Alexandrium pacificum and Alexandrium minutum: Harmful or environmentally friendly?

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Abstract

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

Graphical abstract: Fig. 1

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

1. Introduction

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

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

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

of HABs on the French coasts and other coasts worldwide (John et al. 2014, Lewis et al; 2018). However, no data were reported so far on DMSP production by *A. pacificum*.

At the ecosystem level, both compounds may be part of the range of metabolites that structure relationships between marine organisms through chemical ecology (Fig. 1). For instance, in interactions such metabolites may mediate the interactions between microalgae and their predators, viruses, bacteria and parasites (Barak-Gavish et al., 2018; Evans et al., 2006; Garces et al., 2013; Johnson et al., 2016). Towards higher trophic levels such as zooplankton, fish, fish larvae, seabirds and mammals, DMS, the by-product of DMSP, is known to act as a foraging cue in the marine trophic food web (Foretich et al., 2017; Steinke et al., 2006). By contrast, some marine birds and mammals are able to change their trophic behavior to avoid STX contaminated shellfish (Ferrer and Zimmer, 2013); (Fig. 1).

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

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

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

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

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

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

2. Materials and methods

2.1 Algal cultures and salinity regimes

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

2.2 Experimental design

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

Germany) and cell pellets were stored at -80°C until further chemical analyses. Cell densities and total cell volumes were determined on fresh samples using a particle counter (Coulter Multisizer 3, Beckman, Villepinte, France). Growth rates on the day of sampling were calculated for each strain, 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 (Supplementary data, Table S2).

2.3 DMSP and GBT measurements by LC-MS/MS

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

Fallavier, France) prepared in methanol to cover calibration levels from 10nM to 5000 nM. Values are expressed in fmol $cell^{-1}$, in mM of CV, in pg $cell^{-1}$ and in g L^{-1} of CV.

2.4 Toxin measurements by LC/FLD

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

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

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

alt, REDOX alt, SAMmt (JGI accession numbers 273803, 173405 and 207357) were identified as candidates for the transaminase, reductase and methyltransferase respectively. The proteins DECARB and DIDECARB (JGI accession numbers 238865, 263016) could encode for the decarboxylase oxydative (Lyon et al., 2011). In the diatom *Thalassiosira pseudonana*, the protein TpMMT was identified as encoding for the methyltransferase that converts MTHB to DMSHB (NCBI accession number XP_002291473, Kageyama *et al.,* 2018). In the bacteria *Labrenzia aggregata* strain LZB033, the protein DsyB (NCBI accession number KT989543) was also identified as a methyltransferase that converts MTHB to DMSHB (Curson et al., 2017; Curson et al., 2018); (Fig. 2). For all these proteins a TBLASTN homology analysis (Altschul et al., 1997) with a threshold set at E values < $1e^{-10}$, was performed against the reference transcriptome of *A. minutum* (Le Gac et al. 2016, DOI: 10.1111/mec.13815) to identify transcript homologs for DMSP key genes.

2.6 Statistical analyses

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

3. Results

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

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

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

0.5 fmol cell^{-1} for *A. pacificum* IFR-ACA-15 and *A. minutum* RCC2645 respectively or 0.4 and 0.1 mM per cell volume.

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

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

in fmol cell^{-1} and 6% and 4% decrease respectively in pg cell^{-1}) to the benefit of C1 and GTX2 (3 and 6% increase in fmol cell^{-1} and 4% and 6% respectively in pg cell^{-1}, Fig.5 and Fig S5).

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

4. Discussion

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

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

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

Intracellular DMSP concentration in microalgae may depend on environmental conditions (Stefels, 2000; Stefels et al., 2007). Thus, essentially recognized as an osmolyte, intracellular DMSP concentrations may vary with salt water concentrations (Kirst, 1996; Reed, 1983; Speeckaert et al., 2019; Yang et al., 2011). GBT is another osmolyte produced by plants and some phytoplankton species. Little information available on GBT in dinoflagellates presents GBT as absent or scarce in this phytoplankton group. It was reported to be produced in some dinoflagellate species such as *Amphidinium carterae* (Keller et al., 1999), some *Symbiodinium* sp (Yancey et al., 2010) and *Prorocentrum minimum* (Gebser and Pohnert, 2013). In our study, *Alexandrium pacificum* and *A. minutum* also produce GBT but in much lower amount than DMSP. The osmolyte activity of these two compounds is supported here by an increase in concentrations in high salinity conditions for two of the three strains tested. The other strain *A. minutum* RCC4871 does not respond as a strain being 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^{-1} (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

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

5. Conclusion

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

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

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

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

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

435 Acknowledgements

436 We are grateful to Laure Guillou from the Biological Station of Roscoff who provided the *A. minutum*
437 strains. We express our gratitude to Virginie Raimbault from IFREMER for her support in isolating
438 strains. We also thank Nicolas Chomérat from IFREMER for his support in identifying strains and
439 constructive discussions.

440 Funding: This work was supported by the IFREMER Institute.

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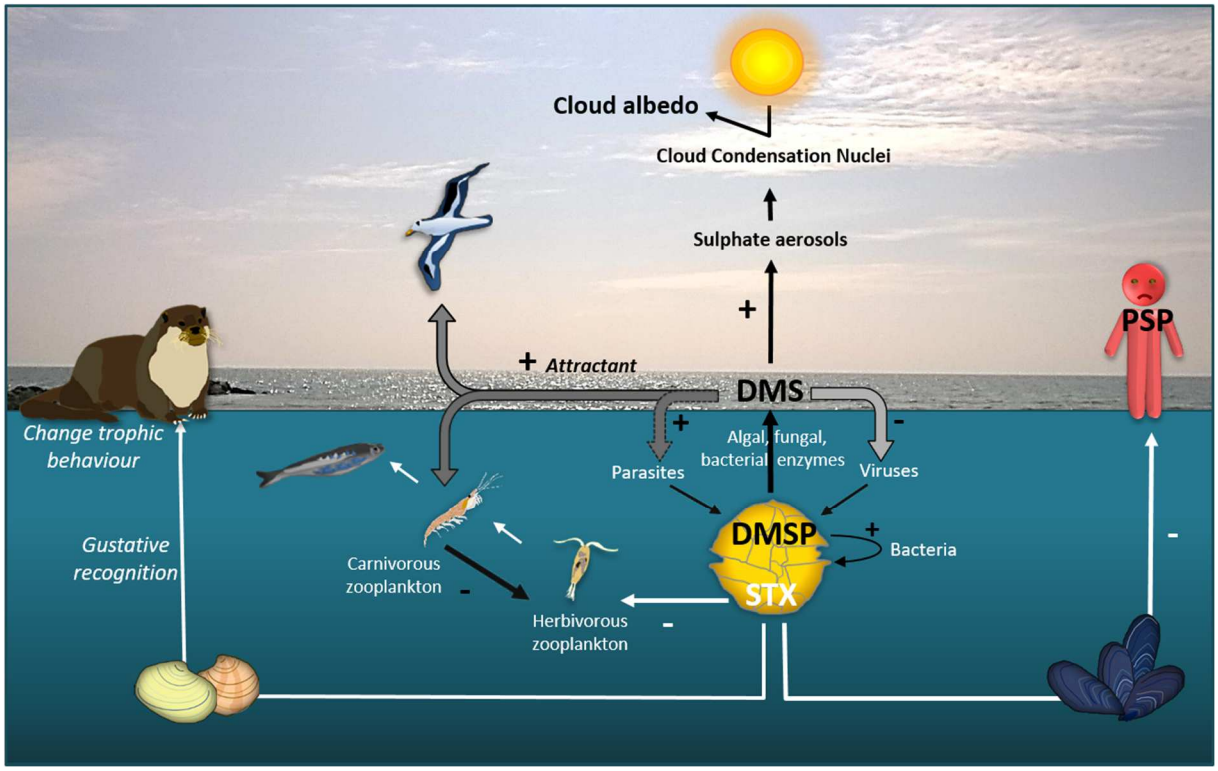

Fig. 1 Summary of the environmental impacts that *Alexandrium minutum* and *A. pacificum* blooms may have on the ecosystem, climate and human health via DMSP and STXs production. STXs may reduce grazing pressure of some copepods, modify trophic behavior of animals capable of gustative recognition of STXs and may generate paralytic shellfish poisoning (PSP) in humans having consumed contaminated shellfish. DMSP 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

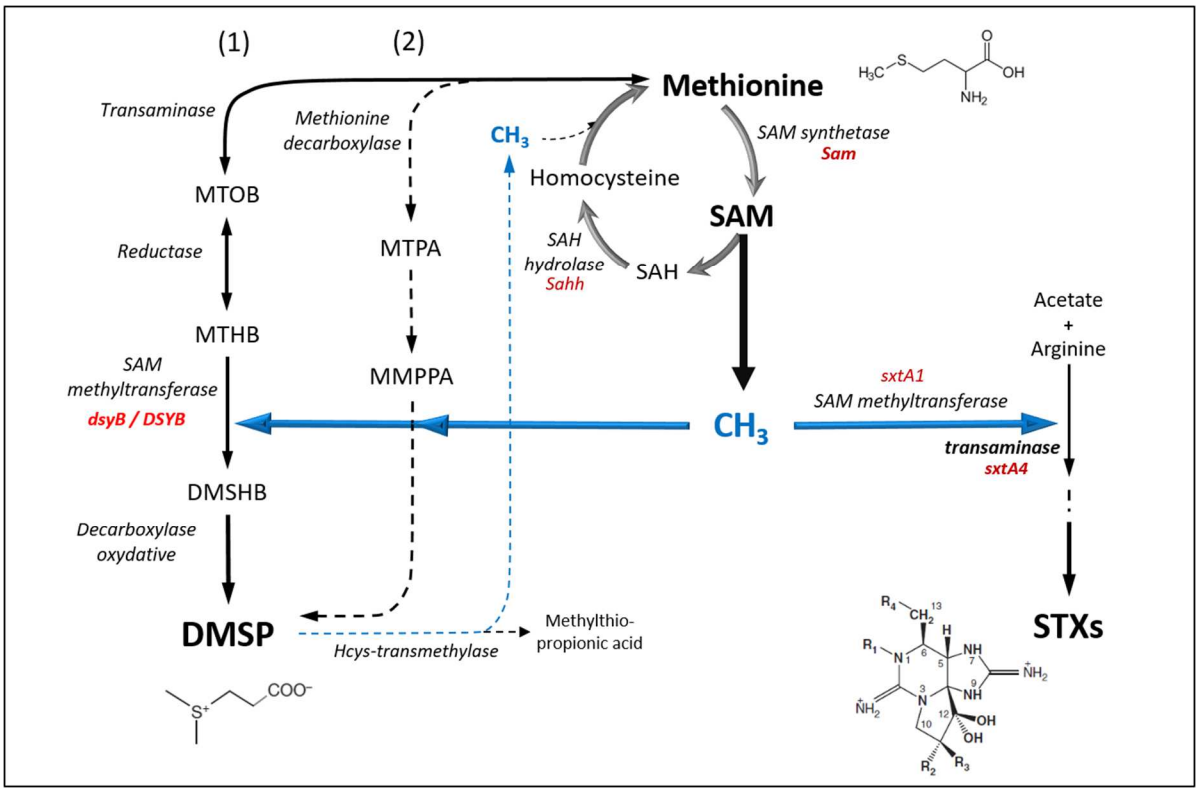

Fig. 2 Central role of methionine in the potential biosynthesis pathways of DMSP and STXs in *Alexandrium*. DMSP may be produced following two ways: (1) described in the green macroalgae *Ulva intestinalis* and (2) proposed in the heterotrophic dinoflagellate *Crypthecodinium cohnii*. The gene identified 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

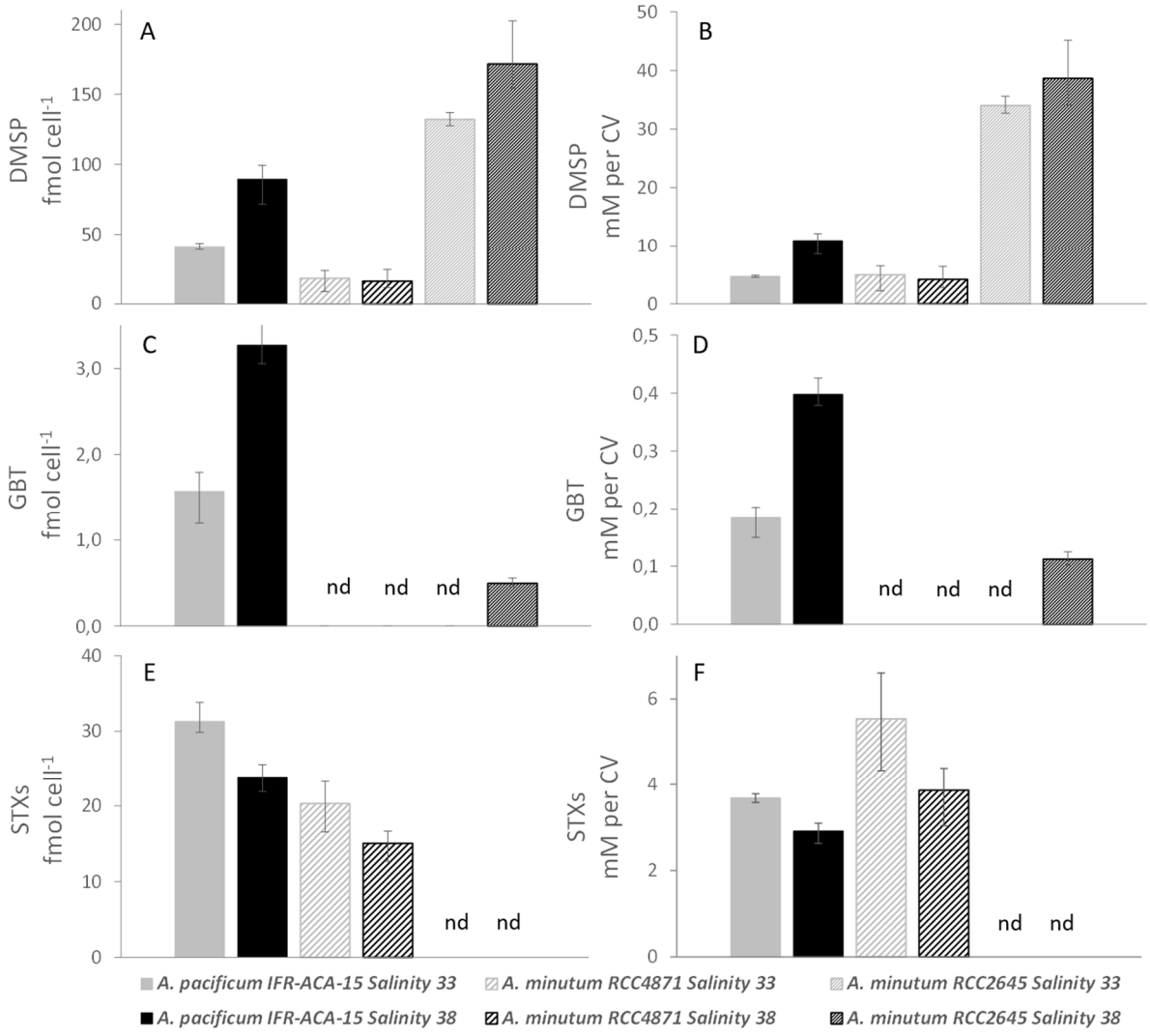

Fig. 3 Concentrations of DMSP in fmol per cell (A) and mM per cell volume-CV (B), glycine betaine - GBT in fmol per cell (C) and mM per CV (D) and toxins of the saxitoxin group - STXs in fmol per cell (E) and mM per CV (F) in three *Alexandrium* species (*A. pacificum* IFR-ACA-15, *A. minutum* RCC4871 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

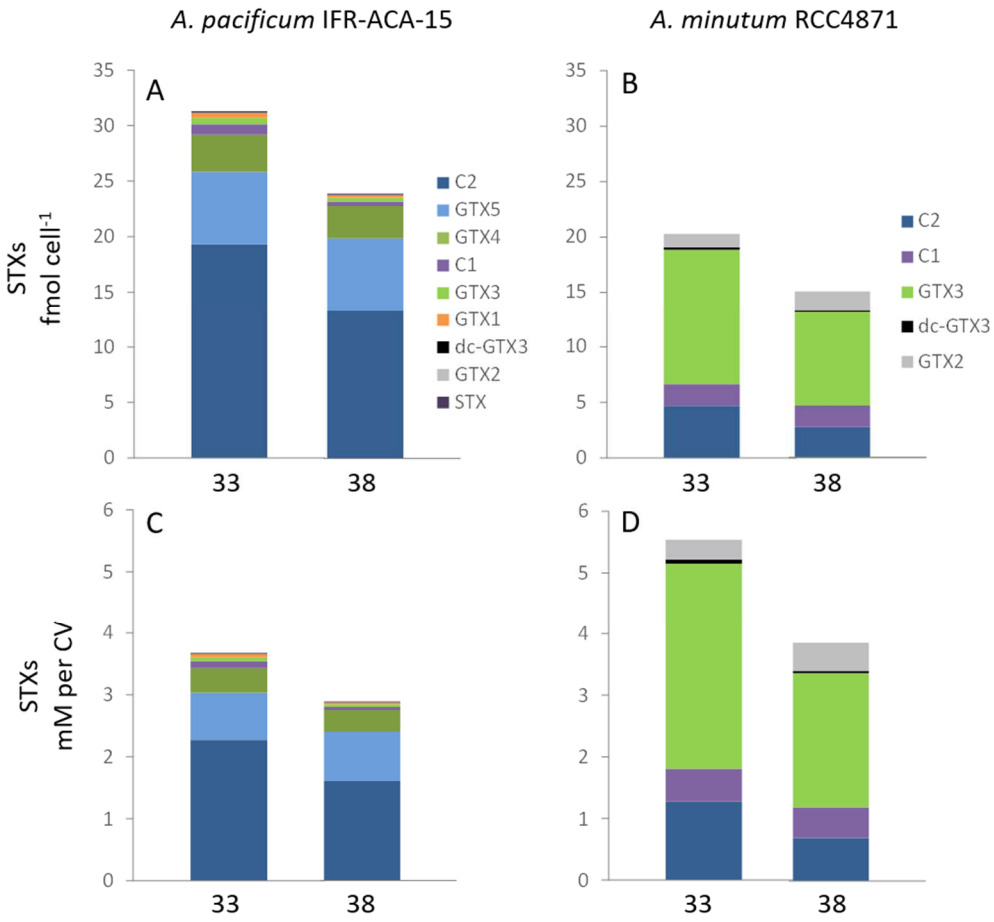

Fig. 4 Toxin profiles (STXs for toxins of the saxitoxin group) in *A. pacificum* IFR-ACA-15 in pmol cell^{-1} (A) and in mM per cell volume –CV (C) and *A. minutum* RCC4871 in pmol cell^{-1} (B) and in mM per CV (D) at salinity regimes 33 and 38. Mean values (n=3) are displayed for each toxin analogue.

1.5-or 2 column fitting image

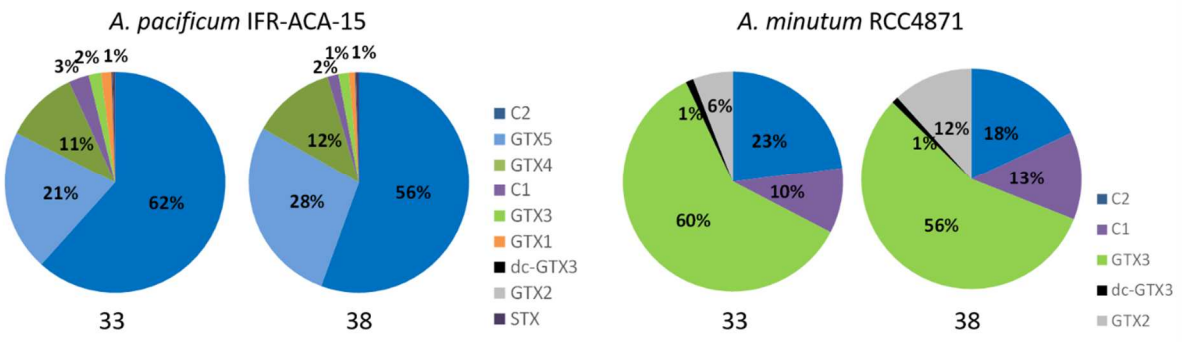

Fig. 5 Toxin profiles at salinity regimes 33 and 38 in *A. pacificum* IFR-ACA-15 (A) and *A. minutum* RCC4871 (B). Mean value (n=3) for each toxin analogue is expressed as a percentage of total toxins of the saxitoxin group expressed in fmol cell^{-1} or mM per cell volume.

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, DIDECARB from *Fragilariopsis cylindrus*	*A. minutum*	0	na	na
TpMMT from *Thalassiosira pseudonana*	*A. minutum*	0	na	na
dsyB from *Labrenzia agregata*	*A. minutum*	3	112841 105760 74202	$4e^{-76}$ $2e^{-59}$ $7e^{-11}$

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