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## Comparative study of domoic acid accumulation, isomer content and associated digestive subcellular processes in five marine invertebrate species

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1 **Comparative study of domoic acid accumulation, isomer content and associated**  
2 **digestive subcellular processes in five marine invertebrate species**

3

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21

22 **Abstract**

23 Despite the deleterious effects of the phycotoxin domoic acid (DA) on human health, and the  
24 permanent threat of blooms of the toxic *Pseudo-nitzschia* sp. over commercially important  
25 fishery-resources, knowledge regarding the physiological mechanisms behind the profound  
26 differences in accumulation and depuration of this toxin in contaminated invertebrates remain  
27 very scarce. In this work, a comparative analysis of accumulation, isomer content, and  
28 subcellular localization of DA in different invertebrate species was performed. Samples of  
29 scallops *Pecten maximus* and *Aequipecten opercularis*, clams *Donax trunculus*, slipper snails  
30 *Crepidula fornicata*, and sea squirts *Asterocarpa* sp. were collected after blooms of the same  
31 concentration of toxic *Pseudo-nitzschia australis*. Differences ( $P < 0.05$ ) in DA accumulation  
32 were found, wherein *P. maximus* showed up to 20-fold more DA in the digestive gland than  
33 the other species. Similar profiles of DA isomers were found between *P. maximus* and *A.*  
34 *opercularis*, whereas *C. fornicata* was the species with the highest biotransformation rate  
35 (~10%) and *D. trunculus* the lowest (~4%). DA localization by immunohistochemical  
36 analysis revealed differences ( $P < 0.05$ ) between species: in *P. maximus*, DA was detected  
37 mainly within autophagosome-like vesicles in the cytoplasm of digestive cells, while in *A.*  
38 *opercularis* and *C. fornicata* significant DA immunoreactivity was found in post-autophagy  
39 residual bodies. A slight DA staining was found free within the cytoplasm of the digestive  
40 cells of *D. trunculus* and *Asterocarpa* sp. The Principal Component Analysis revealed  
41 similarities between pectinids, and a clear distinction of the rest of the species based on their  
42 capacities to accumulate, biotransform, and distribute the toxin within their tissues. These  
43 findings contribute to improve the understanding of the inter-specific differences concerning  
44 the contamination-decontamination kinetics and the fate of DA in invertebrate species.

45 **Keywords:** domoic acid, shellfish, DA isomers, autophagy, interspecific differences.

## 46 1. Introduction

47 Domoic acid (DA) is an extremely dangerous phycotoxin responsible of the illness referred as  
48 amnesic shellfish poisoning (ASP) syndrome in humans (Perl *et al.*, 1990, Pulido, 2008; La  
49 Barre *et al.*, 2014). This highly potent neuroexcitatory amino acid is naturally produced by  
50 some diatoms of the genus *Pseudo-nitzschia* (Bates *et al.*, 1998, 2018), wherein the species  
51 *Pseudo-nitzschia australis* is one of the most toxigenic (Lelong *et al.*, 2012; La Barre *et al.*,  
52 2014). The recurrent presence of toxic blooms of *Pseudo-nitzschia* sp., and the subsequent  
53 production of DA, frequently affect fishery resources on the North Atlantic coasts of France.  
54 Indeed, suspension-feeding invertebrates are capable of ingesting toxic *Pseudo-nitzschia* cells  
55 leading to high amounts of DA accumulated in their tissues (Basti *et al.*, 2018; Dusek  
56 Jennings *et al.*, 2020) seriously threatening human health through contaminated seafood  
57 consumption (Pulido, 2008; La Barre *et al.*, 2014). Over the last two decades, these blooms  
58 have caused numerous and persistent harvest closures for some economically important  
59 species (Amzil *et al.*, 2001; Husson *et al.*, 2016).

60 Notwithstanding, profound inter-specific variability in the toxicokinetics of accumulation and  
61 depuration rates of DA burdens have been reported between several invertebrate species in the  
62 same affected area (Costa *et al.*, 2004, 2005a,b; Bogan *et al.*, 2007a,b,c; Lage *et al.*, 2012;  
63 Ben haddouch *et al.*, 2016; Dusek Jennings *et al.*, 2020; Blanco *et al.*, 2021; Kvirgić *et al.*,  
64 2022). Thus, invertebrates have been broadly classified as “fast” or “slow” DA-depurators  
65 (Blanco *et al.*, 2002a,b; Basti *et al.*, 2018). Larger scallops, such as King scallops *Pecten*  
66 *maximus* (Blanco *et al.*, 2002a; García-Corona *et al.*, 2022) and giant scallops *Placopecten*  
67 *magellanicus* (Gilgan, 1990; Haya *et al.*, 1991), some big-clams, such as razor clams *Siliqua*  
68 *patula* (Horner *et al.*, 1993; Dusek Jennings *et al.*, 2020), and some cephalopod mollusk such  
69 as *Octopus vulgaris* (Costa *et al.*, 2004) and *Eledone moschata* (Costa *et al.*, 2005b) as well as  
70 the common cuttlefish *Sepia officinalis* (Costa *et al.*, 2005a; Ben haddouch *et al.*, 2015) are  
71 capable of accumulating high amounts of DA, principally in the digestive gland, and require  
72 from many months to a couple of years to depurate the toxin from their tissues. Therefore,  
73 these species have been considered as slow DA-depurators. Notwithstanding, during *Pseudo-*  
74 *nitzschia* outbreaks, the king scallop *P. maximus* is usually amongst the most contaminated  
75 species (James *et al.*, 2005; Blanco *et al.*, 2002a, 2021). Levels of DA exceeding up to 5-fold  
76 the European regulatory limit of 20 mg kg<sup>-1</sup> are not unusual in *P. maximus* (Blanco *et*  
77 *al.*, 2006; Bogan *et al.*, 2007a,b; García-Corona *et al.*, 2022). Conversely, mussels (Novaczek  
78 *et al.*, 1992 ; Blanco *et al.*, 2002b; Mafra *et al.*, 2010), and even smaller scallops, such as

79 *Argopecten purpuratus* (Álvarez *et al.*, 2020) are known as fast DA-depurators since they can  
80 depurate up to 90 % of total DA burdens over hours to days. These species-specific  
81 differences in DA accumulation-depuration represent a real issue for fishery economy and  
82 management after ASP-blooms. Thus, understanding the physiological mechanisms behind  
83 this phenomenon is of high interest.

84 Mauriz and Blanco (2010), as well as Lage *et al.* (2012) found that nearly 90% of total DA  
85 accumulated in *P. maximus* and *O. vulgaris*, respectively, was free in a soluble form in the  
86 cytoplasm of the digestive cells. García-Corona *et al.* (2022) observed, using an  
87 immunohistochemical subcellular localization of DA in *P. maximus*, that DA is trapped into  
88 small-spherical membrane-bound vesicles localized in the cytoplasm of digestive cells,  
89 suggesting that autophagy could be one of the potential physiological mechanisms behind the  
90 long retention of a part of DA in this species. Nevertheless, to date, the immunohistochemical  
91 (IHC) localization of DA has not been applied to any other invertebrate species contaminated  
92 with DA, which greatly hinders the comparison of the subcellular mechanisms involved in the  
93 accumulation and retention of this toxin between affected species. Autophagy is a highly  
94 regulated and dynamic “self-eating” catabolic system related to the intracellular ingestion and  
95 digestion (Cuervo, 2004; Wang *et al.*, 2019; Zhao *et al.*, 2021). Through autophagy the  
96 lysosomes receive autophagosomic vesicles (autophagosomes) containing cytoplasmic  
97 cellular components, such as macromolecules, damaged or misfolded proteins, and entire  
98 organelles, as well as extracellular-derived molecular cargo from endocytosis and  
99 phagocytosis for degradation, digestion, recycling, or excretion (Klionsky *et al.*, 2014;  
100 McMillan, 2018; Wang *et al.*, 2019). These distinctive capabilities establish an essential role  
101 of autophagy in maintaining metabolic homeostasis and cellular health in bivalves (Balbi *et*  
102 *al.*, 2018; Picot *et al.*, 2019; Rodríguez-Jaramillo *et al.*, 2022).

103 Not only untransformed DA, but also some structural isomers of the toxin (*i.e.* isoA, isoD,  
104 isoE, and epi-DA) are frequently detected in seafood during ASP-monitoring. The  
105 concentrations of DA-isomers commonly range from 0.5 to ~20% of total DA burdens  
106 (Wright *et al.*, 1990a; Costa *et al.*, 2005; Takata *et al.*, 2009; Zheng *et al.*, 2022). Despite  
107 some studies pointing out some degree of species-specific biotransformation of DA in  
108 bivalves (Wright *et al.*, 1990b; Blanco *et al.*, 2010), fish and shellfish (Vale and Sampayo,  
109 2001), and cephalopods (Costa *et al.*, 2005), no work has ever compared the  
110 biotransformation profiles of DA against the subcellular localization of this toxin in  
111 contaminated invertebrates. This information could be useful to elucidate differences in DA

112 uptake and allocation, as well as the potential implication of subcellular mechanisms on  
113 depuration of this toxin between species.

114 This study compared biotransformation and subcellular localization of DA in five invertebrate  
115 species simultaneously exposed to natural toxic *P. australis* blooms to answer the question:  
116 How do invertebrate species differ in their ability to accumulate, process, and allocate DA in  
117 their tissues?

118

## 119 **2. Materials and methods**

### 120 **2.1. Sample collection and *Pseudo-nitzschia australis* bloom-associated environmental** 121 **data**

122 A total of 38 invertebrate samples were collected in 2021 in the northwest coast of Brittany,  
123 France. The samples consisted in clams *Donax trunculus* (n =11) collected on the 30<sup>th</sup> of  
124 March in the Bay of Douarnenez, and scallops *P. maximus* (n =5), *A. opercularis* (n =10),  
125 slipper snail *Crepidula fornicata* (n =7), and sea squirt *Asterocarpa* sp. (n =5) collected on the  
126 8<sup>th</sup> of April in Camaret-sur-Mer (Fig. 1). Animals were collected eight days after blooms of  
127 similar intensity of the DA-producing *P. australis* according to the French national  
128 phytoplankton monitoring network (French Observation and Monitoring program for  
129 Phytoplankton and Hydrology in coastal waters, REPHY) in both sampling sites ( $[2.6 \times 10^5$   
130  $\text{cell.L}^{-1}]$  on March 23, 2021 in the Bay of Douarnenez), and  $[1.1 \times 10^5 \text{ cell.L}^{-1}]$  on March 30,  
131 2021 (in Camaret-sur-Mer), respectively, <https://bulletinrephytox.fr/accueil>) (Fig 1). Once at  
132 the laboratory, the digestive gland (DG) of the scallops (*P. maximus* and *A. opercularis*) was  
133 carefully dissected from the rest of the tissues, and subsequently sectioned in two halves. For  
134 the rest of the species with diffuse visceral mass (*C. fornicata*, *D. trunculus*, and *Asterocarpa*  
135 sp.) the soft body (*i.e.* total flesh) was divided into two equal portions at the mid visceral  
136 level, including a section of the DG on each. For each individual, one of these DG/visceral  
137 sections was fixed in Davidson's solution (Kim *et al.*, 2006) for histology, and the second  
138 DG/visceral sections section was stored at -20 °C for toxin analysis.

### 139 **2.2. Toxin quantification and DA-isomer analysis by liquid chromatography-tandem** 140 **mass spectrometry (LC-MS/MS)**

141 Since the DG accumulates most of DA (Mauriz and Blanco, 2010), only this tissue was  
142 considered for toxin analysis in this work. For the non-pectinid species, the DG was separated

143 from the rest of the visceral mass once the tissues were frozen. DA was extracted from the  
144 DG following the procedure described by Quilliam *et al.*, (1989). Samples were homogenized  
145 from  $200 \pm 10$  mg of frozen DG in 1 mL of 50% MeOH/H<sub>2</sub>O using a Fastprep-24 5G system  
146 (MP Biomedicals, Sta. Ana, CA, USA). The extract was clarified by centrifugation at 19,000  
147  $\times g$  at 4 °C for 10 min and the supernatant was isolated, filtered through a 0.2  $\mu$ m nylon  
148 centrifugal filter (VWR International, Radnor, PA, USA), and stored at -20 °C until analysis.

149 The quantification of total DA (tDA = ensemble of all DA isomers) and each isomer of the  
150 toxin in the DG was carried out by LC-MS/MS according to Ayache *et al.* (2019) with  
151 modifications, using a Shimadzu UFLCxR system coupled to a quadruple hybrid mass  
152 spectrometer API400Q-Trap (Sciex, Concord, ON, Canada) equipped with a heated  
153 electrospray ionization (ESI) source. Chromatographic separation was carried out on a  
154 reversed-phase column Phenomenex Luna Omega C18 (150  $\times$  2.1 mm, 3  $\mu$ m, Phenomenex,  
155 Torrance, CA, USA). The separation was carried out using a mobile phase consisting of  
156 aqueous eluent A (100% H<sub>2</sub>O + 0.1% H-COOH) and organic eluent B (95% CH<sub>3</sub>CN/ 5% H<sub>2</sub>O  
157 + 0.1% H-COOH). The run started following a gradient from A to B as follows: 5% at min 0,  
158 18.6% at 17 min, 95% at 17.5 min, 95% at 19.5 min, 5% at 20 min, and 5% at 25 min. The  
159 flow rate was 200  $\mu$ L.min<sup>-1</sup> and the injection volume was 5  $\mu$ L. The column temperature was  
160 maintained at 30 °C.

161 The ESI interface was operated with a curtain gas of 20 psi, temperature of 550 °C, gas1 55  
162 psi, gas2 60psi, and an ion spray voltage of 5500 V. The detection of DA was achieved by  
163 multiple reaction monitoring (MRM) in positive ion mode. The transition 312.1 > 266.1  
164 (collision energy = 22 V) was used for quantification and 312.1 > 161.1 (collision energy =  
165 33 V) for confirmation. The quantification was performed relative to the DA standard  
166 (National Research Council Canada, NRCC) with a 6-point calibration curve. The Limit of  
167 Quantification (LOQ) (S/N = 10) and the Limit of Detection (LOD) (S/N = 3) of the method  
168 were 0.25 and 0.08 ng DA mL<sup>-1</sup>, respectively, which corresponded to 1.25 and 0.4 ng DA g<sup>-1</sup>  
169 in tissue.

### 170 **2.3. Immunodetection of DA and quantitative histology**

171 Tissue samples fixed in Davidson's solution were dehydrated in ethanol series of progressive  
172 concentrations (70%, 80%, 95%, and 100%), cleared in xylene, and embedded in paraffin  
173 (Paraplast Plus, Leica Bio-systems, Richmond, IL, USA). Paraffin blocks were cut in 4- $\mu$ m-  
174 thick sections using a rotary microtome (Leica RM 2155, Leica Microsystems) and sections

175 mounted on polylysine-coated glass slides (Sigma-Aldrich, St. Louis, MO, USA). A series of  
176 three consecutive sections was performed for each sample, which were used for (i)  
177 immunohistochemical detection of DA, (ii) multichromic staining, and (iii) hematoxylin/eosin  
178 staining, as described below. Sections were deparaffinized in xylene and rehydrated in ethanol  
179 series of regressive concentrations before staining.

180 In order to detect the presence of DA at the subcellular level in the tissue sections, an  
181 immunohistochemical DA labeling technique was applied following the procedure described  
182 in García-Corona *et al.* (2022) on the first slide of each sample. Briefly, tissue sections were  
183 incubated overnight with a Goat polyclonal anti-DA primary antibody ( $0.01 \text{ mg.mL}^{-1}$ ,  
184 Eurofins Abraxis<sup>®</sup>, Warminster, PA, USA) at  $4^{\circ}\text{C}$ , and the next day the slides were incubated  
185 at  $37^{\circ}\text{C}$  for 2h with an HRP sharped IgG Rabbit anti-Goat secondary antibody ( $0.001 \text{ mg.mL}^{-1}$ ,  
186 abcam<sup>®</sup>, Cambridge, UK). Then, samples were washed and revealed with diaminobenzidine  
187 (DAB+ Chromogen Substrate Kit, abcam<sup>®</sup>, Cambridge, UK) for 1 h in darkness at room  
188 temperature and counterstained with Harry's hematoxylin.

189 The second slide from each sample was stained with a multichromic procedure (Costa and  
190 Costa, 2012). This technique consists of a combination of Alcian Blue and Periodic Acid–  
191 Schiff's for the demonstration of acid mucopolysaccharides and neutral glycoconjugates, in  
192 blue and magenta tones, respectively, Hematoxylin blueing for nuclear materials, and Picric  
193 Acid to identify proteins in yellow hues.

194 The last set of tissue sections was stained with Hematoxylin–Eosin as reference (Kim *et al.*,  
195 2006). The slides were examined under a Zeiss Axio Observer Z1 light microscope.

196 For quantitative histological analysis, five randomly selected regions ( $63\times$ ;  $\sim 1.3 \text{ mm}^2$ ) from  
197 each DG section treated for immunohistochemical DA detection, multichromic, and  
198 hematoxylin-eosin staining were digitized at high resolution (600 dpi). A total of 570 images  
199 (*i.e.* 114 micrographs by species) were used to obtain the following data: (a) DA chromogenic  
200 signal (DAcs) corresponds to the coverage area, in pixels, occupied by the positive anti-DA  
201 staining. This was manually calculated using an operator-driven digital image analysis system  
202 (Image Pro Plus software v. 4.5, Media Cybernetics, Silver Spring, MD, USA) (Gómez-  
203 Robles *et al.*, 2005). The area reported as the DA chromogenic signal was calculated as  $\text{DAcs} =$   
204  $(\text{DA chromogenic signal area} / \text{total area occupied by the DG on the analyzed region of the}$   
205  $\text{slide}) \times 100$ . Since almost all the DA chromogenic signal detected in DG is trapped in  
206 membrane-bound vesicles present in the cytoplasm of digestive cells (García-Corona *et al.*,



207 2022), the (b) Total autophagy (Ta) and total DA autophagy (DAa) were calculated by  
208 counting the total number of autophagosome-like vesicles, and the number of  
209 autophagosome-like vesicles with DA chromogenic signal, respectively, on each digitized  
210 image. A fraction of the DA chromogenic signal is also observed in post-autophagic residual  
211 bodies within the digestive cells (García-Corona *et al.*, 2022), thus the frequencies of (c) Total  
212 residual bodies (Trb) and DA residual bodies (DArb) were assessed as the total number of  
213 residual bodies and the total number of residual bodies with DA chromogenic signal,  
214 respectively, on each digitized image. Finally, (d) Cell vacuolization (Vac), measured as an  
215 indicator of potential histopathologies related to DA accumulation in the DG, represents the  
216 total number of vacuoles within the digestive cells of each invertebrate species on each  
217 digitized image.

218

#### 219 **2.4. Statistical analysis**

220 All statistical analyses were performed in the R computing environment (R v. 4.2.2, R Core  
221 Team, 2022). *A priori* Lilliefors (Kolmogorov-Smirnov) and Bartlett tests were applied to  
222 confirm the normality of frequencies and homogeneity of variances of the residuals of the  
223 data, respectively (Hector, 2015). All data were transformed ( $\log$ ,  $1/\chi$ , or  $\sqrt{\chi}$ ) prior to analysis  
224 to meet a priori assumptions. The percentage-expressed values were also arcsine ( $\arcsin \sqrt{P}$ )  
225 transformed (Zar, 2010), but all data are reported untransformed as the means  $\pm$  standard  
226 errors (SE). Separate one-way analyses of variance (ANOVA, type II Sum of Squares) were  
227 applied to assess statistically significant differences of toxin accumulation in the DG,  
228 proportion of DA isomers, and quantitative histological features between species. As needed,  
229 post hoc comparisons of means with Tukey's honest significance test (HSD) were performed  
230 to identify differences between means (Hector, 2015; Zar, 2010). Principal component  
231 analysis (PCA) was performed using the FactoMineR package with the factoextra package for  
232 data visualization into smaller factorial clusters within a 95% confidence interval. All data  
233 matrices were auto-scaled before PCA analysis. The corrplot package was run to calculate the  
234 correlation coefficients and their significance between variables within their given PCs. All  
235 graphics were generated using the package ggplot2. The level of statistical significance was  
236 set at  $\alpha = 0.05$  for all analyses (Zar, 2010).

237

### 238 **3. Results**

### 239 3.1. Toxin accumulation and biotransformation

240 Significant differences in the amounts of total DA (tDA) accumulated in the digestive glands  
241 (DG) were found between the different invertebrate species sampled after blooms of the toxic  
242 *P. australis* (Fig. 2). The significantly higher burdens of tDA were observed in the scallop *P.*  
243 *maximus*, with  $638.6 \pm 35.5 \text{ mg.kg}^{-1}$ , followed by those of the snail *C. fornicata*, with  $48.5 \pm$   
244  $14.2 \text{ mg.kg}^{-1}$ , the scallop *A. opercularis* ( $22.7 \pm 2.6 \text{ mg kg}^{-1}$ ), and the clam *D. trunculus* ( $12 \pm$   
245  $1.7 \text{ mg kg}^{-1}$ ). The lowest values ( $P < 0.05$ ) of tDA were found in the ascidian *Asterocarpa* sp.  
246 ( $4.2 \pm 1.5 \text{ mg kg}^{-1}$ ). Moreover, as shown in Fig. 2, an important intraspecific variability in  
247 tDA accumulation was also observed in *P. maximus* and *C. fornicata*, with values ranging  
248 from 530 to 731  $\text{mg kg}^{-1}$ , and from 0.2 to 93.8  $\text{mg kg}^{-1}$ , respectively.

249 The toxin analysis carried out by LC-MS/MS revealed differences in biotransformation of DA  
250 in the digestive glands among the different invertebrate species (Table I). For all species,  
251 relative concentration levels of DA isomers were  $< 10\%$  of the tDA burdens. Nonetheless, *C.*  
252 *fornicata* was the species with the highest proportions ( $P < 0.001$ ) of DA isomers ( $9.3 \pm 1.1$   
253  $\%$ ), while *D. trunculus* showed significantly low DA isomer amounts ( $4.2 \pm 0.3\%$ ).

254 Concerning the analysis of DA isomers proportion, *P. maximus* and *A. opercularis* showed  
255 similar biotransformation profiles of the toxin since similar amounts of each DA isomer were  
256 reported in both species. Furthermore, as shown in Table I, among the five species, the lowest  
257 ratio of isoE ( $P < 0.05$ ) was measured in *Asterocarpa* sp., and a significantly higher proportion  
258 of isoD was recorded in *C. fornicata*, while the smallest amounts ( $P < 0.05$ ) of isoA and epi-  
259 DA were quantified in *D. trunculus*.

### 260 3.2. DA subcellular localization and histological measurements

261 The microanatomical observations of histological sections evidenced qualitative differences in  
262 the localization of DA and the subcellular features linked to the accumulation of the toxin  
263 among the invertebrate species analyzed in this study (Fig. 3, and supplementary materials  
264 S1-5). DA detected by immunohistochemistry (IHC) appeared as a brown chromogenic signal  
265 (cs) on slides (Fig 3A, 3D, 3G, 3J, 3M, and S1A-B, S2A-B, S3A-B, S4A-B, S5A-B).

266 In the digestive gland of *P. maximus* DA was detected mainly trapped within small ( $\sim 1\text{-}2.5$   
267  $\mu\text{m}$  diameter) autophagosome-like vesicles (a) distributed throughout the cytoplasm of the  
268 digestive cells (dc). A narrow fraction of DA-immunoreactivity was also observed in residual  
269 bodies (rb) distributed in the acinar region (ar) of the digestive diverticula (dd) (Fig. 3A, S1A-  
270 B). The presence of membrane-bounded vesicles (a) with positive DA-signal (cs) in the

271 tubular region (tr) of the digestive diverticula (dd) was confirmed by means of the  
272 multichromic staining (MC), which produces a dark violet/blue hueing in membrane-bounded  
273 structures (Fig. 3B, S1C-D). Hematoxylin-Eosin (H&E) staining (Fig. 3C, S1E-F) highlighted  
274 a moderate vacuolization (v) within the cytoplasm of the digestive cells of *P. maximus*.  
275 Neither the autophagosomes (a) nor the residual bodies (rb) acquired any coloration with the  
276 H&E staining but residual bodies appeared yellow-green.

277 In the queen scallop *A. opercularis*, a strong DA-chromogenic signal (cs) was found in the  
278 residual bodies (rb) of the digestive diverticula (dd) (Fig. 3D, S2A-B). No DA chromogenic  
279 signal was observed in the autophagosome-like vesicles (a) present in the cytoplasm of the  
280 digestive cells of the digestive diverticula (dd) (Fig. 3E-F, S2A-B). An intense process of  
281 vacuolization (v) of the digestive cells of *A. opercularis* was found (Fig. 3E-F, S2C-D), while  
282 H&E staining (Fig. 3F, S2E-F) showed that the autophagosomes seem to gather giving rise to  
283 the residual bodies (rb) in the cytoplasm of the adipocyte-like digestive cells (al) of the  
284 digestive diverticula (dd).

285 A similar result was found for *C. fornicata*, since most of the brown DA-chromogenic  
286 staining (cs) was found in small residual bodies (rb) present in the basal cytoplasmic region  
287 (bl) of the digestive cells (dc) (Fig. 3G, S3A-B), while autophagosome-like vesicles (a) that  
288 are distributed in the apical region of the digestive cells (dc) (Fig. 3H-I, S3A-B) did not show  
289 any DA-immunoreactivity.

290 A slight-blurred DA-chromogenic signal (cs) was also observed only free in the cytoplasm of  
291 the digestive cells of *D. trunculus* (Fig 3J, S4A-B). The presence of autophagosome-like  
292 vesicles (a, small blue colored vesicles distributed in the cytoplasm, Fig 3K, S4C-D) and  
293 residual bodies (rb, larger round non-colored structures present within adipocyte-like cells,  
294 Fig 3L, S4C-D) was confirmed in the digestive cells (dc) of clams (Fig. 3K-L, S4C-F).

295 Meanwhile, in sea squirts (*Asterocarpa* sp.) DA-chromogenic signal (cs) was rarely identified  
296 and was located as small brown points (Fig. 3M, S5A-B) distributed through the digestive  
297 epithelium (pse) of the blind ampulla (ba) (Fig. 3N-O, S5C-F).

298 The results of the quantitative analysis of histological parameters are shown in Fig. 4. The  
299 coverage area of the DA chromogenic signal (%DAcs, Fig. 4A) was significantly higher in  
300 the most contaminated invertebrate species (*P. maximus* =  $4.8 \pm 0.4$  %, and *C. fornicata* =  $5.3$   
301  $\pm 0.4$  %). In addition, differences ( $P < 0.05$ ) were found in the amount of DA chromogenic

302 signal in *A. opercularis* ( $3.2 \pm 0.2$  %) compared to the species contaminated with the lowest  
303 DA burdens (*D. trunculus* = 0.2 %, and *Asterocarpa* sp. = 0%).

304 On the other hand, as seen in Fig. 4B, total autophagy (Ta) reached its highest values ( $P$   
305  $<0.05$ ) in the bivalve species, with frequencies of  $185.4 \pm 18$  autophagosomes. area<sup>-1</sup> in *P.*  
306 *maximus*,  $123.2 \pm 12.6$  autophagosomes. area<sup>-1</sup> in *D. trunculus*, and  $102.9 \pm 9.7$   
307 autophagosomes. area<sup>-1</sup> in *A. opercularis*. The proportion of total autophagy (Ta) was  
308 significantly lower in *C. fornicata* ( $60.9 \pm 5.8$  autophagosomes. area<sup>-1</sup>) and *Asterocarpa* sp.  
309 ( $18.3 \pm 2.9$  autophagosomes. area<sup>-1</sup>). Nevertheless, the frequency of autophagosomes with  
310 positive DA-chromogenic signal (DAa) significantly peaked in *P. maximus* ( $99.7 \pm 9.7$   
311 autophagosomes. area<sup>-1</sup>, corresponding to 53.8% of the Ta), followed by *C. fornicata* ( $39.8 \pm$   
312  $4.6$  autophagosomes. area<sup>-1</sup>, corresponding to 65.3% of the Ta). The lowest proportions ( $P$   
313  $<0.05$ ) of autophagosomes with positive DA-chromogenic signal (DAa) were observed in *A.*  
314 *opercularis*, *D. trunculus*, and *Asterocarpa* sp, with  $\leq 7$  autophagosomes. area<sup>-1</sup>,  
315 which corresponded to 8.4, 1.2, and 0% of the total autophagy (Ta), respectively (Fig. 4B). In  
316 contrast, the frequencies of total residual bodies (Trb) and residual bodies with DA  
317 chromogenic signal (DArb) significantly peaked in *C. fornicata* ( $92.4 \pm 5.2$  rb. area<sup>-1</sup>, and  
318  $51.9 \pm 4.1$  rb. area<sup>-1</sup>, respectively), while the frequencies of both subcellular parameters  
319 showed their lowest values ( $P <0.05$ ) in the rest of the species (Fig. 4C). It is important to  
320 highlight that the percentage of residual bodies with DA chromogenic signal (%DArb)  
321 compared to total residual bodies (Trb) was significantly higher in *A. opercularis*, with a  $67.1$   
322  $\pm 3\%$ , followed by *C. fornicata* and *P. maximus*, with rates of  $58 \pm 3.8\%$  and  $35.4 \pm 3.3\%$ ,  
323 respectively. The lowest % DArb ( $P <0.05$ ) was reported for *D. trunculus* ( $2.2 \pm 1.3\%$ ) and  
324 *Asterocarpa* sp. (0%). Finally, the highest frequency of cell vacuolization (Vac) of the  
325 digestive cells was measured in *A. opercularis* ( $67.4 \pm 6.7$  vacuoles. area<sup>-1</sup>,  $P <0.05$ ), followed  
326 by *P. maximus* ( $31.6 \pm 2.4$  vacuoles. area<sup>-1</sup>). Significantly lower vacuolization (Vac) rates  
327 were reported for the rest of the species ( $<8$  vacuoles. area<sup>-1</sup>, Fig. 4D).

### 328 **3.3. Integrative analysis compiling DA accumulation/biotransformation and subcellular** 329 **features**

330 A principal component analysis (PCA) was computed to summarize all variables measured in  
331 this study on the five invertebrate species studied: DA accumulation, biotransformation, and  
332 subcellular parameters (Fig. 5). The PCA described two-thirds (66.6 %) of the total variance  
333 of the data along the first two principal dimensions. For the whole data set, the clustering-

334 PCA provided a clear distinction between species, except for the two pectinid species, which  
335 slightly overlapped (Fig. 5A). In the scatter plot, *P. maximus* and *A. opercularis* showed  
336 similar scores on the principal components and were different from the rest of the species.  
337 Meanwhile, *D. trunculus*, *C. fornicata*, and *Asterocarpa* sp., were grouped separately from  
338 each other (Fig. 5A). As shown in Fig. 5B, the dimension/principal component 1 (PC1, 42.3  
339 % of the total variance) mainly explained the accumulated untransformed DA, isoD and isoA,  
340 as well as the histological parameters such as domoic acid chromogenic signal (%DAcs),  
341 domoic acid autophagy (DAa), total residual bodies (Trb), and residual bodies with DA signal  
342 (DARB). In this PC1, the fraction of isoA was strongly and positively correlated to the %DAcs  
343 and DARB ( $r = 0.5$  and  $0.6$ ,  $P < 0.05$ , respectively). Likewise, a strong and significant  
344 correlation was found between the untransformed DA and DAa ( $r = 0.8$ ), and between DARB  
345 and %DAcs ( $r = 0.8$ ) in this dimension. The amounts of isoE and epi-DA, as well as total  
346 autophagy (Ta) and vacuolization (Vac), were the strongest correlated variables to  
347 dimension/principal component 2 (24.3 % of the explained variance). A positive correlation ( $r$   
348  $= 0.5$ ,  $P < 0.05$ ) between total DA (tDA) and isoE was found with Ta within the PC2. As  
349 observed in Fig. 5, *P. maximus* and *A. opercularis* were associated with higher tDA and isoE,  
350 as well as the maximum frequencies of Ta and Vac. Meanwhile, *C. fornicata* was related to  
351 higher amounts of isoD, epi-DA, Trb, and *D. trunculus* with the highest fraction of  
352 untransformed DA.

#### 353 **4. Discussion**

354 In this study, we compared domoic acid (DA) accumulation and isomer profiles with the  
355 subcellular localization of this toxin among naturally contaminated invertebrates to progress  
356 in the understanding of interspecific differences in DA fate in marine invertebrates.

357 The DA contents measured in invertebrate tissues are the result of the accumulated and the  
358 subsequently depurated toxin. Moreover, differences in DA accumulation in the organisms  
359 are strongly dependent on the toxicity of the *Pseudo-nitzschia* cells, the duration of the ASP  
360 blooms, the time through the animals were exposed to toxic microalgae, and the moment at  
361 which the organisms were sampled during the bloom. In this work, DA contaminated animals  
362 were collected 8 days after maximum cell densities of *P. australis* bloom of similar intensity,  
363 duration and origin.

364 Since DA is a highly water-soluble molecule, it is expected to be easily accumulated in the  
365 majority of forager species (Trainer *et al.*, 2012; La Barre *et al.*, 2014). Nonetheless, the  
366 scallops, but notably *P. maximus*, as well as *C. fornicata*, remained significantly more  
367 contaminated than the rest of the species in this study. These important differences in DA  
368 accumulation in the digestive gland at the interspecific level are in accordance with  
369 considerably high variability in DA amounts frequently detected in these species (Bogan *et*  
370 *al.*, 2007a,b,c; Basti *et al.*, 2018, Blanco *et al.*, 2021) resulting from differences in the  
371 accumulation but also in the depuration rates of DA reported mostly for bivalve species (Vale  
372 and Sampayo, 2001; Blanco *et al.*, 2010; Dusek Jennings *et al.*, 2020). Notably, within the  
373 pectinidae family, some large scallops like *P. maximus* can accumulate up to 3,200 mg  
374 DA.kg<sup>-1</sup> in their DG (James *et al.*, 2005; Blanco *et al.*, 2006), which is 5-fold more than the  
375 DA accumulated in the DG of the same species in this work. In contrast, smaller scallops,  
376 such as *A. opercularis* (Ventoso *et al.*, 2019), *A. purpuratus* (Álvarez *et al.*, 2020) and *A.*  
377 *irradians* (O’Dea *et al.*, 2012) accumulate lower DA burdens (~7-30 mg DA.kg<sup>-1</sup>) similar to  
378 those recorded in *A. opercularis* in this work, in the same organs. Depuration kinetics of the  
379 toxin differ also between these species. Whereas *P. maximus* exhibits depuration rates as slow  
380 as 0.007 day<sup>-1</sup> in the DG, remaining highly contaminated for months or even a few years  
381 (Blanco *et al.*, 2002a, 2006), other scallops such as *A. purpuratus* show decontamination  
382 debits near to 10 day<sup>-1</sup> in the DG, allowing to depurate ~90% of total DA burdens within  
383 hours or a couple of days (Álvarez *et al.*, 2020). Thus, after all the differences in  
384 accumulation and depuration rates of DA between invertebrate species discussed above, a  
385 possible event of rapid depuration of DA in *A. opercularis*, *D. trunculus*, and *Asterocarpa* sp.  
386 before sampling can be part of the interspecific differences of DA concentrations measured in  
387 this study. Several factors could explain variability in DA decontamination: the transfer of  
388 DA in other tissues than DG, its biotransformation and its depuration.

389 Differential tissue distribution of DA may not explain more than 20% of the interspecific  
390 variability observed in this study since the digestive gland accumulates more than 80% of  
391 total DA burdens in most invertebrates (Blanco *et al.*, 2002a; Costa *et al.*, 2005a,b). For all  
392 the five species of this study, three bivalve molluscs (*P. maximus*, *A. opercularis* and *D.*  
393 *trunculus*), one gasteropod mollusc (*C. fornicata*) and one ascidian (*Asterocarpa* sp.) DA  
394 isomers were observed in digestive gland with significant interspecific differences between  
395 the proportions of isomers E, D, A and epi-DA; iso-E being more represented in molluscs  
396 compared to ascidian. Although it is known that DA isomerization can occur within toxic

397 *Pseudo-nitzschia* cells (Amzil *et al.*, 2001; Bates *et al.*, 2018; Quilliam *et al.*, 1989; Wright *et*  
398 *al.*, 1990a), in the present study all invertebrate species were exposed to the same *Pseudo-*  
399 *nitzschia* toxic bloom. These two sets of information demonstrate that metabolic conversion  
400 of DA occurs in marine invertebrates as hypothesized first by Vale and Sampayo (2001) and  
401 is species-specific. The integrative analysis revealed a close and significant relationship  
402 between some subcellular features (vacuolization, autophagy, presence of residual bodies) and  
403 the isomer profile of the toxin. Understanding DA compositional changes is important not  
404 only as a means of predicting toxicity, but also because biotransformation could participate in  
405 the prolonged retention of this toxin in invertebrate species by means of some of the  
406 subcellular mechanisms analyzed here. Notwithstanding, biotransformation does not appear to  
407 be the main route of DA elimination in these species since it represents less than 10% of total  
408 DA of the digestive gland measured in these five species, as well as in previous studies (Costa  
409 *et al.*, 2005a; Blanco *et al.*, 2010; Zheng *et al.*, 2022). There is only one study showing some  
410 insights of DA biotransformation linked to apparent augmentation of the overall DA  
411 detoxification rate in the cuttlefish *Sepia officinalis*, wherein DA isomers comprise a relevant  
412 percentage of the toxin profile in the branchial hearts, suggesting that this organ has an  
413 important function in system detoxification of DA (Costa *et al.*, 2005a).

414 Furthermore, it is worth to mention that king scallops were slightly contaminated (~5 mg DA  
415 kg<sup>-1</sup>, data from the REPHY French monitoring program) before the bloom of *P. australis*  
416 occurred in late March 2021, after which they became highly contaminated (~ 650 mg DA kg<sup>-1</sup>  
417 <sup>1</sup>). Therefore, it is inferred that the concentrations of DA isomers found in the digestive glands  
418 of *P. maximus*, and consequently, in all the invertebrate species analyzed in this work, were  
419 the result of the bloom of *P. australis* occurred in late March 2021.

420 Despite the enormous differences in DA concentrations between the marine invertebrates  
421 analyzed in this work, the physiological mechanisms behind this phenomenon remain poorly  
422 understood. To date, only a few hypotheses about the biological processes potentially  
423 involved in the large accumulation and long retention of DA in some bivalve species have  
424 been proposed. On the one hand, Trainer and Bill (2004) characterized tissue-specific  
425 expression of high and low affinity glutamate receptors in *S. patula*, inferring that this species  
426 might selectively express low affinity glutamate receptors in all tissues, and high affinity sites  
427 in specific tissues that retained DA for long periods of time. On another hand, Mauriz and  
428 Blanco (2010) hypothesized that one of the causes of the long retention of DA in the DG of *P.*  
429 *maximus* was not the binding of the toxin to some cellular component as previously discussed,

430 but the lack of efficient membrane transporters in the scallops to excrete the toxin. Recently,  
431 using immunostaining of DA, García-Corona *et al.* (2022) revealed that in *P. maximus*, once  
432 entered the cells, a part of DA was localized in the cytoplasm of digestive cells of the  
433 digestive diverticula, trapped within autophagosome-like vesicles. Moreover, transcriptomic  
434 analyses revealed the upregulation of genes related to autophagy and vesicle-mediated  
435 transport in the DG of *P. maximus* injected with DA in the adductor muscle (Ventoso *et al.*,  
436 2021), as well as in the DG of *A. opercularis* after exposure to DA-producing *Pseudo-*  
437 *nitzschia* (Ventoso *et al.*, 2019). Taken together, these data suggest that the formation of  
438 autophagosomal structures could be part of the explanation for the long retention of DA in *P.*  
439 *maximus*. The results obtained in this work cope with these findings, since most of the DA-  
440 labeling was found within a large number of autophagosomes distributed throughout the  
441 cytoplasm of the digestive cells in *P. maximus*. Additionally, a strong DA-chromogenic signal  
442 was found within the post-autophagic residual bodies present in the adipocyte-like cells in *A.*  
443 *opercularis*, and in the basal region of the digestive diverticula in *C. fornicata*. During  
444 autophagy the lysosomes in the digestive cells of these species receive DA trapped within  
445 autophagosomic-vesicles. Nonetheless, the evidence of this work indicates that a fraction of  
446 DA remains accumulated within autophagosomic structures instead being excreted or used by  
447 the cells, leading to its accumulation within the autophagosomes, and consequently blocking  
448 its excretion outside the cell by exocytosis (Cuervo, 2004; Zhao *et al.*, 2021). This eventually  
449 triggers the aggregation of autophagosomes with sequestered DA to form residual bodies that  
450 can remain in the cytoplasm of the digestive cells indefinitely. There is evidence of the long  
451 retention of exogenous compounds through specialized cellular mechanisms in animals. A  
452 concrete example is the dynamics of phagocytosis displayed by dermal macrophages,  
453 explaining both persistence and strenuous removal of tattoo ink in mammalian skin. Baranska  
454 *et al.* (2018) demonstrated that upon tattooing, pigment particles are captured by dermal  
455 macrophages. Eventually, macrophages laden with tattoo ink die and release the pigment  
456 particles, which remain in an extracellular form at the site of tattooing where they are  
457 recaptured by neighboring or incoming macrophages. Through adult life, several cycles of ink  
458 capture-release-recapture can occur, accounting for long-term tattoo persistence (Baranska *et*  
459 *al.*, 2018). **Macrophagy and autophagy are analogous processes.**  
460 **During macrophagy specialized cells called macrophages use their**  
461 **cytoplasmic membranes to engulf large extracellular particles ( $\geq$**   
462 **0.5  $\mu\text{m}$ , *i.e.* bacteria and debris) via endocytosis, giving rise to internal vesicular**



463 compartments called phagosomes. Phagosomes with cargo materials fuse with lysosomes,  
464 forming phagolysosomes, leading to enzymatic degradation (Flannagan *et al.*, 2012; Gordon,  
465 2016). Like autophagy, macrophagy is a major mechanism used to remove pathogens and  
466 cellular debris for detoxification or nutrient recycling purposes, in which macrophages can  
467 have lifespans of months to a few years (Baranska *et al.*, 2018). The discussion above raises a  
468 new hypothesis suggesting that a part of DA that is not excreted from the cells due to the lack  
469 of efficient membrane transporter (Mauriz and Blanco, 2010), may undergo successive cycles  
470 of capture–release–recapture by autophagosomes through the regenerative cycle of digestive  
471 cells in some invertebrates, without any or very few toxin vanishing from months to years.  
472 Therefore, long-term DA persistence could rely on autophagosome renewal or on potential  
473 longevity of residual bodies. A close relationship between early autophagy and DA  
474 sequestration can be established in *P. maximus*, whereas in *A. opercularis* and *C. fornicata*  
475 toxin accumulation seems to be closely linked to late autophagy and the formation of residual  
476 bodies in the DG. This evidence strengthens the hypothesis stated by García-Corona *et al.*  
477 (2022), where autophagy was proposed as one of the possible causes of the prolonged  
478 retention of part of DA initially accumulated, now not only in *P. maximus*, but also in other  
479 marine invertebrates. The next step is to decipher the fate and life-spent of autophagosomes  
480 and residual bodies with anti-DA immunolabelling within a scenario of contamination and  
481 decontamination.

482 Although the IHC method for the *in situ* detection of DA in contaminated invertebrates used  
483 in this work has a high-sensitivity ( $\sim 1 \text{ mg DA.kg}^{-1}$ , García-Corona *et al.*, 2022) only a slight-  
484 blurred DA chromogenic signal was found in the cytoplasm of the digestive cells of *D.*  
485 *trunculus*, and *Asterocarpa* sp. This would suggest that in these species, intracellular DA is  
486 not bound to any subcellular structure or component. Consequently, the feeble amounts of  
487 toxin free in the cytoplasm of the digestive cells could be quickly depurated after DA  
488 contamination but a part of DA, could also be lost by washing during histological process.  
489 Furthermore, when all species are compared, the proportion of DA chromogenic signal seems  
490 not correspond to the total amount of toxin accumulated in the DG of the animals. Despite the  
491 large difference in DA concentration between *P. maximus* and *A. opercularis* ( $638.6 \text{ mg DA}$   
492  $\text{kg}^{-1}$  vs  $22.7 \text{ mg DA kg}^{-1}$ , respectively), the difference in DA signal was small ( $\sim 2 \%$  between  
493 both species). Therefore, it is possible that a fraction of the DA accumulated in the DG of  
494 both species is free and dissolved in the cytoplasm of the digestive cells as reported for *P.*  
495 *maximus* (Mauriz and Blanco, 2010) and for *O. vulgaris* (Lage *et al.*, 2012), and that *P.*

496 *maximus* effectively lacks efficient membrane transporters to excrete the toxin out of the cell  
497 (Mauriz and Blanco, 2010), thus the chromogenic signal observed in the DG of both pectinids  
498 could correspond to the fraction of DA trapped by the autophagic system, and not to the total  
499 DA burdens in the DG. Further analyzes will be necessary to corroborate all the ideas  
500 discussed above.

501 Scallops, *P. maximus* but even more so *A. opercularis* contaminated by DA in this study have  
502 significantly higher digestive cell vacuolization rates in their digestive gland compared to  
503 other species. Cell vacuolization is a common histopathological lesion in bivalves under  
504 stressful environmental conditions (Rodríguez-Jaramillo *et al.*, 2022). According to Shubin *et al.*  
505 (2016) this is a well-known subcellular phenomenon observed in animal cells which often  
506 accompanies cell death after exposure to artificial or natural low-molecular-weight  
507 compounds, such as DA. The scarce literature related to the effects of *Pseudo-nitzschia spp.*  
508 or DA on invertebrates indicates that DA could potentially disturb behavioral, metabolic,  
509 molecular, and physiological processes in some bivalves such as *P. maximus* (Ventoso *et al.*,  
510 2021; Liu *et al.*, 2007a,b), *A. opercularis* (Ventoso *et al.*, 2019), *A. irradians* (Chi *et al.*,  
511 2019), and some mussels, like *M. edulis* (Dizer *et al.*, 2001) and *M. galloprovincialis* (Pazos  
512 *et al.*, 2017). Nevertheless, no lethal effects resulting from exposure to DA have been reported  
513 in any of these species, suggesting either a low sensitivity to the toxin or yet unnoticed  
514 negative effects. Further research is needed in order to decipher how DA exposure and its  
515 biotransformation modulate cell vacuolization, as well as its potential detrimental effects on  
516 the digestive cells of pectinids, and possibly, over other invertebrates, as reported for other  
517 phycotoxins in other bivalve species (Hegaret *et al.*, 2010; Lassudrie *et al.*, 2014).

518 Furthermore, as discussed above, the highest proportions of total autophagy, and production  
519 of residual bodies reported in *P. maximus*, *A. opercularis*, and *C. fornicata*, seems to directly  
520 correspond to the sequestration of DA within these subcellular structures, which indicates that  
521 autophagy could be also considered as a sign of homeostatic impairment, as reported in other  
522 marine bivalve species when activated as an auxiliary mechanism for recycling internal  
523 energy to cope with detrimental environmental conditions (Moore, 2008; Rodríguez-Jaramillo  
524 *et al.*, 2022), or to deplete toxicological agents (Moore, 2004; Picot *et al.*, 2019). The  
525 particularly highest proportions of DA-autophagy in *P. maximus* analyzed here stress out the  
526 need to carry out the measurement of the frequency of these subcellular features in a DA  
527 contamination and decontamination scenario. This basic knowledge is necessary to confirm

528 these physiological processes are the actual reasons for the long retention of a part of this  
529 toxin in this species.

530 The findings presented in this work put in evidence DA biotransformation in invertebrate  
531 species, and strongly suggest the role of subcellular mechanisms such as early and late  
532 autophagy, in the accumulation, localization and long retention of DA in some marine  
533 invertebrates.

## 534 **5. Conclusions**

535 The evidence presented in this work corroborates the profound interspecific differences in the  
536 accumulation of DA between different species of marine invertebrates, as well as species-  
537 specific profiles of toxin biotransformation among the analyzed species. Similar profiles of  
538 DA isomers were found between *P. maximus* and *A. opercularis*, whereas *C. fornicata* was  
539 the species with the highest biotransformation rate, and *D. trunculus* the lowest. In *P.*  
540 *maximus*, *A. opercularis* and *C. fornicata* the DA chromogenic signal was detected mainly  
541 within autophagosomic-structures in the cytoplasm of digestive cells, while in *D. trunculus*  
542 and *Asterocarpa* sp. DA signal was found free in the cytoplasm of the digestive cells. This  
543 evidence indicates that localization of DA and its effects at the subcellular level appear to be  
544 species-specific, and the integrative analysis revealed that these parameters could be  
545 potentially influenced by the biotransformation profiles of the toxin. All this new information  
546 is highly valuable to strengthen ASP-monitoring systems since most of the invertebrate  
547 species analyzed in this work could be used as sentinels of DA contamination in affected  
548 areas. Furthermore, this study provides a set of innovative histological parameters developed  
549 to assess quantitatively some subcellular mechanisms potentially involved in the  
550 accumulation and long-retention of DA among contaminated invertebrates. This quantitative  
551 information may be integrated into numerical models that allow estimating and predicting  
552 toxicokinetics of contamination and depuration in fishery-stocks frequently affected during  
553 blooms of toxic *Pseudo-nitzschia* sp.

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### 563 **Declaration of competing interest**

564 The authors declare that they have no known competing financial interests or personal  
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### 571 **Data availability statement**

572 The evidence and data that support the findings of this study are available from the  
573 corresponding author upon reasonable request.

### 574 **Ethics statements**

575 The organisms used in this work were transported and handled according to the International  
576 Standards for the Care and Use of Laboratory Animals. The number of sampled organisms  
577 contemplated "the rule of maximizing information published and minimizing unnecessary  
578 studies". In this sense, 38 individuals were considered the minimum number of organisms  
579 needed for this work.

### 580 **Author contributions**

581 Conceived the study: CF, HH, JLGC. Sampling: JLGC, HH, CF, ML, TD, AT. Processed the  
582 samples: JLGC, TD, AD, AT. Analyzed the data: JLGC, AD. Interpretation of data: JLGC,  
583 CF, HH, AD. Contributed reagents/materials/analysis tools: CF, HH, AD, AT, ML. Wrote the  
584 first draft of the manuscript: JLGC. Writing – review & editing: CF, HH, JLGC, ML, AD,  
585 AT.

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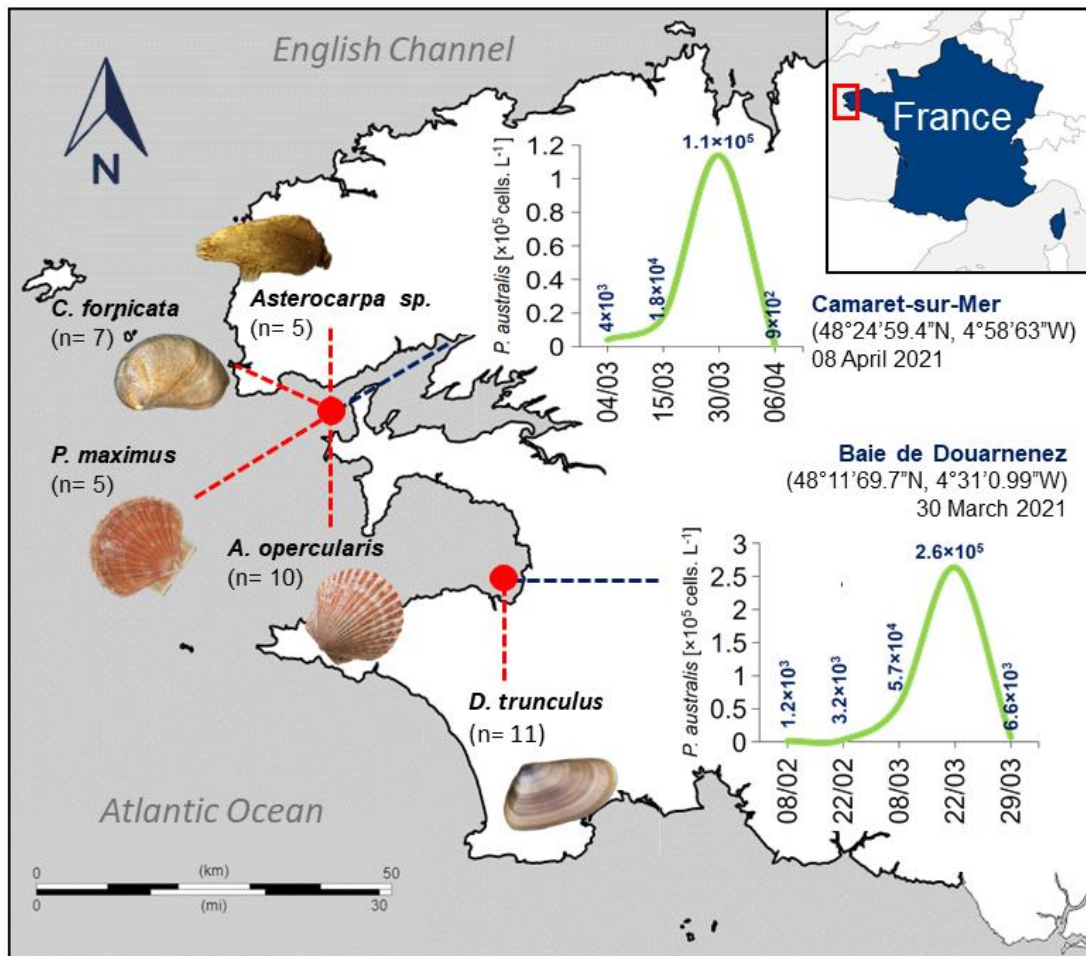
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826 **Table 1.** Relative abundance of DA and its isomers in the digestive glands of the scallops *P. maximus* (n =5) and *A. opercularis* (n =10), the clam  
827 *D. trunculus* (n =11), the slippersnail *C. fornicata* (n =7), and the sea squirt *Asterocarpa* sp. (n =5) contaminated during *P. australis* blooms in  
828 the northwest coast of Brittany, France between March-April 2021.

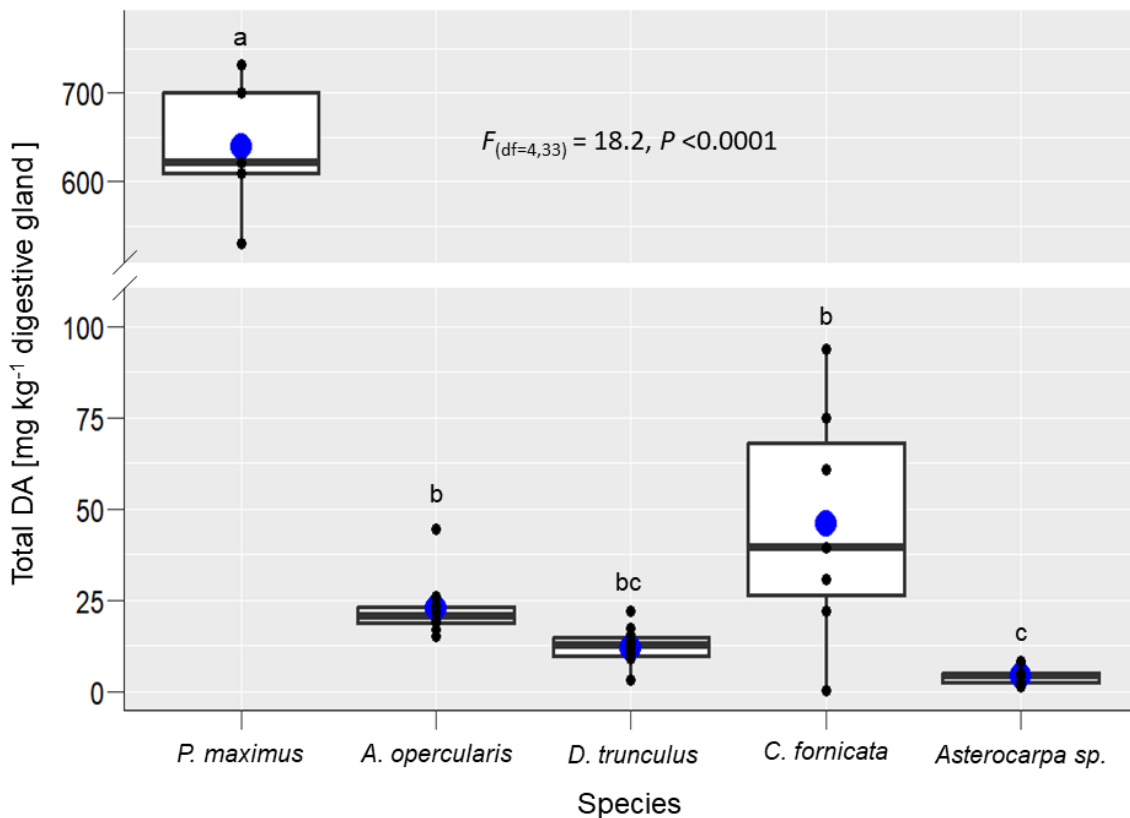
	Species					Statistical analysis	
	<i>P. maximus</i>	<i>A. opercularis</i>	<i>D. trunculus</i>	<i>C. fornicata</i>	<i>Asterocarpa</i> sp.		
DA (%)	93.3 ± 0.6 <sup>b</sup>	93.6 ± 0.3 <sup>b</sup>	95.8 ± 0.3 <sup>a</sup>	90.7 ± 1.1 <sup>c</sup>	94.5 ± 0.1 <sup>ab</sup>	F <sub>(df=4,33)</sub> = 11.8,	P <0.0001
isoE (%)	4.3 ± 0.3 <sup>a</sup>	4.3 ± 0.3 <sup>a</sup>	3.5 ± 0.3 <sup>a</sup>	3.2 ± 0.4 <sup>a</sup>	1.6 ± 0.1 <sup>b</sup>	F <sub>(df=4,33)</sub> = 10.9,	P <0.0001
isoD (%)	1.5 ± 0.3 <sup>bc</sup>	1 ± 0.1 <sup>bc</sup>	0.5 ± 0.1 <sup>c</sup>	4 ± 0.8 <sup>a</sup>	2.1 ± 0.0 <sup>b</sup>	F <sub>(df=4,33)</sub> = 17.3,	P <0.0001
isoA (%)	0.4 ± 0.0 <sup>ab</sup>	0.7 ± 0.0 <sup>a</sup>	0.2 ± 0.0 <sup>b</sup>	0.6 ± 0.1 <sup>a</sup>	0.5 ± 0.0 <sup>a</sup>	F <sub>(df=4,33)</sub> = 10.4,	P <0.0001
epi-DA (%)	0.4 ± 0.1 <sup>b</sup>	0.4 ± 0.0 <sup>0b</sup>	0 ± 0.0 <sup>c</sup>	1.5 ± 0.1 <sup>a</sup>	1.3 ± 0.0 <sup>a</sup>	F <sub>(df=4,33)</sub> = 156.4,	P <0.0001

829 Results are expressed as mean ± SE. Data were analyzed using species (five levels) as factor in separate one-way ANOVAs (P <0.05). The F-test  
830 statistic and degrees of freedom (df) are reported. Different superscript letters indicate significant differences between species. The level of  
831 statistical significance was set at α =0.05.



832

833 **Figure 1.** Sampling sites of the scallops *P. maximus* (n =5) and *A. opercularis* (n = 10), the  
 834 clam *D. trunculus* (n =11), the slippersnail *C. fornicata* (n =7), and the sea squirt *Asterocarpa*  
 835 *sp.* (n =5) and cell densities (cells. L<sup>-1</sup>) of *P. australis* during toxic blooms in the northwest  
 836 coast of Brittany, France between February and-April 2021.

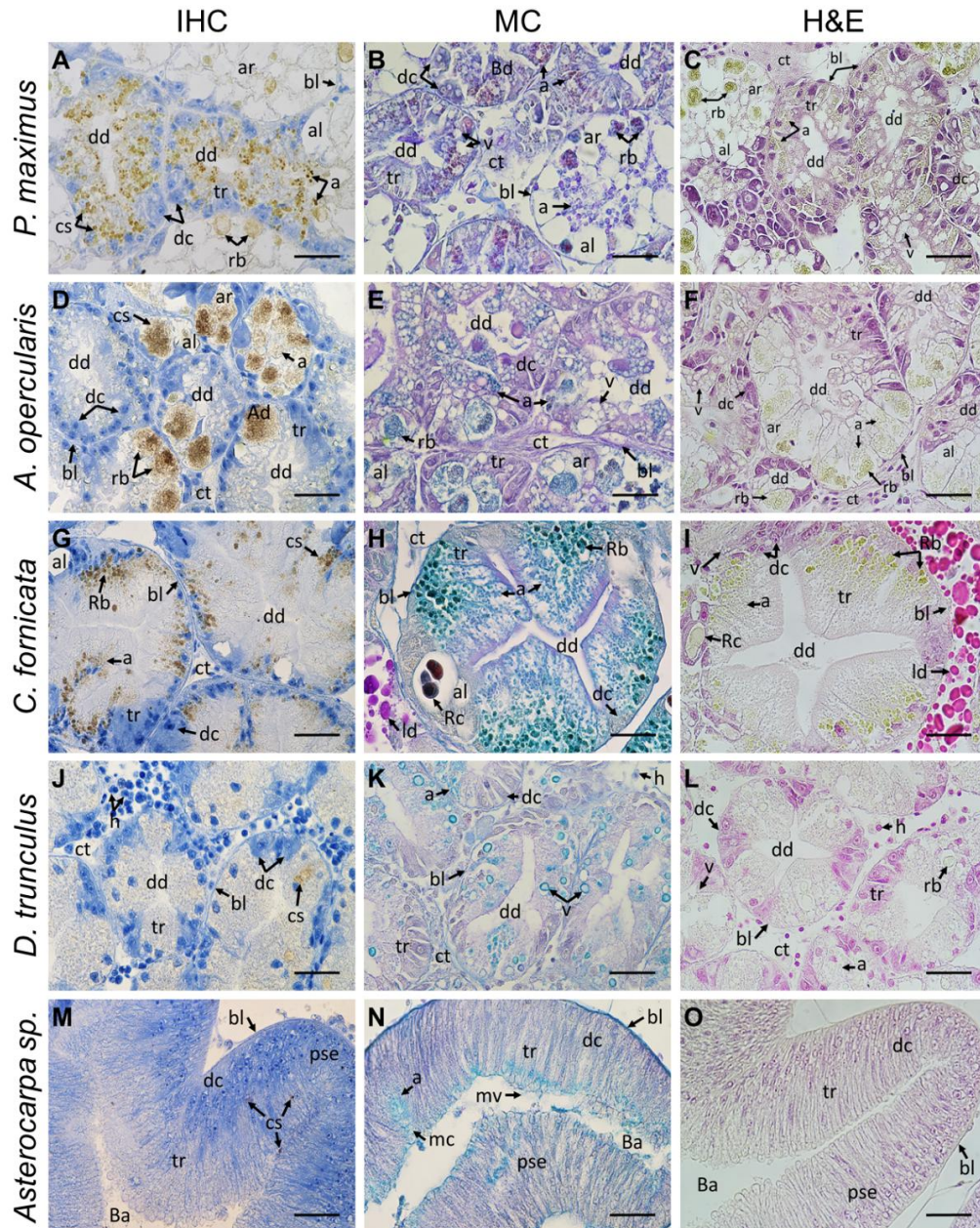


837

838 **Figure 2.** Total domoic acid (tDA) concentration in the digestive glands of the scallops *P.*  
 839 *maximus* (n =5) and *A. opercularis* (n = 10), the clam *D. trunculus* (n =11), the slippersnail *C.*  
 840 *fornicata* (n =7), and the sea squirt *Asterocarpa sp.* (n =5) contaminated during *P. australis*  
 841 blooms in the northwest coast of Brittany, France between on the 30<sup>th</sup> of March (for the  
 842 scallops *P. maximus*, *A. opercularis*, the slippersnail *C. fornicata*, and the sea squirt  
 843 *Asterocarpa sp.*) and on the 8<sup>th</sup> of April, 2021 (for the clam *D. trunculus*). The upper and  
 844 lower limits of the boxes are the quartiles, the middle horizontal line is the median, the  
 845 extremes of the vertical lines are the upper and lower limits of the observations, and black  
 846 dots are the individual observations. The blue dots are the means for each species. Data were  
 847 analyzed using species (five levels) as factor using a one-way ANOVA ( $P < 0.05$ ). The F-test  
 848 statistic and degrees of freedom (df) are reported. Different superscript letters indicate  
 849 significant differences between species. The level of statistical significance was set at  $\alpha = 0.05$ .

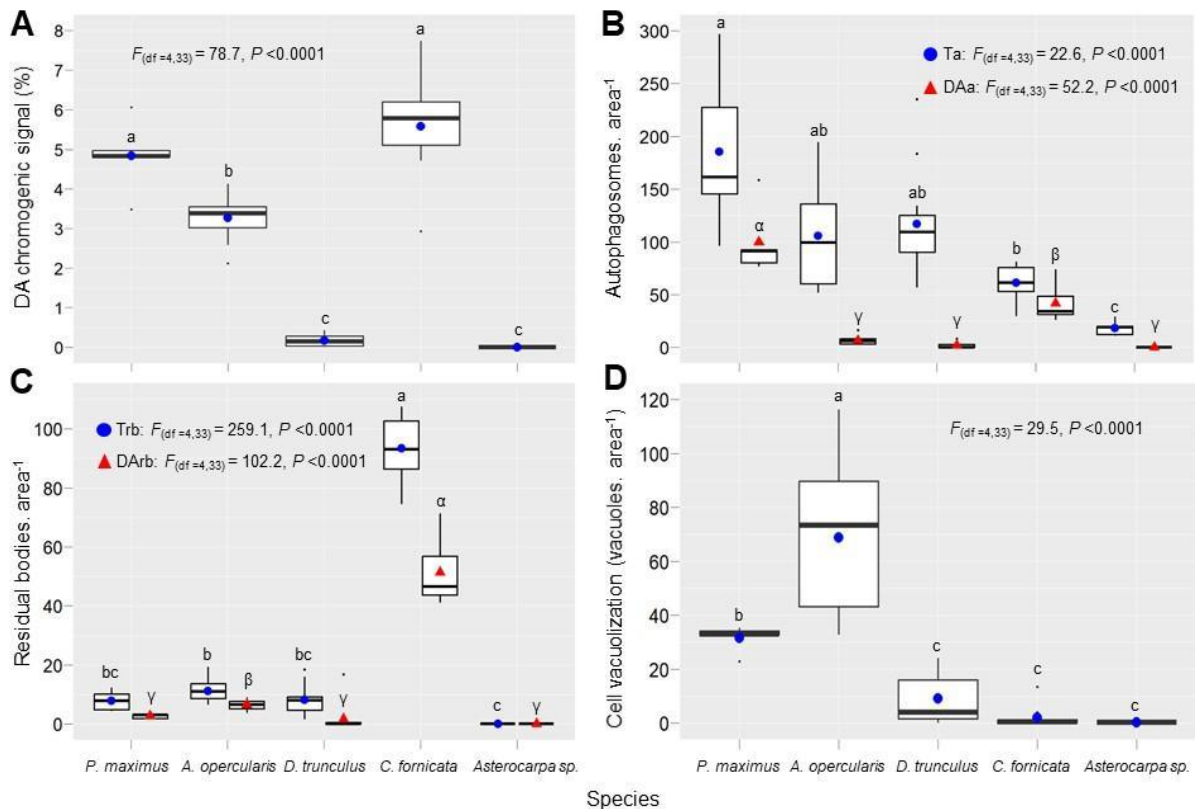
850





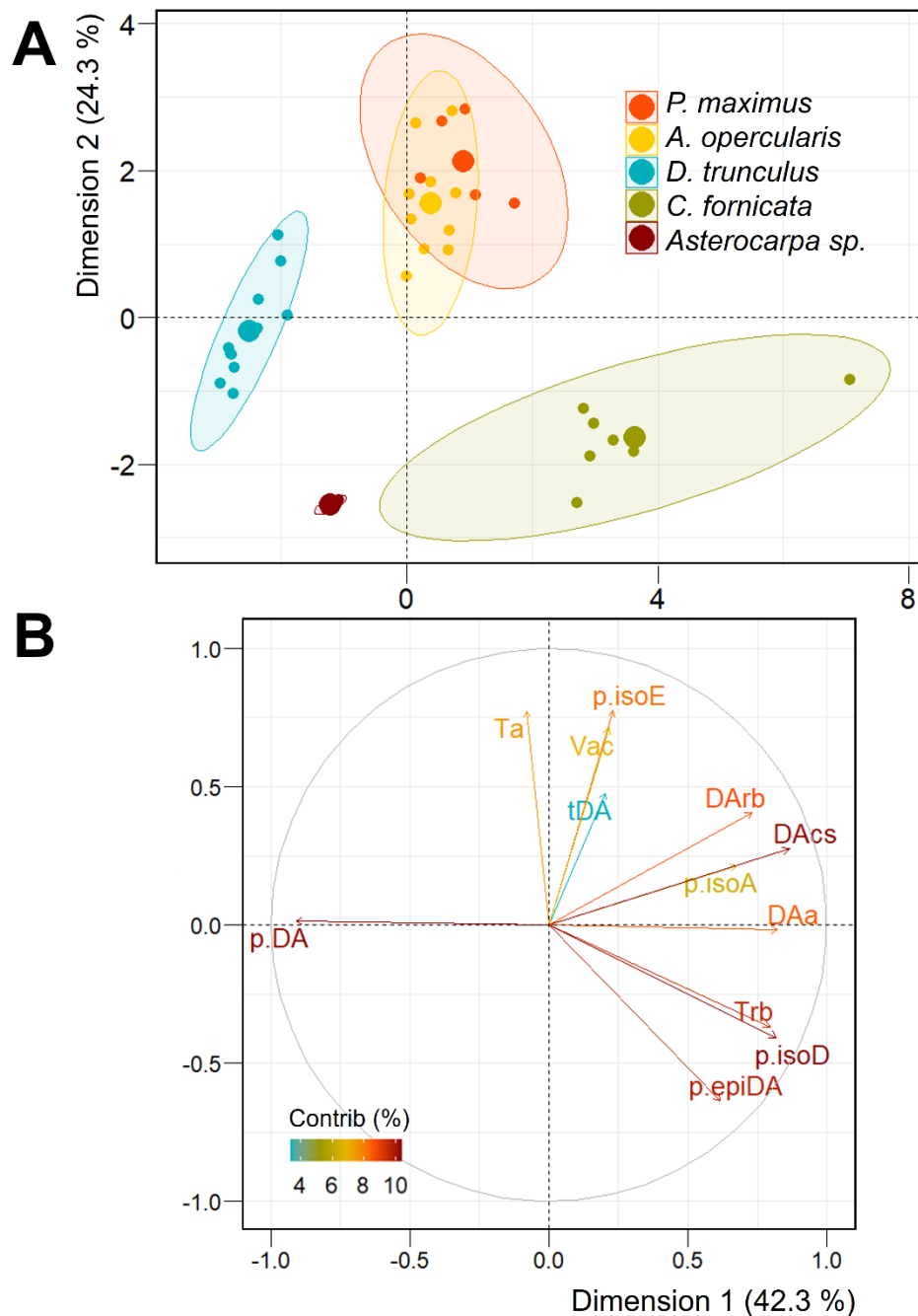
851

852 **Figure 3.** Microphotographs of digestive glands of the scallops *P. maximus* (A, B, C), *A.*  
 853 *opercularis* (D, E, F), the slipper snail *C. fornicata* (G, H, I), the clam *D. trunculus* (J, K, L),  
 854 and the sea squirt *Asterocarpa* sp. (M, N, O) contaminated with domoic acid (DA) during *P.*  
 855 *australis* blooms in the northwest coast of Brittany, France in March-April, 2021. IHC (A, D,  
 856 G, J, M) = Immunohistochemical detection of DA using specific anti-DA antibody (0.08 mg.  
 857 mL<sup>-1</sup>); MC (B, E, H, K, N) = multichromic histochemical staining of neutral carbohydrates  
 858 (violet-magenta dyes), acid glycoconjugates (blue hues), and proteins (yellowish tones); H&E  
 859 (C, F, I, L, O) = conventional histological Hematoxylin-Eosin staining. a = autophagosomic-  
 860 like vesicles, al = adipocyte-like cell, ar = acinar region, Ba = blind ampulla, bl = basal  
 861 lamina, cs = DA chromogenic signal, ct = connective tissue, dc = digestive cells, dd =  
 862 digestive diverticulum, hc = hemocytes, ld = lipid droplets, mc = mucus, mv = microvilli, pse  
 863 = pseudostratified epithelium, rb = residual bodies, rc = residual concretions, tr = tubular  
 864 region, v = vacuoles. Scale bar: 63 × = 30 μm.



865

866 **Figure 4.** Quantitative analysis of DA localization and subcellular features in the digestive  
 867 glands of the scallops *P. maximus* (n =5) and *A. opercularis* (n =10), the clam *D. trunculus* (n  
 868 =11), the slipper snail *C. fornicata* (n =7) and the sea squirt *Asterocarpa* sp. (n =5)  
 869 contaminated with DA during *P. australis* blooms in the northwest coast of Brittany, France,  
 870 in March-April, 2021. (A) DA chromogenic signal (%); (B) Autophagy (autophagosomes. 1.3  
 871 mm<sup>2</sup>, Ta = total autophagy, DAa = DA autophagy); (C) Residual bodies (residual bodies. 1.3  
 872 mm<sup>2</sup>, Trb = total residual bodies, DArb = DA in the residual bodies); (D) Cell vacuolization  
 873 (vacuoles. 1.3 mm<sup>2</sup>). The upper and lower limits of the boxes are the quartiles, the middle  
 874 horizontal line is the median, the extremes of the vertical lines are the upper and lower limits  
 875 of the observations, and black dots are the outliers (values that deviate from the median more  
 876 than 1.5 times the interquartile range). The blue dots and red triangles are the means of each  
 877 variable. Data were analyzed using species (five levels) as factor in separate one-way  
 878 ANOVA's ( $P < 0.05$ ). The F-test statistic and degrees of freedom (df) are reported. Different  
 879 superscript letters indicate significant differences between species. The level of statistical  
 880 significance was set at  $\alpha = 0.05$ .



881

882 **Figure 5.** Principal component analysis (PCA) summarizing data from the scallops *P.*  
 883 *maximus* (n =5) and *A. opercularis* (n =10), the clam *D. trunculus* (n =11), the slippersnail *C.*  
 884 *fornicata* (n =7), and the sea squirt *Asterocarpa sp.* (n =5) contaminated with domoic acid  
 885 (DA) during *P. australis* blooms in the northwest coast of Brittany, France, between March-  
 886 April 2021. Dimension 1 and dimension 2 together describe 66.6 % of the total variance. (A)  
 887 Scatter plot of individuals from each species. Larger symbols are the barycenter of each  
 888 group, confidence ellipses level was fixed at  $\alpha =0.05$ . (B) Variable contribution plot. The  
 889 direction of the arrows shows the correlations of variables (tDA = total DA, DAcs = DA  
 890 chromogenic signal, Ta = total autophagy, DAa = DA autophagy (%), Trb = total residual  
 891 bodies, DArb = DA in the residual bodies (%), Vac = cell vacuolization, and the percentages  
 892 (p) of DA isomers, p.DA = untransformed DA, p.isoE = isoE, p.isoD = isoD, p.isoA = isoA,

893 p.epiDA = epiDA) with given PCs, and its color intensity shows their contribution (Contrib  
894 %) to the explained variance.