Metabolomic responses of an estuarine benthic amphipod to heavy metals at urban-runoff concentrations

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ABSTRACT

Heavy metals released from urban areas have toxic effects on aquatic organisms. Heavy metals in aquatic environments exist in various forms and methods designed to assess their effects need to consider their bioavailability. This study aimed to explore biomarkers in an estuarine amphipod, *Grandidierella japonica*, for exposure to heavy metals using metabolomics. We exposed *G. japonica* to different heavy metals and analyzed their metabolomes using high-resolution mass spectrometry. Partial least squares discriminant analysis (PLS-DA) was used to extract biomarkers of exposure for each heavy metal. As a result, three models were built to predict discrimination based on metabolomic profiles, and 70, 106, and 168 metabolites were extracted as biomarkers for exposure to Cu, Zn, and Cd, respectively. Our results suggest that PLS-DA was effective in extracting biomarkers, and this study demonstrated the usefulness of metabolomics as biomarkers.

Key words | benthic organism, heavy metals, metabolomic biomarker, urban runoff

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INTRODUCTION

Heavy metals produced by urban activities are released into aquatic environments through various pathways, including urban runoff. The concentrations of heavy metals in urban runoff have been summarized by Göbel *et al.* (2007). For example, the concentrations of copper, zinc, and cadmium are 11–2,600 μg/L, 80–6,000 μg/L, and 0.1–3.7 μg/L, respectively. The sources of these heavy metals vary: brake wear for Cu, tire wear for Zn, and wet deposition for Cd (Davis *et al.* 2001; Councell *et al.* 2004).

The toxicity of urban runoff is well-known (Boxall & Maltby 1995; Pitt *et al.* 1995; Marsalek *et al.* 1999; Kayhanian *et al.* 2008) but the primary cause of this toxicity is not yet clearly understood. In order to develop source-control strategies, the toxicity of road dust, a major component of urban runoff, has also been investigated. Road dust has been shown to be toxic for aquatic organisms (Watanabe *et al.* 2011) but it remains difficult to identify the major causes of this toxicity. Heavy metals are present in road dust at high concentrations (Wei & Yang 2010), but it is not clear whether they have severe effects on organisms. Moreover, heavy metals exist in various forms, which may alter their bioavailability and thus their toxicity to organisms.

Using biomarkers is one way of investigating how organisms respond to toxicants. Changes in the expression of biomarkers can be used to indicate changes in reactions within an organism and thus can be used to represent bioavailability. Metallothionein (MT), a well-known biomarker, is a protein that plays a crucial role in the detoxification of heavy metals in the body (Amiard *et al.* 2006). However, we cannot identify which heavy metals are responsible for changes in MT levels. It is thus important to study the possibility of using several biomarkers that are specific to each target heavy metal. To the best of our knowledge, biomarkers for specific heavy metals have not yet been reported.

Metabolomics is an 'omics approach that comprehensively measures biological responses and targets low-molecular-weight compounds such as amino acids and sugars (Viant 2007). Because this method detects compounds using mass spectrometry or nuclear magnetic resonance spectroscopy, information on various compounds can be obtained simultaneously. Therefore, it might be helpful to explore biomarkers for heavy metals by investigating changes in metabolomic profiles. Previous studies have shown that that exposure to heavy metals alters metabolomic profiles in aquatic organisms (Taylor *et al.* 2010; Nagato *et al.* 2013). This suggests that some metabolites could be used to detect heavy metal exposure, but studies on benthic organisms exposed to heavy metals have been

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limited. Benthic organisms are exposed to chemicals both in the water and solid phases, complicating the assessment of chemical toxicity. It is thus important to confirm the applicability of the metabolomic approach to biomarker exploration in benthic organisms.

The specific objective of this study was to explore biomarkers within an estuarine amphipod, *Grandidierella japonica*, which could be used to detect exposure to heavy metals using the metabolomic approach. We investigated metabolomes in amphipods exposed to heavy metals and examined those that were subsequently modified. The metabolomic profiles were analyzed using partial least squares discriminant analysis (PLS-DA) to identify biomarkers specific to each heavy metal. The PLS-DA method has been used in the chemometrics field and also to discriminate metabolomic profiles in many studies. It can handle numerous and noisy data where there is collinearity between variables unlike multiple linear discriminant analysis (Wold *et al.* 2001). *Grandidierella japonica* was selected because it inhabits a wide range of coastal areas (Marchini *et al.* 2016) and is an acceptable species for toxicity assessment (ASTM 2008). Copper, zinc, and cadmium were selected as representative heavy metals contained in urban runoff, and the concentration of Cu and Zn used in the exposure tests was similar to that found in urban runoff (Göbel *et al.* 2007).

MATERIALS AND METHODS

Test organisms and sediment samples

Grandidierella japonica was collected from a tidal flat at the mouth of Nekozane River in Chiba, Japan, and cultured as described previously (Yanagihara *et al.* 2017). Sediment samples were collected from a tidal flat at Komatsugawa River in Tokyo, Japan, using hand shovels and used for culturing and toxicity testing. The collected sediment was sieved (mesh size 2.0 mm) and the amount of organic matter was found to be 3.52 ± 0.39% (regarded as equivalent to loss on ignition at 660 °C). The sediment was stored in a plastic bucket at 4 °C until used.

Four-day exposure to heavy metals

We conducted four-day exposure tests following a standardized method (US EPA 1994). Ten *G. japonica* juveniles were exposed to Cu, Zn, Cd, and a mixture of the three elements (Mix) in separate beakers for four days. The exposure groups consisted of two levels, Low and High. The nominal concentrations of the heavy metals in the test water were 10% of LC50 for the Low groups and 100% of LC50 for the High groups. The concentrations of LC50 for copper, zinc, and cadmium have been reported to be 250 μg/L, 1,560 μg/L, and 340 μg/L, respectively (Boese *et al.* 1997; King *et al.* 2006). For the Mix group, the three heavy metals were mixed at the same concentrations as the other groups. First, three stock solutions were made by dissolving $CuSO_4 \cdot 5H_2O$, $ZnSO_4 \cdot 7H_2O$, or $Cd(NO_3)_2 \cdot 4H_2O$ in Milli-Q water. The test water was then prepared by diluting each stock solution with artificial sea water.

All beakers contained 120 mL of artificial sea water (for the control group) or test water (for the exposure groups) and 30 mL of sediment collected from the environment as described above. There were three beakers for the control group and five each for the Low and High groups in each exposure test. After a day of incubation (25 ± 1 °C, 16 h light/8 h dark cycle), 10 juveniles were added to each beaker. The pH and conductivity were measured daily through the tests. The range of pH value was 7.3–8.1 and the range of conductivity was 38.4–45.3 mS/cm. Four days later, the number of surviving amphipods was recorded and the survivors from each beaker were randomly separated into two groups. Each group contained 3–6 individuals and was transferred into a vial, flash frozen, and stored at −80 °C until metabolome extraction.

At the end of the four-day tests, test water collected from the beakers was filtered using a 0.45 μm filter (PTFE hydrophilic filter, ADVANTEC) and the concentrations of the heavy metals in the water were analyzed using inductively coupled plasma mass spectrometry (ICP-MS). The measured heavy metal concentrations at the end of the tests were lower than the nominal initial concentrations (Table 1). Comparing the concentrations analyzed at Day −1 and those at Day 4, most of the heavy metals were assumed to have transferred to the solid phase. Moreover, the concentrations of heavy metals in the test water of the control group were higher than those of the exposure group in some cases. This suggests that there may be active adsorption and desorption of heavy metals between the water and solid phases. Considering the reported concentrations in urban runoff (Göbel *et al.* 2007), the initial concentrations of Cu and Zn in this test were similar to the levels typically found in urban runoff.

Extraction and analysis of metabolomes from amphipod samples

Metabolomes were extracted from the amphipods after the four-day exposure testing and analyzed using an Orbitrap

Table 1 | Concentrations of dissolved Cu, Zn, and Cd during 4-day exposure tests

		Conc. of Cu in Cu exposure test [μg Cu/L]	Conc. of Zn in Zn exposure test [μg Zn/L]	Conc. of Cd in Cd exposure test [μg Cd/L]	Conc. of Cu, Zn, and Cd in Mix exposure test [μg/L]		
					Cu	Zn	Cd
Control	Day 4	7.87 ± 2.9	15.4 ± 9.9	ND	1.48 ± 0.36	50.9 ± 2.1	0.427 ± 0.15
Low	Day −1	21.9	1.57×10^2	31.5	18.8	92.3	32.0
	Day 4	5.44 ± 2.5	20.6 ± 17	1.39 ± 0.48	2.00 ± 0.51	15.6 ± 24	0.757 ± 0.14
High	Day −1	1.14×10^2	1.40×10^3	3.04×10^2	51.3	1.39×10^3	3.04×10^2
	Day 4	8.64 ± 3.7	27.6 ± 6.2	15.6 ± 6.9	2.38 ± 0.89	27.3 ± 16	4.01 ± 1.2

The concentrations on Day 4 were measured in triplicate and expressed as mean ± SD.

mass spectrometer (Exactive, Thermo Fisher Scientific) following Yanagihara *et al.* (2017). The molecular weight and signal intensity of the detected metabolites were obtained through an analysis using SIEVE software (Version 2.2 SP2, Thermo Fisher Scientific). The average signal intensity for each metabolite was obtained from two groups consisted of amphipods collected from the same test beaker. The signal intensity values were pre-processed using the following process. First, the intensity was normalized to allow the summation of intensity within a sample. Second, the fold change was calculated by comparing the normalized intensity between the control and exposure groups. Finally, the log base 10 of the fold change was auto-scaled. This process is explained in more detail in the supplementary materials alongside the numerical formulas (available with the online version of this paper).

Partial least squares discriminant analysis (PLS-DA) was conducted using the scaled values to observe metabolomic patterns. Three models were constructed based on the PLS-DA for all collected metabolomic data. Each model was designed to discriminate between the metabolomic profiles obtained from the amphipods exposed to copper (model Cu), zinc (model Zn), and cadmium (model Cd) using categorical data. All models contained data from the Mix group because this group was designed to include exposure to all three heavy metals. The number of latent variables was selected following Wold's R criterion (Wold 1978). Q^2 values were calculated for all models to assess their predictive power (Eriksson *et al.* 2006) and the error rate was estimated by comparing the Y variables with the predicted values. The error rate was defined as the ratio of the number of samples which were predicted to belong to the wrong group to the total number of samples. Variable importance on projection (VIP) values were obtained for all compounds in each model and the VIP values were used to select variables which contributed to the difference between the control and exposure groups. Chemical names and formulas were taken from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, based on monoisotopic mass (mass error <10 ppm). Compounds which had a KEGG ID were mapped onto a KEGG pathway (dpx; *Daphnia pulex*) to examine responses related to the compounds.

RESULTS AND DISCUSSION

Metabolomic profiles of *G. japonica* exposed to heavy metals

Through Orbitrap MS analysis, 3,444 metabolites were detected in at least one sample group containing *G. japonica* metabolomes. Putative names and chemical formulae were assigned to 1,469 of these. Based on KEGG pathway analysis, 329 compounds were mapped onto one or more of the 181 pathway types. Dominant pathways included metabolic pathways (ko01100), microbial metabolism in diverse environments (ko01120), and biosynthesis of secondary metabolites (ko01130). Because the pathways used are for *D. pulex*, some compounds assigned with a KEGG ID were not found in the pathways.

Exploring biomarkers for exposure to Cu, Zn, and Cd by constructing PLS-DA models

We conducted PLS-DA to identify important metabolites that can be used to distinguish the effects of different heavy metals. Three models were constructed with all detected metabolites: a model for Cu exposure (model Cu), Zn exposure (model Zn), and Cd exposure (model Cd). The fitted models consisted of 13, 6, and 4 latent variables selected by the R criterion (Wold 1978) and had Q^2 values of 0.88, 0.76, and 0.88, respectively (Table 2). Given that these Q^2 values exceeded 0.5, the models showed good predictive power (Eriksson *et al.* 2006). This suggests that the

Table 2 | Properties of the PLS-DA models

	Model	# of metabolites	# of latent variables	Q^2 value	Error rate of prediction
Models built using all metabolites	Model Cu	3,444	13	0.88	0
	Model Zn		6	0.76	0
	Model Cd		4	0.88	0
Models built using selected metabolites	Model Cu	115	5	0.79	0
	Model Zn	165	7	0.90	0
	Model Cd	214	4	0.91	0

metabolomic profiles obtained from *G. japonica* contained useful information for the detection of heavy metal exposure. The VIP values for each metabolite were calculated for models Cu, Zn, and Cd and it was found that 115, 165, and 214 metabolites, respectively, had VIP values higher than 1.5. Those metabolites were regarded as candidate biomarkers because they contributed to the separation of the exposure groups from the control group. Some of these metabolites were found in more than one of the three models (Figure 1), with 16 found in all three models, thus acting as candidate biomarkers for heavy metal exposure. In total, eight metabolites had intensity values that increased in the Cu, Zn, Cd, and Mix groups compared to the control group, indicating that these metabolites exhibit the same characteristics after exposure to heavy metals. However, the change in the intensity of the other eight metabolites was not consistent among the exposure groups. These results suggest that the combined effect of different heavy metals may not be explained by combining the three models.

New PLS-DA models were constructed using the biomarkers, with 115, 165, and 214 variables for models Cu, Zn, and Cd, respectively. As shown in Table 2, all models had sufficient predictive power with fewer metabolites. This indicates that the metabolites with higher VIP values might be capable of working as biomarkers. The information for all these metabolites is summarized in Tables S1–S3 in the supplementary materials (available with the online version of this paper), while the metabolites with the five highest VIP values are presented in Table 3. The tentative chemical names and formulas for these metabolites were assigned using the database, but some of them were not known. Because each model included data from the Mix group, we proposed 70, 106, and 168 metabolites as possible specific biomarkers for Cu, Zn, and Cd exposure, respectively, from the Venn diagram (Figure 1). Although a previous study that adopted metabolomics and PLS-DA also succeeded in finding novel biomarkers based on VIP values (Yi *et al.* 2014), few studies have discussed the optimal approach to biomarker discovery. More discussion is required to establish a complete methodology, but our results prove the effectiveness of the statistical analysis used in our study. Figure S1 (available online) shows the fold change

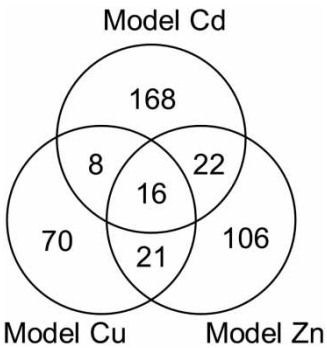

Figure 1 | The commonality of metabolites which had VIP values higher than 1.5 in each model.

Table 3 | The compounds considered as candidate biomarkers of Cu, Zn, Cd, and Mix exposure

Model		VIP value	Molecular weight	Putative name	Putative formula
Model Cu	1	2.15	542.3230	–	–
	2	2.13	234.6631	–	–
	3	2.06	542.3231	–	–
	4	2.03	142.0751	Piracetam	$C_6H_{10}N_2O_2$
	5	2.02	111.0799	Histamine	$C_5H_9N_3$
Model Zn	1	2.47	250.9722	–	–
	2	2.37	221.8215	–	–
	3	2.29	248.9752	–	–
	4	2.29	187.1936	–	–
	5	2.28	248.9752	–	–
Model Cd	1	2.49	414.3204	–	–
	2	2.17	367.0416	(6R,7R) – 7-{[Amino(phenyl) acetyl]amino} – 3-chloro – 8-oxo – 5-thia – 1-azabicyclo[4.2.0] oct – 2-ene – 2-carboxylic acid	$C_{15}H_{14}ClN_3O_4S$
	3	2.15	168.0283	Uric Acid	$C_5H_4N_4O_3$
	4	2.14	141.9035	–	–
	5	2.11	524.2811	–	–

values of the metabolites listed in Table 3. Figure S1 indicates that any single metabolite could not be used to distinguish the toxicant despite its high VIP value, and combinations of several metabolites were required for the biomarkers of exposure for heavy metals. The two pairs of two metabolites, No. 1 and No. 3 in the Cu model and No. 3 and No. 5 in the Zn model, might be identical compounds because they showed similar values in molecular weight and fold change. However, the methodology we employed did not allow us to conclude the identity of the pairs.

The metabolomic biomarkers selected in this study are expected to detect exposure to each heavy metal in urban runoff. However, obtaining biomarkers for other toxicants not tested in this study remains a challenge. In the future, the reproducibility of these candidate biomarkers needs to be tested using urban runoff samples to discuss the availability of the biomarkers for detection of heavy metals.

CONCLUSIONS

We explored biomarkers to detect exposure to heavy metals in the estuarine amphipod *G. japonica* using a metabolomic approach. This study was the first to report metabolomic profiles from amphipods exposed to heavy metals, and the results illustrate the possibility of assessing the toxic effects of heavy metals in urban runoff using metabolomic biomarkers. By obtaining and measuring these biomarkers, the effects of toxicants can be assessed while reflecting bioavailability. Although the applicability to the assessment of the toxicity of road runoff needs to be examined, the use of sets of biomarkers could also be useful for identifying major toxicants in road runoff based on biological effects.

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