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Brevetoxins in marine birds: Evidence of trophic transfer and the role of prey fish as toxin vector

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Brevetoxins in Marine Birds: Evidence of Trophic Transfer and the Role of Prey Fish as Toxin Vector

by

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BREVETOXINS IN MARINE BIRDS: EVIDENCE OF TROPHIC TRANSFER AND THE ROLE OF PREY FISH AS TOXIN VECTOR

Michelle van Deventer

ABSTRACT

Harmful algal blooms (HABs) of the brevetoxin-producing dinoflagellate *Karenia brevis* occur periodically along the central west coast of Florida. Mass mortalities of marine birds have long been associated with these blooms, yet there is little data documenting the accumulation of brevetoxins in the tissues of birds and their prey items. An intense HAB event impacted the region from Tampa Bay to Charlotte Harbor during most of 2005. More than one hundred marine birds, representing twenty three species, were collected during this bloom. All birds sampled were found dead or had died within 24 hours of admittance to local wildlife rehabilitation centers. In order to determine if fish were vectors for brevetoxin ingestion, the stomach contents of all birds were examined and any recovered fish were identified to the extent possible. The gastrointestinal tissues and contents from all avian samples were analyzed for brevetoxin levels, with results ranging from <LD to 9988.62 ng PbTx per gram tissue. Small planktonivorous fish such as thread herring, sardines and anchovies that largely comprise the diet of affected piscivorous birds were also collected and analyzed for brevetoxin content, with results ranging from <LD to 5839.90 ng PbTx per gram tissue. The highest levels of brevetoxins were generally detected in the viscera of fish, with relatively low levels detected in the muscle tissues. These results indicate that piscivorous marine birds, including double-crested cormorants, brown pelicans, terns and gulls, are exposed to a range of brevetoxin levels in their diet during *Karenia brevis* blooms. Ingestion appears to be the primary route of exposure, and brevetoxin-contaminated fish were confirmed in the stomachs of several birds.

Shorebirds and gulls may also be exposed to brevetoxins via scavenging of red tide-killed fish deposited on beaches during blooms. Samples from scavenged fish were found to have brevetoxin levels ranging from 31 to 95,753 ng PbTx per gram tissue.

INTRODUCTION

Background and Motivation

Harmful algal blooms (HABs, or "red tides") of the brevetoxin-producing dinoflagellate *Karenia brevis* (previously *Gymnodinium breve* and *Ptychodiscus brevis*) are frequent events in the Gulf of Mexico. There are numerous accounts of avian mortality events during past HABs along the central west coast of Florida (Quick and Henderson, 1974; Forrester, et al., 1977; Kreuder, et al., 2002). There is also evidence that catastrophic mortalities of sea birds associated with red tides in Florida have occurred as far back in the historical records as the late Pliocene (Emslie, et al., 1996). Today, rehabilitation centers along Florida's Gulf coast describe treating hundreds of birds exhibiting neurological signs during each month of a red tide. With more than one hundred species of seabirds reported for the Southwest Florida region (Owre, 1990 as referenced in Vidal-Hernandez and Nesbitt, 2002), a large variety of both migratory and resident birds can be affected when a bloom occurs along the West Florida Shelf (WFS).

The precise nature of how *K. brevis* blooms impact avian populations has not been thoroughly investigated. Brevetoxins may be a direct cause of death in sea and shorebirds via an acute or lethal exposure, or birds could be exposed to chronic, sublethal levels of toxin over the course of an extended bloom. Subacute doses may also contribute to morbidity and mortality due to an impaired ability to forage productively, disrupted migration behavior, reduced nesting success, or increased vulnerability to predation, dehydration, disease or injury (Shumway, et al., 2003).

Past research into phycotoxins and their cycling in the marine food web has revealed that seabirds and other top-level marine predators can be exposed to toxins that have bioaccumulated in prey items (Anderson and White, 1992; Tester, et al., 2000; Flewelling, et al., 2005). The brevetoxins produced by *K. brevis* have been positively identified through tissue analysis as the causative agent in double-crested cormorant (*Phalacrocorax auritus*) mortalities along Florida's Gulf coast (Krueder, et al., 2002),

and Vargo et al. (2006) detected low levels of brevetoxins in avian tissues collected during beached bird surveys in Pinellas County during a non-bloom period. However, the routes of exposure and specific sources of brevetoxins in avian diets are still unconfirmed and remain poorly understood.

The aim of this study was to record the range of brevetoxins present in the gastrointestinal tissues of piscivorous marine birds during a red tide, and to determine whether phytophagous fish can act as vectors of brevetoxins to sea and shore birds during blooms of *K. brevis*. Piscivorous birds represent apex predators in the marine food web, and understanding sources of brevetoxins in their diet, and the range of brevetoxin levels they might be exposed to, offers insight into potential risks to them and other predatory marine organisms. During this study, samples of common prey fish, including but not limited to thread herring (*Opisthonema oglinum*), Spanish sardines (*Sardinella aurita*, commonly referred to as "whitebait") and anchovies (*Anchoa* spp., commonly referred to as "glass minnows"), were sampled from various sources during a *K. brevis* bloom along the central west coast of Florida. Additionally, the stomach contents of marine birds recovered from this region during a bloom were examined to determine recent foraging activity and identify prey items as possible. It is conceivable that avian predators may avoid contaminated prey items during HABs, and so confirmation of brevetoxin levels in gut contents is an important aspect in determining actual exposure via food items. All gastrointestinal tissues, including liver, stomach and/or stomach contents, intestines and/or intestinal contents, and gallbladder, of collected birds were analyzed for brevetoxin levels using a competitive ELISA (Naar, et al., 2002). Finally, the presence of parent molecules and metabolites were confirmed in representative avian samples with LC-MS analysis.

Harmful Algal Blooms on the West Florida Shelf

Localized *K. brevis* blooms (>5 x 10^3 cell/l) of relatively short duration occur almost annually along the WFS (Steidinger and Ingle, 1972; Vargo, 1999; Tester, et al., 2000). More extensive, longer-term blooms are experienced an average of every three to five years (Steidinger and Ingle, 1972). These larger blooms generally last for two to four months, but can range in duration from a few weeks to as long as 18 months.

Blooms typically initiate offshore during the late summer or early fall, with August to November being the most common period of onset (Steidinger and Haddad, 1981; Vargo, et al., 1987).

HABs precede the urban development of Florida's Gulf coast, with documented reports dating back to the mid-16th century (Steidinger and Ingle, 1972; Turgeon et al., 1998; Vargo, 1999; Kirkpatrick, et al., 2004). While not restricted to the WFS, the region from Tampa Bay to Charlotte Harbor is where blooms of *K. brevis* occur most frequently (Steidinger et al., 1998). However, blooms have also occurred in the northern Gulf of Mexico along the coasts of Alabama, Mississippi and Louisiana. There have been occasional blooms along the North Carolina coast as well, as cells are carried by the Loop Current out of the Gulf of Mexico and along the east coast by the Gulf Stream (Anderson and White, 1989; Turgeon et al., 1998). In addition, fish kills along the Delaware coast from July through September 2000 were attributed to a bloom of the brevetoxin-producing organism *Chattonella cf. verruculosa*, the first confirmed report of brevetoxin-related event in what was previously thought to be an unaffected area (Bourdelais et al., 2002).

Karenia brevis-associated red tides along the WFS are consistent with blooms of other toxin-producing marine phytoplankton in that they often result in massive fish kills and have been implicated in the mortalities of marine wildlife such as dolphins (Anderson and White, 1989; Flewelling, et al., 2005), manatees (Bossart et al., 1998) and birds (Kreuder, et al., 2002; Shumway, et al., 2003). Research has shown that cell counts of $1 – 2.5x10^5$ cells/L can cause fish kills, and brevetoxicosis can occur at $>5x10^3$ cells/L (Vargo, 1999). The general area involved in a red tide can be as large as 30,000 km^2 (Steidinger and Ingle, 1972; Turgeon et al., 1998; Vargo, 1999), but *K. brevis* blooms are considered to be extremely "patchy" in nature (Vargo et al., 1987). HABs on Florida's central west coast typically initiate 10 – 40 miles offshore (Steidinger and Haddad, 1981; Anderson and White, 1989) and records indicate that fish kills can persist nearly twice as long at offshore areas as compared to inshore areas (Smith, 1975).

Brevetoxins and Metabolites

The brevetoxins produced by *K. brevis* are potent lipophilic polyether neurotoxins which bind to voltage-gated sodium channels of nerve and muscle cells, causing the cells to become hyperexcitable (Nakanishi, 1985; Colman and Ramsdell, 2003; Baden, et al., 2005). Investigations over the past thirty years have identified two parent toxins, PbTx-1 and PbTx-2, and at least thirteen derivatives which are produced by alterations to the reactive side chain of the parent toxins (Baden, et al., 2005). *Karenia brevis* cells produce PbTx-1, PbTx-2 and PbTx-3 in ratios that vary from the growth to dissipation phase, but PbTx-2 is the dominant toxin produced at all stages of a bloom (Poli, et al., 2000). PbTx-1 is the most potent brevetoxin and PbTx-3, a reduced form of PbTx-2, is up to ten times more toxic than PbTx-2 when ingested (Baden and Mende, 1982; Landsberg, 2002; Radwan, et al., 2005). Brevetoxin derivatives are produced during metabolism and decomposition, and the toxicity of brevetoxin-containing food and aerosol is believed to depend on the ratios of parent toxins and metabolites present (Baden, et al., 2005; Poli, et al., 2000; Radwan, et al., 2005). The profiles of parent toxins and metabolites in an organism may change over time, and it is also likely that the relative quantities can vary by species (Poli, et al., 2000).

Investigations into the toxicity of brevetoxins have generally involved administration to test subjects via inhalation, intravenous, intraperitoneal or oral routes (Baden and Mende, 1982). Oral exposure is associated with a greater delay in the onset of symptoms versus intravenous or intraperitoneal exposure (5 hours vs. immediate and 30 minutes, respectively; Baden and Mende, 1982). Oral administration of brevetoxins is also associated with a higher LD_{50} in mice than intravenous or intraperitoneal administration (0.520 mg/kg vs. 0.094 mg/kg and 0.170 mg/kg, respectively; Baden and Mende, 1982; Fleming and Baden, 1998; ILO, 1984 as referenced in Kirkpatrick, et al., 2004). Toxicokinetic investigations show that tissue uptake, distribution and elimination of brevetoxins varies by exposure route as well (Poli, et al., 1990; Cattet and Geraci, 1993; Benson, et al., 2005).

In humans, brevetoxin ingestion is responsible for a syndrome referred to as Neurotoxic Shellfish Poisoning, or NSP (Turgeon, et al., 1998; Colman and Ramsdell, 2003; Kirkpatrick, et al., 2004). NSP is characterized by both gastrointestinal and

neurological symptoms, including nausea, abdominal pain, parasthesia and convulsions (Baden and Mende, 1982; Kirkpatrick, et al., 2004). In sea and shore birds, symptoms of brevetoxin exposure often vary among species, but generally include severe ataxia, head tremors, a lack of truncal coordination, lethargy, dehydration and diarrhea (Forrester, et al., 1977; Kreuder, et al., 2002).

From September to December 2001, a period of extremely high *K. brevis* cell concentrations from Tampa Bay to Charlotte Harbor, the Pelican Man's Bird Sanctuary in Sarasota, Florida treated over 350 birds (including white pelicans, brown pelicans, double-crested cormorants, red-breasted mergansers, gulls, terns, skimmers, boobies, plovers, sandpipers, herons, loons and ruddy turnstones) exhibiting symptoms of brevetoxicosis (Table 1). This information is consistent with that from other regions where HABs coincide with increased morbidity and mortality in local bird populations (Hockey and Cooper, 1980; Nisbet, 1983; Work, et al., 1993; Sierra-Beltran, et al., 1997; Kreuder, et al., 2002; Shumway, et al., 2003).

Table 1. Mortality rates for sea and shore birds admitted to the Pelican Man's Bird Sanctuary with neurological symptoms during an intense red tide in the fall of 2001. Numbers represent summary data from patient log book maintained by PMBS staff.

	September	**October**	**November**	**December**
Total No. of Birds	30	60	107	170
No. of Birds Released	9	31	47	68
No. of Birds Expired	20	29	59	102
Mortality Rate	*67%*	*48%*	*55%*	*60%*

Marine Phycotoxins in the Food Web

Phytoplankton are the foundation for the ocean's food web. *Karenia brevis* is one of relatively few species of phytoplankton, mostly dinoflagellates, which produce potent toxins (Turgeon, et al., 1998). It is now understood that marine algal toxins, like other pollutants, are capable of biomagnification in the food web with a devastating affect on top-level predators (Nisbet, 1983; Anderson and White, 1989; Anderson and White,

1992; Tester, et al., 2000). There is a long history of HABs on the central west coast of Florida, yet our understanding of the vectorial transport of brevetoxins in marine ecosystems is relatively limited. Recent advances in analytical techniques now allow detection of toxins in much smaller ranges, providing the opportunity for more thorough investigations. This type of analysis is especially valuable in regions such as the WFS, where algal blooms occur with some frequency, may persist for extended periods, and the possible exposure pathways for organisms are complex (Tester, et al., 2000).

Small, schooling fish play a critical role as food source to predatory fish, mammals and seabirds. They can also provide researchers with a direct link between primary producers and top predators. The liver, kidney and other organs of finfish, such as herring, sardine, anchovy, sandlance and mackerel species, can bioaccumulate certain marine toxins and present a risk to organisms that feed on whole fish (Nisbet, 1983; Anderson, 1994; Turgeon et al., 1998; Lefebvre et al., 1999; Tester, et al., 2000; Lefebvre et al., 2001; Lefebvre et al., 2002b). For example, the northern anchovy (*Engraulis mordax*) has been identified as the primary vector of the Pacific Coast algal toxin, domoic acid, for exposure of pelicans, cormorants, sea lions and other marine animals along the California coast (Lefebvre, et al., 1999; Scholin, et al., 2000; Lefebvre, et al., 2001; Lefebvre, et al., 2002; Lefebvre, et al., 2002b). In the Atlantic Ocean, Atlantic mackerel (*Scomber scombrus*) and sand lance (*Ammodytes* spp.) have been shown to retain and transfer lethal levels of saxitoxins to predatorial organisms (Nisbet, 1983; Castonguay, et al. 1997).

The Gulf of Mexico is critical habitat for over 75% of migratory sea and shore birds (Gore 1992) as well as abundant resident species of birds, yet the primary vectors for HAB-related morbidities and mortalities have not been identified. It has long been believed that bait fish are killed from the lethal effects of brevetoxin exposure prior to bioaccumulation at levels that would be dangerous for piscivorous animals (Baden and Mende, 1982; Flewelling et al., 2005). Recent studies indicate this may not always be the case, underscoring the complex nature of brevetoxins and their role in the region's ecosystem. HABs can be divided into four phases: initiation, growth, maintenance and dissipation (Steidinger and Haddad, 1981; Steidinger and Vargo, 1988; Vargo, 1999). Changes in the frequency, duration, or intensity of one or more phases during a bloom

could influence bioaccumulation of brevetoxins in prey items, and therefore the exposure dynamics for organisms who feed on them.

Biomagnification of brevetoxins in fish exposed to sublethal levels of *K. brevis* is an important area of investigation. Studies in the past that focused on single, large doses of toxin known to impair or kill organisms are unlikely to illustrate the actual impacts of brevetoxin cycling in the environment, and their potential to contribute to illness and death in avian populations (Kimball and Levin, 1985; Anderson and White, 1989). Recent studies have provided information regarding brevetoxin accumulation in fish exposed to low levels of *K. brevis*, or exposure via prey items. Woofter et al. (2005) monitored the blood of striped mullet (*Mugil cephalus*) exposed to sublethal concentrations of *K. brevis* in order to determine uptake and elimination rates of brevetoxins for this species. In this study, brevetoxin was detected fairly rapidly in the blood of exposed mullet, with levels peaking 8 – 12 hours after initializing exposure and leveling off at approximately 24 hours. Once the mullet were removed from the brevetoxin-containing water and placed in control seawater, brevetoxins continued to be detected in the blood for several days. Tester et al. (2000) also verified bioaccumulation of brevetoxins in copepods and subsequent trophic transfer to fish. In this study, fish were not exposed directly to *K. brevis* in the water, but accumulated brevetoxins by feeding upon copepods that had grazed on low concentrations of the dinoflagellate.

There is evidence that various species and stages of fish have different tolerances to *K. brevis* bloom conditions, and these tolerance differences may determine whether they are killed by marine algal toxins or bioaccumulate them (Gunter, et al., 1947; Smith, 1975; Steidinger and Haddad, 1981; Tester, et al., 2000). Smaller, benthic species appear to be more susceptible to brevetoxins as they are generally killed earlier and in larger numbers than other faster-swimming, pelagic species (Steidinger and Ingle, 1972; Quick and Henderson, 1974; Smith, 1975). Similarly, pelagic fish were found to have higher brevetoxin levels than benthic fish in comparative investigations (Landsberg et al., 2000). There also may be variations in depuration rates depending on the lipid composition or metabolic processes of different fish species (Tester et al., 2000; Colman and Ramsdell, 2003). Lefebvre et al. (2002) noted in their study that northern anchovies accumulated

domoic acid toxins to a much more potent level than sardines collected simultaneously under identical exposure conditions.

Evidence that fish are vectoring brevetoxins to predators in the Gulf of Mexico is increasing. During a stranding event of bottlenose dolphins (*Tursiops truncatus*) on the eastern Atlantic coast in 1987, the stomach contents of one dolphin, which contained menhaden (*Brevoortia tyrannus*), tested positive for brevetoxins (Anderson and White, 1992). Similarly, in March and April of 2004, more than 100 bottlenose dolphins died along the panhandle region of Florida. Most of the dolphin's stomachs contained fish with high levels of brevetoxins (Flewelling et al., 2005). Additional fish collected from the Gulf of Mexico during the 2004 dolphin mortality event also tested positive for elevated levels of brevetoxins, causing investigators to suspect that brevetoxin-contaminated prey was an important factor in the stranding of these mammals (Flewelling et al., 2005).

Investigations of brevetoxin accumulation in fish historically focused on accumulation patterns in muscle tissue in order to determine risk to human consumers. Sea and shorebirds, like other marine predators which consume whole fish, may be exposed to higher brevetoxin levels in the viscera of fish, as well as cumulatively harmful amounts as they forage over time (Anderson and White, 1992). Unfortunately, sublethal exposures that could lead to disease or death via secondary effects are difficult to ascertain as these types of ecosystem dynamics are complex and difficult to measure (Quick and Henderson, 1974; Anderson and White, 1992; Tester, et al., 2000). Avian populations, like other wildlife, are vulnerable to numerous stressors and variables such as disease, pollution, parasitism, competition, habitat loss and predation. It is difficult to determine the impact of an environmental perturbation such as red tide without considering such an event in context of these other parameters.

Evidence of HAB Impacts on Marine Birds

Phycotoxins have been implicated in morbidity and mortality events for numerous species of birds in various parts of the world (Gunter et al., 1947; Coulson et al., 1968; Forrester et al., 1977; Armstrong et al., 1978; Nisbet, 1983; Williams et al., 1992; Work et al., 1993; Sierra Beltrán et al., 1997; Krueder et al., 2002; Shumway, et al., 2003).

Records from the Pelican Man's Bird Sanctuary in Sarasota, Florida show that in the fall of 2001, during an intense red tide in the area, there was an observable increase in the number of avian patients admitted exhibiting neurological signs and symptoms following the September initiation of the bloom. The largest number of bird species admitted occurred in the month of December, nearly three months after the start of the bloom (Table 1). Similarly, a time delay of two to ten weeks was observed by Krueder et al. (2002) between outbreaks of cormorants and *K. brevis* blooms, with the greatest positive correlation when bloom levels lagged behind cormorant admittances by eight weeks. Quick and Henderson (1974) reported cormorants, mergansers and lesser scaup dying in red tide-affected areas beginning in late-February 1974, several months after an October 1973 start of a *K. brevis* bloom on the WFS.

Why marine birds die during red tide blooms in southwest Florida has historically been the subject of debate. There are potentially several ways in which birds can be affected: inhalation of airborne brevetoxins, drinking or secondarily ingesting *K. brevis*-containing seawater, ingestion of brevetoxins via contaminated prey items, and/or starvation due to reduced food availability.

Inhalation of aerosolized brevetoxins are known to cause respiratory irritation in humans (Pierce, 1986; Kirkpatrick, et al., 2004), lesions of the upper respiratory tract in manatees (Bossart, et al., 1998), and reduced body weight and impaired immune function in rats (Benson, et al., 2005). A great deal of marine bird behavior occurs at the interface of air and water where concentrations of aerosolized brevetoxins occur. It is probable that sea and shore birds are inhaling aerosolized brevetoxins during red tide events, and so it cannot be assumed that this type of exposure is without consequence. However, tissue analysis of cormorants exhibiting signs of brevetoxicosis and collected during bloom conditions showed only very mild pulmonary congestion and no tracheal lesions or significant lung pathology (Krueder et al., 2002). Similarly, evidence of pulmonary disease was not noted during necropsies of lesser scaup (*Aythya affinis*) found dead or dying during a 1974 red tide in the region (Quick and Henderson, 1974). Benson, et al. (2005) did not observe any neurotoxic effects in rats exposed to brevetoxin-3 via inhalation for an extended period, and while some physiological effects were observed,

no deaths occurred in study subjects. To date, there is no evidence that inhalation of brevetoxins is a primary cause of illness or death of marine birds during red tides.

Seabirds possess special adaptations for salt excretion that allows them to drink seawater for hydration, and it is possible that they ingest *K. brevis* cells in this manner during blooms. However, most reports on this behavior indicate that seabirds are able to manage without relying upon drinking for hydration as they are able to obtain water directly from their diet, as well as use "metabolic water" in fat reserves (Elphick, et al., 2001). Also, seabirds are efficient feeders. Cormorants and other species will shake and manipulate their prey for easy swallowing, minimizing ingestion of water during foraging (Gunter, 1958; Elphick, et al., 2001). Observations of pelican feeding in the Gulf confirm that this species allows water to drain from the bill prior to swallowing, thereby minimizing incidental intake of seawater (Gunter, 1958). In the event that some ingestion of toxic water is occurring, Forrester, et al. (1977) exposed domesticated White Pekin (*Anas domesticus*) ducklings to brevetoxin-containing seawater and toxic clams in an effort to determine effects. A greater number of ducklings died, and in less time, in the group that received toxic clams and toxic seawater, versus the group that received normal clams and toxic seawater.

Close relationships between prey availability and the health of marine bird populations have been confirmed (Anderson, et al., 1982; Schreiber and Clapp, 1987). Red tide blooms on the west coast of Florida are associated with short-term, reduced populations of reef and schooling fish (Gunter, et al., 1947; Steidinger and Ingle, 1971; Smith, 1975). However, necropsies of birds sampled from red-tide associated mortalities have found individuals with adequate subcutaneous fat reserves and normal breast muscle mass which indicates an acute cause of death, and does not support the theory that all red tide-related avian mortalities are a result of starvation (Forrester, et al., 1977; Quick and Henderson, 1974).

The observed delay between initiation of a bloom and outbreaks in birds has been postulated as the result of the time required for bioaccumulation in schooling fish and eventual transfer to predatory marine birds (Krueder, et al., 2002). In an important discovery that sheds light on possible timing and vectorial transport of phycotoxins, Flewelling et al. (2005) found that extracellular brevetoxins in seawater, which occur

following cell lysis, can accumulate in seagrass and fish and pose a serious threat to marine animals well beyond the onset of a red tide.

While it may not be an obvious route of transfer, prey fish could also pose a risk to shorebirds such as sanderlings (*Calidris alba*), ruddy turnstones (*Arenaria interpres*), plovers (*Charadrius* spp.), gulls and other species that forage on beaches. It is commonly believed that the primary risk to shorebirds during a red tide is via contamination of shellfish and other invertebrates that comprise their traditional diet. Coquinas (*Donax variabilis*) and other items that shorebirds feed upon can accumulate marine toxins during red tide blooms and may pose a risk to foraging shorebirds (Cummins et al., 1971; Bretz, et al., 2000). However, this may not be the only route of exposure to brevetoxins for beach dwelling birds. Shorebirds and gulls (*Larus* spp.) have been observed actively consuming dead fish when present onshore (Rand, 1957; Gochfeld and Burger, 1980). The risk to scavenging shorebirds and gulls when dead fish are deposited on beaches during a red tide event is not well understood, nor is the distribution of toxins in beached fish.

Overview of the 2005 *Karenia brevis* Bloom

A widespread and prolonged bloom of *K. brevis* impacted the central west coast of Florida for most of 2005. This event began offshore in early January 2005 and was affecting coastal areas from Tampa Bay to Charlotte Harbor within several weeks (Figure 1). The bloom began dissipating in late fall and cell counts in water samples from the region returned to normal in December 2005. This bloom was notable not only for its intensity and duration, but also because bloom conditions combined with warm Gulf waters in late summer, resulting in a large area of hypoxia/anoxia and benthic community die-offs.

The southwest Florida region is home to over 100 species of marine birds, with brown pelicans (*Pelicanus occidentalus*), double-crested cormorants (*P. auritus*), terns (*Sterna* spp.) and gulls (*Larus* spp.) being the most abundant (Table 2). Species which consume marine fish as a primary prey item, or in combination with other prey items, include pelicans, herons, egrets, gulls, terns, loons, gannets and cormorants. Most piscivorous marine birds rely on schooling bait fish for food. Vidal-Hernandez and

Nesbitt (2002) estimate that daily consumption rates for these species range from 135 to 216 g/day, but this range may underestimate actual daily rations for larger seabirds (Bayer, 1989).

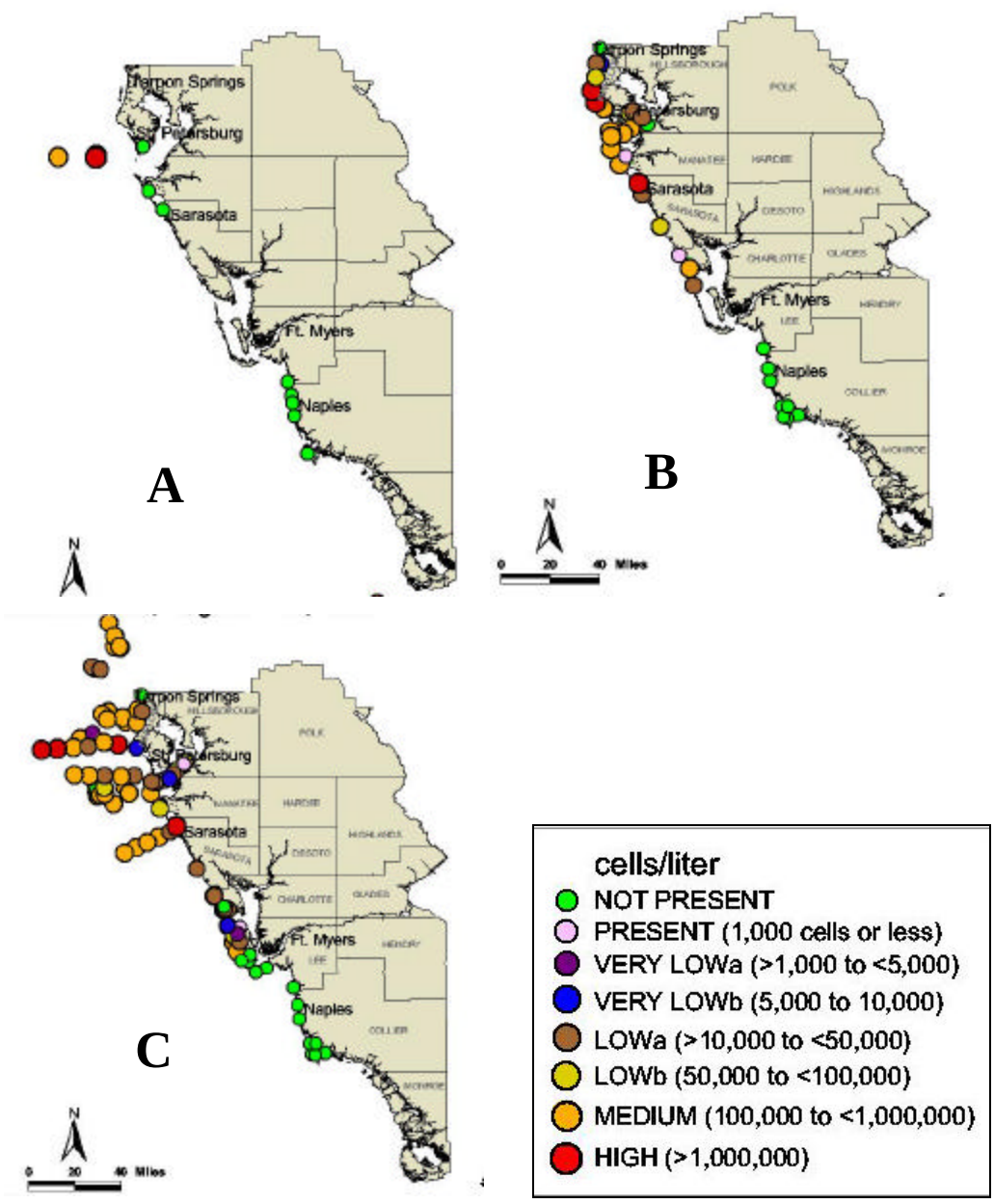
Table 2. Numbers and occurrence of sea birds in Florida whose diet is comprised mainly of small bait fish (reproduced from Vidal-Hernandez and Nesbitt, 2002 with data from Dunning, 1993).

Species	Occurrence	Population Estimate
Brown Pelican	Year round	17,000
White Pelican	September – April	4,000
Double-crested Cormorant	Year round	20,000
Northern Gannet	November – May	15,000
Common Loon	October – April	4,000
Terns and Gulls	Year round	20,000
Red-breasted Mergansers, Ducks and Grebes	October – April	10,000

The very nature of marine birds, predators foraging for prey offshore and then returning to the coast, makes them an excellent sentinel species for monitoring ecosystem health, especially when considering the patchy nature of algal blooms in the eastern Gulf of Mexico and the fact that most blooms initiate offshore. Seabirds are perhaps the most conspicuous of marine predators, and have often been used as effective monitors of the marine environment (Montevecchi, 1993, 2001). Large numbers of dead or dying seabirds can create an awareness of offshore marine events, and provide important clues of ecosystem disturbances. Birds tend to be very sensitive to marine pollutants and toxins, and have frequently provided the initial evidence for pollution or toxin presence in local waters (Shumway, et al., 2003). Mass mortalities of other marine predators, such as whales, dolphins, sharks and large predatory fish, could represent only a fraction of total mortality because most carcasses are likely to drift out to sea or eventually succumb to sinking and scavenging. In contrast, marine birds are capable of flying considerable distances from feeding sites where they may have been exposed to toxins in order to

return to nesting or roosting grounds where morbidities and mortalities are observed (Kreuder, et al., 2002).

Figure 1. Maps of the southwest region of Florida (compiled by the Florida Fish and Wildlife Conservation Commission Fish and Wildlife Research Institute) illustrate the development and progression of the 2005 *Karenia brevis* bloom. Map A indicates the first water samples offshore containing elevated cell counts during the first week of January. Map B shows the presence of the bloom alongshore from Tampa Bay to Charlotte Harbor in late June. Map C, dated mid-August, details the extent of the bloom.



Study Objectives

The objective of this study was to identify and quantify the levels of brevetoxins in the gastrointestinal contents and digestive tissues of sea and shore birds during a bloom of *K. brevis*, and to confirm the potential role of prey fish in vectoring brevetoxins to piscivorous marine birds. Seabirds often represent apex predators in the marine food web, and their conspicuous nature makes them excellent indicators for the integrity of local fish stocks. Identifying sources of brevetoxins in their diet, and the range of brevetoxin levels they might be exposed to, improves our understanding of how periodic blooms impact avian populations in the region, and offers insight into potential risks for other predatory marine organisms.

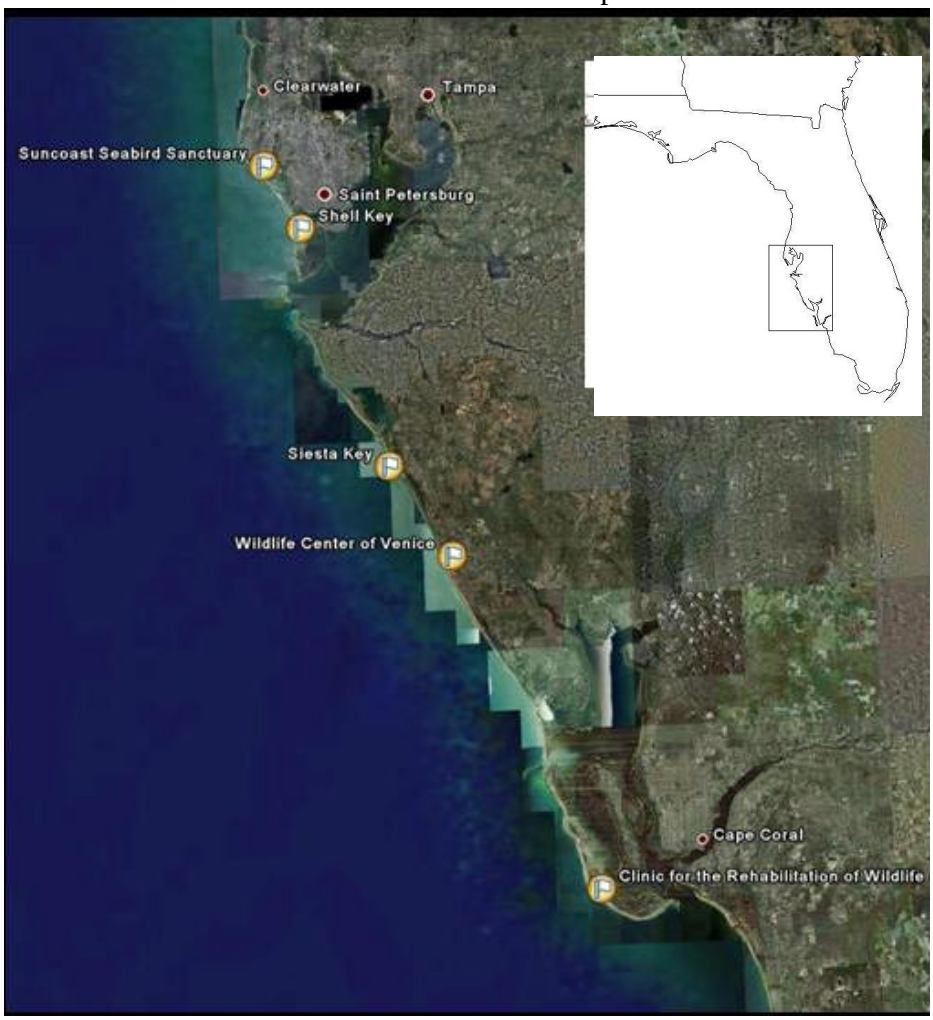
Florida's birds are an integral part of our wildlife communities, and there is evidence that today's populations are only a fraction in size of the colonies that once existed (Runde, 1991; Butcher and Niven, 2007). Verifying the trophic transport of brevetoxins to resident or migratory marine bird populations during *K. brevis* blooms is critical for conservation and management planning, particularly when species affected are known to be vulnerable to perturbations in their diet or whose populations are in decline.

METHODS

Study Region

Historically, the region from Tampa Bay to Charlotte Harbor (Figure 2) along the central WFS is where *K. brevis* blooms have occurred most frequently (Vargo, 1999; Steidinger et al., 1998). During the 2005 bloom, this region experienced massive fish kills as well as reports of marine bird and mammal mortalities. Samples used for this study were gathered from the coastal areas within this region.

Figure 2. Map of central west Florida region from Tampa Bay to Charlotte Harbor. Location of rehabilitation facilities and other avian sample collection sites are indicated.



Avian Sample Collection

Avian samples used for this study were obtained through a variety of sources. The Seabird Ecological Assessment Network (SEANET) provided beached birds that were opportunistically recovered during monthly beach surveys on Shell Key in Pinellas County. Least tern chicks were provided by the Eckerd College/St. Petersburg Audubon Society Least Tern Rooftop Nest Monitoring project, which monitors the rooftop nests of least terns in Pinellas County. All dead least tern chicks observed at nesting sights were collected by observers, labeled, and forwarded to the University of South Florida College of Marine Science (USF CMS) for analysis.

Three independent wildlife rehabilitation centers in the region (Figure 2) provided carcasses of sea and shore birds that were brought to their facilities but did not survive. The Clinic for the Rehabiliation of Wildlife (C.R.O.W.) is located in Sanibel, FL alongside Charlotte Harbor. The Wildlife Center of Venice (W.C.V.) is located in Venice, FL near Sarasota Bay. The Suncoast Seabird Sanctuary (S.S.S.) is located in Indian Shores, FL on Tampa Bay. Combined, these three centers provide wildlife recovery services for nearly 200 miles of coastline along the central west coast of Florida and are staffed with individuals experienced in identifying symptoms consistent with suspected brevetoxicosis in seabirds. Only birds which did not eat while in care at a rehabilitation center and expired within 24 hours of intake were used for this study. Records for each bird include location and date found, symptoms presented if alive at time of recovery, and final disposition (e.g. died or euthanized). All expired animals were stored frozen in a -20^{o}C freezer pending necropsy and tissue analysis.

Avian Necropsies

All avian necropsies were performed in St. Petersburg, FL at USF CMS or at the Florida Fish and Wildlife Conservation Commission Fish and Wildlife Research Institute (FWCC FWRI). Necropsy protocols established by SEANET at Tufts Center for Conservation Medicine and the "Avian Necropsy Manual for Biologists" (Work, 2000) were followed. Notes on carcass and body condition were kept for each bird, including feather condition, muscle mass, subcutaneous fat reserves, stomach contents, and parasite

load. At minimum, ventral and dorsal digital photos of each bird were taken. Approximate age was determined, and measurements of tarsus, wing chord, culmen, beak depth and beak width were recorded. The gastrointestinal tract was examined for parasites, abnormalities, and evidence of recent feeding. All significant or unusual findings were noted and photographed. All stomach contents were photographed, identified to the extent possible, with assistance from staff at FWCC FWRI, and analyzed individually for brevetoxins. All tissue samples and stomach contents collected during necropsy were either immediately extracted or placed in individual Whirlpak bags and frozen at -20^{o}C for future use.

Fish Sample Collection

Fish samples for this study were taken from several sources during the 2005 bloom of *K. brevis*. Locally caught thread herring (*O. oglinum*), Spanish sardines (*H. jaguana*) and bay anchovies ("glass minnows", *A. mitchilli*) were purchased monthly from a large commercial fishery in Cortez, FL which supplies many area facilities with locally caught bait fish. These fish were caught in waters between Sarasota Bay and Tampa Bay but can not be correlated to specific locations or bloom conditions due to lack of records for catch date and location. No fish were collected after the end of September due to a crash in fish stocks as a reported by local fisherman. The lack of available bait fish was attributed by suppliers to red tide conditions and the associated hypoxic/anoxic zone occurring in the region at the time. All fish were stored frozen in a -20^{o}C freezer pending dissection and tissue analysis.

Fish samples were also taken monthly from the food supply of participating rehabilitation facilities. Rehabbers generally rely on locally caught "finger" mullet (*Mugil* spp.), thread herring and Spanish sardines that have been purchased from regional baitfish companies or commercial suppliers. Once each month, fish were taken from the supply offered to patients that day so as to determine the brevetoxin levels present in the food provided injured and sick marine birds treated during a red tide bloom. Samples were stored frozen in a -20^{o}C freezer pending dissection and tissue analysis.

In order to determine the risk to birds foraging on local beaches during a bloom, red tide killed fish deposited onshore during active scavenging by gulls and shorebirds

were also collected. Information about date, location and bloom conditions were recorded for all beached fish collected. Finally, additional small prey fish were collected live from coastal areas, with an effort to collect during observed sea and shore bird feeding, or a lack of obvious foraging activity was noted. Date, location and bloom conditions were recorded for all small minnows collected alongshore, and samples were stored frozen in a -20^{o}C freezer pending dissection and tissue analysis.

Tissue Extractions

Sample extractions for avian and fish tissues were performed in St. Petersburg, FL at the USF CMS or the FWCC FWRI Biotoxins Laboratory. Samples of whole tissues, such as liver and gall bladder, were macerated (Waring Commercial Laboratory Blender) and weighed to ±0.1g. The stomachs of larger birds were opened and contents scraped, combined, weighed and transferred to 50mL Falcon tubes. Any whole fish present in stomach contents were separated and analyzed as described below. Small and large intestines were emptied to create a homogenous mixture and a sample removed and weighed for extraction. Minimum sample size requirements for analysis by ELISA required that stomachs and intestines of smaller birds (species weighing less than 40 - 50g) or partially decomposed samples were macerated whole. All samples were weighed and stored in individual 50 mL Falcon tubes for extraction.

All fish weighing less than 5g were macerated whole and a representative sample taken for extraction and analysis. For larger fish, all viscera was removed from the intraceolomic cavity and macerated for whole viscera analysis. The exception to this was gravid females, for which eggs were removed, macerated, and sampled separately from visceral organs. A sample of the fish muscle tissue was also removed with skin and scales, macerated and a sample weighed out for analysis.

All macerated and weighed tissue samples were extracted twice in 80% methanol at 60ºC and centrifuged twice (10 minutes each, 3,000 x g; Eppendorf Centrifuge 5810). Both methanol extracts were combined and partitioned with hexanes (1:1 v:v). Methanolic fractions were transferred to 8 ml Wheaton glass specimen vials with rubber lined caps and stored at -20ºC until further analysis.

Brevetoxin Analysis

An enzyme linked immunoabsorbant assay (ELISA) was used to test methanol extract samples for brevetoxin content (PbTx). All ELISA analysis was performed by staff at the FWCC FWRI Biotoxins Laboratory in St. Petersburg, FL. This is an FDA-certified laboratory for brevetoxin analysis and the official state laboratory for shellfish testing during HABs. The ELISA recognizes all congeners and metabolites of brevetoxin that have a PbTx-2 type backbone and the lower limit of quantification is 2 - 5 ng/g. Protocols established by Naar et al. (2002) were followed. To summarize, 96-well microplates were pre-coasted with brevetoxin, and any remaining binding sites in the wells were blocked. Samples and controls were then added to the wells and allowed to compete with the plate-bound toxin for anti-brevetoxin antibodies (from a goat). After one hour, the wells were rinsed out, removing the samples and all antibodies except those attached to the plate-bound toxin. The antibodies remaining in the plate were then visualized using a horseradish peroxidase (HRP) conjugated secondary antibody (rabbit anti-goat antibodies) and the HRP substrate TMB (3,3',5,5'-Tetramethylbenzidine). Absorbance of the wells was read at 450 nm.

Selected avian samples were also evaluated with LC-MS in order to confirm the presence of parent toxins (PbTx-1:867, PbTx-2:895, PbTx-3:897) and/or specific brevetoxin metabolites (PbTx-3-Cys:1018, PbTx-2-Ox-Cys:1034). A 0.5g equivalent of the methanol extracts were diluted with de-ionized water and passed through a Strata-X SPE (solid phase extraction) column (60 mg, 3 mL, Phenomenex) that had been preconditioned with 100% MeOH, and then 20% MeOH (9 ml each). Columns were rinsed with 4.5 mL 20% MeOH and the samples were eluted with 4 mL 100% MeOH. Samples were then evaporated to dryness and redissolved in 0.5 mL MeOH. All samples were stored at -20ºC until further analysis. All MS analysis was conducted at the Center for Marine Science, University of North Carolina, Wilmington by using a Waters Alliance 2695 LC (Waters, Milford, MA, USA) coupled to a Micromass ZQ mass spectrometer equipped with an electrospray ionization (ESI) probe (Micromass Inc., UK). All analyses were conducted using electrospray ionization with the probe at 3 kV and 250^{o}C. Samples were first separated on a YMC J'Sphere ODS-L80, S-4 2.0×150 mm column (YMC Inc., Wilmington, NC, USA). The solvent gradient was composed of

acidified (0.3 % acetic acid) ACN/H_2O with initial 50:50 ACN/H_2O to 95:5 ACN/H_2O over 40 min. Parent brevetoxins (PbTx-1:867, PbTx-2:895, PbTx-3:897, PbTx-6:911, PbTx-7:869, PbTx-9:899, PbTx-10:871) and brevetoxin metabolites (Cyst-PbTx-2:1018, Ox-Cyst-PbTx-2:1034) were monitored at indicated masses. The instrument was calibrated with a standard brevetoxin mix containing PbTx-2 and PbTx-3, obtained from the Center for Marine Science, UNC Wilmington, N.C..

Determination of Bloom Conditions

The timing and location of recovery for all avian samples were analyzed with respect to local bloom conditions. Numerical abundance of *K. brevis* cells from water samples collected in the region is available from the FWCC FWRI HAB Monitoring Program. Weekly summary reports are provided for bloom conditions for the southwest region of Florida as part of ongoing monitoring work, including near and offshore water sampling locations and results. *K. brevis* concentrations in water samples are determined by hand counting cells using a dissecting microscope. Measurements are reported as a range, and a midpoint of the range is used to estimate cell concentration. Red tide status reports are issued weekly. Cell concentrations are correlated to terms used to describe bloom conditions, or red tide intensity, according to Table 3.

Table 3. Terms used for describing *K. brevis* bloom conditions in weekly Red Tide Status reports issued by the FWRI HAB Monitoring Program.

***K. brevis* cells per liter seawater**	**Bloom Conditions**
No cells detected	Not Present
1,000 cells or less	Present
>1,000 to 10,000 cells	Very Low
>10,000 to <100,000	Low
100,000 to <1,000,000 cells	Medium
>1,000,000 cells	High

RESULTS

Overview of Avian Samples

One hundred and one birds, representing 23 species, were collected from Tampa Bay to Charlotte Harbor during the *K. brevis* bloom in 2005 (Table 4). Most carcasses were recovered during the summer months (Figure 3), but this was not necessarily a reflection of a red tide-related trend as nesting seasons and other factors may have influenced this pattern.

Figure 3. The number of birds collected each month for necropsy and brevetoxin analysis during the 2005 *K. brevis* bloom.

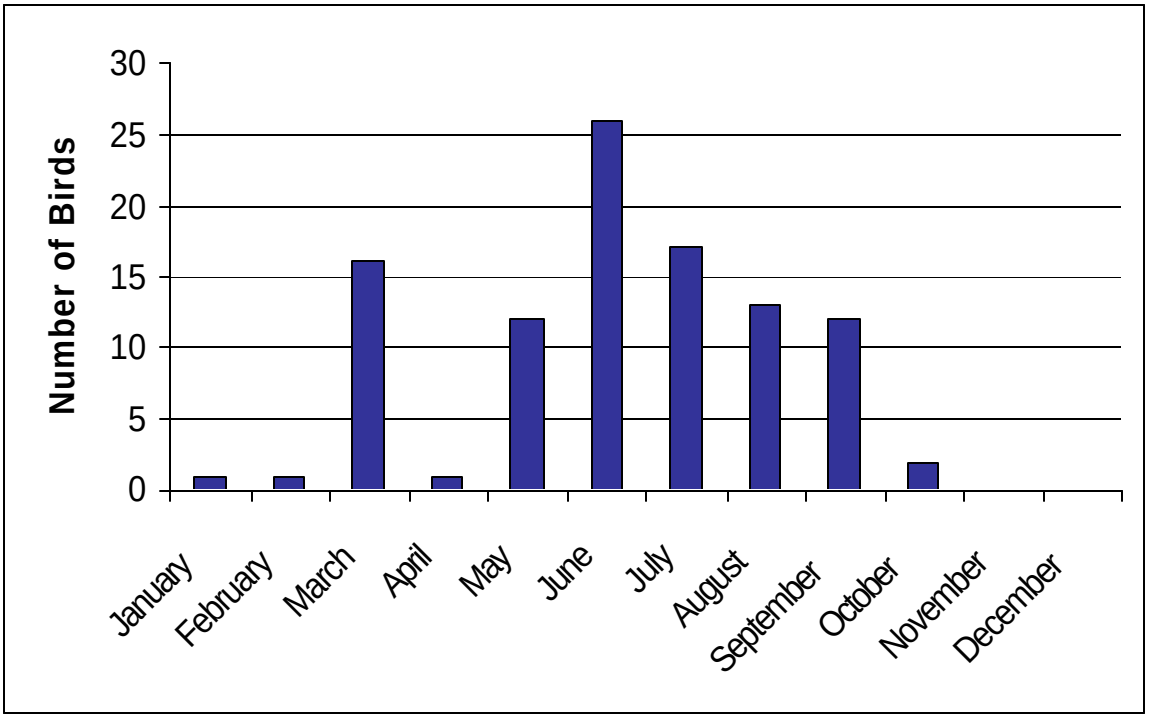


Table 4. Summary data of bird samples collected during the 2005 *Karenia brevis* bloom. Positive results indicate an individual had at least one tissue result above 5 ng PbTx per gram tissue. The ELISA assay used is not able to detect brevetoxins below this level. A result below the limit of detection (<LD) is considered negative.

Species	No. Tested	No. Positive
Black-bellied Plover *(Pluvialis squatarola)*	1	1
Black-crowned Night Heron (*Nycticorax nycticorax*)	1	1
Black Skimmer *(Rhynchops niger)*	1	1
Brown Pelican *(Pelecanus occidentalis)*	10	10
Common Loon *(Gavia immer)*	1	1
Double-crested Cormorant (*Phalacrocorax auritus*)	18	18
Dunlin (*Calidris alpina*)	1	1
Great Blue Heron (*Ardea herodias*)	4	3
Green Heron *(Butorides virescens)*	2	2
Herring Gull (*Larus argentatus*)	2	2
Laughing Gull *(Larus atricilla)*	11	11
Least Tern (*Sterna antillarum*)	15	9
Northern Gannet (*Morus bassanus*)	4	4
Osprey (*Pandion haliaetus*)	4	2
Royal Tern ((*Thalasseus maximus*)	3	3
Ruddy Turnstone (*Arenaria interpres*)	2	2
Sanderling (*Calidris alba*)	11	11
Sandwich Tern (*Thalasseus sandvicensis*)	5	5
Snowy Egret (*Egretta thula*)	1	1
Sora (*Porzana carolina*)	1	1
Yellow Crowned Night Heron (*Nyctanassa violacea*)	1	1
White Pelican (*Pelecanus erythrorhynchos*)	1	1
Willet *(Tringa semipalmata)*	1	1
TOTAL	**101**	**92**

Birds provided by rehabilitation centers were not limited to patients that had displayed symptoms of brevetoxicosis prior to expiring, but red tide-like symptoms were noted in history forms for many birds, especially cormorants. Carcasses collected during beached bird surveys and the Least Tern Rooftop Nesting Study did not include symptoms of the animal prior to death, and these carcasses were in various states of decomposition at time of recovery. Also, due to freezer capacity constraints, we were unable to continue collecting avian samples after October.

Brevetoxin Analysis of Avian Tissues

The digestive tissues tested for each bird included liver, stomach contents or whole stomach, intestinal contents or whole intestine, and gall bladder. Additional tissues were analyzed for the majority of birds examined, with total body burden results to be investigated separately (Karen Atwood, unpublished). Several birds did not have intestinal samples as a result of decomposition, and gall bladders were not recovered from small birds or decomposed carcasses. If multiple stomach content samples were taken from a bird, the specific sample referred to is indicated or results are reported as an average of all stomach content samples. A total of 321 tissues were sampled, with brevetoxins detected in 264 (82%) samples. Brevetoxins were detected in at least one gastrointestinal sample for 92 of the 101 birds tested (91%). Sixty-one of the 321 avian tissues tested (19%) were found to have a brevetoxin level over 200 ng PbTx/g tissue (Table 5). Thirty-four birds (34%) had at least one digestive tissue or gastrointestinal content sample greater than 200 ng PbTx/g tissue. Cormorants were the most frequently occurring species in this group with twelve individuals.

Table 5. Brevetoxin levels in avian tissues collected during from the 2005 *Karenia brevis* bloom, as determined by ELISA. Mean results were determined by considering all results below the limit of detection (<LD) to be zero.

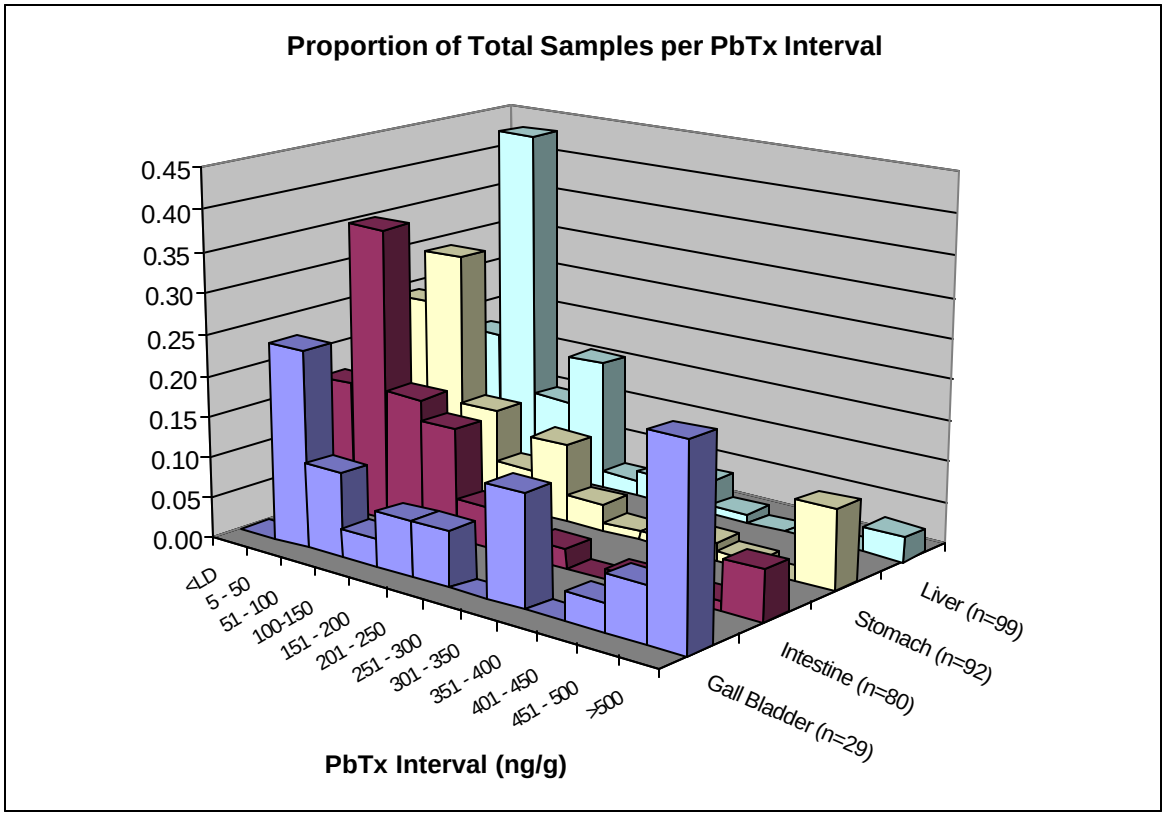
				LIVER			STOMACH CONTENTS			INTESTINAL CONTENTS		
	# tissues	% tissues positive	% tissues >200 ng/g	LOW	HIGH	MEAN	LOW	HIGH	MEAN	LOW	HIGH	MEAN
Cormorants (n=18)	71	92%	27%	<LD	197.67	76.00	<LD	9988.62[†]	416.21	<LD	2645.35	214.80
Gulls and Terns (n=22)*	73	73%	23%	7.53	1355.47	184.54	<LD	4399.5[†]	329.58	<LD	2800.73	328.67
Herons and Egrets (n=9)	31	77%	26%	<LD	295.71	95.36	<LD	727.22	161.58	<LD	510.77	114.26
Ospreys (n=4)	12	50%	0%	<LD	19.45	4.86	<LD	152.48	42.02	<LD	40.67	18.86
Pelicans (n=11)	40	95%	5%	<LD	72.92	21.84	10.06	181.25	64.75	11.08	143.47	39.39
Northern Gannets (n=4)	14	79%	7%	<LD	29.41	10.28	<LD	32.62	9.23	<LD	203.17	59.43
Sanderlings (n=11)	33	100%	27%	38.84	1313.33	239.34	19.01	1071.46	238.89	29.48	1363.08	238.33
Other Shorebirds (n=6)	18	94%	22%	25.73	297.74	117.62	15.78	1267.42	307.36	<LD	151.90	88.08

**Does not include data for least tern chicks.*

[†]*Value is for whole fish recovered from stomach (see Table 9).*

Gall bladder samples were not available for most of the birds sampled due to the relatively small size of this organ and difficulties removing it from previously frozen or partially decomposed carcasses. Of the 29 gall bladders sampled, none fell below the limit of detection for the ELISA, a finding not observed with the liver, stomach or intestinal samples. Also, a greater proportion of all gall bladder samples had very elevated brevetoxin levels in comparison to the frequency distribution for all other tissues sampled (Figure 4).

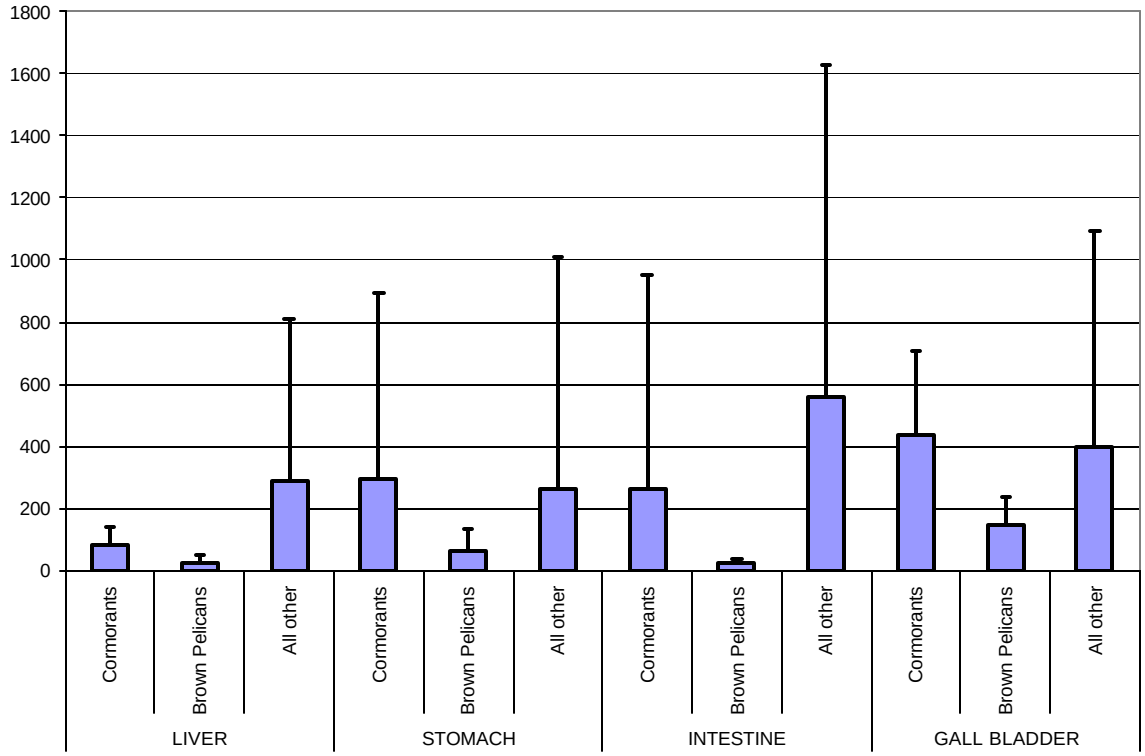
Figure 4. Comparison of the frequency interval for brevetoxin levels in gall bladder samples of marine birds as compared to liver, stomach and intestinal samples.



In a comparison of tissues from the 29 birds for which gall bladders were included, observed individual concentrations in the gall bladder were the highest for all tissues sampled in 20 of the birds (69%). Also for these 29 birds, the mean gall bladder concentration was greater than the mean concentration of the liver, stomach and intestinal samples in both cormorants (n=14) and brown pelicans (n=6), but the same trend was not observed in all other avian species (n=9) for which gall bladders were sampled (Figure

5). For the latter group, mean brevetoxin concentration was greatest for intestinal contents, followed by the mean brevetoxin concentration in gall bladder, liver and stomach contents, respectively.

Figure 5. Distribution of brevetoxins in digestive samples from cormorants (n=14), brown pelicans (n=6) and other avian species (n=9) for which gall bladders were analyzed. Mean brevetoxin levels with standard deviation are shown.



In addition to the seabirds described above, the carcasses of fifteen least tern (*S. antillarum*) nestlings and fledglings were recovered from rooftop nesting sites in Pinellas County between mid-May and early-July in 2005. These chicks ranged in weight from 2g to 27g, and all expired as a result of falling from their rooftop nesting area. Samples for these small chicks frequently required that all gastrointestinal tissues or all viscera be combined for analysis. Twenty nine least tern tissue samples were analyzed, with brevetoxins detected in 13 tissues (45%). All positive tissues were less than 40 ng

PbTx/g tissue, with the exception of one gastrointestinal tract sample from a fledgling collected June 19 which was found to have 218.81 ng PbTx/g tissue (Table 6).

Table 6. Brevetoxin levels found in tissue samples from least tern chicks recovered between mid-May and early-July during the 2005 *K. brevis* bloom.

TISSUE	# samples	% positive	Low	High	Mean
Whole Viscera	2	100%	14.41	17.82	16.12
Liver	13	46%	<LD	37.14	7.17
Stomach Contents	6	17%	<LD	13.17	2.20
Gastrointestinal Tract	8	50%	<LD	218.81	35.44

Fish in the Stomachs of Recovered Birds

Seventeen of the 101 birds necropsied (19%) had whole or partial fish in their gastrointestinal tract (Table 7). Eight of these 17 birds were least tern chicks with small marine silversides (*Menidia* spp.) in their stomach. Most of these small minnows (63%) were below the limit of brevetoxin detection for the assay (Tables 6 and 7). One additional bird, a ring-billed gull (*L. delawarensis*), regurgitated a small mojarra (*Eucinostomus* sp.) while scavenging red tide-killed fish on a Siesta Key beach in February during onshore bloom conditions, but did not expire and was not available for tissue analysis. Gulls and shorebirds were observed feeding upon these dead fish frequently during the 2005 red tide. The results of brevetoxin analysis on the mojarra are included and additional information on analysis of beached fish can be found further in the results section. Detailed information for each of the remaining nine birds, including location recovered, symptoms at time of recovery, bloom conditions at location of recovery, and necropsy findings are as follows:

- A juvenile double-crested cormorant (*P. auritus*) was rescued alive from Casey Key, FL on May 1, 2005. Water sampling reports indicate that *K. brevis* was not present in the area around this time as the bloom was concentrated further north of the Sarasota Bay area. This bird did not display neurological symptoms, but received 24 hours of care for propeller wounds before dying. The cormorant was determined to be of healthy weight during necropsy and the stomach contained numerous bones identified as remnants of a toadfish (*Opsanus* sp.). Brevetoxin

analysis of the liver and stomach contents, including toadfish bones, revealed relatively low levels of brevetoxin present (14.56 ng/g and 16.40 ng/g, respectively) with higher levels detected in the intestinal content sample (103.13 ng/g). The gall bladder ruptured and was therefore not available for analysis.

- A sandwich tern (*T. sandvicencis*) was brought to a rehabilitation center from Stump Pass State Park on Manasota Key, FL on June 13, 2005. This bird was emaciated and unwilling to fly, dying within 24 hours of care. A fishing hook with a small amount of fishing line was found in the stomach of the bird during necropsy. The hook was protruding through the stomach wall causing extensive internal injury. A portion of a Spanish sardine (*H. jaguana*) was also recovered from the tern's stomach. The fish was found to be below the limit of detection for the brevetoxin assay, as were the intestines of the tern, and only a small amount of brevetoxin was identified in the liver (12.39 ng/g). Cell counts for *K. brevis* in water samples collected June 14 from nearby Charlotte Harbor and Gasparilla Sound found no cells present, with higher counts collected further north in Sarasota Bay.
- A juvenile, male brown pelican (*P. occidentalis*) with wing injuries was collected June 25 from coastal waters in St. Petersburg. A whole fish (unidentified species) was recovered from the pelican's esophagus during necropsy, with subsequent analysis confirming that the viscera of this fish contained elevated levels of brevetoxin (Table 7). The pelican was also found to have low levels of brevetoxin in its liver (30.83 ng/g) and intestine (31.65 ng/g) but no neurological symptoms were noted by the rehabilitation handlers.

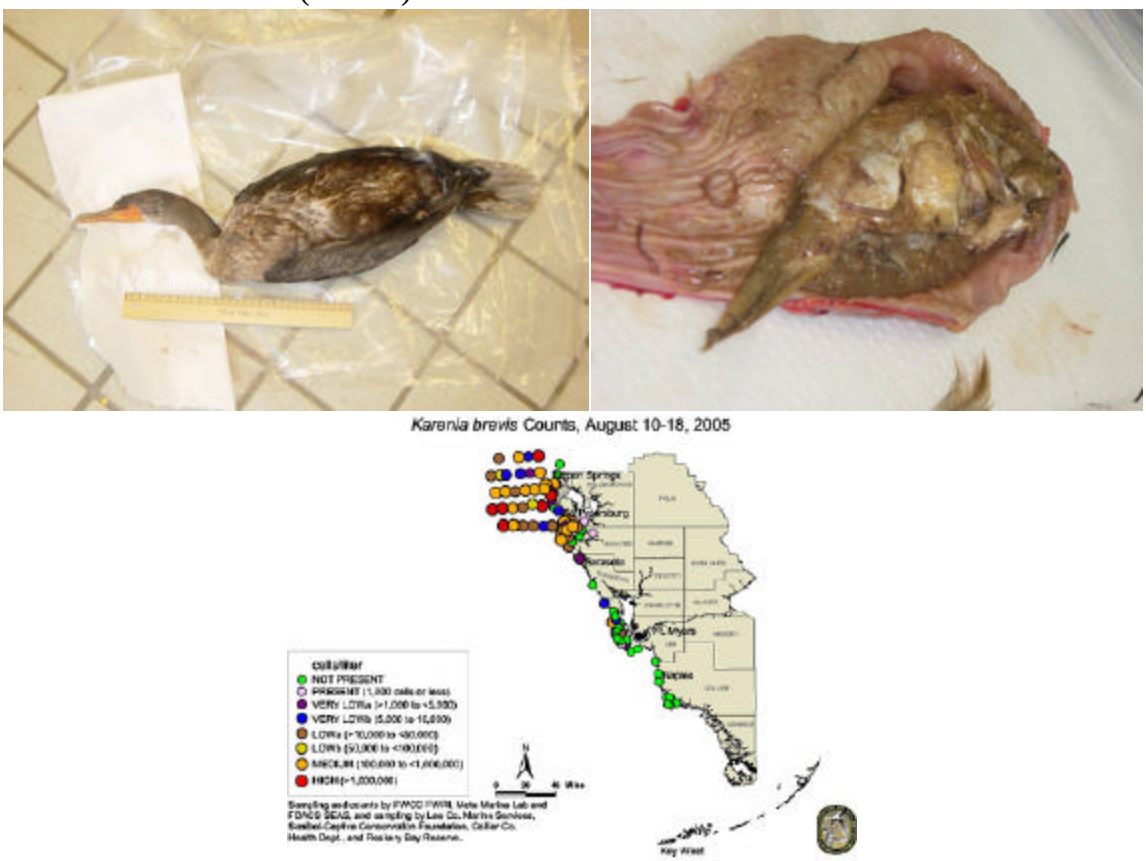
Table 7. Detailed summary of birds found with whole or partial fish in stomach during necropsy. *K. brevis* counts refer to bloom conditions in area at time of recovery of bird. PbTx level is for whole fish unless indicated otherwise.

Species	Recovery Date	Location Found	Stomach Contents	PbTx Level	*K. brevis* levels
Ring-billed Gull (*Larus delawarensis*)*	February 20, 2005	Siesta Key, FL	Mojarra spp. (*Eucinostomus* spp.)	924.41 (381.95)†	Medium to high
Double-crested Cormorant (*Phalacrocorax auritus*)	May 1, 2005	Casey Key, FL	Toadfish (*Opsanus* sp.)	16.40	Not present
Least Tern (*Sternula antillarum*)	May 20, 2005	Madiera Beach, FL	Silverside (*Menidia* sp.)	16.22	Not present to low
Least Tern (*Sternula antillarum*)	May 26, 2005	Madiera Beach, FL	Silverside (*Menidia* sp.)	13.17	Not present to low
Least Tern (*Sternula antillarum*)	May 30, 2005	Clearwater, FL	Silverside (*Menidia* sp.)	<LD	Not present to very low
Least Tern (*Sternula antillarum*)	May 30, 2005	Clearwater, FL	Silverside (*Menidia* sp.)	<LD	Not present to very low
Least Tern (*Sternula antillarum*)	May 30, 2005	Clearwater, FL	Silverside (*Menidia* sp.)	<LD	Not present to very low
Least Tern (*Sternula antillarum*)	June 3, 2005	Madiera Beach, FL	Silverside (*Menidia* sp.)	17.82	Not present to medium
Least Tern (*Sternula antillarum*)	June 7, 2005	Madiera Beach, FL	Silverside (*Menidia* sp.)	<LD	Not present to medium
Least Tern (*Sternula antillarum*)	June 7, 2005	Madiera Beach, FL	Silverside (*Menidia* sp.)	<LD	Not present to medium
Sandwich Tern (*Thalasseus sandvicencis*)	June 13, 2005	Manasota Key, FL	Spanish sardine (*Harengula jaguana*)	<LD	Not present
Brown Pelican (*Pelicanus occidentalis*)	June 25, 2005	St. Petersburg, FL	Unidentified fish	1095.98 (N/A)†	Low to high
Double-crested Cormorant (*Phalacrocorax auritus*)	August 19, 2005	St. Peterburg, FL	Pinfish (*Lagodon rhomboides*)	9988.62	Low to medium
Royal Tern (*Thalasseus maxima*)	August 25, 2005	Siesta Key, FL	Thread Herring (*Opisthonema oglinum*)	650.52	Medium to high
Royal Tern (*Thalasseus maxima*)	August 25, 2005	Siesta Key, FL	Thread Herring (*Opisthonema oglinum*)	4399.50 (153.69)†	Medium to high
Laughing Gull (*Larus atricilla*)	August 25, 2005	Siesta Key, FL	Thread Herring (*Opisthonema oglinum*)	387.43	Medium to high
Laughing Gull (*Larus atricilla*)	August 29, 2005	Tierra Verde, FL	Thread Herring (*Opisthonema oglinum*)	2215.80	Medium to high
Laughing Gull (*Larus atricilla*)	August 29, 2005	Tierra Verde, FL	Thread Herring (*Opisthonema oglinum*)	915.78	Medium to high

Fish was regurgitated, bird did not die.
†Value outside parentheses is for viscera, value inside parentheses is for muscle.

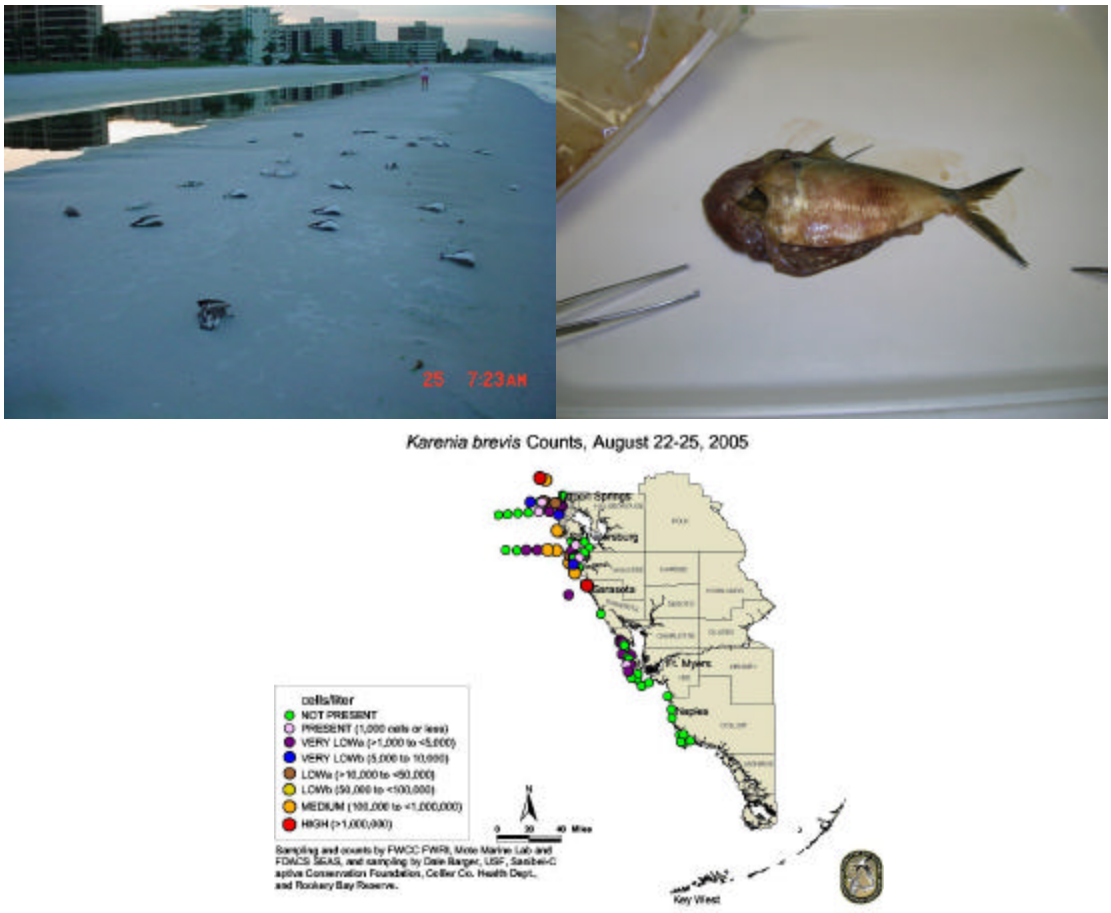
- A juvenile, female double-crested cormorant was discovered alive on a Pinellas County beach of the Gulf coast on August 19, 2005. Water samples taken the day before from nearby Boca Ciega Bay, just north of the mouth of Tampa Bay, found medium levels of *K. brevis* cells for this area. The cormorant was observed displaying symptoms consistent with toxicosis, including severe ataxia and convulsions. The bird died en route to a rehabilitation facility and did not receive any treatment. Subsequent necropsy found two partially digested pinfish (*Lagodon rhomboides*) in the cormorant's stomach (Figure 6). ELISA analysis on the pinfish and digestive tissues of this bird returned high levels of brevetoxin contamination (Table 8).

Figure 6. Juvenile female double-crested cormorant (top left) recovered from coastal area of Tampa Bay after exhibiting signs and symptoms of brevetoxicosis. Partially digested pinfish found in the bird's stomach (top right) tested positive for high levels of brevetoxin. Water samples taken from the area the previous day found medium to high cell counts of *K. brevis* (bottom).



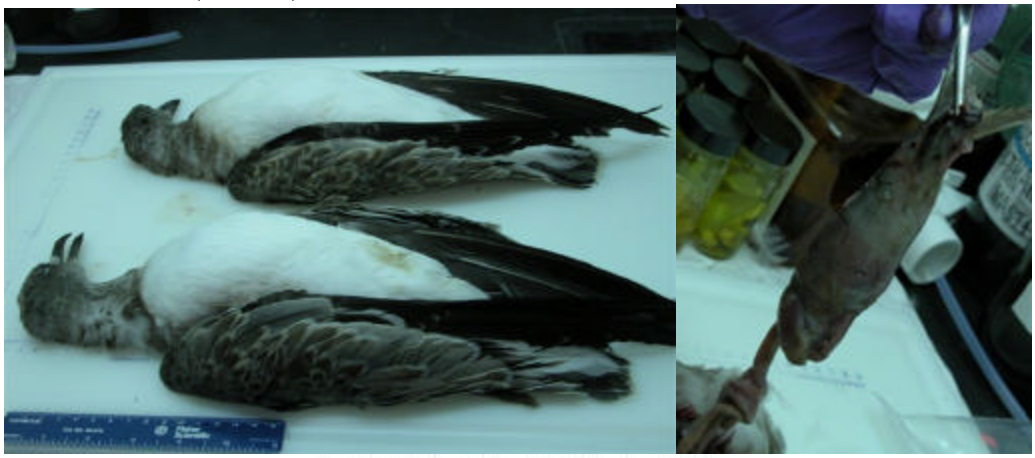
- On the morning of August 25, 2005, twenty laughing gulls (*L. atricilla*) and nine royal terns (*T. maxima*) were discovered dead on Siesta Key in Sarasota County (Figure 7). Necropsies were completed on two royal terns and one laughing gull, and each bird was found to have whole or partial thread herring (*O. oglinum*) in their stomach. ELISA analysis on these stomach contents indicated high levels of brevetoxin, and elevated brevetoxin levels were also found in the liver of each bird (Table 8). Water samples collected in the region that week found low to high levels of *K. brevis* in Sarasota Bay and surrounding coastal areas.

Figure 7. A flock of laughing gulls and royal terns discovered dead on the beach of Siesta Key in Sarasota County, FL on August 25, 2005 (left). While the cause of death is unknown, subsequent necropsy and analysis showed the birds recently fed on thread herring containing high levels of brevetoxins (right).



- On August 29, 2005, visitors to Tierra Verde encountered two laughing gulls in severe distress. Both birds displayed severe neurological symptoms, including difficulty in righting themselves. An observer killed both birds with the objective of ending their suffering. The gulls were found to have thread herring in their stomachs upon necropsy and the fish, which were analyzed whole, were found to have high levels of brevetoxin. The intestinal contents and livers of both birds also were found to have elevated levels of brevetoxin (Table 8). Tierra Verde, which is located at the northern shore of the mough of Tampa Bay, was experiencing medium to high counts of *K. brevis* in the region for the week these gulls were recovered (Figure 8).

Figure 8. Two laughing gulls (top left) recovered from coastal area of Tampa Bay after exhibiting signs and symptoms of brevetoxicosis. Partially digested thread herring (*O. oglinum*) found in the stomachs of both birds (top right) tested positive for high levels of brevetoxins. Water samples taken from the area that week found medium to high cell counts of *K. brevis* (bottom).



Karenia brevis Counts, August 29-September 1, 2005

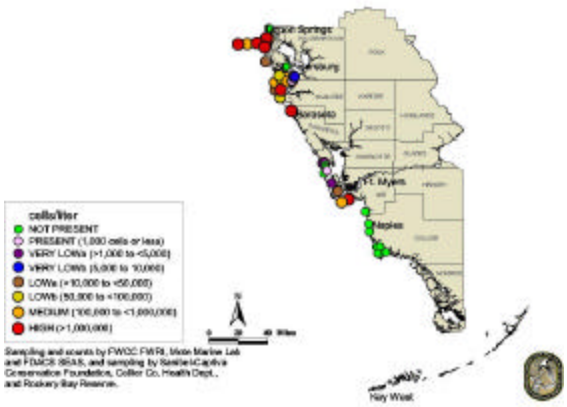


Table 8. Distribution and amount of brevetoxin (PbTx) found in the digestive organs and gastrointestinal contents of six marine birds collected from the central west Florida coast during the 2005 red tide event. Each of these birds had whole, identifiable fish in their stomachs at time of necropsy. These fish and other gastrointestinal tissues were found to contain elevated levels of brevetoxin.

Species	Date collected	Tissue	PbTx (ng/g)	LC-MS[†]
Double-crested Cormorant	19 August	Liver	197.67	
		Stomach contents (whole sample from partially digested pinfish)	9988.62	positive
		Stomach contents (muscle & skin of partially digested pinfish)	4095.08	positive
		Stomach content (all other stomach contents)	2310.32	positive
		Intestinal contents	2645.35	positive
		Gall bladder	718.43	positive
Royal Tern	25 August	Liver	96.86	
		Stomach contents (thread herring viscera)	4399.50	
		Stomach contents (thread herring muscle and skin)	153.69	
Royal Tern	25 August	Liver	140.87	
		Stomach contents (thread herring - whole)	650.52	positive
		Stomach contents (other contents present)	733.61	ND
		Intestinal contents	465.10	ND
Laughing Gull	25 August	Liver	147.11	
		Stomach contents (partially digested thread herring and other contents present)	387.43	
		Intestinal contents	272.89	
Laughing Gull	29 August	Liver	1355.47	ND
		Stomach contents (partially digested thread herring)	2215.80	positive
		Stomach contents (other contents present)	345.17	
		Intestinal contents	2800.73	positive
		Gall bladder	897.11	positive
Laughing Gull	29 August	Liver	1044.38	positive
		Stomach contents (partially digested thread herring)	915.78	positive
		Stomach contents (other contents present)	653.74	positive
		Intestinal contents	2021.28	positive
		Gall bladder	2099.21	positive

[†]Samples indicated were evaluated with LC-MS at UNCW Center for Marine Science in order to confirm the presence of brevetoxins (parent toxins and/or metabolites). Positive indicates confirmation of the presence of brevetoxins without quantification. ND indicates that brevetoxins could not be confirmed by LC-MS, but does not indicate the sample is negative for brevetoxins..

Seven birds, in addition to those listed in Table 8, had brevetoxin levels in their stomach and/or intestinal samples greater than 300 ng/g. No fish or evidence of a recent fish meal was noted during necropsy of these birds as all had empty or mostly empty stomachs. While six of these birds are not strictly piscivorous, fish are known to comprise at least some part of the diet for all these birds and so the elevated levels are shown in Table 9, with any actual stomach contents of each bird noted.

Table 9. Distribution and amount of brevetoxin (PbTx) found in the digestive organs and gastrointestinal contents of seven marine birds collected from the central west Florida coast during the 2005 red tide event. Each of these birds were found to have very high levels of brevetoxin in their stomach or intestines.

Species	Date Collected	Tissue	PbTx (ng/g)	LC-MS[†]
Sanderling	20 February	Liver	269.70	
		Stomach Contents (empty, some sand)	574.67	
		Intestinal Contents	371.86	
Sanderling	6 March	Liver	1313.33	positive
		Stomach Contents (empty)	1071.46	positive
		Intestine (whole)	1363.08	positive
Double-crested Cormorant	9 March	Liver	121.52	
		Stomach Contents (nematodes only)	442.91	
		Intestinal Contents	67.14	
		Gall Bladder	418.13	
Green Heron	25 June	Liver	227.16	
		Stomach Contents (empty)	727.22	positive
		Intestine (whole)	270.76	
Green Heron	5 July	Liver	295.71	
		Stomach Contents (empty)	47.72	
		Intestine (whole)	510.77	positive
Great Blue Heron	10 August	Liver	100.73	
		Stomach Contents (empty)	315.67	
		Intestinal Contents	40.27	
		Gall Bladder	314.93	
Black-bellied Plover	23 September	Liver	53.73	
		Stomach Contents (empty, some shell)	1267.42	positive
		Intestine (whole)	122.88	

[†]Samples indicated were evaluated with LC-MS at UNCW Center for Marine Science in order to confirm the presence of brevetoxins (parent toxins and/or metabolites). Positive indicates confirmation of the presence of brevetoxins without quantification.

Brevetoxin Analysis of Fish Tissues

Twenty five thread herring (*O. oglinum*) were collected from rehabilitation centers and a commercial fishery in the study region between April and October 2005. Whole fish ranged in length from 13.5 cm to 21.5 cm (total length) and in weight from 20g to 90g. Thread herring tissues sampled included eyes, gills, whole viscera and whole muscle (including skin and scales). All thread herring samples analyzed contained detectable levels of brevetoxins as measured by ELISA.

Brevetoxin levels in *O. oglinum* tissues ranged from 24.27 ng PbTx/g tissue to 5839.89 ng PbTx/g tissue. Highest levels were found in the viscera as compared to eyes, gills or muscle of a fish (Table 10 and Figure 9). Sixteen of the 25 viscera samples (64%) had over 1000 ng PbTx per gram tissue, and only one viscera sample was less than 200 ng PbTx per gram tissue. Muscle tissue samples contained the lowest levels of brevetoxins as compared to other tissues sampled, with only one muscle tissue greater than 200 ng PbTx per gram tissue. Monthly mean concentrations are shown in Figure 9.

Two of the twenty five thread herring sampled were gravid females. For these two individuals, eggs were analyzed separately from other viscera and contained 585.42 ng PbTx per gram eggs and 843.17 ng PbTx per gram eggs. In both of these cases the brevetoxin levels for eggs were less than the toxin levels in the whole viscera sample of the same fish (3617.02 ng PbTx/g tissue and 3269.90 ng PbTx/g, respectively).

Only three of the fifteen tissue samples taken from four individual "finger" mullet (*Mugil* spp.) were found to have detectable levels of brevetoxin (Figure 10). Mullet ranged in length from 15cm to 17cm (total length), and in weight from 36g to 42g. For those tissues with detectable levels, all were below 15 ng PbTx per gram tissue (Table 10 and Figure 10). All 39 tissues sampled from nine Spanish sardines (*S. auritus*) and one scaled sardine (*H. jaguana*) contained detectable levels of brevetoxins (Table 10 and Figure 10). Whole sardines ranged in length 11.6cm to 18.5cm (total length) and in weight from 17g to 57g.

Figure 9. Brevetoxin levels detected in locally caught thread herring (*O. oglinum*) obtained from a local commercial fishery and the food supply of participating rehabilitation facilities. All brevetoxin results were determined by ELISA. Tick marks indicate the LD_{50} for oral ingestion of brevetoxins in mice (520 ng PbTx/g mouse) and the level at which oyster beds are closed for human consumption (800 ng PbTx/g oyster tissue).

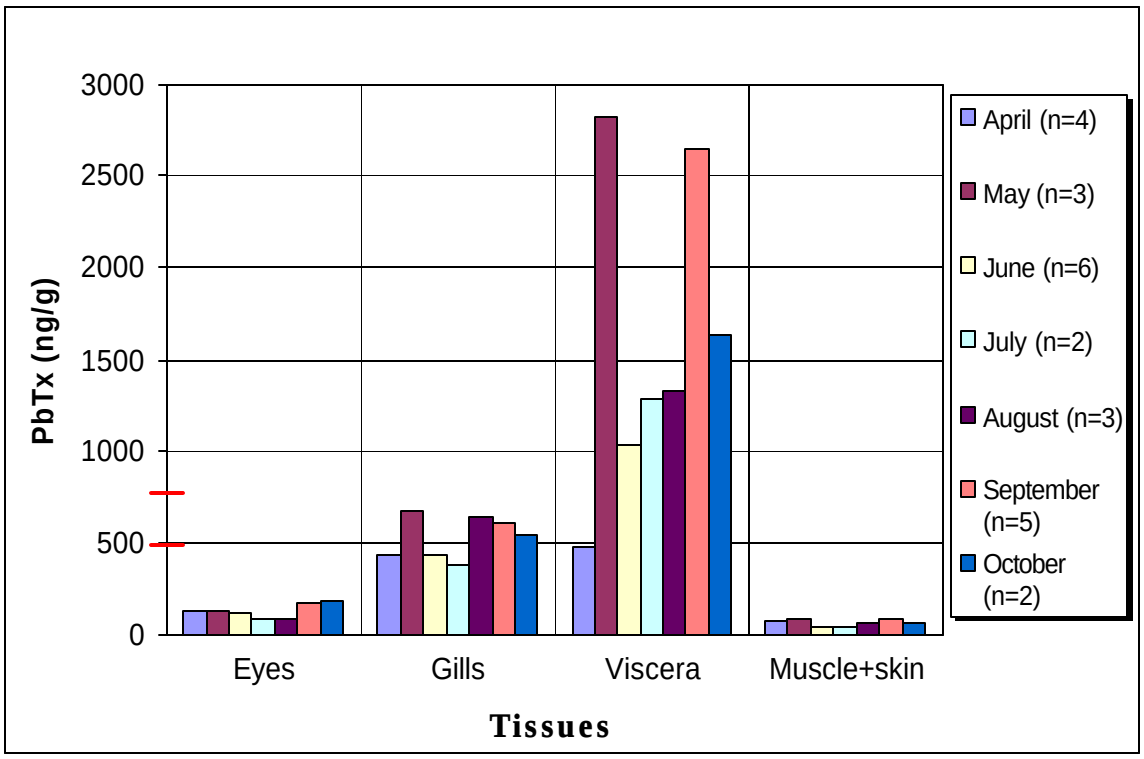


Figure 10. Brevetoxin levels detected in mullet (*Mugil* spp.), Spanish sardines (*S. auritus*) and a scaled sardine (*H. jaguana*) obtained from a local commercial fishery and the food supply of participating rehabilitation facilities. All brevetoxin results were determined by ELISA. Tick marks indicate the LD_{50} for oral ingestion of brevetoxins in mice (520 ng PbTx/g mouse) and the level at which oyster beds are closed for human consumption (800 ng PbTx/g oyster tissue).

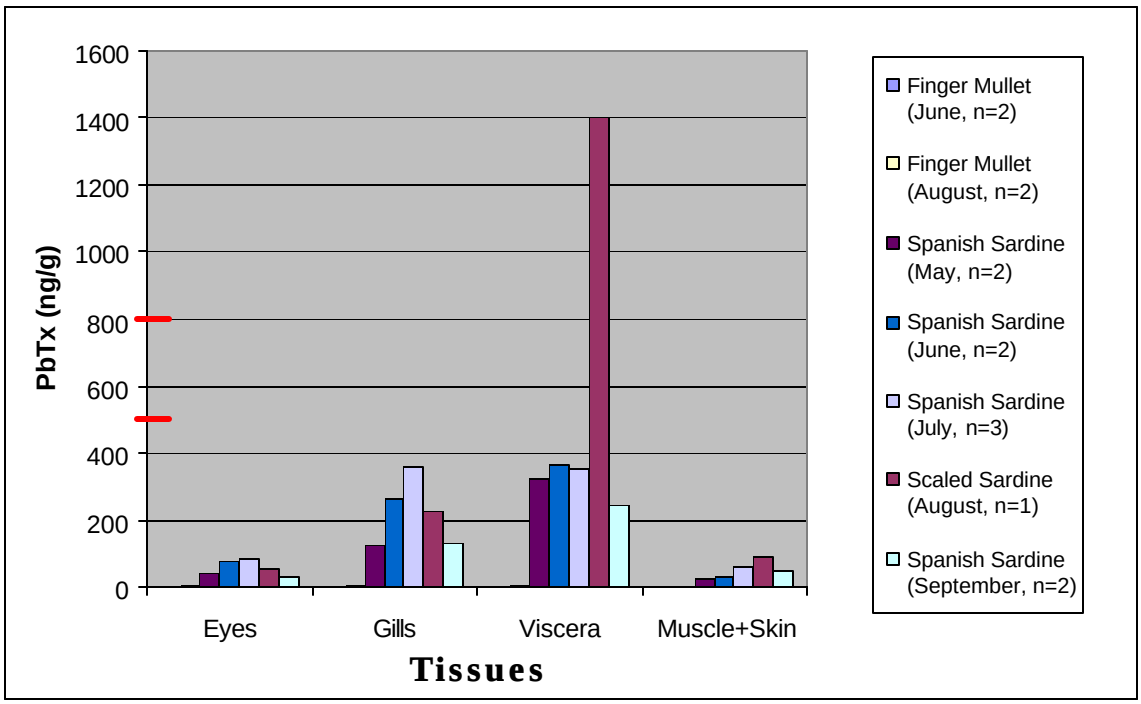


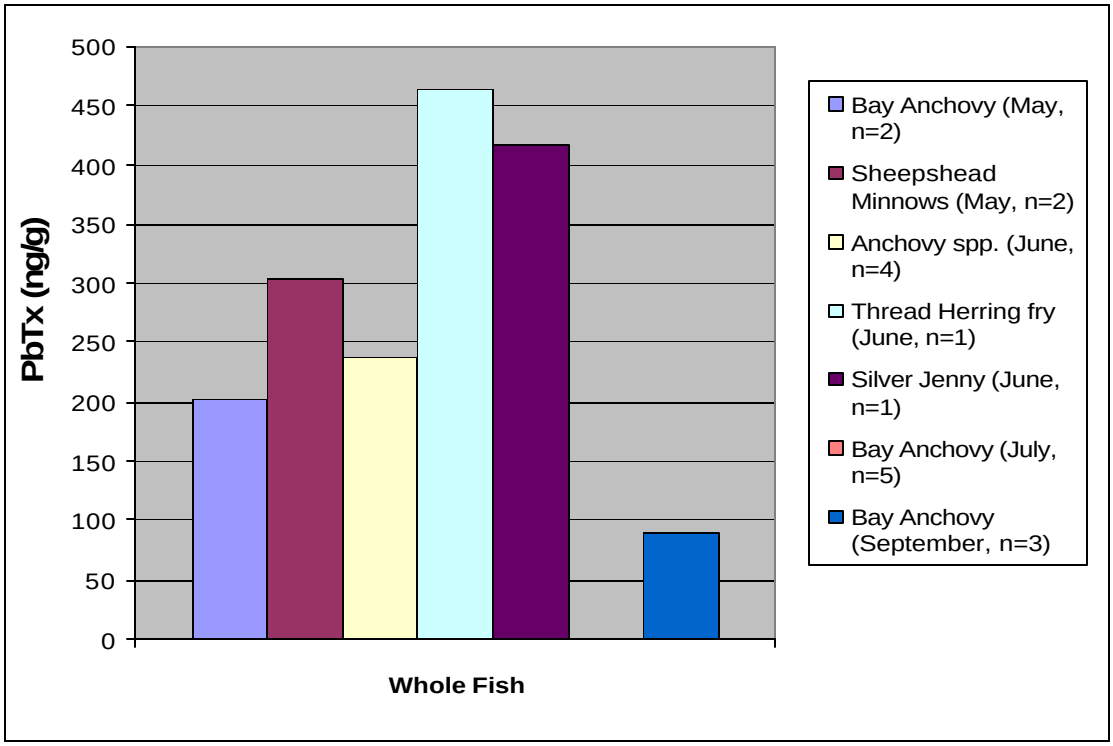
Table 10. Distribution of brevetoxins in tissues of baitfish obtained from a local commercial fishery and the food supply of participating rehabilitation facilities during the 2005 *K. brevis* bloom.

	Thread Herring (n=25)			Sardines (n=9)			Mullet (n=4)		
	low	high	**mean**	low	high	**mean**	low	high	**mean**
Eyes	60.47	259.04	**125.53**	15.51	155.89	**60.16**	<LD	11.02	**2.76**
Gills	113.30	1422.10	**526.30**	50.20	623.91	**223.20**	<LD	14.83	**4.94**
Viscera	70.15	5839.89	**1583.07**	198.80	1402.34	**432.15**	<LD	6.29	**1.57**
Muscle	24.27	202.38	**62.68**	26.60	92.39	**48.77**	<LD	<LD	**<LD**

Eighteen individual small minnows were collected from shoreline sites during the 2005 *K. brevis* bloom. All of these fish were less than 4cm long (total length) and less than 5g in weight each. Species sampled included bay anchovy (*A. mitchilli*, aka "glass minnows"), sheepshead minnows (*Cyprinodon variegatus*), silver jenny (*Eucinostomus*

harengulus), and thread herring fry. Minnow samples represent whole, macerated fish as opposed to individual tissue samples. In some cases two or three individuals were pooled and tested as a single sample (Figure 11). All samples tested were below 500 ng PbTx/g fish.

Figure 11. Brevetoxin levels in small minnows caught along the west central coast of Florida during the 2005 red tide. All brevetoxin results were determined by ELISA.



Twelve dead fish observed being actively scavenged along beaches during the 2005 bloom were collected, dissected and analyzed for brevetoxin content. Birds observed feeding upon red tide-killed fish were generally limited to shorebirds and gulls. Video recordings of scavenging behavior were taken, and a tendency for shorebirds such as ruddy turnstones, sanderlings, and gulls to bore into the eyes, gills and intracoelomic cavity while scavenging was noted. Subsequent dissection of beached fish verified the preferential feeding on eyes, gills and viscera by scavenging birds, with many fish lacking eyes, gills and/or internal organs (Figure 12). Only eight of the twelve beached fish were recovered with eyes intact, and all of the eyes sampled contained detectable levels of brevetoxin. Results for eyes ranged from 55.46 ng/g to 929.43 ng/g, with six of the eight samples (75%) over 400 ng PbTx per gram eye tissue (Figure 13 and Table 11).

Figure 12. Sanderlings observed actively scavenging red tide-killed fish along a beach on the west central coast of Florida during the 2005 *Karenia brevis* bloom (left). Evidence of shorebird preference for eyes, gills, and internal organs during scavenging of beached fish (right).

Three of the twelve beached fish had no recoverable gill tissue remaining. Of the ten gill tissue samples, all had detectable levels of brevetoxins and four (40%) were greater than 1000 ng/g. All viscera samples were also found to have very high levels of brevetoxin, with results ranging from 150.15 ng/g to 95,753.08 ng/g. It should be noted the low finding of 150.15 ng/g was from a mullet (*Mugil* spp.) whose liver had been scavenged through a borehole and was therefore absent in the sample. One thread herring was also found upon dissection to have no viscera present as it had apparently been removed entirely via an opening near the gill region. Brevetoxins were detected in all samples of muscle tissue, with results generally lower than levels found in the viscera and gill tissues of the same fish (Figure 13 and Table 11).

Figure 13. Brevetoxin levels in beached fish collected from beaches along the west central coast of Florida during the 2005 *K. brevis* bloom. All brevetoxin results were determined by ELISA. Tick marks indicate the LD_{50} for oral ingestion of brevetoxins in mice (520 ng PbTx/g mouse) and the level at which oyster beds are closed for human consumption (800 ng PbTx/g oyster tissue).

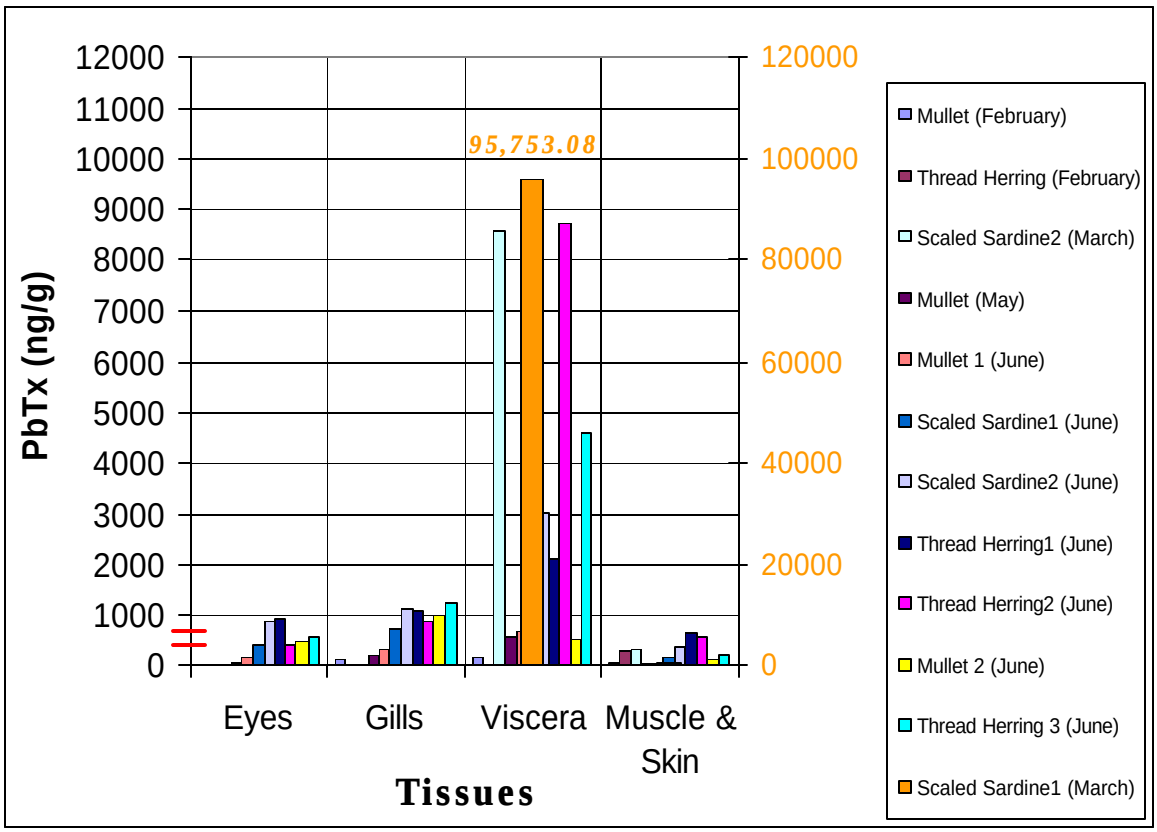


Table 11. Brevetoxin levels in beached fish collected from beaches along the west central coast of Florida during the 2005 *Karenia brevis* bloom. All brevetoxin results were determined by ELISA and reported as ng PbTx per gram tissue.

	Thread Herring (n=4)			Scaled Sardines (n=4)			Mullet (n=4)		
	Low	High	**Mean**	Low	High	**Mean**	Low	High	**Mean**
Eyes (n=8)	431.61	929.43	**644.05**	429.73	856.38	**643.06**	55.46	494.23	**236.97**
Gills (n=9)	855.98	1251.65	**1067.27**	735.56	1119.04	**927.30**	116.40	1007.08	**407.94**
Viscera (n=11)	2127.90	8726.46	**5155.55**	2586.62	95753.08	**27488.64**	150.15	685.08	**479.84**
Muscle (n=12)	302.03	654.77	**437.62**	158.40	692.17	**392.54**	31.63	125.7	**62.77**

DISCUSSION

Brevetoxins in Digestive Tissues of Marine Birds

Results from this study indicate that exposure to brevetoxins is widespread among piscivorous marine birds during *K. brevis* blooms. One or more digestive tissues and/or gastrointestinal content samples contained a detectable level of brevetoxin for more than 90% of the birds sampled. Brevetoxins were detected in at least one tissue for all 23 species of marine birds that were represented in this study, showing that exposure can occur among a variety of birds occupying various feeding niches in the region.

Results of brevetoxin analyses for the stomach, intestine, liver and/or gall bladder of sampled birds support the theory that ingestion is the primary route of exposure for these animals during red tide blooms. A toxicokinetic study of rats exposed to PbTx-3 via ingestion found that the liver concentrated the highest levels of brevetoxins (Cattet and Geraci, 1993), whereas Benson, et al., (2005) determined that no accumulation of brevetoxin or metabolites occurs in the liver of rats repeatedly exposed to toxins via inhalation. In the results presented here, 83% of the liver samples from all birds sampled were positive for brevetoxins.

One hundred percent of the avian gall bladders sampled in this study were positive for brevetoxins, and the gall bladder frequently had the highest mean brevetoxin levels of tissues sampled in brown pelicans and cormorants for which gall bladders were available. Furthermore, in a comparison of all liver, stomach, intestinal and gall bladder samples, a greater proportion of the gall bladder samples was found to have higher (>500 ng/g) levels of brevetoxin than the proportion of other tissues. The mean gall bladder levels were greater than the mean levels in the liver, stomach or intestinal samples for representative cormorants and pelicans, but this trend was not consistently seen in all species or individuals for which gall bladders were sampled. This is likely indicative of metabolic processes as ingested brevetoxins move through the gastrointestinal tract, and also due to differences in the time elapsed between exposure and death in individuals

sampled. It may also, however, provide indications to the exposure sensitivity of different avian species to brevetoxins. A more complete analysis of brevetoxin levels in avian tissues will be presented in greater depth at a later date (Karen Atwood, unpublished).

Poli, et al. (1990) and Kennedy, et al. (1992) concluded that the hepatobiliary system was largely responsible for metabolism and excretion of brevetoxins in toadfish (*O. beta*) and rats following intravenous administration. The results presented here offer evidence that the biliary system of birds also may be an important pathway for metabolism and excretion of ingested brevetoxins. Biotransformation of toxins occurs in the liver, which produces bile that is received by the gall bladder (Berne and Levy, 2000). Feeding stimulates the release of bile via the bile duct into the small intestine and, during periods of fasting, bile is stored and concentrated in the gall bladder (Bowen, 2001). Toxins that pass to the intestine from the gall bladder may be eliminated in the feces or reabsorbed (Duffus and Worth, 2001). Woofter, et al. (2005) suggested that brevetoxin is reabsorbed by the intestines after biliary secretion, and that this would account for the sustained blood levels observed in mullet many days after aqueous exposure. The elevated gall bladder levels observed in birds sampled during this study may reflect a situation in which these birds ingested a sublethal amount of brevetoxin, but were behaviorally impaired and unable to feed as they metabolized the toxin, resulting in storage and concentration in the gall bladder. The birds sampled here obviously did not survive this scenario if in fact it was their experience. It is possible that some birds are exposed to high, but sublethal, doses of brevetoxins during a meal, are then unable to feed during metabolism and recovery, but eventually recover after a period of fasting. This raises questions regarding the toxicity of the brevetoxins and derivatives in the post-exposure bile that is excreted once feeding is resumed, and the potential effects if there is reabsorption in the small intestine.

While all species examined were found to have some detectable level of brevetoxins in at least one digestive tissue or gut content sample, differences could be seen among families of birds. Double-crested cormorants (Family: Phalacrocoradae) are considered one of the avian species most impacted when red tides occur in the region (Quick and Henderson, 1974; Forrester, et al., 1977; Kreuder, et al., 2002). In this study,

cormorants were the most abundant species sampled (Table 4) and were found to have a greater percentage of tissues with more than 200 ng PbTx/g than any other sea bird species sampled. Cormorants are abundant in Florida (Vidal-Hernandez and Nesbitt, 2002). They are efficient predators and considered opportunistic generalists, diving from the surface to pursue schooling or, occasionally, benthic fish and generally feeding on prey that is most abundant (Wires, et al. 2001; Elphick, et al., 2001). During beach surveys in 2005, cormorants could often be seen foraging along local beaches even during intense onshore bloom conditions when brown pelicans, gulls and other species were absent (Figure 14). It is possible that the opportunistic nature of these birds could draw them to the erratic swim patterns of brevetoxin-intoxicated fish. Neurointoxicated fish are potentially easy prey as they often display behaviors attractive to predators, such as floundering at the surface and a depressed flee response (Quick and Henderson, 1974).

Figure 14. Double-crested cormorants congregated along Casey Key Beach in Sarasota during onshore bloom conditions on March 3, 2005. These birds appeared to be foraging alongshore while gulls, terns and pelicans were absent.

The majority of cormorants examined were juveniles (85%), 67% were found to have gastrointestinal nematode infestations, and 38% were noted to be very thin or emaciated (Appendix I lists body weight information). This is consistent with observations made by Kreuder, et al., (2002), as a high occurrence of endoparasitism and immaturity was observed in cormorants with suspected brevetoxicosis. Gastrointestinal parasites are not uncommon in wild animals, but it is possible that a heavy parasite load combined with brevetoxin exposure may overwhelm the immune system of young or weak birds making an otherwise survivable condition more serious.

Pelicans, ospreys and gannets were not found to contain brevetoxins in their gastrointestinal tissues at levels as high as cormorants, gulls and terns, herons and egrets, and shorebirds (Table 5). Perhaps most noteworthy was the difference between mean brevetoxin concentrations in tissues from brown pelicans versus double-crested cormorants. There are similarly-sized, large populations of both these bait fish-consuming species in Florida (Table 2). The results offered here indicate that despite similarities between the two populations, there could be important differences in their exposure to or tolerance of brevetoxins during blooms of *K. brevis* in the region. Possible reasons why brown pelicans may be less vulnerable to brevetoxin exposure during red tide blooms as compared to double-crested cormorants include different physiological tolerances to brevetoxins, or behaviors designed to reduce exposure or vulnerability to toxins, such as prey avoidance or regurgitation of contaminated prey. Also, interspecific differences in body burdens of parasites or other contaminants which could lead to secondary effects following brevetoxin exposure could play a role. As there are numerous, complex factors that can influence fitness in a population, a more thorough examination was beyond the scope of this study.

In addition to cormorants, other avian families from which individuals were found to have elevated levels of brevetoxins were gulls and terns (Family: Laridae), herons and egrets (Family: Ardeidae), and shorebirds (Family: Scolopacidae). Members from these avian families consume a variety of foods and forage in a number of diverse ways. Gulls are generally considered opportunistic omnivores, while terns and skimmers have more specific methods of prey capture and can often be observed foraging on small fish close to shorelines and along beaches (Elphick, et al., 2001). In a comparison of tissue

analyses among larids, the highest levels of brevetoxins were found in laughing gulls and royal terns. These two species are often seen together in flocks on beaches, and the recovery and stomach content findings from the royal terns and laughing gulls on Siesta Key in August 2005 suggests that they forage together, or, more likely, that laughing gulls are "pirating" terns successfully as they forage (Elizabeth Forys, personal communication).

Herons and egrets are found in both fresh and marine habitats, and eat primarily fish, but will also feed on crustaceans, amphibians and occasionally small rodents or the chicks and eggs of other birds (Elphick, et al., 2001). They are wading birds and forage in shallow coastal waters, canals or along mangrove fringe in search of small fish and other prey. The tissue analyses of birds in this family, and the high levels of brevetoxins found in the green and great blue herons (Table 9), indicate ardeids are not avoiding marine prey during red tide blooms in favor of a freshwater diet. However, it cannot be concluded that fish were the source of brevetoxin exposure for these birds as fish were not confirmed in stomach contents during necropsy, and, due to the variety in their diet, exposure may have resulted from consumption of shellfish or other prey items.

Sanderlings, turnstones, dunlins and willets (Family: Scolopacidae), along with a variety of plovers (Family: Charadridae), generally forage for invertebrate prey, such as interstitial insects, amphipods, and polychaete worms, in the intertidal zone of beaches (Elphick, et al., 2001). Some of their prey items, including small bivalves (eg., *Donax* spp.), are known to accumulate brevetoxins (Cummins, et al., 1971; Roberts, et al., 1979). Mass die-offs of invertebrates in the intertidal zone have also been documented immediately following red tides in the region (Gunter, et al., 1947; Simon and Dauer, 1972), and the absence of a number of species which contribute to the diet of shorebirds, such as polychaetes and amphipods, was personally observed on beaches during the 2005 bloom. Therefore, confirmation of shorebirds scavenging red tide killed fish is important because it supports the possibility that shorebirds switch their diets from traditional sources, due to contamination or reduced availability, to abundantly available dead fish during red tide blooms.

Fish deposited onshore could vector brevetoxins to shorebirds, as well as gulls and other scavenging animals, if toxins have bioaccumulated in the fish prior to their

death and deposition. This is an aspect of the food web not generally considered as fish typically do not comprise a large portion of the diet of small shorebirds. Based on my observations, beached fish do appear to be an important alternative food source during red tides. Samples from dead fish being scavenged by shorebirds found some alarmingly high levels of brevetoxins in the parts of fish known to be targeted by shorebirds, such as the eyes, gills and viscera. As further evidence that red tide-killed fish may contain high levels of toxins, there were also anecdotal accounts of terrestrial organisms, including bobcats, rats and pet dogs, exhibiting signs of neurointoxication following scavenging of fish on red tide impacted beaches in 2005 and past red tides (Wildlife Center of Venice, personal communication; Ernst, 2003).

Most of the beached fish sampled, particularly the scaled sardine with 95,000 ng/g brevetoxin in the viscera, would appear to contain lethal levels of toxin for a small shorebird weighing less than 60g. When Aldrich and Ray (1965) exposed two similarly sized chicks to a total ingestion of 198,000 ng PbTx, an amount comparable to what could be consumed from the Scaled sardine by a sanderling assuming a consumption of 2g viscera, both chicks lost equilibrium within 10 hours and expired within 22 hours.

Elevated levels of brevetoxins were found in the digestive tissues and contents of several shorebirds, but symptoms associated with brevetoxicosis such as ataxia and spastic movements (Kreuder, et al., 2002) were not noted in birds provided by rehabilitators. Also, there were no reports of large numbers of dead or dying shorebirds in the hours or days following observed consumption of red tide-killed fish on beaches in February and March of 2005.

A morbidity and mortality event of primarily scolopocids (sanderlings, ruddy turnstones and willets) was reported and observed in the fall of 2005 from Tampa Bay to Charlotte Harbor, nine months after the start of the red tide bloom (Suncoast Seabird Sanctuary and Wildlife Center of Venice, personal communication). Individuals collected by rehabilitation facilities were reported as exhibiting signs and symptoms of severe gastrointestinal distress, lower body paralysis and systemic disease. These birds do not consume fish whole and therefore the nature of their diet was not able to be confirmed in stomach contents during necropsy. Brevetoxins were detected in the digestive tissues of all of these birds, but very high levels of brevetoxins were not

consistently found in all shorebirds associated with the mortality event (Appendix II). In the shorebirds found to have elevated brevetoxins in their tissues, it could not be confirmed that this was the result of scavenging contaminated fish rather than exposure through traditional invertebrate prey. During the course of this study, though, we did document that shorebirds are consuming dead fish onshore during red tide blooms, and that those fish frequently contain elevated levels of brevetoxins in the tissues targeted by scavenging birds.

A few species of birds for which mortalities have been reported during past *K. brevis* blooms were not well represented during in this study. Red-breasted mergansers (*Mergus merganser*) and lesser scaup (*Aythya affinis*) were completely absent among the collected bird carcasses despite reports that these species were heavily impacted during past blooms (Quick and Henderson, 1975; Forrester, et al., 1977). Only one white pelican (*P. erythrohynchos*) was recovered during this study as compared to the 2001 bloom in the region during which time dozens of these birds were treated by local rehabilitators for neurological symptoms (Pelican Man's Bird Sanctuary, personal communication). As previously described, the 2005 bloom reached onshore waters in February and persisted through the spring and summer months. In contrast, the 1974 red tide occurred from approximately October to May and the 2001 red tide lasted from about September to early January. These observations underscore the potential importance of bloom timing as an influence on which avian species are affected when a bloom occurs. A bloom which initiates in the late fall and persists through the winter is likely to contribute to increased mortality and morbidity among Florida's migratory bird species that occur in winter and early spring. It should be noted that least terns, which were included in this study, are a migratory bird occurring in Florida during their nesting season from April to September.

Prey Fish and Brevetoxins in Stomach Contents of Birds

An important objective of this study was to determine the role of prey fish in vectoring brevetoxins to marine birds. Brevetoxins were consistently found in the digestive tissues or gastrointestinal contents of all species and most individual birds examined, and samples of the small schooling fish targeted by piscivorous marine birds

were also shown to carry potentially harmful levels of brevetoxins. In order to make the link between brevetoxin-containing prey fish and their avian predators, it was important to find birds whose last fish meal, and the brevetoxin levels therein, could be confirmed through stomach content analysis.

Six of the 101 seabirds examined were found to have whole or partial fish in their stomachs which contained elevated levels of brevetoxins. Of these, three birds had exhibited signs and symptoms consistent with brevetoxicosis before dying, an indication that their fate directly resulted from poisoning. While toxin levels for sublethal versus lethal exposures for brevetoxins in marine birds are not known, it is possible that the levels found in the double-crested cormorant collected on August 19, 2005 and the laughing gulls recovered on August 29, 2005 (Table 8) are somewhere in the range of an acute exposure. Also, finding brevetoxin-containing fish in the stomachs of these birds confirms that piscivorous birds are feeding on contaminated fish during *K. brevis* blooms and not all individuals are able or willing to avoid red tide impacted fish entirely.

Tester, et al., (2000) documented the transfer of brevetoxins from copepods to the zooplanktivorous pinfish (*L. rhomboides*), and here we present evidence of consumption of brevetoxin-containing pinfish by a double-crested cormorant. These findings offer confirmation of vectorial transport of brevetoxins from dinoflagellate (*K. brevis*) to predatory marine birds via planktivorous fish, an ecologically important link in the Gulf of Mexico food web.

Eight of the 15 least tern chicks sampled were found to have small minnows in their stomachs. Unlike adult seabirds, chicks have their meals brought to them by adults and do not rely on being able to successfully forage for themselves. Analysis on these small minnows and gastrointestinal contents and tissues did not reveal high levels of brevetoxins, with the exception of one gastrointestinal sample for a single fledgling. While nine of the fifteen chicks tested were positive for brevetoxins, results were generally low (Table 6) and do not indicate that brevetoxin exposure was an important contributor to their loss.

For the remaining 83% of the marine birds examined for this study, there was no evidence of a recent fish meal in their stomach contents. This suggests that acute, lethal poisoning soon after feeding may not be the fate of most piscivorous seabirds during

blooms of *K. brevis*. These results could be more consistent with birds being exposed to chronic low levels in their diet via contaminated prey, or acute exposures that result in prolonged illness rather than sudden death. Consumption of brevetoxins via prey fish could potentially impair immune and behavioral functioning in marine birds, triggering a cascade of events in which functional behavior of an individual, such as feeding, preening, and injury aversion, is impaired due to ingestion of contaminated prey (Elphick, et al., 2001; Kreuder, et al., 2002). An investigation into a mortality event of dolphins in 1987-1988 supports such a scenario. Here it was determined that many individuals died as a result of opportunistic infection or other factors that were only fatal due to the physiological weakening of individuals, potentially due to brevetoxin exposure (Anderson and White, 1992).

During this study, there were two examples of birds potentially incapacitated such that survival behaviors may have been compromised. The gulls and terns collected from Siesta Key Beach on August 25, 2005 were found to have recently fed on thread herring, and for all three birds sampled, those fish contained elevated levels of brevetoxins (Tables 7 and 8). While these results offer important information about brevetoxins in the fish that these birds are feeding on during red tide conditions, it does not appear the contaminated fish were the direct cause of the mortality event. There is strong evidence from weather reports and necropsy findings, including sloughing and discoloration of muscle tissue and degradation of intestinal tissues, that these birds were the victim of a lightening strike. As previously described, brevetoxin exposure in birds is associated with "drunken" behavior and a reduced flight response. It is possible that the contaminated fish the gulls and terns recently fed on resulted in their not responding normally to the storm and threat of lightening, thereby contributing to their fate.

The second example is the double-crested cormorant recovered from Sanibel Island on August 2, 2005. This individual may also have been experiencing reduced functional behavior as a result of toxin exposure. This adult bird was hit by a car and euthanized by local rehabilitators due to spinal injuries. Analysis on the gastrointestinal tissues detected 719.25 ng PbTx per gram gallbladder tissue and 28.46 ng/g, 32.08 ng/g, and 31.66 ng/g in the liver, stomach contents, and intestinal contents respectively. This toxin profile of elevated brevetoxins in the gallbladder as well as lower levels in all other

gastrointestinal tissues is consistent with the tissue profiles found in other cormorants collected which were labeled as suffering from red tide syndrome (ataxia, slow blink, head tremor) prior to expiring (Appendix III). Therefore, this bird's nervous system could have been compromised to the point that it was incapable of an appropriate flight response when threatened by an oncoming vehicle.

Brevetoxins in Common Prey Fish of Marine Birds

Results from fish tissue analyses suggest that prey fish can bioaccumulate brevetoxins during *K. brevis* blooms, and piscivorous marine birds may be vulnerable to toxin exposure via their diet. Among the live caught fish sampled, thread herring were found on average to have higher levels of brevetoxins as compared to the sardines and juvenile mullet sampled (Table 10). The levels of brevetoxins detected in the viscera of thread herring were extremely high and would appear to pose a risk to predatory marine birds, as they were similar to the levels in fish recovered from the stomachs of the double-crested cormorant and laughing gulls which exhibited symptoms of brevetoxicosis prior to expiring (see Tables 8 and 10, Figure 8). If it is assumed that birds are not effectively avoiding or regurgitating brevetoxin-containing prey, a potential exposure level from contaminated fish can be calculated simply from the findings and known consumption rates of certain species (Lefebvre, et al., 2002). For example, a thread herring taken from the food supply of the Wildlife Center of Venice in June was found to have 1892 ng PbTx per gram viscera, and a total viscera weight of 6.9 grams, which calculates to a total body burden of more than 13,000 ng PbTx in this 90 gram fish. A 45g thread herring collected in September was found to have 2231 ng PbTx per gram viscera, and a total viscera weight of 4.7 grams, resulting in a body burden of at least 10,000 ng PbTx for the whole fish. Reports of daily consumption rates for double-crested cormorants vary from an estimate of 16% of their total body weight (Schramm, et al., 1987 as referenced in Bayer, 1989) to the more common estimate of approximately a pound (500g) of fish per day (USFWS, 2007). In considering only the toxin levels of the viscera and ignoring all other tissue levels, consumption estimates for birds feeding on 250 – 500g of heavily contaminated thread herring could be estimated at over 35,000 ng PbTx/day.

The sardines sampled, while not generally as high as the thread herring, were also shown to carry a body burden of toxins with higher concentrations in the viscera (Table 10). The juvenile mullet and small minnows collected nearshore were found to have relatively low or no detectable toxin and may pose less of a risk to those birds targeting these small species. This theory is supported by the less frequent occurrence of high brevetoxin levels found in the gastrointestinal tissues of birds known to feed exclusively on small minnow species nearshore and along beaches, such as least terns (Table 6) and black skimmers.

Management Implications

These results provide evidence that small schooling fish such as thread herring and sardine species, which represent an important source of food for marine birds on the central west Florida coast, can contain elevated levels of brevetoxins. Brevetoxins were consistently found in the tissues of live-caught fish, with the highest levels detected in the viscera of thread herring. Facilities utilizing whole bait fish from Florida's Gulf coast for animal consumption should be aware of the implications of these findings. Rehabilitation centers in the region, as well as aquariums, zoos and amusement parks with animals consuming baitfish, may need to consider the potential for these fish to carry potentially harmful levels of brevetoxins (Anderson and White, 1989; Krueder et al., 2002). Facilities may consider importing baitfish from unaffected areas during bloom conditions. Also, there are implications for the practice by fisherman of throwing fish offal and viscera discards to begging seabirds during fish cleanup. Evidence presented here indicates this may pose a health concern for these birds.

In general, there are indications that red tide blooms caused by harmful algae may be increasing in frequency and/or impacting larger or novel coastal areas worldwide (Hallegraeff. 1993; Van Dolah, 2002; Smayda, 2002). In late September 2007, a *K. brevis* bloom began affecting coastal areas of northeast Florida, a region not generally prone to these blooms. Educational efforts regarding risks to marine birds, particularly potential exposure dynamics in their food supply and possible treatment methods would be useful to rehabilitators and others not accustomed to dealing with brevetoxin-impacted animals. Species recovery plans for threatened and endangered sea or shore birds should

also consider the potential risk of spatial or temporal changes to *K. brevis* blooms. Increasing frequency or intensity of blooms may result in greater toxin exposure or impacts on prey species abundance for marine birds.

Recommendations for Future Investigations

While this study shows that brevetoxins can be found in the gastrointestinal contents of marine birds and the fish they prey upon, there is a lack of applicable clinical information for dose-response levels of brevetoxin ingestion in birds. Therefore, we cannot conclusively say that consumption of brevetoxin-contaminated prey directly led to the illness or death of individuals. Any exposure to marine toxins may tip the balance for animals subject to competition for food, loss of habitat, parasites, disease, and increasing boat traffic, as was determined during past investigations of dolphin mortalities in Florida (Anderson and White, 1992). Ingestion of chronic, subacute levels of brevetoxins, or sporadic acute exposures, could impair critical behaviors of birds, including feeding, preening and injury avoidance, and cause individuals to succumb more easily to other factors, such as parasites, disease or injury.

Future studies investigating the impact of sublethal ingestion of brevetoxins on immune and behavioral function would improve our understanding how *K. brevis* blooms impact marine birds. Also, a long-term study tracking fluctuations in levels of brevetoxins in avian populations during non-bloom and bloom periods would help illustrate potential changes in exposures or the number and species of birds impacted over time.

Long-term ecosystem models of baitfish toxicity and abundancy during red tide blooms could also be useful to understanding impacts on avian predators. Thread herring findings presented in this study indicate the ability of this species to accumulate brevetoxins in their viscera and potentially vector those toxins to predatory marine birds and other organisms. It is also important to point out that this species was unavailable from commercial fisheries in the later months of 2005 due to purported red tide related losses. Gulf coast landings of Atlantic thread herring comprise approximately 88% of the fishery for the state of Florida, and the 2005 total landings were reduced by 22% from historical averages from 1982 (FWRI Species Account, 2006). This dramatic reduction

in availability may result in avian stress and disease due to reduced prey availability, or a reduction in potentially contaminated fish could also drive predatory birds from affected areas and result in fewer incidences of exposure.

The impact on organisms that scavenge red tide-killed fish merits further consideration. There are implications for both terrestrial and pelagic scavengers, including sharks which have been documented gorging on floating dead and decomposing fish (Steidinger and Ingle, 1972). The shorebirds of Florida's Gulf coast face numerous pressures as coastlines are urbanized and beaches become increasingly crowded with human traffic. It is important to consider all influences on the health of shorebird populations in order to determine appropriate conservation measures. Investigations into the abundance and toxicity of traditional food sources for shorebirds during bloom and non-bloom periods, and the potential risks or benefits associated with switching to dead fish during red tide blooms, would greatly improve our understanding of how *K. brevis* blooms in this region impact resident and migratory shorebird populations.

Finally, it is possible that periodic red tide events along the WFS are the kind of environmental perturbation that contributes to the diversity of avian populations and other marine organisms (Vargo, 1987). Double-crested cormorants have historically stood out as a species impacted by red tide blooms in the area (Quick and Henderson, 1974; Forrester, et al., 1977; Emslie, et al., 1996; Kreuder, et al., 2002). In other regions, these birds are considered nuisance species for their adept fishing skills and consumption rates, prompting harassment measures and calls for population control by local fishermen (Bayer, 1989 and references therein; Wires, et al., 2001). Cormorants also cause some of the most dramatic impacts on vegetation at nesting colonies, resulting in impacts on other birds due to competition for nesting space and habitat degradation (Wires, et al., 2001). Perhaps red tide blooms in the southwest region of Florida may serve to limit populations of more competitive birds, such as cormorants, creating opportunities for other species to flourish. A long-term investigation into fluctuations of avian populations in context of red tide blooms may offer insight into this possibility.

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APPENDICES

APPENDIX I: Mean weights for all species for which more than one individual was sampled during this study. Healthy adult mean ranges are listed for comparison.

Species	Sample Size	Observed Weights (g) Mean	Std Dev	Range	Adult Mean:Range Weight (g)
Double-crested Cormorant	18	1160	± 249.8	805 - 1560	1800 - 3000[†]
Brown Pelican	10	2140	± 458.1	1420 - 2950	3200 - 3700[†]
Laughing Gull*	11	230	± 58.8	155 - 356	289 - 327[†]
Sanderling	11	52	± 6.2	40 - 63	40 - 100[††]
Sandwich Tern	5	125	± 22.3	93 - 148	140 - 300[†]
Great Blue Heron**	4	1771	± 1036.3	284 - 2686	2100 - 2500[††]
Northern Gannet	4	1713	± 207.4	1517 - 1932	2930 - 3070[†]
Osprey	4	1101	± 196.1	852 - 1307	1400 - 2000[††]
Royal Tern	3	416	± 95.3	306 - 475	320 - 500[†]

[†]Source: Schreiber and Burger, 2002.

[††]Source: Cornell Lab of Ornithology, 2003.

*Some individuals were partially decomposed, therefore listed weights may not provide accurate indication of body condition at time of death.

**Includes one nestling.

APPENDIX II: Brevetoxin findings for shorebirds collected during reported mortality event in the study region from August to October, 2005.

Species	Date of Death	Location Recovered	Liver	Stomach	Intestine
Sanderling	8/12/2005	N. Captiva Beach	202.39	66.40	187.04
Sanderling	8/26/2005	Dunedin	48.75	28.81	29.48
Ruddy Turnstone	8/29/2005	Clearwater Beach	46.26	58.35	44.56
Sanderling	9/4/2005	Sand Key	125.32	167.91	196.12
Sanderling	9/4/2005	Sand Key	38.84	19.01	31.05
Sanderling	9/5/2005	Sand Key	107.30	49.46	57.27
Willet	9/6/2005	Sand Key	254.64	238.60	91.76
Ruddy Turnstone	9/10/2005	Dunedin	25.73	80.99	117.38
Sanderling	9/22/2005	Fort DeSoto	201.90	119.69	129.05
Sanderling	9/22/2005	Fort DeSoto	74.28	105.93	47.97
Black-bellied Plover	9/24/2005	Isla del Sol	53.73	1267.42	122.88
Sanderling	10/12/2005	Fort DeSoto	115.76	185.31	126.18
Sanderling	10/14/2005	Indian Shores	135.19	239.18	82.49
		Mean	***110.01***	***202.08***	***97.17***
		Std Dev (±)	***72.59***	***328.60***	***55.36***

APPENDIX III: Results for all double-crested cormorants (*P. auritus*) collected during this study, and indication of brevetoxicosis diagnosis by attending rehabilitation facility.

	Location Found	Date of Death	Red Tide?	Liver	Stomach Contents	Intestinal Contents	Gall Bladder
DCCO1	Sanibel	1/8/2005	Yes	<LD	8.61	22.24	34.45
DCCO2	Sanibel	3/4/2005	Yes	107.91	161.53	116.57	
DCCO3	Sanibel	3/7/2005	Yes	82.76	30.68	35.94	247.22
DCCO4	Sanibel	3/7/2005	Yes	98.61	61.37	72.70	948.92
DCCO5	Sanibel	3/7/2005	Yes	<LD	<LD	<LD	32.38
DCCO6	Sanibel	3/8/2005	Yes	61.79	182.59	68.16	240.52
DCCO7	Sanibel	3/9/2005	Yes	121.52	442.91	67.14	418.13
DCCO8	Sanibel	3/9/2006	Yes	137.20	163.82	194.85	510.69
DCCO9	Sanibel	3/16/2005	Yes	121.93	88.52	136.14	310.95
DCCO10	Sanibel	3/17/2005	Yes	46.41	131.91	81.40	301.72
DCCO11	Sanibel	3/19/2005	Yes	107.31	237.43	96.25	685.87
DCCO12	Sanibel	3/29/2005	Yes	99.91	383.31	105.78	492.44
DCCO13	Nokomis	5/2/2005		14.56	16.40	103.13	
DCCO14	Indian Rocks Beach	6/22/2005	Yes	29.37	<LD	<LD	
DCCO15	St. Petersburg	6/28/2005		25.07	26.79	11.12	
DCCO16	Sanibel	7/9/2005	Yes	87.46	59.22	77.92	498.86
DCCO17	Sanibel	8/2/2005		28.46	32.08	31.66	719.25
DCCO18	Vina del Mar	8/19/2005	Yes	197.67	2310.32* 9988.62** 4095.08***	2645.35	718.43

*All stomach contents besides whole fish.
**Whole sample from partially digested pinfish.
***Muscle and skin of partially digested pinfish.