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Note

Dynamic of intoxication and detoxification in juveniles of *Mytilus chilensis* (Bivalvia: Mytilidae) exposed to paralytic shellfish toxins

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Abstract – Juveniles of the mussel *Mytilus chilensis* were exposed to a diet containing paralytic shellfish poisoning (PSP) toxins produced by the dinoflagellate *Alexandrium catenella* (strain ACC02). The feeding behaviour and the dynamics of intoxication and detoxification were evaluated over an intoxication period of nine days, followed by a detoxification period of eight days. A significant reduction in the feeding activity was measured during the first days of exposure to the PSP toxins (days 0 and 2), followed by a period of recovery observed on days 5 and 9, when the clearance rate of the contaminated mussels significantly increased. During the detoxification period, the contaminated bivalves showed a total recovery of clearance rate, and no significant differences were observed between contaminated and control groups. The intoxication dynamic was characterised by a rapid and sustained increase in the amount of PSP toxins accumulated in the tissues of the bivalves. Due to this increase, the level of PSP toxins critical for human consumption was reached on the first day, and at the end of the intoxication period, the mussels had accumulated 1601 μg STX eq. 100 g^{-1} tissue. During the detoxification period, a decrease of PSP toxins was observed, down to 319 μg STX eq. 100 g^{-1} tissue. The lower clearance rates observed over the first days of exposure would produce a decrease in the energy intake and could affect the rate of growth of juveniles. Despite this initial effect, the rapid intoxication capacity of *M. chilensis* corroborates that this species is a good indicator for the early detection of harmful algal blooms.

Key words: Paralytic shellfish toxin / Detoxification / Harmful algae / *Alexandrium catenella* / Feeding / Bivalve / *Mytilus chilensis*

1 Introduction

Toxic dinoflagellates (e.g., *Alexandrium*, *Gymnodinium*), commonly produce large blooms in different geographical areas (Hallegraeff 2003). Filter-feeding organisms exposed to these toxic blooms ingest the dinoflagellates and consequently accumulate paralytic shellfish poisoning (PSP) toxins in their tissues. If bivalves accumulate high levels of toxins, they become a risk to human health, especially in the case of commercially-important species (mussels, oysters and scallops). PSP toxins may be accumulated rapidly in the soft tissues of bivalves, but they can also be eliminated gradually, as the toxic cells disappear from the diet available to these filter-feeding organisms.

There are intrinsic (e.g., physiological responses and biotransformation of toxins) and extrinsic factors (e.g., toxic cell density, duration of exposure, toxicity of the phytoplankton species and their relative abundance) involved in the rate of toxin uptake and detoxification of shellfish during a toxic algal bloom (Bricelj and Shumway 1998). Furthermore, the rate of toxin uptake varies among bivalve species (Cucci et al. 1985; Lassus et al. 1989) and between individuals of the same species (White et al. 1993). For instance, bivalves of the genus *Mytilus* have been used as indicator organisms in biotoxin monitoring programmes, as they are not significantly affected by these toxins but quickly show high levels of toxin uptake and reach high levels of toxicity. The mussel *Perna viridis*, fed on the dinoflagellate *Alexandrium fundyense*, accumulated PSP toxins exceeding the safety limit level for human consumption (80 μg STX eq. 100 g^{-1} tissue) on the second day of exposure (Kwong et al. 2006). Other bivalves such as the

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scallop, *Placopecten magellanicus* and the clam, *Spisula solidissima* presented differences in the accumulation of paralytic toxins in their tissues due to species-specific responses in intoxication and detoxification kinetics (Cembella et al. 1993). Different studies have demonstrated that toxic dinoflagellates produce sub-lethal effects in some filter-feeding organisms, affecting byssus production and oxygen consumption, and also reducing feeding activity, which may even be stopped completely due to the closure of the valves (Shumway et al. 1985; Marsden and Shumway 1993). The dinoflagellate *Gyrodinium aureolum* produces a reduction in the clearance and growth rates, as well as intestinal damage, when it is ingested by the bivalve *Mytilus edulis* (Nielsen and Strömgren 1991).

The presence of PSP toxins in southern Chile has been associated with blooms of the dinoflagellate *Alexandrium catenella*, and 387 cases of human poisoning leading to 26 deaths between 1972 and 2000. Harmful algal blooms of this dinoflagellate have occurred with almost annual frequency in the most southerly Regions of Chile (Regions XI and XII) and the phenomenon has recently extended northward, reaching the island of Chiloé (Region X), which is the principal area for bivalve cultivation in Chile. The presence of high levels of PSP toxins in edible bivalves is characteristic of this geographical area. To prevent human poisoning, shellfish are monitored routinely and a quarantine period imposed when the toxin level exceeds 80 μg STX eq. 100 g^{-1} tissue. The presence of harmful algal blooms (HABs) causes a negative socio-economic effect in the Region due to the closure of cultivation and fishing areas, resulting in the temporary shutdown of extraction activities. In southern Chile, *Mytilus chilensis* (Hupé 1954) is a species of great ecological importance and commercial value due to the wide range of activities involved in the culture and extraction of this resource (over 180 000 tons per year). In view of the negative impact that HABs have on human health and the economy of the country, it is important to obtain information available on the different responses that mussels may show to HAB exposure; this would allow better administrative decisions to be taken and so reduce the negative effect that this phenomena has on the Chilean aquaculture sector.

In a previous study, we examined the physiological energetics and intoxication dynamics of adult *Mytilus chilensis* individuals during a period of 21 days of exposure to the toxic dinoflagellate *A. catenella*. The concentration of toxin in the soft tissue reached values of 642 μg STX eq. 100 g^{-1} at day eight, with no significant increase throughout the rest of the experimental period (Navarro and Contreras 2010). The goal of the present study was to identify the dynamics of the intoxication and detoxification of juveniles of *M. chilensis* exposed to a diet containing the dinoflagellate *A. catenella*. For this, measurements of the clearance rate and accumulation of PSP toxins were carried out over experimental intoxication and detoxification periods. These results allowed us to estimate the period of time taken for this species to reach levels that are toxic for human consumption (80 μg STX eq. 100 g^{-1} wet tissue), as well as the time required for them to return to sub-toxic levels at which they could be used to provide seed for other cultivation centres, where no natural settlement of *Mytilus chilensis* occurs.

2 Materials and methods

2.1 Collection and acclimation of mussels

Juvenile individuals of *Mytilus chilensis* (75.5 to 110.1 mg) were collected from culture ropes located in Yaldad Bay, Chiloé (43°08′S, 73°44′W). These mussels were selected by size (1.8 to 2.3 cm), cleaned of epibionts and transported to the laboratory in chilled conditions. Before the experiments, the mussels were acclimated for one week in aquaria containing 30 L of seawater with constant aeration, at 14 °C and 30 psu salinity. Each aquarium was continuously supplied, by peristaltic pump, with a diet containing 80% of the non-toxic microalga *Isochrysis galbana* and 20% of inorganic sediment (1.5–2.5 mg L^{-1}).

2.2 Diet preparation

The monoclonal non-axenic *Alexandrium catenella* (strain ACC02; 32–36 μm spherical diameter) used during the experiments was isolated in the Aysén Region of Chile (Region XI) in 1994 and was cultivated in 0.45 μm filtered seawater enriched with "L1" algae culture medium (Guillard 1995), at 14 °C and 30 psu salinity. The toxicity of *A. catenella* cells was measured over the experiment, and a mean value was obtained from 15 samples. The microalgae *Isochrysis galbana* was cultivated using $f/2$ medium (Guillard 1975). Both species of algae were harvested for use in the experiment when they were in their exponential growth phase. Sediment was added to the diets to emulate the organic/inorganic fractions of the natural suspended particulate matter. This sediment was collected from the upper centimetre of the tidal flat at Yaldad Bay, passed through a 40-μm m sieve, rinsed with distilled water, and ashed in a muffle furnace at 500 °C for 12 h to eliminate the organic fraction. After ashing, the sediment was sieved again (40-μm sieve), to eliminate sediment aggregations.

2.3 Dynamics of intoxication and detoxification

Three replicates containing 266 bivalves each were kept in aquaria of 5 L capacity and fed with the contaminated diet (50% *Alexandrium catenella*, 30% *Isochrysis galbana* and 20% inorganic sediment) for 9 days. In parallel, three other similar aquaria were kept as controls, with the same number of individuals in each (n = 266), and fed on the control diet (a toxin-free diet; 80% *I. galbana* and 20% inorganic sediment). After the intoxication period, the detoxification began: the remaining individuals in each aquarium were fed for 8 days with the control diet, to promote the detoxification of the mussels. The diets were supplied continuously using a Masterflex L/S peristaltic pump model 7519–05, and were kept, at all times, within a particle concentration range similar to that found in the natural environment of the bays of southern Chile (1.5–2.5 mg L^{-1}) (Navarro and Jaramillo 1994). The quantity of food provided daily was equivalent to 6% of the dry weight of the soft tissue of each individual in the experiment (5.5 mg per individual per day). To estimate the total weight and organic content of the diets, a known volume of each was filtered (in triplicate) through Whatman fibreglass GF/C filters

of 47 mm diameter, which had previously been washed, burnt and weighed. Both the blank filter and those containing the samples were washed with an isotonic solution of ammonium formate, to remove the salt and prevent cell lysis. The filters were dried at 80 °C for 24 h, weighed, burnt at 450 °C for 3 h and reweighed after cooling in a desiccator.

2.4 Feeding experiments

To investigate the feeding response of the juveniles *M. chilensis* over the intoxication and detoxification periods, mussels were taken randomly from each of the contaminated aquaria and used to measure the clearance rate (CR) under standardised conditions. Measurements were made during the intoxication period, on day 0 (initial contact with the toxic diet) and on days 2, 5 and 9. During the detoxification period, measurements were taken on days 4 and 8. In parallel, CR measurements were also taken in the control individuals. CR was estimated using a static system homogenised by aeration, containing a food concentration between 1.8 and 2.0 mg L^{-1} dry weight. The composition of the diets used to measure the clearance rate was the same as those supplied to feed the mussels daily. Two juvenile mussels were taken from each replicate and placed in an experimental aquarium (0.8 L filtered seawater) and the CR was measured. The decrease in the number of particles was monitored every 30 min over a period of three hours, using an Elzone 180XY particle counter, equipped with a 120-μm-aperture tube. A control aquarium without mussels was used to discount the sedimentation of particles and the CR (L h^{-1} $mussel^{-1}$) was calculated according to Coughlan (1969). All the experiments were carried out under conditions of controlled temperature (14 °C) and salinity (30 psu).

2.5 Toxin analyses of *Alexandrium catenella* and mussels

Aliquots of 10 mL were recovered from three replicate cultures of *Alexandrium catenella*, fixed with 10% seawater formalin, and allowed to decant in sedimentation chambers for 40 min using the Utermöhl method (Utermöhl 1958). Cell counts were carried out using an inverted microscope, and an average was calculated from the three replicates. The toxicity of *A. catenella* was calculated from samples taken over the whole experimental period. These samples, containing 5×10^5 *A. catenella* cells, were settled by centrifuging at 6000 rpm for 20 min and the pellet was treated with 700 μL 0.1 N HCL and sonicated in a bath (Aquasonic model 75T) for 5 min. The supernatant (500 μL) was removed and made up to 1000 μL with distilled water (400 μL) and 1 N HCL (100 μL). The toxicity of the soft tissue of *M. chilensis* was calculated on three replicates by pooling the wet tissues (4 g approx.) of 27 juvenile mussels from each contaminated aquarium on days 0, 2, 5, and 9. The same procedure was carried out on individuals from the three control aquaria to verify the complete absence of toxin. On days 4 and 8 of the detoxification period, 27 mussels were also taken from each of the 3 contaminated and control aquaria. The extraction of the toxins from the homogenate of mussels tissues was carried out, using 0.1 N hydrochloric acid.

Extracts from the dinoflagellate *A. catenella* and the tissues of the mussels were quantified using the electro-physiological test of Vélez et al. (2001), where HEK 293 cells (Human Embryonic kidney cells) expressing STX-sensitive rat skeletal muscle Na channels were patch clamped in the whole-cell configuration. The equivalent STX concentration was estimated using calibration curves obtained by external perfusion with known concentrations of purified STX; these curves were generated using a stepped series of increasing concentrations of STX-dihydrochloride (US Food and Drug Administration, Office of Seafood). According to Velez et al. (2001), there is a correlation of 0.96 between the mouse bioassay and the electro-physiological test.

2.6 Statistical analysis

The feeding responses of the mussels exposed to the contaminated and control diets were compared with each other for each sampling day (between treatments comparison). The clearance rates of each treatment (control and contaminated groups) measured in the intoxication and detoxification periods were also compared to identify significant differences over the time (within treatment). Comparisons were carried out using one way analysis of variance (ANOVA) followed by "post-hoc" Tukey tests. The significance threshold was $\alpha = 0.05$.

3 Results

3.1 Experimental diets

The experimental diet containing 50% *A. catenella* had an average of 2.5 ± 0.8 mg L^{-1} total dry weight and 79.1 ± 12.7% organic matter, while the control diet presented an average total dry weight of 2.4 ± 0.9 mg L^{-1} and 83.5 ± 4.0% organic matter. The number of cells in the toxic diet was 2.5×10^5 cells L^{-1} *A. catenella* and 2.5×10^7 cells L^{-1} *Isochrysis galbana*. The control diet contained 6.4×10^7 cells L^{-1} *I. galbana*. The mean toxin concentration of *A. catenella* (strain ACC02) was 3.8 ± 1.8 fmol STX eq. $cell^{-1}$. Therefore, the concentration of PSP toxins in the toxic diet was 975 pmol STX eq. L^{-1} (Table 1).

3.2 Feeding experiments

Specimens of *Mytilus chilensis* fed with the diet containing *A. catenella* showed a significant reduction ($p < 0.05$) in the clearance rate during the first days of exposure to the toxins (days 0 and 2) compared with the control group. This behaviour was followed by a recovery period, seen on days 5 and 9, when the clearance rate of the contaminated mussels showed significantly higher values ($p < 0.05$) than those measured on the previous days. During the detoxification period, the contaminated mussels showed a total recovery in clearance rate, with no significant differences ($p > 0.05$) from the control (Fig. 1).

Table 1. Characterization of the experimental and control diets supplied to *Mytilus chilensis*.

Diet	Total dry weight (mg L^{-1})	Organic dry weight (mg L^{-1})	Organic dry weight %	Sediment (mg L^{-1})	*Isochrysis galbana* (cells L^{-1})	*Isochrysis galbana* (mg L^{-1})	*Alexandrium catenella* (cells L^{-1})	*Alexandrium catenella* (mg L^{-1})	Toxin concent. (pmol L^{-1})
Experimental diet (50% *A. catenella*)	2.5 ± 0.8	2.0 ± 0.8	79.1 ± 12.7	0.5	2.5×10^7	0.8	2.5×10^5	1.3	975
Control (0% *A. catenella*)	2.4 ± 0.9	2.0 ± 0.1	83.5 ± 4.0	0.5	6.4×10^7	1.9	0	0	0

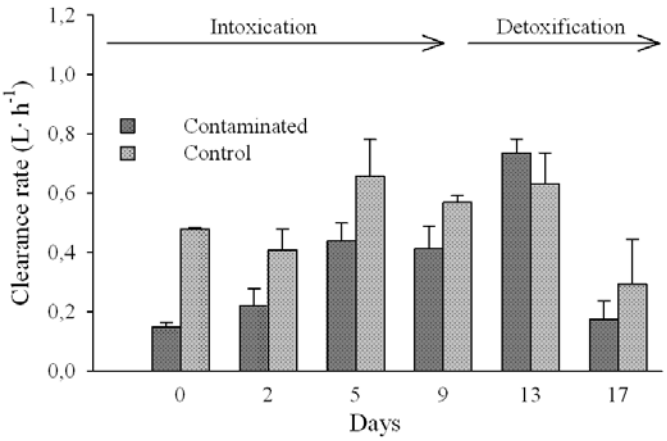

Fig. 1. *Mytilus chilensis*. Clearance rate of individuals exposed to a contaminated or a control diet during periods of intoxication and detoxification. Values are means ±SE.

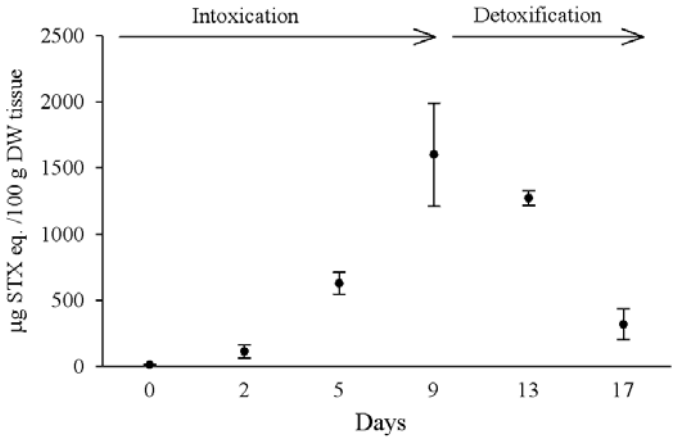

Fig. 2. *Mytilus chilensis*. Dynamics of intoxication and detoxification in mussels exposed to *Alexandrium catenella*. Values are means ±SE.

The PSP toxin analyses of mussels from the control group showed that no trace of toxins was detected in the tissues during the experiment. The intoxication dynamics of the mussels exposed to the contaminated diet over a period of nine days was characterised by a rapid and sustained increase of the PSP toxins in the tissues. On the second day, a concentration of 116 μg STX eq. 100 g^{-1} tissue was measured, which exceeds the quarantine level (80 μg STX eq. 100 g^{-1} tissue). The toxin concentration continued to increase and reached an average maximum value of 1601 μg STX eq. 100 g^{-1} tissue after the nine days of intoxication. During the detoxification period, a reduction in the concentration of toxins was observed, down to 319 μg STX eq. 100 g^{-1} tissue at the end of the experiment (Fig. 2).

4 Discussion

The diet containing *Alexandrium catenella* initially produced a short-term negative effect on the clearance rate of *Mytilus chilensis*, which was reversible over time. These results agree with previous reports on mussel species, which demonstrate a high capacity of acclimation in the presence of diets containing dinoflagellates that produce paralytic toxins. Similar behaviour was described by Navarro and Contreras (2010) in adult specimens of *M. chilensis* when these were exposed to a diet containing *A. catenella*. Wildish et al. (1998) described start/stop behaviour in the clearance rate of *Crassostrea gigas* when it was fed toxic and non-toxic strains of *A. tamarense*, suggesting that periods >48-h were required to achieve acclimation to these diets. Li et al. (2001) described a reduction in the clearance rate in juveniles of the clam, *Ruditapes philippinarum* due to an increase in the toxin concentration in the diets produced by *Alexandrium tamarense*; however, no such effect was observed in the mussel, *Perna perna*. Bricelj et al. (1990) found that *Mytilus edulis* reduced its clearance rate in comparison with control mussels when fed with the dinoflagellate *Alexandrium fundyense*, although no extreme negative effect, such as valve closure or cases of mortality, was observed. Nevertheless, the results of the feeding behaviour of *Mytilus chilensis* differ from those described for other species of bivalves, such as the oyster *Crassostrea gigas* (Bardouil et al. 1993; Gueguén et al. 2008) and the clam, *Mya arenaria* (Bricelj et al. 2005), which are highly sensitive to PSP toxins, resulting in a low accumulation of toxins in their tissues (Blasco et al. 2003). The present study estimated that juveniles of the mussel *Mytilus chilensis* exposed to a diet of 2.5×10^5 cells L^{-1} of *A. catenella* accumulated levels of paralytic shellfish toxins higher than the quarantine level (80 μg STX eq. 100 g^{-1} tissue) after one day, and that after nine days of intoxication, mussels reached a concentration of 1601 μg STX eq. 100 g^{-1} tissue. This is in agreement with other findings on the genus *Mytilus*, with respect to its relative insensitivity to PSP-producing toxic dinoflagellates: these species accumulate high levels of toxins in short periods of exposure (Bricelj et al. 1990; Bricelj and Shumway 1998; Navarro and Contreras 2010). The maximum concentration of

toxin accumulated by *M. chilensis* is lower than that recorded in its natural environment, where values reached 8554 μg STX eq. 100 g^{-1} tissue in Quellón during the bloom of 2002 (Servicio de Salud Llanchipal). This may be partially explained by the greater concentration of *A. catenella* in the natural environment, where it reached concentrations of 7.7×10^5 cells L^{-1} (Clement et al. 2002) and also by the prolonged period of permanence of the *A. catenella* bloom in the natural environment, which exceeded two months (Molinet et al. 2003). These results agree with those described for *Mytilus edulis* in the northern hemisphere, where the toxicity in the tissues is strongly related to the relative abundance of the toxic dinoflagellate *Alexandrium tamarense* in the diet (Lee et al. 2001).

The dynamics of intoxication and detoxification showed that the detoxification of *Mytilus chilensis* seems to be slower than the intoxication process since, after eight days of feeding with the control diet, the concentration was still 319 μg STX eq. 100 g^{-1} tissue. This agrees with the results of Cembella et al. (1993), who showed that the scallop *Placopecten magellanicus* and the clam *Spisula solidissima* present differences in the accumulation-elimination of toxins due to the different intoxication and detoxification dynamics of these species. Although only a small number of data were collected in the present study on juveniles of *M. chilensis* and high variability was observed between replicates, we suggest that the high variation in the concentrations of toxins accumulated in *M. chilensis* could be explained by the marked individual variability observed in feeding behaviour, which agrees with the findings described for *Patinopecten yessoensis* by Sekiguchi et al. (2001). Furthermore, Twarog et al. (1972), Shumway and Cucci (1987) and Bricelj et al. (2005) conclude that previous exposure to PSP toxins affects toxin accumulation responses. This is the case with the clam *Mya arenaria*. When repeatedly exposed to natural events of toxic dinoflagellate blooms this clam has been shown to be more resistant to PSP toxins and accumulate toxins at a higher rate than sensitive clams from areas not exposed to these events (Bricelj et al. 2005).

In agreement with the reports of Li et al. (2002) on the clam *Ruditapes philippinarum*, the presence of a diet containing the toxic dinoflagellate *A. catenella* would have effects on the fitness of juvenile *Mytilus chilensis.* The lower clearance rates observed over the first days of exposure would produce a decrease in the energy intake and could affect the rate of growth in juveniles. Despite this initial effect, the rapid intoxication capacity of *M. chilensis* shown in this study reaffirms that this species is a good indicator for the early detection of harmful algae blooms among other species of bivalve with lower intoxication rates. These findings are important considering the ecological and commercial importance of *M. chilensis* in the austral zone of Chile, where today over 180 000 tons are produced annually in suspended cultivation, and where harmful algal blooms are highly frequent.

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