



# Domoic acid production and elemental composition of two *Pseudo-nitzschia* multiseres strains, from the NW and SW Atlantic Ocean, growing in phosphorus- or nitrogen- limited chemostat cultures.

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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.**

**JOHANNES A. HAGSTRÖM<sup>1</sup>, EDNA GRANÉLI<sup>1\*</sup>, MARIA O. P. MOREIRA<sup>2</sup>, AND CLARISSE ODEBRECHT<sup>3</sup>**

<sup>1</sup>LINNAEUS UNIVERSITY, NATURAL SCIENCES INSTITUTE, MARINE ECOLOGY DEPARTMENT, SE-39182 KALMAR, SWEDEN  
<sup>2</sup> INSTITUTO DE CIÊNCIAS DO MAR (LABOMAR), UFC, 60.165-081 FORTALEZA, CE, BRAZIL  
<sup>3</sup>DEPTO. DE OCEANOGRAPHIA, FURG, 96201-900 RIO GRANDE, RS, BRAZIL

\*e-mail: edna.graneli@lnu.se  
Phone: +46 480 447307 Fax:+46 480 447340  
Keywords: *Pseudo-nitzschia*, Domoic acid, Chemical composition, Toxin, Nutrient limitation

**ABSTRACT**

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 d<sup>-1</sup> 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 X 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 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-deficiency affect DA production and cellular DA concentrations.

1 INTRODUCTION

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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). More than 100 humans consuming the mussels suffered from e.g. 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 e.g. 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<sub>3</sub><sup>-</sup>, NH<sub>4</sub><sup>+</sup> 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 ( $0.2 - 1.2 \text{ d}^{-1}$ ). 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: (1) Are there any physiological differences between two strains of *P. multiseriis* from different origins (SW and NW Atlantic Ocean) concerning DA production when growing in N- and P-deficient conditions? (2) 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 multiseriis* 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 Prince Edward Island (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. multiseriis* 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  $\mu\text{L}$  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 (dNTP), 5% dimethylsulfoxide, 5 pmol each of primers EUK1 and 518r, Taq DNA polymerase reaction buffer with 1.5 mM  $\text{MgCl}_2$  and 1 U of a mixture of Taq DNA polymerase (Roche) in a total reaction volume of 50  $\mu\text{L}$  (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  $94^\circ\text{C}$ . The 30 subsequent cycles were 30 seconds at  $94^\circ\text{C}$ , 30 seconds at  $50^\circ\text{C}$  and 1 min at  $72^\circ\text{C}$ . A final incubation of 5 min at  $72^\circ\text{C}$  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 hours light:dark cycle. Photosynthetically Active Radiation (PAR) was adjusted to 200 μmol photons m<sup>-2</sup> s<sup>-1</sup> (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 one month in f/10 medium, with the same light, temperature and adjustments of nutrient ratio as 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<sub>2</sub>·6H<sub>2</sub>O, CaCl<sub>2</sub>·2H<sub>2</sub>O, Na<sub>2</sub>SO<sub>4</sub>, NaHCO<sub>3</sub> and K<sub>2</sub>SO<sub>4</sub>) 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<sub>3</sub>) was added before the water was autoclaved (90 min., 121°C). Stock solutions of phosphorus (KH<sub>2</sub>PO<sub>4</sub>), iron (FeCl<sub>3</sub>·6H<sub>2</sub>O/EDTA), and trace metals were autoclaved separately. Silicon (Na<sub>2</sub>SiO<sub>3</sub>·5H<sub>2</sub>O, always 140 μM final concentration to avoid limitation), vitamins, and the salt NaHCO<sub>3</sub> 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. multiseriis* were grown in duplicate under N deficiency (36  $\mu\text{M}$   $\text{NaNO}_3$ , 36  $\mu\text{M}$   $\text{K}_2\text{HPO}_4$ ; atomic N:P 1:1) and P deficiency (580  $\mu\text{M}$   $\text{NaNO}_3$ , 2  $\mu\text{M}$   $\text{K}_2\text{HPO}_4$ ; atomic N:P 290:1). All eight 1-L chemostat culture flasks, containing 920 – 996 mL culture, were aerated with sterile air (0.2  $\mu\text{m}$  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 8 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 hours light: dark cycle at 200  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ . The dilution rates (i.e. cell division rate, related to the level of nutrient deficiency) of the cultures were checked daily by measuring the out-flow of each culture. The first dilution rate was set to 0.2  $\text{d}^{-1}$  (most deficient). As soon as steady state conditions had been achieved in all cultures, a sample ( $\approx 50\%$  of volume) from each culture was taken (see below for details about sub-sampling) and the dilution was thereafter adjusted to 0.3  $\text{d}^{-1}$ . Sampling was repeated and the dilution rate was finally adjusted to 0.4  $\text{d}^{-1}$  (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 ( $\text{N-NO}_2^- + \text{N-NO}_3^-$ ,  $\text{N-NH}_4^+$ ,  $\text{P-PO}_4^{3-}$ , 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 domoic acid (DA; particulate and dissolved) concentrations were analyzed (see below).

## Analytical procedures and measurements

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



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series and counting. Cell volume estimation of *P. multiseriata* was based on frustule measurements, assuming a rectangular box shape with double trapezoidal prisms in their extremes (Maldonado *et al.*, 2002). The cell volume

$$V = (A \times B \times C \times D) + [(1 - D) \times A \times (B + b)/2 \times C] \tag{Eq. 1}$$

where V is the cell volume, A the length of the valve (apical axis), B is the valve width (transapical axis in valve view), C is the valve depth (prevalvar axis in girdle view), D is the ratio of the linear part of the cell length to the total cell length, and b is the cell breadth on its extreme, corresponding to the minor base of the trapezoid. The BR strain had an average  $b = 0.5 \mu\text{m}$ , and  $b = 1.0 \mu\text{m}$  in the CA strain. Measurements were made using a light microscope with phase contrast at 1000X 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 Federal de Rio Grande (FURG), Brazil.

Chlorophyll *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 minutes. Extraction was allowed to complete under quiescent conditions for at least 2 hours in the dark. Prior to modification of the original method, tests for comparison with 6 hour extraction in room temperature were done and no significant difference was observed (data not shown). Chlorophyll *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  $\mu\text{m}$  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  $\mu\text{M}$ , 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 fiber filter was used to obtain filtrate for N and P analyses. An all-plastic filtration apparatus and Nuclepore membrane filter (1.0  $\mu\text{m}$  pore size) was used for the reactive silicate samples. Sub-

1 samples for analyses of the particulate fraction were filtered onto pre-combusted (2 hours,  
2 450°C) Whatman GF/C glass fiber filters for total carbon, nitrogen and phosphorus (TPC,  
3 TPN and TPP, respectively). The filters were then dried (24 hours, 60°C) and analyzed using  
4 a CHN elemental auto analyzer (Fisons Instruments model NA 1500) for TPC and TPN.  
5 Determination of TPP followed the method of Solórzano and Sharp (1980). Analysis of BPSi  
6 followed the method originally described by Paasche (1980). The filters, with algal cells, were  
7 stored in plastic petri dishes at -20°C, and dried (2 hours, 80°C) between thawing and  
8 analyses.

9 Domoic acid in the cells was determined by filtering duplicate sub-samples onto  
10 Whatman GF/F glass fiber filters. The filters were freeze-dried to avoid loss of DA during the  
11 transport to the Department of Food Chemistry, Friedrich-Schiller University, Jena, Germany  
12 for analysis. The filtrates of the above samples were also collected for dissolved DA  
13 measurements. Sodium azide ( $\text{NaN}_3$ , final concentration  $10 \text{ mg L}^{-1}$ ), was used to preserve the  
14 samples, which were stored at + 4°C prior to shipping. The DA analyses (particulate and  
15 dissolved) followed Furey *et al.* (2001) using an HPLC coupled to a mass-spectrometer (API  
16 165, Applied Biosystems/MDS Sciex). Total DA was calculated as the sum of particulate and  
17 dissolved DA per cell. Domoic acid production rate ( $\text{pg TDA cell}^{-1} \text{ day}^{-1}$ ) was calculated by  
18 multiplying total DA concentration ( $\text{pg TDA cell}^{-1}$ ) with dilution rate per day.

## 20 Statistics

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22 2-way ANOVA, using GraphPad Prism 5 for Mac OS X, was used to compare results  
23 between level of deficiency and algal strain. Results were considered significantly different at  
24  $P < 0.05$ .

## 26 RESULTS

### 28 Algal cell volume, numbers, chlorophyll *a* concentrations and bacterial abundance

30 Cell volume, calculated as mean of all nutrient treatments and dilution rates for each  
31 strain ( $n = 12$ ), was higher ( $835.4 \pm 108.3 \mu\text{m}^3$ ) for the BR strain (apical axis around  $100 \mu\text{m}$ )  
32 than the CA strain ( $262.2 \pm 33.6 \mu\text{m}^3$ , apical axis around  $32 \mu\text{m}$ ). There was no trend in final  
33 cell abundance between treatments (Fig. 3). The smaller Canadian cells were expected to give  
34 a higher cell yield, and the lack of difference in cell abundance between strains is probably

1 due to the fact that the CA strain had been in culture much longer than the BR strain (see  
2 below). We observed a general increase of chl *a* concentration as the cultures became less N-  
3 deficient, ranging from 24 – 113  $\mu\text{g L}^{-1}$ . Both extremes were observed in the BR strain, with  
4 the lowest concentration during N-deficiency at 0.2  $\text{d}^{-1}$  and highest during P-deficiency at 0.4  
5  $\text{d}^{-1}$  (Fig. 2). There was a significant increase in chl *a*  $\text{C}^{-1}$  (2-way ANOVA,  $P < 0.001$ ), as the  
6 cultures of both strains became less nutrient deficient (Fig. 3). There was on the other hand a  
7 decreasing trend in bacterial abundance with reduced level of nutrient deficiency in all flasks.  
8 The highest abundance,  $12.2 \times 10^6$  bacteria  $\text{mL}^{-1}$ , was found in the N-deficient BR culture at  
9 0.2  $\text{d}^{-1}$  (i.e. high deficiency) whereas the P-deficient CA culture had the lowest recorded  
10 bacterial abundance,  $1.5 \times 10^6$   $\text{mL}^{-1}$ , at the intermediate level of deficiency (Fig. 3).

11  
12 **Nutrients**

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14 The amount of N  $\text{cell}^{-1}$  in the BR strain was higher in the P-deficient than in the N-  
15 deficient treatment (Fig. 4). This was not observed in the CA strain. The CA strain had  
16 highest N content at the intermediate level of deficiency in both nutrient deficient treatments.  
17 No significant difference in the amount of P per cell between the strains within the same  
18 nutrient treatment was found, but a significant difference was observed between treatments  
19 (2-way ANOVA,  $P < 0.001$ ). The N-deficient cultures of both strains had 2 – 4 times more P  
20 per cell than the corresponding P-deficient cultures (Fig. 4). Cellular content of C and Si was  
21 also 2 – 4 times higher in the BR than in the CA strain, in particular during P-deficiency, and  
22 this difference was significant (2-way ANOVA,  $P < 0.05$ ) (Fig. 4). Both C and Si per cell  
23 decreased as level of deficiency was reduced in the BR strain.

24 The atomic C:N and C:P ratios were significantly higher in the BR strain, with the  
25 exception of the C:P ratio under N-deficiency, and this did not change due to level of  
26 deficiency (2-way ANOVA,  $P < 0.05$ ) (Fig. 5). The C:N ratios were similar for the same  
27 strain under both N- and P-deficiency. This shows that the cells contained low amounts of N  
28 even in the P-deficient treatment when N was supplied in excess to their need. The C:P ratios  
29 however, show a “luxury consumption” of P under N-deficiency as the C:P ratios are below  
30 the Redfield atomic ratio of 106. The C:Si ratios decreased in the N-deficient BR culture as  
31 the culture became less nutrient deficient (Fig. 5). There was a significant difference in N:P  
32 ratios between N-deficient and P-deficient cultures (2-way ANOVA,  $P < 0.01$ ). N-deficient  
33 cultures did not show a change in atomic N:P ratios due to level of deficiency, while there  
34 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 domoic acid, and domoic acid production rate

Domoic acid 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  $\text{cell}^{-1} \text{ day}^{-1}$  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  $\text{pg cell}^{-1}$  with values ranging between 0.93 and 3.45  $\text{pg cell}^{-1}$  for cellular N:P ratios  $\geq 14.7$  (Fig. 6). PDA in the BR strain cells ranged between 0.11 and 3.45  $\text{pg cell}^{-1}$ , and PDA concentrations in the CA strain ranged between 0.08 and 2.99  $\text{pg cell}^{-1}$  (Fig. 6). Total DA production  $\text{cell}^{-1} \text{ day}^{-1}$  followed the same trend as PDA  $\text{cell}^{-1}$ . That is, there was a clear difference in TDA production depending on the N:P ratios (0.04 to 0.07 and 0.40 to 0.97  $\text{pg TDA cell}^{-1} \text{ day}^{-1}$  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  $\text{cell}^{-1}$  nor TDA production  $\text{cell}^{-1} \text{ day}^{-1}$  in this experiment since no trends could be observed (Fig. 6). Level of nutrient deficiency ( $\text{d}^{-1}$ ) only had an effect on PDA  $\text{cell}^{-1}$  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 domoic acid 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  $\text{d}^{-1}$ ). 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 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 (Granéli and Johansson, 2003; Granéli and Salomon, 2010; Pan *et al.*, 1996a; Sakshaug *et al.*, 1984). Harrison *et al.* (1977) observed that chl *a*:C ratios were lower under N-deficiency than for NP-sufficient diatom cells (*Skeletonema costatum*, *Chatoceros debilis*, *Thalassiosira gravida*) when growing in chemostats. This was due to a decrease in chl *a* cell<sup>-1</sup> (i.e. not increase in C) the more N-limited the cells were. 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<sup>-1</sup> 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. multiseriis* by others (e.g. Pan *et al.*, 1996a,b).

### Nutrient ratios and Domoic acid

A number of studies have investigated the effect of various sources of N (e.g. urea, NO<sub>3</sub><sup>-</sup>, NH<sub>4</sub><sup>+</sup>) 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. multiseriis* in chemostats. Kudela *et al.* (2004) used chemostat cultures to determine DA production of *P. multiseriis* 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. multiseriis* 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 than 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. multiseriis* (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. multiseriis* cellular DA. Pan *et al.* (1996a) report a range of 0.3 – 4.5 pg DA cell<sup>-1</sup> during P deficiency. Furthermore, they argue that the optimum N:P ratio for *P. multiseriis* 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<sup>-1</sup> with highest concentrations at high N:P ratios. There was no general trend in DA concentration as



1  
2  
3 1 dilution rate changed, i.e. N:P ratio seem to have greater impact on DA production than  
4  
5 2 growth rate. Bates *et al.* (1999), investigating DA toxicity of large new cells of *P. multiseri*  
6  
7 3 after induced sexual reproduction in cultures, observed that the parental clones had almost lost  
8  
9 4 their capacity to produce DA ( $< 0.1 \text{ pg DA cell}^{-1}$ ) while their offspring cells (large new cells)  
10  
11 5 were able to produce up to 33 to 67 (5 months old clones) and  $65 \text{ pg DA cell}^{-1}$  (one 12 months  
12  
13 6 old clone). According to our results, culture age was not the most important factor since the  
14  
15 7 10-year older CA isolate produced similar levels of DA as the 6 months old isolated BR strain  
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17 8 ( $0.08 - 2.99$  and  $0.11 - 3.45 \text{ pg DA cell}^{-1}$ , respectively). DA production in this experiment  
18  
19 9 ( $0.04 - 0.97 \text{ pg DA cell}^{-1} \text{ day}^{-1}$ ) was also found to be similar to what has been reported  
20  
21 10 elsewhere. Lewis *et al.* (1993) found  $0.01 \text{ pg DA cell}^{-1} \text{ day}^{-1}$ , and Subba Rao *et al.* (1990)  
22  
23 11 report a maximum of  $2.0 \text{ pg DA cell}^{-1} \text{ day}^{-1}$ . This variation in DA cellular concentration is  
24  
25 12 within the range found for other *Pseudo-nitzschia* species (Fehling *et al.*, 2004).

26  
27 13 Other authors have found a positive correlation between associated bacterial abundance  
28  
29 14 and DA production. That is, non-axenic cultures had a higher DA production rate than axenic  
30  
31 15 cultures (Douglas and Bates, 1992; Douglas *et al.*, 1993). Stewart (2008), studying bacterial  
32  
33 16 contribution to DA levels in culture, concluded that the total amount of DA at any one given  
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35 17 time is dependent on the *Pseudo-nitzschia* spp. production rate, nutrient status, bacterial DA  
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37 18 production stimulation, and bacterial degradation of DA. In our experiment, however, the  
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39 19 bacteria associated with *P. multiseri* did not seem to affect DA production, neither  
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41 20 positively nor negatively.

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44 21  
45 22 **SUMMARY**

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48 24 Our results show that the level of deficiency did have a significant effect on atomic  
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50 25 N:C, P:C, Si:C and N:P ratios in the *P. multiseri* cells from Brazil or Canada. Furthermore,  
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52 26 DA concentrations per cell and production rates by the CA and BR strains are in the range of  
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54 27 those observed previously by other authors, across all three levels of deficiency and N:P ratios  
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56 28 used in our experiments. However, domoic acid concentration per cell, and DA production  
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58 29 per cell per day, were significantly different depending on level of nitrogen or phosphorus  
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60 30 deficiency, being higher under P-deficiency as expected due to the high content of N in the  
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32 31 toxin. However, DA was always produced in all treatments by *P. multiseri* in this chemostat  
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34 32 culture experiment. Hence, devastating DA contamination of, for example, shellfish can  
35  
36 33 occur at a wide range of growth rates and, in particular, nutrient ratios during and after a  
37  
38 34 prolonged bloom.



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## REFERENCES

- Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (1989) *Current Protocols in Molecular Biology*. John Wiley & Sons, Inc., New York, U.S.A.
- Bates, S.S. (1998) Ecophysiology and metabolism of ASP toxin production. In Anderson, D.M., Cembella, A.D. and Hallegraeff, G.M. (eds.) *Physiological Ecology of Harmful Algal Blooms*. Springer-Verlag, Heidelberg, Germany, pp. 405-426.
- Bates, S.S., de Freitas, A.S.W., Milley, J.E., Pocklington, R., Quilliam, M.A., Smith, J.C. and Worms, J. (1991) Controls on domoic acid production by the diatom *Nitzschia pungens* f. *multiseries* in culture: nutrients and irradiance. *Can. J. Fish. Aquat. Sci.*, **48**, 1136-1144.
- Bates, S.S., Garrison, D.L. and Horner, R.A. (1998) Bloom dynamics and physiology of domoic-acid-producing *Pseudo-nitzschia* species. In Anderson, D.M., Cembella, A.D. and Hallegraeff, G.M. (eds.) *Physiological Ecology of Harmful Algal Blooms*. Springer-Verlag, Heidelberg, Germany, pp. 267-292.
- Bates, S.S., Hiltz, M.F. and Léger, C. (1999) Domoic acid toxicity of large new cells of *Pseudo-nitzschia multiseries* resulting from sexual reproduction. *Can. Tech. Rep. Fish. Aquat. Sci.*, **2261**, 21-26.
- Bates, S.S., Léger, C., Satchwell, M., and Boyer G.L. (2001) The effects of iron on domoic acid production by *Pseudo-nitzschia multiseries*. In Hallegraeff, G.M., Blackburn, S.I.,

- 1
- 2
- 3 1 Bolch, C.J. and Lewis, R.J. (eds.), *Harmful Algal Blooms* 2000. IOC of UNESCO,
- 4 2 Paris. pp. 320-323.
- 5
- 6 3 Bates, S.S. and Trainer, V.L. (2006) The ecology of harmful diatoms. In Granéli, E. and
- 7 4 Turner, J.T. (eds.) *Ecology of Harmful Algae*, Vol. Ecological Studies 189. Springer-
- 8 5 Verlag, Berlin and Heidelberg, Germany, pp. 81-93.
- 9
- 10 6 Brzezinski, M.A. (1985) The Si:C:N ratio of marine diatoms: Interspecific variability and the
- 11 7 effect of some environmental variables. *J. Phycol.*, **21**, 347-357.
- 12
- 13 8 Cusack, C.K., Bates, S.S., Quilliam, M.A., Patching, J.W. and Raine, R. (2002) Confirmation
- 14 9 of domoic acid production by *Pseudo-nitzschia australis* (bacillariophyceae) isolated
- 15 10 from Irish waters. *J. Phycol.*, **38**, 1106-1112.
- 16
- 17 11 Davidovich, N.A. and Bates, S.S. (1998) Sexual reproduction in the pennate diatoms
- 18 12 *Pseudonitzschia multiseriata* and *P. pseudodelicatissima* (Bacillariophyceae). *J. Phycol.*,
- 19 13 **34**, 126-137.
- 20
- 21 14 Douglas, D.J. and Bates, S.S. (1992) Production of domoic acid, a neurotoxin amino acid, by
- 22 15 an axenic culture of the marine diatom *Nitzschia pungens* f. *multiseriata* Hasle. *Can. J.*
- 23 16 *Fish. Aquat. Sci.*, **49**, 85-90.
- 24
- 25 17 Douglas, D.J., Bates, S.S., Bourque, L.A. and Selvin, R.C. (1993) Domoic acid production by
- 26 18 axenic and non-axenic cultures of *Nitzschia pungens* f. *multiseriata*. In Smayda, T.J. and
- 27 19 Shimizu, Y. (eds.) *Toxic Phytoplankton Blooms in the Sea*. Elsevier, Amsterdam,
- 28 20 Netherlands, pp. 595-600.
- 29
- 30 21 Fehling, J., Davidson, K. and Bates, S.S. (2005) Growth dynamics of non-toxic
- 31 22 *Pseudonitzschia delicatissima* and toxic *P. seriata* (Bacillariophyceae) under simulated
- 32 23 spring and summer photoperiods. *Harmful Algae*, **4**, 763-769.
- 33
- 34 24 Fehling, J., Davidson, K., Bolch, C.J. and Bates, S.S. (2004) Growth and domoic acid
- 35 25 production by *Pseudo-nitzschia seriata* (Bacillariophyceae) under phosphate and
- 36 26 silicate limitation. *J. Phycol.*, **40**, 674-683.
- 37
- 38 27 Flynn, K.J. and Martin-Jezequel, V. (2000) Modelling Si-N-limited growth of diatoms. *J.*
- 39 28 *Plankton Res.*, **22**, 447-472.
- 40
- 41 29 Furey, A., Lehane, M., Gillman, M., Fernandez-Puente, P. and James, K.J. (2001)
- 42 30 Determination of domoic acid in shellfish by liquid chromatography with electrospray
- 43 31 ionisation and multiple tandem mass spectrometry. *J. Chromatogr. A*, **938**, 167-174.
- 44
- 45 32 Galtier, N., Gouy, M. and Gautier, C. (1996) SEAVIEW and PHYLO\_WIN: Two graphic
- 46 33 tools for sequence alignment and molecular phylogeny. *Comput. Appl. Biosci.*, **12**, 543-
- 47 34 548.

- 1 Granéli, E. and Flynn, K. (2006) Chemical and physical factors influencing toxin content. In
- 2 Granéli, E. and Turner, J.T. (eds.) *Ecology of Harmful Algae*, Vol. Ecological Studies
- 3 189. Springer-Verlag, Berlin and Heidelberg, Germany, pp. 229-241.
- 4 Granéli E. and Johansson N., (2003) Increase in the production of allelopathic substances by
- 5 *Prymnesium parvum* cells grown under N- or P- deficient conditions. *Harmful Algae*, **2**,
- 6 135-145.
- 7 Granéli, E. and Salomon, P., (2010) Factors influencing allelopathy and toxicity in
- 8 *Prymnesium parvum*. *J. American Water Resources Assoc. (JAWRA)*, **46**, 108-120.
- 9 Guillard, R.R.L. (1995) Culture methods. In Hallegraeff, G.M., Anderson, D.M. and
- 10 Cembella, A.D. (eds.) *Manual on Harmful Marine Microalgae*. IOC Manuals and
- 11 Guides No. 33. UNESCO, Paris, pp. 45-62.
- 12 Harrison, P.J., Conway, H.L., Holmes, R.W. and Davis, C.O. (1977) Marine diatoms grown
- 13 in chemostats under silicate or ammonium limitation. III. Cellular chemical composition
- 14 and morphology of *Chaetoceros debilis*, *Skeletonema costatum*, and *Thalassiosira*
- 15 *gravida*. *Mar. Biol.*, **43**, 19-31.
- 16 Howard, M.D.A., Cochlan, W.P., Ladizinsky, N. and Kudela, R.M. (2007) Nitrogenous
- 17 preference of toxigenic *Pseudo-nitzschia australis* (Bacillariophyceae) from field and
- 18 laboratory experiments. *Harmful Algae*, **6**, 206-217.
- 19 Janson, S., Gisselson, L.-Å., Salomon, P.S. and Granéli, E. (2000) Evidence for multiple
- 20 species within the endoparasitic dinoflagellate *Amoebophrya ceratii* as based on 18S
- 21 rRNA gene-sequence analysis. *Parasitol. Res.*, **86**, 929-933.
- 22 Jespersen, A.M. and Christoffersen, K. (1987) Measurements of chlorophyll-a from
- 23 phytoplankton using ethanol as extraction solvent. *Arch. Hydrobiol.*, **109**, 445-454.
- 24 Jukes, T.H. and Cantor, C.R. (1969) Evolution of protein molecules. In Muntz, H.N. (ed.)
- 25 *Mammalian Protein Metabolism*. Academic Press, New York, U.S.A., pp. 21-132.
- 26 Kotaki, Y., Lundholm, N., Onodera, H., Kobayashi, K., Bajarias, F.F.A., Furio, E.F., Iwataki,
- 27 M., Fukuyo, Y. and Kodama, M. (2004) Wide distribution of *Nitzschia navis-varingica*,
- 28 a new domoic acid-producing benthic diatom found in Vietnam. *Fisheries Science*, **70**,
- 29 28-32.
- 30 Kudela, R., Roberts, A. and Armstrong, M. (2004) Laboratory analyses of nutrient stress and
- 31 toxin accumulation in *Pseudo-nitzschia* species from Monterey Bay, California. In:
- 32 Steidinger, K.A., Landsberg, J.H., Tomas, C.R. and Vargo, G.A. (eds.) *Harmful Algae*
- 33 2002. Florida Fish and Wildlife Conservation Commission, Florida Institute of
- 34 Oceanography, and Intergovernmental Oceanographic Commission of UNESCO, Paris,

- 1 pp. 136-138.
- 2 Laflamme, M.Y. (1993) Contrôle de la croissance et de la production d'acide domoïque par la
- 3 diatomée *Pseudonitzschia pungens* forma *multiseriis* en cultures continues. M.Sc.
- 4 Thesis, Université de Moncton, Moncton, New Brunswick, Canada. 141 p.
- 5 Landsberg, J.H. (2002) The effects of harmful algal blooms on aquatic organisms. *Rev. Fish.*
- 6 *Sci.*, **10**, 113-390.
- 7 Lewis, N.I., Bates, S.S., McLachlan, J.L. and Smith, J.C. (1993) Temperature effects on
- 8 growth, domoic acid production, and morphology of the diatom *Nitzschia pungens* f.
- 9 *multiseriis*. In Smayda T.J. and Shimizu, Y. (eds.) *Toxic Phytoplankton Blooms in the*
- 10 *Sea*. Elsevier, Amsterdam, Netherlands, pp. 601-606.
- 11 Lundholm, N., Skov, J., Pocklington, R. and Moestrup, Ø. (1997) Studies on the marine
- 12 planktonic diatom *Pseudo-nitzschia*. 2. Autecology of *P. pseudodelicatissima* based on
- 13 isolates from Danish coastal waters. *Phycologia*, **36**, 381-388.
- 14 Maldonado, M.T., Hughes, M.P., Rue, E.L. and Wells, M.L. (2002) The effect of Fe and Cu
- 15 on growth and domoic acid production by *Pseudo-nitzschia multiseriis* and
- 16 *Pseudonitzschia australis*. *Limnol. Oceanogr.*, **47**, 515-526.
- 17 Míguez, Á., Fernández, M.L. and Fraga, S. (1996) First detection of domoic acid in Galicia
- 18 (NW Spain). In Yasumoto, T., Oshima, Y. and Fukuyo, Y. (eds.) *Harmful and Toxic*
- 19 *Algal Blooms*. IOC of UNESCO, Paris, France, pp. 143-145.
- 20 Paasche, E. (1980) Silicon content of five marine plankton diatom species measured with a
- 21 rapid filter method. *Limnol. Oceanogr.*, **25**, 474-480.
- 22 Page, R.D.M. (1996) TreeView: An application to display phylogenetic trees on personal
- 23 computers. *Comput. Appl. Biosci.*, **12**, 357-358.
- 24 Pan, Y., Parsons, M.L., Busman, M., Moeller, P.D.R., Dortch, Q., Powell, C.L. and Doucette,
- 25 G.J. (2001) *Pseudo-nitzschia* sp. cf. *pseudodelicatissima* - a confirmed producer of
- 26 domoic acid from the northern Gulf of Mexico. *Mar. Ecol. Prog. Ser.*, **220**, 83-92.
- 27 Pan, Y., Subba Rao, D.V. and Mann, K.H. (1996a) Changes in domoic acid production and
- 28 cellular chemical composition of the toxigenic diatom *Pseudo-nitzschia multiseriis*
- 29 under phosphate limitation. *J. Phycol.*, **32**, 371-381.
- 30 Pan, Y., Subba Rao, D.V., Mann, K.H., Li, W.K.W. and Harrison, W.G. (1996b) Effects of
- 31 silicate limitation on production of domoic acid, a neurotoxin, by the diatom
- 32 *Pseudonitzschia multiseriis*. II. Continuous culture studies. *Mar. Ecol. Prog. Ser.*, **131**,
- 33 235-243.
- 34 Rhee, G.-Y. (1980) Continuous culture in phytoplankton ecology. In Droop, M.R. and

- 1 Jannasch, H.W. (eds.) *Advances in Aquatic Microbiology*, Vol. 2. Academic Press,  
2 London, United Kingdom, pp. 151-203.
- 3 Rue, E. and Bruland, K., (2001) Domoic acid binds iron and copper: a possible role for the  
4 toxin produced by the marine diatom *Pseudo-nitzschia*. *Mar. Chem.*, **76**, 127–134.
- 5 Sakshaug, E., Granéli, E., Elbrächter, M. and Kayser, H., (1984). Chemical composition and  
6 alkaline phosphatase activity of nutrient- saturated and P-deficient cells of four marine  
7 dinoflagellates. *J. Exp. Mar. Biol. Ecol.*, **77**, 241-254
- 8 Solórzano, L. and Sharp, J.H. (1980) Determination of total dissolved phosphorus and  
9 particulate phosphorus in natural waters. *Limnol. Oceanogr.*, **25**, 754-758.
- 10 Stewart, J.E. (2008) Bacterial involvement in determining domoic acid levels in  
11 *Pseudonitzschia multiseriis* cultures. *Aquat. Microb. Ecol.*, **50**, 135–144.
- 12 Subba Rao, D.V., de Freitas, A.S.W., Quilliam, M.A., Pocklington, R. and Bates, S.S. (1990)  
13 Rates of production of domoic acid, a neurotoxic amino acid in the pennate marine  
14 diatom *Nitzschia pungens*. In Granéli, E., Sundström, B., Edler, L. and Anderson, D.M.  
15 (eds.) *Toxic Marine Phytoplankton*. Elsevier, New York, U.S.A., pp. 413-417.
- 16 Thessen, A.E., Bowers, H.A. and Stoecker, D.K. (2009) Intra- and interspecies differences in  
17 growth and toxicity of *Pseudo-nitzschia* while using different nitrogen sources.  
18 *Harmful Algae*, **8**, 792–810.
- 19 Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994) CLUSTAL W - improving the  
20 sensitivity of progressive multiple sequence alignment through sequence weighting,  
21 position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.*, **22**, 4673-  
22 4680.
- 23 Troussellier, M., Courties, C., Lebaron, P. and Servais, P. (1999) Flow cytometric  
24 discrimination of bacterial populations in seawater based on SYTO 13 staining of  
25 nucleic acids. *FEMS Microbiol. Ecol.*, **29**, 319-330.
- 26 Utermöhl, H. (1958) Zur vervollkommnung der quantitativen phytoplankton methodik. *Mitt.*  
27 *Int. Verein. Theor. Angew. Limnol.*, **9**, 1-38.
- 28 Valderrama, J.C. (1995) Methods of nutrient analysis. In Hallegraeff, G.M., Anderson, D.M.  
29 and Cembella, A.D. (eds.) *Manual on Harmful Marine Microalgae*. IOC Manual and  
30 Guides No. 33, UNESCO workshop, Paris, France, pp. 251-268.
- 31 Wells, M.L., Trick, C.G., Cochlan, W.P., Hughes, M.P. and Trainer, V.L. (2005) Domoic  
32 acid: The synergy of iron, copper, and the toxicity of diatoms. *Limnol. Oceanogr.*, **50**,  
33 1908-1917.



**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-nitzschia 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%.

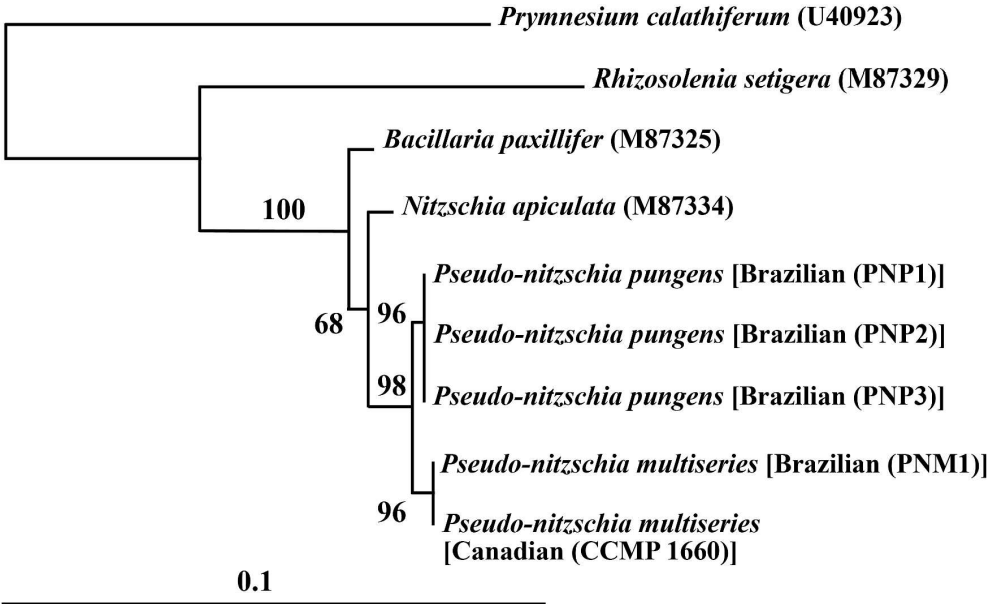
**Fig. 2.** Chlorophyll a concentrations ( $\mu\text{g L}^{-1}$ ) of *Pseudo-nitzschia multiseries* (a Canadian and a Brazilian strain) growing under nitrogen (N) or phosphorus (P) deficiency in chemostat cultures during steady state (mean  $\pm$  SD, n=2). Error bars are occasionally smaller than the 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 ( $0.2 \text{ d}^{-1}$ ), intermediate ( $0.3 \text{ d}^{-1}$ ), and low ( $0.4 \text{ d}^{-1}$ ) N or P deficiency. Only the days when steady-state was obtained for each treatment is plotted.

**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 level of N or P deficiency. Other explanations as in Fig. 2.

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

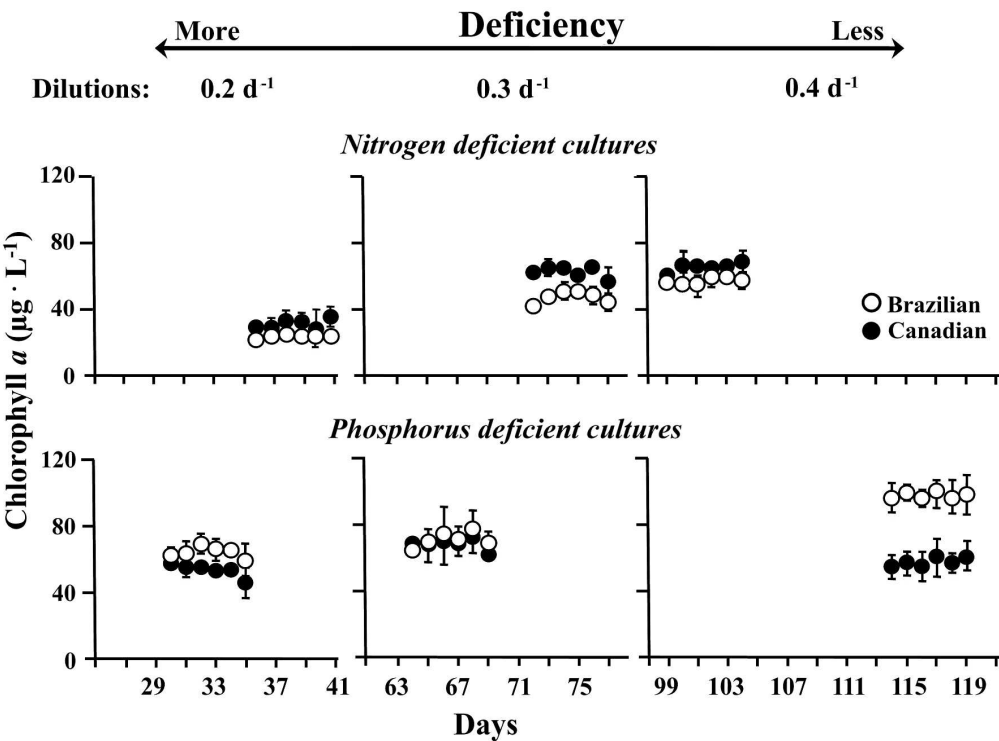
**Fig. 5.** Elemental ratios of C:N, C:P, C:Si, and N:P in relation to level of nutrient deficiency. The elemental value for diatoms (Brzezinski, 1985) of each nutrient ratio is indicated with a horizontal line. Other explanations as in Fig. 2.

**Fig. 6.** Concentration of Domoic acid (PDA)  $\text{cell}^{-1}$ , and total domoic acid  $\text{cell}^{-1} \text{ day}^{-1}$  (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).

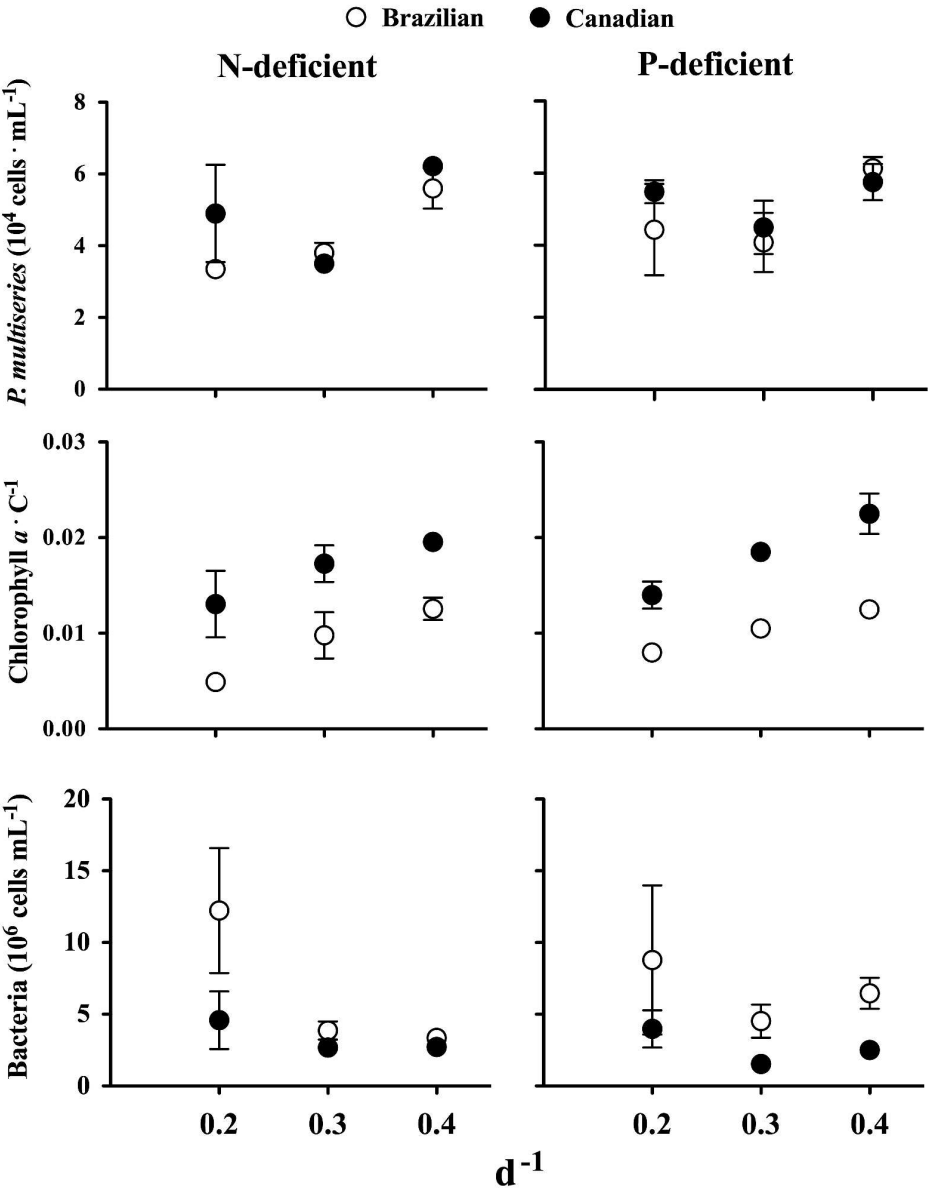


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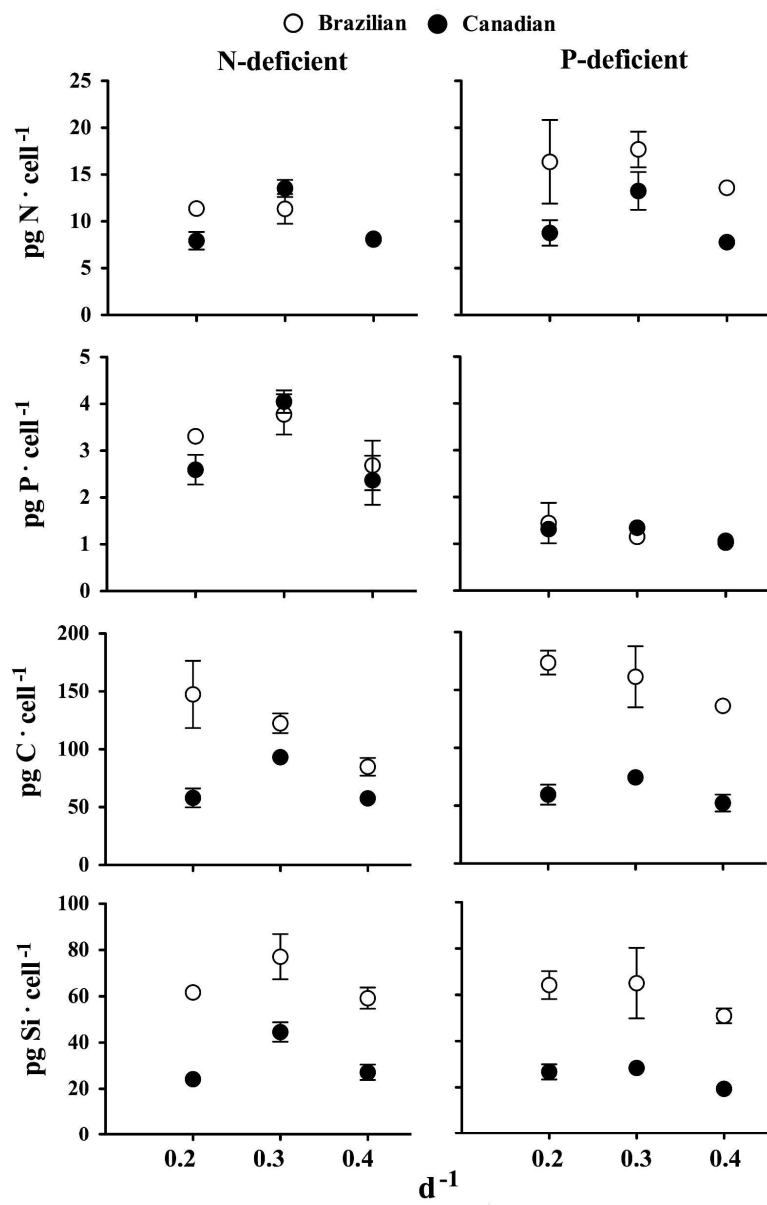




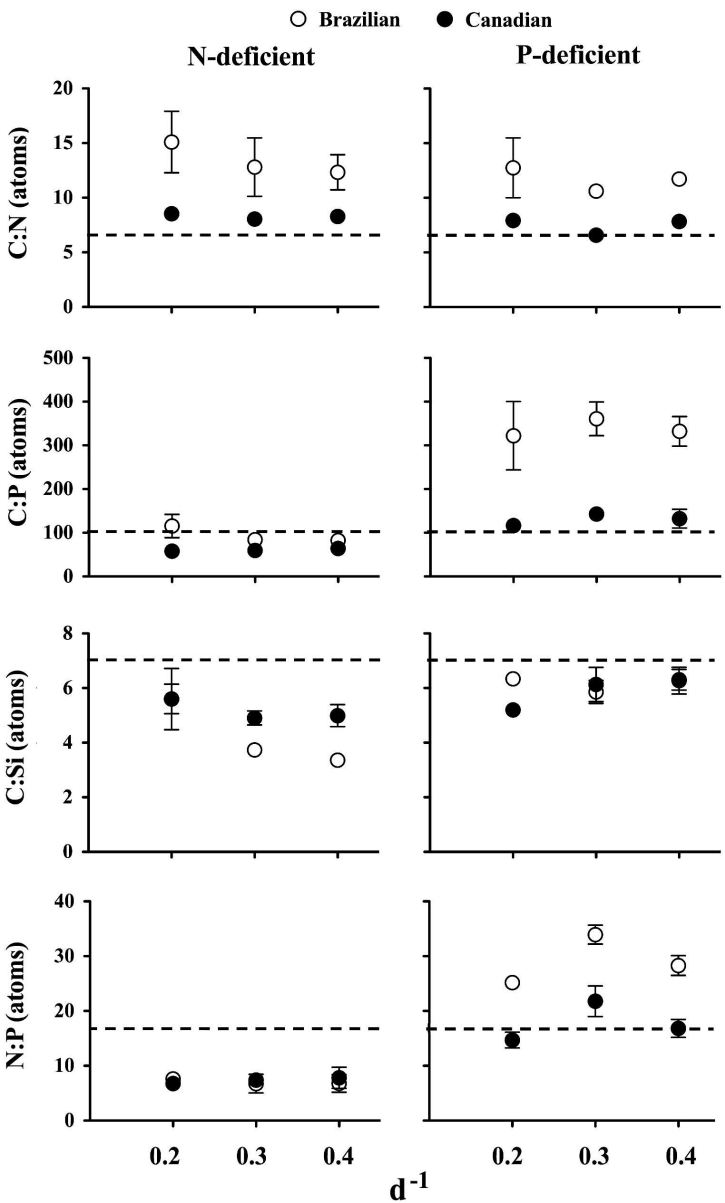
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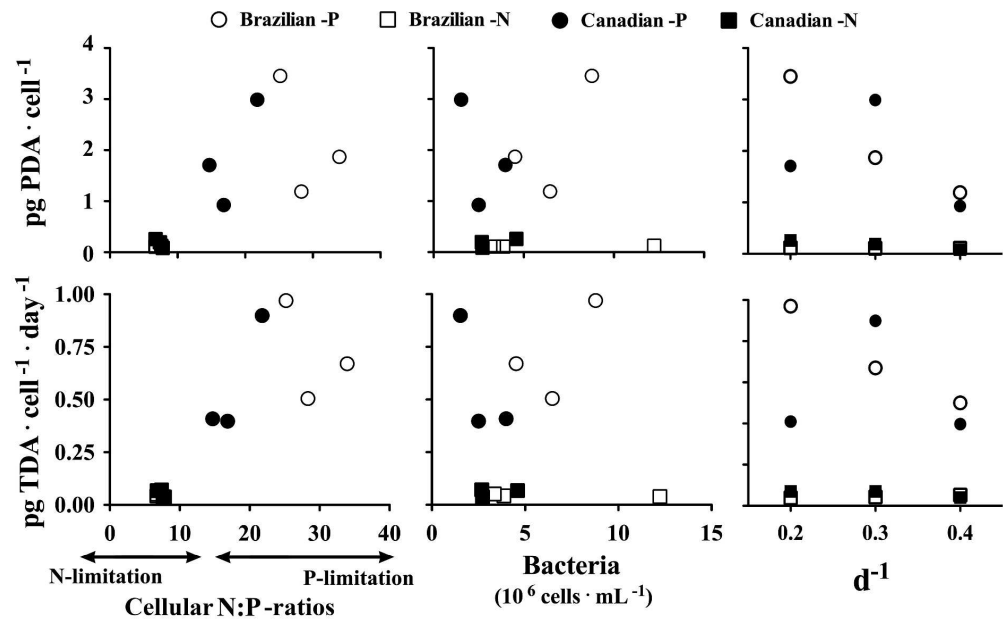
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133x208mm (600 x 600 DPI)



129x213mm (600 x 600 DPI)



159x98mm (600 x 600 DPI)