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Research Paper

Identification of *Azadinium* species and a new azaspiracid from *Azadinium poporum* in Puget Sound, Washington State, USA

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ABSTRACT

The identification of a new suite of toxins, called azaspiracids (AZA), as the cause of human illnesses after the consumption of shellfish from the Irish west coast in 1995, resulted in interest in understanding the global distribution of these toxins and of species of the small dinoflagellate genus *Azadinium*, known to produce them. Clonal isolates of four species of *Azadinium, A. poporum, A. cuneatum, A. obesum* and *A. dalianense* were obtained from incubated sediment samples collected from Puget Sound, Washington State in 2016. These *Azadinium* species were identified using morphological characteristics confirmed by molecular phylogeny. Whereas AZA could not be detected in any strains of *A. obesum, A. cuneatum* and *A. dalianense*, all four strains of *A. poporum* produced a new azaspiracid toxin, based on LC–MS analysis, named AZA-59. The presence of AZA-59 was confirmed at low levels *in situ* using a solid phase resin deployed at several stations along the coastlines of Puget Sound. Using a combination of molecular methods for species detection and solid phase resin deployment to target shellfish monitoring of toxin at high-risk sites, the risk of azaspiracid shellfish poisoning can be minimized.

1. Introduction

In November 1995, a shellfish poisoning event of unknown etiology occurred after several people consumed cultivated mussels (*Mytilus edulis*) from the Irish west coast (McMahon and Silke, 1996). Their symptoms were similar to diarrhetic shellfish poisoning (DSP) and included nausea, vomiting, severe diarrhea and stomach cramps. Diarrhetic shellfish toxins (DSTs; okadaic acid and dinophysistoxin-2) were present at concentrations below the regulatory limit, and thus were less likely to have caused the severe intoxications. Soon after this poisoning event, the first member of a novel group of marine biotoxins, designated as azaspiracids (AZAs), was isolated and characterized from shellfish and named AZA-1 (Satake et al., 1998).

It took over a decade to positively identify a small dinoflagellate (<20 μm) *Azadinium spinosum* from the North Sea on the Scottish East Coast as a source organism for AZA (Tillmann et al., 2009). Since that time, 12 new species within the genus *Azadinium* have been isolated and characterized (Tillmann and Akselman, 2016; Luo et al., 2017). As the study of *Azadinium* intensifies, several species of this genus have been recorded in many countries including Europe (Tillmann et al., 2009, 2012b, 2014a; Percopo et al., 2013), East Asia (Potvin et al., 2012; Gu et al., 2013; Luo et al., 2013), New Zealand (Smith et al., 2015) and Central and South America (Luo et al., 2016; Tillmann et al., 2016, 2017b). Likewise, there are several reports of contamination with AZAs in shellfish from Ireland (Salas et al., 2011), the east coast of England and the west coast of Norway (James et al., 2002), Portugal (Vale et al., 2008), Morocco (Taleb et al., 2006), Canada (M. Quilliam, pers. comm.), Chile (López-Rivera et al., 2010), Japan (Ueoka et al., 2009; reported in a sponge) and China (Yao et al., 2010). Therefore, the distribution of *Azadinium* is a topic of great interest.

To date, the only *Azadinium* species known to produce AZA are *A. spinosum*, *A. poporum* and *A. dexteroporum,* and one species of the closely related genus *Amphidoma*, *Am. languida*, has also been shown to produce AZA (Krock et al., 2012; Tillmann et al., 2017a, 2012a). For many of the species one or few strains have been

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obtained and tested, and thus it is not totally clear if and to what extent toxin production is a stable species-specific trait. For *A. poporum* and *Am. languida*, different strains of the same species may show different toxin profiles (Krock et al., 2014; Tillmann et al., 2017a). Moreover, a few non-toxigenic strains have been described for *A. poporum* (Krock et al., 2014), and a subarctic strain of *A. dexteroporum* lack AZA (Tillmann et al., 2015), whereas the Mediterranean type culture of the species produce a number of different AZA (Rossi et al., 2017).

Azaspiracid-1 is known to be toxic to human cell lines such as B lymphocyte, embryonic kidney, lung ephithelial, monocyte (Twiner et al., 2005), breast cancer (Ronzitti et al., 2007), hepatoblastoma, bladder carcinoma (Flanagan et al., 2001), neuroblastoma (Vilariño et al., 2006) and T lymphocyte (Twiner et al., 2008). AZA-2 has been shown to have very similar toxic activity as AZA-1 in terms of cytotoxicity and cytoskeleton alterations (Vilariño et al., 2008). There are few studies about the toxicity of other AZA analogues (Kilcoyne et al., 2014, 2015; Krock et al., 2015) and all of them had cytotoxic effect on Jurkat T lymphocyte cells being either higher or lower in cytotoxicity compared to AZA-1.

In the USA, diarrhetic shellfish poisoning was first confirmed in June 2011 in 3 people who ate contaminated mussels collected from a public dock in Sequim Bay, Washington (Lloyd et al., 2013; Trainer et al., 2013). Since that time, the Washington State Department of Health has reported over 100 closures of commercial and recreational shellfish harvesting sites annually resulting from shellfish contamination by diarrhetic shellfish toxins (DTX), mostly DTX-1. Anecdotal reports from consumers having DSP-like symptoms after eating Puget Sound shellfish from sites with no DTX or *Vibrio* have caused managers to suspect that additional toxins, such as AZAs, were the cause. Therefore, this study attempted to determine whether *Azadinium* species were present in Puget Sound as a first step to estimating the risk for azaspiracid shellfish poisoning in the region.

In the present study, *Azadinium* spp. were isolated from sediment samples collected in Puget Sound, Washington State. Several *Azadinium* species were positively identified, and their morphology was examined using light and electron microscopy. A new azaspiracid toxin was identified from local *A. poporum* strains and its presence was confirmed at low levels *in situ* using a solid phase resin deployed at several sites along the coastlines of Puget Sound.

2. Materials and methods

2.1. Sediment sampling

Sediment samples were collected from 15 stations in Puget Sound (Fig. 1) in January and February 2016 using a hydraulically dampened Craib corer (Craib, 1965) with a diameter of 6.2 cm or, for some of the stations, using a Van Veen grab. In the case of Dabob Bay, Quilcene Bay and Sequim Bay, samples were obtained at several stations in each bay considering the hydrographical characteristics (e.g., inner/middle/outer stations, edge/central stations). Sediment from the upper 0–1 cm of the core or grab samples was stored in the dark at 4 °C until analysis.

2.2. Detection of Azadinium in sediment samples

2.2.1. DNA extraction from sediment

One gram of sediment was transferred to 15 mL conical tubes and diluted in 10 mL of seawater that had been passed through a 25 mm filter (Whatman GF/F; nominal pore size 0.7 μm). DNA debris remaining in sediment particles was removed to prevent overestimation the result using quantitative real-time polymerase chain reaction (qPCR) analysis. To remove DNA debris, a heating and dilution method was applied (Kim et al., 2015). Briefly, 2 mL of sediment suspension were transferred to 2 mL microfuge tubes and then centrifuged (2,000*g* for 7 min). The pellets were resuspended with 1 mL double distilled water to dilute salts. The suspensions were incubated at 75 °C in a water bath for 10 min and mixed 2–3 × during incubation by inverting tubes. Samples were centrifuged (2,000*g* for 7 min) and supernatants were removed by pipetting. Sediment pellets were mixed with beads and lysis buffer from the PowerSoil® DNA Isolation Kit (MoBio,

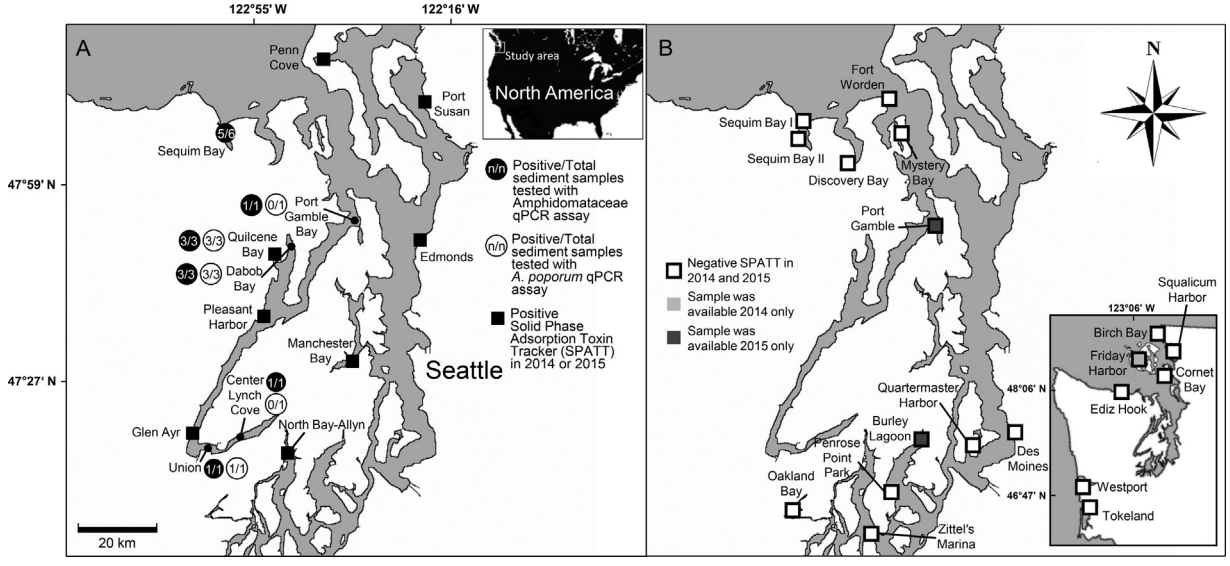

Fig. 1. Sediment sampling stations (2016) for qPCR assay and locations of Solid Phase Adsorption Toxin Tracking (SPATT) sampler deployment (2014–2015). (A) Locations where sediment samples were collected (black circles) and where AZA-59 was detected in SPATT resin (black squares) in 2014 or 2015. Numbers in circles show numbers of positive stations and total stations tested in qPCR assay using probes for Amphidomataceae (black) and *Azadinium poporum* (white). Inset shows the study area. (B) Locations where AZA-59 was not detected in SPATT in both 2014 and 2015 (white squares) with three exception stations (grey and dark grey squares). Inset shows all locations in northern Puget Sound, the Strait of Juan de Fuca and the Washington outer coast.