Bottlenose Dolphins and Brevetoxins: A Coordinated Research and Response Plan

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Introduction

Bottlenose dolphins (*Tursiops truncatus*) are widely distributed medium-sized toothed whales. The Gulf of Mexico coastal stock complex of bottlenose dolphins inhabit coastal, nearshore, and estuarine habitats along the Gulf of Mexico. They are protected under the U.S. Marine Mammal Protection Act (MMPA), which also requires that marine mammal Unusual Mortality Events (UME) be investigated.

One hundred and seven bottlenose dolphins stranded dead on the coast of the Florida Panhandle between March 10 and April 13, 2004 (Flewelling et al., 2005; NOAA, 2004). During this UME, hundreds of dead marine fish and invertebrates also were discovered in the area. Although no bloom was evident in the area concurrent with the mortalities, epidemiological evidence strongly suggests that naturally occurring brevetoxins produced by the dinoflagellate *Karenia brevis* (formerly *Gymnodinium breve* and *Ptychodiscus brevis*) were responsible. Brevetoxin involvement has been implicated in other marine mammal mortality events in the region (Figure 1), including a mortality event that killed 152 bottlenose dolphins in the Florida Panhandle from August 1999 to May 2000 (NOAA, 2004; Mase et al., 2000) and a 1996 bottlenose dolphin mortality event in Mississippi (NOAA, 2004) that coincided with dense blooms of *K. brevis*. It is of note that blooms of *K. brevis* are historically rare in the Panhandle region. In contrast, on the Southwest coast of Florida, *K. brevis* blooms have been documented during 40 of the past 41 years. Yet, large scale red tide associated mortalities of bottlenose dolphins are comparatively rare in Southwest Florida with mortality events recorded only in 1946-47 and 1953-55 (Gunter et al., 1948; Rounsefell and Nelson, 1966 in Landsberg, 2002) and most recently during a prolonged bloom of *K. brevis* that occurred during the fall of 2005 and spring of 2006 (NOAA, Unpublished data). The bloom densities and cellular toxicities do not appear to differ between Southwest Florida and the Florida Panhandle. Overall the factors contributing to brevetoxin associated mortalities of bottlenose dolphins are poorly understood. Furthermore, the impact of repeated sublethal brevetoxin exposure to the health and fitness of bottlenose dolphins is unknown, and the consequences of repeated UMEs on bottlenose dolphin populations, particularly in the Florida Panhandle, has not been assessed.

This research and response plan summarizes the current knowledge of the circumstances resulting in red tide associated bottlenose dolphin mortality events and identifies data needed to better understand the acute and chronic impacts of brevetoxin exposure on individuals and populations of bottlenose dolphins. Because critical information needed to fill these knowledge gaps can primarily be obtained during these unpredictable red tide events, the response plan was developed to facilitate efficient data gathering and integration by the numerous organizations and agencies that participate in such investigations. This bottlenose dolphin – brevetoxin research and response plan is a product of a workshop held at Walt Disney World Resorts in Orlando, Florida between December 13 and 15, 2004 (see Appendix I for workshop participants) and outlines the steps needed to better understand:

1. The role of brevetoxins in causing the 2004 bottlenose dolphin UME.
2. The role of brevetoxins in causing historical and future bottlenose dolphin UMEs.
3. The impact of repeat UMEs on bottlenose dolphin populations in the Florida Panhandle.

Insights Gained from the 2004 Panhandle Mortality Event

Background

Four UMEs involving bottlenose dolphins have occurred in the northern Gulf of Mexico since 1993, almost as frequently as the occurrence of *K. brevis* blooms in this region of the Gulf. In 1993-1994, an UME of bottlenose dolphins attributed to a morbillivirus epizootic started in the Florida Panhandle and spread west with most of the mortalities occurring in Texas (Lipscomb et al., 1996). That mortality event was caused by morbillivirus as determined by histologic lesions, immunohistochemical demonstration of mobilliviral antigen, and detection of morbilliviral RNA by a reverse transcriptase polymerase chain reaction (Lipscomb et al., 1996). In 1996, dolphin mortalities coincided with an unusual bloom of *K. brevis* in Mississippi, but the role of brevetoxin in the fate of these animals was not confirmed (NOAA, 2004). Between August 1999 and May 2000, at least 152 bottlenose dolphins died in the Florida panhandle coincident with extensive and persistent *Karenia brevis* blooms (Mase et al., 2000; NOAA, Unpublished data). In 2004, 107 bottlenose dolphins stranded dead on the coast of the Florida Panhandle between March 10 and April 13, 2004. In contrast with the 1999-2000 event, in 2004 a bloom of *K. brevis* was never detected, despite satellite remote sensing established in the region and extensive surveys funded through the National Oceanic and Atmospheric Administration (NOAA) emergency response funding. During both the 1999-2000 and 2004 bottlenose dolphin UMEs other species of marine life such as fish, invertebrates, sea turtles, and marine birds died. The 2004 UME and the 1999-2000 UME both started in the same location (St. Joseph Bay) and extended along the seven counties of the Florida Panhandle (Escambia, Santa Rosa, Okaloosa, Walton, Bay, Gulf, and Franklin Counties). Unlike the 1999-2000 UME where a high proportion of dolphins stranded in Choctawhatchee Bay, only three animals stranded in this bay during the 2004 UME (NOAA, Unpublished data).

Dolphin Ecology and Behavior in the Florida Panhandle

The historical average of bottlenose dolphin strandings for the seven Panhandle counties (Bay, Escambia, Franklin, Gulf, Okaloosa, Santa Rosa, and Walton) over the last 10 years (1995-2005) is 21.4 animals per year excluding 1999, 2000, 2004, and 2005 during which UMEs occurred (NOAA, Unpublished data). However, little is known of bottlenose dolphin behavior, ecology, and stock structure in the Florida Panhandle, including the region in and around St. Joseph Bay. To the best of knowledge, no systematic research was performed prior to the 2004 UME to determine the daily or seasonal patterns of use of St. Joseph Bay by bottlenose dolphins, or their broader ranging patterns or genetic composition as these might relate to stock structure. The occurrence of 2 die-offs of over 200 animals in the last 5 years, as well as the recent biopsy darting and photographic identification work with dolphins in St. Joseph Bay and vicinity, indicate clearly that the 1993 aerial survey findings of no dolphins present in the Bay are out of date and do not provide sufficient information to determine impacts of these mortality events on coastal populations or stocks.

Necropsy Findings

For animals dying in the 2004 event, sex distribution was approximately equal: 36 females and 46 males, with 25 carcasses of unknown sex. Carcass measurements of 100 animals (not including 2 fetuses) show that length ranged from 76 cm (neonate) to 280 cm (adult). Comparing the results of length classes with historic stranding records for this region (n=51, over 7 years), a higher

number of animals over 210 cm are represented in this die-off. Most animals were found to be in good nutritional condition, however a few animals were considered to be thin for this season as compared to winter season dolphin nutritional condition in Sarasota, Florida. Many animals had evidence of generalized lymphoid stimulation, which was characterized by multiple enlarged lymph nodes with scarce cellularity. Lesions of the upper respiratory tract, indicative of aerosol exposure to brevetoxins as seen previously in manatees (*Trichechus manatus latirostris*), were not observed.

Brevetoxins were found at high concentrations (average 1.8 μg/g, range 0.1 – 10 μg/g) in the stomach contents of all dolphins examined, which is a level greater than or equal to those observed in previous marine mammal mortality events associated with Florida red tides. In most of the dolphins, the first chamber of the stomach was filled with large amounts of fish, largely menhaden (*Brevoortia* spp.), a planktivorous herring-like fish. Some of the fish were partially whole and undigested indicating recent feeding. Mean stomach content values were 773 g, with 10 samples weighing > 1 kg (maximum: 2.258 kg). This suggests an acute intoxication through ingestion of menhaden that had ingested *K. brevis* cells. The fish themselves could have been compromised by brevetoxin intoxication (Steidinger et al., 1973; Lu and Tomchik, 2002) or could have been acting normally and just serving as vectors for the toxin after ingesting *K. brevis* cells.

Although no bloom of *K. brevis* was identified, fish (planktivorous and omnivorous fish species) collected from St. Joseph Bay on March 18, 2004 tested positive for brevetoxins in stomach contents and in muscle, liver, and gill tissues. Fish collected on March 28-31, 2004 also tested positive for brevetoxin but at much lower levels. The identification of brevetoxin and brevetoxin metabolites in the tissues of fish for the first time identifies fish as a vector for transfer of brevetoxins up the food chain to top predators such as dolphins (Flewelling et al., 2005).

Dr. Carlos Romero (University of Florida) tested samples collected during necropsy for viral nucleic acid using PCR and RT-PCR. All samples tested negative for: morbillivirus (50 samples from 24 animals); influenza A virus (14 samples from 8 animals); influenza B viruses (14 samples from 10 animals); calicivirus (26 samples from 15 animals); cetacean poxvirus (3 samples from 3 animals); herpes virus (10 samples); and parapoxvirus (3 samples from 3 animals).

Concentrations of persistent organic pollutants ("POPs", i.e., polychlorinated biphenyl congeners, DDT and its derivatives, and other organochlorine insecticides) in bottlenose dolphin blubber samples collected from the 2004 event were lower than those measured in free-ranging Sarasota Bay dolphins captured during health assessment work. Genetic analyses performed on 64 sampled dolphins using DNA sequencing of a portion of the mitochondrial DNA genome indicated that all 64 animals were of the coastal morphotype.

Conclusion

Overall, it appears that the 2004 bottlenose dolphin UME resulted from the transfer of brevetoxins from an undetected bloom through menhaden to dolphins via ingestion. The bloom possibly occurred offshore given that the usual signs of red tide were absent within St. Joseph Bay, including discolored water and aerosol effects. Bottlenose dolphins affected in this UME appeared to have gorged upon menhaden, a species not considered to be a major prey species for coastal bottlenose dolphins where their diet is known. The occurrence of brevetoxins in fish and seagrass

in St. Joseph Bay suggests that a bloom of *K. brevis* did occur in the region, possibly prior to the onset of the mortality event. Consistent with this hypothesis, large numbers of dead fish, jellyfish, and birds were documented during the same period in and around St. Joseph Bay. This UME identified for the first time the accumulation and persistence of brevetoxin in the muscle and viscera of fish, which can serve as a vector to higher trophic levels. The reason for the brevetoxin-related mortality of bottlenose dolphins in the Panhandle area of Florida, as compared to dolphins living in Southwest Florida, who are exposed annually to red tides, remains unresolved.

Coordinating Approaches

Harmful algal blooms (HABs) are complex in their effects as are the ecosystems in which they occur. Equally complicated is the array of scientists, managers, and private, state and federal agencies involved in studying and responding to HABs. In the U.S., the 1993 *National Plan for Marine Biotoxins and Harmful Algae* has served as a foundation to address HABs and marine biotoxins. Much has been learned about HABs and marine biotoxins since 1993 and agencies and organizations have changed. The national plan developed in 1993 was recently updated to reflect new findings and agency priorities and to redirect future resources to reflect current needs (HARRNESS, 2005). Many of the needs enumerated in the *Harmful Algal Research* & *Response National Environmental Science Strategy 2005-2015* document (HARRNESS, 2005) dovetail with needs required to better understand the impact of brevetoxins on bottlenose dolphins in the Gulf of Mexico.

In the Gulf of Mexico, numerous private, state, and federal agencies respond to HABs and fish and wildlife related mortality events. No one agency or organization has the resources to address the multitude of research and monitoring needs required to understand the impacts of HABs on bottlenose dolphin populations and their significance to dolphin conservation and welfare. A coordinated effort is needed to answer the multitude of research and monitoring requirements. Historically, attempts have been made to bring together individuals and groups studying HABs and wildlife mortality events (e.g., the Gulf of Mexico Aquatic Mortality Network), however federal oversight and inclusion of groups and organizations working with cetacean strandings and pathology have been lacking. The December 2004 workshop held at Disney was the first time that many of these individuals and organizations have met, representing a critical first step in establishing the long-term professional cooperation needed to understand the impact of brevetoxins on bottlenose dolphins. Increased future collaboration and data integration are needed to develop this understanding of brevetoxicosis in dolphins. This document identifies the information, tools, and monitoring needs to do this, as well as the agencies and organizations who can accomplish them.

Critical Research Needs

Bloom Ecology and Dynamics

In Florida, blooms of the dinoflagellate *Karenia brevis* most frequently originate offshore on the west Florida shelf where they can be transported inshore by currents and winds to the west coast between Tampa and Charlotte Harbor. Blooms tend to be seasonal, beginning in late summer and early fall and lasting into January or beyond (Steidinger, 1989). Over a century of data from the Florida Fish and Wildlife Conservation Commission suggest that blooms occur in the fall (Figure 2). Blooms in the Panhandle area are less frequent than along the southwest coast of Florida. As previously stated, we do not currently understand the factors that lead to brevetoxin-associated mortality in bottlenose dolphins during some blooms and not others. Improved understanding will require a more comprehensive monitoring of bloom ecology, particularly in the Panhandle region.

In Florida, HAB monitoring for exposure assessment and physiological responses to exposure for the protection of public health from shellfish toxicity events is conducted jointly by the Florida Fish & Wildlife Conservation Commission's (FWC) Fish and Wildlife Research Institute (FWRI), and the Florida Department of Agriculture and Consumer Services, Division of Aquaculture using fixed sampling sites associated with commercial shellfishing activities. This HAB monitoring program, which has successfully protected public health for 50 years, follows the guidelines of the Interstate Shellfish Sanitation Conference (a voluntary, cooperative association of states, U.S. Food and Drug Administration, National Marine Fisheries Service (NMFS), Environmental Protection Agency (EPA) and the shellfish industry). Shellfish beds are closed in the presence of 5,000 *K. brevis* cells/L and reopened when shellfish contain <20MU/100g using the regulatory mouse bioassay. Test results and shellfish openings and closures are posted on the Internet (http://www.floridaaquaculture.com/RedTide/RedTideInfo.htm). Coastal areas not open to shellfish harvest are not closely monitored (Figure 3). Monitoring does not occur regularly in Florida Panhandle shellfish harvest areas unless there is a known bloom in the region. However, St. Joseph Bay is sampled monthly.

An additional offshore red tide monitoring program for the Gulf coast of Florida has been instituted by FWC and utilizes volunteer charter boat captains to collect offshore water samples for red tide testing. Satellite imagery and red tide test results are routinely reported on the Internet (http://www.floridamarine.org/features/view_article.asp?id=9670#Rpt). On a larger scale, NOAA produces weekly HAB bulletins for the Gulf of Mexico posted on the Internet (http://coastwatch.noaa.gov/hab/bulletins_ns.htm). Satellite imagery, which detects grossly elevated chlorophyll levels (50,000 cells/L), has been successfully implemented as a forecasting and monitoring tool in Southwest Florida. However, the 2004 mortality event is an example in which extensive mortalities occurred in the absence of an observable bloom by either inshore sampling or satellite imagery. Better temporal and spatial documentation of *K. brevis* blooms are needed to understand their causal role in marine mammal mortality events in the Panhandle area. Therefore, it is recommended that additional bloom monitoring efforts be expanded in regions of the Panhandle that are prone to HAB-associated bottlenose dolphin mortality events.

The 2005 Harmful Algal Research and Response: A National Environmental Science Strategy (HARNESS) report calls for establishment of a coast-wide monitoring and data

management system through NOAA's Integrated Ocean Observing System (IOOS). The Gulf of Mexico HAB community and developing regional IOOS regional associations have an existing infrastructure in the Harmful Algal Blooms Observing System (HABSOS) which integrates buoy data, remote sensing, cell counts, and volunteer monitoring. It is recommended that increased surveillance be implemented in the Panhandle region with particular emphasis on understanding the occurrence and food web effects of *K. brevis* blooms in this area.

Using receptor binding assay, ELISA, and LC tandem mass spectroscopy, trace levels of domoic acid were found in the stomach contents and feces of some, but not all, of the stranded dolphins tested from the 2004 event (FWRI, St. Petersburg, FL and NOAA/National Ocean Service [NOS], Charleston, SC). This was the first time that domoic acid had been documented in tissues from marine mammals that have stranded in the Gulf of Mexico, however the concentrations of domoic acid found were much lower than those found associated with marine animal mortalities documented in other regions, such as California (Gulland, 2000). Additionally, *Pseudo-nitzschia delicatissima* was present in water samples, although domoic acid concentrations were below levels detectable by ELISA (FWRI, St. Petersburg, FL and NOAA/NOS, Charleston, SC). Shellfish samples collected from the area had domoic acid concentrations well below the established regulatory limit (20 μg/g) for public health protection. Domoic acid intoxication is not considered as a primary cause of the 2004 dolphin UME. In response to the mortality, a preliminary study aiming to identify possible synergistic effects on mammals of concurrent exposure with domoic acid and brevetoxins was carried out. This study revealed that in mouse models and at the toxin levels observed in dolphins, presence of domoic acid did not increase the potency of the brevetoxins and do not result in synergistic effects (Naar and Flewelling, unpublished data). Studies on *Pseudo-nitzschia* bloom ecology should be combined with efforts to better understand *K. brevis* bloom ecology and dynamics. It is important to note that domoic acid has never been documented to be a problem in the Gulf of Mexico, however proactive efforts to better understand *Pseudo-nitzschia* ecology and the potential for domoic acid toxicity in the region will provide important baseline information this historical situation change.

Work is needed to better understand the potential for oceanographic and other factors to cause brevetoxin-related bottlenose dolphin strandings to be over-represented in Gulf County, Florida (or at least in St. Joseph Bay). St. Joseph Bay is a small embayment (29.80° N 85.36° W),which is partially isolated from the Gulf of Mexico by St. Joseph Peninsula/Spit, which extends from Cape San Blas in the south to St. Joseph Point in the north. It is the only body of water in the eastern Gulf of Mexico not influenced by the inflow of fresh water. In non-UME years, bottlenose dolphin strandings are extremely rare in St. Joseph Bay and on the Coast of Gulf County. However, the 1999-2000 and the 2004 brevetoxin-associated UMEs started in St. Joseph Bay. During the 2004 event, 70 percent of strandings were localized in St. Joseph Bay (NOAA, 2004).

Work is also needed to better understand why brevetoxin-related dolphin strandings were over-represented in Choctawhatchee Bay during the 1999-2000 UME, as well as during the recently declared 2005-2006 Bottlenose Dolphin UME (NOAA, Unpublished data). Choctawhatchee Bay lies west of St. Joseph Bay within the counties of Okaloosa and Walton (30.45° N 86.45° W). Freshwater flow to the bay comes from the Choctawhatchee River, Pea River, Wrights Creek, Sandy Creek, Pine Log, Seven Run, Holmes Creek, and Bruce Creeks. The bay is separated from the Gulf of Mexico along most of its length, but connects through the Pensacola and East Passes entering through the south at Destin Pass.

In addition to increasing baseline monitoring for HABs and background brevetoxin levels in these bays (discussed in detail under Food Web Studies), detailed bathymetric mapping of the region combined with passive oceanographic drift card studies are needed to characterize the bays' oceanographic features as they might relate to brevetoxin bloom or toxin persistence.

Priority Research and Monitoring Needs: Bloom Ecology and Dynamics

- Increase temporal and spatial coverage of routine HAB monitoring in the Florida Panhandle region
- Implement the use of buoys for remote monitoring in and offshore of the St. Joseph Bay and Choctawhatchee Bay areas
- Incorporate the Florida panhandle into the existing HABSOS monitoring network
- Compile or develop a detailed understanding and GIS analysis of St. Joseph and Choctawhatchee Bays' oceanographic features (bathymetry, wind, currents, etc.) and how they influence bloom dynamics and toxin and carcass distribution

Food Web Studies

Evidence from the 2004 dolphin mortality event suggests that brevetoxin can be concentrated in shellfish and fish in the absence of detectable blooms, resulting in dolphin exposure that is delayed or remote from *Karenia* blooms (Flewelling et al., 2005). Therefore, improved efforts to monitor the duration and extent of biotoxins in marine waters also need to document the extent and duration of biotoxins in living marine organisms, including fish and shellfish. Active sampling of organisms within and proximal to defined bloom areas is probably the most effective way to do this.

St. Joseph Bay has been the apparent epicenter of two brevetoxin-induced dolphin mortality events and both St. Joseph Bay and Choctawhatchee Bay have been over-represented with strandings during recent brevetoxin-associated UMEs. Increased baseline monitoring for HABs in these bays is warranted. A suite of biological indicator organisms, including fish and shellfish, should be considered for monitoring background brevetoxin levels. Due to the presence of *K. brevis,* shellfish harvest in St. Joseph Bay was closed on November 19, 2003, four months prior to the 2004 bottlenose dolphin mortality event. Oysters collected and tested on February 2, 2004 were still too toxic to re-open and were not tested again until after the dolphin UME was underway. At that time, levels were <20MU/100g but due to the on-going dolphin mortality in the region, shellfish harvest remained closed until April 29, 2004 for precautionary reasons. Real-time data on toxin levels in shellfish can provide important information on brevetoxin persistence in the Bay. Expanding within-bloom shellfish monitoring to include other organisms such as fish feeding at different trophic levels would provide valuable data on brevetoxin persistence within the ecosystem and help better document potential avenues for bottlenose dolphin or human exposure.

Experimental tracing of brevetoxins from dinoflagellates through copepods grazers to juvenile fish has been conducted, but work needs to be done looking at this transfer in natural settings (Tester et al., 2000; and Landsberg, 2002). Presently it appears that we see parent toxins and metabolites in planktivorous and carnivorous fish (note: PbTx-2, which is rapidly metabolized, is not observed in carnivorous fish; Flewelling et al., 2005 and Flewelling, pers. comm.). Experimental exposure revealed that brevetoxin accumulates in the muscle and viscera of all fish types tested, however levels are higher in viscera than in muscle (Flewelling et al., 2005). It is not known how long fish can remain toxic and what concentrations of which metabolites cause acute or chronic disease in bottlenose dolphins. Field work on biotoxins in the food chain should be closely linked with laboratory work on the toxins and their effects.

The trophic transfer of brevetoxin in the marine ecosystem and the mechanism of bottlenose dolphin exposure could provide important information about when and where *K. brevis* blooms cause brevetoxicosis in bottlenose dolphins. It has been hypothesized and is understood that manatees can be exposed to brevetoxins via ingestion or inhalation (O'Shea et al., 1991; Bossart et al., 1998; Landsberg and Steidinger, 1998; and Flewelling et al., 2005). Unlike the 1996 manatee mortality event where upper respiratory tract lesions suggested inhalation as a route of brevetoxin exposure for manatees (Bossart et al., 1998), data from the 1999-200 and 2004 dolphin mortality events suggest that the route of exposure in bottlenose dolphins was likely oral (Flewelling et al., 2005; Mase et al., 2000). In a 1987-1988 bottlenose dolphin mortality event where morbillivirus was the primary etiology, brevetoxin was detected in dolphin liver samples, as well as in the viscera of migratory menhaden. It was hypothesized that menhaden were likely sources of brevetoxin for dolphins (Geraci, 1989; Steidinger, 1989). Spanish mackerel (*Scromberomorus maculatus*), a preferred food item for dolphins, prey on menhaden and were hypothesized to be another route through which dolphins ingested brevetoxin (Steidinger, 1989). During the 2004 event, preliminary works show that menhaden was the dominant prey species in at least 50 percent of the dolphin stomach's examined. Shrimp, sciaenid fish, and other fish also were found in the stomachs of dead dolphins. Menhaden are not considered a significant dietary item for bottlenose dolphins off the coast of west central Florida (Barros and Wells, 1998), east central Florida (Barros, 1993), North Carolina (Gannon and Waples, 2004), nor at other locations in the Southeastern U.S. (Barros and Odell, 1990). Despite this, menhaden seem to be over-represented in the stomach contents from dolphins in the 1999 and 2004 brevetoxin-associated mortality events.

Clearly, it is important to better understand the preferred diet of bottlenose dolphins inside and outside of regions where brevetoxin causes mortality in dolphins. Understanding the trophic transfer of brevetoxin and its metabolites in the marine ecosystem is also needed. Evaluating the stomach contents of stranded dolphins is one mechanism by which to learn more about dolphin diets, especially when animals die acutely or are determined to die of brevetoxicosis. Stomach contents from all stranded dolphins, however, will likely not be characteristic of preferred prey in many cases, especially if animals show signs of chronic disease. Therefore, it is still critical to identify otoliths and boney remains in the stomachs of all stranded dolphins. Whole stomachs should be sent to a laboratory where contents will be weighed and accurately identified. Currently, this is being performed at Mote Marine Laboratory. When present, gastric juices and fleshy remains of prey will be submitted for brevetoxin analysis with samples being alternately sent to a NOAA/NOS laboratory and the FWC FWRI. Additionally, a project using biopsy and necropsy samples and fatty acid or stable isotope analysis should be developed to learn more about the trophic level at which different dolphin stocks are feeding. Finally, during HAB events with documented dolphin mortality, efforts should be made to recover and analyze brevetoxin levels in

fish in dolphin stomachs, fish caught near mortality sites, dead fish on the beach, and fish bought from local fish markets.

Priority Research and Monitoring Needs: Food Web Studies

- Increase monitoring for HABs and background brevetoxin and domoic acid levels in the St. Joseph Bay region, including monitoring of toxin levels in seagrass, shellfish, and fish from the region
- Understand the feeding ecology of bottlenose dolphins in the region of St. Joseph and Choctawhatchee Bays as compared to dolphins in other regions that do not seem to be as impacted by brevetoxin, especially sites that appear to have more *K. brevis* blooms, but less brevetoxin-associated dolphin mortality
- Compare stomach contents between dolphins diagnosed with brevetoxicosis and dolphins dying from other causes
- Determine the tissue distribution of toxins and metabolites in fish that are positive for brevetoxin

Toxin and Effects

Brevetoxins are neurotoxins and hemolysins produced by *Karenia brevis* (a dinoflagellate) and *Chloromorum toxicum* (Thomas et al., 2005; Landsberg, 2002). Brevetoxin-like compounds have been identified in another *Karenia* sp. and in the raphidophytes *C. antique*, *C. marina*, *Heterosigma akashiwo*, and *Fibrocapsa japonica* (Landsberg, 2002). Historically, brevetoxin-producing *Karenia* blooms have occurred more frequently and impacted a greater range of species than raphidophyte blooms, which seem to be primarily ichthyotoxic. Brevetoxins are complex lipid soluble polycyclic ethers. The nine brevetoxins that have been isolated from *K. brevis* (PbTx-1, 2, 3, 5, 6, 7, 8, 9, and 10) have varying degrees of toxicity and are differentiated into two groups based on whether they have a 10-ring (PbTx-1 type) or 11 ring backbone (PbTx-2 type) (Baden, 1989; Schulman et al., 1990). Twenty-one toxin metabolites also have been identified (Wang et al., 2004).

The mechanism of toxin action is sodium channel activation: toxin binds to site 5 on the voltage gated sodium channel, resulting in channel opening at normal resting potential and prolongation of their open state, leading to uncontrolled Na^+ influx into the cell (Poli et al., 1986; Gawley et al., 1992). Toxin metabolism is not as well understood. In rats, PbTx-3 (Brevetoxin-3) injected intravenously was cleared (90 percent) within 1 minute, but a sub-lethal oral dose remained in serum up to 192 hours post consumption, with maximum concentration found at 48 hours (Poli et al, 1990). However, differences in the rate and mechanism of clearance of PbTx-2 versus PbTx-3 from blood are seen in rats. PbTx-2 is rapidly transformed to a polar metabolite of reduced biological activity that appears in the blood and is retained for up to 4 hours, yet is cleared entirely to

the urine within 24 hours in the form of cysteine adducts (Radwan et al, 2005). At the same dose, only about one-fifth as much PbTx-3 was cleared to urine. The routes of toxin clearance in orally exposed mammals (rats) appear to be fecal, then renal; probably with most metabolism and highest levels occurring in the liver (Cattet and Geraci, 1993). *In vitro* studies with hepatocytes identified the involvement of cytochrome P450 and glutathione in generating metabolites of PbTx-2 in rat (Radwan and Ramsdell, *In Press*) Toxin metabolism, the pathogenesis of toxicosis, and the mechanism of toxin clearance have not been studied in bottlenose dolphins and more research is needed. Experimental dosing of bottlenose dolphins is not an option, so alternate non-invasive and opportunistic strategies such as immunohistochemistry tracking of metabolites in tissues of dead stranded dolphins will have to be employed.

During the 2004 mortality event, metabolites in stomach, liver and kidney were identified in approximately six animals. PbTx-3 and -9 dominated in liver and kidney, with the *cys*-conjugate also present in stomach contents, indicative of the presence of PbTx2 (Radwan et al., in prep). Efforts should be made to compare the PbTx (brevetoxin) types and concentrations in other mortality events to gain further insight into the levels and form that functionally compromise and adversely affect bottlenose dolphins. In addition, the identification and quantization of toxin metabolites present in different dolphin tissues will help to confirm if uptake, metabolism, and clearance patterns are similar to those in rodent models and may yield insight into the contributions of oral versus aerosol routes of exposure. Improved immunohistochemical detection of brevetoxin in tissues of intoxicated dolphins will further serve to refine the understanding of its mechanism of toxicity in stranded animals. Collectively, these data will help to create a case definition for brevetoxicosis in bottlenose dolphins and potentially allow the definition of sub-acute or chronic exposure. Additionally, a better understanding of PbTx types and metabolism should help the understanding of toxin movement and metabolism through marine trophic levels (see food web studies).

In addition to research on brevetoxin metabolism and clearance, additional tools are needed to assist in monitoring of toxin associated with dolphin mortalities. In particular, the investigation of brevetoxin associated mortality events would be greatly facilitated by the development of a rapid portable test to confirm presence/absence of brevetoxin in urine or blood. Confirmation of brevetoxin exposure in a stranded animal by field personnel would aid in initiating collection of the proper evidence of blooms (which may not be evident from shore), could potentially help responders treat live-stranded dolphins, and would facilitate collection of critical tissue samples for the quantitative analyses described above in dead-stranded animals.

Priority Research and Monitoring Needs: Toxin and Effects

- Understand the relative toxicities of brevetoxins and their metabolites in bottlenose dolphins using non-invasive opportunistic investigations or *in-vitro* techniques
- Understand metabolism, retention and clearance of brevetoxins in bottlenose dolphins using non-invasive techniques or a biomarker that acts as a proxy for brevetoxin clearance
- Develop toxin-specific biomarkers of exposure and effects for assessing sub-lethal and chronic exposure
- Develop a case definition for brevetoxicosis in bottlenose dolphins
- Develop a field test for rapid assessment of brevetoxin exposure in bottlenose dolphins

Acute Impact on Bottlenose Dolphins

Just as better documentation of the temporal and spatial distribution of *K. brevis* blooms is needed (see Bloom Ecology and Dynamics), so are better documentation and investigation of cetacean mortality events. The relationship between the blooms and dolphin mortality events will be better understood only with improvement in both of these areas. Marine mammal mortality events in Florida are monitored by NOAA's Marine Mammal Health and Stranding Response Program, which includes a Stranding Network comprised of other government agencies, non-governmental agencies, aquaria, and universities. The historical average of bottlenose dolphin strandings for the seven Panhandle counties (Bay, Escambia, Franklin, Gulf, Okaloosa, Santa Rosa, and Walton) over the last 10 years (1995-2005) is 21.4 animals per year excluding 1999, 2000, 2004, and 2005 during which UMEs occurred (NOAA, Unpublished data). Excluding brevetoxin-associated UMEs, information is not available on the level of brevetoxin (background or other) in stomach contents, feces, or tissues from bottlenose dolphins in the Florida Panhandle. In fact, brevetoxin levels in dolphins stranded off the coast of South Carolina were used as references during the 2004 UME (NOAA, 2004). Despite frequent and numerous documented *K. brevis* blooms in the Gulf of Mexico, few brevetoxin-related bottlenose dolphin mass mortality events have been documented (Figure 1). More work is needed to determine why some *K. brevis* blooms in the Gulf of Mexico result in bottlenose dolphin mortality, and why the majority apparently do not.

The Florida coast along the northern Gulf of Mexico (including the Panhandle and Northwest Florida) currently has some areas where stranding response is well-covered, and others where it is either limited or have no coverage. It is imperative that efforts be made to: (1) improve coverage and response to marine mammal strandings in the region; (2) provide additional training for responders to collect Level B and C data, with an emphasis on collecting samples for prey identification and brevetoxin and domoic acid analysis; and (3) make every effort to determine the cause of death and the level of brevetoxin and domoic acid involvement in all regional marine

mammal strandings. Opportunities (realistic and idealistic) to improve marine mammal stranding response in under-served areas include:

- Fund a stranding response person at the NOAA Panama City laboratory whose job it will be to coordinate more effective response to strandings.
- Pay for weekly aerial surveillance to establish a stranding baseline and facilitate retrieval of carcasses while they are still reasonably fresh (estimated cost of $2,000-4,000/week).
- Develop a standard fly-over and reporting schedule utilizing the U.S. Coast Guard or local Air Force Base (Eglin Air Force Base) to survey and report stranded marine mammals.
- Develop and implement a sustained outreach campaign to educate the public about the importance of reporting live and dead strandings, and how to report stranded marine mammals.
- Provide necropsy training for more Stranding Network volunteers.
- Provision and fund a rapid response necropsy team to assist local stranding response groups with mass strandings.
- Provide freezer storage space to store the volume of tissues that often result from a UME.
- Build capacity for stranding response in Northwest Florida, in areas where there is no stranding response.

In addition to enhancing routine marine mammal stranding response, efforts should be made to enhance surveillance for strandings during documented HABs, which could improve diagnosis of low level, HAB-related mortality. There is concern that *Karenia* spp. other than *K. brevis* are present in the Gulf of Mexico and that they can produce brevetoxin-like compounds. Because there is interest in the impact of *Pseudo-nitzschia* spp. and other toxin-producing algae on bottlenose dolphin populations, all identified HABs, and not just *K. brevis* blooms, need to be investigated for associated marine mammal mortality.

To adequately monitor HABs for associated cetacean mortality there needs to be a mechanism for alerting management agencies and targeted surveillance groups that a bloom has been identified in their region and that they need to heighten their surveillance efforts. An old multi-state HAB alert network called GMNET (Gulf of Mexico Aquatic Mortality Network) sponsored by the EPA used to alert natural resource managers about mortality events and potential or diagnosed HABs. It was suggested that NOAA or the Department of Homeland Security should be asked to resurrect GMNET or possibly ask the EPA if they are interested in resurrecting GMNET. A fish-kill hotline (1-800-636-0511) is currently in operation and is run by the FWRI's Fish and Wildlife Health (FWH) Group. The public can call this number to report fish kills, and often use it to report other wildlife mortality events. Callers are either referred to the appropriate agencies or the appropriate agencies are contacted after FWC receives the call. It could potentially be officially expanded to include reporting of wildlife mortality and integrated with the current system for reporting and responding to marine mammal strandings. As appropriate for the relevant species, unofficially FWRI FWH staff call the pre-established NOAA number for reporting marine mammal mortalities (1-888-404-3922), but perhaps a more official memorandum of understanding should be developed.

An electronic discussion group that alerts people to the presence of HABs and provides a venue for discussion of fish kills also exists and is maintained by the FWC/FWRI. Electronic mail is sent out weekly alerting participants of HAB status and detailed HAB status reports and maps are frequently attached. Weekly maps of HAB distribution are posted on the FWC/FWRI website at http://floridamarine.org/. Marine mammal stranding networks should be linked into the FWC's

discussion group as a means of better understanding when HABs are present in their region. In addition, stranding network responders should sign up to receive the HAB bulletin, which is electronically sent to subscribers twice weekly and reports developing blooms and changes in the location and extent of existing blooms (see website at http://www.csc.noaa.gov/crs/habf/). Electronic communications reporting HAB locations and requests for people to be alert for wildlife mortalities should also target members of state parks, wildlife reserves, wildlife rehabilitation organizations, county sheriff departments, beach watcher programs, the Florida Marine Patrol, and others that have routine beach exposure or are frequently alerted to marine mammal mortality. These people could use the pre-established NOAA number for reporting marine mammal mortalities (1-877-433-8299). Ultimately all cetacean stranding reports should go to NOAA Fisheries. All marine mammal mortality investigations should culminate in a report that is sent to a central clearinghouse, which needs to be established. A dedicated person or group of people should create and maintain a database of HABs with and without reported marine mammal mortality (and wildlife mortality; see next chapter), as well as a database of causes of marine mammal mortality, including information on what proportion are HAB-related. There is on-going work with on electronic databases with the FWC, the Florida Department of Health, and NOAA and new initiatives should build upon these efforts.

In addition to receiving complete post-mortem examinations that include histopathology and ancillary testing directed to determining cause of death, all cetaceans collected for necropsies (presumed HAB-associated and presumed non-HAB associated) should be examined according to similar standardized testing for acute and chronic exposure to HAB-related toxins. This should include toxicological testing performed on a suite of similar tissues, as well as immunohistochemistry on a suite of standardized paraffin-embedded tissues, which help define the presence, abundance, and distribution of brevetoxins in tissues. A standardized necropsy form should be used that includes sampling guidelines, a handling guide for tissues (packaging, shipping, and chain of custody), photographic requirements, and morphometric requirements. Appendix II contains a preliminary description of samples requested by researchers for certain tests and Appendix III contains a list of samples to be collected from every animal that is necropsied.

HAB testing proposed in Appendix II should be limited to all marine mammals in carcass condition 2 or 3. Captive bottlenose dolphins in Florida that die also should be tested for exposure to brevetoxin and domoic acid, especially if locally caught fish make up some portion of their diet. Necropsy and test results (final reports) for every case should be submitted to a central database as described above, which will permit differentiating HABs that have associated fish and wildlife mortality and those that do not.

In addition to expanding the existing HAB electronic discussion group in an effort to identify HAB-associated marine mammal mortality, work needs to done to enhance the recovery and necropsy of stranded marine mammals even when HABs are not identified in the region. Individual stranding networks could be empowered to do this as they see fit or a regional advertising campaign could be considered. All non-HAB related stranded marine mammals also should be subjected to the suite of testing described in Appendix II.

The pharmacokinetics, residence time, and pathogenesis of brevetoxin in bottlenose dolphins are unknown. A validated bottlenose dolphin specific immunohistochemical test for determining the presence of brevetoxin in paraffin-embedded tissues would greatly improve the understanding of the pathogenic mechanisms of lethal and sub-lethal chronic exposure in this

species. Immunohistochemistry that utilizes a primary goat anti-brevetoxin has been developed and has been utilized to identify brevetoxin in tissues of manatees (Bossart et al., 1998). This is a polyclonal antibody that reacts with all nine brevetoxins. It has also been modified to eliminate nonspecific background and used to test tissues of double-crested cormorants (Kreuder et al., 2002). Work is underway to further modify and utilize the technique on tissues of bottlenose dolphins (Bossart, unpub. data). Immunohistochemistry identified brevetoxin in macrophages and lymphocytes from multiple tissues in manatees (Bossart et al., 1998) and in lymphoid cells (spleen), macrophages (spleen and lung), tracheal mucosa, heart, and brain of double-crested cormorants (Kreuder et al., 2002). Once the technique is perfected for bottlenose dolphins, immunohistochemistry performed on brevetoxin and non-brevetoxin associated dolphin mortalities may increase the understanding of pharmacokinetics, residence time, and pathogenesis of brevetoxin in this species, especially if a method is developed to distinguish between toxic and non-toxic metabolites.

Although not discussed in detail at the Orlando meeting, live-stranded bottlenose dolphins provide an additional opportunity to learn more about the acute effects of brevetoxins. The Marine Mammal Stranding Network responds to live stranded animals and rehabilitation facilities including, but not limited to, Gulf World Marine Park, Mote Marine Laboratory, and Clearwater Marine Aquarium will take these animals into rehabilitation when appropriate. Developing and using a sampling protocol for live-stranded dolphins in rehabilitation centers offers a great opportunity to study naturally occurring brevetoxicosis-related morbidity in bottlenose dolphins. It also permits comparisons between dolphins in Sarasota and the Panhandle, which differ dramatically in the number of dolphins dying of brevetoxicosis. A protocol should be developed that permits testing of all live-stranded dolphins for acute brevetoxin and domoic acid exposure, including length of time, course of metal excretion, and clinical symptoms. Also, they may be tested for antibodies to brevetoxins if a test is available. Animals collected during live-capture, sample, and release health assessment projects should also be similarly evaluated when possible.

Priority Research and Monitoring Needs: Acute Impact on Dolphins

- Improve marine mammal stranding response in under-served areas and enhance routine marine mammal stranding response
- Develop toxin-specific biomarkers of exposure and effects for assessing acute (lethal and sub-lethal) and chronic exposure including protein biomarkers in serum and urine as well as the expression of brevetoxin antibodies in serum
- Increase testing of code 1 and 2 stranded bottlenose dolphins for brevetoxin and domoic acid in non-UME situations. Liver, stomach, urine and feces should be tested.
- Perform standardized postmortem examinations on all code 2 bottlenose dolphins including contaminates, infectious diseases, and presence or absence of brevetoxins and domoic acid.
- Develop a case definition for brevetoxicosis in bottlenose dolphins
- Develop improved immunohistochemistry methods for diagnosis of brevetoxicosis in paraffin-embedded marine mammal tissues
- Develop a kinetic model for brevetoxicosis in bottlenose dolphins
- Establish a lethal effect level for brevetoxin in bottlenose dolphins
- Coordinate the reporting of marine mammal disease testing into a central database or sharing of data to permit retrospective analysis of region-wide marine mammal mortality
- Develop and utilize a sampling protocol for testing live-stranded dolphins for acute and chronic brevetoxin exposure

Acute Impact on Other Species

While monitoring for marine mammal mortality, it is also important to monitor documented HABs for associated mortality in birds, sea turtles, fish, and other species. The FWC has been monitoring HABs and networking with local resource agencies, academia, and volunteer groups for associated non-cetacean wildlife mortality events and these efforts should be enhanced. In order to adequately monitor HABs for associated fish and wildlife mortality there needs to be an enhanced mechanism for alerting management agencies and targeted surveillance groups (beyond the FWC) that a bloom has been identified in their region and that they need to heighten their surveillance efforts to document and diagnose associated fish and wildlife mortality (see Acute Impacts on

Bottlenose Dolphins). If they are not already doing so, regional fish, bird, and turtle biologists should be encouraged to join an electronic discussion list or join the HAB bulletin distribution list so that they might have a heightened awareness of mortality during HAB events.

Bird mortalities in the state of Florida are reported to the FWC (see the website at: http://myfwc.com/bird/default.asp). Staff from FWC is available to respond to potential marine bird events and to test for potential HAB involvement. Marine bird mortality calls reported through the fish kill hotline are transferred to Dr. Danielle Stanek (FWC,FWRI) who coordinates response efforts and diagnostics with collaborating organizations including FWC, University of Florida College of Veterinary Medicine (Drs. Spalding and Forrester), the Southeastern Cooperative Wildlife Disease Study (Dr. Kevin Keel, Athens, GA), FDACS Animal Diagnostics laboratory (Kissimmee, FL), Mote Marine Laboratory (Dr. Deb Fauquier, Sarasota, FL), or the National Wildlife Health Center (Dr. Grace McLaughlin, Madison, WI). Bird rehabilitation facilities in the Florida Panhandle should be educated about brevetoxicosis and other HABs in fish-eating marine birds. A list of regional bird rehabilitation facilities could be developed and guidelines could be written directing these facilities to submit appropriate carcasses of fish-eating marine birds to a pre-determined postmortem laboratory or pathologist for necropsy or at least to submit tissues for brevetoxin testing.

Sea turtle mortality should continue to be reported to NMFS or to FWC (Allen Foley, 904-573-3930). Non-marine mammal wildlife collected for necropsies (presumed HAB-associated and presumed non-HAB associated) need to receive standardized testing for acute and chronic exposure to HAB-related toxins, just like marine mammal mortality investigations. Due to the sheer numbers of fish kills and bird mortality reported, HAB testing proposed in Appendix II should be limited to mass mortality of fish-eating marine birds (>5 birds) and all sea turtles.

Fish, bird, and sea turtle mortality and HAB reports are separately housed in the FWC, and plans are being made for integration. These data could also be linked to a federal database that contains marine mammal mortality information. This could be done by linking marine mammal, bird, sea turtle, and fish mortality databases with a HAB database, which would permit evaluation of HABs on causing fish and wildlife mortality.

Priority Research and Monitoring Needs: Acute Impacts on Other Species

- Appropriate agencies should continue to investigate the impact of brevetoxins as a mortality factor for birds, turtles and fish and improve coordination of findings with marine mammal managers
- Fish, bird and turtle mortality reports should be integrated into a federal database with marine mammal mortality information.

Impact of Chronic Brevetoxin Exposure on Bottlenose Dolphins

The lipophilic nature of brevetoxins suggests that they are likely metabolized and stored in the tissues and/or blubber of dolphins for some time after an acute exposure. However, the body burden of brevetoxin in bottlenose dolphins exposed repeatedly to brevetoxin on the Southwest Florida coast has not been established and may be critical to establishing the toxic effect level in acute exposure cases. The use of blood collection cards has shown promise for monitoring exposure in living animals exposed to red tides (Fairey et al., 2001). However, the timeframe in which this method is useful following exposure has not yet been determined.

An alternative to testing toxin itself in living animals is to use protein biomarkers as a proxy for toxin exposure. The development of biomarkers specific for brevetoxin exposure is feasible with the use of toxicogenomic (microarray) analyses or plasma following exposures in or proteomic analyses in serum or urine of exposed animals. Protein biomarkers would be expected to provide a longer-lived signal of toxic exposure or effect than the toxin alone.

The consequences of chronic and repeat exposures to oral brevetoxin exposure have not been investigated, even in rodent models. Brevetoxin-exposure should be evaluated as a risk factor for chronic diseases identified in stranded animals. Aerosol exposures in manatees (Bossart et al., 1998) and rats (Benson et al., 2005) have previously been shown to cause immunosuppression. Many examples exist to suggest that immunosuppression brought on by acute toxicities may facilitate the establishment of secondary infections. Thus investigating the possible immunosuppressive effect of oral exposure to brevetoxin in a rodent model would provide insight into its possible adverse effects in dolphins.

Conversely, given the episodic exposures experienced by dolphins in Southwest Florida, an obvious question arises as to whether repeat exposure confers some level of resistance to brevetoxicosis that the Panhandle dolphins lack. Although the brevetoxin molecule is somewhat small to be predicted to be antigenic, nonetheless exploration of the possibility of an adaptive immune response in Southwest Florida dolphins is warranted and could be addressed using a competitive ELISA to assess anti-PbTx antibodies in dolphin serum.

Priority Research and Monitoring Needs:
Impacts of Chronic Exposure on Dolphins

- Establish brevetoxin body burdens in dolphins repeatedly exposed to brevetoxin
- Develop toxin-specific biomarkers of exposure and effects for assessing sub-lethal and chronic exposure including protein biomarkers in serum and urine as well as the expression of brevetoxin antibodies in serum
- Utilize developed tests to investigate potential chronic impacts
- Evaluate the immunological integrity and potential suppression of brevetoxin-exposed and non-exposed bottlenose dolphin populations
- Determine if brevetoxin exposure has reproductive effects in bottlenose dolphins

Impact on Bottlenose Dolphin Populations

Although the information is integral to understanding brevetoxin-associated mortality and population impacts of brevetoxin UMEs on bottlenose dolphins in the Florida Panhandle, little is known about bottlenose dolphin stock structure in the region. Currently, NMFS designates each of the 33 bays, sounds, and estuaries in the in the Gulf of Mexico as separate stocks, along with 3 separate coastal stocks. This does not include St. Joseph Bay, where dolphins were not observed during the 1993 aerial surveys that were used to designate these putative stocks (NOAA, 2004). Little information exists about the natural history of bottlenose dolphins in the Florida Panhandle. A better understanding of home range and movement patterns for dolphin stocks in the region will allow a more accurate assessment of which stocks could be impacted by blooms. Brevetoxicosis, however, is more complex than dolphin temporal and spatial association with blooms and information is needed beyond just spatial and temporal movement of dolphins. Evidence from the 2004 UME strongly suggests that dolphins orally ingested brevetoxin by feeding on fish that accumulated toxin through the food chain. If bottlenose dolphin feeding ecology varies significantly between stocks, knowledge about preferred diets in different regions or at different times of the year combined with better knowledge of toxin movement through the food chain could give a better understanding of why some dolphin populations are impacted by brevetoxin while others are not.

Work is underway to characterize the bottlenose dolphin population in the St. Joseph Bay region and this information should be included in a comprehensive re-evaluation and possible re-structuring of bottlenose dolphin stocks along the Florida panhandle. All stocks that could potentially be affected by a HAB need to be described. This description should include a description of the stock's geographic range, a minimum population estimate, current population trends, current and maximum net productivity rates, and optimum sustainable population levels. In addition, baseline information about the behavior, ecology, and health status of these stocks should

be gathered. Information such as diet, seasonal home range, and baseline brevetoxin exposure could be instrumental in determining why brevetoxin-associated UMEs have occurred frequently along the coast of Florida's Panhandle, and rarely in areas such as along the Florida west coast.

With the exception of gathering biopsies and conducting live captures of dolphins, detailed research plans for redefining the stock structure and learning more about the natural history of bottlenose dolphin populations in the Florida Panhandle were not discussed. If the sample size is sufficient and genetic markers are adequate, biopsies collected from free-ranging animals along the coast could help provide a genetic picture of the region's stocks. Genetic data from stranded animals could then be compared to the genetic picture developed from free-ranging dolphin samples to confirm animal origin and hopefully get more reliable information on stock identity of stranded animals.

The health assessment of bottlenose dolphins in the Panhandle region using live-capture-release techniques is important to determine other potential health issues and additional biological and chemical toxin exposures that could make them more susceptible to mortality events. Data from these baseline health assessments can be compared to data being collected from other U.S. coastal sites to determine if the Panhandle dolphins differ from dolphins in other sites, such as Sarasota Bay, where the populations do not appear to be as vulnerable to mortality events. Collection of samples listed in Appendix III from live-captured animals will provide important baseline data on background biotoxin levels, pathogen exposure, immune function, hematology, and serum chemistry, as well as genetic material for stock assessment. This data, combined with other information, will help to better understand: (1) underlying health issues of Florida Panhandle dolphins; (2) potential hemolytic actions of brevetoxins; (3) lethal brevetoxin doses for bottlenose dolphins; (4) whether or not acute or chronic sub-lethal brevetoxin exposure can have reproductive impacts or increase dolphin susceptibility to infectious diseases; (5) if sub-lethal exposure confers immunity to subsequent brevetoxin exposure; and (6) how brevetoxin is metabolized, and even if differences in prey exist (or at least if differences trophic level of feeding exist). Additionally, live captures will enable biologists to affix radio-transmitters and time depth recorders to dolphins to learn more about dolphin movements, seasonal habitat use, feeding behavior, and other natural history characteristics.

To adequately determine the impacts of brevetoxin-associated mortality and UMEs on bottlenose dolphin populations on the coast of the Florida Panhandle, changes in the population size over time need to be identified. Spikes in mortality do not necessarily correspond to declines in the population, if the population is growing proportionally to the increases in mortality. Because a true census is not feasible, some sort of population index needs to be established and used to evaluate changes in the population over time. This index should be repeated annually if possible. As future UMEs occur, changes in annual population indices can be evaluated in light of mortality. This was not discussed at the Orlando workshop, but bears consideration.

Priority Research and Monitoring Needs: Impacts on Dolphin Populations

- Determine stock structure of bottlenose dolphins along the Florida panhandle
- Better understand biological and chemical toxin and pathogen exposures of bottlenose dolphins along the Florida panhandle
- Examine differences in health parameters (e.g. immunological measures) between Florida panhandle dolphins and other coastal dolphin populations that appear less vulnerable to mortality events
- Better understand the spatial and temporal movements of bottlenose dolphin stocks that repeatedly experience brevetoxin-associated mortality
- Develop toxin-specific biomarkers of exposure and effects for assessing sub-lethal and chronic exposure including protein biomarkers in serum and urine as well as the expression of brevetoxin antibodies in serum
- Utilize brevetoxin-specific biomarkers to investigate differences in brevetoxin exposure and susceptibility in different bottlenose dolphin populations
- Develop an indexing method to determine changes in dolphin populations that experience brevetoxin-associated mortality
- Utilize new information to develop a long-term risk assessment for dolphin populations experiencing brevetoxin-related mortality

Improving Response to Future Brevetoxin-related UME's

Immediate Needs

There are numerous action items that could improve response efforts during future brevetoxin-associated cetacean UMEs and resources need to be identified to make these happen. These action items include the assessment of existing resources, filling identified gaps, and establishing memorandums of understanding between NOAA and other potential partners such as the U.S. Coast Guard. Although these ideas were discussed at the Workshop, many were not fleshed out regarding responsibility, cost, or benefit, and currently funding needed to make these things happen has not been identified. Items discussed to be done as parts of a preparedness plan include:

1. Designate Marine Mammal Leads who will be responsible for overseeing response efforts in different regions. These people could correspond to pre-established stranding networks and response structure. Alternates should be established in the case that the primary Lead is

unavailable. These designated Leads should help to conduct the additional pre-outbreak steps designated below as they need to be thoroughly knowledgeable about normal stranding patterns and regional stranding response capacity.

2. Develop a list of names and contact numbers for people who are willing and potentially available to respond to a mass mortality event. This should be two-tiered, with the first level being the routine responders and the second level being additional volunteers and professionals who are willing to help. Although routine marine mammal stranding response capabilities need to be enhanced in certain regions as discussed previously, a mass mortality event would overwhelm these resources and require participation of increased numbers of people to assist in search and recovery efforts, conducting necropsies, sample recording and shipment, media relations, etc. Representatives from the National Institute of Standards and Technology (Charleston, SC) should be requested to attend all UMEs to collect samples for the National Marine Mammal Tissue Bank.

3. Develop a similar set of names and contact numbers for people who could make up a HAB-focused team. During documented HAB-related bottlenose dolphin mortality this group would be responsible for monitoring HAB movement using water samples, imaging, and dedicated ships.

4. Conduct a complete survey of marine mammal stranding resources in the Florida Panhandle region. This should include a map of areas covered by specific stranding networks, names and contact numbers of people who participate in those networks, and lists of facilities, freezers, four-wheelers, trucks, refrigerated trucks, boats, planes, and necropsy supplies maintained or available to each network. Gaps in resources should be filled. For example, the Big Bend area has been identified as an area with poor road access where most strandings go unreported. Additionally, it was reported that most first responder partners (Apalachicola National Estuarine Research Reserve, St. Joseph Peninsula State Park, St. Joseph Buffer Preserve State Park, Gulf World, or NMFS Panama City Laboratory, etc.) have limited equipment for stranding response. A list of potential incident command centers should be established. Refrigerators, freezers, and necropsy facilities should be upgraded at each facility (or be ready to be upgraded) for events. Telecommunication options should be evaluated for each potential incident command center. For beach areas where cell phone coverage is poor, plans should be made for communication via radio or satellite phones. Carcass disposal options also should be designated for each facility.

5. Provide search and collection and necropsy training for all stranding participants in the region, as well as for all people who are willing and potentially available to respond to a mass mortality event.

6. Establish standardized aerial survey techniques for enhancing identification and recovery of marine mammals stranded in remote locations. Aerial surveys could be conducted by NOAA contracted pilots, volunteer pilots, the U.S. Coast Guard, or others. This does not have to be done by a single entity and could be done regionally for each stranding network. This should be linked to research efforts previously discussed to determine non-event baseline levels of stranding.

7. Develop a standardized necropsy form and tissue handling guide. The form that exists should be modified to include diagrams of marine mammals as well as all of the tissue and other samples listed in Appendix II of this plan.

Note: It is probably a good idea to replicate steps 1-4 for Alabama, Louisiana and Mississippi where stranding response is weak. NOAA Fisheries may also want to consider making these pre-outbreak preparations in Alabama, Louisiana, Mississippi and Texas.

Event Response Plan

A well-developed response plan could help improve response as well as information learned during future brevetoxin-related bottlenose dolphin mortality events. Such a plan was developed for dealing with manatee mortalities (Geraci and Lounsbury, 1997) and has proved very successful. Described HAB monitoring, marine mammal stranding response, and routine testing of stranded cetaceans for exposure to brevetoxins should adequately alert the NMFS' Marine Mammal Health and Stranding Response Program of a brevetoxin-associated cetacean mortality event in the Florida Panhandle. Although a standard case definition for brevetoxicosis in bottlenose dolphins does not exist, evidence from the 2004 UME suggests that brevetoxicosis acutely kills animals of all age classes and both sexes leaving no pathopneumonic gross or histopathological lesions except levels of brevetoxin exceeding 200 ng/g in stomach contents and feces (NOAA, 2004). Further work must be done to help better understand background brevetoxin levels in bottlenose dolphins and brevetoxin pharmacokinetics, residence time, and pathogenesis. However, a working case definition for brevetoxicosis in bottlenose dolphins might be: any bottlenose dolphin that dies with postmortem evidence that the mortality was acute, no significant underlying pathology is present that could explain death in the animal, and stomach and or fecal brevetoxin levels exceed 200 ng/g. When the Marine Mammal Stranding Network determines there is an increase in strandings, the network participants and investigators should be alerted and sample analyses should be rushed to confirm or deny brevetoxin and domoic acid involvement.

Additionally, the following items need to happen:

1. All of the standard responders, as well as the list of potential additional responders, should be contacted by phone or electronic mail, warning them that reported regional strandings are increased or a dolphin has been diagnosed with brevetoxicosis and that they should be on alert for a potential UME. At this time, it also would be helpful to advise them of the location and status of a red tide, if one has been identified.

2. The Marine Mammal Lead or backup person for the area (both to be determined) needs to discuss the location of the diagnosed mortality with lead HAB people (to be determined) who can provide up to date information on HAB monitoring, including the defined area any identified blooms in the region and levels of brevetoxin in any biological organisms (shellfish or other) being monitored.

3. The Marine Mammal Lead should then alert all stranding response groups in the potentially affected and adjacent regions to mobilize their search and collection people and report findings back to the Marine Mammal Lead. Depending on the location of the bloom, wind direction, and

potential drift time, search and collection response might need to be repeated every day or every other day for multiple days.

4. If mortality is identified, routine but temporally expedited response, necropsy, and testing should be performed. All findings at every stage should be reported to the Marine Mammal Lead. Depending on number of carcasses located and gross necropsy findings (multiple age classes, both sexes, no evidence of chronic disease), the Marine Mammal Lead might want to consider having the UME working group appraised of the situation and determine what other information they need to declare the mortality event a UME.

5. The exact timing of the next step will vary for each unique situation, but at some point, the Marine Mammal Lead will need to determine if the situation is going to overwhelm standard stranding response capacity. If it is, then a command center should be established in reasonable proximity to the center of the mortality event and all response efforts should then be conducted from that site. People with needed skill sets should be called from the previously developed list of potential additional responders.

6. If it is possible, all stranded carcasses should be permanently marked in the field (roto-tags or other identification) and transported to the command center for processing. Refrigerated trucks would be ideal for transportation as they also could double as temporary carcass storage facilities pending necropsy. The lead pathologist present should triage the carcasses, conducting necropsies on carcasses in better condition first and refrigerating marginal carcasses for later. All efforts should be made to collect and archive the uniform set of samples described in Appendix II. Samples taken do not necessarily have to be tested, but samples not taken during necropsy can not be taken later.

7. A person responsible for all record keeping should be designated. This person should develop a system for recording all information for each animal, including: spatially explicit stranding location; permanent number assigned in the field and utilized in labs; carcass measurements; photographs; gross necropsy findings; which samples were taken; location of samples; and which samples were shipped. These datasheets should be created prior to a mass mortality event.
8. Ideally, a person trained in identifying stomach contents should be at the command center with the equipment needed to rapidly identify stomach contents. If it is possible to transport analytical machines from the NOAA/NOS laboratory or the FWC FWRI, they too should be moved to the command center to analyze samples on site. It was recommended that a mobile HAB analytical lab be created (or designed for creation in a rented vehicle when needed) so equipment and personnel could do on-site testing.

9. For monetary and personnel limitations, people often wonder when testing of the standard set of tissue samples, recommended in Appendix II, should be stopped. As discussed earlier, if it is possible all samples should be collected and archived so they can be tested at a later date if determined to be important. Important aspects of all epidemiological investigations are the temporal and spatial descriptions of the mortality event. For this reason, at a minimum routine samples should be tested from all carcasses that expand the known spatial and temporal aspect of the die-off. For example, if all carcasses are coming from St. Joseph Bay and one or two animals also show up in Pensacola Bay or Cedar Key, they definitely should receive complete testing as they might or might not be associated with the event in St. Joseph Bay. Additionally, if 10 carcasses a week are found for a month, it is important to periodically run a complete set of

test on a subset of animals in good condition each week. This will establish continuity of the outbreak by fulfilling case definition criteria and also provide important material for research concerning dolphin diet over the outbreak, levels of toxin relative to time of exposure, and a whole suite of other potentially important information.

10. During a documented HAB-related dolphin mortality event, the HAB-focused response team should be called to monitor HAB movement using water samples, imaging, and dedicated ships. This information should be reported daily to the Marine Mammal Lead, who then could use it to direct daily search and collection efforts. It is imperative that during an event the extent and duration of brevetoxin in the marine ecosystem, as well as the extent and duration of bloom, are determined. Sampling should systematically determine where brevetoxin is present in the water, as well as which species of fish and invertebrate contain toxin and where and for how long is the toxin present in these species. This information on toxin duration and trophic transfer will aid our understanding of why certain *K. brevis* blooms are associated with dolphin mortality while others are not. As an ancillary sampling technique, it was recommended that during a documented brevetoxin-bottlenose dolphin mortality event, fish should even be purchased at local fish markets and tested.

11. During a brevetoxin-associated bottlenose dolphin mortality event, marine mammal stranding responders and pathologists are not likely to have the time to collect and necropsy co-localized dead birds, sea turtles, and fishes. Currently, these animals are collected and sampled by the State of Florida. Ideally, dedicated parallel response groups for different taxa should work cooperatively during mortality events to ensure that all carcasses collected receive a similar suite of tests and data from all species mortalities are used to define the events. This could be done formally or informally and bears further consideration.

12. Media relations during the outbreak can help to generate volunteers to help, educate the public, and galvanize popular support for marine mammal stranding work and ocean health. A specified media relations person (or group) should be identified as soon as an increase in strandings in noted. The FWC/FWRI has history of dealing with HABs, aquatic animal mortality events and has designated point responders and media people. The Marine Mammal Lead could work closely with FWC/FWRI personnel and the media contact person. All new information should be filtered through these people so that the media knows who to contact and the message coming from the response is coordinated and uniform.

Moving Forward

As the database of mortality-free HAB events and HAB-associated marine mammal mortality grows, it should be periodically evaluated for spatial, temporal, and other trends that could suggest why some *K. brevis* blooms in the Gulf of Mexico result in bottlenose dolphin mortality, while others do not. The background brevetoxin tissue levels and stomach content levels, immunohistochemistry patterns, differences in disease and toxin exposure, differences in fish identified in gastric contents, as well as other necropsy and test results also should periodically be evaluated. These data (and data collected from live-caught and captive bottlenose dolphins) will provide insight into background brevetoxin levels, guidelines for diagnosing brevetoxicosis in bottlenose dolphins, and a better understanding as to why and when brevetoxicosis occurs in bottlenose dolphins. This will not happen without somebody to lead this effort. Ideally, a lead person should be contracted to periodically review all new research findings, compare marine mammal strandings and HAB data, and update research and response needs. An alternate strategy would be to assemble a small working group that could meet periodically to perform these tasks. Regardless of the option or combination of options chosen, funding will be necessary to either pay a lead person for their time or at a minimum, pay the travel and meeting expenses of a working group.

Better tools and approaches are needed for understanding the impacts of HABs. The recently published national research and response strategy (HARRNESS, 2005) updates the current state of the HAB problem and identifies the research needs and priorities necessary for better understanding and managing HABs. All of the needs required to better understand the impact of brevetoxins on bottlenose dolphins in the Gulf of Mexico fit with the nation's critical needs for HAB research and response. In that light, elucidating the priority research and monitoring need enumerated in this document will not only benefit marine mammals, they also will gather knowledge and data required by the nation to understand HABS and the impact they have on human health, wildlife health, and ecosystem health.

Acknowledgements:

This research and response plan would not have been possible with the help of many people. Support for this project came from a collaborative effort between the Marine Mammal Health and Stranding Response Program, Office of Protected Resources, NOAA/National Marine Fisheries Service and Disney's Animal Programs who helped sponsor the workshop held at Walt Disney World Resorts in Orlando, Florida between December 13 and 15, 2004. T. Rowles and A. Stamper were the people who made this happen. Many people took the time to attend this workshop and contribute their knowledge and ideas (Appendix I) and many workshop participants provided comments that improved the quality of this plan including N. Barros, G. Bossart, L. Engleby, R. Ewing, S. Fire, L. Flewelling, J. Landsberg, J. Naar, P. Rosel, T. Rowles, L. Schwacke A. Stamper, F. Van Dolah, R. Varela, and R. Wells. Other people also provided important information and help including L. Garrison, F. Gulland, J. Litz, and B. Mase. T. Spradlin did an incredible job coordinating the project. None of this would be possible with the help of the volunteers and staff who make up the Florida Marine Mammal Stranding Network and the scientists that study bottlenose dolphins, harmful algal blooms and all the other disciplines that come together in this plan.

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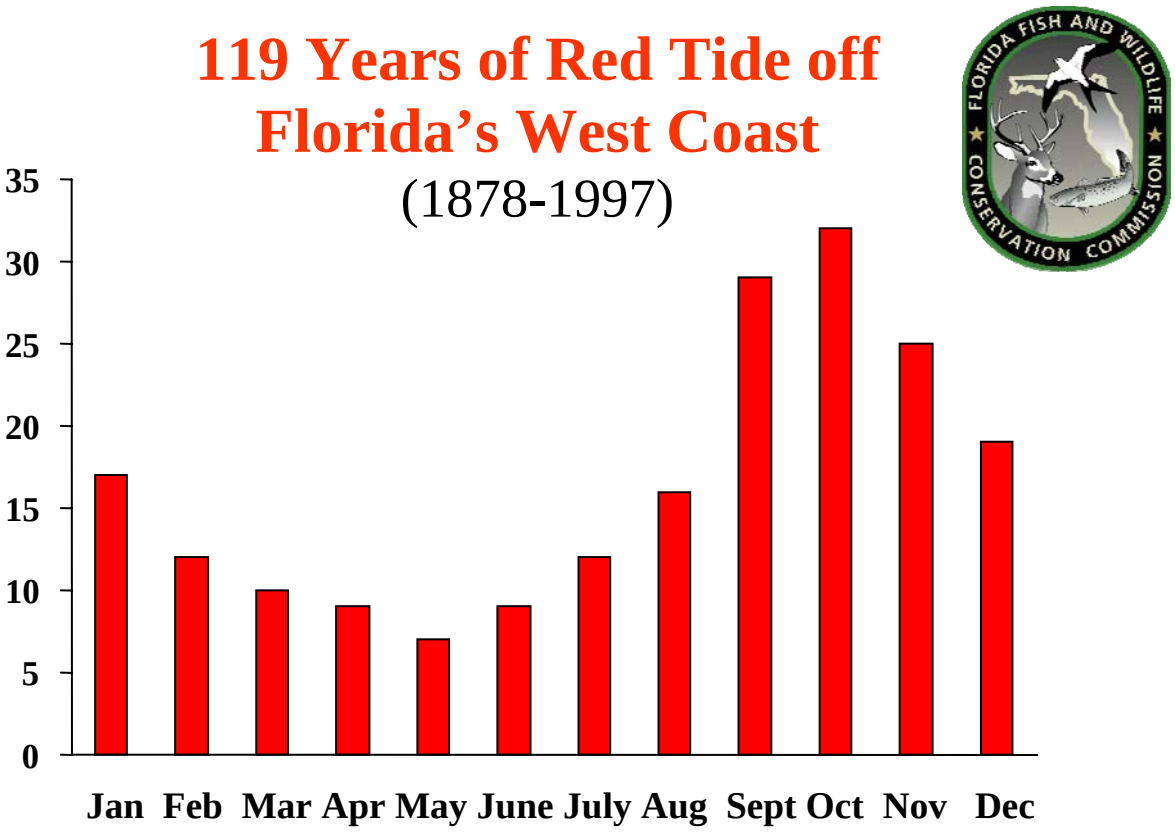
Figure 1: List of Reported Brevetoxin Related Marine Bird or Mammal Mortality Events

Started (month/year)		Ended (month/year)		Species affected	Location	Reference
November	1946	August	1947	Bottlenose dolphins, marine fish and invertebrates	Florida (Sarasota to Keys region)	Gunter et al., 1948
Unknown	1953	Unknown	1955	Bottlenose dolphins, sea turtles, and marine fish	Tarpon Springs to Key West	Rounsefell and Nelson, 1966 in Landsberg, 2002
Unknown	1963	Unknown	1963	Manatees (7), turtles, cormorants, fish, and possible raccoon	Unknown	Layne, 1965; Landsberg, 2002
October	1973	May	1974	Lesser Scaup (approx 2,000), double-crested cormorants, red-breasted mergansers	Florida	Forrester et al., 1977
Unknown	1982	Unknown	1982	Manatees (39)	Florida –Charlotte Harbor	O'Shea and Rathbun, 1982; O'Shea et al., 1991
June	1987	March	1988	Bottlenose dolphins (>740)	Atlantic Coast	Geraci, J. R., 1989; Steidinger, K. A. 1989 (morbillivirus epizootic; brevetoxin detected in stranded animals)
Unknown	1995	Unknown	1999	Double-crested cormorants (360)	Florida - Gulf Coast	Kreuder et al., 2002
March	1996	April	1996	Manatees (149)	Florida	Bossart et al., 1998; Landsberg and Steidinger 1998
Unknown	1996	Unknown	1996	Bottlenose dolphins	Mississippi	NOAA, 2004
August	1999	May	2000	Bottlenose dolphins (at least 152), other cetacean species, marine birds, fish, sea turtles and invertebrates	Florida Panhandle	Mase et al., 2000; NOAA, 2004; NOAA Southeast US Marine Mammal Stranding Database; FWC unpub. data
Unknown	2002	Unknown	2002	Manatees (34)	Florida	FWC unpub data; NOAA, 2004; Flewelling et al., 2005
Unknown	2003	Unknown	2003	Manatees (96)	Florida	FWC unpub data; NOAA, 2004; unpub. data
March	2004	April	2004	Bottlenose dolphins (107), marine birds, fish and invertebrates	Florida Panhandle	NOAA, 2004; Flewelling et al., 2005
July	2005	Unknown	2006	Manatees, bottlenose dolphins (132 as of April 06), birds, fish, sea turtles	Southwest Florida	NOAA and FWC, unpub. data
September	2005	Unknown	2006	Bottlenose dolphins (93 as of April 2006), marine birds, fish (sturgeon, gar, and anchovies)	Florida Panhandle	NOAA and FWC, unpub. data

Figure 2: Red Tide Occurrence off of Florida's West Coast

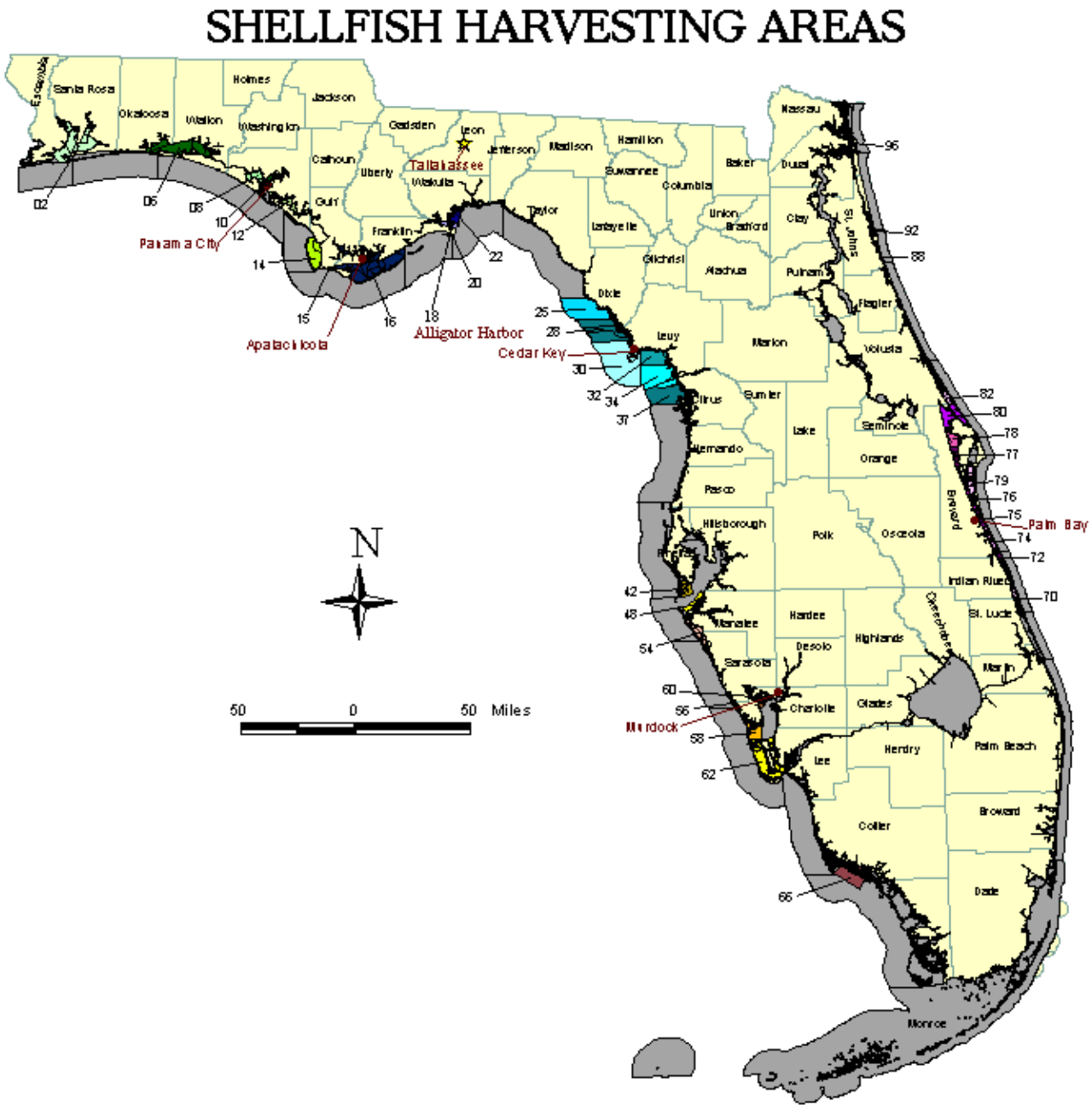


53% of Florida West Coast Red Tide Blooms occur September-December.
46% of the blooms are initiated in September, 21% in October

(Courtesy of Florida Fish and Wildlife Conservation Commission)

Figure 3: Shellfish Harvesting Areas in Florida

(numbered and colored to distinguish sites)



(Courtesy of http://www.floridaaquaculture.com/SEAS/SEAS_SHAmap.htm)

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Appendix I: Participant List for the Disney Workshop on Brevetoxins and Bottlenose Dolphins, December 13-15, 2004

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Appendix II: HAB-related Tissue Disposition List for Marine Mammal Samples

This is a sample wish-list. The following are suggestions for sample to be collected from all marine mammals (code 2 and 3); all mass mortality (>5 birds) of fish-eating seabirds and all sea turtles. If there are fresh dead fish proximal to mass mortality of sea turtles, birds or mammals – put the entire fish in a Ziploc bag and freeze; if the fish is too large, freeze the stomach contents

- Collect the following samples (ideally at least 50 gm each where applicable) in a whirl pack or Ziploc bag and store at -20C: submit alternately to NOAA/NOS laboratory (Charleston, SC) and FWC FWRI (St. Petersburg, FL) for PbTx analysis:
    - Feces, urine, vitreous humor, brain, lung, liver, spleen, kidney (freeze separately)
    - Note: the entire stomach (forestomach, glandular compartment, and pyloric stomach) should be submitted first for contents analysis; when present, gastric juices and fleshy remains of prey will be submitted o NOAA/National Ocean Service lab (Charleston, SC) and FWC FWRI (St. Petersburg, FL) for PbTx analysis
    - Collect postmortem heart blood for PbTx antibodies – spin off serum and freeze at -20C; send to a ½ ml aliquot to Jerome Naar (Center for Marine Science, Wilmington, NC - address in Appendix I)

- Collect and submit 50gm liver sample to Mote Marine Laboratory for fatty acid profiles or stable isotopes

- Collect the following tissue samples, fix in 10% neutral buffered formalin, and embed in paraffin within 6 days. Have pathologist read slides and submit a subset of the paraffin-embedded tissues for immunohistochemistry to Greg Bossart (Harbor Branch Oceanographic Institution).
  Important note: tissues fixed in 10% neutral buffered formalin should be processed (embedded into paraffin) within 10 days of complete formalin fixation. This is critical.
    - For Histopathology: Skin, umbilicus, mammary gland, blubber, tongue, thymus, thyroid, vitreous humor, brain (cerebrum, cerebellum, brainstem), spinal cord, trachea, lung, pulmonary lymph node, heart, liver, spleen, kidney, bladder, adrenal stomach (forestomach, glandular compartment, and pyloric stomach), small intestine, large intestine, mesenteric lymph node, inguinal lymph node, skeletal muscle, reproductive tissues, bone marrow, and multiple other lymph nodes.
    - For Immunohistochemistry, submit the following subset of tissues embedded in paraffin to Dr. Bossart: brain, lung, liver, kidney, spleen, multiple lymph nodes (at least 3 sites), forestomach, fundic stomach, small intestine and large intestine

- Take photographs (high resolution if digital) of the carcass being sure to get the entire body from above and both sides of the dorsal fin.
- Collect the following water samples at the mortality site; store as described: submit alternately to NOAA/NOS laboratory (Charleston, SC) and FWC FWRI (St. Petersburg, FL)

- Take 1 liter of water and freeze it for toxin analysis
- Take three 25 ml water samples (at stranding site and 100 meters to either side of site) and fix for algae identification (using kit provided)

o Not discussed, but should consider: It would be helpful to get a skin biopsy for genetics from all Code 2, 3, and 4 stranded bottlenose dolphins (probably should contact Renee Varela, Harbor Branch Oceanographic Institution).

Note: Additionally, every effort should be made to get a spatially explicit exact reference point (GPS location) for every animal collected for necropsy.

Appendix III: Sample TissueChecklist for Marine Mammal Necropsy

Tissue	10% buffered formalin	HAB samples (Whirl Pack - frozen)	Bacteriology, virology, etc. (Whirl Pack -frozen)	Other
Skin	□			Genetics – 20% DMSO saturated sodium chloride
Umbilicus	□			
Mammary gland	□		□	
Blubber	□	□		D□ L□ V□
Milk		□		
Tongue	□			
Thymus	□			
Thyroid	□			
Vitreous Humor		□ (red-top tube - freeze)		
Brain - cerebrum	□	□	□	□ (freeze in foil for contaminants)
Brain - cerebellum	□	□	□	□ (freeze in foil for contaminants)
Brain - Brainstem	□	□	□	□ (freeze in foil for contaminants)
Spinal cord (thoracic)	□	□		
Trachea	□			
Lung	□	□ □		
L.N. (pulmonary)	□	□		
Blood from heart		□ □ (1/2 ml serum)		□ Red-top tubes – freeze serum □ Blood card
Heart	□			
Liver	□	□ □	□	□ (freeze in foil for trace metals) □ (freeze in foil for fatty acids / stable isotopes)
Spleen	□	□	□	
Kidney	□ □	□ □	□ □	□ (freeze in foil for trace metals)
Bladder	□		□	
Urine	□	□		□ Red top tube - freeze

Tissue	10% buffered formalin	HAB samples (Whirl Pack - frozen)	Bacteriology, virology, etc. (Whirl Pack -frozen)	Other
Adrenals	□ □		□	
Stomach (forestomach, glandular compartment, and pyloric stomach)	□ □ □	□ Ziploc bag with contents		Note: send entire stomach for prey identification; will aliquot HAB samples from there
Small intestine	□		□	
Large intestine	□		□	
Fecal sample		□ □		□ Parasitology
L.N. (mesenteric)	□		□	
L.N. (inguinal)	□		□	
Uterus	□		□	
Testes	□		□	
Muscle (skeletal)	□		□	
Bone marrow	□		□	

Note: where two boxes are present (□□) take 2 samples; please submit desired samples to researchers listed above paying close attention to the following recommendations:

- Label each whirl pack, Teflon bag, vial or aluminum foil packet with the following:
    - Necropsy Number
    - Species
    - Tissue
    - Date of collection
- When taking samples for 10% Buffered Formalin remember to take thin (1cm) sections and be sure there are 10 parts formalin to 1 part tissue in the vial. Note: all tissues collected in 10% neutral buffered formalin must be embedded in paraffin within 6 days!
- Be sure to store all tissue samples as recommended.
- Please call ahead prior to shipping any samples.

Appendix IV: Samples to Take from Live Captured or Live Stranded Bottlenose Dolphins

Note: Contact Dr. Randy Wells for a complete list of samples as well as shipping and handling requirements. This is an abbreviated list

Blood:

- Serum from red top, thrombin or serum separator tubes; freeze in ½ ml aliquots at -20C:
  - Serum chemistry including iron
  - Test for antibodies to PbTx: send a ½ ml aliquot to Jerome Naar (Center for Marine Science, Wilmington, NC - address in Appendix I)
  - Test for antibodies to other select pathogens - including marine *Brucella* (National Veterinary Services Laboratory) Caliciviruses (A. Smith, Oregon State); Eastern Equine Encephalitis; *Erysipelas rhusiopathiae*; Herpesvirus; Influenza A and B; Leptospirosis serovars; Morbilliviruses (CDV, PDV, CMV; J. Saliki, Oklahoma State Diagnostic Laboratory); West Nile Virus; others
  - Hormones: cortisol, T3, T4, fT4, aldosterone, estradiol, progesterone
  - Mercury / Selenium
  - Immunology testing
  - PbTx ELISA – S. Fire's work

- EDTA-anticoagulated blood (purple top)
  - Complete blood count with differential
  - Trace elements
  - Mercury / Selenium

- Heparin-anticoagulated blood (green top):
  - Blood gasses (I-stat machine)
  - Organochlorines
  - Mitochondrial DNA for microsatellite markers

- Citrate-anticoagulated blood
  - Fibrinogen

- Whole blood:
  - Trace elements

- Blood collection card (provided by Tod Leighfield, NOAA/ National Ocean Service, Charleston, SC); collect whole blood, allow to dry then store at room temperature until submission. These are used to test for the presence of brevetoxins in blood (Fairey et al, 2001)

- A PAX gene tube
    - Fran Van Dolah, NOAA/NOS laboratory, Charleston, SC
    - Ryan's RNA work
    - Warr's RNA work

Biopsy Samples:
- Histology
- Stable Isotopes
- Stress proteins
- Trace elements
- Mercury and selenium and other contaminant concentrations
- Genetics

Dorsal Fin Plug:
- Genetics
- Stress proteins

Milk Sample:
- Contaminants
- Fatty acids
- Composition
- PbTx ELISA - Spencer Fire's work

Urine:
- Urinalysis
- Urine electrolytes
- Toxicology
- Reproductive status
- PbTx ELISA - Spencer Fire's work
- Leptospirosis
- Biotoxin analysis
- Contaminants

Tooth:
- Age

Blowhole swabs
- *Nasitrema* sp. cytology
- Microbiological culture

Exhalation sample:
- Microbiological culture
- Red Tide work
- Breath analysis

Fecal Sample:

- Toxicology
- PbTx ELISA - Spencer Fire's work
- Lungworm analysis
- Microbiological culture
- Diet and ageing study

Gastric sample:

- Cytology
- Red Tide Analysis -Discussed, but not deliberated: This should be frozen and submitted for PbTx analysis. This would serve as a baseline for levels of brevetoxin present in stomach contents of bottlenose dolphins that appear healthy. Fluid collected should be frozen at -20C until shipped to one of the labs equipped to test for PbTx (FFWCC and Charleston NOAA lab) and tested by Spencer Fire at Mote Marine Laboratory.
- If ingested material (otoliths or other bony structures are collected, they should be frozen and sent for identification)

Whole body photograph, dorsal fin photographs (both sides) and routine measurements

Discussed, but not deliberated: It would be beneficial to get a small liver biopsy from all live-captured dolphins. A liver biopsy could be divided between Fatty Acid analysis / stable isotope analysis, one of the two labs equipped to test for PbTx (FWC and Charleston NOAA/NOS laboratory) and analyzed for PbTx via ELISA by Spencer Fire. It was recommended that a group of medical professionals experienced in cetacean liver biopsy discuss the risk versus merit of this procedure.

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