Biological effects measured on caged mussels and cod

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1. Introduction

Due to the vast amount of chemical warfare agents (CWAs) detected from the sediments at different dumping areas it is likely that organisms exposed to these chemicals show increased stress responses or even elevated levels of higher physiological alterations, such as diseases. Biological responses methods including antioxidant defence, neurotoxicity, lysosomal membrane stability and bioenergetic status were applied to determine the possible effects of CWAs on biota using blue mussels and cod as sentinel organisms. The main results obtained from the analysis performed in the SYKE laboratory on samples from the mussel caging experiment and cod sampling in December 2011 are presented in this report.
2. Materials and Methods

Experiments to detect biological effects conducted during the CHEMSEA project:

- Blue mussel caging was used to expose mussels exactly at the selected hot spot 1 (WRECK-1G) and hot spot 2 (2359) sites and one reference (TR4) site in the Bornholm dumping area. Caging experiment was performed in May-August 2012 (see Deliverable 5.1.1.3 for details). Samples were taken for biomarker assays and chemical analysis; in addition cages were equipped with temperature, salinity and oxygen data loggers and passive samplers (POCIS).

- Baltic cod was sampled from dumping areas at Bornholm, Gotland and Gdansk and at the reference area during two sampling campaigns that took place in December on 2011 and 2012.

- Blue mussel laboratory exposure experiment was successfully conducted in May-June 2013. Mussels were exposed to mixtures of selected CWAs (Adamsite, Clark 1 and Chloroacetophenone) in different concentrations (low, medium and high) for 96 hours. Additional experiment was performed with mustard gas also for 96 hours. All the analyses are ongoing, detailed description of the experiment and results will be presented in the next report.

Due to the complex stress responses influenced by different chemicals and their mixtures it is important to construct a battery of biomarkers that comprises different biological functions and levels (molecular/biochemical, subcellular/cellular, physiological and individual). A number of different biological responses (see descriptions below) were analysed from blue mussels and cod to determine the possible CWA induced biological effects.

Lysosomal responses

Lysosomes are cellular organelles involved in various physiological and disease responses and in accumulating chemical contaminants. Therefore the stability of the lysosomal membrane has been widely used as a sensitive marker of environmental stress. Lysosomal membrane stability was investigated with Neutral Red Retention (NRR) assay on mussel haemocytes.

Enzymatic biomarkers

Exposure to hazardous chemical substances stimulate the formation of reactive oxygen species (ROS) ensued by oxidative damage to membrane lipids, proteins and DNA. Cells are protected against the deleterious effects of oxyradical generation by maintaining ROS at low levels through the action of several antioxidant enzymes.
Metabolism of certain chemical compounds produces reactive intermediates and ROS connecting biotransformation processes to the oxidative challenge. The levels of glutathione S-transferase (GST) for biotransformation phase II activity and catalase (CAT), glutathione reductase (GR) and superoxide dismutase (SOD) enzyme activity as well as lipid peroxidation (LPO) for antioxidant defence were measured. Inhibition of acetylcholinesterase activity (AChE) was measured to detect the possible neurotoxic effect of various dumped neurotoxic agents.

Bioenergetics and condition index

Exposure to chemical contaminants disturbs the energy balance of organisms, inducing adverse consequences at various levels of biological organisation. Cellular energy allocation (CEA) was applied as a method to investigate effects at the bioenergetics level measuring the relation between energy consumption (mitochondrial electron transport system activity, ETS) versus energy available in proteins, lipids and carbohydrates (total energy available Ea) for growth and reproduction. A morphometric condition index (CI) was applied as a robust indicator of the body condition (dry weight vs. shell length) of mussels.

Environmental factors at the caging sites

Data loggers detecting water temperature, salinity and oxygen were attached to the mussel cages, the data obtained from the site 2359 are presented in the Fig 1.

Statistical analysis

Data was tested for normality and homogeneity of variances. When the assumptions were met, one-way ANOVA (F) followed by multiple comparisons post-hoc test was applied with Bonferroni correction. In the case of non-normal data, the Kruskal-Wallis-test (KW) was applied. Values p < 0.05 were considered statistically different. All statistical analyses were performed with the program R.
3. Results and Discussion

Caging experiment

In the caging experiment highest biomarker responses were generally detected at both hot spot sites at 65 meters. Data loggers attached to the cages reviled high variations in the environmental factors, which probably contribute also to the observed biomarker responses. The water at 35 meters mixes with the surface water resulting in high temperature fluctuations but the salinity remained stable (Fig. 1A). Situation was completely opposite in the 65 meters where temperature was stable, but salinity and the concentration of dissolved oxygen fluctuated showing the mixing of the near bottom water in the sub halocline water layer (Fig. 1B).
This indicates that it is very likely that mussels at 65 meters were exposed to CWAs and other contaminants present in the sediment or near bottom water resulting in the observed biological effects. However, it has to be kept in mind that mussels were also exposed to lower oxygen concentrations that could, at least for a short period of time, induce the change from aerobic to anaerobic metabolism in mussels contributing to the variation of the observed biological responses.

Markedly lower lysosomal membrane stability observed at 65 meter cages at stations WRECK1 and 2359 indicated significantly impaired haemocyte function compared to all other stations (Fig. 2). A somewhat lower LMS value at the 65m reference station compared to 35m stations possibly show the effect of increased general stress due to the eg. lower oxygen at the 65m.

Fig. 2. Lysosomal membrane stabilisation in caged mussels recorded in the hotspot cages hot1 and hot2 (sites WRECK1 and 2359 respectively) and in the reference cage (TR-4) at 35m and 65m. Start and End refer to native mussels sampled before (start) and after (end) of the caging period.

Significant enzymatic responses detected in the caged mussels at hot spot sites compared to the reference site at 65 meters were increased GST activity in the digestive gland at WRECK1 (Fig. 3A), increased CAT activity in gills at WRECK1 and 2359 (Fig. 3D) and increased GR activity at 2359 (Fig. 3E) indicating increased biotransformation and antioxidant system activities due to the contaminant exposure.
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**C**

![Box plots showing GST and CAT activity](chart.png)

**E**

![Box plots showing GSTgill and CATgill activity](chart.png)
Fig. 3. Enzymatic biomarkers measured in mussels caged at 65m. Activity of the biotransformation enzyme GST observed in the digestive gland (A) and gills (C). Antioxidant enzyme activities of CAT measured in the digestive gland (B) and gills (D) and GR in the digestive gland (E). Bh-End stands for the native mussel population sampled at the end of the experiment (same population used for the experiment), hotspot cages hot1 and hot2 refer to sites WRECK1 and 2359 respectively and ref to the reference cage (site TR-4).

Fig. 4. Cellular energy allocation (CEA) (A) and condition index (CI) (B) measured in native (Bh-end) and caged mussels. Hotspot cages hot1 and hot2 refer to sites WRECK1 and 2359 respectively and ref indicates the reference cage (site TR-4). Significantly different groups are indicated with different letter.
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Higher CEA value was observed in mussels caged at 35 meters compared to 65 meter cages possibly indicating better food conditions at 35 meters. No significant differences in the CEA values were observed between cages at the same depth (Fig 4A). Decreased CI value was observed in mussels at WRECK1 compared to other caging stations (Fig. 4B).

Interestingly the native mussels collected from Bornholm right after the experiment (site Bh-end) were markedly in poorer condition (CI and CEA) compared to the caged specimens. This was also seen in the beginning of the experiment when unexpected difficulties were faced in finding large mussels (shell length > 2.5 cm) for the caging.

Cod

Due to high variability in different individuals and the bell-shape nature of enzymatic responses, the mean values of enzymatic markers did not show significant differences between study areas. However, the amount of abnormally high or low values of different enzymes was increased in cod individuals from the dumping areas compared to the reference area (Fig. 5). The state of the cod individuals was assessed based on analysed GST, CAT, GR, total glutathione and AChE responses. Specimens showing abnormal activities in three or more measured enzymes were considered as impacted individuals.

Fig. 5 The percentage (%) of the impacted cod individuals observed at different sampling stations in 2011 based on calculated abnormal values of several enzymatic biomarkers. Stations B13, B15 and B09 refer to the sampling areas of Bornholm, Gdansk and the reference area respectively.
Neurotoxicity

Neurotoxicity was not observed in cod or caged mussels probably due to the rapid degradation of neurotoxic CWAs in water. However, the presence of intact ammunitions containing neurotoxic agents still represents a considerable risk of exposure to humans if accidentally lifted on board.
4. Conclusions

Elevated levels of measured biomarker responses in caged mussels were detected from the hot spot sites at 65 meters, especially from WRECK-1G possibly indicating a contaminant induced stress in mussels.

Biomarker responses detected from mussels caged at 35 meters are most likely due to environmental factors, such as fluctuating temperature and increased metabolic activity caused by higher food availability compared to 65 meter cages. Halocline and thermocline efficiently prohibit mixing of the two water layers and therefore decrease the possibility that mussels at 35 meters were exposed to contaminants present in the sediment.

Cod favours the deep water layers when possible and the better oxygen conditions might draw the cod specimens nearer to the bottom and CWA contaminated sediments. Cod from the Bornholm dumping site showed highest amount of biomarker responses that deviated from the average values indicating increased stress response in some individuals.