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Abstract

The use of detritivores under sea farms is a promising avenue to mitigate the benthic impacts of marine fish farms. Sea cucumbers are interesting candidates for integrated multi-trophic aquaculture (IMTA) due to their prevalence in the marine environment, their diversified diet and their economic value. Yet limited information is available regarding their capacities to be stocked and reared underneath aquaculture cages and the associated effects on their survival, growth rate and body composition. This study focused on *Holothuria tubulosa*, a Mediterranean sea cucumber species candidate for rearing in the vicinity to marine fish cages. We investigated its potential for co-culture on the seabed more or less influenced by marine fish cages. The farm's waste footprint was predicted using a dispersion model (NewDEPOMOD) to estimate the farm's influence along a transect where we also sampled sediment at four distances from the cages (0 m, 25 m, 100 m from the cages, plus a reference

site at 150 m). Organic composition of the sediment was analysed (TOC, TON, TOP, OM, stable isotope signature) and linked to the results from the dispersion model. Based on the model simulation, the maximum flux of matter reached almost 17 kg solids.m^{-2}.$year^{-1}$ below the cages, and gradually decreased with distance from the cages. An isotopic gradient was also found in the sediments according to the distance from the farm, with an enrichment in δN^{15} and a depletion in δC^{13} with increasing proximity to the farm. In parallel we investigated the response of adult sea cucumbers placed at varying distances from the fish cages for a period of one month, measuring their proximate composition, isotopic concentration, and fatty acid and protein composition. We found that despite good survival, growth was null over the experiment. While the isotope signature of the sea cucumbers was significantly affected by distance from the cage, this did not follow the pattern found in sediment. There was a clear difference in fatty acid composition between sites, with sea cucumbers closer to the cages having lower levels of short-chain fatty acids. The protein content was also lower in sea cucumbers reared right below the cages. These results suggest that while adult *H. tubulosa* can survive the environmental conditions below marine aquaculture cages, they do not nutritionally benefit from fish waste over short periods in the stocking conditions we tested. Their use in IMTA requires further investigation to find optimal stocking conditions.

Key words: detritivores, sea cucumber, integrated multi-trophic aquaculture, isotopes, fatty acids, organic footprint

1. Introduction

Detritivores have a crucial role in terrestrial ecosystem processes, as their presence avoids biotic detritus accumulation and facilitates nutrient cycling and further primary production (Schowalter, 2016). Their role is also central in marine ecosystems through their bioturbation

activities, as they can enhance microbial processes that play a major role in organic matter decomposition, mineralization and nutrient production (Göltenboth, 2006).

Among marine detritivores, sea cucumbers (*Echinodermata: Holothuroidea*) are vital members of benthic communities and are present all over the world. They have multiple ecological roles including (a) sediment bioturbation, (b) remediating organic load (Costa et al., 2014), (c) enhancing the productivity of benthic biota (e.g. seagrass, Costa et al., 2014, Wolkenhauer et al., 2010), (d) buffering against ocean acidification, (e) hosting more than 200 symbionts, and (f) acting as food sources for many animals, including humans (Purcell et al., 2016).

Sea cucumbers can have high economic value (Purcell et al., 2018). They are mostly exploited for food, principally in Asia, with body integument the main targeted product, which is dried, boiled, salted or cooked (Conand, 1990). Other food by-products such as fermented intestines and dried gonads are marketed in Japan, Korea and China (Stutterd and Williams, 2003). In East Asia, sea cucumbers are also used as traditional medicine to treat a number of conditions (Pangestuti and Arifin, 2018; Xue et al., 2015), including arthritis and joint pain. Scientific studies confirming the presence of a range of bioactive compounds in sea cucumbers have more recently attracted the attention of the pharmaceutical sector (Kiew and Don, 2012). Their saponins have been found to have anti-inflammatory and anti-cancer properties (Pangestuti and Arifin, 2018), and other molecules have been described as having antibacterial (Santos et al., 2016) and antifungal properties (Hamel and Mercier, 1997). Sea cucumbers are also used in certain cosmetics, including liniment, soap and toothpaste. Depending on the species, the selling price can vary dramatically, with some reaching more than 1000 $.kg^{-1} (e.g. *Apostichopus japonicus* and *Holothuria scabra*, Conand, 2017), which has resulted in strong

74 fishing pressure to supply expanding international demand. It is estimated that 200 million
75 sea cucumbers are extracted from marine ecosystems every year (Purcell et al., 2013; Tanzer
76 et al., 2015) and provide an important source of income to many coastal fishermen, but are
77 consequently threatened by overfishing (FAO, 2008). The development of sea cucumber
78 aquaculture could provide a solution to meet commercial demand on one hand, while equally
79 facilitating conservation by allowing the restocking of some species.

80 In aquaculture systems, particulate wastes, including the faeces of fish and uneaten feed,
81 settle in the vicinity of farms, potentially unbalancing the benthic environment once ecological
82 carrying capacity is exceeded (Hargrave et al., 1997; 2008). This accumulation of organic
83 matter is a major environmental stressor which can induce anoxic zones and increases
84 pathogen pressure, therefore having an impact on the health of both farmed animals and the
85 environment (Chopin et al., 2012; Dauda et al., 2019; Granada et al., 2016; Troell et al., 2009).
86 The organic footprint of an aquaculture farm can be evaluated through environmental
87 sampling or modelled using particle dispersion models such as KK3D, AWATS, or DEPOMOD
88 (Cromey et al., 2012; Dudley et al., 2000; Jusup et al., 2009; Riera et al., 2017).

89 Aquaculture effluents also stimulate biological activity, with organisms of different trophic
90 strategies aggregating in and around cage facilities to consume the waste (Ballester-Molto et
91 al., 2017; Callier et al., 2013; 2018). Integrated multi-trophic aquaculture (IMTA), which co-
92 cultivates species from different trophic levels, has been put forward as a potential tool to
93 mitigate aquaculture footprint (Chopin et al., 2012). Systems including detritivorous species
94 such as sea cucumbers, capable of consuming aquaculture waste material (Nelson et al.,
95 2012), have been successfully combined with many species. For example, associations have
96 been tested between sea cucumber and sea urchin (*Holothuria tubulosa* as extractive species

97 and *Paracentrotus lividus* as primary species, Grosso et al., 2021) or between sea cucumber
98 and fish such as *Sebastes melanops* and *Apostichopus japonicus* (Park et al., 2015),
99 *Anoplopoma fimbria* and *Parastichopus californicus* (Hannah et al., 2013), *Sciaenops ocellatus*
100 and *Isostichopus badionotus* (Felaco et al., 2020), *Dicentrarchus labrax* and *Holothuria forskali*
101 (MacDonald et al., 2013), and *Dicentrarchus labrax* or *Sparus aurata* and *Holothuria tubulosa*
102 (Neofitou et al., 2019; Tolon et al., 2017a,b).

103 The most common sea cucumber species in the Mediterranean are *H. tubulosa*, *H. forskalii*
104 and *H. poli* (Ocana and Sanchez Tocino, 2005). Of these, the deposit feeder *H. tubulosa*
105 (Gmelin, 1790) is the most widespread. Over the last decades, this species has been
106 increasingly harvested in the Mediterranean due to growing consumer demand in Asia, with
107 illegal fishing more and more frequently reported (Meloni and Esposito, 2018). Aquaculture
108 research has demonstrated successful results for the artificial reproduction and larval rearing
109 of *H. tubulosa* (Rakaj et al., 2017; Tolon et al., 2017b). However, little is known about their
110 capacities to survive, and grow on fish effluents in proximity to aquaculture marine cages, and
111 nothing is available on consequences on their body composition and consequently nutritional
112 value.

113 The aim of this study was to use biometric and biochemical measures to explore whether *H.*
114 *tubulosa* can benefit from fish farm waste when stocked at sites under different levels of
115 influence from marine aquaculture cages, identified using a deposition model. We
116 hypothesized that (a) *H. tubulosa* placed underneath fish cages would assimilate waste from
117 the farm, that (b) the distance to the farm would influence *H. tubulosa* isotopic composition
118 proportionally to the organic matter content in the sediment; and that (c) specific fatty acids
119 and higher protein content would be found in individuals in proximity to the farm. To test

120 these hypotheses, over a period of one month, we first identified sites more or less affected
121 by fish cages using the dispersion model DEPOMOD, then stocked *H. tubulosa* on the seabed
122 along a transect starting from the aquaculture cages and analysed isotopic values ($\delta^{15}N$ and
123 $\delta^{13}C$) in the sediment and in sea cucumbers as tracers of fish waste. Complementary analyses
124 of protein content and fatty acid composition in sea cucumbers were performed to obtain
125 information on *H. tubulosa*'s capacity to assimilate a new source of food in an IMTA context.

126 **2. Material and methods**

127 ***2.1 Study site and characteristics***

128 The study was conducted in the bay of Campomoro (Gulf of Valinco, southern Corsica, France),
129 which since 1992 has hosted a marine fish farm producing 150 tonnes of organic sea bass and
130 sea bream each year. The bay is around 1.2 km wide with a maximum depth of 65 m. The two
131 species are reared in separate cages from a body weight of 5 g to a commercial size of 500 g.
132 The farm had 32 square cages (6 x 6 x 9 m, WxLxH) and 2 round cages (16 x 17m, DxH). The
133 fish were fed every day by hand with an organic commercial diet from Gouessant or Skretting.
134 The farm's biological data (feed composition, feed quantity, fish species, fish biomass) were
135 provided by the farm.

136 ***2.2 Modelling of farm waste deposition***

137 The organic footprint of the farm was modelled using NewDEPOMOD software (v1.3.1-
138 patch02, SAMS). NewDEPOMOD is a lagrangian particle dispersion model structured in four
139 modules (i) grid generation, (ii) particle tracking, (iii) resuspension, and (iv) benthic fauna
140 response (benthic impacts).

141 The grid generation module allows to generate a grid domain based on bathymetry data and
142 cage positions and dimensions. Bathymetry data at an initial resolution of 111m were

143 extracted from the SHOM database (SHOM, 2015) and interpolated using the kriging method
144 in ArcGIS software (v.10.6.1) to obtain 10m x 10m resolution data. These bathymetry data
145 were then imported in NewDEPOMOD to create a grid for the entire bay of Campomoro. Cages
146 layout and positions were communicated by the farm and entered in NewDEPOMOD.

147 The particle tracking module calculates particle waste emission using farm rearing data and
148 describes their transport from the surface to the seabed based on current and particle settling
149 velocities. Solid waste emissions for sea bream and sea bass stocks were calculated in the
150 particle tracking module based on the stocking density and feeding ratios. Default waste
151 composition (Percentage of carbon in faeces composition 30%, Percentage of carbon in feed
152 49%, Percentage of water in feed composition 9%) and feed digestibility (Percentage of feed
153 absorbed by fish 85%, Default specific feeding rate - mass of feed in kg as a percentage of
154 biomass in kg 0.7%) data of the software were used. A value of 3% unconsumed feed was
155 assumed, based on previous studies (Cromey et al., 2002; 2012). Faeces settling velocities
156 parameters were set as lognormal distribution with a mean of 0.7 $cm.s^{-1}$ and 0.48 $cm.s^{-1}$ and
157 a distribution of 0.83 and 0.47 for seabass and seabream respectively (Magill et al., 2006). No
158 information on the settling velocity of feed pellets was available, so the software's default
159 value was used: a uniform distribution at 9.5 $cm.s^{-1}$. Current fields over the entire water
160 column were measured at the farm from 24 September to 24 October 2019 using an acoustic
161 current meter (3D-ADCP, WorkHorse Sentinel 600 kHz Teledyne®). The current meter was
162 placed 2 m from the cages at the extreme offshore end of the farm and recorded current
163 velocity and directions at a 5 minutes time step. Current velocity data was averaged at each
164 time point over three water layers (0–3 m, 7–10 m and 17–20 m) representative of the surface,
165 middle and bottom of the water column according to Hills et al. (2005) and used as forcing in
166 the particle tracking module, therefore assuming to be the same over the entire grid.

Dominant currents were found to be mostly flowing south/southeast with a velocity generally below 10 cm.s^{-1}. Default values were used for vertical (kz = 0.001 m^2 s^{-1}) and horizontal (kx = 0.1 m^2 s^{-1}, ky = 0.1 m^2 s^{-1}) dispersion coefficients (Cromey et al., 2002; Gillibrand and Turrell, 1997).

The resuspension module is used to describe seabed processes including erosion, transport, deposition and consolidation of the particles on the seabed. NewDEPOMOD's default resuspension parameters were used (Black et al., 2016).

Waste emission and deposition were simulated with NewDEPOMOD over one month, from 24 September to 24 October 2019. The software provided particulate waste deposition fluxes expressed in g solids.m^{-2}.$year^{-1}$ for each cell of the grid. The results were then transferred to ArcGIS to create the deposition graphs.

2.3 Specimen collection

In July 2020, 42 adult specimens of *H. tubulosa* naturally present in the study area were collected by scuba divers at a depth of 17 m near *Posidonia oceanica* meadows in the bay of Campomoro, outside the influence of the farm.

Before biometric measurements, holothurians (171.4 $\pm$ 64.3 g, mean WW $\pm$ SD) were kept fasted overnight to ensure gut content evacuation, as recommended by Tolon et al. (2017a). They were then anesthetized for 30 minutes in a mixture of menthol and ethanol (5.6 g/L ethanol) diluted in aerated seawater (1 L per 50 L). The body length and diameter (at the largest part) were then measured with a precision of 0.1 mm and 0.01 mm respectively. Wet weight (WW, in g) was measured in a tank filled with clear seawater on a scale with a precision of 0.1 g. Six randomly chosen individuals were then euthanized on ice, opened, and the body wall stored at -20°C for further analyses.

2.4 Experimental design

Four experimental sites to test the effect of aquaculture waste on sea cucumbers were identified along a transect: site “0” under the cages (20.4 m deep), site “25” at 25 m from the cages (21.3 m deep), site “100” at 98 m from the cages (23 m deep), and the “reference” site (19 m deep), located 250 m northwest of the cages, outside the influence of the farm. At each site, triplicate baskets with a triangular-prism shape and rigid 1-cm mesh (commonly called Australian oyster baskets) were fixed in the sediment. The baskets’ length, width and height were respectively 80 cm x 35 cm x 20 cm (effective sediment surface area of 0.28 m^{-2}). At the beginning of July 2020, three individuals were placed in each basket.

One month later, the baskets were brought to the surface, and the animals were anesthetized and weighed using the same protocol as at the start of the experiment. They were then euthanized on ice, opened, and the body wall was stored at -20°C until further analyses. In total, 33 animals out of the 36 originally placed in the baskets were recovered. The 3 lost animals (1 at site “reference”, and 2 at site “25 m”) may have died or escaped during the experiment.

2.5 Environmental data

The bottom water temperature (°C) near the experimental infrastructure was recorded at 30-minute intervals by an NKE-STPS logger fixed to the infrastructure (from 6 July to 6 August 2020). During the entire experiment, there was a stable water temperature of around 21.7 $\pm$ 1.3°C. At the beginning and the end of the experiment, samples of superficial sediment (1–3 cm) were collected by divers at each site using a corer (diameter 20 mm). Samples (3 cores per site, n = 23*) were kept in the dark and on ice before being frozen and processed within

30 days (ISO, 2004). *One sediment sample (at the end of the experiment and from site "reference") was partially spilled after being collected and was therefore discarded.

2.6 Trophic biomarkers

Dry weight (DW) was measured at a precision of 0.1 g after lyophilization for all samples (animals, sediment and fish feed). Lyophilized sea cucumbers (n = 33), sediment (n = 23) and feed (n = 4) were homogenized into powder using a grinder. Powdered samples were combusted in an Integra CN Analyzer and the resultant gases were introduced into a continuous-flow isotope ratio mass spectrometer (SERCON Integra CN) to determine C and N amounts as well as their isotope ratios, according to the procedure described in Raimbault et al. (2008). The stable isotope (SI) data was expressed as the relative difference between samples and standard reference materials as follows:

$$\delta X\ (‰) = \left(\frac{R_{sample}}{R_{standard}} - 1\right) \times 10^3$$

where X is ^{13}C or ^{15}N, and R is the ratio of heavy to light isotope ($^{13}C/^{12}C$ or $^{15}N/^{14}N$). $^{15}N/^{14}N$ gives clues about the animal food source and trophic level, upward movement tending to concentrate $\delta^{15}N$ isotopes by 3–4‰ at each stage in the food chain.

The particulate phosphorus was determined by wet oxidation using potassium persulfate (Raimbault et al., 1999). Carbon (TOC), nitrogen (TON) and phosphorus (TOP) levels were reported in %.

To further explore sea cucumber biochemical composition, crude protein content was determined according to the Kjeldahl method (N × 6.25) (Association of Official Analytical Chemists, 2000).

After extraction by acid transmethylation, fatty acid (FA) composition was analysed using gas chromatography (Varian CP 8400 GC equipped with a splitless injector and a flame-ionization

detector and using hydrogen as the mobile phase: see Mathieu-Resuge et al., 2020 for details). Results (mean values ± SD) were reported in % of total protein or FAs and/or in $mg.g^{-1}$ of sample dry weight (DW).

2.7 Statistical analysis

The results were expressed as mean ± standard deviation. One-way analysis of variance (ANOVA) was carried out with a parametric Fisher test (F) to determine the differences (in the weight, isotopes, protein and lipids of sea cucumbers and in the organic contents of the sediment) between all conditions (distance from farm: 0 m, 25 m, 100 m and reference site).

A linear model was fitted to explain stable isotope values using the site as an explanatory factorial variable. For sediment values, the sampling time (initial: beginning of experiment; final: end of experiment) and the interaction with the site was also added to the model. Non-significant interactions were removed. Post hoc tests were computed using least-squares means (using the "lsmeans" package in R) to compare differences between sites.

3. Results

3.1 Organic footprint: deposition simulations and particulate matter signature

NewDEPOMOD simulations

The deposition of organic matter was estimated to be very localized around the cages, with the deposition of 1000 g of $solids.m^{-2}.year^{-1}$ occurring at a geographical limit less than 20 m away from the cages (Fig. 1). Right below the cages, deposition was estimated to reach a maximum of 16 862 $g.m^{-2}.year^{-1}$.

For the four sites used in the experiment, simulations from NewDEPOMOD estimated a deposition of 6233.5, 882.8, 96.1 and 3.7 $g.m^{-2}.year^{-1}$ for sites "0", "25", "100" and "reference" respectively.

The analysis of the organic contents (TON, TOP, TOC) of the sediment confirmed the gradient simulated by NewDEPOMOD (Fig. 2). All varied significantly according to the distance from the cages – TON (F-value=14.906, df=3, *p*-value=0.002), TOP (F-value=25.358, df=3, *p*-value<0.001) and TOC (F-value=5.430, df=3, *p*-value=0.03) – with the highest values below the cages (0 m), indicating a farm footprint concentrated between 0 and 25 m from the cages. The correlation on a log-scale between TOP and NewDEPOMOD results was significant (p-value<0.001) with a R^2 of 0.99 (Supplementary Fig. 1).

3.2 Stable isotope (SI) signatures

The $\delta^{13}C$ and $\delta^{15}N$ values obtained for the samples of fish feed were −23.0 ± 0.2‰ and 7.9 ± 0.5‰ respectively (Fig. 3). The SI composition of sediment showed that δN^{15} varied significantly according to the sampling date (F-value=44.2, df=1, *p*-value<0.001) and the site (F-value=57.3, df=3, *p*-value<0.001, Fig 3B). No effect of sampling date or site was observed for $\delta^{13}C$. No interaction between sampling date and site was found for $\delta^{13}C$ and $\delta^{15}N$, so all samples were illustrated on the same graph to show general trends (Fig. 3A and B). An isotopic gradient was found in sediments according to the distance from the farm, indicating an enrichment in $\delta^{15}N$ with increasing proximity to the farm (Fig. 3A). Of the 4 sites, site “0” had the highest mean $\delta^{15}N$ value (5.2‰ at final sampling). The correlation (log-based) between sediment $\delta^{15}N$ and NewDEPOMOD results was significant (p-value=0.017) with a R^2 0.97 of (Supplementary Fig. 2).

Concerning the SI signature of *H. tubulosa,* $\delta^{15}N$ was significantly different between sites (F-value=4, df=4, *p*-value=0.009) with the “reference” site being significantly higher than site “0” (Fig. 4B). Stable C isotope ratios ($\delta^{13}C$) were not significantly different between sites (Fig. 4A).

3.3 Weight, fatty acid profiles and proximate composition of H. tubulosa

281 At the start of the experiment, the weight of the 36 living individuals was 171.4 ± 64.3 g (mean
282 WW ± SD). The 6 euthanized individuals weighed 131.5 ± 60.1g (mean WW ± SD) and their dry
283 body wall 12.0 ± 3.3 g (mean DW± SD). At the end of the experiment, animals weighed 158.76
284 ± 95.39g (mean WW ± SD) and their dry body wall 14.47 ± 5.80 g (mean DW± SD). No
285 significant difference between sites was observed for the final body wall weight and dry
286 weight (Fig. 5a and b).
287 A significant difference between sites (F-value= 4.201, df=3, *p*= 0.0364) was observed for
288 protein content (Fig. 6), with higher values for the "reference" site (60.95 ± 1.06%) compared
289 to site "0" (57.09 ± 3.32%).
290 Regarding the fatty acid profile (Table 1), the total fat content varied between 0.56 and 1.29
291 $\mu g.mg^{-1}$, representing 0.13% of the total composition of the animal. We identified 33 fatty
292 acids (FAs) in *H. tubulosa* samples, with high variability between replicates (Fig. 7). The PCA
293 performed on FA profiles showed that sites differed on the second axis (Fig. 7B). The FAs
294 contributing the most to this difference were the shortest FAs measured (Fig. 7C, 15:0, iso15:0,
295 16:0, etc.). These are usually also saturated FAs (SFAs) and this translated to the total SFAs
296 increasing with the distance from the cages (Table 1).
297 Table 1 shows the major classes of FA in the composition of samples, with palmitic acid (16:0),
298 stearic acid (18:0), dimethyl acetal DMA 18:0 (1,1-Dimethoxyoctadecane) and arachidonic acid
299 ARA (20:4n-6) being the most commonly found in the animals.
300 Most FAs in *H. tubulosa* samples were SFAs, representing from 42.4–50.8% of the total. A total
301 of ten monounsaturated fatty acids (MUFAs) were identified: 16:1n-7, 17:1n-x, 18:1n-7,
302 18:1n-9, 20:1n-7, 20:1n-9, 20:1n-11, 22:1n-7, 22:1n-9 and 24:1n-9. The major MUFA was
303 20:1n-11, with values that varied between 3.4% and 6.1%. Eight polyunsaturated fatty acids
304 (PUFAs) were measured, three belonging to the omega 3 category (16:4n-3, 18:4n-3, 20:5n-3)

and four others to the omega 6 category (16:2n-6, 18:2n-6, 20:2n-6, 20:4n-6). The major PUFA was arachidonic acid (ARA 20:4n-6) with a maximal value of 13.7%. The sum of highly unsaturated fatty acids (HUFAs; fatty acids with ≥ 20 carbon chain length and two double bonds, i.e. 20:2n-6, 20:4n-6 and 20:5n-3) varied between 14.4% at 0 m, 11.3% at 25 m, 12.5% at 50 m and 13.1% at 150 m.

Finally, the sea cucumber FA composition also presented micro-organism markers (Table 2), with a gradient that increased from the farm (17.3%, at site "0") to the reference site (22.4%), but that was lower at all sites than the proportion found in wild individuals (30.8%).

4. Discussion

Localized aquaculture footprint

The deposition simulation and the sediment samplings were performed at two separated seasons, but a good relationship was observed between estimated fluxes and sediment element composition, especially for total organic phosphorus and $\delta^{15}N$ values. This link partly validates the modelling outputs. The organic footprint of the fish farm, both through the NewDEPOMOD simulation and through sediment analyses, was found to be very localized, with aquaculture waste settling in the immediate vicinity of the cages (less than 25 m), with a deposition of 1 kg solids.m^{-2}.$year^{-1}$ within a geographical limit of less than 20 m from the cages. These deposition fluxes are within the low range of values demonstrated to have ecological impacts in temperate environments (i.e. 0.1 to 10 kg solids m^{-2}.$year^{-1}$, reviewed by Keeley et al. 2013). An extent of influence of <25 m is consistent with previous studies on the impact of fish farming (Callier et al., 2013; Kutti et al., 2007; Mazolla et al., 2000).

Increased $\delta^{15}N$ signature of the sediment around cages was expected since fish feed and waste generally have enriched levels of ^{15}N (Mazzola and Sarà, 2001) as feed contains fish meal and fish oil. This was confirmed by the feed's SI signature (7.9 ± 0.5‰ $\delta^{15}N$). Fish feed is also composed of terrestrial material (e.g. wheat, soja) and generally has lower levels of ^{13}C (here confirmed by the $\delta^{13}C$ value of −23.0 ± 0.2‰) than other marine sources of organic matter. Therefore $\delta^{13}C$ can be used as an effective tracer of fish waste (Callier et al., 2013). In this study, we confirmed SI to be an effective way to trace farm deposition (Vizzini and Mazzola, 2004; White et al., 2017), as it showed an isotopic gradient in sediment enrichment in $\delta^{15}N$ with increasing proximity to the farm (5.2 ± 0.0‰ at final sampling of site "0" compared to 2.6 ± 0.1‰ at the reference site).

Survival of H. tubulosa below fish cages

A prerequisite of the study was to choose an optimal period for rearing *H. tubulosa*, at a temperature known to be a critical determinant of growth (Hannah et al., 2013). This was the case for the experiment, during which the water temperature was stable in a favourable range of 21.7 ± 1.3°C. We found that *H. tubulosa* survival was high (91.3%), with only 3 animals missing at the end of the period (2 at site "25" and 1 at site "reference"). These were assumed to have escaped or potentially been eaten by a predator (*Pagurus bernhardus* was found in great quantities in the corresponding baskets). This survival rate suggests the feasibility of associating *H. tubulosa* as an extractive species in IMTA under fish cages in a Mediterranean context. Nevertheless, we did not observe any weight differences between sites and sampling times. Multiple reasons can explain these negative results. First, the use of adults reduces growth potential, and therefore capacities to observe weight differences over one month. Indeed, although *H. tubulosa* is known to grow much further than the initial weight of our

specimen (e.g. ultimate weight of 620 g reported in Aydın, 2019), investment in reproduction and higher somatic maintenance in adults necessarily lower growth compared to juveniles. Consequently, the use of juveniles (although difficult to find in nature) or an increase in the duration of the study would have probably helped getting significant growth, and maybe differences between sites. For example, Costa et al. (2014) were able to get a significant growth (0.89 ± 0.29% d^{-1}) with juveniles of *H. tubulosa* (between 23.7 and 24.1 g) kept twice as long (60 days), but in laboratory conditions. In open-water, but with another species (*H. poli*), a recent study also observed significant growth, but low survival, of juveniles over 1 year (Cutajar et al. 2022). Sea cucumbers are stress-sensitive with physiological responses observed in response to environmental changes (Jobson et al., 2021; Hou et al., 2019; Kamyab et al., 2017). Performing a longer experiment allows the sea cucumbers to recover from environmental stress applied and acclimate to the new conditions. We should also acknowledge here the difficulty of getting a reliable wet weight measurement in these species. Along with the random water ejection of alive sea cucumbers, the duration of the fasting period prior measurement is crucial. Here, we applied an overnight fasting period as recommended by Tolon et al., 2017a. Nevertheless, a parallel study (Sadoul et al., to be submitted) showed that the fasting period is temperature-dependent and that 48 hours fasting period ensures complete emptying of the gastrointestinal tract.

Consequently, further experiments, with longer periods of (i) starvation before biometrics and (ii) experimentation, would be needed to demonstrate that cucumbers can grow under such IMTA conditions.

Stocking density is also known to be a critical determinant of sea cucumber growth (Aydin, 2019; Costa et al., 2014). Wild sea cucumbers can be found at densities below 1 individual.m^{-}

^{2} (Aydin, 2019). When kept in captivity, densities higher than 15 ind.m^{-2} lead to weight loss and potentially death, as found with *H. tubulosa* in a prolonged time experiment (Tolon et al., 2017b). Similar results were found after keeping *Cucumaria frondosa* several years downstream from a land-based salmon farm: in that study, individuals lost half of their wet weight (Sun et al., 2020). In our study, for statistical reasons, each basket contained 3 individuals, equivalent to a density of 11 ind (or 1700 g).m^{-2}. This density is in the upper range of what *H. tubulosa* can withstand according to Tolon et al. (2017b), who recommended 6 ind (of 40 g) m^{-2}.

Further experiments are needed to better understand the relationship between growth, density and *H. tubulosa* welfare in an IMTA context.

Assimilation of organic fish effluents

We used a double trophic biomarker approach to verify *H. tubulosa*'s ability to assimilate organic fish waste. Stable isotopes have previously been used as trophic markers to investigate sea cucumber ecology (Costa et al., 2014; Slater and Carton, 2010). Coupling biomarkers with fatty acids allows a refined understanding of trophic relationships, especially in polyculture systems (Feng et al., 2014; Mathieu-Resuge et al., 2020). Associating these methods showed differences in sea cucumber composition according to distance from the farm, suggesting differences in food assimilation.

Several studies on other sea cucumber species co-cultivated in IMTA – *A. japonicus* (Park et al. 2015), *C. frondosa* (Sun et al., 2020), *A. mollis* (Slater and Carton, 2007) – have used SI signatures to indicate assimilation of aquaculture waste. We expected a shift in sea cucumber SI signature towards the SI sediment signature, and towards the SI fish feed signature for individuals stocked in the vicinity of fish cages. Based on the trophic enrichment factor (TEF)

previously measured for *H. tubulosa* (Costa et al., 2014), we were expecting a negligible shift in $\delta^{13}C$ (0.2±0.2 ‰) but a significant enrichment in $\delta^{15}N$ (2.7±0.3 ‰) compared to the sediment. While we did not observe any difference between sea cucumber $\delta^{13}C$ according to distance from the farm, surprisingly the $\delta^{15}N$ decreased for animals closer to the farm (value deviating from the fish feed $\delta^{15}N$ of 7.9 ± 0.5‰). The *H. tubulosa* isotopic pattern was overall the opposite of the sediment isotopic pattern, suggesting that individuals did not assimilate the sediment, and associated organic waste from the farm. It is probable that instead they assimilate lower trophic food, such as bacteria or diatoms growing in the sediment in the vicinity of sea cages, as previously observed in an IMTA context by Hochard et al. (2016).

One explanation of such a pattern can be related to sediment characteristics. Organic matter content and sources (sedimentary, plant or animal material) along with granulometry of sediment are known to play a specific role in sea cucumber feeding behaviour and thus in the ingestion process (Boncagni et al., 2019; Grosso et al., 2021; Mezzali and Soualili, 2013; Ricart et al., 2015; Tolon et al., 2015). According to Boncagni et al. (2019) *H. tubulosa* selectively assimilates food with a preference demonstrated for seagrass detritus in an environment with multiple food sources.

In parallel, Mezali and Soualili (2013) demonstrated *H. tubulosa*'s preference for ingestion of medium sediment fractions (200 to 600 µm). Moreover, mineral and microorganism (benthic microalgae and bacteria) concentrations in the sediment may also play a role in sea cucumber feeding (Hair et al., 2016). Additional studies exploring the sediment characteristics (composition and granulometry) below the cages in more detail would confirm *H. tubulosa*'s ability to grow by assimilating fish waste in an IMTA context. The different potential food sources' contributions (fish waste versus natural resources of the sediment) to the diet of sea

cucumbers would also have been of interest following previously published methods (Boncagni et al., 2019; Parnell and Jackson, 2013; Ricart et al., 2015).

Finally, we worked with wild specimens collected in the studied bay to avoid any risk of introducing pathogens or undesirable organisms. Only large specimens could be found, which is not ideal because they were probably all already investing energy in reproduction rather than growth. Consequently, the isotope measures performed in the body wall probably might lack the capacities to detect diet switches. Measures in the gonads might have provided different results. This problem could be overcome by using juveniles, as has been demonstrated in an IMTA with *P. californicus* (Hannah et al., 2013), where small animals (<100 g WW) presented high potential to assimilate organic components (grew of 27–56%) while large ones (>100 g) decreased in size by 10–33% over a year. That difference may be due to greater competition for food and space for the latter, or different feeding preferences, with small individuals preferring fine particulate material (Yingst, 1982).

Farm effect on biochemical markers of interest

Holothurians are nutritionally interesting because of their low fat and high protein content, as well as they contain amino acids and trace elements essential for human health (Chen, 2003). Regarding *H. tubulosa*'s biochemical profile, we confirmed this, finding a protein content of 57.1±3.3%, similar than the 60.9±0.3% found by Bilgin and Tanrikulu (2018). Fat content was also low, at 0.13% of the total composition (between 0.56 and 1.29 µg/mg), lower than the value of 0.76% found by Bilgin and Tanrikulu (2018) for *H. tubulosa* and the value of 0.1–0.9% found for *Parastichopus spp.* (Chang Lee et al., 1989), and much lower than the fat content of *H. forskali* (4.8±2.3%, Santos et al., 2016).

We identified 33 fatty acids in *H. tubulosa* samples, which is in the same order of magnitude as other species such as *H. forskali* (37 FAs identified in David et al., 2020). The major Fas identified in *H. tubulosa* individuals were SFAs (42.4–50.8%), stearic acid (18:0) and arachidonic acid (20:4n-6) – these were the most commonly found in all samples. Of the SFAs, FA 18:1n9, known to be a tracer of fish feed (Irisarri et al., 2015), was identified. Fish feed increasingly contains plant oils (Sun et al., 2020), which have lower levels of omega-3 PUFAs and higher levels of omega-6 PUFAs (Menoyo et al., 2007). *H. tubulosa* individuals were rich in PUFAs (18%), which are significant for human nutrition. The highest values obtained were for stearic acid C18:0 (25–30%), arachidonic acid C20:4 omega-6 polyunsaturated FA (5–11%) (said to be essential because it is necessary but not synthesized by the human body), palmitic acid C16:0 (6–13%), and eicosapentaenoic acid C 20:5 omega-9 (3–6%). The PUFA content (18%) was lower than other studies on *H. tubulosa* (36% in Bilgin and Tanrikulu, 2018) or *H. forskali* (43% in Santos et al., 2016) but similar for FAs of nutritional interest such as arachidonic and eicosapentaenoic acids (19% and 9.1% respectively for *H. tubulosa* in Bilgin and Tanrikulu, 2018; 20% and 10% respectively for *H. forskali* in Santos et al., 2016). These discrepancies could be due to the sampling season, which took place in the reproduction period for our study, and in the winter, the period of fat storage, for Santos et al. (2016).

We measured between 11.3% and 14.4% of HUFAs, which are known to be vital for their role in membrane properties and immune response (Twining et al., 2016). Other studies have found HUFA values of 23.2–36.6% in *H. forskali* tissues (David et al., 2020) and 21.8% in the foregut of *H. leucospilota* (Mfilinge and Tsuchiya, 2016).

We observed a difference between the four sampling sites in terms of FA content in sea cucumbers, with more FAs observed with increasing distance from the farm. This increase is

mostly explained by higher quantities of saturated, branched-chain and shorter fatty acids (below 17C). Many of these FAs were microbial markers (Salvo et al., 2015). Consequently, in contrast to the $\delta^{15}N$ isotope results, this suggests that sea cucumbers near sea cages ingested less micro-organisms.

5. Conclusion

This study found that *H. tubulosa* sea cucumbers can survive in the vicinity of fish cages in a Mediterranean context. However, individuals overall showed decreased protein and lipid content and a reduced isotopic signature when stocked closer to fish cages. This suggests reduced food assimilation during the period of the experiment. Thus, the findings could neither demonstrate that nutrients in aquaculture waste meet the nutritional needs of *H. tubulosa* nor confirm the possibility of developing *H. tubulosa* aquaculture up to commercial size under such conditions. It would be valuable to carry out further experiments to test the cross effects of sediment characteristics on sea cucumber growth in order to test whether, in an IMTA context, fish waste could provide another food source suitable to sustain *H. tubulosa* survival, growth and reproduction. As seasonal changes in adult physiology influence somatic growth by reducing available energy due to gamete production, we recommend working with juveniles in further research on the association of sea cucumbers in IMTA in order to observe growth in such systems. A priority should be to determine their feeding preferences (i.e. sediment granulometry and composition) and behavioural responses regarding the effect of different stocking densities and captivity on *H. tubulosa* welfare.

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Author’s contributions:

Gloria Maris, a French aquaculture company and employer of J. P. Caprioli (JPC), generated the initial idea of *H. tubulosa* association with sea farm cages. E. Roque d'orbcastel (ERO) conceived the study with post-doctoral researcher Bastien Sadoul (BS) and ERO wrote the initial draft of the manuscript. ERO and JPC created the IMTA experimental design. ERO, BS, C. Barrier-Loiseau (CBL) and M. Callier (MC) developed the experimental design. ERO, JPC, M.O. Guillermard (MOG), N. Cimiterra (NC), T. Laugier (TL) conducted the experiments, diving to collect the samples and to deploy the current meter and environmental probes. F. Lagarde (FL) prepared the current meter and extracted the current data. ERO, BS, JPC, MOG, NC and CBL performed the biometrics. BS and K. Chary (KC) performed the DEPOMOD simulations. CBL, BS, ERO, MC performed lyophilization and sediment analysis. ERO and BS performed statistical analyses. BS, MC, KC, NC and FL contributed to improve the writings of the initial draft. All authors approved the manuscript submission.

Conflict of Interest statement. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethical. Animal handling was performed respecting ethical animal welfare guidelines. The number of sea cucumbers sampled was limited to the strictly necessary and received authorization from the local authority (order no. 01-2019 of 7 August 2019 authorizing the exceptional sampling of *Holothuria tubulosa* for scientific or experimental purposes.)

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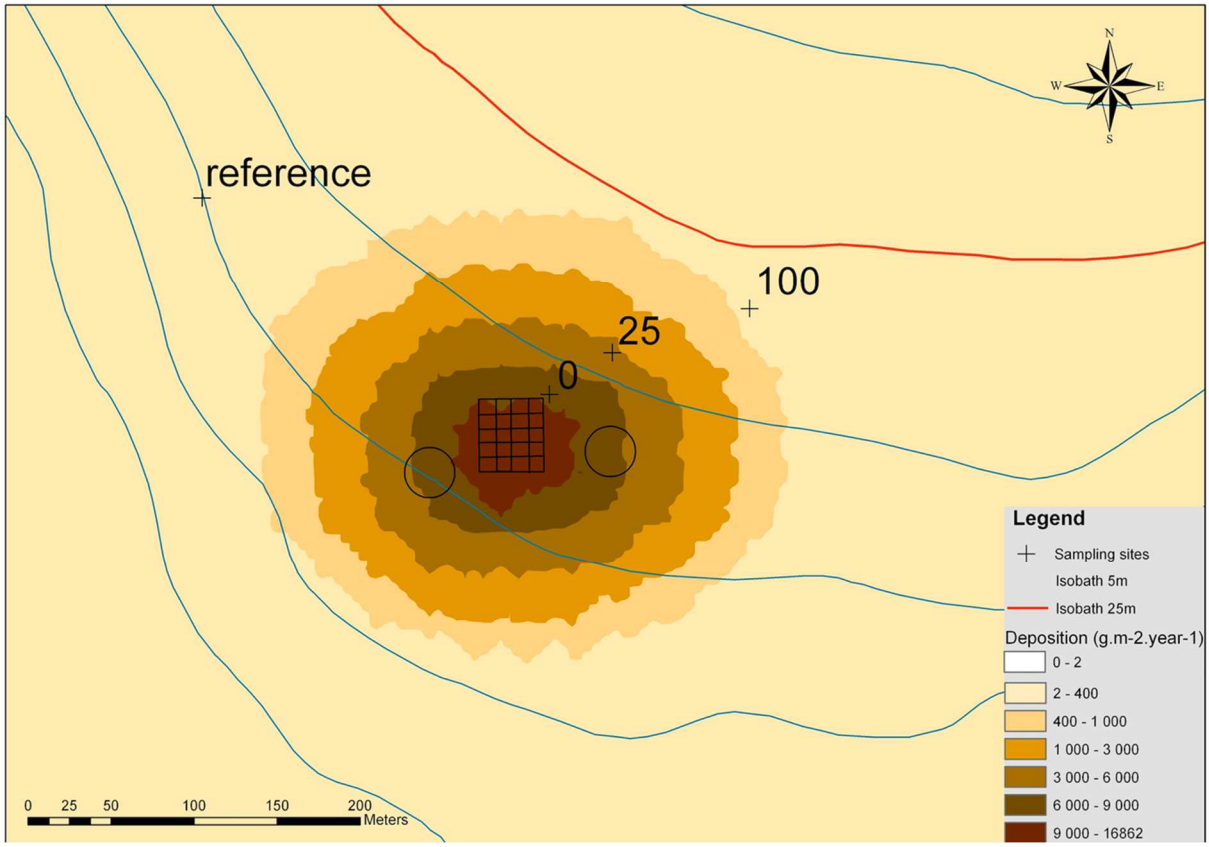

Fig. 1. Deposition of particulate waste from the fish farm (expressed in g of solids.m^{-2}.$year^{-1}$) simulated by NewDEPOMOD (from 24 September to 24 October 2019) for sites “0”, “25”, “100” and the reference site. The cages are represented by black circles and squares. Results were transferred to ArcGIS to create the graph. Black squares represent the 32 squared rearing cages of the farm and circles the 2 round rearing cages.

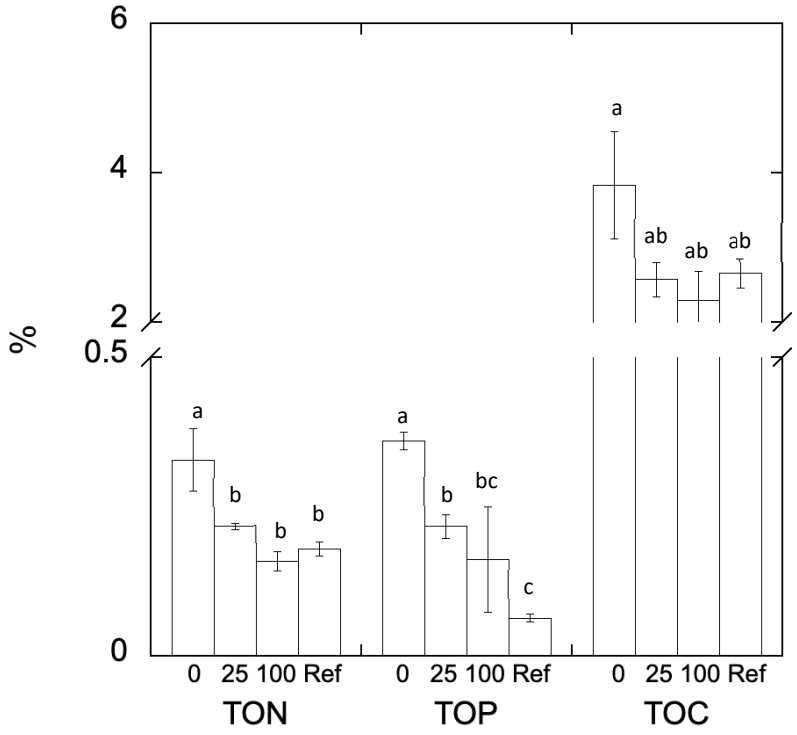

Fig. 2. Total organic nitrogen (TON), phosphorus (TOP) and carbon (TOC), expressed in %, in the final sampled sediment, at different distances from the fish cages (0, 25, 100 m and reference). Letters indicate significant differences between groups (F test, α = 5%).

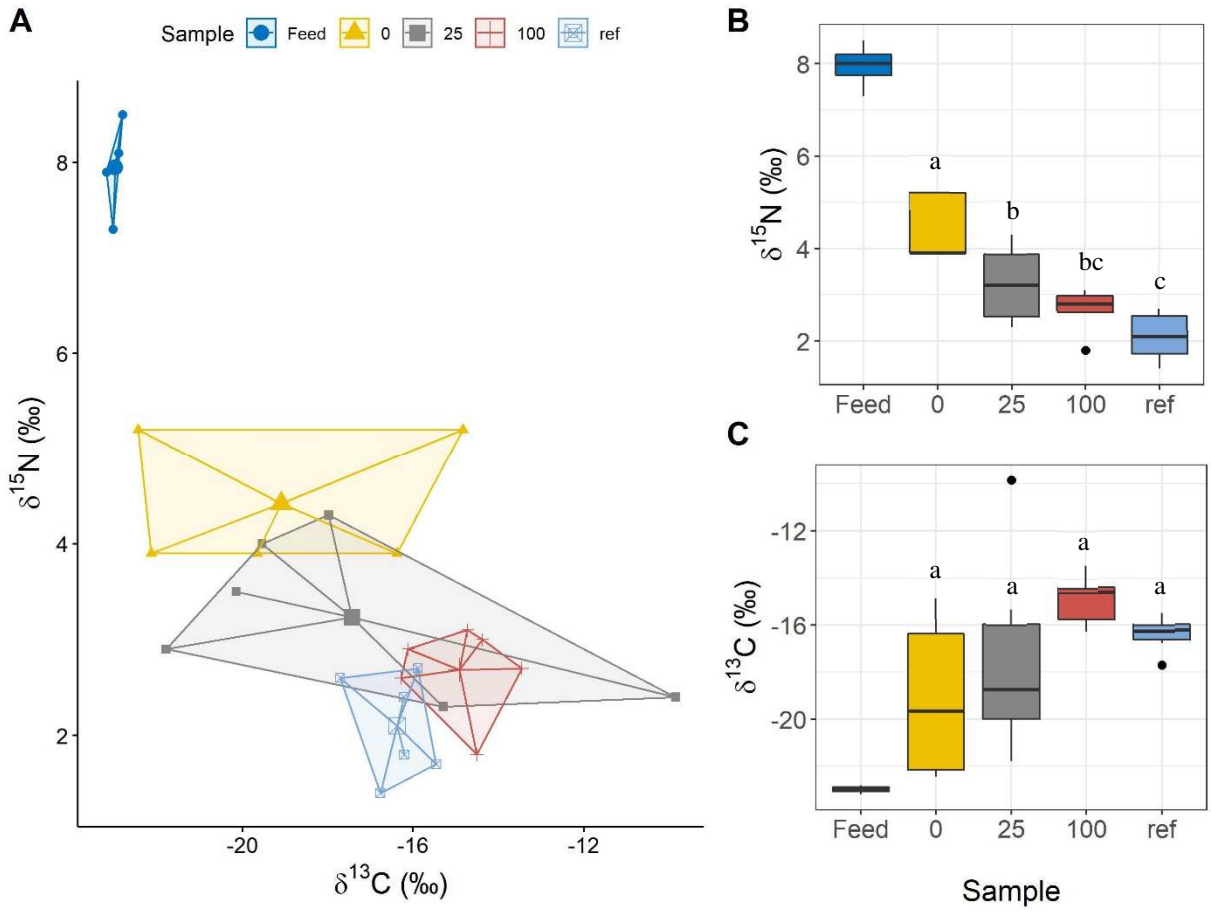

Fig. 3. Stable isotope values ($\delta^{13}C$, $\delta^{15}N$) of the fish feed and sediments sampled at four distances from the fish cages: 0, 25, 100 m and reference (ref). A: Biplot of $\delta^{13}C$ and $\delta^{15}N$ (‰) of the fish feed and sediments sampled at four distances from the fish cages: 0, 25, 100 m and reference. B: $\delta^{15}N$ values (‰) of the fish feed and sediments sampled at four distances from the fish cages: 0, 25, 100 m and reference. C: $\delta^{13}C$ values (‰) of the fish feed and sediments sampled at four distances from the fish cages: 0, 25, 100 m and reference. Letters indicate significant differences between measurements performed on the sediment samples.

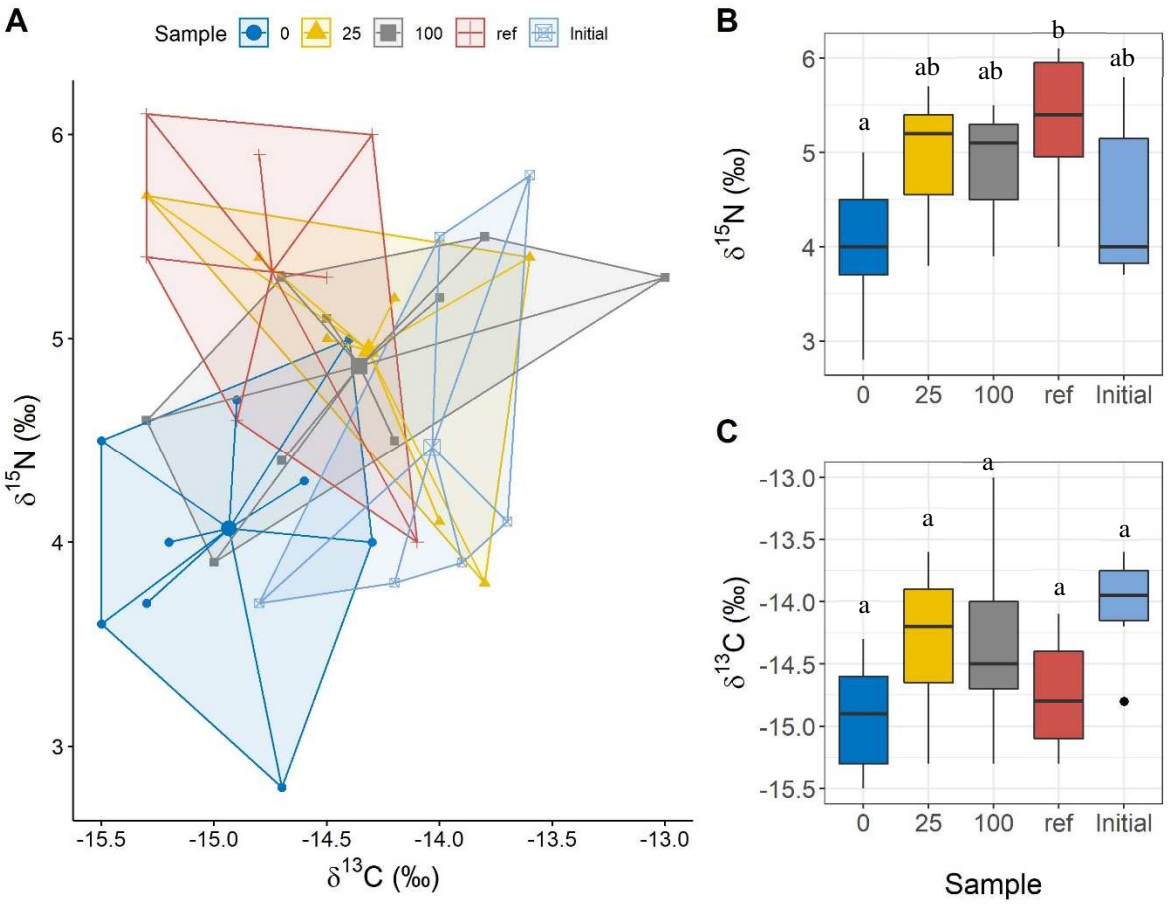

Fig. 4. Mean (±SD) stable isotope values ($\delta^{13}C$, $\delta^{15}N$) of *H. tubulosa* kept for a month at four distances from the fish cages: 0, 25, 100 m and reference (ref), with 'initial' referring to the wild sea cucumber fished at the reference site. A: Biplot of $\delta^{13}C$ and $\delta^{15}N$ (‰) of the sea cucumbers kept at four distances from the fish cages: 0, 25, 100 m and reference. B: $\delta^{15}N$ values (‰) of the sea cucumbers kept at four distances from the fish cages: 0, 25, 100 m and reference. C: $\delta^{13}C$ values of the sea cucumbers kept at four distances from the fish cages: 0, 25, 100 m and reference. Letters indicate significant differences between samples.

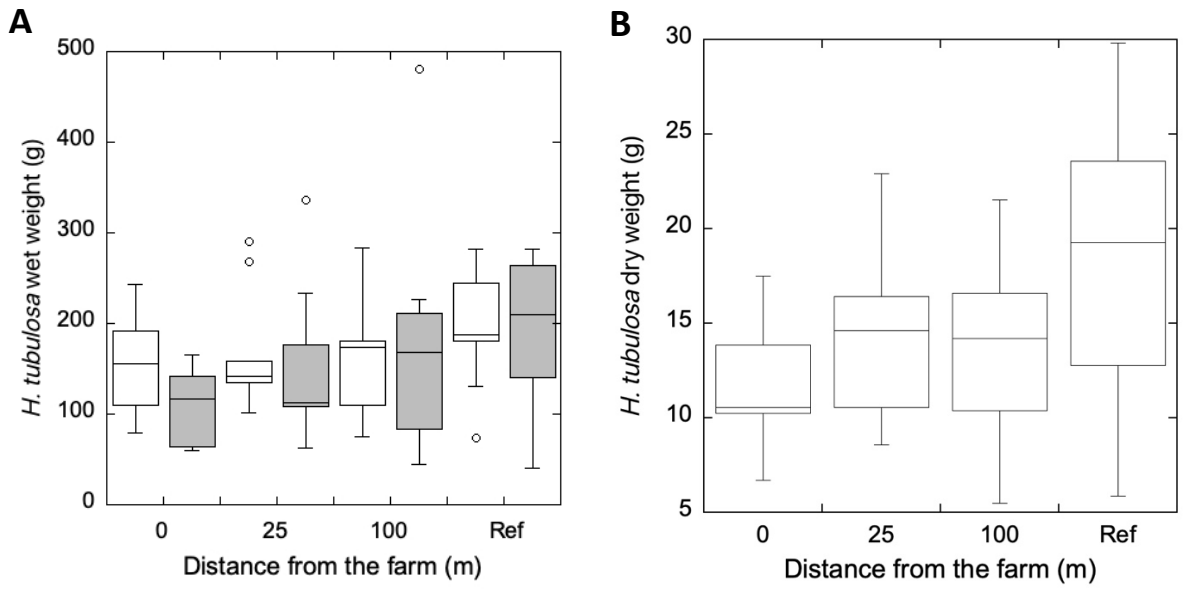

Fig. 5. *H. tubulosa* body weight (mean and SD, in g) after 1 month of exposure at four distances from the farm. A: body wet weight at initial (white box plot) and final sampling (grey box plot), B: body dry weight after 1 month of exposure at four distances from the fish cages (0 m, 25 m, 100 m and Reference).

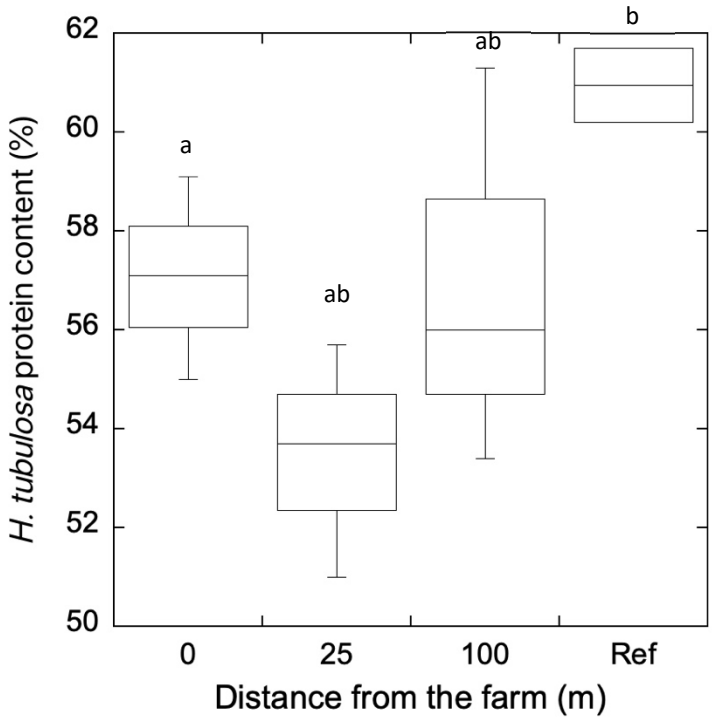

Fig. 6. *H. tubulosa* protein content (in % of dry sample) at four distances from the fish cages (0 m, 25 m, 100 m and reference). Letters indicate significant differences between groups (F test, $\alpha = 5\%$)

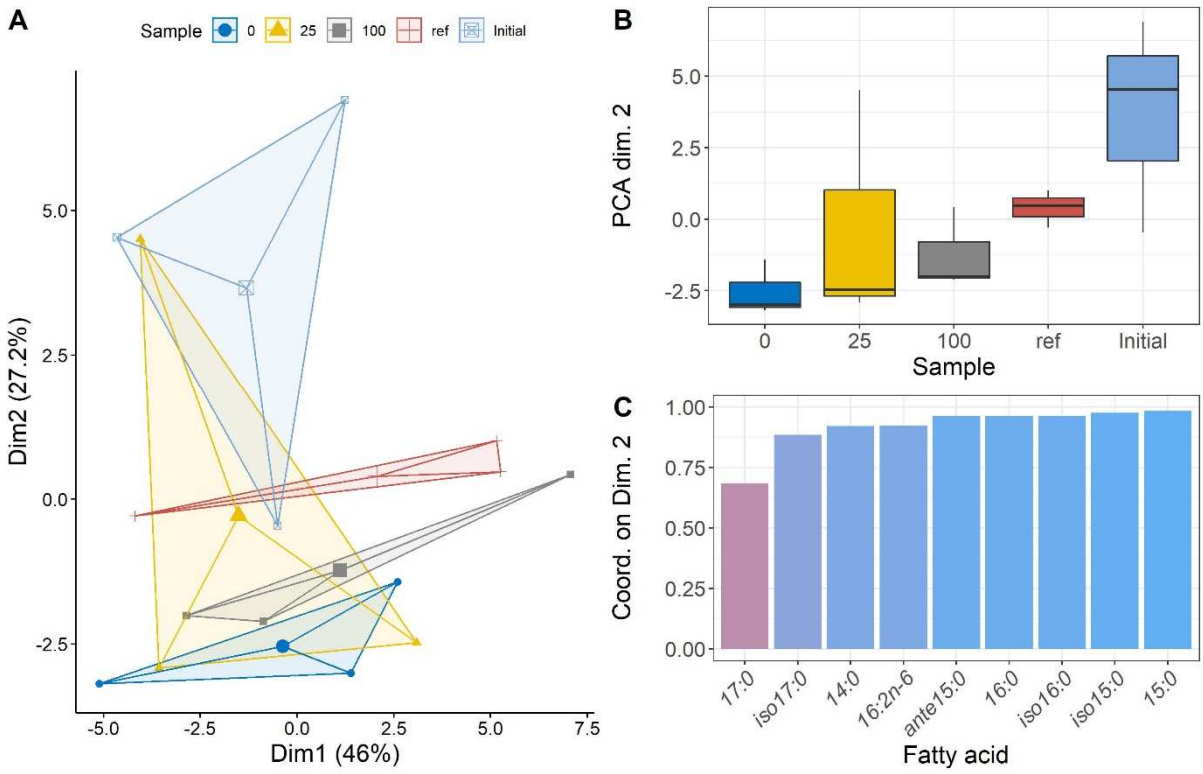

Figure 7. (A) PCA on fatty acid profiles of the sea cucumbers at the 4 distances from the fish cages. (B) Coordinates on the second dimension for each site. (C) Fatty acids contributing the most to the variability on the second dimension of the PCA.

Table 1. Major fatty acids analysed in *H. tubulosa* samples (mean ± SD, in µg.mg^{-1} of dry weight) from the four sites (and baseline wild samples), with the total saturated FAs (SFAs), monounsaturated FAs (MUFAs), polyunsaturated FAs (PUFAs), and dimethyl acetal (DMA).

SAMPLES	0	25	100	REF	BASELINE
NB OF SAMPLES	3	3	3	3	3
14:0	0.01±0.00	0.02±0.01	0.02±0.1	0.03±0.0	0.03 ±0.01
16:0	0.05±0.01	0.06±0.03	0.06±0.02	0.08±0.0	0.12 ±0.05
17:0	0.01±0.00	0.01±0.00	0.01±0.0	0.2±0.0	0.01±0.00
ISO 17:0	0.01±0.00	0.01±0.01	0.01±0.0	0.01±0.0	0.01±0.00
18:0	0.10±0.03	0.11±0.02	0.12±0.02	0.13±0.01	0.12±0.01
20:1N-11	0.05±0.02	0.04±0.03	0.05±0.03	0.06±0.04	0.03±0.02
20:4N-6	0.09±0.04	0.07±0.07	0.09±0.05	0.11±0.08	0.05±0.03
18:0DMA	0.13±0.04	0.13±0.03	0.16±0.03	0.14±0.02	0.12±0.03
TOT. SFAs	**0.35±0.10**	**0.39±0.06**	**0.42±0.08**	**0.46±0.03**	**0.46±0.07**
TOT. MUFAs	**0.15±0.05**	**0.13±0.08**	**0.16±0.09**	**0.17±0.12**	**0.14±0.06**
TOT. PUFAs	**0.16±0.07**	**0.13±0.10**	**0.16±0.09**	**0.19±0.13**	**0.11±0.05**
TOT. DMAs	**0.14±0.04**	**0.14±0.03**	**0.17±0.03**	**0.15±0.02**	**0.13±0.03**
TOTAL FAs	**0.84±0.26**	**0.83±0.20**	**0.94±0.30**	**1.02±0.30**	**0.91±0.17**

Table 2. Bacterial fatty acids, diatom and zooplankton markers analysed in *H. tubulosa* samples (mean ± SD, in % of Total Fatty Acids) at initial sampling for wild animals and final sampling for the experimental sites (0, 25, 100 and reference according to distances to the cages in m).

SAMPLES	0	25	100	REF	INITIAL
BACTERIAL FAS: 15:0, 16:0, 17:0, 16:1N-7, 17:1N-X, 18:1N-7	13.4±1.1	17.9±9.3	15.2±1.7	18.5±1.8	28.2±8.8
DIATOM MARKER 20:5N-3	2.8±0.6	2.3±1.8	2.7±1.2	2.7±2.4	2.0±0.9
ZOOPLANKTON MARKERS: 20:1N-9, 22:1N-9	1.1±0.2	1.1±0.9	1.3±0.6	1.1±0.9	0.6±0.3