



Guideline for Sampling and Sample Processing



Bream (*Abramis brama*)

Roland Klein, Martin Paulus, Kathrin Tarricone, Diana Teubner

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

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**Appendices: Checklist to Prepare and Conduct the Sampling
Specimen Data Sheets**

**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 instrument for environmental monitoring of the Federal Ministry for the Environment, Nature Conservation and Nuclear Safety (BMU) subject to specialist and administrative coordination by the Federal Environment Agency (UBA). The ESB collects ecologically representative environmental and human samples and stores and investigates them for environmentally relevant substances.

Specific operating procedures as well as the conception of the ESB are the basis of the program. (Umweltbundesamt 2008, 2014)

The long-term storage is carried out under conditions which, as much 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 not yet known.

Comprehensive information on the ESB is available at www.umweltprobenbank.de.

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, standardized methods to avoid contamination and inhibit loss of chemical information. The need for an exceptionally high level of quality assurance results 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 Klein *et al.* (2012) version.

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

3 Function of the Specimen Type

In freshwater ecosystems breams (*Abramis brama*) occupy the trophic level of carnivorous consumers. Though as non-predatory fish they do not rank at the bottom of the food chain. Breams mainly feed on benthic organisms, particularly on larvae of chironomids, which rate as decomposers, sludge worms, snails, and mollusks. Plants and plankton are ingested in times of distress. Since breams feed from the bottom they are also related to the sediment and not only to the pelagic zone like the roach (*Rutilus rutilus*).

For a long time, the bream has successfully been used in passive biomonitoring as an accumulation indicator (Brazova *et al.* 2012, Hradkova *et al.* 2012, Miede *et al.* 2012, Fliedner *et al.* 2014). Additionally, in some recent field studies, they have been used as effect indicators (Morozov *et al.* 2012, Giari *et al.* 2012, Silkina *et al.* 2012, 2013, Teubner *et al.* 2015).

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

- wide distribution in Europe, except the extreme north and south,
- one of the most common fish species in Central Europe, therefore available for sustainable and repeatable long-term sampling,
- largest species among the most frequently found fish in Central Europe, making it especially convenient for organ dissection,
- long life span (up to 25 years),
- wide ecological amplitude: occurs in (slow) moving and stagnant waters with differential pollution, and degree of waterway construction and even in the brackish water of the Baltic Sea,
- resistance to high pollution
- relatively resident, although, depending on water conditions and predator pressure, seasonal location changes in parts of the population have been observed (Donnelly *et al.* 1998, Gardner *et al.* 2013),
- mirrors the pollution of the water bed (including the sediment) and the limnetic zone (Farkas *et al.* 2002),

- regionally used for human consumption, which constitutes a direct relation to the human food chain.

Problems arise with the bream in captivity, because they are difficult to rear. However, this is necessary for toxicity and impact tests.

4 Target Compartments

Because a sufficient homogenization of whole fish is not possible (Paulus and Klein 1995), specific suitable organs have to be selected for the purposes of the ESB.

The muscle and liver tissue are chosen for the examination of chemical substances. The former is edible and therefore a link to the human food chain. Furthermore, it is simple to dissect and has a large biomass, allowing for a multitude of chemical analyses even for single specimens.

On the basis of the muscle tissue, only a part of the eco-toxicological relevant substances can be represented. Thus, as the body's main metabolic organ the liver is also collected.

Blood plasma is primarily used for biomarker impact studies (e.g. immunological, hormonal, genotoxic). Due to its lipid content and homeostatic capacity it is also ideal for the quantitative analysis of organic, lipophilic xenobiotics and elements. In clinical diagnostics blood plasma is the most important body fluid.

5 Predefinitions for the Sampling

5.1 Species Determination

The bream is easily confused with *Blicca bjoerkna* (white bream, silver bream, etc.). Basic distinctive features are shown in Fig. 1. In addition, the bream tends to hybridize with this species and other members of the Cyprinids bearing fertile hybrids (Hayden *et al.* 2010, 2014).

5.2 Selection and Definition of Sampling Sites

The sampling sites must represent the respective ecosystem. Meaning that they must not be located close to local emission sources. The distance to pollution sources depends on the type of emissions and on numerous hydrologic and hydrogeographic factors. The distance to the nearest emission source has to be individually determined for each sampling site (chap. 5.5).

5.3 Selection of Individuals and Sample Size

To ensure the comparability of homogenates, the breams must be within a defined age class. This guarantees not only a sufficient availability of samples but also adequate body and organ weight. In the extremely varying waters the listed criteria is most compatible with the eight- to twelve-year-old breams. Screenings have proven that metals accumulate independent of age within this group. There is as yet no knowledge for age-related accumulation of organic pollutants in the target group (Paulus and Klein 1995).

During the sampling, the age of the individual fish is estimated by their length and weight. Because the growth of the bream depends on the specific water conditions and varies greatly, there is no rule of thumb for length and weight. Prior to the first sampling, a screening is carried out to investigate availability and bream growth. This screening has an additional purpose: It enables a survey of at least 20 breams to be conducted in order to determine their individual variance of substance concentration.

The age is determined in the laboratory using scales and opercula for the analysis. (chap. 7). Hence, breams outside of the mentioned age group may be present in the random sample. If there is an insufficient quantity of the target age group, younger or older age groups complement the sample, where necessary. Juveniles can present great physiological variances. Thus, sexually mature individuals should be sampled. The beginning of sexual maturity for bream is listed as 3 or 4 years old.

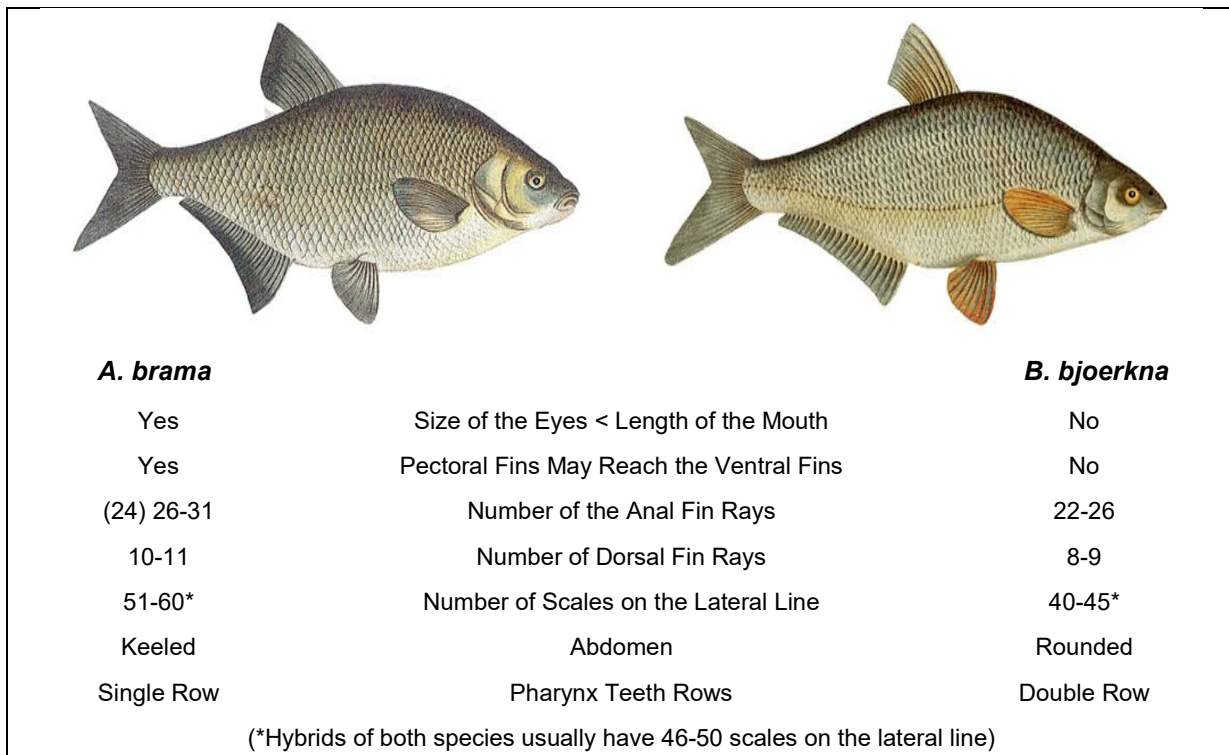


Fig. 1: Distinctive Features of *Abramis brama* and *Blicca bjoerkna*

For statistical purposes, at least 20 breams per sampling must be dissected and stored. This minimum quantity is increased in waters accommodating small, scanty breams, to reach the ESB required quantity of 2,200 g muscle tissue.

The minimal sampling size for a specific chemical substance can be estimated statistically (e.g. by power analysis).

5.4 Sampling Period and Frequency

In long-term programs, such as those of the ESB, sampling should be carried out annually.

The sampling is conducted after the spawn period from mid-July until the end of October depending of the body of water. Later sampling dates hamper the catch of breams because they are relatively inactive during the cold seasons. The spawn season (from April to the beginning of July), a period of permanent physiologic changes for the sexually mature individuals, is also excluded due to dynamics that cannot be standardized.

5.5 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 (i. e. permission by the respective Water and Shipping Authority to use the waterway rights and the adjacent work paths, approval from the respective Fishing Agency or Nature Conservation Agency, a certificate of exemptions issued by the Nature Conservation Agency for containment of the fish until dissection).

At the very least, an agreement with the respective owner of the fishing rights is required.

Here it is important to consider how to ensure a long-term sampling continuity. If changes are made, the document must be updated.

6 Sampling Procedure

All data collected during sampling and biometric sample characterization must be documented in the corresponding specimen data sheets (see appendix). 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.

6.1 Required Equipment and Cleaning Procedures

Field Work:

- fishing tackle depending on the method applied (chap. 6.2),
- species-appropriate net cage,
- species-appropriate transport containers (at least 200 l) with ventilation,
- landing net.

Laboratory:

- clean bench with particle and activated carbon filtration,
- specimen data sheets,
- waterproof pen,
- club,
- electrical system to anaesthetize the fish,
- photographic equipment,
- measuring board (reading 0.5 cm),
- laboratory scale (reading 1 g),
- laboratory scale (reading 0.01 g),
- scale bowl for whole fish,
- 2 stainless steel beakers for dissecting instruments,
- demineralized water (Aqua_{demin}),
- stainless-steel tweezers,
- stainless steel scalpel handle with blades,
- stainless steel scissors,

- stainless steel pliers,
- bone cutter,
- teflon dissecting pad,
- 2.6 ml polypropylene tube with sealing push-in cap, treated with heparin (S-Monovette with 10-30 LE/ml whole blood) (per individual 2 units),
- 0.9 mm hypodermic needle, 38 mm long,
- pipettes with appropriate tips (0.1-1000 µl),
- cryo-tubes (PP) (Ø11 mm, L 47 mm, capacity max. 500 µl), labeled for identification
- cooling centrifuge for laboratory,
- disposable gloves and laboratory clothes,
- tissues,
- stainless steel containers (5.5 l and 3.5 l) with lids and fasteners and cardboard tube storage boxes for blood plasma samples,
- zip lock bags or plastic jars with screw caps for opercula,
- paper bags for scales,
- insulated container to hold stainless steel containers
- liquid nitrogen (LIN),
- tools and protective clothing for liquid nitrogen handling,
- cooling device (dewar) for the rapid deep-freezing and storage of the samples in the gas phase above liquid nitrogen (LIN), corresponding to the number of required stainless steel containers.

In addition at screenings:

- 100 ml Schott Duran bottles for liver samples,
- stainless steel containers (1.5 l),
- stainless steel funnel sieve,
- stainless steel containers (3.5 l).

Sample containers and all equipment is 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 130°C (± 10°) for a minimum of an hour (sterilization). The containers remain in the closed cabinet dryer while they are left to cool. Sterilization is not applied to synthetic materials

6.2 Sampling Technique

The method of catching the eight to twelve-year-old breams generally depends on the local conditions. Thus it is not possible to successfully catch the fish using the same method in every location.

Anchored nets are used in deep, stagnant or slow-moving waters and their branches. Gill or mirror nets are suitable as ground nets with a mesh size of 70-100 mm depending on the size of the eight to twelve-year-old breams. The in situ net time should not exceed a few hours because otherwise the captured fish could suffer too much stress or injury. The advantage of the relatively large mesh and short duration also ensures a minimum impact on the non-target species.

Dragnets are particularly suitable for catching bream in shallow, stagnant waters. In major water flows, using scoop nets or stow nets is the most suitable method for catching.

On demand, electrofishing can be used as suitable technique.

Only when everything else fails and the catch is insufficient is angling considered. In that case the fish usually have to be fed for a successful catch.

Regardless of the catching method and immediately after catching, breams have to be kept in habitat water in a species-appropriate manner. Net cages, keep nets or specific fish transport containers (filled with habitat water and provided with a ventilation system) are suitable for keeping the fish.

It is important that no individual fish remains in captivity for longer than four days, because starvation results in physiological.

For further processing, each fish is individually removed from the net cage or transport container using a landing net. The exact species determination is done before anesthetization through an electrical system. After this at least 2 x 2.5 ml whole blood is taken directly from the heart. To do this, the opercula is opened and the Monovette cannula penetrates the skin under the gill directly above the osseous base.

The anesthetized fish is killed according to the animal protection laws. The following steps are chronologically processed:

- weighing (reading 1 g),
- measuring of the length (cm reading 0.5) from tip of the mouth to end of the compressed tips of the caudal fins (= length complete or LC) and of the length from tip of the mouth to the center between the ends of the caudal bifurcation (= total length or fork length or LT),
- recording of all conspicuous skin features,
- removal of a minimum of six scales (shortly underneath the lateral line between the ventral and the anal fin) with tweezers and transferring to labeled paper bags,
- centrifugation of the blood sample (10 min. at 3000 rpm and $3^{\circ}\text{C} \pm 3^{\circ}$) or direct storage in a cooling device (refrigerator) at $5^{\circ}\text{C} (\pm 3^{\circ})$ until the centrifugation, which must be carried out no later than four hours after the blood withdrawal,
- afterwards, pipettes are used to allocate the blood plasma by quota in PP-cryo-tubes to at least 60 μl per sample-container, and transferred above LIN.

The subsequent dissection is performed on a clean bench with particle and activated carbon filtration. The required instruments are kept in stainless steel beakers filled with deionized water. One contains the instruments required for stripping the skin, and the other one the instruments required for the removal of organs, which are then stored. The following work steps are carried out:

- incision of the skin along the dorsal-ventral line and the operculum on the left body side using a pair of stainless steel scissors to avoid injury of the organs, care must be taken that the incisions do not deeply penetrate the flesh or the abdominal cavity,
- stripping off the skin from the head to the tail using strong stainless steel pliers,
- incision of the muscle tissue along the dorsal line and along the upper edge of the spine and its removal from head to tail using stainless steel tweezers and using a scalpel for further cutting,
- cutting the remaining muscle tissue with a scalpel,

- weighing of the muscle tissue on a PTFE pad (reading 0.1 g and shock-freezing in liquid nitrogen in a stainless steel container (5.5 l) (the muscle tissue of all dissected breams is deep-frozen together),
- opening of the abdominal cavity using stainless steel scissors; care must be taken to avoid damage to the organs,
- removal of the liver using stainless steel tweezers and scissors without damaging other organs (especially the gall bladder),
- weighing of the liver (reading 0.1 g) and shock-freezing in liquid nitrogen in a stainless steel container (3.5 l), the livers of all breams are deep-frozen together,
- removal of the spleen and weighing (reading 0.1 g),
- removal of the remaining innards (excluding the kidney) and weighing (reading 0.1 g)
- determination of the sex,
- dissection of the kidney and weighing reading (0.1 g),
- resection and packing the opercula for maceration,
- documentation of all conspicuous features in the viscera.

Screenings are carried out to determine the individual variation of substance values on at least 20 breams. The dissection of the muscle tissue as well as the packing of the liver differs as follows:

- the muscle tissue from the right side of the body is additionally dissected and weighed,
- each muscle tissue is snap-frozen in liquid nitrogen and packed individually,
- each liver is individually snap-frozen in a stainless steel funnel sieve and packed in a 100 ml Schott Duran bottle.

7 Biometric Sample Characterization

Most of the biometric parameters are obtained during the sampling (chap. 6). Only the exact age determination gained from the scales and the opercula is carried out in the laboratory. Therefore, the opercula are macerated and then cleaned. On the scales as well as the opercula, the annual growth rings are counted, which develop during

winter and are visible as lines. Opercula provide a more reliable result than scales, especially in breams older than 10 years.

Furthermore, the condition index (K) has proved to be trustworthy for the degree of the nutritional status of the fish. It is calculated as follows:

$$K = \frac{100 \times \text{body weight [g]}}{(\text{total length [cm]})^3}$$

In general, a reduced condition index indicates degraded living conditions, possibly caused by e.g. adverse water temperatures, chronic oxygen deficiency, or symptoms of poisoning (Teubner, *et al.* 2015).

The hepatosomatic index (HSI) is used to identify influences of environmental pollutants that lead to an enlargement of the liver (Sloof *et al.* 1983). It is calculated as follows:

$$HSI = \frac{100 \times \text{liver weight [g]}}{\text{total body weight [g]}}$$

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Checklist to Prepare and Conduct the Sampling

Specimen Type	Bream (<i>Abramis brama</i>)
Target Compartments	muscle tissue and liver, blood plasma
Individual Specimens	eight to twelve-year-old individuals (target age group)
Random Sample Number	at least 20 individuals
Sample Quantity for the ESB	<ul style="list-style-type: none"> • muscle tissue from the left side of the body, generally > 2,200 g • entire available liver
Sampling Period	from the middle of July until the end of October
Sampling Frequency	1 sampling per annum
Required Equipment for Field Work	<ul style="list-style-type: none"> • specimen data sheets for documentation during the sampling • fishing equipment depending on the method to be applied (chap 6.2) • species-appropriate net cage or transport containers with ventilation equipment • landing net
Sample Packing Until Further Processing	<ul style="list-style-type: none"> • stainless steel containers (5.5 l and 3.5 l) with lids and fastener • cardboard tube storage boxes for blood plasma samples • paper bags for scales • zip lock bags or sealable plastic containers for opercula • for screenings additional 100 ml Schott Duran bottles and stainless steel containers (1.5 l)
Transport and Interim Storage	cooling device (dewar) for the rapid deep-freezing and storage of the samples in the gas phase above liquid nitrogen (LIN)
Required Equipment for Laboratory Work	<ul style="list-style-type: none"> • specimen data sheets for the biometric sample description • clean bench with particle and activated carbon filtration • club • electrical system to anaesthetize the fish • measuring board (reading 0.5 cm) • laboratory scales (reading 1 g and 0.1 g) and standard weights • scale bowl for whole fish • 2.6 ml polypropylene tube with sealing push-in cap, treated with heparin (S-Monovette with 10-30 LE/ml whole blood) and 0.9 mm cannula, 38 mm length) • pipettes for laboratory use with adequate tips (0.1-1000 µl) • cryo-tubes (PP), labeled for identification • cooling centrifuge for laboratory use with external rotor motor • scalpel holders with stainless steel blades • 2 stainless steel beakers for demineralized water • demineralized water • stainless steel tweezers • stainless steel scissors • stainless steel pliers • stainless steel bone cutters • PTFE pad • disposable gloves and clothes for laboratory work

	<ul style="list-style-type: none">• liquid nitrogen• protective clothing for liquid nitrogen handling• insulated container to hold stainless steel containers
Biometric Sample Characterization:	<ul style="list-style-type: none">• body weight (reading 1 g)• complete length and total length (reading 0.5 cm)• weight of muscle tissue, liver, kidneys, spleen and innards (reading 0.1 g)• age and sex• condition index and hepatosomatic index

GERMAN ENVIRONMENTAL SPECIMEN BANK

Specimen Data Sheet 1: Sampling Location(s)

Bream (*Abramis brama*)

Identification:

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

Sampling Site (plaintext) _____

Sampling Point (number) _____

Sampling Point (plaintext) _____

Remarks _____

Sampling Leader _____

Notes _____

GERMAN ENVIRONMENTAL SPECIMEN BANK

Specimen Data Sheet 4.1: Storage (not for entry in the information system IS UPB)

Bream (*Abramis brama*)– Norm for Mixed Samples

Identification

____ / X / ____ / ____ / ____

Number of the stainless steel container

Matrix

Remarks:

GERMAN ENVIRONMENTAL SPECIMEN BANK

Specimen Data Sheet 4.2: Storage (not for entry in the information system IS UPB)

Bream (*Abramis brama*)– Individual Samples

Identification

____ / X / ____ / ____ / ____

Number of fish	Number of the stainless steel container	Matrix (plain text)
01	_____	
02	_____	
03	_____	
04	_____	
05	_____	
06	_____	
07	_____	
08	_____	
09	_____	
10	_____	
11	_____	
12	_____	
13	_____	
14	_____	
15	_____	
16	_____	
17	_____	
18	_____	
19	_____	
20	_____	

