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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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3 1 **Domoic acid production and elemental composition of two *Pseudo-nitzschia multiseries***
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5 2 **strains, from the NW and SW Atlantic Ocean, growing in phosphorus- or nitrogen-**
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7 3 **limited chemostat cultures.**
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3 **1 ABSTRACT**
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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 d⁻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 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_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

1 different rates in a chemostat ($0.2 - 1.2 \text{ d}^{-1}$). However, to our knowledge, there is a lack of
2 knowledge regarding the possible differences between strains within the same species isolated
3 from different locations and grown under identical continuous culture conditions with
4 different N:P ratios.

5 The questions we focused on during this experiment were therefore: (1) Are there any
6 physiological differences between two strains of *P. multiseriis* from different origins (SW
7 and NW Atlantic Ocean) concerning DA production when growing in N- and P-deficient
8 conditions? (2) What is the effect of growth rate (i.e. level of nutrient deficiency) on cell
9 physiology and DA production for the two strains?

11 METHOD

13 Strains

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

25 The partial 18S rRNA gene was amplified from *Pseudo-nitzschia* spp. cultures by
26 adding 5 μL of cells resuspended in TE buffer (10 mM Tris, 0.5 mM EDTA, pH 8.0) to the
27 polymerase-chain-reaction (PCR) mix. The PCR mix contained 200 mM deoxy-nucleoside
28 triphosphate (dNTP), 5% dimethylsulfoxide, 5 pmol each of primers EUK1 and 518r, Taq
29 DNA polymerase reaction buffer with 1.5 mM MgCl_2 and 1 U of a mixture of Taq DNA
30 polymerase (Roche) in a total reaction volume of 50 μL (Janson et al., 2000). The
31 amplification was performed with a GeneAmp PCR System 2400 (Perkin Elmer, USA) with
32 an initial incubation of 5 min at 94°C . The 30 subsequent cycles were 30 seconds at 94°C , 30
33 seconds at 50°C and 1 min at 72°C . A final incubation of 5 min at 72°C finished the PCR
34 amplification. The gel electrophoresis of the PCR products was performed according to

1 Ausubel *et al.* (1989).

2 The PCR products were later sequenced on both strands after purification with a
3 QIAquick™ PCR kit (Qiagen) using the primers EUK1 and 518r. The sequencing reaction
4 was performed using dye terminator chemicals (BigDye: Perkin Elmer) according to the
5 instructions provided by the manufacturer. Sequences were read using a DNA auto sequencer
6 Model ABI 377-XL (Perkin Elmer). The sequences reported here, and those retrieved from
7 GenBank, were aligned using CLUSTAL W 1.8 (Thompson *et al.*, 1994), with the gap and
8 extension penalty set to 6.6. Phylogenetic analyses were performed on the PHYLO_WIN
9 software (Galtier *et al.*, 1996). The tree was edited using TREEVIEW 1.5 for PC (Page,
10 1996). The Jukes and Cantor method was used to estimate the distances (Jukes and Cantor,
11 1969).

12 **Culture maintenance**

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15 The two strains were maintained in f/2 medium, with the trace metal solution modified
16 according to medium L1 (Guillard, 1995). However, the nitrogen concentration was reduced
17 so that the 16N:1P Redfield ratio was attained (see below). The cultures were grown under a
18 16:8 hours light:dark cycle. Photosynthetically Active Radiation (PAR) was adjusted to 200
19 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (Philips TLD 36W/830 cool white fluorescent tubes) and the
20 temperature was kept at $18 \pm 0.5^\circ\text{C}$. This was followed by sub-culturing for one month in f/10
21 medium, with the same light, temperature and adjustments of nutrient ratio as above. These
22 sub-cultures were later used as inocula for the chemostat cultures. All media were prepared in
23 10 L Nalgene polycarbonate carboys using filtered (Munktel glass fibre filter, 1.2 μm pore
24 size) aged Baltic Sea surface water (6 psu) adjusted to 31 ± 1 psu. Proportional quantities of
25 the major salts (NaCl , $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, Na_2SO_4 , NaHCO_3 and K_2SO_4) were used
26 to achieve the desired salinity. The salinity was checked using a calibrated refractometer. To
27 avoid precipitation in the final media only nitrogen (NaNO_3) was added before the water was
28 autoclaved (90 min., 121°C). Stock solutions of phosphorus (KH_2PO_4), iron
29 ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}/\text{EDTA}$), and trace metals were autoclaved separately. Silicon ($\text{Na}_2\text{SiO}_3 \cdot 5\text{H}_2\text{O}$,
30 always 140 μM final concentration to avoid limitation), vitamins, and the salt NaHCO_3 were
31 filtered through a 0.2 μm pore size non-pyrogenic and hydrophilic sterile single use syringe
32 filter unit (Sartorius Minisart®). Finally, media pH was adjusted to 8.1 ± 0.1 with sterile 1.2
33 M HCl and/or 2 M NaOH, all done aseptically.

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1 Chemostat culture experiments

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7 Both strains of *P. multiseriis* were grown in duplicate under N deficiency (36 μM
8 NaNO_3 , 36 μM K_2HPO_4 ; atomic N:P 1:1) and P deficiency (580 μM NaNO_3 , 2 μM K_2HPO_4 ;
9 atomic N:P 290:1). All eight 1-L chemostat culture flasks, containing 920 – 996 mL culture,
10 were aerated with sterile air (0.2 μm pore size Sartorius Minisart® single use syringe filter
11 unit) to keep algal cells in suspension, and to ensure mixing as new medium was added drop-
12 wise to the surface with peristaltic pumps (see below). Measurements of temperature, pH,
13 chlorophyll *a* (chl *a*), and microscopic observation of cells in all 8 cultures were made daily.

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19 To avoid differences in media composition between culture flasks of one nutrient
20 treatment they were supplied with media from the same bottle. All cultures were kept under a
21 16:8 hours light: dark cycle at 200 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$. The dilution rates (i.e. cell division
22 rate, related to the level of nutrient deficiency) of the cultures were checked daily by
23 measuring the out-flow of each culture. The first dilution rate was set to 0.2 d^{-1} (most
24 deficient). As soon as steady state conditions had been achieved in all cultures, a sample
25 (\approx 50% of volume) from each culture was taken (see below for details about sub-sampling)
26 and the dilution was thereafter adjusted to 0.3 d^{-1} . Sampling was repeated and the dilution rate
27 was finally adjusted to 0.4 d^{-1} (least deficient). The final sampling was conducted after a total
28 of 119 days in chemostat culture (Fig. 2). We considered the cultures to be at steady state
29 when the coefficient of variation of daily measurements of chl *a* of five consecutive days was
30 not greater than 8 % (usually < 5%).

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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 ($\text{N-NO}_2^- + \text{N-NO}_3^-$, N-NH_4^+ , 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).

29 Analytical procedures and measurements

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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

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

$$4 \quad V = (A \times B \times C \times D) + [(1 - D) \times A \times (B + b)/2 \times C] \quad \text{Eq. 1}$$

5 where V is the cell volume, A the length of the valve (apical axis), B is the valve width
6 (transapical axis in valve view), C is the valve depth (prevalvar axis in girdle view), D is the
7 ratio of the linear part of the cell length to the total cell length, and b is the cell breadth on its
8 extreme, corresponding to the minor base of the trapezoid. The BR strain had an average $b =$
9 $0.5 \mu\text{m}$, and $b = 1.0 \mu\text{m}$ in the CA strain. Measurements were made using a light microscope
10 with phase contrast at 1000X magnification (ZEISS, model AXIOVERT). The microscope
11 was equipped with a digital camera and an image processing and measurement program
12 (SPOT Insight, Diagnostic Instruments Inc., version 3.5 for Windows). Cell volume
13 estimation was conducted on the samples preserved in Lugol's solution at the Phytoplankton
14 Laboratory of the Fundação Universidade Federal de Rio Grande (FURG), Brazil.

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

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

28

29 **Nutrient analyses**

30

31 Analyses of dissolved inorganic nutrients in the culture filtrates and inflow media
32 followed the methods modified by Valderrama (1995). A Whatman GF/C glass fiber filter
33 was used to obtain filtrate for N and P analyses. An all-plastic filtration apparatus and
34 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 (NaN_3 , final concentration 10 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 (pg TDA cell^{-1}) with dilution rate per day.

20 **Statistics**

21
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**

29
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

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3 1 due to the fact that the CA strain had been in culture much longer than the BR strain (see
4 below). We observed a general increase of chl *a* concentration as the cultures became less N-
5 2 deficient, ranging from 24 – 113 $\mu\text{g L}^{-1}$. Both extremes were observed in the BR strain, with
6 3 the lowest concentration during N-deficiency at 0.2 d^{-1} and highest during P-deficiency at 0.4
7 4 d^{-1} (Fig. 2). There was a significant increase in chl *a* C^{-1} (2-way ANOVA, $P < 0.001$), as the
8 5 cultures of both strains became less nutrient deficient (Fig. 3). There was on the other hand a
9 6 decreasing trend in bacterial abundance with reduced level of nutrient deficiency in all flasks.
10 7 The highest abundance, 12.2×10^6 bacteria mL^{-1} , was found in the N-deficient BR culture at
11 8 0.2 d^{-1} (i.e. high deficiency) whereas the P-deficient CA culture had the lowest recorded
12 9 bacterial abundance, 1.5×10^6 mL^{-1} , at the intermediate level of deficiency (Fig. 3).
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14 11

12 Nutrients

13
14 The amount of N 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

1 three levels of deficiency (Fig. 5). However, the N:P ratios of all cultures growing under N-
2 deficiency were below, and all but one culture growing under P-deficiency had N:P ratio
3 above, the atomic Redfield ratio.

5 **Particulate, dissolved and total domoic acid, and domoic acid production rate**

7 Domoic acid was detected in both strains and nutrient treatments (Fig. 6). The
8 particulate (PDA) concentration per cell and the total DA (TDA = dissolved + particulate
9 fractions) production $\text{cell}^{-1} \text{ day}^{-1}$ was higher in P-deficient than in N-deficient cultures for
10 both strains. The range in PDA concentrations at cellular N:P ratios ≤ 7.8 were 0.08 – 0.26 pg
11 cell^{-1} with values ranging between 0.93 and 3.45 pg cell^{-1} for cellular N:P ratios ≥ 14.7 (Fig.
12 6). PDA in the BR strain cells ranged between 0.11 and 3.45 pg cell^{-1} , and PDA
13 concentrations in the CA strain ranged between 0.08 and 2.99 pg cell^{-1} (Fig. 6). Total DA
14 production $\text{cell}^{-1} \text{ day}^{-1}$ followed the same trend as PDA cell^{-1} . That is, there was a clear
15 difference in TDA production depending on the N:P ratios (0.04 to 0.07 and 0.40 to 0.97 pg
16 TDA $\text{cell}^{-1} \text{ day}^{-1}$ for cellular N:P ratios ≤ 7.8 and ≥ 14.7 , respectively). Bacterial abundance in
17 the culture flasks did not seem to have an important effect on neither PDA cell^{-1} nor TDA
18 production $\text{cell}^{-1} \text{ day}^{-1}$ in this experiment since no trends could be observed (Fig. 6). Level of
19 nutrient deficiency (d^{-1}) only had an effect on PDA cell^{-1} and TDA production in the P-
20 deficient BR strain with a decrease in toxin as dilution increased. No such trend could be
21 observed in the CA strain or the N-deficient BR strain (Fig. 6).

23 **DISCUSSION**

25 In this paper we present results from chemostat culture studies comparing the cell
26 physiology and domoic acid production for two strains of *Pseudo-nitzschia multiseries*. The
27 strains were isolated from marine waters at two opposite latitudes: Canada (CA) and Brazil
28 (BR). The strains were grown in parallel under N- or P-deficiency at three different levels of
29 nutrient deficiency (0.2, 0.3 and 0.4 d^{-1}). The CA strain had been cultured in laboratory for ca.
30 10 years, whereas the BR strain was isolated 6 months prior to the experiment. Diatoms are
31 known to decrease in cell size while in culture (Davidovich and Bates, 1998). Reduced cell
32 sizes, cell volumes (about 31 % of the BR strain), and number of cells per chain in the CA
33 cultures were therefore expected. The CA strain also had more morphological deformations
34 such as “lobed” or “sickle-shaped” cells, and some with one or more “undulations”, than the

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3 1 BR strain. Thus we considered differences in cell morphology observed between the two
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5 2 strains a consequence of their culture age before the start of the experiment.

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7 3 A deficiency in N or Si in phytoplankton leads to an increase in cellular carbon content,
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9 4 resulting from carbon accumulation when cell division ceases (Flynn and Martin-Jezequel,
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11 5 2000), as well as under P-deficiency (Granéli and Johansson, 2003; Granéli and Salomon,
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13 6 2010; Pan *et al.*, 1996a; Sakshaug *et al.*, 1984). Harrison *et al.* (1977) observed that chl *a*:C
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15 7 ratios were lower under N-deficiency than for NP-sufficient diatom cells (*Skeletonema*
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17 8 *costatum*, *Chatoceros debilis*, *Thalassiosira gravida*) when growing in chemostats. This was
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19 9 due to a decrease in chl *a* cell⁻¹ (i.e. not increase in C) the more N-limited the cells were. In
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21 10 line with the findings by Pan *et al.* (1996a) we also observed an increase in cellular chl *a*:C in
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23 11 both strains and nutrient treatments as the cells became less deficient. The amount of C cell⁻¹
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25 12 in this experiment, on the other hand, decreased as the cells were subject to reduced
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27 13 deficiency in the BR strain, something not observed for CA cells. Cellular N, P, C and Si in
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29 14 the experiment reported here were generally higher in the BR strain; and, especially for the
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31 15 BR strain, decreased as the level of nutrient deficiency decreased. The higher N, P, Si, and C
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33 16 cellular concentrations in the BR strain can be explained by the larger size of this strain in
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35 17 comparison with the CA strain. Furthermore, the elemental ratios measured for both strains
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37 18 are all within the range of what has been reported for *P. multiseriis* by others (e.g. Pan *et al.*,
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39 19 1996a,b).

20 21 **Nutrient ratios and Domoic acid**

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23 23 A number of studies have investigated the effect of various sources of N (e.g. urea,
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25 24 NO₃⁻, NH₄⁺) on the physiology of *Pseudo-nitzschia* spp. and strains in batch cultures (Bates,
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27 25 1998; Bates and Trainer, 2006, Howard *et al.*, 2007; Thessen *et al.*, 2009). Laflamme (1993)
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29 26 studied the effect of Si- and P-limitation on DA production of *P. multiseriis* in chemostats.
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31 27 Kudela *et al.* (2004) used chemostat cultures to determine DA production of *P. multiseriis*
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33 28 strains, from one location, during Si- and N-deficiency. However, until now the effect of N-
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35 29 deficiency on general physiology and DA production in two *P. multiseriis* strains isolated
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37 30 from the NW and SW Atlantic Ocean, and growing in parallel chemostat cultures, has not yet
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39 31 been investigated. Since DA is an amino acid, and N is an essential element required for its
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41 32 synthesis, it has been argued that DA production would cease during N-deficiency (Bates *et*
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43 33 *al.*, 1991). However, Kudela *et al.* (2004) detected low levels of DA in N-limited chemostats.
44
45 34 Kudela *et al.* (2004) and Thessen *et al.* (2009) could also show inter-strain variability in DA

1 production, even when the strains had been isolated from the same location. Our results show
2 that, not only when submitted to P-deficiency but also even under N-deficiency and when
3 there is a continuous cell division (supply of N even in small amounts), both BR and CA
4 strains produced DA. However, DA production was several-fold greater at cellular N:P ratios
5 above 14.7 (P-deficiency and N-sufficiency) than below 7.8 (N-deficiency and P-sufficiency).
6 Further, investigations of environmental conditions that may promote *Pseudo-nitzschia* spp.
7 blooms, affect the physiology, and/or trigger/enhance DA production are therefore necessary.
8 Among these are, light conditions (Bates *et al.*, 2001; Fehling *et al.*, 2005), iron sufficiency or
9 deficiency (Bates *et al.*, 2001; Rue and Bruland, 2001; Wells *et al.*, 2005), several other trace
10 metals (Wells *et al.*, 2005), and the studies must be conducted under identical conditions on
11 several strains. Continuous cultures as an instrument to understand algal ecophysiology have
12 an advantage over batch cultures by allowing a dynamic equilibrium between nutrient input,
13 and cell death and growth; similar in principle to the conditions in natural waters. As in
14 nature, nutrient input rate during continuous culturing, rather than their concentrations at a
15 given time, determines growth limitation (Rhee, 1980).

16 There are several examples from the literature where N- and/or P-deficiency cause an
17 increase in cellular toxin concentration in a number of algal species from different taxa,
18 including *P. multiseriis* (Johansson 2003; Granéli and Flynn, 2006; Granéli and Salomon,
19 2010). It has been argued that DA production in *Pseudo-nitzschia* spp. is associated with Si
20 depletion, as reported by Pan *et al.* (1996b). The results reported here resemble those reported
21 for two clones of *Pseudo-nitzschia* sp. cf. *pseudodelicatissima* isolated from the northern Gulf
22 of Mexico by Pan *et al.* (2001). Their data suggest that a rapidly growing population, i.e. not a
23 stressed population due to nutrient deficiency, yields maximum net DA production rates and
24 DA cell quotas. Production of DA at steady state was also found to be positively correlated to
25 the N:P ratios, suggesting that P-deficiency promoted DA production (Pan *et al.*, 1996a). We
26 found that N was indeed a requirement for high DA production for both the Canadian and
27 Brazilian strain during chemostat culturing since the amount of toxin was low at cellular N:P
28 ratios ≤ 7.8 , i.e. N-deficiency.

29 A literature review reveals a great variation in *P. multiseriis* cellular DA. Pan *et al.*
30 (1996a) report a range of 0.3 – 4.5 pg DA cell⁻¹ during P deficiency. Furthermore, they argue
31 that the optimum N:P ratio for *P. multiseriis* is not the Redfield atomic ratio of 16:1 but that it
32 is closer to 10:1, and that DA production was only halted at N:P ratios less than about 7. This
33 is in line with the findings in our experiment reported here, i.e. 0.08 – 3.45 pg DA cell⁻¹ with
34 highest concentrations at high N:P ratios. There was no general trend in DA concentration as

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

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13 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. multiseriis* did not seem to affect DA production, neither positively nor negatively.

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24 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. multiseriis* 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, domoic acid concentration per cell, and DA production per cell per day, were significantly different depending on 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. multiseriis* 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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3 **Fig. 1.** Phylogenetic analysis of 18S rRNA gene sequences from *Pseudo-nitzschia* spp. and
4 other diatoms. The Jukes and Cantor method was used to estimate the distances. The numbers
5 at the branches indicate the percentage of 500 bootstraps; resamplings that support the branch
6 values below 50% are not shown. The *Pseudo-nitzschia* sp. sequences are a sister group to
7 *Nitzschia apiculata*. Note that the three Brazilian strains of *Pseudo-nitzschia pungens* (PNP1),
8 *Pseudo-nitzschia pungens* (PNP2) and *Pseudo-nitzschia pungens* (PNP3) sequences form one
9 tight cluster and the Brazilian *Pseudo-nitzschia multiseriis* (PNM1) and Canadian *Pseudo-*
10 *nitzschia multiseriis* (CCMP 1660) form a second tight cluster with zero branch lengths. The
11 scale indicates the observed sequence divergence of 10%. Thus the divergence between
12 Brazilian and Canadian *P. multiseriis* is less than 1%.

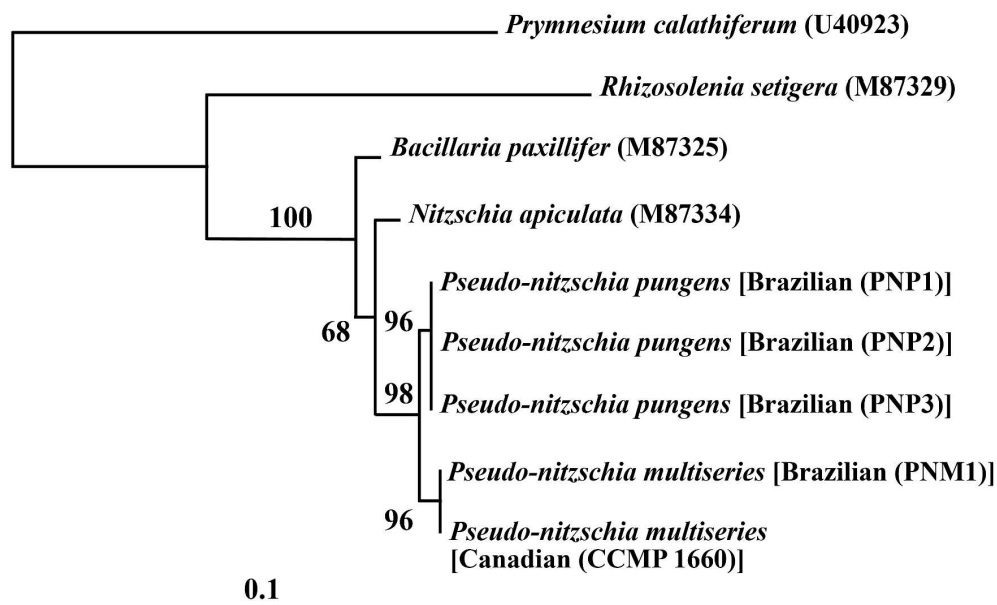
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23 **Fig. 2.** Chlorophyll a concentrations ($\mu\text{g L}^{-1}$) of *Pseudo-nitzschia multiseriis* (a Canadian and
24 a Brazilian strain) growing under nitrogen (N) or phosphorus (P) deficiency in chemostat
25 cultures during steady state (mean \pm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

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

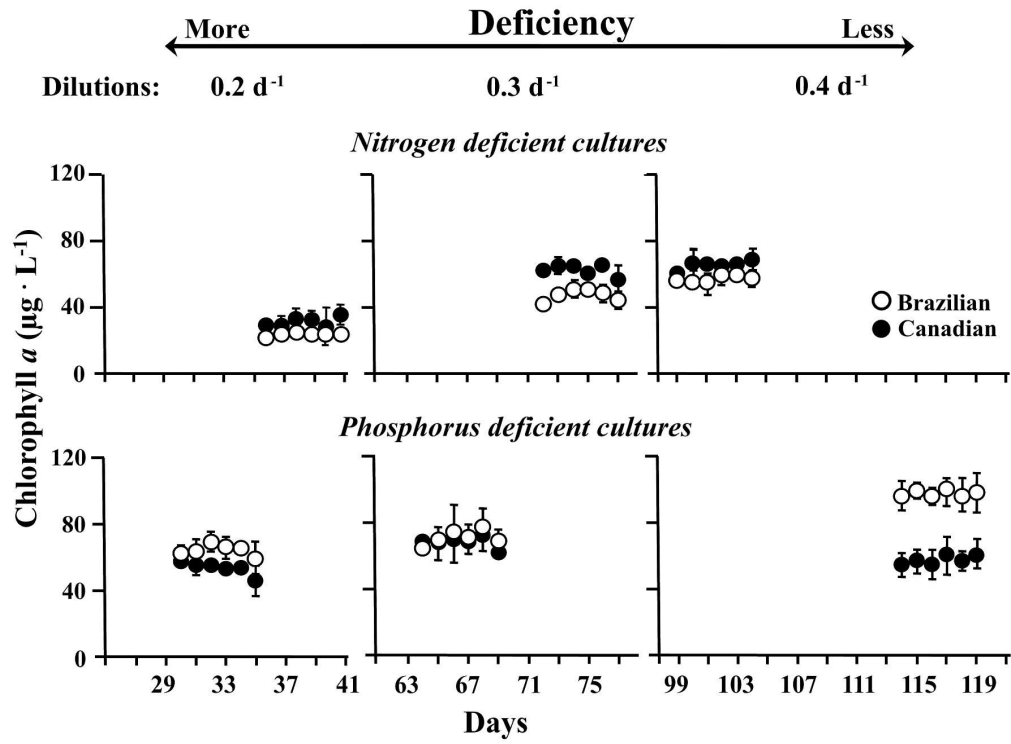
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42 **Fig. 4.** Intracellular concentrations of N, P, C, and Si in relation to level of nutrient deficiency.
43 Other explanations as in Fig. 2.

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

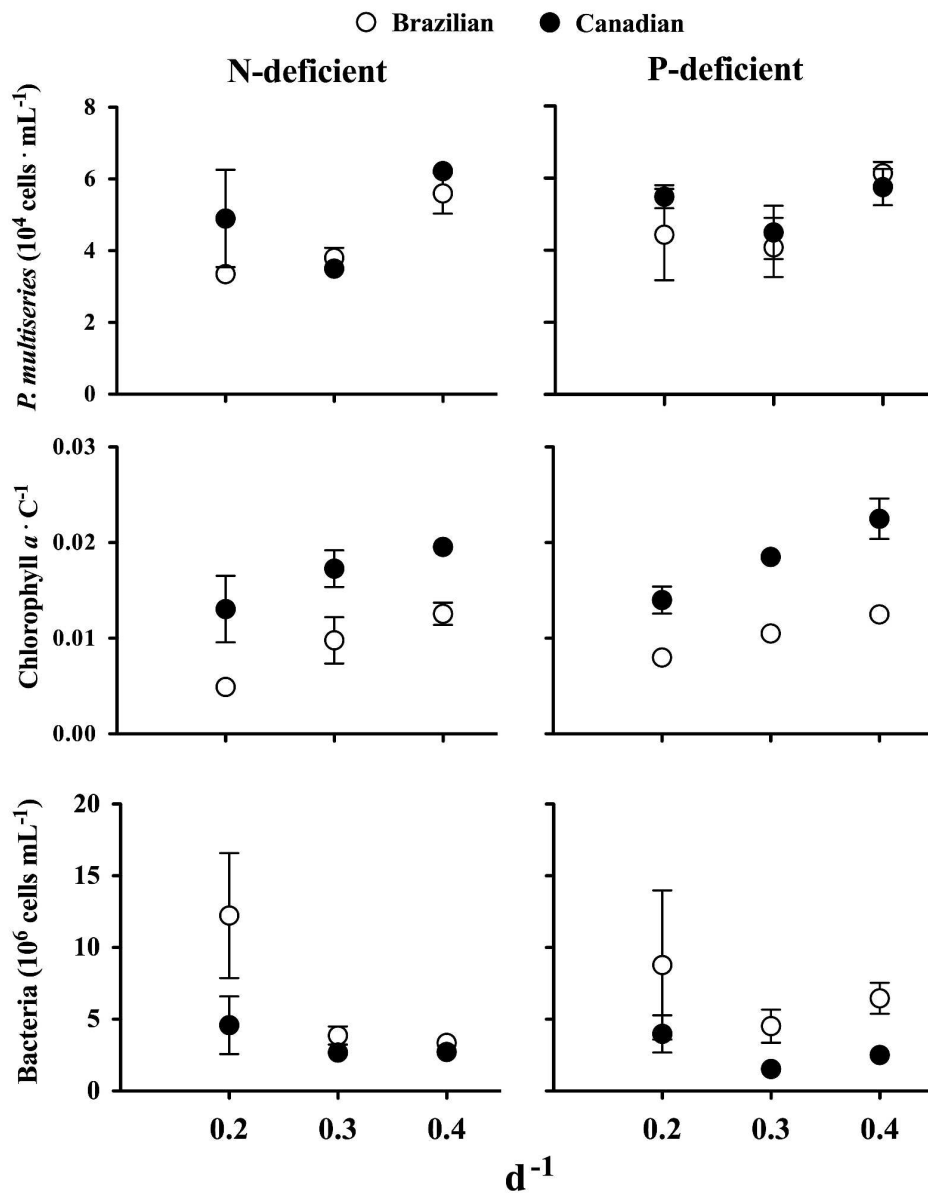
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55 **Fig. 6.** Concentration of Domoic acid (PDA) cell^{-1} , and total domoic acid $\text{cell}^{-1} \text{ day}^{-1}$ (TDA =
56 particulate + dissolved DA in the culture bottles) in relation to cellular (atomic) N:P ratios
57 (left hand panels),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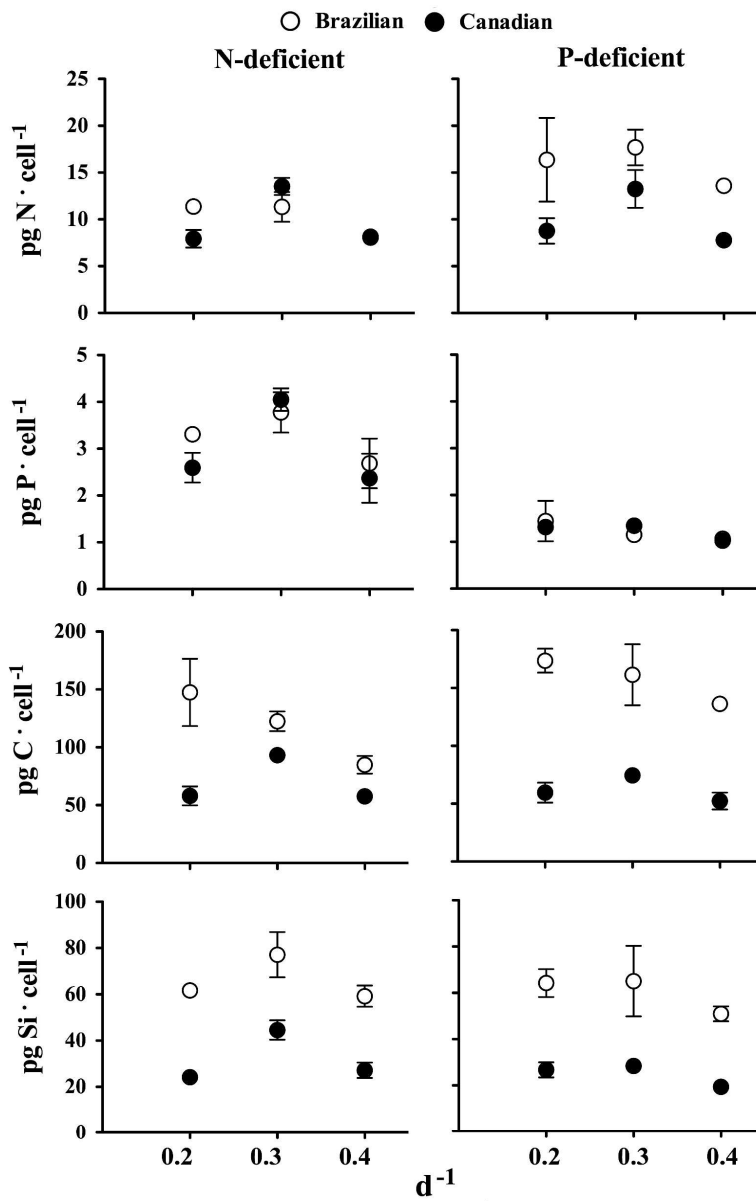
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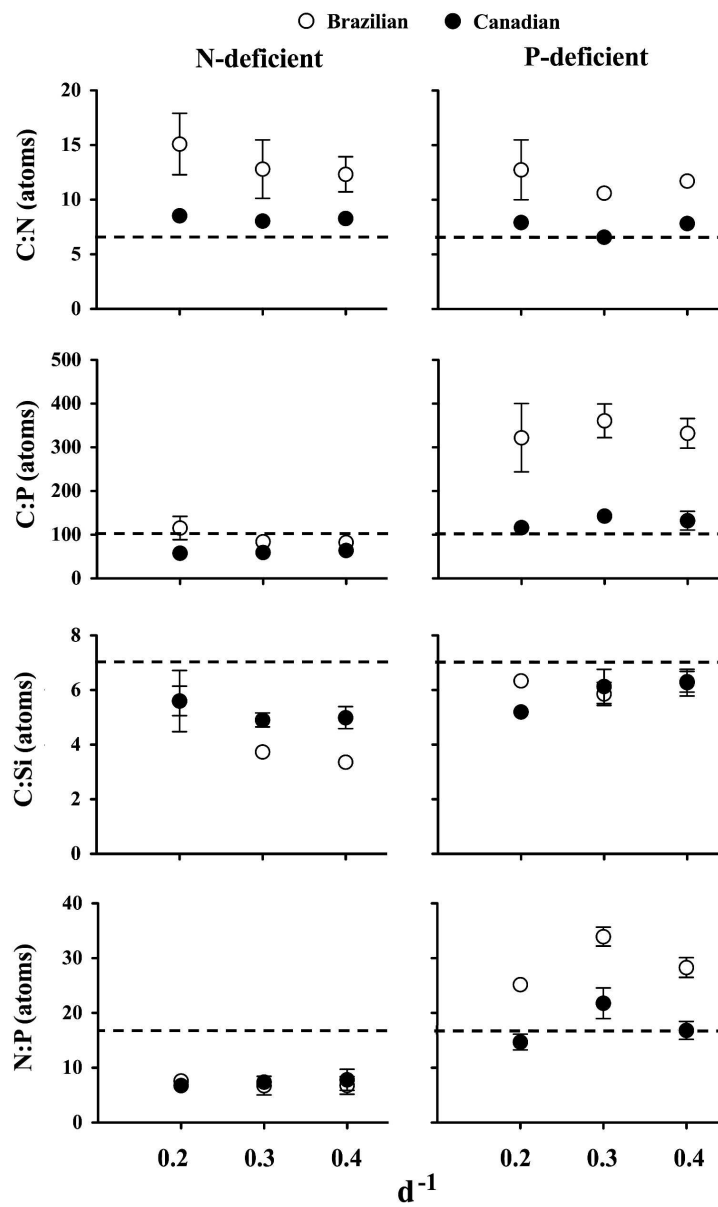
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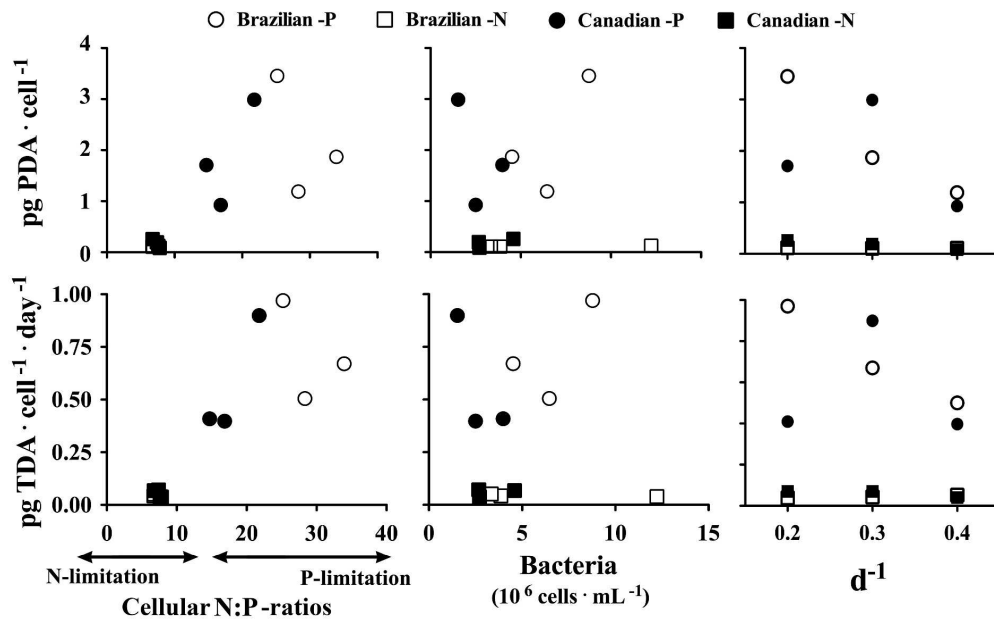
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Review