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Stable Isotopes Analysis of Bioremediating Organisms in an Innovative Integrated Multi-Trophic Aquaculture System

Jacopo Borghese [1,2,*], Adriana Giangrande [1,2,3], Daniele Arduini [1,2], Lorenzo Doria [1], Caterina Longo [2,4], Lucia Rizzo [2,5], Antonio Pennetta [6], Giuseppe E. De Benedetto [7] and Sergio Rossi [1,2,8]

1 Department of Biological and Environmental Sciences and Technologies, University of Salento, 73100 Lecce, Italy; adriana.giangrande@unisalento.it (A.G.); daniele.arduini@unisalento.it (D.A.); lorenzo.doria@unisalento.it (L.D.); sergio.rossi@unisalento.it (S.R.)
2 National Interuniversity Consortium for Marine Sciences (CoNISMa), 00196 Rome, Italy; caterina.longo@uniba.it (C.L.); lucia.rizzo@cnr.it (L.R.)
3 National Biodiversity Future Center, 90133 Palermo, Italy
4 Department of Bioscience, Biotechnology and Environment, University of Bari Aldo Moro, 70125 Bari, Italy
5 Institute of Sciences of Food Production, National Research Council (CNR-ISPA), 73100 Lecce, Italy
6 Institute of Atmospheric Sciences and Climate, National Research Council (CNR-ISAC), 73100 Lecce, Italy; antonio.pennetta@cnr.it
7 Department of Cultural Heritage, University of Salento, 73100 Lecce, Italy; giuseppe.debenedetto@unisalento.it
8 Institute of Marine Sciences (LABOMAR), Federal University of Ceará (UFC), Fortaleza 60455-760, Brazil
* Correspondence: jacopo.borghese@unisalento.it

Abstract: Integrated Multi-Trophic Aquaculture (IMTA) has been demonstrated to be a very useful tool to minimize the waste product production of fish monocultures whilst promoting biomass that can be used for different purposes. The stable isotope analysis ($\delta^{13}C$, $\delta^{15}N$, and C:N ratio) of bioremediating organisms present in an IMTA facility is critical to understanding the nutrient flow between farm food waste and filter-feeding organisms, and hence the bioremediation capability of the IMTA system. Here, we report the isotopic signature of the sediment below the fish cages, the fish artificial food and sixteen different suspension feeding species present in the IMTA system in the Mar Grande of Taranto (Italy). A comparison of the stable isotopes results of the bioremediating organisms with those of the same species collected from a control (Cnt) site, unaffected by the plant discharges, was thus conducted looking for trophic level patterns. This assessment aimed to evaluate the possible influence of aquaculture waste on the diet of the organisms, revealing these findings for the first time. Similar $\delta^{15}N$ values (below 2–3‰ between areas) were found between the IMTA and Cnt sites, while differences in $\delta^{13}C$ values were found among multiple organisms between the two sites, suggesting a possible different primary source of the organic matter that supports the trophic web. Almost all analyzed species in the IMTA site reported $\delta^{13}C$ values lower than Cnt site, being more similar to the isotopic signature of the aquaculture finfish food. However, the wide IMTA isotopic range for both $\delta^{15}N$ and $\delta^{13}C$ suggested a broad spectrum of diets for bioremediating organisms that can actively mitigate the impacts of mariculture by capturing different particles and using various food sources, leading to more sustainable mariculture activities.

Keywords: isotopic signature; filter feeders; mariculture; Integrated Multi-Trophic Aquaculture; bioremediation; Mediterranean Sea

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1. Introduction

Since 2013, aquaculture has become the main fish supply for human consumption [1]. Intensive aquaculture is impacting not only the water column but also the sediments and the surrounding benthic communities in many ways [1]. Sustainable aquaculture practices have become necessary to feed the world's growing population and ensure the sector's profitability and sustainability [2,3], especially because the excess amount of particulate (i.e.,

feces and unfed nutrients) and dissolved (i.e., nitrogen and phosphorus) nutrients produced by mariculture activities are regarded as one of the primary sources of pollution in coastal environments [4,5]. Among the most successful tools used to minimize the monoculture waste products, the Integrated Multi-Trophic Aquaculture (IMTA) has been consolidated during the last couple of decades [6]. In fact, it has been highlighted the importance of IMTA planning not only as an efficient tool for bioremediation, but also as a promoter of biomass that can be used for several purposes [7], including restoration plans [8].

Among potential impacts of fish farming, aquaculture waste products represent a critical issue since they may pollute the area and enter the food web, affecting the natural isotopic composition at both the base and upper trophic levels [9]. As previously mentioned, one potential approach to diminish the anthropogenic pressure due to the sewage is the implementation of the IMTA system, which combines fed aquaculture species with additional commercially relevant organisms (bioremediators) capable of extracting organic and/or inorganic compounds from the seawater (fish farm waste) [10,11]. Indeed, the physiological and ecological properties of the bioremediating organisms (e.g., macroalgae, suspension feeders, deposit feeders, etc.) influence the rate of particle collection in the water column. The importance of these organisms has been crucial to establish different protocols based on calculations of carbon and nitrogen flow from the waste products of the fish to the bioremediating species [12]. An in-depth understanding of the trophic interactions and hierarchy between fed organisms and extractive species is therefore necessary to maximize the biomitigation effect of the IMTA system, increasing the cultivation of the most appropriate organisms related to such bioremediating effects [13].

Applying a stable isotope approach to investigate the dynamics of trophic webs within the IMTA is insightful since this technique is based on the assumption that isotope signatures do not decay over time, integrating long-term diets and giving information on the trophic ecology of an organism over a wide temporal range (from days to months, depending on the tissue turnover) [14]. In a standard food web, $\delta^{15}N$ increases in a stepwise manner from one trophic level to the next, usually resulting in an enrichment of 3.4–5.0‰ in $\delta^{15}N$ [15] from the prey to the consumer, allowing us to estimate the trophic position of the different organisms [16]. Only a shift above the 3‰ is considered a real change in its trophic position [16]. Furthermore, there is a slight enrichment in $\delta^{13}C$ of approximately 1‰ from one trophic level to the next [14], and the $\delta^{13}C$ signature provides information of the primary source of the organic matter supporting the trophic web depending on the photosynthetic source [17]. Hence, the trophic ecology and position of reared organisms in IMTA facilities can be assessed through carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) stable isotope analyses, as they have been used successfully in determining sources of nutrition for consumers, evaluating trophic interactions among species, and determining the impact of aquaculture waste on the environment [13,18–22].

Research on energy fluxes in IMTA systems using stable isotopes is quite limited and primarily available for non-Mediterranean ecosystems. In a study conducted in the Mediterranean region, invertebrates such as *Aplysina aerophoba* (Nardo, 1833), *Balanus perforates* Bruguière, 1789, and *Anemonia sulcata* (Pennant, 1777), influenced by fish farms, were compared to a reference site. The findings indicated that water quality was maintained through IMTA activities [9,23].

Other studies have focused on bivalves, commonly used as extractive organisms due to their economic value, particularly in Northern Europe [24]. Although IMTA systems are less developed in the Mediterranean Sea, some research has investigated the integration of mussel farming into IMTA systems in Mediterranean regions [25,26]. In these cases, stable isotopes were used to analyze the bioremediation activity of *Mytilus galloprovincialis* Lamarck, 1819 [25,27]. Stable isotopes were also employed to study the dispersal area of fish farm waste, showing that sediments around the cages can be organically enriched up to 1000 m from the source [28].

The present study refers to an innovative inshore IMTA rearing model, performed at a preindustrial level within the EU REMEDIA Life project (LIFE16 ENV/IT/000343) in a

semi-enclosed basin within the Gulf of Taranto (Mediterranean Sea, Northern Ionian Sea), historically affected by intense fish farming. Here, a new set of filter-feeding bioremediators, such as polychaetes, sponges, and mussels, coupled with macroalgae and the natural fouling assemblages, have been reared within a fish farm for the first time in Europe [29]. This research focuses on understanding the role of lower trophic levels in the reduction in farm waste. The selected species are composed of active suspension feeders, belonging to all the major taxa present in the area: Porifera, Polychaeta, Mollusca, Tunicata, and Bryozoa. These species have different diets and clearance rates [30], being associated with complex structures that can clear the waters and promote the zooplankton concentration thanks to their forest-like structures [31].

In particular, the main objective was to investigate the carbon and nitrogen flow from the fish farm to the bioremediating organisms, analyzing the isotope composition ($\delta^{13}C$, $\delta^{15}N$, and C:N ratio) of the sediments below the fish cages, the fish artificial food, and sixteen different species living within the IMTA system, including selected bioremediating organisms and associated fouling organisms.

Such an approach will let us compare the stable isotope composition of the bioremediating organisms with that of the same species collected from a control (Cnt) site unaffected by the plant discharges. This comparison aimed to evaluate the possible influence of aquaculture waste on the isotopic composition (and thus its primary food component) of the bioremediating organisms reared in the innovative IMTA system.

2. Materials and Methods

2.1. Study Area

The study area is located on the south-west side of the Mar Grande of Taranto (40°25′56″ N; 17°14′19″ E; Southeast Italy, Ionian Sea; Figure 1). The Mar Grande of Taranto is a semi-enclosed basin reaching a maximum depth of 45 m. The local surface current is directed from the north-east to the south-west at a speed of about 3 cm s^{-1}. At the bottom, the direction of the current is inverted, proceeding from south-west to north-east at a speed of about 1.3 cm s^{-1}.

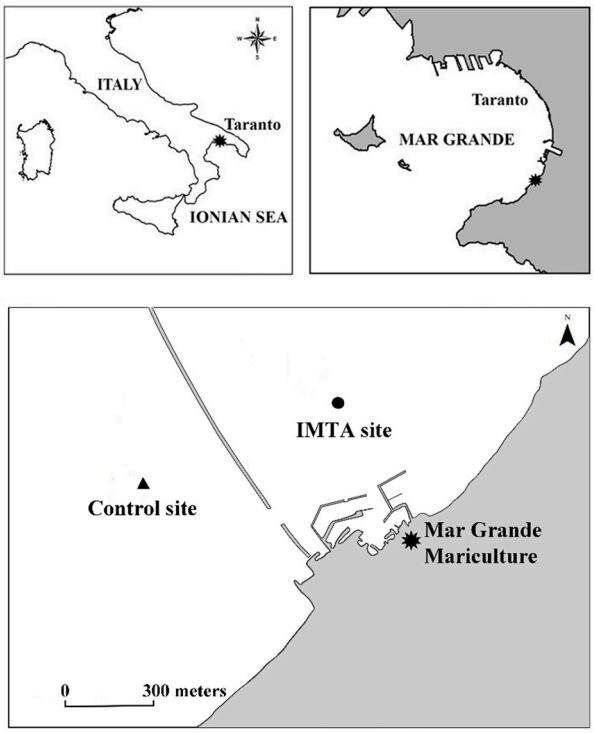

Figure 1. Study area. ✹: Mar Grande Mariculture head office; ●: IMTA site; ▲: control site.

The investigation was performed at the aquaculture plant "Maricoltura Mar Grande", which covers a surface of 0.06 km^2 and it is positioned at about 600 m from the coast. It consists of 15 cages (Ø 22 m), working at a depth ranging from 7 to 12 m and producing about 100 tons $year^{-1}$ of European seabass *Dicentrarchus labrax* (Linnaeus, 1758) or sea bream *Sparus aurata*, Linnaeus, 1758.

At the start of the REMEDIA Life project (2018), half of the mariculture plant was converted into an IMTA system (Figure 2). The IMTA rearing system, utilizing the polychaete *Sabella spallanzanii* (Gmelin, 1791), different sponge species, mainly *Sarcotragus spinosulus* Schmidt, 1862, the mollusc *Mytilus galloprovincialis*, and the macroalgae *Chaetomorpha linum* (O.F. Müller) Kützing, 1845 and *Gracilaria bursa-pastoris* (S.G.Gmelin) P.C.Silva, 1952, is described in [29]. On the collectors, intended for the growth of the project's target organisms, part of the available space is colonized by the fouling organisms, increasing the filtering capacity of the system.

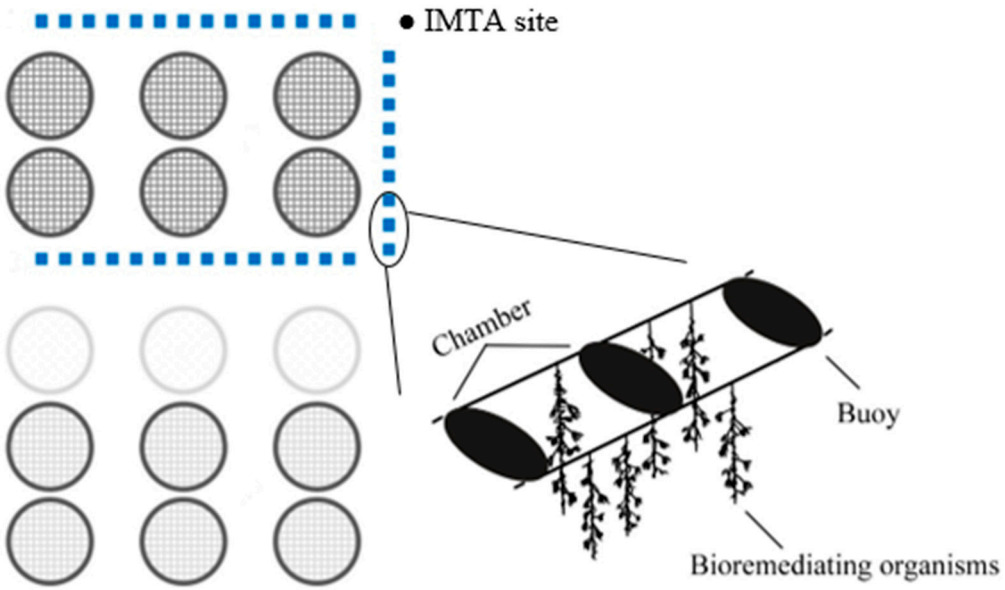

Figure 2. Schematic drawing of the IMTA site. The blue squares represent the arrangement of the breeding chambers within the long lines.

2.2. Sample Collection

Stable isotope analyses were conducted on organisms and soft sediments collected in the IMTA plant and in the control site (Figure 1). A total of 16 species (three replicates for each species) belonging to Porifera, Annelida Polychaeta, Mollusca, Bryozoa, and Tunicata were collected on the same day in July 2022. Specimens at the IMTA site were collected randomly on vertical collectors, such as coconut ropes 10 m long, placed for the REMEDIA Life project in the IMTA rearing system, while those in the control site were collected on artificial substrates (vertical metal poles 10 m long), located ~600 m from the IMTA site and partially sheltered by an artificial barrier.

The organisms and the sediments were collected by scuba divers and stored in ice (6–10 °C) until arrival at the laboratory. The fish feed (pellets) used by the mariculture company was taken from new containers to avoid contamination.

2.3. Stable Isotope Analyses

Solitary ascidians were dissected, and the body wall muscles were used for the stable isotope analysis. Mollusc species were analyzed without shells, while polychaetes were removed from the tubes. The remaining organisms were analyzed in full. Samples were dried (40 °C, 48 h), ground to powder with a mortar, and calcium carbonate-rich species and sediment samples were acidified with the addition of 10% HCl drop-by-drop until effervescence ceased. The acidification of samples is a common practice in stable isotope

analyses and aims to remove inorganic calcium carbonate from the organisms, known to interfere with $\delta^{13}C$ ratios [32]. After acidification, the treated samples were dried at 50 °C for 72 h. Afterwards, three pseudo replicates of each sample were weighed with a precision balance (±0.001 mg) into tin capsules (11 × 4 mm, Elementar Microanalysis, Okehampton, UK) to conduct the carbon/nitrogen (C/N) mass ratio and isotopic composition analyses. Around ~2 mg of each sample and ~10 mg for the sediment samples were analyzed using the Elementar IsoPrime 100 isotope ratio–mass spectrometry (IR–MS) instrument (IsoPrime Ltd., Manchester, UK) coupled to a CNS elemental analyzer (Elementar Vario Pyro Cube EA CNS; Elementar Analysensysteme GmbH, Frankfurt Germany). Sulfanilamide was used as reference material for determining the C/N mass ratio. Stable carbon isotopic values ($\delta^{13}C$) were quality checked and calibrated by using the reference materials Glucose (BCR-657) and Polyethylene (IAEA-CH-7), while for the stable nitrogen isotopic ($\delta^{15}N$) values, potassium nitrate (USGS32), caffeine (IAEA600), and ammonium sulfate (USGS25) were used. Vienna Pee Dee belemnite (V.P.D.B.) for carbon and atmospheric N2 (Air) for nitrogen were used as reference materials, and stable isotope values are here reported with respect to those. Stable isotope ratios ($\delta^{13}C$ and $\delta^{15}N$), in relation to the standards, were calculated as following:

$$\delta^{13}C \text{ or } \delta^{15}N(‰) = [Rsample \times Rstandard - 1] \times 1000 \quad (1)$$

where R corresponds to $^{13}C/^{12}C$ or $^{15}N/^{14}N$ of the analyzed sample (*Rsample*) and standard used (*Rstandard*). Data collection and analysis were performed with IonVantage (version 1,7,3,0) and ionOS (version 4.5.8.26) software (IsoPrime Ltd., Manchester, UK), respectively.

2.4. Statistical Analyses

The variability in $\delta^{13}C$, $\delta^{15}N$ (‰), and C:N ratio values in the investigated species was assessed in the IMTA and in the control sites by permutational analyses of variance [33] based on Euclidean distances of previously normalized data, using 9999 random permutations of the appropriate units [34]. The design consisted of two factors: Aquaculture (AQ, as a fixed factor with 2 levels, i.e., IMTA and control sites) and Species (SP, as a fixed factor with 16 levels) with n = 3. Because of the restricted number of unique permutations in the pairwise tests, p-values were obtained from Monte Carlo tests. The analyses were performed using PRIMER v. 6 software [34], including the PERMANOVA + add-on package [35,36].

3. Results

The list of the sampled species and the results of the stable isotopes analysis of carbon ($\delta^{13}C$), nitrogen ($\delta^{15}N$), and carbon to nitrogen (C:N) ratio values are summarized in Table 1, along with the values found in the literature [37–41]. When multiple data from the same species were present in the literature, only values from studies conducted in similar conditions to the present study were shown in Table 1.

Table 1. List of the species analyzed and comparison of observed $\delta^{13}C$, $\delta^{15}N$ (‰) and C:N ratio values with literature values. Reference [41] refers to *Schizoporella* sp. values.

Taxa	Sampled Species	Variables	Authors	Literature Values	IMTA	Control
Porifera	*Paraleucilla magna* Klautau, Monteiro and Borojevic, 2004	$\delta^{15}N$ (±s.d.) (‰)			6.72 ± 0.23	8.30 ± 0.10
		$\delta^{13}C$ (±s.d.) (‰)			−14.56 ± 2.02	−9.55 ± 1.48
		C:N (±s.d.)			7.45 ± 0.23	12.02 ± 1.63
	Sarcotragus spinosulus Schmidt, 1862	$\delta^{15}N$ (±s.d.) (‰)			2.66 ± 0.10	1.65 ± 0.03
		$\delta^{13}C$ (±s.d.) (‰)			−18.87 ± 0.04	−18.04 ± 0.07
		C:N (±s.d.)			3.34 ± 0.02	3.43 ± 0.04

Table 1. *Cont.*

Taxa	Sampled Species	Variables	Authors	Literature Values	IMTA	Control
Porifera	*Dysidea avara* (Schmidt, 1862)	δ^{15}N (±s.d.) (‰)			7.30 ± 0.19	8.02 ± 0.16
		δ^{13}C (±s.d.) (‰)			−20.41 ± 0.01	−19.99 ± 0.10
		C:N (±s.d.)			4.64 ± 0.11	4.48 ± 0.07
	Haliclona (Reniera) mediterranea Griessinger, 1971	δ^{15}N (±s.d.) (‰)			8.56 ± 0.59	9.36 ± 0.07
		δ^{13}C (±s.d.) (‰)	[37]	−22.50	−20.18 ± 0.16	−20.24 ± 0.11
		C:N (±s.d.)			4.54 ± 0.18	4.48 ± 0.14
Polychaeta	*Branchiomma luctuosum* (Grube, 1870)	δ^{15}N (±s.d.) (‰)			7.28 ± 0.24	8.00 ± 0.11
		δ^{13}C (±s.d.) (‰)			−18.64 ± 0.04	−18.82 ± 0.32
		C:N (±s.d.)			3.44 ± 0.05	3.82 ± 0.28
	Branchiomma boholense (Grube, 1878)	δ^{15}N (±s.d.) (‰)			7.01 ± 0.28	6.81 ± 0.15
		δ^{13}C (±s.d.) (‰)			−19.01 ± 0.06	−18.42 ± 0.12
		C:N (±s.d.)			3.87 ± 0.07	3.75 ± 0.05
	Sabella spallanzanii (Gmelin, 1791)	δ^{15}N (±s.d.) (‰)	[38]	8.85 ± 0.48	6.38 ± 0.30	6.68 ± 0.20
		δ^{13}C (±s.d.) (‰)	[38]	−21.26 ± 0.91	−22.10 ± 0.06	−21.37 ± 0.55
		C:N (±s.d.)			5.72 ± 0.06	5.60 ± 0.40
Mollusca	*Pinctada radiata* (Leach, 1814)	δ^{15}N (±s.d.) (‰)	[39]	11.60	6.05 ± 0.14	6.66 ± 0.12
		δ^{13}C (±s.d.) (‰)	[39]	−17.40	−20.02 ± 0.27	−20.09 ± 0.19
		C:N (±s.d.)			2.80 ± 0.07	3.97 ± 0.34
	Mytilus galloprovincialis Lamarck, 1819	δ^{15}N (±s.d.) (‰)	[38]	6.55 ± 1.20	6.01 ± 0.47	6.10 ± 0.69
		δ^{13}C (±s.d.) (‰)	[38]	−22.61 ± 0.57	−20.65 ± 0.24	−20.46 ± 0.29
		C:N (±s.d.)	[40]	4.31 to 6.01	5.55 ± 0.03	4.91 ± 0.38
Tunicata	*Botryllus schlosseri* (Pallas, 1766)	δ^{15}N (±s.d.) (‰)	[41]	6.40 to 7.30	5.19 ± 0.47	6.04 ± 0.56
		δ^{13}C (±s.d.) (‰)	[41]	−18.30 to −18.70	−19.67 ± 0.08	−18.15 ± 0.36
		C:N (±s.d.)			3.53 ± 0.13	3.96 ± 0.08
	Lissoclinum weigelei Lafargue, 1968	δ^{15}N (±s.d.) (‰)			6.25 ± 0.11	7.13 ± 0.30
		δ^{13}C (±s.d.) (‰)			−19.35 ± 0.07	−15.76 ± 1.11
		C:N (±s.d.)			4.37 ± 0.08	3.99 ± 1.40
	Ciona robusta Hoshino and Tokioka, 1967	δ^{15}N (±s.d.) (‰)			6.81 ± 0.14	6.71 ± 0.13
		δ^{13}C (±s.d.) (‰)			−17.48 ± 0.49	−19.69 ± 0.07
		C:N (±s.d.)			5.54 ± 0.18	4.90 ± 0.04
	Ascidiella aspersa (Müller, 1776)	δ^{15}N (±s.d.) (‰)	[38]	7.30	7.51 ± 0.23	7.91 ± 0.25
		δ^{13}C (±s.d.) (‰)	[38]	−20.86	−16.54 ± 1.30	−18.95 ± 0.13
		C:N (±s.d.)			7.92 ± 1.22	6.90 ± 0.48
	Phallusia mammillata (Cuvier, 1815)	δ^{15}N (±s.d.) (‰)			4.20 ± 1.02	5.70 ± 0.23
		δ^{13}C (±s.d.) (‰)	[37]	−23.60	−21.86 ± 0.34	−20.30 ± 0.16
		C:N (±s.d.)			7.86 ± 0.96	6.03 ± 0.28
	Styela plicata (Lesueur, 1823)	δ^{15}N (±s.d.) (‰)	[38]	6.90	7.52 ± 0.33	6.44 ± 0.09
		δ^{13}C (±s.d.) (‰)	[38]	−22.23	−19.99 ± 0.23	−17.29 ± 0.39
		C:N (±s.d.)			5.71 ± 0.75	6.99 ± 0.75
Bryozoa	*Schizoporella errata* (Waters, 1878)	δ^{15}N (±s.d.) (‰)	[41]	6.70 ± 0.23	4.67 ± 2.01	5.87 ± 0.54
		δ^{13}C (±s.d.) (‰)	[41]	−16.50	−13.33 ± 2.92	−6.11 ± 2.50
		C:N (±s.d.)			25.02 ± 22.96	53.69 ± 60.76

3.1. Porifera

Among analyzed Porifera species, δ^{15}N ranged from 1.65 ± 0.03‰ to 9.36 ± 0.07‰, respectively, found in *S. spinosulus* and *H. mediterranea*, while the lowest measured δ^{13}C value was recorded in *D. avara* (−20.41 ± 0.01‰) and the highest in *P. magna* (−9.55 ± 1.48‰) (Figure 3a). The C:N ratio ranged from 3.34 ± 0.02 (*S. spinosulus*) to 12.02 ± 1.63 (*P. magna*, Table 1). The only value found in the literature for sponges corresponds to the δ^{13}C of *H.*

mediterranea, which in Dauby [37] appeared slightly lower (−22.50‰) than the obtained results (−20.18 ± 0.16‰ at the IMTA site and −20.24 ± 0.11‰ at the Cnt site; Table 1). Significant differences between IMTA vs. control sites were found in $\delta^{13}C$ and $\delta^{15}N$ values of the sponges *P. magna S. spinosulus* and *D. avara*, whilst C:N ratio of *P. magna* and *S. spinosulus* resulted to be significant between IMTA vs. control site (Tables 2 and 3).

Table 2. Results of PERMANOVA testing for the effects of IMTA on $\delta^{13}C$, $\delta^{15}N$ (‰) and C:N ratio values in investigated species. Aq = Aquaculture; Sp = Species; df = degree of freedom; MS = mean squares; Pseudo-*F* = *F* critic; *P*(perm) = permutational level of probability.

Source	**df**	**MS**	**Pseudo-*F***	***P*(perm)**
			$\delta^{15}N$ (‰)	
Source	df	MS	Pseudo-*F*	*P*(perm)
Aq	1	4.72	19.36	0.0002
Sp	15	14.26	58.46	0.0001
AqxSp	15	0.88	3.59	0.0003
Res	64	0.24		
Total	95			
			$\delta^{13}C$ (‰)	
Aq	1	35.22	44.07	0.0001
Sp	15	59.75	74.77	0.0001
AqxSp	15	9.11	11.40	0.0001
Res	64	0.80		
Total	95			
			C:N ratio	
Aq	1	199.26	2.12	0.1628
Sp	15	661.53	7.02	0.0005
AqxSp	15	176.01	1.87	0.0265
Res	64	94.18		
Total	95			

Table 3. Results of the pairwise tests contrasting $\delta^{13}C$, $\delta^{15}N$ (‰) and C:N ratio values in investigated species between IMTA vs. control site (Cnt). *P*(MC) = probability level after Monte Carlo simulations; t = pairwise tests.

	$\delta^{15}N$		**$\delta^{13}C$**		**C:N**	
	t	***P*(MC)**	**t**	***P*(MC)**	**t**	***P*(MC)**
			P. magna			
IMTA vs. Cnt	11.08	0.001	3.45	0.0256	4.79	0.0101
			S. spinosulus			
IMTA vs. Cnt	16.41	0.0001	15.70	0.0002	2.90	0.0442
			D. avara			
IMTA vs. Cnt	4.85	0.008	6.61	0.0032	1.95	0.123
			H. mediterranea			
IMTA vs. Cnt	2.30	0.085	0.81	0.4633	0.71	0.5066
			B. luctuosum			
IMTA vs. Cnt	4.59	0.0107	0.96	0.3904	2.28	0.0907
			B. boholense			
IMTA vs. Cnt	1.06	0.3447	7.15	0.0021	2.00	0.1194
			S. spallanzanii			
IMTA vs. Cnt	1.36	0.2449	2.26	0.0876	0.49	0.6373
			P. radiata			
IMTA vs. Cnt	5.41	0.0052	0.39	0.7164	5.69	0.0035

Table 3. *Cont.*

	$\delta^{15}N$		$\delta^{13}C$		C:N	
	t	***P*(MC)**	**t**	***P*(MC)**	**t**	***P*(MC)**
			M. galloprovincialis			
IMTA vs. Cnt	0.19	0.8562	0.84	0.4441	2.84	0.0457
			B. leachii			
IMTA vs. Cnt	1.98	0.1154	6.99	0.0015	4.79	0.0088
			L. weigelei			
IMTA vs. Cnt	4.66	0.009	5.58	0.0056	0.46	0.669
			C. robusta			
IMTA vs. Cnt	0.83	0.4526	7.69	0.0019	5.94	0.0039
			A. aspersa			
IMTA vs. Cnt	1.97	0.1188	3.18	0.0286	1.35	0.2536
			P. mammillata			
IMTA vs. Cnt	2.47	0.0625	7.11	0.0015	3.12	0.0374
			S. plicata			
IMTA vs. Cnt	5.44	0.0041	10.11	0.0005	2.07	0.1088
			S. errata			
IMTA vs. Cnt	0.90	0.4241	3.25	0.0324	1.36	0.245

3.2. Polychaeta

Regarding polychaetes, $\delta^{15}N$ ranged from 6.38 ± 0.30‰ (*S. spallanzanii*) to 8.00 ± 0.11‰ (*B. luctuosum*), $\delta^{13}C$ ranged from −22.10 ± 0.06‰ (*S. spallanzanii*) to −18.42 ± 0.12‰ (*B. boholense*), and the C:N ratio ranged from 3.44 ± 0.05 (*B. luctuosum*) to 5.72 ± 0.06 (*S. spallanzanii*) (Figure 3b). Carbon and nitrogen values of *S. spallanzanii* were found to be similar to the values reported by Bongiorni et al. [38] (Table 1). Significant differences were found in $\delta^{13}C$ values of *B. luctuosum* in $\delta^{15}N$ values of *B. boholense* between IMTA vs. reference site (Tables 2 and 3).

3.3. Mollusca

Concerning Mollusca, $\delta^{15}N$ ranged from 6.01 ± 0.47‰ (*M. galloprovincialis)* to 6.66 ± 0.12‰ (*P. radiata)*, $\delta^{13}C$ showed similar values ranging from −20.65 ± 0.24‰ (*M. galloprovincialis)* to −20.02 ± 0.27‰ (*P. radiata*) and the C:N ratio ranged from 2.80 ± 0.07 (*P. radiata)* to 5.55 ± 0.03 (*M. galloprovincialis*) (Tables 2 and 3, Figure 3c). In Bouillon et al. [39], *P. radiata* individuals showed higher $\delta^{15}N$ value (11.60‰) and lower $\delta^{13}C$ value (−17.40‰) with respect to our results ($\delta^{15}N$ of 6.05 ± 0.14‰ at the IMTA site and 6.66 ± 0.12‰ at Cnt site and $\delta^{13}C$ of −20.02 ± 0.27‰ at IMTA site and −20.09 ± 0.19‰ at the Cnt site). For *M. galloprovincialis*, the observed $\delta^{15}N$ and C:N ratio values were similar to those reported in the literature [22,30], while slightly lower $\delta^{13}C$ values (−22.61 ± 0.57‰) with respect to our results (−20.65 ± 0.24‰ at the IMTA site and −20.46 ± 0.29‰ at the Cnt site) were found in Bongiorni et al. [38] (Table 1). Significant differences were found in $\delta^{13}C$ values of *P. radiata* and in C:N ratio of *P. radiata* and *M. galloprovincialis* between IMTA vs. reference site (Tables 2 and 3).

3.4. Tunicata

Tunicates $\delta^{15}N$ ranged from 4.20 ± 1.02‰ (*P. mammillata)* to 7.91 ± 0.25‰ (*A. aspersa)*, $\delta^{13}C$ ranged from −21.86 ± 0.34‰ (*P. mammillata)* to −15.76 ± 1.11‰ (*L. weigelei*) and the C:N ratio ranged from 3.53 ± 0.13 (*B. schlosseri)* to 7.92 ± 1.22 (*A. aspersa*) (Tables 2 and 3, Figure 3d). *B. schlosseri* showed $\delta^{13}C$ and $\delta^{15}N$ values similar to those reported in Schaal et al. [41], while *A. aspersa* showed higher $\delta^{13}C$ values (−16.54 ± 1.30‰ at the IMTA site and −18.95 ± 0.13‰ at Cnt site) respect to Schaal et al. [41] (−20.86‰), maintaining similar $\delta^{15}N$ values (Table 1). Significant differences were found in $\delta^{15}N$ values of all investigated

tunicates, in $\delta^{13}C$ values of *L. weigelei* and *S. spicata*, and in C:N ratio of *B. leachii*, *C. robusta* and *P. mammillata* between IMTA vs. reference site (Tables 2 and 3).

3.5. Bryozoa

S. errata was the only representative of the Bryozoa group, with $\delta^{15}N$ ranging from 4.67 ± 2.01‰ to 5.87 ± 0.54‰, $\delta^{13}C$ ranging from −13.33 ± 2.92‰ to −6.11 ± 2.50‰ and the C:N ratio ranging from 25.02 ± 22.96 to 53.69 ± 60.76 (Figure 3e). The $\delta^{13}C$ values resulted in being higher than those reported in Schaal et al. [41] (−16.50‰; Table 1). Significant differences were found in $\delta^{13}C$ values of the investigated bryozoan between IMTA vs. reference site (Tables 2 and 3).

3.6. Aquaculture Feed and Sediment Samples

The aquaculture feed showed an average $\delta^{15}N$ value of 4.46 ± 0.17‰ and an average $\delta^{13}C$ value of −23.51 ± 0.07‰, while IMTA and Cnt sediments showed, respectively, $\delta^{15}N$ average values of 6.28 ± 0.93‰ and 5.20 ± 1.64‰ and, respectively, $\delta^{13}C$ average values of −12.87 ± 3.42‰ and −14.05 ± 4.47‰. The distance between the $\delta^{13}C$ and $\delta^{15}N$ values of the sampled taxa and the sediment and the aquaculture feed values are shown in Figure 4. The carbon and nitrogen stable isotope bi-plot represents a total isotopic range of 7.71‰ for $\delta^{15}N$ and of 17.41‰ for $\delta^{13}C$.

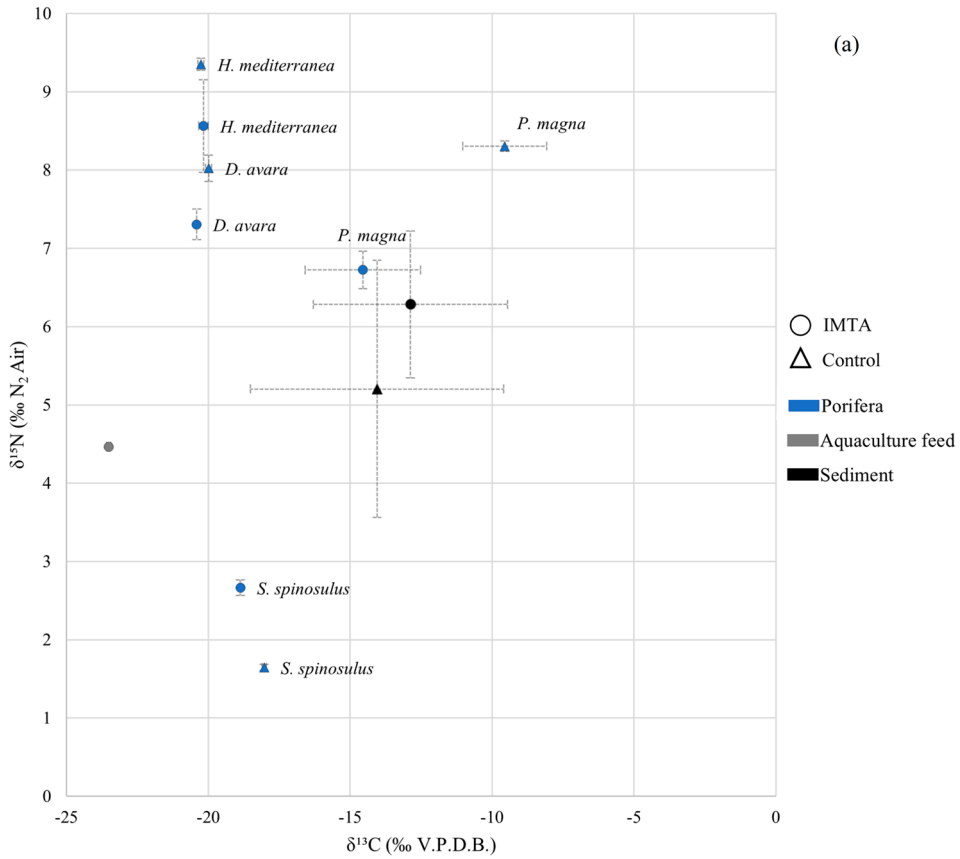

Figure 3. *Cont.*

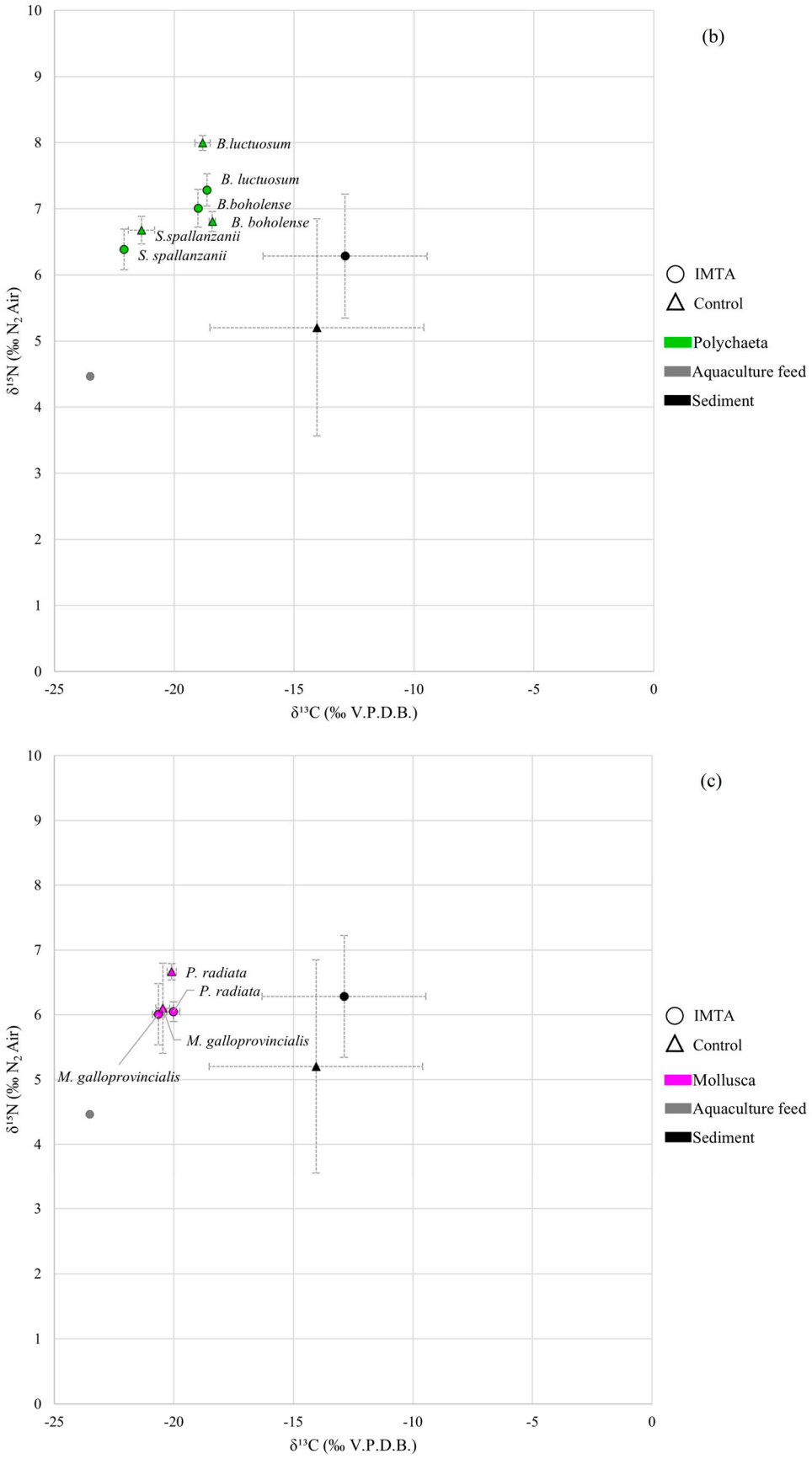

Figure 3. *Cont*.

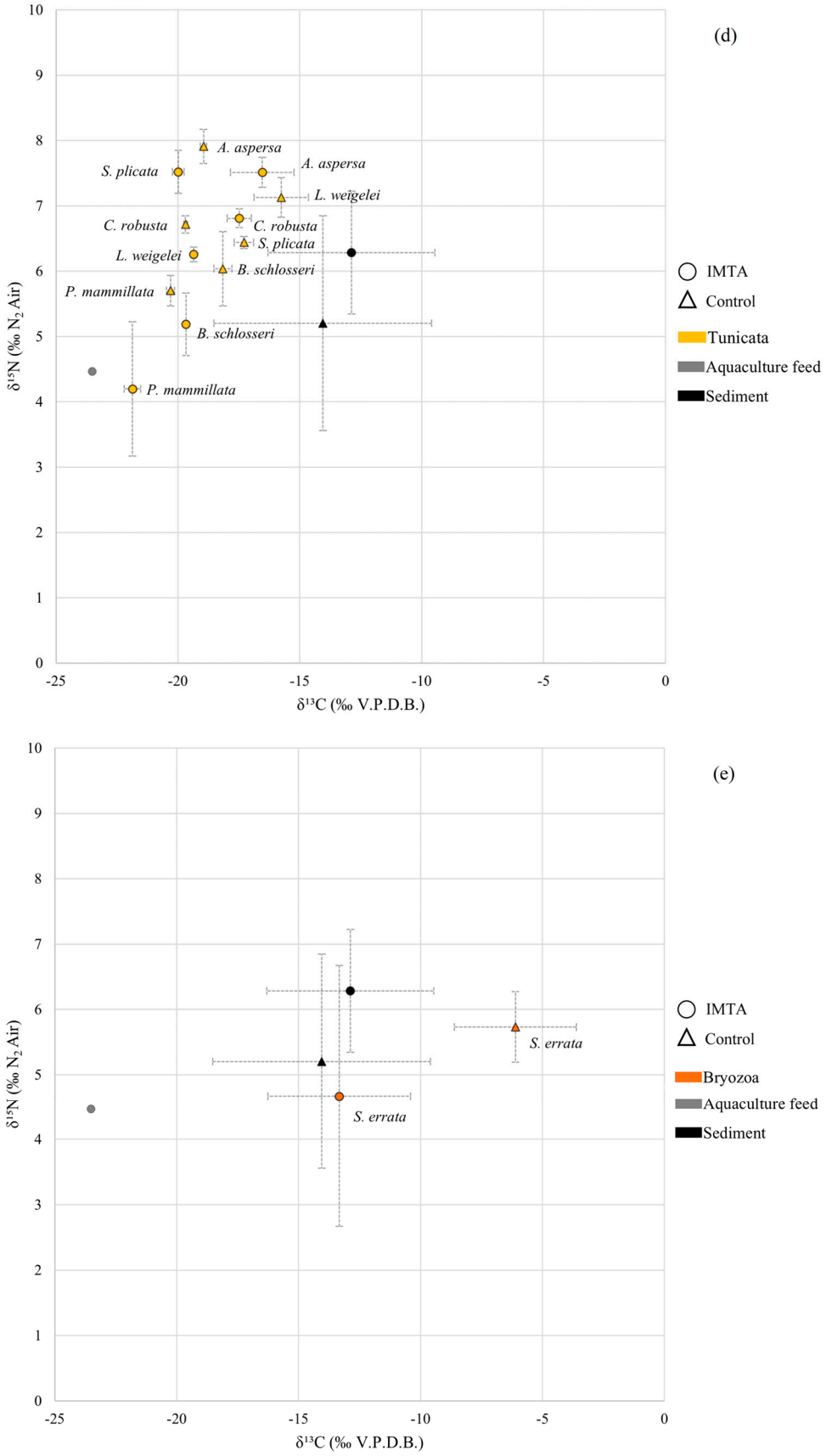

Figure 3. Bi-plots of the mean carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) stable isotopes of Porifera (**a**), Polychaeta (**b**), Mollusca (**c**), Tunicata (**d**), and Bryozoa (**e**) and the sediments sampled at the IMTA and Cnt sites, along with the aquaculture feed. Dashed gray lines represent the standard deviation for each plot.

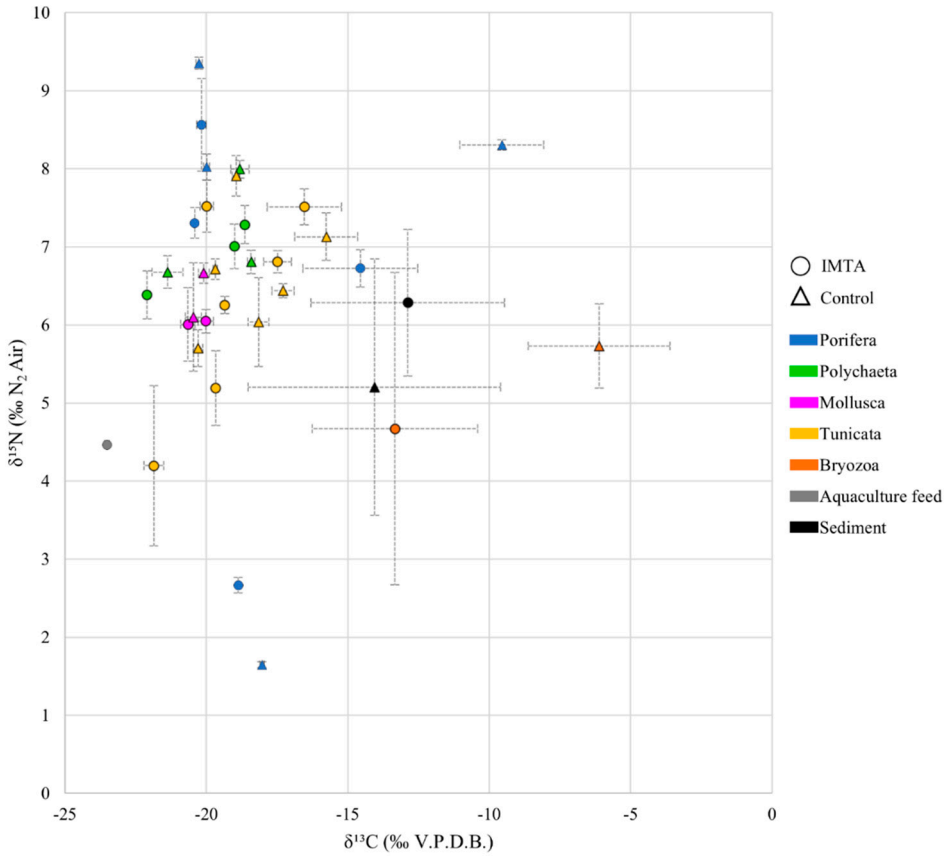

Figure 4. Bi-plot of the mean carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) stable isotopes of the species grouped in higher taxa and the sediments sampled in the IMTA and Cnt sites and the aquaculture feed. Dashed gray lines represent standard deviation.

4. Discussion

Even if there are many significant statistical results when the same species is compared between the IMTA and Control site, we have to be cautious with such comparisons. We have to consider that only when there is a difference above 3‰ in $\delta^{15}N$ can we assess there is a shift in this species in terms of trophic position (e.g., from herbivorous to omnivorous).

Food webs in coastal marine ecosystems are fueled by pools of suspended particle matter, which are often composed of a complex mix of phytoplankton, local benthic primary producers (macroalgae and/or phanerogams), zooplankton, and riverine terrestrial materials [42]. Such a mixture sometimes makes it difficult to discern the primary sources of food and the position of the different organisms [43]. The environments affected by finfish mariculture discharges are exposed to a mixture of the natural seston supplemented with daily fish wastes, especially those from in-shore facilities located in enclosed areas with limited waste dispersion [44]. Such mixture could influence the quantity and quality of seston, affecting the natural isotopic composition of the suspension feeder [9,45]. Such finfish food may have different compositions, mainly depending on the trophic position of the fish [46]. This variable food source may influence the surrounding benthic organisms, especially suspension feeders [47]. Certain species, such as algae and shellfish, may absorb waste nutrients from fish, and stable isotope analysis is instrumental in tracing nutrient pathways and assessing the efficiency of IMTA setups [48].

The first observation to consider in the present study, however, is the lack of information regarding the isotopic composition of several of the sampled species. Thus, these preliminary results can serve as future comparisons to further investigate their ecology, especially those found in the control site. Where information was present, the species analyzed showed similar $\delta^{15}N$ values to the cited literature data (differences were less than

3.4–5.0‰ in $\delta^{15}N$), resulting in the same trophic level, except for *P. radiata*, which showed a lower $\delta^{15}N$ value (~6‰) than what is reported in Bouillon et al. [39] (~11‰, Table 1).

4.1. IMTA and Cnt Site Values

Similar $\delta^{15}N$ values were found between the IMTA and Cnt sites. As expected, organisms in the IMTA system have not changed trophic level with respect to the Cnt site. Most of them are in the range of grazers-omnivores (passive and active suspension feeders) in Mediterranean coastal areas [49]. However, differences in $\delta^{13}C$ values were found in several organisms between the two sites, suggesting a possible different primary source of the organic matter supporting the trophic web [17]. Almost all species on the IMTA site reported $\delta^{13}C$ values lower than the Cnt site, being more similar to the isotopic signature of the aquaculture finfish food. It has been suggested that mobile benthic organisms such as brittle stars, urchins, and brown crabs have similar trophic signatures with respect to aquaculture finfish food [50]. According to Colombo et al. [51], the farm may contribute to the depletion of the isotopic signatures among organisms within the vicinity of the site, in line with the present results. This may also happen in the case of Taranto, as they are suspension-feeding organisms capable of processing an important part of the finfish waste products derived from the daily feed of the cultured fishes. Waste nutrients from fish farming can be effectively absorbed by other organisms within an IMTA system [52]. They used stable isotope analysis to quantify nitrogen flow, confirming that waste from one species can indeed serve as a resource for others in the setup. Interestingly, the macrozoobenthic species in the present study may benefit from reworking of the seston by different protozoans, accumulating $\delta^{15}N$ in their analyzed tissues [53]. In the case of sponges, it seems that the microbiome may be responsible for the shift from the lower $\delta^{15}N$ levels to the moderate high levels, as suggested by Vinha et al. [54] for deep-sea porifera.

The wide IMTA isotopic range for $\delta^{15}N$ and for $\delta^{13}C$ (Figure 3) suggests a broad spectrum of bioremediating organism diets that can then actively mitigate the impacts of mariculture from different fronts by capturing different particles and using various food sources, coming directly or indirectly from the mariculture activity. Nutrient cycling can be visualized through isotopic ratios, helping to optimize the species used within a system for maximum ecological synergy [55]. This point is important because it has been suggested that not all the suspension-feeding organisms may feed on the finfish waste food [27]. Different feeding strategies (passive or active suspension feeding, diet, or the organisms, etc.) [30] may explain such different food capture among the benthic suspension feeders of the present study.

4.2. Porifera Analyses

Polychaeta and Mollusca, but also Tunicata, showed smaller interspecific differences within the taxon than those observed for Porifera (Figure 3). The ability of sponges to incorporate nitrogen and carbon from particulate organic matter is effectively analyzed using stable isotope analysis. This method can trace nutrient pathways and assess the ecological interactions between sponges and other species within the IMTA system, demonstrating the extent of their bioremediation function [56]. Indeed, the stable isotope analysis of sponge species revealed a wide range in $\delta^{13}C$ and $\delta^{15}N$ signatures (Figure 4), confirming interspecific differences in diet and isotopic niche space of sponges [57,58]. Endosymbiont communities also influence the stable isotope signatures of their sponge hosts. Both the sponge host and associated bacteria jointly determine the typical $\delta^{13}C$ and $\delta^{15}N$ signature of the sponge holobiont [54,58,59]. Sponges hosting phototrophic endosymbionts, which fix CO_2 tend to have lower $\delta^{13}C$ values than sponges without phototrophic endosymbionts, and sponges hosting N_2-fixing endosymbionts tend to have lower $\delta^{15}N$ values [60], so the results found in terms of $\delta^{15}N$ for the species *Sarcotragus spinulosus* may be in line with such a mixotrophic trophic strategy [61]. In addition, it is reasonable to hypothesize that the sponges sampled in the control site and in the IMTA site showed different isotopic signatures as they are highly sensitive and adaptable to the available food source, which

is different in the two sampling stations [62]. Although for sponges the isotope signature range is rather wide, the samples collected in the IMTA plant come closer to the isotopic fingerprint here found. In particular, specimens of *P. magna*, the alien invasive sponge largely found and collected in the IMTA plant, showed the closest range of the facility, probably due to its rapid life cycle and Low Microbial Abundance functional trait [63,64]. It is also known that sponges are able to retain smaller particles compare to other filter-feeding organisms [65], although they can assimilate more carbon from larger organisms when available (e.g., nano eukaryotes) [65] as well as a high amount of dissolved organic matter [66,67]. This represents an adaptive advantage that the different isotopic signature detected in the two sampling stations has highlighted.

4.3. Polychaeta Analyses

The polychaete species analyzed belong to the Sabellidae family, characterized by the presence of a branchial crown, which protrudes out of the tube they inhabit to collect and sort material of different sizes [68–72]. Most of the field and laboratory studies on filter feeder polychaetes have been carried out employing phytoplankton and/or bacteria as a trophic source [68–73]. *Sabella spallanzanii* (autochthonous species) have a broad range of trophic plasticity, consuming both phytoplankton and detrital organic materials from the water column [69–73]. This may be evidence that the $\delta^{15}N$ is in line with an omnivorous filter-feeder, capable of processing also the reworked protozoans' detritus material of the near-bottom seston more than the invasive *Branchiomma* species. In fact, this species may be one of the most relevant species in the IMTA facilities of Taranto, probably responsible for a significant seston depletion [31,74]. As bioremediatory organisms, polychaetes also have influence on microplastic retention [75], efficiently assimilating nutrients and contributing to waste mitigation [76].

4.4. Mollusca Analyses

M. galloprovincialis (autochthonous species) and *P. radiata* (invasive species) are two common bivalves abundant in the Mar Grande of Taranto, with the former employed in intensive farming in the area near the fish facilities. The use of bivalves in IMTA systems has produced different results, with a faster rate near fish farms in some circumstances [77,78], but in other cases the fish farms did not seem to influence their growth [54,79,80]. This was also observed in an experiment involving *M. galloprovincialis* farmed in the IMTA system of Taranto [81].

The observed changes in growth rate, however, may not always prove that bivalves are assimilating fish farming wastes. In situ studies have shown that fish farming wastes do not make up a substantial part of their diet [54,82,83].

Mytilus species are known to be generalist suspension feeders. Field studies using stable isotopes have confirmed that mussels can ingest and assimilate organic waste from fish farms [25], suggesting that aquaculture activities play an important role in nutrient cycling. However, some authors offer a contrasting view, arguing that the exclusive use of bivalves is not an effective tool for reducing the environmental impact of fish farming [27,84]. Using stable isotope analysis, these researchers found that mussels did not directly assimilate fish farm waste, and therefore, it did not constitute a major component of their diet.

In the present observations, the $\delta^{13}C$ and $\delta^{15}N$ values of molluscs are similar to those of polychaetes, suggesting that bivalves may be omnivorous. However, there does not appear to be a substantial difference in the values of individuals between the two sites. Further studies are needed to clarify the actual role of phytoplankton in their diet.

4.5. Tunicata Analyses

Tunicates are almost all filter feeders, eating phytoplankton and other small particles such as detritus [85,86]. Among the sampled species, four were solitary ascidians (*C. robusta*, *A. aspersa*, *P. mammilla*, and *S. plicata*) and two were colonial species (*B. schlosseri* and *L.*

weigelei). The sampled solitary ascidians pump water through the inhalant siphon into the pharyngeal basket, where particles remain entrapped on a mucus net [87], while the colonial strategists are encrusting organisms, bearing up to several hundred morphologically identical units (zooids) embedded with a translucent gelatinous matrix. In a single colony, all zooids are parabiosed to each other through a ramified network of blood vessels [88]. All of them seemed to have a similar impact on the seston with also similar $\delta^{13}C$ and $\delta^{15}N$ values (Figure 3d).

4.6. Bryozoa Analyses

The last group studied in the present work were bryozoans. Bryozoans are able to build large masses and be significant rock-former species [89–91], used to capture suspended phytoplankton, protozoa, and detrital organic particles. Species of the genus *Schizoporella* are among the most important builders [92]. *S. errata* together with the sponge *P. magna*, and the sediment samples, showed high standard deviation values both for $\delta^{13}C$ and $\delta^{15}N$. These samples contained significant amounts of nondietary carbon ($CaCO_3$) that may have influenced the results despite the acid treatment [93]. Indeed, *S. errata* showed lower $\delta^{13}C$ values with respect to the literature data, especially in the Cnt samples (Table 1).

5. Conclusions

Stable isotopes are a powerful trophic tool that indicate trophic level and potential trophic sources. In our study, it is clear that none of the organisms has a real shift in the trophic level, most of them relying on a reworked detritus-alive material that is especially abundant in the Gulf of Taranto with a high biochemical quality [31,74]. The detritus from the finfish food has a clear influence on most of the organisms, especially those that rely on detrital food. Thus, an important part of the biomass of bioremediating organisms comes from the seston derived from the aquaculture facility, both directly and indirectly, confirming the effectiveness of the IMTA system in mitigating the negative impacts of mariculture activities. Further studies may elucidate the proportions of the food source that, in any case, will be influenced by the microbial loop associated with the mixture of artificial (fish food) and natural (phytoplankton, zooplankton, and macroalgae-phanerogam detritus) particles.

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