



Utilization of different macroalgae by sea cucumber *Apostichopus japonicus* revealed by carbon stable isotope analysis

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ABSTRACT: Understanding the feeding habit of sea cucumber *Apostichopus japonicus* (Selenka) is crucial for improving aquaculture techniques of this commercially important species. In the present study, carbon stable isotopes were used as trophic tracers to investigate the uptake of different macroalgae, including brown alga *Sargassum muticum*, red alga *Gracilaria lemaneiformis* and green alga *Ulva lactuca* by *A. japonicus*. A 70 d experiment was conducted to examine the carbon isotopic signatures of *A. japonicus* feeding on 6 different types of diets containing either pure powder of a single alga species or mixtures of 2 algae species. After the feeding trial, carbon isotope ratios ($\delta^{13}\text{C}$) of *A. japonicus* showed significant changes and reflected the isotopic compositions of corresponding diets. An isotope mixing model revealed the dietary preferences of *A. japonicus* between the 3 species of macroalgae, suggesting that green alga *U. lactuca* was the preferentially utilized food source of *A. japonicus*, followed by brown alga *S. muticum*. *A. japonicus* tended to reject red alga *G. lemaneiformis* in the presence of multiple macroalgae choices. Moreover, the specific growth rates of *A. japonicus* fed on *S. muticum* and *U. lactuca* were similar, but were both significantly higher than those fed on *G. lemaneiformis*, indicating the direct link between the feeding preferences and growth performance of *A. japonicus*.

KEY WORDS: *Apostichopus japonicus* · Macroalga · Stable isotope · Growth

INTRODUCTION

Sea cucumbers, belonging to the phylum Echinodermata and class Holothuroidea, are distributed worldwide in marine habitats, from shallow to deep seas. As deposit feeders, these organisms actively select organic-rich particles from the upper millimeters of sediments and promote bioturbation, thus playing an important role in detritus food chains (Yingst 1976, Uthicke 1999, Michio et al. 2003, Hudson et al. 2005, Slater & Jeffs 2010, Slater et al. 2011). Moreover, in seagrass ecosystems, holothurians can

effectively accelerate the degradation of organic matter and enhance the transfer of seagrass-derived organic matter to sediments (Liu et al. 2013, Costa et al. 2014). Besides the ecological role in nutrient recycling, several sea cucumber species, including *Apostichopus japonicus*, are rich in a variety of bioactive compounds with nutritional and medicinal values (Aydın et al. 2011, Bordbar et al. 2011). In response increasing demand, an extensive trade in *A. japonicus* has developed and this species is widely cultured in northern China, Japan, Korea and Russia (Chen 2004, Toral-Granda et al. 2008). A better understand-

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ing of the feeding ecology and physiology of *A. japonicus* will provide insights into the vital role they play in nutrient recycling in benthic environments and crucial information for improving the aquaculture techniques of this species (Uthicke 2001, Slater et al. 2011, Zamora & Jeffs 2011).

Deposit-feeding *A. japonicus* ingest sedimentary organic matter, mainly composed of detritus from macroalgae to seagrass, decayed animals and other microorganisms such as benthic diatoms, bacteria and protozoa (Zhang et al. 1995). Previous studies have demonstrated that detritus derived from macroalgae is the major food source of *A. japonicus* (Sun et al. 2013). However, in eelgrass-rich meadows, decayed eelgrass debris such as *Zostera marina* can be an important food source for *A. japonicus* (Liu et al. 2013). Many other sea cucumber species including *Stichopus chloronotus*, *S. tremulus* and *Mesothuria intestinalis* also tend to select sediments containing high macroalgal biomass (Uthicke & Karez 1999, Hudson et al. 2005). Different basal food resources, e.g. brown, red and green algae, generally have distinctive biochemical compositions and consequently different nutritional values and food quality, which could potentially influence the feeding selectivity of consumers (Danovaro et al. 2001, Adin & Riera 2003, Jormalainen et al. 2005, Rodil et al. 2008, Duarte et al. 2010). Although the biology of *A. japonicus* has been studied for several decades, research efforts on the feeding habits and food preferences are relatively scarce to date (Gao et al. 2011, Sun et al. 2013).

Utilization of different food resources by aquatic animals can be directly assessed by stomach content analysis. However, this traditional approach is often destructive for larger animals and is not feasible for smaller organisms, particularly for *A. japonicus* or other aquatic invertebrates due to the small size and the digestive damage caused to the food particles (Galloway et al. 2015, Gao et al. 2011). More importantly, gut content analysis generally only provides a snapshot of recently ingested food and may be biased towards non-digestible food items (Bowen & Iverson 2013, Galloway et al. 2015), thus making it difficult to distinguish the contributions of various potential food sources. More recently, stable isotope analysis of carbon and nitrogen has been used as a powerful tool to determine the food sources and feeding selectivity, trophic positions and movement patterns of both aquatic and terrestrial organisms (Peterson & Fry 1987, Hobson 1999, Adin & Riera 2003, Gao et al. 2006, Boecklen et al. 2011). Stable isotope analysis generally integrates diet information over an extended time period, typically weeks to

months, depending on tissue-specific turnover rates (MacAvoy et al. 2001, Phillips & Eldridge 2006, Logan & Lutcavage 2010). Moreover, the development of mixing models allows us to estimate the relative contributions of potential resources to the consumer's food absorption (Phillips & Koch 2002, Phillips & Gregg 2003).

Simultaneous provision of various food resources is an effective way to examine the feeding selectivity of mobile sea cucumbers (Uthicke & Karez 1999). In this study, 3 species of macroalgae, including brown alga *Sargassum muticum*, red alga *Gracilaria lemaneiformis* and green alga *Ulva lactuca* were mixed with each other as diets to feed *A. japonicus*. The objectives of the present study were to compare the food quality of the 3 macroalgal species and to quantify the relative contributions of different macroalgae to the food uptake of *A. japonicus*. The subsequent effects of food preferences on the growth performance of *A. japonicus* were evaluated to provide scientific evidence for optimizing the ingredients of artificial feed used in *A. japonicus* farming.

MATERIALS AND METHODS

Experimental diets

Three macroalgal species, including brown alga *Sargassum muticum*, red alga *Gracilaria lemaneiformis* and green alga *Ulva lactuca* were used as feed ingredients in the experiment. Dried macroalgae were obtained from Qingdao Great Seven Biotech. Six types of diets were prepared for *Apostichopus japonicus* (Selenka) containing the ingredient of either pure powder of a single alga species or mixtures of 2 algae species, i.e. single *S. muticum* (S), *G. lemaneiformis* (G) and *U. lactuca* (U), and mixtures (1:1) of *S. muticum* and *G. lemaneiformis* (SG), *S. muticum* and *U. lactuca* (SU), and *G. lemaneiformis* and *U. lactuca* (GU). The macroalgae were ground into fine powder through a 150 µm mesh, then well mixed before being pelletized with a feed processing machine and stored at 4°C for future use.

Experiment and sample collection

The experiment was conducted at the laboratory of Qingdao National Ocean Scientific Research Center, Ocean University of China. *A. japonicus* juveniles were collected from a local sea cucumber farm and acclimatized for 14 d before the start of the experi-

ment. After 48 h starvation, a total of 108 juvenile *A. japonicus* with initial body weights of 5.57 ± 0.11 g were randomly distributed into 18 glass aquaria ($50 \times 40 \times 40$ cm) in each of which 6 individuals were cultured. The 18 aquaria were further divided into 6 groups with 3 replicates for each group. *A. japonicus* individuals in each of the 6 groups were fed with one of the 6 diets as described above. Prior to the experiment, 10 additional specimens were stored at -80°C for the initial carbon stable isotope analysis.

During the following 70 d feeding experiment, the *A. japonicus* were fed once a day at 15:00 h with a daily ration of 5% body weight. Water temperature was maintained at $16.5 \pm 0.5^\circ\text{C}$, salinity ranged from 29 to 31, aeration was provided continuously, dissolved oxygen was >6.0 mg l⁻¹. Two-thirds of the volume of the water in each aquarium was exchanged with filtered seawater daily. Uneaten feed residue and feces were collected by siphoning during water exchange.

At the end of the experiment, *A. japonicus* individuals in each aquarium were starved for 48 h to empty the gut content, then weighed to the nearest 0.01 g and dissected. Samples of body wall tissues were kept at -80°C for further analysis.

C/N and carbon stable isotope ratios

Carbon and nitrogen contents of the 3 macroalgal species were measured using an elemental analyzer (Elementar Vario ELIII) and molar ratio of carbon to nitrogen (C/N ratio) was calculated. Samples of collected body tissues were dried at 60°C for 48 h to constant weight and ground into a fine powder for carbon stable isotope measurements. Carbon stable isotope ratios of body tissues and algal powder were determined using an elemental analyzer coupled with an isotope ratio mass spectrometer (EA-IRMS, ThermoFinnigan MAT Delta Plus). A laboratory working standard (glycine) was run for every 10 samples. Analytical precision was $\pm 0.1\%$.

Stable isotope mixing model and statistical analysis

Results of carbon isotope ratios were expressed in standard δ -unit notation, which is defined as:

$$\delta^{13}\text{C} = [(R_{\text{sample}}/R_{\text{standard}}) - 1] \times 1000\% \quad (1)$$

where R is the $^{13}\text{C}/^{12}\text{C}$ ratio. The values were reported relative to the Vienna Pee Dee Belemnite (PDB) standard.

For diet groups SG, SU and GU, a 2-source concentration-weighted isotope mixing model was used to evaluate the contribution of each algal ingredient to the food uptake of *A. japonicus* (Phillips & Koch 2002):

$$(\delta^{13}\text{C}'_{\text{X}} - \delta^{13}\text{C}_{\text{M}})[\text{C}]_{\text{X}}f_{\text{X,B}} + (\delta^{13}\text{C}'_{\text{Y}} - \delta^{13}\text{C}_{\text{M}})[\text{C}]_{\text{Y}}f_{\text{Y,B}} = 0 \quad (2)$$

where $f_{\text{X,B}}$ and $f_{\text{Y,B}}$ represent the fractions of assimilated biomass (B) of sources X, Y, respectively, in the mixture M, therefore $f_{\text{X,B}} + f_{\text{Y,B}} = 1$. $[\text{C}]_{\text{X}}$ and $[\text{C}]_{\text{Y}}$ represent the carbon concentrations in food sources X, Y, respectively. Isotopic signatures for the sources were corrected for trophic fractionation as designated by the prime symbol ('). Average fractionation effects of 1‰ for carbon isotope were used to correct stable isotope shifts for each trophic level (Peterson & Fry 1987, Sun et al. 2013).

The growth performance of *A. japonicus* in each aquarium in terms of specific growth rate (SGR) was calculated as:

$$\text{SGR} (\% \text{ d}^{-1}) = (\ln W_f - \ln W_i)/t \times 100 \quad (3)$$

where W_f and W_i are the final and initial wet body weights, respectively, of *A. japonicus*, and t is the duration of the experiment in days.

The differences in C/N ratios and $\delta^{13}\text{C}$ values between the 3 algal species and $\delta^{13}\text{C}$ values and growth performance of *A. japonicus* between the 6 diet groups were compared with 1-way ANOVA followed by Tukey's post hoc test for multiple comparisons at a significance level of 0.05 ($p < 0.05$). Prior to statistical analyses, raw data were diagnosed for normality of distribution and homogeneity of variance with the Kolmogorov-Smirnov test and Levene's test, respectively. The statistical analyses were performed using SPSS software for Windows (Release 20.0).

RESULTS

Carbon stable isotope and C/N ratios of macroalgae

Carbon stable isotope ratios and C/N ratios of the 3 macroalgae ingredients including brown alga *Sargassum muticum*, red alga *Gracilaria lemaneiformis* and green alga *Ulva lactuca* are presented in Table 1. Significant differences in the $\delta^{13}\text{C}$ values and C/N ratios between the 3 ingredients were observed (ANOVA, $p < 0.05$). *U. lactuca* had the highest $\delta^{13}\text{C}$ values (-13.50%), followed by *S. muticum* (-16.06%), while *G. lemaneiformis* exhibited the lowest values (-20.06%). *U. lactuca* showed the lowest C/N ratios

Table 1. Carbon stable isotope ratios ($\delta^{13}\text{C}$), C and N contents, and C/N ratios of macroalgae used as dietary ingredients in a study of the feeding habit of sea cucumber *Apostichopus japonicus*. Data are presented as mean \pm SD (n = 3). Different letters within the same column indicate significant differences (ANOVA, $p < 0.05$)

Ingredient	$\delta^{13}\text{C}$ (‰)	C content (%)	N content (%)	C/N
<i>Sargassum muticum</i>	-16.06 ± 0.17^b	26.50 ± 1.08^b	2.94 ± 0.24^b	9.04 ± 0.42^{ab}
<i>Gracilaria lemaneiformis</i>	-20.06 ± 0.22^c	23.61 ± 2.04^b	2.56 ± 0.11^b	9.22 ± 0.40^b
<i>Ulva lactuca</i>	-13.50 ± 0.08^a	14.15 ± 0.73^a	1.69 ± 0.08^a	8.36 ± 0.08^a

(8.36), followed by *S. muticum* (9.04), while *G. lemaneiformis* exhibited the highest C/N ratios (9.22).

Carbon stable isotope ratios of *Apostichopus japonicus*

Initial and final carbon stable isotope ratios in different diet groups of *A. japonicus* are shown in Fig. 1. After the 70 d feeding trial, remarkable changes occurred in the $\delta^{13}\text{C}$ values of *A. japonicus* relative to the initial values, particularly those in the diet groups U and SU (ANOVA, $p < 0.05$), and appeared to reflect the isotopic compositions of corresponding diets. For the single algae groups, due to the uptake of relatively isotopically heavy *S. muticum* and *U. lactuca*, the average $\delta^{13}\text{C}$ values of *A. japonicus* in the diet groups S and U (-15.49 ± 0.14 and -14.42 ± 0.05 ‰, respectively [mean \pm SD]) were significantly enriched compared to the initial $\delta^{13}\text{C}$ values ($-17.48 \pm$

0.11‰) (ANOVA, $p < 0.05$). By contrast, *A. japonicus* in diet group G exhibited the most ^{13}C -depleted values (-18.75 ± 0.07 ‰) among the 6 diet groups due to the assimilation of isotopically light *G. lemaneiformis*. Moreover, the average $\delta^{13}\text{C}$ value of -18.75 ‰ for the *A. japonicus* in the diet group G was very near to that of the *G. lemaneiformis* (-20.06 ‰) after the correction of 1‰ carbon isotope fractionation for each trophic level.

As for the mixed diet groups, the mean $\delta^{13}\text{C}$ values of *A. japonicus* in the diet groups SG, SU and GU (-16.81 ± 0.23 , -14.04 ± 0.24 and -16.10 ± 0.06 ‰, respectively) were all located within those of the 2 corresponding macroalgal ingredients after correction of isotope fractionation, indicating the simultaneous absorption of 2 algae species by *A. japonicus*. In particular, *A. japonicus* in the diet group SU showed the most enriched $\delta^{13}\text{C}$ values (-14.04 ‰) among the 6 diet groups as a result of the higher contribution of *U. lactuca*, compared with that of *S. muticum*, to the food assimilation of *A. japonicus*.

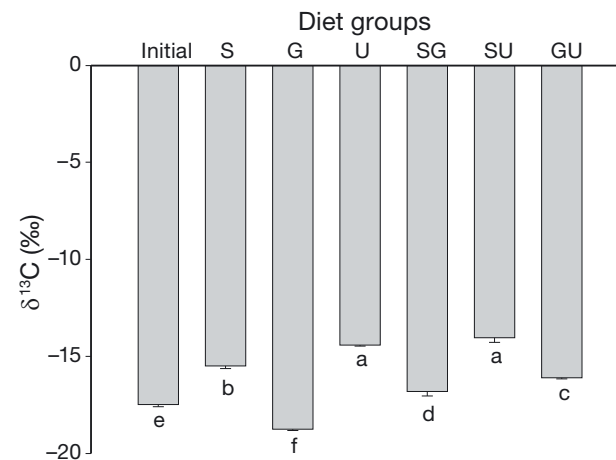


Fig. 1. Carbon stable isotope ratios ($\delta^{13}\text{C}$) of sea cucumber *Apostichopus japonicus* at the beginning of the experiment (initial) and at the end of the experiment after feeding on different diets. S: a diet of *Sargassum muticum*; G: *Gracilaria lemaneiformis*; U: *Ulva lactuca*; S: a mixture of *S. muticum* and *G. lemaneiformis*; SU: *S. muticum* and *U. lactuca*; GU: *G. lemaneiformis* and *U. lactuca*. Error bars represent 1 SD (n = 3). Values with different letters represent significant differences ($p < 0.05$)

Food contributions of different macroalgae

At the end of the experiment, the 2-source concentration-weighted dual isotope mixing model revealed that the proportional contribution of *S. muticum* (53.50 ± 5.99 %) to the diet of *A. japonicus* was higher than that of *G. lemaneiformis* (46.50 ± 5.99 %) in the diet group SG. However, in the diet group SU, the mean contribution of *S. muticum* (44.91 ± 9.97 %) was lower relative to that of *U. lactuca* (55.09 ± 9.97 %). Similarly, the proportional contribution of *G. lemaneiformis* (42.26 ± 0.83 %) was lower compared with that of *U. lactuca* (57.74 ± 0.83 %) in the diet group GU.

Growth of *Apostichopus japonicus*

Initial and final body wet weights of *A. japonicus* in different diet groups are shown in Table 2. At the beginning of the experiment, there were no signifi-

Table 2. Initial and final body wet weight of *Apostichopus japonicus* in different diet groups. Data are mean \pm SD ($n = 3$). Different letters in same row represent significant differences between diets at the significance level of 0.05 ($p < 0.05$). See Fig. 1 legend for descriptions of diets

Diet group	Wet weight (g ind. ⁻¹)	
	Initial	Final
S	5.57 \pm 0.16	15.45 \pm 1.68 ^c
G	5.62 \pm 0.10	10.98 \pm 0.74 ^{ab}
U	5.52 \pm 0.21	14.91 \pm 0.90 ^c
SG	5.60 \pm 0.05	15.21 \pm 1.37 ^c
SU	5.49 \pm 0.02	10.22 \pm 1.21 ^a
GU	5.61 \pm 0.11	14.04 \pm 0.87 ^{bc}

cant differences in the wet body weights of *A. japonicus* between the 6 diet groups (ANOVA, $p > 0.05$). After the 70 d feeding trial, the final body weight of *A. japonicus* in the diet group SU was significantly lower than in other diet groups ($p < 0.05$). Final body weight of *A. japonicus* in diet group G was significantly lower than in the diet groups S and U ($p < 0.05$) and no significant differences were observed between the diet groups S and U ($p > 0.05$). Correspondingly, the SGR of *A. japonicus* in the diet groups G and SU were significantly lower than those in the other diet groups ($p < 0.05$). There were no significant differences in SGR values between the diet groups S and U ($p > 0.05$) (Fig. 2).

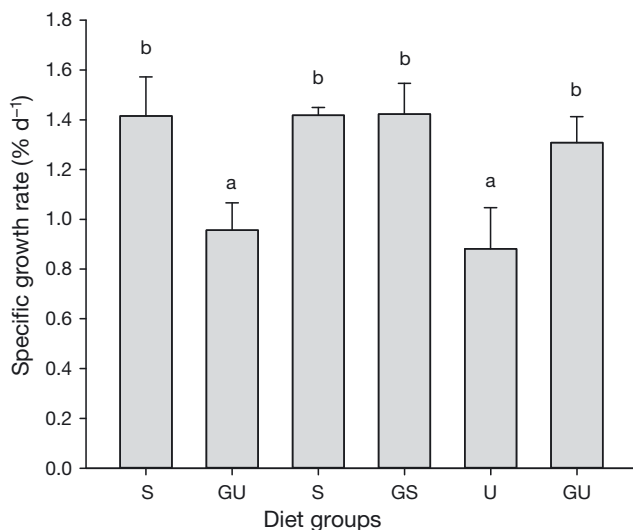


Fig. 2. Specific growth rates of *Apostichopus japonicus* in different diet groups. Error bars represent 1 SD ($n = 3$). Values with different letters represent significant difference at the significance level of 0.05 ($p < 0.05$). See Fig. 1 legend for descriptions of diets

DISCUSSION

Stable isotope analysis, which is based on the predictable trophic enrichment between the isotopic signatures of consumers and their diets, is increasingly being used to investigate the feeding patterns and trophic positions of animals in food webs (Post 2002, Boecklen et al. 2011). Carbon isotope ratios ($\delta^{13}\text{C}$) generally maintain a close similarity between consumers and their diets with 1‰ of fractionation effects and are commonly used to trace the food sources and feeding selectivity of animals (Fleming et al. 1993, Sauriau & Kang 2000). By contrast, nitrogen stable isotopes are frequently used to determine the trophic positions of various species in food webs, since $\delta^{15}\text{N}$ is more enriched than $\delta^{13}\text{C}$ (by a mean of $\sim 3.4\text{‰}$) between each trophic level (Post 2002, McCutchan et al. 2003). In the previous studies, food sources and dietary preferences of *A. japonicus* were successfully determined using single carbon stable isotopes (Gao et al. 2011, Yu et al. 2015). Therefore, nitrogen stable isotopes were not applied in the present experiment.

Applications of stable isotope analysis depend on the distinct differences in the isotopic signatures of various food resources (Peterson & Fry 1987). In the present study, there were significant differences in $\delta^{13}\text{C}$ values between brown alga *Sargassum muticum*, red alga *Gracilaria lemaneiformis* and green alga *Ulva lactuca*. This variation reflected taxonomic differences, probably due to the differences in the mechanisms adopted by the primary producers for use of CO_2 and HCO_3^- for photosynthesis, and their abilities in this regard (Maberly et al. 1992, Michener & Lajtha 2008, Mendonça et al. 2013), as well as the site-specific physicochemistry and biogeochemistry, particularly with regard to concentrations of dissolved inorganic carbon and flow rates (Finlay et al. 2002, Graham et al. 2014). Accordingly, such distinct isotopic signatures provided an opportunity to infer the dietary preferences of *A. japonicus* for the 3 macroalgal species using carbon stable isotope as trophic tracers.

Previous studies have shown that the half-life of carbon isotopic turnover in the body tissue of *A. japonicus* (5.14 ± 0.17 g) is 21.39 d (Sun et al. 2012). In the present experiment, after the 70 d feeding trial, *A. japonicus* with similar sizes (5.57 ± 0.11 g) showed substantial growth and could complete their isotopic turnover after diet switch as a result of growth and metabolic turnover of existing tissues (Phillips & Eldridge 2006, Sun et al. 2012, Xia et al. 2015). For *A. japonicus* in the single algae diet groups, shifts in

$\delta^{13}\text{C}$ values towards those of corresponding macroalgae after isotopic fractionation correction were pronounced, suggesting the assimilation of *S. muticum*, *G. lemaneiformis* and *U. lactuca* by *A. japonicus* in the diet groups S, G and U, respectively.

Analyses of carbon stable isotopes of *A. japonicus* in the mixed diet groups revealed the relative contributions of different macroalgae to the food sources of *A. japonicus*. For diet groups SG and GU, the 2-source mixing models indicated that the proportional contributions of *S. muticum* and *U. lactuca* were obviously higher than that of *G. lemaneiformis*, suggesting that the assimilation of *G. lemaneiformis* was partially replaced by *S. muticum* and *U. lactuca* in these 2 diet groups, respectively, while for diet group SU, the contribution of *S. muticum* was lower relative to that of *U. lactuca*, indicating the relatively higher importance of *U. lactuca* than *S. muticum* as a food source of *A. japonicus*. These results revealed the selective assimilation of different macroalgae by *A. japonicus*. It appeared that *A. japonicus* preferentially utilized *U. lactuca*, followed by *S. muticum*, and tended to reject *G. lemaneiformis* in the presence of multiple macroalgal species. Such results are consistent with the study by Gao et al. (2011) in which *A. japonicus* showed obvious preferences for brown alga *Sargassum thunbergii* relative to *G. lemaneiformis*. Similarly, Yingst (1976) reported that detritus derived from the red algae were not readily assimilated by the epibenthic deposit-feeding holothurian *Parastichopus parvimensis*.

The selective utilization among various basal food resources can be attributed to nutritional quality and the potential availability of different food sources (Boettcher & Targett 1993, Jormalainen et al. 2005, Rodil et al. 2008, Duarte et al. 2010, Seymour et al. 2013). In the present study, *U. lactuca* had lower C/N ratios compared with *S. muticum* or *G. lemaneiformis*, indicating the relatively high food availability of *U. lactuca* for *A. japonicus*, as organic nitrogen is more readily utilized than organic carbon (Danovaro et al. 2001). *A. japonicus* tended to reject *G. lemaneiformis*, probably due to the higher C/N ratios and subsequent lower digestibility of this species relative to *U. lactuca* and *S. muticum*. Similarly, *S. muticum* might contain a high content of low digestible refractory compounds, such as phlorotannins, which could potentially reduce the assimilation efficiencies of *A. japonicus* (Boettcher & Targett 1993, Jormalainen et al. 2005).

As a consequence of the relatively low preference for *G. lemaneiformis*, *A. japonicus* in the diet group G showed the lower SGR compared with those in the

diet groups S or U, suggesting the direct link between the feeding preferences and their growth performance. On the other hand, *A. japonicus* in the mixed diet groups SG and GU achieved a SGR comparable with those in the diet groups S and U and significantly higher SGR than those in the diet group G probably because the tender nature of *S. thunbergii* or *U. lactuca* might, to some extent, enhance the digestibility of *G. lemaneiformis* in the diet groups SG and GU, respectively. Such findings are consistent with the results of the study on the growth performance of another echinoderm species sea urchin *Tripneustes gratilla*, where the addition of brown algae in red algae improved the growth of this species (Seymour et al. 2013).

In conclusion, results of the present study revealed the feeding preference of *A. japonicus* between different macroalgal species. *U. lactuca* was most preferentially utilized as a food source by *A. japonicus*, followed by *S. muticum*. *A. japonicus* tended to reject *G. lemaneiformis* in the presence of multiple macroalgal species. As a result of the preference and selectivity with respect to different food sources, *A. japonicus* showed significant differences in growth performance under various feeding regimes. On the other hand, the feeding selectivity of benthic organisms such as *A. japonicus* might result in substantial changes in ecological conditions. By means of chemically altering (e.g. digesting) and physically redistributing macroalgal detritus in sediments, these organisms are likely to dramatically affect the structure, function and energy flow of benthic ecosystems (Miller et al. 2000, Fornes et al. 2001, Ginger et al. 2001).

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