Original Article

Effect of Control Sediment Composition on the Metabolomic Responses of *Grandidierella japonica* during Toxicity Testing Using Copper at an Acutely Toxic Level

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ABSTRACT

Control sediment in whole-sediment toxicity testing has a wide range of properties, but the effects of the sediment composition at the molecular level have not been investigated. Therefore, we adopted a metabolomic approach to assess the effects of sediment composition at the molecular level, in particular the effects of control sediment composition on the metabolomic responses of test species under control and test conditions. The estuarine amphipod *Grandidierella japonica* was incubated in test chambers with four types of sediment, and the metabolomes of *G. japonica* were analyzed using a high-resolution mass spectrometer. Metabolomes of *G. japonica* exposed to a copper (Cu) solution were also obtained in the same way. We found that the metabolomic profiles were affected by the composition of the control sediment and exposure to Cu and that the exposure to Cu caused a more dominant influence than the change in the compositions of control sediments. However, the metabolites that had key roles to discriminate between the control and Cu exposure groups differed between the tested control sediments. Our results suggest that the effects of control sediment are smaller than those of toxicant exposure, and toxicity assessment using metabolomics is possible regardless of the sediment type.

Keywords: benthic organism; control sediment; metabolomics; sediment toxicity test

INTRODUCTION

The toxicity of sediment for aquatic organisms has traditionally been assessed using bioassays, of which there are several standardized methods available. In principle, however, control sediment needs to be selected to determine the toxicity of the test sediment. According to a proposed test protocol for benthic amphipods [1], sediment that is to be employed as a negative control needs to be free from contamination and be suitable for use in routine tasks. After the testing period, mortality and/or the inhibition of growth or behavior in the test organisms are observed and the outcomes of the control and test sediments compared.

In these tests, the control sediment is not supposed to affect the results itself, but previous studies have indicated that the

composition of a sediment has an influence on the behavior or survival of organisms. For example, the size distribution of sediment affects the burrowing behavior of *Hyaella azteca*, and the number of burrowing individuals decreases when the ratio of sand increases [2]. A higher proportion of sand also leads to greater body length and survival in *Chaetocorophium* *ef. Lucasi* [3]. Another study which investigated the effects of the composition of sand, silt, and clay in control sediments did not find significant changes in the survival of *H. azteca* [4]. These results suggest that the proportion of sand and the quality of the silt or mud in sediments might be important factors that influence endpoints in toxicity testing. Given this, it is possible that sediment composition may have unrecognized effects on test organisms in toxicity tests, thus interfering with the interpretation of the results. Therefore, it

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is necessary to investigate the effects of sediment composition at a molecular level to determine whether they impact on toxicity testing.

Metabolomics – the study of metabolites, which are molecules with a low molecular weight that are typically the intermediates and products of metabolic reactions [5] – has been applied to various aquatic organisms to detect changes in the responses of test organisms. Metabolomics enables physiological status to be observed using non-targeted analysis with the aid of mass spectrometry or nuclear magnetic resonance spectroscopy. When metabolomics is implemented for toxicity assessments, a comparison between control and test cases is essential. Several past studies have reported differences in the metabolomic profiles of control and exposure groups, such as a study that compared changes in the metabolomes within *Daphnia magna* after exposure to heavy metals and insecticides [6]. A review has also summarized instances in which the metabolites of many species were significantly affected by exposure to heavy metals [7]. These metabolites can be used as biomarkers to detect adverse effects on organisms.

Assessing toxicity using metabolomic responses is difficult when other environmental stresses are present, as they will also influence these responses [8,9]. For example, it has been reported that changes in temperature [10–13], salinity [13], and pH [10,11], starvation [12,14,15] and hypoxia [13,15] affect metabolomic responses within aquatic organisms. In addition, control sediments which contain smaller amounts of organic matter, which acts as a food source, may affect metabolomic profiles during toxicity testing. As such, separating the effects of toxicants from those of environmental stressors is crucial when utilizing metabolomics in toxicity assessment. A study that assessed the effects of sediments collected from a harbor on the metabolomes within amphipods reported the clustering of the sediments based on the characteristics of metabolomes, but the effects of specific toxicants were unclear because of the complexity of the tested sediments [16]. It is therefore necessary to discriminate the effects of control sediments and those of target toxicants on metabolomic profiles. However, the number of comparative studies that have addressed this need is limited.

This study aimed to assess the effects of sediment composition on the metabolomic profiles of the estuarine amphipod *Grandidierella japonica* and to compare this with the effects of copper (Cu) exposure. We obtained the metabolomic profiles of *G. japonica* from four types of control sediment using a high-resolution mass spectrometer. The same four sediments were also used for Cu exposure testing and differences

in the metabolomic profiles due to sediment composition and Cu exposure. An inhabitant of coastal waters, *G. japonica* is recommended for use in toxicity testing protocols [17]. Copper was selected as the reference toxicant because it has been reported that it has adverse effects on amphipod species on a molecular [18] and individual level [19].

MATERIALS AND METHODS

Test organisms and sediment samples

Grandidierella japonica was collected from a tidal flat at the mouth of the Nekozone River in Chiba, Japan, in 2009 as described previously [20]. Sediment samples were collected as described in a previous study [20] and used for culturing and toxicity testing. We tested the following four types of sediment: the same sediment as that used for culturing (ES), quartz sand (QS; WS-10BR, HARIO), artificial sediment A (AS-a), and artificial sediment B (AS-b). The composition of the sediments is shown in **Table 1**. AS-a was produced following a previous report [21], and AS-b was prepared following OECD test No. 218 [22], which was developed for toxicity testing with *Chironomus* spp.

Four-day exposure to heavy metals

We conducted four-day exposure tests using a sediment sample from one of the four types of sediment as a control. We followed a test method [1] and Cu was used as the reference toxicant. We set up both control and exposure groups for each sediment type, with three beakers used for the control groups and five for each sediment type. A stock solution of Cu was prepared by dissolving $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in deionized water. The contaminated water for the toxicity testing was then prepared by spiking artificial seawater with the stock solution at 250 $\mu\text{g Cu/L}$, which was the same concentration as the LC50 concentration [19].

Each of the beakers contained either 120 mL of artificial seawater for the control groups or 120 mL of test water for the Cu exposure groups and 30 mL of ES, 30 g of QS, 30 g of AS-a, or 30 g of AS-b. After one day of incubation ($25 \pm 1^\circ\text{C}$, 16 h light/8 h dark cycle), 10 *G. japonica* juveniles were added to each beaker. Four days later, the number of surviving juveniles was recorded and the surviving individuals from each beaker were separated into two groups randomly. The mortality in the exposure test was 0%, 6.4%, 0%, and 6.7% in ES, QS, AS-a, and AS-b group respectively, although we have expected 50%. The groups contained between 3–5 individuals each and were transferred into a vial before being flash-frozen and stored at -80°C until metabolome

Table 1 Composition of each sediment (dry-w/w%)

	ES ^a	QS	AS-a	AS-b
Sediment collected from the river	100	0	0	0
Quartz sand	0	100	80.3	75
Kaolin	0	0	14.2	20
α -Cellulose	0	0	5	0
Dolomite	0	0	0.5	0
Humic acid	0	0	0.01	0
Peat moss	0	0	0	5

^a The loss on ignition of ES was $3.52 \pm 0.39\%$ (regarded as equivalent to loss on ignition at 660°C) [23].

extraction.

Extraction and analysis of the metabolomes from the amphipod samples

Metabolomes were extracted and analyzed using an Orbitrap mass spectrometer (Exactive; Thermo Fisher Scientific, Massachusetts, USA) following the method outlined in a previous report [23]. Briefly, the extraction was conducted using methanol, water, and chloroform, and the extracts were analyzed by flow-injection. The signal intensity values obtained using SIEVE software (Version 2.2 SP2, Thermo Fisher Scientific) were pre-processed as described in the previous research [23].

Principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were conducted using the pre-processed signal values. First, we conducted PCA and PLS-DA using data from the control groups only. The signal intensities were normalized and auto-scaled without transformation to fold change values because the exposure groups were not involved in this analysis. PLS-DA models were constructed to extract characteristic metabolites from each sediment type. Four models, Model_{ES}, Model_{QS}, Model_{AS-a}, Model_{AS-b} were built to separate the ES, QS, AS-a, and AS-b groups, respectively, from the other three groups. Second, PCA and PLS-DA were performed using the pre-processed signal data for all groups. We built four models, Model_{CuES}, Model_{CuQS}, Model_{CuAS-a}, Model_{CuAS-b} to discriminate between the control and Cu exposure groups for each sediment type. The number of latent variables was selected by following Wold's R criterion [24]. Q^2 values were calculated for all models to assess their predictive power, and variable importance on projection (VIP) values were obtained for all compounds within each model (see Supplementary Materials for details). The VIP values were used to identify variables that acted as biomarkers. The metabolites that have higher VIP values are considered as important

variables, and the threshold values used in common is 1 [25]. We defined the threshold as 1.5 to show the most important ones in our results. Chemical names and formulas were taken from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database [26] based on monoisotopic mass (mass error < 10 ppm), as described previously [23]. Then, the compounds which had a KEGG ID were mapped onto the KEGG pathway (dpx; *Daphnia pulex*) to interpret the reactions of the metabolites. Because the information of the *G. japonica* is limited, we adopted the information of *D. pulex* to interpret the metabolomic reactions in *G. japonica* based on the idea that metabolomic reactions have many shared reactions among species [5].

RESULTS AND DISCUSSION

Comparison of the metabolomic profiles of *G. japonica* between different sediments

The number of metabolites detected from one or more samples was 4,060. PCA analysis using the intensity values of the metabolites in the control groups in all sediment groups was conducted. A PCA score plot of PC1 and PC2 exhibited a clear difference between ES and the other groups (Fig. 1). The variance in the metabolomic profile of the ES group was explained by PC2 while PC1 appeared to be contributed to the explanation of variation in all samples. The other groups (QS, AS-a, and AS-b) were not separated by PC1 or PC2. However, PC3 and PC4, which made a lower contribution to the explanation of variance, appeared to be involved in separating the rest of the three groups. Therefore, this PCA showed that the principal components could explain the differences between control sediments.

The PCA thus revealed that incubation in the ES sediment led to *G. japonica* metabolomes with different characteristics to those of the other three sediment types. We hypothesized that the organic carbon content of the tested sediments may

Fig. 1 PCA score plot for control group samples incubated using four different types of sediments. Each plot represents a beaker in the test.

be a major factor in determining metabolomic profiles because it can promote metabolic reactions. Indeed, previous studies have shown that starvation caused disturbances in the metabolomic profiles of amphipods [14,27], suggesting that compounds typically derived from external food sources such as lipids were exhausted. In this study, ES, AS-a, and AS-b sediment contained the organic matter; however, the separation between these three groups and QS sediment group was not apparent. Therefore, the existence of the organic matter itself might not have affected the metabolomic responses dramatically, but other factors of the ES samples such as the concentration of nutrients might have had more significant effects on any metabolomic changes.

We conducted PLS-DA to identify the important metabolomes driving any differences in the characteristics of the

metabolomic profiles. Four models (Model_{ES} , Model_{QS} , $\text{Model}_{\text{AS-a}}$, $\text{Model}_{\text{AS-b}}$) were built; the parameters of those models are shown in **Table 2**. The number of latent variables, Q^2 , R^2X , and R^2Y were used to assess the predictive power of the models. All of the models exhibited sufficient predictive power with high Q^2 values, indicating that the metabolomic profiles could be discriminated based on the sediment type. This suggests that metabolomes might respond to the composition of the control sediments.

We also calculated the VIP values for each model, and the metabolites which contributed to this separation between the different sediment types were extracted. Those metabolites were considered to explain the differences between the sediments. Names or chemical formulas were assigned to the metabolites where possible (**Table 3**), but some could not be identified. The analysis by SIEVE software did not distinguish the metabolites that had similar molecular weight values, and those metabolites were shown as different compounds in **Table 3**. Additionally, the metabolites which had VIP values higher than 1.5 were selected for the comparison between the four types of sediment. As shown in **Fig. 2**, these metabolites tended to differ between the four models, with no single compound being selected for all four models. This could be because we selected 1.5 as the threshold, or that the differences in sediment composition affected the metabolomic responses and the metabolites that were related to the separation between two groups. In particular, the Venn diagram shown in **Fig. 2** shows that there were 143, 192, 352, and 210 metabolites specific to each group, and they might have a strong relationship with a particular sediment composition. According to the KEGG database, some of these compounds mapped onto the metabolic pathways of *D. pulex*. Some pathways, including the arginine and proline metabolism, appeared to overlap between some groups, but

Table 2 The parameters for the PLS-DA models.

	Discriminated groups	# of latent variables	Q^2 value	R^2X	R^2Y
Model_{ES}	ES and the others	12	0.99	88.93	100
Model_{QS}	QS and the others	5	0.93	65.05	98.55
Model_{AS-a}	AS-a and the others	14	0.95	91.93	100
Model_{AS-b}	AS-b and the others	7	0.86	74.48	99.05
Model_{CuES}	Ctr and Cu (ES group)	3	0.77	75.17	99.88
Model_{CuQS}	Ctr and Cu (QS group)	13	0.90	94.64	100
Model_{CuAS-a}	Ctr and Cu (AS-a group)	2	0.68	76.60	98.0

Table 3 Significant metabolites used to discriminate between the control and the exposure group for each model.(1: Model_{ES}, 2: Model_{QS}, 3: Model_{AS-a}, 4: Model_{AS-b}, 5: Model_{CuES}, 6: Model_{CuQS}, 7: Model_{CuAS-a})

<i>m/z</i> ^a	Compo-nent MW ^b	VIP	Compo-und MW ^c	Putative formula ^d	Putative name ^e	Δppm ^f
339.0185	340.0258	2.23	340.0269	C ₁₆ H ₁₄ Cl ₂ O ₄	Diclofop-methyl	3.24
339.0184	340.0256	2.23	340.0269	C ₁₆ H ₁₄ Cl ₂ O ₄	Diclofop-methyl	3.82
341.0153	342.0226	2.22	342.023	C ₁₇ H ₁₁ ClN ₂ O ₂ S	Tilomisolet	1.17
1) 365.1131	366.1203	2.20	366.1191	C ₁₈ H ₁₇ F ₃ N ₂ O ₃	(E)-5-[[[3-Pyridinyl][3-(trifluoromethyl)phenyl]methylene]amino]oxy]pentanoic acid	3.28
307.1000	308.1073	2.20	308.1049	C ₁₉ H ₁₆ O ₄	Warfarin	7.79
229.1546	228.1474	2.59	-	-	-	-
229.1547	228.1474	2.59	-	-	-	-
2) 229.1547	228.1474	2.59	-	-	-	-
229.1547	228.1475	2.59	-	-	-	-
174.1602	173.1529	2.57	-	-	-	-
265.0371	266.0444	3.43	-	-	-	-
349.0754	350.0827	3.42	-	-	-	-
3) 309.0190	310.0263	3.41	310.0251	C ₁₄ H ₁₅ O ₂ PS ₂	Edifenphos	3.87
309.0189	310.0262	3.41	310.0251	C ₁₄ H ₁₅ O ₂ PS ₂	Edifenphos	3.55
425.0616	424.0543	3.41	-	-	-	-
206.9521	207.9594	2.55	-	-	-	-
335.0597	336.0669	2.54	-	-	-	-
4) 335.0597	268.0795	2.54	268.0794	C ₉ H ₁₆ O ₉	3-Hydroxy-2-(α-D-mannopyranosyloxy) propanoic acid	0.37
335.0597	336.0670	2.54	-	-	-	-
273.0440	274.0513	2.53	-	-	-	-
181.0288	147.9953	1.63	-	-	-	-
153.0340	152.0267	1.63	-	-	-	-
5) 250.9922	249.9850	1.62	249.9871	C ₁₀ H ₆ N ₂ O ₂ S ₂	(2Z)-2-(4-Oxo-1,3-thiazolidin-2-ylidene)-1,3-benzothiazol-6 (2H)-one	0.57
126.0527	125.0455	1.62	-	-	-	-
167.0495	134.0161	1.62	-	-	-	-
449.2680	450.2753	2.62	450.2784	C ₃₀ H ₃₄ N ₄	(2S,3R,12bS)-2-[[[(1S)-2-Methyl-2,3,4,9-tetrahydro-1H-beta-carbolin-1-yl]methyl]-3-vinyl-1,2,3,4,6,7,12,12b-octahydroindolo[2,3-a]quinolizine	6.88
6) 189.2075	188.2002	2.54	-	-	-	-
277.0728	278.0800	2.48	-	-	-	-
254.9973	256.0046	2.43	256.0042	C ₁₀ H ₈ O ₆ S	4-Methyl-2-oxo-2H-chromen-7-yl hydrogen sulfate	1.56
127.0007	128.0080	2.42	-	-	-	-
192.9583	193.9656	1.68	193.9657	C ₃ H ₇ CaO ₅ P	Fosfomycin calcium monohydrate	0.52
194.9553	195.9626	1.66	-	-	-	-
7) 281.1498	280.1425	1.66	-	-	-	-
273.6864	272.6792	1.65	-	-	-	-
342.2388	278.2231	1.65	278.2246	C ₁₈ H ₃₀ O ₂	5b-estran-3a,17b-diol	5.39

^a Detected *m/z* value of the component.^b Molecular weight value of the detected compound calculated by SIEVE software considering *m/z* value and adduct ions.^c Molecular weight of the compound found in KEGG database using the component MW.^d Putative formula of the compound found in KEGG database.^e Putative name of the compound found in KEGG database.^f Difference between component MW and compound MW [ppm].

Fig. 2 The commonality of the metabolites that had VIP values higher than 1.5 between the four models using control data.

it was difficult to identify all of the pathways for the selected metabolites. It suggests that we should consider changes in both the metabolites and the pathways and that it is important to continue to add information to the database to allow more accurate analysis.

Effects of sediment composition and Cu exposure on the metabolomes of *G. japonica*

We also compared the metabolomic profiles of *G. japonica* exposed to Cu in different sediments. Though PC1 did not explain differences between the groups, PCA using preprocessed signal values lead to the separation of the control and exposure groups by PC2, except for the AS-b sediment (**Fig. 3**). In other words, when the ES, QS, or AS-a sediments were used, the metabolomic profiles of the Cu exposure groups differed from those of the control groups. It indicated exposure to Cu produced clear differences in the metabolome of *G. japonica* regardless of the effect of sediment type. However, the metabolomic profiles obtained from the AS-b sediment did not discriminate between the control and Cu exposure groups. This might be because the Cu was adsorbed on the peat moss layer that formed on the top of the AS-b during the testing. Peat moss has a metal-binding capacity, and the adsorption of Cu has been reported. A previous study reported that the uptake of Cu reached 0.4 mmol/g peat [28]. It is difficult to estimate the adsorption mass from the previous report because adsorption is affected by pH and the concentration of Cu in the overlying water, but it can be assumed that AS-b had a larger capacity to bind metals.

PCA was then conducted after the removal of the AS-b sediment samples to confirm that the metabolomic profiles observed after Cu exposure could be discriminated from those of the control groups for the other three sediment

Fig. 3 PCA score plot for samples from the control and Cu exposure groups using four different types of sediment. Each plot represents a beaker in the test.

types. **Figure 4** shows the results of clustering analysis (Ward's method) based on the scores along PC1 and PC2. The separation between the control and Cu exposure groups was clear and only one sample from the control group was grouped with the Cu exposure samples. This indicates that the effects of copper exposure caused metabolomic changes in a similar way under ES, QS, and AS-a conditions. These results derive from unsupervised analysis, indicating that the effects of Cu exposure at the acutely toxic level had a larger impact on metabolomic changes than did the effects of sediment composition.

We also conducted PLS-DA to identify important metabolites that could be used to discriminate between the control and Cu exposure groups. Four models were designed for each sediment type, but only Model_{CuES}, Model_{CuQS}, Model_{CuAS-a} were constructed. Model_{CuAS-b} exhibited less predictability than the other models because the differences between the control and Cu exposure groups were unclear, as discussed above. For the ES, QS, and AS-a sediments, discriminant models were built successfully, and the model parameters are shown in **Table 2**. The results were consistent with those of the PCA, confirming that the effects of Cu exposure were able to be detected using metabolomic analysis. A total of 119, 481, and 282 metabolites with a VIP value higher than 1.5 were extracted from Model_{CuES}, Model_{CuQS}, Model_{CuAS-a}, respectively. The top five compounds are summarized in **Table 3**. As illustrated clearly by **Fig. 5**, eight metabolites were found in all three models. This result suggests that

Fig. 4 Cluster analysis based on the PCA scores. The PCA was conducted without the AS-b samples. The number in parentheses indicates the number of samples in a corresponding cluster.

Fig. 5 The commonality of the metabolites that had VIP values higher than 1.5 between the three models used to discriminate between the control and Cu exposure groups.

there are common metabolites that contribute to an explanation of Cu exposure even though different types of sediments are used in the tests. One of the common metabolites was assumed as (2Z)-2-(4-Oxo-1,3-thiazolidin-2-ylidene)-1,3-benzothiazol-6 (2H)-one based on the database. It could be an important metabolite that responds to Cu exposure regardless of sediment types, but the detailed mechanisms were unclear because the compound was not mapped onto the KEGG pathway. Moreover, KEGG pathway mapping showed that the metabolites that had VIP values higher than 1.5 in each model could be found in the same metabolic pathway. However, the reaction of the pathway was not revealed, and the relationship between the pathway and Cu exposure was unclear. This suggests that, even if different types of sediment are used, there may be some pathways that relate to Cu exposure. In Model_{CuQS}, some metabolites that showed high VIP values were related to the pathway of biosynthesis of the amino acids. A previous metabolomic study has reported that this pathway may exist in other species of

amphipod [14], and the present study suggests the relationships between sediment types and metabolic pathways. To further verify this, future work should seek to develop an improved database for annotation of metabolites. Furthermore, the effects of sediment in a test chamber and the effects of feeding activity on the metabolomic reactions should also be examined in the future.

Our results found that PCA score plots can be used to identify differences between control and Cu exposure groups and that PLS-DA followed by VIP analysis is effective in identifying important metabolites. This analysis revealed that the effects of Cu exposure were larger than the effects of sediment composition. Though testing with other sediments and toxicants needs to be examined in future studies, our result leads to the conclusion that, in toxicity testing at an acutely toxic level, the type of control sediment does not affect the separation between control and exposure groups in PLS-DA models.

CONCLUSIONS

We assessed metabolomic changes within *G. japonica* when different types of sediment (ES, QS, AS-a, and AS-b) were used as the control sediment during toxicity testing. These changes were compared with those caused by exposure to Cu. Our results found that sediment composition clearly impacted metabolomic profiles, and the metabolites that were related to each sediment type were extracted. Copper exposure also affected metabolomic profiles, and this was more obvious than the effects of the control sediment, when ES, QS, or AS-a sediment was used. Eight metabolites involved in discriminating between the control and Cu exposure groups overlapped between the different sediment types. It is clear that more knowledge on the metabolic

pathways is necessary for further analysis. In conclusion, the effects of Cu exposure were more dominant than those of control sediment composition under the conditions we investigated. It suggests that the composition of control sediment may not need to be limited to a specific type when assessing metabolomic changes.

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SUPPLEMENTARY MATERIALS

Supplementary Materials file for this article is available at the link below.

https://www.jstage.jst.go.jp/article/jwet/17/6/17_18-104/supplement/_download/17_18-104_1.pdf

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