A multi-phylum study of grazer-induced paralytic shellfish toxin production in the dinoflagellate Alexandrium fundyense: A new perspective on control of algal toxicity

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Project
» Chemical Defenses in a Toxic Dinoflagellate: Mechanisms and Constraints (Chemical Defenses)

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Abstract
Data cover a multi-phyla study of grazer-induced algal toxin production in the marine dinoflagellate Alexandrium catenella (formerly known as A. fundyense). The survey included seven species of grazer representing five phyla: the protists, Polykrikos kofoidii (Dinoflagellata) and Tiarina fusus (Ciliophora) the bivalve molluscs, Mytilus edulis and Mya arenaria (Mollusca), the ascidians, Molgula manhattensis and Botrylloides violaceus (Chordata), and the copepod, Eurytemora herdmani (Arthropoda). Independent variable: seven species of grazer, direct and indirect (kairomone and feeding-related cues) induction of toxin production, and total number of ingested cells ingested (cells per treatment). Dependent variables: toxicity (pgSTXeq per cell) and increase in toxicity (%) Data were published in: Senft-Batoh, C. D., Dam, H. G., Shumway, S. E., & Wikfors, G. H. (2015). A multi-phylum study of grazer-induced paralytic shellfish toxin production in the dinoflagellate Alexandrium fundyense: A new perspective on control of algal toxicity. Harmful Algae, 44, 20-31. http://dx.doi.org/10.1016/j.hal.2015.02.008

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Coverage

Spatial Extent: **Lat**:41.320717 **Lon**:-72.06196
Temporal Extent: 2010-09 - 2012-01

Methods & Sampling

Refer to the Methods section of Senft-Batoh, et al. (2015).

Collection and Maintenance of Grazers:
Zooplankton from Casco Bay, Maine were collected using a 200 µm mesh net suspended in tidal flow. The catch was transferred to a cooler and transported to the laboratory within 7 hours. Adult female copepods, *Eurytemora herdmani*, were separated from the sample and placed in a 4 L container filled with 0.2 µm filtered

seawater from Long Island Sound, Groton, CT. The copepods (approximately 70 individuals) were maintained at 15°C in a temperature- and light-controlled (12 h:12 h light:dark cycle) chamber and fed a non-limiting diet of the diatom, *Thalassiosira weissflogii*, and the prasinophyte, *Tetraselmis* sp. After five days of maintenance in these conditions, adult female copepods to be used in assays were sorted into large petri dishes (10 copepods per dish) containing 80 mL of 0.2 µm filtered seawater. Dishes were set in an environmental chamber (18°C), 24 hours prior to the start of the experiment, to allow the copepods to acclimate to experimental conditions. During this time, the copepods also were starved to ensure complete gut evacuation prior to assays (Dam & Peterson 1988).

Protists were collected from locations in Long Island Sound using a 20 µm mesh net suspended in tidal flow. The heterotrophic dinoflagellate, *Polykrikos kofoidii*, was isolated from microzooplankton samples collected in Northport Harbor, Long Island, New York. The ciliate, *Tiarina fusus*, was isolated from samples collected at Avery Point, Groton, Connecticut. The isolates were maintained in 6-well, polycarbonate culture plates. Each well contained 10 mL of a non-limiting mixed diet of the autotrophic dinoflagellates *Lingulodinium polyedrum* and *Scrippsiella trochoidea*. This diet is sufficient for the maintenance of both *P. kofoidii* and *T. fusus* (Flores et al., 2012). To maintain the microzooplankton populations in culture, subsamples were transferred weekly to new 6-well culture plates containing fresh mixtures of the prey diet. Cultures were kept in a temperature (18°C) and light (12 h:12 h light:dark cycle) controlled incubator. Prior to experiments, *Polykrikos kofoidii* and *Tiarina fusus* were micropipetted from the cultures and transferred gently to small petri dishes containing 10 mL of 0.2 µm filtered seawater. Each dish contained 50 individuals. Dishes were returned to the incubator set to 18°C , and microzooplankton were starved for the next 24 hours to ensure complete evacuation of gut contents prior to assays (Tsuda et al., 1989).

Juvenile softshell clams, *Mya arenaria* (~100 individuals; ~1 cm shell length), were obtained from an aquaculture facility (Downeast Institute for Applied Marine Research and Education) on Beals Island, Maine. Blue mussels, *Mytilus edulis* (~100 juveniles; ~1 cm shell height), were collected directly from a rocky intertidal outcropping at Avery Point, Groton, Connecticut. Byssal threads of individuals were gently cut to separate them from rocks and from each other. Molluscs were placed in 4 L containers of continuously aerated 0.2 µm filtered seawater in a temperature-controlled (15°C) chamber and fed a non-limiting mixture of *Thalassiosira weissflogii* and *Tetraselmis* sp. After 3 days, healthy, actively-feeding clams and mussels were selected for use in experiments. These individuals were moved to 4 L containers of continuously-aerated 0.2 µm filtered seawater and starved for 24 hours prior to assays to ensure complete evacuation of gut contents (Hawkins et al., 1990). Containers were placed in a temperature controlled (18°C) chamber during this 24 hour period to acclimate animals to experimental conditions. Prior to the start of experiments, shell height for mussels and length for clams used in assays was measured. Individuals of near-equal shell height or length were divided among treatments (5 individuals per treatment; mussel mean height= 1.04 (±0.03) cm; clam mean length= 1.07(±0.02) cm). Analysis of variance confirmed that there was no difference in shell height among treatments with mussels (p= 0.232) or shell length among treatments with clams (p= 0.494). Juvenile molluscs were used in the present investigation as juveniles have lower clearance rates than adults; therefore, treatments would not require re-inoculation of the food source as often as would be necessary for adults.

Divers collected ascidians from pilings off of Avery Point, Groton, Connecticut. Individuals of the solitary ascidian, *Molgula manhattensis*, were removed gently from the substrate. Upon transport to the laboratory, specimens were immediately affixed at the base to a 2 x 2 inch tile of polyvinyl chloride (PVC) with cyanoacrylate glue. The colonial ascidian, *Botrylloides violaceus*, was found encrusting the shells of live mussels, *Mytilus edulis*. Colonies cannot be removed from shells without causing significant damage to the organisms. Mussels, therefore, were removed from shells and the valve containing *B. violaceus* was kept for experiments. Forceps were used to clean the ascidians of any encrusting organisms, such as bryozoans and small arthropods. Ascidians were held in 4 L containers of continuously-aerated, 0.2 µm filtered seawater in a chamber set to 15°C and a 12 h:12 h light:dark cycle. For 2 days, ascidians were fed a non-limiting mixture of *Thalassiosira weissflogii* and *Tetraselmis* sp. Individuals then were transferred to 4 L containers of continuously-aerated 0.2 µm filtered seawater and placed in a chamber (18°C) for 24 hours to adjust to experimental conditions.

Direct and indirect induction of toxin production by copepods:
Direct and indirect mechanisms of toxin induction were tested simultaneously using experimental cages. 1 L polycarbonate beakers with bottoms made of 10 μm mesh were nested within another 1 L beaker containing 500 mL of toxic *Alexandrium fundyense* (300 cells mL^{-1}). The mesh isolated these cells from materials within the cage. Adult female *Acartia hudsonica* (15 individuals) from either Maine or New Jersey were added to each cage and offered a diet of toxic *A. fundyense* (300 cells mL^{-1}) or were starved (no addition of algal food). Triplicate treatments (n=3) of the combinations of copepods and algal food within the cages were: 1) Maine copepods fed toxic algae; 2) Maine copepods starved; 3) New Jersey copepods fed toxic algae; 4) New Jersey copepods starved. Control cages (n=3) contained 300 cells mL^{-1} of toxic algae and no copepods. Assays were

run for 72 h, long enough to ensure induction of toxin production, and incubation conditions were identical to those of the algal cultures. Cages were lifted every 12 hours to ensure exchange of cues between compartments. At the termination of the assay, cells of *Alexandrium fundyense* within cages (where applicable) and below cages were collected from treatments and controls for toxin analysis. Cells within cages (treatments and controls) were enumerated microscopically, before and after incubation, to calculate copepod ingestion rates (Frost 1972). Differences in ingestion rate between the populations were assessed by a *t*-test.

Culture of Phytoplankton:
The toxic dinoflagellate, *Alexandrium fundyense* (strain BF-5, isolated from Bay of Fundy, Canada (Anderson et al., 1994)), was grown in semi-continuous culture in F/2 medium without silica (Guillard, 1975). Cultures were maintained in an environmental chamber with fluorescent lighting set to 18°C and a 12 h:12 h light:dark cycle. Toxic cells were used in all assays. Other phytoplankton species used as feed cultures (*Thalassiosira weissflogii*, *Tetraselmis* sp., *Lingulodinium polyedrum,* and *Scrippsiella trochoidea*) were cultured in the same manner and maintained under the same conditions as *Alexandrium fundyense*, except that *T. weissflogii*, a diatom, was grown in F/2 medium containing silica.

Grazer-Induced Toxin Production Assay:
The assay is designed to allow concurrent testing of direct and indirect mechanisms of grazer-induced toxin production on toxin content of *Alexandrium fundyense*. Separation of direct and indirect mechanisms of stimulation required the use of “cage” vessels. A general description of the experimental design for induction assays can be found in the Appendix. The design allows for manipulation of grazers and algal prey within cages. Such treatments can be used to assess the source of cues by which toxin content is increased. For grazers within cages that are starved (provided with no algal prey), indirect stimulation of toxin production of cue-receiving *A. fundyense* (300 cells mL^{-1}) below the cages would indicate that a predator kairomone was the cue responsible for increased production. Indirect enhancement of toxin production by grazers actively feeding on toxic *A. fundyense* suggests that a cue produced by predator interaction with, or digestion of, conspecific cells is a signal for increased toxin production. Increased toxin production in *A. fundyense* exposed directly to grazers (within cages) could be stimulated by chemical signals but may also involve effects of physical handling of cells or feeding selection by grazers for cells of lower-toxin content.

For each species of grazer, there were three treatments each (n=3) of starved grazers and grazers fed toxic algae. The number of grazers per treatment and the cell density of algae offered as prey (within cages) differed based upon the consumer, and were chosen as to approximate natural abundances and conditions. Control vessels (n=3) containing only toxic *A. fundyense*, both within and below cages (at the same concentrations as treatments), were prepared to serve as a baseline to detect increased toxin production. Each phylum of grazer was tested in a separate assay with its own preparation of control cells. Indirect induction of toxin production was measured for all species of grazer, but direct induction was only measured for copepod and microzooplankton grazers. The high clearance rates of the shellfish and ascidian species for *A. fundyense* required re-inoculation of these treatments with algal prey twice daily (12 hours apart). Thus, it was impossible to collect cells that had been directly exposed to grazing from these treatments.

Toxin analysis:
For each assay, extraction of saxitoxins from *Alexandrium fundyense* proceeded as follows. Cells from treatment and control vessels were collected on a 10 µm mesh and resuspended in filtered seawater in 50 mL centrifuge tubes. Subsamples (1 mL replicates) were drawn from each tube and cells were fixed at a final concentration of 2% Lugol’s solution. Cells were enumerated microscopically to determine the number present in each extract. The 50 mL centrifuge tubes containing *A. fundyense* were centrifuged at 4,000 x *g* for 20 minutes. Seawater supernatant was decanted, and the cell pellet was resuspended in 1 mL of 0.1 M acetic acid. Cells were lysed using a probe sonic dismembrator. Sonication was conducted with the tube immersed in ice to prevent heating of the samples. Resulting preparations were centrifuged again at 4,000 x *g* for 20 minutes to remove cell debris. Next, the acetic acid supernatant (extract) was filtered through a 0.45 µm ultracentrifuge filter cartridge to remove any remaining particulates. Samples were stored at -80°C until analysis. High-performance liquid chromatography with fluorescence detection (HPLC-FD; Oshima, 1995) was used to identify the carbamoyl paralytic shellfish toxin derivatives of saxitoxin (STX), neosaxitoxin (NEO), and gonyautoxins 1 through 4 (GTX 1-4), as well as the less toxic sulfamate congeners, C1 and C2 (Indrasena and Gill, 1998). Toxins were quantified by comparison to known standards (Certified Reference Materials Program, NRC Institute for Marine Biosciences, Canada) and expressed as mass of STX equivalents per cell according to conversion factors of Oshima (1995).

Data Processing Description

Data Processing:
For each species, direct induction of toxin content of *Alexandrium fundyense* within cages was assessed by a t-test comparing toxin content of cells exposed directly to grazers to control cells. To measure indirect induction of algal toxin content (also by each separate species), a 1-way analysis of variance (ANOVA) was used. Post hoc Holm-Sidak analysis of means (Sokal and Rohlf, 2012) compared toxin content among treatments (grazers starved or fed toxic algae) and control algae. These analyses were performed using SigmaPlot version 11.0 software. For treatments in which indirect induction of algal toxin occurred, data were examined further to compare magnitude of enhanced toxin production among the treatments. Enhanced toxin content was converted to a percent increase compared to control cells. Because some increases in toxin content were greater than 100% (i.e. toxin content of treatments was more than double that of control cells), values were scaled to the highest percent increase. An arcsine-square-root transformation was performed to normalize the percent values (Sokal and Rohlf, 2012). A 1-way ANOVA of the transformed values and post hoc Tukey analysis of means was performed to detect differences in enhanced toxin content among the multiple grazer-induced treatments tested. Three-way ANOVA of transformed data for *all* treatments was used to determine whether grazer phylum, species (nested within phylum), or diet (starved or fed) affected enhancement of *Alexandrium fundyense* toxin production. The interactions of phylum and diet, (phylum*diet) and species and diet (species(phylum)*diet) also were considered in this test. The interaction of phylum and species (phylum*species) was not considered as these variables are not independent of one another. The analyses involving combined data from multiple phyla were performed using SAS v. 9.1 [SAS Institute].

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Data Files

File
senft-batoh_2015b.csv (Comma Separated Values (.csv), 4.75 KB) MD5:d07a837c3c78b02c9a45600263a9357e Primary data file for dataset ID 853857

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Related Publications

Anderson, D. M., Kulis, D. M., Doucette, G. J., Gallagher, J. C., & Balech, E. (1994). Biogeography of toxic dinoflagellates in the genusAlexandrium from the northeastern United States and Canada. Marine Biology, 120(3), 467–478. doi:10.1007/bf00680222 https://doi.org/10.1007/BF00680222
Methods

Dam, H. G., & Peterson, W. T. (1988). The effect of temperature on the gut clearance rate constant of planktonic copepods. Journal of Experimental Marine Biology and Ecology, 123(1), 1–14. doi:10.1016/0022-0981(88)90105-0
Methods

Flores, H., Wikfors, G., & Dam, H. (2012). Reactive oxygen species are linked to the toxicity of the dinoflagellate Alexandrium spp. to protists. Aquatic Microbial Ecology, 66(2), 199–209. doi:10.3354/ame01570
Methods

Frost, B. W. (1972). Effects of size and concentration of food particles on the feeding behavior of the marine planktonic copepod Calanus pacificus. Limnology and Oceanography, 17(6), 805–815. doi:10.4319/lo.1972.17.6.0805
Methods

Hawkins, A. J. S., Navarro, E., & Iglesias, J. I. P. (1990). Comparative allometries of gut-passage time, gut content and metabolic faecal loss inMytilus edulis andCerastoderma edule. Marine Biology, 105(2), 197–204. doi:10.1007/bf01344287 https://doi.org/10.1007/BF01344287
Methods

Indrasena, W. M., & Gill, T. A. (1998). Fluorometric Detection of Paralytic Shellfish Poisoning Toxins. Analytical Biochemistry, 264(2), 230–236. doi:10.1006/abio.1998.2843
Methods

Oshima, Y. (1995). Post-column derivatization HPLC methods for paralytic shellfish poisons. In: Manual on

Harmful Marine Microalgae (eds. Hallegraeff, G. M., Anderson, D. M., Cembella, A. D. & Enevoldsen, H. O.). IOC manuals and guides No. 33, UNESCO, pp. 81-94.
Methods

Senft-Batoh, C. D., Dam, H. G., Shumway, S. E., & Wikfors, G. H. (2015). A multi-phylum study of grazer-induced paralytic shellfish toxin production in the dinoflagellate Alexandrium fundyense: A new perspective on control of algal toxicity. Harmful Algae, 44, 20–31. doi:10.1016/j.hal.2015.02.008
Results

Tsuda, A., Furuya, K., & Nemoto, T. (1989). Feeding of micro- and macrozooplankton at the subsurface chlorophyll maximum in the subtropical North Pacific. Journal of Experimental Marine Biology and Ecology, 132(1), 41–52. doi:10.1016/0022-0981(89)90175-5
Methods

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Parameters

Parameter	Description	Units
Phylum	Five phyla: Arthropoda, Chordata, Ciliophora, Dinoflagellata, and Mollusca	unitless
Induction	Grazer-induced toxin production: direct induction (within cage), indirect induction (outside cage; cue-receiving cells), and control (no grazers)	unitless
Grazer	Eight grazers: the protists, Polykrikos kofoidii (Dinoflagellata) and Tiarina fusus (Ciliophora) the bivalve molluscs, Mytilus edulis and Mya arenaria (Mollusca), the ascidians, Molgula manhattensis and Botrylloides violaceus (Chordata), and the copepod, Eurytemora herdmani, and Acartia hudsonica (Arthropoda)	unitless
Cues	Cue induction: kairomone and feeding-related cues	unitless
Toxicity	Total cellular toxin content in saxitoxin equivalents	picograms saxitoxin equivalents per cell (pg STXeq cell-1)
Percent_Toxicity	Percent increase in indirect toxin production stimulated by indicated treatments	percent (%)
Ingested_cells	Total number of cells of A. fundyense ingested per treatment	number of cells per treatment (cells treatment-1)

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Instruments

Dataset-specific Instrument Name	HPLC system (Waters, Milford, MA)
Generic Instrument Name	High-Performance Liquid Chromatograph
Dataset-specific Description	High Performance Liquid Chromatography (HPLC) is a form of column chromatography that pumps a sample mixture or analyte in a solvent (known as the mobile phase) at high pressure through a column with chromatographic packing material (stationary phase). Scanning Fluorescence Detector (Waters 474, Waters, Milford, MA) was used for in-vitro diagnostic testing to analyze compounds of STX and its derivatives.
Generic Instrument Description	A High-performance liquid chromatograph (HPLC) is a type of liquid chromatography used to separate compounds that are dissolved in solution. HPLC instruments consist of a reservoir of the mobile phase, a pump, an injector, a separation column, and a detector. Compounds are separated by high pressure pumping of the sample mixture onto a column packed with microspheres coated with the stationary phase. The different components in the mixture pass through the column at different rates due to differences in their partitioning behavior between the mobile liquid phase and the stationary phase.

Dataset-specific Instrument Name	Olympus IX70 inverted system microscope
Generic Instrument Name	Inverted Microscope
Dataset-specific Description	The IX70 inverted tissue culture microscope is a research-level instrument capable of imaging specimens in a variety of illumination modes including brightfield, darkfield, phase contrast, Hoffman modulation contrast, fluorescence, and differential interference contrast.
Generic Instrument Description	An inverted microscope is a microscope with its light source and condenser on the top, above the stage pointing down, while the objectives and turret are below the stage pointing up. It was invented in 1850 by J. Lawrence Smith, a faculty member of Tulane University (then named the Medical College of Louisiana). Inverted microscopes are useful for observing living cells or organisms at the bottom of a large container (e.g. a tissue culture flask) under more natural conditions than on a glass slide, as is the case with a conventional microscope. Inverted microscopes are also used in micromanipulation applications where space above the specimen is required for manipulator mechanisms and the microtools they hold, and in metallurgical applications where polished samples can be placed on top of the stage and viewed from underneath using reflecting objectives. The stage on an inverted microscope is usually fixed, and focus is adjusted by moving the objective lens along a vertical axis to bring it closer to or further from the specimen. The focus mechanism typically has a dual concentric knob for coarse and fine adjustment. Depending on the size of the microscope, four to six objective lenses of different magnifications may be fitted to a rotating turret known as a nosepiece. These microscopes may also be fitted with accessories for fitting still and video cameras, fluorescence illumination, confocal scanning and many other applications.

Dataset-specific Instrument Name	mesh net suspended in tidal flow
Generic Instrument Name	Plankton Net
Generic Instrument Description	A Plankton Net is a generic term for a sampling net that is used to collect plankton. It is used only when detailed instrument documentation is not available.

Dataset-specific Instrument Name	Sonic dismembrator (Model 50, Fisher Scientific)
Generic Instrument Name	ultrasonic cell disrupter (sonicator)
Dataset-specific Description	The Fisher Scientific™ Model 50 Sonic Dismembrator is compact, portable and extremely simple to operate. Weighing less than 4 lb., this model is the smallest unit on the market and is highly effective for cell disruption, sample preparation and many other small volume applications.
Generic Instrument Description	Instrument that applies sound energy to agitate particles in a sample.

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Project Information

Chemical Defenses in a Toxic Dinoflagellate: Mechanisms and Constraints (Chemical Defenses)

Coverage: New England waters from Connecticut to Maine

Description from NSF award abstract:
Species of the dinoflagellate genus Alexandrium occur around the globe, and some species, because of their toxin production, have been hypothesized to be keystone species. Alexandrium produces chemical compounds that appear to target different consumers. Neurotoxins such as PST target metazoan grazers. In preliminary experiments in their laboratory, the investigators also verified the presence of reactive oxygen species that target, at a minimum, protistan grazers. Such compounds reduce grazer fitness, and, at least in the case of PST, have been shown to have profound evolutionary effects on grazers. Grazer adaptation, in turn, can affect Alexandrium population dynamics. A common assumption is that production of toxic compounds in phytoplankton represents an adaptive defense. However, unequivocal experimental evidence in support of this hypothesis is scarce. This project will be a rigorous experimental test of the chemical defense hypothesis. The project's investigators will investigate a series of experimentally falsifiable hypotheses with both metazoan and protistan grazers challenged with Alexandrium. This project will provide novel understanding of, and insight into, the factors that determine grazer-induced toxin production, the relationship between degree of chemical defense and susceptibility to grazing, and the costs and tradeoffs of the purported mechanisms of chemical defense in Alexandrium. Verification or refutation of the chemical defense hypothesis is essential to conceptual models of the formation, control and persistence of toxic algal blooms, and chemically-mediated predator-prey interactions.

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Funding

Funding Source	Award
NSF Division of Ocean Sciences (NSF OCE)	OCE-1130284

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