Effects of salinity and two coastal waters on the growth and toxin content of the dinoflagellate *Alexandrium minutum*

**INTRODUCTION**

*Alexandrium minutum* is a coastal dinoflagellate producing toxins responsible for paralytic shellfish poisoning (PSP). This species can form blooms in estuarine waters (Hallegraeff *et al.*, 1988; Cannon, 1993; Erard-Le Denn *et al.*, 1993; Poletti *et al.*, 1998; Weise *et al.*, 2002), in harbours (Delgado *et al.*, 1990; Zaghoul and Halim, 1992; Forteza *et al.*, 1998), eutrophic brackish lagoons (Giacobbe *et al.*, 1996) and aquaculture ponds (Yoshida *et al.*, 2000). Such waters often are of lowered salinity and contain chemically complex mixtures of dissolved organic matter (DOM) of terrestrial and/or aquatic origin.

Utilization by phytoplankton of DOM as a source of N has been suspected. The direct utilization of dissolved organic N (DON) has been studied by focusing on low molecular weight molecules (Antia *et al.*, 1991). Among PSP toxic dinoflagellates, *Alexandrium fundyense* is capable of taking up a wide range of dissolved amino acids at concentrations (low nM) similar to those found in natural waters; but alone, these did not sustain growth (John and Flynn, 1999). *Alexandrium tamarense* can utilize various dissolved organic P compounds for growth (Oh *et al.*, 2002). DOM of terrestrial origin has also been found to favour growth of auxotrophic algae such as dinoflagellates.
In A. minutum, as in many coastal marine phytoplankton species, growth rate response to salinity changes may be strain specific. In studies on the effect of salinity on paralytic shellfish toxin (PST) content of different strains of A. tamarense, different patterns of variation occur (White, 1978; Parkhill and Cembella, 1999; Hwang and Lu, 2000; Hamasaki et al., 2001). Furthermore, Alexandrium spp. cells from natural populations contain more intracellular PST than cultured cells (Kodama et al., 1982; White, 1986; Cembella et al., 1988; 1990; Hamasaki et al., 2001). This discrepancy suggested to Cembella et al. that the ‘forced growth’ conditions experienced in cultures might not promote high cell content of PSTs (Cembella et al., 1988). Culture conditions are, however, far from those under which toxic blooms occur naturally: they contain high N- and P-nutrient concentrations and a low diversity of micro-nutrients, and lack the complex mixture of DOM present in coastal and estuarine waters.

For >10 years, A. minutum blooms have been a serious concern for public health and the shellfish industry on the coast of Brittany, France. In summer in estuaries of Rance, Aber Wrac’h, Aber Benoit and the Bay of Morlaix, high cell densities (of Rance, Aber Wrac’h, Aber Benoit and the Bay of the coast of Brittany, France. In summer in estuaries concern for public health and the shellfish industry on Hamasaki Cembella et al. content of PSTs (Cembella experienced in cultures might not promote high cell in coastal and estuarine waters. nutrients, and lack the complex mixture of DOM present nutrient concentrations and a low diversity of micro-blooms occur naturally: they contain high N- and P- nutrient concentrations and a low diversity of micro-nutrients, and lack the complex mixture of DOM present in coastal and estuarine waters.

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In this study, we characterized the Brittany strain of A. minutum AM89BM for its genetically determined capacity to adapt to variations in salinity, and examined the effect of salinity conditions on growth rate and cell toxin content in moderately enriched offshore seawater media. We set the experimental conditions at a high N:P mole ratio since P limitation favours PST production in Alexandrium spp. (Boyer et al., 1987; Anderson et al., 1990; Béchemin et al., 1999). In order to investigate the effect of more natural media on growth and cell toxin content in A. minutum AM89BM, we sampled two coastal waters with different salinity and sources of DOM, one from an estuary and the second one from an oyster farming area. These waters were used as growth media after two filtration treatments (<3 μm and <0.2 μm) to consider the microbial transformation of DOM. An offshore seawater moderately enriched with N- and P-inorganic nutrients but no micro-nutrients (vitamins and trace metals), was used as a control, and to test whether these micro-nutrients generally associated with DOM are required by A. minutum. Growth rates and PST contents of A. minutum cells grown in these water treatments were compared with each other and with those obtained in the salinity experiment.

**METHOD**

**Materials and cultures**

The clonal, non-axenic strain, A. minutum AM89BM, was isolated from the Bay of Morlaix, Brittany, France, by E. Erard-Le Denn. AM89BM was maintained in f/2 medium (Guillard and Ryther, 1962) modified as follows: Fe³⁺ concentration lowered to 1.05 μM, and 580 μM NO₃⁻ enrichment to obtain a N:P mole ratio of 16; pH was adjusted to 7.5 before autoclaving. This medium was made with offshore seawater, the salinity of which was lowered to 30 p.s.u. by dilution with ultrapurified water (Milli-Q, Millipore). For all experiments, cultures were grown at 18 ± 1°C under ~100 μmol photon m⁻² s⁻² provided by cool-white fluorescent tubes on a 16:8 h light:dark cycle.

**Capacity of A. minutum strain AM89BM to adapt to salinity variations**

Preliminary work was carried out to determine the tolerance of this strain to salinity variations, before undertaking further experiments. Thirteen salinity treatments, ranging from 12 to 37 p.s.u., were prepared by diluting offshore seawater with ultrapurified water; 30 ml aliquots were distributed into borosilicate glass tubes. Two experiments were conducted. First, cells growing at 30 p.s.u. were inoculated into duplicate tubes at each salinity. In the second series, cells were pre-adapted by two successive subculturings through exponential growth phase in seven media of different salinities (12.5, 15, 20, 25, 30 and 37 p.s.u.); these cells were then inoculated into triplicate tubes containing the media of nearest salinity, in the range going from 12 to 37 p.s.u. Additionally, cells adapted at 12.5 p.s.u. were inoculated in triplicate tubes containing the media of nearest salinity, in the range going from 12 to 37 p.s.u. Furthermore, cells adapted at 12.5 p.s.u. were inoculated in triplicate tubes containing the media of nearest salinity, in the range going from 12 to 37 p.s.u. Growth was monitored daily by the measurement of in vivo fluorescence (see below).

**Influence of salinity on cell growth rate and toxin content**

Based on preliminary work, seven salinity conditions (12.5, 15, 20, 25, 30 and 37 p.s.u.) were chosen. Batch cultures were grown in triplicate in 4-l polycarbonate bottles, filled after filter-sterilization (0.2 μm). Pre-adapted cells (as described above), were inoculated into the media with the seven salinities but with micro-nutrient concentrations lowered to one-fifth of the modified f/2. Cell numbers were measured by cell counts in the seven
stock cultures prior to inoculation: the volumes of the inocula were adjusted to give similar initial cell concentrations at all salinities. After inoculation, initial NO$_3^-$ and PO$_4^{3-}$ concentrations at all the salinities were set to 120 ± 1 μM and 1.57 ± 0.04 μM, respectively (N:P = 76), and analytically verified. In vivo fluorescence was measured daily (see below); 20-ml samples were taken and fixed in Lugol’s iodine solution for direct cell counting. Based on the in vivo fluorescence growth curves, samples were taken during and at the end of the exponential growth phase and during the stationary phase, for the analysis of PST concentrations.

Comparison of estuarine and oyster farming waters

On October 22, 1998, two water samples were collected: (i) from the downstream part of the Charente River estuary (45°57′20″N, 1°38′3″W), and (ii) 5.4 km westward, in the vicinity of an oyster farming area (45°57′47″N, 1°7′12″W) at the mouth of the Charente River estuary on the French Atlantic coast. Salinity and inorganic nutrient concentrations were measured on the day of collection. Particles were allowed to settle for a few hours and then the water was passed sequentially through a 25- and a 3-μm sieve mesh (ESTU<3 and OYST<3 treatments). Then, half of the two 3-μm filtered waters was filtered through a Millipore 0.22 μm GS WP membrane (ESTU<0.2 and OYST<0.2 treatments). A quantity of 0.22 μm filter-sterilized, aged offshore seawater (OFFSH treatment) was enriched with inorganic nutrients to the same concentrations present in the oyster farm water (which had nearly the same salinity, Table II) on the day of collection i.e. NO$_3^-$ (28.9 μM) and PO$_4^{3-}$ (1.0 μM), but no vitamins and trace metals as a control treatment. Each treated water was distributed in triplicate into autoclaved 4-l polycarbonate bottles (<7 mC) and NH$_4^+$, were analysed using standard protocols (Valderama, 1995); total dissolved nitrogen was analysed after UV oxidation and dissolved organic nitrogen (DON) was obtained by subtracting the sum of dissolved inorganic nitrogen (DIN). Salinity was measured with an S/Mill Atago NaCl refractometer.

Cells of A. minutum were collected onto precombusted filters (Whatman GF/F). Chl a was measured after extraction with 6 ml pure methanol at 4°C for 1 h in the dark (Yentsch and Menzel, 1963). Particulate protein was analysed as follows: after hydrolysis with 5.8 M HCl at 100°C for 20–24 h, dissolved free amino acids were analysed using flow injection sample processing (Petty et al., 1982); results are reported as glycine equivalents (Gly eq.).

For intracellular PST analyses, filter samples were stored at −20°C before lyophilization. Lyophilized filters were extracted in 0.05 M acetic acid by ultrasonication on ice (3 × 10 s). The extracts were centrifuged (14000 g, 15 min) and the supernatants decanted and stored at −70°C prior to analysis. PSTs were analysed by high performance liquid chromatography (HPLC) with fluorescence detection according to Oshima (Oshima, 1995), with minor modifications. The mobile phase and post-column derivatization reagents were delivered by three Waters model 510 pumps. Columns used for analyses were Luna C$_{18}$ (150 × 4.6 mm internal diameter, 3 μm particle size; Phenomenex, Macclesfield, UK) for the gonyautoxins (GTX) and C$_{18}$ (5 μm particle size) for saxitoxin, neosaxitoxin and decarbamoyl saxitoxin. Columns were maintained at 40°C in a column oven. The detector was a Waters 470 scanning fluorescence detector. Data were collected and processed by Waters Millenium 32 software. Toxin identification and concentrations were determined by comparison with standards supplied by the National Research Council of Canada (Institute for Marine Biosciences, Halifax, NS) (PSP-1B). Toxin contents were expressed as fmol PST cell$^{-1}$. The net toxin production rate $R_{Tox}$ (fmol PST cell$^{-1}$ day$^{-1}$) for each cell growth period (for periods ≥2 days) was calculated according to (Anderson et al., 1990).

One way ANOVA tests were used to evaluate differences in the mean values.
RESULTS

Adaptation to salinity variations

Cells maintained in the medium at 30 p.s.u. adapted rapidly to salinity changes. At 12 and 15 p.s.u. (after a decrease in salinity of 18 and 15 p.s.u., respectively), growth started after one day of adaptation. At 37 p.s.u., a similar adaptation time was also apparent. Between 17 and 35 p.s.u., growth started immediately. Values of $k_{IVF}$ doubled from 12 to 20 p.s.u., reaching a maximum of 0.6–0.7 doublings day$^{-1}$ between 20 and 35 p.s.u., then decreased at 37 p.s.u. (Figure 1).

With inocula adapted to each salinity, between 12 and 35 p.s.u., growth rates ($k_{IVF}$) were comparable with those previously obtained with non-adapted cells (Figure 1). At 37 p.s.u., adapted cells showed the same growth rate as those grown between 21 and 35 p.s.u. At 10 p.s.u., growth was very slow (~0.15 doublings day$^{-1}$) for 1 week and then stopped. No growth was observed at lower salinities and cells did not survive for a long period under these conditions as indicated by the decrease of in vivo fluorescence from the time of inoculation.

Influence of salinity on cell growth rate and PST content

In all salinity treatments, in vivo fluorescence showed an exponential increase after Day 2 (data not shown). In contrast, cell growth curves were typically sigmoidal, with a 2–4 day lag phase followed by a short period (2–3 days) during which the cell division rate markedly increased (Figure 2). PO$_4^{3-}$, as the limiting nutrient, was exhausted (≤0.04 μM) in all media at salinity ≥15 p.s.u. when the cultures were in early stationary phase (Figure 2). Significant amounts of NO$_3$ remained at stationary phase, ranging between 62.9 ± 4.2 μM (at 37 p.s.u.) and 88.0 ± 5.5 μM (at 15 p.s.u.). In the media of salinity 12.5 p.s.u., 0.20 ± 0.07 μM PO$_4^{3-}$ and 84.0 ± 9.4 μM NO$_3$ remained at the end of the experiment while cultures appeared to be close to entering into the stationary phase. The highest exponential growth rates ($k_{cell}$) were observed at 25 p.s.u., however, between 25 and 37 p.s.u., neither $k_{cell}$ nor maximum cell concentrations were significantly different ($P > 0.16$ and 0.83, respectively) (Table I). At salinities <25 p.s.u., values of $k_{cell}$ greatly decreased following the salinity decrease; however, between 20 and 12.5 p.s.u., maximum cell concentrations did not differ significantly ($P > 0.70$).

Toxin composition in cells was dominated by the α/β epimer pair GTX2 and GTX3, these generally representing >97% of the total PSTs, the remainder comprising the decarbamoyl analogues, deGTX2 and deGTX3. Toxin composition did not vary widely with salinity, nor with the growth phase of the cultures.

Significant variations were observed in the cell PST content and net toxin production rate $R_{Tox}$ (Figure 2; Table I). At all salinity conditions, $R_{Tox}$ was generally higher during the exponential growth, when it was often higher enough to allow the cell toxin content to increase in dividing cells. In the four fastest growing cultures (25–37 p.s.u.), PST content per cell increased progressively during the exponential phase, and generally continued to increase during the early stationary phase. The toxin content increased rather similarly to a maximum of ~10 fmol PST cell$^{-1}$ at 30, 35 and 37 p.s.u., while at 25 p.s.u. it increased at a faster rate to 30.4 ± 9.8 fmol PST cell$^{-1}$. At 25 p.s.u. and lower, $R_{Tox}$ was three to six times higher than at 30–37 p.s.u. (Table I). At 20 p.s.u. the PST content increased to 30.7 ± 6.5 fmol PST cell$^{-1}$ at the end of the exponential phase, before stabilizing. At 12.5 and 15 p.s.u., the PST content remained within the range of 10–20 fmol cell$^{-1}$ during early growth phase (until Day 7). Then, $R_{Tox}$ increased markedly, and consequently the cell PST content, which reached a maximum value of 52.7 ± 1.7 fmol PST cell$^{-1}$ at 15 p.s.u. when the cultures entered stationary phase, and 41.9 ± 8.8 fmol cell$^{-1}$ on Day 18 at 12.5 p.s.u., although these cultures had not reached stationary phase because of their slow growth.

Growth and toxin content of A. minutum strain AM89BM grown in estuarine, oyster farming and offshore waters

Salinities and inorganic nutrient concentrations of the three different seawaters used for this experiment are given in Table II. During the 4-day period between
Fig. 2. Cell growth (black squares) and changes in cell toxin content (open squares) of A. minutum AM89BM grown under seven salinity conditions. Arrows indicate when media were determined to be P-depleted (concentration equal or below the detection limit). Error bars represent the standard deviation ($n = 3$); error bars are absent when the error is smaller than the size of the symbol.
### Table I: Average exponential growth rates ($k_{cell}$), maximum cell concentrations, and net toxin production rate of *A. minutum* grown at seven salinity conditions

<table>
<thead>
<tr>
<th>Salinity (p.s.u.)</th>
<th>$k_{cell}$ (div day$^{-1}$)</th>
<th>Cell concentration ($10^6$ cell l$^{-1}$)</th>
<th>Net toxin production rate ($R_{tox}$ fmol cell$^{-1}$ day$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Period (day)</td>
</tr>
<tr>
<td>12.5</td>
<td>0.26 ± 0.01</td>
<td>2.40 ± 0.28</td>
<td>2.42 ± 0.54</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>0–7</td>
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<tr>
<td></td>
<td>5–14</td>
<td>7.41 ± 1.62</td>
<td>12–18</td>
</tr>
<tr>
<td>15</td>
<td>0.32 ± 0.01</td>
<td>2.41 ± 0.13</td>
<td>1.68 ± 0.90</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>0–7</td>
</tr>
<tr>
<td></td>
<td>5–12</td>
<td>12.01 ± 0.52</td>
<td>7–14</td>
</tr>
<tr>
<td>20</td>
<td>0.43 ± 0.05</td>
<td>2.29 ± 0.10</td>
<td>2.57 ± 0.20</td>
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<td></td>
<td></td>
<td></td>
<td>0–5</td>
</tr>
<tr>
<td></td>
<td>4–7</td>
<td>9.21 ± 2.28</td>
<td>5–10</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>0.91 ± 3.51</td>
</tr>
<tr>
<td>25</td>
<td>0.63 ± 0.07</td>
<td>4.43 ± 0.46</td>
<td>4.28 ± 1.55</td>
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<td></td>
<td></td>
<td></td>
<td>0–5</td>
</tr>
<tr>
<td></td>
<td>4–7</td>
<td>9.91 ± 4.52</td>
<td>5–9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4.01 ± 1.13</td>
</tr>
<tr>
<td>30</td>
<td>0.53 ± 0.03</td>
<td>4.25 ± 0.26</td>
<td>2.35 ± 0.62</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>0–5</td>
</tr>
<tr>
<td></td>
<td>4–7</td>
<td>2.62 ± 0.60</td>
<td>5–9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.97 ± 0.50</td>
</tr>
<tr>
<td>35</td>
<td>0.55 ± 0.05</td>
<td>4.31 ± 0.28</td>
<td>0.91 ± 0.10</td>
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<td></td>
<td></td>
<td></td>
<td>0–5</td>
</tr>
<tr>
<td></td>
<td>4–7</td>
<td>1.42 ± 0.43</td>
<td>5–9</td>
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<tr>
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<td></td>
<td></td>
<td>2.09 ± 0.40</td>
</tr>
<tr>
<td>37</td>
<td>0.58 ± 0.05</td>
<td>4.44 ± 0.16</td>
<td>1.07 ± 0.17</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>0–5</td>
</tr>
<tr>
<td></td>
<td>4–7</td>
<td>2.36 ± 0.39</td>
<td>5–10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.91 ± 0.45</td>
</tr>
</tbody>
</table>

Maximum cell concentrations are values obtained at the stationary phase (salinities from 15 to 37 p.s.u.) or at the end of the experiment (salinity 12.5 p.s.u.) when cultures were approaching stationary phase. Net toxin production rate was calculated for early and late growth periods, and early stationary phase. Data are the mean ± SD, $n=3$.

### Table II: Initial conditions in the five coastal and offshore water treatments

<table>
<thead>
<tr>
<th>Origin of water:</th>
<th>Estuarine</th>
<th>Oyster farm</th>
<th>Offshore</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salinity (p.s.u.)</td>
<td>27</td>
<td>36</td>
<td>37</td>
</tr>
<tr>
<td>Filtration treatment:</td>
<td>3 µm</td>
<td>0.2 µm</td>
<td>3 µm</td>
</tr>
<tr>
<td>Day of collection</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PO$_4^{3-}$ (µM)</td>
<td>2.57</td>
<td>2.74</td>
<td>0.77</td>
</tr>
<tr>
<td>DIN (µM)</td>
<td>94.2</td>
<td>107.9</td>
<td>22.7</td>
</tr>
<tr>
<td>After inoculation ($t_0$)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PO$_4^{3-}$ (µM)</td>
<td>1.54</td>
<td>1.96</td>
<td>0.13</td>
</tr>
<tr>
<td>DIN (µM)</td>
<td>87.7</td>
<td>103.8</td>
<td>1.3</td>
</tr>
<tr>
<td>Inorganic N:P ratio</td>
<td>56.9</td>
<td>53.0</td>
<td>10.0</td>
</tr>
<tr>
<td>DON (µM)</td>
<td>7.9</td>
<td>5.5</td>
<td>6.4</td>
</tr>
<tr>
<td>Chl a (µg l$^{-1}$)</td>
<td>2.12 ± 0.04</td>
<td>2.20 ± 0.13</td>
<td>2.11 ± 0.14</td>
</tr>
<tr>
<td>Protein (µM Gly eq.)</td>
<td>2.57 ± 0.32</td>
<td>2.34 ± 0.16</td>
<td>6.91 ± 0.71</td>
</tr>
</tbody>
</table>

Nutrient concentrations were measured on the day of collection following 3 µm or 0.2 µm filtrations, and after inoculation with *A. minutum* 4 days later ($t_0$). Chl a and particulate protein concentrations were also measured after inoculation. Values at $t_0$ are means ± SD of triplicate cultures. *Measured concentrations after enrichment with PO$_4^{3-}$ and NO$_3^-$, respectively.
filtration and inoculation, nutrient concentrations decreased in processed coastal waters. In the estuarine water, dissolved inorganic PO$_4^{3–}$ (DIP) decreased by 40% in the <3 μm treatment (ESTU <3) and by 28% in the <0.2 μm treatment (ESTU <0.2), while dissolved inorganic nitrogen (DIN) concentrations decreased by 7 and 4%, respectively. In the <3 μm-filtered oyster farm water (OYST <3), DIP and DIN were nearly exhausted, decreasing by 83 and 94%, respectively. In contrast in the 0.2 μm-filtered fraction of this water (OYST <0.2), DIP and DIN only decreased by 25 and 2%, respectively. In the offshore seawater treatment (OFFSH), 1.0 μM PO$_4^{3–}$ and 28.9 μM NO$_3$ were added prior to inoculation to give the same concentrations of DIP and DIN as those measured in the oyster farm water when it was collected; after inoculation, DIP and DIN concentrations increased by <0.2 μM, indicating that only low quantities of nutrients were carried over with the inoculum. Concentrations of dissolved organic nitrogen (DON) (Table II) would have been contributed mostly from carry-over by the inoculum as shown by the value in the OFFSH treatment, and from original coastal DOM and/or microbial production in the four other treatments. DON concentrations were in the same range among the five treatments: the highest DON value measured in the ESTU <3 treatment was less than twice that in the OFFSH treatment.

In samples taken immediately after inoculation ($t_0$) with *A. minutum*, small variation in Chl a concentrations indicated that the inoculum could be considered homogeneous among all experimental bottles (Table II). In contrast, initial concentrations of particulate protein were significantly higher in all coastal water treatments than in the OFFSH treatment (Table II). The origin of these higher values, especially in the OYST <3 treatment, is discussed later.

In both estuarine water treatments, exponential growth began after 1 day of adaptation, according to the in vivo fluorescence data (Figure 3A). DIP was exhausted at the stationary phase (<0.01 μM). DIN was exhausted on Day 8 in the ESTU <3 treatment (<0.2 μM), but remained in excess at stationary phase in the ESTU <0.2 treatment, i.e. 27.8 ± 1.3 μM on Day 13. Although in vivo fluorescence increased up to Day 8, the stationary phase was reached on Day 6 according to direct cell counts (Figure 3B). Cell division rate, $k_{cell}$ was slightly higher in the ESTU <3 treatment than in the ESTU <0.2 treatment, although the maximum cell concentrations were comparable at the stationary phase (Table III). Chl a and particulate protein concentrations of cultures increased with respect to increase of cell concentrations, but maximum values at stationary phase were significantly lower in the ESTU <0.2 treatment than in the ESTU <3 treatment (Table III, Figure 3C). In the ESTU <3 treatment, cell content of Chl a increased during the early log phase (Day 3), then remained stable at a high content (>5 pg cell$^{-1}$) (Figure 4A). In the ESTU <0.2 water, Chl a content peaked at 8.11 ± 0.44 pg cell$^{-1}$ on Day 3, then fell to 3.15 ± 0.20 pg cell$^{-1}$ during the stationary/decay phases. In contrast, cell protein content decreased in both treatments, although to a greater extent in the ESTU <0.2 water than in the ESTU <3 water (Figure 4B).

Fig. 3. Change with time of in vivo fluorescence [A], of cell concentration of *A. minutum* AM89BM [B], and of particulate protein concentration [C] in the five coastal and offshore water treatments. Error bars represent the standard deviation ($n = 3$); error bars are absent when the error is smaller than the size of the symbol.
In the oyster farm water, growth pattern of *A. minutum* cells was significantly different between the two treatments, even though in both cases, stationary phase was reached on Day 6 (Figure 3). In the OYST <0.2 treatment, after one day lag phase, exponential growth lasted 2-4 days; then from Day 3, the cultures grew slowly as DIP was exhausted and only ~12 μM DIN remained, which was exhausted on Day 6. Particulate protein concentration in cultures followed a similar pattern to the cell growth curve (Figure 3C). The Chl a cell content remained stable throughout the culture, and protein content peaked at 6.35 ± 1.61 pmol cell^{-1} Gly eq. on Day 3 during the period of maximum growth (Figure 4).

In contrast in the OYST <3 treatment, after a lag phase of 2-3 days, the maximum growth rate was only maintained for 1-2 days, the fourth day, while DIP and DIN were already exhausted by Day 3. Maximum growth rate was higher, and the maximum cell concentration was almost twice as high in the OYST <3 treatment as in the OYST <0.2 treatment (Table III; Figure 3B).

In the OFFSH treatment, growth of *A. minutum* was slow as indicated by the low maximum values of \( k_{\text{cell}} \) and
biomass (cell, Chl a and particulate protein concentrations) (Figure 3; Table III). On Day 6, the cultures reached stationary phase according to the \textit{in vivo} fluorescence data, although there was a small increase in cell numbers. Cells also continued to take up inorganic nutrients between Days 6 and 10, with DIP decreasing from 0.74 to 0.45 \textmu M and DIN from 26.1 to 22.4 \textmu M. Chl a per cell increased throughout the experiment, while proteins per cell only increased during growth until Day 6, reaching the highest concentration at stationary phase of any of the treatments (Figure 4).

The composition of the intracellular toxin pool was the same as that found in the salinity experiment and did not markedly differ with treatments and over time. The net toxin production rate $R_{\text{Tox}}$ and total PST content of \textit{A. minutum} cells varied greatly between treatments (Figure 5; Table III). In both treatments of the estuarine water, particulate toxin concentration increased to maxima of $\sim 70$ and 60 nmol PST l$^{-1}$ in the ESTU $<3$ and ESTU $<0.2$ treatments, respectively (Figure 5A). In cells, $R_{\text{Tox}}$ was highest during the early exponential growth phase until Day 3, making the cell PST content peak that day at 9.56 $\pm$ 1.06 and 7.63 $\pm$ 0.44 fmol PST cell$^{-1}$ in the ESTU $<3$ and ESTU $<0.2$ treatments, respectively. Then $R_{\text{Tox}}$ dropped during the late growth phase by 58 and 43%, respectively, causing the decrease of cell toxin content, before cells began losing toxins at stationary phase (Figure 5B).

In the OYST $<0.2$ treatment, particulate PST concentration increased three to four-fold during the growth phase (Figure 5A). Inside the cells, toxin content (Figure 5B) first decreased due to early fast growth phase (till Day 3), then increased till Day 6 as $R_{\text{Tox}}$ increased during this period while cell growth rate decreased dramatically, before decreasing again in the stationary phase. In contrast, in the OYST $<3$ treatment, toxin production was very low all along the growth and the particulate PST concentration only increased slightly during growth ($\sim$1.5-fold) (Figure 5A). Consequently, the PST content per cell continuously decreased during the growth phase, down to 1.11 $\pm$ 0.22 fmol PST cell$^{-1}$ on Day 6, then remained constant in the stationary phase (Figure 5B).

The OFFSH treatment was the only one in which the particulate toxin concentration decreased during the growth phase (up to Day 6), falling by $\sim$74%, to 0.92 $\pm$ 0.08 nmol PST l$^{-1}$. Thereafter, it remained almost constant at 0.86 $\pm$ 0.05 nmol PST l$^{-1}$. Therefore, the toxin content per cell decreased by $\sim$78% from the initial value, to 1.44 $\pm$ 0.07 fmol PST cell$^{-1}$ on Day 6, and then to 1.08 $\pm$ 0.14 fmol PST cell$^{-1}$ on Day 10 (Figure 5B).

**DISCUSSION**

**Growth capabilities of Alexandrium minutum strain AM89BM**

The strain AM89BM of \textit{A. minutum} is euryhaline, capable of growing in salinities from 12 to 37 p.s.u. Exponential growth rates were close to the maximum rate (0.5 div. day$^{-1}$) at salinities $>20$ p.s.u. Its capacity to survive at low salinities, down to 10 p.s.u., explains the organism’s ability to withstand the mixing of coastal and river waters in estuaries. This is consistent with the original coastal habitat of this dinoflagellate, in the waters of Brittany, where blooms of \textit{A. minutum} usually occur in estuaries under conditions of lowered salinity (Erard-Le Denn et al., 1993). Similar conditions have also been observed during blooms of \textit{A. minutum} and other \textit{Alexandrium} spp. in various geographic areas (Cembella and Therriault, 1989; Larocque and Cembella, 1990; Franks and Anderson, 1992; Zaghloul and Halim, 1992; Cannon, 1993; Chang et al., 1995; Giacobbe et al., 1996). Furthermore, numerous cultured strains of \textit{Alexandrium} spp. have been shown to be euryhaline, although the...
range of salinity optimum varies (Prakash, 1967; Watras et al., 1982; Cembella and Therriault, 1989; Cannon, 1993; Hamasaki et al., 1998; Parkhill and Cembella, 1999; Hwang and Lu, 2000).

In enriched offshore seawater media, highest average exponential growth rates (0.5–0.7 div day$^{-1}$) of A. minutum AM89BM, observed around the optimum salinity condition (range 20–37 p.s.u.) are comparable with those reported for other strains of Alexandrium spp. (Watras et al., 1982; Anderson et al., 1990; Cannon, 1993; Flynn et al., 1994; Chang and McClean, 1997; Hamasaki et al., 1998; Parkhill and Cembella, 1999). Interestingly, the average exponential growth rates obtained in both estuarine water treatments (~0.8 div day$^{-1}$) were significantly higher than in the enriched offshore seawater medium at the nearest salinity (25 p.s.u., Table I). Moreover, in the ESTU-c3 treatment, as division rate peaked 1 day at 1.21 ± 0.13 div day$^{-1}$, comparable high growth rates (1.3 div day$^{-1}$) have only once been reported for the in situ growth of A. minutum populations in diffusion cages (Garcés et al., 1996). Most values of $k_{cell}$ in the oyster farm water treatments were also higher than those obtained in the enriched offshore seawater medium at the nearest salinities (35–37 p.s.u.). The difference in growth rates observed in the coastal waters, higher in the estuarine water than in the oyster farm water, may be partly the result of the differences in salinity, which was optimal only in the former (27 p.s.u.), and sub-optimal in the latter (36 p.s.u.). Altogether, these observations suggested that our filtered coastal waters were better media for the growth of A. minutum than the usual enriched culture media.

The two coastal waters also contained significantly different nutrient concentrations (Table II). However, in another strain of A. minutum, lower growth rates were reported only when N nutrients (NO$_3^-$, NH$_4^+$ or urea) were provided at <6 µM, whereas only slightly enhanced growth rates were observed at higher NO$_3^-$ concentrations (6–200 µM) (Chang and McClean, 1997). In addition, with the exception of the OYST-c3 treatment, NO$_3^-$ concentrations were well over half-saturation constants ($K_s$) for NO$_3^-$ uptake observed in dinoflagellates; PO$_4^{3-}$ concentrations, however, were in the range of $K_v$ values for PO$_4^{3-}$ uptake by dinoflagellates (Smayda, 1997; Matsuda et al., 1999). Nevertheless, we hypothesize that nutritional factors other than inorganic nutrient concentrations accounted for differences in exponential growth rate and cell biomass obtained at stationary phase.

Dissolved organic matter (DOM) present in the two coastal waters might have been utilized by A. minutum to support growth. As previously experimentally demonstrated, A. tamarense and A. catenella (Ogata et al., 1996; Carlsson et al., 1998; Legrand and Carlsson, 1998), and many other dinoflagellates, can use some organic compounds as sources of N and/or P (Antia et al., 1991; Carlsson and Granéli, 1998), though the organic compounds were generally supplied at very high concentrations unknown in natural environments. In contrast, John and Flynn demonstrated in A. fundyense that the uptake of dissolved amino acids alone at low/natural concentration could not sustain cell growth, and appeared only additional to main DIN sources (John and Flynn, 1999). In our coastal water treatments, the initial concentrations of DON were low compared with DIN, therefore suggesting that DOM might not have significantly contributed to the constitutive nutrient pool of A. minutum cells.

Alternatively, our results suggest that DOM contributed to the enhancement of growth through the supply of substance(s) necessary for auxotrophic growth, contrary to the OFFSH treatment with vitamins and trace metals omitted. In this treatment, growth was very slow then stopped, leaving a lot of NO$_3^-$ in the medium, indicating that a substantial amount of some vitamin(s) and/or trace metal(s) may be required for growth by A. minutum. Many dinoflagellates require indeed at least one vitamin among biotin, thiamin and vitamin B$_{12}$ (Provasoli and Carlucci, 1974; Swift, 1984); for example, A. catenella specifically requires vitamin B$_{12}$ with a half-saturation constant for growth estimated at 0.22 ng l$^{-1}$ (Matsuda et al., 2001). The two coastal waters we used appeared to contain sufficiently auxotrophic compounds required for the growth of A. minutum.

Interestingly, A. minutum appeared to grow somewhat better in the <3 µm than in the <0.2 µm treatment of both coastal waters. The <3 µm treatments were set up to observe the influence of bacterioplankton on dinoflagellate growth. One could think that bacterial degradation of DOM into inorganic nutrients could have stimulated A. minutum growth or contributed to generating a greater algal biomass. On the contrary, DON increased in the <3 µm filtered waters during the 4-day period prior to inoculation with A. minutum (Table II), while inorganic nutrient concentrations decreased much more, probably as a consequence of uptake by heterotrophic bacterioplankton (Kirchman, 1994; Middelburg and Nieuwenhuize, 2000). Subsequently, a certain development of bacterioplankton and/or nanoflagellates that passed through the 3-µm mesh might have occurred, possibly accounting for the higher particulate protein concentrations and cell contents measured after inoculation ($d_0$) in the coastal water treatments than in the OFFSH treatment (Table II; Figure 4). Therefore, rather than playing the role of remineralizing DOM into inorganic nutrients, following
the requirement of *A. minutum* for micronutrients, it is likely that bacterial activity in the <3 μm filtered waters contributed to the production of vitamins or other compounds required for auxotrophic growth (Starr *et al.*, 1957; Haines and Guillard, 1974). This hypothesis concurs with other observations indicating that coastal bacterial assemblages stimulated *A. fundyense* growth in culture (Ferrier *et al.*, 2002).

Surprisingly, in the OYST<3 cultures, despite the initial lack of inorganic nutrients (at 40), a higher cell biomass was obtained than in the OYST<0.2 cultures (Table III). In OYST<3 also, the particulate protein concentration in cultures was initially very high, was thought to be microbial biomass, but subsequently did not increase much throughout the growth of *A. minutum*. A likely explanation for dinoflagellate growth is the mixotrophic utilization of the microbial biomass as food, providing in particulate form the N and P required for growth. This is consistent with mixotrophy widespread among dinoflagellates (Granéli and Carlsson, 1998; Jacobson, 1999; Stoecker, 1999), and especially bactervory previously reported in *A. tamarensis* (Nygaard and Tobiesen, 1993; Ogata *et al.*, 1996). In support of this mixotrophy hypothesis compensating for the lack of inorganic nutrients, our observations are consistent with other reports of mixotrophy in predominantly phototrophic dinoflagellates. For instance, phagotrophy was induced in cells of *Heterocapsa triquetra* grown in nutrient-depleted medium (Legrand *et al.*, 1990). In *Fragilidium subglobosum*, ~24 h were required by phototrophic cells to adapt to phagotrophic metabolism (Skovgaard, 1996). Similarly, the onset of exponential growth in the OYST<3 treatment occurred after a lag-time of 1 day compared with other treatments (Figure 3B). Furthermore, *A. minutum* cells grew more quickly in the presumably mixotrophic OYST<3 than in the phototrophic OYST<0.2 treatment, as did mixotrophic compared with phototrophic cells of *F. subglobosum* (Skovgaard, 1996) and *Gyrodinium galatheanum* (Li *et al.*, 1999).

**Effects of salinity on toxin content in P-limited media**

An initial N:P ratio close to 80 was used in the experiment to result in higher PST content per cell following previous observations with AM89BM (Béchemin *et al.*, 1999). At all salinities tested, cell toxin content increased during the exponential growth phase, which was the more active phase of toxin production. However, cell PST content peaked toward the end of growth phase or in the early stationary phase (Figure 2), i.e. toxin production continued for a few days while growth slowed down or stopped, when PO₄³⁻ became exhausted in the medium and in the presence of a large excess of NO₃⁻. Between 30 and 37 p.s.u., the cell PST content accumulated during the stationary phase, was the lowest among all salinity treatments, and was comparable with that reported in the N:P = 80 nutrient condition at salinity 35 p.s.u. (Béchemin *et al.*, 1999). At these salinities, the stability of growth rate and PST content was somewhat similar to that reported by Anderson *et al.* (Anderson *et al.*, 1990), showing that rapid, up and down changes of salinity between 28 and 38 p.s.u. did not affect growth nor cell PST content of *A. fundyense*. At salinities of <30 p.s.u., the maximum toxin content increased significantly, approximately three-fold at 20 and 25 p.s.u., approximately five-fold at 15 p.s.u., and about four-fold at 12.5 p.s.u., compared with that found at 30–37 p.s.u. Similarly, in two *A. tamarensis* strains grown in 1/2 medium (N:P = 24), cells exhibited increasing toxicity below the optimum growth salinity, and mostly low toxicity at higher salinity (Hamasaki *et al.*, 2001).

However, other studies reported different patterns of variation of cell PST content versus salinity. Parkhill and Cembella (Parkhill and Cembella, 1999) cultured a strain of *A. tamarensis* between 10 and 30 p.s.u. on K medium (Keller and Guillard, 1985) at a high N:P ratio (88) similar to that we used: the PST content of the cells increased during stationary phase, and the highest toxin contents occurred between 20 and 30 p.s.u., corresponding to the optimum growth conditions for the strain. Similarly, but in a medium with a low N:P ratio (9.2), the highest toxicity of *A. minutum* cells coincided with the optimum growth condition at 15 p.s.u., and cell toxicity decreased more at higher salinity (25 p.s.u.) than at lower salinity (7.5 p.s.u.) (Hwang and Lu, 2000). In contrast, White (White, 1978) found in a strain of *Gonyaulax excavata* (i.e. *A. tamarensis*) grown in 1/2 medium, that the highest cell PST content occurred at 37 p.s.u. (higher than the optimum for growth) during mid-log phase, and the lowest at 21 p.s.u. Therefore, these different reports make difficult any generalization, and raise the question of whether these differences may be due to different growth conditions (e.g. N:P ratio or nutrient concentrations) or are strain-specific responses.

**Toxin content in coastal water cultures**

The OFFSH treatment was the only one in which toxin content decreased during the growth period, both on a per culture volume and per cell basis. Therefore, during cell division, the cells did not synthesize PST and lost the toxins. When cells ceased dividing, their toxin content also stabilized at a low value (~1 fmol cell⁻¹). Although a significant amount of NO₃⁻ remained in the medium, consistent with the data of Béchemin *et al.* (Béchemin...
et al., 1999). PST content reflected N-limitation in cells, whereas the Chl a content reflected slightly N-limited cells. Potentially, this may be the result of the lack of micronutrients (vitamins and/or trace metals) involved in the uptake of NO₃⁻ and/or the subsequent synthesis of amino acid precursors of PST. Among these precursors is methionine (Cembella, 1998); the synthesis of which requires a coenzyme derived from vitamin B₁₂ (Swift, 1980). The fate of the lost toxins, whether recycling N by the cells, or both, was not determined. However, recycling these toxins for N (GTX2/3 molecules contain 7 N atoms) would not have contributed more than 1% of 4–20 pmol N cell⁻¹ of constitutive N in these cells (Béchemail et al., 1999).

In the estuarine water, the initial inorganic nutrient content was moderately P-limiting (N:P ≈ 39). Though this ratio increased during the pre-inoculation period, it might explain, however, why in both treatments the PST content of the cells increased only during early log phase (until Day 3, Figure 5), when the rate of toxin production and the cell toxin content were in the range of that found at 25–30 p.s.u. in the previous experiment during the same growth period. Then, toxin production decreased at a rate not compensating for growth rate, resulting in a decrease in cell toxin content until the stationary phase, contrary to that which was found in the salinity experiment (Figure 2). Toxin production was slightly faster in the ESTU <3 treatment compared with the ESTU < 0.2 treatment. In the ESTU < 3 treatment, N-uptake was more efficient (leading to the total DIN exhaustion) than in the ESTU < 0.2 treatment where ~26% of the initial DIN was left at the end of the experiment (Day 13), explaining why higher concentrations of Chl a and protein were obtained in the ESTU < 3 cultures (Table III). Consequently, ESTU < 3 cells maintained a high content of Chl a similar to N-replete cells (Béchemail et al., 1999), a rather high content of protein, even in late stationary phase (Figure 4), and accumulated then retained more toxins. In contrast, in ESTU < 0.2 cells at stationary phase, despite DIN left in the medium, decreasing Chl a and protein contents suggest that cells were facing N-limitation, consistently with lower and decreasing toxin content. Altogether, better NO₃⁻ uptake leading to a higher toxin production in the < 3μm treatment also supports the hypothesis of bacteria producing auxotrophic molecules (e.g. vitamins) that were utilized by the A. minutum in the processes of N uptake and toxin synthesis.

In the OYST < 0.2 treatment, the DIN:DIP ratio was moderately P-limiting. Rates of toxin production during growth were in the range of that found at 35 p.s.u. in the salinity experiment, but lower in early stationary phase, likely due to lower DIN concentration left in the medium. However, cells maintained high contents of protein and chlorophyll indicating N-replete cells (Béchemail et al., 1999). Cell toxin content also reflected N-repletion, remaining much higher than the 1.42 ± 0.58 fmol cell⁻¹ obtained in the N:P balanced condition (N:P ≈ 16) by Béchemail et al. (Béchemail et al., 1999). The decrease in cell toxin content during the early growth was likely due to toxin dilution in dividing cells. At stationary phase (from Day 6), as the medium was depleted from DIN, cell chlorophyll and protein contents tended to decrease. At the same time, toxin production stopped and cells began losing toxins.

Unlike in the three other coastal water treatments, PST production was very low in the OYST < 3 treatment. The toxin concentration in these cultures remained almost constant during growth, and consequently, the toxin content per cell decreased (Figure 5). During the log phase, the rate of decrease in cell toxin content (0.36–0.50 halving day⁻¹) appears to have been inversely proportional to the growth rate (0.44–0.50 div. day⁻¹). It appears, therefore, that the decrease in cellular toxin content was primarily due to dilution during cell division. The cell toxin content indeed stabilized during the stationary phase, at ~1 fmol cell⁻¹, which value was intermediate between those obtained in the N:P balanced condition (N:P ≈ 16; 1.42 ± 0.58 fmol cell⁻¹) and N-limiting condition (N:P ≈ 3.2; 0.58 ± 0.50 fmol cell⁻¹) in this strain by Béchemail et al. (Béchemail et al., 1999). This toxin content indicated moderate N-limitation in these cells, consistent with their low cell Chl a content in the range of N-limited cells in Béchemail et al. (Béchemail et al., 1999) and with their low cell protein content. As DIN was nearly exhausted prior to inoculating with A. minutum, we conclude that the new amino acid production (born from DIN uptake) was minimal in these cells. Alternatively, N may have been obtained mixotrophically, mostly from particulate organic sources, which were then digested and utilized by the dinoflagellate cells. As PST synthesis occurs via an arginine precursor (Cembella, 1998), we propose that these mixotrophically growing cells were unable to obtain sufficient precursor amino acids from the prey material to support PST synthesis in addition to production of new biomass required for cell division. In contrast, during heterotrophic growth of A. tamarense under N- or light-limiting conditions, Ogata et al. (Ogata et al., 1996) reported that the toxin content of the cells was either not affected or increased following the addition of yeast extract or bacteria. This discrepancy suggests that the influence of mixotrophy on PST production might depend on the nitrogenous content of their food, in particular, the amino acid precursors of PST.
Significance of our results for shellfish farming

At the first sight, areas of shellfish farming are not the most favourable for dinoflagellate growth and high PST production/accumulation by AM89BM-type *A. minutum*. High salinity and moderate inorganic nutrient concentrations, and possibly higher DOM concentration and/or microbial biomass favouring mixotrophic nutrition, are all conditions favouring low cell toxin accumulation. The most favourable conditions appeared to be of low salinity in estuaries. However, shellfish farms are generally located in the proximity of estuaries, but how toxic cells grown in estuaries could reach and impact shellfish toxicity may vary depending on environmental conditions specific to each area (such as coastal morphology or hydrological circulation). Locally, rainy periods followed by high runoff may bring lower salinities, and therefore conditions more favourable for *Alexandrium* growth and PST production, to shellfish farm areas. For shellfish farms located in the vicinity of small estuaries, such as those found in Brittany, situations where *Alexandrium* cells might encounter lowered salinity estuarine water for a long enough period might be complex. One possible scenario is that dinoflagellates might be maintained for a few days in the estuarine water during neap tides, which may prevent the rapid dispersion of the river plume. This would allow dinoflagellates to grow and accumulate toxins in optimum conditions. Then, with the transition to spring tides or wind-induced water mixing, highly toxic cells could be transported in the nearby bay harbouring the shellfish farms.

CONCLUSION

*Alexandrium minutum* strain AM89BM was found to be euryhaline, showing fast growth between 20 and 37 p.s.u. Cells grown under a high N:P ratio had the capacity to produce and accumulate high quantities of PST, especially at salinities ≤25 p.s.u. When grown in two coastal waters at similar salinities, *A. minutum* grew much faster than in enriched seawater media, while cell toxin content was significantly decreased. A possible cause is that the initial DIN and DIP concentrations (and subsequent N:P ratios) affected both growth and toxin production. In addition, the DOM present in the two coastal waters may have stimulated growth. In the nutrient-rich estuarine water, the fast growth may have counterbalanced the accumulation of the toxins in the cells, although the high cell biomass resulted in a high quantity of PST produced in the cultures at the end of growth. Our results suggest that *A. minutum* has the capability to grow mixotrophically, and that mixotrophy may result in decreasing cell toxin content. In *A. minutum* cultured in offshore seawater medium without the addition of vitamins and trace metals, the slow growth and the low cellular PST content suggest that some micro-nutrients are required for growth and toxin synthesis.

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