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# Lack of allelopathic effects of the domoic acid-producing marine diatom *Pseudo-nitzschia multiseries*

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ABSTRACT: Many *Pseudo-nitzschia* species produce the toxin domoic acid, which accumulates in the food web during blooms, sometimes causing amnesic shellfish poisoning (ASP) in higher trophic levels, including humans. In addition, *Pseudo-nitzschia* species have been reported to form long-lasting monospecific blooms, and a possible explanation for this could be allelopathic effects of the toxin, since domoic acid has been detected in high amounts in the surrounding medium in stationary growth phase. We therefore examined the potential allelopathic effects of *P. multiseries* and its toxin domoic acid. In mixed-batch culture studies of domoic acid-producing *P. multiseries* and the algal test species *Chrysochromulina ericina*, *Heterocapsa triquetra*, *Eutreptiella gymnastica* and *Rhodomonas marina*, no allelopathic effects of *P. multiseries* were found. Different growth results using 2 different strains of *P. multiseries* grown with *C. ericina* were explained by minor differences in pH. Bioassays testing the effect of domoic acid itself on 9 different phytoplankton species, namely *C. ericina*, *E. gymnastica*, *Karenia mikimotoi*, *H. triquetra*, *Heterosigma akashiwo*, *Prorocentrum minimum*, *P. micans*, *Pyramimonas propulsa* and *R. marina* confirmed a lack of allelopathic effects of the toxin. This lack of allelopathic effect of the shellfish-poisoning toxin domoic acid seems to correspond with the results of phytoplankton species causing DSP (diarrheic shellfish poisoning) and PSP (paralytic shellfish poisoning), where it appears that these shellfish-poisoning toxins do not cause allelopathic effects either.

KEY WORDS: Allelopathy · pH · *Pseudo-nitzschia* · Domoic acid · Diatom · Physiology · Ecology · Inorganic carbon · Phytoplankton



## INTRODUCTION

The availability of nutrients is considered one of the main factors controlling growth of phytoplankton, thus species that are successful in competition for growth-limiting nutrients will potentially become relatively more abundant than their competitors. Another factor influencing succession among phytoplankton is allelopathic interactions. Production of allelopathic substances inhibits growth of competitors and, hence, gives competitive advantages to the species producing the allelopathic substances (e.g. Cembella 2003, Gross 2003).

In marine phytoplankton, production of allelopathic substances is known from the prymnesiophytes, where the ichthyotoxic species *Prymnesium parvum* and *Chrysochromulina polylepis* have been shown to exhibit strong allelopathic effects on a number of other organisms (Myklestad et al. 1995, Schmidt & Hansen 2001, Granéli & Johansson 2003). Differences in sensitivity to allelopathic substances have been found among organisms, e.g. the effect of *P. parvum* filtrate on a natural plankton community varied among groups. The effect was greatest on diatoms and nanoflagellates, and smaller on dinoflagellates and cyanobacteria (Fistarol et al. 2003). Similarly, the allelopathic effect of *C. polylepis* differed among the 15 phytoplankton species tested; a growth depressing allelopathic effect was shown for 10 species. However, the

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dinoflagellate *Prorocentrum minimum* was not affected and in the case of 4 other species, a potential allopathic effect could not be differentiated from a potential inhibiting effect of elevated pH (Schmidt & Hansen 2001).

The allelopathic effects of *Chrysochromulina polylepis* and *Prymnesium parvum* may contribute to the ability of the organisms to form monospecific blooms. *P. parvum* is known to form monospecific blooms (Granéli & Johansson 2003) and a major bloom of *C. polylepis* in the pycnocline in Scandinavian waters in 1988 was observed to be almost monospecific; no other autotrophic organisms or heterotrophic grazers were observed during the peak of the bloom. Only dying cells of the dinoflagellate *Ceratium* were detected (Dahl et al. 1989, Nielsen et al. 1990). Another ichthyotoxic organism, the dinoflagellate *Karenia mikimotoi*, is also known to exert allelopathic effects on a number of other phytoplankton organisms (Gentien & Arzul 1990). The ecological significance of this is supported by field observations of a bloom of *K. mikimotoi*, where reduced algal growth of other organisms was found (Arzul et al. 1993); hence, the allelopathic effects of these ichthyotoxic organisms have been firmly established and may have ecological relevance. On the contrary, reports on allelopathic effects of the shellfish-poisoning toxins causing DSP (diarrheic shellfish poisoning), PSP (paralytic shellfish poisoning) and ASP (amnesic shellfish poisoning) are contradictory, and in the case of ASP, not thoroughly studied (Windust 1992, Subba Rao et al. 1995, Sugg & Van Dolah 1999, Tillmann & John 2002).

Marine diatoms of the genera *Pseudo-nitzschia*, *Nitzschia* and *Amphora* have been recognised as producers of domoic acid, the toxin responsible for ASP (e.g. Bates et al. 1989, Maranda et al. 1990, Lundholm et al. 1994, Kotaki et al. 2000). During blooms of toxin-producing diatoms, domoic acid may accumulate in the food web and an array of different organisms such as shellfish may serve as vectors for the toxin (e.g. McGinness et al. 1995, Bargu et al. 2002). Domoic acid may hereby cause harm to seabirds, mammals and humans (e.g. Bates et al. 1989, Work et al. 1993, Scholin et al. 2000). Hence, accumulation of domoic acid could have serious implications for human health, the environment and the economy (e.g. in small communities, which economically depend on shellfish fishery).

*Pseudo-nitzschia* species are regular components of phytoplankton all over the world (Hasle 2002); however, knowledge about the distribution and abundance of toxic *Nitzschia* species in nature is only just emerging (Kotaki et al. 2004). When blooming, *Pseudo-nitzschia* may form dense blooms, $10^6$ to $10^8$ cells $l^{-1}$. The blooms are sometimes reported to be more or less monospecific, accounting for up to 99% of the total phytoplankton (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). Apart from being monospecific, some of these blooms have been recorded to last for a long time. A monospecific bloom of *P. multiseries* in Cardigan Bay, Canada, in 1987 lasted for 2 mo (Bates et al. 1989).

The ecophysiological role of domoic acid has not yet been elucidated (Bates 1998, Cembella 2003). One of the hypotheses presented suggests that domoic acid could function as an allelopathic substance. This could result in a competitive advantage for *Pseudo-nitzschia* and, hence, explain the existence of monospecific blooms. It could also explain the long duration of blooms of *Pseudo-nitzschia* species (Bates et al. 1989, Martin et al. 1990). In culture experiments, domoic acid has been found to be leaking or actively transported into the surrounding medium/water (Maldonado et al. 2002) and the amount of domoic acid in the medium may account for up to 95% of the total amount of domoic acid (Bates et al. 1991, Maldonado et al. 2002). The high levels of domoic acid in the medium support the hypothesis that an allelopathic effect of domoic acid represents a potential explanation for an ecophysiological function of the toxin.

The allelopathy hypothesis for domoic acid has so far only been examined using diatoms as test species. No allelopathic effects have been found on the 2 diatoms *Chaetoceros gracilis* Schütt and *Skeletonema costatum* (Greville) (Brightwell) Sundström (Windust 1992); hence, any potential effect on phytoplankton organisms other than diatoms has never been examined. In addition, allelopathic effects may vary among test organisms as shown for *Prymnesium parvum* and *Chrysochromulina polylepis* (Schmidt & Hansen 2001, Fistarol et al. 2003).

In culture experiments, the potential effect of elevated pH on growth must be taken into consideration (Schmidt & Hansen 2001, Lundholm et al. 2004). During photosynthesis, phytoplankton takes up dissolved inorganic carbon (DIC) and this may cause an elevation of pH. Elevated pH has, thus, been detected in laboratory experiments and in natural environments during algal blooms (e.g. Hinga 1992, 2002, Macedo et al. 2001, Schmidt & Hansen 2001, Hansen 2002). An example of the importance of pH in allelopathic studies is that of *Phaedactylum tricornutum*, for which the claimed allelopathic effect was later shown to be due to a much higher pH tolerance of *P. tricornutum* than of the species it was supposed to affect (D'Elia et al. 1979, Sharp et al. 1979, Goldman et al. 1981, 1982).

The aim of the present study was to examine the potential allelopathic effect of diatoms producing domoic acid. We studied the potential allelopathic effects

of 2 toxic strains of *Pseudo-nitzschia multiseries* on *Chrysochromulina ericina* in mixed-batch culture experiments. *C. ericina* was chosen as it has an upper pH limit for growth close to that for *P. multiseries*. In this way, we tried to avoid that elevated pH would limit growth of either *P. multiseries* or the test algae. Furthermore, we examined the allelopathic effect of 1 of the *P. multiseries* strains on 3 other test species, which have previously been affected by allelopathic substances (Schmidt & Hansen 2001).

We also examined the potential allelopathic effect of pure domoic acid additions to cultures of 9 different phytoplankton species. The test species were selected in order to represent different algal classes and to represent species that occur in natural environments together with *Pseudo-nitzschia multiseries*.

## MATERIALS AND METHODS

**Algal species, clones and culture conditions.** Two different strains of *Pseudo-nitzschia multiseries* (OKPm013-2 and CL-195) were used as representatives of toxin-producing diatoms. Nine clones of different marine phytoplankton species were included as test-organisms for possible allelopathic effects. Information on strain designation, isolation place and date, and source 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 gain a concentration 3 times higher than originally prescribed for the L1-medium. The 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 the irradiance measured using a Li-1000, Li-Cor sensor equipped with a Li-193SA spherical quantum probe.

**Allelopathic studies using mixed-batch cultures of *Pseudo-nitzschia multiseries* and *Chrysochromulina ericina*, *Heterocapsa triquetra*, *Eutreptiella gymnastica* or *Rhodomonas marina*.** Two different *P. multiseries* strains and the other 4 algal species were grown as monoclonal 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. *P. multiseries* (strain OKPm013-2) was grown together with either one of the cultures *C. ericina*, *H. triquetra*, *E. gymnastica* and *R. marina* in mixed-batch culture under similar conditions. In addition, *P. multiseries* (Strain CL-195) was grown in a mixture with *C. ericina*. The experiments with the monoclonal cultures and the mixed cultures of the respective *P. multiseries* strain were carried out simultaneously. The experiments were carried out at an irradiance of 100 µmol photons $m^{-2}$ $s^{-1}$, to ensure that neither growth nor production of domoic acid was light-limited (Bates 1998), and at 15 ± 1°C. The experiments were carried out in triplicate flasks. Prior to all experiments; the strains were acclimated to the experimental conditions for at least 5 d. Inoculates used for the experiments were taken from exponentially growing cultures and initial experimental concentrations were ca. 2000 cells $ml^{-1}$. The pH was adjusted to ca. 8.0 prior to the experiment by addition of 1 mM HCl and afterwards allowed to drift. 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 7.0 and 10.0. Cell concentration and pH were measured daily until stationary growth phase; thereafter less frequently (1 to 5 d intervals). In the experiments with *C. ericina*

Table 1. Information on strain designation, sampling place and time, and source of the strains. SCCAP: Scandinavian Culture Collection of Algae and Protozoa

| Strain | Species | | Sampling place and date | Source |
|---|---|---|---|---|
| K0562 | *Chrysochromulina ericina* | Prymnesiophyceae | Kattegat, March 1995 | SCCAP |
| K-0333 | *Eutreptiella gymnastica* | Euglenophyceae | Kattegat, Denmark, 1988 | SCCAP |
| K-0481 | *Heterocapsa triquetra* | Dinophyceae | Øresund, Denmark, 1988 | SCCAP |
| K-0246 | *Heterosigma akashiwo* | Raphidophyceae | Unknown | SCCAP |
| K-0260 | *Karenia mikimotoi* | Dinophyceae | Oslofjord, Norway, 1977 | SCCAP |
| K-0335 | *Prorocentrum micans* | Dinophyceae | Kattegat, Denmark, 1989 | SCCAP |
| K-0295 | *Prorocentrum minimum* | Dinophyceae | Kattegat, Denmark, 1989 | SCCAP |
| CL-195 | *Pseudo-nitzschia multiseries* | Bacillariophyceae | Deadman's Harbour, Bay of Fundy, Canada, Oct 9, 2002 | Stephen Bates |
| OKPm013-2 | *Pseudo-nitzschia multiseries* | Bacillariophyceae | Okkiray Bay, Iwate Prefecture, Japan, Sep 20, 2001 | Yuichi Kotaki |
| K-0005 | *Pyramimonas propulsa* | Prasinophyceae | Port Phillip Bay, Australia | SCCAP |
| K-0435 | *Rhodomonas marina* | Cryptophyceae | Kattegat, Denmark, 1990 | SCCAP |

and the 2 *P. multiseries* strains, samples for domoic acid concentration in whole culture and in the filtrate were taken 6 to 10 times during the experiments (30 d). 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 measured. After subsampling, the flasks were refilled with L1-medium of the same pH (±0.05) as the respective flask. Samples for enumeration were fixed in Lugol's solution (final concentration 2%) and counted in a Sedgewick rafter chamber. Each counting was based on approximately 400 cells, corresponding to a deviation of ±10% using 95% confidence limits (Utermöhl 1958). Maximum growth rates (μ) were calculated based on the algorithm $\mu = \ln (N_{t_2}/N_{t_1})/(t_2 - t_1)$, where $N_{t_2}$ and $N_{t_1}$ are the cells numbers at times $t_2$ and $t_1$. Three successive cell counts were used in the calculations. Dilutions due to subsampling were adjusted for in calculations of the growth rates. For each subsampling for toxin analyses, 2 samples of 10 ml were taken. One of the samples was immediately frozen and used for determination of toxin content in the whole culture (cells and medium), while the other sample was filtered using a Nuclepore filter (0.8 μm, 25mm) and the 8 ml filtrate stored at –20°C until analysis.

**Allelopathic studies of the effect of domoic acid on 9 different phytoplankton species.** Nine clones of different phytoplankton species: *Chrysochromulina ericina*, *Eutreptiella gymnastica*, *Karenia mikimotoi*, *Heterocapsa triquetra*, *Heterosigma akashiwo*, *Prorocentrum minimum*, *P. micans*, *Pyramimonas propulsa* and *Rhodomonas marina* were used for the experiments. One set of triplicates of each culture was grown with an addition of 23 μg of domoic acid (BioVectra) $ml^{-1}$. A control set of triplicates of each culture was grown without domoic acid. The experiments were carried out in 25 ml glass vials in volumes of 7.5 ml, with initial cell concentrations of 600 to 2000 cells $ml^{-1}$. Cultures were exposed to 100 μmol photons $m^{-2}$ $s^{-1}$ and kept at 15°C. The pH was adjusted to 8.0 prior to the experiment by addition of 1 mM HCl. Subsamples (0.5 ml), after 0 h, 4 h, 1 d, 2 d, 5 d and 9 d, for cell counting were applied directly to a Sedgewick rafter chamber containing 10 μl Lugol's solution and counted immediately. Counting and calculation of growth rates was carried out as outlined above. At the end of the experiment, pH was measured in all vials. In addition, 4 ml of each toxin-containing vial and 4 ml of 1 of the control triplicates was frozen for toxin analyses. Subsamples of the cultures used for inoculation were also tested for the presence of domoic acid.

To examine the extent of potential photo-degradation of domoic acid during the experiment, a set of triplicate glass vials with 20 ml of medium and 20 μg of domoic acid $ml^{-1}$ was simultaneously set up under the same conditions. Subsamples for toxin analyses (2 ml) were taken at 0 h, 4 h, 1 d, 2 d, 5 d and 9 d, simultaneously with those from Expt 2.

**Toxin analyses.** For Expt 1, the whole culture samples were thawed, sonicated under cool conditions (less than 10°C) and centrifuged (8000 × *g*, 10 min). The obtained supernatant and the filtrate were analysed for domoic acid concentration, applying a slightly modified method of Pocklington et al. (1990), in which a Develosil 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 the filtrate content from the content of whole culture (cells plus medium). The toxin content per cell was calculated by dividing the content in the cellular fraction by the cell number.

For Expt 2, the cell-free medium fraction of each sample was applied for domoic acid measurement. The samples were analysed using a slightly modified HPLC-UV method according to Quilliam et al. (1989), in which the same Develosil ODS-5 column as described in Expt 1 and a mobile phase of 10% acetonitrile in phosphate buffer (pH 2.5) were used. The detection limit was 0.1 μg $ml^{-1}$.

## RESULTS

### Allelopathic studies using mixed batch cultures of *Pseudo-nitzschia multiseries* and *Chrysochromulina ericina*, *Heterocapsa triquetra*, *Eutreptiella gymnastica* and *Rhodomonas marina*

The growth of the *Pseudo-nitzschia multiseries* strains was similar when grown as monoclonal batch cultures and as mixed batch cultures (Fig. 1A,C,G,I). The cultures started exponential growth phase after around 1 d and entered stationary growth phase after 4 to 5 d, after which cell numbers slowly declined. When the cultures entered stationary growth phase, the pH in the medium increased to 9.0 and 8.9 in OKPm013-2 and CL-195, respectively (Fig. 1B,D,H,J). pH remained at these levels for around 14 d, whereafter it slowly decreased.

When grown as a monoclonal batch culture, *Chrysochromulina ericina* entered exponential growth phase after 2 to 3 d (Fig. 1E) and attained a maximum growth rate of 0.41 $d^{-1}$. After around 17 to 22 d, it entered stationary growth phase and simultaneously, the pH in the medium reached a maximum of around 9.0 (Fig. 1F).

The fate of *Chrysochromulina ericina* when grown in mixed cultures differed between the 2 *Pseudo-*

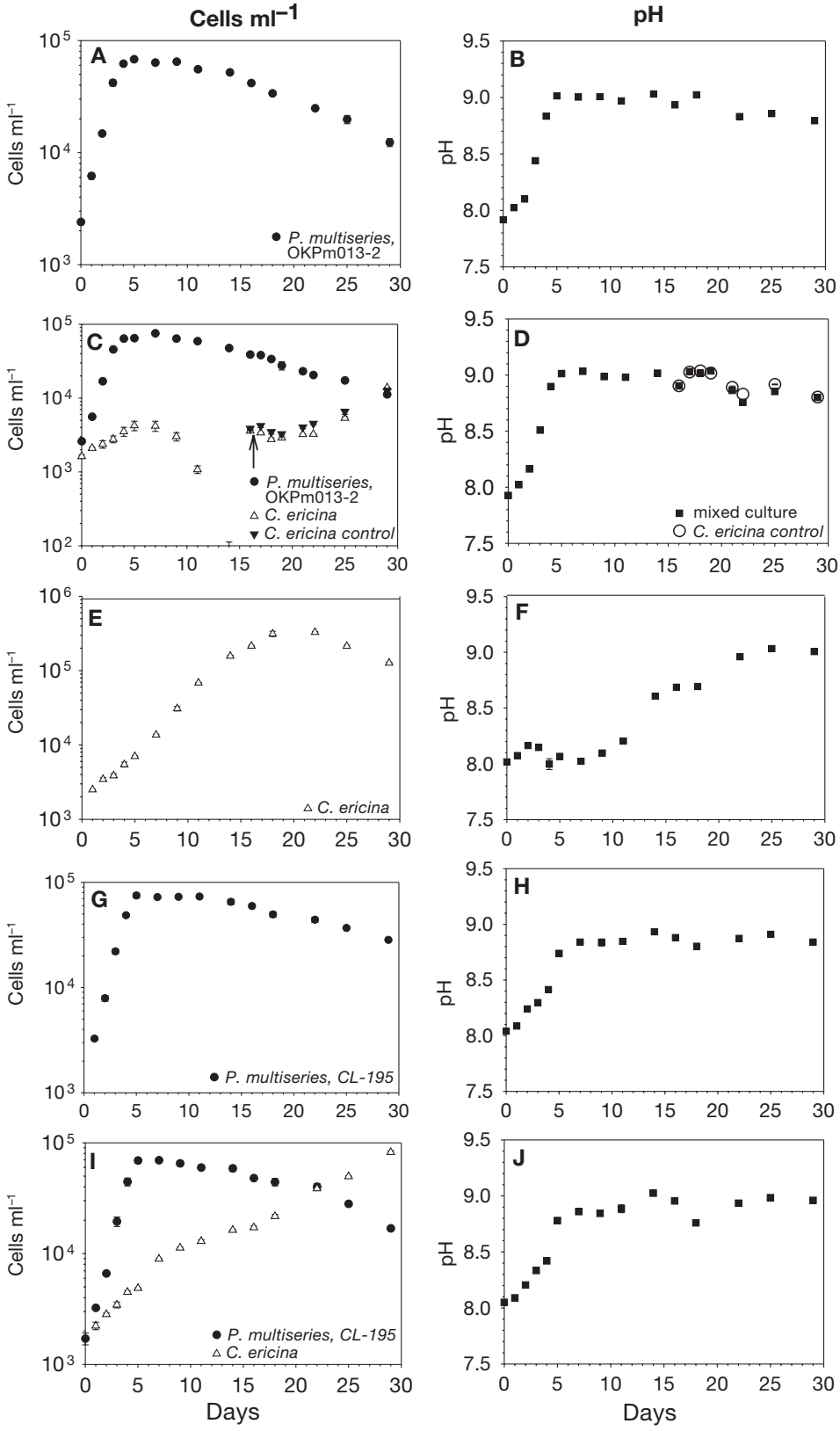


Fig. 1. *Pseudo-nitzschia multiseries*. Changes in cell numbers and pH as a function of time. (A–B) *P. multiseries* strain OKPm013-2. (C–D) Mixed-culture experiment with *P. multiseries* (CL-195) and *Chrysochromulina ericina*. Arrow indicates addition of new *C. ericina* cells. (E–F) *C. ericina*. (G–H) *P. multiseries* strain CL-195. (I–J) Mixed-culture experiment with *P. multiseries* (CL-195) and *C. ericina*. Data points refer to mean ± SE, n = 3

*nitzschia multiseries* clones. Mixed with OKPm013-2, *C. ericina* started exponential growth after 1 to 2 d and reached a maximum growth rate of 0.26 $d^{-1}$ for 3 d. Cell growth then stopped for 2 d and afterwards, cell numbers declined to 75 cells $ml^{-1}$ (Fig. 1C). In order to test the reason for the decline in cell numbers of *C. ericina*, new cells of *C. ericina* were added to the flasks on Day 16, the concentration reaching 3000 cells $ml^{-1}$. Simultaneously, triplicate control flasks with a similar number of *C. ericina* cells $ml^{-1}$ in L-medium of the same pH were set up under the same conditions. The cell numbers remained more or less stable for 3 d (until Day 19) after which they started increasing and reached 10.00 cells $ml^{-1}$ 9 d later, at the end of the experiment (Fig. 1C). The pH increased from 7.9 to 9.0 after 5 d and remained at this level for the next 14 d (until Day 19), after which it declined slightly (Fig. 1D).

In contrast to the experiment with OKPm013-2, *Chrysochromulina ericina* continued to grow throughout the experiment when mixed with CL-195 (Fig. 1I). *C. ericina* attained a maximum growth rate of 0.35 $d^{-1}$; slower (*t*-test: $p < 0.05$) than in the monoclonal culture and cell numbers were never as high as in the monoclonal culture (Fig. 1I). pH increased to 8.8 after 5 d and remained at approximately 8.9 throughout the experiment (Fig. 1J).

In both mixed-culture experiments with *Chrysochromulina ericina*, domoic acid was detected in the medium after 10 to 15 d (around 1 wk after entering stationary growth phase), and the concentrations increased for the remaining 15 d of the experiment (Fig. 2B,D). The concentrations attained were maximally 3 to 12 ng domoic acid $ml^{-1}$ in the medium (Fig. 2). Variations in the concentration of domoic acid detected in the medium were found between monoclonal cultures and mixed cultures; for CL-195, higher concentrations were found in the mixed cultures, whereas in OKPm013-2, the highest concentrations were found in the monoclonal cultures (Fig. 2). In all flasks, however, the amount of domoic acid per cell was more or less similar at 0.85 ± 0.15 pg domoic acid $cell^{-1}$ on Day 29 (data not shown).

In the experiments with the 3 other test species, *Heterocapsa triquetra*, *Eutreptiella gymnastica* and *Rhodomonas marina*, growth of *Pseudo-nitzschia*

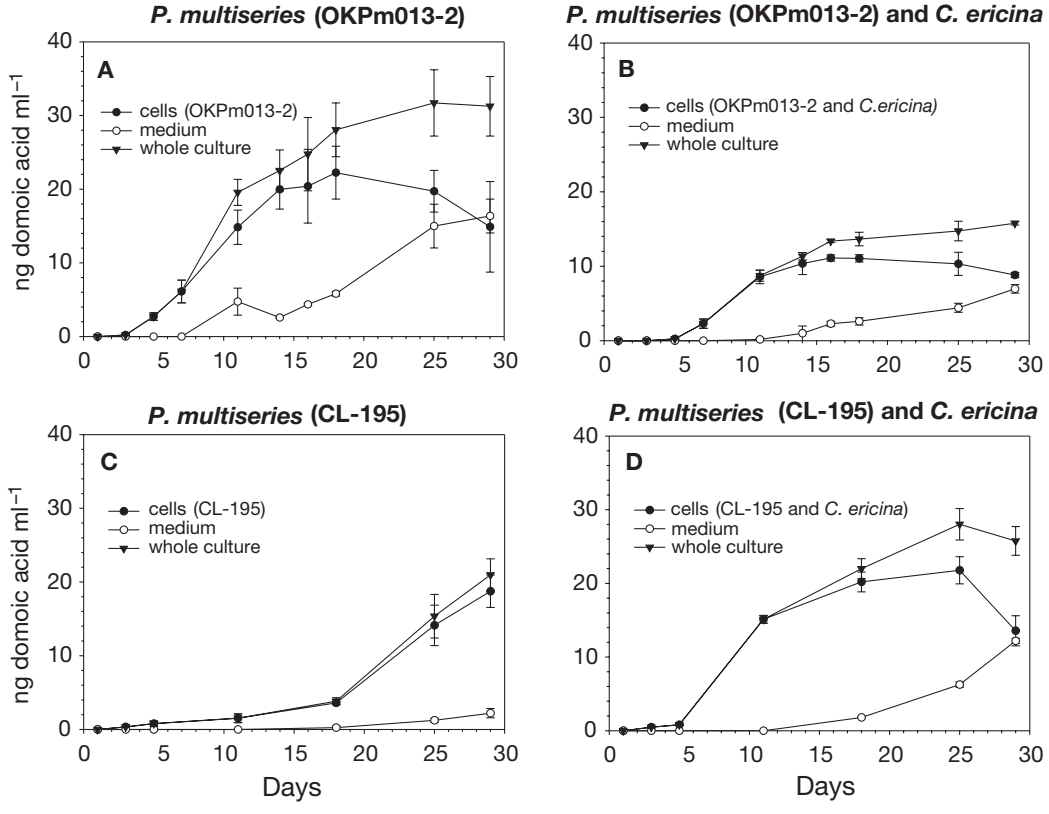


Fig. 2. *Pseudo-nitzschia multiseries*. Content of domoic acid in cell fraction (●), medium fraction (○) and in the whole culture (▼) as a function of time in the monoclonal and mixed culture experiments. (A) *P. multiseries* (OKPm013-2). (B) Mixed culture experiment with *P. multiseries* (OKPm013-2) and *Chrysochromulina ericina*. (C) *P. multiseries* (CL-195). (D) Mixed culture experiment with *P. multiseries* (CL-195) and *C. ericina*. Data points refer to mean ± SE, n = 3

*multiseries* (OKPm013-2) was similar in all 3 mixed experiments until late stationary phase, at which time the cell numbers decreased with a different rate depending on the pH in the experiments. The highest rate of decrease was found in the experiment with *H. triquetra*, where pH reached the highest values (Fig. 3). The fate of the growth of the 3 test species showed similar trends (Fig. 3). Initially, the monocultures of the test species grew exponentially until Day 6 (*H. triquetra* and *E. gymnastica*) or Day 2 (*R. marina*), and attained maximum growth rates of 0.51 ± 0.02, 1.02 ± 0.03 and 1.18 ± 0.08 $d^{-1}$ for *H. triquetra*, *E. gymnastica* and *R. marina*, respectively (Fig. 3A,C,E). The cultures entered stationary growth phase and, more or less simultaneously, pH in the cultures reached a maximum of 9.35, 9.15 and 9.45, respectively (Fig. 3B,D,F).

In the mixed experiments, growth reached maximum growth rates of 0.47 ± 0.05, 1.02 ± 0.05 and 1.08 ± 0.4 $d^{-1}$, respectively for *Heterocapsa triquetra*, *Eutreptiella gymnastica* and *Rhodomonas marina* for the first 2 to 4 d; thereafter, the cultures grew with a lower rate than in the corresponding monocultures (Fig. 3A,C,E). In the mixed experiments, pH increased to values around 9 after 5 to 6 d and only increased or decreased slightly afterwards (Fig. 3B,D,F).

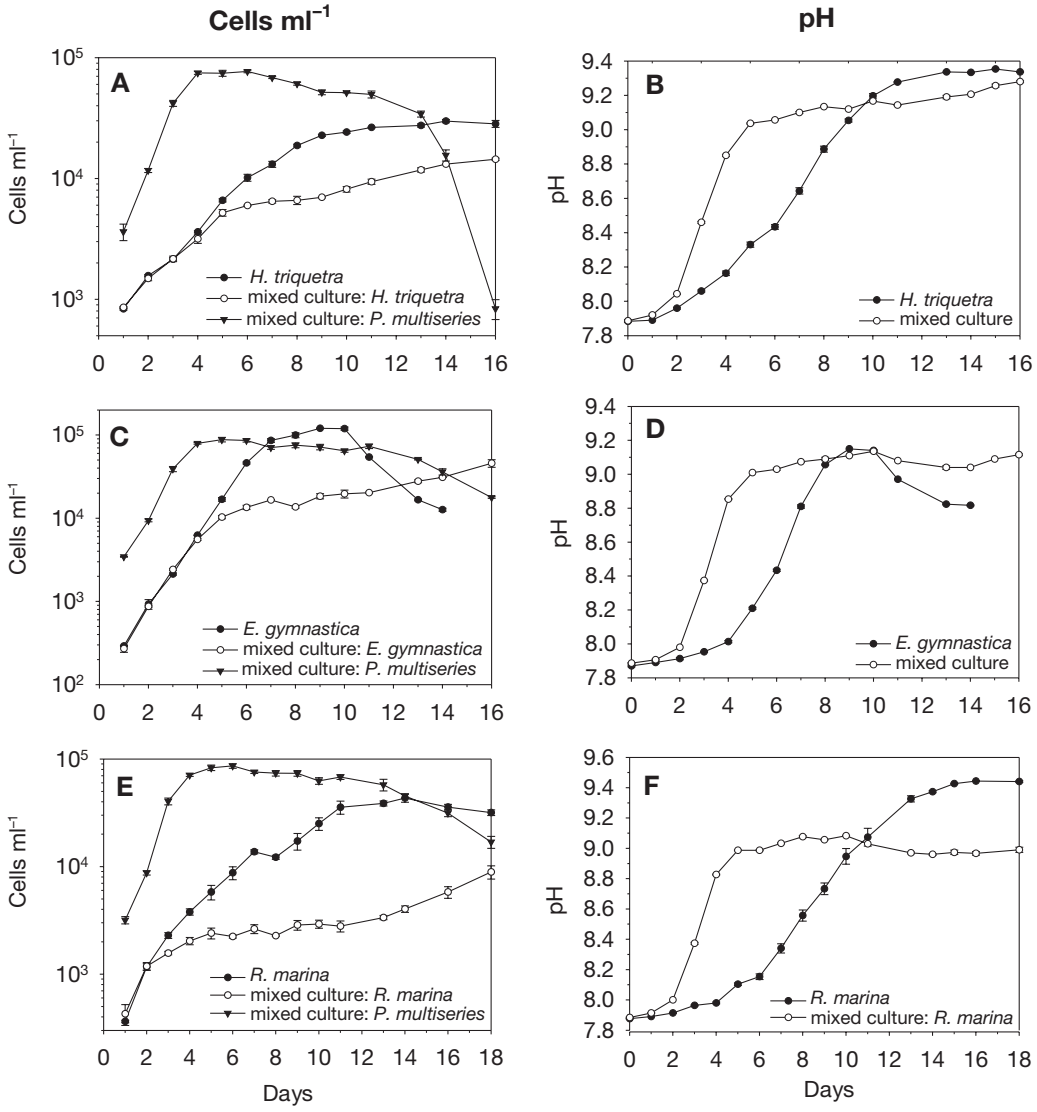


Fig. 3. *Heterocapsa triquetra*, *Eutreptiella gymnastica* and *Rhodomonas marina*. Changes in cell number and pH as a function of time. (A–B) Monoculture and mixed-culture experiment with *Heterocapsa triquetra*. (C–D) Monoculture and mixed-culture experiment with *Eutreptiella gymnastica*. (E–F) Monoculture and mixed-culture experiment with *Rhodomonas marina*. Data points refer to mean ± SE, n = 3

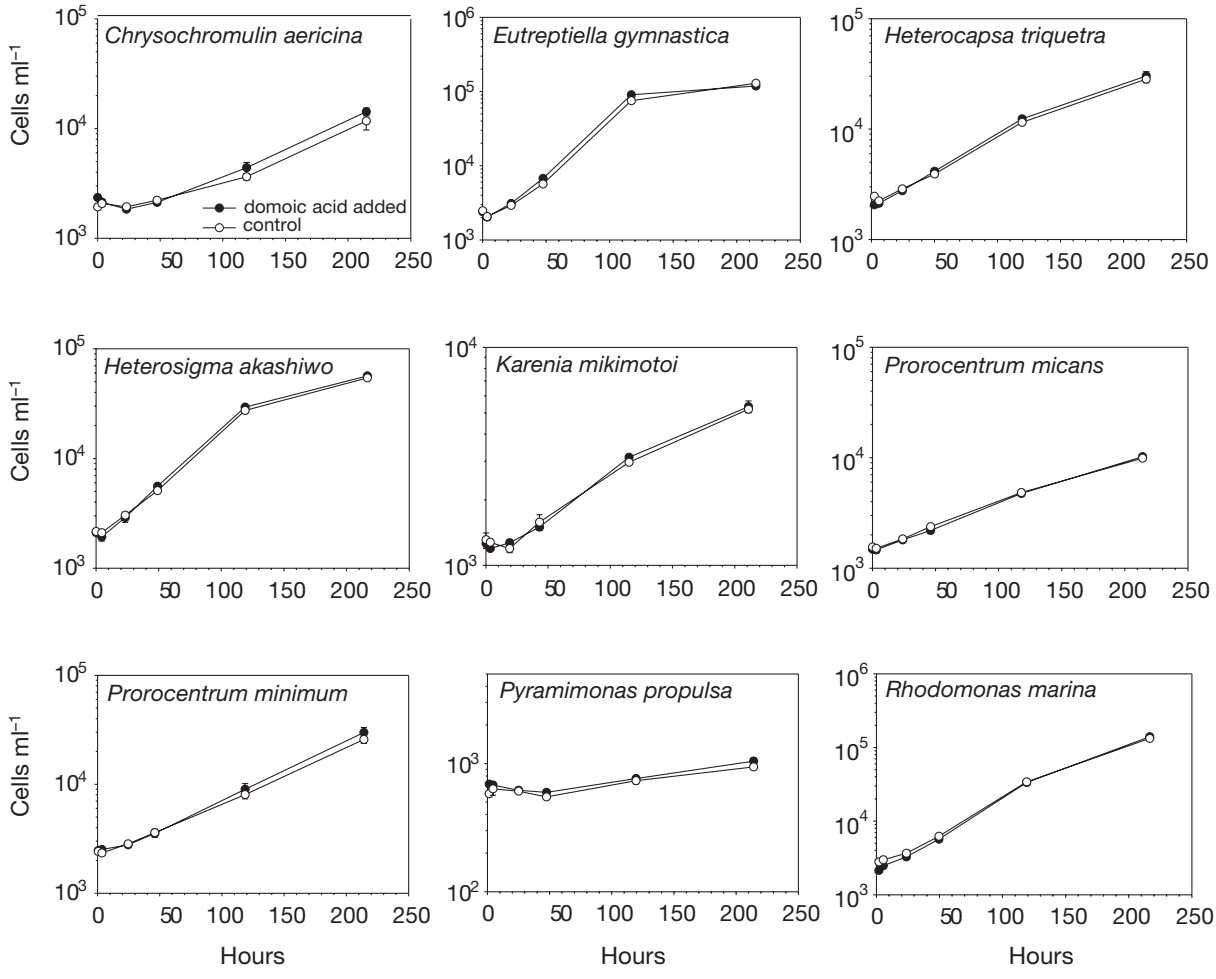


Fig. 4. *Chrysochromulina ericina*, *Eutreptiella gymnastica*, *Heterocapsa triquetra*, *Heterosigma akashiwo*, *Karenia mikimotoi*, *Prorocentrum micans*, *P. minimum*, *Pyramimonas propulsa* and *Rhodomonas marina*. Growth changes as a function of time in batch culture experiments with and without domoic acid. Data points refer to mean ± SE, n = 3

### Allelopathic studies of the effect of domoic acid on 9 different phytoplankton species

The growth of all 9 phytoplankton test species did not differ between vials to which domoic acid had been added and vials without domoic acid (Fig. 4). The exponential growth rates of the test flasks with domoic acid and the control flasks were similar (*t*-test: $p > 0.05$). The pH values measured at the end of the experiment were not significantly different (*t*-test: $p > 0.01$) in the flasks with domoic acid and in the control flasks. Toxin analyses of the cultures at the end of the experiment showed that the amounts of domoic acid detected at the end of the experiment was not significantly different (*t*-test: $p > 0.1$) from the amount detected in the vials with sterile medium (Fig. 5). No domoic acid was detected in the cultures used for inoculation of the test species or in the control flasks. In all flasks, the concentrations of domoic acid were above 20 µg domoic acid ml$^{-1}$ (mean ± SE equals 23.0 ± 0.2 µg domoic acid ml$^{-1}$).

The vials with domoic acid added to sterile L-medium showed that the initial concentration of domoic acid (ca. 23.1 ± 0.4 µg domoic acid ml$^{-1}$) decreased slightly during the experiment. A linear regression showed that the domoic acid concentration decreased to 22.6 ± 0.4 µg domoic acid ml$^{-1}$ after 9 d, corresponding to a decrease of 0.2%.

## DISCUSSION

### Allelopathic effects of 2 strains of *Pseudo-nitzschia multiseries* on *Chrysochromulina ericina*

In the mixed-culture experiments, the fate of the test species *Chrysochromulina ericina* differed between

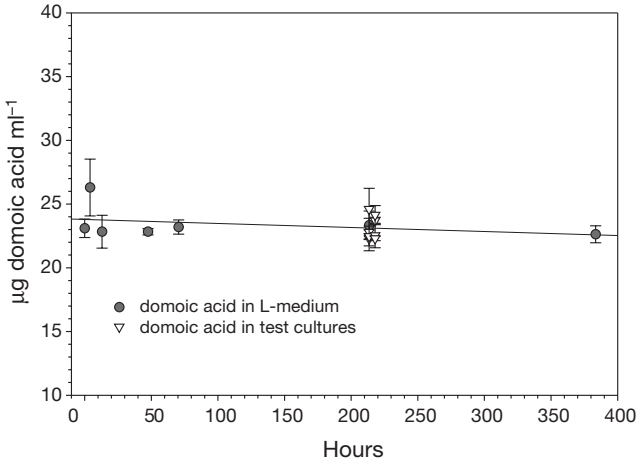


Fig. 5. Domoic acid content in sterile L-medium as a function of time (●) and domoic acid concentration at the end of the experiment of the test species in Expt 2 (▽). Data points refer to mean ± SE, n = 3

the experiments with the 2 different *Pseudo-nitzschia multiseries* strains. In the experiment with *P. multiseries* strain OKPm013-2, the cell numbers of *C. ericina* declined 3 d after *P. multiseries* entered stationary growth phase. This could potentially look like an allelopathic effect of *P. multiseries* on *C. ericina*, as the production of domoic acid is known to begin in the stationary growth phase; however, the amount of domoic acid in the medium was close to the detection limit at the time when cell numbers of *C. ericina* declined. When the growth of *C. ericina* stopped and a few days later declined, pH values simultaneously reached the upper pH limit for growth of *C. ericina* (pH ~9). The upper pH limit for growth of *C. ericina* can be deduced from the monoclonal culture experiment, where *C. ericina* entered stationary growth phase when pH reached a value of ~9. It has previously been shown that in L- or f/2-medium, growth of *P. multiseries* and *C. ericina* is inhibited by elevated pH and not by nutrient limitation (Hansen & Hjorth 2002, Lundholm et al. 2004). The pH value that indicates initiation of stationary growth phase must, therefore, be considered to be the upper pH limit for growth.

In order to ensure that it was elevated pH and not allelopathic substances that caused the decline in cell numbers in the mixed-culture experiment, more *Chrysochromulina ericina* cells were added on Day 16 (pH value ~9). The cell number of *C. ericina* began to increase on Day 20, when pH values declined to around pH 8.8. At the time when the added cells of *C. ericina* began to grow (Day 20), the concentration of domoic acid in the medium had increased to between 1 and 2 ng ml$^{-1}$, and in the remaining part of the experiment, *C. ericina* was growing in spite of an increasing concentration of domoic acid, reaching levels of ca. 3 ng ml$^{-1}$. During this part of the experiment, pH levels were ~8.8. This strongly indicates that the decline in cell numbers was an effect of elevated pH and not inhibition due to the presence of domoic acid or any other allelopathic substance produced by *Pseudo-nitzschia multiseries*.

This conclusion is supported by the mixed-culture experiment with the other *Pseudo-nitzschia multiseries* strain (CL-195) that has an upper pH limit for growth slightly lower than OKPm013-2. In this experiment, growth of *Chrysochromulina ericina* was high for the first 5 d, during which pH values were increasing from 8.0 to between 8.8 and 8.9. During the remaining part of the experiment, the pH values stayed at this level and growth of *C. ericina* continued, but at a lower growth rate, due to the elevated pH. The growth of *C. ericina* was not inhibited, but only reduced, as pH did not reach the upper pH limit for growth of *C. ericina*. The concentration of domoic acid in the medium was higher than in the experiment with OKPm013-2 (Fig. 2B,D); thus, the results of the 2 mixed-culture experiments show that, at the domoic acid concentration levels obtained, *P. multiseries* has no allelopathic effect on *C. ericina*. The experiments also show that no allelopathic effects of *P. multiseries* due to substances other than domoic acid were found.

### Allelopathic effects of *Pseudo-nitzschia multiseries* on *Heterocapsa triquetra*, *Eutreptiella gymnastica* and *Rhodomonas marina*

In the experiments with *Heterocapsa triquetra*, *Eutreptiella gymnastica* and *Rhodomonas marina*, the fate of the 3 test algal species grown mixed with *Pseudo-nitzschia multiseries* was very similar. Initially, when pH values were low, growth in the mixed experiments was similar to that in the monocultures. In the monocultures, maximum growth rates were found until pH reached values of 8.4 (*H. triquetra* and *E. gymnastica*) and 8.2 (*R. marina*). As previously shown, (Lundholm et al. 2004), growth in L-medium is not nutrient-limited; hence, these pH values indicate the pH at which growth of the species becomes limited by elevated pH (Fig. 3B,D,F). When pH in the mixed experiments after 2 to 4 d reached the above-mentioned values, growth was thereafter limited by elevated pH and slower compared to the corresponding monocultures. In the mixed experiments, pH reached values of around 9 after 5 to 6 d and growth of the 3 test species was, hence, limited by pH and attained growth rates of 0.08, 0.10 and 0.16 for *H. triquetra*, *E. gymnastica* and *R. marina*, respectively. When the monocultures reached similar pH values

(Days 9, 10 and 11), the growth rates were not significantly different from the growth rates of the test species in the mixed cultures at similar pH (*t*-test: $p > 0.1$).

The results, hence, clearly show that the limited growth of the test species in the mixed experiments was due to high pH and not caused by allelopathic substances produced by *Pseudo-nitzschia multiseries*.

### Allelopathic effects of domoic acid

Domoic acid was added to the test cultures at a concentration >20 µg $ml^{-1}$; a concentration clearly higher than what would be expected in the field. We tried to calculate the maximum amount of domoic acid that one could potentially find in the field. These calculations were based on the maximum amount of domoic acid detected in the medium in laboratory experiments (3.2 µg domoic acid $ml^{-1}$) at a *Pseudo-nitzschia multiseries* cell concentration of $18 \times 10^7$ cells $l^{-1}$ (Bates et al. 1991). When relating the domoic acid concentration to the maximum cell numbers of *P. multiseries* detected in the field ($1.5 \times 10^7$ cells $l^{-1}$) in a bloom in the Cardigan area in 1987 (Bates et al. 1989), maximum levels of domoic acid of around 0.3 µg $ml^{-1}$ could be expected in water in the field. The amount of domoic acid added in the experiments was more than 60 times higher and should have elicited an allelopathic effect if allelopathic effects of domoic acid were to have an ecological significance.

No significant degradation of domoic acid took place during the experiment in which pure domoic acid was added to sterile L-medium (Fig. 5).

In the experiments with addition of pure domoic acid, the growth rates of the 9 different phytoplankton species in flasks with and without domoic acid were similar (Fig. 4). This shows that no allelopathic effect of domoic acid was found on *Chrysochromulina ericina*, *Eutreptiella gymnastica*, *Karenia mikimotoi*, *Heterocapsa triquetra*, *Heterosigma akashiwo*, *Prorocentrum minimum*, *P. micans* and *Rhodomonas marina*, indicating that no allelopathic effects of domoic acid are to be expected in the field. The experiment with *Pyramimonas propulsa* did not show any allelopathic effect either, but the cells grew very slowly (growth rate: 0.08 $d^{-1}$) and maximally reached cell numbers of 1000 cells $ml^{-1}$. The culture did not attain exponential growth and we can, therefore, only conclude that no acute allelopathic effects of domoic acid were detected.

Similar to our results, Windust (1992) did not detect allelopathic effects on the diatoms *Chaetoceros gracilis* and *Skeletonema costatum* in a bioassay using domoic acid in concentrations from 50 ng $ml^{-1}$ to 50 µg $ml^{-1}$. A study of allelopathic effects of *Pseudo-nitzschia multiseries* on *Proboscia alata* using mixed batch cultures claimed to show allelopathic effects of *P. multiseries* (Subba Rao et al. 1995); however, as the concentrations of domoic acid were never quantified, and the potential inhibiting effect of elevated pH not considered, drawing conclusions on allelopathic effects would be premature. The results in the present paper show how difficult it is to differentiate allelopathic effects from inhibition due to elevated pH, and only a few studies have taken the inhibiting effect of elevated pH into account (Goldman et al. 1981, 1982, Schmidt & Hansen 2001).

As no allelopathic effect of domoic acid was found, monospecific blooms of *Pseudo-nitzschia* observed in the field must be explained by factors other than allelopathic effects of domoic acid. In addition, no allelopathic effects of *P. multiseries* due to substances other than domoic acid were found in the mixed-culture experiments with *Chrysochromulina ericina*, *Heterocapsa triquetra*, *Eutreptiella gymnastica* or *Rhodomonas marina*; hence, we do not consider allelopathic effects of *Pseudo-nitzschia* species a likely explanation for formation of monospecific blooms of *Pseudo-nitzschia*.

### Allelopathic effects of shellfish toxins

The fact that no allelopathic effects of the shellfish-poisoning phycotoxin, domoic acid, were found poses the question: What about the other shellfish-poisoning toxins that also cause effects on organisms at higher trophic levels? Is it a general phenomenon that the toxic effect of shellfish toxins seems to be of no significance for an allelopathic function of the toxin for the organism producing the toxin? Apart from ASP, shellfish toxins associated with DSP, PSP, NSP (neurotoxic shellfish poisoning), and AZP (azaspiracid shellfish poisoning) are known (Landsberg 2002).

DSP is caused by ocadaic acid and dinophysistoxins (DTX-1), potent phosphatase inhibitors produced by marine dinoflagellates of the genera *Dinophysis* and *Prorocentrum* (Dickey et al. 1990, Cembella 2003). In laboratory experiments, ocadaic acid and DTX-1 have been found to inhibit growth of 6 non-DSP-producing organisms (Uchida 1977, Windust et al. 1996). In addition, ocadaic acid has been found to be released to the surrounding water (Rausch de Trauenberg & Morlaix 1995) and could, hence, potentially cause allelopathic effects. The toxin concentrations causing the growth inhibition are, however, much higher (>0.5 µM toxin; Windust et al. 1996) than the concentrations maximally observed in culture medium (~0.005 to 0.01 µM; Rausch de Trauenberg & Morlaix 1995, Sugg & Van Dolah 1999). In addition, Sugg & Van Dolah (1999)

showed that the growth-inhibiting factor eluted in a different fraction than ocadaic acid when *Prorocentrum lima*-conditioned medium was fractionated by HPLC (Sugg & Van Dolah 1999). Sugg & Van Dolah (1999), therefore, concluded that the growth-inhibiting effect on phytoplankton was mainly due to a substance other than ocadaic acid and its precursors. In addition, they stated that the growth-inhibiting substance was not in the ocadaic acid toxin family, as the growth-inhibitory and the protein-phosphatase-inhibitory activity did not co-elute. They did not, however, reject the possibility that ocadaic acid could function as a deterrent of some sort, e.g. a grazing deterrent (Sugg & Van Dolah 1999); hence, the hypothesis that ocadaic acid could act as an allelopathic substance has been difficult to prove.

PSP is caused by saxitoxins and their derivatives, which are produced by a number of dinoflagellates such as *Alexandrium* and *Pyrodinium*. An allelopathic effect of *Alexandrium* species was shown by Blanco & Campos (1988), who found that the PSP-producing *A. lusitanicum* reduced growth of other phytoplankton species, whereas the non-PSP-producing *A. tamarense* did not. They, therefore, suggested that the PSP toxins caused the allelopathic effect. Since then, the studies of allelopathic effects of *Alexandrium* species have been contradictory. Other studies have found that the allelopathic effect varied among strains of *Alexandrium* and did not always correlate with production of PSP-toxins (Arzul et al. 1999, Tillmann & John 2002), indicating that PSP toxins have no allelopathic effect. In addition, the allelopathic effects have been shown to be due to the production of extracellular substances, which have yet to be identified (Arzul et al. 1999, Tillmann & John 2002). A lack of allelopathic effects of the PSP-toxins probably also explains a similar discrepancy in effects of *Alexandrium* on other organisms such as grazing protists and copepods (Huntley et al. 1986, Hansen 1989, Turriff et al. 1995, Bagøien et al. 1996, Teegarden & Cembella 1996, Dutz 1998, Teegarden 1999, see Tillmann & John 2002).

The brevetoxins causing NSP are produced by, for example, the dinoflagellate *Karenia brevis*. *K. brevis* has been reported to cause allelopathic effects on phytoplankton species (Freeberg et al. 1979), but what actually caused the growth inhibition is unclear. No studies on potential allelopathic effects of the azaspirazids causing AZP have to our knowledge been conducted.

To conclude, a picture is emerging showing that the shellfish toxins responsible for at least DSP, PSP and ASP do not apparently cause direct allelopathic effects. In other words, the toxic effect on the higher trophic levels seems not to be related to toxic effects on competing organisms; thus, the allelopathic effects by DSP- and PSP-producing organisms seem to be caused by substances other than the shellfish toxins. Further research is needed to establish the source of the allelopathic effects, which are caused by some of these organisms.

## CONCLUSION

No allelopathic effects due to the shellfish poisoning toxin, domoic acid, were found on a diverse array of autotrophic phytoplankton organisms. In addition, the lack of allelopathic effects of substances other than domoic acid indicate that *Pseudo-nitzschia* do not exert allelopathic effects. The lack of allelopathic effects of the shellfish-poisoning toxin which causes ASP is similar to DSP and PSP, where the shellfish toxins apparently do not cause allelopathic effects either. In fact, the allelopathic effects caused by DSP- and PSP-producing organisms seem to be attributable to other substances.

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