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Seasonal variation in Terrestrial cellulose assimilation of benthic invertebrates inhabiting wetlands in Osaka Bay, Japan

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Abstract: It has become much clearer that benthic invertebrates are involved in terrestrial organic matter (TerrestrialOM) decomposition in wetlands since the discovery of endogenous carbohydrate hydrolases (CHs, e.g., cellulase) in various invertebrates during recent decades. Because the breakdown of high molecular TerrestrialOMs is essential to the circulation of carbon between the terrestrial and marine ecosystems, the level of TerrestrialOMs decomposed by benthic invertebrates should be estimated correctly to evaluate the true TerrestrialOM decomposition capability of a wetland. To estimate invertebrates' contribution to the wetland, both the levels of relevant enzymatic activities (capability) and stable isotope signatures (performance) and their relationship must be investigated. Also, seasonal variation should be taken into consideration to increase the accuracy of the estimation. Here we firstly used the CMC Plate Assay to confirm that 8 dominant benthic invertebrate species that inhabit Onosato River Estuary (ORE) and Kishiwada Artificial Tidal Flat (KATF), two wetlands facing Osaka Bay, possess cellulase activities. Except for *Cerithidea moerchii*, all of these invertebrates showed obvious seasonal variations in cellulase activities and stable isotope signatures, but the variation of enzymatic activities did not always correspond to that of the carbon and nitrogen isotope ratio ($\delta^{13}C$ and $\delta^{15}N$). In addition, variation in $\delta^{13}C$ of the investigated invertebrates was much larger than that of their candidate food source, namely the sedimentary organic matter (SOM) in the sediments and particulate organic matter (POM) in the sea, indicating the existence of selective feeding habits of the invertebrates, or biasing in their processing of TerrestrialOM. Furthermore, the carbon isotope ratio and C/N ratio of the SOM/POM in ORE and KATF corresponded to the $\delta^{13}C$ value of the invertebrates that inhabited each wetland. Additional cellulose-feeding experiments using *Cerithidea moerchii* showed that the $\delta^{13}C$ did not decrease significantly after the cellulose consumption, suggesting that the degradation product (glucose in this case) is not assimilated to its body tissues. In summary, this study revealed that aquatic invertebrates possessing CHs could use TerrestrialOMs as one of their food sources. Seasonal

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variations of enzymatic activity and carbon and nitrogen isotope ratios could indicate whether animals were more/less dependent on TerrestrialOMs, but were insufficient to reveal the true amount of TerrestrialOMs consumption by aquatic invertebrates.

Key words: Benthic invertebrates, cellulase

Introduction

Wetlands are transitional areas between terrestrial and marine ecosystems. In additional to local hydrophytic vegetation, the large amounts of TerrestrialOM that flow in from hinterlands make wetlands big carbon sinks that sometimes even surpass terrestrial forests as carbon sinks (Sudip et al., 2005; Scholz, 2011). It was at first empirically shown that wetlands play an important role in breakdown of high molecular TerrestrialOMs into low molecular nutrients that benefit marine organisms (Hatakeyama, 2019), and more recently endogenous carbohydrate hydrolase (CH) (e.g., cellulase) genes have been found in many aquatic invertebrates and proven to be responsible for this process (Tanimura et al., 2012). Our recent studies summarized benthic invertebrates that have been proven to date to have endogenous cellulase genes, and/or cellulase activity (Liu et al., 2021). Nevertheless, those studies are not able to answer the most-often asked questions from ecologists, such as "do benthic invertebrates consume more TerrestrialOM (e.g., cellulose) than microorganisms?" or "what are the differences between wetlands with different benthic invertebrate species and populations?". To answer these questions, quantification of the TerrestrialOM consumption of benthic invertebrates will be essential.

Stable isotope analysis has some advantages for revealing food web structures. Recent stable isotope analyses have provided evidence that benthic invertebrates inhabiting wetlands assimilate TerrestrialOMs as their food source (Kikuchi and Wada 1996; Doi et al., 2005; Kasai et al., 2005; Antonio et al., 2010; Kawaida et al., 2019), but the level of assimilation of OMs differed between species and locations (Kanaya et al., 2007; Liu et al., 2014). Thus, stable isotope analyses are insufficient to quantify exactly how much TerrestrialOM is consumed/decomposed by each benthic invertebrate. On the other hand, reducing sugar measurements, such as measurements by the tetrazolium method (Jue and Lipke, 1985) or Somogyi-Nelson Method (Green et al., 1989), are very useful tools to quantify the enzymatic activities of polysaccharides decomposition, and thus to estimate the consumption of TerrestrialOM. However, estimations of the TerrestrialOM consumption by enzymatic activities are made under the assumptions that the organisms possessing these enzymes use them all the time, as well as that the TerrestrialOM supply at the locality of the inhabitant is constantly sufficient, which might not always be true for every species of benthic invertebrate and each wetland environment.

In the present study, we focused on cellulose, which is the most abundant TerrestrialOM, and its catabolic enzyme, namely cellulase, to investigate the relationship between the cellulase activity (the capability of decomposing cellulose/TerrestrialOM) and the stable isotope signature (the assimilation level of TerrestrialOM). If these two indexes are in accord, it means that the cellulase activity could represent the real amount of consumption of TerrestrialOM, and thus could be used to roughly estimate the amount of TerrestrialOM consumed by benthic invertebrates. In addition, our previous studies (not published) showed that both the cellulase level and stable isotope signatures could differ according to the season. Therefore, we collected all target benthic invertebrates as far as possible in all four seasons (May, July, October, and December, 2020). Another attempt made in the present study is that we cultured one of the dominant invertebrates, *Cerithidea moerchi*i, in an environment in which only pure cellulose served as the food source, and investigated the relationship between the amount of cellulose consumption and the C and N isotope variation. Names of species in this article basically follow WoRMS (World Register of Marine Species; https://www.marinespecies.org).

Table 1 Sampling site, sampling date and corresponding information for the plate-assay (Fig. 2) of benthic invertebrates collected in ORE and KATF.

Species	Sampling site	Sampling Date	Plate Assay
Cerithidea moerchii	ORE A	May 26th July 30th October 16th, 2020	a
Batillaria multiformis	KATF A	July 30th October 16th December 18th, 2020	b
Tegula argyrostoma	ORE C KATF B	July 30th October 16th December 18th, 2020	c
Cellana nigrolineata	ORE C	May 26th October 16th December 18th, 2020	d
Acanthopleura japonica	KATF B	July 30th October 16th, 2020	e
Capitulum mitella	ORE D	May 26th October 16th December 18th, 2020	f
Chiromantes dehaani	ORE B	May 26th July 30th October 16th December 18th, 2020	g
Fistrobalanus kondakovi	ORE D	October 16th December 18th, 2020	h

Material and Methods

1. Sampling

Target benthic invertebrates were collected from ORE and KATF, both located near Osaka Bay, Osaka, Japan on May 26th, July 30th, October 16th, and December 18th, as shown in Table 1. All maps were acquired from the Geospatial Information Authority of Japan (Fig. 1) (https://www.gsi.go.jp/). The area of the natural wetland ORE is 0.021 km^2. The area near the north shore has sandy mud sediment, while the area near the south has sandy sediment. Dominant vegetation in ORE is *Phragmites australis*, *Zoysia sinica* var. *nipponica*, *Lysimachia mauritiana*, *Calystegia soldanella,* etc. (Biodiversity Center, Ministry of the Environment, 2007). The area of the artificial wetland KATF is 0.05 km^2. Sediment in KATF is sandy. Dominant vegetation in KATF is *Phragmites australis* (majority), *Glehnia littoralis*, *Tetragonia tetragonioides*, *Calystegia soldanella*, etc. (Kishiwada Shizen Tomonokai Higatahozen Kenkyu Gurupu, 2009). In 2020, the highest tide level of these 2 wetlands was 473 cm (March 10th), the lowest was 256 cm (February 11st), and the mean tide level was 376.9 cm (Japan Meteorological Agency). At ORE, 6 dominant macro-benthos were collected, of which two were detritus feeders, two were scraping feeders, and two were filter feeders. Gastropod *Cerithidea moerchii* (A. Adams, 1855, (WoRMs, same below)) (Mollusca) were collected from a reed field (Fig. 1c, site A). Crab *Chiromantes dehaani* (H. Mile Edwards, 1853) (Arthropoda) were captured on a muddy field (Fig. 1c, site B) using a shovel to turn over their hiding nests. Limpet *Cellana nigrolineata* (G. B. Sowerby, 1839) (Mollusca) and gastropod *Tegula argyrostoma* (Gmelin, 1791) (Mollusca) were collected on the quay on the west side (Fig. 1c, site C). Goose barnacle *Capitulum mitella* (Linnaeus, 1758) (Arthropoda) and acorn barnacle *Fistrobalanus kondakovi* (Tarasov and Zevina, 1957) (Arthropoda) were detached from the dents of the quay close to the mouth of the river (Fig. 1c, site D). At KATF, we only found 3 species of dominant macro-benthos, of which one was a detritus feeder and two were scraping feeders. Gastropod *Batillaria multiformis* (Lischke, 1869) (Mollusca) was collected in the sandy shore (Fig. 1d, site A). Gastropod *T. argyrostoma* and chiton *Acanthopleura japonica* (Lischke, 1873) (Mollusca) were collected on

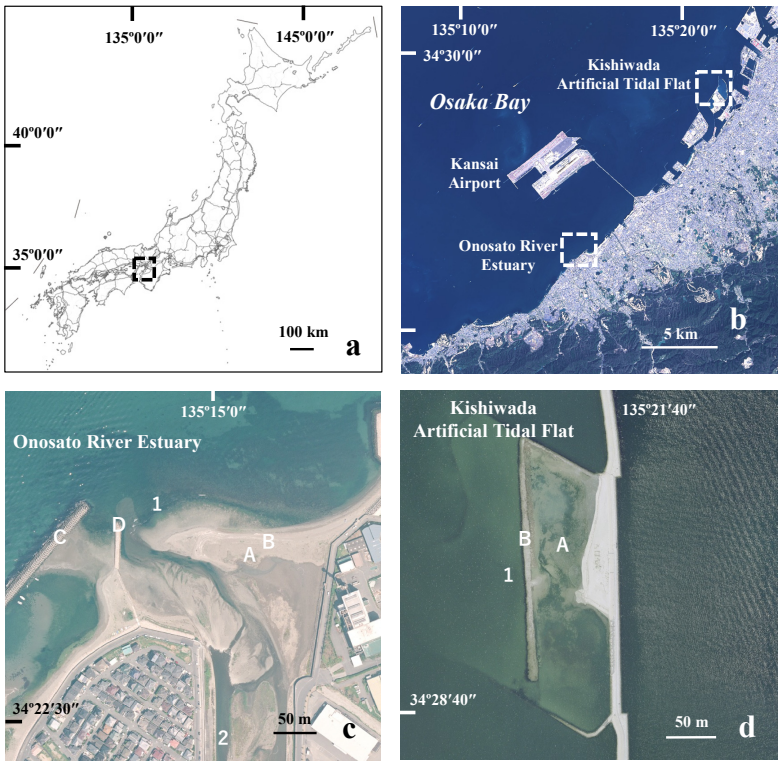

Fig. 1 Sampling sites.

a, Location of Osaka Bay, shown in dashed squares. Latitude and longitude of this area, as well as the scale bar are shown in the margin of the map, same below. **b**, Location of ORE and KATF in the east coast of Osaka Bay. **c**, Detailed map of ORE. Sampling sites of benthic invertebrates and sediments (SOM) are shown by letters, while sampling sites of seawater (POM) are shown in numbers. **d**, Detailed map of KATF. Sampling sites are shown in the same way as in ORE.

Table 2 Carbon and nitrogen concentrations, carbon and nitrogen stable isotope signatures and carbon/nitrogen ratios of SOM and POM in ORE and KATF.

	Date	Wetland	Sampling site	N (%)	C (%)	δ^{15}N (‰)	δ^{13}C (‰)	C/N
SOM (Sediment)	**July 30th, 2020**	**ORE**	Fig.1c, A (Reed field)	0.10	0.87	3.9	-26.5	10.6
	October 16th, 2020	**ORE**	Fig.1c, A (Reed field)	0.13	1.50	3.0	-28.6	13.6
		KATF	Fig.1d, A (Sandy field)	0.03	0.22	7.5	-20.1	7.4
				N (mg/L)	**C (mg/L)**			
POM (seawater)	**May 26th, 2020**	**ORE**	Fig.1c, 1 (River mouth)	0.03	0.23	7.4	-23.6	9.4
			Fig.1c, 2 (Upper stream)	0.04	0.41	6.1	-24.4	12.1
	July 30th, 2020	**ORE**	Fig.1c, 1 (River mouth)	0.43	3.20	7.0	-22.2	8.6
			Fig.1c, 2 (Upper stream)	0.18	1.57	5.6	-24.6	10.0
	October 16th, 2020	**ORE**	Fig.1c, 1 (River mouth)	0.29	2.35	7.4	-23.5	9.5
			Fig.1c, 2 (Upper stream)	0.12	1.10	5.8	-25.1	10.9
		KATF	Fig.1d, 1 (Quay west side)	0.07	0.43	9.3	-21.2	7.3

the quay on the west side of the wetland (Fig. 1d, site B). All benthic invertebrate samples were stored on ice before they were transported to the lab and dissected. Seawater and surface sediment were collected near the sampling sites in both wetlands (Table 2, sampling site) to investigate the characteristics of POM as potential food sources for the targeted species.

2. Plate Assay

One percent carboxymethyl cellulose (CMC) was added to an agar plate for the cellulase activity assay. For *C. moerchii*, *B. multiformis*, *T. argyrostoma*, *C. nigrolineata* and *A. japonica*, digestive glands were separated as the sample for the assay. For *C. mitella* and *F. kondakovi*, the whole body except their exoskeleton was used. The *C. dehaani* were first frozen at –20°C for 2 hours before their digestive glands were separated. All samples were washed with 70% ethanol and dried first, and then homogenized with scissors before they were applied to the agar plate. After a 24-hour incubation, 1mM Tris-HCl buffer (pH = 7.5) containing 1% Congo Red was added to stain the CMC for 2 hours. Then, 1M NaCl was added to de-stain unbound Congo Red in the agar plate. Cellulase activity was shown as a halo around the sample where CMC was decomposed.

3. Cellulase activity quantification

Digestive glands were separated from *C. moerchii*, *C. dehaani*, *C. nigrolineata*, *T. argyrostoma*, *B. multiformis*, and *A. japonica* into a 1.5 ml tube and 0.5 ml Tris-HCl buffer (pH = 7.5) was added. After the sample was homogenized (MH-1000, AS ONE Corp.) for 1 min, it was centrifuged at 8,000 × g for 5 min, and the supernatant was transferred to a new tube as the extract of the digestive glands. The protein concentration of the extracts was measured by the Bradford method (Bradford, 1976). Fifty micrograms of the extract were transferred to a new tube and diluted to 50 µl by adding Tris-HCl buffer (pH = 7.5). Fifty microliters of 1% CMC (in Tris-HCl, pH = 7.5) was then added to start the enzyme reaction. After incubation at 25°C for 30 minutes, 100 µl Somogyi reagent was added and the mixture was heated at 100°C for 10 minutes. After the heating, the tube was cooled down, 200 µl of Nelson reagent was added, and the absorbance at 660 nm was measured by using a spectrometer (NanoPhotometer®N60, WakenBtech Co., Ltd). The cellulase activity was defined as the quantity of reducing sugar (in micrograms) with the same reducing power as glucose generated by each mg of the digestive gland protein in 1 minute (µg glucose/min • mg protein) (Nelson, 1944).

4. Stable isotope analysis

4.1. Pre-treatments of the samples

4.1.1. Benthic invertebrates

Foot muscle from *C. moerchii*, *B. multiformis*, *T. argyrostoma*, and *C. nigrolineata*, mantles from *A. japonica* and *F. kondakovi* (near the basal plate), peduncle muscle from *C. mitella*, and working leg muscles from *C. dehaani* were isolated. No delipidation process was applied because these parts contain very less lipids and have little individual differences. All samples were lyophilized to avoid ammonia volatilization by using a vacuum drying oven (DRV320DA, ADVANTEC) connected to a cold trap (DRT140FB, ADVANTEC) and a diaphragm vacuum pump (MD4C, Vacuubrand) for over 1 day until the weight of the samples stabilized. Dried samples were powdered using a mortar and pestle, and transferred to 2-ml glass screwtop storage bottles that were pre-heated at 550°C for 3 hours. Approximately 0.3–0.6 mg of each sample (which contained 80 ~ 100 µg of nitrogen) was weighed inside a tin-capsule (SA76981101, SANTIS) by using an ultra-micro-electronic balance (XP2U, METTLER TOLEDO), molded into a small pellet and kept dry until analyses.

4.1.2. Sediment organic matter (SOM)

Dried sediment samples were sieved through a 300-µm mesh to remove plant fragments, shells, and coarse sand, and then homogenized with an agate mortar and pestle. Approximately 10 mg of sediment sample was weighed inside a silver capsule (SA76981105, SANTIS) by using an ultra-micro-electronic balance, and acidified with 2–3 drops of 1N HCl to remove $CaCO_3$. More drops of 1N HCl were added to the sample collected from KATF because numerous CO_2 bubbles appeared. Then, samples were transferred onto a 60°C hot plate (C-MAG HP7, IKA) and heated for 5 hours to remove remaining HCl and water. Finally, SOM samples were packed into cubes with tin disks (SA999902, SANTIS) as described above.

4.1.3. Particulate organic matter (POM)

Five liters of seawater from each sampling site were transferred to the lab and filtered within the same day by using a suction pump (DA-30S, ULVAC Inc.). Seawater samples were mixed well before they were applied to the suction filter system until the glass filter was clogged with POM. The glass filter (ϕ=47mm, GF/F, Sigma-Aldrich Co.) was pre-heated at 450°C for 3 hours to remove remaining organic matter before use. Vacuum pressure was fixed at under 0.02 MPa to prevent large size particles from penetrating the glass filter. Collected POM was then transferred to a plastic container and fumigated with 12 N–HCl for 1 day to remove inorganic carbon, and then transferred to a vacuum desiccator with NaOH pellets and allowed to stand for 1 week to remove HCl vapor. The filter was dried again on a hotplate at 60°C for 2 hours to completely remove water and remaining HCl. Margins of the glass filter without POM were removed. The remaining glass filter was cut to one-quarter to one-third of the original size to adjust the nitrogen concentration, and packed with tin-disks (SA999902, SANTIS).

4.2. Elemental analysis and mass spectrometry

Pre-treated samples were then applied to an Organic Elemental Analyzer (Flash 1112, Thermo Fisher Scientific) and an Isotope Ratio Mass Spectrometer (Delta plus XP, Thermo Scientific Inc.) via a Universal Continuous Flow interface (Conflo III, Thermo Fisher Scientific). The carbon concentration (shown as C (%) in SOM and C (mg/L) in POM) and nitrogen concentration (shown as N (%) in SOM and N (mg/L) in POM) were measured, and the carbon stable isotope ratio ($\delta^{13}C$) and nitrogen stable isotope ratio ($\delta^{15}N$) were calculated using the following equation:

$\delta^{13}C$, $\delta^{15}N = (R_{sample}/R_{standard} - 1) \times 1000$ (‰), where the term R denotes the ratios of $^{13}C/^{12}C$ or $^{15}N/^{14}N$, and Vienna Pee Dee Belemnite and atmospheric nitrogen were used as standards for carbon and nitrogen isotopes, respectively.

As the working standard, alanine ($\delta^{13}C$ = –19.6‰, $\delta^{15}N$ = 8.7‰, SI Science Inc.) was regularly analyzed to check the accuracy of the analysis and construct a regression curve between the stable isotope ratio and the amount of sample for the calibration of mass-dependent fluctuation of stable isotopes values (Umezawa, 2020). In addition, alanine ($\delta^{13}C$ = –19.6‰, $\delta^{15}N$ = 13.7‰, SI Science Inc.) was also analyzed to check the accuracy of the regression curve. The precision of the analysis was under ± 0.1‰ for both $\delta^{13}C$ and $\delta^{15}N$ of the samples.

5. Cellulose-feeding experiment

After the surface of shells was cleaned with 70% ethanol, 10 individuals of *C. moerchii* were moved to a plastic culture tank (20 cm length, 20 cm width, 20 cm height) containing 500 ml autoclaved artificial seawater previously poured into it (Fig. 5a). An approximately 100 cm^2 wide stone higher than the surface of the seawater was placed in the tank as a resting scaffold (since *C. moerchii* seems to avoid seawater by climbing reeds in natural wetlands). *Cerithidea moerchii* were fed with laboratory paper tissue (NIPPON PAPER CRECIA CO., LTD, $\delta^{13}C$ = –23.4‰, $\delta^{15}N$ = 4.1‰ analyzed in the present study), which was cut into same-size pieces (each piece weighed approximately 32.8 mg). After 20 days, a total of 18 pieces of paper tissue had been provided and consumed by *C. moerchii*, and three individuals were randomly sampled. After 44 days, an additional nine pieces of paper tissue had been eaten by the remaining seven individuals of *C. moerchii*, and another three were randomly sampled. For the control group, instead of artificial seawater, sediment heated at 600°C for 3 hours was spread in the bottom of the tank, and no paper tissue was provided until Day 44. All sampled *C. moerchii* were dissected and their carbon and nitrogen isotope ratios were analyzed as described above in 4.1.1.

6. Statistical analysis

Statistical analyses were carried out using IBM SPSS, ver.22. Levene's test was used before one-way ANOVA was applied to check the differences in cellulase activities of each invertebrate species collected in ORE and KATF among seasons. If ANOVA showed significance, Tukey Scheffe Post-Hoc analysis was applied. In addition, since we only had data of July and October for *A. japonica* in KATF, bivariate correlation analysis (Pearson correlation coefficients, 2-tailed) was applied instead. Bivariate correlation analysis was also applied in the cellulose-feeding experiment to verify the differences of $\delta^{13}C$ and $\delta^{15}N$ values between cellulose-feeding groups and control groups.

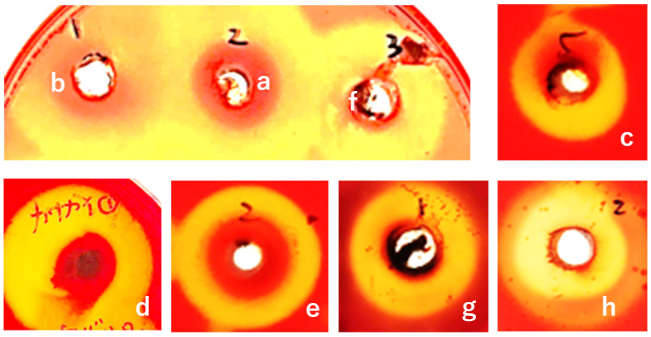

Fig. 2 Plate assay of benthic invertebrates.
Eight species of benthic invertebrates (5 species from ORE, 2 species from KATF, 1 species from both sites) were investigated for their cellulase activities. Digestive glands or the whole body of each species were placed in a well made in a 1% CMC-containing agar plate. Congo Red was used to stain the CMC after the enzymatic reaction. The halos around each well mean no CMC remained, representing the cellulase activity. Corresponding information between the plate assay letters and species is summarized in Table 1.

Results

1. Plate Assay

For purposes of discussion, we categorized all collected invertebrates into three feeding habit groups: detritus feeder, scraping feeder and filter feeder. As shown in Fig. 2, all species showed strong cellulase activity.

2. Composition differences and the seasonal variation of SOM and POM between Onosato River Estuary (ORE) and Kishiwada Artificial Tidal Flat (KATF)

As shown in Table 2, we investigated the carbon and nitrogen concentrations in SOM (C (%), N (%)), carbon and nitrogen concentration in POM (C (mg/L), N (mg/L)), carbon and nitrogen isotopes ratio ($\delta^{13}C$, $\delta^{15}N$) as well as carbon/nitrogen ratio (C/N) of both SOM and POM in ORE and KATF. SOM was sampled in both July and October in ORE, but only in October in KATF. POM was sampled in May, July and October in ORE, but only in October in KATF. $\delta^{13}C$ and $\delta^{15}N$ showed no significant seasonal variation in ORE: $\delta^{13}C$ of SOM showed a 2.1‰, and $\delta^{15}N$ showed a 0.9‰ difference between July and October, while $\delta^{13}C$ of POM showed a maximum of 1.4‰ difference in the river mouth, and δ15N of POM showed a maximum of 0.5‰ difference (in the upper stream) among May, July and October. As for the concentration of C and N, the highest concentration of C (%) and N (%) of SOM in ORE was shown in October. The N concentration (%) of SOM in ORE was about 4.3 times larger than that in KATF in October, and the carbon concentration (%) in ORE was about 6.8 times larger than that in KATF in the same month. POM showed the same tendency: the highest concentration of both C (mg/L) and N (mg/L) in ORE (in both river mouth and upper stream) were shown in July. The C (mg/L, river mouth) in ORE was about 5.5 times higher than that in KATF, and the N (mg/L, river mouth) in ORE was about 4.1 times higher than that in KATF. In addition, the $\delta^{13}C$ values of both SOM and POM in ORE were much smaller than those in KATF (8.5‰ variation in SOM, 2.4–3.9‰ variation in POM). The C/N ratio in ORE was much higher than that in KATF. In ORE, the lowest C/N ratio in SOM (10.6) was found in July, and the lowest C/N ratio in POM (8.6) was found in the same month.

3. Seasonal variation in cellulase activity and stable isotope ratio of benthic invertebrates

3.1. Onosato River Estuary

The stable isotope ratios of benthic invertebrates differed among feeding habit groups (Fig. 3). Usually, the $\delta^{13}C$ values of detritus feeders (i.e., *C. moerchii* and *C. dehaani*) are lower than those of scraping feeders (i.e.,

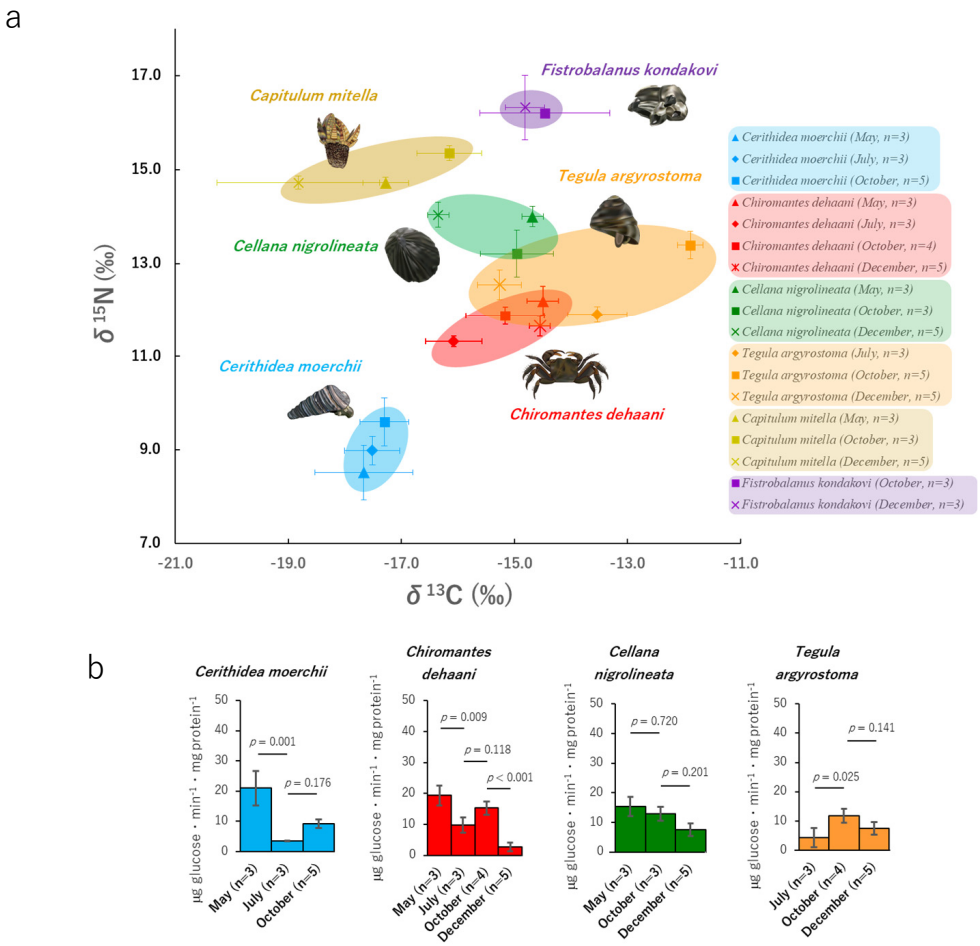

Fig. 3 Carbon and nitrogen stable isotope signatures and cellulase activity level of the benthic invertebrates in ORE.

a. Carbon and nitrogen stable isotope signatures. Ellipse of each color shows the range of the seasonal variation of carbon (horizontal axis) and nitrogen (vertical axis) of each benthic invertebrate species. Four shapes represent the sampling seasons (Triangle: May; Rhomboid: July; Square: October; X mark: December; same in each species). Values are means ± SD.

b. Cellulase activities. The cellulase activity was defined as the reducing sugar produced in unit time (min) and unit tissue (mg protein) that have the same reducing power of unit weight glucose (μg). Values are means ± SD. *p* values of Post Hoc analysis (Scheffe) are shown between adjacent seasons.

T. argyrostoma and *C. nigrolineata*). Among detritus feeders, the $\delta^{13}C$ value of *C. moerchii* stayed in a low and narrow range (–17.5 to –17.7‰) throughout the whole year. Both the $\delta^{13}C$ value (–14.5 to –16.1‰) and $\delta^{15}N$ value (11.3 to 12.2‰) of *C. dehaani*, another dominant detritus feeder, were higher than those of *C. moerchii*. As scraping feeders, *C. nigrolineata* and *T. argyrostoma* showed higher $\delta^{13}C$ and $\delta^{15}N$ values compared to the detritus feeders. *Tegula argyrostoma* showed a very wide range of $\delta^{13}C$ value (–11.9 to –15.3‰), but the variation between individuals in each season was small. Also, the lowest $\delta^{13}C$ values of *C. nigrolineata* and *T. argyrostoma* were both shown in winter (December). The $\delta^{13}C$ values of filter feeders (i.e., *C. mitella* and *F. kondakovi*) showed no big differences compared to those of detritus feeders, but the $\delta^{15}N$ value of the filter feeders was much higher (14.6–15.5‰ in *C. mitella* and 15.4–16.9‰ in *F. kondakovi*). The filter feeders also showed a large

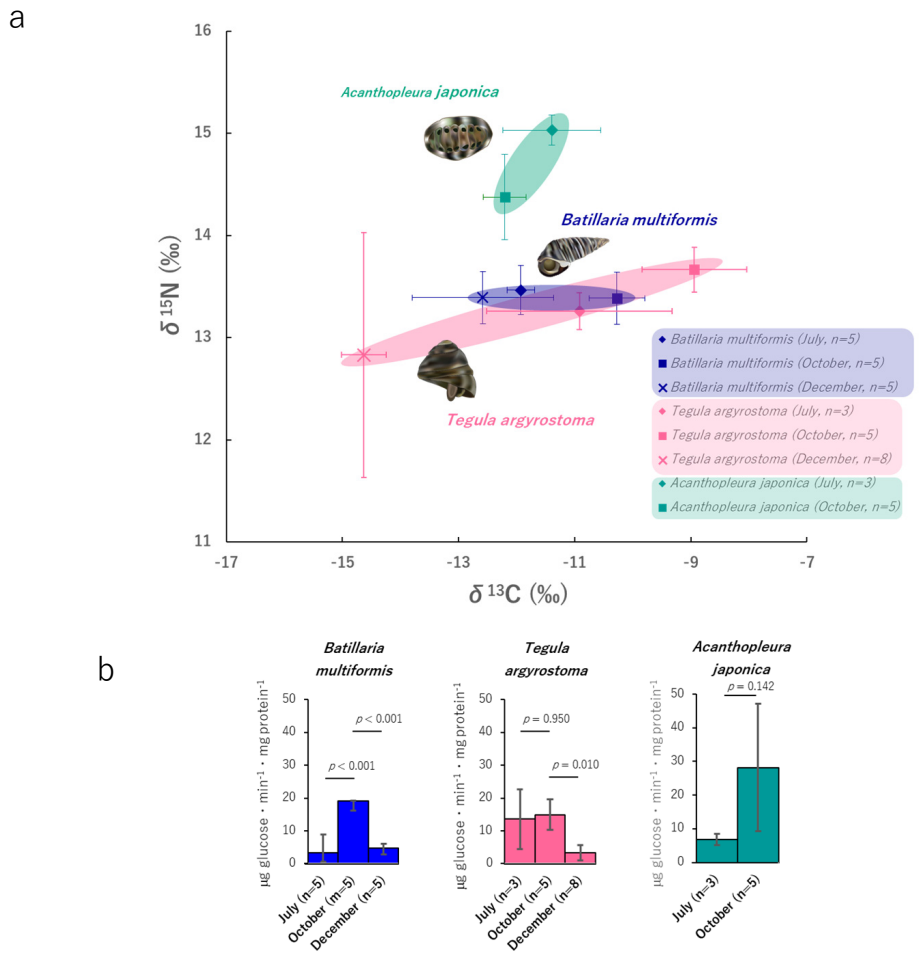

Fig. 4 Carbon and nitrogen stable isotope signatures and cellulase activity level of the benthic invertebrates in KATF.

a. Carbon and nitrogen stable isotope signatures. **b**. Cellulase activities. Legends are the same as for Fig. 3, except *p* values of *A. japonica* are for 2-tailed t test.

variation of $\delta^{13}C$ values between individuals (especially *C. mitella* in December and F. kondakovi in October), which was not seen in the other two feeding habit groups. The cellulase activities of four investigated invertebrates (*C. moerchii*, *C. dehaani*, *C. nigrolineata*, and *T. argyrostoma*) all showed seasonal variation. For example, *C. moerchii* showed the highest level of cellulase activity in May, and the lowest in July. The cellulase activity of *C. dehaani* was kept at a high level from May to October (slightly dropped in July), but suddenly decreased to almost zero in December. For *C. nigrolineata*, the highest cellulase activity was shown in May, it decreased in October, and further decreased in December but still remained at half the activity level of May. *Tegula argyrostoma* showed the lowest cellulase activity in May with a large deviation among individuals, and showed the highest activity in October, and slightly decreased activity in December.

3.2. Kishiwada artificial tidal flat

We found only three dominant species of invertebrates in this tidal flat throughout the year, namely, *B. multiformis*, *T. argyrostoma*, and *A. japonica*. As shown in Fig. 4, overall, the $\delta^{13}C$ value and $\delta^{15}N$ value of these three species were higher than those invertebrates that lived in ORE. Nevertheless, seasonal variation of the stable isotope ratio could still be observed in each species. For *B. multiformis*, the lowest $\delta^{13}C$ value was shown in December, with a large deviation among individuals. The $\delta^{13}C$ value in July was close to the

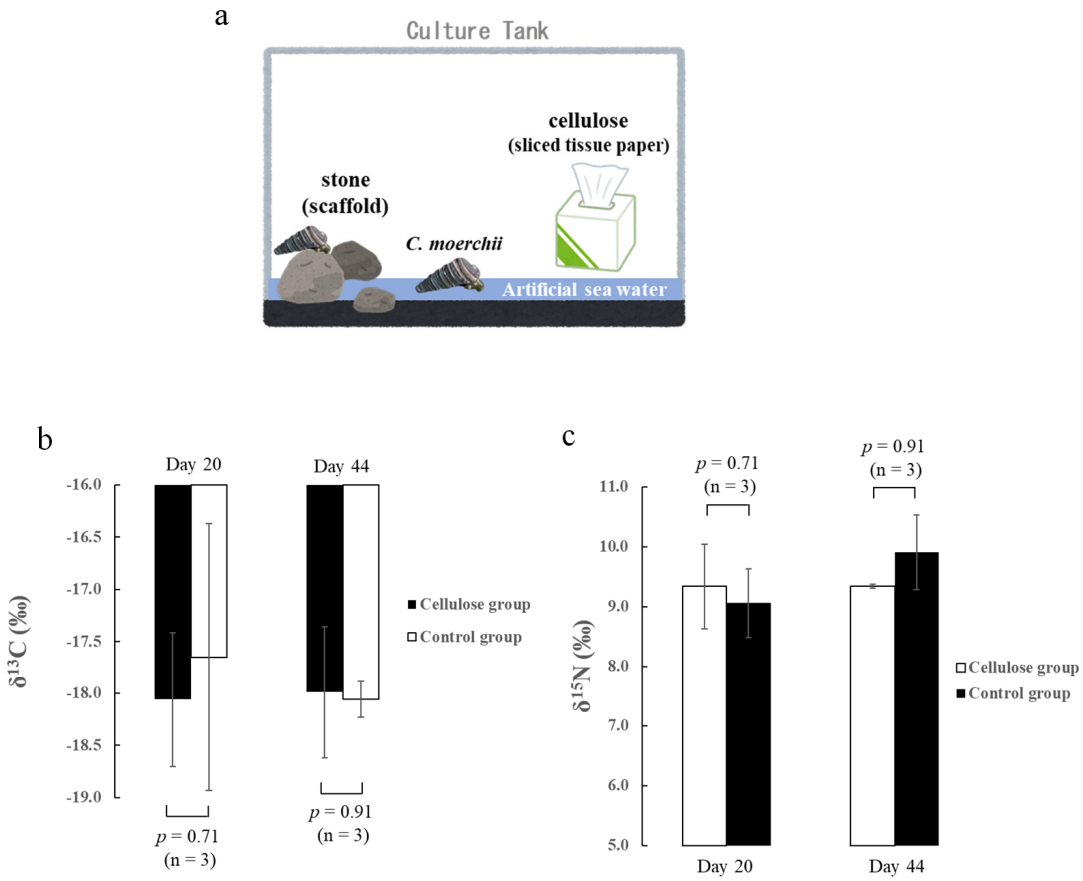

Fig. 5 Cellulose-feeding experiment.
a. Scheme of the culturing system for cellulose-feeding group. **b**. Carbon stable isotope ratio between cellulose-feeding group (black bars) and control group (white bars). Values are means ± SD. p values are for 2-tailed t test between the cellulose-feeding groups and control groups. **c**. Nitrogen stable isotope ratios, legends are the same as for b.

average value in December, with much smaller deviation. The highest $\delta^{13}C$ value was shown in October. The range of $\delta^{13}C$ value of *B. multiformis* at KATF was higher than that of the scraping feeders at ORE (except *T. argyrostoma* in October). *Tegula argyrostoma* living in KATF showed exactly the same pattern of seasonal variation of $\delta^{13}C$ and $\delta^{15}N$, but the values were higher than those in ORE.

For the cellulase activities, it seems that the higher the cellulase activities were, the higher the $\delta^{13}C$ value became in *B. multiformis* and *T. argyrostoma*. For *A. japonica*, however, the lowest cellulase activity and highest $\delta^{13}C$ value were found in July and the opposite pattern appeared in October.

4. Cellulose-feeding experiment

As shown in Fig.5b, the $\delta^{13}C$ value of the cellulose-feeding group did not significantly decrease compared to the control group after 20 ($p = 0.71$, 2 tailed t test, same below) or 44 ($p = 0.91$) days. Similarly, Fig. 5c showed that the $\delta^{15}N$ value of the cellulose-feeding group was not significantly increased at day 20 ($p = 0.69$) or day 44 ($p = 0.53$).

5. Statistical analysis

The p values of Post Hoc analysis between seasons' cellulase activities of each invertebrate, as well as the p values of bivariate correlation analysis (for *A. japonica* only) are shown in Fig. 3b and Fig. 4b. The results of the t test between the $\delta^{13}C$ and $\delta^{15}N$ values of the cellulose-feeding group and control group are shown in Fig.5.

Discussion

Before any investigation of the relationship between the seasonal variations of cellulase activity and isotope ratios, we first validated the cellulase activity in each collected invertebrate species by plate assay. As shown in Fig. 2, all species showed strong activity. The plate assay could not distinguish between whether these activities came from the invertebrates themselves or symbiotic microorganisms in their digestive system. Nevertheless, previous studies revealed a variety of aquatic invertebrates, especially molluscs and arthropods, possessing endogenous cellulase (Watanabe and Tokkuda, 2001; Tanimura et al., 2014; Liu et al., 2021). In the present study, we added several drops of 1mM NaN_3 to each sample to suppress the activity of microorganisms such as bacteria during the enzymic reaction, indicating that the detected cellulase activity was more likely from the invertebrates themselves. With either origin of the cellulase, however, these invertebrates are capable of decomposing Terrestrial cellulose, and thus they are very likely to be responsible for utilizing TerrestrialOMs.

SOM and POM usually contain TerrestrialOMs (Mishima et al., 1999; Ward et al., 2013), and the composition of TerrestrialOMs will affect the carbon and nitrogen isotope ratios of SOM and POM (Ogawa et al., 1994; Wada et al., 1984; Wada et al., 1987). Normally, $\delta^{13}C$ of Terrestrial C_3 plants ranged from –23 to –33‰, $\delta^{13}C$ of marine phytoplankton ranged from –18 to –21‰, and $\delta^{13}C$ of Terrestrial C_4 plants or seagrass ranged from –8 to –15.5‰, Hayami et al., 2019). Thus, in Japan which is dominated by C_3 plants, the higher the Terrestrial OMs contents in SOM and POM, the lower their $\delta^{13}C$ value will be. We investigated the seasonal variation of carbon and nitrogen isotope ratios in ORE. The results showed that most of the time, the $\delta^{13}C$ value of both SOM and POM in ORE was lower than –22‰, and it did not fluctuate very much across seasons (Table 2), indicating that the TerrestrialOM content was lower. On the other hand, the $\delta^{13}C$ values suggested that the TerrestrialOM content in POM was smaller than that in the SOM, which was supported by the fact that the C/N ratio of the POM was lower (normally, the C/N ratio of Terrestrial C_3 plants and soils is 13–25, while that of marine phytoplankton is 8–10.5; Boutton, 1991;Yoneyama, 2008), since previous studies revealed that Terrestrial plant-origin OMs have higher C/N ratios compared to marine phytoplankton and freshwater phytoplankton (Hayami et al., 2019). In addition, the fact that the carbon and nitrogen concentrations (mg/L) in POM in July both suddenly increased almost 14-fold compared to those in May indicated a large marine-origin organic matter (C/N ratio = 8.6) input in that season. In KATF, the carbon and nitrogen concentrations in SOM (0.22% carbon, 0.03% nitrogen) and POM (0.43 mg/L carbon, 0.07 mg/L nitrogen) were lower than those in ORE (0.87–1.5% carbon and 0.1 ~ 0.13% nitrogen in SOM, 0.23–3.2 mg/L carbon and 0.03–0.43 mg/L nitrogen in POM, respectively), suggesting that organic matters used as the food resources for benthic organisms in SOM and POM might be limited in KATF. In addition, the $\delta^{13}C$ values of SOM and POM in KATF (SOM: –20.1‰; POM: –21.2‰) were both higher than those in ORE (SOM: –28.6‰; POM: –23.5 to –25.1‰), and the C/N ratios in KATF (SOM: 7.4; POM: 7.3) were lower than those of ORE (SOM: 13.6; POM: 9.5~10.9), indicating that KATF not only had limited total organic matter, it also lacked TerrestrialOM inputs. This lack of TerrestrialOM might have been because this small tidal flat was newly reclaimed in the middle of the sea, isolated from the land, and thus had no riverine TerrestrialOM inputs as well as limited hinterland for hydrophyte vegetation. In contrast, the Onosato River in ORE carries TerrestrialOM (such as leaves, which can be seen by the eye in the POM samples), which accumulates in the sediment near the river mouth.

As shown in Fig. 3, in ORE, the $\delta^{13}C$ values of *C. moerchii* and *C. dehaani* (both detritus feeders) were lower than those of *T. argyrostoma* and *C. nigrolineataI* (scraping feeders), which is reasonable because the sediment in ORE contains a large amount of TerrestrialOM ($\delta^{13}C$ = –26.5 to –28.6‰, Table 2). Because *C. moerchii* maintained its $\delta^{13}C$ value low in all seasons, it is considered to assimilate more TerrestrialOMs than the other investigated invertebrates. In the present study, we consider the low $\delta^{13}C$ value to be evidence of more TerrestrialOM assimilation. But normally, the $\delta^{13}C$ value will not be much different from that of an animal's food source (trophic enrichment factor (TEF) = 0–1‰, Fry, 2006, sometimes 2–3‰, Busst and Britton, 2016; Umezawa et al., 2018). In ORE, however, a difference in $\delta^{13}C$ between potential food sources (SOM) and *C. moerchii* were large (approx. 10‰), which remains to

be explained. One of the possibilities is the existence of unidentified food source(s) in ORE. Our previous study focused on four gastropod species inhabiting another natural wetland in the east coast of Japan revealed that the $\delta^{13}C$ values of the gastropods were positively related to the cellulase activities, indicating a relationship between the $\delta^{13}C$ value and $_{Terrestrial}OM$ assimilation (Liu et al., 2014). The δ13C value of *C. moerchii* in the previous study was approximately –18 to –19‰, and the microphytobenthos (MPB) investigated in the previous study had a $\delta^{13}C$ value of around –18‰, and were considered to be the most likely food source for *C. moerchii*. Another previous study in Obitsu River, Tokyo Bay also showed that *C. rhizophorarum* (same as *C. moerchii*) had a $\delta^{13}C$ value of –18.8 ± 0.2‰, close to the MPB (–19.8 ± 0.3‰) in the same region (Kon et al., 2012). In the present study, we only investigated the isotope ratio of SOM and POM as a total. The MPB in ORE probably have a similar $\delta^{13}C$ value to these previous studies, and thus might be the main food source of *C. moerchii*. However, it is hard to consider that *C. moerchii* had a selective feeding habit to differentiate high $\delta^{13}C$ value foods from SOM and/or POM, which needs further investigation, and in this case, it is difficult to explain why there would be a need to possess cellulase activity. Another possible explanation is that *C. moerchii* (as well as other investigated invertebrates in the present study) might have an isotope biasing or fractionation in their processing of food. The product of cellulose digestion is glucose, and it can be used in respiration pathways rather than other biochemical synthesis pathways. There might be some unknown bias of metabolizing a particular carbon isotope (^{13}C) in glucose and finally releasing it as carbon dioxide. However, testing this hypothesis will require a series of experiments, probably using isotope techniques, to follow carbon in the metabolic pathways of these invertebrates. Instead, we have conducted a cellulose-feeding experiment in the present study, and the result showed that after cellulose was consumed (eaten) by *C. moerchii* (average 59 mg cellulose/individual after 20 days, average 101 mg cellulose/individual after 44 days), the $\delta^{13}C$ value of *C. moerchii* had a trend, but did not significantly decrease compared to that of the control group (Fig. 5b). This is strong evidence that the cellulose was consumed by *C. moerchii* (we observed that the paper tissue was eaten by *C. moerchii* every time it was provided), but somehow was not assimilated by their body tissue. Note here that this experiment assumed that each *C. moerchii* individual consumed the same amount of cellulose, which might not be true. Since we sampled them randomly, there was a chance that we accidently sampled the individuals that consumed less cellulose.

However, a previous study using grapsid crabs feeding on mangrove leaf litter has raised some other possibilities that should also be considered when looking at our feeding experiment data (Bui and Lee, 2014). First, the time frame of 44 days might not be long enough for *C. moerchii* to show measurable isotopic change in their muscle tissue. Secondly, the food source that *C. moerchii* primarily consumes in the field may have a similar $\delta^{13}C$ value to that of the tissue paper. In fact, *C. moerchii* often resides in or adjacent to reed fields below wetlands. If reed litter dominated their diet before the feeding experiment, the tissue paper provided as food in the experiment may not have produced a change in their body $\delta^{13}C$ value. Thirdly, the TEF of the *C. moerchii* consuming tissue paper was extremely high (c.a. 5‰), and similar to the difference in $\delta^{13}C$ between *C. moerchii* and tissue paper, resulting in a "concealing" of any change in the $\delta^{13}C$ value even if tissue paper was assimilated. Newly designed future experiments such as extending feeding period, changing target animals, and using tissue paper with a higher $\delta^{13}C$ value than that of *C. moerchii* body tissues will be required to inspect these possibilities. On the other hand, the $\delta^{15}N$ values (7.8–10.2‰) of *C. moerchii* in ORE were lower than in the previous study (10–13‰, Liu et al., 2014), which could be ascribed to the lower $\delta^{15}N$ value of POM in the present study (ORE, 5.6–7.4 ‰) compared to the $\delta^{15}N$ value of POM (about 9 ‰) in the previous study. This result suggests that *C. moerchii* from these two studies consume similar amount of MPB.

As for the cellulase activity, we thought at first that the cellulase level would correspond to the seasonal variation of the organisms' carbon isotope ratio, because generally thinking, higher cellulase activity means more cellulose decomposition which could leads to more cellulose ($_{Terrestrial}OM$) assimilation. But there were quite a large number of exceptions in the present study. *C. moerchii*, for example, showed the highest level of cellulase activity in May, the same season when the lowest $\delta^{13}C$ value was shown. However, in October, *C. moerchii*

showed the highest $\delta^{13}C$ value but the cellulase activity was quite low. As described above, the marine-origin OM input in July might deposit in the sediment and increase its $\delta^{13}C$ value, causing the decrease of cellulase activity and the increase of $\delta^{13}C$ and $\delta^{15}N$ values of the detritus feeders living on it from July to October.

Chiromantes dehaani is another detritus feeder investigated in ORE. Both the $\delta^{13}C$ value (–14.5 to –16.1‰) and $\delta^{15}N$ value (11.3 to 12.2‰) of *C. dehaani* were higher than those of *C. moerchii*. Previous studies based on stomach/fecal content analysis or direct observations revealed that aquatic crabs consume a significant amount of leaf litter (Odum and Heald, 1972: Poon et al., 2010; Nordhaus et al., 2011). Another previous study in China showed that *C. dehaani* that inhabit a salt marsh dominated by *Phragmites australis* (reed, C_3 plant) have a significant lower $\delta^{13}C$ value (–22.67 ± 0.58‰) than that (–16.07 ± 0.63‰) of the same species that inhabit a nearby salt marsh dominated by *Spartina alterniflora* (cordgrass, C_4 plant) (Qin et al., 2010). These results are strong evidence that *C. dehaani* assimilates $_{Terrestrial}$OM, resulting in the decrease of $\delta^{13}C$ value. In the case of *C. dehaani* in ORE, the seasonal variation of cellulase activity was basically synchronized with the variation of $\delta^{13}C$ value: the cellulase activity was maintained at a high level from May to October (Fig. 3), while the $\delta^{13}C$ value decreased accordingly. The cellulase activity suddenly decreased to close to zero in December, and the $\delta^{13}C$ value of *C. dehaani* then increased to almost the highest level of the year. *Chiromantes dehaani* was known to consume marine-origin microphytoplankton according to a previous study (Kon et al. 2012). Similar to the values in *C. moerchii*, the $\delta^{13}C$ values in *C. dehaani* in the present study were quite high (–14.5 to –16.1‰), indicating that *C. dehaani* might consume both $_{Terrestrial}$OM and marine-origin microphytoplankton, but assimilate the latter more, supported by the high $\delta^{15}N$ values of *C. dehaani* in ORE (11.2 to 12.6‰) compared to the values in the study in China (7.54 ± 0.21‰ in C_3 plant-dominant salt marsh (Qin et al., 2010). However, the lowest $\delta^{13}C$ value and cellulase activity shown in winter could also be ascribed to their habit of hibernation. Previous studies revealed that starvation could result in an increase of $\delta^{13}C$ and $\delta^{15}N$ values in chironomid larvae (Doi et al., 2007) and salt marsh snail (Kurata and Kikuchi, 2001). *Chiromantes dehaani* individuals were all hidden in nests when we visited ORE in winter. If no food is taken in anymore during the winter, they might start to consume assimilated body parts, which would cause increased $\delta^{13}C$ and $\delta^{15}N$ values, but since this phenomenon depends on the species, further investigation is required.

As scraping feeders, *C. nigrolineata* and *T. argyrostoma* showed higher $\delta^{13}C$ and $\delta^{15}N$ values compared to the detritus feeders, possibly because they consume more microphytobenthos that include heterotrophic ones (e.g., Chlamydomonas, Blifernez-Klassen et al, 2012; Actinophrys, Sakaguchi and Suzaki, 1999) probably with higher $\delta^{13}C$ and $\delta^{15}N$ ratio living on the quay and breakwater. Interestingly, *T. argyrostoma* showed a very wide range of $\delta^{13}C$ values (–11.9 ~ –15.3‰), but the variation between individuals in each season was small. This might because all individuals of *T. argyrostoma* change their feeding habit similarly to each other in different seasons. Also, the lowest $\delta^{13}C$ values of *C. nigrolineata* and *T. argyrostoma* were both found in December. This might be because 1) in winter, the production of microalgae on the quay was decreased (Sutherland et al., 2013) so the scraping feeders who rely on them have to use $_{Terrestrial}$OMs as an alternative food source. There seems to be a large input of $_{Terrestrial}$OM during this month because POM in the upper stream showed the lowest $\delta^{13}C$ value (–25.1‰) then among the values throughout the year, or 2) microphytobenthos is their main food source but the $\delta^{13}C$ value of microphytobenthos decreased in winter. The reason for this is that the microphytobenthos have a larger isotopic fractionation of dissolved inorganic carbon (DIC) uptake in winter, when they have low productivity, as well as that the low temperature will increase the dissolution of atmosphere CO_2 and thus decrease the $\delta^{13}C$ value of seawater DIC (Macleod and Barton, 1998; Finlay, 2004). For cellulase activities, it seems that the higher the activities of *C. nigrolineata* and *T. argyrostoma* are, the higher the $\delta^{13}C$ value becomes, which is very puzzling. One of the possibilities is that, in May, *C. nigrolineata* and *T. argyrostoma* mainly assimilated microphytobenthos, and high cellulase activity was used for decomposing more cellulose but most of the products (i.e., glucose) were used to synthesize ATP for microphytobenthos-searching activities. In winter, cellulase activity decreases because the whole metabolism was decreased by the low temperature.

Instead of being used to synthesize ATP, produced glucose was bypassed to synthesize biochemicals used in body tissues, which cannot be acquired much from microphytobenthos. However, these are all speculations with no support from previous studies, and thus remain to be further investigated.

The filter feeders (i.e., *C. mitella* and *F. kondakovi*) showed high $\delta^{15}N$ values, which might be because of that they assimilate zooplankton in POM as their food source. For *C. mitella*, the $\delta^{13}C$ value was quite low, close to that of *C. moerchii*, indicating they might also assimilate TerrestrialOMs. Also, both *C. mitella* and *F. kondakovi* showed a large variation of $\delta^{13}C$ values between individuals (especially *C. mitella* in December and *F. kondakovi* in October), which was not seen in the other two feeding habit groups. This might be because these two water filtering invertebrates cannot move once they are grounded on the quay and breakwater. The POM composition should have no big differences within such a small area, but microturbulence in narrow spaces between stones of quays and breakwaters might cause unevenness of POM distribution, causing the variation of $\delta^{13}C$ values. Previous studies showed that an approximately 2–4‰ isotope fractionation could appear in phytoplankton in a close water range (Yoshioka et al., 1994), and phytoplankton with small size and short life cycle could receive more influence (Montoya, 2007), and thus accordingly might cause the variation of $\delta^{13}C$ values of *C. mitella* and *F. kondakovi*.

Kishiwada Artificial Tidal Flat (KATF) is a newly reclaimed land close to ORE, but with no river inputs and remarkably less vegetation, which we thought to make it valuable for comparison to ORE. As described above, the total organic matter content is limited, and most of its organic matter is of marine origin. We found only three species of invertebrates that inhabit this tidal flat throughout the year, namely, *B. multiformis*, *T. argyrostoma*, and *A. japonica*. As shown in Fig. 4, overall, the $\delta^{13}C$ value and $\delta^{15}N$ values of these three species were higher than those of invertebrates that live in ORE, which might because of the shortage of TerrestrialOMs. Nevertheless, seasonal variation of the stable isotope ratio could still be observed in each species. For *B. multiformis*, the range of $\delta^{13}C$ value was even higher than that of the scraping feeders (except *T. argyrostoma* in October), indicating that their feeding habit relied on marine-origin OMs, unlike other detritus feeders living in ORE. We have observed that *B. multiformis* fed on sea lettuce (*Ulva spp.*) several times in KATF. According to Matsuo et al., 2009, *Ulva spp.* in Osaka Bay have $\delta^{13}C$ value around 10.5‰ and $\delta^{15}N$ value around 9.0‰, which is quite close to October's values of *B. multiformis* in KATF. Our previous studies showed that in addition to cellulase, *B. multiformis* also has mannanase and laminarinase activities, which could be used to decompose the sea lettuce (Liu et al., 2014). Compared to the *T. argyrostoma* that inhabited ORE, *T. argyrostoma* living in KATF showed exactly the same pattern of seasonal variation of $\delta^{13}C$ and $\delta^{15}N$, but the values were higher. For *A. japonica*, although the $\delta^{15}N$ value (14.4–15.0‰) was similar to that of its related species (*C. nigrolineata*) in ORE (13.2–14.0‰), the $\delta^{13}C$ value was much higher (–11.4 to –12.2‰) compared to that of *C. nigrolineata* (–14.7 to –16.4%). The 2 scraping feeders in KATF seemed to assimilate less TerrestrialOMs compared to the same/related species in ORE, possibly because of the shortage of TerrestrialOMs.

Regarding the cellulase activities, the higher the cellulase activities were, the higher the $\delta^{13}C$ value became in *B. multiformis* and T. argyrostoma. These results might indicate that TerrestrialOMs are acting as an alternative food source of these invertebrates for respiration without assimilating them for making body tissues during the time they could not find high nutritional value foods (i.e., foods with high $\delta^{13}C$ and $\delta^{15}N$ value). For *A. japonica*, however, the lowest cellulase activity and highest $\delta^{13}C$ value was found in July and the opposite pattern appeared in October, indicating that *A. japonica* consumed and assimilated more TerrestrialOMs in in October, possibly because of the limited planktonic and benthic microalgae production caused by the dropping of water temperature.

Conclusion

All dominant invertebrates inhabiting wetlands around Osaka Bay, including detritus feeders, scraping feeders and filter feeders, were found to have cellulase activity. Further investigations are needed to determine whether the cellulases are endogenous or of symbiotic origin. Nevertheless, these invertebrates have the capability of utilizing Terrestrial plant-origin cellulose. In addition, the

high $\delta^{13}C$ value and low C/N ratio of SOM and POM in KATF indicate that KATF lacks TerrestrialOMs inputs, which might cause the higher $\delta^{13}C$ and $\delta^{15}N$ values of the invertebrates inhabit KATF compared to ORE. Those results suggested that the isotope ratio (especially $\delta^{13}C$) could be used as an indicator of the assimilation level of TerrestrialOMs. Seasonal variations of $\delta^{13}C$ value were found in almost all investigated invertebrates, indicating that their level of assimilation of TerrestrialOM changes throughout the year.

We tried to quantify the cellulase activity in order to estimate how much TerrestrialOMs the invertebrates consume, but the results showed that the pattern of the seasonal variation of cellulase activity didn't always match the seasonal variation pattern of the stable isotope ratio, which means that we cannot simply apply the cellulase activity to estimate how much TerrestrialOMs (in this case, cellulose) these invertebrates are consuming. This is an unexpected result. From another point of view, the trophic enrichment factor (TEF) values of carbon and nitrogen are very large (more than 5 ~ 10‰ between invertebrates and SOM/POM) in both ORE and KATF. This might be because invertebrates are picking high $\delta^{13}C$ value food sources such as MPB, or, as indicated by the cellulose-feeding experiment of *C. moerchii*, because of a biasing of processing TerrestrialOMs (i.e., glucose in this case). However, the cellulose-feeding experiment wasn't flawless in the present study: *C. moerchii* individuals might consume cellulose in different amounts. But in any case, these hypotheses need further isotope-tracing experiments to be confirmed. And even if these hypotheses are true, other invertebrates might have different feeding habits and biasing of processing carbon isotopes, which in that case would make it difficult to use only cellulase activity and isotope analysis as a universal tool to estimate the quantity of TerrestrialOM consumption for all invertebrates.

What's important here is that this study might have failed to find a relationship between enzymatic activities (capability of consuming TerrestrialOM) and stable isotope signatures (assimilation level of TerrestrialOM) useful for employing the enzymatic activities as an estimator to calculate the amount of TerrestrialOM consumption, but it also revealed a possibility that aquatic invertebrates could consume more TerrestrialOMs, as represented in the isotope signatures. It will be essential in future research to trace the metabolic pathway of the decomposed/consumed TerrestrialOM to reveal the true role played by aquatic invertebrates in carbon cycling between land and sea.

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