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Trophic ecology of suspension-feeding bivalves inhabiting a north-eastern Pacific coastal lagoon: Comparison of different biomarkers

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Abstract :

Understanding the nature and origins of food sources supporting coastal lagoon-inhabiting organisms is necessary to evaluate the ecological status of such ecosystems. The trophic ecology of a bivalve specie *Spondylus crassisquama* was studied in the Ojo de Liebre lagoon (Baja California, Mexico), combining stable isotope (SI), fatty acid (FA) and sterols analyses along a transect under oceanic influences. The second objective of the study was to investigate if sterols compositions give complementary information to those obtained from FA and SI. Temporal and spatial patterns of the three biomarkers in bivalve tissues suggest oceanic inputs at the mouth of the lagoon, while the inner station was characterized by a contribution of local sources including an important role of micro heterotrophs. This study revealed that the association of lipid biomarkers provide higher taxonomic resolution of the spatiotemporal heterogeneity in nutrient sources influencing the trophic functioning of a large coastal lagoons.

Highlights

► Stable isotopes fatty acids and sterols were used to characterize the diet of bivalves. ► A transect was sampled in a Pacific lagoon with increasing distance from the mouth. ► Bivalves at the entrance of the lagoon relied on oceanic phytoplankton inputs. ► Bivalves in the inner lagoon relied on local microbial food web. ► Lipid biomarkers and SI were complementary to distinguish the food sources origins.

Keywords : coastal zone, Ojo de Liebre, suspension-feeders, multi-tracer analyses, fatty acids, stables isotopes, sterols

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1. Introduction

Coastal areas are highly productive marine ecosystems (Clavier et al., 2014; Nixon, 1982) that provide a variety of ecosystem services, such as nutrient recycling, biogeochemical cycles regulation, or food supply (Gattuso et al., 1998; Martínez et al., 2007). Among coastal ecosystems, lagoons play key ecological and economic roles, providing habitat to many resident and migratory species and hosting commercial, artisanal and subsistence fisheries that support local populations. Their high productivity originates from nutrients inputs of both terrestrial and marine origins (Carlier et al., 2007; Mann, 1982), as well as from local primary productivity, enhanced by the shallow and enclosed nature of these areas. Understanding the relative contribution of autochthonous processes versus allochthonous inputs (lagoon vs oceanic primary production, respectively) for the functioning of coastal lagoons is therefore critical to understand the dynamics of these ecosystems, and how they are likely to react when facing anthropogenic disturbances.

Suspension-feeding bivalves are major components of coastal lagoons, where they can dominate benthic communities and play a key role in the regulation of benthic and pelagic biogeochemical fluxes (Dame, 1993). Because these organisms are primary consumers, they directly reflect processes occurring at the lowest level of food webs (Marchais et al., 2013). Furthermore, suspension-feeders can feed on a wide variety of sestonic particles, hence can be integrative indicators of coastal oceanic versus lagoon autochthonous inputs into coastal ecosystems (Coma et al., 2001; Kraak et al., 1991; Lefebvre et al., 2009).

Since a few decades, trophic studies have evolved from field or laboratory observational approaches (*i.e.* stomach contents, food choice experiments) to biomarker-based approaches (BBA). BBA have been applied to a variety of organisms, from primary consumers (Desvilettes et al., 1997) to marine mammals (Kelly, 2000), as well as at the community level (Alfaro et al., 2006; Vander Zanden and Rasmussen, 2001). Every BBA is based on several prerequisites, which need to be met in order to produce robust trophic information. Uncertainties concerning these prerequisites are common (*i.e.* concerning turnover, metabolic fate of biomarker compounds) and result in less reliable data, and stress the need to couple different BBA to fully understand to trophic dynamics of natural environments. Among the most commonly used BBA, stable isotopes (SI) ($\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$) have been widely and successfully used to characterize the origin of food sources and the trophic level of consumers (Peterson and Fry, 1987; Post, 2002). However, SI analysis requires potential food sources to be discriminated in the two or three dimensional isotopic space, which is not always the case in high diversity systems. Moreover, SI analysis provides a limited taxonomic resolution of the food sources assimilated concerning heterogeneous pools of organic matter (*e.g.* suspended particulate organic matter, sedimentary organic matter). In this context, the combination with others biomarkers can give useful information to complement identifications of food sources. Fatty acids allow the identification of some taxa in the diet of consumers due to the existence of specific FA (Dalsgaard et al., 2003; Parrish et al., 2000). Although primary producers have characteristic FA compositions, most do not possess unique FAs which can cause limitations for identifications of food sources (Bec et al., 2010). In addition, in the specific case of coastal benthic food webs, the important number

71 of potential food sources is a problem for distinguishing their contributions to diets (Kelly and
72 Scheibling, 2012).
73 Sterols are lipid compounds common in all primary producers. Some of them have been
74 reported to allow the characterization of classes, genus and in some cases, species of primary
75 producers that contribute to the diet of filter feeders (Napolitano et al., 1993; Soudant et al.,
76 1998; Volkman, 1986). As for FAs, the different primary producers may display distinct sterols
77 composition, which a necessary condition for using these compounds as trophic biomarkers.
78 Moreover, since the capacity to synthesize *de novo* or modify dietary sterols is absent or
79 considered very low in marine invertebrates (Holden and Patterson, 1991; Kanazawa, 2001),
80 sterols have to be acquired from their diet. The role of sterols is still poorly understood, even if
81 they are recognized as important structural components of cell membranes (Giner et al., 2016)
82 and necessary for growth and larval development (Soudant et al., 1998). Primary producers that
83 constitute the major part of the diet of bivalves contain an important diversity of sterols, named
84 phytosterols (Wasowicz and Rudzinska, 2011), and their presence in consumers could be a
85 reliable complementary biomarkers to identify and distinguish contribution of specific food
86 sources to their diet.
87 The present study aims to characterize the trophic ecology of a suspension-feeding bivalve,
88 *Spondylus crassisquama* (Lamarck, 1819) within the Northeastern Pacific coastal lagoon of Ojo
89 de Liebre (Baja California, Mexico). By sampling this species at two sites, varying in their
90 distance to the open ocean, and at two seasons, characterized by different environmental
91 conditions, we test the hypothesis that oceanic inputs induce a spatial structure in the trophic

ecology of suspension-feeding bivalves within the lagoon, including a higher reliance towards oceanic production in individuals living close to the lagoon mouth. For this purpose, a multi-tracers approach was chosen, combining stable isotopes, fatty acids and sterols composition in bivalve digestive glands as well as in their potential food sources (particulate organic matter, sedimentary organic matter, and seagrass detritus). The potential complementarity among those different biomarkers is also discussed.

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2. Material and methods

2.1 *Sampling*

The lagoon of Ojo de Liebre (Baja California, Mexico) is one of the largest coastal lagoons in the north-east Pacific (≈ 40 km long, ≈ 10 km wide, 446 km2). Its topography is characterized by deep and narrow channels surrounded by large intertidal flats. The tidal range is about 1 to 2.5 meters and currents are more turbulent in channels (maximum 2.5 knots) than intertidal flats (0.35 to 1.5 knots) (Phleger and Ewing, 1962). Two stations, located on historically fished banks, characterized by a depth around 7 to 10 m, were studied into the Ojo de Liebre lagoon (Figure 1). Station 1 (Chocolatero) is a bank located close to the mouth of the lagoon (at 6.1 km from the entrance), while station 2 (Datil) represented the most inner station (15.5 km from the

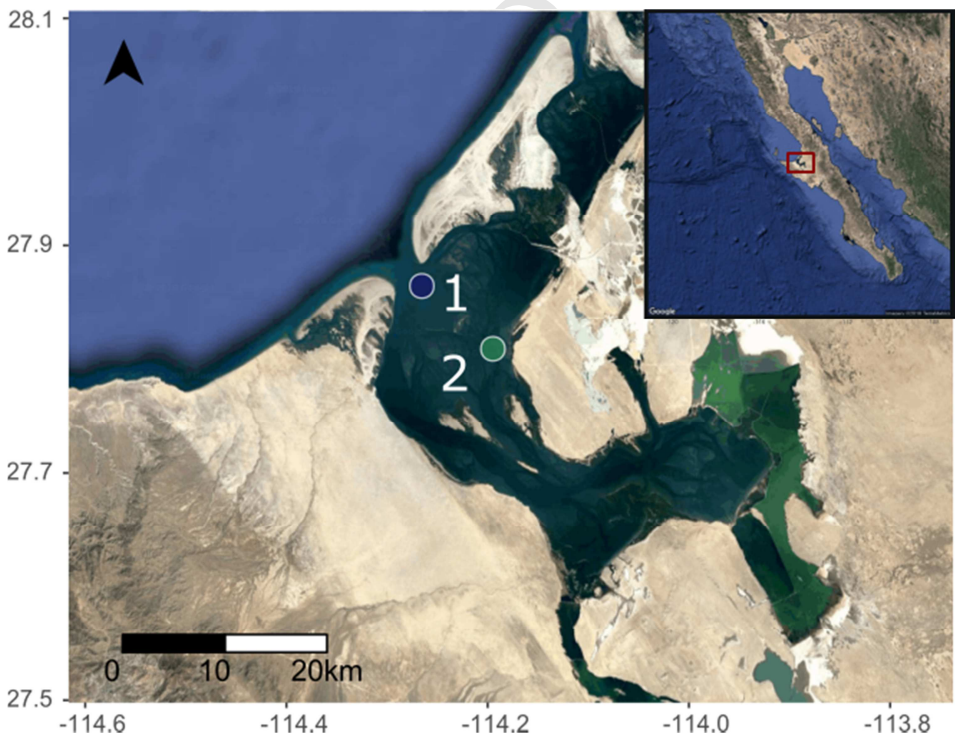

Figure 1. Location of the both sampling stations in the Ojo de Liebre lagoon (Baja California Sur, Mexico).

mouth) and the southern distribution limit of *Spondylus crassisquama* in this bay.

Samples were collected in February and August 2016. For each station, 10 similarly sized individuals were sampled by divers. Potential food sources available for filters feeders were also sampled. One sedimentary core was sampled at each station. The upper centimeter, the most likely to be resuspended by currents and therefore available to suspension-feeders, was collected. Due to the absence of water column vertical stratification into the lagoon (Phleger and Ewing, 1962), one surface water sample (3L) was collected for each site and each date. Because of logistical limitations associated to sampling in the lagoon, we could not replicate our source sampling within each site. Ojo de Liebre contains one of the largest seagrass bed of *Zostera marina* in the Pacific (Cabello-Pasini et al., 2003) which represents one of the dominant primary producer at the bay scale. Because seagrass detritus could contribute to the composition of particulate organic matter (POM) and potentially as a food source for bivalves, stranded *Z. marina* detritus were collected from the shore at the eastern part of the sampling area, and considered as proxies of eelgrass detritus within the lagoon. Indeed, the SI composition of seagrass is known to be little altered during degradation, in particular for $\delta^{13}C$ (Fenton and Ritz, 1988).

After collection, samples were placed in cooler boxes containing water and transported to the laboratory (CIBNOR, Unidad Guerrero Negro). Bivalves were dissected and each tissue of interest (*i.e.* adductor muscle and DG) was sampled, packed into aluminum and stored in liquid nitrogen until further analysis. For each station, surface water samples were filtered on pre-

129 combusted GF/F filters (6 h, 450°C). For each filter 1.5 L of sea water was filtered. Immediately
130 after filtration, filters were frozen in liquid nitrogen. Sediment samples were soaked in local
131 filtered water (GF/F 0.7 µm), and allowed to settle fora few seconds. The supernatant
132 containing fine organic particles, was then collected and filtered on pre-combusted (6 h, 450°C)
133 GF/F filters. Two subsamples of suspended particulate organic matter (POM) (one for SI
134 analyses and one for FA analyses), and four subsamples of sedimentary organic matter (SOM)
135 (two for SI and two for FA analyses) were taken at each sampling station/date. All samples were
136 stored in liquid nitrogen during transportation to the laboratory (CIBNOR, La Paz) and then
137 stored at -80°C until further preparations for analysis.

138 2.2 *Stable isotopes analysis*

139 For SI analyses, muscle samples were freeze dried for 24 h and ground into homogenized fine
140 powder using a ball mill. Muscle samples were not previously delipidated because bivalve
141 muscles generally show a very low content of lipids in this tissue (Lorrain et al., 2002), also C/N
142 ratios were measured and did not exceed 3.5, suggesting delipidation unnecessary (Post et al.,
143 2007). A minimum of 0.250 mg of powder were then placed in tin capsules (8x5 mm) for
144 isotopic analyses. For SOM and POM, filters were scraped, and approximately 1.5 mg was
145 placed in tin capsules.

146 Samples were analyzed by continuous flow on a Flash EA2000 elemental analyzer coupled to a
147 Delta V Plus mass spectrometer (Thermo Fisher scientific). Reference gas calibration was
148 conducted using IAEA-600 Caffeine, IAEA-CH-6 Sucrose and IAEA-N-2 Ammonium

149 Sulphate.Analytical precision, based on repeated analyses of an acetanilide working standard
150 (Thermo scientific), was ± 0.1 ‰ for both $\delta^{13}C$ and $\delta^{15}N$. Results are expressed in δ, with respect
151 to international standards (Vienna Pee Dee Belemnite for $\delta^{13}C$ and atmospheric nitrogen for
152 $\delta^{15}N$), following the equation: $\delta^{13}C$ or $\delta^{15}N = [(R_{sample}/R_{standard})-1].10^{-3}$ (expressed in ‰) and
153 where R is $^{13}C/^{12}C$ or $^{15}N/^{14}N$.

154 2.3 *Lipid analysis*

155 *2.3.1 Lipid extraction*

156 Samples of bivalve DG and *Z. marina* were ground in liquid nitrogen into a fine homogeneous
157 powder. Lipids were then extracted, between 0.2 and 0.4 g of material was placed in 6 ml of
158 chloroform/methanol (2:1; v/v) and sonicated at 4°C during 5 min, to ensure complete
159 extraction of lipids. Lipid extracts were stored at -20°C under nitrogen atmosphere until further
160 analysis. Lipids from the other potential food sources (POM and SOM) were extracted following
161 the same method, by resuspending filters directly in chloroform/methanol (2:1; v/v).

162 *2.3.2 Separation of neutral lipids*

163 Lipids from DG were separated into two fractions (neutral and polar lipids) following the
164 method described by Le Grand et al. (2014). An aliquot (1/6) of total lipid extract was
165 evaporated under nitrogen flux, recovered with 3 washes of 0.5 ml of chloroform/methanol
166 (98:2; v/v) and deposited at the top of a silica gel column (40 mm x 4 mm, silica gel 60A 63-200
167 µm rehydrated with 6% H_2O (70-230 mesh)). NL were eluted with 10 ml of

168 chloroform/methanol (98/2; v/v). NL fractions were collected in 20 ml glass vial containing 2.3
169 µg of each internal standards (tricosanoic acid (C23:0), and cholestane). After evaporation to
170 dryness, NL fraction was recovered with 3 washes of 0.5 ml of chloroform/methanol (98:2; v/v)
171 and separated in two aliquots (1/2), one for FA analysis and the second for sterol analysis.

172 *2.3.3 Methylation of FA*

173 The NL fraction of DG, and an aliquot of the total lipid extract from food sources (POM, SOM,
174 and eelgrass) were evaporated to dryness under nitrogen flux. Acidic transesterification was
175 done to obtain fatty acid methyl esters (FAME). Briefly, 0.8 ml of H_2SO_4/methanol were added
176 (3.4 %; v/v) and lipid samples were then vortexed and heated at 100°C for 10 min. After cooling,
177 0.8 ml of hexane and 1.5 ml of distilled water saturated in hexane were added. Vials were
178 homogenized and centrifuged at 738 g, 1 min at 20°C. The aqueous phase (without FAME) was
179 discarded and the organic phase–containing FAME was washed two more times with distilled
180 water saturated in hexane.

181 *2.3.4 Saponification and silylation of sterols*

182 After evaporation to dryness of NL fraction of DG and of an aliquot of the total lipid extract from
183 food sources (POM, SOM, and eelgrass), 3 ml of KOH/methanol (5.6:94.4; v/v) were added;
184 samples were homogenized and placed under nitrogen atmosphere. Samples were then heated
185 at 100°C during 10 min. After cooling at room temperature, 0.8 ml of hexane and 1.5 ml of
186 distilled water saturated in hexane were added, followed by a homogenization and

187 centrifugation at 738 g during 1 min at 20°C. The aqueous phase was discarded and the organic
188 phase–containing sterols was washed two more times with distilled water saturated in hexane.

189 Before GC analysis, sterol samples were silylated to improve resolution for gas chromatography.
190 For that, organic phases obtained after saponification were evaporated to dryness under
191 nitrogen flux, then 100 µl of reagent (BSTFA:TMCS; 99:1; v/v) and 100 µl of pyridine were
192 added. Sterols samples were then heated at 70°C during 30 min. After cooling at room
193 temperature, 0.8 ml of hexane and 1.5 ml of distilled water saturated in hexane were added,
194 followed by a homogenization and centrifugation at 738 g during 1 min at 20°C. The aqueous
195 phase was discarded and the organic phase–containing silylated sterols was washed two more
196 times with distilled water saturated in hexane.

197 *2.4 Lipid analysis by Gas Chromatographs*

198 *2.4.1 Fatty acid Gas Chromatography analysis*

199 FAME were analyzed in a Varian CP8400 gas chromatograph, programmed in temperature (from
200 0°C to 150°C at 50°C min^{-1}, then to 170°C at 3.5°C min^{-1}, to 185°C at 1.5°C min^{-1}, to 225°C at
201 2.4°C min^{-1}, and finally to 250°C at 5.5°C min^{-1} and maintained for 15 min) and equipped with
202 two splitless injectors and two flame-ionization detectors, with hydrogen as vector gas. FAME
203 were injected and separated simultaneously on two columns, one polar (ZBWAX: 30 m x 0.25
204 mm ID x 0.2 µm) and one apolar (ZB5HT: 30 m x 0.25 mm ID x 0.2 µm), with two different
205 polarities we can easily quantify FA in case of co-elution. FAME were identified by comparisons
206 of their retention time with those of commercial standards (Supelco 37 Component FAME Mix,

207 the PUFA No.1 and No.3, and the Bacterial Acid Methyl Ester Mix from Sigma) and in-house
208 standard mixtures from marine bivalves and micro- and macroalgae. FA compositions were
209 expressed as the mass percentage of the total FA content.

210 *2.4.2 Sterol analysis by Gas Chromatography*

211 Sterols were analyzed in a Shimadzu GC-2010 gas chromatograph (GC). Sterols were injected
212 and separated on a ZB5HT: 30 m x 0.25 mm ID x 0.2 µm (Phenomenex). The GC temperature
213 program was from 40°C to 100°C at 25°C.min^{-1}, then to 250°C at 15°C.min^{-1}, and finally to 315°C
214 at 3°C.min^{-1} and maintained for 10 min. GC was equipped of splitless injector slated at 280°C,
215 and a flame-ionization detector programmed at 250°C, with hydrogen as vector gas and an
216 interface maintained at 315°C. Sterols were then identified by comparisons of their retention
217 time with those of commercial standards and an in-house standard mixtures from marine
218 bivalves. Sterols relative proportions were expressed as the mass percentage of the total sterols
219 content.

220 2.5 *Statistical Analysis*

221 All data were first tested for normality (Shapiro-Wilks test) and homoscedasticity (Bartlett test).
222 If conditions were satisfied, parametric analyses were carried out, alternatively, non-parametric
223 analyses were used. Du to number of replicates, the statistical comparison of food sources were
224 impossible. SI variations between stations of bivalve muscles were compared by Student's test
225 (t-tests). In total, 35 FA and 11 sterols having a mass proportion higher than 0.5 % of total FA or
226 sterol were considered for statistical analyses, and non-identified FA and sterols were grouped

227 as “unknown”. Permutational variance analysis (PERMANOVA) permitted to test the interaction
228 between periods and stations. As the interaction was significant, we analyzed the global spatial
229 difference of both FA and sterol compositions by PERMANOVA tests at each periods. Due to the
230 low proportion of zero in our dataset (less than 5 % for FA and 1.5% for sterols) Euclidean
231 distances or Euclidean-based methods were used throughout our study. To identify which were
232 the most discriminant FA and sterols between stations, a similarity of percentages analyses
233 (SIMPER) was used. Principal component analyses (PCA) were performed to investigate the
234 variation in lipid profiles between stations, for each species and both periods. FA and sterols
235 which accounted for more than 80 % of dissimilarity (SIMPER) were represented on PCA. For
236 each lipid fractions, pairwise comparisons (Wilcoxon tests) were carried out to explore spatial
237 differences for each sterol, at each periods.
238 Firstly, we considered the consistency between trophic structures derived from FA and sterol
239 tracers, at the species levels and at the two periods. Coinertia analyses (Dray et al., 2003) were
240 used to assess whether the same spatial and temporal patterns were retrieved with the two
241 sets of markers. RV coefficient (between 0 and 1) was used to quantify the degree of adequacy
242 between the two datasets, with high values indicating strong concordance. Secondly, we
243 considered the simple relationships among the principal biomarkers using Spearman’s rank
244 correlation (where r_s is the correlation coefficient). When a correlation was detected, the model
245 (linear or not) selected by the Akaike Information Criterion (AIC) was adjusted and the normality
246 of residuals was checked using Q–Q plots.

247 All statistical analyses and graphics were performed with the free software R (Core Team, 2017),

248 with R Version 3.4.1 (2017 06 30).

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3. Results

3.1 Spatial differences in biomarker compositions

3.1.1 Food sources

Spatial and seasonal trends were observed for all biomarker compositions (SI, FA and sterols) of potential food compartments that contribute to bivalve diets. Nevertheless, precautions should be taken as the low number of replicates (n ≤ 2 per stations), did not permit to carry out statistical analyses to reveal spatial differences.

POM was more ^{15}N-enriched in February than in August (Figure 2). For both seasons, the highest δ^{15}N values for POM were recorded from station 1, near the lagoon entrance (8.3‰ in February and 5.5‰ in August) and the lowest at station 2, the furthest site from the bay mouth (5.9‰ in February and 3.7‰ in August). At the both periods, the δ^{13}C values of POM were close between both stations but were quite ^{13}C-depleted compared to SOM (Figure 2). The δ^{15}N and δ^{13}C of SOM did not seem to differ between stations in February (Figure 2). As for POM, SOM samples from station 1 were more ^{15}N-enriched than samples from station 2. The same gradient also occurred for δ^{13}C, with station 1 which seems more ^{15}N-enriched than station 2 (Figure 2). SOM δ^{13}C were close to those of *Zostera marina* detritus (Figure 2), between -13.1‰ and -8.6‰, in February and in August. *Z. marina* detritus did not show big differences in δ^{15}N and δ^{13}C between seasons with an average δ^{13}C of -11.5‰ in February and August and an average δ^{15}N of 2.8‰ in February and 3.4‰ in August.

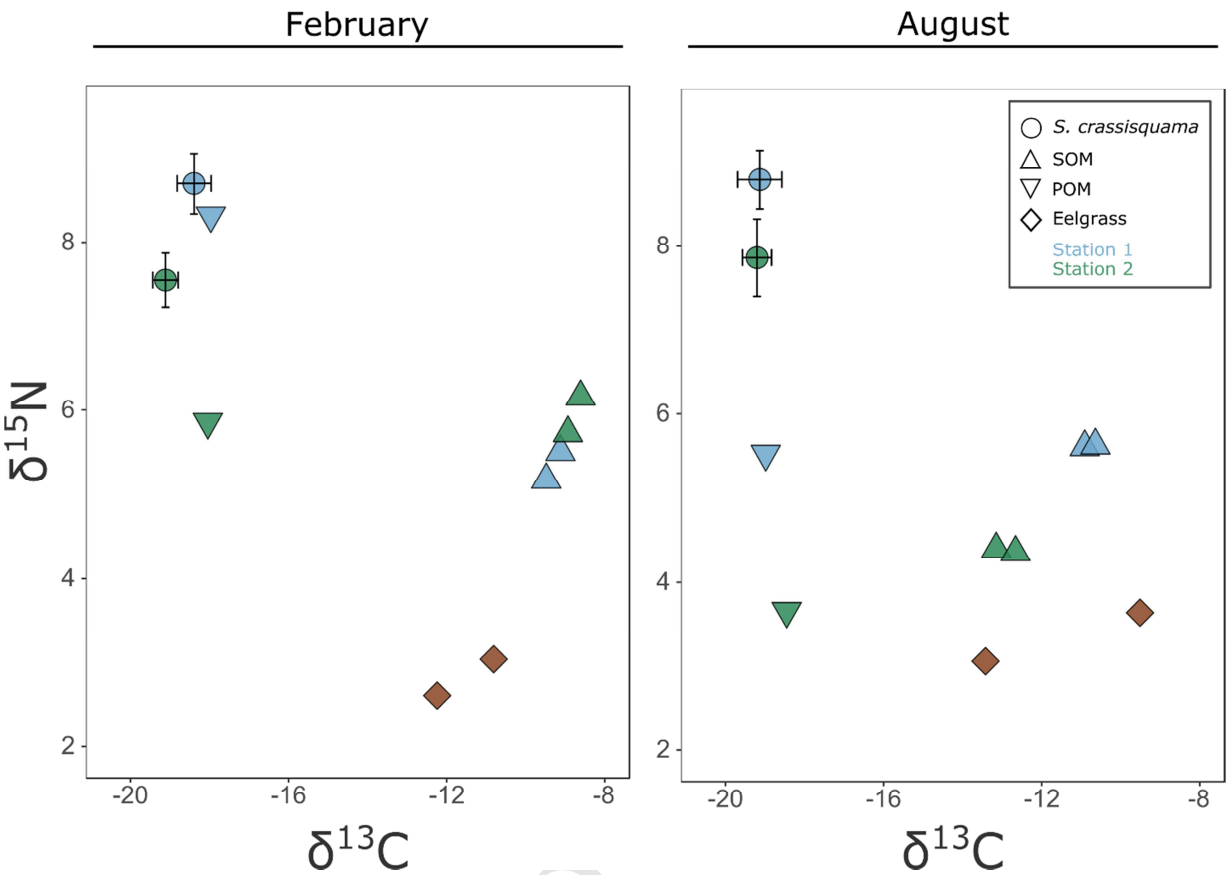

Figure 2. Stable Isotope (SI) bi-plot ($\delta^{13}C$ and $\delta^{15}N$, mean values ±SD when n ≥ 2) of the adductor muscles of Spondylus crassisquama (represented by circles) and of their potential food sources (POM, SOM and seag seagrass), in February and August. Samples from station 1 are shown in light blue and from station 2 in green.

The main FA found in POM and SOM were 16:0 for saturated FA (SFA), and 16:1n-7, 18:1n-9, 18:1n-7 for monounsaturated FA (MUFA) (Supplementary Table 1). For polyunsaturated FA (PUFA), 20:5n-3 and 22:6n-3 were the FA that exhibited the highest abundances in POM and SOM; 18:3n-3 and 18:4n-3 showed highest values in POM and 20:4n-6 in SOM (Supplementary Table 1). Station 2 samples had POM enriched in 18:4n-3 (8.3% in February and 5.3% in August) and in 22:6n-3 (6.9% in February and 8.8% in August) (Supplementary Table 1). SOM showed specifically higher values of 20:4n-6 at station 2 (5.8% in February, and 4.3% in August)

275 (Supplementary Table 1). In February, *Z. marina* was characterized by high levels of 16:3n-3
276 (6.7%) and 18:3n-3 (9.7%) (Supplementary Table 1). In August, the proportion of 18:3n-3
277 reached 33.6% of total FA, whereas 16:3n-3 proportion decreased to 3.7%. The proportion of
278 18:2n-6, which was at low levels in February (1.9%), reached 17.4% in *Z. marina* sampled in
279 August (Supplementary Table 1).

280 The main sterols found in POM were Cholesterol, Brassicasterol, Norcholesterol, 24-
281 Methylenecholesterol, Isofucosterol and β-Sitosterol. At both periods, proportions of
282 Cholesterol in POM collected at station 1 showed higher proportions than station 2 (40.1%
283 against 34% in February, and 56.1% against 38% in August) (Supplementary Table 2).
284 Brassicasterol was present at similar levels in POM at both stations in February (15.4% and
285 15.5%), but was lower at station 1 in August (7.6%) (Supplementary Table 2). The 24-
286 Methylenecholesterol was more abundant in the POM in February (9.0% at station 1 and 12.8%
287 at station 2) (Supplementary Table 2). Stigmasterol was no detected in any samples of POM in
288 February and only 2.9% at station 1 in August, but presented 26.4% of total sterols of POM
289 sample at station 2 in August. In August, the ratio 24-Methylenecholesterol / Brassicasterol was
290 more than twice higher in POM at station 1 (0.8) than at station 2 (0.3). Predominant sterols
291 found in SOM were the same as POM except for Cholestanol, and Campesterol that were
292 principally found in SOM (only detected in SOM for Campesterol). At both months, SOM seemed
293 to present a higher abundance of Norcholesterol and Cholestanolat station 1 than at station 2
294 (Supplementary Table 2). Inversely, Cholesterol, Brassicasterol, and β-Sitosterol were present in
295 higher proportions in SOM collected at station 2 (Supplementary Table 2). The main eelgrass

296 sterols were β-Sitosterol, Stigmasterol and Cholesterol (Supplementary Table 2). Stigmasterol
297 showed higher proportions in eelgrass sampled in August (20.9 vs 10.3% in February), whereas
298 Cholesterol was in much higher proportions in February (16.7% vs 3.8% in August).

299 3.1.2 Bivalves

300 Although spatial differences were quantitatively low, $\delta^{13}C$ values significantly differed between
301 stations in February, (-18.4‰ at station 1 and -19.1‰ at station 2) (t-test, $p < 0.001$, df = 16.70,
302 t = 4.21; Figure 2). In August, $\delta^{13}C$ did not differ significantly between stations (t-test, $p > 0.05$,
303 df = 15.54, t = 0.31), with individuals from station 1 presenting values of -19.1‰ and from
304 station 2 of -19.2‰ (Figure 2). $\delta^{15}N$ differed significantly between stations for both sampling
305 dates, with $\delta^{15}N$ values higher at station 1 than at station 2, this difference being more
306 pronounced in August. In February, $\delta^{15}N$ significantly decreased in muscles along the transect (t-
307 test, $p < 0.001$, df = 17.76, t = 7.56), from 8.7‰ at station 1, to 7.6‰ at station 2 (Figure 2). In
308 August, $\delta^{15}N$ significantly differed between station 1 (8.8‰) and station 2 (7.9‰) (t-test, $p <$
309 0.001, df = 16.7, t = 5.12; Figure 2).

310 Because significant interactions were found for FA proportions between periods and stations
311 (PERMANOVA, p-value < 0.01, df = 1, F = 10.14, $R^2 = 0.13$), and because sterol proportions
312 presented same interactions (PERMANOVA, p-value < 0.01, df = 1, F = 4.45, $R^2 = 0.09$), we
313 compared spatial differences at each period.

314 The PCA based on NL FA and sterols composition shows the differences between stations and
315 lipid biomarkers responsible for 80% of these differences (Figure 3).

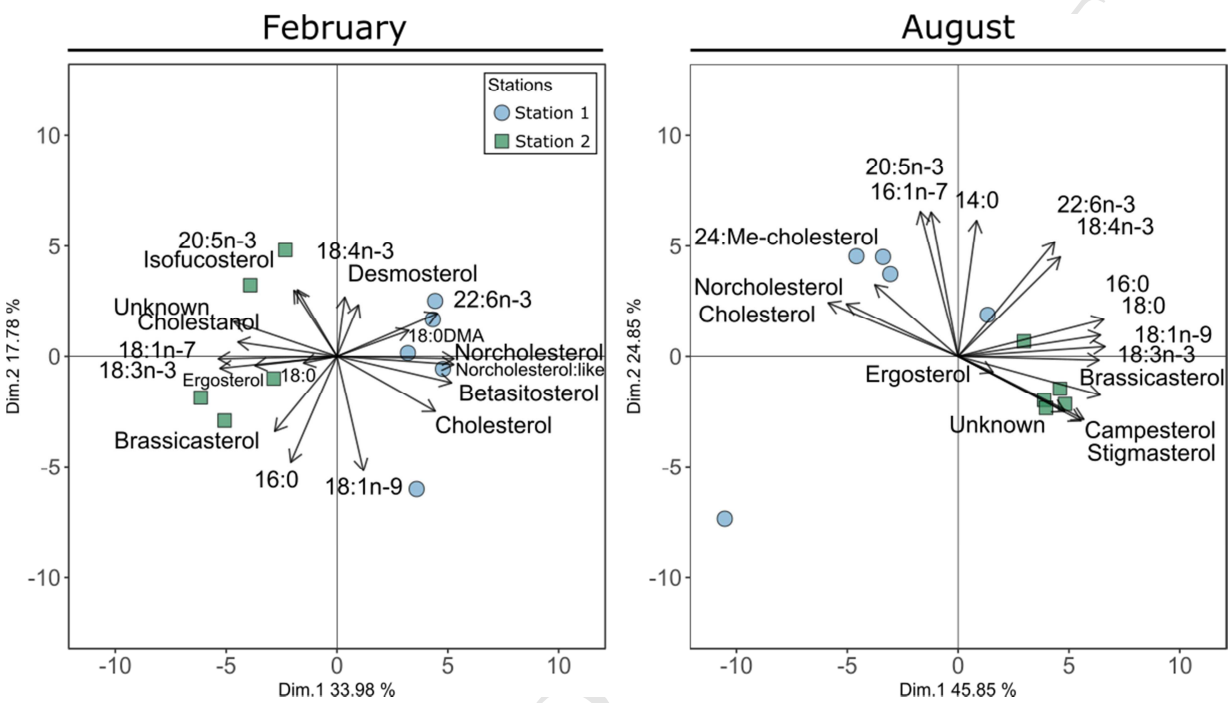

Figure 3. Principal Component Analyses (PCA) of neutral lipids (NL) fatty acids (FA) and sterols compositions (mass %) of Spondylus crassisquama digestive gland in February (A and C) and August (B and D). Only FA and sterols that account for > 80% of the contribution of dissimilarity between stations are shown.

316 In February, significant spatial differences were found for both NL FA composition
317 (PERMANOVA, $p < 0.001$, df = 2, F = 5.45, $R^2 = 0.3$), and sterol composition (PERMANOVA, p-
318 value < 0.05, df = 1, F = 10.02, $R^2 = 0.56$). At this period individuals from station 1 were
319 characterized by higher levels in 20:4n-6 and 22:6n-3 (Figure 3 and Supplementary table 3) and
320 were associated to higher values of β-Sitosterol (Figure 3 and Supplementary table 4).
321 Individuals from station 2 tended to be characterized by FA 18:1n-7, 18:3n-3 (Figure 3 and
322 Supplementary table 3) and were not associated to any particular phytosterol.

323 In August, NL FA compositions differed among sampling location (PERMANOVA, $p < 0.001$, df =
324 2, F = 34.11, $R^2 = 0.74$). The same was found forsterol composition (PERMANOVA, p-value <
325 0.01, df = 1, F = 13.55, $R^2 = 0.63$). Here again, a clear spatial pattern was observed along the first
326 axis of the PCA (Figure 3). We observed significantly decreasing values of 20:5n-3 and 16:1n-7
327 from the mouth to the inner bay (Figure 3 and Supplementary table 3). Even if PCA revealed a
328 contribution of 24-Methylenecholesterol (Figure 3), pairwise comparisons revealed no
329 significant spatial differences (Supplementary table 4). Individuals from station 2 were
330 characterized by higher proportions of 18:1n-9 and 18:3n-3 (Figure 3 and Supplementary table
331 3), and by significantly higher proportions of Campesterol, Stigmasterol, Brassicasterol (Figure 3
332 and Supplementary table 4).

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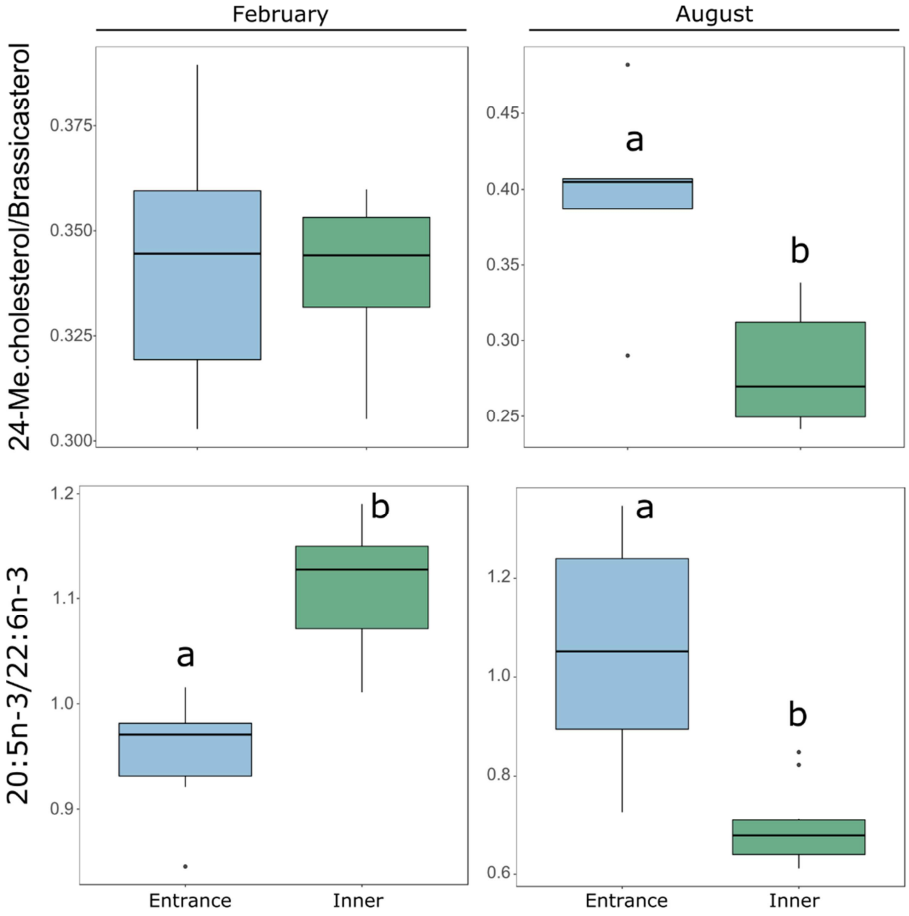

Figure 4. Boxplot of the ratios of 24-Methylenecholesterol/Brassicasterol (n=5) and of 20:5n-3/22:6n-3 (EPA/DHA; n=10) of Spondylus crassisquama at the both periods. Different letters indicate significant differences (Wilcoxon test, at significant level α < 0.05). 24-ME.cholesterol: 24-Methylenecholesterol.

We calculated the ratio between 24-Methylenecholesterol and Brassicasterol and the ratio between 20:5n-3 and 22:6n-3 (EPA/DHA) (Figure 4).In February, the EPA/DHA was significantly higher at the inner site, while no difference was found for the 24-Methylenecholesterol/Brassicasterol. In August, the two ratios displayed the same pattern, being the highest near the entrance of the lagoon.

3.2 Comparisons of different biomarkers

339 We found a robust correlation between the two ordinations of FA and sterols. This relationship
340 was stronger in February (RV = 0.71) than in August (RV = 0.6). Both co-inertia analyses were
341 significant (p-value < 0.01). Significant Spearman's rank correlations were found between
342 EPA/DHA and 24-Me/Bra (r_s = 0.7, p-values < 0.05; Figure 5). Both ratios were positively
343 correlated to the 16:1n-7 (0.33 < r_s < 0.54, p-values < 0.05; Figure 6). Inversely, ratios were
344 negatively correlated to Campesterol (-0.76 < r_s < -0.78, p-values < 0.05; Figure 5), as for
345 Stigmasterol (-0.69 < r_s < -0.91, p-values < 0.05; Figure 5). Interestingly, Stigmasterol biomarker
346 was strongly and positively correlated with Campesterol (r_s = 0.8, p-values < 0.05; Figure 5). In
347 contrast, it was negatively correlated to 20:5n-3 (r_s = -0.48, p-values < 0.05; Figure 5). No
348 correlation was observed between Stigmasterol and 16:1n-7 (r_s= −0.39, P > 0.05; Figure 5). 24-
349 Methylenecholetrol was strongly correlated with EPA/DHA ratio (r_s= 0.73, P < 0.05; Figure 5).

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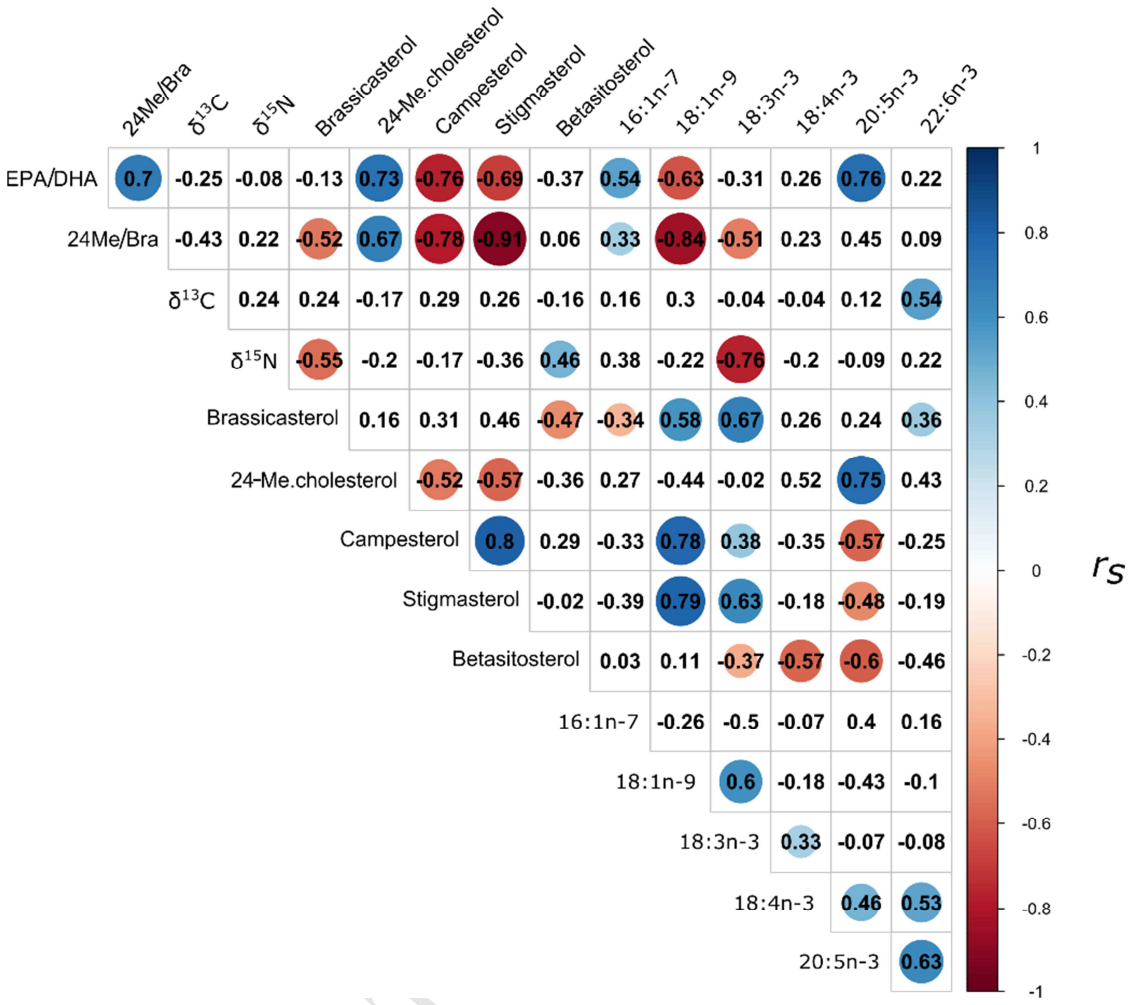

Figure 5. Correlations between the three biomarkers (Spearman's test): stable isotope rations (carbon and nitrogen; ‰), neutral lipid fatty acids, and sterols (mass %) for Spondylus crassisquama. The colored circles represent significant correlations between two tracers (p-value < 0.05). The numbers inside cells are the associated Spearman's correlation.

351

352 **4. Discussion**

353 In coastal lagoons, investigating the nature of food sources consumed by bivalves can provide a
354 spatially and temporally integrated overview of nutrient transfers within the system and across
355 ecosystem boundaries (e.g lagoon primary production vs inputs from coastal ocean). Here, the
356 coupled approach used revealed quite powerful to unravel spatial patterns in the trophic
357 ecology of *S. crassiquama*. Our results point out the significant role played by oceanic inputs in
358 the ecological functioning of coastal lagoons, including the support of bivalve populations. This
359 study also highlights the role played by remineralization in the food web of the inner lagoon.

360 4.1 Spatial differences in biomarker compositions

361 The similarity of carbon SI composition observed between POM and bivalves isotopic
362 composition is in accordance with a direct and non-selective feeding (Vander Zanden and
363 Rasmussen, 2001). Although significant differences were observed between stations especially
364 for $\delta^{15}N$, the extent of these differences was low, typically below 1.5 ‰ suggesting that no
365 major contrast occurred in the diet of this species in the lagoon. Individuals sampled close to the
366 mouth displayed the highest $\delta^{15}N$, while those from the inner lagoon displayed the lowest $\delta^{15}N$.
367 This pattern matches the one displayed in POM, suggesting that bivalve $\delta^{15}N$ directly reflect the
368 composition of POM, and their diet does not drastically differ from its composition (Coma et al.,
369 2001). Consequently, $\delta^{15}N$ indicates that oceanic inputs affecting the composition of POM are
370 transferred into the local food web through their consumption by suspension-feeding bivalves
371 of the lagoon. Ojo de Liebre is located in an arid region, with virtually no freshwater inputs
372 (Phleger and Ewing, 1962). In contrast, the open ocean facing the lagoon can induce ^{15}N-

373 enriched nutrient or plankton pulses that could explain the spatial pattern observed here
374 (Altabet et al., 1999). The higher values at the entrance of the lagoon tend to be close to those
375 described in the region (McMahon et al., 2013; Olson et al., 2010). It seems therefore that the
376 lagoon of Ojo de Liebre, at least in its proximal part, is significantly influenced by its nearshore
377 hydrological conditions, through the entrance of oceanic originating organic matter/nutrients.

378 At both seasons, global FA and sterol compositions differed, revealing spatial differences in the
379 diet of *S. crassisquama*. In February, even though some biomarkers were significantly different
380 between stations, the spatial pattern was quite slight, revealing only small dietary differences.
381 Individuals from the mouth of the lagoon were characterized by higher proportions of 22:6n-3
382 and β-Sitosterol. The presence of 22:6n-3 suggests a dominance of dinoflagellates in the diet of
383 *S. crassisquama* (Alfaro et al., 2006; Parrish, 2009). Although β-Sitosterol was the major sterol
384 found in eelgrass, it is not possible to conclude that β-Sitosterol found in bivalves DG is obtained
385 from eelgrass detritus, as this sterol was also found in POM and SOM. High proportions of β-
386 Sitosterol have also been reported in haptophytes and diatoms in the literature (Napolitano et
387 al., 1993; Soudant et al., 1998). In fact, most phytosterols found in this study were found in all
388 POM, SOM, and eelgrass samples, making difficult to decipher their origin among potential
389 foods sources of bivalves.

390 In August, a clear gradient from the mouth to the center of the bay suggested a more important
391 spatial diet shift. Individuals from the most oceanic stations (station 1) were characterized by
392 higher levels of 16:1n-7 as well as 20:5n-3, but also by higher levels of 24-Methylenecholesterol.

The presence of these lipid biomarker, as well as higher EPA/DHA and 24-Me./Bra ratios, highlights the importance of diatoms in the diet of bivalves from this station during this period (Kharlamenko et al., 2001; Volkman, 2003; Alfaro et al., 2006; Lavaud et al., 2018). This supply in diatoms at the mouth of the lagoon supports the hypothesis of oceanic inputs and intake of primary production from coastal waters suggested by ^{15}N enrichment. Du et al. (2015) showed in the Northern California current that diatoms proliferate during strong upwelling events (from April to September), when nutrients and turbulence are relatively high. The fact that oceanic water masses present the same taxonomic dominances supports again the hypothesis that oceanic organic matter enters the lagoon and supports local the food web. However, the diminution of diatoms importance at the inner station suggests that oceanic inputs do not extend to the whole bay. At both seasons, in the center of the bay, *S. crassisquama* was characterized by high levels of C18 PUFA (18:3n-3) and/or MUFA (18:1n-9), but also by high proportions of Brassicasterol, Campesterol, or Stigmasterol. These FA can be found in various primary producers, such as macroalgae (Nelson et al., 2002), higher plants (Valitova et al., 2016), seagrass (Kharlamenko et al., 2001; Jaffé et al., 2006; Volkman et al., 2008), various phytoplankton taxa (Soudant et al., 1998; Dalsgaard et al., 2003), including dinoflagellates (Soudant et al., 1998; Kharlamenko et al., 2001; Hurtado et al., 2012; Nerot et al., 2015) and are very abundant in various ciliate species, which contribute to the marine microbial food web (Zhukova and Kharlamenko, 1999). Consequently, bivalves from the center of the bay seem to rely, at least partly, on microheterotrophs (dinoflagellates, flagellates, ciliates), hence revealing the influence of the microbial food web in this part of the lagoon in summer. The lower

414 EPA/DHA and 24-Me./Bra ratios also support the hypothesis of the presence of dinoflagellates
415 in the diet of this species (Alfaro et al., 2006; Kelly and Scheibling, 2012; Lavaud et al., 2018).

416 Campesterol, has been reported in small amount in some species of diatoms like *Chaetoceros*
417 *calcitrans* (Yamauguchi et al., 1986; Soudant et al., 1998; Mohammady, 2004). The contrast in
418 the spatial repartition of Campesterol (higher at inner station) and 24-Methylenecholesterol
419 (higher at entrance station) suggests different diatom assemblages and the contribution of
420 different diatom species in the diet of bivalves within the lagoon. In addition, Campesterol was
421 absent from POM samples at both stations and for both seasons, whereas it was present in non-
422 negligible amounts in SOM. The presence of this sterol in bivalves might therefore suggest a
423 contribution of SOM in the diet of bivalves, through the resuspension of sediment.

424 The contribution of eelgrass detritus to the diet of bivalves, although already reported as a
425 secondary dietary source in enclosed coastal systems (Schaal et al., 2008) seems unlikely
426 because of the lack of ^{13}C-enrichment in bivalves. However, lipid biomarkers as 18:3n-3 or
427 Stigmasterol seem to reveal the presence of eelgrass in bivalve diets, and in particular at the
428 inner station. Seagrass cover a surface of more than 590 km² in the Ojo de Liebre lagoon
429 (Riosmena-Rodríguez et al., 2013), and are known to produce large amounts of detritus, which
430 can be buried in the sediment, exported to the adjacent nearshore environment, or locally
431 transferred into the food web (Hyndes and Lavery, 2005; Schaal et al., 2008). Here, SI ratios
432 measured in sedimentary organic matter revealed strong ^{13}C enrichment, suggesting that
433 detritus may be major components of sediment in the Ojo de Liebre lagoon. Although these

434 high $\delta^{13}C$ in SOM could also reveal the importance of epiphytes, which commonly display a SI
435 composition similar to their seagrass host, the highly degraded nature of sediments suggests a
436 dominance of refractory compounds, which are abundant in seagrass (Fourqurean and Schrlau,
437 2003). In contrast with sediment, POM did not seem to reflect any significant contribution of
438 seagrass, which would have resulted in more ^{13}C-enriched SI composition than what was
439 observed. Only a slight ^{13}C-enrichment could be found in POM at stations inside the lagoon that
440 could be due to abundant seagrass detritus in suspension on the water column, but the lack of
441 replicates prevents any definitive conclusion about seagrass contribution to the POM pool.

442

443 4.2 Comparison of different lipid biomarkers

444 Comparing sterol composition with FA and SI analysis brings some interesting insights into the
445 potential contribution of benthic primary producers in the diet of bivalve species. The
446 compositions in FA and sterols were strongly correlated according to the RV coefficient of the
447 co-inertia analyses. The higher RV coefficient in February could be explained by the fact that the
448 diet of bivalves was more spatially homogeneous in the winter period. Spearman's correlations
449 allowed to validate the use of 24Me./Bra as a maker of predominance of diatoms or
450 dinoflagellates in the bivalve diet. Indeed, this ratio was strongly positivley correlated with
451 EPA/DHA ratio, which is currently used in literature (Le Croizier et al., 2016; Parrish, 2009). Both
452 ratios were also positively correlated to 16:1n-7, which confirmed their usefulness to unravel
453 the predominance of diatoms in the diet of bivalves (Dalsgaard et al., 2003; Richoux and
454 Froneman, 2008). However, Campesterol was negatively correlated to both ratios, which could

455 be surprising for a known diatom biomarkers. In fact, Campesterol is reported to be biomarker
456 of Bacillariophyceae (Soudant et al., 1998; Mohammady, 2004), which seems to be a group of
457 diatoms only predominant in the inner lagoon. Besides, Stigmasterol, which could be considered
458 as biomarker of eelgrass (Jaffé et al., 2006; Volkman et al., 2008), is highly and positively
459 correlated to 18:3n-3 also known as biomarker of eelgrass (Kharlamenko et al., 2001). This
460 correlation and high amount of these lipid biomarkers found in seagrass detritus samples,
461 support their utilization as biomarker of seagrass detritus in the lagoon of Ojo de Liebre.

462 **5. Conclusion**

463 Stable isotopes and fatty acids and sterols proved being highly complementary tools to identify
464 spatio-temporal patterns in the Ojo de Liebre lagoon. The entrance of the lagoon seems to
465 reflect coastal oceanic processes, supporting the hypothesis of significant oceanic primary
466 production pulses into the lagoon. Although significant, the influence of oceanic inputs does not
467 extend to the whole lagoon. The center of the lagoon seems to be more local processes-driven,
468 with a recycled organic matter-based food web and the role of seagrass suggested by FA and
469 sterols analysis. Our results revealed strong correlations between FA and sterols in bivalves.
470 Consequently, sterol compositions could be used as complementary and/or substitute
471 biomarkers to FA. The approach used in this study brought some interesting new insights on the
472 potential contribution of primary producers to bivalve diets. This coupling brings new
473 information about phytoplankton assemblages and diet composition differences at low

474 taxonomic level, which seems to be an improvement compared to the broader discrimination

475 power and SI and FA.

476

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Highlights

- Stable isotopes fatty acids and sterols were used to characterize the diet of bivalves
- A transect was sampled in a Pacific lagoon with increasing distance from the mouth
- Bivalves at the entrance of the lagoon relied on oceanic phytoplankton inputs
- Bivalves in the inner lagoon relied on local microbial food web
- Lipid biomarkers and SI were complementary to distinguish the food sources origins

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