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Specimen Banking for Marine Animal Health Assessment

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Abstract—Marine animals are faced with health threats including disease and accumulation of toxic pollutants. There are several efforts in the USA seeking to relate health metrics to the exposure of marine animals to pollution, biotoxins, and disease. The National Institute of Standards and Technology (NIST) supports those efforts through projects directed towards marine mammals and sea turtles. The marine mammal activities conducted by NIST aid multiple investigators engaged in live-capture and release marine mammal health assessments. NIST developed a uniform collection protocol for blood, blubber, skin, and milk samples to be analyzed for persistent organic pollutants (POPs) and trace elements. The protocol includes instructions for collecting samples for immediate analysis as well as sub-samples for long-term archival. For bottlenose dolphins (*Tursiops truncatus*), the protocol was utilized 26 times along the US Atlantic coast, Gulf of Mexico coast, and off Bermuda. The work resulted in publications assessing pollutant exposure to bottlenose dolphins in these areas. NIST also supports efforts to monitor sea turtle health. A uniform protocol for collecting blood and scute samples from live sea turtles was developed and applied to turtles from several locations along the US East Coast resulting in publications detailing pollutant exposure and health effects.

Keywords: cetacean, sea turtle, health, pollution, sampling

INTRODUCTION

Specimen banking for marine animals, in particular cetaceans, sea turtles, and sea birds, provides opportunities to delineate geographic and temporal trends of many different constituents, including organohalogen compounds and trace

elements. Results from archived specimens were particularly useful in demonstrating concentration changes of persistent organic pollutants (POPs) in upper trophic levels in response to changes in POP production (Stapleton *et al.*, 2006). Information from specimens in environmental specimen banks (ESBs) has drawn attention to current use compounds that are increasing in the environment, such as brominated flame retardants and perfluorinated compounds (PFCs).

Historically, banked marine animal tissues are typically limited to blubber, liver, and kidney samples for marine mammals and eggs for seabirds. Samples from marine mammals are generally collected from dead, stranded animals, fisheries bycatch or subsistence hunts. The sea bird egg archive is primarily composed of eggs collected from the Alaska Maritime Wildlife Refuge—a very large wildlife refuge encompassing much of coastal Alaska. Health information is often unavailable for many of these samples.

Unlike traditional banking, marine animal health assessments rely on sample collection from living animals. In this type of assessment, animals are sampled, and then released back into the wild. This precludes the need for lethal sampling. Health assessments generally seek to explain the relationships between health endpoints and environmental toxin exposure. Non lethally-collected marine mammal samples used for pollutant exposure assessment include blood, milk, skin, blubber or fat and blood and scute scrapings from sea turtles. Health assessment studies have been conducted both on bottlenose dolphins and sea turtles (Wells *et al.*, 2004; Keller *et al.*, 2006; Swarthout *et al.*, 2010). Banking from marine animal health studies provides opportunities for future analyses that may not be obvious at collection time. Archived samples can be used for biomarker measurement, new pollutants identification, and the application of new "omic" techniques.

This paper describes the protocols developed by the National Institute of Standards and Technology (NIST) for the collection and archival of bottlenose dolphin and sea turtle samples collected from health assessments. Published studies using samples collected with the protocols are also summarized.

MATERIAL AND METHODS

Strategy for specimen banking for marine animal health assessments

Developing a banking strategy depends both on the health assessment goals and the sample availability. Strategy development starts with assessing project requirements with attention to short term analysis and long term archival. Many health assessment samples are utilized immediately. For example, blood collected for serological endpoints must be processed quickly (usually within 48 hours) after collection and is therefore not amenable to banking. Conversely, persistent organic pollutant (POP) or trace element samples can be held indefinitely at liquid nitrogen vapor temperatures (–150°C). Therefore, the banking component for marine animal health has focused on the archival of samples for POP and trace element analysis.

Uniform sampling protocols are essential to ensure that samples are collected both appropriately and consistently. Uniform protocols help to maintain sample integrity and comparability among samples collected at different times and locations. NIST typically provides trained personnel to participate in health assessment exercises to ensure that the collection protocol is being properly followed. If NIST personnel are unavailable, training of field personnel is undertaken.

Following sample collection, the last step in the strategy incorporates the samples into the specimen banking framework. This includes developing protocols on shipping, record keeping, and sample storage. Cetacean and sea turtle protocols are described below.

Cetacean protocols

The cetacean protocol was first developed for use during bottlenose dolphin health assessments and is the most formalized NIST effort. During these assessments, animals are first encircled with a net in shallow (<2 m) water and then physically restrained by field personnel (Wells *et al.*, 2004). While in the water, approximately 300 mL of blood is collected from the fluke of each animal using a double-ended butterfly catheter into vacuum blood collection tubes. The blood samples are mainly used for clinical health measures including erythrocyte and leukocyte counts, glucose, lipid, electrolyte, hormone and protein levels, and indices of liver and kidney function. Additionally, ~50 mL are used for other purposes including the assessment of POP, PFC, and trace element exposure (Fig. 1). Blood for organic contaminant analysis is collected into 10 mL glass heparin plasma tubes and for trace element analysis into 10 mL plastic EDTA whole blood tubes. The trace element tubes are collected first to avoid contamination by other sample tubes. Samples are kept cool (*ca.* 10°C) in the field and not frozen. Field blanks are collected by drawing deionized water through an unused catheter into a fresh blood collection tube.

Blubber and skin are surgically collected via 3 cm × 5 cm full-blubber depth biopsy following local anesthetia and cleaning of the skin with chlorhexadine 2% and methanol. Supplies, except the scalpel blade, are pre-cleaned with soap and water, rinsed with deionized water, rinsed with residue-grade organic solvent, wrapped in solvent-rinsed aluminum foil and then autoclaved. Individual supply kits are prepared for each dolphin. After collection, the ski/blubber biopsy is placed on a pre-cleaned glass Petri dish and covered. The biopsy is sub-sampled in the field using pre-cleaned scalpel and forceps. The epidermis is dissected, rinsed with deionized water, and sub-sampled into 1 mL or 2 mL cryovials for trace element, genetics, and stable isotope analyses. The blubber is sub-sectioned into two or more full depth pieces and placed in a 10 mL Nalgene jar and a 7 mL Teflon jar (for POP analysis). All epidermis and blubber samples are stored in liquid nitrogen vapor-phase biological dry shippers in the field and at –80°C or below upon return to the laboratory. Milk samples are collected using a method detailed elsewhere (Yordy *et al.*, 2010) and are transferred to a 7 mL Teflon jar or cryovial for POP/mercury analysis.

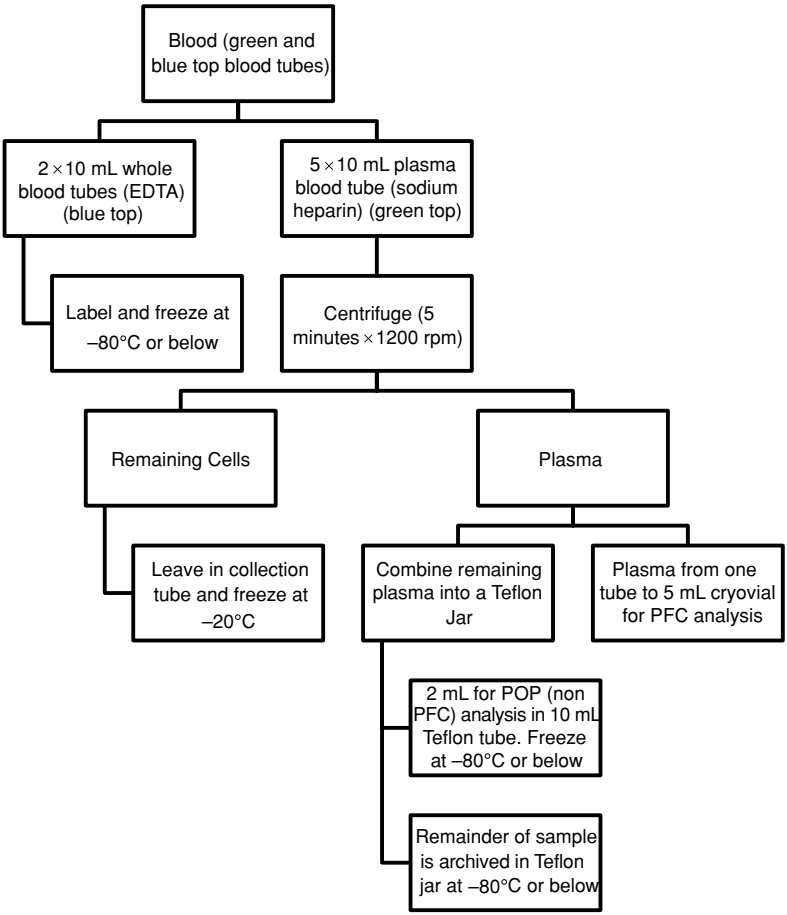

Fig. 1. General protocol for cetacean blood processing.

Sea turtle protocols

NIST collaborates with a variety of partners to sample sea turtles, and in most cases, the primary goals of the field sampling are not for health and contaminant measurements. For this reason, sea turtle protocols are more diverse than those for cetaceans and they require compromises because of field logistics, sample volume limitations, personnel training, and availability of sample storage. The following is a generic protocol description primarily for the collection of samples for contaminant measurements. Sea turtles are listed on the U.S. Endangered Species Act and on the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), so all sampling is performed non-lethally using minimally invasive procedures.

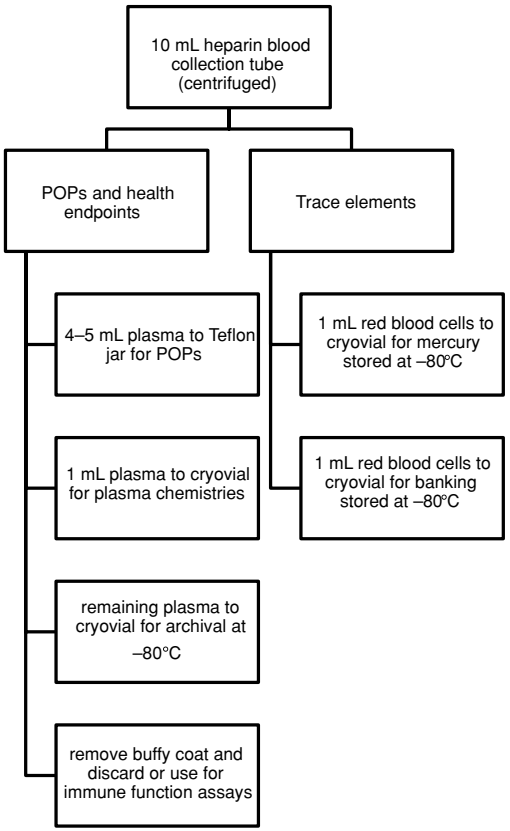

Fig. 2. General protocol for sea turtle blood processing.

Turtles are captured using pound nets, shrimp trawl nets, gill nets, or by hand. All age classes and sexes can be captured during "in-water" studies, while only adult females and eggs can be sampled from nesting beaches. Blood is collected from the dorsocervical sinus using a double-ended stainless steel needle directly into vacuum blood collection tubes. A 10 mL blood sample in a lithium heparin glass tube is shared for health measures (plasma chemistries and immune function assays; see above) as well as for estimating POP and mercury exposure (Fig. 2). If an additional blood tube can be collected, one that is certified trace element-free containing EDTA is typically used. It is important to note that sea turtle red blood cells (RBCs) will lyse in EDTA, so only whole blood can be obtained from this second tube. Often the glass heparin tubes are centrifuged and simply frozen at –20°C standing upright to keep the plasma separate from the RBCs. Later these tubes are thawed carefully and sub-sampled. While this is not an ideal procedure (plasma is best sub-sampled immediately), this level of processing is often not possible because of lack of trained personnel, concern that

the samples will become contaminated in the field, and other concerns. After collection, samples are kept cool (*ca.* 10°C) in the field and blanks are prepared as above. When possible, the 10 mL tube is processed on the same day as collection.

Scute scrapings from the outermost edge of the eight most posterior marginal scutes of the carapace are often collected for mercury measurements. Scutes free of fouling organisms are used or a plastic scraper is used to clear the target area. The marginal scute edge is scrubbed vigorously with plastic scrubbing pad to remove sloughing keratin. The region is rinsed with high purity water and isopropanol, wiped with clean room, particle-free wipers, and then rinsed again. The lateral edges of the prepared marginal scutes are removed by scraping a disposable stainless steel biopsy tool parallel to the scute's edge, allowing the shavings to fall into a polyethylene sample bag. Shavings of keratin ≈ 1 mm in thickness and 10 cm in total length (≈0.2–0.5 g) is targeted. The sample is kept cool until frozen at –80°C in the laboratory.

On rare occasions, fat biopsies (target of 1–2 g) are collected from live sea turtles that are transported to a clean, indoor location. Hexane-rinsed, sterile, stainless steel surgical instruments are prepared for each turtle. The subcutaneous fat sample is surgically removed from the left inguinal region following the administration of a local anesthetic. The incisions are sutured and glued closed, the turtles are monitored for a few hours, and then released. Fat samples are stored in hexane-rinsed aluminum foil at –80°C.

RESULTS AND DISCUSSION

Bottlenose dolphin health assessments

The protocol established by NIST was used on 26 separate bottlenose dolphin health assessment projects resulting in the sampling of several hundred animals for blubber, blood, skin, and in some instances, milk. The following is a brief summary of published findings relating to the pollutant determination in bottlenose dolphin samples collected using the protocol.

Surgical blubber biopsies from 104 juvenile and adult bottlenose dolphins from Sarasota Bay, Florida USA were analyzed for POPs including chlordanes, DDT compounds, polychlorinated biphenyl (PCB) congeners, polybrominated diphenyl ether (PBDE) congeners, dieldrin and chlorobenzenes (Yordy *et al.*, 2010). Concentrations and mixtures of POPs in bottlenose dolphins from this location were strongly influenced by both age and gender. POPs and PFCs were determined in 139 surgical blubber biopsies collected from dolphins living near in Charleston, South Carolina waters and the Indian River Lagoon, Florida. PCBs dominated the POP profile and PFCs represented less than 1% of the blubber organohalogen burden (Fair *et al.*, 2010).

Blood was collected from bottlenose dolphins for the assessment of dolphin exposure to organohalogen pollutants including PFCs and trace elements. PFCs were determined in 109 plasma samples collected from dolphins from two US East Coast locations, Sarasota Bay Florida, and Bermuda (Houde *et al.*, 2005).

Median concentrations of perfluorooctane sulfonate (PFOS) were 1170 ng/g ± 190 ng/g in the blood of dolphins sampled from the Charleston Harbor (South Carolina). The lowest observed concentration of PFOS was a mean of 49 ng/g collected from two dolphins sampled near Bermuda. Perfluorinated carboxylic acids (PFCAs) were also quantified in blood samples. Mercury and other trace elements were determined in 51 whole blood samples obtained from the Sarasota Bay population (Bryan *et al*., 2007). Good correlations ($p < 0.05$) were established between blood and skin from dolphins for V, As, Se, Rb, Sr, and Hg demonstrating that skin is useful for monitoring these elements. Mercury was also determined in bottlenose dolphin blood and skin samples collected from Charleston and Indian River Lagoon (Stavros *et al*., 2007, 2008).

Sea turtle studies

The protocol outlined above has been applied in several studies investigating contaminant exposure in living sea turtles. For example, 44 juvenile loggerhead (*Caretta caretta*) sea turtles were live captured in the pound-net fishery in Core Sound, North Carolina, US. Individual turtles were sampled for blood and adipose tissue. Lipid normalized POP concentrations in blood and adipose tissue were significantly ($p < 0.05$) correlated indicating that POP concentrations in blood are a good proxy for exposure (Keller *et al*., 2004). POP concentrations in sea turtle blood from this study correlated to immune response parameters indicating immunomodulation (Keller *et al*., 2006).

The protocol also was successfully applied for the determination of PFCs and mercury in sea turtles. PFCs, including PFOS and PFCAs, were determined in blood from 73 loggerhead sea turtles and six Kemp's Ridley sea turtles collected in trawl samples conducted from the northeast Florida coast to the central South Carolina coast and from the North Carolina pound net fishery (Keller *et al*., 2005). Plasma PFC concentrations were 2 to 12 times higher than the total PCB concentrations in blood and were significantly higher in northern than southern turtles. Paired blood and scute samples were collected from 34 live captured loggerhead sea turtles from the Southeast US (Day *et al*., 2005). Blood and scute total mercury concentrations were highly correlated ($r^2 = 0.93$). Scute concentrations, representing long-term mercury exposure, were used with blood concentrations, representing recent mercury exposure, to investigate the exposure history of individual turtles to possible mercury sources. The index of recent exposure (residual from the blood mercury versus scute mercury regression) in turtles varied by location generally in accordance with potential industrial sources.

The protocols developed by NIST for the collection and banking of marine animal blood and tissue samples for contaminants and trace element analysis continue to be applied to marine animal health assessments. For example, 29 bottlenose dolphins were sampled from the Georgia, US coast in August 2009 and 18 beluga whales (*Delphinapterus leucas*) from Bristol Bay, Alaska were sampled for blood, blubber, and skin in May and September, 2008. In addition, the

protocols have been used almost annually since 2002 in Sarasota Bay, Florida for samples collected from dolphin health assessments (Wells *et al.*, 2005) and variations of the protocols have been adapted for the monitoring of sea turtles and marine mammals for the impacts of oil resulting from the British Petroleum oil spill in the Gulf of Mexico in 2010.

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