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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.