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Exposure to toxic *Alexandrium minutum* activates the detoxifying and antioxidant systems in gills of the oyster *Crassostrea gigas*

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ABSTRACT

Harmful algal blooms of *Alexandrium* spp. dinoflagellates regularly occur in French coastal waters contaminating shellfish. Studies have demonstrated that toxic *Alexandrium* spp. disrupt behavioural and physiological processes in marine filter-feeders, but molecular modifications triggered by phycotoxins are less well understood. This study analyzed the mRNA levels of 7 genes encoding antioxidant/detoxifying enzymes in gills of Pacific oysters (*Crassostrea gigas*) exposed to a cultured, toxic strain of *A. minutum*, a producer of paralytic shellfish toxins (PST) or fed *Tisochrysis lutea* (*T. lutea*, formerly *Isochrysis* sp., clone Tahitian (*T. iso*)), a non-toxic control diet, in four repeated experiments. Transcript levels of sigma-class glutathione S-transferase (GST), glutathione reductase (GR) and ferritin (Fer) were significantly higher in oysters exposed to *A. minutum* compared to oysters fed *T. lutea*. The detoxification pathway based upon glutathione (GSH)-conjugation of toxic compounds (phase II) is likely activated, and catalyzed by GST. This system appeared to be activated in gills probably for the detoxification of PST and/or extra-cellular compounds, produced by *A. minutum*. GST, GR and Fer can also contribute to antioxidant functions to prevent cellular damage from increased reactive oxygen species (ROS) originating either from *A. minutum* cells directly, from oyster hemocytes during immune response, or from other gill cells as by-products of detoxification.

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1. Introduction

The proliferation of toxic micro-algae in coastal environments has major implications for marine ecosystems and human health (Shumway, 1990; Van Dolah, 2000; Hallegraeff, 2014). The paralytic shellfish toxins (PST) produced by dinoflagellates are the most widespread and among the most potent of shellfish-contaminating biotoxins (Huss, 2002). In France, PST toxic events have been reported since the 1980's, first in Brittany caused by *Alexandrium minutum* and later in the Thau Lagoon with *Alexandrium catenella* (Lassus et al., 2004; Lassus et al., 2007). The last major PST-producing bloom occurred in July 2012 in the Bay of Brest (Brittany) where *A. minutum* cell counts reached 41,740 cells mL^{-1}, a record for the French coast.

During blooms, filter-feeding organisms such as bivalve molluscs can accumulate large amounts of toxins, causing behavioural and physiological symptoms in the shellfish (Shumway, 1990). Some studies have reported that the toxicity of dinoflagellates may be enhanced by, as yet uncharacterized, extracellular products. First demonstrated with planktonic ciliates and other protists (Hansen, 1989; Arzul et al., 1999; Tillmann et al., 2008; Lelong et al., 2011), toxic effects of these compounds have been shown to be haemolytic to mammalian blood cells (Arzul et al., 1999; Emura et al., 2004) and to have deleterious effects upon bivalve hemocyte functions (Hégaret et al., 2011). Studies have demonstrated that *Alexandrium* spp. affect bivalve biology by modifying valve opening behaviour, filtration processes, nutrition, and cardiac activity (Gainey and Shumway, 1988a,b; Shumway, 1990; Shumway and Gainey, 1992; Lassus et al., 1999; Tran et al., 2010). Dinoflagellates also may interfere with reproduction of bivalves, altering spermatozoa quality (Haberkorn et al., 2010a; Le Goïc et al., 2013) or larval survival (Leverone et al., 2006; Shumway et al., 2006) and inducing inflammatory responses in digestive gland and gills of mussels and oysters (Galimany et al., 2008;

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Haberkorn et al., 2010a). The innate immune system of bivalves, based upon hemocytes, can be depressed by exposure to *Alexandrium* spp. (Hégaret et al., 2007; Ford et al., 2008; Galimany et al., 2008; Hégaret et al., 2011; Mello et al., 2013). There are, however, two defence mechanisms that may counteract the effects of dinoflagellate toxins (both PST and non-PST extra-cellular compounds) in shellfish: detoxification pathways for the biotransformation and elimination of phycotoxins, and antioxidant metabolism to neutralize reactive oxygen species (ROS) resulting from the exposure to *Alexandrium* spp. (Kim et al., 1999; Flores et al., 2012).

Bivalves are able to detoxify ingested PST by excreting algal cells or by biotransforming and eliminating these toxins (See for example Bricelj and Shumway, 1998; Jaime et al., 2007). Paralytic shellfish toxins are composed of saxitoxin (STX) and analogues that can be classified into three groups (Wang, 2008): 1) carbamate toxins, including STX, neo-saxitoxin (NEO) and gonyautoxins 1–4 (GTX1-4). The *N*-sulfocarbamoyl group, which includes the C1-4 and B (GTX5 and GTX6) toxins; and the decarbamoyl compounds (dcGTX1-4, dcSTX and dcNEO). GTX3, GTX4, C2, C4 (β epimers) are observed in dinoflagellates producing PST with GTX1, GTX2, C1, C3 (α epimers) increasing with time in toxin-contaminated bivalves (Oshima, 1995a; Bricelj and Shumway, 1998). As a result, the biotransformation of PST in tissues of contaminated bivalves generally corresponds to a decrease in the ratio of β:α epimers. Enzymes involved in PST biotransformation are poorly known, but some studies suggested that glutathione S-transferase (GST) may participate to this mechanism, as in *Mytilus edulis* for which an activation of GST was observed in the digestive gland after injection of crude extracts of toxin (Gubbins et al., 2001). The GST are known, in plants and animals, as key enzymes for the detoxification of toxic compounds. These enzymes can catalyze the conjugation of electrophilic toxic molecules to reduced glutathione (GSH), one major component for the detoxification of harmful molecules (Meister and Anderson, 1983).

These detoxification processes are based mainly upon oxidation/reduction reactions which generate oxidized radicals. These molecules, together with ROS, produced by either hemocytes or dinoflagellates, may lead to disequilibrium of the redox system in bivalves exposed to *Alexandrium* spp. To prevent cellular oxidative damage, the antioxidant system must control the removal of ROS (Lesser, 2006). The antioxidant metabolism appears to be modulated in bivalve tissues by exposure to toxic dinoflagellates, but it is difficult to summarize a general pattern of response, as variations in antioxidant enzymes appeared to be different according to micro-algal species, bivalve species, and experimental design. For example, the expression of extra-cellular superoxide dismutase (ec-SOD), catalase (CAT) and ferritin (Fer) in hemocytes of *Mytilus chilensis* increased 48 h after intra-muscular injection of an extract of *A. catenella* (Núñez-Acuña et al., 2013). In contrast, cytoplasmic Cu/Zn SOD (cy-SOD), CAT and glutathione peroxidise (GPx) were under-expressed in gills of *Crassostrea gigas* exposed to *A. minutum* for 48 h (Mat et al., 2013).

The present study quantified toxin content in the digestive gland and the mRNA levels of the enzymes of the antioxidant/detoxifying system in gills of *Crassostrea gigas* oysters exposed to environmental concentrations of cultures of a toxic strain of the dinoflagellate *Alexandrium minutum*, compared with oysters fed *Tisochrysis lutea*, a non-toxic diet. The gill was chosen as the target organ because this is the first one in contact with *Alexandrium* cells during filtration—the first step of nutrition in bivalves. Further, the gill was previously demonstrated to be directly affected by contact with toxic algae (Haberkorn et al., 2010a). The modulation of mRNA levels of seven genes encoding a CAT, a cy-SOD, an ec-SOD, a selenium-dependent GPx (GPx), glutathione reductase (GR), a sigma-class GST (GST), and a soma ferritin (Fer) were analyzed by real-time PCR. Four exposure experiments were done following the same protocol in April and May 2007 and in May and June 2008 to confirm consistency of oyster responses to *A. minutum*.

2. Materials and methods

2.1. Oysters

Diploid Pacific oysters, *Crassostrea gigas*, were obtained from "île de Kerner" (Morbihan, France) cultured stocks. Oysters sampled in April and May 2007 were 2-years old, and oysters sampled in May and June 2008 were 18-months old. After collection, oysters were transferred to the experimental facilities of the LEMAR Laboratory (Finistère, France). The absence of paralytic shellfish toxins (PST) contamination within oysters before the experiments (no detectable levels of PST by liquid chromatography/fluorescence detection (LC/FLD) analysis) was confirmed in 3 pools of 4 oysters in April and May 2007, and in 3 pools of 10 oysters in May and June 2008.

2.2. Algal cultures

The toxic dinoflagellate *Alexandrium minutum* (strain AM89BM isolated from Bay of Morlaix, France in 1995) was grown according to the protocol described in Haberkorn et al. (2010a) in 10-L batch cultures at 16 ± 1 °C and 100 μmol photon m^{-2} s^{-1}, with a dark:light cycle of 12:12 h. After 12 days, when still in the exponential growth phase, *A. minutum* cultures were harvested. At this point, toxin production of the cultured strain was 1.3 ± 0.1 pg STX eq. $cell^{-1}$ (Haberkorn et al., 2010a,b).

Cultures of *Tisochrysis lutea* (formerly *Isochrysis* sp., clone Tahitian (*T. iso*)) were obtained from the Argenton hatchery of IFREMER (Finistère, France). Cultures were grown as described in Haberkorn et al. (2010a) in 300-L cylinders, at 24 ± 1 °C with continuous light. The cultures were harvested in the exponential growth phase, 6–8 days after inoculation, for the experiments. *T. lutea* was the non-toxic control diet.

2.3. Exposure experiments

For each exposure experiment, in April and May 2007 and in May and June 2008, oysters were distributed randomly into six 15-L tanks (10 oysters per tank in 2007 and 20 oysters per tank in 2008). Oysters were acclimated for 10 days in conditions described by Haberkorn et al. (2010a), and fed *Tisochrysis lutea* at 5×10^5 cells mL^{-1}. After acclimation, oysters were fed continuously for 4 days at 14 mL min^{-1} with 5×10^5 cell mL^{-1} of *T. lutea* (3 control tanks) or with 5×10^3 cell mL^{-1} of *Alexandrium minutum* (3 exposed tanks). The different cell densities were used to provide equivalent bio-volume of micro-algae to oysters as the cellular volume of *A. minutum* is approximately 100× higher than that of *T. lutea*.

2.4. Oyster sampling

At the end of each of the four algal-exposure experiments, the digestive glands of 4 oysters in 2007 and 10 oysters in 2008 were sampled from each tank and pooled for toxin content measurement using LC/FLD. All saxitoxin (STX) analogues were analyzed through three successive runs. Among these oysters sampled for biotoxins, 2 oysters per tank were also sampled for gene expression analyses (6 oysters per treatment). Gills were dissected immediately under RNase-free conditions and frozen in liquid nitrogen. Individual samples were stored at −80 °C for further molecular analyses.

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