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Trends in *Dinophysis* Abundance and Diarrhetic Shellfish Toxin Levels in California Mussels (*Mytilus californianus*) from Monterey Bay, California

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Abstract

Diarrhetic shellfish toxins (DSTs) are produced by the marine dinoflagellate, *Dinophysis*, as well as select species of benthic *Prorocentrum*. The DSTs can bioaccumulate in shellfish and cause gastrointestinal illness when humans consume high levels of this toxin. Although not routinely monitored throughout the U.S., recent studies in Washington, Texas, and New York suggest DSTs may be widespread throughout U.S. coastal waters. This study describes a four-year time series (2013-2016) of *Dinophysis* concentration and DST level in California mussels (*Mytilus californianus*) from Santa Cruz Municipal Wharf (SCMW) in Monterey Bay, California. Results show a maximum *Dinophysis* concentration of 9,404 cells/L during this study and suggest *Dinophysis* persists as a member of the background phytoplankton community throughout the year. In California mussels, DSTs were found at persistent low levels throughout the course of this study, and exceeded the FDA guidance level of 160 ng/g 19 out of 192 weeks sampled. Concentrations of *Dinophysis* alone are a positive but weak predictor of DST level in California mussels, and basic environmental variables (temperature, salinity, and nutrients) do not sufficiently explain variation in *Dinophysis* concentration at SCMW. This study demonstrates that *Dinophysis* in Monterey Bay are producing DSTs that accumulate in local shellfish throughout the year, occasionally reaching levels of concern.

Keywords: *Dinophysis*; Diarrhetic shellfish toxin; Okadaic acid; Monterey Bay; Dinoflagellate; Harmful algal bloom
1. Introduction

Harmful algal blooms (HABs) include any phytoplankton event that negatively impacts human health, socioeconomic interests, or aquatic ecosystems (Anderson et al., 2012). Over the past several decades, negative economic and ecosystem impacts of HABs have been increasingly observed worldwide (Hallegraeff, 1993; Anderson et al., 2012). The majority of toxin-producing HABs in marine waters are caused by dinoflagellates, and include several well-documented syndromes such as paralytic shellfish poisoning (PSP), neurotoxic shellfish poisoning (NSP), Ciguatera fish poisoning (CFP), and diarrhetic shellfish poisoning (DSP; Smayda, 1997; Burkholder, 1998; FDA, 2011). The illness DSP is caused by a suite of lipophilic algal toxins referred to as diarrhetic shellfish toxins (DSTs). The suite of DSTs include okadaic acid (OA) and its analogues: dinophysistoxin 1 (DTX-1) and dinophysistoxin 2 (DTX-2; Reguera et al., 2014). When high levels of DSTs bioaccumulate in seafood and are consumed by humans, they cause nausea, vomiting, diarrhea, abdominal pain, headache, and fever, all of which generally pass within a few days (Cohen et al., 1990; Cordier et al., 2000; FDA, 2011). In addition to causing gastrointestinal illness, okadaic acid has been demonstrated to promote tumors in rodents (Fujiki and Suganuma, 1999).

Diarrhetic shellfish toxin production and contamination in shellfish is mainly associated with toxigenic species within the dinoflagellate genus *Dinophysis* (Yasumoto et al., 1980; Yasumoto et al., 1985; Reguera et al., 2014). In addition, multiple benthic species of *Prorocentrum* have also been found to produce DSTs (Lee et al., 1989; Dickey et al., 1990; Marr et al., 1992) Historically, additional toxins were grouped together with OA, DTX-1 and DTX-2 as DSTs. This included yessotoxins (YTX) produced by the
dinoflagellates *Protoceratium*, *Gonyaulax*, and *Lingulodinium*, which have been associated with HAB issues in California (De Wit et al., 2014), but evidence suggests that these toxins should be excluded from the DST group since YTX does not cause symptoms similar to other compounds in the DST group (Ogino et al., 1997; Tubaro et al., 2010). It has also been suggested that pectenotoxins (PTX), another group of lipophilic toxins often linked with DSTs, be excluded from the DST group, as their mechanisms of action differs from DSTs, and PTXs have not been linked with human intoxication (European Food Safety Authority, 2008; FAO/WHO, 2016).

The most common cause of DSP in humans is consumption of contaminated shellfish, especially mussels, but large-scale DSP outbreaks have also been associated with consumption of other types of seafood, such as brown crabs (*Cancer pagurus*; Reguera et al., 2014; Torgensen et al., 2005). The regulatory limit for DSTs in Europe is 160 ng OA equivalents (combined okadaic acid, dinophysistoxins, and pectenotoxins) per g shellfish meat (=160 ng/g; Regulation (EC) No 853/2004; O’Mahony, 2018), and the regulatory limit in China and Australia is 200 ng/g (Reguera et al., 2014, FSANZ, 2015). The U.S. Food and Drug Administration (FDA) recommends that all shellfish products with DSTs (combined free okadaic acid, dinophysistoxins, and acyl-esters of okadaic and dinophysistoxins) measuring above 160 ng/g be removed from the market (FDA, 2011).

Some of the earliest documentation of DST detected in North American shellfish occurred along the East Coast of Canada. A study published in 1989 detected okadaic acid in plankton tows from the Gulf of Saint Lawrence (Cembella, 1989). In August 1990, 469 ng/g okadaic acid was measured in scallop digestive gland from Bedford Basin (Subba Rao et al., 1993). That same month, DTX-1 reaching 1000 ng/g in edible
cultured mussel tissue was associated with 13 illnesses in Nova Scotia, Canada (Quilliam et al., 1993). These studies generally found correlations to D. norvegica, D. acuminata or P. lima either in the water column or in shellfish gut analysis. A study by Marr et al. in 1992 isolated P. lima off the Atlantic Coast of Nova Scotia and confirmed the production of okadaic acid and DTX-1 in the isolates. While these studies detected relatively high levels of DST, other studies along U.S. coasts found low DST content in shellfish (Maranda and Shimizu, 1987; Morton et al., 1999), and low toxicity in Dinophysis strains (Morton et al., 1999; Hackett et al., 2009). The findings of low toxicity Dinophysis and low levels or no DST in shellfish, combined with a lack of clinical reports of DSP prior to 2011 (Trainer et al., 2013) has resulted in DSTs often being overlooked as a public health hazard in the U.S.

While not the first studies to find DSTs in North American shellfish, multiple recent studies have documented high levels of DST and brought increased attention to this toxin in the U.S. In 2008, the first shellfish harvesting closure as a result of DSTs in the U.S. occurred in Texas. High concentration of DST was found in oysters (Crassostrea virginica) with a maximum concentration of 470 ng/g, almost three times the FDA guidance level of 160 ng/g (Deeds et al., 2010). Oyster harvesting in Texas has been closed multiple times since this first event (Texas Health and Human Services, 2014a, 2014b). In the summer of 2011, high levels of DST in shellfish in the Pacific Northwest led to three illnesses in Washington State, marking the first clinical report of DSP in the U.S., and 62 illnesses in British Columbia, Canada (Trainer et al., 2013; Taylor et al., 2013). During this event, a maximum concentration of 1603 ng/g DST was measured in blue mussels from Washington, resulting in a shellfish harvesting closure.
(Trainer et al., 2013). The maximum DST concentration in mussels from British Columbia during the same time period were found to have a maximum concentration of 860 ng/g DST (Taylor et al., 2013). Following this event, Washington State has included DSTs in their routine biotoxin monitoring program (WDOH website, 2019). In the summer of 2011 in New York, non-commercially harvested mussels were found to have DST over 7 times the FDA guidance level with a maximum concentration of 1245 ng/g (Hattenrath-Lehman et al., 2013). A recent study in San Francisco Bay, California found mussel tissue collected in 2015 to have a maximum DST concentration over 400 ng/g (Peacock et al., 2018). Most recently, in Eastham, Massachusetts, a shellfish harvesting closure during the summer of 2015 occurred as a result of DST contamination (MDMF, 2015). These recent findings demonstrate the need to better understand and measure DST occurrence in all U.S. coastal waters.

The genus *Dinophysis* has historically been recorded as a member of the phytoplankton community in Monterey Bay and along California’s coastline (Jester et al., 2009; Southern California Coastal Ocean Observing System, 2017), yet little is known about the ecology of *Dinophysis* in California’s coastal waters. Multiple factors make *Dinophysis* difficult to study. In Monterey Bay, *Dinophysis* represents a small portion of the phytoplankton population, and does not form dense blooms in surface waters like other dinoflagellate genera, such as *Margalefidinium fulvum* (previously *Cochlodinium fulvum*), *Ceratium spp.*, and *Akashiwo sanguinea* (Ryan et al., 2009). There are also no documented occurrences of DSP in humans within California, although given the symptoms, it is possible that mild DSP events have gone unrecognized. As a result, *Dinophysis* has not attracted attention to the same extent as other toxic
dinoflagellates that have large bloom events and discolor local waters. In addition, *Dinophysis* was not successfully cultured in a laboratory until 2006, when it was found to be a mixotroph requiring a three member trophic chain, where *Dinophysis* obtains chloroplasts by first feeding on the ciliate *Mesodinium rubrum* (previously *Myrionecta rubra*) that itself has fed on the cryptophyte from the *Teleaulax/Plagioselmis/Geminigera* clade (Johnson et al., 2006; Park et al., 2006; Peltomaa and Johnson, 2017; Hernández-Urcera et al., 2018; Smith et al., 2018). This trophic chain continues to make studying *Dinophysis* in culture a challenge.

Four toxic species of *Dinophysis* and one potentially toxic species of *Phalacroma* (previously belonging to the genus *Dinophysis*) have been recorded in samples from Santa Cruz Municipal Wharf in the past twenty years: (*D. acuminata*, *D. caudata*, *D. fortii*, *D. tripos*, and *P. rotundatum* (previously *D. rotundata*; Weber, 2000; Sutherland, 2008). A study by G. Carl Schrader in 1981 found three additional toxic species, *D. acuta*, *D. norvegica*, and *D. ovum*, in Monterey Bay phytoplankton samples. In the summer of 1999, *Dinophysis* cells from Monterey Bay were determined to contain OA and DTX-1 (Weber, 2000). In 2004-2005, California mussel tissue tested for DSTs were positive for both OA and DTX-1 at low levels (Sutherland, 2008). To date, these studies of DST in Monterey Bay have not been published in the peer-reviewed literature, and a baseline of DST level in shellfish of central California marine waters has not been established. In this study, three main questions were proposed to improve the understanding of *Dinophysis* and DSTs in Monterey Bay. First, what levels of toxin were present in local shellfish during the study period at Santa Cruz Municipal Wharf (SCMW) and what were the concurrent concentrations of *Dinophysis*? Second, to what
degree did genus-level *Dinophysis* measurements relate to DST levels in shellfish?

Lastly, what environmental conditions were most associated with local populations of *Dinophysis*?

## 2. Methods

### 2.1 Sampling site and sample collection

Data used in this study were collected weekly at SCMW (36.9573°N, 122.0173°W), from 2013-2016. Phytoplankton and environmental data originated from two methods of collection — a depth integrated whole water sample and a vertical net tow sample. For the depth integrated sample, a Niskin bottle was used to collect equal volumes of water at 0, 1.5, and 3 meters depth, which were then mixed together in a plastic container. To collect a net tow sample, a 20 μm mesh phytoplankton net was vertically dragged through 15.24 m of water (dropped to 3.05 m, then, pulled to the surface 5 times), following standard methods employed by the California Department of Public Health (CDPH) monitoring program.

### 2.2 *Dinophysis* analyses

Cell counts were conducted by settling 50 mL of depth integrated whole water (preserved with Lugol’s iodine solution) in an Utermöhl settling chamber. Counts were done on a Zeiss Axiovert 200 inverted microscope. The entire slide was counted for a majority of samples (N=146), with a detection limit of 20 cells/L. When phytoplankton biomass was unusually high, such as during a bloom, 10 random fields of view were selected for enumeration, resulting in a detection limit of 600 cells/L. This counting method was applied to 54 samples, most of which contained *Dinophysis*. Eleven of these samples had zero *Dinophysis*, but because of the high limit of detection, these eleven
samples were removed from all time series plots and time series analyses of genus-level
_Dinophysis_ data. For samples from 2013-2014, _Dinophysis_ cells were identified to
species. The classification _D. acuminata_ complex was used to include the species _D.
acuminata, D. ovum, and D. sacculus_, which are difficult to distinguish morphologically
using light microscopy (Raho et al., 2008). Species-level _Dinophysis_ concentration data
from 2013-2014 was grouped to genus-level concentrations for statistical comparisons
with DST concentration in mussel tissue to maintain continuity with the rest of the time
series data. The use of ‘_Dinophysis_ concentrations’ in this paper refers to genus-level,
total _Dinophysis_ cell concentrations. The species _Phalacroma rotundatum_ (formerly
_Dinophysis rotundata_) was included in all genus-level _Dinophysis_ concentrations
throughout this study.

Presence/absence of _Dinophysis_ was determined from the net tow sample. A
small portion of the sample (~5 mL) was examined each week on the day of collection
using a Leica MZ 12.5 dissecting microscope. Relative abundance was determined for
each genus present. Relative abundance index (RAI) observations categorized each
genera of phytoplankton by the percent it made up of the whole phytoplankton
community (Jester et al., 2009). The categories were: absent (0%), rare (<1%), present
(1-10%), common (10-50%), and abundant (>50%). For this study, _Dinophysis_ RAI data
was binned into categories of absent (0%) or present (>0%).

### 2.3 Diarrhetic shellfish toxin analyses

California mussels (_Mytilus californianus_) were collected weekly from SCMW as
part of the CDPH Biotoxin Monitoring Program. Santa Cruz does not have any
commercial shellfish growing areas; however mussel beds along this area of the coast are
open to recreational mussel harvesting, except during the annual closure (May 1 – October 31; CDPH website) The mussels deployed at SCMW were initially collected from the intertidal zone at Davenport Landing Beach, put into mesh bags of approximately 30 mussels per bag and maintained for various durations in a flowing seawater table of sand filtered water (30 μm pore size) at University of California, Santa Cruz Long Marine Laboratory. These bags were deployed off a platform at SCMW for at least one week. Each week, one bag of mussels was removed from the wharf and brought into the laboratory for processing. The mussels were not tested for DSP toxin prior to deployment at the wharf, and may have been exposed to DSTs at Davenport Landing Beach.

In the laboratory, mussels were shucked and all tissues from 20-30 mussels, except for the white fibrous muscle tissue, was removed, drained with a colander, and homogenized using a Waring Xtreme Hi-Power Blender. Homogenized tissue was frozen at -20 °C until analysis. A 2 g aliquot of this tissue homogenate was extracted and hydrolyzed following a slightly modified version of the methods described by Villar-Gonzalez et al. (2008). The tissue homogenate was extracted by adding 18 mL 100% MeOH, followed by vortexing, homogenization, centrifugation, and separation of the homogenate, while Villar-Gonzalez et al. 2008 extracted in two steps with 9 mL 100% MeOH for each step, followed by combination of the two extracts (18 mL total).

Hydrolyzed extracts were analyzed on an Agilent 6130 quadrupole liquid chromatography-mass spectrometer (LC-MS) with Select Ion Monitoring (SIM) in negative mode using an Agilent Poroshell 120 SB-C18, 2.1x50mm, 2.7μm (with 1.7μm solid core) particle size column with matching guard column. A gradient elution
(modified from Louppis et al., 2010) started with 95% water with 2 mM ammonium formate and 50 mM formic acid (A) and 5% acetonitrile with 50 mM formic acid (B) for 1 minute, then to 60% A at 6 minutes, and 5% A at 8 minutes, held until 11 minutes before returning to initial conditions. Injection volume was 50 µL and flow rate was 0.85 mL/min. Okadaic acid, DTX-1, and DTX-2 were monitored using masses 803.5 (OA, DTX-2) and 817.5 (DTX-1). Quantification was based on mass and time, with an external standard curve using certified reference material from NRC-Canada. Minimum Detection Limits (MDL) were 0.5, 0.75, and 1.0 ng/mL on-column, equivalent to 5.0, 7.5, and 10.0 ng/g tissue. A chromatogram of the certified reference material standards for okadaic acid, DTX-1, and DTX-2 is provided in Fig. 1.

As previously noted, DSTs are produced by both pelagic Dinophysis and benthic Prorocentrum. While mussels growing in beds on the benthos might be regularly ingesting resuspended benthic phytoplankton (Muschenheim and Newell, 1992), in this experimental design, the degree of spatial separation between mussels deployed on a rope suspended in the water column compared to the benthos suggest that benthic phytoplankton would likely make up a small portion of these mussels’ diet (Nielsen et al., 2016). This would imply that benthic Prorocentrum was relatively unavailable to the mussels in this study.

2.4 Environmental sample collection

The depth integrated whole water sample was used to determine all environmental variables. Water temperature was measured at the time of collection at SCMW using a NIST-traceable digital thermometer. Beginning March 11, 2015, salinity measurements were conducted in the laboratory using an ECOSense EC300A salinometer. Prior to that
date, salinity was calculated from formalin preserved samples using an YSI 3100 Conductivity Meter, cross-calibrated with a YSI 6600v2 sonde deployed at SCMW as part of the Central and Northern California Ocean Observing System (CeNCOOS). A subset of samples was also analyzed using both the formalin-preserved sample and the fresh sample to ensure continuity and intercomparability of the discrete samples.

Chlorophyll $a$ concentration was determined by filtering sample water, in duplicate, onto a ~0.7 μm glass fiber filter (Whatman GF/F) and extracted for 24 hours in 90% acetone. Extracts were read on a Turner 10AU fluorometer using the non-acidification technique (Welschmeyer, 1994). Water samples were analyzed for ammonium concentrations using the OPA method and read on a TD700 fluorometer (Holmes et al., 1999). Urea concentrations were determined using the colorimetric method and read on a Varian Cary 50 Bio UV/Visible Spectrophotometer with a 10 cm pathlength cell (Mulvena and Savidge, 1992). Sample water for nitrate+nitrite, phosphate and silicate was filtered through a Whatman GF/F filter (~0.7 μm) and analyzed using a Lachat QuikChem 8500 Flow Injection Analyst System and Omnion 3.0 software (Lachat, 2010). Nitrate+nitrite is referred to as nitrate for the remainder of the analysis.

2.5 Imaging Flow Cytobot (IFCB) images

Images of *Dinophysis* were obtained using an Imaging Flow Cytobot (IFCB), an automated imaging flow cytometer. The design and capabilities of the IFCB are provided in detail in Olson and Sosik (2007) and Sosik and Olson (2007). The images are from SCMW integrated whole water and net tow samples brought back to the laboratory and run through the IFCB on the benchtop, as well as samples taken at SCMW, where the IFCB samples from a pumped flow through system at approximately 20-minute intervals.
IFCB data are provided primarily to illustrate the presence of various *Dinophysis* species; at the time of this study, there were insufficient data to attempt more sophisticated analysis (e.g. Campbell et al., 2010).

### 2.6 Statistical Analyses

The relationship between *Dinophysis* and DSTs in mussels was evaluated two ways. First, a Wilcoxon rank sum test was used to determine if the median DST concentration in California mussels when *Dinophysis* was present in the net tow sample was greater than when *Dinophysis* was absent. This non-parametric alternative to the t-test was chosen because toxin distribution was not normally distributed. Second, *Dinophysis* cell concentrations were compared to DST concentrations using logistic regression. Logistic regression was chosen because it allowed toxin data to be binned around a relevant threshold and also allowed the data to be modeled without transformation. The concentration of DST in mussel tissue was binned as greater than or less than 100 ng/g. This level was chosen as a way to group toxin into a “low” category and a “higher” category that approached the FDA regulatory limit (160 ng/g). A second logistic regression was run for toxin binned by presence/absence in mussel tissue. The logit link function was used to produce the logistic regression output in terms of the predicted probability of mussel tissue containing toxin greater than 100 ng/g, or presence/absence of toxin, for a given *Dinophysis* concentration.

A stepwise multiple linear regression was used to determine which environmental variables were most associated with *Dinophysis* concentrations. This method was chosen as a way to discern if any statistically significant linear relationships existed between *Dinophysis* and the environmental variables that collected as a part of the weekly SCMW...
time series (Schulien et al., 2017). Variables used in this model were log transformed
\( \log_{10}(x+1) \), excluding temperature and salinity, which did not require transformation. To determine if the mean temperature when \( D. fortii \) was present in the water column was statistically higher than when \( D. acuminata \) complex was present in the water column, a Welch’s two-sample t-test was used. All statistical tests were performed in R (R Core Team, 2017).

Data are presented as boxplots in multiple figures. Boxplots were produced in R using the ggplot2 package. The box is centered on the median, with lower and upper hinges corresponding to the first and third quartiles, and whiskers extending from the hinge to the largest/smallest value 1.5 times the inter-quartile range. Points beyond that range are plotted individually as outliers from a normal distribution.

3. Results

3.1 Time series (2013-2016)

\( Dinophysis \) concentration at SCMW showed consistent seasonal peaks throughout 2013-2016 (Fig. 2A). The time series showed moderate interannual variability in maximum \( Dinophysis \) concentration and in the relative persistence of high cell concentration during each year (Fig. 2A). During this four-year sampling period, the mean \( Dinophysis \) concentration was 754 cells/L and the median was 80 cells/L. The maximum concentration was 9,404 cells/L, and was observed in July 2013. In that same year, cell concentrations above 5,000 cells/L occurred from March through October, making 2013 the year with highest concentration in a single sample and the year with the longest time period with elevated \( Dinophysis \) concentrations.
Detectable concentrations of DSTs were found in 61% of weekly non-commercial California mussel samples collected over the four-year time series (Fig 2B). The FDA guidance level for DSTs (160 ng/g) was exceeded 19 of the 192 weeks sampled during this four-year study period. The mean DST concentration was 51.61 ng/g, the median was 15.5 ng/g, and the maximum concentration was 562.9 ng/g (3.5 times the FDA guidance level). The maximum DST concentration each year was: 562.9 ng/g on 5/1/2013, 439.0 ng/g on 5/21/2014, 377.7 ng/g on 6/10/2015, and 137.0 ng/g on 9/7/2016. Okadaic acid (OA), dinophysistoxin 1 (DTX-1) and dinophysistoxin 2 (DTX-2) were all detected during the study period (Fig. 2B). DTX-2 dominated the toxin profile found in mussel tissue during this study. Okadaic acid was consistently found every year, but at low levels. DTX-1 was only detected during 9 weeks of this study.

3.2 Seasonal Trends

Concentrations of genus-level *Dinophysis* showed a clear seasonal cycle at SCMW (Fig. 3A). The concentration of *Dinophysis* cells generally began to increase in March and peak in the summer months of June and July, with a smaller peak occurring in the fall, around October. Concentrations of *Dinophysis* had a median value of zero for December, January and February.

The seasonal trends of DST in mussel tissue are more variable than the seasonal trends observed in *Dinophysis* cell concentrations (Fig. 3B). Median toxin concentration increases to a detectable level in March, with the highest median values occurring in May and June. Toxin trends from June-November and January-February were characterized by extremely high interannual variability. Interestingly, the month of December had a non-zero median value and low interannual variability. Toxin seasonality appears to
track *Dinophysis* seasonality from January through May, but the relationship was unclear from June through December.

### 3.3 *Dinophysis* as a predictor of DST concentration in California mussels

Concentration of DST in California mussel tissue was significantly greater during weeks when *Dinophysis* was present in a net tow sample (median = 19.99 ng/g, mean = 62.80 ng/g) than in weeks *Dinophysis* was absent from the new tow sample (median = 0 ng/g, mean = 23.87) (Wilcoxon rank sum test: $W=2356$, $p=0.00004$) (Fig. 4A). Despite this difference in median, the range of toxin concentrations in each distribution was not distinct.

The results of a logistic regression using genus-level *Dinophysis* cell concentration as a predictor of toxin level greater than or less than 100 ng/g in mussel tissue is plotted in Fig. 4B. This level (100 ng/g) was chosen as a threshold to mark when toxin in mussel tissue begins to approach unsafe levels. The probability of mussel tissue containing toxin greater than 100 ng/g significantly increases with increasing *Dinophysis* concentration, but there was a high level of uncertainty represented by the wide confidence intervals seen in Fig. 4B (log-odds ratio = 0.00020, $p = 0.0447$). When no cells were present, there was a 16% probability mussel tissue would contain toxin at a concentration greater than 100 ng/g. When cell counts were high, there were fewer data to constrain this relationship and variability became too high to make an accurate prediction. A logistic regression was also conducted with presence/absence of toxin in mussels as the binary response variable. Results of this second logistic regression were not statistically significant (log-odds ratio 0.00017, $p=0.13$), indicating cell concentration data alone were a weak predictor of toxin presence/absence in mussel tissue.
3.4 Environmental predictors of Dinophysis

A stepwise multiple linear regression (MLR), run forward and backward, was used to begin to explore which environmental conditions are associated with Dinophysis concentrations at SCMW. Relevant environmental variables collected as part of the SCMW time series and known to be associated with phytoplankton ecology are shown in Fig. 5. Ammonium, nitrate, phosphate, silicate, urea, water temperature, salinity and nitrate:phosphate ratio were entered into the model. Results of the stepwise MLR are presented in Table 1. The MLR with the lowest Akaike information criterion (AIC) score contained ammonium, silicate, urea, and salinity as predictor variables of Dinophysis concentration. Abundance of Dinophysis increased with decreasing ammonium, decreasing silicate, decreasing urea and increasing salinity. Trends for ammonium and silicate were not significant at p<0.05 (p=0.07, p=0.08, respectively), trends for urea and salinity were significant at p<0.05 (Table 1). The overall adjusted R-squared for the model was 0.24 (p=1.28e-11). When the stepwise regression was run, water temperature was the first variable to be removed from the model, followed by the N:P ratio, nitrate, and phosphate.

3.5 Dinophysis species at SCMW

The IFCB captured images of multiple species of toxigenic Dinophysis at Santa Cruz Municipal Wharf between 2015-2017. Images representing the diversity of species seen by the IFCB are presented in Fig. 6 and include: D. fortii, D. tripos, P. rotundatum (previously D. rotundata), D. caudata, and species in the D. acuminata complex. In the two years of available microscopy data that identify Dinophysis to species level (2013-2014), these five species seen in the IFCB images, as well as D. acuta, D. norvegica, and
D. odiosa were identified (Table 2). The most abundant species in Lugol’s preserved samples were D. acuminata complex and D. fortii, followed by P. rotundatum (previously D. rotundata). In 2013, D. acuminata accounted for 76% of the observed Dinophysis population, while in 2014, D. acuminata accounted for 34% and D. fortii accounted for 45% of the Dinophysis population. It is noted that the genus Dinophysis generally makes up a small fraction of the phytoplankton population at SCMW, and because the Lugol’s preserved cell counts were conducted as an effort to enumerate the total phytoplankton population and were leveraged for use in this study, the total raw count for some of the more rare Dinophysis species from table 2 was often low. For example, D. acuta was present in 12% of the weekly samples, however, there were never more than 1 or 2 cells counted in the 50 mL sample settled onto the slide.

Temperature ranges versus concentration of D. acuminata complex, D. fortii, and total Dinophysis concentration (Dinophysis spp.) are shown in Fig. 7. Visual interpretation of this data suggests D. acuminata complex has a broad temperature range, while D. fortii might favor a higher temperature range of 15-17°C. A Welch’s two-sample t-test was used to determine if the mean temperature when D. fortii was present (14.6°C) was statistically higher than the mean when D. acuminata complex are present (13.9°C). Results show there was no significant difference (t=1.3088, p=0.09681) between the two temperature distributions.

4. Discussion

At Santa Cruz Municipal Wharf (SCMW), DSTs were found at persistent low levels in non-commercial mussel tissue throughout this four-year study (2013-2016), and DST exceeded the FDA guidance level of 160 ng/g during three out of the four years.
The yearly maximum DST concentration from these three years ranged from 2-3.5 times the FDA guidance level (562.9, 439.0, and 377.7 ng/g). During the 2011 event on the West Coast of the U.S. that marked the first clinical report of DSP in the U.S., the maximum DST in blue mussels from Washington State was recorded as 1,603 ng/g, but the concentration for mussels collected within a few days of the reported illnesses were found to range from 2-10 times the FDA guidance level (Trainer et al., 2013). At SCMW, the maximum DST concentration in shellfish was overall lower than the maximum concentration measured in 2011 in Washington, but the SCMW maximum concentrations during 2013, 2014 and 2015 fall within the low end of the range of values seen in Washington mussel tissue measured days after the reported illnesses.

The toxin profile of DSTs observed at SCMW was found to be dominated by DTX-2, with low levels of OA often present, and DTX-1 present during two distinct time blocks in 2013 and 2015. This DST toxin profile differs from that observed during the 2011 event in Washington State, in which DTX-1 was detected, but OA and DTX-2 levels were recorded as being below the limit of detection (Trainer et al., 2013). When considering DST concentration as it pertains to regulatory limits, DTX-2 is often given a lower toxic equivalence factor (TEF) than OA and DTX-1. The European Food Safety Authority (2008) has published the recommend TEF for DSTs as: OA = 1, DTX-1 = 1, DTX-2 = 0.6. A joint Food and Agriculture Organization of the United Nations and World Health Organization Technical Report (FAO/WHO, 2016) has published suggested TEF’s agreed on by an expert group as: OA = 1.0, DTX-1 = 1.0 and DTX-2 = 0.5, stating that on average DTX-2 is half as toxic as DTX-1. Application of these adjusted TEFs to this dataset would reduce potential toxicity.
Cell concentrations of *Dinophysis* at SCMW peaked during the summer months (May-July), but were found in low background concentrations throughout the year. Globally, *Dinophysis* is usually a small fraction of the phytoplankton community, as was observed at SCMW, but contamination of shellfish at low concentrations can occur. The most extreme example is contamination of shellfish in Japan by populations of *D. fortii* with concentrations as low as 200 cells/L (Yasumoto et al., 1980; Yasumoto et al., 1985).

This high toxicity, however, is likely limited to specific species and strains of *Dinophysis*. At SCMW, when *Dinophysis* exceeds 200 cells/L, concurrent DST does not always reach levels of concern.

Early monitoring efforts of *Dinophysis* and DST in Washington adopted a cell concentration threshold of either 20,000 cells/L, or when relative abundance of *Dinophysis* increased from *present* to *common* to provide early warning of shellfish toxicity (Trainer and Hardy, 2015). The maximum *Dinophysis* concentrations at SCMW during 2013-2016 only approached half of that threshold (9,404 cells/L), and *Dinophysis* was described as common in only five weeks of this four-year study, yet, high concentrations of DST were observed in mussel tissue. During those five weeks when *Dinophysis* was “common”, DST concentrations were 21.7, 25.5, 41.39, 150.86, and 334.97 ng/g, indicating that at SCMW, relative abundance measurements of *Dinophysis* are not representative of concurrent DST concentrations in mussels. Currently in Washington State, *Dinophysis* concentrations measured above 1,000 cells/L (considered the Red level) by the SoundToxins monitoring program warrants additional shellfish testing beyond what is regularly conducted by the Washington State Department of Health (V. Trainer, personal communication). Choosing a threshold of *Dinophysis*
concentration as an early warning of DST in shellfish is complicated because the relationship between *Dinophysis* concentration in the water column and toxin level in shellfish is not straightforward. Additionally, toxin concentration per cell is complex and can vary based on time of day, cell division, and genetic differences among strains (Reguera et al., 2014).

Previous studies and publicly available data for Monterey Bay report *Dinophysis* concentrations similar to, and occasionally higher than, those observed in this study (Table 3). In particular, a study from 2005 identified *D. fortii* as the dominant species of *Dinophysis* at SCMW with a mean cell concentration of 2,300 cells/L and a maximum cell concentration of 21,000 cells/L, over double the maximum cell concentration observed in this study (Sutherland, 2008). Two additional records of *Dinophysis* concentration approaching 20,000 cells/L include June 1999 and October 2011 (Weber, 2000; Southern California Coastal Ocean Observing System, 2017). While this study presents the first published long-term record of paired *Dinophysis* concentration and DST level in mussels in Monterey Bay, past data suggests a longer paired record is needed in order to capture the levels of DST in mussels when *Dinophysis* reaches concentrations on the order of 20,000 cells/L, as has been recorded in the past at SCMW.

The relationship between *Dinophysis* cells and toxin levels in shellfish is known to be complex and is dependent on multiple confounding factors. As expected, the distribution of toxin concentrations in mussel tissue when *Dinophysis* is present in a net tow sample is significantly higher than when it is absent, but these distributions overlap. Additionally, when looking at *Dinophysis* concentration data, when the concentration is zero, logistic regression shows there is still a 16% chance mussel toxin will be over 100
ng/g, a concentration approaching the FDA guidance level. Ultimately, this study found that when *Dinophysis* is present at SCMW, that does not mean that toxin will be found in the mussels, and similarly, when *Dinophysis* is absent, that does not mean mussels will be free of toxin. Toxin level in shellfish is affected by the percentage of the mussel’s diet that is composed of *Dinophysis*, mussel depuration rates (which can vary by season), and the toxin quota of the *Dinophysis* cells present (Reguera et al., 2014). With the current state of knowledge for this system, the only way to be sure of the toxin level in mussel tissue is through direct testing.

Although genus level *Dinophysis* concentrations alone are not a strong predictor of DST level in shellfish at SCMW, understanding *Dinophysis* ecology and environmental drivers of *Dinophysis* abundance is integral to fully understanding and eventually predicting DST concentration in shellfish. In this study, *Dinophysis* concentrations at SCMW were not found to correlate strongly with observed environmental parameters that could inform predictive and conceptual models. Stepwise multiple linear regression showed *Dinophysis* has a negative relationship with nutrients (silicate, urea, ammonium) and a positive relationship with salinity. The association of *Dinophysis* with low nutrient levels is consistent with dinoflagellate preference for a stratified water column that develops following upwelling pulses (Smayda and Reynolds, 2001). When diatoms have drawn down surface nutrient concentrations, dinoflagellates such as *Dinophysis* can vertically migrate between deeper waters with ample nutrients and sunlit surface waters; however, this conceptual model for *Dinophysis* is complicated by its dependence on ciliate prey. In addition to preferring a low nutrient environment, a positive relationship with increased salinity suggests that *Dinophysis* concentrations are
associated with upwelling pulses, which introduce cooler, more saline waters to the SCMW site (Anderson et al., 2016). Overall, the regression model has a fairly low $R^2$ of 0.2 ($p<0.05$), indicating that the variables entered into this model (nutrients, temperature, salinity) alone are not enough to predict *Dinophysis* concentrations at SCMW.

Conceptual models of *Dinophysis* abundance in other systems require knowledge of factors beyond temperature, salinity and nutrients. These species-specific models take into account physical transport via upwelling and coastal jets, stratification in the water column, and predator prey population dynamics between *Dinophysis* and *Mesodinium rubrum* (Farrell et al., 2012; Diaz et al., 2013; Velo-Suarez et al., 2014; Harred and Campbell, 2014). A direct relationship between environmental variables and *Dinophysis* concentration may be further obscured by the tolerance of *Dinophysis* to a broad range of environmental conditions, as evidenced by its presence throughout the year. It is suggested that consideration of physical (transport, upwelling, stratification, temperature, salinity), chemical (nutrients) and biological (ciliate prey) variables as they relate to specific species of *Dinophysis* would be required to successfully predict *Dinophysis* abundance at SCMW.

In a two-year (2013-2014) weekly study of species composition of *Dinophysis* at SCMW, *D. acuminata* complex and *D. fortii* were found in the highest abundance, while other potential toxin producers were found in low concentrations (Table 2). The *Dinophysis* population in 2013 was dominated by *D. acuminata* complex, while both *D. acuminata* complex and *D. fortii* dominated in 2014. The presence/absence of each species was compared to water temperature to determine if either of the two most common species was associated with a specific temperature regime. Based on this
study’s observations, it appears that *D. fortii* may prefer a higher temperature range; however, the temperature range for *D. acuminata* complex was not found to be significantly different from that of *D. fortii* (*p*=0.09681). Sutherland (2008) found the average temperature when *D. acuminata* was present to be 15.9°C, while the average temperature when *D. fortii* was present to be 16.1°C; however, no test to determine if there was a significant difference was performed in that analysis. A longer data set or laboratory experiments will be required to describe *Dinophysis* species temperature preferences with more confidence, and to determine if the temperature range for *D. fortii* is significantly different from that of *D. acuminata* complex at SCMW. Understanding species-specific temperature preferences could aid in predictions of *Dinophysis* species concentrations under various scenarios and ultimately inform predictions of DST concentration in seafood.

5. Conclusions

At SCMW, *Dinophysis* is present year-round and DST is present in shellfish at persistent low levels throughout the year, with occasional peaks above the FDA guidance level. The toxin profile was found to consist of OA, DTX-1, and DTX-2. Multiple species of toxic *Dinophysis* were found, mainly *D. acuminata* complex and *D. fortii*, with the highest concentrations of *Dinophysis* occurring throughout the early summer months (May-July). Concentrations of *Dinophysis* at SCMW are not well explained by temperature, salinity, and nutrient data. Future predictive models of *Dinophysis* could benefit from work to understand physical transport of *Dinophysis*, population dynamics of *Mesodinium rubrum*, and potential differences in environmental preference between *Dinophysis* species. While DSTs are not regularly monitored in California, these results
show the potential for DSP outbreaks at relatively low cell abundances, suggesting that a proactive response, such as routine testing as part of the existing California Department of Public Health mussel monitoring program, would be prudent.
Fig. 1. (A) Chromatograms for certified reference material standards of okadaic acid, DTX-1, and DTX-2, analyzed by LCMS with Selected Ion Monitoring as described in the text. Note that OA and DTX-2 were in one run, and DTX-1 was in a second run, with the two sets of chromatograms overlaid to indicate the peak separation. Peak identification for unknowns was based on mass to charge ratio (m/z) and retention time, compared to reference material standards included in each analytical run. Representative chromatograms are shown for peak toxin levels of (B) 28 May 2013, (C) 6 May 2014, and (D) 10 June 2015.
**Fig. 2.** Weekly time series data (2013-2016) at Santa Cruz Municipal Wharf. (A) *Dinophysis* concentrations (cells/L). (B) DST concentration (ng/g) of okadaic acid (OA), dinophysistoxin 1 (DTX-1), and dinophysistoxin 2 (DTX-2) in California mussel tissue. Dashed black line at y=160 ng/g toxin is the FDA guidance level for DST. Dashed grey line at y=100 ng/g toxin represents the cutoff used to signify DST level approaching the FDA guidance level in the logistic regression model.
Fig. 3. Monthly binned seasonal trends for (A) log_{10}(x+1) transformed *Dinophysis* (cells/L) and (B) log_{10}(x+1) transformed DST (ng/g). The box is centered on the median, with lower and upper hinges corresponding to the first and third quartiles.
Fig. 4. Relationship between *Dinophysis* and DST in California mussel tissue. (A) Boxplot of *Dinophysis* presence and absence in net tow sample versus DST in mussel tissue. The box is centered on the median, with lower and upper hinges corresponding to the first and third quartiles. (B) Fit of predicted probabilities from logistic regression model of mussel tissue toxin greater than 100 ng/g in relation to *Dinophysis* concentration (cells/L). This model has a scatterplot overlay with data from SCMW that went into the logistic regression — samples with toxin greater than 100 ng/g are plotted along y=1.00 and samples of toxin less than 100 ng/g are plotted along y=0.00, both in relation to *Dinophysis* concentration. Point size relates to the number of samples (N) for a given *Dinophysis* concentration.
Fig. 5. Data from SCMW time series are provided. (A) *Dinophysis* (closed symbols) and DST (open symbols), (B) temperature, (C) silicate (closed symbols) and phosphate (open symbols), (D) nitrate (closed symbols), ammonium (open symbols), and urea (+ symbols), (E) salinity, and (F) chlorophyll *a*. 
Fig. 6. Images of *Dinophysis* and *Phalacroma* species diversity at SCMW detected by an Imaging Flow Cytobot (IFCB), 2015-2017. (A) *D. fortii*, (B) *D. caudata*, (C) *P. rotundatum* (previously *D. rotundata*), (D-F) *D. acuminata* complex, (G) *D. tripos*
Fig. 7. $\log_{10}(x+1)$ transformed *Dinophysis* concentration data (2013-2014) with one-degree temperature bins for (A) *D. acuminata* complex, (B) *D. fortii*, and (C) genus-level *Dinophysis* *spp*. The box is centered on the median, with lower and upper hinges corresponding to the first and third quartiles.
Table 1. Results from a stepwise multiple linear regression of environmental variables to model *Dinophysis* concentration.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Coefficient</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium (µm)</td>
<td>-0.77</td>
<td>0.07</td>
</tr>
<tr>
<td>Silicate (µm)</td>
<td>-0.55</td>
<td>0.08</td>
</tr>
<tr>
<td>Urea (µm)</td>
<td>-1.28</td>
<td>0.03</td>
</tr>
<tr>
<td>Salinity (ppt)</td>
<td>0.59</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Stepwise multiple linear regression was run forward and backward using the environmental variables ammonium, nitrate, phosphate, silicate, urea, water temperature, salinity and nitrate:phosphate ratio. Model multiple $R^2 = 0.24$, p<0.05.
Table 2. *Dinophysis* species (as well as *P. rotundatum*, formerly *D. rotundata*) identified by microscopy in weekly samples at SCMW over a two-year period, 2013-2014.

<table>
<thead>
<tr>
<th><em>Dinophysis</em> Species</th>
<th>Proportion of weeks present (N=98)</th>
<th>Mean concentration (cells/L)</th>
<th>Maximum concentration (cells/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>D. acuminata</em> complex</td>
<td>0.76</td>
<td>626</td>
<td>8229</td>
</tr>
<tr>
<td><em>D. fortii</em></td>
<td>0.46</td>
<td>230</td>
<td>4114</td>
</tr>
<tr>
<td><em>P. rotundatum</em></td>
<td>0.3</td>
<td>75</td>
<td>1763</td>
</tr>
<tr>
<td><em>D. acuta</em></td>
<td>0.12</td>
<td>10</td>
<td>588</td>
</tr>
<tr>
<td><em>D. caudata</em></td>
<td>0.1</td>
<td>21</td>
<td>1176</td>
</tr>
<tr>
<td><em>D. tripos</em></td>
<td>0.05</td>
<td>7</td>
<td>588</td>
</tr>
<tr>
<td><em>D. norvegica</em></td>
<td>0.05</td>
<td>1</td>
<td>40</td>
</tr>
<tr>
<td><em>D. odiosa</em></td>
<td>0.01</td>
<td>6</td>
<td>588</td>
</tr>
</tbody>
</table>
Table 3. Summary of *Dinophysis* mean, median and maximum abundance recorded in Monterey Bay (Santa Cruz Municipal Wharf = SCMW, Monterey Wharf = MW, Southern California Coastal Ocean Observing System = SCOOS).

<table>
<thead>
<tr>
<th>Source</th>
<th>Location</th>
<th>Species</th>
<th>Date</th>
<th>Mean (cells/L)</th>
<th>Median (cells/L)</th>
<th>Single Measure/Maximum (cells/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weber 2000*</td>
<td>SCMW</td>
<td><em>Dinophysis spp.</em></td>
<td>Jun 1999</td>
<td></td>
<td>20,000</td>
<td></td>
</tr>
<tr>
<td>Sutherland 2008*</td>
<td>SCMW</td>
<td><em>D. acuminata</em></td>
<td>2004</td>
<td>1000</td>
<td>5,000</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SCMW</td>
<td><em>D. fortii</em></td>
<td>2004</td>
<td>140</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>SCMW</td>
<td><em>D. acuminata</em></td>
<td>2005</td>
<td>870</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>SCMW</td>
<td><em>D. fortii</em></td>
<td>2005</td>
<td>2300</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SCOOS</td>
<td>MW</td>
<td><em>Dinophysis spp.</em></td>
<td>2013-2016</td>
<td>263.2</td>
<td>30</td>
<td>7,935</td>
</tr>
<tr>
<td></td>
<td>SCMW</td>
<td><em>Dinophysis spp.</em></td>
<td>Oct 2011</td>
<td></td>
<td></td>
<td>18,900</td>
</tr>
<tr>
<td>This study</td>
<td>SCMW</td>
<td><em>Dinophysis spp.</em></td>
<td>2013-2016</td>
<td>754</td>
<td>80</td>
<td>9,404</td>
</tr>
</tbody>
</table>

*Weber 2000 and Sutherland 2008 are unpublished Master’s theses; SCOOS data is publicly available*
Acknowledgements

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