Stable isotope analyses reveal major nutritional deficiencies in captive vs. field juvenile individuals of *Pinna nobilis*

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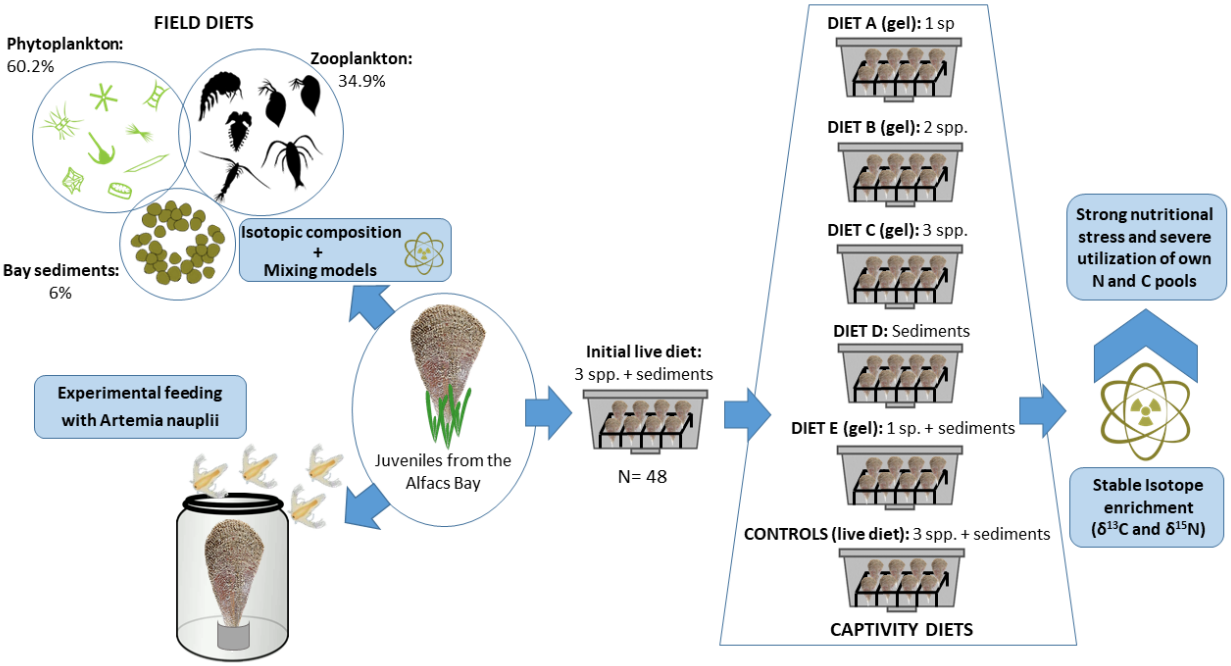
14 GRAPHICAL ABSTRACT



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ABSTRACT

The pen shell, *Pinna nobilis* L. is critically endangered by the spread of a haplosporidan parasite. Stable isotopes have been shown an association with dietary assimilation, trophic level, and body condition, and can provide valuable information for ex situ conservation and breeding. In this context, the aim of this study was to investigate the nutritional status of individuals using $\delta^{13}C$ and $\delta^{15}N$ patterns across pen shell body tissues as tracers of elemental incorporation from treatment diets based on commercial gels and living phytoplankton and/or sediment. Further comparisons were also conducted with field animals and diets to better understand nutritional needs. Captive individuals, were enriched in $\delta^{13}C$ and $\delta^{15}N$ (~18.9 and 1.3‰, respectively), suggesting severe nutritional stress and utilization of own N and C pools, especially muscle. The mixing model for field individuals evidenced a large contribution from the zooplankton fraction (34.9%), which was further confirmed by experimental feeding with *Artemia* nauplii.

Keywords: isotopic enrichment; nutritional stress; pen shell; microalgae gels; zooplankton; dietary formulation

1. Introduction

The fan mussel, *Pinna nobilis*, is a critically endangered bivalve mollusk endemic to the Mediterranean Sea (Kersting et al., 2019). During the fall of 2016, populations started to be seriously affected by the spread of a parasitic disease caused by the protozoan *Haplosporidium pinnae* (Catanese et al., 2018), alone or in combination with a new species of Mycobacteria (Carella et al., 2019, 2020). Mass mortality events close to 100% of the individuals were first observed in Spanish waters during the fall of 2016, and then spread to other Mediterranean countries including France, Italy, Morocco, Tunisia, Turkey, Cyprus, Malta, and Greece, putting the future of species in jeopardy in less than three years (García-March et al., 2020). Given the threatening scenario for the species, the Spanish government implemented a rescue project for 215 individuals from the remaining populations, and 100 adult pen shells from the Alfacs Bay in the Ebro Delta were kept at IRTA facilities. However, pen shells appeared to be stressed under captive conditions and were also prone to mortality by emerging bacterial pathogens such as *Vibrio mediterranei* thus preventing successful breeding (Prado et al., 2020a; Andree et al., 2020). Also, larval cultures using field individuals have so far failed to obtain viable seed, apparently for similar reasons related to bacterial vulnerability (Trigos et al., 2018), thus hindering the species production under captivity conditions. Yet, bivalve aquaculture has provided effective solutions for the rehabilitation of other endangered bivalve populations subjected to overexploitation (Loor et al., 2016; Lodeiros et al., 2016), pointing that further research is needed to establish adequate protocols for pen shell maintenance and growth.

In particular, the proper formulation of diets is generally indicated as a major bottleneck for rearing bivalves in captivity (FAO, 2006). A balanced diet as close as possible to field feeding patterns may reinforce the efficiency of the immune system in cultured bivalves (Hégaret et al., 2004). For instance, distinctive growth patterns and mortality rates by *V. mediterranei* have been observed for juvenile pen shells fed different combinations of phytoplankton gels (1 to 3 species) and an *in situ* produced diet based on three microalgae species, suggesting that diet

quality can mediate disease resistance (Prado et al., 2020b). Similarly, the failure of larval cultures of *P. nobilis* (Trigos et al., 2018) due to an association between disease susceptibility and unbalanced diet formulation cannot be discarded. *P. nobilis* has been indicated to ingest a diversity of phytoplankton species (up to 68 different taxa), along with large quantities of detrital material (up to 95%), and some micro and mesozooplankton species (Davenport et al., 2011; Morton and Puljas, 2019), although the assimilation of the different fractions has not been assessed and dietary contributions are inferred from presence within stomach contents. For logistical reasons, most larval hatcheries including attempts conducted with *P. nobilis* (Trigos et al., 2018), only use 2-3 species of microalgae (at least one Haptophyceae and one Bacillariophyceae; Robert and Gérard, 1999) to feed the bivalves without a clear knowledge of their dietary requirements (Muller-Feuga et al., 2003). Besides, the finding of an adequate formulation could become even more challenging for species such as *P. nobilis* that may use resources other than phytoplankton.

The determination of stable isotope abundances is an important tool for assessing dietary contributions and element assimilation by consumers over long periods of time (months and years; Hobson and Clark, 1992). Isotopic signatures of potential dietary resources can be used to determine their relative contributions to the mixed signature of the animal using mass balance equations (Phillips and Gregg, 2003), but their application relies on the existence of distinctive signatures among diet sources (DeNiro and Epstein, 1978), and on the availability of accurate estimates of consumer-diet fractionation (Prado et al., 2012). Organic matter contents in phytoplankton and zooplankton fractions, and in sediment samples are expected to have locally distinctive isotopic signatures (e.g., Prado et al., 2014a), that may allow the determination of the relative contribution of these broad resources to pen shell diets in situ. Besides, experimental evaluation of isotopic signatures under captivity conditions may also provide useful information about the efficiency of elemental assimilation (C and N) under each dietary condition (Prado et al., 2012), thus aiding dietary formulation. In regard to fractionation, bivalves

are usually considered to be slightly enriched in $\delta^{13}C$ by 0-1‰ (i.e. $\Delta^{13}C$) and to resemble the value of their diet (McCutchan et al., 2003; Post *et al*., 2007; Marín Leal *et al*., 2008). For $\Delta^{15}N$ (i.e. the increase in $\delta^{15}N$ between consumers and their diets), bivalves have been reported lower enrichment values than the usually applied 3.4‰ (Peterson *et al*., 1985; Kwak and Zedler, 1997). Raikow and Hamilton (2001) examined available data on marine bivalves from Minagawa and Wada (1984) and Fry (1988) and suggested that bivalves tend to be only 1.7‰ enriched in $\delta^{15}N$ relative to suspended food resources. Furthermore, Post et al., (2007) indicated a $\Delta^{15}N$ value of only 0.4 for freshwater mussels, and Deudero et al. (2009) consistently found negative values of $\Delta^{15}N$ (from -0.11 to -2.5‰) and $\Delta^{13}C$ (0.27 to -2.29‰) across tissues of *Mytilus galloprovincialis* from an oligotrophic Mediterranean environment.

The population of *P. nobilis* in the Alfacs Bay has been indicated as one of the largest in the Mediterranean within an estimate of over 90,000 individuals (Prado et al., 2014b). Unfortunately, the area was infected by *H. pinnae* in July 2018, although most of the inner bay remains unaffected (Prado et al. in press). A few months earlier, in late January 2018, abundant juvenile recruitment was observed on an emerged sand-bar (less than 10 cm water depth) adjacent to the area of adult collection for the adult rescue project implemented by the Spanish government (Prado et al., 2020a). These juveniles, which would have died in the following months due to desiccation in the sand bar, provided a suitable source of individuals for field and dietary experimentation at the IRTA aquaculture facilities (see also Prado et al., 2020b). In this context, the objectives of this study were twofold: (1) to assess the adequacy of artificial diets for the species maintenance using stable isotope analysis across *P. nobilis* tissues, and when possible, determining the dietary contributions of each diet item; and (2) to evaluate the contributions of the three potential natural resources (i.e., the phytoplankton, and zooplankton fractions and the detrital material) to the diets of field individuals. Besides, a feeding experiment at different concentrations of newly hatched *Artemia* nauplii was conducted to account for possible discrepancies between ingestion and assimilation of zooplankton.

2. Materials and Methods

2.1. Collection of juvenile pen shells and initial keeping conditions.

Juvenile pen shells (N= 48) were collected from a shallow sand bar located in the Alfacs Bay (Fig. 1) and featuring only ≤10 cm water in January 2018, the period of coldest seawater temperature causing exceptionally low tides. Individuals were less than 1 year of age and ranged between 69 to 137 mm total length. Individuals at the study site were in risk of infection by *Haplosporidium pinnae* and *Mycobacteria* sp., which were detected in the bay the following summer (see Carella et al., 2020) coupled with exposure to air desiccation, and consequently, were transported to IRTA's facilities in an attempt to preserve them from death.

Once in the laboratory, individuals were kept in six 50 L tanks featuring an open-water circuit system that uptakes water from the Alfacs Bay. To prevent the entrance of the haplosporidan parasite (ca. 2.8 µm size; Darriba, 2017) seawater was filtered through 10, 5 and 1 µm. Besides, it was disinfected with UV light and subjected to active carbon filtration in order to neutralize agrochemicals discharged to the Alfacs Bay during the rice cultivation period. Physicochemical conditions during the experiment were the same than those indicated in Prado et al., (2020b).

During the ca. 3 initial months (late January to April) individuals were fed twice a day with a mix of three microalgae (*Isochrysis* aff. *galbana* (T-ISO), *Tetraselmis chuii*, and *Chaetoceros calcitrans*), and sediment with ca. 13% organic content. Each tank ration included$9{\cdot}10^9$ cells of T-ISO (1200 ml), $1.2{\cdot}10^9$ (600 ml) cells of *T. chuii*, and $5{\cdot}10^9$ cells of *C. calcitrans* (600 ml) with 1 g of riverine sediments <200 µm. When fed, tanks were closed during 3 h to avoid food loss, and then the tap opened slightly to allow for water renewal. Microalgae species were cultured *in situ* and Ebro River sediments were obtained from Tarragona water consortium (CAT) at the Atmella de Mar (see Prado et al., 2020b for further details).

2.2. Experimental design and diet treatments

Juveniles were allocated within six 50 L tanks in May 2018. Each tank hosted containing 8 individuals, which were evenly distributed across available sizes, and maintained in a vertical position with the aid of a PVC structure. Pen shells were maintained in these tanks for dietary experiments until they died due to infection with *Vibrio mediterranei* (most of them in August-September; see Prado et al. 2020b).

We tested five dietary treatments (A to E) based on different combinations of three species microalgae (*T. chuii*, T-ISO, and *P. tricornutum*) of commercial phytoplankton gel (Easy Reefs, Fitoplancton Marino) featuring a fixed number of cells per ml (Prado et al., 2020b), and/or riverine sediments (see Table 1 for diet details). Also, one group of eight individuals was maintained with the same initial diet based on living microalgae and water renewal conditions and used as an "experimental control". In fact, this group showed the lowest mortality (Prado et al., 2020b), and only 3 individuals could be investigated for stable isotope content. Individuals in diets A to E were fed once in the morning and the tap was maintained close until the following day, when water was fully renovated, and pen shells fed again. Food was provided *ad libitum* by ensuring that microalgae were still available in the water column before water change (5% of supplied doses; > 3000 cell/ ml remaining), this being checked once a week by counting in an Utermöehl plate. This criterion was also supported by parallel experiments with the rough pen shell, *Pinna rudis*, showing that additional quantities of the same gel diet did not result in further growth (S. Hernandis, unpublished data).

The number of dead individuals and the exposure time to each of the diets was registered throughout the stabling period (i.e. from late January to November 2018, when the last individual under experimental diets died). When death occurred, the individual was dissected into different tissues: mantle, gill, adductor muscle, kidney, and digestive gland, and rinsed with ultrapure water to avoid cross contamination. In the occasional instances when some stage of tissue decomposition was observed (e.g. in individuals B1, B5 and E5) the specimen was discarded. This only happened when individuals died in the longest time intervals between

observations (ca. 24 h), which occurred between Sunday morning and Monday morning. Tissue samples and phytoplankton gels (N= 3 replicates of each microalgae species) were dried separately at 60°C until constant weight and then ground to fine powder using a mortar and pestle. Samples of riverine sediments (N= 3) were preserved for further stable isotope analyses. For the initial control diet, volumes of the phytoplankton mix and each separate species (N= 3) were filtered until saturation using a pre-combusted Whatman glass microfiber filter and then dried at 60°C until constant weight. Dried phytoplankton was carefully removed from the filters with a razor blade, and samples examined under the stereomicroscope to ensure the absence of glass fibers prior to homogenization.

In addition to individuals kept in IRTA, six additional healthy juveniles of the same age were collected from the same field site in the Alfacs Bay in summer 2018 and used for comparative purposes. Individuals were transported to IRTA facilities within an icebox and, once in the lab, dissected into different tissues and processed for stable isotope analyses as indicated above. Simultaneously to the collection of individuals, marine sediments (<200 μm fraction), phytoplankton (up to 44 μm fraction), and zooplankton samples (44 to 200 μm fraction) from the Alfacs Bay were also collected for dietary assessment. Phytoplankton samples were collected using a 10 μm net, and once in the lab filtered through a 44 μm gauze for separation of zooplankton and other large, suspended particles (Prado et al., 2017). For zooplankton, samples were collected with a 44 μm net, and once in the lab, filtered through a 200 μm net for separation of particles above that size. The resulting sample material was processed as indicated above.

2.3. Stable isotopes ($\delta^{13}C$ and $\delta^{15}N$) and nutrients contents

Samples of individuals and diets were analyzed with a PDZ Europa 20-20 mass spectrometer connected to a PDZ Europa ANCA-GSL elemental analyzer at the UC Davis Stable Isotope Facility. Isotope ratios in samples were calculated from linear calibration curves constructed with

standard reference materials of known composition and a blank correction. The difference in isotopic composition between the sample and reference materials is determined by the equation:

$$\delta_{sample-standard} = [(R_{sample} - R_{standard})/\ R_{standard}] \text{ x } 1000$$

where R_{sample} is the $^{13}C/^{12}C$ or $^{15}N/^{14}N$ in the sample; $R_{standard}$ is the $^{13}C/^{12}C$ or $^{15}N/^{14}N$ in the calibration material and $\delta_{sample-standard}$ is the difference in isotopic composition of the sample relative to that of the reference (Vienna Peedee Belemnite and atmospheric nitrogen for carbon and nitrogen, respectively). Experimental precision (based on the standard deviation of replicates of an atropine standard and/or IAEA or USGS intercomparison materials) was considered to be adequately high (±0.10‰ for ^{13}C and ±0.12‰ for ^{15}N).

The possible variability in $\delta^{13}C$ signatures induced by uneven storage of lipids (typically depleted in ^{13}C) across tissues was corrected using the equations provided by Post et al. (2007). The threshold for the application of this correction was an increase in corrected signatures of at least 0.1‰ (Prado et al. 2012). $\delta^{13}C$ signatures of pen shell tissues and zooplankton were normalized using the relationship with C:N ratios indicated for aquatic animals (corrected $\delta^{13}C = \delta^{13}C - 3.32 + 0.99 \text{ x C:N}$). For commercial phytoplankton gel diets and field phytoplankton we used the relationship with C:N ratios indicated for plants (corrected $\delta^{13}C = \delta^{13}C + 1.25 + 0.00 \text{ x C:N}$). No correction values were used for riverine and marine sediments since the lipid content was assumed to be very low compared to living organisms.

Elemental contents (C and N) in pen shell tissues and diets were given in total µg per sample and transformed into percent values using sample weights. All values where expressed as C:N. In the particular case of kidney, the presence of large amounts of nephroliths mostly made of a granular inorganic constituent (Ghiretti et al., 1972) yielded very low values of both C (5.6 ± 0.18 %) and N (0.8 ± 0.04 %). Therefore, isotope results for kidney were discarded due to low precision.

214 *2.4. Isotope mixing models*

215 The MixSiar Bayesian mixing model was used to identify the long-term biomass contributions
216 of food items to mixed pen shell diets. This was only possible for the initial vivarium diet and for
217 the field diet, for which there was obvious nutrient incorporation into tissues (see later). The
218 model was first outlined by Moore and Semmens (2008) and modified later to improve the
219 robustness of results. MixSiar v.1.0.4 uses stable isotope signatures with their standard error
220 (SE), and tissue-diet discrimination factors are used as input variables to estimate the probability
221 distributions (5^{th}, 25^{th}, 50^{th}, 75^{th}, and 95^{th} percentiles) of each food item to a mixture and
222 accounts for uncertainty associated with multiple sources. The estimated median contribution
223 (i.e., the 50% percentile) represents the median source contribution value for each source and
224 is usually given for comparative purposes.

225 For mix source values the average of the four tissues investigated per individual (i.e., gill,
226 digestive, muscle, and mantle, featuring similar weight contributions at juvenile stages) was
227 used as a *proxy* of the value in the whole body. For fractionation, we used the 0.4‰ $\delta^{13}C$
228 enrichment (McCutchan et al., 2003; Marín Leal et al., 2008) and the 1.7‰ $\delta^{15}N$ enrichment
229 (Raikow and Hamilton, 2001) indicated for bivalves. Yet, since these fractionation rates
230 constitute a theoretical approximation, runs were conducted using SE of 1‰$\delta^{13}C$ and $\delta^{15}N$ to
231 account for deviations observed in other studies (e.g. Post et al., 2007).

232

233 *2.5.* Artemia *experiment*

234 Adult pen shell individuals of similar size (ca. 50 cm) kept in IRTA as a part of a rescue project
235 of field individuals (see Prado *et al*., 2019 for further details) were used for experimental
236 purposes. Eight pen shells with a healthy appearance were randomly selected in different days
237 and after a 48 h fasting, were placed in a 100 L cilindroconical-tank at concentrations of newly
238 hatched *Artemia* nauplii ranging from ca. 5 to 25 individuals per ml. Individuals remained in the
239 tank for 24 hours (usually from 10 am one day to 10 am of the following day), after which the

240 pen shell was returned to its regular maintenance tank and the nauplii remaining in the 100 L
241 tank, carefully recovered in a 40 µm mesh while opening the tank tap. The collected nauplii were
242 placed in a 2 L water contained and homogenized by vigorous shaking in order to remove
243 agglutinations of individuals due to pseudofeces production. Then, five 1 ml replicates were
244 collected for counting under the stereomicroscope and determination of ingested material as
245 the difference with the initial abundances.
246
247 *2.6. Data analyses*
248 Patterns of variability in the stable isotope composition (lipid corrected values for $\delta^{13}C$) and
249 C: N ratios across Diets and Tissues (fixed factors) were investigated with a two-way factorial
250 ANOVA and Student-Newman-Keuls (SNK) post hoc analyses. The existence of significant
251 differences in $\delta^{13}C$ and $\delta^{15}N$ values among captivity and field diets (fixed factor), respectively,
252 was investigated with a one-way ANOVA and SNK. For tissues, ANOVA assumptions of normality
253 (Chi-square test) and homogeneity of variances (Cochran's test) could not be achieved by
254 transformation and the significance level was fixed to $p < 0.01$ to minimize the risk of making a
255 Type I error.
256 The relationship between the isotopic signature of individuals (average of normalized $\delta^{13}C$
257 and $\delta^{15}N$ values of tissues) and the exposure time to dietary conditions until its death was
258 investigated with correlation analyses. Equally, the association between $\delta^{13}C$ and $\delta^{15}N$ values in
259 pen shell tissues was investigated separately for experimental and field diets in order to assess
260 potential differences in elements' assimilation.
261 The association between the initial *Artemia* nauplii concentration in the water column and
262 the ingestion of individuals over the 24 h was investigated with a non-linear distribution fitting
263 analysis. All statistical analyses were performed using Statistica version 8 software.
264
265 **3. Results**

3.1. Stable isotopes ($\delta^{13}C$ and $\delta^{15}N$) and C: N ratios in consumers

Stable isotope signatures showed significant effects of Diet, Time and their interaction for all variables investigated (Table 2a-b), with the highest differences being found between field and captive pen shells under the initial control diet (Fig. 2, 3a). For $\delta^{13}C$, field pen shells showed the highest values (-19.74 ± 0.12 ‰), and those on the initial control diet the most depleted (-53.64 ± 0.53 ‰), whereas individuals on the other experimental diets showed intermediate values (-31.28 ± 0.57, -32.52 ± 0.82, -32.12 ± 0.60, -29.69 ± 0.53, and -32.91 ± 0.68 ‰, respectively for diets A, B, C and E). Yet, diet D (riverine sediment) was also significantly more enriched than the rest of the diets based on phytoplankton gels (Table 2a). Across tissues, there were significant differences among all factor levels, with muscle showing the highest values (-31.41 ± 0.39 ‰), followed by digestive and mantle (-34.82 ± 0.39‰, and -34.75 ± 0.43‰, respectively), and finally by gill (-36.03 ± 0.30 ‰) (Fig. 3a).

For $\delta^{15}N$, field pen shells also showed the most enriched values (9.24 ± 0.07 ‰), followed by individuals on experimental diets (4.97 ± 0.23, 4.67 ± 0.24, 4.65 ± 0.30, 4.59 ± 0.31, and 4.35 ± 0.13, respectively for Diets B, A, C, D, and E, which was slightly lower than the former four), and lowest on the initial control diet (3.57 ± 0.15 ‰) (Table 2b, Fig. 2, Fig. 3b). $\delta^{15}N$ also showed significant differences across the four investigated tissues, with highest values in muscle (6.01 ± 0.18‰), followed by gill, mantle and digestive (5.04 ± 0.20 ‰, 4.85 ± 0.26 ‰, and 4.69 ± 0.18 ‰, respectively) (Fig. 3b).

Lipid corrected $\delta^{13}C$ and $\delta^{15}N$ values of captive individuals under dietary treatments (i.e., average values of gill, digestive, muscle and mantle) showed no significant association with the exposure time to the diet (r= 0.37 and r= 0.13, p> 0.05, respectively for $\delta^{13}C$ and $\delta^{15}N$), which ranged from 60 to 157 days, depending on survival rates. Also, a significant association between $\delta^{13}C$ and $\delta^{15}N$ values was found across study tissues of all experimental diets supplied in captivity conditions (r_A= 0.58, r_B= 0.64, r_D= 0.47, and r_E= 0.66; p< 0.001) except for diet C (r_E= 0.23; p>

291 0.05) and the initial control diet (r_I= 0.12; *p*> 0.05). No relationship was found for field animals
292 (r_F= 0.38; *p*> 0.05).
293 C: N ratios of individuals also showed significant differences across Diets, Tissues and their
294 interaction (Table 2c; Fig. 4). Field pen shells displayed the highest values (4.47 ± 0.22), followed
295 by diet A and the initial control diet (4.07 ± 0.13 and 3.95 ± 0.14) and experimental diets B to E,
296 which were not significantly different between them (3.87 to 3.85). Across tissues, the highest
297 values were observed in the digestive gland (4.25 ± 0.19), followed by gill and mantle (4.09 ±
298 0.06, and 3.92 ± 0.03, respectively), and the lowest in muscle (3.37 ± 0.17).
299
300 *3.2. Stable isotopes ($\delta^{13}C$ and $\delta^{15}N$) and C: N ratios in diets*
301 *3.2.1. Captivity diets*
302 There were significant differences among the isotopic signatures (both $\delta^{13}C$ and $\delta^{15}N$) of diets
303 used during the captivity period. For $\delta^{13}C$ (Table 3a), the riverine sediment used in the initial diet
304 and in Diets D and E featured the highest signature, with $\delta^{13}C$ values of -14.98 ± 0.14‰. Among
305 experimental gel diets, *P. tricornutum* showed the highest signature (-46.42 ± 0.36‰), followed
306 by *T. chuii* (-47.82 ± 0.15‰), and T-ISO (-49.28 ± 0.40‰). The living phytoplankton species (initial
307 control diet) showed the lowest signatures with values of -52.18 ± 0.18 for *T. chuii*, -55.88 ± 0.28
308 and for T-ISO, and-56.40 ± 0.10 for *C. calcitrans,* with significant differences among them (Table
309 3a, Fig. 2).
310 For $\delta^{15}N$ (Table 3b), higher values were found on living *C. calcitrans* (9.47 ± 0.24‰) and T-ISO
311 (7.47 ± 0.40‰) from the initial control diet, followed by riverine sediments (5.40 ± 0.07 ‰), and
312 living *T. chuii* (3.55 ± 0.34‰). Experimental gel diets displayed considerably lower values, with
313 higher $\delta^{15}N$ signatures in *T. chuii* (1.37 ± 0.17 ‰), followed by T-ISO and *P. tricornutum* (-0.02 ±
314 0.12 ‰ and -0.67 ± 0.26 ‰, respectively (Fig. 2).
315 C: N patterns (Table 3c) also evidenced significant differences among the different diet items.
316 The highest values were observed in riverine sediments (17.89 ± 0.24) followed by gel diets of

317 *P. tricornatum* and T-ISO (6.62 ± 0.18 and 6.36 ± 0.17, respectively) and living T-ISO from the
318 initial diet (5.57 ± 0.26). Living *C. calcitrans* and gel and living *T. chuii* (4.40 ± 0.15, 4.16 ± 0.10,
319 and 4.00 ± 0.13, respectively) displayed the lowest values (Fig. 4).
320
321 *3.2.2. Field diets*
322 For field diets, the three investigated resources displayed significant differences in their
323 stable isotope composition (Table 4a,b; Fig. 2). $\delta^{13}C$ signatures of marine sediments (-1.61 ±
324 0.04‰) were much higher than the phytoplankton (-21.63 ± 0.28‰) and the zooplankton
325 fractions (-19.99 ± 0.08‰).
326 For $\delta^{15}N$, the higher values were found in phytoplankton (7 ± 0.22 ‰) and zooplankton
327 fractions (9.24 ± 0.06‰) and lowest values in riverine and marine sediments (5.40 ± 0.07 and
328 5.03 ± 0.13 ‰, respectively).
329 Patterns of C:N, showed very large values for bay sediments (119.47 ± 1.26), whereas C: N
330 ratios in phytoplankton and zooplankton fractions were much lower (5.84 ± 0.06 and 7.41 ± 0.11,
331 respectively) (Table 4c, Fig. 4).
332
333 *3.3. Mixing models*
334 For the initial control diet, results at the 50% percentile showed that the main dietary item
335 incorporated from mixed captivity diets was the microalgae *T. chuii* (up to 97.9%). T-ISO showed
336 a minor contribution of 1.3%, followed by *C. calcitrans* (0.7%), and the riverine sediment (0.1%).
337 For field diets the model indicated dietary contributions of 60.2% of the phytoplankton
338 fraction (i.e., 10 to 44 µm), 34.9% of the zooplankton fraction (i.e. 44 to 200 µm) and 6% of bay
339 sediments.
340
341 *3.4.* Artemia *experiment*

There was a considerable filtration of nauplii, many of which were rejected as pseudofeces material (Fig. 5a,b), but many were also ingested with increased concentration in the water column, passed through the digestive track and excreted in feces (Fig. 5.c) . The association between the initial nauplii concentration and consumption of individuals after 24 h, showed the best fitting for a logarithmic model (Consumption= a · Log10 (concentration)-b; R= 0.97, p< 0.0001, see Fig. 6).

4. Discussion

Our results for the dietary experiment evidenced an overall enrichment in both $\delta^{13}C$ and $\delta^{15}N$ signatures of consumers across all tissues investigated compared to the initial control diet (by a mean of 18.9 and 1.3‰, respectively for $\delta^{13}C$ and $\delta^{15}N$), which suggest no effective assimilation of phytoplankton gels and/ or sediment diets. Although to some extent it could have been possible that animals were feeding on other non-investigated resource in the water column such as the available bacterial community (Stabili et al., 2005), this seems unlikely due to the lack of growth (<1 mm shell growth · $month^{-1}$) observed across treatments during the dietary experiment (Prado et al. 2020b). This absence of growth coupled with isotopic patterns suggest that the metabolism of pen shells was altered in a similar way to that of fasting animals. In fact, living phytoplankton in the initial control diet had considerably higher elemental content than phytoplankton gels and riverine sediments (ca. 10.2 and 2.2 times more C and 11.7 and 8.5 more N), suggesting that they are both extremely poor nutritional resources. Prolonged fasting has been shown to cause enhanced $\delta^{15}N$ signatures in tissues of birds (Hobson et al. 1993), mammals (Kurle and Worthy, 2001), and invertebrates (Scrimgeour et al., 1995; Oelbermann and Scheu, 2002), due to the progressive utilization of own body pools and preferential recycling of ^{15}N for amino acid synthesis (Minagawa and Wada 1984; Kelly 2000; Fuller et al. 2005). Besides, infection by *Vibrio mediterranei* during the keeping period (Prado et al., 2020a,b; Andree et al., 2020), might have caused additional ^{15}N enrichment, as indicated in animal conditions

associated to disease (Katzenberg and Lovell, 1999). Yet, in the particular case of experimental diets including riverine sediments, the assimilation of some dietary N cannot be excluded, given the more similar signature to pen shell tissues (5.4 vs. ca. 4‰) and the negative values of $\Delta^{15}N$ previously reported for other bivalves (Deudero et al., 2009). For $\delta^{13}C$, enriched values due to starvation and consumption of low-quality food has been documented previously (Webb et al., 1998; Oelbermann and Scheu, 2002; Gaye-Siessegger et al., 2007), but appear to be inconsistent across studies targeting different species (Hobson et al., 1993; Schimidt et al., 1999). In *Pinna nobilis*, enhanced $\delta^{13}C$ signatures across tissues of all experimental diets compared to the initial control diet may be associated to a net catabolic state resulting from muscle degradation and depletion of lipid reserves (Gaye-Siessegger et al., 2007), which play an important role in bivalve physiology during periods of high energy requirement such as reproduction (Ojea et al., 2004; Deudero et al., 2017; Acarli et al., 2018).

Pen shell individuals fed on the initial control diet throughout the stabling period showed an isotopic composition that was very similar to that of the microalgae, *Tetraselmis chuii* (-52.2 and 6.5, for $\delta^{13}C$ and $\delta^{15}N$, respectively), which constituted up to 97.9% of the diet according to the mixing model. Such a large contribution of *T. chuii* was unexpected and suggests preferential assimilation of this species despite the other two microalgae supplied have been shown to be effectively filtered by juvenile *P. nobilis* (Prado et al., 2020b). Unlike individuals fed on phytoplankton gels, control pen shells featured some evident growth (ca. 6 mm · $month^{-1}$), although rates were still ca. 30-40% lower than those observed in field animals of the same age (Prado et al., 2020b) and therefore, they are expected to reflect the stable isotope composition of their diet (Fry, 2006). The extremely low $\delta^{13}C$ values of *in situ* cultured microalgae (*T. chuii*, T-ISO, and *Chaetoceros calcitrans;* -56.4 to -52.2‰) may be connected to the CO_2 source used for cultivation (Messer Ibérica de Gases SA), whereas a more enriched source (-49.2 to -46.4‰) might be used by the company producing phytoplankton gels. For $\delta^{15}N$, differences between gel

393 and living phytoplankton were substantial (from -0.67 to 1.37 and from 6.5 to 12.5‰,
394 respectively) and may reflect differences in the source of commercial fertilizers.

395 Although differences in the isotopic composition of pen shell tissues between the initial
396 control diet and experimental diets where the largest observed during the captivity period, there
397 were other significant differences across treatments. In particular, pen shells fed diet D (riverine
398 sediment) showed higher values than those fed phytoplankton gels, suggesting that this
399 constitutes the most unbalanced diet assayed which is supported by early mortality patterns
400 during the experiment (Prado et al., 2020b). Sediment-based diets showed slightly less enriched
401 $\delta^{15}N$ values than gel diets suggesting that there could have been some dietary uptake of N,
402 although this was not sufficient to preserve the well-being of individuals. Differential element
403 assimilation (N and C) from dietary resources has been previously evidenced for other marine
404 organisms (Tomas et al., 2006) and may also occur in pen shells feeding in low-quality food. For
405 C:N ratios, pen shells from all experimental diets including controls displayed similar values,
406 evidencing that are not a good indicator of nutritional stress compared to stable isotopes (but
407 see Schmidt et al., 1999). Slightly higher values were only detected in field animals (by ~0.4%)
408 suggesting that differences in growth rates might be a mechanism for maintaining certain range
409 of elemental homeostasis (Prado et al., 2014c).

410 At the tissue level, differences in $\delta^{13}C$ signatures have been related to variability in carbon
411 turnover rates, whereas patterns of $\delta^{15}N$ are associated to the metabolic pathways responsible
412 for protein synthesis or degradation (Hobson and Clark, 1992; Sweeting et al., 2007; Fogel and
413 Tuross, 2003). The highest values of $\delta^{15}N$ were observed in muscle, as expected for tissues with
414 enhanced protein turnover (e.g., Pinnegar and Polunin, 1999; Sweeting et al., 2007) followed by
415 gill, mantle and digestive which reported lower variability. For $\delta^{13}C$, the highest signatures were
416 also observed in muscle, which in some vertebrate species is associated to lower metabolic
417 activity and slower carbon turnover (Hobson and Clark, 1992; Tieszen et al., 1983), followed by
418 mantle and digestive tissue, and the lowest in gill. However, the higher $\delta^{13}C$ and $\delta^{15}N$ signatures

in muscle relative to other tissues observed in pen shells from experimental diets compared to control and field animals seems to evidence the use of stored reserves. In fact, the adductor muscle of pen shells has been shown to store energy resources such as glycogen, lipid and carbohydrates are for reproduction and growth (Deudero et al., 2017; Acarli et al., 2018). Therefore, our results suggest that individuals were confronted with strong nutritional stress during the captivity period and forced to consume energy contents in lipids and proteins in the adductor muscle. Remarkably, variability in patterns of $\delta^{13}C$ across tissues of wild, and presumably non-stressed pen shells was very little, which contrast with more distinct patterns reported for pen shells in the Balearic Islands (Cabanellas-Reboredo et al., 2009).

Field pen shells displayed isotopic values that were in the range of variability to those reported previously for *P. nobilis* (Kennedy et al., 2001; Cabanellas-Reboredo et al., 2009; Alomar et al., 2015). According to our results of the mixing model, the phytoplankton fraction constitutes the major dietary resource (ca. 60.2%), but there may also be a significant contribution of the zooplankton fraction (up to 34.9%). These results are in agreement with observations from previous studies suggesting the contribution of micro and mesozooplankton to pen shell diets (Davenport et al., 2011; Morton and Puljas, 2019) and support an overall central role of pelagic versus benthic food sources (Alomar et al., 2015). Our experiment with newly hatched *Artemia* nauplii also confirmed the actual ingestion of animal material, which was increased with the availability in the water column, thus supporting the description of opportunistic predator proposed by Morton and Puljas (2019). According to visual observations under the microscope, *Artemia* nauplii were only partly digested, which might also account for comparatively lower contributions of zooplankton vs. phytoplankton. Yet, the isotopic composition of both fractions was relatively similar (1.63‰ for $\delta^{13}C$ and 2.23‰ for $\delta^{15}N$), so further research is necessary to assess seasonal variation in both resources in order to attain more accurate dietary contributions, which might also account for observed patterns of variability in pen shell tissues (Cabanellas-Reboredo et al., 2009). Besides, it is also necessary

consider that the signatures of phytoplankton and zooplankton corresponded to a size fraction, and that the contribution of suspended organic matter cannot be excluded. For instance, pen shells have been shown to incorporate suspended particulate matter from seagrass epiphytes and macroalgae (Kennedy et al., 2001), so contributions from local beds of *Cymodocea nodosa* cannot be discarded. In regard to bay sediments, however, results from the mixing model returned only minor contributions (ca. 6%) evidencing that this is not a relevant resource for pen shells. These findings are similar to those reported by Kennedy et al., (2001) for surface sediment samples but contrast with results from Davenport et al. (2011), who indicated a major contribution of detritus to the diet, particularly in small individuals living closer to the water-sediment interface. This discrepancy might be due to distinctive assimilations of ingested materials. Thus, sediments may be largely ingested when resuspended from the sea bottom, but animals are only capable of extracting minor components for effective tissue growth.

5. Conclusions

Our study confirms that the use of phytoplankton gel diets is extremely inadequate for the growth and development of juvenile pen shells (Prado et al., 2020b). Individuals fed diets of low nutritional value display enriched patterns of isotopic composition (both $\delta^{13}C$ and $\delta^{15}N$) in their tissues that suggest the undergoing of enhanced catabolic processes and protein degradation (Webb et al., 1998; Oelbermann and Scheu, 2002; Gaye-Siessegger et al., 2007), which accompany a deficient nutritional status. In particular, this enrichment was more severe in muscle, due to its important role in reserve accumulation in *P. nobilis* (Deudero et al., 2017; Acarli et al., 2018). Given the results from field mixing models, the formulation of adequate diets for captivity maintenance of *P. nobilis* appears to be more complex that in other bivalve species regularly cultured for human consumption (FAO, 2006). If pen shells are adapted to assimilate certain elemental contributions of zooplankton, this should be considered in dietary formulations in order to preserve their optimal status and prevent possible nutritional

deficiencies in the long-term (Muller-Feuga et al., 2003). Further research is also needed to investigate the assimilation of different species of phytoplankton, since our results evidence that they can be very variable despite effective filtration and ingestion of different microalgae. Conversely, the supply of sediments appears to be avoidable given its marginal contribution to pen shell diets.

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651

652 **Fig. 1.** Map of the Alfacs showing the position of the pen shell collection site (star) on the
653 emerged sand bar.
654

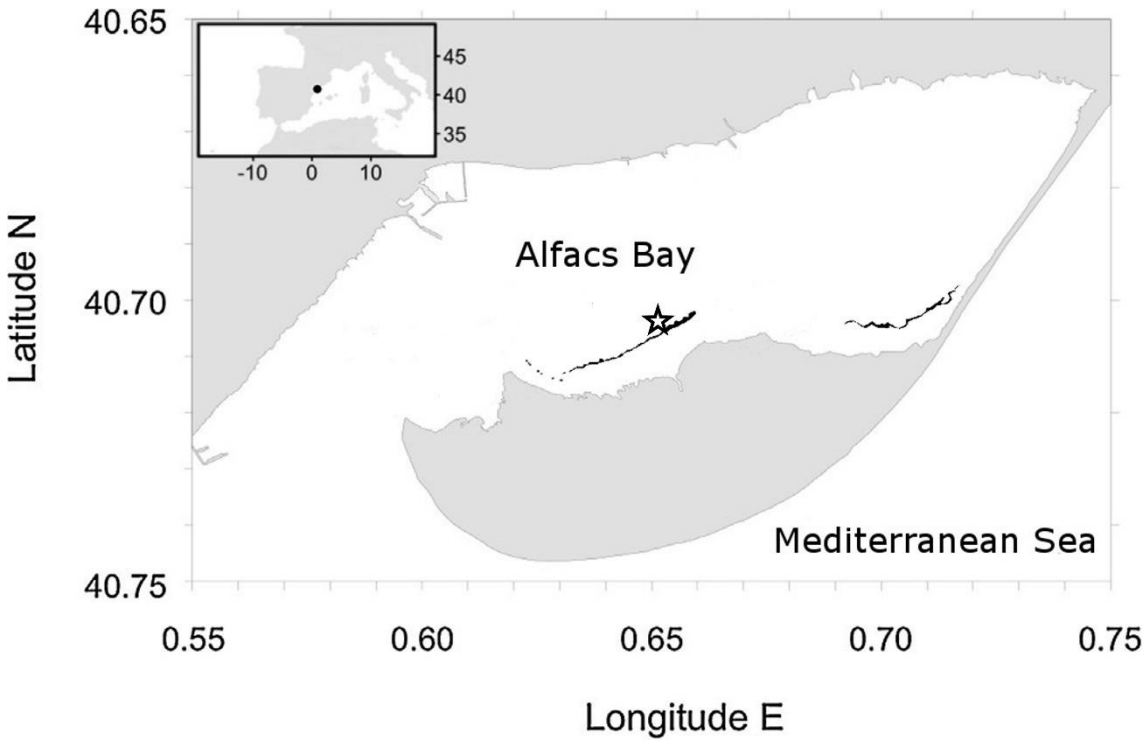

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Fig. 2. $\delta^{13}C$ and $\delta^{15}N$ signatures of field (F) and captive individuals (those on the initial control diet (I), and experimental diets A to E) of *P. nobilis* by tissue type (subscripts g= gill, d= digestive, m= muscle, and ma= mantle) and of the diets in each condition. For the captivity period, the initial control diet (living *T. chuii*, T-ISO, *C. calcitrans* and riverine sediments), and gel signatures (*T. chuii*, T-ISO, and *P. tricornutum*) are indicated with symbols. For field diets, the phytoplankton and zooplankton fractions, and bay sediments are also indicated with symbols. Error bars are SE.

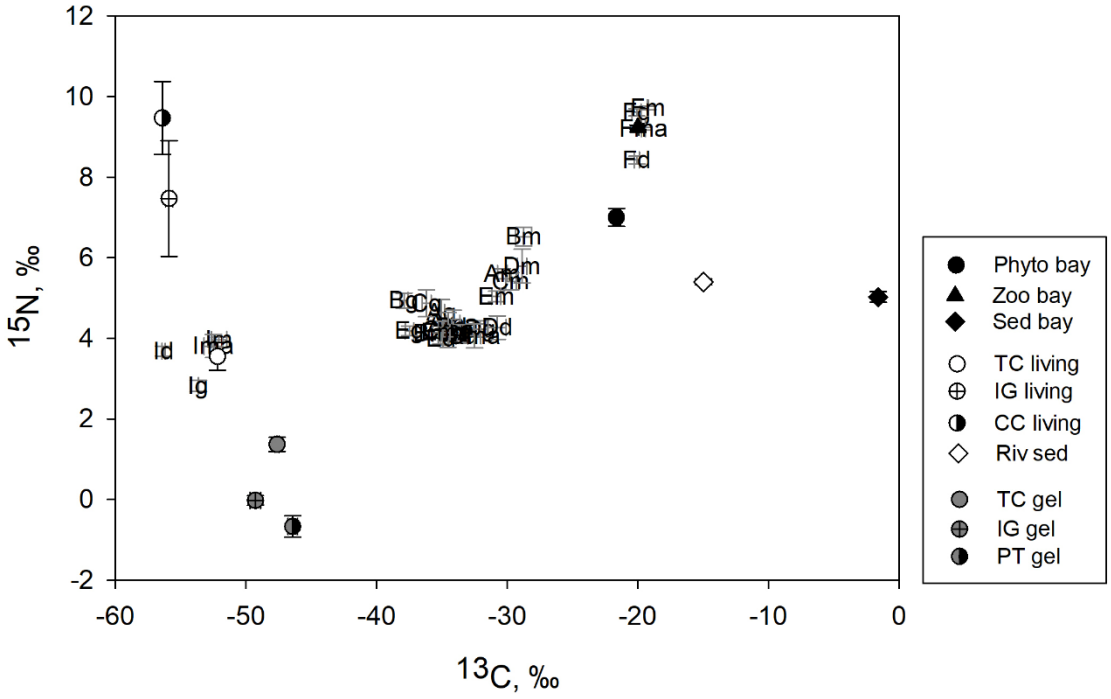
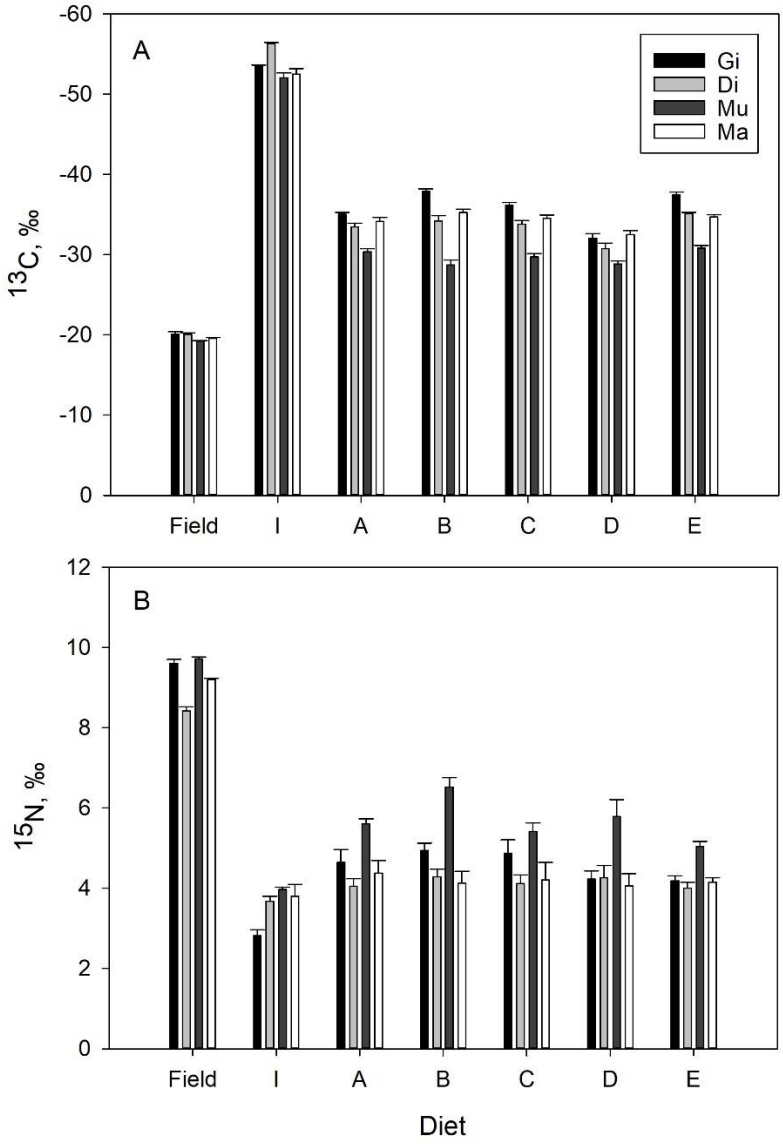


Fig. 3. Stable isotope signatures (mean ± SE) in *P. nobilis* tissues (Gi= gill; Di= digestive; Mu= muscle, and Ma= mantle) under field and captivity dietary conditions (I= initial control diet; A to E= experimental treatments with phytoplankton gels and/or riverine sediments). a) $\delta^{13}C$ and b) $\delta^{15}N$. Error bars are SE.



672 **Fig. 4**. Mean C:N ratios in *P. nobilis* tissues (Gi= gill; Di= digestive; Mu= muscle, and Ma= mantle)
673 under field and captivity dietary conditions (I= initial control diet; Diets A to E= experimental
674 treatments), and in associated diets. Field diets: Alfacs Bay phytoplankton (BF) and zooplankton
675 fractions (BZ) and sediments (BS). Initial control diet: living *T. chuii* (TCi), *T-ISO* (IGi), and *C.*
676 *calcitrans* (CCi), and riverine sediment (RS), and experimental diets based on phytoplankton gel
677 (*T. chuii* (TC gel), *T-ISO* (IG gel), and *P. tricornutum* (PT gel) and/or riverine sediments. Error bars
678 are SE.

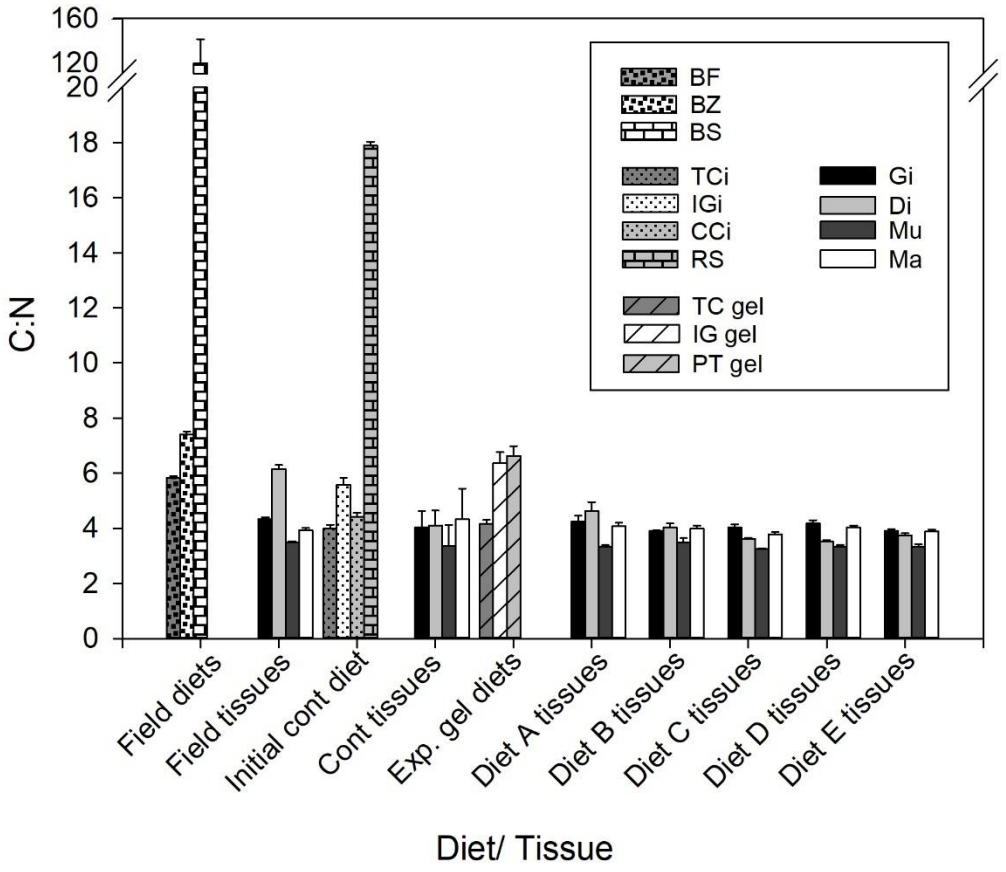


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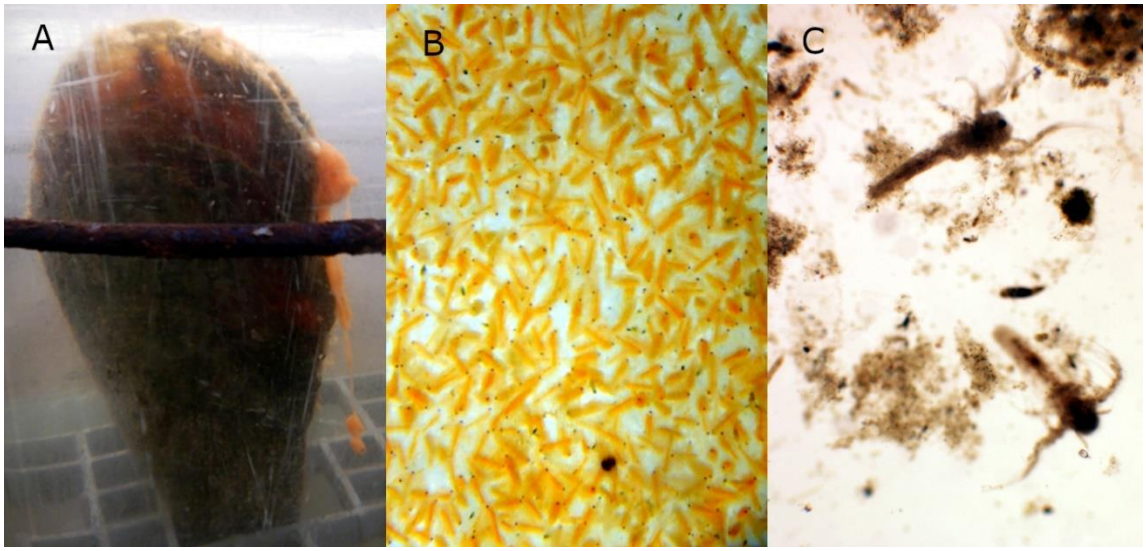
Fig. 5. a) Adult pen shell producing pseudofaeces. b) Pseudofaeces made of living *Artemia* nauplii, and c) close-up of the pen shell feces showing partially digested nauplii.



686 **Fig. 6.** Consumption rates of newly hatched *Artemia* nauplii by adult individuals of *P. nobilis* at
687 increasing concentrations in the water column over a 24 h period considering the numbers
688 rejected in pseudofaeces. Significant fitting results for a logarithmic equation are indicated.
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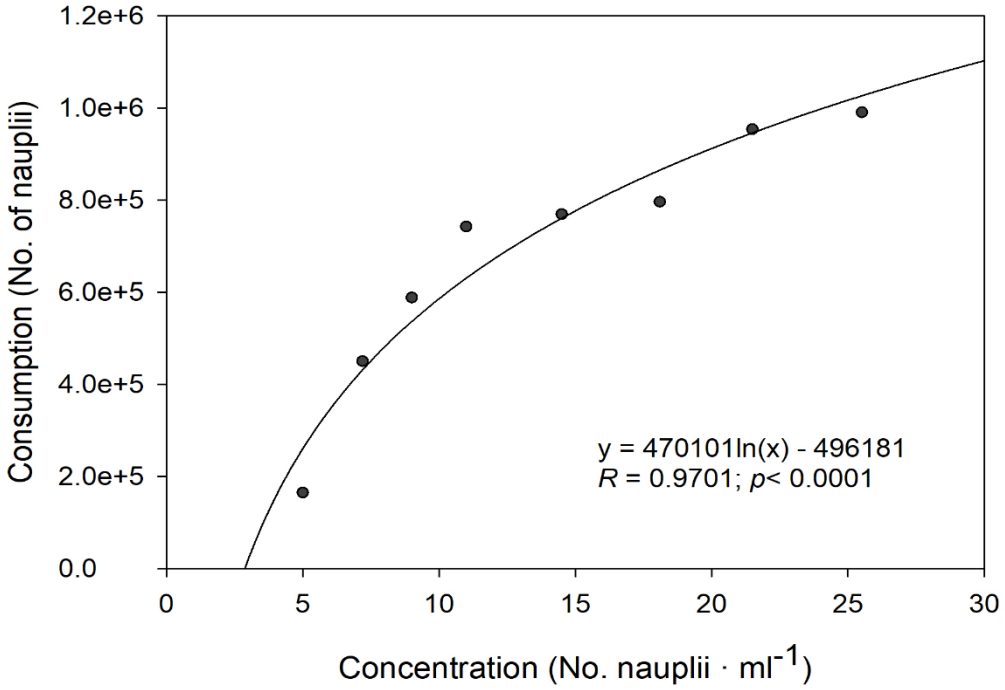

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693 **Table 1.** Description of diets used during the experimental period, after the initial control diet
694 based on living microalgae and sediment (see text for further details). Diets were named
695 alphabetically.
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Diet	Type	Microalgae species	Daily ration
A	Gel	*T. chuii*	7 to 14 ml along the EP
B	Gel	*T. chuii* and T-ISO	3.5 to 7 ml each along the EP
C	Gel	*T. chuii,* T-ISO and *P. tricornatum*	2.4 to 4.7 ml each along the EP
D	Sed	None	2 g DW (13.5% OM)
E	Gel + Sed	*T. chuii*	5 to 10 ml + 0.5 g of sed

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Table 2. Two-way factorial analysis of variance (ANOVA) and post-hoc Student-Newman-Keuls (SNK) for: a) $\delta^{13}C$ signatures; b) $\delta^{15}N$ signatures; and c) C:N ratios of pen shells across diets (F= field individuals; I= control individuals under the *initial* (I) diet with living phytoplankton and riverine sediment; A-E= captive individuals subjected to experimental diets of phytoplankton gel and/ or riverine sediment), and tissues (Mu= muscle; Di= digestive gland; Ma= mantle; and Gi= gill). Significant values are indicated in **bold**.

2-way ANOVA				
A) $\delta^{13}C$	**df**	**MS**	**F**	***p***
Diet (Di)	6	1590.8	1275.9	**0.0000**
Tissue (Ti)	3	164.4	131.9	**0.0000**
Di x Ti	18	11.1	8.9	**0.0000**
Error	156	1.2		
SNK (Di)	F> D> A= B= C= E> I			
SNK (Ti)	Mu> Di= Ma> Gi			
B) $\delta^{15}N$	**df**	**MS**	**F**	***p***
Diet (Di)	6	79.26	178.09	**0.0000**
Tissue (Ti)	3	14.42	32.41	**0.0000**
Di x Ti	18	0.86	1.93	**0.0166**
Error	156	0.44		
SNK (Di)	F> B= A= C= D≥ E> I			
SNK (Ti)	Mu> Gi≥ Ma= Di			
C) C:N	**df**	**MS**	**F**	***p***
Diet (Di)	6	2.009	18.90	**0.0000**
Tissue (Ti)	3	6.233	58.65	**0.0000**
Di x Ti	18	1.261	11.87	**0.0000**
Error	156	0.106		
SNK (Di)	F> A= I ≥ B= C= D= E			
SNK (Ti)	Di= Gi≥ Ma> Mu			

Table 3. One-way analysis of variance (ANOVA) and post-hoc Student-Newman-Keuls (SNK) of pen shells diets used during the experiment (RS= Riverine Sediment; PTgel= *P. tricornut*um gel; IGgel: T-ISO gel; TCgel= *T. chuii* gel; CCi= *C. calcitrans* initial diet; IGi= T-ISO initial diet; and TCi= *T. chuii* initial diet). a) $\delta^{13}C$ signatures; b) $\delta^{15}N$ signatures; and c) C:N ratios Significant values are indicated in **bold**.

One-way ANOVA				
A) $\delta^{13}C$	**df**	**MS**	**F**	***p***
Diet (Di)	6	614.67	1455.4	**0.0000**
Error	23	0.42		
SNK (Di)	RS> PT gel> TC gel> IG gel> TCi> IGi> CCi			
B) $\delta^{15}N$	**df**	**MS**	**F**	***p***
Diet (Di)	6	58.81	282.58	**0.0000**
Error	23	0.20		
SNK (Di)	CCi> IGi> RS> TCi> TC gel> IG gel= PT gel			
C) C: N	**df**	**MS**	**F**	***p***
Diet (Di)	6	76.03	590.44	**0.0000**
Error	23	0.12		
SNK (Di)	RS> PT gel= IG gel> IGi> CCi= TCi= TC gel			

Table 4. One-way analysis of variance (ANOVA) and post-hoc Student-Newman-Keuls (SNK) of field pen shells diets (P=Phytoplankton fraction; Z= Zooplankton fraction; BS= Bay sediment). a) $\delta^{13}C$ signatures; b) $\delta^{15}N$ signatures; and c) C:N ratios. Significant values are indicated in **bold**.

One-way ANOVA				
A) $\delta^{13}C$	**df**	**MS**	**F**	***p***
Diet (Di)	2	555.32	8809.09	**0.0000**
Error	9	0.06		
SNK (Di)	BS> Z > P			
B) $\delta^{15}N$	**df**	**MS**	**F**	***p***
Diet (Di)	2	18.09	192.50	**0.0000**
Error	9	0.09		
SNK (Di)	Z > P > BS			
C) C: N	**df**	**MS**	**F**	***p***
Diet (Di)	2	27839.20	158.36	**0.0000**
Error	9	175.80		
SNK (Di)	BS> P= Z			