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Kidney developmental effects of metal-herbicide mixtures: Implications for chronic kidney disease of unknown etiology

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ABSTRACT

Chronic kidney disease of unknown etiology (CKDu) is an emerging global concern affecting several agricultural communities in the Americas and South Asia. Environmental contaminants such as heavy metals (e.g., Cd, As, Pb, and V) and organic pesticides (e.g., glyphosate) in the drinking water have been hypothesized to play a role in childhood onset and progression of this disease. However, a comprehensive analysis of chemical contaminants in the drinking water and effects of these compounds and their mixtures on kidney development and function remains unknown. Here, we conducted targeted and non-targeted chemical analyses of sediment and drinking water in CKDu affected regions in Sri Lanka, one of the most affected countries. Using zebrafish *Danio rerio*, a toxicology and kidney disease model, we then examined kidney developmental effects of exposure to (i) environmentally derived samples from CKDu endemic and non-endemic regions and (ii) Cd, As, V, Pb, and glyphosate as individual compounds and in mixtures. We found that drinking water is contaminated with various organic chemicals including nephrotoxic compounds as well as heavy metals, but at levels considered safe for drinking. Histological studies and gene expression analyses examining markers of kidney development (*pax2a)* and kidney injury (*kim1)* showed novel metal and glyphosate-metal mixture specific effects on kidney development. Mitochondrial dysfunction is directly linked to kidney failure, and examination of mixture specific mitochondrial toxicity showed altered mitochondrial function following treatment with environmental samples from endemic regions. Collectively, we show that metals in drinking water, even at safe levels, can impede kidney development at an early age, potentiating increased susceptibility to other agrochemicals such as glyphosate. Drinking water contaminant effects on mitochondria can further contribute to progression of kidney dysfunction and our mitochondrial assay may help identify regions at risk of CKDu.

1. Introduction

Chronic kidney disease (CKD) affects ~15% of the global population and is typically associated with systemic disorders (e.g., diabetes) (James et al., 2010). In recent years, CKD of unknown etiology (CKDu) (Gifford et al., 2017; Obrador and Levin, 2019) has emerged across farming communities in Central America and South Asia, with some prevalence data emerging in the United States (Aguilar and Madero, 2019; Kulathunga et al., 2019; Ramirez-Rubio et al., 2013). CKDu is postulated to be multifactorial, involving genetic predisposition, nutritional and dehydration status, and exposure to anthropogenic contaminants (Levine et al., 2016; Friedman and Luyckx, 2019; Kulathunga et al., 2019). Sri Lanka is one of the most affected countries, where CKDu prevalence is over 20% in some communities (Rajapakse et al.,

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2016). Notably, 8.7% of children (ages 5–11) tested positive for kidney injury markers in CKDu endemic regions in Sri Lanka (Agampodi et al., 2018), suggesting a potential early life onset of this disease (Friedman and Luyckx, 2019).

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). Water and soils in CKDu endemic regions are reportedly contaminated with various agrochemicals such as pesticides (e.g., glyphosate) and heavy metals (e.g., cadmium, arsenic, and lead) that are likely introduced via fertilizers (Atafar et al., 2010; Jayasumana et al., 2014). A study done in Nicaragua, a region also suffering from CKDu, found that young adults working primarily in agriculture were exposed to metal and pesticide mixtures, as traces of these elements and compounds were found in their urine samples (Smpokou et al., 2019). Importantly, anthropogenic contaminants (e.g., Cd, As, Pb, glyphosate) in the environment in endemic regions are found at concentrations well-below current maximum allowable limits in the drinking water (Kulathunga et al., 2019; Gunarathna et al., 2018; Levine et al., 2016). However, as demonstrated in a recent rodent study, synergistic effects of chemical mixtures, even at levels considered “safe” are likely to play an important role (Wasana et al., 2017). Given the potential early onset of kidney disease in children (Friedman and Luyckx, 2019; Agampodi et al., 2018; Jayasekara et al., 2013), effects of chemical contaminants and their mixtures on kidney development may play a critical role in initiation and progression of CKDu.

A key limitation to studying mixtures effects on kidney development has been the lack of a higher throughput animal model. Here, we utilized zebrafish (*Danio rerio*), a prominent high-throughput toxicological and kidney injury model (McKee and Wingert, 2015; Tanguay, 2018), to evaluate effects of individual compounds and their mixtures on kidney development. Zebrafish embryonic kidney, or pronephros, is formed by 24 h post fertilization (hpf) (Elmonem et al., 2018), and is structurally and functionally similar to the adult mammalian kidney. The pronephros retains their structure until 10 days post fertilization (dpf) until developing into the mesonephros (Drummond et al., 1998). Given these attributes, we used the embryonic and larval zebrafish to assess effects of chemical contaminants, at levels considered safe for human consumption, and their mixtures on kidney development.

Effect directed analyses (EDA) provide a powerful integrative approach to better determine the complexity of chemical mixture effects. EDA combines the testing of environmental samples for chemical constituents with biological outcome assays, and can include the examination of laboratory derived mixtures to further resolve the toxicity of specific chemical mixtures or concentrations that are found in the environment. Biological assays have previously been conducted on cell lines (Hashmi et al., 2020, Zwart et al., 2018) but *in vivo* models, such as the zebrafish, are increasing in popularity (Di Paolo et al., 2015). Zebrafish embryo toxicity tests (e.g gene expression analysis, histopathology, mitochondrial function) as well as GFP transfected zebrafish for target gene expression have been utilized in EDA studies (Sonavane et al., 2018, Fetter et al., 2014, Chen et al., 2019, Trevisan et al., 2019).

Here, to examine renal specific developmental effects of environmentally relevant chemical mixtures we focused on transcript levels of *pax2a* and *kim1*, histopathology, and mitochondrial function. *Pax2* is critical for precise organ development, including the kidney, whereas *kim1* is a key marker of kidney injury and is associated with the progression of kidney disease (Orisio et al., 1993; Humphreys et al., 2013; Patel & Dressler, 2013). It is also well established that mitochondrial integrity may play a key role in CKDu given that impaired mitochondrial function and increased reactive oxygen species (ROS) production are directly linked to reduced kidney function and increased damage (Dounousi et al., 2006; Granata et al., 2009; Emma et al., 2016; Gamboa et al., 2016). Mitochondrial function was also highlighted as one of the significantly altered biochemical processes in CKDu patients in Sri Lanka (Sayanthooran et al., 2018).

Collectively, our study integrated targeted and non-targeted chemical analyses with toxicity studies to evaluate the impact of “safe” levels of chemical mixtures on kidney development, and examined their potential role in contributing to the progression of kidney dysfunction via mitochondrial impairment.

2. Materials and methods

2.1. Environmental sample characterization

Environmental samples were collected from CKDu endemic and non-endemic regions in Sri Lanka (Fig. 1A). Sediment samples were collected for metal analysis and toxicity studies, and water samples were collected for non-targeted chemical analysis. 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. Patient and non-patient wells were determined based on information from local health officials – none of the researchers herein had access to patient data. Non-endemic 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 stored at 4°C during transportation and processed into extracts within one week of collection. Each composite sediment sample was thoroughly mixed and aliquots of 25 mL of sediment and 25 mL of deionized water were added to a 50 mL centrifuge tube. All tubes were placed horizontally on a rotary shaker for 24 hrs in the dark 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.

2.2. Metal analysis

The sediment extracts were acidified (1% v/v) with optima nitric acid prior to analysis. Samples were analyzed for metal contaminants (As, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, Sb, Se, V) 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.

2.3. Sample collection and organic contaminant analysis via High Resolution Mass Spec

For each reservoir and well, 1 L water samples were collected on site in Sri Lanka and passed through solid phase extraction columns (Waters Oasis HLB Prime cartridges), using vacuum pumps. The columns were frozen and shipped to Duke University for chemical analysis. Samples were extracted from the SPE columns using 10% methyl tert butyl ether/methanol and reconstituted in 1 mL of 5% acetonitrile/water for high resolution mass spectrometry analysis.

The presence of organic contaminants was detected via high performance liquid chromatography (HPLC; Thermo Scientific, Waltham MA) coupled to a high-resolution mass spectrometer (HRMS; Thermo Scientific Orbitrap Fusion Lumos with positive and negative polarity electrospray ionization (ESI)). Both positive and negative electrospray ionization were used to cover a wider area of chemical space. The 1 mL

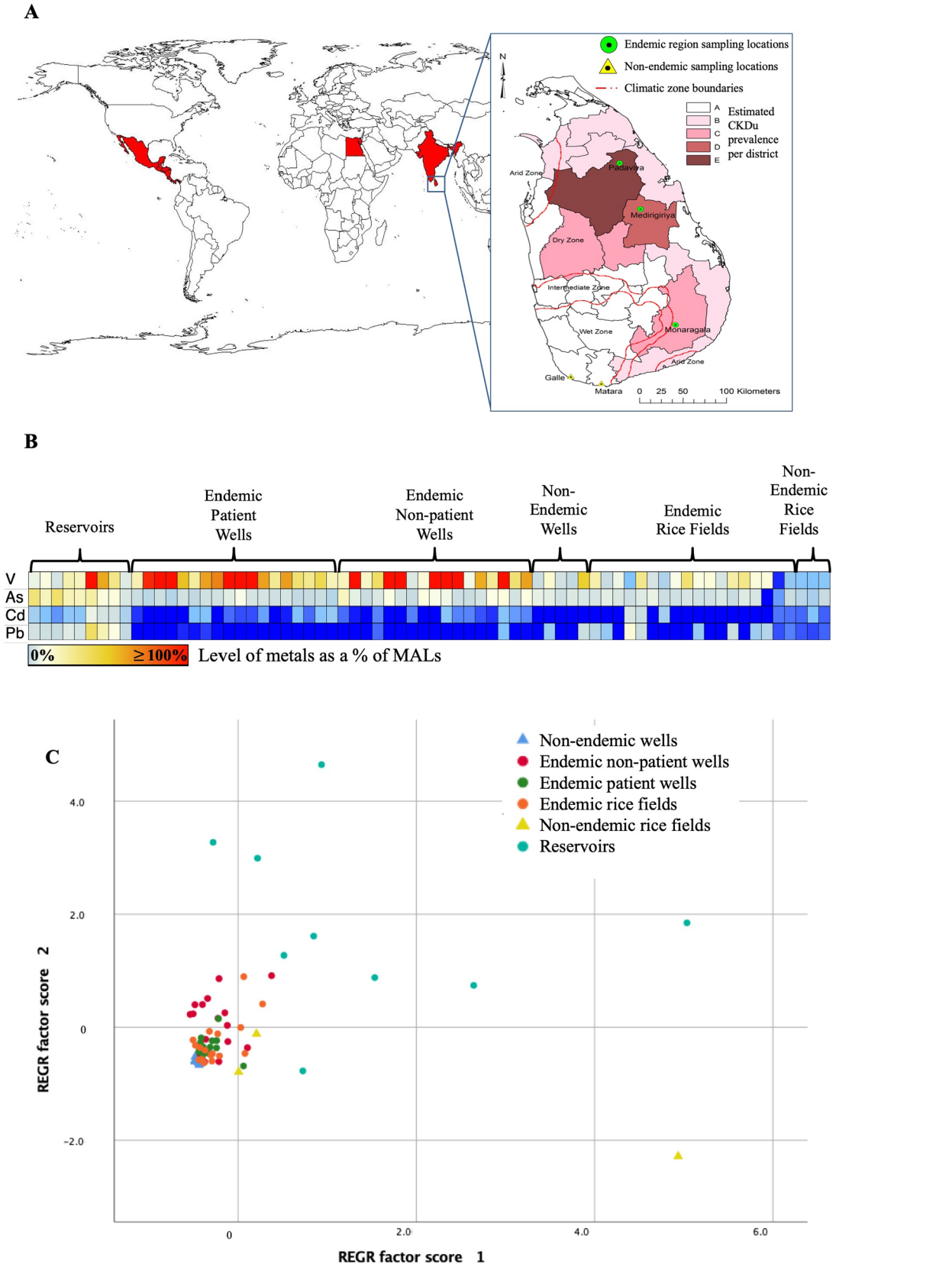

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Fig. 1. Sampling locations and results from heavy metal analysis (measured in ppb) of environmental samples from reservoirs (RSV), CKDu patient associated wells (P), non-patient wells (N), non-endemic wells (Gal), endemic rice fields (RFE), and non-endemic 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 color indicates a lower percentage and red indicates a higher percentage. (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. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

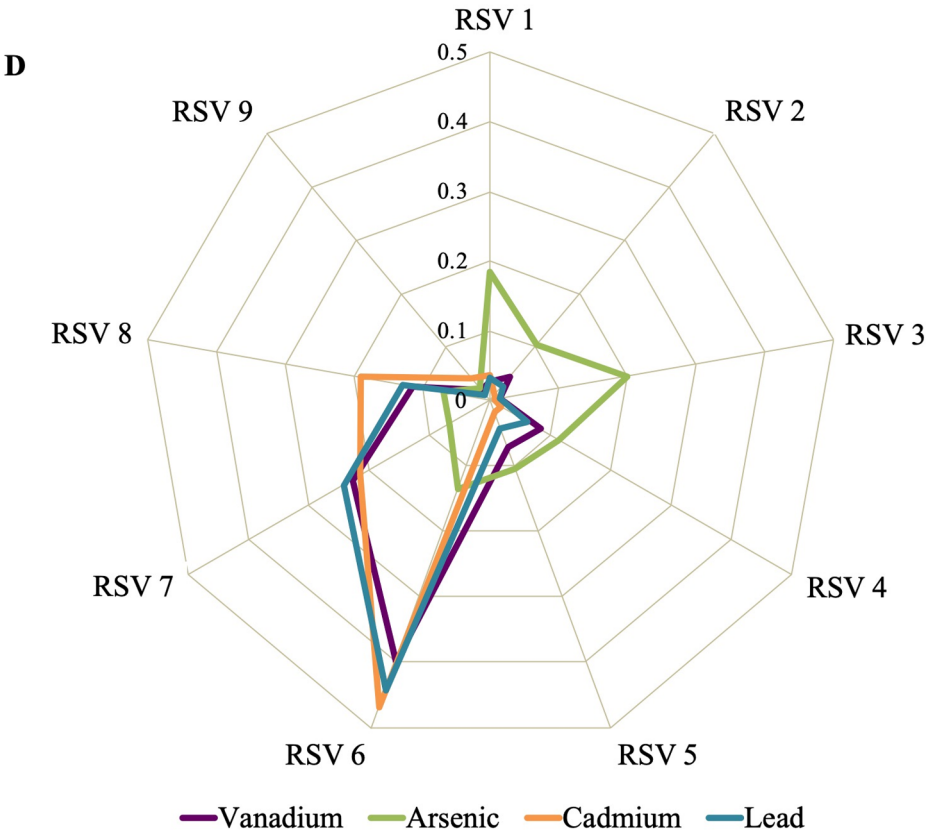


Fig. 1. (*continued*)

aliquot of each sample was prepared for HPLC-HRMS analysis with 10 µL of sample being injected on a Hypersil GOLD aQ column (100 × 2.1 mm, 1.9 µm particle size, Thermo Scientific, Waltham MA).

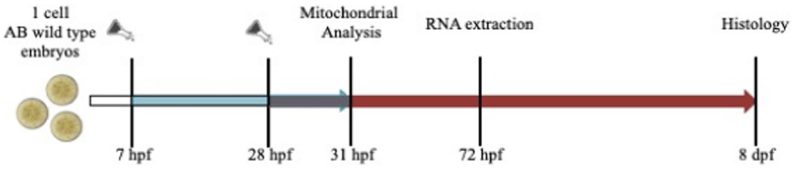
The HPLC was operated at a flow rate of 0.3 mL min^{-1} using a linear gradient from 5% to 99% acetonitrile/water over 30 min with a 5 min hold before a linear gradient from 99% to 5% acetonitrile/water over 5 min. 0.1% formic acid was in the acetonitrile and water mobile phases were used in the positive electrospray runs but not in the negative electrospray runs. MS^1 data acquisition consisted of concurrent full scan (120–1500 *m/z*), high resolution (R = 240,000), accurate mass (< 1 ppm in positive mode and negative mode based on mass labeled surrogate standards) spectra and data-dependent orbitrap tandem high-energy collisional dissociation mass spectra ($OTMS^2$ HCD, R = 15,000). Raw MS files were uploaded and analyzed for non-target screening of organic contaminants in Compound Discoverer 3.1 (Thermo Scientific). Resultant data were subject to peak picking, adduct and isotope detection, molecular formula assignment, and spectral library searching using the mzCloud mass spectral database and mzVault a local mass in-house mass spectral database within Compound Discoverer 3.1.

2.4. 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. 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 1. 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 due to its ubiquitous use and the known chelation properties with metals. The environmental levels of glyphosate in the endemic regions range between 1 ppb and 1000 ppb (Gunarathna et al., 2018). Mixtures of glyphosate and cadmium (LM1), and glyphosate and arsenic (LM2) were used to specifically explore the

Table 1
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). These different dosing times were determined based on previous studies or preliminary analyses.



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).

interactive effects of a chelating compound and a known nephrotoxic metal (Gao et al., 2013, Jayasumana et al., 2014) (Table 1).

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.

This protocol was approved by the University of Maine IACUC, **protocol number A2017-05-04.**

2.5. RT-qPCR

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 to 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 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). 500 ng RNA was then synthesized into cDNA using BioRad iScript cDNA synthesis kit (BioRad, Hercules, CA) for RT-qPCR. Previously, we conducted preliminary studies with a known nephrotoxin (gentamicin) to examine changes in the expression of several genes involved in kidney development and injury. Based on our results, here we focused on *kim1* and *pax2a.* 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 supplemental material for thermocycler parameters and primer sequences (Tables S1 and S2). 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.

2.6. 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 ~0.5μ m 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).

2.7. 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 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. We focused on 7 – 31 hpf window of exposure, since this is a crucial interval in early embryogenesis in which mtDNA copy number decreases offering a potential to accumulate mtDNA mutations (Otten et al., 2016) and was demonstrated to be a sensitive time period for mitochondrial measurements using the flux analyzer assay described below (Stackley et al., 2011).

Embryos were treated with LMs (Table 1) 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 (OCR) 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 OCR) and NaAz as a complete mitochondrial inhibitor (decreasing mitochondrial OCR). The protocol consisted of three measurement types, basal (15 cycles), post injection FCCP (6 cycles), and post injection NaAz (20 cycles) with a 2:00 min mix, 2:00 min wait, and 3:00 min measure period per cycle. Data were downloaded and OCR 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.

2.8. Statistical analysis

A Shapiro-Wilk 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 $p\ value < 0.05$ 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).

3. Results

3.1. Metal levels in environmental mixtures

Irrespective of the region of origin or the source, all of the metals in the environmental samples, with the exception of vanadium, were below current regulatory thresholds (Fig. 1B, Table S3). Notably, each environmental sample had a unique metal profile (Fig. 1B, Table S3). 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 (Fig. 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 (Fig. 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 (Fig. 1C, Fig. S1). 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 (Fig. S2).

Reservoir-derived samples did not cluster with well and rice field samples, indicating a different metal profile (Fig. 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 (Fig. 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) (Fig. 1D).

3.2. Organic contaminant analysis

A non-targeted analysis for organic contaminants in reservoir (data not shown) and well samples resulted in 2646 unique chemical features. The ESI positive and negative analyses detected 1936 and 710 unique chemical features respectively (Fig. 2A, B). The clustering analysis of chemical profiles did not collectively differentiate wells belonging to families with at least one patient and wells without patients. However, it is evident that the majority of patient wells have a considerably higher abundance of organic contaminants compared to the wells without patients. Of all the unique features detected, 152 of these features had a confident mass spectral library match (≥75%), meaning that these features were identified at a level 2 confidence level (Schymanski et al., 2014) in non-targeted environmental mass spectrometry (Table S4). This is the highest confidence level achievable without an authentic standard. A literature search identified many of the chemical compounds as agrochemicals, pharmaceuticals, and those used in industrial applications. According to previous studies, 24 of the 152 chemicals identified have nephrotoxic properties (Table S5).

3.3. Gene expression changes following laboratory-derived mixture treatments

Fig. 3 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 1) and individual compounds. None of the treatments had significant effects on mortality or embryonic development. 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* (Fig. 3A). The effect of glyphosate in *kim1* increase was statistically significant when compared to all other individual metal treatments (Table S6). With metals, Pb and V treatments had a significant effect on *pax2a* levels (> 2-fold increase) at both concentrations tested compared to the control. Two and 20 ppb Cd and 4 ppb As treatment 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 (Fig. 3B).

Gene expression analyses with mixture treatments demonstrated mixture specific 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 (Fig. 3C). 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 (Fig. 3C). Metal mixtures without glyphosate showed a 2-fold increase in *pax2a,* but had no effect on *kim1* compared to the control (Fig. 3D).

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 LM 5 being the only exception. Collectively, these data show highly mixture specific effects on kidney development.

3.4. Gene expression changes following environmental sample treatments

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 (Fig. 4A, 4B). 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 (Fig. 4A). Reservoir sample 9, which borders an endemic region, treatment did not show any effect on *pax2a* expression (Fig. 4A). Compared to the control, *pax2a* expression also increased (~2–6-fold) following well sample treatments from the endemic regions (Fig. 4A). 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 (Fig. 4A). Effects of rice-field derived samples from endemic and non-endemic regions

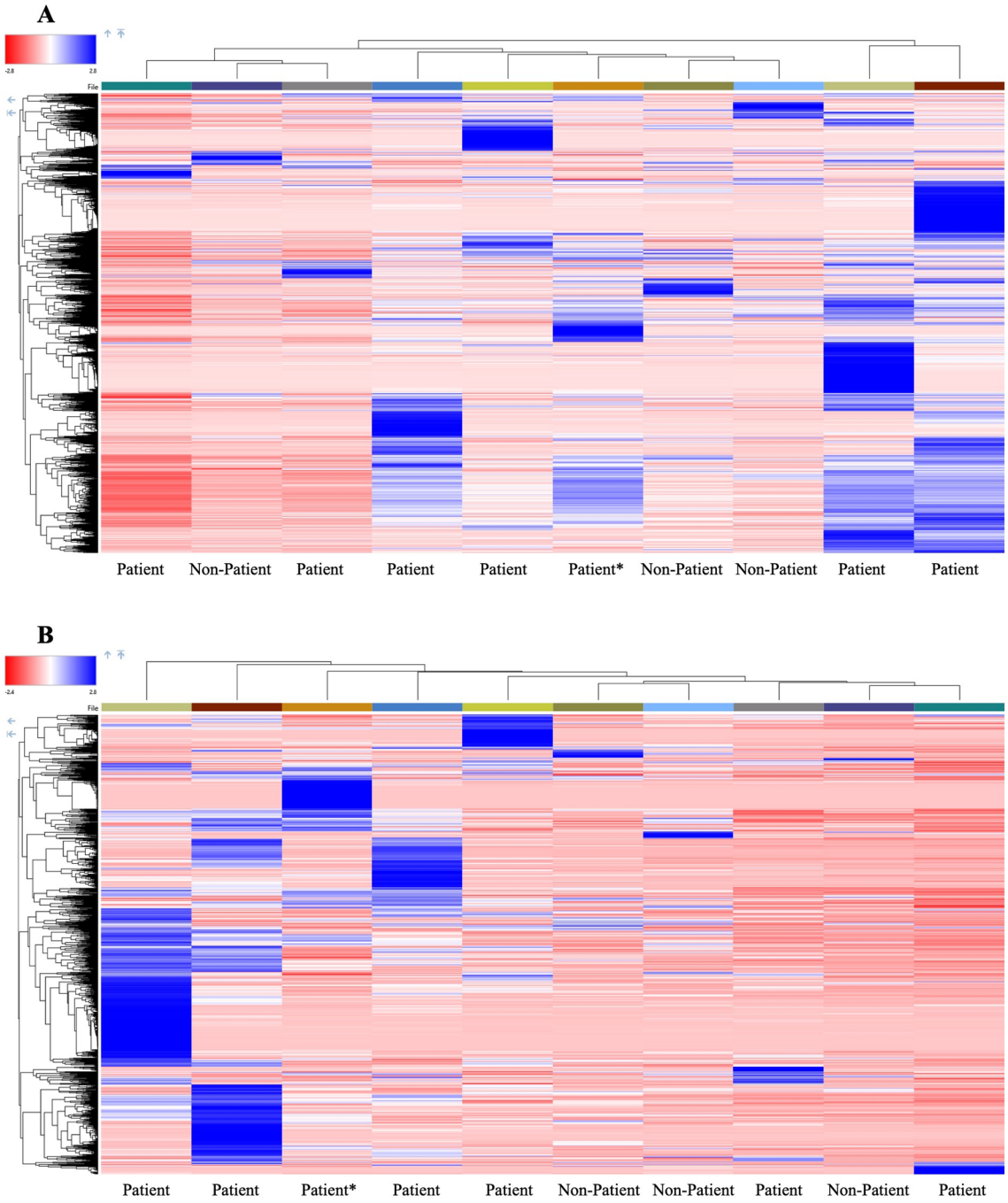


Fig. 2. (A) ESI + and (B) ESI – heat maps displaying 1936 and 710 unique chemical features found in patient and non-patient wells. * denotes a well sample that was originally non-patient but since the time of collection is now associated with a CKDu patient. Both the samples (on the top) and the features (on the left) are clustered based on similarity using a Euclidean distance function within Compound Discoverer 3.1. Blue represents an increase in compound concentration while red represents a decrease (or close to a value of 0). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

were minimal on *pax2a* expression in which only two samples showed a 2-fold increase in expression compared to the control (Fig. 4A).

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 (Fig. 4B).

3.5. Histopathological changes following laboratory-derived mixture treatments

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 (Fig. 5, Fig. S3)

3.6. Mitochondrial analysis following laboratory- and environmentally-derived mixture treatments

Mitochondrial function was significantly altered following exposure to certain chemical treatments. Notably, compared to the impacts of

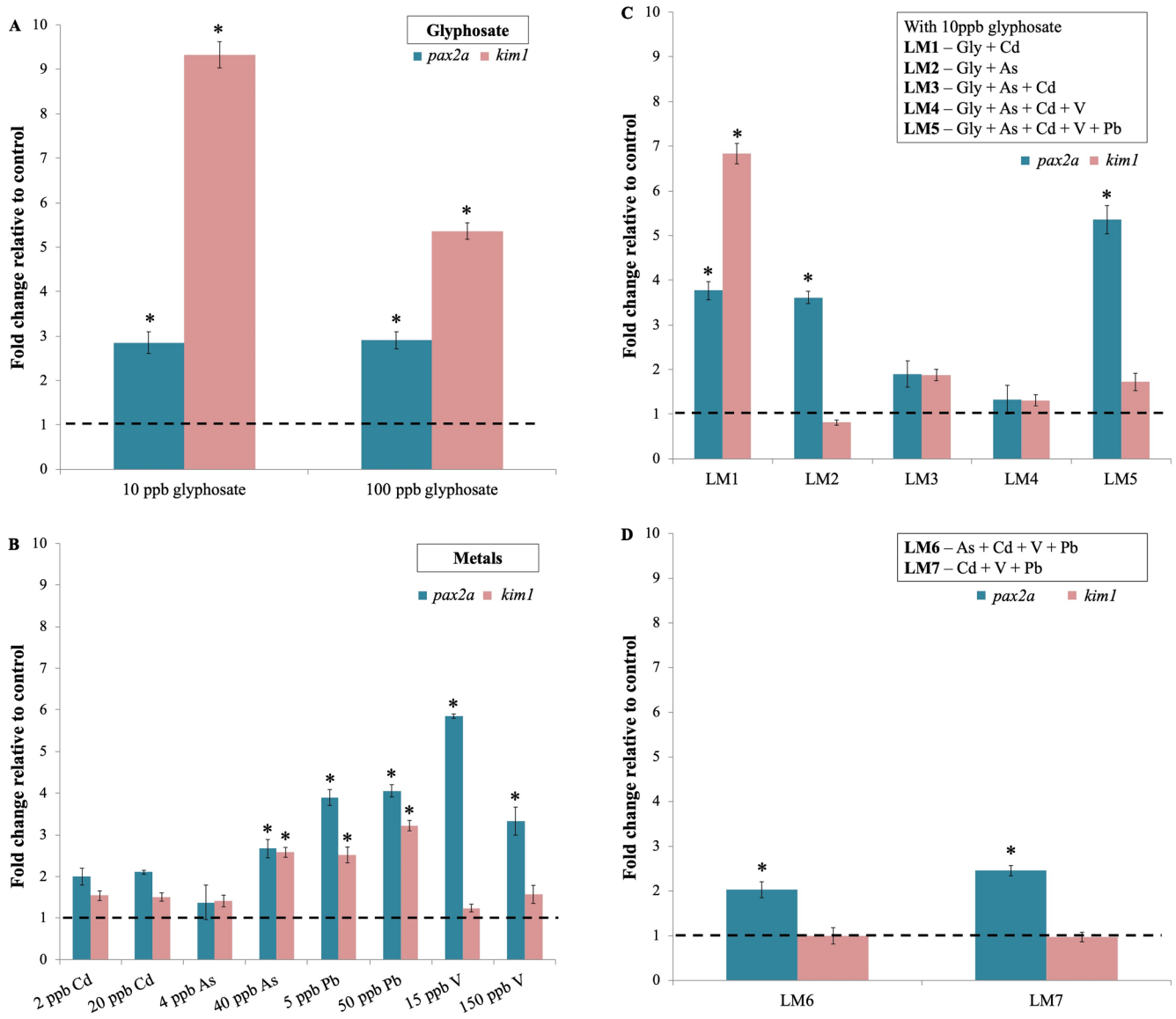


Fig. 3. Gene expression changes associated with kidney development (*pax2a*) and injury (*kim1*), following (A) herbicide glyphosate treatment (B) individual metal treatments (C) lab mixtures (LM) containing 10 ppb glyphosate, and (D) LM with no glyphosate. Embryos were treated from 28 hpf until the 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 S6 for exact p-values), N = 8.

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 arsenic (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 (Fig. 6A, 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 (Fig. 6C). 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 (Fig. 6D).

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 mitochondrial function, which may contribute to the initiation and propagation of kidney dysfunction. This research emphasizes the potential role of chemical mixtures and the importance of effect directed analysis in determining mixture toxicity. This approach in combination with epidemiological studies, may provide a key framework to uncovering the role of environmental contaminants in CKDu.

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. For example, metals

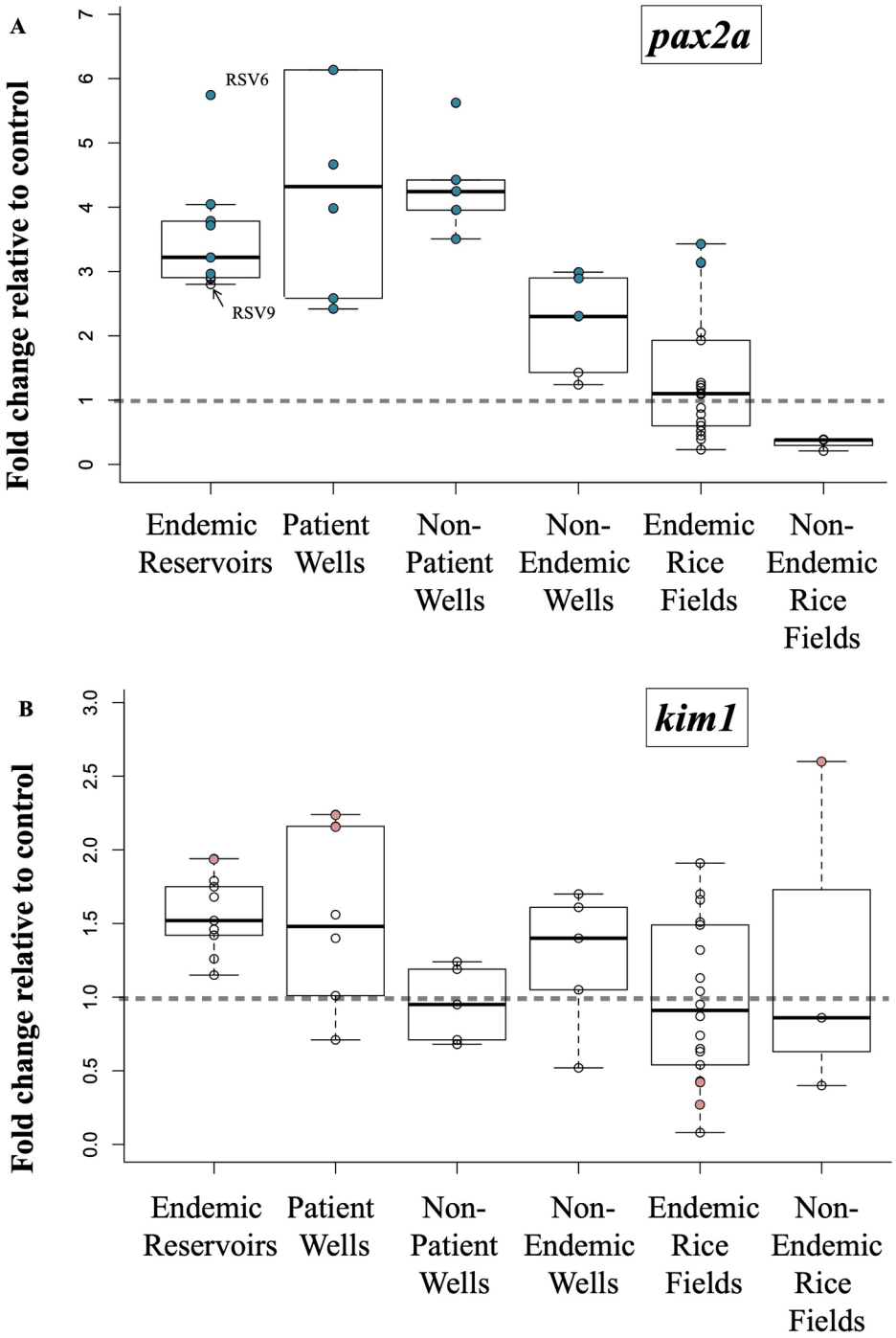


Fig. 4. Gene expression changes associated with kidney development (A) *pax2a* and injury (B) *kim1*, following reservoir sample, endemic region patient and non-patient well sample, non-endemic well sample, endemic rice field, and non-endemic rice field sample treatments from 28 hpf until the time of 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 S6 for exact p-values), N = 8 (reservoir samples), N = 6 (wells), N = 5 (rice fields).

appear variegated in distribution across endemic region samples compared to non-endemic samples which cluster together in a PCA analysis (Fig. 1C). Further, certain metals (As, Cd, V, Pb) are present at slightly higher concentrations in endemic region samples, regardless of a patient or non-patient affiliation, compared to non-endemic region well samples (Fig. 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 (Hsueh et al., 2017) and *en utero* exposure to both As and Pb have influences on birth outcomes (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.

Our non-targeted analysis data show that ~2600 potential organic compounds are present in well water samples in the endemic zone of Sri Lanka. This indicates a highly diverse chemical burden in the drinking water and the potential for human exposure to thousands of chemicals at trace levels (Fig. 2A, B). Multiple classes of chemical compounds are present in the environmental samples, including agrochemicals and pharmaceuticals, some of which have been identified as nephrotoxic compounds (Table S5). 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 (Cleuvers, 2004) and warrants further research to evaluate mixtures with respect to kidney toxicity. This may explain the high heterogeneity of the disease even within regions that are most affected by CKDu. It should also be noted that we

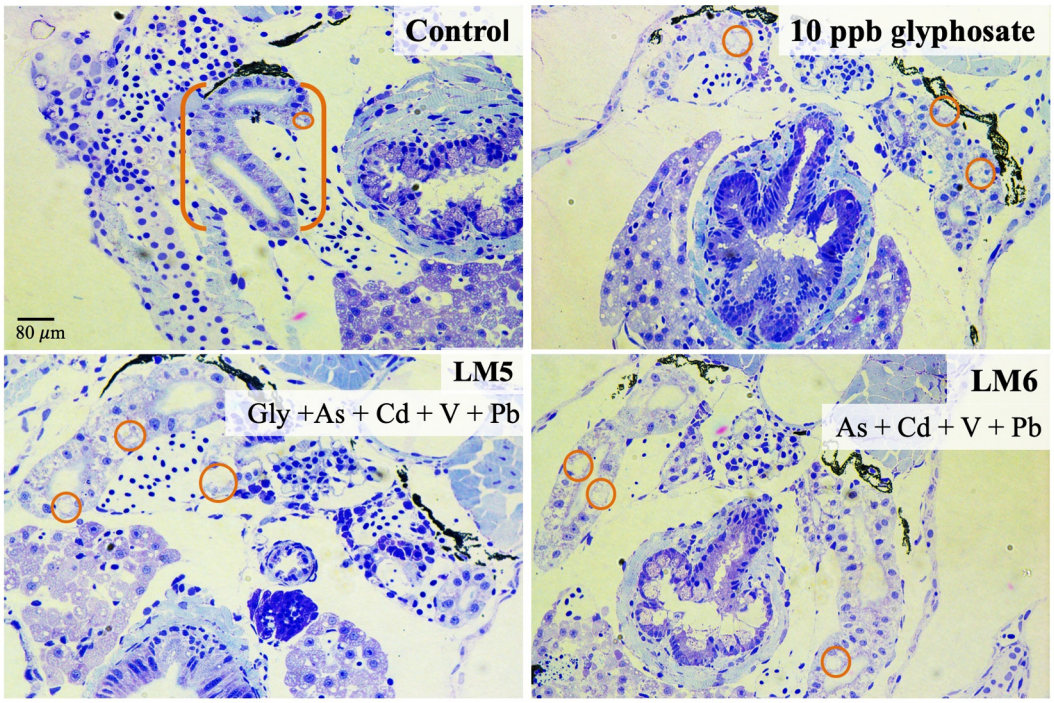


Fig. 5. 0.5μ m resin sections of zebrafish pronephric kidney at 8 dpf under control, 10 ppb glyphosate, LM5 (lab mix 5), and LM6 (lab mix 6) treatment conditions. Sections were stained with toluidine blue, the orange bracket is denoting a kidney tubules. Orange circles are referring to formation of vacuoles within kidney tubules (not all vacuoles are circled), N = 4. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

did not identify glyphosate in this analysis. 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 here. 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.

4.2. Kidney developmental effects 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 (Martínez-Salgado et al., 2004, Gao et al., 2013) to identify appropriate markers of altered kidney development and damage. 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 and 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 (Fabrizio and Fishman, 2001). Loss of *pax2* expression inhibits nephron tubule formation in both zebrafish and mice (Majumdar et al., 2000; Dressler and Woolf, 1999). In contrast, increased expression of *pax2* has been linked to renal cell carcinoma in endothelial cells (Fonsato et al., 2006) and persistent expression leads to abnormal kidney development in mice (Dressler et al., 1993). 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 (Fig. 3). 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 (Fig. 3C). Furthermore, V significantly affects *pax2a* expression, but this effect is not apparent when in mixture with other metals. It should also be noted that individual chemicals, glyphosate and V, produced a greater response at their respective lower concentrations, 10 and 15 ppb compared to 100 and 150 ppb. Similar findings in which lower concentrations of metals or pesticides induce responses relative to their higher counterparts have been previously identified (Woo et al., 2009, Tsave et al., 2018, Velki et al., 2017). It is speculated that lower – mid concentrations may result in the activation of adaptive response pathways compared to higher dosages which may manifest a pathological response and may explain our data.

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 not apparent (potential antagonistic effect) when in mixture with other metals with the exception of Cd (Fig. 3A, C).

Overall, *pax2a* and *kim1* results following exposure to LMs confirm the significance of considering differential developmental renal effects

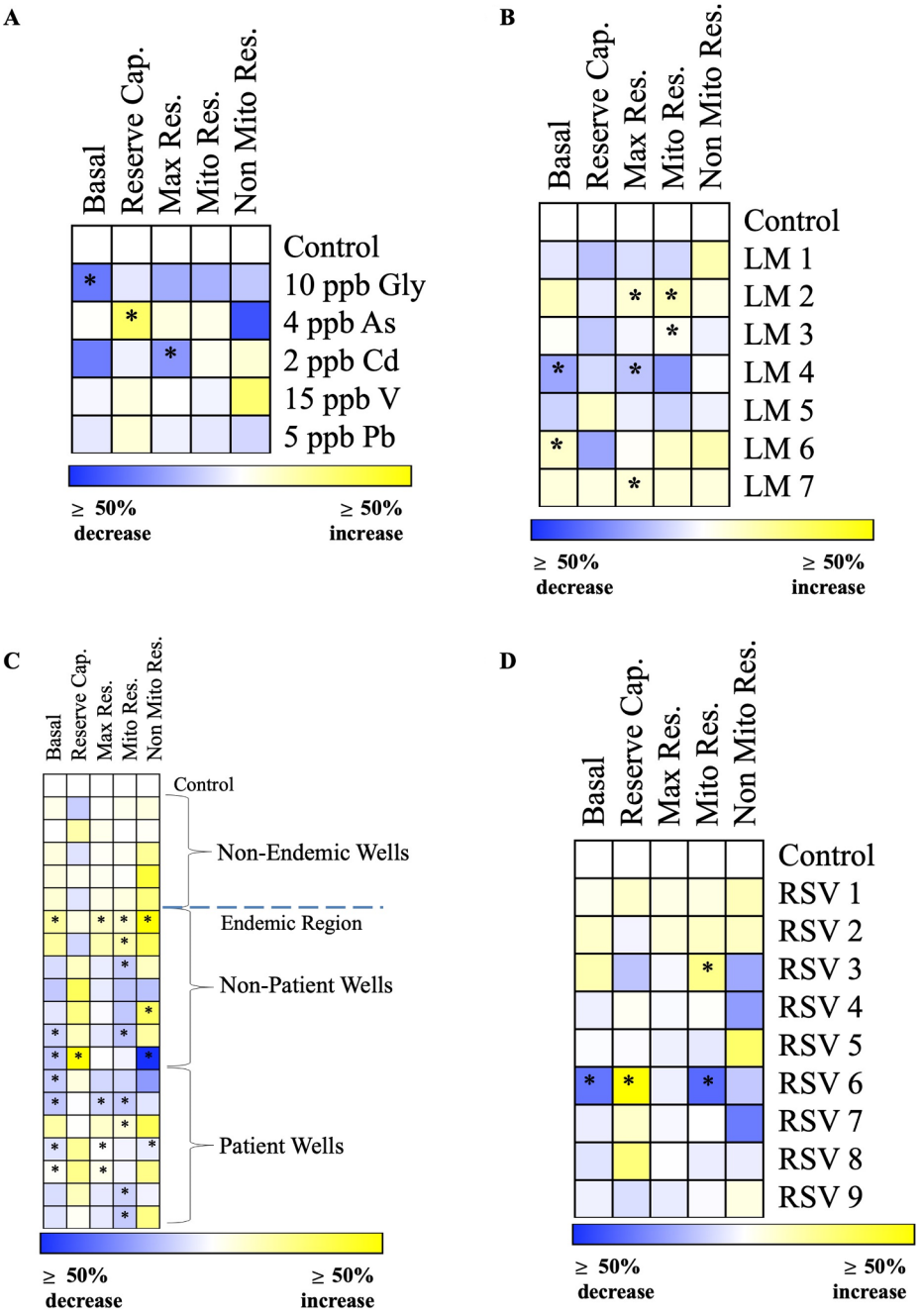


Fig. 6. Heat map representing oxygen consumption rates (OCR) in zebrafish treated with (A) individual chemicals; glyphosate (gly), arsenic (As), cadmium (Cd), vanadium (V), and lead (Pb), (B) lab mixtures (LM) 1–5, (C) well samples from non-endemic wells, non-patient wells, and CKDu patient wells and (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 ANOVA was used to test for significance between treatment and control. Significance is represented by (*), *p-value* < *0.05,* (See Supplemental Material Table S7 for exact p-values), N = 21. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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 (Fig. 5). 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 (Thijssen et al., 2007), as well as in fish exposed to heavy metal toxicity (Zn, Cd, Pb, Cu) (Galindo-Riaño et al., 2015). 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).

4.3. Kidney developmental effects 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 (Fig. 4A). 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 (Figs. 1D, 4A). A similar pattern is detected for non-endemic wells, where metal levels and *pax2a* expression changes are minimal (Figs. 1B, 4A). 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 of the treatment groups, rice field sample treatments, irrespective of the region of origin, showed muted gene expression changes (Fig. 4A). 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 (Fig. 1B). 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 (Fig. 4B). 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. A multifaceted role for agrochemicals in 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. Notably, 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 (15 ppb) and EPA health reference concentration (21 ppb) by close to 10-fold. Rat studies suggest that V is nephrotoxic (National Toxicology Program Report, 2002) but the environmentally safe levels are ambiguous for this metal. Therefore, the role of V warrants further examination in the context of CKDu. We speculate that metals in the endemic region, even at levels considered safe for drinking, can contribute to abnormal kidney development in children (Friedman and 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. We postulate that exposure to glyphosate coupled with individuals with impaired kidney development is likely to increase the initiation and progression of CKDu. (Fig. 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.

4.5. 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 (Granata et al., 2009; Emma et al., 2016; Gamboa et al., 2016).

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 (Fig. 6). 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 (Fig. 1B) 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 (Fig. 6C). As demonstrated by the non-targeted chemical analysis, patient and non-patient well samples also have specific chemical constituent profiles. 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.

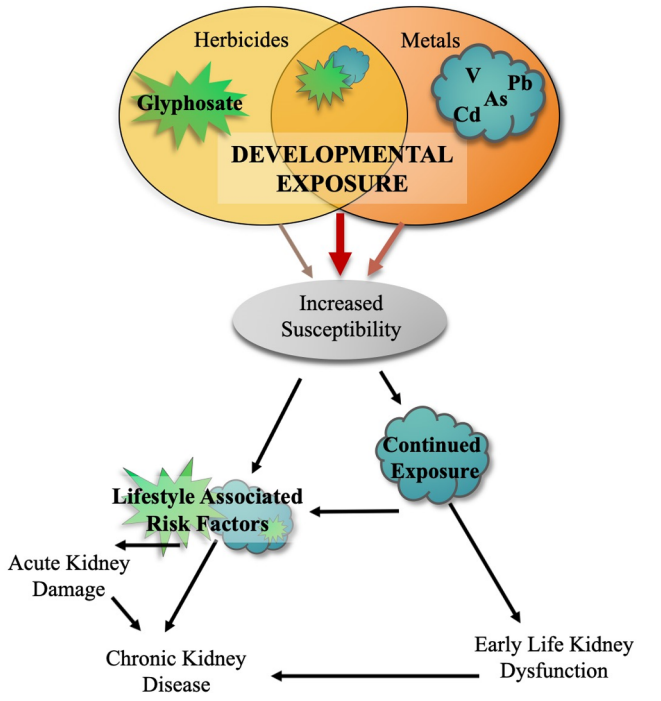


Fig. 7. Conceptual map illustrating the potential progression of impaired kidney development to CKDu, beginning *in utero* through adulthood. Children are exposed to chemicals through development, in which it is most likely that chemical and metal mixtures impact the immature kidney leading to an increased 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.

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 OCR 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 (Yamano and Morita, 1995; Meyer et al., 2018). Kidney tissues are also known to

accumulate or aggregate chemical compounds (Jin et al., 1999; Shen et al., 2011), and continuous perturbation of kidney mitochondria through exposure to chemical mixtures in the drinking water may have deleterious effects on renal structures and function. 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 (Sayanthooran et al., 2018), suggesting a potential mitochondrial etiology for CKDu.

4.6. Implications for CKDu

Our data highlight the need to examine kidney developmental effects of chemical mixtures and provide a possible mechanistic explanation for the increasing number of younger individuals with kidney dysfunction in CKDu impacted regions. 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 (Mertens et al., 2018), 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). 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 (Fig. 7). Potentially nephrotoxic factors include increased exposure to heat stress and mycotoxins which have both been postulated as contributors to the progression of CKD (Herath et al., 2018, Schulz et al., 2018, Desalegn et al., 2011). 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. 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.

CRediT authorship contribution statement

Remy Babich: Methodology, Formal analysis, Investigation, Validation, Writing - original draft, Visualization. **Jake C. Ulrich:** Methodology, Formal analysis, Investigation, Writing - original draft, Visualization. **E.M. Dilini V. Ekanayake:** Investigation. **Andrey Massarsky:** Investigation. **P. Mangala C.S. De Silva:** Investigation, Resources. **Pathmalal M. Manage:** Investigation, Resources. **Brian Jackson:** Formal analysis, Investigation. **P. Lee Ferguson:** Formal analysis, Resources. **Richard T. Di Giulio:** Formal analysis, Resources. **Iain Drummond:** Formal analysis, Resources. **Nishad Jayasundara:** Conceptualization, Methodology, Formal analysis, Investigation, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

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

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2020.106019.

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