

# Distribution of gastropods in a tidal flat in association with digestive enzyme activities

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**Abstract:** We investigated the relationship between digestive function and ecological distribution for four related species of Gastropoda (*Cerithidea rhizophorarum*, *Cerithidea cingulata*, *Batillaria multiformis*, and *Batillaria attramentaria*) inhabiting the Tanaka River estuary (Mie Prefecture, Japan). We compared the digestive enzyme activities for four hard degradable carbohydrates, namely, cellulose, mannan, xylan, and laminarin. Among the investigated four species, *Cerithidea rhizophorarum* showed the highest cellulase, mannanase, xylanase, and laminarinase activities, and was also dominantly distributed in the estuary reed bed. The results of CN stable isotopic analysis suggested that this species assimilated organic matter derived from reeds. Hard degradable carbohydrates, including plant components derived from the reed bed or from dry land, accumulate in the sediment of estuaries. Estuarine benthic animals are assumed to consume these accumulated hard degradable carbohydrates, as well as microphytobenthos and particulate organic matter (including phytoplankton). Our present findings suggest that *Cerithidea rhizophorarum* is dominant in reed beds because it can more efficiently digest plant-derived carbohydrates than can *Cerithidea cingulata*, *B. multiformis*, and *B. attramentaria*. To the best of our knowledge, ours is the first study to demonstrate that the specific ecological distribution of related animal species with similar ecological traits can be explained by the efficiency of their digestive enzyme activity.

**Key words:** cellulase, distribution, estuary, gastropod, reed

## Introduction

Detritus, including organic matter derived from seaweed and terrestrial plants, accumulates in estuaries via tidal flow from the sea and river flow from the land (Wada et al., 1987; Chanton and Lewis, 2002). Within the estuaries, specific ecological systems are formed with organisms that feed on the accumulated detritus (Macintyre et al., 1996; Galvan et al., 2008). Benthic animals are the major organisms contributing to the degradation of organic matter in estuaries (Reise, 1985).

Estuaries are ecologically important areas, because they

are the spawning sites of fish and stopover points for migrating birds (Beukema, 1979; Reise, 1985). However, since the Second World War, land reclamation has greatly reduced the size of estuarine areas in Japan (National Institute for Environmental Studies, 2003). This reduction in habitat has led to a marked decrease in the numbers of estuarine species, such as the crustacean *Uca lactea lactea* and the mollusk *Ellobium chinense*, thereby resulting in the designation of these species as vulnerable (Japan Integrated Biodiversity Information System, 2008). In addition, populations of *Cerithidea rhizophorarum*, *Cerithidea cingulata*, and *Batillaria multiformis* have decreased, and these species have been designated as near threatened species. To prevent the populations from decreasing further, it is important to un-

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derstand the feeding system and distribution of these animals.

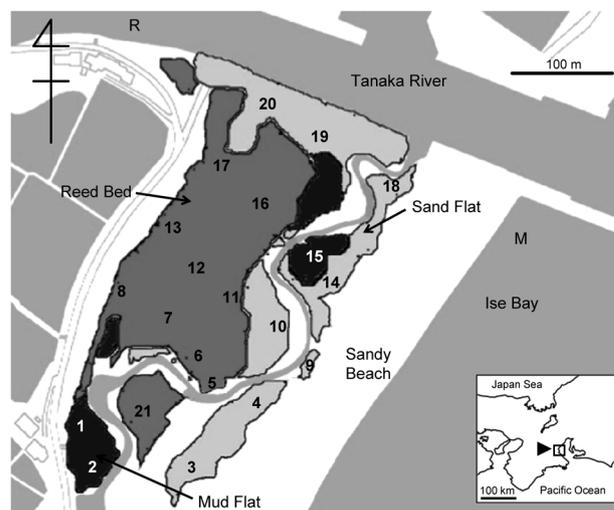
The results of recent stable isotopic studies suggest that benthic animals in estuaries preferentially feed on benthic diatoms or microphytobenthos (MPB; Kang et al. 2003; Kanaya et al. 2005; Yokoyama et al. 2005). Most benthic animals are unable directly to assimilate organic matter derived from land plants, because they lack the enzymes required to digest cellulose, which is one of the primary components of land plants (Kristens 1972). On the other hand, benthic animals inhabiting estuaries and reed beds are assumed to feed on detritus and the remains of land plants that are primarily composed of cellulose (Macintyre et al. 1996; Cividanes et al. 2002; Kasai & Nakata 2005). Therefore, these animals should be equipped with the enzymes necessary for the breakdown of substances such as cellulose, mannan, xylan, and laminarin (Tanimura et al. 2013). The results of stable isotopic analysis suggest that the brackish bivalve *Laternula marilina* and the polychaete *Hediste* spp. assimilate plant detritus (Kanaya et al. 2008). Similarly, the brackish bivalve *Corbicula japonica* assimilates terrestrial organic matter through the utilization of multiple endogenous cellulases (Sakamoto et al. 2007, 2009; Sakamoto & Toyohara 2009; Antonio et al. 2010). Niiyama and Toyohara (2011) detected several plant-degrading enzymes in various benthic animals, including crustaceans and mollusks. In addition, the benthic mollusks *Aplysia kurodai*, *Haliotis discus hannai*, and *Mytilus edulis* are reported to possess several enzymes necessary for digesting cellulose, mannan, and alginic acid in seaweeds (Xu et al. 2002; Shimizu et al. 2003; Suzuki et al. 2003; Rahman et al. 2011).

The distribution of benthic animals in brackish water areas is restricted by factors such as elevation, salt content, substratum, and food (Adachi & Wada 1998; Vizzini & Mazzola 2006; Kanaya & Kikuchi 2008; Weerman et al. 2011). Benthic animals possess digestive enzymes for plant-derived carbohydrates (Davison & Blaxter 2005; Tanimura et al. 2013), and therefore, the activity level of these enzymes may determine the distribution of related benthic animals in estuaries. In the present study, we used distribution surveys, stable isotope analysis, and measurement of enzyme activity to investigate the relationship between feeding characteristics and ecological distribution of four related gastropod species (*Cerithidea rhizophorarum*, *Cerithidea cingulata*, *B. multiformis*, and *B. attramentaria*) inhabiting the mouth of the Tanaka River in Mie Prefecture.

## Materials and Methods

### Study sites and sample collection

The Tanaka River runs through the north of central Mie Prefecture and into the west side of Ise Bay. The estuary includes an oval tidal flat with an area of approximately



**Fig. 1.** Sampling sites in the estuary of the Tanaka River, Mie Prefecture, Japan. Charcoal gray, reed bed; black, mud flat; gray, sand flat.

3600 m<sup>2</sup>, which stretches 200 m in an east-west direction and 300 m in a north-south direction (Kimura & Kimura 1999; Geospatial Information Authority of Japan 2012). This tidal flat is divided into a reed bed containing accumulated litter, and an adjacent field without reeds (Fig. 1). The southern part of the field is closest to the reed bed and is composed of clay soil, and the northeastern part is composed of sandy clay soil. A fish culture pond is located to the west of the estuary, and the river flows out through a gate in the northwestern part of the estuary. The pulmonates *Ellobium chinense* and *Laemodonta siamensis*, as well as >20 gastropod species, inhabit the estuary, mainly in the reed bed (Kimura & Kimura 1999; Suzuki et al. 2006). Tidal range in the estuary varies between -0.04 m and 2.35 m between May and September (Japan Meteorological Agency 2010).

For the analysis of species distribution, samples were collected at 20 stations. For enzyme activity analysis, *Cerithidea rhizophorarum* was sampled at sites 6, 13, 17, and 21; *Cerithidea cingulata* was sampled at sites 10 and 17; *B. multiformis* was sampled at site 20; and *B. attramentaria* was sampled at site 19. For stable isotopic analysis, MPB was collected at sites 2 and 15. Sediment was sampled at sites 2, 10, 15, and 17; riverine particulate organic matter (RPOM) was sampled at point "R"; and marine particulate organic matter (MPOM) was sampled at point "M". The marsh plant, *Phragmites australis*, was sampled at sites 8 and 17, and *Carex scabrifolia* was sampled at site 2.

### Preparation of enzymatic extracts

Gastropods were collected for the measurement of enzyme activity in October 2010. The 21 sampling sites are indicated in Figure 1. The specific collection sites for each species were determined according to environmental characteristics as follows: *Cerithidea rhizophorarum* was col-

lected from reed-bed sites 6, 13, 17, and 21; *Cerithidea cingulata* was collected from sand-flat site 10 and reed-bed site 17; *B. multiformis* was collected from sand-flat site 20; and *B. attramentaria* was collected from sand-flat site 19. Five individuals of each gastropod species were collected and transported on ice to the laboratory at Kyoto University.

Gastropods were dissected on ice to isolate the midgut and crystalline style, which were homogenized with 5 mL of phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 8 mM Na<sub>2</sub>HPO<sub>4</sub>, and 5.5 mM KH<sub>2</sub>PO<sub>4</sub> at pH 7.4). The homogenates were centrifuged at 9100×g for 10 min, and 1 mL of each supernatant was used as enzyme solution. The protein concentration of each enzyme solution was determined according to the method of Bradford (Bradford 1976), by measuring the absorbance at 595 nm (UV mini 1240 spectrophotometer; Shimadzu Corporation). Bovine serum albumin was used as the standard for protein concentration assays.

### Enzyme activity assay

#### Cellulase activity

Enzyme solution (5 μL), 5 μL of 1 M sodium acetate (AcNa) buffer (pH 5.5), and 40 μL of 1% carboxymethyl cellulose (CMC; Sigma) were mixed and incubated at 37°C for 10 min with shaking. After incubation, enzyme reactions were terminated by heating at 100°C for 3 min. Next, 1 mL of blue tetrazolium was added and each sample was heated at 100°C for 3 min. After cooling on ice, the absorbance at 660 nm was measured to determine the amount of reducing sugar produced (Jus & Lipke 1985).

Two types of control were prepared to account for the amount of reducing sugar contained in each enzyme solution and in CMC. To determine the amount of reducing sugar in the enzyme solutions, 5 μL of each enzyme solution, 5 μL of 1 M AcNa buffer (pH 5.5), and 40 μL of distilled water were mixed and measured. To determine the amount of reducing sugar in CMC, 5 μL of distilled water, 5 μL of 1 M AcNa buffer (pH 5.5) and 40 μL of 1% CMC were mixed and measured. The enzyme activity was expressed as the amount of glucose released per min per μg of protein. The control values were subtracted from the sample values, to obtain the enzyme activity for each animal collected.

#### Hemicellulase activity

Mannanase, xylanase, and laminarinase activities were measured by using the same method as for cellulase, but with CMC replaced by 1% locust bean gum (Sigma), xylan (Sigma), and laminarin (Nacalai Tesque), respectively.

#### Zymographic analysis

SDS-PAGE zymographic analysis was performed according to the method of Beguin (1983). Enzyme solutions (protein content per 5 μL: *B. multiformis*, 7 μg; *B. attramentaria*, 7 μg; *Cerithidea cingulata*, 4 μg; *Cerithidea rhi-*

*zophorarum*, 0.25 μg) were applied to 7.5% polyacrylamide gels containing 0.5% w/v CMC. After electrophoresis, each gel was rinsed twice for 30 min in 0.1 M AcNa buffer (pH 5.5) containing 0.1% Triton X-100, and was then incubated in 0.1 M AcNa buffer (pH 5.5) at 37°C for 24 h. Gels were stained with 0.1% Congo Red for 30 min and de-stained with 1 M NaCl. Enzyme activity was detected as de-stained bands in the gel.

### Stable isotopic analysis

Stable isotope ratios increase at each step in the food chain ( $\delta^{13}\text{C}$ , 0‰ to +1‰;  $\delta^{15}\text{N}$ , +3‰ to +4‰) and can be used to estimate the trophic level (Fry & Sherr 1984; Minagawa & Wada 1984).

Samples of four gastropod species were collected by hand at the same locations and at the same time as the samples collected for the measurement of enzyme activity (Fig. 1), and were transported to the laboratory on ice. Foot tissue was removed, washed with deionized water, freeze-dried (24 h), and powdered by using a mortar. Lipids were removed with chloroform–methanol solution (2:1 by volume).

Potential food sources were sampled several times from 2009 to 2010. MPOM and RPOM samples were collected (Fig. 1) in May 2009, August 2009, February 2010, and March 2010. At each station, surface water was sampled by collection in a polypropylene bottle ( $n=1$ ), and was pre-filtered through a 0.125-mm mesh. The POM in each sample was then concentrated onto a pre-combusted Whatman GF/F glass fiber filter (500°C, 2 h). Sediment organic matter (SOM) was collected with a spatula from the top 1 cm at sites 2, 7, 15, and 17 in May and September 2009 and in March 2010, and was placed in three plastic tubes. MPB was collected at sites 2 and 15 (Fig. 1) in May 2009, September 2009, and March 2010, when visible diatom mats developed. In the field, surface sediments (0–5 mm deep) were placed on three polyethylene trays, and MPB samples were extracted in the laboratory by exploiting their positive phototaxis under two fluorescent lamps (see Riera & Richard 1996). Samples were then suspended in deionized water and concentrated onto a pre-combusted GF/F. All filters and sediment samples were acidified with 1 M HCl to remove carbonates, and were then freeze-dried (24 h). In May 2009, two species of marsh plants were collected—*Phragmites australis* was collected from sites 8 and 17, and *Carex scabrifolia* was collected from site 2 (three replicates of each). Samples were cleaned by using a brush dipped in deionized water, freeze-dried (24 h), and powdered.

$\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  were determined by using a mass spectrometer connected to an elemental analyzer (Flash EA1112 and Delta XP; Finnigan Mat). Isotope ratios were expressed in delta notation:

$$\delta^{13}\text{C} \text{ or } \delta^{15}\text{N} (\text{‰}) = (R_{\text{sample}}/R_{\text{standard}} - 1) \times 1000$$

where  $R$  is the  $^{13}\text{C}/^{12}\text{C}$  or  $^{15}\text{N}/^{14}\text{N}$  ratio for  $\delta^{13}\text{C}$  or  $\delta^{15}\text{N}$ , respectively. Pee Dee belemnite and atmospheric  $\text{N}_2$  were used as references for  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ , respectively. D-Alanine and L-histidine were used as working standards. In the analyses, the C/N molar ratio was also calculated for plants, MPB, POM, and SOM samples, based on their total organic carbon and total nitrogen contents.

### Spatial distribution of gastropods

The investigation of species distribution was conducted during the ebb tide on May 18 and 21, 2010. In order to include the various environments of the estuary, 20 sites were located at even intervals along five transect lines (thirteen sites on the sand flat or mud flat, and seven sites on the reed bed; Fig. 1). Gastropods were collected in three quadrats (50 cm×50 cm) laid randomly at each of the 20 sites. All visible gastropods within the quadrats were collected and taken to Mie University, fixed in 80% alcohol, identified, and counted.

### Physical environmental data

Elevation and plant coverage were measured for each of the 20 sites. Elevation was calculated by using coordinates obtained from a GPS survey. Elevation was defined as follows:

$$\text{Elevation (m)} = (e - T) + 1.3 \times T$$

where  $e$  is the measured elevation at the estuary and  $T$  is the mean sea level of Tokyo Bay. The tide level of the intertidal zone ranged from  $-0.04$  m to  $2.35$  m at Toba City, Mie, between May 2010 and September 2010 (Japan Meteorological Agency 2010).

Plant cover was estimated by using the same quadrat method as for the collection of gastropods. In September 2010, the vegetation inside each quadrat was recorded, and the amount of sediment covered by vegetation was expressed as subjective values from 0 to 10 according to visual estimation.

### Ignition loss of organic matter in sediment

On the same day that plant coverage was recorded, sediment samples were collected from each quadrat by using a core sampler (vinyl chloride pipe, diameter 6 cm, height 10 cm). After removing gravel (major axis longer than 2 mm), plants, benthos, artifact fragments such as broken pieces of glass bottles or plastic goods, and sediment were weighed and dried to a constant weight at  $110^\circ\text{C}$  in a drying oven. Approximately ten gram of each sample was well ground, and coarse particles were removed by passing the sample through a 1-mm mesh with shaking. Three grams of each sample were weighed, placed in an oven, and ignited for 2 h at  $850^\circ\text{C}$ . The percentage of organic matter content was calculated as the ratio of the sample weight lost after ignition to the sample weight before ignition. Sample sediment measurements were conducted in triplicate,

and values were expressed as the average  $\pm$  SD.

### Statistical analysis

Differences in enzyme activity level among sites or species, stable isotope ratio among sites or species, and distribution among sampling sites were statistically tested by using one-way ANOVA followed by post-hoc tests (Tukey's HSD) in SPSS (IBM, version 22).

## Results

### Enzyme activities of gastropods

All of the investigated gastropods showed cellulase, mannanase, xylanase, and laminarinase activities (Fig. 2). The level of enzyme activity differed between species and among sampling sites.

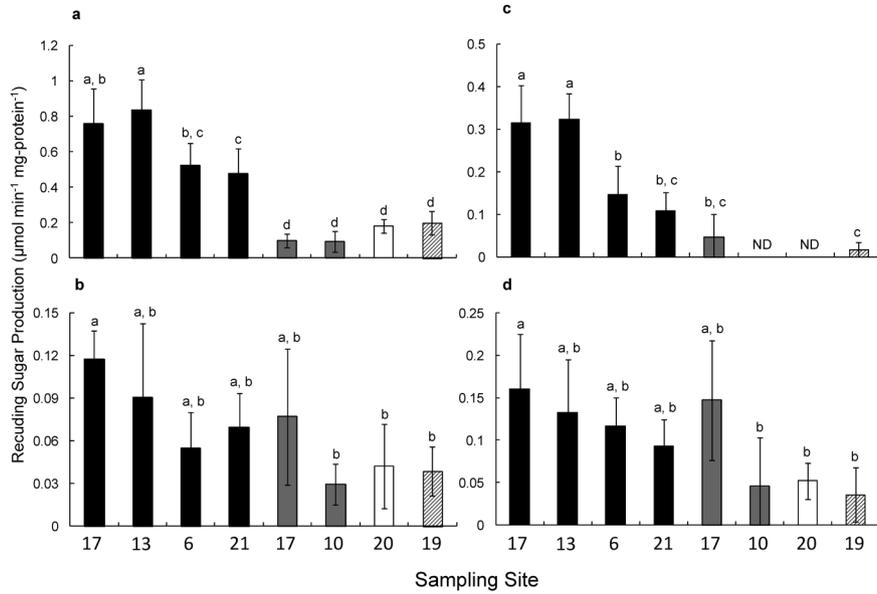
For *Cerithidea rhizophorarum*, individuals collected from reed-bed site 21 showed the lowest level of cellulase activity. In addition, individuals collected from reed-bed site 13 showed significantly higher cellulase activity than did those collected from reed-bed sites 6 and 21 (Tukey's HSD,  $p < 0.05$ ). The cellulase activity of *Cerithidea rhizophorarum* was significantly higher than those of the other three species ( $p < 0.05$ ). However, there were no significant differences among the level of cellulase activity of *Cerithidea cingulata*, *B. multiformis*, and *B. attramentaria*.

For gastropods collected from reed-bed site 6, the mannanase activity of *Cerithidea rhizophorarum* was lower than was that of the other three species; however, this difference was not statistically significant. The mannanase activity level of *Cerithidea rhizophorarum* collected from reed-bed site 17 differed significantly from that of *Cerithidea cingulata* collected from sand-flat site 10, and also from those of *B. multiformis* and *B. attramentaria* (Tukey's HSD,  $p < 0.05$ ).

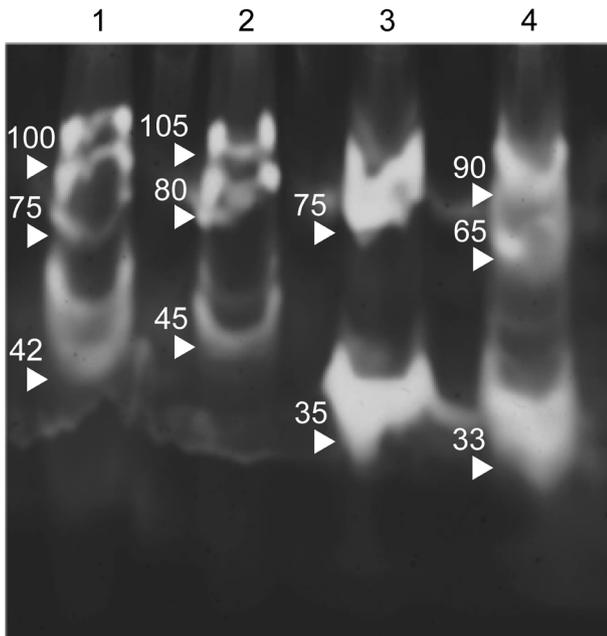
The xylanase activity of *Cerithidea rhizophorarum* was lowest at reed-bed site 21. Similar to the cellulase activity, the xylanase activities of individuals collected from reed-bed sites 13 and 17 were significantly higher than those of individuals collected from reed-bed sites 6 and 21 (Tukey's HSD,  $p < 0.05$ ). In addition, the xylanase activity level of *Cerithidea rhizophorarum* collected from reed-bed sites 17 and 13 differed significantly from that of *Cerithidea cingulata* collected from reed-bed site 17, and also from that of *B. attramentaria*. No xylanase activity was detected in *Cerithidea cingulata* collected from sand-flat site 10, or in *B. multiformis*.

The laminarinase activity level was similar among the investigated sites and species. The laminarinase activity level of *Cerithidea rhizophorarum* collected from reed-bed site 17 differed significantly from that of *Cerithidea cingulata* collected from sand-flat site 10, and also from those of *B. multiformis* and *B. attramentaria*.

The results of zymographic analysis to detect the molecular weight of cellulases in the midguts and crystalline



**Fig. 2.** Cellulase and hemicellulase activities in the digestive glands of *Cerithidea rhizophorarum* after incubation of tissue extracts with appropriate substrates. a, cellulase activity; b, mannanase activity; c, xylanase activity; d, laminarinase activity. Bars represent SD. ND, not detectable. Black, *Cerithidea rhizophorarum*; gray, *Cerithidea cingulata*; white, *Batillaria multiformis*; striped pattern, *B. attramentaria*. Values followed by the same letters do not differ significantly (Tukey's HSD,  $p < 0.05$ ).



**Fig. 3.** Detection of deduced molecular weight of cellulases by using zymographic analysis. 1, *Batillaria multiformis*; 2, *B. attramentaria*; 3, *Cerithidea cingulata*; 4, *Cerithidea rhizophorarum*. White triangle indicates the site of each band. Numbers denote the deduced molecular weight (kDa).

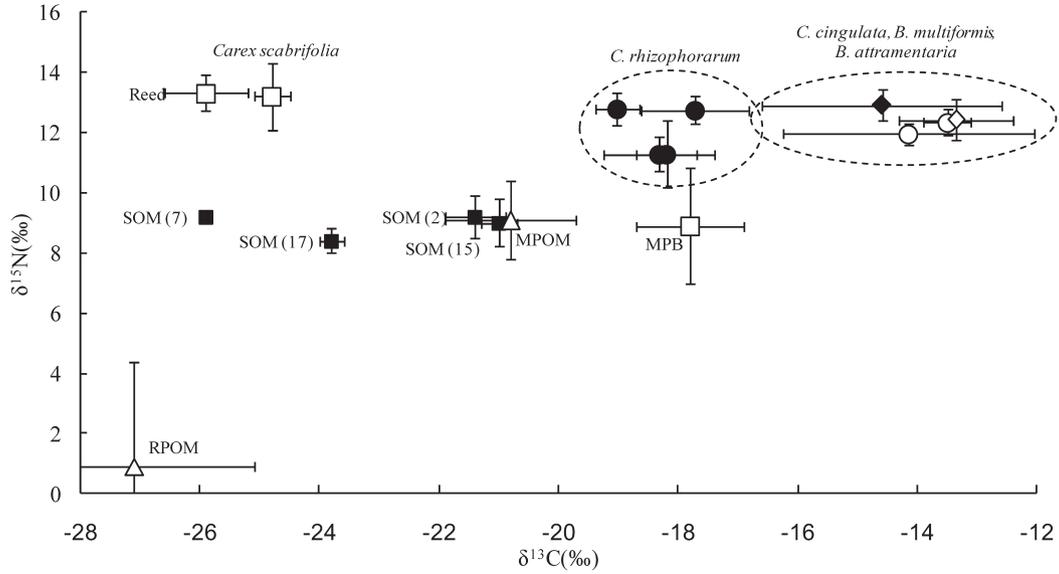
styles of gastropods (Fig. 3) revealed that multiple cellulases were present in all of the gastropods. Activity bands representing approximately 44 kDa, 80 kDa, and 100 kDa

were observed for *B. multiformis* and *B. attramentaria*. Activity bands representing approximately 34 kDa were observed for *Cerithidea rhizophorarum* and *Cerithidea cingulata*.

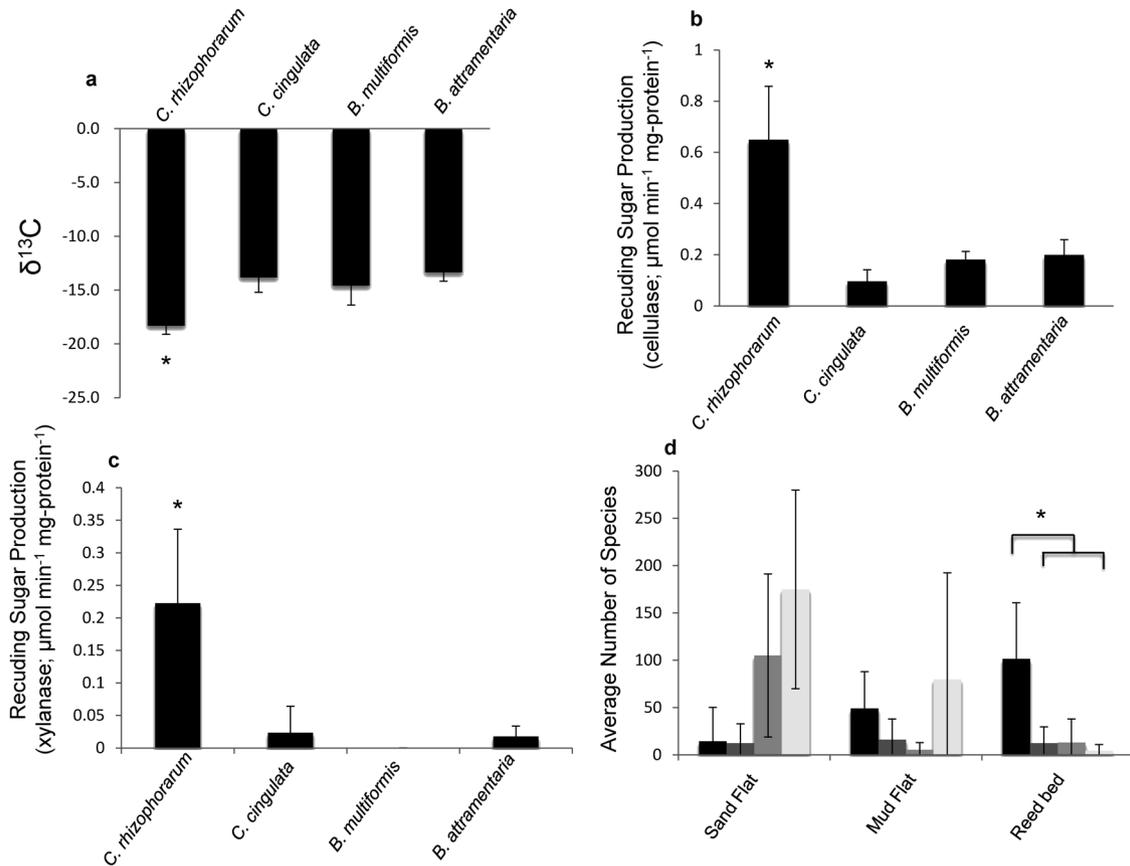
#### Stable isotope ratio and statistical analysis

Figure 4 shows the results of stable isotope ratio analysis. The sediment  $\delta^{15}\text{N}$  values were similar among the sites (8.4–9.2‰), whereas the sediment  $\delta^{13}\text{C}$  values varied markedly (–25.9‰ to –21.0‰). The MPB  $\delta^{13}\text{C}$  value (–17.8 ± 0.9‰) was similar to the *Cerithidea rhizophorarum*  $\delta^{13}\text{C}$  value (–19.0‰ to –17.7‰), but differed from the *Cerithidea cingulata* (–14.1‰ to –13.5‰), *B. multiformis* (–14.6‰), and *B. attramentaria* (–13.4‰)  $\delta^{13}\text{C}$  values. The  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  values for *P. australis* and *C. scabrifolia*, which are major salt marsh herbaceous plants, were approximately equal. The sand-flat sediment  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  values were similar to the MPOM  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  values, whereas the reed-bed sediment  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  values differed from the MPOM  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  values. The C/N ratios of sediment (12.2–15.3) and reeds (13.3) were high. The RPOM  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  values were lower than all of the other  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  values.

The average  $\delta^{15}\text{N}$  value for each gastropod species ranged from 11.9‰ to 13.0‰; these values did not differ significantly between sites or species (Tukey's HSD,  $p < 0.05$ ). On the other hand, the  $\delta^{13}\text{C}$  value of *Cerithidea rhizophorarum* differed significantly from those of the other gastropods (–19.0‰ to –17.7‰ vs. –14.6‰ to –13.4‰; Tukey's HSD,  $p < 0.05$ ). The  $\delta^{13}\text{C}$  value of *Cerithidea cingulata* at reed-bed site 17 was more similar to that of *Cerithi-*



**Fig. 4.** Stable isotope ratios of *Cerithidea rhizophorarum*, *Cerithidea cingulata*, *B. multiformis*, *B. atramentaria*, sediment organic matter (SOM), plants, microphytobenthos (MPB), and particulate organic matter (POM). All samples were collected at the Tanaka River estuary. Black circle, *Cerithidea rhizophorarum*; white diamond, *B. multiformis*; gray diamond, *B. atramentaria*; white circle, *Cerithidea cingulata*; black square, SOM; white square, plants and MPB; white triangle, POM. Bars represent SD.



**Fig. 5.** Comparison of  $\delta^{13}\text{C}$  value, cellulase activity, xylanase activity, and spatial distribution among 4 species of gastropods. (a),  $\delta^{13}\text{C}$  value; (b), cellulase activity; (c), xylanase activity; (d), spatial distribution. Error bars represent SD; asterisks indicate significant differences. Bars in (d): black, *Cerithidea rhizophorarum*; dark gray, *Cerithidea cingulata*; gray, *Batillaria multiformis*; white, *B. atramentaria*.

*dea cingulata* at sand-flat site 10 than to that of *Cerithidea rhizophorarum* in any of the reed-bed sites.

In comparison with *Cerithidea cingulata*, *B. multiformis*, and *B. atramentaria*, the  $\delta^{13}\text{C}$  value of *Cerithidea rhizophorarum* was significantly lower, whereas the activity levels of cellulase and xylanase were significantly higher (Fig. 5; Tukey's HSD,  $p < 0.05$ ); further, the number of *Cerithidea rhizophorarum* individuals in the reed bed was significantly higher (Fig. 5; Tukey's HSD,  $p < 0.05$ ).

**Spatial distribution of gastropods**

Figure 6 shows the total populations of gastropods in each of the investigated sites. *Cerithidea rhizophorarum* was observed in six sand-flat sites and in nine reed-bed sites. The population density of this species was highest in the reed bed (mean population, 101 gastropods per  $\text{m}^2$ ) than in the sand flat or mud flat. The population density was highest in reed-bed site 5 (213 gastropods per  $\text{m}^2$ ), which was located at the edge of the reed bed; this was followed by the sites in the center of the reed bed (reed-bed site 17, 156 gastropods per  $\text{m}^2$ ; reed-bed site 16, 137 gastropods per  $\text{m}^2$ ; and reed-bed site 6, 133 gastropods per  $\text{m}^2$ ). The population density was also relatively high in mud-flat

sites 1 and 2. No *Cerithidea rhizophorarum* individuals were observed in sand-flat sites 9, 10, 15, 19, and 20, all of which were located in the tidal flats.

*Cerithidea cingulata* was observed in five sand-flat or mud-flat sites, and in four reed-bed sites. The gastropod population density was high at reed-bed site 5, mud-flat site 1, and sand-flat site 10. The mean population density in these sites ranged from 40 gastropods per  $\text{m}^2$  to 59 gastropods per  $\text{m}^2$ . *Cerithidea cingulata* was observed in fewer sites than were *Cerithidea rhizophorarum*, *B. multiformis*, and *B. atramentaria*. In addition, the total population of *Cerithidea cingulata* was lower than those of the three other investigated species. *Cerithidea cingulata* tended to be most abundant near the water gate (mud-flat site 1 and reed-bed site 7), along the waterway (sand-flat site 10), and close to the river (reed-bed site 17).

*Batillaria multiformis* was observed in nine sand-flat or mud-flat sites, and in four reed-bed sites. Within these sites, this species tended to be distributed in sandy and sandy-muddy areas. The population density of *B. multiformis* was highest in sand-flat sites 18, 19, and 20; at these sites, the average population density ranged from 151 gastropods per  $\text{m}^2$  to 265 gastropods per  $\text{m}^2$ , and accounted for 70% of the total population.

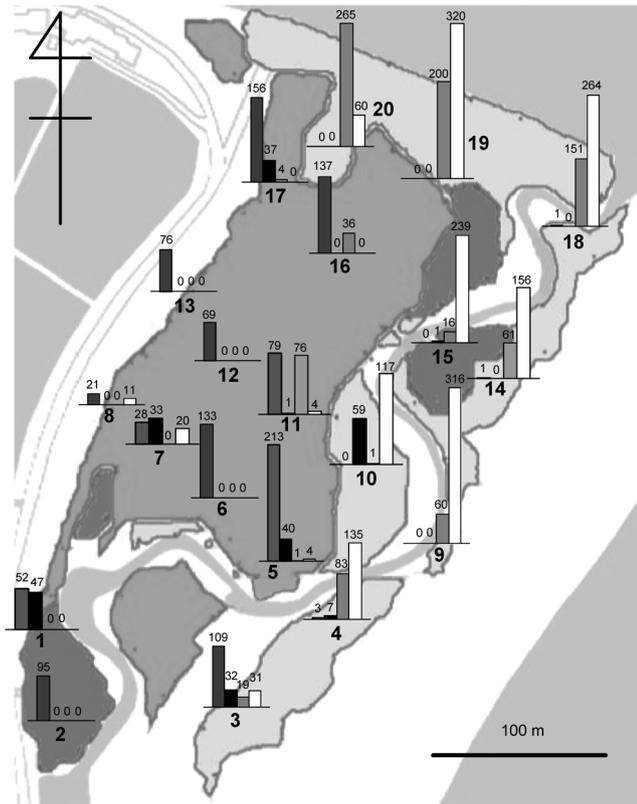
*Batillaria atramentaria* also tended to be distributed in sandy and sandy-muddy areas, and was observed in nine sand-flat or mud-flat sites, and in four reed-bed sites. The overall population density of this species was 1677 gastropods per  $\text{m}^2$ , which was the highest among the four investigated species. The population density was highest along the waterway (sand-flat sites 4, 9, 14, 18, and 19; and mud-flat site 15). Together, these six sites accounted for >50% of the total population. *Batillaria atramentaria* was rarely observed in the reed bed.

**Physical environmental data**

Figure 7 summarizes the physical environmental data for the study sites. The elevation of the 20 sites ranged from 1.0 m to 1.88 m. The mean elevation in the reed bed was 1.3–1.81 m, indicating that the reed bed was located in the intertidal to upper tidal zone. The reed bed was at a higher elevation than were the sand and mud flats (data not shown).

The plant coverage of the sand and mud flats was 0.0–3.0 (mean=0.4). The plant coverage of the reed bed was 2.0–3.7 (mean=2.8; data not shown). On the tidal flat, *Carex scabrifolia* was observed at mud-flat site 1, *Phragmites australis* was observed at reed-bed site 5 and sand-flat site 8, and *Gracilaria vermiculophylla* was observed at sand-flat site 10. On the reed bed, *Phragmites australis* was observed at all of the study sites.

The sediment in the sand and mud flats contained 0.89–4.48% organic matter (mean=2.28%). In the reed bed, the sediment contained 1.53–3.61% organic matter (average=2.39%; data not shown). The percentage organic matter was slightly higher in the reed bed than in the sand or



**Fig. 6.** Distribution of gastropods in the Tanaka River estuary. Charcoal gray, *Cerithidea rhizophorarum*; black, *Cerithidea cingulata*; gray, *B. multiformis*; white, *B. atramentaria*. Population is the sum of 3 quadrats (50 cm×50 cm); 3 core samples were taken at each site.

mud flats. Mud-flat areas (sites 1 and 2) contained high percentages of organic matter (3.58% and 3.05%, respectively). These sites were located near a sluice, where fresh water containing domestic wastewater entered the river. In the reed bed, the percentage of organic matter was particularly high at site 11 (3.61%), which was located on the boundary between a reed bed and a mud flat.

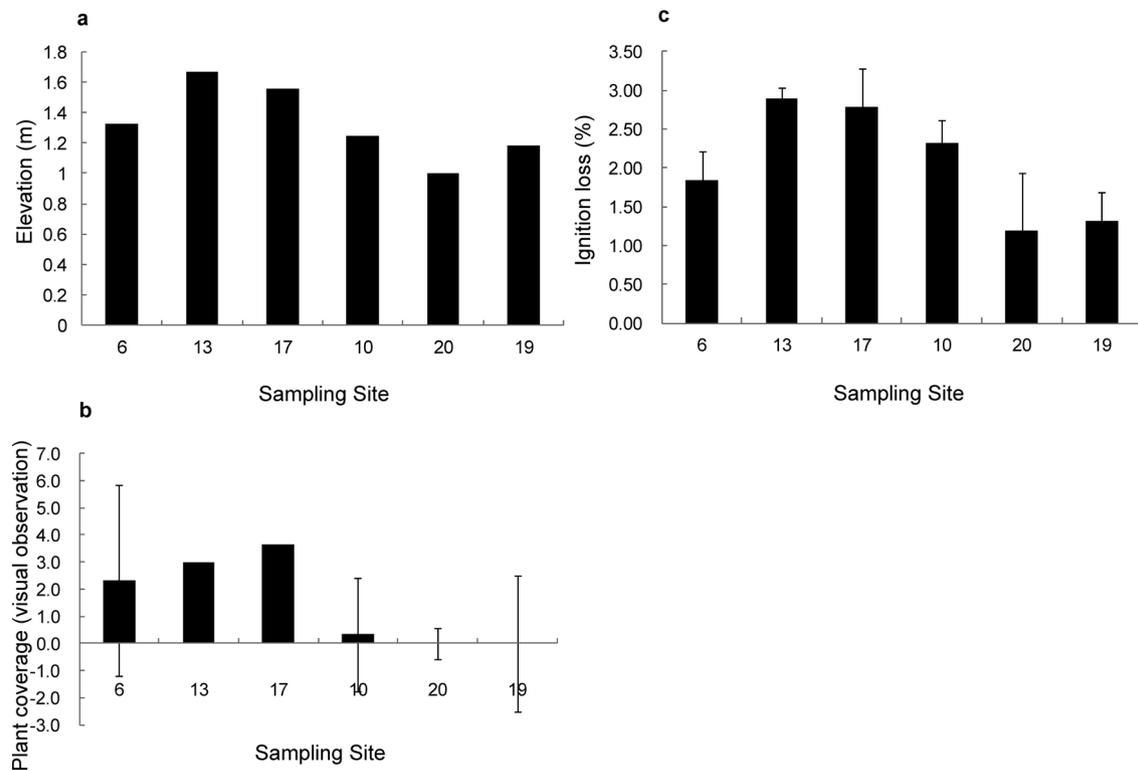
The physical environmental data indicate an increasing tendency of reed-bed spread, plant coverage, and organic matter at higher elevation.

## Discussion

Recent studies on benthic animals have focused either on their plant-digesting enzymes (Davison & Blaxter 2005; Rahman et al. 2011; Tanimura et al. 2013) or on their species distribution (Adachi & Wada 1998; Vizzini & Mazzola 2006; Kanaya & Kikuchi 2008). However, to the best of our knowledge, the relationship between enzyme activity level and species distribution has not previously been investigated. In the present study, we examined the relationship between digestive enzyme activities and the distribution of four species of gastropods (*Cerithidea rhizophorarum*, *Cerithidea cingulata*, *Batillaria multiformis*, and *B. attramentaria*) inhabiting the Tanaka River estuary (Mie Prefecture, Japan), to clarify the species-specific distribution of related gastropods.

We successfully quantified the activities of cellulase and several hemicellulases by measuring the production of reducing sugars. Our results indicated that the investigated gastropod species were able to digest cellulose, mannose, xylan, and laminarin (Fig. 2). Among the four species evaluated, *Cerithidea rhizophorarum* showed particularly high activity for all of the investigated enzymes, implying that this species more efficiently secretes these enzymes than do *Cerithidea cingulata*, *Batillaria multiformis*, and *B. attramentaria*.

The levels of xylanase and cellulase activities differed markedly according to species and sites. Xylan concentrations are particularly high in terrestrial plants; on the other hand, cellulose is included in all plants—including marine algae (McNeil et al. 1984; Wyman et al. 2005). Therefore, significant differences in xylanase activity between gastropods are likely related to the consumption of terrestrial plants. For example, *Cerithidea cingulata* collected from reed-bed site 17 showed xylanase activity, whereas *Cerithidea cingulata* collected from sand-flat site 10 did not. Further, *Cerithidea rhizophorarum* individuals collected from reed-bed sites 17 and 13 showed significantly higher xylanase activity than did those collected from sand flats (Fig. 2). In other words, *Cerithidea cingulata* and *Cerithidea rhizophorarum* collected from the reed bed showed high xylanase activity, and probably ingested and assimilated organic matter derived from reeds in the sediment.



**Fig. 7.** Environmental characteristics of the sampling sites. (a) elevation; (b) plant cover; (c) ignition loss of organic matter in sediment.

Mannan is a hemicellulose found in some species of marine algae (Wyman et al. 2005; Moreira & Filho 2008). Laminarin is a hemicellulose used for storage of carbohydrates by marine algae. Brown algae such as *Laminaria hyperborea* and *Saccharina longicuris* contain large amounts of laminarin, whereas red algae contain relatively low amounts (Nelson & Lewis 1974). In the present study, we observed no marked differences in the levels of mannanase and laminarinase activities among species and sites (Fig. 2). However, all of the gastropods examined showed detectable activities of mannanase and laminarinase. In accordance with previous findings, our results suggest that gastropods ingest algae and benthic diatoms entering the estuary (see, for example, Kang et al. 2003; Kanaya et al. 2005; Yokoyama et al. 2005).

The results of zymographic analysis indicated that the four species of gastropods examined possess multiple cellulases with different molecular weights. The molecular weights of cellulases in *Cerithidea rhizophorarum* and *Cerithidea cingulata* were similar (approximately 34 kDa); however, *B. multiformis* and *B. attramentaria* cellulases had molecular weights of approximately 44 kDa, 80 kDa, and 100 kDa (Fig. 3). Thus, related gastropod species showed similar cellulase patterns—*Cerithidea rhizophorarum* and *Cerithidea cingulata* belong to the family Potamididae, whereas *B. multiformis* and *B. attramentaria* belong to the family Batillariidae (Davison & Blaxter 2005; Species Dictionary 2012). Phylogenetic relationships are not necessarily correlated with digestive enzyme characteristics, but may explain our observed pattern of results. For example, the amino acid sequences of cellulases in *Haliotis discus hannai* and *Haliotis discus discus* tend to be highly homologous (Suzuki et al. 2006; Nikapitiya et al. 2010).

In the present study, we further showed that the cellulase activity levels differed significantly among species (Fig. 2), resulting in different band intensities when the same amount of protein was applied. When the amount of protein required to obtain the clearest band for *B. multiformis* was used for all of the investigated species, the band for *Cerithidea rhizophorarum* was smeared (data not shown). Therefore, we optimized the amount of protein used in the zymographic analysis for each species, in order to obtain clear bands (Fig. 3). We showed that the largest amount of protein was required by *Batillaria multiformis*, followed by *B. attramentaria*, *Cerithidea cingulata*, and *Cerithidea rhizophorarum*. Our results suggest that differences in enzyme activity level among species are not derived from differences in the enzyme itself, but from the amount of enzyme secreted.

We evaluated several potential food sources for the four investigated species of gastropod. We determined the highest  $\delta^{13}\text{C}$  value for MPB (annual mean =  $-17.8\%$ ), followed by MPOM (annual mean =  $-20.8\%$ ), *C. scabrifolia* (annual mean =  $-24.8\%$ ), *P. australis* (annual mean =  $-25.9\%$ ), and RPOM (annual mean =  $-27.1\%$ ). The  $\delta^{13}\text{C}$  of the four gas-

tropod species ranged from  $-19.0\%$  to  $-13.4\%$ , which was more than  $5\%$  higher than the reed  $\delta^{13}\text{C}$  ( $-25.9\%$ ), but similar to the MPB  $\delta^{13}\text{C}$  and the MPOM  $\delta^{13}\text{C}$ . Our results indicate that organic matter from reeds is not usually utilized as a source of nutrients by gastropods. On the other hand, the  $\delta^{13}\text{C}$  of gastropods collected from reed beds was approximately  $5\%$  lower than that of gastropods collected from sand and mud flats, indicating that reeds contribute, at least partially, to the diet of reed-bed inhabiting gastropods. This may be explained by a relatively low availability of MPB because of plant cover, and also an abundance of organic matter from reeds. We further observed that reed beds had a high  $\delta^{13}\text{C}$  value ( $-25.9$  to  $-23.87\%$  at sites 7 and 17), but a low C/N ratio (12.2–15.3). Similarly, reeds had a low  $\delta^{13}\text{C}$  value ( $-25.9\%$ ), but a high C/N ratio (13.3), indicating the presence of abundant organic matter derived from reeds within the reed-bed sediment.

Several studies have shown that the main carbon sources of macrobenthos are benthic diatoms, epiphytic algae, and phytoplankton, whereas the contribution of terrestrial organic matter is low (Hargrave 1970; Kanaya et al. 2005; Yokoyama & Ishii 2007). Kurata et al. (2001) reported that *Assiminea japonica* and *Angustassiminea castanea castanea* inhabiting salt marshes utilize phytoplankton and benthic diatoms. Choy et al. (2008) reported that two species of Batillariidae (*Batillaria attramentaria* and *B. exarata*) mainly assimilated algae and bacteria. Similarly, the gastropods examined in the present study probably eat benthic diatoms and phytoplankton, rather than terrestrial plants. Interestingly, however, in comparison with *Cerithidea cingulata*, *B. multiformis*, and *B. attramentaria*, *Cerithidea rhizophorarum* showed a significantly low  $\delta^{13}\text{C}$  value, high cellulase and/or xylanase activities, and dominance in reed beds (Fig. 5). Taken together, these results suggest that *Cerithidea rhizophorarum* depends more heavily on reeds for its nutrition than do *Cerithidea cingulata*, *B. multiformis*, and *B. attramentaria*.

In the present study, we further observed that *Cerithidea rhizophorarum*, *Cerithidea cingulata*, *B. multiformis*, and *B. attramentaria* tended to have isolated habitats (Fig. 6). *Cerithidea rhizophorarum* had a tendency to dominate reed beds, whereas relatively few individuals were found in the sand and mud flats. Interestingly, at sand-flat site 3, *Cerithidea rhizophorarum* was the most common species. This site was in a sand-flat habitat, but was located very close to the edge of the reed bed, and had a similar biota to that found inside the reed bed.

Wada and Nishikawa (2005) reported that, among *Cerithidea rhizophorarum*, *Cerithidea cingulata*, *B. multiformis*, and *B. attramentaria*, *Cerithidea rhizophorarum* was most likely to be unevenly distributed in the upper intertidal zone. The authors concluded that this tendency was strongly affected by the presence of vegetation. The results of our present study are in accordance with the findings of Wada and Nishikawa (2005), because the reed bed had relatively high elevation, plant coverage, and organic matter

(Fig. 7). *Cerithidea rhizophorarum* is reported to be more tolerant of aridity than are *Cerithidea cingulata*, *B. multiformis*, and *B. attramentaria* (Wakamatsu & Toyama 2000). This attribute may further explain why this species was widely distributed in the reed bed. *Cerithidea rhizophorarum* individuals collected from reed beds (sites 13 and 17) showed higher enzyme activities than did those collected from other sites (Fig. 2). Sites 13 and 17 tended to show relatively high, although not markedly high, levels in environmental parameters (elevation, plant coverage, and organic matter). Enzyme activity level can vary according to the substratum environment, even within the same reed bed; therefore, further detailed environmental research to confirm the species differences observed in the present study is required.

*Cerithidea cingulata*, *B. multiformis*, and *B. attramentaria* were rarely observed in reed beds, but were dominant in the sand and mud flat habitats. According to Wada and Nishikawa (2005), *Cerithidea cingulata* shows a preference for muddy substrata in the middle intertidal zone. The sites where *Cerithidea cingulata* was found in the present study had higher elevation in the intertidal zone than did the sites where *B. multiformis* and *B. attramentaria* were dominant (Fig. 7). This elevation characteristic of *Cerithidea cingulata* is similar to that of *Cerithidea rhizophorarum*. Therefore, *Cerithidea cingulata* and *Cerithidea rhizophorarum* may prefer similar physical environments. These two species were generally found at the same sites in the present study (Fig. 6). It is possible that the observed differences in the number of these two species were derived from the considerable gap between their enzyme activities, and therefore the resulting differences in dietary habits (Figs 2 & 3).

In comparison with *Cerithidea cingulata* and *Cerithidea rhizophorarum*, *B. multiformis* and *B. attramentaria* showed a preference for sites with lower elevation, less plant coverage, and smaller amounts of organic matter (Fig. 7). The enzyme activities of *B. multiformis* and *B. attramentaria* were significantly lower than those of *Cerithidea rhizophorarum* (Figs 2 & 3). These differences in enzyme activity level and food habits likely determine substratum preference, thereby contributing to the observed habitat distribution. *Batillaria multiformis* and *B. attramentaria* preferred similar habitats, but showed differing density distributions—*B. multiformis* preferred the riverbank, whereas *B. attramentaria* preferred sites along the waterway (Fig. 6). According to Magi et al. (2002), the distribution of these two species is regulated by distribution preferences, and substrate and salt tolerance. Further detailed research to facilitate detailed elucidation of their habitat requirements is required.

Habitat isolation of related species has been extensively studied. For example, the mytilid mollusks *Musculista senhousia* and *Xenostrobus securis* show habitat segregation in the larval stage, based on bottom sediment characteristics (Gofas & Zenetos 2003). *Batillaria multiformis* and *B.*

*attramentaria* prefer sand and mud substrata, whereas *Cerithidea rhizophorarum* prefers elevated locations (Magi et al. 2002; Wada & Nishikawa 2005). However, research on mollusk habitats has mainly focused on the relationship between species distribution, and ecology or gene diversity. Few studies have compared digestive enzymes, feeding habits, and habitat isolation of related species inhabiting a relatively small area.

In the present study, *Cerithidea rhizophorarum* showed relatively high cellulase and xylanase activities (Figs 2 & 3). In addition, the results of stable isotopic analysis indicated that *Cerithidea rhizophorarum* assimilated more terrestrial plant material than did *Cerithidea cingulata*, *B. multiformis*, or *B. attramentaria* (Figs 4 & 5). Furthermore, our species distribution investigation revealed that *Cerithidea rhizophorarum*, *Cerithidea cingulata*, *B. multiformis*, and *B. attramentaria* were distributed differently inside and outside the reed bed (Figs 6 & 7). It is possible that habitat is determined not only by environmental characteristics such as substratum, but also by the activity of digestive enzymes. Based on the results of our present study, and also on the findings of several previous studies, we draw the following conclusions. (1) *Cerithidea cingulata*, *B. multiformis*, and *B. attramentaria* have lower abilities to digest cellulose than does *Cerithidea rhizophorarum*, and therefore cannot settle inside reed beds, which contain abundant reed-derived carbohydrates that are difficult to degrade. Thus, as previously reported, these three gastropod species feed mainly on benthic diatoms or seaweed in tidal flats (Kang et al. 2003; Kanaya et al. 2005; Yokoyama et al. 2005). (2) In order to degrade plant-derived carbohydrates from reeds, *Cerithidea rhizophorarum* secretes cellulase and xylanase more efficiently than do *Cerithidea cingulata*, *B. multiformis*, and *B. attramentaria*. Therefore, *Cerithidea rhizophorarum* is able to inhabit reed beds that are inhospitable to the other three gastropod species.

Thus, the evaluation of digestive enzyme activities can clarify the detailed habitat use of gastropods inhabiting the same area. *Cerithidea rhizophorarum* likely uses reed beds as its habitat, because it can degrade plant-derived carbohydrates, which other gastropods cannot. Reed bed areas have been shown to support many rare benthic animals (Kimura & Kimura 1999; National Institute for Environmental Studies 2003). The results of our present study indicate that the physiological characteristics of polysaccharide degradation are important factors for the expansion of benthic animals into reed beds. Various mollusks inhabit the estuary environment, including many endangered species, e.g., *Ellobium chinense* (Kimura & Kimura 1999; Japan Integrated Biodiversity Information System 2008). *Batillaria multiformis*, *Cerithidea cingulata*, and *Cerithidea rhizophorarum* are major gastropods inhabiting estuaries of Japan, but are listed as near threatened species in the Red Data Book (National Institute for Environmental Studies 2003; Japan Integrated Biodiversity Information System

2008). Populations of these species are decreasing markedly, because of the destruction, burial, and pollution of their habitats through land reclamation (Lee et al. 1997; National Institute for Environmental Studies 2003). Therefore, clarification of food habits and ecological distribution is essential for the preservation of Japanese domestic biodiversity.

In the present study, the observed differences in digestive enzyme activity level may determine the detailed distribution of related species inhabiting the same environment. For example, *Cerithidea rhizophorarum* assimilated larger amounts of terrestrial plant materials than did *Cerithidea cingulata*, *B. multiformis*, and *B. attramentaria*, and therefore tended to dominate reed beds. We believe that our findings will facilitate a detailed understanding of estuarine environments and the preservation of benthic animals.

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