Vol. 273: 1–15, 2004 | MARINE ECOLOGY PROGRESS SERIES
Mar Ecol Prog Ser | Published June 8

Effect of pH on growth and domoic acid production by potentially toxic diatoms of the genera *Pseudo-nitzschia* and *Nitzschia*

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ABSTRACT: The effect of elevated pH on growth and on production of the neurotoxin domoic acid was studied in selected diatoms belonging to the genera *Pseudo-nitzschia* and *Nitzschia*. Growth of most of the 11 species studied stopped at pH values of 8.7 to 9.1. However, for *P. delicatissima* and *N. navis-varingica* the pH limit for growth was higher, 9.3 and 9.7 to 9.8, respectively. A compilation of all available data on the pH limits for growth of marine planktonic diatoms suggests that species from ponds and rock pools all have higher limits than coastal and oceanic species. Taking only coastal and oceanic species into account, the data suggest that smaller species have a higher upper pH limit for growth than larger species. Elevated pH induced production of domoic acid in *P. multiseries* in amounts comparable to those detected previously under silicate and phosphate limitation. As *Pseudo-nitzschia* species are found in high concentrations in nutrient-enriched areas, high pH and hence induction of the production of domoic acid would be expected during blooms. These results may help to understand when and why *Pseudo-nitzschia* species produce domoic acid in the field.

KEY WORDS: Cell volume · Diatom · Domoic acid · Growth rate · pH · Phytoplankton · *Pseudo-nitzschia* · Toxin production

INTRODUCTION

Diatoms are receiving increasing attention worldwide due to the ability of some species to produce the neurotoxic amino acid, domoic acid (Hasle 2002). At present, at least 9 different *Pseudo-nitzschia* species (*P. australis*, *P. calliantha*, *P. delicatissima*, *P. fraudulenta*, *P. multiseries*, *P. multistriata*, *P. pseudodelicatissima*, *P. seriata* and *P. turgidula*) and 1 *Nitzschia* species (*N. navis-varingica*) have been reported to produce domoic acid (e.g. Bates 2000, Kotaki et al. 2000, Lundholm et al. 2003). Domoic acid (DA) may accumulate in the food web, and organisms such as mussels, scallops, clams, crabs, krill and plankton-feeding fishes may serve as vectors for the toxin (e.g. McGinness et al. 1995, Bargu et al. 2002). Domoic acid may thereby cause harm to seabirds, mammals and humans (e.g. Bates et al. 1989, Work et al. 1993, Scholin et al. 2000, Gulland et al. 2002). Among gastrointestinal and neurological symptoms in humans, one of the distinct symptoms is amnesia; hence the use of the term 'amnesic shellfish poisoning (ASP)' for poisoning with DA.

Some research has been directed towards resolving which factors might influence toxin production. Laboratory experiments have shown that depletion of silicate, phosphate and iron enhance the production of DA (Bates et al. 1991, Pan et al. 1996a, b, c, Maldonado et al. 2002), whereas depletion of nitrogen in batch cultures results in a reduction in DA production (Bates et al. 1991). As for several other toxins, photosynthesis is required for production of DA (Pan et al. 1998) and periods of darkness result in cessation of toxin production (Bates et al. 1991). Experiments have shown that irradiance of ≥100 µmol photons m^{-2} s^{-1} ensures that neither growth nor production of DA is limited (Bates 1998).

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Pseudo-nitzschia spp. blooms are often found in coastal areas enriched by nutrient-rich freshwater runoff (Smith et al. 1990, 1993, Horner & Postel 1993, Dortch et al. 1997, Scholin et al. 2000, Odebrecht et al. 2001) or in upwelling areas (Abrantes & Moita 1999, Trainer et al. 2000, Villac & Tenenbaum 2001). A positive relationship between increasing eutrophication of coastal waters and the abundance of *Pseudo-nitzschia* spp. was found in present and historical data from the coast of Louisiana and Texas (Dortch et al. 1997, Parsons et al. 2002). Hence, nutrient enrichment may result in increased densities of phytoplankton organisms such as *Pseudo-nitzschia* spp.

In seawater, pH has generally been recognised as being very stable (pH ≈ 8.2), as it is buffered by the carbonate system. However, uptake of dissolved inorganic carbon (DIC) during photosynthesis by high densities of phytoplankton may actually cause an increase in pH, and thus high pH values (around pH 9) have been recorded in natural environments during algal blooms (Hinga 1992, 2002, Macedo et al. 2001). For instance, in Narrangansett Bay, USA, 18% of the samples had pH values above 8.7 (Hinga 2002). Similarly, during the development of a bloom of the haptophyte *Phaeocystis* spp. in the North Sea, Brussard et al. (1996) found an increase in pH from 7.9 to 8.7. In fjords and coastal lagoons, pH values can attain even higher levels, and pH values up to around 10 may then be found during bloom periods (e.g. Marshall & Orr 1948, Macedo et al. 2001, Hansen 2002). The only study that we have been able to find in which pH, growth of potentially toxic diatoms and DA content has been studied is that of Douglas et al. (1993). However, the scope of that study was confined to comparing production of DA in non-axenic and axenic strains, and it was not possible to conclude what caused the growth limitation recorded in that study; as indicated by the authors, no conclusions could be made about the effect of pH on growth or on DA cell content and production.

As *Pseudo-nitzschia* spp. comprise bloom-forming species that are often found in nutrient-enriched areas, and as pH is expected to increase during algal blooms, we found it appropriate to conduct a study on the effect of pH on growth and toxin production of potentially toxic strains of *Pseudo-nitzschia*. Thus, the aim of the present study was to examine the effect of elevated pH on growth and toxin production of different potentially toxic diatoms in batch culture supplied with a surplus of nutrients. We also investigated the pH limits for growth of different strains of *Pseudo-nitzschia* and of a single *Nitzschia* species (*N. navis-varingica*) isolated from environments of different levels of trophy (oligotrophy-hypertrophy) to evaluate their tolerance to high pH.

MATERIALS AND METHODS

Algae species, clones and culture conditions. Different strains of *Pseudo-nitzschia* and *Nitzschia* were used. Information on strain designation, isolation place and date, and on the donors of the strains is given in Table 1. All strains were clonal and non-axenic and grown in L1-medium (Guillard & Hargraves 1993) based on autoclaved seawater with a salinity of 32 psu. Extra silicate was added to achieve a concentration (321 µM) 3 times higher than originally described for the L1-medium. The stock cultures were maintained at 15 ± 1°C and 15 to 25 µmol photons m^{-2} s^{-1} following a light:dark cycle of 16:8 h. Illumination was provided by cool fluorescent lamps and irradiance was measured using a Li-1000, Li-Cor sensor equipped with a Li-193SA spherical quantum probe.

Calculations of cell volumes were based on measurements on 10 to 20 cells of each strain, picked in exponential growth phase. Cell volumes were calculated according to Hillebrand et al. (1999) but with modifications for *Pseudo-nitzschia* spp. It was assumed that the linear part of *Pseudo-nitzschia* cells accounts for 60% of the cell length; hence this part of the cell volume was calculated as a rectangular box. Calculation of the remaining part of the cell volume was based on a prism on a parallelogram-base at each end. An additional assumption was that the width and height of *Pseudo-nitzschia* cells were similar. Thus the cell volume of *Pseudo-nitzschia* spp. was calculated using the equation:

$$\text{Volume} = (0.6 \times L \times W^2) + (0.4 \times 0.5 \times L \times W^2)$$

where L is the cell length and W is the width of the cell.

General experimental conditions. The experiments were carried out at an irradiance of 100 µmol photons m^{-2} s^{-1} to ensure that neither growth nor production of DA was light-limited (Bates 1998). Otherwise conditions were as described above. Prior to all experiments, the strains were acclimated to the experimental conditions for at least 7 d. Inoculates used for the experiments were taken from exponentially growing cultures. Initial experimental concentrations were 2000 cells ml^{-1}, and all experiments were carried out in triplicate flasks containing the volumes given below.

The medium used for the experiments was adjusted to the experimental pH by addition of 1 mM HCl or NaOH. pH was measured using a Sentron pH meter (Model ArgusX) equipped with a red line probe with a relative accuracy of ±0.01%. The pH meter was calibrated (2-point calibration) using Sentron buffers of pH 7 and 10.

Subsamples for cell counting (5 ml) and toxin analyses (2 × 10 ml) were taken at approximately the same time every day, and the pH of the medium was mea-

Table 1. *Pseudo-nitzschia* spp. and *Nitzschia* spp. Information on strain designations, sampling locations and times, and donors

Strain	Species	Sampling location and date	Donor
CL-193	*P. pungens*	Deadmans Harbour, Bay of Fundy, Canada, Oct 9, 2002	S. Bates
CL-195	*P. multiseries*	Deadmans Harbour, Bay of Fundy, Canada, Oct 9, 2002	S. Bates
OKPm013-2	*P. multiseries*	Okkiray Bay, Iwate Prefecture, Japan, Sep 20, 2001	Y. Kotaki
VHL987	*N. navis-varingica*	Ha Long Bay, Vietnam, Dec 5, 1998	Y. Kotaki
PS111V	*P. australis*	Baiona, Ría de Vigo, Spain, Sep 20, 2001	S. Fraga
NWFSC095	*Pseudo-nitzschia* sp.	Sequim Bay, Washington, USA, Aug 8, 2002	B. Bill
CL-190	*P. calliantha*	Baie- Sainte-Anne, New Brunswick, Canada, Sep 30, 2002	S. Bates
Tasm10	*P. delicatissima*	Hobart Harbour, Tasmania, Feb 13, 2000	N. Lundholm
CL-192	*P. fraudulenta*	Deadman's Harbour, Bay of Fundy, Canada, Oct 9, 2002	S. Bates
PG	*P. granii*	Ocean Station Papa, NE Pacific (latitude 50.0° N, longitude 145.0° W), Jun 2000	R. El Sabaawii
CL-159	*P. seriata*	Tracadie Harbour, Prince Edward Island, Canada, Apr 9, 2002	S. Bates
PT	*P.* cf. *turgidula*	Ocean Station Papa, NE Pacific (latitude 50.0° N, longitude 145.0° W), Aug 2002	A. Marchetti

sured. After subsampling, the flasks were refilled with L1 medium of the same pH (±0.05). Samples for enumeration were fixed in Lugol's solution (final concentration 2%) and counted in a Sedgewick rafter chamber. Counts were based on approximately 400 cells, corresponding to a deviation of ±10%, using 95% confidence limits (Utermöhl 1958). Maximum growth rates (μ) were calculated using the algorithm:

$$\mu = \ln (N_{t_2}/N_{t_1})/(t_2 - t_1)$$

where N_{t_2} and N_{t_1} are cell numbers at times t_2 and t_1. We used 3 successive cell counts in the calculations. Dilutions due to subsampling were adjusted for in the calculations of the growth rates.

Subsamples (50 ml) for determination of NH_4^+, NO_3^-, NO_2^-, PO_4^{3-} and SiO_4^- were taken from the medium used for inoculation at the beginning (Day 0) and at the end (Day 29) of the experiment with *Pseudo-nitzschia multiseries* (Strain CL-195). For silicate analyses, 50 ml were passed through a 5.0 μm polycarbonate filter and the filtrate was frozen immediately. For analyses of N and P, 50 ml of each nutrient was filtered through a GF/C filter and the filtrate was frozen. The nutrients were determined according to the method of Valderrama (1995) using a Beckman DU 640 spectrophotometer. Simultaneously, samples for measurements of dissolved inorganic carbon content were taken and measured immediately using a 225 MK3 infrared gas analyser (IRGA, ADC, Hoddesdon). We carried out 3 different experiments (Expts 1 to 3) to study the effects of elevated pH on the growth and DA production in selected potentially toxic diatom species.

Expt 1: Effect of pH on growth and DA production in pH-drift experiments. We grew 2 *Pseudo-nitzschia multiseries* strains (CL-195 and OKPm013-2) as batch cultures in 260 ml Nunclon polystyrene flasks (260 ml medium) mounted on a plankton wheel (1 rpm) in order to keep the cells in suspension. The pH was adjusted to 8.0 prior to the experiment and afterwards allowed to drift. Cell concentration and pH were measured daily until stationary growth phase, and thereafter less frequently (1 to 4 d intervals). Samples for DA concentration in the whole culture and in the filtrate were taken 7 to 10 times during the duration of the experiments (30 d). Samples for nutrient and dissolved inorganic carbon analyses were taken prior to and at the end of the experiment for Strain CL-195.

Expt 2: Effect of different initial pH levels on growth and culture cell yield. In order to demonstrate that elevated pH and not depletion of vitamins, micro- or macronutrients were inhibiting growth, *Pseudo-nitzschia multiseries* Strain OKPm013-2 was grown in batch culture in 260 ml Nunclon polystyrene flasks (260 ml medium) mounted on a plankton wheel

(1 rpm). The pH was adjusted to 4 different initial values (7.9, 8.3, 8.6 and 8.8) prior to the experiment, and subsequently pH was allowed to drift. Cell concentration and pH were followed daily until stationary growth phase, and thereafter less frequently (2 to 3 d intervals).

Expt 3: Role of environment and cell volume in pH tolerance of potentially toxic diatoms. The effect of elevated pH on the growth of 10 *Pseudo-nitzschia* species and *Nitzschia navis-varingica* (Table 1), isolated from temperate-subtropical areas with different nutrient levels, were studied in pH-drift experiments. All species were grown as previously described with the exception of *P. seriata* (Strain CL-159), a cold-water species which was grown in 62 ml Nunclon flasks at 4°C and an irradiance of 55 µmol photons m^{-2} s^{-1}. In all experiments, the pH was adjusted to ca. 8.0 before the experiment and afterwards allowed to drift. Cell concentration and pH were followed daily until stationary growth phase, and thereafter less frequently (1 to 3 d intervals). Samples for DA concentration in the whole culture and in the filtrate were taken 6 times during

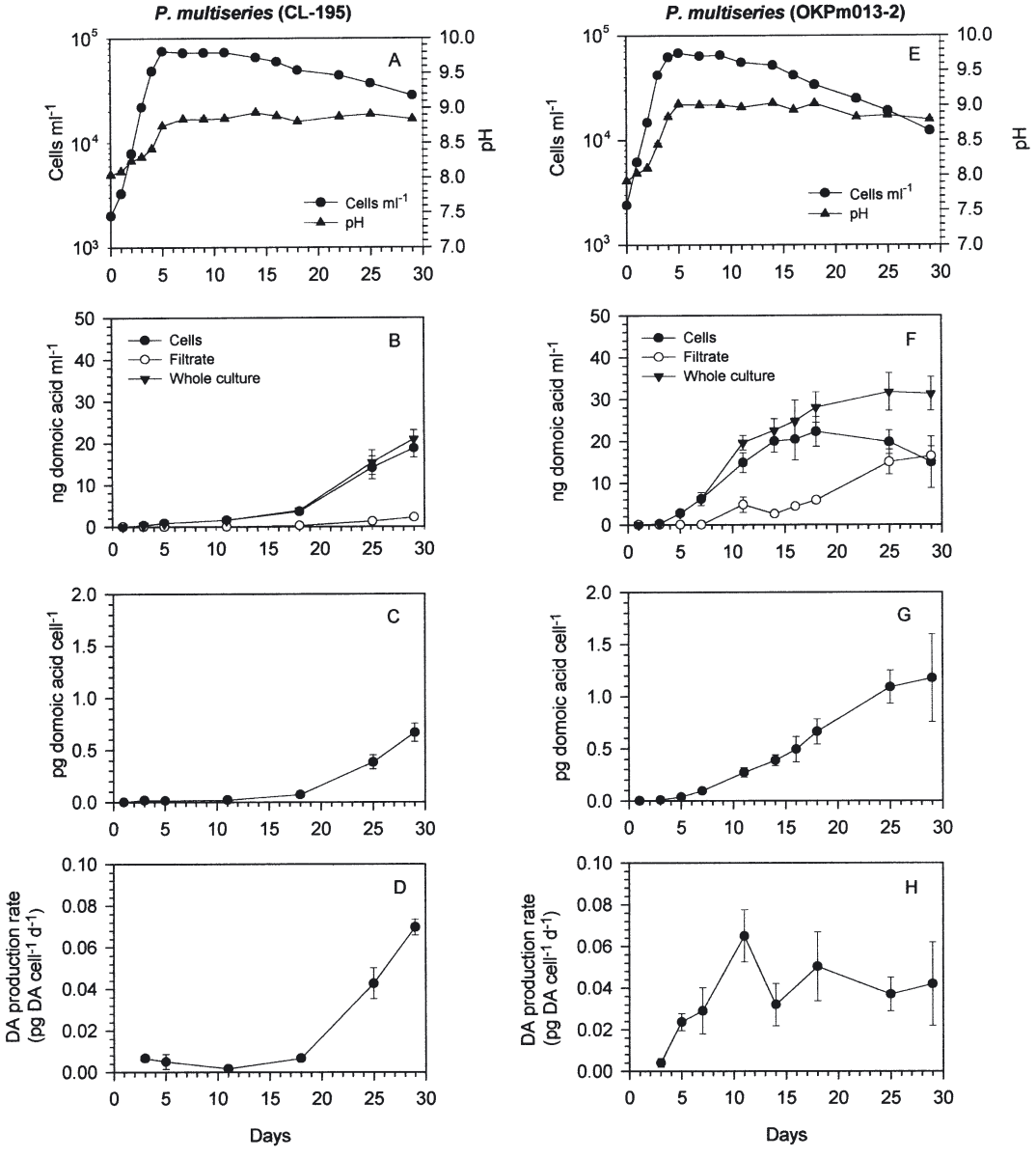

Fig. 1. *Pseudo-nitzschia multiseries.* Growth and toxin production of 2 strains: (A–D) Strain CL-195; (E–H) Strain OKPm013-2. (A,E) Cell concentration and pH as a function of time; (B,F) domoic acid content in cell fraction, medium and in whole culture as a function of time; (C,G) content of DA per cell as a function of time; (D,H) domoic acid production rate as a function of time. Data points are means ± SE, n = 3 (where SE bars are not visible, SE values were smaller than symbols)

the duration (30 d) of the experiment with *P. pungens* (Strain CL-193).

Toxin analyses. At each subsampling, 2 samples of 10 ml were taken. One was immediately frozen and used for determination of toxin content in the whole culture (cells plus medium), the other was filtered using a Nuclepore filter (0.8 µm pore size, 25 mm diameter) and the 8 ml filtrate was stored at –20°C. After each experiment, the frozen samples were immediately sent for toxin analyses.

The whole-culture sample was thawed, sonicated under cool conditions (<10°C) and centrifuged (8000 × *g*, 10 min). The supernatant and the filtrate were analysed for DA concentration using a slightly modified method of Pocklington et al. (1990) in which an ODS-5 column (4.6 × 250 mm, Nomura Chemical) and a mobile phase of 40% acetonitrile in phosphate buffer (pH 2.5) were used. The detection limit was 0.3 ng ml^{-1}.

Toxin content in the cellular fraction was calculated by subtracting DA in the filtrate from that in the whole culture. The toxin content per cell was calculated by dividing the DA in the cellular fraction by the cell number. The DA production rate was calculated using the formula:

$$\text{DA production rate (pg DA cell}^{-1}\text{ d}^{-1}) = \frac{DA_2 - DA_1}{0.5(N_1 + N)(t_2 - t_1)}$$

where DA_n is the domoic acid concentration (pg DA ml^{-1} in the whole culture), N is cell concentration (cells ml^{-1}) and t is time (d) (modified from Pan et al. 2001). Dilutions through subsampling were adjusted for in the calculation of the DA production rate. According to Pan et al. (2001), breakdown of DA can be ignored.

RESULTS

Effect of pH on growth and DA production in pH-drift experiments (Expt 1)

For the first 5 d, 2 *Pseudo-nitzschia multiseries* strains (CL-195 and OKPm013-2) grew exponentially with growth rates of ~1 d^{-1}. During this period, pH values increased from 8.0 to 8.5 (Fig. 1A,E, Table 2). After 5 d, cells entered the stationary phase and pH stabilised at a maximum pH value (Fig. 1A,E). pH values at which no positive growth took place (µ = 0) were between 8.8 and 9.0 (Table 2). The values of pH at which maximum growth rates were reduced by more than 20% were above 8.5 to 8.6. The concentrations of the inorganic nutrients (N, P, Si) were measured in the experiment with *P. multiseries* (Strain CL-195), and the results showed that the nutrients were in excess both at the beginning and at the end of the experiment (Table 3). A calculation of the estimated total uptake of inorganic nutrients by the cells using the maximum cell yield and the cellular content of N, P and Si supports this conclusion (Table 3).

Domoic acid was detected during the experiments with the 2 *Pseudo-nitzschia multiseries* strains CL-195 and OKPm013-2 (Fig. 1B,F). At the beginning of the experiment, on Day 1, no DA was detected (Fig. 1B,F), while low toxin concentrations in the whole culture were detected in the exponential growth phase (Day 3). At the end of exponential growth phase (Day 5; Fig. 1B,F) toxin concentrations (whole culture) had increased and continued doing so even after many days in the stationary phase.

The toxin was found in the filtrate during the stationary growth phase in all cultures (Days 11 to 18). During the remainder of the experiments, the toxin concentra-

Table 2. *Pseudo-nitzschia* spp. and *Nitzschia navis-varingica*. Maximum growth rates, cell volumes and pH limits for exponential growth for several strains. Environment: data from Hasle (1965, 2002), Kaczmarska et al. (1986), Hasle & Syvertsen (1997), Kotaki et al. (2000), and Lundholm et al. (2003); Max. pH: maximum pH when growth rate µ = 0. nd: no data

Species (Strain)	Environment	Cell volume (μm^3 ± SE)	Max. specific growth rate (d^{-1} ± SE)	Max. pH
N. navis-varingica (VHL987)	Marine ponds	5427 ± 196	0.51 ± 0.02	9.7–9.8
P. delicatissima (Tasm 10)	Coastal + oceanic	128 ± 13	1.47 ± 0.01	9.3
P. australis (PS)	Coastal + oceanic?	1307 ± 73	0.77 ± 0.05	8.9
Pseudo-nitzschia sp. (NWFSC095)	Coastal + oceanic?	306 ± 25	1.12 ± 0.05	8.9
P. calliantha (CL-190)	Coastal + oceanic?	250 ± 12	0.74 ± 0.02	9.0
P. fraudulenta (CL-192)	Coastal + oceanic	1536 ± 103	1.07 ± 0.02	8.8
P. multiseries (CL-195)	Coastal + oceanic?	nd	1.01 ± 0.02	8.8–8.9
P. multiseries (OKPm013-2)	Coastal + oceanic?	1122 ± 60	1.01 ± 0.05	9.0
P. pungens (CL-193)	Coastal + oceanic	nd	1.14 ± 0.04	8.8–8.9
P. seriata (CL-159)	Coastal + oceanic	1105 ± 50	0.65 ± 0.03	9.0
P. granii (PG)	Mainly oceanic	425 ± 5	1.81 ± 0.05	9.1
P. cf. *turgidula* (PT)	Mainly oceanic	479 ± 73	0.90 ± 0.02	8.7

Table 3. *Pseudo-nitzschia multiseries* (Strain CL-195). Contents of inorganic N, P, Si and C in L1-medium at inoculation (Day 0) and in filtrate of batch cultures at end (Day 29) of experiment. Bottom of table shows calculations of total N, P, Si and C uptake using maximal cell concentrations in experiment with Strain CL-195. Total N, P, Si and C uptake calculated on the basis of cellular contents measured in *P. multiseries* by Pan et al. (1996c) in an experiment without nutrient limitation (Si: 43.78 pg cell^{-1}, N: 11.23 pg cell^{-1}, P: 1.65 pg cell^{-1}; C: 94.99 pg cell^{-1})

Sample	NH_4^+ (µM)	NO_2^- (µM)	NO_3^- (µM)	PO_4^{3-} (µM)	SiO_4^- (µM)	C (mM)
Day 0	0.23 ± 0.01	1.49 ± 0.02	1111.4 ± 12.8	47.0 ± 0.2	430.7 ± 3.5	1.9
Day 29	0.43 ± 0.04	9.69 ± 0.20	1042.1 ± 5.1	39.0 ± 0.2	356.2 ± 0.2	1.73 ± 0.03

	Max. cell conc. (cells ml^{-1})	Estimated total uptake (µm) of: NO_3^-	PO_4^{3-}	SiO_4^-	C
CL-195	75000	60	4	117	593

tion in the filtrate continued to increase (Fig. 1B,F). The amount of toxin in the cellular fraction increased from the exponential growth phase and continued to do so in the stationary phase.

The cellular toxin content (pg DA cell^{-1}) increased throughout the experiment in Strain CL-195, whereas it seemed to reach a plateau in the late stationary phase in Strain OKPm013-2 (Fig. 1C,G). The production rates of DA, which represent the amount of DA produced between 2 sampling dates, were initially (exponential growth phase, Days 1 to 3) slightly positive (Fig. 1D,H). For Strain CL-195, this coincided with a slight increase in the amount of DA in the cells (Fig. 1D). The time course of DA production differed between the 2 strains. In Strain CL-195, the DA production rate increased during the stationary phase, especially during the latter part (Fig. 1D). In contrast, in Strain OKPm013-2 the DA production rate increased as early as the end of the exponential growth phase, and further increased at the beginning of the stationary phase; later in the stationary growth phase it decreased slightly and seemed to stabilise (Fig. 1H). Both strains reached maximum DA production rates of 0.07 pg DA cell^{-1} d^{-1}.

Effect of different initial pH levels on growth and culture cell yield (Expt 2)

Pseudo-nitzschia multiseries (Strain OKPm013-2) inoculated at different initial pH levels showed exponential growth for the first 2 or 3 d in the flasks inoculated at the 3 lowest pH values (Fig. 2A). The flasks initiated at pH 8.8 showed a lag phase of 1 d before the cultures started to grow exponentially. In all flasks, cultures entered stationary growth phase when pH reached 8.9 to 9.0. Hence, a negative linear relationship was found between maximum cell yield and pH; a higher initial pH resulted in a lower maximum cell yield (Fig. 2C). For initial pH values of 7.9 to 8.6, the stationary phase was reached at Days 4 to 5. The flask that had an initial pH value of 8.8 experienced a slower growth rate and reached the stationary phase later.

The maximum growth rates at the different pH levels were not statistically different at pH values of 8.0 and

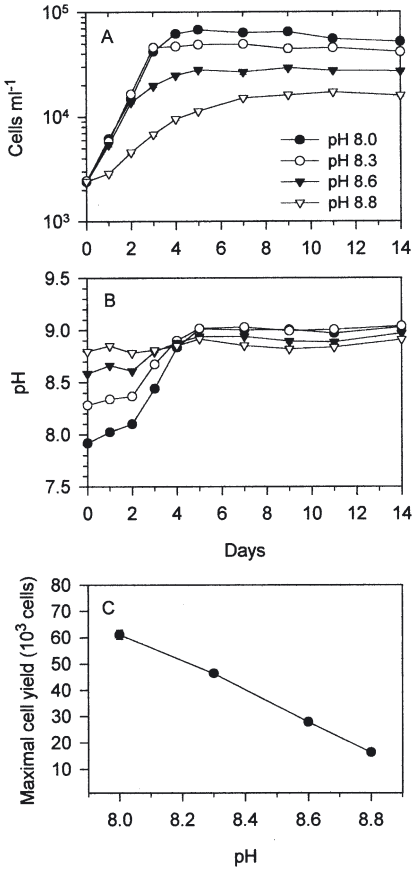

Fig. 2. *Pseudo-nitzschia multiseries*. Strain OKPm013-2 grown at different initial pH levels. (A) Changes in cell concentration as a function of time from inoculation at 4 different initial pH levels; (B) changes in pH as a function of time; (C) maximum cell yield in stationary phase as a function of time. Data points are means ± SE, n = 3 (where SE bars are not visible, SE values were smaller than symbols)

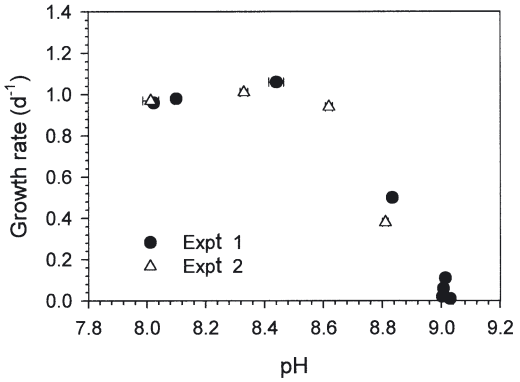

Fig. 3. *Pseudo-nitzschia multiseries.* Growth rate of Strain OKPm013-2 as a function of pH. (●) Data from pH drift experiments with initial pH of 8 (Expt 1); (△) data from experiments with different initial pH levels (Expt 2). Data points are means ± SE, n = 3

8.3, with a maximum of 1.02 d^{-1} at pH 8.3 (Student's *t*-test: $p > 0.05$), and slightly lower at pH 8.6 (*t*-test: $p < 0.05$) (Fig. 3). Above pH 8.6, a dramatic decrease in maximum growth rates was found and a maximum growth rate of 0.38 d^{-1} was attained in the flask inoculated at pH 8.8 (*t*-test: $p < 0.001$). Similar results could be obtained from the pH-drift experiments with *Pseudo-nitzschia multiseries* (Fig. 1E), using average values of pH and corresponding growth rates extracted day-by-day throughout the experiment. Combining both data sets, it is evident that growth rates were not affected significantly by pH below 8.6 (Fig. 3). When pH exceeded 8.6, the growth rate decreased until pH 9.0, above which no further positive growth was observed (Fig. 3).

Role of environment and cell volume in pH tolerance of potentially toxic diatoms (Expt 3)

The exponential growth phase occurred immediately or after a short lag-phase in all strains (Fig. 4). Maximum growth rates are shown in Table 2. The beginning of the stationary growth phase corresponded to the stabilisation of the pH values at a certain pH maximum (Fig. 4). The pH maximum, after which no further positive growth took place, varied among strains (Table 2). The large majority of species and clones had a pH limit for growth around 8.8 to 9.1. However, some exceptions were found. The highest pH limits for growth were reached by a strain of *Nitzschia navis-varingica* (9.7 to 9.8) isolated from a shrimp pond, and a coastal strain of *Pseudo-nitzschia delicatissima* (9.3). The oceanic *P.* cf. *turgidula* had the lowest pH limit for growth (8.7), whereas the other oceanic strain, *P. granii*, had a higher maximum pH (9.1).

Data on pH limits for growth and cell volume of planktonic diatoms were collected from the literature and combined with our own data. This gave a significant semi-logarithmic relationship (linear regression: $p < 0.001$ and $r^2 = 0.54$) between the upper pH limit and the cell volumes of the diatoms (Fig. 5), which can be described by the equation:

$$pH = 9.76 - 0.281 \times \log \text{cell volume } (\mu m^3)$$

Thus, small diatoms have a higher upper pH limit for growth than large diatoms. No DA was detected in any of the subsamples of *Pseudo-nitzschia pungens* collected throughout the experiment.

DISCUSSION

Effect of high pH on growth of *Pseudo-nitzschia* spp.

It was our intent to study how pH affected both growth and toxin production in DA-producing diatoms. Thus, we had to ensure that other factors such as macronutrients, micronutrients, vitamins and light were not limiting algal growth in our study. Irradiance was provided at an intensity that satisfies both growth and the photosynthetic energy requirement for production of DA by *Pseudo-nitzschia multiseries* (Bates 1998). We could directly demonstrate that nitrate, phosphate, silicate and total dissolved inorganic carbon were available in non-limiting concentrations both before and after the experiments (Table 3). The concentrations found are clearly above the concentrations previously reported to limit growth of *P. multiseries* and correspond to or exceed concentrations used in non-nutrient limiting experiments (Bates et al. 1993, Pan et al. 1996a,b,c). Nutrient excess in the present study is also indicated when comparing the total amount of nutrient uptake with the amount left at the end of the experiment (Table 3). To ensure that micronutrients and vitamins were not limiting growth, we ran an experiment in which *P. multiseries* (Strain OKPm013-2) was grown at different initial pH levels. In this experiment, it was evident that whenever pH reached values of about 8.9 to 9.0 growth stopped, independent of the cell concentration, and therefore independent of the concentration of macro- and micronutrients and vitamins. Hence, an inverse linear relationship was observed between initial pHs and maximum cell yield (Fig. 2C). Thus, our results of nutrient measurements as well as the experiment with different initial pH demonstrate that neither micro- or macronutrients nor vitamins were limiting growth of *P. multiseries*, and that pH was the limiting factor. These results support previous findings of pH as a limiting factor for growth of other microalgae grown in

nutrient-rich media such as f/2 growth medium (e.g. Schmidt & Hansen 2001, Hansen 2002, Hinga 2002).

Although positive growth of *Pseudo-nitzschia multiseries* continued until the pH reached 8.9 to 9.0, the growth rate became reduced at lower pHs. Maximum growth rates were retained at pHs below 8.6 (Fig. 3). These results show that the pH in nutrient-enriched coastal waters may potentially affect growth

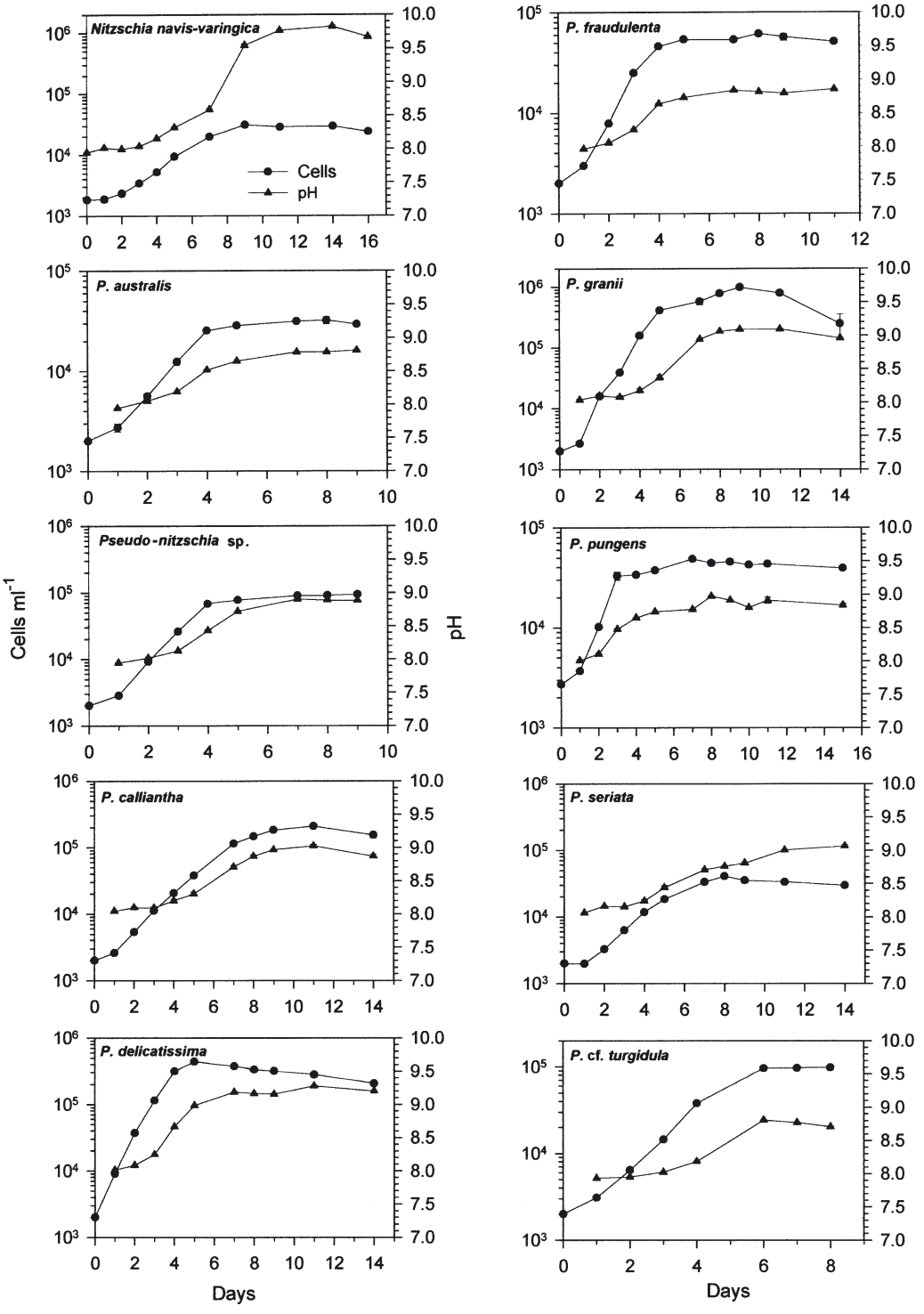

Fig. 4. *Nitzschia navis-varingica*, *Pseudo-nitzschia australis*, *Pseudo-nitzschia* sp., *P. calliantha*, *P. delicatissima*, *P. fraudulenta*, *P. granii*, *P. pungens*, *P. seriata*, *P.* cf. *turgidula*. Growth and pH changes as a function of time in batch-culture experiments. Data points are means ± SE, n = 3 (where SE bars are not visible, SE values were smaller than symbols)

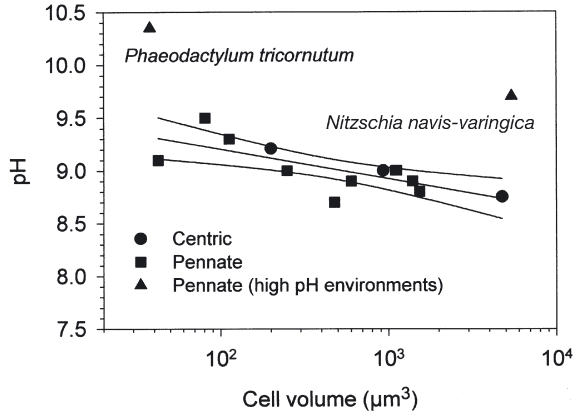

Fig. 5. pH as a function of cell volume of diatoms based on results from present study (Table 2) and data from other studies (Table 4) shown as a linear regression with 95% CI

of *P. multiseries* in the field and that not only nutrient depletion but also high pH may be the reason that a bloom does not increase further. *P. pungens*, which often co-occurs in nature with *P. multiseries*, had the same pH limit for growth as *P. multiseries* (Table 2).

Possible mechanisms responsible for effect of high pH on growth of marine microalgae

Several explanations of how pH in general might influence growth have been suggested, but the physiological mechanisms are still not fully understood. High pH may result in carbon limitation of species that do not utilise HCO_3^- effectively. The changes in the speciation of inorganic carbon as CO_2, HCO_3^- or CO_3^- that follow changes in pH may therefore affect the growth of the algae. At pH 8.0, about 1% of the dissolved organic carbon (DIC) is present as CO_2, whereas at a pH value of 9.0 only about 0.1% of DIC is available as CO_2 (Hinga 2002). This lower availability of CO_2 may potentially reduce photosynthesis and hence growth. Many diatoms have, however, been found to utilise HCO_3^-; either by taking up HCO_3^- actively and converting it to intracellular CO_2 using carbonic anhydrase, or transforming it to CO_2 using extracellular carbonic anhydrase and then utilising it for carbon fixation (e.g. Korb et al. 1997, Tortell et al. 1997). Some diatoms have even been found to be able to utilise HCO_3^- directly for carbon fixation through C_4-photosynthesis (Tortell et al. 1997, Reinfelder et al. 2000).

Another reason for the effect of elevated pH on phytoplankton is that pH may affect the bioavailability and/or toxicity of metals to marine microalgae (Sunda & Guillard 1976, Gensemer et al. 1993, Granéli & Haraldsson 1993, Craig et al. 2003). Conclusions on the effect of pH on metals and their toxicity, however, usually cannot be firmly established, since several studies have recorded opposite results (Peterson et al. 1984). For example, despite constant concentrations of copper ion, an increase in pH from 7.7 to 8.7 decreased copper toxicity in the diatom *Thalassiosira pseudonana*, possibly due to increased chelation of copper ions (Sunda & Guillard 1976). However, another study found no changes in copper toxicity for *Pyrocystis lunula* in the 8 to 10 pH interval and a decrease in pH to below 6 even counteracted copper toxicity (Craig et al. 2003). This clearly illustrates that final conclusions with respect to the effect of pH changes on the toxicity or availability of a metal are often difficult to make. In addition, there is evidence that the effect of pH on metal toxicity depends on the metal species itself (Wang 1987).

Apart from the 2 mechanisms suggested above, high pH may also influence the processes and energy involved in maintaining intracellular pH and cause changes to membrane transport processes (Raven 1980). Enzymatic processes in cells have different pH optima, and hence a shift in intracellular pH may affect the rate of the metabolic processes (Taraldsvik & Myklestad 2000). The existing knowledge on the effect of pH on the maintenance of intracellular pH and transport processes across the membrane in microalgae is sparse and further studies are greatly needed.

Upper pH limits for growth and their relationship with cell volume and the environment

Examination of the data for all the potentially toxic diatoms included in this study indicates a relationship between the environment from which the algae are isolated and their pH limit for growth. The highest pH limit for growth (9.7 to 9.8) was found in *Nitzschia navis-varingica* isolated from a brackish (27 psu) shrimp pond in Vietnam (Kotaki et al. 2000). In such shrimp ponds, pH values of up to 9.1 to 10.7 have been found (N. N. Lam pers. comm.). Of the remaining 10 *Pseudo-nitzschia* species, the maximum pH for growth was below 9.3. This may reflect the fact that all these species typically exist as planktonic life forms (Hasle 2002), e.g. none of them are benthic or found in rock pools or ponds, environments that often experience very high pH conditions.

Taking not only potentially toxic diatoms, but diatoms in general into consideration, is it possible to determine a relationship between environment and pH limitations for growth? Only a limited number of diatoms have been studied with respect to effect of pH on growth, and among those studied, the pH limits for

growth vary (Table 4). Some diatoms such as *Cerataulina pelagica* and *Thalassiosira punctigera* cannot grow when the pH exceeds 8.5 to 9.0 (Elzenga et al. 2000, Pedersen & Hansen 2003). The growth of other diatoms such as *Skeletonema costatum*, *T. pseudonana* and *T. oceanica* is limited at pH values above 8.5 or 8.8 and is inhibited when pH exceeds 9.4 (Chen & Durbin 1994, Taraldsvik & Myklestad 2000, Schmidt & Hansen 2001). The diatom species with the highest pH limit for growth is *Phaeodactylum tricornutum*, which is able to grow even when the pH exceeds 10 (Humphrey 1975, Goldman et al. 1982, Nimer et al. 1997). This species is mainly found in intertidal rock pools (Hasle & Syvertsen 1997), and pH values up to 9.3 have been found in such rock pools (see Knopp et al. 2003, www.hut.fi/~tjmatti2/projektit/Tvar2003_kirppu.pdf). Thus, diatoms like *Nitzschia navis-varingica* and *P. tricornutum* that grow in environments such as marine ponds and rock pools appear to have higher pH limits (Fig. 5). Both environments are characterised by periods of very high pH. It seems therefore likely that these organisms have evolved a way to exist in such environments through a yet unknown mechanism.

We found a significant semi-logarithmic relationship between the upper pH limit for growth and cell volume for diatoms with a planktonic lifestyle (Fig. 5). This relationship, which is valid for both centric and pennate diatoms, shows that smaller diatoms have a higher upper pH limit for growth than larger diatoms. The mechanism most probably responsible for the observed relationship is the regulation and maintenance of intracellular pH. The larger surface/volume of smaller cells size may allow them to better regulate their intracellular pH, because the maintenance of intracelular pH is presumed to be controlled by surface-associated ion-exchange processes (e.g. Gatti & Christen 1985, Boron 1986, Biagini et al. 2001). At high pH levels, smaller cells thus have a relatively larger surface area and hence a larger potential for maintaining a stable intracellular pH.

Effect of high pH on DA production

In 2 strains of *Pseudo-nitzschia multiseries*, elevated pH resulted in limitation of growth. Simultaneously to the increase in pH, DA was detected (Fig. 1). Domoic acid production began during late exponential growth phase (Fig. 1D,H) and was only found in the cells during this phase (Fig. 1B,F). During late exponential phase, pH increased and hence elevated pH is a possible explanation for the induction of production of DA. Neither nutrients nor vitamins were limiting. Salinity, temperature and light were unchanged and bacteria were present during the whole experiment although they were not enumerated. The variable parameter was pH and an increased pH in the late exponential phase was followed by an increase in production of DA. Thus, we suggest that elevated pH accounts for the production of DA. Similarly, studies on *Oscillatoria laetevirens* and *Chrysochromulina polylepis* found that increasing pH from 7 to 10 led to increased toxin concentration or increased toxicity of exudates, respectively (Ray & Bagchi 2001, Schmidt & Hansen 2001).

Table 4. Upper pH limits for growth of diatoms. Data from studies in which growth was not considered as being nutrient-limited. nd means no data. Cell volumes based on measurements of Hasle & Syvertsen (1997), Schmidt & Hansen (2001) and Pedersen & Hansen (2003) and M. F. Pedersen (pers. comm.)

Species	pH limit	Cell vol. (μm^3)	Source
Phaeodactylum tricornutum	>10, 10.4, 10.3	38	Humphrey (1975), Goldman et al. (1982), Nimer et al. (1997)
Asterionellopsis glacialis (as *Asterionella japonica*)	ca. 9.2[a]	nd	Kain & Fogg (1958)
Cerataulina pelagica	8.5–9.0	4762	Pedersen & Hansen (2003)
Chaetoceros didymus	9.2	nd	Humphrey (1975)
Cylindrotheca closterium	>9.5, 8.5[b], 9.5[b]	81	Humphrey (1975), Pedersen & Hansen (2003)
Leptocylindrus minimus	9.0	932	Pedersen & Hansen (2003)
Nitzschia sp.	9.5	nd	Humphrey (1975)
Odontella aurita (as *Biddulphia aurita*)	9.2	nd	Humphrey (1975)
Skeletonema costatum	9.21, ca. 9.5	200	Schmidt & Hansen (2001)
Thalassiosira oceanica	9.4–9.5	nd	Chen & Durbin (1994)
Thalassiosira pseudonana	9.4–9.5, 9.77	nd	Chen & Durbin (1994), Elzenga et al. (2000)
Thalassiosira punctigera	8.9	nd	Elzenga et al. (2000)

[a]Extrapolated
[b]Represents 2 different strains: *Cylindrotheca closterium* and *Nitzschia closterium* in Humphrey (1975)

Small amounts of DA were detected in the mid- to late-exponential growth phase (Fig. 1). DA has previously been detected in this phase in *Pseudo-nitzschia multiseries*, *P. australis*, *P. seriata* and *P. pungens* (e.g. Garrison et al. 1992, Lundholm et al. 1994, Pan et al. 1996b, 2001, Trainer et al. 1998). This growth phase has been explained either as (1) a transition stage between exponential and stationary growth during which some cells stop growth while others just slow division, or (2) as a general decrease in growth rate. In both instances, nutrient limitation or some other factor has been considered responsible (see Bates 1998). The production and concentration of DA in both medium and cells in the present study reached levels similar to those recorded in previous laboratory experiments (Bates et al. 1991, Bates 1998).

Scenarios where nutrients were not limiting but production of DA did occur have been described previously (see Bates et al. 1993, Pan et al. 1996c). A possible explanation for these scenarios could be elevated pH. We suggest that production of DA is influenced by several factors, including pH and depletion of silicate and phosphate, and that elevation of pH may interact in different ways in different scenarios. Pan et al. (1996b, c) suggested the existence of 2 phases (I and II) in the production of DA. Phase II corresponded to the typical nutrient stress situations in which growth was evidently limited by depletion of nutrients and production of DA was therefore enhanced; e.g. the chemostat experiments of Pan et al. (1996c). Phase I was in batch-culture experiments ascribed to the mid- to late-exponential growth phase in which nutrients were not yet evidently limiting growth. In a continuous culture experiment (Pan et al. 1996c), Phase I was described as a situation in which growth was reduced and production of DA occurred under conditions where neither silicate nor any of the other nutrients, vitamins or light were limiting. The population size stayed at a certain level and low amounts of DA were produced. This was explained as being due to an 'intrinsic physiological mechanism' (Pan et al. 1996c). We suggest that a potential explanation for the production of DA under the conditions where nutrients were not limiting could be the effect of elevated pH. (It should be mentioned that in Pan et al.'s [1996c] study the production of DA was higher in the continuous cultures limited by silicate than in the cultures limited by the intrinsic mechanism, possibly pH.) Further studies examining the combined effects of elevated pH and nutrient levels are under way by the authors.

In *Pseudo-nitzschia pungens*, no production of DA was detected (detection limit 0.3 ng ml^{-1}) at elevated pH. This is not surprising, as both toxic and non-toxic clones of *P. pungens* from geographically diverse locations have been found (e.g. Bates et al. 1993, MacKenzie et al. 1993, Villac et al. 1993, Lundholm et al. 1994, Rhodes et al. 1996, Trainer et al. 1998). This scenario, with the existence of both toxic and non-toxic strains of the same species, is also known for other *Pseudo-nitzschia* species. Both toxic and non-toxic strains of (e.g.) *P. australis* (Villac et al. 1993), *P. calliantha* (Lundholm et al. 2003), *P. multiseries* (e.g. Bates et al. 1989, Villareal et al. 1994), and *P. seriata* (Bates et al. 1989, Lundholm et al. 1994) have been found. These differences among strains could be a question of detection limit, or could be due to intraspecific genetic differences among strains or to differences in the factors controlling the production of DA. An experiment with one *P. pungens* strain therefore does not exclude the possibility that other strains of the same species might produce DA under conditions in which elevated pH is the inducing factor.

Possible mechanisms of DA production

It has generally been concluded that physiological stress caused by silicate and phosphate limitation enhances DA production (Bates 1998, Pan et al. 1998). Physiological stress arising through temperature or light conditions, however, does not enhance DA production (Bates et al. 1991, Bates & Leger 1992, Lewis et al. 1993, Lundholm et al. 1994). This indicates that different mechanisms may be responsible for the production of DA. Silicate has been hypothesized to promote production of DA indirectly by arresting cells in the cell cycle at 'a particular phase that is conducive to DA production' (Pan et al. 1998). On the other hand, phosphate limitation leading to decreased use of precursors such as acetyl CoA for lipid synthesis has resulted in an increased availability of these precursors for the production of DA (Pan et al. 1996a).

One of the possible explanations for a mechanism mediating the effect of pH on the DA production could be that enzymatic processes involved in production of DA have a certain pH optimum, and hence a shift in intracellular pH may affect the production of DA. In fact, it was shown by Taraldsvik & Myklestad (2000) that the composition of the cellular content of 19 different amino acids changed due to elevated extracellular pH. The concentration of some of the amino acids was constant over a broad pH interval, whereas the concentrations of others varied as a function of pH.

Another explanation for the effect of pH on DA production could be carbon limitation at high pHs. However, if carbon were depleted, one would expect a decrease, not an increase, in production of DA, as carbon is needed for the synthesis of the toxin. In addition, we do not consider this mechanism very likely, as most diatoms have been found to be able to

utilise HCO_3^- as carbon source in one way or another (e.g. Nimer et al. 1997, Tortell et al. 1997, Hobson et al. 2001).

A third potential explanation for the effect of pH on DA production could be that pH- mediated changes in speciation of metals affect the production of DA due to increased toxicity or reduced bioavailability of the metal. No study has examined the possibility 'elevated pH → increased metal toxicity → induction of DA production'. It is therefore necessary to combine the results of various studies examining individual processes. Studies on the effects of pH on metal toxicity are, as mentioned, conflicting. A study on copper toxicity to a diatom showed that increased pH resulted in reduced, not increased, toxicity of copper (Sunda & Guillard 1976). Studies on the induction of DA by metals have shown that copper toxicity (and iron limitation) induce production of DA (e.g. Maldonado et al. (2002). Maldonado et al. (2002) also found that copper toxicity (and iron limitation) in the exponential growth phase induced active transport of DA to the medium. Such active transport of DA was not seen in the present study; on the contrary, DA was only found in cells in the exponential phase. This indicates that copper toxicity (and iron limitation) is probably not the explanation for the pH-mediated induction of DA. On the other hand, studies on diatoms at higher pH levels are lacking, and in general studies on pH-related toxicity of copper on living organisms are contradictory (Craig et al. 2003). Therefore, we cannot reject the possibility that pH-mediated changes in the speciation of other metals could explain the production of DA at elevated pHs, since pH is known to affect the bioavailability and toxicity of metals such as Zn, Mn and Al (Gensemer et al. 1993).

A fourth possibility is that bacterial composition could change following changes in the pH. This hypothesis also cannot be rejected, but we find it unlikely as the only explanation, since even axenic cultures have been found to produce DA (Bates 1998).

pH and natural occurrences of *Pseudo-nitzschia* spp. and DA

In general, measurements of pH during algal blooms have shown that in open coastal areas, blooms of microalgae may result in pH values of up to 8.7 (e.g. Pegler & Kempe 1988, Brussard et al. 1996, Hinga 2002). In semi-enclosed coastal areas such as fjords and lagoons, pH may attain even higher values, up to around 10 during blooms (e.g. Marshall & Orr 1948, Macedo et al. 2001, Hansen 2002).

We have not been able to find any field data connecting pH with the occurrence of *Pseudo-nitzschia* or DA. However, *Pseudo-nitzschia* species are often found in large densities in coastal areas and often in areas that receive nutrient input from either upwelling or freshwater run-off (e.g. Yuzao et al. 1996, Dortch et al. 1997, Scholin et al. 2000, Trainer et al. 2000, Odebrecht et al. 2001, Stonik et al. 2001, Villac & Tenenbaum 2001). *Pseudo-nitzschia* spp. may form dense blooms (10^6 to 10^8 cells l^{-1}), during which they account for up to 99% of the total microalgal biomass (e.g. Subba Rao et al. 1988, Martin et al. 1990, Walz et al. 1994, Dortch et al. 1997, Fryxell et al. 1997, Gallacher et al. 2001, Stonik et al. 2001). Such dense blooms of *Pseudo-nitzschia* spp. in nutrient-enriched areas are expected to increase the pH to levels that could limit growth and also enhance production of DA. The most probable scenario is that an interaction of several different factors determines the production of DA in the field.

Conclusions

In batch-culture experiments, pH values above 8.6 reduced growth rates of *Pseudo-nitzschia multiseries* and pH levels of 8.8 to 9.0 inhibited growth. In addition, elevated pH levels induced production of DA in *P. multiseries*. These pH values are within levels previously observed during blooms of microalgae in coastal areas. Thus, inhibition of growth and induction of DA production due to elevated pH may potentially occur in blooms of *Pseudo-nitzschia* spp. in the field. Using a combination of results of the present study and data from the literature, a relationship between cell volume and the upper pH limits for growth was found for these diatoms, indicating that smaller cells have higher pH limits for growth. A possible explanation for this relationship could be related to the regulation of intracellular pH.

Acknowledgements. We wish to thank P. M. Jönsson and E. Granéli for nutrient analyses, A. Bisgaard for help in the laboratory, N. N. Lam for access to unpublished data on pH in shrimp cultures, A. Marchetti, R. E. Sabaawii, S. Bates, S. Fraga and B. Bill for providing cultures and M. F. Pedersen for access to cell measurements. The work was funded by a grant to N.L. from the Carlsberg Foundation (grant 0656/20) and a grant to P.J.H. from the Natural Research Council (grant 21-03-0449).

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Editorial responsibility: Otto Kinne (Editor), Oldendorf/Luhe, Germany

Submitted: December 1, 2003; Accepted: March 16, 2004
Proofs received from author(s): May 19, 2004