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Accumulation, transformation and breakdown of DSP toxins from the toxic dinoflagellate *Dinophysis acuta* in blue mussels, *Mytilus edulis*

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ABSTRACT

Okadaic acid (OA), dinophysistoxins (DTX) and pectenotoxins (PTX) produced by the dinoflagellates *Dinophysis* spp. can accumulate in shellfish and cause diarrhetic shellfish poisoning upon human consumption. Shellfish toxicity is a result of algal abundance and toxicity as well as accumulation and depuration kinetics in mussels. We mass-cultured *Dinophysis acuta* containing OA, DTX-1b and PTX-2 and fed it to the blue mussel, *Mytilus edulis* under controlled laboratory conditions for a week to study toxin accumulation and transformation. Contents of OA and DTX-1b in mussels increased linearly with incubation time, and the net toxin accumulation was 66% and 71% for OA and DTX-1b, respectively. Large proportions (≈50%) of both these toxins were transformed to fatty acid esters. Most PTX-2 was transformed to PTX-2 seco-acid and net accumulation was initially high, but decreased progressively throughout the experiment, likely due to esterification and loss of detectability. We also quantified depuration during the subsequent four days and found half-life times of 5–6 days for OA and DTX-1b. Measurements of dissolved toxins revealed that depuration was achieved through excreting rather than metabolizing toxins. This is the first study to construct a full mass balance of DSP toxins during both accumulation and depuration, and we demonstrate rapid toxin accumulation in mussels at realistic *in situ* levels of *Dinophysis*. Applying the observed accumulation and depuration kinetics, we model mussel toxicity, and demonstrate that a concentration of only 75 *Dinophysis* cells l^{-1} is enough to make 60 mm long mussels exceed the regulatory threshold for OA equivalents.

1. Introduction

Contamination of shellfish with various biotoxins can lead to several different shellfish poisoning syndromes following human consumption (Landsberg, 2002). In all cases, the causative toxins are *de novo* produced by certain photo- or mixotrophic microalgae – not by the shellfish (Landsberg, 2002; Lewitus et al., 2012). Filter-feeding transfers the toxins to the shellfish, where they may accumulate to high concentrations. Symptoms range from nausea over paralysis and amnesia to death depending on the involved toxins. Consequently, commercial shellfish harvesting (fisheries or aquaculture) are subject to extensive monitoring of *in situ* concentrations of causative algae and/or toxicity of harvested shellfish.

Diarrhetic shellfish poisoning (DSP) syndrome is one such syndrome, and the causative organisms are a frequent cause for concern in shellfish industries, as they may cause prolonged closures of mussel harvesting, sometimes lasting several months, with severe economic repercussions (Hinder et al., 2011). Predicting DSP toxicity in mussels would be a powerful mitigation tool, but has so far proved difficult (Reguera et al., 2014). Identification of the causative toxins and variability in algal toxicity has received the bulk of the attention, but shellfish toxicity is also a result of toxin accumulation and depuration kinetics. Nevertheless, comparably little is known about accumulation and depuration of DSP toxins in any shellfish species.

DSP toxins are produced by species of the two marine dinoflagellate genera *Dinophysis* and *Prorocentrum* (Hoppenrath and Elbrächter, 2011; Reguera et al., 2014). The toxin producing *Prorocentrum* species are benthic, and thus unavailable for suspension-feeding mussels in most cases. Hence, *Dinophysis* is considered the main source of DSP toxins in marine shellfish. Acute effects of DSP toxins include diarrhea, nausea, vomiting and cramps, but chronic

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effects have also been reported, including carcinogenic effects and effects on the immune- and nervous systems and alterations in DNA and cellular components (Valdiglesias et al., 2013).

The DSP toxin group includes okadaic acid (OA) and its analogues the dinophysistoxins (DTX) as well as pectenotoxins (PTX). From the OA-group, OA, DTX-1, DTX-1b, and DTX-2 and their diol-ester precursors have been found in the toxin-producer *Dinophysis* spp. (Miles et al., 2006a; Hackett et al., 2009; Fux et al., 2011; Nielsen et al., 2013; Reguera et al., 2014). From the PTX-group, PTX-2 and PTX-11 – PTX-14 have been found in plankton samples or *Dinophysis* spp. cultures (Draisci et al., 1996; Miles et al., 2004b, 2006b; Suzuki et al., 2006). In shellfish, on the other hand, OA/DTX toxins are often transformed to fatty acid esters (collectively known as DTX-3), and these frequently comprise more than half the total OA/DTX in mussels (Vale and de M. Sampayo, 2002a; Vale, 2006). Likewise, mussels are known to transform PTX-2 to PTX-2 seco acid (PTX-2sa), but data on PTX in shellfish are scarce (Vale and de M. Sampayo, 2002b). PTX-2sa may also be transformed to numerous different fatty acid esters, further adding to the complexity (Wilkins et al., 2006; Torgersen et al., 2008).

Our knowledge on accumulation of DSP toxins in mussels comes almost exclusively from field populations; controlled laboratory experiments are very scarce (Bauder et al., 2001; Rossignoli et al., 2011b). This owes primarily to the fact that culturing of *Dinophysis* spp. was only recently made possible (Park et al., 2006). Depuration (or detoxification) has been studied more intensely using mussels contaminated *in situ*. Effects of various parameters, including food availability (Blanco et al., 1999; Svensson, 2003; Svensson and Förlin, 2004; Marcaillou et al., 2010), temperature (Shumway and Cembella, 1993; Blanco et al., 1999) and mussel size and lipid content (Svensson and Förlin, 2004; Duinker et al., 2007) have been evaluated using different mussel species. Reported toxin half-life times vary substantially, from less than a day to 25 days, but despite the efforts, depuration kinetics of DSP toxins is far from well understood. Accumulation kinetics remain virtually unstudied.

With the recent discovery of suitable culturing techniques, it is now possible to study accumulation kinetics of DSP toxins from the prime *in situ* source, *Dinophysis* spp. Thus, for the first time, we studied the intoxication of mussels with OA, DTX-1b and PTX-2 supplied via mass cultured *Dinophysis acuta*. We chose the blue mussels, *Mytilus edulis*, as the target organism due to its commercial importance and well-studied physiology. The aim was to establish a mass balance under controlled laboratory conditions, quantifying accumulation, transformation, depuration and excretion of DSP-toxins from *D. acuta*. Based on these results, we model the toxin content of various-sized *M. edulis* under different *Dinophysis* spp. concentrations.

2. Materials and methods

2.1. Culture conditions and specimen collection

A laboratory culture of the marine DSP producing dinoflagellate *Dinophysis acuta* (Strain DANA-2010) was established from water samples collected in the North Atlantic during a research cruise in June 2010 (60°24′ N; 6°58′ W) (Nielsen et al., 2013). Cells were isolated under a dissection scope by micro manipulation. The culture was non-clonal, as ≈10 cells were picked and grown together in 1 ml 0.2 μm filtered seawater. To facilitate the mixotrophic growth of *D. acuta,* the ciliate *Mesodinium rubrum* was added as prey twice a week at a prey:predator ratio of 10:1. The *M. rubrum* culture was fed the cryptophyte *Teleaulax amphioxeia* at similar intervals and ratios. All protist cultures were grown in a temperature controlled room at 15 °C in f/2 seawater-based growth medium (Guillard and Ryther, 1962) with a pH of 8.0 ± 0.05, a salinity (psu) of 30.0 ± 1.0 and a dissolved inorganic carbon content of 2.3 ± 0.1 mmol l^{-1}. A Sentron ArgusX pH-meter equipped with a Hot-line cupFET probe was used to determine pH (NBS scale), and salinity was measured with a handheld visual refractometer. Dissolved inorganic carbon was measured with an infrared gas analyzer as described in detail elsewhere (Nielsen et al., 2007). An irradiance of 130 μmol photons m^{-2} s^{-1} was provided by Osram 58W/640 cool-white fluorescent tubes at a 16:8 h light:dark cycle. Previous studies of the *Dinophysis acuta* culture had shown that it produced OA and PTX-2 as well as a novel isomer of DTX-1 tentatively termed DTX-1b (Nielsen et al., 2013).

The mussel *Mytilus edulis* was collected in Oresund, Denmark with a dredge deployed from boat during autumn 2011. The mussels were brought back to the laboratory, where they were kept for at least a month in a continuous flow of aerated sea water (30 ± 1.0 psu, 10 °C) before use in experiments. The collection site typically has low abundances (<1 cell ml^{-1}) of *Dinophysis* spp. almost year round, but only rarely experiences blooms. The mussels were thus not naïve. On the other hand, one month in the laboratory may have removed any adaptations towards DSP toxins before the onset of the experiment.

2.2. Accumulation, transformation and breakdown of DSP toxins

The experiment was designed to study accumulation and depuration rates of different DSP toxins in *Mytilus edulis*. Several initial attempts to intoxicate *M. edilus* with DSP toxins from *Dinophysis acuta* resulted in production of pseudo-faeces and correspondingly low (<1%) net toxin retention. These observations were most likely due to high concentrations of *D. acuta* (>20,000 cells l^{-1}), and the present experiment was thus designed to work with low *D. acuta* concentrations.

Four replicate 12 l aquaria were setup in a 15 °C temperature controlled room. Five litre GF/C filtered seawater (salinity = 30 ± 1) and 12 *M. edulis* (length 23.4 ± 1.0 mm) were added to each. Mussels were allowed 6 h to attach to the bottom, after which centrifugal aquarium pumps were deployed to ensure adequate mixing. The aquaria were all aerated with atmospheric air (≈40 ml min^{-1}). To limit the growth of *D. acuta* in both supply cultures and the four aquaria, the experiments were conducted at relatively low irradiance levels (≈10 μmol photons m^{-2} s^{-1}).

2.3. Dinophysis acuta inflow

Once the experiment started, *D. acuta* was pumped into each aquarium by a Gilson Minipuls 3 peristaltic pump (Biolab A/S, Denmark), equipped with a 4 channel pump head. A well-mixed *D. acuta* supply culture was ensured by continuous stirring with a magnetic stirrer. Glass funnels, custom-made from Pasteur pipettes, were positioned in the narrow intake of the tubes to avoid clogging of *D. acuta* cells. In order to design the experiment with low cell concentrations of *D. acuta*, a simple model was developed to predict the steady-state concentration of *D. acuta* as a function of *D. acuta* inflow rate:

$$A_t = \frac{A_{t-1} \times V_{t-1} + F - (M \times C \times A_{t-1})}{V_t} \quad (1)$$

where A is *D. acuta* concentration (cells l^{-1}) at times t and t-1, V is total water volume (l), F is inflow of *D. acuta* in (cells h^{-1}), M is number of mussels and C is clearance rate (l ind^{-1} h^{-1}). For designing the experiment, C was, according to Kiørboe and Møhlenberg (1981), assumed to be 1.0 l ind^{-1} h^{-1} for the size of *M. edulis* used.

The experiment was designed to produce an asymptotic rise to a