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Influence of sudden salinity variation on the physiology and domoic acid production by two strains of *Pseudo-nitzschia australis*

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Abstract :

Several coastal countries including France have experienced serious and increasing problems related to *Pseudo-nitzschia* toxic blooms. These toxic blooms occur in estuarine and coastal waters potentially subject to fluctuations in salinity. In this study, we document for the first time the viability, growth, photosynthetic efficiency and toxin production of two strains of *Pseudo-nitzschia australis* grown under conditions with sudden salinity changes. Following salinity variation, the two strains survived over a restricted salinity range of 30 to 35, with favorable physiological responses, as the growth, effective quantum yield and toxin content were high compared to the other conditions. In addition, high cellular quotas of domoic acid (DA) were observed at a salinity of 40 for the strain IFR-PAU-16.1 in comparison with the other strain IFR-PAU-16.2 where the cell DA content was directly released into the medium. On the other hand, the osmotic stress imposed at lower salinities, 20 and 10, resulted in cell lysis and a sudden DA leakage in the medium. Intra-specific variability was observed in growth and toxin production, with the strain IFR-PAU-16.1 apparently able to withstand higher salinities than the strain IFR-PAU-16.2. On the whole, DA does not appear to act as an osmolyte in response to sudden salinity changes. Since most of the shellfish harvesting areas of bivalve molluscs in France are located in areas where the salinity generally varies between 30 and 35, *Pseudo-nitzschia australis* blooms might potentially impact public health and commercial shellfish resources in these places.

Keywords : Amnesic shellfish poisoning, domoic acid, harmful algae, *Pseudo-nitzschia australis*, salinity stress

Abbreviations

API 4000 Trap, triple quadrupole mass spectrometer; ASP, amnesic shellfish poisoning; DA, domoic acid; dDA, dissolved domoic acid; F_v/F_m, effective quantum yield,; LOQ, Limit of quantification; LOD, Limit of detection; QY, quantum yield; UFLC, ultra-fast liquid chromatography

Introduction

The genus *Pseudo-nitzschia* is distributed throughout the world. At least 52 species are currently described of which 26 are known to produce a neurotoxin called domoic acid (DA; Ajani et al. 2018, Gai et al. 2018, Lundholm 2018). The consumption of DA-contaminated shellfish at concentrations higher than the EU-regulatory limit of 20 mg DA · kg^{-1}, can cause severe amnesic shellfish poisoning (ASP; Visciano et al. 2016). The human illness associated with DA intoxication is characterized by gastrointestinal symptoms like nausea, cramps, vomiting and diarrhoea followed by neurological symptoms including severe headache, seizures, short term memory loss, coma and in severe cases, death (Lefebvre and Robertson 2010, La Barre et al. 2014, Tasker 2016). In addition to public health impacts, mortality events of marine birds and mammals linked to DA in the food web are also a serious ethical and economic issues (Beltrán et al. 1997, Gulland 2000, Pulido 2008). In recent decades, several species of toxic *Pseudo-nitzschia*, including *P. australis*, have been repeatedly reported along French coastlines (Amzil et al. 2001, Nezan et al. 2006, Husson et al. 2016) as well as in many other parts of the world such as the North and South Americas (Anderson et al. 2006, Bargu et al. 2008, Mather et al. 2010), other parts of Europe (Lundholm et al. 2006, Churro et al. 2009, Zapata et al. 2011, Orive et al. 2013), Asia and Africa (Hallegraeff 2004, Hasle and Lundholm 2005, Teng et al. 2016, Louw et al. 2018). Moreover, the species *P.*

australis is considered one of the most toxic species worldwide (Thessen et al. 2009, Trainer et al. 2012). It was identified for the first time in France in 2004 as the main source of DA contamination in king scallops (*Pecten maximus*; Nezan et al. 2006, Amzil et al. 2007, Klein et al. 2010). Toxic blooms of *Pseudo-nitzschia* species appear to be increasing in frequency and toxicity (Trainer et al. 2009). Several studies have examined the environmental factors that may affect toxin production in *Pseudo-nitzschia*, such as pH, salinity, temperature, irradiance, nutrient availability (silicate, phosphate, nitrate, and iron) and the presence of bacteria and copepod grazers (Pan et al. 1998, Cusack et al. 2002, Howard et al. 2007, Lelong et al. 2012, Tatters et al. 2012, Haroardóttir et al. 2015, Tammilehto et al. 2015). In addition, DA production may vary according to the inter- and intra-specific diversity commonly highlighted for the *Pseudo-nitzschia* genus, along with several physiological factors such as the age of the culture and the growth phase (lag, exponential and stationary phases). This highlights the difficulties in predicting the initiation of *Pseudo-nitzschia* blooms and DA production in the environment (Lundholm et al. 1997, Bates and Trainer 2006, Lelong et al. 2012, Kim et al. 2015, Godhe and Rynearson 2017).

Most of the *Pseudo-nitzschia* species are euryhaline (i.e., able to grow in a wide range of salinities, and can inhabit coastal areas exposed to significant and long lasting changes in sea water salinity; Jackson et al. 1992, Lundholm et al. 1997, Bates 1998, Thessen et al. 2005). The increase in frequency of storm tides, rise in water temperatures, altered precipitation and run-off can all cause the salinity level of coastal aquatic and estuarial ecosystems to change, and major alterations might be expected in phytoplankton diversity, dominance and toxin production (Stocker et al. 2013). According to several studies on climate change, salinity alterations have already been observed and are predicted to intensify worldwide (IPCC 2007). The consequences of such changes in water salinity may have an impact on the frequency, intensity and the duration of *Pseudo-nitzschia* blooms and their

ability to produce toxins (Hallegraeff 2010, Wells et al. 2015, Van Meerssche and Pinckney 2017). To our knowledge, very few studies have been conducted on the effect of salinity variation on growth and DA production by other *Pseudo-nitzschia* species (*P. multiseries*, *P. delicatissima*, *P.pungens*, *P. calliantha*), and those studies that were carried out tested only one acclimated strain as a representative of the studied species (Lundholm et al. 1997, Thessen et al. 2005, Doucette et al. 2008, Van Meerssche and Pinckney 2017, Pednekar et al. 2018). During cell growth in hypersaline conditions, diatoms can establish cellular adaptation mechanisms in order to compensate the osmotic pressure (Schobert 1974). The osmotic balance can be achieved by ion accumulation mechanisms (sodium uptake) or by the synthesis and accumulation of osmolytes, which are low-molecular weight organic solutes (Liu and Hellebust 1976, Schobert 1977, Masmoudi et al. 2013). In diatoms, osmolytes are commonly amino acids like proline, lysine, taurine, alanine, arginine, glutamic acid and aspartic acid (Fujii et al. 1995). However, while Doucette et al. (2008) and Pednekar et al. (2018) noticed an increase in DA production with increasing salinity in *P. multiseries* and *P. pungens* cells, the potential physiological role of DA as an osmolyte have not yet been fully confirmed (Doucette et al. 2008, Lelong et al. 2012, Pednekar et al. 2018).

According to an FAO survey, France is the top shellfish producer in the EU, with an estimated production of 187,420 tons in 2002 for a turnover of 371 million euros (Food and Agricultural Organization FAO 2005). The present article reports for the first time, the effect of salinity shock on two strains of *P. australis* isolated from French waters and grown in batch cultures. The two studied strains were subjected to five different salinity conditions (10, 20, 30, 35 and 40) and their growth, effective quantum yield (F_v/F_m) and toxin production were measured following the salinity shock. The aim of this study is to better understand the physiology of these toxic *Pseudo-nitzschia* strains, the possible role of DA as an osmolyte in the cells and to test whether the toxicity of *Pseudo-nitzschia* cells might be dependent on

salinity. The overall objective is to investigate the effect of instantaneous salinity changes on *P. australis* cells and their ability to produce toxin. The acquired knowledge will be made available to help predict potential *P. australis* toxic blooms and their economic and health impacts on shellfish resources and local communities.

Materials and methods

Culture conditions

Two strains of *Pseudo-nitzschia australis* were used in this study, strains IFR-PAU-16.1 and IFR-PAU-16.2, which were isolated in May 2016 from the Bay of Arcachon, located on the south-western Atlantic coast of France (44°40' N, 1°10' W). Both strains were confirmed to produce domoic acid. The cultures were grown in 1 L polystyrene culture flasks containing sterile L1 medium (Guillard and Hargraves 1993). Natural seawater was filter-sterilized through a 0.22 µm polycarbonate membrane filter (Corning, NY, USA) and enriched with L1 media with additional silicate for a final concentration of 100 µM as described by Guillard and Hargraves (1993). The *P. australis* strains were grown in batch cultures and routinely maintained in a temperature-controlled room at 16°C, exposed to a photon flux density of 120 µmol photons $\cdot$ m^{-2} $\cdot$ s^{-1} (fluora and cool-white fluorescent light, Osram, Germany) in a 12 : 12 h light:dark cycle. Natural seawater obtained from the English Channel, with a salinity of 35, was selected as a control medium for comparison of salinity-induced stress. The natural seawater was diluted with distilled water to reach the salinities 10, 20 and 30. To provide a salinity treatment of 40, seawater was freeze-concentrated.

Experimental set-up

Pseudo-nitzschia australis strains IFR-PAU-16.1 and IFR-PAU-16.2 were cultured for one week in L1 growth medium at salinity 35 (i.e., the same salinity that was subsequently used as a control condition in the experiment). The cells were then centrifuged for 5 min at 150*g* and 25°C and transferred into new culture media of different salinities (10, 20, 30, 35 and 40). Initial inocula concentrations for strains IFR-PAU-16.1 and IFR-PAU-16.2 before centrifugation were 17×10^3 cells $\cdot$ mL^{-1} and 16×10^3 cells $\cdot$ mL^{-1}, respectively. Triplicate cultures of each salinity condition were incubated in 1 L polystyrene bottles (Nest Biotechnology, China) at 16°C, with 120 µmol photons $\cdot$ $m^{-2} \cdot s^{-1}$ photon flux density and 12:12 h light – dark cycle. Monitoring was carried out 4 h after the salinity shock (T_0), than daily for 4 d (T_1, T_2, T_3 and T_4) at a fixed time corresponding to the middle of the light cycle. Percentage cell mortality was calculated with data collected four h after the transfer of cultures to the different salinities. Calculation was based on the differences between cell counts of the control culture at salinity 35 and those at the different salinities (10, 20, 30, 35 and 40).

Physiological measurements

A daily sampling of 35 mL of each culture was used in order to measure cell growth, effective quantum yield (QY) F_v/F_m, and DA content. Growth was monitored by daily cell counts of Lugol fixed samples (1% final concentration). A minimum of 400 viable cells were counted using a Nageotte counting chamber with a light microscope (Leica Microsystems Switzerland) at 10x magnification. When growth occurred, the specific growth rate for the mean value of the triplicate cultures ($\mu \cdot d^{-1}$) was calculated for the period of the first three

days. When the strains did not grow, negative growth rates were calculated one day after the salinity stress according to the equation given in by Guillard (1973):

$\mu = (\ln N_1 - \ln N_3)/ (T_1 - T_3)$

where N is cell density at a given time (T).

A PhytoPAM Phytoplankton fluorometer (Walz, Effeltrich, Germany) was used for the measurements of the effective quantum yield (QY) of the primary photosystem PSII (F_v/F_m). For each culture, F_v/F_m was calculated via an assessment of the chlorophyll fluorescence yield and the saturation pulse using the following equation (Schreiber et al. 2002):

$QY = (F_m - F_0)/F_m$

where F_0 is the minimum fluorescence measured after a period of complete darkness, F_m is the maximum fluorescence obtained during a saturating light pulse when all reaction centers are closed, and $F_v = F_m - F_0$.

Toxin analyses

In order to determine the toxin content (particulate and dissolved in the medium) of the two *Pseudo-nitzschia australis* strains in different salinity treatments, 15 mL of culture were centrifuged (5 min, 3600*g*, 4°C) and separated into cell pellet and filtrate. DA concentrations in the particulate fraction were determined by extraction of the cell pellet in 1 mL methanol/water mixture (50/50: v/v) and stored at -80°C. Before analysis, the extraction was prolonged by 15 min of sonication in an ice-cold bath then centrifuged and filtered (15 min, 8000*g*, 4°C, 0.2 µm) to recover the supernatant (Amzil et al. 2007).

DA content in the dissolved fraction was extracted by solid phase extraction (SPE) using Agilent Bond Elut C18 200 mg cartridges. The SPE column was conditioned with 10 mL methanol followed by 10 mL of ultra-pure water. The sample was then passed through the SPE column after acidifying with 20% aqueous formic acid to give 0.2% formic acid. The cartridge was rinsed with 10 mL of 0.2% aqueous formic acid then dried for 1 minute. DA, adsorbed on the cartridge, was eluted with 1.5 mL of methanol/water (50/50: v/v) into a glass vial and stored at -80°C for later analysis.

DA analyses were performed using Ultra-Fast Liquid Chromatography (UFLC, Shimadzu) coupled to an ABSciex API 4000QTrap triple quadrupole mass spectrometer. The chromatographic separation was carried out on a Kinetex C18 column (150 × 2.1 mm, 2.6 µm, Phenomenex) equipped with a pre-column. Certified DA standard (CNRC, Halifax, Canada) was used for external calibration range in order to quantify DA in sea water. LOD and LOQ are respectively 0.1 and 0.25 ng · mL^{-1}.

Statistical analyses

The experiment was carried out in triplicate and the data presented are mean standard deviation (SD) for n=3. Statistical analyses were performed to determine whether the growth, effective quantum yield (F_v/F_m), or the DA content of the two tested strains significantly varied ($p < 0.05$) for the different salinity conditions. Data were analysed statistically using nonparametric Kruskal-Wallis tests followed by multiple comparison tests (H tests) to determine the source of significant differences. All comparisons were made against the salinity 35 control data and analyses were performed using R software version 3.4.1, 2017.

Results

Cell growth, effective quantum yield and domoic acid content (particulate, dissolved and total domoic acid) of the two strains of IFR-PAU-16.1 and IFR-PAU-16.2 were all influenced by the sudden changes in salinity conditions (Figs. 1-4).

The salinity stress led to an instantaneous decrease in cell density for all cultures, with a percentage of mortality ranging from 18% to 93% (Fig. 1). At salinities 20 and 10, the two strains showed a sharp increase in mortality (84% to 93% cell mortality), while small increases were observed at salinities 40 and 30 (18% to 38% cell mortality; see Table S1 in the Supporting Information, Fig. 1). Similarly, control cultures, at 35 salinity, showed a moderate cell mortality of 20% and 18% for strains IFR-PAU-16.1 and IFR-PAU-16.2, respectively (Table S1, Fig. 1). This was likely due to culture centrifugation and transfer to new media at the beginning of the experiment. At salinity 30, the two strains IFR-PAU-16.1 and IFR-PAU-16.2 showed high growth rates of $0.39 \cdot d^{-1}$ and $0.43 \cdot d^{-1}$, respectively. Moderately lower growth rates of $0.35 \cdot d^{-1}$ and $0.19 \cdot d^{-1}$ were observed at salinity 35 for strains IFR-PAU-16.1 and IFR-PAU-16.2, respectively. However, at salinities 10, 20 and 40, neither strain showed any growth at all (Figs. 1, 2), and negative growth rates were observed (e.g.,$-0.29 \cdot d^{-1}$, $-0.39 \cdot d^{-1}$, and $-0.04 \cdot d^{-1}$, respectively) in IFR-PAU-16.2 . After the salinity shock, growth recovery was observed at salinity 30 during the four days of culture, and a slight and progressive decrease in cell density was observed at salinity 40 (Fig. 2). In transfers to salinities 10 and 20, the osmotic shock led to cell lysis and very low cell densities. The highest cell concentrations were reached on days 3 and 4 for both strains at salinities 30 (26×10^3 and 21×10^3 cells $\cdot$ mL^{-1}) and 35 (26×10^3 and 20×10^3 cells $\cdot$ mL^{-1}; Fig. 2).

The salinity shocks of 20 and 10 for the two *Pseudo-nitzschia* strains also had a negative effect on the cellular photosynthetic efficiency (Fig. 3). The effective quantum yield F_v/F_m of the stock culture was 0.65 for both strains (Fig. 3). Following the salinity stress treatments, the F_v/F_m values declined in both strains, possibly as a consequence of the cell transfer process. However, cultures transferred at salinities 40, 35 and 30 showed a recovery of the effective quantum yield, as the F_v/F_m value remained stable throughout the growth period (Fig. 3). The F_v/F_m of cultures at salinity 40 decreased on the last two days of the experiment to minima of 0.47 and 0.38 for IFR-PAU-16.1 and IFR-PAU-16.2, respectively. The F_v/F_m yield was greatest for cultures at salinity 30 with values equal to 0.67 for IFR-PAU-16.1 and IFR-PAU-16.2, and similar for cultures at 35 salinity (0.65 for both strains; Fig. 3). However, cultures subjected to salinity stress of 20 underwent a drop in the F_v/F_m just after the transfer (0.11 and 0.09 for IFR-PAU-16.1 and IFR-PAU-16.2, respectively), whereas at salinity 10, photosynthetic efficiency was reduced to almost nothing (0.01 and 0.02, respectively).

The initial particulate domoic acid content of all cultures was 0.25 pg · cell^{-1} and 0.16 pg · cell^{-1} for IFR-PAU-16.1 and IFR-PAU-16.2, respectively. Neither strain survived the osmotic shock when subjected to salinity stress at 10 or 20 as this resulted in cell lysis (Fig. 2). Cell lysis effects were also observable on the dissolved DA concentration (Fig. 4, C and D), as high amounts of dissolved DA were detected in the culture medium from the beginning of the experiment and remained constant over the four next days (almost 3 ng · mL^{-1} for IFR-PAU-16.1 and IFR-PAU-16.2 at salinities 10 and 20). This shows that the entire DA cell content had been released into the culture medium. At salinity 40, particulate DA showed a slow and steady increase for IFR-PAU-16.1 (0.24 pg · cell^{-1} at day 4) and very low levels of particulate DA for IFR-PAU-16.2 (0.03 pg · cell^{-1} at day 4; Fig. 4, A and B). In addition, the highest total DA content (particulate and dissolved) differed substantially between the two

lowest salinities and other salinities tested. At salinities 30, 35 and 40, high amounts of DA were detected after the salinity shock. The highest total DA concentrations were reached in cultures at salinity 35 for strain IFR-PAU-16.1 (13.8 ng · mL^{-1}) and at salinity 30 for IFR-PAU-16.2 (15.2 ng · mL^{-1}). An increase was observed during the two first days, which corresponds to the early exponential growth phase (Fig. S1 in the Supporting Information, Fig. 4, E and F). This was followed by a phase where DA production slowed down and stabilized at days 3 and 4, corresponding to the late exponential and stationary growth phases (Fig. S1). A slow and steady increase in DA was observed for strain IFR-PAU-16.1 at salinities 30 and 40, and for IFR-PAU-16.2 at salinities 35 and 40 (Fig. 4, E and F). The large increase of total DA observed at the beginning of the experiment for salinities 35 and 30 resulted from the accumulation of DA inside the cell during the first day for IFR-PAU-16.1 (from 0.19 to 0.46 pg · $cell^{-1}$ for salinity 35) and the two first d for IFR-PAU-16.2 (from 0.10 to 0.78 pg · $cell^{-1}$ for salinity 30; Fig. 4, A and B). This was followed by the decrease of intracellular DA content until the end of the experiment due to toxin release into the culture medium (Fig. 4, C and D). Dissolved DA showed a rapid and progressive increase after the medium change for the salinities 30, 35 and 40 and reached elevated levels once cell concentrations reached a plateau. Dissolved DA reached its highest levels at salinity 35 for strain IFR-PAU-16.1 (7.8 ng · mL^{-1}) and 30 for strain IFR-PAU-16.2 (7.4 ng · mL^{-1}; Fig. 4, C and D). The concentration of dissolved DA in the medium was also high at salinity 40 for strain IFR-PAU-16.1 (5.1 ng · mL^{-1}). In contrast, the dissolved DA value for strain IFR-PAU-16.2 at salinity 40 remained steady and low (1.3 ng · mL^{-1} at day 4) throughout the experiment (Fig. 4, C and D).

Discussion

This study was the first investigation into the effects of salinity stress on two strains of the diatom *Pseudo-nitzschia australis*, considered to be one of the main species responsible for amnesic shellfish poisoning incidents worldwide (Bates 2000, Campbell et al. 2001, Husson et al. 2016). As reported in several studies, the expected future increases in salinity fluctuations in coastal and estuarine areas, brought about by climate change and anthropogenic pressure, could potentially affect the frequency and intensity of *Pseudo-nitzschia* blooms (IPCC 2007, McCabe et al. 2016). Our results indicate that such salinity fluctuations may also affect domoic acid (DA) production by *Pseudo-nitzschia* cells. *Pseudo-nitzschia australis* blooms have been reported every year in spring and autumn in many estuarine and coastal waters in France and other parts of the world (Hallegraeff 2004, Hasle and Lundholm 2005, Nezan et al. 2006, Husson et al. 2016). In particular, our experiments raise concern about the potential effects of rapid fluctuations of sea surface salinity as a result of rainfall or river discharge into the coastal areas and the effect of salinity increase during drought periods on cell viability and domoic acid content of *P. australis* cells. The results of this experiment clearly demonstrate that the studied strains can resist sudden salinity change over only a small range, here from 35 to 30.

Previous field and laboratory studies on other *Pseudo-nitzschia* species (*P. multiseries*, *P. pungens*, *P. calliantha*, *P. cuspidata*, and *P. fraudulenta*) have also reported impacts of salinity variation on growth and DA production (Lundholm et al. 1997, Thessen et al. 2005, Doucette et al. 2008, Thessen and Stoecker 2008, Markina and Aizdaicher 2016, Van Meerssche and Pinckney 2017, Pednekar et al. 2018). The results of these previous studies showed that high cell abundances and growth rates of *Pseudo-nitzschia* cells were associated with high salinity levels. A recent field study by Van Meerssche and Pinckney (2017) showed a positive correlation between dissolved DA concentrations and salinity. Their

findings showed an increase in *Pseudo-nitzschia* abundance with increasing salinity but with less DA release in the water. Our results are in agreement with the laboratory studies on *P. multiseries* and *P. pungens* strains acclimated to different salinities (Doucette et al. 2008, Pednekar et al. 2018). Doucette et al. (2008) concluded that the *P. multiseries* strain was able to maintain high growth rates and its greatest cellular DA concentrations at higher salinities (three- to seven-fold higher total DA concentrations at salinities 30 and 40 compared with salinities 10 and 20). Additionally, a recent study by Pednekar et al. (2018) showed that the highest growth rates were obtained at salinities of 15 to 25, and that higher and lower salinities resulted in a reduction of growth rate. This salinity range also corresponded to the area where this species was isolated and is commonly present in the Zuari and Mandovi estuaries in western India (Pednekar et al. 2018). The authors also found that DA production increased as salinity increased with the most produced at salinity 35. However, the lack of laboratory studies on the effect of sudden salinity changes on toxic *Pseudo-nitzschia* species makes comparisons of our finding difficult, especially knowing the wide variability within and between *Pseudo-nitzschia* species (Casteleyn et al. 2010, Moschandreou et al. 2012, Tesson et al. 2014).

In comparison to previous studies examining *P. australis* growth rates with different nitrogen sources, the growth rates observed in our study at salinities 35 and 30 (0.39 and 0.43 $\cdot$ d^{-1} respectively) were similar to those observed by Martin-Jézéquel et al. (2015; 0.33 to 0.60 $\cdot$ d^{-1}), and lower than those in Howard et al. (2007; 0.52 to 0.93 $\cdot$ d^{-1}). In addition, the cells maintained high effective quantum yield (F_v/F_m). However, cultures at salinity 40 showed a slow decrease in F_v/F_m while the biomass was rapidly decreasing suggesting that the effect of salinity stress was greater on cell division than on the effective quantum yield. Furthermore, in response to salinity stress at 10 and 20, the effective quantum yield (F_v/F_m) decreased severely, suggesting that the salinity drop to 10 and 20 was too excessive to allow the

maintenance and protection of the cell membrane and caused direct cell lysis. These responses are common in plants and microalgae under severe environmental stress (Pancha et al. 2015, Chokshi et al. 2017).

Likewise, salinity stress also had an impact on toxin production of particulate and dissolved DA. High amounts of dissolved DA were detected four hours after cultures were transferred from salinity 35 to salinities 10 and 20 due to cell lysis (lysed cells were observed under the microscope). The DA content of these cultures remained stable until the end of the experiment, while for salinities 30, 35 and 40, a progressive release of DA was observed over the four days of the experiment (Fig. 4, C and D). The highest cell particulate DA contents detected for strains IFR-PAU-16.1 and IFR-PAU-16.2 (0.46 pg · cell^{-1} and 0.76 pg · cell^{-1}, respectively) were lower than the values previously reported for *P. australis* strains studied by Buck et al. (Buck et al. 1992; 3 to 31 pg · cell^{-1}) and Trainer et al. (2000; 0.1 to 78 pg · cell^{-1}) and comparable to those measured by Campbell et al. (2001; 1.2 to 1.32 pg · cell^{-1}), Cusack et al. (2002; 0.21 to 0.98 pg · cell^{-1}) and Rhodes et al. (2002; 2 pg · cell^{-1}). The particulate DA content measured in *P. australis* strains by Martin-Jézéquel et al. (2015; 10.8 to 30.3 fg · cell^{-1}) and Howard et al. (2007; 0.48 fg · cell^{-1}), however, were much lower than the detected concentrations in our experiment. Furthermore, the highest cellular DA contents were observed in the cultures at salinities 35 and 30, where growth rates and the effective quantum yield were high. In suitable growth conditions, the DA content of *P. australis* seemed to appear in two stages: a first one where the DA content (particulate) is greater than the release (dissolved), (until days 1 and 2 for both strains) and a second where the release in the culture medium prevails. Similar patterns of DA production in *P. australis* strains have also been reported in previous studies (Garrison et al. 1992, Howard et al. 2007, Martin-Jézéquel et al. 2015), where the highest cell DA contents were measured during the exponential or late-exponential growth phases. It was suggested that during active growth

(exponential growth phase) in which no nutrients were limiting and the effective quantum yield (F_v/F_m) was elevated, a large amount of metabolic energy was generated to fulfil the requirements of both growth and DA biosynthesis (Bates 1998, Maldonado et al. 2002). It is noteworthy that the majority of previous studies (Pan et al. 1996, Bates 1998, Doucette et al. 2008, Martin-Jézéquel et al. 2015) usually reported enhanced cellular DA levels during the mid-stationary growth phase in *P. multiseries* strains, and it has been speculated that the reduction or cessation of cell division during this phase could allow the cells to accumulate a higher particulate DA content, especially when nutrients (i.e., silicate and phosphate) are depleted in the medium. This variability in toxin production phase may be attributable to the wide interspecific diversity of *Pseudo-nitzschia* genus as well as changes in cell physiology (cell size, age of the culture) or their initial environmental conditions (Bates and Trainer 2006, Thessen et al. 2009, Moschandreou et al. 2012, Trainer et al. 2012, Gai et al. 2018). This exacerbates the difficulties of comparing different studies and deriving conclusions about the behavior observed.

The stress imposed by raising the salinity up to 40 induced the accumulation of high amounts of particulate DA in the strain IFR-PAU-16.1, while for the strain IFR-PAU-16.2, a much lower particulate DA content were observed, associated with a rapid increase of DA in the medium. These results suggest that IFR-PAU-16.1 had higher salinity tolerance (salinity 30 to 40) than IFR-PAU-16.2 (30 to 35) in terms of toxin production. Likewise, previous laboratory experiments conducted by Parkhill and Cembella (1999) on the marine dinoflagellate *Alexandrium tamarense* also showed that the sudden transfer of cells from salinity 30 into medium of 10 and 15 reduced toxin production, and caused an instantaneous leakage of toxins from the cells. Guerrini et al. (2007) found that the amount of yessotoxin released into the medium by the dinoflagellate *Protoceratium reticulatum* decreased as

salinity increased , the highest amounts of cellular toxin was produced at intermediate salinity, with decreases at both lower and higher salinity levels.

Following a fluctuation in salinity, diatoms have been shown capable of producing and accumulating organic osmolytes in order to maintain cellular osmotic balance (Masmoudi et al. 2013). For example, Schobert (1974) reported high levels of proline in *Cyclotella meneghiniana* subjected to high salt concentrations. The levels of proline and lysine in *Nitzschia ovalis* were likewise found to increase as salinity went up (Garza-Sánchez et al. 2009). A study by Jackson et al. (1992) reported an increase in glutamic acid and taurine production in *Pseudo-nitzschia pungens* and *P. multiseries* following an increase in salinity conditions. Given that DA is highly structurally similar to common osmolytes in diatoms, like glutamic acid and aspartic acid (Lefebvre and Robertson 2010), it is tempting to suggest that DA acts as an osmolyte in response to increasing salinity as put forward by Bates (1998). In addition, Doucette et al. (2008), Van Meerssche and Pinckney (2017) and Pednekar et al. (2018) showed an increase in DA production with rising salinity levels, reinforcing the hypothesis of Bates (1998). In our study, the concentration of any osmolyte would be expected to decrease in cells transferred to lower salinity and to increase in cells transferred to higher salinity. Whereas a decrease in particulate DA was observed for strain IFR-PAU-16.1 when the salinity was decreased to 30, and a slow increase was found when cells were transferred to salinity 40. Particulate DA content remained lower at salinity 40 than at salinity 35. Likewise, higher particulate DA content were observed at salinity 30 than at salinities 35 for the strain IFR-PAU-16.2. When strain IFR-PAU-16.2 was grown at salinity 40, it produced lower amounts of DA that were directly released into the culture medium (no accumulation observed), probably because the salinity stress was too severe for this strain and damaged the cells. Therefore, with regard to abrupt salinity changes, DA does not seem to act as an osmolyte in *P. australis* cells in either of the two strains studied here.

Furthermore, intra-specific variability was observed, as strain IFR-PAU-16.1 seemed to withstand higher salinities than strain IFR-PAU-16.2 in terms of growth capacities and DA content, with an optimal salinity at 35, whereas this was 30 for IFR-PAU-16.2. The two strains of *P. australis* tested withstand and tolerate fluctuation in the salinity range 30 to 35, in which cells continued to proliferate and produce high amounts of DA. As these salinities generally correspond to the areas along the French coast most heavily harvested areas for bivalve molluscs, ASP could be expected in these places. However, the osmotic stress imposed at salinities 20 and 10 resulted in cell lysis, inhibition of toxin biosynthesis and an immediate DA leakage into the medium. Thus, further investigations on the effect of salinity variation, done in acclimation mode rather than with a shock, would provide interesting complementary knowledge on the potential response of *P. australis* cell physiology, particularly regarding DA production in an environment of variable salinity.

Conclusion

In recent decades, 26 toxic *Pseudo-nitzschia* species have been reported in the world's oceans (Amzil et al. 2001, Trainer et al. 2009, Gai et al. 2018). These habitats are widely used as shellfish growing and harvesting areas and are often subjected to large fluctuations in salinity. These variations in salinity are influenced by various natural and/or anthropogenic environmental factors (heavy rain, river discharge, high evaporation). The results of our study revealed that the two studied strains show similar behaviour in response to sudden salinity variations, with a limited range of salinity tolerance between 30 and 35, and an intra-specific variability in term of optimal growth and DA content. Our result does not support the hypothesis of DA acting as an osmolyte in *Pseudo-nitzschia australis* cells subjected to rapid salinity change. Moreover, deviation from optimal salinities towards lower and higher levels (salinity 20 and 40) can stress cells, leading potentially to cell lysis, reduced growth, photosynthetic efficiency, and effects on DA production and release. These results indicates

that *P. australis* is a salinity-sensitive species that will not thrive in coastal areas after large salinity fluctuations. It would now be useful to perform further laboratory studies on the impact of salinity variation on cell physiology and DA production mechanisms in acclimation mode.

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Figure 1: Percentage mortality and growth rate (μ) in strains IFR-PAU-16.1 and IFR-PAU-16.2 exposed to a broad range of salinities: 10, 20, 30, 40 and 35 (control). Data are means ± SD, n = 3. (μ calculated between day 1 and 3 for salinities 30 and 35, and between day 0 and 1 for salinities 10, 20 and 40). * Significant difference between cultures grown at salinities 10 and 20 compared with the control at 35 (for strain IFR-PAU-16.1: $p = 3\times10^{-6}$, and $p = 6\times10^{-6}$, respectively and for strain IFR-PAU-16.2: $p = 3\times10^{-7}$, and $p = 2\times10^{-6}$, respectively).

Figure 2: Effect of different salinity conditions: 40, 35, 30, 20 and 10 on cell densities for in strains IFR-PAU-16.1 (A) and IFR-PAU-16.2 (B). Time 0 refers to the cell transfer to the new salinity treatment media. Data are means ± SD, n = 3.

Figure 3: Variation in the effective quantum yield $QY = F_v/F_m$ in strains IFR-PAU-16.1 (A) and IFR-PAU-16.2 (B) in response to salinity conditions 10, 20, 30, 35 and 40. Data are means ± SD, n = 3. * Significant difference between cultures at salinities 10 and 20 compared to the control at salinity 35 (for strain IFR-PAU-16.1: $p = 9\times10^{-25}$, and $p = 9\times10^{-23}$, respectively and for strain IFR-PAU-16.2: $p = 4\times10^{-26}$, and $p = 6\times10^{-11}$, respectively).

Figure 4: Particulate, dissolved and total domoic acid concentrations in strains IFR-PAU-16.1 (A, C, E) and IFR-PAU-16.2 (B, D, F) subjected to different salinity conditions: 40, 35, 30, 20 and 10. Data are means ± SD, n = 3.

Table S1: Individual data point of the percentage of mortality and growth rate (μ) in strains IFR-PAU-16.1 and IFR-PAU-16.2 exposed to a broad range of salinities: 10, 20, 30, 40 and 35 (control). Data are means ± SD, n = 3. (μ calculated between day 1 and 3 for salinities 30 and 35, and between day 0 and 1 for salinities 10, 20 and 40).

Figure S1: Semi-log plots of cell counts measured for the two strains IFR-PAU-16.1 and Ifr-PAU-16.2 grown at salinities 35 and 30.