Production of Paralytic Shellfish Toxin (PST) by *Alexandrium tamarense* in response to Copepod grazing

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Introduction

Dinoflagellates are one of the largest groups of protozooplankton in the sea, comprising an estimated 2000 extant species (Taylor, 1987). The dinoflagellates include the largest number of toxic species associated with harmful algal blooms (HABs) (Anderson, 1998). Some planktonic dinoflagellates produce a suite of toxin associated with paralytic shellfish toxins (PSTs), a group of highly potent neurotoxic alkaloids (Selander et al. 2006, Selander et al. 2008), especially the genus Alexandrium, have a more or less cosmopolitan distribution (Hallegraeff, 2003). The toxins accumulate in shellfish feeding on the algae often with no apparent negative effect on the shellfish. However, these contaminated shellfish pose a serious threat to human health and economic losses for shellfish industries worldwide (Asp, 2004). Also, PST may cascade through the food web and intoxicate higher trophic levels and occasional mass mortalities of marine mammals and seabirds have been attributed to PST (Landsberg, 2002).

PSTs are neurotoxins which primarily act through inhibition of sodium ion influx through neuronal sodium channels. The mode of action is well known on a molecular level, much due to the use of PSTs as a manipulating agent in neurobiology (Llewellyn, 2006). The toxins have been hypothesized to act as feeding deterrents; many studies have shown that toxic algae are ingested by grazers (Frangópulos et al. 2000, Cástor et al. 2002, and Selander et al. 2006). The copepods are one of the zooplankton groups that prey on toxic dinoflagellates (Turner & Tester 1997) and can be negatively affected by toxic marine dinoflagellates. Ingestion rate, eggs production, hatching success naupliar fitness has been reported to decrease in response to toxic dinoflagellates (Frangópulos et al. 2000).

Toxin synthesis in marine phytoplankton species is not a constitutive component of algal metabolism, but both toxin content and toxin composition of algae are influenced by environmental growth conditions (Plumley, 1997). Some dinoflagellates species produce more toxins under phosphate limitation (Guisande et al. 2002).

The ultimate cause for PST production is not clear. Cembella (2003) suggested that the toxins may act as a protection against grazers. An ecological advantage of toxin production by toxic dinoflagellates is probably to offset interspecific competition by redirecting grazing pressure onto non-toxic phytoplankton species that are potential competitors.
Selander (2007) shows that some dinoflagellates are able to sense and respond to waterborne cues from their natural enemies. *Alexandrium minutum* responds to waterborne cues from copepod grazers with increased toxicity and copepod grazers avoid the more toxic *A. minutum* which together suggest that PST formation constitutes an inducible defense towards copepod grazers. Also, this indicates that not only resource availability and physical stress, but also biological interactions may be important in determining the toxicity of *Alexandrium* spp. All previously published work on grazer induced toxin formation concerns the warm water species *A. minutum* and that it has been suggested that this species mainly responds to warm water copepods species (Bergkvist *et al.*, 2008). In Sweden PSTs are mainly produced by *A. tamarense*, and it is not known if *A. tamarense* responds to copepods grazers. Here we evaluate the following hypothesis:

H1: Cultures of *Alexandrium tamarense*, exposed to copepod grazers, produce a larger amount of PST compared with individuals not exposed to copepods grazers (control)

The general objective is to determine the production of PST of *Alexandrium tamarense* cultured with and without different species of copepods grazer.
Materials and methods

Algae
The strains of *Alexandrium tamarense* used in this study were originally obtained from the Gullmarsfjord, Sweden. Two experiments were performed. The first experiment was carried out with *A. tamarense* #3 and the second with *A. tamarense* #1 now available at Goteborg University Culture Collection (GUMACC). Cultures were grown in sterile cotton-plugged Erlenmeyer flasks (250 ml) by inoculating approximately one fifth of the cultures into fresh K medium every second week (Selander *et al*, 2006).

Zooplankton
The calanoid marine copepods, *Acartia clausi*, *Centropages typicus*, *Acartia tonsa* and *Calanus finmarchicus* were used as predators in the experiments. *C typicus* and *A. tonsa* samples were filtered from a culture (200 µm mesh size filter) and *A. clausi* and *C. finmarchicus* used for the experiment were collected from the sea at 20 m depth from the Gullmarsfjord, Sweden, with a plankton net (90 µm mesh size). The plankton sample was diluted with surface water and transported to the laboratory where the sample was further diluted with filtered seawater before the experiments were conducted.

Induction Experiment
The experiment was carried in 250 ml glass bottles. 5 replicates were used for controls (no copepods added) and for each copepods species treatment, a total of 20 bottles were used in the first and 15 in the second induction experiment (because *C. finmarchicus* was not available for this experiment). Copepod species differed in size and the surface area of the copepods was calculated to achieve a similar exposure, using the formula:

\[
\text{Surface Area} = 2\pi \left( a^2 + \frac{ab\phi}{\sin(\phi)} \right)
\]

Where: \( \phi = \arccos \left( \frac{a}{b} \right) \), \( a = \left( \frac{\text{Width}}{2 \times \text{Length}} \right) \) and \( b = \left( \frac{\text{Length}}{2} \right) \).
In the first experiment: 4 C. typicus, 1 C. finmarchicus and 15 A. clausi were added to the different copepod treatments and in the second experiment; 4 C. typicus and 15 A. tonsa, was used since no C. finmarchicus was available at the time.

The cell concentration in the A. tamarense culture was determined using a particle counter (Elzone 5380) before addition to the experimental containers. At the start in the first experiment, the A. tamarense culture (181 cells ml\(^{-1}\)) was added to each glass bottle and A. tamarense culture (184 cells ml\(^{-1}\)) for the second experiment.

The first experiment was terminated after 5 days, and the second experiment was terminated after 3 days, because A. tamarense cells seldom divide faster than every second day, so experiments should not be shorter than three days. Samples for toxin analysis were prefiltred through a 63\(^\mu\)m sieve to eliminate copepods and copepod eggs and were transferred individually to clean beakers. 50 ml of each sample was preserved with a couple of drops of lugols solution, in the first experiment, samples were counted with a particle counter (Elzone 5380) to determined the algal cell concentration and in the second experiment, the cells were counted manually, in an inverted microscope at 100\(^*\) magnification to verify the readings from the Elzone 5380, because there were additional non A. tamarense material detected in these samples. The rest of live samples were weighed and suctioned filtered onto 25 mm glass fibre filters for further toxin analysis. These samples were put in an eppendorf tube and frozen directly. The filters were freeze-dried (Lyovac GT2, Leybold-Heraeus) to eliminate dilution of the extract from remaining water. PST was extracted through three freeze-thaw cycles in 1 ml 0.05 M acetic acid. The extracts were filtered through glass fiber filters and frozen in high performance liquid chromatography (HPLC) glass vials until analysis by HPLC with fluorescent detection.

Chemical analysis

The chemical analyses were carried out according to the method described by Asp et al (2004) using High Performance Liquid Chromatography (HPLC). This is basically liquid chromatography performed at high pressure. The toxins were separated on a silica based reversed phase column (150 X 3 mm i.d., 5 \(\mu\)m particles, Chrompack C8). Seven carbamates were determined, since they were commercially available as standards. C toxins were detected after extraction with 0.05M acetic acid why all samples were
hydrolyzed in 0.1M HCl at 100 ºC for 10 minutes to convert C toxins to the corresponding carbamates.

Statistical Analysis
Data on the total cell specific toxin content and growth of *A. tamarense* in the induction experiment was statistically analysed using a one-way analysis of variance (ANOVA). The Student-Newman-Keul’s (SNK) multiple comparison procedure was used to detect statistically significant differences between groups.

**Results**

**Average diameter of cells**
In the first experiment, the average diameter of *Alexandrium tamarense* cells that did not receive copepods (controls) was significantly higher compared to all treatments with copepods (Fig. 1; ANOVA F$_{3,11}$=16.3, P=0.001, SNK p<0.05), but did not differ between copepods treatment (ANOVA F$_{3,11}$=16.3, P=0.104, SNK p=0.10)
In the second experiment there was a reduction in size of the cells in response to copepod additions, but this was only significant for *C. typicus* (Fig.2; ANOVA F$_{2,12}$=5.68, P=0.018, SNK p<0.05) while the size of *Alexandrium* cells in *A. tonsa* treatments was not statistically different from controls (SNK p=0.38)
Fig. 1: Equivalent spherical diameter of cell (µm) in the first experiment. In all treatments with copepods the average diameter is significantly lower compared to controls (SNK p<0.05) and *C. typicus* decreased the average diameter significantly more compared to *C. finmarchicus* and *A. clausi* (SNK p<0.05) treatments.

![Spherical diameter of cell (Experiment N° 2)](chart)

Fig 2: Equivalent spherical diameter of cell (µm) in the second experiment. The bars indicate the average diameter of cells error bars denote standard deviation. The average diameter of the cells is significantly smaller compared to controls for *C. typicus* (SNK p<0.05) but not for *A. tonsa* treatment (SNK p=0.38)

Feeding experiment
The concentration of *Alexandrium tamarense* cells was measured when the experiments were terminated, for each treatment. In the first experiment, the number of cells ml⁻¹ is less in treatment exposed to copepods compared to control treatments (Fig. 3), indicating that copepods were actively feeding during this experiment (ANOVA F₃,₁₁ = p<0.001). The proportion of ingested cells is similar between copepods species. However, in the second experiment, the number of cells ml⁻¹ is similar between treatments (Fig. 4), including controls, this indicates that copepods did not feed significantly on *A. tamarense* cells in the second experiment (ANOVA F₂,₁₂ = 0.574)
Fig. 3: Cell abundance (cells ml$^{-1}$) at the end of the induction experiment. The bars show the average concentration of cells in the different treatments. The concentration of *A. tamarense* was significantly lower in grazed treatments compared to controls, but not between copepods species.

Fig. 4: Concentration of *Alexandrium tamarense* cells (cells ml$^{-1}$) at the end of the second experiment. The bars show the average concentration of cells in the different treatments. The number of cells is not significantly different between the different treatments.
Toxin analysis
Toxicity of *A. tamarense* was calculated from the HPLC chromatograms. Six different PSTs, C toxins, gonyautoxin (GTX) 1, 2, 3, 4, saxitoxin (stx) and neosaxitoxin (neo stx), were detected in the *A. tamarense* cell (Fig. 5) and seven different toxin in the standard, gonyautoxin (GTX) 1, 2, 3, 4, saxitoxin (stx), neosaxitoxin (neo stx) and dcsaxitoxin (dc STX). The C toxins were hydrolysed to corresponding carbamates before analysis.

![Chromatogram of paralytic shellfish poison (PSP), extracted from *Alexandrium tamarense* strain #3 and an analytical standar containing. GTX: gonyautoxin 1, 2, 3 and 4; STX: saxitoxin; NEO: neosaxitoxin and dc STX: dcsaxitoxin. The C-toxin peak from algal samples was hydrolyzed to corresponding carbamates before analysis.](image)

Fig. 5: Chromatogram of paralytic shellfish poison (PSP), extracted from *Alexandrium tamarense* strain #3 and an analytical standar containing. GTX: gonyautoxin 1, 2, 3 and 4; STX: saxitoxin; NEO: neosaxitoxin and dc STX: dcsaxitoxin. The C-toxin peak from algal samples was hydrolyzed to corresponding carbamates before analysis.
Cells-specific toxicity

Six different PSTs, GTX 1-4, STX and NEO STX were detected after hydrolysis in the first experiment, where we used *A. tamarense* cells #3 strain, and seven different toxin GTX 1-4, STX, NEO and dc STX were detected in the second experiment where we used *A. tamarense* cells #1 strain.

In the first experiment, the results for cells-specific toxicity is not significantly different between treatments (Fig. 6, ANOVA $F_{3.15}= 1.45$, $p=0.267$). However, there is a trend towards higher toxicities in copepod treatments. GTX3 and STX were dominating toxin in this experiment, followed by neo STX (Fig. 6).

In the second experiment, all copepod treatments were significantly more toxic (Fig. 7, ANOVA $F_{2.11}=22.53$, $p=0.001$), but toxicity was not different between *Acartia tonsa* and *Centropages typicus* treatments (SNK $>0.05$). GTX3 and STX were dominating toxin in this experiment, followed by GTX4 (Fig. 7)

![Toxin per cell (Experiment N° 1)](image)

Fig. 6: Toxin content per cell in the first experiment. The difference between bars indicates the relative increase average toxin content per cell. There appears to be a trend towards higher toxicities in copepod treatments, but it is not statistically significant (SNK $p=0.267$; n=20)
Fig. 7: Toxin content per cell in the second experiment. The bars indicate relative increase in cell specific PST content relative to control samples. The controls are significantly lower than both copepod treatments (SNK p=0.001; n=15)

**Discussion**

In this study, the second experiment has shown that *Alexandrium tamarense* #1 produce a larger amount of PSTs when they are exposed to copepod grazers, but not for the first experiment with *A. tamarense* #3, where the toxin content is not statistically different from controls. There is, however, a trend towards increased toxicity also in *A. tamarense* #3 in response to copepod grazers (Fig. 6) and it is likely that an experiment with higher statistical power would reveal grazer induced increase in toxicity also in *A. tamarense* #3. Thus, it seems that *A. tamarense*, similar to *A. minutum* (Bergkvist *et al.*, 2008) is able to sense and respond to grazer presence with increased toxin formation, but with some interesting differences. In similar experiments *A. minutum* responds to waterborne cues from several species of calanoids copepods with increased cell specific toxin content (Selander, 2007) which is only observed in the second experiment in my study. Also, in the first experiment it is possible to observe that there is an increasing trend to increase of toxin from *A. tamarense* in response to *Centropages typicus* and *Calanus finmarchicus*. In the second experiment, *A. tamarense* responds to *C. typicus* and *Acartia tonsa* with a similar increase in toxicity, but the controls are significantly lower. In a similar experiment with *A. minutum C. typicus* induced a substantially stronger response
compared to *A. tonsa*, (Selander *et al*. 2006; Bergkvist *et al*. 2008). Bergkvist *et al* (2008) suggest that the effect is strongly dependent on the species composition of the grazing community. *Centropages sp.* is one of the most abundant species in the north-western Mediterranean (Calbet *et al*. 2001) but usually only present in Swedish waters during the warm summer and autumn months, i.e. not during early spring when *A. tamarense* most commonly appears, while *C. finmarchicus* is present all year in the Swedish coast, i.e. if *C. finmarchicus* and *A. tamarense* coexist more time together in the sea, it is possible that *A. tamarense* responds with increased toxicity more than to other copepods species. Moreover, this assumption is only for the first experiment, because in the second experiment *C. finmarchicus* was unfortunately not available, for this reason it is not possible to verify the hypothesis of Bergkvist *et al* (2008) without additional experiments including both warm water and cold water species of grazers and *Alexandrium* spp.

Grazer induced toxicity does, however not seem to be as species specific in *A. tamarense* as in *A. minutum*, because in my experiment *A. tamarense* does not react significantly to different to different copepod species. These results are different compared to *A. minutum* that reacts differently to different copepod species; strong to *C. typicus*, that induce a more than twenty fold increase in toxicity, intermediate to *Acartia clausi* and not significantly detectable to *Pseudocalanus sp*. (Bergkvist *et al*. 2008)

Furthermore, the first experiment showed a significant decrease in the average diameter of *A. tamarense* cells exposed to different copepods grazers. The diameter of *A. tamarense* cells exposed to *C. typicus*, decrease 2 µm to compared to control cultures corresponding to a 7.4% decrease in diameter and which corresponds to a 21 % decrease in volume, which is actually quite a lot. In the second experiment, however, the reduction in the diameter of the cells is significant only for *C. typicus* treatments and not in *A. tonsa* treatments. The reduction in size may result from a cost associated with the formation of additional paralytic shellfish toxins or alternatively, it may be a response that in itself reduce the risk to encounter predators, because smaller cells are harder to detect than larger (Svensen and Kiorbøe, 2000). If we consider the results from feeding experiment, for the first experiment, copepods consumed a substantial part of the *A. tamarense* cells, and it is also possible that the change in size only reflects that copepods more selectively grazed on the larger *A. tamarense* cells, leaving smaller cells. Selective grazing on less
toxic cells could also have contributed to the trend towards higher toxicity in this experiment if less toxic cells were preferred by the grazers, leaving more toxic cells in the culture. In the second experiment, however, it is not possible to observe a significant difference in the number of cells between different treatments including controls, i.e. the copepods did not feed significantly on *A. tamarense*. Based on these results, we can conclude that for the first experiment there is no induction (significantly) of toxin production and the copepods fed on *A. tamarense*, in the second experiments is possible to observe a strong induction and the copepods did not feed on *A. tamarense*. Also, the toxicity in the less grazed strain # 1 was approximately twice as high as in the other strain # 3. It implies that copepod grazers avoid feeding on the strain that produce more toxins in response to copepod grazing. It has previously been suggested that PST formation constitutes an inducible defense towards copepod grazers in *A. minutum* (Selander, 2007) and it has been shown that some copepods prefer feeding on non toxic or less toxic cells when they are offered an option (Guisande *et al.* 2002; Teegarden, 1999)

In conclusion, there is variability between strains within species, sometimes larger than the variation between species. So it is not all that surprising that they respond differently. The present study demonstrates that *A. tamarense* exposed to copepods grazing, may respond with increasing PST production, but the response may be strain specific, because for different strains of *A. tamarense* the results for PSTs are different.

Also, *A. tamarense* did not react differently to different copepod species in any of the experiments; it does not seem to be as grazer specific as shown for *A. minutum* (Bergkvist *et al.* 2008)

Further experiments are however needed to test if *A. tamarense* is more tuned to local cold water grazers compared to the warm water. The recommendation for future work is to use warm and cold water copepods, ideally include *Calanus finmarchicus*, and repeat similar conditions in the experiments using both cold and warm water species of *Alexandrium spp.*
References


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