NATIONAL SEA GRANT FINAL REPORT

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Project Title:
The purple hinged rock scallop, a promising aquaculture species with a toxic algal problem

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OBJECTIVES:

1) Examine uptake and accumulation of PSP toxins in rock scallops through lab-based feeding experiments with *Alexandrium catenella.*

2) Examine bioaccumulation and detoxification of biotoxins in rock scallops exposed to naturally occurring biotoxins (PSP, ASP and DSP).

3) Evaluate accumulation of saxitoxin in different components (adductor muscle and viscera) through receptor binding assay (RBA).

4) Produce validated data to support the NSSP approval process for RBA for toxicity determination of PSP toxins in rock scallops.

5) Observe and evaluate grow out conditions at the project study sites.

Table of Contents

List of Figures

List of Tables

EXECUTIVE SUMMARY

Purple hinge rock scallop (*Crassadoma gigantea*) is a promising native species for aquaculture production, with strong market potential and substantial interest by the shellfish industry. The most serious unresolved issue with rock scallop aquaculture potential is the lack of information on biotoxin retention and detoxification. The overarching goal of this project was to address the needs of public health agencies and the shellfish industry to: 1) enhance understanding of uptake and depuration of biotoxins by rock scallops, and 2) establish approved National Shellfish Sanitation Program (NSSP) laboratory tests for marine biotoxin testing for this species.

Research was conducted under laboratory conditions to investigate the uptake of the cultured dinoflagellate *Alexandrium catenella*. Feeding behavior exhibited during uptake studies were not apparently inhibited by the presence of toxic *A. catenella*. Scallops readily fed on high concentrations (> 2000 cells L^{-1}) of *A. catenella* under laboratory conditions. While intact dinoflagellate cells were observed in pseudofeces produced by rock scallops (and not ingested), the fact that intact *A. catenella* cells were not generally observed in the fecal ribbons produced by scallops, indicated that ingestion of *A. catenella* had occurred. Rock scallops, however, showed no behavioral changes (e.g. valve clapping, valve closure, cessation of feeding activities) when fed toxic *A. catenella*.

The pattern of toxification and detoxification in rock scallops is similar to other studies of bivalves fed toxic dinoflagellates, with bivalves increasing toxin loads in digestive gland tissues at significantly higher rates than for other tissues (Bricelj and Shumway, 1998). Detoxification studies in rock scallops demonstrated that this species attains very high toxin loads for long time periods. The high toxin loads associated with the visceral mass of rock scallops and time frames associated with toxin uptake, and particularly rates of detoxification, will likely result in regulatory concerns over aquaculture development for the whole scallop market. While whole body toxin loads decreased over detoxification timeframes measured in weeks, the overall toxicity of rock scallops considered in these studies remained well above regulatory thresholds. The market for adductor muscle only will also require careful scrutiny, as this study has demonstrated.

Temporal field exposure trials examined bioaccumulation and subsequent detoxification of biotoxins in whole rock scallops for diarrhetic shellfish toxins (DSP) and in selected component parts of individual scallops, adductor muscle and viscera for saxitoxin (STX). The extended toxicity in rock scallops during cold winter months may be an effect of slower metabolic rate during this time, influencing the rate of toxin depuration in this species. While active cells of *Alexandrium* were not reported in the water column during winter months, toxicity in scallops persisted in individual adductors and well above the paralytic shellfish poison (PSP) thresholds in viscera.

It is not clear from any of our datasets, field exposure or laboratory, how long rock scallops take to completely depurate saxitoxin from all tissues. Given the high level of STX in the viscera at the end of each time series and relatively slow depuration rate in this component and species in general, it is reasonable to discern that complete depuration of toxins may extend for many more months, quite possibly a year or longer if scallops still high in STX encounter the following

spring bloom season. Implications for management of future commercial culture, harvest and processing of rock scallops will need to be determined to ensure a safe seafood product can be sold to consumers. Furthermore, the general trend of rock scallop adductor following uptake and depuration rates closely with blue mussels across all the time series, could be quite valuable for future regulation and monitoring of rock scallop and should be examined more closely with additional analyses and data collection.

This project provided a focused opportunity to validate data, assess feasibility, and realize the benefits of receptor binding assay (RBA) as an alternative method to measure saxitoxin in shellfish from the state of Washington. RBA is more sensitive than the mouse bioassay (MBA), but remains approved only for limited use for regulatory testing of mussels and for limited use as a screening tool for clams and scallops. As was the case when this project's research was first funded, RBA is designated as an Official Method of Analysis (OMA 2011.27) for Limited Use Method for clams and scallops for the purpose of screening and precautionary closure for PSP. This project produced additional data, including spike recovery for STX from rock scallops, but the ultimate decision to move to RBA is contingent on NSSP inclusion of the method for regulatory applications for commercial shellfish, including rock scallop, geoduck and oysters.

The overall species richness and diversity observed on the submerged scallop cages over the duration of this project period suggests that the cages provide refuge and nursery areas sought by many marine species. However, the level of fouling presents the potential of reducing scallop viability and marketability. This study provides a baseline assessment of the presence of biofouling organisms specific to the Puget Sound, Washington, with the intent to inform future research on commercial scale growout methods in the region. Growth and mortality for multiple cohorts of hatchery-produced scallops were also monitored at multiple sites in Puget Sound and are reported here. Mortality events observed to occur in scallops over this project do not follow patterns observed in other commercially significant species on the West Coast of North America.

RATIONALE

Purple hinge rock scallop (*Crassadoma gigantea*) are emerging as a promising new native species for aquaculture production. The species has strong market potential, and substantial interest and investment has already been realized by the shellfish industry. The most serious unresolved issue with rock scallop aquaculture potential is the lack of information on biotoxin retention and detoxification. This is critical because toxins associated with paralytic shellfish poisoning (PSP), e.g. saxitoxin and its derivatives, are currently the most widely reported toxins in bivalve molluscs along the west coast of North America. The overarching goal of this project is to address the needs of public health agencies and the shellfish industry to: 1) enhance understanding of uptake and depuration of biotoxins by rock scallops, and 2) establish approved National Shellfish Sanitation Program (NSSP) laboratory tests for marine biotoxin testing methods for this new aquaculture species. This research will also enhance understanding of optimal grow-out methods for purple hinge rock scallops in Washington State.

Objective 1: Examine uptake and accumulation of PSP toxins in rock scallops through lab based feeding experiments with *Alexandrium catenella*.

Research was conducted on purple hinge rock scallops (*Crassadoma giganteus*) under laboratory conditions to investigate the uptake of the cultured dinoflagellate *Alexandrium catenella*. The investigation was prompted by prior research that suggested rock scallops would likely feed indiscriminately upon toxic dinoflagellates and then subsequently retain saxitoxins produced by *A. catenella* into different tissues. This work represents the first attempt to induce toxicity in purple hinge rock scallops under controlled conditions of algal cell density, temperature and salinity. Three trials were conducted at the NOAA Northwest Fisheries Science Laboratory located in Manchester, WA and where Baywater, Inc. (now Pacific Hybreed, Inc.) maintains and operates a combined hatchery and marine laboratory facility. The third study describes results associated with research on detoxification rates in rock scallops. In this case, rock scallops that were known to contain significant amounts of saxitoxin were brought into the laboratory from Sequim Bay, WA, maintained there on filtered seawater and sampled weekly to chart the time course of detoxification in this species.

The specific research tasks were:

1. Will purple hinge rock scallops feed on and retain toxic *A. catenella* cells
2. What is the time course of toxin uptake in different scallop tissues
3. What is the time course of scallop detoxification

Biotoxin Uptake and Detoxification Study 1

Methods and Materials

Sub-adult purple hinge rock scallops were obtained from a cultured population of hatchery produced rock scallops maintained at Hood Head, Hood Canal. The group of scallops had been produced 2.5 years prior as part of an investigation of growth and survivorship of rock scallops maintained under different culture conditions. These rock scallops were returned to the

EPA/NOAA pier in Manchester, WA and maintained in suspended trays there until the research was initiated on July 9, 2017.

To assess dinoflagellate uptake, 20-25 rock scallops (30-40 ml in shell length) were placed onto the bottom of 20-liter trays (30" x 12" x 4") (**Figure 1**) containing seawater filtered to 1 micron. Seawater flow through was maintained at approximately 1.5L per minute except when scallops were feeding on dinoflagellates. Each tray was held at a temperature of 16 °C and salinity of approximately 30 ppt. over the duration of toxification (4 day) and detoxification (28 day) time frames.

Dinoflagellates were cultured in the laboratory of NOAA researcher Dr. Vera Trainer by her technician, Brian Bill. Dinoflagellates needed for the feeding studies were transported on a daily basis during the two feeding experiments.

Figure 1. Sub-adult rock scallops in flow-through tray during biotoxin uptake feeding study.

Scallops were introduced to trays on July 9, 2017 with feeding of dinoflagellates initiated on July 10 for each of four days (e.g. July 10-13). Briefly, for each day of the trial, dinoflagellate cultures were gently poured into each tray when scallops were observed to be actively filtering seawater (valves open and mantle tentacles extended (Figure 2). Prior to the addition of dinoflagellates, 10 liters of seawater were siphoned from the trays. Each tray was gently aerated to assist in keeping dinoflagellates suspended in the water column. When dinoflagellate cultures were introduced to the tray the flow was turned off to enable scallops to feed under static conditions (with gentle aeration supplied). The approximate number of dinoflagellates supplied to the tray was approximately 50,000 cells per day, based on

Figure 2. Open rock scallop feeding on toxic dinoflagellates, *A. catenella*.

a culture volume of 10 liters and counts of 5000 cells per liter made daily. Scallops were initially observed for changes in feeding behavior using video, however once it was observed that feeding behaviors were not altered in the presence of dinoflagellates, video monitoring was discontinued. Approximately 30 minutes following initial feeding scallops produced pseudofeces. These were examined on a daily basis to observe for the presence of intact dinoflagellates within the pseudofecal mass. Similarly, 4-6 hours after feeding was initiated the trays were examined for the presence of fecal ribbons on a daily basis. These were similarly microscopically examined for the presence of intact dinoflagellates.

Scallops (N=5) were sampled 24 hours following the 4-day toxification phase (24 hours after the fourth day of feeding) for toxin load (7/14/2017). Briefly, scallops were removed from trays and shucked. Two tissue types were dissected: adductor muscle and digestive gland. The remainder of the scallop was discarded. Tissues were stored separately in small zip lock bags at -20 C until assessed for saxitoxin by Tod Leighfield, NOAA National Ocean Service, Charleston, S.C.

The detoxification phase was initiated following the first sample with remaining scallops placed into a flow through seawater tank and supplied a hatchery diet consisting of diatoms and dinoflagellates over the next 30 days. Scallops (N=5) were similarly sampled during the detoxification phase after 7 days (7/21/17) and after 28 days (8/11/17). Tissues were similarly stored at -20 C prior to analysis by the Charleston NOAA Lab.

All tissue samples were extracted and extracts analyzed using a saxitoxin receptor binding assay (RBA). The saxitoxin RBA measures the competition between radiolabeled STX and sample or FDA standard for binding to the voltage-gated sodium channel, the pharmacological target of paralytic shellfish toxins, to determine the total saxitoxin-like activity of the sample. The RBA method is described in Van Dolah et al. (2012). Two extracts from the 2018 uptake & depuration study were additionally analyzed by tandem mass spectrometry coupled to liquid chromatographic separation (LC-MS). Sample clean-up and the LC-MS analysis method is described in Hattenrath-Lehmann et al. (2018). Briefly, acidic extracts were prepared for LC-MS analysis by using C18 solid phase extraction cartridges. The LC-MS method utilized an acidified water/acetonitrile mobile phase and a TSK-gel Amide-80 column, using an Agilent 1100 HPLC coupled to an ABI-SCIEX 4000Qtrap triple quadrupole mass spectrometer using multiple reaction monitoring (MRM). MRM transitions were developed using certified PSP standards from the National Research Council of Canada.

Results

Rock scallops were uniformly observed to remove dinoflagellates from the water column and exhibited normal feeding behaviors. Scallops exhibited no change in valve gape nor changes in mantle behavior (e.g. extension of mantle tentacles during feeding) when fed toxic *A. catenella* under controlled conditions. **Figure 2** shows a rock scallop actively feeding on cultured microalgae. Note the band of eyes on mantle margin and mantle tentacle extension beyond the margin of the valves.

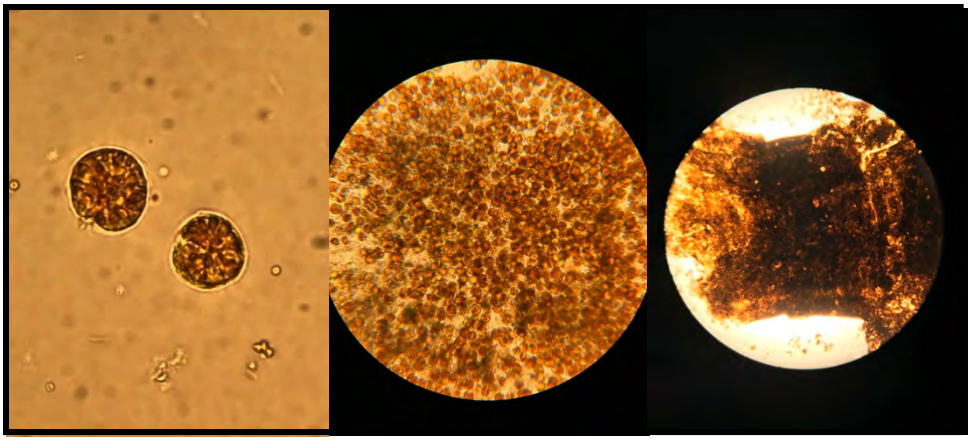


Figure 3. a) Two Alexandrium cells, b) *A. catenella* cells wrapped in mucus and other detritus in pseudofeces, c) fecal ribbons collected 6-8 hours after toxification.

Examination of biodeposits indicated that dinoflagellates were readily taken in but rejected prior to ingestion as pseudofeces. **Figure 3b**, shows *A. catenella* cells (as bright orange spheres) wrapped in mucus and other detritus in pseudofeces collected from rock scallops shortly after toxification studies were initiated. Fecal ribbons collected 6-8 hours following the initiation of toxification studies show no indication of intact dinoflagellates contained in the fecal ribbon (**Figure 3c**). That intact cells appear absent or sparse is an indication that ingested cells were lysed during digestive activities.

The 2017 uptake and deputation experiment yielded small sample sizes with an average sample size of 1.6 grams for adductor and 0.34 for digestive gland. The extraction procedure used the entire sample in preparation for analysis by the saxitoxin RBA. Using the entire sample was a modification of the standard method where a fixed sample weight for all samples would have been extracted in acid. Specifically, the entire sample was added to 3 volumes of 0.1N aqueous hydrochloric acid for the adductor muscle and 6 volumes of 0.1N aqueous hydrochloric acid for the digestive gland. The sample was sonicated for 2 minutes, pH confirmed to be <4, then boiled for 5 minutes. Samples were then centrifuged at 1700 xg for 15 minutes and supernatant collected. It was noted that the adductor matrix type produced a polymerized gel-like consistency and the remaining liquid phase of the supernatant was small relative to other shellfish species. The pH of the resulting sample was confirmed to be <4 for each acidic extract prior to analysis.

Analyses of these extracts in the saxitoxin RBA yielded the data shown on **Figures 4 and 5**. Unfortunately, it had been assumed that saxitoxin presence would be very low to non-detectible in rock scallops brought in from the specific field location used for this study. It turned out that this may have not been the case as it was noted initial samples contained saxitoxin-like activity in both the digestive gland and adductor muscle. Unfortunately, scallops were not sampled initially (prior to feeding on cultured dinoflagellates) and it was difficult therefore to assess whether rock scallops had contained residual toxicity prior to the toxification phase. Because the purple hinge rock scallop is known to retain saxitoxin for months following uptake, it is possible that the rock scallops used for the uptake/detoxification study already had residual toxin loads prior to initiating the work. Note that adductor muscle toxin loads rarely exceeded the 80 µg/100 g regulatory tissue threshold while digestive gland toxin loads remained high over the toxification/detoxification timeframe.

Biotoxin Uptake and Detoxification Study 2

A second toxification/detoxification study was completed in September 2018 at the Baywater (now Pacific Hybreed) hatchery facility. Details of the experimental design were similar to those described above for Study 1, completed in 2017.

Methods and Materials

In contrast to feeding studies executed in 2017 the decision was made to dose rock scallops at a higher rate with *A. catenella* in order to potentially increase the toxification signal. The feeding experiment commenced on September 10, 2018 with 25 rock scallops introduced to a single tray at the Manchester laboratory facility maintained at 15-16 °C. Scallops were acclimated to the tray for one hour prior to adding a 10 liter volume of *A. catenella*. Relative density of *A. catenella* cells varied slightly over the four day feeding period but were in the range of 4500-5200 cells per L over the four day trial (see **Table 1** for *Alexandrium* cell densities for AM and

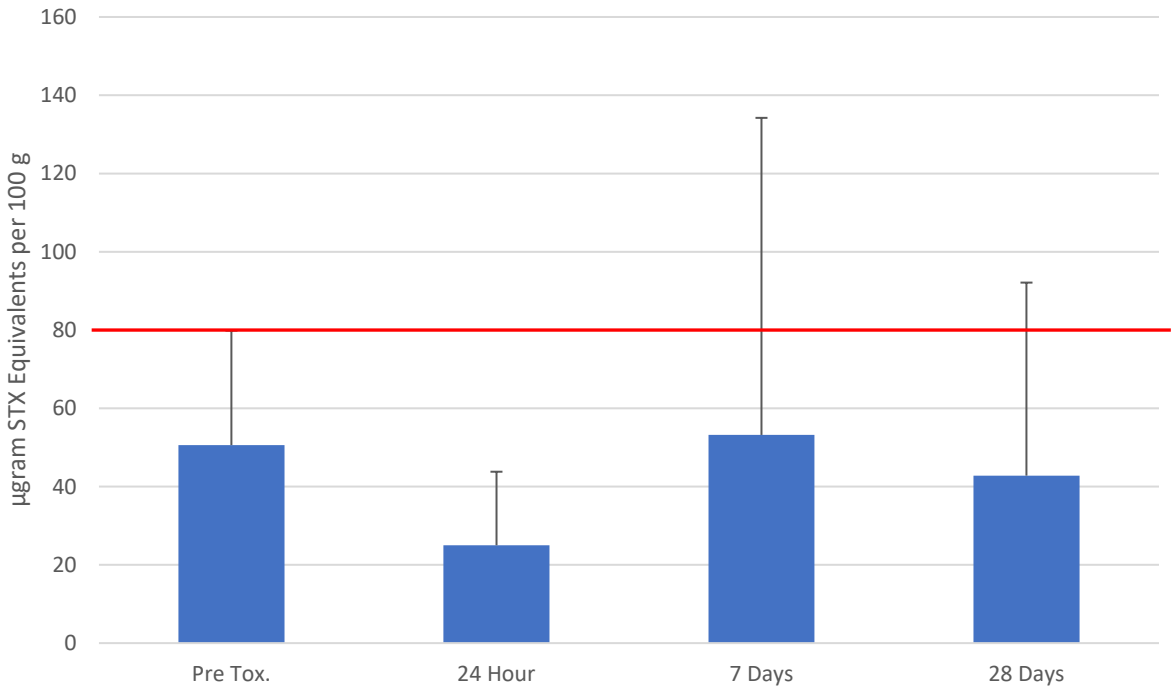


Figure 4. Saxitoxin load over time in rock scallop adductor muscle following 4-day toxification phase. The red line at 80 µgram STX equivalents per 100 g. tissue represents the threshold for the regulatory biotoxin limit in shellfish tissues. Data represent mean plus standard deviation.

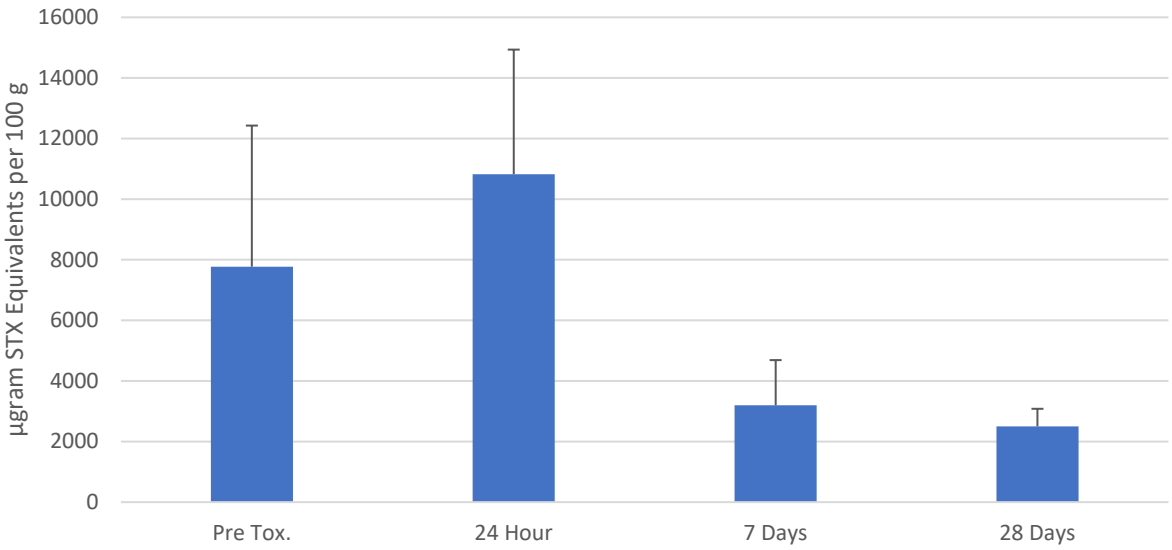


Figure 5. Saxitoxin load in STX equivalents per 100 g tissues over time in rock scallop digestive glands following 4-day toxification phase. Data represent mean plus standard deviation.

PM feedings on days 1-3). The initial densities of *Alexandrium* cells encountered by scallops on average was therefore between 2000-2218 per liter. It was observed that cell density was quickly grazed down over 2-3 hours during the feeding trial (note that tray was maintained on static conditions during the daily trials in order to retain all *Alexandrium* cells). A second carboy of A. catenella was added daily between 2-3 hours later to replenish the availability of dinoflagellate cells to feeding scallops. Total duration of the feeding phase was on average 7 hours on a daily basis for each of four days. Scallops were otherwise maintained on flowing seawater filtered to 5 microns before and following feeding trials. Accumulated biodeposits (feces and pseudofeces) were collected daily and examined microscopically for the presence of intact *Alexandrium* cells. Biodeposits were subsequently treated with a chlorine bath prior to disposal.

Table 1. *Alexandrium catenella* cell density during days 1-3 of the 2018 toxification study.

Date	*A. catenella* Culture Density (cells per L)	*A. catenella* Tray Density (cells per L)
9/10/18AM	5000 in 9.5 L	2065
9/10/18PM	5000 in 10.0 L	2174
9/11/18AM	5100 in 10 L	2218
9/11/18PM	4600 in 10 L	2000
9/12/18AM	4900 in 9.5 L	2024
9/12/18PM	5200 in 10.0 L	2261

Rock scallops dosed 2X daily with *A. catenella* over a period of four days (September 10-13) were subsequently sampled following the toxin dosing phase at 1 day, 7 days, 14 days, 28 days and 58 days post toxification phase. As was the case in 2017, tissues were dissected from several rock scallops per sampling event. Unlike 2017 studies, additional scallops were sampled at 14

days (N=2) and 58 days (N=3) post toxification. Data on wet weight of scallop tissues was recorded prior to freezing of samples for later biotoxin analysis (**Table 2**).

Table 2. Tissue mass data for dissected tissues associated with 2018 toxification study.

Date	Post Toxification	AM (g)	DG (g)	Rest (g)	Total (g)
9/15/18	1 Day	6.84	2.13	9.00	17.97
9/15/18	1 Day	5.82	2.41	9.00	17.23
9/15/18	1 Day	5.75	2.01	7.31	15.07
9/15/18	1 Day	7.79	2.13	11.54	21.46
9/15/18	1 Day	8.76	2.85	13.13	24.74
9/21/18	7 Days	5.93	1.51	6.92	14.36
9/21/18	7 Days	11.65	2.47	13.34	27.46
9/21/18	7 Days	5.63	2.04	10.72	18.39
9/21/18	7 Days	10.37	3.10	13.98	27.45
9/21/18	7 Days	6.33	1.48	7.09	14.90
9/28/18	14 Days	9.26	1.50	9.90	20.66
9/28/18	14 Days	10.32	1.99	12.26	24.57
9/28/18	14 Days	6.45	1.75	9.80	18.00
10/12/18	28 Days	11.33	4.04	18.06	33.43
10/12/18	28 Days	8.88	5.06	16.05	29.99
10/12/18	28 Days	9.24	3.17	11.77	24.18
11/12/18	58 Days	2.79	4.06	9.49	16.34
11/12/18	58 Days	3.01	5.62	22.16	30.79
11/12/18	58 Days	4.62	5.18	22.25	32.05
11/12/18	58 Days	4.45	2.67	10.67	17.79

Results

Results of STX toxicity indicated, similar to 2017 studies, that adductor muscle toxin loads were low compared to STX loads in digestive glands (**Figures 6 & 7**). The 2018 uptake and depuration experiment yielded larger sample weights, so for this study the entire sample was homogenized and 1 gram of digestive gland or 5 grams of adductor was extracted. Specifically, for each sample 5 grams of adductor muscle was added to 5 milliliters of 0.1N aqueous hydrochloric acid, and 3 milliliters of 0.1N aqueous hydrochloric acid was added to 1 gram of each digestive gland sample. Each sample was vortexed, pH confirmed to be <4, then boiled for 10 minutes. Samples were then centrifuged at 1700 xg for 15 minutes, the supernatant collected. The collected supernatant was centrifuged a second time at 10000 xg for 20 minutes. This second supernatant was filtered using a 0.45µm syringe filter (Whatman GxF). The pH of the resulting sample was confirmed to be <4 for each acidic extract prior to analysis. It should be noted that the control samples contained saxitoxin-like activity in extracts prepared from the digestive gland, whereas the control adductor were below the limit of detection for the saxitoxin RBA. The unexpected findings of the presence of saxitoxin-like activity in the digestive gland extracts from the initial time point led to the analysis of two extracts, 9/10 control, and 9/21 one week exposure, using LC-MS. LC-MS provided chemical composition

analysis based on the parent mass and molecular fragments of saxitoxin congeners as compared to certified reference material. The LC-MS showed the presence of a variety of saxitoxin congeners in digestive gland extracts in both the control and 1 week samples. These data show that the initial samples contained saxitoxin and some of its congeners. Specifically, the control digestive gland extract contained STX > GTX1 > GTX2 > GTX3 > GTX4 (multiple congeners were not detected) with a total summed amount of the control digestive gland approximating that observed by saxitoxin RBA (471 vs. 948 µg STX equivalents/100 grams).

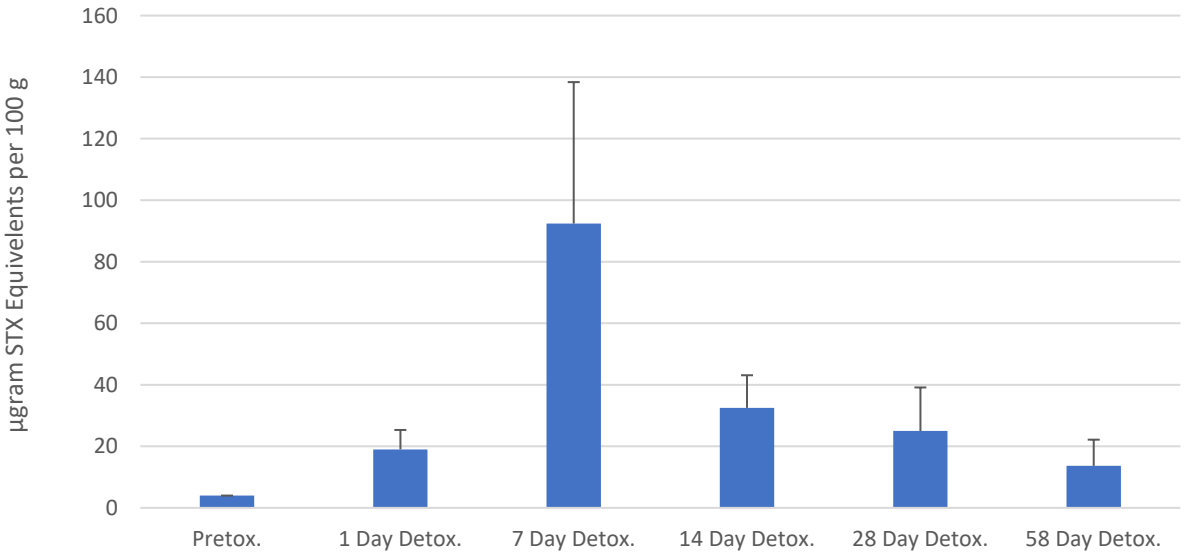


Figure 6. Saxitoxin load in STX equivalents per 100 g tissues over time in rock scallop adductor muscle following 4-day toxification phase. Data represent mean plus standard deviation for sampled rock scallops. The red line indicates the regulatory biotoxin threshold limit for toxicity in bivalve molluscs at 80 µgrams per 100 grams tissue.

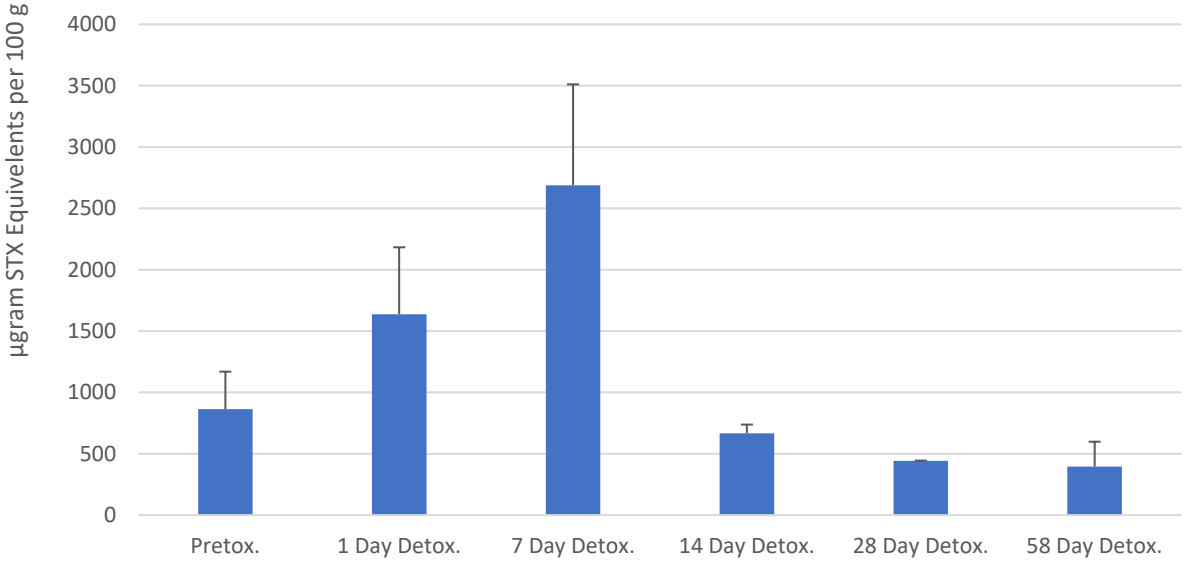


Figure 7. Saxitoxin load in STX equivalents per 100 g tissues over time in rock scallop digestive gland following 4-day toxification phase. Data represent mean plus standard deviation for sampled rock scallops.

The 1 week digestive gland extract contained saxitoxins, but with a different chemical composition, and at increased concentrations with a different rank concentration order relative to the control sample NEO > STX > GTX2 > 11-hydroxySTX > GTX3 > M3 > M1 > M5 > dcGTX2 > C2 > dcGTX3 > GTX5 > GTX4 > C1 (only GTX1, dcSTX, dcNEO were not detected) (**Table 3**). The total summed amount of the 1 week digestive gland measured by LC-MS approximated that observed by saxitoxin RBA (2168 vs. 2064 µg STX equivalents/100 g).

Table 3. LC-MS results corrected for toxin equivalency factors (TEFs) in µM saxitoxin equivalents, with a summed conversion of the TEF corrected value to µg saxitoxin equivalents per 100 grams for direct comparison to saxitoxin RBA values.

control DG - 9/10/2018

STX	1.105
GTX1	0.617
GTX2	0.596
GTX3	0.583
GTX4	0.265
NEO	not detected
11-hydroxySTX	not detected
dcGTX2	not detected
dcGTX3	not detected
M3	not detected
GTX5	not detected
C1	not detected
C2	not detected
M1	not detected
M5	not detected
dcSTX	not detected
dcNEO	not detected
Σ µg/100g= 471	

1 week DG - 9/21/2018

NEO	3.288
STX	2.989
GTX2	2.671
11-hydroxySTX	1.884
GTX3	1.614
M3	0.455
M1	0.411
M5	0.403
dcGTX2	0.273
C2	0.185
dcGTX3	0.155
GTX5	0.100
GTX4	0.090
C1	0.055
GTX1	not detected
dcSTX	not detected
dcNEO	not detected
Σ µg/100g= 2169	

Detoxification Studies in a Controlled Environment

Following regular sampling by PSI biologists as part of the overall biotoxin project, remaining rock scallops that were part of this sampling program were transferred to the Pacific Hybreed Laboratory in Manchester for detoxification studies under controlled conditions in September, 2017.

Methods and Materials

Rock scallops previously held in trays in Sequim Bay were placed into seawater tanks with filtered seawater under ambient condiitons of temperature and salinity for a period of 7 weeks. Scallops had been previously determined to contain a high load of biotoxin per field sampling results by PSI biologists and WA Department of Health determinations. The transfer of scallops to Manchester was accomplished on September 14, 2017 with sampling of tissues commensing immediately (Day 0). Sampling consisted of dissecting 15 rock scallops selected haphazardly from the pool of available scallops in the cohort. Three tissue types were selected: *adductor muscle*, *digestive gland* and *rest of tissues* from each scallop. Rest of tissues included gonad, mantle and stomach material. Tissues were dissected from scallops by Pacific Hybreed personnel and placed into plastic bagging and maintained in the freezer at the Pacific Hybreed hatchery and later at the PSI Laboratory in Olympia, WA. Scallops were sampled weekly from the cohort for a period of six weeks.

Results

Analysis of tissues for biotoxins was performed by the Bigelow Laboratory for Ocean Sciences in Boothbay Harbor, ME (Bigelow Analytical Services) as it was no longer feasible for the Charleston SC NOAA Laboratory to perform the analysis. Overall results for detoxification in scallop tissues for samples assessed by Bigelow Analytical Services indicated that total STX equivalent toxicity for scallop tissues declined significantly over the six week detoxification period with the vast majority of decrease occurring in the digestive gland (**Figure 8**). Total

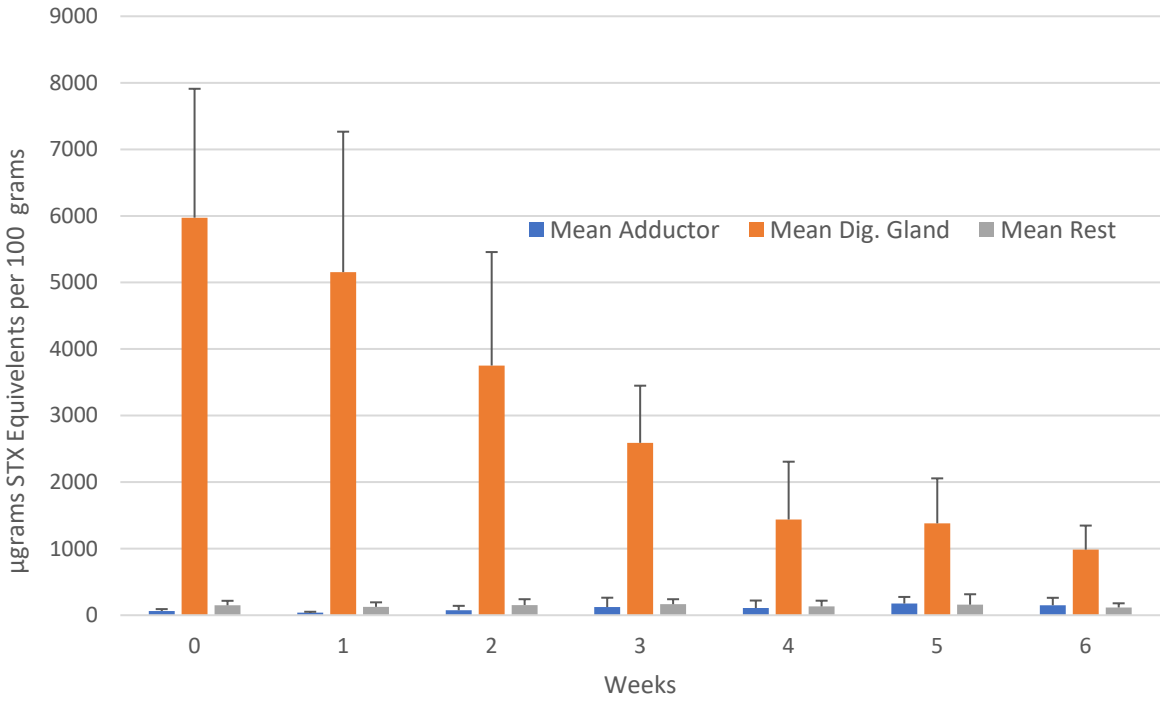


Figure 8. Saxitoxin load in STX equivalents per 100 g tissues over time in combined rock scallop tissues over a six week detoxification period. Data represent mean plus standard deviation for tissues dissected from individual rock scallops. Note that after 6 weeks of detoxification that whole body toxicity remained over ten times the regulatory threshold (80 µg per 100 g tissue) for bivalves.

toxin load in this tissue decreased from a mean of 5973 µg equivelents at day 0 to about 985 µg STX equivelents per gram after 6 weeks – a 6-fold decrease (**Figure 9**). The large decline in toxicity in the digestive gland masked, however a modest and unexpected increase in toxin load of the adductor muscle. At the beginning of the detoxification study mean adductor muscle toxicity was 63.8 µg STX equivelents per gram. After 6 weeks mean adductor toxicity was 149 µg STX equivelents per gram (**Figure 10**). Toxin load in the "rest" of tissue component was not significantly different at the conclusion of the study from initial levels (**Figure 11**). Toxin load in the adductor muscle was below the regulatory threshold of 80 µg STX equivalents per 100 g tissue at the onset of the detoxification phase. After 7 weeks, however total toxin load had increased to nearly 150 µg STX equvelents, a 2.3 fold increase in toxin load. The analysis of rock scallop tissues for toxin load included assessing 12 of the more than 20 PSP congeners that have been identified in toxigenic dinoflagellates and the bivalves that feed on them (Bricelj and Shumway, 1998). For scallop adductor muscle, the increase in mean toxicity over the detoxification phase was associated mainly with an increase in average toxin loads of two PSP congeners neosaxitoxin (NEO) and saxitoxin (STX), strongly indicating that biotoxin load within individual rock scallops is labile and may transform among different tissues (**Figure 12**). For the three rock scallop tissue types assessed in this study, only NEO and STX *increased* in adductor muscle over the detoxification period. Otherwise, and as expected, overall mean biotoxin levels assessed as PSP congeners in digestive gland and "rest" of tissues generally decreased over the 49-day detoxification period (**Figures 13 and 14**).

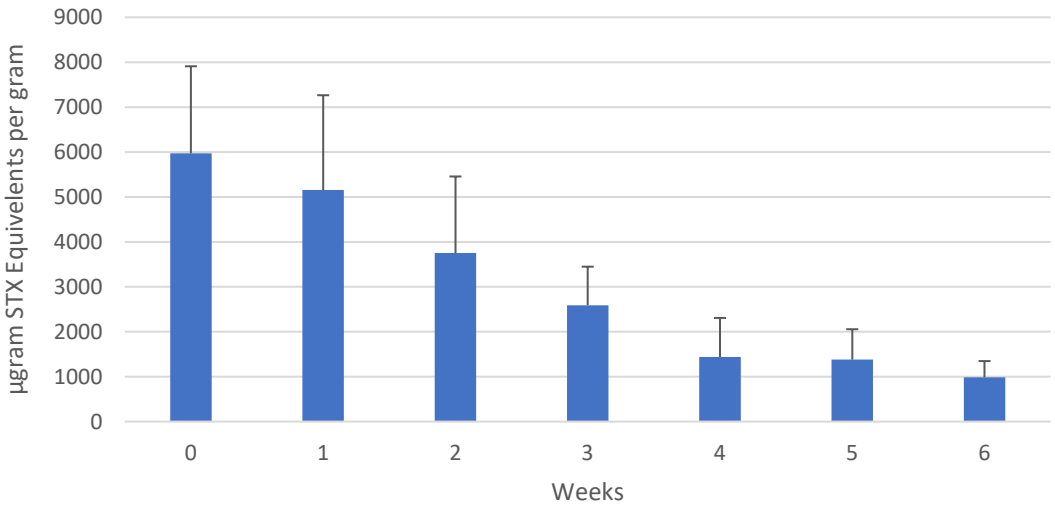


Figure 9. Saxitoxin load in STX equivalents per 100 g tissues over time in rock scallop digestive gland over a six week detoxification period. Data represent mean plus standard deviation for digestive glands dissected from individual rock scallops.

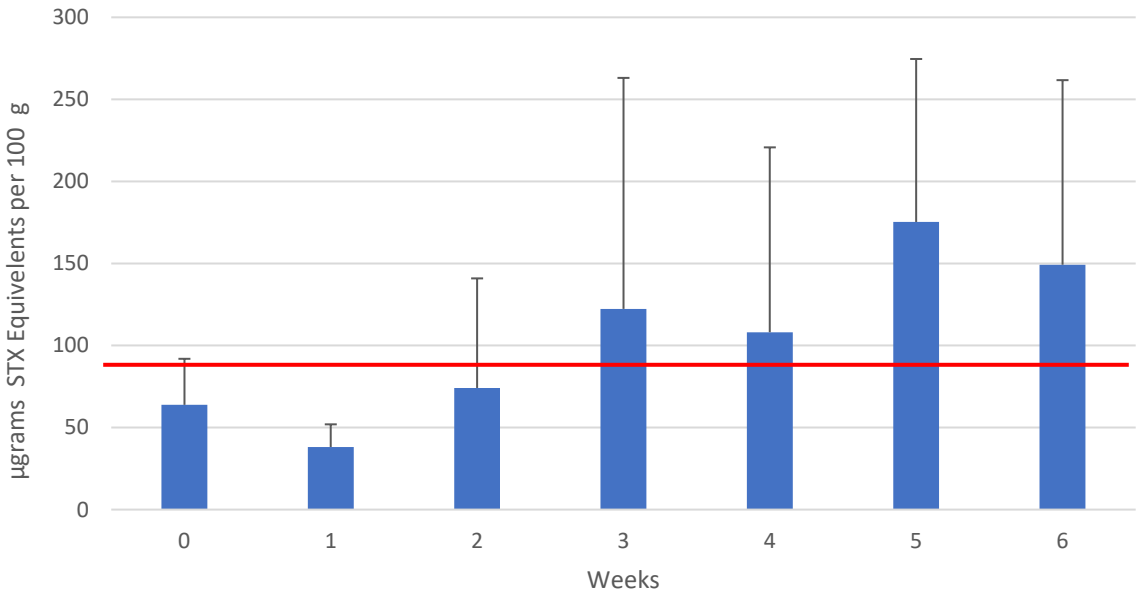


Figure 10. Saxitoxin load in STX equivalents per 100 g tissues over time in rock scallop adductor muscle over a six week detoxification period. Data represent mean plus standard deviation for adductor muscles. Red line indicates the regulatory biotoxin threshold limit for toxicity in shellfish.

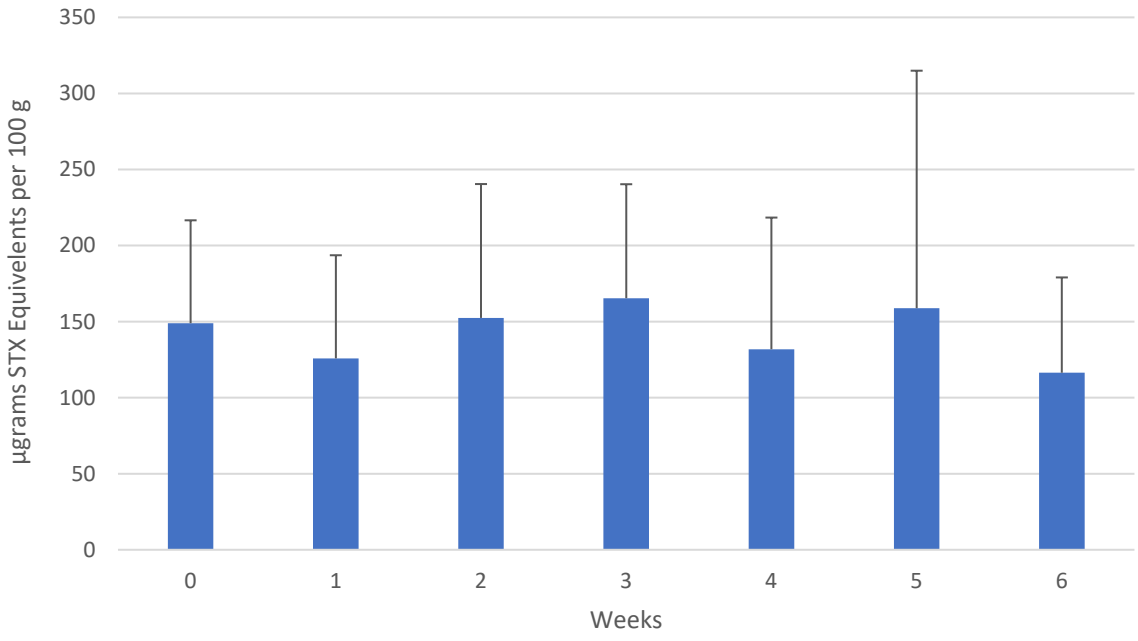


Figure 11. Saxitoxin load in STX equivalents per 100 g tissues over time in rock scallop “rest of tissues” over a six week detoxification period. Data represent mean plus standard deviation for tissues dissected from individual rock scallops.

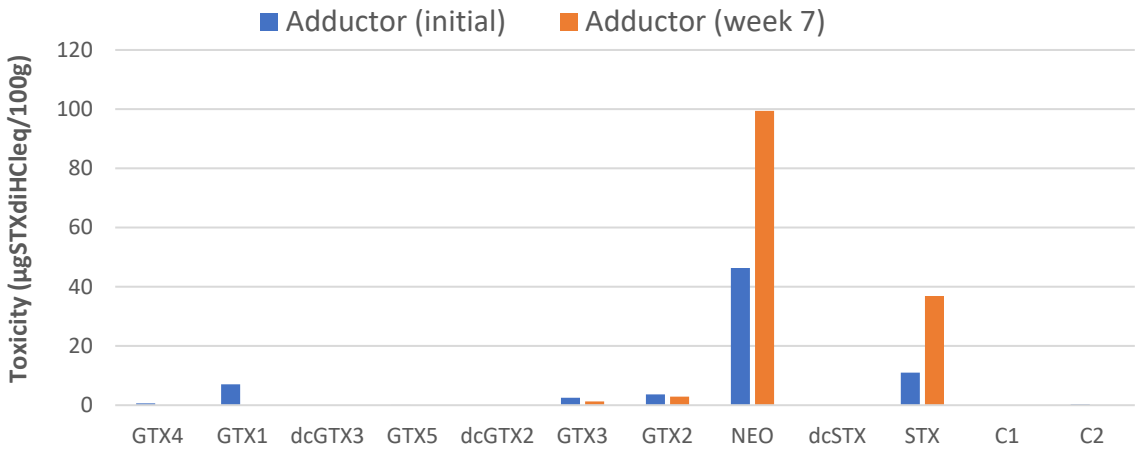


Figure 12. PSP congeners assessed for adductor muscle at day 0 and six weeks in purple hinge rock scallops.

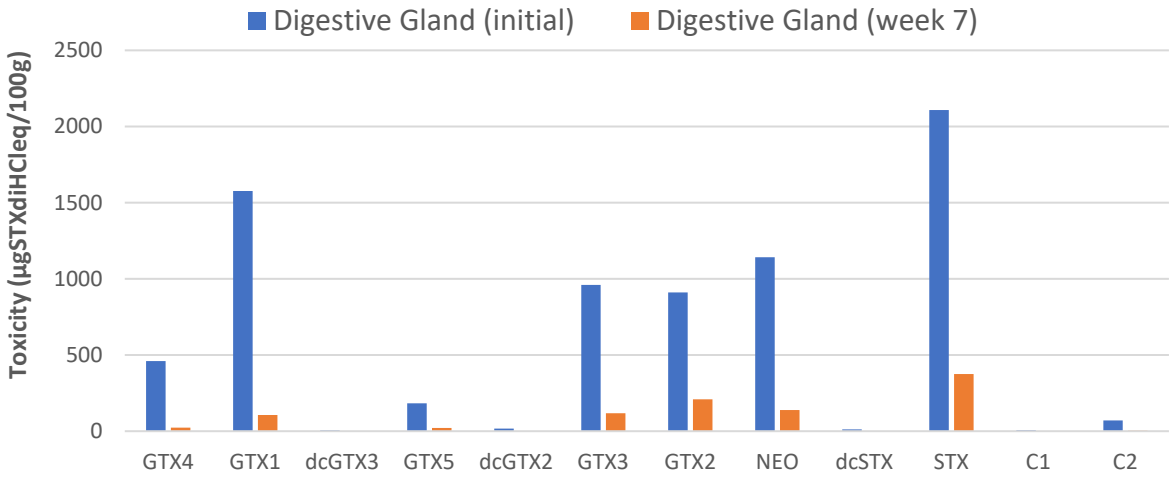


Figure 13. PSP congeners assessed for digestive gland at day 0 and six weeks in purple hinge rock scallops.

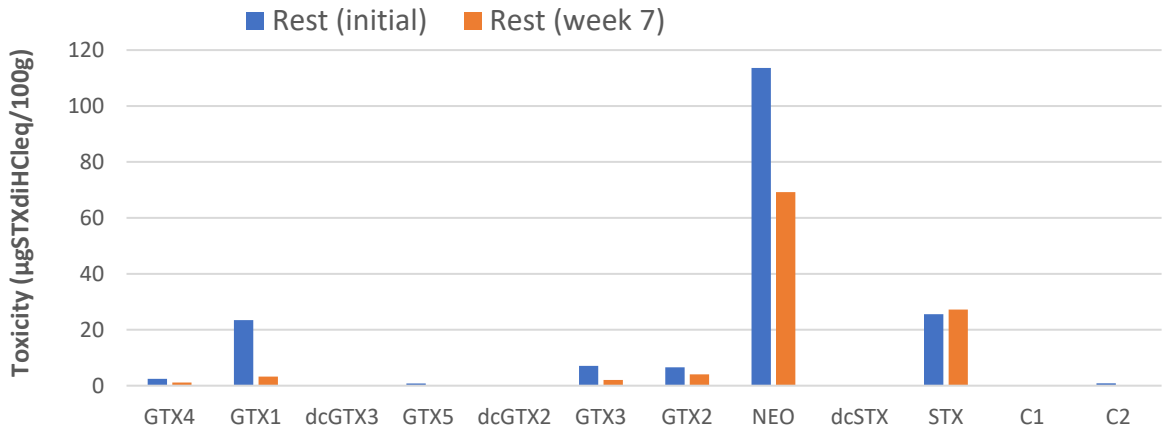


Figure 14. PSP congeners assessed for Tissue Rest at day 0 and six weeks in purple hinge rock scallops.

Discussion

Feeding Behavior in Rock Scallops Fed Toxic Alexandrium catenella

Feeding behavior exhibited by purple hinge rock scallops during uptake studies were not apparently inhibited by the presence of toxic *A. catenella*. Scallops readily fed on high concentrations (> 2000 cells L^{-1}) of *A. catenella* under laboratory conditions. While intact dinoflagellate cells were observed in pseudofeces produced by rock scallops (and not ingested), the fact that intact *A. catenella* cells were not generally observed in the fecal ribbons produced by scallops, indicated that ingestion of *A. catenella* had occurred. This of course was confirmed by the presence of biotoxin (expressed as STX equivalents in different PSP congeners) in tissues following uptake studies. *Mytilus* mussels readily uptake PSP and show no inhibitions, including nerve sensitivity associated with the presence of toxic dinoflagellates (Bricelj et al., 1990), while cupped oysters including *Crassostrea gigas* show some inhibition to PSP uptake in the laboratory. On the other hand, *Mercenaria mercenaria*, exhibit distinct behavioral changes in the presence of toxic dinoflagellates, including valve closure and cessation of filtration activities (Bricelj et al., 1991), that would appear in part to preclude significant toxin uptake during dinoflagellate blooms (Twarog et al., 1972). Rock scallops, however showed no behavioral changes (e.g. valve clapping, valve closure, cessation of feeding activities) when fed toxic *A. catenella* and readily ingest dinoflagellates, individual scallops themselves becoming toxic as a direct consequence (studies reported here). Rock scallops hold the distinction among bivalves of attaining and retaining very high toxin loads, mainly in the digestive gland, but also in adductor muscle tissues at generally lower concentrations (Beitler, 1991).

PSP Uptake and Detoxification Studies

Rock scallops increased overall tissue toxicity following 4-day toxification experiments in 2017 and 2018, with toxin loads increasing after 24 hours in both adductor muscle and digestive gland tissues. Uptake studies in 2017 were hampered by high initial toxin loads in scallops that increased over the first 24 hours of toxification. Scallops appeared to rapidly detoxify in 2017 but toxin load in tissues remained high throughout the detoxification period. After four weeks toxin load in digestive gland tissues were reduced from pre-toxification levels but still well above the regulatory limit. Adductor muscle toxicity also increased in the 2017 toxification study in the days following the feeding phase but never exceeded the regulatory threshold. In the 2018 experiments, the pattern was different with rock scallops exhibiting peak toxin loads in both adductor muscle and digestive gland tissues seven days following the initiation of a four day toxification period. Again, pre-toxification of digestive gland tissues was high prior to initiating the toxification work, indicating that rock scallops had been previously exposed to PSP and had retained toxin for an unknown period of time. In 2018 studies, following peak toxin load in both adductor muscle and digestive gland tissues after one week, tissues detoxified over the ensuing 28 days with adductor muscle tissue falling well below the regulatory level of 80 µg STX equivalents per 100 g tissue after the first week post toxification (**Figures 6 & 7**).

The pattern of toxification and detoxification in rock scallops is similar to other studies of bivalves fed toxic dinoflagellates with bivalves increasing toxin loads in digestive gland tissues at significantly higher rates than for other tissues (Bricelj and Shumway, 1998).

Detoxification Following Natural Toxification in Rock Scallops

Rock scallops that were naturally toxified during a large bloom of *A. catenella* in Sequim Bay, WA in 2018 and transferred to Manchester, WA for 6 weeks of depuration in toxin free water clearly demonstrated rapid detoxification over this time frame. Whole scallop tissue toxicity was very high at the onset of detoxification with toxin loads in excess of 6000 µg STX equivalents per 100 g tissue, with the majority of toxin load located in the digestive gland (**Figure 8 & 9**). Whole adductor muscle toxicity was significantly lower at the onset of detoxification at 63.8 µg STX equivalents per 100 g. tissue, a level below the regulatory threshold. Interestingly, adductor muscle toxicity increased over the ensuing weeks with toxicity increasing to well over 100 µg STX equivalents after three weeks detoxification (**Figure 10**). The toxin profile also changed over time during detoxification with the PSP congeners NEO and STX increasing in adductor muscle tissue over time (**Figure 11**). Importantly both the STX and NEO PSP congeners are the most toxic carbamate based neurotoxins (Cembella et al., 1994). Changes in the toxin profile in bivalves during both toxification and detoxification phases are well known. Generally, changes come about as a result of biotransformation processes that include congener conversion in the presence of natural reductants in vitro or to epimerization, the change in physical form from one congener to its chiral counterpart (see reviews by Cembella et al., 1993 and 1994; Bricelj and Shumway, 1998). The present study demonstrated that toxin profiles change over the detoxification phase, however additional analysis is necessary to affirm both process and pattern in rock scallops.

Detoxification studies in rock scallops demonstrated that this species attains very high toxin loads for long time periods compared to other bivalves, including other scallops following exposure to bloom conditions of toxic *A. catenella*. In addition, unlike some other scallops including the commercially valuable *Placopectin magellanicus*, toxin uptake and retention in the adductor muscle may remain high for significant time frames.

The high toxin loads associated with the visceral mass of rock scallops and time frames associated with toxin uptake and particularly rates of detoxification will likely result in regulatory concerns over aquaculture development for the whole scallop market. While whole body toxin loads decreased over detoxification timeframes measured in weeks, the overall toxicity of rock scallops considered in these studies remained well above regulatory thresholds. The market for adductor muscle only will also require careful scrutiny as this study has demonstrated. It will be vitally important that PSP testing be done on adductor muscles on a time frame that accommodates potential changes in toxin load following toxification in tissues generally.

Objective 2: Examine bioaccumulation and detoxification of biotoxins in rock scallops exposed to naturally occurring biotoxins (PSP and DSP)

Method and Materials

Purple hinge rock scallops previously produced from Washington broodstock at the Taylor Shellfish hatchery in Quilcene, Washington, through a Western Regional Aquaculture Center (WRAC) funded project were deployed at four sites with a history of biotoxin blooms and at locations routinely monitored by WDOH Sentinel Mussel Monitoring Program. In North Puget Sound, WA: Sequim Bay (John Wayne Marina and private residence, Crowell House) and Discovery Bay (**Figure 15**). Sequim Bay has one of the longest recorded histories of PSP in the state (Trainer 2002, Trainer et al. 2003) and is considered a hot spot for PSP due to frequently elevated levels of saxitoxin in mussels (Lefebvre 2008). The fourth site was located in South Puget Sound, 90 miles south of the northern sites at West Bay Marina in Budd Inlet. This site was selected based on its history of *Dinophysis spp.* blooms and regular biotoxin monitoring by the WDOH Sentinel Mussel Monitoring Program.

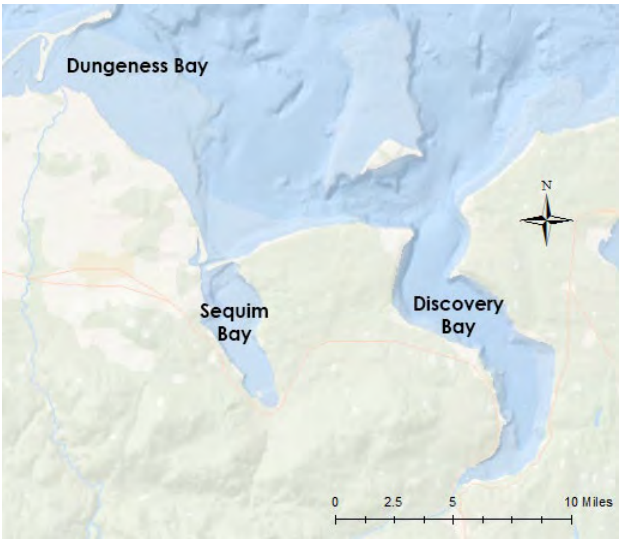


Figure 15. Northern sites, Sequim Bay and Discovery Bay were selected for field exposure trials based on a history of biotoxin blooms.

Figure 16. a) Project partners Jamestown S'Klallam Tribe, Baywater Inc. and PSI prepare scallops for outplant in Sequim Bay, WA. b) Replicate bags in a single array with PVC frame for initial grow out of rock scallops for biotoxin research. c) Plastic trays with wider gauge to increase flow and improve routine maintenance and monitoring.

On February 24, 2017, in partnership with Jamestown S'Klallam Tribe, Baywater Inc. and PSI, sub-adult scallops (~40-60mm SL) were deployed at all four sites (**Figure 16a**) and maintained until early 2020. Scallops were placed in cages made from polyethylene bags commonly used for bivalve mariculture, supported by PVC frames to provide structure for suspending the cages from floating subtidal dock sites (**Figure 16b**). Scallops were deployed 55 to a cage, 4 cages to an array, and 2 arrays per site. Arrays were suspended approximately 3m below the surface at each site. After eight months of grow out in the polyethylene bags, it was evident that gear fouling was a challenge. Heavy fouling in warm summer months restricted water flow and feeding for growing scallops and increased the overall weight of the setup, making the arrays difficult to monitor and manage. Therefore, scallops were transferred in fall 2017 to replicate grow out trays (**Figure 16c**) with larger mesh size and a removable lid for easy maintenance for the duration of the project.

Through collaboration with two existing early-warning biotoxin monitoring programs: WDOH Sentinel Mussel Monitoring Program and the Jamestown S'Klallam Tribe's monitoring prior to subsistence shellfish digs, timing for rock scallop collection and shipment for biotoxin testing was determined. For the Sentinel Mussel Monitoring Program, mussels are placed in cages and collected biweekly for PSP testing, and rising PSP levels trigger more targeted and frequent sampling regimens in other shellfish species. When existing monitoring indicated rising or sustained biotoxin levels, 10 animals were removed from culture cages and shipped whole on ice via FedEx overnight to either the Sitka Tribe of Alaska Environmental Regulatory Lab (STAERL) or the WDOH Public Health Lab, depending on biotoxin present. Jamestown S'Klallam Tribe biologists collected and shipped scallop samples from the northern sites and PSI collected and shipped scallops from Budd Inlet. From the first indication of PSP or DSP, 10 scallops were sampled each week, for the duration of the bloom or until the animals no longer indicated toxins above the FDA thresholds, DSP 16µg/100g tissue and PSP 80µg/100g tissue. STAERL conducted saxitoxin analysis using receptor binding assay (RBA) as described in Obj. 4. STAERL technicians dissected whole animals into two component parts (adductor muscle, viscera) for two separate RBA analyses per animal. Individual scallops (n=10) collected from early 2017 – October 2, 2017 were homogenized in the lab for a "pooled" sample resulting in a single data point of saxitoxin (STX) for a particular location/time point. From October 9, 2017 and onward (through early 2020), all 10 scallops and component parts were analyzed individually for each time point. The change in method was improved and made possible through project funds that allowed STAERL to

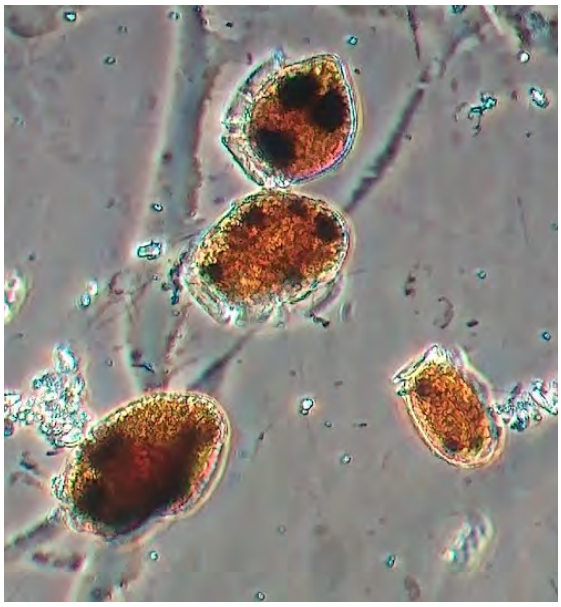


Figure 17. Several species of Dinophysis collected from Budd Inlet, (top) *D. norvegica*, (middle) *D. fortii*, (right) D. acuminata and HAB species, (bottom left) *Akashiwo sanguinea*. Photo: A. Christy (PSI)

purchase new equipment to run smaller tissue samples using the RBA method. When monitoring programs indicated DSTs (diarrhetic shellfish toxins), scallops were shipped to WDOH's Public Health Lab for testing using liquid chromatography-tandem mass spectrometry (LCMS/MS) in whole animals.

Results

The purpose of the temporal field exposure trials was to examine bioaccumulation and subsequent detoxification of biotoxins in whole animals for diarrhetic shellfish toxins (DSP) and in selected component parts of individual scallops, adductor muscle and viscera for saxitoxin (PSP). The following time series include biotoxin levels in rock scallop and blue mussels. Blue mussel toxicity data was provided by WDOH through the Sentinel Mussel Monitoring Program. Cell counts of harmful algae species, *Dinophysis spp*. (DSP) and *Alexandrium catenella* (PSP) was collected from the SoundToxins database and PSI data collection. Water quality data including water temperature (ºC) and salinity (ppt) was collected from SoundToxins (biweekly) and PSI (bimonthly) monitoring at scallop sites. PSI collects phytoplankton and water quality data for SoundToxins in Budd Inlet including *Dinophysis spp.* cell counts and speciation included in the following results. SoundToxins data for Discovery Bay, WA is collected by Port Townsend Marine Science Center and data for Sequim Bay, WA is collected by Jamestown S'Klallam tribe biologists.

Diarrhetic Shellfish Toxins (DSTs)

Dinophysis is a genus of mixotrophic dinoflagellate that produces a suite of toxins including dinophysistoxins (DTX) and okadaic acid (OA), collectively referred to as DSTs, diarrhetic shellfish toxins that cause diarrhetic shellfish poisoning (DSP) when consumed. Consuming contaminated shellfish tissue can lead to symptoms including; nausea, vomiting, abdominal pain, and diarrhea. Species of *Dinophysis* have been in WA waters for many years, but only recently has the toxicity increased to levels of concern to public health (WDOH). In June 2011, the first US confirmed clinical report of DSP illnesses occurred in Washington where three people became ill from contaminated blue mussels harvested in Sequim Bay (WDOH). Blue mussels collected a few days following the illnesses contained DSTs 2-10 times the action threshold, resulting in recreational and commercial shellfish closures. Following this event, monitoring for *Dinophysis* in Washington state became formalized in 2012 in partnership through existing programs, SoundToxins in Puget Sound and Olympic Region Harmful Algal Bloom (ORHAB) on the outer coast (Trainer et al. 2013). Washington Department of Health began regularly monitoring for DSTs in recreational and commercial shellfish species in 2013. WDOH routinely monitors shellfish throughout the state and closes growing areas when DSP toxins exceed 16µg/100 grams of shellfish tissue. Since 2013, approximately 34 shellfish growing areas and 10 counties have been affected by DSP related closures (WDOH). **Figure 17**, features several species of *Dinophysis* in our region that are known to produce DSTs. Understanding what conditions are causing the recent blooms, toxicity and resulting DSP closures is an area of active research in Puget Sound, led by scientists at NOAA Fisheries. The following datasets collected for rock scallop will provide more information on accumulation and depuration of DSTs in this

native shellfish species through time in comparison with blue mussels, while also furthering the collective knowledge of *Dinophysis* toxicity and interaction with changing water temperature and salinity in our region.

A limited number of blooms and subsequent DSP related closures occurred during the project period for northern sites, Sequim Bay (**Figure 18**) and Discovery Bay. **Figure 18**, illustrates a DSP related shellfish closure in Sequim Bay, WA during early fall in 2017. High cell counts of *Dinophysis* (7000 cells/L) on 10/3 precede high DSP toxicity in manila clams (19µg/100g tissue), initiating the WDOH shellfish closure on 10/4. Rock scallops (n=10) sampled on 10/17 tested over the DSP action level at both sites, 17µg/100g (JWM) and 59µg/100g (CH). Rock scallops sampled each week following the closure remained well above the action level. Rock scallops reached peak toxicity on 11/1, (108) following high *Dinophysis* cell counts (10000 cells/L) on 10/10. After this peak, scallops detoxed precipitously over the course of several weeks until toxins were below the action level on 12/13, (4). In contrast, blue mussels seemed to exhibit a marked delay in uptake of DSTs, remaining very low during the initial closure on 10/4, (3) and did not exceed the action level until 11/21, (25). Blue mussels detoxed in December initiating the bay to be opened for all shellfish species on 12/14. From 12/6, through the following winter months, *Dinophysis* is reported "absent" in SoundToxin plankton samples. This dataset shows how multiple species of shellfish may illicit differential responses to DSTs in initial uptake, peak toxicity and depuration rates all within the same water body and within close proximity. While manila clams grow at the sediment/water interface, it's notable that scallops and blue mussels sampled for this time series were growing at comparable depth at the same location. With that said, *Dinophysis* can exist in thin layers and when mixed through storms and currents can become spatially patchy throughout the water column, even on small spatial scales (Reguera *et al*. 2012), which may have affected the observed differences in uptake and toxicity in blue mussels and rock scallops during this time period.

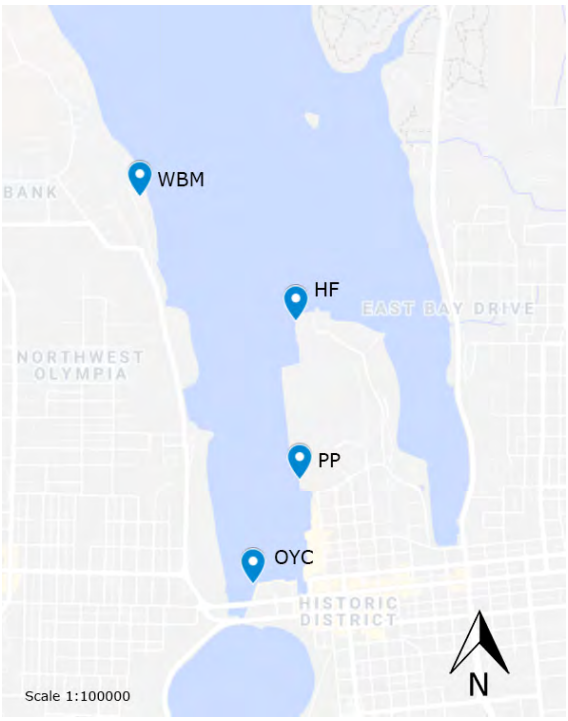


Figure 19. Sample sites in Budd Inlet for DSP biotoxin monitoring. Rock scallop grow out site at West Bay Marina (WBM), DOH Sentinel Mussel site at Olympia Yacht Club (OYC), SoundToxins monitoring sites at Port Plaza (PP) and Hearthfire (HF).

Two time series were collected for Budd Inlet, 2017-2018 and 2019-2020, **Figure 19** shows sample locations. These datasets augment the existing long-term dataset of water quality, *Dinophysis* cell counts (PSI) and blue mussel toxicity data (WDOH) for Budd Inlet dating back to 2013 (**Figure 20**). The first dataset begins in August 2017 after blue mussels test over the action level on 8/24/2017, (19) prompting a

shellfish closure for all species (**Figure 21**). Rock scallops (n=10) were sampled weekly until the bay was opened to all species on 3/7/18. Budd Inlet was only briefly open, experiencing another DSP closure two weeks later on 3/22 when blue mussels tested over the action level (19). The last scallops collected for this early spring period was on 4/3. The gap in scallop data was a result of all animals being sampled from the grow out trays. Re-stocking this site was not logistically feasible until July when scallops from a previous research project (J. Davis) were transferred from nearby Totten Inlet to Budd Inlet for continued grow out and DSP testing/sample collection. After re-stocking, rock scallops were sampled on 7/10 and tested below the action level, 2µg/100g one day after blue mussels on 7/9 at 5µg/100g for DSP. WDOH opened Budd Inlet to all species shortly thereafter on 7/12/18. During this time series, blue mussels maximum toxicity level was 31µg/100g on 12/28/17 compared to 54µg/100g in rock scallops on 12/4/17. From the date of maximum toxicity, rock scallops detoxed below the action level in 16 days in contrast to a longer 5-week detox in blue mussels. Relatively high cell counts of *Dinophysis* in August 2017 (768 cells/L) likely increased shellfish toxicity in blue mussels and rock scallops, prompting the 2017 DSP closure. Interestingly, the only *Dinophysis* species found within the samples at this time was *D. fortii* producing DSTs. In colder months, cells tapered off and reappeared in the summer with high cell counts of the species, *D. norvegica* on 7/3/18 (3798 cells/L). While high cell counts occurred at this time, toxicity in both species, blue mussels and rock scallops remained low.

The following data series from November 2019 – April 2020 is illustrated in **Figure 22** for DSP in rock scallops, blue mussels and *Dinophysis* cell counts. A *Dinophysis* bloom on 11/19, (2113 cells/L) increased toxicity in blue mussels (76), prompting a DSP closure. Rock scallops (n=10) were sampled on 11/25 and tested well above the action level at 42µg/100g tissue. Scallops were shipped and tested weekly following the closure. At the date of maximum toxicity, 12/3 (46) rock scallops depurated the toxins for 9.5 weeks, falling below the action level at the end of January 2020. In contrast, peak toxicity in blue mussels was significantly higher than rock scallops at two time points in December (85) and early March (151). After rock scallops depurated the DSTs and were no longer toxic after January, the DSP shellfish closure continued due to extended toxicity in blue mussels. In early 2020 through spring months, *Dinophysis* cell counts remained low and had a mixed composition of multiple species known to produce DSTs, *D. fortii* and *D. acuminata*. It is interesting to note the low toxicity in rock scallops during periods of high toxicity in blue mussels when *Dinophysis* was not blooming. Reported cell counts from this time period were collected at the scallop grow out location (WBM) and not at the nearby WDOH Sentinel Mussel site (OYC), see site map, **Figure 19**, therefore it's possible given the spatial variability of *Dinophysis* cells and closer proximity to freshwater inputs (Deschutes River) at OYC, toxicity of existing cells may have increased toxicity in blue mussels at this site and not within the nearby scallops just 1.5 miles north. It is unclear however from our data what variables may be creating this distinct species specific response to uptake of DSTs at this time. Grow out conditions of blue mussels, environmental variables and any additional stressors at OYC during early 2020 time points will need to be explored further.

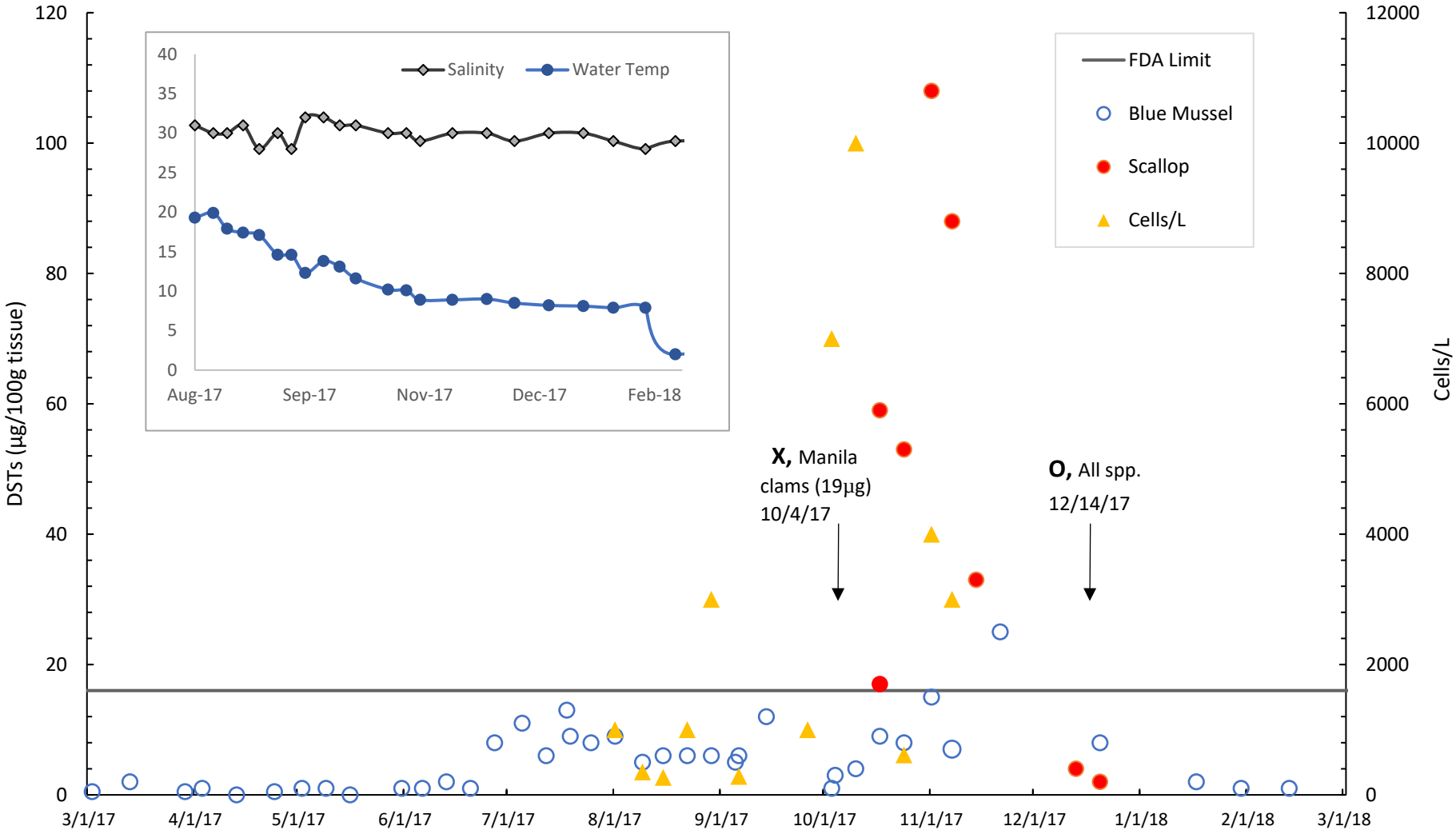


Figure 18. Time series of DSP in whole blue mussels and whole rock scallops (n=10) from Sequim Bay, WA for 12 months, 2017-2018. Blue mussel data courtesy of WDOH. Dinophysis spp. cell counts, water temperature and salinity data from SoundToxins (N. Harrington, Jamestown S'Klallam). FDA limit = 16µg/100g tissue. WDOH shellfish closure (X) and open dates (O). Water temperature (ºC) and salinity (ppt) data provided in subset for period of Dinophysis bloom/closure and scallop sampling only.

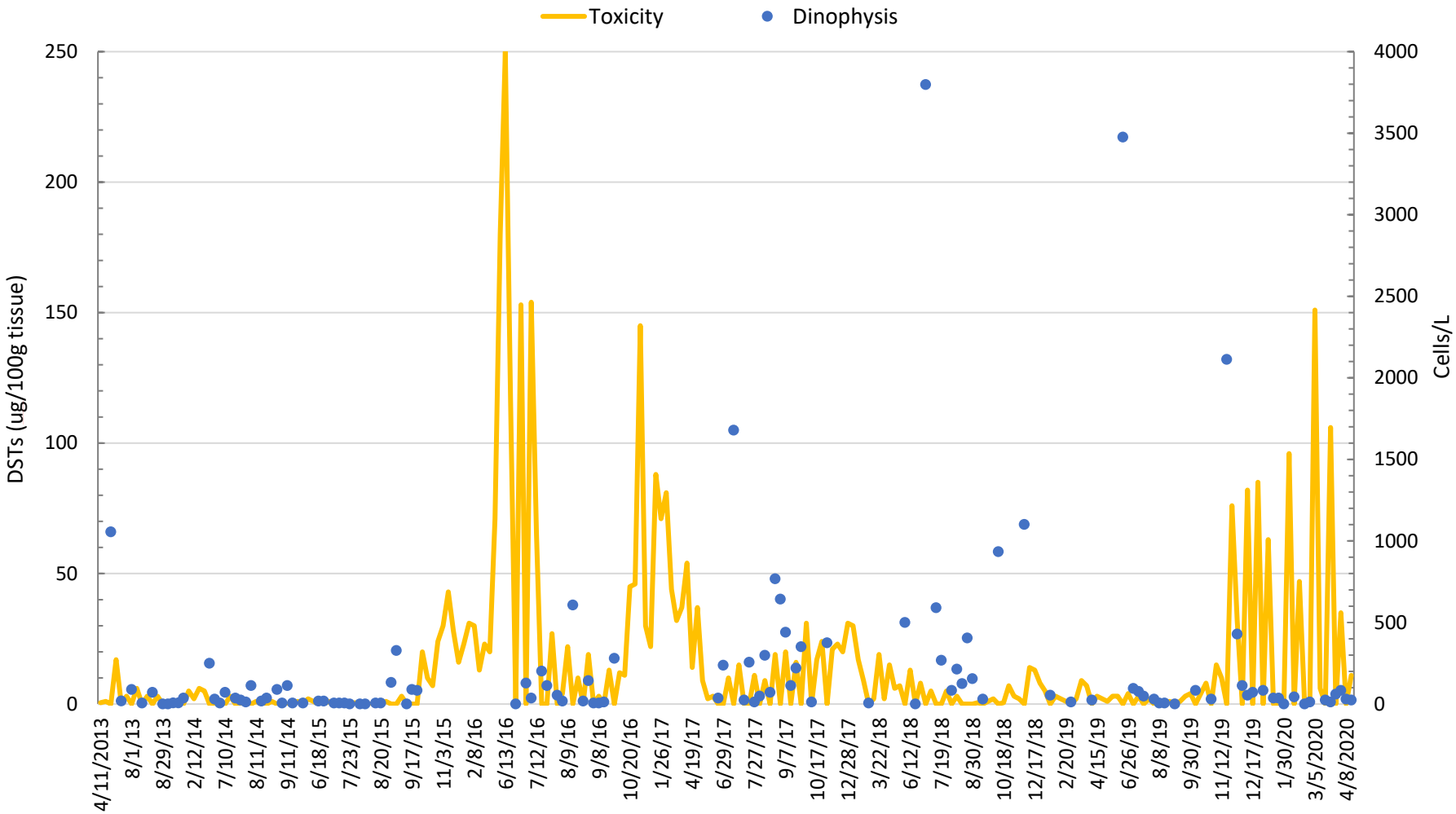


Figure 20. Long-term data set of Dinophysis spp. and DSP in blue mussels from Budd Inlet, WA. Blue mussel data provided by WDOH, cell counts by PSI (A. Christy). Rock scallops were collected and tested for DSP from Aug 2017 – Aug 2018 and Nov 2019 – April 2020.

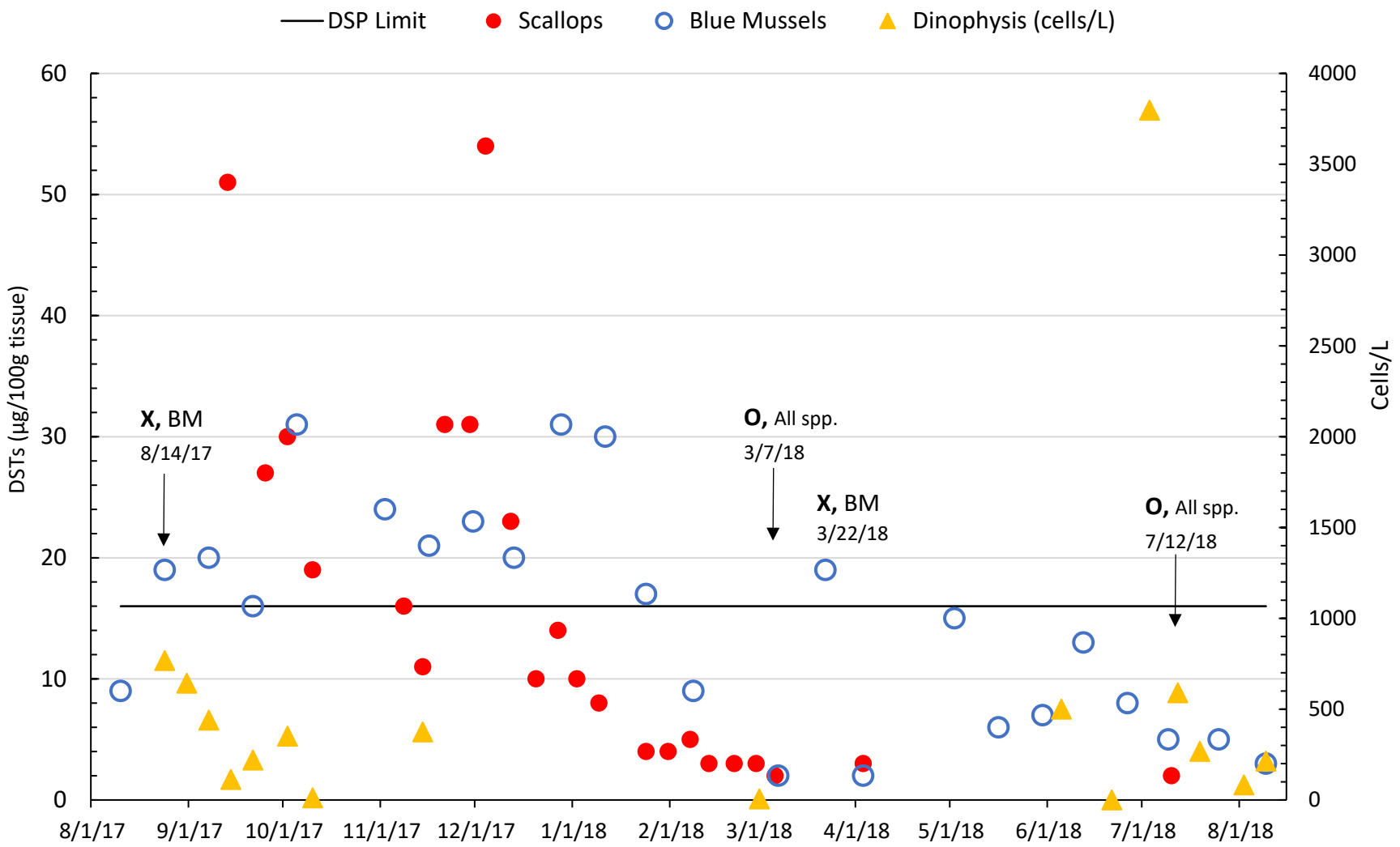


Figure 21. DSP toxicity in whole rock scallops and whole blue mussels (WDOH) sampled from Budd Inlet, WA in South Puget Sound from Aug 2017 – Aug 2018. Dinophysis cell counts reported from concentrated 3m vertical plankton net tows at the scallop grow out location (PSI, A. Christy). DSP limit = 16µg/100g tissue. WDOH shellfish closure (X) and open dates (O).

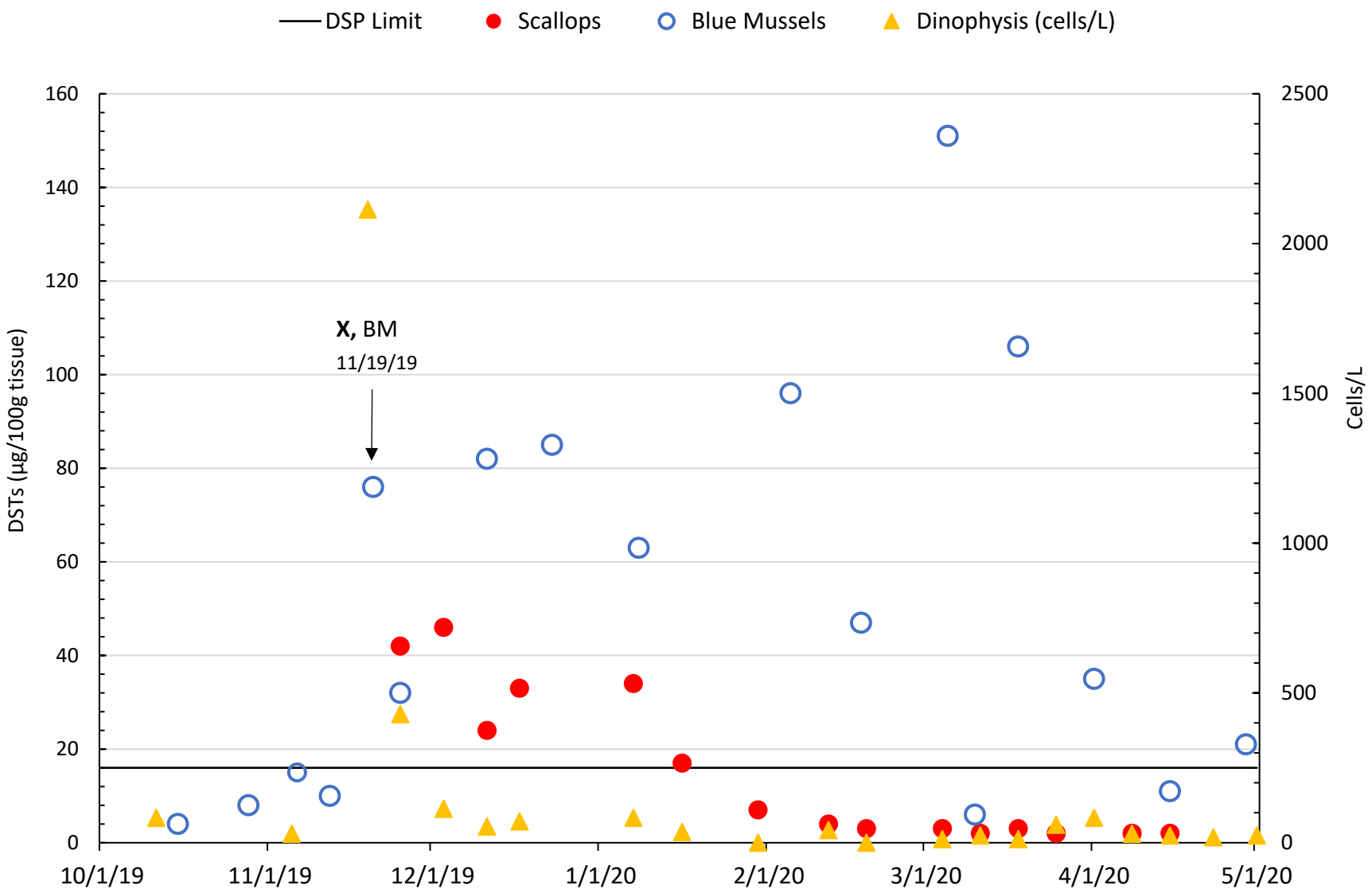


Figure 22. DSP toxicity in whole rock scallops and whole blue mussels (WDOH) sampled from Budd Inlet, WA from Nov 2019 – April 2020. Dinophysis cell counts reported from concentrated 3m vertical plankton net tows at the scallop grow out location, WBM. Note: Plankton collected on 11/5, 11/19 at PP and 4/23, 5/1, 5/7 collected at HF (see map, Figure 19). DSP limit = 16µg/100g tissue. WDOH shellfish closure (X) date. Note: Budd Inlet opened late June, after the project ended.

In general, the time series of DSP for rock scallops and blue mussels indicate high levels of toxicity in shellfish during fall months with a varied response of subsequent depuration rates for each species. Cell counts of *Dinophysis* preceding all three DSP closures ranged from 768cells/L – 7000cells/L, indicating high cell counts may lead to toxicity in shellfish. However, our data series also show that high cell counts of *Dinophysis* may *not* lead to toxicity in either species of shellfish, as was true in summer 2018. This outcome may be due to the species composition of *Dinophysis* and environmental stressors at the time of the bloom, influencing toxicity of algae and uptake. This phenomenon needs further investigation. Furthermore, high toxicity in blue mussels in spring 2020 when cell counts were low and toxicity in rock scallops were also low, creates additional questions. To reliably predict DSP related closures for rock scallop, it's clear more research is needed to de-couple the complex interactions of *Dinophysis*, DSTs, physiology and water quality.

Paralytic Shellfish Toxins (Saxitoxin)

The toxic dinoflagellate *Alexandrium catenella* is a common bloom-forming dinoflagellate on the west coast of North America. This species produces a suite of marine biotoxins referred to as, paralytic shellfish toxins (PSTs). PSTs are 1000 times stronger than cyanide, and the symptoms of poisoning occur soon after ingestion of toxic shellfish resulting in the deadly paralytic shellfish poisoning (PSP) in humans. The dosage necessary to induce fatal poisoning in humans is small (500µg in 1g of tissue), thus the consequences of eating toxic shellfish constitute a major public health concern (Kao 1993). Human health impacts including the only human death specifically associated with ingesting rock scallops have been well documented (Price et al. 1991), and whole scallop fisheries for pink and spiny scallops (*Chlamys rubida*, *C. hastata*, respectively) have been largely curtailed in Washington State due mainly to the propensity for biotoxin uptake and long-term retention (Washington Administrative Code, WAC 220-52-069). The toxins associated with PSP are the most widely reported toxins in bivalve molluscs along the west coast of North America. Of these toxins, saxitoxin (STX) is the most potent and therefore prioritized for testing in shellfish biotoxin monitoring programs, including Washington State. Rock scallops are not among the species routinely tested, and the standing recommendation from WDOH is for no recreational harvest of rock scallops from Washington State waters at any time.

Preliminary data produced by WDOH in the 1980s indicate that saxitoxin is not generally distributed in body tissues, but remains concentrated in the viscera, with smaller amounts of toxin accumulating in the adductor muscle (Beitler 1991) (**Figure 23**). The following results of field exposure trials in Sequim and Discovery Bays from 2017 – 2020, supports and expands on previously reported findings of STX in rock scallop adductor muscle and viscera tissues from Sequim Bay, WA (WDOH, **Figure 23**).

Time Series - Sequim Bay

The first PSP time series from Sequim Bay was initiated on 8/3/2017 with high levels of STX in blue mussels (181µg/100g) well above the action level of 80µgSTX/100g tissue, resulting in a shellfish closure that persisted for 4.5 months (**Figure 24**). Shortly after the closure on 8/7, rock scallops were collected (n=30) from both Sequim Bay grow out sites and shipped to the Sitka

Tribe of Alaska Environmental Research Lab for dissection and analysis of STX in viscera and adductor tissues. In the early stages of lab analysis, tissue samples were "pooled" to equate to 100g of each viscera and adductor muscle resulting in one STX value for each time point/location sampled. Four days following the shellfish closure, rock scallops tested extremely high in the viscera at both Sequim locations, CH (297) and JWM (500). In contrast, adductors were significantly lower in toxins, well below the FDA limit at both sites, CH (8) and JWM (31).

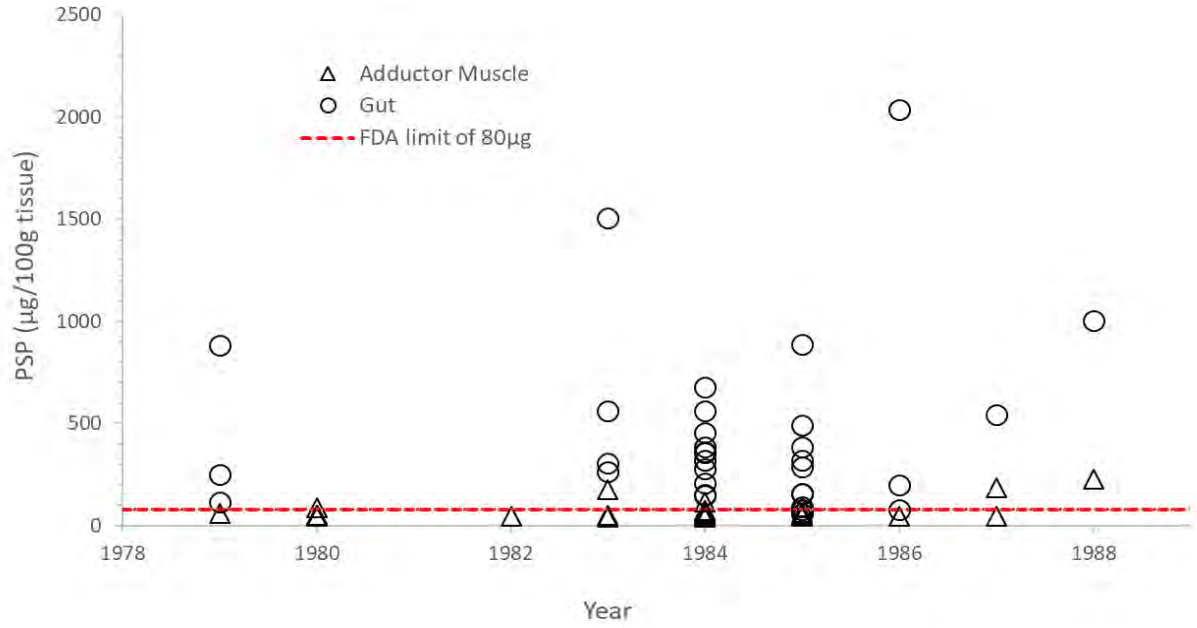


Figure 23. Saxitoxin levels in rock scallops collected from Sequim Bay, WA. The dashed line corresponds with the FDA limit of 80µgSTX/100g shellfish tissue, the threshold that results in PSP related shellfish closures. Historical data courtesy of WDOH.

Alexandrium cells were reported "present" in Sequim Bay water samples by SoundToxins near the grow out location for both scallops and blue mussels at the time of the closure, but no indication of bloom conditions. On 8/9, 88cells/L of *Alexandrium* were reported in SoundToxins samples. By the following week of sampling on 8/14, water temperatures dropped significantly by two degrees Celsius and toxicity continued to increase in rock scallop viscera and adductor tissues at both sites as blue mussels showed depuration of toxins. At the end of August, peak toxicity in scallop adductor at JWM (162) was observed with proportionately high toxins in viscera (1671). Water temperature (17.1ºC) and salinity (28ppt) continued to fall during this time. The following week, 9/5 blue mussels reached peak toxicity (346) for the data series, which coincides with a steep drop in water temperature (14ºC) following peak toxicity in rock scallop viscera on 9/11 at both locations, CH (6450) and JWM (4567), while adductors hovered around the threshold at CH (73) and JWM (93). After max toxicity in blue mussels and rock scallops, a slow depuration period extended through the rest of the time series during the cold winter months. Blue mussels depurated all toxins by the end of the year, WDOH reported Sequim Bay open for blue mussel harvest on 12/14. Rock scallops, however persisted with high STX in the viscera, well above the PSP threshold, for the duration of the 6-month time series. It's unknown from our collected data exactly how long rock scallops can persist with STX in the viscera. Future monitoring will be necessary to acquire that information.

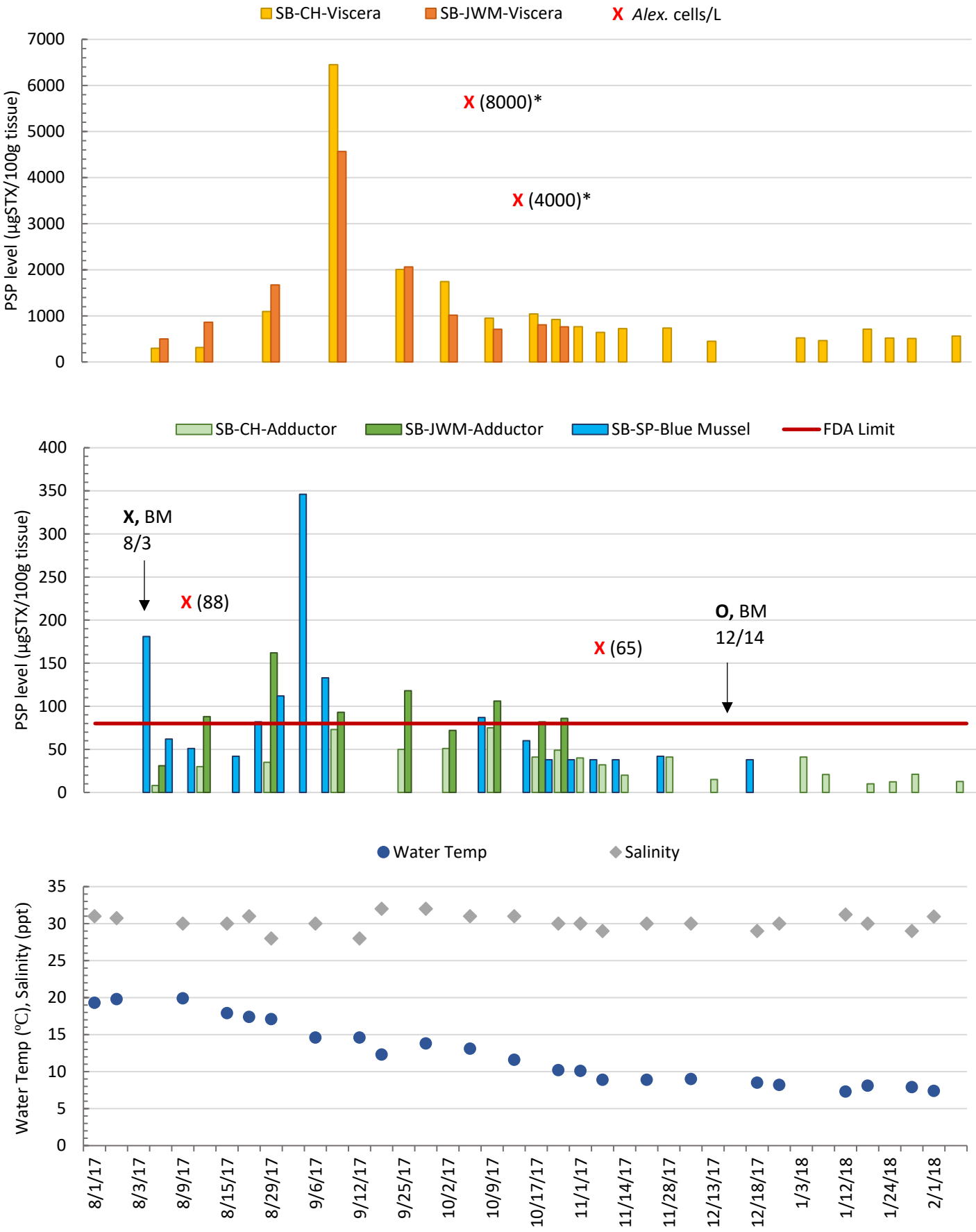


Figure 24. PSP in rock scallop viscera (top), and adductor muscle and blue mussels (middle) for Sequim Bay sites (CH & JWM), Aug 2017 – Feb 2018. Water temperature, salinity and cell count data (bottom) collected from SoundToxins (N. Harrington) and PSI during the time series. Note: Mean PSP values (n=10) for rock scallop after Oct 2017 presented. WDOH shellfish closures (X), and opening (O) dates. Whole water sample method for cell counts indicated by (*).

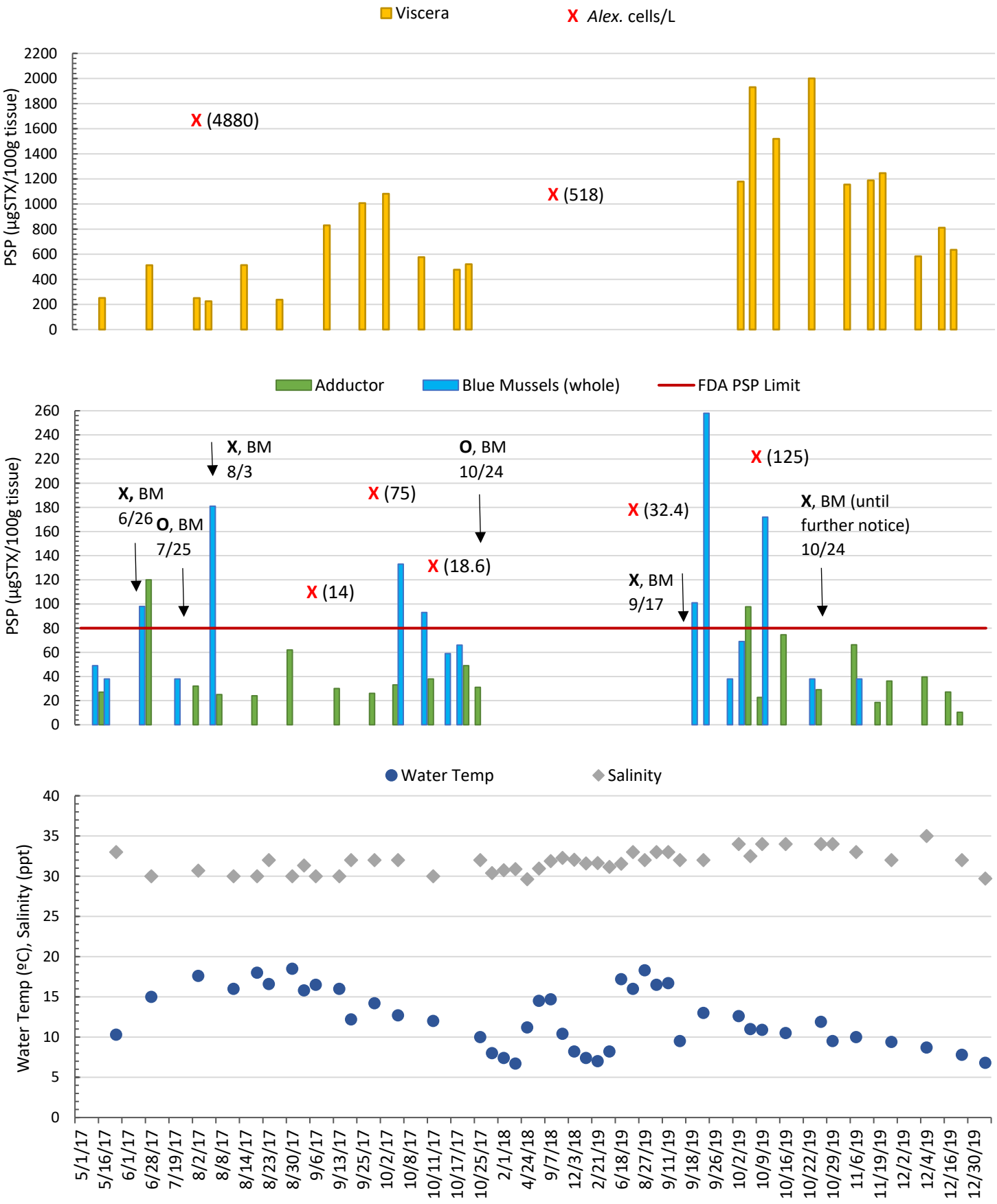


Figure 25. PSP in rock scallop viscera (top), adductor muscle and blue mussels (middle) for Discovery Bay, May 2017 – Dec 2019. Water temperature, salinity and cell count data (bottom) collected by SoundToxins (PTMSC, J. Landry) and PSI during the time series. Note: Mean PSP values (n=10) for rock scallop presented for 2019 sampling dates. WDOH shellfish closures (X), and opening (O) dates.

Table 4. PSP shellfish harvest closure and opening dates for Discovery and Sequim Bays from WDOH marine biotoxin monitoring program during field exposure trials, 2017-2020.

Location	Date	Closure	Opened
Discovery Bay	5/4/2017	Geoduck PSP 135	
	5/18/2017		Geoduck
	6/23/2017	Geoduck PSP 118	
	6/26/2017	Blue mussels PSP 99	
	7/25/2017		Blue mussels
	8/3/2017	SB State Park blue mussels PSP 181	
	8/31/2017	Blue mussels DSP 21	
	10/24/2017		Blue mussels
	9/13/2019	Geoduck PSP 110	
	9/17/2019	Blue mussels PSP 101	
	10/14/2019	Blue mussels closed until further notice	Geoduck
	8/5/2020	All species	
	9/24/2020		Geoduck
Sequim Bay	8/3/2017	Blue mussels PSP 181	
	9/7/2017	All species, blue mussels PSP 133	
	10/6/2017	Manila clams DSP 17	
	12/14/2017		Blue mussels
	11/21/2018	Blue mussels PSP 370	
	12/7/2018		Blue mussels

Time Series - Discovery Bay

The time series presented in **Figure 25**, describes STX in rock scallop adductor, viscera and blue mussels from Discovery Bay at the same grow out location for two periods, June 2017- Oct 2017 and Sept 2019-Dec 2019. The initial shellfish closure occurred on 6/26/17 when blue mussels tested over the action level (98). Scallops were promptly sampled following the closure on 6/28 (n=30), pooled samples reported values of STX over the limit for both adductor (120) and viscera (513) tissue. *Alexandrium* cells were reported "absent" from SoundToxins sampling at this location for all of May, June and July. Following the initial closure, weekly sampling showed quick depuration in blue mussels and rock scallop adductor, viscera however remained high (251). The bay opened for blue mussel harvest on 7/25/17, followed by a large *Alexandrium* bloom (4880 cells/L) on 8/2. Toxins from the bloom were ingested by blue mussels (181) on 8/3 which resulted in a second closure. At this time point, rock scallop viscera remained high (226), while adductors tested low for STX (25). *Alexandrium* cells persisted at low levels in the water column throughout the fall months, while rock scallop viscera increased in toxicity and persisted at low levels in adductor muscle. Water temperature dropped two degrees Celsius between the end of August and mid-September, which coincided with the increased toxicity within rock scallop viscera seen on 9/11- 10/2. After peak toxicity in scallop viscera on 10/2 (1082), scallops began to slowly depurate toxins, while blue mussels detoxed below the threshold in late October. Discovery Bay opened for blue mussel harvest on 10/24/2017, ending the time series. Rock scallop adductor remained below the threshold for STX throughout the 2017 time series, except

for the initial closure date on 6/28, while viscera remained persistently high with STX and did not indicate a clear depurating trend throughout the summer – fall months.

Beginning in fall 2019, a blue mussel closure for Discovery Bay on 9/17 (101) followed low cell counts of *Alexandrium* (32.4 cells/L) reported on 8/21 and persistent cells in the water, reported as "present" in SoundToxins samples from 8/27 – 9/25. This period of increased toxicity also coincided with a significant drop in water temperature from 16.7ºC on 9/11 to 9.5ºC on 9/17. Max toxicity in blue mussels were seen shortly after the closure and temperature change on 9/18 (258). Logistics delayed the coordination of sampling rock scallops immediately after the closure, however on 10/2 scallops were collected and tested at very high levels in viscera (1179) and above the threshold in adductor (97).

In general, blue mussels and rock scallops (both tissue types) followed a depurating trend in late 2019. Mean STX in scallop viscera during the 2019 period (1248±793) was more than twice the mean STX in 2017 scallop viscera (540±295), while mean STX in adductor were very similar between the two years 2017 (41±27), 2019 (43±48) with comparatively less variability than viscera.

Summary

Table 5 summarizes results from the field exposure trials for both blue mussel (BM) and rock scallop (RS) for 2017 and 2019 in Sequim and Discovery Bays, including toxification (number of days from closure to max toxicity), max toxicity (µg STX), detox period (in some cases inconclusive) and end toxicity/date. In some cases, the end toxicity (µg STX) and date correspond to the opening for blue mussel harvest (see **Table 4** for WDOH open date and species), no remaining scallops to sample or end of project period. Mean PSP values are shown in **Table 6**.

The time period of increasing accumulation of toxins and maximum toxicity coincide with an environmentally dynamic time during the seasonal shift from warm summer temperatures to cooler fall conditions. From the initial closures in late summer to peak toxicity in mid – September, water temperatures drop significantly. It's possible that the drop in temperature may play a role in *A. catenella* cell toxicity and subsequently high STX in blue mussels and rocks scallops at this time of year. A controlled laboratory study (Navarro et al. 2006) showed a Chilean strain of *A. catenella* had highly variable toxicity within cells at all different temperatures tested, however results trended toward highest toxicity at lower temperatures of 10°C and lowest toxicity at warmer temperatures of 16°C. Another controlled laboratory study found that a Hong Kong strain of *A. catenella* populations were the least dense with the highest toxin/cell at colder temperatures of 10°C verse highest cell density and lowest toxin/cell at 30°C (Siu et al. 1997).

The extended toxicity in rock scallops during cold winter months may be an effect of slower metabolic rate during this time, influencing the rate of toxin depuration in this species. *Alexandrium* cells were reported in water samples from Sequim Bay SoundToxins sampling through the end of November (8.9°C) and remained absent thereafter. While active cells of *Alexandrium* were not reported in the water column during winter months, toxicity in scallops

persisted in individual adductors and well above the PSP thresholds in viscera. Further assessment of environmental conditions and depuration rates in this species will be essential for future marketability and harvest.

Table 5. PSP/STX toxification, max toxicity and detoxification in whole blue mussels, and rock scallops (viscera/adductor) from Sequim and Discovery Bays, 2017 and 2019. Definitions: toxification (=closure -> max STX), max toxicity (= peak STX in time series), detox (= max STX -> opening/or end of sampling).

Location	Year	Species	Tissue	Closure Date	Toxification (days)	Max toxicity (µg)	Detox (days)	End Toxicity (µg), Date
Sequim	2017	BM	Whole	8/3/17	32	346	100	38, 12/20/17
CH		RS	Viscera		34	6450	>148*	562, 12/13/17
JWM		RS	Viscera		34	4567	>41*	762, 10/24/17
CH		RS	Adductor		56	75	72	15, 12/13/17
JWM		RS	Adductor		22	162	>61*	86, 10/24/17
Discovery	2017	BM	Whole	8/3/17	-	181	82	66, 10/15/17
		RS	Viscera		56	1082	>22¥	521, 10/24/17
		RS	Adductor		27	62	56	31, 10/24/17
	2019	BM	Whole	9/17/19	1	258	>46×	38, 11/4/19
		RS	Viscera		33	2001	>26*	635, 12/16/19
		RS	Adductor		15	98	75*	10, 12/16/19

(*) end sampling, no more scallops
(¥) end sampling, bay open for blue mussels (WDOH)
(×) closed until further notice for blue mussels (WDOH), end project

Objective 3: Evaluate accumulation of saxitoxin in different components (adductor muscle and viscera) through receptor binding assay (RBA)

The following results focus on the statistical analysis of STX in rock scallop adductor muscle and viscera as determined through receptor binding assay. A comparison of means of STX (µg/100g tissue) in viscera and adductor was assessed using Welch's two-sample t-test after meeting assumptions. Data that was not normally distributed was log transformed. Results are detailed in **Table 6**. Across all time series and locations, mean STX in viscera tissue (1074±925) were significantly higher ($p<0.05$; n=308 for both) than mean adductor muscle tissue (52±32). These results support previous reports for rock scallops in Sequim Bay by the WA Department of Health (**Figure 23**). Data from the current study also show that STX concentrations and depuration rates are variable among individuals as seen in time series from both Sequim and Discovery Bays (**Figures 26, 27**). In a single sample period, some scallops tested had toxin levels in adductor muscle over the FDA approved limit for PSP (80µg/100g tissue), while the toxin was not detected (NTD) in others (Sequim Bay, **Figure 26**, Jan '18) or very low (Discovery Bay, **Figure 27**, Dec '19). In the time series from Sequim Bay '17-'18, a subset of adductor muscles from individual scallops tested above the limit after the bay opened for blue mussel harvest on 1/3/18 (20% of tested individuals) and on 1/9/18 (10% of individuals tested). Mean values for these time points show adductors below the limit at Sequim Bay, CH site

(**Figure 28**). During the Discovery Bay time series on 12/3/19, one individual adductor tested above (108µg) and one just below (78µg) the PSP limit, while the mean value for adductors at this time point was below the threshold (39µg) (**Figure 29**). This is a critical finding from our data series, and important to note that this occurred in two different years in two separate bays. This indicates more species specific testing for rock scallop and monitoring may be necessary to assess duration of STX in rock scallop adductors, mean and variance in populations and also in relation to blue mussels and scheduled openings. While response of scallop adductor seems to follow the general depuration trend of blue mussels, the implications of the data points above the threshold in the cited examples above need to be further evaluated as this native species is considered for commercial culture and harvest in Washington State.

Table 6. Mean PSP values ± SD (STXug/100g tissue) in rock scallop viscera and adductor tissue during the project period, 2017 – 2019 at Sequim and Discovery Bays, WA. (*) indicates significant difference between means ($p<0.05$).

Location	Year	Site	Viscera	N=	Adductor	N=	p-value
Sequim Bay	2017 – 2018	Crowell	1071 ± 1338	150	34 ± 20	150	*
		JWM	1439 ± 1275	36	93 ± 35	36	*
Discovery Bay	2017	Condos	540 ± 295	39	41 ± 27	39	*
	2019	Condos	1248 ± 793	83	43 ± 48	83	*
		Total Means	**1074 ± 925**	**308**	**52 ± 32**	**308**	*****

Conclusions

It is not clear from any of our datasets, field exposure or laboratory, how long rock scallops take to completely depurate saxitoxin from all tissues. Given the high level of STX in the viscera at the end of each time series and relatively slow depuration rate in this component and species in general, it's reasonable to discern that complete depuration of toxins may extend for many more months (**Table 6**), quite possibly a year or longer if scallops still high in STX encounter the following spring bloom season. This aspect of research will need to be further explored with future monitoring efforts to identify complete depuration rates. This data gap aside, it's clear that very high, persistent levels of STX in scallop viscera will likely preclude this species from safe, whole product consumption and will have to be marketed as a shucked, adductor only product. Implications for management of future commercial culture, harvest and processing of rock scallops will need to be determined to ensure a safe seafood product can be sold to consumers. Furthermore, the general trend of rock scallop adductor following uptake and depuration rates closely with blue mussels across all the time series, could be quite valuable for future regulation and monitoring of rock scallop and should be examined more closely with additional analyses and data collection. As a collective, these PSP/STX time series from Sequim and Discovery Bays comparing uptake, accumulation and depuration in blue mussels and rock scallop tissues are absolutely critical to further develop a robust biotoxin monitoring program for this species.

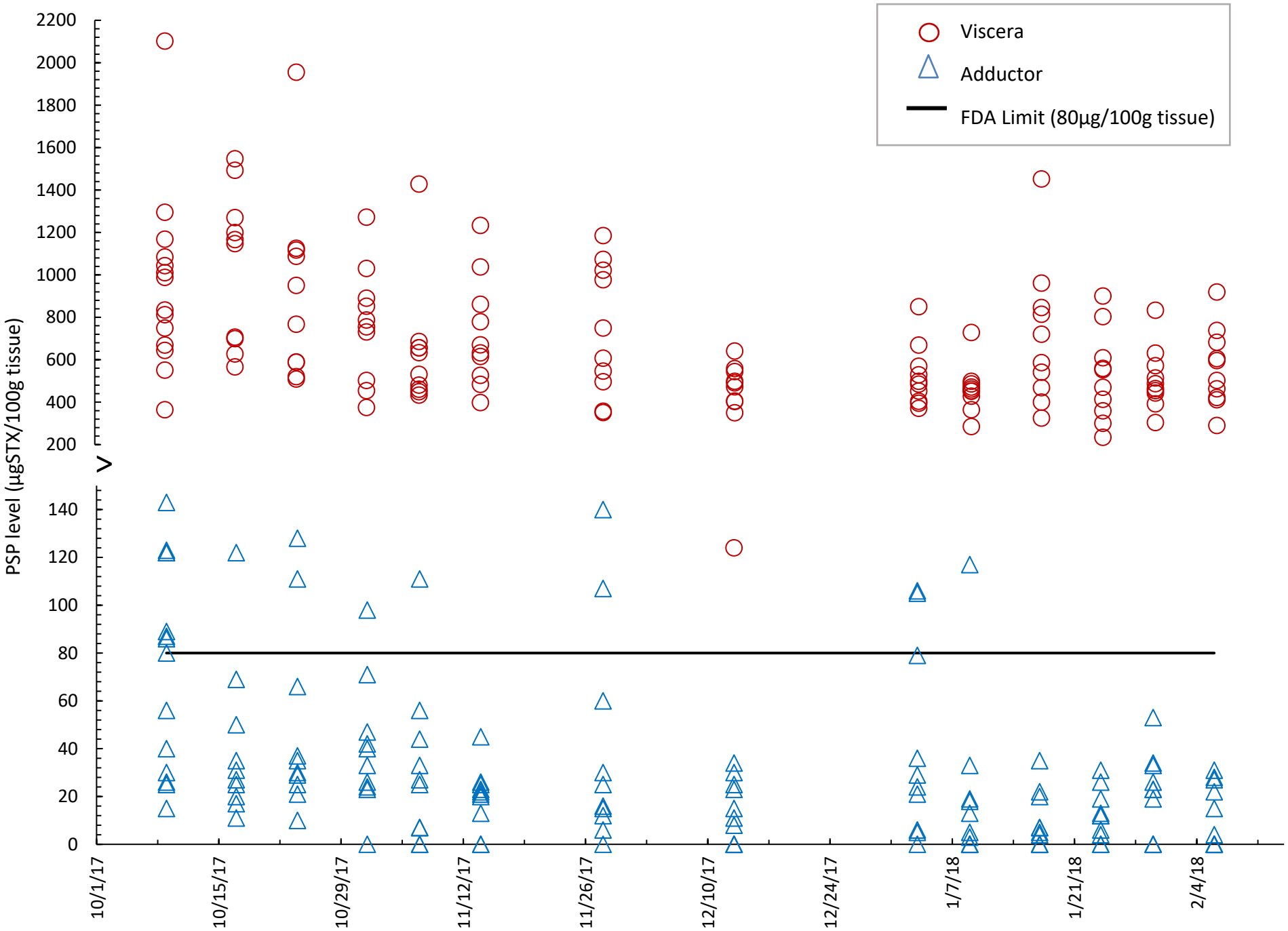


Figure 26. PSP in rock scallop adductor and viscera during Oct 2017 – Feb 2018 sampling events from Sequim Bay, WA. Individual scallops (n=14 10/9/17 only, n=10 all others) from each sample date are represented. A comparison of means for both tissues = viscera 687±27, adductor 32±2.9µgSTX/100g tissue ($p<0.05$, N=144, both).

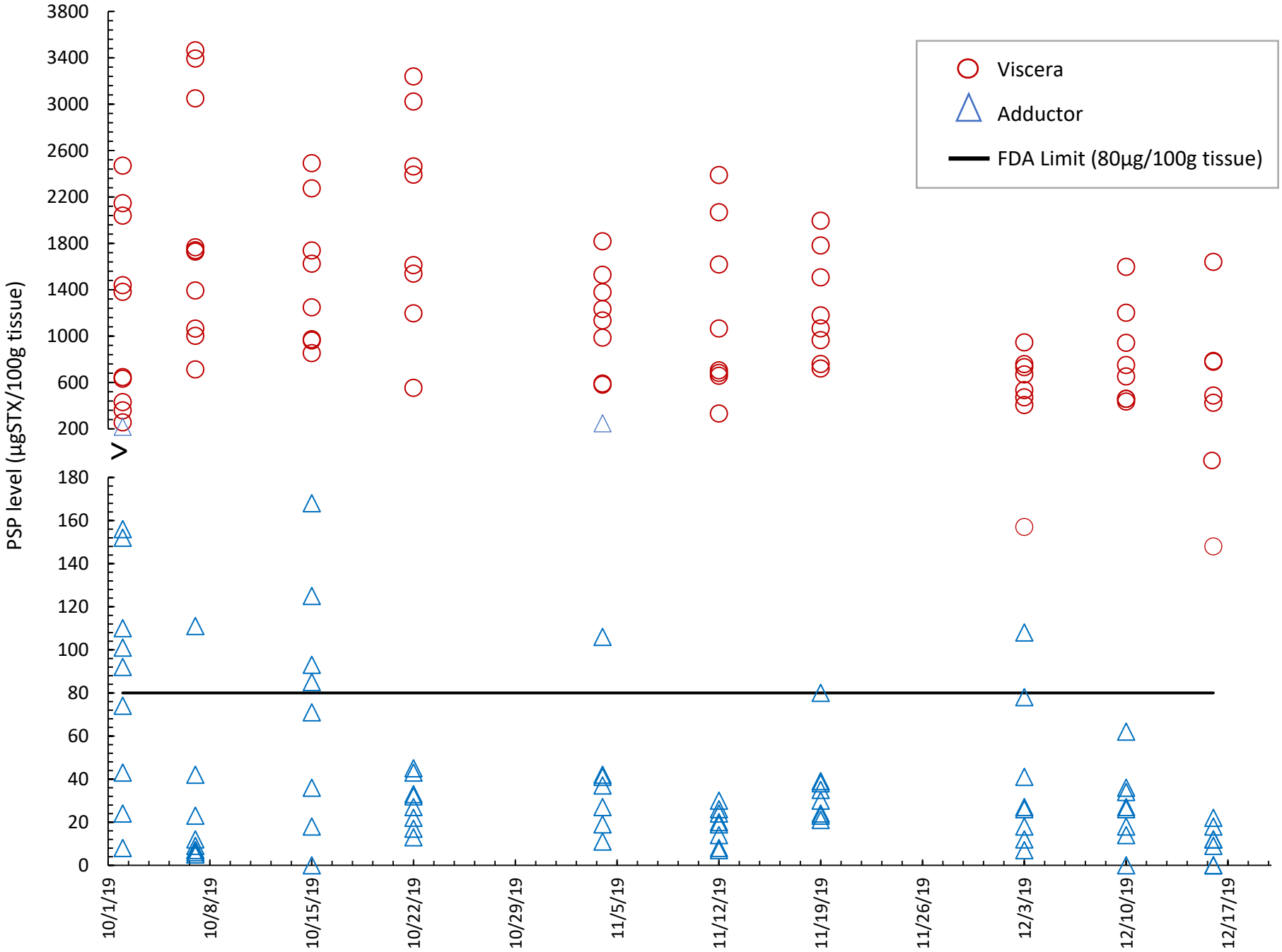


Figure 27. PSP in rock scallop adductor and viscera during Oct 2019 – Dec 2019 sampling events from Discovery Bay, WA. Individual scallops (n=10) from each sample date are represented. A comparison of means for both tissues = viscera 1248±793, adductor 43±48µgSTX/100g tissue ($p<0.05$, N=83, both).

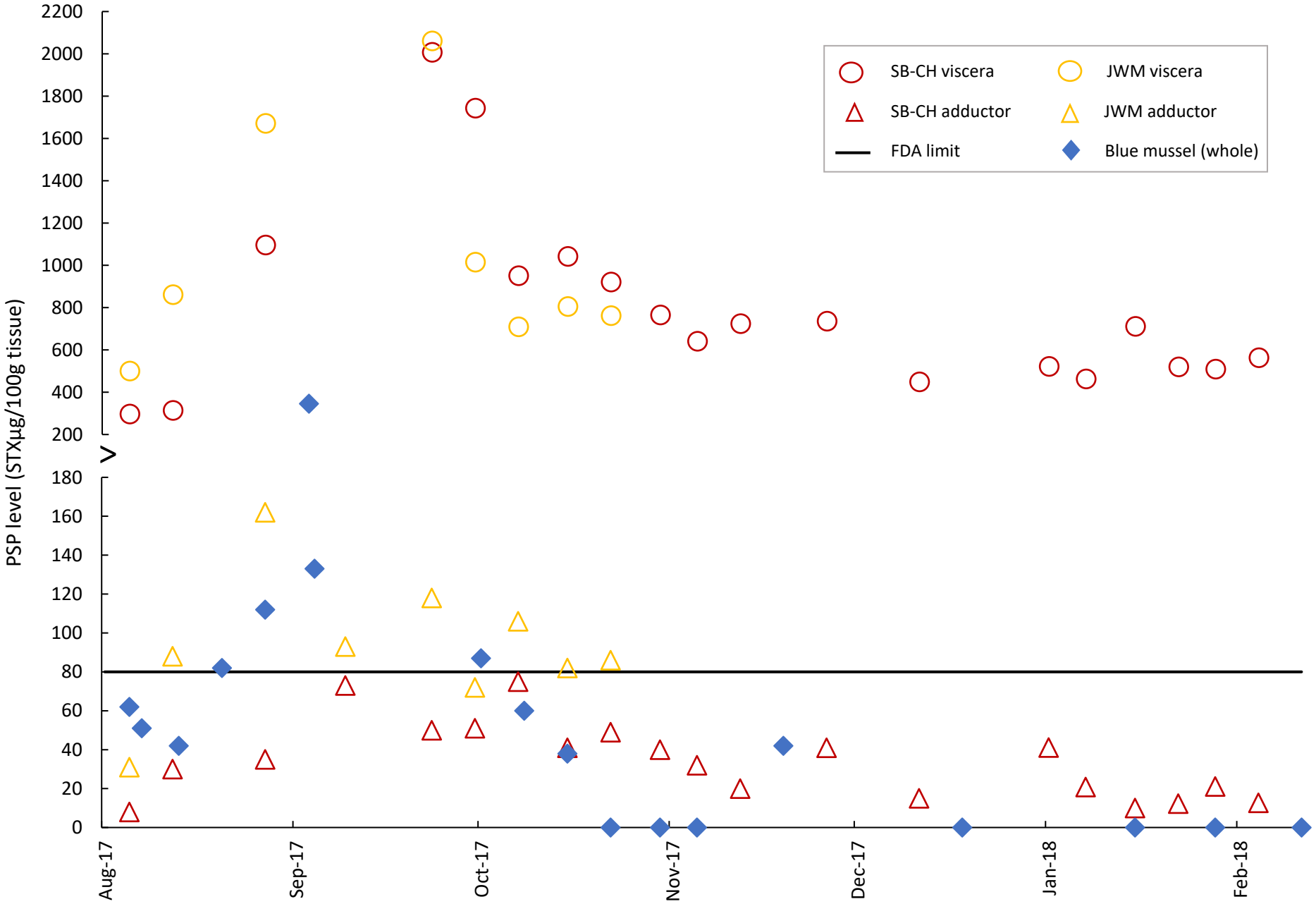


Figure 28. PSP levels in scallop adductor and viscera tissue from two Sequim Bay sites, Crowell House (CH) and John Wayne Marina (JWM), Aug 2017- Feb 2018. Data points from Aug – Oct 2017 represent a single "pooled" value from multiple individuals that equated to 100g of tissue for adductor and viscera. Note: two outliers on 9/11/17, CH-viscera (6450) and JWM-viscera (4567) removed. Data points from Oct 2017 –Feb 2018, represent a mean of individual samples (n=10). Whole blue mussel PSP toxicity data from WDOH.

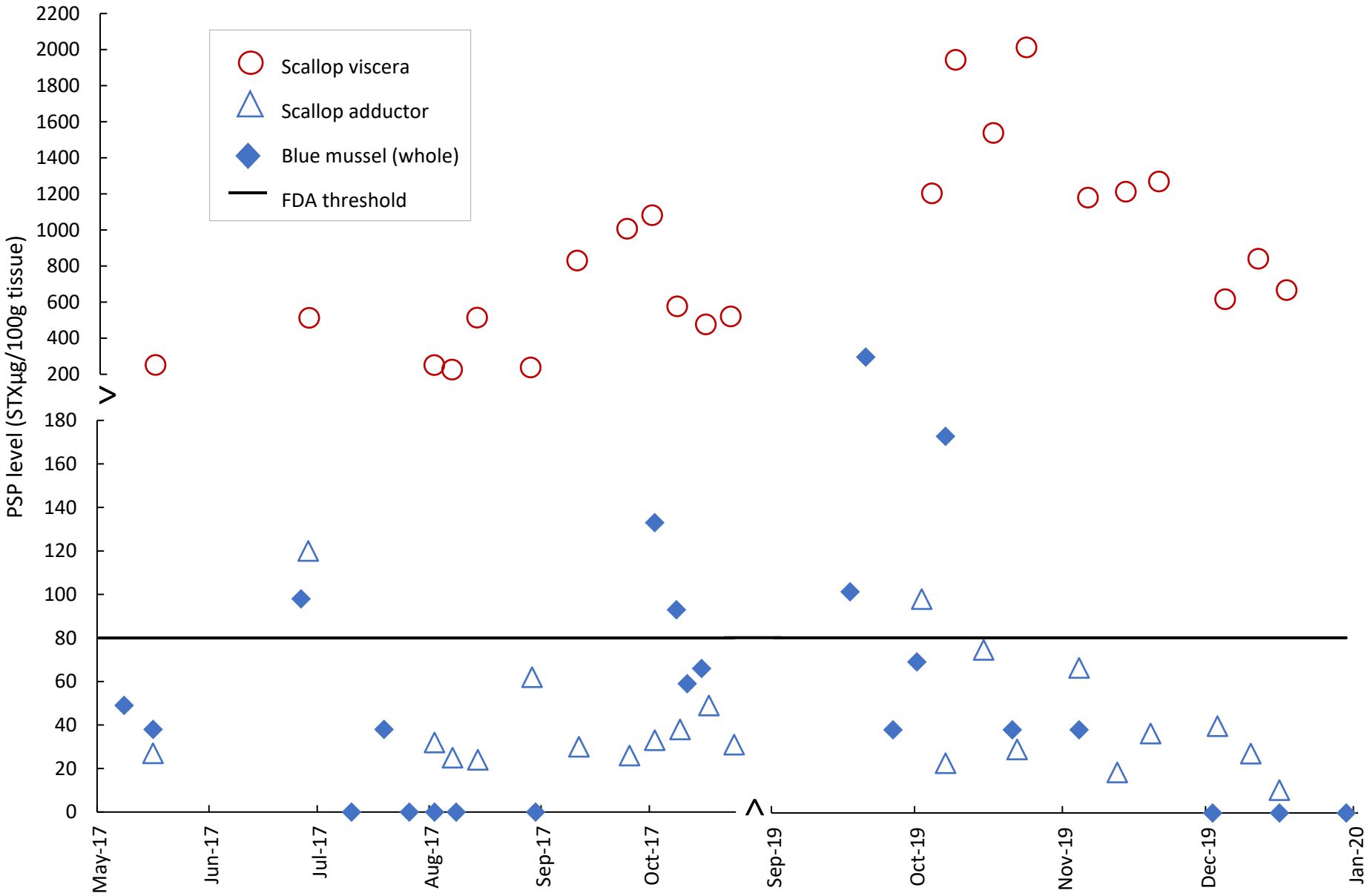


Figure 29. PSP levels in rock scallop adductor and viscera tissue from Discovery Bay, WA. Data points from May – Oct 2017 represent a single "pooled" value from multiple individuals that equated to 100g of tissue for adductor and viscera. Data points from Sept 2019 –Jan 2020, represent a mean of individual samples (n=10). Whole blue mussel PSP toxicity data from WDOH.

Objective 4: Produce validated data to support the NSSP approval process for RBA for toxicity determination of PSP toxins in rock scallops

Mouse bioassay (MBA) was adopted as an official Association of Official Analytical Collaboration (AOAC) International method for STX determination in 1965 and was the only method available for PSP testing for five decades (AOAC 2005, 2006 and 2011). However, regulatory agencies expressed the desire to transition to a method that does not require the use of live animals and is not subject to the matrix effects documented for the MBA (Turner 2012).

Receptor binding assay (RBA) eliminates the need for live animals for PSP testing and is more sensitive than the MBA. RBA is a competition-based assay that employs radiolabeled saxitoxin (3H-STX) to compete with PSP toxins present in standards/samples for binding sites on natural receptors in the assay. Following incubation with the receptors, unbound 3H-STX is removed and the remaining labeled toxin is measured with a scintillation counter. The amount of remaining 3H-STX is inversely proportional to standard/sample toxicity. RBA eliminates the use of live animals for detection, and although it still uses receptors prepared from animals, the number of animals required for RBA is significantly reduced. RBA also allows for a composite measure of overall toxicity, as opposed to toxin concentrations measured by liquid chromatographic methods, such as High Performance Liquid Chromatography (HPLC), Liquid Chromatography Mass Spectrometry (LCMS/MS) or Liquid Chromatography Post Column Oxidation (LC-PCOX), which require conversion factors of equivalent toxicity to calculate the overall toxicity.

The National Shellfish Sanitation Program (NSSP) recently approved both postcolumn oxidation liquid chromatographic (PCOX) method and receptor binding assay (RBA) as alternatives to the MBA for PSP toxicity determination. The PCOX method is approved for full use in the United States, but RBA remains approved only for limited use for regulatory testing of mussels and for limited use as a screening tool for clams and scallops. As was the case when this project's research was first funded by NOAA, RBA has undergone AOAC single and multi-laboratory validation and is designated through AOAC as an Official Method of Analysis (OMA 2011.27) and was validated for ten species of shellfish in a collaborative study completed by NOAA in 2011 (Van Dolah et al. 2012). The RBA is an NSSP approved for Limited Use Method for clams and scallops for the purpose of screening and precautionary closure for PSP (adopted by the FDA Action on January 11, 2016, following ISSC 2015 Summary of Actions Proposal 13-114).

Expanded NSSP acceptance of the RBA will provide an additional tool for monitoring toxin levels and making regulatory decisions. In the US, the long-standing regulatory method for paralytic shellfish toxins (PSTs) is the MBA (AOAC Method 959.08) with a regulatory limit of 80µg of toxin per 100g of shellfish tissue. However, the MBA has several downsides including a high limit of detection (40µg/100g), the requirement of many animals used on negative samples, and, at concentrations near the regulatory limit, an under-estimation of the toxins in shellfish (Van Dolah et al. 2012, Turner et al. 2015). As described by Masias et al. (2019), the United States, Canada, Australia and Norway began to use alternative analytical methods, such as the LCPCOX method AOAC 2011.02 (Hignutt 2014, Rourke and Murphy 2014, Burrel et al. 2016)

or LCMS/MS to determine and quantify each of the toxic analogues associated with PST (Boundy et al. 2015; Turner et al. 2015). The PCOX HPLC method requires costly equipment and skilled personnel and offers low throughput. Conversely, RBA is an ideal method for high throughput monitoring of PSTs in a regulatory setting and can better detect toxins near or below the regulatory limit (with a limit of detection of 6.4 µg/100g and a limit of quantitation of 13.1 µg/100g.) While quantitative, the assay is also a high throughput method. Using a 96 well plate format, the RBA assay is capable of testing as many as 23 samples in less than 3 hours. Like the mouse bioassay, RBA measures the integrated toxic potency of a sample, a valuable characteristic for assessing food safety. RBA also tends to overestimate this toxic potency, minimizing the chance of false negatives.

For these reasons, the Sitka Tribe of Alaska, through the Pacific Rim Shellfish Sanitation Association, submitted a "Proposal for Task Force Consideration at the ISSC 2019 Biennial Meeting". The proposal presents a 'Matrix Expansion for the Receptor Binding Assay (RBA) for Paralytic Shellfish Poisoning (PSP) Toxicity Determination to Allow Use with Geoduck' for consideration as an NSSP Approved Method for Marine Biotoxin Testing for PSP in geoduck (*Panopea generosa*). This species of shellfish was chosen for RBA because geoduck clams are the target of lucrative commercial dive fisheries in both southeastern Alaska and Washington state, and constitute significant economic value among Washington aquaculture production (Decker 2015).

While the Sitka Tribe of Alaska Environmental Regulatory Lab (STAERL) pursued RBA for PSP detection in geoduck, a similar effort for Pacific oysters (*Crassostrea gigas*) was initiated by a regulated lab in the state of California. Unfortunately, their proposal for NSSP consideration did not advance through the Interstate Shellfish Sanitation Committee (ISSC) and the California lab's RBA research and pursuit of validation data is currently stalled due to a lack of funding.

Validation data supporting the use of the RBA for testing in a regulated environment of oysters, clams and scallops is still lacking. The STAERL has completed single lab validation as proposed in the 'Matrix Expansion for the Receptor Binding Assay (RBA) for Paralytic Shellfish Poisoning (PSP) Toxicity Determination to Allow Use with Geoduck' and is currently awaiting verification by NOAA's National Ocean Service, Charleston Laboratory (once COVID-19 related restrictions are lifted for non-essential activities.) This is expected to be completed during 2021. Rock scallop validation will also be completed, utilizing 90 tissue samples collected by STAERL during this research project. Samples include 30 each of concentrations near the saxitoxin RBA detection limit (10µg/100g), within the regulatory limit (<80µg/100g) and higher (>>80µg/100g).

Once the validation is completed by NOAA's Charleston Laboratory, STAERL will submit the validation package to ISSC to amend the AOAC method to include geoduck and rock scallop as a matrix for RBA for PSP toxicity determination. The amended RBA matrix for these species of shellfish will then be presented at the annual ISSC meeting where final regulatory approval will be considered, following review by the ISSC Biotoxin Committee (see http://www.issc.org/biotoxin for the committee's charge, reports, and a current roster of

members.) Ultimately, the amended AOAC method and approval of the ISSC will incorporate the RBA in the NSSP for each species of shellfish for which a validation package is submitted and approved by ISSC.

To refine rock scallop validation, a spike recovery study was conducted at the NOAA National Ocean Service, Charleston Laboratory to determine the relative efficiencies of two different extraction methods in a rock scallop matrix and their performance in the saxitoxin RBA. As described above, two saxitoxin extraction procedures have been validated for application in seafood safety regulatory settings in the United States. Given the goals of producing rock scallops for commercial harvest with likely PSP monitoring needs and the difficulties in preparing an extract using the hydrochloric acid method, an alternative extraction solvent was employed. This alternative extraction solvent spike recovery study is detailed in a July 2019 memo titled "Pacific Shellfish Institute, purple hinged rock scallops - Saxitoxin analyses" from Dr. Leighfield, also amended to this final report. NOAA's Dr. Leighfield analyzed three purple hinged rock scallop sample sets comprised of:

1) an uptake and depuration study completed in 2017;
2) a spike recovery study prepared in Charleston using pooled material; and
3) an uptake and depuration study completed in 2018.

The first and third components of Dr. Leighfield's analysis are provided in previous sections of this final report. The second component, the spike recovery, is presented here, and were detailed in PSI's "Progress Report for February 1, 2019 through January 31, 2020" to Washington Sea Grant. As detailed in that early 2020 progress report, pooled rock scallop material (separate adductor and digestive gland) was homogenized and spiked at four concentrations. The concentrations chosen were to be near the saxitoxin RBA detection limit (10µg/100g), half the regulatory limit (40µg/100g), at the regulatory limit (80µg/100g) and higher (300µg/100g). Purified FDA saxitoxin dihydrochloride standard was added to each sample type, homogenized, and an aliquot prepared for extraction using either 0.1N hydrochloric acid or 1% acetic acid. Extracts were prepared by adding the respective acid to the spiked material, boiling, centrifugation, and collection of the supernatant. A gel-like matrix formed with both acids.

The extracts were analyzed using the saxitoxin receptor binding assay and shown as a % recovery relative to the spiked amount (**Figure 30**). Zero values were below the limit of detection in the saxitoxin RBA. The recovery of saxitoxin from rock scallop matrix was 66% to 132% for digestive gland and 63% to 133% for adductor muscle. No obvious differences were observed when comparing the two acids used, nor across spike concentrations. These values are representative of one homogenate, and replication of extraction would likely improve the observed variability. The observed recovery was similar to other matrix types suggesting that either acid (0.1N hydrochloric acid or 1% acetic acid) is appropriate for use in preparing rock scallops for analysis by saxitoxin RBA.

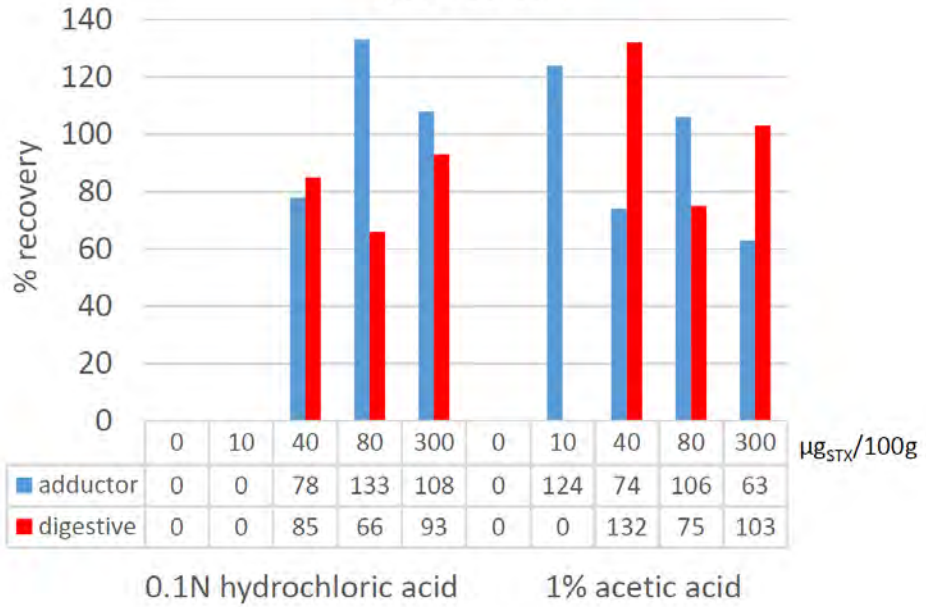


Figure 30. Spike recovery results in *Crassadoma gigantea* adductor and digestive gland tissue. Sample analysis and data presentation by Dr. Tod Leighfield of the NOAA National Ocean Service, Charleston Laboratory.

A noteworthy final goal of this project's research was to aid the Washington Department of Health (WDOH) decision to move from the mouse bioassay to RBA for saxitoxin analysis. WDOH is the state of Washington's ISSC designated shellfish authority. WDOH has been considering transitioning to RBA for saxitoxin analysis for required monitoring of commercial and recreational shellfish harvest, in consultation and collaboration with STAERL and Drs. Steve Morton and Tod Leighfield of NOAA's Charleston Laboratory. This project provided a focused opportunity to validate data, assess feasibility, and realize the benefits of RBA as an alternative method to measure saxitoxin in shellfish from the state of Washington. Currently, WDOH remains interested in pursuing RBA, but the ultimate decision to move to RBA is contingent on NSSP inclusion of the method for regulatory applications for commercial shellfish, including rock scallop, geoduck and oysters. Furthermore, WDOH utilization of RBA will require equipment acquisition which has yet to occur, despite attempted funding requests by the agency.

Objective 5: Observe and evaluate the grow out conditions at the project study sites

Water Quality

Methods

The four project sites; John Wayne Marina (JW) and Crowell's house (CR) in Sequim Bay, Discovery Bay (DB), and lower Budd Inlet (BI); were sampled every couple of months between February 2018 and February 2020 for a total of 12 visits. During each visit, water quality parameters (temperature, salinity, dissolved oxygen and pH) were measuring at the depth of the submerged scallop cages (2-meters) using a YSI Professional Plus instrument. Water clarity was sampled using a secchi disk and a 3-meter vertical net (20 µm) tow was performed for species

composition and quantification of the harmful algal bloom (HAB) species *Dinophysis*, *Pseudo-nitzschia* and *Alexandrium*. Plankton samples were preserved in Lugol's solution, loaded onto a Palmer Maloney counting chamber and viewed at 200X magnification using an Olympus Inverted microscope (IMT-2). HAB species were quantified (cells/L) and observed species were recorded along with their relative abundance (bloom, common, present).

Results – Water Quality

The following data was collected between June 2018 and June 2019 (8 sampling visits). Due to instrument-related gaps in data during initial sampling visits and gaps in time between the final two visits, four sampling dates were not included in this analysis. Budd Inlet data was collected on different sampling days than JW, CR and DB and was graphed separately.

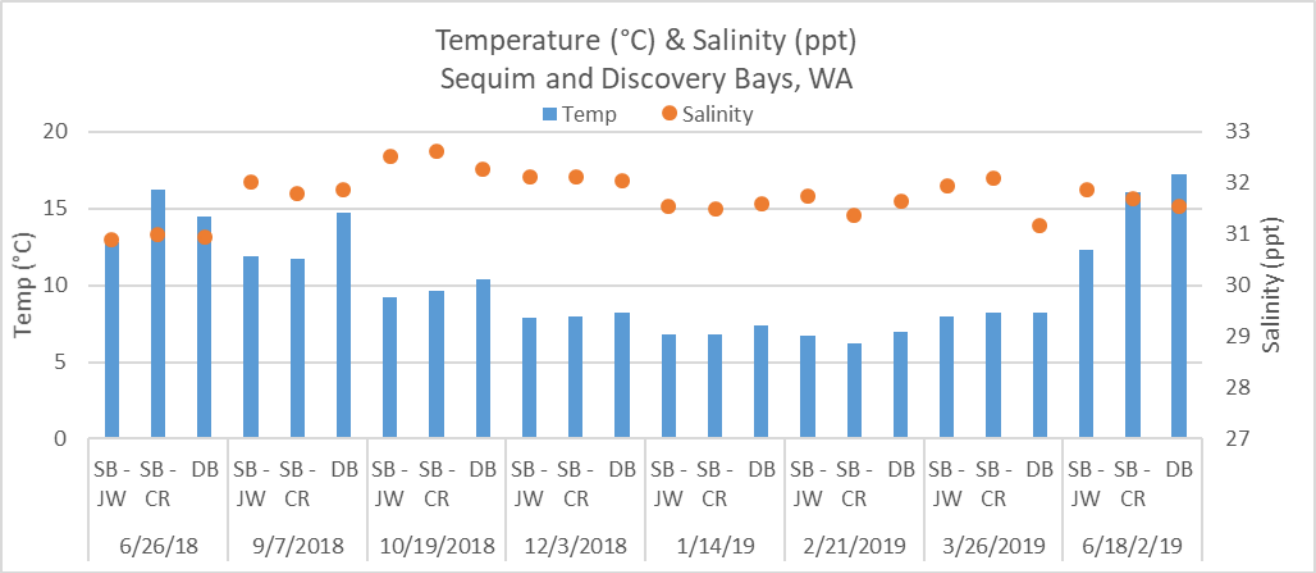


Figure 31. Seasonal temperature and salinity data from Sequim and Discovery Bays, June 2018 to June 2019.

Water temperature at 2-meters followed a typical profile with the warmest values recorded during the summer and coolest during the winter. Temperatures ranged from 6.2 °C at CR (2/21/19) to 17.2 °C at DB (6/18/19) (**Figure 31 & 32**). Mean temperatures were coolest at JW (10.0 °C), followed by CR (10.4 °C), DB (11.0 °C), and BI (11.6 °C) (**Figure 33**).

Water salinity collected at a 2-meter depth eliminated surface fluctuations imposed by fresh water lenses, especially at BI. Values at all sites were highest during early fall and lowest during the winter months due to seasonal fluctuations in rainfall and snowmelt. All sites experienced a slight decrease on 6/26/18 after a rain event occurring the day prior. Salinity values ranged from 25.02 ppt at BI (1/15/19) to 32.62 ppt at CR (10/19/18). Mean salinities were fairly similar at CR (31.77 ppt), JW (31.72 ppt) and DB (31.64 ppt), but significantly lower at BI (27.98 ppt) near the mouth of the Deschutes River.

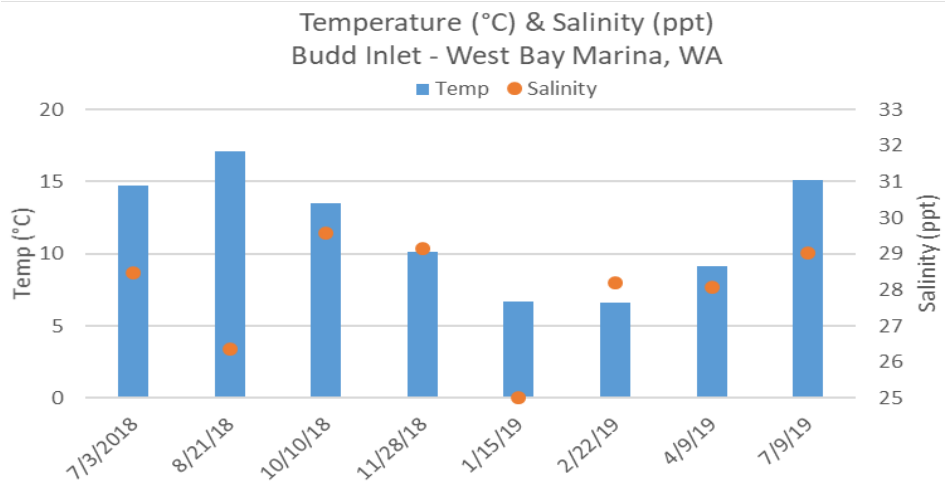


Figure 32. K. Houle collecting YSI data in lower Budd Inlet and seasonal temperature and salinity data.

Dissolved oxygen (DO) concentrations were highest during spring and summer due to photosynthesis, and lowest during early fall and winter due to phytoplankton decomposition at depth and decreased phytoplankton concentrations. DO values ranged from 5.53 mg/l at JW (10/19/18) to 13.65 mg/l at DB (10/19/18) (**Figure 34-35**). These two extremes occurred on the same day, clearly demonstrating the influence that phytoplankton have on oxygen and pH (Figure 5). Mean DO concentrations were lowest at JW (8.7 mg/l) followed by BI (8.9 mg/l), CR (9.1 mg/l) and DB (10.8 mg/l) (**Figure 36**). While BI experiences high plankton concentrations and DO levels throughout much of the year, lower BI also suffers from resulting periods of extended low DO due to eutrophication.

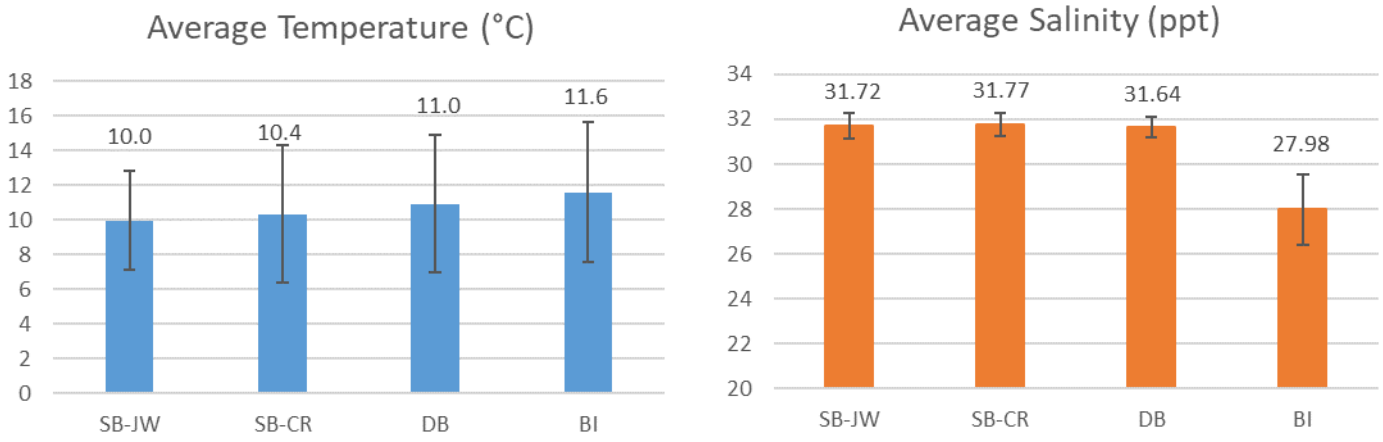


Figure 33. Average Temperature and Salinity over 1-year period.

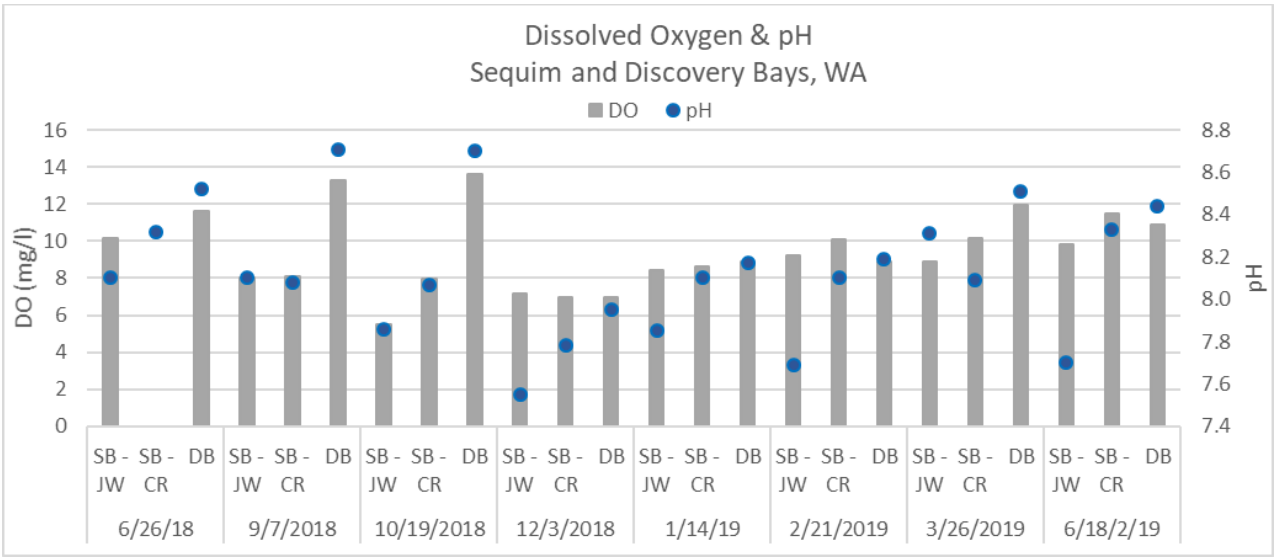


Figure 34. Seasonal oxygen and pH from Sequim and Discovery Bays, June 2018 to June 2019.

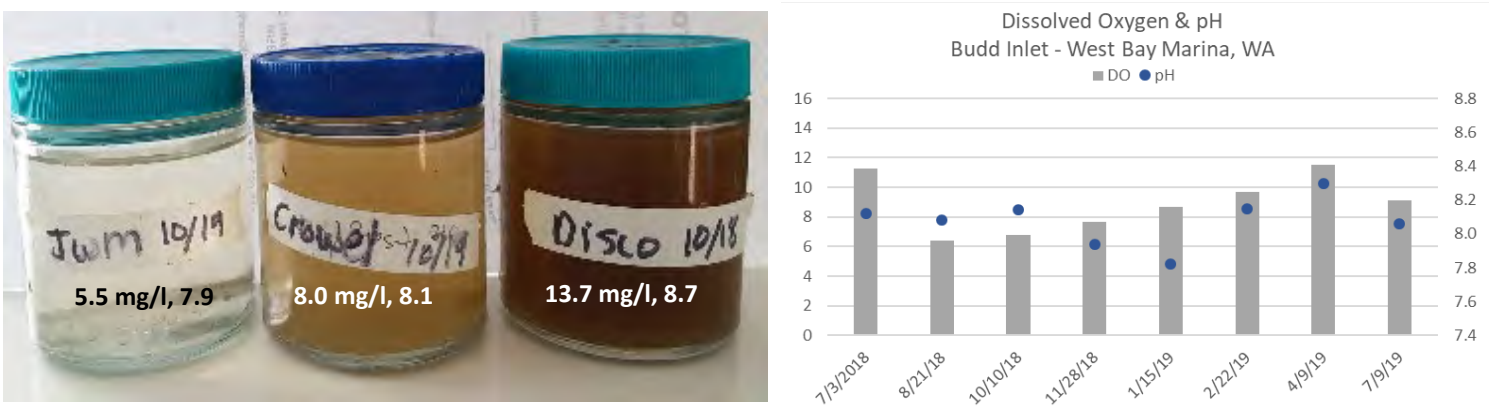


Figure 35. Plankton net tow samples and associated DO (mg/L and pH values collected from JW, CR and DB on 10/19/18 within one hour of each other (left). Seasonal oxygen and pH data at Budd Inlet (right).

The pH typically tracked DO with the highest values recorded in the spring and summer and lowest values in the winter. Driving forces included phytoplankton concentrations, late season decomposition, and likely combination of filtering and respiration from a shellfish FLUPSY (floating upweller system) located immediately adjacent to scallop cages at JW. The pH values ranged from 7.5 at JW (12/3/18) to 8.7 at DB (9/7/18). Mean pH values were lowest at JW (8.0), similar at CR and BI (8.1) and highest at DB (8.4).

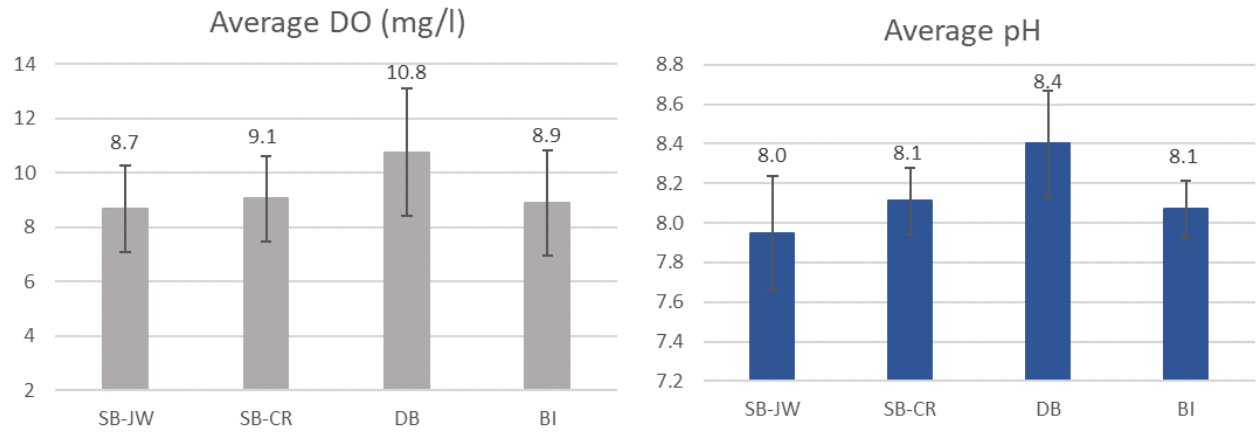


Figure 36. Average Dissolved Oxygen and pH over 1-year period.

Results – Plankton & HABs

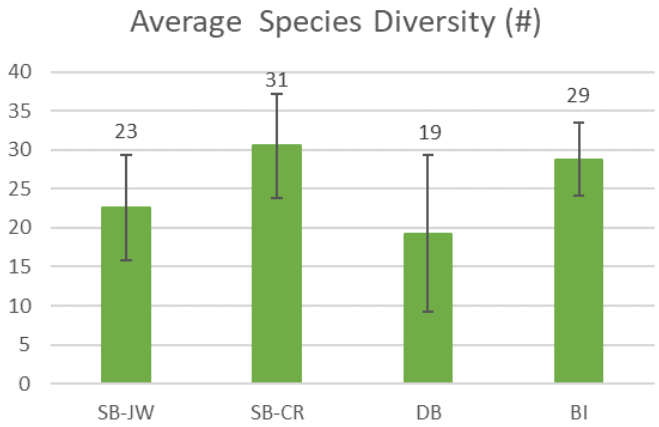


Figure 37. Average Species Diversity over 1-year period.

A secchi disk could not be used to measure water clarity due to insufficient water depth at the 3 northern sites. The disk hit bottom during all but a few readings. A better, albeit non quantitative indicator of plankton concentration, was simply a visual hue comparison of side-by-side net tow samples as shown in Figure 5. Typically, phytoplankton levels were most concentrated at DB, followed by CR and JW. Based on 8 years of Budd Inlet monitoring, phytoplankton levels are fairly rich, typically ranging between 1 and 3.5 meters throughout the summer. The mean number of species detected per site visit was highest at CR (31 spp.), followed by BI (29 spp.), JW (23 spp.) and DB (19 spp.) (**Figure 37**). Plankton diversity counts included both phytoplankton and zooplankton species (**Figure 38**). Species diversity was lowest at DB, despite having the highest plankton concentration; and highest at CR and BI.Plankton community composition was often similar at each of the 3 northern locations extending to BI in some, but not all, instances. For example, a spring and summer bloom of the diatom *Rhizosolenia* was encountered in Sequim and Discovery Bays, but not in BI. Whereas the fall blooms of *Ditylum* and *Prorocentrum* extended their range to all 4 sites. BI experienced a higher number of dinoflagellates such as *Akashiwo*, *Ceratium* and *Noctiluca*, particularly during stratified summer and fall months. *Dinophysis* blooms were most common during summer and fall; *Alexandrium* during fall; and *Pseudo-nitzschia* during all four seasons (**Figure 39**).

Diatoms - Centrics	*Proboscia*	**Dinoflagellates**	**Other Flagellates**
Actinoptychus	*Rhizosolenia spp.*	*Akashiwo sanguinea*	*Small naked flagellates*
Asteromphalus	*Skeletonema*	*Alexandrium spp.*	*Dictyoca spp.*
Aulacodiscus	*Stephanopyxis spp.*	*Amphidinium*	*Ebria*
Cerataulina	*Thallassiosira spp.*	*Amylax*	*Euglenoids*
Chaetocreros spp.	*Unidentified centrics*	*Ceratium spp.*	**Zooplankton**
Corethron	**Diatoms - Pennates**	*Dinophysis spp.*	*Barnacle nauplii*
Coscinodiscus spp.	*Achnanthes*	*Gonyaulax spp.*	*Bivalve larve*
Dactyliosolen spp.	*Asterionellopsis*	*Gymnodinium*	*Copepods*
Detonula	*Cylindrotheca*	*Gyrodinium spp.*	*Crustacean nauplii*
Ditylum	*Fragilaria*	*Heterocapsa*	*Larvaceans*
Eucampia	*Fragillariopsis*	*Minuscula*	*Polychaete larvae*
Grammatophora	*Licmophora*	*Nematodinium*	*Rotifers*
Guinardia	*Navicula spp.*	*Noctiluca*	*Tiarina*
Hemiaulus	*Nitzschia spp.*	*Oxyphysis*	*Tintinnids*
Lauderia	*Petrodictyon*	*Polykrikos*	*Other (No. different species)*
Leptocylindrus spp.	*Pleurosigma*	*Prorocentrum spp.*	
Melosira	*Pseudo-nitzschia spp.*	*Protoceratium*	
Odontella	*Thalassionema*	*Protoperidinium spp.*	
Paralia	*Tropidoneis*	*Pyrophacus*	
	Unidentified pennates	*Scrippsiella*	
		Unidentified Dinos	

Figure 38. Total plankton genera identified at the 4 study sites.

	SP - 18	**SU - 18**	**F - 18**	**W - 18/19**	**SP - 19**	**SU - 19**
	Mar-May	**Jun-Aug**	**Sep-Nov**	**Dec-Feb**	**Mar-May**	**Jun-Aug**
JW	*Rhizosolenia - B*	No Data	*Ditylum - B*	*Pleurosigma - C*	*Thalassiosira - B*	*Rhizosolenia - B*
	Ceratium - C		*Prorocentrum - B*	*Prorocentrum - C*	*Thalassionema - C*	*Cylindrotheca - C*
	Chaetoceros - C		*Dinophysis - P*	light mix *spp. - P*		*Pseudo-nitzschia - B*
						(small type)
CR	*Rhizosolenia - B*	No Data	*Ditylum - B*	*Pleurosigma - C*	*Thalassiosira - B*	No Data
	Ceratium - C		*Prorocentrum - B*	*Prorocentrum - C*	*Chaetoceros - C*	
	Chaetoceros - C		*Pseudo-nitzschia - B*	*Cylindrotheca - C*		
	Pseudo-nitzschia - B		*Alexandrium - P*	light mix *spp - P*		
	(small type)		*Dinophysis - P*			
DB	*Rhizosolenia - B*	No Data	*Ditylum - B*	light mix *spp. - P*	*Thalassiosira - B*	*Rhizosolenia - B*
	Chaetoceros - C		*Prorocentrum - B*		*Thalassionema - C*	*Dinophysis - P*
	Pseudo-nitzschia - C		*Chaetoceros - B*		*Chaetoceros - C*	
	(small type)		*Alexandrium - C*			
			Dinophysis - P			
BI	*Thalassiosira - B*	*Ceratium - B*	*Ditylum - B*	*Chaetoceros - C*	*Chaetoceros - B*	*Ceratium - B*
	Scripsiella - B	*Noctiluca - C*	*Ceratium - B*	*Skeletonema - C*		*Thalassiosira - C*
		Akashiwo - C	*Prorocentrum - C*	*Pseudo-nitzschia - C*		
		Dinophysis - B	*Akashiwo - C*			
			Dinophysis - B-C			

Figure 39. Species recorded as Blooming (B) or Common (C) at each of the study sites. HAB species (red) that are Present (P) may also be included if their cell counts are notable.

Dinophysis

Dinophysis, the species responsible for Diarrhetic Shellfish Poisoning (DSP), is not uncommon in Puget Sound and was found at elevated concentrations at all 4 sites during summer and fall. Of the more notable spikes, *Dinophysis* was present in Sequim and Discovery Bays in September of 2018 (200 cells/L) and again the following June (500 cells/L) (**Figure 40**). Sequim Bay was closed in September of 2018 and again in June of 2019 due to DSP toxins in shellfish exceeding the state limit of 18 µg/100g. Of the *Dinophysis* species, *D. acuminata* was dominant in September, a 50:50 mixture of *D. acuminata* and *D. fortii* in October and an 80:20 mixture of *D. fortii* and *D. acuminata* in June of 2019 (**Figure 41**).

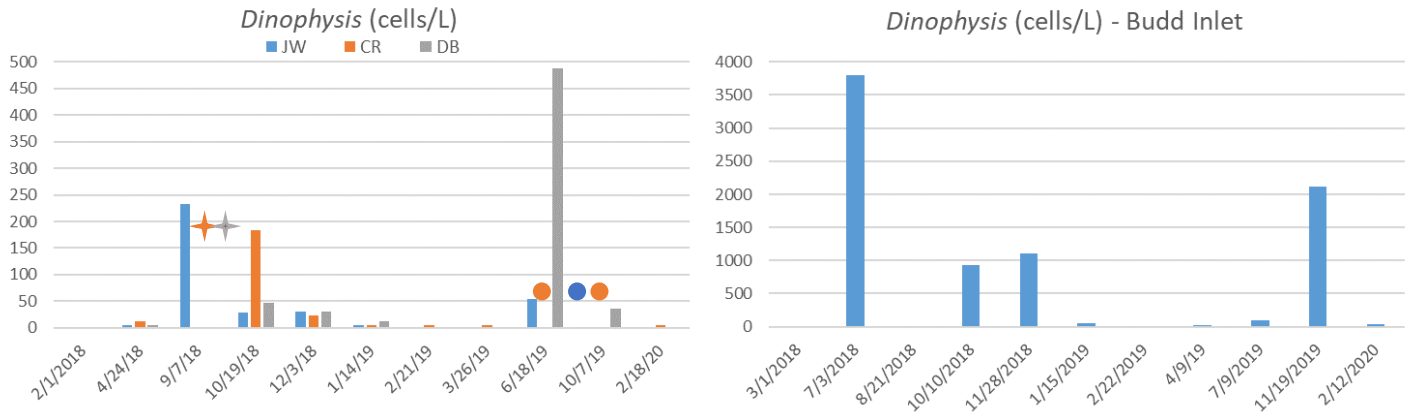


Figure 40. Dinophysis cell counts for Sequim (JW, CR) and Discovery Bays (left) and Budd Inlet (right). The star symbols indicate that Dinophysis was Present, but cell counts were not performed. The circles indicate missing samples.

Cell numbers were higher in BI with concentrations reaching almost 4,000 cells/L during summer of 2018 and 1,000 and 2,000 cells/L during fall of 2018 and 2019 respectively. *Dinophysis* also reached 3,500 cells/L at Port Plaza on 6/20/19, but was not included in this data set. The summer *Dinophysis* spikes in BI consisted primarily of *D. norvegica*, whereas the fall spikes consisted of a mixture of *D. acuminata* and *D. fortii*. No toxins were detected during the summer blooms, however DSP toxins were detected (76 µg/100g) during the *D. acuminata* and *D. fortii* bloom (2,000 cells/L) on 11/20/19. DSP remained low during the other BI spikes.

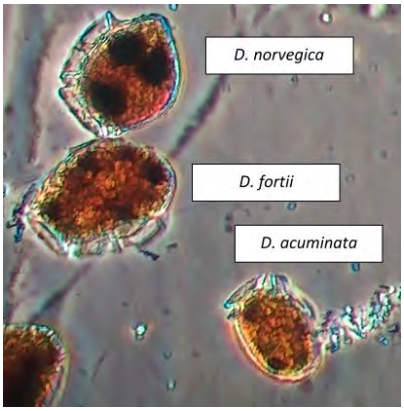


Figure 41. Dinophysis (spp.) in Budd Inlet, June 2020. Photo: A. Christy

A positive relationship was observed between water temperature and *Dinophysis* cell counts for the combined northern bays (R^2=0.625, n=29) (**Figure 42**). This relationship did not hold true when BI was included (R^2=0.162, n=48) or analyzed separately (R^2=0.167, n=19). The June *Dinophysis* bloom in DB occurred during peak temperatures (17°C), whereas spikes in BI occurred during early summer and fall when water temperatures remained under 15°C.

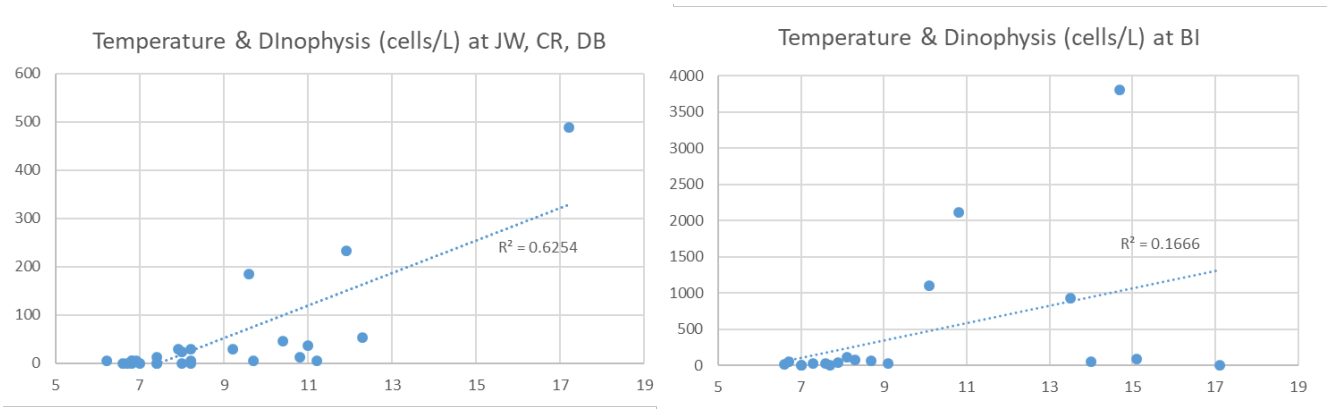


Figure 42. Relationship between Temperature & Dinophysis in Sequim (JW, CR) and Discovery Bays (left) and Budd Inlet (right).

Warmer ocean temperatures have been shown to increase the range of dinoflagellates like *Dinophysis*, *Prorocentrum*, *Ceratium* and *Noctiluca* in the north Atlantic (Hallegraef 2010). The temperature-induced stratification is believed to offer dinoflagellates a competitive advantage over diatoms in that their flagellas allow them to access nutrients beyond the reach of diatoms. Between 2013 and 2020, the highest DSP concentrations (250 µg/100g) in Budd Inlet were detected during 2015 and 2016, when a warm water mass nicknamed "the Blob," increased temperatures on the coast and Puget Sound by 2°C (**Figure 43**). Only summer cell count data was collected during this time.

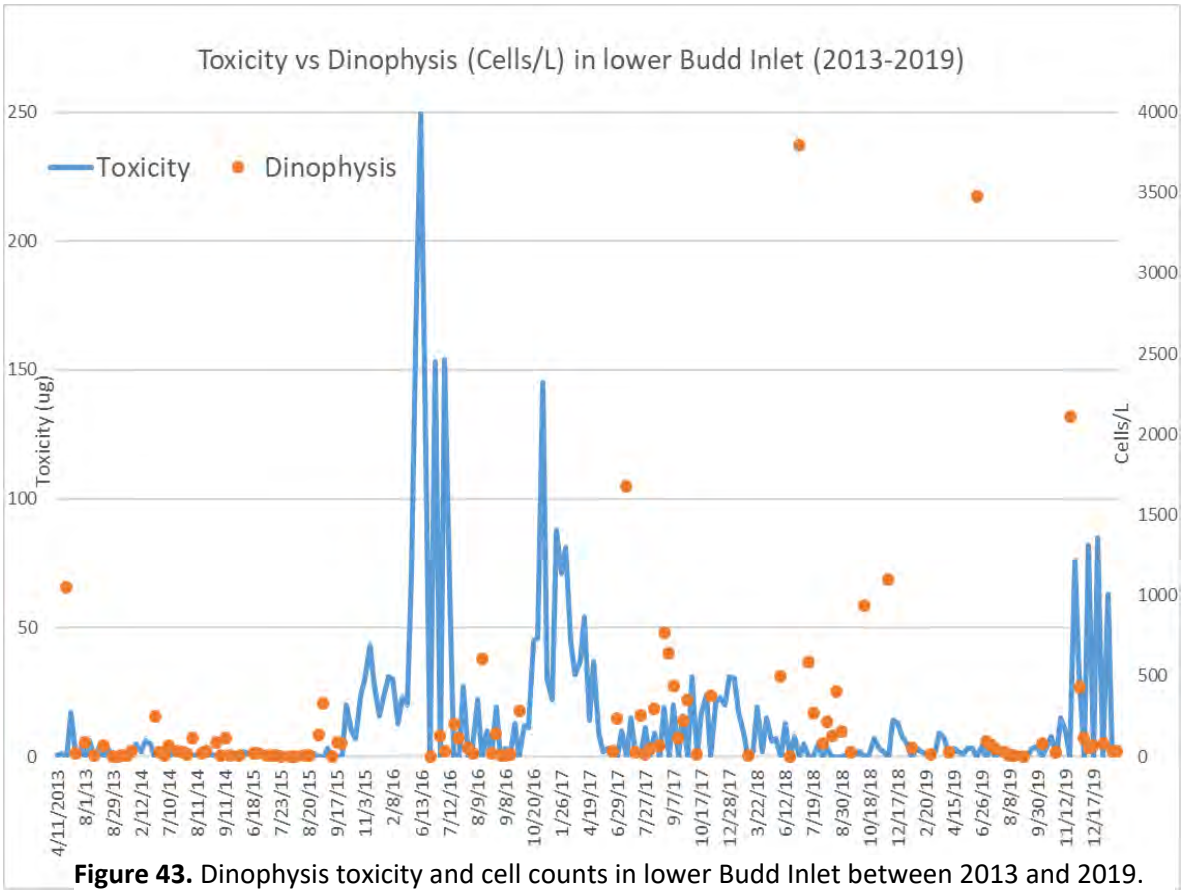


Figure 43. Dinophysis toxicity and cell counts in lower Budd Inlet between 2013 and 2019.

No relationship was observed between *Dinophysis* and DO (R^2=0.004, n=24), pH (R^2=0.062, n=28), or salinity (R^2=0.062, n=29) at the northern sites, or BI.

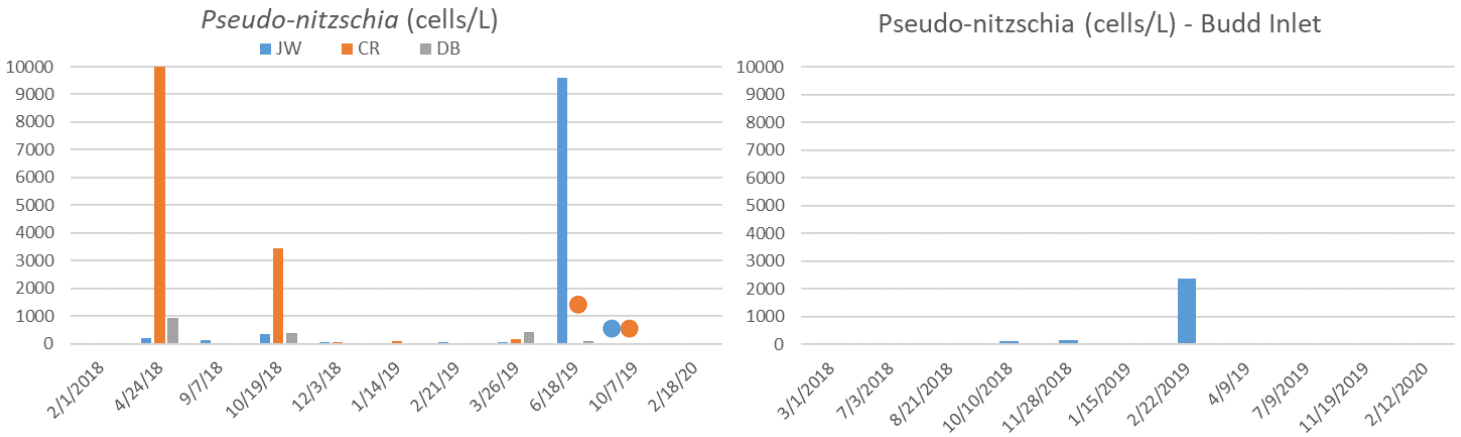


Figure 44. Pseudo-nitzschia cell counts for Sequim (JW, CR) and Discovery Bays (left), and Budd Inlet (right). Circles indicate missing samples.

Pseudo-nitzschia

Similar to *Dinophysis*, *Pseudo-nitzschia*, the species responsible for Amnesic Shellfish Poisoning (ASP), is frequently observed throughout Puget Sound. Unlike *Dinophysis*, however, ASP toxicity is rare within Puget Sound. *Pseudo-nitzschia* blooms were encountered at all four sites during all four seasons (**Figure 44**). The most notable spikes were detected at CR and JW, and consisted predominantly of the smaller cell type (*P. delicatissima* or *P. pseudodelicatissima) (Figure 15).* Cell counts on 4/24/18 were recorded as "too many to count" and estimated to be 10,000 cells/L of the smaller type and 242 cells/L of the larger type (*P. pungens/multiseries* or *P. australis/fraudulenta*). The 6/18/19 bloom (9,595 cells/L) also consisted of the smaller cell type.

According to literature, *Pseudo-nitschia* cell growth, toxin production and fishery closures have been associated with an increase in water temperature. During 2015, the anomalous increase in temperature along the Pacific west coast was linked to unprecedented toxic algal blooms (McCabe et al 2016). Laboratory studies also demonstrated an increase in *Pseudo-nitzschia australis* cell growth and domoic acid production with increased temperature (Zhu et al. 2017).

No significant relationship was detected between *Pseudo-nitzschia* cell counts and temperature at the northern sites (R^2=0.075, n=29) or Budd Inlet (R^2=0.063, n=19). The highest cell count detected in Budd Inlet was 2,300 cells/L (large cell type) during February when the water temperature was 6.6°C. These data sets, however, are too small to base conclusions off of. They do, however, demonstrate that blooms can occur during all four seasons.

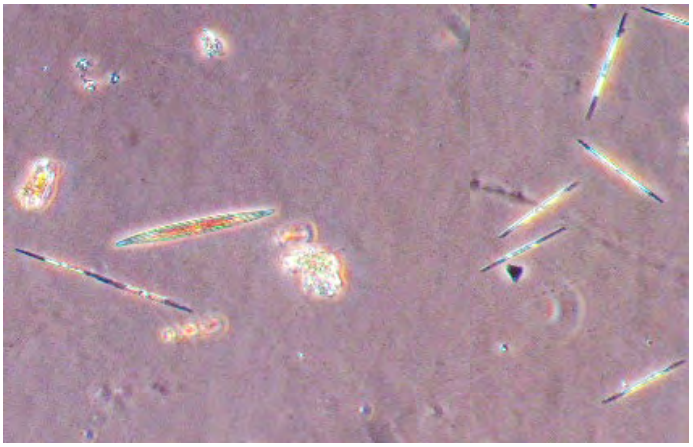


Figure 45. *Pseudo-nitzschia* (*delicatissima/ pseudo-delicatissima*) cells at CR, April 24, 2018. Photo: A. Christy

No relationship was observed between *Pseudo-nitzschia* and DO (R^2=0.000, n=24), pH (R^2=0.002, n=28), or salinity (R^2=0.007, n=29) at the northern sites, or BI.

Alexandrium

Alexandrium, the species responsible for Paralytic Shellfish Poisoning (PSP), routinely results in shellfish bed closures throughout north and central Puget Sound. Closures in south Puget Sound, particularly BI, are rare. *Alexandrium* was detected in Sequim and Discovery Bays during fall of 2018 (400-500 cells/L) and to a lesser extent during fall of 2019 (100 cells/L) (**Figure 46**). Sequim Bay was closed in November 2018 and Discovery Bay in September 2019 due to PSP toxin levels exceeding the state limit of 80 µg/100 g. *Alexandrium* cell counts remained low in Budd Inlet during both years. This pattern is typical of Budd Inlet which may, on occasion, experience small blooms of solitary *Alexandrium* cells, but rarely chain forming *Alexandrium catenella* (**Figure 47**).

Modeling has indicated that increased surface temperatures in Puget Sound would widen the window of accelerated growth (>13°C) for *Alexandrium catenella* (Moore et al. 2008). No relationship, however, was found between temperature and *Alexandrium* at the northern sites (R^2=0.017, n=29) or BI (R^2=0.022, n=19) based on this study's limited data set. Additionally, no significant relationships were detected between *Alexandrium* and DO, pH, or salinity at the four sites (**Table 7**). That said, *Alexandrium* cell counts were highest during fall when salinity was also at its highest (**Figure 48**).

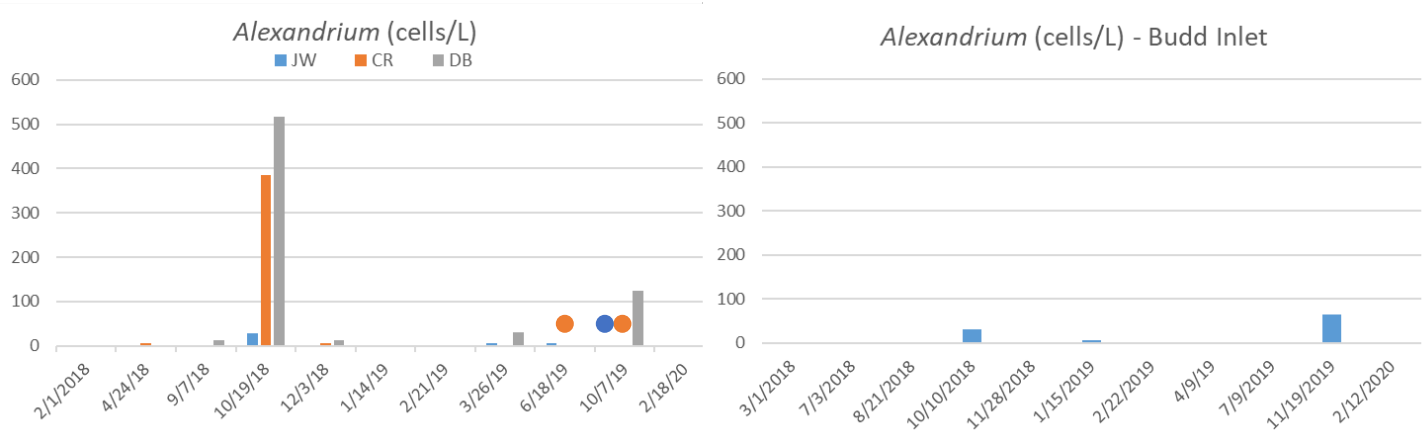


Figure 46. Alexandrium cell counts for Sequim (JW, CR) and Discovery Bays (left), and Budd Inlet (right). Circles indicate missing samples.

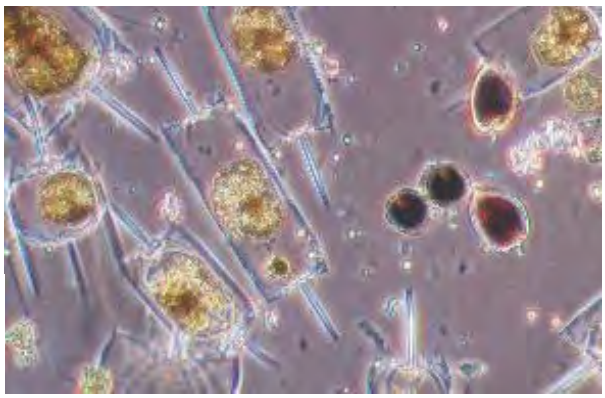


Figure 47. A 2-cell chain of *Alexandrium catenella* during a bloom of Dytilum and Prorocentrum at Discovery Bay, 10/19/18. Photo: A. Christy

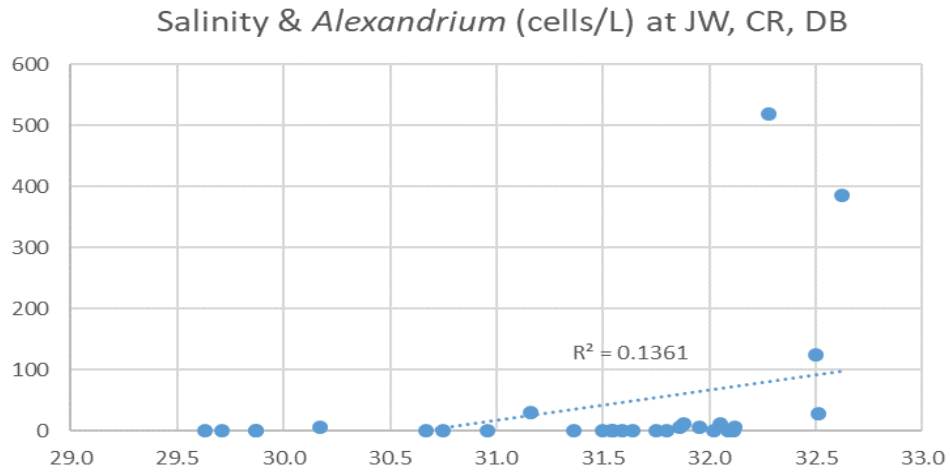


Figure 48. Relationship between Salinity and Alexandrium at JW, CR, DB.

Table 7. R^2 values between HAB species and water quality parameters. Red values indicate a negative relationship.

		Temp	Salinity	DO	pH
Dinophysis	**JW/CR/DB**	0.625	0.062	0.004	0.062
	BI	0.167	0.124	0.002	0.015
Pseudo-nitzschia	**JW/CR/DB**	0.075	0.007	0.000	0.002
	BI	0.063	0.006	0.010	0.101
Alexandrium	**JW/CR/DB**	0.017	0.136	0.108	0.113
	BI	0.022	0.122	0.235	0.050

Summary of Site Conditions

Sequim Bay - John Wayne Marina

Site selection at JWM made this location a poor place to grow scallops. Because the scallop cages were positioned at the discharge end of a shellfish FLUPSY, the site experienced low concentrations of plankton and low plankton diversity, low DO and low pH. Otherwise, the water was cool with high salinity. Water quality conditions likely contributed to both slow growth rates and high shellfish mortality. HAB species were present at this site, but tended to be lower in concentration, perhaps an artifact of lower plankton concentrations overall.

Sequim Bay – Crowell Property

Water quality at CR was perhaps the best of the 4 sites. Water temperatures were cool, salinity high, and DO and PH elevated, but not to the extent that it may lead to eutrophic conditions. Plankton concentrations were rich and diverse. As a result, growth rates were high and scallop mortality was the lowest of all sites. Scallop density was ideal, also contributing to strong growth rates and low mortality. That said, HABs do exist at this site, particularly *Dinophysis* and *Alexandrium* in the fall resulting to DSP and PSP closures.

Discovery Bay

DB was the warmest of the 3 northern sites. The site also experienced extremely elevated plankton concentrations, DO levels and pH. While one would expect scallop growth rates to be high, they were likely inhibited by overcrowding in the cages. What started as a winter mortality event was exacerbated by the quick spread to neighboring scallops over the following few months. HAB species were detected at elevated concentrations, both *Alexandrium* in the fall and *Dinophysis* in the summer, both resulting in DSP and PSP closures. According to news reports, summer shellfish mortality events have also been a recent issue in DB.

Budd Inlet

Budd Inlet, was the warmest and freshest of the 4 sites. Phytoplankton concentrations were rich and diverse with oxygen and pH levels on par with the Crowell site. Oxygen was slightly less due to late summer eutrophication in lower Budd. Scallop growth rates were a bit slower at this site perhaps due to the lower salinity and/or sedimentation on the cages. PSP historically has not been a concern at this site. DSP, however, is a concern, particularly in the fall with closures sometimes extending into the following summer.

Growth Rates

Scallop lengths (mm) were measured at the four study sites every 1-2 months. At each site, 20 randomly selected scallops were removed from each of the multiple trays totaling 40-60 length readings, and as high as 80 readings at the start of the project. Scallops lengths were measured by placing individuals on a measuring board with a ruler affixed to it (**Figure 49**). Scallop growth rates were calculated by subtracting the mean scallop lengths between 2 sampling periods and dividing by the number of days within that period.

Figure 49. K. Houle measuring scallop lengths at BI, January 2018.

Comparing scallop growth rates over time and between sites was challenging due to the addition of new scallop cohorts to cages throughout the 3-year period. Only trays at the Sequim Bay, CR site housed the same cohort of scallops for the entire duration of the project. The following charts represent the most comparable data over both time and space.

Mean scallop lengths increased during summer and fall and leveled off during the winter, with the exception of CR where increased growth continued during winter of 2019. During the 3-year period, maximum mean shell lengths were measured at CR (117mm) followed by JW (106mm), DB (99mm) and BI (94mm) (**Figure 50**). Because the most comparable data for DB began in July 2018, mean growth rates were calculated between summer 2018 and summer 2019. During this period, mean growth rates were highest at CR (0.07mm/day) followed by BI (0.05mm/day), JW (0.01mm/day) and DB (-0.01mm/day) (**Figure 50**). Mean seasonal shell lengths between 2017-2020 are displayed in **Figure 51**.

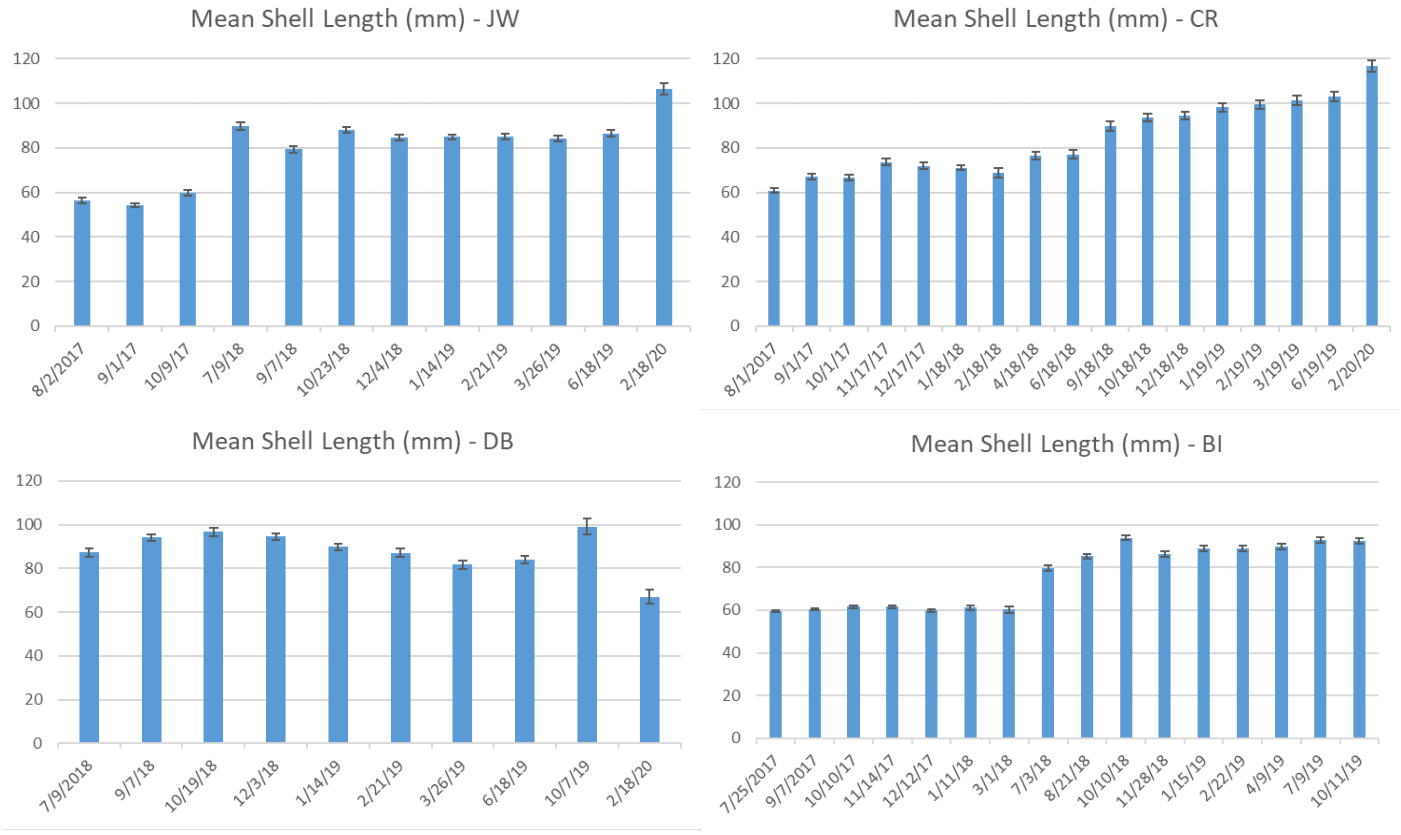


Figure 50. Maximum Mean Shell Lengths (mm) during entire project period (left), and Mean Growth Rates (mm/day) over 1-year period (right). Scale bars indicate ±1SD.

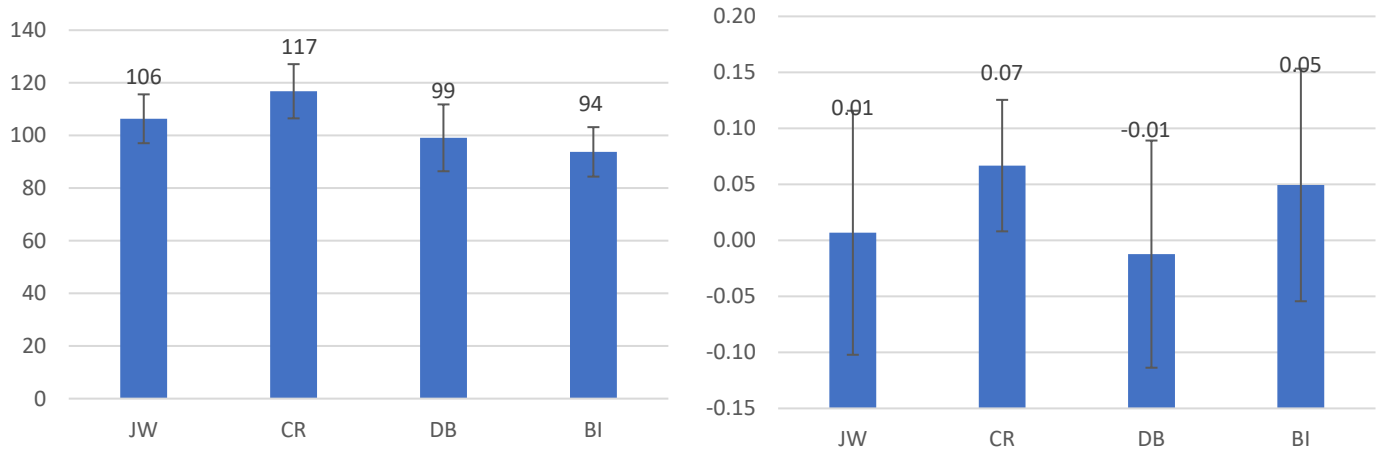


Figure 51. Mean Shell Length (mm) at the 4 sites between 2017 and 2020. Error bars represent ±1SE.

Several factors likely impacted scallop growth rates including food availability, stocking densities, mortality events, and sedimentation. For example, despite having the highest concentration of plankton, growth rates at DB were lowest (-0.01mm/day) where overcrowding was a significant issue. Higher stocking densities have been shown to decrease growth rates in many species as individuals compete for limited resources. Scallops at this site also experienced mortality rates as high as 38% during winter of 2019 which may have disproportionately removed the larger individuals.

Growth rates were also low at JW (0.01mm/day) which similarly experienced high mortality rates. Unlike DB, plankton concentrations were the lowest of all sites due to the proximity of the adjacent FLUPSY. Despite the slow growth rates, mean scallop lengths jumped to 106mm during the final sampling event in February 2020. Stocking density decreased considerably during this last time period.

Growth rates were highest (0.07mm/day) at CR due to both rich plankton concentrations and low stocking density. At this site, 34 to 36 scallops coexisted in one cage for almost a year experiencing continual growth and minimal mortality.

Growth rates were also fairly high at BI (0.05mm/day) despite the fact that maximum scallop lengths remained the lowest of all sites (94mm). Scallops grew quickly between spring and early fall of 2018 and then leveled off for the final duration. While plankton concentrations were high at BI, factors that may have limited final scallop length include sedimentation and stocking density. Of the 4 sites, BI scallops experienced the most sedimentation, particularly during winter months, often requiring thorough flushing between visits. Sediment can clog gills requiring a high level of metabolic energy for removal – energy that would otherwise be used for growth (Volety, A.K. 2006).

Throughout the project, the addition of new scallop cohorts to trays made it difficult to compare growth rates among the 4 sites. Even so, growth rates were strongest at sites with high plankton concentrations and lower stocking densities. Other factors that negatively impact growth rates included mortality events and sedimentation accumulation.

Figure 52 provides growth rate data for a comparative time period of approximately 2.5 years for rock scallop grow out from a previous study (J. Davis, in prep) at several Puget Sound locations. Peak growth from 25mm shell length to ~100mm shell length indicates daily growth of ~0.08mm/day at the top performing locations from this previous study. Compared to our grow out locations, the best performing site (CR) in Sequim Bay was 0.07mm/day shell length. These results indicate location and grow out methods for our field exposure trials could have been further optimized to promote growth in this species.

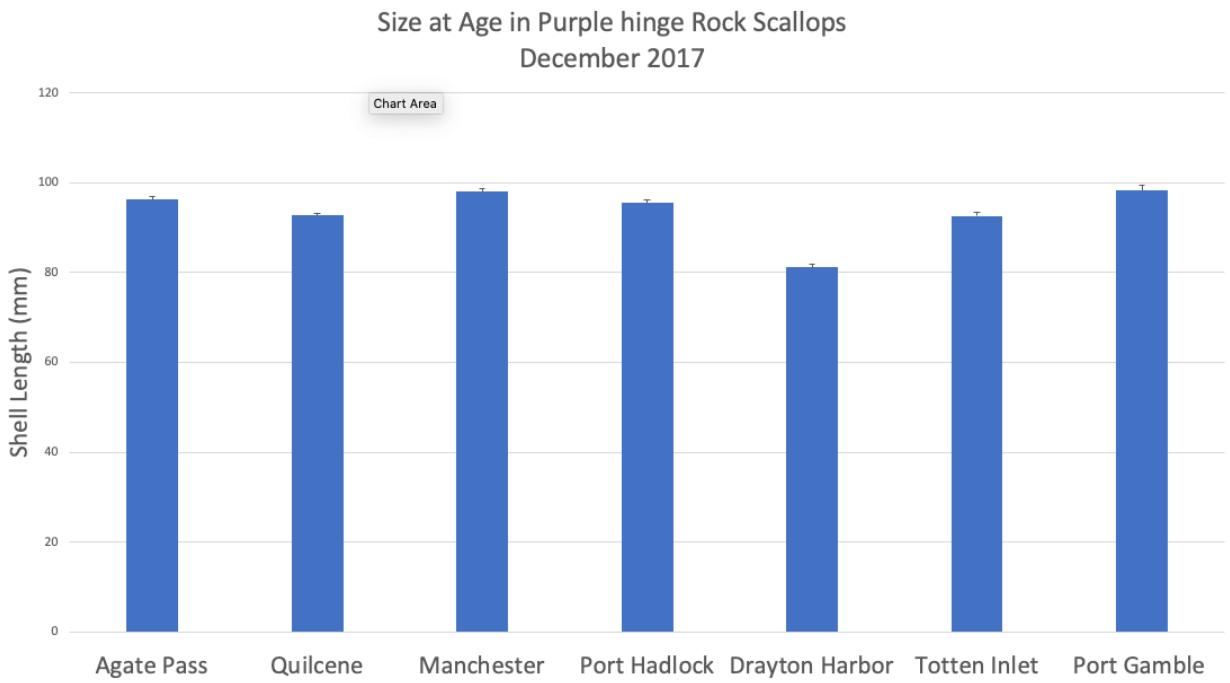


Figure 52. (J. Davis, unpublished) Mean size at age in WRAC study rock scallops in December 2017 that had been out planted at an approximate SL of 25mm in July, 2015 in 7 Puget Sound sites. Mean and S.E. taken on replicate shellfish cages of rock scallops maintained at an approximate 3m depth.

Survival & Mortality Rates

Throughout the duration of the three year project period, the scallops amongst the four sites experienced differential levels of mortality. **Figure 53** summarizes the total percent mortality across the four sites. Scallop mortality was lowest at the Budd Inlet (BI) Site located at the West Bay Marina followed by the Crowell Residence (CR) site in Sequim Bay. The highest rates of mortality were observed at the John Wayne Marina (JW) sampling site followed by the Discovery Bay (DB) site. Average percent mortality over the three-year period ranged from 2.1%±2.0 SD at Budd Inlet to 14.0%±11.5 at John Wayne Marina in Sequim Bay.

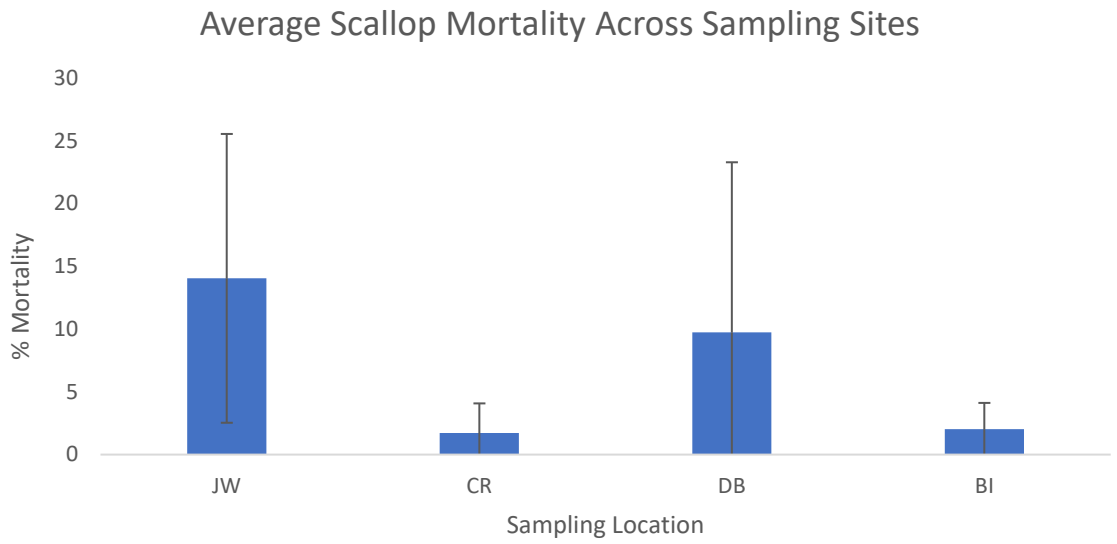


Figure 53. Average mortality across the four sampling sites from 2017 to 2019. Highest mortality rate occurred at John Wayne marina sampling site (r=14.0% ± 11.5) and lowest at Crowell Residence sampling site (r= 1.7% % ± 2.0). Error bars represent ±1SD.

A discussion of potential contributing factors to mortality will focus on John Wayne Marina and Discovery Bay where average observed mortality rates exceeded 10% over the project period. At John Wayne Marina, the highest mortality rates were recorded during October 2018, December 2018 and June 2019. Discovery Bay mortality rates were the highest in February and April 2018 and again during February, March and June 2019. In general, scallops exhibited very little mortality during the peak of summer and early fall.

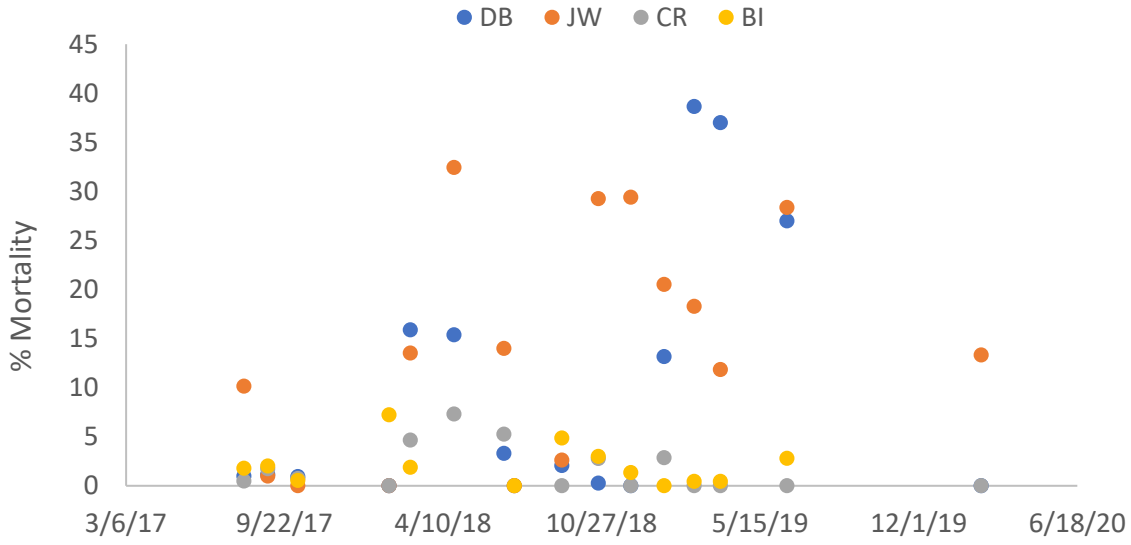


Figure 54. Mortality rates as measured across all four sampling sites at each sampling event from 2017-2019. Peak mortality occurred at Discovery Bay (DB) in February 2019 (r=38%). High mortality was observed most commonly across all four sites from October to June.

Water quality parameters and plankton sampling that occurred concurrently with scallop sampling at the two sites with lowest survivorship rates indicate a possible link between mortality events and stressors such as nefarious phytoplankton blooms, low food availability, cold temperatures, low DO concentrations and low pH. Siting in relation to sub-optimal water quality parameters may have also played a role in the observed mortality rates at the John Wayne Marina Site. Mortality events observed at both sites from October through March in 2018 and 2019 occurred simultaneously with, or immediately following, one or more of the following conditions: water temperature ≤7 ºC, DO ≤5.5 mg/l, pH≤7.6, as well as qualitative observations of low plankton and POM abundance.

The largest recorded mortality events occurred at Discovery Bay during the months of February to March 2019 (38% and 37%, respectively) (**Figure 54**). Low temperatures recorded in February in addition to qualitatively clear water indicate the presence of temperature and starvation stressors that may have contributed to mortality. It's possible that stressful conditions during February continued to effect scallop survivorship as observed during March sampling where no parameters indicative of stress were measured.

In John Wayne Marina, Sequim Bay, the highest mortality rate of 29% was observed in both October 2018 and December 2018. The October mortality event occurred concurrently with low DO, whereas the December event occurred in the presence of combined low pH and low food availability. Proximity of the John Wayne Marina site to a commercial FLUPSY, likely reduced food and oxygen availability and could have been a factor in locally decreasing pH at this sampling station. Proximity to seed operations likely will not be applicable to commercial scallop grow out scenarios in the Puget Sound.

Although stocking density did not have a direct correlation with mortality events over this project period, it is possible that the relationship between stocking density and food availability could have been a contributing stressor to observed mortality across sites. A full analysis of the interrelation and contribution of multiple extrinsic stressors to mortality is outside the scope of this project. However, understanding the effects of environmental stressors on grow out scenarios could help inform future best practices to reduce mortality.

Across both sites, April and June 2018 and 2019 mortality events occurred during blooms of *Rhizosolenia setigera*. *Rhizosolenia setigera* is a species of diatom that is suspected to cause mechanical damage and prevent normal feeding in shellfish seed and adults. Although not known to result in adult shellfish mortality, these blooms have been observed to slow adult growth (McIntyre, 2013). In combination with other stressors, such as high water temperature (17.2 ºC) as observed during the June 2019 mortality event in Discovery Bay, these plankton blooms may have the potential to act as mortality-inducing stressors.

Currently the relationship between shellfish harming algae blooms and commercially devastating mortality events in species such as Pacific oysters and Manila clams is a source of interest and research on the West Coast. Diagnostic testing performed following hatchery and farm mortality events over the past three year period have yielded results that indicate mechanical damage from feeding and other adverse symptoms related to secondary infection of irritated tissues and

unknown possible toxic effects on shellfish from blooms of plankton species such as: *Protoceratium reticulatum*, *Noctiluca scintillans*, *Cochlodinium fulvescens*, *Heterosigma akashiwo*, *Dictyocha fibula*, *D. speculum*, *Rhizosolenia setigera*, and large *Chaetoceros* species (McIntyre et al, 2013). For producers interested in pursuing scallop farming on the West Coast, close attention should be paid to the timing of mortality events and the plankton species composition to further elucidate the observed relationship between bloom events and scallop survivorship.

Mortality events observed to occur in scallops over this project do not follow patterns observed in other commercially significant species on the West Coast. Significant effort over the past decades has been put into elucidating the mechanism(s) behind catastrophic oyster mortality events, which usually occur during the peak of summer when temperatures are the highest. Although poor water quality conditions are known extrinsic stressors to Pacific oysters, winter to early summer mortality of this species, as observed in scallops, is generally uncommon.

Manila clam mortalities on the West Coast, similar to scallop mortalities, are observed to occur in mid-late winter. These mortalities are often associated with a combination of freshwater influx in addition to consistent freezing temperatures and tidal exposure. Although a similar negative response to cold temperatures is consistent between scallops and manila clams, the cultivation of scallops will likely not occur near high inputs of fresh water, nor will air exposure be a factor in scallop grow out. Initial comparisons between commercially significant species and scallop mortality events suggest that more research is necessary to further inform the optimization of survivorship in scallop cultivation in relation to the environmental stressors present in the Puget Sound.

Biofouling: Ecological and Commercial Implications

Substantial accumulation of biofouling species on the scallop cages and the utilization of the cages by several motile species as a nursery site and refuge was observed amongst the four sampling sites. A combined total of over 80 species were observed and recorded to either permanently or transiently utilize the experimental submerged cage structures throughout the duration of the project period. Observed species abundance and diversity suggest that scallop cage structures, as would be deployed in commercial growout applications, could result in structured habitat for ecologically significant species.

Table 8. Comprehensive list of all the species found over the four project sites over the three-year study period:

ALGAE	**Brown algae**	Sugar Kelp, *Saccharina latissima*
	Green Macroalgae	Filamentous Green, *Ulva spp.*
	Red Macroalgae	
BRYOZOANS		Arborescent bryozoan, *Bugula spp.*
		Encrusting bryozoan, *Membranipora sp.*
		Orange Encrusting Bryozoan, *Schizoporella spp.*

CNIDARIANS	**Hydroid**	Hydroid, *Obelia spp.*
	Jellyfish	
	Sea Anenomes	
CRUSTACEANS	**Amphipods**	Skeleton Shrimp, *Caprellid spp.*
	Barnacles	
	Crab	Decorator Crab, *Oregonia gracilis*
		Dungeness crab, *Cancer magister*
		Hermit Crab, *Pagurus spp.*
		Kelp Crab, *Pugettia producta*
		Pea Crab, *Pinnixa faba*
		Porcelain Crab, *Petrolisthes spp.*
		Pygmy Rock Crab, *Glebocarcinus oregonensis*
		Red Rock, *Cancer productus*
		Sharp Nosed Crab, *Scyra spp.*
		Shorecrab, *Hemigrapsus spp.*
	Isopods	
	Shrimp	Dock Shrimp, *Pandalus danae*
		Bay Shrimp, *Crangon crangon*
		Grass Shrimp, *Hippolyte clarki*
		Barred Shrimp, *Heptacarpus pugettensis*
ECHINODERMS	**Seastars**	Brittle stars, *Ophiuroidea spp*.
		Mottled Sea Star, *Evasterias troschelii*
		Purple Sea Star, *Pisaster ochraceus*
		Six-rayed Sea Stars, *Leptasteris hexactis*
	Sea Cucumbers	Orange Sea Cucumber, *Cucumaria miniata*
		White Sea Cucumber, *Eupentacta sp.*
	Urchins	Green Urchin, *Strongylocentrotus droebachiensis*
FISH		Green Sculpin, *Oligocottus maculatus*
		Juvenile rock fish, *Sebastes spp.*
		Kelp Perch, *Brachyistius frenatus*
		Northern Clingfish, *Gobiesox meandricus*
		Pacific staghorn sculpin, *Leptocottus armatus*
		Penpoint gunnel, *Apodichthys falvidus*
		Saddleback gunnel, *Pholis ornata*
		Shiner Perch, *Cymatogaster aggregata*
		Three Spined Stickleback, *Gasterosteus aculeatus*
MOLLUSKS	**Bivalves**	Blue Mussels, *Mytilus edulis*
		European Flat Oyster, *Ostrea edulis*
		Jingle Shell, *Pododesmus macrochisma*
		Macoma Clams, *Macoma nasuta*
		Pink Scallops, *Chlamys rubida*
		Soft Shell Clam, *Mya arenaria*

	Nudibranchs	Sea Lemon, *Anisodoris nobilis*
		Barnacle Eating Nudibranch, *Onchidoris bilamellata*
		Dorid nudibranchs, *Doridoidea spp*.
		Giant Nudibranch, *Dendronotus iris*
		Shaggy Nudibranch, *Aeolidia papillosa*
		Opalescent Nudibranch, *Hermissenda crassicornis*
		White-lined Dirona, *Dirona albolineata*
	Snails	Shield limpet, *Lottia pelta*
		Plate limpet, *Tectura scutum*
		Periwinkles, *Littorina spp*.
		Dogwinkles, *Nucella spp.*
		Slipper Shell, *Crepidula fornicate*
	Chitons	Hairy Chiton, *Mopalia sp.*
SPONGES		Breadcrumb Sponge, *Halichondria* sponge
		Elegant Branching Sponge, *Haliclona spp.*
		Yellow boring sponge, *Cliona sp.*
TUNICATES	**Colonial**	*Colonial orange tunicate, Botrylloides spp.
		*Colonial star tunicate, *Botryllus spp.*
	Solitary	Brooding transparent tunicate, *Corella inflata*
		Red solitary tunicate, *Cnemidocarpa sp.*
		Sea Grape, *Mogula sp.*
		*Solitary club tunicate, *Styela clava*
WORMS	**Flatworms**	
	Nemerteans	Green ribbon worm, *Emplectonema sp*.
		Orange Ribbon Worm, *Tubulanus polymorphus*
		Six-lined ribbon worm, *Tubulanus sexlineatus*
	Polychaetes	Pile worms, *Nereis spp.*
		Green polychaete, *Nereis brandti*
		Feather Duster Worm, *Eudistylia polymorpha*
		Mud Tubeworm spp.
		Calcarious Tube Worms, *Serpula vermicularis*
		Iridescent worms, *Lumbrinerid spp.*
		Scaleworms, *Polynoidea spp.*
	Sipunculids	Peanut worms

The level of biofouling as observed throughout the project period, especially at northern sites, adjacent to potential scallop cultivation sites, presents a potential challenge to cultivation and processing of native scallops. During sampling, the colonization of the cages by tunicates, barnacles, and encrusting bryozoans heavily fouled the cages (**Figure 55a & b**) obstructing the flow of water and increasing the weight of the cages so that the retrieval of the cages resulted in damage to the suspended gear. The heavy and restrictive fouling observed initially on the 10mm mesh resulted in a transition to the wider 25mm mesh for the duration of the project period in an

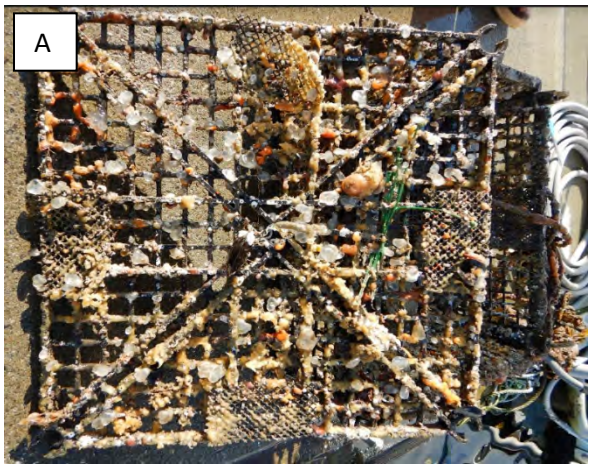
effort to increase access by the scallops to POM and plankton. Despite the transition of gear, obstructive fouling continued at all locations, albeit with lesser assumed impact to flow with the larger mesh.

Cages were defouled at irregular intervals corresponding to toxicity sampling year-round. It is our observation and recommendation that to maintain water flow to the scallops, the cages should be defouled every 2-months throughout the year, increasing to monthly in the late spring and summer months (May-September). Our project sampling team of 1-2 individuals underwent the defouling process using paint scrapers and hard-bristled brushes and averaged 30-min of cleaning time per 24 inch x 24 inch x 12 inch cage. In commercial growout scenarios in deepwater lease areas, where scallop cages could be stacked in large arrays, 10-20 feet deep over 40 foot x 40 foot areas, this would translate into an unfeasible maintenance scenario.

Personal communication with a commercial scallop operation in British Columbia (BC), where scallops are grown in fully submerged stacked cage systems, as well as a review of the literature, indicate that the scallops attract high densities of fouling organisms. One hypothesis offered from BC producers is that scallops are at an ecological advantage from predation if disguised in a reef-like structure. At select operations in BC, fouling organisms were reported to be removed from the organisms during processing. For scallops that are processed for their adductor muscle, fouling evidence on the shell is of less commercial concern. However, higher end restaurants and retailers feature scallops in an open half-shell presentation. This high end commercial retail is where fouling organisms such as calcareous tube worms (**Figure 55h**) may reduce marketability.

One study from a commercial scallop cultivation site in Spain suggests that biofouling on cages was found to have an inverse relationship to stocking density (Lauro et al, 2007) in the great scallop, *Pecten maximus*. Both stocking density and biofouling were inversely related to growth rate in the same study. Since biofouling was observed to increase with temperature in our Puget Sound study sites, consistent with the literature on biofouling in aquaculture (Lauro et al, 2007; Ross et al, 2004, Watson et al, 2009), it could be a consideration to increase stocking density during summer when biofouling rates are highest and reduced during winter months, so as to encourage growth rate when biofouling is low. Exploring the relationship between stocking density, as well as the presence of urchins and other organisms that may act as biological controls to fouling, would inform cost and efficiency measures relating to biofouling in the Puget Sound.

The overall species richness and diversity observed on the submerged scallop cages over the duration of this project period suggests that the cages provide refuge and nursery areas sought by many marine species. However, the level of fouling presents the potential of reducing scallop viability and marketability. This study provides a baseline assessment of the presence of biofouling organisms specific to the Puget Sound with the intent to inform future research on commercial scale growout methods in the region.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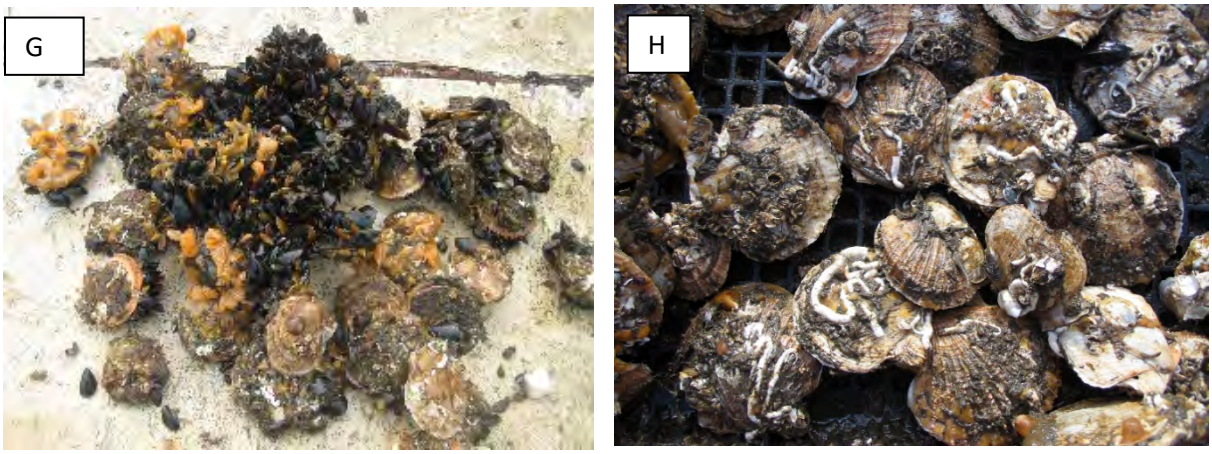

Figure 55. Biofouling and species utilization of scallop cages amongst the four study sites.

A. Anemone and tunicate fouling on cage lid at Crowell Residence, Sequim Bay.
B. Mussel and encrusting bryozoan heavily foul cages at Budd Bay site.
C. Penpoint Gunnels utilizing cage as structure for nursey at Crowell Residence, Sequim Bay
D. Sculpins predate on small organisms found in the scallop cages
E. Juvenile rock fish found regular refuge among scallop trays at Discovery Bay.
F. Brittle star found among scallops at Crowell Residence, Sequim Bay.
G. Heavy native mussel set and encrusting bryozoans foul scallops at Budd Bay site.
H. Calcareous Tube worms and barnacles encrust scallops.

OUTREACH

Houle, K., Hudson, B., Davis, J., Shumway, S., Morton, S., Borchert, J., Vadopalas, B.BIOTOXIN ACCUMULATION AND DEPURATION IN FIELD EXPOSED ROCK SCALLOPS *Crassadoma gigantea*: A PREREQUISITE FOR CULTURE. World Aquaculture Annual Meeting, New Orleans, LA, March 2019.

Houle, K., Hudson, B., Davis, J., Shumway, S., Morton, S., Borchert, J., Vadopalas, B.BIOTOXIN ACCUMULATION AND DEPURATION IN FIELD EXPOSED ROCK SCALLOPS *Crassadoma gigantea*: A PREREQUISITE FOR CULTURE. Pacific Coast Shellfish Growers Conference/National Shellfish Association Pacific Coast Section Annual Meeting, Portland, OR, September 2019.

Houle, K., Hudson, B., Davis, J., Shumway, S., Morton, S., Borchert, J., Vadopalas, B.BIOTOXIN ACCUMULATION AND DEPURATION IN FIELD EXPOSED ROCK SCALLOPS *Crassadoma gigantea*: A PREREQUISITE FOR CULTURE. Pacific Coast Shellfish Growers Conference/National Shellfish Association Pacific Coast Section Annual Meeting, Virtual, October 2020.

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