Original Article

Antioxidant Responses of a Brackish Water Clam to Sediment Composition and Water Quality: a Field Experiment

Preeti Pokhrel ^a, Hiroki Machida ^a, Shumona Akther ^a, Jumpei Suzuki ^b, Masafumi Fujita ^a

^a Graduate School of Science and Engineering, Ibaraki University, Hitachi, Japan

^b Environmental Science Research Laboratory, Central Research Institute of Electric Power Industry, Abiko, Japan

ABSTRACT

Field experiments were conducted at brackish-water sites (Lake Hinuma, Hinuma River, and Naka River) to investigate oxidative stress responses of a brackish-water clam, *Corbicula japonica*, to sediment composition and water quality. To ascertain the influence of the sediment composition, clams were subjected to three test sediments with different contents of clay/silt and organic matter from the same site. Sediment with ~33% of clay/silt content and ~10% of ignition loss exhibited decreased oxygen radical absorbance capacity (ORAC) within two weeks ($p < 0.05$). However, sediment with less than ~20% of clay/silt content and/or ~7% of ignition loss had no influence on ORAC ($p > 0.05$). To ascertain the influence of the water quality, clams were placed in baskets without sediment at four brackish-water sites. Significant differences in ORAC were observed in response to salinity and chlorophyll a (Chl a) ($p < 0.05$). Multiple regression analysis indicated that the levels of salinity and water temperature as well as variations in Chl a, dissolved oxygen, and turbidity for 1 d prior to the sampling date affected ORAC. We concluded that natural influencing factors need to be considered in ORAC assays for *C. japonica*, when anthropogenic impacts are assessed.

Keywords: *Corbicula japonica*, oxygen radical absorbance capacity, sediment, salinity, chlorophyll a

INTRODUCTION

The influence of natural and anthropogenic disturbances is high in brackish-water areas due to their distinct characteristics [1,2]. Fluctuations in salinity during high tides, changes in freshwater flow in the upstream and downstream parts of rivers [3], spatial and temporal variations in water temperature [2], and seasonal variations in food abundance [4] negatively affect brackish clam species. In coastal brackish-water bodies, suspended sediment concentrations can increase with sediment runoff [5], and these changes may vary the composition of the sediment, affecting food availability and habitats of different bivalve species [6]. Because the hydrochemistry of rivers and lakes are influenced by spatial and temporal variations, regular inspections of water quality are essential [7]. To maintain the sustainability of brackish-

water areas, assessments are required from the viewpoint of ecosystem conservation. However, prior to the assessment of anthropogenic disturbances, that of natural ones should be clarified, because of the above mentioned unique characteristics of brackish-water areas.

Oxidative stress biomarkers are becoming valuable tools for revealing biological responses influenced by environmental factors in aquatic organisms [4,8,9]. Previous studies have used oxidative stress biomarkers and the total oxyradical scavenging capacity (TOSC) for the estimation of antioxidant capacity against varied environmental stresses [10,11]. In recent years, the oxygen radical absorbance capacity (ORAC) has been used because of its simple and rapid determination using a microplate reader [12] and has been widely accepted as a biomarker for the direct assessment of the antioxidant capacity [13]. The ORAC analysis measures

Corresponding author: Masafumi Fujita, E-mail: masafumi.fujita.wenv@vc.ibaraki.ac.jp

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Fig. 1 Locations of the experimental sites: (A) Naka River; (B) Ohnuki Bridge; (C) the north part of Lake Hinuma; and (D) the south part of Lake Hinuma.

peroxyl radical-induced oxidation of a fluorescent probe via the change in its fluorescence intensity using a microplate reader [4,13]. High values of ORAC indicate a high antioxidant capacity against environmental stresses.

Globally, brackish-water diversity accounts for several thousand species [14]. Brackish-water areas, where seawater and freshwater are mixed, provide habitats for fish and benthic invertebrates and are characterized by a rich biodiversity. The brackish-water clam, *Corbicula japonica*, inhabits rivers and brackish waters [15]. Owing to their commercial importance [15,16] and filter-feeding capabilities via the consumption of particulate organic matter, these bivalves are considered to be a good bioindicator for the assessment of natural environmental factors [4,17].

In recent years, multivariate statistical techniques have been used to identify and evaluate spatial and temporal variations in water quality [7,18,19]. Previous studies have examined sediment quality and its effect on the *C. fluminea* clam species, which exhibits significant biological stress [20]. Other studies have reported similar effects in the settlement of different clam species [21,22], and few studies have examined survival following eutrophic-driven changes in the food supply and habitat [6,23–26] as well as biochemical alterations due to salinity and food availability [4] on brackish-water clams. However, to the best of our knowledge, the ORAC responses of *C. japonica* to sediment composition and water quality have not yet been investigated in actual brackish environments. In other words, there are

limited findings concerning the effects of sediment composition and water quality on the antioxidant capacity of these clams, in spite of the fluctuating environmental conditions in brackish-water areas.

This study focuses on the brackish-water clam, *C. japonica*, which is found in Lake Hinuma, Hinuma River, and Naka River. Our research objectives are (1) to investigate the effects of different sediment compositions on ORAC and (2) to determine the water quality parameters affecting ORAC.

MATERIALS AND METHODS

Experimental methodology to ascertain influence of sediment composition

Field experiments were conducted at Site D (Lake Hinuma) from September 30, 2017 to October 27, 2017 to ascertain the influence of the sediment composition (**Fig. 1**). This experimental period witnessed a typhoon from October 22 to 23, 2017. Previous studies [24,25] have reported that clay/silt and organic matter contents in the sediment influence the mortality of *C. japonica*. Therefore, three test sediments with different contents of clay/silt and ignition losses were prepared. The sediments were collected from Site D and an affluent of the Naka River and were air dried for a week and then sieved (< 2 mm). Subsequently, the sediments were blended to assemble the test sediments. The contents of clay/silt were measured using 75- μ m sieves. For the ignition loss test, the test sediments were dried at $750 \pm 50^\circ\text{C}$ for 1 h. At

Table 1 Composition of the sediments used for the experiments.

Run	Clay/silt content (%)	Ignition loss (%)
1	3.7	2.8
2	20.2	7.1
3	33.1	10.4

the start of the experiment, plastic cages (35 cm × 65 cm × 30 cm) containing the test sediments with clay/silt contents of 3.7%, 20.2%, and 33.1% and ignition losses of 2.8%, 7.1%, and 10.4% (**Table 1**) were fixed on the site separated by ~1 m. For each run, 50 individuals were placed in the cages, with 10 individuals taken out once every two weeks. Five samples of gill were used and stored immediately at -80°C for the ORAC analysis.

Experimental methodology to ascertain influence of water quality

Field experiments were conducted at Sites A, B, C, and D (**Fig. 1**) from October 2, 2017 to December 10, 2017 to ascertain the influence of the water quality. At Site A, because of technical problems, continuous water quality data were not recorded from October 27, 2017 to November 1, 2017. Clams were placed in baskets (100 individuals) without sediment from October 13, 2017 to November 24, 2017 at each survey site for the ORAC assays. At the same time, water quality parameters, such as the water temperature (°C), salinity (psu), dissolved oxygen (DO) (mg/L), turbidity (FTU), and chlorophyll a (Chl a) (µg/L), were measured every 30 min at the sites. Five individuals were collected for the initial ORAC analysis on October 13, 2017. The sampling dates for the ORAC analysis at the different sites were October 27, November 10, and November 24, 2017.

ORAC analysis

The gills of five clams were homogenized in 20 mL of a buffer solution (20 mM Tris-HCl, 1 mM EDTA, and 50 mM KCl) for 1 min (GLH-115, Yamato Scientific, Tokyo, Japan) and centrifuged at 10,000 g for 10 min at 4°C (H-1500Ra, Kokusan, Saitama, Japan). The supernatants were subjected to the total antioxidant capacity and protein assays. The total antioxidant capacity was determined using ORAC [27] with minor modifications. A total of 30 µL of the samples was added to each well and mixed with 150 µL of fluorescein (40 nM), as a fluorescent probe, and 60 µL of 2,2-azobis (2-amidinopropane) dihydrochloride (100 mM), as a source of peroxy radicals. The fluorescence was continuously mea-

sured using a microplate reader (Infinite F200 PRO, Tecan Japan, Tokyo, Japan). ORAC was expressed as micromoles of trolox equivalent per milligram of protein [28]. The proteins were quantified via the Bradford method [29] using the Protein Assay CBB Solution (Nacalai Tesque, Kyoto, Japan).

Statistical analysis

Tukey's test was conducted to detect significantly different means ($p < 0.05$) in the ORAC analysis. Water quality data at Sites A, B, C, and D were analyzed via principal component analysis (PCA) and cluster analysis. To understand the effect of the independent water quality variables on ORAC, multiple regression analysis was performed. The average values of ORAC were used for each sampling date and site as the response variable. The average and standard deviation values for the water temperature, salinity, DO, turbidity, and Chl a were calculated using the data for 1 d prior to the sampling date and were treated as explanatory variables. For multiregression analysis, 12 average values in the ORAC (3 sampling dates × 4 sites) and 60 each average and standard deviation values in water quality (3 sampling dates × 5 water quality parameters × 4 sites) were used. All statistical analyses were performed with R (version 3.6.1) and R studio (version 1.2.5019).

RESULTS AND DISCUSSION

Sediment compositions and ORAC responses

The effect of different sediment compositions (clay/silt content and ignition loss) on the ORAC responses are shown in **Fig. 2**. Run 1 (clay/silt content of 3.7% and ignition loss of 2.8%) was selected as the control because this condition is common for the *C. japonica* clams inhabiting Lake Hinuma. Run 3 (clay/silt content of 33.1% and ignition loss of 10.4%) had a significantly decreased ORAC from 58.2 µmol TE/mg protein to 31.9 µmol TE/mg protein ($p < 0.05$), whereas runs 1 and 2 (clay/silt content of 20.2% and ignition loss of 7.1%) experienced no change ($p > 0.05$). A previous study reported that clay/silt sediments are unfavorable environmental substrates for brackish-water bivalves [23]. Foe and Knight [30] reported that the concentration of the silt is independent of the tissue growth in the *C. fluminea* clam species. Nakamura [24] reported that the clay/silt content and ignition loss were 50% and 14% for the habitat limitation of the *C. japonica* clam species, respectively. According to Pracoyo *et al.* [26], the effect of clay/silt contents lower than 26% could not be identified in terms of the mortality rate of the clams. These results support the findings of the present study and suggest

Fig. 2 Antioxidant response to the varying sediment composition. The values indicate the mean ($\pm$ standard deviation) of two weeks of data. Significant differences between the experimental sites are indicated by the different letters ($p < 0.05$).

that sediments with less than ~20% clay/silt content and/or ~7% ignition loss should not be considered in ORAC assays for *C. japonica*. However, it is unknown that initial clay/silt and ignition loss contents had been certainly maintained throughout the experimental period because they were not assessed routinely.

Water quality characteristics and ORAC responses

Evaluation of the water quality parameters for each site

The data in **Table 2** provide the average values of the various water quality parameters measured at Sites A, B, C, and D (**Fig. 3**). The PCA results (**Fig. 4**) reveal that the first principal component, which accounted for 65.7% of the total variance, correlated with the water temperature [factor loading (FL) = -0.87], salinity (FL = -0.99), and Chl a

(FL = 0.90) and that the second principal component, which accounted for 32.1% of the total variance, correlated with DO (FL = -0.76) and turbidity (FL = 0.81). Therefore, the first component favored the characterization of the most important water quality parameters in the study area. The cluster analysis divided the four sites into three groups on the PCA biplot: Group I (Site A), Group II (Site B), and Group III (Sites C and D). In the present study, a remarkable variation in the salinity at Sites A and B was observed compared with Sites C and D. Site A was in the Naka River near the Pacific Ocean. Similarly, Site B was in the Hinuma River, whereas Sites C and D were in Lake Hinuma. The highest turbidity was seen at Sites B, C, and D. The turbidity rapidly increased over an approximately one-week period. The average value of the turbidity at Site B was higher than Site A. The average value of Chl a was higher at Sites C and D,

Table 2 Water quality measured at the different sites: (A) Naka River; (B) Ohnuki Bridge; (C) the north part of Lake Hinuma; and (D) the south part of Lake Hinuma.

Site	Water quality variables				
	Water temperature (°C)	Salinity (psu)	Dissolved oxygen (mg/L)	Turbidity (FTU)	Chlorophyll a (μg/L)
A	15.5 $\pm$ 3.2	9.0 $\pm$ 8.0	8.8 $\pm$ 1.2	3.0 $\pm$ 1.4	1.6 $\pm$ 1.5
B	15.7 $\pm$ 3.1	8.1 $\pm$ 7.0	7.8 $\pm$ 2.0	13.0 $\pm$ 16.0	6.0 $\pm$ 5.8
C	15.3 $\pm$ 3.9	2.3 $\pm$ 1.5	8.7 $\pm$ 3.2	14.0 $\pm$ 10.0	23.0 $\pm$ 14.0
D	15.1 $\pm$ 3.7	1.8 $\pm$ 1.2	9.1 $\pm$ 3.0	13.0 $\pm$ 8.4	16.0 $\pm$ 10.0

Fig. 3 Variations in the water quality parameters, such as a) water temperature; b) salinity; c) dissolved oxygen; d) turbidity; and e) chlorophyll a, at the experimental sites.

Fig. 4 Biplot of the first and second principal components (PC1 and PC2) for the experimental sites and variables. Groups I (Site A), II (Site B), and III (Sites C and D) (shown within dotted ellipses) were categorized via cluster analysis of the responses of the variable.

Table 3 Oxygen radical absorbance capacity (ORAC) values measured at the different sites: (A) Naka River; (B) Ohnuki Bridge; (C) the north part of Lake Hinuma; and (D) the south part of Lake Hinuma.

Site	ORAC ($\mu\text{mol TE/mg protein}$)			
	October 13, 2017	October 27, 2017	November 10, 2017	November 24, 2017
A	58.2 ± 5.4	40.3 ± 4.0^a	37.5 ± 6.7^a	31.2 ± 6.2^a
B	↓	63.9 ± 7.0^b	58.0 ± 8.3^b	70.6 ± 5.0^b
C	↓	38.8 ± 7.0^a	45.7 ± 6.1^{ab}	53.0 ± 7.1^c
D	↓	57.4 ± 8.7^b	47.7 ± 6.6^{ab}	48.6 ± 6.0^c

Different letters indicate significance levels ($p < 0.05$) for the ORAC values on each sampling date.

indicating a higher food supply to these sites than Sites A and B. This is due to the greater growth of phytoplanktons in Lake Hinuma compared with the two rivers. The PCA and cluster analysis demonstrated that Sites A and B have distinct features, whereas Sites C and D have similar characteristics. Collectively, the water quality at the lake (Sites C and D) was found to be different in terms of salinity, turbidity, and Chl a compared with the two rivers (Sites A and B).

Impact of water quality parameters on ORAC

Differences in ORAC were found after two weeks of exposure at the four sites (**Table 3**). On comparing all the sites after two weeks of exposure (**Fig. 5**), significant differences were observed in the ORAC values at Sites A and B ($p < 0.001$), Sites A and C ($p < 0.05$), Sites A and D ($p < 0.001$), Sites B and C ($p < 0.001$), and Sites B and D ($p < 0.001$); however, no differences were observed between Sites C and

Fig. 5 Oxyradical-absorbance capacity (ORAC) responses of *C. japonica* when exposed to different sites: (A) Naka River; (B) Ohnuki Bridge; (C) the north part of Lake Hinuma; and (D) the south part of Lake Hinuma. The values indicate the mean ($\pm$ standard deviation) after two weeks of exposure. Significant differences between experimental sites are indicated by different letters ($p < 0.05$).

D ($p > 0.05$). The lack of a significant difference between these sites was due to their similar characteristics of the water quality.

The ORAC value in the clams was the lowest at Site A. Site A has the highest salinity and the lowest Chl a (food) concentration, as described above. Weiss *et al.* [31] suggested that clams near oceans have limited food supply, which would reduce their growth. In addition, Pokhrel *et al.* [4] reported lower ORAC values for *C. japonica* under high salinity and limited food supply conditions. A decline in antioxidant capacity (TOSC) was also observed in the mussel, *Mytilus galloprovincialis*, suggesting that oxidative stress is higher in such organisms [32]. According to Gossett *et al.* [33], high salinity leads to production of reactive oxygen species. Considering these previously reported findings, the low ORAC value at Site A can be attributed to high salinity and low food availability.

Although Sites A and B had high salinity, ORAC significantly increased at Site B. This difference can be attributed

to the high food availability at Site B. According to Dickinson *et al.* [34], invertebrates may increase their metabolic activity to activate defense mechanisms under stressful conditions. Clausen and Riisgård [35] reported that the bivalve growth rate increased at high food availability ($5 \mu\text{g Chl a/L}$). Pokhrel *et al.* [4] reported experimental results under food-rich conditions in which *C. japonica* maintained high ORAC values to counter environmental stress. Our field observations also demonstrate that clams with high food availability maintained high ORAC values at high salinity. The reason for the lower ORAC values at Sites C and D might be due to considerably higher food availability (Chl a concentration, 2.5–4.0 times higher than site B) and lower salinity. The clam, *C. japonica*, species could maintain the ORAC values at Sites C and D and did not require synthesizing more ORAC because of food abundances and low stressful conditions as compared to Sites A and B. Marsden [36] reported that the combination of salinity and food availability affects the growth and conditions of clam species. In the present

Table 4 Effect of natural variables on the ORAC response (W_{avg} , average water temperature; W_{std} , standard deviation of the water temperature; S_{avg} , average salinity; S_{std} , standard deviation of salinity; D_{avg} , average dissolved oxygen; D_{std} , standard deviation of dissolved oxygen; T_{avg} , average turbidity; T_{std} , standard deviation of turbidity; C_{avg} , average chlorophyll a; and C_{std} , standard deviation of chlorophyll a). R-square = 0.77.

Variables	Regression coefficients	Standard Error	<i>t</i> value	Probability
W_{avg}	16.8	6.25	2.70	0.010 *
W_{std}	16.7	23.2	0.72	0.47
S_{avg}	8.21	2.13	3.85	< 0.001 ***
S_{std}	-14.2	7.76	-1.83	0.07
D_{avg}	4.44	4.51	0.99	0.33
D_{std}	52.1	10.3	5.06	< 0.001 ***
T_{avg}	-3.33	4.13	-0.81	0.42
T_{std}	9.70	4.06	2.39	0.02 *
C_{avg}	0.09	0.55	0.16	0.87
C_{std}	-10.7	2.90	-3.71	< 0.001 ***
Intercept	-232	67.1	-3.50	

Asterisks (*) indicate the significance levels (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$).

study, the tested combination affected the *C. japonica* clam species such that they exhibited unstable ORAC values. Our results suggested that salinity and food availability are natural influencing factors for responses in clams in terms of ORAC.

The effects of abiotic and biotic factors need to be considered in environmental studies [37–39]. Multiple regression analyses revealed that the *t* values were significant for average salinity, S_{avg} ($p < 0.001$); average water temperature, W_{avg} ($p < 0.05$); standard deviation chlorophyll a, C_{std} ($p < 0.001$); standard deviation dissolved oxygen, D_{std} ($p < 0.001$); and standard deviation turbidity, T_{std} ($p < 0.05$) (Table 4). In other words, the salinity and water temperature levels and the variations in Chl a, DO, and turbidity, 1 d prior to the sampling date, significantly influenced ORAC. Several previous studies have reported impact of natural factors, such as water temperature, salinity, and oxygen saturation on the antioxidant activity of bivalve species [40,41]. Irato *et al.* [42] reported that the *Tapes philippinarum* clam species showed a negative correlation between the superoxide dismutase activities and DO levels. In our study, variations in DO affected the ORAC responses in *C. japonica*. A major food source for *C. japonica* is diatoms [16]. Chl a and turbidity can reflect the amount of food sources available for clams. The present study also showed that the variations in Chl a and turbidity affected the ORAC responses in *C. japonica*. Pokhrel *et al.* [4] provided evidence that the levels of ORAC in *C. japonica* vary with variations in food. These results agree with the findings of the present study that show an impact on ORAC

linked to variations in the food concentration. As discussed above, it is interpreted that salinity and important influencing factors determine the ORAC responses in *C. japonica* from the multiple regression analyses. We conclude that natural factors in brackish environments need to be considered in ORAC assays for *C. japonica*, when anthropogenic impacts are assessed.

CONCLUSIONS

In this study, the effects of sediment composition and water quality on antioxidant responses were assessed for the *C. japonica* clam species. Experimentally, it was observed that sediments with less than ~20% clay/silt content and/or ~7% ignition loss had no influence on the ORAC values for *C. japonica*. Moreover, significant differences were observed in ORAC values in response to the salinity and Chl a (food availability). Multiple regression analysis showed that the levels of salinity and water temperature and variations in Chl a, DO, and turbidity, 1 d prior to the sampling date, significantly influenced the ORAC responses. We concluded that natural influencing factors need to be considered in ORAC assays for *C. japonica*.

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