

Development of harmful algal blooms species responsible for lipophilic and amnesic shellfish poisoning intoxications in southwestern Mediterranean coastal waters

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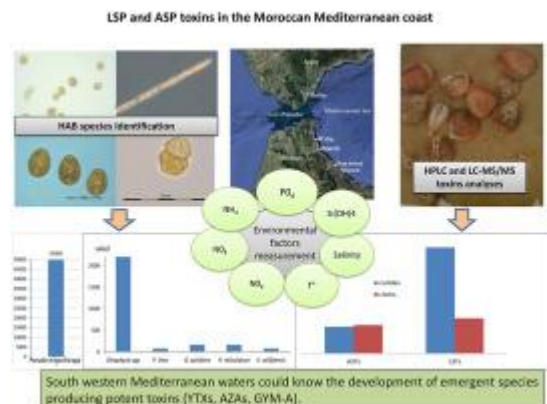
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Abstract :

Mediterranean waters have undergone environmental changes during the last decades leading to various modifications of the structure of phytoplankton populations, especially Harmful Algal Blooms (HABs) species. Monitoring of the potentially toxic phytoplankton species was carried out biweekly in the western Mediterranean coast of Morocco from March 2018 to March 2019. Lipophilic Shellfish Toxins (LSTs) using LC-MS/MS and Domoic Acid (DA) using HPLC-UV were measured in the exploited mollusks, the cockle *Acanthocardia tuberculata* and the smooth clam *Callista chione*. We also determined the prevailing environmental factors in four surveyed sites (M'diq bay, Martil, Kaa Asras, and Djawn) selected to cover a variety of coastal ecosystems. Results showed that *Pseudo-nitzschia* spp. A DA producer species, was abundant with a pick of 50×10^3 cells l⁻¹ on October 2018 in Djawn. *Dinophysis caudata* was the dominant *Dinophysis* species and showed a maximum density of 2200 cells l⁻¹ on July in Djawn. *Prorocentrum lima*, an epibenthic dinoflagellate, appeared rarely in the water column with densities <80 cells l⁻¹. *Gonyaulax spinifera* and *Protoceratium reticulatum* were found occasionally with a maximum density of 160 cells l⁻¹. *Karenia selliformis* was detected only five times (<80 cells l⁻¹) throughout the survey period. LC-MS/MS analyses revealed the presence of OA/DTX3, PTX-2, PTX-2 sa, and PTX-2 sa epi in the cockle at concentrations of up to 44.81 (OA/DTX-3+PTXs) ng g⁻¹ meat. GYM-A was detected in the clam at concentrations of up to 4.22 ng g⁻¹ meat. For the first time, AZAs and YTXs were detected in the southwestern Mediterranean with maximum values of 2.49 and 10.93 ng g⁻¹ meat of cockle, respectively. DA was detected in moderate concentrations not exceeding 5.65 µg g⁻¹ in both mollusks. Results showed that the observed toxic algae in the water column were responsible from the analysed toxins in the mollusks. It is likely that the southwestern Mediterranean waters could see the

development of emergent species producing potent toxins (YTXs, AZAs, GYM-A). These dinoflagellates have to be isolated, ribotyped, and their toxin profiles determined.

Graphical abstract



Highlights

► Many HAB species were encountered regularly along the year southwestern Mediterranean. ► Presence of OA/DTX3, Pectenotoxins (PTX-2, PTX-2 sa, PTX-2 sa epi), AZA-2, YTXs and GYM-A in shellfish. ► Cockles were much more contaminated than the *Smooth clams* which accumulated only GYM-A.

Keywords : southwestern Mediterranean, lipophilic and amnesic toxins, environmental factors, HABs, mollusk's contamination, LC-MS/MS analyses.

51 **1. Introduction**

52 The impact and spread of Harmful Algal Blooms (HABs) are increasing worldwide
53 (Hallegraeff, 2010; Fu et al., 2012; Wells et al., 2015; Gobler, 2020). The acceleration of
54 human-induced global warming is a major concern as it leads to the modification of the
55 dynamics of HABs (Wells et al., 2020). This dynamic is controlled by several abiotic
56 (temperature, salinity, nutrients) and biotic (allelopathy, parasitism, predation) environmental
57 factors (Madhu et al., 2007; Laabir et al., 2011, 2013; Wang et al., 2014; Vajravelu et al., 2018;
58 Wells et al., 2020). Phytoplankton react rapidly and specifically to environmental changes
59 (Hattich et al., 2021). The acquisition of knowledge on the environmental factors that regulate
60 the density and distribution of phytoplankton species is necessary to understand the functioning
61 of a determined ecosystem (Smayda and Reynolds, 2001; Reiss et al., 2011). Toxins produced
62 by HABs species cause health problems for consumers of contaminated marine organisms,
63 especially mollusks, as well as considerable economic losses due to the closing of shellfish
64 breeding and harvesting areas (Belin et al., 2021). Phycotoxins are classified into three main
65 groups of regulated marine biotoxins in the European Union (EU): Saxitoxins (STXs) and its
66 derivatives causing Paralytic Shellfish Poisoning (PSP), Domoic Acid (DA) generating
67 Amnesic Shellfish Poisoning (ASP), and the Lipophilic Shellfish Toxins (LSTs) group,
68 counting Dinophysistoxins (DTXs) and associated esters, Okadaic Acid (OA) and its
69 derivatives, causing Diarrhetic Shellfish Poisoning (DSP). Yessotoxins (YTXs), Gymnodimins
70 (GYMs), Azaspiracids (AZAs), Spirolides (SPXs), and Pinnatoxins (PnTXs) are considered as
71 emerging toxins (Vilariño et al., 2018; Wu et al., 2019). Among LSTs, only OA/DTXs, YTXs

72 and AZAs groups are regulated by EC Regulation N° 2021/1374 and thus evaluated in all
73 programs of mollusks surveyed in the European Union Member States (Dhanji-Rapkova et al.,
74 2018). However, the global occurrence in shellfish of YTXs and AZAs is less prevalent. The
75 other groups of LSTs are not regulated due to a lack of toxicological data. OA/DTXs and PTXs
76 groups are considered to be the most detected toxins in the Mediterranean (Faust and Gullidge,
77 2002; Dominguez et al., 2010; Anon, 2011; Cusick and Sayler, 2013; Zingone et al., 2021)
78 (Table 1), they are produced mainly by species of *Dinophysis* genera (Hallegraeff and Lucas,
79 1988). However, toxicology studies indicate that PTXs are much less toxic via the oral route
80 and have been de-regulated in some countries outside the EU (Codex Alimentarius, 2015).
81 *Dinophysis caudata* and *Dinophysis sacculus* were found in the Mediterranean more than one
82 century ago and were frequently observed in natural phytoplankton samples (Zingone et al.,
83 2021). In the 1980s, the first threat to human health had been described in the Gulf of Lion,
84 France (Belin et al., 1995). Low densities of *Dinophysis* spp. (< hundred cells per liter) could
85 generate LSP intoxications (Yasumoto et al., 1985). The highest densities of *Dinophysis* spp.
86 in the Mediterranean of up to 85000 cells l⁻¹ were reported intermittently in Greece between
87 2003 and 2008 and generated a shellfish harvesting ban period exceeding 150 days (Vlamiš and
88 Katikou, 2014). In northern Tunisia (Bizerte lagoon, Mediterranean), Turki et al. (2014)
89 reported concentrations of 25000 cells l⁻¹ of *Dinophysis* and *Phalacroma* species including *D.*
90 *sacculus*, *Dinophysis acuminata*, and *Phalacroma rotundatum*. Additionally, a *Dinophysis*
91 bloom dominated by *D. sacculus* reached high densities of up to 2 x 10⁵ in March 1998 in the
92 western Mediterranean in Sicily, Italy (Giacobbe et al., 2000). In Slovenian coastal waters, an
93 important development of *Dinophysis fortii*, reaching 2000 cells l⁻¹, caused the closure of
94 shellfish exploitation from May 2010 to March 2011 (Francé et al., 2018). Cañete et al. (2008)
95 reported that *Dinophysis* densities of 2200 cells l⁻¹ were associated with the 11 weeks of harvest
96 ban of bivalves in Alfacs Bay (Catalonia in the NW Mediterranean Sea, Spain) during 2012.

97 Several arrests of mollusk harvesting were caused by frequent and high amounts of DTXs and
98 OA in France (Belin et al., 2021) and Spain (García-Altarets et al., 2016; Fernandez et al., 2019).
99 OA was also detected in Sardinia, Italy (Mudadu et al., 2021) and in the Thermaikos Gulf in
100 Greece (Ciminiello et al., 2006; Reizopoulou et al., 2008) (Table 1). An important amount (1.1
101 $\mu\text{g g}^{-1}$ meat) of OA was recorded in *Mytilus galloprovincialis* in the Northern Adriatic Sea
102 (Ciminiello et al., 1997). *Dinophysis* is also responsible for the production of pectenotoxins
103 (PTXs) in the Mediterranean Sea. PTX-2 was recorded for the first time in France in 2005 in
104 the oysters with a maximum of $22 \mu\text{g Kg}^{-1}$ and in mussels with a maximum of $26 \mu\text{g kg}^{-1}$ in
105 Thau Lagoon (Amzil et al., 2007), as well as found in oysters in Sardinia (Italy) during 2019
106 (Mudadu et al., 2021) and in mussels in Catalonia (Spain) in 2012 (Garcia Altarets et al., 2016).
107 Belin et al. (2021) revealed frequent detection of high levels of PTX2-seco-acid in various sites
108 of shellfish aquaculture in the French Mediterranean lagoons. The azaspiracids toxins are the
109 latest LSTs which were found first in Irish mussels in 1995 (James et al., 2002). *Azadinium* and
110 *Amphidoma* genera have been known to be responsible for the production of these toxins
111 (Tillmann et al., 2017, 2021; Ozawa et al., 2021). Bacchiocchi et al. (2015) demonstrated the
112 presence of AZAs in *M. galloprovincialis* in the Mediterranean Sea. The YTX group is linked
113 to the dinoflagellates *Gonyaulax spinifera*, *Protoceratium reticulatum*, and *Lingulodinium*
114 *polyedra* which are frequently reported in the Mediterranean. The presence of these toxins
115 produces the same positive results of LSP in mouse bioassay, but no intoxications were
116 confirmed in humans (Tubaro et al., 2010). Pinzaru et al. (2018) detected YTXs in mussels in
117 the Mali Ston Bay (Croatia) in the Adriatic Sea. Many studies have showed the dominance of
118 YTXs among the accumulated lipophilic toxins in mollusks in the Adriatic Sea (Ciminiello et
119 al., 1997). Long periods of mussel harvesting bans have been associated with the contamination
120 by YTXs in the northwestern Adriatic Sea from 2002 to 2007 (Pistocchi et al., 2012).

121 Mediterranean marine ecosystems have not only faced LSPs intoxications but also ASP events
122 that have been attributed to many species of the genus *Pseudo-nitzschia* known to produce DA
123 (Zingone et al., 2021). High abundances ($>10^6$ cells l^{-1}) of *Pseudo-nitzschia* spp. were registered
124 in the Mediterranean (Caroppo et al., 2005; Cerino et al., 2005; Marić et al., 2011; Cabrini et
125 al., 2012; Ruggiero et al., 2015; Taş and Lundholm, 2017; Totti et al., 2019; Belin et al., 2021).
126 However, DA events caused only few shellfish farm closures in Spain (HAEDAT: Harmful
127 Algal Event Database) and France (Amzil et al., 2001; Belin et al., 2021). Low DA
128 concentrations were detected in shellfish from the southern Mediterranean coast in Tunisia
129 (Sahraoui et al., 2012; Turki et al., 2014), Morocco (Rijal Leblad et al., 2013, 2020), Greece
130 (Kaniou-Grigoriadou et al., 2005), and the Adriatic Sea (Ciminiello et al., 2005; Ujević et al.,
131 2010; Arapov et al., 2016). The EU regulation determined the permitted limits of toxins in
132 bivalve mollusks must not exceed ASP: 20 mg DA eq kg^{-1} edible part (e.p.) (Regulation EC N°
133 853/2004), OA and DTX: 160 μg OA eq/kg e.p., AZA: 160 μg AZA eq kg^{-1} e.p. (Regulation
134 (EU) 2021/1374), and YTX: 3.75 mg YTX eq kg^{-1} e.p. (Regulation EC No 786/2013).

135 In Morocco, the first case of poisoning due to the ingestion of shellfish accumulating algal
136 toxins was recorded in 1966 in Atlantic waters (Essaid, 1977). The years 1971, 1975, 1981, and
137 1994 were marked by several episodes of food poisoning by phycotoxins (Bourhili, 1982; Tber,
138 1983; Taleb et al., 2003). *Dinophysis* spp., *Prorocentrum lima*, *Gonyaulax spinifera*,
139 *Protoceratium reticulatum*, *Karenia* spp., *Ostreopsis* cf. *ovata* and *Pseudo-nitzschia* spp. were
140 reported from the Mediterranean coast of Morocco (El Madani et al., 2011; Daoudi et al., 2012;
141 Rijal Leblad et al., 2013, 2020). *Dinophysis* spp. has been identified in the Moroccan Atlantic
142 coast (Bennouna et al., 2005; Abouabdellah et al., 2011). LSTs were reported from the
143 Moroccan Atlantic waters, the main identified toxins were DTX-1, DTX-2, and OA as well as
144 their esterified forms (Taleb et al., 2006; Elgarch et al., 2008; Abouabdellah et al., 2011).
145 Amnesic shellfish toxins have been detected in bivalves from the Moroccan coasts since 2003

146 (INRH-monitoring program). During 1978, DA caused the first case of human intoxication after
147 the consumption of the mussel *M. galloprovincialis* in the eastern Mediterranean of Morocco
148 (Rijal Leblad et al., 2013). Bennouna et al. (2002) revealed the presence of LSTs using mouse
149 bioassay in 1998 in bivalves along the Atlantic coast of Morocco from the blooms of
150 *Lingulodinium polyedrum* accompanied with *D. acuta* and *D. acuminata*.

151 The present study was carried out in the Moroccan Mediterranean coast in various ecosystems
152 under anthropogenic pressure located in M'diq bay, Martil, Kaa Asras, and Djawn. Shellfish
153 exploitation in this region is hampered by the frequent occurrence of toxin events, especially
154 PSP and LSP, that caused frequent and long closures of mollusk farming and harvesting zones
155 generating important socio-economic problems (Rijal Leblad et al., 2020). Until now, few
156 studies have been conducted on the diversity and dynamic of HABs species and the related
157 toxins using modern chemical analyses such as LC-MS/MS in the southern Mediterranean
158 coast. Additionally, the emergent lipophilic toxins including AZAs, YTXs and GYM-A have
159 not been yet investigated. The objectives here were to 1) identify the phytoplankton species
160 producing LSTs and DA in the southwestern Moroccan Mediterranean where exploited
161 mollusks were regularly intoxicated, 2) evaluate the role of the main environmental factors in
162 the dynamic of those HABs species, and 3) investigate the contamination by DA, LSTs and
163 other emergent lipophilic toxins such as AZAs, YTXs and GYM-A of two commercially
164 exploited shellfish species, the cockle *Acanthocardia tuberculata* and smooth clam *Callista*
165 *chione*.

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167

168

2. Materials and Methods

2.1. Sampling area

The Moroccan Mediterranean coastline extends 512 km, from Tangier in the west to Saïdia in the east. During the last decades, this region has undergone a remarkable development at the economic and touristic levels as well as fishing and aquaculture. Each year, this coast receives important amounts of different types of discharges (Nakhli, 2010). The Tétouan coast is among the most affected areas in the Moroccan Mediterranean, the amount of discharge in Tétouan and Tangier exceed 232 tons' day⁻¹ of suspended matter (Er-Raioui et al., 2012). The study area is located within the coastline of Tétouan-Chefchaouen characterized by shallow sloping coastal topography, a rocky coastline, and sedimentary bottoms (El Hmaïdi et al., 2002; Laouina, 2010). Martil River (35 km long) and the Oued Laou river (65 km long) directly discharge their contents in the study area. Four sites were selected: M'diq, Martil, Kaa Asras, and Djawn (Fig. 1). They are part of the zones monitored by the INRH (Moroccan Institute of Fisheries Research) for toxic phytoplankton and shellfish toxin contamination. They are known to have placed numerous ban periods of shellfish harvesting (Table 2). This region has a large number of shellfish production zones for bivalve mollusks. It holds important economic activities and is of considerable ecological interest because of its richness in species of fauna (e.g., fish, bivalve mollusks). The depth of the sampling sites ranged between 7 and 10 m. Sampling was conducted from March 2018 to March 2019.

2.2. Phytoplankton sampling

For the identification and quantification of microphytoplankton species, 200 ml of seawater was sampled biweekly at integral samples of the water column from 0–7 m at all sites. The samples were preserved adding 2–3 ml Lugol iodine acidic solution. After depositing the

193 samples in Hydro-bios 25 ml chamber for one day, the identification and counting of potentially
194 toxic species (*Pseudo-nitzschia* spp., *Dinophysis* spp., *Prorocentrum lima*, *Protoceratium*
195 *reticulatum*, *Gonyaulax spinifera*, *Karenia selliformis*) were carried out using a Leica DMIL
196 inverted photonic microscope (Uthermöl, 1958). These HABs species are related to the
197 production of domoic acid, okadaic acid/dinophysistoxins/pectenotoxins, and of emergent
198 toxins such as yessotoxins, azaspiracids, and gymnodimins which were recently highlighted in
199 the Mediterranean marine ecosystems (Zingone et al., 2021).

200

201 2.3. Environmental abiotic factors

202 Seawater was taken biweekly from the four stations at subsurface -0.5 m, using a 500 ml
203 polypropylene bottles and were filtered (0.45 µm). The samples were stabilized by freezing at
204 -20 °C before analysis. The nutrients (nitrite NO₂⁻, nitrate NO₃⁻, ammonium NH₄⁺, phosphate
205 PO₄³⁻, and silicate Si(OH)₄) were measured using a spectrophotometer (Unico SQ4802 UV/VIS
206 double Beam spectrophotometer model) following the method of Aminot & Chaussepied
207 (1983). Temperature and salinity were measured using the probe Cond 3310 SET 1
208 (https://www.geotechenv.com/Manuals/WTW_Manuals/WTW_Cond_3310.pdf).

209

210 2.4. Shellfish sampling

211 At the same time of seawater sampling, cockles *A. tuberculata* (N = 25 individuals) and smooth
212 clams *C. chione* (N = 25 individuals) were collected from the seabed using a dredge. Back in
213 the laboratory, animals were frozen at -20 °C until toxin analysis.

214

215 *2.5. Lipophilic toxins analysis using mouse bioassay*

216 The analyses of lipophilic shellfish toxins were carried out using the method of EU harmonized
217 Standard Operating Procedure for detection of lipophilic toxins by Mouse Bioassay (EURLMB,
218 2013). This method consists of injecting an extract into the intraperitoneal cavity of mice
219 weighing between 18 g and 20 g. This extract was obtained after a double extraction, first using
220 acetone, and then second with dichloromethane and water. Twenty grams of hepatopancreas
221 was mixed with 50 ml of acetone and crushed, then filtered and collected in a 500 ml flask. The
222 residue remaining in the filter was taken up in 50 ml of acetone, then grounded and filtered.
223 This filtrate was recovered in the same flask. This step was repeated three times until a final
224 volume of 150 ml of acetone extract was obtained. In order to obtain a residue, the acetone was
225 evaporated at a temperature of 42 ± 2 °C. This mixture was treated with 50 ml of
226 dichloromethane. The two phases were separated, organic fractions (containing lipophilic
227 toxins) and aqueous (elimination of Paralytic Shellfish Poisoning toxins). This last phase was
228 taken up with 50 ml of dichloromethane; this step was repeated twice. The final volume
229 obtained was 150 ml. The dichloromethane was evaporated off in vacuo using the rotary
230 steamer at a temperature of 42 ± 2 °C. The obtained residue was dissolved in 5 ml of tween 60
231 (1%), then 1 ml of the extract was injected into the intraperitoneal cavity of an albino Swiss
232 mouse. After a 24 h observation period, if at least 2/3 of the mice died, the result would be
233 considered positive.

234

235 *2.6. Chemical analysis of toxins in shellfish*

236 *2.6.1. LC-MS/MS analysis of lipophilic toxins*

237 In accordance with the European method validated internally, lipophilic toxins were extracted
238 from 2 ± 0.05 g of hepatopancreas homogenate of shellfish. Then, the homogenate was

239 extracted in duplicate with 9 ml methanol. After the first addition of methanol, the mixture was
240 homogenized with an Ultra-Turrax mixer for 2 min at 10.000 rpm and the extract was
241 centrifuged for 8 min at 8000 g, after the supernatant was transferred to a 20 ml volumetric
242 flask. After the second addition of methanol, the mixture was homogenized with an Ultra-
243 Turrax, and centrifuged for 8 min at 8000 g. The supernatant was combined with the first extract
244 and methanol was added to reach a final volume of 20 ml. For the determination of the esters
245 of OA and its analogs DTX-1 and DTX-2, alkaline hydrolysis of the extracts was carried out.
246 For every 1 ml of methanolic extract, 125 μ l of 2.5M NaOH was added. The whole mixture was
247 put in a vortex for 30 sec and then heated for 40 min at 76 °C. After cooling in an ice bath, the
248 mixture was neutralized and mixed with 125 μ l of 2.5M chlorohydric acid. All samples were
249 filtered (0.2 μ m, Nanosep, MF, Pall) and stored at -20 °C before analysis.

250 Sample analyses (two aliquots per each shellfish sample) was performed on a UFLC (model
251 UFLC, Shimadzu) coupled to a triple-quadrupole mass spectrometer (4000 Qtrap, ABSciex)
252 equipped with a turboV® ESI source. Chromatographic separation was carried out on a C18
253 Kinetex column (100 Å, 2.6 μ m, 100 \times 2.1 mm, Phenomenex) with a C18 guard column (4 \times
254 2.0 mm, 2.6 μ m, Phenomenex). A binary mobile phase was used, phase A (100% aqueous) and
255 phase B (95% aqueous acetonitrile), both containing 2 mM ammonium formate and 50 mM
256 formic acid. The flow rate was 0.4 ml min⁻¹ and injection volume at 5 μ L. The column and
257 sample temperatures were 40 °C and 4 °C, respectively. A gradient elution was employed,
258 starting with 20% B, rising to 95% B over 8 min, held for 3 min, then decreased to 20% B in
259 0.5 min and held for 3 min to equilibrate the system.

260 For quantitation, the mass spectrometer was operated in multiple reactions monitoring (MRM)
261 acquisition mode, scanning two transitions for each toxin. Negative acquisition experiments
262 were established using the following source settings: curtain gas set at 20 psi, ion spray at -
263 4500 V, temperature of 550 °C, gas 1 and 2 set, respectively, at 40 and 55 psi, and an entrance

264 potential of 13 V. In the positive mode: curtain gas was set at 30 psi, ion spray at 5500 V,
265 temperature of 350 °C, gas 1 and 2 set, respectively, at 40 and 50 psi, and an entrance potential
266 of 10 V. These parameters had been previously optimized using toxin standards. The mass
267 spectrometer was operated in multiple reaction-monitoring (MRM), analyzing the two product
268 ions per compound; for each toxin, the first transition, the most intense, is used for
269 quantification (Supplementary Tables S-2a and S-2b). Certified calibration solutions of OA,
270 DTX-2, DTX-1, AZA-1, AZA-2, AZA-3, YTX, homo-YTX, PTX-2, SPX-13-desMeC and
271 GYM-A, PnTX-A, and PnTX-G were obtained from the National Research Council Canada
272 (NRCC, Halifax, NS, Canada).

273

274 *2.6.2. Amnesic shellfish toxins analyses*

275 DA concentrations were analysed by a high-performance liquid chromatography-UV detector
276 (HPLC-UV) (Shimadzu 10vp type). DA was analysed biweekly following Quilliam's (1995)
277 method according to standard EN 14176:2017. About 100 g of shellfish meat were shredded
278 and homogenated, then 4 g were mixed with 16 ml of solvent extraction (methanol: water 1:1)
279 homogenized for 3 min at 10,000 rpm (Ultra-Turrax) and centrifuged for 10 min at 4000 rpm.
280 Then the sample was analysed using the following chromatographic conditions: Column C₁₈ (5
281 µm, 250 mm x 4,6 mm), mobile phase flow rate of 1 ml min⁻¹, detector wavelength of 242 nm,
282 injection volume of 20 µL, and an oven temperature for the column of 40 °C. The determination
283 of DA content in samples was done with a detection limit of 0.05 mg kg⁻¹.

284

285 *2.7. Statistical analysis*

286 Canonical Correspondence Analysis (CCA) using the XLSTAT 2022.2.1.1312 software was
287 conducted to investigate the relationship between HABs species densities and environmental

288 factors (temperature, salinity, and nutrients). One-way ANOVA analysis was performed to
289 show any significant correlation between phytoplankton taxa and environmental factors
290 considered individually. Significant differences were obtained when $p < 0.05$.

291

292 **3. Results**

293 *3.1. Environmental abiotic factors*

294 *3.1.1. Temperature and salinity*

295 The variations of temperature and salinity are illustrated in Figure 2 and correspond to
296 temperate Mediterranean climate. The study area was characterized by seasonal variation of the
297 temperature, but this never goes below 15 °C. The lowest values, 15.5 °C in M'diq, 15.6 °C in
298 Kaa Asras and Djawn, and 15.7 °C in Martil were recorded during winter 2019. The highest
299 values were registered in summer 2018, 23.7 °C (July 23rd) in Djawn, 23.6 °C (August 13th) in
300 Kaa Asras, 23.5 °C (August 13th) in Martil, and 23.2°C (July 23rd and August 13th) in M'diq.
301 Temperature did not vary significantly between the surveyed stations (Fig. 2a). Salinity showed
302 the lowest values of 33.7 °C in Kaa Asras and Martil on April 10th 2018 and December 25th
303 2018, respectively. The highest values were recorded in Martil (36.2 °C on February 4th 2019)
304 and 36 °C in Kaa Asras and Djawn on October 10th 2018. Salinity showed an almost stable
305 curve, most of the time the values ranged from 35.4 and 36.2. However, we noted a drop in
306 salinity during the rainfall period (from November 2018 to January 2019) in M'diq and Martil
307 and during April in Djawn (Fig. 2b).

308

309 *3.1.2. Nutrients*

310 Nutrient concentrations in seawater fluctuated during our monitoring and did not showed any
311 clear tendency (Fig. 3). Ammonia showed a wide spatiotemporal variation. The highest
312 concentration was measured in Kaa Asras with values of $17.04 \mu\text{mol l}^{-1}$ and $11.64 \mu\text{mol l}^{-1}$ on
313 July 10th 2018, and January 7th 2019, respectively (Fig. 3a). The lowest concentration of 0.09
314 $\mu\text{mol l}^{-1}$ was registered in M'diq on April 10th. For the phosphorus, the highest concentrations
315 were measured during Spring in all sites with a maximum of $1.85 \mu\text{mol l}^{-1}$ in Martil and Djawn
316 on April 30th and May 22nd, respectively. Nitrate showed the highest concentration of $2.85 \mu\text{mol}$
317 l^{-1} on December 11th in M'diq, this may be linked to the river discharge near M'diq during the
318 rainy period. The highest concentrations of nitrite (up to $0.82 \mu\text{mol l}^{-1}$) were measured in Djawn
319 during winter in January. The lowest concentration ($\leq 0.01 \mu\text{mol l}^{-1}$) was registered in late
320 summer and early autumn. Silicates showed spatiotemporal fluctuations with the highest
321 concentrations of $16.75 \mu\text{mol l}^{-1}$ and $15.00 \mu\text{mol l}^{-1}$ measured in Martil in Jun 11th and
322 November 5th, respectively.

323

324 3.2. Spatiotemporal variation of potentially toxic phytoplankton species.

325 Six different species of *Dinophysis* and *Phalacroma* were observed and identified: *D.*
326 *acuminata*, *D. fortii*, *D. caudata*, *D. diegensis*, *Phalacroma rotundatum*, and *D. mitra*. Globally,
327 *Dinophysis* species showed an increase in their densities from the end of May to the end of
328 Summer, except *D. fortii*, which developed at the beginning of Spring (Fig. 4). The rest of the
329 year, *Dinophysis* spp. concentration stayed low and did not exceed 60 cells l^{-1} . Among the three
330 species (*D. caudata*, *D. acuminata*, and *D. fortii*) regularly observed, *D. caudata* was the most
331 abundant reaching $2080 \text{ cells l}^{-1}$ and $1980 \text{ cells l}^{-1}$ on July 23rd 2018 in Djawn and on August
332 13th 2018 in M'diq, respectively.

333 The benthic dinoflagellate *Prorocentrum lima* was observed in the water column at moderate
334 densities not exceeding 80 cells l⁻¹, registered on August 13th in Djawn (Fig. 5a), cells
335 correspond to the detached ones from the colonized biotope. *Protoceratium reticulatum*
336 developed only in the eastern stations (Kaa Asras and Djawn) with the highest value in late
337 spring and early summer (160 cells l⁻¹ in Djawn on June 25th) (Fig. 5b). *Gonyaulax spinifera*
338 was detected regularly during the survey, the highest value (160 cells l⁻¹) was recorded once
339 in Martil on August 6th 2018 (Fig. 5c). *Karenia selliformis* was detected only five times in the
340 surveyed stations with the highest abundance of 80 cells l⁻¹ in M'diq and Martil (Fig. 5d).
341 *Pseudo-nitzschia* spp. was found in all of the samples in the surveyed sites (Fig. 6a). The spatial
342 distribution did not indicate a remarkable difference between the sites. Autumn seems to be the
343 optimal growth period for this genus reaching values of 50 x 10³ cells l⁻¹ at Djawn during the
344 first week of October.

345

346 3.3. Toxin accumulation in shellfish

347 The concentrations of DA found in the cockles and smooth clams were similar (Fig. 6b and 6c).
348 In cockles, DA concentration showed maximum values on October 8th 2018 reaching 2.68 µg
349 DA g⁻¹ meat, 2.60 µg DA g⁻¹ meat, and 2.59 µg DA g⁻¹ meat in Kaa Asras, Djawn, and M'diq,
350 respectively. In smooth clams, DA concentration was maximum on July 10th 2018 reaching
351 5.65 µg DA g⁻¹ meat in M'diq, subsequently to the highest abundance of *Pseudo-nitzschia* spp.
352 of 29 x 10³ cells l⁻¹. Interestingly, each peak of DA in both shellfish species was preceded by a
353 development of *Pseudo-nitzschia* spp. which is shown in Figure 6, suggesting the presence of
354 toxic *Pseudo-nitzschia* species in the water column.

355 In the same way, Figure 7 showed that the maximum densities of dinoflagellate species in
356 seawater were registered in the 15 days before LST pics (*Dinophysis* spp., *P. lima*, *P.*
357 *reticulatum*, *G. spinifera*, *K. selliformis*) which signifies that these dinoflagellate species could
358 be responsible for the production of the measured LSTs in the mollusks.

359 Lipophilic toxins were found five times in the cockles and clams in M'diq and Djawn (Fig. 8),
360 from May 31st to November 5th 2018. Five groups of toxins were detected: acyl-derivative-
361 okadaic acid (dinophysistoxin-3, DTX-3), Yessotoxins (YTXs), Gymnodimins (GYMs),
362 Azaspiracids (AZAs), and Pectenotoxins (PTXs). Results showed that the cockles accumulated
363 all of the found LSTs. In contrast, the clams accumulate only GYM-A, which suggests different
364 physiological behavior of these mollusks. Total OA/DTX3 was detected only in the cockles
365 with concentrations up to 18.55 ng g⁻¹ meat in Djawn site on July 23th and September 3th 2018.
366 YTXs were detected only in the cockles at highest concentrations of 10.93 ng g⁻¹ meat in Djawn
367 on July 23rd 2018. GYM-A was the only toxin found in both mollusk species, with maximum
368 concentration of 4.22 ng g⁻¹ meat measured in the clam on September 3rd 2018, and the lower
369 concentration of 1.03 ng g⁻¹ meat measured in cockles on May 31st 2018. AZA-2 was found in
370 the cockles one time at the M'diq site on November 5th 2018 at a concentration of 2.49 ng g⁻¹
371 meat and two times at the Djawn site on June 25th and November 5th 2018 at a concentration of
372 1.29 ng g⁻¹ meat and 2.02 ng g⁻¹ meat, respectively. PTX-1 and PTX-6 were not detected in the
373 investigated area. PTX-2 sa and PTX-2 sa epi were detected in cockles with maximum
374 concentrations of 25.24 ng PTX-2 sa g⁻¹ meat and 3.95 ng PTX-2 sa epi g⁻¹ meat on July 23rd
375 in Djawn. PTX-2 was found only in cockles from the Djawn site with a concentration of 1.40
376 ng g⁻¹ meat on July 23rd 2018. Shellfish developing in Djawn were more contaminated
377 compared to those at M'diq in term of toxin profiles and levels which denotes an East-Ouest
378 gradient (Fig. 8).

379

380 3.4. Correlation between environmental factors and potentially toxic species

381 CCA was performed to investigate the relationships between HABs species densities and
382 physicochemical parameters considering all data from M'diq, Martil, Kaa Asras, and Djawn.
383 The first two axes explained 90.24% and 5.18% of the total variance ($p < 0.001$), respectively
384 (Fig. 9). *Ostreopsis* spp., *P. lima*, and *Dinophysis* spp. appeared to be affected by temperature.
385 *P. reticulatum* was associated with ammonia and dissolved inorganic nitrogen. *Pseudo-*
386 *nitzschia* spp. was not correlated to any of the measured parameters. A one way ANOVA
387 analyses considering individual relationships of taxa with the measured physicochemical
388 parameters showed that *Dinophysis* spp. ($F = 2.113$, $p < 0.05$) and *P. lima* ($F = 1.967$, $p < 0.05$)
389 were correlated to temperature, whereas *P. reticulatum* was significantly correlated to ammonia
390 ($F = 3.175$, $p < 0.05$).

391 4. Discussion

392 4.1. Potentially toxic microalgae species density in relation to environmental factors

393 We studied the development of HABs species in relation to environmental abiotic factors in the
394 western Moroccan Mediterranean coast. The one-year monitoring (March 2018–March 2019)
395 showed the presence of several potentially toxic species namely *Pseudo-nitzschia* spp., *D.*
396 *acuminata*, *D. fortii*, *D. caudata*, *D. diegensis*, *P. rotundatum*, *D. mitra*, *P. lima*, *G. spinifera*,
397 *P. reticulatum*, and *K. selliformis*. These species have been previously observed within the
398 southern Mediterranean in Moroccan (El Madani, 2011; Daoudi et al., 2012; Rijal Leblad et al.,
399 2013, 2020), Algerian (Frehi et al., 2007; Draredja et al., 2019), and Tunisian coastal waters
400 (Turki et al., 2014; Smida et al., 2014).

401 Species in the *Pseudo-nitzschia* genera were the most abundant during this survey with a
402 maximum of 50×10^3 cells l^{-1} on October 7th 2018 in Djawn. They were reported on the

403 Moroccan Mediterranean coast by Rijal Leblad et al. (2020). *Pseudo-nitzschia* is known to
404 bloom regularly in the Mediterranean Sea (Cerino et al., 2005; Quiroga, 2006; Quijano-
405 Scheggia et al., 2008; Marić et al., 2011; Cabrini et al., 2012; Sahraoui et al., 2012; Turki et al.,
406 2014; Ruggiero et al., 2015; Taş and Lundholm, 2017; Totti et al., 2019). In our survey, the
407 highest densities of *Pseudo-nitzschia* spp. were observed in autumn while the lowest were
408 observed in June–July and during winter. This agrees with Turki et al. (2014), who showed the
409 quasi-absence of *Pseudo-nitzschia* species during the summer from 2007 to 2011 in Bizerte
410 lagoon, Tunisia, and with Sahraoui et al. (2012) who showed that *Pseudo-nitzschia* spp.
411 exhibited a seasonal pattern with early fall being the optimum period of its development.

412 CCA analysis showed no clear correlation between *Pseudo-nitzschia* with silicates, this could
413 be explained by the uptake of this nutrient by these diatoms when they develop. However,
414 Thorel et al. (2017) and Melliti Ben-Garali et al. (2019) showed that the abundance of *Pseudo-*
415 *nitzschia* was correlated with silicates in the Bay of Seine in France and in the Bizerte Bay in
416 Tunisia, respectively. Our results showed that species of *Dinophysis* genera developed in
417 summer (Fig. 4) and *D. caudata* was the most abundant species. This corroborates other works
418 conducted in the same area during 2008 and 2009 (Rijal Leblad et al., 2020). Zingone et al.
419 (2021) reported that *D. caudata* and *D. sacculus* were the most frequent species of *Dinophysis*
420 genera in the Mediterranean Sea. The maximum density of *D. caudata* in our survey was 2080
421 cells l⁻¹ recorded in Djawn on July 23rd 2018, while the highest abundance recorded by Rijal
422 Leblad et al. (2020) was of 560 cells l⁻¹ of all *Dinophysis* species. *D. caudata* densities of up to
423 2000 cells l⁻¹ were found in western Adriatic (Ingarao et al., 2009). Garcia Altares et al. (2016)
424 reported a bloom of *Dinophysis* spp. dominated by *D. sacculus* in the Mediterranean coast of
425 Catalonia (Spain) during 2012 with a maximum abundance of 2200 cells l⁻¹. During a bloom of
426 *D. acuminata*, a close abundance of 2200 cells l⁻¹ was registered in the Thermaikos Gulf
427 (Greece) (Reizopoulou et al., 2008). Francé et al. (2018) reported densities of up to 2000 cells

428 l^{-1} of *D. fortii* in the northern Adriatic. An exceptionally high abundance of 25×10^4 cells l^{-1} of
429 *Dinophysis* spp. was reported in January 2008 in Bizerte lagoon, Tunisia (Turki et al., 2014).
430 This dinoflagellate was detected in other Mediterranean ecosystems such as on the Sardinia
431 coast in Italy during 2019 (Mudadu et al., 2021), France (Séchet et al., 2021), Greece
432 (Ciminiello et al., 2006), and Tunisia (Aissaoui et al., 2014).

433 Here, the CCA and one-way ANOVA statistical treatments revealed significant and positive
434 correlations between *Dinophysis* spp. and temperature. This agrees with Smayda (1980) who
435 showed the importance of warm waters and the absence of turbulence (from late spring to early
436 autumn) for the growth of dinoflagellates. Reguera et al. (2014) indicated the presence of *D.*
437 *sacculus* in the Mediterranean Sea with abundances over 3000 cells l^{-1} only in warm-temperate
438 coastal zones with freshwater inputs. A bloom of *D. fortii* was reported in the Adriatic Sea when
439 temperatures reached 30 °C during June–July (Francé et al., 2018). In contrast, high *Dinophysis*
440 spp. abundances of up to 85.4×10^3 cells l^{-1} were recorded in Greek coastal waters during winter
441 (Koukaras and Nikolaidis, 2004) and in early winter (January) in Tunisian Waters with
442 abundances of up to 3×10^4 cells l^{-1} (Smida et al., 2014). In our study, *Dinophysis* spp. was
443 positively correlated to salinity. In contrast, *D. fortii* blooms occurred during the period of low
444 salinity in Adriatic waters (Francé et al., 2018). Caroppo et al. (2001) showed that maximum
445 abundances of *Dinophysis* species were observed in the Adriatic Sea at low salinity (Caroppo
446 et al., 2001). Aissaoui et al. (2014) showed a tolerance of *D. sacculus* and *D. acuminata* to wide
447 variations of temperature and salinity ranging from 11.6 °C to 30 °C and 32 to 40.3,
448 respectively. Here, nitrate and nitrite were negatively correlated to *Dinophysis* spp. In contrast,
449 Caroppo et al. (2001) showed positive correlations between *Dinophysis* developing in the
450 Adriatic Sea with nitrite and phosphorus and negative correlation with ammonia (Caroppo et
451 al., 2001).

452 *P. lima* was detected in the water column only a few times with densities lower than 80 cells l⁻¹
453 ¹. This is not surprising because *P. lima* is a benthic dinoflagellate (Pearce et al., 2005). Ingarao
454 et al. (2009) showed the absence of *P. lima* in the water column along the coast of the Abrazo
455 region in the western Adriatic, while it was detected with abundances of up to 4.7x10⁵ cells l⁻¹
456 in Ortora harbor on macroalgae. Rijal Leblad et al. (2020) showed in a field study during 2008–
457 2009 that *P. lima* was found in the water column of Oued Laou with densities of up to 1280
458 cells l⁻¹. CCA analysis showed that *P. lima* density was significantly and positively correlated
459 with temperature, nonetheless *P. lima* is considered as a thermophilic species (Ben-Gharbia et
460 al., 2016). In the present study, *P. lima* abundance was not correlated with salinity. Ingarao et
461 al. (2009) showed that *P. lima* was negatively correlated with salinity in the northern Adriatic.
462 In our study, nitrate was negatively linked to *P. lima*. *P. reticulatum*, and *G. spinifera* species,
463 producers of yessotoxins, were observed sporadically. *P. reticulatum* appeared between late
464 spring and summer and was found only in the east part of our study area (Kaa Asras and Djawn).
465 *G. spinifera* developed in all of the surveyed sites during all the year with densities ranging
466 between 40 and 80 cells l⁻¹ and a maximum of 160 cells l⁻¹ on August 6th 2018 in Martil. These
467 dinoflagellate species were reported to be present in other Mediterranean area (Zingone et al.,
468 2021).

469 Our results showed that the HABs species developing in the southwestern Mediterranean
470 respond differently to environmental factors particularly for nutrients (Fig. 9). This could be
471 explained by inter and intraspecific variability in the physiology and growth requirements of
472 each species in the colonized ecosystem.

473

474 4.2. Accumulation of toxins in shellfish

475 4.2.1. Amnesic shellfish toxins

476 Our results showed that chemically measured DA (LC-UV method) was present in the
477 investigated mollusks with maximum concentrations of $5.65 \mu\text{g g}^{-1}$ clam meat on July 9th 2018
478 in Djawn. Rijal Leblad et al. (2013) showed that DA concentrations detected on the
479 Mediterranean coast of Morocco in clams sampled in 2007 ranged between 0.71 to $4.9 \mu\text{g g}^{-1}$
480 clam meat. Higher amounts with a maximum of $16.88 \mu\text{g DA g}^{-1}$ clam meat in Oued Laou and
481 $15.94 \mu\text{g DA g}^{-1}$ clam meat in M'diq bay were detected in 2008–2009 (Rijal Leblad et al.,
482 2020). Different amounts of DA in various shellfish species were recorded in the Mediterranean
483 (Table 1). Ujević et al. (2010) showed that the mussel *M. galloprovincialis* in the Adriatic Sea
484 was contaminated in 2006 with DA during *Pseudo-nitzschia* spp. bloom with a maximum of
485 $6.54 \mu\text{g g}^{-1}$ meat. In the same area, but in 2009, low DA levels were measured in the cockles
486 and the clams with a maximum of $0.77 \mu\text{g g}^{-1}$ meat (Ujević et al., 2019). The variability in DA
487 concentrations could be related to the shellfish species, the abundance of toxic species and
488 strains of *Pseudo-nitzschia* during the boom period. Interestingly, in the present study, the
489 increase of DA concentrations in the cockles and the clams was preceded by an increase
490 *Pseudo-nitzschia* spp. densities (Fig. 6) which suggests the presence toxic species of *Pseudo-*
491 *nitzschia* developing in the Moroccan western Mediterranean coast.

492 4.2.2. Lipophilic Shellfish Toxins

493 The impact of LSTs on human health have been reported in the Mediterranean, specifically the
494 Thermaikos Gulf in Greece, in 2000 where this contamination caused the hospitalization of 200
495 people from a *Dinophysis* spp. bloom (Koukaras and Nikolaidis, 2004) and the other one in
496 Piemonte (northwestern Italy) where the consumption of contaminated mussels from the
497 Northern Adriatic Sea led to the intoxication of 150 people (Pistocchi et al., 2012). Other cases

498 of human illness were reported in the Tyrrhenian (Lugliè et al., 2011) and Adriatic Seas (Boni
499 et al., 1990).

500

501 4.2.2.1. LSP mouse bioassay

502 During our survey, the DSP mouse bioassay for lipophilic toxins shellfish revealed no positive
503 animals despite the presence in the water column of the toxic *D. caudata* up to 2080 cells l⁻¹
504 and of the toxic *P. lima* which produces LSTs. The same results were obtained during a 2008–
505 2009 survey in M'diq bay and Oued Laou estuary (Rijal Leblad et al., 2020). However, during
506 the period 2015–2019, the DSP mouse bioassay carried out in the same area revealed several
507 bans of shellfish harvesting because of the contamination of two mollusk species *A. tuberculata*
508 and *C. chione* by LSTs beyond the sanitary threshold (Table 2). Shellfish farms closing due to
509 LSTs were also reported several times in the Adriatic Sea (Della Loggia et al., 1993; Bernardi
510 Aubry et al., 2000; Francé and Mozetič, 2006; Marasović et al., 2007; Ninčević Gladan et al.,
511 2008).

512

513 4.2.2.2. LC-MS/MS toxin analyses

514 In our study, in M'diq bay, the OA/DTX-3 group was not detected in the cockles or the clams.
515 In contrast, this OA/DTX-3 was found two times in Djawn cockles with concentrations of 14.2
516 ng g⁻¹ meat and 18.6 ng g⁻¹ meat. In the Mediterranean Sea, OA and DTXs toxins were detected
517 in mollusks at different concentrations (Table 1). Séchet et al. (2021) evidenced the absence of
518 OA and DTXs in cells of monoclonal cultures of *D. caudata* and *D. triplos* isolated from
519 different ecosystems in Atlantic and French Mediterranean waters. Sibat et al. (2021) showed

520 the absence of OA and DTXs but the presence of PTX-2 in *D. caudata* strain isolated from the
521 French Atlantic Ocean. These authors also showed that *D. acuminata* produces OA but not
522 PTX-2. Campas et al. (2021) reported that *P. lima* from the NW Mediterranean Sea produces
523 OA and DTX-1.

524 OA and DTXs intoxications of oysters and mussels causing the closure of the shellfish breeding
525 zones occurred frequently in France (Belin et al., 2021) and Spain (Fernández et al., 2019).
526 García-Altare et al. (2016) reported that mussels (592 μg OA kg^{-1} meat) and oysters (49 μg
527 OA kg^{-1} meat) were contaminated during a bloom of *Dinophysis* spp. dominated by *D. sacculus*
528 in the Catalanian coasts in Spain. *P. lima* and *Dinophysis* spp. have been shown to be
529 responsible for high concentrations of OA (up to 1244 μg OA kg^{-1} meat) measured in oysters
530 in Sardinia, Italy. A bloom of *D. acuminata* occurred in the Thermaikos Gulf (Greece) in 2003
531 and 2004 with high amounts of OA measured in mussels around 684 ng OA g^{-1} meat and 2123
532 ng OA g^{-1} meat, respectively (Reizopoulou et al., 2008; Ciminiello et al., 2006). *Dinophysis*
533 and *Prorocentrum* genera have been related to the oyster's contamination by PTX-2 on the
534 Sardinia Coast (Italy) with concentrations up to 173 μg PTX-2 kg^{-1} meat (Mudadu et al., 2021)
535 and in mussels of the Catalanian coast (Spain) with concentrations of up to 61 μg PTX-2 kg^{-1}
536 meat (Garcia Altare et al., 2016). PTX-2 was found in cockles from Djawn on July 23rd 2018
537 suggesting that the contamination was related to the presence of *Dinophysis* spp. and *P. lima* in
538 the water column. PTX-2-sa and PTX-2-sa-epi were found in M'diq with concentrations lower
539 than those of Djawn and were present only in the cockles. The higher amounts of LSTs in
540 Djawn could be explained by the higher densities of *P. lima* and *Dinophysis* species in this area
541 when compared to M'diq (Figs. 4, 5, 7). To resume, as shown in Table 1, the quality and the
542 quantity of LSTs is clearly specific to each toxic microalgae species and strain, and shellfish
543 contamination is different in each ecosystem.

544 For the first time in the SW Mediterranean waters of Morocco, we revealed the presence of
545 YTXs in mollusks and cockles, with values reaching 10.93 ng g⁻¹ meat. The measured YTXs
546 in mollusks was probably related to the presence of *G. spinifera* and *P. reticulatum* observed
547 during our monitoring (Figs. 6 and 8). These species were shown to produce YTXs in the
548 Mediterranean (Ciminiello et al., 1997; Pinzaru et al., 2018). High amount of YTXs up to 57.9
549 mg kg⁻¹ meat exceeding the sanitary threshold were found in mussels from Mali Ston Bay in
550 Croatia (Pinzaru et al., 2018) and the French Mediterranean Sea (Amzil et al., 2008), while
551 oysters and clams showed low contamination level (Amzil et al., 2008).

552 Azaspiracids toxins were occasionally detected during our monitoring and only in cockles with
553 a maximum AZA-2 amount of 2.49 ng g⁻¹ meat. The AZA-producing species *Azadinium spp.*
554 were not identified during our monitoring. However, *Azadinium spp.* was observed on
555 November 20th 2017 in our study area, with densities of about 1000 cells l⁻¹ (INRH Monitoring).
556 Bacchiocchi et al. (2015) detected the traces of AZA-2 for the first time in the Mediterranean
557 in Italy. Percopo et al. (2013) described *Azadinium dexteroporum* as a new species in the
558 Mediterranean that can be harmful to mollusks (Giuliani et al., 2019; Rossi et al., 2017). Luo
559 et al. (2018) showed the presence of AZA-2 in *Azadinium poporum* strain isolated from Gulf
560 of Amvrakikos, Greece.

561 Our results showed that, despite the measured low concentrations of GYM-A (<4.22 ng g⁻¹
562 meat), this toxin was detected in all samples both in cockles and clams during our monitoring.
563 In our study, the producing species *K. selliformis* was recorded only five times with densities
564 not exceeding 80 cells l⁻¹. In the Gulf of Gabes (Tunisia), GYM-A reached high levels up to
565 2136 ng g⁻¹ in clams in 2009, simultaneously, with high abundances of *K. selliformis* (Ben Naila
566 et al., 2012). The other species known to produce GYM-A is *Alexandrium ostenfeldii* (Lamas
567 et al., 2021). No intoxication events related to AZAs and GYMs were documented in the

568 Mediterranean (Zingone et al., 2021). However, works on these toxins are scarce in the
569 Mediterranean and further studies are needed for the assessment of their impacts on the food
570 components and the dynamic of the producing dinoflagellate species.

571 Given the high inter- and intra-specific genetic and physiological diversity in *Dinophysis* genera
572 (Guiry, 2020), and over 200 identified species (Hallegraeff and Lucas, 1988) with 10 toxic
573 species (Séchet et al., 2021; Zingone et al., 2021), the accumulated toxin levels will depend on
574 the blooming species and recruited populations of *Dinophysis*. Séchet et al. (2021)
575 demonstrated notable variation of toxin profile and quantity in *D. caudata*, *D. tripos*, *D. acuta*,
576 and *D. acuminata*.

577 Rijal Leblad et al. (2020) showed that, contrary to *C. chione*, the cockle *A. tuberculata* showed
578 a permanent and extremely high toxicity level during the 15-month survey with concentration
579 ten times higher than the sanitary threshold (800 $\mu\text{g eq STX Kg}^{-1}$ meat). Our results showed
580 that the LSTs, including OA/DTX-3, PTXs, YTXs, AZAs, and GYM-A, were present in the
581 cockles but absent in the clams, except GYM-A. This could be due to a more efficient
582 depuration activity of the clams and/or the avoidance of the toxic species by this mollusk when
583 filtrating phytoplankton. Other physiological mechanisms such as the specific fixation of the
584 toxins or their biotransformation/detoxication could explain these observed differences (Sagou
585 et al., 2005; Takati et al., 2007; Rijal Leblad et al., 2017).

586

587 **5. Conclusion**

588 For the first time, this study evaluated LSTs accumulation in shellfish in the Moroccan western
589 Mediterranean waters using sensitive and modern methods (LC-MS/MS). The generated data
590 are necessary to understand the DSP mouse bioassay method that is used in these ecosystems.

591 Many LSTs, OA/DTX3, Pectenotoxins (PTX-2, PTX-2 sa, PTX-2 sa epi), AZA-2, YTXs, and
592 GYM-A were measured in shellfish. The cockles were much more contaminated than the
593 smooth clams which accumulated only GYM-A. The highest concentrations were those of
594 PTX-2 sa with up to 25.24 ng g⁻¹ meat of clams. The potentially toxic species responsible for
595 the highlighted LSTs namely, *Dinophysis* spp., *P. lima*, *P. reticulatum*, *G. spinifera*, and *K.*
596 *selliformis*, were identified in the water column of the surveyed ecosystems. DA was detected
597 periodically following the development of *Pseudo-nitzschia* spp.

598 In further studies, the identified toxic species producing LSTs and DA must be isolated and
599 monoclonal cultures established. This will allow ribotyping of the isolated strains and the
600 measurements of the toxins they produce to compare them with the measured toxins by LC-
601 MS/MS in mollusks. Here we showed that Moroccan Mediterranean waters could house the
602 development of emergent species with potent toxins (YTXs, AZAs, GYM-A) which could
603 threaten culture and harvesting of mollusks together with human health.

604

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612

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616

617 **Tables and figures legends**

618 Figure 1: Investigated area with the localization of sampling sites (M'diq bay, Martil, Kaa
619 Asras and Djawn) in the western Moroccan Mediterranean sea.

620

621 Figure 2: Temporal variation of temperature (a) and salinity (b) in the surveyed stations
622 (western Mediterranean, Morocco)

623

624 Figure 3: Temporal variations of nutrients ($\mu\text{mole l}^{-1}$) in the water column of the sampled
625 stations (M'diq, Martil, Kaa Asras and Djawn). a: Ammonia (NH_4^+), b: Nitrate (NO_3^-), c:
626 Nitrite (NO_2^-), d: Phosphorus (PO_4^{3-}), e: Silicates ($\text{Si}(\text{OH})_4$). *: Data not available.

627

628 Figure 4: Temporal variation of *Dinophysis* species potentially responsible of Lipophilic
629 Shellfish Poisoning in the Moroccan Mediterranean coastline, a: *Dinophysis caudata*; b:
630 *Dinophysis acuminata*; c: *Dinophysis fortii*.

631

632 Figure 5: Temporal variation of potentially toxic phytoplankton species in the studied stations
633 (M'diq bay, Martil, Kaa Asras, Djawn), Western Moroccan Mediterranean coast.

634

635 Figure 6: Temporal variations of *Pseudo-nitzschia* spp. densities (cells l⁻¹) (a) and domoic
636 acid ($\mu\text{g DA g}^{-1}$ shellfish meat) concentrations in the cockle *Acanthocardia tuberculata* (b)
637 and the smooth clam *Callista chione* (c) in M'diq bay, Martil, Kaa Asras, and Djawn
638 (Western Mediterranean sea).

639

640 Figure 7: Densities in cells l⁻¹ of potentially toxic phytoplankton species (*Dinophysis* spp.,
641 *Karenia selliformis*, *Gonyaulax spinifera*, *Protoceratium reticulatum*, *Prorocentrum lima*) in
642 M'diq (a) and Djawn (b) during the periods when the cockle *Acanthocardia tuberculata* and
643 the smooth clam *Callista chione* were harvested for toxins analyses by LC-MS/MS.

644

645 Figure 8: Lipophilic shellfish toxins amount (ng g^{-1} shellfish meat) in the cockle
646 *Acanthocardia tuberculata* (a-1: M'diq; a-2: Djawn), and in the smooth clam *Callista chione*
647 (b-1: M'diq; b-2: Djawn). Pectenotoxins (PTX-2 sa epi, PTX-2 sa, PTX-2), Azaspiracids
648 (AZA-2), Gymnodimins (GYM-A), Yessotoxins (YTX) and total Okadaic
649 acid/Dinophysistoxins (Total OA/DTX-3)

650

651 Figure 9: Canonical Correspondence Analysis ordination diagram showing the correlation
652 between harmful algae species and physicochemical parameters prevailing in the water column
653 during sampling period. Data used was from the whole survey of the four stations (M'diq bay,
654 Martil, Kaa Asras, and Djawn).

655 Table 1: Domoic acid and lipophilic toxins in shellfish, in the producer's phytoplankton
656 species and in sea water from various ecosystems in the Mediterranean Sea. To facilitate the

657 comparison of toxins amounts in different matrices and ecosystems, the units are expressed as
658 $\mu\text{g g}^{-1}$ shellfish flesh and pg cell^{-1} . * no data available.

659

660 Table 2: Bivalve mollusks harvesting ban during the period of 2015-2019 in the
661 Mediterranean Moroccan coast. Lipophilic Shellfish Toxins (LSTs) were measured using the
662 DSP mouse bioassay. Data are from RSSL (Réseau de Surveillance de Salubrité du Littoral,
663 Institut National de Recherche Scientifique). Sc and Ck refer to the smooth clam *Callista*
664 *chione* and the cockle *Acanthocardia tuberculata*, respectively.

665

666 Supplementary material

667 Table S1: Location, depth and distance from the shore of the sampling stations and the main
668 characteristics of the corresponding areas.

669 Table S-2a: Transitions and mass spectrometer parameters used for the negative ion mode
670 MRM detection of AO, DTXs and YTXs.

671 Table S-2b: Transitions and mass spectrometer parameters used for the positive ion mode
672 MRM detection of PTXs, SPXs, PnTXs, GYMs and AZAs.

673 Table S3: Values of physicochemical environmental parameters at the surveyed stations, n =
674 number of samples, Avg: Average value, Min = minimum value; Max = maximum value. T:
675 Temperature, Sal: Salinity, TN: Total Nitrogen, NH_4 : ammonia, NO_2 : nitrite, NO_3 : nitrate,
676 PO_4 : phosphorus, SiO_4 : silicate.

677

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Table 2: Bivalve mollusks harvesting ban during the period of 2015-2019 in the Mediterranean Moroccan coast. Lipophilic Shellfish Toxins (LSTs) were measured using the DSP mouse bioassay. Data are from RSSL (Réseau de Surveillance de Salubrité du Littoral, Institut National de Recherche Scientifique). Sc and Ck refer to the smooth clam *Callista chione* and the cockle *Acanthocardia tuberculata*, respectively.

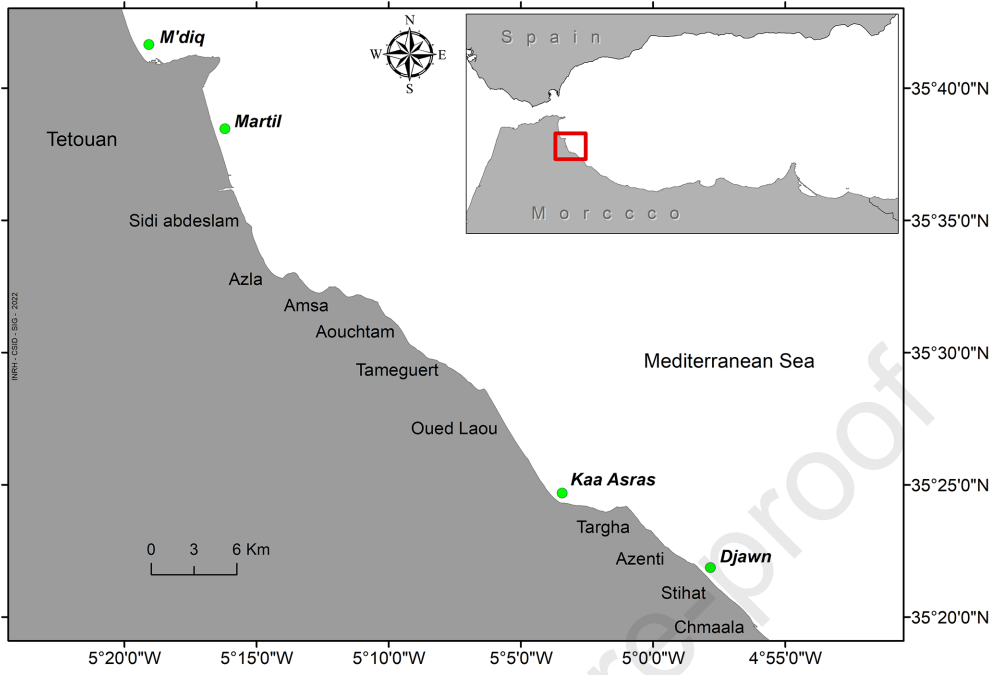
Ban zone	Bivalve species	Type of analysis	Start date of the ban	End date of the ban
Targha – Djaoun (Djawn)	Sc	LSTs	05/10/2015	19/11/2015
Oued Negro – M'diq (M'diq)	Sc	LSTs	06/10/2015	25/01/2016
Cabo negro – Martil (Martil)	Sc	LSTs	06/10/2015	25/01/2016
Cabo Negro – Martil (Martil)	Ck	LSTs	06/10/2015	19/11/2015
Oued Negro – M'diq (M'diq)	Ck	LSTs	06/10/2015	19/11/2015
Targha – Chmaâla (Djawn)	Sc	LSTs	07/05/2019	28/06/2019

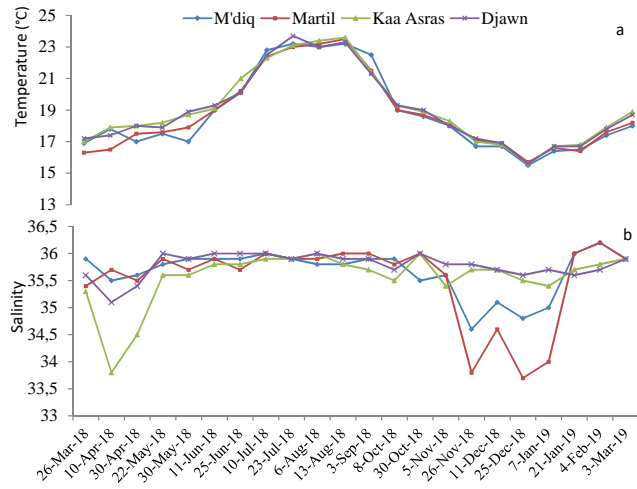
Table 1: Domoic acid and lipophilic toxins in shellfish, in the producer's phytoplankton species and in sea water from various ecosystems in the Mediterranean Sea. To facilitate the comparison of toxins amounts in different matrices and ecosystems, the units are expressed as $\mu\text{g g}^{-1}$ shellfish meat and pg cell^{-1} . * no data available.

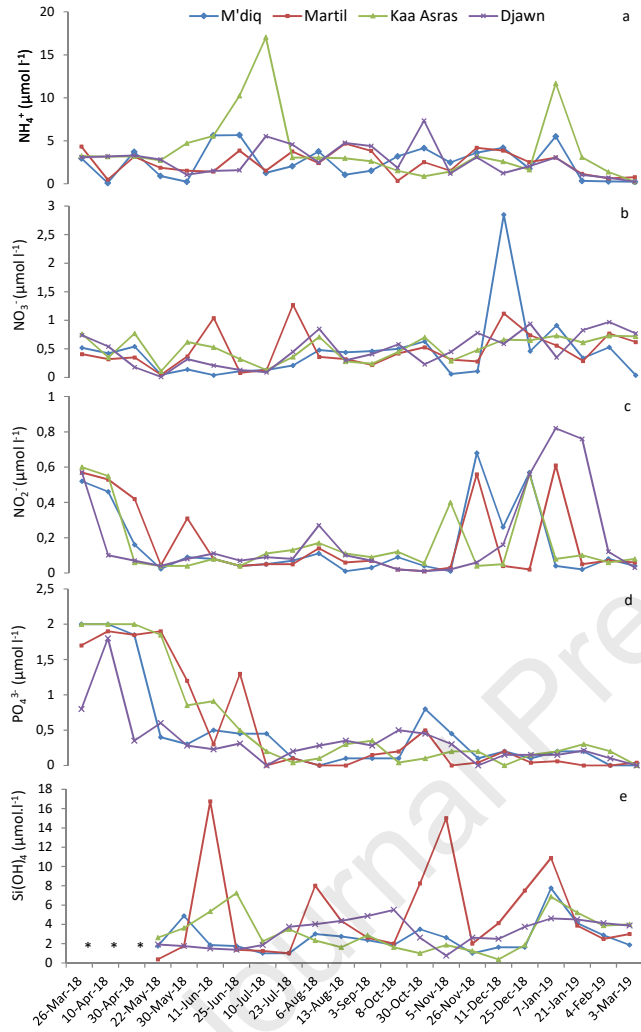
Toxins	Species	Area	Maximum abundance (cells l^{-1})	Year	Matrices	Maximum concentration	Reference
DA	<i>Pseudo-nitzschia</i>	Morocco	35×10^4	2008	<i>C. chione</i>	$16.88 \mu\text{g g}^{-1}$	Rijal Leblad et al., 2020
				2009	<i>Acanthocardia tuberculatum</i>	$4.73 \mu\text{g g}^{-1}$	
		Croatia	*	2009	<i>Acanthocardia tuberculata</i>	$0.77 \mu\text{g g}^{-1}$	Ujević et al., 2019
					<i>Callista chione</i>	$0.28 \mu\text{g g}^{-1}$	
		Tunisia	21×10^6	(2007 - 2011)	<i>Mytilus galloprovincialis</i>	$1.8 \mu\text{g g}^{-1}$	Turki et al., 2014
					<i>Crassostera gigas</i>	$2.62 \mu\text{g g}^{-1}$	
		Spain	*	2008	<i>C. chione</i>	$23.3 \mu\text{g g}^{-1}$	Papiol et al., 2013
					2010	<i>Donax trunculus</i>	
Tunisia	49×10^5	2006	Seawater	$2 \mu\text{g l}^{-1}$	Sahraoui et al. 2012		
Croatia	$>10^6$	2006	<i>M. galloprovincialis</i>	$6.5486 \mu\text{g g}^{-1}$	Ujević et al., 2010		
OA	<i>Dinophysis</i> spp & <i>Prorocentrum</i>	Italy	*	2015	<i>C. gigas</i>	$1.24 \mu\text{g g}^{-1}$	Mudadu et al., 2021
		Spain	2200	2012	<i>M. galloprovincialis</i>	$592 \times 10^3 \mu\text{g g}^{-1}$	Garcia Altares et al., 2016
	<i>C. gigas</i>				$49 \times 10^3 \mu\text{g g}^{-1}$		
	Phytoplankton cells				461 pg cell^{-1}		
	Sea water				$0.11 \mu\text{g l}^{-1}$		
	<i>P. lima</i>	Tunisia	32019×10^3	2014	Culture	$28.33 \text{ pg} \cdot \text{cell}^{-1}$	Benghabia et al., 2016
<i>Dinophysis</i> spp. <i>Prorocentrum</i> <i>P. minimum</i>	Tunisia	25×10^4 5×10^3 413	(2007 - 2011)	<i>M. galloprovincialis</i>	$1.3 \times 10^{-3} \mu\text{g g}^{-1}$	Turki et al., 2014	
				<i>C. gigas</i>	$1.3 \times 10^{-3} \mu\text{g g}^{-1}$		

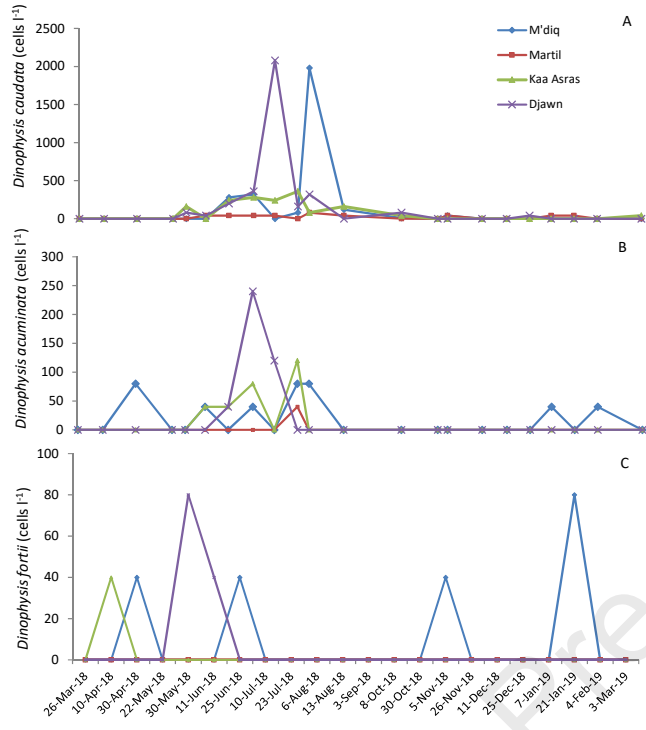
	<i>P. lima</i>	Italy	*	2002	Culture	15.89 pg cell ⁻¹	Vanucci et al., 2010
	<i>D. acuminata</i>	Greece	2200	2003	Phytoplankton cells	4.4 pg cell ⁻¹	Reizopoulou et al. 2008
			10700	2004	<i>M. galloprovincialis</i>	0.684 µg g ⁻¹	
					phytoplankton cells	14 pg cell ⁻¹	
					<i>M. galloprovincialis</i>	2.123 µg g ⁻¹	
	<i>D. acuminata</i>	Greece	*	2004	<i>M. galloprovincialis</i>	0.2 µg g ⁻¹	Ciminiello et al., 2006
	*	Italy	*	1995	<i>M. galloprovincialis</i>	1.1 µg g ⁻¹	Ciminiello et al., 1997
DTX-1	<i>P. lima</i>	Tunisia	32019x10 ³	2014	Culture	7.4 pg·cell ⁻¹	Benghabia et al., 2016
	<i>P. lima</i>	Italy	*	2002	Culture	0.39 pg·cell ⁻¹	Vanucci et al., 2010
	<i>D. sacculus</i>	Italy	2x10 ⁵	1998	Phytoplankton cells	65x10 ⁻³ pg cell ⁻¹	Giacobbe et al., 2000
PTX-2	<i>D. caudata</i>	France	*	2019	Culture	39 pg cell ⁻¹	Sechet et al., 2021
	<i>D. sacculus</i>		*	2018		113 pg cell ⁻¹	
	<i>Dinophysis</i> & <i>Prorocentrum</i>	Italy	*	2019	<i>C. gigas</i>	0.17 µg g ⁻¹ .	Mudadu et al., 2021
	<i>D. sacculus</i>	Spain	2200	2012	<i>M. galloprovincialis</i>	61x10 ⁻³ µg g ⁻¹	Garcia Altares et al., 2016
					<i>C. gigas</i>	52x10 ⁻³ µg g ⁻¹	
					Sea water	0.15 µg l ⁻¹	
					Phytoplankton cells	668 pg cell ⁻¹	
AZA-2	<i>Azadinium</i>	Greece	*	2014	Culture	2.6x10 ⁻³ pg cell ⁻¹	Luo et al., 2018
	*	Italy	*	(2012-2013)	<i>M. galloprovincialis</i>	7x10 ⁻³ µg g ⁻¹	Bacchiocchi et al., 2015
YTX	*	Croatia	*	2015	<i>M. galloprovincialis</i>	57.91 µg g ⁻¹	Pinzaru et al., 2018
	*	Italy	*	1995	<i>M. galloprovincialis</i>	1.8 µg g ⁻¹	Ciminiello et al., 1997
GYM	*	Croatia	*	2009	<i>A. tuberculata</i>	15.77x10 ⁻³ µg g ⁻¹	Ujević et al., 2019
					<i>C. chione</i>	6.14x10 ⁻³ µg g ⁻¹	
GYM-A	<i>Karenia</i>	Tunisia	3929600	2009	<i>Ruditapes decussatus</i>	2.136 µg g ⁻¹	Ben naila et al., 2012

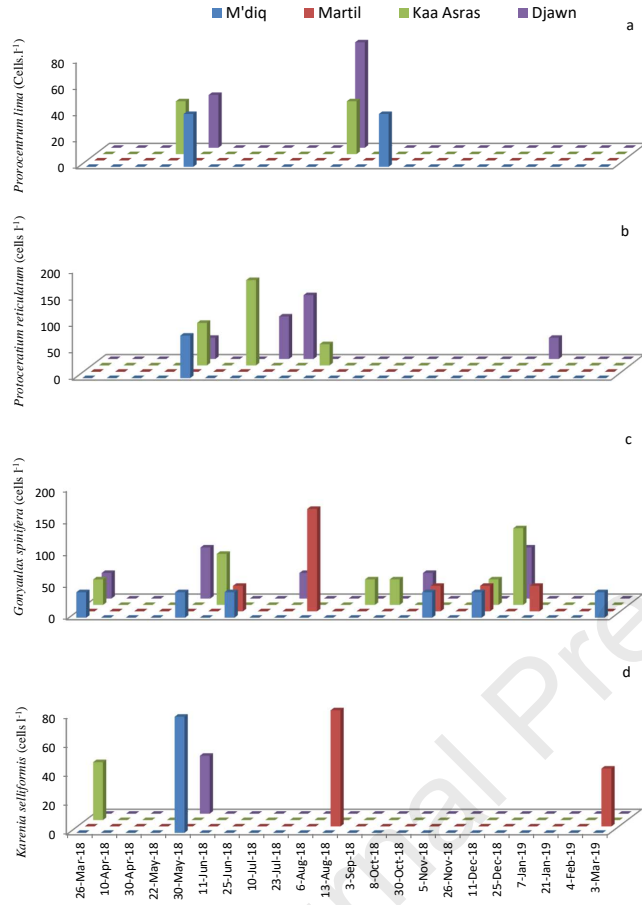
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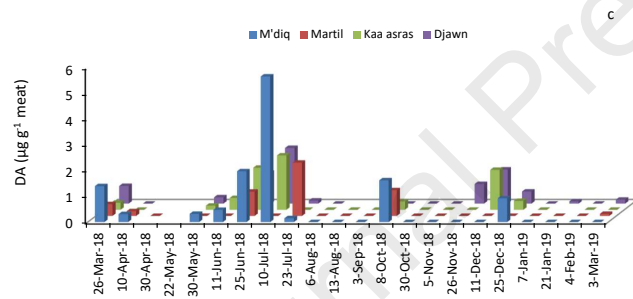
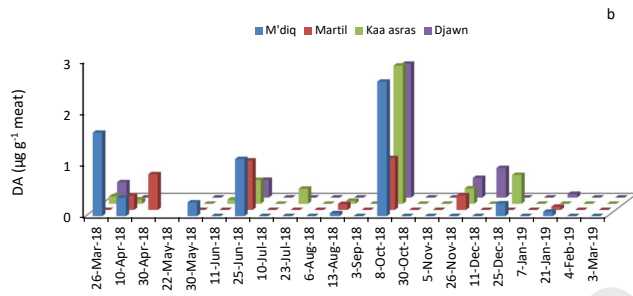
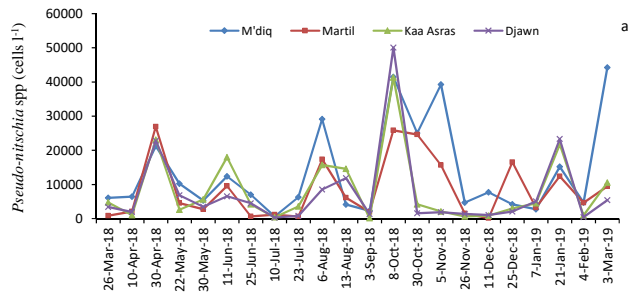


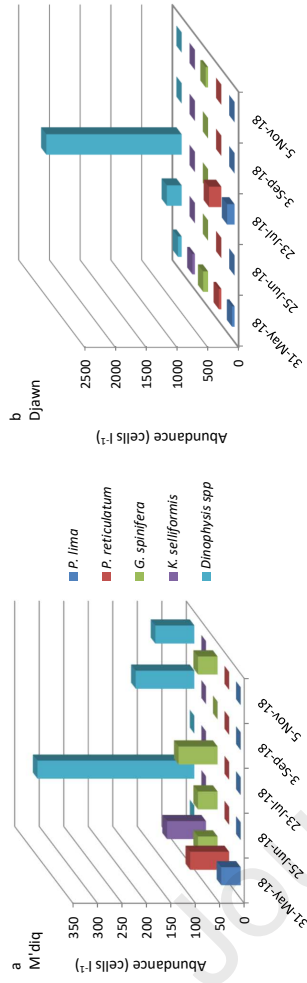


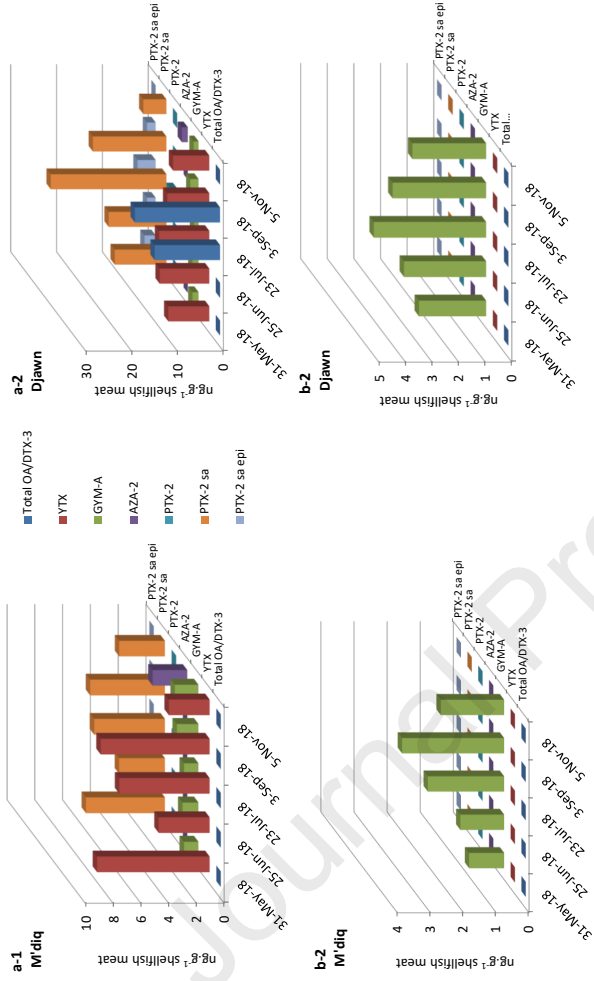


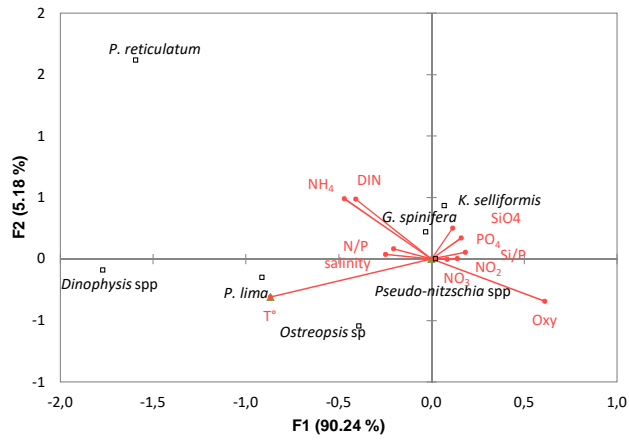












Highlights:

- Many HAB species were encountered regularly along the year southwestern Mediterranean.
- Presence of OA/DTX3, Pectenotoxins (PTX-2, PTX-2 sa, PTX-2 sa epi), AZA-2, YTXs and GYM-A in shellfish.
- Cockles were much more contaminated than the *Smooth clams* which accumulated only GYM-A.

Ethical statement:

This paper has not been published in or submitted to any other journal. Any use of animals has been subject to ethical approval prior to experimentation.

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Declaration of interests

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

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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