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Phosphorus limitation might promote more toxin content in the marine invader dinoflagellate *Alexandrium minutum*

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Abstract: *Alexandrium minutum* (strain AM89BM) has been grown in semi-continuous culture (0.2 vol. d^{-1}) in N-limiting (NO_3^- : PO_4^{3-} = 1.6 and 3.16), in P-limiting (N : P = 160 and 80), and in N and P balanced (N : P = 16) media. The toxin content in cells changed greatly according to the N : P ratio. Cells grown in N : P balanced condition showed an average total paralytic shellfish poisoning (PSP) content of 1.24 fmol $cell^{-1}$. In N-limiting conditions, cells contained ca. 3 times less toxin with mean values of 0.41–0.45 fmol $cell^{-1}$. In contrast, cells grown in P-limiting conditions contained on average 3.5 and 7 times more toxins than in the balanced N : P condition: 4.31 fmol $cell^{-1}$ in the N : P = 160 medium and 8.01 fmol $cell^{-1}$ in the N : P = 80 medium. The toxin content per carbon unit shows the same trend; the lowest content in N : P < 16 conditions was 6.5 fmol PSP nmol C^{-1}, whereas in the N : P = 16 condition it was 3.3 times higher at 21.8 fmol PSP nmol C^{-1} and 9 times higher in N : P > 16 conditions at 59.0 fmol PSP nmol C^{-1}. Since present trends in nutrient loading are assumed to have skewed some historically stable situations towards higher N : P ratios, the stimulation of toxin production by P-limiting media has important ecological consequences; in niches where DIN : DIP is >16, populations of *A. minutum* sufficiently concentrated to represent a significant fraction of shellfish food could pose a serious problem.

Key words: *Alexandrium minutum*, phosphorus limitation, toxin content, PSP

Introduction

The marine dinoflagellate *Alexandrium minutum* Halim produces toxins responsible for paralytic shellfish poisoning (PSP) in vertebrates, particularly human consumers of molluscs having eaten the alga (Oshima et al. 1989). Until 1988, *A. minutum* had been known only from Egypt (Halim 1960), but has since been recorded in many other locations, such as the Mediterranean Sea (Forteza et al. 1998; Poletti et al. 1998), the Atlantic coastal waters of Spain (Delgado et al. 1998), France and Ireland (Erard-Le Denn 1997), the Baltic Sea (Nehring 1994), Australia, North America, Thailand, Taiwan and Japan (Hallegraeff 1995). Dispersal by human means, mainly deballasting (Hallegraeff 1998) and inadvertent transfer of detrimental species with translocation of shellfish stocks (Honjo et al. 1998), is well documented. Respective importances of increased scrutiny and dispersal are still unclear, however.

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It remains controversial whether certain environmental conditions favour toxin occurrence in cells. Saxitoxins' content being enhanced by phosphorus deficiency has been observed in *Alexandrium fundyense* and *A. tamarense* (Anderson et al. 1990), whereas Matsuda et al. (1996) reported that the cell toxin content decreased as the cell quota for nitrogen decreased in *A.catenella*. Previous batch-culture experiments with *Alexandrium minutum* have shown no conclusive differences between the toxin contents in cells grown under different nutrient regimes (Flynn et al. 1994, 1995).

By using semi-continuous cultures, we have cultured *A. minutum* in nitrogen-limiting and phosphorus-limiting media. Toxin contents of N- or P-deprived cells have been compared with those of control cells.

Materials and Methods

Five NO_3^- : PO_4^{3-} (atom : atom) regimes were established, each in 5 replicates: phosphorus limiting (N : P = 160

Table 1. Mean carbon (POC), nitrogen (PON) and phosphorus (POP) cell contents in *Alexandrium minutum* grown under different NO_3^-:PO_4^{3-} conditions. Numbers between parentheses are SD (n=30).

N:P	POC (pmol cell^{-1})	PON (pmol cell^{-1})	POP (pmol cell^{-1})
160	193 (62)	19.5 (6.1)	0.46 (0.24)
80	144 (27)	16.5 (3.3)	0.28 (0.11)
16	76 (19)	8.6 (2.2)	0.41 (0.08)
3.16	81 (19)	5.5 (1.3)	0.31 (0.09)
1.6	83 (20)	5.1 (1.4)	0.45 (0.20)

and 80; NO_3^-: 60 μM), balanced N and P condition (N:P=16; NO_3^-: 60 μM; PO_4^{3-}: 3.75 μM), and nitrogen limiting (N:P=3.16 and 1.6; PO_4^{3-}: 3.75 μM). Four-liter cultures were run as batch cultures for five days. Then a semi-continuous-culture regime was established: during the first three days, 500 ml of culture per bottle were removed and replaced by fresh medium (0.125 volume d^{-1}), and for the remainder the replaced volume was increased to 800 ml (0.2 volume d^{-1}). On Days 9, 10, 11, 12, 27, 29, 33 and 36, the removed culture fractions were used for analyses. Subsamples for cell counts (Utermöhl 1931), nutrient concentrations (Strickland & Parsons 1972; Valderrama 1995) and toxin analyses (Hummert et al. 1997; Yu et al. 1998) were taken for all these fractions; subsamples for biomass analyses (POC, PON: Bendschneider & Robinson 1952; POP: Pujo-Pay & Raimbault 1994) were taken for all fractions but two: on Days 10 and 11. More details can be found in Bechemin et al. (1999).

Results

When limiting, nutrients added daily within the renewal volume were taken up until exhaustion; most residual concentrations (culture medium with cells removed) were near the limits of detection. In the two nitrogen-limiting media (N:P=1.6 and 3.6), the values were 0.21 (±0.26, n=30) and 0.24 (±0.25, n=30) μM N-NO_3^-, respectively. Similar reciprocal trends occurred in the two phosphorus-limiting media (N:P=160 and 80); residual PO_4^{3-} concentrations were 0.08 (±0.05, n=30) and 0.08 (±0.11, n=30) μM, respectively. In contrast, large amounts of non-limiting nutrients remained: 39.68 (±12.98, n=30) and 28.22 (±15.35, n=30) μM N-NO_3^-, in the two nitrogen-rich media, and 2.66 (±0.58, n=30) and 3.03 μM (±0.81, n=30) μM P-PO_4^{3-}, in the two phosphorus-rich media, respectively. In the nitrogen:phosphorus balanced medium (N:P=16), residual NO_3^- concentration always remained very low, 0.15 (±0.09, n=30) μM, whilst the mean remaining PO_4^{3-} concentration was somewhat higher, 0.27 (±0.20, n=30) μM.

The cell content of C and N varied considerably according to the N:P growth conditions (Table 1). Cells growing in phosphorus-limiting media (N:P=80 and 160) contained 2–3 times more carbon and nitrogen than cells grown in the N:P balanced medium. Additionally, cells that grew in nitrogen-limiting media (N:P=3.16 and 1.6) contained 1/3 less nitrogen. In contrast, the phosphorus content per cell decreased by 30% in the N:P=80 treatment only (Table 1).

The toxin content of cells changed considerably according to the N:P ratio. For total PSP toxin concentrations, cells grown under the N:P balanced condition (N:P=16) had a content of 1.24±0.32 fmol cell^{-1} (n=34). At lower N:P ratios the cell contents were ca. 3 times lower: 0.41±0.26 fmol cell^{-1} (n=35) for N:P=3.16, and 0.45±0.26 fmol cell^{-1} (n=36) for N:P=1.6. At the two higher N:P ratios, the cell contents were 3.5 and 6.5 times higher than at the balanced N:P value, but the highest content was not recorded for the highest N:P treatment; for the medium concentration treatment (N:P=160), the toxin cell content was 4.31±2.52 fmol cell^{-1} (n=31), while it peaked at 8.01±2.96 fmol cell^{-1} (n=35) in the N:P=80 medium.

Discussion

Both the carbon and nitrogen contents of cells grown under the P-limiting conditions were significantly higher than those in cells grown under the N-limiting conditions: roughly twice as much carbon, and three times more nitrogen (Table 1). Flynn et al. (1994) also observed almost exactly the same difference. Cells grown under the N:P balanced condition had a carbon content similar to cells grown under N-limiting conditions, while their nitrogen content was in-between those of cells grown under P-limiting or N-limiting conditions. Our values for cells grown in P-limiting media are higher than those reported by Flynn et al. (1994); we have no clear explanation for this difference. The PON:POP ratio was >50 for cells grown in phosphorus-limiting media, <21 for cells grown in nitrogen-limiting media, and 21 for cells grown in the N:P balanced condition (Table 2).

Sakshaug & Holm-Hansen (1977) concluded that the point of change from N- to P-deficiency in cells would be PON:POP=23 for *Skeletonema costatum* and 45 for *Pavlova lutheri*. Maestrini & Kossut (1981) recorded PON:POP=11.5–19.7 in N-deficient, and PON:POP=31.8–35.5 in P-deficient cells of *Thalassiosira pseudonana*. According to Healey & Hendzel (1980), who reviewed a large array of results, a C:P >260 and a N:P ratio >43 (atom:atom) reflect a severe phosphorus deficiency in the cell content, whereas a C:N >14.3 reflects a severe nitrogen deficiency. Moreover, Hillebrand & Sommer (1999) concluded that with a C:N>10 and a N:P<13 in cells, the microalgae are nitrogen limited, whereas with a C:P>180 and a N:P>22 they are phosphorus limited.

Accordingly, the mean values we obtained for *A. minutum* grown in N-limiting media (POC:PON=15.0 and 16.4, N:P=12 and 18) would reflect a nitrogen deficiency

Table 2. Mean POC : PON, POC : POP and PON : POP of *Alexandrium minutum* grown under different NO_3^- : PO_4^{3-} conditions. Numbers between parentheses are SD (n=30).

N : P	POC : PON	POC : POP	PON : POP
160	10.0 (1.4)	516 (267)	50 (20.0)
80	8.9 (1.0)	560 (285)	62 (26.6)
16	8.8 (2.5)	184 (25)	21 (2.9)
3.16	15.0 (1.4)	266 (32)	18 (3.4)
1.6	16.4 (1.8)	203 (52)	12 (3.5)

in the cell contents, whereas values for the P-limiting media (POC : POP=516 and 560, N : P=50.3 and 61.7) would reflect a phosphorus deficiency. Nevertheless, the fact that the phosphorus content per cell decreased by 30% in the N : P=80 treatment only, prompts us to be cautious. Altogether, the results indicate that cells grown under the two N : P<16 conditions were nitrogen-deficient, while it is unclear whether contents of cells grown in N : P=80 and N : P=160 conditions were phosphorus-deficient in the full sense—namely that the cell metabolism was affected by a lack of phosphorus. It remains a fact, however, that the organic content of cells grown in the high N : P media was skewed towards an accumulation of carbon and nitrogen; accordingly, we conclude that these cells were phosphorus-deprived and nitrogen-surfeit.

Whether expressed per cell or per unit carbon biomass, there was a big increase in toxin content as P deprivation and N supply were increased (Fig. 1). Increases in cell volume and carbon content have frequently been reported to co-occur with P deficiency (Fuhs 1969; Fuhs et al. 1972). Likewise, Flynn et al. (1996) showed that *A. minutum* cells enlarged by up to 37% during prolonged P deprivation. Nevertheless, such changes in the cell volume cannot explain the ca. 20-fold higher toxin content in the cells grown under the N : P>16 conditions compared to N : P<16 conditions we observed; 8.0 and 0.4 fmol cell^{-1}, respectively. Moreover, the toxin content per carbon unit followed the same trend; for the N : P>16 condition it was 9 times higher than for the N : P=16 condition. Hence, we conclude that cells growing in high N : P media did indeed synthesise PSP toxins more actively than other cells.

This finding is in agreement with the results of Boyer et al. (1987) in *A. tamarense* and those of Anderson et al. (1990) in *A. fundyense*. In contrast, it differs somewhat from that of Flynn et al. (1994, 1995), who reported that toxin content in *A. minutum* declined with N or P deprivation. This discrepancy might partly be due to the different experimental designs. In Flynn et al.'s experiments, *A. minutum* was grown in batch culture, in the presence of high initial NO_3^- and PO_4^{3-} concentrations. All the highest toxin contents per cell were recorded in the early growth stages, when only a fraction of the nutrient reservoirs had been taken up. In contrast, during our experiments, cells exhausted daily the reservoir of the limiting nutrient; at the

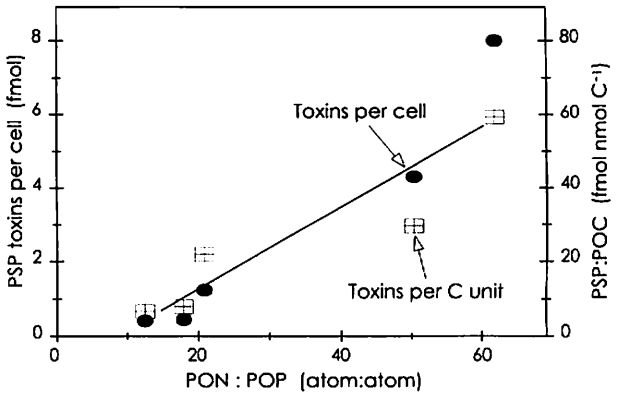

Fig. 1. PSP toxin content per cell and per POC unit in *Alexandrium minutum*, according to the PON : POP ratio in cells.

time of harvesting, concentrations of the limiting nutrients were below the detection levels.

The stimulation of toxin production by phosphorus deficiency in *A. minutum* has potentially important ecological consequences. In recent decades, proliferation in coastal waters of toxin-producing algae with accumulation of toxins in shellfish and fish has emerged as a major nuisance, affecting the shellfish farming and fisheries industries, as well as posing a danger to public health (Anderson 1997). Although toxic algal episodes are not new, anthropogenic activities appear to be increasing the extent and intensity of harmful algal blooms (Anderson 1997).

Nitrogen has for long been invoked as the principal nutrient limiting algal growth potential (Ryther & Dunstan 1971). However, human activities have significantly increased the input of nitrogenous and phosphorous nutrients to estuarine and coastal waters, while the silicon concentration has remained constant or has even decreased in river discharges as a result of large blooms of freshwater diatoms stimulated by the loading of nitrogen and phosphorus in inland waters (Schelscke & Stoermer 1972; Egge & Aksnes 1992). Hence, by and large, along with increased eutrophication in coastal waters, N : Si and P : Si ratios have increased (Conley et al. 1993; Rahm et al. 1996), thus favouring the proliferation of organisms having little or no requirement for silicon such as flagellates and cyanobacteria (Schöllhorn & Granéli 1996; Sandén & Håkansson 1996; Wasmund et al. 1998).

Moreover, decade-long data sets have led to the hypothesis for a shift from nitrogen to phosphorus limitation for large oceanic areas, such as the North Pacific gyre (Karl 1998). In coastal waters, current trends in nutrient loading are assumed to have skewed historically stable situations towards higher N : P ratios, thus pushing some coastal systems to phosphorus limitation (Rabalais et al. 1996; Billen & Garnier 1997; Hessen et al. 1997). Herbland et al. (1998), for instance, have reported NO_3^- : PO_4^{3-} values ranging from 62 to 161 in shelf waters of the Gulf of Biscay that are influenced by the River Gironde. In these areas, populations of *A. minutum* sufficiently concentrated to rep-

resent a significant fraction of shellfish food could pose a serious problem.

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