Aquaculture 358-359 (2012) 108–115

Contents lists available at SciVerse ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

Spirolide uptake and detoxification by *Crassostrea gigas* exposed to the toxic dinoflagellate *Alexandrium ostenfeldii*

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ARTICLE INFO

Article history:
Received 6 October 2011
Received in revised form 15 May 2012
Accepted 21 June 2012
Available online 3 July 2012

Keywords:
Spirolides
Alexandrium ostenfeldii
Contamination
Depuration
Crassostrea gigas
Inflammatory responses

ABSTRACT

Oysters (*Crassostrea gigas*) were exposed 4 days to cultures of the toxic dinoflagellate *Alexandrium ostenfeldii* (strain CCMP1773) that produces spirolides belonging to fast acting toxins (FAT) and let depurate for 7 days. During depuration, oysters were either fed the non-toxic algae Isochrysis galbana Tahitian clone (T. Iso) or starved. The objectives of this experiment were to evaluate (i) spirolide uptake and depuration by oysters (ii) spirolide effects on oysters and (iii) oyster recovery according to food supply during depuration.

A. ostenfeldii cells were filtered and ingested by oysters while faeces contained numerous intact cells of the toxic diet. This suggested that ingested cells were not totally digested by oysters. Contents of spirolides (SPX) in digestive gland and remaining tissues during contamination and detoxification periods were measured by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). Four different SPX analogues (13,19-didesMeC, 13-desMeC, 13-desMeD and trace of SPX-D) were detected. The 13,19-didesMeC-SPX dominated in both digestive gland and remaining tissues. After four days exposure, digestive gland (DG) contained 83% of the total initial spirolide concentration, whereas remaining tissues contained only 17%. During detoxification, spirolide content in DG was lower in fed than in unfed oysters but similar in remaining tissues.

Exposure to *A. ostenfeldii* resulted in an inflammatory response consisting of hemocyte infiltration and diapedesis into the intestinal tract of the oysters. Percentage of active digestive tubules in oysters fed *A. ostenfeldii* was significantly lower than in control (prior exposition) oysters (36% and 61%, respectively). At the end of the detoxification period, there was a significant difference in the percentage of active digestive tubules ($P<0.001$) between fed and unfed oysters. When oysters were fed T-Iso following the *A. ostenfeldii* exposure, 80% of digestive tubules were active, thus revealing a rapid recovery after toxic algae exposure.

Overall, both spirolide detoxification and recovery from their toxic effects are almost complete within 7 days after exposure to spirolide producers. Such information may help to resume faster oyster sales after toxic events involving FAT implying thus more frequent chemical analysis.

1. Introduction

The marine dinoflagellate *Alexandrium ostenfeldii* has been identified as the only micro-organism producing spirolide toxins (Cembella et al., 2000a,b). This species has been found in Denmark (Moestrup and Hansen, 1988), Scotland (John et al., 2003a), Norway (Balech and Tangen, 1985) and Spain (Fraga and Sánchez, 1985), and it produces either saxitoxin analogues, i.e. paralytic shellfish poisoning (PSP) toxins, or spirolide toxins, which belong to cyclic imine group. Some *A. ostenfeldii* strains can produce spirolides and PSP toxins at the same time, as demonstrated in Denmark (Hansen et al., 1992; MacKinnon et al., 2004) with local strains.

Compared to other *Alexandrium* species, relatively little is known to date about the biology and ecology of *A. ostenfeldii*. This may be partly attributed to the fact that it typically occurs at low concentrations of

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doi:10.1016/j.aquaculture.2012.06.023

10^2 to 10^3 cells L^{-1} in phytoplankton mixed assemblages which already contain other bloom-forming dinoflagellates (Balech and Tangen, 1985; Gribble et al., 2005; John et al., 2003b; Moestrup and Hansen, 1988). *A. ostenfeldii* is known for its capability to behave as a mixotrophic species (Gribble et al., 2005; Jacobson and Anderson, 1996) and to produce allelochemical compounds (Hansen et al., 1992; Tillmann et al., 2007).

A. ostenfeldii life cycle includes two types of cysts. Temporary cysts are commonly formed when cells are subjected to stress either in cultures or at field (Cembella et al., 2001; Østergaard-Jensen and Moestrup, 1997). Resting cysts are formed in winter and are generally isolated from coastal sediment (Bravo et al., 2006; MacKenzie et al., 1996b).

Like other *Alexandrium* species, *A. ostenfeldii* can produce paralytic shellfish toxins (PSTs) (Hansen et al., 1992; MacKenzie et al., 1996b). PST profiles and specific toxicities in culture may vary largely according to strains and localities. It has been noticed that Danish *A. ostenfeldii* strains, predominantly, produce low potency saxitoxin derivatives (Hansen et al., 1992), whereas in New Zealand strains PSP toxicity ranged from 0 to much higher levels (up to 217 pg STX eq $cell^{-1}$) than usually observed in cells of other potent *Alexandrium* species (MacKenzie et al., 1996a,b,c). North Atlantic populations of *A. ostenfeldii* do not seem to produce PST's at all (Cembella et al., 2000b; Gribble et al., 2005). Instead, spirolides, a recently isolated group of highly potent neurotoxins (Hu et al., 1995; Richard et al., 2000), are commonly found in cultured strains and natural samples (Cembella et al., 2000b; Gribble et al., 2005; John et al., 2003b).

In June 2005, the French Phytoplankton and Phycotoxin monitoring Network (REPHY) revealed the presence of *A. ostenfeldii* for a short period in Arcachon Bay (French S.W coasts). For the first time, spirolides were detected (Amzil et al., 2007) in local oyster digestive glands. When shellfish are contaminated by this toxic microalga at a level giving a positive DSP mouse bioassay, they are no longer harvested and marketed resulting in serious economic losses. Spirolides are 'Fast Acting Toxins' (FAT), causing neurotoxic symptoms and rapid death of laboratory mice when intraperitoneally (*i.p*) injected or administered orally (Hu et al., 1995). However, no adverse effects on humans have been reported so far. Although their toxicity to humans and their mode of action are still under investigation (Gribble et al., 2005), spirolides are known to produce an antagonistic effect at the muscarinic acetylchloline receptor (Richard et al., 2000).

The characterization of the most common spirolides and their des-methyl derivatives from toxic plankton and contaminated shellfish has recently been reported by different authors (Hu et al., 2001; MacKinnon et al., 2006; Sleno et al., 2004). Toxicological studies on mice revealed that toxicity of pure 13-desMeC is much lower when administered by gavage than when *i.p* injected, with LD 50 of 150 μg kg^{-1} and 5–8 μg kg^{-1}, respectively (Munday, 2008).

Insofar as no regulatory limits have been established for macrocyclic imines (FAO/IOC/WHO report, 2004), no data were available about spirolide bioaccumulation and elimination pathways in shellfish.

The present study investigated (i) the uptake and detoxification of spirolides by oysters, (ii) the impact of non-toxic algal food on detoxification rates, testing the hypothesis that oysters fed *I. galbana* detoxified faster than unfed (control) oysters (iii) the effects of *A. ostenfeldii* experimental exposure on tissue structures and recovery ability of contaminated oysters during depuration.

2. Materials and methods

2.1. Experimental animals before exposure to A. ostenfeldii

Triploid oysters, *Crassostrea gigas* (64.3 ± 2.8 mm mean shell length), were obtained from the IFREMER coastal laboratory located in Bouin (French Atlantic coast). All oysters (n = 108) were cleaned from epibionts and other encrusting organisms. Prior exposure to *A. ostenfeldii*, oysters were acclimatized for 3 days in a raceway filled with seawater at 16 °C and fed on T.*Iso* for only one day. Mean of oyster total fresh tissues weight was 6.26 ± 0.91 g/individual (n = 10). The same sample (non exposed control) was used for the histology study.

Eight other oysters were also used for extraction and analysis of lipophilic toxins (okadaic acid, pectenotoxins, azaspiracides, yessotoxines, spirolides and gymnodimines).

2.2. Algal cultures

The harmful algal species used in this study was the CCMP1773 strain of *A. ostenfeldii* (Balech) isolated from Limfjorden (Denmark) and obtained from the Provasoli Guillard National Center for Culture of Marine Phytoplankton. In addition, the non-toxic Tahitian clone of *Isochrysis galbana* (T. *Iso)* acclimated in IFREMER laboratory was used as a non-toxic detoxification diet. *A. ostenfeldii* was grown in 10-liter batch cultures and 100-liter photo-bioreactors using sterile sea water at a salinity of 35 psu supplemented with L1 nutrient enrichment (Guillard and Hargraves, 1993). T. *Iso* was grown in 10-liter culture vessels filled with seawater previously autoclaved (121 °C, 2 bars, 2 h), filtered through a 0.22 μm membrane (Whatman allipore filter) and enriched with Conway medium.

Batch cultures were maintained at 16 °C under a 12 h:12 h L:D cycle at 150 μmole photon/m^2/s and was used to inoculated a 100 litre photobioreactor filled with 35 psu sterile seawater prepared as above and enriched with L1 medium (Guillard and Hargraves, 1993). Culture were maintained at 17 °C under a 16 h:8 h (L:D) cycle and at 170 μmole photon/m^2/s.

T. *Iso* cultures were harvested for oyster feeding during the exponential phase of growth (days 14).

2.3. Experimental design for A. ostenfeldii exposures

After the 3 days acclimation, 90 oysters were distributed randomly into two raceways filled with 150 l seawater at a salinity of 35 psu and a temperature of 16 °C. The experimental setting was similar to that previously described in Lassus et al. (1999). A 40 l buffer tank placed within the circuit just after the flume outlet contained the heat exchanger and the pumps, ensuring circulation of the water and continuous measurement of chlorophyll with a Turner Design fluorometer equipped with 340–500 nm excitation and 665 nm emission filters. Toxic and non-toxic micro-algae inputs at the flume inlet were supplied by an Ismatec peristaltic micropump with flow rate regulated according to the settings established at the beginning of the experiment. Continuous measurements provided by the fluoremeter were integrated via a LABVIEW® acquisition and control card and a data-logger connected to a microcomputer.

Oysters were fed *A. ostenfeldii* at a concentration of 200 $cell.ml^{-1}$ in a recirculated seawater circuit (flow rate of 800 $l.h^{-1}$) for 4 days. Sea water was totally renewed every two days. Following *A. ostenfeldii* exposure, oysters were divided in two groups: 45 oysters were fed T.*Iso* at 12000 $cell.ml^{-1}$ for 7 days, whereas, another 45 oysters group was not fed (sea water only) during the same period. Faeces and pseudo-faeces were removed every day from each raceway with a pipette and examined to verify the status of the cells using light microscopy.

Oysters samples were collected after 4 days exposure to the toxic dinoflagellate (day 0), and during detoxification period, i.e. on days 1, 4, and 7. At each sampling time, 8 oysters were removed from the raceway, pooled and processed for spirolide extraction and analysis. Ten oysters were also sampled for the histopathology study after exposure to *A. ostenfeldii*, and after 7 days detoxification with or without food supply.