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# Compound-specific stable isotope analysis of amino acid nitrogen reveals detrital support of microphytobenthos in the Dutch Wadden Sea benthic food web

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The Wadden Sea is the world's largest intertidal ecosystem and provides vital food resources for a large number of migratory bird and fish species during seasonal stopovers. Previous work using bulk stable isotope analysis of carbon found that microphytobenthos (MPB) was the dominant resource fueling the food web with particulate organic matter making up the remainder. However, this work was unable to account for the trophic structure of the food web or the considerable increase in  $\delta^{15}\text{N}$  values of bulk tissue throughout the benthic food web occurring in the Eastern regions of the Dutch Wadden Sea. Here, we combine compound-specific and bulk analytical stable isotope techniques to further resolve the trophic structure and resource use throughout the benthic food web in the Wadden Sea. Analysis of  $\delta^{15}\text{N}$  for trophic and source amino acids allowed for better identification of trophic relationships due to the integration of underlying variation in the nitrogen resources supporting the food web. Baseline-integrated trophic position estimates using glutamic acid (Glu) and phenylalanine (Phe) allow for disentanglement of baseline variations in underlying  $\delta^{15}\text{N}$  sources supporting the ecosystem and trophic shifts resulting from changes in ecological relationships. Through this application, we further confirmed the dominant ecosystem support by MPB-derived resources, although to a lesser extent than previously estimated. In addition to phytoplankton-derived particulate, organic matter and MPB supported from nutrients from the overlying water column there appears to be an additional resource supporting the benthic community. From the stable isotope mixing models, a subset of species appears to focus on MPB supported off recycled (porewater) N and/or detrital organic matter mainly driven by increased phenylalanine  $\delta^{15}\text{N}$  values. This additional

resource within MPB may play a role in subsidizing the exceptional benthic productivity observed within the Wadden Sea ecosystem and reflect division in MPB support along green (herbivory) and brown (recycled/detrital) food web pathways.

#### KEYWORDS

trophic discrimination, intertidal, diatoms, microbial loop, permeable sands

## Introduction

Tracing the sources and fate of primary productivity as it supports an ecosystem or food web is challenging in coastal ecosystems, and stable isotope techniques have been useful tools that have allowed for further insight into these processes (Fry, 1984; Middelburg et al., 2000; Evrard et al., 2010). As shallow intertidal ecosystems are important sites for both primary production and the interception and reworking of carbon and nitrogen prior to export to shallow seas (Bauer et al., 2013a,b; Chua et al., 2022), it is important to resolve the relative incorporation of these elements into the wider food web. The world's largest intertidal ecosystem (Compton et al., 2013), the Wadden Sea, stretches from Netherlands to Denmark behind a barrier island chain with connection to the North Sea (Postma, 1996). This intertidal ecosystem has been designated as a culturally significant UNESCO World Heritage site due to considerable biodiversity within the system as benthic productivity supports an estimated 10–12 million migratory birds across each year (Reise et al., 2010). The Wadden Sea has a long history of multiple direct impacts from human activity (Eriksson et al., 2010; Wolff, 2013) that includes land reclamation, partial damming and hydraulic changes, eutrophication, overfishing, and extensive dredging for shellfish. These impacts have resulted in a shift from a benthos dominated by seagrass, extensive bivalve reefs, and supporting apex predators toward one dominated by polychaetes, with minimal fringing bivalve reefs, and largely devoid of apex predators (Philippart et al., 2007). Despite historical and recent anthropogenic impacts, the Wadden Sea remains a very productive intertidal ecosystem and direct support of macrozoobenthos by phytoplankton has been identified as a controlling factor of benthic biomass in the Wadden Sea (Beukema et al., 2002). However, changes in higher trophic level species have coincided with shifts in the benthic community as inferred from bird species shifting from primary bivalve carnivores toward polychaete carnivores (Van Roomen et al., 2005; Eriksson et al., 2010) and the drivers of these shifts remain unclear.

Analysis of the stable isotope composition of carbon ( $\delta^{13}\text{C}$ ) and nitrogen ( $\delta^{15}\text{N}$ ) in animal tissues is routinely used to identify underlying resource use and trophic relationships within ecosystems (Minagawa and Wada, 1984; Fry, 1988).

Through the application of trophic discrimination factors (TDFs) for carbon and nitrogen ( $\Delta^{13}\text{C}$  and  $\Delta^{15}\text{N}$ ) stepwise isotopic increases that occur between a consumer's diet and their tissues during metabolism can be accounted for (Post, 2002; McCutchan et al., 2003), allowing for the identification of the animal's trophic level. Accounting for isotopic changes across trophic levels allows for the application of both simple and complex stable isotope mixing models (SIMMs) to be used to resolve the relative use of food resources by consumers within a food web (Fernandes et al., 2014; Stock et al., 2018). Approaches for characterizing resource use using SIMMs have typically been based on either bottom-up or top-down approaches to examine consumer resource use. Bottom-up approaches combine measured dietary resources and clearly defined TDFs to estimate resource use within ecosystems [McCormack et al. (2019) and references within, Cresson et al. (2020) and Kahma et al. (2020)] while top-down approaches rely on identification of unique consumer values within an ecosystem to identify cryptic or under-sampled resources without which the mixing envelope for all consumers in that ecosystem cannot be adequately resolved [Supplementary material 6 and references within Chi et al. (2021) and Then et al. (2021)]. For the Dutch part of the Wadden Sea, Christianen et al. (2017) identified that the majority of the biomass is supported by both pelagic phytoplankton and microphytobenthos (MPB). Their study used a two-source mixing model with a single isotopic tracer ( $\delta^{13}\text{C}$ ) and clearly identified benthic productivity from MPB as an important resource supporting the benthic community. However, they may have overestimated the amount of support due to compression of the MPB end member through the use of an animal proxy instead of directly measured resources (Post, 2002).

Thus far, it has not been possible to assess the trophic structure within the Dutch Wadden Sea benthic community due to the large variability in  $\delta^{15}\text{N}$  values observed in multiple benthic species. Christianen et al. (2015) found clear trends of increased  $\delta^{15}\text{N}$  values for filter feeders (*Mytilus edulis*) in the eastern Dutch Wadden Sea indicating the potential for either a more positive  $\delta^{15}\text{N}$  baseline value or altered trophic structure. Potential causes for locally increased  $\delta^{15}\text{N}$  values could be (1) unaccounted for source of N to the ecosystem (e.g., terrestrial input), (2) altered trophic structure resulting in regionally increased  $\delta^{15}\text{N}$  values in benthic consumers

(Durante et al., 2022), or (3) altered nitrogen cycling, e.g., increased denitrification resulting in increased  $\delta^{15}\text{N}$  values in residual pool of N supporting remineralization (Vokhshoori and McCarthy, 2014). Terrestrial inputs have been found to be limited within the Dutch Wadden Sea (Christianen et al., 2017). Intertidal areas within the German Wadden Sea have been identified as having high rates of denitrification associated with tidal pumping and long exposure times within intertidal sediments (Marchant et al., 2016, 2017, 2018). Increased porewater processing of nitrogen may coincide with the unique distribution of sandy sediments and pore sizes due to the parallel flow of tidal waters through the Wadden Sea basin. These physical attributes result in reverse grading of sediments with coarser grains toward the basin edges and finer sediment centrally deposited along with longer exposure times in the East than in West (Otto et al., 1990; Compton et al., 2013). This combination of unique physical and biogeochemical settings may contribute to increased underlying  $\delta^{15}\text{N}$  values but identifying any use of denitrification-affected N porewater resources by MPB and the food web has remained largely intractable solely using traditional bulk isotope techniques.

Microphytobenthos thrive in unvegetated sandy sediments where they can contribute significantly to the primary production supporting an ecosystem (Miller et al., 1996) as benthic diatoms fix carbon and take up nitrogen from the overlying water column and porewaters (Cook et al., 2007; Oakes et al., 2012; Riekenberg et al., 2020a). Much of the fixed carbon is excreted as extracellular polymeric substances (EPS), which are sticky, sugar-rich substrates that help to stabilize sediment and facilitate diatom motility throughout sand (Goto et al., 1999; Stal, 2010), but also serve as a labile carbon source for heterotrophic bacteria (Taylor et al., 2013). Diatom motility in sandy sediments is an adaptation to the physiochemical variations that occur within intertidal settings. Diel vertical migration of diatoms coincides with tidal cycles and available sunlight to support favorable conditions for photosynthesis at the surface (Barnett et al., 2020) and to maximize nutrient availability for cell growth and division in the subsurface (Saburova and Polikarpov, 2003). Due to the diel vertical migration of several centimeters and a strong coupling between diatoms and heterotrophic bacteria, MPB actively straddle the boundary between water column and sediment porewaters to maximize resource availability.

Food webs are often described as either green or brown depending on whether consumers are supported by herbivory of primary producers or supported by detrital reworking through the microbial loop (Middelburg, 2014; Potapov et al., 2019). MPB-supported food webs blur the line between green (MPB<sub>green</sub>) and brown (MPB<sub>brown</sub>) designations as MPB-derived material that can be used by consumers comes in several forms: (1) newly fixed organic matter from water column nutrients

(MPB<sub>green</sub> throughout), (2) MPB-derived organic matter formed from recycled detrital material by heterotrophs through oxic or anoxic pathways in sediment porewaters, or (3) detrital MPB-derived material such as reworked EPS that is still labile within the sediment (MPB<sub>brown</sub> throughout). Microbial subsidies to MPB of both C and N from heterotrophic bacterial reworking of organic matter in porewaters blur the line between primary production and detrital resource use. Consumers such as deposit feeders have access to MPB biomass (living or dead) and EPS made using a mixture of resources derived directly from the water column or from heterotrophic processing within porewaters (e.g., denitrification affected N pools). This biogeochemical complexity can make identification of carbon and nitrogen food web support from MPB difficult to identify using bulk isotope techniques.

Application of compound-specific stable isotope analysis techniques (e.g., analysis of individual amino acid  $\delta^{15}\text{N}$  values) could allow for identification of the trophic structure of the benthic community in the Wadden Sea without requiring measurement of all primary producers and all N sources within the ecosystem. This is because amino acid  $\delta^{15}\text{N}$  values provide additional information for each sample analyzed in comparison with traditional bulk analysis due to the different fractionations that occur during metabolic reworking of trophic and source amino acids (O'Connell, 2017). Trophic amino acids such as glutamic acid (Glu) fractionate considerably between diet and the consumer resulting in a TDF ranging from 3–3.8‰ in marine mammals and sharks (Whiteman et al., 2018; Ruiz-Cooley et al., 2021) to as high as 11‰ in teleost fish fed a diet of low protein quality (McMahon et al., 2015b).  $\delta^{15}\text{N}$  values for source amino acids such as phenylalanine (Phe) are relatively preserved as they are processed throughout food webs due to a low and consistent TDF of ~0–0.5‰ (Chikaraishi et al., 2009; McMahon et al., 2015a). Integration of source amino acid values into trophic level estimates removes the effects from baseline N variations across an ecosystem using a single chemical analytical method (Vokhshoori et al., 2019; Xing et al., 2020) instead of requiring multiple comparisons and adjustments using resource or primary consumer measurements.

In this study, we apply natural abundance stable isotope analysis of amino acid nitrogen to resolve the trophic structure for 28 species from within the Dutch part of the Wadden Sea benthic ecosystem, which was previously not possible using solely bulk isotope values. Through the application of this amino acid-based method, it should be possible to further investigate the widespread nature of the driver causing increased variability in  $\delta^{15}\text{N}$  across the ecosystem, which we have postulated as potentially being either caused by (1) changed food web complexity, (2) widespread variability in underlying biogeochemical processes (denitrification), or (3) unique sources such as freshwater inflow or groundwater influx coming from terrestrial sources.

## Materials and methods

### Study site

The Wadden Sea is a large intertidal ecosystem formed behind a chain of 12 sand barrier islands that stretch from the Netherlands to Denmark (Christianen et al., 2017). It is an highly productive estuarine ecosystem formed of sedimentary tidal flats that receive direct and indirect terrestrial inputs from the Ems, Weser, Elbe, IJssel, Muse, and Rhine rivers (Eriksson et al., 2010). Extensive long-term sampling of the benthic community has occurred in the Dutch Wadden Sea with spatial coverage extending from the Marsdiep (52° 58' 12" N, 4° 44' 24" E) to the Ems River mouth at the border of Germany (53° 19' 48" N, 7° 1' 12" E; Figure 1 and Supplementary Figure 1).

### Sampling

Sampling for macrozoobenthic species occurred between June and October of 2011–2014 as part of a yearly spatially extensive long-term monitoring campaign within the Dutch Wadden Sea (SIBES, Synoptic Intertidal Benthic Survey). The SIBES program performs core sampling of the intertidal mudflats either by foot or with the use of a rigid hulled inflatable boat, with the aim of comprehensive identification and collection of benthic species in a gridded pattern (500 m separation) throughout the Dutch Wadden Sea that are spatially randomly sampled across the sampling season to minimize the possibility of temporal effects between the years with additional random sample points that result in ~4,500 samples per year (Bijleveld et al., 2012; Compton et al., 2013). Samples were sieved using 1-mm mesh size and all retained animals were stored frozen after collection. Identification and dissection was done either aboard the ship or in the laboratory. In addition, samples of the filter feeding community (e.g., *Mytilus edulis* and *Balanus crenatus*) were obtained from buoys in the main tidal channels in 2014 to constrain regional N variability throughout the Dutch Wadden Sea. Encrusting communities were scraped from the sides of free-standing anchored buoys and were collected, dissected, and frozen on board (Christianen et al., 2015). Fish were collected from long-term monitoring efforts from the NIOZ Fyke. For all sampling campaigns, samples of muscle tissue (fish, crustaceans, and bivalves), soft tissue (invertebrates), or whole animals (smaller specimens) were freeze-dried, homogenized, and placed in sample vials prior to bulk tissue or amino acid stable isotope analysis. Species were selected for bulk stable isotope analysis based on their contribution to total biomass within the Dutch Wadden Sea ecosystem (Christianen et al., 2017), with 35 species contributing 99% of the total benthic biomass for the system. From these samples, 28 species (25 consumers, three macroalgae) were selected for amino

acid isotope analysis based on tissue availability (>3 mg), spatial representation across the Dutch Wadden Sea, and adequate replication (>5 individuals) within the data set. Also samples for particulate organic matter (POM) were collected and filtered through combusted GFF filters (Whatman). MPB were filtered onto GFF filters after migration into combusted sand through a 100- $\mu$ m mesh (Eaton and Moss, 1966). This method will preferentially select for motile (epipellic) diatoms vs. sessile (episammic) diatoms. Additional sampling details for the SIBES campaigns can be found in Bijleveld et al. (2012), Compton et al. (2013), and Christianen et al. (2017).

### Isotope analyses

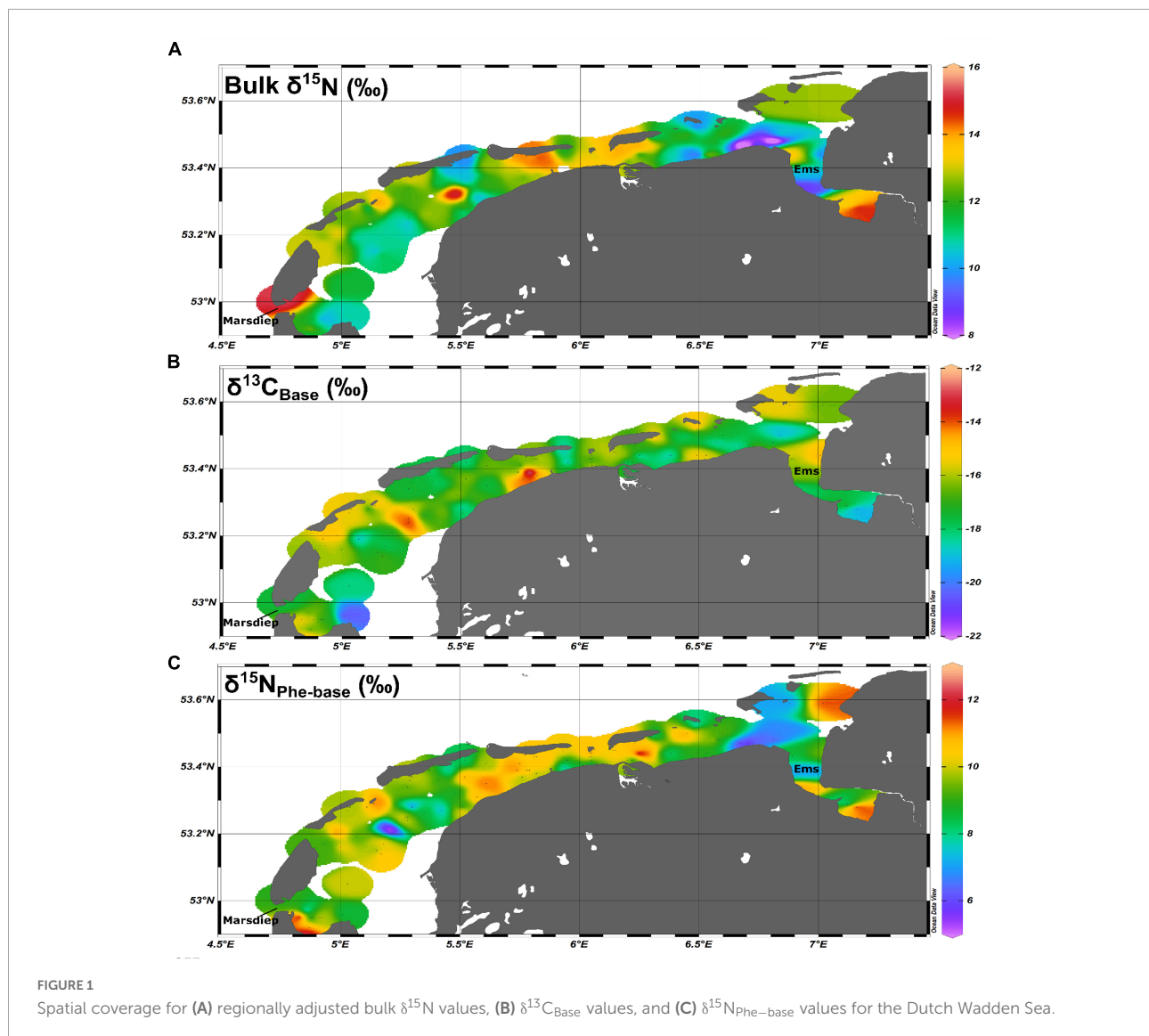
For bulk stable isotope analysis of  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values, 0.4–2 mg of freeze-dried, homogenized, and non-lipid extracted animal tissue was loaded into tin capsules. Because the presence of inorganic carbonates can greatly influence  $\delta^{13}\text{C}$  values (Androuin et al., 2019; Sturbois et al., 2022), samples were initially analyzed without acidification for  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  values and then re-analyzed after acidification for a  $\delta^{13}\text{C}$  value of the organic matter in tissue if (1) the animal has a shell, (2) if the animal is in contact with sediment, or (3) if C/N ratios were higher than eight. Acidification was performed through an initial addition of two drops of 2N hydrochloric acid, gentle shaking to mix, allowing time for bubbling to evolve (15 min), and then adding two more drops and gently shaking until the addition of acid does not cause bubbling. After a final addition of a few drops of acid, sample vials were then shaken overnight, repeatedly rinsed with double distilled water to a pH of five, and then freeze-dried to completely remove any remaining acid prior to analysis. Samples were analyzed with a Flash 2000 elemental analyzer coupled to a Delta V Advantage isotope ratio mass spectrometer (Thermo Scientific, Bremen, Germany). Stable isotope ratios are expressed using the  $\delta$  notation in units per mil:

$$\delta(\%) = ((R_{\text{sample}}/R_{\text{standard}}) - 1) \times 1000, \text{ where}$$

$$R = {}^{13}\text{C}/{}^{12}\text{C} \text{ or } {}^{15}\text{N}/{}^{14}\text{N} \quad (1)$$

respectively, reported relative to Vienna Pee Dee belemnite and atmospheric  $\text{N}_2$ . Certified laboratory standards of acetanilide, urea, and casein with known  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values and known %TOC and %TN values calibrated against NBS-22 and IAEA-N1 were used for calibration with each sample run. Average precision for standards and replicate samples was  $\pm 0.1\%$  for  $\delta^{13}\text{C}$  and  $0.2\%$  for  $\delta^{15}\text{N}$ .

For analysis of  $\delta^{15}\text{N}$  values in individual amino acids, 2–5 mg of tissue was hydrolyzed and derivatized to



*N*-pivaloyl/isopropyl (NPIP) derivatives. Analysis occurred using two different gas chromatography combustion isotope ratio mass spectrometers with similar ramp schedules, flow rates, and combustion temperatures measured at the NIOZ Royal Netherlands Institute for Sea Research between 2012 and 2019. ~90% of the samples were analyzed in duplicate with a Trace 1310 gas chromatograph coupled to a Delta V advantage isotope ratio mass spectrometer through an IsoLink II using a modified version of the amino acid preparation and analysis method used by Chikaraishi et al. (2007) that is described in further detail in Riekenberg et al. (2020b). From this analysis, we report 12 amino acid  $\delta^{15}\text{N}$  values including alanine, aspartic acid, Glu, glycine, isoleucine, leucine, lysine, methionine, Phe, serine, threonine, tyrosine, and valine with a precision for samples and standards of  $<\pm 0.5\%$ .

Due to the conditions during hydrolysis, the terminal amines contained in glutamine and asparagine are cleaved thereby converting to glutamine to glutamic acid and asparagine to aspartic acid; so Glu represents a pool of combined material in this work. The remaining 10% of the samples were analyzed in duplicate on an Agilent 6890 gas chromatograph coupled to a Delta V advantage isotope ratio mass spectrometer through a combustion III interface using the method presented in Svensson et al. (2016) reporting five amino acids (alanine, glycine, norleucine, Glu, and Phe) with an average precision for standards and samples of  $\pm 1\%$ . We, therefore, present only Glu and Phe for the 338 amino acid analyses presented here due to the reduced precision and reduced number of reported amino acids present in 10% of the data set.



## Trophic-level calculations

Trophic levels were estimated using single trophic and source amino acids (Glu and Phe, respectively) with the equation presented in Chikaraishi et al. (2009) modified with an ecosystem-specific TDF and  $\beta$  following Bradley et al. (2015) to account for the range of TDFs present from primary producers to tertiary consumers within the benthic community.

$$\text{Trophic level} = (\delta^{15}\text{N}_{\text{Glu}} - \delta^{15}\text{N}_{\text{Phe}} - \beta) / \text{TDF} + 1 \quad (2)$$

where TDF, the stepwise increase in  $\delta^{15}\text{N}$  value between a consumer and their diet, is 4.9‰ and  $\beta$ , the difference between Glu and Phe in the ecosystem's primary producers, is 4.3‰. These values are obtained from the slope and intercept of the linear relationship between the difference between  $\delta^{15}\text{N}$  values for Glu and Phe and trophic position as determined by stomach content and ecological observation. This relationship was then adjusted to place primary producers at the intercept of 0 using a re-arranged version of Equation 2 as further described in Bradley et al. (2015):

$$\Delta_{\text{Glu-Phe}} = (TP_{\text{SCA}} - 1) * \text{TDF} + \beta \quad (3)$$

where  $\Delta_{\text{Glu-Phe}}$  is the measured isotopic difference between Glu and Phe for each species and  $TP_{\text{SCA}}$  is the trophic level as indicated by stomach content analysis or ecological determination of direct feeding and was determined for each species using FishBase for fish species (Froese and Pauly, 2000), literature determinations for invertebrates (Christianen et al., 2015, 2017; Borst et al., 2018), and estimation through interpolation of centroid placement for the remaining species relative to ecosystem trends (Table 1).

Adjustment to account for the small trophic increase in  $\delta^{15}\text{N}$  values of the source AA phenylalanine to establish baseline  $\delta^{15}\text{N}$  values for individual species was calculated as:

$$\delta^{15}\text{N}_{\text{Phe-Base}} = \delta^{15}\text{N}_{\text{Phe}} - (0.2 * (\text{trophic level} - 2)) \quad (4)$$

which accounts for the small increase observed in the  $\delta^{15}\text{N}_{\text{Phe}}$  (‰) value that is associated with consumer fractionation of diet during metabolism of Phe (0–0.4‰) (Chikaraishi et al., 2009; McMahon and McCarthy, 2016) and trophic level is the trophic level estimate for each individual species. Correction for the trophic increase in bulk  $\delta^{13}\text{C}$  values was calculated as:

$$\delta^{13}\text{C}_{\text{Base}} = \delta^{13}\text{C}_{\text{Bulk}} - (0.5 * (\text{trophic level} - 2)) \quad (5)$$

where 0.5 is the increase observed in bulk  $\delta^{13}\text{C}$  values between diet and consumer. This value falls between the TDF values observed for bulk  $\delta^{13}\text{C}$  for whole tissue from consumers ( $0.3 \pm 0.1\text{‰}$ ) and consumer muscle tissues ( $1.3 \pm 0.3\text{‰}$ )

(McCutchan et al., 2003). Due to the wide range of animals included in this analysis ranging from invertebrate primary consumers to teleost tertiary consumers, we have found it appropriate to use a single intermediate TDF value for bulk  $\delta^{13}\text{C}$  for all consumers across the trophic structure.

## Data analysis

Statistical analyses were performed in R (v 4.0.3) using RStudio (v 1.3.1056), OriginLab 2020b, or in Ocean Data View (5.6.0) (Schlitzer, 2022). Mixing models were performed using Food Reconstruction Using Isotope Transferred Signals (FRUITS v 2.1) and the MixSIAR package in R (Stock et al., 2018). We first used FRUITS to model the species means against measured values for end members (Tables 1, 2) for two sources (POM and MPB global averages) using  $\delta^{13}\text{C}_{\text{Base}}$  values only and then three sources (POM, MPB<sub>green</sub>, and MPB<sub>brown</sub>) using  $\delta^{13}\text{C}_{\text{Base}}$  and  $\delta^{15}\text{N}_{\text{Phe-Base}}$  values with SD values set to 0.2‰ for  $\delta^{13}\text{C}$  and 0.5‰ for  $\delta^{15}\text{N}$  reflecting global measurement errors. Both MPB<sub>green</sub> and MPB<sub>brown</sub> end members from minimum and maximum bulk  $\delta^{15}\text{N}$  values for MPB and POM are the average measured during the sampling campaigns. Models in FRUITS were run using 50,000 updates, a burn-in of 10,000 and a minimum uncertainty of 0.001. We then proceeded to modeling individuals within species groupings in MixSIAR using end member values for  $\delta^{15}\text{N}_{\text{Phe}}$  that allowed for the resolution of individuals within the mixing envelope between end members (Table 2b) with minimal exclusion of consumer data points (4% excluded,  $n = 302$  remaining). For individual models using three source end members, values for N were expanded for both MPB-derived end members with  $\delta^{15}\text{N}$  values of 5.0‰ for MPB<sub>green</sub> and 13.0‰ for MPB<sub>brown</sub> taken from values observed for  $\delta^{15}\text{N}_{\text{Phe-Base}}$  for individuals from *Peringia ulvae* (Cone mudsnail) and *Littorina littorea* (Periwinkle). Models in MixSIAR were run using the “long” setting, where three Markov chain Monte-Carlo algorithms with a length of 300,000 run with a burn-in of 200,000. Results are presented from the species averages along with correlation indices comparing the two modeling approaches. Additional details about model fit are provided in the Supplementary material.

## Results

Bulk  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values and standard errors (SE) for the 28 species included in this study are listed in Table 1 and bulk  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values and standard errors (SE) for resources identified as supporting the ecosystem are listed in Table 2a. We confirmed that bulk  $\delta^{15}\text{N}$  values were increased for samples taken from the Ems-Dollard region (eastern Dutch Wadden Sea) in comparison with other regions in the Dutch Wadden Sea and have now adjusted for this regionally observed increase in

**TABLE 1**  $\delta^{15}\text{N}$  values for bulk material, glutamic acid, and phenylalanine along with  $\delta^{13}\text{C}$  values, trophic position from stomach content and trophic position calculated from amino acids (AAs) for the subset of 25 consumers and three macroalgae species examined in this study sampled from the Dutch Wadden Sea benthic food web during SIBES and Waddenseutels campaigns spanning from 2011 to 2014 and in the NIOZ Fyke during routine monitoring.

Species name	Common name	<i>n</i>	$\delta^{15}\text{N}$ (%)	SE	$\delta^{13}\text{C}$ (%)	SE	Trophic position stomach content	Trophic position from AAs	SE	Glutamic acid $\delta^{15}\text{N}$ (%)	SE	Phenylalanine $\delta^{15}\text{N}$ (%)	SE	Glu-phe $\delta^{15}\text{N}$ (%)	SE
<i>Arenicola marina</i>	Lugworm	14	12.8	0.4	-16.0	0.5	1.9	2.1	0.1	21.0	0.7	11.2	0.5	9.8	0.4
<i>Balanus crenatus</i>	Acorn barnacle	13	12.5	0.2	-17.4	0.4	1.9	2.4	0.2	20.7	1.0	9.3	0.4	10.9	0.8
<i>Carcinus maenas</i>	Green crab	18	14.0	0.3	-15.8	0.2	3	3.2	0.1	24.2	0.7	8.9	0.3	14.5	0.6
<i>Cerastoderma edule</i>	Common cockle	21	11.0	0.4	-18.8	0.1	1.8	2.2	0.1	20.1	0.6	9.8	0.5	10.0	0.5
<i>Chrysaora hysoscella</i>	Compass jellyfish	8	13.4	0.3	-19.2	0.5	3.4	3.5	0.1	26.7	0.3	10.4	0.6	15.8	0.5
<i>Clupea harengus</i>	Herring	10	14.6	0.2	-18.0	0.3	3.4	3.4	0.0	23.6	0.7	7.4	0.7	15.3	0.2
<i>Crangon crangon</i>	Shrimp	17	13.6	0.3	-14.9	0.3	3.2	3.5	0.1	26.2	0.4	9.7	0.4	15.7	0.5
<i>Magallan gigas</i>	Pacific oyster	13	12.3	0.3	-17.9	0.2	1.8	2.2	0.1	20.0	0.6	9.6	0.3	10.2	0.6
<i>Crepidula fornicata</i>	Slipper limpet	8	10.5	0.3	-17.4	0.1	2	1.7	0.1	17.1	0.3	9.0	0.5	7.9	0.3
<i>Dicentrarchus labrax</i>	European bass	10	15.9	0.5	-15.9	0.5	3.5	4.0	0.2	27.8	0.6	8.3	0.3	17.9	0.7
<i>Ensis directus</i>	Razor clam	9	9.9	0.4	-17.8	0.3	2	1.2	0.1	14.0	0.3	8.3	0.4	5.5	0.3
<i>Pomatoschistus</i> sp.	Goby	8	14.6	0.3	-14.9	0.5	3.2	3.6	0.1	24.7	0.4	8.8	0.3	15.9	0.4
<i>Hediste diversicolor</i>	Ragworm	17	12.3	0.4	-16.5	0.3	2.6	2.6	0.1	20.1	0.7	7.8	0.3	11.6	0.6
<i>Hydrobia ulvae</i>	Cone mudsnail	10	9.2	0.3	-15.1	0.8	2	1.4	0.1	15.3	0.5	8.2	0.7	6.7	0.5
<i>Lanice conchilega</i>	Sand mason worm	11	11.1	0.5	-18.1	0.3	1.8	2.2	0.1	19.3	0.7	9.1	0.5	9.9	0.6
<i>Liocarcinus holsatus</i>	Flying crab	7	13.7	0.4	-18.1	0.5	3	3.3	0.1	26.5	0.4	11.7	0.6	14.8	0.3
<i>Littorina littorea</i>	Periwinkle	17	11.5	0.3	-14.5	0.4	2.3	2.3	0.1	20.7	0.4	9.8	0.2	10.6	0.4
<i>Macoma balthica</i>	Baltic clam	20	11.9	0.3	-16.2	0.2	1.8	1.7	0.1	18.5	0.6	10.1	0.4	7.8	0.5
<i>Mytilus edulis</i>	Blue mussel	30	11.1	0.3	-18.6	0.2	2	1.7	0.1	17.2	0.3	8.7	0.3	8.1	0.3
<i>Osmerus eperlanus</i>	European smelt	8	16.4	0.4	-17.2	1.0	3.5	3.8	0.1	27.0	0.5	9.4	0.4	17.1	0.3
<i>Platichthys flesus</i>	European flounder	8	15.1	0.5	-14.5	0.4	3.5	3.4	0.1	25.7	0.7	9.9	0.3	15.3	0.4
<i>Pleuronectes platessa</i>	European plaice	10	13.4	0.3	-15.6	0.3	3.2	3.2	0.1	22.7	0.5	7.4	0.5	14.2	0.4
<i>Rhizostoma pulmo</i>	Barrel jellyfish	7	13.0	0.3	-20.5	0.9	3	2.9	0.2	23.5	0.6	10.1	0.3	13.1	0.7
<i>Solea solea</i>	Flatfish	11	14.8	0.2	-16.8	0.3	3.2	3.0	0.1	25.5	0.6	11.4	0.5	13.6	0.6
<i>Zoarcis viviparus</i>	Eelpout	8	15.9	0.2	-15.0	0.2	3.5	3.8	0.1	27.6	0.5	10.4	0.3	16.8	0.4
<i>Ceramium rubrum</i>	Red horn weed	8	11.6	0.3	-18.4	0.9	1.5	1.7	0.1	14.5	0.7	6.6	0.8	7.7	0.3
<i>Fucus vesiculosus</i>	Bladderwrack	8	8.0	0.9	-15.3	0.6	1	0.3	0.1	12.1	0.8	10.3	0.8	1.8	0.3
<i>Ulva</i> sp.	Sea lettuce	8	11.1	0.7	-13.5	0.6	1	0.8	0.1	15.7	0.6	11.5	0.4	4.1	0.4

TABLE 2 Bulk  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  values for the end member values supporting the Dutch Wadden Sea benthic food web used for two and three source mixing models on species means.

End members	<i>n</i>	$\delta^{15}\text{N}$ (%)	SE	$\delta^{13}\text{C}$ (%)	SE
Particulate organic matter	101	8.6	0.1	-21.2	0.1
Microphybenthos average	95	9.2	0.2	-13.4	0.2
Microphybenthos green	20	7.3	0.2	-14.2	0.3
Microphybenthos brown	25	11.3	0.3	-13.4	0.2
End members		$\delta^{15}\text{N}$ (%)	SE	$\delta^{13}\text{C}$ (%)	SE
Particulate organic matter		8.6	0.1	-21.2	0.1
Microphybenthos average		9.2	0.2	-13.4	0.2
Microphybenthos green		5.0		-14.2	0.3
Microphybenthos brown		13.0		-13.4	0.2

bulk  $\delta^{15}\text{N}$  values for filter feeders [ $-2.3\text{‰}$ ; One-way ANOVA,  $F_{(2,43)} = 6.8$ ,  $p = 0.003$ ; [Supplementary Table 1](#)]. Adjustment of  $\delta^{15}\text{N}$  values was identified as necessary in both *M. edulis* (blue mussel) and *B. crenatus* (acorn barnacle) from samples pooled into regional categories within the Wadden Sea (West  $< 5.5^\circ\text{E}$ , East  $> 5.5^\circ\text{E}$ , and Ems-Dollard associated with the mouth of the river Ems). Despite application of a regional adjustment, ranges for bulk  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values remained large for consumers ([Figures 1A,B](#)) as well as for POM and MPB sampled from across the Dutch Wadden Sea (POM  $-15.4$  to  $-21.7\text{‰}$ ;  $13.7$  to  $5.7\text{‰}$  and MPB  $-10.4\text{‰}$  to  $-16.2\text{‰}$ ;  $13.6$  to  $6.5\text{‰}$ ).

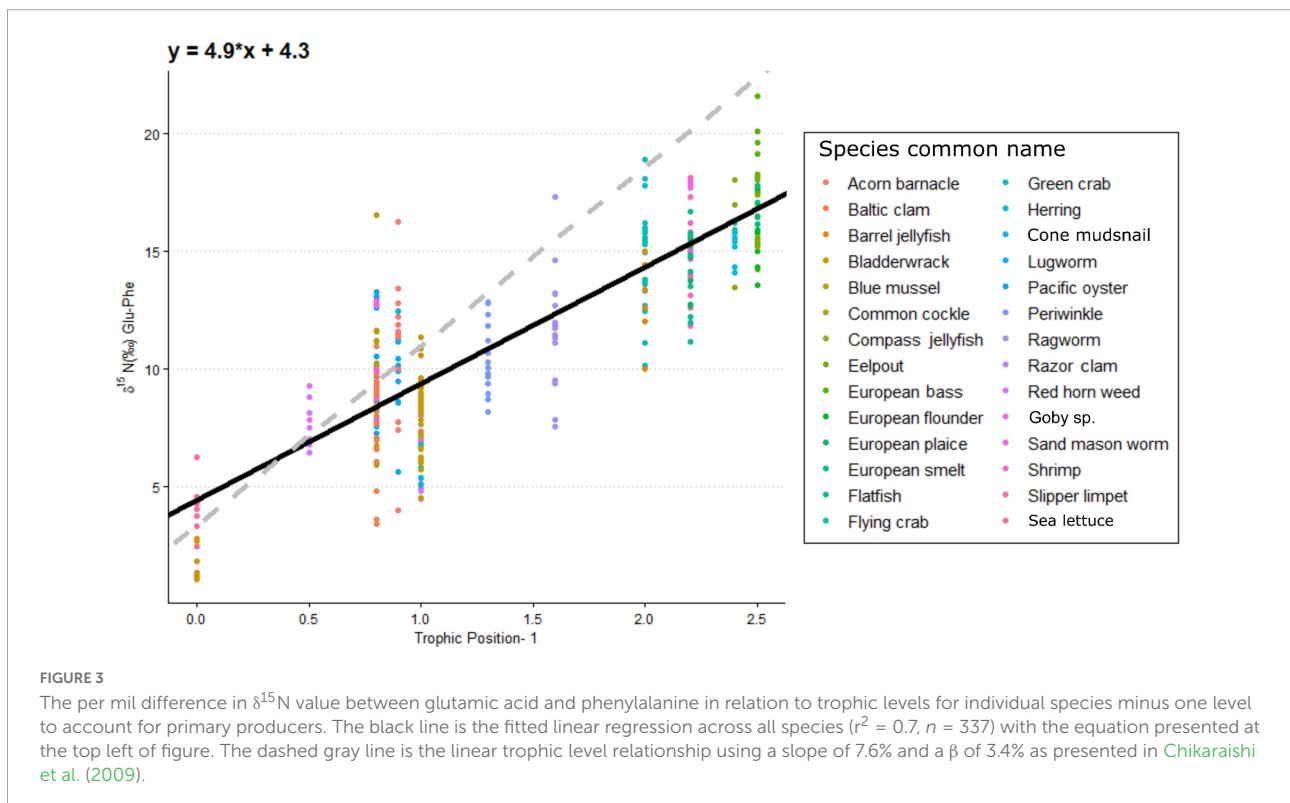
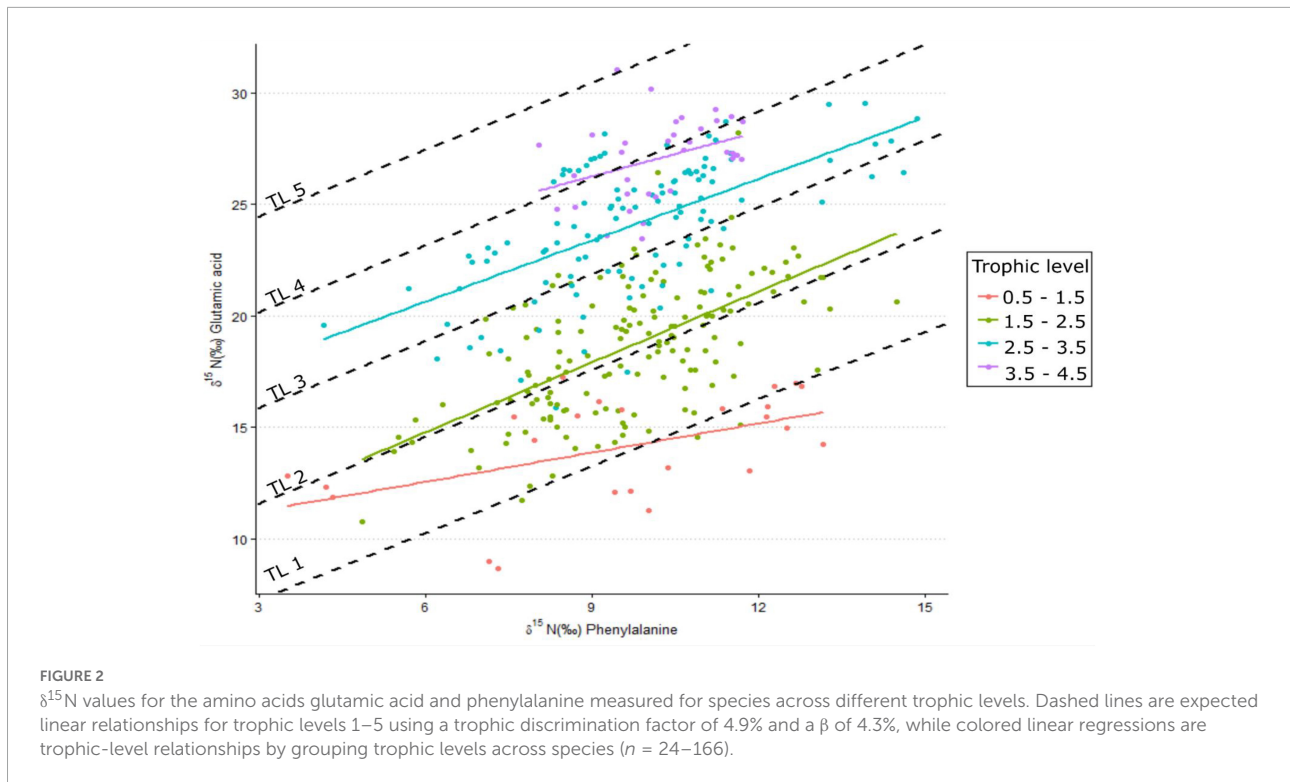
Increased  $\delta^{15}\text{N}_{\text{Phe}}$  values were observed for filter feeders sampled from Ems-Dollard in comparison with the rest of the Dutch Wadden Sea and values were adjusted in a similar manner as the bulk  $\delta^{15}\text{N}$  values [ $-2.1\text{‰}$ ; One-way ANOVA,  $F_{(2,43)} = 5.7$ ,  $p = 0.006$ ; [Supplementary Table 1](#)]. Values for  $\delta^{15}\text{N}_{\text{Phe}}$  ranged from  $6.6\text{‰}$  observed for *Ceramium vigatum* (Red horn weed) to  $11.7\text{‰}$  for *Liocarcinus holsatus* (Flying crab) and mean values for species correspondingly increased with larger Glu-Phe differences reflecting the minor trophic increases expected for source amino acids ([Table 1](#)).  $\delta^{15}\text{N}_{\text{Glu}}$  values ranged from  $12.1\text{‰}$  for *Fucus vesiculosus* (Bladderwrack) to  $27.8\text{‰}$  for *Dicentrarchus labrax* (European bass) and were higher than  $\delta^{15}\text{N}_{\text{Phe}}$  values as expected for the larger fractionation associated with trophic amino acids.  $\delta^{15}\text{N}$  values for Glu and Phe were correlated across trophic levels (Pearson's correlation coefficient; [Figure 2](#); TL 1  $R^2 = 0.26$   $n = 24$ ,  $p < 0.01$ ; TL 2  $R^2 = 0.37$   $n = 166$ ,  $p < 0.001$ ; TL 3  $R^2 = 0.39$   $n = 96$ ,  $p < 0.002$ ; TL 4  $R^2 = 0.14$   $n = 34$ ,  $p = 0.03$ ), but the relationships were weaker for both TL 1 and TL 4 when compared to TL 2 and TL 3. The relationship between  $\Delta_{\text{Glu-Phe}}$   $\delta^{15}\text{N}$  values and  $\text{TP}_{\text{SCA}} - 1$  had a strong correlation ([Figure 3](#);  $n = 337$ ,  $R^2 = 0.7$ ,  $p < 0.001$ ) with a slope of  $4.9 \pm 0.3\text{‰}$  and an intercept of  $4.3 \pm 0.2\text{‰}$  that reflect TDF and  $\beta$ , respectively, for the Dutch Wadden Sea benthic food web. These values for TDF and  $\beta$  are different than the canonical values of  $7.6$  and  $3.4\text{‰}$  found by [Chikaraishi et al. \(2009\)](#)

and resulted in a slope of  $0.99$  for TL vs.  $\text{TP}_{\text{SCA}}$  while using the canonical TDF and  $\beta$  resulted in a slope of  $0.64$  (both  $R^2 = 0.72$ ).

Calculated trophic levels for primary producers ranged from  $0.3$  for *Fucus vesiculosus* (Bladderwrack) to  $1.7$  for *C. rubrum* ([Figure 4](#)). Primary consumers ranged from  $1.2$  for *Ensis leii* (Razor clam) to  $2.3$  for *Littorina littorea* (Periwinkle), secondary consumers from  $2.6$  for *Hediste diversicolor* (Ragworm) to  $3.5$  for *Crangon crangon* (shrimp) and tertiary consumers from  $3.6$  for *Pomatoschistus* sp. (Goby sp.) to  $4$  for *D. labrax* (European bass, [Figure 4](#)). No amino acid measurements were possible for POM or MPB as part of this study as the sample weights remaining after the initial bulk analysis between 2012 and 2015 were below detection limits for this analysis. Consumer  $\delta^{13}\text{C}$  values fell between the measured values for POM and MPB ( $-21.2$  and  $-13.4\text{‰}$ ). We therefore used the trophic level estimates calculated using system-specific TDF and  $\beta$  to account for fractionation while estimating values for  $\delta^{13}\text{C}_{\text{Base}}$  and  $\delta^{15}\text{N}_{\text{Phe-base}}$ .

We then used  $\delta^{13}\text{C}_{\text{Base}}$  values from POM and MPB ([Table 2](#)) and each consumer to calculate dietary contributions for species averages ([Figure 5A](#)) and for all individuals within each species. Dietary contributions from these two source mixing models are presented in [Supplementary Table 2A](#) and indicated use of POM ( $>0.5$ ) for 11 of the 25 consumer species examined with the highest contribution being observed for *Rhizostoma pulmo* (Barrel jellyfish,  $0.86 \pm 0.09$  and  $0.76 \pm 0.04$ ). To further investigate the use of MPB within the ecosystem, we used  $\delta^{13}\text{C}_{\text{Base}}$  and  $\delta^{15}\text{N}_{\text{Phe-Base}}$  values from POM,  $\text{MPB}_{\text{green}}$ , a proxy of freshly fixed MPB-derived material, and  $\text{MPB}_{\text{brown}}$ , a proxy of MPB-derived material using reworked N and OM associated with heterotrophic reworking of detrital OM and denitrification occurring across the tidal cycle ([Table 2b](#)), to calculate dietary contributions from species average values ([Figure 5B](#)). Similar to the two source models, dietary contribution





from POM was considerable ( $>0.5$ ) for 11 of the 25 consumer species with *R. pulmo* (Barrel jellyfish) having the highest contribution ( $0.78 \pm 0.1$ ). For the two MPB end

members, dietary contribution of  $\text{MPB}_{\text{green}}$  was considerable for six species with *Pleuronectes platessa* (European plaice) having the highest contribution ( $0.75 \pm 0.1$ ) and dietary

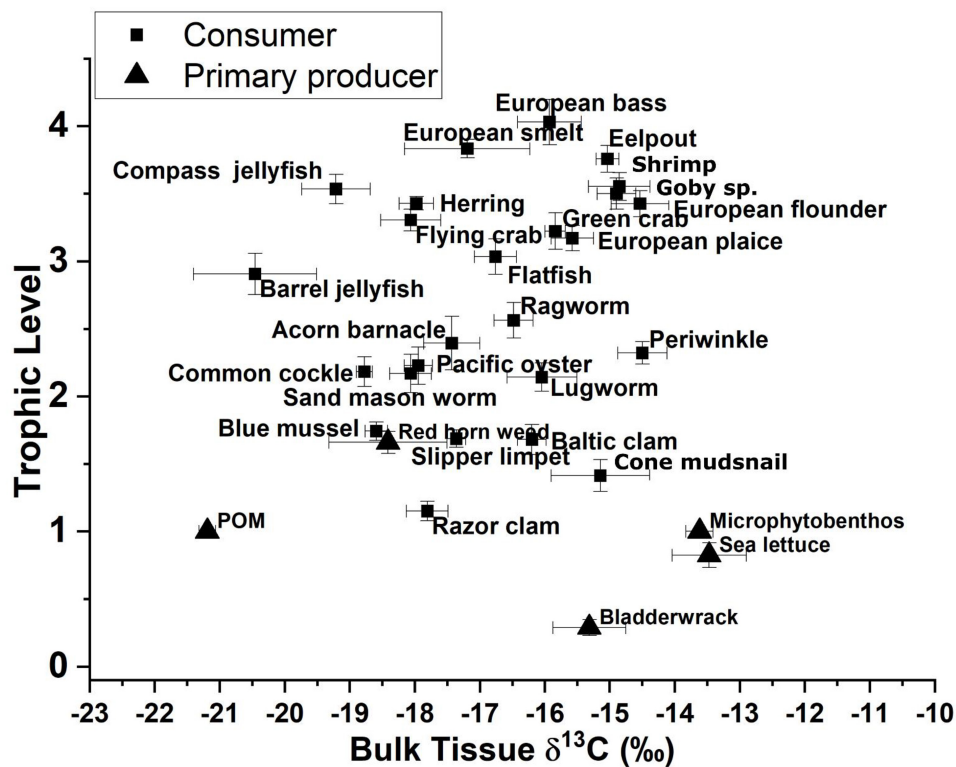


FIGURE 4

Trophic-level estimates from amino acid  $\delta^{15}N$  values for glutamic acid and phenylalanine vs. bulk  $\delta^{13}C$  values for both consumers and primary producers.

contribution from  $MPB_{brown}$  was considerable for five species with *Zoarcetes viviparus* (Eelpout) having the highest contribution ( $0.57 \pm 0.13$ ). Dietary contributions from POM were  $> 0.5$  for 10 species with *Chrysaora hysoscella* (Compass jellyfish) having the highest contribution ( $0.78 \pm 0.04$ ), while contributions for  $MPB_{green}$  were lower with no species  $> 0.5$ , but with four species having a contribution of  $> 0.4$  with *H. diversicolor* (Ragworm) having the highest contribution ( $0.47 \pm 0.05$ ), and contributions for  $MPB_{brown}$  being  $> 0.5$  for six species with *Arenicola marina* (Lugworm) having the highest contribution ( $0.67 \pm 0.05$ ). Both two-source and three-source mixing models indicated good agreement between the use of POM with a correlation between the models using species averages and individual values of 0.88 and 0.89, respectively ( $R^2$ , Figure 5C).

## Discussion

In the Dutch Wadden Sea, we found that using amino acid  $\delta^{15}N$  values allowed for more detailed and reliable identification of the trophic structure of the benthic food web than possible in previous work using solely bulk isotope methodologies. This improvement allowed for the identification

of a subset of species that rely on microbially reworked materials supported through active tidal pumping. Across all four simple and complex SIMMs, mean consumer reliance on MPB was 0.55–0.6, but fell short of the 0.7 previously assessed (Christianen et al., 2017). This smaller contribution from MPB is likely due to the combined use of  $\delta^{13}C$  and  $\delta^{15}N$  values to assess resource contributions instead of using consumer integrators as a proxy for MPB resource values (Post, 2002). Additionally, analysis of individual amino acid  $\delta^{15}N$  values allowed for trophic adjustment of  $\delta^{13}C$  and  $\delta^{15}N$  values to account for trophic discrimination for the 25 benthic consumer species prior to analysis with SIMMs. Through the application of source amino acids, we were able to construct a trophic structure within the Dutch Wadden Sea despite the considerable underlying variability present in bulk  $\delta^{15}N$  values across the area (Figure 1A). This variability was potentially caused by widespread biogeochemical processing in porewaters unique to regions of intense tidal flushing within intertidal sands (e.g., denitrification). Analysis of trophic and source amino acid  $\delta^{15}N$  values allowed for the development and application of a system-specific TDF and  $\beta$  to determine the trophic structure of the Wadden Sea benthic food web that was previously impossible using solely bulk isotopic techniques.

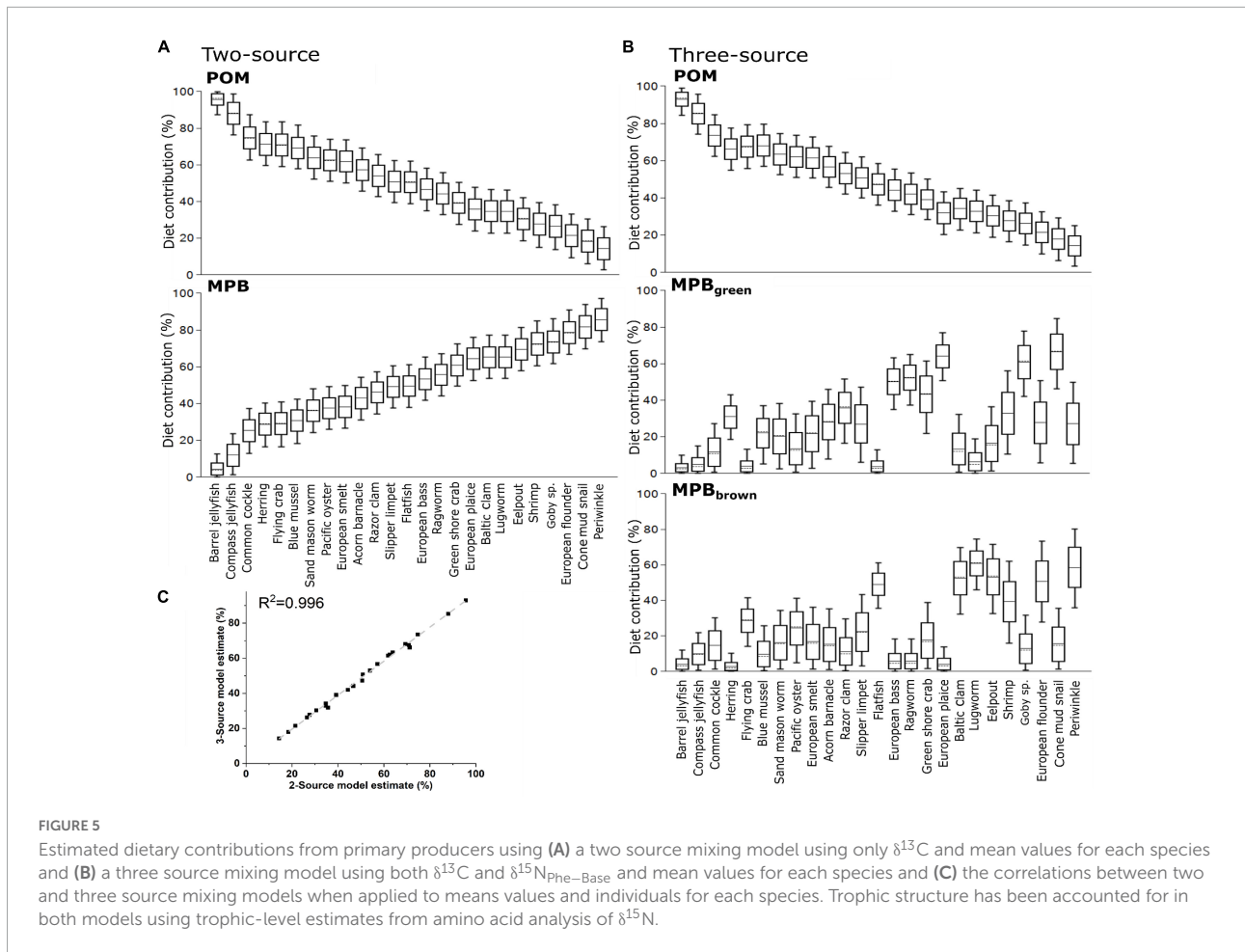


FIGURE 5

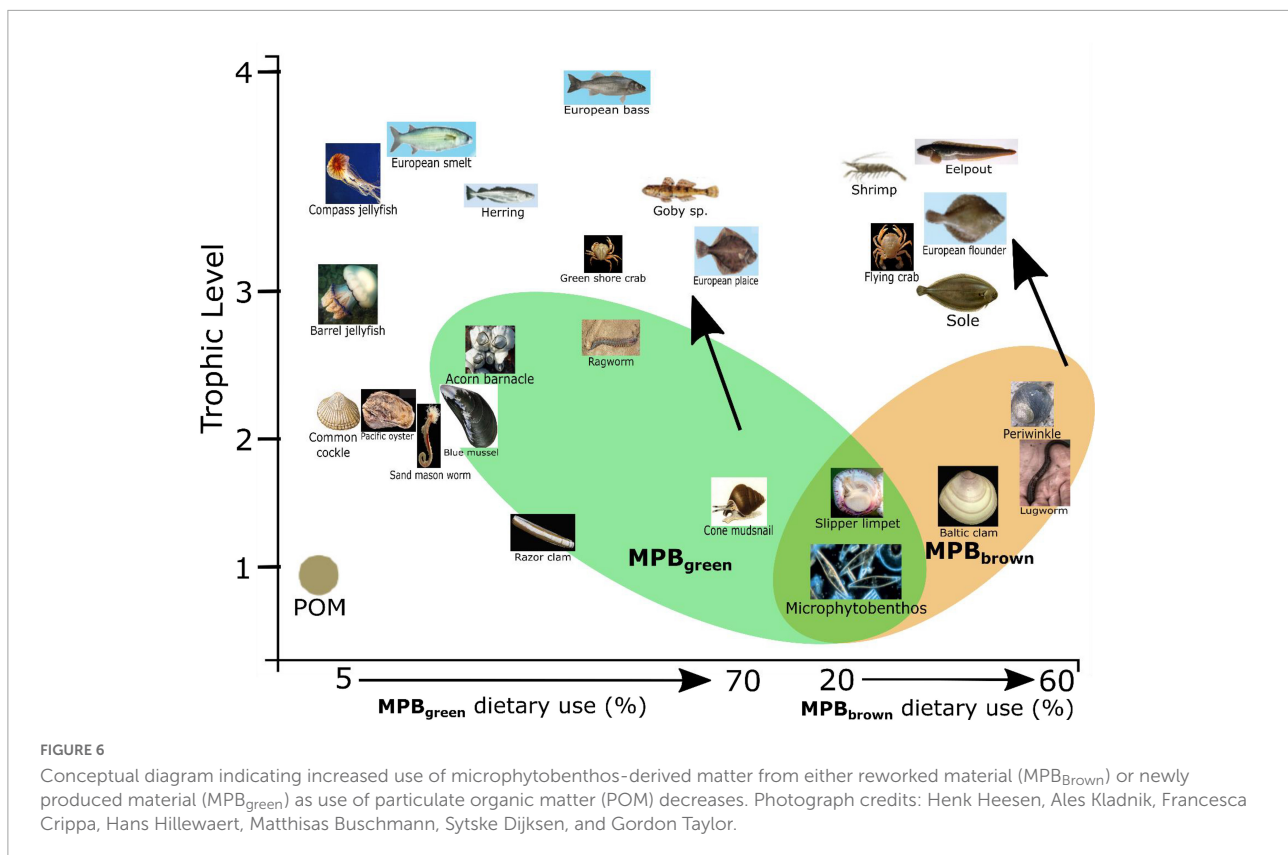
Estimated dietary contributions from primary producers using (A) a two source mixing model using only  $\delta^{13}\text{C}$  and mean values for each species and (B) a three source mixing model using both  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}_{\text{Phe-Base}}$  and mean values for each species and (C) the correlations between two and three source mixing models when applied to means values and individuals for each species. Trophic structure has been accounted for in both models using trophic-level estimates from amino acid analysis of  $\delta^{15}\text{N}$ .

## A bulk isotope approach and its complications

We confirm that MPB is a major source of productivity that widely supports the food web in the Dutch Wadden Sea (Christianen et al., 2017). The majority of consumers examined here (21 out of 25 consumer species) have  $\delta^{13}\text{C}$  values higher than  $-18\text{‰}$ , indicating reliance on a resource with a higher value than POM ( $-21.2 \pm 0.2\text{‰}$ ;  $n = 101$ ). MPB ( $-13.4 \pm 0.2\text{‰}$ ;  $n = 95$ ) is a widely available resource across the Dutch Wadden Sea (Figure 1B) with variable  $\delta^{13}\text{C}$  values indicating the use of both dissolved inorganic carbon (DIC) from the overlying water column and remineralized organic matter from porewater DIC. Due to the sandy nature of the sediment and extensive tidal pumping that occurs in the basin, benthic-associated MPB, primarily pennate diatoms (Scholz and Liebezeit, 2012) are widely available to consumers during diel inundation and exposure. Other potential candidate resources available to consumers include sediment organic matter, terrestrial input, macroalgae, or seagrasses, but are unlikely to play a significant role in supporting production.

Sediment organic matter ( $-21.8 \pm 0.3\text{‰}$ ;  $n = 117$ ) and terrestrial inputs [ $-27\text{‰}$  (Middelburg and Herman, 2007) and  $-23\text{‰}$  (Jung et al., 2019)] have lower  $\delta^{13}\text{C}$  values than POM and therefore cannot contribute to explain the higher values widely observed across consumers in the Dutch Wadden Sea. Green macroalgae such as *Ulva* sp. (Sea lettuce;  $-13.5 \pm 0.6\text{‰}$ ,  $n = 8$ ) have a higher value that could meaningfully contribute but are spatially limited due to their need for hard anchoring points such as exposed rocks within the larger landscape of mud and sand. This limited distribution constrains the amount of contribution from this resource as blooms of *Ulva* or so-called green tides (Charlier et al., 2008) did not occur in the period between 2011 and 2014 in the Dutch Wadden Sea. This makes *Ulva* sp. an unlikely resource to support more than half of the food web. Similarly, seagrasses have previously occurred extensively in the Wadden Sea, but due to their current extremely limited spatial ranges in the German and Danish Wadden Sea (Folmer et al., 2016) are unlikely to be a large source of productivity supporting consumers in the Dutch Wadden Sea.

Further examination of MPB as a resource reveals substantial variability in  $\delta^{15}\text{N}$  values ranging from 7.3 to 11.3‰



when grouping the minimum and maximum measurements taken from across the study site (Table 2a; MPB<sub>green</sub> and MPB<sub>brown</sub>). This variability could potentially reflect the input from an unaccounted resource or a biogeochemical process that is occurring across the basin. Although freshwater input from terrestrial sources has a  $\delta^{15}\text{N}$  of 10–14‰ (Jung et al., 2019), the limited inputs to the Wadden Sea and  $\delta^{13}\text{C}$  value of  $-23\text{‰}$  indicate at best a regionally confined contribution to the ecosystem. Another possible unaccounted source includes submarine groundwater discharge, which due to the relatively porous nature of the sandy sediment in the Wadden Sea basin could contribute reworked N with higher  $\delta^{15}\text{N}$  values to the benthos. Previous work has identified freshwater groundwater discharge as a minor input (Santos et al., 2015) that is unlikely to have a basin wide impact. However, this work did confirm considerable porewater exchange resulting in efflux of waters with higher total dissolved nitrogen and lower dissolved oxygen saturation as tidal exchange flushes previously isolated porewaters that have accumulated products from remineralization. Porewater exchange across the sediment surface during tidal flushing resulted in porewater total dissolved nitrogen that decreased with depth that is expected with efflux into the overlying water column. Tidal pumping through permeable sands (Marchant et al., 2018) is a potentially region-wide process providing nutrients that have undergone considerable denitrification that may provide substrate pools

in porewaters with increased  $\delta^{15}\text{N}$  values that then support MPB production within the Wadden Sea. Increased  $\delta^{15}\text{N}$  values are most apparent in the Ems-Dollard region and required adjustment for both bulk  $\delta^{15}\text{N}$  and  $\delta^{15}\text{N}_{\text{Phe-Base}}$  values (Supplementary Table 1). Despite this adjustment within the wider data set, there remains considerable variability in  $\delta^{15}\text{N}$  values (Figure 1A) across the basin that has made identifying a trophic structure using bulk  $\delta^{15}\text{N}$  values alone difficult without a method to integrate underlying shifts in baseline  $\delta^{15}\text{N}$  values across the Dutch Wadden Sea.

## Trophic discrimination factor and ecosystem baseline using amino acids

The TDF value from analysis of the difference between Glu and Phe throughout the food web is lower in this study than the most often applied canonical value (4.9‰ compared to 7.6‰) (Chikaraishi et al., 2009); as has been previously observed in multiple studies (McMahon and McCarthy, 2016; Hebert et al., 2016; Lemons et al., 2020; Nuche-Pascual et al., 2021). The lower TDF value reflects an ecosystem-wide application to species ranging from macroalgae to teleosts, and represents a compromise between the use of a single canonical TDF (Chikaraishi et al., 2009; Kato et al., 2021; Vokshoori et al., 2021), application of multiple species-specific TDFs as

determined by feeding studies (28×, 1 per species) or feeding group-dependent strategies based on the relative diet vs. tissue quality comparisons (McMahon et al., 2015b; McMahon and McCarthy, 2016; Bode et al., 2021; Le-Alvarado et al., 2021). Using wild caught animals across an entire ecosystem to derive a system-wide TDF, we relied on measured variations in the difference between Glu and Phe between individuals in each species sampling to estimate the TDF value that was then applied to estimate the trophic structure within the ecosystem. To this end, we combined trophic position estimates of teleosts from FishBase (Froese and Pauly, 2000), literature values for trophic position of invertebrate species (Christianen et al., 2015) and measured  $\delta^{15}\text{N}_{\text{Glu-Phe}}$  values for primary producers to ensure representation across the food web and found a TDF that is broadly comparable to one developed using solely marine teleosts (5.7‰). Application of a system-specific TDF was possible here due to the relatively large number of replicates for each species examined across the food web. Developing the TDF was necessary due to the presence of a large number of species for which no controlled feeding studies have been performed where the stable isotope composition of amino acids have been analyzed (e.g., a wide range of benthic invertebrates).

Application of  $\delta^{15}\text{N}$  values from amino acids allows for the integration of underlying variability in N sources when characterizing the trophic relationships within the benthic community. The large range observed for  $\delta^{15}\text{N}_{\text{Phe-Base}}$  (Figure 1C) in consumers further confirms that use of N with a high  $\delta^{15}\text{N}$  value is not regionally confined and occurs across the basin. In contrast, variability in the  $\delta^{13}\text{C}_{\text{Base}}$  values is more regionally confined, likely indicating the widespread use of MPB-derived carbon that is more consistently available across the basin and therefore less variable. These contrasting patterns and wide range of  $\delta^{15}\text{N}$  values observed across the MPB sampling support splitting of the MPB end member into two distinct resources: (1) MPB<sub>green</sub> which reflects newly fixed organic matter from MPB supported by dissolved inorganic C (DIC) and N substrates from the overlying water column and (2) MPB<sub>brown</sub> which reflects newly fixed organic matter from MPB supported from bacterially reworked C and N substrates provided from porewater associated materials (Table 2a). Furthermore,  $\delta^{15}\text{N}_{\text{Phe-Base}}$  values for a subset of the measured individuals of *Peringia ulvae* (Cone mudsnail;  $5.6 \pm 0.2\text{‰}$ ;  $n = 3$  vs.  $8.2 \pm 0.7\text{‰}$ ;  $n = 10$ ) indicated the individual specialization on a specific resource in a species previously used as an end member proxy for MPB (Christianen et al., 2017) while *Littorina littorea* (Periwinkle;  $9.8 \pm 0.2\text{‰}$ ;  $n = 17$ ; Table 1) had a substantially higher  $\delta^{15}\text{N}_{\text{Phe-Base}}$  value despite similar use of MPB between these two species indicated by the two source mixing model (Figure 5A; 71 and 82%, respectively). A 5‰ difference between source amino acid  $\delta^{15}\text{N}$  values between two primary consumers using similar amounts of MPB-derived carbon further confirms the substantial variation in MPB- $\delta^{15}\text{N}$  values due to availability of porewaters that have been impacted by denitrification. This

difference warranted further examination using three-source mixing models with two distinct MPB sources after adjustment for trophic position indicated using the ecosystem-dependent TDF from amino acid  $\delta^{15}\text{N}$  values.

## Identifying detrital contributions from microphytobenthos into the wider food web

Food webs are often split into herbivory (green) and detrital (brown) pathways depending on whether newly fixed productivity or detrital reworking are the predominate basal resource supporting them (Odum, 1969; Middelburg, 2014). These are often not clear-cut distinctions, but rather a spectrum of resource use split between multiple sources depending on the diet of each individual species. Application of the three source mixing models (Figure 5B) reveals separation between species groups that use the MPB<sub>green</sub> and the MPB<sub>brown</sub> resources. Species using MPB<sub>green</sub> include *Carcinus maenas* (Green crab), *Pomatoschistus* sp. (Goby sp.), *P. platessa* (European plaice), and *P. ulvae* (Cone mudsnail), indicating reliance on newly fixed MPB-derived organic matter supported from the overlying water column (DIC and available N). Species using MPB<sub>brown</sub> include *M. baltica* (Baltic clam), *A. marina* (Lugworm), *L. littorea* (Periwinkle), *C. crangon* (Shrimp), *Z. viviparus* (Eelpout), *Platichthys flesus* (European flounder), and *Solea solea* (Sole) indicating reliance on MPB-derived organic matter supported-off of microbially reworked N substrates that are readily available due to tidal pumping (Figure 6). The separation observed between these two groups of species appears to be along the boundaries of green vs. brown food webs (Evans-White and Halvorson, 2017; Cordone et al., 2020), where green food webs are directly supported by newly fixed OM from primary producers and brown food webs have detrital and microbial support through the breakdown, processing, and reuse of organic matter through an efficient microbial loop (Azam et al., 1983; Fenchel, 2008). The re-entry and reuse of detrital products (MPB<sub>brown</sub>) in this system appear to be mediated by MPB and may reflect either (1) support of live MPB using reworked nutrients and porewater DIC or (2) the direct use of reworked dead MPB-derived organic matter by deposit feeders. MPB samples in this study required migration across a permeable filter pressed against the sand so both MPB<sub>green</sub> and MPB<sub>brown</sub> end members reflect live-sampled MPB.

The large variability for both bulk  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values found in the MPB end members points to the need for further investigation into how MPB are supported across the tidal cycle as well as better characterization for all primary producers using compound-specific techniques (PLFAs, AA-C, and AA-N) within the Wadden Sea. Partitioning support from MPB throughout the food web into green and brown pathways highlights that each individual resource in this shallow system



(POM, MPB) likely has multiple forms due to the shallow nature of the basin as green and brown food webs interact and exchange materials (Krumins et al., 2013). The various forms of organic matter reflect the stages of processing that have occurred: (1) direct initial use of phytoplankton (green POM), (2) deposition and partial reworking of phytoplankton that are resuspended and used (brown POM/SOM), (3) direct uptake of water column N and DIC (MPB<sub>green</sub>), (4) use of reworked nutrients and porewater DIC (MPB<sub>brown</sub>), or (5) direct use of detrital MPB biomass (MPB<sub>brown</sub>). Environmental bulk isotope samples can often have quite variable values, but this variance likely represents inputs from a variety of resource pools and the combined application of other compound-specific techniques may be able to contribute to teasing apart individual contributions from these pools.

Due to the diversification of MPB resources, there is support of two distinct food web pathways in the Dutch Wadden Sea for MPB-derived resources as indicated by the three source mixing models (Figures 5B, 6): newly fixed production from the overlying water column (MPB<sub>green</sub>) and use of microbially reworked substrates from porewaters (MPB<sub>brown</sub>). This resource diversification likely contributes to the outstanding productivity that has been observed within the Wadden Sea. Specialization on the two different MPB types allows for further feeding niche differentiation beyond the classic model of deposit and surface/particulate feeders through identification of MPB specialist consumers that predominantly rely on either newly fixed or reworked MPB-derived organic matter. This specialization likely reduces competition between feeding types in a highly productive ecosystem and reflects efficient use of not only newly fixed organic matter but also efficient use of reworked OM as either direct uptake or through nutrient and DIC support of MPB through tidal pumping.

## Conclusion

Compound-specific analysis of amino acid nitrogen allowed for further identification and adjustment for underlying baseline shifts in  $\delta^{15}\text{N}$  values within the Dutch Wadden Sea that were not possible using solely bulk stable isotope methods. The resulting regional adjustments allowed for the development and application of a system-specific TDF that could then successfully be applied to estimate the trophic level of 28 species within the food web. Further application of SIMMs confirmed the dominant support of the food web by MPB (2-source models) and identified biogeochemical pathways supporting MPB along separate green and brown pathways (3-source models). This subsidy of nutrients derived from microbial processing in permeable sands may account for some of the exceptional productivity supporting the Wadden Sea fishery and seabird populations. Separate support of MPB between newly fixed and reworked organic matter showcases that diatom connectivity

with underlying porewaters needs to be further considered in food web studies examining the considerable productivity that occurs within intertidal ecosystems.

## Data availability statement

The original contributions presented in this study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author. The data is available at [data.4tu.nl](https://data.4tu.nl), the amino acid data is available at doi: 10.4121/21207470, and bulk carbon and nitrogen data are available at doi: 10.4121/21213113.

## Ethics statement

This study used animals previously obtained as part of the SIBES and NIOZ Fyke projects in 2011 to 2014, so no further ethical approval was needed for samples associated with this study.

## Author contributions

PR, SH, HV, and MM contributed to conception and design of the study. SH, HV, and TH contributed to sample design and collection of the samples through the SIBES and Waddensleutels projects that support this study. PR and SH organized the databases. PR performed the statistical analysis and wrote the first draft of the manuscript. All authors contributed to manuscript revision and read and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fevo.2022.951047/full#supplementary-material>

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