Vol. 9: 223–230, 2017
https://doi.org/10.3354/aei00226

AQUACULTURE ENVIRONMENT INTERACTIONS
Aquacult Environ Interact

Published July 6

Uptake of farming wastes by sea cucumber *Apostichopus japonicus* in polyculture systems of abalone *Haliotis discus hannai*: evidence from C and N stable isotopes

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ABSTRACT: With the development of intensive aquaculture, worldwide concerns are increasingly focused on the biological control of farming wastes for the purpose of reducing nutrient loadings. In the present study, 5 experimental polyculture systems combining abalone and sea cucumber (SC) as different inter-species biomass ratios, i.e. 100:0, 70:30, 50:50, 30:70 and 0:100 (referred to as Group A, B, C, D and E, respectively), were set up to evaluate the potential of sea cucumber *Apostichopus japonicus* as scavengers to alleviate the farming wastes derived from the co-culture of abalone *Haliotis discus hannai*. Growth performance and significant isotopic shifts of the SCs in Groups B, C and D demonstrated the uptake of farming wastes, i.e. feces and uneaten feed residues. The proportional contributions of SC assimilated from uneaten feed residues, feces and sea mud were 21.7, 62.5 and 15.8% in Group B, 18.5, 54.3 and 27.2% in Group C, 11.5, 44.4 and 44.1% in Group D, respectively. The results revealed that sea cucumber had great potential to become an effective organic extractive species and aid in the concordant reduction of farming wastes in polyculture systems. The biomass ratio of abalone to sea cucumber 70:30 was recommended for aquaculture practice as providing the greatest economic and environmental benefits.

KEY WORDS: Abalone · Sea cucumber · Polyculture · Isotope mixing model · Nutrient loadings

INTRODUCTION

Abalone is one of the most highly valued seafood treasures across the globe. Because of over-exploitation, the wild catches of abalone seriously diminished in many traditional natural harvest locations, which has resulted in the rapid expansion of abalone aquaculture in recent decades (Qi et al. 2010, O'Mahoney et al. 2014). In China, the production of abalone reached 1.10×10^5 t covering a total farming area of 1.34×10^4 ha in 2013 (MOAC 2014). To maximize commercial profit, the stocking density of abalone has been continuously growing, leading to substantial discharge of farming pollutants, such as uneaten feed and feces (Porter et al. 1987, Wang & Yan 2012, Kemp et al. 2015). Accumulation of organic matter in both farming and adjacent waters should be prevented, because it can bring about a series of harmful issues including oxygen depletion, eutrophication and toxicity of ammonia and nitrite. As such, environ-

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mental deterioration is of increasing concern worldwide because the continuous discharge of farming wastes causes substantial ecological impacts and economic losses (Wu et al. 1994, Cao et al. 2007).

Sea cucumber has been recorded to effectively remove the nutrient accumulation derived from farming wastes, e.g. feces, pseudofeces and feed residues, and consequently reduce nutrient loadings in polyculture systems (Zhou et al. 2006, Hannah et al. 2013, Kim et al. 2015). For example, Paltzat et al. (2008) found significant reduction of sedimentary organic matter as a result of the active selection by sea cucumber *Parastichopus californicus* in a sea cucumber-oyster polyculture system in British Columbia, Canada. Slater & Carton (2009) also reported that the feeding activity of sea cucumber *Australostichopus mollis* led to an obvious decrease in accumulation of both organic carbon and phyto-pigments associated with biodeposition from green-lipped mussel *Perna canaliculus* farms. More recently, a field experiment demonstrated that sea cucumber *Apostichopus japonicus* cultured in the water column below fish cages could utilize the fish feed residues (Yokoyama 2013). These studies provided evidence that sea cucumber can be a good co-culture candidate in integrated multi-trophic aquaculture systems.

Previous studies have proved the possibility that sea cucumber utilizes farming wastes derived from the culture of abalone, including feed residues and feces (Kang et al. 2003, Kim et al. 2015). Quantitative evaluation of the removal efficiency of farming wastes in polyculture systems of abalone *Haliotis discus hannai*, however, has not been reported. Ingestion and utilization of various potential food sources by aquatic animals have traditionally been evaluated by direct gut analysis, i.e. morphological identification of the food items taken up by consumers. Application of direct gut analysis only represents an instant snapshot of the food ingested by consumers (Gao et al. 2006, Sun et al. 2013). Furthermore, taxonomical identification was sometimes impossible because of the small size of the gut contents and the damage to the gut contents due to food digestion. Stable isotope analysis can overcome such limitations because (1) quantification of food sources by stable isotopes is based on the assimilated instead of ingested constituents; and (2) the utilized matter represents the time-integrated assimilation of food (Hobson & Welch 1992, Xia et al. 2013a). As a result, stable isotopes have been increasingly used as biomarkers to understand the flux of organic matter or pollutants along food chains and food webs in both terrestrial and aquatic environments (McClelland & Valiela 1998, Gao et al. 2006, 2011, Sarà et al. 2007, Du et al. 2015). Sun et al. (2013) successfully used a stable isotope technique to quantify the seasonal fluctuations in the relative contributions of potential food sources to *Apostichopus japonicus* in an *in situ* field investigation.

In the present study, polyculture systems combining abalone and sea cucumber at different inter-species biomass ratios were set up to investigate the co-culture potential of these 2 species. We determined the C and N stable isotope values of sea cucumber and its potential food sources. The objectives of this study were (1) to quantify the relative proportional contributions of potential food sources to sea cucumber in polyculture systems of abalone; and (2) to evaluate the capacity of sea cucumber to reduce nutrient loadings based on C and N stable isotope analysis.

MATERIALS AND METHODS

Experimental design and sample collection

The experiment was carried out at the Laboratory of the National Marine Science Research Centre, Qingdao, China. Juvenile sea cucumbers and abalones were collected from a commercial farm. The water on the surface of the animals was absorbed by blotting paper and weighed using a balance to a precision of 0.01 g. Initial wet weights (mean ± SD) of sea cucumber and abalone were 1.84 ± 0.01 g and 2.58 ± 0.09 g, respectively. Prior to the experiment, the sea cucumbers and abalones were acclimated in the laboratory for 15 d at 15–16°C and salinity of 30–32.

A total of 15 fiber reinforced plastic tanks (diameter: 80 cm, height: 100 cm) were used as experimental tanks in the present study. Juvenile abalones and sea cucumbers were cultured at 5 inter-species ratios in terms of animal biomass, i.e. 100:0, 70:30, 50:50, 30:70 and 0:100 (referred to as Group A, B, C, D and E, respectively), and each group had 3 replicates. The average total biomass (mean ± SD) in each tank was 160.58 ± 0.48 g, which did not differ significantly among the experimental tanks (ANOVA, $p > 0.05$). To comply with the feeding habit of sea cucumber, sea mud was collected from an intertidal zone, dried and ground to powder (<0.2 mm). Equal quantities were then spread on the bottom of each tank to a depth of 5 cm before introducing the sea cucumber. The abalones were bred in plastic cages (length × width × height = 50 × 30 × 10 cm, 0.5 cm mesh), and one piece of wave-shaped tile (30 × 20 × 1 cm) was installed for abalone attachment. The cages were

hung 40 cm above the sea mud at the bottom of each tank. In addition to the 15 experimental tanks as described above, 3 control tanks without animals were used. All setups and maintenance of the control tanks were the same as in the experimental tanks except that no sea cucumber or abalone was cultured in the control tanks. The experiment lasted for 12 wk. At the beginning of the experiment, 10 sea cucumber individuals were collected as initial samples for stable isotope analysis.

During the experiment, commercial pellet feed for juvenile abalone (length × width × height = 5 × 1.5 × 0.2 cm, Weihai Jin Feed) was placed directly in the cages once per day at 17:00 h and amounted to up to 2% of the abalones' total biomass. The feed used fish meal, fermented soybean meal and algae powder as protein sources and squid liver oil as a lipid source and contained 42% crude protein, 2% crude fat and 30% carbohydrate. The uneaten feed was removed from the cages every 2 d. No additional feed was supplied to the sea cucumbers. During the experiment, 1/5 of the water in each tank was changed daily using sand-filtered seawater, and aeration was provided continuously. The experimental temperature was 15–16°C, salinity 30–32, dissolved oxygen (DO) > 5 mg l^{-1}, and a 14 h light: 10 h dark photoperiod was applied.

At the end of the experiment, the abalones and sea cucumbers were collected, starved for 24 h, and weighed for the calculation of growth performance. The sea cucumber body samples as well as samples of its potential food sources, including uneaten feed residues, feces egested by abalone and sea cucumber, and sea mud, were collected for stable isotope analysis. Five sea cucumbers from the same tank were pooled as 1 replicate and those from the 3 different tanks were used as 3 replicates. To avoid any isotopic shifts, 3 replicates of the abalone feed were soaked in sea water from the same plastic tank with aeration for 48 h and used for uneaten feed residue analysis. The feces egested by abalone and sea cucumbers were collected by siphoning 20 h after feeding. To gain original isotopic profiles for sea mud as a food source for sea cucumber, sea mud for stable isotope analysis was collected from the 3 control tanks and pooled. The samples were taken from the surface to ~0.3 cm depth using a syringe with the luer-lock end cut off. All the samples were dried at 55°C for at least 72 h to constant weight, and ground into fine and homogeneous powder using a micro-grinder and a sieve. Then, the sample was tightly sealed in a glass Petri dish and freezer-stored at –80°C for further analysis.

Measurements of stable isotopes

For carbon stable isotope analysis, the samples were decarbonated using HCl (35%) fume for at least 24 h until no more CO_2 bubbles were produced. For nitrogen stable isotope analysis, the food source samples were washed 3 times with KCl solution (30%) to remove inorganic nitrogen.

Carbon and nitrogen isotope ratios were determined using an elemental analyzer coupled with an isotope ratio mass spectrometer (EA-IRMS, Thermo Finnigan MAT Delta-plus). Results of isotope ratios were expressed in standard δ-unit notation, which is defined as follows:

$$\delta X = [(R_{sample} / R_{standard}) - 1] \times 1000‰ \quad (1)$$

where $X = {}^{13}C$ or ^{15}N, and R is either ^{13}C:^{12}C ratio for carbon or ^{15}N:^{14}N ratio for nitrogen. The values were reported relative to the Vienna Pee Dee Belemnite (PDB) standard for carbon and relative to air N_2 for nitrogen. A laboratory working standard (glycine) was run for every 10 samples. Analytical precision was ±0.1‰ and ±0.2‰ for carbon and nitrogen, respectively.

Statistical analysis and isotope mixing model

One-way analysis of variance (ANOVA) with Duncan's test for multiple comparisons was used to compare growth, stable isotopic changes of sea cucumber and its potential food sources among the treatments. The difference between the initial and final sea cucumber isotope ratios was compared using paired Student's *t*-test. Prior to analysis, normality and variance equality of raw data was tested with Kolmogorov-Smirnov test and Levene's test, respectively (Zar 2009). All statistical analyses were performed with the software SPSS for Windows release 16.0 (SPSS Inc 2008).

The relative contributions of food sources to sea cucumber assimilation were calculated by the software 'Isosource' (Phillips & Gregg 2003). In the present study, average fractionation effects of 1.20 and 1.90‰ were used for carbon and nitrogen stable isotopes, respectively (Sun 2012).

RESULTS

Growth performance

Growth in terms of the difference between final and initial biomass of abalone and sea cucumber is presented in Table 1. Both abalone and sea cucumber

Table 1. Growth in terms of biomass gain (final biomass – initial biomass; mean ± SD) for abalone *Haliotis discus hannai* and sea cucumber *Apostichopus japonicus* in different experimental groups. AB/SC: biomass ratios of abalone to sea cucumber. Values with different superscripted letters indicate a significant difference ($p < 0.05$)

Group	AB/SC	Abalone (g)	Sea cucumber (g)	Total biomass gain (g)
A	100:0	51.24 ± 3.01^{a}	–	51.24 ± 3.01^{a}
B	70:30	75.71 ± 2.40^{b}	46.82 ± 4.43^{a}	122.52 ± 5.84^{b}
C	50:50	68.05 ± 1.26^{b}	34.60 ± 8.57^{a}	102.65 ± 9.56^{c}
D	30:70	58.58 ± 2.39^{a}	1.37 ± 1.58^{b}	59.96 ± 3.83^{a}
E	0:100	–	-109.14 ± 3.20^{c}	-109.14 ± 3.20^{d}

showed significant discrepancy in biomass gain among the experimental groups (ANOVA, $F_{3,11}$ = 20.72, $p < 0.05$, for abalone; $F_{3,11} = 190.58$, $p < 0.05$, for sea cucumber). Total biomass gain of abalone and sea cucumber also exhibited significant differences among the experimental groups (ANOVA, $F_{4,14} = 261.29$, $p < 0.05$). For abalone, the highest biomass gain of 75.71 g appeared in Group B. For sea cucumber, Group E showed negative growth, with the lowest value of −109.14 g. The maximum and minimum values of total biomass gain were observed in Group B and E, respectively.

Isotopic compositions and food contributions

The $\delta^{13}C$ and $\delta^{15}N$ values of sea cucumber and its potential food sources, i.e. feces egested by abalone and sea cucumber, uneaten abalone feed residues and sea mud in the experimental groups, are summarized in Table 2. After 12 wk culture, the carbon isotope ratios of sea cucumber increased significantly in Groups B, C, D and E, while the nitrogen isotope ratios decreased significantly in Groups B, C and D (paired Student's *t*-test, $p < 0.05$). The $\delta^{13}C$ and $\delta^{15}N$ values of sea cucumber ranged from −18.92 to −18.25‰ and 6.80 to 10.09‰, respectively. Significant differences in the $\delta^{13}C$ and $\delta^{15}N$ values of sea cucumber were observed among the experimental groups (ANOVA, $F_{3,11} = 6.64$, $p < 0.05$, for $\delta^{13}C$; $F_{3,11}$ = 15.67, $p < 0.05$, for $\delta^{15}N$).

For abalone feces, both $\delta^{13}C$ and $\delta^{15}N$ values did not show any statistical difference among the experimental groups (ANOVA, $F_{2,8} = 0.39$, $p > 0.05$, for $\delta^{13}C$; $F_{2,8} = 0.74$, $p > 0.05$, for $\delta^{15}N$) (Table 2). For feces egested by sea cucumber, the $\delta^{13}C$ values showed significant difference among the groups, with the highest value of −19.72‰ in Group C and the lowest value of −21.32‰ in Group E (ANOVA, $F_{3,11} = 7.26$, $p < 0.05$). Similarly, the $\delta^{15}N$ values also exhibited significant difference among the groups, with the highest value of 4.87‰ in Group D and the lowest value of 4.10‰ in Group E (ANOVA, $F_{3,11} = 6.20$, $p < 0.05$).

The dual stable isotope plots illustrated the isotopic shifts between the final and initial sea cucumber isotopic profile and among the food sources uptake by sea cucumber (Fig. 1). The calculation of the relative contributions of food sources in Group E was unavailable because the sea cucumbers exhibited negative growth. The proportional contributions of uneaten abalone feed residues and feces egested by abalone decreased with the increase of biomass ratios of abalone to sea cucumber; however, the contributions of feces egested by sea cucumber and sea mud increased from 16.5 to 42.0% and from 15.8 to 44.1%, respectively (Table 3). Total contributions of the farming wastes (i.e. feces and residual diets) decreased as the biomass ratios of abalone to sea cucumber increased in the present study.

DISCUSSION

The results showed wide ranges of carbon and nitrogen isotope signatures of potential food sources

Table 2. Carbon and nitrogen stable isotopic ratios (mean ± SD) of sea cucumber *Apostichopus japonicus* and its potential food sources in the experimental groups (A–E: abalone *Haliotis discus hannai* to sea cucumber biomass ratios of 100:0, 70:30, 50:50, 30:70 and 0:100, respectively). ISC: initial sea cucumber, FSC: final sea cucumber, AF: abalone feces, SCF: sea cucumber feces, AUF: uneaten abalone feed residues, SM: sea mud. Values with different superscripted letters indicate a significant difference ($p < 0.05$)

Sample	Group	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)
ISC	All	-19.44 ± 0.28	9.89 ± 0.16
FSC	B	-18.25 ± 0.34^{a}	6.80 ± 0.86^{a}
	C	-18.43 ± 0.12^{a}	6.86 ± 1.08^{a}
	D	-18.92 ± 0.08^{b}	7.14 ± 0.15^{a}
	E	-18.30 ± 0.15^{a}	10.09 ± 0.11^{b}
AF	B	-18.75 ± 0.12^{a}	5.13 ± 0.10^{a}
	C	-18.70 ± 0.09^{a}	5.02 ± 0.20^{a}
	D	-18.63 ± 0.24^{a}	5.15 ± 0.10^{a}
SCF	B	-20.81 ± 0.49^{ab}	4.64 ± 0.38^{a}
	C	-19.72 ± 0.61^{c}	4.70 ± 0.16^{a}
	D	-20.00 ± 0.11^{bc}	4.87 ± 0.10^{a}
	E	-21.32 ± 0.52^{a}	4.10 ± 0.19^{b}
AUF	All	-19.09 ± 0.19	3.89 ± 0.06
SM	All	-20.56 ± 0.25	5.85 ± 0.10

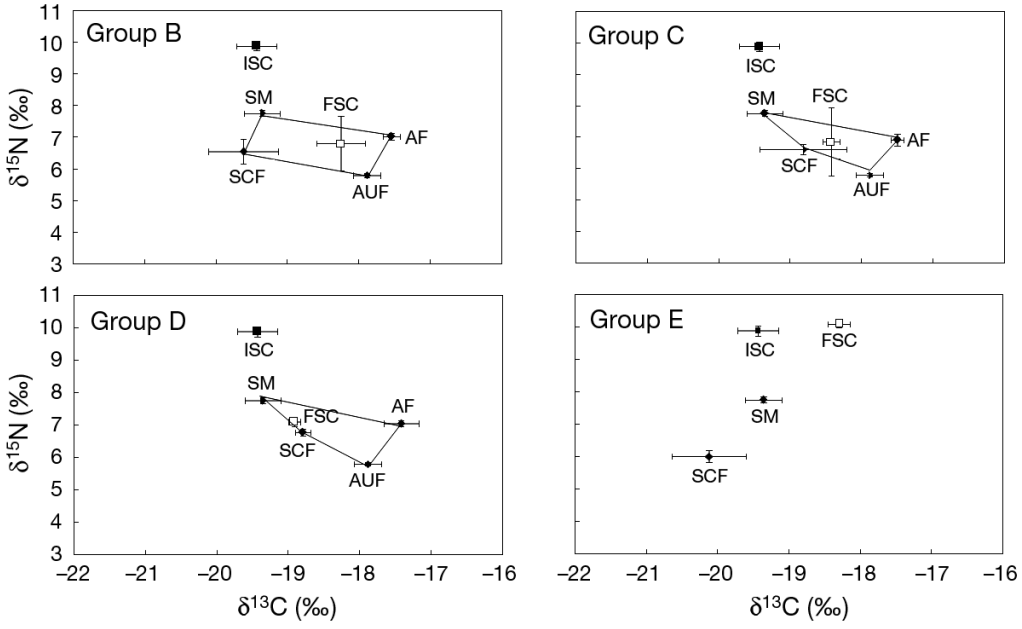

Fig. 1. Dual stable isotope plots of $\delta^{13}C$ and $\delta^{15}N$ values (mean ± SD) for sea cucumber *Apostichopus japonicus* and its potential food sources in the experimental groups (B, C, D and E: abalone *Haliotis discus hannai* to sea cucumber biomass ratios of 70:30, 50:50, 30:70 and 0:100, respectively). ISC: initial sea cucumber, FSC: final sea cucumber, AF: abalone feces, SCF: sea cucumber feces, AUF: abalone uneaten feed residues, SM: sea mud

and sea cucumber, which implied the feasibility of using stable isotope ratios to calculate the proportional contributions of food sources to sea cucumber assimilation (Peterson & Fry 1987). Compared to abalone feed, feces egested by abalone were lightly, but not significantly enriched in ^{13}C, while being significantly enriched in ^{15}N due to the possibility of preferential absorption of isotopically lighter ^{14}N in metabolic processes. Similarly, the $\delta^{13}C$ values of abalone feces in Groups B, C and D indicated the consistent isotopic fractionation under the condition of sufficient carbon sources (Sun et al. 2012, Xia et al. 2013b). No significant discrepancy was found in the $\delta^{13}C$ and $\delta^{15}N$ values of abalone feces among the experimental groups (B, C and D), which demonstrated that stocking density and food competition had no effect on the assimilation of abalone carbon and nitrogen isotopes. However, significant differences in isotopic signatures of sea cucumber feces among the groups might be due to the fluctuation in various food sources with different stable isotope ratios (Xia et al. 2015b).

At the end of the experiment, the C and N stable isotopes of sea cucumber showed obvious shifts among the experimental groups. Significant isotopic ^{13}C enrichments and ^{15}N depletions of sea cucumber in Groups B, C and D, might imply the uptake of various food sources with higher $\delta^{13}C$ values and lower $\delta^{15}N$ values relative to the initial values for sea cucumbers. However, the significant enrichments of both ^{13}C and ^{15}N isotopes in sea cucumbers in Group E could mainly be attributed to the excessive expenditure of nutritional reserve. Previous studies also demonstrated that the prior consumption of isotopically light ^{12}C and ^{14}N in tissues of sea cucumber could lead to the enrichment of ^{13}C and ^{15}N when the

Table 3. Percent contributions of potential food sources to sea cucumber *Apostichopus japonicus* based on Isosource software. For groups and food sources abbreviations see Table 2. Average fractionation effects of 1.20‰ and 1.90‰ for carbon and nitrogen stable isotopes, respectively, were used in the present study. Data are mean ± SD

Group	AF		SCF		AUF		SM		AF + SCF + AUF
	Average (%)	Range (%)	Average (%)	Range (%)	Average (%)	Range (%)	Average (%)	Range (%)	Average (%)
B	46.0 ± 9.4	25.0–66.0	16.5 ± 9.8	0–36.0	21.7 ± 9.7	1.0–42.0	15.8 ± 9.5	0–34.0	84.2
C	27.6 ± 4.9	15.0–39.0	26.7 ± 15.8	0–60.0	18.5 ± 11.1	0–42.0	27.2 ± 9.0	8.0–45.0	72.8
D	2.4 ± 2.0	0–9.0	42.0 ± 16.4	0–72.0	11.5 ± 8.1	0–33.0	44.1 ± 9.3	27.0–68.0	55.9

organisms showed net consumptions in nutritional budget (Sun et al. 2013). Different proportions of biological components including proteins, lipids and carbohydrates in tissues potentially result in various isotopic fractionations (Peterson 1999). For instance, the tissues with higher lipid contents usually show more negative carbon isotopic values as a result of the discrimination against isotopically heavy ^{13}C in lipid synthesis (Tieszen et al. 1983). During the processes of biomass loss, net consumption of energy substrates including lipids and proteins substantially reduced the contents of biological components in Group E compared to those in other groups (Bao et al. 2010, Sun et al. 2012). Hence, different biological compositions of sea cucumber might be another factor contributing to the isotopic enrichments in Group E.

Calculated by the software 'Isosource', our results revealed that the farming wastes including animal feces and residual feed were the main food source for sea cucumber. The proportional contributions of various food sources to sea cucumber growth and metabolism depended on the biomass ratio of abalone to sea cucumber. Along with the decrease in abalone stocking density, the availability for sea cucumber of uneaten feed residues and feces egested by abalone decreased. The contributions of residual feed and feces to sea cucumber were accordingly reduced, which implied opportunistic feeding habit of sea cucumber to optimize its energetic and nutritional requirements responding to the fluctuations in food quality and quantity in the natural environment (Canuel et al. 1995, Gannes et al. 1998, Schmidt et al. 1999, Shimoda et al. 2007, Wen et al. 2016). Previous studies on the acclimation of physiological processes and digestive enzyme activities of sea cucumber to different food conditions also suggested its opportunistic feeding habit (Wang et al. 2007, Liu et al. 2009, Gao et al. 2011).

As an obligate deposit-feeding species, sea cucumbers have been identified as bioturbators and scavengers that can reduce nutrient loadings in organically enriched costal sediments and aquaculture systems (Zhou et al. 2006, Slater & Carton 2009, MacTavish et al. 2012, Ren et al. 2012). Previous studies found that sea cucumber co-cultured with filter-feeding mussel or predatory fish species can offer a balanced ecological system, in which inter-specific competition was minimal (Kang et al. 2003, Ren et al. 2012). Therefore, the introduction of sea cucumber to polyculture systems of abalone as a biological measure could effectively eliminate farming wastes and improve the aquatic environment. Additionally, the culturing of sea cucumber could increase economic returns due to its high nutritional value and curative effects (Xia et al. 2015a,b, Yang et al. 2015).

In the present study, the C and N isotopic profiles from abalone and sea cucumber co-culture suggest that sea cucumbers have great potential to become an effective organic extractive species and aid in the concordant reduction of farming wastes in polyculture systems of abalone. Both abalone and sea cucumber showed the best growth performance at an abalone to sea cucumber biomass ratio of 70:30, at which sea cucumbers consumed the highest proportion of farming wastes. We therefore recommend this biomass ratio in aquaculture practice, for both economic and environmental benefits. However, due to the experimental limits of the present study, further research at much larger scales, e.g. enclosures and ponds, is warranted.

Acknowledgements. The present study was funded by grants from the National Natural Science Foundation of China (Project No. 31672657 and 31372549). Thanks to Ian Charles for his assistance in reviewing the manuscript.

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Editorial responsibility: Adam Hughes, Oban, UK

Submitted: September 7, 2016; Accepted: May 4, 2017
Proofs received from author(s): June 18, 2017