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Uptake and depuration of anatoxin-a by the mussel *Mytilus galloprovincialis* (Lamarck, 1819) under laboratory conditions

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ABSTRACT

Cyanobacterial blooms tend to be more common in warm and nutrient-enriched waters and are increasing in many aquatic water bodies due to eutrophication. The aim of this work is to study the accumulation and depuration of anatoxin-a by *Mytilus galloprovincialis* a widespread distributed mussel living in estuarine and coastal waters and recognized worldwide as a bioindicator (*e.g.* Mussel Watch programs). Research on the distribution and biological effects of anatoxin-a in *M. galloprovincialis* is important. Nevertheless, the risk of human intoxication due to the consumption of contaminated bivalves should also be considered. A toxic bloom was simulated in an aquarium with 5×10^5 cell ml^{-1} of *Anabaena* sp. (ANA 37), an anatoxin-a producing strain. Mussels were exposed to *Anabaena* for 15 days and then 15 days of depuration followed. Three or more animals were sampled every 24 h for total toxin quantification and distribution in soft tissues (edible parts). Water samples were also taken every 24 h in order to calculate total dissolved and particulate anatoxin-a concentrations. Anatoxin-a was quantified by HPLC with fluorescence detection. No deaths occurred during accumulation and depuration periods. One day after the beginning of depuration, the toxin could not be detected in the animals. Anatoxin-a is distributed in the digestive tract, muscles and foot and is probably actively detoxified.

1. Introduction

Cyanobacterial blooms have been increasing with eutrophication of freshwater systems all over the world. The main concern about these events is related to the production of cyanotoxins that are lethal for aquatic and terrestrial organisms.

There are several types of cyanobacterial toxins: neurotoxins, hepatotoxins, cytotoxins and irritant and gastrointestinal toxins (Chorus, 2001; Codd et al., 2005). These toxins have been responsible for several human and animal poisoning, some of them with a fatal outcome (Falconer, 2005). Neurocyanotoxins comprise anatoxin-a, homoanatoxin-a and anatoxin-a(s). Anatoxin-a(s) is a potent organophosphate firstly found to be produced by a Canadian cyanobacterial strain. Presently, few reports of this toxin have been registered in United States and Denmark (Matsunaga et al., 1989; Henriksen et al., 1997; Monserrat et al., 2001). Homoanatoxin-a is a chemical homologue of anatoxin-a that was detected in France, New Zealand and Ireland (Furey et al., 2003; Cadel-Six et al., 2007; Wood et al., 2007), and anatoxin-a, the object of our study, was the first cyanotoxin to be chemically characterized (Devlin et al., 1977). It is an alkaloid and a potent neurotoxin (mice LD_{50} of 250 μg kg^{-1} *i.p.* (Rogers et al., 2005)), which can be produced by several cyanobacterial genera:*Anabaena, Aphanizomenon, Microcystis, Planktothrix, Raphidiopsis, Arthrospira, Cylindrospermum, Phormidium* and *Oscillatoria* (Park et al., 1993; Bumke-Vogt et al., 1999; Namikoshi et al., 2003; Viaggiu et al., 2004; Ballot et al., 2005; Gugger et al., 2005; Araós et al., 2005).

As it happens with some other cyanotoxins, anatoxin-a has been reported mainly in fresh waters but also in brackish waters (Mazur and Plinski, 2003). Although this toxin is very potent, it has received less scientific attention compared to other cyanotoxins such as microcystins and cylindrospermopsin. These have already caused serious human intoxications, including deaths, in Brazil (Carmichael et al., 2001; Byth, 1980). The lack of toxicological studies with anatoxin-a is probably due to its chemical characteristics which make it very unstable and labile in the water and therefore difficult to detect (Stevens and Krieger, 1991). Because no chronic effects have been associated with anatoxin-a, human health aspects associated with this toxin have been disregarded. Nevertheless, some animal fatalities have occurred, stressing the need to investigate anatoxin-a effects on aquatic organisms and communities. In recent study, we found that anatoxin-a may be bioaccumulated by carps in significant levels (0.768 μg of

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anatoxin-a per gram of dry weight of carp) (Osswald et al., 2007). Whether this may have an impact in aquatic food webs is not yet known.

In this experiment, we wanted to study the accumulation and depuration of anatoxin-a by *Mytilus galloprovincialis*, a very widespread mussel in estuarine and coastal waters and recognized worldwide as a bioindicator (*e.g.* Mussel Watch programs) (Izquierdo et al., 2003; Catsiki and Florou, 2006). This mussel is an important component of estuarine and marine food webs and because it is a sessile filter feeder, it may be exposed to high density of cyanobacteria and their toxins. Several studies with cyanotoxins have shown that bivalves are able to accumulate cyanotoxins, like microcystin-LR (Vasconcelos, 1995; Pires et al., 2004), paralytic shellfish toxins (Pereira et al., 2004), nodularin (Karlsson et al., 2003) and cylindrospermopsin (Saker et al., 2004). This later toxin is also an alkaloid like anatoxin-a but it is very stable. As far as we know there is no scientific literature about the effects of anatoxin-a in mussels. It is important to know the biological effects and the distribution of anatoxin-a in *M. galloprovincialis*. On the other side we should also consider the risk of human intoxications due to the consumption of bivalves contaminated with anatoxin-a. Dinoflagelates and diatoms are not the only toxin producers in estuarine and marine environments, so health authorities should also be aware of cyanobacteria. In this work, we studied the accumulation and depuration of anatoxin-a by *M. galloprovincialis* mimicking a bloom (10^5 cells ml^{-1}) of an anatoxin-a producing *Anabaena* sp. strain.

2. Material and methods

M. galloprovincialis was exposed to live cells of an *Anabaena* sp. toxic strain (ANA 37) producer of anatoxin-a in an aquarium during 15 days (accumulation phase). A depuration period of 15 days followed, with the mussels without ANA 37 suspension (depuration phase).

2.1. Cyanobacterial culture and preparation of ANA 37 suspensions

To simulate the toxic cyanobacteria bloom, an anatoxin-a producing strain was cultivated in batch cultures. The cyanobacterium*Anabaena* sp. (strain ANA 37) was isolated from Lake Sääskjärvi, Finland by Professor Kaarina Sivonen from the University of Helsinki (Department of Applied Chemistry and Microbiology) who kindly supplied us with one culture in solid medium (Sivonen et al., 1989). We cultured ANA 37 in liquid Z8 media (Kotai, 1972) in aerated batch cultures (monocyanobacterial, non-axenic) (20 ± 1 °C, photoperiod of 14 h light – PAR of 10 μmol m^{-2} s^{-1}). Initial inoculums of 50 ml were used and they were inoculated in larger flasks. After 1 month of growth, 4 l of culture in exponential phase in 6 l flasks were obtained. The number of cells per ml, of lugol fixed samples of the culture, was determined in a Sedgewick-rafter counting cell. In order to obtain number of cells per counting transect and to convert it to number of cells per ml, total length of trichomes was measured and divided by average cell diameter (5.68 μm ± 0.67, $n = 81$) (Lawton et al., 1999). Measurements were attained with the aid of software Leica QWin®, version 1, connected to an optical microscope. Suspensions of ANA 37 were prepared by concentration of the ensuing cultures through a plankton net. In order to have cell suspensions ready every day during the accumulation period, it was necessary to prepare it 12 h in advance. To avoid the introduction of an excess of Z8 medium and dilution of the seawater, we prepared *Anabaena* sp. concentrated cell suspensions at days 1, 3, 6 and 10 by gentle filtration of the cultures; the volume of the inoculum was always under 0.2% of the total volume of the aquarium. The cell suspensions were maintained in the dark at 4 °C in such a way that cell growth and anatoxin-a degradation was limited. The toxin content of ANA 37 was always monitored by anatoxin-a quantification and cell viability by microscopic observation immediately after adding the suspension to the aquarium.

2.2. Experimental set up

Specimens of *M. galloprovincialis* were obtained from a commercial depuration plant: Ñncoramar – mariscos Lda. situated at Viana do Castelo, Portugal. Eighty two animals were used in this experiment: average valve length was 7.63 ± 0.52 cm and average total fresh weight, with shell, was 33 ± 7.05 g. Before the experiment, the molluscs were acclimatized for one week to the experimental conditions: they were all maintained in one glass aquarium with natural seawater (filtered through 0.2 μm mesh), aerated, at constant temperature of 18 °C, photoperiod with 14 h light (PAR 1.9×10^{-6} E m^{-2} s^{-1}). Loading was 5.4–9.8 animals per litre (489–1078 animal m^2). These animal densities may be found in nature and do not represent the highest possible density found in *M. galloprovincialis* populations (up to 50 000 ind m^2) (Rius and Cabral, 2004).

The experiment consisted of two phases:

Accumulation phase (I) – *M. galloprovincialis* was exposed to live cells of the cyanobacterial strain ANA 37 (3.27×10^5 cell ml^{-1} ± 1.63×10^5 cell ml^{-1}) for 15 days. This density simulated ecological relevant densities of cyanobacteria (Bartram et al., 1999; Briand et al.,2002; Pereira et al., 2004). During phase I, the sea-water was replaced every 24 h, the volume readjusted according to the number of animals in the tank and a new volume of cyanobacterial cell suspension was added.

Depuration phase (II) – during 15 days, the mussels were maintained in the same conditions as in the phase I but without cyanobacteria.

2.3. Sampling

2.3.1. Water

During phase I, the water from the aquarium was sub-sampled every 24 h immediately before and after adding a new volume of cyanobacterial cells.

A volume of 100 ml of water was filtered through GF/C filters (Whatman®) and frozen (−22 °C) for quantification of dissolved anatoxin-a. The corresponding GF/C filters were frozen to quantify anatoxin-a in the suspended matter. Ten millilitres of water were fixed with lugol solution to quantify cell density and thus to determine filtering rate.

During phase II, water sampling was carried only before the daily water change and 250 ml were sampled instead of 100 ml because expected anatoxin-a concentration was lower than in phase I. The filtered water, as well as its respective filter, was kept frozen until anatoxin-a extraction.

2.3.2. Mussels

Mussels were collected randomly from the tank every 24 h. Maximum shell length and weight were recorded. Depending on the phase of the experiment, a different number of animals were taken. During the accumulation period, three animals; during depuration we expected a lower concentration of anatoxin-a and therefore five animals were collected and at day 10 of the accumulation phase, 10 animals. The mussels were dissected in four parts: foot plus other muscles, gills, digestive tract plus heart and mantle plus rest. Soft tissues from the same sampling day were pooled together and freeze-dried until extraction of anatoxin-a as described below.

2.4. Anatoxin-a extraction and purification

All procedures of extraction and purification of anatoxin-a described below as well as detailed evaluation of the method were previously described by Rellán et al. (2007). Filtrated water samples, previously adjusted to pH 7, were extracted by solid phase extraction (SPE) using weak cation exchange (WCX) in a vacuum manifold of Millipore (Waters™, Sep-Pak®). Supelco's Supelclean LC-WCX cartridges (3 ml) were conditioned with 6 ml of methanol followed by 6 ml of ultra-pure water (Milli-Q®, Millipore). Samples were then loaded into the cartridge and washed with methanol–water (1:1, 3 ml). After air-drying the cartridges, the anatoxin-a was eluted with 10 ml of methanol (0.2% TFA). The solvent of the eluates was removed by evaporation in a sample concentrator with nitrogen flux (Tecnhne Dri-block® DB.3) at 40 °C. Extracts thus obtained were reconstituted in methanol and kept at −22 °C in amber vials until HPLC–FLD analysis.

Lyophilized mussels and dissected parts were weighed in order to register dry weight. After breaking the soft tissues in small pieces with a scalpel, they were grinded and homogenized with an Ultraturrax homogenizer for 5 min with 10 ml of methanol (1% HCl, 1 M). The suspension was then sonicated with an ultrasonic processor for 2 min at *ca.* 80 amplitude (Sonics Materials, Vibra Cell 50). Finally the suspension was clarified by centrifugation at 3000*g* for 10 min. This procedure was repeated twice and the three supernatants were pooled together before evaporation and dissolution in 5 ml of ultra-pure water (pH 7). The aqueous extracts were adjusted to pH 7 and then subsequently purified by SPE as described before for water samples. Washing step was done with 3 ml of 100% methanol instead of methanol–water (1:1) due to higher complexity of the matrix of these samples comparing to the water ones (Rellán et al., 2007).

2.5. HPLC–FLD analysis

Procedures for HPLC–FLD analysis of anatoxin-a in mussels and water have been previously described in detail by Rellán et al. (2007). SPE extracts were evaporated under nitrogen stream and then reconstituted with 100 μl of sodium borate 0,1 M (pH 10). 50 μl of NBD-F (1 mg ml^{-1} in acetonitrile) was added and the vial was put aside in the dark for 10 min at room temperature for the derivatization of the anatoxin-a to take place. Hydrochloric acid (50 μl, 1 M) was added to finish the reaction. 20 μl of the derivative were injected in the HPLC system to perform the analysis (James et al., 1998). Three injections per sample were performed. The instrument consisted of an HPLC–FLD Lachrom by Merck Hittachi (Interface D-7000, Fl detector L-7480, Auto sampler L-7200 and Pump L-7100) with a LC reversed phase, Luna C_{18} column (5 μm, 250 mm × 4.6 mm i.d., Phenomenex®). Analysis was carried out at room temperature; the mobile phase was acetonitrile-water (50:50) with a flow rate of 0.8 ml min^{-1}. The excitation and emission wavelengths for fluorimetric detection were set at 470 and 530 nm, respectively.

Derivatized standard solutions containing anatoxin-a fumarate (Tocris Bioscience, Ref. 0789), ranging from 0.5 to 20 μg ml^{-1} were used for system calibration. Triplicate HPLC–FLD analyses of the prepared calibration levels were performed. Good linearity was achieved with a correlation coefficient $R^2 = 0.9948$.

The evaluation of the method resulted in method detection limits (based on a signal-to-noise ratio of 3) close to 25 ng l^{-1} for water and 15 ng g^{-1} for mussel tissue. Method quantification limits (based on a signal-to-noise ratio of 10) were 70 ng l^{-1} and 50 ng g^{-1} for water and mussel tissue, respectively. Adequate method repeatability values in terms of RSD were obtained (2.4–5.7%).

The recovery rates obtained by using uncontaminated river water sample and freeze-dried mussel spiked with two different concentrations of anatoxin-a standard, were 73% and 86% for mussels and water, respectively.

For routine use in sample monitoring by SPE and HPLC–FLD analysis, concentration of anatoxin-a was corrected for recovery. The fact of using anatoxin-a fumarate instead of pure anatoxin-a was also taken into account for quantitative results. Sample derivatives containing anatoxin-a at concentrations above the calibrated range were diluted with water.

As an example, it is shown an overlaid chromatogram of anatoxin-a standard with one sample of mussels soft tissue, after exposure to ANA 37 cells during the 15 days of accumulation in this experiment (Fig. 1).

3. Results

The mussels showed no significant behavioural alterations during the two phases of the experiment, except that pseudo faeces production decreased markedly during the last 8 days of the experiment. This was expected since the last 15 days corresponded to depuration phase (Phase II) when there was no cyanobacteria supplied to the aquarium. At the tested cell density, no deaths were observed during the 30 days of the experiment and there was no evidence of any adverse effects on *M. galloprovincialis* exposed to anatoxin-a.

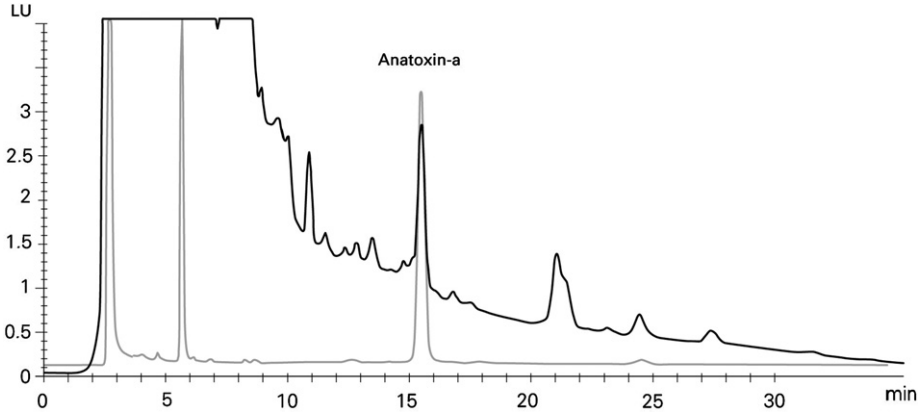

Fig. 1. Overlaid HPLC–FLD chromatogram. Black – one sample of soft tissue of *M. galloprovincialis* at day 15 of the accumulation period. Gray – anatoxin-a standard (11.74 ng of anatoxin-a in column).

Average weight of soft tissue per mussel was 5.99 g (relative standard of deviation (RSD) = 20%). Enumeration of ANA 37 cells in the aquarium was done in triplicates (RSD < 26%). Evolution of ingestion of toxic cyanobacteria by *M. galloprovincialis* in phase I is shown in Fig. 2. The ingestion rate reached its maximum at the 3rd day of Phase I (2×10^7 cell g^{-1} f.w. per day) then decreased, reaching a dynamic equilibrium with an average of 7×10^6 cell g^{-1} f.w. soft tissue per day (Fig. 2). Daily clearance rate ($100 \times$ (removed cells)/added cells) of the cells was always 100%.

Anatoxin-a concentration in the particulate matter (GF/C filters) was always under the lower limit of detection (1 ng l^{-1}).

Replicates of HPLC injections presented a RSD < 7%. Dissolved anatoxin-a was monitored during both phases (anatoxin-a cell quota varied between 1×10^{-5} and 8×10^{-5} ng $cell^{-1}$). Because different ANA 37 suspensions had to be used throughout the experiment, there are fluctuations of the dissolved anatoxin-a (Fig. 3). During the depuration, very low amounts of dissolved anatoxin-a were found varying from undetectable to 0.143 ng ml^{-1} (limit of detection = 9.6×10^{-4} ng ml^{-1}).

Anatoxin-a concentration in the soft tissue and the corresponding accumulated cells of ANA 37 are represented in Fig. 4. A dynamic decrease of anatoxin-a concentration in the soft tissue of *M. galloprovincialis* is observed with a maximum concentration of 6.6 ng of anatoxin-a per gram (d.w.) at day 4, after day 3 when the maximum of cell removal was observed (Fig. 2). This variation of anatoxin-a concentration in soft tissue follows the variation of removed anatoxin-a during phase I (Fig. 5), except for the last days when mussels did not accumulate any toxin. During Phase I, accumulation efficiency ((total toxin accumulated by the mussels/total dissolved toxin removed from the aquarium) × 100) varied between 0 (at days 2, 14 and 15) and 10.92 (at day 10) (Fig. 6).

The distribution of the toxin in the soft tissue (edible parts) shows that half the toxin (53% of total) is accumulated in the digestive tract and heart and the other half is distributed mostly in foot, muscles and gills (Fig. 7).

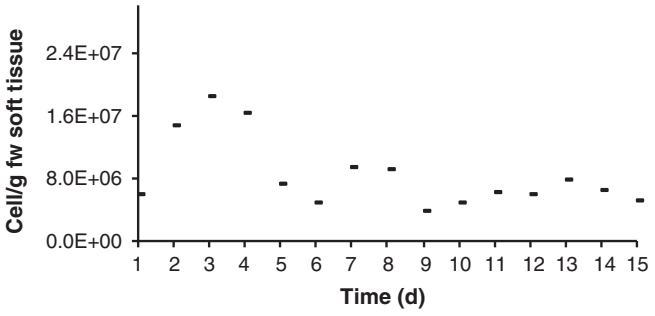

Fig. 2. Evolution of cyanobacterial cell ingestion by *M. galloprovincialis*. Removed cells (number of cells per gram f.w.) at 24 h interval during the 15 days of accumulation phase.

4. Discussion

No death occurred during the accumulation and depuration experiments, showing that mussels are very resistant to anatoxin-a and are good toxin vectors, as it was shown in previous experiments using other cyanotoxins (Vasconcelos, 1995; Amorim and Vasconcelos, 1999).

The fact that 100% of the cells were filtered by the mussels every day demonstrates that probably the mussels could have filtered more cyanobacteria and probably would have accumulated higher concentration of anatoxin-a, if a higher cell density was provided. In other experiments with cyanobacteria and *M. galloprovincialis* (Vasconcelos, 1995; Amorim and Vasconcelos, 1999) no clearance rate was determined, so that we can not compare our results.

The absence of anatoxin-a in the filters suggests that *Anabaena* cells liberated the toxin when placed in salt water. If this is the case, *M. galloprovincialis* is incorporating the toxin that is dissolved in the water and not the one that is in the ANA 37 cells. This would imply different exposure routes of the organisms to cyanotoxins, depending on the salinity.

Anatoxin-a level in the mussels did not increase steadily during the accumulation period until a maximum value was reached. This pattern happens with other toxins in other bivalves such as PSP in *Hiatula dophos* L. (Chou et al., 2005), PST in *Anodonta cygnea* (Pereira et al., 2004) and microcystin-LR in *Unio douglasiae* (Yokoyama and Park, 2003) and *M. galloprovincialis* (Vasconcelos, 1995). This might be explained by degradation of the toxin through a biochemical process undertaken by the mussels or by the short half-life of anatoxin-a due to its chemical instability, photodegradation and microbial degradation (Kiviranta et al., 1991; Stevens and Krieger, 1991; James et al., 1998; Rapala et al., 1994; Smith and Sutton, 1993). Further investigations should be considered in order to know if there is any physiological process of detoxication of anatoxin-a by *M. galloprovincialis*.

The low values of dissolved anatoxin-a observed during our experiments, could be explained by the anatoxin-a instability in water (Stevens and Krieger, 1991) or due to some physiological process that would actively metabolize and eliminate the toxin. A similar situation was observed with *Anodonta cygnea* and

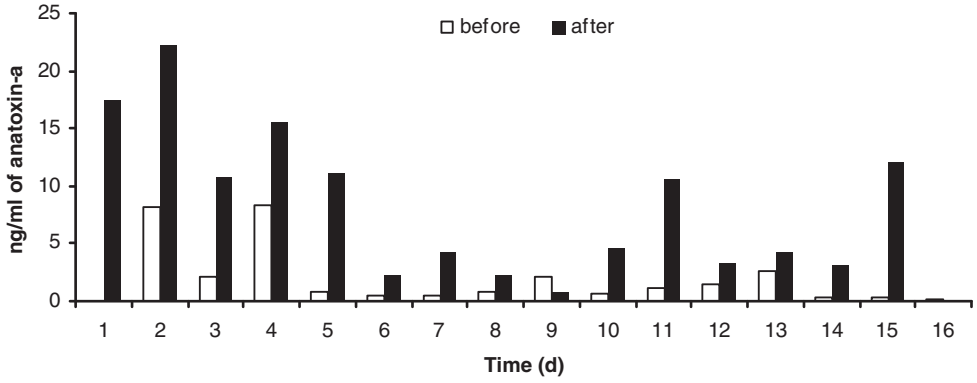

Fig. 3. Dissolved anatoxin-a (ng ml^{-1}) in the aquarium during phase I. HPLC analysis of three injections per sample with RSD < 7%. "Before" and "after" corresponds to samples taken immediately before and after addition of cyanobacteria every 24 h, along the 15 days of accumulation. Time is given in days after beginning of phase I.

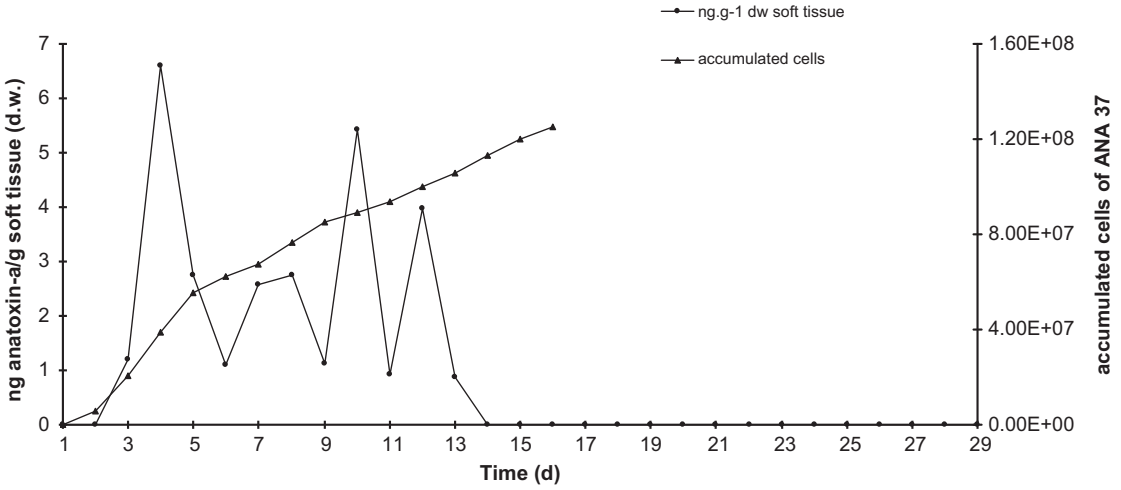

Fig. 4. Detected anatoxin-a concentration in the soft tissue of mussels and corresponding accumulated cells of ANA 37. HPLC analysis of three injections per sample with RSD < 7%. The last adding of ANA 37 occurred on day 15.

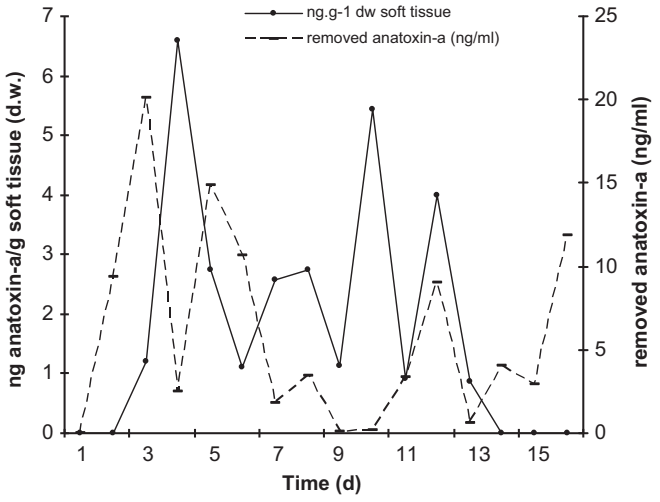

Fig. 5. Detected anatoxin-a concentration in the soft tissue of mussels and corresponding removed anatoxin-a from water. HPLC analysis of three injections per sample with RSD < 7%.

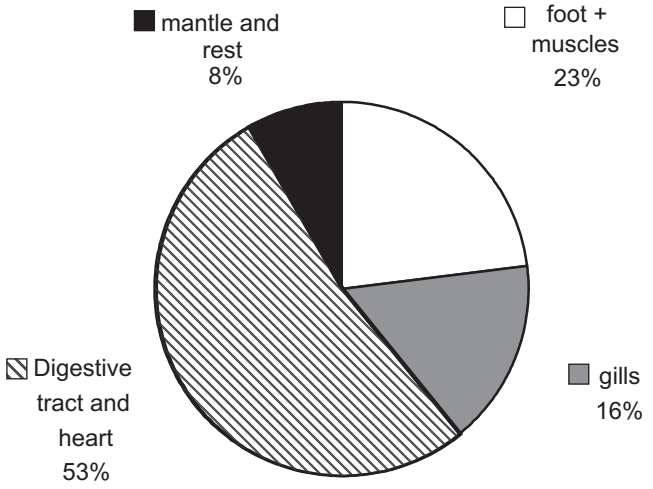

Fig. 7. Concentration of anatoxin-a and its distribution in soft tissue of *M. galloprovincialis* at day 10 of the accumulation experiment. HPLC analysis of three injections per sample with RSD < 2%.

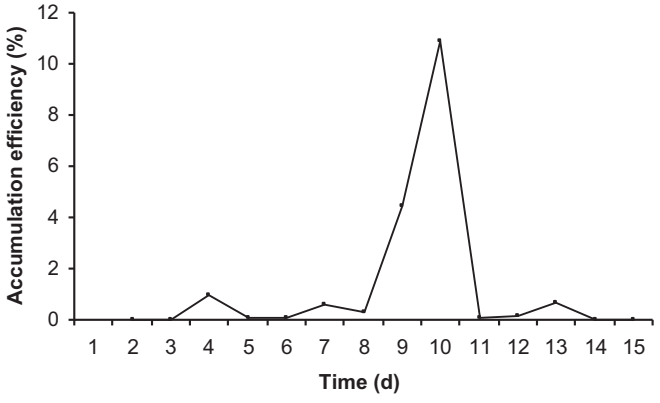

Fig. 6. Accumulation efficiency ((total toxin accumulated by the mussels/total dissolved toxin removed from the aquarium) × 100) of anatoxin-a by *M. galloprovincialis* during Phase I of the experiment.

cylindrospermopsin, also a cyanobacterial alkaloid but more stable than anatoxin-a (Saker et al., 2004). Maybe these two mussels genera have a similar metabolism for elimination of the alkaloids, anatoxin-a and cylindrospermopsin.

The maximum concentration of anatoxin-a detected in the soft tissue (6.6 ng of anatoxin-a per gram of soft tissue d.w.) is low when compared to other accumulation experiments of cyanotoxins by bivalves (Negri and Jones, 1995; Vasconcelos, 1995; Yokoyama and Park, 2003; Pereira et al., 2004; Pires et al., 2004; Saker et al., 2004), crayfish (Vasconcelos et al., 2001) and fish (Osswald et al., 2007). Our results show no bioaccumulation of anatoxin-a by *M. galloprovincialis.* Although there was some uptake, this was rapidly corrected by the depuration process in phase II. The pattern of anatoxin-a accumulation in the various mussel organs is similar to what happens to other cyanotoxins such as PST (Paralytic Shellfish Toxin) in *Anodonta cygnea* in which most of the toxin (between 50% and 78%) accumulated in the viscera (Pereira et al., 2004). Other two experiments had more evident results showing the importance of the viscera: Negri and Jones (1995) detected 96% of PSP

(Paralytic Shellfish Poisoning) toxin in the viscera of *Alathyria condola* and Vasconcelos (1995) showed that 95% of the microcystin was present in the viscera. Different results were reported by Saker et al. (2004) who was the first author to evaluate the toxin content in the haemolymph of *Anodonta cygnea*, and found that most of cylindrospermopsin was accumulated in this fluid that accounts for *ca.* 50% of the animal bodyweight. Optimization for the detection of anatoxin-a in the haemolymph is under study in our laboratory.

5. Conclusions

At ecologically relevant density of an anatoxin-a producing cyanobacteria (10^5 cell ml^{-1}), *M. galloprovincialis* did not show any behaviour alteration and no deaths were registered. Because clearance rates were always near 100%, this mussel is probably able to filter higher cell densities and thus to accumulate higher amounts of anatoxin-a. The maximum value of anatoxin-a accumulated by *M. galloprovincialis* (6.6 ng per gram of dry weight) was lower than reports for other toxins. Half of the toxin was accumulated in the digestive tract. After 15 days of an accumulation phase followed by other 15 d of depuration, the mussels contained no toxin and this fact could be explained either by detoxication of anatoxin-a by the mussel and/or due to the chemical instability of this alkaloid in water.

Although *M. galloprovincialis* is able to uptake anatoxin-a it does not accumulate it in very high rates (maximum accumulation efficiency=10.92%) and depurates it fast. These two aspects highly reduce the possible concerns about health risk due to consumption of contaminated *M. galloprovincialis*.

This work left some issues that need further investigation: quantification of accumulation of anatoxin-a by the haemolymph and what is the role of the shells. Although the later are not edible parts, in terms of aquatic ecosystem and interaction with other organisms, namely parasites, this issue should be explored.

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