Chemical Analysis of *Karenia papilionacea*

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Abstract

One of the most widely studied organisms responsible for Harmful Algal Blooms (HABs) is the marine dinoflagellate *Karenia brevis*. This organism produces neurotoxic compounds known as brevetoxins. A related dinoflagellate, *Karenia papilionacea*, has been reported to occasionally co-bloom with *Karenia brevis* but has received little attention as a possible toxin producing species. Therefore, our aim was to investigate the toxin profile for *K. papilionacea*. A toxic fraction was identified using a cell based cytotoxicity assay and the toxin was isolated and identified as the ladder frame polyether brevetoxin-2 (PbTx-2) using mass spectrometry (MS) and nuclear magnetic resonance (NMR). Toxin production in *K. papilionacea* increased in response to hypoosmotic stress, as previously observed in *K. brevis*.

Keywords: *Karenia papilionacea*, *Karenia brevis*, brevetoxin, toxin isolation, HAB, osmotic stress

22 1. Introduction

23 Although most HAB events are comprised of a single dominant algal species, there is
24 often more than one species of algae present during a single event. For instance, *Karenia brevis*
25 blooms generally contain *K. brevis* as the dominant species but other *Karenia* species such as *K.*
26 *mikimotoi* are also found in significant numbers (Heil & Steidinger 2009). Often, the toxicity of
27 a bloom is associated with a single HAB species, however, at times there may be additional
28 species present for which very little is known, including their toxicity. This is especially notable
29 for the dinoflagellate *Karenia papilionacea*, that is known to co-occur with *K. brevis* during
30 HAB events and has also been associated with toxic blooms in the absence of *K. brevis* (McNabb
31 *et al.* 2006).

32 *Karenia papilionacea* has two different cell forms, a large and small cell (Figure 1). Both
33 are very similar to *K. brevis* and have been observed in the same bloom. The larger butterfly
34 form has a pronounced hypothecal excavation (Haywood *et al.* 2004) and has been shown to
35 contract horizontally allowing the left and right sides to almost touch, similar to a butterfly
36 flapping its wings. The smaller cell form has a slight hypothecal excavation making it look very
37 similar to *K. brevis* (Haywood *et al.* 2004) (Figure 1). In fact, until *K. papilionacea* was formally
38 identified as a separate species in 2004 (Haywood *et al.* 2004), it was often mistakenly identified
39 as *K. brevis*. There are early reports of a ‘butterfly’ shaped *K. brevis* species (Steidinger 1979)
40 which is now thought to actually have been *K. papilionacea*. *K. papilionacea* has been reported
41 to be able to change cell shape quickly when under stressed growth conditions (Gómez, 2006).

42 Compared to many marine dinoflagellates, *K. papilionacea* has a wide distribution. *K.*
43 *papilionacea* is a neritic species also found in oceanic gyre systems (Gómez, 2006). Although, it

is most commonly found in coastal New Zealand waters, it has also been observed in the Gulf of Mexico, Florida Straits, Delaware Bay, Mediterranean, North Atlantic, South China Sea, and the Caribbean Sea.

Figure 1: Comparison of *K. papilionacea* cellular morphologies to *K. brevis*.

Fig. 1: Comparison of two cellular morphologies to *K. brevis*. **A.** *K. papilionacea*, *K. brevis*-like form; **B.** *K. papilionacea* butterfly form; **C.** *K. brevis*.

To date, all *Karenia* species that have been studied in depth have been associated with fish kills and marine mammal mortality, which supports the designation as a toxic genus. For example, *K. brevis* produces a family of ladder frame polyethers known as brevetoxins (Baden *et al.* 1979, Lin 1981)), *Karenia selliformis* produces the neurotoxin gymnodimine (Seki *et al.* 1995), *Karenia mikimotoi* produces ladder frame polyethers called gymnocins (Satake *et al.* 2002, Satake *et al.* 2005), and *Karenia brevisulcata* produces *Karenia brevisulcata* toxins (KBT) and brevisulcata acids (BSX) (Holland *et al.* 2012). *K. papilionacea*, *K. umbella*, and *K. bidigitata* are considered to be toxic but the toxins associated with each species are yet to be identified (Brand *et al.* 2012).

Haywood *et al.* (2004) reported that different algal species can bloom together and *K. brevis* and *K. papilionacea* are often found in the same bloom. During toxic events that contain both *K. brevis* and *K. papilionacea*, the toxicity has generally been attributed to *K. brevis* because it is known to produce brevetoxins. However, in blooms that were composed primarily of *K. papilionacea* in the absence of *K. brevis*, brevetoxins (50 – 110 pg/mL) were detected using an ELISA with antibodies specific for brevetoxins containing the PbTx-2 backbone. Toxin isolation and characterization was not done for samples from this bloom (Haywood *et al.* 2004). Toxin identification was not confirmed by liquid chromatography-mass spectrometry (LC/MS) either. More recently, blooms of *K. papilionacea* in New Zealand and *K. umbella* in Tasmania, Australia, have been associated with salmonid aquaculture mortality (de Salas *et al.* 2004). These blooms consisted of several different *Karenia* species and showed the presence of several toxins. Due to the complex species profile of most blooms attributing a particular toxin to one species has been difficult. For example, brevetoxins, gymnodimine and domoic acid were isolated from shellfish from the 1992-1993 biotoxin event in New Zealand (Landsberg 2002, Seki *et al.* 1995, 1996, Mackenzie *et al.* 1996, Chang *et al.* 1995, Rhodes *et al.* 1996). There were numerous *Karenia* species present, all initially identified as *K. brevis* (Chang *et al.* 1995, Haywood *et al.* 1996) but later reclassified as several new species (Haywood *et al.* 2004). One of these newly described species could be responsible for the production of one or many of these toxins

Brand *et al.* (2012) reported that both Mass Spectrometry (MS) and Nuclear Magnetic Resonance (NMR) techniques are essential to confirm toxin identity in a bloom. These two methods have become the standard for identifying and characterizing new toxins. Harwood *et al.* (2014) recently used these techniques to quantify and create a sensitive detection assay for the recently described brevisculatic acids (BSXs) and brevisculcenals (KBTs) from the toxic

dinoflagellate *K. brevisculata*. Mooney *et al.* (2007) reported that *K. papilionacea*, along with other *Karenia* species, produces unusual lipids, sterols, and PUFAs that have been shown to be ichthyotoxic. Research by Chang (2011) showed that *K. brevis* and other *Karenia* species (*K. concordia*, *K. brevisulcata*, *K. mikimotoi*) produce unidentified allelochemical compounds that can suppress the growth of most other species of algae, such as raphidophytes, diatoms and other photosynthetic organisms but are not effective against other dinoflagellates. Mooney *et al.* (2007) reported *K. papilionacea* showed high levels of 23-methyl-27-norergosta-8(14), 22-dien-3b-ol (59%–66%), and an unknown C28 sterol with three double bonds (Trimethylsilyl ether MW 468, 16%–17%). These compounds were shown to be present with a suite of other, unknown, C28 unsaturated sterols. Although, *K. papilionacea* has been associated with toxic blooms and fish kills no toxin has been identified from mono-blooms or cultures of this species. The current study is designed to determine the in vitro cytotoxicity of *K. papilionacea* using bioassay guided fractionation of *K. papilionacea* extract and subsequent identification of the toxin(s) produced by *K. papilionacea* using a combination LC/MS and NMR experiments.

2. Materials and Methods

Methanol, acetonitrile, 0.1% formic acid in acetonitrile, acetone, and ethyl acetate were purchased from Honeywell Burdick and Jackson (Muskegon, MI). Petroleum ether and molecular grade ethanol were purchased from Fisher Scientific (Fair Lawn, NJ). Chloroform was purchased from Mallinckrodt Chemicals (Phillipsburg, NJ). Sodium nitrate, sodium phosphate, disodium salt dihydrate, ferric chloride, boric acid, manganese chloride, cobalt chloride, and thiamine hydrochloride were purchased from Fisher Scientific (Fair Lawn, NJ). Tetra deuteromethanol, sodium selenite, cyanocobalamin (vitamin B₁₂), and biotin (vitamin H) were

108 purchased from Sigma–Aldrich (St. Louis, MO) and the zinc chloride was purchased from
109 Mallinckrodt Chemicals (Phillipsburg, NJ).

110 2.1 Culture

111 *K. papilionacea*, (CAWD-9 (Cawthron Micro-Algae Collection, Hawkes Bay, New
112 Zealand) and (KP-0709-2) Rehoboth Bay, Delaware) were grown in LH media for 4 weeks in a
113 10 L bioreactor (IKA Works, Wilmington, NC) at 22°C with 16hr light/8hr dark light regiment.
114 The LH media was composed of L1 media using filtered sea water (Guillard and Hargraves
115 1993) with a 50% PII metal mix (Na₂EDTA, 2H₂O - 6g; FeCl₃, 6H₂O - .290g; H₃BO₃ - 6.850g;
116 MnCl₂, 4H₂O; ZnCl₂ - .060g; CoCl₂, 6H₂O - .026g) with the addition of sodium selenite, .01 μM
117 (Loeblich 1975; Haywood et al. 2004). These alterations were referred to by Tatters *et al.* (2010)
118 as a modified L1 media but have since been renamed LH media. The seawater was collected
119 from the Gulf Stream near Wilmington, NC and transported back to the culture facility to be
120 filtered using a Whatman GF/F paper filter (Buckinghamshire, UK). The salinity of the seawater
121 was adjusted to a salinity of 31 by adding filtered deionized freshwater to filtered seawater prior
122 to nutrient addition. After preparation, the LH media was inoculated with approximately 100,000
123 cell/L from the stock cultures of *K. papilionacea*. Cell counts using 5 ml of culture from the
124 bioreactor were conducted using the Utermöhl method, (Utermöhl 1931) and were monitored
125 and recorded every 3-4 days from time of inoculation until the culture reached senescence, at
126 which point, it was harvested.

127 2.2 Culture Extraction

128 The *K. papilionacea* cultures were extracted using a chloroform:water extraction
129 technique as described by Baden and Mende (1982). Particulate matter was removed from the

130 chloroform extract by filtration using Whatman 542 paper filter (185mm, Buckinghamshire, UK)
131 and Celite 545 (EMD Chemicals, Gibbstown, NJ). After filtration through the Celite, the extract
132 was dried under vacuum and resuspended in 100 ml ethyl acetate. The ethyl acetate solution was
133 washed with water (3 x 100 mL) and separated. The combined aqueous extracts were
134 backwashed with fresh ethyl acetate to recover any residual lipophilic material. The ethyl acetate
135 layers were collected and dried down with a rotor evaporator (RV10 with HB10 digital water
136 bath, IKA works, Wilmington, NC). The residue obtained from the ethyl acetate extracts was
137 further partitioned based on polarity using a petroleum ether/MeOH/H₂O (50:40:10, v/v/v)
138 mixture (Truxal et al. 2010). The MeOH/H₂O layer was collected and dried under vacuum and
139 used for the following experiments.

140 2.3 Fractionation of the *K. papilionacea* Extract for Cytotoxicity Studies

141 Prior to the cytotoxicity studies the methanol extract was filtered through a 0.2 micron
142 polypropylene filter (Whatman, Buckinghamshire, UK) and then dried under vacuum. The
143 filtrate was the redissolved in 90% acetonitrile and 10% water and separated using High
144 Performance Liquid Chromatography (HPLC). The solvent delivery system was composed of a
145 Shimadzu Scientific Instruments HPLC consisting of 2 LC-10AS Liquid handling pumps, a
146 SCL-10A System Controller, and SPD-10AVUV-VIS Dectector. The HPLC conditions for the
147 initial HPLC separation were achieved using a Phenomenex Phenyl-Hexyl column, 5 μ M, 100
148 A, 8 X 250 mM reversed-phase column at a flow rate of 3.4 mL/min. A binary gradient starting
149 at 50% MPA proceeding to 0% MPA over 45 minutes was used, MPA (100% water), MPB
150 (100% MeOH). Fractions were separated into 45 one minute fractions using a Foxy Jr. Fraction
151 collector (ISCO, Lincoln, NE). The collected fractions were put into scintillation vials and dried

152 using a Speed-Vac (Savant Instruments, Speed VacPlus SC210A and Refrigerated Vapor Trap,
153 Holbrook, NY).

154 2.4 Cytotoxicity Assay

155 SJCRH30 cells (ATCC, Manassas, VA) were seeded at a density of 5,000 cells/well in
156 Greiner CELLcoat poly-D-lysine-coated 96-well plates and incubated at 37°C overnight. The
157 following day, cells were treated with 1 μ l of sample fractions suspended in 1ml of ethanol and
158 cell nuclei were stained with a 0.1 μ g/ml final concentration of Hoechst 33342 (Invitrogen) that
159 was added to the sample/medium solution (RPMI 1640 supplemented with 10% FBS) and
160 incubated at 37°C for 48 hrs.

161 After incubation the growth medium containing the Hoechst dye and fraction was
162 removed from the wells, and the cells rinsed once with warm HBSS (100 μ l per well). The
163 HBSS rinse was replaced with warmed 200 nM TT488 staining solution (200 nM TT488 in
164 HBSS, 100 μ l per well) to stain the microtubules. The plate was then returned to the 37°C
165 incubator for 30 minutes before imaging on an Image Xpress Micro system equipped with an
166 environmental control chamber warmed to 37°C. Hoechst 33342 and TT488 staining was
167 visualized using a DAPI filter and an FITC filter, respectively. Transmitted light images were
168 also collected to assess cell morphology.

169 Cytotoxicity of each fraction was determined using the ImageXpress Micro (Molecular
170 Devices, Sunnyvale, CA) using the multi-wavelength cell scoring macro. For this macro the
171 number of nuclei, cell size, and cell count for each well was determined. Fractions with
172 differences of more than 30% from the control wells were chosen for further purification.

173 2.5 Purification Techniques

174 Further purification was performed on biologically active fractions from the cytotoxicity
175 assay using a reverse-phased HPLC Prontosil 200-5 C30, 8.0 x 250mm (Bischoff
176 Chromatography, Leonberg Germany) or a microsorb-MV 100-5 C18, 250 x 4.6mm from Varian
177 (Walnut Creek, CA) column using a isocratic mobile phase (ACN or MeOH) and collected by
178 hand.

179 2.6 Mass Spectrometry

180 Low resolution $M + 1$ was obtained for all fractions of pure compounds using an AB
181 Sciex Q Trap 2000 MC/MS/MS system, Turbo spray @ 300° C. The experiment was a Q1 MS,
182 positive ion mode with a sweep width of 110.0 – 1700.0 amu (ionization parameters: curtain gas:
183 25.0 psi; temperature: 300.0 °C; ion source gas 1: 35.0 psi; ion source gas 2: 30.0 psi; interface
184 heater: on; ion spray voltage: 4500.0 V; declustering potential: 100.0 V; entrance potential: 11.0
185 V). Three μ L of sample was injected into the Qtrap using an Agilent 1100 series G1312A
186 Binary pump, G1313A Autosampler, and G1379A Degasser. The solvent conditions for the
187 HPLC were 20% mobile phase A isocratic (MPA (98% H₂O, 2% ACN, 0.1% FA), MPB (98%
188 ACN, 2% H₂O, .1% FA)), flow rate 110 μ L/min with a 4 minute run time. Samples were
189 dissolved in a mixture of 50% acetone:50% MPB prior to injection.

190 2.7 High Resolution Mass spectrometry

191 Accurate mass of the toxins isolated from the *K. papilionacea* was performed using Ultra
192 Performance Liquid Chromatography-High Resolution Mass Spectrometry (UPLC/HRMS).
193 UPLC/HRMS was performed on an Agilent Infinity 1290 UPLC consisting of a binary pump, a
194 degasser, a diode array detector (λ =215), thermostatted column compartment (40°C), and
195 autosampler. The column used for separation was an Agilent Eclipse plus C18 column (1.8 mm,

2.1 X 50 mm) protected by an EC C18 guard column (2.7 μ m, 2.1x5 mm). An isocratic mobile phase was used consisting of 80:20 v:v (LCMS grade Acetonitrile: HPLC grade H₂O + 0.1% formic acid) with a flow rate of 300 μ l/min and a 4 min run time. HRMS of target compounds was performed on a Bruker micrOTOF-Q II HRMS using an Electrospray ionization (ESI) source set in positive mode, dry heater setting of 200C, capillary voltage of 4000V, nebulizer pressure of 3.0 Bar, and drying gas flow of 10.0 l/min. The end plate offset was set to -500V, the collision cell RF was set to 60.0 Vpp and the scan with was set from 50 m/z to 3000 m/z. The whole system was run by Hystar 3.2 (Bruker Biospin, Billerica, MA) and the data was processed and analyzed using Compass (Bruker Biospin, Billerica, MA).

2.8 NMR

A Bruker Avance 500 MHz NMR equipped with a 1.7 mm TXI probe was used for structure elucidation of isolated compounds. The sample was dissolved 100 μ l CD₃OD NMR solvents (Sigma – Aldrich, St. Louis MO). Both 1-D (¹H and ¹³C and 2-D spectra ¹H -¹H COSY, ¹H -¹H TOCSY, ¹H -¹³C HMBC, and ¹H -¹³C HSQC experiments were performed.

2.9 Toxin Production per Cell

1.5L cultures of both strains were grown specifically for the use of determining how much PbTx-2 is produced on a per cell basis. The cultures were extracted in ethyl acetate as mentioned above and the samples were run through a Phenomenex Kinetex 2.6u PFP, 100A, 150 x 3.00mm column on the mass spectrometer using a Multiple Reaction Monitoring (MRM) method in a positive ion mode (ionization parameters: curtain gas: 23.0 psi, temperature: 425.0 °C, ion source gas 1: 30.0 psi, ion source gas 2: 75.0 psi, interface heater: on, ion spray voltage: 5300.0 V, declustering potential: 85.0 V, entrance potential: 11.0 V) . Four μ l of sample was

218 injected into the Qtrap using an Agilent 1100 series G1312A Binary pump, G1313A
219 Autosampler, and G1379A Degasser. The solvent conditions for the HPLC were 40% MPA from
220 .10 to 5 min, 1% MPA from 5.1 to 15 min, and 40% MPA from 12.1 to 20 min. The flow rate
221 ramped from 110 μ l/min (.10 to 5 min) to 140 μ l/min (5.1 to 15 min) and back to 110 μ l/min (15.1
222 to 20 min). Retention times for the brevetoxin standards and samples were compared to
223 determine if the correlating mass is a brevetoxin. Brevetoxin standards were created from
224 purified unialgal *Karenia brevis* (Wilson strain) cultures as previously described (Bourdelaïs *et*
225 *al.*, 2005; Truxal *et al.*, 2010). The brevetoxin standards were made by adding 977 μ l of MPB to a
226 .0977mg vial of PbTx-2 to make a concentration of 40,000 pg/ μ l. Serial dilutions were done to
227 create a final concentration of 4 pg/ μ l. These serial dilutions were used to calculate how much
228 toxin was present per injection to be able to calculate the toxin production per cell.

229 2.10 Test for Contamination – Raw Culture Samples

230 Given that much of the research conducted within our laboratories is concerned with the
231 culturing and isolation of compounds from *K. brevis*, it was necessary to ascertain that any toxins
232 associated with *K. papilionacea* were not merely contaminants from the *K. brevis* research. To
233 ensure that the presence of any toxin was not due to contamination from the laboratory
234 equipment, the use of virgin glassware as well as a series of raw culture samples was taken.
235 Briefly, 25ml of raw culture sample was taken from each carboy and filtered through a 3 ml
236 Strata X column. The column was preconditioned by washing with 10ml of MeOH, followed by
237 10ml of H₂O. The 25ml of culture was then added and washed with 10ml of H₂O, followed by
238 20ml of acetone. The acetone layer was collected and filtered using a 0.2 micron filter
239 (polypropylene, Whatman, Buckinghamshire, UK) and then prepared for mass spectroscopy in
240 the same manner as previously described.

In addition, samples were prepared from a *K. papilionacea* (CAWD-91) culture grown in the Campbell laboratory in L1 medium at a salinity of 35 and extracted using the ethyl acetate method, as previously described by Errera *et al.* (2010). Response to hypoosmotic stress was conducted by diluting a log-stage culture with MilliQ water (Millepore) containing L1 nutrients to a salinity of 27 and comparing the results to a control culture diluted with L1 medium.

3. Results and Discussion

3.1 Cytotoxicity

Using the HPLC fractionation method described, 45 fractions were obtained from each culture. The Delaware culture (DE#1) showed cytotoxicity in four fractions. The Delaware culture (DE #2) showed biological activity in five fractions. These fractions all came off during minute 32 through 37 in the crude sample fractionation suggesting these are fairly non-polar compounds. All toxic fractions were further separated using HPLC and were tested in a second cytotoxicity assay. This second assay showed significant cytotoxic activity in four subfractions.

3.2 LC-MS

The biologically active DE fractions were analyzed using low resolution mass spectrometry and showed a mass of 896.1, similar to the mass for PbTx-2 (895.4). There was also the presence of the mass 877.4, which is consistent with the daughter ion of PbTx-2 and is attributable to the loss of water (18 amu). This resulted in all of the 896.1 mass containing fractions being chosen for further purification and characterization using NMR. Similar masses were observed in DE #2

The New Zealand cultures (NZ#1 and NZ#2) showed cytotoxicity in five fractions and twelve fractions respectively. The NZ #1 fractions showing bioactivity were analyzed using low resolution mass spectrometer. The results of these experiments showed a mass of 896.1 for five fractions. There was also the presence of the mass 877.4, which again is the loss of 18 amu (H₂O) and is also very characteristic of PbTx-2. The 896.1 mass was also observed in the NZ #2 culture in numerous fractions. Due to the results from the DE culture suggesting that the 896.1 mass was PbTx-2, fractions containing this mass were selected for further purification. The purified compounds were then subjected to high resolution mass spectroscopy and NMR analysis.

High resolution mass spectrometry was done on three samples (30-8 (NZ#1), 38-3 (NZ #2), and 37-2-1 (DE #1)). Sample nomenclature signifies the Nth fraction and the Nth peak collected during purification; ex: 30-8 signifies the 30th fraction and the 8th peak collected during purification. All three of the samples showed the exact same m/z value of 895.48383 and a molecular formula of C₅₀H₇₁O₁₄, which match the high resolution m/z value and molecular formula of PbTx-2. The library monoisotopic mass for PbTx-2 was declared as 894.476563 Da and our compound showed the monoisotopic mass of 894.476557 Da. There is only one other compound in the library with a mass similar and it is that of (10'E,14'E,16'E)-6-sec-Butyl-24'-hydroxy-5,11',13',22'-tetramethyl-2',21'-dioxo-5,6-dihydrospiro[pyran-2,6'-[3,7,19]trioxatetracyclo[15.6.1.1^{4,8}.0^{20,24}]pentacosa[10,14,16,22]tetraen]-12'-yl 2,6-di-

deoxy-4-O-[(5E)-4-methoxy-6-methyl-5-(2-oxoethylidene)tetrahydro-2H-pyran-2-yl]-3-O-methylhexopyranoside, which will not have a similar ¹H NMR spectrum.

The results from the DE experiment showed the 895.4/895.4 mass, the ion extraction mass for PbTx-2, in the DE sample to have a retention time of 11.70 minutes. This value, when

compared to the retention time collected from the PbTx-2 standard spectrum was consistent for the known brevetoxin. The results from the NZ experiment showed the 895.4/895.4 mass in the NZ #1 sample to have a retention time of 9.02 minutes and for NZ #2, 8.99 minutes. This value, when compared to the retention time collected from the PbTx-2 standard spectrum, 9.03 minutes, was consistent for the known brevetoxin. Further support that the isolate was PbTx-2 was offered by spiking pure isolate samples with a PbTx-2 standard. Complete overlap between the signals was observed conclusively supporting the compound isolated from the DE and NZ strain was PbTx-2.

Using the MRM method described above the NZ strain had a value of 5.48 and 8.49 fg cell⁻¹, while the DE strain had a much lower concentration of .13 and .263 fg cell⁻¹. Ranges reported for cultures grown in the Campbell laboratory was 2-9 fg cell⁻¹ with the average brevetoxin production average of 5 fg cell⁻¹. After hypoosmotic stress, toxin production increased 2- to 3-fold over control cultures.

Raw culture samples and .001nm concentration of brevetoxin standards were taken and column retention times of specific peaks were compared. The presence of a mass of 895.4 and 877.7 was observed in the raw samples. The retention time of the compound suspected as PbTx-2 from the raw samples showed strong correlation to that observed for the brevetoxin standard (NZ: 9.48 mins and 9.49 mins respectively; DE: 9.35 mins and 9.36 mins respectively).

3.3 ¹H NMR

The structure of the compounds isolated from the New Zealand strain with masses indicative of PbTx-2 (.268 mg isolated from NZ #1 and .509 mg isolated from NZ #2) were determined using ¹H NMR spectroscopy. Comparison of the H-NMR spectrum from the NZ

unknown and the spectrum of a PbTx-2 standard showed strong overlap (Figure 2). The peaks 1-4 on the spectrum showed signals that correlated well with PbTx-2. Peak 1 correlates to the aldehyde proton, peaks 2 and 3 correlate to the exomethylene protons and peak 4 is a combination of the protons on the unsaturated bonds in the A and H-rings.

Figure 2: NMR spectra and correlating PbTx-2 molecule.

Fig. 2: NMR spectrum above shows critical correlating peaks that are identified on the PbTx-2 molecule.

3.4 Discussion

PbTx-2 is a known neurotoxin produced by *Karenia brevis*, but previously not known to be produced by *K. papilionacea*. Brevetoxins are known to cause massive fish kills and marine mammal mortality in Florida coastal waters as well as neurotoxic effects in humans (Baden *et al.* 1979, Poli *et al.* 2000, Wu *et al.* 1988, McFarren *et al.* 1965, Hughes *et al.* 1976, Sakamoto *et al.* 1987).

The results from mass spectrometry and the NMR experiments suggest that *Karenia papilionacea* produces the neurotoxin PbTx-2. When compared to *K. brevis*, *K. papilionacea* does not produce as much PbTx-2 on a per cell basis. *Karenia papilionacea* was believed to produce brevetoxin due to low positive ELISA tests (Naar *et al.* 2002) but it was never confirmed. The presence of PbTx-2 that has been observed in New Zealand without the presence of *K. brevis* can now be explained by the production of this toxin by *K. papilionacea*. In Delaware, brevetoxins have been detected and have been contributed to the presence of a

340 *Chattonella* species that has been shown to also produce PbTx-2 (Bourdelaïs *et al.* 2002). It is
341 possible that *K. papilionacea* could also have been contributing to the brevetoxin concentrations
342 that were observed during that bloom. Additionally, brevetoxin has been observed at times when
343 blooms of *Chattonella* have not been observed, so it is plausible that *K. papilionacea* produced
344 the brevetoxin.

345 The response of *K. papilionacea* to hypoosmotic stress is similar to previous observations
346 for *K. brevis* (Errera & Campbell 2012). Although the function of brevetoxin in *Karenia* cells is
347 unknown, the similar increase in brevetoxin production in both of these two species in response
348 to rapid decrease in salinity suggests a common role.

349 The production of brevetoxins by *K. papilionacea* is significant because this species has
350 been shown to bloom in areas with extensive shellfish beds (as is seen in Delaware) and may
351 pose a threat to humans through consumption of contaminated shellfish. The shellfish beds that
352 occur in areas that *K. papilionacea* is endemic, may will need to be monitored for brevetoxin
353 accumulation.

354 **4. Conclusions**

355 With the use of HPLC, cytotoxicity assays, Mass Spectrometry, and NMR, it was
356 determined that the New Zealand and Delaware strains of *Karenia papilionacea* produce PbTx-
357 2. The New Zealand strain produced the most out of the two at approximately 5 fg cell⁻¹, where
358 the Delaware only produced approximately 0.2 fg cell⁻¹. Appropriate monitoring measures for *K.*
359 *papilionacea* should be undertaken in locations with prominent shellfish industry to prevent
360 consumers from being exposed to potentially toxic of brevetoxin which could lead to Neurotoxic

361 Shellfish Poisoning. As a result of this study we now have a better understanding of another
362 toxic *Karenia* species, which further supports the notion of *Karenia* as being a toxic genus.

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369 Literature Cited:

- 370 Baden, D.G., Mende, T.J., Block, R.E., 1979. Two similar toxins isolated from *Gymnodinium breve*. Toxic
371 Dinoflagellate Blooms. Elsevier, New York, pp. 327–334.
- 372 Baden D.G. and T.J. Mende. 1982. Toxicity of two toxins from the Florida red tide marine dinoflagellate,
373 *Ptychodiscus brevis*. Toxicon. 20, 457–461.
- 374 Bourdelais, A.J., Tomas, C.R., Naar, J., Kubanek, J., Baden, D.G. 2002. New fish-killing alga in coastal Delaware
375 produces neurotoxins. Environ. Health Prospect. 110, 465–470.
- 376 Bourdelais, A.J., Jacocks, H.M., Wright, J.L.C., Bigwarfe, P.M., Baden, D.G., 2005. A new polyether ladder
377 compound produced by the dinoflagellate *Karenia brevis*. J. of Nat. Prod. 68, 2–6.
- 378 Brand, L.E., Campbell, L., Bresnan, E. 2012. *Karenia*: The biology and ecology of a toxic genus. Harmful Algae.
379 14, 156–178.
- 380 Chang, F.H., 2011. Toxic effects of three closely-related dinoflagellates, *Karenia concordia*, *K. brevisulcata* and *K.*
381 *mikimotoi* (*Gymnodiniales*, *Dinophyceae*) on other microalgal species. Harmful Algae 10, 181–187.
- 382 Chang, F.H., MacKenzie, L., Till, D., Hannah, D., Rhodes, L. 1995. The first toxic shellfish outbreaks and the
383 associated phytoplankton blooms in early 1993 in New Zealand. Harmful Marine Algal Blooms. Lavoisier
384 Intercept Ltd., New York, pp. 145–50.
- 385 De Salas, M.E., Bolch, C.J.S. Hallegraeff, G.M. 2004. *Karenia asterichroma* sp nov (*Gymnodiniales*, *Dinophyceae*),
386 a new dinoflagellate species associated with finfish aquaculture mortalities in Tasmania, Australia.
387 Phycologia. 43, 624–631.
- 388 Errera, R. M., Bourdelais, A., Drennan, M. A., Dodd, E. B., Henrichs, D. W. & Campbell, L. 2010. Variation in
389 brevetoxin and brevenal content among clonal cultures of *Karenia brevis* may influence bloom toxicity.
390 Toxicon. 55, 195–203.
- 391 Errera, R. M. & Campbell, L. 2012. Osmotic stress triggers toxin production by the dinoflagellate *Karenia brevis*.
392 *Proceedings of the National Academy of Sciences of the United States of America*. 109, 17723–24.
- 393 Gómez, F. 2006. The Dinoflagellate Genera *Brachidinium*, *Asterodinium*, *Microceratium* and *Karenia* in the Open
394 SE Pacific Ocean. Algae. 21, 445–452.
- 395 Guillard, R.L., Hargraves P.E. 1993 *Stichochrysis immobilis* is a diatom, not a chrysophyte. Phycologia. 32, 234–
396 263.
- 397 Harwood, D.T., Feng, S., Masayuki, S., Holland, P.T. 2014. A sensitive LC-MS/MS assay for brevisulcinal and
398 brevisulcatic acid toxins produced by the dinoflagellate *Karenia brevisulcata*. Toxicon, 84, 19–27.
- 399 Haywood, A., Mackenzie, I., garthwaite, I., Towers, N. 1996. *Gymnodinium breve* ‘look-alikes’: three *Gymnodinium*
400 isolates from New Zealand. Harmful and Toxic Algal Blooms, pp. 227–230.

Haywood, A.J., Steidinger, K.A., Truby, E.W., Bergquist, P.R., Bergquist, P.L., Adamson, J. & Mackenzie, L. 2004. Comparative morphology and molecular phylogenetic analysis of three new species of the genus *Karenia* (Dinophyceae) from New Zealand. *J. Phycol.* 40, 165-179.
 Heil, C. A. & Steidinger, K. A. 2009. Monitoring, management, and mitigation of *Karenia* blooms in the eastern Gulf of Mexico. *Harmful Algae*. 8, 611-617.
 Holland, P.T., Shi, F., Satake, M., Hamamoto, Y., Ito, E., Beuzenberg, V., McNabb, P., Munday, R., Briggs, L., Truman, P., Gooneratne, R., Edwards, P., Pascal, S.M. 2012. Novel toxins produced by the dinoflagellate *Karenia brevisulcata*. *Harmful Algae*. 13, 47-57.
 Hughes, J.M. and M.H. Merson. 1976. Fish and Shellfish Poisoning. *N Engl J Med*. 295, 1117-1120.
 Landsberg, J.H. 2002. The effects of harmful algal blooms on aquatic organisms. *Rev. Fish. Sci.* 10, 113-390.
 Lin Y.Y., Risk, M.A., Ray, S.M., Van Engen, D., Clardy, J., Golik, J., James, J.C., Nakanishi, K. 1981. Isolation and structure of brevetoxin B from the "red tide" dinoflagellate *Ptychodiscus brevis*. *JACS*; 103, 6773-6775.
 Loeblich, A.R. 1975. A seawater medium for dinoflagellates and the nutrition of *C. nlei*. *J Phycol.* 11, 80-86.
 Mackenzie, A.L., Haywood, A.J., Adamson, J., Truman, P., Till, D., Seki, T., Satake, M., Yasumoto, T. 1996. Gymnodimine contamination of shellfish in New Zealand. In: Yasumoto, T., Oshima, Y., Fukuyo, Y. (Eds.), *Harmful and Toxic Algal Blooms*. Intergovernmental Oceanographic Commission of UNESCO, pp. 97-100.
 McFarren, E.F., Silva, F.J., Tanabe, H., Wilson, W.B., Campbell J.E., Lewis, K.H. 1965. The Occurrence of Ciguatera-like Poison in Oysters, Clams and *Gymnodinium Breve* Cultures. *Toxicon*. 3, 111-123.
 McNabb, P., Rhodes, L., Adamson, J., Holland, P. 2006. Brevetoxin – an elusive toxin in New Zealand waters. *African J. Mar. Sci.* 28, 375 -377.
 Mooney, B.D., Nichols, P.D., de Salas, M.F., Hallegraeff, G.M. 2007. Lipid, fatty acid and sterol composition of eight species of *Kareniaceae* (Dinophyta): chemotax-onomy and putative lipid phycotoxins. *J. Phycol.* 43, 101-111.
 Naar, J., Bourdelais, A., Tomas, C., Kubanek, J., Whitney, P.L., Flewelling, L., Steidinger, K., Lancaster, J., Baden, D.G. 2002. A competitive ELISA to detect brevetoxins from *Karenia brevis* (formerly *Gymnodinium breve*) in seawater, shellfish, and mammalian body fluid. *Environ. Health Perspect.* 110, 179-185.
 Poli, M.A., Musser, S.M., Dickey, R.W., Eilers, P.P., Hall, S. 2000. Neurotoxic Shellfish Poisoning and Brevetoxin Metabolites: A Case Study from Florida. *Toxicon*. 38, 981-993.
 Rhodes, I., White, D., Syhre, M., Atkinson, M. 1996. *Pseudonitzschia* species isolated from New Zealand coastal waters: domoic acid production *in vitro* and links with shellfish toxicity. *Harmful and toxic Algal Blooms*, pp. 155-158.
 Sakamoto, Y., Lockey, R.F., Krzanowski, J.J. Jr. 1987. Shellfish and Fish Poisoning Related to the Toxic Dinoflagellates. *S. Med J.* pp. 866-872.
 Satake, M., Shoji, M., Oshima, Y., Naoki, H., Fujita, T., Yasumoto, T. 2002. Gymnocin-A a cytotoxic polyether from notorious red tide dinoflagellate *Gymnodinium mikimotoi*. *Tetrahedron Lett.* 43, 5829-5832.
 Satake, M., Tanaka, Y., Ishikura, Y., Oshima, Y., Naoki, H., Yasumoto, T. 2005. Gymnocin-B with the Largest Contiguous polyether rings from the red tide dinoflagellate *Karenia* (formally *Gymnodinium*) *mikimotoi*. *Tetrahedron Lett.* 46, 3537-3540.
 Seki, T., Satake, M., Mackenzie, L., Kaspar, H. and Yasumoto, T. 1995. Gymnodimine, a new marine toxin of unprecedented structure isolated from New Zealand oysters and the dinoflagellate, *Gymnodinium* sp. *Tetrahedr. Lett.* 36, 7093-7096.
 Seki, T., Satake, M., Mackenzie, L., Kaspar, H. and Yasumoto, T. 1996. Gymnodimine, a novel toxic imine isolated from the Foveaux strait oysters and *Gymnodinium* sp. *Harmful and Toxic Algal Blooms*. pp. 495-498.
 Steidinger, K.A. 1979. Collection, enumeration and identification of free living marine dinoflagellates. In Taylor, F. J. R. & Seliger, H. H. (Eds.) *Toxic Dinoflagellate Blooms*. Elsevier Science Publisher, New York, pp. 435-442.
 Tatters, A.O., Muhlstein, H.J., Tomas, C.R. 2010. The hemolytic activity of *Karenia selliformis* and two clones of *Karenia brevis* throughout a growth cycle. *J. App. Phycol.* 22, 435-442.
 Truxal, L., Bourdelais, A.J., Jacocks, H., Abraham, W.M., Baden, D.G. 2010. Characterization of Tamulamides A and B, Polyethers Isolated from the Marine Dinoflagellate *Karenia brevis*. *J. Nat. Prod.* 73, 536-540.
 Wu, C.H. and T. Narahashi. 1988. Mechanism of Action of Novel Marine Neurotoxins in Ion Channels. *Annu Rev Pharmacol Toxicol.* 28, 141-161.