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Detection of cyanotoxins (microcystins/nodularins) in hepatic tissues and epidermal mats of stranded bottlenose dolphins (Tursiops truncatus) in Northeast Florida

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DETECTION OF CYANOTOXINS (MICROCYSTINS/NODULARINS) IN HEPATIC TISSUES AND EPIDERMAL MATS OF STRANDED BOTTLENOSE DOLPHINS (*TURSIOPS TRUNCATUS*) IN NORTHEAST FLORIDA

by

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Abstract

The St. Johns River (SJR; Jacksonville, FL, USA) is a large, brackish, estuarine system characterized by considerable anthropogenic pollution, recurrent harmful algal blooms (HABs), and diverse toxin-producing cyanobacteria. The most prevalent toxins in SJR water samples are microcystins/nodularins (MCs/NODs). Additionally, the SJR provides critical habitat for a genetically and behaviorally distinct estuarine community of bottlenose dolphins (*Tursiops truncatus*) that routinely uses and strands in low mesohaline and oligohaline areas of the river. This population has been subject to two unusual mortality events (UME) since 2010 and has since been described as having substantial declines in population health, characterized by widespread dermatitis and emaciation. Additionally, three dolphins have been recovered from low salinity habitats with epidermal algal mats. Because dolphin illness and strandings overlapped temporally and spatially with confirmed cyanobacterial blooms in the SJR, there is concern that estuarine dolphin health may be declining due to exposure to toxic cyanobacteria and HAB events. Specific to this study, the SJR estuarine community was considered a high-risk group for cyanotoxin exposure in relation to coastal animals. This study analyzed all available hepatic tissues for estuarine dolphins, and used samples from coastal individuals that stranded outside of the known cyanotoxin bloom season as controls. Three analytical methods were used to determine MCs/NODs presence in dolphin liver and epidermal algal mat samples. An Adda ELISA and LC-MS/MS were used to determine free MCs/NODs presence while the MMPB technique was used to determine total (bound and free) concentrations and as confirmatory analyses. ELISA analyses produced high values that were not supported by concurrent LC-MS/MS or MMPB analyses, indicative of false positives. MMPB testing resulted in low-level

total MCs/NODs detection in some specimens. Results indicate that both estuarine and coastal dolphins are exposed to MCs/NODs, with potential toxic and immune health impacts.

Introduction

Cyanobacteria are diverse, photosynthetic, prokaryotic organisms that are integral to most aquatic ecosystems (Dvorák et al., 2017). Many cyanobacterial species have the ability to produce toxic secondary metabolites, which function as neurotoxins, hepatotoxins, and/or dermatoxins, that are most associated with harmful algal blooms (HABs; Watson et al., 2015). Recent research has shown that epiphytic cyanobacteria are also able to produce significant concentrations of cyanotoxins leading to death of avians such as bald eagles, American coots, Canada geese, and mallard ducks after consumption (Rocke et al. 2002, Augspurger et al. 2003; Williams et al., 2007; Mohamed & Shehri, 2010; Wilde et al., 2014). Presence of HABs and cyanotoxins have severely impacted local and state economies (Anderson et al., 2000), caused temporary closure of public water systems (McCarty et al., 2016), led to declarations of municipal emergencies (Executive Order, 2016), and have prolonged, deleterious impacts on aquatic animals and ecosystems (Paerl et al. 2001; Miller et al., 2010).

An excellent example of an ecosystem experiencing recurrent HAB events is the large, north-flowing, brackish St. Johns River (SJR; Jacksonville, FL, USA). The SJR is characterized by considerable anthropogenic pollution (*e.g.*, high nutrient load, chemical runoff, heavy metal pollution, and septic system failure), recurrent HABs, and diverse toxin-producing cyanobacterial taxa (Aubel et al., 2006; Dunn et al., 2008; Environmental Protection Board, 2017). Water sampling in the SJR has documented recurrent cyanobacterial blooms throughout the year since at least 2005, with many species (*e.g. Microcystis, Anabaena/Dolichospermum,*

Aphanizomenon/Chrysosporum, and *Cylindrospermopsis*) having the ability to produce potent toxins (Aubel et al., 2006; Environmental Protection Board, 2017). Additionally, the State of the River Report summarized the ten year status trend of HABs in the SJR as "unsatisfactory" and "unchanged" and with no projected decrease (Environmental Protection Board, 2017). Environmental exposure to cyanotoxins can pose both acute and chronic adverse health impacts. Symptoms of exposure in mammals include dermatitis, gastroenteritis, anorexia, impaired immune function, respiratory compromise, tumor production, and death, depending on the cyanotoxin type, concentration, route, and duration of exposure (Codd et al., 1995; Codd, 2000; Dow and Swoboda, 2000; Zhou et al., 2002; Shen et al., 2003).

Regardless of bloom presence, the most prevalent and persistent toxins in SJR water samples are microcystins/nodularins (MCs/NODS), whose concentrations have ranged from 0.15 to >2,000 $\mu l\ L^{-1}$ (ppb; SJWMD, unpublished). The SJR also provides critical habitat for a genetically and behaviorally distinct estuarine community of bottlenose dolphins (*Tursiops truncatus,* Montague 1821; Caldwell, 2001; Gubbins, 2002; Ermak et al., 2017). This population routinely uses mesohaline (5-18ppt) and oligohaline (0-3 ppt) areas of the river and has been subject to two unusual mortality events (UME) since 2010 (Borkowski, personal comm.; Gibson, unpublished; Environmental Protection Board, 2017). Since the 2010 UME, behavioral and stranding data suggest substantial declines in population health, characterized by widespread dermatitis, emaciation, and routinely utilizing and stranding in low mesohaline and oligohaline areas of the river (Borkowski , unpublished; Gibson, unpublished). Because dolphin illness and strandings overlapped temporally and spatially with confirmed cyanobacterial blooms in the SJR

ecosystem, there is concern that estuarine dolphin health may be declining due to chronic or intermittent exposure to HAB events and associated cyanotoxins.

One of the most important aspects of analyte determinations in biological matrices is pairing extraction methodology with appropriate analytical techniques. MCs (heptapeptides) and NODs (pentapeptides) are structurally similar hepatotoxins that possess the unique Adda moiety ([2S,3S,8S,9S,4E,6E,]-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid, Rinehart and Harada, 1988; Namikoshi et al., 1990; Mazur-Marzec et al., 2006; Bortoli and Volmer, 2014; Niedermeyer, 2014). All MC/NOD variants share the same mechanisms of toxicity, though the specific amino acid combinations on the peptide ring affect LD50 of MCs (MC-LR = 50 μg kg-1; MC-RR = 600 μg kg-1) and most NOD congeners have comparable LD50 to MC-LR (Krishnamurthy et al., 1986; Watanabe et al., 1988; Ohta et al., 1994; Pearson et al., 2010). Similarity in structure and methodologies that target the Adda result in the two classes being jointly classified in quantification in some analyses, such as the Adda ELISA and the MMPB technique. Traditional methodologies used (*i.e.*, ELISA and LC/MS-MS) are limited to the ability of detecting only of free forms of MCs/NODs for detection. More recently, the MMPB technique has been used to measure free, bound, and degraded forms of MCs/NODs (Sano et al., 1992; Foss and Aubel, 2015). Specific to this study, there is the potential that MCs/NODs may be degraded through environmental exposure, tissue decomposition, sample preservation, or present as unavailable, conjugated forms.

The core objective of the first chapter was to compare MCs/NODs liver burdens for estuarine and coastal bottlenose dolphin populations using the most reliable methodology available, and with direct comparison of findings from several analytical techniques. The

primary objectives of the second chapter was to identify the species of, and to assess the toxin producing potential of the epidermal algal mat found on a stranded SJR resident. For both studies, three methods were used in confirmatory analyses due to the complex nature of the target matrices. Results from chapter one provided evidence of cyanobacterial hepatotoxin exposure to both estuarine and coastal populations of bottlenose dolphins. Three analytical techniques were used to screen for MCs/NODs in liver samples. MMPB was detected in oxidized liver samples of six wild dolphins, with levels ranging from 1.3-19.9 ng g^{-1} d.w. total MCs/NODs. The Adda ELISA revealed high detects, including a sample negative for total MCs/NODs via MMPB (8.1 – 487 ng^{-1}). These results support that the ELISA is prone to false positive data for dolphin tissue extracts. Individual variant analysis (LC-MS/MS) did not result in free MC (14 variants tested) nor NOD-R detections, suggesting that the MMPB response was due to partially degraded, bound, conjugated, or untested MCs/NODs variants. Results of the second study support the erection of a new species of potentially toxin producing cyanobacteria (*Komarekiella delphikthonos*) based on a total evidence approach of molecular (toxin analysis), genetic (16S rDNA and 16-23S ITS), and ecological (*e.g*., growing as an epidermal mat on *T. truncatus*) characters under the International Code of Nomenclature for Algae, Fungi, and Plants. Results from these studies provides more information on the determinations of MCs/NODs concentrations in bottlenose dolphin liver tissues as well as adds to the literature previously uninvestigated toxin potential and species identification of epidermal mats to aid effective management of these important ecological sentinels.

Detection of cyanotoxins (microcystins/nodularins) in livers from estuarine and coastal bottlenose dolphins (*Tursiops truncatus*) from Northeast Florida

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Highlights

- First report of MCs/NODs detected in cetacean liver samples from Florida, USA.
- MMPB technique should be strongly considered for screening MCs/NODs in liver samples.
- Adda-ELISA analyses are unreliable for screening MCs/NODs in dolphin liver samples.
- Hepatic MCs/NODs were found in both coastal and estuarine dolphin populations.
- Expanded monitoring is advised for animals living in cyanotoxin-impaired habitats.

Abstract

Microcystins/Nodularins (MCs/NODs) are potent hepatotoxic cyanotoxins produced by harmful algal blooms (HABs) that occur frequently in the upper basin of the St. Johns River (SJR), Jacksonville, FL, USA. Areas downstream of bloom locations provide critical habitat for an estuarine population of bottlenose dolphins (*Tursiops truncatus*). Since 2010, approximately 30 of these dolphins have stranded and died within this impaired watershed; the cause of death was inconclusive for a majority of these individuals. For the current study, environmental exposure to MCs/NODs was investigated as a potential cause of dolphin mortality. Stranded dolphins from 2013-2017 were categorized into estuarine (n=17) and coastal (n=10) populations. Because estuarine dolphins inhabit areas with frequent or recurring cyanoblooms, they were considered as a comparatively high-risk group for cyanotoxin exposure in relation to coastal animals. All available liver samples from estuarine dolphins were tested regardless of stranding date, and samples from coastal individuals that stranded outside of the known cyanotoxin bloom season were assessed as controls. The MMPB (2-methyl-3-methoxy-4-phenylbutiric acid) technique was used to determine total (bound and free) concentrations of MCs/NODS in liver tissues. Free MCs/NODs extractions were conducted and analyzed using ELISA and LC-MS/MS on MMPB-positive samples to compare test results. MMPB testing resulted in low-level total MCs/NODs detection in some specimens. The Adda ELISA produced high test values that were not supported by concurrent LC-MS/MS analyses, indicative of false positives. Our results indicate that both estuarine and coastal dolphins are exposed to MCs/NODs, with potential toxic and immune health impacts.

Key words: ELISA, HABs, microcystins, MMPB, *Tursiops truncatus*

Introduction

1.1 Background

Harmful algal blooms (HABs) are large, toxic and/or nuisance algae blooms that can negatively impact aquatic habitat and threaten human and animal health. Due to substantial human health risks associated with cyanotoxin ingestion, respiration, or skin contact (WHO, 1998), detection of these toxins above recommended guidance levels has resulted in prolonged closure of public water supplies (McCarty et al., 2016), municipal or regional emergency declarations (Executive Order, 2016), and substantial economic losses (Anderson et al., 2000). Environmental exposure to cyanotoxins can pose both acute and chronic adverse health impacts. Symptoms of exposure in mammals include dermatitis, gastroenteritis, anorexia, impaired immune function, respiratory compromise, tumor production, and death, depending on the cyanotoxin type, concentration, route, and duration of exposure (Codd et al., 1995; Codd, 2000; Dow and Swoboda, 2000; Zhou et al., 2002; Shen et al., 2003). With increased anthropogenic watershed manipulation, nutrification, and urbanization altering flow and natural filtration of waterways worldwide, humans are becoming more susceptible to adverse health effects posed by more frequent and severe cyanobacterial blooms. Moreover, warmer temperatures associated with climate change are hypothesized to increase toxic HAB events (Paerl, 2008; Davis et al., 2009; Ye et al., 2011).

An excellent example of an ecosystem experiencing recurrent HAB events is the large, north-flowing, brackish St. Johns River in the southeastern United States (SJR, Jacksonville, FL, USA). The SJR is severely impacted by anthropogenic pollution (*e.g.*, noise, chemical and nutrient runoff, septic system failure, and heavy metal pollution), which can accentuate existing

ecological stressors (*e.g.*, seasonal freshwater discharge, harmful algal blooms, daily pH and salinity fluctuations). In this complex river system, northward flow plus strong tidal influences prevent natural flushing and cause periodic reverse directional flow (up to 161 miles upstream), allowing for prolonged retention of pollutants and biotoxins (Environmental Protection Board, 2017).

Water sampling in the lower SJR and surrounding freshwater tributaries has documented recurrent cyanobacterial blooms throughout the year since at least 2005 (Aubel et al., 2006). Many of the cyanobacteria species that predominate during blooms (*e.g. Microcystis, Anabaena/Dolichospermum, Aphanizomenon/Chrysosporum,* and *Cylindrospermopsis*) can produce potent toxins that can adversely affect public health, and cause widespread morbidity and mortality of aquatic organisms (Environmental Protection Board, 2017). Since 2005, the St. Johns Water Management District (SJWMD) has documented cyanotoxins in all areas of the river, from low salinity tributaries, to the brackish main channel (SJWMD, unpublished). Regardless of bloom presence, the most prevalent and persistent toxins in SJR water samples are microcystins/nodularins (MCs/NODS), whose concentrations have ranged from 0.15 to >2,000 $\mu g\ L^{-1}$ (ppb) (SJWMD, unpublished). MCs and NODs are classified together as they are biochemically similar, potent and environmentally persistent hepatotoxins that share the unique Adda moiety (Rinehart et al., 1988). The U.S. Environmental Protection Agency (EPA) has drafted a nationally recommended ambient water quality criterion for recreational exposure to MCs/NODs at 4 ng mL^{-1} (EPA, 2016). Under these guidelines, waterbodies exceeding this limit may require swimming and recreational advisories to protect human health (EPA, 2016).

In contrast, health risks for animals continuously or periodically inhabiting the river and feeding in cyanotoxin-impaired riverine systems are less well characterized, although their MC/NODs exposure may be substantial (D'Anglada, 2017). The SJWMD did not consistently collect toxin data or track toxin prevalence following dissipation of visible bloom events. However, detection of MCs/NODs throughout the year suggests that the SJR ecosystem retains cyanotoxins long after visible blooms have dissipated (SJWMD, unpublished). As a result, marine mammals utilizing this habitat could be repeatedly or chronically exposed to MCs/NODs, including levels exceeding human recreational exposure limits.

Although the precise metrics of post-bloom toxin persistence are unknown, marine mammals utilizing this habitat can serve as sentinels for the SJR ecosystem, and for associated human health risks, due to their high trophic level and ecological and physiological similarities to humans (Bossart, 2011). The SJR provides critical habitat for >300 common bottlenose dolphins (*Tursiops truncatus*), approximately half of which inhabit the river year round (Gibson, unpublished). This population has suffered two unusual mortality events (UME) since 2010 (Environmental Protection Board, 2017). The cause of the 2010 UME was not determined, but followed a significant harmful algal bloom of *Aphanizomenon flos-aquae*, and a mass fish die-off as cited in the State of the River Report for the St. Johns River (Environmental Protection Board, 2017). Dolphins stranding during the 2010 UME exhibited mild to severe skin lesions, and a few individuals exhibited strange swimming patterns (TtNEFL1021, TtNEFL1024, TtNEFL2027) prior to stranding (FWC-FWRI, unpublished). Skin lesions in cetaceans can be associated with environmental and anthropogenic pollution and/or prolonged freshwater exposure (Wilson et al., 1999; Fury and Reif, 2012). Exposure to bloom byproducts may be associated with skin and

respiratory disease, although specific byproducts remain poorly characterized. Exposure to water-borne cyanotoxins has been associated with development of a rash and other skin ailments in humans and terrestrial animals (Torokne et al., 2001; Stewart et al., 2006a; Stewart et al., 2006b). Pre-existing or concurrent skin disease in dolphins could further enhance susceptibility to freshwater cyanotoxin exposure, and associated health impacts. Since the 2010 UME, behavioral and stranding data suggest substantial declines in population health, characterized by widespread dermatitis, emaciation, and routinely utilizing and stranding in oligohaline (0.5-5 ppt) and low mesohaline (5-18 ppt) areas of the river (Gibson, unpublished; Borkowski, personal comm.; Environmental Protection Board, 2017). Although most of these stranded estuarine dolphins had no definitive cause of death, gross necropsy findings included dermatitis, congested and hemorrhagic-appearing livers, kidneys, and lungs, and pneumonia (FWC-FWRI, unpublished data), all of which are non-specific symptoms, but are all ailments associated with cyanotoxin exposures (Codd et al., 1995; Codd, 2000; Dow and Swoboda, 2000; Zhou et al., 2002; Shen et al., 2003) Because dolphin illness and strandings overlapped temporally and spatially with confirmed cyanobacterial blooms and cyanotoxin presence in the SJR ecosystem (Unpublished data; report received 3/28/2017 per request to the SJRWMD), there is concern that estuarine dolphin health may be declining due to chronic or intermittent exposure to HAB events and associated cyanotoxins.

The high anthropogenic activity and additional environmental stressors that characterize the SJR could enhance the susceptibility of estuarine dolphins to the adverse effects of cyanotoxin exposure both during bloom and non-bloom periods, similar to findings from Grasman (2002), who documented enhanced wildlife disease impacts in pollution-impaired

ecosystems. Exposure to pollution and environmental stressors has been associated with increased frequency and severity of UME events in eastern US estuarine dolphins, indicated by a higher proportion of infectious disease, physiological stress, and exposure to algal toxins (Hohn, 2002; Van Bressem et al., 2009). Marine mammals could also be exposed to biotoxins outside of bloom periods due to toxin persistence in sediment and biota, bioaccumulation, biomagnification, and disturbance of contaminated sediment during foraging activity (Welker and Steinberg, 2000; Miller et al., 2010;; Papadimitriou et al., 2012; Corbel et al., 2014; Zastepa et al., 2017). Direct and recurrent exposure to biotoxins have been associated with 50% of declared marine mammal UMEs (NOAA, 2010). The SJR dolphins are classified as a genetically unique population, so continued deaths could result in significant loss of genetic diversity (Caldwell, 2001).

Microcystins (MCs) are a class of heptapeptides, consisting of over 150 structurally-related variants, while nodularins (NODs) are pentapeptides with over ten naturally occurring variants (Namikoshi et al., 1990; Stirling and Miles, 1999; Mazur-Marzec et al., 2006; Bortoli and Volmer, 2014; Niedermeyer, 2014). Both MCs/NODs are hepatotoxins that share the unique Adda moiety ([2S,3S,8S,9S,4E,6E,]-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid) (Rinehart and Harada, 1988) and are jointly classified due to lack of specificity in some analyses. For instance, the Adda-specific enzyme-linked immunosorbent assay (ELISA) (Fischer et al., 2001) and the MMPB (2-methyl-3-methoxy-4-phenylbutiric acid) technique (Foss et Aubel, 2015) detect MCs/NODs indiscriminately. MCs/NODs are actively absorbed by liver hepatocytes through a specific energy-dependent transport process involving the rifampicin-sensitive hepatic bile acid carrier (Eriksson et al., 1990; Hooser et al., 1991; Runnegar et al.,

1991; Fischer et al., 2010). This process allows MCs/NODs to concentrate in hepatocytes (Yu, 1995) as free, covalently bound, and conjugated forms (Kondo et al., 1992; MacKintosh et al., 1995; Buratti et al., 2011). Not only does this make the liver a key indicator for MCs/NODs exposure, but the change in toxin availability also influences methodologies used in analyte determination.

One of the most important aspects of analyte determinations in biological matrices is pairing extraction methodology with appropriate analytical techniques. Traditional methodologies used such as ELISA and LC/MS-MS rely on the availability of free forms of MCs/NODs for detection. More recently, the MMPB technique has been used to measure free, bound, and degraded forms of MCs/NODs (Foss and Aubel, 2015; Sano et al., 1992). The MMPB technique uses oxidation to cleave the Adda moiety unique to MCs/NODs to create the MMPB molecule which can be monitored using mass spectrometry (Sano et al., 1992; Harada et al., 1996; Williams et al., 1997a; Williams et al., 1997b). To date, there has been no reported MMPB equivalent found in nature, and is strongly supported to only be formed when oxidizing MCs/NODs, allowing for method specificity in recovering all forms (i.e. free, conjugated, or degraded) of these toxins (Sano et al., 1992; Williams et al., 1997a; Williams et al., 1997b; Foss et Aubel., 2015; Foss et al., 2017). Specific to this study, there is the potential that MCs/NODs present in hepatic samples have been degraded through decomposition, as evidenced by Code 3 (moderate decomposition) and late Code 3 (moderate to severe decomposition) stranded individuals (Dierauf et al., 2001). In forensic toxicology, analyte recovery is affected by extent of tissue decomposition and the time between sampling and analyses (Kastrissios et al., 2005). Bacterial degradation of the analyte and putrefaction of the tissue are both sources that affect

retrieval in decomposing tissues (Kastrissios et al., 2005). With these influences, total MCs/NODs concentrations may not be accurately quantified using ELISA or LC-MS/MS methodologies and thus the MMPB method was used in confirmatory analyses to account for all forms of MCs/NODs.

The core objective of our study was to compare MCs/NODs liver burdens for estuarine and coastal bottlenose dolphin populations using the most reliable methodology available, and with direct comparison of findings from several analytical techniques. Our diagnostic approach provides more information on the determinations of MCs/NODs concentrations in bottlenose dolphin liver tissues to aid effective management of these important ecological sentinels.

2.0 Methods

2.1 Selection Criteria of Estuarine and Coastal Individuals

Using unique dorsal fin characteristics (*i.e.* fin shape, nick and notch patterns), bottlenose dolphins from the Florida Fish and Wildlife Conservation Fish and Wildlife Research Institute's (FWC-FWRI) North East Field Lab (NEFL) mortality database (2013-2017) were identified as either "estuarine" or "coastal" individuals. Dorsal fins that matched with the University of North Florida's (UNF) dolphin photo-identification catalog (n = 17) were included in the study as the "estuarine" population. Cryoarchived liver samples from all estuarine dolphins, regardless of stranding date or season, were analyzed in attempts to determine MCs/NODs exposure throughout the year.

Dorsal fin images of dolphins that were not identified in the UNF catalog, stranded outside of typical bloom seasons, and were recovered from the coastline were submitted to the Northeast Florida Dolphin Research Consortium to be compared to all estuarine dolphin

catalogs. Dolphins not identified as estuarine dolphins within any of the Consortium catalogs were considered a part of the "coastal" population (n=10) and their cryopreserved liver samples were used as MCs/ NODs controls. These selection criteria were based on the hypothesis that coastal-dwelling animals that stranded outside of typical bloom seasons would have lower hepatic burdens of MCs/NODs. Tested livers were limited to Code 2 (fresh dead), Code 3 (moderate decomposition), and late Code 3 (moderate/advanced) stranded dolphins (Dierauf et al., 2001), and decomposition status was assessed as part of toxin retrieval efforts.

2.2 Retrieval and Necropsy Protocols

For the above 17 cases and 10 controls, FWC-FWRI provided archived liver samples, all available necropsy reports, necropsy photographs, and GPS coordinates of stranding locations under permission from NOAA's Code of Federal Regulations 50CFR216.22. All samples were stored at -20° C in sterile whirlpak bags. The weight of individual samples varied from <5-70gm.

Full necropsy reports with histology were available for 8 dolphins (5 cases and 3 controls). Results of diagnostic testing for potential pathogens, such as cetacean morbillivirus and *Brucella* sp. were available for 16 individuals (12 cases and 4 controls). All available data were used to assess health status and identify potential histological lesions that could be associated with MCs/NODs exposure at the time of stranding.

2.3 Sample Preparation

All toxin analyses were conducted at Greenwater Labs/CyanoLab located in Palatka, FL. Initially, homogenization of >30 gm wet weight (w.w.) samples was attempted using a bead ruptor (Omni Bead Ruptor 24, Omni Kennesaw, GA, USA), but this sample size was too large and fibrous to provide sufficient homogenization. Thereafter, samples, including the attempted

homogenized samples, were lyophilized (Thermo Savant Modulyo freeze dryer), and homogenized into fine powder via a coffee grinder. The stainless-steel coffee grinder was thoroughly cleaned between samples to prevent cross-contamination. To equate results produced by testing lyophilized samples to wet tissue concentrations, wet to dry ratios were determined by adding known wet weight (w.w.) aliquots to vials, lyophilizing, and determining water loss.

2.4 Total Microcystins/Nodularins by MMPB

2.4.1 – Liver samples

Chemical oxidations and extractions were conducted as in Foss et al. (2017). Quality control included pre-oxidation spikes of all samples (2- 20 ng g^{-1}), matrix curves of 3 samples (TtNEFL1624, TtNEFL1701, TtNEFL1703) and lab duplicates (TtNEFL1460, TtNEFL1510, TtNEFL1517, TtNEFL1610). Spikes were prepared using certified MC-LR (National Research Council, CRM, Halifax, Nova Scotia, CA). Liver samples were oxidized as 0.100 ± 0.005 gram subsets dry weight (d.w.) using 5 mL of oxidant solution (0.2 M K_2CO_3, 0.1 M $KMnO_4$, 0.1 M $NaIO_4$) for 2.5 hours. The reaction was stopped with drop-wise addition of 40% (w/v) sodium bisulfite until solutions turned opaque. Samples were centrifuged (1,500 xg; 10 minutes) and pellets were rinsed with 2 mL DI. Pooled supernatants were sent through pre-conditioned solid phase extraction (SPE) (Strata™-X; PN 8B-S100-FCH, Phenomenex, Torrance, CA, USA), rinsed (2 mL; 5% MeOH), eluted (5 mL; 90% acetonitrile) and blown to dryness at 60°C with N_2 (Zymark LV TurboVap® evaporator, Biotage, Charlotte NC). Extracts were reconstituted in 1.5 mL of 5% MeOH, pH adjusted using 1 N HCl (< 3) and loaded onto 12 cc Novum simplified liquid extraction (SLE) tubes (PN 8B-S138-KDG, Phenomenex, Torrance, CA, USA). Elutions

(ethyl acetate; 10 mL) were blown to dryness (60ºC; N_2), reconstituted (5% MeOH; 200 mg mL^{-1}) and filtered (0.2 µm polyvinylidene fluoride - PVDF).

2.4.2 Extracts of Free MCs/NODs & Standards

External standard curves (0.5 - 100.0 ng g^{-1}) of certified MC-LR were oxidized and extracted following protocols in Foss and Aubel (2015). Subsamples of all 'free MCs/NODs' extracts analyzed using ELISA (section 2.5.2) were oxidized in the same manner. Extracts (100 µL) and MC-LR standards (1 mL) were oxidized 0.5 hours at final concentrations of 15 mM $KMnO_4$, 15 mM $NaIO_4$, and 100 mM K_2CO_3. The oxidation was stopped with dropwise addition of 40% (w/v) sodium bisulfite and clarified using Strata X Polymeric SPE, as previously described.

2.4.3 LC-MS/MS Analysis MMPB

The $[M-H]^-$ ion of MMPB (*m/z* 207) was fragmented and the product ion *m/z* 131 was monitored. External standard curves of oxidized MC-LR (0.5 - 100 ng g^{-1}) were analyzed daily and used to determine recoveries, verify instrument response and to quantitate 'free MCs/NODs' in extracts. Quantification of liver samples was conducted using matrix spikes and matrix standard curves. The method detection limits (MDL) for both matrices were calculated using a signal to noise ratio of 3, with quantification limits set to 3x that of the MDL.

2.5 Free MCs/NODs

2.5.1 Individual Variant Analysis (LC-MS/MS)

A single sample from individual (TtNEFL1435) tested >10 ng g^{-1} for MMPB and was analyzed for intact individual variants of microcystins/nodularin. The liver sample was extracted in 0.100 ± 0.005 gram subsets (d.w.). Quality control included the pre-extraction fortification of

isotopically labelled standards d7-MC-LR & d5-MC-LF (Abraxis Kits, Warminster PA, USA) and a matrix spike containing 14 variants of MC and NOD-R. NOD-R and MC standards were purchased from National Research Council Canada / Certified Reference Materials (MC-LR, MC-RR, Nodularin & [Dha^7]MC-LR; Ottawa, Ontario, CA), Enzo Biochem Inc. (MC-WR, [DAsp^3]MC-RR, [DAsp^3]MC-LR, MC-HtYR, MC-LF, MC-LW & MC-HilR; Farmingdale, NY, USA) and GreenWater Laboratories (MC-YR, MC-LA & MC-LY; Palatka, FL, USA).

Subsets were extracted (5 mL; 75% methanol in 0.1 M acetic acid) in a sonicating water bath (25 minutes) followed with centrifugation (10 minutes; 1,500 xg). The supernatants were retained and pellets rinsed with five mL 75% acidified methanol. Methanolic supernatants were pooled and treated separately from a second 1 mL rinse utilizing n-butanol (certified ACS, Thermo Fisher, Waltham, MA, USA). Supernatants were blown to dryness using N_2 at 60°C, reconstituted (5 mL; deionized water), and clarified using preconditioned Strata X Polymeric SPE (200 mg for methanolic extracts, 100 mg for butanol extracts). The SPE columns were rinsed with 5% MeOH (1-2 mL), and followed by an elution with 90% acetonitrile (column volume). Elutions were blown to dryness (60° C, N_2) and reconstituted (1 mL, 5% MeOH) and filtered 0.2 µm PVDF.

LC-MS/MS analysis used a Thermo Scientific Surveyor HPLC system coupled with a LTQ XL™ Linear Ion Trap Mass Spectrometer as described in Foss et al. (2017). Transitions monitored are summarized in Table 4. Separation of analytes was achieved using a Phenomenex Kinetex™ 2.6 µm C18 100 Å, LC Column (150 x 2.1 mm) over a linear gradient with mobile phase A (2 mM formic acid and 3.6 mM ammonium formate in deionized water) and B (95% acetonitrile (v/v) in 2 mM formic acid and 3.6 mM ammonium formate). Acetonitrile (Optima

LC/MS), water (HPLC), ammonium formate (ACS grade), and formic acid (98%) were purchased from Thermo Fisher Scientific (Waltham MS). The gradient running at 0.2 mL min^{-1} was as follows: Solvent A held 70% for 5 minutes, 70-65% A over 8 minutes, held 65% A for 2 minutes, 65-30% A over 4 minutes, 30-70% A over 2 minutes and held 70% A for 3 minutes. Each chromatographic run was 24 minutes and 20 µL full loop injections were employed. MDLs were determined using the pre-extraction matrix spike and a signal to noise value of 3. Seven-point external standard curves ranging from 1-100 ng g^{-1} using the suite (14 variants of MCs and NOD-R) were used in spike return assessments. The internal standard d7-MC-LR was used to calibrate analytes detected in positive ionization mode ([$DAsp^3$] MC-RR, MC-RR, NOD-R, MC-YR, MC-HtYR, MC-LR, [$DAsp^3$]MC-LR, [Dha^7]MC-LR, MC- HilR, MC-WR, [Leu^1]MC-LR). The internal standard d5-MC-LF was used to calibrate analytes monitored in negative ionization mode (MC-LA, MC-LY, MC-LW, MC-LF). An external standard curve was also used to assess recoveries without internal standard correction.

2.5.2 Adda Enzyme-linked Immunosorbent Assay (ELISA)

Free MCs/NODs were extracted for all hepatic samples that were MMPB-positive, and one MMPB-negative sample was analyzed for comparison. Liver samples were extracted in 0.250 ± 0.005 g subsets (d.w.) with pre-extraction spikes (MC-LR) prepared of two samples (TtNEFL1501 & TtNEFL1701) at 20 and 100 ng g^{-1}, respectively. Samples were extracted using the same methods as in the Individual Variant Analysis (2.5.1), but had an additional butanol rinse combined in the final extract. The samples were reconstituted at a concentration of 500 mg/mL and further diluted 10 fold for analysis at final sample concentrations of 50 mg/mL. A polyclonal Adda ELISA (Abraxis Kits, PN 520011, Warminster, PA, USA) was used to analyze

extracted MCs/NODs as described in Foss et al. 2017Additional dilutions were required to achieve final ELISA values for samples reported greater than 80 ng/g. TtNEFL1503 and TtNEFL1507 samples were diluted 50-fold from the extract, a final 100-fold dilution from the sample (10 mg/mL).The MDL was determined to be 3 ng g^{-1} (ppb) based on dilution factors and kit sensitivity.

2.5 Dolphin Habitat Analyses

Stranding data provided by FWC-FWRI were used to map stranding locations for all MMPB-positive dolphins (n=6) using ArcGIS 10.4.1 (ESRI; Redlands, California). Additionally, to assess associations between pre-stranding habitat use and presence of MCs/NODs in hepatic subsamples for stranded estuarine dolphins, the sighting histories (2011-2016) were compared between MMPB-positive and MMPB-negative estuarine dolphins. Photo identification surveys have been conducted weekly in the SJR since March 2011 along a fixed 40-km transect from Mayport Inlet (N 30.39904, W -81.39396) to downtown Jacksonville (N 30.31479, W -81.62987) as described in Ermak et al., (2017). Spatial analyses were limited to individuals sighted ≥10 times (MMPB–positive, n=3; MMPB-negative, n=12). After same day resights had been removed, mean sightings per individual was 28.1 (SD= 12.5) with a mean sighting duration (time between first and last sighting) of 3.51 years.

To enable comparisons of individual home ranges and critical habitat areas, 95% and 50% utilization distributions were calculated. Univariate kernel density estimate (KDE) analyses were preferred over bivariate KDE because it decreases the amount of uninhabitable areas (*i.e.* land) included in analyses of narrow habitats (Rayment et al., 2009; Nekolny et al., 2017), such as the St. Johns River (narrowest along route = 381 m, Water-Resources Investigations Report,

1995; widest along route = 1.25 km; Gibson, unpublished). In order to generate univariate datasets, a midline was mapped throughout the survey route where downtown Jacksonville was assigned as location "0", and the Mayport Inlet was defined as "40". Sighting locations were plotted and transformed to the midline using the tool "locate features along routes". Data were then input into SAS (version 9.4; SAS Institute, Cary, NC, USA) and the function PROC KDE computed the 95% and 50% utilization distributions. Three different computational methods were compared (*i.e.* simple normal reference, Silverman's rule of thumb, and the Sheather-Jones plug in) to select bandwidth; Silverman's rule of thumb (SROT) was chosen, as it moderately smoothed the data in comparison to other methods.

3.0 Results

3.1 Total MCs/NODs measured as MMPB

For hepatic subsamples tested from 27 dolphins (17 estuarine and 10 coastal controls), six resulted in the detection of MMPB, indicating the presence of total (bound, conjugated and free) MCs/NODs. The MDL was determined to be 1.3 μg g^{-1} d.w. with a method quantification limit (MQL) of 3.9 μg g^{-1}. The average ratio of wet to dry weight was calculated as 3.856 ± 0.333 $\frac{wet\ weight}{dry\ weight}$ grams.

Three MMPB-positive dolphin livers were from estuarine dolphins, while three were from the coastal population. One female and two male estuarine dolphins were MMPB-positive, while two female and one male coastal animals were MMPB-positive. A larger sample size would be needed to determine whether sex, age, or other attributes are significant risk factors for MCs/ NODs exposure for dolphins. Five of the hepatic subsamples had concentrations of MMPB between the MDL and the MQL (1.3 – 3.9 ng g^{-1}), while one hepatic subsample tested above the

MQL at 14.3±5.6 ng g^{-1} (Table 1). A representative chromatogram of Individual TtNEFL1701, with overlaid matrix spikes can be viewed in Figure 1. The standard addition matrix curve generated from chromatograms in Figure 1 data can be viewed in Figure 2.

3.2 Free MCs/NODs

3.2.1 Individual Variant Analysis LC-MS/MS

LC-MS/MS analysis of the only hepatic subsample whose MMPB concentration was > 10 ng g^{-1} (dolphin TtNEFL1435) did not reveal intact free MCs or NOD-R present above MDLs (1.6 - 11.5 ng g^{-1}; Table 3), suggesting that the MCs/NODs in this sample were derived from bound, conjugated or partially degraded MCs/NODs variants. Alternatively, the MMPB may have come from variants not tested for in the current study, or from multiple variants with no single congener exceeding current MDLs.

The two-step extraction with 75% acidified methanol followed by a butanol rinse resulted in recovery of additional, less polar, and later eluting analytes from the spike (*i.e.* MC-WR, MC-LA, MC-LY, MC-LW & MC-LF; Table 3). Although examination of variant-specific recoveries in relation to extraction solvent was brief, recovery of additional MC variants following addition of a butanol rinse to the original extraction implies that detection of less polar MC variants, when present in the sample, requires application of a stronger solvent in order to be released from dolphin liver. Additional study is warranted to optimize free MCs/NODs extraction from mammalian livers.

3.2.2 ELISA

ELISA testing of all MMPB-positive and one MMPB-negative livers yielded levels of MCs/NODs 3.5-244 times higher than total MCs/NODs measured as MMPB (Table 1). A liver

subsample that had been considered <MDL via MMPB analysis (TtNEFL1610; <1.3 ng g^{-1} total MCs/NODs) resulted in detection of 12.8 ng g^{-1} free MCs/NODs by ELISA. Our results indicate that ELISA testing yielded false positive data due to matrix effects, potentially through non-specific binding with kit antibodies. Although spikes were recovered, the returns were below most acceptable ranges at 13% for the 20 ng g^{-1} spike, and above most acceptable ranges at 169% for the 100 ng g^{-1} spike (Table 2).

3.3 MMPB of ELISA Extracts

MMPB analysis of the above ELISA extracts supported that ELISA analyses yielded false positive data, with all extracts registering <MDL (2.5 ng g^{-1}) for total MCs/NODs (Table 1). The low spike returns (below detection and 39%) indicate that the matrix retained the analytes and supports that the ELISA overestimated MCs/NODs concentrations (Table 2).

3.5 Dolphin Habitat Analyses

Dolphins whose liver samples were MMPB-positive stranded in multiple areas of the SJR, as well as along the coast (Figure 3). Two MMPB-positive estuarine dolphins stranded in oligohaline areas, while one stranded in a low mesohaline area (Figure 3). Individual 95% utilization distributions (home ranges) showed that a majority of both MMPB-positive (n=3) and MMPB-negative (n=12) dolphins utilize almost the full extent of the SJR study area, including low mesohaline areas of the river. However, 50% utilization distributions (core areas; Figure 4) showed that two MMPB-positive dolphins (CLSA, NIKE) were habitually utilizing low mesohaline areas. The one MMPB-positive animal (MKNA) whose 50% utilization distribution was not primarily found in low mesohaline areas stranded in oligohaline waters. Limitations of the 40 km survey route prevent further analyses of how far MMPB-positive dolphins routinely

travel upriver. Interestingly, core areas of four MMPB-negative dolphins (U141, Q132, Q175, Q58) were also located in low mesohaline waters.

4.0 Discussion

4.1 MCs/NODs retention and exposure

MCs/NODs are well known to persist in natural systems due to resistance in degradation by sunlight, fluctuating temperatures, or changing pH (Tsuji et al., 1994; World Health Organization, 1998; Song et al., 2006). Additionally, the half-life of one of the most toxic variants (MC-LR) is 90-120 days per meter water depth (Welker and Steinberg, 2000), allowing for persistence in deeper water systems, such as the SJR (channel average depth ~12.2 m from Jacksonville to the mouth; Environmental Protection Board, 2017). Unlike a previous study where passive adsorptive systems (SPATT bags) were deployed to track toxin movement (Miller et al., 2010), cyanotoxin data taken in the SJR did not determine toxin retention or dissipation from blooms. Water sampling for cyanotoxins (2005-2016) in the SJR were inconsistent with respect to sample site, depth, testing method, and sampling intervals. Despite these shortcomings, analytical data have confirmed the presence of MCs/NODs in water collected throughout the SJR ecosystem, including the river mouth (SJWMD, unpublished). Toxin presence in the lower basin may be due to blooms occurring in freshwater tributaries close to the river mouth, or due to prolonged retention of contaminated water originating upstream. Due to the combination of an extremely low slope gradient and reverse directional flow, the retention time of SJR water is approximately three to four months (Benke and Cushing, 2005). Previous studies conducted by Anderson and Goolsby (1973) determined that tidal inflow into the mouth

of the river is seven times greater than the volume of freshwater discharged from the SJR, potentially facilitating toxin retention long after toxic blooms have dissipated.

It is unknown whether MCs/NODs can bioaccumulate throughout the SJR food web or biomagnify in higher predators. Typical MCs/NODs exposure paths include inhalation, possible dermal contact, ingestion through contaminated food, and drinking contaminated water (World Health Organization, 1998). For this pilot study, we were unable to determine the route of MCs/NODs exposure for dolphins, as most animals stranded without stomach contents. Estuarine and coastal dolphins inhabiting the SJR ecosystem may be chronically exposed to MCs/NODs or cyanobacterial byproducts via multiple routes, including toxin ingestion in water or biota, inhalation, or dermal contact. Due to the environmental persistence of MCs/NODs and limited flushing in the SJR, estuarine dolphins could be exposed to persistent MCs/NODs following bloom events (De Maagd et al., 1999; Welker and Steinberg, 2000;; Miller et al., 2010; Papadimitriou et al., 2012; Corbel et al., 2014; Zastepa et al., 2017). A potential explanation of the MCs/NODs in coastal animal hepatic tissue is transient exposure through coastal stocks migrating into estuaries (Griffin et al., 2018) or exposure to estuarine or marine, MCs/NODs producing cyanobacterial species (Pearson et al., 2010)).

4.2 Microcystins as potential immunosuppressants

The results of this study support that MMPB-positive animals did not die as a result of acute MCs/NODs exposure. Rather, the low MC/NOD exposure may have contributed to health decline or mortality through synergetic effects due to immune system compromise. All six dolphins that stranded with MMPB-positive liver samples exhibited skin lesions, confirmed cetacean morbillivirus infection, pneumonia, and/or poor nutritional condition (Table 1).

Although effects of cyanotoxins on the immune system have not been characterized in cetaceans, development of skin lesions has been associated with impaired immune function (Aguilar and Raga, 1993; Schulman and Lipscomb, 1999; Venn-Watson et al., 2015). Chronic sublethal exposure to MCs/NODs can impair immune function in mammals and facilitate more severe infection by viruses and other pathogens in mammals and birds (Shen et al., 2003; Pikula et al., 2010; Palikova et al., 2012). For example, concurrent exposure to MCs and low concentrations of heavy metals (*i.e.*, lead) and Newcastle disease virus (NDV) resulted in enhanced morbidity and mortality of Japanese quails (*Coturnix japonica*; Pikula et al., 2010). Japanese quails ingesting 0.045 – 46.044 μg MCs per 10 mL in water daily for 10 - 30 days did not exhibit mortality, despite analytically-confirmed MC accumulation, oxidative stress, and microscopic evidence of hepatic damage (Pikula et al., 2010). In contrast, MC exposure in combination with NDV or Pb resulted in 20-40% mortality of exposed animals around 10 days post-exposure (Pikula et al., 2010). Quail exposed to both MCs and Pb, or MCs, Pb, and NDV showed minimal NDV seroreactivity, suggesting that exposure to cyanobacterial biomass altered humoral immune responses (Pikula et al., 2010).

In a similar study, common carp (*Cyprinus carpio*) were exposed to either cyanobacterial biomass (0.4 mg MCs/kg total fish weight), white spot disease (*Ichthyophthirius multifiliis*), or both physiological stressors for 20 days (Palikova et al. 2012). Exposure to cyanobacterial biomass alone led to a significant increase in white blood cell count without significant mortality, while 32.5% mortality was observed in the combined exposure group (Palikova et al., 2012). These studies suggest that sub-lethal MCs exposure can substantially elevate mortality in animals that are concurrently exposed to anthropogenic pollutants or pathogens (Pikula et al.,

2010; Palikova et al., 2012). Concurrent pathogen and MCs exposure may pose significant additive impacts on non-specific immune responses (Palikova et al., 2012); when hosts are faced with multiple environmental or immune stressors, including cyanotoxins, opportunistic pathogens may be better able to penetrate host defenses and disseminate.

A similar scenario may be affecting dolphins in the SJR. In addition to chronic or recurrent MCs/ NODs exposure (SJWMD, unpublished), estuarine dolphins are exposed to other potential immune suppressors, such as heavy metals (e.g. lead, copper, silver), viruses (e.g. cetacean morbillivirus), anthropogenic pollution (e.g. leaking septic tanks, noise) (Environmental Protection Board, 2017; King, 2017). While exposure to separate health threats at low concentrations are unlikely to cause mortality, simultaneous exposure to multiple stressors in the SJR may be enhancing dolphin mortality.

4.3 Habitat Utilization

Although this study was limited in survey area and sample size, GIS analyses suggest that MMPB-positive estuarine dolphins and some MMPB-negative dolphins are consistently using lower salinity habitats in the SJR ecosystem. Habitat use and home range vary greatly among individual bottlenose dolphins, so a much larger sample size will be needed to draw definitive conclusions. Maintenance dredging occurs frequently in the SJR, and deepening the channel through expanded dredging has been proposed (Environmental Protection Board, 2017), possibly re-suspending sediment containing cyanotoxins and other pollutants. Additional research to assess pollutant burdens in SJR sediment could enhance protections for estuarine and coastal dolphin populations.

4.4 MMPB use for Biological Matrices

Due to the lack of a distinctive shoreline with a low slope gradient, dolphins that die in the SJR are unable to beach and are typically found floating in the water. As a result, carcasses may float in the water for hours or days, actively decomposing prior to carcass recovery. Partial decomposition in water should not allow MCs/NODs present in the water column to enter the liver, as there is strong scientific evidence that MCs/NODs cannot passively diffuse across cell membranes. MCs require active uptake by cells due to the large molecular size and logDow ratio (Opperhuizen et al., 1985; Pouria et al., 1998; De Maagd et al., 1999). The logDow is the comparison of the log of the octanol/water distribution ratio, or the ratio that correlates the ability of a ML-LR to concentrate or bind into lipids of the cell wall (Karickhoff et al., 1979; Mackay, 1982). The MC-LR logDow is approximately -1 at physiological pH, signifying that MC-LR is highly unlikely to passively diffuse into tissues from surrounding contaminated water, or diffuse from the liver into adjacent tissues during decomposition (De Maagd et al., 1999). This knowledge is beneficial in selection of an analytical technique to determine total MCs/NODs concentrations in stranded and decomposing animals.

In this study, the MMPB technique provided low level detection limits for total (*e.g.* free, bound, conjugated, degraded, partially metabolized) MCs/NODs in livers from fresh or moderately decomposed, stranded bottlenose dolphins. Although individual MCs/NODs congeners cannot be identified using the MMPB technique, it can be used to successfully quantify a broad spectrum of harder-to-detect MCs/NODs congeners (*e.g.* conjugated, bound, more hydrophobic variants). An individual variant analysis (LC-MS/MS) did not confirm the presence of intact MCs or NOD-R in this study. The loss of less polar microcystins during free analyte extraction, such as MC-LW & MC-LF, highlights the need to optimize the efficacy of

congener extraction in biological matrices, such as mammalian liver. The suspected false positive data from the ELISA for free MCs/NODs requires additional attention as well, as many studies are prone to utilize the ELISA without additional or secondary confirmation, which may lead to erroneous conclusions.

The disparity in ELISA and MMPB analyses highlights the importance of conducting secondary/confirmatory analyses when testing novel biological matrices, such as dolphin liver. Heussner et al. (2014) conducted a study to assess the suitability of monoclonal and polyclonal Adda ELISAs to screen MCs/NODs exposure across multiple mammalian species. They confirmed that use of human-sera specific Adda ELISAs for screening MCs/NODs presence in bovine or canine sera resulted in extreme deviations from spiked concentrations, suggesting high matrix effects. Assessing the reliability of Adda ELISAs against MCs/NODs recovery from standard cell culture media also yielded high matrix effects and significant overestimation of true MCs/NODs concentrations (Heussner et al., 2014). Additionally, Meissner et al. (2013) and Ernst et al. (2005) demonstrated that conventional extraction procedures used for ELISA are not suitable for extracting bound MCs. Although ELISA analyses have been successful for screening some biological matrices, such as smallmouth bass livers (Foss et al. 2017), analysis of mammalian, and potentially other animal tissues for MCs/ NODs, may require higher detection limits coupled with more rigorous clean up to minimize matrix effects. The lack of a consistent, sensitive and reference-validated biochemical assay for MCs/ NODs detection in biological samples has greatly limited prior efforts to study the impacts of these potent hepatotoxins on mammals and birds.

Another interesting discovery was that MCs/NODs were detected in archival liver samples of <5 gm w.w. During dolphin necropsies, liver sampling techniques were not standardized to a single hepatic lobe or location, and the size of the archived sample also varied. Because MCs/NODs are not lipid soluble, samples <5-10 grams may not be representative of MCs/NODs concentrations in the liver.MC-LR has been shown to specifically and preferentially accumulate in hepatic cells and do not localize in non-parenchymal cells (Yoshida et al., 2001). Furthermore, Yoshida et al. (2001) determined that MC-LR distribution in the liver was heterogeneous after intraperitoneal exposure in mice. Results from this study demonstrated MC-LR accumulated most in the centrilobular region and least in the perilobular region (Yoshida et al., 2001). Thus, samples under a certain weight, or sampled from a nonspecific location may not allow for detection of MCs/NODs, and could under-estimate actual/ total MCs/NODs concentrations.

It is possible that additional dolphins in this study could have been affected by MCs/NODs but were false-negative. Some factors include the elimination of the MCs/NODs prior to death or the hepatic sampling technique used (e.g. small size, non-representative sampling). For example, MMPB-negative dolphins (*i.e.* U141, Q132, Q175, Q58, DUSK) also shared characteristics of MMPB-positive dolphins, such as skin lesions, cetacean morbillivirus infection, emaciation, and hepatic necrosis, unusual freshwater stranding locations and low mesohaline critical habitat use areas (FWC-FWRI, unpublished; Bossart, unpublished; Figure 3). Standardized tissue sampling techniques could help address this question from a scientific and management perspective.

4.5 Future Analyses of MCs/NODs

Future application of MMPB testing, to allow for all bound, degraded, and free MCs/NODs recovery, will greatly facilitate efforts to test biological tissue matrices, and assess animal exposure to hepatotoxic MCs/NODs. Prior studies have demonstrated inconsistent detection of MCs/NODs in biological matrices such as liver, even when cyanotoxin ingestion was confirmed via biochemical tests of gastrointestinal content, and animal exposure was known or highly suspected (Palikova et al., 2012; Mittelman et al., 2016). For many prior studies, LC-MS/MS was the preferred method of analysis, and targeted intact and free MCs/NODs. However, LC-MS/MS analysis of intact MCs/NODs is likely to underreport congeners in biological matrices such as dolphin livers. Thus, reliance on one analytical method may be insufficient in determining total MCs/NODs concentrations in biological matrices.

This study demonstrated the successful detection of MMPB in all assessed postmortem decomposition codes, with detection of MCs/ NODs in liver samples from Code 2 (n=2), Code 3 (n =2) and late Code 3 (n= 2) dolphins. The highest hepatic MMPB concentration was retrieved from a Code 3 (moderately decomposed) individual. The ability of MMPB methodology to detect bound, degraded, and partially metabolized MCs/NODs removes some of the current obstacles for assessing cyanototoxin exposure using biological matrices, including samples from both fresh dead and more severely decomposed animals. According to current sampling protocols, the amount of tissue archived as part of gross necropsy is lower for more decomposed specimens. Our research demonstrates that both fresh and more autolyzed animals can be successfully analyzed for MCs/NODs using MMPB methodology and that MMPB could serve as an optimal screening test for assessing MCs/NODs exposure in animals in various states of decomposition. We have also demonstrated that Adda-ELISA analyses are unreliable as a

screening test for MCs/NODs in dolphin liver samples. In order for ELISA to be an effective screen for MCs/NODs, further research and method improvement is needed.

The MCs/NODs measured in this study did not exceed 20 ng g^{-1} d.w., which would roughly equate to 5 ng g^{-1} w.w. when using the average wet to dry weight ratio. It is difficult to directly relate the data from this study to others determining MCs/NODs in hepatic tissue due to the differences in methodologies used. Miller et al., 2010, whom extracted wet weight, free MCs from sea otter hepatic tissue, and quantified via LC-MS/MS, had reported MC concentrations ranging from 1.6 ng g^{-1} to approximately 390 ng g^{-1}. The concentrations of total MCs/NODs in this study were comparative to the lower concentrations of free MCs in the sea otters, though size of sample, methodologies used, and inability to determine individual MCs/NODs congeners prohibit direct comparisons between sea otter and dolphin toxin exposures**.** Although the concentration of MCs/NODs in estuarine and coastal dolphin liver samples were comparatively low, the potential for chronic, recurrent, sublethal exposure, and the demonstrated ability of these toxins to accentuate the negative impacts of anthropogenic pollutants, pathogens, and environmental stressors is of significant concern. Chronic exposure to low concentrations to MCs/NODs may be contributing to low population fitness and increased mortality of the SJR bottlenose dolphins. These effects may not be raising immediate concern, however sub-lethal effects will become an important issue in management of this population if it continues to decline.

5.0 Conclusions

This study provided evidence of cyanobacterial hepatotoxin exposure of wild bottlenose dolphins, a sentinel species. Three analytical techniques were used to screen for MCs/NODs in

liver samples of SJR estuarine and coastal stranded bottlenose dolphins, including a more recent technique (MMPB) known to detect free, bound, and degraded MCs/NODs in biological samples. MMPB was detected in oxidized liver samples of six wild dolphins, with levels ranging 1.3-19.9 ng g^{-1} d.w. total MCs/NODs. Individual variant analysis (LC-MS/MS) did not result in free MC (14 variants tested) or NOD-R detections, suggesting that the MMPB response was due to partially degraded, bound, conjugated or untested MCs/NODs variants.

ELISA analysis may not be an acceptable screening mechanism for (MCs/NODs) in dolphin liver samples, especially for low level detection (<15 ng g^{-1}). In this study, MMPB detections were not confirmed using traditional analysis techniques geared towards free MCs/NODs (ELISA & LC-MS/MS). Additional approaches that may support MMPB results include techniques demonstrated to measure bound sources of MCs/NODs in biological matrices, such as thiol-deconjugation followed by extraction and LC-MS/MS analysis (Miles et al., 2016). Every effort was employed to verify the MMPB data in this work, including replicate oxidations and standard addition. However, since low level detections in complex matrices may be due to artifacts, it should be a priority to investigate alternate approaches to detecting low level MCs/NODs in marine mammalian tissues. While this study encompasses a small sample size, it supports MCs/ NODs uptake by wild dolphins in a cyanotoxin-impaired watershed. This study also provides a clear representation of the obstacles faced when analyzing complicated biological matrices from animals, such as liver, for MCs/NODs analytes. Analyses incorporating a larger sample size coupled with systematic antemortem and postmortem health assessments may help clarify whether cyanotoxin exposure can also impair immune function in bottlenose dolphins.

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Tables

Table 1. Abnormalities noted as part of postmortem examination or diagnostic testing, and hepatic concentrations of total microcystins (MCs) and nodularins (NODs) reported as ng g^{-1} d.w. for estuarine and coastal populations of bottlenose dolphins (*Tursiops truncatus*) using the 2-methyl-3-methoxy-4-phenylbutiric acid (MMPB) or ELISA techniques. Basic Level A and stranding data was provided by FWC-FWRI and Bossart, unpublished data.

Population	Stranding Date	Decomp Code	Sex	Necropsy evidence	Total MCs/NODs (MMPB)	Free MCs/NODs (ELISA)	MCs/NODs (MMPB) of ELISA Extracted Material
Estuarine							
TtNEFL1435	7/7/2014	3	M	Cetacean morbillivirus (PCR+)	14.3±5.6	54.1	ND
TtNEFL1460	10/21/2014	Late 3	F	Splenomegaly, thin blubber	1.3-3.9[a]	12.7	ND
TtNEFL1501	1/15/2015	Late 3	M	Thick algal mat on epidermis	1.3-3.9 [a]	8.1	ND
TtNEFL1610*	6/6/2016	3	F	Stranded in lake (salinity ~ 0 ppt)	ND	12.8	ND
Coastal							
TtNEFL1503	2/5/2015	2	F	Emaciation, dermatitis, angiomatosis, pneumonia, possible intestinal obstruction	1.3-3.9 [a]	239	ND
TtNEFL1507	3/5/2015	3	M	Emaciation	1.3-3.9 [a]	487	ND
TtNEFL1701	1/14/2017	2	F	Pneumonia, dermatitis, angiomatosis, emaciation	1.3-3.9 [a]	59.8	ND
MDL					1.3	3.0	2.5
MQL					3.9	3.0	7.5

The average ratio of wet to dry weight was calculated as 3.856 ± 0.333 $\frac{wet\ weight}{dry\ weight}$ grams.

* Represents MMPB negative individual used as a negative control for ELISA analyses

[a] =MMPB detected above the MDL but below the MQL

Table 2. Recoveries of lab fortified sample matrices measured using an Adda ELISA and as Total MCs/NODs via the oxidation of ELISA extracts and analysis for MMPB" for clarification

Analyte	Spike Concentration (ng g^{-1})	Sample ID	ELISA Returns	MC-LR Returns (as MMPB)
MC-LR	20	TtNEFL1501	13%	Not Recovered
MC-LR	100	TtNEFL1701	169%	39%

Table 3. Recoveries of the MC and NOD-R suite using 75% acidified MeOH and an additional butanol rinse. Method detection limits (MDLs) are shown.

Variant:	75% Acidified MeOH	Butanol Rinse	SUM	Uncorrected Returns (sum)	MDL (ng g^{-1})
[DAsp^3]RR	75%	0%	75%	33%	5.3
RR	69%	0%	69%	37%	5.8
NOD-R	129%	0%	129%	45%	1.6
YR	113%	0%	113%	50%	4.5
HtYR	85%	0%	85%	41%	2.9
LR	105%	0%	105%	46%	2.4
[DAsp^3]LR	109%	0%	109%	45%	2.8
[Dha^7]LR	92%	0%	92%	36%	3.3
HilR	71%	0%	71%	38%	7.0
WR	39%	4%	43%	17%	11.5
[Leu^1]LR	59%	0%	59%	30%	4.2
LA	122%	4%	126%	30%	1.6
LY	51%	6%	56%	18%	3.5
LW	18%	19%	37%	4%	5.4
LF	22%	30%	53%	12%	3.8

Table 4: Transitions monitored for MCs and NOD-R analysis

Analyte	Precursor Ion (*m/z*)		Fragment Ions (*m/z*)
[DAsp^3]MC-RR	$[M+2H]^{2+}$	512.9	291, 426, 446 ,499, 504
MC-RR	$[M+2H]^{2+}$	519.9	298, 440, 453, 455, 504, 511
NOD-R	$[M+H]^+$	825.5	599, 674, 776, 781
MC-YR	$[M+H]^+$	1045.5	599, 710, 1027
MC-HtYR	$[M+H]^+$	1059.5	599, 584, 567, 484
MC-LR	$[M+H]^+$	995.5	553, 599, 867, 968, 978
[DAsp^3]MC-LR	$[M+H]^+$	981.5	539, 599, 954, 964
[Dha^7]MC-LR	$[M+H]^+$	981.5	539, 599, 954, 964
MC-HilR	$[M+H]^+$	1009.5	484, 567, 599
MC-WR	$[M+H]^+$	1068.6	599, 626, 940, 1041, 1051
[Leu^1]MC-LR	$[M+H]^+$	1037.6	599, 1019 ,612
MC-LA	$[M-H]^-$	908.5	780, 797, 878, 891
MC-LY	$[M-H]^-$	1000.5	872, 889, 970, 984
MC-LW	$[M-H]^-$	1023.6	1005.6
MC-LF	$[M-H]^-$	984.6	966.6
Internal Standards			
d7-MC-LR	$[M+H]^+$	1002.5	599.5
d5-MC-LF	$[M-H]^-$	989.6	971.5

Figures

Figure 1: The TtNEFL1701 liver sample chemically oxidized, extracted, and analyzed for MMPB (blue chromatogram) with overlaid matrix spikes in red (2, 10, 20 ng g^{-1}) of MC-LR.

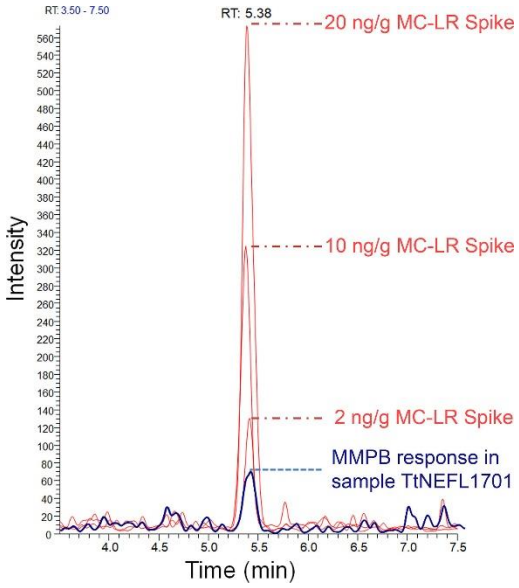


Figure 2: The standard addition curve of dolphin liver sample (TtNEFL1701) constructed using MC-LR spiked at 2, 10 & 20 ng g^{-1}. The sample was oxidized, extracted and analyzed for MMPB. Corresponding chromatograms can be viewed in Figure 1.

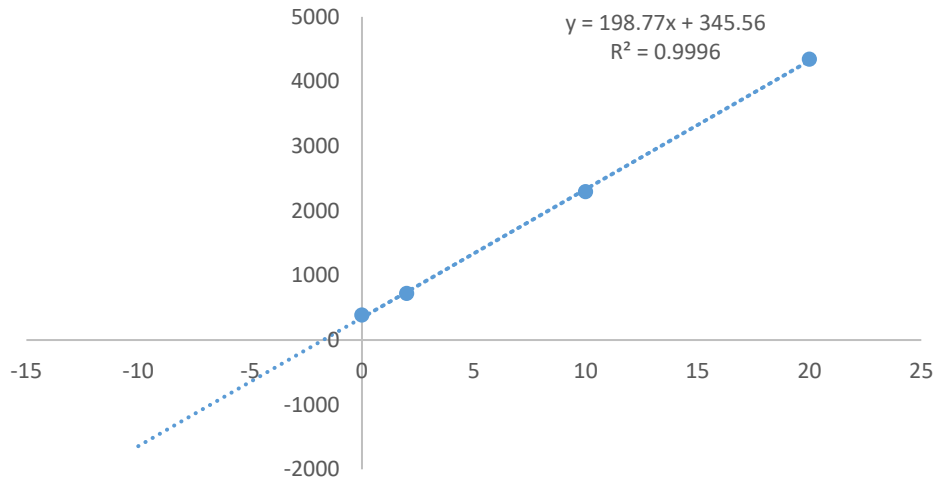


Figure 3. Locations of MMPB-positive bottlenose dolphins (*Tursiops truncatus*) stranding in northeast Florida from 2014-2017. Black circles indicate stranded MMPB-positive coastal dolphins, and black stars indicate stranded MMPB-positive estuarine dolphins that stranded within the St. Johns River.

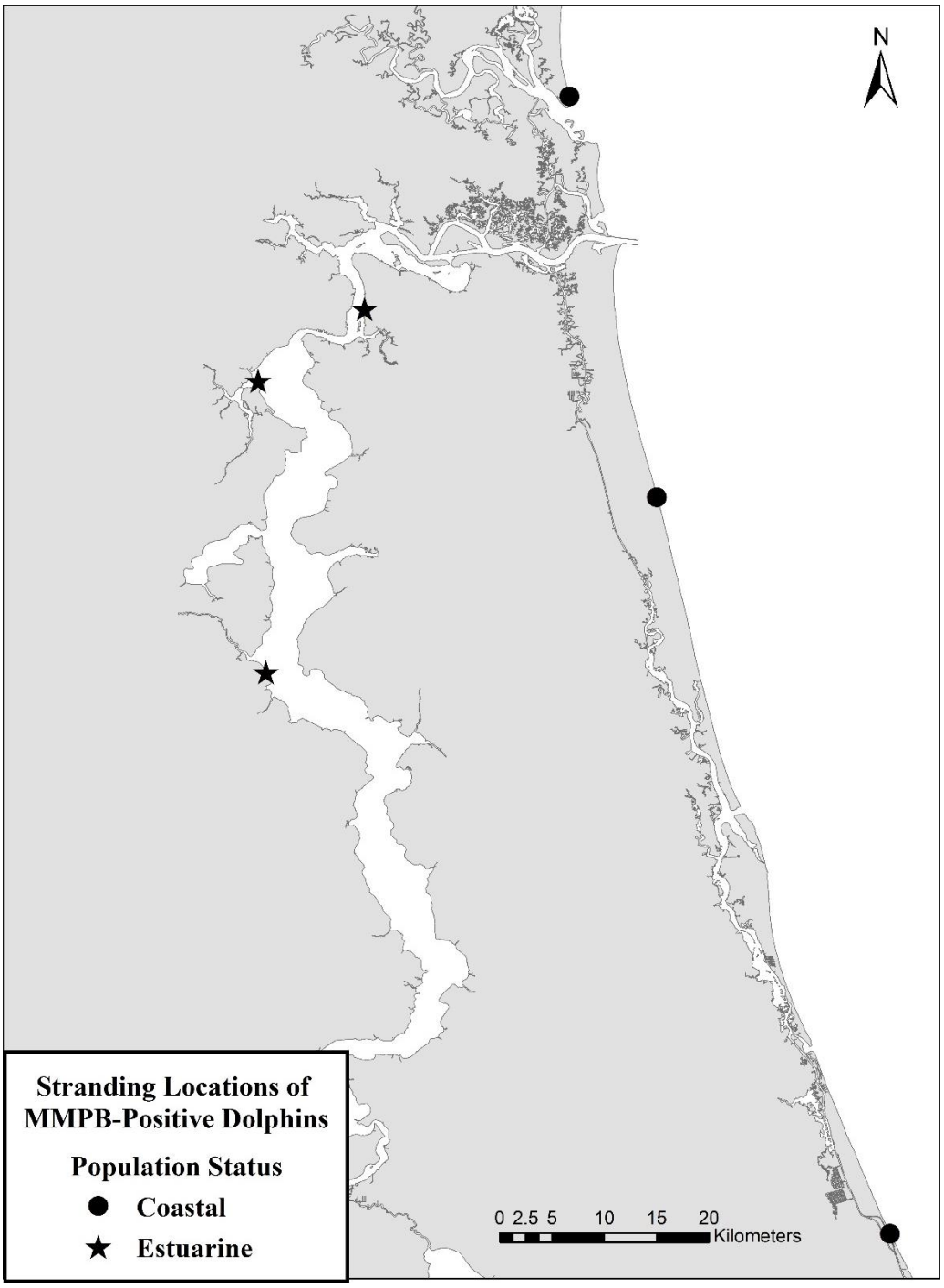
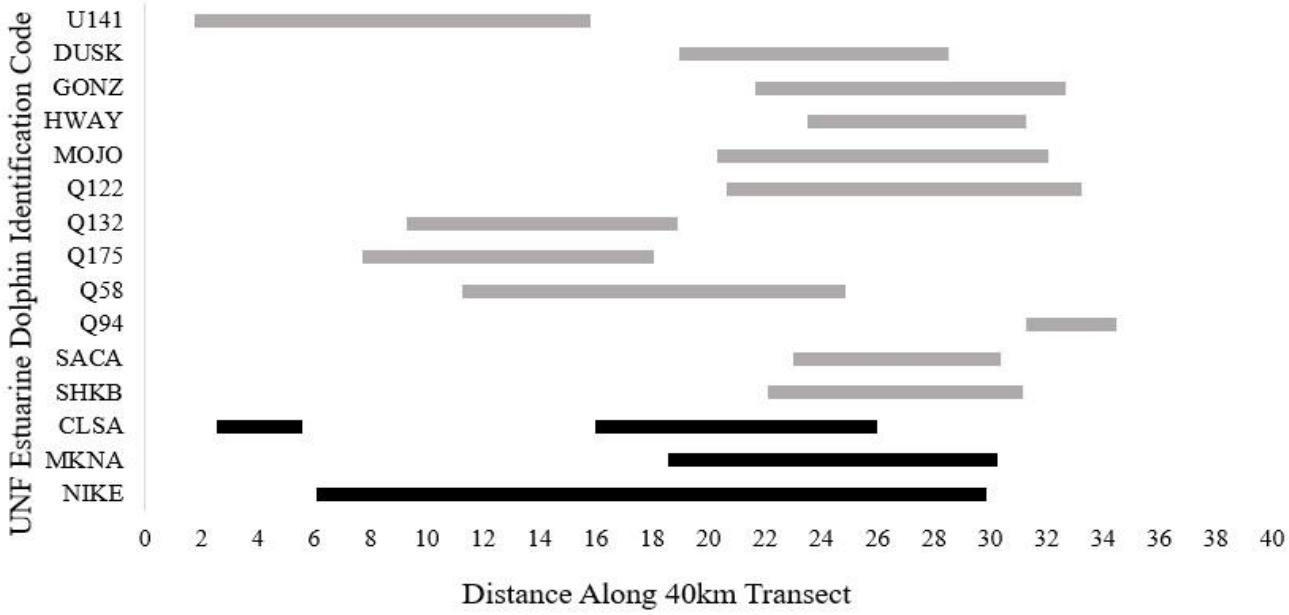


Figure 4. 50% utilization distributions (core areas) of individual MMPB-positive estuarine bottlenose dolphins (black), and individual MMPB-negative estuarine dolphins (gray) calculated using univariate Kernel Density estimates The x-axis represents distance along the 40km transect where 0 represents the stop location in downtown Jacksonville (ogliohaline), and 40 represents the mouth of the SJR near Mayport (oceanic salinity).



Komarekiella delphikthonos sp. nov. (Cyanobacteria): an epidermal cyanobacterium implicated in an estuarine bottlenose dolphin (*Tursiops truncatus*) fatality.

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Highlights

- Proposed new taxon of epiphytic cyanobacteria with unknown toxin potential
- Confirmed epiphytic cyanobacterial growth on a stranded dolphin
- Identification of *Komarekiella* sp. in Florida
- Expanded monitoring is advised for epiphytic growth on skin lesioned dolphins

Abstract

On January 15, 2015, an adult male bottlenose dolphin (*Tursiops truncatus*), identified as "MKNA" from the UNF photo-identification catalog, stranded in an unusual, oligohaline habitat with an epidermal algal mat present on the dorsal fin, both flanks, peduncle, and fluke. Microscopic investigation of the composition of this mat revealed cyanobacterial dominance with the presence of other bacteria and fungi. While gross necropsy was unable to determine cause of death, subsequent toxin analysis revealed low levels of microcystins/nodularins (MCs/NODs) in MKNA's hepatic tissue. Cultures of the epidermal mat produced a Nostocalean isolate that was closely aligned (97% 16S rDNA) with the recently erected genus, *Komarekiella*. Immunoassay (ELISA) and 2-methyl-3-methoxy-4-phenylbutyric acid (MMPB) techniques were used to test the original mat sample and subsequent culture samples for microcystins/nodularins (MCs/NODs). Concentrations of MCs/NODs could not be confirmed between methodologies, but data generated using the MMPB technique suggested toxin concentrations in all samples were below detection. A total evidence approach of molecular, genetic, and ecological examination determined this isolate to have many unique characters, constituting the erection of a new species within the *Komarekiella* genus, *Komarekiella delphikthonos.* This discovery warrants an area of further investigation as toxic, epiphytic cyanobacteria may be new source of health decline and mortality in bottlenose dolphins utilizing low salinity habitats.

Key words: 16S rDNA, microcystin, oligohaline stranding, *Tursiops truncatus*

1. Introduction

Cyanobacteria are diverse, photosynthetic, prokaryotic organisms that are integral to most aquatic ecosystems (Dvorák et al., 2017). Many cyanobacterial species have the ability to produce toxic secondary metabolites, cyanotoxins, which function as neurotoxins, hepatotoxins, and/or dermatoxins. Most notably, these toxins are associated with harmful algal blooms (HABs), described generally as the visual domination of planktonic and benthic cyanobacteria (Watson et al., 2015). Presence of HABs and cyanotoxins have severely impacted local and state economies (Anderson et al., 2000), caused temporary closure of public water systems (McCarty et al., 2016), led to declarations of municipal or regional emergencies (Executive Order, 2016), and have prolonged, deleterious impacts on aquatic animals and ecosystems (Paerl et al. 2001; Miller et al., 2010). Toxin-producing cyanobacteria have also been identified as causative agents of health decline and mortality to such diverse taxa as Caribbean coral reefs (Casamatta et al. 2012), dogs (Edwards et al., 1992; Gugger et al., 2005; Wood et al., 2010; Backer et al., 2013), cattle (Galey, 1987; Mez et al., 1997), terrapins (Nasri et al., 2008), and sea otters (Miller et al., 2010). Recent research has shown that epiphytic cyanobacteria are also able to produce significant concentrations of both hepatotoxins (Mohamed & Shehri, 2010) and neurotoxins, leading to death of avians such as bald eagles, American coots, Canada geese, and mallard ducks after consumption (Rocke et al. 2002, Augspurger et al. 2003; Williams et al., 2007; Wilde et al., 2014). Although ingestion of contaminated water or food sources is the most common route of toxin exposure (Pitosis et al., 2000; Sharma et al., 2008; Cox et al., 2016), no prior research has been conducted on the potential of direct toxin transfer from epiphyte to host.

The St. Johns River (SJR, Jacksonville, FL, USA) is a large, brackish, estuarine system characterized by considerable anthropogenic pollution (*e.g.*, high nutrient load, chemical runoff, heavy metal pollution, and septic system failure), recurrent HABs, and diverse toxin-producing and epiphytic cyanobacterial taxa (Environmental Protection Board, 2017; Aubel et al., 2006; Dunn et al., 2008). The State of the River Report summarized the ten year status trend of HABs in the SJR as "unsatisfactory" and "unchanged" and with no projected decrease (Environmental Protection Board, 2017). Additionally, the SJR provides critical habitat for a genetically and behaviorally distinct estuarine community of bottlenose dolphins (*Tursiops truncatus;* Montague 1821) (Caldwell, 2001; Gubbins, 2002; Ermak, 2017). This dolphin population routinely uses mesohaline (5-18 ppt) and oligohaline (0-3 ppt) areas of the river and has been subject to two unusual mortality events (UME) since 2010 (Borkowski, personal comm.; Gibson, unpublished; Environmental Protection Board, 2017). Additionally, the SJR community has been recently described as being exposed to hepatotoxic microcystins/nodularins (MCs/NODs), classes of potent hepatotoxins, produced by cyanobacteria (Brown et al., 2018).

On January 15, 2015, an adult male dolphin carcass (TtNEFL1501) was recovered from an oligohaline habitat in Green Cove Springs, FL (N 30.029086, W -81.696062) (FWC-FWRI, unpublished). The stranded animal was later identified as "Makena" (MKNA) from the University of North Florida (UNF) dolphin photo-identification catalog, was discovered with a thick mat of fungi, bacteria, and cyanobacteria covering his left and right flank, dorsal fin, peduncle, and fluke. TtNEFL1501 was last seen alive on August 28, 2014 with extensive skin lesions in mesohaline waters located near the Hart Bridge (N 30.3154, W -81.62584) in the SJR. The gross necropsy was unable to determine cause of death for TtNEFL1501 with the only major

findings being lack of stomach contents and PCR negative for cetacean morbillivirus (FWC-FWRI, unpublished). Subsequent toxin analysis revealed TtNEFL1501 had low concentrations of MCs/NODs present in hepatic tissue at the time of stranding (Brown et al., 2018).

Because of the MCs/NODs present in TtNEFL1501's hepatic tissue at the time of stranding, toxin analyses were performed on both the original mat sample and isolated cultures. MCs (heptapeptides) and NODs (pentapeptides) are structurally similar hepatotoxins that possess the unique Adda moiety ([2S,3S,8S,9S,4E,6E,]-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid) (Rinehart and Harada, 1988; Namikoshi et al., 1990; Mazur-Marzec et al., 2006; Bortoli and Volmer, 2014; Niedermeyer, 2014). All MC/NOD variants share the same mechanisms of toxicity, though the specific amino acid combinations on the peptide ring affect LD_{50} of MCs (MC-LR = 50 μg kg^{-1}; MC-RR = 600 μg kg^{-1}) while most NOD congeners have comparable LD_{50} to MC-LR (Krishnamurthy et al., 1986; Watanabe et al., 1988; Ohta et al., 1994; Pearson et al., 2010). Similarity in structure and methodologies that target the Adda result in the two classes being jointly classified in quantification in some analyses, such as the Adda enzyme-linked immunosorbent assay (ELISA) and the 2-methyl-3-methoxy-4-phenylbutyric acid (MMPB) technique. The polyclonal Adda ELISA nonspecifically measures free MCs/NODs that have a methylated, unmodified (*e.g.* [$DMAdda^5$] and [$ADMAdda^5$]) Adda moiety through antibody binding (Samdal et al., 2014). The MMPB technique is used to detect the same unmodified MCs/NODs, but includes free, bound/conjugated, or degraded MCs/NODs by oxidative cleavage of the Adda moiety and analysis through mass spectrometry (Sano et al., 1992; Foss & Aubel., 2015; Foss et al., 2017; Brown et al., 2018).

The unusual conditions surrounding TtNEFL1501's stranding (*i.e.* location, hepatic MCs/NODs, epidermal mat) warranted further investigation to determine whether the epiphytic cyanobacteria played a role in the stranding of this animal. Morphologic, genetic (16s rDNA gene), and molecular (secondary folded structures of the 16S-23S ITS region) methods were used to elucidate cyanobacterial species identity. Toxin analyses (*i.e.* ELISA and MMPB) were conducted to determine MCs/NODs presence in the epidermal mat sample at the time of stranding and the potential for the isolates to produce toxins in culture.

2.0 Methods

2.1 Sample Collection/Dorsal Fin Identification

The cryoarchived epidermal mat sample, necropsy report, associated histopathology reports, necropsy photographs, and GPS coordinates of stranding location for TtNEFL1501 were provided by Florida Fish and Wildlife Conservation Fish and Wildlife Research Institute (FWC-FWRI) under permission from NOAA's Code of Federal Regulations 50CFR216.22. TtNEFL1501 was identified as MKNA in the UNF dolphin photo-identification catalog by comparing distinctive dorsal fin features (*i.e.* nick and notch patterns, fin shape) from photos taken from the necropsy to photos taken during behavioral surveys. Two algal samples (0.05 g and 0.06 g w.w.) were excised during routine necropsy and stored at -20° C in a sterile whirlpak bag for one year and 9 months before culture.

2.2 Culturing

A small piece of the cryoarchived epidermal mat sample was removed and initially plated on BG-11 agar (Allen and Stanier, 1968) until enough growth was achieved for sub-culturing. Subcultures were then isolated using standard isolation techniques (Anderson, 2005) and were

plated on nitrogen-free, BG-11, and Z-8 media (Staub 1961) to assess for morphological differences. Cultures were grown for approximately two months at room temperature and maintained in 100 mm Petri dishes.

2.3 Microscopic Identification

Samples from the BG-11 and Z-8 culture samples were prepared and photographed using an Olympus Fluoview FV1000 (Olympus Corporation, Center Valley, PA) confocal laser microscope. Photos were taken using brightfield, phase contrast, and epifluorescence at 400x.

2.3.1 Extraction and Cloning

DNA from cyanobacterial isolates was extracted with the PowerSoil™DNA Kit from 0.25 g of culture samples (Mo Bio Laboratories Inc., Carlsbad, CA). DNA quality was checked on an ethidium bromide stained 1.5% agarose gel. Polymerase chain reaction (PCR) amplification of the partial 16S rDNA and the whole 16S–23S ITS was performed using primers forward 8F (5'–AGTTGATCCTGGCTCAG–3'), and reverse B23S (5'-125CTTCGCCTCTGTGTGCCTAGG –3'), previously described in Lane (1991). ITS PCR amplification was performed using primers forward VRF5 (5' –TGTACACACCGGCCCGTC – 3') and reverse VRF1 (5' – CTCTGTGTGCCTAGGTATCG – 3') as previously described (Ororio-Santos et al. 2014). The 50 µl PCR reaction contained 19 µl sterile water, 2 µl of each primer (0.01 mM concentration), 25 µl PCR Master Mix (Promega, Madison, WI) and 2 µl template DNA (50 ng μl^{-1}). PCR amplification proceeded as detailed in Casamatta et al (2005). Amplified rDNA was cloned into pGEM® T Vector System I and JM-109 High Efficiency Competent Cells (Promega, Madison, WI) and cultured using carbenicillin infused LB media. Plasmid DNA was purified from eight replicate transformed competent cell colonies per isolate,

using QIAprep® Spin Miniprep Kits (QIAGEN, Hilden, Germany). Sequencing of cDNA libraries from two operons of varying size was performed by Eurofins Genomics (MWG Operon Inc., Louisville, KY).

2.4 Toxin analyses

2.4.1 Sample Preparation

Cyanobacterial growth was removed from the agar of both BG-11 and Z-8 culture samples using a razor blade and were extracted as 0.20 g (w.w.) subsets for ELISA and oxidized as 0.25 g (w.w.) subsets for MMPB. Sample spikes of culture material (20 ng g^{-1} for ELISA; 2 & 5 ng g^{-1} for MMPB) were prepared pre-extraction/pre-oxidation using certified reference material of MC-LR (National Research Council, CRM, Halifax, Nova Scotia, CA). The epidermal mat sample was extracted free as a 0.06 g subset and oxidized for total as a 0.05 g subset. Both the mat sample and the culture samples were homogenized in 7 mL tubes with 0.01 M phosphate buffer (pH = 7) and metal beads (2.4 mm) using an Omni Bead Rupter 24 (Omni Kennesaw, GA, USA) for 15 seconds. The mat sample subset extracted for ELISA analysis was not pre-treated prior to extraction. Extractions were conducted using 5 mL of extraction solution (75% MeOH in 0.1M acetic acid) and water bath sonication for 25 mins. Samples were centrifuged (10 min; 1,500 × g), supernatants retained and the pellet was rinsed with extraction solution via vortex mixing (1 mL). Solutions were re-centrifuged (10 minutes at 1,500 × g) and resulting supernatants were combined. Methanol was removed using N_2 at 60°C (Zymark LV TurboVap® evaporator, Biotage, Charlotte NC) and samples were reconstituted to ca 5 mL using deionized water (DI). Samples were loaded onto preconditioned (3 mL MeOH followed by 3 mL DI) solid phase extraction (SPE, 100 mg) columns (Strata™-X; PN 8B-S100-FCH,

Phenomenex, Torrance, CA, USA), rinsed (2 mL DI) and eluted (3 mL 90% acetonitrile). Elutions were blown to dryness (60° C, N_2), reconstituted (DI), and filtered (0.2 µm polyvinylidene fluoride).

2.4.2 Adda Enzyme-linked Immunosorbent Assay (ELISA)

A polyclonal Adda ELISA (Abraxis Kits, PN 520011, Warminster, PA, USA) was used for free MCs/NODs analysis as described in Foss et al. (2017). Culture samples were analyzed at a sample concentration of 50 mg mL^{-1}, resulting in a detection limit of 3 ng g^{-1} (ppb) based on kit sensitivity (0.15 ng mL^{-1}) and sample dilution factor (DF=20). The mat sample was analyzed at 100 mg mL^{-1}, resulting in a detection limit of 1.5 ng g^{-1} (ppb).

2.4.3 Total Microcystins/Nodularins Oxidation (MMPB) & Analysis

Two sample sets were oxidized to create MMPB; the first were the extracts prepared and analyzed using ELISA and the second were whole samples not previously extracted. Oxidations of ELISA extracts were conducted similar to those described in Brown et al. (2018). Extracts (100 µL) were oxidized at final oxidant concentrations of 100 mM K_2CO_3, 15 mM $KMnO_4$, 15 mM $NaIO_4$ for 30 min. Unextracted samples (0.05 – 0.25 g) were oxidized using 5 mL of oxidant solution (0.2 M K_2CO_3, 0.1 M $KMnO_4$, 0.1 M $NaIO_4$) for 2 hours. Oxidations for both reactions were stopped via dropwise addition of 40% (w/v) of sodium bisulfite. Preconditioned Strata X Polymeric SPE (3 mL MeOH followed by 3 mL DI) was used to clean extracts (Sample followed by 3 mL DI and eluted with 5 mL ACN). The samples were blown to dryness (N_2 60°C) and reconstituted in DI.

Samples were analyzed as described in Foss et al. (2017) using a Thermo Scientific Surveyor HPLC system coupled with a LTQ XL™ Linear Ion Trap Mass Spectrometer. The

MMPB $[M-H]^-$ ion, (*m/z* 207), was fragmented and the resulting ion (*m/z* 131) was monitored. The method detection limit (MDL) was determined using a signal to noise ratio of 3. External curves generated of oxidized MC-LR (0-10 ng mL^{-1}) were used to determined spike recoveries.

2.5 Home Range/Core Area Determination

TtNEFL1501 (MKNA)'s home range and core area was determined to assess associations between pre-stranding habitat use and abnormal, oligohaline stranding location. Weekly dolphin photo-identification surveys have been conducted in the SJR since March 2011 along a fixed 40-km transect from Mayport Inlet (N 30.39904, W -81.39396) to downtown Jacksonville (N 30.31479, W -81.62987) (Ermak et al., 2017). MKNA had 20 sightings after same-day resights were removed. Sighting locations were plotted in ArcGIS 10.4.1 (ESRI; Redlands, California) and were transformed into a univariate dataset. Univariate kernel density estimate (KDE) methodology is preferred when determining the home range and core areas of populations that inhabit narrow, aquatic habitats, such as the SJR, as it prevents the inclusion of uninhabitable areas, (*i.e.* land) in analyses and allows for more accurate habitat use calculation (Moyer et al., 2007; Rayment et al., 2009; Nekolny et al., 2017). A midline was run through the middle of the survey route and downtown Jacksonville was designated as location 0 while the Mayport Inlet was designated as location 40. Sighting locations were transformed to the midline via the "locate features along routes" tool. The generated univariate dataset was input into SAS (version 9.4; SAS Institute, Cary, NC, USA) and the PROC KDE function was used to calculate MKNA's 95% (home range) and 50% (core area) utilization distributions. To select bandwidth, the simple normal reference (SNR), Silverman's rule of thumb (SROT), and the Sheather-Jones plug in

(SJPI) methodologies were compared. SROT was selected as the appropriate bandwidth as it smoothed the data moderately in relation to the other two methods.

3.0 Results

3.1 Phylogeny

The new isolate fell within a highly supported (97% ML bootstrap support, 96% MP bootstrap support) clade containing other species of *K. atlantica* (Figure 1). All *K. atlantica* strains formed a highly supported clade (100% ML support), with *K. delphikthonos* falling as a sister taxon. As a clade, *K. atlantica* and *K. delphikthonos* were sister to a clade with *Halotia*, *Nostoc*, and *Trichormus* on one branch and *Goleter*, *Cronbergia*, *Hydrocoryne*, and *Nodularia* on the other (Figure 1).

3.2 ITS data

Comparisons of folded ITS structures showed slight variations between the new taxon and available structures from sister species, *K. atlantica*. The D1-D1′ helix for *K. delphikthonos* had a single nucleotide change, which altered the basal clamp from 4 b.p. to 5 b.p., constricting the basal unilateral bulge (Figure 2A). Further, an internal bilateral bulge, offset by 2 b.p. on either side, distinguished this structure from *K. atlantica*.

Analyses of the Box-B helix revealed a 2 b.p. insert between the internal and terminal bulge, as well as two nucleotide substitutions in the terminal bulge (not affecting structure) in the Box-B helix of K. *delphikthonos* (Figure 3A).

The V3 helix showed the greatest deviation in structure in relation to the other strains. The *K. delphikthonos* V3 helix was 83 nucleotides in length as compared to 54 nucleotides for similar strains (Table 1). There was one large insertion located within the internal portion of the

helix, as where the basal clamp and the basal bilateral bulge were highly conserved (Figure 4). This insert also facilitated several changes in the internal secondary structure of the stem. Additionally, a single nucleotide polymorphism was discovered in the terminal bulge for *K. delphikthonos* (Figure 4A).

3.3 Morphology

Komarekiella delphikthonos was morphologically similar to other *K. atlantica* strains. This taxon exhibits unusual features, such as radical morphologic change as a component of the life-history stage, as previously described by Hentschke et al. (2017). Hormogonia resemble fragments as seen in other *Nostoc* taxa, and other cells congregate into pseudo-sarconoid packs (Figure 5). Additionally, *K. delphikthonos* has single cells that vary in size and color (Figure 5). Some stages of colonial development are morphologically similar to *Pseudanabaena* (Figure 5D), while other cells resemble unicellular *Synechococcus,* or as aggregates of filamentous and/or packet-like cells typically associated with *Chlorogloeopsidaceae* morphology. Filaments were either uni- or multi-serate, with a mixture of both structures found within the same culture. Unlike *Chlorogloeopsis* and *Komarekiella*, however, *K. delphikthonos* had heterocysts that were both similar in size, and at times larger than, vegetative cells (Figure 5B). It is important to note that akinetes were not present within either the epidermal or culture samples.

3.4 Toxin Analyses

3.4.1. ELISA

ELISA analysis for free MCs/NODs resulted in absorbencies relating to concentrations just above the MDLs (1.5 & 3 ng g^{-1}), with assay values for all samples below 0.50 ng mL^{-1}. Resultant concentrations were 4.6, 5.0 and 4.5 ng g^{-1} (w.w.) for the epidermal mat, BG-11

culture and Z-8 culture samples, respectively (Table 2). ELISA determined spike returns for the free extracted culture samples were low (2% - 6%), potentially due to retention of co-extracted agar.

3.4.2. MMPB of free (ELISA extracts) and total MCs/NODs

MMPB analysis of the oxidized extracts did not support the ELISA data, as all samples tested were below detection, even though the MDL was lower than ELISA responses (MDL= 2.5 ng g^{-1} total 'free' MCs/NODs). MMPB of oxidized whole samples supported samples were below detection for total MCs/NODs, with the samples all < 1.3 ng g^{-1} total MCs/NODs. A small peak was observed at the MMPB retention time for both oxidized whole and extracted ELISA epidermal mat samples, however the levels were below the established MDLs.

Spikes for the ELISA culture extracts were not returned post oxidation and MMPB analysis, supporting losses were to the agar. In contrast, when culture samples were spiked and oxidized directly, without a free MCs/NODs extraction, spikes were recovered 92% & 95%.

3.5 Home Range and Core Area

Home range analyses demonstrated that MKNA utilized almost the entirety of the 40 km study area, including mesohaline areas of the SJR. However, Kernel density analyses indicated that MKNA's core area (50% UD) was located in mesohaline (5-18ppt) waters. MKNA stranded approximately 44 km upriver from the furthest inland extent of his documented home range in oligohaline water (0-3 ppt) (Figure 6).

Komarekiella delphikthonos **A.O. Brown, A.D. Garvey, C.D. Villanueva et D.A. Casamatta (Figure 5)**

Description: Thallus at first sandy-brown on dolphin epidermal samples, then dark blue-green, later turning jade green in culture. Thallus consists of subspherical colonies and aggregates of filaments enveloped in a common, mucilaginous sheath. Colonies initially consist of mainly unicellular aggregates later differentiating into either filaments via cell division or as fascicles of filaments, occasionally in multiple planes. Hormogonia frequent in culture, small (ca. 3 μm), and few-celled (3-8). Filaments both uni- and multiseriate. Vegetative cells mainly spherical, with compressed spherical to roughly isodiametric forms common, 3-5 μm. Heterocytes intercalary, 3-6 μm. Akinetes were not observed.

4.0 Discussion

Though it was not determined whether the *K. delphikthonos* mat was present on TtNEFL1501 (MKNA)'s epidermis while alive, there have been many reports of epidermal algal-type mats associated with skin lesions on live dolphins (Barry et al., 2008; Riggin & Maldini, 2010; Mullin et al., 2015; Nokes, personal comm.; Mazzoil, personal comm.; FWC, unpublished). Dolphin skin lesions have been associated with, and are known to, manifest and worsen due to prolonged low salinity exposure, potentially leading to opportunistic infections (Greenwood et al. 1974; Colbert et al. 1999; Wilson et al., 1999; Dierauf and Gulland 2001;). Additionally, it is suspected that as the skin condition worsens and becomes more textured, microorganisms, such as bacteria, algae, or water molds, may aggregate (Mullin et al., 2015). Though it was determined that MKNA stranded 44km upriver from the furthest inland extent of his home range, this could be an artifact due to the limitations of the 40km survey route. The home range for MKNA, and other dolphins, may extend much further into low salinity habitats, which are not routinely surveyed.

In addition to TtNEFL1501, two other animals have been reported with algal-type epidermal mats in the SJR (HBOI-0806 and TtNEFL1511); both animals were described as "out of habitat" and were recovered from low salinity waters (HBOI 0806, a rescue attempt from Trout River; TtNEFL1511, stranded N 30.264378, W -81.697820). Epidermal algal-type mats have been reported on dolphins utilizing low salinity waters in Lake Pontchartrain (Louisiana, USA; Barry et al., 2008) and in the Tomoka River (Ormond Beach, FL; Hubbs-1037-Tt; Noke, personal comm.). A case study from Monterey Bay, California documented an "orange film" present on the dorsal surface of a calf that also had large, necrotic lesions (Riggin & Maldini, 2010). Salinity measurements were not taken, however this animal was described as having marked health decline (emaciated) with irregular swimming patterns (unable to sustain proper body orientation) (Riggin & Maldini, 2010). All three animals with algal-type mats in the SJR expired (HBOI-0806 - euthanasia; TtNEFL1501and TtNEFL1511 – carcasses; FWC-FWRI unpublished) as well as the dolphin, Hubbs-1037-Tt. The fates of the Lake Pontchartrain animals and the calf in Monterey Bay are unknown to the authors. Though the health and behavioral decline of these animals is likely attributed to a multitude of factors, it is a possibility that some of deleterious effects could be associated from an epiphytic toxin producing cyanobacterium.

The MCs/NODs producing capability of *K. delfikthonos* was not confirmed in this study, though that does not exclude this species from being a toxin producer. Cyanobacterial species have the ability of producing multiple toxins, of which many were not targeted in this work. For instance, the Florida manatee (*Trichechus manatus latirostris*) is known to be a host to dermal flora, with one instance of cyanotoxin associated dermal disease. Toxin producing epiphytic cyanobacteria (*Lyngbya* spp. dominated) were collected from manatees inhabiting Homosassa

Springs, FL, USA (Harr et al., 2008). Dermatoxins (e.g. aplysiatoxins & lyngbyatoxins) were detected in associated cyanobacteria mats, on the mat samples growing on the manatee dorsum and in the feces (Harr et al., 2008). Direct exposure to these toxins hypothetically resulted in the dermatological disease associated with this population of manatees. Though toxin concentrations for both epidermal and fecal samples were low, there are still potential health implications , such as immunosuppression, that may make the animal more susceptible to secondary infections. Though it is unlikely that cyanobacterial species constituting epidermal mats will ever be a source of mass mortality, this information provides relevance to the management of these species, especially in assessing the importance of intervening when dolphin has had prolonged freshwater exposure.

Photos taken from the TtNEFL1501's necropsy suggest the *K. delphikthonos* mat penetrated the epidermis. In culture, *K. delphikthonos* also permeates, degrades, and produces bubbles in culture media, indicating the ability to interact with its surrounding matrix. This characteristic will be relevant for future studies determining and assessing health effects associated with dolphin epidermal mats. Future sampling of these epidermal mats is recommended as epiphytic cyanobacteria may be a new source of health decline and mortality in dolphins inhabiting freshwater areas.

Toxin analyses on isolates supported that these samples are not producing MCs/NODs in culture. ELISA analyses from culture samples resulted in false positives and spike loss, which was likely due to interference from the culture agar. In culture, *K. delphikthonos* was embedded within agar, which could not be fully removed prior to toxin extraction. Although a trace level of MMPB was detected in the epidermal mat sample, it is possible that it was contaminated by

native MCs/NODs in the field or concomitant cyanobacteria. These findings, however, do not eliminate the potential of cyanotoxin production. It has been documented that some cyanobacteria produce MCs with modifications to the Adda which would not be observed using these methodologies (Sivonen et al., 1992). Additionally, other cyanotoxins may have been present, but were not tested for (*e.g.* anatoxin-a, cylindrospermopsin, saxitoxin), as evident in other studies (Harr et al., 2008). In light of these observations, further toxin and genome analyses are warranted.

K. delphikthonos has an unusual ability to prosper in cooler temperatures, as evidenced by the extent of growth found on MKNA in January, as well as its ability to survive being frozen for approximately two years. A previous survey of epiphytic cyanobacteria in the Lower St. Johns River showed greatest algal richness beginning in winter with majority of the identified species being described as filamentous with the potential to be mat forming (Dunn et al., 2008).

5.0 Conclusions

Culturing of the epidermal mat yielded a Nostocalean isolate, with subsequent 16S rDNA gene analysis revealing close similarity (97%) to the recently erected genus, *Komarekiella* (Hentschke et al. 2017). Based on a total evidence approach of molecular (toxin analysis), genetic (16S rDNA and 16-23S ITS), and ecological (e.g., growing as an epidermal mat on *T. truncatus*) characters, we propose the erection of *K. delphikthonos* under the International Code of Nomenclature for Algae, Fungi, and Plants. To the authors' knowledge, this is the first time MCs/NODs analyses have been conducted on an epidermal mat recovered from a bottlenose dolphin. This discovery warrants an area of further investigation as toxic, epiphytic

cyanobacteria may be new source of health decline and mortality in bottlenose dolphins utilizing low salinity habitats.

When initially described, this genus was recovered as epiphytes from subtropical and tropical trees of the Atlantic Rainforest, as well as from wet cement in Hawaii (Hentzscke et al. 2017). In a potentially prescient insight echoing the Baas-Becking hypothesis (Baas-Becking 1934), the authors of Hentzchke et al., 2017 noted that "everything is *not* everywhere, *but* humans will soon fix that". It may be that, like *Cylindrospermopsis* (Padiak 1997), *Komarekiella* may be potentially increasing distribution perhaps triggered by anthropogenic factors (e.g., ballast water, eutrophication, invasive vectors). It may also be that this taxon has historically been misidentified. Depending on the life history cycle, this taxon could easily be mistaken for *Pseudanabaena*, *Nostoc*, or even any number of colonial, coccoid cyanobacteria. The presence on the epidermis of the dolphin may also be much more common than currently described. For example, images of wild dolphins from Lake Pontchartrain and the St Johns River contain grossly similar epidermal mats (Barry et al., 2008; FWC-FWRI, unpublished data). The biotic component of these mats has not been studied but may prove fruitful to further investigation for the biogeography of this lineage.

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Tables

Table 1. Comparison of nucleotide lengths of conserved ITS domains for *K. delphikthonos* sp. nov. and closest relatives with available ITS data.

Strain	Leader	D1-D1' Helix	spacer+D2+spacer	D3+spacer	tRNA Ile gene	spacer+V2+spacer	tRNA Ala gene	Spacer	Box-B+spacer	Box A	D4+spacer	V3+ITS end (partial)
K. delphikthonos clone A	8	65	39	25	64	92	73	38	48	11	21	100
K. atlantica CCIBT 3483	7	66	39	12	64	92	73	40	44	11	20	71
K. atlantica CCIBT 3481	7	65	39	12	64	92	73	40	43	11	20	71
K. atlantica CCIBT 3487	7	66	39	12	64				47	11	20	71
K. atlantica CCIBT 3552	8	102	38	11	64	84	73	133	47	11	20	71
K. atlantica CCIBT 3486	1	67	38	139					47	11	20	71
K. atlantica HA4396-MV6 clone C10B	8	67	38	129					49	11	20	71
K. atlantica HA4396-MV6 clone C10A	8	79	38	11	74	60	73	126	49	11	20	71

Table 2. Concentrations of microcystins/nodularins (MCs/NODs) reported as ng g^{-1} w.w. for cultures and the epidermal mat sample. The samples were analyzed using an Adda ELISA (first column) with additional confirmatory analyses of ELISA extracts using the MMPB technique (middle column). Whole samples were also oxidized (last column) and analyzed for MMPB.

Sample ID	**Free MCs/NODs (ELISA)** ng g^{-1} (ppb)	**Free MCs/NODs (MMPB)** ng g^{-1} (ppb)	**Total MCs/NODs (MMPB)** ng g^{-1} (ppb)
Culture Sample Z8	4.5	ND	ND
Culture Sample BG11	5.0	ND	ND
Original Epidermal Mat Sample	4.6	ND*	ND*
Method Detection Limit (MDL)=	*3.0*	*2.5*	*1.3*

** = trace level observed below MDL*

Figures

Figure 1. Maximum Likelihood tree of *K. delphikthonos* and closest relatives.

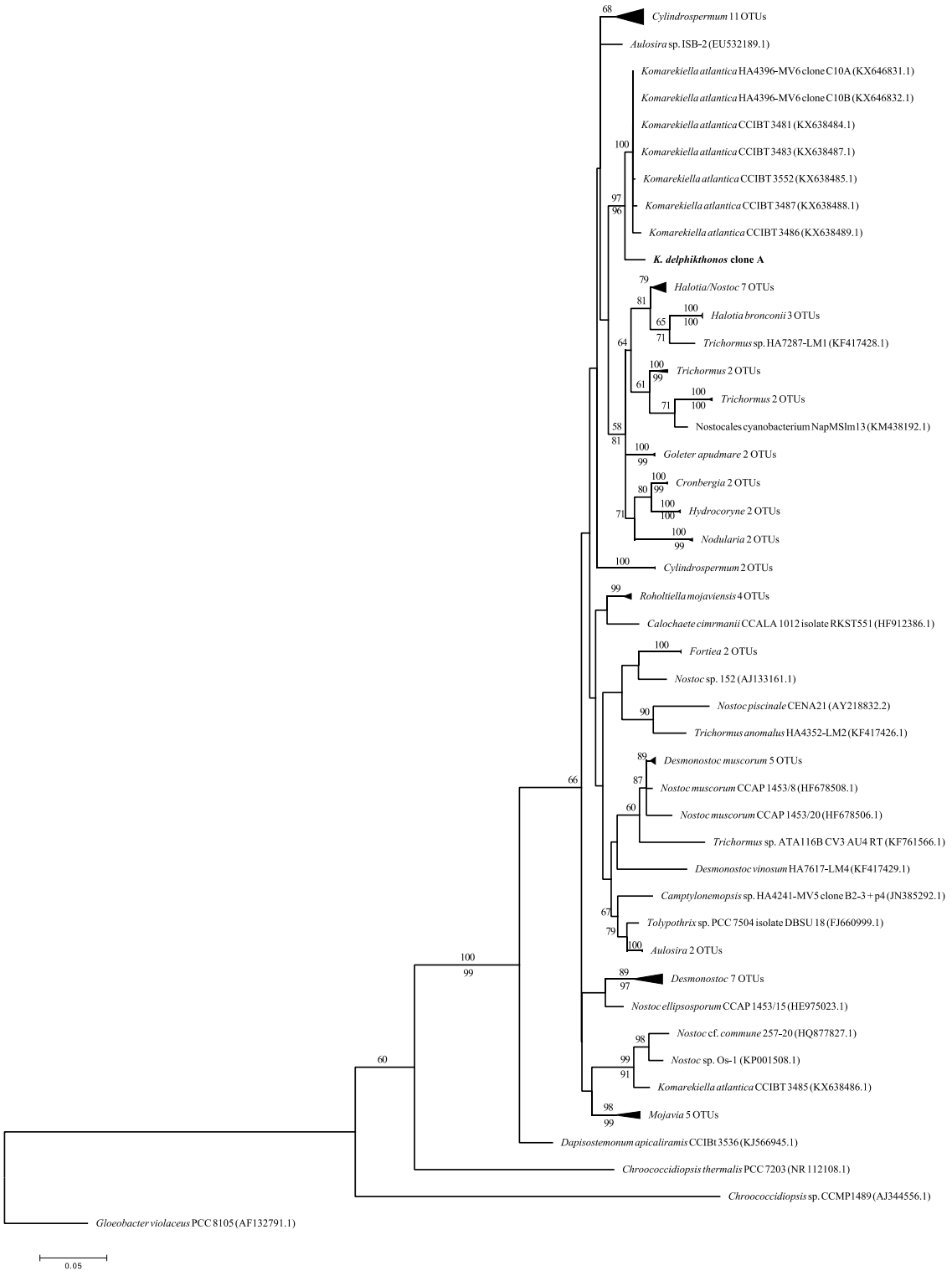


Figure 2. Folded ITS D1-D1′ helices for A) *K. delphikthonos*, and B-H) *K. atlantica*, B) CCIBT 3483 (KX638487.1), C) CCIBT 3481 (KX638484.1), D) CCIBT 3487 (KX638488.1), E) CCIBT 3552 (KX638485.1), F) CCIBT 3486 (KX638489.1), G) HA4396-MV6 clone C10B (KX646832.1), H) HA4396-MV6 clone C10A (KX646831.1).

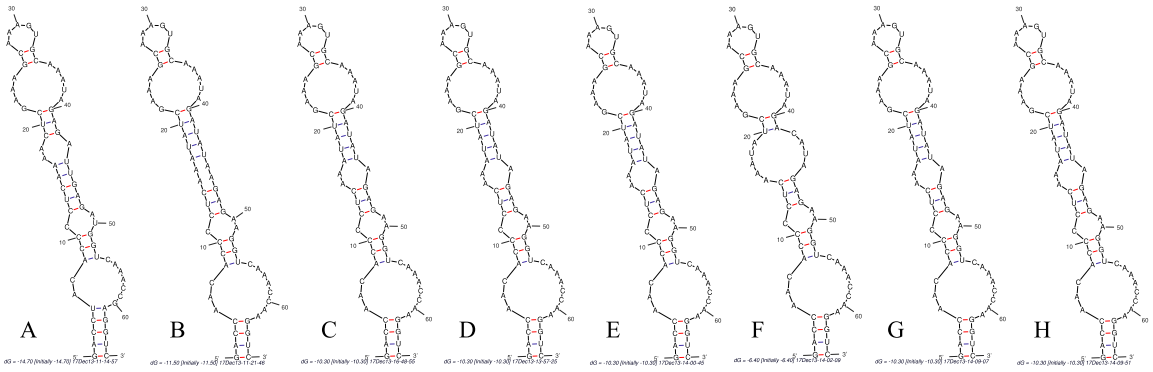


Figure 3. Folded ITS Box B helices for A) *K. delphikthonos* and B-H) *K. atlantica*, B) CCIBT 3483 (KX638487.1), C) CCIBT 3481 (KX638484.1), D) CCIBT 3487 (KX638488.1), E) CCIBT 3552 (KX638485.1), F) CCIBT 3486 (KX638489.1), G) HA4396-MV6 clone C10B (KX646832.1), H) HA4396-MV6 clone C10A (KX646831.1)

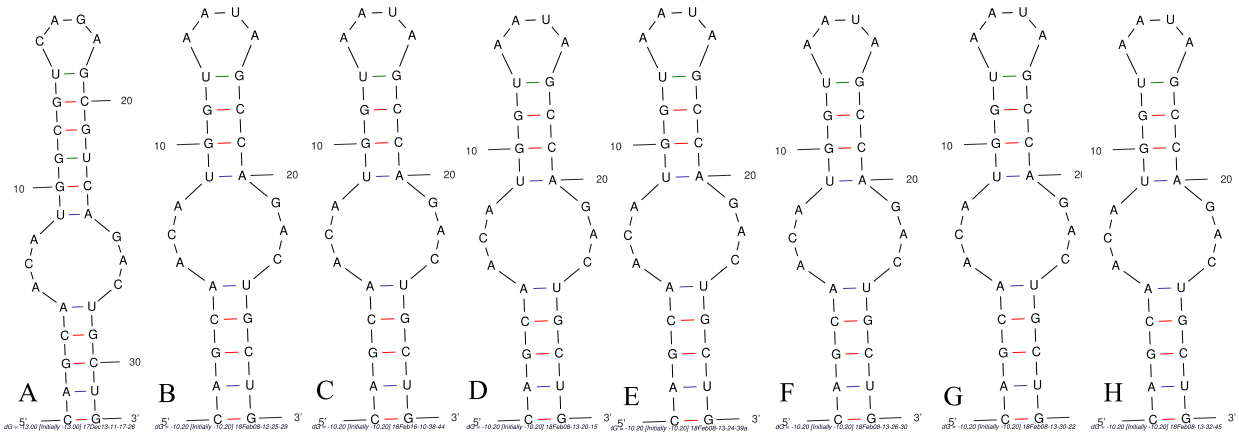


Figure 4. Folded ITS V3 helices for A) *K. delphikthonos* and B-H) *K. atlantica*, B) CCIBT 3483 (KX638487.1), C) CCIBT 3481 (KX638484.1), D) CCIBT 3487 (KX638488.1), E) CCIBT 3552 (KX638485.1), F) CCIBT 3486 (KX638489.1), G) HA4396-MV6 clone C10B (KX646832.1), H) HA4396-MV6 clone C10A (KX646831.1)

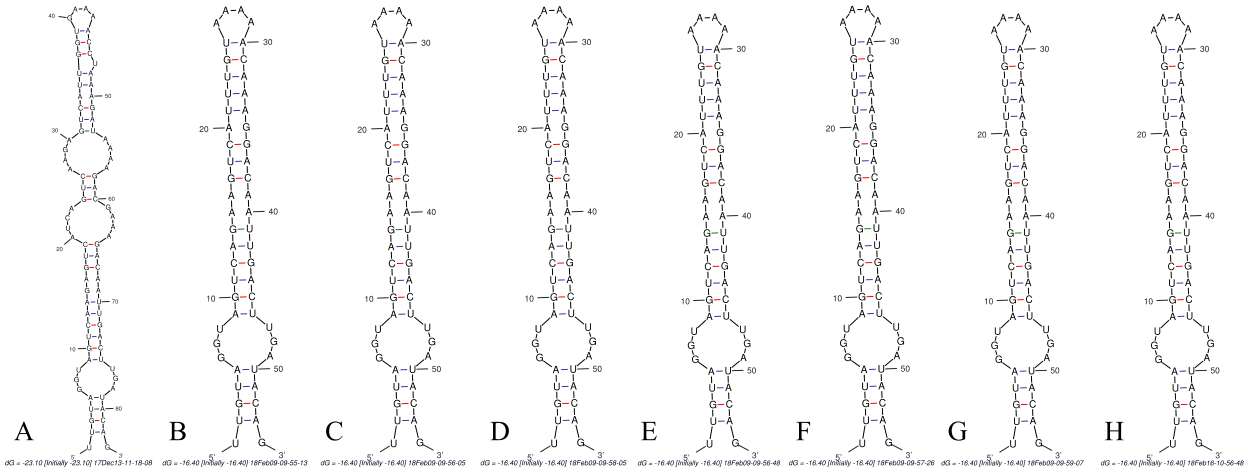


Figure 5. Morphological assessment of *K. delphikthonos*. A) Colony appearance after initial isolation, B) appearance of heterocytes after culturing on nitrogen free medium, C) mixture of both filamentous and individual cells common after culturing, D) *Pseudanabaena*-like filament production. Scale bars = 10 μm.

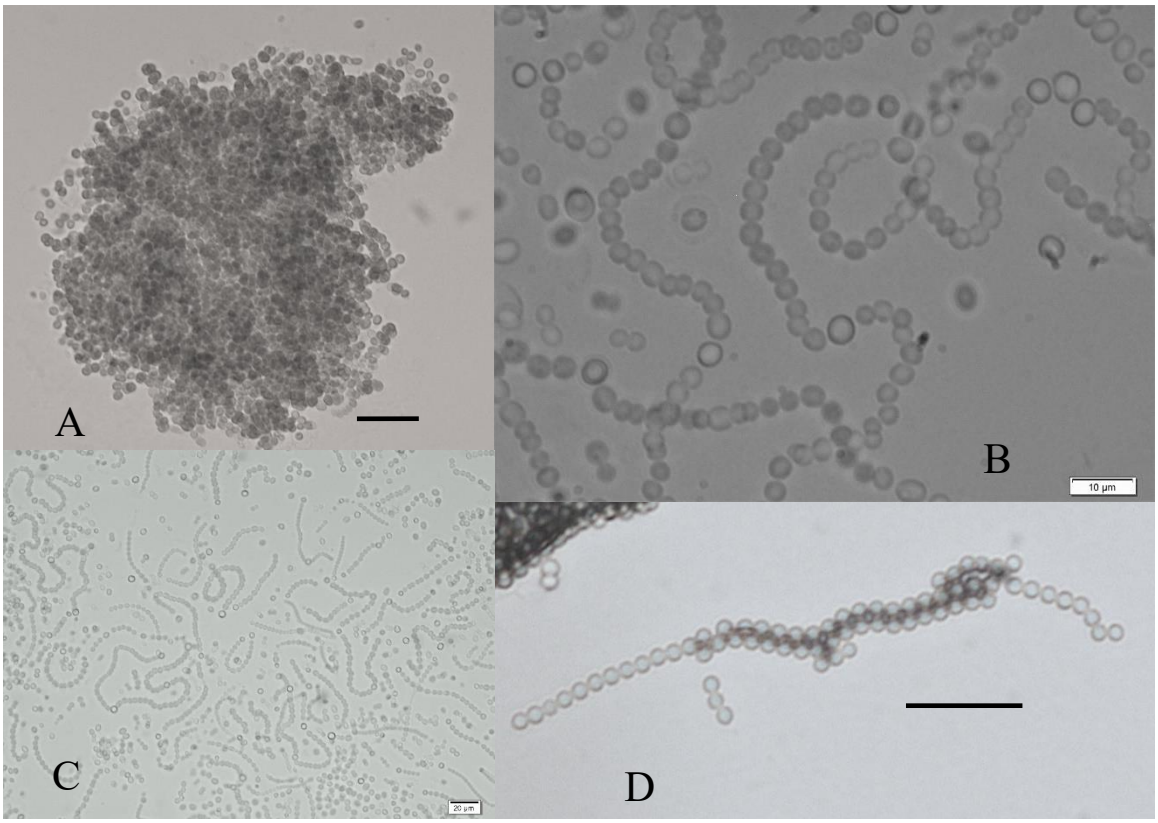
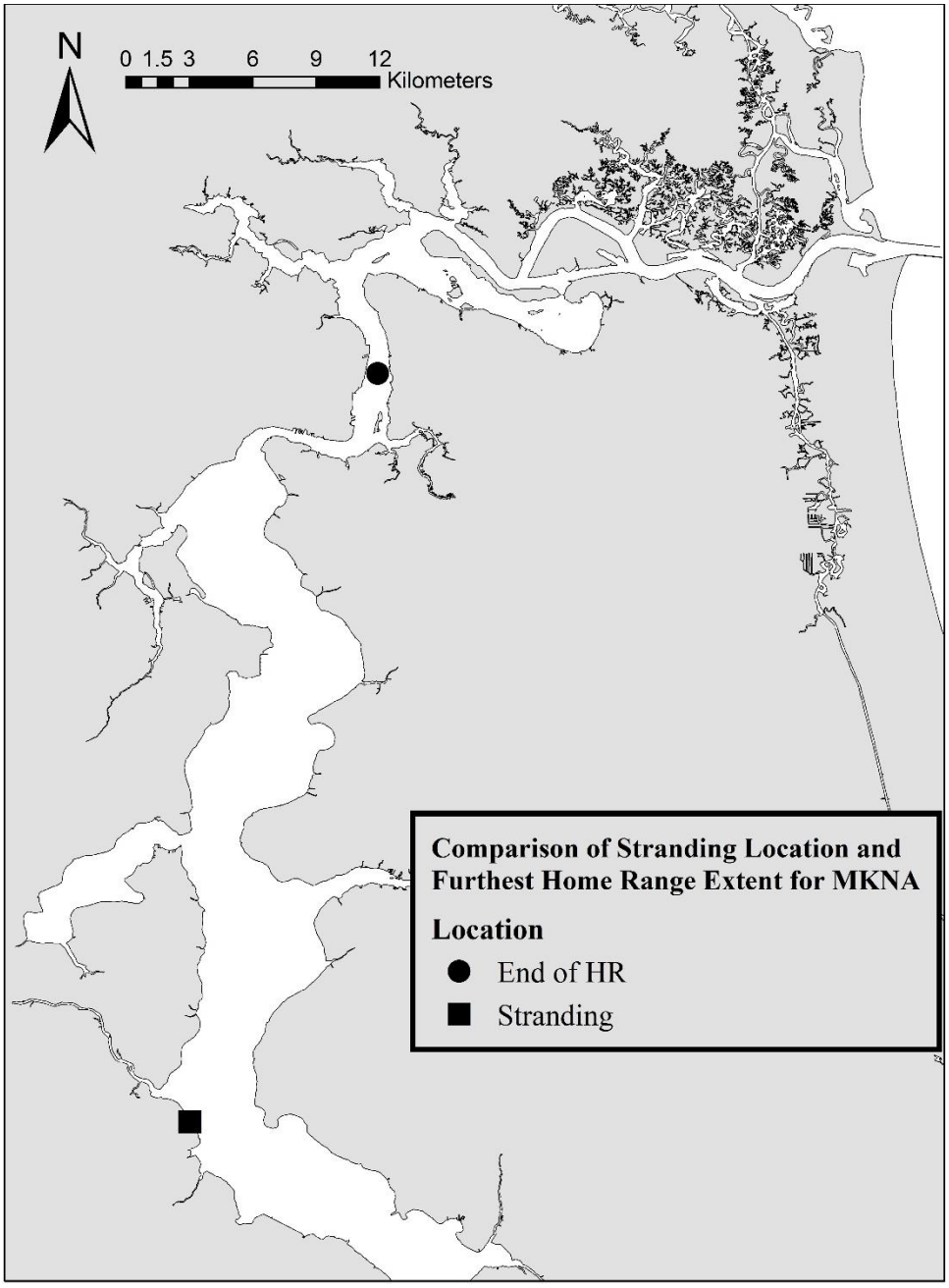


Figure 6. Plotted stranding location (black square) and furthest extent of home range (black circle) of MKNA, St. Johns River, Jacksonville, FL.



VITA

Amber graduated with her B.S. from the University of Virginia (UVA) with a major in Environmental Science and Specialization in Biological and Environmental Conservation. During her time at UVA, Amber became very interested in toxicology and conducted an independent study investigating the environmental and biological degradation of the Macondo Well (BP) Oil spill. After moving to Jacksonville, she has been able to pursue her passion of toxicology while working with one of her favorite marine mammals. Amber has presented her research at both domestic and international conferences and has been active in expanding her skillsets as Florida Fish and Wildlife Conservation Commission intern, assisting in strandings, rescues, necropsies, and public outreach. Amber has published her first chapter of her thesis and plans to publish her second. After graduation, Amber is moving to Sydney, Australia, with her husband where she will continue her career in marine biology.

Amber Brown

Education

M.S. Biology Candidate Expected May 2018
University of North Florida, Jacksonville, Florida
Determining microcystin/nodularin presence in stranded Tursiops truncatus in Northeast Florida

B.S. Environmental Science, Ecology 2013
University of Virginia, Charlottesville, Virginia
Specialization in Environmental and Biological Conservation

Professional Experience

Student Ambassador to the Board of Trustees March 2018
University of North Florida, Jacksonville, Florida
- Selected as student ambassador for giving tours of the Biology Department to the UNF Board of Trustees.

Animal Care Technician Aug 2017 – Current
University of North Florida, Dr. Judith Ochrietor, Jacksonville, Florida
- Responsible for general husbandry and maintenance of laboratory mouse records.

Exotic Animal Veterinary Technician Jan 2017 – Current
Riverside Animal Hospital, Dr. John Rossi, DVM, Jacksonville, Florida
- Responsible for initial evaluation of animal's condition, cleaning and wrapping wounds, collecting samples and administering medications and vaccinations.
- Proficient in urinalyses, CBC chemistry, ear cytology, and X-rays.
- Help during patient examinations and surgical procedures by restraining animals when necessary and communicating with pet owners.

Intern Feb 2017 – Aug 2017
GreenWater Labs/Cyanolab, Amanda Foss MS, MS and Mark Aubel, PhD, Palatka, Florida
- Conducted microcystin analyses including ELISA and MMPB techniques.

Marine Mammal Research Volunteer Sep 2016 – Current
Florida Fish and Wildlife Conservation Commission Research Institute (FWC-FWRI), Jacksonville, Florida
- Necropsy experience in bottlenose dolphins (*Tursiops truncatus*), West Indian manatees (*Trichechus manatus*), pygmy sperm whales (*Kogia breviceps*), and sea otters (*Enhydra lutris*; under direction of Dr. Melissa Miller – California Fish and Wildlife).

Marine Mammal Intern Jun 2016 – Sep 2016
FWC-FWRI, Nadia Gordon and Allison Perna, Jacksonville, Florida
- Assisted supervisors with marine mammal carcass salvage, necropsy, rescue, interventions, research, and public outreach in Northeast Florida.
- Completed mortality reports, equipment maintenance, and assisted with data entry and collection.
- Presented findings on benefits of local collaboration.
    - *Tursiops truncatus information sharing in northeast Florida: catalog and stranding data sharing between FWC-FWRI and UNF*

Environmental Educator Jan 2015 – May 2015
Driftwood, St. Simons Island, Georgia
- Lead staff responsible for creating, organizing, and implementing schedules.
- Responsible for teaching students marine sciences in an outdoor environment.

- Husbandry experience of various reptiles (*i.e.* venomous and non-venomous snakes, terrapins, alligators).

Environmental Educator Aug 2014 – Dec 2014
Jekyll Island 4H Center, Jekyll Island, Georgia
- Constructed lesson plans using the University of Georgia's Extension Program curriculum.
- Lead staff responsibilities and husbandry of various reptiles.

Relevant Research Experience

University of North Florida
Dolphin Behavioral Ecology Lab, Dr. Quincy Gibson Jun 2016 – Current
- Conducting research on dolphin health using both photographic and GIS analyses.
- Leadership role training undergraduate research assistants and conducting boat-based surveys in the St. Johns River, Florida.
- Proficient in data collection (behavioral and environmental) and small boat handling and maneuvering around marine mammals.
- Public outreach and education of the St. Johns River bottlenose dolphins at conferences and festivals.

University of North Florida
Cyanobacteria Research Lab, Dr. Dale Casamatta Aug 2016 – Current
- Experience in bacteria culturing, DNA extraction, PCR, and sterile techniques in order to determine microbial communities present on both stranded and live bottlenose dolphins.

University of Virginia
Dr. Aaron Mills 2013
- Literary study focusing on the environmental, microbial, and chemical degradation of crude oil released by the *Deepwater Horizon* oil spill.
    - *Succession and Degradation of the Macondo Oil from the Deepwater Horizon Oil Spill*

University of Richmond 2012
Gottwald Science Center, Department of Neuroscience, Dr. Craig Kinsley
- Assisted in behavioral research of virgin and maternal rats correlating empathy levels to hormonal changes associated with motherhood.

Teaching Experience

Science Instructor College of Arts and Sciences Dean's Council Feb 2018
University of North Florida, Jacksonville, Florida
- Nominated to lead a DNA extraction experiments for the Arts and Sciences Dean Council.

BioFLITE Instructor Aug 2017
University of North Florida, Jacksonville, Florida
- Nominated to lecture and conduct laboratory experiments for an intensive biology course for incoming freshman.

Graduate Teaching Assistant Jan 2017 – Current
University of North Florida, Jacksonville, Florida
- Facilitated understanding of scientific development through experimentation and writing assignments.
- Implemented lessons efficiently while maintaining discipline in the classroom.
- Designed, administered, and graded examination papers and lab reports to evaluate student performance.
- Organized and prepared course materials.

Middle School and High School Biology Teacher May 2015 – Jun 2016

Somerset Preparatory Academy. Jacksonville, Florida

- Curriculum and laboratory development for middle and high school science.
- Organized and fundraised field trips to Jekyll Island, Cumberland Island, and SeaWorld.
- Organized and led multi-grade dissections in which elementary, middle, and high school students could participate at varying levels of spiny dogfish, sheep brain, and pig heart dissections.

Research Presentations

Brown, A., Foss, A., Garvey, A.G., Gibson, Q., Villanueva, C., Casamatta, D.A. 2018. *Komarekiella delphikthonos* sp. nov. (Cyanobacteria): an epidermal cyanobacterium implicated in an estuarine bottlenose dolphin (*Tursiops truncatus*) fatality. Southeast & Mid-Atlantic Marine Mammal Symposium. Oral Presentation. Conway, SC.

Brown, A., Foss, A., Gibson Q. 2017. Using three comparative analyses to determine microcystin exposure in stranded bottlenose dolphins (*Tursiops truncatus*) in Northeast Florida. 22nd Biennial Conference on the Biology of Marine Mammals. Oral Presentation. Halifax, Nova Scotia.

Brown, A., Gibson, Q., Casamatta, D. 2017. Determining microcystin presence in stranded *Tursiops truncatus* in Northeast Florida.

- Showcase of Osprey Advancements in Research and Scholarships. Poster Presentation. Jacksonville, Fl.
- Statewide Graduate Student Research Symposium. Poster Presentation. Jacksonville, Fl.

Brown, A., Gibson, Q., Casamatta, D. 2017. Determining cyanotoxin (microcystin) exposure in stranded bottlenose dolphins (*Tursiops truncates*) in Northeast Florida. Phycological Society of America. Oral Presentation. Monterey Bay, Ca.

Brown, A., Gibson, Q., Casamatta, D. 2017. Microcystin exposure in resident and coastal *Tursiops truncatus* in Northeast Florida. Southeast and Mid-Atlantic Marine Mammal Symposium. Poster Presentation. Beaufort, NC.

Brown, A., Gibson, Q., Casamatta, D. 2016. Novel water mold and cyanobacteria on two stranded *Tursiops truncatus.* Society for Marine Mammology. Southeast Student Chapter. Oral Presentation. Gainesville, Fl.

Honors and Awards

Best Graduate Student Oral Presentation, Southeast and Mid-Atlantic Marine Mammal Symposium	2018
Student Government Graduate Scholarship	2018
Hoshaw Travel Grant Award	2017
Summer Biology Graduate Research Award	2017
Research Recognition Award, Showcase of Osprey Advancements in Research and Scholarships	2017
Top Research Project Representative for UNF at Statewide Research Symposium	2017
Graduate Student Research Grant	2017
Biology Graduate Research Enhancement Scholarship Recipient	2016
Environmental Center SEED Grant Recipient	2016
Remarkable Achievement, Orange Academic Stole	2013
Pi Epsilon, Environmental Science Honor Society	2013
Phi Eta Sigma, Honor Society	2011
William C. Folkes Academic Scholarship Recipient	2010 – 2013
Landstar Fund Scholarship Recipient	2010 – 2013

Grant Writing Experience

EPA STAR Grant, Co-Author	2016
Pass the Torch Grant, Co-Author	2016
Environmental Center SEED Grant, Co-Author	2016

Certifications

Florida State Temporary Teaching Certifications 2015-2017

- Middle Grades General Science and Biology 6-12

CPR/AED & First Aid
Florida Boating License
Institutional Animal Care and Use Committee Training