Harmful Algae 80 (2018) 15–34

Contents lists available at ScienceDirect

Harmful Algae

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

Diversity, distribution, and azaspiracids of Amphidomataceae (Dinophyceae) along the Norwegian coast

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ARTICLE INFO

Keywords:
Azadinium
Amphidoma
Azaspiracids
Norway
New strains
New toxins

ABSTRACT

Azaspiracids (AZA) are a group of lipophilic polyether compounds which have been implicated in shellfish poisoning incidents around Europe. They are produced by a few species of the dinophycean genera *Azadinium* and *Amphidoma* (Amphidomataceae). The presence of AZA toxins in Norway is well documented, but knowledge of the distribution and diversity of *Azadinium* and other Amphidomataceae along the Norwegian coast is rather limited and poorly documented. On a research survey along the Norwegian coast in 2015 from the Skagerrak in the South to Trondheimsfjorden in the North, plankton samples from 67 stations were analysed for the presence of *Azadinium* and *Amphidoma* and their respective AZA by on-board live microscopy, real-time PCR assays specific for Amphidomataceae, and liquid chromatography-tandem mass spectrometry (LC–MS/MS).

Microscopy using live samples and positive real-time PCR assays using a general family probe and two species specific probes revealed the presence of Amphidomataceae distributed throughout the sampling area. Overall abundance was low, however, and was in agreement with a lack of detectable AZA in plankton samples. Single cell isolation and morphological and molecular characterisation of established strains revealed the presence of 7 amphidomatacean species (*Azadiniun spinosum*, *Az. poporum*, *Az. obesum*, *Az. dalianense*, *Az. trinitatum*, *Az. polongum*, *Amphidoma languida*) in the area. Azaspiracids were produced by the known AZA producing species *Az. spinosum*, *Az. poporum* and *Am. languida* only. LC–MS/MS analysis further revealed that Norwegian strains produce previously unreported AZA for Norway (AZA-11 by *Az. spinosum*, AZA-37 by *Az. poporum*, AZA-38 and AZA-39 by *Am. languida*), and also four novel compounds (AZA-50, -51 by *Az. spinosum*, AZA-52, -53 by *Am. languida*), whose structural properties are described and which now can be included in existing analytical protocols. A maximum likelihood analysis of concatenated rDNA regions (SSU, ITS1-ITS2, partial LSU) showed that the strains of *Az. spinosum* fell in two well supported clades, where most but not all new Norwegian strains formed the new Ribotype B. Ribotype differentiation was supported by a minor morphological difference with respect to the presence/absence of a rim around the pore plate, and was consistently reflected by different AZA profiles. Strains of *Az. spinosum* from ribotype A produce AZA-1, -2 and -33, whereas the new strains of ribotype B produce mainly AZA-11 and AZA-51. Significant sequence differences between both *Az. spinosum* ribotypes underline the need to redesign the currently used qPCR probes in order to detect all AZA producing *Az. spinosum*.

The results generally underline the conclusion that for the Norwegian coast area it is important that amphidomatacean species are taken into account in future studies and monitoring programs.

1. Introduction

Norway has one of the longest coastlines in the world and the sea is a key source of income and an important part of the national culture. Norwegian seafood exports have always been important for the country's economy, and - next to oil and gas - seafood is the most important exported product with a $ 6.4 billion (7.2% of total export) in 2016 (source: https://atlas.media.mit.edu/de/resources/about/). In addition

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https://doi.org/10.1016/j.hal.2018.08.011
Received 11 July 2018; Received in revised form 27 August 2018; Accepted 30 August 2018

to wild fisheries, a rapidly growing aquaculture industry is gaining an increasing importance. Salmon, rainbow trout, and several marine finfish (cod, halibut) are by far the most important farmed species, but shellfish farming (blue mussels, oysters) is of increasing importance. A major threat to the growing aquaculture and shellfish industries are marine biotoxins produced by harmful algal species, which can cause large fish kills or which accumulate in marine animals creating potential health problems in human seafood consumers.

Regular or sporadic toxic microalgal blooms in Norwegian waters are mainly formed by dinophytes, haptophytes, or dictyochophytes. Among haptophytes, the ichthyotoxic species *Prymnesium parvum* frequently has caused recurrent fish kills in fjords of the west coast of Norway (Johnsen and Lein, 1989; Larsen et al., 1993), and the vast bloom of *Prymnesium polylepis* (as *Chrysochromulina polylepis*) in 1988 was an outstanding and devastating event for marine life all along the Skagerrak and the Norwegian coast (Dundas et al., 1989). Since the late 1990s, massive fish-killing blooms formed by members of the dictyochophyte genus *Pseudochattonella* recur along the Scandinavian coasts (Edvardsen et al., 2007; Jakobsen et al., 2012). The dinophyte *Karenia mikimotoi* caused significant blooms and mortality of farmed salmon mainly in the 1970s–1980s (Dahl and Tangen, 1993). Another concern for Norway are species of *Dinophysis* which are the cause of diarrhetic shellfish poisoning (DSP). In Norwegian blue mussels DSP has been detected annually since 1984 (Underdahl et al., 1985; Naustvoll et al., 2012). Elevated abundance of *Dinophysis* spp. in Norway severely affect shellfish production, and on the south and west coast of Norway harvesting of mussels due to elevated toxin content can be prevented for several months each year (Aune et al., 1996; Dahl and Johannessen, 2001). In addition, significant shellfish levels of paralytic shellfish poisoning toxins (PSP toxins) produced by species of *Alexandrium* have been observed since the 1980s in Norway (Tangen, 1983). The yessotoxins, produced by the dinoflagellate *Protoceratium reticulatum*, have been responsible for numerous closures of Norwegian commercial shellfisheries (Aasen et al., 2005) since their first identification in 1988 (Lee et al., 1988). Its toxicity to humans, however, is presently uncertain (Tubaro et al., 2010).

Driven by the significant economic losses, strategies on tackling and mitigating the threat and problems from harmful algae have been on the agenda in Norway since the 1980s (Dahl and Tangen, 1999). Among other aspects, the regular monitoring of algae and rapid distribution of this information to the aquaculture industry and public has been identified and have led to the establishment of a commendable monitoring system along the whole Norwegian coast (see http://algaeinfo.imr.no).

One of the more recently discovered algal toxins are azaspiracids (AZA), a group of lipophilic polyketides that can accumulate in seafood and upon human consumption can cause severe gastrointestinal health problems (azaspiracid shellfish poisoning; AZP) (Twiner et al., 2014). Azaspiracids were first recognized in the 1990s after an outbreak of human illness in the Netherlands following the consumption of blue mussels originating from Killary Harbour, Ireland (McMahon and Silke, 1996). Since the structural elucidation of the compound (Satake et al., 1998), elevated AZA levels in shellfish above the EU regulatory level (0.16 mg kg^{-1} mussel meat) has been a recurrent and major problem in Ireland (Salas et al., 2011). The planktonic source of the toxins was first identified in 2007 when the small thecate dinoflagellate *Azadinium spinosum* was described as a new species and as the first primary source of AZA (Tillmann et al., 2009). Since that time, a number of subsequent studies revealed a high biodiversity within the genus *Azadinium,* with 12 new species isolated and characterized to date (Tillmann and Akselman, 2016; Luo et al., 2017). Moreover, morphological and molecular data for a new species of the genus *Amphidoma*, *Am. languida*, revealed a sister group relationship between *Azadinium* and *Amphidoma* (Tillmann et al., 2012a). Toxin production is now known for some but not all species in both genera, with AZA detected in strains of *Az. spinosum* (Tillmann et al., 2009), *Az. poporum* (Krock et al., 2012), *Az. dexteroporum* (Rossi et al., 2017), and *Am. languida* (Krock et al., 2012).

Although Ireland seems to be most seriously affected by AZA contamination events, the compounds have been documented over the last decade in shellfish from numerous geographical sites around the globe (James et al., 2002; Taleb et al., 2006; Vale et al., 2008; Álvarez et al., 2010; Yao et al., 2010; Trainer et al., 2013; Massucatto et al., 2014; Turner and Goya, 2015; Smith et al., 2016; Blanco et al., 2017). In recent years, AZA are now known to also be present and problematic in Norway (James et al., 2002). James et al. (2002) detected the first occurrence of AZA-1, -2 and -3 in blue mussels collected in 1998 in Sognefjorden, western Norway. Moreover, AZA were detected in Norwegian brown crabs collected along the north and north-west coast of Norway (Torgersen et al., 2008). Although the majority of samples were below the regulatory limit, levels up to 733 µg kg^{-1} meat were observed, underlining the necessity of surveillance for AZA along the Norwegian coast. This was emphasized in 2002/2003 by the first closures of mussel farming due to the presence of AZA above the regulatory limit in blue mussels, covering the entire south coast of Norway up to the area around Sognefjorden and two locations at the most northern part of the country (Aasen et al., 2006). In 2005, AZA were detected in water samples from southern Norway at Flødevigen by the solid phase adsorption toxin tracking (SPATT) technique, and levels of AZA of 20–50 µg kg^{-1} were recorded at the same site in blue mussels (Rundberget et al., 2009). Azaspiracid-1 was present in plankton samples collected in June 2007 in the Skagerrak area and at all stations off the Southern Norwegian coast (Krock et al., 2009).

Whereas the presence of AZA toxins in Norway is thus well documented, knowledge of the distribution and diversity of *Azadinium* and other Amphidomataceae along the Norwegian coast is rather limited and poorly documented. The species *Azadinium caudatum* (as *Amphidoma caudata*) was described from the Norwegian Sea (Halldal, 1953), and this species is known to be common and fairly numerous along the Norwegian coast in some years (Throndsen et al., 2007), but based on a single strain investigated so far this is a non-toxigenic species (Tillmann et al., 2014b). One strain of *Azadinium* isolated in 2010 from the Oslofjorden has been identified as *Az. spinosum* based on SSU rDNA sequence and morphology viewed in the electron microscope (Ota & Edvardsen pers. comm.). Although a whole suite of other molecular markers (HSP90, COB, COX, etc.) are available for that strain (Orr et al., 2012), its morphology or AZA production potential has not been reported.

For a sound monitoring system targeting AZA, more detailed information on the species diversity and the toxin profile of local populations of Amphidomataceae is needed. Monitoring and surveillance of the AZP risk potential with the need to unambiguously identify and quantify the source organisms of AZA is challenging and hindered by a number of facts. First, within Amphidomataceae there is a high diversity of very morphologically similar species. Species of *Azadinium* are generally small, inconspicuous, and thus difficult to detect and identify by regular light microscopy. Moreover, the co-existence of both toxigenic and non-toxigenic species in the same area has been repeatedly documented (Tillmann et al., 2010, 2012b; Kim et al., 2017). Amphidomataceae are thus a good example for the necessity of applying molecular detection methods in monitoring and early warning systems. A general molecular probe detecting all Amphidomataceae is available (Smith et al., 2016). Moreover, species specific molecular probes have been developed for the first three described species, *Az. spinosum*, *Az. poporum*, and *Az. obesum* (Toebe et al., 2013), but many new species and strains potentially affecting specificity and cross-reactivity have been found since then and it is likely that the diversity of the group is not yet fully explored. A significant number of described species (mainly of *Amphidoma* spp.) have not yet been cultured and analysed for their AZA production potential. Establishment of new strains has revealed a wide diversity in AZA profiles at least for some species such as *Az. poporum* (Krock et al., 2014) and *Am. languida* (Tillmann et al., 2017a) so that today more than 30 AZA analogues of