ANNEX II

PROTOCOL FOR TAKING BIOLOGICAL SAMPLES IN THE FIELD FROM LIVE BIRDS, FOR TOXICOLOGICAL AND PARASITOLOGICAL ANALYSIS

INTRODUCTION

Although the project foresees only toxicological analysis we consider it to be important to include also parasitological samples/analysis that can also be easily collected while sampling for toxicology (only small additional effort is needed and the cost of these analyses is nearly insignificant compared to the cost of the toxicological analyses).

The results of the toxicological and parasitological analysis can provide important information regarding the physical condition of the animal but could also identify some unknown threat/problem.

The protocol is offering recommendations and guidelines for taking biological samples from birds (target species: Egyptian Vulture and Bonelli's Eagle) while capturing adults/immature or marking of juvenile birds at nests and provides information on the proper conservation and transport of these samples to the lab where the analysis will be performed.

* A different protocol will be prepared for collecting samples from dead animals (birds).

IMPORTANCE OF THE QUALITY OF THE SAMPLES AND MEDICAL HISTORY

The most important for the laboratory analysis is the sample. From a well-selected sample in sufficient quantity, well preserved and sent in a correct time to the lab, all information related to the health of the animal (clinical condition, pathological and toxicology) can be obtained.

GENERAL RULES

Sampling:

- The staff responsible for sampling must use (be provided with) **personal protective equipment** (**PPE**) during the sampling process (at least gloves and masks, disposable overalls is also recommended).
- It is essential to collect **samples aseptically** especially for microbiological analysis due to possible interference with the isolates. Avoid contact between the soil and the samples (very important for toxicological analysis).
- Samples **must be individually identified** (labelling, seal, sticker, etc.) clearly indicating the material (tissue, organ, etc.), animal species and sampling date and location, before sending to the lab.

Conservation:

- **Freezing** of samples should be done immediately after the sampling. Recommended freezing temperature is -20 ºC. For samples such as:
    - blood in heparin for toxicological analyses
    - blood in EDTA (to study various microorganisms by molecular diagnosis PCR),
    - clean plasma and serums (after centrifugation of the blood samples)
    - swabs in virus medium
- **Refrigeration** of samples can be used for not more than 24-48 hours until the shipment:
    - 24 hours: blood in EDTA and heparin if it is for toxicological, haematological or biochemical analysis.
    - 24-48 hours: swabs in conservation medium for microbiology (Amies) and virology (specific medium for virus).
    - 24-48 hours: faeces.

** Also check table 1 at the end of this document.*

SAMPLING PROCEDURES

1. **Collection of samples by swabs from cloaca in Amis medium and virus medium**

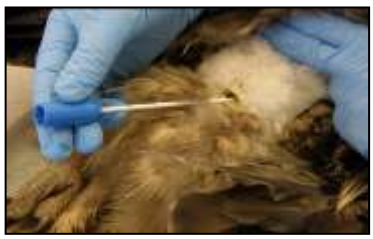
   - Use the swab to collect cloacal and / or oropharynx samples. Insert and turn the swab in rubbing effort to obtain content.

   - Number of swabs: ideally 1 per organism (bacteria) for study

   - Analysis:

- Conservation in Amies medium (blue swab):
  - Microbiology: diverse bacteria species: *Salmonella, Campylobacter, Escherichia coli O157*.

- Conservation in virus medium (pink swab):
  - Virology (molecular diagnostics PCR, virus cultivation). Different viruses.



Collection of samples from cloaca with swab in Amies medium (blue)

Collection of samples from cloaca with swab in virus medium (pink)

Collection of samples from oropharynx with swab in virus medium (pink)

** Also check table 1 at the end of this document.*

2. Sample collection: FAECES

- Collect fresh faeces. Try to discard the urea phase (the white or yellow content).
- Use a sealed, sterile container.
- Possible analysis:
  - Parasitology.
  - Microbiology: Microbiology: diverse bacteria species: *Salmonella, Campylobacter, Escherichia coli O157*.

3. Sample collection: BLOOD IN EDTA AND HEPARIN

- Take the sample by venepuncture, previously disinfect the area with 70% alcohol and wait at least 30 seconds.
- Fill in the sample tube with blood. Overturn smoothly 3-4 times for mixing the blood and the EDTA / heparin, to prevent lyse (blood cell destruction) of the erythrocytes.
- Recommended volume: 1 tube of 1 ml heparin to study toxicology: heavy metals, Non Steroid Anti-Inflammatory Drugs (NSAIDs), antimicrobials and pesticides; 1 tube of 1 ml EDTA to study molecular diagnostics (PCR); 1 tube EDTA for haematology and sex determination (few blood drops in alcohol can also do); 1 tube heparin for biochemistry and proteinogram.
- Analysis from EDTA:
  - Sex determination
  - Molecular diagnostics (PCR): different microorganisms (bacteria, virus, parasites).
  - Metals: lead, cadmium.
  - Non-steroid anti-inflammatory NSAIDs
  - Antimicrobials
  - Pesticides
- Analysis from heparin: In this case it is recommended to centrifuge the tube and recover the blood plasma. This plasma can be refrigerated if the shipment is carried out frozen.
  - Biochemistry
  - Proteinogram (serum protein profile)

IMPORTAN NOTES:

In practice it is difficult to extract big quantities of blood from relatively small animals (Egyptian Vultures or Bonelli's Eagle) therefore we need to think about priorities. The most important blood samples are the ones collected in 1ml tube with heparin, meant for the toxicological analysis.

- Proceed filling the other 1ml tubes only if you have taken sufficient quantity in the 1ml heparin tube for toxicology!

Following this protocol we are suggesting total blood extraction quantity of 3 ml, which is much smaller amount considering the maximum of blood quantity that can be extracted for this species (Egyptian Vulture and Bonelli's Eagle) that can be up to 20ml (about 10% of the body weight).

4. Sample collection: SERUM

- Take blood into a tube for serum.
- To facilitate clot retraction leave reverse or leaning tube at room temperature about 30 minutes. If possible centrifuge (2500 rpm 10 minutes), and collect the clean serum in other tube aside. It can be frozen until its shipment to the lab. If there is no centrifuge available please just extract the serum after the clot retraction.
- Recommended volume: 0,2-0,5 ml of clean serum for each analysis.
- Analysis: detection of antibodies against different microorganisms.

Decanting and passage of the clean serum in new tube (eppendorfs or other containers can be used for maintaining the serum, for

5. Sample collection: FEATHERS

- Collect the feathers from the nest of below the nest (during ringing or marking of juvenile birds). Store in paper or plastic sealable bags.
- Analysis that can be performed:

- Heavy metals and metalloids (lead, mercury, zinc, cadmium, selenium and arsenic)
- Non-steroid anti-inflammatory NSAIDs, Antimicrobials and Pesticides – difficult to detect.

6. Sample collection: Eggshell/s or eggs

- Collect the Eggshells from the nest (during ringing or marking of juvenile birds), if whole egg found in late breeding season please also collect. Store in paper bag or paper box (even better to avoid breaking to smaller pieces).

- Analysis that can be performed:
  - Heavy metals (lead, zinc, cadmium)
  - Pesticides

Table 1: Specific details about the conservation and transport preparation of all different samples

Sampling procedure, C section	Sample	Conservation medium	Analysis	Conservation by refrigeration (4ºC)*	Conservation by freezing (-20ºC; -80ºC)	Comments
1	Swab	Amies	Microbiological	24-48 hours	No	
1	Swab	Virus	Virus study (PCR/ cell cultivation)	24-48 hours	-80ºC (recommendable)	Freezing tº can interfere with the viability of some viruses (e.g. Influenza)
2	Faeces	No medium	Parasitological	24-48 hours	No	Freezing is not recommended, but some parasites and bacteria are viable after freezing on -20ºC to -80ºC
			Microbiological			
3	Blood	EDTA	PCR	Until 3 days	Yes	
			Metals (lead, cadmium)	Until 3 days	Yes	
			Sex (PCR)	Until 3 days	Yes	
			Lead, antibiotics, NSAIDs	24-48 hours	Yes	
		Heparin	Proteinogram	24 hours	No	The plasma can be frozen after centrifugation. Should not be hemolysate (destroyed erythrocytes)
			Biochemical	24 hours	No	
4	Serum	No medium	Immunological (antibodies)	Until 3 days	Yes	Is recommended to freeze if not immediate shipment
5	Feather	Paper bag/- no medium	Heavy metals and pesticides	24-48 hours	Yes	Important to note: please avoid humidity, samples must be dry.
6	Eggshells	Paper bag/box - no medium	Heavy metals and pesticides	24-48 hours	Yes	Important to note: please avoid humidity, samples must be dry.

* Only when really necessary