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Changes in toxins, intracellular and dissolved free amino acids of the toxic dinoflagellate *Gymnodinium catenatum* in response to changes in inorganic nutrients and salinity

Kevin J.Flynn[1,5], Krystyna Flynn[1], Eurgain H.John[1], Beatriz Reguera[2], Maria I.Reyero[2] and José M.Franco[3,4]

[1]*Swansea Algal Research Unit, School of Biological Sciences, University of Wales Swansea, Singleton Park, Swansea SA2 8PP, UK,* [2]*Instituto Español de Oceanografía, Apdo 1552, 36280, Vigo, and* [3]*Instituto de Investigaciones Marinas (CSIC), Eduardo Cabello, 6, Vigo 36208, Spain*

[4]*Present address: Instituto Español de Oceanografía, Apdo 1552, 36280, Vigo, Spain*

[5]*To whom correspondence should be sent*

Abstract. The paralytic shellfish poison producing dinoflagellate *Gymnodinium catenatum* was subjected to changes in salinity, phosphate, ammonium and nitrate using continuous culture and batch culture methods. In contrast with other algae, this species showed very slow changes in the concentration of intracellular amino acids, in the Gln:Glu ratio, and, in contrast with *Alexandrium* spp., only slow changes in toxin content, during such events as N-feeding of N-deprived cells or during nutrient deprivation. This organism was found to be very susceptible to disturbance; maximum growth rates, around 0.25–0.3 day^{-1} with a minimum C:N mass ratio of 5.5, were attained when cultures were only disturbed by sampling once a day. P-deprived cells were larger (twice the usual C content of 4 ng C $cell^{-1}$ and volume of 20 pl). The content of free amino acids was always low (5% of cell-N), with low contributions made by arginine (the precursor for paralytic shellfish toxins). Cells growing using ammonium had the lowest C:N ratios and the highest proportion of intracellular amino acids as arginine. The toxin profile (equal mole ratios of dcSTX, GTX_5, $dcGTX_{2/3}$ C_1 and C_2, and half those values for C_3 and C_4) was stable and the toxin concentration varied between 0.2 and 1 mM STX equivalents (highest when ammonium was not limiting, lowest in P-deprived cells, though as the latter were larger toxin per cell was not so variable). Decreased salinity did not result in increases in toxin content. Significant amounts of amino acids (mainly serine and glycine, with a total often exceeding 4 μM) accumulated in the growth medium during batch growth even though the cultures were not bacteria free.

Introduction

Gymnodinium catenatum Graham is an unarmoured (or naked) chain-forming dinoflagellate that was first reported by Graham (1943) from the Gulf of California, and later by Balech (1964) from Argentina. This species was first suspected to be involved with paralytic shellfish poisoning (PSP) outbreaks in the Galician rias of northwest Spain in 1977 (Estrada *et al.*, 1984), and was associated with human fatalities in 1979 in the Gulf of California (Mee *et al.*, 1986). Confirmation of its toxicity by chemical analysis was achieved in specimens from Australia (Oshima *et al.*, 1987) and from Spain (Anderson *et al.*, 1989). Ever since then, reports of PSP outbreaks associated with *G.catenatum* have appeared from a growing number of localities in northern (Franca and Almeida, 1989) and southern Atlantic waters (Brazeiro *et al.*, 1996), the Mediterranean Sea (Bravo *et al.*, 1990), the Pacific Ocean (Hallegraeff and Sumner, 1986; Yuki and Yoshimatsu, 1987; Fukuyo *et al.*, 1993) and the Caribbean (LaBarbara-Sánchez *et al.*, 1993).

Despite its wide geographical distribution and its serious impact on many shellfish resources, data on the physiology of this organism, which are required in order to help in the understanding of toxic events, are limited. Published experimental studies on *G.catenatum* mainly deal with the life history (Blackburn *et al.*, 1989), the cyst phase (Bravo and Anderson, 1994) and with a comparison of toxic profiles under batch culture conditions (Oshima *et al.*, 1993a,b; Sousa *et al.*, 1995).

Several mechanisms have been advanced to explain the origin of the inoculum populations and bloom dynamics of the *G.catenatum* responsible for PSP outbreaks in the Galician rias. Fraga *et al.* (1988, 1993) suggest that major proliferations of *G.catenatum* are initiated by means of warm surface water transport, induced by upwelling relaxation, of shelf populations into the rias. Figueiras *et al.* (1994, 1995) maintain that the outer parts of the rias are suitable places for active *in situ* growth of *G.catenatum* during periods of weak upwelling. Blanco (1995) developed a theoretical model where population growth results from a combination of advection by the poleward surface slope current and cyst germination.

The main form of nitrogen supporting the growth and development of *G.catenatum* populations is unknown. Results from field experiments, based on the stoichiometric balance of nitrogen compounds at the time of a *G.catenatum* bloom (Figueiras and Fraga, 1990; Fraga *et al.*, 1992), lead to the hypothesis that this species is able to exploit the nutrient rich layer of the pycnocline, by virtue of its vertical migratory capacity, with the nitrate generated by upwelling as the main nitrogen source. However, preliminary results from batch cultures indicate a better growth rate and yield of cells when using ammonium as the nitrogen source when compared with nitrate (Reguera, 1993) and Prego (1992) suggests that the growth of *G.catenatum* in the rias is enhanced by ammonium-N.

In order to attempt a simulation of possible changes in nutrient availability during the advection of *G.catenatum* populations into coastal waters (such as rias) we have employed a culture system in which test growth medium was introduced so that 90% of the original medium was replaced by day 10, and 95% by day 14. This method (the first time that continuous culture methods have been employed for this organism) has the additional advantage that biomass is maintained at a relatively high level. This paper reports some preliminary results using this approach which indicates that this dinoflagellate, like *Alexandrium* spp., appears rather unique in its N-physiology as indicated by intracellular amino acids, although it contrasts with *Alexandrium* spp. in certain respects.

Method

Cultures of *G.catenatum* (strain GC21V from I.E.O., Vigo, Spain, isolated by I.Bravo) were grown in a modified K-medium (Keller *et al.*, 1987), prepared according to Flynn *et al.* (1994), in static 2-l ballon flasks filled to 1 l, with light supplied in a 12 h/12 h light/dark cycle at 180 μmol m^{-2} s^{-1} ('day-light' fluorescence tubes), at 18°C. This type of flask was used rather than conical flasks because swirling to mix a culture in the latter often leads to cavitation of the media which may damage chain forming organisms. *Gymnodinium catenatum* appears to be extremely sensitive to disturbance in culture; Hallegraeff *et al.*

(1995) report the importance of low turbulence for field populations of this organism. It was found that any stirring of the culture (even slowly for just 10 min every 6 h with a magnetic bar) or the dropping of fresh medium onto the surface of the culture (where cells accumulated at least during the first half of the light phase) affected growth rates or killed cells.

Several types of experiments were conducted, including refeeding of N-stressed cells (with an estimation of nutrient uptake kinetics by incubation with different concentrations of ^{15}N-labelled ammonium or nitrate), growth under batch culture conditions, and growth in cultures into which fresh (test) media were introduced at a rate of 0.2 day^{-1}. The various treatments are summarized in Table I. Where fresh medium was supplied, this was pumped continuously (Watson–Marlow pump with silicon pump tubing via capillary Teflon tubing to the bottom of the culture flasks) to give a dilution rate of 0.2 day^{-1}; this gave a gradual change in the nutrient or salinity regime over the experimental period. Excess medium was removed manually once or twice a day using aseptic technique after gentle swirling of the flask.

Flasks were sampled, after gentle swirling to mix the suspension, by pouring using aseptic technique. Samples were taken and analysed for nutrients (ammonium, nitrate, nitrite and phosphate), cell number and biovolume. Biovolume and number of chains were determined with live samples using an Elzone PC286 particle analyser (Particle Data, Europe) with a 240 μm orifice tube (calibrated with latex beads), and cell number by microscopy. The average chain length was thus obtained by dividing the cell count (from microscopy) by number of chains (from the Elzone). Cells were collected onto ashed 13 mm Gelman A/E glassfibre filters under low vacuum (<75 mm Hg) and maintained frozen (–20°C) until required.

Intracellular free amino acids (InAA) were determined using the method of Flynn and Flynn (1992), and the secondary amine proline determined using the FMOC methodology (60 s derivatization with 3 mM 9-fluorenylmethyl chloroformate terminated with 40 mM adamantaneamine, separation on a Merck Superspher 60 RP8 column using a linear gradient over 25 min, at a flow rate of 1.25 ml min^{-1}, from 100% 100 mM pH 4 acetate buffer with 5% v/v tetrahydrofuran to 80% methanol, with fluorescence detection using excitation at 262 nm and emission at 314 nm). Dissolved free amino acids (DFAA) were determined by HPLC using the method of Flynn (1988) or using the fluorescamine method of North (1975) with glutamate as the standard. Amino acids are referred to using their standard abbreviations (alanine, Ala; arginine, Arg; aspartate, Asp; glutamate, Glu; glutamine, Gln; glycine, Gly; ornithine, Orn; proline, Pro; serine, Ser; taurine, Tau; valine, Val; mole ratio of glutamine:glutamate, Gln:Glu).

Toxins were analysed by HPLC using the post-column oxidation method of Franco and Fernández-Vila (1993), and using the pre-column oxidation method of Flynn and Flynn (1996) where quantification was made relative to the peak area of saxitoxin (STX) standards. Although the post-column method is more laborious and time-consuming, its main advantage is its capacity for good separation of the toxins. In contrast the precolumn method does not give a proper separation but is ideal for rapid screening and where the toxin profile is stable.

Table I. Experimental conditions. For the continuous cultures, 5 l of culture were grown on medium N as the start inoculum for all treatments. The test media were pumped into the cultures at a dilution rate of 0.2 day^{-1}; the residual concentration of PO_4^{3-} in all low-phosphate continuous cultures was below detection after day 6

Experiment type	Figures	Treatments	Abbreviation	Nitrate (μM)	Ammonium (μM)	Phosphate (μM)	Salinity (‰)
N-refeeding	1	Nitrate-fed	NO_3^--fed	50	0	10	33
		Ammonium-fed	NH_4^+-fed	0	50	10	33
Batch-culture	4	Nitrate–phosphate	NP	100	0	20	33
Continuous culture: low biomass series	5	Nitrate–phosphate	NP	50	0	10	33
		Nitrate–low phosphate	NLP	50	0	0	33
		Ammonium–phosphate	AP	0	50	10	33
		Ammonium–low phosphate	ALP	0	50	0	33
		Nitrate–low salinity	NLS	50	0	10	22
		Ammonium–low salinity	ALS	0	50	10	22
Continuous culture: high biomass series	6,7	Nitrate (control)	N	200	0	20	33
		Nitrate–low salinity	NLS	200	0	20	22
		Nitrate–low phosphate	NLP	200	0	0	33
		Ammonium–nitrate	AN	100	100	20	33
		Ammonium–nitrate–low-phosphate	ANLP	100	100	0	33

Nevertheless, to apply the latter method it is necessary to have a previous knowledge of the toxin profile of the samples to ensure the reliability of the quantitative results. These conditions, undoubtedly, are followed in this paper. Concentrations of toxins and InAA are expressed on a cell volume basis assuming equal distribution throughout the cells. Toxin concentrations as STX equivalents have been computed using the toxicity data of Oshima (1995).

Cellular carbon and nitrogen, and values of δ ^{13}C and ^{15}N, were determined using Europa Scientific RoboPrep and TracerMass instruments with isoleucine as the standard. Uptake of ^{15}N-labelled ammonium or nitrate (98 atom% additions at a range of substrate concentrations and sampled at 20, 40 and 60 min) are expressed as N-specific uptake rates using the equation of MacIsaac *et al.* (1985). Growth rates were computed (with correction for continuous dilution as appropriate) from the formula:

$$\mu = \mathrm{Ln}(p_1) - \mathrm{Ln}(p_0)/(t_1 - t_0)$$

where p_1 and p_0 are parameter values at times (in days) t_1 and t_0.

All experiment formats have been replicated, giving the same qualitative results. Replication between cultures grown using the same batch of sea water was also good quantitatively.

Results

Nitrogen refeeding of N-starved cells

Following the addition of 50 μM nitrate or ammonium to stationary phase cells, the uptake of N was at a similar rate for both (Figure 1a), but the C:N ratio fell (from 10) more rapidly for the ammonium-fed cells reaching a value of 5.5 after 6 days. The uptake kinetics of N-starved cells (C:N > 10) as determined using ^{15}N substrates are shown in Figure 2a. The time course for nitrate uptake showed evidence of induction, with the rate of nitrate uptake increasing over the 1 h incubation period. Ammonium uptake rates were generally three to four times greater than for nitrate, and N-starved cells had higher values for V_{max} (Figure 2a, cf. Figure 2b). K_S values for nitrate uptake (0.42 ± 0.37 μM) were half those for ammonium (1.02 ± 0.42 μM).

During refeeding, the Gln:Glu ratio varied little although it was slightly higher in the ammonium-fed cells, as was the concentration of Glu (Figure 1b). However, Tau concentrations were lower in the ammonium-fed cells, and Arg levels remained low (Figure 1c). The dissolved free amino acid concentration (DFAA measured as fluorescamine-positive substances) varied from 3 to 15 μM for nitrate-fed cells, and remained around 7 μM for ammonium-fed cells. In both cases there was evidence of a diel cycle with increased DFAA at the end of the light phase and decreased at the end of the dark (not shown). Toxin concentrations in nitrate-fed cells showed a marked increase during D-phase and decreasing during L-phase (Figure 1d), while ammonium-fed cells showed very little change in toxin concentration during this period (Figure 1e). The toxin profile changed little during this refeeding period (Figure 1d and e).

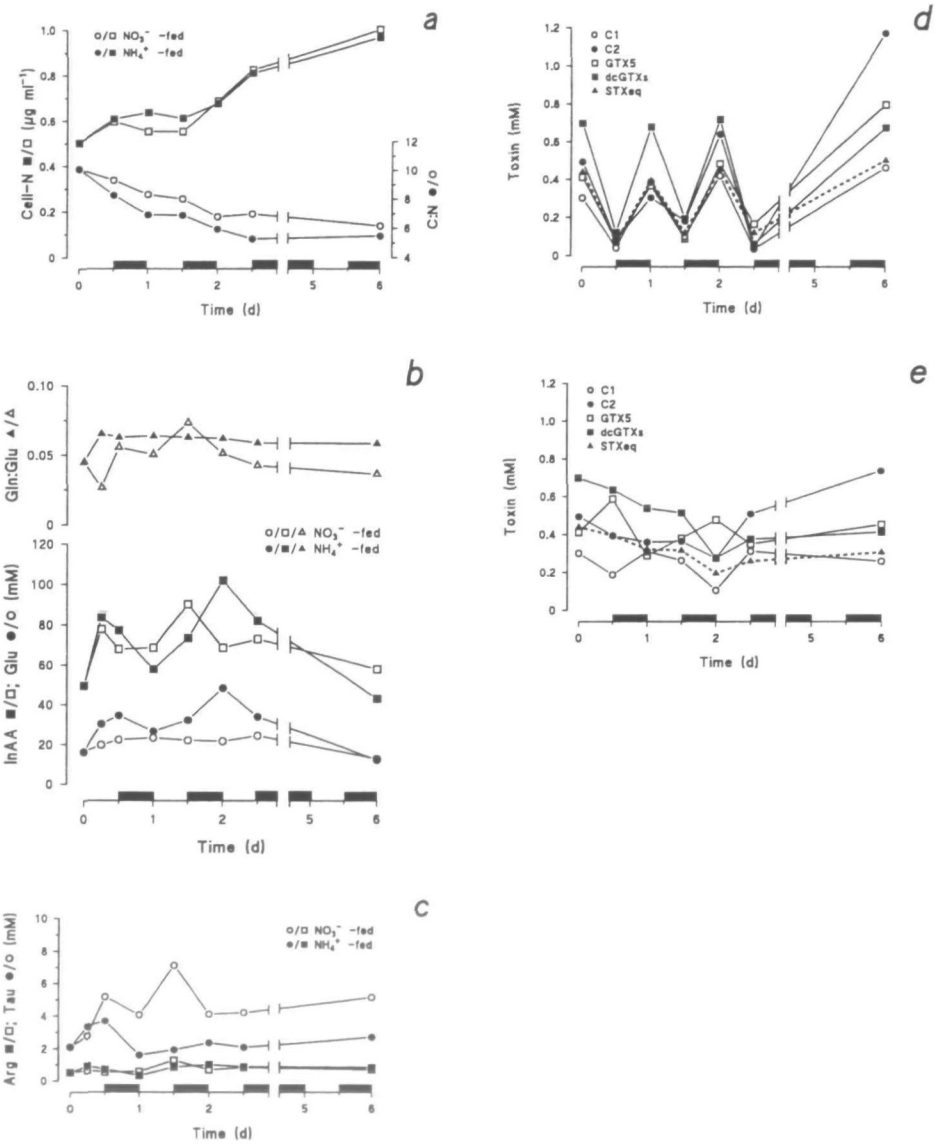

Fig. 1. Nitrate and ammonium refeeding of N-starved *G.catenatum*. N-uptake and C:N ratio (**a**), Gln:Glu ratio, Glu and intracellular amino acids concentration (**b**), Arg and Tau concentrations (**c**), and toxin concentrations for nitrate-fed (**d**) and ammonium-fed cells (**e**). The light/dark cycle is indicated on the *x*-axis.

Figure 3 shows average toxin profiles for all of the toxin samples, from all but the high biomass continuous culture experiment, analysed using HPLC with post-column oxidation. As these profiles did not vary significantly with the nutrient status of the cells, all other toxin data are given as concentration of STX (toxicity) equivalents.

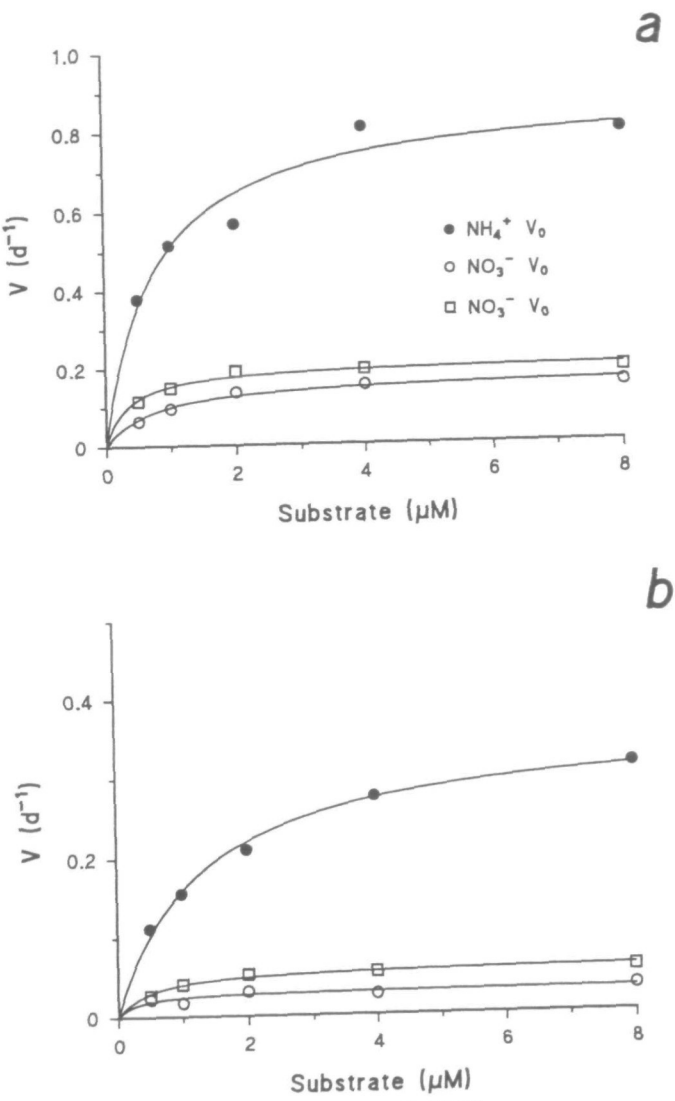

Fig. 2. Uptake kinetics for N-starved cells (C:N > 10; **a**) and N-sufficient cells (C:N = 5; **b**). Samples were taken at 20, 40 and 60 min following addition of [^{15}N]nitrate or ammonium, a quadratic line was fitted through the data, differentiated and the rates of uptake determined at time 0 (V_0, initial uptake rate) and at 1 h (V_1). For ammonium there was no difference between V_0 and V_1.

Batch growth

N-stressed (NP and AP) and NP-stressed (NLP and ALP) cells were inoculated into media containing either nitrate or ammonium at a concentration of 100 μM and 20 μM PO_4^-. The ammonium cultures (AP and ALP) did not grow; this concentration of ammonium appears toxic to the cells. The NP and NLP cells showed the same trends but in the latter changes in concentrations of metabolites

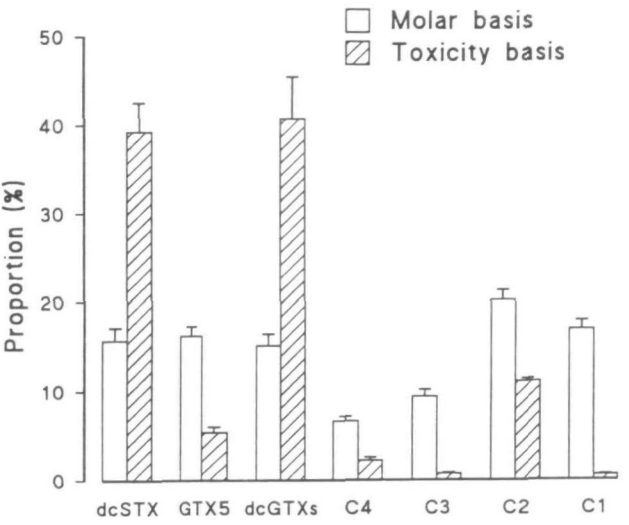

Fig. 3. Bar-chart representing the proportions of individual toxins averaged from all HPLC analyses conducted using postcolumn oxidation according to their molar concentrations (open bars) and their toxicities (in STX equivalents; hatched bars). Error bars are of the 95% confidence limits (n = 160).

were slower and/or of lesser magnitude. The maximum growth rate was around 0.2–0.3 day^{-1} (Figure 4a). The C:N ratio decreased slowly over the duration of the growth cycle to a minimum of 5.5 at day 22 when nitrate became exhausted. The C:N ratio then increased to around 8 (higher for NP than NLP) when the cells reached the stationary phase (Figure 4a). During batch growth the Gln:Glu ratio gradually increased to exceed 0.5 in both instances, and reached a value of 1.5 in the culture inoculated with NP cells (Figure 4b), and then started to decline as the nitrate concentrations decreased below 30 μM. Intracellular concentrations of Glu and amine-X (defined by Flynn and Flynn, 1992) were highest during the early part of growth, but Arg concentrations, and the contribution that Arg made to total amino-N gradually increased until day 16 (mid-exponential phase) reflecting the changes in Gln:Glu (Figure 4c). DFAA concentrations (fluorescamine-positive substances) were generally <5 μM but with a maximum of 12 μM at the end of the exponential phase (Figure 4b). NLP-cells always had a higher toxicity per unit of cell volume after day 5 but toxin concentration changed little during batch growth (Figure 4d).

Changes in culture regime

This was studied using dilute cultures with low substrate concentrations, and also using more dense cultures with higher nutrient concentrations (Table I). Exponentially growing NP cultures were grown for several weeks prior to the start of the treatments. In both low and high biomass series the dilution rate (0.2 day^{-1}) was close to the maximum growth rate (cf. Figure 4a).

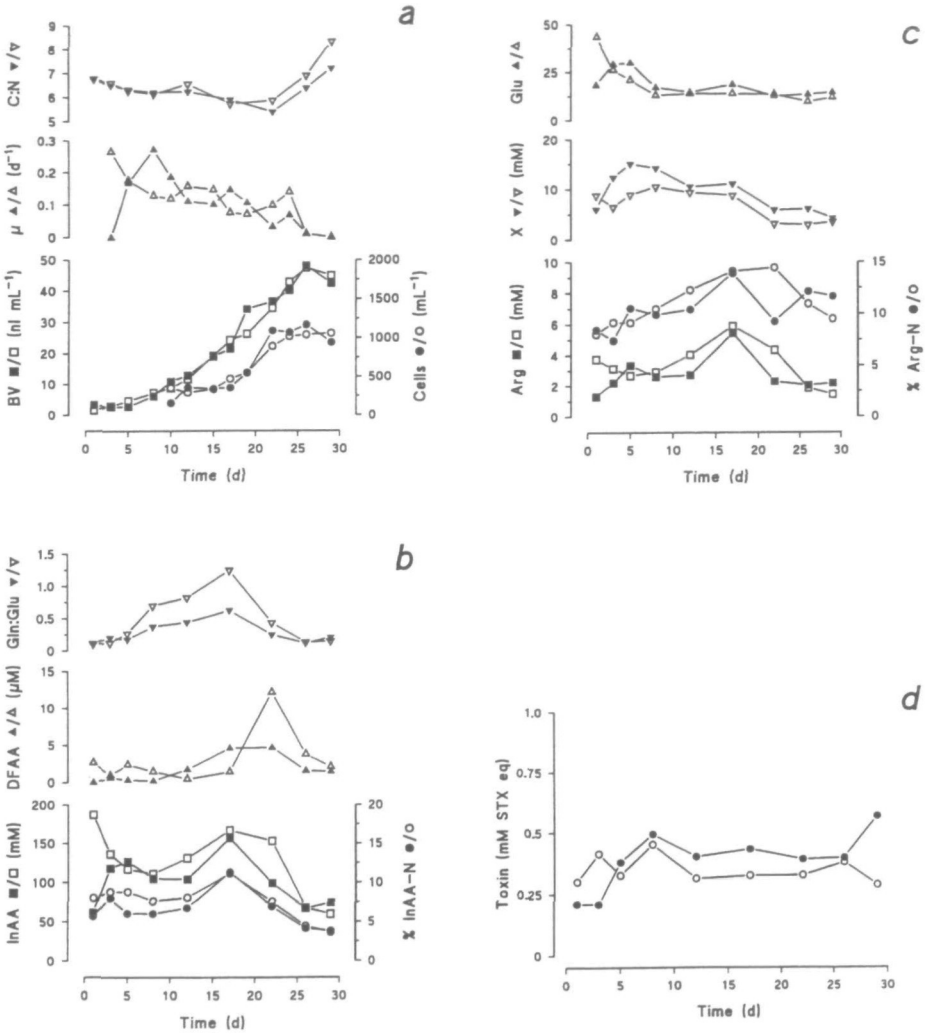

Fig. 4. Batch growth of *G.catenatum* which were initially N-stressed (grown on nitrate in NP media; open symbols) or NP-stressed (NLP; closed symbols) on NP media (Table I). Changes in the C:N ratio, μ (growth rate), biovolume (BV) and cell numbers (**a**), Gln:Glu ratio, DFAA (measured as fluorescamine-positive substances), InAA concentrations and the percentage contribution of InAA-N to total cell-N (**b**), Glu, amine-X, Arg, and the contribution of Arg-N to total InAA-N (**c**), and in the toxicity of the cells as calculated in STX equivalents (**d**). Nitrate was exhausted by day 22 for both cultures; phosphate levels were never <5 μM.

Low biomass series

Cell numbers (ml^{-1} of culture) remained at around 200, with chain lengths varying from two to eight cells; due to patchiness at this density it was not possible to obtain reliable cell counts by microscopy so data are expressed on a biovolume basis using results from the Elzone particle analyser which gave reliable

consistent readings as it sampled 1-ml volumes. Cells supplied with ammonium showed a progressive fall in C:N (Figure 5a) and increasing concentrations of InAA in comparison with nitrate-fed cells (Figure 5b), consistent with the results of the refeeding experiment (cf. Figure 1a). However, nitrate-fed cells at full salinity had a higher Gln:Glu ratio (Figure 5c) with a maximum of 0.45 obtained for NLP and NLS cells; ammonium-fed cells had higher concentrations of Glu (not shown) as noted before (Figure 1b). Arg concentrations increased in ammonium-fed cells, especially in ALP cells (Figure 5d), but as a percentage of total InAA-N the contribution of Arg-N remained at ~10% of InAA-N (Figure 5e). Total toxin content (as STX equivalents per unit of biovolume) varied by at most a factor of 5; lowest values were seen in NLP cells and the highest in AP cells early during the test (Figure 5f).

High biomass series

The cell density was approximately an order of magnitude greater in these cultures than in the low biomass series enabling reliable cell counts (Figure 6a). For the first 10 days, cultures were sampled twice daily but the difference in growth rates before and after day 10 suggest that even this frequency of disturbance may adversely affect the growth rates of this organism.

The C-quota of P-replete cells was 4 ng cell^{-1} (Figure 6b), with a cell volume around 20 pl (not shown); biovolume correlated with cell-C (0.2 μg C nl^{-1}, covariance = 16.5%), though NLS cells were of lower density by 10–20%. NLP and ANLP cells gradually became larger (eventually attaining double the typical C-quota, Figure 6b, with twice the cell volume) and formed shorter chains (average of 1.8 cells rather than 4 cells per chain) after depletion of phosphate at day 6. The C:N mass ratio in nutrient-replete cells was 5.5 (Figure 6c) rising on phosphate exhaustion in NLP and ANLP. Values of $\delta^{13}C$ during the first 10 days were around –28 for all cultures, rising as growth rate increased after this period to reach –22 at day 20 (not shown).

Concentrations of InAA were generally low (amounting to only 5% of cell-N in all instances) and changing only slowly in composition (not shown). The values of Gln:Glu were usually very low, typically below 0.1 even at growth rates over 0.3 day^{-1}. The only time that Gln:Glu was elevated (reaching 0.6) was in the AN and ANLP cells around day 10 when residual amounts of ammonium exceeded 10 μM (Figure 7a); between day 10 and day 20 these cultures only used ammonium with nitrate levels remaining above 100 μM. The maximum amount of inorganic N removed (achieved in the NLS and AN cultures) was 160 μM from the 200 μM supplied and there was an excess of nitrate present in all cultures. Arg-N contributed <4% of InAA-N in the control cells using nitrate only (N), increased to twice that in NLS cells by day 8, and NLP cells also attained 8% for a short period. When a surplus of ammonium was present in the growth medium around day 10 (Figure 7a), cells attained over 16% as arginine-N (AN and ANLP in Figure 7b). Concentrations of intracellular Pro were similar in all treatments (around 10 mM, not shown), with no evidence for a role in osmoregulation.

Toxin analysis for these cultures was performed using low temperature HPLC

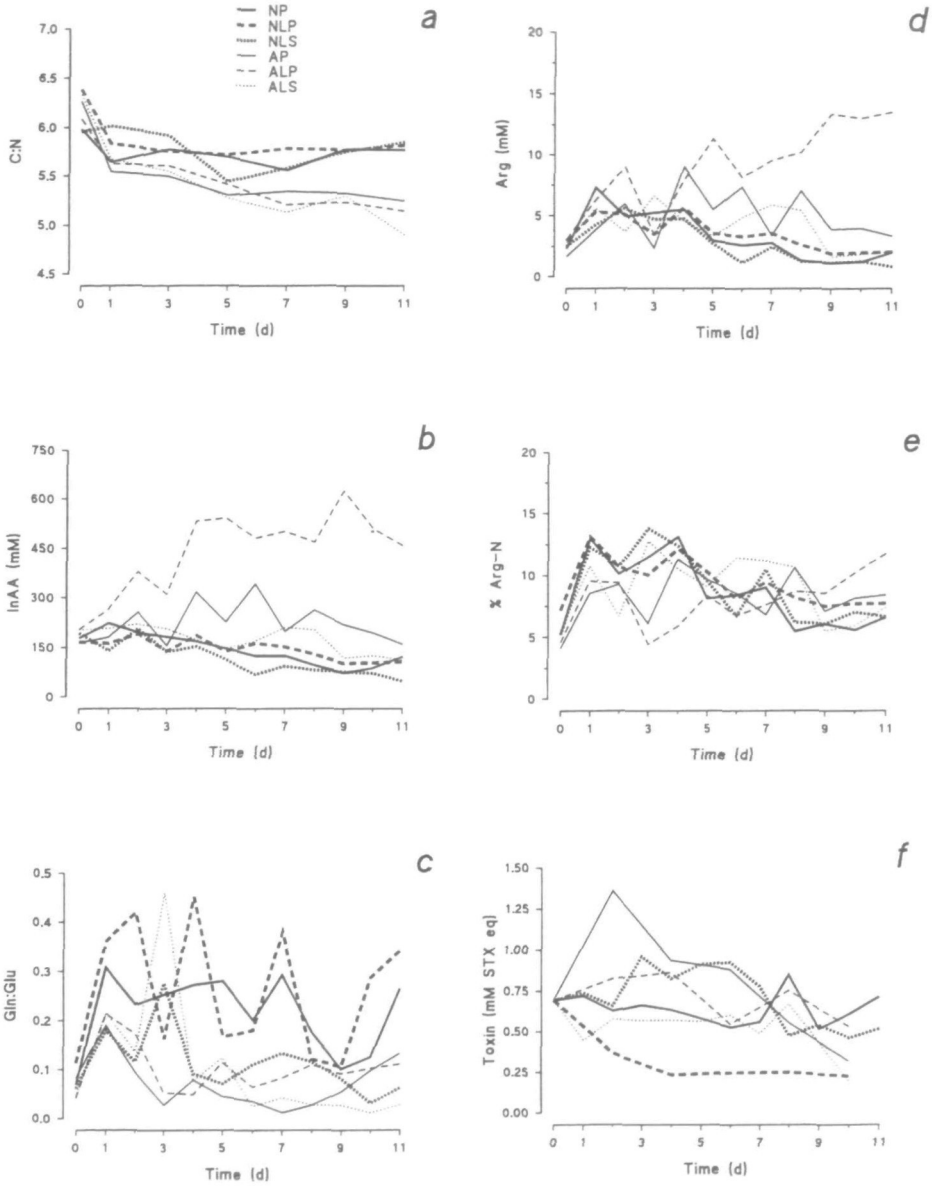

Fig. 5. Low biomass cultures of *G.catenatum* subjected to gradual changes in nutrition or salinity (see Table I). C:N mass ratio (**a**), InAA concentration (**b**), Gln:Glu ratio (**c**), Arg concentration (**d**), contribution of Arg-N to total InAA-N (**e**) and the total toxicity (as STX equivalents; **f**). Abbreviations of culture conditions explained in Table I. Samples were taken once daily during the experiment.

with pre-column oxidation method (Flynn and Flynn, 1996). This technique showed four major peaks, two of which coeluted with GTX_1 and the C-toxins, but the other two were not identifiable due to a lack of suitable standards. The first

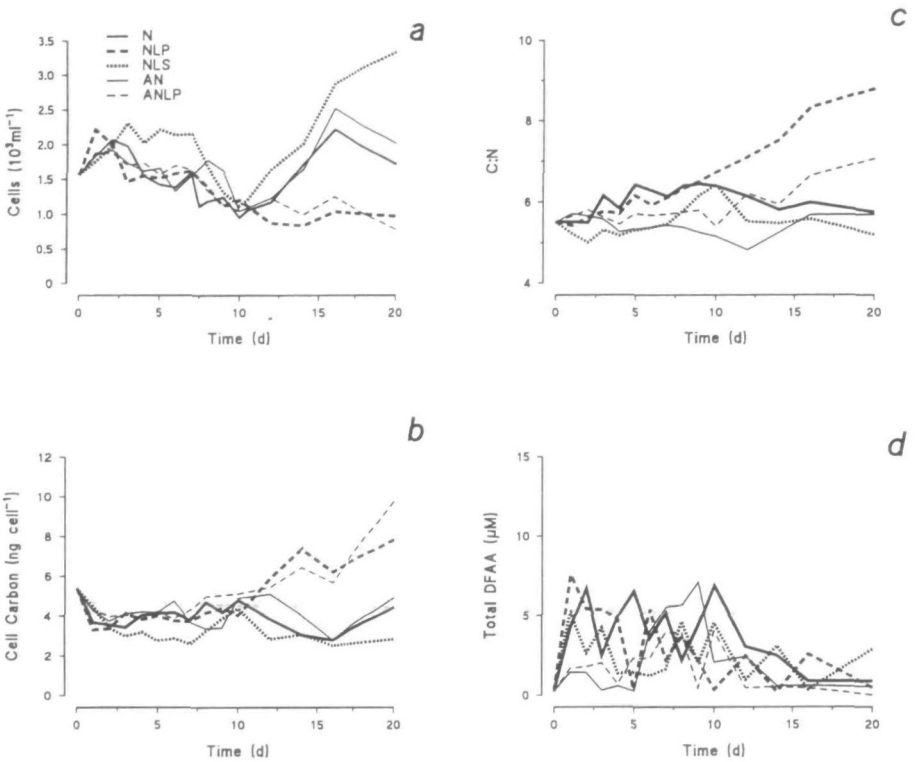

Fig. 6. High biomass cultures of *G.catenatum* subjected to gradual changes in nutrition or salinity (see Table I). Cell numbers (**a**), C-quota (**b**), C:N mass-ratio (**c**) and DFAA (measured by HPLC; **d**). Abbreviations of culture conditions explained in Table I; sampling prior to day 10 was every 0.5 days, and then once a day afterwards.

of these peaks (peak [1] in Flynn and Flynn, 1996) was not acid-hydrolysable and perhaps is due to dcGTX, while the second ([2] in Flynn and Flynn, 1996) degraded with a simultaneous appearance of STX suggesting the presence of GTX_5. The proportions of these four peaks remained constant (cf. toxin profiles obtained using the post-column methodology; Figure 3). The areas for these peaks have been summed and referenced to STX for the figures; this has given an estimated concentration about twice that obtained using the post-column method (i.e. ~2 mM rather than 1 mM).

Total toxin per cell (Figure 7c) increased in ammonium-fed cells, especially in ANLP which were larger (Figure 6b). The toxin concentration was highest in AN cells (Figure 7d), especially around the time when ammonium was detectable in the growth medium (Figure 7a) and Arg content increased (cf. Figure 7b). NLS and NLP cells had the lowest toxin concentration; NLP cells in the low biomass series also had the lowest toxin concentration (cf. Figure 5f).

Except for a period between days 6 and 12, DFAA in the AN and ANLP

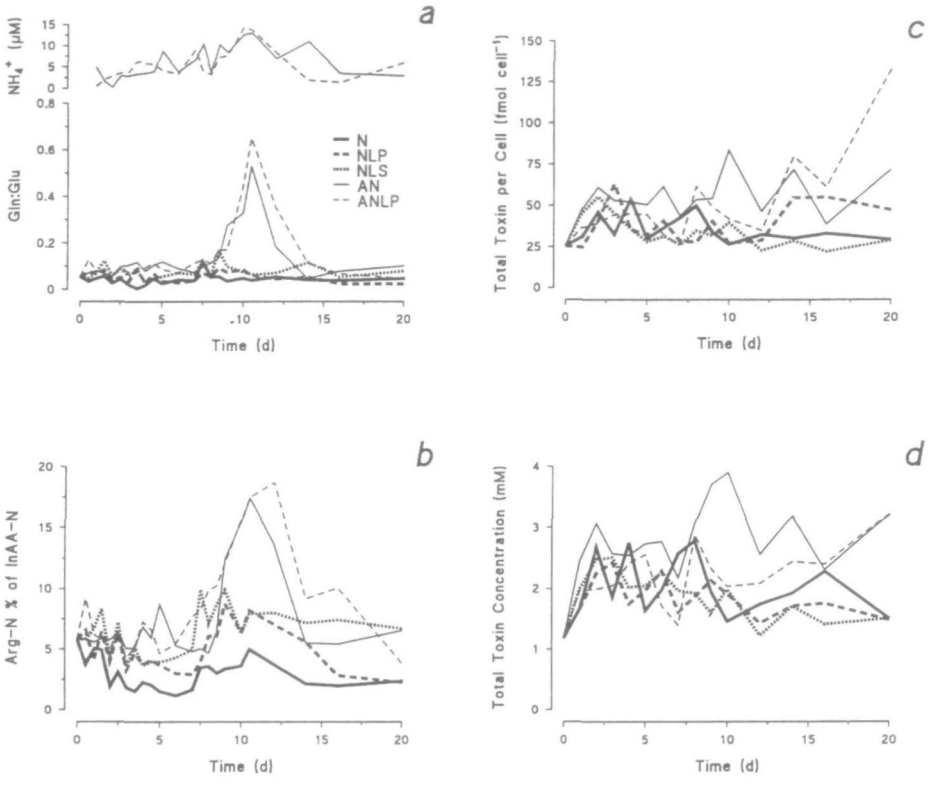

Fig. 7. As Figure 6. Changes in extracellular ammonium and Gln:Glu (**a**), contribution of intracellular Arg-N to total InAA-N (**b**), total toxin content per cell (**c**), and in intracellular toxin concentration (**d**). In the absence of suitable standards toxin concentrations, determined by precolumn HPLC, were estimated by application of the response factor for STX for all unknown peaks. Other details as for Figure 6.

cultures were much lower than in cultures which were not supplied with ammonium (Figure 6d). Although the total concentration of DFAA was rather variable, generally between 2 and 5 μM, the composition was remarkably constant with major components at 5% Val, 10% each of Ala, Asp and Orn, 15% Gly and 30% Ser.

Discussion

Gymnodinium catenatum, at least the strain used (which is the most robust of those tested), appears very sensitive to disturbance of the culture. Excessive disturbance results in a decreased growth rate, formation of shorter chains and cells of abnormal appearance. Undisturbed cultures may have chain lengths of 16 or 32 cells; some of the chains in our cultures were as long as eight or so, though the average was closer to half that. We saw no evidence of an induction of gamete and cyst formation, which may be induced by nutrient deprivation in non-clonal

culture mixtures (Blackburn *et al.*, 1989). Cysts of the dinoflagellate can in any case germinate after only a few days (Bravo and Anderson, 1994). The susceptibility to turbulence appears to be not uncommon in dinoflagellates (Berdalet, 1992; Thomas and Gibson, 1992; Thomas *et al.*, 1995) and has important implications both for ecology (Hallegraeff *et al.*, 1995), and the design and execution of laboratory studies.

The culture protocol employed would appear suitable for use in other experiments where the effects of alternative growth media are being studied, being preferable to the use of a batch culture system because it enables the removal of a relatively constant amount of material each day. The dense accumulations of cells near the surface (which occurs whenever sufficient inorganic nitrogen is available) and the consequences of stirring prevent the automation of culture removal during continuous culturing of these organisms. The low values of $\delta^{13}C$ which we determined (indicative of a ready availability of CO_2 (aq) for RUBISCO; Johnston and Raven, 1992) are consistent with cells residing at the culture surface in order to minimize DIC-stress. The same result was seen with cultures of *Alexandrium minutum* (Flynn *et al.*, 1996). It does not seem likely that DIC-stress was the cause for the premature cessation of growth for *G.catenatum* noted above.

In common with several other dinoflagellates in culture (Dixon and Syrett, 1988), *G.catenatum* only grew to a relatively low biomass even in the presence of excess macronutrients. In the presence of soil extract and in small containers, higher densities may be attained (B.Reguera, unpublished data). However, it is clearly important to use low nutrient concentrations if one wishes to grow cultures of this organism through to a reasonable degree of N-stress (Flynn and Flynn, 1995); the maximum concentration of inorganic-N which should be employed is ~50 μM. This creates additional problems with large motile organisms or chains because the low cell or chain densities lead to patchiness and hence sampling problems.

The intracellular amino acid composition in *G.catenatum* contrasts with that of *Alexandrium* spp. (Flynn *et al.*, 1993). *Alexandrium* spp. have much higher concentrations of glutamine and higher ratios of glutamine:glutamate (Gln:Glu) than other algae we have examined (Flynn *et al.*, 1996). *Gymnodinium catenatum*, in contrast, often has much lower values of Gln:Glu than seen in other algae, attaining less than 0.1 even in the presence of high external nitrate or ammonium concentrations for several days. Such low values of Gln:Glu are usually only seen in cells which are N-deprived (Flynn, 1990), though the C:N ratio in these cells of *G.catenatum* was low. Only when ammonium was not limiting (Figure 7a) or after prolonged growth at high nitrate concentrations (Figure 4b) did Gln:Glu rise to levels more typical of N-replete cells. The reason for this abnormality may be due to the use of the glutamine dehydrogenase (GDH) pathway for ammonium assimilation rather than the GS-GOGAT pathway; the former yields Glu rather than Gln as the first product of ammonium assimilation (Syrett, 1981).

The slow response of Gln to N-feeding (Figure 1b; Flynn *et al.*, 1993) may explain the problems in growing *G.catenatum* using ammonium when the external concentration is elevated and the cells initially N-starved. Under such conditions the uptake regulation of ammonium, which is likely to be by an initial product of

ammonium assimilation such as Gln (Flynn, 1991), may be too slow to prevent the toxic accumulation of ammonium within the cells. This may be a particular problem in N-deprived cells where ammonium V_{max} is high (Figure 2a). Ammonium-fed cells of *G.catenatum* appear to have a higher N-status than those using nitrate, as indicated by a lower C:N ratio and higher concentrations of InAA and Arg. This is also seen in the raphidophyte *Heterosigma carterae* (Wood and Flynn, 1995), suggesting that cells growing on nitrate are more N-stressed than those using ammonium. In conjunction with reports indicating that ammonium may support higher growth rates (Reguera, 1993) and be of more importance in red-tide formation of *G.catenatum* (Prego, 1992; Ríos *et al.*, 1995), it appears that studies of this organism should make use of ammonium as the N-source.

Another difference between *G.catenatum* and *Alexandrium* spp. is in the concentration of intracellular Arg, which is a precursor for the synthesis of paralytic shellfish toxins (Shimizu *et al.*, 1990). The contribution of InAA-N to cell-N, at ~5%, is similar in both *G.catenatum* and *A.minutum*, though the concentration of InAA is higher by a factor of 1.5–2 in the latter (Table II). However, while the Arg content in *A.minutum* attains 30% of InAA-N when growing on nitrate, in *G.catenatum* it is usually ~5–10% (Figures 4c, 5e and 7b) and not dissimilar to that in other algae (Flynn and Flynn, 1995). Concentrations of Pro, an amino acid close to Arg biochemically, and suggested to have a role in osmoregulation (Karsten *et al.*, 1991), did not vary significantly under any condition tested. A similar result was obtained in *Alexandrium* spp. (Flynn *et al.*, 1996).

Quantification of the toxins found in this dinoflagellate is complicated by the lack of suitable standards. The toxin composition of *G.catenatum*, according to Reguera and Oshima (1990) and Oshima *et al.* (1993a), is dominated by GTX_5 and GTX_6, while Franco Fernández-Vila (1993) shows the importance of C_2, $dcGTX_3$ and GTX_5. The profiles obtained in the present work are more similar to the latter, but dcSTX is also important. Acid hydrolysis of our extracts confirmed the presence of C_2 and GTX_5, but not of GTX_6 which should degrade to give neoSTX. The stability of the toxin profile in our results is in keeping with results of Oshima *et al.* (1993a) reporting that the toxin composition in *G.catenatum* is not affected significantly by temperature, light or nutrient status.

Table II. Summary table of comparisons between culture strains of *Gymnodinium catenatum* and *Alexandrium minutum* isolated from the Ría de Vigo, Spain. Summary data from current study and from Flynn *et al.* (1993, 1994 and 1996)

	Gymnodinium catenatum		*Alexandrium minutum*
Minimum C:N	5.5		5.5
Growth rate (μ_{max})	0.25		0.35
Sensitivity to disturbance		>>	
Exponential phase Gln:Glu	typically < 0.5		typically > 1.5
Arg concentration (mM)	5		20
Arg: % of InAA-N	5–10		30
Amine-X concentration (mM)	15		0
Toxin types	dcSTX, GTX_5, $dcGTX_5$, C_1, C_2, C_3, C_4		GTX_1, GTX_2, GTX_3, GTX_4
Toxin concentration (mM STX eq)	0.25 to 1.3		0.3 to 3.0

Riverine inputs have been reported to be beneficial for bloom development of *G.catenatum* (Fraga *et al.*, 1993; Hallegraeff *et al.*, 1989). Exposure to low salinity decreased the concentration of Tau, and of InAA in general, though the proportion of InAA as Arg may increase on prolonged exposure. It is apparent that low salinity does not enhance toxicity in *G.catenatum*, as it may in *Alexandrium fundyense* (Anderson *et al.*, 1990). As the experimental data presented for the low salinity cultures do not show any significant effects on growth or toxicity of *G.catenatum* cells, the influence of fresh water inputs in nature may be interpreted as benefits associated with increased water column stability or introduction of various growth factors.

Enhancement of toxicity in *G.catenatum* by assimilation of ammonium, or by the refeeding of N-stressed cells is certainly not rapid if it occurs at all (Figures 1e, 5f and 7d). The apparent diel variation in toxin content during nitrate refeeding (Figure 1d) suggests that toxins are subject to turnover. However, in contrast with the situation in *Alexandrium* spp., where toxin content may change rapidly with nutrient status (Anderson *et al.*, 1990; Flynn *et al.*, 1994), toxin content in *G.catenatum* changes relatively slowly, if at all, during batch growth (Figure 4d).

Preliminary studies of *G.catenatum* suggested changes in the composition of toxins per cell when the N:P nutrient ratio was changed (Reguera and Oshima, 1990). However, in the current study there were no significant changes in the toxin profile during P-stress. Also, while toxin per cell increased, this simply reflected an increase in cell volume in these cells rather than an increase in toxin concentration. Anderson *et al.* (1990) reported a similar event in *Alexandrium* spp., while in *A.minutum* P-stress appears to affect toxin turnover during N-stress (Flynn *et al.*, 1994). All of this suggests that attempts to link nutrient status to toxin content are at best difficult, and that such associations may be species-specific.

The concentration of DFAA noted in our cultures of *G.catenatum* was higher than seen in cultures of other algae (Flynn, 1990). The composition must reflect changing rates of release by the dinoflagellate and uptake by the bacteria which would have been present (*G.catenatum* is not yet available as an axenic culture). The composition of DFAA was in total contrast with that of InAA. However, it is likely that the composition of DFAA reflects bacterial utilization rather than release from the phototroph. Whether any of the bacteria growing in our cultures are capable of producing toxins (Gallacher *et al.*, 1996) is unknown.

In conclusion, the content of InAA and toxins in *G.catenatum* offers an interesting contrast with those of *Alexandrium* spp. In general *G.catenatum* responds slowly to changes in the nutrient regime and its toxin content is more stable rather than showing rapid changes of an order of magnitude as may be seen in *A.minutum* (Flynn *et al.*, 1994). For coastal zone management, the implications of the N:P ratio of anthropogenic input may thus be rather less important for *G.catenatum* blooms which are transported into coastal waters, than for other species such as *A.minutum*, because of the slower response rates of the former. However, that is not to say that high levels of ammonium or imbalanced N:P ratios arising from anthropogenic inputs do not have significant effects on *in situ* growth rates and toxicity of *G.catenatum* within coastal embayments.

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