivity

associated mixture subsets. Arsenic that was present in mixtures was found at low levels, with the majority of concentrations found below the MCL of 10 ppb. These data, in combination with previous epidemiological and model organism studies, emphasizes the need to address the potential toxicity of low levels of As, both individually and in mixture, especially in the contexts of drinking water. For example, it was found that women who have had trouble conceiving in the past, that were exposed to As at 1 μg/L in their drinking water had a significant decrease in likelihoods for pregnancy (Susko et al., 2017). Another study showed that a median of 1.2 μg/L As in maternal drinking water correlated with decreased birth weight in smaller infants (Rahman et al., 2017). Further, it was found that zebrafish exposed to environmentally relevant levels of As (50 and 500 ppb) caused a more severe change in activity when in a zinc-deficient mixture compared to those supplemented with zinc, suggesting that As mediated toxicity is highly dependent upon the presence or absence of other chemicals (Beaver et al., 2017). Given that we show As is an important factor in mixture toxicity, even at levels ranging from 0.03 – 5.1 μg/L, low levels of As, between 1 and 5 μg/L, should be considered when determining water quality standards.

It should also be observed that the prevalence of some metals, in the subsets of mixtures resulting in distinct hypo or hyperactive clusters, change dramatically based upon activity level. For example, Cr is seen in 52% of mixtures associated with hypoactivity and 1% of mixtures associated with hyperactivity. Pb and Ni are also found in many of the mixtures associated with hypoactivity (67% and 64%) compared to hyperactivity (26% and 26%). This supports the notion that biological endpoints are highly sensitive to metal mixtures and their corresponding concentrations and that the mechanism of action can potentially change given what is present in a mixture.

There is a clear link between activity, mortality, and hatching inhibition at 5 dpf. Mortality, as well as hatching inhibition, after exposure to samples that induced hypoactivity significantly increased compared to egg water controls and those samples that resulted in hyperactivity (Figure 6.4). This phenotypic response is indicative that other developmental consequences may be occurring in response to mixture exposure besides neurobehavioral effects. Additionally, changes in phenotype may be contributing to changes seen in behavior (Ogungbemi et al., 2020). Broadly, our findings indicate a necessity to consider potential developmental impacts of all metal contaminants in drinking water supplies, even at levels considered safe for human consumption.

The zebrafish has been used to test toxicity of many environmental contaminants, such as As and U, during early development (Ali et al., 2012; Armant et al., 2017; Babich & Van Beneden, 2019). It has also been a model, alongside rodent species, for evaluating neurotoxic effects and later in life consequences of heavy metals (Sonnack et al., 2015; Torrente et al., 2002). While zebrafish are established as a relevant toxicological model certain parameters such as exposure time and route of exposure during embryogenesis are variable compared to human fetal exposure and should be taken into consideration when drawing conclusions (Bambino & Chu, 2017). Nonetheless, we believe data collected using the zebrafish model is highly informative and research combining water chemistry and epidemiological studies followed with multivariate analyses, such as described here, can provide key insights into adverse health outcomes of chemical mixture exposure.

Through complementing water analyses with behavioral outcomes we have shown here that chemicals such as As, Cd, Pb, and U, even at low-levels, should be taken into special consideration when evaluating results from regular water testing. More importantly, we

emphasize that other metals that are not typically associated with adverse effects (e.g., Ni and Sb) should be considered in risk assessment when in mixture with chemicals such as Pb and U. Determining safe levels of these chemicals as well as potential mixture effects warrant further study and should be examined when implementing federal regulations. Our study has merged citizen science and toxicology research lending itself to the development of improved testing and assessment methodologies, and to better inform households in Maine and New Hampshire, as well as policy makers, of their local quality of water. Through our synergistic efforts with local schools, we are able to inform teachers and students about new ways to assess water quality and generate engaging and in-depth discussion on chemical mixture effects through fish behavior visualizations following well water exposure.

CHAPTER 7
CONCLUSIONS

Collectively, this work provides the foundation for understanding chemical mixture effects on zebrafish development with a specific focus on the role of mitochondrial function. Here, mitochondria were subjected to a variety of anthropogenic chemicals at varying concentrations and unique responses, such as recovery, mitohormesis and non-monotonic dose responses, were identified. When a subset of chemicals both individually and in mixture (As, Cd, V, Pb, and Gly), that are often found in drinking water were explored, impacts on eOCR were mixture specific and trends among mitochondrial eOCR parameters were observed. Specifically, small (2) and complex mixtures (4+), appeared to have a dampening effect on eOCR parameters where mixtures of 3 or 4 chemicals often had a mitohormetic effect, increasing eOCR. This suggests an overall non-monotonic mitochondrial eOCR response to the number of contaminants in a mixture, and that even at low levels overall chemical burdens will alter an organisms cellular respiratory capacity. There is also the potential that, with an increasing chemical burden, additive or synergistic effects of more complex mixtures are occurring.

The non-monotonic response of variations in chemical burden was also observed in the CKDu case study when exploring mitochondrial eOCR after exposure to lab mixtures and endemic region well samples (where the chemical burden is likely very high).

To understand underlying mechanisms of developmental mixture toxicity, and the potential role of mitochondria, RNA seq was explored. Here, mixtures of glyphosate & metals compared to metals alone, shared biological function terms related to the ECM. Given the importance of the ECM to cell structure, this supports our preliminary histopathology results, that mixtures have an impact on kidney tissue morphology. Upon further investigation, gly &

metals uniquely had terms related to the ECM components, where terms associated with transcription regulation were unique to metal mixture exposure. This differentiation is distinct, and suggests a divergence in responses to metal mixtures with (phenotypic) and without glyphosate (adaptive). However, it is interesting to note that the same mixtures, although with slight variability in concentration and timing of exposure, do not produce the same substantial difference in mitochondrial eOCR.

Therefore, it speculated that complex metal and organic mixtures, at low levels, alter mitochondrial function, which may subtly alter the abundance of ROS. Given that, especially during early development, ROS is involved in cellular signaling and regulating gene expression, it may be responsible for the alterations in gene expression associated with ECM pathways that are shared by exposure to metals (+ / - glyphosate). Metals are known to interact directly with DNA, modify histones, and alter expression of proteins responsible for methylation. Given that glyphosate is a known metal chelator, it is possible that glyphosate is forming complexes with metals such as As, so that they may not act on DNA (and resulting changes in transcript levels). Therefore, early changes in gene expression may be more sensitive to changes in complex mixtures, and slight changes in ROS generation through altered mitochondrial function, may also serve to regulate gene expression pathways. The dysregulation of the ECM, along with a chronic low-dose exposure to mixtures, could exceed the mitochondria's capacity to respond to stressors (as seen in the pre-exposure and acutes stressor study), which may ultimately result in an accumulation of ROS, cellular apoptosis, tissue degeneration, and disease.

Additional studies to confirm these hypotheses may include isolated mitochondrial OCR functional assays after exposure to mixtures. This would confirm that the inclusion of glyphosate does not alter known metal impacts on ETC complexes, and would also shed light if there were

any additive or synergistic effects on specific ETC complexes that could not be captured in the current eOCR assays explored. Here, the gene set for kidney was differentially expressed compared to controls under mixture exposure. A grow out study, beginning with a developmental exposure to low level mixtures, and subsequent kidney tissue exploration as adults as well as kidney organ mitochondrial function would confirm developmental alterations to kidney tissue function and integrity. Given that ROS is known to contribute to tissue pathology and disease, monitoring lipid peroxidation and the presence of antioxidants would provide additional insights into the long-term consequences of developmental exposures.

This body of work also provided valuable information on glyphosate and vanadium toxicity, both of which are just beginning to be explored in scientific literature. Here, we show that glyphosate, when in mixture with chemicals, not only results in altered mitochondrial toxicity profiles but also alters gene expression as well. We have also uniquely identified that, on it's own, glyphosate impacts expression levels of genes associated with development of the central nervous system. This is a critical finding and should be highlighted for women and young children in agricultural communities.

Vanadium was found to show similar mitochondrial toxicity both alone and when in mixture, to that of Pb. No level of Pb is considered safe as Pb is known to have severe neurological impacts and impede mitochondrial function. This body of work also highlights, that Pb (in particular) in mixture may be a driver of behavioral changes seen in the zebrafish model. Given the similarities on mitochondrial function observed between V and Pb, as well as the high concentration of V found in CKDu endemic region wells, V may play an important role in developmental mixture toxicity and disease progression and warrants further exploration.

This work highlights the importance of equitable access to clean drinking water, contributes to the hypothesis of developmental origins of health and disease, and provides evidence to encourage consideration of chemical mixture effects when determining drinking water standards.

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APPENDIX
SUPPLEMENTAL MATERIAL FOR A GREATER UNDERSTANDING OF FINDINGS FROM THE CURRENT STUDY

Table A1: Chemicals, manufacturer, concentrations used in the study, and literature review for concentrations used and known mitochondrial effects of a given chemical.

Chemicals added to egg water (μM) *Initial chemical stock solutions were diluted in 100% DMSO					Literature search for chemical concentrations	Literature search for mitochondrial effects
Amiodarone Hydrochloride (Sigma, 19774824)	1	0.1	0.01	0.001	(Jomaa et al. 2014)	Uncoupler (Spaniol et al. 2001)
Arsenic (Sigma, S7400)	6.67	0.667	0.133	0.026	(Carlson and Van Beneden 2014)	Disrupts MMP, (Y. Wang et al. 2009)
Azoxystrobin (Sigma, 131860338)	12.39	8.67	4.96	1.24	U.S EPA. 1997. Pesticide fact sheet	Inhibits complex III, (Gao et al. 2014)
BDE-47 (Sigma, 5436431)	20.6	2.058	0.205	0.0205	(Tanaka et al. 2018)	
Benzo(A)pyrene (Sigma, 50328)	0.079	0.0079	0.00079	0.000079	(Hoffmann and Oris 2006)	
Ezetimibe (Sigma, 163222331)	50	5	0.5	0.05	(Baek et al. 2012)	
FCCP (Sigma, 370865)	0.15	0.05	0.025	0.0125	(Conlin et al. 2018)	Mitochondrial uncoupler, (To et al. 2010)
Fenpyroximate (Sigma, 134098616)	0.4	0.2	0.1	0.05	(McCollum et al. 2017)	Complex 1 inhibitor, (Nakamaru-Ogiso et al. 2003)

Table A1 Continued

Fenazaquin (Sigma, 120928098)	65.27	16.32	1.63	0.163	(Alinejad, Kheradmand, and Fathipour 2014)	Complex 1 inhibitor, (Höllerhage et al. 2009)
Fluoranthene (Sigma, 206440)	0.741	0.148	0.029	0.005	Ohio EPA. 2006. Surface water quality criterion fact sheet	
Naproxen Sodium (Sigma, 26159342)	4.34	0.434	0.043	0.004	(Dhir, Naidu, and Kulkarni 2005)	
Nitrobenzene (Sigma, 98953)	406.1	40.61	4.061	0.406	US EPA. 1995. OPPT chemical fact sheet.	
Oligomycin (Sigma, 75351)	1	0.5	0.25	0.125	Agilent seahorse cell mito stress kit. User guide kit 103015-100	Inhibits mitochondrial ATP synthase, (Devenish et al. 2000)
Oxybenzone (Sigma, 131577)	17.53	1.753	0.1753	0.0175	(Blüthgen, Zucchi, and Fent 2012)	
PFOA (Sigma, 335671)	0.00169	0.00017	0.000017	0.000008	U.S EPA. 2016. Fact sheet	
PFOS (Sigma, 77283)	0.00139	0.00014	0.000014	0.000007	U.S EPA. 2016. Fact sheet	Decreased MMP (X. Wang et al. 2013)
Pyraclostrobin (Sigma, 175013180)	0.052	0.010	0.002	0.0004	(Levina et al. 2012)	Inhibits ETC complex III, (Luz et al. 2018)

Table A1 Continued

Pyridaben (Sigma, 96489713)	0.004	0.0008	0.0002	0.00003	Pesticide Action Network	Inhibits complex I, (Navarro et al. 2010)
Rosiglitazone (Sigma, 122320734)	10	1	0.1	0.01	(Tingaud-Sequeira, Ouadah, and Babin 2011)	
Rotenone (Sigma, 557369)	0.0126	0.0013	0.00013	0.00001	(Melo et al. 2015)	Inhibits complex I, (Li et al. 2003)
Sodium Azide (Sigma, 26628228)	0.0154	0.0015	0.0002	0.00002	(Yuwen et al. 2000)	Inhibits complex IV, (Bennett et al. 1996)
TDCPP (Sigma, 13674878)	1	0.1	0.01	0.001	(X. Wang et al. 2013)	
Toxaphene (Supelco, N13586)	48.57	24.28	12.14	2.428	Toxicological profile for toxaphene, ATSDR	
Tributyltin (Sigma, 7486353)	0.1	0.01	0.001	0.0001	(Liang et al. 2017)	Decreases MMP, (Nishikimi et al. 2001)
Trifloxystrobin (Sigma, 141517217)	0.0245	0.0025	0.0003	0.00003	U.S EPA. 1999. Pesticide fact sheet	

Table A2: BioRad thermocycler protocol. BioRad thermocycler protocol used for RT-qPCR analysis.

Thermocycler protocol					
Step 1	Step 2	Step 3	Step 4	Step 5	Step 6
95°C for 30 sec	95°C for 5 sec	Annealing temp for 45 sec	Repeat steps 2 – 3 39x	95°C for 10 sec	Melt curve analysis

Table A3: qPCR specifications for *Pax2a and Kim1*. List of genes, reverse and forward primers, annealing temperature, primer nm used, and % efficiency (E) for all genes analyzed by RT-qPCR.

Gene	F primer 5'→ 3'	R primer 5'→ 3'	Annealing Temperature C°	Reaction Quantity (nm)	% E
Pax2a	TCTCACCCGCAGTACACAAC	CTAGTGGCGGTCATAGGCAG	59.0	300	102
Kim1	ATCGTCTTCTGCGGAAAGCA	ACATGAAAGCAGCGGCAATG	56.3	300	102

Table A4: Significant p-values associated with Pax2a and Kim1 gene expression. Statistical analysis was completed using SPSS software ANOVA and Tukey post-hoc.

	Pax2a	*Kim1*
10 ppb glyphosate	0.015	0.000
100 ppb glyphosate	0.003	0.000
40 ppb As	0.01	0.000
5 ppb Pb	0.000	0.000
50 ppb Pb	0.000	0.000
15 ppb V	0.000	-
150 ppb V	0.000	-
LM1	0.000	0.000
LM2	0.001	-
LM5	0.000	-
LM6	0.008	-
LM7	0.001	-
RSV1	0.024	0.021
RSV2	0.029	-
RSV3	-	-
RSV4	0.015	-
RSV5	0.007	-
RSV6	0.002	-
RSV7	0.006	-
RSV8	0.004	-
RSV9	-	-
1P	0.001	0.003

Table A4 Continued

2P	0.004	-
3P	0.000	-
4P	0.000	0.006
5P	0.000	-
6P	0.000	-
8P	0.003	-
1N	0.000	-
2N	0.000	-
3N	0.000	-
4N	0.000	-
5N	0.000	-
Gal2	0.013	-
Gal3	0.042	-
Gal4	0.011	-
RFE3	-	0.049
RFE5	-	0.003
RFE9	0.009	-
RFE12	0.014	-
RFNE3	-	0.050

Table A5: Significant p-values from Tukey post hoc associated with gene expression analysis of 10 ppb glyphosate compared to other individual metals. Statistical analysis was completed using SPSS software. For a full Tukey post-hoc table for gene expression please see supplemental excel file Table S7.

		Pax2a	*Kim1*
10 ppb glyphosate	2 ppb Cd	0.982	0.000
	4 ppb As	0.267	0.000
	5 ppb Pb	0.906	0.000
	15 ppb V	0.101	0.000
	20 ppb Cd	1	0.000
	40 ppb As	1	0.000
	50 ppb Pb	0.837	0.000
	100 ppb glyphosate	1.00	0.041
	150 ppb V	0.998	0

Table A6: Significant p-values associated with mitochondria toxicity analysis. Statistical analysis was completed using SPSS ANOVA and games-howell post hoc For a full Games-howell post-hoc table for gene expression please see supplemental excel file Table S9.

	Basal	RC	Max Res	Mito Res	Non Mito Res
10 ppb gly	0.000	-	-	-	-
2 ppb Cd	-	-	0.033	-	-
4 ppb As	-	0.003	-	-	-
LM2	-	-	0.002	0.044	-
LM3	-	-	-	0.013	-
LM4	0.008	-	0.016	-	-
LM6	0.033	-	-	-	-
LM7	-	-	0.013	-	-
1N	0.000	-	0.009	-	0.06
2N	-	-	-	0.000	-
3N	-	-	-	0.022	-
4N	-	-	-	-	-
5N	-	-	-	-	-
14N	0.017	-	-	0.001	-
15N	0.000	0.000	-	-	0.000
1P	0.004	-	-	-	-
2P	0.000	-	0.004	0.04	-
3P	-	-	-	0.03	-
5P	0.000	-	0.000	-	-
6P	0.024	-	0.000	-	0.001
9P	-	-	-	0.045	-
10P	-	-	-	0.002	-
RSV3	-	-	-	0.004	-
RSV6	0.000	0.000	-	0.000	-

Table A7: Metal analysis of well water samples and impact on zebrafish. Summary of metal analysis of a given well sample and the effect of exposure on zebrafish larval mortality, hatching inhibition rate, and total distance (TD) traveled. Fish were exposed to 50% well water sample: egg water solution from 24 hours post fertilization (hpf) to 5 days post fertilization (dpf). Three categories of data are presented depicting samples that produced no significant change (control-like), a significant decrease (hypoactive), or a significant increase (hyperactive) in TD traveled relative to 100% egg water controls (p-value < 0.05, n=24). Chemical composition of As, Cd, Pb, and U (µg/L) of a given sample are also included. These data can be visualized in Figure 1. For the 14 chemical panel of each sample, see Appendix table A8.

Sample	As (µg/L)	Cd (µg/L)	Pb (µg/L)	U (µg/L)	TD (mm)	% mortality	% chorion
Egg water control	0.00	0.00	0.00	0.00	7535.11	1.70	0.00
Control-like: No significant change in TD relative to egg water control							
479	0.10	0.00	0.48	0.05	8855.29	0.02	0.00
487	717.91	0.00	0.51	0.34	8930.76	0.07	0.00
489	0.12	0.08	1.07	0.28	6223.95	0.08	0.25
504	2.44	0.00	0.09	8.49	6054.51	0.00	0.09
506	1.57	0.24	0.31	42.58	3864.54	0.91	0.00
516	0.74	0.00	0.08	0.01	5424.11	0.07	0.00
517	1.27	0.05	2.01	4.19	6053.60	0.53	0.42
518	0.96	0.00	0.14	81.01	5451.83	0.02	0.00
522	4.95	0.01	1.36	23.17	7042.62	0.02	0.00
526	0.26	0.00	0.03	0.22	7685.84	0.00	0.00
841	6.23	0.00	0.67	2.08	6555.46	0.00	0.11
894	6.73	0.00	0.01	5.63	9515.57	0.03	0.00
895	24.11	0.00	0.03	0.02	9461.67	0.00	0.00
897	0.03	0.01	0.58	0.01	8108.86	0.36	0.09
904	4.31	0.00	0.04	30.42	6360.82	0.04	0.00
908	0.52	0.00	0.07	0.52	7008.03	0.00	0.00
909	0.40	0.00	0.54	0.28	6953.07	0.04	0.00
1230	0.07	0.01	0.11	0.96	8630.19	0.07	0.00
1237	0.10	0.02	2.04	26.32	7729.85	0.18	0.00
1258	0.11	0.07	7.14	0.48	5594.31	0.36	0.51
1260	3.64	0.00	0.14	0.66	5618.73	0.02	0.73
1264	0.43	0.02	0.30	0.25	8193.32	0.07	0.09
1270	0.68	0.00	0.12	0.55	8004.28	0.53	0.31

Table A7 Continued

1271	0.18	0.01	1.25	69.07	6204.89	0.53	0.31
1272	0.40	0.01	0.11	8.08	5627.45	0.00	0.00
1541	0.18	0.01	0.21	0.05	8048.06	0.31	0.28
1544	0.10	0.06	6.55	0.03	3960.31	0.00	0.02
1554	0.52	0.10	0.31	0.67	6525.81	0.89	0.04
1555	0.08	0.01	0.69	3.40	5858.85	0.04	0.00
1556	8.87	0.01	0.46	0.14	8230.31	0.16	0.00
1561	1.88	0.00	0.07	2.87	7408.15	0.02	0.00
1598	0.09	0.00	0.20	2.31	6131.45	0.07	0.02
Hypoactive: Significant decrease in TD relative to egg water control							
481	0.00	0.01	5.57	0.12	4908.57	0.33	0.56
500	0.00	0.01	0.78	5.58	3973.08	0.00	0.09
505	1.08	0.03	0.76	210.81	3795.27	0.04	0.93
507	5.26	0.01	4.07	629.40	5255.01	0.02	0.60
508	2.20	0.41	4.74	15.43	4441.57	0.62	0.27
510	0.00	0.00	1.89	0.26	4338.62	0.00	0.22
511	0.07	0.19	0.38	0.03	3619.22	0.04	0.07
512	2.85	0.02	0.71	4.94	4642.59	0.02	0.58
513	0.43	0.04	8.16	14.50	3767.88	0.64	0.36
515	59.39	0.00	0.17	2.13	4196.32	0.00	0.00
519	0.08	0.01	0.38	2.07	4398.03	0.16	0.69
520	4.46	0.00	0.27	388.17	3635.68	0.00	0.24
524	0.03	0.20	12.16	0.01	3963.78	0.40	0.49
755	0.08	0.07	1.12	0.10	1511.76	0.04	0.27
890	0.24	0.00	0.49	0.09	5085.91	0.14	0.39
891	0.06	0.02	3.77	0.02	5142.79	0.22	0.28
896	0.08	0.01	0.59	0.04	4495.62	0.22	0.64
900	0.09	0.28	3.04	0.04	3891.62	0.31	0.67
902	0.31	0.00	0.03	5.82	5282.96	0.00	0.27
903	0.09	0.01	1.38	0.02	3156.87	0.02	0.93
906	0.09	0.02	0.90	0.00	3568.68	0.62	0.18
907	0.33	0.14	20.09	0.07	1036.05	0.47	0.33
910	5.07	0.00	0.45	30.42	5102.11	0.31	0.38
911	0.04	0.00	0.82	0.02	4172.73	0.31	0.40
1248	0.74	0.02	4.75	0.49	4139.45	0.58	0.04
1249	0.08	0.01	11.83	0.02	4755.77	0.07	0.00

Table A7 Continued

1250	7.51	0.03	0.93	86.24	4516.01	0.56	0.42
1259	0.06	0.07	5.73	0.01	4204.23	0.11	0.27
1261	0.18	0.03	5.36	0.06	4007.04	0.00	0.00
1265	0.27	0.01	0.34	0.03	4595.61	0.00	0.00
1267	0.07	0.06	3.12	0.70	4852.35	0.00	0.36
Hyperactive: Significant increase in TD relative to egg water control							
482	0.28	0.00	0.04	0.40	13465.28	0.16	0.00
485	8.17	0.00	0.10	1.35	14699.09	0.02	0.00
490	11.16	0.00	0.26	1.04	16170.37	0.00	0.00
492	2.18	0.00	0.07	52.49	11467.34	0.00	0.00
496	0.30	0.02	0.29	2.42	14403.15	0.00	0.00
498	1.17	0.00	0.10	2.91	16274.85	0.00	0.00
501	13.69	0.00	0.03	0.94	13212.47	0.00	0.00
502	0.27	0.01	0.66	7.76	10408.18	0.00	0.00
509	1.45	0.03	9.60	9.91	10243.90	0.00	0.00
514	25.00	0.00	0.09	4.40	10581.52	0.00	0.02
840	6.44	0.00	0.19	2.15	14229.56	0.00	0.00
847	2.51	0.00	0.03	4.69	9657.94	0.06	0.00
858	5.67	0.00	0.05	0.62	11916.76	0.04	0.02
867	8.85	0.00	0.02	10.68	11460.87	0.04	0.00
892	0.13	0.00	0.07	0.26	16949.24	0.00	0.00
893	0.52	0.10	1.02	0.17	13494.12	0.53	0.11
898	0.71	0.00	0.04	9.88	17504.06	0.04	0.00
901	0.12	0.00	0.10	13.00	12958.65	0.02	0.00
950	11.66	0.00	0.07	1.31	10332.80	0.09	0.00
1226	0.13	0.01	0.78	0.03	14724.24	0.07	0.00
1228	6.70	0.00	0.15	3274.37	18890.70	0.02	0.00
1229	0.05	0.02	0.31	0.23	16348.05	0.04	0.00
1238	1.61	0.02	0.10	13.64	12274.28	0.07	0.00
1245	14.91	0.00	1.84	0.28	15390.33	0.11	0.00
1247	1.31	0.00	0.20	5.54	15938.81	0.00	0.00
1252	0.56	0.00	0.03	41.45	14170.24	0.02	0.00
1523	1.16	0.01	0.18	31.11	18955.95	0.00	0.19
1548	8.60	0.00	0.08	1.48	11334.50	0.00	0.00
1589	2.70	0.00	0.10	0.05	21595.08	0.00	0.00

Table A8: Additional supplemental excel file

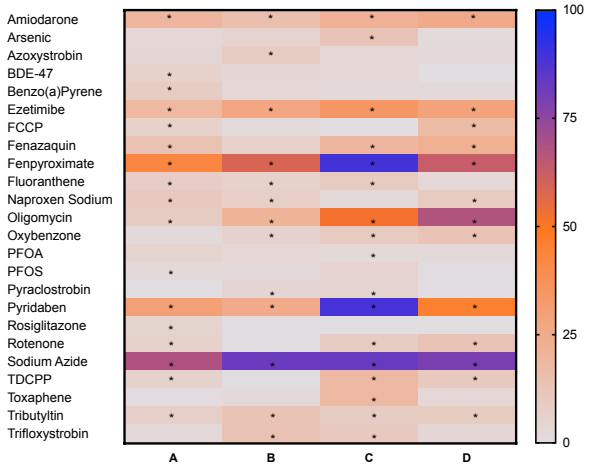

Figure A1: Heat map representing area under the curve (AUC) of the recovery trajectory based on a non-linear regression analysis conducted on embryonic oxygen consumption rates (eOCR) over 510 minutes after zebrafish embryos were removed from a 24 hour chemical treatment. Embryos were treated at 1 hpf. Heat map squares represent % difference from control, with white representing 0%, orange as a mid-range % change, and blue as maximum % change. A, B, C, D represents the concentrations used for treatment with each chemical as listed in Table 1. An independent T-Test was used to test for significance between AUC of the recovery trajectory of treatment and control. Significance is represented by (*), *p-value* < *0.05,* N = 9.

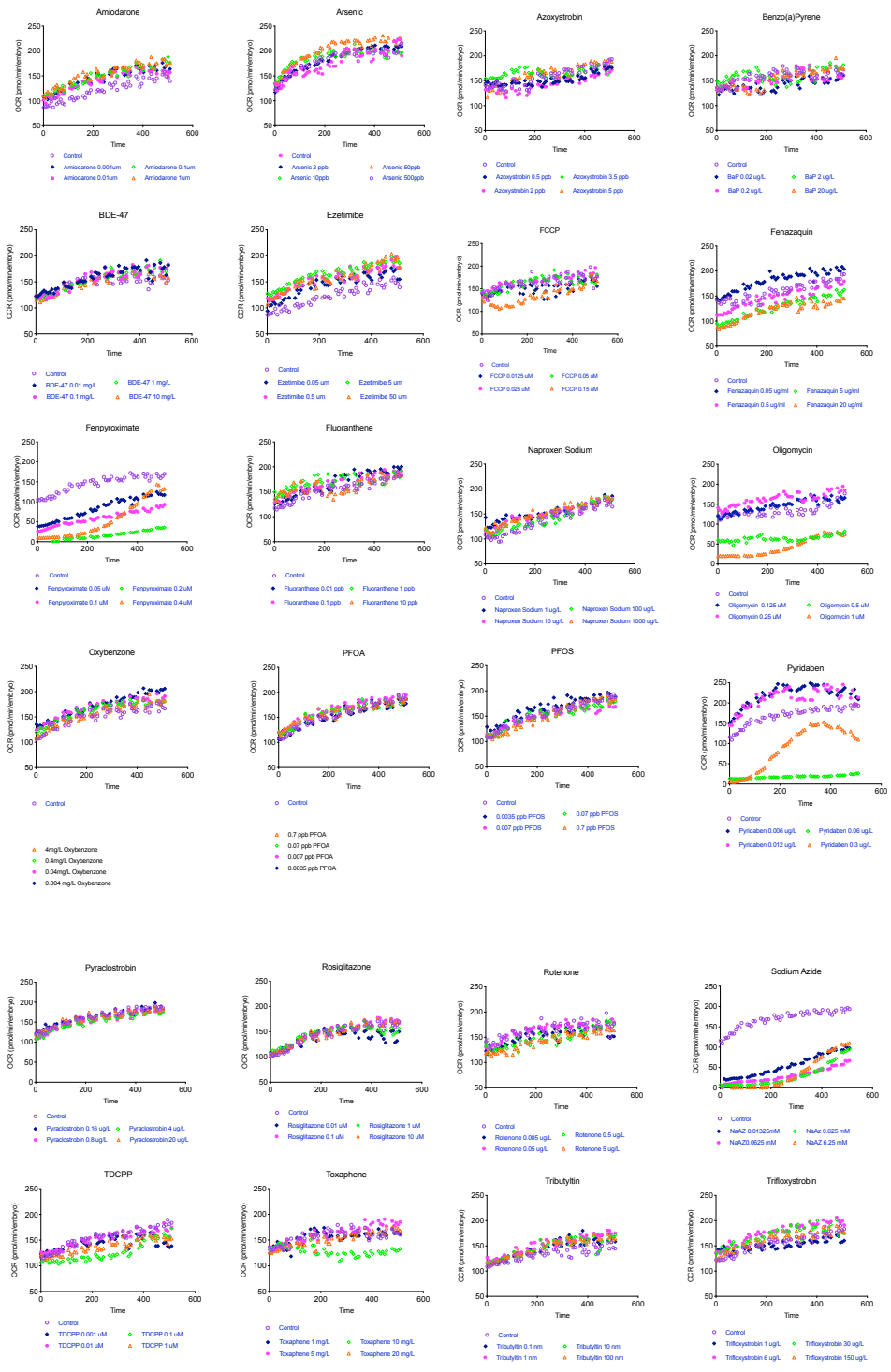

Figure A2: Time series data representing basal embryonic oxygen consumption rate (eOCR) over ~ 510 minutes of 25 hpf zebrafish after 24 hours of treatment for 24 different chemicals tested.

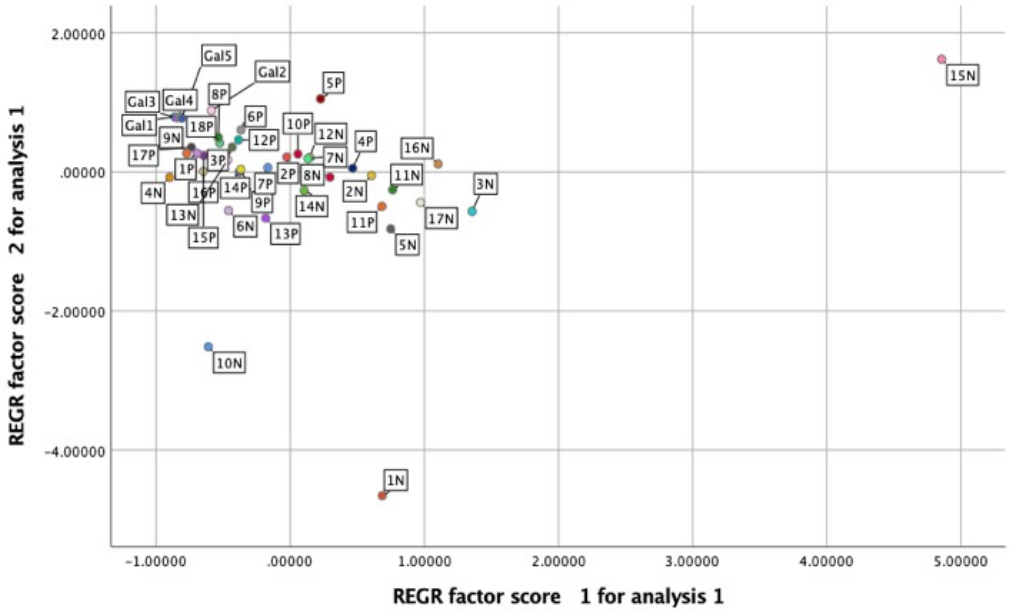

Figure A3: Clustering of patient vs non-patient wells. PCA plot representing relationships among patient wells (#P), non-patient wells (#N), and non-endemic wells (Gal#) generated using metal values, designed by SPSS software.

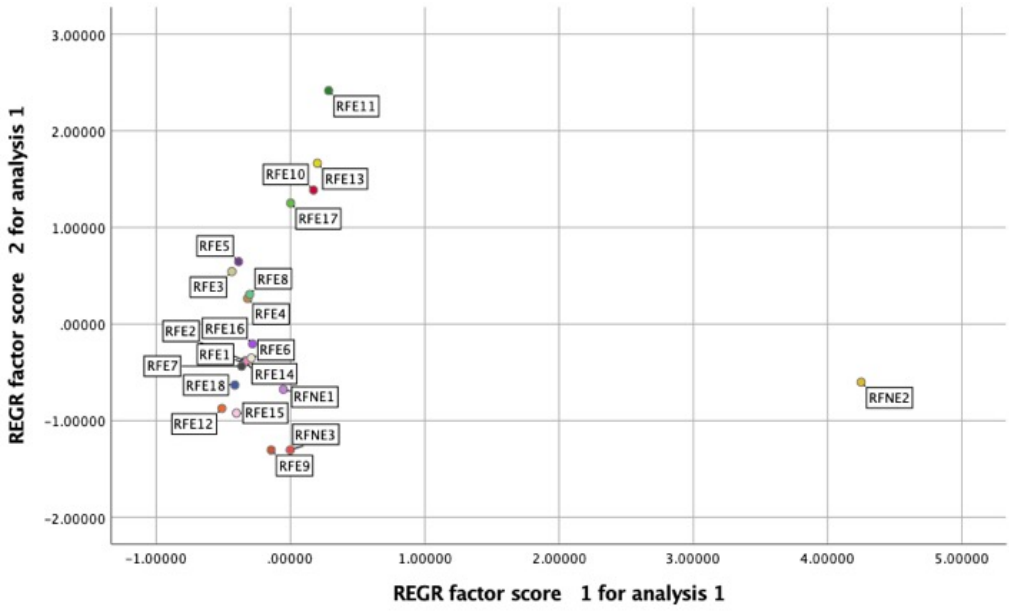

Figure A4: Clustering of endemic vs non-endemic rice fields. PCA plot representing relationships among endemic rice fields (RFE#) and non-endemic rice fields (RFNE#) generated using metal values, designed by SPSS software.

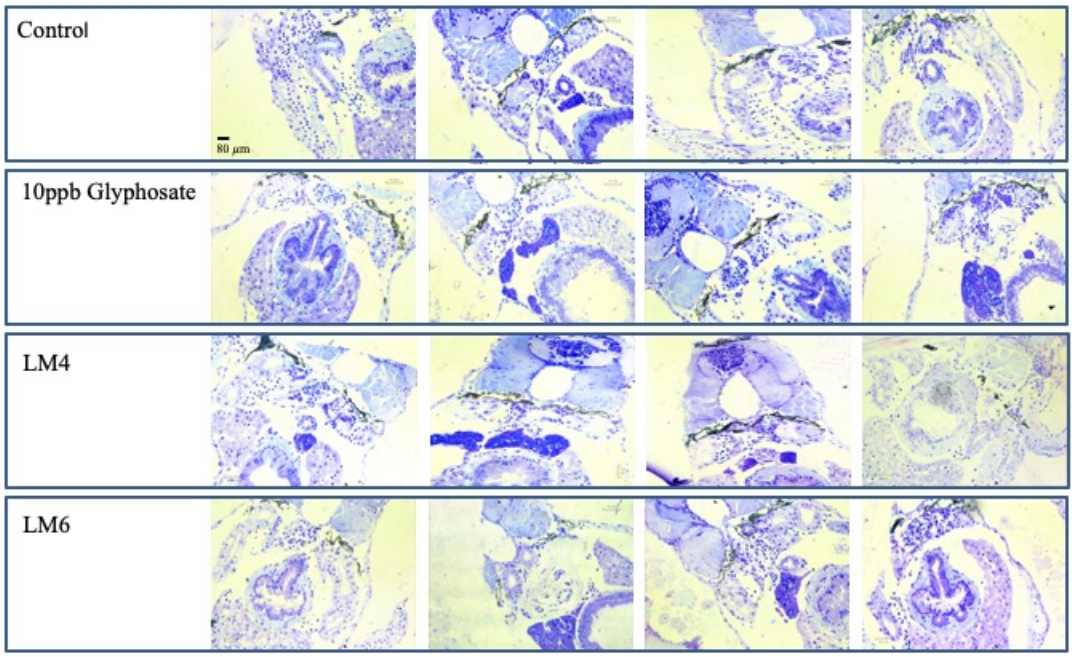

Figure A5: Histopathology of zebrafish pronephros after exposure to lab mixtures.Total 0.5 um resin sections of larval zebrafish body at 8 dpf under control, 10 ppb glyphosate, LM5, and LM6 treatment conditions. Sections were stained with toluidine blue.

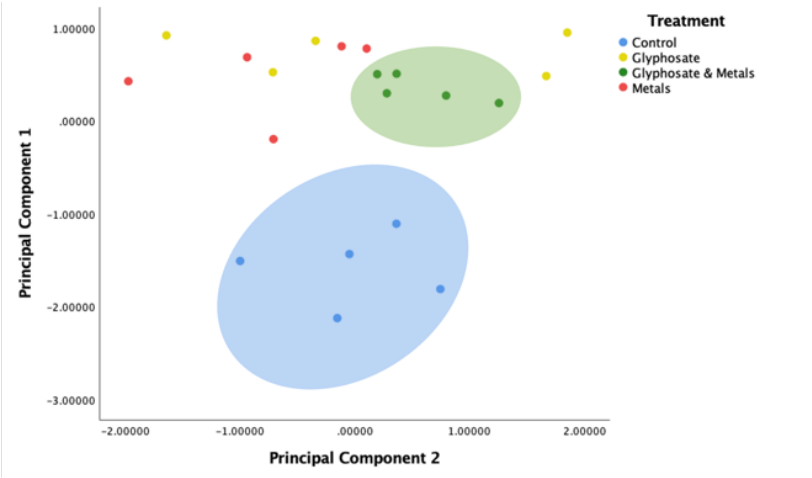

Figure A6: RNA seq cluster of DEGs shared by treatments. Principal component plot representing clustering of gene expression where gene expression values inserted into the PCA include only the DEGs shared by all treatments. Clusters are labeled by treatment condition for control (blue), glyphosate (yellow), glyphosate & metals (green), metals (red).

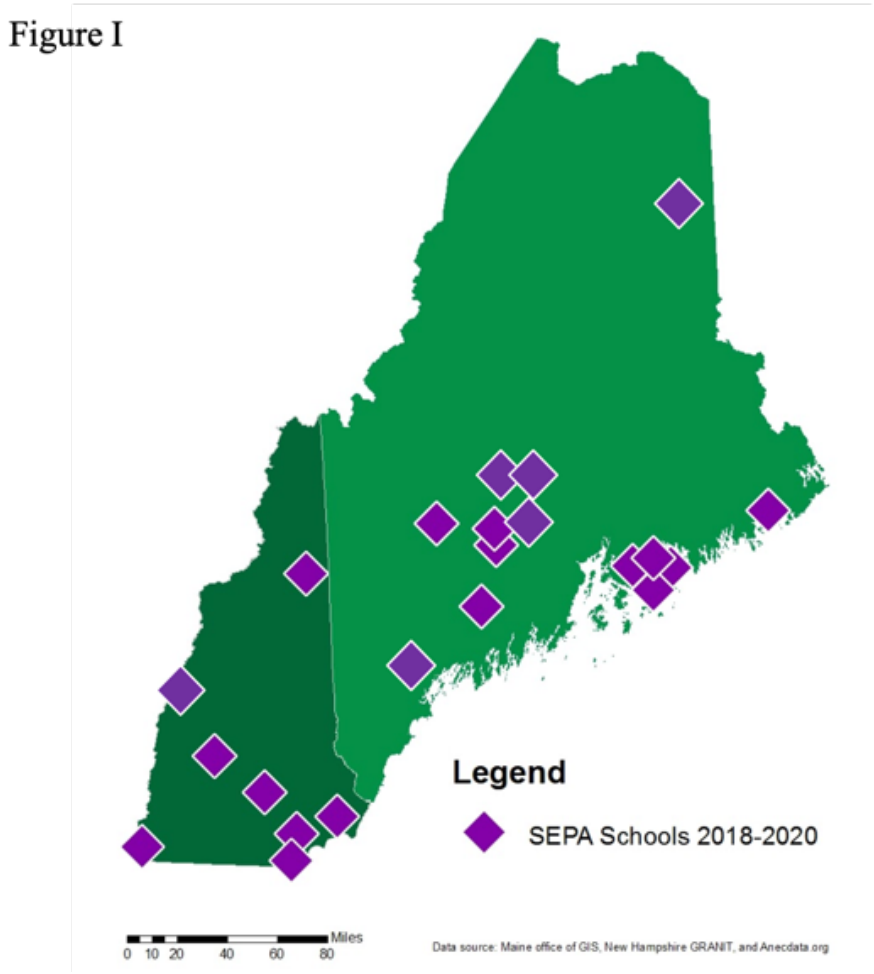

Figure A7: Water sample collection sites in Maine and New Hampshire. Map of Maine and New Hampshire depicting schools (purple diamonds) involved in the SEPA program. Five schools from Maine and 3 schools from New Hampshire contributed drinking water samples used in this study.

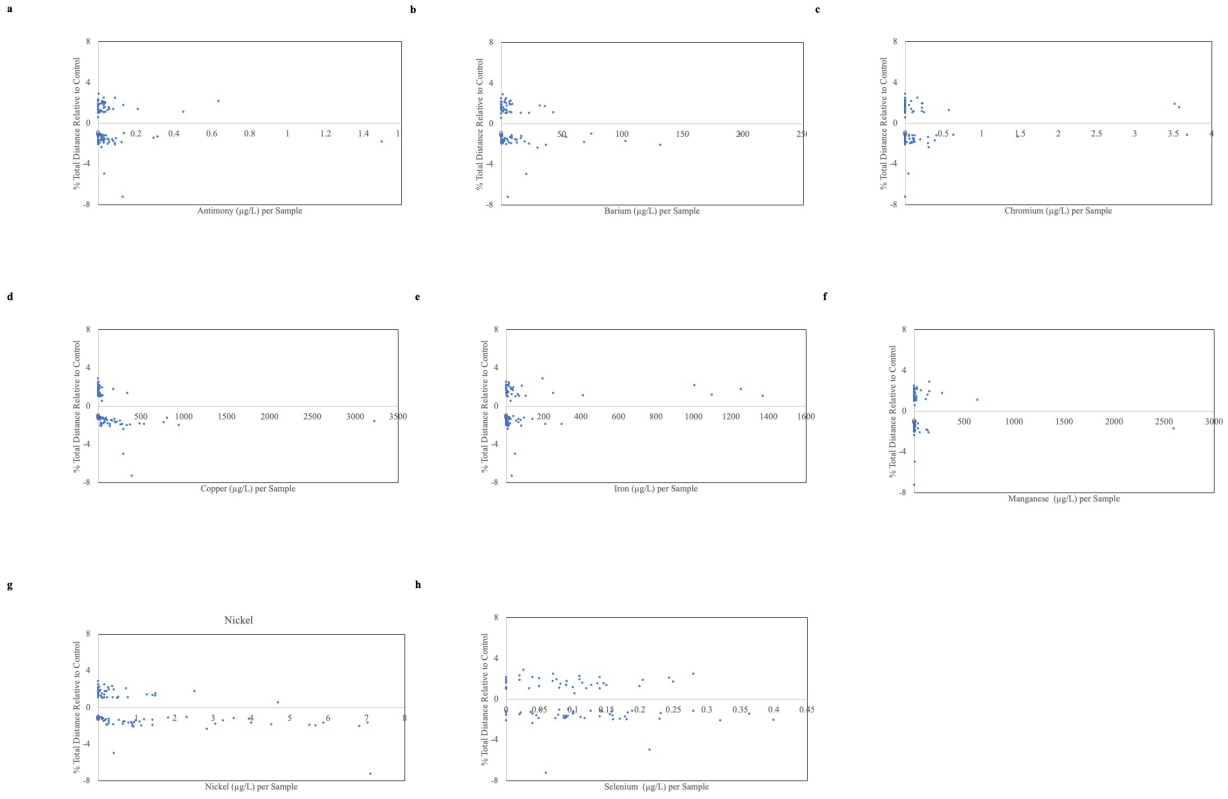

Figure A8: Dot plots comparing metal concentration and total distance traveled. Dot plot representing total distance after a 1:1 sample / egg water exposure as a percent of 100% egg water control against the amount of A) antimony (µg/L), B) barium (µg/L), C) chromium (µg/L), D) copper (µg/L), E) iron (µg/L), F) manganese (µg/L), G) nickel (µg/L), and H) selenium (µg/L), present in a given sample. Samples that induced significant hyper or hypoactivity can be found in Table SI, p-value < 0.05, ANOVA, n=24.

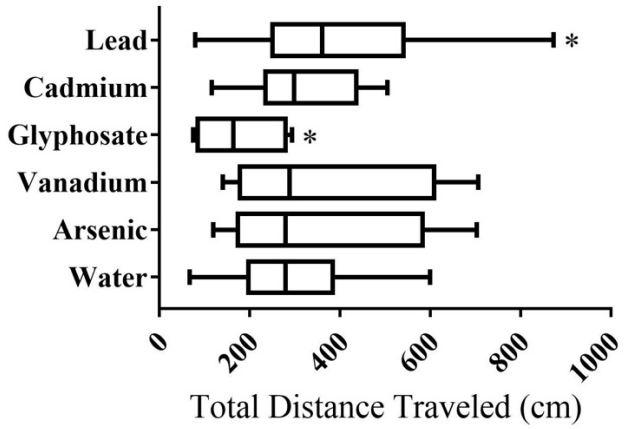

Figure A9: Changes in zebrafish behavior after individual metal treatments. Box and whisker plot showing distribution of TD travelled by individual zebrafish at 5 dpf after exposure to arsenic (4 µg/L), vanadium (15 µg/L), glyphosate (10 µg/L), cadmium (2 µg/L), or lead (5 µg/L) supplemented to egg water from 24 hpf to 5 dpf. The box plot depicts the range, 1st and 4th quartile, and average. Those exposed to water represent control embryos that were reared in egg water. Asterisks indicate a significant difference in TD travelled between exposure group and control, one-way Anova LSD Fishers test p–*value* < *0.05.*

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BIOGRAPHY OF THE AUTHOR

Remy Babich was born in Pittsburgh, Pennsylvania on December 4th 1992. Remy and her family shortly thereafter moved to Harrisburg, Pennsylvania where she lived her childhood. She graduated from Central Dauphin East High in May 2011. During this time Remy also pursued Cosmetology, and received her Pennsylvania Cosmetologists license in 2012. Upon graduation, Remy attended Penn State University Harrisburg campus for 2 years and continued her career as a beautician. She was then transferred to Penn State University Park where she finished her B.S. degree in Biology. Remy then continued to the University of Maine to pursue her Master's Degree in Biochemistry in the Department of Molecular and Biomedical Sciences, and received her Master's in August of 2018. Remy worked for 1.5 years as a research assistant for Nishad Jayasundara, and ultimately decided to rejoin the University of Maine as a Ph.D student in Biochemistry as a One Health NRT fellow. Remy is a candidate for the Doctor of Philosophy degree in Biochemistry and Molecular Biology from the University of Maine in August 2022.