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Original Article

Hiding in plain sight: Shellfish-killing phytoplankton in Washington State

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ABSTRACT

Summer bivalve shellfish mortalities have been observed in Puget Sound for nearly a century and attempts to understand and mitigate these losses have been only partially successful. Likewise, the understanding of the environmental conditions triggering shellfish mortalities and successful strategies for their mitigation are incomplete. In the literature, phytoplankton have played only a cursory role in summer shellfish mortalities in Washington State because spawning stress and bacteria were thought to be the primary causes. In recent years, the occurrence of *Protoceratium reticulatum* (Claparede & Lachmann) Buetschli and *Akashiwo sanguinea* (Hirasaka) Hansen & Moestrup, have been documented by the SoundToxins research and monitoring partnership in increasing numbers and duration and have been associated with declining shellfish health or mortality at various sites in Puget Sound. Blooms of these species occur primarily in summer months and have been shown to cause mass mortalities of shellfish in the U.S. and other parts of the world. In 2016-2017, yessotoxins (YTX) were measured in several species of Puget Sound bivalve shellfish, with a maximum concentration of 2.20 mg/kg in blue mussels, a value below the regulatory limit of 3.75 mg/kg established by the European Union for human health protection but documented to cause shellfish mortalities in other locations around the world. In July 2019, a bloom of *P. reticulatum* coincided with a summer shellfish mortality event, involving a dramatic surfacing of stressed, gaping Manila clams, suggesting that YTX could be the cause. YTX concentrations in their tissues were measured at a maximum of 0.28 mg/kg and histology of these clams demonstrated damage to digestive glands. A culture of *P. reticulatum*, isolated from North Bay during this massive bloom and shellfish mortality event, showed YTX reaching 26.6 pg/cell, the highest recorded toxin quota measured in the U.S. to date. Concentrations of YTX in phytoplankton samples reached a maximum of 920 ng/L during a *P. reticulatum* bloom in Mystery Bay on 13 August 2019 when cell abundance reached 1.82 million cells/L. The highest cellular YTX quota during that bloom that lasted into September was 10.8 pg/cell on 3 Sept 2019. Shellfish producers in Washington State have also noted shellfish larvae mortalities due to *A. sanguinea* passing through filtration intake systems into hatchery facilities. Early warning of shellfish-killing harmful algal bloom (HAB) presence in Puget Sound, through partnerships such as SoundToxins, provides options for shellfish growers to mitigate their effects through early harvest, movement of shellstock to upland facilities, or enhanced filtration at aquaculture facilities.

1.0. Introduction

1.1. Set the stage – Washington is a vital shellfish growing region

A national leader in shellfish farming, Washington State has been cultivating shellfish for more than 160 years, providing a safe, sustainable food to the region, the nation, and the world. Washington State is the largest producer of farmed bivalve shellfish in the United States (USDA (United States Department of Agriculture) 2018). It is estimated that Washington shellfish growers directly and indirectly employ more than 3,200 people and provide an estimated total economic contribution of $270 million (Washington Sea Grant, 2015).

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Pacific oysters (*Crassostrea gigas*) Manila clams (*Venerupis philippinarum*), geoduck (*Panopea generosa*) and mussels (*Mytilus galloprovincialis, Mytilus trossulus*) are the primary shellfish species grown in Washington State (Washington Sea Grant, 2015).

1.2. Shellfish mortalities

Shellfish mortalities in Puget Sound have been studied and documented for over 50 years (Glude, 1974; Cardwell et al., 1977; Cardwell et al., 1979). As early as the 1930s, mass mortalities of adult and juvenile bivalve shellfish in Puget Sound were noted by shellfish growers and researchers (Nightingale, 1936). The number of annual mortality events and shellfish size classes affected by these events has varied from year to year. Pathogens, predators, spawning stress, and sudden environmental changes, such as salinity, harsh winters, warm temperature, low dissolved oxygen, and toxic algal blooms have been identified as causes of mortality in oysters (Cheney et al., 2000; Mackin, 1961). Symptoms of compromised health in shellfish include gaping (increased distance between valve pairs), surfacing (digging out of sediment), and sloughing (mussels falling off their lines).

In the 1960s, the Washington State Department of Fisheries established a monitoring program to evaluate water quality conditions in select shellfish growing embayments that sustained oyster larvae development and growth. The locations associated with historical summer and fall oyster larvae mortality included Sequim, Discovery and Liberty Bays, Dyes Inlet, Hood Canal, and waters south of Tacoma (summarized in Cardwell et al., 1977). Among other things, these studies noted the presence of exceptional algal blooms during the time when the majority of oyster mortalities occurred. At that time, the dominant algal blooms included *Gymnodinium* spp. (synonym *A. sanguinea*) and *Gonyaulax* spp. (synonym *A. catenella*) in Sequim and Discovery Bays and *Ceratium fusus* in the Puget Sound basin. The researchers concluded that Pacific oyster larvae mortality was most likely linked to bacteria, toxic metabolites of phytoplankton, or both.

1.3. Attempts to reduce mortalities

Earnest attempts have been made to address these summer occurring shellfish mortalities by methods such as the creation of triploid oysters to reduce spawning stress, and the adoption of shellfish farm best management practices to address *Vibrio* bacteria and other bacteria-related mortality concerns (Leibovitz, 1978, Cheney et al., 2000). Specifically, the control of temperature for shellfish storage was used to slow *Vibrio* bacterial growth. However, despite extensive research, these techniques did not eliminate shellfish mortalities. Investigations of summer shellfish mortality in France demonstrated that the presence of bacteria alone did not consistently cause mortality (Houssin et al., 2019). In fact, one of the solutions implemented to reduce summer mortalities due to spawning stress, the triploid oyster, has been shown to result in higher mortality rates at some sites compared to diploid oysters (Wadsworth, 2018). Thus, aquaculturists and scientists have concluded that additional studies are needed to consider other factors that could be contributing to shellfish deaths.

1.4. Have harmful phytoplankton been overlooked as a cause of mass shellfish mortalities in Washington State?

After the initial research of Cardwell et al. (1977; Cardwell et al., 1979) on summer mortality events, there has been no comprehensive follow up research or year-round monitoring at the many important shellfish growing areas in Puget Sound until the creation of the SoundToxins partnership in 2006. SoundToxins was established to train shellfish growers, environmental learning centers, Native Tribes, universities, Federal, State and local governments, and private residents to monitor phytoplankton cells routinely at multiple sites in Puget Sound to benefit the Washington State Department of Health. Specifically, SoundToxins warns managers of the presence of harmful algae, primarily those of human health concern and documents other phytoplankton present throughout the year. With unusual mortality events of bivalve shellfish, the program expanded to include weekly monitoring and reporting for phytoplankton that have been shown to harm shellfish in other parts of the world. Due to the changing naming convention for these species, at times it has been difficult to reconcile historical reports of phytoplankton blooms in Puget Sound with present day observations. Here we connect those past records with current observations and provide evidence that *Protoceratium reticulatum* (Claparede & Lachmann) Bütschli (synonymous with *Gonyaulax grindleyi* Reinecki, *Operculodinium centrocarpum* [Diflandre et Cookson] Wall, *Protoceratium aceros* [Bergh]) and *Akashiwo sanguinea* (Hirasaka) Hansen & Moestrup (synonymous with *Gymnodinium splendens* [Lebour], *Gymnodinium sanguineum* [Hirasaka], *Gymnodinium nelson* [Martin]) are associated with mass mortality of shellfish in Puget Sound.

1.5. Some harmful phytoplankton that affect shellfish health

Some dinoflagellate species, *Protoceratium reticulatum, Lingulodinium polyedrum*, and *Gonyaulax spinifera*, produce yessotoxin (YTX), a sulfated polyether compound and a number of hydroxylated and sulfated analogues, which are either produced by the dinoflagellates or are transformed in the shellfish (summarized in Paz et al., 2008). The mechanism of action of YTX specifically in shellfish is poorly understood, however immunochemical studies have demonstrated involvement of YTX in immune system and digestive function (Franchini et al., 2003; Franchini et al., 2010). *Protoceratium reticulatum* was first confirmed as a YTX producer in Washington State in 2004 (Howard et al., 2008). Prior to that, *Lingulodinium polyedra* was found to produce YTX, primarily in south-central California (Armstrong and Kudela, 2006). Mass mortalities of shellfish and other invertebrates along the California coast in 2012 were associated with *Gonyaulax spinifera*, another confirmed YTX producer (Rogers-Bennett et al., 2012). Further north in British Columbia, Canada, Cassis (2005) observed a mass mortality of juvenile Pacific oysters (*C. gigas*) at a farm site in Jervis Inlet, British Columbia, Canada, in the northern Salish Sea (Puget Sound is in the southern part of the Salish Sea). A bloom of *P. reticulatum* was observed just prior to and during the mortality events and was identified as the probable cause of the die off. Exposure of juvenile oysters to *P. reticulatum* "bloom" water resulted in strong rejection of feeding, lack of particle clearance, and complete closure of shells. However, feeding resumed when other phytoplankton food was provided.

In Puget Sound, reports of "red tides" associated with Olympia oyster (*O. lurida*) mortalities in 1929, 1934, 1935 in Oakland Bay, south Puget Sound, were associated with an elevated abundance of *A. sanguinea* (previously named *Gymnodinium sanguineum, Gymnodinium splendens*) at densities reaching 30,000 cells/mL (Nightingale, 1936). Laboratory exposure experiments demonstrated responses in oysters ranging from excessive mucus production to cessation of feeding (Nightingale, 1936). The mechanism of action of *A. sanguinea* toxicity is not known although Kim et al. (1999) found that *A. sanguinea* can produce reactive oxygen species that may be toxic. More recent studies with cultured isolates of *A. sanguinea* demonstrate that, although toxicity is variable from isolate to isolate, they produce a toxic chemical or chemicals (Xu et al., 2017) that are believed to be responsible for mass mortalities of marine animals observed in Texas, USA (Harper and Guillen, 1989), China (Wu et al., 2001), Peru (Kahru et al., 2004), and other locations worldwide.

The goals of our study were to determine the linkage between phytoplankton species not typically monitored for the protection of human health and shellfish mortalities in Puget Sound. This was achieved by connecting routine monitoring data collected by SoundToxins personnel with observations of illness and mortalities by shellfish growers and farmers in the region. The measurement of toxins produced by specific phytoplankton species and in shellfish in the areas where mortalities occurred established a connection between phytoplankton

blooms and compromised shellfish health. Here we present SoundToxins monitoring results and associated research data that confirm the linkage between phytoplankton and shellfish mortalities in Puget Sound.

2.0. Methods

2.1. SoundToxins research and monitoring

SoundToxins is a monitoring and research program that has grown from 4 partners in 2006 to 25 partner organizations in 2021, some of which monitor multiple sites. These partners include state shellfish managers, environmental learning centers, tribes, residents, and commercial fish and shellfish farmers in Puget Sound, an inland fjord in western Washington State (Fig. 1). Puget Sound is part of a larger body of water called the Salish Sea, which also includes the waters of British Columbia, the Strait of Georgia. One of the objectives of the SoundToxins program is to document unusual bloom events and new species entering the Salish Sea. Surface seawater samples were collected weekly from 1 March to 1 October and biweekly during all other months for analysis of harmful phytoplankton relative and quantified abundance, particulate and dissolved toxins (Trainer et al., 2016). In addition, environmental parameters, including temperature and salinity were monitored. Phytoplankton cells were isolated from water samples to establish cultures for research purposes, in particular to establish cellular toxin quotas produced by Puget Sound strains.

2.2. Phytoplankton counts

Seawater was collected in 2018 and 2019 at SoundToxins sites (Fig. 1) from the surface using a bucket, then transferred to a 2-liter bottle. A fixed whole water sample was prepared by aliquoting 20 mL seawater into a labeled scintillation vial, adding 1 mL of formaldehyde fixative (~1% final concentration), capping the vial and mixing by inversion. A 10x concentrated whole water sample was prepared by

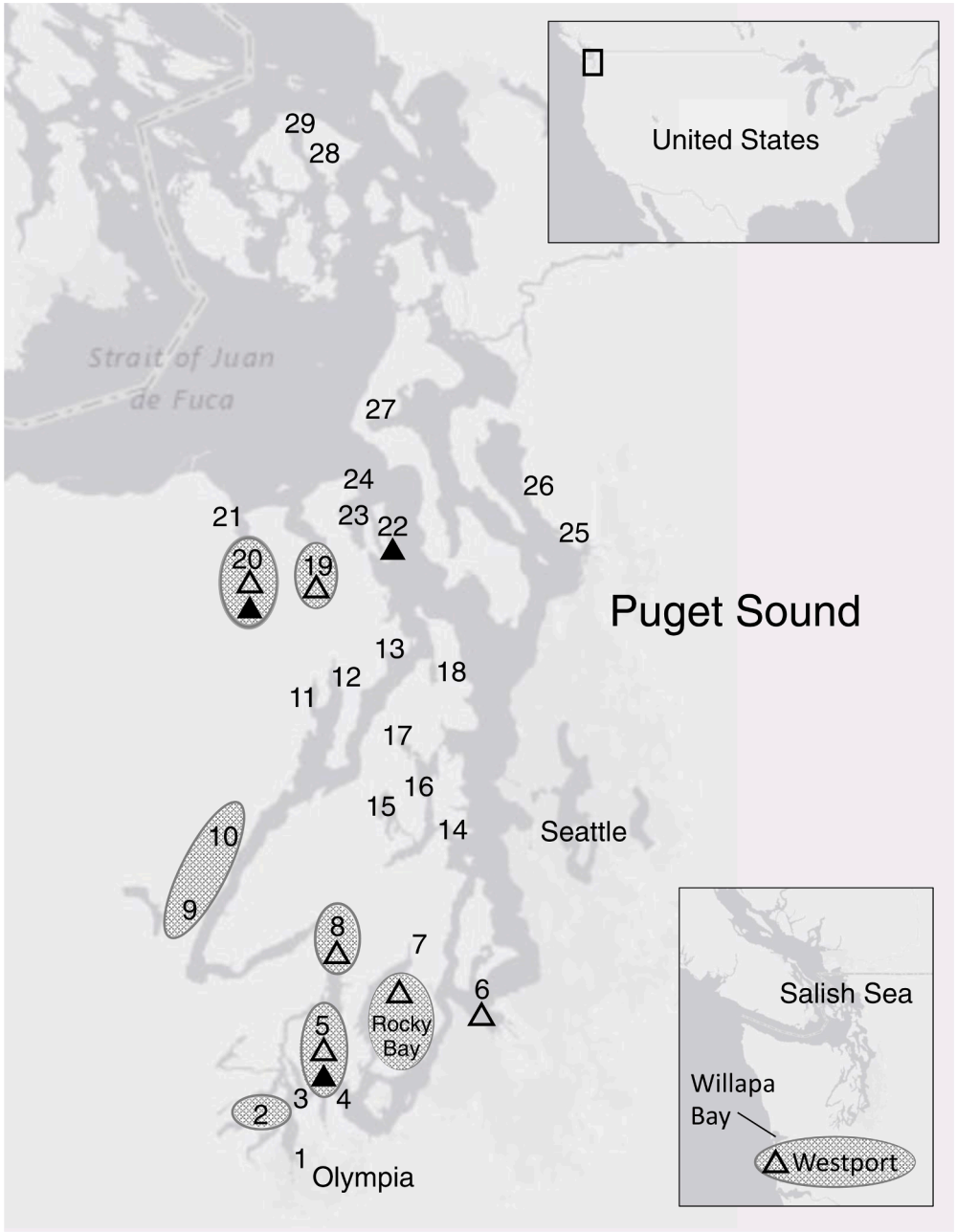

Figure 1. Numbered SoundToxins sampling sites. SoundToxins sites are: 1. Budd Inlet South, 2. Totten Inlet, 3. Budd Inlet North, 4. Nisqually Reach, 5. Spencer Cove, 6. Quartermaster Harbor, 7. Burley Lagoon, 8. North Bay, 9. Glen Ayr, 10. Hama Hama, 11. Quilcene Bay, 12. Dabob Bay, 13. Hood Head, 14. Clam Bay, 15. Dyes Inlet, 16. Brownsville, 17. Liberty Bay, 18. Port Gamble, 19. Discovery Bay, 20. Sequim Bay South, 21. Sequim Bay North, 22. Mystery Bay, 23. Port Townsend, 24. Fort Worden, 25. Tulalip Bay, 26. Port Susan, 27. Penn Cove, 28. Glenwood Springs, 29. East Sound. Locations where YTX was measured in seawater (solid triangles; data shown in Table 1) and shellfish (open triangles in Figure 1; data shown in Table 2). The solid triangle at North Bay indicates YTX measured in *P. reticulatum* (NWFSC 614) isolated from this site. Sites where shellfish mortalities were observed are shaded. Rocky Bay and Willapa Bay are not routine monitoring locations for SoundToxins but are sites where shellfish mortalities occurred. Upper inset: the location of Puget Sound in Washington State, USA. Lower inset: the Salish Sea is a transboundary sea that spans British Columbia, Canada and Washington State, USA, including the Strait of Georgia, the Strait of Juan de Fuca and Puget Sound. The location of the outer coast town of Westport, Willapa Bay is shown.

Table 1
Protoceratium reticulatum abundance and cellular yessotoxin quotas

Location	Date	*P. reticulatum* (relative abundance)	*P. reticulatum* (cells/L)	YTX (ng/L)	YTX (pg/cell)
Mystery Bay	06-08-2019	Common	115000	0	0
Mystery Bay	13-08-2019	Bloom	1820000	920	0.5
Mystery Bay	20-08-2019	Bloom	41000	160	3.9
Mystery Bay	27-08-2019	Bloom	41000	330	8
Mystery Bay	03-09-2019	Bloom	36000	390	10.8
Mystery Bay	10-09-2019	Common	67500	260	3.8
Sequim Bay	06-09-2017	Present		850	
Sequim Bay	12-09-2017	Bloom		470	
Sequim Bay	18-09-2017	Present	1100000	170	0.2
Sequim Bay	24-09-2019	Common		220	
NWFSC 614*			170000	4520	26.6
NWFSC 614*			195000	4360	22.4

* cultured isolate of *P. reticulatum* from North Bay. When data are not shown, no samples were analyzed.

inverting the 2-liter bottle 3x to mix and pouring a 50 mL aliquot into a glass test tube, and mixing by gentle inversion. After 24 h of settling without disturbance, 45 mL of liquid were aspirated from the top of the tube using a pipette without disturbing the settled cells. The remaining 5 mL of sample were transferred into a labeled 20 mL scintillation vial for sample archiving and preserved cell counting. Cell counts were performed using a Zeiss Axiovert 135 inverted light microscope on either 1 mL of well-mixed 10x or unconcentrated whole water using a gridded Sedgewick-Rafter counting slide.

2.3. *Protoceratium reticulatum* isolation and culturing

A *P. reticulatum* strain (NWFSC614) was isolated from North Bay, WA (Fig. 1) on 7 July 2019 associated with the massive shellfish mortality event in order to confirm the production of YTX in a lab isolate. An individual cell was picked using a flame-drawn capillary tube and aseptically transferred to growth medium. The isolate was grown in nutrient-enriched, filter-sterilized (0.2 μm, PES, Nalgene) Puget Sound seawater as described by Bill et al. (2016). Culture flasks used were Corning™ Falcon™ 50 mL polystyrene flasks. Cultures were maintained in an environmental incubator at 14°C on a 14:10 h light:dark cycle and illuminated by soft-white fluorescent bulbs at a photosynthetic photon flux density (PPFD) of ~ 80–100 μmol photons/m^2/s^1. A 50 mL aliquot was filtered, extracted, and analyzed by liquid chromatography–mass spectrometry (LC-MS) as described below.

2.4. Relative abundance estimates

Phytoplankton samples were collected in 2018 and 2019 at SoundToxins sampling sites (Fig. 1) using a weighted 20 μm-mesh plankton net by allowing the net to drop to near the bottom. The net was pulled up to the surface at a rate of ~1 m/sec, and the total number of meters vertically towed was documented. The cod end was removed from the net and the sample was swirled to mix before pouring into a glass jar. Twenty mL of the well-mixed net tow sample were transferred into a labeled scintillation vial. Fixative was added to a final concentration of ~1% formaldehyde and inverted to mix. A 0.1 mL aliquot of live or preserved net tow material was placed on a Palmer-Maloney slide for examination under a light microscope. Relative abundance was noted as present, common, or bloom (Trainer et al., 2016; in which example micrographs are provided for each relative abundance category).

2.5. Phytoplankton sample preparation and extraction

Particulate (cellular) toxin samples were prepared by filtering 1 L of whole seawater using up to two filters (47 mm, 0.45 μm; Millipore, HAWP). Filters were folded in half with forceps, wrapped into aluminum foil packets and stored at -20°C until analysis at the Northwest Indian College lab. These filter(s) for a single sample were placed into a 15 mL polypropylene centrifuge tube (BD Falcon), covered with 2 mL of 80% methanol, macerated by hand using a spatula for about 3 min and bath sonicated for 60 min (Branson 5510 sonicator). Samples were then vortexed, centrifuged for 10 min at 2500 × *g*, and the supernatant transferred to a glass vial. The residual pellets were re-extracted with 1 mL of 80% methanol by vortexing for 3 min, followed by centrifugation for 10 min at 2500 × *g*. The resulting supernatant was combined with the first extract and evaporated to less than 2 mL with nitrogen at 25°C. The final extract was brought to 2 mL with 100% methanol, filtered through a 0.22 μm PTFE syringe filter (Fisher Scientific), and stored at 4°C in glass vials until analyzed by LC-MS/MS.

For all data shown in Table 1, filters were prepared as above and stored at -20°C until analysis. The filters were analyzed following Shultz et. al (2019) and Villar-Gonzalez et al. (2008), modified for filters instead of shellfish tissue. Briefly, filters were placed in 50 mL falcon tubes (BD falcon) with 18 mL of MeOH, vortexed for 3 min, then sonicated on ice at 60% power using a Fisherbrand 120 Sonic Dismembrator (Fisher Scientific) until the filter was broken up (~60 s). Samples were then centrifuged for 8 min at 2500 x *g* and filtered through a PTFE syringe filter. About 2 mL of MeOH were added to the filtrate to bring the sample volume up to 20 mL, and then briefly vortexed. An aliquot of 2.5 mL was placed in a glass culture tube, and the remaining filtrate was archived and kept frozen. A 2.5N NaOH solution (313 μL) was added to the aliquot, then vortexed for 30 s. The mixture was incubated at 76°C in a Poly Science 10L Water Bath (Poly Science, USA) for 40 min with a lid to avoid evaporation. Samples were then removed and cooled to room temperature (about 30 min), before 313 μL of 2.5N HCL were added, and again vortexed for 30 s. Samples were then verified that the pH was between 4 and 5 and adjusted as necessary. Finally, the hydrolyzed extract was filtered through a PTFE syringe filter, aliquoted to a LC-MS vial, and stored at -80°C until analysis.

Table 2
Concentrations of yessotoxins in Puget Sound shellfish.

Shellfish Species	Date Collected	Site	Total YTX equiv. (mg/kg)	YTX (mg/kg)	45-OH-YTX (mg/kg)	45-OH-YTX (%)
Blue mussel	14-06-2016	Sequim Bay South	0.033	0.026	0.007	21.2
Pacific oyster	21-06-2016	Sequim Bay South	ND	ND	ND	ND
Manila clam	21-06-2016	Sequim Bay South	ND	ND	ND	ND
Littleneck clam	21-06-2016	Sequim Bay South	ND	ND	ND	ND
Blue mussel	05-07-2016	Sequim Bay South	0.030	0.024	0.006	20.0
Blue mussel	12-07-2016	Sequim Bay South	0.049	0.042	0.007	14.3
Blue mussel	19-07-2016	Sequim Bay South	0.068	0.051	0.017	25.0
Blue mussel	25-07-2016	North Bay	0.032	0.028	0.004	12.4
Blue mussel	25-07-2016	Sequim Bay South	0.093	0.075	0.019	20.4
California mussel	01-08-2016	Westport	0.047	0.042	0.005	10.6
Blue mussel	02-08-2016	North Bay	0.162	0.135	0.027	16.7
Blue mussel	02-08-2016	Sequim Bay South	0.155	0.136	0.020	12.9
Pacific oyster	02-08-2016	Sequim Bay South	0.004	0.004	< LOQ	NA
Blue mussel	16-08-2016	Sequim Bay South	0.472	0.384	0.088	18.6
Pacific oyster	16-08-2016	Sequim Bay South	0.013	0.008	0.005	39.4
Blue mussel	24-08-2016	Westport	0.027	0.027	< LOQ	NA
California mussel	29-08-2016	Westport	0.570	0.441	0.129	22.6
Blue mussel	31-08-2016	Sequim Bay South	0.403	0.324	0.079	19.6
Blue mussel	07-09-2016	Sequim Bay South	0.914	0.687	0.227	24.8
California mussel	12-09-2016	Westport	0.026	0.026	< LOQ	NA
Blue mussel	12-09-2016	Quartermaster Harbour	0.006	0.006	< LOQ	NA
Blue mussel	14-09-2016	Sequim Bay South	0.816	0.637	0.179	21.9
Manila clam	14-09-2016	Sequim Bay South	ND	ND	ND	ND
Pacific oyster	14-09-2016	Sequim Bay South	0.010	0.007	0.004	38.5
Blue mussel	19-09-2016	Quartermaster Harbour	0.005	0.005	< LOQ	NA
California mussel	19-09-2016	Westport	0.016	0.016	<LOQ	NA
Blue mussel	20-09-2016	Sequim Bay State Park	0.022	0.022	<LOQ	NA
California mussel	25-09-2016	Westport	0.018	0.018	<LOQ	NA
Blue mussel	26-09-2016	Quartermaster Harbour	0.004	0.004	ND	ND
Blue mussel	27-09-2016	Sequim Bay South	0.701	0.548	0.153	21.8
Littleneck clam	27-09-2016	Sequim Bay South	ND	ND	ND	ND
Manila clam	27-09-2016	Sequim Bay South	ND	ND	ND	ND
Pacific oyster	27-09-2016	Sequim Bay South	0.009	0.005	0.004	44.4
California mussel	05-07-2017	Sequim Bay South	0.046	0.042	0.005	10.9
Blue mussel	02-08-2017	Discovery Bay	0.471	0.421	0.051	10.8
Blue mussel	03-08-2017	Sequim Bay South	0.193	0.159	0.034	17.7
Manila clam	17-07-2019	Rocky Bay	0.069	0.069	NA	NA
Manila clam	18-07-2019	Rocky Bay	0.281	0.281	NA	NA
Pacific oyster	17-08-2019	Spencer Cove	0.037	0.037	NA	NA

ND=Not detectable; NA=Not Analyzed or Not Applicable; For locations of sample sites, see Fig. 1.
Both the detection limit and limit of quantitation (LOQ) were 0.004 mg/kg; homo-YTX and 45-OH-homo-YTX were not detected.

2.6. Shellfish collection and preparation

Shellfish were collected and analyzed in 2016 and 2017 as part of a separate NOAA Monitoring and Event Response for Harmful Algal Blooms (MERHAB) project, "Clear and present danger: monitoring and management of lipophilic shellfish toxins in Washington State" to quantify diarrhetic shellfish toxins in various shellfish species. Shellfish were collected by Washington Sea Grant at shellfish mortality sites in 2019. Shellfish tissue was thawed, rinsed briefly to remove sand and debris, and drained before being homogenized by a high-powered blender and stored at -20°C until analysis. A 2 g aliquot of this tissue homogenate was extracted and hydrolyzed following the same method described for particulate toxin samples above (Shultz et al., 2019). All samples were aliquoted into LC-MS vials and were stored at -80°C until analysis, within 2-3 months of sample collection.

2.7. Analysis of yessotoxins (YTXs) in shellfish tissue and phytoplankton

For samples collected in 2016-2017 in Table 2, shellfish homogenates were precooked at 70°C for 20 min, extracted with methanol twice, and defatted with hexane (Wang and Doucette, 2021). YTX extracts from shellfish or phytoplankton were analyzed by LC-MS using an HP1100 system (Agilent Technologies, USA) coupled to an API4000 mass spectrometer (AB Sciex, USA). LC separation was performed on an Xbridge C18 column (150 × 3 mm, 5 μm; Waters, USA) at a temperature of 30°C using mobile phases water (A) and 90% acetonitrile/water (B), both containing 6.7 mM ammonium hydroxide, at a flow rate of 0.4 mL/min (Gerssen et al., 2009). YTX congeners were detected in negative ion mode using the following MRM transitions: m/z 570.2 → 467.2 and 502.2 for YTX, m/z 577.2 → 474.2 and 403.1 for homo-YTX, m/z 578.2 → 467.2 and 396.1 for 45-OH-YTX, m/z 585.2 → 474.2 and 403.1 for 45-OH-homo-YTX.

Hydrolyzed extracts for YTX from shellfish (2019 samples in Table 2) or phytoplankton (Table 1) were analyzed by LC–MS with a 1290 Infinity II System coupled to a 6460A Triple Quadrupole Mass Spectrometer using Electrospray Ionization (ESI JetStream Source; Agilent Technologies, USA). LC separation was performed on a Zorbex Eclipse Plus C8 column (4.6 × 75 mm, 3.5 μm; Agilent Technologies, USA) at a temperature of 30°C using 2 mM ammonium acetate in water (A) and MeOH (B) at a flow rate of 0.2 mL/min on a linear gradient (Keuth and Glauner, 2010). YTX congeners were detected in negative ionization mode using the following MRM transitions: m/z 1141.5 → 1061.3 and 925.5 for YTX and m/z 1155.4 → 1075.5 for homo-YTX. No YTX congeners were detected in phytoplankton (Table 1). Standards were from the National Research Council (NRC, Canada).

2.8. Shellfish histology

Histology was performed based on the method described in Friedman et al. (2005). Briefly, a transverse section was cut through the anterior portion of the visceral mass including mantle, gonad, stomach, and digestive gland. Tissue samples were preserved in Davidson's

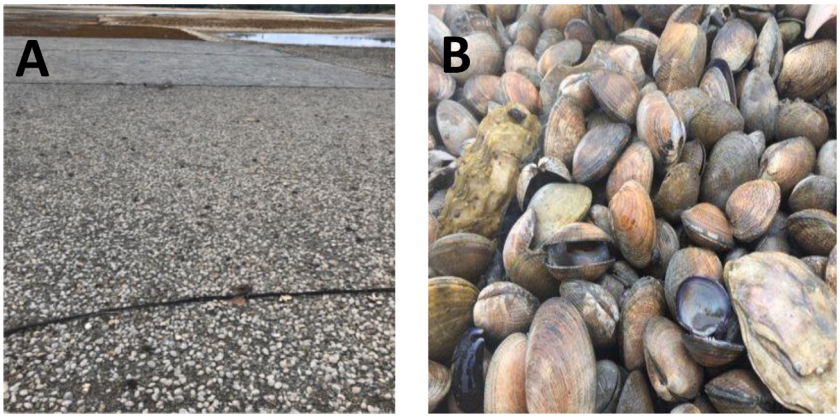

Figure 2. Surfacing, dying and dead Manila clams Rocky Bay, 2019. A. Section of beach with surfacing clams, B. Close view of gaping clams.

solution (Shaw and Battle, 1957) and processed using routine paraffin histology. Sections were stained with hematoxylin and eosin (Luna, 1968) and viewed by light microscopy to characterize morphological changes.

3.0. Results

3.1. Shellfish mortalities and histology

On July 15, 2019 in Rocky Bay, a dramatic surfacing of stressed, gaping clams, predominantly Manila clams (*V. philippinarum*), was noted by community residents and shellfish producers (Fig. 2A, B).

On July 16, a whole water sample from that site revealed a large bloom of *P. reticulatum* (Fig. 3A), including a massive number of cysts (Fig. 3B). After dissection of shellfish tissues in the laboratory, cysts of *P. reticulatum* were isolated from the Manila clam digestive tract (Fig. 3C).

On July 17, more of the clams began to die, yet on July 19 some of the clams were able to dig back into the sediment and on July 22, the bloom was dissipating. Shellfish samples were submitted on July 18 for histological examination, which indicated that the clam digestive glands had substantial damage, suggesting a toxic insult and blood borne bacterial colonies and occasional dying blood cells (Fig. 4). The analysis of YTX in Manila clams collected from Rocky Bay showed 0.07 mg/kg on July 17 and 0.28 mg/kg total YTX equiv. in clams harvested the next day (Table 2).

During this event, residents of Rocky Bay noted that seagulls and other foraging wildlife were not scavenging on the meats of the dead clams at low tide (T. King, pers. comm.). A shellfish farmer in Rocky Bay noted that the mortality event impacted approximately 30% of their standing stock and included all age classes, with higher mortality closer to shore in the intertidal area. They also noted that all species of clams were impacted and suggested that native species may have suffered higher mortality rates.

3.2. SoundToxins observations

SoundToxins partners observed varying abundances of *P. reticulatum* and *A. sanguinea* at the majority of sites sampled throughout Puget Sound in 2018 and 2019 (Fig. 5).

Most of the blooms of both species occurred in summer months, though these species were reported throughout Puget Sound during most of the year (Fig. 6). Shellfish mortalities and observations, including surfacing and gaping, were compiled by SoundToxins for several locations in Puget Sound (Table 3). In 2010 and 2017, mussel farmers in Totten Inlet noted the dramatic sloughing of Mediterranean mussels from the outer lines on their rafts in concert with a bloom of *A. sanguinea.*

In 2011, SoundToxins partners and members of the Jamestown S'Klallam Tribe in Sequim Bay observed an increase in *P. reticulatum* cells and a large die-off of various clam species, where morbid clams were observed surfacing out of the substrate (Table 3). Concentrations of YTX up to 0.89 mg/kg were measured in shellfish, primarily blue mussels, in July and August, 2012 (Trainer et al., 2013). In 2017, SoundToxins personnel at shellfish farms in Hood Canal and south Puget Sound witnessed an increasing number of *P. reticulatum* cells in their water samples and simultaneously observed increased shellfish mortality of both juvenile and adult bivalves in 2017 (Table 3). One shellfish farm observed an unexpected die-off of juvenile geoduck (*Panopea generosa*) and Pacific oysters (*Crassostrea gigas*) at their hatchery in Spencer Cove during a bloom of *P. reticulatum*. In 2017 and 2018, another shellfish company noted large unexplained die-offs of juvenile Pacific oysters in Discovery Bay and varnish clams (*Nuttallia obscurata*) in Hood Canal associated with the presence of a large *P. reticulatum* bloom. In the spring and summer, coincident with observances of *A. sanguinea*, a shellfish company in Totten Inlet suffered juvenile and adult Pacific oyster mortality in their hatchery system and farm. In summer of 2018, a grower in Hood Canal experienced a high mortality

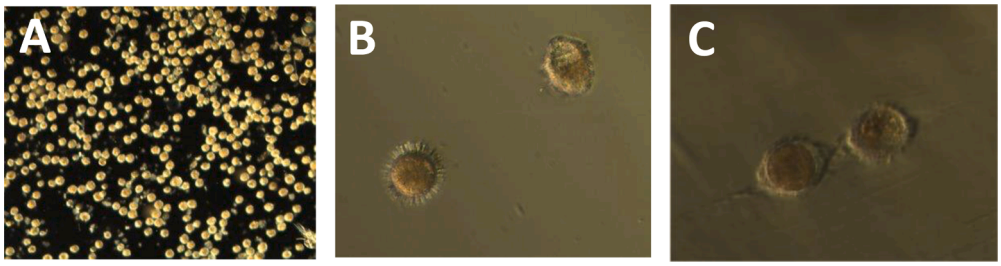

Figure 3. Light microscope images of *P. reticulatum* in North Bay and Rocky Bay, Case Inlet. A. *P. reticulatum* cells in whole water sample collected on 7 July 2019 (80x magnification), B. Vegetative cell (upper right) and cyst with spines (400x magnification) from a whole water sample from Rocky Bay, C. *P. reticulatum* cysts isolated from the digestive tract of a Manila clam collected in July 2019. The cysts have a pinched appearance due to partial digestion by the clam (400x magnification).

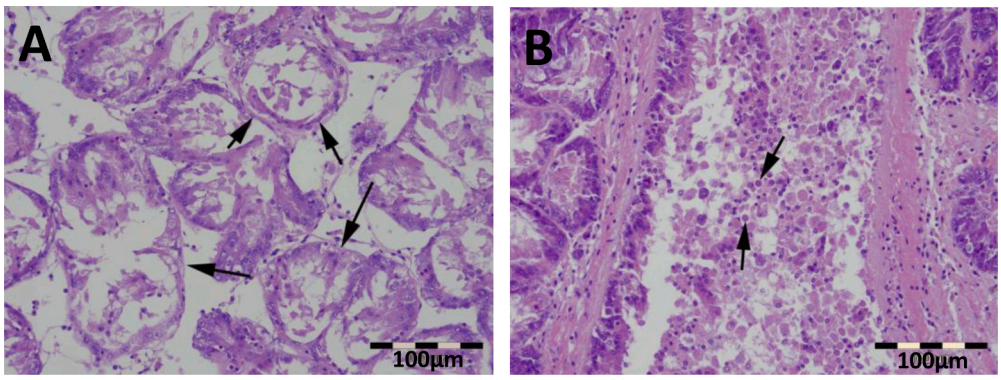

Figure 4. Histological images from Rocky Bay Manila clams, collected in July 2019. A. Abnormal digestive gland tubules with examples shown at arrows. The digestive gland tubules have markedly reduced height and sloughing of epithelial cells that can indicate a lack of feeding possibly due to toxic exposure. B. Black arrows show examples of dying clam blood cells indicated by pycnotic nuclei.

event of all age classes of cultured and wild Pacific oysters on their farm that was correlated to an unprecedented bloom of *Phaeocystis*. In the spring of 2018, shellfish growers in Willapa Bay reported "unusual" Manila clam (*V. philippinarum*) mortality events and, similar to previous reports, morbid clams were observed surfacing before their death (Table 3). A number of growers reported Manila clam losses of 30%-90% on commercially cultivated beds in Willapa Bay. A pathological investigation of shellfish from Willapa Bay suggested an algal bloom may have produced a toxin or substance that induced massive tissue inflammation leading to the death of affected clams (Zachary Forster, WFDW, pers. comm.).

3.3. Seasonal occurrence and toxin content of shellfish-killing algae

Increased observations of shellfish mortality since 2017 (Table 3) motivated the inclusion of suspected shellfish-killing phytoplankton for mandatory reporting within SoundToxins identification protocols beginning in 2018. Weekly SoundToxins data provide relative abundance information describing the frequency and intensity of *A. sanguinea* and *P. reticulatum* in Puget Sound (Fig. 6). The most frequent observations of *P. reticulatum* in 2018 and 2019 were in July, August, and September, ranging from 15-23 sites reporting observations during each of those months in 2018 and 24-32 sites in 2019 from a total number of 824 observations at the sites sampled for those species in 2018 and 894 observations in 2019. *A. sanguinea* was observed most frequently in August through October, with 19-24 sites reporting observations each month in 2018 and 23-32 sites in 2019. Blooms of *P. reticulatum* were observed most frequently in July in both years, whereas blooms of *A. sanguinea* were observed most often in July in 2018 and September in 2019. To provide insight into the geographical distribution of these two species, the reported maximum relative abundance at each SoundToxins site during summer 2018 and 2019 is shown in Fig. 5. Blooms of *P. reticulatum* were observed in Mystery Bay and North Bay in summer 2018 and 2019, with additional blooms observed in 2019 at Glenwood Springs on Orcas Island as well as Spencer Cove and Nisqually Reach. Blooms of *A. sanguinea* were observed in Spencer Cove and Budd Inlet near Olympia in both 2018 and 2019 (Fig. 5).

A bloom of *P. reticulatum* (Table 1, Fig. 5) in 2019 in Mystery Bay was observed on August 13, 20, 27, and September 3. Cell counts performed on 10x concentrated whole water samples ranged from 36,000 cells/L to >1.8 million cells/L. Corresponding YTX concentrations in filtered phytoplankton samples reached 920 ng/L during the Mystery Bay bloom on August 13. The highest cellular YTX quota was 10.8 pg/cell, similar to measurements for the cultured isolate, NWFSC 614, which ranged from 22.4-26.6 pg/cell (Table 1).

YTX was measured in various Puget Sound shellfish from 2016-2019 (Table 2). Blue mussels (*Mytilus edulis*) and California mussels *(Mytilus californianus)* contained the highest concentrations of YTX, reaching 0.91 mg/kg in blue mussels from Sequim Bay State Park on 7 Sept 2016 and 0.57 mg/kg in California mussels from Westport on the Pacific coast of Washington State on 29 Aug 2016. For shellfish collected on the same date and analyzed for YTX, mussels often showed measurable concentrations of YTX, whereas Pacific oysters (*C. gigas*), littleneck clams (*Leukoma staminea*) and Manila clams (*V. philippinarum)* showed lower levels or no measurable YTX (Table 2; data from various shellfish species collected on the same date). The majority of YTX was the parent compound, while an average of 22% of the total measured YTX was 45-OH-YTX, the shellfish metabolite.

4.0. Discussion

4.1. P. reticulatum impacts worldwide

Our data suggest that a number of recent shellfish mortality events in Puget Sound are attributable to HABs that are not traditionally monitored. This is supported by observations of shellfish mortalities in other regions of the world due to *P. reticulatum,* confirmed to be a YTX producer by Satake et al. (1997). *P. reticulatum* has been associated with shellfish mortalities in Chile (Álvarez et al., 2011; Álvarez et al., 2020), Japan (Koike et al., 2006), Canada (Cassis, 2005), Norway (Aasen et al., 2005), New Zealand (MacKenzie et al., 1998), and South Africa (Grindley and Nel, 1970; Horstman, 1981). Suzuki et al. (2005) demonstrated that an injection of YTX into the adductor muscle of scallops caused excessive mucus production and mortality.

Devastating abalone (*Haliotis midae*) mortalities in South Africa resulted in losses of 250 tons of product at different aquaculture farms due to YTX production by *G. spinifera*. Severe damage to abalone gill epithelia was observed, including degeneration, necrosis and inflammation, with detritus observed between filaments (Pitcher et al., 2019). However, some abalone recovered from this insult 2-3 weeks later, once other phytoplankton food became available. In 1966, the death of hundreds of thousands of white mussels (*Donax serra*) on the west coast of South Africa was associated with a bloom of *G. grindleyi* (synonymous with *P. reticulatum*); (Reinecke, 1967; DeVilliers, 1979) that was later confirmed to be toxic (Fawcett et al., 2007; Krock et al., 2008). More recently in 2005, a bloom of *P. reticulatum* was associated with YTX in plankton samples and mussels with concentrations of YTX in mussels reaching 3.6 mg/kg (Pitcher et al., 2019). The presence of *P. reticulatum* cysts in shellfish guts suggest that they are also present in nearby sediment as these cysts are known to be a common means of cell overwintering and survival in sediment (Matsuoka et al., 1990). These cysts have been found in estuarine environments around the world and have been documented as one of the most abundant cysts in the northeast Pacific region since the last glaciation (Dobell, 1978).

Figure 5. Spatial distribution and relative abundance of *P. reticulatum* (A, C) and *A. sanguinea* (B, D) in summer 2018 (A, B) and 2019 (C, D). Colored symbols indicating "present (blue), common (purple), bloom (orange)" are maximum relative abundances at each site from net tow samples using the method described in Trainer et al. (2016).

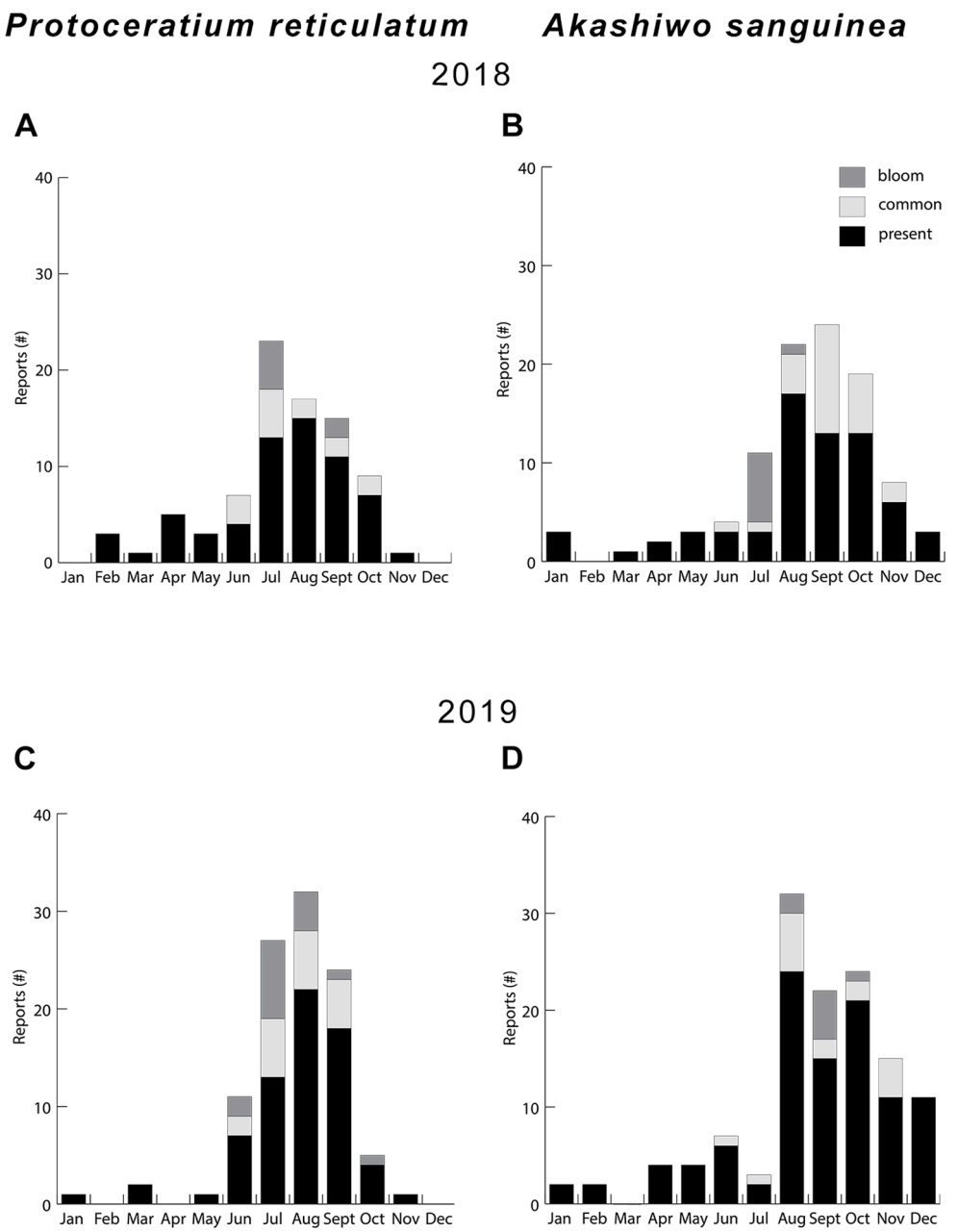

Figure 6. Monthly reports of relative abundance of *P. reticulatum* (A, C) and *A. sanguinea* (B, D) in 2018 (A, B) and 2019 (C, D) at all SoundToxins sites throughout Puget Sound. Shaded bars indicate the number of reports "present, common, bloom" estimated from net tow sample using the method described in Trainer et al. (2016).

4.2. YTX cell quotas and seawater YTX

Cellular quotas of YTX for *P. reticulatum* range from 0-79 pg/cell in isolates from Canada, Italy, Norway, Japan, New Zealand, Spain, the United Kingdom and the United States, with the maximum concentration measured in an isolate from Norway (Samdal et al., 2004) although most cell quotas in YTX-producing cultures do not exceed 14 pg/cell (Howard et al., 2008). The single isolate from Puget Sound (NWFSC 614; Table 1) showed YTX quotas reaching 26.6 pg/cell, higher than most other *P. reticulatum* isolated around the world. Other isolates from the U.S. examined previously had YTX quotas ranging from 0-2.1 pg/cell (Paz et al., 2004; Paz et al., 2007; Cassis, 2005). To date, the Puget Sound isolate has the highest cellular YTX quota recorded in the U.S.

Brief exposure to a relatively small *P. reticulatum* bloom (2200 cells/L) in Norway was shown to be sufficient to cause contamination of blue mussels, with YTX above regulatory closure levels (Aasen et al., 2005), not surprising given that isolates from this country have the highest YTX quotas measured to date. However, YTX concentrations in Puget Sound shellfish have reached 0.9 mg/kg (Table 1), with concentrations of *P. reticulatum* exceeding 1.1 million cells/L. These data demonstrate that although this toxin can be present at high concentrations in Puget Sound (Trainer et al., 2013), levels in shellfish have thus far remained below the EU regulatory limit of 3.75 mg/kg (European Union, 2015; Mahoney, 2018). Nonetheless, the cell concentrations that toxify or cause mortalities in shellfish clearly will depend upon the relative toxicity of local *P. reticulatum* populations. Lab experiments with cultured isolates are needed to determine the following: 1. the concentrations of cells that cause mortality in shellfish to best inform an early warning system, 2. the suite of environmental factors that promote maximum toxicity in this harmful phytoplankton taxon, and 3. genetic variability in strains

Table 3
Shellfish observations associated with phytoplankton blooms in WA State since 2010.

Year	Plankton species	Location*	Shellfish affected	Observations
2010	*A. sanguinea*	Totten Inlet	mussels	sloughing off lines
2011	*P. reticulatum*	Sequim Bay	Manila clams	mortality
2012	*P. reticulatum*	Sequim Bay	clams	surfacing, mortality
2017	*P. reticulatum*	Hood Canal	varnish clams	mortality
2017	*P. reticulatum, A. sanguinea*	Spencer Cove	Pacific oysters, geoduck	mortality
2017	*A. sanguinea*	Totten Inlet	mussels/Pacific oysters	sloughing, mortality
2017	*P. reticulatum*	Sequim Bay	Manila clams	surfacing, mortality
2018	*P. reticulatum, Phaeocystis*	Discovery Bay	Pacific oysters	mortality
2018	*A. sanguinea*	Totten Inlet	Pacific oysters	mortality
2018	*P. reticulatum*	North Bay	cockles	mortality
2018	*Phaeocystis*	Hood Canal	Pacific oysters	mortality
2018	unknown	Willapa Bay	clams, oysters	surfacing, mortality
2019	*P. reticulatum*	Rocky Bay	Manila clams	stressed, gaping, mortality

*Locations are shown in Fig. 1.

and the difference in toxin content among isolates.

4.3. Shellfish YTX

Our data show that the parent compound, YTX, is the major isomer, whereas 45-OH-YTX, the shellfish metabolite (Aasen et al., 2005; Röder et al., 2011) of this toxin, comprises a minor component of the total YTXs in Puget Sound shellfish (Table 2). In a recent study, the maximum concentration of 12 mg YTX eq./kg (the sum of 45-OH-YTX and YTX) was measured in mussels from Jervis Inlet, B.C., western Canada with *P. reticulatum* abundance increasing prior to observation of the elevated YTX levels (Rourke et al., 2020). In abalone that died from YTX exposure in South Africa, toxins in the digestive gland averaged 0.73, 0.21, and 0.09 mg/kg of the three forms, homo-YTX, 45-OH-YTX and YTX, respectively, and in gill tissue (the most contaminated tissue) averaged 1.1, 0.33, and 0.11 mg/kg of homo-YTX, 45-OH-YTX and YTX, respectively. Similarly, 0.42 mg YTX eq./kg was observed in clams killed during a bloom of *P. reticulatum* in Chile in 2019 (Álvarez et al., 2020). Our study shows that YTX and 45-OH-YTX are present at concentrations similar to those shown in shellfish that died in South Africa and Chile, measuring a maximum of 0.69 and 0.23 mg/kg YTX and 45-OH-YTX, respectively, in whole blue mussels collected from Sequim Bay in 2016 (Table 2). These concentrations of YTX in Puget Sound shellfish appear to be sufficient to cause their mortality. However, the range of YTX concentrations required to cause shellfish mortality should be determined through continuous monitoring by partnerships such as SoundToxins and through laboratory exposure studies.

4.4. Causes of damage to shellfish

The specific mechanism of YTX toxicity in shellfish is not known. However, laboratory exposure studies using a variety of cell lines can provide some insights. Previous work suggests that YTX affects the digestive and immune system (Franchini et al., 2003; Franchini et al., 2010) and the cytoskeleton, primarily the components actin and cadherin (Tubaro et al., 2010; Franchini et al., 2010), which help give cells their shape and structure. The abnormal digestive gland tubules shown in the histology of clams collected from Rocky Bay during the mortality event in July 2019 (Fig. 4) is a sign of cytoskeletal degradation that could be due to YTX. However, this must be confirmed in controlled studies where Puget Sound shellfish are exposed to cultured isolates of *P. reticulatum* in the laboratory. In addition, because YTX causes apoptosis (programmed cell death) by activating caspase proteins (Korsnes and Espenes, 2011), shellfish exposed to YTX will be vulnerable to cell death in affected tissues. It is interesting that although YTX has been shown to be harmful to shellfish, it has also been studied as a potential therapeutic drug to treat cancer, immune disease, allergies and Alzheimer's disease, and is a powerful research tool for the study of intracellular pathways (summarized in Alfonso et al., 2016).

4.5. Akashiwo sanguinea

Other phytoplankton have been associated with shellfish mortalities in Puget Sound (Table 3). *Akashiwo sanguinea*, a common species in upwelling systems, in particular the California and Benguela Upwelling Systems (Trainer et al., 2010), is known to be toxic to oysters (*C. gigas* and *O. lurida*) in their larval through adult stages of development (Nightingale, 1936; Woelke, 1961; Cardwell et al., 1979). Shellfish producers in Washington State have noted mortality of larvae in their hatchery facilities as the concentration of *A. sanguinea* increases, despite thorough intake water processing for their operations. In fact, some shellfish producers call this cell, "the octopus", as it slimes its way through various filter systems. The "breakthrough" or introduction of harmful phytoplankton or their toxins into hatchery water has recently been observed in other regions, where water treatment at hatcheries does not remove toxins or cells from the incoming water (Pease et al., 2021). Over the past few years, shellfish hatchery facilities have worked with SoundToxins scientists to develop proper water exchange procedures to ensure a sufficient supply of the beneficial phytoplankton species for maintenance of healthy shellfish larval tank cultures. Additionally, Puget Sound mussel farmers have been adapting to recent increasing abundances of *A. sanguinea* to employ mitigation procedures to minimize shellfish loss. Specifically, when high concentrations of *A. sanguinea* are in the water column, the outward facing lines on the mussel rafts see a dramatic increase in mussel sloughing from the lines. These outer lines are sacrificed as a protective barrier in order to allow the innermost mussels to survive.

Other marine animals have suffered ill effects of *A. sanguinea* blooms. Off the U.S. west coast, these blooms have resulted in massive bird deaths, associated with the production of a surfactant-like compound that strips bird feathers of their oils, causing wing fouling, hypothermia, and death (Jessup et al., 2009; Du et al., 2011; Jones et al., 2017). The discoloration of the seaweed, specifically nori, by blooms of *A. sanguinea* has been reported in Japan (Matsubara et al., 2007). Abalone (*H. midae*) larvae and spat have suffered mortalities due to *A. sanguinea* blooms in South Africa (Botes et al., 2003). Recent studies demonstrate that *A. sanguinea* produces resting cysts (Tang and Gobler, 2015) and is tolerant to a broad range of temperatures (Matsubara et al., 2007, Boyd et al., 2013), suggesting that this species can grow and survive over long periods to permit slow accumulation into blooms (Menden-Deuer and Montalbano, 2015). These factors suggest that *A. sanguinea* will become a more persistent global problem in the future.

4.6. Phaeocystis

Phaeocystis globosa has also been associated with Puget Sound shellfish mortalities (Table 3). Observations in other parts of the world include the massive mortality of more than 10 million kg mussels that occurred in the Netherlands in spring 2001 following a bloom of

Phaeocystis globosa (Peperzak and Poelman, 2008). However, the mussel deaths were eventually attributed to a lack of oxygen, and not a toxin associated with the algal bloom. This type of high-biomass algal bloom that, upon decay, provides dead organic matter as food for bacteria, results in the non-specific impacts of oxygen depletion on shellfish health.

4.7. The future

A balanced phytoplankton community structure plays a critical role in the future success of shellfish aquaculture around the world. Early warning of shellfish-killing HAB presence in Puget Sound provides options for shellfish growers to mitigate their effects (Wells et al., 2019) through actions to reduce the incidence of summer mortality events. However, in order to optimize the early warning of shellfish-killing HABs provided by partnerships such as SoundToxins, it is crucial, through culture studies and field observations, to determine the threshold numbers of cells that cause shellfish mortalities and the stages of shellfish growth that are most susceptible. By recognizing the threat of *P. reticulatum, A. sanguinea*, and potentially other HABs that have devastating effects on shellfish populations, growers can make farm-based management decisions such as to harvest early, move shellstock, or enhance filtration at aquaculture facilities, thereby protecting the shellfish from mortality.

Both *P. reticulatum* and *A. sanguinea* are dinoflagellates, phytoplankton that are able to swim through the water column to access nutrients, especially under stratified conditions. Studies on phytoplankton community structure have suggested that the California Current System is shifting to more dinoflagellate-dominated communities, including notable HABs (e.g. Jester et al., 2009; Fischer et al., 2020), and even returning to the "age of dinoflagellates", related to the interaction between regional upwelling and local wind forcing (Fischer et al., 2020). Many of the extreme HAB events noted around the world are due to dinoflagellates that cause devastating economic losses to coastal communities (Trainer et al., 2019). Toxic flagellates will do extremely well under future climate change conditions, including increased stratification and temperature in coastal oceans (summarized in Wells et al., 2015). These factors point to the importance of persistent monitoring and exploring potential mitigation strategies, such as planting eelgrass beds near shellfish operations to reduce harmful dinoflagellate occurrence (Inaba et al., 2017; Jacobs-Palmer, 2020).

Dedication

We dedicate this publication to Dr. Kenneth K. Chew, for teaching us to never stop investigating until we have an answer and to not overlook the obvious.

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.

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