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Comparisons among species of *Alexandrium* (Dinophyceae) grown in nitrogen- or phosphorus-limiting batch culture

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Abstract Three species of the dinoflagellate genus *Alexandrium* (Halim) – two strains of toxic. *A. minutum*, one each of nontoxic *A. tamarense* and *A. affini* – were grown in batch culture in either a low-nitrogen or a low-phosphate medium. Maximum carbon-specific growth rates for *A. tamarense* were lower (at $< 0.25\ d^{-1}$) than for the other strains, which all exceeded $0.38\ d^{-1}$. C-quotas (C content per cell) during exponential growth were similar for all strains (~ 2.5 ng C cell^{-1}), with cells becoming smaller during the N-limiting stationary phase, but enlarging during prolonged P-deprivation. Values of $\delta\ ^{13}C$ during the exponential phase were low (-25 to -30), with most cells during the light phase swimming at the surface when nutrient-replete and migrating to the bottom of the flasks when nutrient-deplete with $\delta\ ^{13}C$ rising to around -15. Biomass could not be estimated reliably from pigmentation, but could be estimated from biovolume ($r > 0.95$), although this was complicated in cultures of *A. minutum* by the presence of particles comprized of thecal plates of a similar size to intact cells. Alkaline phosphatase activity was not a reliable indicator a P-status. The most toxic strain tested (*A. minutum* AL1V) contained the highest concentrations of free amino acids, of arginine (a precursor of paralytic shellfish toxins) and of proline, and also had the lowest C:N mass ratio (at 4.3). *A. affini* contained the lowest concentrations of arginine, and *A. tamarense* the highest exponential phase C:N (7.8). For all strains, the mole ratio of intracellular glutamine:glutamate (Gln:Glu, which was abnormally high compared to other algae) could only be used to indicate the presence or absence of N-stress rather than the degree of stress. Additions of ammonium and phosphate resulted in increases in Gln:Glu within 20 min in N-stressed cells and also enhanced toxin content in *A. minutum* (mainly gonyautoxin) 4 over a 24 h period.

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Introduction

Species of the genus *Alexandrium* (Halim) (previously *Gonyaulax* or *Protogonyaulax*; Steidinger and Moestrup 1990) form an important dinoflagellate group of global distribution (Balech 1988; Hallegraeff et al. 1991). Many strains are also toxic, producing paralytic shellfish toxins (PST), and are thus potentially damaging to public health and to shellfish industries (White et al. 1993). Various aspects of the ecophysiology of *Alexandrium* spp. have been reported, including stages of encystment and excystment (Lirdwitayaprasit et al. 1990; Bolch et al. 1991; Cannon 1993), toxin synthesis (Anderson et al. 1990), and ecology (Franks and Anderson 1992).

In this work we examined four strains of *Alexandrium* spp.: two toxic strains of *A. minutum* (isolated from Vigo, Spain), the Plymouth (UK) strain of *A. tamarense*, and one strain of *A. affini* (also from Vigo). The aims of this study were to compare these species and strains growing in nitrogen- or phosphate-limited batch culture with respect to (1) changes in growth rates, cell size, carbon and nitrogen quotas, values of $\delta\ ^{13}C$, alkaline phosphatase activity, and pigment content; (2) changes in the quantitative and qualitative content of intracellular amino acid (InAA), to see if the previously noted anomaly (absence of the non-protein amine X) in *A. tamarense* compared to other algae (Flynn et al. 1993) was typical of just that strain or of the genus; (3) testing the usefulness of measuring the initial response of the glutamine:

glutamate ratio (Gln:Glu, Flynn 1990) to a short-duration pulse of nutrients as an indicator of nutrient stress in *Alexandrium* spp. [this approach had been used on field populations of dinoflagellates (Flynn et al. 1994b) but not on cultured algae]; (4) testing the response of cells exposed to elevated nutrients with respect to toxin content; having identified N-upshock (i.e. N-refeeding of N-starved cells) as a factor promoting net toxin synthesis in *A. minutum* (Flynn et al. 1994a), we wished to test if a change in toxin content was apparent after exposure of this organism to ammonium and phosphate for only 24 h.

Materials and methods

Strains of *Alexandrium minutum* (AL1V and AL2V), and *A. affini* (PA5V) from Instituto Español de Oceanografia, Vigo, Spain, and *A. tamarense* (173) from Plymouth Marine Laboratory, Plymouth, UK, were grown as described by Flynn et al. (1994a) in modified K-medium (Keller and Guillard 1985) containing no organic buffer, ammonium or glycerophosphate, with either 100 μM nitrate (KNO_3) plus 20 μM phosphate (KH_2PO_2), hereafter termed "low-N medium", or 300 μM nitrate plus 5 μM phosphate ("low-P medium"). All media were sterilized by passage through 0.2 µm-pore Durapore (Millipore) filters. Cultures were grown at 17 °C in a 12 h light:12 h dark cycle at a photon flux density of 180 $\mu mol\, m^{-2}\, s^{-1}$, in static, non-aerated flasks. Even gentle aeration or stirring was found to adversely affect the growth of these dinoflagellates. Flasks were plugged with foam bungs, allowing some degree of gas exchange. The experimental flasks were all inoculated from early stationary-phase cultures grown in the low-N medium. Samples were withdrawn via a syphon tube after the culture had been mixed by gently swirling the flasks; sampling was performed at 6 h into the light phase.

In order to test if short-term additions of ammonium and phosphate promoted changes in glutamine:glutamate (Gln:Glu) which could be related to the C:N ratio, 20 ml volumes were withdrawn and spiked by the addition of 10 μM ammonium plus 2 μM phosphate, incubated in darkness for 20 min, and then sampled for intracellular amino acids (InAA). This was essentially the same protocol as that used on field populations by Flynn et al. (1994b). At dates selected to be representative of exponential, post-exponential and stationary phases, a similarly spiked sample of 100 ml was incubated for 24 h in the normal light/dark cycle and then sampled for InAA and, for strains of *Alexandrium minutum*, also for toxins.

Routine cell counts and cell-sizing were made on live cells using an Elzone 282 PC analyzer (Particle Data Inc.) with a 76 µm orifice and hydropulser mixer, calibrated with latex beads. Additional counts were made by microscopy using a Sedgewick-Rafter chamber, after killing the cells with Lugol's iodine. Cell suspensions were filtered at < 75 mm Hg through pre-ashed (450 °C for 6 h) 13 mm Gelman GA/E filters held in a custom-made holder. Cells and supernatants were frozen at − 20 °C until required. Nitrate and phosphate were measured in duplicate within 20 d of sampling by a micro-segmented flow analyzer (Alpkem RFA/2). The pigment content of extracts in dimethyl formamide (DMF) were estimated using the equations of Strickland and Parsons (1972); we have noted no significant difference in the absorbance coefficients between DMF and acetone, but DMF is a more efficient extractant. Cellular carbon and nitrogen, and the value of $\delta\ ^{13}C$, were determined in duplicate using Europa Scientific RoboPrep and Tracer Mass instruments with isoleucine as the standard. Primary amine components of intracellular amino acids were determined using the o-phthalatdehyde methodology described by Flynn and Flynn (1992). In addition, the secondary amine proline was determined in selected samples by the (fluoronylmethyl chloroformate)- FMOC-derivatization procedure and high-performance liquid chromatography, (HPLC) using methods supplied by Merck–Hitachi for use with the Hitachi AS-4000 preparative autosampler. Quantification of unidentified non-protein amines assumed one nitrogen atom per molecule, and an integration response-factor the same as for glutamate. Concentrations of InAA are given as mM with respect to cell volume. Alkaline phosphatase (AlkPase) activity was measured using p-nitrophenol phosphate as the substrate (Kuenzler and Perras 1965). Levels of AlkPase were the same in both fresh and frozen cells; any intracellular phosphatase released as a consequence of freezing presumably had insignificant activity at pH 9.8. The stability of AlkPase activity in frozen cells of *Alexandrium* spp. was found to decrease by only 5% over a storage period of up to 8 wk at − 20 °C. PSTs were estimated by HPLC after precolumn oxidation with periodate (Flynn and Flynn 1996).

The data presented here were obtained simultaneously for all treatments so as to remove any variability due to differences in sea water quality. The results are consistent with those obtained from studies of individual processes in these organisms on other occasions. The data for changes in toxins per cell for the cultures of *Alexandrium minutum* described in this paper have been presented in Experiment III of Flynn et al. (1994a).

Cell and biomass-specific growth rates (cell-μ and biomass-μ, respectively) were computed from $(\ln\ P_{t(n)}) - (\ln\ P_{t(n-1)}) \cdot (t(n) - t(n-1))^{-1}$, where P is the parameter measured at times $t(n)$ and $t(n-1)$ (in days).

Results

Nutrients

The times at which the *Alexandrium* spp. cultures exhausted their nitrate (thus becoming N-deprived) or phosphate (becoming P-deprived) are indicated in Figs. 2 to 5 and Fig. 7. For both strains of *A. minutum* growing in the low-P medium, P-deprivation was followed some time later by N-deprivation.

Cell counts and sizes

Alexandrium spp. released thecal plates into the growth medium during division. This material was responsible for a significant distribution in the size analysis of suspensions of these cells. For *A. minutum*, these plates remained together leaving "ghost cells" that appeared in the size spectrum as a distribution which overlapped with that of the living cells (Fig. 1). The total count of cells plus debris for *A. minutum* was 2 to 2.5 times the cell count, as determined by microscopy (increasing with age of the culture as an increasing number of cells died with the onset of the stationary phase). Corrected counts and biovolumes were determined by splitting the distribution at an inflection point judged from distribution on a population and on a biovolume basis (as indicated in Fig. 1), with verification from microscope counts every third day. Cell counts for the two strains of *A. minutum* in both nutrient regimes reached 25 000 to 30 000 ml^{-1}, with exponential-phase values of cell-μ exceeding 0.45 d^{-1}.

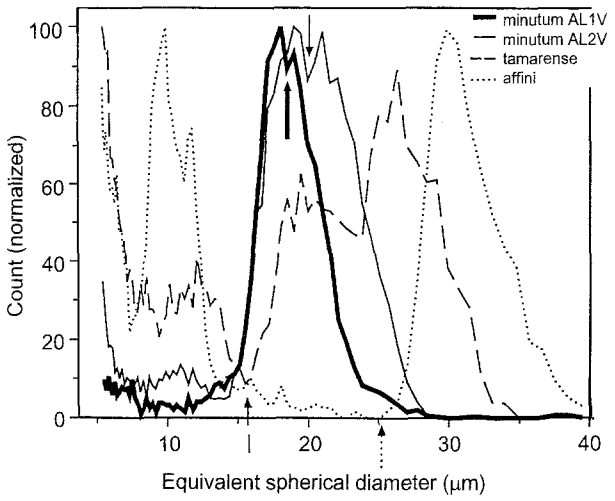

Fig. 1 *Alexandrium* spp. Particle-size distributions on population (count) basis for exponentially growing cultures (*Arrows* lower channel-settings for the various cell distributions; see "Results–Cell counts and sizes" for further details)

For *Alexandrium tamarense*, the debris of thecal plates formed a peak well separated from that of the cells, but this strain had two distinct size distributions (also visible my microscopy) with diameters around 20 and 27 µm (Fig. 1). *A. tamarense* grew at only half the rate of *A. minutum* (cell-μ of 0.23 d^{-1}) attaining 17 000 cells ml^{-1} in the low-N medium, and twice that number in the low-P medium.

The major proportion of *Alexandrium affini* grew either as single cells or as pairs (with fewer longer chains); in the same sample there could be large single cells similar in size to small paired cells. For *A. affini* it was therefore not possible to obtain true cell counts using the electronic counter, although the debris field was well separated from that of the cells (Fig. 1). *A. affini* growing in the low-N medium attained higher maximum counts than those in the low-P medium, with a maximum cell-μ similar to that of the *A. minutum* strains (Table 1).

Carbon and nitrogen

Carbon-specific growth rates, presented in Fig. 2a, b, were similar to cell-specific growth rates. Only *Alexandrium tamarense* maintained exponential growth for more than a few days under the batch-culture conditions employed.

Carbon $cell^{-1}$ for the strains of *Alexandrium minutum* attained a maximum value of 2.5 ng; in both strains, this value decreased to 20% of this value after prolonged N-deprivation but increased during N-deprivation following P-deprivation (Fig. 2c, d; Table 1). Cells of *A. tamarense* contained a similar amount of C as *A. minutum* (Fig. 2c, d). Values of C $cell^{-1}$ for *A. affini* were difficult to determine, because the paired or single cells were not discriminated by the counter. However, during N-deprivation, single cells of *A. affini* predominated, giving a value of 1.5 to 2 ng C $cell^{-1}$, while the C-content of the predominately paired cells in the P-deprived culture were twice this value (Fig. 2d), thus suggesting that the values of C $cell^{-1}$ for *A. affini* were similar to those for the other species.

Table 1 gives values of C $biovolume^{-1}$ for the cultures. Values were the same (Student's *t*-test $P < 0.05$) in cells of any one strain grown in low-N and in low-P media, except for *Alexandrium affini*, in which C $biovolume^{-1}$ was statistically higher (by 15%) in low-P cells after the lag phase. Beyond the lag phase, and considering the total populations for each strain, cells of *A. affini* had statistically lower values of C $biovolume^{-1}$ than *A. tamarense*. Cells of *A. minutum* AL1V contained 18% more C $biovolume^{-1}$ than AL2V; in general *A. minutum* cells contained almost

Table 1 *Alexandrium* spp. Biomass and growth parameters. Minimum cell quotas (C and N content per cell) and C:N (mass ratio) values are means of duplicate or triplicate estimates for at least three consecutive days, with a coVar covariance (standard deviation as % of mean) of < 10% (*Values in brackets* values for C $biovolume^{-1}$ computed with exclusion of data points before Day 10 for *A. tamarense* and before Day 5 for *A. affini*, when cells were recovering from lag phase) Cell volumes are given for cells grown in low-N and low-P conditions

	A. minutum		*A. tamarense*	*A. affini*
	AL1V	AL2V		
Cell-μ_{max} (d^{-1})	0.5	0.45	0.23	0.5
Minimum C-quota (ng $cell^{-1}$)	0.7	0.7	0.8	1.4
Minimum N-quota (ng $cell^{-1}$)	0.042	0.038	0.073	0.25
Minimum C:N	4.3	6.4	7.8	4.8
C $biovolume^{-1}$ (µg nl^{-1})	0.314	0.267	0.169 [0.14]	0.161 [0.15]
coVar (%)	(14%)	(13%)	(34%) [11%)	(20%) [10%)
Cell volume (fl) + coVar (%)				
low-N cells	4165 (43%)	4006 (53%)	7997 (14.8%)	14520 (23%)
low-P cells	4817 (28%)	5072 (37%)	7865 (9.3%)	18316 (17%)

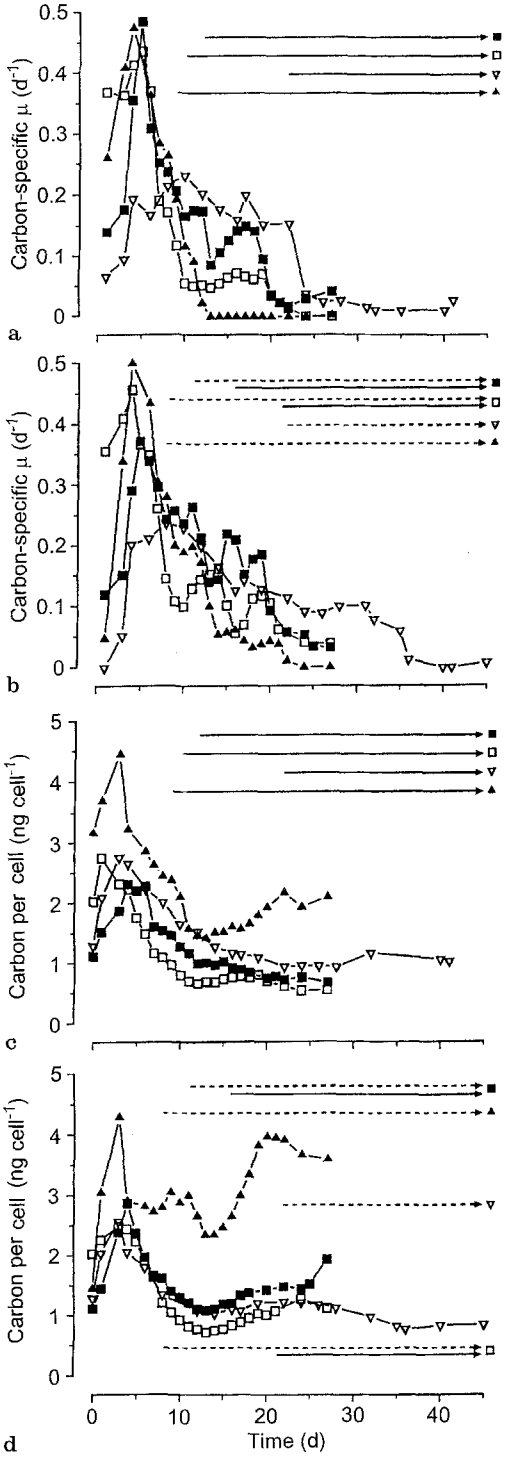

Fig. 2 *Alexandrium minutum* AL1V (■), *A. minutum* AL2V (□), *A. tamarense* (▽) and *A. affini* (▲). Carbon-specific growth rates (**a**, **b**) and carbon-quotas (**c**, **d**) of cultures grown in low-N medium (**a**, **c**), and low-P medium (**b**, **d**) (*Black arrows* nitrate-exhaustion; *dashed arrows* phosphate exhaustion)

twice the C biovolume^{-1} of the other species, were smaller (Fig. 1), and were thus presumably less vacuolate.

Values of cellular δ ^{13}C for all cultures except the low-N culture of *Alexandrium affini* (which remained

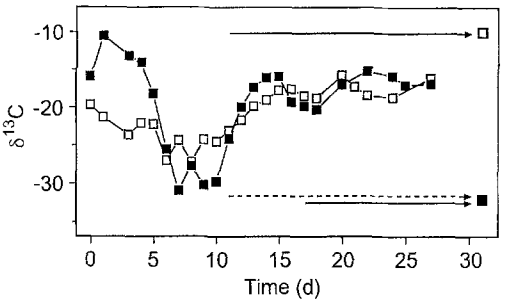

Fig. 3 *Alexandrium minutum* AL1V. Cellular δ^{13}C (□ cultures grown in low-N medium; ■ cultures grown in low-P medium; *arrows* as Fig. 2)

constant at −15) declined from −15 to around −25 to −30 during the exponential part of the growth cycle, increasing to around −20 afterwards. Data for *A. minutum* AL1V are shown in Fig. 3. The period of exponential growth was also marked for each culture by an increase in pH of the media from 7.9 to a maximum of 8.6 by the end of exponential growth, and then a drop to pH 8. During exponential growth the dinoflagellates were usually at the surface of the medium, while once the nutrients had been depleted they were mainly at the bottom of the flasks.

Minimum values of the mass C:N ratio (Fig. 4a, b; Table 1) show significant variation between the strains. For all cells in low-N medium, C:N increased on exhaustion of nitrate (Fig. 4a). P-deprivation caused an increase in C:N in *Alexandrium affini*, but initially a decrease in *A. tamarense* (Fig. 4b). C:P ratios (P content being computed from decreases in soluble reactive phosphate content in the media) were ~20 to 30 in P-sufficient cells (Fig. 4c, d) rising to a maximum of > 200 in *A. minutum* (AL1V) (Fig. 4d). *A. affini* did not show an elevated C:N ratio after exhaustion of the nitrate, although C:P values did increase during P-deprivation (Fig. 4d).

Pigments

In all flasks, the values of chlorophyll *a* C^{-1} (Table 2) and carotenoid C^{-1} (data not shown) increased during exponential growth and then declined to (< 20%) of the maximum. In all instances, chl$_a$ C^{-1} declined more rapidly in cells which had exhausted their nitrate. Values of chl$_a$:chl$_c$ remained constant at ~6 for the *Alexandrium minutum*, ~4 for *A. tamarense* and ~7 for *A. affini*. Values of the pigment-extraction absorbance-ratio A_{480}:A_{665} (suggested by Health et al. 1990 to correlate with C:N) did not correlate with C:N ratios for any of the cultures (data not shown).

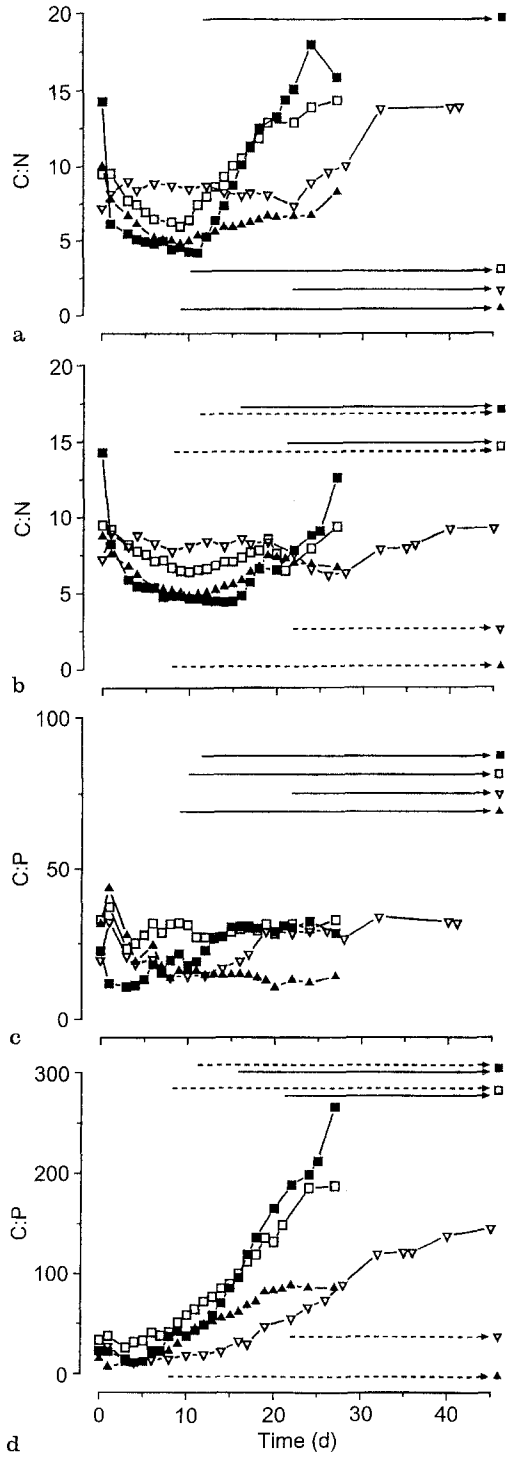

Fig. 4 *Alexandrium minutum* AL1V (■), *A. minutum* AL2V (□), *A. tamarense* (▽) and *A. affini* (▲). C:N mass ratios (**a, b**) and C:P mass ratios (**c, d**) for cultures grown in low-N medium (**a, c**), and low-P medium (**b, d**) (*Arrows* as in Fig. 2)

Alkaline phosphatase (AlkPase)

For both strains of *Alexandrium minutum*, AlkPase C^{-1} remained constant in cells growing in the low-N medium, but increased upon P-deprivation (Table 2). The increase in activity developed a few days after phosphate disappeared from the external medium. In *A. tamarense*, AlkPase C^{-1} remained relatively constant in both cultures, although it was always considerably higher (2 to 7 times) in the low-P than in the low-N culture. In *A. affini*, activity was relatively high at all time points, but it increased further on P-deprivation.

Intracellular amino acids

Following the rapid rise in InAA concentrations seen in all cultures during the first 5 d or so in response to the inoculation of previously N-deprived cells into fresh media, the highest concentrations of total InAA (200 m*M*) were seen in *Alexandrium minutum* AL1V (Fig. 5a, b). The other strains contained ~100 m*M* InAA during the exponential phase before exhaustion of the nutrients (Fig. 5). As a percentage of cell-N, however, the strains of *A. minutum* contained only ~4 to 5% as InAA-N, *A. tamarense* ~8%, and *A. affini* 6%. In *A. minutum* AL1V, this percentage declined in both low-N and low-P cells, while in the other strains during P-deprivation the percentage either remained stable or increased (Table 2). Only in *A. tamarense* did the concentration of InAA increase during P-deprivation (Fig. 5b).

The concentration of arginine, the most important component of InAA-N and a precursor in toxin synthesis (Shimizu et al. 1990), was highest in *Alexandrium minutum* AL1V, rising with P-deprivation to a maximum of > 30 m*M*. N-deprivation led to a decline in arginine (Fig. 5c). P-deprivation of *A. tamarense* led to an increase in arginine concentrations to levels similar to those seen in exponentially-growing *A. minutum* AL1V. *A. affini* had the lowest concentrations of arginine. As a percentage of InAA-N, arginine constituted ~30% for *A. minutum* and *A. tamarense*, but less (10 to 20%) for *A. affini*. In all instances the value fell during N-deprivation.

During the exponential phase (Fig. 6a), the InAA composition was dominated by glutamate, glutamine, arginine, alanine, the non-protein amine taurine and γ-aminobutyric acid (GABA). Proline was highest in *Alexandrium minutum* AL1V. During N-deprivation (Fig. 6b), these concentrations declined (except in *A. affini* which showed little evidence of developing pronounced N-stress – see C:N values in Fig. 4a). P-deprivation, however, did not lead to such decreases, and concentrations of some amino acids, notably arginine, increased (Fig. 6c).

The non-protein amine, taurine (Tau) and the amino acid GABA, were always present, together with small amounts of unidentified amine X2 (Flynn and Flynn 1992). Concentrations of taurine, reaching 20 m*M*, where highest in *Alexandrium minutum* AL1V, and consistently lowest (4 m*M*) in *A. tamarense*, although

Table 2 *Alexandrium* spp. Changes in chlorophyll *a* (*Chla* C^{-1}; ng μg^{-1}), alkaline phosphatase activity (AlkPase C^{-1}; arbitrary units) and intracellular amino acid (*InAA-N*) content (as % of cell nitrogen) in cultures at time points (days, *D*) selected to be representative of exponential phase and periods of increasing N and/or P-stress

	A. minutum								*A. tamarense*				*A. affini*			
	AL1V				AL2V											
	D6	D13	D17	D22	D6	D13	D16	D21	D10	D19	D27	D40	D6	D12	D17	D24
Chla C^{-1}																
Low-N	5.0	7.2	3.0	0.1	8.5	9.0	5.3	2.0	3.6	4.5	2.2	0.8	8.6	7.5	3.5	2.2
Mean (coVar)		3.5 (65%)				6.0 (59%)				3.3 (40%)				5.3 (55%)		
Low-P	5.0	7.2	5.3	2.8	8.0	13	11	6.0	3.6	4.6	3.0	1.6	10	12	6.0	2.0
Mean (coVar)		4.6 (49%)				8.3 (42%)				3.5 (43%)				6.5 (54%)		
AlkPase C^{-1}																
Low-N	2	2	3	1	6	6	5	5	2	1	3	1	10	10	10	14
Low-P	2	7	15	13	5	11	11	8	14	6	6	6	10	11	22	19
InAA-N																
Low-N	3.5	3.2	3.5	4.0	5.3	3.6	4.0	4.2	8.2	7.0	6.0	7.0	5.0	4.0	4.3	6.5
Low-P	4.0	4.0	3.8	2.8	5.0	4.0	5.7	5.5	10.0	9.5	11.0	18.0	5.2	3.5	6.3	4.8

values in all N-deprived cells became very low (< 1 m*M*). Concentrations of GABA attained higher concentrations in *A. minutum* AL1V (reaching 12 m*M*), than in *A. minutum* AL2V and *A. affini* (both 6 m*M*), with *A. tamarense* (4 m*M*) lower still. Again, concentrations fell during N-stress. The non-protein amine X found in other species of phototrophic dinoflagellates (Flynn and Flynn 1992) was not detected.

Glutamine:glutamate (Gln:Glu)

Exponential phase values of the mole ratio Gln:Glu were between 1 and 2 for all cultures (Fig. 7a, b), declining on N-deprivation to between 0.25 and 0.5 (Fig. 7a). P-deprivation also lead to a decline in Gln:Glu in *Alexandrium minutum* AL2V and *A. affini* (Fig. 7b).

Response of InAA to short-term nutrient pulsing

Changes in concentrations of most components of InAA following a short (20 min) incubation in the presence of 10 μM ammonium plus 2 μM phosphate were generally insignificant. Only Gln and the Gln:Glu ratio showed a response. Fig. 7c and d show the ratio of Gln:Glu before:after the incubation, where a value of 1 indicates no change. The greatest response was seen with cells grown in the low-N medium following exhaustion of the nitrate (e.g. *Alexandrium minutum* AL1V and *A. tamarense* – Fig. 7c), but responses were also seen in both N-deprived and P-deprived cells of *A. minutum* AL2V and *A. affini*. For both the latter strains, the magnitude of the increase in Gln:Glu diminished as the cultures passed into stationary phase (Fig. 7c, d).

Longer-term incubations after nutrient-pulsing

Incubations of 24 h following the addition of the ammonium plus phosphate pulse showed increases in concentrations of especially glutamate, glutamine, arginine and alanine of the order of 150% (data not shown). Generally the response was more marked in N-stressed cells (from the low-N medium) than in P-stressed cells (from the low-P medium).

Measurements of the toxin content of the two strains of *Alexandrium minutum* before and after a 24 h incubation following a spike of ammonium plus phosphate suggest that toxicity may be enhanced most in response to N-refeeding, although there were also increases in cells still growing in the presence of nitrate (Table 3). There was no clear relationship between changes in the toxin and arginine contents of these spiked cells.

Discussion

This study suggests that the amino acid physiology of *Alexandrium tamarense* (Flynn et al. 1993) is typical of the genus; the work of Scholin and Anderson (1994) shows that the species of *Alexandrium* we have used are not closely related. (*A. lusitanicum* strain AL2V referred to by Scholin and Anderson has subsequently been reclassified as *A. minutum*.) This genus has two unique features in this respect: (1) In *Alexandrium* spp. levels of Gln:Glu rise during the relief of N-stress (N-upshock) and fall during N-stress (as is normal in algae), but the values are always higher than seen in other algae, which have Gln:Glu values of ~0.5 and 0.1 for N-replete and N-deplete cells, respectively (Flynn 1990), in contrast with values of 1 to 2 and 0.3, respectively, in *Alexandrium* spp. (Fig. 7). (2) Of all dinoflagellates we have

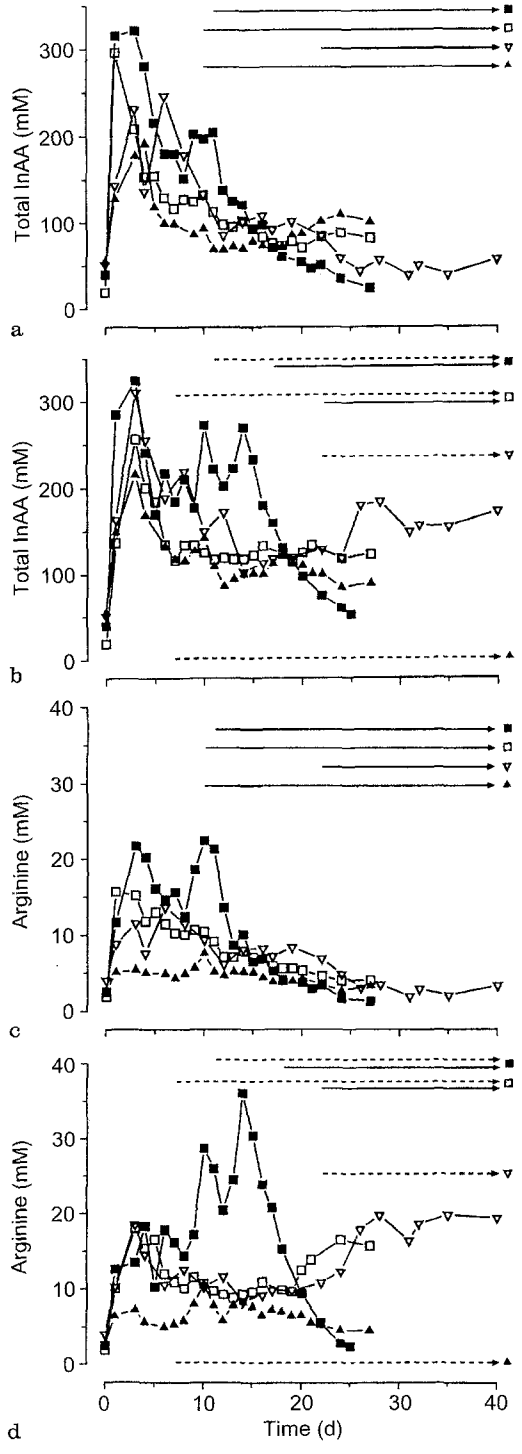

Fig. 5 *Alexandrium minutum* AL1V (■), *A. minutum* AL2V (□), *A. tamarense* (▽) and *A. affini* (▲). Concentrations of total intracellular free amino acids (**a**, **b**) and of arginine (**c**, **d**) for cultures grown in low-N medium (**a**, **c**), and low-P medium (**b**, **d**) (*Arrows* as in Fig. 2)

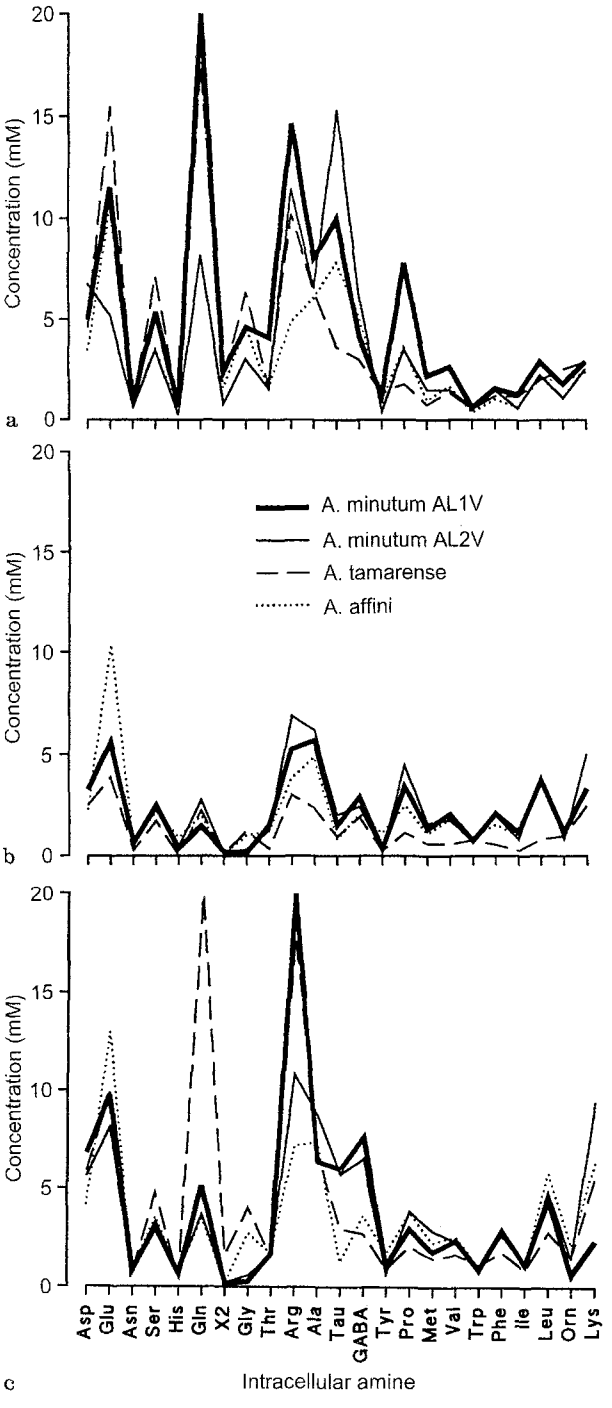

Fig. 6 *Alexandrium* spp. Polygons showing concentrations of intracellular amino acids during exponential growth (**a**), N-deprivation (**b**), and P-deprivation (**c**). Order of amines corresponds with their elution order from HPLC (*Asp* aspartate; *Glu* glutamate; *Asn* asparagine; *Ser* serine; *His* histidine; *Gln* glutamine; *X2* unidentified amino acid (see Flynn and Flynn 1992); *Gly* glycine; *Thr* threonine; *Arg* arginine; *Ala* alanine; *Tau* taurine; *GABA* γ amino butyric acid; *Tyr* tyrosine; *Pro* proline; *Met* methionine; *Val* valine; *Trp* tryptophan; *Phe* phenylalanine; *Ile* isoleucine; *Leu* leucine; *Orn* ornithine; *Lys* lysine)

examined, the *Alexandrium* spp. are unique in that no detectable amounts of the non-protein amine X (Flynn and Flynn 1992) were present. They did, however, contain the non-protein amines taurine (typical of dinoflagellates) and γ-aminobutyric acid (GABA, which has a role in polyamine metabolism in plants; Flores et al. 1989). The concentrations of both these amines declined to very low levels during N-deprivation of *Alexandrium* spp. Although the most toxic of the strains

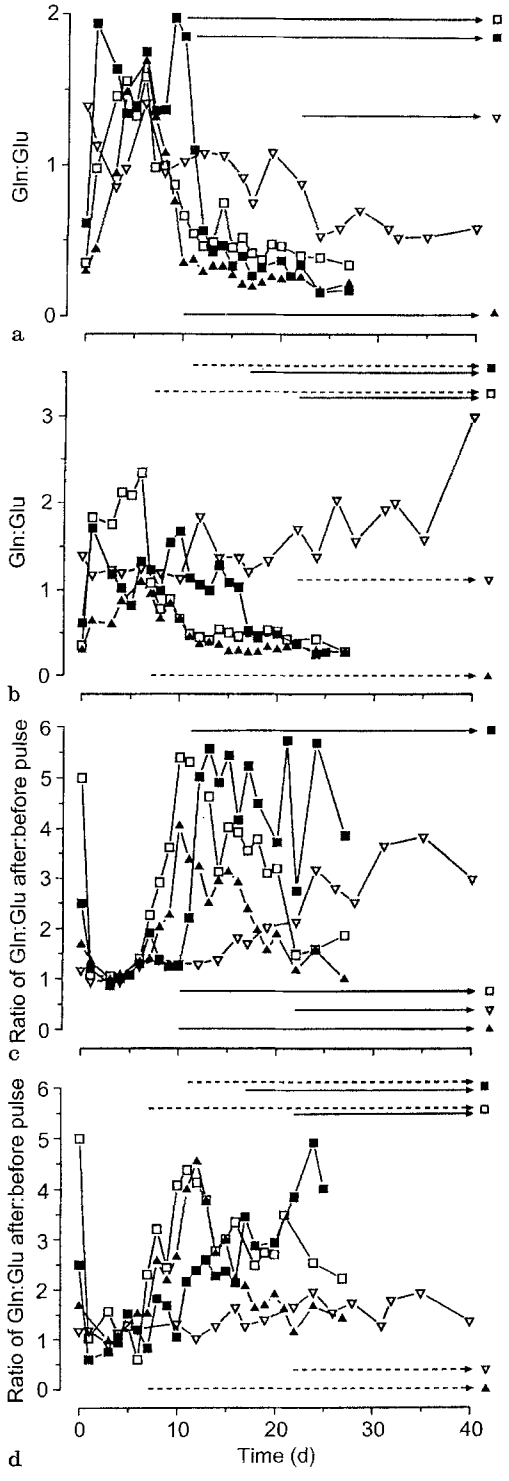

Fig. 7 *Alexandrium minutum* AL1V (■), *A. minutum* AL2V (□), *A. tamarense* (▽) and *A. affini* (▲). Glutamine:glutamate (*Gln:Glu*) ratios (**a**, **b**) and changes in Gln:Glu ratios following 20 min incubation in darkness with added ammonium and phosphate (**c**, **d**, where value of 1 indicates no change). Cultures were grown in low-N medium (**a**, **c**), or low-P medium (**b**, **d**) (*Arrows* as in Fig. 2)

tested (*A. minutum* AL1V) contained the highest concentration of GABA, there is no reason to suspect that its presence is related to toxicity, as GABA was also present in nontoxic strains.

Table 3 *Alexandrium minutum* AL1V and AL2V. Ratios of toxin content (95% gonyautoxin 4) and free arginine content in cells after and before 24 h incubation with added ammonium plus phosphate. Value of 1 indicates no change

AL1V, after:before			AL2V, after:before		
Day	Toxin	Arginine	Day	Toxin	Arginine
Low-N culture					
8	0.84	1.12	6	1.73	0.68
17	1.25	1.10	13	2.7	1.24
22	1.47	0.47	20	1.21	0.93
Low-P culture					
8	1.06	0.09	6	1.42	1.11
18	0.64	1.07	13	1.17	1.28
25	0.8	1.34	16	1.27	1.16
			21	0.84	1.12

The concentration of arginine, a precursor in the synthesis of paralytic shellfish toxins (Shimizu et al. 1990), was also highest during exponential growth in the more toxic *Alexandrium minutum* AL1V. As this strain also had higher values of C biovolume^{-1} (Table 1), and was thus presumably less vacuolate, the real cellular concentrations of arginine were possibly higher again in comparison with Strain AL2V and with the other species. Flynn et al. (1994a) considered that there was no simple relationship between the presence of toxin and arginine in *A. minutum*; that P-stressed, non-toxic, *A. tamarense* could have arginine concentrations as high as in toxic *A. minutum* supports this contention. Proline, an amino acid closely related to arginine in biosynthesis, was also present at highest levels in the most toxic strain during exponential growth (Fig. 6a). While the concentrations of arginine in *Alexandrium* spp. are higher than is typical for microalgae, the situation with proline is not known, as it is not normally determined. The problem remains, however, that in the absence of information concerning the localized concentration of these compounds, it is not possible to rule out a link between high concentrations of arginine, proline and PSTs.

The composition of InAA, and the concentration of its components, generally decreased in response to both N-stress and P-stress. Anderson et al. (1990) suggest that InAA concentrations may increase in response to P-stress. Such increases are reflected in our data, although the changes were limited to a few components, notably arginine (Fig. 5). Given the important role of phosphate in cell metabolism and its regulation, one may expect only a transient increase in amino acids in response to P-stress. However, differences may become apparent over longer periods of P-stress (for example when growing in continuous culture with a medium of high N:P ratio).

The response of Gln:Glu in N-deprived cells to the addition of ammonium was, predictably (Flynn 1990), an increase in the ratio (Fig. 7). However, the

magnitude of changes in Gln:Glu could not be used as a predictive tool to determine the N-status or C:N ratio of the organisms tested. Thus, determination of the Gln:Glu ratio after:before spiking with ammonium (Fig.7c, d) can only be used as an "all or nothing" indicator of N-stress in *Alexandrium* spp., as may methods measuring the dark enhancement of C-fixation on enrichment in other algae (Gilbert et al. 1985). Interestingly, P-stressed cells in the presence of excess nitrate also showed increases in Gln:Glu when pulsed with ammonium plus phosphate, although whether this indicated relief of P-stress or "preferential" uptake of ammonium is not known.

There were changes in toxin content in *Alexandrium minutum* following a 24 h incubation with added ammonium plus phosphate (Table 3). These responses were seen following the addition of relatively small amounts of ammonium (10 μM to cultures grown on 100 or 300 μM nitrate), and it is possible that proportionately larger additions would have stimulated more toxin synthesis. Although these are only preliminary results, they are consistent with our previous suggestion that the development of toxicity in *A. minutum* is an "upshock" phenomenon during the recovery from stress (Flynn et al. 1994a).

Cell sizes of *Alexandrium* spp. varied more in the low-N cultures (Table 1), with N-deprived cells being smaller. The C:N ratios for all of the cultures showed such variability (exponential phase ratios between 4.3 and 7.8) that it would be difficult to reliably ascribe a N-status to a species of *Alexandrium* simply from knowledge of the C:N ratio. Likewise, the variability of the activity of alkaline phosphatase makes the use of this as an indicator of P-stress less than straightforward. Further, in the cultures of *A. minutum* which exhausted phosphate, phosphatase activity either fell (AL2V) or remained constant (AL1V) on subsequent exhaustion of the nitrate (Table 2).

Our results indicate that pigments should not be used to estimate biomass, cell number, or the C:N ratio (from pigment-extraction absorbance-ratio $A_{480}:A_{665}$; Heath et al. 1990) for *Alexandrium* spp. In contrast, good correlations were obtained between C-biomass and biovolume ($r > 0.99$); this was so even when the "ghosts" formed by thecal plates within the biovolume for *A. minutum* were included, although the value of C biovolume^{-1} would then be incorrect. However, particle sizes such as the Particle Data or Coulter instruments for counting and sizing *Alexandrium* spp. must be used with caution, especially when employing simpler, older instruments (such as that used by Montagnes et al. 1994), since these do not provide full size distributions.

Our data indicate that for *Alexandrium minutum* the ratio of cells:"ghosts" is 1 for exponentially growing cells. In the stationary phase, where some cells may have been dying, the proportion of "ghosts" increased, with no evidence of a significant disintegration of the "ghosts" liberating smaller particles. The liberation of thecal plates in this form may complicate the use of flow cytometers to identify some species of immunolabelled toxic dinoflagellates (Adachi et al. 1993; Vrieling et al. 1994). Antibodies may be raised inadvertently both to intact cells and to "ghosts", while those raised to intact cells may bind to external features which may remain present in "ghosts". Chlorophyll fluorescence would separate the two particle types, but often it is desirable to bleach out the pigment because it masks fluorescence from the immunolabel.

Dinoflagellates are relatively large algae which, especially when present at high cell densities, may be expected to be more prone to dissolved inorganic carbon (DIC)-stress due to the thickness of their boundary layer (Raven 1991). Growth on nitrate (in contrast to growth on ammonium) will increase pH and drive the DIC dissociation to even lower proportions of dissolved CO_2. Anderson et al. (1990) mention that some of their cultures of *Alexandrium* spp. appeared to stop growing due to DIC exhaustion, although they were not sure if pH was also implicated in the event. A common problem encountered during studies of dinoflagellates is the premature cessation of growth in the presence of excess macronutrients (Dixon and Syrett 1988; K.J. Flynn unpublished data). The ability of cultures of *Alexandrium* spp. to attain low values of $\delta^{13}C$ (elevated values indicate increasing utilization of ^{13}C relative to ^{12}C as DIC-stress increases: Johnston and Raven 1992) during exponential growth may be attributable to the accumulation of cells at the surface of the medium during that period. Given the initial concentration of DIC in sea water (2 mM, or 24 $\mu g\,C\,ml^{-1}$), the concentration of nitrate (100 or 300 μM, 1.4 or 4.2 $\mu g\,N\,ml^{-1}$), and a final C:N ratio of $\simeq 15$ (Fig. 4), it is apparent that growth during the later stages of the experiments may have been rate-limited by the entry of atmospheric CO_2 into the flasks (flasks were not sealed, thus allowing such gaseous exchange). Aeration of cultures of dinoflagellates is often not an option, because the turbulance created may adversely affect growth. A solution is not to use N-substrate concentrations $> 100\ \mu M$ and to consider the addition of bicarbonate to raise the DIC content above the normal 2 mM (a value which will be decreased very considerably if autoclaving rather than filter-sterilization is employed during media preparation).

In conclusion, the genus *Alexandrium* appears to have an abnormal amino acid physiology compared to other dinoflagellates, and neither pigmentation or Alk-Pase activity were found to be of use as indicators of biomass or phosphate-stress, respectively. There were no simple remarkable differences between the growth dynamics and nutrient physiology between toxic and non-toxic strains, except that cells of the toxic species *A. minutum* contained more nitrogen at the whole-cell level and higher concentrations of some amino acids during exponential growth.

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References

Adachi M, Sako Y, Ishida Y (1993) Application of monoclonal antibodies to field samples of *Alexandrium* species. Jap Soc Scient Fish 59: 1171–1175

Anderson DM, Kulis DM, Sullivan JJ, Lee C (1990) Dynamics and physiology of saxtitoxin production by the dinoflagellates *Alexandrium* spp. Mar Biol 104: 511–524

Balech E (1988) Los dinoflagelados del Atlantico Sudoccidental. Publnes esp Inst españ Oceanogr, Madrid 1: p 310

Bolch CJ, Blackburn SI, Cannon JA, Hallegraeff GM (1991) The resting cyst of the red-tide dinoflagellate *Alexandrium minutum* (Dinophyceae). Phycologia 30: 215–219

Cannon JA (1993) Germination of the toxic dinoflagellate *Alexandrium minutum* from sediments in the Port River, South Australia. In: Smayda TJ, Shimizu Y (eds) Toxic Phytoplankton blooms in the sea. Elsevier, Amsterdam, pp 103–107

Dixon GK, Syrett PJ (1988) The growth of dinoflagellates in laboratory cultures. New Phytol 109: 297–302

Flores HE, Protacio CM, Signs MW (1989) Primary and secondary metabolism of polyamines in plants. Recent Adv Phytochem 23: 329–393

Flynn K, Flynn KJ (1996) An automated HPLC method for the rapid analysis of paralytic shellfish toxins from dinoflagellates and bacteria using precolumn oxidation at low temperature. J exp mar Biol Ecol (In press)

Flynn K, Flynn KJ, Jones KJ (1993) Intracellular amino acids in dinoflagellates; effects of diural changes in light and of N-supply. Mar Ecol Prog Ser 100: 245–252

Flynn K, Franco JM, Fernandez P, Reguera B, Zapata M, Wood G, Flynn KJ (1994a) Changes in toxin content, biomass and pigments of the dinoflagellate *Alexandrium minutum* during nitrogen refeeding and growth into nitrogen or phosphorus stress. Mar Ecol Prog Ser 111: 99–109

Flynn KJ (1990) The determination of nitrogen status in microalgae. Mar Ecol Prog Ser 61: 297–307

Flynn KJ, Flynn K (1992) Non-protein amino acids in microalgae: consequences for the estimation of the glutamine/glutamate ratio. Mar Ecol Prog Ser 89: 73–79

Flynn KJ, Jones KJ, Raine R, Richard J, Flynn K (1994b) Use of intracellular amino acid analysis as an indicator of the physiological status of natural dinoflagellate populations. Mar Ecol Prog Ser 103: 175–186

Franks PJS, Anderson DM (1992) Alongshore transport of a toxic phytoplankton bloom in a buoyancy current: *Alexandrium tamarense* in the Gulf of Maine. Mar Biol 112: 153–164

Glibert PM, Dennett MR, Goldman JC (1985) Inorganic carbon uptake by phytoplankton in Vineyard Sound, Massachusetts. II. Comparative primary productivity and nutritional status of winter and summer assemblages. J exp mar Biol Ecol 86: 101–118

Hallegraeff GM, Bolch CJ, Blackburn SI, Oshima Y (1991) Species of the toxigenic dinoflagellate genus *Alexandrium* in southeastern Australian waters. Botanica mar 34: 575–587

Heath MR, Richardson K, Kioerboe T (1990) Optical assessment of phytoplankton nutrient depletion. J Plankton Res 12: 381–396

Johnston AM, Raven JA (1992) Effect of aeration rates on growth rates and natural abundance $^{13}C/^{12}C$ ratio of *Phaeodactylum tricornutum*. Mar Ecol Prog Ser 87: 295–300

Keller MD, Guillard RRL (1985) Factors significant to marine dinoflagellate culture. In: Anderson DM, White AW, Baden DG (eds) Toxic dinoflagellates. Elsevier, New York, pp 113–116

Kuenzler EJ, Perras JP (1965) Phosphatases of marine algae. Biol Bull mar biol Lab, Woods Hole 128: 271–284

Lirdwitayaprasit T, Nishio S, Montani S, Okaichi T (1990) The biochemical processes during cyst formation in *Alexandrium catanella*. In: Granelli E, Sundström B, Edler L, Anderson DM (eds) Toxic marine phytoplankton. Elsevier, New York, pp 294–297

Montagnes DJS, Berges JA, Harrison PJ, Taylor FJR (1994) Estimating carbon, nitrogen, protein, and chlorophyll *a* from volume in marine phytoplankton. Limnol Oceanogr 39: 1044–1060

Raven JA (1991) Implications of inorganic carbon utilization: ecology, evolution and geochemistry. Can J Bot 69: 908–924

Scholin CA, Anderson DM (1994) Identification of group-specific and strain-specific genetic markers for globally distributed *Alexandrium* (Dinophyceae). I. RFLP analysis of SSU ribosomal-RNA genes. J Phycol 30: 744–754

Shimizu Y, Gupta S, Pradad AVK (1990) Biosynthesis of dinoflagellate toxins. In: Granelli E, Sundström B, Edler L, Anderson DM (eds) Toxic marine phytoplankton. Elsevier, New York, pp 62–71

Steidinger KA, Moestrup Ø (1990) The taxonomy of *Gonyaulax*, *Pyrodinium*, *Alexandrium*, *Gessnerium*, *Protogonyaulax* and *Goniodoma*. In: Granelli E, Sundström B, Edler L, Anderson DM (eds) Toxic marine phytoplankton. Elsevier, New York, pp 522–523

Strickland JDH, Parsons TR (1972) A practical handbook of seawater analysis. 2nd edn. Bull Fish Res Bd Can 167: 1–310

Vrieling EG, Peperzak L, Gieskes WWC, Veenhuis M (1994) Detection of the ichthyotoxic dinoflagellate *Gyrodinium* (cf.) *aureolum* and morphologically related *Gymnodinium* species using monoclonal antibodies: a specific immunological tool. Mar Ecol Prog Ser 103: 165–174

White AW, Nassif J, Shumway SE, Whittaker DK (1993) Recent occurrence of paralytic shellfish toxins in offshore shellfish in the northern United States. In: Smayda TJ, Shimizu Y (eds) Toxic phytoplankton blooms in the sea. Elsevier, Amsterdam, pp 435–440