

TOXIN VARIABILITY IN Alexandrium SPECIES

DONALD M. ANDERSON*

*Biology Department, Woods Hole Oceanographic Institution,
Woods Hole, MA 02543 USA

INTRODUCTION

One of the more important yet least understood aspects of the ecology and physiology of the dinoflagellate Alexandrium is that toxicity is highly variable both between isolates of the same species and in single isolates grown under different conditions [1-9]. The following summary focuses on some of the more important aspects of toxin variability, not in the form of a comprehensive review, but rather as an indication of the types of insights such variability provides on important issues in dinoflagellate taxonomy, population dynamics, toxin biosynthesis, and general physiology.

VARIABILITY BETWEEN POPULATIONS

In regions subject to recurrent episodes of Paralytic Shellfish Poisoning (PSP), it is now clear that toxicity is not caused by one widespread, homogeneous species but instead by a variety of different subpopulations that may be varieties of one species or a group of closely-related species. Two regional populations of Alexandrium have been well characterized in this respect, one on the northeast [6,16] and the other on the northwest [5,7,15] coast of North America. Despite the inherent limitations of the mouse bioassay for saxitoxin, a clear indication of the extent of Alexandrium population heterogeneity over large geographic areas resulted from a study that quantified the toxicity of 34 strains isolated from the region between New York and Nova Scotia [6]. This study is noteworthy because it not only documented a two order of magnitude variation in toxicity among isolates, but it also revealed that this toxicity followed a decreasing trend from north to south.

Toxicity was expressed as toxin content (μ mouse units per cell), so the differences between isolates could have been due to variability in the total number of moles of toxin contained in a cell or in the mixture of toxins (toxin composition) of variable potency that each isolate produced [10,11]. Although the former possibility remains to be rigorously tested, extensive differences in toxin composition have since been demonstrated in this collection of isolates using HPLC [12]. It is now clear that the high toxicity of the northern isolates reflects their production of the highly potent carbamate toxins (e.g. saxitoxin (STX) and gonyautoxins I-IV (GTX I-IV)), whereas southern isolates typically contain the weaker N-sulfocarbamoyl toxins (e.g. B1, B2, C1-4). Although the toxin content data suggest a continuous north to south trend, cluster analysis (based on toxin composition differences) suggests the existence of three related groups within this regional population - a distinct cluster of

northern isolates and two more geographically-diverse clusters of southern isolates [12]. The observed north to south trend in toxin content could thus result from latitudinal differences in environmental parameters (such as temperature) and their influence on the establishment of genotypically different blooms. Alternatively, the clustering of toxin composition types might reflect species dispersal from several origins or spreading centers.

On the west coast of North America, studies by Hall [5] and Cembella et al., [7] also document considerable variability in toxin composition between regional populations of Alexandrium. Some regional populations were found to be more genetically diverse than others, possibly reflecting the extent of advection and mixing of populations in those areas. Most importantly, there was no correlation between toxin composition and the morphological characteristics typically used to separate tamarenoid from catenelloid morphotypes [7]. A similar finding resulted from toxin composition analyses of the east coast Alexandrium populations [12]. Some strains identified as A. fundyense had toxin profiles that were more closely related to those of A. tamarense isolates than to other A. fundyense strains. These two studies, both based on toxin composition differences, have important implications relative to the species concept in dinoflagellates [13]. Morphologists have consistently had difficulties discriminating between species in the "tamarensis complex" [13,14], and biochemical information from isozyme electrophoresis and toxin composition [15,16] should therefore be useful in resolving the confusion. Since isolates were grown under identical conditions in each of the toxin composition studies described above, the observed differences are presumed to be genetic. To some, such differences should be sufficient for the definition of separate species, yet others argue that such distinctions are too restrictive and that a better alternative is to recognize the existence of physiological races or varieties within one "supraspecies" [13]. Resolution of this important taxonomic controversy will take time, but it is clear that toxin variability will be a major factor in the debate.

VARIABILITY IN INDIVIDUAL ISOLATES

Toxin Composition. Variability in the toxicity of a single isolate is generally attributed to differences in the rate of toxin production or accumulation under different growth conditions and not to any differences in toxin composition. Until recently, those studies that have used chromatographic methods to quantify saxitoxin and its derivatives have all concluded that toxin composition is a relatively stable or conservative property in Alexandrium species [5,7-9]. It is now clear, however, that toxin composition changes in a single isolate do occur under different growth conditions. This was first noted by Boczar et al., [17] using batch cultures of A. tamarense and A. catenella. In one strain that produced only neosaxitoxin (NEO) and STX, the mole % of NEO increased from 8 to 44% as

the culture aged, with STX decreasing proportionally. Another culture that produced a more diverse array of toxins also showed variability through time, with GTX I and IV increasing as GTX II and III decreased. These changes were only significant 10 days or more after cell division had ceased - the toxin composition remained relatively constant during exponential growth. The cultures were nutrient replete, so growth presumably ceased due to CO₂ depletion.

In a more recent study of A. fundyense in both batch and semi-continuous cultures where growth was limited by factors other than CO₂, these same trends and some new ones were verified [12]. Nitrogen limitation in semi-continuous culture favored the production of toxins Cl,2 and GTX I,IV, whereas phosphorus limitation produced cells with high relative abundance of GTX II,III. As batch cultures aged, the interconversion between STX and NEO was again observed. In addition, the accumulation of GTX II,III in cells that had ceased dividing due to phosphorus depletion was noted [12]. In all cases, STX reached its highest relative abundance when growth was most rapid.

These are all relatively new data, so their implications are only now being considered. At first glance, these results raise doubts as to the fundamental validity of using toxin composition as a chemotaxonomic marker in the context described above for the east and west coast North American populations of Alexandrium. However, on closer examination, it is clear that the changes in batch culture occur only after exponential growth has stopped and the cells become senescent. Replicate cultures assayed at the same stage of growth have essentially identical toxin profiles. Since toxin composition comparisons have always been made between cultures grown under identical conditions and harvested at the same stage of exponential growth [7,12], the conclusion that the toxin differences have a genetic basis is still correct and the clustering analyses and other comparisons still valid. It should also be noted that it was the relative abundance (mole %) of the different toxins that changed - the specific array of toxins produced did not vary in these studies.

From a chemical standpoint, most of the compositional changes observed in the semi-continuous cultures [12] are reasonable, in that as growth rate increased, a decreasing trend in one toxin was typically associated with an increase in another toxin that could result from a relatively simple chemical transformation. For example, with nitrogen-limited growth, a decreasing trend in the relative abundance of GTX I,IV and Cl,2 as growth rate increased was associated with an increasing trend in STX, NEO and GTX II,III. We know that Cl and C2 are easily hydrolyzed to GTX II and GTX III in vitro. Likewise, oxidation at the N-1 position converts STX to NEO, and conversion of GTX I and GTX IV to GTX II and GTX III requires only the cleavage of the N-1-hydroxyl group. We must be careful here, as these statements imply interconversions between toxins (as might occur in a batch culture approaching stationary phase), whereas each toxin profile at a given growth rate in the semi-continuous culture study [12] was from a culture that was essentially in steady state. The more

correct way to view the compositional trends would be to recognize that differences in cell physiology under the growth conditions of each culture altered either the rate of biosynthesis of each toxin or the extent of conversions between toxins at equilibrium. Given our relative ignorance of the pathways involved in synthesis of saxitoxin and its derivatives [18], it is not yet possible to explain these compositional changes on the basis of specific biochemical reactions that might be expected under different growth or physiological conditions (i.e. why would a cell growing slowly due to lack of phosphorus produce predominantly 11-hydroxysulfate toxins?). We are thus in the awkward position of knowing that the compositional changes described above will someday be logical given a more complete knowledge of biosynthetic pathways and cell physiology during growth, but at present, this background knowledge is lacking and we are left with unexplained observations.

Toxin Content and Net Toxin Production Rates. The toxin content of individual isolates has been shown to vary with temperature, salinity, irradiance, degree of nutrient limitation, and with growth stage in culture, [1-9,17,19,20]. In an attempt to synthesize these diverse observations, all of which are from batch cultures, it is first necessary to recognize the dynamic nature of these batch cultures. Growth conditions are initially optimal, but then rapidly change as one nutrient is depleted or when growth is limited by other variables such as light or CO₂ depletion. Generally, toxin content is highest in mid-exponential growth, decreasing thereafter as the cells enter stationary phase [1,3,5,8,17,19]. This means that during the early stage of growth with optimum conditions with no nutrient limitation or stresses, toxin accumulates because it is produced faster than it is transferred to daughter cells during division. The optimum conditions are short-lived, however, and the cells soon experience nutrient limitation or the effects of CO₂ depletion (which presumably causes the eventual cessation of growth in nutrient-replete cultures). The subsequent decrease in toxin content that is often observed indicates that the rate of toxin production decreases even though cell division continues. This occurs with both nitrogen limitation and CO₂ limitation [1,3,5,8,19], but when phosphorus is limiting, toxin content continues to rise, even after the cells have stopped dividing [5,18,19]. These changes in late stages of growth indicate several important points: (a) the pathways of toxin synthesis are more easily inhibited by nitrogen limitation or CO₂ depletion (pH or CO₂ limitation) than are those involved in cell replication; and (b) cell division is more sensitive to phosphorus limitation than is toxin synthesis.

Cultures grown under sub-optimal temperatures show the same convex toxin content curve through time described above for many batch cultures, but the entire curve is elevated. This type of enhanced toxicity superimposed on the growth stage variability described above may also be the case with light limitation, although published data are not sufficiently

detailed in time [9] for this to be verified.

The first attempt to synthesize these different observations was by Proctor et al., [1] and Ogata et al., [9] who argued that toxin content was inversely proportional to growth rate. This conclusion was based on the high toxin content of cells grown at sub-optimal temperatures and light. It is now clear that this is not a general relationship that applies to all growth conditions, since recent studies using nitrogen-limited semi-continuous cultures of Alexandrium revealed a direct proportionality between growth rate and toxin content [19], the opposite of the hypothesized inverse relationship. Phosphorus limited semi-continuous cultures did follow an inverse relationship, however, so at least three variables (temperature, light, phosphorus) seem to affect growth rate and toxin accumulation in a similar manner. This relationship is only relevant in comparisons between growth treatments, such as with cultures grown at different temperatures. Within one culture, the opposite generally applies, namely that toxin content is highest when the cells are growing fastest.

But why would cells growing slowly due to sub-optimal temperatures contain more toxin than faster growing ones? Are they producing toxin at the same rates, but retaining more due to a lower rate of cell division? How and why does the rate of toxin production vary with growth rate under different conditions? To address these questions it is necessary to move beyond toxin content as a measure of toxicity. Toxin content reflects the difference between toxin production and a series of loss terms that include catabolism, leakage into the medium, and most importantly, toxin transferred to new cells during division. A more useful measure for comparing the dynamics of toxin accumulation under different conditions would be based on the rate of production ($\text{fmol toxin cell}^{-1} \text{ day}^{-1}$). This approach was taken in an extensive study of several Alexandrium isolates to be published elsewhere [19]. A few conclusions from that study should be emphasized. In batch culture, there is a direct proportionality between growth rate and net toxin production rate (i.e. as growth rate increases, so does the rate of toxin production). This is not a 1:1 relationship, however, as toxin is consistently produced in excess of the amount transferred to daughter cells when nutrients are abundant and growth rapid. As CO_2 depletion or nitrogen limitation occurs in late exponential growth, toxin is "lost" to daughter cells faster than it is produced. These toxin production excesses or deficits are generally within a factor of 2 of levels needed for balanced growth, but the differences are sufficient to produce the typical convex toxin content curve through time in batch culture. In the approximate steady state of semi-continuous cultures, the net toxin production rate is also directly proportional to growth rate, again indicating that the faster the cells grow, the faster they produce toxin. Since there are conditions under which toxin synthesis is blocked but cell division continues and other conditions where the opposite occurs, there is no simple relationship between growth rate and toxin content.

By themselves, these observations tell us little about the regulation of toxin production. However, in conjunction with a series of concurrent physiological measurements, some useful relationships emerge [19]. For example, in most of the batch cultures, free arginine (the amino acid precursor to saxitoxin [18]) varied as the mirror image of toxin content, being very low when toxin content peaked and increasing rapidly when toxicity declined. Arginine synthesis thus continued and accumulated in the cell under conditions that inhibited toxin synthesis. In contrast, arginine pools remained low in the P limited culture that increased so dramatically in toxin content during stationary phase. Phosphorus limitation thus blocked pathways necessary for cell division but did not affect toxin synthesis. Interestingly, the low temperature culture that had elevated toxin content contained high arginine levels during early exponential growth. Since the protein level of these cells was low, it may be that the low temperature inhibited protein synthesis (and thus growth rate), resulting in a surplus of arginine within the cell that could be used for toxin synthesis. The general hypothesis that emerged was that enhanced toxin production is associated with increased availability of arginine in the cell at times when the toxin biosynthetic pathway is still functional [19]. Arginine can be made more available through the blockage of competing pathways (e.g. for general cell division or protein synthesis) or from a reorganization of N metabolism that results in greater activity of the arginine synthetic pathway. The latter has been suggested as an explanation for enhanced de novo synthesis and accumulation of arginine in P-deficient citrus leaves, possibly as a mechanism for detoxifying excess ammonia [21]. If this mechanism holds for phosphorus deficient Alexandrium cells, arginine would not accumulate due to the existence of an alternative pathway that requires arginine (toxin synthesis).

The variable toxin contents of cells at different stages of growth and under different growth conditions clearly reflect a number of different mechanisms and physiological conditions. The discussion above emphasizes the complexity of the toxin biosynthetic process and stresses the need for concurrent studies of the pool sizes and regulation of arginine and other important metabolites.

CELL CYCLE VARIABILITY

All studies of toxin variability in Alexandrium species have examined toxin changes on time scales from days to weeks. The discussion above demonstrates how toxin production and growth can be closely coupled. Without knowledge of the metabolic role of the saxitoxins in the dinoflagellate, however, such data once again remain observations without physiological explanations. Reasoning that we need to know more about the finer details of toxin synthesis as cells divide, we initiated a cell cycle study of one A. fundyense isolate for which the preliminary data can be summarized here.

The generalized cell division cycle for eukaryotic cells is usually depicted with 4 discrete sequential intervals called G_1 , S, G_2 , and M (Fig. 1). G_1 and G_2 are the gaps separating DNA synthesis (S) and cell division or mitosis (M). The time it takes to traverse one complete cell division is the generation time of the cell; the duration of each cell cycle stage varies with cell type and with different growth conditions.

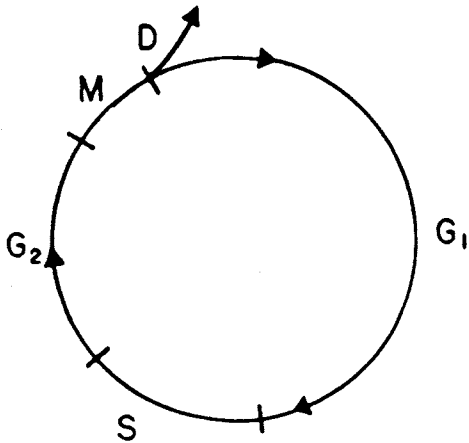


FIG. 1. Generalized cell cycle diagram. S corresponds to DNA synthesis, M to mitosis, D to cytokinesis and daughter cell separation. G_1 and G_2 are gaps between these events.

The objective of our study, was to see whether toxin synthesis is continuous during the cell cycle, or occurs instead during discrete intervals. In order to conduct a study of this type, the culture had to be synchronized such that the divisions of individual cells in a population were aligned as tightly as possible to result in simultaneous division. In essence, the objective was to induce an entire culture to express the behavior of a single cell. This required prolonged storage in constant darkness, after which the culture was released to a normal 14:10 L:D cycle. Cell concentration, cell volume, toxicity, and some physiological parameters were measured at hourly intervals. The number of cells in each cell cycle stage was also determined using propidium iodide stain and flow cytometry. As seen in Fig. 2A, a small amount of division occurred 15-20 hrs after the normal photocycle was re-established, but the major division burst caused the cell concentration to double over a 6 hr interval beginning 45 hours after the lights were first turned on. As seen in Figure 2C, toxin analyses are not yet complete, but those samples that have been run suggest some important relationships. Figure 2C shows that toxin content began to increase slightly before the increase in DNA synthesis that was first apparent at hr 34 and peaked at hr 40. The subsequent decrease in toxin content through hr 48 coincided with the division burst. Simple book-keeping of the total toxin in the culture shows that toxin synthesis occurred during the first half of the S phase, but then completely stopped for more than 10 hrs as the cells went through mitosis and divided at the end of our experiment. Toxin synthesis was thus not continuous throughout the cell cycle, but was temporally linked to the pulse of DNA synthesis. Other

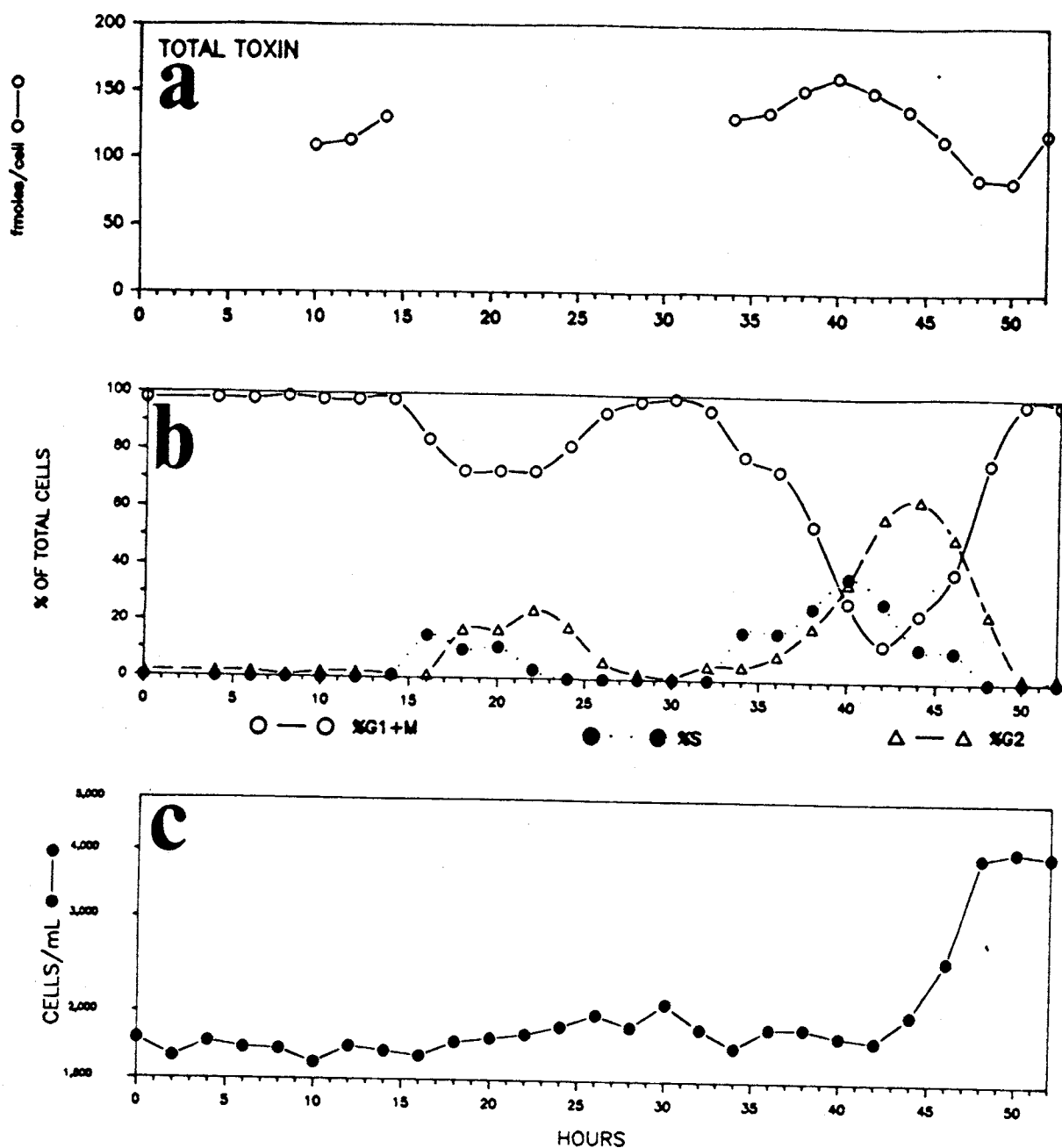


FIG. 2. Toxin production during the Alexandrium cell cycle. Starting at time 0, the cells experienced a regular 14:10 L:D photocycle. (a) Total toxin (fmol/cell) (missing points represent samples not yet analyzed due to a shortage of standards). (b) Percentage of cells in each cell cycle stage. ○ = G₁+M, ● = S, and Δ = G₂. Note that prior to the major division burst, DNA synthesis began at hour 34. (c) Alexandrium fundyense cell concentration. Note the major division burst beginning hour 44.

experiments with two complete division bursts are underway to test the general validity of this important observation.

We now can hypothesize that some of the variability in toxicity under different growth conditions may be due to cell cycle variability. For example, Olson et al., [22] showed that low temperatures caused an increase (up to 5 fold) in the duration of all cell cycle phases of a diatom and a coccolithophore, but increases in only G₁ when nitrogen was limiting. If these relationships are valid for Alexandrium, we could explain the elevation in toxin content observed at low temperatures [5,9,19,20]. Specifically, when low temperature slows growth, the S phase will be proportionately longer, allowing toxin to be produced for a longer time between divisions and resulting in an elevated toxin content. (Recall that batch culture experiments showed that toxin synthesis could proceed at high rates even at low temperatures that inhibited protein synthesis and growth [19]). There are no published data showing the effect of P limitation on cell cycle stage durations in phytoplankton, but it is possible that the S phase might be lengthened (and possibly other stages as well) due to a shortage of P for energy and for nucleic acid synthesis. Thus the enhanced toxicity in P-limited Alexandrium might also be cell cycle related. These are clearly speculations based on a rather limited dataset, but they do demonstrate the level of knowledge that we must have if we are to understand toxin variability over small time scales. Cell cycle studies will not answer all of our questions about toxin variability, but they should provide insights relevant to many of our presently unexplained observations.

OVERVIEW

Toxin variability in Alexandrium isolates takes a variety of forms. Differences in total potency or toxin content between populations can now be attributed in part to heterogeneity in toxin composition, but explanations for those differences and for geographic trends in toxicity remain out of reach. Such trends may reflect environmental selection of certain genotypes, but it's too soon to even guess what role the different toxins might play (if any) in that selection. Alternatively, geographic patterns in toxin content or composition might simply reflect dispersal patterns. For individual isolates, we now see that the commonly accepted paradigm of invariant toxin composition is wrong and that dramatic changes are possible with different growth conditions. Here again, explanations for the observed compositional changes await more knowledge of organism physiology and biochemistry. In addition to toxin compositional changes, the number of moles of toxin in individual isolates (toxin content) can vary with growth conditions. This variability takes two forms in batch culture. One is a growth stage effect that reflects the balance between variable rates of toxin synthesis and the dilution of that toxin through cell division. A direct

proportionality between growth rate and the rate of toxin production is generally observed, even though the rate of toxin production can vary by more than two orders of magnitude under different growth conditions. The second type of toxin variability in batch culture is a general enhancement of toxicity due to temperature or light effects - an enhancement seen in addition to the growth stage variability described above. There are no physiological explanations for this enhancement, but one possibility is that variability in the duration of the different cell cycle stages might regulate the amount of toxin produced in each division cycle, and thus determine the toxin content. Alternatively, the combined effects of these environmental variables on the pool size of free arginine and the activity of the toxin biosynthetic pathway could determine the level of toxin accumulation. As we learn more about toxin variability, these mechanisms will undoubtedly become clearer, and hopefully will enable us to address the fundamental question of whether the saxitoxins are important in Alexandrium metabolism and cell replication or are instead only secondary metabolites with no essential functional role.

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