



## Research article



# Stable isotope and fatty acid analysis reveal the ability of sea cucumbers to use fish farm waste in integrated multi-trophic aquaculture

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## ABSTRACT

Stable isotope ratios, carbon ( $\delta^{13}\text{C}$ ) and nitrogen ( $\delta^{15}\text{N}$ ), and fatty acids validated the trophic connection between farmed fish in a commercial nearshore fish farm and sea cucumbers in the Mediterranean Sea. This dual tracer approach evaluated organic matter transfer in integrated multi-trophic aquaculture (IMTA) and the ability of sea cucumbers to incorporate fish farm waste (fish faeces and uneaten artificial fish feed) into their tissue. Between October 2018 and September 2019, *Holothuria (Roweothuria) poli* Delle Chiaje, 1824, co-cultured at IMTA sites directly below one of the commercial fish cage, at 10 m and 25 m from the selected fish cage, and at two reference sites over 800 m from the fish farm. Sea cucumbers were sampled from each site in February, May and September, except at 0 m due to mass mortalities recorded here in the first month of study. Isotopic mixing models revealed that fish farm organic waste was the dominant dietary source for *H. poli* in IMTA at 10 m and 25 m from the cage. The contribution of marine plant-derived organic matter, *Posidonia oceanica* leaves and rhizomes, was least important. The isotopic signatures of sea cucumber tissues at reference sites were not explained by the sampled food resources. Importantly, fatty acid profiling revealed a high abundance of individual terrestrial plant fatty acids, such as oleic (18:1n-9), linoleic (18:2n-6) and eicosenoic (20:1n-9) acids in sea cucumber tissue at 10 m and 25 m from the fish cage, presumably linked to the terrestrial plant oil content of the fish feeds. At the reference sites, sea cucumber tissues were characterised by higher relative abundance of arachidonic acid (20:4n-6) acid, and the natural marine-based eicosapentaenoic (20:5n-3) and docosahexaenoic (22:6n-3) acids. These analyses revealed important differences in the composition of *H. poli* between the IMTA and reference locations, driven by aquaculture-derived waste near fish cages. Moreover, this study revealed temporal variation in food availability and quality, and possible differences in the physiological responses of *H. poli*. Stable isotope analysis and fatty acid profiling provided complementary evidence for the important dietary preferences of *H. poli* and validated the potential of sea cucumbers to uptake aquaculture organic waste as part of inshore fish–sea cucumber IMTA. It reveals the important implications that an established trophic link has on the viability of using sea cucumbers for the development of IMTA and the sustainable expansion of aquaculture.

## 1. Introduction

Sea cucumbers are receiving increased attention for use in integrated multi-trophic aquaculture (IMTA) not only for their high commercial

value on the seafood market (Toral-Granda et al., 2008; Purcell, 2015), but also for their ability to process enriched sediments (Cubillo et al., 2016; Zamora et al., 2016; Chary et al., 2020). Earlier research demonstrated the capacity of sea cucumbers to utilise and reduce

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organic-rich aquaculture waste in experimental open-water IMTA (Yu et al., 2012; Yu et al., 2014; Tolon et al., 2017; Neofitou et al., 2019). If these IMTA systems are to be developed, there is a need to establish a trophic link between fed and extractive species and to verify the ability of sea cucumbers to assimilate organic matter from aquaculture-derived waste.

Deposit-feeding aspidochirotid holothurians can rework benthic sediment to ingest inorganic matter, detrital organic matter (algae, plants and decaying animals) and microorganisms (diatoms, protozoa and bacteria) (Belbachir and Mezali, 2018, 2020). These studies have shown that food sources for holothurians can be diverse and that uptake varies with season depending on the availability and nutritional quality of food items. The ability of *Holothuria (Roweothuria) poli* Delle Chiaje, 1824 to selectively ingest and assimilate organic-rich particles (Mezali and Soualili, 2013) promotes this species as a potential candidate to uptake nutrients from organic waste produced by commercial aquaculture.

Mediterranean countries (e.g., Greece, Spain and France) have increasingly exploited sea cucumbers for exportation to meet the growing demands of seafood markets in Asia (González-Wangüemert et al., 2018). For instance, the annual catches in Turkey, a major producing country in the Mediterranean region, increased to peak 550 000 kg (80% of catch being *H. poli*) in 2012 (González-Wangüemert et al., 2014) and then 150 250 kg in the first six months of 2016 (Aydın, 2017). As different Mediterranean Sea cucumbers become increasingly popular target species (González-Wangüemert et al., 2018), overfishing of wild populations is a risk, so they are favourable candidates for aquaculture (Rakaj et al., 2019), and fish-sea cucumber IMTA may be a novel means of production in the Mediterranean region.

Traditionally, the use of different food sources by sea cucumbers has been assessed through stomach and gut content observations (Belbachir and Mezali, 2018). However, this conventional approach fails to account for indigestible particles (Dalsgaard et al., 2003) and does not provide an accurate time-integrated picture of food source contribution to the consumer diet. Moreover, this approach might not be suitable to process stomach and gut contents from small marine organisms because of the physical limitations in sampling smaller organisms (Gao et al., 2011). The use of stable isotope and fatty acid (FA) analysis is an alternative approach to this conventional techniques to trace organic matter pathways and food webs in coastal systems (Vizzini et al., 2002; Fry, 2006; Gao et al., 2011; Wen et al., 2016; Signa et al., 2017).

Stable isotopes are widely used in food web studies because isotopic ratios change in a predictable manner as nutrients are transferred across trophic levels (Fry and Sherr, 1989). Stable isotopes can provide valuable information on the transfer of organic matter and nutrients from aquaculture waste to the natural environment because natural marine resources and organic fish farm waste have different isotopic signatures (Vizzini and Mazzola, 2004). Particularly, isotopic studies have revealed the ability of the sea cucumber, *Apostichopus japonicus*, to take up organic matter from aquaculture waste (Gao et al., 2006; Yokoyama, 2013). However, quantitative evaluation of food source contribution is technically difficult from stable isotope analysis alone, particularly to distinguish between benthic sources that have similar isotopic signatures (Sun et al., 2013; Wen et al., 2016). The contextual use of other biochemical tracers, such as fatty acids (FAs), can help to overcome this constraint (Kelly and Scheibling, 2012; Signa et al., 2017). The biological specificity and the trophic stability of FAs as they are incorporated conservatively into consumer tissue make FAs highly suitable to distinguish among organic matter sources (Kelly and Scheibling, 2012; Signa et al., 2017) and to assess dietary contribution to sea cucumbers in IMTA (Wen et al., 2016). The incorporation of terrestrial lipid sources in commercial aquaculture feeds provides the opportunity to use specific terrestrial plant FAs to trace fish farm-derived sources in the marine environment (Redmond et al., 2010; Parrish, 2013; White et al., 2019). Stable isotopes and FAs have been widely used to provide dietary information on marine organisms (Gao et al., 2006; Redmond et al., 2010;

Wen et al., 2016). Essentially, the simultaneous use of stable isotopes and FAs can provide complementary evidence to distinguish between food sources and substantiate the dietary preferences of marine organisms, that would otherwise be obtained from one technique alone (Gao et al., 2006; Wen et al., 2016).

This study combines carbon ( $\delta^{13}\text{C}$ ) and nitrogen ( $\delta^{15}\text{N}$ ) stable isotopes and FA profiling to overcome the limitations of the individual techniques and mutually confirm the dietary information of sea cucumbers in IMTA. The aim of this study is to validate the ability of *H. poli* to incorporate dietary organic matter from aquaculture waste into the sea cucumber body wall. Secondly, this study elucidates the important implications that a validated trophic link in fish-sea cucumber IMTA, particularly in a coastal environment, has on the viability of using sea cucumbers to recycle aquaculture-derived organic wastes and the relevance for the development of inshore IMTA.

## 2. Materials and methods

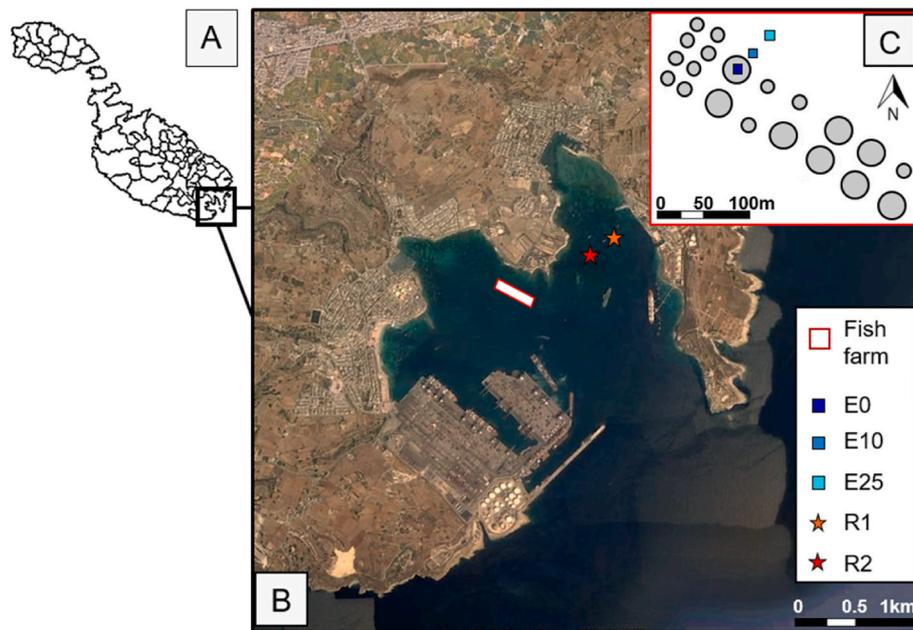
### 2.1. Study site

The study was set up at an inshore commercial fish farm (Malta Fish Farming Ltd.), located in Marsaxlokk Bay (35°49'39.90"N, 14°32'30.73"E), in southeast Malta, central Mediterranean Sea (Fig. 1A) and that has been in operation since 1993. The cage-based fish farm is a nursery and juvenile facility primarily for gilthead sea bream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*) where a total annual production of 719 t and a feed conversion ratio of 1.7 was recorded during the study period using commercially available formulated feeds. The fish farm has 20 round cages that are 12 m or 25 m in diameter. The fish cages are located in shallow waters between 8 m and 12 m depth and have net heights between 7 m and 10 m. Fingerlings are transferred from commercial hatcheries to the fish farm where they are ongrown for a few months before being transferred to the offshore aquaculture site of the company, in deeper and less sheltered waters. During this short period, fish cages at this inshore aquaculture facility are relocated periodically within the fish farm layout to accommodate different transactions (e.g. batch input, batch transfer). Moreover, small quantities of greater amberjack (*Seriola dumerili*) juveniles were produced at this location using baitfish, at the time of this study.

The fish farm is surrounded by patchy and reticulate *Posidonia oceanica* beds on the periphery of the aquaculture site (Adi Associates Environmental Consultants Ltd, 2007; MEPA, 2014). The seabed is characterised by heterogeneous soft sediments that range from very fine to medium grain size. The reference sites, R1 (35°49'55.1"N, 14°32'59.3"E) and R2 (35°49'53.5"N, 14°32'54.5"E), were situated over 800 m northeast of the fish farm where no aquaculture activity was present but had the same depth and seafloor sediment type (Fig. 1B).

### 2.2. Sea cucumbers and acclimation period

Juvenile specimens of *H. poli*, a native sea cucumber species in the Mediterranean Sea (González-Wangüemert et al., 2018), were collected by SCUBA divers along the coast of Palermo, Sicily (Italy), and shipped to Malta. On arrival, the sea cucumbers were housed in a land-based flow-through tank setup that used seawater from the bay. The sea cucumbers were fed with a commercially available artificial microalgal diet (Algamac Protein Plus, Pacific Trading, Ireland) and acclimated for two weeks. Water quality was measured daily, while faeces and unused feed were removed every two days to ensure optimal conditions for the sea cucumbers. All sea cucumbers were free of apparent diseases during the acclimation period. *H. poli* juveniles were blotted in paper and weighed to the nearest  $\pm 0.1$  g after 30 s of removal from seawater to allow body water to drain. Two hundred sea cucumbers of initial wet weight ( $\pm$  standard deviation, SD)  $24.6 \pm 2.1$  g individual<sup>-1</sup> were selected for the field experiment.



**Fig. 1.** A. Location of the experimental sites within Marsaxlokk Port, southeast Malta. B. Zoomed Google Earth image of location showing the IMTA and reference sites, R1 and R2. C. Sea cucumber cage positions at the experimental sites (E0, E10 and E25) in IMTA.

### 2.3. Experimental setup and sampling

In October 2018, a total of 20 cylindrical sea cucumber cages (1 m × 0.2 m ( $d \times h$ )) made of 0.8 cm galvanised mesh wiring and a synthetic rope mesh bottom were placed directly on seafloor sediments, moored to the bottom at a depth of 8 m; four cages of which were deployed directly below a fish cage (E0), then four cages at 10 m (E10) and another set of four cages at 25 m (E25) from the centre of the selected fish cage (Fig. 1C). The same number of sea cucumber cages were used at the reference sites R1 and R2 (Fig. 1B), with four cages set up at each of these sites. At each site, three of the four cages were used to collect specimens for laboratory analyses of stable isotopes and FAs, while the fourth additional cage was used to replace individuals sacrificed from the other three experimental cages to avoid density-dependent effects on growth. All the cages were randomly stocked with 10 juvenile sea cucumbers each, for an initial cage biomass of 310 g m<sup>-2</sup>. Further information on the production of *H. poli* in this field experiment is provided in Cutajar et al. (2022).

Throughout 2019, sampling of sea cucumbers and potential food sources for stable isotope ( $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ ) and fatty acid (FA) analyses were carried out simultaneously in February, May, and then at the end of the experiment in September. Two sea cucumbers were collected from each of the three experimental cages at each site and sampling time. Three farmed sea bream were collected from the fish cages to sample fish faeces after evacuation. The commercial fish feeds administered to the farmed fish were also sampled. The 2 mm feeds, 'A' (Pre-Grower 16; Alltech Coppens, Germany) and 'B' (Proactive 2; Veronesi, Italy), and the 3 mm feed 'C' (Supreme; Alltech Coppens, Germany) were supplemented in succession or in combination as a mixture between sampling times throughout the study. Feeds 'A' and 'C' were administered before the first sampling time (i.e. February), feeds 'A' and 'B' between the first and second sampling time (i.e. February–May), whereas a combination of all feeds was then used until the end of the experiment (i.e. September). These feeds included blends of nitrogen-rich fish oils and terrestrial plant derivatives. The composition of feeds 'A' and 'C' included marine sources (fishmeal and fish oils); terrestrial plant-based sources (sunflower meal, rape oil, wheat, maize gluten and soya protein); and the derivatives of terrestrial animals (poultry meal and blood meal, haemoglobin powder, and hydrolysed feather meal, in no

particular order. Feed 'B' contained fishmeal, fish oil, wheat, wheat gluten meal), and manufactured using soya and corn. The seagrass *P. oceanica* was collected, where present, near the fish farm and at the reference sites. *H. poli* shows preference for marine plant-derived organic matter, including dead and live *P. oceanica* leaves (Belbachir and Mezali, 2018, 2020) and is known to feed on seagrass detritus in the natural environment (Boncagni et al., 2019). Suspended and seafloor sediments were collected in triplicates from each IMTA and reference site using sediment traps and 5 cm diameter sediment corers, respectively, during the same sampling times. The sediment traps had four PVC collection tubes with a height to diameter ratio of 7.4: 1 (77.3 : 10.5 cm). The collection tubes were affixed vertically to a stainless-steel frame and the openings of the sediment traps were situated almost 3 m off the seabed. Sediment traps were retrieved 6 days after deployment, or as otherwise necessary depending on weather conditions. Seagrass, suspended and seafloor sediments were sampled at the same time as the sea cucumbers. All samples were transferred in a cool box to the laboratory for processing.

### 2.4. Sample processing, isotopic and fatty acid analysis

In the laboratory, sea cucumber samples were processed to extract the body wall, specifically composed of connective tissue and muscle tissue together. Fish samples were dissected to extract faeces after careful gut dissection. The top 3 cm layer of sediment from the cores was extracted immediately whereas the entire particulate samples from sediment traps was retained. Suspended and sedimentary samples from the sediment traps and the cores respectively were homogenised separately. Seagrass samples were washed with distilled water and separated further into leaves and rhizomes, removing any epiphytes by scraping. *P. oceanica* leaves and rhizomes have different signatures (Vizzini et al., 2003) so their contribution to the sea cucumber diet was assessed separately. All samples were stored at -20 °C until further processing.

For stable isotope analysis, settling particulate matter and sediment sub-samples were first acidified dropwise using 2N HCl to eliminate carbonates and then washed to retain only the fraction of settling particulate organic matter (SPOM) and sedimentary organic matter (SOM). Sub-samples from each type of sample were then dried at 60 °C to constant weight and ground to fine powder using a micro-mill (Retsch

MM200). Aliquots of each ground sample were packed in tin capsules and analysed for  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  using an isotope ratio mass spectrometer (Thermo Delta Plus XP) coupled with an elemental analyser (Thermo Flash EA 1112). The isotope ratios were defined in equation (1) as:

$$\delta X (\text{‰}) = [(R_{\text{sample}} / R_{\text{standard}}) - 1] \times 10^3 \quad [\text{Eq 1}]$$

where X is  $^{13}\text{C}$  or  $^{15}\text{N}$  and R is the  $^{13}\text{C}/^{12}\text{C}$  or  $^{15}\text{N}/^{14}\text{N}$  ratio. The values are expressed in the standard  $\delta$ -unit notation (as parts per mil) on international reference standard scales (Vienna-Pee Dee Belemnite for  $\delta^{13}\text{C}$ ; atmospheric  $\text{N}_2$  for  $\delta^{15}\text{N}$ ). Analytical precision was based on the standard deviation of internal standards (International Atomic Energy Agency IAEA-CH-6) replicates and within  $\pm 0.2\text{‰}$  for both  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ .

For lipid and FA analysis, frozen sub-samples of sea cucumber body wall, fish faeces, fish feeds, settling particulate matter, sediments, and *P. oceanica* leaves and rhizomes, were freeze-dried (ALPHA 1–4 LD plus, Martin Christ) and ground into fine powder. A modified Bligh and Dyer (1959) method was used to extract lipids from samples using a mixture of MilliQ distilled water, methanol and chloroform (1:2:1 v:v:v) with 0.01% BHT (butylated hydroxyl toluene) to avoid lipid oxidation. Samples were sonicated and centrifuged to separate the lipid and aqueous phases during extraction. Fatty acid methyl esters (FAMES) were then isolated from the lipid extract through an acid-catalysed transesterification with methanolic hydrogen chloride as described in Christie (1993). FAMES were subsequently analysed by a gas chromatograph (GC-2010, Shimadzu) equipped with a BPX-70 capillary column (30 m length  $\times$  0.25 mm ID  $\times$  0.25  $\mu\text{m}$  film thickness, SGE Analytical Science), and detected by a flame ionisation detector. Peaks were identified by using the retention times from mixed commercial standards (37FAME and BAME from Supelco; BR1 and QUALFISH from Larodan). Tricosanoic acid (C23:0) was used as an internal standard for FAME quantification. Lipid concentration and relative abundance of individual FAs were expressed as the percentage of the total FAs. These are presented in the supplementary material as mg 100 g<sup>-1</sup> of dry sample.

## 2.5. Data analysis

$\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  data were used to run Bayesian mixing models to estimate the median ( $\pm 95\%$  credible interval) dietary contribution of food sources to *H. poli*, using the R (v. 4.0.2) (R Development Core Team, 2015) package MixSIAR (Stable Isotope Analysis in R) (Stock and Semmens, 2016; Stock et al., 2018) in RStudio (v. March 1, 1073).

The isotopic values for fish feed and fish faeces were pooled *a priori* when they were not statistically distinguishable and considered cumulatively as a farm-derived source of waste to allow mixing models to converge on a unique solution. This pooling increased isotopic variability and could influence the certainty in estimates of source contributions from the models (Phillips and Gregg, 2003). *P. oceanica* (leaves and rhizomes) and sediments (SPOM and SOM), were also used as potential food sources in the model to assess dietary contribution to *H. poli*. Before running the mixing models, significant differences in the  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  of sources were assessed separately per site for each sampling time in SPSS (v. 1.0.0.1327) using a general linear model (GLM) when residuals followed normal distribution, and a generalised linear model when data violated the assumption of normality. Data were assessed for normality with Kolmogorov–Smirnov test and homogeneity of variances using Levene's test.

Mixing models were fitted separately for each individual level of factor 'site' (E0, E10, E25, R1 and R2), and for each 'time point' (February, May, and September). TEF of  $4.2 \pm 0.5\text{‰}$  for  $\delta^{13}\text{C}$  from the body wall of sea cucumbers was used to account for calcareous spicules (Watanabe et al., 2013), and TEF of  $3.4 \pm 1.0\text{‰}$  for  $\delta^{15}\text{N}$  per trophic level was applied (Peterson and Fry, 1987; Post, 2002).

Permutational multivariate analysis of variance (PERMANOVA;

PRIMER-e Ltd., UK) was used to test for differences in FA profiling between food sources (fish feeds, fish faeces, *P. oceanica* leaves and rhizomes, and sediments SPOM and SOM) and the sea cucumber consumer, *H. poli*, between sites. A two-factorial design, with fixed factors 'source' and 'site', was used for each sampling time. PERMANOVA was performed with Monte Carlo corrected *p*-values on FA data previously transformed with arcsine function and then resembled to generate the Euclidean distance matrix. Analysis of percentage similarity (SIMPER) was used on untransformed resembled data to identify the FAs that contributed most to similarities within and between sites. Principal component analysis (PCA) was performed to compare *H. poli* FA data between sites (MVSP 3.22, Kovach Computing Services, Wales). Data were compared with a one-way analysis of variance (ANOVA) followed by Tukey's test for *post hoc* comparisons.

A significance value of  $p < 0.05$  was applied to all statistical tests.

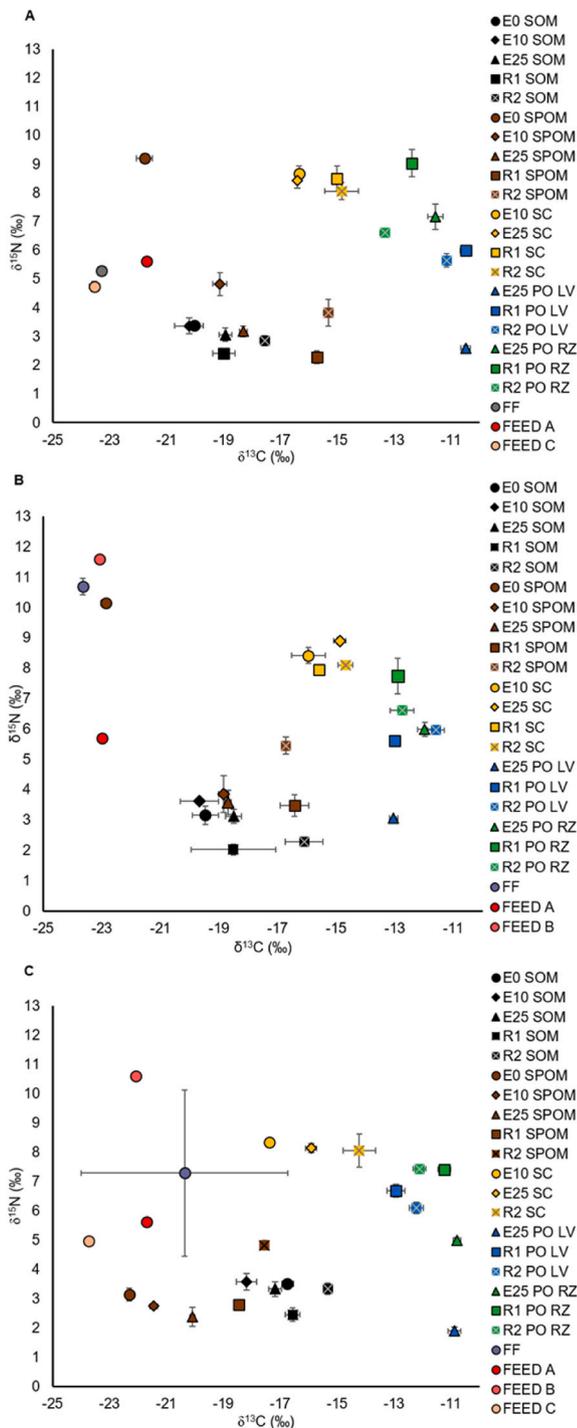
## 3. Results

### 3.1. Stable isotopes

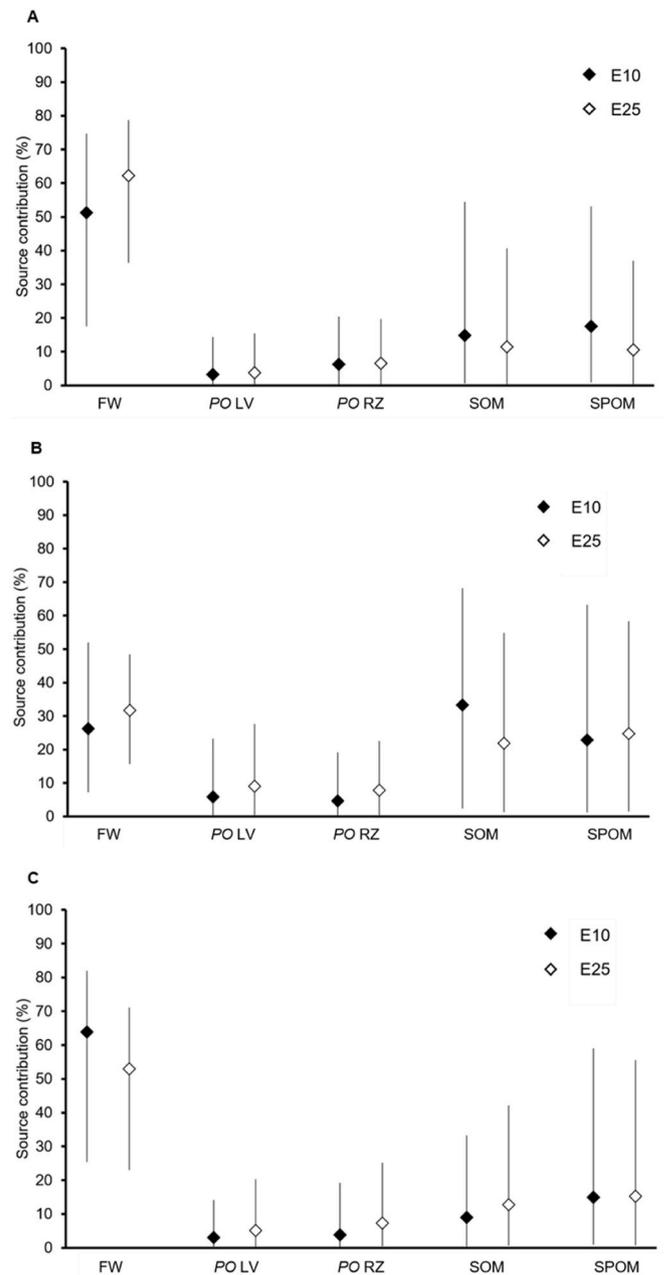
The significant differences between the isotopic ratios of aquaculture-derived waste (fish feed and faeces), and *P. oceanica* and sediments (SPOM and SOM) ( $p < 0.001$ ), allowed the application of stable isotope mixing models to assess food source contribution to the diet of sea cucumbers in integrated multi-trophic aquaculture (IMTA) and at the reference sites. Fish feed and faeces deposited near the fish cages, were the most  $\delta^{13}\text{C}$ -depleted sources, followed overall by SPOM and SOM, and *P. oceanica* leaves and rhizomes (Fig. 2). The different administered feeds throughout the study varied in pellet size and isotopic composition ( $p < 0.001$ ). Fish faeces were generally similar in isotopic composition to the fish feeds ( $p > 0.05$ ). The sampled fish faeces had a wide range of isotopic signatures over time and heterogeneous composition in September (Fig. 2). *P. oceanica* leaves and rhizomes were the most  $\delta^{13}\text{C}$ -enriched sources with significant differences between sampling sites and times ( $p < 0.001$ ), except for  $\delta^{15}\text{N}$  in leaves across time ( $p = 0.117$ ). SPOM deposited near the cages and at the reference sites showed a wider range of  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values than SOM (Fig. 2). The isotopic composition of both SPOM and SOM differed significantly across sites ( $p < 0.05$ ) throughout the study. SOM was more  $\delta^{13}\text{C}$ -depleted and  $\delta^{15}\text{N}$ -enriched close to the fish cages with the reference sites more  $\delta^{15}\text{N}$ -depleted than the IMTA sites, E0 and E10 ( $p < 0.001$ ) (Fig. 2). The isotopic signatures of SPOM varied between sites, with those near fish cages more  $\delta^{13}\text{C}$ -depleted ( $p < 0.001$ ) especially at E0. SPOM varied in  $\delta^{15}\text{N}$  between sites, where E0 was the most  $\delta^{15}\text{N}$ -enriched site ( $p < 0.001$ ) in February and May.

Mass mortalities were recorded directly below the fish cage (E0) within the first month of study (for further information see Cutajar et al., 2022); therefore, the organic source contribution to *H. poli* diet was not assessed by stable isotope and FA analyses for this site. The isotopic signatures of sea cucumbers varied significantly between the other sites near the fish cages (E10 and E25) and the reference sites ( $p < 0.05$ ) at all sampling times. Conversely, the isotopic signatures of *H. poli* were similar between the individual sites, E10 and E25 ( $p > 0.05$ ), and between the reference sites, R1 and R2 ( $p > 0.05$ ), except in May. The mixing model converged to provide the contribution of different organic sources to the sea cucumber diet at E10 and E25 (Fig. 3). The isotopic signatures of the sea cucumber consumers at the reference sites did not fall within the mixing polygon of sampled sources. Since isotopic mass balance was not established, the signatures of *H. poli* at R1 and R2 could not be explained by the proposed model and consequently they were rejected from subsequent analysis.

Farm-derived waste (fish feed and faeces) was the dominant dietary source of *H. poli* although with varied estimates of contribution to the sea cucumber diet at E10 (26.2%–63.9%) and E25 (31.7%–62.2%) (Fig. 3). The contribution of farm waste to the diet of *H. poli* decreased from 54.6% to 25.7% at E10, and 47.0%–27.0% at E25 between

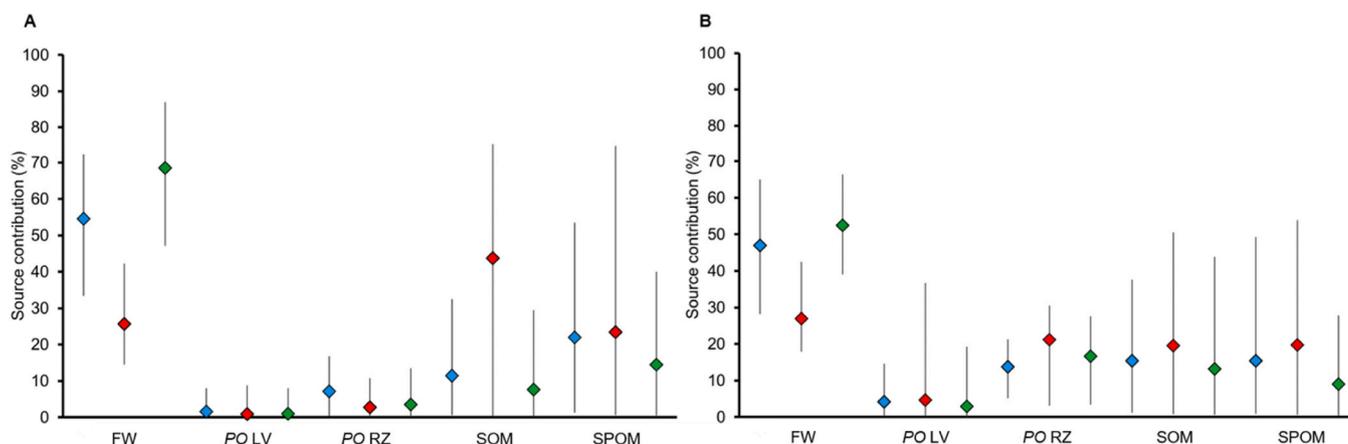


**Fig. 2.** Stable isotope biplot indicating the mean  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  composition of the different sources and the sea cucumber, *Holothuria poli*, where present at the IMTA sites (E0, E10, and E25) and reference sites (R1 and R2) in A. February, B. May, and C. September 2019. Standard deviation is indicated by error bars. Sample typologies represented by different symbol colour and sites represented by different symbol shape. SOM: sedimentary organic matter, SPOM: suspended particulate organic matter, SC: sea cucumbers, PO LV: *Posidonia oceanica* leaves; PO RZ: *Posidonia oceanica* rhizomes; FF: fish faeces. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)



**Fig. 3.** Organic matter source contribution (median  $\pm$  95 credible intervals) to *Holothuria poli* diet at E10 and E25 in A. February, B. May, and C. September. FW: farm waste, PO LV: *Posidonia oceanica* leaves; PO RZ: *Posidonia oceanica* rhizomes; SOM: sedimentary organic matter; SPOM: settling particulate organic matter.

February and May. The contribution then reached peak estimates of 68.5% and 52.5% at the respective sites by September (Fig. 4). The contribution of *P. oceanica* to the diet of *H. poli* was least important at each time (3.0%–9.0%) and with no apparent differences between the contributions of *P. oceanica* leaves and rhizomes. The contribution of *P. oceanica* slightly increased when moving from E10 to E25, away from the fish cages. The dietary contribution of *P. oceanica* leaves was comparable within the narrow range of 0.9% and 4.7%, whereas that of rhizomes varied between 2.8% and 21.2%, across the different sampling times. The dietary contribution of sediments, SOM and SPOM, to the diet of *H. poli* ranged between 9.0% and 33.3%, with different estimates of contribution between E10 and E25. Peak values of contribution from SOM and SPOM, were recorded in May at both sites that then decreased



**Fig. 4.** Organic matter source contribution (median  $\pm$  95 credible intervals) to *Holothuria poli* diet during the sampling times, February (in blue), May (in red), and September (in green) in A. E10, and B. E25. FW: farm waste, PO LV: *Posidonia oceanica* leaves; PO RZ: *Posidonia oceanica* rhizomes; SOM: sedimentary organic matter; SPOM: settling particulate organic matter. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

in dietary contribution by September.

### 3.2. Fatty acids (FAs)

The fatty acid (FA) profile of organic matter sources at each site varied significantly ( $p < 0.05$ ), albeit with similarities between SPOM and SOM ( $p > 0.05$ ).

Fish feeds included high proportions of the FAs, oleic (OA, 18:1*n*-9) (369.6–2190.6 mg 100 g<sup>-1</sup>), linoleic (LA, 18:2*n*-6) (714.0–1382.6 mg 100 g<sup>-1</sup>), and  $\alpha$ -linolenic (ALA, 18:3*n*-3) (132.5–414.9 mg 100 g<sup>-1</sup>) acids to a lesser extent (Supplementary 1). The feed composition also included saturated fatty acids (SFAs) (621.3–1004.2 mg 100 g<sup>-1</sup>), palmitic (16:0) (405.1–642.1 mg 100 g<sup>-1</sup>) and stearic (18:0) (98.5–197.0 mg 100 g<sup>-1</sup>) acids, and lower abundance of marine *n*-3 polyunsaturated fatty acids (PUFAs), particularly eicosapentaenoic acid (EPA, 20:5*n*-3) (109.2–296.6 mg 100 g<sup>-1</sup>) and docosahexaenoic acid (DHA, 22:6*n*-3) (147.5–298.0 mg 100 g<sup>-1</sup>). Fish feeds had higher lipid content (207.3  $\pm$  54.9 mg 100 g<sup>-1</sup>) than fish faeces (89.4  $\pm$  29.1 mg 100 g<sup>-1</sup>), which in turn were more heterogeneous in FAs and characterised by varied abundances of OA (18:1*n*-9) (26.4–978.1 mg 100 g<sup>-1</sup>), palmitic acid (16:0) (15.7–541.1 mg 100 g<sup>-1</sup>) and LA (18:2*n*-6) (18.8–538.8 mg 100 g<sup>-1</sup>).

The lipid levels in SPOM close to the fish cage, specifically at E0, were significantly higher than the other sites ( $p < 0.001$ ) (Supplementary 1). The concentration of lipids in SPOM at the IMTA sites and reference sites varied throughout the year ( $p < 0.001$ ). SPOM and SOM had a high abundance of SFAs particularly at the reference sites whereas OA (18:1*n*-9) and LA (18:2*n*-6), which are associated with the vegetable oils in fish feed, were dominant closer to the fish cages. On the other hand, *P. oceanica* leaves and rhizomes comprised ALA (18:3*n*-3), LA (18:2*n*-6) and SFAs, in proximity to fish cages as well as at the reference sites. These distinctly different FA profiles of fish feeds and the natural marine resources were fundamental to assess the dietary relationship between sea cucumbers and food sources.

The analysis of percentage similarity (SIMPER) confirmed these patterns revealing that the IMTA sites were mainly characterised by OA (18:1*n*-9) (9.3%–30.9%), LA (18:2*n*-6) (7.2%–23.7%) and SFAs (Supplementary 2). In particular, an average similarity over 95% was recorded for organic matter sources in the site directly below the fish cage (E0), which was mainly driven by OA (18:1*n*-9) (21.5%–30.9%), LA (18:2*n*-6) (13.8%–21.9%) and 16:0 (13.6%–19.3%). E10 and E25 were characterised by the same FAs albeit in lower contributions and with higher abundance of the marine *n*-6 PUFA ARA (20:4*n*-6) and the sum of *n*-3 PUFAs. Moreover, the *n*-3 PUFA ALA (18:3*n*-3) was particularly more abundant at E25 (6.9%–15.1%) than at the other IMTA sites across

all sampling times. On the other hand, the reference sites showed less homogeneity at R1 (85.51%–86.71%) and R2 (81.12%–91.13%), and a greater variation in the relative contribution of FAs between sampling times. These sites were characterised by *n*-3 PUFAs, primarily ALA (18:3*n*-3) (7.8%–17.5%) and EPA (2.8%–9.4%), the *n*-6 PUFAs ARA (20:4*n*-6) (7.6%–14.3%) and LA (18:2*n*-6) (7.1%–22.3%), and the ubiquitous SFA 16:0 (10.9%–21.6%).

The dissimilarity between sites increased with increasing distance from E0, with a 25% range of dissimilarity between E0 and E25. The range of dissimilarity was between 42.45% and 55.16% when comparing E0 and the reference sites, R1 and R2, throughout the study (Supplementary 2), providing the opportunity to distinguish between these sites. The extent of dissimilarity between the other IMTA sites (E10 and E25) and the reference sites decreased with increasing distance from the fish cages. This was generally driven by the monoenoic FAs OA (18:1*n*-9) (8.2%–24.2%) and palmitoleic acid (16:1*n*-7) (3.4%–11.0%), the *n*-6 PUFAs LA (18:2*n*-6) (4.7%–13.7%) and ARA (20:4*n*-6) (4.6%–11.2%), and the SFAs 16:0 (3.7%–10.1%) and 18:0 (3.4%–15.8%). The contributions of OA (18:1*n*-9) and LA (18:2*n*-6), the primary drivers of these spatial dissimilarities, were lowest in May with an increased contribution from SFAs, 16:0 and 18:0, and monounsaturated fatty acids (MUFAs), 16:1*n*-7 and 18:1*n*-7.

Sea cucumbers from the sites near fish cages (E10 and E25) and those at the reference sites had similar lipid levels ( $p = 0.201$ ) (Supplementary 3), but showed significant differences between sampling times ( $p < 0.05$ ). The FA profiles of sea cucumbers were similar between E10 and E25, and between R1 and R2, with significant differences between the IMTA and the reference sites ( $p < 0.05$ ). The PCA ordination showed a clear grouping of the sea cucumbers sampled at the sites near fish cages (E10 and E25) in IMTA and the reference sites based on the differences in FA profiles (Fig. 5). Along the first principal component (PC1, 70.5% of the total variance) E10 and E25 clustered driven by OA (18:1*n*-9), LA (18:2*n*-6) and eicosenoic acid (20:1*n*-9), and the reference sites driven by ARA (20:4*n*-6). *Post hoc* analysis ( $p < 0.05$ ) confirmed a higher relative abundance of OA (18:1*n*-9) (5.8–137.2 mg 100 g<sup>-1</sup>), LA (18:2*n*-6) (8.5–98.2 mg 100 g<sup>-1</sup>) and eicosenoic acid (20:1*n*-9) (8.8–101.1 mg 100 g<sup>-1</sup>) in the FA profiles of sea cucumbers at the IMTA than the reference sites throughout the study (Supplementary 3). SFAs 16:0 (1.3–60.3 mg 100 g<sup>-1</sup>) and 18:0 (3.1–69.0 mg 100 g<sup>-1</sup>) explained the second principal component (PC2), which described 12.7% of total data variance.

## 4. Discussion

This study provides complementary evidence for the ability of sea

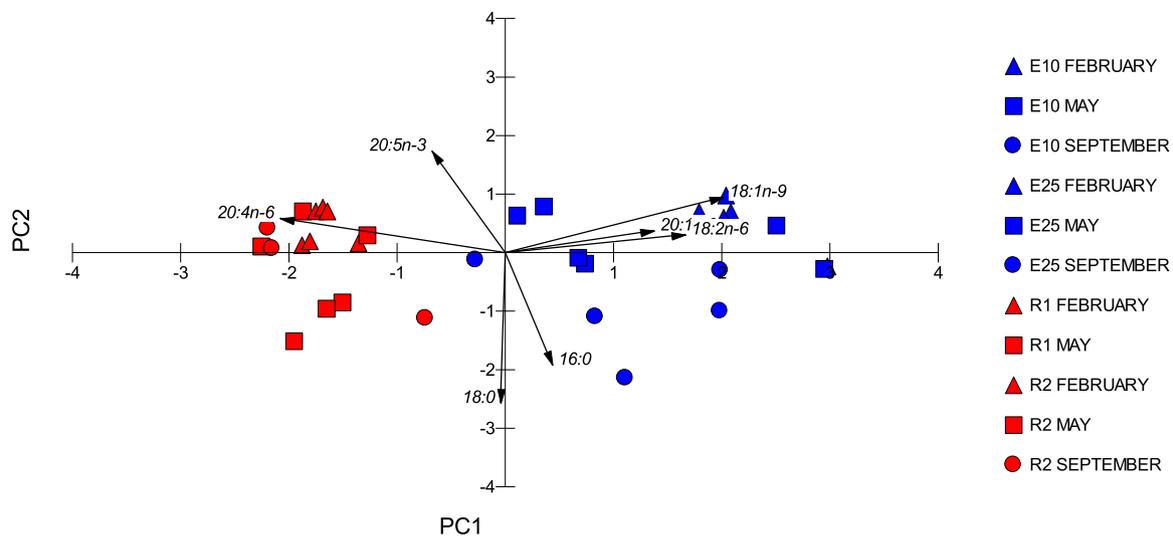


Fig. 5. Principal component analysis of fatty acid composition of sea cucumbers from the IMTA (E10 and E25), shown in blue, and reference sites (R1 and R2), shown in red, in February, May, and September. Fatty acids driving the dietary differences are represented by vectors. Eigen analysis tolerance set as  $1 \times 10^{-7}$ . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

cucumbers to assimilate and mitigate aquaculture-derived organic waste inputs, from a nutritional perspective. The combined stable isotope analysis and fatty acid (FA) profiling mutually validated the transfer of aquaculture-derived organic waste from inshore aquaculture to sea cucumber tissue, when other food sources were available.

#### 4.1. Stable isotopes

The distinctly different isotopic signatures of fish feed and faeces from other marine resources provided the opportunity to trace nutrients and organic matter from aquaculture-derived organic waste to sea cucumbers co-cultured in IMTA. This approach provided isotopic evidence for the long-term assessment of food utilisation and dietary preferences of *H. poli* in nearshore IMTA. The fish feeds administered to the farmed fish included blends of nitrogen-rich fish oils and vegetable sources. This could explain the more  $\delta^{13}\text{C}$ -depleted and  $\delta^{15}\text{N}$ -enriched sediments near the fish farm, similar to the isotopic composition described for sediments near fish cages in other isotopic studies (Yokoyama et al., 2006; Holmer et al., 2007; Yokoyama, 2013). These organic matter sources near fish cages could provide food of adequate nutritional value for the value-added production of sea cucumbers in IMTA (Yokoyama, 2013). However, the temporal differences in food availability and quality in this study could reportedly influence the feeding selectivity and the assimilation of food sources in holothurians (Mangion et al., 2004; Mezali and Soualili, 2013), that could add to changes in the biochemical composition of sea cucumbers when constituents (e.g., lipid and protein) are accumulated or when energy substrates are consumed (Peterson and Fry, 1987; Sun et al., 2013).

This study substantiates the ability of holothurians to use fish feed and faeces as a nutritional source in isotopic studies (Yokoyama, 2013; Park et al., 2015; Xia et al., 2015). There was mass mortality of sea cucumbers placed directly below the fish cage, due to smothering (see Cutajar et al., 2022 for further details), but the results also show that sea cucumbers at 10m and 25m from the centre of the cage were able to utilise aquaculture waste. Evidently, when setting up commercial scale IMTA systems, there would be a need to consider how waste is dispersed and deposited around the farm. The significance of aquaculture-derived organic matter for sea cucumber production near fish cages were substantiated by findings showing that the nutritional value of sources available at the reference sites, away from the fish farm, could not explain the isotopic signatures of *H. poli* tissue and was not able to sustain sea cucumber production, with low survivability compared to

E10 and E25 (Cutajar et al., 2022). Basis for Bayesian model rejection for the dietary contribution of nutritional sources to *H. poli* at the reference sites could suggest missing food samples and may be indicative of a wider diet strategy and preferences for other organic matter sources, consistent with Belbachir and Mezali (2018, 2020). Evidently, sea cucumber production in coastal areas in the Mediterranean could be limited without aquaculture-derived organic inputs.

#### 4.2. Fatty acids (FAs)

Alternative dietary lipid sources have been adopted as replacement for marine-derived oils in artificial feeds (Turchini et al., 2009; Betancor et al., 2016; Sprague et al., 2016). Addition of vegetable oils in commercial feeds influences the biochemical composition of marine organisms (White et al., 2019). The vegetable oils that supplemented dietary fish oils in feeds are characterised by abundant proportions of mono-unsaturated fatty acids (MUFAs), primarily OA (18:1n-9), LA (18:2n-6) and ALA (18:3n-3), in decreasing order of proportion (Turchini and Mailer, 2011). However, LA and ALA are also the dominant FAs in *P. oceanica* (Viso et al., 1993; Kelly and Scheibling, 2012; Signa et al., 2017), which verifies the importance of the complementary tracer approach taken in this study. For these reasons, OA and LA were considered important FA biomarkers, particularly when a higher abundance and contribution characterised the sites near fish cages relative to the reference sites. This was substantiated by lower proportions of LA and ALA in *H. poli* at the reference sites, even though sea cucumbers inhabit *P. oceanica* habitats and take up seagrass-derived organic matter in the natural environment (Belbachir and Mezali, 2018, 2020).

Previous studies showed that holothurians are mainly characterised by PUFAs (53.0%–59.1%), primarily arachidonic acid ARA (20:4n-6), and lesser relative proportions of SFAs and MUFAs (Aydin et al., 2011; David et al., 2020), as in the results here. Findings revealed the relative dominance of ARA and the significance of marine n-3 PUFAs (EPA and DPA) in *H. poli*, corroborating studies which show that holothurians take up higher proportions of highly unsaturated fatty acids (HUFAs) ( $\geq \text{C}_{20}$ ) that are attributed to detrital particles, rich in algae and bacteria (Yu et al., 2016; David et al., 2020). In the natural environment, benthic diatoms are the dominant food source of *H. poli* (Belbachir and Mezali, 2020); however, since diatoms are usually poor in ARA and this FA was in low abundance (<5%) in the organic food sources, the elevated levels of ARA in *H. poli* may be evidence for selective uptake of HUFAs or the

capacity of FA biosynthesis as revealed for other echinoids (Carboni et al., 2012, 2013). The high abundance of plant-derived FAs and low relative proportions of ARA in sea cucumbers near fish cages (E10 and E25) confirms the nutritional benefits of IMTA for sea cucumber production. Moreover, the elevated DHA in sea cucumbers at E10 and E25 reveals the supplement use of *n*-3 PUFA for better growth in IMTA, as reported in Cutajar et al. (2022). On the other hand, the low dietary contribution from dead *P. oceanica* leaves and rhizomes substantiates findings of Belbachir and Mezali (2018, 2020) for *H. poli* in the natural environment. Aquaculture-derived organic inputs could support production of sea cucumbers where the natural marine resources are not able to sustain survival and growth under culture conditions in these coastal environments.

#### 4.3. Wider implications and considerations

This study shows that *H. poli* relied on the organic fish farm inputs to grow effectively at 10 m and 25 m from fish cages and demonstrates the viability of growing sea cucumbers near fish farms. The validated uptake of aquaculture-derived organic waste and the increasing preferences for eco-labelled aquaculture products (van Osch et al., 2017; Xuan, 2021) present economic opportunities for producers that might be interested to adopt environmentally friendlier technologies in the Mediterranean region. These findings are encouraging for product diversification through the development of IMTA and should be coupled with important consideration for the willingness of consumers to pay a price premium for sustainably farmed seafood. Furthermore, the nutritional benefits of sea cucumbers produced near fish cages could create economic value for producers to invest in sustainably farmed products. However, fish-sea cucumber IMTA development should be with a better understanding of the market opportunity and the social acceptability towards these sustainable seafood products, primarily in terms of food safety and quality.

In addition, the development of IMTA should be with recognition for the expected effects that changes in fish farm production would have on food availability and quality. Notably, this temporal variation would have substantial implications for the transfer of dietary organic matter between farmed fish and sea cucumbers, the uptake and removal of organic wastes, sea cucumber production, and other practices (e.g. optimum period of harvest operation) that need to be considered for IMTA development. These fluctuations in waste output and uptake in fish-sea cucumber IMTA need to be appreciated in predictive modelling and then in practice, especially if producers are to invest in IMTA and scale-up towards commercial production.

#### 5. Conclusions

This study validates the trophic connectivity in fish-sea cucumber IMTA in the nearshore environment. It demonstrates the dietary significance of aquaculture-derived organic matter for the production of *H. poli* when cultured near fish cages, as opposed to being placed directly below the fish cages and at the reference sites, away from this source of organic deposits. Lastly, these findings reveal important design and functional requirements of IMTA, and considerations that are required for the development of fish-sea cucumber IMTA for sustainable seafood production.

#### Credit author statement

**Karl Cutajar** Conceptualization, Methodology and investigation, analysis and interpretation. Writing of the original article. **Lynne Falconer** Supervision and conceptualization. Critical revision and contributed towards the writing - Review & Editing. **Alexia Massa-Gallucci** Conceptualization, Investigation and writing - Review & Editing. **Rachel E. Cox** Contributed towards the methodology and investigation, **Lena Schenke** Contributed towards the investigation,

**Tamás Bardócz** Contributed towards conceptualization and supervision, **Cristina Andolina** Formal analysis and writing - Review & Editing, **Geraldina Signa** Investigation, Formal analysis and writing - Review & Editing, **Salvatrice Vizzini** Resources and methodology. Writing - Review & Editing, **Matthew Sprague** Formal analysis and writing - Review & Editing, **Trevor C. Telfer** Supervision and conceptualization. Critical revision and contributed towards the writing - Review & Editing.

#### Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Karl Cutajar reports financial support was provided by ENDEAVOUR SCHOLARSHIP SCHEME. Karl Cutajar reports financial support was provided by EU Horizon 2020 Research and Innovation.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jenvman.2022.115511>.

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