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

70 cultured mussel tissue was associated with 13 illnesses in Nova Scotia, Canada (Quilliam
71 et al., 1993). These studies generally found correlations to D. norvegica, D. acuminata or
72 P. lima either in the water column or in shellfish gut analysis. A study by Marr et al. in
73 1992 isolated P. lima off the Atlantic Coast of Nova Scotia and confirmed the production
74 of okadaic acid and DTX-1 in the isolates. While these studies detected relatively high
75 levels of DST, other studies along U.S. coasts found low DST content in shellfish
76 (Maranda and Shimizu, 1987; Morton et al., 1999), and low toxicity in *Dinophysis* strains
77 (Morton et al., 1999; Hackett et al., 2009). The findings of low toxicity *Dinophysis* and
78 low levels or no DST in shellfish, combined with a lack of clinical reports of DSP prior to
79 2011 (Trainer et al., 2013) has resulted in DSTs often being overlooked as a public health
80 hazard in the U.S.

81 While not the first studies to find DSTs in North American shellfish, multiple
82 recent studies have documented high levels of DST and brought increased attention to
83 this toxin in the U.S. In 2008, the first shellfish harvesting closure as a result of DSTs in
84 the U.S. occurred in Texas. High concentration of DST was found in oysters
85 (*Crassostrea virginica*) with a maximum concentration of 470 ng/g, almost three times
86 the FDA guidance level of 160 ng/g (Deeds et al., 2010). Oyster harvesting in Texas has
87 been closed multiple times since this first event (Texas Health and Human Services,
88 2014a, 2014b). In the summer of 2011, high levels of DST in shellfish in the Pacific
89 Northwest led to three illnesses in Washington State, marking the first clinical report of
90 DSP in the U.S., and 62 illnesses in British Columbia, Canada (Trainer et al., 2013;
91 Taylor et al., 2013). During this event, a maximum concentration of 1603 ng/g DST was
92 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 fulvescens* (previously *Cochlodinium fulvescens*), *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

185 open to recreational mussel harvesting, except during the annual closure (May 1 –
186 October 31; CDPH website) The mussels deployed at SCMW were initially collected
187 from the intertidal zone at Davenport Landing Beach, put into mesh bags of
188 approximately 30 mussels per bag and maintained for various durations in a flowing
189 seawater table of sand filtered water (30 μm pore size) at University of California, Santa
190 Cruz Long Marine Laboratory. These bags were deployed off a platform at SCMW for at
191 least one week. Each week, one bag of mussels was removed from the wharf and brought
192 into the laboratory for processing. The mussels were not tested for DSP toxin prior to
193 deployment at the wharf, and may have been exposed to DSTs at Davenport Landing
194 Beach.

195 In the laboratory, mussels were shucked and all tissues from 20-30 mussels,
196 except for the white fibrous muscle tissue, was removed, drained with a colander, and
197 homogenized using a Waring Xtreme Hi-Power Blender. Homogenized tissue was
198 frozen at -20 °C until analysis. A 2 g aliquot of this tissue homogenate was extracted and
199 hydrolyzed following a slightly modified version of the methods described by Villar-
200 Gonzalez et al. (2008). The tissue homogenate was extracted by adding 18 mL 100%
201 MeOH, followed by vortexing, homogenization, centrifugation, and separation of the
202 homogenate, while Villar-Gonzalez et al. 2008 extracted in two steps with 9 mL 100%
203 MeOH for each step, followed by combination of the two extracts (18 mL total).
204 Hydrolyzed extracts were analyzed on an Agilent 6130 quadrupole liquid
205 chromatography-mass spectrometer (LC-MS) with Select Ion Monitoring (SIM) in
206 negative mode using an Agilent Poroshell 120 SB-C18, 2.1x50mm, 2.7μm (with 1.7μm
207 solid core) particle size column with matching guard column. A gradient elution

208 (modified from Louppis et al., 2010) started with 95% water with 2 mM ammonium
209 formate and 50 mM formic acid (A) and 5% acetonitrile with 50 mM formic acid (B) for
210 1 minute, then to 60% A at 6 minutes, and 5% A at 8 minutes, held until 11 minutes
211 before returning to initial conditions. Injection volume was 50 µL and flow rate was 0.85
212 mL/min. Okadaic acid, DTX-1, and DTX-2 were monitored using masses 803.5 (OA,
213 DTX-2) and 817.5 (DTX-1). Quantification was based on mass and time, with an external
214 standard curve using certified reference material from NRC-Canada. Minimum Detection
215 Limits (MDL) were 0.5, 0.75, and 1.0 ng/mL on-column, equivalent to 5.0, 7.5, and 10.0
216 ng/g tissue. A chromatogram of the certified reference material standards for okadaic
217 acid, DTX-1, and DTX-2 is provided in Fig. 1.
218 As previously noted, DSTs are produced by both pelagic *Dinophysis* and benthic
219 *Prorocentrum*. While mussels growing in beds on the benthos might be regularly
220 ingesting resuspended benthic phytoplankton (Muschenheim and Newell, 1992), in this
221 experimental design, the degree of spatial separation between mussels deployed on a rope
222 suspended in the water column compared to the benthos suggest that benthic
223 phytoplankton would likely make up a small portion of these mussels' diet (Nielsen et al.,
224 2016). This would imply that benthic *Prorocentrum* was relatively unavailable to the
225 mussels in this study.
226 **2.4 Environmental sample collection**
227 The depth integrated whole water sample was used to determine all environmental
228 variables. Water temperature was measured at the time of collection at SCMW using a
229 NIST-traceable digital thermometer. Beginning March 11, 2015, salinity measurements
230 were conducted in the laboratory using an ECOSense EC300A salinometer. Prior to that

231 date, salinity was calculated from formalin preserved samples using an YSI 3100
232 Conductivity Meter, cross-calibrated with a YSI 6600v2 sonde deployed at SCMW as
233 part of the Central and Northern California Ocean Observing System (CeNCOOS). A
234 subset of samples was also analyzed using both the formalin-preserved sample and the
235 fresh sample to ensure continuity and intercomparability of the discrete samples.
236 Chlorophyll *a* concentration was determined by filtering sample water, in duplicate, onto
237 a ~0.7 μm glass fiber filter (Whatman GF/F) and extracted for 24 hours in 90% acetone.
238 Extracts were read on a Turner 10AU fluorometer using the non-acidification technique
239 (Welschmeyer, 1994). Water samples were analyzed for ammonium concentrations
240 using the OPA method and read on a TD700 fluorometer (Holmes et al., 1999). Urea
241 concentrations were determined using the colorimetric method and read on a Varian Cary
242 50 Bio UV/Visible Spectrophotometer with a 10 cm pathlength cell (Mulvena and
243 Savidge, 1992). Sample water for nitrate+nitrite, phosphate and silicate was filtered
244 through a Whatman GF/F filter (~0.7 μm) and analyzed using a Lachat QuikChem 8500
245 Flow Injection Analyst System and Omnion 3.0 software (Lachat, 2010). Nitrate+nitrite
246 is referred to as nitrate for the remainder of the analysis.

247 **2.5 Imaging Flow Cytobot (IFCB) images**

248 Images of *Dinophysis* were obtained using an Imaging Flow Cytobot (IFCB), an
249 automated imaging flow cytometer. The design and capabilities of the IFCB are provided
250 in detail in Olson and Sosik (2007) and Sosik and Olson (2007). The images are from
251 SCMW integrated whole water and net tow samples brought back to the laboratory and
252 run through the IFCB on the benchtop, as well as samples taken at SCMW, where the
253 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

277 time series (Schulien et al., 2017). Variables used in this model were log transformed
278 ($\log_{10}(x+1)$), excluding temperature and salinity, which did not require transformation.
279 To determine if the mean temperature when *D. fortii* was present in the water column was
280 statistically higher than when *D. acuminata complex* was present in the water column, a
281 Welch's two-sample t-test was used. All statistical tests were performed in R (R Core
282 Team, 2017).

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

288 **3. Results**

289 **3.1 Time series (2013-2016)**

290 *Dinophysis* concentration at SCMW showed consistent seasonal peaks throughout
291 2013-2016 (Fig. 2A). The time series showed moderate interannual variability in
292 maximum *Dinophysis* concentration and in the relative persistence of high cell
293 concentration during each year (Fig. 2A). During this four-year sampling period, the
294 mean *Dinophysis* concentration was 754 cells/ and the median was 80 cells/L. The
295 maximum concentration was 9,404 cells/L, and was observed in July 2013. In that same
296 year, cell concentrations above 5,000 cells/L occurred from March through October,
297 making 2013 the year with highest concentration in a single sample and the year with the
298 longest time period with elevated *Dinophysis* concentrations.

299 Detectable concentrations of DSTs were found in 61% of weekly non-commercial
300 California mussel samples collected over the four-year time series (Fig 2B). The FDA
301 guidance level for DSTs (160 ng/g) was exceeded 19 of the 192 weeks sampled during
302 this four-year study period. The mean DST concentration was 51.61 ng/g, the median
303 was 15.5 ng/g, and the maximum concentration was 562.9 ng/g (3.5 times the FDA
304 guidance level). The maximum DST concentration each year was: 562.9 ng/g on
305 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.
306 Okadaic acid (OA), dinophysistoxin 1 (DTX-1) and dinophysistoxin 2 (DTX-2) were all
307 detected during the study period (Fig. 2B). DTX-2 dominated the toxin profile found in
308 mussel tissue during this study. Okadaic acid was consistently found every year, but at
309 low levels. DTX-1 was only detected during 9 weeks of this study.

310 **3.2 Seasonal Trends**

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

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

322 track *Dinophysis* seasonality from January through May, but the relationship was unclear
323 from June through December.

324 **3.3 *Dinophysis* as a predictor of DST concentration in California mussels**

325 Concentration of DST in California mussel tissue was significantly greater during
326 weeks when *Dinophysis* was present in a net tow sample (median = 19.99 ng/g, mean =
327 62.80 ng/g) than in weeks *Dinophysis* was absent from the new tow sample (median = 0
328 ng/g, mean = 23.87) (Wilcoxon rank sum test: W=2356, p=0.00004) (Fig. 4A). Despite
329 this difference in median, the range of toxin concentrations in each distribution was not
330 distinct.

331 The results of a logistic regression using genus-level *Dinophysis* cell concentration as
332 a predictor of toxin level greater than or less than 100 ng/g in mussel tissue is plotted in
333 Fig. 4B. This level (100 ng/g) was chosen as a threshold to mark when toxin in mussel
334 tissue begins to approach unsafe levels. The probability of mussel tissue containing toxin
335 greater than 100 ng/g significantly increases with increasing *Dinophysis* concentration,
336 but there was a high level of uncertainty represented by the wide confidence intervals
337 seen in Fig. 4B (log-odds ratio = 0.00020, p = 0.0447). When no cells were present, there
338 was a 16% probability mussel tissue would contain toxin at a concentration greater than
339 100 ng/g. When cell counts were high, there were fewer data to constrain this
340 relationship and variability became too high to make an accurate prediction. A logistic
341 regression was also conducted with presence/absence of toxin in mussels as the binary
342 response variable. Results of this second logistic regression were not statistically
343 significant (log-odds ratio 0.00017, p=0.13), indicating cell concentration data alone were
344 a weak predictor of toxin presence/absence in mussel tissue.

345 **3.4 Environmental predictors of *Dinophysis***

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

361 **3.5 *Dinophysis* species at SCMW**

362 The IFCB captured images of multiple species of toxigenic *Dinophysis* at Santa
363 Cruz Municipal Wharf between 2015-2017. Images representing the diversity of species
364 seen by the IFCB are presented in Fig. 6 and include: *D. fortii*, *D. tripos*, *P. rotundatum*
365 (previously *D. rotundata)*, *D. caudata,* and species in the *D. acuminata* complex. In the
366 two years of available microscopy data that identify *Dinophysis* to species level (2013-
367 2014), these five species seen in the IFCB images, as well as *D. acuta*, *D. norvegica*, and

368 *D. odiosa* were identified (Table 2). The most abundant species in Lugol's preserved
369 samples were *D. acuminata* complex and *D. fortii*, followed by *P. rotundatum*
370 (previously *D. rotundata*). In 2013, *D. acuminata* accounted for 76% of the observed
371 *Dinophysis* population, while in 2014, *D. acuminata* accounted for 34% and *D. fortii*
372 accounted for 45% of the *Dinophysis* population. It is noted that the genus *Dinophysis*
373 generally makes up a small fraction of the phytoplankton population at SCMW, and
374 because the Lugol's preserved cell counts were conducted as an effort to enumerate the
375 total phytoplankton population and were leveraged for use in this study, the total raw
376 count for some of the more rare *Dinophysis* species from table 2 was often low. For
377 example, *D. acuta* was present in 12% of the weekly samples, however, there were never
378 more than 1 or 2 cells counted in the 50 mL sample settled onto the slide.

379 Temperature ranges versus concentration of *D. acuminata* complex, *D. fortii*, and
380 total *Dinophysis* concentration (*Dinophysis* spp.) are shown in Fig. 7. Visual
381 interpretation of this data suggests *D. acuminata* complex has a broad temperature range,
382 while *D. fortii* might favor a higher temperature range of 15-17°C. A Welch's two-
383 sample t-test was used to determine if the mean temperature when *D. fortii* was present
384 (14.6°C) was statistically higher than the mean when *D. acuminata* complex are present
385 (13.9°C). Results show there was no significant difference ($t=1.3088$, $p=0.09681$)
386 between the two temperature distributions.

387 **4. Discussion**

388 At Santa Cruz Municipal Wharf (SCMW), DSTs were found at persistent low
389 levels in non-commercial mussel tissue throughout this four-year study (2013-2016), and
390 DST exceeded the FDA guidance level of 160 ng/g during three out of the four years.

391 The yearly maximum DST concentration from these three years ranged from 2-3.5 times
392 the FDA guidance level (562.9, 439.0, and 377.7 ng/g). During the 2011 event on the
393 West Coast of the U.S. that marked the first clinical report of DSP in the U.S., the
394 maximum DST in blue mussels from Washington State was recorded as 1,603 ng/g, but
395 the concentration for mussels collected within a few days of the reported illnesses were
396 found to range from 2-10 times the FDA guidance level (Trainer et al., 2013). At
397 SCMW, the maximum DST concentration in shellfish was overall lower than the
398 maximum concentration measured in 2011 in Washington, but the SCMW maximum
399 concentrations during 2013, 2014 and 2015 fall within the low end of the range of values
400 seen in Washington mussel tissue measured days after the reported illnesses.
401 The toxin profile of DSTs observed at SCMW was found to be dominated by
402 DTX-2, with low levels of OA often present, and DTX-1 present during two distinct time
403 blocks in 2013 and 2015. This DST toxin profile differs from that observed during the
404 2011 event in Washington State, in which DTX-1 was detected, but OA and DTX-2
405 levels were recorded as being below the limit of detection (Trainer et al., 2013). When
406 considering DST concentration as it pertains to regulatory limits, DTX-2 is often given a
407 lower toxic equivalence factor (TEF) than OA and DTX-1. The European Food Safety
408 Authority (2008) has published the recommend TEF for DSTs as: OA = 1, DTX-1 = 1,
409 DTX-2 = 0.6. A joint Food and Agriculture Organization of the United Nations and
410 World Health Organization Technical Report (FAO/WHO, 2016) has published
411 suggested TEF's agreed on by an expert group as: OA = 1.0, DTX-1 = 1.0 and DTX-2 =
412 0.5, stating that on average DTX-2 is half as toxic as DTX-1. Application of these
413 adjusted TEFs to this dataset would reduce potential toxicity.

414 Cell concentrations of *Dinophysis* at SCMW peaked during the summer months
415 (May-July), but were found in low background concentrations throughout the year.
416 Globally, *Dinophysis* is usually a small fraction of the phytoplankton community, as was
417 observed at SCMW, but contamination of shellfish at low concentrations can occur. The
418 most extreme example is contamination of shellfish in Japan by populations of *D. fortii*
419 with concentrations as low as 200 cells/L (Yasumoto et al., 1980; Yasumoto et al., 1985).
420 This high toxicity, however, is likely limited to specific species and strains of *Dinophysis*.
421 At SCMW, when *Dinophysis* exceeds 200 cells/L, concurrent DST does not always reach
422 levels of concern.
423 Early monitoring efforts of *Dinophysis* and DST in Washington adopted a cell
424 concentration threshold of either 20,000 cells/L, or when relative abundance of
425 *Dinophysis* increased from *present* to *common* to provide early warning of shellfish
426 toxicity (Trainer and Hardy, 2015). The maximum *Dinophysis* concentrations at SCMW
427 during 2013-2016 only approached half of that threshold (9,404 cells/L), and *Dinophysis*
428 was described as common in only five weeks of this four-year study, yet, high
429 concentrations of DST were observed in mussel tissue. During those five weeks when
430 *Dinophysis* was "common", DST concentrations were 21.7, 25.5, 41.39, 150.86, and
431 334.97 ng/g, indicating that at SCMW, relative abundance measurements of *Dinophysis*
432 are not representative of concurrent DST concentrations in mussels. Currently in
433 Washington State, *Dinophysis* concentrations measured above 1,000 cells/L (considered
434 the Red level) by the SoundToxins monitoring program warrants additional shellfish
435 testing beyond what is regularly conducted by the Washington State Department of
436 Health (V. Trainer, personal communication) . Choosing a threshold of *Dinophysis*

437 concentration as an early warning of DST in shellfish is complicated because the
438 relationship between *Dinophysis* concentration in the water column and toxin level in
439 shellfish is not straightforward. Additionally, toxin concentration per cell is complex and
440 can vary based on time of day, cell division, and genetic differences among strains
441 (Reguera et al., 2014).

442 Previous studies and publicly available data for Monterey Bay report *Dinophysis*
443 concentrations similar to, and occasionally higher than, those observed in this study
444 (Table 3). In particular, a study from 2005 identified *D. fortii* as the dominant species of
445 *Dinophysis* at SCMW with a mean cell concentration of 2,300 cells/L and a maximum
446 cell concentration of 21,000 cells/L, over double the maximum cell concentration
447 observed in this study (Sutherland, 2008). Two additional records of *Dinophysis*
448 concentration approaching 20,000 cells/L include June 1999 and October 2011 (Weber,
449 2000; Southern California Coastal Ocean Observing System, 2017). While this study
450 presents the first published long-term record of paired *Dinophysis* concentration and DST
451 level in mussels in Monterey Bay, past data suggests a longer paired record is needed in
452 order to capture the levels of DST in mussels when *Dinophysis* reaches concentrations on
453 the order of 20,000 cells/L, as has been recorded in the past at SCMW.

454 The relationship between *Dinophysis* cells and toxin levels in shellfish is known
455 to be complex and is dependent on multiple confounding factors. As expected, the
456 distribution of toxin concentrations in mussel tissue when *Dinophysis* is present in a net
457 tow sample is significantly higher than when it is absent, but these distributions overlap.
458 Additionally, when looking at *Dinophysis* concentration data, when the concentration is
459 zero, logistic regression shows there is still a 16% chance mussel toxin will be over 100

460 ng/g, a concentration approaching the FDA guidance level. Ultimately, this study found
461 that when *Dinophysis* is present at SCMW, that does not mean that toxin will be found in
462 the mussels, and similarly, when *Dinophysis* is absent, that does not mean mussels will be
463 free of toxin. Toxin level in shellfish is affected by the percentage of the mussel's diet
464 that is composed of *Dinophysis,* mussel depuration rates (which can vary by season), and
465 the toxin quota of the *Dinophysis* cells present (Reguera et al., 2014). With the current
466 state of knowledge for this system, the only way to be sure of the toxin level in mussel
467 tissue is through direct testing.

468 Although genus level *Dinophysis* concentrations alone are not a strong predictor
469 of DST level in shellfish at SCMW, understanding *Dinophysis* ecology and
470 environmental drivers of *Dinophysis* abundance is integral to fully understanding and
471 eventually predicting DST concentration in shellfish. In this study, *Dinophysis*
472 concentrations at SCMW were not found to correlate strongly with observed
473 environmental parameters that could inform predictive and conceptual models. Stepwise
474 multiple linear regression showed *Dinophysis* has a negative relationship with nutrients
475 (silicate, urea, ammonium) and a positive relationship with salinity. The association of
476 *Dinophysis* with low nutrient levels is consistent with dinoflagellate preference for a
477 stratified water column that develops following upwelling pulses (Smayda and Reynolds,
478 2001). When diatoms have drawn down surface nutrient concentrations, dinoflagellates
479 such as *Dinophysis* can vertically migrate between deeper waters with ample nutrients
480 and sunlit surface waters; however, this conceptual model for *Dinophysis* is complicated
481 by its dependence on ciliate prey. In addition to preferring a low nutrient environment, a
482 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

529 show the potential for DSP outbreaks at relatively low cell abundances, suggesting that a
530 proactive response, such as routine testing as part of the existing California Department
531 of Public Health mussel monitoring program, would be prudent.
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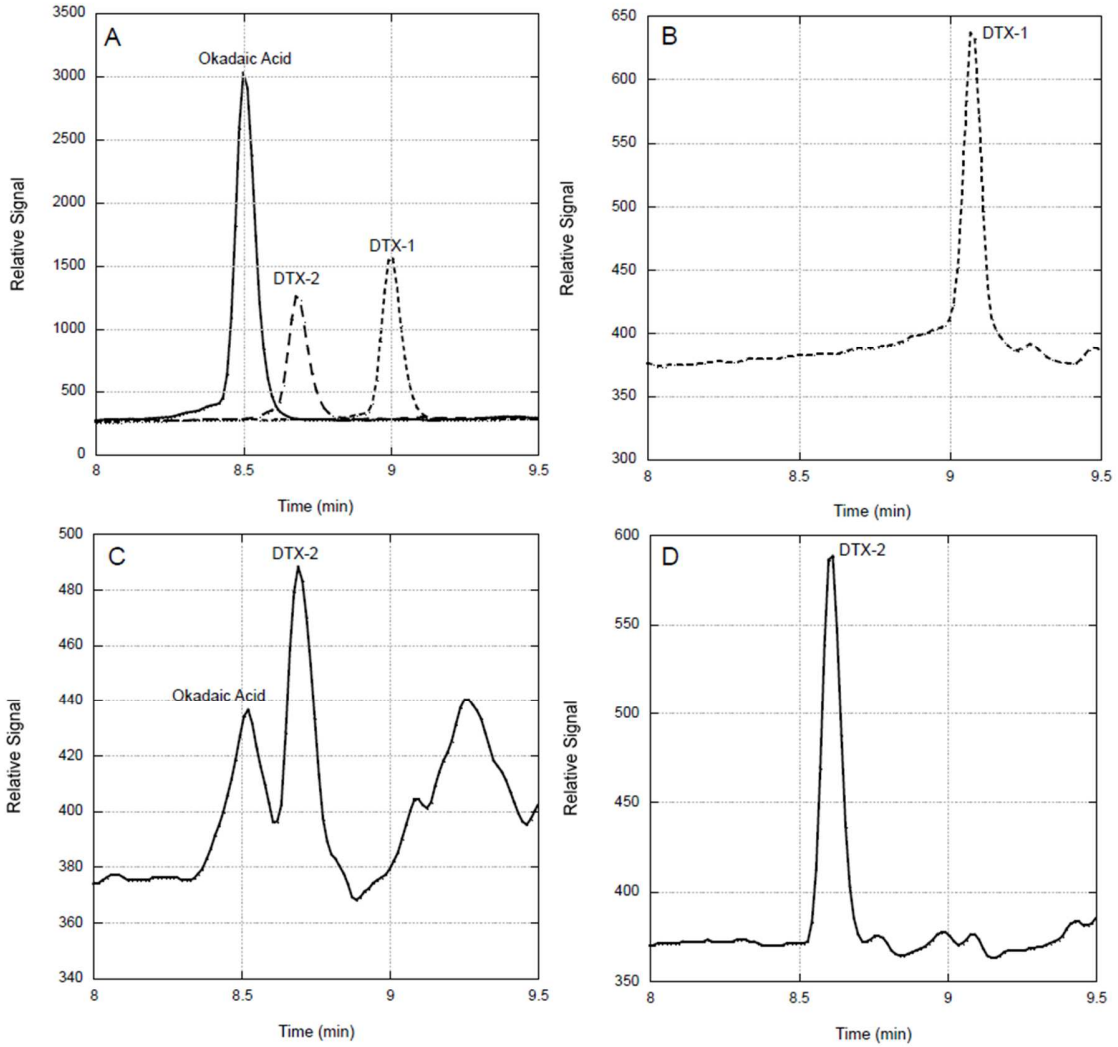

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553 **Fig. 1.** (A) Chromatograms for certified reference material standards of okadaic acid,
554 DTX-1, and DTX-2, analyzed by LCMS with Selected Ion Monitoring as described in the
555 text. Note that OA and DTX-2 were in one run, and DTX-1 was in a second run, with the
556 two sets of chromatograms overlaid to indicate the peak separation. Peak identification
557 for unknowns was based on mass to charge ratio (m/z) and retention time, compared to
558 reference material standards included in each analytical run. Representative
559 chromatograms are shown for peak toxin levels of (B) 28 May 2013, (C) 6 May 2014,
560 and (D) 10 June 2015.

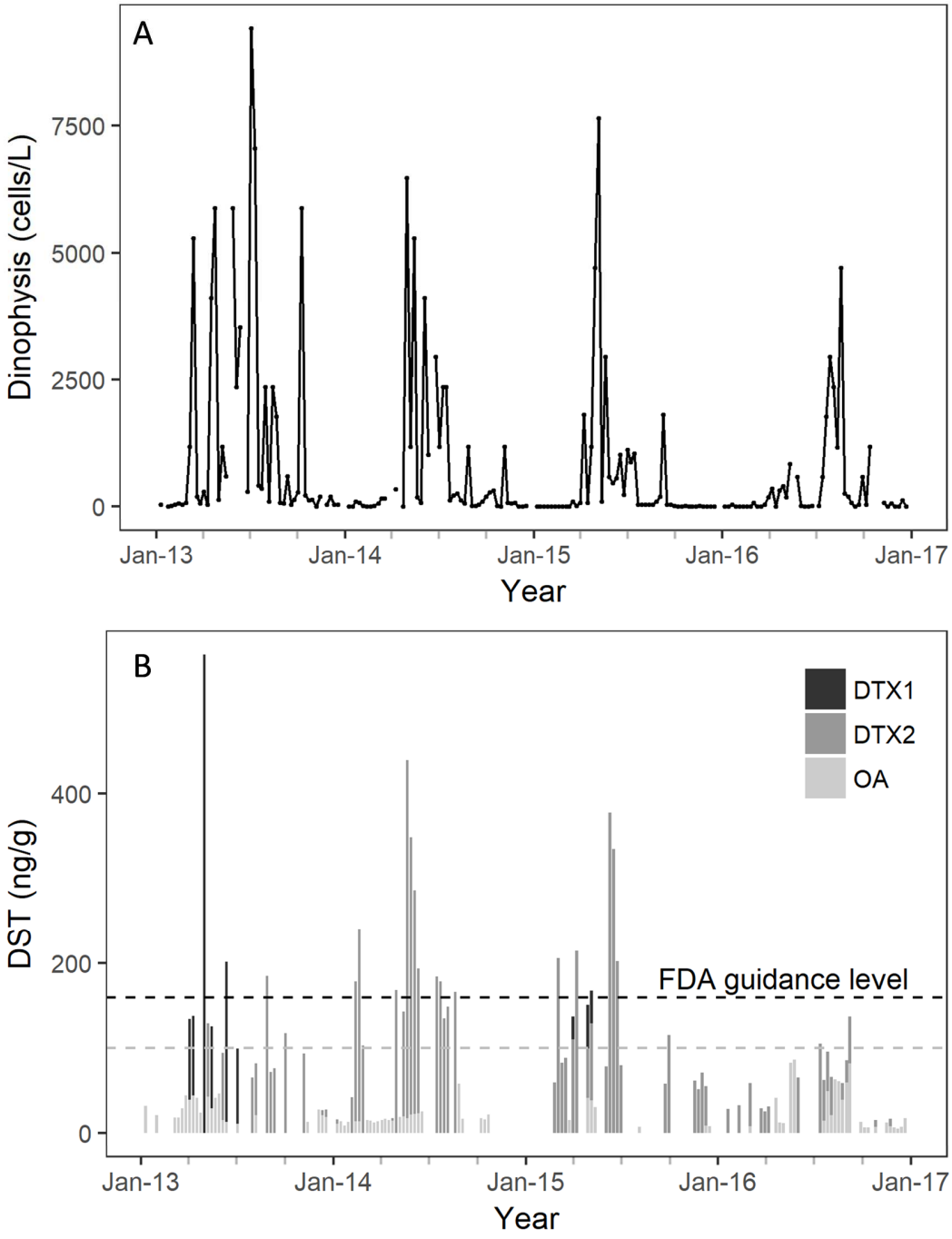

Fig. 2. Weekly time series data (2013-2016) at Santa Cruz Municipal Wharf. (A) *Dinophysis* concentrations (cells/L). (B) DST concentration (ng/g) of okadaic acid (OA), dinophysistoxin 1 (DTX-1), and dinophysistoxin 2 (DTX-2) in California mussel tissue. Dashed black line at y=160 ng/g toxin is the FDA 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 logisitic regression model.

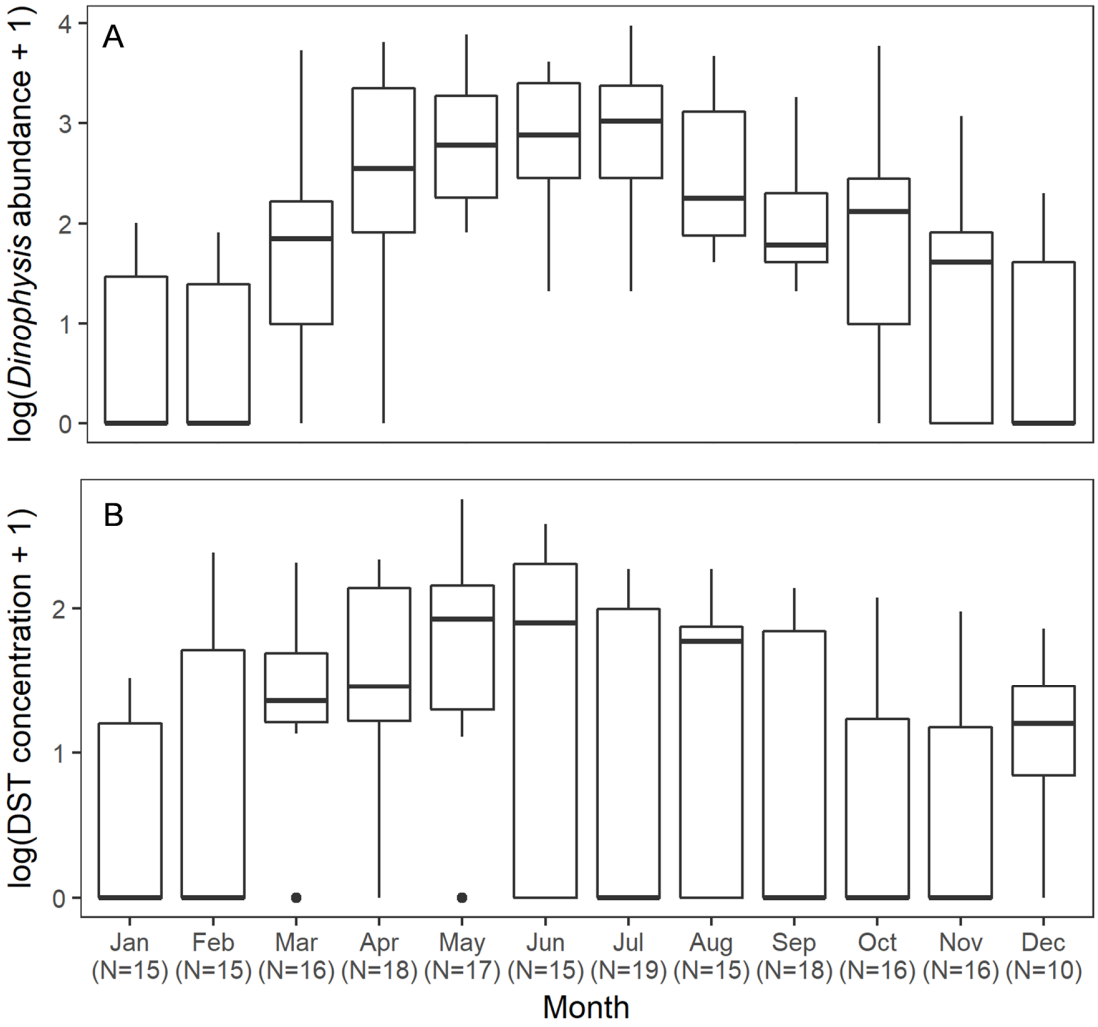

Fig. 3. Monthly binned seasonal trends for (A) $\log_{10}(x+1)$ transformed *Dinophysis* (cells/L) and (B) $\log_{10}(x+1)$ transformed DST (ng/g). The box is centered on the median, with lower and upper hinges corresponding to the first and third quartiles.

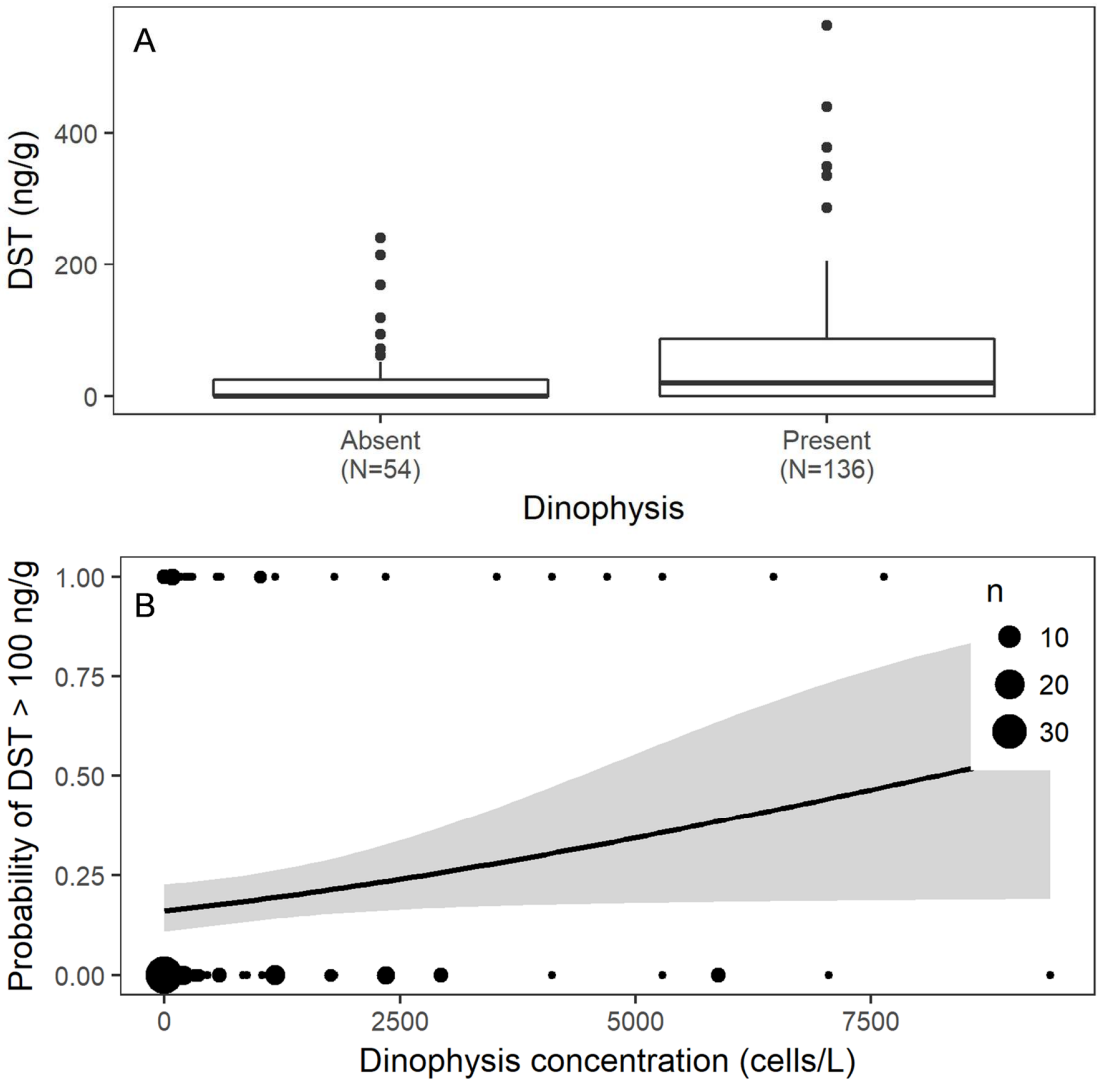

Fig. 4. Relationship between *Dinophysis* and DST in California mussel tissue. (A) Boxplot of *Dinophysis* presence and absence in net tow sample versus DST in mussel tissue. The box is centered on the median, with lower and upper hinges corresponding to the first and third quartiles. (B) Fit of predicted 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.

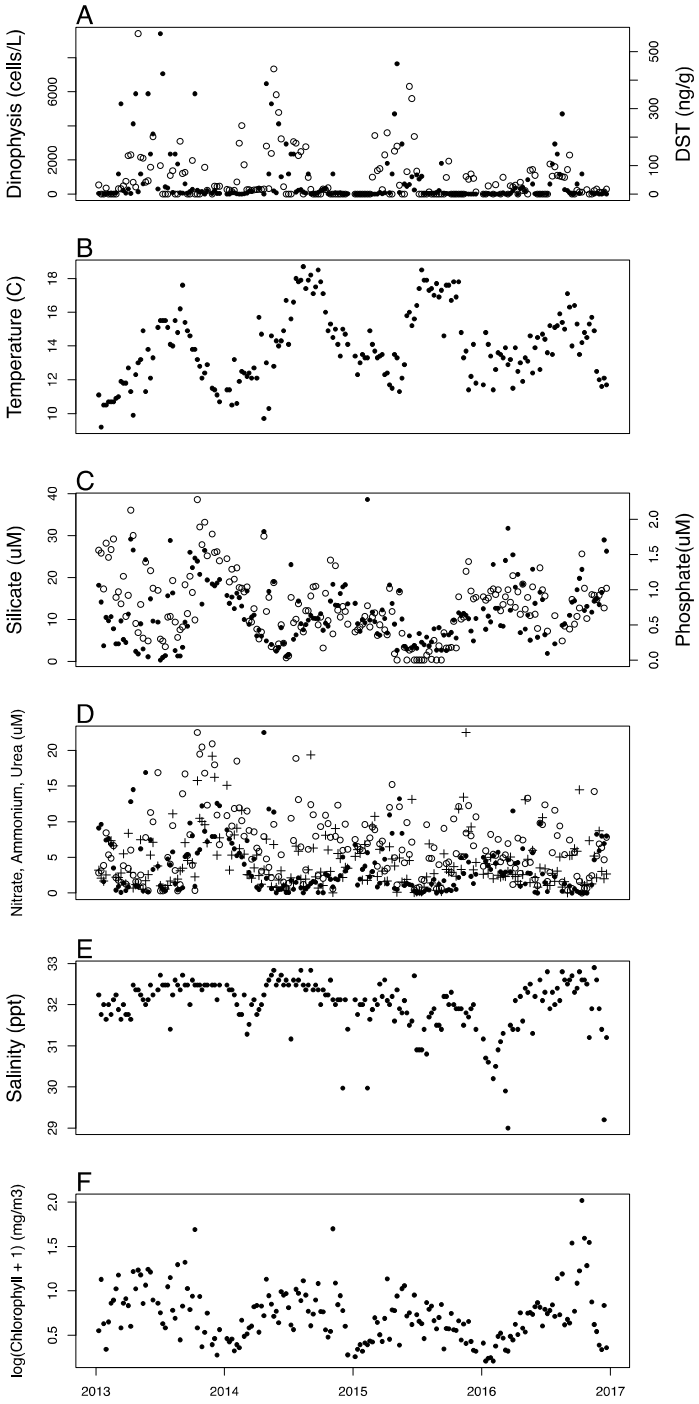

Fig. 5. Data from SCMW time series are provided. (A) *Dinophysis* (closed symbols) and DST (open symbols), (B) temperature, (C) silicate (closed symbols) and phosphate (open symbols), (D) nitrate (closed symbols), ammonium (open symbols), and urea (+ symbols), (E) salinity, and (F) chlorophyll *a*.

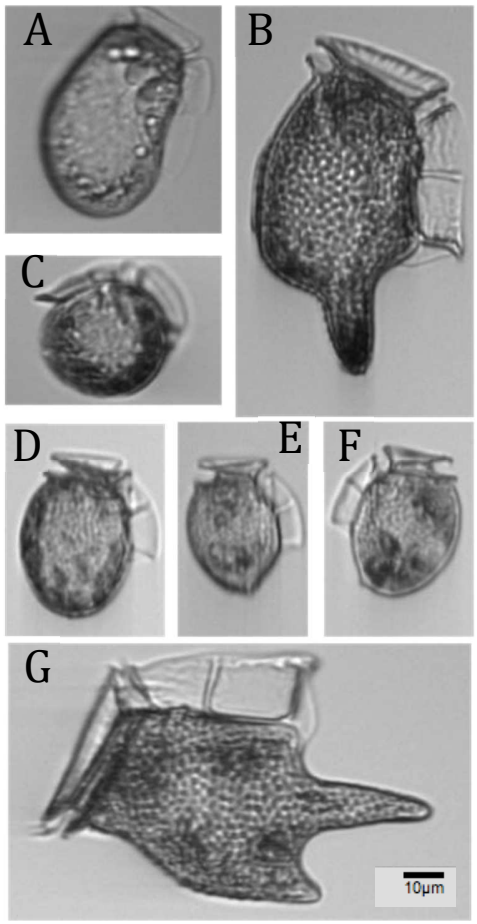

Fig. 6. Images of *Dinophysis* and *Phalacroma* species diversity at SCMW detected by an Imaging Flow Cytobot (IFCB), 2015-2017. (A) *D. fortii*, (B) *D. caudata*, (C) *P. rotundatum* (previously *D. rotundata)*, (D-F) *D. acuminata* complex, (G) *D. tripos*

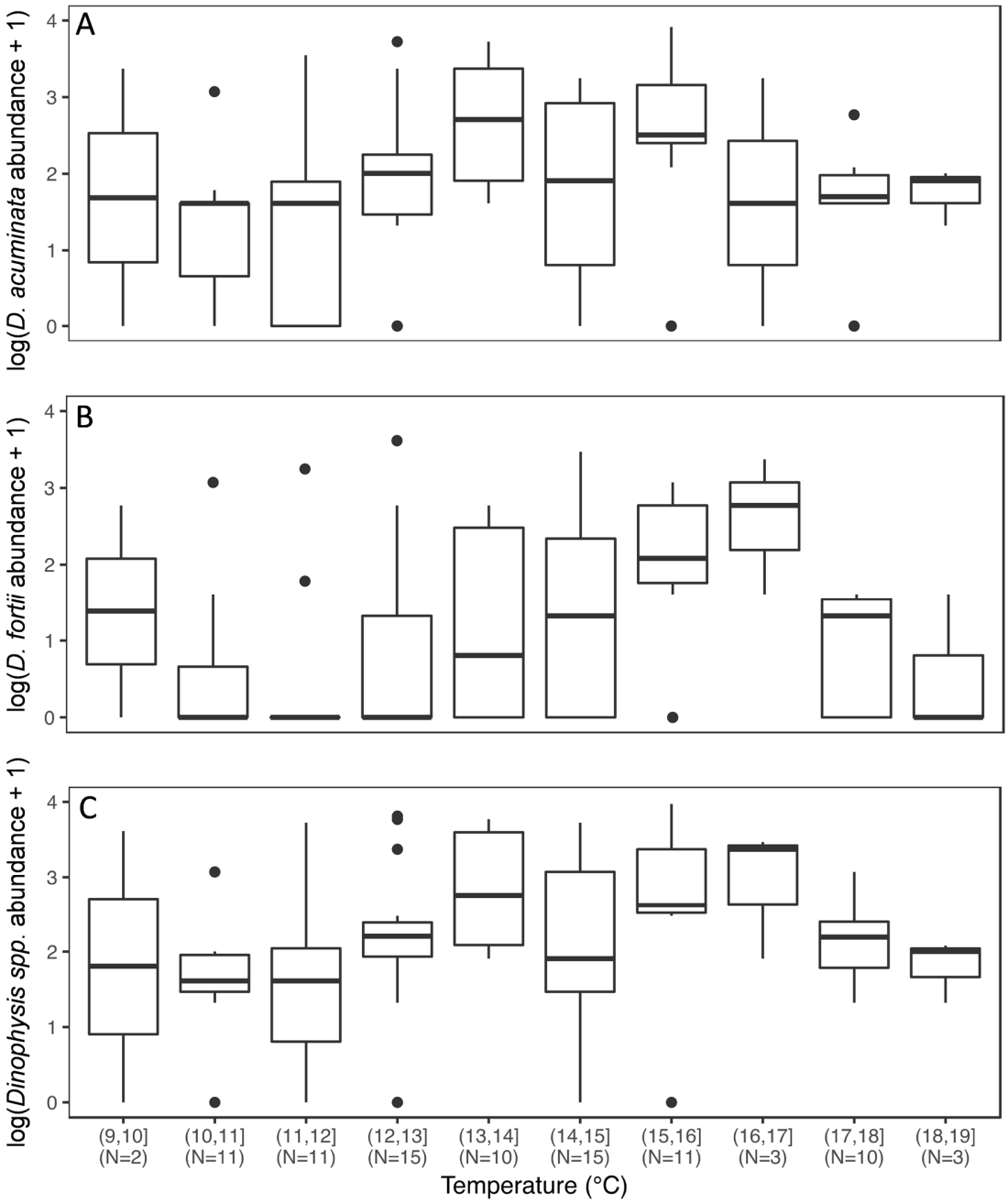

Fig. 7. $Log_{10}(x+1)$ transformed *Dinophysis* concentration data (2013-2014) with one-degree temperature bins for (A) *D. acuminata* complex, (B) *D. fortii*, and (C) genus-level *Dinophysis spp*. The box is centered on the median, with lower and upper hinges corresponding to the first and third quartiles.

Table 1. Results from a stepwise multiple linear regression of environmental variables to model *Dinophysis* concentration.

Variable	Coefficient	p Value
Ammonium (µm)	-0.77	0.07
Silicate (µm)	-0.55	0.08
Urea (µm)	-1.28	0.03
Salinity (ppt)	0.59	0.00

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.

Dinophysis Species	Proportion of weeks present (N=98)	Mean concentration (cells/L)	Maximum concentration (cells/L)
D. acuminata complex	0.76	626	8229
D. fortii	0.46	230	4114
P. rotundatum	0.3	75	1763
D. acuta	0.12	10	588
D. caudata	0.1	21	1176
D. tripos	0.05	7	588
D. norvegica	0.05	1	40
D. odiosa	0.01	6	588

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).

Source	Location	Species	Date	Mean (cells/L)	Median (cells/L)	Single Measure/ Maximum (cells/L)
Weber 2000*	SCMW	*Dinophysis spp.*	Jun 1999			20,000
Sutherland 2008*	SCMW	*D. acuminata*	2004	1000		5,000
	SCMW	*D. fortii*	2004	140		
	SCMW	*D. acuminata*	2005	870		
	SCMW	*D. fortii*	2005	2300		21,000
SCOOS	MW	*Dinophysis spp.*	2013-2016	263.2	30	7,935
	SCMW	*Dinophysis spp.*	Oct 2011			18,900
This study	SCMW	*Dinophysis spp.*	2013-2016	754	80	9,404

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

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