EFFECT OF CILIATE STRAIN, SIZE, AND NUTRITIONAL CONTENT ON THE GROWTH AND TOXICITY OF MIXOTROPHIC *DINOPHYSIS ACUMINATA*

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ABSTRACT

Previous studies indicate differences in bloom magnitude and toxicity between regional populations, and more recently, between geographical isolates of *Dinophysis acuminata*; however, the factors driving differences in toxicity/toxigenicity between regions/strains have not yet been fully elucidated. Here, the roles of prey strains (i.e., geographical isolates) and their associated attributes (i.e., biovolume and nutritional content) were investigated in the context of growth and production of toxins as a possible explanation for regional variation in toxicity of *D. acuminata*. The mixotrophic dinoflagellate, *D. acuminata,* isolated from NE North America (MA, U.S.) was offered a matrix of prey lines in a full factorial design, 1 x 2 x 3; one dinoflagellate strain was fed one of two ciliates, *Mesodinium rubrum*, isolated from coastal regions of Japan or Spain, which were grown on one of three cryptophytes (*Teleaulax/Geminigera* clade) isolated from Japan, Spain, or the northeastern USA. Additionally, predator: prey ratios were manipulated to explore effects of the prey's total biovolume on *Dinophysis* growth or toxin production. These studies revealed that the biovolume and nutritional status of the two ciliates, and less so the cryptophytes, impacted the growth, ingestion rate, and

maximum biomass of *D. acuminata*. The predator's consumption of the larger, more nutritious prey resulted in an elevated growth rate, greater biomass, and increased toxin quotas and total toxin per mL of culture. Grazing on the smaller, less nutritious prey, led to fewer cells in the culture but relatively more toxin exuded from the cells on per cell basis. Once the predator: prey ratios were altered so that an equal biovolume of each ciliate was delivered, the effect of ciliate size was lost, suggesting the predator can compensate for reduced nutrition in the smaller prey item by increasing grazing. While significant ciliate-induced effects were observed on growth and toxin metrics, no major shifts in toxin profile or intracellular toxin quotas were observed that could explain the large regional variations observed between geographical populations of this species.

KEYWORDS

Dinophysis, okadaic acid, pectenotoxin, *Mesodinium*, nutrition, mixotrophy

1. INTRODUCTION

Diarrhetic shellfish poisoning (DSP) toxins, i.e., okadaic acid (OA) and dinophysistoxins (DTXs), and/or the less-potent pectenotoxins (PTXs) have been detected in ten of the 75+ species of *Dinophysis* identified worldwide (Reguera et al., 2012; Gómez 2012). While other DSP toxin-producing species of this genus appear to have a more limited geographical range (e.g., *D. ovum*, Raho et al., 2008, Campbell et al., 2010), *Dinophysis acuminata* poses a threat to seafood safety along most major coastlines, including European, Atlantic coasts, Adriatic Sea, NE Japan, Australia, New Zealand, South Africa, California, Tasmania, NE and Mid-Atlantic North America (Reguera et al.,

50 2014 and references therein). Previous field and culture studies indicate significant differences in DSP
51 toxin content associated with *D. acuminata*, i.e., over an order of magnitude difference in amount of
52 DSP toxin per *D. acuminata* cell, among geographical populations and/or isolates (Lee et al., 1989,
53 Cembella 1989, Masselin et al., 1992, Tango et al., 2002; Park et al., 2006, Lindahl et al., 2007, Kim
54 et al., 2008, Kamiyama and Suzuki 2009, Riisgaard and Hansen 2009, Hackett et al., 2009, Suzuki et
55 al., 2009, Hattenrath-Lehmann et al., 2013, Trainer et al., 2013, Tong et al., 2015b). Even within a
56 region, significant variation exists; for example, *D. acuminata* populations from NE North America
57 (i.e., coasts of ME, MA and NY, U.S.) contain DSP toxins and PTX2, however, the relative
58 contributions of the toxin congeners varied between isolates: e.g., one isolate did not produce OA
59 (Tong et al., 2015b), and the intracellular level of OA was similar, greater, or less than DTX1,
60 depending on the isolate (Tong et al., 2015b, Hattenrath-Lehmann and Gobler 2015, Hattenrath-
61 Lehmann et al., 2015). Further emphasizing intraspecific variability, seven isolates of *D. acuminata*
62 from Denmark and isolated cells from Chile contained only PTX2, with no DSP toxins present
63 (Blanco et al., 2007, Fux et al., 2011, Nielsen et al., 2012). These inconsistencies in toxin profile and
64 significant differences in toxin content between regions are reflected in the observed, cross-regional
65 variations in incidence of shellfish harvesting closures due to DSP toxins (Reguera et al., 2014). The
66 factors driving these differences in geographical toxicity of *D. acuminata*, however, have not yet been
67 completely explained.

68 Laboratory studies into the physiology of *Dinophysis* spp. were logistically impossible until a
69 critical discovery by Park et al. (2006) led to the successful isolation and culturing of this genus in the
70 laboratory; mixotrophic *Dinophysis* require a unique multi-stage feeding regime whereby a
71 cryptophyte (photosynthetic nanoflagellate of the *Teleaulax/Geminigera* clade) is fed to *Mesodinium*

72 *rubrum* (a photosynthetic, mixotrophic ciliate) before the ciliate is fed to *Dinophysis*. Over the
73 subsequent decade, there has been a surge in laboratory studies investigating the relative importance
74 of prey, light, and dissolved nutrients in cell growth and/or toxin production by *Dinophysis*
75 *acuminata*. As a mixotrophic species, *D. acuminata* requires both prey, i.e., for particulate nutrients
76 and pigment function, and light to sustain photosynthesis, growth, and toxin production when
77 incubated in nitrate- and phosphate-rich medium (Park et al., 2006; Kim et al., 2008; Riisgaard and
78 Hansen 2009, Tong et al., 2011, Nielsen et al., 2012). Cells, however, could survive on reserves (with
79 no toxin production) for an additional two months after prey were removed as long as sufficient light
80 was provided, or only one month without light (Smith et al., 2012). More research is required to
81 investigate the importance of dissolved inorganic and organic nutrients in toxin production, but in
82 regards to growth, recent studies indicate that ammonium likely plays a direct role in *D. acuminata*
83 growth and bloom development (Hattenrath-Lehmann et al., 2013, 2015, Hattenrath-Lehmann and
84 Gobler 2015). Elevated levels of phosphate and nitrate, however, may indirectly impact *D. acuminata*
85 by promoting blooms of prey, *M. rubrum*, capable of rapid assimilation (Tong et al. 2015a,
86 Hattenrath-Lehmann et al., 2015b). The dinoflagellate may also be impacted by elevated levels of
87 dissolved organic nutrients, as growth increased when provided filtered, lysed ciliates (Nagai et al.,
88 2011), urea, an amino acid, or waste water organic matter (Hattenrath-Lehmann and Gobler 2015,
89 Hattenrath-Lehmann et al., 2015). While Kim et al., (2008) and Riisgaard and Hansen (2009) clearly
90 demonstrated a direct relationship between prey abundance and *D. acuminata* growth rate, the effects
91 of prey abundance, prey nutrition, or prey strain, on DSP toxin production remain uncharacterized.
92 The latter, i.e., prey strain, is of particular interest as 1) a possible impediment to invasion if *D.*
93 *acuminata* is a highly selective grazer on *M. rubrum* strains or is unable to sustain growth equally

amongst strains, or 2) a driver of regional toxicity due to variability in nutrition, e.g., a more or less nutritious prey strain leads to more or less toxic *D. acuminata* in that region. Recent molecular evidence also supports this line of investigation as it points toward a more diverse array of cryptophyte-ciliate prey than originally proposed (Kim et al., 2012a, b).

The effect of prey strain, prey nutritional content, and prey biovolume on the growth, toxin production, and toxin exudation by an isolate of *D. acuminata* were investigated. The overall goal of this work was to assess if intrinsic differences between geographically-isolated prey strains (e.g., differences in maximum cell abundances, cell size, and nitrogen, carbon and phosphorus content) could potentially account for the observed variability in toxin profiles and bloom toxicity levels observed across regions. From these data, new hypotheses can be formed regarding whether local prey species could serve as barriers to *D. acuminata* immigration.

2. METHODS

An isolate of *Dinophysis acuminata* from the northeastern USA was offered a matrix of prey lines in a full factorial design, 1x2x3; where one dinoflagellate isolate was fed one of two ciliates as prey, at a ratio of 1:15 predator:prey. The two ciliates, *Mesodinium rubrum*, were isolated from coastal regions of Japan or Spain, which were grown on three cryptophytes, *Teleaulax/Geminigera* clade, isolated from Japan, Spain, or the northeastern USA. As the biovolume of *M. rubrum* from Japan was 3.3x greater than the isolate from Spain, a second set of experiments was conducted where predator to prey ratios were changed from 1:15, to represent equal prey biovolume (1:33 in the Spanish treatment and 1:10 in the Japanese treatment). Intracellular and extracellular toxin levels, cell abundances, grazing rates, and the nutritional content of the ciliate prey were monitored over time, with a focus on exponential and plateau growth phases of *D. acuminata*.

2.1 Culture maintenance

The mixotrophic dinoflagellate *D. acuminata* (DA) used in these experiments was isolated from Eel Pond, MA U.S. in 2006 (strain DAEP01, Hackett et al., 2009). Two isolates of the ciliate *Mesodinium rubrum* (MR), and three isolates of cryptophyte, identified as either *Teleaulax amphioxeia* (TA) or *Geminigera cryophila* (GC) were also cultured for the experiments (Table 1). Two prey lines were utilized, consisting of *T. amphioxeia* and *M. rubrum* from Japan (JA, Nishitani et al., 2008), and *T. amphioxeia* and *M. rubrum* from Spain (SP, Rodriguez et al., 2012). An isolate of *G. cryophila* isolated from the U.S. (strain USGC, originally isolated as GCEP02 from Eel Pond, MA in 2008) was also included in the experimental design. A local ciliate was not utilized as attempts to isolate from this location have been unsuccessful.

Two additional isolates, from Antarctica, were utilized in maintenance culturing only (Table 1); *D. acuminata* cultures were maintained at 6ºC with the addition of Antarctic *Mesodinium rubrum* (=*Myrionecta rubra*, CCMP2563) at a ratio of 1:10 predator:prey. *M. rubrum* was, in turn, maintained using Antarctic *G. cryophila* (CCMP2564) at a ratio of 1:10 predator:prey (Tong et al., 2010). The isolates of ciliate used in the experiments, originating from Japan and Spain, were maintained at 19ºC under 50 µmol photons m^{-2} sec^{-1} of light on a 14:10 hr light:dark photocycle (Table 1), and fed their respective cryptophyte prey from Japan or Spain, respectively.

In preparation for the experiment, *D. acuminata* cultures were starved 40-54 days with the goal of clearing internal reserves previously accumulated from the maintenance prey line. Two days before the beginning of the experiment, cultures of *D. acuminata* were 10-µm sieved to concentrate cells and remove debris. Dinoflagellate cells were resuspended in clean filtered seawater, and cultures were warmed stepwise from the maintenance temperature, 6°C, to the experimental temperature, 15°C. To

prepare the ciliate isolates for the experiment, two strains of *M. rubrum* (MR), from Japan (JA) and Spain (SP), were inoculated into multiple 2.8-L Fernbach flasks with fresh f/12-Si medium, starved for one week, and then fed with the experimental cryptophyte prey from Japan, Spain, or the U.S. at a ratio of 1:10 predator:prey. The ciliates then fed on the experimental prey lines for two months prior to the beginning of the experiments. The ciliate cultures were cooled to the experimental temperature and held for one week before being fed to *D. acuminata*, marking the beginning of the experiment.

2.2 Experimental design

Experiment 1: Effect of prey strain on Dinophysis *growth and toxigenicity.*

To begin the first experiment, *D. acuminata* culture was divided between six treatments, conducted in triplicate, consisting of various combinations of ciliate and cryptophyte prey: (1) JAMR grown on JATA, (2) JAMR grown on SPTA, (3) JAMR grown on USGC, (4) SPMR grown on JATA, (5) SPMR grown on SPTA, and (6) SPMR grown on USGC (Table 1). The dinoflagellates were fed JAMR or SPMR at the time of inoculation, at a ratio of 1:15, and then allowed to deplete the food source. Experimental flasks, containing f/12-Si medium, predator, and prey, were incubated at 15°C with 65 µmol photons m^{-2} sec^{-1} of light on a 14:10 hr light:dark photocycle and randomized daily on the shelf to account for any minor light variability. The abundances of prey and predator, nutrient content of the prey types, and intracellular and extracellular toxin levels were monitored over exponential and plateau growth phases.

Experiment 2: Effect of prey biovolume on Dinophysis *growth and toxigenicity.*

The biovolume of the Japanese ciliate was 3.3x greater than the Spanish ciliate prompting us to conduct a second set of experiments varying predator to prey ratios to account for biovolume and

159 nutritional differences. A subset of *D. acuminata* treatments from the first experiment, i.e., JAMR +
160 JATA and SPMR + SPTA, were re-fed at mid-plateau phase with ratios of predator:prey matching the
161 first feeding, 1:15, and ratios that represented an equal amount of prey biomass, i.e., 1:33 in the
162 Spanish treatment and 1:10 in the Japanese treatment. Two controls were also included in the second
163 experiment, in which *D. acuminata* treatments from the original feeding experiment, JAMR + JATA
164 and SPMR + SPTA, were not re-fed, but allowed to continue without additional food during this
165 period. As such, this experiment included six treatments in total: 1) 1:15 of JAMR grown on new
166 JATA, 2) 1:10 of JAMR grown on new JATA, 3) 1:15 of SPMR grown on new SPTA, 4) 1:33 of
167 SPMR grown on new SPTA, and the 2 controls without new prey. The other four treatments from the
168 first experiment were not carried into the second.

169 ***2.3 Growth rate and biovolume***

170 Triplicate 1.5-mL subsamples were taken for *M. rubrum* and *D. acuminata* enumeration;
171 subsampling occurred every other day throughout the experiments. Subsamples were removed
172 directly from the flasks, fixed with a 0.2% v/v Acid Lugol's (Tong et al., 2010), and enumerated for
173 cell concentrations using a Sedgewick-Rafter chamber and microscope at 100X magnification.

174 The average growth rates of *D. acuminata* and the ciliate prey, *M. rubrum,* were calculated over
175 exponential growth (spanning 5 time points) using the formula by Guillard (1973):

176 $$\mu = \frac{\ln(C_2 / C_1)}{t_2 - t_1} \qquad (1)$$

177 In this equation, C_1 and C_2 are the concentrations of cells at time 1 and time 2 (cells/mL), respectively,
178 t is the experimental time (day), and μ (day^{-1}) is the growth rate.

179 The ingestion rate of *D. acuminata*, U (cells/predator/d), was calculated using the model developed

by Jakobsen and Hansen, (1997):

$$\frac{dx}{dt} = \mu_x \cdot x - U \cdot y \quad (2)$$

$$\frac{dy}{dt} = \mu_y \cdot y \quad (3)$$

The ingestion rate calculation assumes that the predator concentration y (*D. acuminata*) and prey concentration x (*M. rubrum*) grew exponentially, with growth rate constants of μ_y and μ_x, respectively.

The diameters of 20-30 ciliate cells of JAMR and SPMR, fed JATA and SPTA respectively, were measured using the software of Carl Zeiss AxioVision Rel. 4.8 and a microscope at 100X magnification. The average ciliate volume was calculated using the following formula, assuming *M. rubrum* cells are spheres;

$$V = 4\,\pi\,r_3/\,3 \quad (4)$$

where r is the radius of a cell.

2.4 Particulate nutrient composition

In the first experiment, the six treatments of *M. rubrum*, fed various cryptophyte prey, were harvested for particulate nutrient analyses and replicate flasks were processed separately. Ciliate harvesting occurred after cryptophytes were removed via grazing to assure that the nutrient composition reflected only the ciliate.

For nutrient analyses, 10 mL of culture were collected through pre-combusted GF/F filters (450°C for 4 hours, 0.8 μm, 25 mm) for total particulate organic carbon/nitrogen analysis (CHN). Another 10 mL of culture was collected through membrane filters (PALL Supor R-800, 0.8μm, 25mm) for the determination of total particulate phosphorus. After collection, all filters were placed in

a 60°C drying oven for 24 hours and stored at -20°C. Potassium persulfate, 5 mL of 5%, and 10 mL of Milli-Q water were added to the particulate phosphorus filters and autoclaved (121°C) for 20 min. After hydrolization, all particulate phosphorous was converted to, and was measured as, dissolved orthophosphate (PO_{43-}). Solid phase carbon, i.e., particulate carbon, and nitrogen samples were analyzed on a Flash EA1112 Carbon/Nitrogen Analyzer at WHOI using a Dynamic Flash Combustion technique.

2.5 Toxin extraction and analysis

Harvesting of the cultures for toxin analysis occurred during two growth phases in the first experiment, on days 13 (late exponential) and 32 (plateau), and days 16 (late exponential) and 38 (plateau), for the Japanese and Spanish treatments, respectively (Figure 1). In the second experiment, cells were harvested prior to refeeding and then at the transition between late exponential and early plateau growth phases. To harvest, batch cultures were gently swirled and the appropriate volume of culture (i.e., equivalent to 100,000 *Dinophysis* cells) was aseptically removed from each flask and then sieved through a 13-µm mesh adhered to a PVC tube (diameter of 3 cm). To minimize cell damage, no vacuum was applied and the mesh and cells were kept wet at all times in a glass petri dish filled with fresh filtered seawater. The desired volume of culture was poured through a sieve and collected in a beaker. Media and cells were hereafter separated and processed independently for toxin concentration.

The *Dinophysis* cells were washed from the sieve into 15-mL falcon centrifugation tubes using 14 mL of fresh filtered seawater. Subsamples were collected from the tubes for cell enumeration. Tubes were frozen overnight (-20 °C) and then thawed at room temperature for 24 h, in the dark, to allow for enzymatic hydrolysis of diol-esters previously identified in this isolate (Fux et al., 2011) to

222 OA and DTX1 (Quilliam et al., 1996). All cell samples were bath sonicated for 15 min (Fisher
223 ultrasonic cleaner, Model FS30H) and loaded onto an Oasis HLB 60 mg cartridge (Waters, Millford,
224 MA) that was previously equilibrated with 3 mL of methanol (MeOH) and 3 mL of Milli-Q water. The
225 cartridge was washed with 6 mL of Milli-Q water, blown dry, and eluted with 1 mL of 100% MeOH
226 into a glass 1.5-mL high recovery LC vial and stored at -20°C until analysis by LC-MS/MS (liquid
227 chromatography coupled to tandem mass spectrometry).

228 The sieved media were loaded onto an Oasis HLB solid phase extraction (SPE) cartridge (60 mg,
229 Waters, Millford, MA) immediately after separation from cells. Media samples did not undergo the
230 24-hr enzymatic hydrolysis step as the majority of extracellular toxins were assumed to have already
231 converted to the parent toxins, OA and DTX1; this was not confirmed empirically. The SPE cartridge
232 was equilibrated with 3 mL of MeOH and 3 mL of Milli-Q water prior to loading. After loading, the
233 SPE cartridge was washed with 3 mL of Milli-Q water, blown dry, and the toxins eluted with 1 mL of
234 100% MeOH into a glass 1.5-mL high recovery LC vial and stored at -20°C (modified from Suzuki et
235 al., 2009).

236 To confirm that toxins were not produced by the prey, maintenance cultures were harvested to
237 produce samples of *ca.* 200,000 ciliates or *ca*. 1,000,000 cryptophytes in 15-mL tubes. Samples were
238 centrifuged at 4,200 x g for 5 min, and the overlaying seawater discarded. The remaining cell pellets
239 were then extracted using four cycles of 1) bath sonication with 200 µL of MeOH for 15 min, 2)
240 centrifugation at 4,200 x g for 5 min, and 3) transfer of the MeOH supernatant to a tube. The methanol
241 extracts were pooled, and pushed through a syringe filter (0.2 µm) into a glass 1.5-mL high recovery
242 LC vial and stored at -20°C (modified from Hackett et al., 2009; Rodriguez et al., 2012).

243 To reduce any error associated with varying eluate volumes from the SPE clean-up step, all

MeOH eluates were heated to 40°C using a heating block, taken to dryness under a stream of N_2, and resuspended in 1 mL of MeOH prior to LC-MS/MS analysis. Analysis of medium and cell samples was performed on a Quattro Ultima (Micromass, Waters) coupled with an 1100 Agilent HPLC. Separation was achieved on a C8 Hypersil column (50 x 2.1 mm; 3.5 µm particle size) maintained at room temperature. The flow rate was set at 0.25 mL/min and a volume of 10 µL was injected. Binary mobile phase was used, with phase A (100% aqueous) and phase B (95% aqueous acetonitrile) both containing 2 mM ammonium formate and 50 mM formic acid (Quilliam et al., 2001). A gradient elution was employed, starting with 30% B, rising to 100% B over 9 min, held for 3 min, then decreased to 30% B in 0.1 min and held for 3 min to equilibrate at initial conditions before the next run started. The triple quadrupole was operated in multiple reaction monitoring (MRM) mode and the following transitions were monitored in two runs: OA, m/z 803.5>255.5 and 803.5>803.5; DTX1, m/z 817.5>255.5 and 817.5>817.5 in negative ionization mode and PTX2, 876.5>213.0 in positive ionization mode. OA and DTX1, or PTX2 were quantified using the daughter transitions, against 7 level calibration curves using OA or PTX2 reference solutions (NRC- Canada), ranging from 6 – 500 ng OA/µL or ng PTX2/µL, respectively. Toxin data are expressed as toxin content (pg/cell) or toxin concentration (ng/mL of culture).

The net toxin production rate, R_{tox}, (toxin units/cell/d) was calculated for each toxin (OA, DTX1, and PTX2) between the initial and first sampling point, during late exponential growth, for each treatment using the following equation (Anderson et al., 1990):

$$R_{tox} = \frac{(C_2T_2 - C_1T_1)}{(\overline{C})(t_2 - t_1)} \quad (5)$$

where $\overline{C}$ is the ln average of the cell concentration,

$$\overline{C} = \frac{C_2 - C_1}{\ln(C_2 / C_1)} \quad (6)$$

In this equation, C_1 and C_2 are the concentrations of cells at time 1 and time 2 (cells/mL), respectively, and t is the experimental time (day). The toxin concentration, C_tT_t (toxin units/mL culture), was determined by multiplying C_t (cells/mL) by T_t, the cellular toxin content (toxin units/cell) at time t.

2.6 Statistical Analysis

After the determination of normality, all toxin data, growth rate, and cell biomass data were subjected to one-way repeated measures ANOVA (Systat Software 9.0) with Holm-Sidak pairwise comparisons to test for effects of time or treatment. Repeated measures analyses were chosen because the same flasks were sampled over time. Primary statistical analysis did not detect a difference in toxin quotas or total toxin concentrations between cryptophyte treatments within a ciliate strain, i.e., no effect of cryptophyte strain, and so the three cryptophyte treatments were grouped by ciliate (i.e., n = 9 for each ciliate strain) for all later statistical analyses, unless otherwise noted. Alpha was set at 0.05 for all analyses.

3. RESULTS

3.1 Prey strain and nutritional content (Experiment 1)

In the first experiment, an isolate of *Dinophysis acuminata* from the northeastern U.S. was offered a matrix of prey lines consisting of two ciliates, i.e., *Mesodinium rubrum,* isolated from coastal regions of Japan or Spain, which were grown on one of three cryptophytes isolated from

285 Japan, Spain, or the northeastern U.S (Table 1). The origin or strain of ciliate, and less so the
286 cryptophyte, directly impacted the growth of *Dinophysis* (Table 2). More specifically, when provided
287 the same ratio of predator:prey (1:15), the dinoflagellate grew significantly faster in the three
288 treatments offering a Japanese ciliate as prey, versus those three treatments grown on a Spain-derived
289 ciliate ($p<0.001$, $n=9$). This effect on *Dinophysis* growth was independent of the cryptophyte strain
290 provided to the ciliate as prey (Table 2).

291 After the complete consumption of ciliates, all *Dinophysis* cultures began their transition into late
292 exponential growth and then plateau phase (Figure 1). Faster *Dinophysis* growth rates in the Japanese-
293 ciliate treatments resulted in greater *Dinophysis* biomass; the average maximum cell concentration of
294 *Dinophysis* in the Japanese-ciliate treatments (3,099 ± 277 cells/mL) was more than twice that of the
295 Spanish-ciliate treatments (1,314 ± 300 cells/mL; Figure 1, Table 2). The average ingestion rate,
296 calculated over the period of *Dinophysis* exponential growth, was significantly lower, however, when
297 the dinoflagellate grazed on the Japanese ciliates (0.08 ± 0.07 cells/d) rather than the Spanish ciliates
298 (1.15 ± 0.2 cells/d, Table 3).

299 The ciliate from Japan was 3.3x larger in volume than the strain from Spain, a significant size
300 difference that may be responsible for the observed differences in *Dinophysis* growth rate, biomass,
301 and ingestion rate between ciliate lines in Experiment 1 (Tables 2 and 3). The average cell diameters
302 and biovolumes (25 ± 5 μm; 7,940 μm^3 and 17 ± 2 μm; 2,390 μm^3, respectively) were determined for
303 the Japanese and Spanish ciliates based on the assumption that a ciliate is a sphere. The average
304 quotas of particulate carbon (C), nitrogen (N), and phosphorous (P) were also approximately three
305 times greater in the Japanese ciliate strain than in the Spanish ciliate. While the nutrient contents of
306 the two ciliates were significantly different, this difference was not due to the cryptophyte strain that

was fed to the ciliate; i.e., the nutrient content was similar within a ciliate strain despite which of the three cryptophyte strains were consumed, and the two ciliates strains had significantly different nutrient contents even when feeding on the same cryptophyte (Table 2).

Diarrhetic shellfish poisoning toxins and pectenotoxins were present in *Dinophysis* cells and media over the entire growth cycle in all treatments, and as expected, no toxins were detected in any ciliate and cryptophyte monocultures. Intracellular OA and DTX1 toxin quotas in *Dinophysis* were significantly greater during plateau phase than exponential growth phase; this pattern was consistent within each ciliate strain (Figures 2A, 2B, 2C, 2D; RM ANOVA). When comparing between ciliate strains, *Dinophysis* cells grown on the Japanese ciliate contained significantly more OA and DTX1 per cell than *Dinophysis* grown on the Spanish ciliate (Figures 2A, 2B, 2C, 2D; RM ANOVA). This effect was growth-phase dependent, with the elevated OA and DTX1 quotas in the Japanese-ciliate treatments occurring during late exponential and plateau phases, respectively. Pectenotoxin-2 quotas in *Dinophysis* were similar between ciliate strains; however, the highest toxin quota was observed during plateau phase in culture of *Dinophysis* fed the Spanish ciliate (Figures 2E, 2F).

When extracellular toxins were considered in the analysis, i.e., when intra and extracellular toxins were summed per milliliter of culture, the link between the consumption of Japanese ciliates and increased DSP toxicity was further strengthened; there was significantly more *total* OA and DTX1 in the culture when *Dinophysis* was fed the Japanese ciliate, relative to the Spanish ciliate (Figures 3A, 3B, 3C, 3D; RM ANOVA). Similarly, Japanese-ciliate treatments contained significantly higher total concentrations of PTX2 than *Dinophysis* cultures fed the Spanish ciliate (Figures 3E, 3F).

Overall, there was no difference in the daily production rates of intracellular OA, DTX1, and PTX2 between treatments, i.e., no detectable effect of ciliate or cryptophyte on *intracellular* toxin

329 production rates by *D. acuminata* (Table 4). Once extracellular toxin levels were accounted for in the
330 rate calculation, however, the *Dinophysis* cultures fed the Spanish ciliate demonstrated elevated *total*
331 toxin production rates of OA and PTX2, but not DTX1 (Table 4). This general trend of increased total
332 toxin production rates (Table 4) within the Spanish ciliate treatments was not due to a greater amount
333 of extracellular toxin in the medium (toxin/mL of culture, Table 5), as these cultures actually
334 contained lower extracellular concentrations during exponential and plateau growth phases compared
335 to the Japanese-ciliate treatments (Figures 3A, 3B, 3C, 3D, 3E, 3F, Table 5). The enhanced *total* toxin
336 production in the Spanish-ciliate treatments is instead due to elevated levels of extracellular toxin *per*
337 *cell* (Table 5).

338 By transforming the intracellular and extracellular toxin concentrations to percentages, it
339 becomes apparent that *Dinophysis* cultures fed the Spanish ciliate also had a slightly higher
340 percentage of their total DSP toxins external to the cell, compared to the Japanese-ciliate treatments,
341 when averaged over the two growth phases and all cryptophyte treatments (Table 5). The proportion
342 of the PTXs external to the *Dinophysis* cells, 32%, was similar between ciliate strains, but overall
343 much lower than extracellular DSP toxins, 65 – 80%.

344 The single, but notable, exception to these trends in total toxin and production rates occurred in
345 one of the six ciliate+cryptophyte treatments: *Dinophysis* grown on the pure Spanish prey line
346 (SPMR+SPTA). The pure Spanish-line treatment, with a final *Dinophysis* concentration of 1,210 ±
347 137 cells/mL, contained similar amounts of total OA during plateau phase as the more-dense
348 treatments of *Dinophysis* fed the Japanese ciliate, containing 3,425 ± 186 cells/mL. When this one
349 treatment was excluded from the statistical analysis, the OA results fell in line with general findings,
350 i.e., that *Dinophysis* grown on the Japanese ciliate contained significantly more total toxin than those

grown on the Spanish ciliate (Figures 2A, 2B). Similarly, the pure Spanish-line treatment (SPMR+SPTA) stood out as an anomaly when extracellular toxin concentrations were included in toxin production rates; the SPMR+SPTA treatment consistently showed the highest total OA, DTX1, and PTX2 production rates over all six treatments (Table 4). This pure Spanish-line treatment also contained more extracellular OA in a milliliter of medium than the Japanese equivalent treatment (JAMR+SPTA) (Figures 3A, 3B), despite having a reduced biomass of *Dinophysis* (Figures 1A, 1B). These two anomalies, both tied to extracellular toxin concentrations and the pure Spanish-line treatment, are the only example of a perceived cryptophyte-induced effect in this study.

Overall, Japanese-ciliate treatments had significantly higher OA and DTX1 toxin quotas and lower PTX2 quotas relative to Spanish-ciliate treatments (Figure 2). This pattern, however, was weakened once quotas were averaged across all treatments and growth phases, and the data transformed into percent toxin composition. More specifically, ciliate strain did not have a detectable effect on *Dinophysis* toxin profile in this experiment (Table 5), as all *Dinophysis* intracellular toxin profiles were dominated by PTX2 (92 – 96 %), with much smaller contributions by DSP toxins: DTX1 (3 – 6 %) and OA (≤1 %).

3.2 Prey biovolume (Experiment 2)

In a second set of experiments, the ratios of predator to prey were altered in an attempt to correct for differences in biovolume and/or nutrition between the two ciliate strains. When the first experiment reached plateau phase (Figure 1), the *Dinophysis* cultures from two treatments (JAMR+JATA and SPMR+SPTA) were diluted with fresh medium and refed their respective prey at dinoflagellate:ciliate abundance ratios of 1:10 and 1:15 for *Dinophysis*:JAMR, and 1:15 and 1:33 for *Dinophysis*:SPMR, marking the beginning of the second experiment.

As in the first experiment, when both ciliates were offered at a 1:15 ratio, *Dinophysis* grew significantly faster when fed the Japanese versus the Spanish ciliate (Table 6). When the dinoflagellate was fed equal biovolumes of the two prey types, i.e., predator:prey ratios of 1:10 for Japan and 1:33 for Spain, the ciliate-induced effect on *Dinophysis* growth was removed. The dinoflagellate, therefore, may be able to compensate for inferior nutrition in the Spanish ciliate by increasing their consumption of the smaller ciliate (Table 3).

The *Dinophysis* cells and medium were harvested in the second experiment once treatments reached late exponential growth, intracellular and extracellular levels of OA, DTX1, and PTX2 were quantified, and toxin production rates calculated. A main finding from the first experiment, i.e., elevated total DSP toxin production rates by *Dinophysis* fed the Spanish ciliate and Spanish cryptophyte, continued into the second experiment (Table 7). Adjusting the predator:prey ratios to balance prey biovolume further enhanced this effect; the *Dinophysis* culture fed the greater number of smaller Spanish ciliates, the 1:33 treatment, showed the overall highest rates of total OA, DTX1, and PTX2 production amongst all the treatments in both experiments.

4. DISCUSSION

Overall, the U.S. isolate of *Dinophysis* grew faster and achieved greater maximum cell concentrations when fed equal numbers of the Japanese ciliate as opposed to the ciliate from Spain. The Japanese ciliate was 3x larger in biovolume and was more nutritious, i.e., 3X more C, N, and P per cell (Table 2, Figure 1). Together this suggests that the Japanese ciliate was a more beneficial prey item for the U.S. *Dinophysis* isolate. It is important to point out that the ciliate-specific effects on *Dinophysis* growth and biomass held true over all cryptophyte strains for each ciliate strain, meaning that the ciliates, and not the cryptophytes, were responsible for any observed effects. When the

predator:prey ratio of *Dinophysis* and ciliate was then adjusted in the second experiment to equate the preys' biovolume and bulk C, N, and P nutrition, *Dinophysis* grew equally well on both ciliate strains (Table 6). It, therefore, appears that *Dinophysis* can increase prey consumption to compensate for nutritional deficiencies due to small prey size and/or lower nutritional content; more simply, they can eat more nutrient-poor prey to achieve the same growth rates as when eating fewer nutrient-rich prey. It can also be inferred from this study that any energy costs associated with increased grazing on the less nutritious prey (e.g., extra effort needed in searching, catching, and feeding on additional small prey) did not have a measurable, detrimental effect on overall *Dinophysis* growth.

The ability of the dinoflagellate to compensate for less nutritious prey through increased grazing will likely be limited by the cell concentration of its prey. A comparison of ingestion rates between the first and second experiment (Table 3), for example, demonstrates that rates increased in all ciliate treatments once the initial prey concentration was increased at inoculation. Similarly, Kim et al., (2008) found that prey concentrations of 1000 cells/mL or less appear to have a dampening effect on ingestion rate by *Dinophysis* in culture. Given that bloom concentrations of *M. rubrum* are typically below 200 cells/mL but can occasionally reach above 3,000 cells/mL in U.S. coastal waters (Johnson et al., 2013, Harred and Campbell 2014), there is a need to further investigate a possible lower threshold at which *Dinophysis* grazing upon *M. rubrum* is insufficient for growth and toxin production in the field. A threshold may exist whereby *Dinophysis* spp. turn to other ciliates (Harred and Campbell 2014) or dissolved nutrients (ammonium and/or urea, Hattenrath-Lehmann and Gobler 2015, Hattenrath-Lehmann et al., 2015) for their nutritional requirements. Defining the thresholds of these possible drivers of *Dinophysis* abundance and toxicity would be important for future DSP management and mitigation strategies.

417 Intracellular levels of OA and DTX1 were also influenced at the ciliate (not cryptophyte) level,
418 with the consumption of larger, more nutritious Japanese ciliates leading to more intracellular and
419 total (intracellular + extracellular) DSP toxins in the *Dinophysis* cultures (Figures 2, 3). This pattern
420 was again consistent across cryptophyte treatments within a ciliate strain (Figure 2, Table 5), meaning
421 cellular toxigenicity was independent of what cryptophyte was previously fed to the ciliate.

422 In general, more DSP toxins were contained in each cell during plateau phase, relative to
423 exponential growth phase, consistent with previous reports that the uncoupling of cell division and
424 toxin production after exponential growth leads to increased toxin quotas (Tong et al., 2011, 2015b).
425 Similarly, the ciliate strain also influenced the concentration of total toxin in the culture (ng/mL); the
426 consumption of the larger, more-nutritious Japanese ciliate led to increased dinoflagellate growth rates
427 and biomass, and therefore, more cells and significantly more total OA and DTX1 in the *Dinophysis*
428 culture. Together with the growth data, this suggests that it is the nutritional content and/or biovolume
429 of the ciliate, and less so the cryptophyte, that determines *Dinophysis* growth rates and maximum
430 biomass, and therefore, total toxins in the culture. Other unmeasured attributes of *M. rubrum* (e.g.,
431 stoichiometry beyond C, N, and P, and associated bacteria), should also be considered in future
432 studies focused on understanding the relationship between predator and prey as they relate to
433 *Dinophysis* growth and toxin production.

434 Interesting, however, it was the *Dinophysis* cultures that consumed the less nutritious, smaller
435 Spanish ciliate that actually produced significantly more total DSP toxins and PTXs per cell per day,
436 (Table 4). This may seem contrary to what was expected given that the Japanese treatments contained
437 significantly more total toxins in the culture and had higher toxin quotas, but the *Dinophysis* fed the
438 Spanish ciliate exuded more toxins relative to the Japanese-ciliate treatments. Specifically, more DSP

toxins and PTX2 were found external to the cell, on a per cell basis, when *Dinophysis* was fed the Spanish ciliate. Spanish-fed cultures also had a greater proportion of DSP toxins associated with the dissolved fraction of the culture, i.e., percent external to the cell (Table 5). In other words, the slower growing, lower biomass *Dinophysis* cultures that consumed the less nutritious Spanish ciliate passively or actively released more toxin extracellularly per cell, and therefore, may have produced elevated amounts of new toxin per day to maintain intracellular quotas (Table 4). When the ratio of predator to prey was altered in the second experiment to deliver an equal total biovolume of Spanish ciliates (1:33 treatment) as in the Japanese-ciliate treatments (1:10 treatment), the exudation effects of the Spanish ciliate further intensified. Therefore, in the case where a smaller, less nutritious ciliate is present, but in high abundance, *D. acuminata* has the potential to reach high bloom densities and produce more DSP toxins (per cell per day), however, the majority of these DSP toxins may be extracellular and potentially less available for trophic transfer to humans.

On the other hand, increased extracellular toxin levels may have allelopathic consequences for the phytoplankton community. Okadaic acid and DTX1 are inhibitors of serine and threonine protein phosphatases, and are capable of negatively impacting the growth of microalgae (Windust et al., 1996, Windust et al., 1997). These effects may also extend to *Dinophysis* prey, as problems isolating *M. rubrum* from field samples were attributed to detrimental or allelopathic activity associated with *Dinophysis* (Nagai et al., 2008, Hansen et al., 2013). *Mesodinium rubrum* cells exhibited abnormal behavior when exposed to high densities of *D. fortii*, such as forming clumps or rotating in-place, followed by cell mortality (Nagai et al., 2008). It is not yet known if stressed *D. acuminata* produce and exude more toxin to enhance prey capture. Toxin exudation has previously been reported in cultures of *D. acuminata* and *D. fortii*, with 79.5 – 86.6% of the total OA and DTX1 being external to

461 the cells during exponential growth (Nagai et al., 2011). Results presented here show a similar
462 proportion of DSP toxins external to the cell, 65 – 80% (Table 5), but one cannot conclude that the
463 increased extracellular toxins present in the Spanish-ciliate treatments were due to active or passive
464 exudation. Additional measurements and treatments, e.g., cell viability/permeability assays,
465 transporter inhibition assays, and treatments with cultures in exponential growth incubated with and
466 without prey, would be necessary to conclude active exudation and target the mechanism. Active
467 toxin exudation has also been suggested in other toxic phytoplankton such as *Prymnesium parvum,*
468 *Alexandrium pseudogonyaulax*, and *Karlodinium veneficum*, species which release bioactive
469 chemicals into the environment to "trap" or "immobilize" their prey and assist in feeding (Skovgaard
470 and Hansen 2003, Sheng et al., 2010, Blossom et al., 2012). Alternatively, DSP toxins may be
471 passively released by *D. acuminata* during feeding, cell division, impaired cell viability, or as a result
472 of cell death.

473 This exudation effect was further enhanced in one cryptophyte treatment, when *Dinophysis* were
474 fed the Spanish ciliate and cryptophyte combination (SPMR+SPTA), thereby providing the only
475 evidence in this work for an effect of cryptophyte. This treatment showed the highest rate of total OA
476 production over all cryptophyte and ciliate treatments (Table 4) and contained similar amounts of total
477 OA per milliliter of culture to the Japanese-ciliate treatments despite having fewer dinoflagellate cells
478 in the culture (Figure 3). As with all other Spanish-ciliate treatments, this extra toxin was found to be
479 external to the *Dinophysis* cell, i.e., associated with the dissolved fraction in the medium. This
480 observed effect of cryptophyte hints at an underlying importance of the nutritional content or quality
481 of cryptophytes; however, a specific macro-elemental factor was not borne out by ciliate CHN
482 measurements presented here (Table 2), and as such, other chemical factors could be contributing.

Previous studies have shown an effect of cryptophyte strain and cell concentration on *M. rubrum* growth (Yih et al. 2004, Park et al. 2007), suggesting a cascading effect on *Dinophysis* growth should be further considered, but instead focused on other nutritional elements or geographical isolates.

Despite showing a significant effect of ciliate strain on *D. acuminata* toxin content and total toxin, the observed level of effect does not explain the vast differences in isolate and bloom toxicity found across the globe (i.e., an order of magnitude increase in toxin content or a shift in toxin profile). From this, it appears that cross-regional differences in toxin profile and bloom toxicity are not due to the strain of *M. rubrum* or cryptophyte consumed. This conclusion is supported by the results of Nagai et al., (2011) and Gao et al., (2017) who fed the same Japanese cryptophyte and ciliate strains used in this study to a *D. acuminata* isolate from Japan and China. The Japanese and Chinese *D. acuminata* isolates produced much higher maximum toxin quotas of PTX2 (73.3 pg/cell and 18.5 pg/cell, respectively) and OA (58.8 pg/cell and 0.54 pg/cell, respectively), than were quantified in the U.S. *D. acuminata* cultures tested here (10.39 pg PTX2/cell and 0.11 pg OA/cell, Table 5). Maximum toxin quotas of DTX1 measured in the U.S. isolate (0.67 pg DTX1/cell, Table 5) were moderate, however, being greater than maximum levels measured in the Chinese isolate (0.05 pg DTX1/cell), but less than maximum toxin quotas measured in the Japanese isolate (9.6 pg DTX1/cell). In other words, feeding upon the same isolates of cyrptophyate + ciliate did not result in the same toxigenicity across U.S., Japanese, and Chinese isolates of *D. acuminata*, suggesting a more intrinsic attribute of these dinoflagellate strains is responsible for toxicity.

4.1 Conclusions

Surprisingly, relatively minimal changes to potential toxicity were detected when an isolate of *Dinophysis acuminata* was fed *Mesodinium* strains of varying size and nutritional status, suggesting

505 intracellular toxin production rates, quotas, and profiles are largely intrinsic to the dinoflagellate strain
506 or population. Instead, *D. acuminata* appear able to compensate for differences in biovolume and/or
507 nutritional content by simply consuming a greater number of smaller, less nutritious prey to achieve
508 the same growth rate and maximum cell concentration. Large differences in bloom toxicity between
509 regions, is therefore, more likely due to the strain(s) of *Dinophysis* spp. present, with prey abundance
510 and/or environmental factors largely affecting local *Dinophysis* growth rates and bloom magnitude,
511 and possibly toxicity. The demonstrated ability of *D. acuminata* from the U.S. to grow and produce
512 consistent toxin profiles whether fed prey isolated from Spain, Japan, or the U.S., suggests that prey
513 strain is likely not an impediment to invasion and that efforts to thwart the expansion or introduction
514 of *D. acuminata* to new regions should be continued. The spreading of more toxic strains to regions
515 such as NE and Mid-Atlantic North America could be devastating for aquaculture sustainability and
516 seafood safety given the regions' elevated production of clam, oyster, and/or blue mussels.
517 Additionally, the strain of *Mesodinium rubrum* present in a region, and less likely the strain of
518 *Teleaulax* or *Geminigera* present, may play a role in local bloom magnitude and toxicity, as blooms
519 supported by high abundances of small, less nutritious ciliates may result in more extracellular toxins
520 with largely unexplored allelopathic effects and trophic transfer. Conversely, blooms supported by an
521 abundant population of large, nutritious *M. rubrum* can lead to elevated toxin quotas (i.e., intracellular
522 toxins), more *Dinophysis* cells, and consequently elevated toxicity in filter-feeding seafood products.
523

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FIGURE LEGENDS

Figure 1.

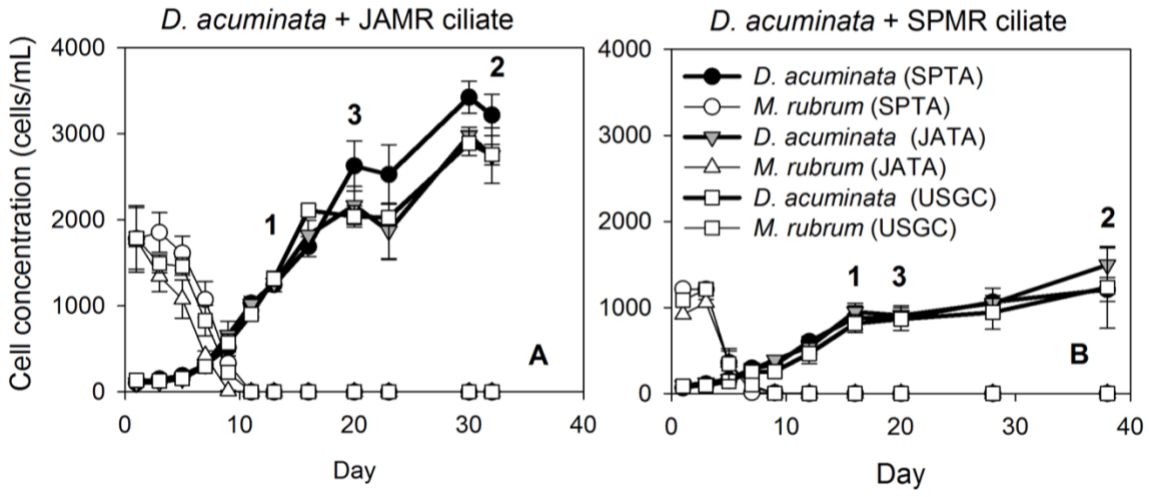
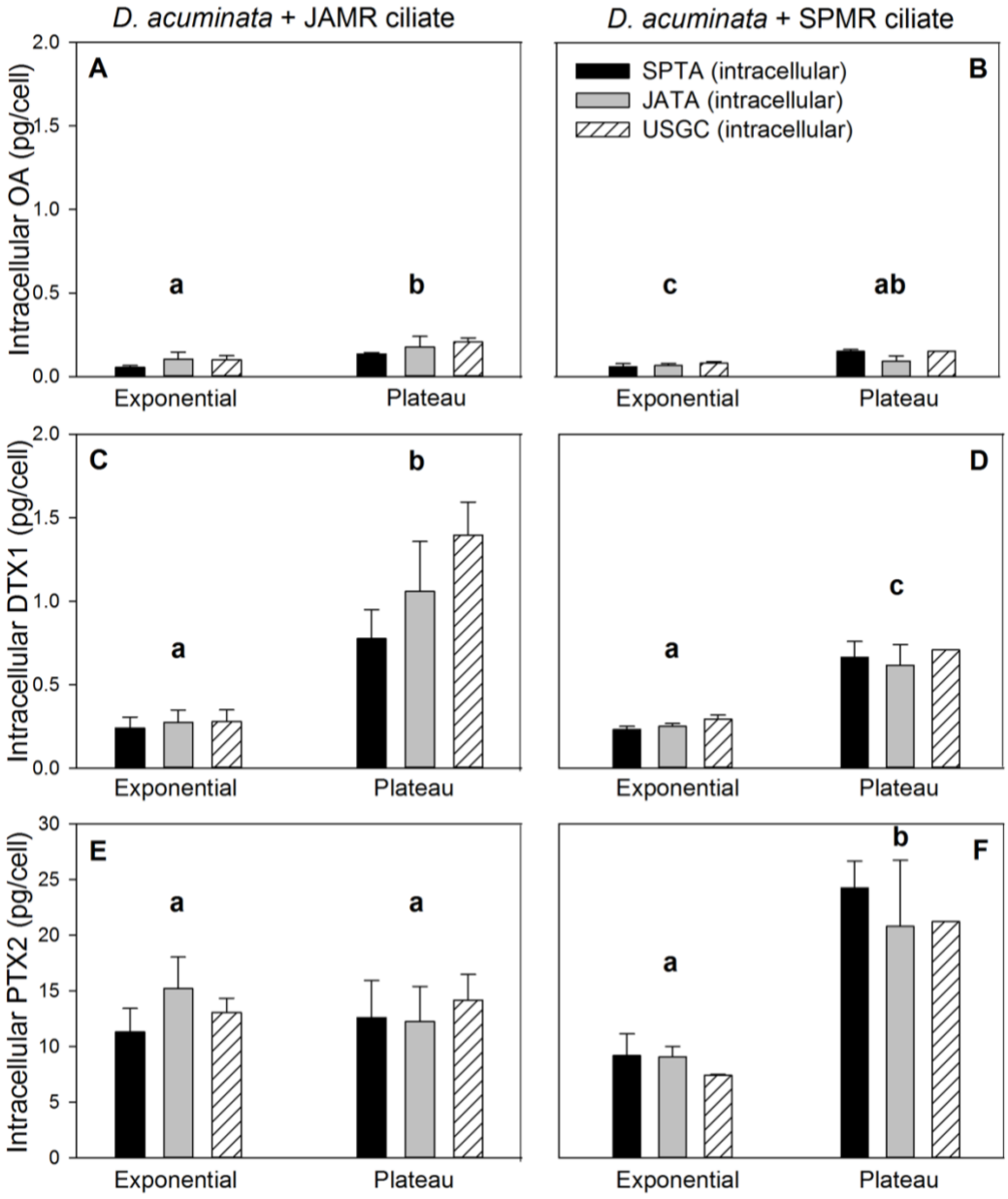


Figure 1. The growth of *Dinophysis acuminata* and consumption of *Mesodinium rubrum*, isolated from Japan (A) and Spain (B), when fed a variety of cryptophytes from Japan (JA), Spain (SP), and

541 the United States (US). Cells and medium were harvested during exponential (1) and plateau (2)
542 growth phases for toxin quantification. Triplicates of two treatments, JAMR + JATA and SPMR +
543 SPTA were refed (3) to begin the second experiment. Means were plotted with standard deviation.
544 Abbreviations include MR = *Mesodinium rubrum* (ciliate), TA = *Teleaulax amphioxeia* (cryptophyte),
545 GC = *Geminigera cryophila* (cryptophyte).

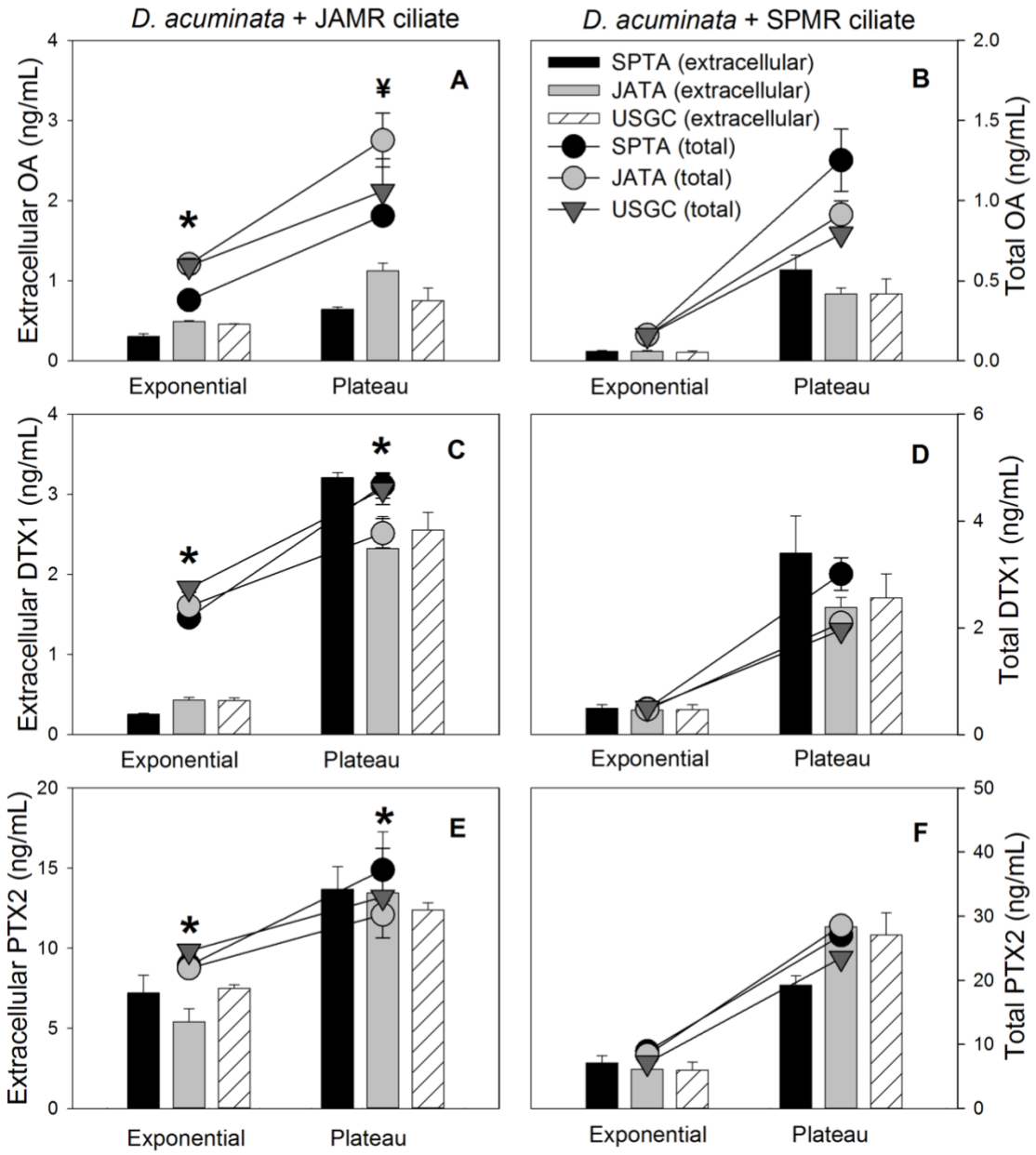
Figure 2.



546

Figure 2. Intracellular toxin quotas of okadaic acid (OA), dinophysistoxin-1 (DTX1), and pectenotoxin-2 (PTX2), when *Dinophysis acuminata* were fed a combination of ciliate and cryptophyte isolates from Japan (JA), Spain (SP), and the United States (US). Treatments were grouped by ciliate and statistically analyzed across growth phases within each toxin group. Significance is indicated with unshared letters. Means were plotted with standard deviation. Abbreviations include MR = *Mesodinium rubrum* (ciliate), TA = *Teleaulax* sp. (cryptophyte), GC = *Geminigera cryophila* (cryptophyte).

Figure 3.



554

555 Figure 3. Extracellular and total toxin concentrations (intracellular + extracellular) of okadaic acid
556 (OA), dinophysistoxin-1 (DTX1), and pectenotoxin-2 (PTX2) in the medium during late exponential
557 and plateau growth phases after *Dinophysis acuminata* was fed a combination of ciliate and
558 cryptophyte isolates from Japan (JA), Spain (SP), and the United States (US). Means were plotted
559 with standard deviation. Treatments were grouped by ciliate and statistically analyzed for differences

560 in total toxin within each growth phase and toxin group. The total toxin concentrations of OA (A, B),
561 DTX1 (C, D), and PTX2 (E, F) were significantly (*) greater in cultures fed the Japanese ciliate than
562 those fed the Spanish ciliate. When comparing total OA concentrations between *Dinophysis* cultures
563 in plateau phase (A, B), significance (¥) was detected when the Spanish line (SPMR + SPTA) was
564 excluded from the analysis. Otherwise, there was no detectable difference in total OA concentrations
565 between Japanese (JAMR) and Spanish ciliates during plateau phase. Abbreviations include MR =
566 *Mesodinium rubrum* (ciliate), TA = *Teleaulax amphioxeia* (cryptophyte), GC = *Geminigera cryophila*
567 (cryptophyte).
568

Table 1. Maintenance culturing conditions for isolates, including two lines of *Teleaulax amphioxeia* (TA), two lines of *Geminigera cryophila* (GC), three lines of *Mesodinium rubrum* (MR) and one strain of *Dinophysis acuminata* (DA).

Origin	Culture ID	Taxonomic group	Species	Medium[a]	Light[b] (uE)	Temperature (°C)
Antarctic	CCMP2564[c]	cryptophyte	*Geminigera cryophila*	f/2-Si	50	4
	CCMP2563[c]	ciliate	*Mesodinium rubrum*	f/2-Si	50	4
Japan	JATA	cryptophyte	*Teleaulax amphioxeia*	f/2-Si	50	15
	JAMR	ciliate	*Mesodinium rubrum*	f/12-Si	50	15
Spain	SPTA	cryptophyte	*Teleaulax amphioxeia*	f/2-Si	50	19
	SPMR	ciliate	*Mesodinium rubrum*	f/12-Si	50	19
USA	USGC	cryptophyte	*Geminigera cryophila*	f/2-Si	50	15
	DAEP01	dinoflagellate	*Dinophysis acuminata*	filtered seawater	65	6

JA = Japan, SP = Spain, US = United States; [a] Culture medium, f/2-Si and f/12-Si, modified as described in Anderson et al., 1994; [b] All cultures grown on a 14h light:10h dark photocycle; [c] Identifies isolates not used in any experiments, only in maintenance culturing.

Table 2. Nutritional content of the ciliate prey, expressed as particulate carbon (C), nitrogen (N), and phosphorus (P), when grown on three different cryptophytes, and the resulting growth rates of *Dinophysis acuminata* when grown on these food sources. Values represent means ± SD, averaged over triplicates within each cryptophyte treatment.

Ciliate + cryptophyte	Ciliate carbon (pg C/cell)	Ciliate nitrogen (pg N/cell)	Ciliate phosphorus (pg P/cell)	*Dinophysis* growth rate (div/day)	
JAMR+SPTA	2532.4±48.8	359.5±3.6	32.7±6.3	0.20 ± 0.02	a
JAMR+JATA	1746.8±296.6	262.7±64.5	28.2±6.0	0.23 ± 0.01	a
JAMR+USGC	1907.9±310.1	269.2±46.8	27.6±4.5	0.24 ± 0.01	a
SPMR+SPTA	758.9	84.25±4.27	8.58±0.58	0.16 ± 0.04	b
SPMR+JATA	768.8	86.39±4.98	8.42±0.03	0.16 ± 0.02	b
SPMR+USGC	842.9	95.14±3.62	12.86±0.09	0.16 ± 0.02	b

JA = Japan, SP = Spain, US = United States; MR = *Mesodinium rubrum* (ciliate), TA = *Teleaulax amphioxeia* (cryptophyte), GC = *Geminigera cryophila* (cryptophyte); Significance is indicated with unshared letters.

579 Table 3: Ingestion rate of *Dinophysis* when feeding on different ratios and strains of ciliate, *Mesodinium rubrum* (Experiments 1 and 2).

Ciliate + cryptophyte	Ratio *Dinophysis*:ciliate	Initial concentration of ciliate (cells/mL)	*Dinophysis* ingestion rate (ciliate/*Dinophysis*/d)	
JAMR + JATA$_1$	1:15	1500	0.8±0.07	a
SPMR + SPTA$_1$	1:15	1200	1.51±0.20	b
JAMR + JATA$_2$	1:10	6000	1.61±0.24	b
JAMR + JATA$_2$	1:15	9000	1.64±0.05	b
SPMR + SPTA$_2$	1:15	3500	2.57±0.41	c
SPMR + SPTA$_2$	1:33	7900	2.82±0.28	c

580 Superscripts 1 and 2 indicate if values are associated with Experiments 1 or 2, respectively. JA = Japan, SP = Spain, US = United States; MR = *Mesodinium rubrum* (ciliate),
581 TA = *Teleaulax amphioxeia* (cryptophyte); Significance is indicated with unshared letters (T-Test).
582
583 Table 4. Average intracellular and total toxin (intracellular + extracellular) production rates by *D. acuminata* during exponential growth. The six treatments,
584 making up a 2 x 3 factorial design, included a combination series of two ciliates and three cryptophyte strains (Experiment 1). Values represent means ± SD,
585 averaged over triplicates within each cryptophyte treatment.

Ciliate + cryptophyte	**OA** (pg/cell/day)		**DTX1** (pg/cell/day)		**PTX2** (pg/cell/day)	
	Intracellular	Total	Intracellular	Total	Intracellular	Total
JAMR+SPTA	0.004±0.001 a	0.015±0.004 a	0.025±0.007 a	0.059±0.009 a	0.83±0.16 a	1.08±0.14 ab
JAMR+JATA	0.004±0.001 a	0.019±0.004 ab	0.027±0.008 a	0.090±0.008 a	0.80±0.05 a	1.06±0.04 ab
JAMR+USGC	0.004±0.001 a	0.016±0.005 a	0.027±0.006 a	0.084±0.012 a	0.70±0.13 a	0.93±0.14 a
SPMR+SPTA	0.008±0.001 a	0.030±0.001 b	0.030±0.007 a	0.087±0.018 a	1.16±0.17 a	1.72±0.05 c
SPMR+JATA	0.007±0.001 a	0.026±0.002 ab	0.026±0.004 a	0.070±0.001 a	0.95±0.11 a	1.35±0.13 b
SPMR+USGC	0.010±0.001 a	0.029±0.001 b	0.034±0.003 a	0.085±0.016 a	0.86±0.04 a	1.31±0.11 b

586 JA = Japan, SP = Spain; MR = *Mesodinium rubrum* (ciliate), TA = *Teleaulax amphioxeia* (cryptophyte), GC = *Geminigera cryophila* (cryptophyte); OA = okadaic acid, DTX1
587 = dinophysistoxin-1, PTX2 = pectenotoxin-2; Significance is indicated with unshared letters.; Values were statistically analyzed within each column only.

588 Table 5. Intracellular and/or extracellular measurements of toxin per cell, toxin profile, and toxin in the medium for cultures of *D. acuminata*; values represent
589 means ± SD, averaged over all cryptophyte treatments and both growth phases, and grouped by ciliate strain. (Experiment 1).

Ciliate	Toxin	Intracellular Toxin per Cell (pg/cell)	Intracellular Toxin Profile (%)	Extracellular Toxin Concentration (ng/mL)	Extracellular Toxin per Cell (pg/cell)	Proportion External to Cell (%)
	OA					
JAMR		0.11±0.02	1.0±0.2	0.46±0.42	0.44±0.58	76±6
SPMR		0.10±0.01	0.6±0.1	0.55±0.44	0.99±1.96	80±10
	DTX1					
JAMR		0.67±0.15	5.9±1.3	1.53±1.24	1.29±1.05	65±7
SPMR		0.46±0.05	2.8±0.3	1.15±0.93	2.11±4.05	69±10
	PTX2					
JAMR		10.39±1.95	91.7±17.2	7.41±6.11	6.28±5.42	32±9
SPMR		15.59±1.66	96.2±10.3	6.25±4.03	9.03±11.40	32±8

590 JA = Japan, SP = Spain; MR = *Mesodinium rubrum* (ciliate); OA = okadaic acid, DTX1 = dinophysistoxin-1, PTX2 = pectenotoxin-2.
591

592 Table 6. Average intracellular toxin production rates, toxin quotas, and growth by *D. acuminata* while incubated with different ratios of predator to prey
593 (Experiment 2). Values represent means ± SD, averaged over triplicates within each treatment.

Ciliate + cryptophyte	Ratio *Dinophysis* to ciliate	Intracellular OA (pg/cell)	Intracellular DTX1 (pg/cell)	Intracellular PTX2 (pg/cell)	Intracellular OA Production (pg/cell/day)	Intracellular DTX1 Production (pg/cell/day)	Intracellular PTX2 Production (pg/cell/day)	*Dinophysis* growth rate (div/day)
JAMR+JATA	1:10*	0.07±0.02 a	0.50±0.02 a	8.09±0.74 a	0.005±0.003 a	0.027±0.012 a	0.29±0.05 a	0.28 ± 0.04 a
JAMR+JATA	1:15	0.07±0.02 a	0.54±0.14 a	10.49±1.71 a	0.005±0.001 a	0.042±0.007 a	0.86±0.08 b	0.26 ± 0.03 a
SPMR+SPTA	1:15	0.09±0.04 a	0.64±0.15 a	7.79±1.64 a	0.006±0.005 a	0.037±0.022 a	0.37±0.16 a	0.18 ± 0.01 b
SPMR+SPTA	1:33*	0.11±0.06 a	0.88±0.33 a	11.59±2.82 a	0.007±0.004 a	0.063±0.023 a	0.83±0.19 b	0.24 ± 0.01 a

JA = Japan, SP = Spain; MR = *Mesodinium rubrum* (ciliate), TA = *Teleaulax amphioxeia* (cryptophyte); OA = okadaic acid, DTX1 = dinophysistoxin-1, PTX2 = pectenotoxin-2; *indicates ratios that represent equal biomass of Japanese and Spanish ciliates by adjusting for a difference in biovolume of 3.3.; Significance is indicated with unshared letters.; Values were statistically analyzed within each column only.

Table 7. Average extracellular toxin concentration in the medium and total toxin production rates (intracellular + extracellular) by *D. acuminata* while incubated with different ratios of predator to prey (Experiment 2). Values represent means ± SD, averaged over triplicates within each treatment.

Ciliate + cryptophyte	Ratio *Dinophysis* to ciliate	Extracellular OA (ng/mL)	Extracellular DTX1 (ng/mL)	Extracellular PTX2 (ng/mL)	Total OA Production (pg/cell/day)		Total DTX1 Production (pg/cell/day)		Total PTX2 Production (pg/cell/day)	
JAMR+JATA	1:10*	0.27±0.01	0.94±0.13	4.23±0.82	0.017±0.003	a	0.082±0.014	a	0.90±0.003	a
JAMR+JATA	1:15	0.31±0.05	1.18±0.08	5.97±0.56	0.014±0.003	a	0.089±0.011	a	1.18±0.12	a
SPMR+SPTA	1:15	0.37±0.03	0.63±0.04	0.73±0.10	0.10±0.02	b	0.25±0.013	b	0.84±0.18	a
SPMR+SPTA	1:33*	0.79±0.24	1.69±0.78	1.61±0.81	0.19±0.07	b	0.37±0.14	b	1.23±0.10	a

JA = Japan, SP = Spain; MR = *Mesodinium rubrum* (ciliate), TA = *Teleaulax amphioxeia* (cryptophyte); OA = okadaic acid, DTX1 = dinophysistoxin-1, PTX2 = pectenotoxin-2; *indicates ratios that represent equal biomass of Japanese and Spanish ciliates by adjusting for a difference in biovolume of 3.3.; Significance is indicated with unshared letters.; Values were statistically analyzed within each column.

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