4. NASTNet Protocol on Sea turtle Blood and Tissue sampling + Epibionts (Final version)

Introduction

The blood and tissue specimens collected from Sea turtles can provide an important biological sample for scientific investigations, such as genetic studies, environmental contamination, pathological studies, tissue banking, etc.

Any sampling program should be conducted by qualified and trained personnel in the project team. Taking specimens is usually invasive and, if not completed in a precise manner, may lead to injuries and later inflammations for the affected turtle organs. Such inflammations may cause the turtle to become sick and lead to death.

The following protocol describes the basic blood and tissue sampling procedures requirements.

Sampling Goal

Taking blood or tissues from turtles for conduction of either scientific investigation or building a tissue bank for future usage should take into account the exact goal of this activity.

Some goals may include having a representative collection of tissues on an annual basis from prioritized sea turtle species and geographic locations for real-time and retrospective contaminant, health-related research studies, phylogenetic studies to investigate population structure, stable isotope analyses, and physiological studies to understand the health condition of laying or bycaught Sea turtle.

Sample preservation considerations

1. According to scientific protocols, sample collection, packaging, transport, cataloging, homogenizing, archiving, and sample sharing should be performed. If available, storage is preferably in liquid nitrogen vapour-phase temperatures (-150 °C), which provides the best conditions for minimizing sample degradation. Ethanol 70% should be used if freezing nitrogen is unavailable on site. No Formalin should be used if the study is related to the genetic investigation, as it alters the structure of DNA.
2. Preserved tissue or blood samples in a national tissue bank (or country collection) should be available to researchers/ partners for future studies.
3. Before collecting samples, the technical and financial capacities should be considered, i.e., available funds, equipment, skilled and available personnel.
4. Sterile surgical gloves should always be worn. Blood is usually collected from the cervical sinus of adults after cleaning the site with ethanol before sampling.
5. The sinus is on the neck sides, 1/3 - 1/2 way toward the back of the head from the carapace anterior edge.
6. Depending on the size of the turtle, the sinus is from 0.5-3 cm lateral to the midline.
7. With practice, the sample can be taken within 30 seconds.
8. Lithium or sodium heparin is best for an anticoagulant. EDTA (also an anticoagulant) should be avoided since it causes hemolysis in sea turtle blood.
9. It is important to position the turtle so that the sinus fills with blood. For this reason, consistent results have been obtained when the turtle's head is lower than the body. An angled restraining rack, a slanted table or bench, or an inclined nesting beach (with assistants doing the restraining) work well.
10. Always carefully clean the neck with alcohol (containing at least 70% concentration of ethanol) or other antiseptics before sampling.

11. The most common solid tissue biopsied is the skin (Jacobson 1999).

12. The biopsy site is treated with a local anesthetic and then a surgical scrub (including ethanol and iodine). The sample can be obtained using a scalpel blade (#10 or #15) or a disposable biopsy punch. Subsequently, the site should be cleaned and then sutured or left to heal (Jacobson 1999).

13. For histologic evaluation, a portion of each sample should be fixed in neutral buffered 10% formalin (NBF), with tissue to fixative volume ratio of 1:10 within 24 hours and ethanol beyond 48 hours.

14. They should be cleaned with sterile saline for microbial isolation and then placed in the specified transport media. Samples should never be frozen.

15. Tissue and blood samples can be collected from live and dead hatchlings. Skin is probably the best tissue type for stable isotope analysis and can be sampled easily from both dead and live individuals and integrated diet over several months.

16. In nesting areas, hatchlings and eggs could be sampled as proxies of samples from females. This information can confirm the sex and infer multiple paternities of the nests.

17. Most genetic data are based on mitochondrial haplotypes and nuclear microsatellites, which allow the individual assignment of loggerhead and green turtles to major nesting/foraging areas.

18. The use of SNPs (Single nucleotid polymorphism) is recommended to improve individual assignation

19. All samples should be centrally stored in each country to ease the access for national or regional studies using the samples.

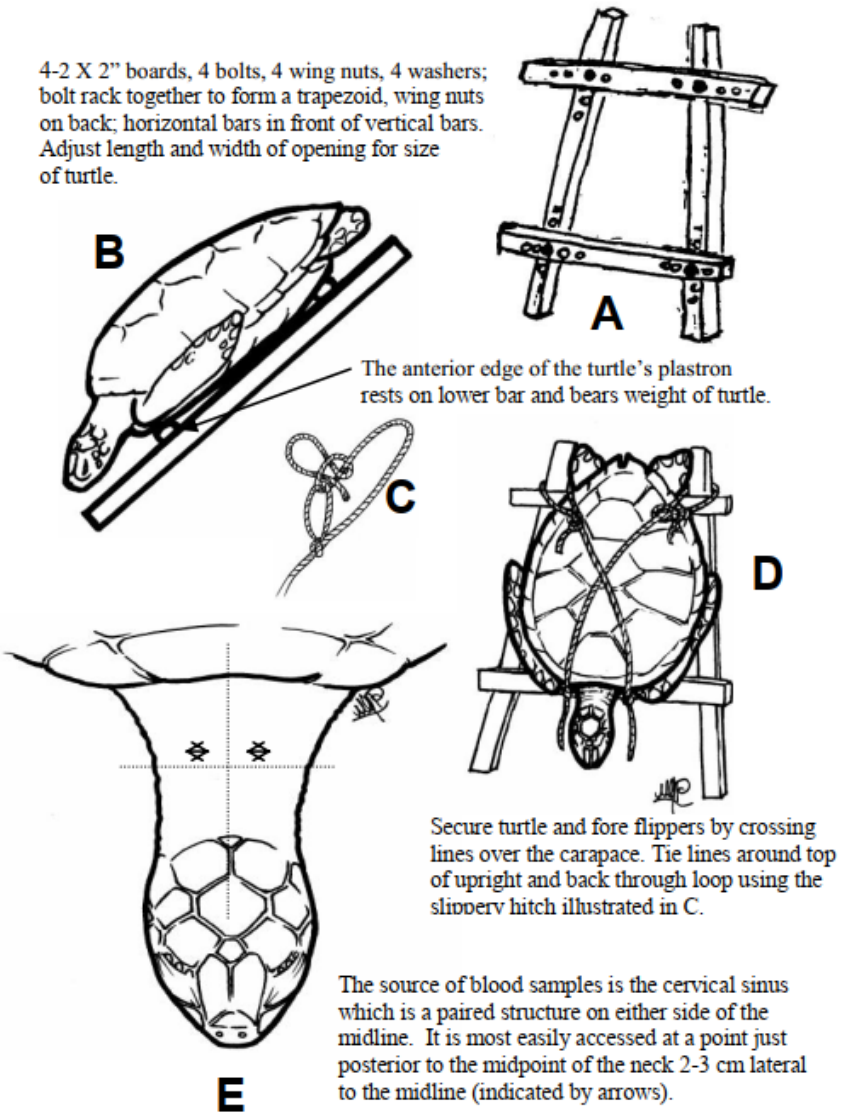


Figure 1. Illustrations for blood collection from the cervical sinus. A) bleeding rack; B) lateral view of correct placement of turtle on rack; C) slippery hitch for tying turtle; D) frontal view of a turtle on rack; E) detail of neck with the site of the cervical sinus (after: Meylan et al., 2016)

Epibiont Sampling Sheet (After Pinou et al., 2019).

COLLECTOR _____ *SAMPLING NUMBER* _____

Epibiont Sampling Sheet (ENGLISH)

HOST INFORMATION (injury information on back)

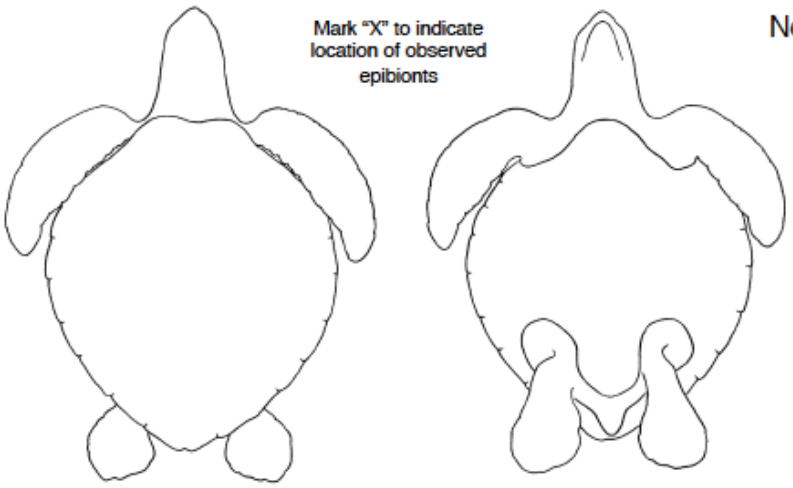
Turtle species: _____ ☐ Positive ☐ Probable ☐ Unsure

Morphometrics: SCL _____ SCW _____ CCL _____ CCW _____

Tags: ☐ Present ☐ Absent LFF _____ RFF _____ LRF _____ RRF _____

State of turtle: ☐ Free-roaming ☐ Nesting ☐ Stranded [☐ Alive ☐ Dead ☐ Cold-stunned]

EPIBIONT INFORMATION Epibionts observed: ☐ Present ☐ Absent



Notes:

Epibionts collected: ☐ All ☐ Some ☐ None Diatoms collected: ☐ Yes ☐ No

BODY REGION	COLLECTED?	COMPLETE?	NUMBER OF CONTAINERS
Head (H)	☐ Yes ☐ No	☐ Yes ☐ No	
Neck (N)	☐ Yes ☐ No	☐ Yes ☐ No	
Shoulders (SH)	☐ Yes ☐ No	☐ Yes ☐ No	
Front flippers (FF)	☐ Yes ☐ No	☐ Yes ☐ No	
Rear flippers (RF)	☐ Yes ☐ No	☐ Yes ☐ No	
Inguinal (ING)	☐ Yes ☐ No	☐ Yes ☐ No	
Carapace (C)	☐ Yes ☐ No	☐ Yes ☐ No	
Plastron (P)	☐ Yes ☐ No	☐ Yes ☐ No	

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COLLECTOR _____ *SAMPLING NUMBER* _____

FIELD INFORMATION DATE _____ TIME _____

Latitude / Longitude: _____ / _____

Site/Beach _____ City/Town _____ Country _____

Habitat: ☐ Beach [☐ Nesting ☐ Stranded] ☐ Inwater [☐ Nearshore ☐ Open Ocean]

Description _____

Capture method: ☐ Beach patrol ☐ Hand-capture ☐ Fishing gear [☐ Net ☐ Hook/line]

Description _____

Estimated time between turtle capture and epibiont survey/collection: _____

Temperature: Water _____ Air _____ Sand _____

Rainfall: Date of most recent rainfall (>5cm) _____ Amount _____

INJURY INFORMATION Injuries: ☐ Present ☐ Absent

Body part: ☐ H ☐ N ☐ SH ☐ LFF ☐ RFF ☐ LRF ☐ RRF ☐ ING ☐ C ☐ P

Description _____

Missing appendages: ☐ Yes ☐ No Description _____

Lesions: ☐ Yes ☐ No Description _____

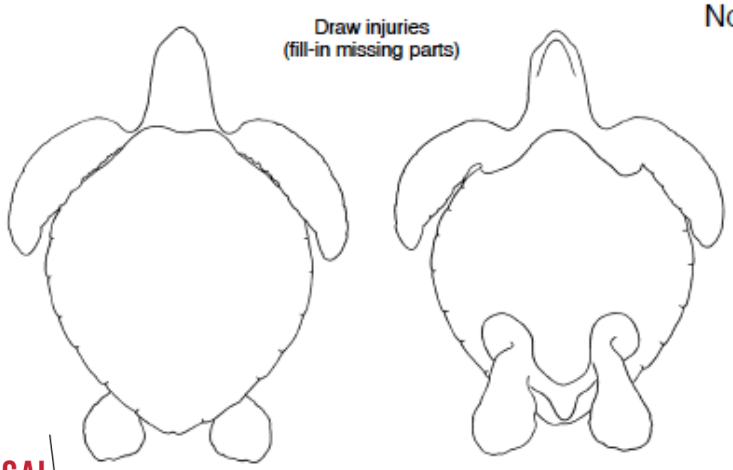
Tumors: ☐ Yes ☐ No Description _____

Hooks or fishing line: ☐ Yes ☐ No Description _____

Cracked carapace or plastron: ☐ Yes ☐ No Description _____

Notes:

Draw injuries
(fill-in missing parts)



CRITICAL ECOSYSTEM PARTNERSHIP FUND

Specific biopsy sampling protocol *(after Poppi and Marchiori, 2013)*

3.1 Life History

- Age determination –
  - ❑ Humerus, should be kept frozen.
- Reproductive Status –
  - ❑ Gonads and Uterine samples fixed in 10% NBF.
- Feeding Habits –
  - ❑ Stomach contents can be collected into a sealable plastic bag or jar, freeze.
  - ❑ Carapace keratin, for stable isotopes should be kept frozen. What the animal has been eating recently.

3.2 Genetics

- Internal organs, Muscle, Bone - Better to freeze tissue samples, in case the tissue is used for something other than genetics. Genetic tissue samples can be fixed in DMSO saturated with NaCL.
- Blood – can only be collected from Code 1 and 2 animals. Minimum amount is ~10 ml; 50-100 ml is optimal for DNA studies.

3.3 Microbiology

Take separate samples for bacteriology and virology. Lesions should be sampled from several distinct locations, include normal tissue with the infected tissue sample.

- Bacteriology – Avoid freezing samples for bacteriology if avoidable. Refrigerate samples at 4 C. Freezing at –70°C is preferable to decomposition.
  - ❑ External samples can be taken with a swab from the eye, and vent. Culture swab in a bacterial transport medium.
  - ❑ Internal samples can be taken from the intestine, heart, kidneys, lungs, liver, spleen, bone with marrow, and tissues showing pathological changes. Culture swab in a bacterial transport medium or 6 x 6 cm sample placed in a sterile container.
  - ❑ Fluid samples can be taken from the pleural fluid, peritoneal fluid, urine, blood, fluid from abscesses. Store in appropriate aerobic or anaerobic vial.

- Virology – Refrigerate samples at 4° C.
  - ❑ External samples use a sterile swab dipped in viral transport medium. Take samples from the eye and vent. Place swabs in

the vial that **contains** the viral transport medium.

- ❑ Internal samples can be taken from the CNS tissues, lungs, liver, spleen, kidneys, tissues with pathological changes, intestinal contents. 6 x 6 cm sample placed in a sterile container.
- ❑ Fluid samples from pleural fluid, peritoneal fluid, pericardial fluid, urine, blood from heart. Store in sterile container.

3.4 Parasites

- Barnacles – first fix in 10% NBF, for no more than 24 hrs, then transfer to 70 % EtOH.
- Copepods & Amphipods – place directly into 70% EtOH.
- Nematodes (roundworms) – Fix in GAA for 5-10 minutes first if possible. Otherwise use 70% EtOH or 10 % NBF. If formalin is used, fix only for a few hrs. Then transfer to 70% glycerin alcohol.
- Trematodes (flukes/flatworms) – Dead or alive, fix in AFA for up to 3 days, transfer to 70% EtOH. Do Not use glycerin alcohol.
- Cestodes (tapeworms) – Fix for 5-10 min. in AFA solution and water, 4:1 ratio. Transfer to 70% EtOH. Include cestode head when removing from the host, if necessary cut host tissue.
- Acanthocephalans – Fix in AFA for up to 24 hrs. Then transfer to 70% glycerin alcohol.

3.5 Histopathology

Lesions, fractures, lacerations, and gunshot wounds of any code should be sampled in this manner.

- Tissues should be preserved in 10% NBF.
- Tissue samples should be no larger than 3 x 3 cm and approximately 1 cm in thickness.
- Ideally, histo samples should be cassetted and placed into a labeled jar for the appropriate Institution / researcher. Individual requests should be noted and tracked.
- Samples of gross lesions should include abnormal and normal tissue.

3.6 Contaminants / Biotoxins:

(organochlorines, heavy metals)

- Biotoxin Analysis – Code 2 animals only. Collect stomach contents, liver and/or kidney tissue. Freeze.
- Contaminant Analysis – All of the samples should be frozen in plastic zip lock bags and/or wrapped in aluminum foil.
  - ❑ Skin and subcutaneous tissue, muscle, liver, kidney, and

brain (if possible include cerebrum and cerebellum).

SAMPLES SCHEMA (*after: Poppi and Marchiori, 2013*)

	Standard Samples							
	Life History	**Genetics**	**Parasites**	**Histo.**	**Contam.**	**EnteroBT**	**HerpesV R**	**Biotox**
Tissue*	(Frozen or fixed)	(Frozen &/or DMSO)	(70% EtOH)	(10% Formalin)	(Foil wrapped and frozen)	(Culture swab)	(Frozen)	(Frozen)
Adrenal								
Blood/Serum								
Brain								
Carapace								
Esophagus								
Fat deposition								
Feces								
Heart								
Humerus								
Intestine								
Kidney (R)								
Kidney (L)								
Liver								
Lung (R)								
Lung (L)								
Muscle								
Oral Mucosa								
Ovary								
Pancreas								
Skin								
Spleen								
Stomach								
Stomach Contents								
Testis								
Thyroid								
Trachea								
Urine								

* Only white cells should be filled

Refernces:

Meylan, P., Gary, J., Hardy, R & A., Meylan. 2016. Proceedures Manual For The Bermuda Turtle Project. 6th revision. pp.27. available from http://bermudaturtleproject.org/wp-content/uploads/01-BTP_Procedures_Manual_2016_revision.pdf

Pinou, T., Domenech, F., Lazo-Wasem, E. A., Majewska, R., Pfaller, J. B., Zardus, J. D., & Robinson, N. J. (2019). Standardizing sea turtle epibiont sampling: outcomes of the epibiont workshop at the 37th International Sea Turtle Symposium. *Marine Turtle Newsletter*, (157), 22-32.

Poppi, L., & Marchiori, E. (2013). Standard protocol for post-mortem examination on sea turtles. *Network for the Conservation of Cetaceans and Sea Turtles in the Adriatic*. https://www.plavi-svijet.org/bw/wp-content/uploads/2017/05/NETCET_Standard-protocols-for-post-mortem-examination-of-sea-turtles.pdf