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Effect of a short-term salinity stress on the growth, biovolume, toxins, osmolytes and metabolite profiles on three strains of the *Dinophysis acuminata*-complex (*Dinophysis* cf. *sacculus*)

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Abstract :

Dinophysis is the main dinoflagellate genus responsible for diarrheic shellfish poisoning (DSP) in human consumers of filter feeding bivalves contaminated with lipophilic diarrheic toxins. Species of this genus have a worldwide distribution driven by environmental conditions (temperature, irradiance, salinity, nutrients etc.), and these factors are sensitive to climate change. The D. acuminata-complex may contain several species, including D. sacculus. The latter has been found in estuaries and semi-enclosed areas, water bodies subjected to quick salinity variations and its natural repartition suggests some tolerance to salinity changes. However, the response of strains of D. acuminata-complex (D. cf. sacculus) subjected to salinity stress and the underlying mechanisms have never been studied in the laboratory. Here, a 24 h hypoosmotic (25) and hyperosmotic (42) stress was performed in vitro in a metabolomic study carried out with three cultivated strains of D. cf. sacculus isolated from the French Atlantic and Mediterranean coasts. Growth rate, biovolume and osmolyte (proline, glycine betaine and dimethylsulfoniopropionate (DMSP)) and toxin contents were measured. Osmolyte contents were higher at the highest salinity, but only a significant increase in glycine betaine was observed between the control (35) and the hyperosmotic treatment. Metabolomics revealed significant and strain-dependent differences in metabolite profiles for different salinities. These results, as well as the absence of effects on growth rate, biovolume, okadaic acid (OA) and pectenotoxin (PTXs) cellular contents, suggest that the D. cf. sacculus strains studied are highly tolerant to salinity variations.

Highlights

► The three *D.* cf. *sacculus* strains tolerated hypo- and hyperosmotic conditions (25 and 42 vs. 35). ► Higher proline, glycine betaine and DMSP contents of *D.* cf. *sacculus* at the highest salinity. ► Growth, biovolume, toxin contents and profiles were not affected by salinity stress. ► Metabolomics showed intraspecific variability of *D.* cf. *sacculus* but no clear salinity effect.

Keywords : Dinoflagellates, DMSP, Glycine betaine, Okadaic acid, Pectenotoxins, Proline

Abbreviations

DSP (diarrheic shellfish poisoning); DMSP (dimethylsulfoniopropionate); DTX1 (dinophysistoxin 1); DTX2 (dinophysistoxin 2); DTXs (dinophysistoxins); ESI (electrospray ionization); GBT (glycine betaine); HAB (harmful algal bloom); OA (okadaic acid); PTX2 (pectenotoxin 2); PTX2eq (pectenotoxin 2 eq); PTXs (pectenotoxins); QC (pool samples); QTOF (quadrupole-time of flight mass spectrometer; LC-HRMS (liquid chromatography coupled to high resolution mass spectrometry); LC-LRMS/MS (liquid chromatography coupled to low resolution tandem mass spectrometry); REPHY (Observation and Surveillance Network for Phytoplankton and Hydrology in coastal waters).

1. Introduction

Diarrhetic shellfish poisoning (DSP) – a human intoxication due to the consumption of contaminated mollusks – is characterized by nausea, abdominal pain, vomiting and diarrhea (Yasumoto et al., 1985). This intoxication is mainly caused by lipophilic toxins synthetized by species of the genus *Dinophysis* (Ehrenberg, 1841), namely okadaic acid (OA) and dinophysistoxins (DTXs). In addition, some species of *Dinophysis* can also produce pectenotoxins (PTXs), another group of non-diarrheic toxins (Ito et al. 2008, Reguera et al. 2012). Since the description of the syndrome in 1976 (Yasumoto et al., 1978, 1980), worldwide observations of DSP outbreaks and indirect impacts on human health have been reported (Reguera et al., 2014). Consequently, national surveillance programs have been implemented in many countries, as the REPHY/REPHYTOX (hereafter named REPHY) in France that monitors toxic phytoplankton species in seawater and their toxins in seafood since 1984 (Belin et al., 2020). Shellfish farming and recreational harvesting are forbidden when the concentration of regulated toxins exceeds sanitary thresholds (e.g. a limit of 160 µg OA eq. per kg of bivalve meat in Europe, America and Japan; DeGrasse and Martinez-Diaz, 2012; EU.Commission, 2011; Hess, 2012; National Shellfish Sanitation Program, 2017; Suzuki and Watanabe, 2012).

Moreover, recent studies highlighted direct effects of *Dinophysis* on commercial bivalve shellfish species, such as hypersecretion of mucus and paralysis in the Japanese (*Mizuhopecten yessoensis*) and noble (*Mimachlamys nobilis*) scallops (Basti et al., 2015), reduction in clearance rate of the blue mussel (*Mytilus edulis*) (Nielsen et al., 2020) or an increase in oocyte mortality and a decrease of fertilization success of the Pacific oyster (*Crassostrea gigas*) (Gaillard et al., 2020). Altogether, the effects of *Dinophysis* blooms could lead to environmental, sanitary, societal and economic issues (Reguera et al., 2012; Van Dolah, 2000).

Numerous field studies described the habitat of toxic species of the genus *Dinophysis* and showed both positive and negative significant correlations between *Dinophysis* abundance and distribution and salinity (Ajani et al., 2016; Alves et al., 2018; Caroppo et al., 2001; Ninčević-Gladan et al., 2008). Moreover, *Dinophysis* can be found in salinity stratified-areas (Alves-de-Souza et al., 2014; Diaz et al., 2011; Peperzak et al., 1996), over a wide range of salinities (Alves and Mafra, 2018; Hoshiai et al., 2003), e.g. *D. acuminata* was found at salinities ranging from 5 to 31 in Chile (Diaz et al., 2011).

Since the successful mixotrophic culture of *D. acuminata* by Park et al. in 2006, several laboratory studies have been carried out on *Dinophysis* about the effects of (1) abiotic factors, such as nutrient availability, concentration and uptake (e.g. Nagai et al., 2008, García-Portela et al., 2020), temperature (e.g. Basti et al., 2018, Kamiyama et al., 2010), salinity (Fiorendino et al., 2020), light intensity and quality (e.g. Kim et al., 2008, García-Portela et al., 2018) or (2) biotic factors, such as type of prey (e.g. Smith et al., 2018), prey concentration (e.g. Kim et al., 2008, Smith et al., 2018) and prey exudates (Gao et al., 2019; Nagai et al., 2011), nutritional status (García-Portela et al., 2020) or (3) geographical origin (Fux et al., 2011) on the physiology and/or toxicity. Nevertheless, laboratory studies exploring the short-term effect of salinity stress on physiology and metabolites production (including toxins) are still lacking.

D. acuminata and *D. sacculus* were described on the basis of their morphology only, but several studies pointed out their considerable morphological plasticity (Lassus and Bardouil, 1991; Zingone et al., 1998). In addition, their nuclear (e.g. D1/D2 regions of the LSU or ITS part of the rRNA operon) or mitochondrial (*cox*1) genes are closely related and do not support discrimination into several species (Wolny et al., 2020). Indeed, although *D. sacculus* seems to have a different ecological niche compared to *D. acuminata*, its taxonomical identity is not clear

and genetic data (LSU, ITS rRNA or *cox*1 genes) could not separate it clearly from other species of the complex (Séchet et al. 2021).

Indeed, *D. sacculus* found on European Atlantic coasts and in the Mediterranean Sea, is likely to perform well in closed or semi-enclosed areas, e.g. lagoons, rias or bays (Ninčević-Gladan et al., 2008; Zingone et al., 1998), where shellfish farming activities are located. In coastal waters, salinity can rapidly change over short time spans due to runoff, precipitation, evaporation and extreme events, likely to be more frequent with global change (Kirst 1989, IPCC 2013, Wells et al. 2019).

Salinity stress can affect microalgae, impacting their osmotic potential, ion ratios (uptake and loss) due to the difference in osmolarity between the intra- and extracellular environment (Kirst, 1989). In order to maintain an equilibrium, cells rely on different physiological responses to salt stress, such as modification of growth rate, inhibition of photosynthesis or respiration, reactive oxygen species (ROS) production and osmoregulation (Borowitzka, 2018; Mayfield and Gates, 2007). This last process has been described as a possible threestep process, i.e. involving three mechanisms in sequence: microalgae (and cyanobacteria) can (1) adjust their turgor pressure by fluxes of water, (2) adjust their ion concentrations and (3) synthetize osmolytes (i.e. compatible solutes) (Hagemann, 2016, 2011; Kirst, 1989), which are typically small, water-soluble compounds containing nitrogen and sulfur (Keller et al., 2004; Rhodes et al., 2002). The most commonly found osmolytes are amino acids (e.g. proline) (Hagemann, 2016; Stefels, 2000), quaternary ammonium derivatives (glycine betaine, GBT) (Dickson and Kirst, 1986), as well as the tertiary sulfonium compound dimethyl sulfonio-propionate (DMSP) (Keller et al. 1999), which has been found in massive blooms of coccolithophores (Malin et al., 1993) but is also commonly produced by dinoflagellates (Caruana and Malin, 2014). However, no laboratory

studies addressed the production of proline, GBT and DMSP by *Dinophysis* and especially in a context of sudden changes in salinity.

In addition to the quantitation of known metabolites, untargeted LC-HRMS approaches such as metabolomics (i.e. metabolic fingerprinting) can be used to study the response of the organisms, including microalgae, to salinity changes. Indeed, metabolomics allows to get a fingerprint of a large set of small metabolites affected by the environmental changes in any organism (Bundy et al., 2009; Fiehn, 2002). Other molecules acting like osmolytes have been described (Rhodes et al., 2002) and characterized after salinity changes by liquid chromatography coupled to high resolution mass spectrometry (e.g. sucrose; Georges des Aulnois et al. 2020).

In this study, well-fed cultures of *D.* cf. *sacculus* isolated from French coastal waters (two from a semi-enclosed and one from an estuarine system) in exponential growth were subjected to a short-term (i.e. 24 h) hypoosmotic and hyperosmotic stress. In addition to growth and biovolume measurements, toxin and osmolyte (proline, GBT and DMSP) contents were quantified using ultra high performance liquid chromatography coupled to low resolution tandem mass spectrometry (LC-LRMS/MS) to determine *D.* cf. *sacculus* putative mechanisms of osmoregulation under salinity stress conditions. Finally, a non-targeted metabolomic approach was also used in an attempt to identify any other osmolyte or molecule potentially involved in the response of the three strains of *D.* cf. *sacculus* to salinity changes.

2. Materials and methods

2.1 Culture maintenance

Three strains of *D. acuminata*-complex isolated from the French Atlantic coast at Loscolo (Vilaine Bay, strain IFR-DSA-01Lo = Dsa-Lo) and Meyran (Arcachon Bay, strain IFR-DSA-

01Me = Dsa-Me) and one strain from Thau Lagoon (Crique de l'Angle, strain IFR-DSA-02Th = Dsa-Th) in the Mediterranean Sea were used. Genetic characterization of these strains has been realized by Séchet et al., (2021) who investigated morphology and genetics and showed that they all clustered in *D. acuminata*-complex subclade in the *cox*1 phylogenetic analysis, corresponding presumably to *D.* cf. *sacculus* (Séchet et al., 2021).

The three mixotrophic *Dinophysis* strains were cultivated according to a classic three-step culture method (Park et al., 2006) with the ciliate *Mesodinium rubrum* (Lohmann, 1908) (strain MBL-DK2009) and the cryptophyte *Teleaulax amphioxeia* (Conrad) Hill (Hill, 1992) (strain AND-0710) as described in Gaillard et al. (2020). *Dinophysis* cultures were maintained in sterilized seawater at salinity 35, 17.8 ± 0.6 °C, and light intensity of ~ 100 µmol photons m^{-2} s^{-1} with a circadian cycle of 12: 12 (L: D; Table 1). All the cultures were monoclonal and xenic.

2.2 Experimental design

Cultures of *D.* cf. *sacculus* in exponential growth phase were filtered on a nylon sieve (mesh 11 µm) and gently rinsed with sterilized seawater to remove any cryptophyte and ciliate cells. Each filtered strain was then resuspended in nine replicates of 10 mL of sterilized seawater (salinity 35), at a cell density of 2200 ± 350 cells mL^{-1} and fed *M. rubrum* at a 6: 1 (prey: predator) ratio. Growth was monitored for seven days until the total consumption of *M. rubrum* and the exponential growth phase was reached.

Then, for each strain, the same volume of either milliQ water or salted-milliQ water was added in three replicates for each treatment, to decrease the salinity down to 25, increase the salinity up to 42 and maintain the salinity at 35 (control condition). NaCl was chosen because it is the main dissolved salt in natural seawater (Hagemann, 2016).

Salinities 25 and 42 correspond approximately to the minimum (at Loscolo) and maximum (at Thau Lagoon) salinities observed in the field sampling locations (Figure 1) (REPHY 2019). Moreover, a preliminary experiment on the same three strains revealed no positive growth rate at salinity 45.

One day (24 h) after the salinity stress and control treatments, cultures were filtered on a nylon sieve (mesh 11 µm) and the filtrates were filtered on 0.2 µm (cellulose) syringe filters to remove any potential cryptophyte and ciliate or bacteria. Filters, with *D.* cf. *sacculus* cells were stored at -80 °C for further toxin and metabolomic analyses. Supernatants, stored at -80 °C, were analyzed for their extracellular toxin contents.

2.3 Growth rate calculation and biovolume measurement

A duplicate sample (i.e. 2 replicates at the same time) of 90 µL each of *D.* cf. *sacculus* cultures were fixed in acidic Lugol solution (1% final concentration) and a minimum of 100 cells of *D.* cf. *sacculus* or *M. rubrum* (before full consumption) were counted using a Nageotte cell counting chamber with a light microscope. The growth rates were calculated from the slope of the linear regression for the natural logarithm-transformed values of population size during the time interval of exponential growth phase during the salinity stress (i.e. 24 h) (Guillard, 1973).

To assess the biovolume, micrographs of at least 30 individuals of each *D.* cf. *sacculus* strain were made under a light microscope (Leica DMR, Germany). Cells of *D.* cf. *sacculus* correspond to a flattened ellipsoid (Olenina et al., 2006), thus the cells biovolume was calculated after measuring height and large diameter (see details in Olenina et al., 2006) using ImageJ software. The small diameter was estimated according to Olenina et al. (2006).

2.4 LC-LRMS/MS and LC-HRMS

Intracellular metabolites were extracted with methanol (at a ratio of 0.5 mL for 25 x 10^3 cells) and sonicated at 25 kHz for 15 min. Extracellular metabolites were recovered from the supernatant after liquid-liquid extraction with dichloromethane, evaporated under a flow of nitrogen (Gaillard et al., 2020) and resuspended in 0.5 to 1.4 mL of methanol. All the samples were filtered (0.2 µm, Nanosep, MF, Pall) before analysis.

2.4.1 Toxin analysis

Toxins were quantified as in Gaillard et al. (2020). Analysis was performed by liquid chromatography coupled to low resolution tandem mass spectrometry (LC-LRMS/MS) with a UHPLC system (UFLC XR Nexera, Shimadzu, Tokyo, Japan) coupled to a triple quadrupole/ion-trap mass spectrometer (API 4000 QTrap, Sciex, Redwood City, CA, USA), equipped with a turboV® electrospray ionization (ESI) source. Certified calibration solutions of pectenotoxin 2 (PTX2), okadaic acid (OA), dinophysistoxin 1 and 2 (DTX1 and DTX2) were obtained from the National Research Council Canada (NRCC, Halifax, NS, Canada). Pectenotoxin 2 equivalent (PTX2eq) was the sum of pectenotoxin 2, pectenotoxin 2b, pectenotoxin 2 seco-acid and 7-*epi*-pectenotoxin-2 seco-acid, all quantified with the PTX2 standard by assuming similar molar responses. As toxins were mostly intracellular (≈ 91 and 97 % for OA and PTX2eq, respectively, see Table S1) and that the cultures were exponentially growing (without cell death), the toxin contents were expressed in total, corresponding to the sum of intracellular and extracellular in a per-cell basis. The detection/quantification limits were 1/3 ng mL^{-1} for OA, DTX1 and DTX2, and 0.2/0.6 for PTXs.

2.4.2 Proline, glycine betaine and DMSP analysis

Quantification of intracellular proline, glycine betaine and DMSP was adapted from Georges des Aulnois et al. (2019) and performed on the analytical system used for toxin analysis. The chromatographic column was a Hypersil GOLD HILIC (150 x 2.1 mm; 3 µm; ThermoScientific, Waltham, MA, USA) and the flow rate was 0.25 mL min^{-1}. Compounds were quantified using external 5-point calibration curves of standards (Sigma-Aldrich, Saint-Quentin Fallavier, France) solubilized in methanol, with concentrations from 50 nM to 5000 nM. As biovolumes were similar among conditions (see section 3.1), concentrations were expressed in a per-cell basis.

2.4.3 Metabolomics

Metabolomic profiles were acquired by liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS) as in Estevez et al. (2020). The system was a UHPLC (1290 Infinity II, Agilent technologies, Santa Clara, CA, USA) coupled to a quadrupole-time of flight mass spectrometer (QTOF 6550, Agilent technologies, Santa Clara, CA, USA) equipped with a Dual Jet Stream ESI interface. Both positive and negative full scan modes were used over a mass-to-charge ratio (*m/z*) ranging from 100 to 1700. The only difference was that the injection volume used here was 10 µL. Pool samples (QC) were prepared and injected ten times at the beginning of the batch sequence and then every five samples (including blanks). Blanks were prepared as *D.* cf. *sacculus* samples.

LC-HRMS raw data (.d) were converted to .mzXML format using MS-Convert (ProteoWizard 3.0) (Chambers et al., 2012) and pre-processed with theWorkflow4Metabolomics (W4M; http://workflow4metabolomics.org) e-infrastructure (Guitton et al., 2017). Peak picking, grouping, retention time correction, and peak filling were performed with the “CentWave”, “PeakDensity”, “PeakGroups”, and “FillchromPeaks” algorithms. Annotation (isotopes, adducts) was conducted with the “CAMERA” algorithm (Kuhl et al., 2012). Intra-batch signal intensity

drift was corrected by fitting a locally quadratic (loess) regression model to the QC values (Dunn et al., 2011; Van Der Kloet et al., 2009).

Three successive filtering steps using in-house scripts on R were applied, as in Georges des Aulnois et al. (2020). Pre-processing of +MS and –MS data matrices led to 8458 and 2651 variables respectively, and 540 and 203 variables remained after the filtrations. The two matrices were concatenated, log transformed and Pareto scaled before statistical analyses.

In parallel, HRMS data were acquired by autoMS/MS in an attempt to annotate the significant features revealed by the metabolomic approach, as in Georges des Aulnois et al., (2019).

2.5 Statistical analysis

Statistical analyses were performed on RStudio v 1.1.463. When the assumptions of independence (Durbin-Watson test), homoscedasticity (Bartlett test) and normality (Shapiro-Wilk test) of the residuals were validated, a two-way ANOVA was computed on the two factors location and salinity. When a significant difference was highlighted for one factor, a one-way ANOVA followed by a Tukey post hoc test was performed. When the previous assumptions were not met, data were log-transformed. Differences were considered statistically significant when $P < 0.05$, for a significance level of $\alpha = 0.05$. Values were expressed as mean ± SD.

For metabolomics, the statistical analyses were carried out with MetaboAnalyst 4.0 (Chong et al., 2018). As the PLS-DA models were not validated (determined by a permutation test), ANOVA followed by a Tukey post hoc test (for the strains Dsa-Lo and Dsa-Me) and t-tests (for the strain Dsa-Th) were used to assess the features significantly affected between the three salinity conditions.

3. Results

3.1 Growth rate and biovolume

The three strains of *D.* cf. *sacculus* (Table 1) survived to the 24 h hypoosmotic (25) and hyperosmotic (42) stress conditions and maintained a positive growth but neither growth rates nor biovolumes were significantly different between salinities and locations (Table 2). The growth rates ranged from 0.16 ± 0.04 to 0.33 ± 0.06 d^{-1} and the biovolumes from 12.4 ± 1.50 x 10^3 to 14.5 ± 1.65 x 10^3 μm^3 (Table 2). However, variations between replicates have been observed and may be due to the relatively small number of *Dinophysis* cells counted or reflect different stages of the exponential growth phase between replicates.

3.2 Toxins

The three strains of *D.* cf. *sacculus* exhibited the same qualitative toxin profile constituted by 80-91% of PTX2eq and 9-20% of OA (Figure 2), with some quantitative differences. The strain Dsa-Lo synthetized in total significantly less OA (2.0 ± 0.4 pg $cell^{-1}$; $P < 0.001$; mean of the three salinities) than Dsa-Me and Dsa-Th (8.4 ± 1.3 and 10 ± 0.8 pg $cell^{-1}$, respectively; Figure 2A) and the highest PTX2eq concentration was found in the strain Dsa-Me (83 ± 9.0 pg $cell^{-1}$; $P < 0.001$; Figure 2B).

However, no significant effect of hypoosmotic and hyperosmotic stress was observed on toxin contents after this 24 h experiment. Furthermore, the proportion of intracellular toxins was high for both OA (> 85%) and PTX2eq (> 88%) but similar among strains and salinity conditions (Table S1).

3.3 Proline, GBT and DMSP

The three strains of *D.* cf. *sacculus* synthetized proline, GBT and DMSP and the origin of the strains had no effect on the concentrations (i.e. no intraspecific variability) (Figure 3).

The concentration of proline, GBT and DMSP appeared to be greater at the highest salinity tested (42; Figure 3). The mean concentration of proline in control (35) for the three strains was 4.0 ± 0.6 fmol cell^{-1} and the salinity stress led to 1.1-fold less and 1.6-fold more proline at salinity 25 and 42, respectively (Figure 3A). More specifically, for the strains Dsa-Lo and Dsa-Me, a 2-fold significantly higher concentration ($P < 0.05$) was observed between salinity 42 and 25 (6.2 ± 1.4 vs. 2.7 ± 0.8 fmol cell^{-1} and 4.7 ± 0.7 vs. 2.0 ± 1.2 fmol cell^{-1} respectively; Figure 3A).

For GBT, the mean concentration in the controls was 3.2 ± 1.4 fmol cell^{-1} and was similar at the lowest salinity (25) (Figure 3B). However, for the strain Dsa-Lo a significant 6.3-fold higher GBT concentration was observed at salinity 42 (10 ± 5.0 fmol cell^{-1}) compared to control (1.7 ± 0.3 fmol cell^{-1}) and around 2-fold for the strain Dsa-Th and Dsa-Me (Figure 3B). For this last strain, a 4-fold higher accumulation of GBT was observed at 42 compared to 25 (Figure 3B).

In control conditions (salinity 35), *D.* cf. *sacculus* synthetized 1.0 ± 0.2 pmol cell^{-1} of DMSP (Figure 3C) with a non-significant trend of accumulation at 42 compared to 35 (1.1-fold) and at 35 compared to 25 (1.2-fold). The only significant difference was for the strain Dsa-Lo, with a 1.4-fold higher DMSP content at salinity 42 compared to 25 ($P < 0.05$; Figure 3C).

3.4 Metabolomics

Metabolomics and the resulting PCA illustrated the intraspecific variability of the three strains of *D.* cf. *sacculus* (Figure 4). We could not however discriminate the control from the hypoosmotic and hyperosmotic conditions, thus no clear salinity effect was observed despite the presence of six hundred features in the data matrix (Figure 4). Nevertheless, 19, 2 and 32 features were

significantly affected by salinity in the strain Dsa-Me, Dsa-Lo and Dsa-Th respectively (Table S2).

Among them, adenosine and eicosapentaenoic acid (EPA) (features M268T51 and M301T600 in Table S2 C), were identified in Dsa-Th (Table S2C, Figure S1) based on Georges des Aulnois et al. (2020) and by comparison with a standard (same retention time, mass and similar MS/MS spectra), respectively. These two compounds were significantly less synthetized at the highest salinity (i.e. 42; Figure S1; $P < 0.05$).

4. Discussion

This study highlighted the high tolerance of *D.* cf. *sacculus* to abrupt changes in salinity after 24 h exposure to salinity stress treatments. Indeed, growth rates and biovolumes were not significantly affected and there was no release of toxins (i.e. 85-99% were intracellular) that could be synonym of cell lysis and death (Smith et al., 2012). Moreover, neither the metabolomic approach nor the toxin concentrations, could not discriminate the control from the hypoosmotic and hyperosmotic stressed cells. These results suggest that only minor metabolic modifications occurred. However, some osmoregulation possibly occurred since cells maintained a positive growth.

Similar to our observations, Errera and Campbell (2011) and Sunda et al. (2013) did not report any significant difference in the growth rates of several strains of *Karenia brevis* during a hypoosmotic stress (from 36 to 27). However and unlike our results, both studies noted a quick (i.e. in 3 minutes) increase in biovolume, significant for Sunda et al. (2013) but not for Errera and Campbell (2011). It should be noted that dinoflagellates of the genus *Karenia* are naked cells, while those belonging to the genus *Dinophysis* have a theca made of cellulosic thecal plates

(Jensen and Daugbjerg, 2009). Thus, these solid plates and the rapidity of water flux exchanges (Kirst, 1989) may explain the absence of biovolume modification under hypoosmotic and hyperosmotic conditions after 24 h of treatment.

The role of DSTs to act like osmolytes in *Dinophysis* is questionable. In toxic phytoplankton, *de novo* synthesis has been suggested to play a role in osmoregulation during salinity stress. Indeed Errera and Campbell (2011) suggested that toxins of *K. brevis* (brevetoxins) may "facilitate osmoregulation" because of their interaction with sodium channels that can affect ion concentrations. Indeed, these authors pointed out an important (up to 14-fold compared to control) and rapid (< 3 h) accumulation of brevetoxins in several strains of *K. brevis* subjected to a hypoosmotic stress. Nonetheless, Sunda et al., 2013 repeated the original experiments (using the same strains) of Errera and Campbell (2011) and found contradictive results (i.e. no increase in brevetoxins production during hypoosmotic stress) and refuted the involvement of brevetoxins in osmoregulation in *Karenia*. Accumulation of another phycotoxin, domoic acid, has been shown for the toxic diatom *Pseudo-nitzschia australis* after 24 and 48h hypoosmotic stress (35 to 30), which suggests that this toxin did not act like an osmolyte (Ayache et al., 2019).

Our study indicated that the two *D.* cf. *sacculus* toxins, i.e. OA and PTX2, were not involved in osmoregulation since no significant change in their concentrations was observed after either hypo- or hyperosmotic stress, as would be expected for osmolytes (Kirst, 1989).

A crucial step in the osmoregulation of photosynthetic microorganisms is the synthesis of osmolytes (Kirst, 1989). This work shows that three strains of *D.* cf. *sacculus* were able to accumulate nitrogen and sulfur containing osmolytes -proline, GBT and DMSP respectively- at the highest salinity (42) tested, when compared to the lowest (25), and even during hyperosmotic

(from 35 to 42) stress condition with GBT (strain Dsa-Lo) (Hagemann, 2011; Keller et al., 2004, 1999; Kirst, 1989).

To our knowledge, there is a lack of short-term (i.e. sampling times ≤ 24 h) studies on proline, GBT and DMSP contents in dinoflagellates subjected to rapid salinity stress conditions. However, time is a key parameter in osmoregulation (Gwinn et al., 2019; Hagemann, 2011). After acclimation of a strain of *Prorocentrum minimum* (salinities from 16 to 36), Gebser and Pohnert (2013) showed a constant level of DMSP, while a 20-fold increase in GBT was observed after hyperosmotic acclimation. Caruana et al. (2020) observed an increase in DMSP in one strain of *Alexandrium minutum* (only on a per-cell basis) after hyperosmotic acclimation (38), whereas GBT content decreased after hypoosmotic acclimation (33) in one strain of *A. pacificum*. Overall, these studies showed the important role of GBT, and to a lesser extent of DMSP, in the osmoregulation process of toxin-producing phytoplankton, even after acclimation. This observation may be explained by the fact that DMSP is a less efficient osmolyte than GBT and proline (Kirst, 1996; Rhodes et al., 2002), the latter being one of the most efficient osmolytes (Kirst, 1989). Caruana and Malin (2014) classified DMSP as a "major osmolyte" for *D. acuminata* but according to our study, DMSP was likely to be a "medium to minor osmolyte" in *D.* cf. *sacculus*, with a maximum increase of 16 mM between salinity 35 and 42 for Dsa-Lo. Data from Caruana and Malin (2014) were obtained from a field study on a natural population of *D. acuminata* by Jean et al. (2005), who reported 477 mM of DMSP. The difference could be attributed to the fact that in the environment, sulfur-derived compounds (e.g. DMSP) may represent a more important part of synthetized osmolytes than nitrogen-derived compounds (e.g. proline or GBT), because nitrogen is usually more limiting than sulfur (Gwinn et al., 2019; Keller et al., 1999; Raven and Giordano, 2016; Rhodes et al., 2002).

Whether the adjustment of proline, GBT and DMSP concentrations by *D.* cf. *sacculus* is the only mechanism of osmoregulation for this species/genus and whether this synthesis is effective enough to maintain intracellular homeostasis during salinity stress remains to be elucidated.

Despite the absence of discrimination between control and hypo- hyperosmotic stress conditions by the LC-HRMS analysis, several features were significantly affected by the different salinities (Table S2 and Figure S1), including EPA and adenosine. The polyunsaturated fatty acid (PUFA) EPA is mainly observed in marine eukaryotic organisms (especially diatoms and dinoflagellates) (Peltomaa et al., 2019) and is an essential fatty acid for heterotrophic consumers. It has for instance a beneficial effect on growth and fecundity of marine organisms, and it prevents or treats several human diseases (Bajpai and Bajpai, 1993; Galloway and Winder, 2015; Okuyama et al., 2008). EPA produced by toxic dinoflagellates and raphidophytes displayed toxic effects when associated with ROS and were suggested to be involved in prey capture mechanism of *M. rubrum* by *Dinophysis* (Mafra et al., 2016 and references therein). A meta-analysis on phytoplankton fatty acid profiles according to environmental conditions by Galloway and Winder (2015), showed a negative correlation between dinoflagellates long-chain essential fatty acids (e.g. EPA) and salinity. These results support our observation, and may be explained by modification of fatty acid metabolism by salinity variation (Kirst, 1990).

The other unidentified features may also play a role in the osmoregulation process. In the literature, some other potential osmolytes such as carbohydrates (Rhodes et al., 2002) or polyols (Borowitzka, 2018; Gebser and Pohnert, 2013) have been described but their mechanism of action are still unknown. Still, the absence of effects of salinity on metabolic profiles consistent between strains may also be attributed to the fact that: (1) cell densities of *D. sacculus* cultures were low, possibly giving rise to a lack of sensitivity with our LC-HRMS analysis and/or (2) only

the methanol-soluble part of the metabolome was analyzed which may not be optimal for the extraction of highly polar molecules. Indeed, one of the main difficulties of this experiment was to obtain dense cultures before the experiment, a fact that limited the number of extractions.

The dinoflagellate *D. sacculus* is known for its preference for semi-enclosed areas (Zingone et al., 1998) which are, as many coastal areas, dynamic environments subjected to rapid changes in salinity, notably during extreme events which are likely to be more frequent with global change (Fu et al., 2012; IPCC, 2013; Skliris et al., 2014). Two out of the three strains of *D.* cf. *sacculus* used in this study were isolated from semi-enclosed environments, from the Atlantic (Dsa-Me, Arcachon Bay) and Mediterranean (Dsa-Th, Thau Lagoon) coasts respectively, while the third strain (Dsa-Lo, Loscolo) originated from an estuarine region from the Atlantic coast (i.e. Vilaine estuary). These three sites show salinity fluctuations (Figure 1). Indeed, while the mean salinity was around 35 and quite stable in Meyran-Arcachon (between 30.1-35.7), it could decrease down to 25.3 at Loscolo, a location subjected to fresh water releases from a dam. On the opposite, the salinity at Thau Lagoon (Crique de l'Angle) could reach up to 41.3. Salinity in this Mediterranean lagoon is influenced by the balance between evaporation, rainfall and exchange with the sea among other factors (Collos et al., 2009). Importantly, *Dinophysis*, including *D.* cf. *sacculus*, were observed in these locations at all salinities reported (Gaillard et al., 2020; REPHY, 2019). Other *Dinophysis* species, such as *D. acuminata*, a species genetically close to *D.* cf. *sacculus* (Séchet et al. 2021), have been shown to occur in a wide range of salinities. Indeed, in Chile, *D. acuminata* has been found at salinities between 5 to 31 (Diaz et al., 2011) and at salinities 8 to 27 in North East USA (Hattenrath-Lehmann and Gobler, 2015), reflecting an important tolerance to salinity fluctuations. Moreover, in a recent study (Fiorendino et al., 2015), the *in vitro* salinity tolerance after acclimation of two strains of *D. acuminata*-complex, *D. ovum*

(DoSS3195) and *D. acuminata* (DAVA01) was highlighted by a positive growth rate over a range from 22 to 34, although the optimal growth was observed between 22 and 26. Those results are consistent with our observations, showing the tolerance of coastal species of the *D. acuminata*-complex, including *D.* cf. *sacculus*, to abrupt changes in salinity, which seems not surprising knowing their habitats (e.g. thin layers, estuaries and lagoons). In addition, our data showed that even after years of acclimation in the lab at salinity 35, the three strains were not affected by salinity in terms of growth, biovolume toxin and other metabolite contents after short-term stress, which suggested that they can maintained their ability to cope with sudden salinity fluctuations. For the first time, we highlighted that *D.* cf. *sacculus* can respond to rapid environmental changes by displaying mechanisms of osmoregulation. Whether this response is solely due to variations of the known measured osmolytes (i.e. proline, GBT and DMSP) remains to be demonstrated, as these variations were also sometimes not significantly different from the control condition (except for GBT content in the strain Dsa-Lo), but simply showed a coherent trend from the lowest to the highest salinity.

In future studies, it would be interesting to measure the ion concentrations, such as sodium, potassium and ion fluxes, as well as the activity of ion channels, known to mediate a rapid response to salinity stress (Hagemann, 2011), with epifluorescence, NMR or radiotracer methods during osmotic stress (Hagemann, 2016; Louzao et al., 2006). It also appears fundamental to investigate kinetics of the synthesis of osmolytes with longer-term (> days) responses to salinity stress measurement and/or acclimation of several species of the genus *Dinophysis* including different chemotypes and non-toxic species. Finally, identification of all molecular features significantly affected by salinity in this study is challenging but necessary in order to better understand and characterize the osmoregulation of *Dinophysis* and other toxic phytoplankton.

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Figures and tables

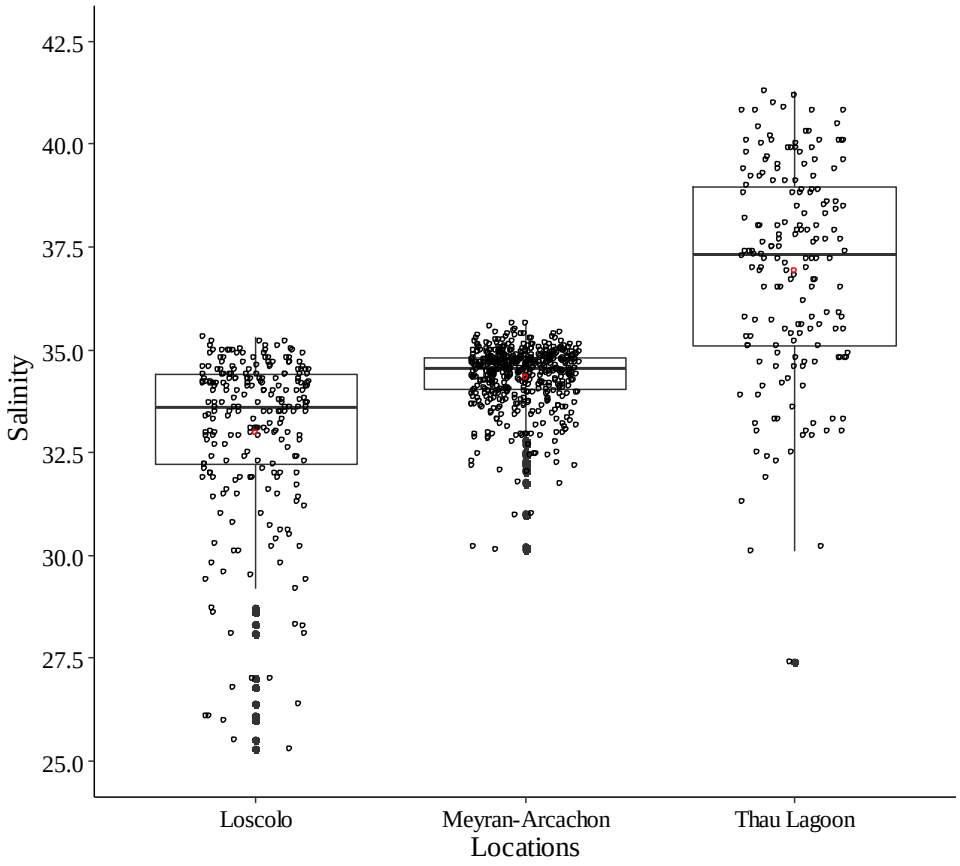

Figure 1: Box plot of the sea water salinity at the three locations where the studied strains of *Dinophysis* cf. *sacculus* were isolated. The 1st and 3rd quartiles are represented by the lower and upper limits of the box, respectively, with the median in bold horizontal line. Error bars represent both lower and higher values within 1.5 times interquartile range below the 1st and above the 3rd quartiles, with outside values (plain dot) below or above these limits. Mean salinities are represented by red circles. Data were extracted from the REPHY database from 2015 to 2018 and correspond to weekly (September to May) or biweekly (June-July-August) salinity measurements of sea water samples by a conductivitymeter. n = 238, 500 and 174 for Loscolo, Meyran-Arcachon and Thau Lagoon, respectively.

Table 1: Origin and culture conditions of the strains used in this study

Species	Location	Isolation date	Strain ID	Code	Medium	Temperature (°C)	Irradiance [a] (µmol photons m^{-2} s^{-1})
Teleaulax amphioxeia	Huelva (Spain)	2007	AND-A0710	-	L1-Si	17.8 ± 0.60	~ 100
Mesodinium rubrum	Helsingør Harbor (Denmark)	2009	MBL-DK2009	-	L1/20-Si	17.8 ± 0.60	~ 100
Dinophysis cf. *sacculus*	Loscolo-Vilaine estuary (French Atlantic)	2016	IFR-DSA-01Lo	Dsa-Lo	Sterilized sea water	17.8 ± 0.60	~ 100
	Meyran-Arcachon (French Atlantic)	2015	IFR-DSA-01Me	Dsa-Me	Sterilized sea water	17.8 ± 0.60	~ 100
	Thau Lagoon (Crique de l'Angle) (French Mediterranean)	2017	IFR-DSA-02Th	Dsa-Th	Sterilized sea water	17.8 ± 0.60	~ 100

[a] Cultures were subjected to light in the PAR domain with a circadian cycle 12 h: 12 h (light: dark)

Table 2: Growth rate (μ, d^{-1}) and biovolume ($x10^3$ μm^3) of the 3 strains of *Dinophysis* cf. *sacculus* after 24 h of salinity stress at 25 and 42 and for the control. Values are expressed as mean ± SD (n = 3 for μ and n ≥ 30 for biovolume).

Strain	Salinity	μ (d^{-1})	Biovolume ($x10^3$ μm^3)
Dsa-Lo	25	0.17 ± 0.06	13.2 ± 1.67
	35	0.23 ± 0.13	14.1 ± 1.70
	42	0.33 ± 0.06	12.4 ± 1.50
Dsa-Me	25	0.25 ± 0.04	14.0 ± 1.75
	35	0.16 ± 0.04	13.4 ± 1.77
	42	0.20 ± 0.06	13.8 ± 1.75
Dsa-Th	25	0.28 ± 0.10	14.1 ± 1.77
	35	0.18 ± 0.05	14.2 ± 2.29
	42	0.20 ± 0.11	14.5 ± 1.65

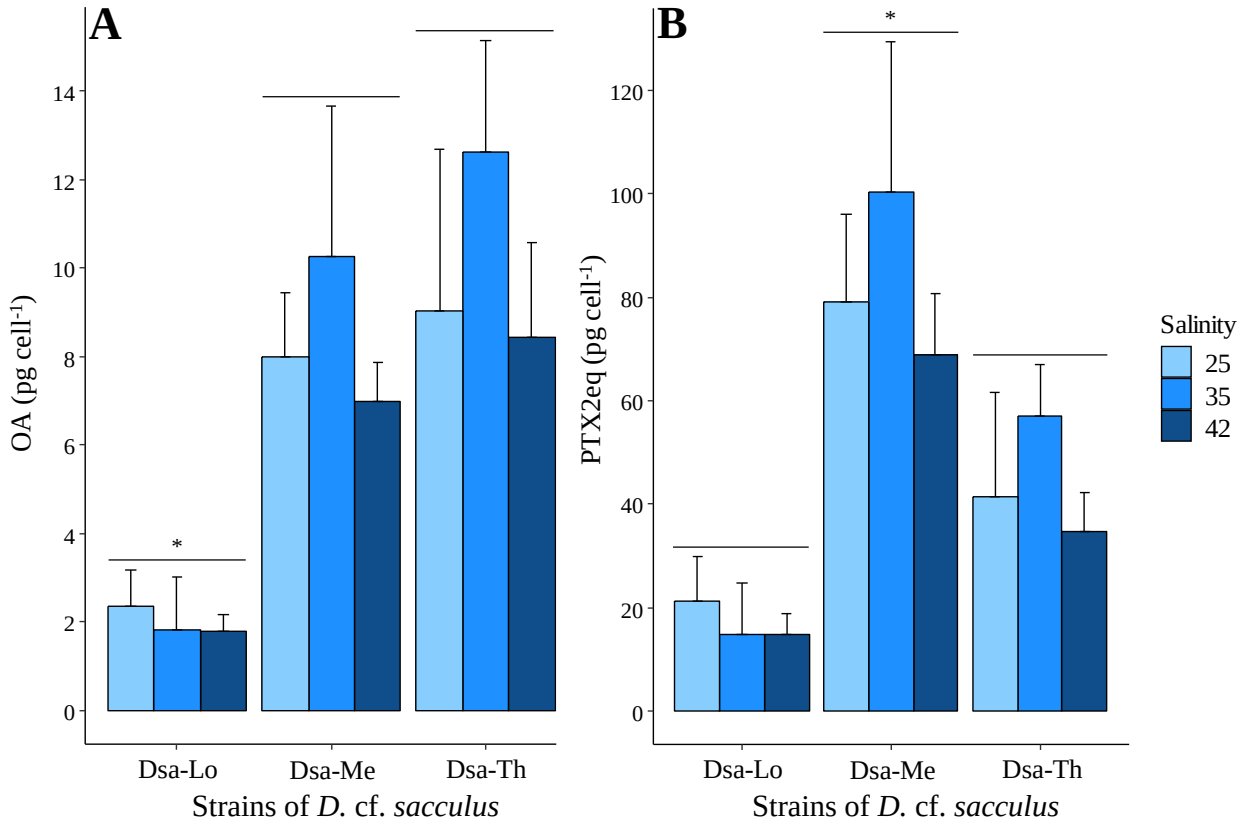

Figure 2: Total (sum of intracellular and extracellular in pg cell^{-1}) of **A.** okadaic acid (OA) and **B.** pectenotoxin 2 eq (PTX2eq) in the three strains of *Dinophysis* cf. *sacculus* after 24 h of salinity stress at 25 and 42 and for the control. Values are expressed as mean ± SD (n = 3). Treatments with asterisks were significantly different.

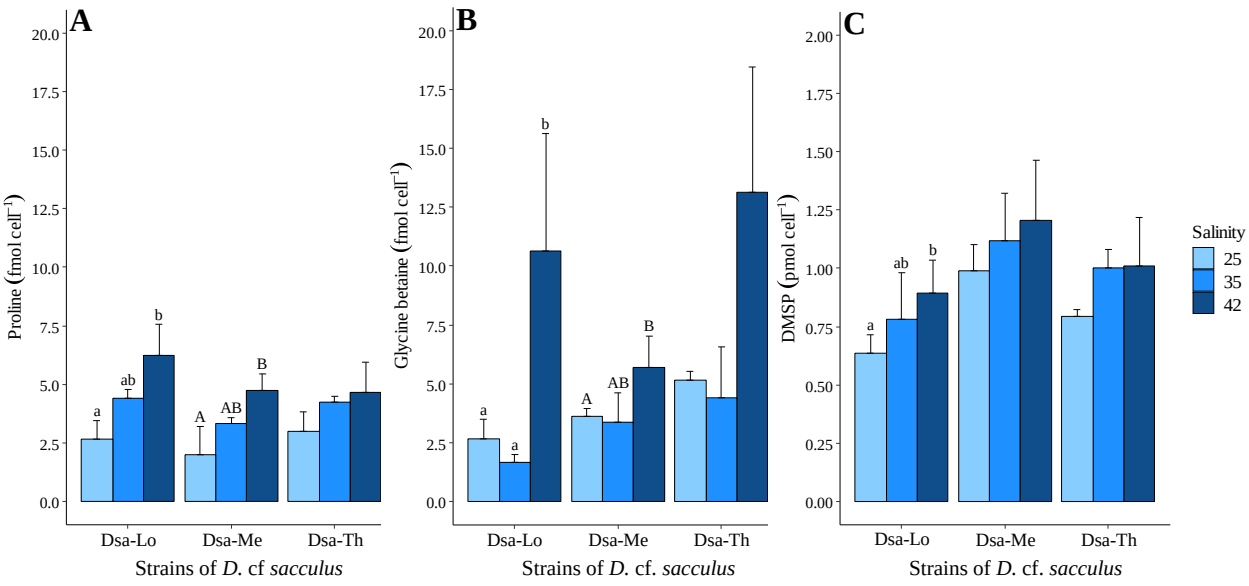

Figure 3: Intracellular concentration of **A.** proline (fmol cell^{-1}), **B.** glycine betaine (fmol cell^{-1}) and **C.** DMSP (pmol cell^{-1}) in the three strains of *Dinophysis* cf. *sacculus* after 24 h of salinity stress at 25 and 42 and for the control. Values are expressed as mean ± SD (n = 3). Conditions with different superscript letters were significantly different.

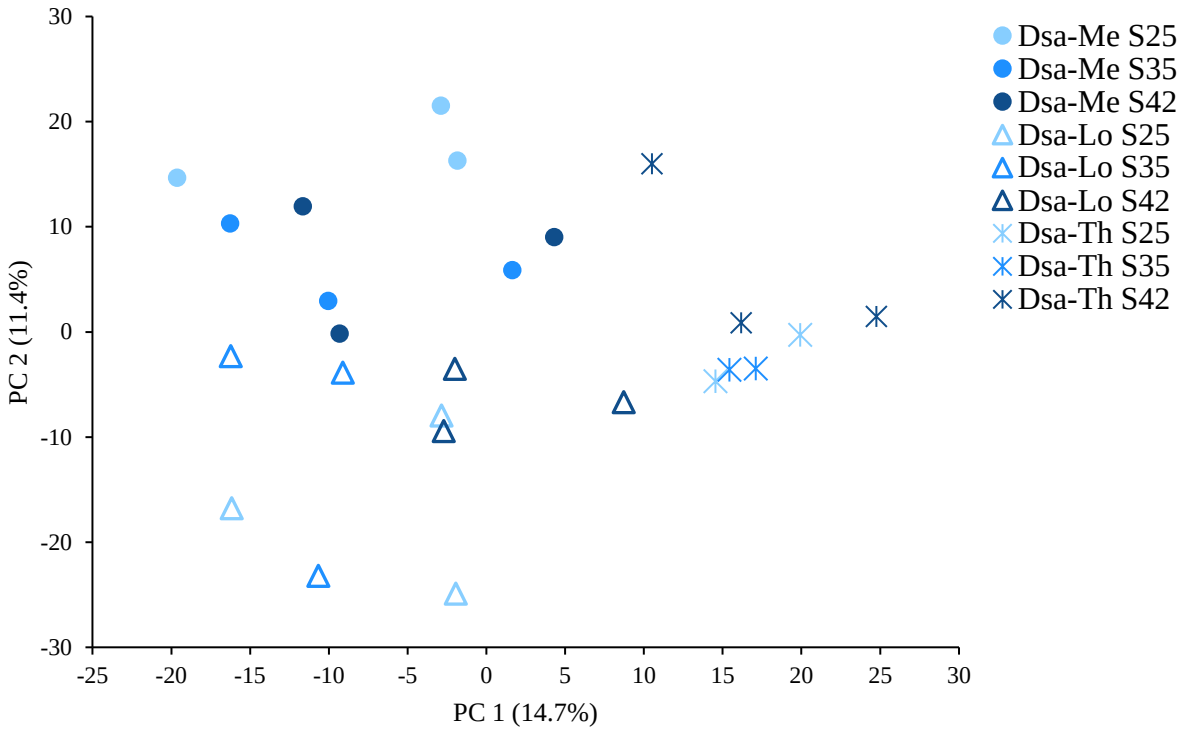

Figure 4: Principal component analysis (PCA) score plot obtained from LC-HRMS profiles of the three *Dinophysis* cf. *sacculus* strains exposed to the three salinities (S)

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