Untargeted NMR-based metabolomics for field-scale monitoring: Temporal
reproducibility and biomarker discovery in mosquitofish (*Gambusia holbrooki*) from
a metal(loid)-contaminated wetland

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26 **Abstract**

27 There is considerable interest in applying omics techniques, which have
28 proven extremely valuable for laboratory-based toxicology studies, towards field-
29 scale ecotoxicology and environmental monitoring. Concerns that confounding
30 factors in natural ecosystems may exacerbate variability in omics datasets must be
31 addressed to validate the transition from laboratory to field. This study explores how
32 temporal variability related to seasonal and climatic trends influence qualitative and
33 quantitative metabolomics outcomes, in fish from reference and metal(loid)-polluted
34 wetlands in Australia. Female mosquitofish (*Gambusia holbrooki*) were sampled on
35 two separate occasions, from a rehabilitated tailings wetland at the site of historic
36 antimony (Sb) processing and a reference wetland with comparable water quality. The
37 first sampling coincided with greater monthly rainfall and colder water temperature,
38 whereas the second sampling was drier and water was warmer. Despite temporal
39 changes and associated differences in metal(loid) concentrations, site differences in
40 metabolite profiles were qualitatively very similar between sampling events.
41 However, quantitative differences were observed, with a greater number of
42 significantly altered metabolites identified during the second sampling event, which
43 coincided with greater metal(loid) concentrations in both water and fish. The majority
44 of identified metabolites were elevated in fish from the contaminated wetland, but
45 with notable decreases in several metabolites that are known to play a role in various
46 aspects of metal(loid) binding, detoxification and excretion. Specifically, decreased
47 aspartate, histidine, myo-inositol, taurine and choline were observed in fish from the
48 contaminated wetland, and may therefore represent a metabolite suite that is broadly
49 indicative of metal toxicity. Quantitative differences between sampling events are
50 suggestive of a dose-response relationship observable at the cellular level which, if

51 harnessed, may be useful for assigning levels of concern based on the degree of
52 change in a multi-parameter set of metabolite biomarkers.

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54 **Capsule**

55 NMR-based metabolomics of fish from reference and metal(loid)-polluted wetlands
56 demonstrate reproducibility and identify potential multi-parameter biomarker suite.

57

58 **Keywords**

59 Metals; Metabolomics; Environmental Monitoring; Biomarkers; Field sampling

Introduction

Innovative analytical technologies and computational platforms are more accessible than ever, bolstering our ability to understand chemical toxicity and effectively monitor the health of aquatic environments (Brockmeier et al., 2017). Ecotoxicology has increasingly benefitted from applications of the various omics techniques (i.e., genomics, transcriptomics, proteomics and metabolomics), due to their strength as diagnostic tools and suitability for exploring the mechanistic underpinnings of toxicological injury (Sturla et al., 2014). The majority of omics research in ecotoxicology has thus far occurred under controlled laboratory conditions, but there is growing interest and considerable scope for applying these tools in complex natural settings (Hines et al., 2007; Fasulo et al., 2012; Capello et al., 2013; Leung, 2018). However, there are some basic questions that need to be answered for omics to successfully transition from laboratory to field. Are omics tools sufficiently robust to allow meaningful relationships to be distinguished from normal background variation in physiology and metabolism (Denslow et al., 2012; Simmons et al., 2015)? Do these techniques yield reproducible results when applied to samples collected from complex natural systems (Sauer et al., 2017)? The present study aims to address these questions.

Metals and metalloids are important contaminants due to their prevalence and persistence in the environment (USEPA, 2015). They can regulate numerous cellular and biological processes, playing roles in the functioning of various enzymes, oxygen transport systems, redox reactions and more (García-Sevillano et al., 2014; Wood et al., 2011). Metabolomics is, therefore, well suited for exploring metal(loid) toxicity, since these various mechanisms are generally associated with biochemical alterations detectable at the cellular level. Indeed, several studies have observed alterations to

endogenous metabolites in aquatic species exposed to metal(loid)s (Kim et al., 2016; Li et al., 2016; Nagato et al., 2013; Spann et al., 2011; Wu et al., 2011), many of which are symptomatic of metabolic disruption and oxidative stress (Voie and Mariussen, 2010; Brandão et al., 2015; Cappello et al., 2016a,b). Nuclear Magnetic Resonance (NMR) spectroscopy is a powerful metabolomics tool that has previously been used to shed light on both metabolic disruption (Melvin et al., 2017; Ortiz-Villanueva et al., 2017) and oxidative stress (Andrisic et al., 2018; Melvin et al., 2018; Yang et al., 2017), and produces timely results due to relatively simple and fast sample processing. These attributes make NMR well suited for field-based toxicological analysis of organisms from metal(loid) contaminated sites, and a promising option for high-throughput applications in environmental monitoring.

Antimony (Sb) is an example of a priority metalloid that has been widely exploited for industrial use, with increasing global extraction to satisfy demands for its use in flame-retardants, lead hardeners, battery components, and other applications (Obiakor et al., 2018). The lowest reported median lethal concentration (96 h LC₅₀) for fish exposed to Sb is 2.46 mg/L for *Danio rerio* (Yang and Chen, 2018), however, higher toxicity thresholds have been reported for other species, ranging from 238 mg/L (48 h LC₅₀) for *Oryzias latipes* (Nam et al., 2009) to more than 530 mg/L (96h LC₅₀) for *Lepomis macrochirus* (Buccafusco et al., 1981). There have been no studies, to our knowledge, exploring sub-lethal biochemical or metabolic endpoints in fish exposed to relevant concentrations of Sb, which is a necessary step to identify potentially useful biomarkers for environmental monitoring. Importantly, the extraction and processing of Sb is commonly associated with increased concentrations of other toxic metal(loid)s, such as arsenic (As), since these elements tend to co-occur in the earth's crust (Obiakor et al., 2018). Evaluation of Sb toxicity, and indeed other

metal(loid)s, should therefore involve field-scale sampling from contaminated systems where mixture effects and other confounding variables are incorporated.

Here we apply NMR spectroscopy as a platform for untargeted metabolomics, to explore potential metabolite biomarkers in mosquitofish (*Gambusia holbrooki*) inhabiting a reclaimed wetland that was historically impacted by Sb processing activities. We further explored the reproducibility of metabolomics for field-based monitoring by incorporating temporal variation, including associated differences in fish population demographics and fluctuating environmental factors, in our sampling.

Materials and Methods

Site selection and mosquitofish sampling

The Urunga wetland (UW) is a reclaimed tailings pond that received heavy inputs from an adjacent Sb processing operation in northern New South Wales, Australia ($-30^{\circ}30'19''\text{S}$; $153^{\circ}00'35''\text{E}$), for a five-year period during the early 1970s (Warnken et al., 2017). The site was, until recently, one of the most heavily polluted natural environments in the region, but has since undergone major rehabilitation into a recreational area. The wetland supports a large population of mosquitofish (*Gambusia holbrooki*), a robust invasive species with a wide distribution throughout Australia and globally (Pyke, 2008). The presence of mosquitofish provides an opportunity to apply metabolomics to evaluate sub-lethal biochemical effects related to metal(loid) toxicity in a natural system, since this species is quite prevalent and has a wide distribution in Australia and globally. To achieve this, we identified a reference wetland (RW) in southern Queensland, Australia, containing mosquitofish and exhibiting similar water quality characteristics ($27^{\circ}57'58''\text{S}$; $153^{\circ}22'40''\text{E}$) despite having significantly different metal(loid) concentrations (Table 1). Fish were

collected from the wetlands during two separate sampling events spaced approximately four months apart, in June and October 2017. These sampling events were selected to capture seasonal differences in weather conditions, including temperature and rainfall. The first sampling (June 2017) followed a major rainfall event, whereas the second sampling (October 2017) followed several weeks with considerably less rainfall.

Eighteen fish from each wetland were collected during both sampling events, using a dip-net and immediately euthanized via submersion in a solution of buffered 3-aminobenzoic acid ethyl ester (MS-222; Sigma Aldrich, Castle Hill, Australia). All analyses were performed on juvenile female fish, to ensure that differences related to sex did not introduce additional variance in the data (Hines et al., 2007). Mosquitofish are a sexually dimorphic species so females were visually identified in the field based on the absence of gonopodium. Once euthanized, photos were immediately taken of each fish on laminated grid paper for length measurements (mm), and carcasses were transferred to 2 mL cryo-vials and snap-frozen in a Taylor-Wharton[®] liquid nitrogen dry-shipping container. Average length (mm $\pm$ S.D.) and dry-weight (mg $\pm$ S.D.) of all collected fish was 16.29 ± 2.41 and 100.22 ± 28.52 , respectively. Samples were transported to the laboratory where they were stored at -80°C until further processing and analysis. Experimental work was performed in accordance with Griffith University Animal Care Protocol ENV/03/16/AEC.

Trace element analysis of water and fish using ICP-MS

Triplicate water samples were collected during each sampling event, syringe filtered (0.45 μm mixed cellulose ester, Millipore), and acidified (2% v/v ultrapure HNO_3). Samples were analysed for a suite of trace metal(loid)s by inductively

coupled plasma – mass spectrometry (ICP-MS, Agilent 7900) with external calibration using a multi-element standard (28 element custom standard, High Purity Standards). An internal standard, consisting of Sc, Y, In, Tb, and Bi, was added in-line to the sample flow to correct for instrument drift and matrix effects. River water certified reference material (SLRS-6, National Research Council Canada) was analysed to verify the accuracy of the analysis. Typical CRM recoveries were between 80-120% of the certified value (see Supporting Table 1 and 2 for specific values).

Twelve mosquitofish were also collected from each wetland during the first sampling event, and ten during the second sampling event, for trace element analysis. The fish were lyophilised, weighed (mg), and then placed directly into clean perfluoroalkoxy (PFA) digestion vessels (MAR SXpress, CEM) with HNO₃ (sub-boiling distilled, DST-1000, Savillex), HCl (instrument quality, Seastar), and H₂O₂ (Baseline, Seastar) in a 9:2:1 ratio. The samples were digested in a microwave digestion system (Mars 6, CEM) for 15 min at 180°C (as per EPA 3052; USEPA 1996). Digested samples were diluted with deionised water (to ~2% v/v HNO₃) and analysed by ICP-MS. A dogfish liver certified reference material (DOLT-5, National Research Council Canada) was digested using the same method, and analysed to verify the accuracy of the procedure. Typical CRM recoveries were between 80-120% of the certified value (see Supplementary Table 3 and 4 for specific values).

Extraction of whole-body polar metabolites

Prior to extraction of metabolites, fish were freeze-dried and weighed (mg) before being coarsely ground with a ceramic pestle. Metabolites were extracted from ground carcasses using a modified Bligh-Dyer methanol:chloroform extraction (Bligh

and Dyer, 1959), as previously described (Lin et al., 2007; Melvin et al., 2017). Individual fish were homogenized in 400 µg/L ice-cold methanol using an Ultra-Turrax® T10 tissue homogenizer (IKA®, Selangor, Malaysi) and subsequently ultrasonicated using a Q55 probe sonicator (Qsonica, USA). Following 1 h incubation at -20°C for 1 h, 800 µL chloroform and 200 µL deionised water were added and samples were vortexed and then centrifuged (10 min, 16,000×g, at 4°C). The upper phase containing polar metabolites and lower phase containing lipids were carefully partitioned into separate glass amber vials, and the extraction was repeated with the remaining protein pellet to maximise recovery. The combined yields from two extractions were stored at -80°C until further processing.

¹H NMR spectroscopy

The extracted polar metabolites were dried using a Series II centrifugal vacuum concentrator (GeneVac Technologies, England), and then lyophilized. Polar metabolites were re-suspended in 200 µL phosphate buffer (K₂HPO₄ and NaH₂PO₄; pH 7.4) made with deuterium oxide (D₂O), which contained 0.05% sodium-3-(trimethylsilyl)-2,2,3,3-tetradeuteriopropionate (TSP) as an internal reference. Samples were vortexed and centrifuged to remove debris (5 min, 2,500×g, at 4 °C), and the supernatants were transferred to 3 mm NMR tubes using glass Pasteur pipettes.

NMR spectra were acquired using an 800 MHz Bruker® Avance III HDX spectrometer equipped with a Triple (TCI) Resonance 5 mm Cryoprobe with Z-gradient and automatic tuning and matching. The instrument was controlled using IconNMR™ automation software (Bruker Pty Ltd., Victoria, Australia) and included a SampleJet automatic sample changer. Spectra were acquired at 298°K with D₂O used for field locking and TSP (¹H δ 0.00) as an internal reference. Proton (¹H)

spectra were acquired for all samples using zg30 pulse program with 128 scans, 1.0 s relaxation delay, 8.75 μ s pulse width and a spectral width of 16 kHz (^1H δ -3.75 – 16.28). Edited ^1H - ^{13}C (Heteronuclear Single Quantum Coherence (HSQC) spectra were acquired for pooled samples with 210 scans and 128 experiments, 1.0 s relaxation delay, 8.75 μ s pulse width and spectral widths of 12.8 kHz (^1H δ -3.23 – 12.79) and 33.1 kHz (^{13}C δ -9.40 – 155.2).

Peak assignments and processing of ^1H NMR spectra

Post processing was performed with MestReNova v11.0.3 (Mestrelab Research S.L., Spain). ^1H NMR FIDs were Fourier transformed with a 0.3 Hz line broadening and HSQC spectra were processed with default Bruker post-processing parameters. All spectra were manually phase corrected, automatically baseline adjusted (ablative) and referenced to TSP (^1H δ 0.00, ^{13}C δ 0.0). Spectra were normalized to the chemical reference and binned into 0.02 ppm buckets. Integrated peak areas of each bucket were exported for further processing and multivariate statistical analysis. Metabolites were tentatively assigned to ^1H NMR spectra using Chenomx NMR suite 8.2 software (Chenomx Inc., Edmonton, Canada). Metabolite identification was further verified by comparison of HSQC NMR spectra with published reference spectra from the Human Metabolome Database (HMDB).

Statistical analysis

Prior to statistical analysis, exported ^1H NMR data were normalized to individual fish weight, the residual water signal (4.67 – 5.05 ppm) was excluded, and spectra were visually inspected to remove regions devoid of chemical signals. These processing steps are important for ameliorating the quality and validity of subsequent

multivariate analysis (Rubingh et al., 2006). Multivariate analysis was performed using MetaboAnalyst 3.0 (Xia et al., 2015; Xia and Wishart, 2016). Imported spectra were normalised via log transformation and auto-scaling, and examined by Principal Components Analysis (PCA) to explore differences in metabolite profiles between the UW and RW sites. Orthogonal Partial Least-Squares Discriminant Analysis (oPLS-DA) was subsequently applied to further characterize the separation between sites. Differences in the abundance of specific metabolites were identified using the non-parametric Wilcoxon rank-sum test, with an adjusted p-value (FDR) threshold of 0.05.

Results and Discussion

Water chemistry and ICP-MS analysis of metal(loid)s in water and fish

Water quality parameters were similar between sites during both sampling events (Table 1). However, both UW and RW had lower specific conductivity, pH, dissolved oxygen, and slightly higher turbidity during the first sampling event, which likely reflects that considerably greater rainfall occurred in the days prior to that sampling. Rainfall was approximately 62 mm/d in June (first sampling) compared to 7 mm/d in October (second sampling) in the 72 h prior to sampling in the Urunga region (www.bom.gov.au). Water temperature was also approximately 5°C higher at both sites during the second sampling due to normal seasonal warming and a transition into summer, which would result in greater evaporation.

Analysis of dissolved metal(loid)s revealed a dilution effect due to rainfall, with lower concentrations of most analytes observed in water during the first sampling event compared to the second sampling (Table 1). Trigger values established for the protection of freshwater biota (ANZECC and ARMCANZ 2000) were exceeded for Cr and Zn at the reference wetland and Cu and Zn at the

contaminated wetland during the first sampling event (Supplementary Table 1). Trigger values for Cr, Cu, Zn, As and Pb were exceeded in the contaminated wetland during the second sampling event (Supplementary Table 2). The apparent dilution effect was similarly reflected in accumulated metal(loid)s measured in fish, which similarly exhibited lower levels during the first sampling event compared to the second sampling (Table 2). Results of PCA of whole-body metal(loid) burdens in fish from the first (n = 12 per site) and second (n = 10 per site) sampling event revealed key differences between wetlands. Specifically, the sites formed distinct groupings during both events, predominantly influenced by higher concentrations of Sb, Cd, As and Cs at the contaminated wetland and marginally higher concentrations of Ba, Sr, Cu and Zn at the reference wetland (Figure 1a,b). These patterns reveal that concentrations of trace elements measured in water provide a poor representation of the potential for bioaccumulation in fish, particularly in complex environments, since the presence of certain metal(loid)s can influence the uptake, bioconcentration and depuration of others (Shuhaimi-Othman and Pascoe, 2007).

Metabolite profiles of fish from contaminated and reference wetlands

The representative HSQC spectrum including identified metabolites is provided in Supplementary Figure 1. We identified 42 metabolites from the ^1H and HSQC spectra, which largely consisted of various amino acids, carbohydrates and nucleotides. Metabolite profiles between the two wetlands exhibited clear separation during both the first and second sampling events based on unsupervised PCA (Supplementary Figure 2a,b). Supervised oPLS-DA was therefore applied, and this further revealed marked site differences during both sampling events (Figure 2a,b). Analysis by Wilcoxon rank-sum test (FDR cut-off 0.05) identified 18 metabolites

with significantly different relative abundance between sites during the first sampling event, all of which were also significantly different during the second sampling (Figure 3). An additional 16 metabolites were identified as significantly different between sites during only the second sampling event, as well as several unknown features representing unidentified metabolites (Figure 4). Site differences included representative metabolites from all classes, including amino acids, carbohydrates and nucleotides. The difference between sampling events are consistent with the higher metal(loid) concentrations observed during the second sampling, when conditions were drier. This could be taken as anecdotal evidence of a dose-response relationship in metabolite responses (Brockmeier et al., 2017), but further sampling paired with metal(loid) analysis is required to characterise such trends in field-scale sampling (García-Sevillano et al., 2014). Irrespective of statistical significance, a high level of reproducibility was observed in metabolite profiles between the two sampling events (Figures 3 and 4). Specifically, the directionality (i.e., relative increase or decrease in abundance) of site differences was consistent for the majority of metabolites between the two sampling events.

This high level of reproducibility, considering that the sampling events were spaced approximately four months apart, is promising evidence of the applicability of metabolomics towards environmental monitoring (Hines et al., 2007). Indeed, outcomes were qualitatively similar despite differences in temperature, water quality parameters, metal(loid) concentrations, and other potentially unidentified variables between sampling events (Simmons et al., 2015). However, our results also suggest that the timeframe for sampling can impact quantitative outcomes of metabolomics analysis, since a substantial number of metabolites were identified as significantly different between sites during the second sampling event that were not observed

during the first sampling (Figure 4). It may therefore not be appropriate to make direct comparisons between sites if sampling is carried out over broad temporal scales, since (as we observed) doing so can introduce seasonal variability that may influence physiology and metabolism (Myrick and Cech, 2000; Singh et al., 2013). Such variability may consequently influence the extent of sub-lethal toxicological responsiveness (Carvalho and Fernandes, 2006; Patra et al., 2015). Realistically, the most appropriate sampling strategy for field-based monitoring studies would be to collect data representative of a range of environmental variables (Bidwell and Heath, 1993).

Biomarker discovery and mechanistic insights

There are trade-offs associated with the application of metabolomics and other omics techniques towards field-based toxicity evaluation and monitoring, when compared to toxicity exposures in controlled laboratory settings. On the one hand, collecting free-living organisms from polluted ecosystems ensures a more realistic representation of the variability that is expected in nature, including that related to both environmental variables and to the co-occurrence of multiple contaminants (García-Sevillano et al., 2015). On the other, the inherent variability of natural ecological systems can also complicate efforts to attribute observed alterations as being mechanistically linked to specific stressors (Simmons et al., 2015). Despite these concerns and considerable differences in environmental parameters and metal(loid) concentrations between sampling events in the present study, distinct metabolite profiles were observed between mosquitofish inhabiting the contaminated UW and the (comparatively) uncontaminated RW. Importantly, the observed differences are quite rational based on our understanding of how metal(loid)s exert

their toxic effects, and how basic physiological processes work to mitigate harmful toxicity. As such, our results highlight the potential benefits associated with using untargeted metabolomics as a tool for field-scale toxicity evaluation and environmental monitoring.

Numerous studies have described a general increase in diverse assortments of endogenous metabolites in aquatic species exposed to meta(loid)s, with far fewer studies observing metabolites as decreasing (Brandão et al., 2015). One of the best examples are amino acids which, for example, have broadly been observed to increase following exposure to toxic metal(loid)s like As and Cd (Kumari and Ramashankar, 2016; Wu et al., 2011). Numerous amino acids were indeed increased in animals collected from UW in the present study, where both As and Cd occurred at much higher concentrations in both water and fish (Figure 1). Amino acids serve as the building blocks for proteins, so increased levels may reflect decreased protein biosynthesis, as has indeed been previously reported as a consequence of metal(loid) exposure (Kumar and Banerjee, 2016; Viarengo et al., 1980). Reductions in amino acids may be mechanistically linked with alterations to protein folding (Tamás et al., 2014) or repair capacity (Meng et al., 2017), or with alterations to the activity of various enzymes (Kumar and Banerjee, 2013; Mizrahi and Achituv, 1989). The latter is largely consistent with the overall trends observed in the present study, specifically in terms of a well-established relationship between metal(loid) exposure and increased aspartate amino-transferase (AST) enzymatic activity (Kumari et al., 2016).

Decreased aspartate would be expected as a consequence of increased AST activity, and was observed at UW in the present study (Figure 3b) and following exposure to various metal(loid)s (including both Cd and As) in previous studies (Bao et al., 2016; Jayawardena et al., 2017; Wu et al., 2011). Enzymes like AST are commonly used as

indicators of hepatic injury, including that associated with metal(loid) toxicity (Al-Asgah et al., 2015; Firat and Kargin, 2009; Omar et al., 2013), but their detection is neither cost-effective nor particularly rapid. Analysis of associated metabolites such as aspartate may therefore represent an economical option for identifying stressed organisms in metal(loid) contaminated ecosystems.

Only a handful of metabolites were significantly reduced at the UW site compared to RW, however, several of these are indicative of various processes related to metal(loid) detoxification and excretion. For example, histidine binds with a high affinity to metal(loid)s (Nair et al., 2010) and this chelation increases excretion and thus represents a protective mechanism against toxicity in fish (Jiang et al., 2016). Similar chelating properties have been demonstrated for myo-inositol in studies with mice and rats (Glover and Wood, 2008; 2007; Nadella et al., 2006), and both histidine and myo-inositol were significantly reduced in fish from UW (Figure 4j,n). Taurine mitigates the toxicity of various metals through diverse mechanisms that may relate to enhanced detoxification and protection against oxidative stress (El-Sayed et al., 2011; Ghosh et al., 2009; Oudit, 2004). Taurine also has other biological roles including involvement with maintenance of cellular homeostasis and membrane stabilisation (Brandão et al., 2015; Cappello et al., 2016). In this study, taurine was significantly lower in fish at the UW site during the second sampling event (Figure 3p), which could be a response to metal(loid)-induced oxidative stress. Finally, choline was reduced in fish from UW (Figure 4f), and is known to play an important role in the biotransformation and methylation of metal(loid)s such as As (Peraza et al., 1998; Vahter and Marafante, 1987; Vahter, 2007). These various metabolites are involved with different mechanisms that serve to decrease toxicity, reduce uptake and accumulation of metal(loid)s into tissues, or increase the rate and efficiency of

elimination. We therefore suggest that coordinated decreases in aspartate, histidine, myo-inositol, taurine and choline, may be considered as a metabolite profile that is broadly characteristic of metal(loid) toxicity in fish collected from complex environments. As previously discussed, the fact that only aspartate was significantly reduced during the first sampling is indicative of a potential dose-response relationship at the cellular level. Further research is necessary to characterise this relationship, since this could be used to assign levels of concern based on the degree of alteration observed in a multi-parameter set of metabolite biomarkers.

Conclusions

There is growing interest in using advanced analytical techniques like metabolomics for environmental monitoring and risk assessment, due to the sensitivity and comprehensive nature of the data they provide. However, there has been some hesitation surrounding the use of such tools for samples collected from complex natural environments, since they have yet to be proven sufficiently robust or repeatable to ensure the provision of meaningful information. The present study demonstrates a high level of temporal reproducibility in qualitative differences in metabolite profiles between fish collected from a reference wetland (RW) and metal(loid)-polluted wetland (UW). Similar trends were observed from sampling events spaced several months apart, and encompassing a considerable level of environmental variability associated with seasonal changes. Overall differences in metabolite profiles in fish from UW compared to RW were also consistent with expectations based on previous studies documenting biochemical alterations in fish exposed to metal(loid)s. A general increase in many amino acids combined with notable decreases in aspartate, histidine, myo-inositol, taurine and choline, may

represent a metabolic fingerprint of stress induced by sub-lethal metal(loid) toxicity. These results demonstrate great potential for using untargeted NMR-based metabolomics to achieve a cost-effective multi-parameter biomarker suite for environmental monitoring. Future research should scale-up the present study to include multiple polluted and reference sites with distinct contaminant profiles, and explore metabolomic dose-response relationships in fish exposed to complex mixtures in the laboratory.

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Table 1. Water quality parameters and metal(loid) concentrations ($\mu\text{g/L} \pm 1\text{SD}$) in water samples collected from the Urunga Wetland (UW) and Reference Wetland (RW) during the two sampling events ($n = 3$).

Parameter	Urunga Wetland		Reference Wetland	
	June 2017	October 2017	June 2017	October 2017
<i>Physico-chemical</i>				
Temperature (C°)	15.2	20.4	15.6	20.8
DO (mg/L)	5.91	6.19	4.27	6.44
EC ($\mu\text{S/cm}$)	50.7	266.3	101	267.1
pH	5.56	6.81	5.58	6.43
Turbidity (NTU)	21.6	17.2	33.4	18.2
V	1.46 ± 0.06	2.16 ± 0.08	1.84 ± 0.24	0.36 ± 0.08
Cr	0.87 ± 0.01	1.48 ± 0.05	1.39 ± 0.09	0.59 ± 0.08
Mn	14.86 ± 0.05	108.48 ± 1.58	14.72 ± 0.96	7.08 ± 0.24
Fe	820.28 ± 27.35	2745.73 ± 142.09	1657.11 ± 112.14	796.80 ± 155.04
Co	0.33 ± 0.00	0.93 ± 0.02	0.43 ± 0.03	0.18 ± 0.01
Ni	0.65 ± 0.00	1.60 ± 0.05	0.62 ± 0.03	0.36 ± 0.05
Cu	3.48 ± 0.03	14.49 ± 0.30	1.08 ± 0.05	0.53 ± 0.02
Zn	12.87 ± 4.30	27.19 ± 0.56	18.72 ± 1.54	17.66 ± 3.23
As	1.40 ± 0.07	139.33 ± 6.41	1.04 ± 0.09	0.74 ± 0.02
Se	0.26 ± 0.04	0.41 ± 0.07	0.51 ± 0.09	0.18 ± 0.10
Cd	0.01 ± 0.00	0.03 ± 0.01	0.01 ± 0.00	0.00 ± 0.00
Sb	4.29 ± 0.02	273.45 ± 2.25	0.12 ± 0.00	0.09 ± 0.02
Pb	0.65 ± 0.06	6.82 ± 0.41	0.60 ± 0.05	0.13 ± 0.03

DO = dissolved oxygen

EC = electrical conductivity

431 Table 2. Concentration of metal(loid)s ($\mu\text{g/g}$ dry weight $\pm$ 1SD) in fish collected from
 432 the Urunga Wetland (UW) and Reference Wetland (RW) during the two sampling
 433 events.

Parameter	Urunga Wetland		Reference Wetland	
	June 2017	October 2017	June 2017	October 2017
	<i>n</i> = 12	<i>n</i> = 10	<i>n</i> = 12	<i>n</i> = 10
V	0.27 ± 0.10	0.21 ± 0.05	0.26 ± 0.11	0.08 ± 0.03
Cr	0.23 ± 0.13	0.25 ± 0.17	0.25 ± 0.28	0.06 ± 0.07
Mn	70.89 ± 10.02	64.59 ± 3.62	59.30 ± 15.16	48.05 ± 17.18
Fe	132.98 ± 14.45	226.70 ± 66.14	188.58 ± 78.48	185.27 ± 60.98
Co	0.20 ± 0.04	0.15 ± 0.01	0.20 ± 0.05	0.19 ± 0.05
Ni	<LOD	0.56 ± 0.36	<LOD	0.32 ± 0.24
Cu	5.75 ± 0.63	9.43 ± 2.73	9.37 ± 2.73	10.76 ± 2.55
Zn	268.02 ± 18.27	225.56 ± 10.92	303.26 ± 33.26	376.89 ± 83.02
As	4.17 ± 0.41	8.11 ± 1.50	0.26 ± 0.10	0.49 ± 0.19
Se	2.01 ± 0.81	1.84 ± 0.25	1.57 ± 0.49	1.91 ± 0.34
Sr	116.71 ± 11.35	108.86 ± 6.75	245.33 ± 34.84	216.45 ± 52.15
Mo	0.16 ± 0.06	0.10^a	0.18 ± 0.26	0.14 ± 0.02
Ag	<LOD	0.04 ± 0.01	0.12^a	0.06 ± 0.01
Cd	0.08 ± 0.03	0.13 ± 0.02^b	0.03 ± 0.02	0.04 ± 0.01
Sb	0.31 ± 0.07	2.06 ± 0.59	0.01 ± 0.01	0.04 ± 0.05
Cs	0.14 ± 0.03	0.10 ± 0.01	0.07 ± 0.07	0.05 ± 0.02
Ba	17.30 ± 3.04	8.00 ± 1.51	92.37 ± 22.76	80.19 ± 23.35
Pb	0.32 ± 0.15	0.58 ± 0.18	0.22 ± 0.07	0.38 ± 0.17

434
 435 <LOD = below instrument limit of detection

436 ^a = all but one animal <LOD

437 ^b = calculated from only two animals above LOD

Figure Captions

Figure 1. Bi-plots from PCA analysis of metal(loid) burdens measured in mosquitofish (*Gambusia holbrooki*) collected from the Urunga Wetland (UW) and Reference Wetland (RW) during the a) first (June 2017) and b) second (October 2017) sampling events.

Figure 2. Results of supervised oPLS-DA of polar metabolites from mosquitofish (*Gambusia holbrooki*) collected from the Urunga Wetland (UW) and Reference Wetland (RW) during the a) first (June 2017) and b) second (October 2017) sampling events.

Figure 3. Polar metabolites identified in mosquitofish (*Gambusia holbrooki*) exhibiting significantly different relative abundances between the Urunga Wetland (UW) and Reference Wetland (RW) during both sampling events. Significance was determined by non-parametric Wilcoxon rank-sum test, with an adjusted p-value (FDR) threshold of 0.05.

Figure 4. Polar metabolites identified in mosquitofish (*Gambusia holbrooki*) exhibiting significantly different relative abundance between the Urunga Wetland (UW) and Reference Wetland (RW) during only the second (October 2017) sampling event. Significance was determined by non-parametric Wilcoxon rank-sum test, with an adjusted p-value (FDR) threshold of 0.05.

Figures

Figure 1. Bi-plots from PCA analysis of metal(loid) burdens measured in mosquitofish (*Gambusia holbrooki*) collected from the Urunga Wetland (UW) and Reference Wetland (RW) during the a) first (June 2017) and b) second (October 2017) sampling events.

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Highlights

- There is interest in omics for monitoring, but reproducibility and robustness must be validated
- NMR metabolomics was used for fish from metal-polluted and reference site
- Sampling incorporated temporal variability in climatic conditions
- Metabolite profiles were qualitatively similar over time
- A multi-parameter suite of metabolite biomarkers was identified

Accepted Manuscript

Untargeted NMR-based metabolomics for field-scale monitoring: Temporal reproducibility and biomarker discovery in mosquitofish (*Gambusia holbrooki*) from a metal(loid)-contaminated wetland

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