UMWELTPROBENBANK
DES BUNDES

Guideline for Sampling and Sample Processing

Blue Mussel (*Mytilus edulis complex*)

Martin Paulus, Roland Klein, Diana Teubner

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

Umwelt Bundesamt

Contents

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 Wagner *et al.* (2011) 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

The blue mussel *Mytilus edulis* (Linnaeus 1758) inhabits hard structures on marine tidal coasts of the North Sea and the North Atlantic, often with a high density and high biomass. Along with the species related to it, it represents the level of first-order consumers in marine coastal ecosystems, which, in the limnetic ecosystems, is represented by the zebra mussel (*Dreissena polymorpha*) (Binelli *et al.* 2015.)

Blue mussels (family Mytilidae) feed by filtering plankton and detritus from the seawater and thus belong to the sessile marine organisms that absorb and accumulate a wide range of organic and inorganic substances in dissolved, but also in particulate, form from the surrounding seawater. Recent studies indicate that blue mussels also pick up and partially incorporate microplastic particles as well as concentrate the pollutants accumulated from the plastics in their soft bodies (Avio *et al.* 2015, van Cauwenberghe *et al.* 2015.

Mussels are therefore suitable for detecting the bioavailability of substances from the marine environment (Sondergaard *et al.* 2014) and are used in many national and international marine environmental monitoring programs, e.g. International Corporation for the Exploration of the Seas (Davis and Vethaak 2012), Mussel Watch (Farrington *et al.* 1987, Seranico *et al.* 1995), Arctic Monitoring and Assessment Program (AMAP) (Christensen *et al.* 2002, Riget *et al.* 2010) and Prestige Oil Spill Biomonitoring (Marigomez *et al.* 2013). In addition to the accumulation of substances, mussels are also used to study the biological effects of marine ecosystem pollution based on various biomarkers (Brooks *et al.* 2011, Li *et al.* 2013, Suarez-Ulloa *et al.* 2013, Lehtonen *et al.* 2014).

As mussels are consumed by humans, their levels of various groups of substances are monitored worldwide, e.g., pharmaceuticals (Ericson *et al.* 2010, Quinn *et al.* 2015), radionuclides (Kilic *et al.* 2014, Bode *et al.* 2015) and POPs (Widdows *et al.* 1995, Webster *et al.* 2003, 2009, Dondero *et al.* (2006).

The following criteria underline the use of the blue mussel as an indicator organism (q.v. Einsporn *et al.* 2009, Brooks *et al.* 2015):

- It is widely spread along temperate climatic coastal regions.
- It often occurs in high population densities and biomasses.
- Due to the sessile lifestyle of the adult mussels, they guarantee location loyalty for their multi-year lifetime.
- It accumulates dissolved and particulate substances through filtration from the surrounding medium.
- It has a strong resistance to a variety of pollutants.
- It is easy to manipulate, i.e. suitable for active monitoring (exposure of blue mussels colonizing substrates) as well as for toxicity and effect tests.
- Mussels are consumed by humans and thus provide a link between the marine environment and human beings.

4 Target Compartments

The soft body, including the respiratory water contained and the gut contents, is used as a sample. Respiratory water and gut contents remain in the mussels as sample components, as it is not practicable to store the mussels in order to remove the respiratory water or to evacuate the intestines and would be associated with a contamination risk for the samples.

5 Predefinitions for the Sampling

5.1 Species Determination

The common mussel native to the North and Baltic Seas (*Mytilus edulis* Linnaeus 1758) has blue-black shells that are up to 10 cm long and 4 cm wide, which consist of two almost-equal valves. The color scheme of the shells varies from dark blue/gray to black/dark gray, often with beige to gold colored zones; damaged areas are silvery white.

M. edulis, together with the bay mussel (*Mytilus trossulus* Gould 1850), common in the Baltic Sea, and the mediterranean mussel (*Mytilus galloprovincialis* Lamarck 1819), common in the Mediterranean and on the European Atlantic form a superspecies complex (Gosling 1992). *M. galloprovincialis* has a tendency to spread north and east (Hilbish *et al.* 2012, Steinert *et al.* 2012). *M. trossulus* is native to the Pacific Ocean and populates the Baltic Sea (Zbawicka *et al. 2014). M. edulis* forms hybrid zones with both taxa (Rawson *et al.* 1996, Hilbish *et al.* 2002, Dias *et al.* 2011, Zbawicka *et al*. 2014).

M. galloprovincialis grows to about the same size as *M. edulis,* but usually has broader shells, while *M. trossulus* is much smaller and mostly slender and thin-shelled. Typical shells of the three taxa are shown in Fig. 1.

It is not possible to clearly distinguish the three taxa and their hybrids in the field on the basis of external shell morphological features, since both their shell morphology and the color patterns have enormous plasticity and the most differentiating features (relative size of the anterior hinged plate and the adductor muscle scar) are only accessible after careful dissection and optical magnification (Asmus 1984, Gosling 1992, Daguin *et al.* 2001, Baird 2012). Therefore, precise species identification during sampling, especially for larger sample sizes is not possible.

However, the fact that there are indications that the three taxa have different accumulation potential (Chernova 2010, Brooks *et al.* 2015) means it is important for the interpretation of monitoring results to consider the genetic structure of the mussel populations from which the samples are taken.

Investigations on the ESB sampling sites have shown that mussel populations in the North Sea are pure *M. edulis*. Screening of the Baltic Sea individuals showed the expected intermixing of *M. edulis* (approx. 80%) und *M. edulis* × *M. trossulus* hybrids (approx. 20%) (Quack and Kosuch 2005, Quack *et al.* 2010). Since the superspecies complex has many dynamic zones of hybridization, a periodic check of the taxonomic affiliation of the populations to be sampled is recommended.

Fig. 1: Comparative representation of the shells of *Mytilus edulis* (top), *Mytilus trossulus* (middle) and *Mytilus galloprovincialis* (bottom)

5.2 Selection and Definition of Sampling Sites

Since the sampling sites have to be representative for the ecosystem, the vicinity to local sources of emissions must be avoided. If *Mytilus* stocks on artificial substrates are selected, care must also be taken to ensure that no contamination occurs due to the nature of the substrate. Substrate type, location and condition of the sampling sites as well as possible changes are to be documented in the specimen data sheets.

The selection of sampling sites is primarily determined through the incidence and accessibility of natural mussel banks with a sufficient quantity and long-term stability. When selecting, current and previous mappings and observations are consulted to increase the probability of long-term stability of the presumed sampling banks (Common Wadden Sea Secretariat 2008, Dolch and Reise 2010).

The mussel populations are exposed to high temporal and spatial dynamics, which has a particularly great effect in the sublittoral zone of the Wadden Sea (Nehls and Thiel 1993, Nehls and Sach 2000, Millat *et al.* 2009, Dolch and Reise 2010, Büttger *et al.* 2011, Nehls *et al.* 2011). In particular, the strong spread of the pacific oyster (*Crassostrea gigas*) has contributed to a large decline in mussel populations and a reduction in growth rates (Reise 1998, Diederich *et al.* 2005). However, a general threat to mussel populations in the Wadden Sea is not currently expected (Kochmann *et al.* 2008, Markert *et al.* 2010, Troost 2010, Eschweiler and Christensen 2011, Millat *et al.* 2012, Bray *et al.* 2015).

5.3 Selection of Individuals and Sample Size

A suitable criterion for reducing the natural variability is the shell length of the mussels. For practical reasons, relatively large shells are sought, for biological reasons, adult mussels older than 2 years should be sampled.

The length class corresponding to the respective body of water must be determined before sampling by means of a screening for each sampling site, since the growth rates of the mussels and age structure of the banks may be subject to spatial and temporal variations.

For longer-term sample series, the size distributions must be reviewed over several years and the size class to be collected must be readjusted if necessary.

The quantity and the size class of the individuals to be collected for each sampling site are to be set according to the results of the preliminary investigations in the respective area-related sampling scheme (see Chap. 5.5). To carry out the sampling, it is recommended that a template be used to test the minimum size of the mussels to be collected.

There is no minimum sample size valid for all substances for the determination of temporal and spatial concentration differences. Based on preliminary investigations, the minimum sample size can be estimated statistically (e.g. by power analysis) for a specific substance. For the ESB and along with the consideration of pragmatic aspects, the recommended minimum sample size is 50 individuals of a defined size class per sampling date. In order to obtain a sufficient amount of sample material, larger quantities may be required, depending on the individual weights and thus the defined size class.

5.4 Sampling Period and Frequency

The pollutant concentration in the common mussel depends on a variety of parameters, such as the pollutant concentration in the circumfluent water, the time of year, the water temperature, and the salinity. Since the development and release of gametes has a decisive influence on the material composition of the mussels and the mussels are extremely variable in their spawning behavior (Gosling 1992), the sampling period for each sampling site must also be adjusted to the respective local spawning cycle.

If, as in the Federal Environmental Specimen Bank, a representative sample of one year is sought, as a result of the above-mentioned annual rhythm of the parameters influencing the substance content, the sampling must be carried out several times a year. Because of the large dynamics involved (tides) and the constant substance exchange associated with it, this is especially true for Wadden Sea ecosystems.

The samples are combined into an annual homogenate, each with the same weight proportions (Knopf 2012).

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,
- size class to be collected,
- required sample size,
- sampling period,
- appropriate authorities.

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:

- scales (weighing range up to at least 5 kg, reading 1 g),
- template or gauge for the minimum size,
- stainless steel wire baskets,
- disposable gloves,
- stainless steel containers with lids and fasteners,
- cooling device for immediate deep freezing and transport of the samples in the gas phase above liquid nitrogen (LIN),
- specimen data sheets,
- protective clothing for handling liquid nitrogen.

Laboratory:

- clean bench with particle and activated carbon filtration,
- protective clothing for handling liquid nitrogen,
- stainless steel containers with lids and fasteners,
- insulated container to hold stainless steel containers,
- liquid nitrogen,
- scales (weighing range up to at least 5 kg, reading 1 g) to determine the soft body weight,
- scales (reading 0.01 g) to determine the biometric parameters,
- caliper (reading 0.1 mm),
- weighing pan,
- pincers, oyster knife and stainless steel scalpel,
- absorbent laboratory paper,
- disposable gloves and protective clothing,
- specimen data sheets.

Sample containers and all equipment are cleaned in a laboratory washer using a chlorine-free powerful washing agent in the 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

In general the collecting is carried out in eulittoral tidal waters by hand, in sublittoral commonly by divers or by using a dredge. Individual blue mussels of the predefined size classification are carefully removed by hand, washed in seawater and laid in the stainless steel basket. For this powder-free laboratory gloves are to be worn. When using dredges, the selection takes place after the landing of the mussels. As far as possible all adherents, plants and lightly attached animals are removed manually. Byssus threads, which protrude from the shell and firmly bonded sea urchins, or similar, are not removed.

The entire sample material is multiply rinsed in the sea to eliminate sediment residuals. Then the shells are set up to drain the water. The sample is then transferred to stainless steel containers and weighed. Immediately after the sampling take, the sample material is deep frozen in the gas phase above liquid nitrogen in a transport Dewar flask, to instil instant death and conserve the mussels. If, for logistical reasons, the mussels must be frozen and stored at -20°C, this intermediate storage should not exceed four weeks.

Work-Up in the Laboratory

The stainless steel container designated for the soft bodies is pre-weighed, marked with the associated sample identification and pre-cooled with liquid nitrogen.

Since the respiratory water could only be removed by thawing the mussels, which would be contrary to maintaining an uninterrupted cryogenic storage in accordance with ESB requirements, the entire contents of the mussel shells are sampled.

The separation of shell and soft body including respiratory water is done in the laboratory at a pure-air workstation with particle and activated carbon filtration (clean bench) in the frozen state, without thawing the soft bodies during dissection. For this, three to four of the cryogenically frozen mussels are carefully removed from the sample vessel and placed on the work surface of the pure-air workstation for superficial thawing.

When the frost formed on the shells begins to thaw, the shells are opened with tweezers or an oyster knife. The still solidly frozen body is removed with a pair of tweezers, freed with a second pair of tweezers of any residual shell still attached and collected in the sample container.

If upon opening the soft body has started to thaw, the specimen is discarded.

The soft body samples of the individual sampling dates are merged and homogenized at the end of the sampling year in compliance with the cryogenic conditions with equal proportions by weight.

7 Biometric Sample Characterization

Biometric sample characterization is performed on 50 frozen mussels in the laboratory. The length, width and height of the shells as well as the fresh weight of the entire mussel with respiratory water, the fresh weight of the soft body as well as that of the shell are determined.

Since the determination of the soft body fresh weight of frozen mussels is subject to significant error risks, a precise standardization of the determination method must be followed to avoid systematic random errors and to minimize them, as described below:

To determine the fresh weight with respiratory water, the mussels are freed of attached barnacles after removal from the sample container in the frozen state. Once the surface has defrosted and becomes moist, the mussel is wiped off with absorbent laboratory paper and weighed immediately (reading 0.01 g).

After determining the fresh weight with respiratory water, the mussels are individually laid out on absorbent laboratory paper in consecutively numbered places. The beginning of the thawing time (= removal of the mussel from the cooled sample vessel) is recorded in order to be able to comply with the thawing time determined for each mussel according to its fresh weight. Subsequently, the length, width and height of the shells are measured by means of a caliper (reading to 0.1 mm) and recorded in specimen data sheet 3. To thaw, the mussels are placed with the ventral side down on the paper.

The soft body weight is defined as the fresh weight of the soft body when the body of the mussel has fully thawed and the respiratory fluid fully emitted, but the loss of tissue fluids is minimal.

The thawing time depends on the size of the mussel as well as the ambient temperature. It is reached at the time when rapid weight loss due to leaking of the thawed respiratory water changes to a much slower weight loss caused by evaporation of the tissue water.

When the shells have opened, the mussels are rotated several times to allow the contained water to drain completely. At a room temperature of 20 – 22°C, the following thawing times were determined in relation to the fresh weight with respiratory water (Tab. 1).

Tab. 1: Thawing times as a function of the fresh weight with respiratory water for the determination of the soft body weight

Fresh weight with respiratory water [g]	Thawing time [min]	Fresh weight with respiratory water [g]	Thawing time [min]
5,0	56	20	83
7,5	64	25	86
10	69	30	92
12,5	73	35	95
15	77	40	99
17,5	80		

After the thawing time of 56 to 99 minutes (Tab. 1), the soft body weight defined above is determined. For this, the soft body is removed after thawing by means of a scalpel and tweezers, quantitatively collected in a pre-weighed dish and weighed immediately to avoid evaporation losses (dripping water is removed, reading to 0.01 g). After that, the shell is also weighed (reading to 0.01 g).

It should be noted that the stored mussel samples provided for chemical characterization contain the respiratory water. Therefore, the concentrations of substances of the UPB samples are diluted by a factor of about 2.4 to 3, depending on the sampling site, compared to mussels that are often dissected in fresh condition in other studies.

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Checklist to Prepare and Conduct the Sampling

Specimen Type	**Blue mussel (*Mytilus edulis* complex)**
Target Compartment:	soft body (deep frozen, dissected, including respiratory water and gut content)
Individual Specimens	mussels of the size class specified in the area-related sampling scheme
Sample Number	at least 50 individuals
Sample Quantity for the ESB	for a sample volume of 1,000 g of soft bodies, the removal of 6 x approx. 350 g or 2 x approx. 1,000 g of mussels per year is required
Sampling Period	for 6 sampling dates, every 2 months from February to December for 2 sampling dates, in June and November
Sampling Frequency	2 or 6 samplings per year as an annual mixed sample
Required Equipment for Field Work	• scales (weighing range up to at least 5 kg, reading accuracy 1 g) • template or gauge for the minimum size • stainless steel wire baskets • disposable gloves • specimen data sheets • protective clothing for handling liquid nitrogen
Sampling Packing	• stainless steel containers with lids and fasteners
Transport and Interim Storage	• cooling device for immediate deep freezing and transport of the samples in the gas phase above liquid nitrogen (LIN)
Required Equipment for Laboratory Work	• clean bench with particle and activated carbon filtration • protective clothing for handling liquid nitrogen • stainless steel containers with lids and fasteners • insulated container to hold stainless steel containers with liquid nitrogen • liquid nitrogen • scales (weighing range up to at least 5 kg, reading 1 g) • scales (reading accuracy 0.01 g) • caliper (reading 0.1 mm) • weighing pan • pincers, oyster knife and stainless steel scalpel, • absorbent laboratory paper, disposable gloves • specimen data sheets
Biometric Sample Characterization of 50 Mussels	• length, width, and height of the shell (reading 0.1 mm) • fresh weight including respiratory water (reading 0.01 g) • weight of the soft body (reading 0.01 g) • weight of the shell (reading 0.01 g)

GERMAN ENVIRONMENTAL SPECIMEN BANK

Specimen Data Sheet 1: Sampling Location

Blue mussel (*Mytilus edulis* complex)

Identification:

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

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

Sampling Site (plaintext) _____

Sampling Point (number) _____

Sampling Point (plaintext) _____

Remarks _____

_____

_____

_____

Sampling Leader _____

Notes _____

_____

_____

_____

_____

_____

_____

_____

GERMAN ENVIRONMENTAL SPECIMEN BANK

Specimen Data Sheet 2: Sampling Method, Sample Description and Storage
Blue mussel (*Mytilus edulis* complex)

Identification: __ __ __ __ **/ X /** __ __ __ __ **/** __ __ __ __ __ **/** __

from: __ __ . __ __ . __ __ Sampling Date to: __ __ . __ __ . __ __

Start: __ __ : __ __ Time End: __ __ : __ __

Substratum

☐ Groyne

☐ Mussel Bed

☐ Other: _____
(Description of Structure and Kind)

Sampling Technique

☐ Manually Collected

☐ Collected by Use of a Dredge

☐ Other: _____

Sample Description

Mussels Polluted by

☐ Sediment: _____

☐ Other: _____
(Description of Structure and Kind)

Periphyton

☐ Barnacles ☐ sporadic ☐ middle rate ☐ plenty

☐ Other: _____ ☐ sporadic ☐ middle rate ☐ plenty

Storage

Number of stainless steel containers	Weight Empty [g]	Weight Filled [g]	Weighted Sample [g]	Priority	Remarks

GERMAN ENVIRONMENTAL SPECIMEN BANK

Specimen Data Sheet 3: Sample Description

Blue mussel (*Mytilus edulis* complex)

Identification: __ __ __ __ **/ X /** __ __ __ __ **/** __ __ __ __ __ **/** __ __

No.	Time Weighed Soft Body _ _ : _ _	Fresh Weight (Resp. Water incl. _ _ , _ _ g	Length _ _ , _ mm	Width _ _ , _ mm	Height _ _ , _ mm	Weight of the Soft Body _ _ , _ _ g	Weight of the Shell _ _ , _ _ g

Processing Status:

from No.	to No.	Date: dd.mm.yy	Reviser	Signature

GERMAN ENVIRONMENTAL SPECIMEN BANK

Sampling Protocol

Blue mussel (*Mytilus edulis* complex)

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
- ☐ 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 (clear text):

_____

_____

_____

_____

Remarks: _____

_____

_____ __ __ . __ __ . __ __ _____

Recorder Date Signature