
# Plan and report of the *in situ* mussel caging experiment

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**Anu Lastumäki, Raisa Turja and Kari Lehtonen**[1]

**Katja Broeg and Matthias Brenner**[2]

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[1] Finnish Environment Institute (SYKE)

[2] Alfred Wegener Institute for Polar and Marine Research (AWI)

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## Contents:

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# 1. Introduction and objectives of the experiment

Blue mussels (*Mytilus* spp.) are widely used sentinel species for studying accumulation and biological effects of a variety of contaminants present in the marine environment. Mussel caging studies have been successfully conducted in different coastal areas worldwide, and the method has also been tested and further developed in the Finnish Environment Institute (SYKE) for many years in several areas of the Baltic Sea.

In these *in situ* experiments the mussels are deployed in specifically developed cages at the desired study sites for a certain period of time (several weeks/months). The on-site exposed organisms are subsequently analysed for tissue accumulation of chemical substances and biological effects (biomarkers). The cage anchoring system has been designed as sufficiently robust to be coupled with other kinds of oceanographic equipment including hydrography sensors with automatic data loggers (e.g., salinity, temperature and oxygen) and passive samplers of chemicals. The locations now selected as mooring stations for CHEMSEA project were based on previous studies carried out during the MERCW –project (INCO-CT2005-013408) as follows:

1. WRECK-1G (N55.19.07` E015.37.61`), depth 96m: highest concentrations of CWA degradation products found from the sediment.

2. 2359 (N55.20.95´ E015.38.82`), depth 95m: highest concentrations of CWA degradation products found from the sediment.

3. TR4-10KM (N55.22.94` E015.44.26`), depth 93m: supposedly less contaminated area.

4. BY 5 (N55.15.00` E015. 59.00`), depth 90m: COMBINE monitoring station, reference site.

The chemical warfare agents (CWA) present in the sea bottom of the Bornholm Basin study area can induce a variety of adverse biological effects in organisms exposed to them. The target of the study was to use various biomarker methods representing different biological functions and levels of biological organization that allows us to predict the potential risk of dumped chemicals to aquatic organisms using the mussel as a model organism.

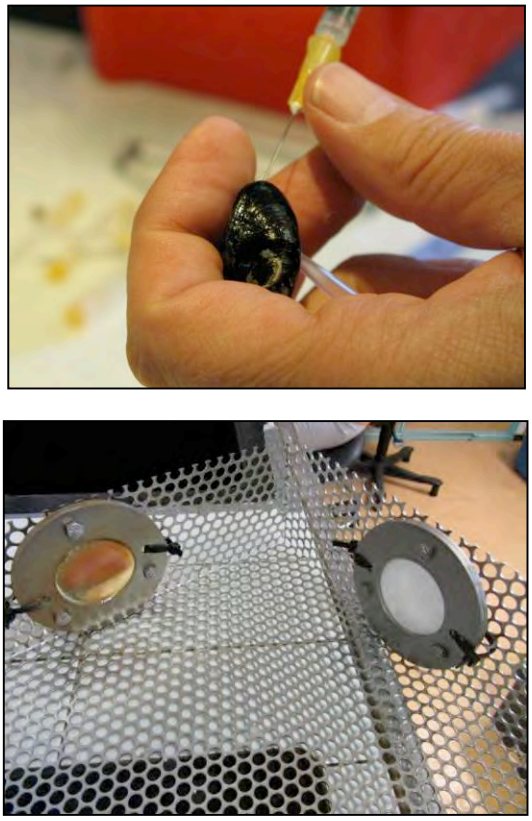


**Figure 1.** *Top left*: Collection of a haemolymph (blood) sample from a mussel. Various biomarkers (e.g. immunological) can be measured from the blood cells of organisms. *Down left:* POCIS passive samplers to collect waterborne chemicals were attached to cages. *Right:* Deployment of mussel cages aboard RV *Aranda*.

# 2. Material and methods

## 2.1 Start of the experiment: COMBINE 2 cruise onboard RV *Aranda*

Mussels for the caging experiment were collected by scuba diving in the east coast of Bornholm in Svenske Havn by four scientific divers during 24.-25.5.2012. The exact location of the diving place was N55°05, 33`, E15°09, 50`. The mussels were collected from the depth of 18 m where they were found to be within the required size range of 2.5-3.5cm (Fig. 2). Mussels in shallower areas were too small for the experiment. The target was to include 400 mussels per cage, and an additional 400 mussels were collected to be dissected immediately for the "start" situation. Thus, a large amount of mussels were sampled and from these a total of 3600 individuals were sorted out as large enough for the caging experiment (Fig. 2).

**Figure 2.** *Left*: Diving for blue mussels in Svenske Havn (Bornholm) for the caging experiment. CTD Chief Tuomo Roine with his catch of the day, local diver Allan Olsen further back. *Right*: Cleaning up the mussels for the caging. Chief chemist Pia Varmanen is giving the biologists a much-welcomed helping hand.

Hydrographical parameters were measured from the water column before the deployment of the cages. The parameters included nutrients, salinity, pH, temperature, oxygen content and fluorescence as well as Secchi depth.

Based on this hydrographical data, the cages were decided to be deployed at 35 and 65 meters depth at all four study stations. The poor oxygen conditions (<1 mg $O_2$ $l^{-1}$) prevailing in the main CWA dumping area (average depth ca. 95 m) made caging closer to the sea bottom unfeasible. The cages were deployed during 25.-26.5.2012.

The mussel cages were deployed in the Bornholm Basin in May and recovered in August (total exposure time 2.5 months). The cages were positioned at four sites with two cages at each site (35m and 65m depth) (Table 1, Fig. 4 and 5). Four hundred mussels were placed in each cage (200 for biomarker and 200 for chemical analyses). In addition, the cages were equipped with POCIS (Polar Organic Chemical Integrative Sampler) passive samplers to record the accumulation of selected organic contaminants and hydrography sensors with automatic loggers measuring temperature, salinity and oxygen at 30 min intervals (Fig. 3).

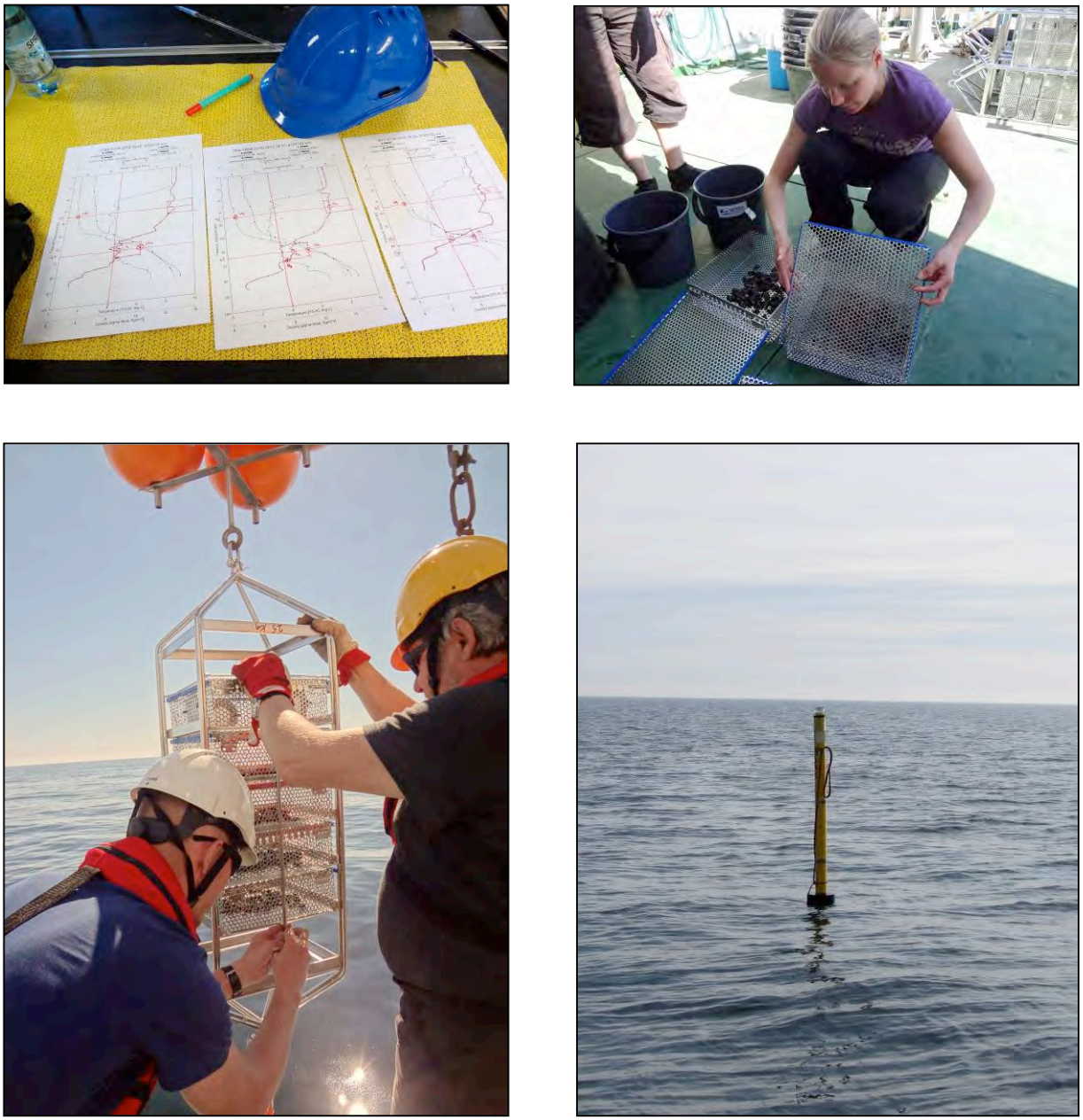

**Figure 3.** *Up left*: Fixing the caging depths by examining the previously run CTD data. *Up right:* Scientist Raisa Turja introducing the mussels to their new quarters. *Down le*ft: Tuomo Roine and technician Juhani

Rapo fixing a mussel cage ready for deployment. *Down right:* The cages were marked with ODAS buoys equipped with flashing light signal and radar reflector.

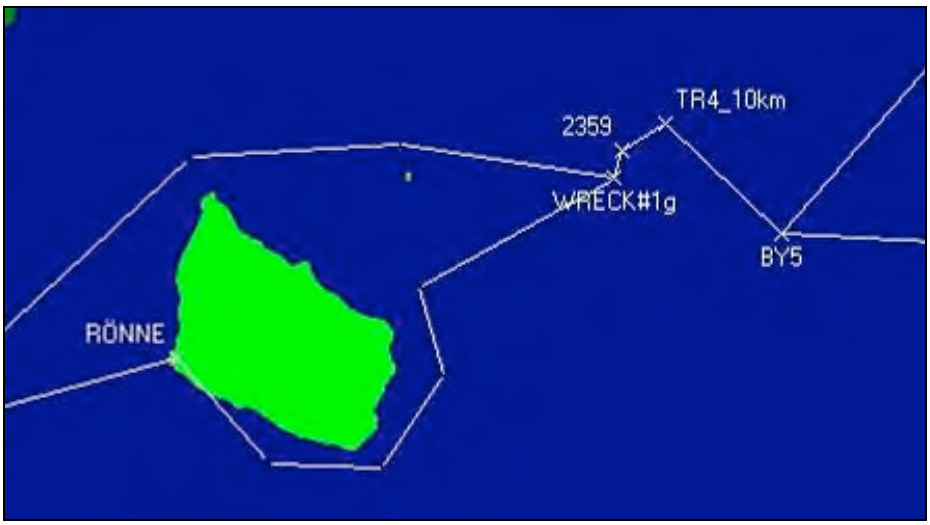


**Figure 4.** The mussel caging stations were located in NE of Bornholm.

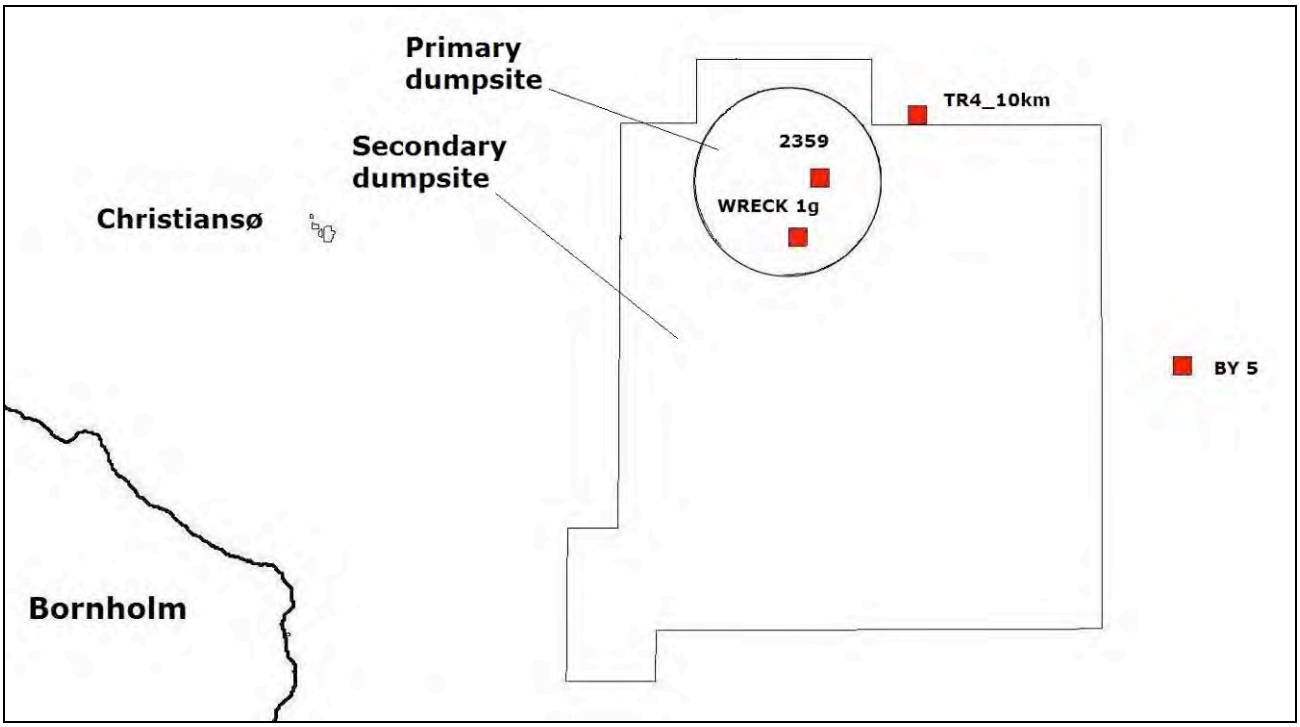


**Figure 5.** A simplified map of the CWA dumpsite area, indicating the primary and secondary dumpsites as well as the stations of the mussel cage moorings.

**Table1.** Codes and geographic positions of the caging stations. Two cages were deployed at all stations with the upper one to 35m and the lower to 65m depth. Six of the cages contained hydrographical sensors with automatic data loggers.

| | | | Loggers | |
|---|---|---|---|---|
| Station code | Latitude | Longitude | Salinity Temperature Pressure | Oxygen |
| WRECK-1G | N55°19.07` | E015°37.61` | | |
| 2359 | N55°20.95` | E015°38.82` | 35m + 65m | 65m |
| TR4-10KM | N55°22.94` | E015°44.26` | 35m + 65m | |
| BY5 | N55°15.00` | E015°59.00` | 35m + 65m | 65m |

## 2.2 End of the experiment: COMBINE 3 cruise onboard RV *Aranda*

The mussel cages deployed in the Bornholm Basin in late May 2012 were successfully retrieved during the COMBINE 3 cruise of RV *Aranda* in late August 2012. Mussel samples (400 individuals) for recording the "end" condition of the natural population were collected on 16.8.2012 by scuba diving from the same place (Svenske Havn) as in May 2012. Retrieval of the cages took place on 18.8.2012. At one of the four caging sites, the original reference site BY5 the installations had disappeared, probably due to a collision by a fishing or cargo vessel. After retrieval all the mussels were kept in cold rooms in vented water collected from the sampling site. Mortality of the mussels in all 6 cages was in general very low and oxygen conditions were relatively good also at the depth of 65m (ca. 4.9 mL/L). Dissecting of the mussels for chemical and biomarker analyses started immediately and all the samples were stored as appropriate for each analysis type on 20.8.2012 (Fig. 6).

**Figure 6**. *Up left*: Laboratory technician Anna Korpela is dissecting the mussels for biomarker analyses. *Up right:* Senior scientist Kari Lehtonen is examining lysosomal membrane stability of mussel blood cells. *Down left and right:* Scientist Anu Lastumäki is taking the mussels from the cages and placing them in a cooling box immediately after retrieval.

# 3. Samples collected and some preliminary results

Even though the cages at one of the stations (BY5) had disappeared, altogether 8 full sample sets were obtained, with 6 cages and samples also from ”start” and ”end” mussels collected from Bornholm sampling site.
Samples were taken for the analysis of CWA and degradation products in mussel tissues and selected biomarkers (Tables 2-4).

*Biochemical and cellular biomarkers*

- Antioxidant enzyme system activity: an important molecular defence mechanism scavenging reactive oxygen radicals excessively formed by exposure to harmful compounds. Several enzymatic measurements.

- Lipid peroxidation: the impaired function of the antioxidant system results in, e.g., peroxidised cellular membrane lipids, causing serious damage to normal cell function.

- Acetylcholinesterase activity: exposure to neurotoxic chemicals inhibits the proper function of cholinergic neurotransmission mediated by the acetylcholinesterase enzyme.

- Micronuclei and chromosomal aberrations: widely used universal indicators of genotoxicity.

- Lysosomal membrane stability (Neutral Red retention): a sensitive marker of impaired lysosomal function in cells reflecting general health status.

- Lipofuscin accumulation: Lipofuscin, also known as “aging pigment”, is widely regarded as an end product of protein and lipid peroxidation due to oxidative stress. Increased accumulation of lipofuscin in lysosomes of the digestive gland of mussels has been shown to be associated with contamination by anthropogenic pollutants (e.g. metals).

- Neutral lipid accumulation: Accumulation of neutral lipids in cellular vacuoles is used as an indicator of lipidosis induced by toxic chemicals.

- G6PDH-activity: G6PDH is an enzyme found especially in red blood cells and it dehydrogenates glucose-6-phosphate in a glucose degradation pathway. During this process NADPH and glutathione are produced, helping to protect membranes from oxidative damages. G6PDH itself is very sensitive to inactivation by xenobiotics and therefore a useful biomarker for pollution-induced carcinogenesis.

- p53 activity: p53 is a tumour suppressor protein and crucial in multicellular organisms in which it induces growth arrest or apoptosis depending on the physiological circumstances and cell type. p53 becomes activated in response to various types of stress, which include DNA damage (induced by e.g. UV or chemical agents), oxidative stress or osmotic shock.

The severity of the effects of exposure to contaminant stress increases when the organism is not able anymore to energetically maintain the molecular and cellular defence and repair mechanisms, or the energetic cost results reduction of normal functions such as growth and fecundity.

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*Higher level biomarkers*

- Cellular energy allocation (CEA): The amount of proteins, lipids and carbohydrates are measured together with respiratory electron transport system (ETS) activity in mitochondria. Reduced CEA value indicates that mussels are spending increasing amounts of energy on survival under stressful conditions.

- Condition index: (soft tissue dry weight versus shell length) is measured as a general parameter of mussel health, which is also related to the prevailing food conditions for mussels in the caging area. Dry weight is also used for calculating the accumulation of chemicals in tissues of mussels.

- Histopathology of digestive gland: Assessment of histopathological alterations such as degeneration of membranes and epitheliums, cellular debris, hemic neoplasia, inflammation, etc.

The on-site measurement of lysosomal membrane stability by the application of the Neutral Red retention test on blood cells of mussels showed a significantly lower dye retention time (=bad condition) in individuals in the deeper cage (65m) at the ”hot spot“ sites compared to the "reference" site (Fig. 7). Other biomarkers from mussels are still to be analysed.

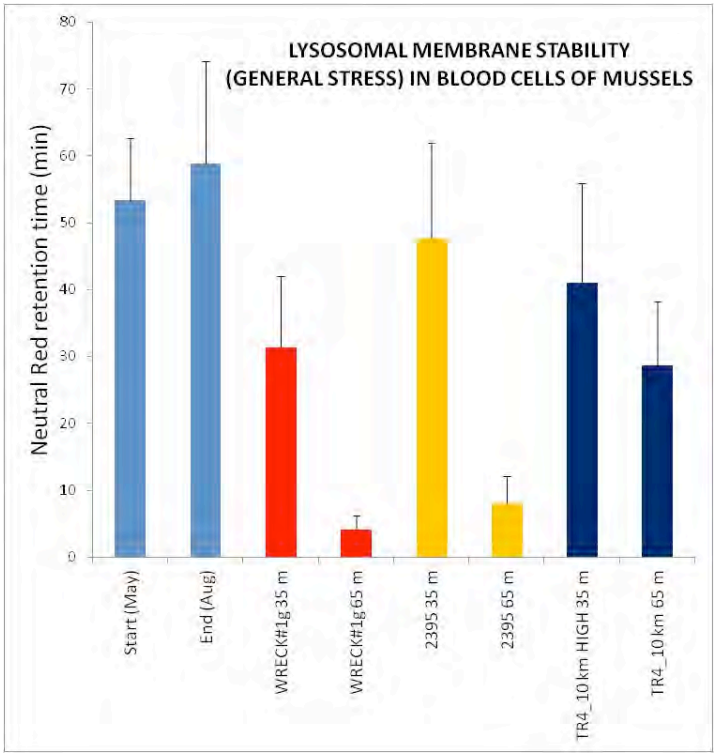


**Figure 7**. Lysosomal membrane stability (Neutral Red retention assay) was lower in the deeper cages (65m) deployed in the "hot spot" area (stations WRECK 1g and 2359) than in the “reference” site (TR4_10km).

**Table2.** Samples for biomarker analyses were taken for SYKE, AWI and NRC. Samples indicated with the same colour in the column are taken from the same individual. Different analysis are indicated as follows: glutathione S-transferase (GST), catalase (CAT), glutathione reductase (GR), lipid peroxidation (LPO), total glutathione (GSH), acetylcholinesterase (AChE), cellular energy allocation (CEA), lysosomal membrane stability (LMS), glucose-6-phosphate dehydrogenase (G6PDH) and p53 protein activity (p53).

| | SYKE | | SYKE | SYKE | SYKE | AWI | AWI | NRC | AWI |
|---|---|---|---|---|---|---|---|---|---|
| **Cage** | **GST, CAT, GR, LPO, GSH** | **AChE, GST, CAT** | **sex determination** | **CEA** | **Condition index** | **Histology, sex, developmental status** | **Lipofuchin, Neutral lipids, G6PDH, p53** | **Micronuclei** | **Histopathology** |
| WRECK-1 (35m) | DG 1-25 | Gill 1-25 | Mantle 1-25 | DG 1-30 | whole soft tissue 1-25 | Mantle 1-20 | DG 1-20 | Gill 1-10 | DG 1-20 |
| WRECK-2 (65m) | DG 1-25 | Gill 1-25 | Mantle 1-25 | DG 1-30 | whole soft tissue 1-25 | Mantle 1-20 | DG 1-20 | Gill 1-10 | DG 1-20 |
| 2359-1 (35m) | DG 1-25 | Gill 1-25 | Mantle 1-25 | DG 1-30 | whole soft tissue 1-25 | Mantle 1-20 | DG 1-20 | Gill 1-10 | DG 1-20 |
| 2359-2 (65m) | DG 1-25 | Gill 1-25 | Mantle 1-25 | DG 1-30 | whole soft tissue 1-25 | Mantle 1-20 | DG 1-20 | Gill 1-10 | DG 1-20 |
| TR4-1 (35m) | DG 1-25 | Gill 1-25 | Mantle 1-25 | DG 1-30 | whole soft tissue 1-25 | Mantle 1-20 | DG 1-20 | Gill 1-10 | DG 1-20 |
| TR4-2 (65m) | DG 1-25 | Gill 1-25 | Mantle 1-25 | DG 1-30 | whole soft tissue 1-25 | Mantle 1-20 | DG 1-20 | Gill 1-10 | DG 1-20 |
| | | | | | | | | | |
| Start | DG 1-25 | Gill 1-25 | Mantle 1-25 | DG 1-30 | whole soft tissue 1-25 | Mantle 1-20 | DG 1-20 | Gill 1-20 | DG 1-20 |
| End | DG 1-25 | Gill 1-25 | Mantle 1-25 | DG 1-30 | whole soft tissue 1-25 | Mantle 1-20 | DG 1-20 | Gill 1-10 | DG 1-20 |
| Number of samples | 200 | 200 | 200 | 120 | 200 | 160 | 160 | 90 | 160 |

Baltic Sea Region Programme 2007-2013

**Table 3.** Samples were also taken for the development of new methods using DG, gill and mantle tissue. Method development to investigate more closely the metabolic processes involved in stress adaptation and anaerobic conditions in Baltic blue mussels.

| | New methods; samples for metabolic enzymes | | |
|---|---|---|---|
| **Cage** | **newDG** | **newGill** | **newMantle** |
| WRECK-1 (35m) | newDG 1-30 | newGill 1-30 | newMantle 1-30 |
| WRECK-2 (65m) | newDG 1-30 | newGill 1-30 | newMantle 1-30 |
| 2359-1 (35m) | newDG 1-30 | newGill 1-30 | newMantle 1-30 |
| 2359-2 (65m) | newDG 1-30 | newGill 1-30 | newMantle 1-30 |
| TR4-1 (35m) | newDG 1-30 | newGill 1-30 | newMantle 1-30 |
| TR4-2 (65m) | newDG 1-30 | newGill 1-30 | newMantle 1-30 |
| | | | |
| Start | newDG 1-30 | newGill 1-30 | newMantle 1-30 |
| End | newDG 1-30 | newGill 1-30 | newMantle 1-30 |

**Table 4.** Samples for chemical analyses were taken for VERIFIN and IOPAS. 30 individuals (whole soft tissue) were pooled in one sample and 3 pools per cage were taken (except "End" samples for IOPAS, where 50 individuals were stored for one pool and station 2359-2, where only 18 individuals were pooled to the last sample).

| **Cage** | **VERIFIN** | **IOPAS** |
|---|---|---|
| WRECK-1 (35m) | 3x30 | 3x30 |
| WRECK-2 (65m) | 3x30 | 3x30 |
| 2359-1 (35m) | 3x30 | 3x30 |
| 2359-2 (65m) | 3x30 | 2x30, 1x18 |
| TR4-1 (35m) | 3x30 | 3x30 |
| TR4-2 (65m) | 3x30 | 3x30 |
| | | |
| Start | 3x30 | 3x30 |
| End | 3x30 | 3x50 |