Guideline for Sampling and Sample Processing

Roe Deer (*Capreolus capreolus*)

Roland Klein, Kathrin Tarricone, Diana Teubner, Martin Paulus

Trier University, FB VI – Biogeography
Universitätsring 15, 54296 Trier

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Guidelines for Sampling, Transport, Storage and Chemical Characterization of Environmental and Human Samples

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1 German Environmental Specimen Bank

The German Environmental Specimen Bank (ESB) is an environmental monitoring instrument of the German Federal Ministry for the Environment, which is under the technical and administrative coordination of the Umweltbundesamt (UBA), Germany's environmental protection agency. The ESB collects ecologically representative environmental and human samples, stores and investigates them for environmentally relevant substances.

Specific operating procedures and the concept of the ESB are the basics for the ESB operations (Umweltbundesamt 2008, 2014, 2023).

The long-term storage is carried out under conditions which, as far as possible, exclude a change in state or a loss of chemical characteristics over a period of several decades. The archive therefore provides samples for retrospective investigations of substances for which the potential risk for the environment or human health is yet unknown. Comprehensive information on the ESB is available at **Error! Hyperlink reference not valid.**.

2 Objective of this Guideline

Sampling is the first and most important step to safeguard the quality of samples and data. It is the result of science-based and standardized methods, to avoid contamination and inhibit loss of chemical information. The exceptionally high demand of true quality results derives from the extraordinary value of the samples as archive material. Representativeness and reproducibility of the samples are the basis for spatial and temporal comparison.

The current guideline is an update of the Tarricone *et al*. (2018) version.

Transport, further sample treatment and storage, as well as chemical analysis must be carried out according to current ESB guidelines.

3 Function of the Specimen Type

The European roe deer (*Capreolus capreolus)* has been investigated as a subject for environmental monitoring since the beginning of the 1970s and has been emphasized as a qualified bioindicator (Holm *et al*., 1990, Hecht 2001, Tataruch 2001, Kierdorf *et al*. 2008, Pokorny *et al*. 2009, Duralec *et al*. 2015, Bakowska *et al.* 2016).

As selective herbivores, roe deer take the position of first-order consumers in terrestrial ecosystems. Sufficient knowledge concerning food consumption and nutrition of roe deer is available due to several research studies (Hespeler 1999, 2016).

The following criteria underline the appropriateness of the use of the roe deer as an indicator organism:

- Wide geographic distribution. It is spread from South-Western Europe through Central and Northern Europe to Russia (Ural Mountains) and Asia Minor (Turkey, Caucasian region, Iran). Other subspecies substitute the species in Siberia and Eastern Asia (Hespeler 2016).
- Most commonly distributed, free-living, tall herbivore in Europe, within Germany alone more than 1,000,000 deer are shot every year (Deutscher Jadgschutzverband 2023 = German Hunting Association 2023).
- Distributed throughout almost all terrestrial ecosystems in Central Europe (Hespeler 2016).
- Considerable location loyalty and therefore an appropriately limited activity range (Debeffe *et al.* 2014, Hespeler 2016), between 10–40 ha for variable territory size, depending on game density, age, sex, gender ratio, regional habitat structure, food supply and season.
- Physiologically and eco-physiologically well-investigated species (Frölich *et al*. 2001, Gehrke 2001, Wisser *et al*. 2001).
- Comprehensive experience concerning the accumulation behavior of wild roe deer for elements and organic substances (Draghi *et al.* 2023, Cygan-Szczegielniak and Stasiak 2021).
- Popular for human nourishment.

Within the ESB program, the roe deer represents the level of consumers in terrestrial ecosystems.

4 Target Compartments

ESB sampling aims at the liver of the roe deer as the target compartment:

- Most substances are best traceable in the liver. This applies to all thus-far-investigated Chlorinated Hydrocarbons and for elements such as Cr, Mo, Mn, Cu and Fe. The elements Cd, Pb, V, Zn and Ca accumulate more in the kidneys than in the liver. Hg, Al and Mg presumably present equal concentrations in both organs (Klein and Nentwich 1995).
- Substances are very homogeneously distributed.
- With approx. 300–500 g, the liver provides a sufficiently large sample amount, approx. four to five times the amount from kidneys.
- Liver fat contents are subject to less seasonal variation than those in the kidneys (Klein and Nentwich 1995).
- Removal of the liver does not decrease the value of the venison.
- The liver is located in the abdomen of the roe deer, which is not damaged by a precise heart shot. The risk of unwanted contamination by the bullet can be drastically reduced by exclusively using roe deer with undamaged abdomens for pollutant studies.

5 Predefinitions for the Sampling

5.1 Selection and Definition of Sampling Sites

As a consequence of the deer's mobility, sampling sites are relatively large. Roe deer are shot during a normal hunt by authorized hunters. In Germany, hunting is organized in a district system. Each hunting district tenant holds the right to hunt in his own district, therefore hunting districts are the most important organizational subdivision. Roe deer density determines the number of districts per sampling region. These are included in the area-related sampling scheme, in order to assure long-term sample consistency.

In case of an extremely low roe deer density and/ or in small-sized sampling regions, it may become necessary to define the whole investigated area as the sampling site.

5.2 Selection of Individuals and Sample Size

For statistical reasons, the storage of the livers of at least 10 animals (yearlings, of both sexes) is required per sampling site and sampling period.

Territorially, yearlings are closely bound by the doe. At the sampling date, they are of a uniform age and have been exposed to the pollution over the course of approximately one year.

Exclusively livers of healthy animals are collected. These must not exhibit any variations of the normal condition of venison and organs. Sicknesses, depending on their kind and manifestation, alter the physiology of the organism. Sick animals can be identified prior to shooting by conspicuous appearance (shaggy coat, body mass losses) and/or abnormal behavior.

Therefore, sampling is to be carried out by experienced hunters, who are able to distinguish these criteria and guarantee adherence to the sampling guideline. Subsequent to shooting and gutting, other manifestations of sickness are detectable, e.g. deviation from the normal size, shape, and color of organs, deposits at organs, increased body fluids, abnormal smell, or extreme ecto- or endoparasite infestation. Livers from non-healthy animals are not used for ESB storage.

5.3 Sampling Period and Frequency

The yearlings are sampled starting at the beginning of the hunting season in early April until the start of the rutting season, which, depending on weather conditions, begins about mid-July (BJagdG 2020). Sampling can be conducted yearly, without serious consequences for the natural populations to be expected.

5.4 Area Related Sampling Scheme

Based on the sampling guidelines, specific definitions for the individual sampling areas and sites must be made and documented in an area-related sampling scheme. These include, but are not limited to:

- location and demarcation of the sampling sites,
- required sample size,
- time frame for sampling,
- appropriate authorities.

The objective of long-term and continuous sampling should be taken into account when drawing up and updating the area-related sampling schemes.

6 Sampling Procedure

All data collected in the course of sampling and throughout the biometric sample description are documented in the respective specimen data sheets (see appendix).

For each roe deer, a separate record (specimen data sheet 2) containing the following information is created by the hunter:

- date and time of the kill,
- name of the hunter,
- if applicable, the Game Origin Certificate number to identify the animal that has been shot,
- chronological sampling procedure,
- position of the bullet hole, exit wound and bullet type,
- description of the health status of the animal.

Thereby a deviation from the guideline can be identified for each individual.

In addition, a protocol must be prepared for each sampling with the following information:

- persons that participated in the sampling,
- chronological sequence of the sampling,
- the underlying version of the sampling guideline and the area-related sampling scheme for the current sampling as well as
- deviations from the sampling guideline and the area-related sampling scheme.

Preparation for roe deer sampling requires comprehensive organization. First, with the help of local contact persons, reliable hunters are selected. Preceding initial sampling, these particular hunters must be briefed on all steps in the course of sampling. Instruction thereby focuses on the selection of animals complying with the guidelines, a contamination-free kill and proper sample packaging. At the start of each sampling interval the necessary packaging materials must be compiled and handed over to the hunters.

It is also important to always stay in contact with the local contact persons in order to be able to quickly react if problems arise and to be able to comlete the sampling including pickup of the samples within the time period allotted in the guideline.

6.1 Required Equipment and Cleaning Procedures

Field work:

- 15 specimen data sheets 2,
- 15 instructions for sampling procedure,
- 15 plastic bags,
- 15 linen bags,
- cooling devices for liver transport and intermediate storage.

For the sampling of a roe deer, the hunter needs to obtain the following materials/documents in advance:

- one specimen data sheet 2,
- checklist to Prepare and Conduct the Sampling,
- one plastic bag for packaging the liver.

For packaging the roe deer livers, only plastic materials that are suitable for cryogenic storage conditions (up to -200°C) are used. They must not contain any additives and should be non-reactive to lipophilic substances in the sample (suitable plastic bags are made of fluorinated ethylene propylene [FEP]).

Laboratory:

- specimen data sheets,
- clean bench with particle and activated carbon filtration,
- stainless steel containers with lid and clips,
- scale (reading 1 g) to determine liver weight,
- waterproof pen to label the stainless steel containers,
- laboratory clothing and disposable gloves,
- cooling device for the storage of the samples in the gas phase above liquid nitrogen (LIN),
- protective clothing for liquid nitrogen handling.

Sample containers and all equipment are cleaned in a laboratory washer using a chlorine-free powerful washing agent in a first step. After cold and hot (90 – 95°C) rinsing, neutralization using 30% phosphorus acid in warm water is performed, followed by hot and cold rinsing with deionized water. After this procedure, the containers are dried in a cabinet dryer at a temperature of at least 120°C for a minimum of one hour (sterilization). Subsequently, the vessels cool in a closed drying oven. Sterilization is not applied to synthetic materials.

6.2 Sampling Technique

The roe deer are killed by means of an aimed bullet shot during stand hunting or stalking. Organs of roe deer shot in the course of a drive hunt are not suitable as sample specimen. Pre-mortal stress caused by hounding or not-directly-lethal shots may influence the pollution concentration within the organs. The abdomen of the animal should not be injured by the shot. Neck shots and precise chamber shots result in the lowest bullet contamination hazards, and are therefore the preferable manner of shooting. In the specimen data sheet for sample description (specimen data sheet 2), the exact position of the bullet's point of entry and exit must be recorded.

During the sampling process, extremely close attention must be paid to avoid contact of the samples with hair, plants, soil particles, etc. Sampling should be carried out as follows:

- the roe deer must be gutted within a timeframe of 30 minutes following the kill,
- the uninjured liver must be placed in the plastic bag which is placed into the portable linen bag,
- alternatively, the whole corpse can be immediately transported to the collection point, where the liver is removed and subsequently placed inside the plastic bag. In this case the Order on Meat Hygiene (VO (EG) 583/2004) must be followed,
- the specimen data sheet for sample description must be filled out and placed inside the repective linen bag,
- livers need to be deep-frozen within the portable bag, together with the respective specimen data sheet 2 within 24 hours at the latest (min. -15°C).

The interim storage of the samples in the deep-freezer must not exceed 4 weeks.

If necessary, sample collectors are equipped with a deep-freezer. Transport from the sample collection point to the laboratory is conducted via portable deep freezers at a minimum temperature of -15°C or inside portable dewars in the gas phase above liquid nitrogen. The data of the steps
- Sample verification and pick-up
- Storage above liquid nitrogen
are to be entered into specimen data sheet 3.

The transfer into labeled (container ID, sample ID) stainless steel containers will take place under clean air conditions in the laboratory as follows:

- under clean air conditions, the liver samples are weighed inside a clean stainless steel container without their plastic bags,
- afterwards they are transferred to the pre-cooled stainless steel containers designated for storage,
- subsequently, the containers will be stored in the gas phase above liquid nitrogen,
- the ID of the container will be recorded together with the weight of the liver and the storage date and time on the specimen data sheet 3.

7 Biometric Sample Characterization

During sampling, data concerning weight, state of heath, and infestation with parasites are collected on the roe deer. All data gained in course of the sampling are to be documented in the respective specimen data sheet. The determination of the liver weight is performed as described in Chap. 6.2. For a better interpretation of the values of the chemical analysis, additional data of the roe deer's browsing behavior is collected. Thus every five years a browsing survey is performed in the sampling areas. The respective instruction can be found in the appendix ("Browsing Survey").

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Checklist to Prepare and Conduct the Sampling

Specimen Type	**Roe deer (*Capreolus capreolus*)**
Target compartment	liver
Individual specimens	yearlings (both sexes)
Random sample number	at least 10 animals
Sample quantity for the ESB	2,200 g liver
Sampling period	early April, to the beginning of the rutting season (about mid-July)
Required equipment for sampling	annually
Sample packing	• specimen data sheets • instruction for the sampling procedure • PTFE bags and portable linen bags
Transport and interim storage	• PTFE bags for interim deep freeze storage • stainless steel containers with lids and clips
Required equipment for laboratory work	• cooling device (at least -15°C) at the collection point • cooling device (at least -15°C) for transport from the collection point to the laboratory • cooling device (dewar) for the storage of the samples in the gas phase above liquid nitrogen (LIN)
Sample characterization	• specimen data sheets • clean bench with particle and activated carbon filtration • scale (reading 1 g) • laboratory clothing and disposable powder-free gloves • waterproof pen to label the stainless steel containers • cooling device (dewar) for the storage of the samples in the gas phase above liquid nitrogen (LIN) • protective clothing for liquid nitrogen handling
Target compartment	• sex • body weight (reading 1 kg) • state of health • infestation with parasites • liver weight (reading 1 g)

GERMAN ENVIRONMENTAL SPECIMEN BANK

Specimen Data Sheet 1: Sampling Location(s)

Roe deer (*Capreolus capreolus*)

Identification:

__ __ __ __ / X / __ __ __ __ / __ __ __ __ __ / __

	Specimen Type
	Specimen Condition
	Collection Date (MM/YY)
	Sampling Area (SA)
	Sampling Region (SR)
	Sampling Site (SS)
	Additional information

Sampling Site (plain text) _____

Sampling Leader _____

Remarks _____

_____

_____

_____

_____

_____

Notes _____

_____

_____

_____

_____

_____

_____

_____

GERMAN ENVIRONMENTAL SPECIMEN BANK

Specimen Data Sheet 2: Sample Description

Roe deer (*Capreolus capreolus*)

Identification: **Sampling Site:**

__ __ __ __ **/ X /** __ __ __ __ / __ __ __ __ __ / __ _____

No. of Proof of Origin __ __ __ __ District/Division: _____

Name of the Hunter: _____

Date of Kill: __ __ . __ __ . __ __

Time: shot: __ __ : __ __ perished: __ __ : __ __

gutting: __ __ : __ __ storage: (deep freezer) __ __ : __ __

Bullet Type: ☐ lead ☐ no lead | Shooting Distance: __ __ __ m

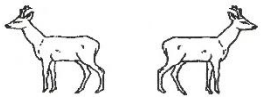
Injured Organs: ☐ lung ☐ spleen ☐ gut

☐ none ☐ heart ☐ rumen

☐ liver ☐ other: _____

Bullet Hole: ☐ heart shot ☐ chamber shot ☐ neck shot ☐ gut shot

Entry/Exit Entry/Exit



(please mark the bullet's point of entry and exit)

Sex: ☐ male ☐ female

Weight: __ __ kg ☐ with head ☐ without head entered in ISESB * _ _ kg

(gutted, with skin) *not filled out by hunter

State of Health: ☐ healthy ☐ sick ☐ emaciated

Injuries: _____

Parasitic Infestation:

Ectoparasites: ☐ none ☐ scarce ☐ average ☐ extreme

Endoparasites: ☐ none ☐ scarce ☐ average ☐ extreme

Organs infected: _____

Date, Signature of the Hunter

GERMAN ENVIRONMENTAL SPECIMEN BANK

Specimen Data Sheet 3: Storage

Roe deer (*Capreolus capreolus*)

These fields are filled out by Trier University

Identification:

__ __ __ __ / X / __ __ __ __ / __ __ __ __ __ / __

Sample: __ __ __

Date of sample pickup and verification: __ __ . __ __ . __ __

Signature: _____

Date of storage above liquid nitrogen: __ __ . __ __ . __ __

Signature: _____

Date of transfer to stainless steel container: __ __ . __ __ . __ __

Liver weight: __ __ __ g

Number of stainless steel container: __ __ __ __ __ __ __

Signature _____

GERMAN ENVIRONMENTAL SPECIMEN BANK

Sampling Protocol

Roe deer (*Capreolus capreolus*)

Sampling Area: _____ Identification: __ __ __ __ __

Underlying version of the sampling guideline __ __ . __ __ . __ __

Underlying version of the sampling scheme __ __ . __ __ . __ __

1. Objective of the Sampling: _____

_____

2. Actual Timeframe of the Sampling:

Start		End		Sample no.		Sampling Leader	Remarks
date	time	date	time	from	to		

3. Participants: internal _____

_____

external _____

4. Checklist Referring to Sampling Scheme and Sampling Guideline: ☒ as prescribed

- ☐ 4.1 Sampling period
- ☐ 4.2 Sampling site and sampling point (selection/definition)
- ☐ 4.3 Selection of the individual specimens and sample size
- ☐ 4.4 Technical preparations
- ☐ 4.5 Cleaning procedure for the packages
- ☐ 4.6 Sampling technique/method of capture
- ☐ 4.7 Sample amount
- ☐ 4.8 Data collection
- ☐ 4.9 Transport and interim storage

Number, kind and reason for deviation (plain text):

_____

_____

_____

_____

Remarks: _____

_____

_____ __ __ . __ __ . __ __ _____

Recorder Date Signature

Browsing Survey

In order to describe the roe deer's habitat quality, every five years a mapping of deer browsing activity needs to be carried out within the perimeters of the sampling areas. Browsing intensity determination should be scheduled/undertaken almost at the end of the vegetation period. At this point in time it is possible to record the herbaceous and woody plants, which have been browsed during summer, as well as the woody plants used as a food resource over the course of the winter. Mast years of forest trees and the composition of additional winter feed must also be recorded.

The methods cited for characterizing roe deer browsing behavior include: Zai 1964, Al-Kittani 1973, Voser-Huber and Niervergelt 1975, Petrak 1982, 1987 and Guthörl 1990. The investigation should be carried out by skilled persons.

The browsing survey, whose results are documented in the respective specimen data sheets, should be carried out as follows:

In forest areas:

First, information is obtained on the (approximate) shooting site locations of the roe deer culled for the ESB within a sampling area. Subsequently, suitable browsing grounds in the immediate surroundings are identified on a map, such as clear-felled areas, (natural) regeneration sites, windthrow sites, pipeline routes, forest and path edges, conservation areas, and thickets. The homogeneity of the vegetation on these sites and their size determines the number of mapping plots (generally between 1 and 3, based on practical experience). The minimum size of each mapping plot is 25 m². To determine the **grazing offer** (occurence of plants), vegetation analyses are conducted on each mapping plot according to the method laid out by Braun-Blanquet (1964). The cover value is defined as the area which would be covered if all above-ground parts of the recorded plants were projected vertically onto the ground. The estimation of the plant species cover value (Tab. 1) is performed for a stratum of up to 1.5 m height (grazing height of the roe deer).

Tab. 1: Scale for the estimation of cover values (Schmidt 1974)

< 1	up to 1	up to 2	up to 3	up to 8	up to 10
up to 15	up to 20	up to 25	up to 30	up to 40	up to 50
up to 60	up to 70	up to 80	up to 90	up to 100	

In contrast to the browsing investigations conducted for forest concerns, which aim to gather information about the state of forest rejuvenation and therefore only consider the young forest trees (Schwab 1999), investigations concerning the grazing behavior include all existing plants. For each plant species on the recorded site, the browsing intensity is determined. When doing so, it is important to differentiate between different browsing animal species (Reimoser and Reimoser 1998). While it is easy to differentiate between the feeding marks of ruminants and rodents (e.g. jack rabbits), it is normally not possible to distinguish between the different ruminant species by their feeding marks (Petrak 1982). The bite patterns caused by ruminants are uneven and fibered, but not smooth like those produced by the jack rabbit (Bang and Dahlström 2000). The specific grazing species can be identified by the different browsing height, which corresponds to body height, the appearance of browsing marks and unambiguous characteristics, such as droppings and footprints. However, it must be kept in mind that

individuals can considerably enlarge their upwards grasp by standing on their hind legs. Additionally a closed snow cover, provided that it bears the burden of the animals, can also increase upwards browsing range (Petrak 1982).

A restriction of the browsing survey to a vegetation height of 1.5 m may lead to an underestimation of the browsing degree caused by the roe deer.

For each particular site, the browsing degree for each plant species is assessed according to a five-class scale of browsing intensity (Tab. 2) developed by Klötzli (1965). By averaging the values of the browsing intensity ascertained from the recorded sites/grazing grounds, the browsing intensity (= browsing rate) of the sampling area is calculated.

Tab. 2: Browsing intensity classes for gramineae, herbs, and young trees (Klötzli 1965)

Browsing intensity class	Browsing intensity	Gramineae and herbs	Shrubs or young trees
0	none		
1	low	1-5% of the plant bitten to a minor degree	only about 1-5 browsing marks per plant
2	moderate	6-20% of the plant bitten to a minor degree, plant inhibited in growth	6-20 browsing marks per plant, no inhibition of growth
3	severe	20-50% bitten in a striking degree, e.g. sprouts decapitated, growth often stopped	> 20 browsing marks per plant, plant inhibited in growth (browsing of the top shoot will be especially noted)
4	extreme	> 50% bitten in a striking degree, plants often ± completely destroyed	> 20 browsing marks per plant, plant without noteworthy sprout growth in the present vegetation period

The browsing continuity for each species is calculated as follows:

$$\frac{\text{No. of surveys in which the species was found browsed}}{\text{No. of surveys in which the species occurred}} \times 100$$

The (specific) grazing proportion for each species is calculated by multiplying the browsing intensity by the cover value and summed across all browsing plots.

In purely field areas:

In purely field areas, the grazing offer on the utilized agricultural area must be registered and recorded in the respective specimen data sheet. It is important to distinguish between fallow land, grassland, and the main field crop species. Groves and adjacent edges must also be recorded. On fallow lands, phytosociological surveys as well as browsing surveys should be performed according to the method described above.