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Prorocentrum lima (Microalga: Dinoflagelleta): killer food for zooplankton

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Abstract

The toxicity of the microalga *Prorocentrum lima* and its probable toxic effects on zooplankton were tested using actively growing cells of *P. lima* and filtered culture medium of *P. lima* on *Artemia* nauplii (brine shrimps). Filtered *P. lima* culture medium at concentrations $\geq$ 50 % (in filtered sea water) killed the brine shrimps within 24 hours. The brine shrimps consumed the *P. lima* cells within 30 minutes following the introduction of the latter into wells holding the animals. Death of brine shrimps occurred from around 90 minutes onwards, following the introduction and consumption of the toxic cells. Older nauplii (3 days old) reacted readily and showed early responses from intoxications than younger nauplii (1 day old). A single *P. lima* cell in the gut of brine shrimp is enough to kill the animal. The probable cause of death in intoxicated brine shrimp is discussed.

Introduction

Prorocentrum lima is a highly reputed toxic microalgae. It produces the toxins okadaic acid (OA) and dinophysistoxins (DTXs) responsible for the human phycotoxin syndrome termed diarrhoetic shellfish poisoning (DSP). This benthic microalga is a cosmopolitan species present in both temperate and tropical waters (see Faust, 1993a,b; Rausch de Traubenberg and Morlaix, 1995; Ajuzie, 2000).

P. lima can cause harm to aquaculture and fisheries in a variety of ways. It may kill the zooplankton grazer outright (e.g. Demaret *et al*., 1995), thereby impacting secondary productivity. On the other hand, the zooplankton grazer may accumulate the toxins in its tissues, which it transfers to organisms up the food chain. Thus, the zooplankton grazer acts as a phycotoxin vector. Fish that consumes phycotoxin-ladened zooplankton may die or remain alive with the toxins stored in its body. Such contaminated fish are not fit for the market.

Many studies, most of which were involved with paralytic shellfish poisoning species, have shown that certain microalgae exert harmful effects on zooplankton. Such studies include White (1981), Fiedler (1982), Boyer *et al*. (1985), Ives (1985, 1987), Edvardsen (1993), Demaret *et al*. (1995). While Demaret *et al*. (1995) observed that *P. lima* kills brine shrimps, they also reported that filtered media induces no mortality in the animal. However, Rausch de Traubenberg and Morlaix (1995) reported that the extracellular medium of *P. lima* cultures contains 19 to 29 % OA. Thus, one of the aims of this research was to re-evaluate the effects of *P. lima* culture medium on *Artemia salina*. The other aim involved the determination of how *P. lima* cells induce mortality in this animal; since Demaret *et al*. (1995) hinted that DSP toxins in brine shrimp bodies might induce specific physiological disturbances.

In view of the above motives, experiments were conducted to:

- investigate the presence of dissolved toxins in *P. lima* cultures
- investigate the least number of *P. lima* cells that, if ingested, will kill brine shrimps
- investigate the time required by the ingested cell(s) to kill the grazer
- trace the possible mechaninsm of action of *P. lima* toxins in brine shrimps.

Materials and Methods

Cells of *Prorocentrum lima* were obtained from two different microalgal culture facilities. These are Instituto Espafiol de Oceanografía, Vigo in Spain and the Botanical Institute, University of Copenhagen, Denmark. The Spanish strain (PL2V) originated

from Vigo (I. Bravo, *pers com*) while the Danish strain originated from Florida in the United States of America (J. Larsen, *pers com*). The microalgae were cultured in bacteria-free K-medium enriched seawater (Keller *et al*., 1987). The toxic potentials of the two strains were tested on *Artemia* nauplii.

Natural seawater was obtained from the English Channel, filtred under low vacuum on Whatman GF/C filters and autoclaved for 1 hour before use. The cells were grown at 24 $\pm$ 2 °C and 60.19 µmol s^{-1} m^{-2} PAR on a 12:12-hr light and dark cycle. The growth rate of the microalgal cells was followed for one month.

The brand name of the *Artemia* cysts used in this research is Novotemia from JBL®. The *Artemia salina* nauplii, hereinafter referred to as brine shrimp(s), were obtained after cysts were treated for hatching. The cysts were incubated at 27 to 29 °C in a mixture of filtered natural sea water and distilled water at 20 ‰. Hatching occurred within 24 hours.

Investigation on the presence of extracellular toxins in *P. lima* cultures

Two different media (10 days old and 8 months old) with *P. lima* cells actively growing in them were used after the cells were filtered out on GF/C filters under low vacuum. 2 ml of the following concentrations (100, 90, 70, 50, 20, 10 and 1 %) of each filtered medium in filtered seawater were poured in 3 ml clear plastic wells, in three replicates. The control was 100 % filtered natural seawater. In each well was placed 10 brine shrimps. The brine shrimps were left in the test media for 24 hours, after which time wells were checked for survivors.

Investigation on the least number of *P. lima* cells that, if ingested, will kill brine shrimp

Cells in 10 days old cultures were employed in feeding the 1-3 days old brine shrimps. Different concentrations of cells (20, 8, 4 cells/well)were introduced into wells containing 4 brine shimps each, in triplicate. Also, in a 4th set, 4 brine shrimps were allowed to feed on a higher *P. lima* cells concentration (400-500 cells per well). The duration of the test was 24 hours, at the expiration of which wells were checked for survivors.

Investigation on the time required by the ingested *P. lima* cells to induce mortality on brine shrimp

For this investigation, we also employed 1-3 days old brine shrimps. The brine shrimps were distributed (8 individuals of each age group per well), and about 400 *P. lima* cells introduced into the wells. The wells were monitored every 30 minutes and the individual time of death for brine shrimps in each age group per well was recorded. In addition, wells were set aside in which 1 day and 3 days old brine shrimps were killed , using 4 % formalin, after 30 and 60 minutes intervals. The killed animals were then observed under the microscope at 100x to check for the presence of *P. lima* cells in their guts.

In these experiments it was necessary to use relatively small brine shrimp populations since such a design erased the possibility of wastes building up in the study wells which otherwise could have influenced the results. Also, since the effects of the two *P. lima*

strains on brine shrimps were similar, we will present the following results and discussions under the umbrella of the species (i.e., *P. lima*) without mentioning the strains.

Results

Investigation on the presence of extracellular toxins in *P. lima* cultures

For the two media (10 days old and 8 months old) no brine shrimp survived the 24 hours test where the test medium contained 50 % or more of the filtered culture medium (Table 1). On the other hand, all brine shrimps in test solutions containing 20 % or less of the filtered culture medium survived the ordeal.

Table 1. **Death of brine shrimp exposed to different concentrations of filtered culture medium of *P. lima* in filtered sea water.**

Medium concentration (%)	Brine shrimps per well	Observation after 24 hours
100	10	all dead
90	10	all dead
70	10	all dead
50	10	all dead
20	10	all survived
10	10	all survived
1	10	all survived

Least number of ingested *P. lima* that will kill brine shrimp

In all the treatments, brine shrimp that had consumed the *P. lima* cells died within 24 hours. Whereas brine shrimps without a single *P. lima* cell in the gut survived the 24 hour ordeal. Just one ingested *P. lima* cell was enough to kill the grazer. Some brine shrimps had up to seven *P. lima* cells in their gut. All brine shrimps in study wells with high *P. lima* cell concentration (i.e. 20 and 120 cells) had food in their gut as a consequence, they all perished within the 24 hours test period (Table 2). In wells with eight and four *P. lima* cells, the results were different. In each of these sets, there were wells where brine shrimps, probably, did not locate (or refused to ingest) the toxic cells. Such brine shrimps survived the test period.

Table 2. ***P. lima* concentrations (in triplicates) and brine shrimp survival after 24 hours**

Cells per well	Brine shrimps per well	Observation after 24 hrs
120	4	all dead
120	4	all dead
120	4	all dead
20	4	all dead
20	4	3 dead

20	4	no death
8	4	2 dead
8	4	2 dead
8	4	3 dead
4	4	2 dead
4	4	1 dead
4	4	no death

Time lapse between the introduction of *P. lima* cells and death of brine shrimp

The 30 minutes intervals investigation on the time of death of brine shrimp, from the time *P. lima* cells were introduced into the study wells, revealed that 1 day old brine shrimp started dying at about 2.5 hours, while the 2 and 3 days old brine shrimps started dying an hour earlier (at about 1.5 hours). The 3 days old brine shrimp died relatively faster than the brine shrimps in the two other age groups- all the 3 days old brine shrimp died within 2 hours (Table 3).

Table 3. **Time of death of day 1 to day 3 brine shrimp fed *Prorocentrum lima* cells**

		Nauplii stage					
		Day 1 nauplii (No. dead*)		Day 2 nauplii (No. dead*)		Day 3 nauplii (No. dead*)	
Time (hour)	Replicates	a	b	a	b	a	b
0.5	0	0	0	0	0	0	
1.0	0	0	0	0	0	0	
1.5	0	0	1	1	6	5	
2.0	0	0	3	2	8	8	
2.5	1	0	4	3			
3.0	3	2	4	3			
3.5	4	4	5	4			
4.0	6	5	5	5			
4.5	7	8	8	6			
5.0	7	8	8	6			

* Equals cumulative number
N = 8 brine shrimps in each replicate group

A further investigation with 1 day and 3 days old nauplii revealed that 3 days old brine shrimps in contact with *P. lima* cells ingested at least a cell within 30 minutes of encounter with the cells. Within this period, 90 to 100 % of the brine shrimps had at least a *P. lima* cell in the gut. There were no dead brine shrimps within this period. Within a period of 60 minutes 98 % (at least) of the 3 days old brine shrimp population had ingested *P. lima* cells. But a 0 to 10 % mortality was noticed within the 60 minutes period. On the other hand, no cells were observed in the guts of 1 day old brine shrimps within the hour the investigation lasted.

Behaviour of intoxicated brine shrimp

Normal and healthy brine shrimp is an active swimmer; swimming on its back as though it was hopping, and seemingly without a direction. It uses its head appendages- the second antennae and the mandibles- as "paddles". However, once brine shrimp had consumed the toxic cells, it gradually lost its hopping-like swimming habit. It then settled at the bottom making a number of intermittent crawling movements, with attempts to venture into the water column again. Gradually all movements that could help displace the animal were lost. The animal can only move parts of the body (like the antennae and mandibles) at this juncture, with the heart still beating (seen at 100x magnification) and the gut contracting rhythmically until death finally sets in.

Mechanisms of action of *P. lima* toxins on brine shrimps

Following the ingestion of *P. lima* cells, the intoxicated animal finally settles to bottom. The moribund animal was observed to be making periodic contractions of the gut (seen at 200x magnification). This resulted in the defecation (false fecal pellets) of some ingested cells in certain of the animals before they finally died.

Discussion

Investigation on the presence of extracellular toxins in *P. lima* cultures

This study showed that *P. lima* leaked toxins into the extracellular medium. This is a highly probable assertion, especially if it is considered that Rausch de Traubenberg and Morlaix (1995) also reported the presence of okadaic acid in the dissolved phase in *P. lima* cultures. These authors observed that the extracellular medium conains 19 to 29 % okadaic acid which they attributed to the partial water-solubility of OA molecule owing to its hydrophilic functions (1 carboxylic function, 4 hydroxyl functions).

Filtered media from both the 10 day and the 8 months old cultures contained leaked toxins; since both media killed brine shrimps. It should be noted that the 10 days old culture was habouring cells still in the latent growth phase. Some metabolic processes, during this phase, may have warranted the cells to leak out their toxins. Rausch de Traubenberg and Morlaix (1995) also observed that *P. lima* cells released OA into the extracellular during the latent growth phase (days 1 to 6).

With respect to the older culture, the hypothesis could be that during mitosis and the resulting cell division, mother cells released toxins as the daughter cells were liberated. Another hypothesis is that as senesced cells lysed they released toxins into the culture medium. These two hypotheses could explain why the 8 months old culture contained extracellular toxins.

Though the cultures were not axenic, these findings cannot be linked to the presence of the free living bacteria in the medium. As shown by Rausch de Traubenberg and Morlaix (1995), free bacteria contains a maximum of 1 % OA which may have adsorbed (by means of its hydrophobic moiety) to the bacteria from the medium.

***P. lima* cells vs brine shimp**

The results on the time lapse between the introduction of *P. lima* cells into wells containing brine shrimps and the time of death of the brine shrimps showed that older brine shrimps were readily impacted-upon by the presence of the toxic cells. The 2 and 3 days old brine shrimps did not receive any food prior to the tests. So, they were starving. This caused them to feed readily on the *P. lima* cells and to develop early responses to the toxins. On the other hand, the 1 day old brine shrimps still had some food reserves in their system (squashed individual emptied to the exterior some fatty substances). The food reserve they contained caused them to delay feeding on the *P. lima* cells. So they responded to the effects of the toxins slowly.

Mechanisms of action of *P. lima* toxins on brine shrimps

Apart from the stimulation of defecation in the animal by the ingested cells, it was also envisaged that the animal lost some quantity of body fluids during the proccess of gut contraction and the resultant defecation. In other words, the intoxicated animal probably became dehaydrated. This observation was supported by the fact that the gut shrank continuously from the abdominal wall and became narrower and shorter, as it was separated more and more from the wall of the abdomen, until the animal died. Following the observation that ingested *P. lima* cells caused the brine shrimps to indulge in periodic contractions of the gut which finally led to the shrinking of the latter, we theorize that the shrinking of the gut resulted from excessive loss of body fluids, and that dehydration could have been one major cause of death in affected brine shrimp.

P. lima produces a number of toxins, including okadiac acid (OA), which are responsible for diarrhetic shellfish poisoning (DSP) syndrome in man. One of the symptoms of DSP is abdominal cramp. For muscles to contract ATP (which is the immediate source of energy for muscle contraction) must be generated. An important process of ATP generation is termed phosphorylation. Rapid stimulation of phosphorylation in intact cells is characteristic of OA (Baden and Trainer, 1993). Additionally, Windust *et al.* (1996) noted that OA can cause hyperphosphorylation of a broad range of animal (and even higher plants) proteins. Conversely, phosphatases (hydrolytic enzymes) uncouple oxidative phosphorylation. And it has been postulated that AO inhibits phosphatase activity on phosphorylation (Takai *et al*., 1987; Bialojan and Takai, 1988; Bialojan *et al.*, 1988).

It has also been suggested that OA probably causes diarrhoea by stimulating phosphorylation of proteins that control sodium secretion by intestinal cells (Cohen *et al.*, 1990) or by enhancing phosphorylation of cytoskeletal or junctional elements which regulate permeability to solutes, thereby resulting in passive loss of fluids (Dho *et al.*, 1990). Demaret *et al.* (1995) demonstrated the transfer of OA from *P. lima* to brine shrimps. Thus, these findings may, meanwhile, help explain why the gut of the brine shrimps that had consumed the toxic *P. lima* cells shrank, and why it is most probable that intoxicated brine shrimps died from dehydration; since fluids dynamics in the affected brine shrimps was no longer under any physiological control.

Conclusion

Prorocentrum lima is, indeed, a killer food for zooplankton, considering the fact that some brine shrimps engorged their gut with several *P. lima* cells before their death. This means that unlike *Alexandrium tamarense* which warns its predator by imposing on it a physiological incapacitation which causes the predator to refrain from consuming it

(Ives, 1987; Demaret *et al.*, 1995), *P. lima* appeared palatable to the unsuspecting zooplankton.

Again, in view of the fact that one ingested *P. lima* cell is enough to kill brine shrimp, as well as the fact that *P. lima* leaks its toxins to the extracellular medium, the ecological impacts of *P. lima* blooms (as well as blooms of other DSP producers like the *Dinophysis* spp) may be tremendous, especially on secondary producers.

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