Effects of Ammonium and Nitrate on Growth and Domoic Acid Production by *Nitzschia pungens* in Batch Culture

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Four clones of *Nitzschia pungens* f. *multiseries* (toxin-producing form) and two of *N. pungens* f. *pungens* (nontoxic) were grown in batch culture at initial nitrogen concentrations of 55, 110, 220, 440, and 880 μM in the form of either nitrate or ammonium. As expected, only *N. pungens* f. *multiseries* clones produced the neurotoxin, domoic acid (DA). Ammonium at 880 μM prevented the growth of all *N. pungens* clones but not of *Skeletoneuma costatum*, although division rate was reduced. At 440 μM, ammonium lowered the cell yield obtained during the stationary phase, inhibited photosynthesis, and caused a lag phase during which DA was produced (by f. *multiseries*). At 220 and 440 μM ammonium, in contrast with the same concentrations of nitrate, stationary phase cellular DA production was enhanced by two- to fourfold. At 110 and 55 μM nitrogen, cell yield and DA production were equivalent for nitrate and ammonium but less DA was produced relative to the higher nitrogen concentrations, possibly due to nitrogen depletion. Enhanced production of DA at elevated ammonium concentrations may be a response to ammonia toxicity. This could be a useful tool for studying mechanisms of DA production and for maximizing the yield of this valuable toxin in large-scale cultures.

Quatre clones de *Nitzschia pungens* f. *multiseries* (forme toxique) et deux de *N. pungens* f. *pungens* (forme non toxique) ont été élevés en cultures discontinues avec des concentrations initiales d'azote de 55, 110, 220, 440 et 880 μM sous forme de nitrate ou d'ammonium. Comme prévu, seuls les clones de *N. pungens* f. *multiseries* ont produit de l'acide domoïque, une neurotoxine. Une concentration en ammonium de 880 μM empêche toute croissance des clones de *N. pungens* mais pas celle de *Skeletoneuma costatum*, dont le taux de croissance a été diminué. Une concentration en ammonium de 440 μM entraîne une réduction de la densité cellulaire obtenue en phase stationnaire ainsi qu'une inhibition de la photosynthèse, et provoque une phase de latence pendant laquelle il y a production d'acide domoïque (chez f. *multiseries*). Aux concentrations en ammonium de 220 et de 440 μM, la production d'acide domoïque par cellule pendant la phase stationnaire a été de deux à quatre fois supérieure par rapport aux mêmes concentrations en nitrate. Aux concentrations de 55 et de 110 μM, le rendement cellulaire et la production d'acide domoïque ont été équivalents pour le nitrate et l'ammonium, mais moins d'acide domoïque a été produit comparativement aux plus hautes concentrations d'azote, signe possible d'une insuffisance de cet élément. L'augmentation de la production d'acide domoïque aux haute concentrations d'ammonium est peut-être une réaction à la toxicité de l'ammonium. Cela pourrait s'avérer utile d'une par dans l'étude des mécanismes de biosynthèse de l'acide domoïque, et d'autre part pour maximiser la production à grande échelle de cette toxine.

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In December 1987, an outbreak of food poisoning due to the consumption of blue mussels (*Mytilus edulis*) from Prince Edward Island (P.E.I.), Canada, led to the identification of a novel marine phytoxin, domoic acid (DA) (Wright et al. 1989), and the demonstration that the toxin was produced by the pennate diatom *Nitzschia pungens* forma *multiseries* (Bates et al. 1989). The continuing problem of DA toxicity and *N. pungens* in P.E.I. during the autumns of 1988–91 allowed the study of the diatom's population dynamics and the factors influencing its growth and DA production in the field (Smith et al. 1990a). This problem now has worldwide implications because DA has appeared on the west coast of the United States (Buck et al. 1992; Fritz et al. 1992; R. Horner, University of Washington, Seattle, Wash., personal communication), and DA-producing *N. pungens* f. *multiseries* has been isolated from coastal waters of Texas (Fryxell et al. 1990) and Massachusetts (Villareal et al. 1993).

Recent laboratory investigations have shown that external nitrogen is required for DA production by nitrate-grown *N. pungens* f. *multiseries* in silicate-limited batch cultures (Bates et al. 1991). Blooms of *N. pungens* in the field are closely associated with pulses of nitrate (Smith et al. 1990a). Nitrate and other inorganic nitrogen sources probably play an important role in controlling growth and DA production by *N. pungens* f. *multiseries* in both the field and the laboratory. Ammonium is another form of combined nitrogen that is commonly available in coastal environments, especially in the presence of ammoniotelic marine animals (Dame et al. 1991). The growth of *N. pungens* on ammonium has not yet been studied. It is necessary to know which nitrogenous nutrient conditions favor *N. pungens* growth and DA production to better understand its cell physiology and to produce reliable supplies of *N. pungens* of known toxin content for studies of uptake and depuration of DA by shellfish and for toxicity experiments using zooplankton or larval fish.

The goal of this laboratory study was to compare the ability of four clones of *N. pungens* f. *multiseries* to grow and to produce DA using ammonium compared with nitrate as the nitro-
gen source. Two clones of the nontoxic *N. pungens* f. *pungens* (Smith et al. 1990b) and one of *Skeletonema costatum* from P.E.I. were included in the comparison. Several of the batch cultures contained elevated concentrations of nitrogen and therefore information on ammonium toxicity was also obtained.

**Materials and Methods**

The diatom clones and their origins are shown in Table 1. *Nitzschia pungens* f. *multiseries* is the DA-producing form (Bates et al. 1989), and *N. pungens* f. *pungens* is the form with no confirmed DA production (Smith et al. 1990b). The response of *N. pungens* to nitrate and ammonium was compared with that of *S. costatum*, a centric diatom also found in P.E.I. embayments. The taxonomic status of the two forms of *N. pungens* was confirmed by scanning electron microscopy (Fryxell et al. 1990; Smith et al. 1990b). The unialgal cultures were nonaxenic; both axenic and nonaxenic cultures produce DA but not at the same rate (Douglas and Bates 1992; Douglas et al. 1993). Cultures were maintained and experiments were carried out in medium f/2 (Guillard and Ryther 1962) made with filtered seawater. Stock cultures for the first ammonium experiment were maintained on 880 μM nitrate, the nitrogen concentration found in medium f/2. For subsequent ammonium experiments, cells were conditioned on 220 μM ammonium for at least 7 wk, during which the cultures were serially transferred into fresh medium a minimum of three times. Late exponential phase cultures were used to inoculate each experiment. This ensured that no DA was present in the inoculum and that the nutrient and irradiance histories of the cultures were similar. The initial concentrations of nitrogen, calculated based on dilution, were 55, 110, 220, 440, and 880 μM, provided in the form of nitrate or ammonium, as indicated. Ammonium, as ammonium chloride, was added aseptically after the f/2 medium was autoclaved. The growth temperature was 17°C and the photon flux density, provided by a bank of "cool-white" fluorescent bulbs (10 h light : 14 h dark cycle), was approximately 100 μE m⁻² s⁻¹ (Li-Cor, model LI-189; 4π sensor), a level sufficient to saturate growth.

Experiments were carried out in triplicate culture vessels in either screw-capped test tubes containing 20 mL of medium, Fernbach flasks containing 1.5 L of medium, or Erlenmeyer flasks containing 250 mL of medium, as indicated. Cultures were agitated daily, and the position of the culture vessels was randomized to achieve even illumination. For experiments carried out in test tubes, growth was monitored as the change in optical density as measured with a spectrophotometer (Spectronic 20; Bausch & Lomb) at 800 nm. Cell concentration was then computed from the regression of optical density versus cell number, determined separately for each clone of each diatom species. Cell concentrations for experiments carried out in flasks were measured by triplicate culture aliquots. cell yields (cells per millilitre) were computed by averaging at least five data points taken during the stationary phase. Chlorophyll a (Chl a) from cells collected on a glass-fibre filter (Whatman GF/F) was extracted in 90% acetone for 24 h in darkness and then measured fluorometrically (Perkin-Elmer LS-3 fluorescence spectrometer); pure Chl a (Sigma Chemical Co., St. Louis, Mo.) was used as a standard. The rate of photosynthesis as a function of incident irradiance was determined using a photosyntheseter (Lewis and Smith 1983). Samples (1.0 mL) were incubated for 30 min with approximately 20 kBq ¹⁴C-labelled bicarbonate·mL⁻¹ (New England Nuclear Corp.), after which 50 μL of concentrated hydrochloric acid was added and the scintillation vials placed on a shaker table overnight to remove inorganic ¹⁴C. Radioactivity was determined with a scintillation spectrometer (Rack Beta, model 1410; Pharmacia/LKB). The maximum rate of photosynthesis at light saturation (P_max) was calculated using the photosynthesis-irradiance model of Platt et al. (1980), fitted with the program "FitAll" (MTR Software, Toronto, Ont.). DA samples were prepared by sonicating 15 mL of "whole culture" (i.e., cells plus medium) for 45 s at 210 W using a Vibra-Cell high-intensity ultrasonic processor (model VC-300; Sonic & Materials, Inc., Danbury, Conn.), followed by filtration through a disposable polycarbonate syringe filter (0.45-μm pore size, 25-mm diameter, Nuclepore) to remove the debris. DA was analyzed (limit of detection = 0.05 ng·mL⁻¹) on a high-performance liquid chromatograph (Beckman System Gold) equipped with a Shimadzu fluorescence detector, using the FMOC fluorescence derivatization technique of Pocklington et al. (1990). Cellular DA from the "whole culture" (picograms per cell) was calculated by attributing the total DA produced in the culture to the cells.

**Results**

**Cell Yield**

The effects of three initial concentrations of nitrate (Fig. 1A) and ammonium (Fig. 1B) on cell growth are shown for *N. pungens* f. *multiseries* (clone NPARL) in batch culture. Similar results were found for *N. pungens* clones TKA-2, MD-1, BRUD-B, and BRUD-C (Table 2). None of the *N. pungens* clones grew when nitrogen was supplied in the form of ammonium at 880 μM (i.e., the nitrate-N concentration found in medium f/2). Consequently, this concentration was not used in subsequent experiments. At 440 μM ammonium, a lag phase was observed and the stationary phase cell yield was significantly lower (P < 0.05; paired Student's t-test) than

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**Table 1. Diatom clones used in this study.**

<table>
<thead>
<tr>
<th>Species</th>
<th>Clone</th>
<th>Origin</th>
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<tbody>
<tr>
<td><em>Nitzschia pungens</em></td>
<td>NPARL</td>
<td>Cardigan Bay, P.E.I.</td>
</tr>
<tr>
<td>f. multiseries</td>
<td></td>
<td>(Bates et al. 1989)</td>
</tr>
<tr>
<td><em>N. pungens</em> f. pungens</td>
<td>POM</td>
<td>Pomquet Harbour, N.S.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Seguel 1991)</td>
</tr>
<tr>
<td><em>N. pungens</em> f. pungens</td>
<td>MD-1</td>
<td>Galveston Channel, Tex.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Fryxell et al. 1990)</td>
</tr>
<tr>
<td><em>N. pungens</em> f. pungens</td>
<td>TKA-2</td>
<td>Galveston Channel, Tex.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Fryxell et al. 1990)</td>
</tr>
<tr>
<td><em>Skeletonema costatum</em></td>
<td>SCOST</td>
<td>Brudenell River, P.E.I.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(C. Léger, Gulf Fisheries Centre, Moncton, N.B., August 1989)</td>
</tr>
</tbody>
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that achieved with the same nitrogen concentration in the form of nitrate. At 220 μM, ammonium still resulted in a significantly lower ($P \leq 0.05$) cell yield when compared with the equivalent nitrate-grown culture. By comparison, there was no significant difference ($P \leq 0.05$) in cell yield due to nitrogen concentration among the three nitrate-grown *N. pungens* cultures and no indication of growth inhibition at the highest nitrate concentration used (Fig. 1A). Compared with the results for *N. pungens*, *S. costatum* showed little difference in cell yields for growth with nitrate (Fig. 1C) or ammonium (Fig. 1D), although the division rate was lower with 880 μM ammonium.

Repeating the experiments using ammonium-grown cells preconditioned with 220 μM ammonium rather than 880 μM nitrate did not alter the growth patterns shown above. The growth curves for all six *N. pungens* clones showed similar patterns for each nitrate and ammonium concentration tested. For this reason, clone NPARL is considered representative. Mean cell yields, total DA in the culture, and DA per cell on day 18 for clone NPARL are plotted in Fig. 2 for each initial nitrogen concentration used in the form of nitrate and ammonium. Cell yields were about 25% lower in the 220 and 440 μM ammonium cultures than in the corresponding nitrate cultures, and a lower cell yield was found with 440 than with

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**Fig. 1.** Growth of (A and B) *N. pungens* f. *multiseries* (clone NPARL) and (C and D) *S. costatum* (clone SCOST) in the presence of (A and C) nitrate or (B and D) ammonium at initial nitrogen concentrations of 880 μM (squares), 440 μM (triangles), and 220 μM (circles). Values are means of triplicate cultures.
220 μM ammonium (Fig. 2A). At 110 and 55 μM nitrogen, cell yields were equivalent for nitrate and ammonium but became diminished at the lowest nitrogen concentrations, despite the form of nitrogen used.

Domoic Acid Production

Each N. pungens f. multiseries clone showed a similar pattern of DA production with increasing nitrogen concentration; f. pungens clones produced no DA, as expected. On day 18, the yield of DA in the "whole culture" (i.e., cells plus medium) expressed per millilitre increased with increasing nitrogen concentration but levelled off or decreased in the cultures grown with 440 μM nitrogen (Fig. 2B). The DA yield per millilitre was similar at the two lower concentrations (55 and 110 μM) of nitrate and ammonium but was higher by twofold for clone NPARL growing with 220 and 440 μM ammonium than with the same concentrations of nitrate (Fig. 2B). The concentration of DA per cell increased with an increase in the initial nitrogen concentration (Fig. 2C). DA production was two- to fourfold greater in cells grown in 440 μM ammonium than in those grown on the same concentration of nitrate. There was no evident distinction in cell size between the nitrate- and ammonium-grown cultures to account for the differences in DA per cell.

In a time-course experiment, N. pungens f. multiseries (clone NPARL) was cultured in f/2 with 440 μM nitrogen in the form of ammonium or nitrate (Fig. 3). As in the previous experiments, the cell yield was consistently lower in the ammonium-grown than in the corresponding nitrate-grown culture (Fig. 3A). In addition, a lag phase was observed in the ammonium-grown culture, during which the DA concentration increased from 4 to 22 ng·mL⁻¹ (Fig. 3B), corresponding to an increase from 0.6 to 4.2 pg·cell⁻¹ (Fig. 3C). A net increase in DA concentration (from 22 to 56 ng·mL⁻¹) was also observed during an interval of near-exponential growth (days 8–13) of the ammonium-grown culture (Fig. 3B). However, the rate of increase in cell number was greater than the rate of DA production, so that the DA content per cell decreased during the exponential phase (Fig. 3C). In contrast, significant levels of DA did not appear in the nitrate-grown culture until after day 11, the beginning of the stationary phase, as expected (Bates et al. 1989). The rate of DA production decreased after day 27 in the nitrate-grown culture, perhaps due to exhaustion of nitrogen. Consistent with the above experiments, DA yield per cell was two- to threefold greater in the ammonium-grown than in the nitrate-grown culture during the stationary phase. The results were essentially the same as above when the time-course experiment was repeated with clone POM growing with 440 μM ammonium or nitrate. At 110 μM nitrogen, clone POM exhibited equivalent cell yield and DA production for both nitrate and ammonium, as was also shown in Fig. 2 for clone NPARL.

Effects on Photosynthesis

Growth at an initial concentration of 440 μM ammonium in the above time-course experiment with clone NPARL severely lowered Pmax (the maximum rate of photosynthesis at light saturation), whether expressed per litre (Fig. 4A), per cell (Fig. 4B), or per unit Chl a (Fig. 4C). Compared with the nitrate-grown culture, photosynthetic inhibition in the ammonium-grown culture was most evident during the lag and exponential growth phases.

Discussion

Results of this study show that nitrate and ammonium gave equivalent growth and DA production for several clones of N. pungens growing in batch cultures, if the initial nitrogen concentration was 110 μM or less. When supplied at initial concentrations of greater than 110 μM, ammonium, in contrast with nitrate, appeared to be toxic to N. pungens: a lag phase in growth was observed, photosynthetic rates were decreased, and stationary phase cell yields were reduced. Under such conditions, DA was produced during the lag phase and a two- to fourfold enhancement in DA yield was seen during the stationary phase. A meaningful comparison of division rates could not be made because of the low sampling frequency during the exponential phase, coupled with the complex lag phase found in ammonium-grown cultures. The similarity in the response of six clones of N. pungens to the form and concentration of nitrogen contrasts with that of S. costatum, which showed little difference in cell yield with nitrate or ammonium, even at 880 μM nitrogen.

The ability to increase the DA per cell by growth on high concentrations of ammonium could be very useful for growing reliable supplies of the toxic form of N. pungens. Such cells could then be used for studies of DA uptake and depuration by
shellfish and of DA toxicity to fish and invertebrates. Our results suggest that, when excreted by ammoniotelic animals under experimental conditions, ammonium may become toxic to *N. pungens* if concentrations begin to exceed 100 μM.

The toxicity of certain forms of ammonium to higher plants and marine animals is well documented (Warren 1962), although there is less information concerning phytoplankton. ZoBell (1935) found that the upper concentration limit for optimum growth of *Nitzschia closterium* (= *Cylindrotheca closterium*) was 50 μM ammonium. The growth of certain diatoms, including *Nitzschia seriata*, was inhibited by 100 μM ammonium (Guillard 1963). In contrast with our results with *N. pungens*, *S. costatum* showed less of a growth inhibition or lag period at initial concentrations of up to 880 μM ammonium (Fig. 1D). Thomas et al. (1980) similarly found no inhibition of growth or photosynthesis in *S. costatum* at 200 μM, the highest ammonium concentration they studied. As in invertebrates, fish, and mammals, toxicity to phytoplankton may involve the passage of aqueous ammonia (NH₃(aq)) through membranes (Warren 1962).

The lowered cell yields of *N. pungens* at 220 and 440 μM ammonium compared with those obtained with the same concentrations of nitrate do not appear to be related to the production of DA. This is because the same phenomenon is observed for both *N. pungens f. multiseries*, a proven DA producer (Bates et al. 1989), and *N. pungens f. pungens*, the form with no DA production (Smith et al. 1990b). It is possible, however, that *f. pungens* produces a nitrogen-containing secondary metabolite other than DA. The observed lower yields of both cells and DA at the lower concentrations (55 and no μM) of both nitrate and ammonium (Fig. 2) are consistent with the hypothesis that these cultures were nitrogen limited by day 18. This is supported by the finding of similar cell yields per unit nitrogen initially added for both forms and both concentrations of nitrogen: 1.42 × 10⁶ cells·μmol⁻¹ nitrate-N or ammonium-
accompanied by DA production. Douglas and Bates (1992) and Douglas et al. (1993) also reported DA production during the lag phase. Thus, DA production may be an indication that the cells are not growing optimally. An exception to this, however, is that toxin production decreases under nitrogen deficiency (Bates et al. 1991), as also suggested in Fig. 2. Experiments are under way to study DA production in silicate- and phosphate-limited chemostats.

There was no difference (P < 0.05) in DA production between cultures grown with low concentrations of nitrogen (i.e., 55 or 110 µM) in the form of ammonium or nitrate (Fig. 2). This is consistent with the hypothesis that the enhanced production of DA in our experiments only occurs at initially high, potentially toxic levels of ammonium. This was observed for ammonium concentrations at or exceeding 220 µM. It is possible that ammonium toxicity may redirect some intracellular glutamate from the pathway of “normal” amino acid synthesis to that of DA synthesis (cf. Laycock et al. 1989; Douglas et al. 1992), perhaps as a response to detoxify intracellular ammonium. The increased production of DA at high concentrations of ammonium may thus be a useful tool for studying the biosynthesis of this toxin.

It is not possible to extrapolate results of this laboratory study to a field situation because of differences in the concentrations of both nitrogen and N. pungens cells and in the frequency and rates of supply of nitrogen. Coastal environments such as Cardigan Bay, P.E.I., nevertheless have an abundance of ammonium from such sources as agricultural runoff, remineralization, and excretion from wild and cultivated bivalves. We have started chemostat experiments with ecologically relevant supply rates of nitrogen to provide insight into the relationship between nitrogen concentration, division rate, and DA production. In addition, phytoplankton in the field are exposed to nitrate, ammonium, urea, and other organic nitrogen compounds and any of these could be used for growth (Guillard 1963). Our laboratory is also studying the effect of mixtures of these nitrogen sources on DA production.

NOTE ADDED IN PROOF: The Section Pseudonitzschia was recently removed from Nitzschia and was promoted to generic status (Hasle 1993). The new nomenclature for Nitzschia pungens is Pseudonitzschia pungens.

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References


