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Bacterial involvement in determining domoic acid levels in *Pseudo-nitzschia multiseries* cultures

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ABSTRACT: This study examining factors contributing to the production or elimination of domoic acid (DA) in cultures of *Pseudo-nitzschia multiseries* showed that in axenic cultures doubling the silicate concentrations increased growth, but not DA levels. DA concentration for axenic cultures was increased by the addition of gluconolactone (GlcA), especially in cultures with increased silicon. In non-axenic cultures, there were similar increases in growth with increased silicon, but a reduction of DA production in the presence of GlcA. Detailed examinations confirmed these findings and also showed that in non-axenic cultures, glucose alone resulted in a reduction of DA while a combination of glucose with gluconolactone resulted in a complete elimination of DA. Subsequent trials with axenic *P. multiseries* cultures showed that additions of DA or DA plus glucose introduced at the early stationary growth phase and incubated for 5 d had no impact on DA concentrations. In contrast, a 6 d incubation of the associated bacteria separated from the non-axenic diatom cultures showed reductions of added DA concentrations ranging from 46 to 72%, depending upon co-additives. The diatom does not use extracellular DA present in surrounding culture medium whereas bacteria associated with the diatom can utilize DA readily. Reductions in the production of DA by aging *P. multiseries* cultures appear to be the result of changing balances over time among bacteria associated with the diatom. These data coupled with results from other studies indicate that the amount of DA measured in *P. multiseries* cultures is a result of competitive interaction, i.e. a function of the diatom's production rate versus the extra-cellular utilization of DA by associated bacteria.

KEY WORDS: Domoic acid · Diatom · *Pseudo-nitzschia multiseries* · Silicon · Gluconolactone · Glucose

INTRODUCTION

In 1987, the neurotoxic properties of domoic acid (DA) were revealed by a major episode of acute human poisoning (Bird et al. 1988, Subba Rao et al. 1988, Bates et al. 1989, Wright et al. 1989, Perl et al. 1990, Todd 1990, 1993). This poisoning resulted from consumption of farmed blue mussels *Mytilus edulis* from Cardigan, Prince Edward Island, Canada that possessed concentrations of DA ranging up to 900 µg g^{-1} of soft tissue (Quilliam et al. 1989, Wright et al. 1989). The source of the DA was the diatom (Subba Rao et al. 1988, Bates et al. 1989) *Pseudo-nitzschia multiseries* (Hasle 1995) upon which the mussels had been feeding. At least 3 people died as a result of the intoxication and 150 others were considered to have been poisoned. As one of the effects of the severe poisonings was loss of short-term memory, the DA intoxication was named Amnesic Shellfish Poisoning (ASP) (Perl et al. 1990).

Subsequently, DA has been found widely in the marine environment (Villac et al. 1993, Rhodes et al. 1998, Bates 2000) largely, but not exclusively, produced by *Pseudo-nitzschia* species. The production of DA has been reported also for one strain of a pennate diatom, *Amphora coffeaeformis* (Shimizu et al. 1989) and from *Nitzschia navis-varingica* isolated from a shrimp culture pond in Vietnam (Kotaki et al. 2000, Lundholm & Moestrup 2000).

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Pseudo-nitzschia multiseries usually proliferates at a time of year when conditions are unfavourable for other algal species, i.e. light is low and most species of algae are unable to grow optimally (Mos 2001, Hagström et al. 2007). Blooms occur usually in spring and late fall when phosphorous and silicate are depleted in surface waters and nitrogen is relatively high as a result of rain and melting snow increasing its transport from land to sea (Bates 1998).

DA is produced in volume by *Pseudo-nitzschia multiseries* only after it has reached the stationary phase of growth, i.e. upon depletion of nutrients (Subba Rao et al. 1990, Bates et al. 1991). Importantly, the DA produced is not retained by diatom cells, but instead most is excreted to the surrounding medium (Subba Rao et al. 1990, Bates et al. 1991). Imposing nutrient stress on cells growing non-axenically by limiting nitrate and silicate (Bates et al. 1991), silicate (Pan et al. 1996a,b) phosphate (Pan et al. 1996c), or by supplying nitrogen in the form of ammonium ions in excess of 110 µM (Bates et al. 1993) slows the rate of cell division and concomitantly increases DA production. Conversely, DA production is increased without affecting the growth rate of non-axenically grown cells by adding LiCl to the medium at a concentration of 385.6 µM; this induces a DA increase in the stationary phase considerably earlier than DA increased in control cultures (Subba Rao et al. 1998). In addition, Douglas & Bates (1992) and Bates et al. (1995) showed that the low production of DA by axenic cultures of *P. multiseries* was enhanced by introduction of particular bacteria to these cultures.

Following the identification of gluconolactone in the fluid of only those mussels containing DA (McLachlan et al. 1993), Stewart et al. (1997) demonstrated that certain bacteria associated with each *Pseudo-nitzschia multiseries* strain examined were strong producers of gluconolactone. Exposure of *P. multiseries* during axenic growth to gluconolactone, or more precisely to the resultant powerful, sequestering gluconic acid/gluconolactone (GlcA) mixtures, produces no interference with growth, but enhances DA production in the stationary phase in proportion to the concentrations of GlcA added (Osada & Stewart 1997). Osada & Stewart (1997), on the basis of the antagonistic, sequestering nature of the GlcA mixtures and the observations by others of the effects of nutrient stress, suggested that the DA could be generated by the diatom as an external chemical scavenger to counter nutrient stress.

DA is utilized by bacteria isolated from shellfish (blue mussels and soft shell clams, *Mya arenaria*) and by bacterial strains isolated from *Pseudo-nitzschia multiseries* cultures [strains N-7, a *Moraxella* species and N-9, an *Alteromonas* species (Stewart et al. 1997)] (Stewart et al. 1998). All of these bacteria supplied with low concentrations of growth factors readily cause the disappearance of DA presented in moderate concentrations of 40 or 80 nM. Thus this information (and the studies by Hagström et al. (2007) showing the ultimate destruction of DA by bacteria associated with the diatom in aged and decaying cultures) suggest that the bacteria associated with the diatom can be factors in determining the production levels of DA and its elimination under particular circumstances.

Bacterial associations and their involvement with DA-producing diatoms were examined by Douglas & Bates (1992), Bates et al. (1995), Stewart et al. (1997, 1998), Osada & Stewart (1997), Kaczmarska et al. (2005) and Hagström et al. (2007). In this study, questions on the dynamics of DA formation by *Pseudo-nitzschia multiseries* and the various factors (including bacteria) which may influence its production and elimination are considered. These include: (1) As a wide variety of nutrient stresses contribute to DA production, i.e. reduced silicate, phosphorous, nitrate etc., what effect would increases in various factors, e.g. silicate, an energy source (glucose) or a sequestering agent (gluconic acid/gluconolactone) have on diatom growth and DA production in axenic and non-axenic cultures? (2) Are declines in DA production in culture a result of its re-absorption by the diatom or degradation by associated bacteria?

MATERIALS AND METHODS

The DA-producing strain (designated NpH) of *Pseudo-nitzschia multiseries* used throughout this work and in previous studies (Osada & Stewart 1997, Stewart et al. 1997) was isolated originally by K. E. Pauley (strain KP59) from New London Bay, Prince Edward Island, Canada on October 18, 1991.

All diatom growth studies were carried out in triplicate (except in one instance as noted) in the appropriate medium usually dispensed in 20 ml quantities in 50 ml capacity polystyrene sterile tissue culture flasks (Nunc). Cultures were maintained at 20°C in a continuous cool-white fluorescent light regime at 170 (range 140–200) µmol photons m^{-2} s^{-1} (QSL light meter, Biospherical Instruments). The growth medium was a modification of that proposed by Harrison et al. (1980); steam-sterilized trace metals and nutrients (minus silicate and boric acid, and with NaH_2PO_4 substituted for glycerophosphate) were added aseptically to steam-sterilized natural sea water (taken from bulk supplies collected from Bedford Basin, Nova Scotia, Canada in the fall of 1990, passed through a 53 µm pore size filter and stored unsterilized in closed containers at room temperature) followed by the aseptic addition of a vitamin mixture and 1 ml l^{-1} of a (0.1 M)

$Na_2SiO_3 \cdot 9H_2O$ sterile solution to give a final silicate concentration of 0.1 mM in the basal medium, the salinity of which was approximately 33 psu.

Axenic *Pseudo-nitzschia multiseries* subcultures were prepared as described earlier (Osada & Stewart 1997) by treating non-axenic liquid cultures with ampicillin (1 mg ml^{-1} of culture medium) overnight, or with mixtures of ampicillin (1 mg ml^{-1}) and streptomycin (2 mg ml^{-1}) for 72 h, followed by subculture in fresh antibiotic-free medium. Subcultures were examined microscopically and bacteriologically using DIFCO Marine Agar and Broth (#2216). The subcultures in which no bacteria were observed or could be cultured were used for axenic studies; identical examinations were carried out after the completion of studies to ensure that the cultures were still axenic.

From each culture flask, 2 ml samples were removed on the sampling days indicated for determination of diatom numbers and chemical concentrations. Diatom numbers were determined by direct microscopic counts using 0.5 ml of the material removed from the growth flasks, mixed with fixative (2% final concentration of 1:1 formalin:glacial acetic acid) and settled in glass-bottomed counting chambers.

Additives for use in growth experiments were dissolved in distilled water to give concentrated solutions which when introduced would result in minimal dilution of the media (<0.5%), i.e. GlcA adjusted to pH 8.0 with NaOH and made up to 100 mM and glucose prepared as a 1.0 M solution. These were filter sterilized (0.22 µm pore size filter) and added as required to sterile growth media to give the desired final concentrations. Silicate concentrations were increased by addition of the appropriate amounts of the sterile silicate medium supplement always added to the medium prior to inoculation with *Pseudo-nitzschia multiseries*.

For tracking DA concentrations in the diatom growth studies, samples were withdrawn aseptically, and stored frozen for later treatment. The cells were ruptured by treatment for 1 min with a Sonic Dismembrator (Artek System) equipped with a titanium microtip and operated at 35% power (about 100 W). The resulting homogenate was centrifuged (10 000 × *g* for 10 min) to remove debris; the supernatant fluids containing the extra- and/or intra-cellular DA were stored frozen to await analysis.

Using sterile techniques to avoid contamination, mixtures of bacteria associated with *Pseudo-nitzschia multiseries* cultures were separated from the diatom by filtration through a 3 µm pore size membrane filter. Upon microscopic examination of the filtrate, no intact diatom cells were detected; aliquots of this filtrate transferred to the standard diatom medium did not exhibit growth of diatom cells even after 10 d incubation at 20°C.

The bacterial mixtures obtained were used to inoculate the yeast extract broth basal medium of MacLeod (1968) dispensed in quadruplicate in 225 µl quantities into sterile 96-well microplates. Each well was inoculated with 25 µl of the freshly prepared bacterial mixtures and incubated in the dark at 20°C in parallel with control wells containing 250 µl of the uninoculated medium. Growth was recorded as optical density at 590 nm using a microplate reader (Molecular Devices). On the 6th day of incubation, the contents of the wells were subjected to centrifugation (10 000 × *g*) to remove bacteria, and the DA concentrations in the supernatant fluids were determined.

DA was determined using the enzyme-linked immunosorbent assay (ELISA) specific for DA (Osada et al. 1995) except for the trials in which the DA was added to axenic cultures of *Pseudo-nitzschia multiseries* (determined by the High Pressure Liquid Chromatography (HPLC) as described by Gilgan et al. 1990). For ELISA analyses, 180 µl of 10-fold PBS dilutions of the supernatant fluids from sonic treated whole cultures were dispensed into each well of the 96-well microplates previously coated with DA conjugated with ovalbumin and blocked with bovine serum albumin. The contents were then treated as described by Osada & Stewart (1997).

Glucose and GlcA concentrations were measured in culture filtrates according to the manufacturers' directions using the appropriate specific enzymatic combinations supplied by Boehringer Mannheim Canada; glucose supplied to the axenic cultures was determined using the Megazyme International Ireland Assay Kit.

RESULTS

The growth of axenic *Pseudo-nitzschia multiseries* (strain NpH) cultures alone or in the presence of additives is shown in Fig. 1 along with data on the influence of these additives on DA production. As expected, the doubling of the silicate concentration increased cell production although at neither level of silicate did the axenic culture prove to be robust or stable. As observed initially by Osada & Stewart (1997), the 0.25 mM GlcA concentrations had no apparent effect upon growth. There were differences, however, in the DA production. When 0.25 mM GlcA and 0.2 mM silicate were combined, the DA production was tripled (Fig. 1). Significant DA production with these cultures actually began considerably after cell division had peaked and did not reach maximum values until the cultures were in severe decline.

As preliminary trials with axenic *Pseudo-nitzschia multiseries* cultures indicated that concentrations of

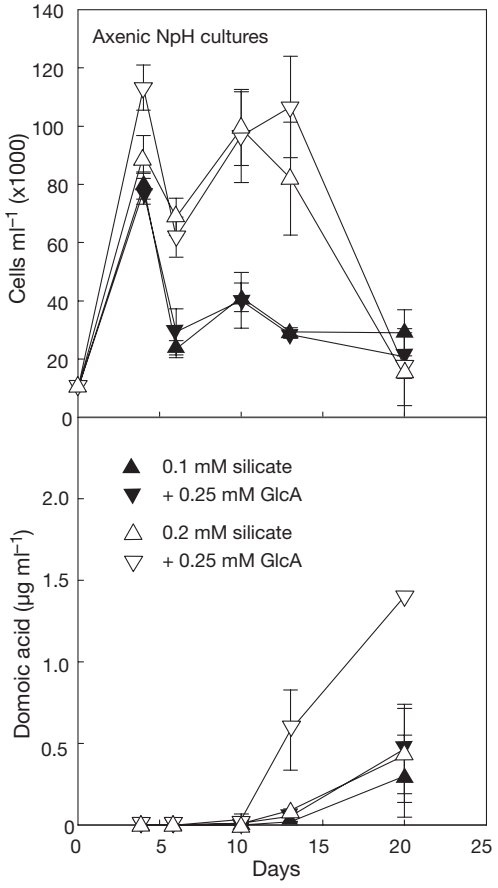

Fig. 1. *Pseudo-nitzschia multiseries*. Growth of axenic cultures (strain NpH) and production of DA (domoic acid) at 20°C in standard diatom medium alone (0.1 mM silicate) and when supplemented to concentrations of 0.25 mM gluconolactone (GlcA)/gluconic acid, or also doubling the silicate concentration (0.2 mM silicate). All supplements were present from the initiation of the growth period. Means ± SE (n = 3)

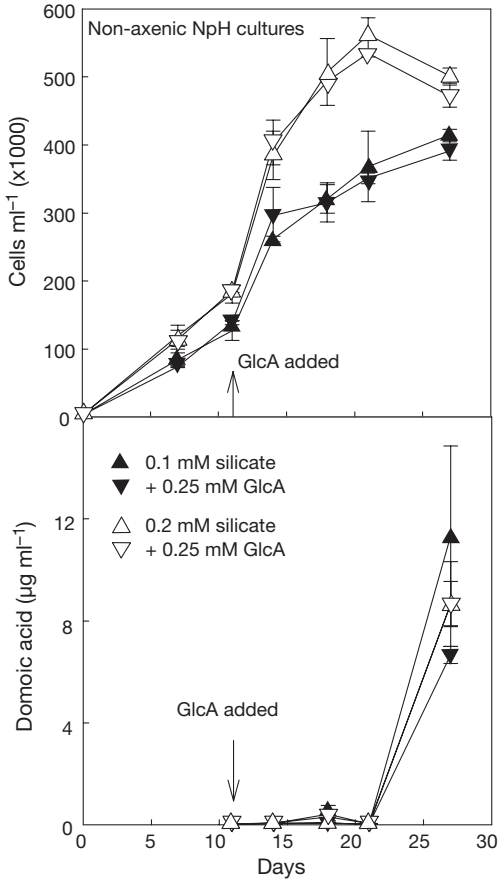

Fig. 2. *Pseudo-nitzschia multiseries*. Growth of non-axenic cultures (strain NpH) and production of DA (domoic acid) at 20°C under the influence of additives indicated in Fig. 1. The GlcA (gluconolactone), however, was added on Day 11 post-inoculation. Means ± SE (n = 3)

added GlcA remained unchanged, a virtual repetition of part of the previous experiment was run for confirmation. Axenic *P. multiseries* was grown in the standard diatom medium increased to 0.2 mM silicate and half of the flasks (4) were brought to 0.5 mM GlcA on the 8th day post inoculation (the remainder (4) had no additives). The measurements made on the 18th day of growth are shown in Table 1. GlcA concentrations (repeated in 2 separate determinations) in the axenic cultures remained essentially unchanged.

When an experiment almost identical to that illustrated in Fig. 1 (except that the GlcA was added 11 d post inoculation) was run with non-axenic cultures of the same strain of *Pseudo-nitzschia multiseries*, some of the results were quite different (Fig. 2). The growth was robust, stable and proportionately responsive to the effect of doubled silicate; the GlcA had no apparent effect on cell production or appearance of individual diatom cells. The major differences occurred in the rates and amounts of DA produced. As expected, significant DA production was not apparent until after the stationary growth phase had been reached. The maximal production of DA recorded on the 27th day of growth (much greater than produced

Table 1. *Pseudo-nitzschia multiseries*. Effects of GlcA (gluconolactone) addition on DA (domoic acid) production in axenic culture. Means ± SE, n = 4

Cell density (cells ml^{-1})	GlcA added on Day 8	DA (ng ml^{-1}) at Day 18	GlcA (µg ml^{-1})	
			Initial (Day 8)	Final (Day 18)
71 610 ± 11 685	0.0	830 ± 387	0.0	0.0
81 840 ± 5391	0.5 mM (133 µg ml^{-1})	1600 ± 533	133 ± 2.1 134 ± 8.4	124.3 ± 3 121.3 ± 19.4

in the axenic culture) actually occurred at the lower or standard level of silicate (0.1 mM) where the cell growth was considerably less than that at 0.2 mM silicate. Moreover DA concentration on the 27th day in 0.1 mM silicate cultures containing 0.25 mM GlcA was 40% less than that in matching GlcA-free culture. However, addition of the same concentration of GlcA to cultures containing 0.2 mM silicate had no effect. Comparisons of DA values (Fig. 2) on Day 27 using a paired *t*-test (Sokal & Rohlf 1995) showed the concentrations for the two 0.1 mM silicate growth curves were significantly different from one another and from concentrations for the 0.2 mM silicate growth curves ($p < 0.05$). Trials with GlcA additions to non-axenic cultures during their logarithmic phase of growth led directly to the following experiment and the decision to increase the GlcA concentration to 0.5 mM.

My aim was to present the additives to cultures at the beginning of the stationary phase or when slowed cell division would be expected to occur (judging by previous trials with 0.1 mM and 0.2 mM silicate concentration diatom media). The impacts of the additives and their fates were then examined in more detail over a shorter time period (about 48 h) than previously . The additives were presented to *Pseudo-nitzschia multiseries* cultures growing in 0.1 or 0.2 mM silicate concentration culture medium (otherwise standard diatom medium) on Day 22 day post-inoculation. On that day, 3 culture flasks were left untreated and the remaining flasks in groups of 3 were supplemented to concentrations of 0.5 mM GlcA, 5 mM glucose or a combination of both (Fig. 3). DA concentrations measured in samples drawn at 0, 2, 4, 18 and 49 h following the additions on Day 22 are presented in Fig. 3B,C. The amounts of GlcA or glucose remaining in the cultures after 49 h incubation are given in Table 2.

Significantly increased amounts of DA were apparent after 18 h in those cultures to which no additions had been made, and amounts were proportional to the numbers of cells produced (Fig. 3). Cell production in 0.2 mM silicate medium was approximately double that in the 0.1 mM silicate medium, as were the DA levels 49 h post addition.

Between 18 and 49 h post addition, the additives had markedly negative influences on DA concentrations. The addition of GlcA alone strongly retarded DA production at both silicate levels. Glucose alone brought about a reduction of DA from those levels present at zero time, while the combination of GlcA with glucose resulted in complete elimination of DA already present

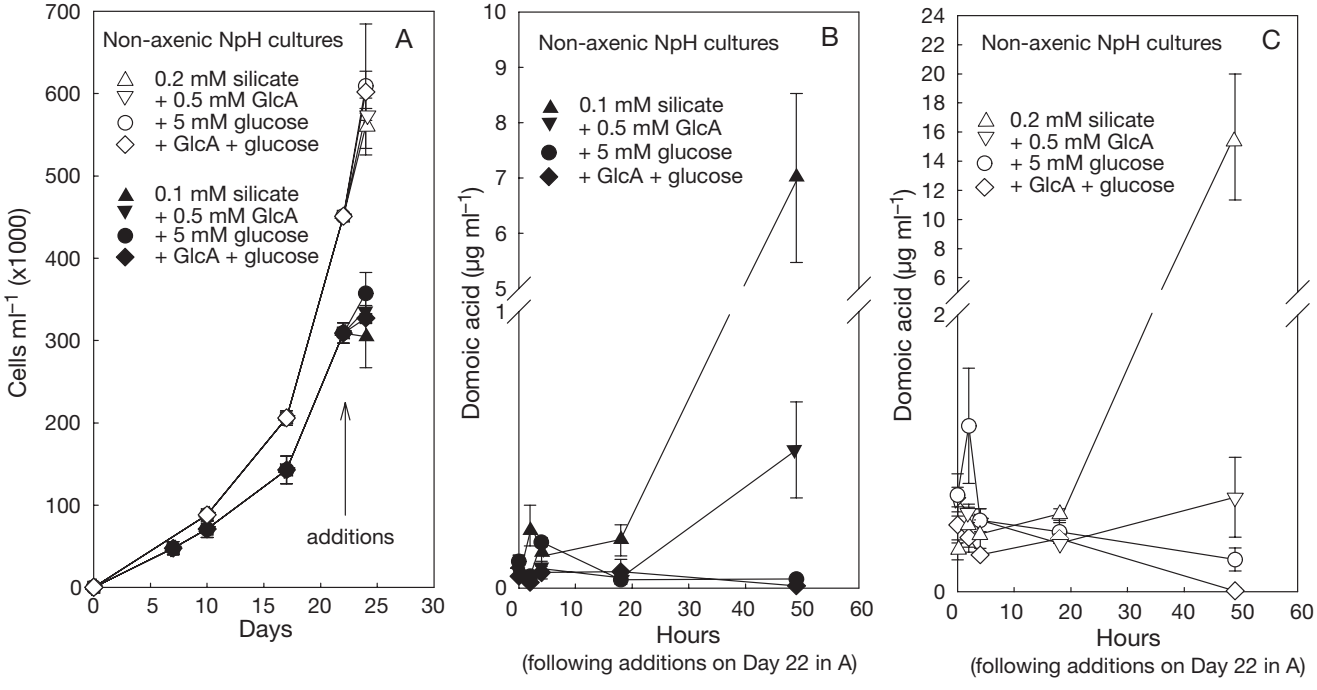

Fig. 3. *Pseudo-nitzschia multiseries*. (A) Growth of non-axenic cultures (strain NpH) at 20°C in single and double silicate concentration media for 22 d, at which time additives (GlcA [gluconolactone], glucose) singly and in combination were introduced to both sets of cultures and their influence followed over the ensuing 49 h. (B) Effect on DA (domoic acid) production of the single additions or combinations of GlcA and glucose with the standard diatom medium (0.1 mM silicate) in the 49 h period following the additions made on Day 22 (Fig. 3A). (C) Effect on DA production of the same additives at the same concentrations added to the standard diatom medium containing double the concentration of silicate (0.2 mM) in the 49 h period following the addition on Day 22 (Fig. 3A). Means ± SE (n = 3)

Table 2. *Pseudo-nitzschia multiseries* concentrations (µg ml^{-1} ± SE, n = 3) of supplements added to 0.1 and 0.2 mM silicate cultures on Day 22 of incubation (Initial) and after 49 h (Final). See Fig. 3. GlcA was prepared as 0.5 mM solution, but as enzymatic determinations gave a higher value, these were used throughout as the base for comparison. GlcA = gluconolactone/gluconic acid; Gluc = glucose

Additives to:	Concentrations			
	Initial GlcA	Final GlcA	Initial Gluc	Final Gluc
0.1 mM silicate cultures				
a) GlcA	116 ± 2	106 ± 4.9		
b) Gluc			900	480 ± 68.2
c) GlcA + Gluc	116 ± 2	109 ± 5.2	900	645 ± 62.4
0.2 mM silicate cultures				
a) GlcA	116 ± 2	113.7 ± 3.2		
b) Gluc			900	718.7 ± 68.2
c) GlcA + Gluc	116 ± 2	111.7 ± 4.2	900	534.7 ± 65.8

at the zero hour addition mark. GlcA concentration remained essentially unchanged over the 49 h examination period; depending on the particular culture, between one third and one half the glucose added disappeared (Table 2).

To determine whether the reduction and elimination of these DA concentrations was a result of (1) absorption and utilization of the DA by the diatom or (2) elimination of the DA by bacteria associated with the diatom, the following examinations were carried out. An axenic diatom culture in 4 flasks each charged with 30 ml of standard diatom medium at a concentration of 0.2 mM silicate was grown for 20 d. Near the beginning of the stationary growth phase (i.e. on Day 20), 2 of the flasks were exposed to DA (5 µg ml^{-1}) plus glucose (5 mM) and 2 flasks to DA alone (5 µg ml^{-1}) (Fig. 4). The addition of glucose restored *Pseudo-nitzschia multiseries* to a logarithmic growth phase resulting in a 50% greater cell production than in those cultures supplied with DA alone. The amount of glucose remaining at the end of the 5 d exposure was 605 ± SE = 1 µg ml^{-1} (n = 3), approximately a one third utilization. The amounts of DA added were not changed significantly at any point in the 5 d exposure in either of the 2 sets of cultures (Table 3).

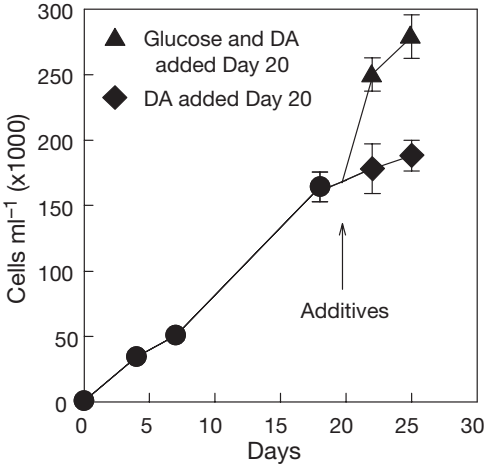

Fig. 4. *Pseudo-nitzschia multiseries*. Growth of axenic cultures in standard diatom medium containing 0.2 mM silicate with addition on Day 20 of DA (5µg ml^{-1}) or DA plus glucose (5 µg ml^{-1} and 5 mM, respectively) (see also Table 3). Means ± SE (n = 2)

To test the possibility of DA elimination by bacteria associated with the diatom, the mixture of bacteria separated from the diatom by filtration was inoculated into yeast extract broth medium (MacLeod 1968) with DA (12.5 µg ml^{-1}) and without DA, glucose (5 mM), gluconic acid/gluconolactone (0.5 mM) or various combinations of these. In all instances, bacteria grew over the 6 d incubation period (Fig. 5). In contrast to axenic *Pseudo-nitzschia multiseries* cultures (Table 3), the bacterial mixture separated from the non-axenic diatom culture reduced the DA concentrations by 46 to 72%, depending upon the co-additives (Table 4).

The growth levels and DA production of one of the *Pseudo-nitzschia multiseries* cultures transferred frequently over a period of approximately 10 yr were markedly reduced (Fig. 6). Its growth was enhanced significantly by increasing the silicate levels in the medium, and DA levels also increased in relation to the increased number of cells. While concentrations of DA in the cells were very low to non-existent, peak levels in the culture filtrate, although low, were significant and generally related to the diatom's growth until Day 16 of incubation. By then the DA produced had virtually disappeared from the culture.

Table 3. *Pseudo-nitzschia multiseries* DA (domoic acid) residues (µg ml^{-1} ± SE, n = 2) remaining in axenic cultures after 5 d incubation (see Fig. 4). There were no significant differences between initial and final DA concentrations in either trial (0 h vs. 5 d; paired *t*-tests [Sokal & Rohlf 1995] $p < 0.05$)

Duration of exposure	Additives	
	DA	DA + glucose
0 h	5.04 ± 0.16	4.82 ± 0.10
24 h	4.76 ± 0.00	4.86 ± 0.18
48 h	4.68 ± 0.04	4.66 ± 0.06
70 h	4.82 ± 0.26	4.58 ± 0.14
5 d	4.40 ± 0.08	4.58 ± 0.38

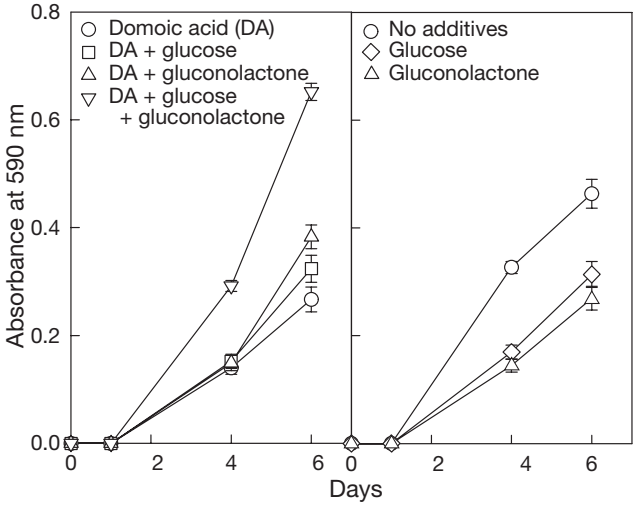

Fig. 5. Growth curves at 20°C of bacterial mixtures from cultures of *Pseudo-nitzschia multiseries* (strain NpH) in yeast extract broth medium (MacLeod 1968) supplemented as indicated. Initial concentrations of DA (domoic acid), glucose and gluconic acid/gluconolactone were 12.5 µg ml^{-1}, 5.0 mM and 0.5 mM, respectively. In mixtures of supplements (DA + glucose, DA + gluconic acid/gluconolactone, DA + glucose + gluconic acid/gluconolactone), these concentrations were maintained. Means ± SE (n = 4)

Thus, although the DA outputs had declined greatly compared to the culture's capacity earlier in its history, they were still appreciable. If however, the measurements had been made only at the end of the incubation period or on a culture grown with the lowest level of silicate when the small, but measurable amount of DA produced had been eliminated, the diatom would have been considered a non-producer.

In the culture grown with the highest levels of silicate (0.4 mM) (Fig. 6), the cells in the stationary phase were distorted and incomplete, conforming to protoplast formation as described by Lewin (1962) and von Stosch (1942). As reported by von Stosch (1942), when the medium was supplemented with manganese (133 µg l^{-1}) this deficiency was overcome and the frustule formation proceeded normally.

Table 4. Bacterial utilization of domoic acid (DA, µg ml^{-1}) at 20°C; initial concentration for all culture additives was 12.5 µg DA ml^{-1}. Confidence intervals (CI) calculated for the final concentrations indicate significant declines in domoic acid following the 6 d incubation ($p < 0.05$, n = 4). Bacteria isolated from non-axenic cultures of *Pseudo-nitzschia multiseries*

Culture additive	DA final conc.	CI
1) DA	3.50	1.43–5.57
2) DA + glucose (5 mM)	4.75	3.95–5.55
3) DA + gluconic acid/ gluconolactone (0.5 mM)	6.75	5.95–7.55
4) DA + glucose (5 mM) + gluconic acid/ gluconolactone (0.5 mM)	5.88	4.00–7.98

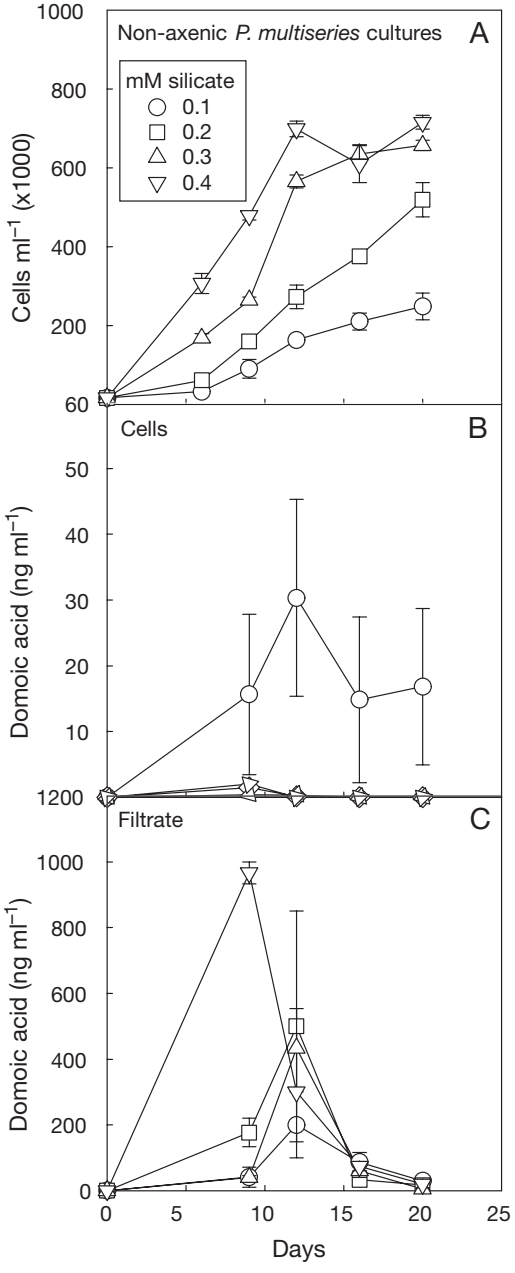

Fig. 6. *Pseudo-nitzschia multiseries*. (A) Growth curves and (B,C) DA (domoic acid) production at 20°C of an aging non-axenic culture. Medium supplemented with silicate as indicated. (B) DA concentrations within diatom cells; (C) DA concentrations in cell-free filtrates prepared from whole cultures. Means ± SE (n = 3)

DISCUSSION

Studies by earlier workers such as Jørgensen (1952) and Lewin (1955, 1957) showed that in media composed of sea water supplemented with N, P, trace metals and vitamins, silicon supply was deficient and diatom growth was directly, immediately and proportionately increased by raising the silicon concentrations. This proved to be true with the medium used in these studies; growth of both axenic and non-axenic *Pseudo-nitzschia multiseries* cultures responded directly and proportionately to a doubling of the silicate concentrations although the non-axenic culture was, by far, the more robust and stable.

Again, as shown by Osada & Stewart (1997), GlcA added to the axenic cultures, although not affecting growth, increased the amount of DA present. In this case (Fig. 1) doubling the silicate alone matched the influence of GlcA alone; when the presence of the GlcA was coupled with increased silicate, however, there was a tripling of the DA concentration. Under the circumstances prevailing here, i.e. where substantial quantities of the sequestering agent gluconic acid/gluconolactone were present after growth had peaked, the resulting enhanced accumulation of DA would appear to be consistent with the earlier hypothesis (Osada & Stewart 1997) that DA may act on behalf of *Pseudo-nitzschia multiseries* as a chemical scavenger.

Although the presence of GlcA in the non-axenic cultures did not appear to affect its growth (Fig. 2), its effect on the production of DA seemed to be the reverse of that observed in the axenic cultures. Somewhat less DA was produced in its presence rather than more. An explanation for this is not readily available and must await further investigation; presumably the effect arises from the interaction of GlcA with certain of the bacteria present.

When these aspects were examined in detail over a much more restricted time period (Fig. 3) it was apparent that, in fact, increases in silicate concentrations resulted in enhanced DA accumulations directly related to increases in diatom cell production. In marked contrast to results with axenic cultures, where GlcA stimulated DA production, the GlcA introduced alone to non-axenic cultures appeared to have retarded DA production in the period between the 18th and 49th hours post-addition time. Glucose added alone resulted in a subsequent significant reduction in DA present at the time the additions were made, but when coupled with GlcA, there was complete disappearance of DA formed at and subsequent to the introduction of the additives. The speed with which the various major changes in DA concentrations occurred, both positive and negative was impressive. These results immediately raised questions as to the reasons for DA disappearance. Previously Stewart et al. (1998) showed bacteria isolated from association with *Pseudo-nitzschia multiseries* had the capacity to grow at the expense of DA. As bacteria mutate rapidly and constantly, the resulting populations that already have this basic capacity would be expected to become ever more capable of successfully exploiting products found in their particular niche. Long-term culture of *P. multiseries* provides just such a niche for enhancement of bacteria initially endowed with the capability of utilizing DA.

Exposure of axenic *Pseudo-nitzschia multiseries* to DA, either alone or with added glucose over a 5 d period did not result in any significant changes in the concentrations of the added DA (Table 3). The added glucose, however, did stimulate the culture's growth, restoring it to the logarithmic phase, demonstrating that the diatom could use the glucose directly as an energy source to overcome impediments to growth. In contrast, when bacteria previously associated with *P. multiseries* were grown in the basal medium of MacLeod (1968) to which 12.5 µg ml^{-1} DA was added, they grew readily utilizing substantial amounts of DA (Table 4).

These results, taken together with those of Subba Rao et al. (1990), Douglas & Bates (1992), Bates et al. (1995), Osada & Stewart (1997), Stewart et al. (1997) and Hagström et al. (2007), suggest a dynamic and competitive set of interactions influenced especially by the confining and artificial constraints imposed by batch culture conditions, operating as follows. DA is produced not at all or only in very small quantities through the diatom's logarithmic growth phase. As its growth slows upon entering the stationary phase through outgrowing one or another nutrient, it experiences nutrient stress and DA production begins or is greatly enhanced. In axenic culture, its production is also stimulated by specific bacteria acting in concert with nutrient stress (Douglas & Bates 1992, Bates et al. 1995). At this point, with production of DA in appreciable quantities, the bacteria associated with the diatom and capable of utilizing moderate concentrations of DA would be enhanced and begin using the DA released to the medium. Thus the amount of DA measured in the culture medium at any one time would be a balance between the diatom's DA production rate enhanced by bacterial stimulation and bacterial consumption of DA. If, however, the nutrient stresses are relieved by the addition of nutrients, e.g. glucose as in this study, or by proline or glutamic acid in axenic cultures (Osada & Stewart 1997), the production of DA ceases or slows. Following the cessation of its production, DA-utilizing bacteria in non-axenic cultures would eliminate amounts already produced. A continuing apparent increase of DA in a non-axenic culture medium indicates vigorous production by the diatom

exceeding the amount used by the bacteria. By contrast, in an aging culture in which the specific bacteria responsible for stimulating DA production have declined or disappeared (Douglas & Bates 1992, Bates et al. 1995), a lessened DA production occurs coupled with a continued or increased rate of bacterial utilization leading to complete elimination similar to the results presented in Fig. 6. It is also logical to suggest that the bacteria isolated earlier from mussels and soft shell clams and capable of growing at the expense of DA (Stewart et al. 1998) might in fact have been bacteria originally associated with *Pseudo-nitzschia multiseries* and thus consumed by the bivalves.

This suggested explanation for the disappearance of the DA is also consistent with reports (Bates 1998) that as *Pseudo-nitzschia multiseries* cultures age, they gradually lose the ability to produce DA. Such an explanation raises a number of questions including the merits of the common practice of rating *P. multiseries* DA production by the number of picograms of toxin produced per cell. The number of cells can alter dramatically and suddenly. Much of the DA is released by the diatom to the aquatic medium, especially in the late logarithmic and stationary phases of growth, where it could become generally available to bacterial attack. Rating the production only or mainly by the picograms per diatom cell apparent at the time of measurement would be misleading as it implies that the number of diatom cells is the sole determinant of DA concentrations in the cultures.

Thus the production of DA is complex involving the diatom, nutrient stress and at least 2 quite different bacterial influences, one stimulatory and the other degradative. The information currently available suggests that a more detailed investigation of the ever-changing interactions of bacteria associated with the diatom is essential to fully understand the dynamics of DA production.

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