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Toxicity of *Alexandrium lusitanicum* to gastropod larvae is not caused by paralytic-shellfish-poisoning toxins

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Abstract

Laboratory grazing experiments compared ingestion of two subclones of the dinoflagellate *Alexandrium lusitanicum* by gastropod veliger larvae (*Nassarius* sp.). While the two prey subclones originated from the same monoclonal isolate of *A. lusitanicum*, one possessed the ability to produce paralytic-shellfish-poisoning toxins (PSTs), while the other did not. Ingestion rates on the two *Alexandrium* subclones were not significantly different over a range of prey concentrations (approximately 100 – 660 cells ml⁻¹), indicating that PSTs did not serve as a grazing deterrent for these larvae. However, ingestion rates on both subclones were low at the higher prey concentrations tested. Mortality of the predators also increased linearly with concentration of either subclone. These observations indicated that both *A. lusitanicum* subclones produced an unknown substance that inhibited and killed the grazers. Veliger mortality was not induced by culture filtrates or lysates, suggesting either that the substance was either highly labile or that contact with intact cells was required. Because toxic algae can produce multiple bioactive substances, experimental demonstrations of allelopathic effects of toxic species should not be assigned to known toxins without supporting evidence. In addition, the results show that the effectiveness of algal grazing deterrents can increase with cell concentration, which may have implications for bloom dynamics.

Keywords

Alexandrium lusitanicum; Dinoflagellate; grazing; Harmful algal blooms (HABs); *Nassarius*; Paralytic shellfish poisoning (PSP); Red tide; saxitoxin; veliger

1. Introduction

Several species within the dinoflagellate genus *Alexandrium* produce saxitoxin and related toxic compounds. During blooms of toxic *Alexandrium* species, the toxins can accumulate in filter-feeding shellfish. Subsequent ingestion of such contaminated shellfish by humans can result in serious illness or death through paralytic shellfish poisoning (PSP; Shumway,

1990; Van Dolah, 2000). For this reason, saxitoxin and related derivatives (Shimizu, 1996; Cembella, 1998) can be collectively referred to as paralytic shellfish toxins (PSTs). Reducing PSP occurrence requires shellfish testing, and closures of shellfish beds and aquaculture facilities, all of which carry substantial economic costs (Shumway et al., 1988; Hoagland et al., 2002; Anderson et al., 2005). Furthermore, PSTs can also accumulate in organisms other than shellfish, potentially affecting the entire food web. For example, copepods carrying PSTs were identified as the secondary toxin vector in fish kills (White, 1980) and in mortality of endangered whales (Geraci et al., 1989). Concerns related to *Alexandrium* blooms have been heightened in recent years because these dinoflagellates can spread to previously unaffected areas through both natural and anthropogenic means (Anderson, 1997; Hallegraeff, 1998; Anderson et al., 2005).

While maintenance and regulation of a metabolically complex biosynthetic pathway suggests that there must be a selective advantage associated with PST production (Cembella, 1998), there is no consensus on what that advantage might be. Several potential functions for PSTs have been hypothesized. PSTs could play a role in DNA metabolism or structure (Mickelson and Yentsch, 1979; Anderson and Cheng, 1988), in N storage (Anderson et al., 1990), as allelochemicals against competing phytoplankton (Blanco and Campos, 1988), or as pheromones (Wyatt and Jenkinson, 1997). However, the most common hypothesis is that PSTs provide a chemical defense against predators (see reviews by Turner and Tester, 1997; Turner et al., 1998 and additional references below). In analogy, evidence is accumulating for the production of other anti-predation chemicals by other planktonic microalgae (Strom et al., 2003a; Strom et al., 2003b; Cembella, 2003). Nevertheless, experimental evidence of an antipredatory role for PSTs remains equivocal (Turner and Tester, 1997; Turner et al., 1998). Typical experiments present a given predator with a PST-producing *Alexandrium* prey and a non- (or low-) PST-producing prey, either separately or mixed. Many such studies have shown negative responses of grazers to PST-producers. Grazers may change their behavior (Hansen, 1989; Tillmann and John, 2002), reject toxic cells (Teegarden, 1999), or reduce clearance or ingestion rates (Ives 1985; Turriff et al., 1995; Barreiro et al., 2006; Teegarden et al., 2007). Grazer mortality (Hansen, 1989; Tillmann and John, 2002; Tillmann et al., 2007) or decreased reproduction (Dutz, 1998; Frangopulos et al., 2000; Barreiro et al., 2006) can also occur. However, some studies have shown no effects of PST producers on at least some of the grazers tested (Teegarden et al., 2001; Colin and Dam, 2003; Kamiyama et al., 2005; Tillmann et al., 2007), or conversely found that only some (but not all) PST-producers had an effect on a particular grazer (Tillmann and John, 2002; Teegarden et al., 2007; Tillmann et al., 2007).

Such prey-selection studies come with a variety of caveats. In cases of selection against a PST producer, it is certainly possible that the toxin itself explains the preference for the non-PST prey. However, other, unknown differences between toxic and non-toxic prey could also account for the result, even if the prey are closely related. In cases where PST producers were eaten without negative selection, it may be that the incubation conditions were inappropriate to detect the natural effect of the toxins. For example, a predator might be induced to ingest undesirable prey if it has few, or no, other food options. Also, the toxins may only be effective against certain predators, or under special environmental conditions,

not included in the study. The diversity of results related to an antipredator function for PSTs is therefore not surprising.

One way to improve the experimental approach is to minimize potential differences between the PST- producing and non-PST-producing prey. In this study, two subclones of the same parental culture of *Alexandrium lusitanicum* were used as a novel experimental prey pair for veliger larvae. Although derived from the same clonal isolate, over many years, one of the subclones lost the ability to produce PSTs (Martins et al., 2004). Excepting PST production, these two subclones were probably as similar as two different prey cultures could be. Even under these carefully controlled conditions, the results illustrate the difficulties in interpreting simple grazing experiments, suggest that chemical defense in dinoflagellates can involve substances other than the obvious toxins, and point out the important role of cell concentration for predator deterrence by phytoplankton.

2. Methods

2.1. Culture history

Details of the culture history, and taxonomic and toxin analyses are given in Martins et al. (2004). To summarize, the original, monoclonal *A. lusitanicum* culture was isolated in 1962 from a bloom in Lagoa de Obidos, Portugal (strain 18-1). It has since been continuously maintained at the Laboratório de Microbiologia e Ecotoxicologia in Lisbon. In 1992, a subculture was sent to Dr. Greg Doucette's Laboratory (Marine Biotoxins Program, National Ocean Service Center for Coastal Environmental Health and Biomolecular Research, Charleston, SC, USA), where PST production was confirmed (therefore designated strain 18-1T). Between 1995 and 2000, the original culture maintained in Portugal stopped producing PST (now designated strain 18-1NT to distinguish it from the original and the Doucette subcultures). Most recently, the two strains have been maintained for several years side by side, under identical growth conditions, in the Anderson laboratory (Woods Hole Oceanographic Institution). Plate morphology of strains 18-1T and 18-1NT from the Anderson lab confirm that both are *A. lusitanicum*. Ribosomal gene sequences (of the D1/D2 and D8/D10 regions of the large ribosomal subunit, and of the ITS1, 5.8S, and ITS2 regions) of the two strains are identical to each other and match known sequences for *A. lusitanicum*. Mouse bioassays, HPLC assays, and mouse neuroblastoma assays all show that 18-1T produces PST, while 18-1NT does not. The toxin profile of 18-1T matches that of the original isolate, dominated by gonyautoxins 1 and 4, with lesser amounts of gonyautoxins 2 and 3. Based on these results and other information, Martins et al., (2004) concluded that the two strains were subclones, derived from the original clonal culture.

From the perspective of grazing experiments, these two subclones are arguably as similar as two prey types can be, with the critical absence of PSTs in 18-1NT, and thus provide an excellent experimental system to test for potential effects of PSTs on grazers. For this study, field-collected veliger larvae were used as the experimental grazers. The two subclones were presented to veligers in separate monocultural suspensions to test the hypothesis that ingestion rates on the two subclones were equal. If the null hypothesis was rejected, the presence of PSTs in clone 18-1T could be responsible.

2.2. Prey culture conditions

Both subclones were maintained in modified f/2 medium: as in Guillard and Ryther (1962), but without silicate, only 1×10^{-8} M Cu^{2+} (final conc.) and the addition of 1×10^{-8} M SeO_3^{-2} (final conc.). Cultures were grown at 18°C with a 12:12 h light:dark cycle (approximately 200 – 250 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ with the lights on) provided by cool-white fluorescent lights. Only exponential-phase cultures were used in experiments.

2.3. Grazing experiments

Grazing experiments were conducted in June 2003, using zooplankton collected from Great Pond, a coastal embayment along the south shore of Cape Cod (MA, USA). On the day before an experiment, a plankton tow was made using a 64- μm mesh plankton net with a solid cod end. Contents of the cod end were diluted into chilled, filtered seawater for transport to the Anderson laboratory (~15 min). Veliger larvae, identified as *Nassarius* sp. (H. Fuchs, personal comm.), and *Polydora* sp. larvae (Spionid polychaetes) dominated the mesozooplankton at the time of the study. Initial trials with *Polydora* larvae revealed that they were highly cannibalistic so further tests focused only on the veliger larvae. Using a dissecting microscope, 20-30 ml subsamples of the zooplankton tows were inspected and veligers were individually pipetted into 0.2- μm filtered seawater (18°C, FSW) using drawn-out glass Pasteur pipettes. After 50-300 veligers were isolated, they were added to a polystyrene tissue culture flask (280 ml). The flasks were then filled with FSW and the veligers were allowed to clear their guts overnight (at 18°C). The next morning, most of the water was siphoned off from each flask using tubing fitted with 64- μm mesh netting to exclude the larvae. An aliquot of the appropriate prey algae and enough FSW to top off the flask were added. Two to four replicate flasks were made of each prey concentration (Table 1). For each prey concentration (except zero prey), two no-grazer control flasks were set up for each subclone to determine growth rate of the dinoflagellates without grazing. All flasks were attached to a plankton wheel and rotated end over end at 1 rpm for approximately 48 h.

Subsamples for determining cell concentration were collected at the beginning of each incubation and again after approximately 24 and 48 h. At each time point, the plankton wheel was stopped, each flask was gently rotated several times, and three, 0.5-ml subsamples were pipetted out, pooled and preserved with Utermöhl's solution (2% final concentration) (Utermöhl, 1958). Prey cells were counted at 100 $\times$ magnification using a gridded Sedgewick-Rafter counting chamber (Wildco). The entire chamber (1 ml), or at least 300 cells within a known fraction of the chamber, was counted per sample.

Grazer mortality was estimated after 24 and 48 h of incubation by counting the number of veligers within each flask that settled to the bottom of the flask. Veligers found on the bottom were partitioned into several categories: actively moving, non-moving/intact, and non-moving/decaying. Only non-moving/decaying veligers were considered unquestionably dead, but even individuals with the most decayed soft parts could be counted because of their shell. The living veliger number was then estimated by difference. Veliger mortality rates were calculated assuming an exponential decay of live veligers over time. The total number of veligers per flask was recounted on several occasions by reisolation. The count only deviated from the expected number by 2-3 larvae. Using the counts of grazers and prey

over time, mean ingestion rates of prey cells per veliger per hour were calculated using the equations of Frost (1972). To account for grazer mortality, the mean living veliger number in each flask during the incubation was used in calculations. In the incubations with the highest prey level (950 cells ml⁻¹) grazer mortality was particularly high and ingestion rates were not estimated.

2.4. Effect of prey filtrates and lysates on veliger mortality

A separate set of experiments determined the effect of filtrates and lysates of the 18-1T strain on veliger larvae. To test the effect of the filtrate, 4 flasks were prepared as for the grazing experiments, each with 50 veligers suspended in *A. lusitanicum* fresh culture filtrate (approximately 1500 cells ml⁻¹, GF/F filter). Similarly, another 4 flasks were made with 50 veligers each suspended in cell lysate. The cell lysate was obtained by centrifuging down 1.5 L of culture (approximately 1500 cells ml⁻¹) into a dense pellet that was sonicated on ice for a total of 3 min. in 30 s bursts. Lysis of the majority of dinoflagellate cells was confirmed by microscopic observation. FSW was used to reconstitute the original volume for suspending the larvae. Finally, 4 control flasks used 50 veligers each in GF/F filtered seawater. As in the grazing experiments, veligers that settled to the bottom of each flask were counted and categorized after 24 and 48 h. Only non-moving/decaying individuals were considered dead. As before, mortality rate calculations assumed an exponential decay of live veligers over time.

2.5. Prey toxin profiles

To confirm that toxin profiles of the two subclones remained consistent with previous analyses (Martins et al., 2004), even in the presence of grazers, toxin analyses were conducted on samples of each subclone from stock cultures and from two flasks used in the highest cell concentration treatments (see Table 1). For each subclone, fifteen ml of cell suspension were harvested by centrifugation (5000g; 10 min) followed by resuspension of the pellet in 0.05M acetic acid. Toxins were extracted by sonicating (10W, three 10s cycles) the acid slurry in an ice bath followed by 3 cycles of freezing/thawing. Samples were stored at -20°C prior to analysis. Toxin content and profile of each sample was obtained by HPLC according to the method of (Oshima, 1995) as described in (Anderson et al., 1994). PSTs were identified and quantified by comparison with reference standards of GTX 1-5, kindly provided by Prof. Y. Oshima (Tohoku University, Sendai, Japan).

3. Results

HPLC analysis of extracts from the two subclones, collected both from stock cultures and from experimental incubations, confirmed that the 18-1T subclone produced PSTs with a profile matching previous analyses (primarily GTX1 to 4), while 18-1NT lacked retention peaks matching any known saxitoxin. Chromatograms were essentially identical to previous analyses and are therefore not shown (see Martins et al., 2004). As mentioned above, the toxicity of 18-1T (and lack of toxicity of 18-1NT) has been previously confirmed with mouse bioassays and mouse neuroblastoma assays (Martins et al., 2004). Assuming approximately 3785 fg saxitoxin equivalents cell⁻¹ (Martins et al., 2004), total PSTs in the

18-1T treatments of different experiments ranged approximately 0.38 – 3.6 ng saxitoxin equivalents ml^{-1} .

Ingestion rate data were first analyzed separately for each subclone using linear regression of ingestion rate as a function of prey concentration. The regression intercepts for both subclones were significantly different than zero (18-1NT: $P = 0.046$, 18-1T: $P < 0.0001$), indicating that significant ingestion was occurring at the lower prey concentrations. The linear regression slope of ingestion rate vs. prey concentration for 18-1NT was not significantly different from zero ($y = -0.001 \times + 8.46$, $r^2 = 0.002$, $F_{1,10} = 0.016$, $P = 0.90$), while ingestion rate on the toxic subclone declined significantly with increasing prey concentration ($y = -0.018 \times + 18.1$, $r^2 = 0.644$, $F_{1,10} = 18.1$, $P = 0.002$). However, neither the slopes nor intercepts for the two subclones were significantly different from each other (m : $F_{1,20} = 2.78$, $P = 0.11$; b : $F_{1,21} = 3.08$, $P = 0.094$).

An additional comparison of ingestion rates on the two subclones was made using a 2-way analysis of variance (ANOVA). The ingestion rate data were parsed into 4 categories of approximate prey concentration: 100, 200, 450, and 600 cells ml^{-1} , with 2 - 4 replicate flasks per category. The ANOVA used ingestion rate as the dependent variable with subclone (2 categories) and prey concentration (four categories) as the factors. No significant differences in mean ingestion rates by subclone ($F_{1,16} = 3.275$, $P = 0.089$) or prey concentration ($F_{3,16} = 2.386$, $P = 0.107$) were found, and there was no significant interaction between the factors ($F_{3,16} = 0.976$, $P = 0.43$). Figure 1 shows the mean ingestion rates by veliger larvae on each subclone as a function of prey concentration.

The regression and ANOVA analyses indicated that pooling ingestion rate data for the two subclones was justified. After pooling, the function of ingestion rate vs. prey concentration appeared unimodal in shape (Fig 1). A Gaussian function fitted to the mean ingestion rate data for both subclones resulted in an r^2 of 0.60 (solid line in Fig. 1).

Mortality of veliger larvae during grazing experiments was linearly related to the prey concentration for both subclones (Fig. 2). However, there were no significant differences in either the slopes ($F_{1,36} = 1.15$, $P = 0.29$) or intercepts ($F_{1,37} = 0.325$, $P = 0.57$) of the relationship for either subclone.

The mean daily mortality rate of larvae incubated in FSW, cell filtrate, and cell lysate of *A. lusitanicum* (18-1T) were compared using a one-way ANOVA. No significant differences in mortality rates were found ($F_{2,11} = 0.264$, $P = 0.77$).

4. Discussion

The two *A. lusitanicum* subclones provided the opportunity to experimentally present a grazer with prey that were as similar as possible except for the presence of PSTs. The objective was to test the effect of PSTs, rather than to quantify potential grazing in the field. These veliger larvae are likely to encounter PST-producing *Alexandrium* sp. in their natural habitat, though not *A. lusitanicum*. Contrary to the hypothesized role of PSTs as a grazing inhibitor, veliger larvae ingested both subclones at comparable rates over a range of prey concentrations. PSTs clearly did not function to inhibit grazing for these larvae. However,

this aspect of the results should not be generalized, as other grazers may be affected by PSTs. Further use of these *A. lusitanicum* subclones in experiments with other grazers may shed light on this potential ecological function for PSTs.

Zooplankton ingestion rates generally increase with prey concentration. However, in these experiments, ingestion rates either did not change or fell significantly as prey concentrations rose. Overall, the relationship between ingestion rate and prey concentration appeared to be unimodal. One possibility for low ingestion rates at higher prey concentrations is grazer saturation. To test this possibility, ingestion rates of veliger larvae on *A. lusitanicum* were compared to veliger ingestion rates on other algae. Fig. 3 shows the C-based ingestion rates on *A. lusitanicum* from this study, along with C-based ingestion rates of *Nassarius* sp. veliger larvae on three other algal species (Pechenik and Fisher, 1979). The veliger larvae used in the Pechenik and Fisher (1979) study were collected at the same time of year and within a few km of our study site. In addition, their grazing experiments were conducted at a similar temperature and salinity (19 °C, 30 ppt). The two data sets should therefore be quite comparable. Cell-based ingestion rates from figure 3 of Pechenik and Fisher (1979) were converted using C cell⁻¹ values for each of the 3 prey species listed in their Table 1. To convert our data to C units, we used a value of 0.6 ng C cell⁻¹ for *A. lusitanicum*, based on a biovolume to C relationship for dinoflagellates (Menden-Deuer and Lessard, 2000).

At low cell concentration, ingestion of *A. lusitanicum* was comparable to the ingestion rate on the other algae (see inset box of Fig. 3). However, the Pechenik and Fisher (1979) data show that veliger larvae similar to those used in this study clearly have the capacity to graze algal prey at C-based rates that are as much as an order of magnitude greater than those measured for either *A. lusitanicum* subclone. More importantly, veligers can also maintain such high ingestion rates with prey concentrations as much as an order of magnitude higher than the highest *A. lusitanicum* concentrations used in this study. The comparison of our result with those of Pechenik and Fisher (1979) discounts the likelihood of grazer saturation as an explanation for the low ingestion rates at the higher concentration of either *A. lusitanicum* subclone.

Although the results do not support a role for PSTs in inhibiting grazing, several lines of evidence indicate that both *A. lusitanicum* subclones did produce an allelopathic substance that affected the grazers. First, ingestion rates at higher prey concentration were low, although the predators should not have been saturated. Qualitative observations of veliger behavior during incubations suggest that these low ingestion rates were related to incapacitation of the larvae. In low prey concentration incubations, and in cell-free controls, nearly all larvae maintained normal swimming behavior throughout the experiment. In high prey concentration incubations, by contrast, many veligers did not swim or swam only weakly. These observations are consistent with the linear rise in veliger mortality with increasing concentration of either subclone. We conclude that both *A. lusitanicum* subclones produced a substance that depressed grazing by veliger larvae and caused larval mortality. However, the substance was not active in culture filtrates or lysates, which may indicate that it is highly labile or that it functions after ingestion.

Several other studies have found evidence for production of “toxins” or allelopathic compounds other than PSTs in *Alexandrium* sp., including production of hemolytic substances (Bass et al., 1983; Ogata and Kodama, 1986; Simonsen et al., 1995; Arzul et al., 1999; Tillman and John, 2002; Fistarol et al., 2004; Tillman et al. 2007). Specifically, investigations of the toxic effects of *A. lusitanicum* potentially indicate multiple toxins in this species. Perovic et al. (2000) found that supernatants from cultures of two strains of *A. lusitanicum* (K-2 and BAH ME-091) were neurotoxic and caused an influx of Ca^{+2} to isolated neuronal cells. Similarly, Blanco and Campos (1988) found that *A. lusitanicum* filtrates (“culture 54”) killed 3 other species of microalgae. While these cultures were all described as PST producers, toxicity in cell-free medium and the effects on Ca^{+2} transport described in Perovic et al. (2000) are not consistent with PSTs, which are generally intracellular and function as Na-channel blockers. The results of Blanco and Campos (1988) and Perovic et al. (2000) also contrast with this study which found no toxic effects in culture filtrates. Dütz (1998) found that copepod fecundity dropped when fed *A. lusitanicum* (BAH ME-091). The fecundity effect was attributed to PSTs, though other substances could have been responsible. Interestingly, Tillmann and John (2002) found no effect of the BAH ME-091 *A. lusitanicum* strain on swimming and cell integrity of two heterotrophic dinoflagellate grazers, although other *Alexandrium* sp. strongly affected these grazers. The effects of other *Alexandrium* cultures on the grazers were attributed to unknown toxins, apparently lacking in BAH ME-091. It is likely that toxic algae produce multiple bioactive compounds in addition to any known toxins. The results of this study point out that apparent toxic effects by harmful algae should not be assigned to the known toxins without supporting evidence.

A grazing deterrent does not necessarily have to increase in effectiveness as cell concentration rises. However, it is not surprising that the *A. lusitanicum* deterrent described here functions in this manner. Higher cell numbers means that more of the deterrent will be available. Several other studies have shown similar cell-concentration dependence in grazing deterrents produced by harmful algae (Tracey, 1988; Hansen, 1989; Nielsen et al., 1990; Hansen et al., 1992; Turriff et al., 1995; Teegarden et al., 2003; Juhl and Franks, 2004). During bloom formation, greater inhibition of grazers as cell concentration rises could provide a positive feedback, enhancing net algal growth rates. Similarly, once a dense patch of cells is established through local growth or aggregation, it would be less susceptible to attack by grazing until physically dispersed. Such feedbacks on algal net growth and mortality rates may be important in bloom formation and termination.

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Figure 1.

Mean ingestion rates of veliger larvae fed PST-producing and non-PST-producing subclones of *A. lusitanicum* as a function of prey concentration. Further experimental details listed in Table 1. Error bars = 1 standard error. The solid line is a Gaussian function fitted to the mean values for both subclones ($r^2 = 0.60$).

Figure 2.

Exponential mortality rate of veligers fed on either *A. lusitanicum* subclone as a function of prey concentration. Data are from the same experiments shown in Fig. 1 with the addition of data at zero (FSW) and 950 *A. lusitanicum* cells ml⁻¹.

Figure 3.

C-based ingestion rates of *Nassarius* sp. veliger larvae on four species of algae. Ingestion rates of *A. lusitanicum* from this study (same data as Fig. 1, converted to C units), rates for the other algae taken from Pechenik and Fisher (1979). As in Fig. 1, the solid curve in the inset graph is a Gaussian function fitted to the mean ingestion rates on both *A. lusitanicum* subclones ($r^2 = 0.60$).

Table 1

Experimental conditions for different treatments

Prey	Mean prey concentration (cells ml ⁻¹)	Veligers per flask	Number of replicate flasks
No prey control	0	50	4
18-1T (PST)	116	50	3
18-1T (PST)	201	100	3
18-1T (PST)	426	100	2
18-1T (PST)	660	300	4
18-1T (PST)*	950	50	4
18-1NT (no PST)	97	50	3
18-1NT (no PST)	205	100	3
18-1NT (no PST)	487	100	2
18-1NT (no PST)	559	300	4
18-1NT (no PST)*	950	50	4