Qualitative and quantitative analysis of hemolytic toxins from dinoflagellates specifically associated with fish kills by mass spectrometry

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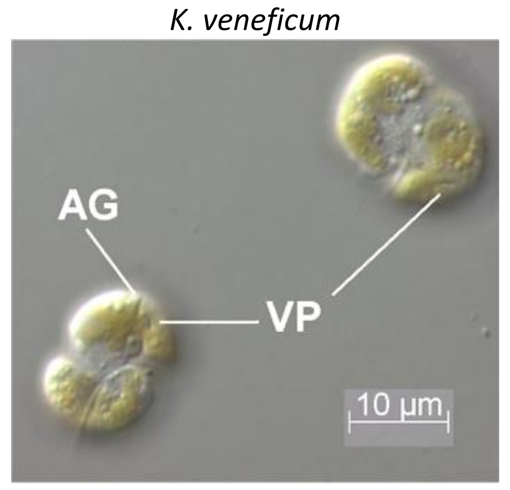
Introduction:

Dinoflagellates are motile, unicellular protists found in many aquatic environments and capable of causing harmful blooms, sometimes referred to as "red tide [1]." Karlotoxins and amphidinols are hemolytic polyol toxins (> 1000 Da) produced by *Karlodinium* and *Amphidinium* dinoflagellate species, respectively, that have been associated with fish kills throughout the world [2-4]. Many species, and even strains of the same species, seem to make unique toxin structures. However, very little genomic data exist to delineate strains of these species, partially due to their very large genomes [1]. The goal of this research was to develop a comprehensive mass spectrometric methodology to identify and define primary chemical structures of polyol toxins for support of applied attribution studies and basic dinoflagellate biology studies.

K. veneficum



A. carterae

Methods:

Previously archived extracts and newly acquired water samples, from areas in which fish kills were observed, were analyzed. All samples were extracted using the method previously published by Bachvaroff, *et al*. [5] Eluents were injected onto a Phenomenex (Torrance, CA) Kinetex core shell C8 column (2.1 mm i.d. x 100 mm, 2.6 um particle) and subjected to a ten minute, linear, acidic acetonitrile-water gradient from 20% to 95% organic composition. A hybrid 3D ion trap-time of flight mass spectrometer coupled to an analytical flow HPLC with an online degasser and diode array detector (Shimadzu, Columbia, MD) was used for the analyses. Source and collision parameters were optimized by direct infusion. A data dependent acquisition tandem MS strategy was employed for sample screening, followed by targeted tandem experiments to achieve optimal spectral quality. Ultra-high resolution, accurate mass spectra were acquired on a hybrid linear ion trap – 21 T Fourier transform-ion cyclotron resonance mass spectrometer, including several tandem experiments with various activation methods.

Results:

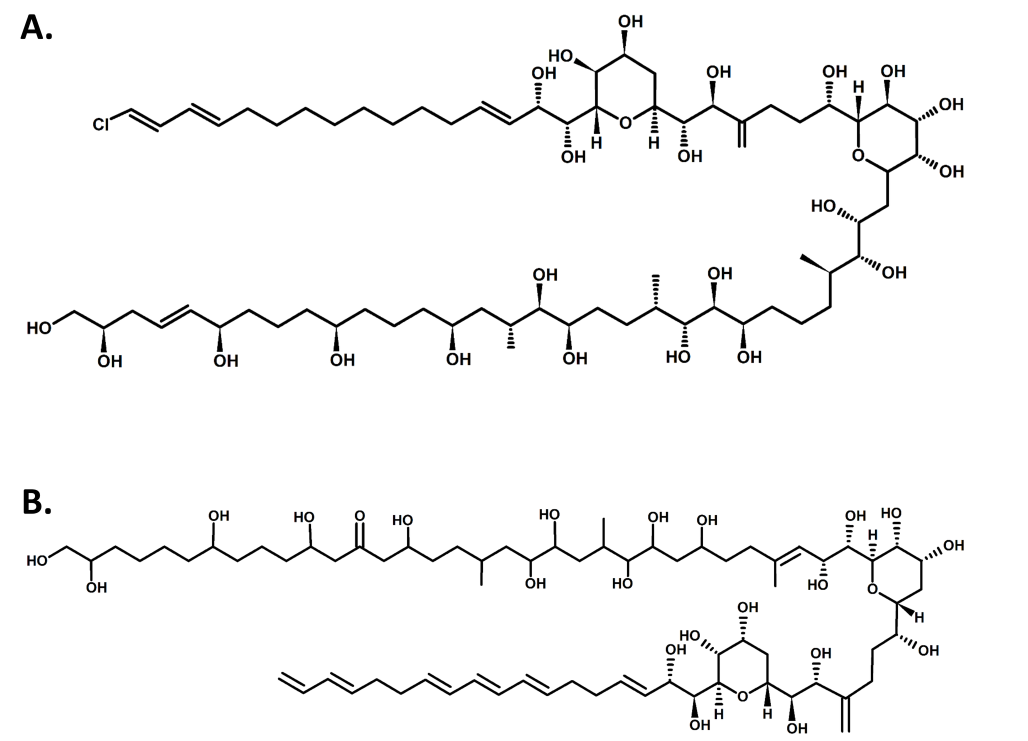


Figure 1. Representative polyol dinoflagellate toxin structures. A.) karlotoxin 2, made by *K. veneficum* and, B.) amphidinol 18, made by *A. carterae*.

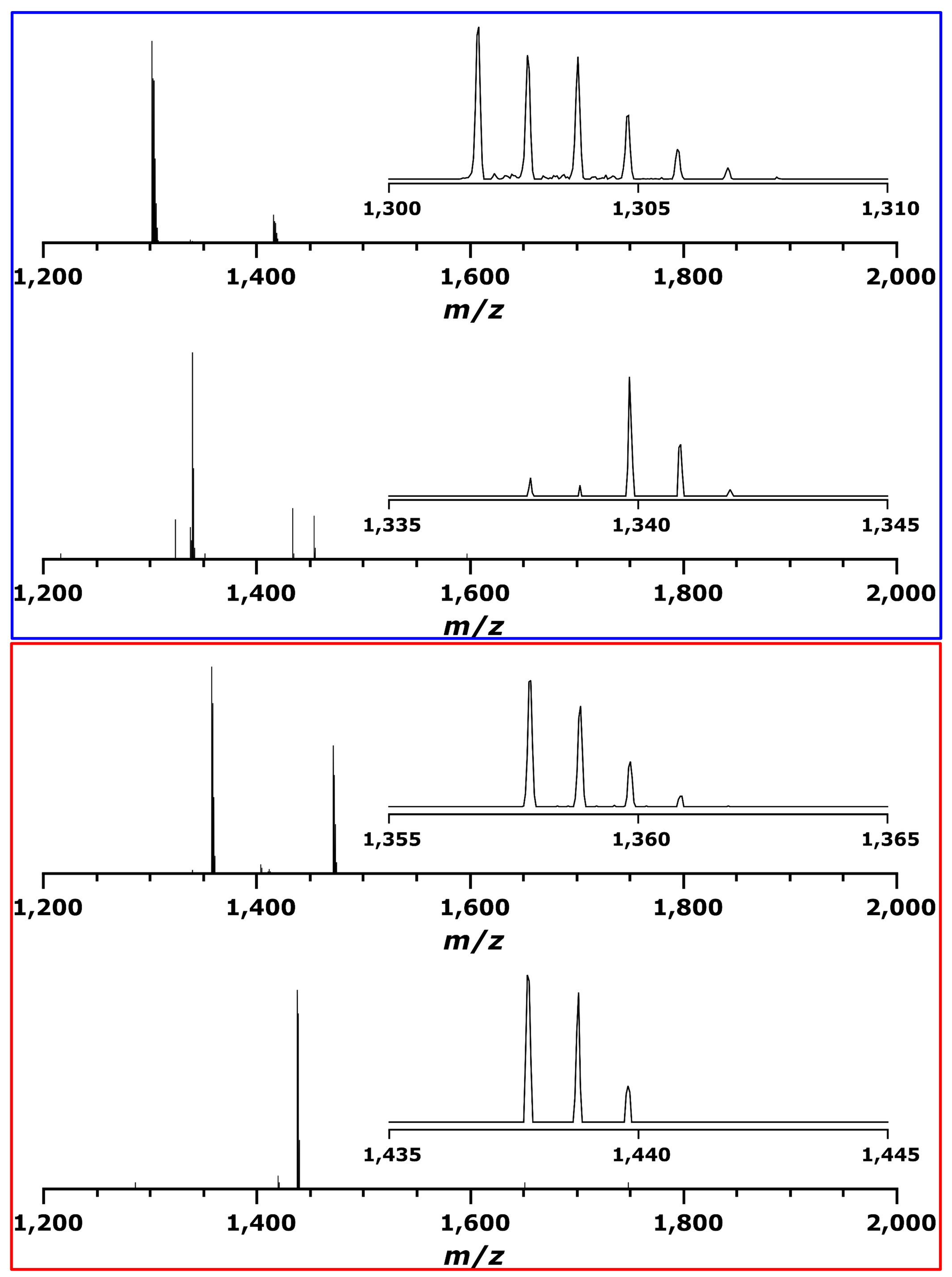


Figure 2. ESI-TOF mass spectra of toxin extracts from four water samples after possible dinoflagellate fish kills. (blue box) Two spectra from *Karlodinium* species. (red box) Two spectra from *Amphidinium* species. The last spectrum is from a sulfated analogue of amphidinol.

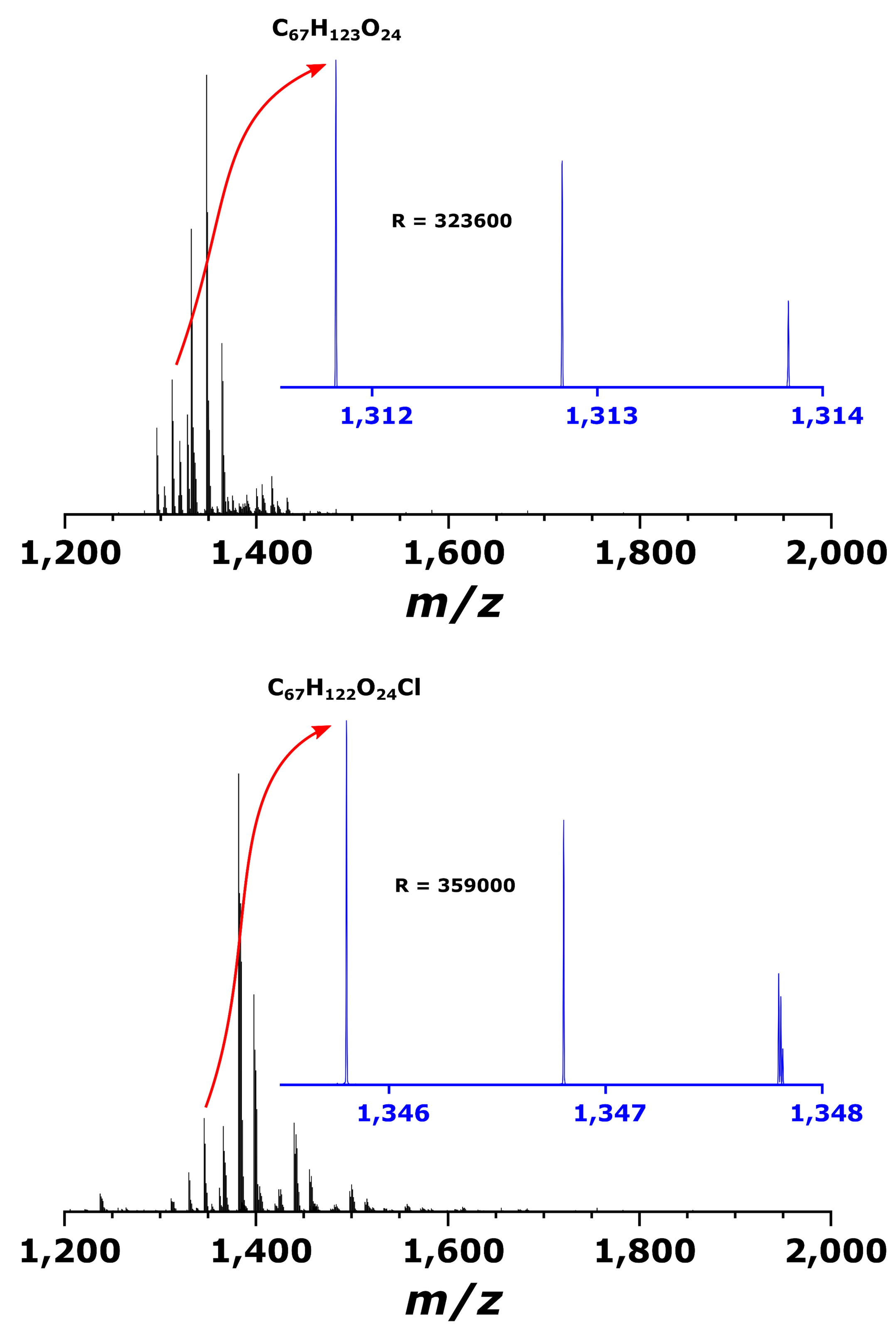
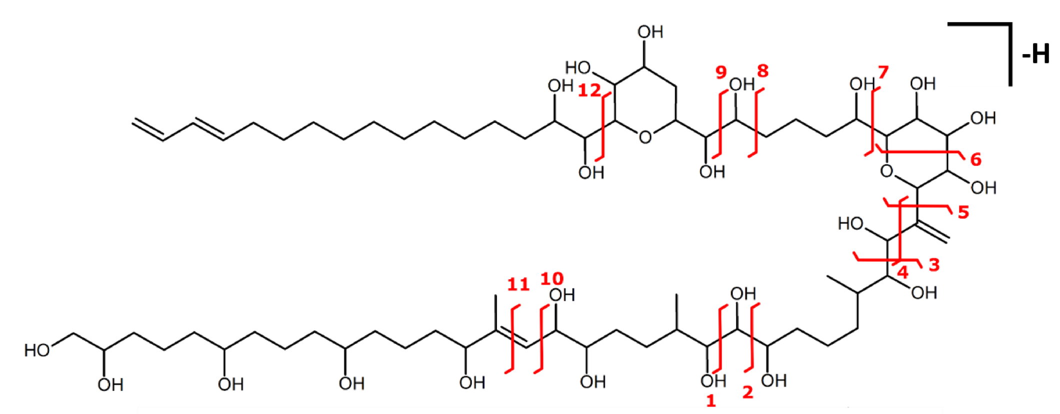


Figure 3. ESI (-) FT-ICR broadband mass spectra of two karlotoxins purified from a *K. veneficum* isolate (GM2) from the East China Sea.



m/z	Interpretation	Empirical formula	Error (ppm)	DBE	Bond # in figure
463.3276	aliphatic cleavage	$C_{24}H_{47}O_8$	-0.1	1	1
493.3382	aliphatic cleavage	$C_{25}H_{49}O_9$	0.0	1	2
623.4376	aliphatic cleavage	$C_{32}H_{63}O_{11}$	0.0	1	3
653.4482	aliphatic cleavage	$C_{33}H_{65}O_{12}$	0.1	1	4
679.4638	aliphatic cleavage	$C_{35}H_{67}O_{12}$	0.0	2	5
691.4638	ring cleavage + DB formation	$C_{36}H_{67}O_{12}$	0.0	3	cross ring
703.4638	ring cleavage + 2DB formation	$C_{37}H_{67}O_{12}$	0.0	4	cross ring
709.4744	ring cleavage	$C_{36}H_{69}O_{13}$	0.0	2	cross ring
739.4849	ring cleavage	$C_{37}H_{71}O_{14}$	0.0	2	6
811.5061	aliphatic cleavage	$C_{40}H_{75}O_{16}$	0.0	3	7
885.5792	aliphatic cleavage + ring opening	$C_{44}H_{85}O_{17}$	0.0	2	8
913.5742	aliphatic cleavage	$C_{45}H_{85}O_{18}$	0.1	3	9
995.6160	aliphatic cleavage	$C_{50}H_{91}O_{19}$	0.0	5	10
1007.6160	aliphatic cleavage + deprotonated	$C_{51}H_{91}O_{19}$	0.0	6	11
1061.6477	aliphatic cleavage + ring opening	$C_{51}H_{97}O_{22}$	0.0	3	12
1293.8304	precursor - H2O	$C_{67}H_{121}O_{23}$	0.0	7	N/A
1311.8408	deprotonated ion	$C_{67}H_{123}O_{24}$	-0.1	6	N/A

Figure 4. CID interpretation from FT-ICR MS/MS data for a KmTx2 variant obtained after a fish kill in China. All product ions' *m/z* values were determined at < 150 ppb mass accuracy.

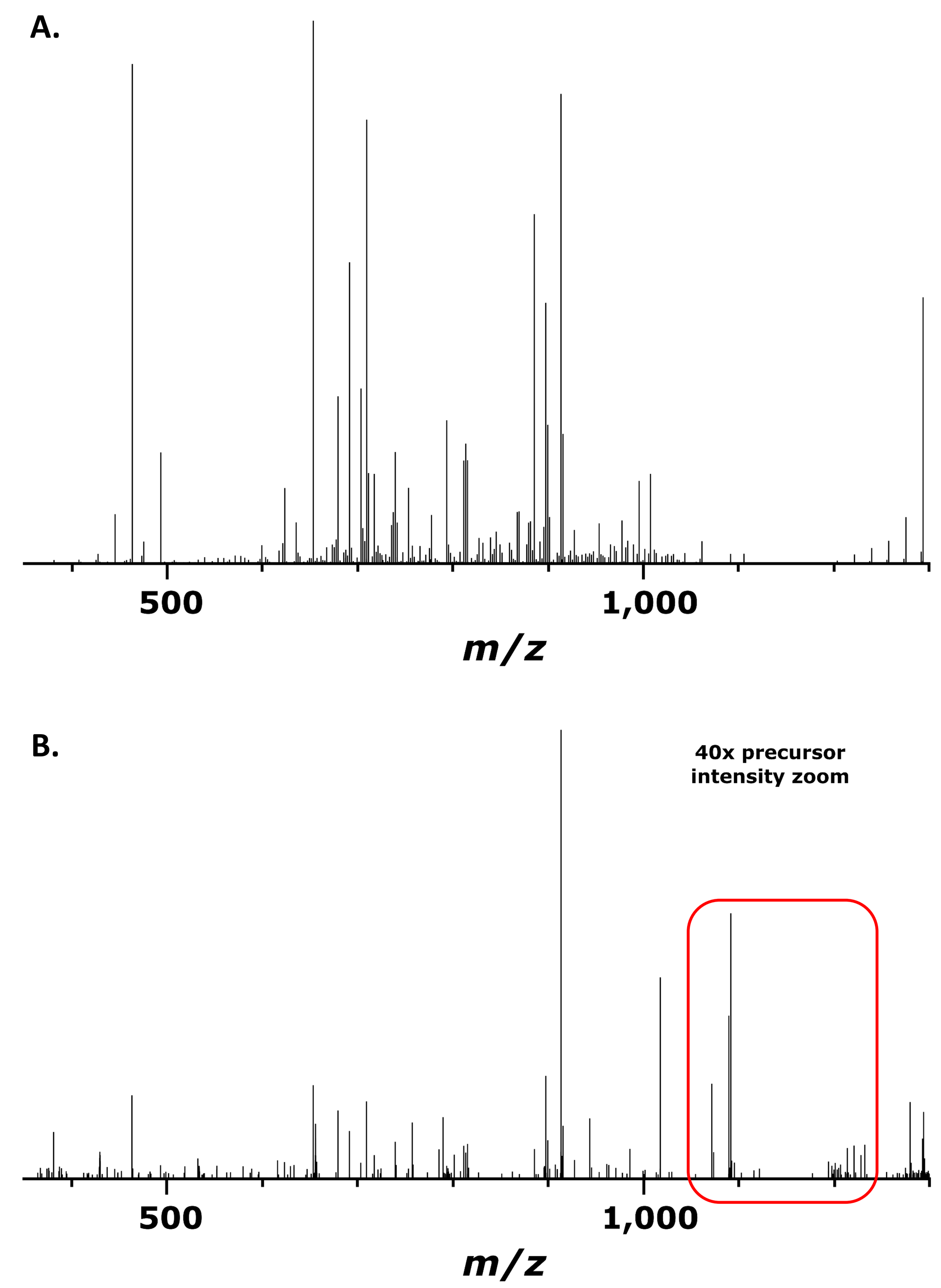
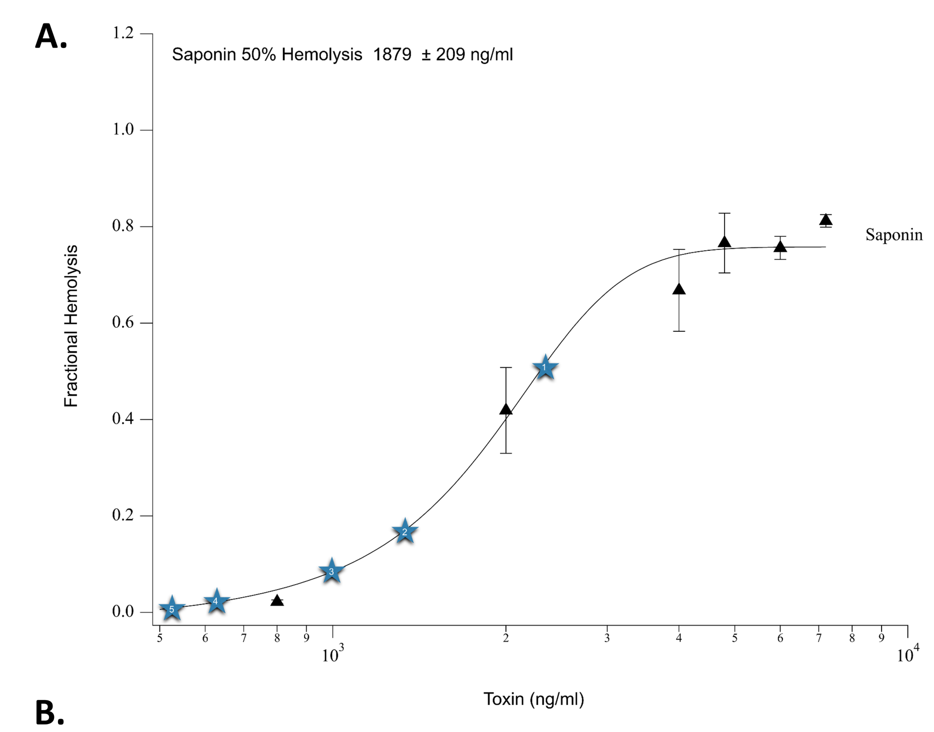


Figure 5. KmTx2 variant dissociation using A.) trap CID @ NCE = 50%, and B.) UVPD @ 1115 µJ. (red box) Additional ions detected after UVPD.



B.

Sample Name	Cell Count (cells/mL)	Volume (mL)	UV Area	MS Area	m/z	Total KmTx (ng)	Concentration (ng/mL)	Hemolytic?	Number symbols
Bottle 1	4282	300	15880.5	765867	690.3977	13897.8	46.33	+	
Bottle 6475	4040	300	12715.8	545107	690.3962	11128.2	37.09	+	6
Bottle 007	3394	300	57618.8	2051719	690.3970	50424.9	168.08	+	3
Bottle 3118	3232	300	22201.8	861004	690.3965	19429.8	64.77	+	4
Bottle 4771	3151	300	6255.0	253033	690.3943	5474.0	18.25	++	2
Bottle 91	2424	300	11160.3	394222	690.3980	9766.9	32.56	+(-)	1
Bottle 5912	1454	300	8476.0	396306	690.3965	7417.7	24.73	+	5

Figure 6. A.) Hemolytic data from water sample extracts of a karlotoxin variant. B.) Quantitative measures of dinoflagellate toxin levels in water samples and sample information from a recent fish kill event.

Discussion and Conclusions:

- Comprehensive LC-MS/MS methods were developed to identify and quantify dinoflagellate polyol toxins in water samples.
- Tandem, accurate mass spectra from both IT-TOF and FT-ICR instruments were used to confidently assign empirical formulae and primary structures.
- For a newly discovered karlotoxin, CID performed with ultra-high mass accuracy detection allowed for localization of functional groups previously assigned to other loci by NMR spectroscopy.
- Multiple dissociation techniques provided more complete coverage for structural inferences.
- Fish kills presumed to be associated with coincident dinoflagellate blooms were confirmed by accurate mass LC-MS/MS detection of hemolytic toxins.
- Toxin levels were quantified by LC-DAD area under the curve for characteristic absorption maxima.
- Hemolytic data supported the conclusion that a karlotoxin was responsible for a recent fish kill in the Gunpowder River, MD, USA.

Acknowledgment:

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5. Bachvaroff, T. R., Adolf, J. E., Squier, A. H., Harvey, H. R. and Place, A. R. (2008). Characterization and Quantification of Karlotoxins by Liquid Chromatography-Mass Spectrometry. *Harmful Algae*. 7; 473-484.

Presentations from Goodlett Lab and Collaborators

TOF pm 03:30 Detecting Antibiotic Resistance by MALDI-TOF Analysis of Bacterial Membrane Glycolipids, William Fondrie
WOD pm 04:10 Comparison of Quadrupole and Ion Trap Collision Induced Dissociation for Structure Determination of *Francisella Novicida* Lipid A Variants, David Goodlett
WP409 Molecular Structural Analysis of the Gram-positive Bacterial Membrane – *Enterococcus faecium* and *Staphylococcus aureus*, Sung Hwan Yoon
WP421 Structure Activity Relationship Elucidation of *Pseudomonas aeruginosa* Lipopolysaccharide Variants Associated with Cystic Fibrosis using a Multivaried Mass Spectrometric Approach, Mohsin Khan
WP488 Identification of ESKAPE Pathogens by MALDI-TOF MS Analysis of Microbial Membrane Glycolipids, Lisa Leung
WP491 Ultra-Rapid Identification of Bacteria by MALDI-TOF MS, Tao Liang
WP589 A SRM/MRM Based Targeted Proteomics Strategy for Quantification of Potential Biomarkers of TKI Sensitivity in EGFR Mutated Lung Adenocarcinoma, Shivangi Awasthi

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