

1 *Alexandrium pacificum* and *Alexandrium minutum*: harmful or environmentally friendly?

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13 Abstract

14 *Alexandrium minutum* and *Alexandrium pacificum* are representatives of the dinoflagellate genus  
15 that regularly proliferate on the French coasts and other global coastlines. These harmful species  
16 may threaten shellfish harvest and human health due to their ability to synthesize neurotoxic  
17 alkaloids of the saxitoxin group. However, some dinoflagellates such as *A. minutum*, and as reported  
18 here *A. pacificum* as well, may also have a beneficial impact on the environment by producing  
19 dimethylsulfoniopropionate-DMSP, the precursor of dimethylsulfur-DMS and sulfate aerosols  
20 involved in climate balance. However, environmental conditions might influence *Alexandrium*  
21 physiology towards the production of harmful or environmentally friendly compounds. After  
22 assessing the influence of two salinity regimes (33 and 38) relative to each species origin (Atlantic  
23 French coast and Mediterranean Lagoon respectively), it appears that DMSP and toxin content was  
24 variable between the three experimented strains and that higher salinity disadvantages toxin  
25 production and tends to favor the production of the osmolytes DMSP and glycine betaine. Hence,  
26 this key metabolite production is strain and species-dependent and is influenced by environmental  
27 conditions of salinity which in turn, can diversely affect the environment. Widespread coastal blooms  
28 of *A. minutum* and *A. pacificum*, although being a risk for seafood contamination with toxins, are also  
29 a DMSP and DMS source that potentially contribute to the ecosystem structuration and climate.  
30 Regarding recent advances in DMSP biosynthesis pathway, 3 *dsyB* homologs were found in *A.*  
31 *minutum* but no homolog of the diatom sequence TpMMT.

32

33 Graphical abstract: Fig. 1

34 Keywords: DMSP, algal toxins, saxitoxin, paralytic shellfish toxins, PST, glycine betaine, dinoflagellate,  
35 phytoplankton, salinity, *dsyB* gene

36

## 37 1. Introduction

38 Similarly to land plants, algal cells have developed chemical strategies to communicate, defend and  
39 adapt to their aquatic environment. They are able to produce a range of metabolites that are  
40 involved in cell functioning, ecosystem interactions and climate regulation (Ferrer and Zimmer, 2013;  
41 Ianora et al., 2011). In this regard, some species of the cosmopolitan toxic bloom forming  
42 dinoflagellate *Alexandrium* are unique in their ability to produce two key molecules: toxins of the  
43 saxitoxin group (named hereafter STXs) and dimethylsulfoniopropionate (DMSP); (Fig. 1).

44 The saxitoxin group gather neurotoxic alkaloids with paralytic actions that may accumulate in  
45 shellfish and affect food safety for human and marine predators (Anderson et al., 2012). About 12  
46 species of *Alexandrium* are able to produce STXs. Their toxin profile and concentrations may differ  
47 among *Alexandrium* species and abiotic conditions (Caruana and Amzil, 2018). Nevertheless, the  
48 biological role of STXs and its benefit for dinoflagellate cells remain unclear. STXs production is  
49 induced by the presence of certain grazers and may provide defence properties for the dinoflagellate  
50 prey (Selander et al., 2015; Selander et al., 2006; Senft-Batoh et al., 2015). For instance, *A. minutum*  
51 appears more resistant to grazing following increased toxin content in presence of the copepod  
52 *Acartia tonsa* (Selander et al. 2006).

53 DMSP is a molecule that may influence climate via DMS emissions (Charlson et al., 1987). At the cell  
54 level, DMSP is a multifunctional compound with potential as an osmolyte, a cryoprotectant, a  
55 compound involved in thermal stress, an antioxidant, a methyl donor and an overflow metabolite for  
56 sulfur excess (Zhang et al., 2019). It is often compared to the nitrogen-containing osmolyte glycine  
57 betaine (GBT) in some other algae and plants (Keller et al., 1999). Among dinoflagellate species, *A.*  
58 *minutum*, *A. tamarense* and *A. fundyense* were reported to produce DMSP, notably in considerable  
59 amount for *A. minutum* (Caruana and Malin 2014). *A. minutum* and *A. pacificum* (previously named *A.*  
60 *catenella* regarding the Mediterranean subgroup, John et al., 2014) are two major causative agents

61 of HABs on the French coasts and other coasts worldwide (John et al. 2014, Lewis et al; 2018).  
62 However, no data were reported so far on DMSP production by *A. pacificum*.  
63 At the ecosystem level, both compounds may be part of the range of metabolites that structure  
64 relationships between marine organisms through chemical ecology (Fig. 1). For instance, in  
65 interactions such metabolites may mediate the interactions between microalgae and their predators,  
66 viruses, bacteria and parasites (Barak-Gavish et al., 2018; Evans et al., 2006; Garces et al., 2013;  
67 Johnson et al., 2016). Towards higher trophic levels such as zooplankton, fish, fish larvae, seabirds  
68 and mammals, DMS, the by-product of DMSP, is known to act as a foraging cue in the marine trophic  
69 food web (Foretich et al., 2017; Steinke et al., 2006). By contrast, some marine birds and mammals  
70 are able to change their trophic behavior to avoid STX contaminated shellfish (Ferrer and Zimmer,  
71 2013); (Fig. 1).

72 Beside their ecological implications, these two relevant metabolites have methionine as a common  
73 precursor and unclear biosynthesis pathways (Berdalet et al., 2011; Curson et al., 2018; Murray et al.,  
74 2016), (Fig. 2). A relevant question concerns the independence or the connection between these two  
75 pathways and how methionine may be used depending on cell requirements.

76 In particular, two synthesis pathways of DMSP have been described: (1) one pathway identified in  
77 the green macroalgae *Ulva intestinalis* including 4-methylthio-2-oxobutyrate (MTOB), 4-methylthio-  
78 2-hydroxybutyrate (MTHB), 4-dimethylsulfonio-2-hydroxybutyrate (DMSHB) with the intermediate  
79 DMSHB shown in several microalgae such as a diatom, a chlorophyte and a haptophyte (Gage et al.,  
80 1997), (2) a theoretical pathway proposed for the heterotrophic dinoflagellate *Cryptocodinium*  
81 *cohnii* including methylthiopropylamine (MTPA) and methylmercaptopropionic acid (MMPAA),  
82 (Uchida et al., 1996). So far, the synthesis pathway of DMSP in dinoflagellates and *Alexandrium*  
83 remains to determine. DMSP might also supply a methyl group to recycle methionine (Ishida and  
84 Kadota, 1968; Maw and Du Vigneaud, 1948); (Fig. 2).

85 Recent advances in molecular analyses have pointed out several putative genes that could be  
86 involved in DMSP metabolic pathways in diatoms, corals and bacteria and more recently in a few  
87 dinoflagellates species. The *dsyB* gene identified in the bacterium *Labrenzia aggregata* was shown to  
88 be responsible for the methylation of MTHB to DMSHB as in the “Ulva DMSP pathway” (Pathway 1,  
89 Fig.2), (Curson et al., 2017). Homologs for this gene (named *DSYB*) were found in several  
90 phytoplankton species including 26 dinoflagellates such as *Alexandrium tamarense* and *A. monilatum*  
91 and shown to be functional in *Alexandrium tamarense* (Curson et al., 2018). Another gene was also  
92 identified in the diatom *Thalassiosira pseudonana*, with the same function but encoding for a non-  
93 homologous methyltransferase (Kageyama et al., 2018).

94 For STXs, the proposed synthesis pathway includes the incorporation of methionine in the first steps  
95 of synthesis in dinoflagellates and cyanobacteria (Harlow et al., 2007b; Shimizu, 1993); (Fig. 2). The  
96 detailed model of STX biosynthesis pathway for cyanobacteria is initiated by the methylation of an  
97 acetate unit with a methyl group supplied by S-adenosyl methionine - SAM, which undergoes a  
98 condensation on arginine (D'Agostino et al., 2014). This leads to the intermediate Int-A' and further  
99 steps allow to form the various saxitoxin analogs produced by *Alexandrium* (Cho et al., 2019). The  
100 expression of genes involved in the methionine cycle (*Sam*, *Sahh*, Fig. 2) to produce SAM appears to  
101 be correlated with STXs production in *A. catenella* (Harlow et al., 2007a).

102 First, we tested whether *A. pacificum* (*A. catenella*) is a DMSP-producing dinoflagellate and thus, a  
103 potential DMS contributor. Then, we investigated how environmental conditions such as salinity  
104 conditions may influence the production of the key metabolites DMSP and STXs and may determine  
105 whether *Alexandrium* blooms might have a noxious or beneficial impact on the surrounding waters.  
106 We experimented with two species of *Alexandrium*, *A. minutum* and *A. pacificum* that proliferate on  
107 the French coasts and also major global coastlines (John et al., 2014; Lewis et al., 2018). In particular,  
108 we investigated how the salinity regime (33 and 38, relevant to each species origin), which is stated  
109 to control the osmolyte cell concentration may affect DMSP, GBT and STXs content. Additionally, for

110 improving knowledge of the DMSP biosynthesis pathway, we searched for homologs of proposed and  
111 identified genes in the available *A. minutum* transcriptome.

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## 113 2. Materials and methods

### 114 2.1 Algal cultures and salinity regimes

115 Three monoclonal strains of *Alexandrium* including *A. pacificum* IFR-ACA-15, *A. minutum* RCC2645  
116 (AM233) and *A. minutum* RCC4871 (AM1249) were grown in batch cultures under illumination of 75  
117  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  supplied by cool day light tubes (Philips) over a 12:12 light:dark cycle at 17°C  
118 temperature. The strains were cultivated in L1 medium without silicate, which is not required for  
119 dinoflagellates, (Guillard and Hargraves, 1993) prepared with filtered (0.2  $\mu\text{m}$ , Filter bottle, Corning,  
120 Amsterdam, the Netherlands) and autoclaved seawater. Two salinity regimes 38 and 33 approaching  
121 environmental salinity conditions of the French strain origins for *A. pacificum* (Thau Lagoon,  
122 Mediterranean Sea, October 2015) and *A. minutum* (Bay of Concarneau, Atlantic Ocean, 2010 for  
123 strain RCC2645 and Bay of Brest 2013 for strain RCC4871, (Le Gac et al., 2016)) respectively. These  
124 two salinity conditions were set up by preparing media with natural seawater of salinity 38 (collected  
125 from the Mediterranean Sea) diluted with Milli-Q water, checked for pH (7.84) and supplemented  
126 with nutrients. The Mediterranean seawater was naturally at salinity 38 and allowed to have the  
127 same water background for all strains. Strains were maintained during 10 weeks in these two salinity  
128 conditions prior to the experiment to prevent any salinity shock.

### 129 2.2 Experimental design

130 Triplicate batch cultures of each strain were inoculated at a cell density of 9000 cell  $\text{ml}^{-1}$  in 500 ml  
131 conical flasks filled with medium to reach 300 ml of culture volume. After 9 days, growing cultures of  
132 cell density that ranged between  $2.0 \times 10^4$  and  $3.9 \times 10^4$  cell  $\text{ml}^{-1}$  were sampled for cell densities,  
133 total cell volume (CV), DMSP, GBT and toxin measurements to characterize their metabolite content  
134 at the two salinity regimes tested. Growths over 9 days for the three *Alexandrium* strains  
135 (supplementary data, Fig. S1) were similar at salinity 33 and 38 (ANOVA test,  $P > 0.05$ ) allowing the  
136 comparison of metabolite production between the two salinity regimes. For metabolite analyses,  
137 culture samples were centrifuged (3000 G, 10 min, 4°C, centrifuge 3-18K, Sigma, Osterode am Harz,

138 Germany) and cell pellets were stored at -80°C until further chemical analyses. Cell densities and  
139 total cell volumes were determined on fresh samples using a particle counter (Coulter Multisizer 3,  
140 Beckman, Villepinte, France). Growth rates on the day of sampling were calculated for each strain,  
141 based on the formula  $\mu = \ln(N_2/N_1)/(t_2 - t_1)$  where  $N_2$  and  $N_1$  were respectively, day 9 and day 6  
142 (Supplementary data, Table S2).

### 143 **2.3 DMSP and GBT measurements by LC-MS/MS**

144 Metabolites were extracted from algal cell pellets in ice by methanol addition (1ml) and sonication  
145 (30 min, Branson Ultrasonic Cleaner 2510EDTH, Branson, Eemnes, Netherlands). Supernatant was  
146 collected after centrifugation (3,600 G, 5 min, 4°C; Centrifuge 3-18K, Sigma, Osterode am Harz,  
147 Germany) and filtration (0.2 µm membrane filters, Nanosep, Pall, Saint-Germain-En-Laye, France)  
148 then stored at -20°C until analyses by LC-MS/MS. Analyses of dimethylsulfoniopropionate (DMSP),  
149 glycine betaine (GBT) were performed on a LC System (model UFLC XR, Shimadzu, Marne-La-Vallée,  
150 France) coupled to a triple-quadrupole mass spectrometer (4000Qtrap, ABSciex, Les Ulis, France).  
151 Chromatographic system was equipped with a Hypersil GOLD HILIC column (150 × 2.1 mm, 3µm,  
152 ThermoScientific, Thermo Fisher Scientific, Whaltam, USA) with a guard column (10 × 2.1 mm, 3 µm),  
153 based on Curson *et al.*, (2018). A binary mobile phase was used, 10% aqueous acetonitrile containing  
154 4.5 mM ammonium formate (phase A) and 95% aqueous acetonitrile containing 5mM ammonium  
155 formate (phase B). The flow rate was 0.25 ml min<sup>-1</sup> and injection volume was 5 µl. The column and  
156 sample temperatures were 30°C and 4°C, respectively. An elution gradient was employed, starting  
157 with 90% B during 1 min, falling to 45% B over 7 min, held for 4 min, then increased to 90% B in 0.1  
158 min and held for 5 min to equilibrate the system. The LC-MS/MS system was used in positive  
159 ionization and multiple reaction monitoring (MRM) mode, with the two following transitions per  
160 compound: DMSP  $m/z$  134.9→73.1 and 134.9→63.1; GBT  $m/z$  118.1→58.0 and 118.1→59.0. The  
161 most intense transition, first transition mentioned for each compound, was used to quantify  
162 compounds. Compounds were quantified against their standards (Sigma Aldrich, Saint-Quentin-



163 Fallavier, France) prepared in methanol to cover calibration levels from 10nM to 5000 nM. Values are  
164 expressed in fmol cell<sup>-1</sup>, in mM of CV, in pg cell<sup>-1</sup> and in g L<sup>-1</sup> of CV.

#### 165 **2.4 Toxin measurements by LC/FLD**

166 For STXs extraction, defrost cell pellets were suspended in 1 ml of 0.1 N acetic acid. To release toxins,  
167 cells were lysed by grinding with 250 mg glass beads (150 µm, VWR, France) in a mixer mill (Retsch  
168 MM400, Haan, Germany) for 30 min at 30 Hz. Then, lysates were centrifuged (17000 G, 10 min, 4 °C;  
169 centrifuge 3-18K, Sigma, Osterode am Harz, Germany) and supernatants filtered through a 0.2 µm  
170 inert filter (Nanosep, Pall, Saint-Germain-En-Laye, France). Then samples were analyzed or stored at -  
171 20°C until analyses. Toxin analyses are based on the Post-Column Oxidation method (PCOX) (Van de  
172 Riet et al., 2009) and was performed using the analytical system LC/FLD Agilent 1200 series (Agilent  
173 Technologies, Massy, France). Two groups of toxins were separated by reversed-phase  
174 chromatography using two different columns. A C18 column (Zorbax Bonus RP, 150 × 4.6 mm,  
175 3.5 µm) was filled with a step gradient of a heptane sulfonic acid/ phosphoric acid buffer system and  
176 acetonitrile for the analysis of GTXs, dc-GTXs, dc-STXs and STX. A C8 column (BetaBasic, 8.5 µm, 250 ×  
177 4.6 mm) with an isocratic tetrabutylammonium phosphate buffer system and acetonitrile was used  
178 for the C toxins (analytical conditions are detailed in supplementary data, Table S3). A derivation of  
179 toxins was carried out by post-column oxidation with a phosphoric acid/ periodic acid buffer solution  
180 at 85°C. This oxidized eluent was acidified using nitric acid, and the derivatives were detected by  
181 fluorescence (excitation: 330 nm, emission: 395 nm). Toxin concentrations were calculated based on  
182 standard curves performed using certified reference standards obtained from CNRC (Halifax, Nova  
183 Scotia). Concentrations of the following STX variants: STX, C1, C2, GTX1, GTX2, GTX3, GTX4, GTX5 and  
184 dc-GTX2 are displayed in fmol cell<sup>-1</sup>, in mM of CV, in pg cell<sup>-1</sup> and in g L<sup>-1</sup> of CV.

#### 185 **2.5 Search for DMSP key genes in *A. minutum* reference transcriptome**

186 Several proteins and protein candidates were reported to be involved in the “pathway 1” of DMSP  
187 biosynthesis in several phytoplankton and bacterial species. In the diatom *Fragilariopsis cylindrus*, AT

188 alt, REDOX alt, SAMmt (JGI accession numbers 273803, 173405 and 207357) were identified as  
189 candidates for the transaminase, reductase and methyltransferase respectively. The proteins  
190 DECARB and DIDECARB (JGI accession numbers 238865, 263016) could encode for the decarboxylase  
191 oxydative (Lyon et al., 2011). In the diatom *Thalassiosira pseudonana*, the protein TpMMT was  
192 identified as encoding for the methyltransferase that converts MTHB to DMSHB (NCBI accession  
193 number XP\_002291473, Kageyama *et al.*, 2018). In the bacteria *Labrenzia aggregata* strain LZB033,  
194 the protein DsyB (NCBI accession number KT989543) was also identified as a methyltransferase that  
195 converts MTHB to DMSHB (Curson et al., 2017; Curson et al., 2018); (Fig. 2). For all these proteins a  
196 TBLASTN homology analysis (Altschul et al., 1997) with a threshold set at E values  $< 1e^{-10}$ , was  
197 performed against the reference transcriptome of *A. minutum* (Le Gac et al. 2016, DOI:  
198 10.1111/mec.13815) to identify transcript homologs for DMSP key genes.

## 199 **2.6 Statistical analyses**

200 The effects of salinity regimes were assessed by performing statistical analyses using Statgraphics  
201 software version 18. Data were assessed for normal distribution by performing the Normality test  
202 Shapiro-Wilk. Then, data of DMSP per cell, GBT per cell and STXs per cell that followed a normal  
203 distribution were submitted to an analysis of variance ANOVA. The ANOVA includes the factors  
204 salinity and strain to identify a putative effect of this two factors on the variables measured. Data  
205 that did not follow a normal distribution (DMSP per cell volume and cell volume) were analyzed using  
206 the non-parametric tests Kruskal-Wallis and Mann-Whitney. Significant effect was obtained and  
207 reported when  $P < 0.05$ .

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### 211 3. Results

212 The three *Alexandrium* strains present significant different cell volume (Kruskal-Wallis test,  $P < 0.01$ ),  
213 in particular *A. pacificum* has a two-fold higher cell volume than *A. minutum* (Table S2). The strain *A.*  
214 *pacificum* IFR-ACA-15 shows no significant difference in cell volume between the two salinity  
215 regimes, though higher cell volume was observed at high salinity for the two *A. minutum* strains  
216 (Mann-Whitney test,  $P < 0.05$ ).

217 The three experimented *Alexandrium* strains are able to produce DMSP (Figs 3A and B). There is  
218 significant difference in DMSP production per cell between strains and between salinity regimes  
219 (ANOVA,  $P < 0.05$ ; Fig. 3A). For instance at salinity 33, *A. minutum* RCC2645 contains higher DMSP  
220 concentration per cell ( $132.1 \text{ fmol cell}^{-1}$ ) than *A. pacificum* IFR-ACA-15 ( $41.2 \text{ fmol cell}^{-1}$ ) and *A.*  
221 *minutum* RCC4871 ( $18.1 \text{ fmol cell}^{-1}$ ), (Fig. 3A). However, due to the two-fold higher cell volume of *A.*  
222 *pacificum* (Table S2), DMSP concentration per cell volume at salinity 33 is rather equivalent for *A.*  
223 *pacificum* IFR-ACA-15 and *A. minutum* RCC4871 ( $4.7\text{-}5.0 \text{ mM per total cell volume - CV}$ ) and higher  
224 for *A. minutum* RCC2645 ( $34.0 \text{ mM per CV}$ ), (Fig. 3B). Nevertheless, the differences in DMSP per CV  
225 are significant between strains (Kruskal-Wallis  $P < 0.01$ ) but not significant between the two salinity  
226 regimes tested (Mann-Whitney test,  $P > 0.05$ ). DMSP concentrations are 1.3 to 2.2-fold higher at  
227 salinity 38 than 33 for the strains *A. pacificum* IFR-ACA-15 and *A. minutum* RCC2645 reaching at  
228 salinity 38,  $89.5$  and  $171.6 \text{ fmol cell}^{-1}$  respectively (or  $10.9$  and  $38.6 \text{ mM per CV}$  respectively) while it  
229 remains roughly stable for the strain *A. minutum* RCC4871 (Figs. 3A and B).

230 Glycine betaine (GBT) is detected in *A. pacificum* IFR-ACA-15 at both salinities and in *A. minutum*  
231 RCC2645 at salinity 38. In these two strains, GBT concentrations follow the same trend as DMSP  
232 concentrations among salinity regimes, but in 1 to 3 orders of magnitude lower concentrations per  
233 cell or CV (Figs. 3C and D). GBT concentrations per cell and per cell volume are significantly higher at  
234 salinity 38 than 33 (ANOVA test,  $P < 0.05$ ). At salinity 38, GBT concentrations reach  $3.3 \text{ fmol cell}^{-1}$  and

235 0.5 fmol cell<sup>-1</sup> for *A. pacificum* IFR-ACA-15 and *A. minutum* RCC2645 respectively or 0.4 and 0.1 mM  
236 per cell volume.

237 DMSP and STXs are produced in the same order of magnitude in *Alexandrium* cells. DMSP values  
238 extended in the following ranges of 2-23 pg cell<sup>-1</sup>, 16-172 fmol cell<sup>-1</sup> and 4-39 mM per cell volume  
239 (Figs 3A, B and S4A, C) and STXs values such as 6-14 pg cell<sup>-1</sup>, 15-31 fmol cell<sup>-1</sup> and 3-5 mM per cell  
240 volume (Figs. 3E and F, S4B, D). The strain *A. minutum* RCC2645 does not produce the targeted STXs.  
241 Significant difference in STXs concentrations per cell and per cell volume is obtained between species  
242 (ANOVA tests, P<0.01) and between salinity regimes (ANOVA tests, P<0.05). The strain *A. pacificum*  
243 IFR-ACA-15 produces higher STXs content per cell (31.3 and 23.9 fmol cell<sup>-1</sup> at salinity 33 and 38  
244 respectively or 13.9 and 10.5 pg cell<sup>-1</sup>) than *A. minutum* RCC4871 (20.3 and 15.0 fmol cell<sup>-1</sup> at salinity  
245 33 and 38 respectively or 8.5 and 6.3 pg cell<sup>-1</sup>), (Fig. 3E). However, the opposite trend is observed  
246 when expressed per cell volume with higher STXs concentrations per CV in *A. minutum* RCC4871 (5.5  
247 and 3.9 mM at salinity 33 and 38 respectively or 2.33 and 1.62 g L<sup>-1</sup> of CV) than in *A. pacificum* IFR-  
248 ACA-15 (3.7 and 2.9 mM at salinity 33 and 38 or 1.64 and 1.27 g L<sup>-1</sup> of CV), (Fig. 3F). The toxin content  
249 is significantly lower at salinity 38 than 33 for the two toxic strains *A. pacificum* IFR-ACA-15 and *A.*  
250 *minutum* RCC4871 (Fig. 3E and F).

251 The toxin profile of *A. pacificum* IFR-ACA-15 is composed of the following variants in descending  
252 order of abundance: C2, GTX5, GTX4, C1, GTX3, GTX1, dcGTX3, GTX2, STX (Fig. 4A and C). The toxin  
253 profile of *A. minutum* RCC4871 is composed of GTX3, C2, C1, GTX2, dcGTX3 in descending order of  
254 abundance (Fig. 4B and D). The toxin profile show minor variations between the two salinity regimes  
255 either expressed per mol or per g (Fig. 5 and S5). At salinity 38, the proportion of the variant C2 is  
256 lower (8% in fmol cell<sup>-1</sup>, 6% in pg cell<sup>-1</sup>) in favor of GTX5 and GTX4 (7 and 1% increase respectively in  
257 fmol cell<sup>-1</sup> and 6 and 2% increase respectively in pg cell<sup>-1</sup>) in *A. pacificum* IFR-ACA-15. Similarly for *A.*  
258 *minutum* RCC4871, C2 and GTX3 proportions are lower at salinity 38 (5 and 3% decrease respectively

259 in fmol cell<sup>-1</sup> and 6% and 4% decrease respectively in pg cell<sup>-1</sup>) to the benefit of C1 and GTX2 (3 and  
260 6% increase in fmol cell<sup>-1</sup> and 4% and 6% respectively in pg cell<sup>-1</sup>, Fig.5 and Fig S5).

261 No homologs of the five proteins (AT, REDOX, SAMmt, DECARB and DIDECARB) proposed in the  
262 diatom *Fragilariopsis cylindrus* (Lyon et al., 2011) for accomplishing the four steps of DMSP synthesis  
263 (pathway 1, Fig. 2) is found in *A. minutum* reference transcriptome. Similarly, no homolog for the  
264 TpMMT protein from the diatom *Thalassiosira pseudonana* is found in *A. minutum*. Nevertheless,  
265 three transcripts identified in *A. minutum* display homology with the DSYB protein previously  
266 identified in some dinoflagellates including *A. tamarense* (Table 1).

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268

#### 269 4. Discussion

270 The species *A. pacificum* (formerly called *A. catenella* for the Mediterranean strains) is shown for the  
271 first time to produce DMSP. This species appears to contain low DMSP concentrations in comparison  
272 with other dinoflagellate representatives (Caruana and Malin, 2014). However, it is a bloom forming  
273 species with a large global distribution (John et al., 2014; Lilly et al., 2002) that have the potential to  
274 contribute to the DMS load in the field. The two French strains of *A. minutum* also produce DMSP as  
275 previously shown for this species (Berdalet et al., 2011; Caruana et al., 2012; Deschaseaux et al.,  
276 2019; Jean et al., 2005), though at lower concentrations in our assays (0.02-0.13 pmol cell<sup>-1</sup> in  
277 comparison with 0.4-1.6 pmol cell<sup>-1</sup> in other laboratory experiments and 14 pmol cell<sup>-1</sup> from field  
278 sample, Table 2). The DMSP content is variable between strains and species as this has been  
279 previously highlighted for many dinoflagellates species (Caruana and Malin, 2014). For instance  
280 DMSP content was reported to extend in *Symbiodinium* cultures from 0.03 to 0.32 pmol cell<sup>-1</sup> and  
281 from 0.04 to 1.96 in *Symbiodinium* cultures and coral extracts, 169-600 mM in *Scripsiella trochidea*,  
282 57-377 mM in *Amphidinium carterae* (Caruana and Malin, 2014) and from 3.6 to 18.9 pmol cell<sup>-1</sup> or  
283 50-242 mM in the coccolithophore *Emiliana huxleyii* (Steinke et al., 1998). The variability in DMSP  
284 content observed for *A. minutum* potentially results from their genetic and phenotypic diversity,  
285 their geographic origin as well as their physiological state and potentially growth rate, both resulting  
286 from their culture conditions (medium, light cycle, light intensity, temperature, salinity as described  
287 in Table 2). Also, field sampling has led to outstanding DMSP value potentially resulting from  
288 environmental conditions that are more complex in coastal seawater than in controlled laboratory  
289 cultures and from different sample preparations that might introduce a bias in DMSP measurement  
290 (Caruana and Malin, 2014; Jean et al., 2005). Consequently, the investigation towards a higher  
291 number of strains is necessary to give an accurate description of the intraspecific variability that may  
292 exist in the capacity of DMSP production by *A. pacificum* and *A. minutum* and thus, the potential  
293 contribution of these species to the DMS input in the marine environment. Since *A. minutum* and *A.*  
294 *pacificum* are able to produce DMSP, as well as *A. tamarense* and *A. fundyense* (Caruana and Malin,

295 2014), and according to the presence of DMSP genes (*DSYB*) in *A. monilatum* (Curson et al., 2018),  
296 further studies would allow to determine whether this DMSP production capacity is widespread to all  
297 representatives of the *Alexandrium* genus. Therefore, screening for a larger set of *Alexandrium*  
298 species would give a better view of the interspecific variability in DMSP production and the DMS load  
299 of *Alexandrium* group to the field.

300 Besides, we showed in our study that several homologs of transcripts for the *DSYB* gene are also  
301 present in *A. minutum*. Thus, the MTHB-DMSHB conversion step in the “Ulva pathway” (Pathway 1,  
302 Fig. 2) for DMSP synthesis exists and is potentially utilized for DMSP production in *A. minutum*.  
303 Nevertheless, these results do not exclude the other putative DMSP biosynthesis pathway described  
304 by Uchida *et al.* (1996), (Fig. 2). Furthermore, the absence of diatom genes for DMSP synthesis (as  
305 proposed by Lyon *et al.* 2011 or evidenced by Kageyama *et al.* (2018) in *Alexandrium* suggests that  
306 *Alexandrium* possesses a distinctive DMSP pathway from certain diatoms, while a few other diatoms  
307 also possess *DSYB* gene (Curson et al., 2018).

308 Intracellular DMSP concentration in microalgae may depend on environmental conditions (Stefels,  
309 2000; Stefels et al., 2007). Thus, essentially recognized as an osmolyte, intracellular DMSP  
310 concentrations may vary with salt water concentrations (Kirst, 1996; Reed, 1983; Speeckaert et al.,  
311 2019; Yang et al., 2011). GBT is another osmolyte produced by plants and some phytoplankton  
312 species. Little information available on GBT in dinoflagellates presents GBT as absent or scarce in this  
313 phytoplankton group. It was reported to be produced in some dinoflagellate species such as  
314 *Amphidinium carterae* (Keller et al., 1999), some *Symbiodinium* sp (Yancey et al., 2010) and  
315 *Prorocentrum minimum* (Gebser and Pohnert, 2013). In our study, *Alexandrium pacificum* and *A.*  
316 *minutum* also produce GBT but in much lower amount than DMSP. The osmolyte activity of these  
317 two compounds is supported here by an increase in concentrations in high salinity conditions for two  
318 of the three strains tested. The other strain *A. minutum* RCC4871 does not respond as a strain being  
319 in a higher saline environment in terms of DMSP and GBT concentrations, suggesting that this strain

320 is not sensitive to this salinity interval (5 units) or that it might produce other osmolytes not searched  
321 here. Indeed, other substances such as trigonelline, gonyol, dimethylsulfonioacetate or  
322 trimethylammonium propionate have been proposed and occurred in the dinoflagellate  
323 *Prorocentrum minimum* (Gebser and Pohnert, 2013). Osmolytes may help phytoplankton cells to  
324 respond to hyperosmotic conditions of high salinity regime and maintain their cell volume (Kirst,  
325 1996). Indeed, in condition of higher salinity, water outflow might lead to a decrease in cell volume.  
326 However, the three strains experimented here does not show a decrease in cell volume which  
327 implies that their cell volume is not affected by the higher salinity regime, and suggests that  
328 osmolytes may compensate for the hyperosmotic conditions.

329 The toxin profile of *A. pacificum* may vary among cells within a population (based on variations  
330 reported among monoclonal strains isolated from a unique bloom) and between populations from  
331 different geographic origins (Laabir et al., 2013). In close culture conditions, the toxin profile of *A.*  
332 *pacificum* IFR-ACA-15 (isolated in 2015 in Thau Lagoon, France) mainly dominated by C2 > GTX5 >  
333 GTX4 approaches the one of strain ACT03 (isolated in 2003 in the same location) composed in  
334 majority by GTX5 > C2 > GTX4 (18°C and 35-40 salinity; Laabir et al., 2013), however with different  
335 proportions. The toxin analogs C2, GTX5, GTX4 represents 66%, 18% and 10% respectively of total  
336 STXs in *A. pacificum* IFR-ACA-15 cells at salinity 33 and 23%, 13% and 58% respectively in *A. pacificum*  
337 ACT03 cells at salinity 35. Minority toxin variants differs in *A. pacificum* IFR-ACA-15 with the absence  
338 of C3, C4 and the detection of GTX1, STX, GTX2 and dc-GTX3 that were not reported in *A. pacificum*  
339 ACT03.

340 At elevated salinity regime in our assays, the toxin content is lower for *A. pacificum* and *A. minutum*.  
341 Similarly, Grzebyk et al. (2003) observed in *A. minutum* cultures that reducing salinity led to an  
342 increase in toxin content. Also, Lim and Ogata (2005) found that higher salinity conditions tend to  
343 reduce toxin content in three species of *Alexandrium* (*A. minutum*, *A. tamarense* and *A.*  
344 *tamiyavanichii*) but tend to increase in *A. peruvianum*. Therefore, different responses to salinity



345 variations may be observed for other strains and species of *Alexandrium*. For instance, *A. pacificum*  
346 ACT03 produced higher toxin concentrations in higher salinity medium from 10 to 40 and generally  
347 from 35 to 40 as well (Laabir et al., 2013). The Chilean strains *A. fundyense* (*A. catenella* group I)  
348 show various responses of higher or lower STX content with high or low salinity conditions. For  
349 instance, strain PFB38 cultivated at 10°C contained higher toxin content at salinity 35 rather than at  
350 salinity 15 while the opposite occurred at 15°C (Aguilera-Belmonte et al., 2013). The investigation of  
351 salinity influence associated with temperature variations or other environmental parameters would  
352 help in understanding the diverse responses of *Alexandrium* toxin content. Furthermore, testing  
353 short-term variations in salinity is necessary to assess how cells would respond to rapid  
354 environmental changes.

355 The two salinity regimes also led to minor variations in toxin profiles. In particular at salinity 38 the  
356 variant C2 and GTX3 are slightly compensated by their respective epimers C1 and GTX2 in *A.*  
357 *minutum* cells and the di-sulfated variant C2 is reduced to the benefit of mono-sulfated variants  
358 GTX4 and GTX5 in *A. pacificum* cells. These changes in *A. pacificum* toxin profile associated with the  
359 lower toxin content tend to save some S atoms and would theoretically allow some S deviation  
360 towards other molecules such as DMSP and proteins for instance, required for maintaining cell  
361 homeostasis at salinity 38. Similarly, Laabir *et al.* 2013 observed for *A. pacificum* ACT03 an increase in  
362 GTX4 variant at salinity 40 in comparison to salinity 35 when grown at 18°C and that GTX4 was  
363 dominant at higher salinities (30-40 at 18-30°C and 10-40 at 21°C) and temperatures (18-30°C). Also  
364 the toxin variant C2 was unique to predominant in the toxin profile at lower temperatures (12 and  
365 18°C) and lower salinities (10-25 at 18°C and 27°C) and appears minority at higher salinities (Laabir *et*  
366 *al.* 2013). Therefore, the toxin profile and the associated toxicity of *A. pacificum* may be altered by  
367 the salinity regime, as particularly observed for the C2 and the more toxic GTX4 analogs.

368 Both metabolites, DMSP and STXs, are synthesized in a close range of values. DMSP and STXs  
369 contents may vary with salinity conditions. It appears that salinity regime may exert an antagonistic

370 effect on DMSP and STXs content of *A. pacificum* IFR-ACA-15. However, it is not yet clear whether it  
371 is a direct relationship between DMSP and STXs synthesis pathway or an indirect response to the  
372 salinity conditions.

373 In Thau Lagoon (South of France, Mediterranean Sea), *A. pacificum* may form recurrent blooms in  
374 spring or preferentially in autumn (Genovesi et al., 2011) in water of which salinity reaches 38 in  
375 average and may extend between 31 and 41 (for the periods of October-November 2011-2019,  
376 (REPHY – French Observation and Monitoring program for Phytoplankton and Hydrology in coastal  
377 waters, 2019). According to our results, such environmental conditions might enhance the DMSP  
378 production capacity of *A. pacificum* and moderate the toxin production capacity. However, increase  
379 in salinity up to 40 might also lead to an increase in toxin production of *A. pacificum* in several  
380 temperature conditions (18, 27, 30°C), (Laabir et al. 2013). Furthermore, the presence of other  
381 phytoplankton species potentially producing DMSP or interacting with *Alexandrium*, as well as other  
382 organisms of the bacterio-zooplankton component might influence the DMSP load in this ecosystem  
383 (Stefels et al., 2007). Consequently, more data on the influence of other abiotic and biotic factors  
384 would help in describing *Alexandrium* behavior in its natural environment. Also, direct measurements  
385 of DMSP in *A. pacificum* blooms are necessary to assess the DMSP concentration that may be  
386 accumulated in Thau Lagoon waters as this dinoflagellate may reach  $10^6$  to  $10^7$  cell L<sup>-1</sup> (Genovesi et  
387 al., 2011). This species is also known to form blooms in other coastal areas of the globe as for  
388 instance in Asian coasts of the North Pacific Ocean, Australian and New Zealand coasts of the South  
389 Pacific and Antarctic Ocean (John et al., 2014). Therefore, the global contribution of *A. pacificum* in  
390 terms of DMSP and climate equilibrium could not be negligible and deserves further scientific  
391 investigation.

392 In the French Atlantic and English Channel coast, *A. minutum* potentially form blooms in spring and  
393 summer periods in water of salinity that mainly ranges between 30 and 35 (Guallar et al., 2017).  
394 Based on our results, these conditions may promote toxin production and support mild DMSP

395 production, though also depending on other environmental conditions. This species have a global  
396 distribution (Lewis et al., 2018) and may also contribute to the DMS load in the ocean and the  
397 atmosphere.

398

## 399 **5. Conclusion**

400 *Alexandrium pacificum* and *A. minutum* are pointed out as blooming toxic species that may impact  
401 seafood harvest and commercializing to protect human health, however these phytoplankton may  
402 also have a positive impact on climate regulation via DMSP and DMS production. Depending on their  
403 environmental conditions and cell requirements, *Alexandrium* blooms may have noxious or beneficial  
404 impacts on the ecosystem, human health and climate. On the French coasts, the ecological niches of  
405 *A. minutum* on the Atlantic side and *A. pacificum* in the Thau Lagoon on the Mediterranean side are  
406 likely to differently condition DMSP and STXs productions in phytoplankton cells. In particular, the  
407 salinity regime appears to affect DMSP content and STX content in two *Alexandrium* strains over  
408 three tested strains. Therefore, the DMS load in the field may vary with bloom species composition,  
409 species physiology and environmental characteristics of bloom location. Such variation in DMS  
410 concentrations in seawater would be further described by direct field measurements during  
411 *Alexandrium* blooms.

412 Since DMSP and STX appear to have an antagonistic response to salinity condition in *A. pacificum* IFR-  
413 ACA-15 and due to slight changes in STXs ratio with salinity regime, further studies are necessary to  
414 verify the assumption of a potential trade-off between the use of methionine and the use of S atoms  
415 in DMSP and/or STX synthesis pathways.

416 Otherwise, the role of DMSP and the underlying molecular processes that governs DMSP production  
417 in dinoflagellates are not yet fully elucidated. DMSP acts obviously as an osmolyte in some strains of  
418 *Alexandrium*. Nevertheless, the cell homeostasis under high salinity regime is maintained by a pool of  
419 osmolyte compounds including among other potential compounds, GBT, though in much lower

420 concentrations. Since *DSYB* gene was shown to be present and expressed in *A. minutum*, allowing the  
421 existence of the “*Ulva* pathway”, it remains to assess the functionality of this gene and to clarify  
422 whether the alternative biosynthesis pathway (“*Cryptocodinium cohnii* pathway”) also exists or not  
423 in dinoflagellates. Nevertheless, DMSP synthesis in dinoflagellates is distinguished from its synthesis  
424 in diatoms by the use of alternative genes.

425 Finally, scientists launched a distress call on the crucial role of microorganisms in climate change and  
426 under climate change and the sustainability of our planet (Cavicchioli et al., 2019). In this way, we  
427 encourage further studies to better understand the conditions and processes that influence the  
428 production of harmful or environmentally friendly compounds from *Alexandrium*. This refers to the  
429 inter- and intra-specific variability in DMSP and STXs production within *Alexandrium* genus, as well as  
430 external stressors that may impact cell physiology and affect the production of these key  
431 compounds, internal molecular processes that regulate their cellular production and release as well  
432 as field investigation of *Alexandrium* blooms.

433

434

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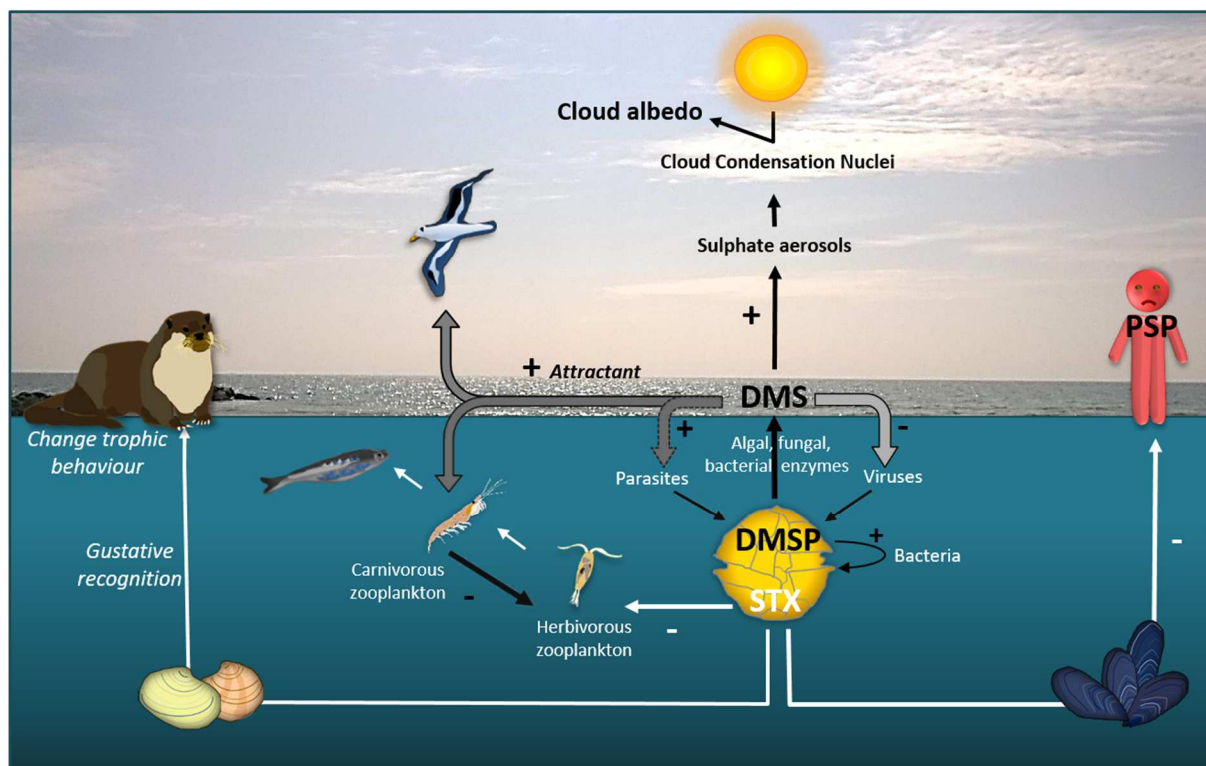


Fig. 1 Summary of the environmental impacts that *Alexandrium minutum* and *A. pacificum* blooms may have on the ecosystem, climate and human health via DMSP and STXs production. STXs may reduce grazing pressure of some copepods, modify trophic behavior of animals capable of gustative recognition of STXs and may generate paralytic shellfish poisoning (PSP) in humans having consumed contaminated shellfish. DMS and its by-product DMS may reduce grazing pressure by attracting second order of predators, restrain viruses, stimulate parasites and bacteria, signalize biological productive zone for ending food chain predators, and may increase cloud albedo via sulfate aerosol formation (references are cited in the text).

2-column fitting image

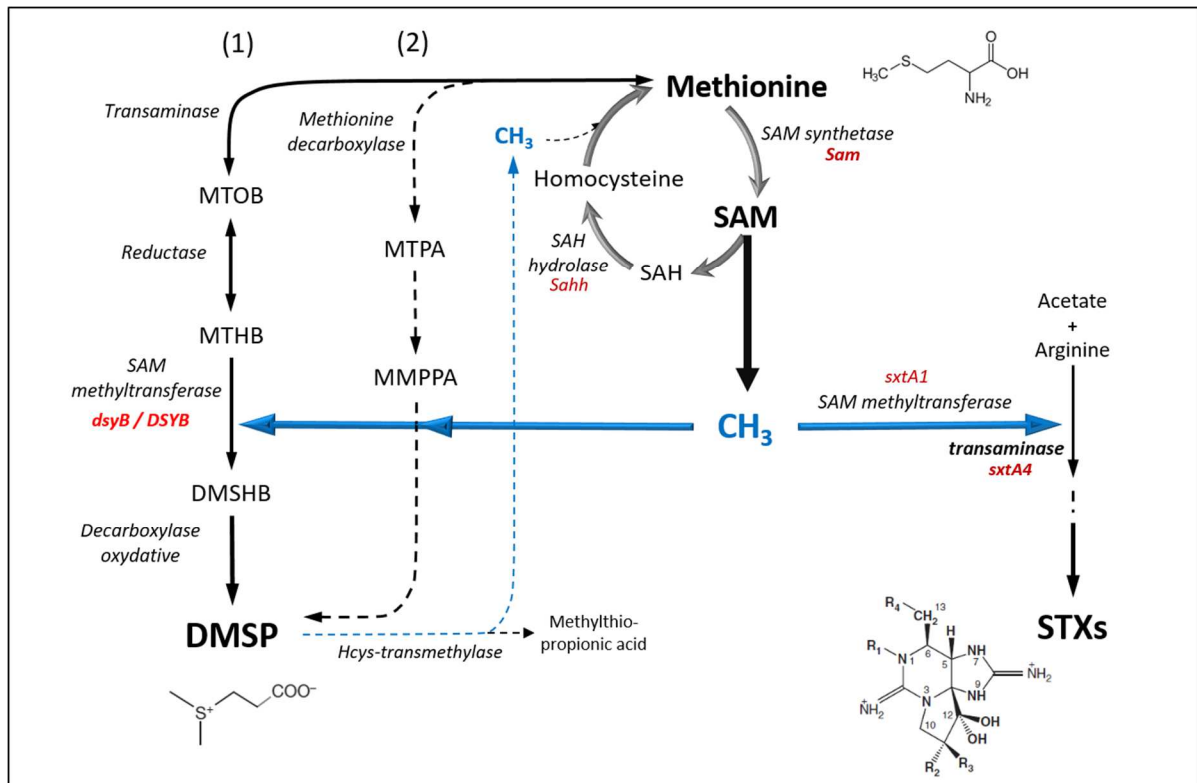


Fig. 2 Central role of methionine in the potential biosynthesis pathways of DMSP and STXs in *Alexandrium*. DMSP may be produced following two ways: (1) described in the green macroalgae *Ulva intestinalis* and (2) proposed in the heterotrophic dinoflagellate *Cryptocodinium cohnii*. The gene identified in some bacteria (*dsyB*) and phytoplankton (*DSYB*) species encodes for a SAM methyltransferase that converts MTHB in DMSHB in pathway 1. Another SAM methyltransferase encoded by gene *sxtA1* operates in the first steps of STXs synthesis in dinoflagellates.

2-column fitting image

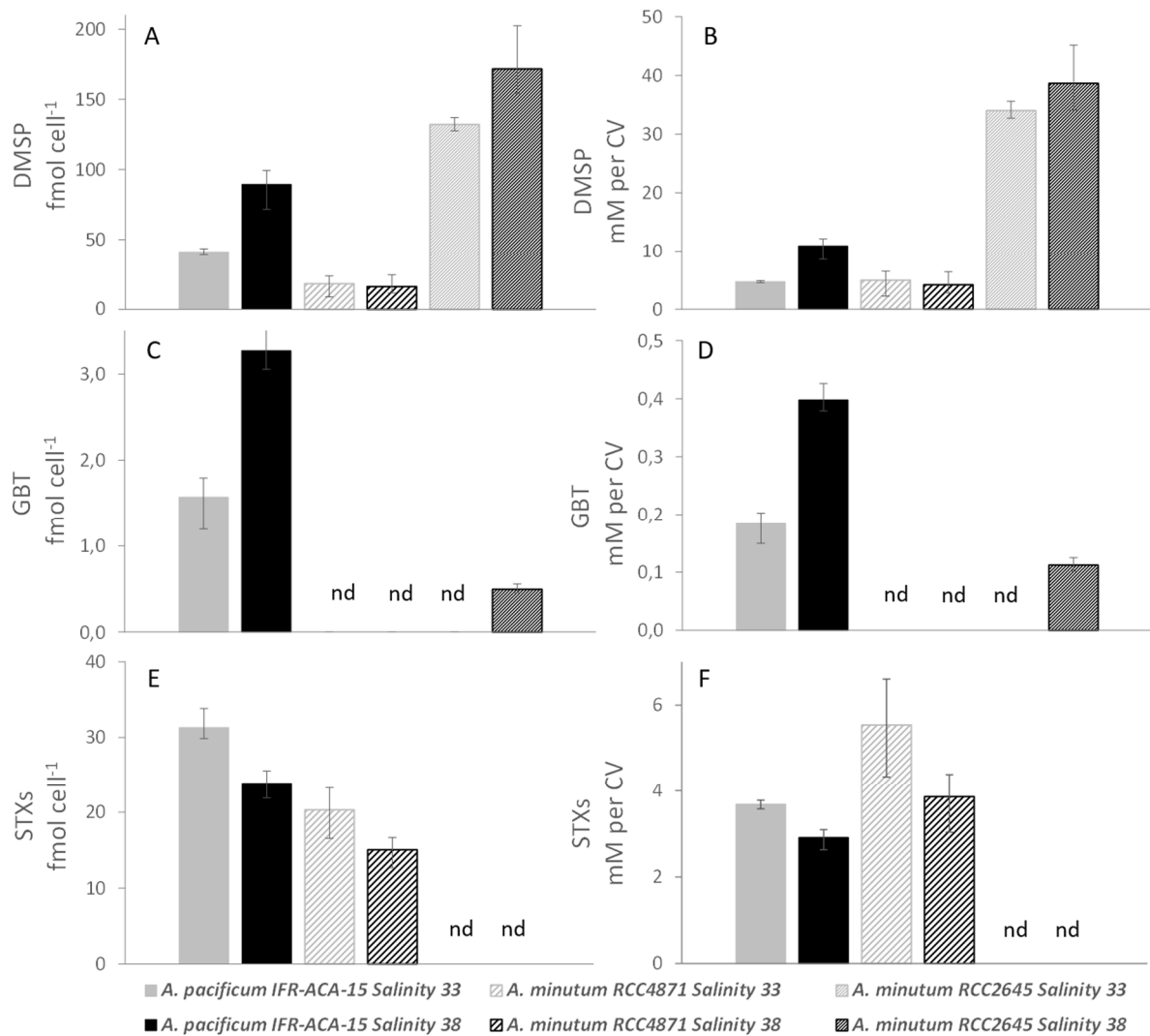


Fig. 3 Concentrations of DMSP in fmol per cell (A) and mM per cell volume-CV (B), glycine betaine - GBT in fmol per cell (C) and mM per CV (D) and toxins of the saxitoxin group - STXs in fmol per cell (E) and mM per CV (F) in three *Alexandrium* species (*A. pacificum* IFR-ACA-15, *A. minutum* RCC4871 and RCC2645) under two salinity regimes of 33 (grey and dashed grey) and 38 (black and dashed black). Values represent means (n=3) and error bars display maximum and minimum values, except n=2 for the first data point of DMSP dataset. Note that Y axis scales may differ for each panel.

2-column fitting image

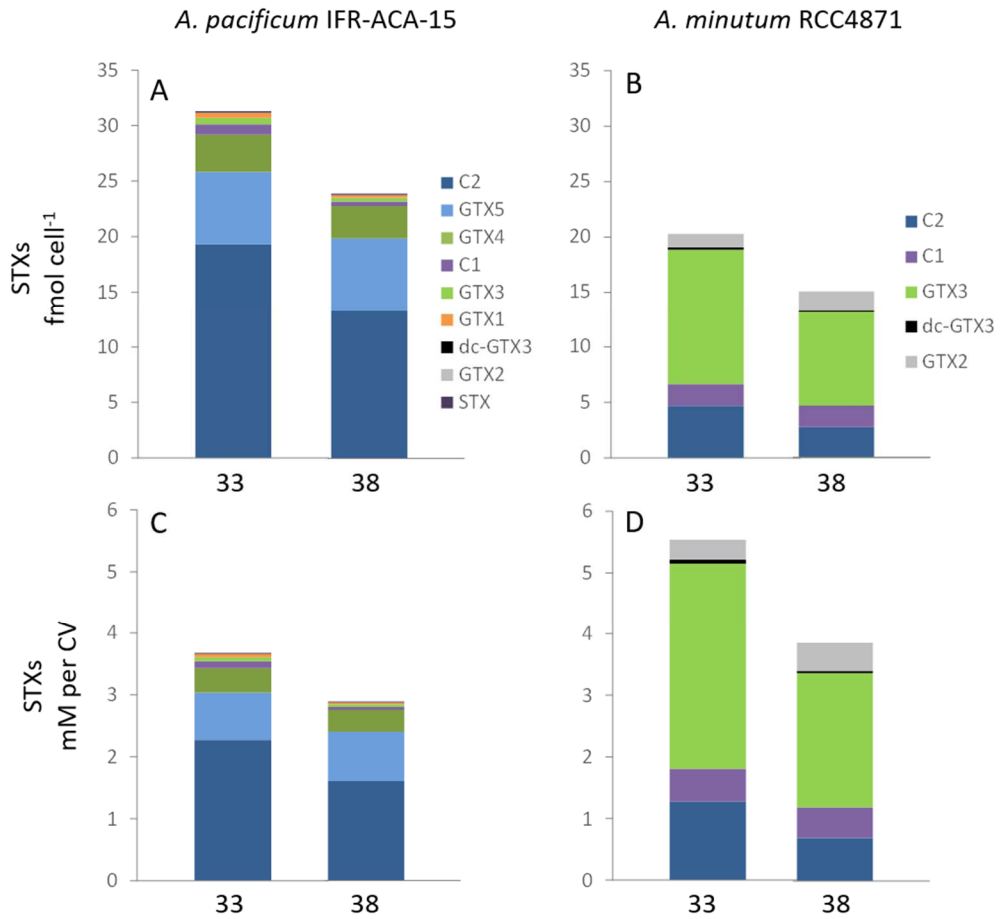


Fig. 4 Toxin profiles (STXs for toxins of the saxitoxin group) in *A. pacificum* IFR-ACA-15 in pmol cell<sup>-1</sup> (A) and in mM per cell volume –CV (C) and *A. minutum* RCC4871 in pmol cell<sup>-1</sup> (B) and in mM per CV (D) at salinity regimes 33 and 38. Mean values (n=3) are displayed for each toxin analogue.

1.5-or 2 column fitting image

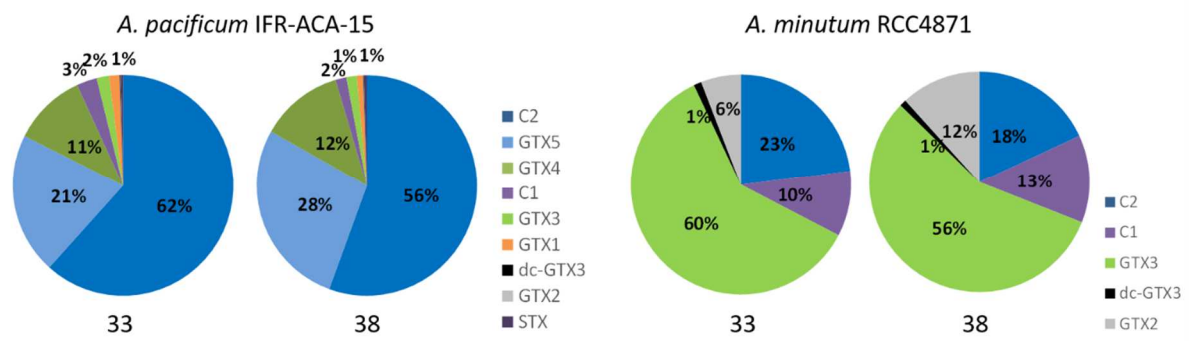


Fig. 5 Toxin profiles at salinity regimes 33 and 38 in *A. pacificum* IFR-ACA-15 (A) and *A. minutum* RCC4871 (B). Mean value (n=3) for each toxin analogue is expressed as a percentage of total toxins of the saxitoxin group expressed in fmol cell<sup>-1</sup> or mM per cell volume.

*2-column fitting image*

**Table1** Number of homologues for proteins potentially involved in DMSP biosynthesis in *A. minutum* reference transcriptome. The associated E value determines the probability for homology with the original sequence. “na” means not applicable.

Searched protein sequences	Organism	Number of homologues	Homologue identification	E value
AT, REDOX, SAMmt, DECARB, DIDEARB from <i>Fragilariopsis cylindrus</i>	<i>A. minutum</i>	0	na	na
TpMMT from <i>Thalassiosira pseudonana</i>	<i>A. minutum</i>	0	na	na
<i>dsyB</i> from <i>Labrenzia agregata</i>	<i>A. minutum</i>	3	112841 105760 74202	4e <sup>-76</sup> 2e <sup>-59</sup> 7e <sup>-11</sup>

**Table 2 DMSP values reported in this study and in literature for *A. minutum* and associated environmental conditions including strain name, geographic origin, light cycle and intensity, medium category and salinity, temperature, growth phase, growth rate, DMSP content. “n. a.” means not available data.**

Strain, geographic origin	Light cycle/ intensity  h:h/ $\mu\text{mol photons m}^{-2} \text{s}^{-1}$	Medium/ Salinity	Temperatu re  $^{\circ}\text{C}$	Growth phase / Growth rate  $\text{d}^{-1}$	DMSP	Reference
<i>A. pacificum</i> IFR-ACA-15, South France, Mediterranean Lagoon	12:12 / 75	L1-Si 33 38	17	Exponential 0.14 0.18	0.041 $\text{pmol cell}^{-1}$ 5 mM 0.089 $\text{pmol cell}^{-1}$ 11 mM	This study
<i>A. minutum</i> RCC2645, France, Atlantic coast	12:12 / 75	L1-Si 33 38	17	Exponential 0.20 0.20	0.132 $\text{pmol cell}^{-1}$ 34 mM 0.171 $\text{pmol cell}^{-1}$ 39 mM	This study
<i>A. minutum</i> RCC4871, France, Atlantic coast	12:12 / 75	L1-Si 33 38	17	Exponential 0.08 0.06	0.018 $\text{pmol cell}^{-1}$ 5 mM 0.016 $\text{pmol cell}^{-1}$ 4 mM	This study
<i>A. minutum</i> CS324, South Australia	14:10 / 200	GSe medium	20, 24, 32	Exponential (except at 32°C) 2.4-2.6	0.4-1.6 $\text{pmol cell}^{-1}$ decrease with heat stress	Dechaseaux et al. 2019
<i>A. minutum</i> CCMP113/AL1V Spain Vigo, Atlantic coast	14 :10 / 156	Enriched seawater L1-Si	15	Exponential 0.15	0.8 $\text{pmol cell}^{-1}$ 290 mM,	Caruana et al. 2012
<i>A. minutum</i> VGO651, France, Brittany, Atlantic coast	12:12/ 120	F/2 – Si 38	20	Exponential, 0.37 0.20 under turbulence	0.7-1.0 $\text{pmol cell}^{-1}$ 220 mM Increase with turbulence to 1.3 $\text{pmol cell}^{-1}$ 227 mM	Berdalet et al. 2011
<i>A. minutum</i> cells from field sample, South France, Mediterranean Sea	n. a.	n. a.	n. a.	Isolation of 61 cells	14.2 $\text{pmol cell}^{-1}$ 3388 mM	Jean et al. 2005