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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

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Here we compare cell physiology and domoic acid (DA) production for two strains of the diatom *Pseudo-nitzschia multiseries* originating from two opposite latitudes: Canada (CA) and Brazil (BR). The algae were grown as chemostat cultures at 0.2, 0.3 and 0.4 day^{-1} under nitrogen (N)- and phosphorus (P)-deficient conditions. The level of deficiency significantly affected the atomic C:N, C:P, C:Si and N:P ratios in both strains. In both strains, P per cell was 2–4× higher in the N- than in the P-deficient cultures. The opposite was not found for N in the P-deficient cultures, as shown by the N:P ratios and C:N ratios. The C:N and C:P ratios were significantly lower in the CA strain, and this did not change due to the level of deficiency. The concentration and production of DA per cell per day were significantly higher for both strains under P deficiency as expected since the toxin is rich in N. However, DA was also produced by both strains during continuous cell division under N deficiency. High or low bacterial densities associated with *P. multiseries* did not increase or decrease DA production. Our data imply that more attention needs to be given to the N:P ratios and concentrations in the waters where these algae occur, as both N and P deficiencies affect DA production and cellular DA concentrations.

KEYWORDS: *Pseudo-nitzschia*; Domoic acid; Chemical composition; Toxin; Nutrient limitation

INTRODUCTION

Mass mortalities of marine birds and mammals occur yearly in many coastal areas of the world during and after blooms of the diatom *Pseudo-nitzschia* spp., especially during strong upwelling. Several *Pseudo-nitzschia* spp. produce the responsible neurotoxin domoic acid (DA), which causes amnesic shellfish poisoning (ASP) in humans (cf. Bates and Trainer, 2006). The most severe incident to date, from a human point of view, occurred in 1987 off Prince Edward Island (PEI), SE Canada. DA from *P. multiseries* Hasle (Bacillariophyceae) bio-accumulated in blue mussels (*Mytilus edulis* Linnaeus).

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More than 100 humans consuming the mussels suffered from, for example, nausea and amnesia, and 3 people died (cf. Landsberg, 2002). In the Galician Rias (NW Spain), DA originating from blooms of *Pseudo-nitzschia* spp. was first detected in 1994 (Míguez *et al.*, 1996). *Pseudo-nitzschia* spp. and *Nitzschia navis-varingica* from brackish waters in Vietnam, Japan and the Philippines, and from marine or brackish waters in Ireland, Denmark, Spain and Sweden are also known to produce DA (Lundholm *et al.*, 1997; Cusack *et al.*, 2002; Kotaki *et al.*, 2004). This shows that a possible ASP outbreak with devastating effects on both ecosystems and economies is a worldwide problem.

Published research has shown that a *Pseudo-nitzschia* species may be toxic in one part of the world but not in another (Bates *et al.*, 1998). Studies on the influence of nutrient availability on DA production by *P. multiseries* have so far been focused on phosphorus (P) and silicon (Si) deficiencies (Bates *et al.*, 1998). These studies have most of the time been performed in batch cultures. This was, for instance, done when investigating the effect of various nutrient deficiencies, including nitrogen (N), and when comparing toxin production in different strains of the same *Pseudo-nitzschia* species (Bates *et al.*, 1991; Fehling *et al.*, 2004). Continuous cultures on the other hand allow a dynamic equilibrium between nutrient input and growth, thereby simulating natural conditions in the lab better than most other methods. Continuous culturing has been successfully used to study, for example, the effect of P deficiency on *P. multiseries* (Pan *et al.*, 1996b). Since DA is an amino acid, i.e. N is an essential element, it has been argued that some strains stop producing the toxin during N deficiency (Bates, 1998). Thessen *et al.* (2009) studied the effect of NO_3^-, NH_4^+ and urea on 19 different strains of three *Pseudo-nitzschia* species in batch culture. Kudela *et al.* (2004) studied the effect of Si limitation on different clones of *P. australis* and *P. multiseries* growing in batch, semicontinuous and continuous cultures. They also studied the effect of N deficiency on a *P. multiseries* clone isolated from Monterey Bay, California, growing at different rates in a chemostat ($0.2-1.2\ day^{-1}$). However, to our knowledge, there is a lack of knowledge regarding the possible differences between strains within the same species isolated from different locations and grown under identical continuous culture conditions with different N:P ratios.

The questions we focused on during this experiment were therefore: (i) are there any physiological differences between two strains of *P. multiseries* from different origins (SW and NW Atlantic Ocean) concerning DA production when growing in N- and P-deficient conditions? (ii) What is the effect of growth rate (i.e. level of nutrient deficiency) on cell physiology and DA production for the two strains?

METHOD

Strains

The experiment was carried out using two non-axenic strains of the toxic diatom *Pseudo-nitzschia multiseries* Hasle. One strain originating from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP 1660), USA and had been isolated from waters of eastern PEI (Canadian SE coast) in December 1993. The other strain was isolated in June 2001 from the southern part of the Atlantic Ocean (Brazil) and was obtained from the Phytoplankton Laboratory of the Fundação Universidade Federal do Rio Grande (FURG), Brazil. Both strains had been identified as *P. multiseries* using electron microscopy. In addition, an 18S rRNA gene sequence analysis of both strains was done prior to the experiment in order to verify that the two strains indeed are the same species (Fig. 1).

The partial 18S rRNA gene was amplified from *Pseudo-nitzschia* spp. cultures by adding 5 μL of cells resuspended in TE buffer (10 mM Tris, 0.5 mM EDTA, pH 8.0) to the polymerase chain reaction (PCR) mix. The PCR mix contained 200 mM deoxy-nucleoside triphosphate, 5% dimethylsulfoxide, 5 pmol each of primers EUK1 and 518r, *Taq* DNA polymerase reaction buffer with 1.5 mM $MgCl_2$ and 1 U of a mixture of *Taq*

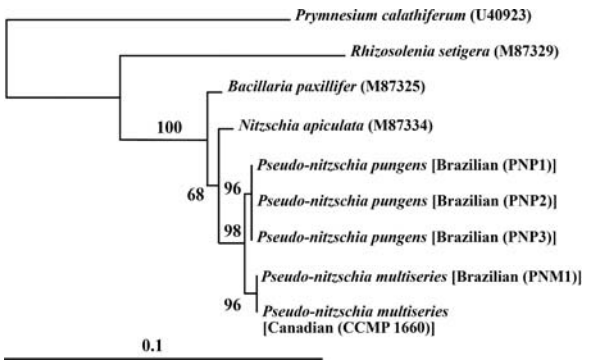

Fig. 1. Phylogenetic analysis of 18S rRNA gene sequences from *Pseudo-nitzschia* spp. and other diatoms. The Jukes and Cantor method was used to estimate the distances. The numbers at the branches indicate the percentage of 500 bootstraps; resamplings that support the branch values below 50% are not shown. The *Pseudo-nitzschia* sp. sequences are a sister group to *Nitzschia apiculata*. Note that the three Brazilian strains of *Pseudo-nitzschia pungens* (PNP1), *Pseudo-nizschia pungens* (PNP2) and *Pseudo-nitzschia pungens* (PNP3) sequences form one tight cluster and the Brazilian *Pseudo-nitzschia multiseries* (PNM1) and Canadian *Pseudo-nitzschia multiseries* (CCMP 1660) form a second tight cluster with zero branch lengths. The scale indicates the observed sequence divergence of 10%. Thus the divergence between Brazilian and Canadian *P. multiseries* is less than 1%.

DNA polymerase (Roche) in a total reaction volume of 50 μL (Janson *et al.*, 2000). The amplification was performed with a GeneAmp PCR System 2400 (Perkin Elmer, USA) with an initial incubation of 5 min at 94°C. The 30 subsequent cycles were 30 s at 94°C, 30 s at 50°C and 1 min at 72°C. A final incubation of 5 min at 72°C finished the PCR amplification. The gel electrophoresis of the PCR products was performed according to Ausubel *et al.* (1989).

The PCR products were later sequenced on both strands after purification with a QIAquick® PCR kit (Qiagen) using the primers EUK1 and 518r. The sequencing reaction was performed using dye terminator chemicals (BigDye: Perkin Elmer) according to the instructions provided by the manufacturer. Sequences were read using a DNA auto sequencer Model ABI 377-XL (Perkin Elmer). The sequences reported here, and those retrieved from GenBank, were aligned using CLUSTAL W 1.8 (Thompson *et al.*, 1994), with the gap and extension penalty set to 6.6. Phylogenetic analyses were performed on the PHYLO_WIN software (Galtier *et al.*, 1996). The tree was edited using TREEVIEW 1.5 for PC (Page, 1996). The Jukes and Cantor method was used to estimate the distances (Jukes and Cantor, 1969).

Culture maintenance

The two strains were maintained in f/2 medium, with the trace metal solution modified according to medium L1 (Guillard, 1995). However, the nitrogen concentration was reduced so that the 16N:1P Redfield ratio was attained (see below). The cultures were grown under a 16:8 h light:dark cycle. Photosynthetically active radiation was adjusted to 200 μmol photons m^{-2} s^{-1} (Philips TLD 36W/830 cool white fluorescent tubes) and the temperature was kept at 18 ± 0.5°C. This was followed by sub-culturing for 1 month in f/10 medium, with the same light, temperature and adjustments of nutrient ratio as mentioned above. These sub-cultures were later used as inocula for the chemostat cultures. All media were prepared in 10 L Nalgene polycarbonate carboys using filtered (Munktel glass fibre filter, 1.2 μm pore size) aged Baltic Sea surface water (6 psu) adjusted to 31 ± 1 psu. Proportional quantities of the major salts (NaCl, $MgCl_2 \cdot 6H_2O$, $CaCl_2 \cdot 2H_2O$, Na_2SO_4, $NaHCO_3$ and K_2SO_4) were used to achieve the desired salinity. The salinity was checked using a calibrated refractometer. To avoid precipitation in the final media, only nitrogen ($NaNO_3$) was added before the water was autoclaved (90 min., 121°C). Stock solutions of phosphorus (KH_2PO_4), iron ($FeCl_3 \cdot 6H_2O$/EDTA) and trace metals were autoclaved separately. Silicon ($Na_2SiO_3 \cdot 5H_2O$, always 140 μM final concentration to avoid limitation), vitamins and the salt $NaHCO_3$ were filtered through a 0.2 μm pore size non-pyrogenic and hydrophilic sterile single use syringe filter unit (Sartorius Minisart®). Finally, media pH was adjusted to 8.1 ± 0.1 with sterile 1.2 M HCl and/or 2 M NaOH, all done aseptically.

Chemostat culture experiments

Both strains of *P. multiseries* were grown in duplicate under N deficiency (36 μM $NaNO_3$, 36 μM K_2HPO_4; atomic N:P 1:1) and P deficiency (580 μM $NaNO_3$, 2 μM K_2HPO_4; atomic N:P 290:1). All eight 1-L chemostat culture flasks, containing 920–996 mL culture, were aerated with sterile air (0.2 μm pore size Sartorius Minisart® single use syringe filter unit) to keep algal cells in suspension, and to ensure mixing as new medium was added drop-wise to the surface with peristaltic pumps (see below). Measurements of temperature, pH, chlorophyll *a* (Chl *a*) and microscopic observation of cells in all eight cultures were made daily.

To avoid differences in media composition between culture flasks of one nutrient treatment, they were supplied with media from the same bottle. All cultures were kept under a 16:8 h light:dark cycle at 200 μmol photons m^{-2} s^{-1}. The dilution rates (i.e. cell division rate, related to the level of nutrient deficiency) of the cultures were checked daily by measuring the outflow of each culture. The first dilution rate was set to 0.2 day^{-1} (most deficient). As soon as steady state conditions had been achieved in all cultures, a sample (~50% of volume) from each culture was taken (see below for details about sub-sampling) and the dilution was thereafter adjusted to 0.3 day^{-1}. Sampling was repeated and the dilution rate was finally adjusted to 0.4 day^{-1} (least deficient). The final sampling was conducted after a total of 119 days in chemostat culture (Fig. 2). We considered the cultures to be at steady state when the coefficient of variation of daily measurements of Chl *a* of five consecutive days was not greater than 8% (usually <5%).

The sample collected from each culture at steady state was sub-sampled in order to determine abundance, volume and morphology of the cells. Concentrations of Chl *a*, dissolved inorganic nutrients ($N\text{-}NO_2^- + N\text{-}NO_3^-$, $N\text{-}NH_4^+$, $P\text{-}PO_4^{3-}$ and reactive silicate) and bacterial abundance in the cultures were also determined. Finally, total particulate carbon (TPC), nitrogen (TPN), phosphorus (TPP), biogenic particulate silicon (BPSi) and DA (particulate and dissolved) concentrations were analysed (see below).

Analytical procedures and measurements

Samples for estimation of cell densities and determination of cell volume were preserved with acid Lugol's

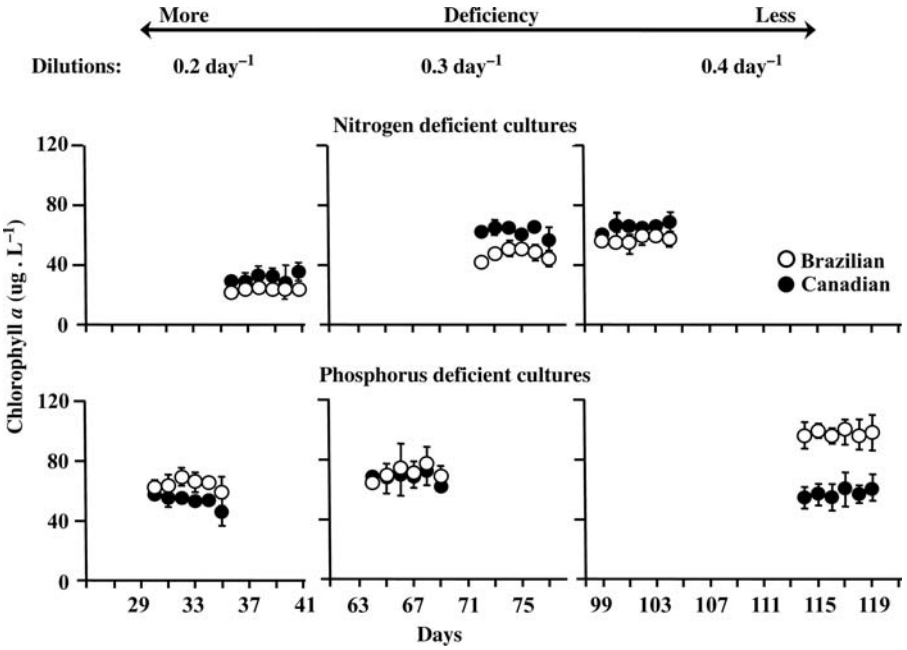

Fig. 2. Chlorophyll *a* concentrations (μg L^{-1}) of *Pseudo-nitzschia multiseries* (a Canadian and a Brazilian strain) growing under nitrogen (N) or phosphorus (P) deficiency in chemostat cultures during steady state (mean ± SD, $n = 2$). Error bars are occasionally smaller than the size of the symbol, and symbols are superimposed. The dilutions used were 0.2, 0.3 and 0.4 per day in order to obtain high (0.2 day^{-1}), intermediate (0.3 day^{-1}) and low (0.4 day^{-1}) N or P deficiency. Only the days when steady state was obtained for each treatment is plotted.

solution. Cells were counted according to Utermöhl (1958), using a Nikon Diaphot-300 inverted microscope. The samples were homogenized using a mini shaker (MS2, IKA Tamro Med-Lab), which also disrupted any chains, before making dilution series and counting. Cell volume estimation of *P. multiseries* was based on frustule measurements, assuming a rectangular box shape with double trapezoidal prisms in their extremes (Maldonado *et al.*, 2002). The cell volume

$$V = (A \times B \times C \times D) + [(1 - D) \times A \times \frac{(B + b)}{2} \times C] \quad (1)$$

where V is the cell volume, A the length of the valve (apical axis), B the valve width (transapical axis in valve view), C the valve depth (pre-valvar axis in girdle view), D the ratio of the linear part of the cell length to the total cell length and b the cell breadth on its extreme, corresponding to the minor base of the trapezoid. The BR strain had an average $b = 0.5$ μm, and $b = 1.0$ μm in the CA strain. Measurements were made using a light microscope with phase contrast at ×1000 magnification (ZEISS, model AXIOVERT). The microscope was equipped with a digital camera and an image processing and measurement program (SPOT Insight, Diagnostic Instruments Inc., version 3.5 for Windows). Cell volume estimation was conducted on the samples preserved in Lugol's solution at the Phytoplankton Laboratory of the Fundação Universidade FURG, Brazil.

Chl *a* was determined by filtering sub-samples through a Gelman Sciences type A/E glass fibre filter. Extraction was done in 6 mL 95% ethanol according to Jespersen and Christoffersen (1987) with the following method modifications. The filter in ethanol was sonicated in an ice-cooled water bath in the dark for 15 min. Extraction was allowed to complete under quiescent conditions for at least 2 h in the dark. Prior to modification of the original method, tests for comparison with 6 h extraction in room temperature were done and no significant difference was observed (data not shown). Chl *a* was measured using a Turner Designs Fluorometer model 10 AU.

Bacterial abundance in the cultures was determined using a flow cytometer (FACSCalibur, Becton Dickinson). Culture sub-samples of 1 mL (duplicates) were fixed with pre-filtered (0.2 μm pore size filter) formaldehyde (2% final concentration) and stained for 30 min at room temperature in the dark with SYTO 13, a nucleic acid stain (Molecular Probes) at a final concentration of 5 μM, following the method by Troussellier *et al.* (1999).

Nutrient analyses

Analyses of dissolved inorganic nutrients in the culture filtrates and inflow media followed the methods modified by Valderrama (1995). A Whatman GF/C glass fibre filter was used to obtain filtrate for N and P analyses. An all-plastic filtration apparatus and Nuclepore membrane filter (1.0 μm pore size) was used for the reactive silicate samples. Sub-samples for analyses of the particulate fraction were filtered onto pre-combusted (2 h, 450°C) Whatman GF/C glass fibre filters for total carbon, nitrogen and phosphorus (TPC, TPN and TPP, respectively). The filters were then dried (24 h, 60°C) and analysed using a CHN elemental auto analyser (Fisons Instruments model NA 1500) for TPC and TPN. Determination of TPP followed the method of Solórzano and Sharp (1980). Analysis of BPSi followed the method originally described by Paasche (1980). The filters, with algal cells, were stored in plastic petri dishes at −20°C, and dried (2 h, 80°C) between thawing and analyses.

DA in the cells was determined by filtering duplicate sub-samples onto Whatman GF/F glass fibre filters. The filters were freeze-dried to avoid the loss of DA during the transport to the Department of Food Chemistry, Friedrich-Schiller University, Jena, Germany for analysis. The filtrates of the above samples were also collected for dissolved DA measurements. Sodium azide (NaN_3, final concentration 10 mg L^{-1}) was used to preserve the samples, which were stored at +4°C prior to shipping. The DA analyses (particulate and dissolved) followed Furey *et al.* (2001) using an HPLC coupled to a mass-spectrometer (API 165, Applied Biosystems/MDS Sciex). Total DA was calculated as the sum of particulate and dissolved DA per cell. DA production rate (pg TDA $cell^{-1}$ day^{-1}) was calculated by multiplying total DA concentration (pg TDA $cell^{-1}$) with dilution rate per day.

Statistics

Two-way ANOVA, using GraphPad Prism 5 for Mac OS X, was used to compare results between the level of deficiency and algal strain. Results were considered significantly different at $P < 0.05$.

RESULTS

Algal cell volume, numbers, Chl *a* concentrations and bacterial abundance

Cell volume, calculated as mean of all nutrient treatments and dilution rates for each strain ($n = 12$), was higher (835.4 ± 108.3 μm^3) for the BR strain (apical axis around 100 μm) than the CA strain (262.2 ± 33.6 μm^3, apical axis around 32 μm). There was no trend in final cell abundance between treatments (Fig. 3). The smaller Canadian cells were expected to give a higher cell yield, and the lack of difference in cell abundance between strains is probably due to the fact that the CA strain had been in culture much longer than the BR strain (see below). We observed a general increase of Chl *a* concentration as the cultures became less N deficient, ranging from 24 to 113 μg L^{-1}. Both extremes were observed in the BR strain, with the lowest concentration during N deficiency at 0.2 day^{-1} and highest during P deficiency at 0.4 day^{-1} (Fig. 2). There was a significant increase in Chl *a* C^{-1} (two-way ANOVA, $P < 0.001$), as the cultures of both strains became less nutrient deficient (Fig. 3). There was, on the other hand, a decreasing trend in bacterial abundance with the reduced level of nutrient deficiency in all flasks. The highest abundance, 12.2×10^6 bacteria mL^{-1}, was found in the N-deficient BR culture at 0.2 day^{-1} (i.e. high deficiency), whereas the P-deficient CA culture had the lowest recorded bacterial abundance, 1.5×10^6 mL^{-1}, at the intermediate level of deficiency (Fig. 3).

Nutrients

The amount of N $cell^{-1}$ in the BR strain was higher in the P-deficient than in the N-deficient treatment (Fig. 4). This was not observed in the CA strain. The CA strain had highest N content at the intermediate level of deficiency in both nutrient deficient treatments. No significant difference in the amount of P per cell between the strains within the same nutrient treatment was found, but a significant difference was observed between treatments (two-way ANOVA, $P < 0.001$). The N-deficient cultures of both strains had two to four times more P per cell than the corresponding P-deficient cultures (Fig. 4). Cellular content of C and Si was also two to four times higher in the BR than in the CA strain, in particular during P deficiency, and this difference was significant (two-way ANOVA, $P < 0.05$) (Fig. 4). Both C and Si per cell decreased as the level of deficiency was reduced in the BR strain.

The atomic C:N and C:P ratios were significantly higher in the BR strain, with the exception of the C:P ratio under N deficiency, and this did not change due to level of deficiency (two-way ANOVA, $P < 0.05$) (Fig. 5). The C:N ratios were similar for the same strain under both N and P deficiencies. This shows that the cells contained low amounts of N even in the P-deficient treatment when N was supplied in excess to their need. The C:P ratios, however, show a "luxury consumption" of P under N deficiency as the C:P ratios are below the Redfield

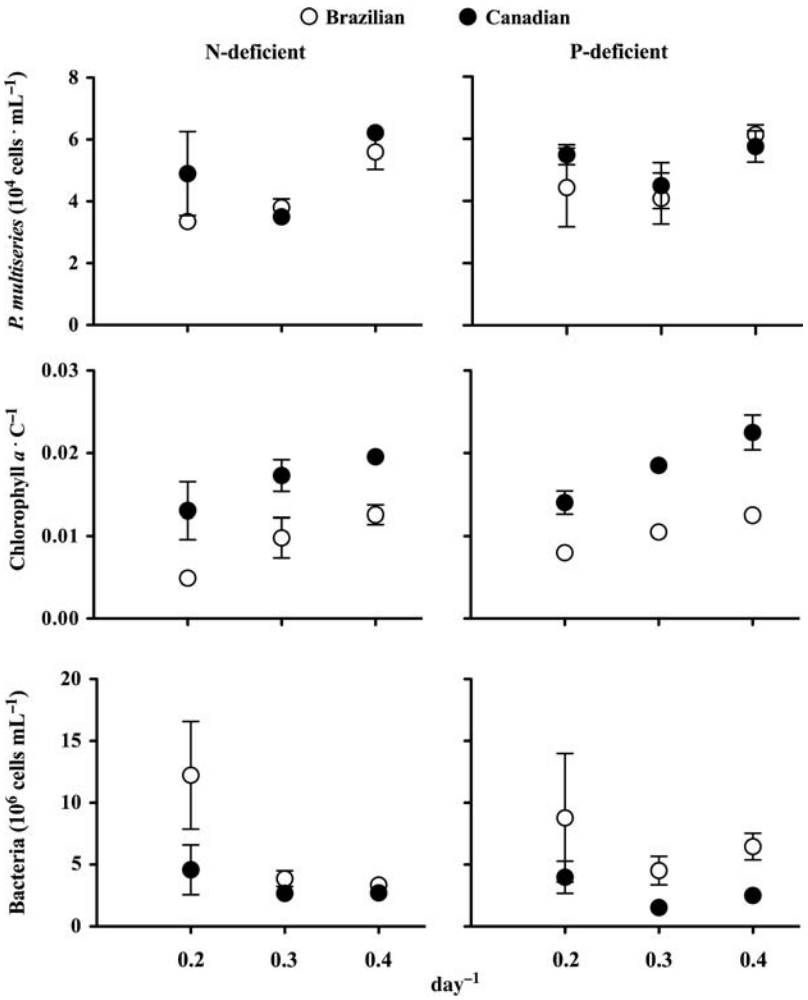

Fig. 3. Observed *Pseudo-nitzschia multiseries* cell abundance (upper panel), chlorophyll *a* per unit cellular C (middle panel) and bacterial abundance (lower panel) in relation to the level of N or P deficiency. Other explanations as in Fig. 2.

atomic ratio of 106. The C:Si ratios decreased in the N-deficient BR culture as the culture became less nutrient deficient (Fig. 5). There was a significant difference in N:P ratios between N- and P-deficient cultures (two-way ANOVA, $P < 0.01$). N-deficient cultures did not show a change in atomic N:P ratios due to the level of deficiency, while there was an increase in the two P-deficient cultures with highest ratio in the central point of the three levels of deficiency (Fig. 5). However, the N:P ratios of all cultures growing under N deficiency were below, and all but one culture growing under P deficiency had N:P ratio above, the atomic Redfield ratio.

Particulate, dissolved and total DA, and DA production rate

DA was detected in both strains and nutrient treatments (Fig. 6). The particulate (PDA) concentration per cell and the total DA (TDA = dissolved + particulate fractions) production cell^{-1} day^{-1} was higher in P-deficient than in N-deficient cultures for both strains. The range in PDA concentrations at cellular N:P ratios $\leq$7.8 were 0.08–0.26 pg cell^{-1} with values ranging between 0.93 and 3.45 pg cell^{-1} for cellular N:P ratios $\geq$14.7 (Fig. 6). PDA in the BR strain cells ranged between 0.11 and 3.45 pg cell^{-1}, and PDA concentrations in the CA strain ranged between 0.08 and 2.99 pg cell^{-1} (Fig. 6). Total DA production cell^{-1} day^{-1} followed the same trend as PDA cell^{-1}. That is, there was a clear difference in TDA production depending on the N:P ratios (0.04–0.07 and 0.40–0.97 pg TDA cell^{-1} day^{-1} for cellular N:P ratios $\leq$7.8 and $\geq$14.7, respectively). Bacterial abundance in the culture flasks did not seem to have an important effect on neither PDA cell^{-1} nor TDA production cell^{-1} day^{-1} in this experiment, since no trends could be observed (Fig. 6). Level of nutrient

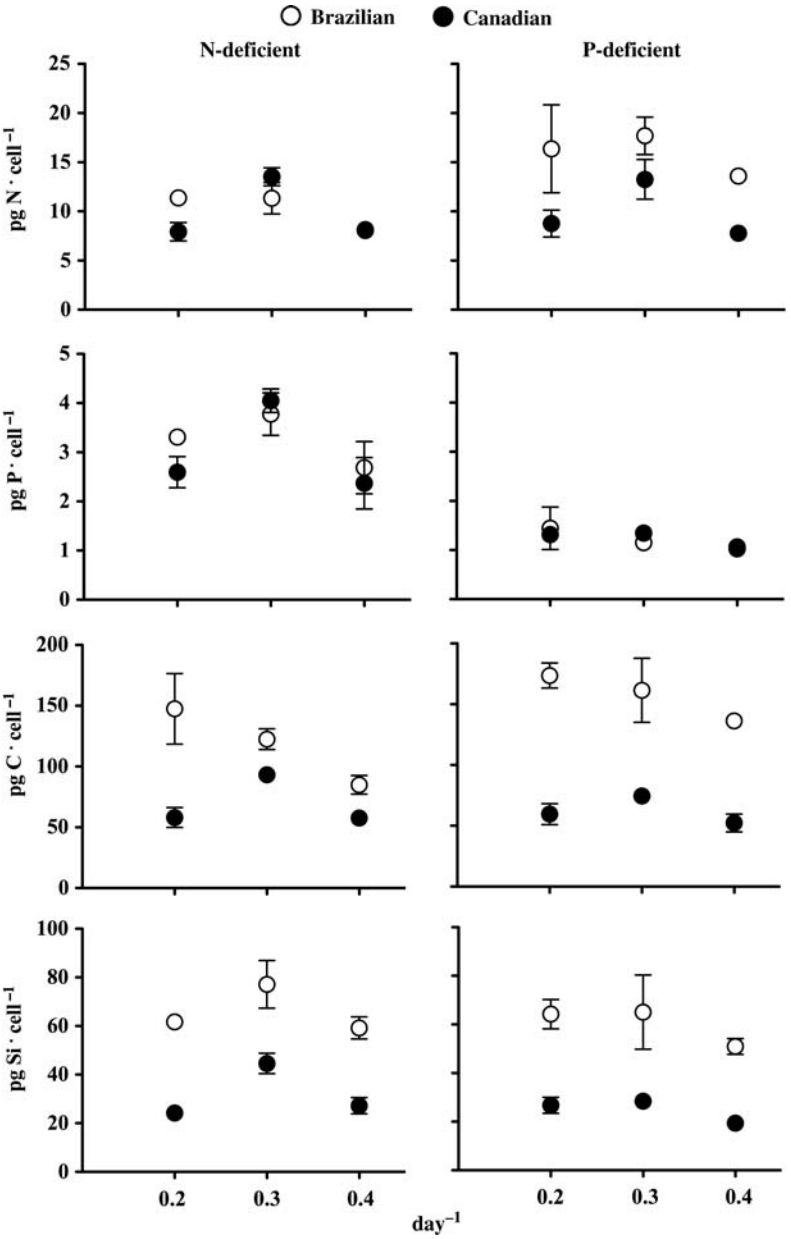

Fig. 4. Intracellular concentrations of N, P, C and Si in relation to the level of nutrient deficiency. Other explanations as in Fig. 2.

deficiency (day^{-1}) only had an effect on PDA $cell^{-1}$ and TDA production in the P-deficient BR strain with a decrease in toxin as dilution increased. No such trend could be observed in the CA strain or the N-deficient BR strain (Fig. 6).

DISCUSSION

In this paper, we present results from chemostat culture studies comparing the cell physiology and DA production for two strains of *Pseudo-nitzschia multiseries*. The strains were isolated from marine waters at two opposite latitudes: Canada (CA) and Brazil (BR). The strains were grown in parallel under N or P deficiency at three different levels of nutrient deficiency (0.2, 0.3 and 0.4 day^{-1}). The CA strain had been cultured in laboratory for ca. 10 years, whereas the BR strain was isolated 6 months prior to the experiment. Diatoms are known to decrease in cell size while in culture (Davidovich and Bates, 1998). Reduced cell sizes, cell volumes (about 31% of the BR strain) and number of

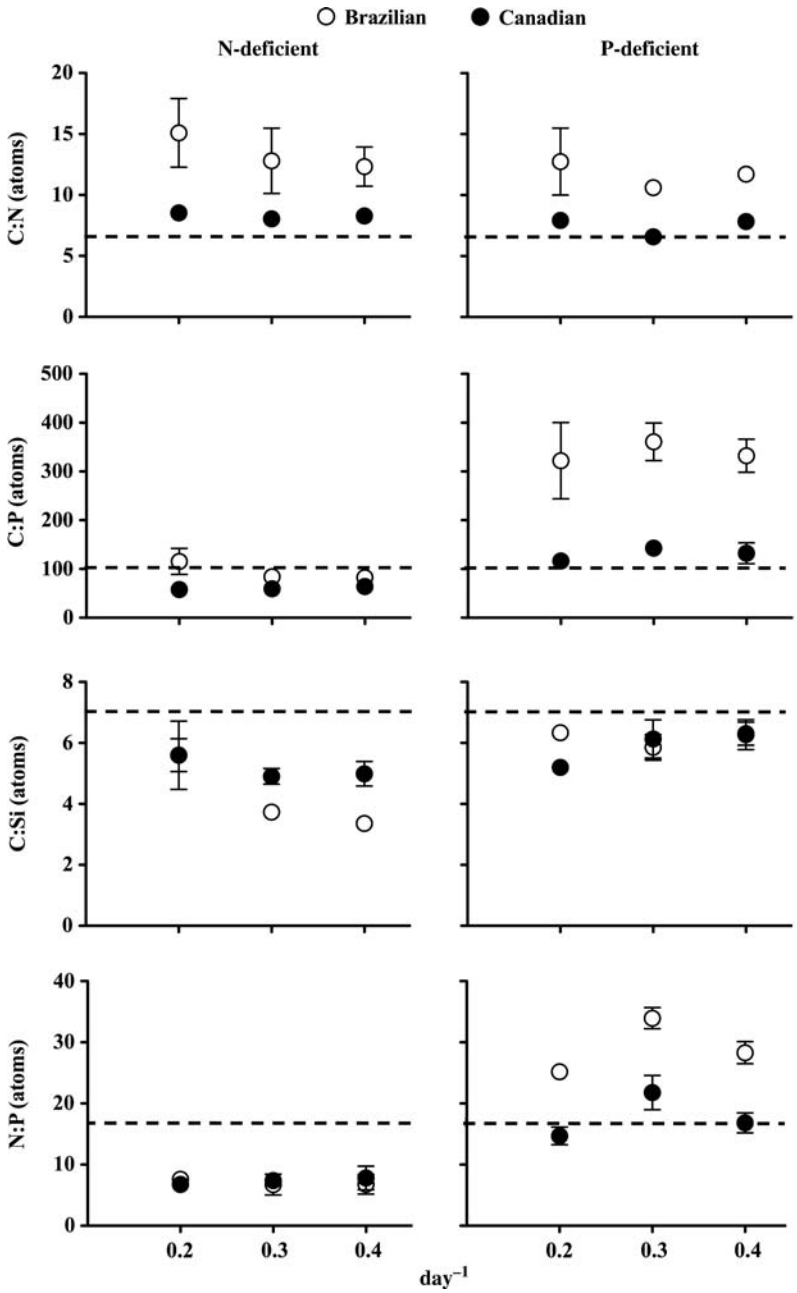

Fig. 5. Elemental ratios of C:N, C:P, C:Si and N:P in relation to the level of nutrient deficiency. The elemental value for diatoms (Brzezinski, 1985) of each nutrient ratio is indicated with a horizontal line. Other explanations as in Fig. 2.

cells per chain in the CA cultures were therefore expected. The CA strain also had more morphological deformations such as "lobed" or "sickle-shaped" cells, and some with one or more "undulations", than the BR strain. Thus we considered differences in cell morphology observed between the two strains a consequence of their culture age before the start of the experiment.

A deficiency in N or Si in phytoplankton leads to an increase in cellular carbon content, resulting from carbon accumulation when cell division ceases (Flynn and Martin-Jezequel, 2000), as well as under P deficiency (Sakshaug *et al.*, 1984; Pan *et al.*, 1996a; Granéli and Johansson, 2003; Granéli and Salomon, 2010). Harrison *et al.* (1977) observed that Chl *a*:C ratios were lower under N deficiency than for NP-sufficient

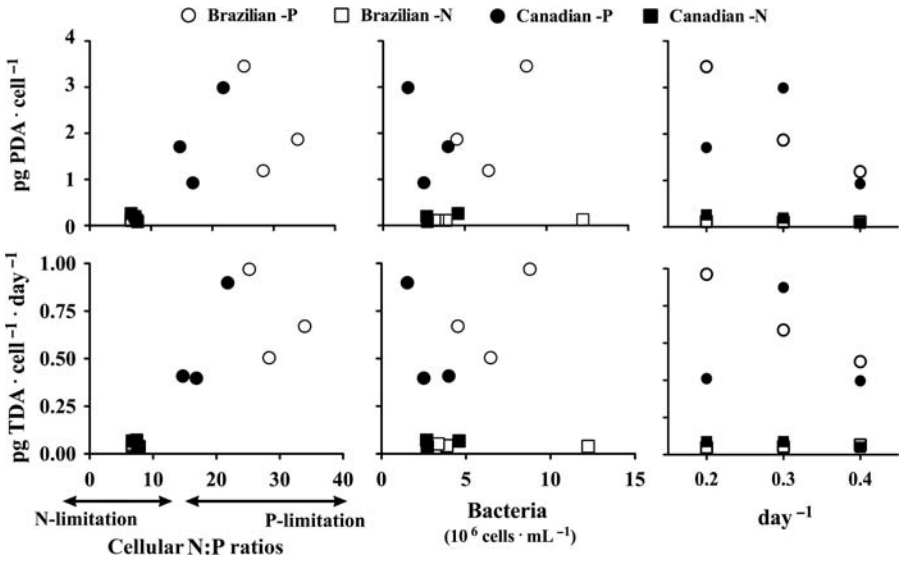

Fig. 6. Concentration of DA (PDA) cell^{-1}, and total DA cell^{-1} day^{-1} (TDA = particulate + dissolved DA in the culture bottles) in relation to cellular (atomic) N:P ratios (left hand panels), bacterial abundance (middle panels) and dilutions (right hand panels) (mean, $n = 2$).

diatom cells (*Skeletonema costatum*, *Chatoceros debilis*, *Thalassiosira gravida*) when growing in chemostats. This was due to a decrease in Chl *a* cell^{-1} (i.e. not increase in C) as the cells became more N limited. In line with the findings by Pan *et al.* (1996a), we also observed an increase in cellular Chl *a*:C in both strains and nutrient treatments as the cells became less deficient. The amount of C cell^{-1} in this experiment, on the other hand, decreased as the cells were subject to reduced deficiency in the BR strain, something not observed for CA cells. Cellular N, P, C and Si in the experiment reported here were generally higher in the BR strain; and, especially for the BR strain, decreased as the level of nutrient deficiency decreased. The higher N, P, Si, and C cellular concentrations in the BR strain can be explained by the larger size of this strain in comparison with the CA strain. Furthermore, the elemental ratios measured for both strains are all within the range of what has been reported for *P. multiseries* by others (e.g. Pan *et al.*, 1996a, b).

Nutrient ratios and DA

A number of studies have investigated the effect of various sources of N (e.g. urea, NO_3^-, NH_4^+) on the physiology of *Pseudo-nitzschia* spp. and strains in batch cultures (Bates, 1998; Bates and Trainer, 2006; Howard *et al.*, 2007; Thessen *et al.*, 2009). Laflamme (1993) studied the effect of Si and P limitation on DA production of *P. multiseries* in chemostats. Kudela *et al.* (2004) used chemostat cultures to determine DA production of *P. multiseries* strains, from one location, during Si and N deficiency. However, until now, the effect of N deficiency on general physiology and DA production in two *P. multiseries* strains isolated from the NW and SW Atlantic Ocean, and growing in parallel chemostat cultures, has not yet been investigated. Since DA is an amino acid and N is an essential element required for its synthesis, it has been argued that DA production would cease during N deficiency (Bates *et al.*, 1991). However, Kudela *et al.* (2004) detected low levels of DA in N-limited chemostats. Kudela *et al.* (2004) and Thessen *et al.* (2009) could also show inter-strain variability in DA production, even when the strains had been isolated from the same location. Our results show that, not only when submitted to P deficiency but also even under N deficiency and when there is a continuous cell division (supply of N even in small amounts), both BR and CA strains produced DA. However, DA production was several-fold greater at cellular N:P ratios above 14.7 (P deficiency and N sufficiency) than below 7.8 (N deficiency and P sufficiency). Further, investigations of environmental conditions that may promote *Pseudo-nitzschia* spp. blooms affect the physiology and/or trigger/enhance DA production are therefore necessary. Among these are light conditions (Bates *et al.*, 2001; Fehling *et al.*, 2005), iron sufficiency or deficiency (Bates *et al.*, 2001; Rue and Bruland, 2001; Wells *et al.*, 2005), several other trace metals (Wells *et al.*, 2005) and the studies must be conducted under identical conditions on several strains. Continuous cultures as an instrument to understand algal ecophysiology have an advantage over batch cultures by allowing a dynamic equilibrium between nutrient input, and cell death and growth;

similar in principle to the conditions in natural waters. As in nature, nutrient input rate during continuous culturing, rather then their concentrations at a given time, determines growth limitation (Rhee, 1980).

There are several examples from the literature where N and/or P deficiency cause an increase in cellular toxin concentration in a number of algal species from different taxa, including *P. multiseries* (Granéli and Johansson, 2003; Granéli and Flynn, 2006; Granéli and Salomon, 2010). It has been argued that DA production in *Pseudo-nitzschia* spp. is associated with Si depletion, as reported by Pan *et al.* (1996b). The results reported here resemble those reported for two clones of *Pseudo-nitzschia* sp. cf. *pseudodelicatissima* isolated from the northern Gulf of Mexico by Pan *et al.* (2001). Their data suggest that a rapidly growing population, i.e. not a stressed population due to nutrient deficiency, yields maximum net DA production rates and DA cell quotas. Production of DA at steady state was also found to be positively correlated to the N:P ratios, suggesting that P deficiency promoted DA production (Pan *et al.*, 1996a). We found that N was indeed a requirement for high DA production for both the Canadian and Brazilian strain during chemostat culturing since the amount of toxin was low at cellular N:P ratios $\leq$7.8, i.e. N deficiency.

A literature review reveals a great variation in *P. multiseries* cellular DA. Pan *et al.* (1996a) report a range of 0.3–4.5 pg DA cell^{-1} during P deficiency. Furthermore, they argue that the optimum N:P ratio for *P. multiseries* is not the Redfield atomic ratio of 16:1 but that it is closer to 10:1, and that DA production was only halted at N:P ratios less than about 7. This is in line with the findings in our experiment reported here, i.e. 0.08–3.45 pg DA cell^{-1} with highest concentrations at high N:P ratios. There was no general trend in DA concentration as dilution rate changed, i.e. N:P ratio seem to have greater impact on DA production than growth rate. Bates *et al.* (1999), investigating DA toxicity of large new cells of *P. multiseries* after induced sexual reproduction in cultures, observed that the parental clones had almost lost their capacity to produce DA (<0.1 pg DA cell^{-1}), while their offspring cells (large new cells) were able to produce up to 33–67 (5-month-old clones) and 65 pg DA cell^{-1} (one 12-month-old clone). According to our results, culture age was not the most important factor since the 10-year older CA isolate produced similar levels of DA as the 6-month-old isolated BR strain (0.08–2.99 and 0.11–3.45 pg DA cell^{-1}, respectively). DA production in this experiment (0.04–0.97 pg DA cell^{-1} day^{-1}) was also found to be similar to what has been reported elsewhere. Lewis *et al.* (1993) found 0.01 pg DA cell^{-1} day^{-1}, and Subba Rao *et al.* (1990) report a maximum of 2.0 pg DA cell^{-1} day^{-1}. This variation in DA cellular concentration is within the range found for other *Pseudo-nitzschia* species (Fehling *et al.*, 2004).

Other authors have found a positive correlation between associated bacterial abundance and DA production. That is, non-axenic cultures had a higher DA production rate than axenic cultures (Douglas and Bates, 1992; Douglas *et al.*, 1993). Stewart (2008), studying bacterial contribution to DA levels in culture, concluded that the total amount of DA at any one given time is dependent on the *Pseudo-nitzschia* spp. production rate, nutrient status, bacterial DA production stimulation and bacterial degradation of DA. In our experiment, however, the bacteria associated with *P. multiseries* did not seem to affect DA production, neither positively nor negatively.

Summary

Our results show that the level of deficiency did have a significant effect on atomic N:C, P:C, Si:C and N:P ratios in the *P. multiseries* cells from Brazil or Canada. Furthermore, DA concentrations per cell and production rates by the CA and BR strains are in the range of those observed previously by other authors, across all three levels of deficiency and N:P ratios used in our experiments. However, DA concentration per cell, and DA production per cell per day, were significantly different depending on the level of nitrogen or phosphorus deficiency, being higher under P deficiency as expected due to the high content of N in the toxin. However, DA was always produced in all treatments by *P. multiseries* in this chemostat culture experiment. Hence, devastating DA contamination of, for example, shellfish can occur at a wide range of growth rates and, in particular, nutrient ratios during and after a prolonged bloom.

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