Toxic effects of *Alexandrium* spp. on heterotrophic dinoflagellates: an allelochemical defence mechanism independent of PSP-toxin content

**Urban Tillmann**, Uwe John

Alfred Wegener-Institute for Polar and Marine Research, Am Handelshafen 12, 27570 Bremerhaven, Germany

ABSTRACT: Sixteen strains of the red tide dinoflagellate *Alexandrium* spp. were tested for their short-term effects on the heterotrophic dinoflagellates *Oblea rotunda* and *Oxyrrhis marina*. Some *Alexandrium* strains, but not others, caused loss of motility and cell lysis of the heterotrophic dinoflagellates. A live counting procedure using *O. marina* was developed to quantify these toxic effects, which were compared with HPLC estimates of paralytic shellfish poisoning (PSP) toxin content. Within 5 strains, for which PSP toxins could be verified, both non-effective as well as effective strains were present and the same holds true for the other strains without detectable PSP toxins. This clearly indicates that the toxic effects are not due to PSP toxins. The observed effects are caused by extracellular substances, because *O. marina* did not ingest *Alexandrium* spp. and lytic effects are also found in cell-free culture medium. The immobilisation effect was strongly dependent on the *Alexandrium* spp. cell concentration. EC_{50} concentration (*Alexandrium* spp. cell concentration which caused 50% immobilisation after 1 h exposure), as estimated for 5 effective strains, ranged from 2.1 × 10^{3} cells ml^{-1} down to 0.6 × 10^{3} cells ml^{-1}. A quantitative comparison experiment showed that both heterotrophic dinoflagellate species are immobilised, with the thecate species (*O. rotunda*) being even more affected compared to the athecate *O. marina*.

KEY WORDS: *Alexandrium* · Heterotrophic dinoflagellates · Allelochemicals · Lytic activity · PSP toxins

INTRODUCTION

Dinoflagellates of the genus *Alexandrium* are responsible for the occurrence of paralytic shellfish poisoning (PSP), a neurological affliction that has caused human illness for centuries (Prakash et al. 1971). As a consequence, there have been a large number of investigations spanning morphology, biochemistry, toxicity, genetics, bloom dynamics and evolution, making the genus *Alexandrium* arguably the best characterised harmful algal species known (Cembella 1998, Scholin 1998). Toxicity research has focused on the phycotoxin saxitoxin and its more than 20 naturally occurring derivates (Shimizu 1996). Their specific significance as compounds of cellular metabolism, however, is poorly understood. The frequent co-occurrences of HAB blooms and shellfish-borne toxicity or fish kills have fostered the notion that such blooms develop because the bloom species are phycotoxic. In this regard, the idea that PSP toxin production in *Alexandrium* spp. might be an adaptation for grazer defence has received great attention, as reduced or inhibited grazing is generally believed to be an important factor in harmful bloom dynamics (Fiedler 1982, Smayda 1997). Indeed, Shaw et al. (1997) recently showed that a mixture of pure PSP toxins, dissolved in seawater, behaved as a feeding deterrent without lethal effects to the copepod *Tigriopus californicus*. Recent results of Teegarden (1999) suggest that cells containing PSP toxins can be discerned by copepod grazers prior to ingestion and thus can be rejected without mortal damage. This is, however, in contrast to earlier observations, which showed that PSP-toxicity of
food items was apparently not a factor in food selection for the copepod species *Acartia tonsa* and *Eurytemora herdmani* (Teegarden & Cembella 1996). Huntley et al. (1986) found that some dinoflagellate species caused inhibition of grazing in the copepods *Calanus pacificus* and *Paracalanus parvus*. Three of the rejected dinoflagellate species produced known neurotoxins. However, other species not known to be toxic also were rejected and some species, which produce PSP toxins, were not rejected as food. Based on that evidence, they hypothesized that substances not associated with PSP toxins were inhibiting grazing. From experiments using filtrates of the rejected algal species they further concluded that potential inhibitory substances were extracellular exudates. Other studies strengthened the view that grazing interaction between *Alexandrium* spp. and copepods are highly variable and can vary greatly among zooplankton species. PSP-toxic strains of *Alexandrium* spp. were rejected (Turriff et al. 1995) or ingested at lower rates in response to increasing toxicity (Ives 1985, 1987). Enhanced mortality upon exposure to *Alexandrium* spp. (Bagoien et al. 1996), reasonable high ingestion rates of toxic *Alexandrium* spp. with no apparent physiological effects (Teegarden & Cembella 1996) as well as negative long term effects such as reduction of fecundity or lower hatching success (Dutz 1998, Frangopulos et al. 2000) have been observed.

Although there are many papers on copepod grazing studies, there are only a few dealing with grazing interactions of protozoa and *Alexandrium* spp. Ciliates (Prakash 1963, Watras et al. 1985) and heterotrophic dinoflagellates (Carreto et al. 1986, Sampayo 1998, Matsuyama et al. 1999) can be found at high concentrations during field blooms of PSP-producing dinoflagellates. Subsequent laboratory growth experiments using protozoan cultures yielded mixed results; the tintinnid *Favella ehrenbergii* is able to grow on *A. tamarense* at low concentrations (Stoecker et al. 1981), but the growth response of *F. ehrenbergii* on *A. tamarense* is clone-specific (Hansen 1989). At higher *Alexandrium* spp. concentrations, however, toxic effects became apparent, which, based on indirect evidence, were attributed to PSP toxins (Hansen et al. 1992). Matsuoka et al. (2000) conducted laboratory experiments using the heterotrophic dinoflagellate *Polykrikos kofoidii* and noted widely varying feeding and growth responses to various strains of *Alexandrium* spp.: some strains, including both PSP-toxic and non-toxic strains, supported rapid growth, whereas others rapidly caused cell death of the heterotrophic dinoflagellate. In a subsequent paper, ingestion of PSP-toxic *Alexandrium* spp. cells was thought to be the cause of *P. kofoidii* cell lysis (Cho & Matsuoka 2000).

In order to clarify the role of PSP toxins or potentially of other substances in *Alexandrium* spp./protozoa interactions, the present paper analyses the effects of *Alexandrium* spp. on heterotrophic dinoflagellates using a wide range of different *Alexandrium* spp. strains, for which PSP toxins were simultaneously analysed.

**MATERIALS AND METHODS**

*Alexandrium* cultures. Table 1 gives an overview of the different strains of *Alexandrium* spp. tested in the present study.

All strains were grown non-axenically with IMR 1/2 medium (Eppley et al. 1967), supplemented with selenite (Dahl et al. 1989), or K-medium (Keller et al. 1987) (see Table 1) under controlled conditions at 15°C with

<table>
<thead>
<tr>
<th><em>Alexandrium</em> species</th>
<th>Strain no.</th>
<th>Origin (yr), collector</th>
<th>Culture medium</th>
<th>Doubling time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. affine</em></td>
<td>CCMP112</td>
<td>Ria de Vigo, Spain (1985); I. Bravo</td>
<td>K</td>
<td>63</td>
</tr>
<tr>
<td><em>A. catenella</em></td>
<td>BAH255</td>
<td>Spain; M. Delgado</td>
<td>IMR 1/2</td>
<td>40</td>
</tr>
<tr>
<td><em>A. lusitanicum</em></td>
<td>BAH91</td>
<td>Laguna de Obidos, Portugal (1996)</td>
<td>K</td>
<td>77</td>
</tr>
<tr>
<td><em>A. minutum</em></td>
<td>AL1T</td>
<td>Mediterranean, Gulf of Trieste; A. Beran</td>
<td>K</td>
<td>41</td>
</tr>
<tr>
<td><em>A. minutum</em></td>
<td>AL3T</td>
<td>Mediterranean, Gulf of Trieste; A. Beran</td>
<td>K</td>
<td>32</td>
</tr>
<tr>
<td><em>A. ostenfeldii</em></td>
<td>BAH136</td>
<td>New Zealand, Timaru (1992); N. Berkett</td>
<td>K</td>
<td>150</td>
</tr>
<tr>
<td><em>A. ostenfeldii</em></td>
<td>k-0324</td>
<td>Limfjord, Denmark</td>
<td>K</td>
<td>82</td>
</tr>
<tr>
<td><em>A. ostenfeldii</em></td>
<td>k-0287</td>
<td>Limfjord, Denmark</td>
<td>IMR 1/2</td>
<td>95</td>
</tr>
<tr>
<td><em>A. pseudogonyaulax</em></td>
<td>AP2T</td>
<td>Mediterranean, Gulf of Trieste; A. Beran</td>
<td>K</td>
<td>75</td>
</tr>
<tr>
<td><em>A. tamarense</em></td>
<td>GTFP01</td>
<td>Perch Pond, Falmouth, MA (1984); D. Kulis</td>
<td>IMR 1/2</td>
<td>50</td>
</tr>
<tr>
<td><em>A. tamarense</em></td>
<td>SZNB01</td>
<td>Mediterranean, Gulf of Naples (1999); M. Montresor</td>
<td>IMR 1/2</td>
<td>58</td>
</tr>
<tr>
<td><em>A. tamarense</em></td>
<td>BAH181</td>
<td>Orkney Island (1997); M. Elbrächter</td>
<td>IMR 1/2</td>
<td>40</td>
</tr>
<tr>
<td><em>A. tamarense</em></td>
<td>CCMP115</td>
<td>Tamar estuary, UK (1957); I. Adams</td>
<td>IMR 1/2</td>
<td>59</td>
</tr>
<tr>
<td><em>A. tamarense</em></td>
<td>31/9</td>
<td>Southern England; W. Higman</td>
<td>IMR 1/2</td>
<td>44</td>
</tr>
<tr>
<td><em>A. tamarense</em></td>
<td>GTLI21</td>
<td>Mud Creek, Long Island (1981); D. Anderson</td>
<td>IMR 1/2</td>
<td>55</td>
</tr>
<tr>
<td><em>A. taylori</em></td>
<td>AYT1</td>
<td>Mediterranean, Lagoon of Marano; A. Beran</td>
<td>K</td>
<td>130</td>
</tr>
</tbody>
</table>
artificial light at 100 µE m\(^{-2}\) s\(^{-1}\) and a light:dark cycle of 16:8 h. Growth of algal cultures was followed by cell counts using the Utermöhl-technique to ensure that cells taken for experiments or for filtration for toxin analysis were in exponential growth phase. The corresponding doubling times of all strains are listed in Table 1.

**Cultures of heterotrophic dinoflagellates.** The heterotrophic dinoflagellate *Oblea rotunda* was isolated by capillary isolation from a brackish pond near Büsum (Germany) in 1993. Stock cultures held in multiwell plates or a 100 ml flask were fed with the raphidophyte *Fibrocapsa japonica*, which recently was shown to sustain rapid growth of *O. rotunda* (Tillmann & Reckermann 2002). Cultures were transferred about once a week to fresh medium containing late exponential *F. japonica* cells. *Oxyrrhis marina* (Göttingen culture collection, Strain B21.89) was grown with *Dunaliella* sp. as food algae. Stock cultures of both heterotrophic dinoflagellates were maintained at 20°C and natural light. Cultures of heterotrophic dinoflagellates used in the experiments were grown to high densities until they became almost deprived of food.

**PSP toxin analysis.** For toxin analysis, 30 to 50 ml of exponentially growing *Alexandrium* culture were gently filtered through 0.2 µm polycarbonate membrane filters (Nuclepore). The sample preparation protocol by Hummert et al. (1997) was applied for extraction. Briefly, 1 ml of acetic acid (0.03 N) was added to the algal filter, which was homogenized for 2 min using a Sonopuls HD 70 ultrasonic probe (Bandelin), and centrifuged for 10 min (2980 \( \times \) g). The supernatant was passed through a 0.45 µm, 25 mm diameter PTFE filter (No. H250.1, Carl Roth) and subsequently injected into the LC equipped with a fluorescence detector. For determination of N-sulfolcarbamoyltoxins 150 µl of the acetic acid extract were mixed with 37 µl of 1.0 N hydrochloric acid and heated for 15 min at 90°C. After cooling down to room temperature the mixture was neutralized with 75 µl of 1.0 N sodium acetate. N-sulfolcarbamoyltoxins concentrations were calculated by the difference (increase) of the peak areas to those obtained by acetic acid extract. The toxin analyses were performed by automated HPLC applying ion-pair chromatographic separation, post-column oxidation with periodic acid, and fluorescence detection, based on the method of Thiebert et al. (1991) with modifications as described detailed in Hummert et al. (1997) and Yu et al. (1998). LC was performed with an SIL-10A intelligent autosampler, an LC-10ATvp intelligent pump, an SCL-10Avp system controller, a 1 ml CRX400 post-column reaction unit (Pickering Laboratories), two LC-9A pumps for delivery of post-column reaction solutions, and an RF-10Axl fluorescence detector (all Shimadzu). Data were analyzed with Class-vp 5.3 software from Shimadzu. Saxitoxin (STX), neosaxitoxin (NEO) and gonyautoxins (GTXs) as PSP toxin standards were purchased from the National Research Council, Marine Analytical Chemistry Standards Program (NRC-PSP-1B), Halifax, Nova Scotia, Canada. The standard solutions of GTX2 and GTX3 contained dcGTX2 and dcGTX3 as minor components, but the exact content of these toxins was not given. dcSTX was provided by the European Commission (BCR, The Community Bureau of Reference, Brussels), for use as a standard during an intercalibration exercise from 1995 to 1996. All chemicals used were analytical grade.

**Effects of *Alexandrium* spp.** Initial experiments were carried out in order to qualitatively monitor the effects of different strains of *Alexandrium* spp. on *Oblea rotunda*. *O. rotunda* mixed with *Alexandrium* spp. (3000 cells ml\(^{-1}\) final concentration) were injected under a stereomicroscope after 1 and 24 h of exposure. This visual inspection, however, was unsatisfactory since most *Alexandrium* spp. strains and *O. rotunda* are very similar in size and swimming behaviour, and making a clear differentiation between both cell types extremely difficult. In order to quantitatively study the effects of different strains of *Alexandrium* spp., the following experimental procedure was developed. We used the heterotrophic dinoflagellate *Oxyrrhis marina*, which can be easily distinguished from *Alexandrium* spp. by size, cell shape, and swimming pattern, even at low magnification under a dark-field stereomicroscope. Aliquots of 0.5 ml of a dense *O. marina* culture (3000 to 6000 cells ml\(^{-1}\)) were mixed with 1 ml of sample. After a defined exposure time at ambient light and room temperature, the number of moving *O. marina* cells was estimated using a droplet-counting procedure: 100 µl of cell suspension was separated into 25 to 30 small droplets in a petri dish and the number of swimming *O. marina* cells was counted under a stereo microscope. The original concentration of *O. marina* initially resulted in a total of 100 to 200 moving cells, which could be counted within 3 to 5 min. Reproducibility of this counting procedure as checked in a preliminary experiment using filtered seawater as sample was satisfactory; repeated subsample counts of the same sample yielded a SD of 5.9% (115.3 ± 6.8; n = 10) and triplicate subsample counts of 10 different samples one after the other pipetted in different wells yielded a SD of 8.2% (115.2 ± 9.4; n = 10). In addition to the live counting procedure, subsamples were fixed with lugol’s solution and counted with an inverted microscope. After exposure to some of the *Alexandrium* spp. strains, *O. marina* lost its motility, thereafter became rounded, and then increased in size due to swelling and finally lysed. In the fixed samples, a cell was only scored if the normal cell shape was still visible.
Based on this counting procedure the following experiment was carried out: In multidish wells, 1.5 ml of *Oxyrrhis marina* cells were mixed with 3 ml of *Alexandrium tamarense* (Strain 31/9), resulting in a final concentration of \( 3.9 \times 10^5 \) *A. tamarense* ml\(^{-1}\). One-quarter ml was fixed for cell counts, and 0.1 ml was counted using the droplet method outlined above at time 0, 20, 40, 60, 120 and 180 min. A control experiment was carried out in a similar way using IMR 1/2 medium instead of the *A. tamarense* culture. The experiment was carried out in triplicate.

Based on the results from these experiments, the following procedure was used in all subsequent experiments: 1ml of algal sample was mixed to 0.5 ml of *Oxyrrhis marina* culture. IMR 1/2 medium was used as a control. After 1 h of exposure at ambient light and room temperature, the number of moving *O. marina* was counted using the droplet method. Results are always expressed as percentage of moving cells compared to the control. After 3 h of exposure, 0.25 ml were removed into small settling-chambers (diameter 10 mm), fixed with lugol’s solution and counted under an inverted microscope. To compare different *Alexandrium* spp. strains, the effects on *O. marina* were tested using exponentially growing algal cultures when they reached the control. After 3 h of exposure, 0.25 ml were removed into small settling-chambers (diameter 10 mm), fixed with lugol’s solution and counted under an inverted microscope.

Dependence of cell concentration. For some strains of *Alexandrium*, algal cell concentration-dependence of the immobilisation effect on *Oxyrrhis marina* (droplet counts after 1 h incubation) was analysed. Therefore, exponentially growing cultures of *Alexandrium* spp. were tested as described above at several different points of the growth curve representing different cell concentrations (note that algae taken at different stages of the growth curve may differ in the per cell activity potential). Percentages of immobilisation were transformed to probits (Hewlett & Placklet 1979). EC\(_{50}\) values, defined as the amount of algae needed to induce 50% immobilisation after 1 h of incubation, were calculated using linear regression analysis of probits against log-transformed *Alexandrium* spp. concentrations.

Test of cell-free filtrate. In some experiments, the effect of culture filtrate of *Alexandrium* spp. on *Oxyrrhis marina* was tested. A few ml of exponentially growing *Alexandrium* spp. culture (same cell concentration as used for parallel whole cell incubations) were gently filtered using either 10 \( \mu \)m gauze or 0.2 \( \mu \)m membrane filters (Sartorius Minisart filters). The filtrate was added to *O. marina* and the samples analysed as described before.

Quantitative comparison between *Oxyrrhis marina* and *Oblea rotunda*. One experiment was conducted in order to quantitatively compare the immobilisation effect on *O. marina* and *O. rotunda*. A quantitative estimate using *O. rotunda* was possible using *Alexandrium tamarense* Strain 31/9, because its dark pigmentation and slow swimming speed allowed for a tedious but reliable application of the life counting droplet method. One ml of a dense *O. rotunda* culture (ca. 500 cells ml\(^{-1}\)) was mixed with 1 ml of *A. tamarense* 31/9 (final algal concentration in the mixture: 3200 cells ml\(^{-1}\)) or 1 ml of 0.2 \( \mu \)m filtrate, respectively. After 1 h of exposure at ambient light and room temperature, 500 µl of the mixture was separated into 25 to 30 small droplets in a petri dish and the number of swimming *O. rotundata* was counted under a stereomicroscope. For *O. marina*, 0.5 ml (ca. 5000 cells ml\(^{-1}\)) were mixed with 0.75 ml of *A. tamarense* culture (Strain 31/9) and 0.25 ml IMR 1/2 medium (resulting as above in a likewise final algal concentration of 3200 ml\(^{-1}\)) or 0.75 ml of filtrate and 0.25 ml IMR 1/2 medium. The experiment then proceeded as described above. For both heterotrophic dinoflagellates a control experiment was carried out in a similar way using IMR 1/2 medium instead of the *A. tamarense* culture. All treatments were done in triplicate.

**RESULTS**

**Toxin analysis**

PSP toxins could be detected in 5 of the 16 *Alexandrium* species/strains (Table 2). All these strains produced only small amounts of PSP toxins, with total

<table>
<thead>
<tr>
<th><em>Alexandrium</em> spp. strain</th>
<th>PSP-toxin content (mol cell(^{-1}))</th>
<th>GTX1</th>
<th>GTX2</th>
<th>GTX3</th>
<th>GTX4</th>
<th>Neo</th>
<th>STX</th>
<th>B(_1)</th>
<th>B(_2)</th>
<th>C(_{1+2})</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. minutum</em> (AL3T)</td>
<td>3.0</td>
<td>56.6</td>
<td>5.3</td>
<td>3.3</td>
<td>34.7</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>A. lusitanicum</em> (BAH91)</td>
<td>16.6</td>
<td>51.5</td>
<td>4.1</td>
<td>3.7</td>
<td>41.4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>A. catenella</em> (BAH255)</td>
<td>9.9</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.6</td>
<td>20.2</td>
<td>26.1</td>
<td>53.1</td>
<td></td>
</tr>
<tr>
<td><em>A. tamarense</em> (BAH181)</td>
<td>42.3</td>
<td>11.9</td>
<td>6.9</td>
<td>7.6</td>
<td>7.2</td>
<td>12.8</td>
<td>11.6</td>
<td>3.1</td>
<td>3.5</td>
<td>35.5</td>
</tr>
<tr>
<td><em>A. tamarense</em> (GTPP01)</td>
<td>33.4</td>
<td>14.2</td>
<td>0.9</td>
<td>1.2</td>
<td>16.0</td>
<td>3.6</td>
<td>0.3</td>
<td>7.0</td>
<td>1.5</td>
<td>55.4</td>
</tr>
</tbody>
</table>
PSP-toxin content ranging from 3.0 fmol cell\(^{-1}\) (\textit{A. minutum} AL3T) to 42.3 fmol cell\(^{-1}\) (\textit{A. tamarense} BAH181). The toxin profile was quite similar for \textit{A. minutum} AL3T and \textit{A. lusitanicum} BAH91 with a predominance of GTX1 and GTX4. For \textit{A. catenella} BAH255 the sulfoxycarbamyl toxins B\(_{1,2}\) and C\(_{1,2}\) were predominant, but small amounts of STX also were found. The PSP-toxin profiles of the 2 strains of \textit{A. tamarense} (BAH181, GTPP01) were quite similar, but showed a high percentage of Neo and STX for the clone isolated near the Orkney Islands (BAH181) (Table 2).

**Effects of \textit{Alexandrium} spp. on heterotrophic dinoflagellates**

Preliminary qualitative observations revealed rapid negative effects on the thecate heterotrophic dinoflagellate \textit{Oblea rotunda} when exposed to different \textit{Alexandrium} spp. strains. As observed under a stereomicroscope, cells tended to disappear from the water column and concentrate at the bottom, either immobilized or only slow moving. Rapidity and strength of effects appeared to vary among different \textit{Alexandrium} spp. strains. With some algal strains, \textit{O. rotunda} seemed to be less affected and grazer attacks and pallium feeding events were observed. However, most \textit{Alexandrium} spp. strains and \textit{O. rotunda} are very similar in size and swimming behaviour, making a clear differentiation between the species extremely difficult. Although rapid immobilisation effects were intuitively apparent for some \textit{Alexandrium} spp. strains, the approach using \textit{O. rotunda} was not practical for detailed quantification of short-term effects. We therefore used the heterotrophic dinoflagellate \textit{Oxyrrhis marina} as a test organism. It can be easily distinguished from \textit{Alexandrium} spp. by size, cell shape, and swimming pattern, even at low magnification under a dark-field stereomicroscope.

Microscopic observations showed that certain \textit{Alexandrium} spp. strains caused the heterotrophic dinoflagellate \textit{Oxyrrhis marina} to lose motility and then become rounded. Subsequently, cells swelled and finally lysed. The time course of these effects was followed after addition of \textit{O. marina} to \textit{A. tamarense} (Strain 31/9, final concentration of 3.9 \(\times\) 10\(^3\) cells ml\(^{-1}\)) (Fig. 1). The effect of immobilisation, as estimated by the life-counting droplet method, was very rapid. The first sample could not be processed before 3 min after mixing, and by this time motility was significantly less than that of controls mixed with IMR 1/2 medium (\(t\)-test, \(p < 0.05\)). Results of the following experiments therefore are always expressed as percentage of moving cells compared to controls. After 20 min of exposure the number of moving \textit{O. marina} was drastically reduced and an exposure time of 60 min resulted in a nearly 100\% immobilisation. Cell lysis, as derived from counts of visible intact cells after fixation, was slower than immobilisation (Fig. 1B) with a nearly complete lysis after 2 to 3 h of exposure. Effects of different \textit{Alexandrium} spp. strains thus were subsequently studied using live counts (droplet method) after 1 h exposure and fixed cell counts after 3 h of exposure.

**Comparison of different \textit{Alexandrium} spp. strains**

Effects of different \textit{Alexandrium} spp. strains were analysed at comparable final cell concentrations of about 3 \(\times\) 10\(^3\) ml\(^{-1}\). The exact numbers are listed in Table 3. The percentage of \textit{Oxyrrhis} which became immobilised after 1 h exposure varied considerably among the different \textit{Alexandrium} spp. strains tested (Fig. 2A). The response varied from unaffected (e.g. \textit{A. minutum} AL3T) to a nearly 100\% immobilisation in some strains (e.g. \textit{A. tamarense} SZNB01). Cell
counts of fixed samples after 3 h exposure showed comparable results (Fig. 2B). No negative effects could be observed for the non-toxic control species Scrippsiella trochoidea. Differences in the strength of effects could not be related to estimates of PSP toxin content (Fig. 3). The most effective strains included both strains without PSP toxins (e.g. SZNB01) and with PSP toxins (e.g. Strain BAH181). On the other hand, strains with (e.g. AL3T) and without PSP toxins (e.g. GTL129) had no effect at the cell concentration tested.

### Dependence of cell concentration

The immobilisation effect was strongly dependent on the *Alexandrium* spp. cell concentration for all 5 tested lytic strains (Fig. 4). Data shown in Fig. 4 are pooled from several experiments using different culture runs. Although all cultures were exponentially growing, there is some scatter in the immobilisation effect, which might be due to small differences in algal physiological conditions or in toxin sensibility of *Oxyrrhis marina* utilised in the different experiments. However, the data clearly show that significant negative short-term effects began to occur at cell concentrations of about 0.2 to 1 × 10³ ml⁻¹, depending on the strain. EC₅₀ values, defined as the amount of algae needed to induce 50% immobilisation after 1 h of incubation, of the 5 tested strains were 2.1 × 10³ ml⁻¹ (*A. catenella* BAH255), 1.6 × 10³ ml⁻¹ (*A. tamarense* 31/9), 1.5 × 10³ ml⁻¹ (*A. tamarense* BAH181), 1.0 × 10³ ml⁻¹ (*A. tamarense* SZNB01) and 0.6 × 10³ ml⁻¹ (*A. tamarense* GTPP01).

### Table 3. Final cell concentrations of *Alexandrium* species/strains tested for their effects on *Oxyrrhis marina* (see Fig. 2).

<table>
<thead>
<tr>
<th><em>Alexandrium</em> species</th>
<th>Strain no.</th>
<th>Final conc. (10⁴ ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. affine</em></td>
<td>CCMP112</td>
<td>2.6</td>
</tr>
<tr>
<td><em>A. catenella</em></td>
<td>BAH255</td>
<td>2.6</td>
</tr>
<tr>
<td><em>A. lusitanicum</em></td>
<td>BAH91</td>
<td>4.0</td>
</tr>
<tr>
<td><em>A. minutum</em></td>
<td>AL1T</td>
<td>3.0</td>
</tr>
<tr>
<td><em>A. minutum</em></td>
<td>AL3T</td>
<td>3.2</td>
</tr>
<tr>
<td><em>A. ostrenfeldii</em></td>
<td>BAH136</td>
<td>2.9</td>
</tr>
<tr>
<td><em>A. ostrenfeldii</em></td>
<td>k-0324</td>
<td>2.7</td>
</tr>
<tr>
<td><em>A. ostrenfeldii</em></td>
<td>k-0287</td>
<td>3.0</td>
</tr>
<tr>
<td><em>A. pseudogonyaulax</em></td>
<td>AP2T</td>
<td>2.4</td>
</tr>
<tr>
<td><em>A. tamarense</em></td>
<td>GTPP01</td>
<td>3.0</td>
</tr>
<tr>
<td><em>A. tamarense</em></td>
<td>SZNB01</td>
<td>3.8</td>
</tr>
<tr>
<td><em>A. tamarense</em></td>
<td>BAH181</td>
<td>3.4</td>
</tr>
<tr>
<td><em>A. tamarense</em></td>
<td>CCMP115</td>
<td>3.0</td>
</tr>
<tr>
<td><em>A. tamarense</em></td>
<td>31/9</td>
<td>2.8</td>
</tr>
<tr>
<td><em>A. tamarense</em></td>
<td>GTL121</td>
<td>4.0</td>
</tr>
<tr>
<td><em>A. taylori</em></td>
<td>AY1T</td>
<td>3.2</td>
</tr>
</tbody>
</table>

Finally, the cell concentrations of fixed *Alexandrium* species/strains tested are listed in Table 3. Results expressed as triplicate mean ± 1 SD.
Effect of culture filtrate

A negative effect on *Oxyrrhis marina* also was obvious when testing *Alexandrium* spp. cell free culture filtrate (Fig. 5). The immobilisation effect of cell free filtrate, however, was lower compared to the effect of algal suspensions (same cell concentration as used for the filtrate). There was no difference between the short-term immobilisation effect of filtrate through either gauze (10 µm) or a 0.2 µm membrane.

Quantitative comparison between *Oxyrrhis marina* and *Oblea rotunda*

In one experiment, the immobilisation effect of *Alexandrium tamarense* (Strain 31/9) filtrate (<0.2 µm) and cells was estimated for both heterotrophic dinoflagellates, *Oxyrrhis marina* and *Oblea rotunda* (Fig. 6). Both species are immobilised with the thecate species *O. rotunda* being even more affected compared to the athecate *O. marina*. Here again, negative effects of the cell-free filtrate were less pronounced for both target species.

---

Fig. 4. Percentage of motile *Oxyrrhis marina* after 1 h exposure (live counts) as a function of algal cell concentration for (A) *Alexandrium tamarense* SZNB01, (B) *A. tamarense* GTPP01, (C) *A. tamarense* 31/9, (D) *A. tamarense* BAH181 and (E) *A. catenella* BAH255. Results expressed as triplicate mean ± 1 SD.
DISCUSSION

The results clearly show immobilising/lytic effects of several species/strains of the genus *Alexandrium*, which could not be explained by PSP-toxin content. The observed toxic effects are caused by extracellular toxins, because (a) *Oxyrrhis marina* did not ingest *Alexandrium* spp. and (b) lytic effects also are found in cell-free culture medium. Using non-axenic algal cultures it is important to consider the potential role of bacteria for the observed effects, since it is well known that bacteria may be either directly or indirectly associated with algal toxin production (Doucette et al. 1998). The immobilisation effect of whole algal culture (including bacteria) was higher compared to the effect of culture filtrate, indicating a continual release of toxic substances. However, there was no difference between the effect of filtrate gained either by gauze (10 µm, free bacteria should be unalteredly present) or membrane-filters (0.2 µm, should remove most bacteria), making involvement of extracellular toxins produced by free bacteria unlikely.

The noxious exudates released by *Alexandrium* spp. may generally be classified as allelochemicals. Allelochemical secondary metabolites are mainly distinguished from phycotoxins (like PSP toxins), in that phycotoxins can be vectored through the food web, accompanied by broad-based trophodynamic effects, whereas allelochemicals are usually directly targeted. The presented evidence of allelochemical activity of *Alexandrium* not related to PSP toxins validates a couple of related indications, which can be found widespread in the literature: Ogata & Kodama (1986) described ichthyotoxic and haemolytic effects in the culture medium of *Protogonyaulax* (= *Alexandrium*) *catenella* and *P. tamarense*. As they did not find any PSP toxins in the medium, they concluded that the observed effect was caused by factor(s) other than PSP toxins. Haemolytic effects of *Alexandrium* spp. cell extract were confirmed later (Simonsen et al. 1995, Eschbach et al. 2001). Based on a comparison with the low haemolytic activity of purified STX and GTX1-5 standards, Simonsen et al. (1995) suggested that components other than PSP toxins are likely to be responsible for the haemolytic effects of *A. tamarense*. Likewise, Lush & Hallegraeff (1996) attributed toxic effects of whole cells and cell-free culture medium, of *A. minutum* to *Artemia salina*, to a fast acting toxin distinct from PSP. Similar toxicity of exudates of *A. minutum* has been reported upon exposure to the copepod *Euteripa acutifrons* (Bagoien et al. 1996).

In addition, there are a few papers reporting negative effects of *Alexandrium* spp. filtrate on other algal species. Blanco & Campos (1988) showed that culture filtrate of a PSP-toxin-containing *A. lusitanicum* adversely affected several flagellates (in fact most of the cells were killed), whereas growth of the algal species was not affected by filtrate of a non-PSP-toxin-producing *A. tamarense*. Based on that comparison, Blanco & Campos (1988) tentatively ascribed the toxic effect of culture filtrate to PSP toxins. More recently, Arzul et al. (1999) investigated the allelopathic properties in 3 *Alexandrium* species. They found that the filtrate of all 3 species repressed growth of certain algal species. Allelopathic activity of exponentially growing *Alexandrium* spp. was positively related to both haemolytic activity and published values of PSP toxicity, probably reflecting correlated metabolic activity. The observed increase in allelopathy at the senescent growth phase led Arzul et al. (1999) to the suggestion, that substances other than STX are present.
In contrast to the large body of literature with copepods, there are only a few reports dealing with impacts of *Alexandrium* spp. on heterotrophic protists. *A. tamarense* has been described as deleterious to the marine heliozoa *Heterophrys marina* (Tobiesen 1991). Hansen (1989) and Hansen et al. (1992) described effects of *Alexandrium* spp. on the tintinnid ciliate *Favella ehrenbergii*. Hansen (1989) studied the behaviour and growth of *F. ehrenbergii* fed with 6 clones of *A. tamarense* producing different levels of PSP toxin. He clearly showed that algae are ingested by the ciliate, but the latter is only affected by exudates in the medium. The exudates induce ciliary reversal resulting in continuous backward swimming, swelling of the ciliate and subsequent cell lysis. Based on indirect evidence, Hansen (1989) suggested that the toxic effect of *Alexandrium* spp. on ciliates is caused by PSP toxins. However, the same immobilisation effects on *F. ehrenbergii* were caused by *A. ostenfeldii*, which contained only very small amounts of PSP toxins (Hansen et al. 1992). Four out of 7 *Alexandrium* tested species/strains sustained growth of the heterotrophic dinoflagellate *Polykrikos kofoidii*, whereas the protozoan grazer was rapidly killed by 3 strains (Matsuoka et al. 2000). Although Matsuoka et al. (2000) did not measure toxin content, they designated these 3 strains (*A. fundyense, A. lusitanicum, A. monilatum*) to be toxic (presumably based on literature data). *A. monilatum* is not known to produce PSP toxins but it synthesizes an unknown ichthyotoxin (Aldrich et al. 1967). Moreover, Matsuoka et al. (2000) reported that both toxic and non-toxic strains (based again on literature data) of *A. tamarense* sustained moderate to rapid growth of *P. kofoidii*, suggesting that, in accordance with the present findings, factors other than PSP toxins are responsible for killing this heterotrophic dinoflagellate. Cho & Matsuoka (2000) described cell lysis of *P. kofoidii* feeding on a PSP-toxic *A. tamarense* strain. According to their descriptions and microphotographs, cell lysis occurs after the ingestion of an *A. tamarense* cell, which was rapidly followed by egestion. In line with the present findings, Cho & Matsuoku (2000) observed a fast and nearly complete lysis of *P. kofoidii* within 1 h exposure to an *A. tamarense* concentration of 2000 ml⁻¹. However, in view of the present results, it seems unlikely that cell lysis of *P. kofoidii* is causatively linked to ingestion.

Quantitative findings in the present study were mainly based on *Oxyrrhis marina*, a species rather atypical for the marine plankton. However, detection of negative effects on *O. marina*, a marine protozoa, are likely of greater ecological significance compared to the rather unspecific impact on blood cells, as obtained with standard haemolytic tests. Both heterotrophic dinoflagellate species are immobilised, with the thecate species *Oblea rotunda* being even more affected compared to the thecate *O. marina*. Additional qualitative observations of the short term effects of *Alexandrium* spp. on the thecate species *Oblea rotunda* as well as the similarity of cell lysis observed for *Polykrikos kofoidii* (Cho & Matsuoka 2000), suggest that other heterotrophic dinoflagellates are probably affected in the same way. Moreover, it may be attractive to ascribe the whole range of observed negative effects of *Alexandrium* spp. culture medium on blood cells (haemolytic effects), heterotrophic protzoa (immobilisation/cell lysis; Hansen 1989, this study), algae (growth repression/cell lysis; Blanco & Campos 1988, Arzul et al. 1999) or copepods (grazing inhibition; Huntley et al. 1986) to one single chemical compound. However, Arzul et al. (1999) concluded that the allelopathic activity of *Alexandrium* spp. is caused by a complex of chemicals, rather than by a specific substance. Almost nothing is known about the chemical composition of such compounds. The experiments with culture filtrate at least indicate that these substances are water-soluble but labile in culture media. Immobilisation activity of whole cells was higher compared to culture filtrate (Figs. 5 & 6; see also Hansen 1989) and thus probably due to a continual release of substances by the cells. Comparable lytic or allelopathic effects caused by other algal species are mainly thought to be due to glycolipids and polyunsaturated fatty acids. For example, digalactosylglycerol and octapentaenoic acid isolated by Yasumoto et al. (1990) have been shown to be both haemolytic (Yasumoto et al. 1990) and inhibitory to diatom growth (Gentien & Arzul 1990, Arzul et al. 1995).

The strength of immobilisation/lytic activity at comparable cell concentrations varied considerably among the different *Alexandrium* species/strains tested. It has been repeatedly established that cultured strains of toxic algae, such as *Alexandrium* spp. or *Chrysochromulina* spp., are typically less toxic than those collected from natural populations (White 1986, Cembella et al. 1988, Edvardsen 1993). They also may vary considerably with respect to cellular toxin content. Ander- son 1990, Chang et al. 1997, Edvardsen & Paasche 1998, Parkhill & Cembella 1999) and toxin profile (Cembella 1998). Within the genus *Alexandrium*, lytic activity of the tested strains seems not to be related to certain species. Even though allelochemical effects are insignificant for all tested species of the *A. minutum/lusitanicum* species complex (Strains AL3T, AL1T, BAH91, see Fig. 2), there is evidence that other strains of the same species complex exude lytic compounds (*A. minutum*: Lush & Hallegraeff 1996; *A. lusitanicum*: Blanco & Campos 1988). For the tested strains which appeared to be less effective, it can not be excluded that lytic effect may be apparent at higher cell concentrations and/or longer exposure times.
Our results confirm that a large variety of combinations of PSP and lytic compounds may occur among different *Alexandrium* spp. strains. Ecophysiological consequences of allelochemicals, however, may mimic phycotoxin effects, making it difficult to trace back observed effects to single compounds, unless many strains covering a whole range of combinations of phycotoxins and allelochemical compounds are tested. Evidence that toxic effects, grazing inhibition, and prey selection in crustacean grazers exposed to *Alexandrium* spp. are caused by substances not associated with PSP (Huntley et al. 1986, Bagoien et al. 1996, Lush & Hallegaef 1996, Teegarden & Cembella 1996) has already been noted above. In addition, there are a couple of reports indicating that copepods are affected by lytic exudates of other toxic algae (Gill & Harris 1987, Nielsen et al. 1990, Uye & Takamatsu 1990). Species/strain specific production of allelochemicals in addition to or instead of PSP toxins thus might partly explain some of the contradictory results on copepod grazing of *Alexandrium* spp. (reviewed in Turner & Tester 1997).

The power to immobilise or kill potential predators surely is of adaptive significance for a HAB species to form dense and long lasting blooms. To assess the potential impact *in situ*, however, a comparison between cell concentrations used in the present laboratory experiments and those occurring during *Alexandrium* spp. blooms, is needed. *Alexandrium* spp. are often considered to be ‘background’ bloom species, in that they often are outnumbered by co-occurring phytoplankton (Anderson 1998). Indeed, a compilation presented by Wyatt & Jenkinson (1997) indicated relatively low numbers of 20 to 400 cell ml\(^{-1}\) as peak cell concentrations reached by *Alexandrium* spp. during blooms in different regions. However, high-biomass, monospecific blooms that discolour the water do occur, including those of *A. minutum* in south Australia (Hallegraeff et al. 1988), or dense blooms (>1000 ml\(^{-1}\)) of *A. tamarense* (= *Gonyaulax excavata*) in the Argentine Sea (Carreto et al. 1986). In the Mediterranean, dense blooms of *Alexandrium* spp. with maximum concentrations up to 60,000 cells ml\(^{-1}\) are repeatedly observed (Vila et al. 2001, and references therein). For the short-term immobilisation effect as observed in the present study, EC\(_{50}\) cell concentrations (defined as the cell concentration which caused 50% of immobilisation) were in the range from 1600 ml\(^{-1}\) down to 600 ml\(^{-1}\) for the 5 tested strains. This is in the same range of reported *Alexandrium* spp. cell concentrations required to induce 50% backwards swimming of the ciliate *Favella ehrenbergii* within 10 min (Hansen 1989). Comparing these numbers with bloom concentrations cited above, we suggest that once a bloom reaches sufficiently high *Alexandrium* spp. concentrations, allelochemicals indeed can prevent the population from any substantial protozoan grazing. A ‘blow out’ of at least the protozoan grazers may explain why some *Alexandrium* spp. blooms can persist for months (Mortensen 1985, Carreto et al. 1986). Sublethal, long-term negative effects of lower *Alexandrium* spp. cell concentrations on protozoans are poorly known. According to Hansen (1989) *F. ehrenbergii* is killed by a PSP-toxic *A. tamarense* strain at high cell concentrations but showed ingestion and rapid growth when fed with low concentrations (<1000 ml\(^{-1}\)) of the same strain. This suggests that grazing by protozooplankton might be of significance in controlling the development of a bloom if the concentration of algae is low and the concentration of predators is sufficiently high. However, extensive investigations on potential long-term effects of sublethal concentrations of allelochemicals on protozoan grazers still have to be carried out.

In addition to eliminating competitors and/or grazers, allelochemical activity may be coupled with mixotrophic nutrition, as was suggested for the haptophytes *Prymnesium patelliferum* (Tillmann 1998) and *Chrysochromulina* (Estep & MacIntyre 1989). *Alexandrium* spp. are generally considered to be mainly autotrophic, but food vacuoles containing ciliates or phytoplankton cells have been observed in *A. ostefeldii* (Jacobson & Anderson 1996). Moreover, it recently was shown that some species of *Alexandrium* have the capacity to take up high molecular weight organic molecules (Carlsson et al. 1998, Legrand & Carlsson 1998), to utilize organic N-substances for growth and toxin production (Ogata et al. 1996) and to remove dissolved free amino acids down to concentrations similar to those found in natural waters (John & Flynn 1999). It thus might be speculated that *Alexandrium* spp. probably benefits from enhanced concentrations of dissolved organic matter in consequence of its lytic activity.

There can be no doubt that PSP toxins, through their neurotoxic effects on sea animals and humans, represent the most threatening property of *Alexandrium* spp. However, for the ecological success of *Alexandrium* spp., that is, to produce dense and long-lasting blooms, it is suggested that the allelochemical potential of *Alexandrium* spp., through its direct destructive effects on competing algae or unicellular grazers, is of much greater significance.

Acknowledgements. The authors gratefully acknowledge Elke Jaim (Friedrich-Schiller University Jena) for the PSP-toxin measurement. We greatly appreciate the effort of all people that have isolated and made available the strains listed in Table 1. Thanks are due to Allan Cembella for constructive criticism and suggestions, and to Steve Pueppke for correcting the English. This work was partly supported by the German BMBF (TEPS project 03F0161) and by the European Commission (Research Directorate General-Environment...
Programme-Marine Ecosystems) through the BIOHAB project ‘Biological control of Harmful Algal Blooms in European coastal waters: role of eutrophication’ (contract EVK3-CT99-00015). The BIOHAB project is part of the EC EUROHAB cluster.

**LITERATURE CITED**


