Acclimation of the marine diatom *Pseudo-nitzschia australis* to different salinity conditions: effects on growth, photosynthetic activity and domoic acid content

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Abstract :

oxic *Pseudo-nitzschia australis* strains isolated from French coastal waters were studied to investigate their capacity to adapt to different salinities. Their acclimation to different salinity conditions (10, 20, 30, 35 and 40) was studied on growth, photosynthetic capacity, cell biovolume and domoic acid (DA) content. The strains showed ability to acclimate to a salinity range from 20 to 40, with optimal growth rates between salinities 30 and 40. The highest cell biovolume was observed at the lowest salinity 20 and was associated with the lowest growth rate. Salinity did not affect the photosynthetic activity; Fv/Fm values and the pigment contents remained high with no significant difference among salinities. An enhanced production of zeaxanthin was, however, observed in the late stationary and decline phases in all cultures except for those acclimated to salinity 20. In terms of cellular toxin content, DA concentrations were 2 to 3-fold higher at the lowest salinity (20) than at the other salinities and were combined with a low amount of dissolved DA. The fact that *P. australis* accumulate more DA per cell in less saline waters, illustrates that climate-related changes in salinity may affect *Pseudo-nitzschia* physiology through direct effects on growth, physiology and toxin content.

Keywords : Acclimation, climate change, domoic acid, *Pseudo-nitzschia australis*, physiology, salinity

List of abbreviations

API 4000 Trap, triple quadrupole mass spectrometer; ASP, amnesic shellfish poisoning; DA, domoic acid; dDA, dissolved domoic acid; pDA, particulate domoic acid; tDA, total domoic acid; F_v/F_m , effective quantum yield,; LOQ, Limit of quantification; LOD, Limit of detection; QY, effective quantum yield of the primary photosystem PSII; UFLC, ultra-fast liquid chromatography; μ , specific growth rate; chl *a*, chlorophyll *a* ; MgDVP, divinyl protochlorophyllide

Introduction

The genus *Pseudo-nitzschia* includes several toxic species that produce the toxin domoic acid (DA) which is harmful to humans. The toxigenic species are responsible for amnesic shellfish poisoning (ASP) events worldwide (Bates et al. 2018). The symptoms of such intoxications are characterized by nausea, vomiting, abdominal cramps, diarrhea, short-term memory loss, seizures, disorientation and in extreme cases, coma and death (Pulido 2008, Ramsdell and Gulland 2014, Schwarz et al. 2014). In recent decades, numerous reports have linked mortality events of sea birds, fish and sea mammals to the presence of toxic *Pseudo-nitzschia* blooms and the accumulation of high DA concentrations in these organisms (Scholin et al. 2000, Goldstein et al. 2008, Lefebvre et al. 2012). The worldwide distribution and occurrence of *Pseudo-nitzschia* outbreaks have been reported to appear on larger spatial and temporal scales in recent decades (Lim 2012, Husson et al. 2016, Louw et al. 2017, Bates et al. 2018). This expansion is likely associated with the accentuated effects of climate change and anthropogenic activities in aquatic ecosystems (Durack and Wijffels 2010, Skliris et al. 2014). For instance, Atlantic coastal waters of France were subjected to a destructive climate event when the storm Xynthia occurred in February 2010. The storm triggered highly toxic *Pseudo-nitzschia australis* blooms and afterwards caused an unusual DA accumulation in king scallops of $445 \text{ mg DA} \cdot \text{kg}^{-1}$, that greatly exceeded the safety thresholds ($20 \text{ mg} \cdot \text{kg}^{-1}$ of shellfish meat) in several areas (Pertuis Breton, Concarneau bay and Quiberon bay; Nézan et al. 2010, Ryckaert et al. 2010, Husson et al. 2016). Thereafter, the recurrence of toxic *Pseudo-nitzschia* blooms combined with a slow depuration of DA in king scallop tissues, extended the closures of the harvest areas for two years in Quiberon Bay (Ryckaert et al. 2010). Monitoring and temporal closures of contaminated shellfish grounds have proven to be beneficial for protecting human health because no further confirmed human DA poisoning have been reported since the 1987 Prince Edward Island (Canada) ASP outbreak (Todd 1993, Bates et al. 2018). The strategy has, however, also contributed to negative economic impacts on shellfish industries and local communities (Hoagland and Scatista 2006) due to closures of harvesting areas when DA concentrations in shellfish exceed the safety thresholds ($20 \text{ mg DA} \cdot \text{kg}^{-1}$; Anderson et al. 2000, Hoagland and Scatista 2006, Visciano et al. 2016).

In order to identify the environmental changes that trigger *Pseudo-nitzschia* occurrence, proliferation as well as toxin content, research have explored the effects of various physical, chemical

and biological factors such as photoperiod, pH, temperature, macronutrient concentrations (silicate, phosphate, and nitrogen), micronutrient availability (copper and iron), life stages and the presence of bacteria and grazers (Lundholm et al. 2004, Fehling et al. 2005, Kaczmarek et al. 2005, Hansen et al. 2011, Lelong et al. 2012, Thorel et al. 2014, Hardardóttir et al. 2015, Martin-Jézéquel et al. 2015, Sobrinho et al. 2017, Ayache et al. 2019). Few laboratory studies have, however, addressed the effect of salinity on the proliferation and DA content in *Pseudo-nitzschia* species, and most studies tested only one strain as a representative of the studied species (Lundholm et al. 1997, Doucette et al. 2008, Pednekar et al. 2018).

Given their wide distribution along most of the world's coastal and estuarine systems including Arctic and Antarctic waters (Almandoz et al. 2008, Hansen et al. 2011), *Pseudo-nitzschia* species might be considered able to adapt to a large salinity range (from salinity 6 to 45) (Thessen et al. 2005). In addition, climate change scenarios suggest that salinity variation may accentuate in the coming decades. Analyses of trends in global sea surface salinity changes over the period 1950-2008 have revealed that regions that are dominated by net evaporation are getting more saline, while regions dominated by net precipitation and runoff are getting less saline (Durack and Wijffels 2010, Terray et al. 2012). They also suggest that the Arctic Ocean becomes gradually less saline and the Atlantic waters become warmer and more saline throughout most of the basins. Salinity fluctuations are mostly related to changes in precipitation patterns, storm frequencies, melting of glaciers, river discharge, changes in wind direction and evaporation (IPCC 2007, Jewett and Romanou 2017). Such climate disturbances may result in salinity changes in coastal and estuarine waters, thereby potentially affecting *Pseudo-nitzschia* blooms and their capacity to produce toxin. Previous laboratory and field studies have shown that salinity may affect growth and DA content of *Pseudo-nitzschia* species (Lundholm et al. 1997, Thessen et al. 2005, Doucette et al. 2008, Van Meerssche and Pinckney 2017, Pednekar et al. 2018, Ayache et al. 2019), but with apparently contradictory results on toxin content, particularly in terms of salinity conditions. Some laboratory studies have e.g. shown increasing salinity to induce DA production rate in *P. multiseriata* and *P. pungens*, hypothesizing that DA may be implicated in osmoregulatory mechanisms in these species (Doucette et al. 2008, Pednekar et al. 2018), and a field study conducted by Van Meerssche and Pinckney (2017) have shown that *Pseudo-*

nitzschia species reduce the amount of DA found into the extracellular medium with increasing salinity levels. Cellular DA content was unfortunately not measured in the latter study.

A high genetic and physiological intra- and interspecific variability exists among strains of *Pseudo-nitzschia*. Among strains used in laboratory experiments, parameters such as strain age, cell size and geographical origin of the strain may also vary (Thessen et al. 2009, Moschandreu et al. 2012, Trainer et al. 2012, Gai et al. 2018). Therefore, comparisons among studies as well as extrapolations to other *Pseudo-nitzschia* species have to be done with care. A recent study conducted on the effect of sudden salinity changes on the physiology and toxin content of two *P. australis* strains showed that the favourable salinity range for growth, photosynthetic capacity and DA content was restricted to a salinity range of 30 to 35 (Ayache et al. 2019). In addition, high cellular DA quotas were found at salinity 40 for one strain, whereas in the other strain most of the DA was found in the medium at the same salinity, illustrating the intra-specific variability. A sudden change in salinity to higher or lower salinity levels (10, 20 and 40) resulted in growth inhibition, cell lysis and a larger proportion of DA found in the water (Ayache et al. 2019).

The present study was initiated to study, for the first time, the effect of salinity variation on growth and DA content of acclimated *Pseudo-nitzschia australis* strains. Two strains were acclimated for several months to different salinity conditions and afterwards monitored for two weeks in batch cultures. The effects of salinity change on growth, cell biovolume, photosynthetic capacity, nutrient consumption as well as DA content were assessed in order to increase our knowledge on which salinity conditions that trigger proliferation and toxicity of *P. australis*.

Materials and methods

Two strains of *Pseudo-nitzschia australis*, IFR-PAU-16.1 and IFR-PAU-16.2, were isolated in May 2016 from the Bay of Arcachon, Atlantic coast of France (44°40' N, 1°10' W), with a water temperature of 16°C and 35 of salinity. The two strains were grown in 1L polystyrene flasks containing sterile L1 medium (Guillard and Hargraves 1993). The *P. australis* strains were grown as batch cultures at 16°C exposed to a photon flux density of 120 $\mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ (fluora and cool-white fluorescent light, Osram, Germany) on a 12:12 h light:dark cycle. For culture medium,

natural seawater with a salinity of 35 obtained from the English Channel was filter-sterilized through a 0.22 μm polycarbonate membrane filter (Corning, NY, USA) and enriched with L1 medium solutions; but adding silicate for a final concentration of 220 μM (Guillard and Hargraves 1993). The medium with a salinity of 35 was considered a control for comparison of salinity induced effects, as it had the same salinity as the sea water from which the strains were isolated. Hence cells acclimated to salinities other than 35 can be compared to cells which stay at a salinity of 35 and thus do not need to acclimate. The seawater was diluted with distilled water to reach salinities: 10, 20 and 30, and freeze-concentrated to increase the salinity up to 40. All salinity treatments were amended with L1 nutrients to the same levels as the control.

Experimental set-up

Prior to the experiment, cells were acclimated to the different salinities by inoculating exponentially growing stock cultures at salinity 35 into media with salinities of 40, 30, 20 and 10. Salinities were increased or decreased by a salinity increment of 5, and only after cultures had reached a constant growth rate at the previous salinity. At low salinity conditions (20 and 10), a salinity increment of 2 was needed to avoid cell death. However, even with this careful handling, cultures at salinity 10 did not exhibit growth. Cultures were grown and acclimated for four months in order to ensure a steady growth rate at the respective experimental salinities before starting the experiments. The initial growth rates before the acclimation period (salinity 35) were 0.56 and $0.47 \cdot \text{d}^{-1}$ for IFR-PAU-16.1 and IFR-PAU-16.2, respectively.

For the experiments, the two strains were cultured in triplicates. Culture monitoring was carried out daily for 13 days at a fixed time of the day, corresponding to the middle of the light cycle, by sampling 35 mL of each flask in order to measure cell growth, cell biovolume, effective quantum yield F_v/F_m , pigment and DA content.

Growth rate

Growth was monitored every day by cell counts of Lugol fixed samples (1% final concentration) using a light microscope (Leica Microsystems Switzerland) at x10 magnification. For each sample, a minimum of 400 cells were counted using a Nageotte cell counting chamber. The specific growth rates (μ , d^{-1}) were calculated using a minimum of three data points of the exponential growth phase based on linear regression according to the equation given in by Guillard (1973):

$$\mu = \frac{1}{t_2 - t_1} \times (\ln x_2 - \ln x_1)$$

where t is the time in days and x is the number of cells.

Cell biovolume

The cell biovolume of *Pseudo-nitzschia* cells was calculated based on a prism of a parallelogram base shape, as defined in Vadrucci et al. (2007). Therefore, for each experimental condition, a minimum of 40 cells in exponential growth phase were selected and measured using a Leica light microscope at x100 magnification equipped with a Leica digital camera and imaging software (Leica application suite 3. 1. 0, Switzerland). Cell biovolume measurement were made as mean $\pm$ standard deviation of 50 cells with the assumption that the width and thickness of *Pseudo-nitzschia* cells are similar (Lundholm et al. 2004) using the following equation.

$$V = \frac{1}{2} a \times b^2$$

With a = length, b = width

Nutrient analyses

Dissolved inorganic nitrogen (nitrate, nitrite, and ammonium), phosphorus and silicate were measured on the last day of the experiment in order to determine the amount of remaining nutrients in the culture. Samples were obtained by filtering 10 mL of culture onto 25 mm Whatman GF/F filters under low filtration pressure. The filtrate was stored frozen at -20°C except for silicate which was stored at

4°C until analyses. Analyses were carried out using a Seal Analytical auto analyzer using colorimetric techniques (Bendschneider and Robinson 1952) optimized by Aminot et al. (Aminot et al. 2009).

Toxin analyses

In order to determine the toxin content (particulate and dissolved fractions), 10 mL of culture was centrifuged (5 min, 3600g, 4°C) and separated into pellet and supernatant. DA concentration in the particulate fraction was determined by extraction of the residue in 1 mL methanol/water mixture (50/50: v/v). The extraction was prolonged by 15 min of sonication in an ice-cold bath then centrifuged and filtered (15 min, 8000g, 4°C, 0.2 µm) to recover the supernatant (Calu 2011). Samples were then stored at -20°C for later analyses.

DA content in the dissolved fraction was extracted by solid phase extraction SPE using Agilent Bond Elut 200 mg C₁₈ cartridges. The SPE column was conditioned with 10 mL of methanol followed by 10 mL of ultra-pure water. The sample was acidified with 20% aqueous formic acid (100 µL) then passed through the SPE column. 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 reserved at -20°C for later analyses. DA analyses were performed using Ultra-Fast Liquid Chromatography (UFLC, Shimadzu) coupled to ABSciex API 4000QTrap triple quadrupole mass spectrometer. The separation was carried out on a Kinetex C18 column (150x2.1 mm, 2.6 µm, Phenomenex) equipped with a pre-column. Certified DA standard (CNRC, Halifax, Canada) was used for external calibration in order to quantify DA in seawater. Limit of detection (LOD) and limit of quantification (LOQ) were respectively 0.1 and 0.25 ng mL⁻¹. The particulate (pDA), dissolved (dDA) and total DA (tDA; tDA = dDA + pDA) content were expressed on a per cell basis (pg · cell⁻¹).

Effective quantum yield F_v/F_m

The effective quantum yield of the primary photosystem PSII ($QY=F_v/F_m$), which is an indicator of the cellular physiological condition, was measured every day (Maxwell and Johnson 2000). QY

measurements were obtained using a PhytoPAM Phytoplankton fluorometer (Walz, Effeltrich, Germany). For each culture, F_v/F_m was calculated using the following equation:

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

Pigment measurements

To further assess the photosynthetic condition of cells exposed to different salinity treatments, pigment concentrations were measured by filtering 10 mL culture samples onto 25 mm Whatman GF/F filters. Filters were immediately frozen in liquid nitrogen and stored in the dark at -80°C.

Pigments were extracted with 1 mL 100% acetone and the mixture was sonicated for 20 min in an ice-cold bath then incubated overnight at -20°C. Afterwards, the extract was filtered through a 0.2 µm syringe PTFE fiber filter (VWR International, Radnor, PA, USA) to remove any particulate and filter residues and stored at -80 °C until analysis. High-performance liquid chromatography (HPLC) analyses were performed on an Agilent 1200 HPLC instrument with a diode-array detector (Agilent Technologies, Massy, France) controlled by an OpenLab CDS Chemstation software. The separation was carried out using a C18 XSelect HSS T3 column (150 × 3 mm, 3.5 µm, Waters) equipped with a pre-column. The method used for the analysis was adapted from the protocol of Zapata et al. (2012). Pigment identification and quantification was done at 436 nm corresponding to the maximum absorbance peaks of chl *a* and its degradation product, chlorophyllide *a* (chlide *a*) and at 450 nm for other pigments (fucoxanthin, chl *c*₁, chl *c*₂, divinyl protochlorophyllide (MgDVP), diadinoxanthin, diatoxanthin, zeaxanthin and β,β - carotene). Pigments were then identified by comparison of the retention time and the UV spectra with commercial standards obtained from DHI (Denmark), except for the chl *c*₁ quantified with the chl *c*₂ spectra. The pigment concentrations were expressed as a percentage of the total cell pigment content according to the following equation:

$$\% = \left(\frac{\text{concentration of cell pigment (fg } \mu\text{m}^{-3})}{\text{concentration of total cell pigment (fg } \mu\text{m}^{-3})} \right) \times 100$$

Statistical analyses

The experiments were carried out in triplicate and the data presented as mean values $\pm$ standard deviation (SD). Statistical analyses were performed to determine whether growth, effective quantum yield (F_v/F_m), cell biovolume, pigments and DA content of the two strains varied significantly ($p < 0.05$) between the different salinity conditions. All data were subjected to one-way analysis of variance (ANOVA) followed by a Tukey HSD post-hoc test for multiple comparisons, in order to determine the source of significant differences. Data were normally distributed, as determined by the Shapiro-Wilk test, thus permitting the use of parametric statistical analyses. Analyses were performed using R software (version 3.4.1, 2017).

Results

Among the five salinity treatments tested, the two strains of *Pseudo-nitzschia australis*, IFR-PAU-16.1 and IFR-PAU-16.2, were able to acclimate to salinities 20, 30, 35 and 40, whereas they did not grow below salinity 20 (data not shown).

Effect of salinity variation on growth and cell biovolume

The initial growth rates before the acclimation period (salinity 35) were 0.56 and 0.47 per day for IFR-PAU-16.1 and IFR-PAU-16.2, respectively. During the 4 months of acclimation, growth rates decreased for both strains and reached 0.29 and 0.31 d^{-1} for IFR-PAU-16.1 and IFR-PAU-16.2, respectively.

In this experiment, an increase in growth rate and cell density were observed with increasing salinity in both strains (Figs. 1 and 2). The highest growth rate (μ) was observed at salinity 40 (0.29 $\cdot \text{d}^{-1}$ and 0.31 $\cdot \text{d}^{-1}$ for strain IFR-PAU-16.1 and IFR-PAU-16.2 respectively (Fig. 2, A and B). At the lowest salinity condition tested (20), growth rates were significantly lower (one-way ANOVA, $F_{3,8}=12.56$, $p = 0.002$; Tukey HSD test, $p < 0.05$) than at the other salinity conditions, which were not

significantly different. The lowest cell densities were observed in the cultures acclimated to salinity 20 (12×10^3 and 14×10^3 cells $\cdot$ mL $^{-1}$ for IFR-PAU-16.1 and IFR-PAU-16.2, respectively) and the maximum cell concentrations were obtained in cultures with high salinities, 40 and 35 for the strains IFR-PAU-16.1 and IFR-PAU-16.2, respectively (59×10^3 cells $\cdot$ mL $^{-1}$). Overall, the maximum growth rate ranged between $0.18 \cdot$ d $^{-1}$ and $0.29 \cdot$ d $^{-1}$ for strain IFR-PAU-16.1 and between $0.17 \cdot$ d $^{-1}$ and $0.31 \cdot$ d $^{-1}$ for strain IFR-PAU-16.2 (Fig. 1, A and B).

The strains were isolated at the same time from the same geographical region and were grown in parallel under the four different salinity conditions. The initial cell biovolumes of the stock cultures for the control condition (salinity 35) were 1277 and 1702 μ m 3 for IFR-PAU-16.1 and IFR-PAU-16.2, respectively. During the acclimation period (4 months), cell biovolumes decreased with percentages of 62% and 43% for the strains IFR-PAU-16.1 and IFR-PAU-16.2, respectively, when grown at salinity 35; this corresponds to a decrease by 3.8% and 2.6% per week, respectively. Among all salinity treatments, cell biovolumes ranged from 478 to 998 μ m 3 for the strain IFR-PAU-16.1 and from 932 to 1352 μ m 3 for strain IFR-PAU-16.2 (Fig. 3). The highest cell biovolume were obtained at salinity 20 for both strains and were significantly higher (one-way ANOVA, $F_{3,156} = 22.4$, $p = 3.9 \times 10^{-12}$; Tukey HSD test, $p < 0.05$) than at salinities 30, 35 and 40, which did not differ significantly from each other (one-way ANOVA, $F_{2,117} = 0.95$, $p = 0.39$; Tukey HSD test, $p > 0.05$).

Effect of salinity variation on photosynthetic capacity

In exponential growth phase, the two studied strains displayed high effective quantum yield F_v/F_m values, ranging between 0.60 and 0.67, with no significant differences among all salinity treatments (one-way ANOVA, $F_{3,96} = 0.08$, $p = 0.21$; Fig. S1, A and B in the Supporting Information). Otherwise, the photosynthetic capacity profile followed the growth, as high F_v/F_m values were observed during the exponential growth phase and then slowly decreased when the cells entered the stationary phase and reached a minimum during the last day of the experiment for all the salinity conditions (Fig. S1, A and B). Among all the salinity treatments, no significant differences in photosynthetic capacity were found among exponential (one-way ANOVA, $F_{3,60} = 0.077$, $p = 0.71$) and stationary (one-way ANOVA, $F_{3,36} = 0.075$, $p = 0.29$) growth phases for both tested strains.

The pigments chl *a* and its degradation product chl *ide a*, along with the accessory pigments (MgDVP, chl *c*₁, chl *c*₂, fucoxanthin, diadinoxanthin, diatoxanthin, and β , β - carotene) were detected at all salinities for both *Pseudo-nitzschia australis* strains (Fig. S2 in the Supporting Information). Pigment compositions as well as their temporal evolution during growth stages were similar for both strains, therefore only the results for the strain IFR-PAU-16.1 are shown (Fig. S3, A-D in the Supporting Information). In both strains, the major identified pigments were chl *a*, chl *ide a* and fucoxanthin and they comprised an average of 85% of the total pigment content in the cultures (Fig. S3, A-C). In addition, the temporal evolution of these pigments as well as the other minor ones (MgDVP, chl *c*₁, chl *c*₂, diadinoxanthin, diatoxanthin, and β , β - carotene) showed no significant changes between the growth stages (exponential and stationary; Fig. S3, A-C). Furthermore when comparing the pigment profiles among the different salinity conditions, no significant differences were found ($p > 0.05$). Interestingly, during the stationary and decline phase, the pigment zeaxanthin appeared above detection levels (LOD and LOQ were respectively 0.025 and 0.050 mg · L⁻¹) at salinity conditions 30, 35 and 40 (between 0.5 and 1% of the total pigment content), but at salinity 20 this pigment was not detected (Fig. S3D).

Effect of salinity variation on toxin content

At salinity 20, both strains contained a high pDA content that remained relatively stable throughout the experimental period (one-way ANOVA, $F_{2,8}=522$, $p = 1.6 \times 10^{-9}$ and $F_{2,8}=522$, $p = 4.8 \times 10^{-8}$, respectively), and ranged between 1.81 and 1.64 pg · cell⁻¹ for IFR-PAU-16.1 and IFR-PAU-16.2 strains, respectively (Fig. 4, A and B). Stable pDA contents were also observed for the other salinities 30, 35 and 40, with average values of 0.19 pg · cell⁻¹ and 0.17 pg · cell⁻¹ throughout the experiment for strains IFR-PAU-16.1 and IFR-PAU-16.2, respectively, but with almost 10-fold lower pDA content than detected at salinity 20. At salinity 35 for strain IFR-PAU-16.1 and salinity 40 for strain IFR-PAU-16.2, a minor increase in pDA was noted during the exponential growth phase, followed by a progressive decrease thereafter until the end of the experiment (0.61 pg · cell⁻¹ and 0.45 pg · cell⁻¹, respectively at day 4; Fig. 4, A and B). At salinity 35, pDA content for strain IFR-PAU-16.2 remained

very low ($0.02 \text{ pg} \cdot \text{cell}^{-1}$) throughout the experiment, although with a slight increase ($0.07 \text{ pg} \cdot \text{cell}^{-1}$) when cell concentration started to decline (days 11 to 13; Figs. 1B and 4B).

The initial particulate DA content (pDA) before the acclimation period (salinity 35) was $0.46 \text{ pg} \cdot \text{cell}^{-1}$ and $0.20 \text{ pg} \cdot \text{cell}^{-1}$ for IFR-PAU-16.1 and IFR-PAU-16.2, respectively (data not shown). During the acclimation period (4 months), pDA content decreased with a percentage of 24% and 65% for the strains IFR-PAU-16.1 and IFR-PAU-16.2, respectively, when grown at salinity 35.

Overall, dissolved DA contents remained essentially constant (one-way ANOVA, $F_{4,15} = 0.21$, $p = 0.92$, and $F_{2,9} = 0.11$, $p = 0.89$, respectively) during the exponential growth phase for both strains grown at all salinities, and ranged between 0.24 and $0.63 \text{ pg} \cdot \text{cell}^{-1}$ for IFR-PAU-16.1 and between 0.21 and $0.32 \text{ pg} \cdot \text{cell}^{-1}$ for IFR-PAU-16.2 from day 1 to 7 (Fig. 4, C and D). Thereafter, dDA content increased and reached the highest level during late exponential and stationary growth phases with concentrations ranging from 0.48 to $1.65 \text{ pg} \cdot \text{cell}^{-1}$ for the strain IFR-PAU-16.1 (Fig. 4C) and from 0.25 to $0.80 \text{ pg} \cdot \text{cell}^{-1}$ for IFR-PAU-16.2 at day 13 (Fig. 4D). At salinity 20, dissolved DA contents for the two strains remained relatively stable and low, with no significant difference (one-way ANOVA, $p > 0.05$) between growth phases (Fig. 5, A and B). The highest dDA values at salinity 20 measured at day 13 were 6 and 3-fold lower than at salinity 30 (which overall had the highest dDA content) for strains IFR-PAU-16.1 ($0.26 \text{ pg} \cdot \text{cell}^{-1}$) and IFR-PAU-16.2 ($0.25 \text{ pg} \cdot \text{cell}^{-1}$), respectively (Fig. 4, C and D). Dissolved DA content of the two strains grown at salinities 30, 35 and 40, represented up to 33 and 70% of the tDA content during both growth phases (exponential and stationary; Fig. 5, A-D). At salinity 20, dDA content accounted, however, only for 13% and 15% of the tDA concentrations during both exponential and stationary growth phase, respectively (Fig. 5, A-D). Likewise, despite the difference in biovolume between strains grown under the different salinities, the pDA and dDA contents showed the same trend when expressed per cell or per biovolume (Fig. S4 in the Supporting Information), therefore only the per cell data are presented here.

Effect of salinity variation on nutrient consumption

Dissolved nitrogen (N), phosphorus (P) and silicate (Si) concentrations were measured on the first, but also on the last day of the experiment in order to determine whether nutrients limited the growth and possibly influenced DA content in stationary growth phase. The initial concentrations of dissolved N, P and Si measured in the growth L1 medium were equal to 883 μM , 35.8 μM and 206.5 μM , respectively (Fig. S5, A-C in the Supporting Information). Cultures of both strains acclimated to salinity 20 showed low nitrogen, phosphate and silicate consumption equal to 16%, 45% and 50%, respectively (Fig. S5, A-C). The concentrations of dissolved nitrogen (nitrate, nitrite) remained high (ranging from 442 μM to 740 μM) at all conditions in both strains and were therefore not limiting. Dissolved P (2.6 - 5.6 μM) and Si (2.4 - 4.4) concentrations became low but probably not limiting for both strains grown in all other salinity conditions (30, 35 and 40) as concentrations were still >1 μM (See Egge and Aksnes 1992, Fehling et al. 2004, Hagström et al. 2011; Fig. S5, A-C).

Discussion

Pseudo-nitzschia species inhabit estuarine and coastal areas exposed to temporal and long-term changes in sea water salinity (Hallegraeff 2004, Hasle and Lundholm 2005, Thessen et al. 2005). Salinity fluctuations in these regions are mainly due to river discharge, rainfall or evaporation, and seasonal or diurnal tidal amplitudes (Skliris et al. 2014, Jewett and Romanou 2017). Climate change is expected to further intensify salinity fluctuations, leading to either higher salt content or increased freshwater inputs in coastal and estuarine waters worldwide (IPCC 2007, McCabe et al. 2016). Consequently, such changes in salinity are likely to impact physiological and biochemical mechanisms in *Pseudo-nitzschia* cells, leading to differences in cell survival (Bates 1998, Thessen et al. 2005).

Salinity effects on growth

Our results demonstrate a growth tolerance of the two tested *Pseudo-nitzschia australis* strains in a salinity range from 20 to 40. None of the strains survived at salinities below 20, most probably due to osmotic stress resulting in cell lysis. This is not surprising as the two strains were isolated from the

Bay of Arcachon (France) with seasonal salinity fluctuations between 20 and 36 (REPHY 2017). The highest cell densities and growth rates for both strains were found at salinities 30, 35 and 40. These salinities agree with salinities recorded in several areas of the French Atlantic coast and the English Channel during blooms of *P. australis* (Nézan et al. 2010, Husson et al. 2016). By contrast, in a previous study, the same *P. australis* strains only grew at salinities 30 and 35, not at 20 or 40, following an abrupt transfer from 35 (Ayache et al. 2019). This suggests that salinity change needs to be progressive with small salinity intervals to allow potential adaptation. Previous laboratory and field studies report high growth rates across the same salinity range for several other *Pseudo-nitzschia* species. For example, Thessen et al. (2005) and Doucette et al. (2008) showed higher growth rates (0.2 and $0.87 \cdot \text{d}^{-1}$, respectively) for *P. multiseriata* grown at salinity 30 (Thessen et al. 2005), compared to growth rates at salinities below 20. Although the positive relationship between the *Pseudo-nitzschia* growth with salinity established in this study is in agreement with Pednekar et al. (2018), Lundholm et al. (1997), and Jackson et al. (1992) observations, in which the highest growth rates of *Pseudo-nitzschia pungens* and *multiseriata* in cultures were detected at the highest salinity levels tested. Our results also agree with several field studies showing increasing abundance of *Pseudo-nitzschia* spp. with salinity in different parts of the world (Thessen and Stoecker 2008, MacIntyre et al. 2011, Trainer et al. 2012, Liefer et al. 2013, Bargu et al. 2016, Tas et al. 2016, Van Meerssche et al. 2017, Pednekar et al. 2018). Similarly, reports on single *Pseudo-nitzschia* species like *P. brasiliensis*, *P. calliantha*, *P. delicatissima*, *P. fraudulenta*, *P. fryxelliana*, *P. pungens*, *P. multistriata* and most relevant also *P. australis* have found a positive correlation between cell density and salinity (Buck et al. 1992, Mendez et al. 2012, Sahraoui et al. 2012, Downes-Tettmar et al. 2013, Hubbard et al. 2014, Tas et al. 2016,) although the opposite, a negative correlation between salinity and *P. pungens* abundance, has also been reported (Kaczmarek et al. 2007).

The growth rates calculated in this study for IFR-PAU-16.1 and IFR-PAU-16.2 strains (0.29 and $0.31 \cdot \text{d}^{-1}$ respectively) were 1.3- to 2.1-fold lower than those measured for the stock cultures four months prior to the experiments (0.39 and $0.65 \cdot \text{d}^{-1}$ respectively). This is possibly due to a change in cell physiology (age of the culture, cell size) as previously pointed out (Thessen et al. 2009, Casteleyn et al. 2010, Lelong et al. 2012, Moschandreu et al. 2012, Tesson et al. 2014), and emphasizes that meaningful comparison of growth rates among strains is challenging and should be done with caution.

Cell biovolume changes

Over time, diatoms decrease in cell size during vegetative cell divisions. Reduced cell size, and therefore cell biovolume was thus expected, and found, for all cultures during the acclimation period. Across salinities, larger cell biovolumes were observed in cultures acclimated to the lowest salinity (20) compared to all other salinities. These high cell biovolumes (about 52% and 27% higher at salinity 20 than at salinity 35 for IFR-PAU-16.1 and IFR-PAU-16.2, respectively) were associated with low growth rates (about 1.6-fold lower growth rates than at salinity 35 for both strains). One explanation for this may be that the osmotic pressure at salinity 20 induced a lower growth rate, and that the larger biovolumes at salinity 20 may result from influx of water into the cells in order to restore the internal turgor pressure. Another possible explanation is that the higher cell biovolumes at salinity 20 may result from the lower growth rates and hence a slower decrease in cell size. Similar inverse correlations between growth rates and cell biovolumes have previously been reported in 14 different diatom species (Williams 1964). Similarly, Roubex and Lancelot (2008) found that at lower salinity, the growth rate of *Cyclotella meneghiniana* was lower and the cell size was higher compared to the other high salinity levels tested.

Nutrient analyses

The concentrations of remaining nutrients (Si, P and N) at salinity 20 were approximately 2- to 3-fold higher than all the other salinity treatments, corresponding to slower growth rates. At high salinities (30, 35 and 40), inorganic phosphate and silicate concentrations became low but probably not limiting in the medium (the average of remaining P and Si at these salinities was 3.1 μM and 3.3 μM , respectively; Egge and Aksnes 1992, Fehling et al. 2004, Hagström et al. 2011). We cannot exclude that other factors such as high pH, limitation of light due to high cell density or certain essential nutrients (vitamins and trace metals) may have become limiting (Pan et al. 1998, Lundholm et al. 2004, Martens et al. 2016, Lema et al. 2017), although phytoplankton growth in L1 medium is usually limited by pH (Lundholm et al. 2004).

Response in photosynthetic capacity and pigments

In the present study, the salinity variation did not affect the photosynthetic capacity and thus the two strains were well acclimated and in a favourable physiological state at the four salinities (F_v/F_m before and during the acclimation period ranged from 0.63 to 0.67, data not shown). Similarly, no differences in pigment profiles were observed among the four tested salinities. This confirms earlier findings of high efficiency of the photosynthetic apparatus of *Pseudo-nitzschia* species for adaptation to environments of variable salinity (Aizdaicher and Markina 2011). The detection of zeaxanthin in late stationary and decline phase at salinities ranging from 30 to 40, however, suggests the initiation of stress behaviour of the *Pseudo-nitzschia* cells due to unfavourable conditions. High salinity conditions may trigger the formation of intracellular reactive oxygen species (ROS; Rout and Shaw 2001, Chakraborty et al. 2011), which can be critical since the generation of ROS may be irreversible and cell destructive. As a part of the antioxidative mechanism, diatoms are known to be able to increase the production of xanthophylls including zeaxanthin, another photoprotective mechanism, in order to avoid the ROS formation (Pelah et al. 2004, Chakraborty et al. 2011, Markina and Aizdaicher 2016). The increased production of zeaxanthin might hence be related to the prevention of cell damage and hence maintenance of the photosynthetic abilities at high salinities (30, 35 and 40). Whereas, the absence of zeaxanthin at salinity 20 may suggest that the dissipation of excess energy associated with the synthesis of this pigment is probably compensated to fulfil other important cell functions necessary for the osmoregulatory mechanisms for instance at this low salinity level. Another plausible reason for the lack of zeaxanthin production in the cultures could be explained by the fact that cultures acclimated at the lowest salinity may have not been subjected to an increase in the pH in the culture media in comparison with the other salinity conditions (cell density was 2- to 5-fold lower at salinity 20 than the other salinities). Therefore, cells were probably not subjected to stress conditions that would trigger zeaxanthin biosynthesis.

Domoic acid content in response to salinity changes

Salinity variation had an impact on the content of particulate and dissolved DA for cells acclimated to different salinities. The two *Pseudo-nitzschia australis* strains produced DA throughout the different growth stages, and pDA and dDA concentrations clearly differed between the lowest and the other salinities. In both strains, tDA content calculated on per cell basis was 2.5- to 4- fold higher at salinity 20 than at the other salinities (30, 35 and 40), and pDA content at salinity 20 were 7- to 12- fold higher compared to all other salinities. These differences could probably be associated with the lower growth rate at salinity 20 (hence a slower “dilution” rate) and/or a larger cell biovolume. Despite the difference in biovolume between cells grown at salinity 20 and all the other salinity levels, the pDA contents for both strains showed the same trend when expressed per cell or per biovolume (Figs. 4 and S4). Thus, the highest pDA content at the lowest growth rate (salinity 20) both on a per biovolume and per cell basis may possibly result from the accumulation of DA in the cells. These high pDA amounts at salinity 20 were also associated with low dDA concentrations measured in the culture medium (up to 2- to 3-fold lower dDA than the highest values at salinity 30 for both strains).

Domoic acid has previously been hypothesized to play a role in the osmoregulation process of *Pseudo-nitzschia* cells (Jackson et al. 1992, Bates 1998). The authors suggested that *Pseudo-nitzschia* cells increase DA synthesis in order to adjust and maintain their effective cellular functions in hypersaline conditions as the external osmotic pressure rises. In studies by Doucette et al. (2008), Van Meerssche and Pinckney (2017) and Pednekar et al. (2018), an increase in DA content was observed with increasing salinity, reinforcing this hypothesis. A *P. multiseries* strain acclimated to salinity 20 was e.g. able to maintain high growth rates (similar to salinities 30 and 40) and was associated with lower particulate DA concentration ($4.3 \text{ fg} \cdot \text{cell}^{-1}$) compared to the higher salinities (30 and 40; 26.1 and $20.4 \text{ fg} \cdot \text{cell}^{-1}$, respectively; Doucette et al. 2008). This assumption of importance of osmotic balance is in disagreement with our results, as we found lower growth rate and higher DA content at low salinity compared to the higher salinities. Similarly, in a study on instantaneous variation of salinity, DA did not seem to act as an osmolyte in *P. australis* strains as increasing salinity did not lead to an increase in cell DA concentration (Ayache et al. 2019). The higher pDA content detected at salinity 20 in our experiment was associated with high cell biovolume and may hence be explained by reduction of the division rate allowing the accumulation of DA in the cells.

The overall toxicity per water volume was also calculated, since the severity of toxic ASP events will depend heavily on the total numbers of cells present and their specific toxicity per cell. Therefore, despite the low cell densities observed at salinity 20 for both strains compared with the other salinity treatments, the highest pDA concentrations calculated on per water volume (rather than per cell) were also obtained at this low salinity level 20 (Fig. S6 in the Supporting Information).

Previous laboratory and field studies have shown that *Pseudo-nitzschia* growth, seasonal occurrence as well as DA content (particulate and dissolved) are strongly correlated with salinity fluctuations particularly in estuarine and coastal areas (Lundholm et al. 1997, Thessen et al. 2005, Doucette et al. 2008, Quijano-Scheggia et al. 2008, Downes-Tettmar et al. 2013, Van Meerssche and Pinckney 2017, Pednekar et al. 2018). In a field study, Van Meerssche and Pinckney (2017) showed a positive correlation between the abundance of *Pseudo-nitzschia* species, dDA concentrations and salinity. Similarly, *P. brasiliensis* populations along with total DA concentrations measured in the Bizerte Lagoon (Tunisia) were strongly influenced by salinity (Sahraoui et al. 2012). The toxic *P. brasiliensis* became dominant in the fall in high salinity waters associated with high nutrient input, potentially leading to a high risk of shellfish contamination in this region. However, the abundance of *P. multistriata*, in the same lagoon was negatively correlated with salinity and positively related with other environmental variables such as temperature and turbidity (Sahraoui et al. 2012). Hence, the effect of different environmental factors on abundance and toxin content seem to differ among species. This highlights the importance of testing several environmental factors that might influence growth and toxicity and testing these factors on multiple species and strains of *Pseudo-nitzschia* in order to more precisely assess the threat of ASP events.

The total cellular DA contents measured in this study at the different salinities (0.39 to 2.10 pg · cell⁻¹ for IFR-PAU-16.1 and 0.06 to 1.92 pg · cell⁻¹ for IFR-PAU-16.2) were similar to other *P. australis* strains grown under different culture conditions: Anderson et al. (2006; 0.14 to 2.1 pg · cell⁻¹), Alvarez et al. (2009; 0.04 to 1.74 pg · cell⁻¹), Santiago-Morales et al. (2011; 0.81 to 1.87 pg · cell⁻¹) but lower than levels reported by Buck et al. (1992; 3 to 31 pg · cell⁻¹) and Cusack et al. (2002; 0.65 to 26 pg · cell⁻¹). This variation in toxicity is not surprising as intra-specific variability within *Pseudo-nitzschia* species has been reported previously, even among strains of the same species isolated from the same geographical (Bates 1998, Gai et al. 2018, Ayache et al. 2019).

Domoic acid content and growth phases

Several studies on *Pseudo-nitzschia* species have reported increasing total DA content during stationary phase (e.g., for *P. multiseriata*; Bates 1998, Maldonado et al. 2002, Lundholm et al. 2004, Bates and Trainer 2006, Doucette et al. 2008). The authors associated increases in cellular DA quotas with reduction in growth rate in stationary growth phase due to the limitation of several macro- and micro-nutrients (P, Si, Cu and Fe), excluding nitrogen limitation as DA is a nitrogen-containing toxin. On the contrary, in the present study, the total cellular DA content of the two *Pseudo-nitzschia* strains grown at salinity 20 were not varying much between growth phases, and growth was probably not restricted by limitation of nutrients (but rather by pH). However, for the rest of the treatments, an increase in cellular DA contents was seen during the late exponential and stationary growth phases. These results are similar to most studies using *P. australis* strains, where an increase in pDA content was detected when cells were growing during both exponentially and stationary growth phases in which nutrients and pH became limiting (Garrison et al. 1992, Cusack et al. 2002, Meredith D. Armstrong Howard et al. 2007, Martin-Jézéquel et al. 2015).

Maximum dDA in IFR-PAU-16.1 and IFR-PAU-16.2 cells (at day 13) was detected at salinity 30, where dDA represented 77% and 63% respectively, of the total cellular DA content. At salinity 20, maximum dDA represented, however, only 13% and 15%, respectively (0.28 and 0.34 pg · cell⁻¹, respectively) of the total DA. For both strains, the highest dDA content was measured during stationary growth phase in most of the salinity conditions tested (except for salinity 20), reaching their maximum values in late stationary phase, as seen in several other species (Lelong et al 2012, Trainer et al. 2012).

For both strains, the pDA concentrations remained relatively similar and constant over time throughout the experiment, and at the high salinities 35 and 40 (for strains IFR-PAU-16.1 and IFR-PAU-16.2, respectively), a small increase in pDA content was observed during exponential growth phase. A study of Ayache et al. (Ayache et al. 2019) also reported higher pDA content for *Pseudo-nitzschia australis* strains during exponential growth phase at salinities ranging from 30 to 35. These results are in agreement with previous hypotheses stating that in nutrient-replete conditions

(exponential growth), *Pseudo-nitzschia australis* cells are able to maintain high growth rates and high pDA content (Howard et al. 2007, Cochlan et al. 2008, Martin-Jézéquel et al. 2015). In contrast, studies on other species (*P. multiseriata*) usually report lower pDA content during exponential phase and higher pDA content during mid-stationary growth phase (Pan et al. 1996, Bates 1998). This diversity in DA content during growth stages may be accounted to the large inter-specific variability that characterizes *Pseudo-nitzschia* (Lundholm et al. 2006, Thessen et al. 2009, Kim et al. 2015).

CONCLUSIONS

After several months of culturing at different salinities, the two toxic strains of *Pseudo-nitzschia australis* showed capacity to acclimate to salinities ranging from 20 to 40, and no growth was detected below this range. An increase in salinity was associated with increases in cell densities and growth rates. The lowest salinity had a lower growth rate, a larger cell biovolume, as well as high contents of pDA and tDA per cell and per water volume basis. Hence, a decrease in salinity can potentially be problematic as *Pseudo-nitzschia australis* cells might be able to survive (although with a slower growth) and at the same time contain high amounts of DA in the cells. Whether this increases the risk of toxic ASP events depends on *Pseudo-nitzschia* cell concentrations as well as the fraction that *Pseudo-nitzschia* makes up of the total phytoplankton community. Such an event may have the greatest potential impact on harvesting areas in coastal and estuarine areas and on the shellfish industries in these areas.

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REFERENCES

- Aizdaicher, N.A. & Markina, Z. V. 2011. Influence of changes in sea water salinity on the growth, photosynthetic pigment content, and cell size of the benthic alga *Attheya ussurensis* Stonik, Orlova et Crawford, 2006 (Bacillariophyta). *Russ. J. Mar. Biol.* 37:472–7.
- Almandoz, G.O., Ferreyra, G.A., Schloss, I.R., Dogliotti, A.I., Rupolo, V., Paparazzo, F.E., Esteves, J.L. & Ferrario, M.E. 2008. Distribution and ecology of *Pseudo-nitzschia* species (Bacillariophyceae) in surface waters of the Weddell Sea (Antarctica). *Polar Biol.* 31:429–42.
- Álvarez, G., Uribe, E., Quijano-Scheggia, S., López-Rivera, A., Mariño, C. & Blanco, J. 2009. Domoic acid production by *Pseudo-nitzschia australis* and *Pseudo-nitzschia calliantha* isolated from North Chile. *Harmful Algae* 8:938–45.
- Anderson, C.R., Brzezinski, M.A., Washburn, L. & Kudela, R. 2006. Circulation and environmental conditions during a toxigenic *Pseudo-nitzschia australis* bloom in the Santa Barbara Channel, California. *Mar. Ecol. Prog. Ser.* 327:119–33.
- Anderson, D.M., Hoagland, P., Kaoru, Y. & White, A.W. 2000. Estimated annual economic impacts from harmful algal blooms (HABs) in the United States. *Woods Hole Oceanographic Institution Technical Report: WHOI-2000–2011*. Woods Hole, MA: Woods Hole Oceanogr Inst.
- Ayache, N., Hervé, F., Martin-Jézéquel, V., Amzil, Z. & Caruana, A.M.N. 2019. Influence of sudden salinity variation on the physiology and domoic acid production by two strains of *Pseudo-nitzschia australis*. *J. Phycol.* 55:186–95.
- Bates, S.S. 1998. Ecophysiology and metabolism of ASP Toxin production. In Anderson, D. M., Cembella, A. D. & Hallegraeff, G. M. [Eds.] *Physiological Ecology of Harmful Algal Blooms*. Springer-Verlag, Berlin, Germany, pp. 405–26.
- Bates, S.S., Hubbard, K.A., Lundholm, N., Montresor, M. & Leaw, C.P. 2018. Pseudo-nitzschia, Nitzschia, and domoic acid: New research since 2011. *Harmful Algae* 79:3–43.

- Bates, S.S. & Trainer, V.L. 2006. The ecology of harmful diatoms. In Graneli, E. & Turne, J. T. [Eds.] *Ecology of Harmful Algae*. Springer Berlin Heidelberg, Germany, pp. 81–93.
- Bendschneider, K. & Robinson, R.J. 1952. A new spectrophotometric method for the determination of nitrite in sea water. *J. Mar. Res.* 11:87–96.
- Buck, K.R., Uttal-Cookel, L., Pilskaln, C.H., Roelke, D.L., Villac, M.C., Fryxell, G.A., Cifuentes, L. & Chavez, F.P. 1992. Autecology of the diatom *Pseudonitzschia australis*, a domoic acid producer, from Monterey Bay, California. *Mar. Ecol. Ser.* 84:293–302.
- Casteleyn, G., Leliaert, F., Backeljau, T., Debeer, A.E., Kotaki, Y., Rhodes, L., Lundholm, N., Sabbe, K. & Vyverman, W. 2010. Limits to gene flow in a cosmopolitan marine planktonic diatom. *Proc. Natl. Acad. Sci. USA* 107:12952–7.
- Chakraborty, P., Acharyya, T., Raghunadh Babu, P. V & Bandhyopadhyay, D. 2011. Impact of salinity and pH on phytoplankton community in a tropical freshwater system: An investigation with pigment analysis by HPLC. *J. Environ. Monit.* 13:614–20.
- Cochlan, W.P., Herndon, J. & Kudela, R.M. 2008. Inorganic and organic nitrogen uptake by the toxigenic diatom *Pseudo-nitzschia australis* (Bacillariophyceae). *Harmful Algae* 8:111–8.
- Cusack, C.K., Bates, S.S., Quilliam, M.A., Patching, J.W. & Raine, R. 2002. Confirmation of domoic acid production by *Pseudo-nitzschia australis* (Bacillariophyceae) isolated from Irish waters. *J. Phycol.* 38:1106–12.
- Doucette, G.J., King, K.L., Thessen, A.E. & Dortch, Q. 2008. The effect of salinity on domoic acid production by the diatom *Pseudo-nitzschia multiseries*. *Nov. Hedwigia* 133:31.
- Downes-Tettmar, N., Rowland, S., Widdicombe, C., Woodward, M. & Llewellyn, C. 2013. Seasonal variation in *Pseudo-nitzschia* spp. and domoic acid in the Western English Channel. *Cont. Shelf Res.* 53:40–9.
- Durack, P.J. & Wijffels, S.E. 2010. Fifty-Year trends in global ocean salinities and their relationship to broad-scale warming. *J. Clim.* 23:4342–62.
- EGGE, J.K. & AKSNES, D.L. 1992. Silicate as regulating nutrient in phytoplankton competition. *Mar.*

Ecol. Prog. Ser. 83:281–9.

Fehling, J., Davidson, K. & Bates, S.S. 2005. Growth dynamics of non-toxic *Pseudo-nitzschia delicatissima* and toxic *P. seriata* (Bacillariophyceae) under simulated spring and summer photoperiods. *Harmful Algae* 4:763–9.

Fehling, J., Davidson, K., Bolch, C.J. & Bates, S.S. 2004. Growth and domoic acid production by *Pseudo-nitzschia seriata* (Bacillariophyceae) under phosphate and silicate limitation. *J. Phycol.* 40:674–83.

Gai, F.F., Hedemand, C.K., Louw, D.C., Grobler, K., Krock, B., Moestrup, Ø. & Lundholm, N. 2018. Morphological, molecular and toxigenic characteristics of Namibian *Pseudo-nitzschia* species – including *Pseudo-nitzschia bucculenta* sp. nov. *Harmful Algae* 76:80–95.

Garrison, D.L., Conrad, S.M., Eilers, P.P. & Waldron, E.M. 1992. Confirmation of domoic acid production by *Pseudo-nitzschia australis* (Bacillariophyceae) cultures. *J. Phycol.* 28:604–7.

Goldstein, T., Mazet, J.A.K., Zabka, T.S., Langlois, G., Colegrove, K.M., Silver, M., Bargu, S., Van Dolah, F., Leighfield, T., Conrad, P.A., Barakos, J., Williams, D.C., Dennison, S., Haulena, M. & Gulland, F.M.D. 2008. Novel symptomatology and changing epidemiology of domoic acid toxicosis in California sea lions (*Zalophus californianus*): An increasing risk to marine mammal health. *Proc. R. Soc. B Biol. Sci.* 275:267–76.

Guillard, R.R.L. 1973. Methods for microflagellates and nanno- plankton. In Stein, J.R. [Ed.]. *Handbook of phycological methods. Culture methods and growth measurements*. Cambridge University Press, New York, pp. 289–312.

Guillard, R.R.L. & Hargraves, P.E. 1993. *Stichochrysis immobilis* is a diatom, not a chrysophyte. *Phycologia* 32:234–6.

Hagström, J.A., Granéli, E., Moreira, M.O.P. & Odebrecht, C. 2011. Domoic acid production and elemental composition of two *Pseudo-nitzschia multiseries* strains, from the NW and SW Atlantic Ocean, growing in phosphorus or nitrogen-limited chemostat cultures. *J. Plankton Res.* 33:297–308.

Hallegraeff, G.M. 2004. Harmful algal blooms: a global overview. In *Manual on Harmful Marine Microalgae*. pp. 25–50.

Hansen, L.R., Soylu, S. í, Kotaki, Y., Moestrup, Ø. & Lundholm, N. 2011. Toxin production and temperature-induced morphological variation of the diatom *Pseudo-nitzschia seriata* from the Arctic. *Harmful Algae* 10:689–96.

Hardardóttir, S., Pančić, M., Tammilehto, A., Krock, B., Møller, E.F., Nielsen, T.G. & Lundholm, N. 2015. Dangerous relations in the arctic marine food web: Interactions between toxin producing *Pseudo-nitzschia* diatoms and *Calanus* copepodites. *Mar. Drugs*. 13:3809–35.

Hasle, G.R. & Lundholm, N. 2005. *Pseudo-nitzschia seriata* f. *obtus*a (Bacillariophyceae) raised in rank based on morphological, phylogenetic and distributional data. *Phycologia* 44:608–19.

Hoagland, P. & Scatasta, S. 2006. The economic effects of harmful algal blooms. In Granéli E. & Turner J.T. [Eds]. *Ecology of Harmful Algae. Ecological Studies (Analysis and Synthesis)*, vol 189. Springer, Berlin, Heidelberg, pp. 391–402.

Howard, M.D.A., Cochlan, W.P., Ladizinsky, N. & Kudela, R.M. 2007. Nitrogenous preference of toxigenic *Pseudo-nitzschia australis* (Bacillariophyceae) from field and laboratory experiments. *Harmful Algae* 6:206–17.

Howard, M.D.A., Cochlan, W.P., Ladizinsky, N. & Kudela, R.M. 2007. Nitrogenous preference of toxigenic *Pseudo-nitzschia australis* (Bacillariophyceae) from field and laboratory experiments. *Harmful Algae* 6:206–17.

Husson, B., Hernández-Fariñas, T., Le Gendre, R., Schapira, M. & Chapelle, A. 2016. Two decades of *Pseudo-nitzschia* spp. blooms and king scallop (*Pecten maximus*) contamination by domoic acid along the French Atlantic and English Channel coasts: Seasonal dynamics, spatial heterogeneity and interannual variability. *Harmful Algae* 51:26–39.

IPCC 2007. Climate Change 2007: The Physical Science Basis. In Solomon, S., Qin, D. M., Manning, Z., Chen, M., Marquis, K. B., Averyt Tingor, M. & Miller, H. L. [Eds.] *Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change*.

Cambridge University Press, Cambridge. United Kingdom and New York, NY, USA, p. 996.

Jackson, A.E., Ayer, S.W. & Laycock, M. V. 1992. The effect of salinity on growth and amino acid composition in the marine diatom *Nitzschia pungens*. *Can. J. Bot.* 70:2198–201.

Jewett, L. & Romanou, A. 2017. Ocean acidification and other ocean changes. In Wuebbles, D.J., D.W. Fahey, K.A. Hibbard, D.J. Dokken, B.C. Stewart, & T.K. Maycock [Eds.] *Climate Science Special Report: Fourth National Climate Assessment, Volume I*. U.S. Global Change Research Program, Washington, D.C., USA, pp. 364-392.

Kaczmarska, I., Ehrman, J.M., Bates, S.S., Green, D.H., Léger, C. & Harris, J. 2005. Diversity and distribution of epibiotic bacteria on *Pseudo-nitzschia multiseries* (Bacillariophyceae) in culture, and comparison with those on diatoms in native seawater. *Harmful Algae* 4:725–41.

Kim, J.H., Park, B.S., Kim, J.H., Wang, P. & Han, M.S. 2015. Intraspecific diversity and distribution of the cosmopolitan species *Pseudo-nitzschia pungens* (Bacillariophyceae): morphology, genetics, and ecophysiology of the three clades. *J. Phycol.* 51:159–72.

Lefebvre, K.A., Frame, E.R. & Kendrick, P.S. 2012. Domoic acid and fish behavior: A review. *Harmful Algae* 13:126–30.

Lelong, A., Hégaret, H., Soudant, P. & Bates, S.S. 2012. *Pseudo-nitzschia* (Bacillariophyceae) species, domoic acid and amnesic shellfish poisoning: revisiting previous paradigms. *Phycologia* 51:168–216.

Lema, K.A., Latimier, M., Nézan, É., Fauchot, J. & Le Gac, M. 2017. Inter and intra-specific growth and domoic acid production in relation to nutrient ratios and concentrations in *Pseudo-nitzschia*: phosphate an important factor. *Harmful Algae* 64:11–9.

Louw, D.C., Doucette, G.J. & Voges, E. 2017. Annual patterns, distribution and long-Term trends of *Pseudo-nitzschia* species in the northern Benguela upwelling system. *J. Plankton Res.* 39:35–47.

Lundholm, N., Hansen, P.J. & Kotaki, Y. 2004. Effect of pH on growth and domoic acid production by potentially toxic diatoms of the genera *Pseudo-nitzschia* and *Nitzschia*. *Mar. Ecol. Prog. Ser.* 273:1–15.

- Lundholm, N., Moestrup, Ø., Kotaki, Y., Hoef-Emden, K., Scholin, C. & Miller, P. 2006. Inter- and intraspecific variation of the *Pseudo-nitzschia delicatissima* complex (Bacillariophyceae) illustrated by rRNA probes, morphological data and phylogenetic analyses. *J. Phycol.* 42:464–81.
- Lundholm, N., Skov, J., Pocklington, R. & Moestrup, Ø. 1997. Studies on the marine planktonic diatom *Pseudo-nitzschia*. 2. Autecology of *P. pseudodelicatissima* based on isolates from Danish coastal waters. *Phycologia* 36:381–8.
- Maldonado, M.T., Hughes, M.P., Rue, E.L. & Wells, M.L. 2002. The effect of Fe and Cu on growth and domoic acid production by *Pseudo-nitzschia multiseriis* and *Pseudo-nitzschia australis*. *Limnol. Oceanogr.* 47:515–26.
- Markina, Z. V. & Aizdaicher, N.A. 2016. The effect of lowered salinity of sea water on the growth and photosynthetic pigment content in three strains of the microalgae *Pseudo-nitzschia pungens* (Grunow ex. P.T. Cleve) Hasle, 1993 (Bacillariophyta). *Russ. J. Mar. Biol.* 42:414–8.
- Martens, H., Van de Waal, D.B., Brandenburg, K.M., Krock, B. & Tillmann, U. 2016. Salinity effects on growth and toxin production in an *Alexandrium ostenfeldii* (Dinophyceae) isolate from The Netherlands. *J. Plankton Res.* 38:1302–16.
- Martin-Jézéquel, V., Calu, G., Candela, L., Amzil, Z., Jauffrais, T., Séchet, V. & Weigel, P. 2015. Effects of organic and inorganic nitrogen on the growth and production of domoic acid by *Pseudo-nitzschia multiseriis* and *P. australis* (Bacillariophyceae) in culture. *Mar. Drugs* 13:7067–86.
- Maxwell, K. & Johnson, G.N. 2000. Chlorophyll fluorescence—a practical guide. *J. Exp. Bot.* 51:659–68.
- McCabe, R.M., Hickey, B.M., Kudela, R.M., Lefebvre, K.A., Adams, N.G., Bill, B.D., Gulland, F.M.D., Thomson, R.E., Cochlan, W.P. & Trainer, V.L. 2016. An unprecedented coastwide toxic algal bloom linked to anomalous ocean conditions. *Geophys. Res. Lett.* 43:10366-10376.
- Moschandreou, K.K., Baxevas, A.D., Katikou, P., Papaefthimiou, D., Nikolaidis, G. &

- Abatzopoulos, T.J. 2012. Inter-and intra-specific diversity of *Pseudo-nitzschia* (Bacillariophyceae) in the northeastern Mediterranean. *Eur. J. Phycol.* 47:321–39.
- Nézan, E., Chomérat, N., Bilien, G., Boulben, S. & Duval, A. 2010. *Pseudo-nitzschia australis* on French Atlantic coast, an unusual toxic bloom. *Harmful Algae News* 41:1-2.
- Pan, Y., Bates, S.S. & Cembella, A.D. 1998. Environmental stress and domoic acid production by *Pseudo-nitzschia*: a physiological perspective. *Nat. Toxins* 6:127–35.
- Pan, Y., Subba Raol, D. V, Mann, K.H., Brown, R.G. & Pocklington, R. 1996. Effects of silicate limitation on production of domoic acid, a neurotoxin, by the diatom *Pseudo-nitzschia multiseries*. I. Batch culture studies. *Mar. Ecol. Prog. Ser.* 131:225–33.
- Pednekar, S.M., Bates, S.S., Kerkar, V. & Prabhu Matondkar, S.G. 2018. Environmental factors affecting the distribution of *Pseudo-nitzschia* in two monsoonal estuaries of Western India and effects of salinity on growth and domoic acid production by *P. pungens*. *Estuar. Coast.* 41:1448–62.
- Pelah, D., Sintov, A. & Cohen, E. 2004. The effect of salt stress on the production of canthaxanthin and astaxanthin by *Chlorella zofingiensis* grown under limited light intensity. *World J. Microbiol. Biotechnol.* 20:483–6.
- Pulido, O.M. 2008. Domoic acid toxicologic pathology: A review. *Mar. Drugs* 6:180–219.
- Quijano-Scheggia, S., Garcés, E., Sampedro, N., Van Lenning, K., Flo, E., Andree, K., Fortuño, J.M. & Camp, J. 2008. Identification and characterisation of the dominant *Pseudo-nitzschia* species (Bacillariophyceae) along the NE Spanish coast (Catalonia, NW Mediterranean). *Sci. Mar.* 72:343–59.
- Ramsdell, J.S. & Gulland, F.M. 2014. Domoic acid epileptic disease. *Mar. Drugs* 12:1185–207.
- REPHY – French Observation and Monitoring program for Phytoplankton and Hydrology in coastal waters. 2019. REPHY dataset - French Observation and Monitoring program for Phytoplankton and Hydrology in coastal waters. 1987-2018 Metropolitan data.
- Roubex, V. & Lancelot, C. 2008. Effect of salinity on growth, cell size and silicification of an

- euryhaline freshwater diatom: *Cyclotella meneghiniana* Kütz. *Transitional Waters Bull.* 2:31–8.
- Rout, N.P. & Shaw, B.P. 2001. Salt tolerance in aquatic macrophytes: ionic relation and interaction. *Biol. Plant.* 4:95–9.
- Ryckaert, M., Nézan, E., Chomérat, N., Le Gal, D., Genauzeau, S., Derrien, A., Schmitt, A., Fillon, A. & Gaignon, J.L. 2010. *Pseudo-nitzschia australis* occurrence and A.S.P. toxicity in Pertuis Charentais (France) after Xynthia storm (28 Feb. 2010). In ICES Annual Science Conference, p. 1.
- Sahraoui, I., Grami, B., Bates, S.S., Bouchouicha, D., Chikhaoui, M.A., Mabrouk, H.H. & Hlaili, A.S. 2012. Response of potentially toxic *Pseudo-nitzschia* (Bacillariophyceae) populations and domoic acid to environmental conditions in a eutrophied, SW Mediterranean coastal lagoon (Tunisia). *Estuar. Coast.* 102:95–104.
- Santiago-Morales, I.S. & García-Mendoza, E. 2011. Growth and domoic acid content of *Pseudo-nitzschia australis* isolated from northwestern Baja California, Mexico, cultured under batch conditions at different temperatures and two Si:NO₃ ratios. *Harmful Algae* 12:82–94.
- Scholin, C.A., Gulland, F., Doucette, G.J., Benson, S., Busman, M., Chavez, F.P., Cordaro, J., DeLong, R., De Vogelaere, A., Harvey, J., Haulena, M., Lefebvre, K., Lipscomb, T., Loscutoff, S., Lowenstine, L.J., Marin, R., Miller, P.E., McLellan, W.A., Moeller, P.D.R., Powell, C.L., Rowles, T., Silvagni, P., Silver, M., Spraker, T., Trainer, V. & Van Dolah, F.M. 2000. Mortality of sea lions along the central California coast linked to a toxic diatom bloom. *Nature* 403:80–4.
- Schwarz, M., Jandová, K., Struk, I., Marešová, D., Pokorný, J. & Riljak, V. 2014. Low dose domoic acid influences spontaneous behavior in adult rats. *Physiol. Res.* 63:369–76.
- Skirris, N., Marsh, R., Josey, S.A., Good, S.A., Liu, C. & Allan, R.P. 2014. Salinity changes in the World Ocean since 1950 in relation to changing surface freshwater fluxes. *Clim. Dyn.* 43:709–39.
- Sobrinho, B.F., De Camargo, L.M., Sandrini-Neto, L., Kleemann, C.R., Da Costa Machado, E. & Mafra, L.L. 2017. Growth, toxin production and allelopathic effects of *Pseudo-nitzschia*

multiseries under Iron-enriched conditions. *Mar. Drugs* 15.

- Terray, L., Corre, L., Cravatte, S., Delcroix, T., Reverdin, G. & Ribes, A. 2012. Near-surface salinity as nature's rain gauge to detect human influence on the Tropical water cycle. *J. Clim.* 25:958–77.
- Tesson, S.V.M., Montresor, M., Procaccini, G. & Kooistra, W.H.C.F. 2014. Temporal changes in population structure of a marine planktonic diatom. *PLoS ONE* 9:e114984.
- Thessen, A.E., Bowers, H.A. & Stoecker, D.K. 2009. Intra-and interspecies differences in growth and toxicity of *Pseudo-nitzschia* while using different nitrogen sources. *Harmful Algae* 8:792–810.
- Thessen, A.E., Dortch, Q., Parsons, M.L. & Morrison, W. 2005. Effect of salinity on *Pseudo-nitzschia* species (Bacillariophyceae) growth and distribution. *J. Phycol.* 41:21–9.
- Thorel, M., Fauchot, J., Morelle, J., Raimbault, V., Le Roy, B., Miossec, C., Kientz-Bouchart, V. & Claquin, P. 2014. Interactive effects of irradiance and temperature on growth and domoic acid production of the toxic diatom *Pseudo-nitzschia australis* (Bacillariophyceae). *Harmful Algae* 39:232–41.
- Todd, E.C. 1993. Domoic acid and amnesic shellfish poisoning - A review. *J. Food Prot.* 56:69–83.
- Trainer, V.L., Bates, S.S., Lundholm, N., Thessen, A.E., Cochlan, W.P., Adams, N.G. & Trick, C.G. 2012. *Pseudo-nitzschia* physiological ecology, phylogeny, toxicity, monitoring and impacts on ecosystem health. *Harmful Algae* 14:271–300.
- Vadrucci, M.R., Cabrini, M. & Basset, A. 2007. Biovolume determination of phytoplankton guilds in transitional water ecosystems of Mediterranean Ecoregion. *Transitional Waters Bull.* 1:83–102.
- Van Meerssche, E. & Pinckney, J.L. 2017. The influence of salinity in the domoic acid effect on estuarine phytoplankton communities. *Harmful Algae* 69:65–74.
- Visciano, P., Schirone, M., Berti, M., Milandri, A., Tofalo, R. & Suzzi, G. 2016. Marine biotoxins: occurrence, toxicity, regulatory limits and reference methods. *Front. Microbiol.* 7:1051.
- Williams, R.B. 1964. Division rates of salt marsh diatoms in relation to salinity and cell size. *Ecol. Soc. Am.* 45:877–80.

Zapata, M., Fraga, S., Rodríguez, F. & Garrido, J. 2012. Pigment-based chloroplast types in dinoflagellates. *Mar. Ecol. Prog. Ser.* 465:33–52.

Figure 1: Growth curves for the two strains of *Pseudo-nitzschia australis* IFR-PAU-16.1 (A) and IFR-PAU-16.2 (B) acclimated at different salinity conditions (20, 30, 35 and 40); y axis is displayed with semi-log scale. Error bars are not visible as they are smaller than the dots. * Significant difference existed between cultures grown at salinities 20 compared with the other cultures at salinities 30, 35 and 40 ($p < 0.05$).

Figure 2: Specific growth rate (μ) calculated during the exponential growth phase for each salinity condition. Data are means $\pm$ SD, $n=3$. * Significant difference existed between cultures grown at salinities 20 compared with the other cultures at salinities 30, 35 and 40 ($p < 0.05$).

Figure 3: Mean cell biovolume of IFR-PAU-16.1 (white bars) and IFR-PAU-16.2 (grey bars) acclimated to diverse salinity conditions: 20, 30, 35 and 40. BA refers to the initial cell volume before acclimation. Data are expressed as mean $\pm$ SD, $n=50$. * Significant difference existed between cultures grown at salinities 20 compared with the other cultures at salinities 30, 35 and 40 ($p < 0.05$).

Figure 4: Particulate and dissolved domoic acid concentrations for the IFR-PAU-16.1 (A-C) and IFR-PAU-16.2 (B-D) strains subjected to different salinities: 20, 30, 35 and 40. Data are means $\pm$ SD, $n=3$.

Figure 5: Total DA concentrations for the IFR-PAU-16.1 (A-C) and IFR-PAU-16.2 (B-D) strains expressed on a per cell basis ($\text{pg} \cdot \text{cell}^{-1}$) and as a percentage (%) of particulate and dissolved DA concentrations during exponential (Expo) and stationary (Stat) growth phases at the different salinities: 20, 30, 35 and 40. Data are means $\pm$ SD, $n=3$. * Significant difference existed between cultures grown at salinities 20 compared with the other cultures at salinities 30, 35 and 40, and for IFR-PAU-16.2 strain grown at salinity 35 ($p < 0.05$).

Figure S1: Variations in maximum photosynthetic activity (F_v/F_m) for the strains IFR-PAU-16.1 (A) and IFR-PAU-16.2 (B) in response to the salinity conditions 20, 30, 35 and 40. Data are means $\pm$ SD, $n=3$. Error bars are not visible as they are smaller than the dots.

Figure S2: High performance liquid chromatogram obtained for the strain IFR-PAU-16.1 at 450 nm on the last day of the experiment showing all the identified pigments including the zeaxanthin.

Figure S3: Temporal variations in pigment concentrations (chl *a*, chl *b*, fucoxanthin and zeaxanthin) observed during the growth of the strain IFR-PAU-16.1 under different salinity conditions: 20, 30, 35 and 40. Data are means $\pm$ SD, $n=3$.

Figure S4: Particulate (pDA) and dissolved (dDA) domoic acid content expressed per cell biovolume basis for the IFR-PAU-16.1 (A-C) and IFR-PAU-16.2 (B-D) strains subjected to different salinities: 20, 30, 35 and 40. Data are means $\pm$ SD, $n=3$.

Figure S5: Dissolved nitrogen (A), phosphate (B) and silicate (C) concentrations measured on the first day (L1) and the last day of the experiment for the strains IFR-PAU-16.1 and IFR-PAU-16.2 grown under different salinity conditions: 20, 30, 35 and 40. Data are means $\pm$ SD, $n=3$. Error bars are not visible as they are smaller than the dots.

Figure S6: Particulate (pDA), dissolved (dDA) and total (tDA) domoic acid content expressed per water volume basis for the IFR-PAU-16.1 (A-C-E) and IFR-PAU-16.2 (B-D-F) strains subjected to different salinities: 20, 30, 35 and 40. Data are means $\pm$ SD, $n=3$.