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1 **Is *Holothuria tubulosa* the golden goose of ecological aquaculture in the Mediterranean**
2 **Sea?**

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14

15 **Abstract**

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

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

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

46

47 **1. Introduction**

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

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

53 Among marine detritivores, sea cucumbers (*Echinodermata: Holothuroidea*) are vital
54 members of benthic communities and are present all over the world. They have multiple
55 ecological roles including (a) sediment bioturbation, (b) remediating organic load (Costa et al.,
56 2014), (c) enhancing the productivity of benthic biota (e.g. seagrass, Costa et al., 2014,
57 Wolkenhauer et al., 2010), (d) buffering against ocean acidification, (e) hosting more than 200
58 symbionts, and (f) acting as food sources for many animals, including humans (Purcell et al.,
59 2016).

60 Sea cucumbers can have high economic value (Purcell et al., 2018). They are mostly exploited
61 for food, principally in Asia, with body integument the main targeted product, which is dried,
62 boiled, salted or cooked (Conand, 1990). Other food by-products such as fermented intestines
63 and dried gonads are marketed in Japan, Korea and China (Stutterd and Williams, 2003). In
64 East Asia, sea cucumbers are also used as traditional medicine to treat a number of conditions
65 (Pangestuti and Arifin, 2018; Xue et al., 2015), including arthritis and joint pain. Scientific
66 studies confirming the presence of a range of bioactive compounds in sea cucumbers have
67 more recently attracted the attention of the pharmaceutical sector (Kiew and Don, 2012).
68 Their saponins have been found to have anti-inflammatory and anti-cancer properties
69 (Pangestuti and Arifin, 2018), and other molecules have been described as having antibacterial
70 (Santos et al., 2016) and antifungal properties (Hamel and Mercier, 1997). Sea cucumbers are
71 also used in certain cosmetics, including liniment, soap and toothpaste. Depending on the
72 species, the selling price can vary dramatically, with some reaching more than 1000 \$.kg⁻¹ (e.g.
73 *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 ($\delta^{15}\text{N}$ and
123 $\delta^{13}\text{C}$) 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.

167 Dominant currents were found to be mostly flowing south/southeast with a velocity generally
168 below $10 \text{ cm}\cdot\text{s}^{-1}$. Default values were used for vertical ($k_z = 0.001 \text{ m}^2 \text{ s}^{-1}$) and horizontal ($k_x =$
169 $0.1 \text{ m}^2 \text{ s}^{-1}$, $k_y = 0.1 \text{ m}^2 \text{ s}^{-1}$) dispersion coefficients (Cromey et al., 2002; Gillibrand and Turrell,
170 1997).

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

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

178 **2.3 Specimen collection**

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

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

190 **2.4 Experimental design**

191 Four experimental sites to test the effect of aquaculture waste on sea cucumbers were
192 identified along a transect: site “0” under the cages (20.4 m deep), site “25” at 25 m from the
193 cages (21.3 m deep), site “100” at 98 m from the cages (23 m deep), and the “reference” site
194 (19 m deep), located 250 m northwest of the cages, outside the influence of the farm. At each
195 site, triplicate baskets with a triangular-prism shape and rigid 1-cm mesh (commonly called
196 Australian oyster baskets) were fixed in the sediment. The baskets’ length, width and height
197 were respectively 80 cm x 35 cm x 20 cm (effective sediment surface area of 0.28 m²). At the
198 beginning of July 2020, three individuals were placed in each basket.

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

205 **2.5 Environmental data**

206 The bottom water temperature (°C) near the experimental infrastructure was recorded at 30-
207 minute intervals by an NKE-STPS logger fixed to the infrastructure (from 6 July to 6 August
208 2020). During the entire experiment, there was a stable water temperature of around 21.7 ±
209 1.3°C. At the beginning and the end of the experiment, samples of superficial sediment (1–3
210 cm) were collected by divers at each site using a corer (diameter 20 mm). Samples (3 cores
211 per site, n = 23*) were kept in the dark and on ice before being frozen and processed within

212 30 days (ISO, 2004). *One sediment sample (at the end of the experiment and from site
213 “reference”) was partially spilled after being collected and was therefore discarded.

214 **2.6 Trophic biomarkers**

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

$$223 \quad \delta X (\text{‰}) = \left(\frac{R_{\text{sample}}}{R_{\text{standard}}} - 1 \right) \times 10^3$$

224 where X is ¹³C or ¹⁵N, and R is the ratio of heavy to light isotope (¹³C/¹²C or ¹⁵N/¹⁴N). ¹⁵N/¹⁴N
225 gives clues about the animal food source and trophic level, upward movement tending to
226 concentrate δ¹⁵N isotopes by 3–4‰ at each stage in the food chain.

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

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

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

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

238 **2.7 Statistical analysis**

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

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

248 **3. Results**

249 **3.1 Organic footprint: deposition simulations and particulate matter signature**

250 *NewDEPOMOD simulations*

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

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

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

265 **3.2 Stable isotope (SI) signatures**

266 The $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values obtained for the samples of fish feed were $-23.0 \pm 0.2\text{‰}$ and $7.9 \pm$
267 0.5‰ respectively (Fig. 3). The SI composition of sediment showed that δN^{15} varied
268 significantly according to the sampling date (F-value=44.2, df=1, p -value<0.001) and the site
269 (F-value=57.3, df=3, p -value<0.001, Fig 3B). No effect of sampling date or site was observed
270 for $\delta^{13}\text{C}$. No interaction between sampling date and site was found for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, so all
271 samples were illustrated on the same graph to show general trends (Fig. 3A and B). An isotopic
272 gradient was found in sediments according to the distance from the farm, indicating an
273 enrichment in $\delta^{15}\text{N}$ with increasing proximity to the farm (Fig. 3A). Of the 4 sites, site “0” had
274 the highest mean $\delta^{15}\text{N}$ value (5.2‰ at final sampling). The correlation (log-based) between
275 sediment $\delta^{15}\text{N}$ and NewDEPOMOD results was significant (p -value=0.017) with a R^2 0.97 of
276 (Supplementary Fig. 2).

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

280 **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 \pm SD). The 6 euthanized individuals weighed 131.5 ± 60.1 g (mean WW \pm SD) and their dry
283 body wall 12.0 ± 3.3 g (mean DW \pm SD). At the end of the experiment, animals weighed 158.76
284 ± 95.39 g (mean WW \pm SD) and their dry body wall 14.47 ± 5.80 g (mean DW \pm 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 ($60.95 \pm 1.06\%$) compared
289 to site “0” ($57.09 \pm 3.32\%$).

290 Regarding the fatty acid profile (Table 1), the total fat content varied between 0.56 and 1.29
291 $\mu\text{g}\cdot\text{mg}^{-1}$, 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)

305 and four others to the omega 6 category (16:2n-6, 18:2n-6, 20:2n-6, 20:4n-6). The major PUFA
306 was arachidonic acid (ARA 20:4n-6) with a maximal value of 13.7%. The sum of highly
307 unsaturated fatty acids (HUFAs; fatty acids with ≥ 20 carbon chain length and two double
308 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%
309 at 50 m and 13.1% at 150 m.

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

313

314 **4. Discussion**

315 *Localized aquaculture footprint*

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

327 Increased $\delta^{15}\text{N}$ signature of the sediment around cages was expected since fish feed and waste
328 generally have enriched levels of ^{15}N (Mazzola and Sarà, 2001) as feed contains fish meal and
329 fish oil. This was confirmed by the feed's SI signature ($7.9 \pm 0.5\text{‰}$ $\delta^{15}\text{N}$). Fish feed is also
330 composed of terrestrial material (e.g. wheat, soja) and generally has lower levels of ^{13}C (here
331 confirmed by the $\delta^{13}\text{C}$ value of $-23.0 \pm 0.2\text{‰}$) than other marine sources of organic matter.
332 Therefore $\delta^{13}\text{C}$ can be used as an effective tracer of fish waste (Callier et al., 2013). In this
333 study, we confirmed SI to be an effective way to trace farm deposition (Vizzini and Mazzola,
334 2004; White et al., 2017), as it showed an isotopic gradient in sediment enrichment in $\delta^{15}\text{N}$
335 with increasing proximity to the farm ($5.2 \pm 0.0\text{‰}$ at final sampling of site "0" compared to 2.6
336 $\pm 0.1\text{‰}$ at the reference site).

337 *Survival of H. tubulosa below fish cages*

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

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

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

371 Stocking density is also known to be a critical determinant of sea cucumber growth (Aydın,
372 2019; Costa et al., 2014). Wild sea cucumbers can be found at densities below 1 individual.m⁻²

373 ² (Aydin, 2019). When kept in captivity, densities higher than 15 ind.m⁻² lead to weight loss
374 and potentially death, as found with *H. tubulosa* in a prolonged time experiment (Tolon et al.,
375 2017b). Similar results were found after keeping *Cucumaria frondosa* several years
376 downstream from a land-based salmon farm: in that study, individuals lost half of their wet
377 weight (Sun et al., 2020). In our study, for statistical reasons, each basket contained 3
378 individuals, equivalent to a density of 11 ind (or 1700 g).m⁻². This density is in the upper range
379 of what *H. tubulosa* can withstand according to Tolon et al. (2017b), who recommended 6 ind
380 (of 40 g) m⁻².

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

383 *Assimilation of organic fish effluents*

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

391 Several studies on other sea cucumber species co-cultivated in IMTA – *A. japonicus* (Park et al.
392 2015), *C. frondosa* (Sun et al., 2020), *A. mollis* (Slater and Carton, 2007) – have used SI
393 signatures to indicate assimilation of aquaculture waste. We expected a shift in sea cucumber
394 SI signature towards the SI sediment signature, and towards the SI fish feed signature for
395 individuals stocked in the vicinity of fish cages. Based on the trophic enrichment factor (TEF)

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

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

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

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

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

432 *Farm effect on biochemical markers of interest*

433 Holothurians are nutritionally interesting because of their low fat and high protein content, as
434 well as they contain amino acids and trace elements essential for human health (Chen, 2003).
435 Regarding *H. tubulosa*'s biochemical profile, we confirmed this, finding a protein content of
436 $57.1 \pm 3.3\%$, similar than the $60.9 \pm 0.3\%$ found by Bilgin and Tanrikulu (2018). Fat content was
437 also low, at 0.13% of the total composition (between 0.56 and 1.29 $\mu\text{g}/\text{mg}$), lower than the
438 value of 0.76% found by Bilgin and Tanrikulu (2018) for *H. tubulosa* and the value of 0.1–0.9%
439 found for *Parastichopus spp.* (Chang Lee et al., 1989), and much lower than the fat content of
440 *H. forskali* ($4.8 \pm 2.3\%$, Santos et al., 2016).

441 We identified 33 fatty acids in *H. tubulosa* samples, which is in the same order of magnitude
442 as other species such as *H. forskali* (37 FAs identified in David et al., 2020). The major FAs
443 identified in *H. tubulosa* individuals were SFAs (42.4–50.8%), stearic acid (18:0) and
444 arachidonic acid (20:4n-6) – these were the most commonly found in all samples. Of the SFAs,
445 FA 18:1n9, known to be a tracer of fish feed (Irisarri et al., 2015), was identified. Fish feed
446 increasingly contains plant oils (Sun et al., 2020), which have lower levels of omega-3 PUFAs
447 and higher levels of omega-6 PUFAs (Menoyo et al., 2007). *H. tubulosa* individuals were rich
448 in PUFAs (18%), which are significant for human nutrition. The highest values obtained were
449 for stearic acid C18:0 (25–30%), arachidonic acid C20:4 omega-6 polyunsaturated FA (5–11%)
450 (said to be essential because it is necessary but not synthesized by the human body), palmitic
451 acid C16:0 (6–13%), and eicosapentaenoic acid C 20:5 omega-9 (3–6%). The PUFA content
452 (18%) was lower than other studies on *H. tubulosa* (36% in Bilgin and Tanrikulu, 2018) or *H.*
453 *forskali* (43% in Santos et al., 2016) but similar for FAs of nutritional interest such as
454 arachidonic and eicosapentaenoic acids (19% and 9.1% respectively for *H. tubulosa* in Bilgin
455 and Tanrikulu, 2018; 20% and 10% respectively for *H. forskali* in Santos et al., 2016). These
456 discrepancies could be due to the sampling season, which took place in the reproduction
457 period for our study, and in the winter, the period of fat storage, for Santos et al. (2016).

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

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

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

468 **5. Conclusion**

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

484

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495 ***Author’s contributions:***

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

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

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

515

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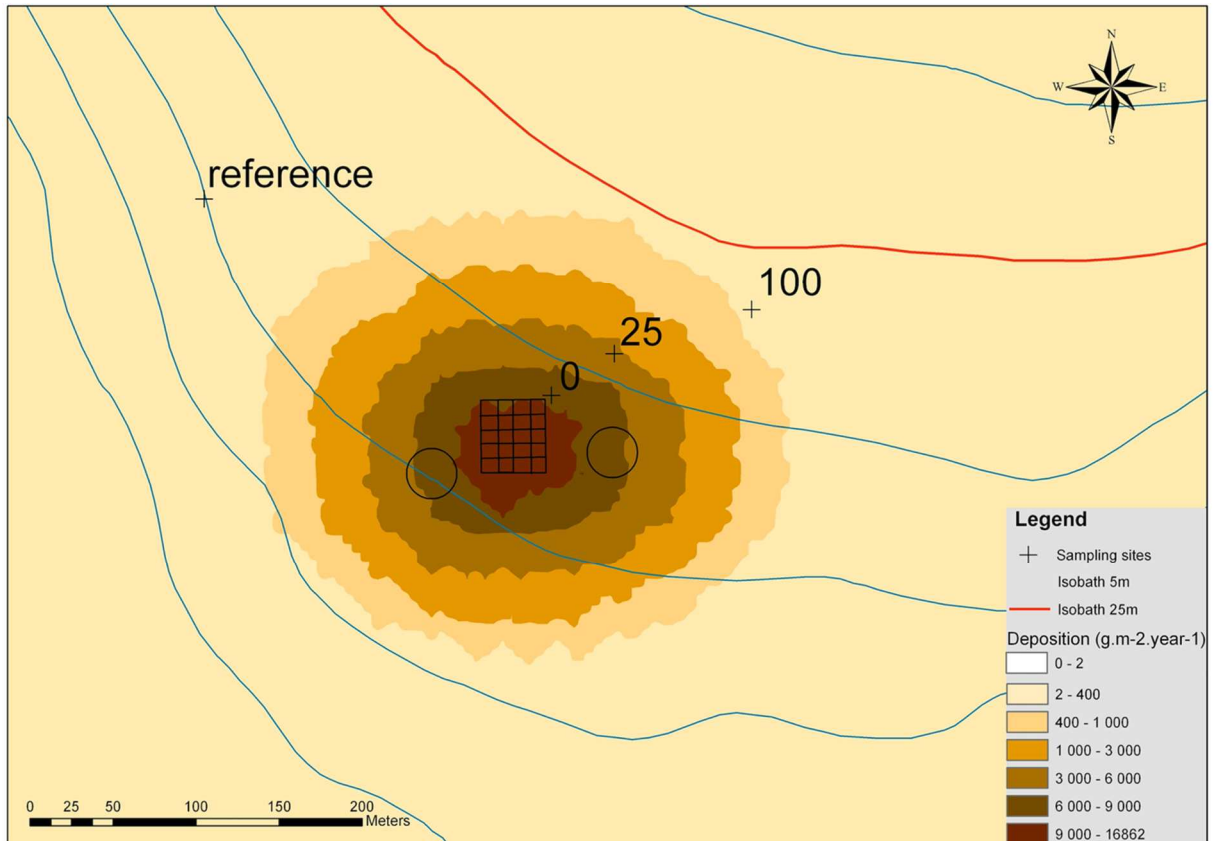


Fig. 1. Deposition of particulate waste from the fish farm (expressed in g of solids.m⁻².year⁻¹) simulated by NewDEPOMOD (from 24 September to 24 October 2019) for sites “0”, “25”, “100” and the reference site. The cages are represented by black circles and squares. Results were transferred to ArcGIS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

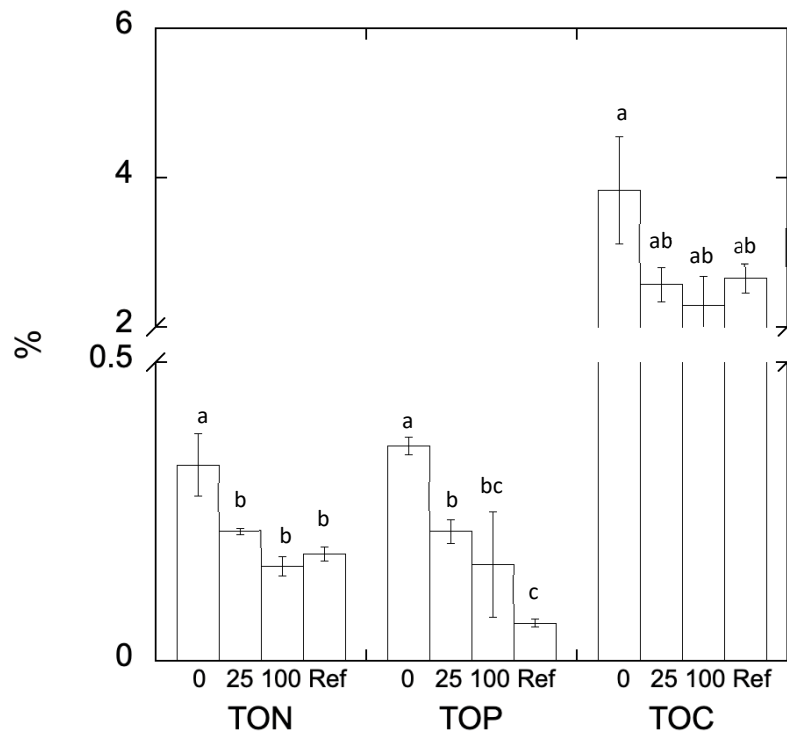


Fig. 2. Total organic nitrogen (TON), phosphorus (TOP) and carbon (TOC), expressed in %, in the final sampled sediment, at different distances from the fish cages (0, 25, 100 m and reference). Letters indicate significant differences between groups (F test, $\alpha = 5\%$).

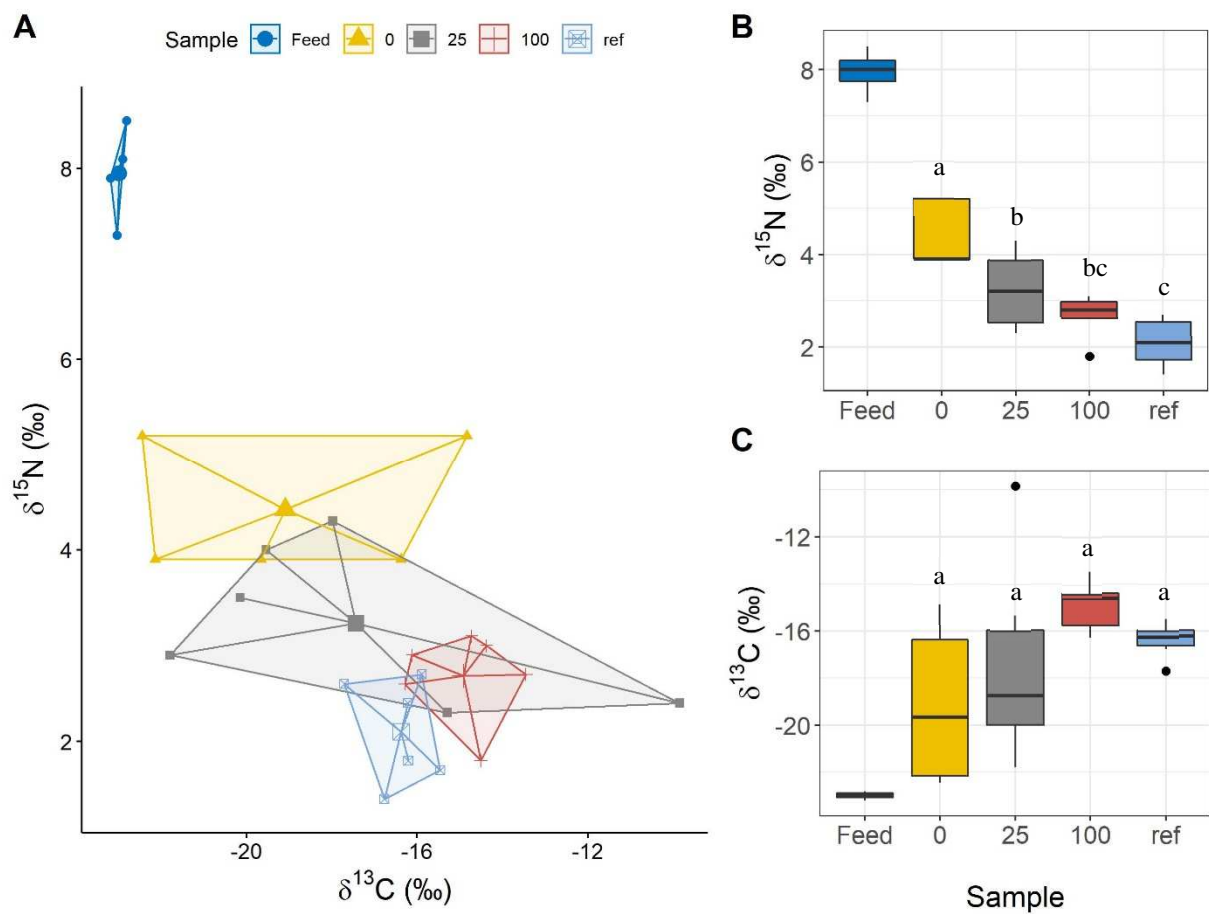


Fig. 3. Stable isotope values ($\delta^{13}\text{C}$, $\delta^{15}\text{N}$) of the fish feed and sediments sampled at four distances from the fish cages: 0, 25, 100 m and reference (ref). **A:** Biplot of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ (‰) of the fish feed and sediments sampled at four distances from the fish cages: 0, 25, 100 m and reference. **B:** $\delta^{15}\text{N}$ values (‰) of the fish feed and sediments sampled at four distances from the fish cages: 0, 25, 100 m and reference. **C:** $\delta^{13}\text{C}$ 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.

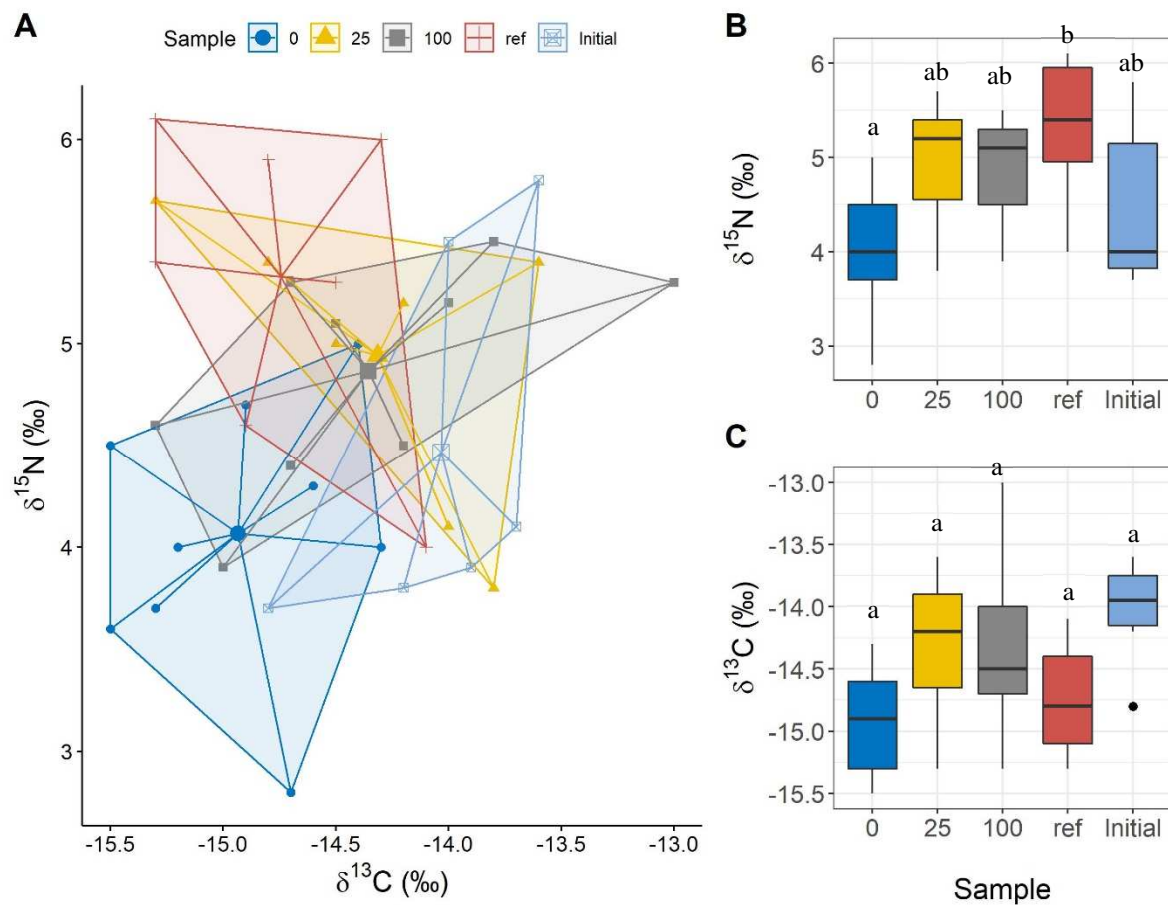


Fig. 4. Mean (\pm SD) stable isotope values ($\delta^{13}\text{C}$, $\delta^{15}\text{N}$) of *H. tubulosa* kept for a month at four distances from the fish cages: 0, 25, 100 m and reference (ref), with 'initial' referring to the wild sea cucumber fished at the reference site. **A:** Biplot of $\delta^{13}\text{C}$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

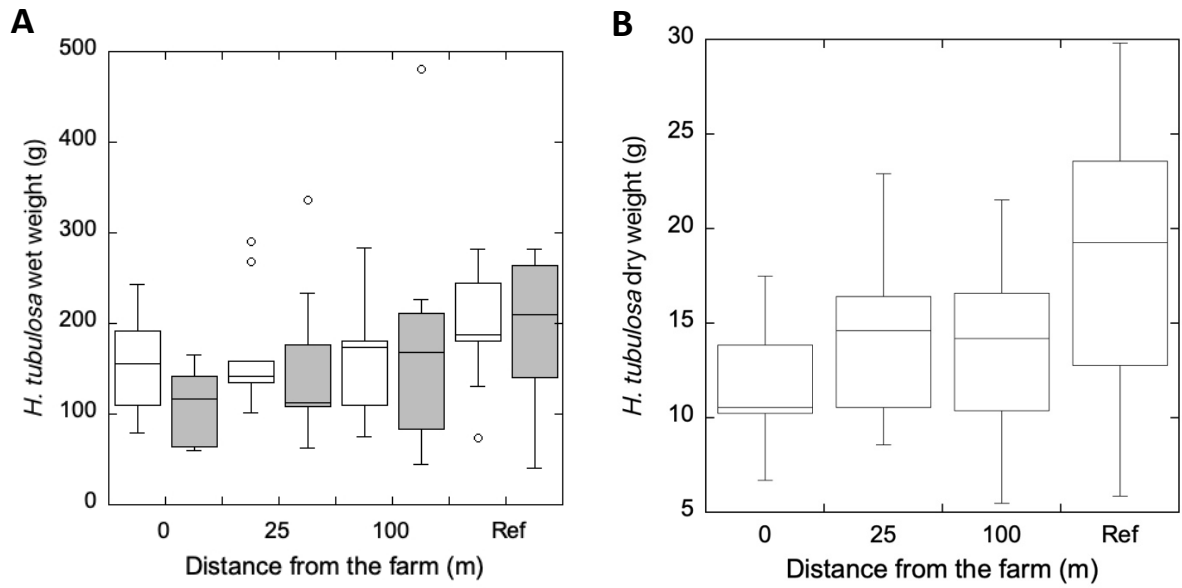


Fig. 5. *H. tubulosa* body weight (mean and SD, in g) after 1 month of exposure at four distances from the farm. A: body wet weight at initial (white box plot) and final sampling (grey box plot), B: body dry weight after 1 month of exposure at four distances from the fish cages (0 m, 25 m, 100 m and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

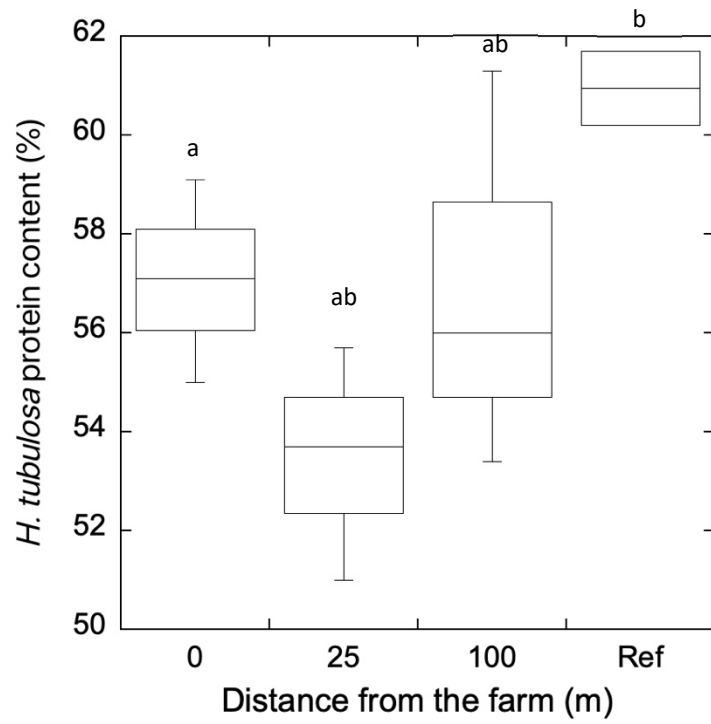


Fig. 6. *H. tubulosa* protein content (in % of dry sample) at four distances from the fish cages (0 m, 25 m, 100 m and reference). Letters indicate significant differences between groups (F test, $\alpha = 5\%$)

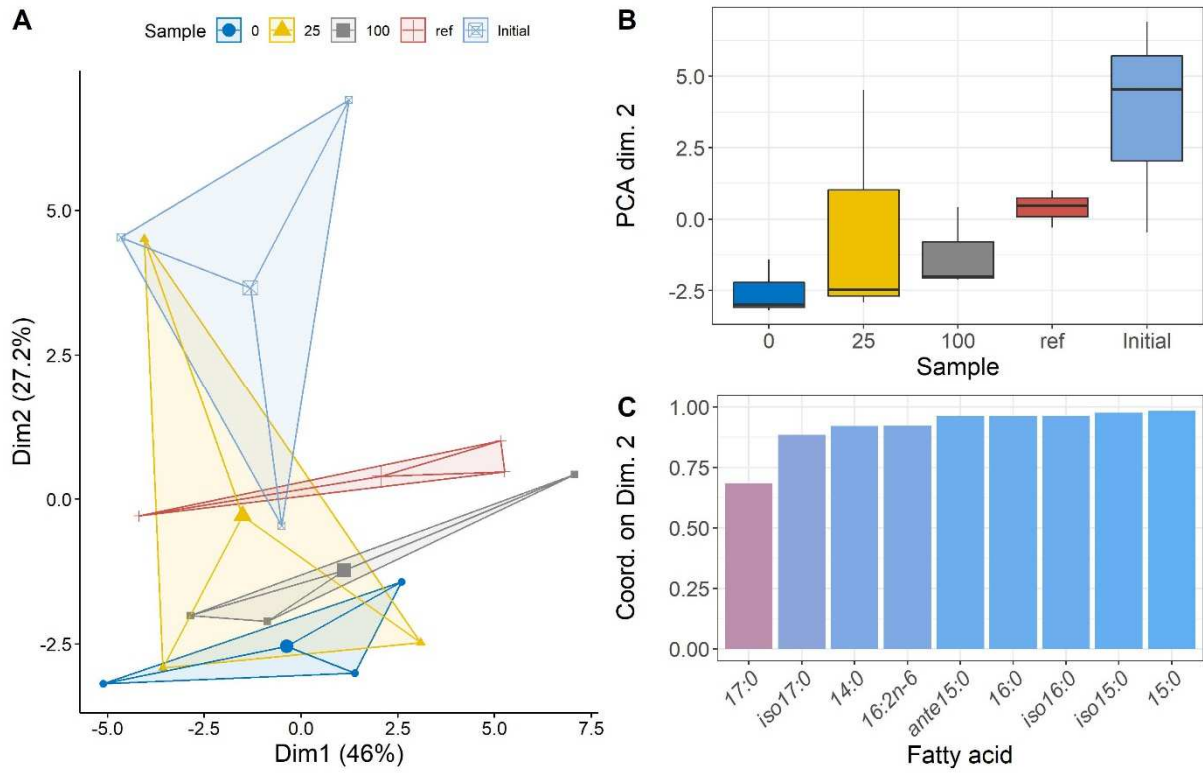


Figure 7. (A) PCA on fatty acid profiles of the sea cucumbers at the 4 distances from the fish cages. (B) Coordinates on the second dimension for each site. (C) Fatty acids contributing the most to the variability on the second dimension of the PCA.

Table 1. Major fatty acids analysed in *H. tubulosa* samples (mean \pm SD, in $\mu\text{g}\cdot\text{mg}^{-1}$ 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 \pm 0.00	0.02 \pm 0.01	0.02 \pm 0.1	0.03 \pm 0.0	0.03 \pm 0.01
16:0	0.05 \pm 0.01	0.06 \pm 0.03	0.06 \pm 0.02	0.08 \pm 0.0	0.12 \pm 0.05
17:0	0.01 \pm 0.00	0.01 \pm 0.00	0.01 \pm 0.0	0.2 \pm 0.0	0.01 \pm 0.00
ISO 17:0	0.01 \pm 0.00	0.01 \pm 0.01	0.01 \pm 0.0	0.01 \pm 0.0	0.01 \pm 0.00
18:0	0.10 \pm 0.03	0.11 \pm 0.02	0.12 \pm 0.02	0.13 \pm 0.01	0.12 \pm 0.01
20:1N-11	0.05 \pm 0.02	0.04 \pm 0.03	0.05 \pm 0.03	0.06 \pm 0.04	0.03 \pm 0.02
20:4N-6	0.09 \pm 0.04	0.07 \pm 0.07	0.09 \pm 0.05	0.11 \pm 0.08	0.05 \pm 0.03
18:0DMA	0.13 \pm 0.04	0.13 \pm 0.03	0.16 \pm 0.03	0.14 \pm 0.02	0.12 \pm 0.03
TOT. SFA_S	0.35\pm0.10	0.39\pm0.06	0.42\pm0.08	0.46\pm0.03	0.46\pm0.07
TOT. MUFA_S	0.15\pm0.05	0.13\pm0.08	0.16\pm0.09	0.17\pm0.12	0.14\pm0.06
TOT. PUFA_S	0.16\pm0.07	0.13\pm0.10	0.16\pm0.09	0.19\pm0.13	0.11\pm0.05
TOT. DMA_S	0.14\pm0.04	0.14\pm0.03	0.17\pm0.03	0.15\pm0.02	0.13\pm0.03
TOTAL FA_S	0.84\pm0.26	0.83\pm0.20	0.94\pm0.30	1.02\pm0.30	0.91\pm0.17

Table 2. Bacterial fatty acids, diatom and zooplankton markers analysed in *H. tubulosa* samples (mean \pm 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 \pm 1.1	17.9 \pm 9.3	15.2 \pm 1.7	18.5 \pm 1.8	28.2 \pm 8.8
DIATOM MARKER 20:5N-3	2.8 \pm 0.6	2.3 \pm 1.8	2.7 \pm 1.2	2.7 \pm 2.4	2.0 \pm 0.9
ZOOPLANKTON MARKERS: 20:1N-9, 22:1N-9	1.1 \pm 0.2	1.1 \pm 0.9	1.3 \pm 0.6	1.1 \pm 0.9	0.6 \pm 0.3