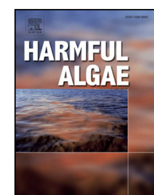
Harmful Algae 98 (2020) 101874

Contents lists available at ScienceDirect

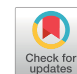
Harmful Algae

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



Original Article

Temporal and spatial distribution of *Azadinium* species in the inland and coastal waters of the Pacific northwest in 2014–2018



Nicolaus G. Adams[a,*], Urban Tillmann[b], Vera L. Trainer[a]

[a] *Environmental and Fisheries Science Division, Northwest Fisheries Science Center, National Marine Fisheries Service, National Oceanic and Atmospheric Administration, 2725 Montlake Blvd. E., Seattle, WA 98112, USA*
[b] *Alfred Wegener Institute for Polar and Marine Research, Am Handelshafen 12 D-27570 Bremerhaven, Germany*

ARTICLE INFO

Keywords:
Azadinium, Azaspiracid
qPCR
AZA
AZP

ABSTRACT

Azaspiracids, produced by some species of the dinoflagellate genera *Azadinium* and *Amphidoma*, can cause a syndrome in humans called azaspiracid shellfish poisoning (AZP). In 1995, mussels from the Irish west coast contaminated with azaspiracids were, for the first time, linked to this human illness that has symptoms of nausea, vomiting, severe diarrhea, and stomach cramps. The only confirmed cases of AZP to date in the United States occurred in Washington State in 2008 from mussels imported from Ireland. Shortly after this case, several others involving similar gastrointestinal symptoms were reported by shellfish consumers from Washington State. However, no detectable diarrhetic shellfish toxins or *Vibrio* contamination were found. Cursory analysis of Solid Phase Adsorption Toxin Tracking (SPATT) samplers suggested the presence of azaspiracids in Washington State waters and motivated a study to evaluate the presence and distribution of *Azadinium* species in the region. During the spring and summer months of 2014–2015, quantitative polymerase chain reaction (qPCR) analyses detected the presence of the toxigenic species *Azadinium poporum* and *A. spinosum* on the outer coast and throughout the inland waters of Washington State. In 2016–2018, standard curves developed using *A. poporum* isolated from Puget Sound and *A. spinosum* isolated from the North Sea were used to quantify abundances of up to 10,525 cells L^{-1} of *A. poporum* and 156 cells L^{-1} of *A. spinosum* at shore-based sites. Abundances up to 1,206 cells L^{-1} of *A. poporum* and 30 cells L^{-1} of *A. spinosum* were measured in the coastal waters of the Pacific Northwest in 2017. Other harmful genera, including *Alexandrium, Dinophysis*, and *Pseudo-nitzschia*, were observed using light microscopy at coastal sites where *A. poporum* was also observed. In some samples where both *A. poporum* and *A. spinosum* were absent, an Amphidomataceae-specific qPCR assay indicated that other species of *Azadinium* or *Amphidoma* were present. The identification of *Azadinium* species in the PNW demonstrates the need to assess their toxicity and to incorporate their routine detection in monitoring programs to aid resource managers in mitigating risks to azaspiracid shellfish poisoning in this region.

1. Introduction

Harmful algal events in the Pacific Northwest (PNW) resulting from paralytic shellfish toxins and domoic acid produced by species of *Alexandrium* and *Pseudo-nitzschia*, respectively, have been well documented (Trainer et al., 2003; Adams et al., 2006; Bill et al., 2006; Lewitus et al., 2012; Trainer et al., 2012; Hickey et al., 2013). However in Washington State in 2009 and 2011, humans became ill with diarrhetic shellfish poisoning (DSP)-like symptoms after eating locally harvested shellfish (Trainer et al., 2013). The 2011 event was eventually linked to diarrhetic shellfish toxins (Lloyd et al., 2013) but the shellfish from the 2009 incident did not contain marine toxins that were routinely monitored in the State. These results suggested the 2009 illnesses were due to the presence of a previously unobserved class of toxins that were present in local shellfish. Azaspiracids (AZAs) are toxins that can cause symptoms similar to those of DSP including nausea, vomiting, stomach cramps, and diarrhea in human consumers of shellfish (Satake et al., 1998; Twiner et al., 2008) and are produced by some species of the dinoflagellate genera *Azadinium* and *Amphidoma* from the family Amphidomataceae (Tillmann, 2018). Azaspiracids were responsible for illnesses suffered by individuals with these DSP-like symptoms in Washington State after they consumed mussels imported from Ireland in 2008 (Klontz et al., 2009) and were therefore the toxins suspected in the undiagnosed illnesses in 2009.

Azaspiracids were first identified in 1998 following a poisoning event in the Netherlands in 1995 where people became ill with DSP-like

* Corresponding author.
E-mail address: Nicolaus.Adams@noaa.gov (N.G. Adams).

https://doi.org/10.1016/j.hal.2020.101874
Received 7 February 2020; Received in revised form 20 July 2020; Accepted 22 July 2020
1568-9883/ Published by Elsevier B.V.

symptoms after consuming mussels harvested in Ireland (Satake et al., 1998). However, the shellfish they consumed contained DSP toxins at concentrations that were not sufficient to cause the illnesses. Following the characterization of AZAs in the Irish shellfish from the 1995 incident in the Netherlands (Ofuji et al., 1999), the toxins have been detected in shellfish from Great Britain (Dhanji-Rapkova et al., 2019), continental Europe (James et al., 2002; Braña Magdalena et al., 2003; Vale et al., 2008; Blanco et al., 2017), northwest Africa (Taleb et al., 2006), China (Yao et al., 2010), Chile (Alvarez et al., 2010), and Argentina (Turner and Goya, 2015), although often at low concentrations. Low concentrations of AZA-1 and AZA-2, as well as associated shellfish metabolites, have also been observed in shellfish in eastern Canada but have not yet been detected in shellfish from western Canada (Wade Rourke, personal communication). While the cases of azaspiracid shellfish poisoning (AZP) in the United States were not due to toxins from local shellfish, AZAs have been detected in the USA. Luo et al. (2016) detected AZA-2 in a culture of *Azadinium poporum* Tillmann & Elbrächter from the US Gulf of Mexico and Kim et al. (2017) found a novel azaspiracid, AZA-59, in *A. poporum* cultures and in Solid Phase Adsorption Toxin Tracking (SPATT) samplers from Washington State.

To date, >60 AZA analogues have been identified with 26 confirmed to be produced directly by Amphidomataceae while others are shellfish metabolites (Krock et al., 2019). Those AZAs that have been tested can have varying levels of toxicity (Hess et al., 2014; Twiner et al., 2014). Both the European Union (EU) and the U.S. regulate AZAs in harvested shellfish with an action level of 160 μg kg^{-1} shellfish meat (European Commission, 2002; U.S. Food and Drug Administration, 2019) but only AZA-1, −2, and −3 are quantified for regulatory purposes (Van Egmond et al., 2004; Kilcoyne et al., 2014b). However, azaspiracids are not routinely monitored in the United States as these toxins have not yet been found in any commercially harvested products.

In 2007, *Azadinium spinosum* Elbrächter & Tillmann was identified as the first organism capable of producing AZAs (Tillmann et al., 2009). To date, 14 species of *Azadinium* have been described. In addition to *A. spinosum*, two other species of *Azadinium, A. poporum* and *Azadinium dexteroporum* Percopo & Zingone, have been shown to produce AZAs (Gu et al., 2013; Percopo et al., 2013; Rossi et al., 2017), and AZA production is also known in the closely related Amphidomataceae species *Amphidoma languida* Tillmann, Salas & Elbrächter (Krock et al., 2012; Tillmann et al., 2017a). Until recently, *A. spinosum* was considered to have a consistent AZA profile (i.e. all strains produced the same AZA analogues), while the AZA profile in *A. poporum* was variable (Tillmann et al., 2014b). However, Tillmann et al. (2018) subsequently found strains of *A. spinosum* from Norwegian waters with variable toxin profiles. Some toxin profile variability in *A. poporum* may be related to geographic origin (Tillmann et al., 2017c), however, individual strains of *A. poporum* collected from the same area have also been shown to contain different AZA profiles (Gu et al., 2013).

Since their initial identification, *Azadinium* and *Amphidoma* spp. have been observed around the world, including in the North Atlantic (Tillmann et al., 2009, 2010; Nezan et al., 2012; Tillmann et al., 2012, 2014a, 2018; Wietkamp et al., 2019), Mediterranean (Percopo et al., 2013), Western Pacific (Potvin et al., 2012; Gu et al., 2013), Eastern North Pacific (Hernandez-Becerril et al., 2012; Kim et al., 2017), eastern South Pacific (Tillmann et al., 2017b, 2017c) and eastern South Atlantic (Akselman and Negri, 2012; Akselman et al., 2014; Tillmann and Akselman, 2016; Cavalcante et al., 2018; Tillmann, 2018). The small size of *Azadinium* makes their detection challenging using light microscopy. In contrast, molecular methods have been used to reliably detect and enumerate Amphidomataceae, in particular the azaspiracid-producing *A. poporum, A. spinosum* (Toebe et al., 2013) and *Amphidoma languida* (Wietkamp et al., 2019).

While *A. poporum* and *A. spinosum* have been detected in many regions, few studies have reported abundances of these organisms. Routine monitoring in Ireland using the real-time quantitative PCR (qPCR) assay of Toebe et al. (2013) found abundances of *A. spinosum* at Killary Harbor that ranged from 17 cells L^{-1} to 7.32 × 10^4 cells L^{-1} with associated azaspiracid concentrations of >2.5 × 10^4 μg kg^{-1} in blue mussels (*Mytilus edulis*) (Kilcoyne et al., 2014a). During a comprehensive survey of Norwegian waters during the summer of 2015, Tillmann et al. (2018) estimated low abundance of Amphidomataceae with light microscopy (1–100 cells L^{-1}) in samples collected from both deep-branching fjords and offshore coastal waters. While they did not specifically enumerate *A. poporum* or *A. spinosum*, these species were detected in almost all DNA extracts using a presence/absence approach of the specific qPCR assays. High bloom densities of Amphidomataceae are also reported in the literature (Tillmann et al., 2017b; Tillmann, 2018). Retrospective studies of blooms from November 1990 and September 1991 by Akselman and Negri (2012) using light microscopy showed high abundances of what was initially reported as *A.* cf. *spinosum* (up to 9.0 × 10^6 cells L^{-1}) in coastal waters of the western South Atlantic. Tillmann and Akselman (2016) have subsequently used scanning electron microscopy to identify a new species, *Azadinium luciferelloides* Tillmann & Akselman and not *A.* cf. *spinosum* as originally reported, as the causative species in the September 1991 bloom in the western South Atlantic. Additionally, Akselman et al. (2014) observed abundances of *A.* cf. *spinosum* (likely to be again *A. luciferelloides*) quantified using light microscopy, ranging from 1.0–4.7 × 10^6 cells L^{-1} during a bloom in the western South Atlantic in August-September 1998.

Azadinium spp. can be found in a variety of environments, from tropical to temperate to sub-arctic climates as well as in inland, coastal and open ocean waters. Although the presence of *A. poporum* has been confirmed by Kim et al. (2017) in Washington State waters, azaspiracids are not routinely monitored in shellfish nor are *Azadinium* spp. part of routine phytoplankton monitoring in the region mainly due to the difficulty of identification by light microscopy. In a companion to the present study, shellfish were tested for AZAs and dissolved toxins were measured using SPATT samplers (MacKenzie et al., 2004; Rundberget et al., 2006) from 2014 to 2018 (Stutts et al. in prep). In the present study, *A. poporum* and *A. spinosum* were observed at shore-based sites on the outer coast and inland waters of Washington State from 2014 to 2018 as well as in offshore waters during two research cruises in the summer of 2017. The presence or absence of these species was evaluated in 2014–2015 and then enumerated in 2016–2018 using qPCR, a technique that could ultimately be integrated into an early warning system for azaspiracid poisoning events.

2. Materials and methods

2.1. Sample sites

Seawater was sampled from the shore of the inland and outer coast waters of Washington State from 2014 to 2018 (see Fig. 1 for numbers of sampling sites named in Tables 1 and 2). Monthly samples were collected at up to 28 sites from June-August in 2014 and 2015. In 2016–2018, specific sites were chosen for weekly sampling based on their proximity to shellfish harvesting areas or their history as locations where blooms of other harmful species have occurred. Samples were collected weekly at six sites from June-September in 2016; eight sites from June into October in 2017 and May-September in 2018 (Fig. 1b). In addition to weekly sampling, opportunistic monthly collections were made at up to 24 additional sites during the sampling periods in 2016 and 2017. Seawater was collected by local collaborators and shipped via overnight delivery to the NOAA-Northwest Fisheries Science Center in Seattle, WA for processing.

Seawater samples were also collected on board the NOAA Ship Bell M. Shimada during two research cruises in the summer and early fall of 2017 that spanned both US and Canadian territorial waters. During cruise 1, from 4 August to 11 September 2017, seawater samples were

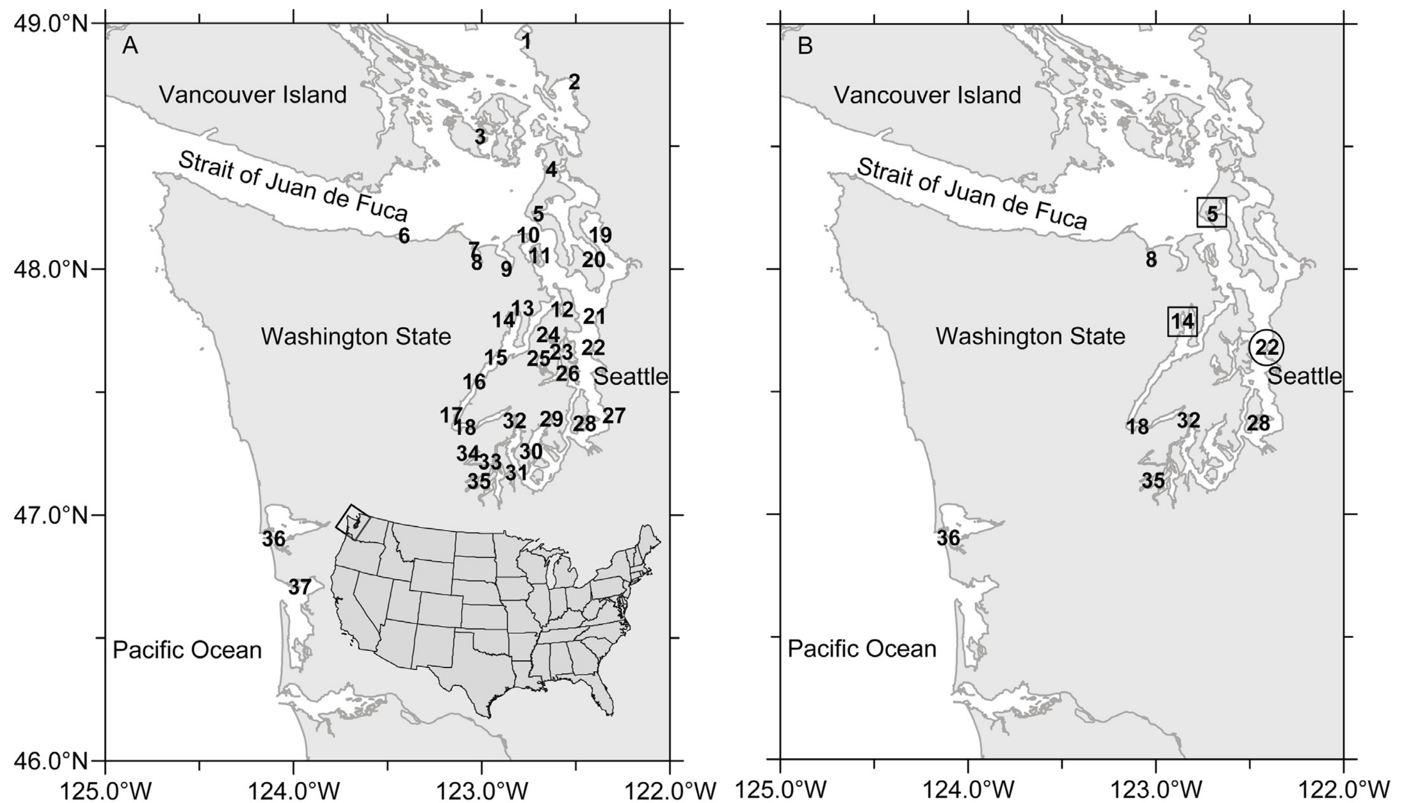


Fig. 1. (A) Locations of all sites sampled. Site names corresponding to the numbered sites are shown in Tables 1 and 2. (B) Locations of weekly sample sites. Site 22 (circled) was only sampled on a weekly basis in 2016. Sites 5 and 14 (boxed) were sampled weekly beginning in 2017. All other sites were sampled weekly beginning in 2016.

collected from the ship's scientific seawater supply that had an intake at 3.5 m depth. During cruise 2, from 18 to 24 September 2017, seawater was collected using Niskin bottles on the CTD rosette from 2 m depth and, when present, at the chlorophyll maximum.

2.2. Sample processing

Up to one liter of seawater was filtered from the shore-based sites, and 500 mL was filtered on both cruises. Samples were collected on 47 mm (shore-based sites) or 25 mm (cruises) diameter 3.0 µm pore size polycarbonate filters (MilliporeSigma, Burlington, MA, USA). Filtration was conducted under low (< 0.017 MPa) vacuum. The filters were folded and placed into plastic 2.0 mL microcentrifuge tubes and frozen at −80 °C until DNA extraction. Samples collected on the cruises were stored at −80 °C on the vessel and were transported to the lab for DNA extraction at the end of the cruise.

2.3. DNA extraction

DNA was extracted from the material on the filters using DNeasy Plant Mini Kits (Qiagen, Hilden, Germany) with some modifications of the manufacturer's protocol. Briefly, 500 µL of Buffer AP1 (https://www.qiagen.com/kr/shop//sample-technologies/dna/dneasy-plant-mini-kit/) and 5 µL of RNase A were added to the tube containing the filter, tubes were vortexed for 30 s then incubated at 65 °C for 30 min. After addition of Buffer P3 and a 5 min incubation on ice, the filters were removed from the tube, placed into the QiaShredder spin column, and the lysate was then added to the QiaShredder spin column containing the filter. The manufacturer's protocol was then followed as written, except two eluates with 200 µL AE buffer were collected. DNA was prepared for qPCR analyses by mixing equal volumes of the two eluates from the DNA extraction process.

Toebe et al. (2013) used DNeasy Plant Mini Kits to extract DNA from both cultured cells for standard curves as well as from concentrated natural samples spiked with *Azadinium* cells where they demonstrated recovery of 80–106% and 80–108% of *A. poporum* and *A. spinosum* cells, respectively. Therefore based on the assumption of high recovery derived from Toebe et al. (2013), neither extraction efficiency nor PCR inhibition were assessed and cell abundances were not adjusted for these factors. Additionally, if inhibition occurred, the cell abundances reported in the present study would be underestimates.

2.4. Shore-based samples

Triplicate species-specific analyses were conducted for *A. poporum* and *A. spinosum* following the procedure in Toebe et al. (2013). In 2014 and 2015, the presence or absence of *A. poporum* and *A. spinosum* was determined, while in 2016–2018 cell abundances were quantified using standard curves. Analyses for members of the family Amphidomataceae (see below) were performed in 2016–2018, in duplicate, on samples negative for both *A. poporum* and *A. spinosum* following the procedure in Smith et al. (2016). However, DNA was analyzed with the Amphidomataceae assay without standardizing to 2.0 ng μL^{-1} as in Smith et al. (2016). Positive control DNA and samples that tested positive using the Toebe et al. (2013) assay for *A. poporum* and *A. spinosum* were also analyzed to ensure that the Amphidomataceae assay was performing as expected. Quantitative PCR results were evaluated as described below.

2.5. Cruise samples

For the cruise samples, DNA was first screened in duplicate, as described below, using the Amphidomataceae assay (Smith et al., 2016). Samples positive for Amphidomataceae were then analyzed using the *A.*

Table 1
Azadinium poporum monthly detections in 2014–2018. Open circles indicate no detection, solid black circles indicate positive detection, and gray shading indicates no sampling. In 2016–2018, *A. poporum* cell concentrations were quantified (numerical value, cells/L), with an open circle indicating no detection, and closed black circle indicating detection below the limit of quantification. Site numbers in parentheses next to the site name correspond to the numbers in Figs. 1 and 2. Sites that were sampled weekly at some point during the study period are noted in bold, and monthly sites are in plain text. The highest abundance in a given month is shown for weekly sites.

A. poporum	2014			2015			2016				2017				2018					
Site Name (#)	Jun	Jul	Aug	Jun	Jul	Aug	Jun	Jul	Aug	Sep	Jun	Jul	Aug	Sep	Apr	May	Jun	Jul	Aug	Sep
Birch Bay (1)	○	●		●	○	●	○	○	○	○	○	○	○	○						
Squalicum Harbor (2)	○	○		○	○	●	●	○	○	○	○	○	○	○						
Friday Harbor (3)	●	○	○	○	○	○	7	●	●	○	●	○	○	○						
Cornet Bay (4)		○		○	○	●	9	●	●	○	●	●	○	○						
Penn Cove (5)	●	○		●	●		348	8	8	274	○	17	○	○	○	○	25	○	42	
Ediz Hook (6)	●	○		●		○														
Sequim Bay Entrance (7)	●	○		●	●	●	38	18	○	○	●	○	○	○						
Sequim Bay State Park (8)	●	○		●		●	417	85	●	●	26	○	●	●	○	249	562	○	○	○
Discovery Bay (9)	●	○		○	○	●	122	○	○	●	●	○	○	○						
Fort Worden (10)	○	○		●	●	●	19	139	○	●	●	●	○	○						
Mystery Bay (11)	○	○		●	○	●	12	●	○	○	○	9	18	●						
Port Gamble (12)				●		○	53	○	○	○	66	○	○							
Dabob Bay (13)				●	○															
Quilcene Bay (14)				●		○					8	○	○	○	○	27	10	○	○	○
Pleasant Harbor (15)	○	○		○	○	○														
Hama Hama (16)							●	○	○	○	○	○	○	○						
Glen Ayre (17)	●	○		○	○	○														
Union (18)							53	○	○	○	○	●	●	○		○	○	○	○	○
Port Susan-Kayak Point (19)	●	○	○	●	○		60	●	○	●	○	○	○	○						
Langley Marina (20)								○	●	24										
Edmonds Marina (21)		●	●	●	●	○	●	7	●	○	●	8	○	○						
Shilshole Marina (22)	●	●		●			○	18	9	●	●	○	●	●						
Brownsville Marina (23)							8	12	○	●	76	○	○	○						
Poulsbo Marina (24)							8	●	○	○	○	○	○	○						
Silverdale Waterfront (25)							17	○	●	●	8	○	●	○						
Manchester-Clam Bay (26)	●	○		●	●	○	●	●	●	○	●	○	●	●						
Des Moines Marina (27)	○	○	●	●	●	●	59	●	●	●	12	15	●	108						
Quartermaster Harbor-Dockton (28)	○	○		●	●	●	84	341	24	89	36	56	●	○	○	●	12	24	○	52
Burley Lagoon (29)				●	○	●														
Penrose Point (30)	●	●		○	●	●	○	28	○	○	○	●	○	○						
Zittel's Marina (31)	○					○					○	7	12							
North Bay-Allyn (32)	○	●		○	○	○	28	●	●	●	○	○	○	○	○	○	○	○	○	○
Hammersley Inlet (33)					○	○	○	○	●	●		○	○	○						
Oakland Bay (34)	○	○		○																
Totten Inlet (35)											●	●	○	○		○	○	○	○	○
Westport (36)	○	○		○	○	○	141	201	9	○	○	○	○	●	○	125	10,525	○	○	○
Tokeland (37)	○	○		●	○	○	262	○	●	○	○	○	○	○						

poporum and *A. spinosum* specific qPCR assays (Toebe et al., 2013). Quantitative PCR results were evaluated as described below.

2.6. Quantitative PCR assay standard curves

DNA standard curves were constructed using clonal cultures of *A. poporum* (967g9) which was obtained by isolation of single cells from incubated sediment samples collected from Washington State (Kim et al., 2017) and *A. spinosum* (UTHE2) based on isolation of a single cells from the North Sea (Krock et al., 2013). Known quantities of *A. poporum* and *A. spinosum* were filtered and DNA was extracted in the same manner as field samples. Ten-fold serial dilutions were analyzed to generate a standard curve. The *A. poporum* and *A. spinosum* standard curves spanned ranges of four to 44,000 cells and seven to 70,000 cells, respectively. Amplification efficiency was calculated from the slope of the standard curve for each run.

2.7. Quantitative polymerase chain reaction analyses

The MIQE (Minimum Information for Publication of Quantitative Real-time PCR Experiments, Bustin et al., 2009) checklist for qPCR assays performed in the present study is provided in Supplementary Table S1. Quantitative PCR analyses were performed on a Stratagene Mx3005P (Stratagene, La Jolla, CA, USA) using the primers, probes and thermal profile for *A. poporum* and *A. spinosum* in Toebe et al. (2013). A standard curve, positive control and a no template control were run with each set of environmental samples. Quantitative PCR reactions contained 2.0 μL of template, 0.6 μL of each forward and reverse primers (10 μM), 10 μL of TaqMan Gene Expression Master Mix (ThermoFisher Scientific, Waltham, MA, USA), either 0.1 μL for *A. spinosum* or 0.5 μL for *A. poporum* of TaqMan probe (10 μM), and brought to a final volume of 20 μL with molecular grade water. An average and standard deviation were calculated using the quantification cycle (*Cq*) values for the three technical replicates. Abundances of *A. spinosum* and *A. poporum* were determined by correcting the output from the MX pro software (version 4.10) with the volume of seawater filtered.

The Stratagene Mx3005P was also used for the Amphidomataceae assay using the primers and thermal profile described in Smith et al. (2016). Quantitative PCR reactions contained 5.0 μL of template, 0.4 μL each of forward and reverse primers (10 μM), 10 μL Platinum SYBR Green qPCR SuperMix-UDG (ThermoFisher Scientific, Waltham, MA, USA), 0.8 μL bovine serum albumin, 0.04 μL ROX reference dye, and brought to a final volume of 20 μL with molecular grade water. Averages were calculated for both the *Cq* values and melting temperatures for the two technical replicates. A positive control of *A. poporum* DNA and a no template control were included with each set of environmental samples.

Table 2

Azadinium spinosum monthly detections in 2014–2018. Open circles indicate no detection, solid black circles indicate positive detection, and gray shading indicates no sampling. In 2016–2018, *A. spinosum* cell concentrations were quantified (numerical value, cells/L), with an open circle indicating no detection, and closed black circle indicating detection below the limit of quantification. Site numbers in parentheses next to the site name correspond to the numbers in Figs. 1 and 2. Sites that were sampled weekly at some point during the study period are noted in bold and monthly sites are in plain text. The highest abundance in a given month is shown for weekly sites.

A. spinosum	2014			2015			2016				2017				2018					
Site Name (#)	Jun	Jul	Aug	Jun	Jul	Aug	Jun	Jul	Aug	Sep	Jun	Jul	Aug	Sep	Apr	May	Jun	Jul	Aug	Sep
Birch Bay (1)	○	●		○	○	○	○	●	●	●	○	○	○	○						
Squalicum Harbor (2)	○	○		●	○	○	○	●	●	○	●	○	○	○						
Friday Harbor (3)	○	○	●	●	●	○	○	○	●	●	○	○	○	○						
Cornet Bay (4)		●		○	○	○	○	●	●	○	○	○	○	○						
Penn Cove (5)	○	○		○	○		139	●	●	●	○	○	○	○	○	○	○	○	○	
Ediz Hook (6)	○	○		●		○														
Sequim Bay Entrance (7)	●	●		○	○	○	○	●	●	●	○	○	○	○						
Sequim Bay State Park (8)	○	●		○		○	●	23	●	●	○	○	○	○	○	○	○	○	○	○
Discovery Bay (9)	○	●		●	○	○	○	37	●	●	○	○	○	○						
Fort Worden (10)	○	●		●	○	●	○	●	●	●	○	○	○	○						
Mystery Bay (11)	○	○		○	○	○	○	●	○	●	○	○	○	○						
Port Gamble (12)				○	○	○	○	●	○	●	○	○	○							
Dabob Bay (13)				○	○															
Quilcene Bay (14)				○		●					○	○	○	○	○	○	○	○	●	○
Pleasant Harbor (15)	○	●		○	●	○														
Hama Hama (16)							●	●	○	○	○	○	○	○						
Glen Ayre (17)	○	●		●	○	○														
Union (18)							●	●	●	●	●	○	○	○		●	47	○	○	○
Port Susan-Kayak Point (19)	●	○	○	●	●		●	●	●	●	○	○	○	○						
Langley Marina (20)								9	●	●										
Edmonds Marina (21)		○	●	○	○	○	○	●	●	○	○	○	○	○						
Shilshole Marina (22)	●	●		●			8	10	●	●	○	○	○	○						
Brownsville Marina (23)							○	●	○	32	○	○	○	○						
Poulsbo Marina (24)							○	●	○	●	○	○	○	○						
Silverdale Waterfront (25)							●	●	●	●	○	○	○	○						
Manchester-Clam Bay (26)	●	●		○	○	○	●	●	●	●	○	○	○	○						
Des Moines Marina (27)	○	●	●	●	●	○	○	●	12	○	○	○	○	○						
Quartermaster Harbor-Dockton (28)	●	○		●	○	○	●	●	●	156	○	○	○	○	○	○	●	○	○	○
Burley Lagoon (29)				○	○	○														
Penrose Point (30)	○	●		○	○	○	○	●	●	●	○	○	○	○						
Zittel's Marina (31)	○				○						○	○	○	○						
North Bay-Allyn (32)	○	●		○	●	○	22	65	●	●	○	○	○	○	●	22	○	16	○	○
Hammersley Inlet (33)				○	○	○	○	●	●	●		○	○	○						
Oakland Bay (34)	○	○																		
Totten Inlet (35)											○	○	○	○		○	○	○	○	○
Westport (36)	○	○		○	○	○	●	●	●	●	○	○	○	○	○	○	○	○	○	○
Tokeland (37)	○	●		○	○	●	○	●	●	●	○	○	○	○						

2.8. Quantitative PCR acceptance criteria

Assays for *A. poporum* and *A. spinosum* were considered to be positive if there was amplification in all three technical replicates. However, if the calculated cell abundances using qPCR were less than the lowest point on the standard curve they were considered to be below the limit of quantification. Limits of quantification ranged from 4 to 108 cells L^{-1} for *A. poporum* and from 7 to 190 cells L^{-1} for *A. spinosum*, depending on the amount of seawater filtered for a given sample (volumes filtered ranged from 0.037–1.0 L). Samples were negative if there was amplification in two or fewer of the technical replicates and the calculated cell abundances were below the lowest point on the standard curve. When the calculated cell abundances were within the limits of the standard curve but there was amplification in only one or two of the three technical replicates, the samples were reanalyzed and the acceptance criteria were re-evaluated. Samples with amplification in two or fewer of the technical replicates in repeated runs were considered to be negative. Samples with calculated cell abundances greater than the highest value of the standard curve were diluted and re-analyzed.

Results from the Amphidomataceae assay were considered positive if there was amplification in both technical replicates and the *Cq* was ≤ 32.2 with the melting temperature ≥ 82.5 °C and ≤ 84.5 °C. Samples were considered negative detections if the above acceptance criteria were not met in samples with amplification in both technical replicates. Samples with amplification in only one technical replicate that met the above acceptance criteria, were reanalyzed. If there was amplification in only one of the technical replicates of the reanalyzed sample, it was considered to be a negative detection for Amphidomataceae in that sample. Additionally, the no template control (NTC) included with a particular set of samples must have been "no *Cq*" for the results of that sample set to be considered valid.

2.9. Primer/Probe cross reactivity tests

Primer and probe cross reactivity tests were performed for the species-specific qPCR assays. The primer sets for *A. poporum* and *A. spinosum* were tested using DNA from other Amphidomatacean strains, including *A. spinosum, A. poporum, A. caudatum* var. *margalefii* (Rampi) Nézan & Chomérat, *A. concinnum* Tillmann & Nézan, *A. cuneatum* Tillmann & Nézan, *A. dexteroporum, A. obesum* Tillmann & Elbrächter, *A. polongum* Tillmann, *A. trinitatum* Tillmann & Nézan, *A. dalianense* Z.Luo, H.Gu & Tillmann and *Amphidoma languida* Tillmann, Salas & Elbrächter, to assess cross reactivity with other species of *Azadinium* and *Amphidoma* (Supplementary Table S2). Non-Amphidomatacean DNA obtained from a strain of *Heterocapsa minima* A.J.Pomroy was also included in the cross reactivity analyses. DNA from all isolates of *A. poporum* and *A. spinosum* amplified with the corresponding primer and probe sets. Primer and probe sets for *A. poporum* and *A. spinosum* generally did not react with other *Azadinium* spp., *Amphidoma languida* or

Heterocapsa minima. Only single replicates cross amplified in four strains using the *A. spinosum* primer and probe sets where the *Cq* values for three of the replicates were >38.7 and the other was 36.1, which suggests non-specific amplification or primer-dimers. The *A. poporum* primer and probe set amplified single replicates in three strains with *Cq* values >38.8. The results of the cross-reactivity tests indicate that there is high confidence that the primer and probe sets for *A. poporum* and *A. spinosum* are indeed amplifying the correct targets in the present study.

3. RESULTS

3.1. qPCR assay assessments

The results for the NTCs included in each qPCR run, both for the TaqMan and SYBR Green (Amphidomataceae) assays, were all "No *Cq*". Amplification efficiencies calculated from standard curves run with each set of samples ranged from 80 to 100% and 85 to 105% for the *A. poporum* and *A. spinosum* TaqMan assays, respectively. All standard curves had $r^2 > 0.99$ and the calculated cell abundances did not exceed the highest value of the standard curve for any of the samples in the present study.

3.2. Detection of Azadinium and Amphidomataceae at shore-based sites

For the shore-based sampling, 57 seawater samples were collected and analyzed in 2014, 82 samples in 2015, 233 samples in 2016, 269 samples in 2017 and 181 samples in 2018. Monthly detection and maximum abundances of *A. poporum* and *A. spinosum* are shown in Tables 1 and 2, respectively. In 2014, *A. spinosum* was detected in 44% of the samples and *A. poporum* was detected in 37%, while in 2015, *A. spinosum* was detected in 22% and *A. poporum* in 51%. In 2016–2018, *A. spinosum* was detected (including quantifiable samples and those below the limit of quantification) in 24% (2016), 1% (2017) and 4% (2018) of the samples while *A. poporum* was detected in 30% (2016), 21% (2017) and 12% (2018) of the samples. Abundances of *A. spinosum* were quantified in 6% (2016), <1% (a single sample in 2017) and 2% (2018) of samples and *A. poporum* in 25% (2016), 8% (2017) and 10% (2018) samples from 2016 to 2018. *Azadinium poporum* and *A. spinosum* were detected in all months sampled in 2014 and 2015. From 2016–2018 when *A. poporum* and *A. spinosum* were quantified, the highest abundance of *A. poporum* was 10,525 cells L^{-1} at Westport in June 2018 and the highest abundance of *A. spinosum* was 156 cells L^{-1} at the Quartermaster Harbor–Dockton site in September 2016. *Azadinium poporum* was generally observed more frequently and at higher abundances than *A. spinosum*. In most cases, the highest abundances of both *A. poporum* and *A. spinosum* were observed at the weekly sampling sites. However, abundances >100 cells L^{-1} of *A. poporum* were measured at monthly shore-based sampling sites including Discovery Bay, Fort Worden, Des Moines Marina and Tokeland (Table 1).

3.3. Azadinium spinosum at shore-based sites

Figs. 2a, 3a and 4a show weekly *A. spinosum* abundances in 2016–2018. In 2016, *A. spinosum* were detected during most of the sampling season at Quartermaster Harbor-Dockton, Sequim Bay State Park, Shilshole Marina and Union. *Azadinium spinosum* was detected in 2016 most often in June and July, however the highest abundance measured in 2016 was in September. In 2017, quantifiable abundances of *A. spinosum* were observed in April at Sequim Bay State Park but no samples collected after 15 June contained *A. spinosum*. In 2018, *A. spinosum* was observed only in May at North Bay-Allyn and June at Union.

3.4. Azadinium poporum at shore-based sites

Except for April 2018, *A. poporum* was observed somewhere in the study region during all months sampled with the highest abundances generally found from May-July (Table 1). The lack of *A. poporum* observations in April 2018, could have been the result of sampling only on a weekly basis in 2018. Figs. 2b, 3b, and 4b show weekly *A. poporum* abundances in 2016–2018. In 2016, *A. poporum* was observed beginning in June at all sites except Shilshole Marina where it was first observed in July. Abundances of *A. poporum* were below 200 cells L^{-1} at all sites in June 2016 but increased to >400 cells L^{-1} at Sequim Bay State Park and >325 cells L^{-1} at Quartermaster Harbor-Dockton before decreasing to <100 cells L^{-1} for the remainder of the sampling season. *Azadinium poporum* densities did not exceed 60 cells L^{-1} in 2017 and this species was undetectable in the majority of the weekly samples, while monthly sample abundances exceeded 60 cells L^{-1} at only three sites (Table 1). In 2018, sampling began at some sites in March, however, *A. poporum* was not observed until May (Fig. 4b). The majority of

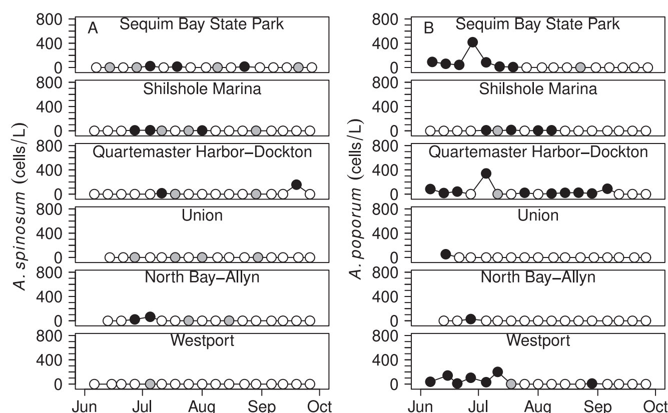


Fig. 2. *A. spinosum* (A) and *A. poporum* (B) abundances from weekly sampling in 2016. Open circles indicate samples below the limit of detection, gray circles indicate samples where the species was detected but were below the limit of quantification, and black circles are samples where cell abundances were quantified. Error bars on quantifiable samples represent one standard deviation based on triplicate analyses. If error bars are not visible they are contained within the symbol.

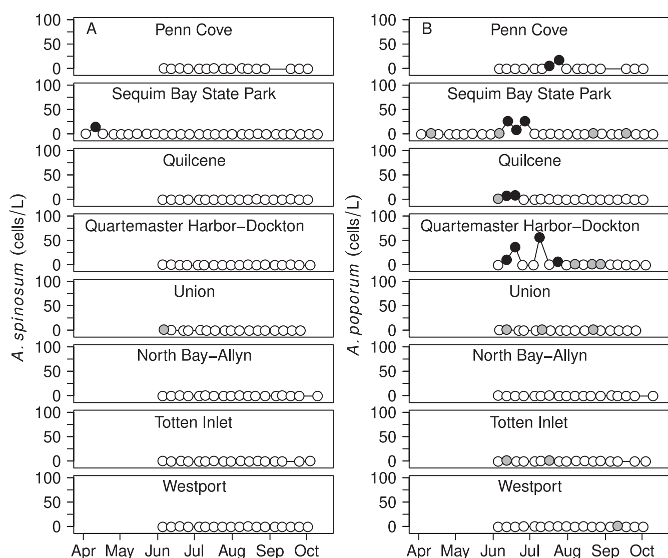


Fig. 3. *A. spinosum* (A) and *A. poporum* (B) abundances from weekly sampling in 2017. Open circles indicate samples below the limit of detection, gray circles indicate samples where the species was detected but were below the limit of quantification, and black circles are samples where cell abundances were quantified. Error bars on quantifiable samples represent one standard deviation based on triplicate analyses. If error bars are not visible they are contained within the symbol.

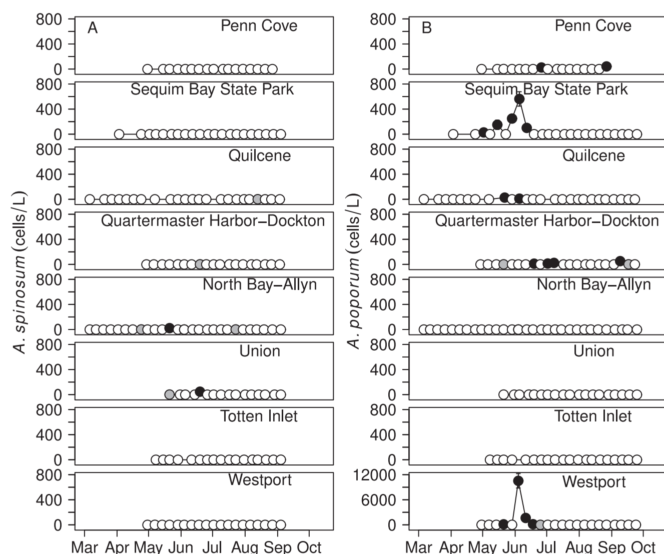


Fig. 4. *A. spinosum* (A) and *A. poporum* (B) abundances from weekly sampling in 2018. Open circles indicate samples below the limit of detection, gray circles indicate samples where the species was detected but were below the limit of quantification, and black circles are samples where cell abundances were quantified. Error bars on quantifiable samples represent one standard deviation based on triplicate analyses. If error bars are not visible they are contained within the symbol. Note the different y-axis for Westport *A. poporum*.

of *A. poporum* were observed prior to 10 July in 2018, except for samples from Penn Cove on 27 August 2018 and Quartermaster Harbor-Dockton on 9 September 2018. At Sequim Bay State Park, *A. poporum* increased from May to early June in 2018 and reached a maximum abundance of 562 cells L^{-1} before decreasing to undetectable levels for the remainder of the sampling season. At Westport, *A. poporum* was 125 cells L^{-1} on 21 May 2018 and abruptly increased to 10,525 cells L^{-1} on 4 June 2018 then decreased in subsequent weeks to undetectable levels in the first week of July and remained below detection for the rest of the sampling season.

3.5. Amphidomataceae assay at shore-based sites

In 2016, *A. poporum* and *A. spinosum* were both undetectable in 27 of the shore-based samples. These samples were analyzed using the Amphidomataceae assay where seven of the samples tested positive. In 2017, 59 of 211 samples and in 2018, 36 out of the 190 samples analyzed with the Amphidomataceae assay were positive. All sites that were sampled tested positive for either *A. poporum, A. spinosum* or Amphidomataceae at some point during the study period.

3.6. Cruise samples

Thirty two samples were analyzed for cruise 1 and 92 samples were analyzed for cruise 2. The results for the Amphidomataceae and the species-specific assays are shown in Fig. 5a (cruise 1) and Fig. 5b (cruise 2). Seventeen of the samples from cruise 1 and 45 samples from cruise 2 met the acceptance criteria for the Amphidomataceae assay. Of these samples, 12 from cruise 1 and 32 from cruise 2 had abundances of both *A. poporum* and *A. spinosum* below the limits of detection, suggesting the presence of other members of the family Amphidomataceae in these samples.

Samples that were either quantifiable or detectable but below the limit of quantification for cruises 1 and 2 are summarized in Table 3. For cruise 1, three samples were quantifiable and two were below the limit of quantification for *A. poporum* while the rest of the samples were below detection (Fig. 5a). Quantifiable abundances of *A. poporum* were observed in both US and Canadian waters. *Azadinium spinosum* was not detected in any of the cruise 1 samples.

For cruise 2 (Fig 5b), *A. poporum* was quantified in 10 samples and *A. spinosum* in two samples. The remaining cruise 2 samples were below the limit of detection for both species. The majority of quantifiable *A. poporum* were observed in surface samples, however, *A. poporum* was also observed at site 13 at both the surface and chlorophyll maximum and at the chlorophyll maximum only at site 28 (Table 3). The highest abundance of *A. poporum* measured on cruise 2 was at station 17 in Canadian waters (1206 cells L^{-1}).

4. DISCUSSION

Routine monitoring of AZAs occurs in the EU as well as on both the east and west coasts of Canada while monitoring of both AZAs and *Azadinium* spp. for resource management purposes has been used successfully for the protection of human health in Ireland (Kilcoyne et al., 2014a). In 2012, AZA-2 was first detected in Sequim Bay, Washington State in several filtered phytoplankton samples, although shellfish samples collected at the same time were found to contain only DSP toxins and no AZAs (Trainer et al., 2013). Kim et al. (2017) later isolated several species of *Azadinium*, including four strains of the known toxigenic species *A. poporum* which were also shown to exclusively produce the novel azaspiracid, AZA-59. Dai et al. (2019) used an additional PNW *A. poporum* strain which was derived from the same source as the strains in Kim et al. (2017) and it also exclusively produced AZA-59. Currently, neither *Azadinium* nor AZAs are included in official monitoring programs in Washington State, although the initial detection of azaspiracids prompted research into the temporal and spatial distribution of potentially toxic *Azadinium* species in the region.

To assess the distribution of *Azadinium* spp. in Washington State, an initial survey of the presence or absence of *A. poporum* and *A. spinosum* was conducted at multiple sites in 2014 and 2015. This survey indicated that both species were present in Washington State waters, based on amplification of their molecular targets. The subsequent isolation and identification of *A. poporum* from Hood Canal in Washington State (Kim et al., 2017) enabled the use one of these local strains to construct a standard curve for use with the species-specific qPCR assay. The *A. poporum* strain used for the standard curve was of ribotype A (Kim et al., 2017). There are three main ribotypes within *A. poporum* with additional ribotype substructure (Luo et al., 2018), and especially ribotype C strains of *A. poporum* from Greece were shown to have mismatches in the TaqMan probe sequence from Toebe et al. (2013). All strains obtained so far from the present study area were of ribotype A, but it cannot be excluded that other *A. poporum* ribotypes were present as well, and that mismatches with the probe sequence may have caused *A. poporum* abundance to be underestimated.

Although attempts were made to isolate *A. spinosum* from this region over the last 2 years using both sediment and water samples, no strain has yet been obtained, highlighting the difficulty of isolating this small and inconspicuous species (Tillmann, 2018). Therefore, a North Sea strain of *A. spinosum* was used to prepare the standard curve for the *A. spinosum*-specific qPCR assay in the present study. *Azadinium spinosum* has not previously been reported in PNW waters and only its molecular target has been detected. Consequently, the identification and quantitation of this species in the present study is considered presumptive until *A. spinosum* can be isolated or definitively identified in phytoplankton samples using SEM.

Azadinium poporum, A. spinosum and other members of the family Amphidomataceae were distributed widely throughout PNW waters, including inland, offshore and coastal marine waters during all years sampled. In general, *A. poporum* was observed more often and in higher densities than *A. spinosum* (Tables 1-3) and quantifiable abundances of *A. poporum* and *A. spinosum* were observed at a number of sites throughout the PNW study area mostly prior to mid-July in each year. At sites that were sampled on a weekly basis, quantifiable abundances generally were not observed until May and only one sample, at Sequim

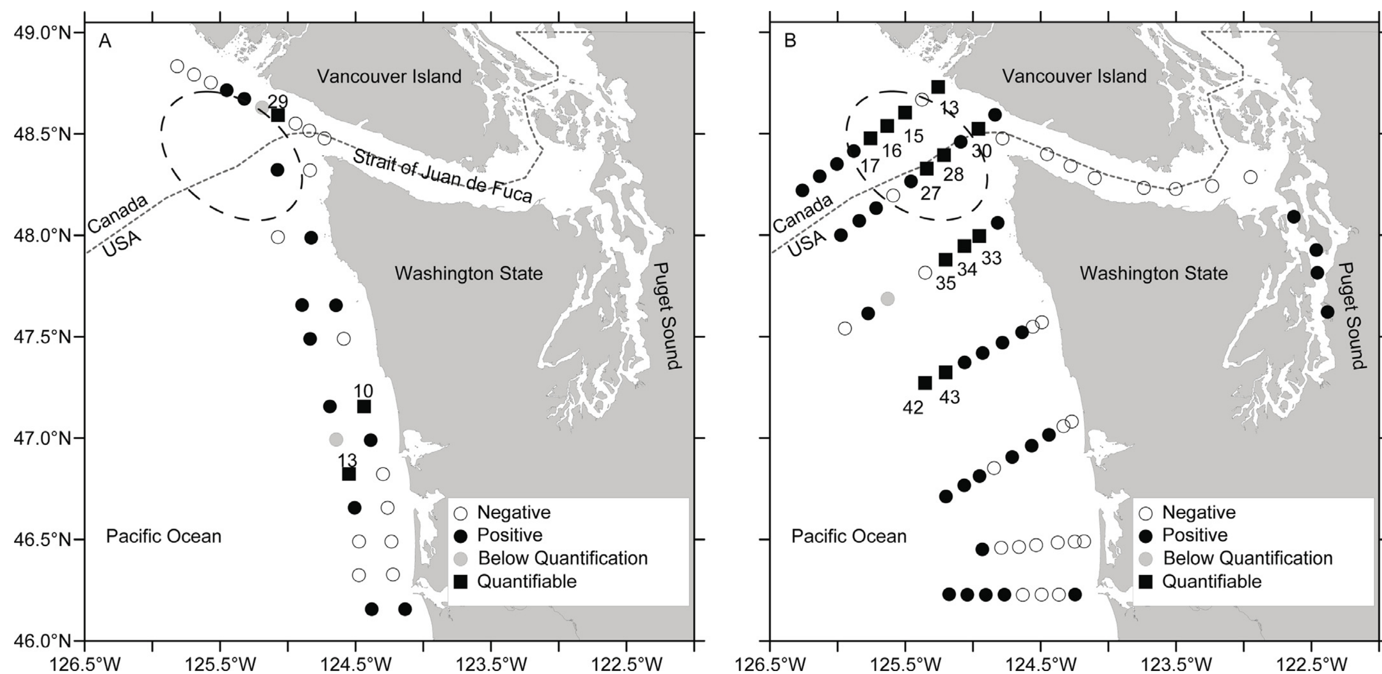


Fig. 5. Results of Amphidomataceae specific assay and subsequent quantification analyses for *A. spinosum* and *A. poporum* for cruise 1 (A) and cruise 2 (B). The approximate location of the Juan de Fuca eddy is noted with the dashed ellipse and the US/Canadian border is noted with the gray dotted line. Open circles indicate stations where surface water samples were negative for the Amphidomataceae assay. Black filled circles are stations where the Amphidomataceae assay was positive but neither *A. spinosum* nor *A. poporum* were detected. Filled gray circles indicate stations where *A. spinosum* or *A. poporum* were detected but were below the limit of quantification (BLQ). Filled black squares labeled with station numbers are stations where quantifiable abundances of either *A. spinosum* or *A. poporum* were measured (See Table 3 for abundances of *A. spinosum* and/or *A. poporum*).

Table 3
Samples for which *A. poporum* or *A. spinosum* were detectable or quantifiable from cruise 1 and cruise 2 during 2017 (black squares in Fig. 5). “BLQ” = Below Limit of Quantification and indicates that a particular species was detected but the abundances observed were below the lowest limit of the standard curve.

	Station ID	Sample depth (m)	*Azadinium* species	Concentration (cells/L)
Cruise 1	10	3	*A. poporum*	28
	11	3	*A. poporum*	BLQ
	13	3	*A. poporum*	20
	28	3	*A. poporum*	BLQ
	29	3	*A. poporum*	30
	13	2	*A. poporum*	108
	13	16	*A. poporum*	56
	15	2	*A. poporum*	198
	16	2	*A. poporum*	134
	17	2	*A. poporum*	1206
	28	10	*A. poporum*	24
Cruise 2	30	2	*A. poporum*	84
	33	2	*A. poporum*	154
	34	2	*A. poporum*	64
	35	2	*A. poporum*	28
	27	2	*A. spinosum*	24
	43	2	*A. spinosum*	30

Bay State Park, had detectable *A. poporum* prior to May (Figs 3-4).

In addition to within-year variation, some year-to-year differences were observed. For example in 2016 and 2017, the two years when both weekly and monthly sampling were conducted, there were markedly fewer detections of *A. spinosum* (only the two noted in Table 2 as well as another from a sample collected in April) and *A. poporum* in 2017 compared to 2016. Additionally, despite analyzing fewer samples in 2016, a higher number of samples contained quantifiable abundances of both *A. poporum* and *A. spinosum* when compared to 2017. However, at some of the monthly sampling sites (e.g. Mystery Bay and Des Moines Marina) in 2016 and 2017, the highest abundance of *A. poporum* was actually observed in 2017 (Table 1) but at relatively low densities (<110 cells L^{-1}). At the weekly sampling sites, the highest abundances of *A. poporum* and *A. spinosum* were measured in 2016 and 2018.

Similar inter-annual variability in AZAs was observed by Salas et al. (2011) and Kilcoyne et al. (2014a) in Ireland from 2002 to 2013 as well as in Great Britain from 2011 to 2016 (Dhanji-Rapkova et al., 2019). Dhanji-Rapkova et al. (2019) observed relatively high concentrations and frequency of observations of AZAs in shellfish from 2011 to 2013 when compared to fewer from 2014 to 2016. While they did not enumerate *Azadinium* spp., Dhanji-Rapkova et al. (2019) suggested that the presence or absence of AZAs in Scotland were linked to observations in Ireland during the same time periods. The inter-annual variability observed in the present study as well as that in Dhanji-Rapkova et al. (2019) is noteworthy, yet both datasets of <5 years are not sufficient for assessing long term trends in association with environmental data.

The higher abundances observed for *A. poporum* and *A. spinosum* in 2016 and 2018 suggest that these were years in which conditions in the region were more favorable to the growth of *Azadinium* spp. than 2017. In the laboratory, growth of local *A. poporum* isolates occurred at temperatures ranging from 6.7–25 °C and salinities of 15–35 (Dai et al., 2019). While the present study did not aim to assess the effects of environmental drivers on the growth of *Azadinium* spp., temperature and salinity measurements were made on a weekly basis by the SoundToxins project (https://www.soundtoxins.org/) at some sites where water samples were collected for *Azadinium* spp. analyses. At the three sites sampled weekly where *A. poporum* were observed at elevated abundances (Sequim Bay, Quartermaster Harbor-Dockton, and Westport), the measured in situ temperatures and salinities were within the ranges shown by Dai et al. (2019) to support growth of *A. poporum* but there were not marked differences among the three years sampled (data not shown). However, the 2017 Puget Sound Marine Waters report (PSEMP Marine Waters Workgroup, 2018) showed that integrated

water temperatures, calculated as thermal energy content, in the 0–50 meter water layer in Puget Sound were warmer than normal in 2016. In early 2017, Puget Sound waters were cooler than normal but were warmer than normal by May, but not as warm as in 2016 (PSEMP Marine Waters Workgroup, 2018). The warmer temperatures in 2016 compared to 2017 may be a contributing factor to the higher abundances of *A. poporum* and *A. spinosum* observed in 2016.

Sequim Bay State Park, Quartermaster Harbor-Dockton and Westport stand out as sites where elevated abundances of *A. poporum* were measured while relatively low abundances were observed at the other sites (Figs 2-4). The Sequim Bay and Quartermaster Harbor areas are well known "hot-spots" for blooms of other harmful algal genera including *Alexandrium* (Trainer et al., 2003; Horner et al., 2011), *Pseudo-nitzschia* (Trainer et al., 2007) and *Dinophysis* (Trainer et al., 2013), so it is interesting that *Azadinium* were found at these sites as well.

The highest observed abundance of *A. poporum* was at Westport in June 2018 and California mussels (*Mytilus californianus*) from this location were tested for AZA-1, −2, −3, and −59 as part of the companion toxin study. In the Westport samples, Stutts et al. (in prep) measured exclusively AZA-59 on 11 June at 0.3 μg kg^{-1} while AZA-2 was measured exclusively on June 25 at 0.6 μg kg^{-1}, concentrations that are well below the U.S. guidance level of 160 μg kg^{-1} AZA-1 equivalents. While AZA-59 was measured in low concentrations in shellfish several times at various locations during the companion toxin study, this was the only time AZA-2 was detected in shellfish (data not shown). AZA-2 is known to be produced by three different Amphidomataceae species. All strains of *A. spinosum* ribotype A consistently have detectable AZA-2 only when higher levels of AZA-1 are measured (Krock et al., 2019; Tillmann et al., 2019). There is a single strain of *A. spinosum* from a different ribotype (B) isolated from the Argentine South Atlantic that produces only AZA-2 (Tillmann et al., 2019). Similarly, strains of *A. poporum* have been shown to have variable strain-to-strain toxin profiles (Gu et al., 2013) where *A. poporum* from Argentina and from the Gulf of Mexico produce exclusively AZA-2 (Luo et al., 2016; Tillmann et al., 2016). As a third potential source organism of AZA-2, one strain of *Amphidoma languida* from Spain has also been shown to produce AZA-2 as the main AZA isomer (Tillmann et al., 2017a). The source organism of AZA-2 detected in *M. californianus* from Westport, Washington thus cannot be identified, therefore future studies are needed to fully describe the Amphidomatacean species diversity and their complete toxin profiles in the area.

Previous studies have shown that *Azadinium* spp. abundances can range from relatively low levels (Hernandez-Becerril et al., 2012; Kilcoyne et al., 2014a) to millions of cells L^{-1} (Akselman and Negri, 2012). With the exception of the 10,525 cells L^{-1} of *A. poporum* measured at Westport in early June 2018, abundances of both *A. poporum* and *A. spinosum* in the PNW were <600 cells L^{-1} for the duration of the current study and are comparable to those observed in other parts of the world. During mid-Spring to early winter from 1994 to 2000, Akselman et al. (2014) measured *Azadinium* spp. abundances, using light microscopy, at a site in the southwestern Atlantic that were below 960 cells L^{-1}, and generally below 400 cells L^{-1}, but they did not measure AZAs associated with *Azadinium* cell counts. In Ireland, Kilcoyne et al. (2014a) found *A. spinosum* abundances of 480 cells L^{-1} with associated AZA concentrations exceeding the European Union regulatory action level of 160 μg kg^{-1} in *M. edulis*. However, a time delay between *Azadinium* in the water and appearance of AZA in shellfish has to be taken into account and direct associations between measurements of cell abundances and toxin concentrations from the same day should be made with caution. Although similar abundances of *A. poporum* and *A. spinosum* have been observed in the PNW, only trace levels of AZAs were found in a small number of shellfish samples (Stutts et al. in prep). The observed AZA concentrations in PNW shellfish suggest that the abundances and/or the toxin production potential of Amphidomataceae in the region were insufficient to cause toxification of shellfish resulting in human illnesses. However, the presence of both species of the family Amphidomataceae as well as AZAs in the PNW indicates that future monitoring should be conducted for both the species and toxins in this region.

Azadinium poporum, A. spinosum, and other members of the family Amphidomataceae were also detected during two research cruises in 2017 (Fig. 5a, b). Abundances of *A. poporum* up to 30 cells L^{-1} were measured on cruise 1 whereas abundances of up to 1.2×10^3 cells L^{-1} were measured on cruise 2. Quantifiable abundances of *A. poporum* were observed throughout the study area, which included US and Canadian waters, on both cruises. *Azadinium spinosum* was only observed on cruise 2, with abundances of up to 30 cells L^{-1}. The wide distribution of *A. poporum* in the PNW region illustrates the importance of cross-boundary coordination of both US and Canadian authorities with respect to early warning systems for harmful algal blooms.

Samples from Puget Sound on cruise 2 (Fig. 5b) showed positive results for the Amphidomataceae assay. However, *A. poporum* and *A. spinosum* were below the limits of detection for the species-specific qPCR assays, consistent with the absence or low abundances of these species in 2017 at shore-based sites. Interestingly, samples collected in the surface waters of the Strait of Juan de Fuca were all negative for the Amphidomataceae assay (Fig. 5b), however other dinoflagellate genera were present in these samples (data not shown).

The highest concentration of *A. poporum* measured in offshore waters was in the Juan de Fuca eddy region (Fig. 5b), an area known to be an incubation site for blooms of *Pseudo-nitzschia* spp. (Trainer et al., 2009). In fact at sites where *A. poporum* was measured, analysis of net tow samples using light microscopy revealed the presence of other harmful algal genera, including *Pseudo-nitzschia, Alexandrium* and *Dinophysis*, suggesting that shellfish may accumulate multiple marine toxins when these cells are advected to coastal shellfish beds at high enough densities. The co-occurrence of multiple toxin groups has been reported in several regions, however the studies evaluating the effects of toxin mixtures are limited (Twiner et al., 2014). In the PNW, the potential exists for shellfish to be contaminated with azaspiracids, domoic acid, paralytic shellfish toxins, and diarrhetic shellfish toxins at the same time. It remains unclear if there is a synergistic effect with respect to the toxins affecting the health of human consumers. Aune et al. (2012) did not find evidence for synergistic effects of okadaic acid (OA, a diarrhetic shellfish toxin) and AZA-1 when mice were exposed to sublethal doses of these toxins. Similarly, Ferron et al. (2016) found increasing antagonism between AZA-1 and OA with increasing toxin concentrations when exposed to human intestinal cell models. However, Ferron et al. (2016) did find increasing synergism with increasing toxin concentrations of AZA-1 and yessotoxin in the same human intestinal cell models, suggesting that the effects of multiple toxins in a system depend on the specific toxin mixture. More research into the effects of mixtures of marine toxins on mammalian systems is needed in order for resource manager to assess risks of combinations of toxins to human consumers of shellfish.

In summary, *A. poporum* and *A. spinosum* have been detected with measurable abundances observed in the inland and coastal waters of Washington State as well as offshore in US and Canadian territorial waters. Both species were found throughout the study area and, depending on the site, they were detected from April through September, but the majority of the detections occurred prior to mid-July. These two species were more abundant in some years than others and abundances were generally low, but were comparable to measurements made in other areas of the world. Shellfish tested as part of a companion study were only found to contain low amounts of the newly described AZA-59 (max. 2.4 μg kg^{-1}) in a small number of samples and AZA-2 (0.6 μg kg^{-1}) in one sample with the concentration of *A. poporum* observed during this study (1.1×10^4 cells L^{-1} measured at Westport in early June 2018) indicating that the risk to consumers for AZP is low at the present time. During the course of these studies, the Washington Department of Health investigated three isolated incidents of

gastrointestinal distress associated with shellfish consumption (one in 2014 and two in 2016), similar to the one in 2009 that helped to inspire this work. Several of these samples were found to contain low levels of DSP toxins but not enough to explain the illnesses. These shellfish were subsequently tested for AZA-1, −2, −3, and −59 and only one sample was found to contain a trace concentration of AZA-59, while no AZA-1, −2, or −3 were detected (Jonathan Deeds, personal communication). However, positive results from the Amphidomataceae assay suggests the presence of additional *Azadinium* spp. or *Amphidoma* spp. in the region, although it cannot be excluded that such species produce new and not yet described AZA derivatives. The detection of toxigenic *A. poporum* and potentially toxigenic *A. spinosum* in Washington State inland and coastal waters, as well as in Canadian territorial waters, along with the quantification of low levels of azaspiracids in shellfish highlights the importance of continued monitoring for these organisms and toxins as a proactive approach to human health protection.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We would like to thank the following organizations for their support with sample collection and delivery: the SoundToxins partnership, Olympic Region Harmful Algal Bloom partnership, Washington State Department of Health, Pacific Northwest National Laboratory, Stillaguamish Tribe, Port Townsend Marine Science Center, Washington Sea Grant, Jamestown S'Klallam Tribe, Taylor Shellfish, Port Gamble S'Klallam Tribe, University of Washington Friday Harbor Laboratories, Coast Seafoods, Hama Seafood Co., Nisqually Indian Tribe, WA Sea Grant, Penn Cove Shellfish, Kitsap Public Health District, Washington State Department of Fish and Wildlife, Vashon Island residents (Karlista Rickerson and Mary Kelly), South Sound Mariculture, and Clam Fresh, Inc. We also thank the Captain and crew of the NOAA Ship Bell M. Shimada for assistance in sample collection during research cruises as well as Tracie Barry, Brian Bill, Lindsey Claasen, Daniel Cox, Jennifer Hagen, Chris Ikeda, Anthony Odell and Lynn Scamman for collecting and processing samples on the ship. Five College Coastal and Marine Sciences Summer intern, Erin Jones (Mount Holyoke College) and NOAA Hollings scholars James Garlant (University of Arizona), Annibel Rice (University of Maryland), Kyla Kelly (University of New Haven), and Alexis Bantle (University of South Carolina) were instrumental in sample processing and analysis. We acknowledge Wade Rourke from the Canadian Food Inspection Agency for providing information regarding AZAs in Canada and Jonathan Deeds of the US FDA for reviewing this manuscript and providing helpful comments. This work was supported by a Monitoring and Event Response to HABs (MERHAB) grant from the NOAA National Centers for Coastal Ocean Science Centers for Sponsored Coastal Ocean Research (Internal transfer to NWFSC) for the project, "Clear and Present Danger: Monitoring and Management of Lipophilic Shellfish Toxins in Washington State". This is NOAA MERHAB publication #219.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.hal.2020.101874.

References

Adams, N.G., MacFadyen, A., Hickey, B.M., Trainer, V.L., 2006. The nearshore advection of a toxigenic *Pseudo-nitzschia* bloom and subsequent domoic acid contamination of intertidal bivalves. Afr. J. Mar. Sci. 28 (2), 271–276.

Akselman, R., Negri, R.M., 2012. Blooms of *Azadinium* cf. *spinosum* Elbrächter et Tillmann (Dinophyceae) in northern shelf waters of Argentina, Southwestern Atlantic. Harmful Algae 19, 30–38.

Akselman, R., Negri, R.M., Cozzolino, E., 2014. *Azadinium* (Amphidomataceae, Dinophyceae) in the Southwest Atlantic: in situ and satellite observations. Rev. Biol. Mar. Oceanogr. 49 (3), 511–526.

Alvarez, G., Uribe, E., Avalos, P., Marino, C., Blanco, J., 2010. First identification of azaspiracid and spirolides in *Mesodesma donacium* and *Mulinia edulis* from Northern Chile. Toxicon 55 (2–3), 638–641.

Aune, T., Espenes, A., Aasen, J.A.B., Quilliam, M.A., Hess, P., Larsen, S., 2012. Study of possible combined toxic effects of azaspiracid-1 and okadaic acid in mice via the oral route. Toxicon 60 (5), 895–906.

Bill, B.D., Cox, F.H., Horner, R.A., Borchert, J.A., Trainer, V.L., 2006. The first closure of shellfish harvesting due to domoic acid in Puget Sound, Washington, USA. Afr. J. Mar. Sci. 28 (2), 435–440.

Blanco, J., Arevalo, F., Morono, A., Correa, J., Muniz, S., Marino, C., Martin, H., 2017. Presence of azaspiracids in bivalve molluscs from Northern Spain. Toxicon 137, 135–143.

Braña Magdalena, A., Lehane, M., Krys, S., Fernandez, M.L., Furey, A., James, K.J., 2003. The first identification of azaspiracids in shellfish from France and Spain. Toxicon 42 (1), 105–108.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T., 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin. Chem. 55 (4), 611–622.

Cavalcante, K.P., Susini-Ribeiro, S.M.M., Tillmann, U., 2018. First detection of species of the potentially toxic genus *Azadinium* (Amphidomataceae, Dinophyceae) in tropical coastal waters of Brazil. Brazilian Journal of Botany 41 (1), 209–218.

Dai, X., Bill, B.D., Adams, N.G., Tillmann, U., Sloan, C., Lu, D., Trainer, V.L., 2019. The effect of temperature and salinity on growth rate and azaspiracid cell quotas in two strains of *Azadinium poporum* (Dinophyceae) from Puget Sound, Washington State. Harmful Algae 89, 101665.

Dhanji-Rapkova, M., O'Neill, A., Maskrey, B.H., Coates, L., Swan, S.C., Teixeira Alves, M., Kelly, R.J., Hatfield, R.G., Rowland-Pilgrim, S.J., Lewis, A.M., Turner, A.D., 2019. Variability and profiles of lipophilic toxins in bivalves from Great Britain during five and a half years of monitoring: azaspiracids and yessotoxins. Harmful Algae 87, 101629.

European Commission, 2002. Commission decision 2002/225/EC of 15 March 2002 laying down detailed rules for the implementation of Council Directive 91/492/EEC as regards the maximum levels and the methods of analysis of certain marine biotoxins in bivalve molluscs, echinoderms, tunicates and marine gastropods. (L75), 62–64.

Ferron, P.J., Dumazeau, K., Beaulieu, J.F., Le Hegarat, L., Fessard, V., 2016. Combined effects of lipophilic phycotoxins (okadaic acid, azapsiracid-1 and yessotoxin) on human intestinal cells models. Toxins (Basel) 8 (2), 50.

Gu, H., Luo, Z., Krock, B., Witt, M., Tillmann, U., 2013. Morphology, phylogeny and azaspiracid profile of *Azadinium poporum* (Dinophyceae) from the China Sea. Harmful Algae 21-22, 64–75.

Hernandez-Becerril, D.U., Baron-Campis, S.A., Escobar-Morales, S., 2012. A new record of *Azadinium spinosum* (Dinoflagellata) from the tropical Mexican Pacific. Rev. Biol. Mar. Oceanogr. 47 (3), 553–557.

Hess, P., McCarron, P., Krock, B., Kilcoyne, J., Miles, C.O., 2014. Azaspiracids: chemistry, biosynthesis, metabolism and detection. In: Botana, L.M. (Ed.), Seafood and Freshwater Toxins: Pharmacology, Physiology, and Detection, 3rd ed. CRC Press, Boca Raton, FL, pp. 799–821.

Hickey, B.M., Trainer, V.L., Kosro, P.M., Adams, N.G., Connolly, T.P., Kachel, N.B., Geier, S.L., 2013. A springtime source of toxic *Pseudo-nitzschia* cells on razor clam beaches in the Pacific Northwest. Harmful Algae 25, 1–14.

Horner, R.A., Greengrove, C.L., Davies-Vollum, K.S., Gawel, J.E., Postel, J.R., Cox, A.M., 2011. Spatial distribution of benthic cysts of *Alexandrium catenella* in surface sediments of Puget Sound, Washington, USA. Harmful Algae 11, 96–105.

James, K.J., Furey, A., Lehane, M., Ramstad, H., Aune, T., Hovgaard, P., Morris, S., Higman, W., Satake, M., Yasumoto, T., 2002. First evidence of an extensive northern European distribution of azaspiracid poisoning (AZP) toxins in shellfish. Toxicon 40 (7), 909–915.

Kilcoyne, J., Jauffrais, T., J. Twiner, M., J., Doucette, G., Bunæs, J.A., Sosa, S., Krock, B., Sechet, V., Nulty, C., Salas, R., Clarke, D., Geraghty, J., Duffy, C., Foley, B., John, U., Quilliam, M., McCarron, P., Miles, C., Silke, J., Tillmann, U., Hess, P., 2014a. ASTOX 2 Final Report.

Kilcoyne, J., Nulty, C., Jauffrais, T., McCarron, P., Herve, F., Foley, B., Rise, F., Crain, S., Wilkins, A.L., Twiner, M.J., Hess, P., Miles, C.O., 2014b. Isolation, Structure Elucidation, Relative LC-MS Response, and in Vitro Toxicity of Azaspiracids from the Dinoflagellate *Azadinium spinosum*. J. Nat. Prod. 77 (11), 2465–2474.

Kim, J.H., Tillmann, U., Adams, N.G., Krock, B., Stutts, W.L., Deeds, J.R., Han, M.S., Trainer, V.L., 2017. Identification of *Azadinium* species and a new azaspiracid from *Azadinium poporum* in Puget Sound, Washington State, USA. Harmful Algae 68, 152–167.

Klontz, K.C., Abraham, A., Plakas, S.M., Dickey, R.W., 2009. Mussel-associated azaspiracid intoxication in the United States. Ann. Intern. Med. 150, 361.

Krock, B., Tillmann, U., Voß, D., Koch, B.P., Salas, R., Witt, M., Potvin, E., Jeong, H.J., 2012. New azaspiracids in Amphidomataceae (Dinophyceae): proposed structures. Toxicon 60 (5), 830–839.

Krock, B., Tillmann, U., Alpermann, T.J., Voß, D., Zielinski, O., Cembella, A.D., 2013. Phycotoxin composition and distribution in plankton fractions from the German Bight and western Danish coast. J. Plankton Res. 35 (5), 1093–1108.

Krock, B., Tillmann, U., Tebben, J., Trefault, N., Gu, H.F., 2019. Two novel azaspiracids

from *Azadinium poporum*, and a comprehensive compilation of azaspiracids produced by Amphidomataceae, (Dinophyceae). Harmful Algae 82, 1–8.
Lewitus, A.J., Horner, R.A., Caron, D.A., Garcia-Mendoza, E., Hickey, B.M., Hunter, M., Huppert, D.D., Kudela, R.M., Langlois, G.W., Largier, J.L., Lessard, E.J., RaLonde, R., Rensel, J.E.J., Strutton, P.G., Trainer, V.L., Tweddle, J.F., 2012. Harmful algal blooms along the North American west coast region: history, trends, causes, and impacts. Harmful Algae 19, 133–159.
Lloyd, J.K., Duchin, J.S., Borchert, J., Quintana, H.F., Robertson, A., 2013. Diarrhetic shellfish poisoning, Washington, USA, 2011. Emerg. Infect. Dis. 19 (8), 1314–1316.
Luo, Z., Krock, B., Giannakourou, A., Venetsanopoulou, A., Pagou, K., Tillmann, U., Gu, H., 2018. Sympatric occurrence of two *Azadinium poporum* ribotypes in the Eastern Mediterranean Sea. Harmful Algae 78, 75–85.
Luo, Z.H., Krock, B., Mertens, K.N., Price, A.M., Turner, R.E., Rabalais, N.N., Gu, H.F., 2016. Morphology, molecular phylogeny and azaspiracid profile of *Azadinium poporum* (Dinophyceae) from the Gulf of Mexico. Harmful Algae 55, 56–65.
MacKenzie, L., Beuzenberg, V., Holland, P., McNabb, P., Selwood, A., 2004. Solid phase adsorption toxin tracking (SPATT): a new monitoring tool that simulates the biotoxin contamination of filter feeding bivalves. Toxicon 44 (8), 901–918.
Nezan, E., Tillmann, U., Bilien, G., Boulben, S., Cheze, K., Zentz, F., Salas, R., Chomerat, N., 2012. Taxonomic revision of the dinoflagellate *Amphidoma caudata*: transfer to the genus *Azadinium* (Dinophyceae) and proposal of two varieties, based on morphological and molecular phylogenetic analyses. J. Phycol. 48 (4), 925–939.
Ofuji, K., Satake, M., McMahon, T., Silke, J., James, K.J., Naoki, H., Oshima, Y., Yasumoto, T., 1999. Two analogs of azaspiracid isolated from mussels, *Mytilus edulis*, involved in human intoxication in Ireland. Nat. Toxins 7 (3), 99–102.
Percopo, I., Siano, R., Rossi, R., Soprano, V., Sarno, D., Zingone, A., 2013. A new potentially toxic *Azadinium* species (Dinophyceae) from the Mediterranean Sea, *A. dexteroporum* sp. nov. J. Phycol. 49 (5), 950–966.
Potvin, E., Jeong, H.J., Kang, N.S., Tillmann, U., Krock, B., 2012. First report of the photosynthetic dinoflagellate genus *Azadinium* in the Pacific Ocean: morphology and molecular characterization of *Azadinium* cf. *poporum*. J. Eukaryot. Microbiol. 59 (2), 145–156.
PSEMP Marine Waters Workgroup. 2018. Puget Sound Marine Waters: 2017 Overview. Moore, S.K., Wold, R., Stark, K., Bos, J., Williams, P., Hamel, N. et al. (Eds.). URL:www.psp.wa.gov/PSmarinewatersoverview.php. Contact email: marinewaters@psemp.org.
Rossi, R., Dell'Aversano, C., Krock, B., Ciminiello, P., Percopo, I., Tillmann, U., Soprano, V., Zingone, A., 2017. Mediterranean *Azadinium dexteroporum* (Dinophyceae) produces six novel azaspiracids and azaspiracid-35: a structural study by a multi-platform mass spectrometry approach. Anal. Bioanal. Chem. 409 (4), 1121–1134.
Rundberget, T., Sandvik, M., Hovgaard, P., Nguyen, L., Aasen, J.A.B., Castberg, T., Gustad, E., Miles, C.O., 2006. Use of SPATT Disks in Norway: Detection of AZAs & DTXs and Comparison With Algal Cell Counts and Toxin Profiles in Shellfish. NZFSA, Wellington, New Zealand, pp. 35–38 Marine Biotoxin Science Workshop No. 23.
Salas, R., Tillmann, U., John, U., Kilcoyne, J., Burson, A., Cantwell, C., Hess, P., Jauffrais, T., Silke, J., 2011. The role of *Azadinium spinosum* (Dinophyceae) in the production of azaspiracid shellfish poisoning in mussels. Harmful Algae 10 (6), 774–783.
Satake, M., Ofuji, K., James, K.J., Furey, A., Yasumoto, T., 1998. New toxic event caused by Irish mussels. In: Reguera, B., Blanco, J., Fernandez, M.L., Wyatt, T. (Eds.), Harmful Algae. Santiago de Compostela: Xunta de Galicia and Intergovernmental Oceanographic Commission of UNESCO, pp. 468–469.
Smith, K., Rhodes, L., Harwood, D.T., Adamson, J., Moisan, C., Munday, R., Tillmann, U., 2016. Detection of *Azadinium poporum* in New Zealand: the use of molecular tools to assist with species isolations. J. Appl. Phycol. 28 (2), 1125–1132.
Taleb, H., Vale, P., Amanhir, R., Benhadouch, A., Sagou, R., Chafik, A., 2006. First detection of azaspiracids in mussels in north west Africa. J. Shellfish Res. 25, 1067–1070.
Tillmann, U., Elbrächter, M., Krock, B., John, U., Cembella, A., 2009. *Azadinium spinosum* gen. et sp. nov. (Dinophyceae) identified as a primary producer of azaspiracid toxins. Eur. J. Phycol. 44 (1), 63–79.
Tillmann, U., Elbrächter, M., John, U., Krock, B., Cembella, A., 2010. *Azadinium obesum* (Dinophyceae), a new nontoxic species in the genus that can produce azaspiracid toxins. Phycologia 49 (2), 169–182.
Tillmann, U., Soehner, S., Nezan, E., Krock, B., 2012. First record of the genus *Azadinium* (Dinophyceae) from the Shetland Islands, including the description of *Azadinium polongum* sp. nov. Harmful Algae 20, 142–155.
Tillmann, U., Gottschling, M., Nezan, E., Krock, B., Bilien, G., 2014a. Morphological and molecular characterization of three new *Azadinium* species (Amphidomataceae, Dinophyceae) from the Irminger Sea. Protist 165 (4), 417–444.
Tillmann, U., Salas, R., Jauffrais, T., Hess, P., Silke, J., 2014b. AZA: the producing organisms—biology and trophic transfer. In: Botana, L.M. (Ed.), Seafood and Freshwater Toxins: Pharmacology, Physiology, and Detection, 3rd ed. CRC Press, Boca Raton, FL, pp. 733–798.
Tillmann, U., 2018a. Electron microscopy of a 1991 spring plankton sample from the Argentinean Shelf reveals the presence of four new species of the Amphidomataceae (Dinophyceae). Phycol. Res. 66 (4), 269–290.
Tillmann, U., 2018b. Harmful Algae species fact sheet: amphidomataceae. In: Shumway, S.E., Burkholder, J.M., Morton, S.L. (Eds.), Harmful Algal Blooms: A Compendium Desk Reference. John Wiley & Sons, Hoboken, pp. 575–582.
Tillmann, U., Akselman, R., 2016. Revisiting the 1991 algal bloom in shelf waters off Argentina: *azadinium luciferelloides* sp nov (Amphidomataceae, Dinophyceae) as the causative species in a diverse community of other amphidomataceans. Phycol. Res. 64 (3), 160–175.
Tillmann, U., Borel, C.M., Barrera, F., Lara, R., Krock, B., Almandoz, G.O., Witt, M., Trefault, N., 2016. Azadinium poporum from the Argentine continental shelf, Southwestern Atlantic, produces azaspiracid-2 and azaspiracid-2 phosphate. Harmful Algae 51, 40–55.
Tillmann, U., Jaen, D., Fernandez, L., Gottschling, M., Witt, M., Blanco, J., Krock, B., 2017a. *Amphidoma languida* (Amphidomatacea, Dinophyceae) with a novel azaspiracid toxin profile identified as the cause of molluscan contamination at the Atlantic coast of southern Spain. Harmful Algae 62, 113–126.
Tillmann, U., Sanchez-Ramirez, S., Krock, B., Bernales-Jimenez, A., 2017b. A bloom of *Azadinium polongum* in coastal waters off Peru. Rev. Biol. Mar. Oceanogr. 52 (3), 591–610.
Tillmann, U., Trefault, N., Krock, B., Parada-Pozo, G., De la Iglesia, R., Vasquez, M., 2017c. Identification of *Azadinium poporum* (Dinophyceae) in the Southeast Pacific: morphology, molecular phylogeny, and azaspiracid profile characterization. J. Plankton Res. 39 (2), 350–367.
Tillmann, U., Edvardsen, B., Krock, B., Smith, K.F., Paterson, R.F., Voß, D., 2018. Diversity, distribution, and azaspiracids of Amphidomataceae (Dinophyceae) along the Norwegian coast. Harmful Algae 80, 15–34.
Tillmann, U., Gottschling, M., Krock, B., Smith, K.F., Guinder, V., 2019. High abundance of Amphidomataceae (Dinophyceae) during the 2015 spring bloom of the Argentinean Shelf and a new, non-toxigenic ribotype of *Azadinium spinosum*. Harmful Algae 84, 244–260.
Toebe, K., Joshi, A.R., Messtorff, P., Tillmann, U., Cembella, A., John, U., 2013. Molecular discrimination of taxa within the dinoflagellate genus *Azadinium*, the source of azaspiracid toxins. J. Plankton Res. 35 (1), 225–230.
Trainer, V., Moore, L., Bill, B., Adams, N., Harrington, N., Borchert, J., da Silva, D., Eberhart, B.-.T., 2013. Diarrhetic shellfish toxins and other lipophilic toxins of human health concern in Washington State. Mar. Drugs 11 (6), 1815–1835.
Trainer, V.L., Eberhart, B.T.L., Wekell, J.C., Adams, N.G., Hanson, L., Cox, F., Dowell, J., 2003. Paralytic shellfish toxins in Puget Sound, Washington State. J. Shellfish Res. 22 (1), 213–223.
Trainer, V.L., Cochlan, W.P., Erickson, A., Bill, B.D., Cox, F.H., Borchert, J.A., Lefebvre, K.A., 2007. Recent domoic acid closures of shellfish harvest areas in Washington State inland waterways. Harmful Algae 6 (3), 449–459.
Trainer, V.L., Hickey, B.M., Lessard, E.J., Cochlan, W.P., Trick, C.G., Wells, M.L., MacFadyen, A., Moore, S.K., 2009. Variability of *Pseudo-nitzschia* and domoic acid in the Juan de Fuca eddy region and its adjacent shelves. Limnol. Oceanogr. 54 (1), 289–308.
Trainer, V.L., Eberhart, B.T.L., Moore, L., Baugh, K., O'Rourke, L., Borchert, J., Cox, F., 2012. Diarretic shellfish toxins in washington state: a new threat to the shellfish industry. J. Shellfish Res. 31 (1) 354-354.
Turner, A.D., Goya, A.B., 2015. Occurrence and profiles of lipophilic toxins in shellfish harvested from Argentina. Toxicon 102, 32–42.
Twiner, M.J., Rehmann, N., Hess, P., Doucette, G.J., 2008. Azaspiracid shellfish poisoning: a review on the chemistry, ecology, and toxicology with an emphasis on human health impacts. Mar. Drugs 6, 39–72.
Twiner, M.J., Hess, P., Doucette, G.J., 2014. Azaspiracids: toxicology, pharmacology, and risk assessment. In: Botana, L.M. (Ed.), Seafood and Freshwater Toxins: Pharmacology, Physiology, and Detection, 3rd Ed. CRC Press, Boca Raton, FL, pp. 823–855.
U.S. Food and Drug Administration, 2019. Fish and Fisheries Products Hazards and Controls Guidance, 4th Ed. US Department of Health and Human Services, FDA, pp. 501. https://www.fda.gov/food/seafood-guidance-documents-regulatory-information/fish-and-fishery-products-hazards-and-controls-guidance.
Vale, P., Botelho, M.J., Rodrigues, S.M., Gomes, S.S., Sampayo, M.A.D., 2008. Two decades of marine biotoxin monitoring in bivalves from Portugal (1986-2006): a review of exposure assessment. Harmful Algae 7 (1), 11–25.
Van Egmond, H.P., Van Apeldoorn, M.E., Speijers, G.J.A., 2004. Marine Biotoxins. FAO Food Nutr Pap 80, 287.
Wietkamp, S., Tillmann, U., Clarke, D., Toebe, K., 2019. Molecular detection and quantification of the azaspiracid-producing dinoflagellate *Amphidoma languida* (Amphidomataceae, Dinophyceae). J. Plankton Res 41 (2), 101–113.
Yao, J.H., Tan, Z.J., Zhou, D.Q., Guo, M.M., Xing, L.H., Yang, S.G., 2010. Determination of azaspiracid-1 in shellfishes by liquid chromatography with tandem mass spectrometry. Chin. J. Chromatogr. 28 (4), 363–367.