Harmful Algae 55 (2016) 191–201

Contents lists available at ScienceDirect

Harmful Algae

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

Toxigenic algae and associated phycotoxins in two coastal embayments in the Ebro Delta (NW Mediterranean)

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ARTICLE INFO

Article history:
Received 21 August 2015
Received in revised form 24 February 2016
Accepted 29 February 2016
Available online 31 March 2016

Keywords:
HAB surveillance
Semi-enclosed embayment
Light microscopy
Quantitative PCR
LC–MS/MS
LSU rDNA

ABSTRACT

Harmful Algal Bloom (HAB) surveillance is complicated by high diversity of species and associated phycotoxins. Such species-level information on taxonomic affiliations and on cell abundance and toxin content is, however, crucial for effective monitoring, especially of aquaculture and fisheries areas. The aim addressed in this study was to determine putative HAB taxa and related phycotoxins in plankton from aquaculture sites in the Ebro Delta, NW Mediterranean. The comparative geographical distribution of potentially harmful plankton taxa was established by weekly field sampling throughout the water column during late spring–early summer over two years at key stations in Alfacs and Fangar embayments within the Ebro Delta. Core results included not only confirmed identification of HAB taxa that are common for the time period and geographical area, but also provided evidence of potentially new taxa. At least 25 HAB taxa were identified to species level, and an additional six genera were confirmed, by morphological criteria under light microscopy and/or by molecular genetics approaches involving qPCR and next generation DNA pyrosequencing. In particular, new insights were gained by the inclusion of molecular techniques, which focused attention on the HAB genera *Alexandrium*, *Karlodinium*, and *Pseudo-nitzschia*. Noteworthy is the discovery of *Azadinium* sp., a potentially new HAB species for this area, and *Gymnodinium catenatum* or *Gymnodinium impudicum* by means of light microscopy. In addition, significant amounts of the neurotoxin domoic acid (DA) were found for the first time in phytoplankton samples in the Ebro Delta. While the presence of the known DA-producing diatom genus *Pseudo-nitzschia* was confirmed in corresponding samples, the maximal toxin concentration did not coincide with highest cell abundances of the genus and the responsible species could not be identified. Combined findings of microscopic and molecular detection approaches underline the need for a synoptic strategy for HAB monitoring, which integrates the respective advantages and compensates for limitations of individual methods.

1. Introduction

Species-specific information is crucial for surveillance of harmful algal blooms (HABs), a proposition recently defended in a comprehensive review (see Pitcher, 2012 and article therein). Adequate responses to such events are complicated by a large diversity of harmful species, especially as algal communities known to be delineated within geographic areas are subject to changes. Both records of harmful algal blooms and toxigenic species are apparently increasing worldwide—a phenomenon linked to the Global Spreading Hypothesis (Hallegraeff, 1993). Reasons for this apparent expansion may include not only a naturally increasing scientific and cultural awareness about HABs, and discovery and description of new species, e.g., of the genus *Azadinium* as novel toxin producer Tillmann et al. (2009), but also the actual introduction of novel taxa and associated phycotoxins to new areas. Range expansion of HAB species is enhanced by multiple vectors, amongst these ballast water discharge from ship traffic and inadvertent introduction via transfer of aquaculture stocks.

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http://dx.doi.org/10.1016/j.hal.2016.02.012
1568-9883/

Up-to-date description of species biogeography is important for both the interpretation of distributional patterns of taxa from time-series data (of which few exist), as well as formulation of management actions to react to or prevent adverse effects on human health, especially in coastal zones linked to tourism and aquaculture activities. As such, two embayments of the Ebro Delta undergo long-term routine monitoring to ensure seafood safety for the major Catalonian shellfish cultivation zones. In Alfacs and Fangar bays, the Mediterranean mussel *Mytilus galloprovincialis* is cultured on wooden fixed constructions (Ramón et al., 2007). Due to harvest closures dictated by the detection of toxins above the regulatory limit in shellfish flesh or the mere presence of toxic species above critical cell abundances, the aquaculture industry faces financial penalties. High levels of paralytic shellfish poisoning (PSP) toxins, produced by the dinoflagellate *Alexandrium minutum*, as well as of diarrhetic shellfish poisoning (DSP) toxins, attributed to members of the dinoflagellate genus *Dinophysis* (Fernández-Tejedor et al., 2008, 2010) are the major reasons for these biotoxin closures. Other dinoflagellates known to produce lipophilic phycotoxins have also been detected in both bays, including *Protoceratium reticulatum*, a possible source of yessotoxins in Ebro Delta shellfish (Diogène et al., 2008), *Lingulodinium polyedrum* and *Prorocentrum lima*. In addition, high biomass blooms dominated by the ichthyotoxic dinoflagellates *Karlodinium armiger* and *Karlodinium veneficum* (formerly known collectively as *Gyrodinium corsicum* in this area, compare Garcés et al., 2006) has led to severe fish-kills in Alfacs Bay and land-based aquaculture facilities (Fernández-Tejedor et al., 2007). In Fangar Bay, no high cell abundances were detected until June 2010 (Busch et al., 2012; Fernández-Tejedor et al., 2010; Llebot et al., 2011). The putative fish-killing agents known as karlotoxins have been identified for *K. veneficum* (Place et al., 2012) but not for *K. armiger*.

Since the inclusion of monitoring for the phycotoxin domoic acid (DA) in routine shellfish toxin sampling in 2001, DA was not detected in the area until 2008–2011, during which DA was found in several harvested mollusc species from production sites along the Catalan coast, including the Ebro embayments (Giménez Papiol et al., 2013). In a few samples, DA even exceeded regulatory limits of 20 mg kg^{-1} shellfish flesh. As yet, the origin of the toxin has not been attributed to a known producer species, despite high cell abundances of the diatom genus *Pseudo-nitzschia*. Nor could the presence of DA in shellfish be clearly related to high cell abundances of this diatom, as the toxin was not detected in *Pseudo-nitzschia*-rich plankton samples (or only at low levels of maximum 0.05 pg $cell^{-1}$; Fernández-Tejedor, pers. comm.). During the past 50 years, as abundances of this diatom have been increasing in the Ebro Delta area (Fernández-Tejedor et al., 2010), knowledge on its distribution and toxin production is becoming increasingly important.

Besides monitoring efforts, detailed targeted studies of cell morphology by electron microscopy, and molecular diversity with genetic probes, have been conducted for a few HAB taxa in the Ebro Delta. For example, such techniques aided in the discrimination of *Pseudo-nitzschia* populations and cultured isolates at the species level over time (Andree et al., 2011; Quijano-Scheggia et al., 2008). Nevertheless, analyses of field samples were based mainly on integrated samples throughout the water column or on a crude distinction between surface and bottom samples. As species of *Pseudo-nitzschia* are known to form thin layers of concentrated cells in the water column (Rines et al., 2002) more detailed insights can be expected by a depth resolved sampling method. Furthermore, molecular screening in this region has only been conducted for specific target genera, and therefore may not include newly introduced or overlooked taxa that are not clearly distinguishable by light microscopy.

Accordingly, the overarching objectives of the current study were to contribute to defining and describing the distribution of harmful planktonic algae and toxins, as well as applying and testing traditional and new observational methods. These objectives were achieved by identification and assignment of HAB taxa and toxins in corresponding samples over the entire water column and with high depth resolution (0.5 m) at two key stations in Alfacs and Fangar bays in the Ebro Delta. Besides novel findings on species of HAB genera known to the area, in particular on *Karlodinium* and *Pseudo-nitzschia* species, emphasis was set upon the identification of cryptic (or previously absent) but potentially harmful emerging species. A number of alternative approaches for surveillance of HAB events were integrated to confirm the presence of HAB taxa that are not routinely resolved by light microscopy or targeted molecular methods.

2. Material and methods

2.1. Location and field sampling of the study sites

Water samples for all analyses were collected during weekly sampling excursions (n = 30) from May to July in 2010 and 2011 at two key stations within the Ebro Delta system of the NW Mediterranean (Fig. 1). In both embayments, Alfacs (40.620083°N, 0.658167°E) and Fangar (40.778767°N, 0.749233°E) bays, the water column was sampled from surface to bottom at 0.5 m intervals for taxon and toxin analysis, with a submersible pumping system (weighted hose) from a small vessel. Sub-samples (100 mL) were collected directly into screw-cap plastic vials and fixed with neutral-buffered Lugol's iodine solution for identification and enumeration of phytoplankton by light microscopy. Water for high-throughput DNA sequencing was collected in 1 L plastic bottles, whereas samples for polymerase chain reaction (PCR) analysis were collected in 50 mL plastic centrifuge tubes, and immediately fixed with neutral Lugol's solution. Plankton for toxin analysis was obtained directly from the on-board pumping system; water volume (1 L in 2010 and 2 L in 2011) was gently transferred to a filter tower sealed at one end with 10 μm Nitex gauze mesh. The retentate on the gauze was re-suspended and washed off the filter with 0.2 μm-filtered seawater and collected in a 50 mL plastic centrifuge tube. All samples on board were stored and transported at <10 °C and under dark conditions and then processed immediately in the laboratory or archived within several hours for future processing.

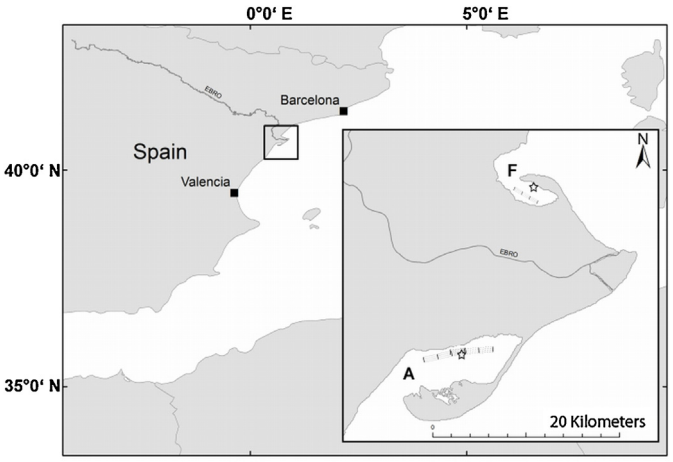

Fig. 1. Location of the study sites, Alfacs Bay (A) and Fangar Bay (F), in the Ebro Delta on the Catalan coast (NW Mediterranean). Key sampling stations in both embayments are marked with a star. Shellfish aquaculture of the Mediterranean mussel *Mytilus galloprovincialis* is conducted on fixed constructions in both bays (dashed lines).