Harmful Algae 7 (2008) 782–797

Contents lists available at ScienceDirect

Harmful Algae

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

Morphogenetic diversity and biotoxin composition of *Alexandrium* (Dinophyceae) in Irish coastal waters

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ARTICLE INFO

Article history:
Received 7 August 2007
Received in revised form 27 March 2008
Accepted 8 April 2008

Keywords:
Alexandrium
HAB
PSP toxins
rDNA
Resting cysts
Spirolides

ABSTRACT

The diversity of *Alexandrium* spp. in Irish coastal waters was investigated through the morphological examination of resting cysts and vegetative cells, the determination of PSP toxin and spirolide profiles and the sequence analysis of rDNA genes. Six morphospecies were characterised: *A. tamarense*, *A. minutum*, *A. ostenfeldii*, *A. peruvianum*, *A. tamutum* and *A. andersoni*. Both PSP toxin producing and non-toxic strains of *A. tamarense* and *A. minutum* were observed. The average toxicities of toxic strains for both cultured species were respectively 11.3 (8.6 S.D.) and 2.3 (0.5 S.D.) pg STX equiv. cell^{-1}. *Alexandrium ostenfeldii* and *A. peruvianum* did not synthesise PSP toxins but HPLC–MS analysis of two strains showed distinct spirolide profiles. A cyst-derived culture of *A. peruvianum* from Lough Swilly mainly produced spirolides 13 desmethyl-C and 13 desmethyl-D whereas one of *A. ostenfeldii*, from Bantry Bay, produced spirolides C and D. Species identification was confirmed through the analyses of SSU, ITS1-5.8S-ITS2 and LSU rDNA genes. Some nucleotide variability was observed among clones of toxic strains of *A. tamarense*, which all clustered within the North American clade. However, rDNA sequencing did not allow discrimination between the toxic and non-toxic forms of *A. minutum*. Phylogenetic analysis also permitted the differentiation of *A. ostenfeldii* from *A. peruvianum*. Resting cysts of PSP toxin producing *Alexandrium* species were found in Cork Harbour and Belfast Lough, locations where shellfish contamination events have occurred in the past, highlighting the potential for the initiation of harmful blooms from cyst beds. The finding of supposedly non-toxic and biotoxin-producing *Alexandrium* species near aquaculture production sites will necessitate the use of reliable discriminative methods in phytoplankton monitoring.

1. Introduction

Harmful algal blooms (HABs) have become a rising issue worldwide for regulating and managing authorities as they represent a potential threat for human health and have been affecting the finfish and shellfish aquaculture industries (Hallegraeff, 1993; Hoagland et al., 2002). Many studies have reported human intoxications after consumption of contaminated shellfish (FAO, 2004) and have documented blooms of flagellates such as *Heterosigma*, *Chrysochromulina* and *Chattonella* as the causes of massive mortalities of caged finfish stocks (Chang et al., 1990; Edvardsen and Paasche, 1998; Hallegraeff et al., 1998). The increasing media coverage of harmful events has also amplified the public awareness of these issues by highlighting the potential socio-economical threats of temporary closures of production sites. Monitoring and research programmes have in response been implemented to estimate the contribution of physico-chemical and biological variables to proliferations of harmful algae (Andersen et al., 2003; Smayda, 2003). These have allowed a basic understanding of the biogeography and ecology of key harmful species and an estimate of their diversity through local field investigations (Usup et al., 2002b; McKenzie et al., 2004; Anderson et al., 2005).

Only a few phytoplankton species can reach high cell densities and synthesise dangerous substances towards humans (Smayda, 1997). Among these are some species from the genus *Alexandrium* which produce potent neurotoxins that are responsible for paralytic shellfish poisoning (PSP), an intoxication syndrome affecting mammals in general (Wright, 1995; Cembella, 1998). PSP events have been well documented and have sometimes resulted in human fatalities, such as those which occurred during recent incidents recorded in Alaska and Chile (Gessner et al., 1997; Garcia et al., 2004). The intoxication pattern is usually charac-

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doi:10.1016/j.hal.2008.04.001

terised by muscular paralysis and locomotion disorders; severe intoxications lead to death through respiratory paralysis and asphyxia (Kao, 1993). Structural derivatives of the saxitoxin molecule, a low molecular weight alkaloid, have been characterised and proved responsible for these intoxications. About 20 variants have been described which display distinct toxicities with the mouse bioassay (Luckas et al., 2003). Another type of biotoxin was recently discovered in *Alexandrium ostenfeldii* cell extracts. The species potentially synthesises saxitoxin derivatives but also bioactive macrocyclic imines known as spirolides (Cembella et al., 2000). These compounds seem to interact with muscarinic receptors in nervous cells and have displayed an acute neurotoxic activity (40 μg kg^{-1} i.p.) in the mouse model (Richard et al., 2000).

More than 27 species of *Alexandrium* have been described (Balech, 1995). A central concern has been the assessment of their biogeography to reveal how natural scattering, human assisted dispersion or developments of endemic populations have contributed to the apparent extent of their distribution (Hallegraeff, 2003). A better understanding of the lifecycles has also focused attention as for example, the significance during the bloom termination of transitions between vegetative cells, ecdysal and resting cysts, along with their respective contribution to dispersal mechanisms, are scarcely documented. The taxonomic diversity of *Alexandrium* species has mainly been assessed by microscopy through examination of morphological features such as size, shape and organisation of the plate tabulation of vegetative cells (Balech, 1995; Usup et al., 2002b). Many studies have aimed at developing other criteria to improve the discrimination of species and populations. Some authors have attempted to infer phylogenetic relationships according to toxin profiles, immunological properties or isozyme patterns (Cembella et al., 1987; Sako et al., 1990; Anderson et al., 1999). However, along with morphology, these are subject to phenotypic plasticity, whose expression can vary according to fluctuations of environmental conditions. Molecular biology techniques based on the variability of the rDNA gene have enabled a better characterisation of the phylogeny of the genus and permitted the partitioning of species assemblages into different clusters and species complexes (Hansen et al., 2003; Lilly et al., 2005). The most documented example is the morphological segregation of the 'tamarensis complex' into three different species (*A. tamarense*, *A. catenella* and *A. fundyense*) whereas the analysis of rDNA defines that complex as six different evolutionary lineages reflecting the geographic distribution of populations rather than their morphotype designations (Scholin et al., 1995; John et al., 2003a).

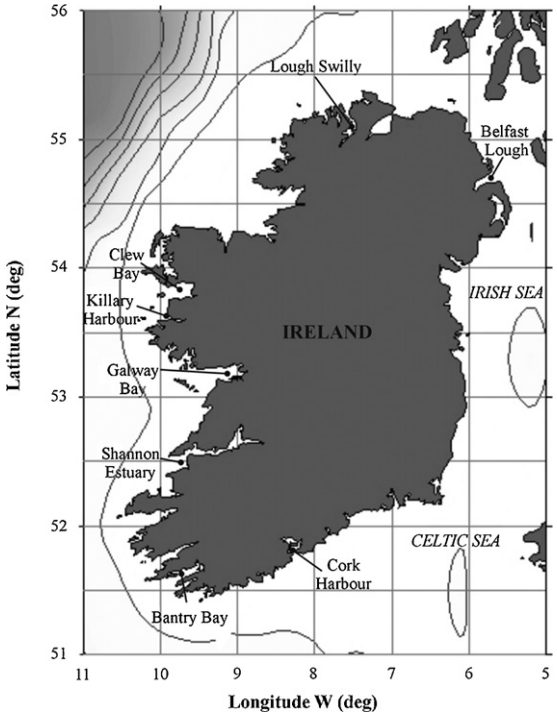

Fig. 1. Map of Ireland showing the geographical origin of collected *Alexandrium* spp. isolates and the locations referred to in the text.

In Europe, PSP events have mostly been associated with occurrences of *A. tamarense* and *A. minutum* (Franco et al., 1994; Higman et al., 2001). Increasingly, research is focusing on the assessment of the diversity within the genus and on the development of new detection and quantification methods (Galluzzi et al., 2004; John et al., 2005). For example, the presence of at least nine *Alexandrium* species in the Mediterranean Sea has now been confirmed (Maso et al., 2004). In Ireland, blooms of *Alexandrium* have been recorded along the south, west and northeast coasts of the island. The present study emphasises what is currently understood of the diversity of the genus in terms of morphogenetics and toxin composition. The main goal of the research was to provide a scientific base for the development of a comprehensive monitoring strategy for *Alexandrium* in Irish coastal waters.

2. Materials and methods

2.1. Isolation and germination of Alexandrium spp. resting cysts

Surface sediments were collected from locations along the Irish coastline (Fig. 1) and stored at 5 °C in the dark and under anoxic conditions. Samples were processed by sieving 1 cm^3 of wet weight sediment material through an 80 μm mesh filter and collecting it onto a 20 μm mesh filter. After backwashing into 15 ml tubes, the suspension was centrifuged (3000 × *g*, 10 min) and the supernatant carefully removed by aspiration. *Alexandrium* resting cysts were extracted using a density gradient, which was prepared by mixing pellets with 2 ml of sodium polytungstate (d~2) and by carefully adding 10 ml distilled water to allow the sedimentation of the material remaining on the tube wall. After centrifugation (700 × *g*, 10 min), the cyst-containing fractions located at the gradient interface were carefully removed, washed onto a 20 μm filter with 0.22 μm filter-sterilised seawater and finally backwashed into suitable containers for analysis.

Material was transferred into Utermöhl chambers, from which *Alexandrium* spp. cysts were isolated using an inverted microscope with a capillary micro-pipette. Each cyst was rinsed once with autoclaved GF/C-filtered seawater and placed into a well of a 96 well-plate containing 200 μl f/2 medium minus silicates (Guillard, 1975). Incubation conditions were a temperature of 15 °C, a photon flux density of 75 μmol m^{-2} s^{-1} and a 14:10 light/dark photoperiod cycle. Cyst germination was periodically checked and once achieved single cells were isolated and placed into different wells to generate mono-clonal cultures.

2.2. Isolation of vegetative cells and culturing

Vertical phytoplankton net hauls were collected onboard the *RV Celtic Voyager* in August 2003 along the west coast of Ireland (Fig. 1). Samples were passed through a 100 μm mesh filter to remove coarse material and stored unpreserved in culture bottles at 15 °C. Within 1 week, isolations of *Alexandrium* vegetative cells were performed with a capillary micro-pipette. Cells were individually placed in wells of a 96 well-plate as described with the cyst isolation method. After a few divisions, cell suspensions