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Dynamics and physiology of saxitoxin production by the dinoflagellates *Alexandrium* spp.

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Abstract

Toxin content (fmol cell^{-1}) and a suite of elemental and macromolecular variables were measured in batch cultures of the dinoflagellates *Alexandrium fundyense, A. tamarense* and *Alexandrium* sp. from the southern New England region, USA. A different perspective was provided by semi-continuous cultures which revealed sustained, steady-state physiological adaptations by cells to N and P limitation. Two types of variability were investigated. In batch culture, changes in nutrient availability with time caused growth stage variability in toxin content, which often peaked in mid-exponential growth. A second type of variability that could be superimposed on growth stage differences is best exemplified by the high toxin content of cells grown at sub-optimal temperatures. Calculations of the net rate of toxin production (R_{tox}; fmol cell^{-1} d^{-1}) for these different culture treatments and modes made it possible to separate the dynamics of toxin production from cell division. Over a wide range of growth rates, cells produced toxin at rates approximating those needed to replace "losses" to daughter cells during division. The exception to this direct proportionality was with P limitation, which was associated with a dramatic increase in the rate of toxin production as cells stopped dividing due to nutrient limitation in batch culture. Growth stage variability in batch culture thus reflects small imbalances (generally within a factor of two) between the specific rates of toxin production and cell division. N limitation and CO_2 depletion both affect pathways involved in toxin synthesis before those needed for cell division; P limitation does the opposite. The patterns of toxin accumulation were the same as for major cellular metabolites or elemental pools. The highest rates of toxin production appear to result from an increased availability of arginine (Arg) within the cell, due to either a lack of competition for this amino acid from pathways involved in cell division or to increased de novo synthesis. There were no significant changes in toxin content with either acclimated growth at elevated salinity, or with short term increases or decreases of salinity. These results demonstrate that toxin production is a complex process which, under some conditions, is closely coupled to growth rate; under other conditions, these processes are completely uncoupled. Explanations for the observed variability probably relate to pool sizes of important metabolites and to the differential response of key biochemical reactions to these pool sizes and to environmental conditions.

Introduction

Several marine dinoflagellates of the genus *Alexandrium* (formerly *Protogonyaulax*) produce a family of potent neurotoxins called the saxitoxins (reviewed by Shimizu 1987), and it has long been recognized that toxicity is variable within this species complex. Isolates of one species can differ in toxin composition, (i.e., they produce different combinations of saxitoxin and its 11 derivatives) and toxin content (the integrated potency or total molar content cell^{-1}) (Alam et al. 1979, Shimizu 1979, Hall 1982, Maranda et al. 1985, Cembella et al. 1987). Single isolates can vary dramatically in toxin content under different growth conditions, and toxin composition has been reported to be invariant by some workers (Hall 1982, Boyer et al. 1987, Cembella et al. 1987, Ogata et al. 1987) or variable by others (Boczcar et al. 1988, Anderson et al. in press). Toxin content in batch culture varies with growth stage (Prakash 1967, Proctor et al. 1975, White and Maranda 1978, Oshima and Yasumoto 1979, Schmidt and Loeblich 1979), salinity (White 1978), temperature (Hall 1982, Ogata et al. 1987), light intensity (Ogata et al. 1987), and nutrient limitation (Hall 1982, Boyer et al. 1987). Toxin content is generally high in exponential growth, decreasing as cultures reach stationary phase. Low temperature and low P concentrations both result in increased toxicity cell^{-1}; N limitation may cause a decrease (Boyer et al. 1987).

The above studies describe general patterns of dinoflagellate toxicity, but physiological or biochemical mechanisms

underlying the observed variations remain unknown. Proctor et al. (1975) hypothesized that toxin content was inversely proportional to growth rate. Ogata et al. (1987) then showed that toxin content increased as growth rate decreased due to temperature or light limitation. Others have looked for a consistent relationship between toxin content and growth rate but have found none (White 1978, Hall 1982).

These studies all focused on toxin content – a highly variable parameter that reflects the balance between toxin production, and losses due to catabolism, leakage into the medium, and toxin transfer to new cells during division. No toxin production rates have been calculated, nor have physiological data been obtained concurrently with toxin measurements and cell counts. Thus there is no way to know whether the toxin increase at low temperature is physiologically related to the increase seen with P limitation, for example. In this study, we have grown *Alexandrium fundyense* under a variety of conditions in batch and semi-continuous culture and monitored changes in toxicity and in several important biochemical parameters. For comparative purposes, some of these measurements were also made for weakly-toxic and non-toxic species of *Alexandrium*. Our objectives were to quantify and compare the rates of toxin production and to look for physiological explanations for the observed variability. Since it is currently not possible to quantify toxin leakage to the medium or toxin breakdown within the cell, measurements were of net toxin production. The corresponding rate calculations are thus limited by the assumption that these toxin loss terms are small, but the information gained adds much to our knowledge of toxin biosynthesis in this important dinoflagellate genus.

Materials and methods

Three experiments are described. One examined the rates of toxin accumulation and the general physiology of *Alexandrium fundyense* and two closely-related species or strains in large batch cultures (hereafter termed batch-culture experiment). The second, salinity-change experiment, examined the effects of short-term changes in salinity on toxin accumulation and the internal pool sizes of free Arg and total amino acids in batch culture. The third, the semi-continuous culture experiment, documented toxicity variations under approximate steady-state P- or N-limited growth.

Organisms

Three *Alexandrium* isolates (Balech 1985) were used in this study. All were unialgal cultures established from germinated cysts and then rendered clonal by isolating single swimming cells, and are available from D. M. Anderson. The most toxic was *A. fundyense* Balech (Strain GtCA29), established in January 1985 from a cyst isolated from Gulf of Maine sediments 20 miles east of Portsmouth, New Hampshire, USA. The weakly-toxic *A. tamarense* Lebour (Strain GtLI22) was established in December 1981 from a germinated cyst from Mud Creek, Long Island, New York, USA. The non-toxic isolate, *Alexandrium* sp. (Strain GtM242), has provisionally been designated a new species by E. Balech (unpublished data). It was isolated using a cyst germinated from Town Cove, Orleans, Massachusetts, USA, in January 1980.

Unless specified otherwise, all cultures were maintained and grown at 15 °C in *K*-medium (Keller et al. 1987) using 0.45 μm filtered Vineyard Sound seawater (31‰ salinity), with 36.3 μM NaH_2PO_4 as the phosphate source. Illumination was on a 14 h light : 10 h dark cycle (ca 125 $\mu E\ m^{-2}\ s^{-1}$ irradiance provided by cool white fluorescent bulbs). All culture glassware was autoclaved partially filled with distilled, deionized water which was decanted after sterilization. Autoclaved medium was then added aseptically.

Batch-culture experiment

This experiment consisted of seven cultures, each in 17 l of medium in 20 l Pyrex carboys. Five carboys were inoculated with the high toxin *Alexandrium fundyense* strain and grown under conditions designated as control: nutrient replete *K*-medium; low PO_4^{3-}: 1/40 of *K*-medium PO_4^{3-}; low NO_3^-: 1/40 *K*-medium NO_3^-, no NH_4^-; high salinity: 38‰, increased from 31‰ by the addition of NaCl; low temperature: 8 °C in *K*-medium. Two additional carboys were inoculated, one with *A. tamarense* and one with *Alexandrium* sp. Both were grown in the same medium and under the same conditions as the control carboy. Each inoculum was grown in 1 l of *K*-medium in a 2 l flask. The low temperature and high salinity treatments were equilibrated to experimental conditions for one transfer prior to inoculation. The medium was gently bubbled with sterile air prior to inoculation; gentle bubbling was continued after inoculation and was increased to moderate bubbling as the cell density exceeded 1 000 ml^{-1}. Cell densities were determined each day by counting at least 200 cells in a Sedgwick-Rafter counting chamber. Average cell volumes were calculated assuming spherical shape and using measurements of the thecal diameter of at least 30 formalin-preserved cells measured microscopically at 400 ×.

At inoculation, several times during exponential growth, and once during the plateau phase, samples were aseptically removed for analysis. Cells for amino acid analysis were filtered onto pre-combusted Gelman A/E filters and placed in cleaned 15 × 150 mm borosilicate screw cap tubes containing 15-ml of boiling double-distilled water. These samples were then placed in a boiling water bath for 3 min and stored frozen at −20 °C. Dissolved free amino acid (FAA) concentrations were determined in the filtered boiling water extract by HPLC analysis of *o*-phthalaldehyde derivatives (Mopper and Lindroth 1982). Total cellular P was assayed using methods described in Anderson and Lindquist (1985). N and C were determined for cells concentrated on pre-combusted 25 mm Gelman A/E filters using a Perkin Elmer 240C Elemental Analyzer. Protein, carbohydrate and

chlorophyll samples were collected on pre-combusted 13 mm Gelman A/E filters, placed in teflon-capped borosilicate glass vials containing 2 ml of 2:1 (v/v) chloroform/methanol and stored at −20 °C. These samples were extracted and analyzed following a sequential extraction scheme (Binder 1986) adapted from Li et al. (1980). Briefly, samples were centrifuged at 2 000 × *g* for 20 min at room temperature. The supernatant was saved and the pellet resuspended in a few ml of chloroform/methanol rinsed twice from the original sample vial and centrifuged again, as above. This supernatant was combined with the first and used for determination of chlorophyll *a* (chl *a*). The pellet was then resuspended in 1.5 ml 5% (w/v) trichloroacetic acid (TCA), incubated at 90 °C for 20 min, and centrifuged as above. The supernatant was used for carbohydrate analysis. The pellet was resuspended in 2 ml of 0.1 *N* NaOH and digested for 20 min at 90 °C, then centrifuged. Next, 1.5-ml of supernatant was removed, neutralized with 150 µl of 1 *N* HCl and used for protein and carbohydrate analyses.

Chl *a* was measured fluorometrically (Strickland and Parsons 1972), with no correction for phaeophytin. Protein was determined with an adaptation (Spector 1978) of Bradford's (1976) assay using Coomassie Blue dye binding. Bovine serum albumin was employed as a standard. Carbohydrate was measured in both the TCA and NaOH fractions using the phenol-H_2SO_4 assay of Dubois et al. (1956). For toxin analysis, 0.5 to 5×10^6 cells were collected in a 10 µm Nitex sieve, washed with filtered seawater into a 15-ml polypropylene centrifuge tube and centrifuged for 3 min at 1 700 × *g* at 20 °C. The supernatant was aspirated and discarded, and 1.0 ml of 0.05 *M* acetic acid added to the pellet. Samples were frozen and thawed three times prior to 0.45 µm filtration into 1.5 ml teflon capped borosilicate autoanalyzer vials. This procedure proved to be as efficient as sonication in disrupting cells for toxin extraction. Most toxins were analyzed by HPLC using the method of Sullivan and Wekell (1988). The C toxins (21-sulfo-1-hydroxysulfate derivatives of saxitoxin and neosaxitoxin) were determined in a separate HPLC run (Sullivan in press).

Salinity-change experiment

Alexandrium fundyense was maintained in normal salinity *K*-medium. Approximately 600 ml of late exponential phase cells were inoculated into two 20 l Pyrex carboys (one experimental, one control) containing 1 l of *K*-medium. The salinity was then reduced from 31 to 28‰ by the addition of nutrient-enriched distilled water. After 2 d cell growth, the salinity of the experimental carboy was increased to 38‰ by the addition of a sea-salt solution (evaporated seawater salts dissolved in 1 l of nutrient-enriched distilled water, ca 3 *M* NaCl) that was gradually added using a peristaltic pump, over a period of 24 h. One liter of *K*-medium was added to the control culture in a similar manner. After experimental cells had grown for 2 d at 38‰, the salinity was reduced to 28‰ by the addition of nutrient-enriched distilled water while the control received an equal volume of *K*-medium (both added in the same manner as previously described). Throughout, samples were taken for cell concentration, toxin and FAA, and analyzed as described for the batch culture experiment. Cell concentrations were adjusted for the different dilutions so that growth curves would be continuous.

Semi-continuous culture experiment

Initial attempts to use true continuous cultures failed due to the sensitivity of *Alexandrium fundyense* to stirring and the need to stir vigorously to ensure uniform withdrawal of cells and medium from the chemostat vessel. Consequently, a semi-continuous mode was adopted whereby medium was pumped into the growth chamber continuously at the desired dilution rate, but cells and medium were removed manually once a day following brief stirring. Each culture was initiated with 5 ml of exponentially growing, axenic, *A. fundyense* (GtCA29) cells inoculated into 1 l water-jacketed reactor vessels (Bellco Glass Inc., Vineland, New Jersey; custom crafted vessels by Anderson Glass Co., Fitzwilliam, New Hampshire). Each vessel contained 1 l of modified *K*-medium (Keller et al. 1987), prepared with 0.2 µ*M* filtered Sargasso seawater reduced in salinity to 30‰ with distilled water. Seawater was autoclaved in teflon bottles and nutrients were later added aseptically (Brand et al. 1981). Modification of the *K*-medium consisted of a decrease in the NaH_2PO_4 and $NaNO_3$ concentration to 0.91 µ*M* and 44.2 µ*M* for the P-limited and N-limited series, respectively, with NH_4^+ also omitted in the latter. Cultures were continuously bubbled with sterile laboratory air and maintained at 15 °C with continuous illumination of ca 100 µE m^{-1} s^{-1} provided by cool white bulbs.

Before these cultures reached maximum cell density, a continuous supply of fresh medium was initiated from a 4 l polypropylene reservoir stored at room temperature. This medium was fed through a network of borosilicate glass, teflon and silicone tubing to the reactor vessels via a peristaltic pump. All components of the semi-continuous culture assembly were autoclaved and aseptically connected prior to inoculation. At the same time each day, cultures were mixed for a few seconds using a magnetic stir bar, and a sample withdrawn (equivalent to the volume of fresh medium pumped in during the previous 24 h). Daily, triplicate, cell counts were performed on this sample. The culture was considered to be at steady state and was harvested when cell density remained constant for a minimum of 2 wk. Toxin samples were processed and analyzed by HPLC as previously described for batch culture.

Calculations

In the batch-culture experiment, a specific growth rate μ (units $time^{-1}$) was calculated over the entire growth exponential phase using the equation:

$$\mu = \frac{\ln (N_1/N_0)}{t_1 - t_0} \quad (1)$$

where N_t is cell concentration at time t. The subscripts denote values at two times. A similar calculation was performed to determine the specific growth rate μ_c and the specific toxin production rate μ_{tox} between successive sampling points within a growth curve. For the latter, toxin content (fmol cell^{-1}) determined from HPLC analysis was multiplied by N_t to yield T_t, the total toxin concentration (pmol toxin ml^{-1} culture) at time t. Values of T were then used instead of the cell concentrations N in Eq. 1 to calculate μ_{tox} over each interval. Using Eq. 1, μ_c was calculated from successive cell concentrations. The net toxin production rate R_{tox} (fmol toxin cell^{-1} d^{-1}) was determined for specific intervals Δt ($=t_1-t_0$) of growth in the batch-cultures using the equation

$$R_{tox}=\frac{(T_1-T_0)}{(\bar{N})(\Delta t)} \quad (2)$$

where $\bar{N}$ is the ln average of the cell concentration,

$$\bar{N}=\frac{N_1-N_0}{\ln N_1-\ln N_0}. \quad (3)$$

It is necessary to use the ln average concentration because the dinoflagellate is growing exponentially between t_1 and t_0 (e.g. Heinbokel 1978).

For semi-continuous cultures, μ was equivalent to the dilution rate. The R_{tox} values were calculated by multiplying the measured toxin content (toxin cell^{-1}) by μ (d^{-1}) for each steady-state.

Results

Batch-culture experiment

Before describing each batch-culture treatment, some general features should be noted. In most cases, cultures experienced a short lag before entering exponential growth. The plateau phase was well-defined for all, except control and non-toxic cultures which exhibited a reduced, but non-zero, growth rate when sampling was terminated. Most cultures had a characteristic convex pattern of toxin content with time (B of Figs. 1 to 7), rising to a peak in mid-exponential growth and decreasing rapidly during the plateau phase. The low PO_4^{3-} culture began with a similar increase in toxin content in the mid-exponential phase, but thereafter continued to increase well into plateau phase (Fig. 2 B). The low temperature culture also exhibited this pattern (Fig. 5), but levels were elevated (ca three-fold over control). In most cultures, Arg cell^{-1} increased as exponential growth ended. Arg often followed a concave pattern with time, mirroring the convex toxin content curve (Figs. 4 B, 5 B and 6 B).

Alexandrium fundyense (GtCA29)

Control culture. These cells grew exponentially between Days 1 and 7 ($\mu=0.5$ d^{-1}; Fig. 1 A). The total culture toxin concentration (Fig. 1 A) closely followed the cell concentration curve, increasing throughout the entire experiment. Toxin content (Fig. 1 B) peaked at ca 140 fmol cell^{-1} between Days 2 and 4 and then decreased to 100 fmol cell^{-1} or less for Days 6 and 7. Arg cell^{-1} was low and relatively constant to Day 6, after which it increased dramatically (15 ×) (Fig. 1 B). P, N, C, protein, and carbohydrate cell^{-1} (Figs. 1 C, D, and E) followed the same general convex pattern as for toxin cell^{-1}.

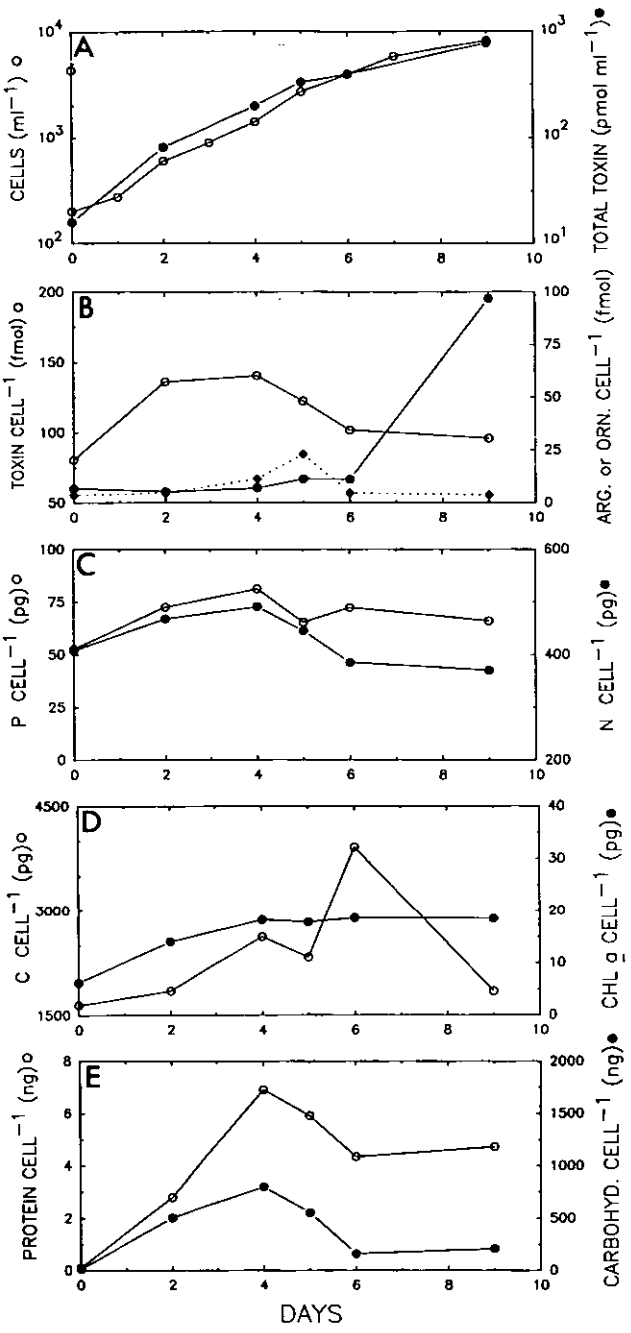

Fig. 1. *Alexandrium fundyense.* Variation in toxicity and general cell physiology of Strain GtCA29 in nutrient replete control culture (batch-culture experiment). A: cell concentration (o—o), total toxin concentration (●—●), inoculum cell concentration (o only); B: toxin content (o—o) and free cellular arginine (●—●) or ornithine (◆···◆); C: phosphorus (o—o) and nitrogen (●—●) cell^{-1}; D: carbon (o—o) and chlorophyll *a* (●—●) per cell; E: protein (o—o) and carbohydrate (●—●) cell^{-1}

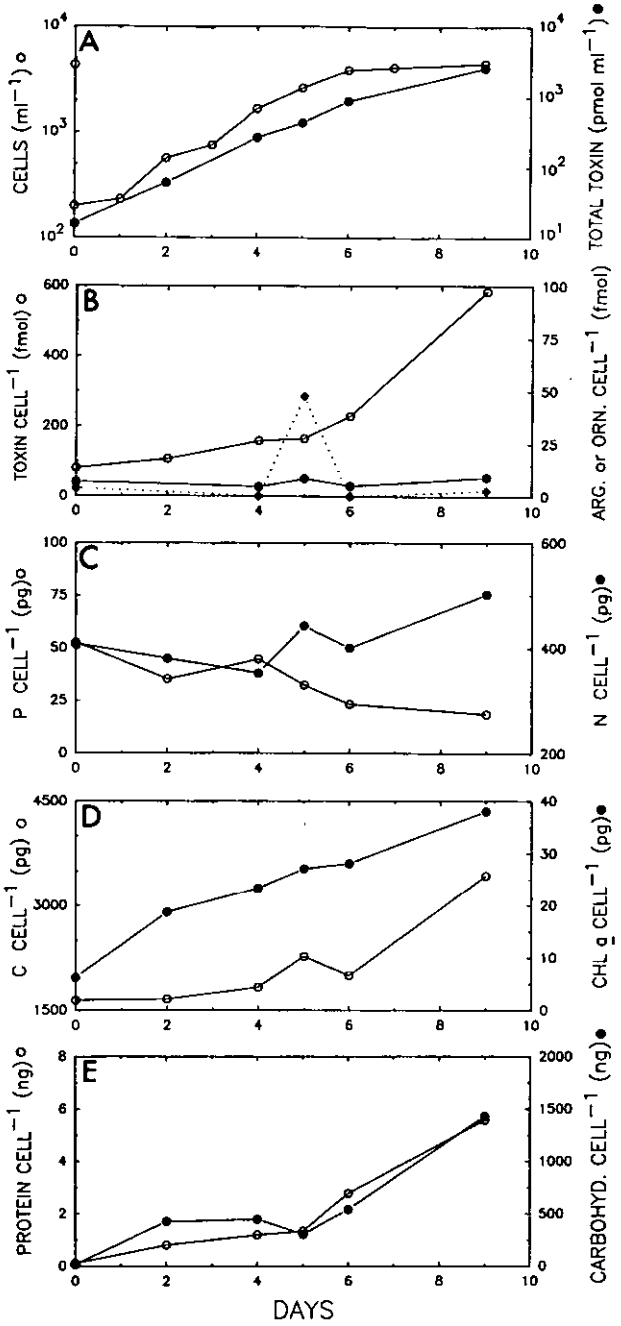

Fig. 2. *Alexandrium fundyense.* Low PO_4^{3-} batch culture experiment. For further explanation see Fig. 1

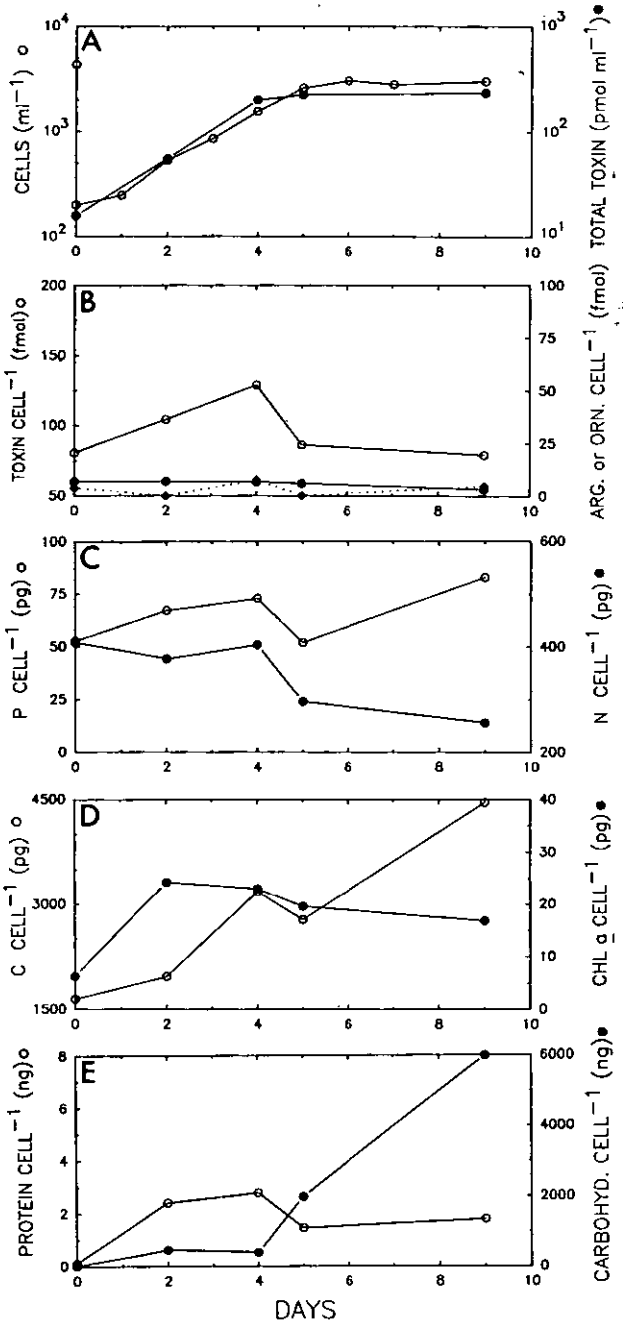

Fig. 3. *Alexandrium fundyense.* Low NO_3^- batch culture experiment. For further explanation see Fig. 1

Low PO_4^{3-} culture. During the exponential phase $\mu = 0.5\ d^{-1}$. A distinct stationary phase was observed (Fig. 2A) due to P limitation, evidenced by decreasing P cell^{-1} (Fig. 2C). Toxin cell^{-1} increased three-fold during stationary phase (Fig. 2B). As a result, the total toxin concentration (Fig. 2A) increased to the end of the experiment, even though the cell concentration had stabilized. Arg cell^{-1} remained low and constant throughout. The trend in toxin content was matched by N, C, Chl *a*, protein and carbohydrate cell^{-1}, all of which increased at the end of the experiment rather than decreasing as in the control.

Low NO_3^- culture. During the exponential phase $\mu = 0.5\ d^{-1}$. A distinct stationary phase was also observed (Fig. 3A), this time due to N limitation (Fig. 3C). The total toxin concentration followed the growth curve (Fig. 3A), and toxin content was similar to the control, peaking at 130 fmol cell^{-1} and decreasing to 80 by Day 9 (Fig. 3B). Arg cell^{-1} was low and essentially invariant throughout. Only chlorophyll and protein cell^{-1} (Fig. 3D to E) followed the toxin content's convex pattern. P, C, and carbohydrate cell^{-1} continuously rose, with the bulk of the increases occurring during plateau phase (Fig. 3C to E).

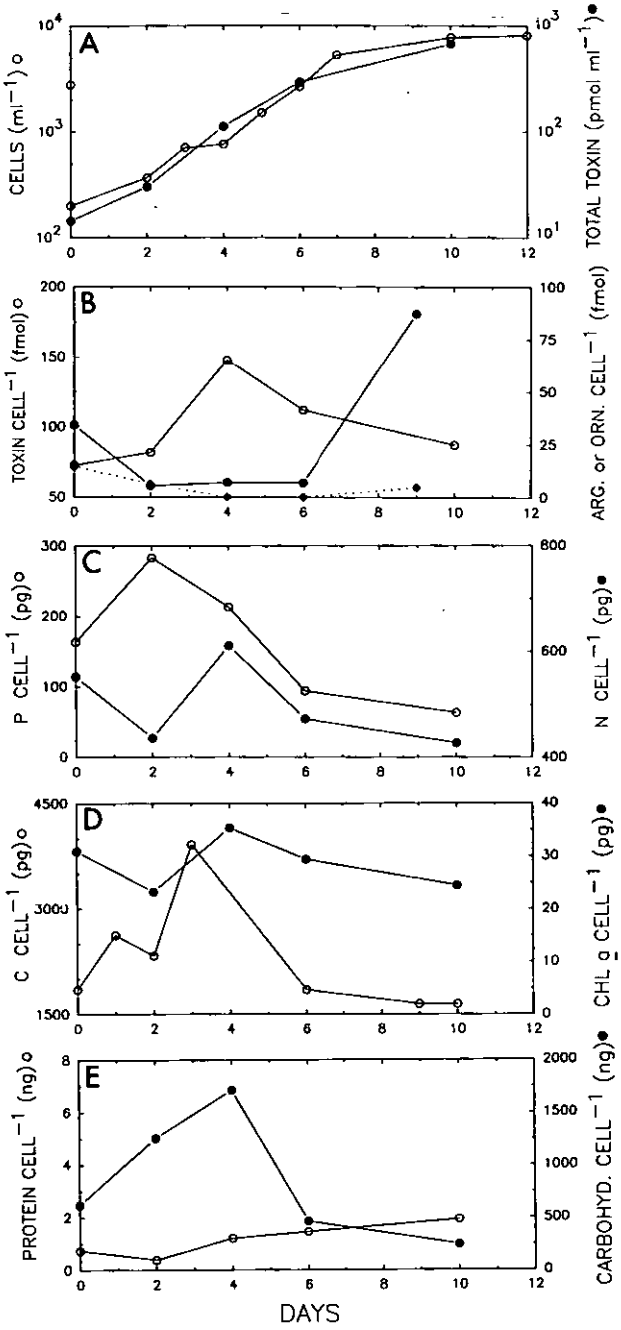

Fig. 4. *Alexandrium fundyense.* High salinity batch culture experiment. For further explanation see Fig. 1

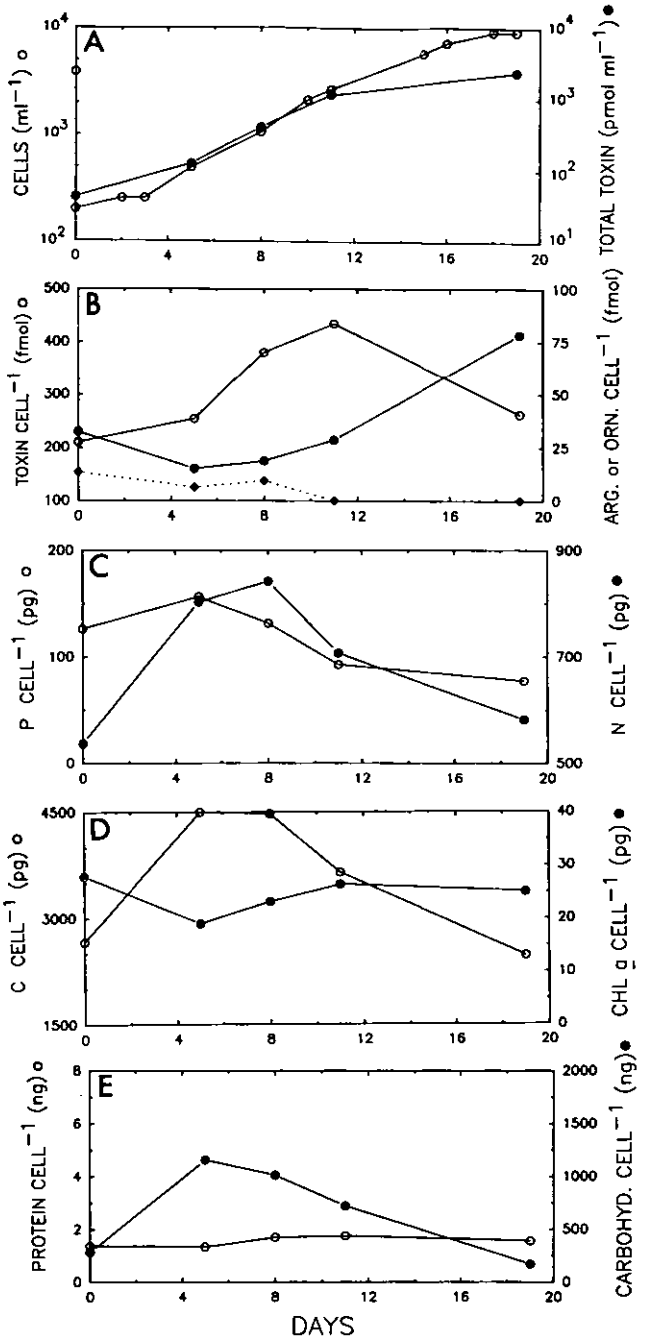

Fig. 5. *Alexandrium fundyense.* Low temperature batch culture experiment. For further explanation see Fig. 1

High salinity culture. During the exponential phase $\mu = 0.5\ d^{-1}$; stationary phase began on Day 10 (Fig. 4A). Total toxin concentration (Fig. 4A) was similar to the growth curve, increasing through Day 10. Toxin content followed the typical convex pattern seen in most other treatments, peaking at 148 and decreasing to 87 fmol cell^{-1} (Fig. 4B). Free cellular Arg (Fig. 4B) was a concave mirror image of the convex toxin content curve. Once again, the patterns of P, N, C, Chl *a* and carbohydrate cell^{-1} over time were similar to that of toxin cell^{-1} (Fig. 4C to E). In contrast, protein cell^{-1} did not peak but increased steadily through time (Fig. 4E).

Low temperature culture. This culture grew at half the rate of the others (0.29 d^{-1}), and the experiment accordingly lasted twice as long. Toxin ml^{-1} increased with time, tracking the growth curve well except at the very end where data points were limited (Fig. 5A). Toxin cell^{-1} was elevated relative to the control (211 fmol cell^{-1} vs 80 at the start of the experiment) and remained high, following the typical convex pattern (Fig. 5B). Again, free cellular Arg was almost a mirror image of the toxin content, but at much higher concentrations than in the control culture (Fig. 5B). P, N, C and carbohydrate cell^{-1} also exhibited the same convex pattern (Fig. 5C to E), whereas protein remained low and invariant (Fig. 5E).

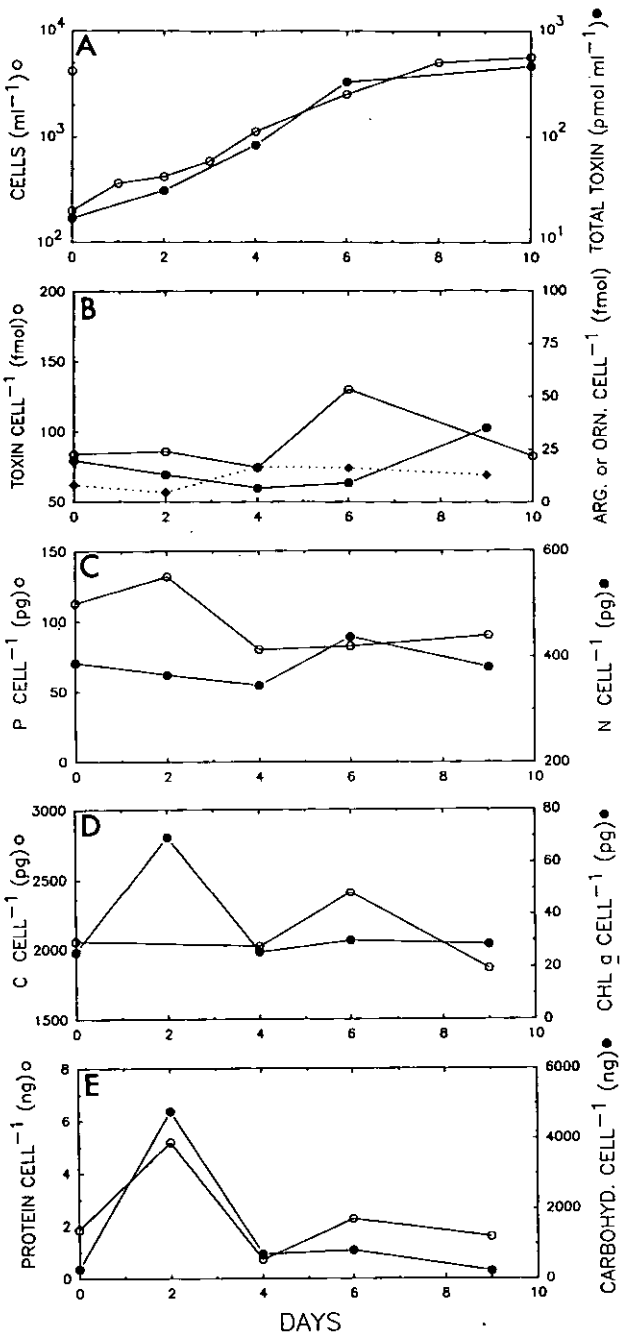

Fig. 6. *Alexandrium fundyense*. Low toxicity culture batch culture experiment. For further explanation see Fig. 1

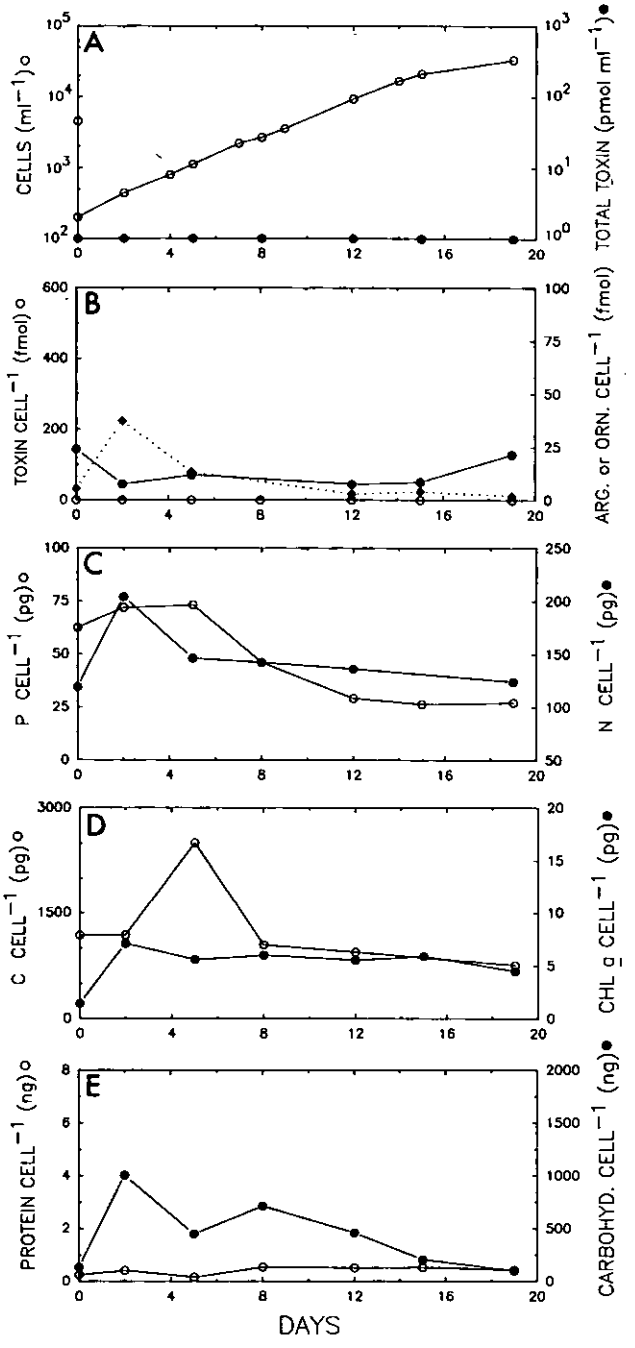

Fig. 7. *Alexandrium sp.* Non-toxic culture, batch culture experiment. For further explanation see Fig. 1

Alexandrium tamarense (GtLI22)

The low toxicity isoplate in nutrient-replete medium grew at $\mu = 0.5\ d^{-1}$ reaching stationary phase on Day 8 (Fig. 6A). Toxin ml^{-1} followed a similar pattern (Fig. 6A). Toxin cell^{-1} rose to a peak at 130 fmol cell^{-1} and then decreased, the peak occurring several days after the maxima observed in the other biochemical parameters (Fig. 6B to E). Free cellular Arg decreased to a minimum on Day 4, increasing gradually thereafter as toxicity decreased (Fig. 6B).

Alexandrium sp. (GtM242)

This *non-toxic* strain grew relatively slowly in nutrient-replete medium (0.32 d^{-1}; Fig. 7A), producing no toxin detectable by HPLC. Arg cell^{-1} was slightly elevated at the beginning and end of the experiment (Fig. 7B). P, N, C and carbohydrate cell^{-1} increased and then decreased (Fig. 7C to E) in the convex pattern-seen in other treatments.

Table 1. *Alexandrium fundyense* and *A. tamarense*. Toxin content expressed on the basis of cells (fmol toxin cell^{-1}), cell volume [fmol toxin (10^3 μm^3)$^{-1}$], cell phosphorus [fmol toxin (ng P)$^{-1}$], cell nitrogen [fmol toxin (ng N)$^{-1}$], cell carbon [fmol toxin (ng C)$^{-1}$], cell chlorophyll *a* [fmol toxin (ng chl *a*)$^{-1}$], cell protein [fmol toxin (ng protein)$^{-1}$], and cell carbohydrate [fmol toxin (ng carbohydrate)$^{-1}$]. All treatments used Strain GtCA29 of *A. fundyense*, except low toxicity culture where Strain GtLI22 of *A. tamarense* was used. N.M. = Not measured

Experiment	Day	Toxin content							
		Cell	Cell vol.	P	N	C	Chl *a*	Protein	Carbohydrate
Control	0	80.6	6.7	1529	198	48.9	13.0	706.2	5.86
	2	136	9.3	1874	291	73.6	9.7	48.6	0.27
	4	140.6	9.8	1731	286	53.6	7.7	20.3	0.17
	5	122.4	8.2	1874	275	52.5	6.9	20.7	0.22
	6	101.6	8.1	1405	264	26.0	5.5	23.3	0.63
	9	95.7	8.0	1450	259	51.9	5.2	20.2	0.46
Low PO_4^{3-}	0	80.5	6.7	1529	198	48.9	13.0	706.2	5.86
	2	106.2	7.1	3007	279	63.9	5.7	128.6	0.25
	4	158.2	11.1	3531	449	86.5	6.8	131.8	0.35
	5	165.0	12.4	5089	373	72.6	6.1	121.7	0.54
	6	229.0	17.4	9827	572	114.6	8.2	82.3	0.42
	9	584.6	35.1	13431	1165	170.3	15.4	104.9	0.41
Low NO_3^-	0	80.6	6.7	1529	197	48.9	13.0	706.2	5.86
	2	104.4	6.4	1552	277	53.0	4.3	43.2	0.22
	4	129.0	9.2	1768	319	40.6	5.6	46.2	0.32
	5	86.3	6.1	1660	292	31.0	4.4	59.2	0.04
	9	79.1	4.3	955	309	17.8	4.8	43.9	0.01
High salinity	0	72.7	5.7	446	131	21.3	2.4	98.6	0.12
	2	81.9	4.5	289	187	26.8	3.5	210.4	0.07
	4	148.0	8.9	694	242	34.2	4.2	121.6	0.09
	6	111.9	6.5	1192	237	41.9	3.8	76.5	0.24
	10	87.3	6.6	1382	205	39.9	3.6	45.1	0.36
Low temperature	0	210.7	10.9	1667	392	79.0	7.5	153.2	0.74
	5	254.2	10.2	1623	317	55.8	13.4	189.9	0.22
	8	380.7	17.4	2890	452	85.0	16.5	223.0	0.38
	11	434.8	17.6	4690	615	119.2	16.5	247.6	0.60
	19	262.5	14.7	3391	451	106.0	10.5	167.5	1.52
Low toxicity	0	84.0	5.4	743	216	40.9	3.3	45.5	0.31
	2	85.9	5.5	651	N.M.	N.M.	1.2	16.6	0.02
	4	74.4	4.9	931	216	36.9	2.9	101.5	0.11
	6	129.9	7.4	1581	297	54.0	4.3	57.3	0.16
	9	30.6	3.1	339	80	16.4	2.9	51.6	0.37
	10	24.3	1.7	N.M.	N.M.	N.M.	N.M.	N.M.	N.M.

General observations – all batch-culture treatments

The toxin data above have been presented in terms of toxin cell^{-1} or toxin ml^{-1} of culture. Table 1 illustrates toxin normalized to other measured parameters. In every treatment except one, normalizing toxin content to cell volume reduced, but did not eliminate, the variability seen on a cell^{-1} basis. For example, in the *Alexandrium fundyense* control culture, toxin cell^{-1} increased 75% from initial levels to a peak on Day 4, whereas toxin (cell vol)$^{-1}$ increased only 46%. Likewise, the percent increases from initial to peak toxin contents on cell^{-1} or (cell vol)$^{-1}$ bases were 625 vs 423% for the low PO_4^{3-} culture, 60 vs 37% for low NO_3^-, 104 vs 56% for high salinity, 106 vs 61% for low temperature, and 55 vs 55% for the *A. tamarense* low toxicity strain.

When toxicity was normalized to other parameters such as P or protein, no consistent pattern emerged (Table 1). Toxin per unit P, for example, had values of 694, 1731, 1768, and 3531 fmol ng^{-1} at the relatively constant toxin contents of 148, 141, 129, and 158 fmol cell^{-1}, respectively, in four of the treatments on Day 4. These toxin cell^{-1} levels corresponded to toxin protein^{-1} values of 121.6, 20.3, 46.2, and 131.8 fmol ng^{-1} protein.

Since there was always a good match between cell concentrations (cells ml^{-1}) and toxin concentrations (fmol toxin ml^{-1}) in each treatment (A of Figs. 1 to 7), an attempt was made to compare the specific rates of increase of these parameters. Rates were calculated between each pair of successive toxin measurements and between each pair of successive cell counts in all batch-culture experiments, including the salinity change experiment. When these specific rates (μ_{tox} and μ_c) were plotted against each other, a linear, direct proportionality was suggested with the highest toxin production occurring at the fastest growth rates (Fig. 8A). No relationship was observed when R_{tox} (fmol cell^{-1} d^{-1} calculated between successive points) was plotted versus μ_c for these same experiments (Fig. 8B).

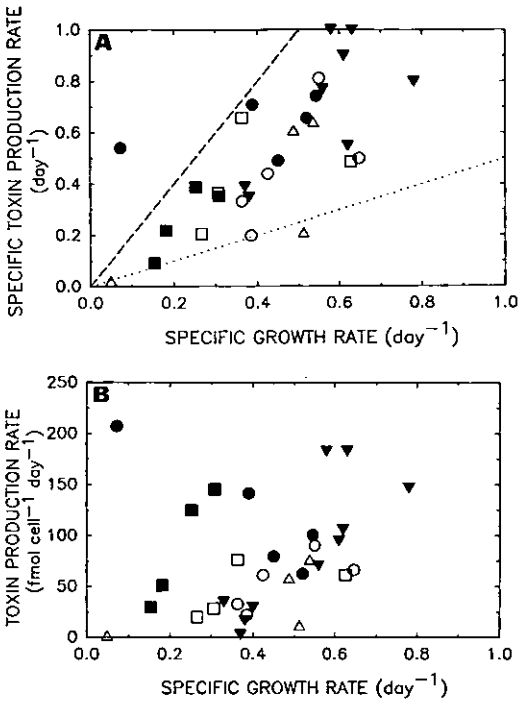

Fig. 8. *Alexandrium fundyense*. Toxin production rates vs specific growth rate (μ_c) calculated between successive data points for all treatments in batch-culture and salinity-change experiments using Strain GtCA29. Symbols are o: control; ●: low PO_4^{3-}; ▵: low NO_3^-; □: high salinity; ■: low temperature; ▾: salinity-change experiment. (A) Specific toxin production rate (μ_{tox}) vs μ_c; dashed and dotted lines represent toxin production rates 2 × and ½ × μ_c, respectively. (B) Net toxin production rate (R_{tox}) vs μ_c

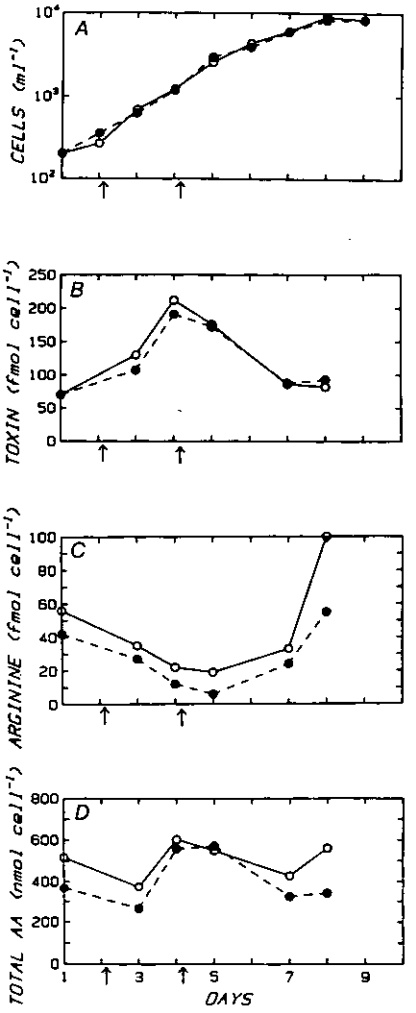

Fig. 9. *Alexandrium fundyense* (GtCA29). Effects of rapid changes in salinity on toxin content and amino acid concentrations. Cells grown initially at 31‰ and inoculated into 28‰ at start of experiment. First arrow indicates when salinity was increased from 28 to 38‰ and the second 38 back to 28‰. Cell concentrations adjusted for dilutions associated with salinity changes. o: control; and ●: experimental culture. (a) Cell concentration; (b) toxin content; (c) free cellular arginine; (d) total cellular free amine acids

Another perspective on the dynamics of toxin accumulation in batch-cultures comes from calculations of R_{tox} for two stages of growth – the interval when toxin cell^{-1} increased in early exponential growth and the interval when it decreased in late exponential, early plateau phase (Table 2). In the former, toxin was produced at rates between 58 and 107 fmol cell^{-1} d^{-1}; in plateau phase, these rates decreased by 50 to 75%, except for the low PO_4^{3-} culture which increased by over 80%. The highest rates were during plateau phase in the low PO_4^{3-} culture and during exponential growth in the low temperature culture (160 and 107 fmol cell d^{-1}, respectively).

Salinity change experiment – *Alexandrium fundyense* (GtCA29)

Cell counts for this experiment, adjusted for the dilutions occurring with each salinity change, showed steady exponential growth for both the control and experimental cultures (Fig. 9A). Toxin cell^{-1} increased to a peak and then decreased, with both cultures having the same general pattern and level of toxicity at each data point (Fig. 9B). Free cellular Arg decreased and then increased in both cultures, mirroring the toxin content curve (Fig. 9C). Arg levels were slightly higher in the control culture that had no changes in

Table 2. *Alexandrium fundyense*. Net toxin production rates (R_{tox}) in batch culture during different growth stages. (Intervals define early exponential growth and late exponential/early stationary phase growth)

Treatment	Interval (d)	R_{tox}
Control	0–4	73.5
	4–6	30.0
Low PO_4^{3-}	0–5	88.6
	5–9	160.6
Low NO_3^-	0–4	69.7
	4–5	11.7
High salinity	0–4	58.3
	4–10	31.2
Low temperature	0–11	106.8
	11–19	29.6

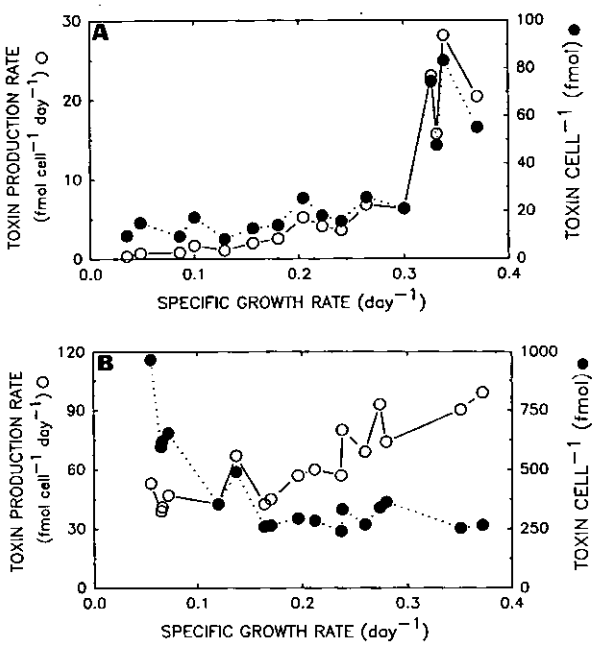

Fig. 10. *Alexandrium fundyense* (GtCA29). Net toxin production rate (R_{tox}; o) and toxin content (●) as function of specific growth rate in (A) N-limited and (B) P-limited semicontinuous cultures

salinity. Total FAA (Fig. 9D) did not vary in a systematic manner.

Semi-continuous cultures – *Alexandrium fundyense* (GtCA29)

In N-limited semi-continuous cultures, both R_{tox} and toxin content increased as growth rate increased (Fig. 10A). These relationships were not linear, however, as both R_{tox} and toxin content increased sharply at and above a μ of 0.3 d^{-1}. With P as the limiting nutrient (Fig. 10B), R_{tox} increased with increasing growth rate in a steady and apparently linear fashion. In contrast, toxin content not only changed in the opposite direction, being highest at the lowest growth rates, but the relationship was clearly non-linear with growth rate.

Discussion

Variability in the toxin content of batch-cultures of *Alexandrium* species can take several forms. One type can be termed "growth stage variability", as it reflects the changing physiological condition of the cells as growth conditions vary through the lag, exponential and plateau stages of batch-culture growth. In nutrient replete cultures with no environmental stresses (e.g. optimal light, temperature, etc.), toxin content is low in lag phase, peaks during early exponential growth and decreases as the cells approach and enter plateau phase. This decrease (and the initial low levels in the lag phase, inoculum cells) presumably reflect the effects of CO_2 depletion, although it is not known whether this is a pH effect or a shortage of CO_2 for photosynthesis in dense cultures. Other examples of growth stage variability are clearly linked to nutrient availability. Examples include the dramatic enhancement in toxicity seen in P-limited cultures (Hall 1982, Boyer et al. 1987, our Fig. 2B) and the low toxin content of plateau phase cells in N-limited cultures (Boyer et al. 1987, our Fig. 3B).

The second type of batch-culture toxin variability is seen as an enhancement in toxin content superimposed on the growth stage variability just described. The cause of this enhancement is not directly nutritional, but is instead a result of physical, environmental variables. The effect is best seen in low temperature cultures, which show the same convex toxin content pattern through time as control cultures grown at a more favorable temperature, but which are typically two to three times more toxic (Fig. 5B). A similar "environmental enhancement" may occur under sub-optimal light or salinity conditions, although the available data are either insufficiently detailed through time in the case of light effects (Ogata et al. 1987), or inconsistent in the case of salinity (see "salinity effects" below) to be conclusive at this time.

It is certainly possible that the physiological mechanisms underlying these two categories of toxin variability in batch-culture are similar, but it is important to recognize that the "signatures" or patterns of that variability are fundamentally different. Likewise, in the discussion to follow, it is important to distinguish the dynamic nature of batch-cultures from the relative steady state of semi-continuous cultures. The two series of semi-continuous cultures in this study isolate the effects of nutrient limitation on toxin production. Each data point represents a degree of intracellular and extracellular nutrient availability to which the cells have adapted. These data thus provide a valuable perspective on growth stage variability not possible in the variable environment of batch-cultures.

Toxin content

Proctor et al. (1975) and Ogata et al. (1987) showed that toxin content in *Alexandrium* species was inversely proportional to growth rate when batch-cultures at different temperatures or light levels were compared. We observed this type of environmental enhancement as well; our low temperature culture grew half as fast as the control culture but accumulated two to three times as much toxin. However, this inverse relationship does not apply to growth stage variability. For example, not only our data (Figs. 1 to 7), but results of Prakash (1967), Proctor et al. (1975), White and Maranda (1978), Oshima and Yasumoto (1979), Hall (1982), Boyer et al. (1987), Cembella et al. (1987), and Boczar et al. (1988) show that within individual batch cultures, toxin content is typically highest when cells are growing fast in early exponential phase and lowest as growth slows and eventually stops in plateau phase. Furthermore, the large differences in toxin content between the phosphorus and nitrogen limited semi-continuous cultures at any given growth rate (e.g.

967 vs 15 fmol cell^{-1} at $\mu = 0.05$ d^{-1}; Fig. 10A, B) clearly preclude a relationship if the data sets are combined.

Toxin content reflects the initial amount of toxin in a cell and the balance between toxin synthesis and toxin losses due to catabolism and leakage (true losses) or cell division (toxin transferred to new daughter cells, hereafter termed division "losses"). A high toxin content is thus not necessarily the result of rapid toxin synthesis, nor is a low toxin content always the result of slow toxin synthesis, although these combinations may occur. It is also possible for a relatively low rate of toxin production to lead to high toxin content in slowly-dividing cells (e.g. Fig. 10B, $\mu = 0.055$), and for a high rate of toxin production to be associated with low toxin content in rapidly dividing cells (e.g. Fig. 10B, $\mu = 0.38$). We conclude that the inverse relationship between toxin content and growth rate observed by Proctor et al. (1975) and Ogata et al. (1987) is only valid for certain types of environmentally-enhanced toxicity; it is not valid across all culture conditions, nor is it applicable to growth stage variability.

Net toxin production rates

We also looked for a relationship between μ and R_{tox} and found none when data from either all batch-culture treatments or both semi-continuous cultures were combined. As with toxin content then, there is no general relationship between the net rate of toxin production and growth rate that applies across all growth conditions. However, within each batch-culture treatment (except low PO_4^{3-}), and in each semi-continuous culture, R_{tox} generally increased with increasing growth rate. The characteristics of this relationship varied with culturing mode and with individual limiting nutrients. One way to visualize and quantify this general relationship in the batch-cultures is to express growth and toxin production in the same units using the specific rates μ_c and μ_{tox} (μ_{tox} can be interpreted as the amount of toxin produced in one day normalized to the amount of toxin present). Fig. 8A shows a general 1:1 proportionality between these two rates, suggesting that toxin was produced at rates approximating those needed to replace division "losses". However, the pooled data obscure subtle but systematic differences inherent in this growth stage variability. The lines in Fig. 8A indicate where toxin production would be two times and one-half times the growth rate. Since all but two points fall within these limits, we see that toxin production excesses or deficits were generally within a factor of two of division "losses". Although small, these differences were systematic and sufficient to cause toxin content to increase and decrease in the convex pattern seen so consistently in batch-cultures. We can generalize that most growth stage variability in toxicity is caused by toxin production rates that are in excess of those needed to offset division "losses" in early exponential growth but less than these losses as plateau phase is approached.

The rates calculated for the semi-continuous cultures reflect approximate steady-state physiological and nutritional conditions. They thus provide useful comparisons with nutrient limitation effects in the low NO_3^- and low PO_4^{3-} batch-cultures. In the low NO_3^- batch-culture, the shortage of nitrogen in plateau phase resulted in a low toxin content and a low R_{tox} (11.7 fmol cell^{-1} d^{-1}). Results from Boyer et al. (1987) suggest that if our experiment had been extended, toxin content might have decreased even further. This is not surprising, since saxitoxin is 33% N on a molecular weight basis. At low dilution rates where N-limitation was most severe in the semi-continuous cultures, toxin content and net toxin production rates were both extremely low but still positive. These data suggest that 10 fmol cell^{-1} represents the lowest toxin content obtainable in dividing cells of *Alexandrium fundyense* (GtCA29). They also show that new toxin continued to be synthesized even when growth was severely limited by N availability, although the rates were the lowest observed in this study (0.35 fmol cell^{-1} d^{-1}). N-limitation in both culturing modes thus resulted in low toxin production rates and low toxin contents since saxitoxin synthesis must compete with other important metabolic pathways for scarce nitrogen atoms.

The value of the semi-continuous cultures is perhaps most evident in the case of phosphorus limitation. Where the low PO_4^{3-} batch-culture showed a very high R_{tox} rate once limitation occurred (161 fmol cell^{-1} d^{-1}), cells adapted to a very low supply of P in the semi-continuous cultures had only moderate R_{tox} rates (45 fmol cell^{-1} d^{-1}). Toxin content still reached high levels similar to those in batch-culture, but the mechanism was different. In the batch-culture, R_{tox} was sustained at high levels for a few days when there was essentially no cell division, producing high toxicity cells. In the semi-continuous cultures, a lower R_{tox} that was sustained over a longer interval (10 to 15 d division^{-1} at low dilution rates) produced the high toxicity cells. In the former case, the important features are that all of the precursors and necessary enzymes were available for rapid toxin synthesis even though phosphorus limitation shut down cell division. This exceptionally high batch-culture R_{tox} was thus a transient event – a doubling of the already rapid rate associated with unstressed, exponential growth (Table 2). As discussed in the section below on cell physiology, this enhanced rate probably reflects both the continued synthesis of the precursor Arg (as occurs in P-limited higher plants; Rabe and Lovatt 1986) and the lack of competition for that amino acid from metabolic pathways specific to cell division. The lower R_{tox} values in the semi-continuous cultures reflect balanced growth where the toxin biosynthetic machinery and the necessary precursors were in equilibrium and competing pathways for cell division and general protein synthesis still operative.

Batch-culture growth stage variability is clearly a disequilibrium phenomenon. Although toxin synthesis often varies in direct proportion to μ, (within a factor of two of levels needed to replace division losses) it can also be completely uncoupled from cell division as a result of nutritional stresses, leading to highly variable rates of accumulation in batch-cultures. The semi-continuous cultures provide a related but fundamentally-different perspective on these nutritional effects since the data represent sustained, steady state

levels. Under those conditions, considerable variability in toxin production rates is still observed, spanning more than two orders of magnitude. It is not a transient imbalance between toxin synthesis and cell division that causes the variability, however, but rather the different physiology of cells under varying degrees of nutrient limitation. Future semi-continuous culture studies are needed that examine other limiting variables such as light or temperature and that extend our N and P results to higher growth rates where nutrients are less limiting.

Salinity effects

White (1978) reported that the toxin content of *Gonyaulax excavata* (= *Alexandrium fundyense*) increased with increasing salinity. This environmentally-enhanced toxicity (between the control culture at 31 and one at 37‰) was by a factor of ca 2. In the two experiments that we conducted in which salinity was varied, no difference in toxin content was detected over control cultures (Figs. 1, 4 and 9). The discrepancy between our results and those of White may reflect differences in experimental design. His high salinity cultures were inoculated with cells acclimated to normal salinity, whereas in our batch-culture experiment (Fig. 4), cells were acclimated to high salinity seawater before the experiment was initiated. In our salinity change experiment, cells experienced short term exposure to higher and lower salinities. With either acclimated growth at 38‰ or with short term changes between 28 and 38‰, we saw no significant changes in toxin content. We also saw no changes in free Arg pools or in total FAA (Fig. 9C, D) with the short term changes. The difference between these results and White's probably reflects the time scales for acclimation. Until White's (1978) experiments are repeated exactly, the effects of high salinity on toxicity must remain an open question.

Cell physiology

In the dynamic environment of a batch-culture, the many metabolic processes involved in nutrient uptake and growth all change rapidly with time. This is called "unbalanced growth" and is associated with major fluctuations in the chemical composition of cells, including growth stage variability in toxicity. Balanced growth occurs when the nutrient supply and environment are invariant (as in a continuous culture), resulting in a stable cellular composition. We have shown that growth stage variability results from major imbalances between the rates of net toxin production and cell division. Before discussing the physiological condition of the cells while these changes were occurring, it is important to note that in the control, high salinity, and low temperature cultures of *Alexandrium fundyense*, and in the low toxicity culture of *A. tamarense*, the final cell yield was limited by CO_2 depletion. This was verified by the addition of CO_2 to carboys grown under our standard experimental conditions, resulting in a three-fold increase in cell number. We do not know if the growth limitation was a direct pH effect or a lack of C.

There are several ways to express toxicity. All studies to date have measured toxin cell^{-1}, but some that have also normalized the data to cell volume have reported no significant change in the patterns of toxicity (Boyer et al. 1987, Ogata et al. 1987). Hall (1982) reported that some observed differences in potency were reduced, but not eliminated, when expressed on a cell volume basis. Our data make this case even stronger, as differences among and within cultures were significantly reduced when toxin was normalized to cell volume (Table 1). For example, peak toxicity in the low temperature culture was three times higher than the control on a cell^{-1} basis, whereas it was only 1.8 times higher on a volume basis. General trends or patterns of toxicity through time were still evident even though the differences between extremes were smaller.

Similarly, when toxin was normalized to each of the measured physiological parameters (Table 1), toxicity was highest in the low PO_4^{3-} and low temperature cultures and the patterns through time showed a peak in early exponential growth for most treatments. One striking aspect of these data is that cells with similar toxin contents in the different treatments were physiologically quite different from each other. For example, cells containing between 130 and 160 fmol toxin cell^{-1} differed five-fold in toxin P^{-1}, six-fold in toxin protein^{-1}, and two to three-fold for the other parameters. A noteworthy consistency is that the highest toxicity cultures both had high toxin protein^{-1}.

In other cultures where cell yield was limited by CO_2 depletion, several metabolic parameters followed the same convex pattern through time as toxin content (Figs. 1, and 4 to 7). The inflection points were not always identical, but cellular P, N, C, protein and carbohydrate all peaked during exponential growth and decreased thereafter. Toxin production thus followed some of the same patterns as major cellular metabolites.

The amino acids Arg and Orn (ornithine) are precursors in saxitoxin biosynthesis (Shimizu et al. 1984). In most batch-cultures, free Arg cell^{-1} varied as the mirror image of changes in toxin content (B of Figs. 1, and 4 to 7; Fig. 8C), being low when toxin content peaked and increasing rapidly as toxicity declined. This Arg increase was not solely the result of a decrease in toxin production, since protein synthesis and other processes requiring that amino acid also decreased as the cells entered plateau phase. Nevertheless, it is of note that Arg synthesis continued under conditions that inhibited toxin synthesis. When N was limiting in batch-culture (Fig. 3), Arg cell^{-1} remained constant and low at all stages of growth.

Cell physiology during the early stages of exponential growth in the low PO_4^{3-} culture (Fig. 2) was the same as in the control and N-limited cultures. However, in a marked departure from the other cultures, there was no increase in the free arginine pool in late exponential/early stationary phase (Fig. 2B). The rapid increase in toxicity at that time demonstrates that the toxin biosynthetic pathway (and thus

the ability to utilize Arg) was still functioning under severe P limitation, even though the cells were unable to divide.

Arg accumulation is characteristic of P deficiency in higher plants. Achituv and Bar-Akiva (1973) proposed that this accumulation could be related to a shortage of pyridoxal phosphate that decreases the activity of aminotransferases. Rabe and Lovatt (1986) argued that accumulation of arginine in citrus leaves during P-deficiency was a result of de novo synthesis, possibly as a mechanism for detoxifying leaf tissue of excess ammonia. If these or other related processes result in increased arginine availability in P-limited *Alexandrium fundyense* cells, the existence of a saxitoxin biosynthetic pathway that is operative even when cell division has ceased can explain the continued toxin production, high toxin content and depleted free Arg pool that we observed in the low PO_4^{3-} batch-culture. The very high R_{tox} value in that culture may reflect this increased Arg synthesis and a lack of competition for this amino acid from pathways involved in cell division. In the latter context, the R_{tox} levels observed for cells that were dividing, albeit slowly, in balanced growth under severe P limitation in the semi-continuous cultures were ¼ the batch-culture rate (Fig. 10 B). Although these R_{tox} levels were low compared to the PO_4^{3-} batch-culture, the associated toxin contents of these semi-continuous culture cells were considerably higher than all other growth treatments in this study. This suggests that there is an enhancement of toxin production due to phosphorus limitation even under steady-state conditions, perhaps due to the excess production of Arg as described above.

The low temperature batch-culture also had elevated toxin content and high R_{tox}. Toxin content was generally two to three times higher than in the control, but it changed through time with the same convex pattern. Protein remained low and constant, but Arg concentrations were generally five to ten times higher than in other batch-cultures throughout early exponential growth (Fig. 5). The explanation given above for the surge in toxicity in the low PO_4^{3-} culture would seem appropriate here as well. We offer the hypothesis that the effect of low temperature was to reduce protein synthesis, resulting in a surplus of arginine within the cell that could be used for toxin synthesis. It follows that the enzymatic reactions necessary for both Arg and toxin biosynthesis would be less affected by low temperature than those involved in general protein synthesis.

An alternative explanation could simply be that low temperature slows cell division more than toxin sythesis, such that the longer duration of each cell generation allows more time for toxin to accumulate. Note that this is a different way of expressing the inverse relationship between toxin content and growth rate proposed by Proctor et al. (1975) and Ogata et al. (1987). One indication that this is not the entire explanation is that the R_{tox} calculated for the exponential growth phase of our low temperature batch-culture was 20 to 40% higher than rates calculated for the same phase in other treatments (Table 2). More data are clearly needed, but if verified, these results suggest that the rate of toxin synthesis is somehow enhanced by cold temperatures, perhaps due to increased arginine availability as discussed above.

Other isolates

This study focused on one isolate of *Alexandrium fundyense*, but two other cultures were included for comparative purposes. One is a "low toxicity" isolate of *A. tamarense*, so called because its toxin composition is dominated by low potency sulfamate toxins (unpublished data). In general, the physiology of this isolate, its toxin content, and the trends in these parameters (Fig. 6) were similar to those of the *A. fundyense* control culture. The non-toxic isolate of *Alexandrium* sp. used in this study (GtM242) is smaller than the *A. fundyense* (GtCA29) strain, averaging one-third to one-half the cell volume. We thus expected a proportionately lower cell content of each of the physiological parameters, but the same general patterns through time as for the *A. fundyense* cultures. This is generally what was observed for N, C, chl *a* and carbohydrate. However, Arg cell^{-1} was virtually the same, while protein cell^{-1} was less than one-tenth the *A. fundyense* values. The non-toxic culture grew relatively slowly (0.3 vs 0.5 d^{-1}) and thus may have been stressed in some unknown way. This may be a situation analagous to the low temperature culture in which there was low protein synthesis and a corresponding increase in the pool of free Arg. The physiological differences between the non-toxic culture of *Alexandrium* sp. and the toxic *A. fundyense* may not be related to whether they made toxin or not, but more likely reflect a difference in the experimental growth rates.

Overview

In this study, we tried to separate and quantify the processes of toxin production and cell division and then looked for physiological patterns that might provide insights into the mechanisms of toxin variability. This is a difficult task because many different types of toxin variability are observed. We have shown that under most growth conditions, toxin is produced at rates that vary in direct proportion to growth rate. Non-nutritional variables such as temperature (and perhaps light) can elevate toxin production in what we have termed environmentally-enhanced variability. Mechanisms underlying this enhancement are unknown, but may include increased availability of the precursor arginine or the differential inhibition of pathways for toxin synthesis and cell division. Nutritional stresses (from N, P, and perhaps C limitation) also affect pathways for toxin synthesis differently from those involved in cell division, leading to the imbalances that are responsible for growth stage variability in batch cultures. When cells are acclimated to varying degrees of nutrient limitation in semi-continuous culture, toxicity is still variable between treatments. Low toxin production rates and toxin contents under N limitation presumably result from competition for that element between saxitoxin and proteins and other essential N-containing compounds. Varying degrees of P-limitation in semi-continuous cultures also cause toxin production to vary. One notable characteristic of this variability is the high toxin content of cells under severe P limitation, possibly resulting from de novo synthesis of arginine at levels in excess of cell division needs.

Future studies should not ignore the complication and importance of intracellular bacteria as potential toxin progenitors inside the dinoflagellate (Kodama and Ogata 1988). If bacteria can be verified in all saxitoxin-producing dinoflagellate strains, their response to the external environment and to the physiological condition of the host dinoflagellate will be important considerations. Regardless of the genetic source of the toxin, however, explanations for the patterns and trends of toxin variability reported here are likely to be found in studies of the pool sizes of arginine and other important metabolites and in the biochemical reactions that are differentially affected by particular limiting nutrients or by environmental factors such as temperature.

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