



Stable Isotope Tracer Addition Reveals the Trophic Status of Benthic Infauna at an Intertidal Area Adjacent to a Seagrass Bed

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Stable isotope tracer addition can enhance the isotopic differences of primary producers for a wider food-web resolution than the use of natural abundance stable isotopes (NASIs) alone, particularly in ecosystems where primary producers have similar NASIs values. To investigate the food sources and the trophic status of benthic infauna in an intertidal area near a *Halophila minor* seagrass bed within inner Tai Tam Bay, Hong Kong, China, a ¹⁵N addition experiment was conducted, and the results were compared with those from NASIs data. Only benthic microalgae (BMA) were labeled by applying ¹⁵N-enriched NH₄Cl to the sediment daily for the first 7 days during a 21-day study. In contrast to the NASIs results, Bayesian mixing models based on the isotope tracer experiment suggested a larger dietary contribution of BMA for nematode *Daptonema* sp. and copepods, whereas a higher reliance on phytoplankton and seagrass detritus was noted for polychaete *Neanthes* sp. However, both NASIs and isotope tracer addition demonstrated that seagrass detritus was a major food source for nematode *Spilophorella* sp. The present isotope tracer experiment also revealed a contrasting result of the relatively lower contribution of meiofauna in the diets of carnivores/omnivores as compared to the results of NASIs. This finding suggested that the isotope values in these consumers may have not reached an equilibrium with the added isotope in the study period. Thus, there is a need for applying NASIs coupled with isotope tracer addition in the investigation of ecosystems in which primary producers have similar isotope values, especially in ecosystems with lower tissue turnover rates, in order for a more accurate determination of dietary contribution and trophic status of consumers in the food-web study.

Keywords: stable isotope, trophic study, meiofauna, nematodes, polychaetes, isotope tracer addition, infauna

INTRODUCTION

Intertidal seagrass habitat offers a variety of primary producers that support secondary production. Apart from seagrass, benthic microalgae (BMA) and phytoplankton transported by incoming tides may also serve as food sources for a wide range of consumers (Lebreton et al., 2012; Ouisse et al., 2012). Being a primary consumer and serving as prey for larger animals such as fish, shellfish, and infauna (Gee, 1989), meiofauna acts as a key intermediate consumer linking the basal primary food sources to higher trophic levels within an ecosystem. Meiofauna also exhibits a range of feeding modes such as microvorous, deposit feeding, and epigrowth feeding (Wieser, 1953; Moens and Vincx, 1997), which allow them to exploit a variety of living or detrital primary producers from local sediment or imported by tides (Buffan-Dubau and Carman, 2000; Lebreton et al., 2012; Cnudde et al., 2015). As the food sources of seagrass ecosystems typically show large variations of quality and quantity depending on locations and seasons (Lebreton et al., 2009; Ha et al., 2014), such natural variability coupled with the small size of meiofauna has made it difficult to identify the food sources supporting the secondary production in seagrass ecosystems. However, the determination of food sources and trophic status of benthic infauna is important to understand the food-web linkage and ecology in seagrass habitat (Lebreton et al., 2012).

The use of multiple natural abundance stable isotopes (NASIs) has become an important tool in investigating the food sources and trophic interactions in marine benthic food webs (Herman et al., 2000; Vafeiadou et al., 2014; Braeckman et al., 2015; Qu et al., 2019; Yang et al., 2020). Based on such stable isotope data, new Bayesian mixing models (e.g., MixSIR, SIAR, and IsotopeR) have also been developed to overcome the limitation of the simple linear mixing models (e.g., IsoError, IsoConc, and IsoSource), allowing to determine the contributions of more than $n + 1$ food sources (n = number of elemental tracers) (Moore and Semmens, 2008; Jackson et al., 2009; Parnell et al., 2010; Solomon et al., 2011; Hopkins and Ferguson, 2012). These models can also incorporate uncertainty and variation in input parameters, such as changes in the consumers, food sources, and trophic discrimination factors, which often occur in natural ecosystems, and thus provide a more accurate assessment of consumer diets (Moore and Semmens, 2008; Swan et al., 2020). However, the application of NASIs is most useful in ecosystems in which primary producers have distinct isotope values from their consumers (Moncreiff and Sullivan, 2001). The utility of NASIs to identify dietary sources may be reduced within systems where primary producers have similar isotope values. One way of solving this problem is to use NASIs coupled with isotope tracer addition (Carman and Fry, 2002; Levin et al., 2006; Mäkelä et al., 2017; Braeckman et al., 2018; Yorke et al., 2019). This is based on the rationale that isotope tracer addition can enhance the differences in the isotope values of the primary producers by creating a unique isotope label, and the label then enters the food web through consumption of the labeled primary producers. Hence, in combination with NASIs, isotope tracer addition allows for a better resolution of food source contribution and determination of the trophic status of

consumers in marine food webs where primary producers have similar isotope values (Galván et al., 2011).

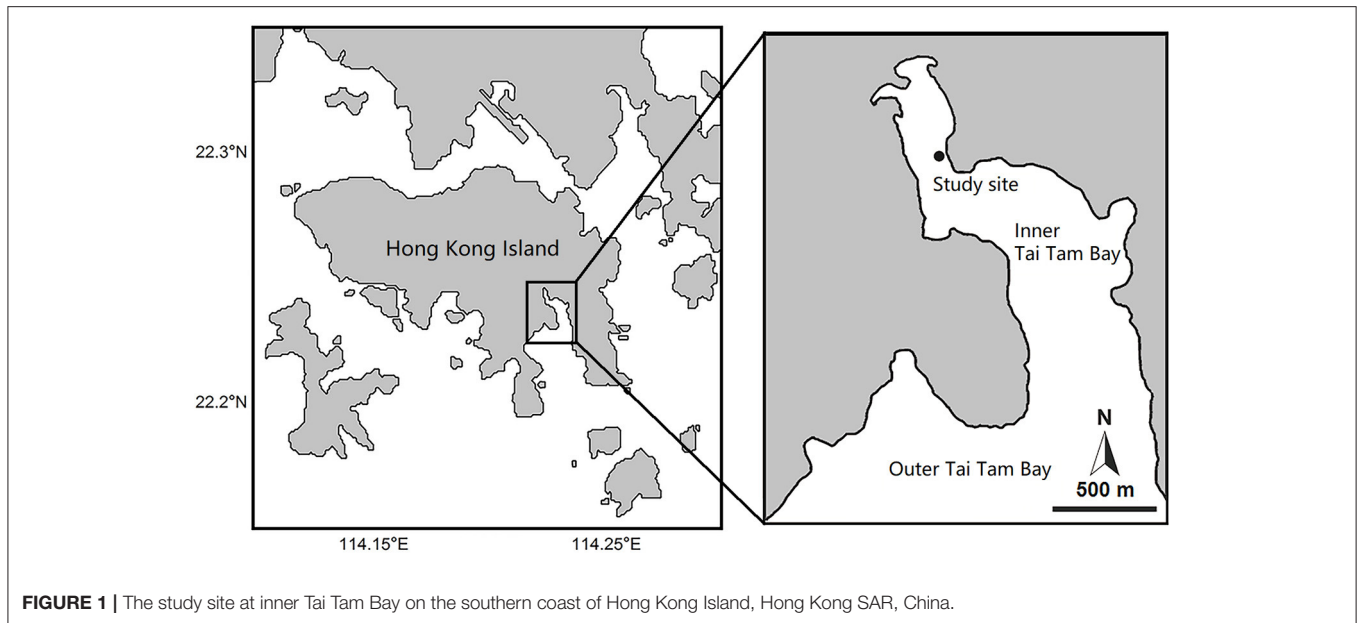
In this study, a ^{15}N addition experiment was conducted in combination with the analysis of NASIs data to reveal the contribution of primary producers to the diets of marine infauna and the meio-macrofaunal trophic linkage in a subtropical intertidal area adjacent to a *Halophila minor* seagrass bed. The NASIs data have been published, showing that benthic microalgae (BMA) were a dominant food source for most consumers, except for nematode *Spilophorella* sp., which mainly relied on seagrass detritus (Xu et al., 2018). The isotope mixing model results based on the NASIs data also indicated a higher contribution of nematodes and copepods to the diet of carnivorous polychaete *Eunice* sp. and a relatively lower proportion of meiofauna in the diets of omnivorous polychaete *Neanthes* sp. and carnivorous/omnivorous nematode *Ditlevsenella* sp. However, the similar NASIs values of BMA and particulate organic matter (POM) in the study area may preclude an accurate identification of the diet sources from the isotope mixing model. Our aims of this study were to examine the importance of primary producers (i.e., from local sources and that transported by the incoming tide) to infauna and the dietary contribution in key predatory infauna, using the mixing model based on stable isotope tracer data, in an intertidal area near a seagrass bed. The results were then compared with the same model output from NASIs. As the study area is exposed at low tide and only has intermittent access to food resources imported by tides, we hypothesized that most infauna will have enriched isotope tracer values that more closely reflect the use of isotope-labeled BMA similar to the results from NASIs.

MATERIALS AND METHODS

Experiment Setup

The investigation was carried out in an intertidal area ($22^{\circ}14'32.6''$ N, $114^{\circ}13'31.8''$ E) within inner Tai Tam Bay, a semi-enclosed embayment on the southern side of Hong Kong Island, Hong Kong, China, during the summertime (Figure 1). Hong Kong experiences a subtropical climate with an average summer temperature of around 28°C , and the study area is pristine without much human disturbance, with mean dissolved oxygen of about 9.67 mg/L, pH 8.0, and salinity 32 (Chiu et al., 1994; Chan and Morton, 2001; Cheung et al., 2003; Liu et al., 2011). Inner Tai Tam Bay has been designated as a Site of Special Scientific Interest by the Hong Kong government, as the area covers a diversity of intertidal habitats with various plant and animal communities (Che and Morton, 1991). The investigation site chosen for this study was an intertidal area adjacent to an *H. minor* seagrass bed.

The field investigation was conducted during the summer (August), in which potential food sources including BMA, POM, and seagrass detritus of *H. minor*, as well as consumers including dominant meio- and macrofauna, were collected during the pre- and post-isotope tracer addition at the study site. Isotope tracer addition was carried out in three 1×1 m experimental plots in the first 7 days during a 21-day experiment. Each plot was separated by a linear distance of 10 m and was marked at



the corners with PVC poles. Within each plot, the sediment was directly sprayed with 250 ml of $^{15}\text{NH}_4\text{Cl}$ solution ($18.5 \text{ mg } ^{15}\text{NH}_4\text{Cl m}^{-2}$, Carman and Fry, 2002) daily at low tide during the 7-day isotope tracer addition. $^{15}\text{NH}_4\text{Cl}$ (Sigma-Aldrich, St. Louis, USA) was dissolved in Whatman GF/F filtered seawater collected on site and applied in the field using a common garden sprayer. The tracer was unlikely to be absorbed by phytoplankton and seagrass detritus because of the dilution of the isotope addition by the tide, and there was no take-up of isotope label by the dead detritus as shown by Galván et al. (2008).

Samples of potential food sources and consumers were collected adjacent to the experimental plots prior to the daily isotope tracer spray, so as to determine the food sources of infauna by NASIs. Results from the NASIs in this experiment have been published (Xu et al., 2018). To determine the changes of ^{15}N isotope values for primary producers and benthic infauna over the 7-day ^{15}N tracer addition, triplicate samples were collected from each sprayed plot on Days 3 and 7. Samples were also collected on Days 14 and 21, 1 and 2 weeks after the tracer addition was stopped. To obtain a sufficient amount, BMA and infaunal samples collected from the three plots were pooled together for stable isotope analysis.

Benthic microalgae were collected following the method of Wainright et al. (2000). The sediment from an area of $20 \times 20 \text{ cm}$ in each experimental plot was first covered by a thin layer of pre-combusted silica sand, and then, a $63 \mu\text{m}$ nylon mesh was placed above the silica sand. Another thin layer of silica sand was applied to the mesh and moistened with seawater filtered by pre-combusted Whatman GF/F filters ($0.7 \mu\text{m}$ porosity). The mesh together with the upper silica sand was retrieved after 2 h during which the microalgae would migrate onto the mesh. After returning to the laboratory, BMA and silica sand were rinsed from the mesh by distilled water and swirled in a beaker, and

the suspended material (without sand) was filtered onto a pre-combusted Whatman GF/F filter with $0.7 \mu\text{m}$ retention. Filter papers were acidified in HCl vapor for 24 h to remove carbonates and dried at 60°C to a constant weight prior to stable isotope analysis. POM was collected as an indication of phytoplankton by filtering near-bottom seawater *in situ* on pre-combusted Whatman GF/F filter ($0.7 \mu\text{m}$ porosity). Water samples were collected at high tide ($\pm 1 \text{ h}$) by hand pumping near-bottom water into nine 2 L Nalgene bottles near the experimental area where isotope tracer addition was conducted. Samples were sieved by a $63 \mu\text{m}$ mesh to remove large zooplankton, and the residues were then examined microscopically. The portion $<63 \mu\text{m}$ was dominated by phytoplankton with few zooplankton. Hence, POM could be used as a proxy for phytoplankton in our analysis. POM samples on filters were rinsed with distilled water to remove salt on the particle surface, then treated with HCl vapor for 24 h to remove carbonates, and dried at 60°C to a constant weight. Triplicate leaf samples of dead *H. minor* were manually collected, cleaned with distilled water, and then oven-dried and powdered using a grinder. The leaf samples were not acidified as suggested by Bunn et al. (1995) to avoid the effect on $\delta^{15}\text{N}$ values.

For the collection of infauna, one large ($20 \times 20 \text{ cm}$) and one small ($15 \times 15 \text{ cm}$) area of sediment in each experimental plot was sampled randomly using a hand-held trowel at each sampling time. Macrofaunal samples were collected from sediment down to 20 cm depth in the large areas, while meiofaunal samples were obtained within the upper 8 cm sediment from the small areas. In the laboratory, sediment samples for macrofaunal analysis were sieved through a 0.5 mm screen. Polychaetes were handpicked from residues on the screen and identified to genus level. Adhered materials on the polychaete body were stripped off carefully using a needle and a pair of forceps, then rinsed in filtered seawater, and checked using a dissecting microscope to make sure all adhering materials were removed. The whole body

TABLE 1 | Natural abundance stable isotope $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of primary producers and infauna (data from Xu et al., 2018), and δ^E (highest observed enrichment values minus natural abundance isotope values) over the 21-day experiment and average natural abundance (NA) $\delta^{13}\text{C}$ values from the 21-day experiment.

Natural abundance	$\delta^{13}\text{C}$ (n)	$\delta^{15}\text{N}$ (n)	Feeding type
Food sources			
Seagrass	-10.0 ± 0.4 (3)	5.8 ± 0.5 (3)	
BMA	-22.3 ± 0.3 (9)	6.4 ± 0.4 (9)	
POM	-23.6 ± 0.5 (9)	5.5 ± 0.4 (9)	
Consumers			
(1) Nematodes			
<i>Daptonema</i> sp.	-19.6 ± 0.4 (3)	8.7 ± 0.4 (3)	Non-selective deposit feeder ^a
<i>Ditlevsenella</i> sp.	-16.6 ± 1.3 (3)	9.9 ± 0.2 (3)	Carnivore/omnivore ^a
<i>Spilophorella</i> sp.	-9.8 ± 0.4 (3)	8.7 ± 0.0 (3)	Epigrowth feeder (grazer) ^a
Bulk nematodes	-19.7 ± 0.7 (3)	8.9 ± 0.6 (3)	–
(2) Copepods	-20.9 ± 0.2 (3)	9.7 ± 0.6 (3)	Herbivore/omnivore ^b
(3) Polychaetes			
<i>Neanthes</i> sp.	-16.9 ± 1.2 (5)	9.7 ± 0.6 (5)	Omnivore ^{c,d}
<i>Eunice</i> sp.	-15.4 ± 0.7 (3)	15.2 ± 1.1 (3)	Carnivore ^{c,d}
¹⁵N-enriched	Average NA $\delta^{13}\text{C}$	Highest δ^E	
Food source			
BMA	–22.1	2578.9	
Consumers			
(1) Nematodes			
<i>Daptonema</i> sp.	–19.5	973.0	Non-selective deposit feeder ^a
<i>Ditlevsenella</i> sp.	–16.4	115.3	Carnivore/omnivore ^a
<i>Spilophorella</i> sp.	–9.7	514.0	Epigrowth feeder (grazer) ^a
Bulk nematodes	–14.4	876.8	–
(2) Copepods	–20.7	2,046.0	Herbivore/omnivore ^b
(3) Polychaetes			
<i>Neanthes</i> sp.	–16.7	174.4	Omnivore ^{c,d}
<i>Eunice</i> sp.	–15.7	150.7	Carnivore ^{c,d}

^a(Wieser, 1953); ^b(De Troch et al., 2006); ^c(Fauchald and Jumars, 1979); ^d(Jumars et al., 2015). Single values represent pooled samples; other values are means \pm SD of pooled samples (number of replicates are shown in parentheses).

of polychaetes was used for stable isotope analysis. At least five individuals were pooled, oven-dried at 60°C, and homogenized through grinding. Then, 1 mg of homogenized materials was transferred to a pre-combusted tin capsule for stable isotope analysis. Sediments for meiofaunal samples were processed according to Heip et al. (1985) by centrifugation with colloidal Ludox solution diluted to gravity of 1.15 with distilled water, in which the process would not significantly change the ^{13}C and ^{15}N isotope values of meiofauna (e.g., nematodes) as demonstrated by Moens et al. (2002). The supernatant containing meiofauna was then rinsed through a 38 μm screen with distilled water. The residues on the screen were washed onto a Petri dish, then, nematodes and copepods were handpicked and rinsed to remove adhering particles, and transferred to a drop of distilled water in pre-combusted tin capsules. Sorting of nematodes was conducted under a stereomicroscope only for those dominant and easily recognizable specimens that could be identified to genus level. Other small-sized nematodes that could not be identified to genus level were pooled together as bulk nematode samples. To obtain enough biomass for NASI analysis, for large-sized nematodes

such as *Ditlevsenella* sp., at least 100 individuals were pooled to represent one replicate. For average-sized nematodes such as *Daptonema* sp. and *Spilophorella* sp., at least 1,000 individuals were pooled as one replicate. For harpacticoid copepods, at least 1,000 individuals were also pooled as one replicate. While for isotope-enriched samples, the number of individuals of the same meiofaunal species/group required per sample was half of that used for NASIs. Dried biomass of the same genus of polychaetes and nematodes and all the harpacticoid copepod individuals were pooled together to obtain adequate sample mass for stable isotope analysis and to homogenize spatial variability in the study area.

Stable Isotope Analysis

All the samples including NASIs and stable isotope tracer were analyzed in the Stable Isotope Facility, University of California, Davis, USA. Isotope values were reported in the standard δ -unit notation:

$$\delta X = [(R_{\text{sample}}/R_{\text{reference}}) - 1] \times 1,000\text{‰}$$

TABLE 2 | ^{15}N enrichment and natural abundance $\delta^{13}\text{C}$ in primary producers and infauna over the 21-day isotope tracer study.

	Day 3		Day 7		Day 14		Day 21	
	$\delta^{13}\text{C}$	δ^{E}	$\delta^{13}\text{C}$	δ^{E}	$\delta^{13}\text{C}$	δ^{E}	$\delta^{13}\text{C}$	δ^{E}
BMA	-21.9	825.4	-22.9	2578.9	-22.0	131.9	-21.6	16.1
<i>Daptonema</i> sp.	-19.7	24.9	-18.9	762.8	-19.4	973.0	-19.9	373.9
<i>Ditlevsenella</i> sp.	-16.7	6.6	-16.3	76.4	-16.7	91.0	-16.0	115.3
<i>Spilophorella</i> sp.	-9.2	69.5	-9.7	514.0	-9.8	337.4	-10.0	56.9
Bulk nematodes	-14.7	59.9	-13.6	876.8	-16.7	409.5	-12.5	135.4
Copepods	-21.2	480.7	-21.1	2046.0	-20.3	972.3	-20.3	330.9
<i>Neanthes</i> sp.	-17.0	34.5	-16.4	136.7	-16.8	174.4	-16.4	138.4
<i>Eunice</i> sp.	-15.7	7.4	-15.9	48.1	-16.2	111.1	-14.9	150.7

Day 7 was the last day of ^{15}N addition; Days 14 and 21 were the first and second weeks after cessation of ^{15}N addition. δ^{E} is the enriched $\delta^{15}\text{N}$ value for that sampling day minus natural abundance $\delta^{15}\text{N}$ from Day 0.

where X is ^{13}C or ^{15}N , and R is $^{13}\text{C}:^{12}\text{C}$ or $^{15}\text{N}:^{14}\text{N}$, respectively. These values were reported relative to standards, the Vienna-PeeDee Belemnite (V-PDB), and air N_2 .

Data Analysis

The possible contributions of potential food sources to the consumers (nematodes, copepods, and polychaetes) from the NASI data before isotope tracer addition (published in Xu et al., 2018) and from the ^{15}N addition experiment were determined using the Bayesian mixing model developed in the software IsotopeR 0.4 package (Hopkins and Ferguson, 2012) under the R 3.10 software (R Core Development Team, 2014). Trophic enrichment factors used for computations were based on the published values from various sources reported in Vander Zanden and Rasmussen (2001). For herbivores, the values adopted were $-0.4\text{‰} \pm 1.1$ (Mean \pm SD) for $\delta^{13}\text{C}$ and $2.5\text{‰} \pm 2.5$ for $\delta^{15}\text{N}$. For carnivores/omnivores, the values used were $0.9\text{‰} \pm 1.0$ for $\delta^{13}\text{C}$ and $3.2\text{‰} \pm 0.4$ for $\delta^{15}\text{N}$. The proportional contribution of food sources in the isotope tracer addition experiment was calculated following the method by Galván et al. (2008), in which δ^{E} (highest $\delta^{15}\text{N}$ observed during the 21-day experiment minus natural abundance $\delta^{15}\text{N}$ before isotope tracer addition) of BMA and benthic consumers were used instead of natural abundance $\delta^{15}\text{N}$ in the Bayesian mixing model. The highest observed values were used because the tissue turnover rates of the consumers are assumed to be rapid and that all the fauna can reach an isotope steady-state equilibrium with the new labeled food sources. The interspecific differences in ^{15}N addition during the 21-day experiment may be resulted from different tissue turnover times or different labeled and unlabeled food source contributions in their diets. Consumers with smaller body sizes are considered to reach their tissue equilibrium more quickly than larger ones and may assimilate more ^{15}N -enriched isotopes over a short period of time. The duration of the present experiment was based on information from a previous stable isotope study with a tissue turnover time of about 3 days for meiofauna (Carman and Fry, 2002). For larger-sized consumers, such as polychaetes, the tissue turnover times were assumed in the range of 6–9 days, based on the findings by Doi et al. (2007) for stable isotopes in deposit-feeding chironomids with a body

mass similar to polychaetes studied in the present experiment. Moreover, it tends to take a longer time for carnivores to reach tissue equilibrium with isotope addition than herbivores, due to their higher trophic levels. To determine the food source contribution of diets in the isotope tracer addition experiment, mean natural abundance $\delta^{13}\text{C}$ values and highest $\delta^{15}\text{N}$ values were used from the results of the 21-day study.

RESULTS

Natural abundance stable isotope values of primary producers and consumers prior to the isotope tracer addition experiment are listed in **Table 1**, as extracted from Xu et al. (2018). Briefly, the NASI values of the food sources ranged from -23.6‰ (POM) to -10.0‰ (seagrass detritus) for $\delta^{13}\text{C}$ and ranged from 5.5‰ (POM) to 6.4‰ (BMA) for $\delta^{15}\text{N}$ before the isotope tracer addition commenced. BMA and POM had similar NASI values, while seagrass detritus showed more ^{13}C enriched than other food sources. The $\delta^{13}\text{C}$ signatures of the consumers ranged from -20.9‰ (copepods) to -9.8‰ (nematode *Spilophorella* sp.). The $\delta^{15}\text{N}$ signatures of the consumers spanned 6.5‰ and their $\delta^{15}\text{N}$ values were at least 3‰ higher than their food sources. The carnivorous polychaetes *Eunice* sp. showed an increase in $\delta^{15}\text{N}$ compared with other consumers, which reflected a higher contribution of nematodes and copepods to their diets. However, the omnivorous polychaete *Neanthes* sp. and carnivorous/omnivorous nematode *Ditlevsenella* sp. showed relatively lower $\delta^{15}\text{N}$ than polychaete *Eunice* sp., indicating a lower dependence of animals in their diets.

For the results of the ^{15}N addition experiment (**Table 2**), the ^{15}N -enriched label was taken up by the BMA quickly, as the enrichment above NASI values was observed on Day 3, the earliest sampling time after the start of the tracer addition. The $\delta^{15}\text{N}$ isotope values of BMA increased over the 7-day addition period, with a peak enrichment value of $2,578.9\text{‰}$ higher than the NASI levels (**Tables 1, 2**). All the nematodes, copepods, and polychaetes investigated became ^{15}N -enriched beyond the NASI values during the 21-day study (**Table 2**). Copepods were highly enriched and quickly reached a value of $2,046.0\text{‰}$ on

TABLE 3 | Mixing model results from natural abundance stable isotopes (data from Xu et al., 2018) and isotope tracer addition data for mean percentage (SD) of food sources in the diet of animals (– = food source was not included).

	BMA	POM	Seagrass detritus	Nematodes	Copepods
Natural abundance					
<i>Daptonema</i> sp.	40.9 (39.3)	31.1 (33.8)	28.0 (17.9)	–	–
<i>Ditlevsenella</i> sp.	23.0 (36.6)	15.5 (30.2)	31.4 (38.2)	22.1 (37.3)	8.0 (22.8)
<i>Spilophorella</i> sp.	1.3 (10.9)	1.0 (9.8)	97.7 (14.5)	–	–
Copepods	59.4 (45.8)	33.1 (43.0)	7.5 (11.5)	–	–
<i>Neanthes</i> sp.	34.9 (20.7)	11.3 (21.1)	38.0 (13.4)	8.4 (15.5)	7.3 (11.8)
<i>Eunice</i> sp.	0.3 (5.0)	0.5 (4.9)	4.8 (15.1)	76.4 (41.1)	18.0 (34.5)
¹⁵N-enriched					
<i>Daptonema</i> sp.	69.1 (29.2)	10.1 (22.9)	20.8 (11.8)	–	–
<i>Ditlevsenella</i> sp.	0.6 (3.7)	38.5 (15.6)	53.7 (10.2)	0.4 (2.3)	6.8 (17.8)
<i>Spilophorella</i> sp.	0.5 (6.5)	0.6 (7.5)	98.9 (9.9)	–	–
Copepods	92.5 (21.8)	4.2 (18.5)	3.3 (8.6)	–	–
<i>Neanthes</i> sp.	1.2 (6.6)	40.8 (17.1)	44.3 (20.4)	12.8 (30.4)	1.1 (8.61)
<i>Eunice</i> sp.	0.4 (2.5)	15.5 (18.9)	46.9 (25.9)	18.4 (37.2)	18.8 (24.6)

Day 7, the last day of isotope tracer addition, and the values decreased to 972.3 and 330.9‰, respectively, on 7 and 14 days after the addition of isotope tracer was stopped. Nematodes *Daptonema* sp. and *Spilophorella* sp. were enriched to 973.0 and 514.0‰ on Day 14 and Day 7, respectively. Polychaetes *Eunice* sp. and *Neanthes* sp. showed an increase in their enriched isotope values progressively and reached the highest values of 150.7 and 174.4‰, respectively, on Days 21 and 14, 2 weeks and 1 week after ¹⁵N addition was completed.

Results of the Bayesian mixing model from the isotope tracer addition data in **Table 3** suggested a larger contribution of BMA in the diets of nematode *Daptonema* sp. and copepods than the model output from NASI data alone. For nematode *Spilophorella* sp., both model results from NASIs and isotope tracer addition data supported a dominant diet of seagrass detritus. However, for polychaete *Neanthes* sp., model results from the isotope tracer addition data did not reveal the importance of BMA in their diet, which contradicted the model output from the NASI values. Although data from isotope tracer addition indicated the importance of both POM (40.8%) and seagrass detritus (44.3%) to the diet of *Neanthes* sp., results of the mixing model based on NASI data revealed that POM contributed little to its diet. For polychaete *Eunice* sp., the model output from NASI data showed that nematodes and copepods supported more than 94% of its diet; however, model results from the isotope tracer addition study indicated that meiofaunal prey only constituted <38% of the *Eunice* diet in the study area.

DISCUSSION

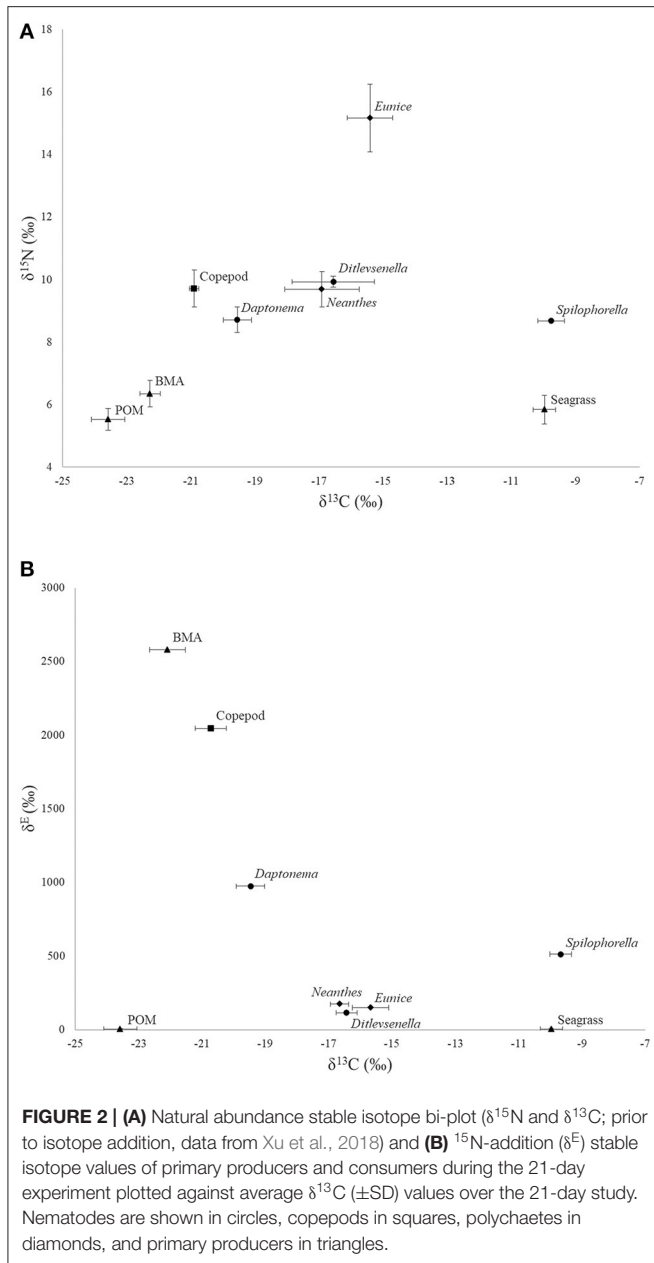
Food Sources for Benthic Consumers

As compared to NASI data, the addition of ¹⁵N tracer to the surface sediment and subsequent uptake by BMA appeared to widen the isotope signatures of primary producers (**Figure 2**), which could enhance the resolution of food sources and thus improve our understanding of the contribution of basal food

resources to the benthic food web. This can be illustrated by the results of the isotope tracer addition that BMA was in fact an important diet for some infauna only. Hence, such isotope tracer addition experiments can allow for a wider food-web resolution than the use of NASIs alone, particularly in ecosystems where NASI values of primary producers are similar (Galván et al., 2008).

A high ¹⁵N label uptake found in nematode *Daptonema* sp. and copepods confirmed the importance of BMA and indicated a higher contribution of BMA in their diets than based on NASI alone. The present findings agreed with many studies showing that grazing on diatoms is a common feeding strategy of harpacticoid copepods and nematode *Daptonema* sp. in shallow sediments (Moens and Vincx, 1997; Moens et al., 2005; De Troch et al., 2006; Cnudde et al., 2015; van der Heijden et al., 2019). The ¹⁵N label uptake by nematode *Spilophorella* sp. was found much lower compared with its counterpart nematode *Daptonema* sp. and copepods, suggesting a much lesser contribution of BMA and the importance of other isotope-unlabeled food sources in its diet. Mixing model outputs from both NASI and isotope tracer addition data also supported the finding that seagrass detritus contributed the most in the food source of nematode *Spilophorella* sp., with more than 97% in its dietary contribution (**Table 3**). However, with a heavily cuticularized mouthpart structure, nematode *Ditlevsenella* sp. is regarded as carnivorous/omnivorous in food-web ecology (Wieser, 1953). This can be reflected in the results of the present isotope tracer addition, as the ¹⁵N value of *Ditlevsenella* sp. only reached a maximum of 115.3‰ during the 21-day study (**Table 2**), suggesting that this species may prey on organisms that consumed isotope-labeled BMA.

Polychaete *Neanthes* sp. is classified as an omnivore (Fauchald and Jumars, 1979; Jumars et al., 2015), with the ability to utilize a variety of primary producers and to prey on small animals. The present isotope tracer addition experiment indicated that



the contribution of ^{15}N -labeled BMA to the diet of *Neanthes* sp. was minimal (circa 1%), implying a greater reliance on isotope-unlabeled food sources, such as POM and seagrass detritus in this study (Table 3). The model output from isotope tracer addition data also revealed a certain amount of nematodes and copepods in the diet of polychaete *Neanthes* sp. The progressive increase of the ^{15}N -enriched values in the present isotope tracer addition experiment could be a result of its feeding on the ^{15}N -enriched prey. *Neanthes* sp. is an opportunistic feeder. Its jawed, eversible proboscis is used primarily to ingest sediment deposits, but it is also capable of grazing some plant material and facultatively capturing small invertebrates (Craig et al., 2003). In agreement with our findings, phytoplankton was identified

as the major contribution to the diet of *Neanthes japonica* in an estuarine environment (Kikuchi and Wada, 1996), while the same species also consumed decomposed macrophytes much faster than fresh materials (Tsuchiya and Kurihara, 1979). As reported in the literature, meiofauna, small crustaceans, and polychaetes are also found consumed by various *Neanthes* species [Kay and Brafield, 1973; Cammen, 1980; Pardo and Dauer, 2003; National Introduced Marine Pest Information System (NIMPIS), 2006]. The polychaete *Eunice* sp. is classified as a carnivore, which has a pair of mandibles and a complex set of maxillae with a strong muscular, eversible pharynx (Fauchald and Jumars, 1979; Jumars et al., 2015). The relatively high natural abundance $\delta^{15}\text{N}$ in *Eunice* sp. also suggested a predatory feeding mode (Figure 2). During the isotope tracer addition experiment, *Eunice* sp. reached a maximum nitrogen value of 150.7‰ (Table 2), suggesting that it fed on ^{15}N -enriched prey in the experimental plots.

Seagrass as a Food Source

Epigrowth and deposit-feeding infauna are widely distributed on shallow intertidal mudflats, regardless of whether they were proximate to macrophytes (Da Rocha et al., 2006; Galván et al., 2008). Epigrowth feeders, however, are characterized by the presence of a buccal armature, supposedly used to either scrape off particles from a substrate or to damage and open food items before emptying them (Moens and Vincx, 1997). Most published isotope studies showed that they mainly feed on BMA and phytoplankton (van Oevelen et al., 2006; Kanaya et al., 2008; Lebreton et al., 2011; Christianen et al., 2017). However, some studies have indicated macrophyte detritus in the diets of meiofauna as well as some polychaetes (Lebreton et al., 2012; Vafeiadou et al., 2013; Ha et al., 2014; Rossi et al., 2015). Most of these previous studies revealed limited assimilation of macrophyte detritus by the benthos. However, this study showed a very high contribution of seagrass detritus in the diet of nematode *Spilophorella* sp. from the model results of both NASI and isotope tracer addition data. The present observations, which are also indicated by other studies (Carman and Fry, 2002; Galván et al., 2008; Vafeiadou et al., 2014), suggested that infaunal organisms have flexible dietary needs and feeding strategies that may change spatially and temporally as well as vary among species, making a generalization of the food-web position of infauna difficult. Thus, the feeding ecology of benthic infauna should be interpreted with caution and cannot be relied on buccal morphology alone.

The refractory nature of macrophytes may make them difficult to serve as a food source for the infauna (Campanyà-Llovet et al., 2017). However, microbial activities are shown to be able to enhance nutritional quality (Holmer et al., 2004; Guy, 2010). Microbial decomposition of macrophyte detritus has been widely reported, and there are also studies illustrating the consumption of such bacteria by infauna through microbial grazing (Danovaro, 1996; Vafeiadou et al., 2013). Majdi et al. (2012) found that grazing on BMA only covered a small part of the energetic requirements for Chromadoridae nematode *Chromadorina* sp., suggesting that

this species probably feeds on other biofilm food sources. Nevertheless, the isotope values of these bacteria which can decompose macrophyte detritus and are subsequently consumed by bacteria-feeding infauna should reflect the isotope values of the macrophyte detritus originally present in the sediment. Our mixing model results from both NASIs and isotope tracer addition also revealed that nematode *Spilophorella* sp., belonging to the same family Chromadoridae, consumed a large part of seagrass (>97%) in their diet. Hence, it is possible that *Spilophorella* may also graze on a large amount of bacteria associated with the decomposition of seagrass in the study area.

Comparison of Isotope Tracer Addition and Natural Abundance Stable Isotopes

For nematode *Daptonema* sp. and copepods, the model output from the NAsI values overestimated the proportion of POM but underestimated the proportion of BMA in its diets comparing with the model results from isotope tracer addition data (Table 3). However, for polychaete *Neanthes* sp., only the addition of a ^{15}N -enriched tracer elucidated the dietary importance of phytoplankton and seagrass detritus. These findings highlight the difficulty for the mixing model to output accurate assessments when food sources have similar isotope values and may lead to a false conclusion in the dietary composition if using NASIs alone. However, the isotope tracer addition method may also provide confusing information if it is not applied carefully. For nematode *Ditlevsenella* sp. and polychaete *Eunice* sp., the present isotope tracer data indicated a contrasting result of the relatively lower contribution of meiofauna in its diets as compared to the model results from NASIs (Table 3). An overestimation of POM and seagrass by the present isotope tracer addition experiment might be caused by the error that an equilibrium of isotopic signatures has not been reached in these consumers during the 21-day study period. There are also possibilities that the labeled BMA was not utilized by some consumers, and/or BMA produced before the application of ^{15}N tracer addition contributed to the diet of some consumers. In the present experiment, overestimations of POM were found in the omnivorous/carnivorous species (nematode *Ditlevsenella* sp. and polychaete *Eunice* sp.), with relatively larger body size and lower tissue turnover rate. There is most likely a time lag in the isotope enrichment of their body tissues because they have not reached tissue equilibrium with ^{15}N over a short-term (7-day) provision of the isotope-labeled food sources.

Overall, there are complementary strengths and weaknesses for the NAsI and isotope tracer addition approaches. The isotope tracer addition is capable of distinguishing the importance of the isotope-labeled source in ecosystems where primary producers have similar isotope values. However, isotope tracer does not readily infiltrate in systems with lower tissue turnover rates, especially in short-term experiments. Hence, in this regard, the use of NASIs can provide a better solution to identify food sources and trophic pathways in

such slow-turnover ecosystems (Fry, 2006). The application of NASIs also helps distinguish trophic levels in food-web ecology. A joint strategy of using NASIs coupled with isotope tracer addition is thus recommended in the investigation of ecosystems where primary producers have similar isotope values, especially in ecosystems in which lower tissue turnover rates are apparent.

CONCLUSION

This study demonstrated that the application of ^{15}N -enriched NH_4Cl to the surface sediment created distinct isotope values of primary producers, which increased the resolution for the determination of the dietary contribution of the consumers. However, it should be noted that isotope addition may also generate confusing information if the results are not interpreted carefully. One problem is the time lag of enrichment of stable isotope tracer, as shown in the findings of carnivorous/omnivorous infauna in this study, as tissue equilibrium with the isotope tracer addition may not have been reached over a short period of time. As such, a combination of NASIs coupled with isotope tracer addition would be a better approach to investigate dietary contributions and trophic status of consumers in ecosystems where primary producers have similar isotope values, especially in ecosystems with lower tissue turnover rates.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

PS designed the experiment and revised and edited the manuscript. WX conducted the experiment with the help of AT and wrote the manuscript. YD, SC, ZZ, and JS gave constructive comments and revised the manuscript. All the authors approved the final manuscript.

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