

Agenda Item 2

Review of New Information on Threats to
Small Cetaceans (reporting cycle 2017 only)

Use of Bycatches and Strandings

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**Cetacean Pathology: Necropsy
Technique & Tissue Sampling**

Action Requested

- Take note
- Comment

Submitted by

IJsseldijk & Brownlow



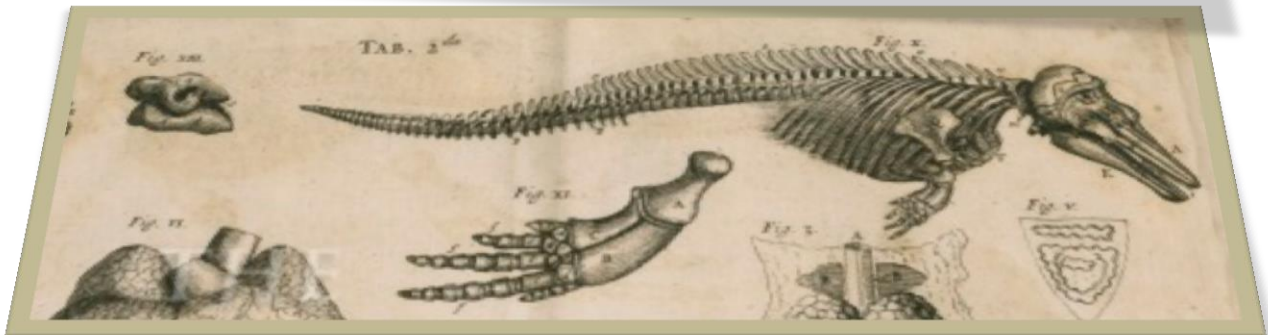
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Secretariat's Note

The Rules of Procedure adopted at the ASCOBANS 8th Meeting of Parties remain in force until and unless an amendment is called for and adopted.

CETACEAN PATHOLOGY: NECROPSY TECHNIQUE & TISSUE SAMPLING

Based on proceedings of the ECS workshop on cetacean
pathology, Funchal Madeira, April 2016



Editors

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PROTOCOL FOR THE BASIC POSTMORTEM EXAMINATION AND TISSUE SAMPLING OF CETACEANS

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1. Introduction and background

Monitoring health in cetaceans is critical to identify and quantify the role of disease, trauma, and environmental and ecological factors affecting these populations. At a basic level, information derived from strandings can give an indication of the composition of the cetacean fauna in an area when other sources of information are not available. For less common species, it may even be the only source of information available. Coherent investigation of carcasses can assist in the determination of cause of death and can provide general surveillance on the incidence of disease in stranded cetaceans. It can identify existing and emerging threats due to human impact, such as bycatch and marine pollution, as well as providing tissues and data for subsequent analysis into a range of biological and ecological parameters.

Several European countries have operating stranding networks around their coastlines as part of their obligations to international agreements, such as the “Agreement on the Conservation of Small Cetaceans of the Baltic, North East Atlantic, Irish and North Seas” (ASCOBANS), the “Agreement on the Conservation of Cetaceans in the Black Sea, Mediterranean Sea and contiguous Atlantic Area” (ACCOBAMS), the EU Habitats Directive, the OSPAR Commission and the “Baltic Marine Environment Protection Commission” (HELCOM). Many of these networks are well established, owning long-term datasets comprising extensive biological and pathological information from systematic collation and investigation of mortalities. In addition, many network curate extensive tissue and pathogen archives spanning many years. In other regions, stranding networks are less extensive or longstanding, but nonetheless informative on the health of the marine environment. Properly collected and analysed data derived from marine animal strandings can provide vital insights into cetacean biology and ecology and quantification of the health of, and threats to key marine species. Many cetacean species are highly mobile, ranging widely across ecosystems that bestride international borders. Establishing if any observed trends are consistent across or between countries therefore requires a harmonisation of data collection and agreement of data sharing.

In September 1991 a cetacean pathology workshop was held to standardise the measurements, sampling protocol and methodology for the diagnostic post mortem examination of small cetaceans. Initiated by Thijs Kuiken and Manuel Garcia-Hartmann, the proceedings from this workshop were published by the European Cetacean Society (in 1993) and proved to be an essential resource to those working in the field. Twenty-five years later, knowledge on many aspects of the causes of cetacean mortality has increased and there have been significant technological advances in the acquisition of stranding data, such as mobile phones, social media and digital photography. Sample analysis and diagnostics, particularly those related to the fields of ‘omics’ research have developed significantly since the original protocol was created. It was considered timely to revise the protocol and a second workshop was organised in Funchal, Madeira on 17th March 2016, after the 30th annual conference of the European Cetacean Society. Representatives from seven stranding networks attended and the list of attendees is given in Appendix 1 of this document. The workshop aimed to:

- 1) review the existing standardised protocol of the diagnostic post mortem examination of cetaceans;
- 2) update the existing protocol on tissue sampling for microbiology, parasitological, virology, toxicology and histopathology;

- 3) investigate scope to extend the protocol to aid additional research and include protocols currently used to diagnose specific causes of death, including those of anthropogenic origin and to provide necropsy guidance for mass mortalities and large whale strandings.

During the workshop, a review of the existing protocol was undertaken following aims one and two of the workshop. Additionally, during the workshop experts were identified to describe advanced topics, following aim three, and it was discussed how protocols describing specific causes of death could be accessible for both existing as well as new stranding networks. This latter aim is still being discussed among the group of experts and therefore not further included in this document. This document presents the update of the existing protocol. The contributors as listed on the cover provided comments on this document after the workshop.

It should be emphasised that this protocol is not designed to supplant existing protocols of established laboratories or stranding networks. It serves two aims: 1) Highlighting areas where harmonisation of data from existing networks can allow for analysis and inference to be made between networks, and 2) Offer a technical framework for those planning to develop or expand new strandings monitoring networks.

This document has been written under the assumption that the time available and circumstances suitable to undertake a full necropsy are available and that definitive diagnosis of the health status and possible cause of death is prioritised over tissue collection for additional research.

1.1 Sample triage

Although this protocol has been written under the assumption that there is sufficient time and resources to carry out a full necropsy, the description of conducting a necropsy and sampling procedures can be seen as a suggestion. Necropsy steps may differ depending on e.g. logistics and species assessed, whilst sampling may differ depending on institutes, laboratories, and researchers involved or research priorities that require different sampling approaches.

If a full post mortem examination cannot be carried out, one should always attempt to minimally collect the following data: species, gender, stranding location, stranding date and (approximate) body length to assess age class. Additionally, teeth (for ageing), skin (for genetic analysis) and swabs of genital slit and blowhole (for e.g. virological analysis) can be relatively easily collected. However, to be able to assess cause of death and health status, a full post mortem investigation with additional testing as proposed below is deemed necessary and therefore highly recommended.

The extent of investigation into stranded animals is contingent on a range of factors; logistics, resources, funding, carcase condition and the scientific, social and political priorities in operation in that region. It may be useful however to consider each investigation as hierarchical, and triage individual cases into one of three tiers:

Tier One: Basic morphometric and descriptive data- “what-where-when?”, assessment of body condition, carcase condition, sex and age determination, and collection of samples for genetic analysis.

Data and samples can be collected at this level with basic training and equipment and may be suitable to be collected by suitably trained members of the public, or in situations where time or

logistics or carcase condition preclude more detailed investigation. A Tier one examination often forms the initial part of a post mortem investigation, where subsequent examination of all internal organs and tissues and sampling in combination with additional analyses aims to diagnose a cause of death. When due to practical, financial or logistical reasons it is decided to only conduct an external examination at this level, this will severely limit the ability to generate conclusions on health status and cause of mortality of these cases assessed.

Tier Two: Post mortem investigation: This involves a thorough pathological necropsy on the animal, involving the visualisation and gross inspection of all organ systems and appropriate subsequent diagnostic testing. This potentially allows for a detailed assessment of indications of trauma and the burden of disease, assessment of the ante mortem health of the animal and likely cause of death.

Tier Three: Ancillary tests and population-level analysis: This involves additional and more detailed analysis of the data and samples collected by post mortem and can be useful to identify subclinical health effects and potentially infer effects at a group or population level. Analysis can include life history and diet analysis, age determination, assessment of contaminant burdens and identification of infectious agents, both as historic exposure and active infection. This stage often requires specialised laboratories and can be carried out in collaboration with other stranding investigation groups.

1.2 Interpretation and biases of strandings

It should be emphasized that while strandings provide a unique sample and data of the living population that is difficult to obtain by most other means of surveillance, there is uncertainty around the extent to which strandings investigation can inform on the at-sea population. Strandings are a complex function of biological, physical and social (effort) processes that influence observed (causes of) mortality. Each process introduces a level of bias, and these should be taken into account when making population level inferences based on the examination of stranded individuals. Where an increase in strandings could indicate increased mortality or increased abundance (biological variation), it may well be a function of unusual variation in environmental conditions (referring to physical aspects) or observer efforts (social). It is encouraged to conduct full characterisation of all three components of the stranding process during (unusual) mortality investigations.

1.3 Social media

The past few years has seen an explosion in digital technology which is fundamentally changing how investigators respond to cetacean stranding events. Information can flow faster and more accurately between first responders and expert teams than ever before, and this can revolutionise how these time-critical incidents can be successfully managed. Such connectivity can however broadcast any stranding event, in real time, to a global audience, and this presents an increasing requirement to effectively manage the public expectations of how such events are managed. Stranding response needs to be done with a clear idea of the welfare and conservation priorities for casualty animals whilst maintaining public safety and sensitivity to the needs and expectations of local community. Nonetheless, these digital technologies are generally useful, allowing for species, body and carcase condition, sex, age class, location and some causes of death to be reliably established to a level of accuracy not previously possible.

1.4 Health and Safety

The collection, transport and post mortem examination of marine mammals potentially exposes several risks. It is strongly advised that each scheme conducts a review of their health and safety protocols according to their local or national operating procedures and legislations.

It is outside the scope of this protocol to provide comprehensive assessments for all possible hazards, however a few key principles and factors are highlighted below:

Biological risks: The zoonotic risk from marine mammals is reported to be low. There is an infection hazard from cuts and penetrating injuries and/or exposure of broken skin to fluid from a marine animal. Any such exposure should be reported to a medical professional. Immediate disinfect, or in case of direct eye contact eye rinsing is necessary and possibly merit appropriate antibiotic prophylaxis. It is advisable to highlight the potential risks from *Brucellosis* and *Mycoplasma* inoculation ('seal finger' where initial symptoms may include redness and swelling of the hand), *Erysipelothrix rhusiopathiae*, and the possibility of hitherto unknown pathogens.

It is strongly advised that a hygiene protocol is put in place for all individuals that are involved with the retrieval, transportation and investigation of (samples derived from) marine mammal carcasses. All sampling should be done using gloves and appropriate personal protective equipment and, in the case of live animals, with suitable respiratory protection to guard against aerosol inhalation. Humans with impairment of the immune system (e.g. due to cortisol treatment, pregnancy, certain viral infections etc.) have a higher susceptibility to (zoonotic) infections and should therefore avoid contact with (samples derived from) stranded marine mammals.

Environmental risks: Hazards of working on remote locations, possibly with poor cell phone reception, hazards of the coastal environment such as slips and trips, tide, weather, low visibility and risk of hypo- and hyperthermia. The physical and machinery hazards associated with moving a large marine animal should also be considered.

Post mortem risks: Prior to any examination involving the moving or opening of a carcase, it is essential to assess and mitigate any likely environmental or public health hazards. Biosecurity factors to consider should include risks from the leakage of fluids, noxious odours or aerosols from the carcase and the aesthetic and social impact on civil society and individual members of the public. It is advised that wherever possible post mortem examinations are conducted in a secure area with adequate access to light, clean water and effective containment of effluent.

2. Tier one: Basic morphometrics and external examination

2.1 Morphometric studies and imaging

If the capacity exists, assessing carcasses by Computed Tomography (CT) and/or Magnetic Resonance Imaging (MRI) prior to necropsy will allow imaging and evaluation of a range of morphometrics and identification of potential pathologies. CT-scanning will allow visualisation of skeletal anatomy and lesions, assay bone density, assess joints and inter vertebral discs. MRI allows the assessment of internal anatomy (including position of organs), all soft tissues and potential pathologies. In addition, the collection and maceration of skeletal material, or the entire skeleton is of value for stable isotope analysis, morphometric studies or accession to museum collections. Definitive diagnosis of cause of death should however take preference over maintaining skeletal integrity for museum collections, and histology samples should be taken of any bone abnormalities that may be present in an animal.

2.2 Condition scoring

The carcase condition / decomposition condition code (DCC) should be judged at the time of the necropsy. It should be judged on an evaluation of all organ systems. If the carcase shows a varying state of preservation throughout the carcase (e.g. due to partly scavenging) an average state of preservation should be assigned. The external status can be ascribed using the following categories (Figure 1):

- 0) Live (becomes code 1 at death);
- 1) Extremely fresh (as if just died, no bloating);
- 2) Slight decomposition (slight bloating, blood imbibition visible);
- 3) Moderate decomposition (bloating, skin peeling, penis may be extended in males, organs still intact, excluding post mortem damage);
- 4) Advanced decomposition (major bloating, skin peeling, penis extended in males, organs beyond recognition, bones exposed due to decomposition);
- 5) Indeterminate (mummified carcase or skeletal remains).

Carcasses frozen prior to examination (see Tier Two, chapter 3.1) are automatically at minimum code 2 (or higher), due to the artefacts invariably induced by the freeze/ thaw process.

Carcase condition dictates which samples are possible and/or useful to take. Clinical examination and sampling of live stranded cetaceans is beyond the scope of this report and not discussed further. In general samples for:

Histology	codes 1-3*
Virology	codes 1-3*

Bacteriology	codes 1-3*
Parasitology	codes 1-4*
Toxicology	codes 1-4*
Genetics	codes 1-5
Skeletal material	codes 1-5

* The suitability and relevance of different sampling in animals that are in advanced decomposition needs to be evaluated on a case-by-case basis and according to the laboratories or research priorities involved.



Figure 1: Carcase Decomposition Condition Codes (DCC). Pictures: Utrecht University

2.3 Photographs

Clear photographic documentation is an important part of any pathological assessment. Images should contain a scale bar and method to unambiguously attribute the carcase to the unique identifier for the case. Given the storage requirements of high resolution digital media it is advised to consider archiving to a secure location e.g. cloud based storage.

The following photographs should be taken:

- Lateral overviews of the whole body, both sides;
- Genital slit region;
- Skyline image outlining end on profile (Figure 2A);
- Close-up of the fins and fluke (Figure 2B);
- Close-up from head region and teeth (Figure 2C);
- Close-up of any lesions or abnormalities with suitable scale bar.

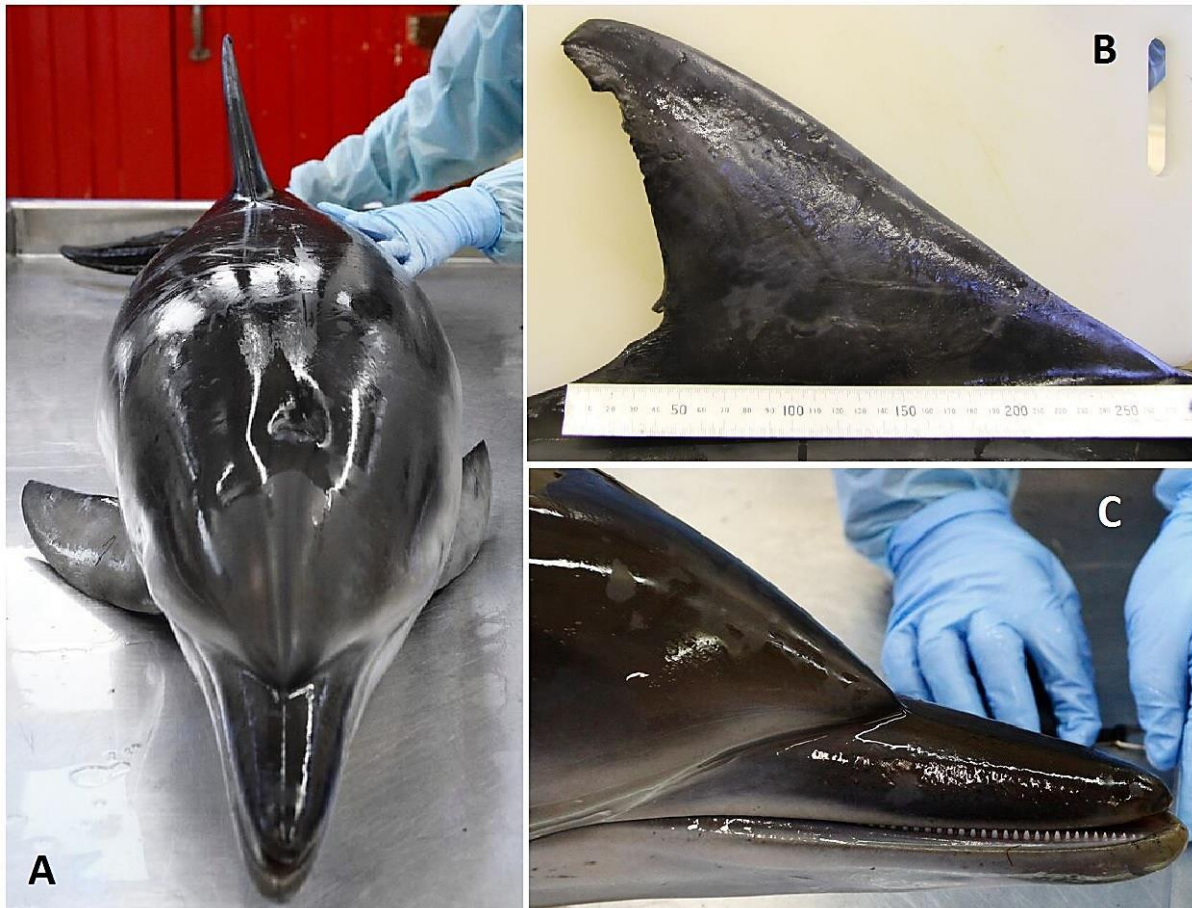


Figure 2: Carcase photograph examples. A: Skyline image outlining end on profile. B: Close-ups of the extremities. C: Close-up showing teeth. Images: Charlie Philips (A,C) and Mariel ten Doeschate (B)

2.4 Body measurements

Two principal measurements should be taken: total length and girth. Measure the length by placing the animal on its belly (if possible), holding a measuring tape or ruler next to the carcase in a **straight line parallel to the longitudinal body axis** and measuring the distance between the notch (if present) in the tail fluke and the tip of the rostrum (Figure 3). Measure the girth, in cm, by placing the measuring tape around the carcase **immediately cranial to the dorsal fin** without compressing the body. A second or girth measurement may be taken at the level of the axilla, **immediately caudal to the pectoral fin** (Figure 3). If it is not possible to take an encircling measurement (e.g. if dealing with a large whale), take a half girth measurement and double it.

To measure the blubber thickness, cut a cross sectional strip of blubber a few cm wide and a few cm long at the level of the cranial insertion of the dorsal fin. Make sure to cut at right-angles to the surface of the skin. Measure the thickness of the blubber strip, in mm, just beside the dorsal mid-line (using this method, the tension of the blubber tissue is relieved before measuring, which guarantees a more accurate reading). Take blubber thickness measurements from three locations (as shown in Figure 3): dorsal (A), lateral (B) and ventral (C). Note that the epidermis is not measured. Additional measurements can be taken accordingly, depending on different research questions and working methods.

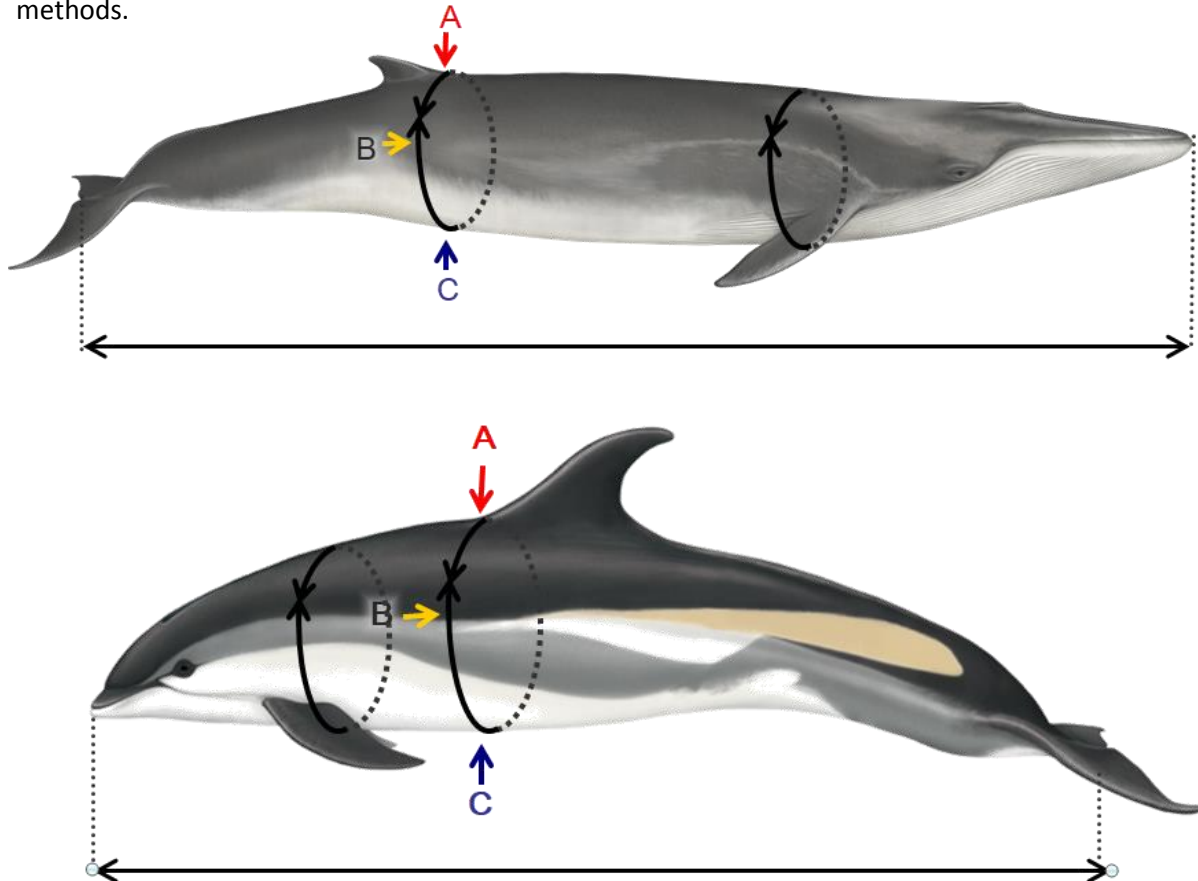


Figure 3: Images indicating the location of necessary measurements to be taken of stranded cetaceans, on the example of a fin whale (*Balaenoptera physalus*) and an Atlantic white sided dolphin (*Lagenorhynchus acutus*). The letters indicate the location of blubber thickness measurement sites, A (red) =dorsal, B (yellow) =lateral and C (blue) =ventral. Images: © CSIP/Lucy Molleson (top image) and WDC/Lucy Molleson (bottom image).

In addition to the measurements described, the complete carcass should be weighed in kg. It should be noted if the animal is not intact and an estimate weight is taken. If weighing is not possible, weigh the heart and use this as a proxy for body weight using the formula:

$$\log W = (\log H + 2.2) / 0.984, \text{ with } H = \text{heart weight and } W = \text{body weight, both in kg}$$

Weighting or other/all organs can be taken accordingly, depending on different research questions and working methods.

3. Tier Two: Gross post mortem examination

This section is modified from the document composed by Kuiken and García Hartmann (1993). For specific organ sample collection for histology or additional examinations, including microbiology, virology and parasitology, see the appropriate subsection in Tier Three of this document.

The aim of a post mortem examination is to establish a cause of death and assess the health status of the individual investigated. This process is as much about excluding potential aetiologies as identifying them. Whilst a basic set of morphometrics can be collected by any suitably trained personnel (Tier One), a complete necropsy examination is a specialist undertaking requiring experienced personnel, a systematic approach and adherence to relevant safety protocols.

During a necropsy, all structures must be examined visually in situ, including the vascular system, by palpation and by making incisions into the organs, and all findings must be recorded, including noting of 'no abnormalities detected' (NAD) and any organ systems not examined (NE). The presence and sampling of gas bubbles within the cardiovascular system should be carried out following specific protocols. Lesions in any organs should be described including the size, location, colour, texture, shape and the nature of the transition from normal to abnormal tissue i.e. how well or poorly demarcated the lesion is. Photographs of lesions should be taken and it is essential that a ruler or scale bar is included to indicate the size of the lesion¹. Representative samples should be placed into fixative for histological examination ensuring inclusion of the transition from normal to abnormal tissue and should also include tissue samples with no gross lesions. Samples should be collected for additional testing and stored appropriately according to the suspected aetiology of any lesion (see also Tier Three).

The necropsy does not necessarily need to take place in the order described below. However, samples for microbiological and virological examination need to be taken as early as possible to avoid contamination. Care should be taken to prevent cross-contamination with enteric micro-organisms and the examination of the gastrointestinal tract should therefore be performed last.

3.1 Freezing

Freezing of a carcase is a valuable alternative when there is no possibility of direct or full examination. This option should only be employed when there is no possibility of examining the carcase fresh or storing it chilled at around 4°C. If immediate necropsy examination is not possible, chilling the carcase is preferable to freezing due to artefacts induced unavoidably by the freeze-thaw process. Ice should be packed around the carcase during prolonged transit with focus given to blood, ear and brain preservation. If a carcase was however frozen prior to examination, clearly state this in the post mortem report.

3.2 External and subcutaneous examination

Photographs, body measurements and carcase condition/state of decomposition are described for Tier One.

¹ For data integrity, the scale bar should state the examining institution, the animal species and the case identification numbers as well as the date of necropsy.

3.2.1 External examination

Examine the animal for external lesions and ectoparasites and sample appropriately. Ectoparasites are most likely to be found in or near the body openings, in crevices or adjacent to and on the fins and flukes. Take a 4 cm² piece of full thickness skin, excluding the blubber layer, for DNA-studies (see Tier Three, chapter 4.4.3).

Examine the oral cavity (including teeth or baleen, tongue, gingiva and lymphatic tissue), eyes, blowhole, anus, genital slit and mammary slits (when present) for lesions, discolorations and discharges. Push the skin in the area cranial to the mammary slits in a caudal direction to express any content present in the mammary glands. If liquid can be extruded take a sample for organochlorine analysis (see Tier Three, chapter 4.9). Record the volume, colour and consistency of liquid. Any abnormalities should also be sampled for microbiology and/or virology.

3.2.2 Subcutaneous examination

Measure the blubber thickness (as described in Tier One). Take toxicological samples of blubber and muscle (as described in Tier Three, chapter 4.9). Record the colour of the blubber (e.g. white, yellow, pink) on the necropsy form.

Position the animal in right lateral recumbency; make a mid-line ventral incision from the symphysis of the mandible to a short distance posterior of the anus circumventing the umbilical region, genital slit and anus. From the posterior end of this ventral incision, make a second incision almost to the dorsal mid-line. Separate blubber from the underlying muscle tissue and remove the skin and blubber from the uppermost side. Examine all blubber by cutting ~2 cm wide strips and note the colour, presence of any discoloration (e.g. haemorrhages). The presence of any parasites or lesions in the blubber should be recorded and a representative sample should be collected (as described in Tier Three, chapter 4.8). Parasites may appear as white cysts of less than 1 cm in diameter, often in the ano-genital region or the dorsal aspect of the chest wall. Also examine the subcutaneous tissue for the presence of bruises and/or gas bubbles.

In females, section the mammary gland and record the presence of liquid, parasites or gross lesions and collect this.

3.2.3 Body condition state

Assessing the body condition state is an important metric as it provides a general indicator for the ante mortem health of the animal. It should be judged based on the blubber thickness, lipid composition and back muscle mass. The physiological blubber thickness is difficult to assess in isolation as it is naturally influenced by a range of factors including species, season, region, sex, age, reproductive status and environmental temperature. An emaciated animal will however have lost all fat reserves and muscle mass; this is most notable in the blubber and lumbar muscles dorsal to the spine. To judge an abnormal blubber thickness, experience in assessment of the species in relation to its environment is needed.

Based on the state of blubber and muscle the nutritional condition code (NCC) can be ascribed as 'emaciated', 'poor', 'moderate' or 'good' (Figure 4):

- 1) **Good:** the animal's outlining on a cranial perspective is convex; no hollow appearance caudal to the skull and lateral to the dorsal fin visible; subcutaneous- and pleural fat can be present.
- 2) **Moderate:** the animal's outline on a cranial perspective is not fully round; a slight hollow appearance caudal to the skull and lateral to the dorsal fin is visible; no internal fat is observed;
- 3) **Poor:** the animal's outline on a cranial perspective shows moderate concavity, and outline of lateral aspects of the vertebrae; a hollow appearance caudal to the skull and lateral to the dorsal fin is visible; scapula's can be observed sticking out;
- 4) **Emaciated:** the animal's outlining on a cranial perspective is very concave and the lateral aspects of the vertebrae are easily palpable; an extremely hollow appearance caudal to the skull and lateral to the dorsal fin is visible; scapula's can be observed sticking out.



Figure 4: Example pictures of three harbour porpoises in good (bottom), moderate, poor and emaciated (top) body condition. Pictures: Utrecht University.

3.3 Examination of internal organs

When opening the body cavities, note any abnormal presence of gas, liquid or lesions and make sure that such are carefully assessed to be able to establish the origin/etiology. The physiological position of the organs should be verified. Protocols might be adjusted during necropsy based on the findings an depending upon people and labs involved.

After removing the left abdominal wall, collect any free fluid in the thoracic and abdominal cavity. Locate the **pre scapular lymph node**, cranial to the pectoral fin, and sample for histopathology and as appropriate in freezer(s).

Free, when present, the testes or ovaries and uterus. A representative sample of any parasites in the abdominal wall (e.g. cysts under the peritoneum) should be collected. Remove the left thoracic wall, for example with bone shears (depending upon procedures for museum collection).

Before handling, inspect and photograph the organs *in situ* and sample the lung, spleen and kidney tissue for virological examination (as described in Tier Three, chapter 4.6) and lung tissue, preferably from the cranio-ventral part of the left lung, the liver spleen and kidney for bacteriological examination (as described in Tier Three, chapter 4.7).

To thoroughly assess macroscopic changes in the organs discussed below, it is recommended to slice all tissues. Additionally, photograph of abnormalities should be taken as mentioned before.

3.3.1 Examination of abdominal organs (except GIT, spleen and pancreas)

Sever the rectum as close to the anus as possible and the abdominal oesophagus as close to the diaphragm as possible, preferable after tying off both sides to prevent leakage of content. Working backwards along the dorsal aspect of the abdominal cavity, remove the **stomachs, mesenteric lymph nodes** with **intestines, pancreas and spleen** from the carcase and either leave further examination of the GIT to the end of the post mortem examination or perform it in a completely separate area to prevent contamination of other tissues with enteric micro-organisms. The spleen and pancreas should be separated from the stomach and intestines and examined prior to examination of the stomach and intestine, to prevent contamination with digesta and/or intestinal content (see in this Tier, chapter 3.3.3).

Examine the **urinary bladder** *in situ*, and sample any urine present with a syringe or after opening the urinary bladder, noting the volume, colour and nature of any contents present. Collect content for biotoxin examination and e.g. virology at -80°C.

In females remove the entire reproductive tract and open the **vagina and uterus**. Record the presence of any corpora lutea, albicantia or follicles on each **ovary** and then store the ovaries appropriately for reproductive studies (see Tier Three, chapter 4.4.4). Additional sampling of the reproductive tract of females for histology, virology and microbiology should be conducted when abnormalities are observed or depending upon required sampling for additional research or assessment which may differ per network. If one is not familiar with the techniques to assess ovary scarring, both ovaries can be stored as a whole and assessment of scars can be done at a later stage. Make sure to be able to differentiate left from right ovary after fixation, e.g. by making a small cut in one side.

In gravid females, photograph the uterus with a ruler in the frame or measure the uterus. Note the side of the uterus horn in which the foetus is present. When the **foetus** is of sufficient size to examine the individual organs, a necropsy and tissue sampling (incl. for toxicological assessment) should be performed. If the foetus is too small for a full post mortem examination, the whole foetus and its placenta could be frozen for organochlorine analysis or fixed and kept as a museum

collection. Note the sex, weight, length and decomposition code of the foetus in either instances. Also note whether the cervix appears to be dilated.

In males remove the **testes**, check for symmetry and make cross-sectional incisions at 1 cm intervals to examine. Store the testes appropriately for reproductive studies (see Tier Three, chapter 4.4.4). Examine the **penis, epididymis** and accessory glands, and additional sampling for histology, virology and microbiology should be conducted when abnormalities are observed.

Remove and examine the **adrenal glands**, located cranial to the kidneys. Collect a cross-sectional slice approximately 1 cm thick of the centre of both adrenal glands for histological examination. Measure the cortex:medulla ratio directly or photograph the cross section through the mid-adrenal gland with an appropriate scale bar. Slice and investigate the adrenal glands for presence of cysts, nodules and/or other abnormalities.

Remove the **kidneys** from the body cavity. Incise both kidneys longitudinally and, if possible, strip the capsule. Sample 1 cm³ of tissue from each kidney for histological examination (if no gross lesions). Assess the urethra for any abnormalities and sample as appropriate.

Remove and weigh the **liver**, examine all surfaces and make multiple incisions into the parenchyma. Examine the bile ducts for abnormalities and parasites (note that cetaceans do not have a gall bladder). Take samples for toxicological examination (see Tier Three, chapter 4.9). Sample 1 cm³ of liver tissue from two regions of the liver (if no gross lesions).

3.3.2 Examination of head and neck region, and thoracic organs

On a ventral approach, incise along the lingual aspects of both mandibles to free the **tongue**, reflect caudally and cut the hyoid bones close to where they attach to the skull.

Locate the larynx and note its position. If the larynx is not retracted, free the larynx from the sphincter muscle that holds it in place and, whilst pulling the tongue caudally incise along the neck, free the trachea. Then, incising dorsally and ventrally within the thoracic cavity, free the heart and lungs. Check for adhesions between lungs and thoracic wall and note and sample any free fluids in the pericardial sac and thoracic cavity. This procedure should give you the tongue, larynx, trachea, oesophagus, thymus, thyroid gland, heart and lungs (pluck) altogether. Examine the **ribcage** for fractures or bruising and, ventral to the thoracic spine, the **rete mirabilis** for evidence of haemorrhage. Examine all organs for gross lesions and sample for histopathology, and as appropriate. At least try assessing the following:

Examine the surface of the **tongue**. Note the presence of neonatal papillae and incise into the tongue muscle for examination. Also examine the lymphatic tissue of the tonsillar ring and sample any abnormalities for histology.

Open the **oesophagus** longitudinally and check for lesions, foreign bodies, food remains and/or parasite presence, and record these.

Using a pair of scissors, larynx, trachea and major bronchi longitudinally check and record for lesions, foreign bodies, food remains and parasite burden. Make multiple incisions into the parenchyma of

both lungs. The presence of any parasites should be recorded and a representative sample should be collected. A minimum of two pieces of each lung (approximately 1 cm thick), one from the hilus (tracheal bifurcation) and one from near the surface of each lung should be taken for histological examination. The samples should include part of the major bronchial tree.

Open all major branches of the pulmonary veins and examine and record the presence of parasites. Examine the bronchial and pulmonary associated lymph nodes. The latter can be found approximately halfway along the ventral edge of each lung. Cut a 1 cm thick cross-sectional central slice of the left pulmonary associated lymph node, including a small amount of the adjacent lung tissue attached, for histological examination.

Examine the **thymus** and place a sample in formalin for histology and upon required sampling for additional research or assessment which may differ per network. In some individuals the thymus may be atrophied or absent (this also varies among species and with age) - if so, note the relative degree of atrophy and whether any cysts are present.

Collect any blood present in the heart lumina (or elsewhere) for serological and/or virological examination. Serum cannot be obtained after freezing of the carcass. Here, a sample of whole blood could be collected and stored similarly. Pericardial fluid could also be collected (note that this should be done prior to opening the pericardial sac, e.g. using a syringe).

Separate the **heart** from the lungs, check the heart for any vascular malformation before cutting through the major blood vessels at the base of the heart. Also examine the myocardium by cutting into the right and left papillary muscles and the septal myocardium. Open the left and right ventricles and atria for examination and record and collect a representative sample of any parasites present. Rinse the heart and weigh it. Sample heart tissue and include a piece of the left and right ventricle wall and atrioventricular septum, for histological examination. Check to ensure no defects (e.g. holes) are present in the septum. Record any gross defects and then sample for histological examination. In case of neonates, assess whether there are any abnormalities in the foetal/neonatal physiological anatomies (e.g. foramen ovale, ductus botalli).

Examine the **ear sinuses** around the tympanic bullae, the Eustachian tube and the nasal sacs. A swab should be taken from the ear sinuses before the ear is removed. Each ear bone should be removed carefully noting any signs of bleeding or trauma in the surrounding tissue. Use forceps to collect a representative sample of any parasites present. After parasite collection, rinse each ear cavity with water to assess the severity of the parasitic burden. In carcass condition 1 and 2 cases, ears should be removed for determination of lesions in the middle and inner ear, as well as the surrounding tissue and in the ear sinuses. The inner ear of one side should be fixed by carefully injecting formalin into the oval window. With electron microscopy techniques, the inner ears of carcasses in decomposition condition 1 can be assessed for malformation in the hair cells. Ears of carcasses in decomposition condition 2 are still valuable for histological assessment to investigate hearing damage.

For odontocetes, examine the **teeth**. Note the down stage of tooth wear, integrity of dentition and state of gingiva. Remove at least 4 intact teeth (if present) from the middle of the lower jaw for aging (see Tier Three, chapter 4.4.1). Check for swelling in or around the jaws or other changes in the jaw articulations.

For mysticetes, examine the baleen for gross lesions and trauma, and collect up to six baleen plates ensuring each is removed as close to the gum line as possible. These can be used for stable isotope analysis and can be dried and stored at room temperature, or frozen, depending onto preferences of laboratories involved.

Next, remove the head and, using a Pasteur pipette through the foramen magnum, sample the cerebral spinal fluid (CSF) for microbacteriological culture prior to opening the skull to examine the **brain**. Sample the **spinal cord** after dislocating the head from the foramen for histopathological assessment.

In odontocetes the skull can be opened by making a vertical cut parallel, and approximately 2 cm posterior, to the transverse dorsal ridge (visible and palpable on dorsum of the skull). A second cut should be made in the horizontal plane through the occipital condyles, making sure to leave the posterior portion of the condyles on the skull. Both cuts should be extended until they meet each other. The separated piece of skull can then be removed using a chisel or flat bladed screwdriver. Care should be taken to avoid human exposure to aerosolized material during this process.

Take a sample of brain for virological and microbiological examinations (see Tier Three, chapters 4.6 and 4.7 respectively) in situ and remove the brain from the skull. Place the remaining whole brain, including the hypophysis which can be found caudally to the optic chiasm within the cranium, in formalin (see Tier Three, chapter 4.5). Once fixed, make multiple slices into the brain tissue to look for lesions, including the presence of parasites. Take samples of any gross lesions and 1 cm³ each of the cortex (minimum 4, preferably more), midbrain, cerebellum, brainstem and medulla for histological examination. Skulls can be retained for morphometric studies (see Tier One, chapter 2.1). Resources permitting, the brain should be fixed entire and processed using large tissue cassettes, to allow full lesion profiling throughout the organ.

3.3.3 Examination of GIT, spleen and pancreas

Examine and incise the **spleen** and accessory spleens for gross lesions. Collect a sample (approximately 1 cm³) from the main spleen including capsule for histological examination, virology and microbiology.

Examine and incise the **pancreas**. Look for parasites, especially within the pancreatic ducts. Sample a 1 cm³ piece of tissue for histological examination.

Examine the **mesenteric lymph node** and sample a 1 cm thick cross-sectional slice from halfway along its length for histological examination.

Open the **stomach**; note that cetaceans generally exhibit three stomach compartments, but there is species-specific variation in compartment numbers. In general, the following three compartments can be recorded: the forestomach (cardiac), the main stomach (fundic) and the pyloric stomach. Record the presence of any ingested prey species, digesta, otoliths, parasites etc. Record the presence of any macro-plastics or other ingested marine debris in the stomachs and retain for future studies. Any parasites should be recorded and a representative sample should be collected from each stomach compartment. Describe any gross lesions, including the distribution and size of any ulcerations for each separate stomach compartment.

Collect the entire GIT contents (including all fish bones, otoliths and all other food remains for prey studies) and freeze (Tier Three, chapter 4.4.2). If micro-plastic analyses are to be undertaken, the GIT, or the parts analysed for micro-plastics, must remain unopened to prevent sample contamination. As this precludes detailed examination of the digestive contents, this should only be undertaken in those cases where diagnosis is likely to be unaffected by unopening the GIT.

Open the entire length of the **intestine**, preferably at the mesenteric attachment, or several parts throughout the entire length of the intestine. Check for lesions, foreign bodies and/or parasite presence, and record and sample these. Any parasites present should be collected. Note the content colour. In very fresh cases, samples of intestine can be taken for microbiome studies.

4. Tier Three: Sampling and storage

4.1 Tissue sampling and storage

The nature and storage requirements for samples taken for subsequent analysis are contingent on the requirements of the testing laboratory, and it is advised to check local requirements and protocols prior to sampling. In general however, the following principles apply:

- Swabs or tissue stored for subsequent microbiological assay should be collected as soon as practical, as aseptically as possible and with an appropriate cold chain between sampling and testing.
- Samples for subsequent assay for persistent organic pollutants should be wrapped in foil to prevent sample contamination from plastic in the storage containers and frozen at -20°C or colder.
- Most samples destined for DNA molecular testing can be stored frozen or in ethanol, however samples collected for other genomic, metabolomics or proteomic work are likely to be more labile and require specific transport and storage media.

4.2 Labelling of samples

Samples from each animal are examined by a number of people, often in different laboratories, under different conditions, and therefore it is vital that they are properly labelled for traceability, also taking into account long-lasting labelling and labelling in freezers (e.g. up to -80°C). This requires that:

- Every animal should be assigned a unique reference number;
- Every sample/ sample container should have a firmly attached label;
- Permanent ink or pencil should be used to write labels;
- Labels should include the unique reference number and tissue sample identifier.

4.3 Tissue archive

To ensure availability of tissues for future studies, it is recommended to store samples in a sample archive. An effective sample archive is very important when working with rare species, so that a larger sample size can be built up over time, but also as new pathogens may be detected in the future and reassessment is required. With availability of a range of samples from multiple individuals and species, long-term studies can be facilitated.

It strongly depends upon the available facilities, but in general, all tissues and body fluids could be sampled and stored at -80°C preferable and/ or at -20°C. As a minimum, we recommend to sample at least: lung, liver and spleen for microbiological assessment (at -20°C) and brain, lung, kidney and spleen for virology (-80°C or colder). Additionally, we recommend storage of blood and/or serum and CSF at -80°C (or colder) as a minimum.

4.4 Life history samples

4.4.1 Age determination

Teeth collection: a longitudinal cross section through odontocete cetacean teeth reveals annual growth rings which can be used to determine age. To ensure all rings are visible on cross section, a minimum of four whole, undamaged or least worn teeth need to be collected from each individual for repeated estimates if necessary. Preferentially take the teeth from the middle of the lower jaw as these are usually the straightest and make age determination more accurate. From the harbour porpoise, teeth can be extracted easily by inserting a sharp knife or scalpel either side of the gums in-between the teeth and the connective tissue. In species such as the bottlenose dolphin, teeth can be loosened initially by levering the space between the tooth and the socket with a sharp pointed instrument. If not possible a piece of mandible containing at least 4 teeth should be removed.

Storage: Loose teeth and jaw sections can be stored frozen. The temperature at which they are frozen is not critical. Alternatively, they can be fixed in 70% ethanol. They should not be stored dry as they may dehydrate which may lead to cracking of the teeth.

4.4.2 Diet analysis and micro-plastics

The best practice is to collect the entire gastrointestinal tract (GIT) (unopened) by removal from the oesophagus to the rectum, to ensure all content is collected. The GIT is then placed on a non-plastic surface for gross examination of digesta, gross lesions, parasites and marine debris. Facilities permitting, the whole GIT can be stored frozen in a plastic bag for subsequent analysis for the presence of microplastics and dietary analysis, which can be undertaken concurrently. Samples for microplastic analysis may also require samples taken from the air at the time of sampling to act as a negative control. The temperature at which the GIT is frozen is not critical.

4.4.3 Genomic (DNA) studies

DNA is most readily recovered from skin, muscle or kidney. These samples can be stored frozen at -20°C or in 70% ethanol. Facilities permitting, storage of duplicate samples in RNAlater at -80°C enables more advanced genetic sequencing techniques to be employed. Leave samples in RNAlater approximately 24 hours on room temperature to ensure fixation of the tissue prior to moving it to a -80°C freezer.

4.4.4 Reproduction studies

Female gonads: Both ovaries should be placed, whole, in 10% neutral-buffered formalin or other fixatives if available or preferable. It is vital to differentiate between the left and the right ovary; this can be done by using separate containers, or, if storage does not allow this, by making a small incision in the left ovary before collecting them together in a container.

Male gonads: Each testis should be weighed separately after removal of the epididymis. Subsequently, place a cross-sectional slice approximately 1 cm thick from midway along the organ's length in 10% neutral-buffered formalin or other fixatives if available or preferable. Fix at ratio of 1 volume tissue: 10 volumes fixative and once fixed, after about 24 hours, the samples can be stored in a smaller volume of fixative.

4.5 Histology samples

Collection: In grossly normal organs take a random cross-sectional sample of 1 cm unless specified otherwise. All grossly observed lesions should be sampled to include an adjacent piece of normal tissue, if present. Always use a sharp scalpel to take the samples. Careful handling of tissues for histology is important, particularly lymphoid tissue as manipulation artefacts frequently resemble lymphoid depletion. Use of individually labelled containers/permeable bags (e.g. tea bags) should be used to aid differentiation when multiple samples are taken from gross or histologically similar organs (e.g. lymphoid tissues) and to be able to identify samples and lesions also after fixation.

Fixation: 4% neutral buffered formaldehyde or 10% neutral-buffered formalin are both suitable fixatives. Formalin will only penetrate approximately 1 cm in any direction, so perfuse or slice samples larger than 2 cm in diameter to expose more tissue surface area to the fixative. Use formalin at a ratio of 1 volume of tissue: 10 volumes of fixative. The tissues should remain in this fixative for a minimum of three days (except the brain, see below) at room temperature. If tissues have a high blood content, the fixative should be changed each 24 hours until it is no longer discoloured.

The brain should be placed in at least 20x as much formalin as tissue when fixing it whole, and should remain in this fluid for at least a week. To allow faster fixation, several transverse/coronal incisions should be made through the frontal lobes to expose the lateral ventricles. This facilitates more rapid penetration of fixative to all area of the brain. In really fresh cases, ensure that the brain has cooled to near room temperature prior to immersion in fixative to avoid artefacts such as dark neurons and contraction of arterioles.

Storage: Once fixation has taken place, the samples may be stored in a smaller volume of 10% neutral buffered formalin. If the time to processing is in excess of three months: change to formal saline at this point to avoid saponification. To minimise fixation artefacts it is imperative that neurological samples are processed within three months. Samples stored in excess of two years will be of limited diagnostic value and wholly unsuitable for immunohistochemistry. Store the histology samples at room temperature.

4.6 Virology samples

Collection: The choice of sampling for virology diagnostics should be made in collaboration with the laboratory undertaking the processing of the samples, taking into account the target virus, the (clinical and) gross lesions of the stranded animal(s) and the state of decomposition of the carcase. The tissues listed in these guidelines- lung, spleen, brain and kidney - are a good range of samples required for several known viruses, e.g. morbillivirus, influenza and herpes detection. Any lesions of suspected viral aetiology should be sampled similarly. For virus isolation, samples of 1x1x1 cm from the relevant organs should be collected aseptically and placed into sterile containers with viral transport medium (VTM).

Storage: If samples are collected for molecular detection of pathogens by PCR and are submitted to the laboratory within 24 hours of sampling, they should be stored at 0-4°C, otherwise they should be frozen at -80°C until analysis. If freezing at -80°C, or even at -20°C, is not possible, then the samples

can be placed in RNAlater and stored at -20°C (or even at room temperature for up to two weeks). Frozen samples can be transported worldwide on dry ice (solid CO₂) or using biofreeze bottles. For situations where large number of samples are being collected in the field or transported distance, it is advised to use an IATA approved LN2 Vapour/Dry shipper.

Serological examination for viral and other diseases requires a blood sample of at least 7 ml collected and centrifuged to obtain serum. Serum should be separated from whole blood as soon as possible and stored at -80°C. Even if the serum contains some haemolyses it can still be of value. Where a blood sample is unobtainable, a sample of pericardial fluid (PCF) can be a suitable substitute.

4.7 Microbiology samples

The choice of sampling for microbiological diagnostics should be made in collaboration with the laboratory undertaking the processing of the samples. Preferable, microbiological examination should be carried out on a minimum of tissues including samples of lung, liver, spleen, kidney, brain, CSF and any gross lesions of suspected microbiological aetiology.

Collection: Ideally, samples should be taken using an aseptic technique whenever possible. Tissue samples (solid or liquid) or swabs, such as nylon flocked swabs, are both appropriate for microbiological examination. Each has advantages and disadvantages and the choice depends on the preference of the person performing the microbiology and also the target microorganism(s) e.g. CSF for *Brucella* spp.

For a solid tissue sample, cut a block approximately 4x4x4 cm using a sterile scalpel and forceps and place this into a sterile container. Samples should be kept at 0-4°C until processed, which should be carried out preferably on the same day or at least within 24 hours after collection. If analysis is not possible <24 hours, samples could be frozen at -70°C until further processed.

For swabs, sear the surface of the organ with a hot blade, then incise with a sterile scalpel and place a sterile swab into the incision. The swab is then inserted into a tube containing transport medium and stored at 4°C until processing or applied directly onto culture medium. Swabs should be processed within 24 hours. If this is not possible samples of solid tissue are preferred.

For diagnosis of neuro-brucellosis, CSF is the sample of choice, collected with a Pasteur pipette through the foramen magnum immediately after removal of the head to reduce the chance of contamination.

Processing: This should be undertaken after consultation with the pathologist/ bacteriologist, as different media and methods are employed dependant on the suspected target microorganisms. Many pathogens require specialist growth media or specialised containment due to zoonotic potential for example *Brucella* spp. or *Mycobacterium* spp.

4.8 Parasitology samples

A thorough external examination of the carcass needs to be performed to detect ectoparasites. All main organs/organ systems, including skin and blubber, should be systematically opened or incised and screened for parasites and associated lesions along the cutting surface. With blunt forceps, collect as many intact specimens with anterior and posterior ending as possible, even if their host animal is decomposed or the specimen appears broken. Parasite specimens should be isolated from the organs and cleaned from blood, dietary residues and adhering tissue in tap water (e.g. with a soft paint brush). Then preserve the specimen in 70% Ethanol or freeze it.

If the total number (intensity) of parasites cannot be recorded, a semi-quantitative assessment of the level of parasitic infection into none = no parasites visible, mild, moderate or severe infection should be recorded for each organ. Documenting the infection and associated lesions through photographs is recommended. Furthermore, the macroscopically detectable severity of lesions associated with parasites should be recorded (no lesion, mild, moderate, severe) and the tissue sampled in 4% neutral-buffered formalin for histopathological examination.

4.9 Toxicology samples

Collection: The choice of sampling for toxicological assessment should be made in collaboration with the laboratory undertaking the processing of the samples. It is recommended to duplicate samples of blubber, muscle and liver, kidneys and brain should be collected for persistent organic pollutant (POP) and trace metal analysis. These samples should only come into contact with stainless steel, aluminium, glass or Teflon and are most conveniently stored in aluminium foil (shiny side out) with a second sample in plastic.

For lactating females, a milk sample should be collected for organochlorine analysis. This must be stored in a glass container. If the container has a plastic cap, it is recommended to cover the opening with aluminium foil (shiny side out) to keep the sample from contacting the (plastic) cap, as this could invalidate the results of any analysis.

If a foetus is present but too small for full post mortem examination, the whole foetus and (parts of) its placenta can be wrapped in aluminium foil (shiny side out) for POP analysis (or collected as museum specimen in formalin if this is preferable).

Samples of muscle and liver can be sampled for trace metal analysis. These samples should not come into contact with any metals other than stainless steel and thus can be stored in plastic containers. Bone samples are also suitable for heavy metal analysis.

Biotoxins: a sample of stomach fluid, urine, faeces and muscle should be collected and stored in plastic containers (e.g. Eppendorf tubes) and stored at -20°C or -80°C for subsequent analysis.

Storage: The minimal size of samples for heavy metal and organochlorine analysis is 10g of solid tissue or 10 ml for milk. Samples should be stored frozen at -20°C until analysis.

Appendix 1. List of workshop participants, Funchal, Madeira

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