Predator Cues Target Signaling Pathways in Toxic Algal Metabolome Protocol

1. Phytoplankton culturing

Cultures of the dinoflagellate *Alexandrium minutum* strain CCMP 113, acquired from NCMA Bigelow Laboratory, were grown in filtered seawater from the Gulf of Maine (NCMA Bigelow Laboratory, 35 ppt) amended with full strength K media [21]. Cultures were arbitrarily arranged in a Percival incubator at 15 °C set to a 12:12 h light/dark cycle with irradiance of 89 100 µmol m 2 s 1. Cell density was quantified by visual enumeration of cells in a Palmer Maloney chamber on an Olympus IX 50 inverted microscope after preservation with 1% acidified Lugol's solution.

2. Metabolomics experimental design and execution

To investigate the effect of copepod chemical cues on the metabolome of *A. minutum*, cultures in exponential growth phase at 15,000 cells mL^{-1} were exposed to copepodamides. Twice, first at the start of the experiment and again 24 h later, 1.5 mL of either 0.2 µM copepodamides in DMSO (treatment, n=20) or DMSO alone (control, n=20) were added aseptically to 300 mL cultures of *A. minutum* resulting in a final concentration of 1 nM copepodamides. A blank media sample consisting of seawater amended with K media at a 1.7-fold dilution was also incubated for the same length of time. At both the start, prior to the first addition of copepodamides, and end of the experiment, prior to harvesting, a 1.0 mL aliquot from each culture was preserved with Lugol's solution to measure cell concentrations, which was used to calculate *A. minutum* growth over the course of the experiment and to normalize sample for metabolomics analysis.

3. Harvesting and extraction of *Alexandrium minutum* for metabolomics experiment

After about 40 h, all cultures were harvested with processing of treatments and controls intermixed over about 2.5 h within 4 h of the start of the light cycle. A 50 mL aliquot from each culture and the blank media sample was collected in a 50 mL centrifuge tube for toxin analysis. The remaining *A. minutum* cells were harvested by vacuum onto GF/F filters (Whatman #1825-110, previously muffled for 3 h at 450 °C) and quenched with liquid nitrogen. Frozen cells with filters were stored in foil (previously muffled for 3 h at 450 °C) at -80 °C until extraction. Frozen filters containing cells were ground with a liquid nitrogen-chilled mortar and pestle and extracted with 30 mL of ice-cold 3:2:1 methanol/acetone/acetonitrile (OmniSolve, >99.5%, Millapore Sigma). Filter particulates were removed by centrifugation (800 x *g* for 15 min) and the supernatant was transferred to a new vial. The filter pellets were rinsed with fresh solvent and centrifuged three times with a total of 23 mL of extraction solvent. All

supernatants for each replicate culture were combined and filtered with a 0.2 μm nylon syringe filter (Acrodisc, Pall Laboratory) to remove remaining small particulates, and then dried *in vacuo* using a Thermo Savant speedvac. Dried extracts were dissolved in a biphasic mixture of 9:10:15 water/methanol/chloroform (OmniSolve, >99.5%, Millapore Sigma) to separate polar (water/methanol) and non-polar (methanol/chloroform) metabolites, and then dried again *in vacuo*.

Polar extracts were re-dissolved completely in 1.0 mL 50% aqueous methanol, with 25% of the mixture aliquoted for UPLC/MS and the other 75% reserved for ^{1}H NMR spectroscopy. Both the UPLC/MS samples as well as the ^{1}H NMR spectroscopy samples were then dried *in vacuo*. Excess, insoluble inorganic salts were removed from polar ^{1}H NMR spectroscopy samples by twice triturating with ice-cold methanol and filtering through combusted glass pipettes with glass wool plugs. Methanol was removed from the triturated polar ^{1}H NMR spectroscopy *in vacuo*. Non-polar extracts were not aliquoted as 100% of the extracts were analyzed first by ^{1}H NMR spectroscopy followed by UPLC/MS analysis.

4. NMR Metabolomic sample preparation, spectral data acquisition, and data processing and analysis

(a) Sample preparation and spectral data acquisition

In order to compare metabolomes without the confounding factor of different number of cells having been extracted across replicates, each sample was dissolved in a volume of solvent for ^{1}H NMR spectroscopy analysis that accounted for its cell concentration at the time of harvest.

(i) Non-polar extracts

Non-polar extracts were dissolved at a concentration equivalent to 2.27 x 10^{7} *A. minutum* cells mL^{-1} in dimethyl sulfoxide-d_6 (99.9% atom d_6-DMSO; Cambridge Isotope Labs) containing 0.1% trimethylsilane (TMS), as internal standard. The media blank extract was prepared in the smallest volume of solvent possible (175 μL). The non-polar extracts were transferred to 3 mm NMR tubes, and analyzed using a Bruker Avance IIIHD 800 MHz NMR spectrometer equipped with a 3 mm triple resonance broadband cryoprobe. Spectra of non-polar extracts were acquired using a simple ^{1}H NMR experiment (Bruker zg30 pulse sequence) with a spectral width of 9.6 kHz centered at 4400.00 Hz over 320 scans. One treatment replicate was lost and therefore its spectrum was not acquired.

(ii) Polar extracts

Polar extracts were dissolved at a concentration equivalent to 1.70 x 10^{7} *A. minutum* cells mL^{-1} in 90:10 H_2O/D_2O (99.96% atom D_2O; Cambridge Isotope Labs) with 0.2 mM phosphate buffer (pH 7.4) and 0.25 mM 3-(trimethylsilyl)propionic-2,2,3,3d-$_4$ acid (TMSP), as internal standard, for ^{1}H NMR spectroscopy analysis. The media blank extract was prepared in the smallest volume of solvent possible (175 μL). Particulates were excluded by briefly centrifuging the samples and then carefully transferring the extracts into 3 mm NMR tubes. The extracts were analyzed using a Bruker Avance IIIHD 800 MHz NMR spectrometer equipped with a 3 mm triple resonance broadband cryoprobe. Spectra for the polar extracts were acquired using a 1D water presaturation (Bruker zgpr pulse sequence) with a spectral width of 12 kHz centered at 3758.36 Hz, with a relaxation delay 3 s, over 480 scans.

(b) Data processing and analysis

^{1}H NMR spectra were pre-processed using Metabolab version 2019.12.08.1237 (Ludwig and Günther 2011) in Matlab R2013a version 8.1.0.604. Only extracts derived from *A. minutum* cultures, not the blank media extracts, were analyzed using Metabolab.

(i) Non-polar extracts

Spectra from non-polar extracts were aligned to TMS at 0.00 ppm, manually phased, spline baseline corrected, and segmentally aligned. The spectral regions associated with TMS (-0.50 to 0.60 ppm), the residual DMSO signal (2.45-2.65 ppm), water (3.30-3.46 ppm), and the excess downfield region that contained no signals (10.40-11.50 ppm) were removed and noise filtration applied. Spectra were then binned (0.005 ppm), probabilistic quotient normalized to account for minute concentration differences (Dieterle et al. 2006), generalized logarithmic transformed to reduce bias toward metabolites with high concentrations ($\lambda = 6.5012 \times 10^{-9}$) (Parsons et al. 2007), and mean centered.

(ii) Polar extracts

Similar to the non-polar extract analyses, NMR spectra from polar extracts were aligned to TMSP at 0.00 ppm, manually phased, spline baseline corrected, and segmentally aligned. For polar extracts the spectral regions around TMSP (-2.60 to 0.50 ppm), processing contaminant (2.63-2.84 ppm), water (4.71-5.23 ppm), and the excess downfield region (9.50-12.50 ppm) were removed and noise filtration applied. Spectra were then binned (0.005 ppm), probabilistic quotient normalized, generalized logarithmic transformed ($\lambda = 5.210 \times 10^{-9}$), and mean centered.

(iii) Analysis

PLS-Toolbox version 8.0.2 (Eigenvector Research) in Matlab was used to generate an oPLS-DA model which was cross-validated using Venetian blinds methods with seven data splits to capture the maximum variance between control and treatment samples in the first latent variable (LV). During model generation for the polar extracts, spectra for two control replicates and one treatment replicate were removed due to poor NMR spectral quality. Differences between metabolomes of *A. minutum* exposed and not exposed to copepodamides were examined using the oPLS-DA model with the loadings of the first latent variable used to identify spectroscopic features that were significantly different between treatments.

5. UPLC/MS Metabolomic sample preparation, spectral data acquisition, and data processing and analysis

(a) Sample preparation and spectral data acquisition

Similar to ^{1}H NMR sample preparation and spectroscopic analysis, non-polar and polar extracts were prepared for and analyzed by UPLC/MS separately. Each extract was dissolved in a volume of solvent appropriate for its final cell concentration, and all samples were centrifuged at 21,100 x *g* for 5 min to pellet minute particulates. During analysis of both non-polar and polar extracts, treatment and control extracts and the media blank extract were randomly interspersed with pooled quality control samples acquired after every 10 sample injections. The pooled sample was used for quality control monitoring, drift correction, and MS/MS collection.

Additionally, a sample blank was created using the same volume of solvent as the pooled quality control sample.

(i) Non-polar extracts

Non-polar extracts were dissolved at a concentration equivalent to 4.54 x 10^6 *A. minutum* cells mL^{-1} in LCMS grade 2:1 isopropyl alcohol/acetonitrile (OmniSolv, >99.5%, MilliporeSigma). The media blank extract was prepared in the smallest volume of solvent possible (1000 µL). Eight pooled quality control samples were then generated by combining a small portion of all samples. Extracts were analyzed using a Q Exactive HF Hybrid Quadrupole-Orbitrap Mass Spectrometer (ThermoFisher Scientific) coupled to a Vanquish UPLC system (ThermoFisher Scientific). Chromatographic separation of the extracts was accomplished by injecting of 2.0 µL onto an Accucore C30 column (2.1 x 150 mm, 2.6-µm particle size; ThermoFisher Scientific) fitted to the UPLC system. The column was operated at a temperature of 50 °C with a flow rate of 0.4 mL min^{-1}. Mobile phase A was 40:60 water/acetonitrile and mobile phase B was 10:90 acetonitrile/isopropyl alcohol. Both mobile phases contained 10 mM ammonium formate and 0.1% formic acid buffer. All solvents and modifiers were LCMS grade (Optima, LCMS, Fischer Scientific). The following mobile phase gradient was used: equilibration at 80% A; 0-1 min ramp to 40% A; 1-5 min ramp to 30% A; 5-5.5 min ramp to 15% A; 5.5-8 min ramp to 10% A; 8-8.2 min ramp to 0% A; 8.2-10.5 min hold at 0% A; 10.5-10.7 min return to 80% A and re-equilibration at 80% A until 12 min.

Mass spectrometric analysis of the non-polar extracts was performed using the Q Exactive HF Hybrid Quadrupole-Orbitrap which possesses a resolution power of 240,000 FWHM at 200 *m/z* and mass accuracy of <3 ppm. Each extract was analyzed twice, once in negative ionization mode and then in positive ionization mode using similar instrument settings except that a spray voltage of 3.5 kV was used for positive mode whereas 2.8 kV was used for negative mode. The heated electrospray ionization (HESI) source was operated at a vaporizer temperature of 425 ºC. The sheath, auxiliary, and sweep nitrogen gas flows were 60, 18, and 4 L h^{-1}, respectively. The mass spectrometric scan range was 150-2000 *m/z*. Tandem MS data were acquired in a data-dependent manner with a resolution of 120,000 and the dd-MS^2 collected at a resolution of 30,000 with an isolation window of 0.4 *m/z* with a loop count of top 5. Stepped normalized collision energies were set at 10, 30, and 50 eV for fragmentation in the higher-energy collisional dissociation (HCD) cell prior to ion analysis in the orbitrap. Dynamic exclusion was set at 10 s and ions with charges greater than 2 omitted. Data acquisition was undertaken using Compound Discoverer version 3.0 (ThermoFisher Scientific).

(ii) Polar extracts

Polar extracts were dissolved at a concentration equivalent to 4.54 x 10^6 *A. minutum* cells mL^{-1} in 30% aqueous acetonitrile (OmniSolv, LCMS MilliporeSigma). The media blank extract was prepared in the smallest volume of solvent possible (250 µL). As with non-polar extracts, prior to analysis seven pooled quality control samples consisting of a small portion of all samples were generated. Analysis was completed using an Orbitrap ID-X Tribrid Mass Spectrometer (ThermoFisher Scientific) coupled to a Vanquish UPLC system. Chromatographic separation of the extracts was achieved using 2.0 µL injections on a Waters Acquity UPLC BEH HILIC column (2.1 x 150 mm, 1.7-µm particle size; Waters Corporation) fitted to the UPLC system. The column was operated at a temperature of 40 °C with a flow rate of 0.4 mL min^{-1}. Mobile phase A was 80:20 water/acetonitrile with 10 mM ammonium formate and 0.1% formic

acid and mobile phase B was acetonitrile with 0.1% formic acid. All solvents and modifiers for both mobile phases were LCMS grade (Optima, LCMS, Fischer Scientific). The following mobile phase gradient was used: equilibration at 5% A; 0-0.5 min hold at 5% A; 0.5-9 min ramp to 70% A (curve 7); 9-9.4 min hold at 70% A; 9.4-9.5 min return to 5% A and re-equilibration at 5% A until 12 min.

The Orbitrap ID-X Tribrid used to analyze the polar extracts possesses a resolution power of 500,000 FWHM at 200 *m/z* and mass accuracy of <1 ppm. Extracts were analyzed first in negative ionization mode and then in positive ionization mode using similar instrument settings except that a spray voltage of 3.5 kV for positive mode and 2.5 kV for negative mode. The HESI source was operated at a vaporizer temperature of 320 ºC. The sheath, auxiliary, and sweep nitrogen gas flows were 40, 8, and 1 L h^{-1}, respectively. The mass spectrometric scan range was 70-1050 *m/z* with an automatic gain control target set at 1 x 10^5 ions. Tandem MS data was acquired in a data-dependent manner with a full scan resolution of 120,000 and dd-MS^2 at a resolution of 30,000 with an isolation window of 0.8 *m/z* with a cycle time of 1.5 s. Stepped normalized collision energies were set at 15, 30, and 45 eV for fragmentation in the HCD cell prior to ion analysis in the orbitrap. Dynamic exclusion was set at 8 s and ions with charges greater than 2 omitted. As with the non-polar extracts, data acquisition was carried out using Compound Discoverer.

(b) Data processing and analysis

After UPLC/MS acquisition, spectral features (retention time, *m/z*) were extracted from chromatograms via alignment, peak picking and integration, and peak area extraction using Compound Discoverer version 3.1 (ThermoFisher Scientific) and XCMS Online [9]. The data were then normalized and scaled by the median peak area across all samples. Spectral features that were not five times greater than background peaks, based on the media blank sample, were removed from the dataset. Additionally, features that were present in fewer than 50% of the pooled quality control samples and had a relative standard deviation of greater than 30% were excluded from the dataset.

PCA was used to examine differences in polar and non-polar metabolomes, separately, of *A. minutum* (control) and *A. minutum* exposed to copepodamides (treatment) using both Compound Discoverer and XCMS Online. Using XCMS platform spectral features were identified as being significantly different between treatment and controls using Welch's t-test [9]. In Compound Discoverer, the data was log 10 transformed. The PCA model was then generated using spectral features with an absolute value fold change greater than or equal to 1.1 in metabolomes of *A. minutum* exposed to copepodamides when compared with standard *A. minutum* metabolomes and p values less than 0.05 based on a t-test.

6. Metabolite annotation

Structure identification was attempted for all features that were identified as significantly different between *A. minutum* exposed versus not exposed to copepodamides. Identification was predominately dependent on MS/MS data with ^{1}H NMR spectroscopy being largely used for confirmation of functional groups. From the UPLC/MS data, elemental formulae were generated based on exact mass and isotopic patterns. The elemental formulae along with $[M-H]^-$ and $[M+H]^+$ ions, MS/MS fragmentation patterns, retention time, isotopic patterns, and significant

^{1}H NMR chemical shifts were compared to a local spectral database, built from curated experimental data, as well as these publicly available databases: Metlin database (Smith et al. 2005), MassBank of North America (MoNA)(2020), the human metabolome database (HMDB) (Wishart et al. 2007), ChemSpider (Pence and Williams 2010), Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Ogata et al. 1999), LIPID Metabolites and Pathways Strategy (LIPID MAPS) database (Sud et al. 2006), SpectraBase (John Wiley & Sons 2020), mzVault (in house database), and mzCloud. Additionally, *in silico* fragmentation of potential structural matches was calculated and scored against experimental data using FISH coverage scoring in Compound Discoverer. Select metabolite identities were further confirmed via comparisons of retention times, $[M-H]^-$ and $[M+H]^+$ ions, and MS/MS fragmentation patterns to purchased standards. KEGG (Ogata et al. 1999) and MetaCyc (Caspi et al. 2017) were used to determine pathways in which significant metabolites were involved. Additionally, a review by Bromke (2013) was consulted when examining the amino acid biosynthetic pathways and a review by Wasternack and Strnad (Wasternack and Strnad 2018) was consulted for biosynthesis of jasmonic acid.

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