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Spatial Variability and Seasonal Patterns of Co-Occurrence between two Marine
Biotoxins in the Puget Sound and Implications for Public Health Management

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Abstract

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Shellfish are of utmost cultural, recreational, and commercial importance in Washington State. However, the accumulation of marine algal biotoxins in shellfish can pose severe health risks to shellfish consumers. While Paralytic Shellfish Toxins (PSTs) have been well monitored in the state for decades, Diarrhetic Shellfish Toxins (DSTs) were only recently detected in Washington but are now widespread throughout the Puget Sound and neighboring marine basins. This study investigates the frequency, intensity, and variability of PSTs, DSTs, and their co-occurrence across various marine basins in Washington State's Puget Sound during the first decade of co-monitoring. Biotxin monitoring data for PST and DST were sourced from the Washington Department of Health and were used to identify when and where toxin co-occurrence was observed from 2012 to 2022. Additionally, Bayesian Hierarchical autoregressive models were created to parse out the effects of month, year, and marine subbasin on PST and DST

concentrations. The findings reveal distinct geographic patterns in toxin occurrences, indicating basin-specific environmental influences on toxin concentration. Additionally, concentrations of both toxins showed seasonal variation, though temporal peaks were not often synchronous, resulting in back-to-back toxin events and prolonged shellfish harvesting closures. This thesis provides a baseline assessment of toxin co-occurrence during the first decade of co-monitoring for PSTs and DSTs and characterizes patterns underlying toxin co-occurrence, highlighting the need for further study into the human health effects of co-exposure to these toxins.

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Chapter 1. INTRODUCTION

Shellfish are central to Washington state's culture and economy. Coast Salish people have harvested shellfish, including intertidal clams, geoduck, oysters, and mussels in the Puget Sound region since time immemorial (Lyall, 2022). Today, shellfish harvesting provides food for subsistence and recreational harvesters and over 3,200 jobs for commercial fishermen employed by Puget Sound's \$180 million dollar shellfish industry (Washington Sea Grant, 2015).

However, the accumulation of marine biotoxins in shellfish, which often occurs after a bloom of toxin-producing algae, can pose severe health risks to shellfish consumers (Louzao et al., 2022). While deaths from biotoxin poisoning have not occurred in Washington since the 1940s, 35 illnesses occurred between 1985 and 2022, often after consumers harvested shellfish recreationally (Trainer et al., 2003; Washington State Department of Health, 2024). The high rate of shellfish consumption in Washington makes early-warning systems, biotoxin monitoring, and fishery closures critical for protecting the health of consumers (Trainer & Hardy, 2015).

In Washington, three types of marine biotoxins that produce toxic effects in humans occur in waters along the outer coast and in the Puget Sound: saxitoxin and its analogs, hereafter referred to as paralytic shellfish toxins (PSTs); okadaic acid and dinophysistoxins, hereafter referred to as diarrhetic shellfish toxins (DSTs); and domoic acid (Trainer & Hardy, 2015). All three biotoxins are biosynthesized by marine algal species: PSTs are produced by dinoflagellate species in the genus *Alexandrium*, DSTs are produced by dinoflagellate species in the genera *Dinophysis* and *Prorocentrum*, and domoic acid is produced by diatoms in the genus *Pseudonitzschia*, among others (Louzao et al., 2022). Consumption of shellfish tissue contaminated with unsafe levels of PSTs can cause the neuromuscular paralysis syndrome called

Paralytic Shellfish Poisoning (PSP) (Louzao et al., 2022). Symptoms of PSP include tingling lips and tongue, headache, nausea, vomiting, paralysis, and even death, depending on the concentration consumed (Louzao et al., 2022). Diarrhetic Shellfish Poisoning (DSP) results from ingestion of unsafe levels of DSTs and presents as diarrhea, nausea, vomiting, and abdominal pain, though is not known to be fatal in humans (Louzao et al., 2022). Domoic acid is the causative agent of Amnesic Shellfish Poisoning (ASP) which is characterized by vomiting, diarrhea, and abdominal pain, followed, in severe cases, by neurological symptoms such as temporary or permanent short-term memory loss, seizures, coma, and possible death (Louzao et al., 2022). To protect human health, the Washington Department of Health (WDOH) will close harvesting areas to recreational and/or commercial shellfisheries if a toxin ‘event’ occurs at that site, wherein any of the following toxin action thresholds (ATs) are reached or surpassed: 80 μ g of PSTs per 100g of mussel tissue, 16 μ g of DSTs per 100g of mussel tissue, or 20ppm of okadaic acid in mussel tissue (Trainer & King, 2023; Wekell et al., 2004).

Paralytic shellfish poisoning has occurred in the Pacific Northwest region for centuries, although an expansion in the toxin’s geographic range within Washington and an increase in the length and intensity of toxin events in the latter half of the 20th century has been documented (Nishitani & Chew, 1984; Trainer et al., 2003). Early reports of PST poisoning in the region include four members of Captain George Vancouver’s crew who presented PSP symptoms after consuming contaminated shellfish in 1793 (Quayle, 1969). The WDOH began monitoring for PSTs in the 1930s and has maintained a consistent monitoring program since 1957 (Trainer et al., 2003). However, PSTs above the action threshold, and concurrent illnesses, were not documented in Washington until 1942 in the Western Strait of Juan de Fuca (Quayle, 1969). Between 1942 and 1997, the occurrence of PST events above the AT appeared to expand east

into the Puget Sound and gradually extend southward (Trainer et al., 2003). PSTs above the AT were first detected in the Eastern Strait of Juan de Fuca in 1957, followed by the San Juan Islands in 1958, North Puget Sound in 1973, Whidbey Basin and Central Basin in 1978, Northern Hood Canal in 1987, and South Puget Sound in 1988, despite previous monitoring at these sites in which PST was not detected or only detected at low levels (Trainer et al., 2003). Trainer et al. (2003) hypothesized that this progression resulted from north-to-south transport of *Alexandrium catenella* cells from the outer coast of Canada. Furthermore, Trainer et al. (2003) hypothesized that the temporal lags between PST expansion into different basins may have been due to mixing conditions at the sills separating the major basins of Puget Sound, as *A. catenella* are known to prefer stratified waters.

In contrast to PSTs, the first recorded illness associated with DSTs in Washington State did not occur until 2011, when 3 people became ill following consumption of mussels harvested recreationally at Sequim Bay State Park (Lloyd et al., 2013). Soon after, DST concentrations were found to be 2-10 times the AT (Trainer et al., 2013). From June to August of the same summer, the first known DSP illnesses in western Canada occurred on Salt Spring Island, British Columbia, after roughly 60 people fell ill following consumption of Pacific coast mussels (Taylor et al., 2013). *Dinophysis* species in North America and associated DST events have been referred to as ‘late-comers to the harmful algae world,’ as the first confirmed incidence of DSTs in the US occurred on the Gulf Coast of Texas in 2008 (Díaz & Reguera, 2023). Even on a global scale, DST events and associated DSP illnesses are considered a new phenomenon, with the first DSP illness recorded in the Netherlands in 1961 and the first DSTs identified in 1976 in Japan (Van Egmond et al., 2004; Yasumoto et al., 1985). However, DSP has become a

widespread problem since then, with large illness case numbers and impacts to bivalve fisheries in countries including Japan, Spain, France, and Sweden (Lloyd et al., 2013).

During the first four months of regular DST monitoring in Washington State in 2012, toxin levels above the AT were detected at more than 20 sites throughout the Puget Sound (Trainer et al., 2013). Detections of DST above the AT were not as widespread in the years following, and it has been hypothesized that above-average freshwater plumes from the Fraser River entering the Salish Sea in 2011 and 2012 may have created conditions to which *Dinophysis* is particularly well adapted (Reguera et al., 2012; V. Trainer et al., 2013). Region-specific strains of *D. acuminata*, which differs from its Atlantic counterparts in toxin profile and morphology, and *D. fortii* are the species primarily responsible for DSTs in Washington State (Ayache et al., 2023). Of a survey of 20 *Dinophysis* strains from five species and three US regions, Pacific Northwest strains of *D. acuminata* and *D. fortii* were found to have the highest DST concentrations on a per-cell basis (Ayache et al., 2023). Furthermore, this survey found that while Pacific Northwest *Dinophysis* blooms tended to be less dense in cell concentration than East Coast blooms, they resulted in higher levels of DSTs and more consistent, prolonged closure events.

Recent studies suggest that it is not uncommon for multiple biotoxins to co-occur in the water column or within shellfish tissues (Hattenrath-Lehmann et al., 2018; Onofrio et al., 2021; Peacock et al., 2018; Pease et al., 2023). However, there is a lack of understanding regarding the human health implications of poisoning by multiple toxins simultaneously, such as whether the toxins may have additive, synergistic, or antagonistic effects (Alarcan et al., 2018; Hattenrath-Lehmann et al., 2013). If toxins have synergistic effects, causing toxic effects to occur in smaller doses of each toxin than would ordinarily induce poisoning when ingested separately, it may be

necessary to lower the threshold levels at which fisheries are closed when those toxins are simultaneously elevated in the environment.

The potential human health effects of PST and DST co-occurrence are unknown. However, interactive effects of various other marine biotoxins have been shown. An *in vitro* study using human intestinal cells demonstrated that okadaic acid acts antagonistically with azaspiracid-1 and yessotoxin, while mixtures of azaspiracid-1 and yessotoxin exhibit increasing synergy with increasing concentrations (Ferron et al., 2016), and an *in vivo* study showed saxitoxin and tetrodotoxin to have additive effects in mice (Finch et al., 2018).

Determining whether the biotoxins found in Washington have synergistic or additive effects, and then identifying the precise amount of each toxin that could cause harm to a consumer under co-occurrence scenarios, would require extensive experimentation, time, and effort. For this research to be warranted, there would first have to be clear evidence that toxins do co-occur regularly in Washington. While it is suspected that toxin co-occurrence has been increasing in recent years (Jerry Borchert, WDOH, personal communication), this has not been confirmed by statistical analysis of the available biotoxin testing data.

In this study, I aim to characterize co-occurrence between PSTs and DSTs in a subregion of Washington's marine waters – the Puget Sound and adjacent basins. I ask the following questions: (1) Where were PST, DST, and co-occurrence events observed, or not observed, in the Salish Sea during the first decade of co-monitoring? (2) Do temporal trends underlying PST and DST events facilitate or preclude co-occurrence at sites of geographic overlap? To answer these questions, I first look at maximum toxin concentrations by month and year at the site level to assess the local-scale variation in PSTs, DSTs, and co-occurrence from 2012 to 2022. I then

model each toxin separately to identify basin-specific seasonal effects on toxin concentration to understand the likelihood of spatial and temporal co-occurrence between the two toxins.

Chapter 2. METHODS

2.1 SHELLFISH COLLECTION & BIOTOXIN TESTING

Biotoxin monitoring data for PST and DST were provided by WDOH's Biotoxin Monitoring Program. The WDOH began monitoring for PSTs in shellfish in the 1930s and has maintained a consistent monitoring program since 1957 (Moore et al., 2009). Prior to the 1990s, monitoring focused on shellfish species of commercial importance, as the WDOH was not responsible for monitoring toxins at beaches open to recreational sport harvesting of shellfish until 1986 (Trainer et al., 2003). To effectively monitor PST in shellfish species of recreational importance, WDOH established a Sentinel Monitoring Program in 1989 in which mussels are placed in cages and suspended from floats or docks, roughly 1 meter below the surface, throughout the Puget Sound, Strait of Juan de Fuca, and Outer Coast (Trainer & King, 2023). Since the early 1990s, *Mytilus edulis* has been the sentinel species sampled at most sites throughout the Puget Sound, due to its rapid accumulation and depuration of biotoxins compared to other bivalve species (Moore et al., 2009). Mussels are collected from sentinel sites for toxin testing at two-week intervals, year-round, though not all sites have been actively sampled for the entirety of the program. Shellfish samples from non-sentinel sites are also sent to WDOH by local health departments, Tribes, commercial shellfish growers, community organizations, and volunteers for testing and the results are incorporated into the biotoxin monitoring dataset used in this analysis. In 2006, the SoundToxins phytoplankton monitoring program was established to provide an early warning mechanism allowing WDOH to increase shellfish testing at sites if harmful algal species, including *Alexandrium catenella* and *Dinophysis acuminata*, are found to be increasing or occur at densities that could indicate a developing bloom at that site (Trainer &

King, 2023). Aside from the Sentinel Monitoring Program, sites are not tested for biotoxins at consistent intervals. Widespread DST testing in shellfish at sentinel and non-sentinel began in the summer of 2012, following the first DST illnesses in 2011 (Trainer et al., 2013).

Shellfish samples are sent to the WDOH laboratory and are prepared for the type of testing being conducted. To test for presence of PSTs, the mouse bioassay method is used, which yields a PST concentration represented in μg PST/100g of shellfish tissue, with a lower limit of quantification of $38\mu\text{g}$ PST/100g tissue. PST extracted from homogenized tissue is injected intraperitoneally into three mice and median time to death, ranging between 5 and 7 minutes, is converted to mouse units (MU) using an interpolated version of Sommers table, mouse weight, a correction factor for the mouse colony used, and a dilution factor. To test for the presence of DSTs, including okadaic acid (OA) and dinophysis toxins (DTX-1 and DTX-2), toxins extracted from homogenized tissues are analyzed with ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) using the methods described in Trainer et al., (2013). The unit of the resulting DST concentration is μg DST/100g shellfish tissue, with a lower limit of quantification of $1\mu\text{g}$ PST/100g tissue.

2.2 DATA COLLECTION & CLEANING

Given differences between shellfish species in the rate at which toxins are accumulated and depurated from tissue, only PST and DST tests on *M. edulis* tissues were used for statistical analysis. Sites in the US portion of the Salish Sea were aggregated into the 8 marine basins used by the Puget Sound Ambient Monitoring Program (Ebbesmeyer et al., 1984) (Figure 1). The Western Strait of Juan de Fuca was not included in the analysis due to lack of data from sites within this basin. Because fewer tests were conducted in the early years of the Sentinel Monitoring Program, PST tests conducted before 1998 were not included in the analysis. The

greatest number of PST and DST samples annually originated from Central Puget Sound and South Puget Sound (Figure 2).

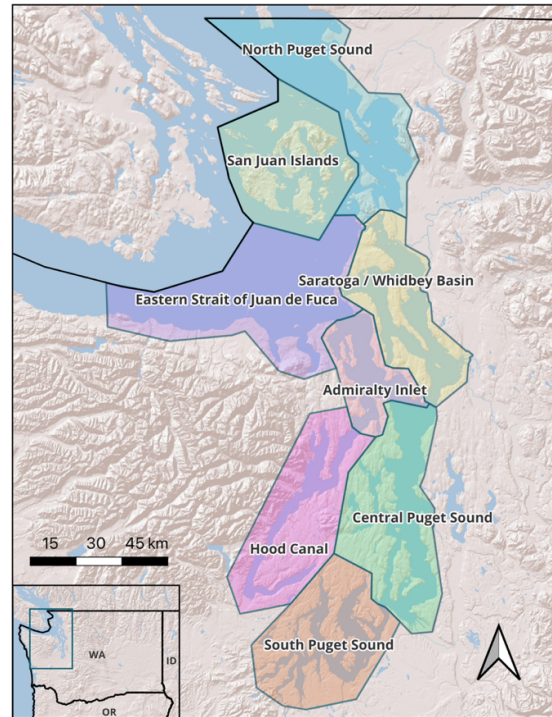


Figure 1. Oceanographic basin delineations of the Salish Sea used in statistical analyses.

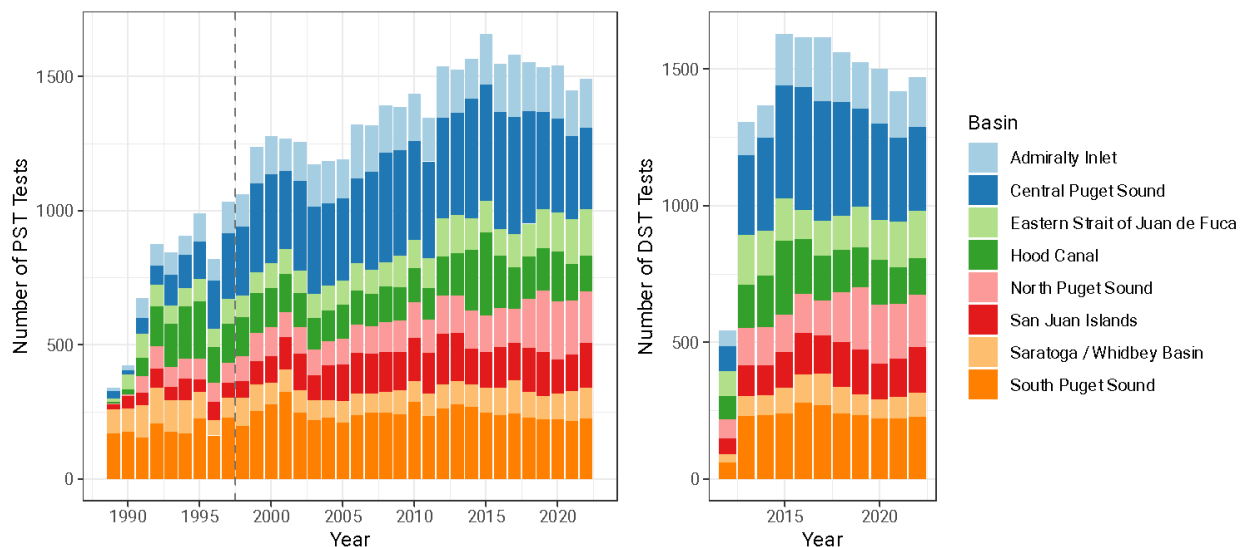


Figure 2. The number of PST and DST tests conducted by basin from 1989 to 2022 for PST (since the establishment of the Sentinel Monitoring Program) and from 2012 to 2022 for DST. Only samples collected since 1998 are used in the PST analysis, represented by the dotted vertical line on the left plot.

2.3 STATISTICAL ANALYSES

2.3.1 *Site-Specific Case Studies for PST, DST, and Co-Occurrence*

To investigate PST trends at individual sites, *M. edulis* samples collected from all sites within the 8 basins displayed in Figure 1 from 2012 to 2022, excluding 2020 due to inconsistencies in data, were assessed. Results in which PST was detected but was below the limit of quantification ($38\mu\text{g}/100\text{g}$ tissue) were replaced with a value of $19\mu\text{g}/100\text{g}$ tissue. Samples were grouped by month, year, and site to determine the maximum concentration of PST at the site during that month. If no PST tests were conducted at a site during a given month, the maximum detection of PST was marked as “NA”. Maximum concentrations of PST per month were binned into the following 6 categories: $0\mu\text{g}$ (no toxin), $1\text{-}79\mu\text{g}$ (low, below AT), $80\text{-}499\mu\text{g}$ (above AT, moderate), $500\text{-}999\mu\text{g}$ (above AT, moderately severe), $1000\text{-}4999\mu\text{g}$ (above AT, severe), $5000+\mu\text{g}$ (above AT, extreme). If the maximum PST concentration at a site during a given month was at or above the AT of $80\mu\text{g}/100\text{g}$ shellfish, that site was said to have experienced a ‘PST event.’

To investigate DST trends at individual sites, similar methods were used. Results from 2012 to 2022 (excluding 2020) in which DST was detected but was below the limit of quantification ($1\mu\text{g}/100\text{g}$ tissue) were reassigned a value of $0.5\mu\text{g}/100\text{g}$ tissue. Maximum concentrations of DST per month at sites were binned into the following 6 categories: $0\mu\text{g}$ (no toxin), $1\text{-}15\mu\text{g}$ (below AT, low), $16\text{-}74\mu\text{g}$ (above AT, moderate), $75\text{-}149\mu\text{g}$ (above AT, severe), $150+\mu\text{g}$ (above AT, extreme). If the maximum DST concentration at a site during a given month was at or above the AT of $16\mu\text{g}/100\text{g}$ shellfish, that site was said to have experienced a ‘DST event.’

The resulting datasets were then used to determine for each toxin, separately, the following measures: (1) toxin “hotspots” at which sites experienced at least one toxin event in 5 or more years; (2) the number of years during which a toxin event was detected for each site; (3) the proportion of sites sampled per year which experienced a toxin event; and (4) the maximum toxin value per site and per year. Binned toxin detections were represented on heatmaps to visualize trends by site within basins and over time.

To assess co-occurrence of PST and DST, samples collected between 2012 and 2022 (excluding 2020) were aggregated by site, month, and year to determine the maximum detection of each toxin for each site/month/year combination. Site/month/year combinations were excluded from the dataset if one of the toxins was not tested for during that month. Using the maximum concentrations of PST and DST per month at sites, each site/month/year combination was assigned a co-occurrence event status: no event (neither toxin exceeded the AT at that site during that month); PST event only; DST event only; or co-occurrence event (both toxins exceeded the AT at that site during that month). Furthermore, each site/month/year combination was also assigned a co-detection status: no toxin (neither toxin was detected at any concentration at that site during that month); only PST detected at any non-zero concentration; only DSP detected at any non-zero concentration; or both toxins detected at any non-zero concentration. The resulting dataset was then used to assess how often co-occurrence and co-detection of toxins was observed, by basin and site.

2.3.2 *Quantitative Analysis of Paralytic Shellfish Toxin*

Samples collected between 2013 and 2019 were used as input observations to inform the PST model. Samples collected in 2020 were removed from the model input due to a disruption in toxin testing in 2020 which may have affected the quality of results, and the years after 2020

were removed to maintain a continuous time series. PST samples collected between 2013 and 2019 were treated as a noisy autoregressive process, and observation and process variability were modeled separately as described below.

Process Model. For each month-long time step t in each of the 8 basins k , the mean toxin concentration, $\mu_{t,k}$, was modeled as an additive combination of lag-1 autocorrelation ($\beta\mu_{t-1,k}$), a monthly (seasonal) effect that varied across basins but was fixed across years ($\gamma_{m,k}$, where m indexes month of the year), and a residual term reflecting unexplained process variability ($\omega_{t,k}$). Because each time step t occurs within a month m and a year j , these latter two subscripts were omitted on μ for clarity, and the prediction for mean concentration was:

$$\mu_{t,k} = \beta\mu_{t-1,k} + \gamma_{m,k} + \omega_{t,k}$$

The month effects ($\gamma_{m,k}$) were hierarchical, such that basins shared a mean effect θ_m in each month of the year. Individual realizations of the month effect, $\gamma_{m,k}$, were drawn from a normal distribution of mean θ_m and standard deviation τ^2 – the latter of which quantified the variation of the month effect among basins within a month m .

Observation Model. Each observation i reported either a toxin detection ($z_i = 1$) or non-detection ($z_i = 0$). Consequently, the probability of detection was modeled as the outcome of a Bernoulli trial, where the probability of a detection in observation i at time step t in basin k was given as $p_{i,t,k}$.

$$z_{i,t,k} \sim \text{Bern}(p_{i,t,k})$$

$$p_{i,t,k} = \text{cloglog}^{-1}(-2 + \psi\mu_{t,k})$$

The inverse cloglog link function kept probability $p_{i,t,k}$ between zero and one, and the probability of detection was otherwise a linear function of the mean log toxin concentration present. The fixed intercept of -2 reflected a low (but nonzero) probability of false-positive; the

cloglog transform was used (rather than the more common logit link) to reflect a steep gradient in $p_{i,t,k}$ as $\mu_{t,k}$ increased.

Log concentrations of observations of positive detections ($z_{i,t,k} = 1$) reported as being below the limit of toxin quantification were treated as parameters to be estimated, $\hat{y}_{i,t,k}$, where plausible values for the parameters vary between zero and the reported assay limit of quantification (LOQ). Given that the probability of detection increases with the underlying concentration of toxin, values of $\hat{y}_{i,t,k}$ were drawn from a prior distribution somewhat skewed toward the upper limit of this range.

$$\hat{y}_{i,t,k}/\text{LOQ} \sim \text{Beta}(3, 1)$$

Finally, log toxin concentrations from individual observations ($y_{i,t,k}$) were modeled as random samples from a normal distribution.

$$y_i \sim N(\mu_{t,k}, \sigma_{t,k}^2)$$

$$\sigma_{t,k}^2 = e^{-2+\phi\mu_{t,k}}$$

The standard deviation around the expected value of toxin concentration was allowed to vary as a function of the mean, and because ϕ was positive, the standard deviation among observations at time t in basin k increased as the mean concentration increased. Exponentiating this function kept the value of σ strictly positive. The intercept was fixed at a low value (here, -2, so that when μ was 0 (on a log scale, and so 1 on a natural scale), $\sigma = e^{-2} = 0.135$) rather than estimated, to improve model-fitting efficiency.

Because the model was fit in a Bayesian framework, prior distributions were chosen to reasonably inform – but not to unduly influence – the posterior probability estimate for each parameter. The priors were as follows

$$\beta \sim \text{Beta}(2, 2)$$

$$\varphi \sim \text{Gamma}(1, 3)$$

$$\omega \sim N(0, 2)$$

$$\psi \sim \text{Gamma}(1, 2)$$

$$\theta \sim N(0, 2)$$

$$\tau \sim \text{Gamma}(1, 2)$$

2.3.3

Quantitative Analysis of Diarrhetic Shellfish Toxin

Samples collected between 2013 and 2019 were used as input observations to inform the DST model. Samples collected in 2012 were not used as model input because sampling was only conducted in the latter half of this year, and samples collected in 2020 – 2022 were removed for the same reasons described in section 2.3.1. Due to a lack of DST tests on *M. edulis* from the San Juan Islands and Saratoga/Whidbey Basin during winter months, samples from these two basins were also removed from the model input.

Process Model. The observed mean log DST concentrations, concentrations, μ , as an additive combination of lag-1 autocorrelation β , month effect γ , and unexplained process variability ω , with subscripts indexing the same quantities as described above. Again, month effects were hierarchical with individual realizations, $\gamma_{m,k}$ drawn from a normal distribution with a shared mean across basins of θ_m but with standard deviation τ^2 fixed at 3 rather than estimated, given the smaller number of basins in the DST model.

Observation Model. In contrast to PST, DST was observed during the great majority of sampling events. Moreover, the limit of quantification of for DSP is quite low – $1 \mu\text{g} / 100 \text{g}$ tissue – and the overall range of DSP observations ($0 - 250\text{ug}/100\text{g}$ shellfish tissue) is more

limited than that of PST ($0 - 30,000+ \mu\text{g}/100\text{g}$ shellfish tissue). For these reasons and for ease of model fitting, observations of DST were set as being present but below the limit of quantification at $1\mu\text{g}/100\text{g}$ tissue, avoiding the need to infer concentration values for these observations.

As for PST, model toxin detection ($z_i = 1$) or non-detection ($z_i = 0$) were again modeled as the outcome of a Bernoulli trial, where the probability of a detection in observation i at time step t in basin k is given as $p_{i,t,k}$. Again, the probability of detection p was a linear function of the underlying toxin concentration μ , using a cloglog link function and a specified low probability of false-positive detection.

As above, the log toxin concentrations from individual observations ($y_{i,t,k}$) were modeled as random samples from a normal distribution, again letting the standard deviation increase with the mean.

The priors for the DSP model were as follows:

$$\beta \sim \text{Beta}(2, 2)$$

$$\omega \sim N(0,1)$$

$$\psi \sim \text{Gamma}(1, 2)$$

$$\theta \sim N(0,5)$$

$$\varphi \sim \text{Gamma}(1,1)$$

Chapter 3. RESULTS

3.1 FREQUENCY OF PST, DST, AND CO-OCCURRENCE EVENTS

Co-occurrence events, when both toxins exceeded the AT during the same month at a given site, were recorded at least once during eight of ten years between 2012 and 2022 (Figure 3; 2020 removed from analysis). The site with the most consistent number of co-occurrence events, observed in 6 out of 10 years sampled, was Squalicum Harbor in the North Puget Sound. Dockton and Poulsbo Marina, both in the Central Puget Sound, each exhibited co-occurrence events in 3 out of 10 years, and Cape George, Sequim Bay, and Portage Bay each exhibited co-occurrence events in 2 out of 10 years. The highest number of co-occurrence events took place in 2012, observed at 11 out of 63 sites. All 11 sites were in the Eastern Strait of Juan de Fuca, Central Puget Sound, or the North Puget Sound. From 2013 to 2021, co-occurrence events were comparatively infrequent, occurring at 0 to 3 sites per year and persisting for a maximum of 2 months per site. In 2022, co-occurrence events took place at 4 out of 82 sites and were observed for the first time in the San Juan Island basin with a co-occurrence event lasting 3 months. No co-occurrence events were documented in Admiralty Inlet, Hood Canal, Saratoga/Whidbey Basin, or South Puget Sound between 2012 and 2022 (Figure 3).

To assess the spatial overlap of sites where toxin events – not necessarily co-occurrence events – were consistently observed, hotspot sites for each toxin, or sites where the toxin exceeded the AT in 5 or more years between 2012 and 2022 (excluding 2020), were identified and compared. Of the 158 sites sampled for PST at least once during this period, 92 sites experienced a PST event, and 26 of those were identified as PST hotspots (Figure 4). Out of 154 sites sampled for DST between 2012 and 2022 (excluding 2020), DST exceeded the AT at least

once at 34 of those sites and 7 were identified as DST hotspots (Figure 4). Of the 7 DST hotspots identified, 6 were also PST hotspots. Toxin events were observed most consistently in the Eastern Strait of Juan de Fuca, with 7 PST hotspots and at least one PST event in 9 of 10 years, and 2 DST hotspots and a DST event in all 10 years examined. Activity of both toxins was similarly consistent in Central Puget Sound and North Puget Sound with 5 PST and 2 DST hotspots, and 4 PST and 2 DST hotspots, respectively. While 7 PST hotspots were located in the San Juan Islands and 3 were located in Admiralty Inlet, no DST hotspots were located in these basins, and only a DST hotspot was located in South Puget Sound. No hotspots for either toxin were located in the Hood Canal or Saratoga/Whidbey Basin.

To understand if PST and DST events were observed at higher rates during the same or different years, the per-year proportion of sites at which PST and DST events were recorded was compared. Both toxins displayed the most widespread number of events in 2012, with PST and DST events recorded at 63.1% of sites sampled (53 out of 84 sites) and 25.4% of sites sampled (17 out of 67), respectively. However, 2014, the year with the second highest percentage of sites exhibiting DST events (19.5%; 16 of 82) was also the year with the least percentage of sites exhibiting PST events (20.4%; 19 of 93). Similarly, the year with the second highest proportion of sites exhibiting PST events (51.7%; 46 of 89), 2017, had a relatively low proportion of sites exhibiting DST events (9.0%; 8 of 89).

Simultaneous detection of both toxins (co-detection at any non-zero concentration) was observed in all basins, and of the 154 sites sampled for both toxins between 2012 and 2022 (excluding 2020), both toxins were detected during the same month at least once in 105 sites (Figure 5). The average proportion of months sampled in which both toxins were detected was highest for the Eastern Strait of Juan de Fuca (57.2% of months sampled) and North Puget Sound

(50.1% of months sampled). In contrast, Saratoga/Whidbey basin (10.3%), Hood Canal (8.6%), and South Puget Sound (8.5%) had the least number of months sampled in which both toxins were detected.

Of the 6,600 site/month/year combinations tested for PSTs, PSTs were detected in 36.5% (n = 2,007) of combinations, and PST events were detected in 9.8% (n = 649), indicating that about one-third of detections were above the action threshold. Of the 6079 site/month/year combinations tested for DSTs, DSTs were detected in 89.9% (n = 5466), while a DST event occurred in only 3.5% (n = 213). Thus, DST presence alone is not a strong predictor of a site's probability of experiencing a DST event, as it may be for PST.

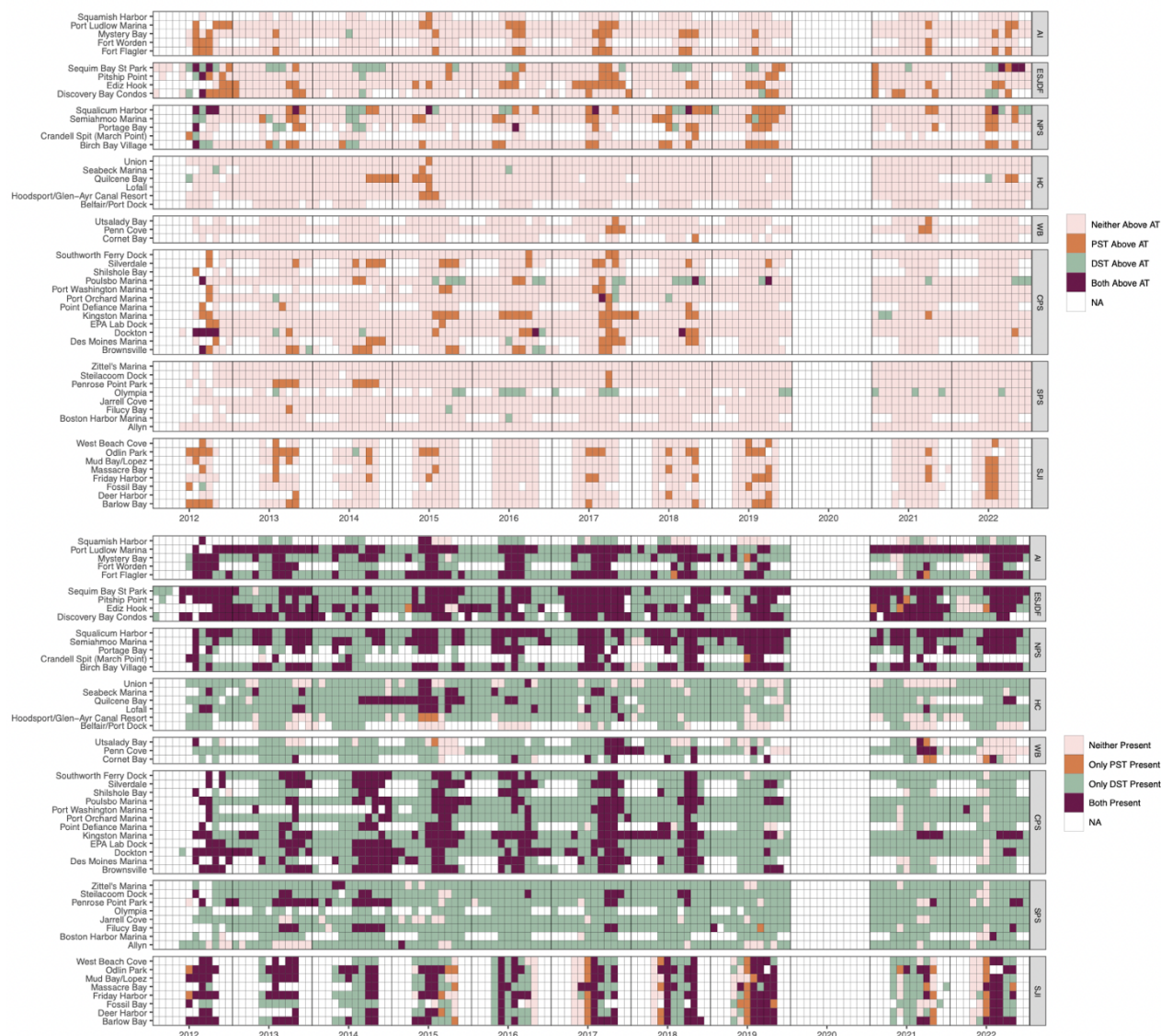


Figure 3. Co-occurrence (top panel) and co-detection (bottom panel) of PSTs and DSTs in a subset of sites which were tested at least once for PST and DST in the same month during all 10 years. Basins are labeled with abbreviations: AI = Admiralty Inlet, ESJDF = Eastern Strait of Juan de Fuca, NPS = North Puget Sound, HC = Hood Canal, WB = Saratoga/Whidbey Basin, CPS = Central Puget Sound, SPS = South Puget Sound, SJI = San Juan Islands.

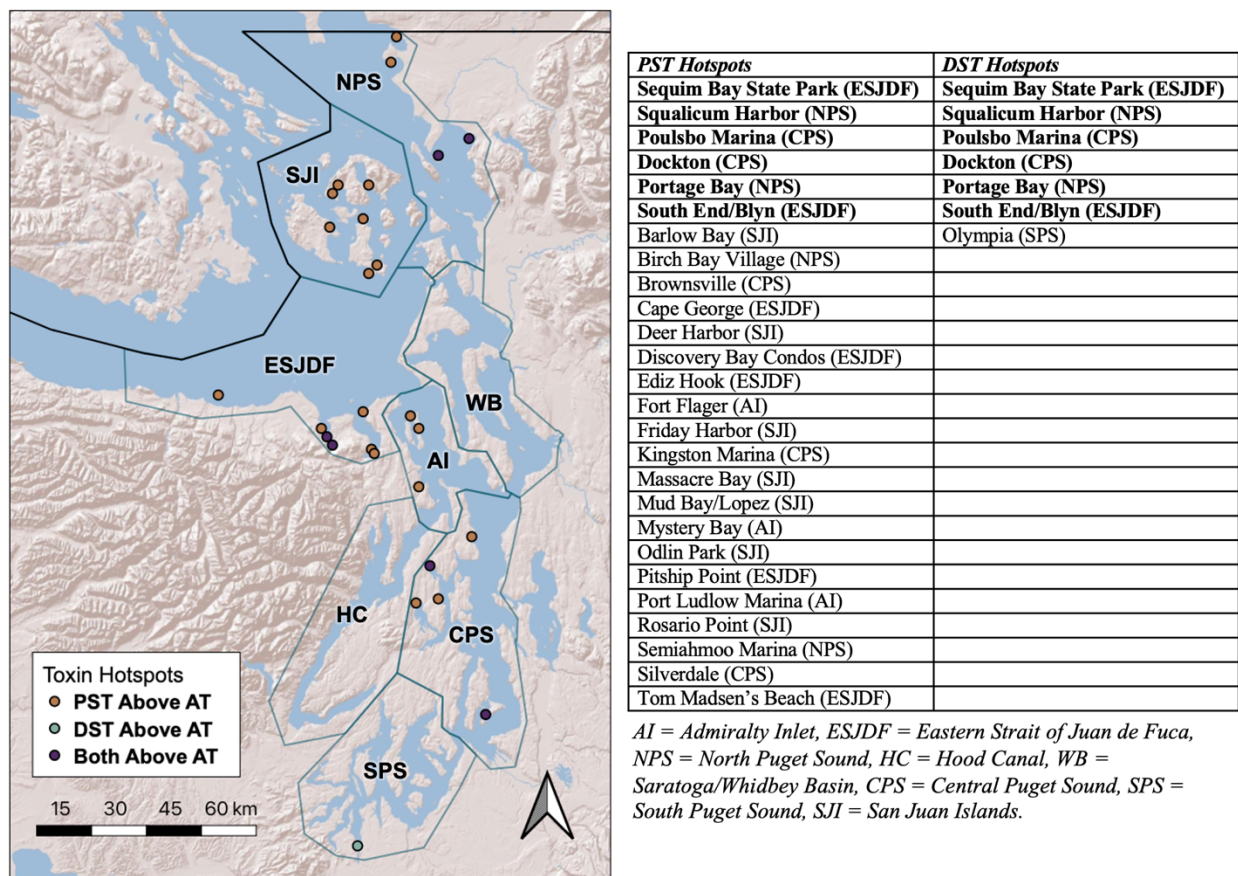


Figure 4. Sites within each basin identified as “hotspots” for either PST, DST, or both toxins. Sites were identified as hotspots if they exceeded their respective action thresholds in at least 5 or more years out of 10 years sampled.

3.2 SEASONAL AND GEOGRAPHIC VARIABILITY UNDERLYING PST AND DST CONCENTRATIONS

3.2.1 Modeled Mean Toxin Concentrations by Basin

The modeled mean concentrations of PST for each month and year from 2013 to 2019 show that PST events differed in frequency and intensity by basin, though all basins exhibited months with means over the action threshold (AT) in at least 1 year (Appendix II, top panel). The modeled mean DST concentrations by month and basin from 2013 to 2019 show that, while DST is consistently present in very low levels in all basins, DST activity tends to be most

variable throughout the year and reach highest concentrations in the Eastern Strait of Juan de Fuca, followed by North Puget Sound (Appendix II, bottom panel).

3.2.2 *Effect of Month on Toxin Concentration*

Across the 8 basins examined, PST concentration appears to be driven by a strong underlying seasonal effect, suggesting a PST ‘season’ in the spring through early fall. For all basins combined, the mean PST concentration explained by month was lowest in January and then increased sharply from February to April (Figure 5, top left). After the April peak, the month effect fell slightly before peaking twice more in July and September, and then gradually decreasing from October to December.

Month effects within individual basins, which were drawn from a normal distribution with a shared mean and standard deviation for each month, varied both in range and shape. North Puget Sound displayed the most distinct bimodal monthly effect, characterized by an earlier peak in April and a later peak in September, both peaks differing significantly from the lowest month (Figure 5, top right). This pattern was also identified for the Hood Canal, though a lower peak in July suggests that PST concentration is less bimodal and rather that concentrations remain elevated throughout the spring to fall months relative to the winter. Central Puget Sound also exhibited a bimodal monthly effect, with a first peak in April and a more prolonged secondary peak from July to August. Finally, the San Juan Islands also displayed a bimodal monthly effect, but the second peak did not differ significantly from any other month, suggesting that this is a weaker pattern. In contrast, Admiralty Inlet displayed more of a unimodal month effect, wherein only one month differed significantly from the lowest month, peaking in April and then decreasing gradually throughout the rest of the year (Figure 5, top right). No month effects differed significantly from one another for the Eastern Strait of Juan de Fuca, Saratoga/Whidbey

Basin, or the South Puget Sound, though higher month effects in the spring to fall months were seen in all basins.

The overall month effects did not differ significantly between any months for the 6 basins included in the DST model, nor did month effects differ significantly from one another within any basin (Figure 5, bottom). However, the effect of month on DST concentration for all basins combined was highest in May through July (Figure 5, bottom left). The Eastern Strait of Juan de Fuca and North Puget Sound displayed the most variability by season, with a peak month effect in June which decreased gradually through the rest of the year (Figure 5, bottom right).

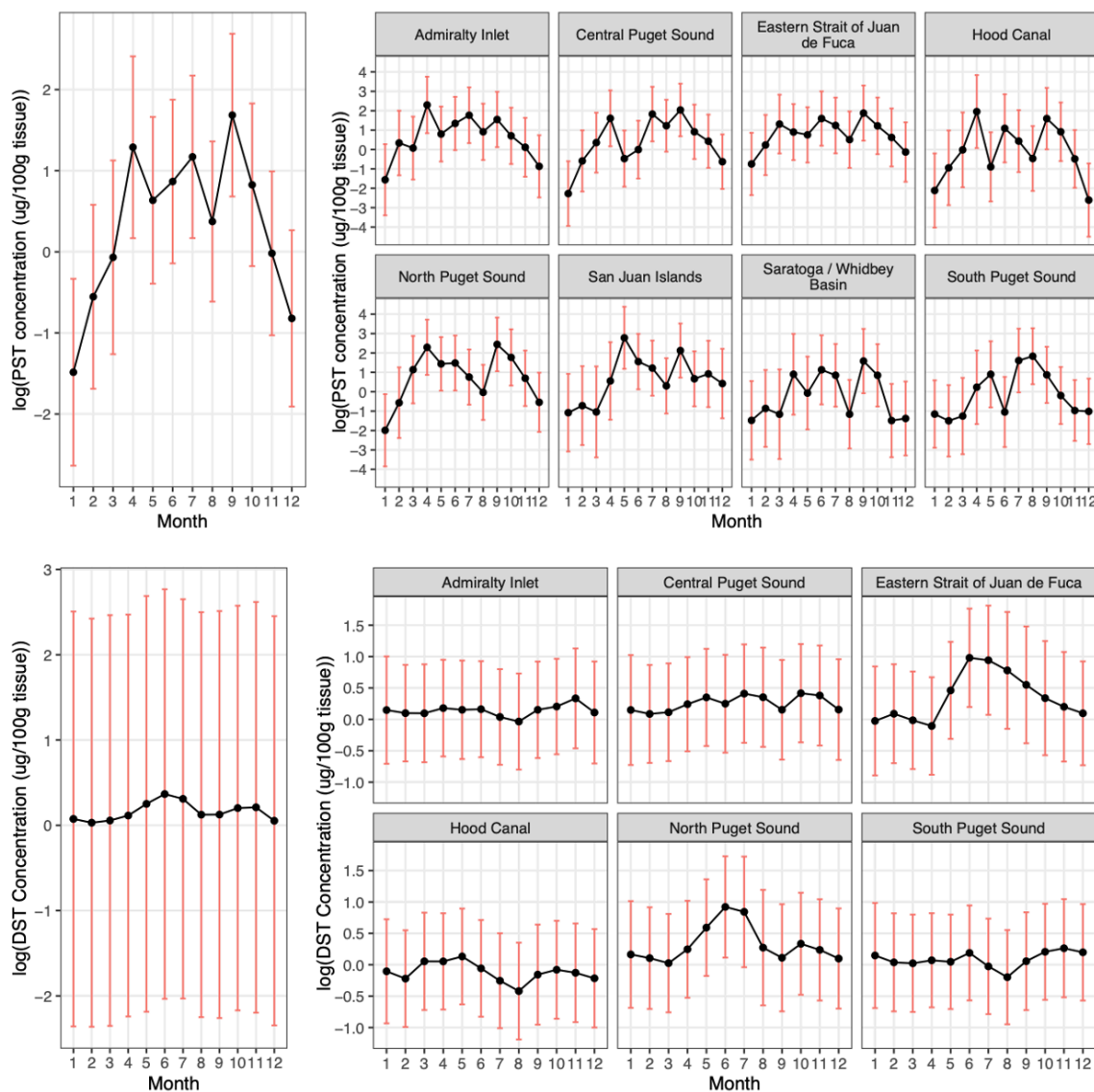


Figure 5. The modeled mean amount of PST and DST concentrations (ug DST/100g shellfish tissue) explained by month for all basins (left panels) and broken down by basin (right panels). Points represent means and error bars represent +/- 2 SD from the mean.

3.2.3

Basin-Specific Autocorrelation of Toxin Concentrations

PST concentrations in all basins were autocorrelated, but the mean toxin concentration explained by autocorrelation differed by basin. Temporal autocorrelation was lowest in North Puget Sound (mean autoregressive correlation coefficient of 0.62), the San Juan Islands (0.63), and Admiralty Inlet (0.63) meaning that observations from these basins were less similar from

any one time point to the next (Figure 6, left). In contrast, PST concentrations in Hood Canal were highly autocorrelated (0.90), meaning that time points tended to be more similar to those preceding them due to the lack of variability in PST activity in most years. The remaining basins displayed levels of autocorrelation similar to one another, with mean autoregressive correlation coefficients ranging between 0.68 and 0.77. DST concentrations in all basins were found to be autocorrelated, but the mean amount of toxin concentration explained by autocorrelation did not differ significantly between basins (Figure 6, right).

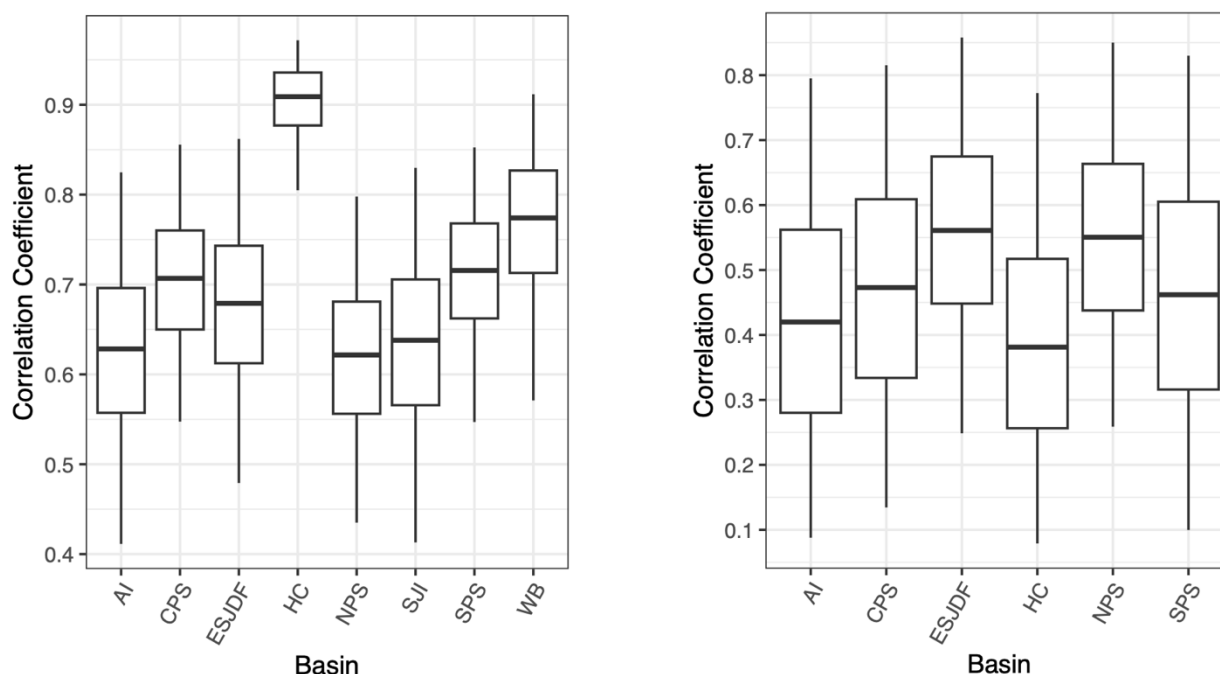


Figure 6. PST (left) and DST (right) autoregressive correlation coefficients for each basin. The midpoint of the box represents the median, the box extents represent the interquartile range (IQR) and the whisker extents represent the 2.5th and 97.5th percentiles.

3.2.4 Mean Residual Variability

The large range of residuals for both the PST and DST models, and non-random patterns that can be observed in the residuals, indicates that other factors not captured here likely have great explanatory power on PST and DST concentrations in most basins. This is especially the case for basins with the greatest ranges of residuals, such as Central Puget Sound, Hood Canal,

Saratoga/Whidbey Basin, and South Puget Sound for PST, and nearly all basins for DST.

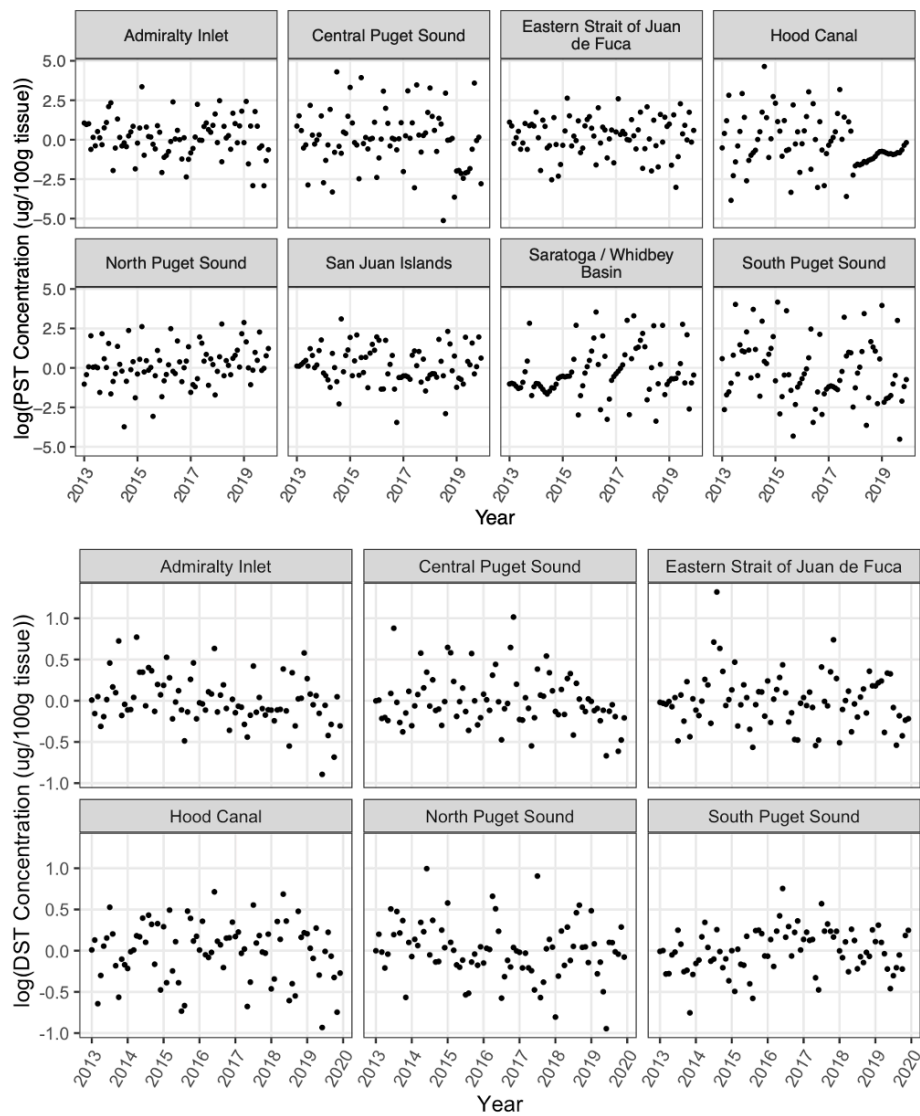


Figure 7. Residual variability for PST (top) and DST (bottom) on a log scale for every month and year for each basin.

Chapter 4. DISCUSSION

The geographic and temporal distribution of PST and DST concentrations between 2012 and 2022 reveal distinct patterns underlying each toxin which appear to facilitate co-occurrence events in several basins and years but preclude co-occurrence events in others. The Eastern Strait of Juan de Fuca, Central Puget Sound, and North Puget Sound contained hotspot sites for both toxins, while the San Juan Islands, Admiralty Inlet, and South Puget Sound only contained hotspots for one toxin, suggesting that PST and DST are driven by distinct environmental conditions that do not consistently overlap in space and time. Spring to fall months had a greater effect on PST and DST concentrations than winter months. PST concentrations remained elevated throughout the spring and summer and peaked in early fall, while DST concentrations peaked in mid-summer and fell more rapidly, though a second peak in late fall was also observed. However, modeled month effects differed geographically, so these trends were not observed in all basins. Additionally, co-detection at low levels underscores the ubiquitous presence of both toxins across all basins, albeit often at low levels and with varying seasonal patterns. While PST typically fell to undetectable levels in the winter months, DST was detected at low levels in almost 90% of samples, regardless of season. However, PST was much more likely to exceed the AT than DST when present. These findings highlight that unique patterns underly PST and DST concentrations throughout the Puget Sound, suggesting that a greater understanding of their individual dynamics may inform more customized management plans for each toxin. Furthermore, consistent co-occurrence events in several basins and a prolonged co-occurrence event in another basin in 2022 emphasizes the need for additional research into the human health effects of co-toxicity with PST and DST.

4.1 WHERE WERE PST, DST, AND CO-OCCURRENCE EVENTS OBSERVED, OR NOT OBSERVED, IN THE PUGET SOUND DURING THE FIRST DECADE OF CO-MONITORING?

PST, DST, and co-occurrence activity was consistently observed at sites throughout the Eastern Strait of Juan de Fuca, North Puget Sound, and Central Puget Sound from 2012 to 2022. Six sites within these three basins were identified as hotspots for both PST and DST, indicating that the environmental conditions of these sites are especially conducive to biotoxin production and accumulation. While environmental conditions were not considered in this study, further research should investigate commonalities between these sites such as oceanography, water chemistry, or phytoplankton community composition. Though no studies have surveyed the long-term distribution of DST in the Puget Sound, sites in the Eastern Strait of Juan de Fuca and Central Puget Sound were previously identified as PST hotspots using earlier decades of data (Moore et al., 2009), suggesting geographic continuity of PST hotspots once established. The non-adjacent nature of these basins suggests that transport of toxins between basins is not a main driver of toxin abundances, and rather that resident source populations of cells in each of these basins exist in the sediment as resting cysts for PST (Dyhrman et al., 2010) or in the water column or sediment for DST (Reguera et al., 2012).

In contrast to these three basins, Saratoga/Whidbey Basin and Hood Canal were distinguished by their low frequency of toxin events. No DST events were reported for Saratoga/Whidbey Basin and only a few isolated DST events were reported from Hood Canal, which were restricted to three sites in the northern portion of the basin – Seabeck Marina, Brinnon, and Quilcene Bay. PST events in both basins were infrequent, with event years often separated by multiple event-free years. In the PST model, Hood Canal showed the highest degree

of autocorrelation, consistent with the finding that PST levels do not shift greatly from one time point to the next. Both of these basins appear to be characterized by multi-year stretches with little or no PST activity, punctuated by sudden and extreme, yet short-lived, PST events. Interestingly, despite infrequent PST events and low PST activity overall, the highest concentrations of PST ever recorded in the Puget Sound were detected in 1978 in Whidbey Basin, when 30,360 μ g PST/100g shellfish tissue was detected at Holmes Harbor, and similarly extreme levels were detected simultaneously at at least 6 other sites within the basin. Similarly, the highest PST concentration observed during a given month between 2012 and 2022 occurred in Hood Canal, when 12,688 μ g PST/100g shellfish tissue was detected in samples from Quilcene Bay in September 2014. The continuation of this punctuated pattern between 2012 and 2022 offers supporting evidence for the current management approach of consistent PST monitoring at sites of commercial and recreational importance, such as Penn Cove, since PST events occur at unpredictable intervals with rapid onset.

Other basins displayed different patterns for each toxin, diverging from the pattern seen in the aforementioned five basins, where toxin activity is either high or low for both toxins. While sites in Admiralty Inlet exhibited low DST concentrations, and only one DST event between 2012 and 2022, frequent PST activity was recorded in this basin and 3 sites were identified as PST hotspots. Annual PST events were observed in the San Juan Islands, which contained 7 PST hotspots, while DST events in this basin only occurred in 3 of 10 years and were short-lived. However, the San Juan Islands first experienced a co-occurrence event in 2022, lasting three months at Rosario Point. This event suggests that co-occurrence is not restricted only to basins with frequent events in both toxins and supports the need for ongoing monitoring

of both toxins at all sites where elevated levels of toxin have been observed in the past, even if infrequently.

South Puget Sound rarely experienced PST events, but DST events were consistently observed at one site. Olympia, a site within the far-south Budd Inlet in western South Puget Sound, was identified as a DST hotspot and was the only site that was identified as a DST hotspot but not a PST hotspot. Interestingly, PST activity has been very low at this site – despite frequent monitoring, the last time it was detected was in 2002 at the low level of $41\mu\text{g}/100\text{g}$ shellfish tissue. In contrast, DST events at this site occurred in 24 out of 30 months tested between October 2015 and March 2018, with a maximum detection of $250\mu\text{g}/100\text{g}$ shellfish tissue in June 2016 – the highest DST value recorded in this dataset. A survey of *Dinophysis* spp. and toxin profiles identified three *Dinophysis* species at Olympia, two of which were characterized by high per-cell toxin concentrations (Ayache et al., 2023). The presence of multiple DST-producing species at this site, perhaps each able to utilize different resources to sustain toxin abundance, may contribute to the relatively high DST concentrations, though further research is required to assess this. The uniquely low PST activity and uniquely high DST activity seen at Olympia, as well as the high PST activity and low DST activity in the San Juan Islands and Admiralty Inlet, raise the question of whether distinct environmental conditions or biological interactions at these sites favor the presence of one toxin and/or limit the survivability of the other.

4.2 DO TEMPORAL TRENDS UNDERLYING PST AND DST EVENTS FACILITATE OR PRECLUDE CO-OCCURRENCE AT SITES OF GEOGRAPHIC OVERLAP?

Co-occurrence events require that toxins accumulate not only at the same place, but at the same time, requiring synchronous seasonal and interannual patterns in PST and DST. On an

annual scale, synchronous events in 2012 initially indicated a potential common driver or environmental trigger for both toxins. However, discrepancies observed in subsequent years, such as in 2014 when the lowest proportion of sites exhibiting PST events coincided with the second highest proportion of sites exhibiting DST events, do not support the idea of a common environmental driver. This lack of a consistent correlation between the proportion of sites exhibiting PST and DST events on an interannual basis, which was also documented in other studies throughout North America (T. K. Hattenrath-Lehmann et al., 2013), means that the annual likelihood of co-occurrence events is unpredictable.

In most basins, the concentration of both toxins was seasonal, resulting in a greater frequency of toxin events and elevated toxin concentrations in the spring through fall compared to winter month. The modeled mean effect of month on PST concentration throughout the study area was greatest from April through October, with peaks in April, July, and September. These results identify an earlier peak PST season than described by Trainer et al. (2003), who documented that the most closures during each decade from the 1950s to the 1990s occurred from July to November. These findings support the continuation of Moore et al. (2009)'s finding of a trend towards shellfishery closures due to PST occurring earlier in the year. While no modeled mean month effects on DST concentration differed significantly from one another, the highest month effects were identified in May through July and October through November, with the greatest month effect in June. While generally, concentrations of both toxins increase during the spring to fall months, basin-specific month effects lead to different implications for co-occurrence.

Temporal non-overlap of PST and DST appeared to preclude co-occurrence on several occasions at several sites within North Puget Sound, Central Puget Sound, and the Eastern Strait

of Juan de Fuca. Most notably, the modeled month effects for the North Puget Sound identify a bimodal effect for PST and a unimodal effect for DST, suggesting that PST events and DST may not overlap. Indeed, this pattern, wherein two separate PST events occurred in the same year, separated by a mid-summer DST event, was observed at Semiahmoo Marina in 2013 and 2019, Birch Bay Village in 2013, and Portage Bay in 2018 (see Figure 3, top). In Central Puget Sound, though both toxins displayed unimodal trends, an earlier PST event followed a later DST event also precluded co-occurrence on several occasions. This was observed at Shishole Bay in 2016 and 2017, Port Washington Marina in 2016, Brownsville in 2013 and 2016. At Sequim Bay State Park, in the Eastern Strait of Juan de Fuca, there were 5 years in which events for both toxins were recorded but a co-occurrence event was never observed due to asynchronous peaks (see Figure 3, top). However, temporal overlap was sometimes observed, resulting in co-occurrence events that typically lasted only one month and were preceded and/or proceeded by a single-toxin event. In general, temporally and spatially non-concurrent PST and DST events were observed more often than co-occurrence events, though co-occurrence events were still detected consistently in these three basins.

4.3 GENERAL DIFFERENCES BETWEEN PST AND DST

A notable distinction between PST and DST is the frequency at which the toxin is detected in low levels versus the frequency at which the toxin exceeds its action threshold. DST was detected 89.9% of the time it was tested for but was only found above the action threshold 3.5% of the time. PST, however, was only detected in 33.1% of tests, but was found above its action threshold in 10.1% of the tests. As seen in Appendix I, DST tends to be present at low levels all the time, while PST tends to disappear and reappear on an annual basis but is more likely to exceed the action threshold when it is present. This difference may be due to the mechanism

through which the algal species produces toxins, and whether toxin production is constant or responsive to environmental signals. A survey found that DST profiles from regional *Dinophysis* strains tended to be conserved and suggested that strain type may be more predictive of toxin production than environmental conditions (Ayache et al., 2023). Furthermore, the authors found that *D. acuminata* and *D. fortii* strains from the Puget Sound region had the highest DST concentrations per-cell of all strains surveyed, and that toxins were produced even at low cell abundances, which may explain why DST is detected throughout the region even in winter months. Contrastingly, Dyhrman et al. (2010) found that it was not uncommon for abundant *A. catenella* cells to be detected in the absence of PSTs in the Puget Sound. Another difference between *Alexandrium spp.* and *Dinophysis spp.* which may contribute to the observed pattern is that *Alexandrium spp.* overwinter as resting cysts in the sediments, while the overwintering mechanism of *Dinophysis spp.* is unresolved and cysts are not thought to play a critical role in reseeding annual cell populations (Reguera et al., 2012). Regardless of mechanism, our findings suggest that DST presence is not strongly associated with a greater likelihood of DST events, while PST is.

The addition of DST events as a mechanism which can prompt shellfish harvesting closures throughout the Puget Sound has not only increased management and monitoring burden for WDOH but has most likely also increased the length of time each year during which areas are closed to shellfish harvesting (Ayache et al., 2023; T. K. Hattenrath-Lehmann et al., 2013). This was demonstrated at Squaticum Harbor in the North Puget Sound wherein non-overlapping, back-to-back toxin events resulted months-long closures in 2014, 2018, and 2022. Furthermore, areas which were previously unaffected by biotoxin-induced shellfish harvesting closures may now be susceptible due to varying geographic patterns between DST and PST, as was the case at

Olympia in the South Puget Sound. While most of the sites where DST events occur have been long-term hotspots for PST events, local health departments and shellfish harvesters in areas previously unaffected by biotoxins may be unprepared to navigate DST events.

The large residual process variability of both the PST and DST models suggests that other, non-seasonal factors that were not captured here have explanatory power on both PST and DST concentrations. Nutrient availability, specifically nitrogenous nutrients, has been identified as a factor affecting of both *Dinophysis spp.* and *Alexandrium spp.* cell accumulation and toxicity (Hattenrath et al., 2010; T. K. Hattenrath-Lehmann et al., 2015). Warm air and water temperatures, low winds, low streamflow, and small tidal variability have all been proposed as elements that play a role in the initiation of PST events (Moore et al., 2009, 2011). Upwelling processes, thermal stratification, water temperature, and prey availability are factors which have been hypothesized to play a role in *Dinophysis spp.* growth, bloom development, and/or toxicity (Gao et al., 2019; Reguera et al., 2012; Trainer et al., 2013). The concentration of PST and DST hotspots in certain basins, and the large residual variability observed in my models, underscores the localized nature of toxin dynamics and highlights the need for more research into the shared and/or distinct factors that drive toxin concentrations and may contribute to co-occurrence events.

Chapter 5. CONCLUSIONS

Co-occurrence between PSTs and DSTs does occur in the Puget Sound and neighboring basins, particularly in the three basins with the highest levels of toxin activity overall – the Eastern Strait of Juan de Fuca, North Puget Sound, and Central Puget Sound. Despite the spatial and temporal overlap between the toxins in these basins, PST activity was high in the San Juan Islands and Admiralty Inlet, where DST activity was low. In South Puget Sound, a DST hotspot exists in the southernmost portion of the basin, where PST has been largely undetectable for the last two decades, indicating unique environmental drivers for each toxin. Hood Canal and Saratoga/Whidbey Basin are characterized by their exceptionally low toxin activity, with few, isolated toxin events recorded. The absence of hotspots in these basins suggests that environmental conditions here may mitigate toxin production or accumulation. The seasonal non-overlap of the two toxins seems to preclude co-occurrence during some years in some basins, such as North Puget Sound and Central Puget Sound, but back-to-back rather than synchronous toxin events are also of concern since they result in lengthy shellfish harvesting closures. My results also identified differences between PST and DST related to the relationship between toxin presence at low levels and toxin events, perhaps due to variations in species' mechanisms of toxin production and life history. As highlighted by this study, co-occurrence is a phenomenon observed in the Puget Sound and further research into the human health impact of co-exposure to PSTs and DSTs should be undertaken in order to evaluate the strength of the current regulatory action thresholds under co-occurrence conditions.

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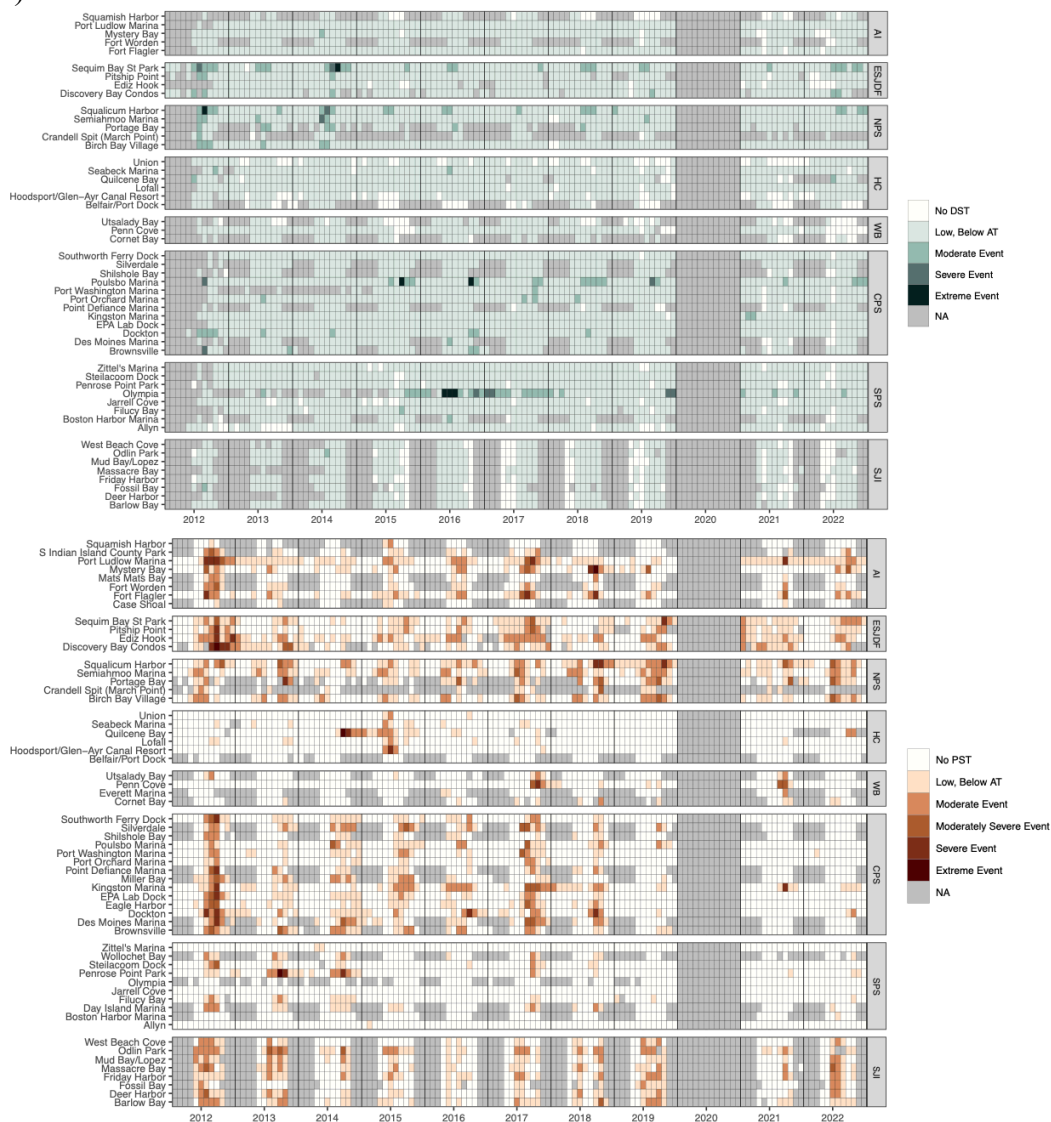
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APPENDIX

APPENDIX I

PST (top panel) and DST (bottom panel) in a subset of sites which were tested at least once per year (2012 to 2022, excluding 2020). Basins are labeled with abbreviations: AI = Admiralty Inlet, ESJDF = Eastern Strait of Juan de Fuca, NPS = North Puget Sound, HC = Hood Canal, WB = Saratoga/Whidbey Basin, CPS = Central Puget Sound, SPS = South Puget Sound, SJI = San Juan Islands. Toxin values for PST are: $0\mu\text{g}$ (No DST), $1\text{-}79\mu\text{g}$ (Low, below AT), $80\text{-}499\mu\text{g}$ (Above AT, Moderate), $500\text{-}999\mu\text{g}$ (Above AT, Moderately Severe), $1000\text{-}4999\mu\text{g}$ (Above AT, Severe), $5000+\mu\text{g}$ (Above AT, Extreme). Toxin values for DST are: $0\mu\text{g}$ (No PST), $1\text{-}15\mu\text{g}$ (Low, below AT), $16\text{-}74\mu\text{g}$ (Above AT, Moderate), $75\text{-}149\mu\text{g}$ (Above AT, Severe), $150+\mu\text{g}$ (Above AT, Extreme).



APPENDIX II

Modeled monthly means of PST concentrations (top) and DST concentrations (bottom) by month from 2013 to 2019. Means were converted from log scale to natural scale. The horizontal red line represents the toxin's action threshold ($80\mu\text{g}$ per 100g shellfish tissue for PST; $16\mu\text{g}$ per 100 grams of shellfish tissue for DST).

