Harmful Algae 8 (2009) 279–290

Contents lists available at ScienceDirect

Harmful Algae

journal homepage: www.elsevier.com/locate/hal

Gonyaulax spinifera from the Adriatic sea: Toxin production and phylogenetic analysis

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ARTICLE INFO

Article history:
Received 4 December 2007
Received in revised form 20 June 2008
Accepted 22 June 2008

Keywords:
Adriatic sea
Dinoflagellate
Gonyaulax spinifera
Phylogenetic analysis
Yessotoxin

ABSTRACT

The north-western coasts of the Adriatic sea have long been subjected to recurring cases of harmful algae blooms and concomitant mussel contamination. In 1995, yessotoxins (YTXs) were detected for the first time in Adriatic mussels and their presence was subsequently linked to the presence of *Protoceratium reticulatum* and occasionally *Lingulodinium polyedrum* in seawater. Since then, farmed molluscs are yearly found to contain YTXs for long periods with a severe impact on aquaculture activities. In 2004, unusually high amounts of homoYTXs were detected in mussels. In this period, a careful look at the phytoplankton composition highlighted that *P. reticulatum* and *L. polyedrum* were nearly absent in seawater whereas the dinoflagellate *Gonyaulax spinifera* was present at high densities. *G. spinifera* has been recently associated with YTX production in New Zealand. The present work was performed in order to ascertain whether Adriatic *G. spinifera* was a toxic species. Determination of the toxin content of natural samples (mussels and net haul plankton samples) as well as monitoring of the dinoflagellate species present in seawaters allowed us to correlate the presence of *G. spinifera* to that of homoYTX in mussels. Cultures of *G. spinifera* were set up which gave us the opportunity to investigate toxin content and profile of the dinoflagellate, for the first time, by liquid chromatography–mass spectrometry (LC–MS) and to perform a molecular characterization of the species through rDNA sequencing. Two *G. spinifera* isolates, sampled in different years, were analyzed and resulted to be different in both toxin content and gene sequence: the isolate sampled in 2005 produced only low levels of YTX (5.4 pg cell^{-1}) while that sampled in 2006 contained higher toxin amounts, namely 33.4 pg cell^{-1} of homoYTX and 3.6 pg cell^{-1} of YTX; the two isolates also differed in 111 and 176 nucleotide positions, respectively, in the SSU and partial LSU rDNA genes. Comparing similar sequences of *G. spinifera* strains contained in GenBank, divergences among the Adriatic strains and strains of different geographical origin emerged; on the contrary, a good similarity was evidenced (1.3%) between the Adriatic isolate sampled in 2006 and two New Zealand strains; the similarity was confirmed also by the presence in all strains of YTXs, although the New Zealand strains were analyzed by ELISA which could not distinguish among the different YTX analogues. Due to an originally confusing description of the species and to the availability of very low number of deposited sequences the doubt arises whether the studied *G. spinifera* strains belong to the same species. However, although the taxonomy of the species complex *G. spinifera* remains largely unresolved, this study allowed to unambiguously link the presence of *G. spinifera* to recurring mussel toxicity episodes.

1. Introduction

Yessotoxin (YTX) is a disulfated polyether toxin (Fig. 1) which was first isolated from scallops (*Patinopecten yessoensis*) collected in Japan (Murata et al., 1987). In the late 1990s, the marine

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doi:10.1016/j.hal.2008.06.008

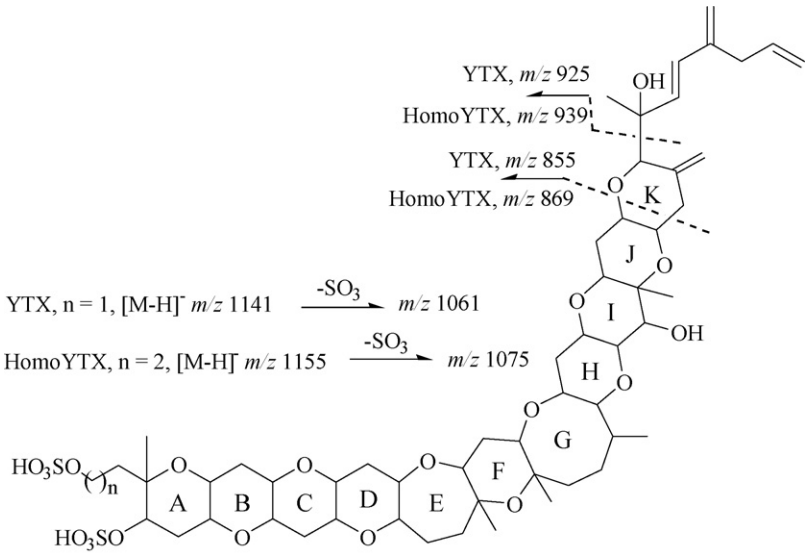

Fig. 1. Structure of YTX and homoYTX and associated $[M-H]^-$ ions and fragment ions.

microalga *Protoceratium reticulatum* from New Zealand (Satake et al., 1997) was identified as the biogenic origin of YTX. Since then, in several European countries, such as Norway (Samdal et al., 2004), Italy (Ciminiello et al., 1997; Boni et al., 2001) and Spain (Paz et al., 2004), the presence of *P. reticulatum* in seawater was found to be associated with the highest concentration of YTX in shellfish with a severe impact on aquaculture industry.

Mussel extracts contaminated by YTX cause high acute toxicity in mice after intraperitoneal (i.p.) injection, however this toxin, administered via the oral route, is much less potent and it does not induce diarrhoea. In addition, there are no reports of human intoxication caused by yessotoxins (Toyofuku, 2006). Although yessotoxin and its metabolites may be of limited public health significance, at present the European legislation sets a limit of 1 mg YTX equivalent/kg shellfish tissue. The use of traditional mouse bioassays (MBAs) (e.g. Yasumoto et al., 1984) instead of alternative quantitative methods, leads to consistent damages to the shellfish industry, as the occurrence of false positives, which these methods are prone to, is directly related to the high acute i.p. toxicity of yessotoxins.

Over the last years, other microalgal species which produce YTX and/or its analogues have been identified: in 1996, homoYTX was detected in a net haul sample from the Adriatic sea dominated by *Lingulodinium polyedrum* (Tubaro et al., 1998); YTX production by cultured strains of this species is however controversial (Boni et al., 2001; Paz et al., 2004). Recently, ELISA analyses on single cells indicated *Gonyaulax spinifera* (Claparède et Lachmann) Diesing 1866 from New Zealand as a YTX producer (Rhodes et al., 2006), thus correlating for the first time the occurrence of such microalga in seawater to mussel toxicity.

The north-western coasts of Adriatic sea, a commercially important area in Italy for covering about 90% of national mussels production, have long been subjected to recurring cases of harmful algae blooms and concomitant shellfish contamination. YTX and its analogues were detected for the first time in Adriatic mussels in 1995 (Ciminiello et al., 1997) and their presence was subsequently linked to the presence of *P. reticulatum* (Boni et al., 2001) in seawater. Since then, every year farmed molluscs have been found to be positive for YTXs for prolonged periods; this well matches both the presence of *P. reticulatum* in seawater and its toxin profile determined by liquid chromatography–mass spectrometry (LC–MS) (Ciminiello et al., 2003).

In Autumn 2004, the production of a number of mussel farms along Emilia-Romagna coasts (north-western Adriatic sea) was suspended by the Regional Competent Authority, due to YTXs levels exceeding the regulatory limit. Shellfish analyses were carried out by mouse bioassay, as official method in use (Gazzetta Ufficiale della Repubblica Italiana, 2002). The observed survival times and symptoms were consistent with those due to yessotoxins, whose presence was subsequently confirmed by HPLC-FLD and LC–MS analyses. A careful look at the phytoplankton composition brought to light that, during the mussel farms closure period, *P. reticulatum* and *L. polyedrum* were nearly absent in seawater whereas *G. spinifera* was present at increasing densities. This suggested that *G. spinifera* could be responsible for shellfish toxicity. The occurrence of YTXs in mussels associated with the presence of *G. spinifera* in seawater was observed also in late 2006.

In the present work, we report on qualitative and quantitative LC–MS analyses of batch cultures of *G. spinifera* from the Adriatic sea, as well as on the analyses of some toxic mussel samples collected during the *G. spinifera* bloom, in order to verify whether *G. spinifera* was the causative agent of mussel contamination. The 18S rDNA (SSU) and partial 28S rDNA (LSU) of *G. spinifera* were also characterized in order to compare the Adriatic *G. spinifera* with strains from different geographical areas.

2. Materials and methods

2.1. Chemicals

All organic solvents were of distilled-in-glass grade and were purchased from either VWR (Milan, Italy) or Carlo Erba (Milan, Italy). Water was distilled and passed through a MilliQ water purification system (Millipore Ltd., Bedford, MA, USA). Formic acid (95–97%, laboratory grade) and ammonium formate (AR grade) were purchased from Sigma–Aldrich (Steinheim, Germany); ammonium hydrate was purchased from Carlo Erba. Yessotoxin standard was purchased from the Institute of Environmental Science & Research Limited (Wellington Science Center, New Zealand) and used for external calibration in HPLC-FLD and LC–MS analyses. Individual standard solutions were obtained from pure