Biological Effects of Contaminants in Mussels *(Mytilus trossulus)* Transplanted in Northern Baltic Sea Coastal Areas

Raisa Turja

Faculty of Biological and Environmental Sciences
Department of Environmental Sciences
Division of Aquatic Sciences
University of Helsinki

Academic dissertation

To be presented for public examination with the permission
of the Faculty of Biological and Environmental Sciences
of the University of Helsinki in Auditorium 132,
Siltavuorenpenger 1A on 20th of March 2015 at 12 noon.

Helsinki 2015

SUPERVISOR
Dr. Kari K. Lehtonen
Finnish Environment Institute, Marine Research Centre, Helsinki, Finland

THESIS SUPPORT GROUP
Prof. Jussi Kukkonen
Department of Biological and Environmental Science, University of Jyväskylä, Finland

Dr. Olli-Pekka Penttinen
Department of Environmental Sciences, University of Helsinki, Finland

REVIEWERS
Prof. Jussi Kukkonen
Department of Biological and Environmental Science, University of Jyväskylä, Finland

Prof. Ketil Hylland
Department of Biosciences, University of Oslo, Norway

OPPONENT
Prof. Ibon Cancio
Department of Zoology and Cell Biology, University of the Basque Country, Spain

CUSTOS
Prof. Jorma Kuparinen
Department of Environmental Sciences, University of Helsinki, Finland

FUNDED BY
Onni Talas Foundation

ISBN 978-951-51-0818-0 (paperback)
ISBN 978-951-51-0819-7 (PDF)
http://ethesis.helsinki.fi
Dissertationes Schola Doctoralis Scientiae Circumiectalis, Alimentariae, Biologicae.
Universitatis Helsinkiensis.
Cover photo: Raisa Turja

CONTENTS

LIST OF ORIGINAL PUBLICATIONS AND AUTHOR'S CONTRIBUTION

This thesis is based on the following original publications and manuscripts, which are referred to in the text by the Roman numerals.

I Lehtonen KK, **Turja R**, Budzinski H, Devier M-H. Chemical contaminants and biomarker responses in caged and native mussels in the Archipelago Sea (SW Finland, Baltic Sea), *submitted manuscript to Marine Environmental Research.*

RT was responsible of the field sampling of native mussels and most of the biomarker analyses in both caged and native mussels, performed most of the data analyses and participated in manuscript preparation.

II **Turja R**, Soirinsuo A, Budzinski H, Devier M-H, Lehtonen KK. 2013. Biomarker responses and accumulation of hazardous substances in mussels (*Mytilus trossulus*) transplanted along a pollution gradient close to an oil terminal in the Gulf of Finland (Baltic Sea). Comp. Biochem. Physiol. C-Toxicol. Pharmacol. 157, 80-92.

RT participated in the designing of the study, performed most of the biomarker analyses and all the data analyses, and was the main person responsible for the manuscript preparation.

III **Turja R**, Lehtonen KK, Meierjohann A, Brozinski J-M, Vahtera E, Soirinsuo A, Sokolov A, Snoeijs P, Budzinski H, Devier M-H, Pääkkönen J-P, Viitasalo M, Kronberg L. The mussel caging approach in assessing biological effects of wastewater treatment plant discharges in the Gulf of Finland (Baltic Sea), *submitted manuscript to Marine Pollution Bulletin.*

RT designed the study with KL and J-PP, was responsible of the field study, performed the analysis of antioxidant defense biomarkers, GST, AChE and CI, was responsible for the CEA analysis, performed most of the data analyses, and was the main person responsible for the manuscript preparation.

IV **Turja R**, Höher N, Snoeijs P, Baršienė J, Butrimavičienė L, Kuznetsova T, Kholodkevich S, Devier M-H, Budzinski H, Lehtonen KK. 2014. A multibiomarker approach to the assessment of pollution impacts in two Baltic Sea coastal areas in Sweden using caged mussels (*Mytilus trossulus*). Sci. Total Environ. 473, 398-409.

RT designed the study with KL, performed the field sampling and participated in the field study, was responsible for most of the biomarker analyses and data analysis of the biomarkers, and was the main person responsible for the manuscript preparation.

ABBREVIATIONS

AC	assessment criteria
AChE	acetylcholinesterase
B(a)A	benzo(a)antracene
B(a)P	benzo(a)pyrene
BAC	background concentration
BSAP	Baltic Sea Action Plan
CAT	catalase
CEA	cellular energy allocation
CI	condition index
CSB	computational systems biology
DBT	dibutyltin
DDE	dichlorodiphenyldichloroethylene
DDT	dichlorodiphenyltrichloroethane
DEHP	diethylhexyl phtalate
dw	dry weight
Ea	energy available
EC	effective concentration
Ec	energy consumption
EE2	ethinylestradiol
EROD	ethoxyresorufin-O-deethylase
ETS	electron transport system
Fluo	fluoranthene
GES	Good Environmental Status
GPx	glutahione peroxidase
GR	glutathione reductase
GSH	glutathione
GSSG	glutathione disulfide
GST	glutathione S-transferase
H.chlor	heptachlor
HBCD	hexabromocyclododecane
HCB	hexacholorobenzene
LC	lethal concentration
LMS	lysosomal membrane stability
LPO	lipid peroxidase
MBT	monobutyltin
MDA	malondialdehyde
MSFD	Marine Strategy Framework Directive
MT	metallothionein
NRR	neutral red retention
OCP	organochlorine pesticides
PAH	polycyclic aromatic hydrocarbons
PBDE	polybrominated diphenyl ethers
PCB	polychlorinated biphenyls
Phe	phenanthrene
POCIS	polar organic chemical integrative sampler
POP	persistent organic pollutant
PUFA	polyunsaturated fatty acid
ROS	reactive oxygen species
SfG	scope for growth
SOD	superoxide dismutase
SoS	stress on stress
TBT	tributyltin
WHO	World Health Organization
VTG	vitellogenin
ww	wet weight
WWTP	wastewater treatment plant

Biological Effects of Contaminants in Mussels (*Mytilus trossulus*) Transplanted in Northern Baltic Sea Coastal Areas

Raisa Turja

University of Helsinki, Faculty of Biological and Environmental Sciences,

Academic dissertation in Environmental Sciences 2015

ABSTRACT

Biomarkers measured in organisms are sensitive molecular, cellular or individual level biological effects, which can be applied as "early-warning" signals of environmental contamination before damage occurs at population, community or ecosystem levels. In this thesis, a suite of biomarkers and tissue concentrations of chemicals were measured in mussels (*Mytilus trossulus*) as indicators of environmental pollution. The mussels were transplanted in specially made cages in coastal areas of the northern Baltic Sea influenced by different types of contamination and environmental factors. The aims of the research were to apply the biomarker approach to (1) assess the impact of contaminants on the health status of mussels, (2) investigate the effects of seasonal variability in biotic and abiotic factors, and low salinity, and (3) validate the usefulness of the mussel caging method for biomonitoring of chemical contamination in the northern Baltic Sea.

The results showed marked biomarker responses coinciding with higher concentrations of contaminants, such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), organochlorine pesticides (OCPs), organotins and trace metals, in mussels caged at the most contaminated study sites. At the contaminated sites, for example the enzymatic biomarkers of the antioxidant defense system indicated higher stress level, which was closely linked to elevated level of DNA-damage, increased biotransformation activity and decreased general health status of the cells.

Biomarker responses and tissue contaminant concentrations were also related to the seasonal variability in growth and especially in soft tissue weight of the mussels. In the spring, high tissue contaminant levels were connected to low soft tissue weight and increased activities of biotransformation and antioxidant enzymes. In the northern Baltic Sea the mussels almost fully deplete their energy stores during the winter; in the spring mussels efficiently feed on the fresh phytoplankton, which forms the main source of energy for their reproduction, energy metabolism and growth. Natural environmental factors caused less variation in the biomarker responses in the late summer and autumn suggesting that this time period is the most suitable for studying contaminant induced effects in mussels.

More pronounced effects were observed in mussels exposed jointly to low salinity and chemical contamination, indicating that increased environmental stress reduces the tolerance of mussels towards anthropogenic pressures. This work showed that the mussel caging approach is an efficient biomonitoring method to assess biological effects and tissue accumulation of complex mixtures of contaminants as long as the effects of seasonal variability and low salinity are taken into account.

Keywords: biomarkers, biological effects, oxidative stress, contamination, *Mytilus*, mussel caging, marine environment, Baltic Sea, biomonitoring, ecotoxicology

Haitallisten aineiden biologiset vaikutukset häkitetyissä sinisimpukoissa (*Mytilus trossulus*) pohjoisen Itämeren rannikkoalueilla

Raisa Turja
Helsingin yliopisto, Bio- ja ympäristötieteellinen tiedekunta, Väitöskirja ympäristötieteestä 2015

TIIVISTELMÄ

Biomarkkerit ovat eliöistä mitattavia molekyyli-, solu- ja yksilötason biologisia vaikutuksia, joita voidaan käyttää ympäristön likaantumisen varhaisina varoitussignaaleina ennen kuin vaikutukset näkyvät populaatio-, yhteisö- ja ekosysteemitasoilla. Tässä väitöskirjatyössä tutkittiin useita biomarkkerivasteita ja haitallisten aineiden kudospitoisuuksia Itämeren sinisimpukassa (Mytilus trossulus), jota käytettiin mallieliönä arvioitaessa kemikaalien vaikutuksia meriympäristöön. Sinisimpukat sijoitettiin erikoisvalmisteisissa häkeissä erilaisen kemikaalikuormituksen sekä vaihtelevien ympäristötekijöiden vaikutusten alaisina oleville tutkimusalueille pohjoisen Itämeren rannikoilla. Työn tavoitteena oli (1) tutkia sinisimpukoiden terveyttä käyttäen useaa eri biomarkkerimenetelmää yhdessä haitallisten aineiden kudospitoisuusmääritysten kanssa, (2) tutkia bioottisten ja abioottisten ympäristötekijöiden vaikutuksia biomarkkerivasteisiin, ja (3) arvioida häkitysmenetelmän käyttökelpoisuutta haitallisten aineiden vaikutusten ja pitoisuuksien ympäristöseurannassa pohjoisella Itämerellä.

Tutkimuksissa havaittiin merkittäviä biologisia vaikutuksia likaantuneemmille alueille häkitetyissä sinisimpukoissa, joista mitattiin myös korkeampia haitallisten aineiden kuten polysyklisten aromaattisten hiilivetyjen (PAH), polykloorattujen bifenylien (PCB), orgaanisten tinayhdisteiden ja raskasmetallien kudospitoisuuksia. Likaantuneilla alueilla esimerkiksi antioksidanttipuolustuksen entsymaattiset biomarkkerit osoittivat suurempaa stressitasoa ja ne kytkeytyivät tiiviisti myös muihin mitattuihin biomarkkerivasteisiin kuten lisääntyneisiin DNA-vaurioihin, vierasainehajotuksen kiihtymiseen ja solun yleisen terveydentilan heikentymiseen.

Haitallisten aineiden biologiset vaikutukset ja kudospitoisuudet kytkeytyivät myös vuodenaikaisvaihtelun aiheuttamiin muutoksiin sinisimpukoiden kasvussa ja etenkin pehmytkudosten painossa. Keväällä mitatut korkeat kudospitoisuudet olivat yhteydessä alhaiseen pehmytkudosten painoon, aktivoituneeseen vierasaineiden hajotukseen sekä kohonneisiin antioksidanttivasteisiin. Pohjoisella Itämerellä sinisimpukat kuluttavat energiavarantonsa vähiin pitkän talven aikana, joten kasviplanktonin kevätkukinnasta saatava ravinto on avainasemassa niiden lisääntymisen, energia-aineenvaihdunnan ja kasvun kannalta. Loppukesällä ja syksyllä luonnollisten ympäristötekijöiden aiheuttama biomarkkerivasteiden vaihtelu oli sinisimpukoissa vähäisintä, josta voidaan päätellä tämän ajankohdan sopivan parhaiten haitallisten aineiden vaikutusten seurantaan.

Suurimmat biologiset vasteet mitattiin sinisimpukoissa, jotka altistuivat yhtä aikaa alhaiselle suolapitoisuudelle ja haitallisille aineille; tämä viittaa siihen, että korkeampi ympäristötekijöiden aiheuttama stressi vähentää niiden kykyä sietää ihmisen toiminnasta johtuvaa kemikaalipainetta. Tutkimus osoitti, että sinisimpukoiden häkitys on tehokas menetelmä Itämeren kemikaalikuormituksen vaikutusten arvioinnissa kun otetaan huomioon vuodenaikaisvaihtelun ja alhaisen suolapitoisuuden vaikutukset mitattaviin muuttujiin.

Avainsanat: biomarkkerit, biologiset vaikutukset, haitalliset aineet, sinisimpukka, Mytilus trossulus, simpukkahäkitys, ympäristöseuranta, Itämeri, ekotoksikologia

1. INTRODUCTION

Anthropogenic chemical contaminants and their impacts on organisms possess a constant threat for the marine environment and especially coastal and estuarine ecosystems (HELCOM 2007; 2010). For many decades, assessments of ecological risk of pollutants in marine ecosystems have been solely based on chemical analysis of environmental samples, i.e., concentrations of selected hazardous substances in sediments, water and organisms. However, a routine analysis of thousands of different toxic chemicals and a countless number of their mixtures continuously released into the environment is clearly too expensive and even impossible task (Devier et al. 2011). Importantly, chemical analysis alone does not provide information on the adverse biological effects of pollutants on the health of organisms.

This has been recognized already for many years (Depledge 1994; Lowe et al. 1995) and the integrated use of chemical analysis and several biological effects methods has been suggested as a holistic approach to assess ecosystem health (Cajaraville 2000, Davies and Vethaak 2012). In aquatic ecotoxicology, a considerable amount of work has been done to develop suitable biomarkers, which have now become an important tool in risk assessment of the marine environment (ICES 2011). Recently, to achieve "Good Environmental Status" (GES) the importance of integrated monitoring approach was addressed in the European Union Marine Strategy Framework Directive (MSFD) Descriptor 8 "Concentrations of contaminants are at levels not giving rise to pollution effects" (Lyons et al. 2010).

In the Baltic Sea region, the Baltic Sea Action Plan (BSAP) of the Helsinki Commission (HELCOM) signed in 2007 commits the coastal governments and the European Commission to carry out actions to achieve the "Baltic Sea in Good Environmental Status" (Backer et al. 2010). Hazardous substances and their effects is one of the ecological objectives emphasized in the BSAP, and the development of tools for monitoring biological effects in Baltic Sea organisms is urgently required. Compared to other European sea areas, research on the effects of pollutants has been relatively scarce in the Baltic Sea (Lehtonen and Schiedek 2006). The BEEP (Biological Effects of Environmental Pollution in Marine Coastal Ecosystems, EU 5FP) was the first extensive international project to develop tools for biological effects monitoring in the Baltic Sea (Lehtonen et al. 2006). The work was continued in the BEAST project (Biological Effects of Anthropogenic Chemical Stress: Tools for the Assessment of Ecosystem Health, Baltic Sea BONUS program supported by the EU) by identifying a set of core and

candidate biomarkers and integrated strategies for the use of, e.g., BSAP and MSFD (Lehtonen et al. 2014).

The Baltic Sea has long been stated as the most contaminated sea in the world. Risk assessment of contaminants requires special attention in this sea area due to the unique brackish-water ecosystem with very few bioindicator species (Box 1). Underlining the importance of this, the potentially higher sensitivity of Baltic Sea organisms to pollution over full marine species has been recognized for decades (Carr and Linden 1984; Tedengren et al. 1988).

BOX 1. Baltic Sea is unique in terms of its hydrography and biology

The Baltic Sea is one of the largest brackish water bodies in the world and is situated in northern Europe between 10°-30°E and 54°-66°N. The average depth of only 55 m makes the Baltic Sea much shallower compared to world's oceans. The deepest basins with a maximum depth of 495 m are located in the Baltic Proper region. The Baltic Sea receives fresh water from numerous rivers of the catchment area approximately four times larger than the sea itself. Connected to the northern Atlantic only via narrow and shallow Danish Straits, the water exchange in the Baltic Sea is restricted. Unique features of the area's hydrography are the decreasing water salinity from south to north and the permanent halocline prevailing at the depths of ca. 60 to 100 m separating the surface water from the more saline deep water layer. A Halocline prevents vertical water circulation and therefore oxygenation of the deep water is limited to occasional inflows of fully marine water from the North Sea (Leppäranta and Myrberg 2009). Hypoxic or anoxic conditions are often present in areas below the halocline, limiting the life of bottom communities (Ojaveer et al. 2010).

During the summer the water column is also stratified by temperature, and the thermocline at the depth of 10-30 m separates the warm upper water layer from the underlying cold waters. Rapid cooling of the surface water in the autumn allows the mixing of the layers. During the winter a partial or complete ice-cover is formed in the northern parts of the sea, and in the Gulf of Finland and the Bothnian Sea it lasts for 2-4 months. Marked temperature fluctuations during the season are characteristic especially to the northern part with warming of the surface water from the close to zero temperatures observed in early spring to temperatures ca. 20°C in July (Leppäranta and Myrberg 2009). The sudden cooling of the surface water is caused by upwelling, which temporarily

mixes the water masses in vertical direction. Upwelling is typically caused by winds and most often occurs in waters close to the coast (Omsted et al. 2014).

The fauna of the Baltic Sea comprises marine and freshwater species, making salinity often the most critical factor determining the distribution range of the inhabiting organisms. As a consequence of adaptation to brackish water conditions that cause osmotic stress, many of the marine species are "dwarfed", i.e., markedly reduced in size compared to the same species in oceanic environments. Moreover, the biodiversity of higher organisms is markedly lower compared to true marine or freshwater ecosystems, and many of the vital ecosystem services are supported by only one key species (Koivisto and Westerbom 2012, Ojaveer et al. 2010). Organisms are also challenged by restricted light and low temperature conditions that slow down the entire ecosystem during the winter. Extensive peak in primary production in the spring and the abundant daylight as well as rising water temperature in the summer activate the feeding and metabolism of heterotrophic organisms.

The above mentioned characteristics make the Baltic Sea sensitive to disturbances, and the sea is suffering from eutrophication, toxic algal blooms, hazardous substances, acidification, spreading of anoxic bottoms and the introduction of harmful alien species (HELCOM 2010a,b). Health of the Baltic Sea has been assessed using several integrative classification tools (HELCOM 2010a). Regarding concentrations of hazardous substances, biodiversity and eutrophication the status of the Gulf of Finland and the Baltic Proper have recently been classified as "moderate", "poor" or "bad" in a five step criteria from "high" to "bad". A "good" status in regard to some of the aspects assessed was recorded only for the Gulf of Bothnia (Fig. 2.1 in HELCOM 2010b). Integrated assessment and classification of ecosystem health showed "poor" to "bad" status for the coastal area of Finland as well as to most parts of the Baltic Proper (Fig. 2.2 in HELCOM 2010b). Objectives of the Baltic Sea Action Plan (BSAP) agreement with regard to hazardous substances have been stated as 1) "Concentrations near background levels", 2) "No health problems among animals", and 3) "All fish safe to eat" (HELCOM 2007). The integrated assessment showed that a lot of work is still remaining to achieve these objectives. Monitoring of the concentrations of hazardous substances should be strengthened and the agreed development of HELCOM biological effects monitoring needs to be undertaken in all HELCOM Contracting Parties (HELCOM 2010b).

1.1 Contaminants in the aquatic environment

Industrial and municipal waste waters and agricultural runoff shipping activities, and atmospheric input are the main pathways for chemical contaminants into the marine environment. The Baltic Sea receives high amounts of contaminants from a large urban and agricultural catchment area, which is home to over 85 million people.

Deleterious effects of trace metals and "classical" persistent organic pollutants (POPs) such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), dioxins, organochlorine pesticides (OCPs), polybrominated diphenyl ethers (PBDEs) and organotins (tributyltin [TBT]) on the local ecosystem has been recognized for long time. Even though the manufacturing and use of a number of "old" persistent chemicals, such as PCBs, DDT and TBT have now been banned they continue to exist in the environment and possess a constant threat for decades to come (Nizzetto et al. 2010).

The currently not regularly monitored contaminants shown to induce toxic effects include phthalates (e.g., diethylhexyl phthalate, DEHP), perfluorinated compounds, nonylphenols and nonylphenol ethoxylates, octylphenols and octylphenol ethoxylates, and short chained chlorinated paraffins (HELCOM 2009, 2010). Moreover, many of the so-called "emerging contaminants" such as pharmaceuticals, hormones, personal care products and artificial sweeteners discharged in waste waters have been shown to cause biological effects in aquatic organisms in very small concentrations (Boxall et al. 2012, Lange et al. 2012). The "new" threats also include nanomaterials and microplastics (Andrady 2011, Klaine et al. 2008). Furthermore, marked increases in oil tanker traffic generate a growing threat of accidents and oil spillages, especially in the northern Baltic Sea (HELCOM 2010). Finally, natural compounds such as algal toxins can exert harmful effects in aquatic organisms (Ferrao-Filho and Kozlowsky-Suzuki 2011, Kankaapää et al. 2007).

1.1.1 Toxicity of chemicals

Toxicity of chemicals in the aquatic environment depends on their chemical and physico-chemical characteristics, and biological processes. The bioavailability and spatial distribution of contaminants are highly regulated by hydrodynamics, biogeochemical processes and environmental conditions (e.g., redox, pH, salinity and temperature) prevailing in the ecosystem. Accumulation of chemicals depends on the chemical properties of the compound (e.g., polarity, lipophilicity, molecular weight) as well as various biological processes regulating food availability and the efficiency of metabolism and excretion (Dachs and

Mejanelle 2010, Schwarzenbach et al. 2006). Contaminant uptake mechanisms and their rates vary among and within species, and depend on development stage, season, behavior, reproductive condition, and history of contaminant exposure. The main routes of contaminant uptake in fish and aquatic invertebrates are via gills (respiration) and liver or digestive gland/hepatopancreas (feeding). Chemicals cross cellular membranes in several ways including passive or facilitated diffusion, active transport through membrane proteins, and endocytosis (Newman and Clements 2008).

In the aquatic environment, bioavailable trace metals are present in a soluble form and are therefore mainly accumulated via gills (Marigómez et al. 2002). For organic contaminants, increased hydrophobicity is described by an increased octanol/water partitioning coefficient (K_{ow}) value. The more hydrophobic (high K_{ow}) compounds have a high tendency to partition from water to the lipids of the organisms, are more readily associated with particulate matter both in water and sediments, and are therefore generally accumulated in the food and enriched along the food chains (Walker 2009).

The effects of chemicals at the level of individual cells are mediated by a finite, small number of innate toxicity pathways involving receptor molecules, downstream signaling and transcriptional networks across organisms in different phyla. Even pharmaceuticals designed to act on very specific molecules in humans or domestic animals do exert harmful effects in non-target species through these evolutionally conserved molecular and cellular mechanisms. Examples include responses of oxidative stress, heat-shock, DNA-damage, hypoxia, and endoplasmic reticulum stress pathways, all of which are present in all cell types of an organism. Typically these pathways are activated at concentrations of chemicals significantly lower than those leading to adverse effects at the organism level (Krewski et al. 2010).

Toxicology and ecotoxicology are challenged by potential mixture effects of chemicals, which has been ranked as one of the major sources of uncertainty for appropriate management strategies by governmental, industrial and academic stakeholders (Backhaus 2014). In the environment, organisms (including humans) are not exposed to single isolated chemicals but to complex chemical mixtures where the individual components are often present at very low (non-toxic, no-effect) concentrations. However, additive and synergistic effects can render such mixtures dangerously potent (Smith et al. 2013). For example, when five estrogenic compounds were mixed in concentrations all below levels at which their individual effects can be detected, their cumulative impact on fish was

detrimental (Brian et al. 2005).

Despite of the common occurrence of chemical mixtures, even new legislations such as EU REACH focus almost exclusively on the assessment of individual chemicals (EU 2009). Concentration addition and independent action have been the two major concepts available to predict mixture toxicity on the basis of known toxicities of individual components in the mixture (Backhaus and Faust 2012; Brian et al. 2005). However, limited amount of experimental and modelled data is available on mixture toxicity effects in non-standard species such as ecologically relevant marine organisms (Backhaus 2014). Furthermore, in field conditions the full composition of the toxic mixture is rarely known and the exposed organisms are always subjected also to natural environmental stressors such as temperature, salinity, oxygen and pH fluctuations, infections, shortage of food, predation, reproductive cycle, etc., which need to be considered in the assessment of the observed biological effects.

Information on the toxicity of chemicals is mostly derived from standardized laboratory toxicity tests using selected model species exposed to a range of concentrations of a single chemical. Toxicity testing is routinely

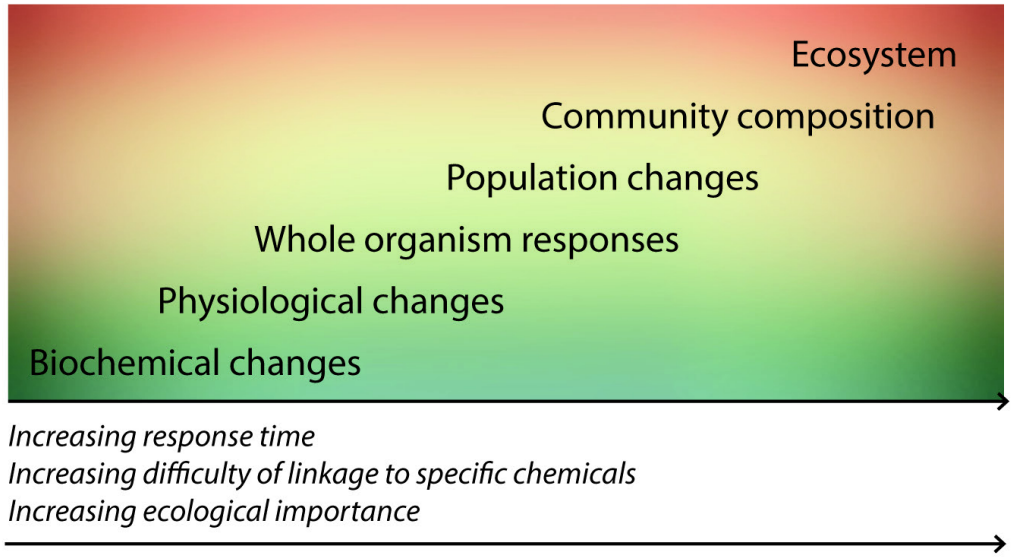


Figure. 1. Different levels of biological organization. Biomarkers are biochemical to whole organism level biological effects induced by exposure to contaminants. Detecting changes already at these lower levels of biological organization can help to prevent irreversible damage at the higher levels (from populations to ecosystem).

based on selected endpoints such as mortality, or sublethal effects such as immobility, reproduction failures or malformations in homogenous animal groups. Among the different parameters of toxicity, the widest range of toxicity data is available as lethal or effect concentrations to 50% of the test organisms (LC_{50} and EC_{50}, respectively) in the external medium (e.g., water, sediment) (Walker et al. 2001).

Traditional toxicity testing methods rely on conservative extrapolations from results on high-dose exposures and therefore often result in questionable relevance predicting the risk of chemicals to humans and to the environment normally experiencing much lower exposure concentrations. Moreover, if only critical endpoints such as lethality are applied, they do not provide information on effects at the target biomolecular sites and the mode of action of the chemical (Krewski et al. 2010).

In the European Union there are more than 100 000 registered chemicals, of which 30 000 to 70 000 are in daily use (EINECS, European Inventory of Existing Chemical Substances). In the global scale 300 million tons of synthetic compounds are annually used in industrial and consumer products and a substantial part of that finds its way into natural waters (Schwarzenbach et al. 2006). New chemicals are discovered and described at an enormous rate of more than 30 new chemicals per hour (Backhaus et al. 2012).

The number of chemicals evidently requires new strategies for toxicity testing such as preferring efficient high throughput *in vitro* techniques in human cells over *in vivo* animal studies as well as the use of computational systems biology (CSB) for cellular toxicity pathway analysis and modelling tools such as "virtual tissue" models for predicting toxicity (Bhattacharya et al. 2011). Similar (high throughput and computational) methodology should be applied and developed to test and predict the toxicity of chemicals and their mixtures in different ecologically relevant species (Moore 2002; Storey and Wu 2013).

1.2 Biomarkers as tools for studying the effects of contaminants

Signs of adverse health effects have been used throughout the history of medical practice. Originally the term "biomarker" comes from the medical science to describe a measurable indicator such as blood cholesterol profile connected to relevant clinical endpoints such as atherosclerosis and heart attack. The World Health Organization (WHO) has defined a biomarker as "any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease" (WHO 1993). Most of the biochemical biomarker methods applied in environmental science

such as acetylcholinesterase inhibition (AChE) for exposure to neurotoxic compounds, cytochrome P450 for detoxification of PAHs and PCBs, and the different methods to detect genotoxicity (see Table 1) have been adapted from the medical science and they still are extensively studied and used in humans (Collins et al. 2014; Decleves et al. 2011; Strelitz et al. 2014). In the future, ecotoxicogenomics will most certainly further tighten the relationship of environmental and medical biomarker research by providing information on gene function in relation to exposure to toxic chemicals (Nikinmaa and Rytkönen 2011; Piña and Barata 2011).

In ecotoxicology, biomarkers can be defined as a change in a biological response related to exposure or toxic effect of environmental chemicals (Peakall 1994). The most famous ecotoxicological biomarkers include the egg-shell thinning in raptors in response to DDT and its metabolites resulting in devastating decreases in population sizes. As a result, eggshell thinning is now a widely used marker of organochlorine exposure. Another well-described ecotoxicological biomarker is imposex, i.e., the development of male sexual structures in females causing infertility in gastropod mollusks exposed to TBT (Walker et al. 2001). Both of these examples are exceptional biomarkers due to their direct link to a specific contaminant and their clear ecological relevance at population and even at ecosystem levels. More commonly, however, biomarkers are not specific to any contaminant in particular but indicate that general stress/toxicity mechanisms have been activated in the exposed organisms.

Biomarkers at lower levels of biological organization (molecular, cellular and individual) are generally reversible and rapid (from hours to weeks) responses to environmental and anthropogenic stress, serving as early-warning indicators of adverse health effects before irreversible damage occurs at higher organizational levels (populations, communities, and ecosystems) (Lam 2009; Moore et al. 2004) (Fig. 1).

Since most of the molecular and cellular level biomarkers represent vital cellular functions they generally show some background levels at all times. Different biomarkers have characteristic response profiles that can be increasing, decreasing or bell-shaped; in the latter case the response is increased until a certain point, followed by a decrease to the background level or even below (Dagnino et al. 2007). Furthermore, the response time and intensity is influenced by exposure conditions, rendering the response curve rarely clearly dose-dependent (see Fig. 2). The highest molecular and cellular responses are often observed in low to medium exposure concentrations since high concentrations tend to collapse the measured biological

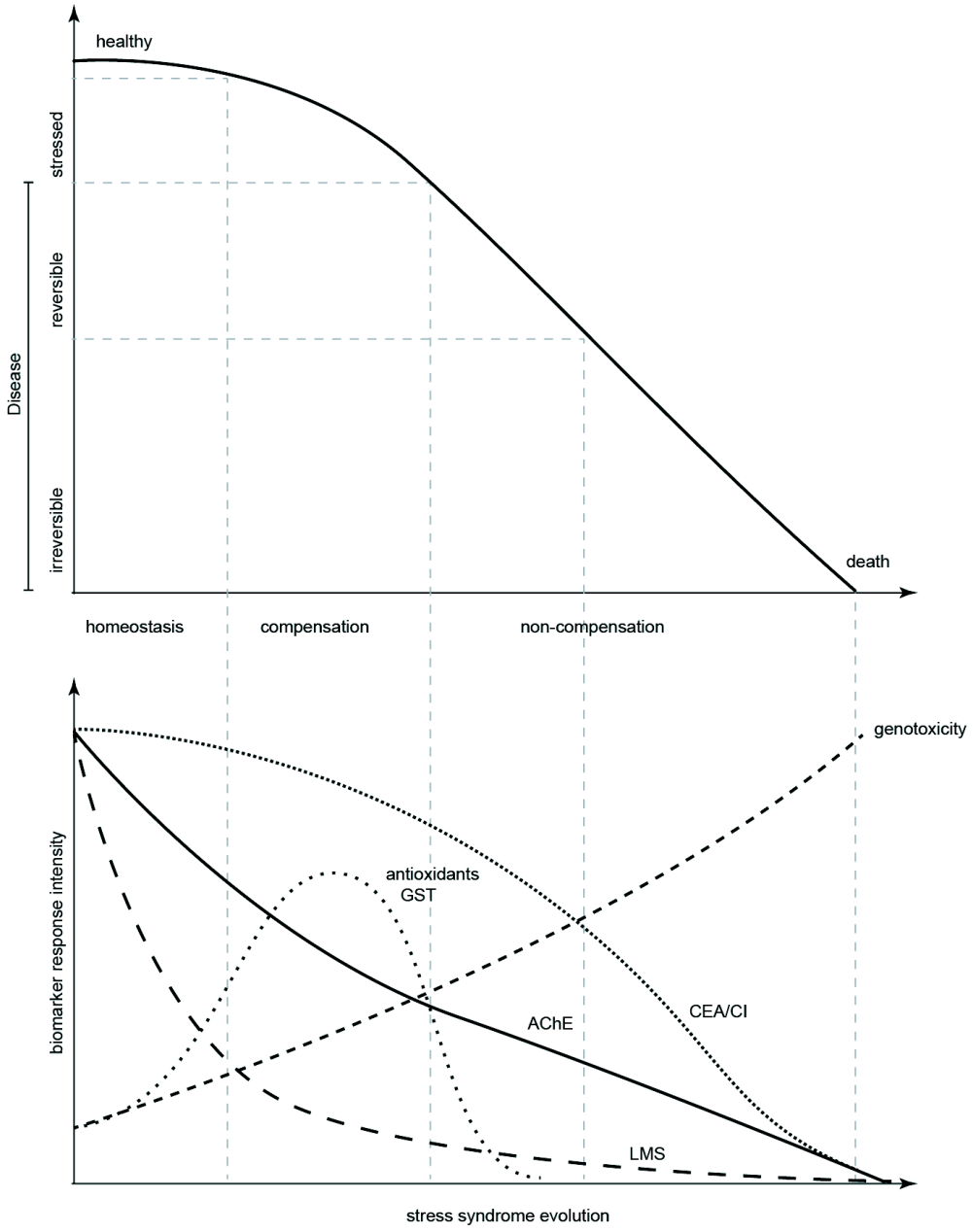


Figure 2. Hypothetical relationships between health status and biomarker responses in organisms exposed to contaminants (stress syndrome evolution, e.g., in time or increasing concentration) (redrawn from Depledge et al. 1993, Dagnino et al. 2007). Different biomarker response patterns are detected during the stress syndrome evolution, showing the importance of measuring several biomarkers to assess the level of stress.

mechanisms (Schmitt et al. 2010).

The use of biomarkers in environmental monitoring and research has been questioned due to their low ecological relevance and non-linear dose response curves (Forbes et al. 2006). To deal with these uncertainties, instead of measuring one single biomarker it is strongly advised to use a selected biomarker battery encompassing biomarkers in different functions and at different levels of biological organization. Enzymatic responses are often bell-shaped and therefore other non-bell-shape biomarkers are needed to describe the intensity of the effect (Dagnino et al. 2007). It is unlikely to see significant differences in all measured biomarkers at each investigated time-point and/or exposure concentration (see Fig. 2); however, significantly varying biomarker patterns are likely to be observed depending on exposure conditions (Depledge 1993, Viarengo et al. 2007).

Current recommendations for the monitoring of the state of the marine environment comprises the assessment of several selected biomarker responses representing different biological levels and functions (Table 1) and the analysis of tissue concentrations of several priority substances in an integrated way (HELCOM 2010, Davies and Vethaak 2012). The Oslo-Paris convention for protection of the marine environment of the North-East Atlantic (OSPAR) and the Programme for the Assessment and Control of Marine and Coastal Pollution in the Mediterranean Region (MEDPOL) recommend the use of several suitable biomarkers (listed in Table 1) in biomonitoring programs utilizing mussels as sentinel species. Additionally, various other biomarkers has been applied to gain more holistic understanding on biological effects in response to different contamination profiles (MEDPOL 2006, OSPAR 2013).

Moreover, using the integrated approach and mussels as sentinel species in biomonitoring have been suggested by various authors including Viarengo et al. (2007) (2-tier approach), Hagger et al. (2008) (assessing environmental quality for the Water Framework Directive), Martinez-Gómez et al. (2010) and Garmendia et al. 2011 (monitoring of oil spill effects), Marigómez et al. (2014) (assessing the effects of contaminants using caged and native mussels), and Dondero et al. (2010) (assessing the effects of contaminants at gene transcription and catalytic levels).

According to the recommendations of HELCOM, priority substances measured in environmental samples include PBDEs, PAHs, PCBs, dioxins and furans, hexabromocyclododecane (HBCD), perfluorooctane sulphonate (PFOS), trace metals (lead, cadmium and mercury), radioactive cesium-137, TBT, and pharmaceuticals (diclofenac and 17α-ethinylestradiol [EE2]) (HELCOM 2010).

Table 1. List of biological effects methods internationally recommended for integrated environmental monitoring. Method guidelines and assessment criteria are available for all the methods mentioned in the table (OSPAR 2013).

Biomarker	Species	Response to	Recommended by
PAH metabolites	Fish	PAHs	OSPAR, HELCOM
CYP450/EROD activity	Fish	PAHs, PCBs	OSPAR, MEDPOL
AChE inhibition	Fish, bivalves	Neurotoxic compounds, general stress	OSPAR, HELCOM, MEDPOL
Lysosomal membrane stability (LMS)	Fish, bivalves	General stress	OSPAR, HELCOM, MEDPOL
Micronuclei test (MN)	Fish, bivalves	Genotoxic compounds, general stress	OSPAR, HELCOM
Comet Assay	Fish, bivalves	Genotoxic compounds, general stress	OSPAR
DNA adducts	Fish, bivalves	Genotoxic compounds	OSPAR
Vitellogenin (VTG)	Fish, bivalves (males)	Estrogenic compounds	OSPAR
Metallothionein (MT)	Bivalves	Metals (e.g. Zn, Cu, Cd, Hg), general stress	OSPAR, MEDPOL
Stress on Stress (SoS)	Bivalves	General stress	OSPAR, MEDPOL
Scope for Growth (SfG)	Bivalves	General stress	OSPAR, MEDPOL
Fish diseases	Fish	General stress	OSPAR, HELCOM
Histopathology	Fish, bivalves	General stress	OSPAR
Eelpout and amphipod embryo malformations	Fish, amphipods	General stress	OSPAR, HELCOM

1.2.1 Biomarkers representing different biological functions

Sufficient energy reserves are vital for the fitness of organisms, i.e., growth, reproduction and survival. Exposure to contaminants can strongly affect the energy balance of an individual due to the additional energy needed to maintain homeostasis at the expense of, i.e., growth and gamete development (Smolders et al. 2004) or resistance to environmental stressors such as anoxia or sub-optimal temperatures (Gagné et al. 2006; Gorokhova et al. 2010).

As an indicator of fitness of individuals within a population, Scope for Growth (SfG) measures the physiological energy balance (i.e., the difference between energy intake and metabolism) which can range from maximum positive values under optimal conditions to negative values when the organism is severely stressed and utilizing body reserves for energy (Widdows and Donkin 1992). At the cellular level the energetic status can be measured with the Cellular Energy Allocation (CEA) method, which quantifies the available energy stores (lipid, protein and glycogen) and energy consumption (measured as mitochondrial electron transport system activity, ETS) (De Coen and Janssen 2003).

Generally, stress depletes first the glycogen and lipid reserves, and, depending on the level of stress, the protein reserves can be either increased (protein synthesis, low to intermediate level of stress) or decreased (protein degradation under high stress) (De Coen and Janssen 2003; McVeigh et al. 2006; Smolders et al. 2004). Stress induced effects to mitochondrial function and ETS activity can be manifold, generally resulting in an increased formation of reactive oxygen species (ROS) and oxidative damage (Fig. 3) (Murphy 2009). The degree of stress-induced disturbance on energy balance defines the change from moderate stress still enabling the persistence of populations (induced biological responses to maintain the homeostasis) to extreme stress where only time-limited existence is possible (decreased energy reserves leading to reduced growth) (Sokolova et al. 2012).

Metallothioneins (MT) are low-molecular-weight, cystein-rich metal-binding proteins involved in the uptake, regulation and detoxification of essential and non-essential metals. Metal-MT complexes are transported to lysosomes for degradation (Marigómez et al. 2002); this system can become impaired in stressed organisms, reducing the MT turnover rate in impacted individuals. MTs are also known to function as oxyradical scavengers and may thus also be connected to other chemical stressors than trace metals, as suggested in the field study by Lehtonen et al. (2006) on bivalves in the Baltic Sea.

Lysosomal membrane stability (LMS) is one of the most documented biomarkers in bivalves and fish in

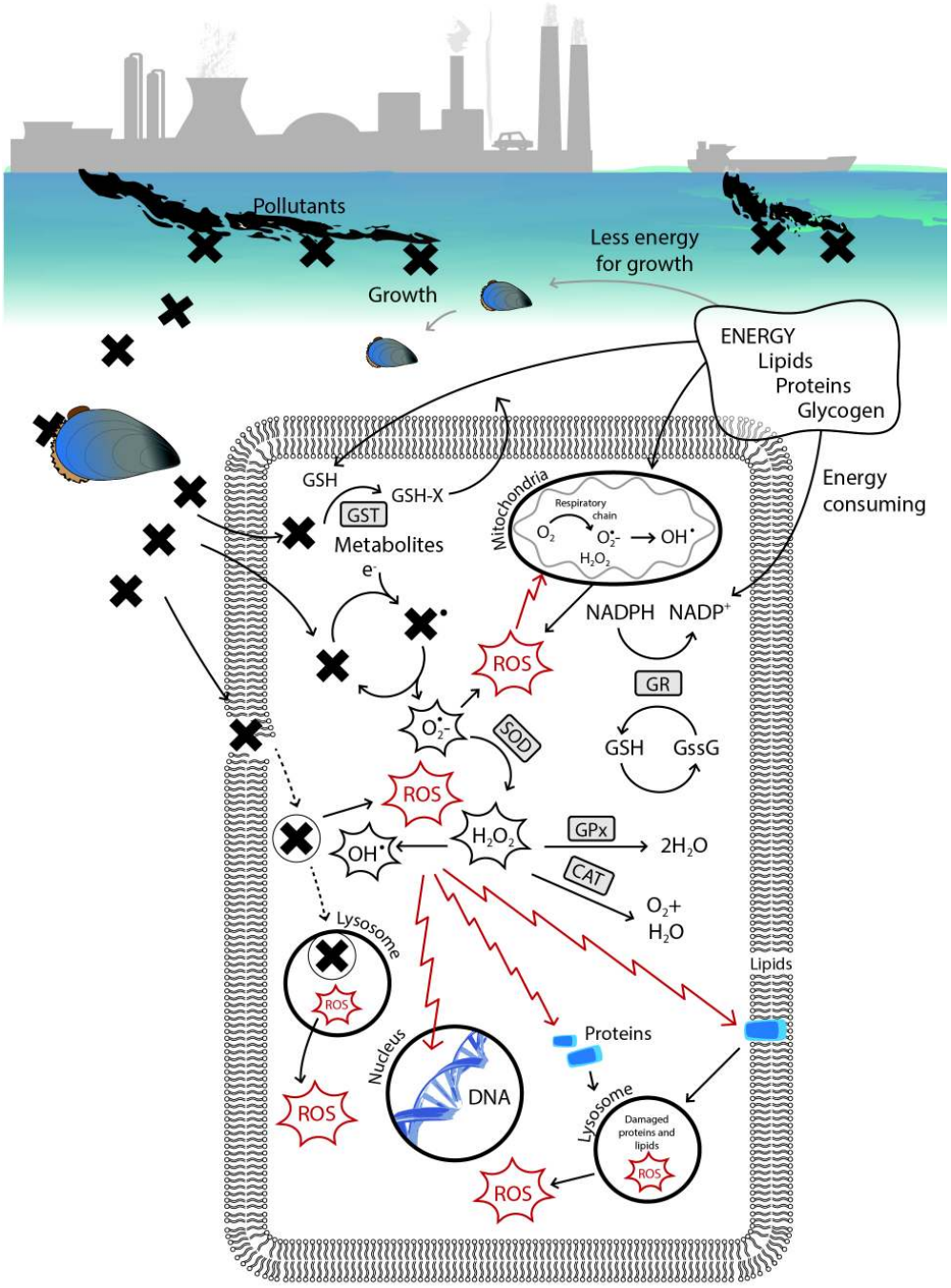


Figure. 3. Reactive oxygen species (ROS) formation increases in mitochondria, lysosomes and metabolic reactions upon exposure to pollutants. The function of antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) and glutathione peroxidase (GPx) protect cells by scavenging excess ROS. If the antioxidant capacity is exceeded ROS induce oxidative damage to lipids, proteins and DNA. The effects of pollutants consume cellular energy stores resulting in less energy for growth and reproduction of the organism.

response to pollution (Moore 2007, and references therein). Lysosomes are organelles involved in a range of cellular processes such as uptake, degradation and storage of different compounds, including contaminants. In the bivalve digestive gland, the highly developed lysosomal system is primarily involved in intracellular digestion of endocytosed food. Lysosomes in hemocytes (blood cells) function in the immune defense to destroy bacteria and other pathogens. Lysosomal membranes are damaged (destabilized) by ROS formed during contaminant exposure or by direct action of toxic chemicals. Organisms in poor condition show faster lysosomal membrane destabilization, after which acidic lysosomal contents leak into the cytosol inducing cell lysis.

A widely used biomarker for neurotoxic effects is the inhibition of AChE activity. AChE hydrolyses the neurotransmitter acetylcholine in the synaptic clefts, this being essential for the normal neuronal function in the cholinergic system. Inhibition of AChE activity is mediated by neurotoxic compounds (mainly by organophosphates and carbamates, but also by some trace metals, detergents and algal toxins) resulting in continuous neuron stimulation, paralysis and even death (Bocquené and Galgani 1998).

Genotoxicity can be defined as potentially harmful effects on genetic material in the cells (e.g., Fernández et al. 2011). Genotoxic damage to the DNA sequence and structure can be produced endogenously due to the attack of ROS and free radicals produced as by-products in normal metabolic processes, or exogenously by exposure to radiation, pollutants or natural toxins. Genotoxic chemicals are able either to act directly on DNA or produce metabolites that cause DNA damage, increase the production of ROS, or inhibit DNA synthesis and repair (Lee and Steinert 2003), and the effects can be detected with several methods (Bolognesi and Cirillo 2014).

Single-cell electrophoresis or the so-called Comet Assay shows the cells containing DNA with an increased number of double-strand breaks. DNA adducts are formed when a genotoxic chemical bounds covalently to the DNA molecule, often resulting in impaired DNA function. Genotoxic disturbance on mitosis results in chromosomal fragmentation and occurrence of micronuclei (MN), which are small chromatin-containing bodies remaining in the cytoplasm of the cells after the cell division (Bolognesi and Cirillo 2014).

Detoxification of organic compounds proceeds generally in two stages; phase I, where the pollutant is converted into more water-soluble metabolites by adding one or more hydroxyl group, and phase II where a water-soluble endogenous compound (such as glutathione [GSH]) is attached to the metabolite. Phase I reactions are carried out by various monooxygenases, which have the protein cytochrome

P450 in their catalytic center. Glutathione *S*-transferase (GST) is one of the most important groups of phase II enzymes catalyzing the conjugation of a wide variety of hazardous substances with GSH to facilitate their solubility and excretion (Fig. 3).

Detoxification reactions can sometimes lead to the formation of more toxic or reactive compounds than the original compound (Fig. 3), leading to an increased amount of ROS and subsequent damage to cellular macromolecules. Most forms of contaminant induced stress eventually cause imbalance between ROS-producing (pro-oxidant) and ROS-scavenging (antioxidant defense) processes (Lushchak 2011), and therefore biomarker responses representing other mechanisms of toxicity are either directly or indirectly (e.g., through mitochondrial dysfunction or damaged biomolecules) linked to oxidative stress (Fig. 3). Since being one of the main subjects of the present study, the antioxidant defense system and its possible use as a biomarker are explained more thoroughly in the next section.

1.2.2 Antioxidant defense biomarkers

Oxygen is vital for all aerobic organisms. The paradox of the oxygen requirement is the high reactivity of the molecule leading to the formation of radical and non-radical ROS, which can cause damage in cells and tissues (Livingstone 1990, Lushchak 2011). Organisms are efficiently protected against ROS induced damage with the help of the ROS scavenging antioxidant defense system. Oxidative stress is a state where the generation of ROS overwhelms the antioxidant capacity, and free ROS are able to cause oxidative damage to proteins, lipids and DNA (Livingstone et al. 1990; Valavanidis et al. 2006). This makes oxidative stress a common denominator underlying many other stress responses and diseases (Moore et al. 2007, Valavanidis et al. 2006).

Cellular respiration in the mitochondria produces ROS such as superoxide ($O2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydrogen radical ($HO^{\bullet}$), which are under normal conditions neutralized by the antioxidant system (Fig. 3) (Lushchak 2011, Murphy 2009). Another classic example of vital cellular ROS production is via phagocytic ROS-generating enzymes possessing an essential role in immune defense. Antioxidants are indispensable in the prevention of oxidative cellular damage, the common pathway for aging and a variety of diseases, such as cancer. Non-enzymatic antioxidants comprise a variety of molecules such as vitamins A, B (thiamine), C and E, flavonoids, and GSH. The latter is considered as one of the most important antioxidant agents involved in the protection of cellular membranes against lipid peroxidation by efficiently scavenging ROS. GSH also functions as an essential cofactor of

many GSH dependent enzymes catalyzing the detoxification of ROS and toxic compounds (Regoli and Principato 1995).

Several enzymes generally considered as the most prominent ones of the antioxidant system include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR) (Fig. 3). SOD is a first line antioxidant defense enzyme that efficiently detoxifies $O2^{\bullet-}$ to less reactive H_2O_2. This molecule is readily diffuses across biological membranes and may directly cause significant cellular damage. H_2O_2 is neutralized by CAT, which most importantly prevents the further reduction of H_2O_2 to the highly reactive $HO^{\bullet}$, which is the most harmful of all ROS. GR is responsible for reducing GSSG to GSH, which is used by GPx that catalyzes the reduction of H_2O_2 to H_2O, by GST in biotransformation reactions, and in numerous other cellular processes. Pro-oxidant conditions rapidly deplete the cellular GSH stores, and GR is a crucial enzyme in maintaining cellular GSH homeostasis. The possible use of antioxidant enzyme activities as biomarkers has been suggested in numerous studies; however, due to the large variability in these responses (see Box 2 for further explanations) more research is still needed to establish reliable assessment criteria for these parameters to be used in monitoring programs.

The reaction of ROS with membrane lipids of mitochondria, lysosomes and peroxisomes is the most predominant mechanism of cellular injury (Fig. 3). Lipid peroxidation of polyunsaturated fatty acids (PUFA) present in membrane lipids generates lipid hydroperoxides that can be further broken down to aldehydes such as malondialdehyde (MDA) (Halliwell and Gutteridge 1999). MDA readily reacts with proteins and DNA, damaging these macromolecules.

Cold adapted organisms increase the amount of PUFAs to maintain the function of cellular membranes, making them more susceptible to lipid peroxidation compared to organisms living in warmer environments. Moreover, it has been shown that higher rates of ROS induced damage occurs after melting of the sea ice exposing organisms to H_2O_2 and contaminants released from the ice, as well as to increased UV-radiation (Regoli et al. 2012). These processes are likely to influence the amount of lipid peroxidation in Baltic Sea organisms.

1.3 Baltic mussels and the mussel caging approach

Mussels (genus *Mytilus*) are ubiquitous keystone species living in coastal and estuarine rocky shores throughout temperate and polar regions. They are among the most commonly used marine bioindicator species of environmental contamination (Goldberg 1975). Their specific biological characteristics

BOX 2. Cellular regulation pathways related to oxidative stress

Reactive oxygen species (ROS) are formed by all aerobic organisms in concentrations required in the normal cellular processes, or in excessive quantities leading to a state called oxidative stress. ROS are vital for normal cellular function as important secondary messengers in cell signaling and regulation in all cell types; however the "redox homeostasis" has to be tightly maintained by the antioxidant system to avoid oxidative stress.

Antioxidant molecules and enzymes are dynamically regulated through complex phosphorylation/ dephosphorylation reactions (Bartosz 2009) at gene transcription and/or catalytic level. Any alteration in the normal phosphorylation/dephosphorylation pathways caused by pollutants can impair ROS regulation by decreasing or increasing antioxidant enzyme activities, leading to abnormal ROS levels. Among the various cellular pathways targeted by ROS, the up-regulation of the Nrf2 and NF-kb transcription factors are related to contaminant induced stress responses and involved in the regulation of GST and antioxidant genes.

However, gene transcription and catalytic levels do not vary in a synchronous way, i.e., an up-regulated gene transcription does not necessarily mean observable increase in the corresponding enzymatic activity (Giuliani et al. 2013). Therefore, it is important to measure also the actual activity of the antioxidant defense system, and particularly when assessing the biological impacts of environmental contamination (Regoli et al. 2014). Furthermore, it is important to consider that other factors, including, e.g., cyanobacterial toxins such as microcystins and nodularins in aquatic systems, are phosphatase inhibitors and can therefore modulate and/or disrupt antioxidant responses in organisms (Amado and Monserrat 2010).

Contaminant induced changes in gene transcription patterns encompassing the expression of the antioxidant genes have been successfully investigated, e.g., in mussels (*Mytilus* spp.) (Dondero et al. 2006, 2011, Regoli et al. 2014, Shaw et al. 2011). Future work is needed to identify the toxicologically relevant genes and their function to establish links between transcriptomic changes and those observed at the cellular and tissue level.

including longevity, tolerance to harsh environmental conditions while still showing sensitive biological responses to stress, bioaccumulation capacity, as well as global distribution and abundance make them ideal bioindicators. The mussel species living in the Baltic Sea is a hybrid of *Mytilus trossulus* and *Mytilus edulis* but represents a locally uniform and geographically relatively homogenous gene pool; therefore, the species name based on the latest genomic investigations has been suggested as the Baltic mussel or Baltic *M. trossulus* (Väinölä and Strelkov 2011).

Ecologically, the Baltic mussel is a key species of the low diversity Baltic Sea coastal brackish-water communities (Westerbom et al. 2002). It is an indispensable prey species for many fish and avian predators, and it also creates important underwater habitats for other species (Koivisto and Westerbom 2010). It has been estimated that Baltic mussels filtrate annually an amount of water corresponding to the entire volume of the Baltic Sea (Kautsky and Kautsky 2000). As a result, mussels efficiently circulate nutrients between the pelagic and benthic compartments of the marine ecosystem as well as accumulate contaminants and natural toxins in their tissues with subsequent consequences in the transfer of these substances along the food chain.

In the northern Baltic Sea mussels have adapted to live at the very limit of their low-salinity tolerance (5–6; e.g., Johannesson and André 2006). As osmoconformers mussels consume energy to actively regulate their internal concentration of free amino acids and ions to match the osmolarity of the surrounding environment. Baltic mussels have evolved characteristic traits adapted to the energetically stressful environment, including a maximum size of only ca. 3.5 cm, slow growth rate, low byssus production and very thin and weak shells (Kautsky 1982, Westerbom et al. 2002).

The salinity-related physiological stress is likely to increase in the future; the currently most supported climate change scenario predicts that in the northern Baltic Sea an increased riverine inputs of freshwater will result in a further reduction in salinity (Johannesson et al. 2011). The predicted salinity changes will also affect bioavailability and toxicity of many chemicals (Hall and Anderson 1995). Moreover, mussels inhabiting temperate and boreal regions are probably not able to cope with the elevated energetic costs caused by the foreseen increase in water temperature; the distribution of *M. trossulus* and *M. edulis* has been shown to be restricted by negative energy balance above the water temperature of 17°C and 23°C, respectively (Fly and Hilbish 2013). Therefore, information on the effects of current and changing environmental conditions on mussels is critically needed.

To investigate tissue contaminant levels and biological effects organisms can be collected directly from the areas

of interest or they can be transplanted to the area in cages for a certain time period (e.g., Marigómez et al. 2013).

In the caging method, organisms are collected from one (reference) location, which reduces the adaptive traits related to local environmental conditions as well as genetic variability of the studied individuals (Matozzo et al. 2013). This is an important benefit in environmental monitoring of hazardous substances, since organisms collected directly from polluted areas have often adapted their stress responses to the prevailing chronic chemical exposure. Mytilid mussels are especially suitable for caging studies since by filtering their food from the surrounding water and using the cage as a substrate to attach to they are able to live in the cages with minimal additional stress. The cages can be placed exactly to the selected locations characterized by similar hydrographical conditions to reduce the variability caused by environmental parameters and to better reflect the different contaminant profiles prevailing at the study sites. With the mussel caging method it is also possible to investigate areas where mussels are not naturally present; however, it has to be considered that this might also expose mussels to an unfavorable habitat and increase the negative effects of environmental conditions unrelated to contamination in these areas (Marigómez et al. 2013). A general challenge in field studies is to find a "pristine enough" reference site that has similar environmental conditions compared to the contaminated sites of interest.

1.4 Aims of the study

The general aim of the work performed in this thesis was to develop the biomarker methodology and investigate the effects of contaminants on various biomarker responses in Baltic mussels. Regarding the selection of the battery of the biomarkers analyzed the work carried out was designed to provide more insight to the applicability of especially of the antioxidant defense responses in the assessment of the impact of contaminants, and, furthermore, to explain the sources of variability in the catalytic level of antioxidant enzymes in Baltic mussels.

The specific aims of the study were threefold. Firstly, the goal was to assess the impact of contaminants on mussels in different areas of the northern Baltic Sea using an integrated approach, i.e., measuring selected biomarkers and tissue concentrations of contaminants in mussels. Secondly, the aim was to investigate the effects of seasonal variability and low salinity, both critical natural factors in the brackish-water northern Baltic Sea environment, on the measured biomarker responses and tissue contaminant concentrations. Finally, the most practical aim was to validate the use of the mussel caging method as a tool for environmental monitoring in the northern Baltic Sea.

2. MATERIALS AND METHODS

This thesis consists of four mussel caging experiments to study the biomarker responses and tissue concentrations of contaminants in different areas with distinct pollution sources and environmental conditions.

2.1 Collection and caging of the mussels

Mussels were collected by scuba diving in the coastal area of Hanko, western Gulf of Finland, from a site away from contamination sources (Fig. 4). Only mussels caged in the Archipelago Sea area (Paper I) were collected from another site in the outer archipelago (island of Utö) due to the ice conditions in April. Salinity at the collection sites was ca. 6.0. Adult mussels of similar size (2.2-3.0 cm shell length) were used in all experiments.

The collected animals were immediately transported to the laboratory in thermo-insulated water buckets and placed in aerated tanks filled with water from the collection site until deployment in cages. For the determination of biomarker levels and contaminant concentrations at Day 0 (prior to experiment) a group of animals was sampled in the laboratory directly after the arrival from the collection site.

The deployment and withdrawal of the cages were operated aboard R/V *Aranda*. Also the immediate preparation of the samples was carried out in the laboratory facilities of the vessel. The only exception was the caging in the coastal area of Helsinki (III) where a smaller vessel was used and the samples were immediately transported to the land-based laboratory. The cages used were cylindrical in shape with 80 cm height and a 40 cm radius, consisting of a steel net with a mesh size of 10×10 mm and equipped with five removable trays. The mussels were cleaned from epibionts and 300 to 400 individuals were placed in each cage depending on the experiment. The cages were placed at the depths of 7 to 8 m, anchored to the bottom with a rope attached to a mass of ca. 350 kg, and held in a stable vertical position by submerged buoys. Data loggers to measure temperature (I, IV: Starmon) and salinity (IV: RBR-logger) as well as passive samplers to detect contaminants in water (III: POCIS) were attached to the cages. A new cage type was designed to make the deployment possible also using smaller vessels (decreasing the operation costs), improve the handling of mussels and to facilitate the attachment of additional equipment to the cages. When needed, the new caging system enables divers to sample a part of the mussels (or passive samplers) underwater by removing the individual boxes and bringing them to the surface leaving others undisturbed.

The caging lasted from one (I, II) to three or four months (III, IV),

depending on the experiment. One month has been regarded as a sufficient time for mussels to adjust their physiological responses to the new environment (Viarengo et al. 2007). After the withdrawal of the cages, mussel tissues (digestive gland and gills) were dissected and stored at -80°C for the biomarker analyses. Samples for the chemical analyses consisted of pooled whole soft tissues of ca. 50 mussels for organic contaminants and 15 for trace metals. The samples were freeze-dried (Edwards Super Modulyo freeze dryer) before prior to analysis at LPTC Université Bordeaux I (organic compounds) and SYKE Laboratory Centre (trace metals).

Mussels were sampled again from the collection site after the caging periods ("End") to examine seasonal variation of the measured biomarkers and tissue contaminant concentrations (III, IV). The "Day 0" and "End" values from the separate caging experiments were combined in the first experiment (I) to study more closely variability in a native mussel population.

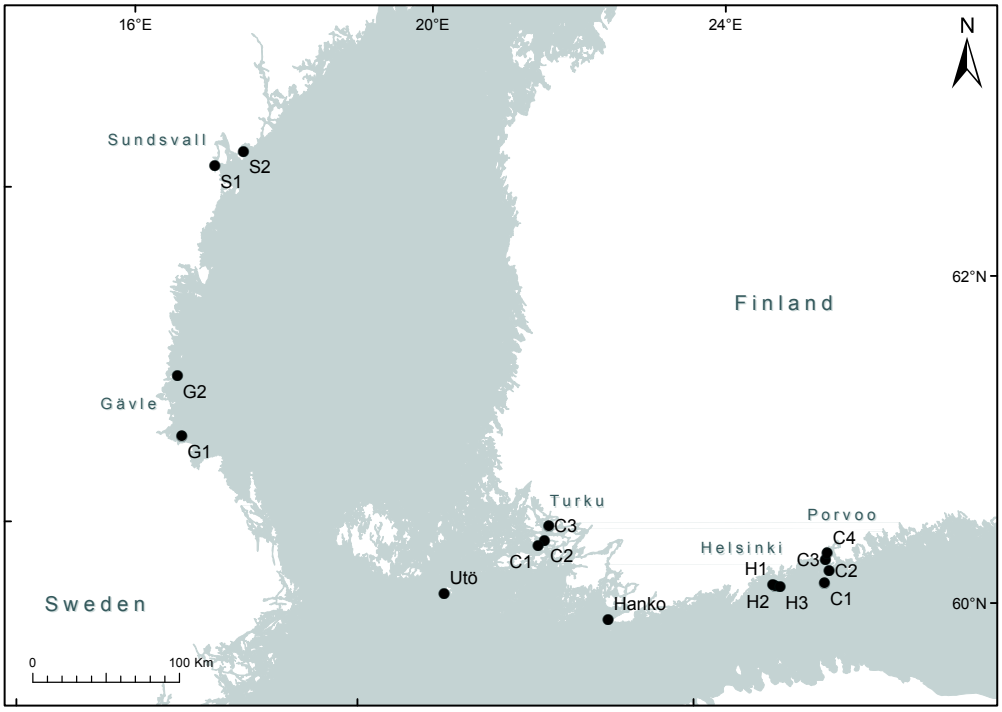


Figure. 4. Map of the study area in the northern Baltic Sea showing the mussel caging sites for the different experiments: Turku, C1-C3 (I); Porvoo, C1-C4 (II); Helsinki, H1-H3 (III); Gävle, G1-G2 and Sundsvall, S1-S2 (IV). Collection site of the native mussels in Hanko was used in all experiments, except in (I) where they were collected near the island of Utö.

2.2 Biomarker assays

For the enzymatic assays, digestive glands and gills of 15-25 mussels per site were individually homogenized using Tissue Lyser II (QIAGEN®), and the S9 supernatants were divided for individual assays and stored at -80°C until analysis. CEA was analyzed individually from the whole mussel soft tissue (IV) or from pooled digestive gland samples (III) consisting of five individuals due the amount of tissue needed for the different analyses. The CEA samples were homogenized, divided for the individual assays and stored at -80°C until analysis.

All enzyme activity rates, LPO and CEA were measured in 96-well plates using the TECAN Infinite 200 microplate reader equipped with Magellan software (TECAN). Protein concentrations were determined using the Bradford (1976) method. For the LMS analysis, microscopic evaluation of living mussel hemocytes was carried out with a microscope (Leica Aristoplan). Mussels sampled for the determination of the condition index were measured for shell length (mm) and the whole soft tissue was stored at -80°C. The samples were then freeze-dried (Edwards Super Modulyo) and weighed obtain their dry weights (mg) needed for the calculations.

In addition to the biomarkers listed in Table 2, a number of others were measured by different laboratories to investigate different biomarker batteries selected for each specific experiment and underlying research hypotheses. Metallothioneins (MT) in the digestive gland were measured to examine a response to increased trace metal contamination based on earlier research in the Archipelago Sea study area (Lehtonen et al. 2006) (I). The Comet Assay was applied to detect DNA damage induced by possible genotoxic compounds present in the discharge of a major waste water treatment plant (WWTP) (III). In (IV), geno- and cytotoxicity, phagocytosis, thiamine levels, and heart rate recovery time were studied in addition to all the biomarkers in Table 2 to apply wider battery of biomarkers representing different biological levels. All these "additional" biomarker methods are described in detail in the respective papers. Finally, the Integrated Biomarker Index (IBR) was calculated according to the original protocol of Beliaeff and Burgeot (2002) with modifications presented in Broeg and Lehtonen (2006) (I-IV).

2.3 Chemical analysis and accumulation of contaminants in mussels

Tissue concentrations of PAHs, PCBs, OCPs, PBDEs, butyltins (ng^{-1} g dw^{-1}) and trace metals (μg^{-1} g dw^{-1}) in mussels were analyzed using accredited methods in the LPTC Université Bordeaux I and SYKE Laboratory Centre.

The possible toxic effect of

contaminants depend also on their actual body burden in individual mussels, influenced by the change in the in the amount of soft tissue during the experimental period. Therefore, in some cases the accumulation rates of contaminants (pg day^{-1} $individual^{-1}$) related to the body burden were calculated (II). Accumulation rate calculations were not possible in all experiments due to the slow soft tissue growth and depuration of some of the contaminant compounds compared to the relatively high "Day 0"-values.

Table 2. Biomarker assays applied in the different studies.

Biomarker	Assay	Reference	Applied in
Catalase (CAT)	Degradation rate of hydrogen peroxide (H_2O_2)	Claiborne 1985	I-IV
Glutathione reductase (GR)	Consumption rate of NADPH in the reduction of oxidized glutathione (GSSG) to GSH	Carlberg and Mannervik 1975	I-IV
Superoxide dismutase (SOD)	Inhibition rate of cytochrome C reduction	McCord and Fridovich 1969	I-IV
Lipid peroxidation (LPO)	Amount of thiobarbituric acid reactive substances (TBARS)	Ohkawa et al. 1979	II-IV
Glutathione S-transferase (GST)	Formation rate of the GSH conjugated substrate	Habig et al. 1974	I-IV
Acetylcholinesterase (AChE)	Hydrolysis rate of acetylthiocholine	Bocquené and Galgani 1998	I-IV
Lysosomal membrane stability (LMS)	Neutral red retention time in hemocytes	Lowe and Pipe 1994	II-IV
Cellular energy allocation (CEA)	Available energy in lipids, protein and glycogen (E_a) vs. energy consumption (E_c); E_a/E_c=CEA	Verslycke and Janssen 2002	III,IV
Condition index (CI)	Ratio between soft tissue dry weight and shell length; $(W/L^2)*100$	Bayne 1976	I-IV

2.4 Statistical analysis

Statistical analysis was carried out using R statistical package (R Core Team 2013). Statistical differences in biological responses between the study sites were analyzed with ANOVA followed by Tukey's post-hoc test with Bonferroni correction. Normality of data and homogeneity of variances were checked using the Kolmogorov-Smirnov and Bartlett's test, respectively. In the case of non-normal data distribution, log10 transformation followed by Kruskal-Wallis (KW) or Mann-Whitney U-test (MW) test were applied. Principal Component Analysis (PCA) was conducted to assess relations between the measured biological parameters.

3. RESULTS AND DISCUSSION

3.1 Seasonal variability in biomarkers and tissue contaminant levels in native mussels

Some of the measured biomarkers and tissue contaminant levels showed marked changes during the spring and summer. In April, the mussels collected in the Archipelago Sea (I) were characterized by low soft tissue weight and CI, the highest seasonal tissue concentrations of several organic contaminants (high molecular weight [HMW] PAHs, PCBs, OCPs and TBT) as well as some trace metals (Cd, Hg and Pb). Moreover, high biotransformation (GST) and antioxidant enzyme activities (SOD and CAT) were recorded. Body condition of mussels was drastically reduced after the extensive winter period, indicating highly depleted energy stores and tissue loss. As a result, tissue concentrations of PAHs and other lipophilic contaminants were markedly increased as the winter progressed although their actual amount (i.e., body burden) may have remained more or less the same.

During the winter period in the ice-covered northern Baltic Sea, several kinds of airborne contaminants (e.g., PAHs, PCBs, trace metals, sulphuric compounds) are deposited on the ice surface and snow; in spring, the melting of ice causes a rapid pulse of these contaminants into the water phase (Granskog and Kaartokallio 2004). During the spring bloom and shortly afterwards mussels feed intensively on the newly-produced phytoplankton and other food items present in the water column. Subsequently, in addition to the harmful chemicals dissolved in the water phase, mussels are exposed also to large amounts of particle-bound contaminants (Riisgård et al. 1987). Moreover, increased riverine inflow

in the spring brings organic matter, nutrients and hazardous substances to coastal waters. Thus, the contaminant exposure situation shows a rapid change during this period of the year.

High interannual and seasonal variations were observed in tissue concentrations of low molecular weight (LMW) PAHs and the butyltins MBT and DBT. In the early summer mussels accumulated high concentrations of phenanthrene, fluoranthene, pyrene and anthracene; however, a loss of these substances from mussel tissues was detected in the late summer. In contrast to this, tissue concentrations of MBT and DBT were lowest in June (3.1-8.6 μg^{-1} g dw^{-1}) and highest in August and September (15.8-77.7 μg^{-1} g dw^{-1}). TBT concentration was highest in April (17.3 μg^{-1} g dw^{-1}) but after that it was detected only at lower concentrations (2.6-9.7 μg^{-1} g dw^{-1}).

Metabolism of TBT can occur in mussel tissues; however, because of the above mentioned differences in the concentration ranges of TBT vs. MBT and DBT, it seems more likely that photosynthetic microalgae and abiotic processes (photolysis) are responsible for the degradation of TBT to DBT and MBT (Rüdel 2003), resulting in their direct up-take and accumulation in mussels. Moreover, for the unusually high MBT and DBT values 324-345.7 μg^{-1} g dw^{-1} detected in September 2009, it is difficult to find other explanations than occurrence of an unrecognized contamination event in the area.

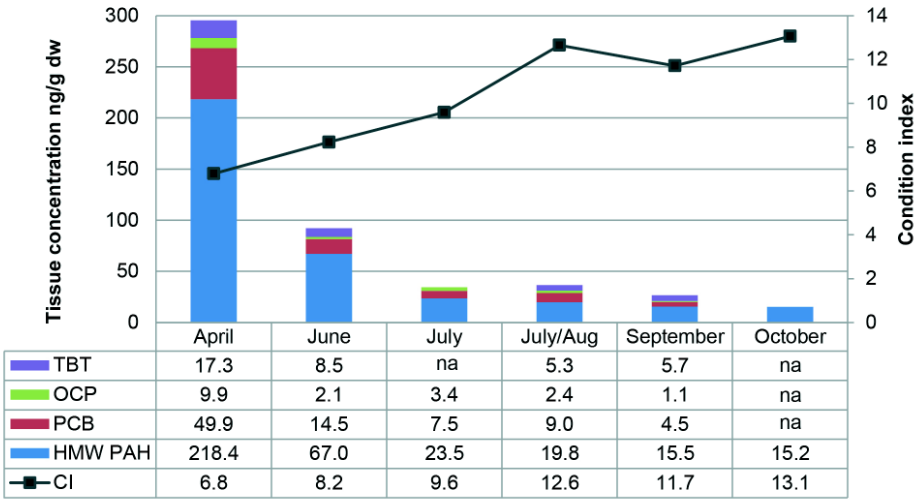
The coincidence of high GST activity and high tissue levels of ∑PAH, consisting especially of HMW-PAH, and PCBs observed in April is worth a closer inspection. During the summer GST activity decreased significantly coinciding with increasing CI and decreasing tissue contaminant concentrations (Fig. 5). Water temperature cannot explain the observed variability in GST activity, being close to 0.5°C in April and between 18-20°C in early July and late August, and has been shown to not to regulate GST activity of mussels in this sea area (Leiniö and Lehtonen 2005).

Elevated GST activities in mussels have been reported under field exposure to PAHs with a strong correlation to tissue concentrations of 5- and 6-ring PAHs (Gowland et al. 2002, Bocchetti et al. 2008). Therefore, the elevated xenobiotic conjugation rate indicated by the high GST activity is most likely associated with the concurrent high tissue levels of HMW-PAHs (Bocchetti et al. 2008). The ca. 10-fold elimination of HMWs observed by early July compared to the tissue concentrations measured in April was apparently caused by active depuration processes possibly fuelled by the rapidly increased energy intake (seen as an elevated CI) leading to effective elimination of PAHs and also PCBs (Fig.5).

Table 3. Concentrations of selected contaminants measured in caged and native (Hanko) mussels in different experiments (I-IV) in late summer and autumn. Caging sites C3, C4, H1, H2, G1 and S1 represent the contaminated study sites and C1, H3, G2 and S2 represent the local reference sites (see Fig. 4). AC refers to international assessment criteria for background contaminant concentrations (BAC) in mussels (OSPAR 2012), except for TBT where the AC value# represents a limit of "concern" (HELCOM 2010, OSPAR 2008). More detailed tables of the measured contaminant concentrations are presented in papers I-IV. Tissue concentrations were measured in one pooled sample and are shown as μg^{-1} g dry $weight^{-1}$ for trace metals and ng^{-1} g dry $weight^{-1}$ for organic compounds.

	Turku Aug		Porvoo Oct			Helsinki Oct			Gävle Sept		Sundsvall Sept		Hanko Sept	OSPAR AC
	C1	C3	C1	C3	C4	H1	H2	H3	G1	G2	S1	S2	Native	BAC
Cd	2.1	3.1	1.9	0.8	0.9	1.0	0.86	1.1	2.8	3.9	3.6	4.2	1.4	0.96
Hg	0.04	0.05	0.04	0.05	0.05	na	na	na	0.09	0.09	0.14	0.08	0.07	0.09
Pb	0.4	0.6	0.3	0.5	0.6	0.3	0.2	0.4	0.6	0.5	0.5	0.4	0.4	1.3
Fluo	3.2	3.1	5.6	7.5	7.6	8.2	18.1	10.5	18.9	19.6	23.8	23.1	2.5-22.2	12.2
Phe	3.4	2.6	14.0	15.3	12.8	26.0	47.2	40.5	69.0	64.7	66.7	72.3	6.9-77.1	11.0
Pyrene	3.5	3.2	4.3	5.0	5.6	14.4	38.5	24.0	35.6	37.7	44.2	44.4	1.6-49.2	9.0
B(a)A	1.6	2.3	1.5	2.4	3.0	0.6	1.1	0.9	2.7	2.8	7.0	3.7	1.5	2.5
B(a)P	nd	nd	1.0	1.4	1.4	nd	nd	nd	0.5	0.9	1.7	1.2	0.4	1.4
B(ghi)P	nd	1.5	2.2	2.9	3.1	1.8	1.5	1.5	1.4	1.5	7.5	1.4	0.9	2.5
∑PCB	16.2	25.0	12.0	12.4	21.6	4.1	9.8	4.7	7.8	7.2	7.0	7.4	4.5	3.3
p,p'DDE	2.5	2.5	na	na	na	0.8	1.2	0.7	0.8	0.6	0.6	0.8	0.7	0.63
HCB	nd	nd	na	na	na	nd	nd	nd	0.4	nd	0.3	nd	nd	0.63
Lindane	0.17	nd	na	na	na	0.3	0.4	0.3	0.3	0.1	0.1	0.1	nd	0.97
H.chlor	0.18	nd	na	na	na	na	na	na	0.1	nd	0.1	0.1	nd	na
TBT	11.1	32.8	12.4	30.8	40.0	na	na	na	2.5	2.5	1.8	2.3	5.7	30#

Similar to GST, the observed high activity of SOD in April appears to be related to the observed high tissue concentrations of various contaminants at the time. SOD activity is also closely associated with oxidative energy metabolism, which is the major producer of endogenous ROS in cells. Its activity is needed to maintain normal ROS balance during respiration while increased metabolic and feeding rates after the winter period



	April	June	July	July/Aug	September	October
TBT	17.3	8.5	na	5.3	5.7	na
OCP	9.9	2.1	3.4	2.4	1.1	na
PCB	49.9	14.5	7.5	9.0	4.5	na
HMW PAH	218.4	67.0	23.5	19.8	15.5	15.2
CI	6.8	8.2	9.6	12.6	11.7	13.1

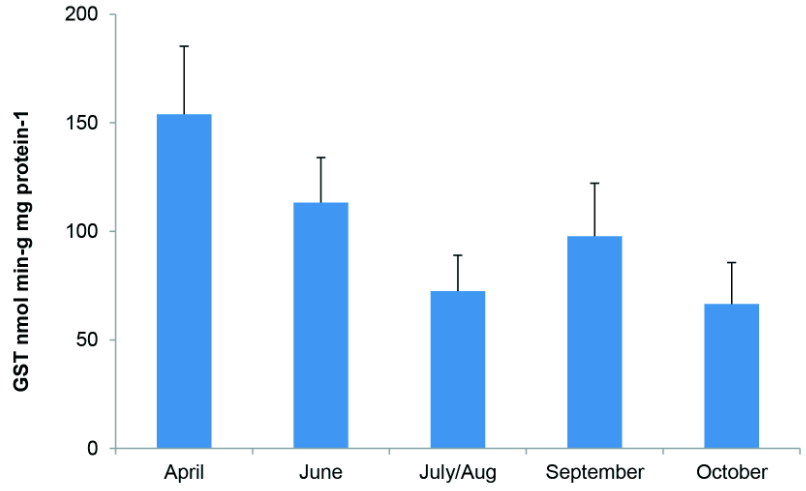


Figure. 5. Relationship between total tissue concentration (ng^{-1} g dry $weight^{-1}$) of high molecular weight polycyclic aromatic hydrocarbons (HMW-PAH), polychlorinated biphenyls (PCB), organochlorine pesticides (OCP) and tributyltin (TBT), condition index (CI) and glutathione S-transferase (GST) activity measured in native (Hanko) mussels from April to October. na=not analysed.

can also upregulate the levels of SOD markedly. This probably contributed also to the high annual variability observed in SOD activity (14-92.7 U^{-1} mg $protein^{-1}$). Conclusively, the observed patterns in SOD activity may be influenced both by contaminants (e.g., redox cycling HMW-PAHs) as well as seasonal food availability prevailing in the environment.

CAT activity seemed to be variable in early summer and started to decrease gradually in late July. The seasonal pattern depicted slightly decreasing values from early summer towards the autumn and agrees with previous studies in the area suggesting that the activity of CAT in mussels (and also in the Baltic clam *Macoma balthica*) responds almost immediately to the increased availability of quality food in the spring (Leiniö and Lehtonen 2005). Variability in the CAT response in mussels is also influenced by the time of reproduction (Devier et al. 2005), which in the northern Baltic is in May-June (Kautsky 1982b).

GR and AChE did not show significant variations between April and October. Whereas GST and CAT activities relate to variability in contaminant and ROS levels, the constant GR activity acts to maintain normal cell functions (Vidal-Liñán et al. 2010). The AChE activity level was somewhat lower (18.8-21.6 nmol min^{-1} mg $protein^{-1}$) compared to those reported in North Atlantic waters, suggesting a baseline activity of 35 nmol min^{-1} mg $protein^{-1}$ (OSPAR 2013). However, AChE levels of mussels in low salinity areas are possibly lower in general compared to full marine conditions (Lehtonen et al. 2006; Leiniö and Lehtonen 2006; Rank et al. 2007). Moreover, in this study AChE did not show similar temperature dependent variation as observed previously in Baltic mussels (Leiniö and Lehtonen 2006).

The low AChE activity observed in the present study could be explained by the generally deteriorated physiological condition of the mussels since the shallow (3 to 6 m) collection area is likely subjected to higher temperatures and different hydrographical and feeding conditions compared to the previously studied areas in the coast of Finland (i.e., Leiniö and Lehtonen 2006). Moreover, the low AChE can also be related to the elevated tissue concentrations of different contaminants, especially LMW PAHs (Table 3), suggesting the presence of unexpected contamination in the Hanko area (I).

The poor post-winter condition of mussels was most critically reflected in soft tissue weight and, therefore, in the CI, showing lowest values in April and followed by a marked increase until the end of the summer (Fig. 5). Bioenergetic status of mussels seemed to follow the same pattern as described for CI. Lower values for the available energy stores (glycogen and lipid) and higher energy consumption (ETS)

were observed in June compared to September ("Day 0" and "End" in Table 2 in IV). Interannual variability in environmental conditions had a significant effect on soft tissue growth and CI of the mussels. The variability was most prominent in 2008 when the weight of the native mussels in Hanko in June and August was almost double compared to the other sampling years (143.3-178.6 and 76.8-116.7 mg dw, respectively). During the exceptionally mild winter of 2007/2008 (Omstedt et al. 2014) there was no ice formation in the natural distribution area of mussels in the northern Baltic Sea; marked changes were also observed in the spring bloom phytoplankton community (Kaitala and Hällfors 2008), which is crucial for the growth of the mussels. In addition, relatively low temperatures during the whole summer of 2008 prevented the occurrence of a major bloom of toxic blue-green algae (Kaitala and Hällfors 2008).

The low summer temperatures are also most likely beneficial for the growth of the mussels (Fry and Hillbish 2013). In June 2008, an elevated activity and variability in CAT (52.1 ± 15.1 μmol min^{-1} mg $protein^{-1}$) and GR (20.0 ± 5.8 nmol min^{-1} mg $protein^{-1}$) compared to the mean levels measured in June of the other study years (30.3 ± 2.2 μmol min^{-1} mg $protein^{-1}$ and 15.6 ± 2.8 nmol min^{-1} mg $protein^{-1}$, respectively) were most likely related to an increased feeding and metabolism during that time. However, in August all the measured biomarkers and tissue concentrations showed "normal" levels in 2008 (data not shown), suggesting that despite of the observed interannual variability the influence of natural factors on biomarkers and contaminant concentrations seem to decrease towards late summer.

3.2 General observations in caged mussels

The decrease in the background concentrations of contaminants in the mussel tissues observed in spring-early summer seems to level off in late summer. This suggests that the mussels should optimally be collected for the caging experiment in August at the earliest and transplanted until October to be able to follow the accumulation of contaminants in tissues. If performed earlier, interpretation of the chemical analysis data obtained from caged mussels is blurred by the clearly higher "Day 0" values, leading to an observed depuration rather than accumulation of contaminants as observed here in mussels collected in April (I) and June (IV).

Similarly to native mussels, the caged mussels showed elevated responses of GST, CAT and CI related to higher contamination indicated by the elevated tissue concentrations of HMW PAHs, PCBs and OCPs. However, whereas native mussels showed no change in the GR and AChE levels, the caged mussels exhibited

somewhat different responses. Some general observations can be made over the whole data set of caged mussels (see 3.2.1 and 3.2.2) although annual and regional variability was clearly observed (see 3.3).

3.2.1 Lysosomal membrane stability

According to the OSPAR Joint Assessment and Monitoring Program (JAMP) assessment criteria for LMS measured using the NRR method, organisms are considered to be healthy if NRR time is > 120 minutes (min), stressed but compensating if < 120 but > 50 min, and severely stressed and probably exhibiting pathologies if < 50 min (OSPAR 2013). In this work the measured mean NRR time of 28-29 min indicated highest stress effect in mussels caged in the vicinity of the city of Gävle (site G1) and Kilpilahti oil terminal in Porvoo (C4) (see the caging sites in Fig. 4). A moderate stress level (47-57 min) was observed in mussels caged near the city of Sundsvall (S1 and S2) and in the area affected by the WWTP discharge in Helsinki (H1 and H2). Mussels caged at the other sites in the Porvoo area (C1-C3) and at the reference site in Gävle (G2) were healthiest showing NRR times in the same range with those measured in the native field population at Hanko (69-87 min). Conclusively, the OSPAR/JAMP assessment criteria seems to apply also to Baltic mussels since the observed "healthiest" mussels in the present study (NRR time 69-87 min) showed some stress effects also according to the other measured biomarkers, and could therefore be classified as "stressed but compensating".

LMS has been used a sensitive first tier biomarker to assess contaminant induced health effects (OSPAR 2013; Viarengo et al. 2007). In the present studies, a higher contamination of the impacted sites at each study region was demonstrated by the elevated tissue levels of HMW PAHs and Cd in mussels caged near Gävle and Sundsvall, and HMW PAHs, PCBs and TBT in the vicinity of Porvoo (Table 3). However, mussels showing reduced LMS values outside Helsinki did not contain elevated levels of any of the above mentioned contaminants (Table 3); therefore, the observed biomarker responses indicated mainly the presence of other toxic compounds that were potentially originating from the WWTP discharge, including pharmaceuticals detected using passive samplers attached to the mussel cages (III).

3.2.2 Remarks on glutathione reductase activity and condition index

A higher level of contaminant induced ROS was indicated by the increased GR activity in mussels caged at the contaminated sites compared to the

reference sites similar to earlier studies using caged mussels (e.g. Box et al. 2007; Regoli et al. 2004). Using the whole data set of caged mussels a significant correlation was observed between GR and CI, suggesting that GR and GSH play an important role not only in maintaining the various GSH dependent defense mechanisms but also in bioenergetics of the organism (Heiss et al. 2013).

The caged mussels showed generally higher CI values and GR activities compared to the native mussels, most likely indicating better food conditions at the caging sites. It is of note that the correct interpretation of the CI values requires information of the food conditions and bioenergetic status of the mussels since high CI is often observed in contaminated areas near-shore or inside a river plume where the eutrophicated waters contains better food conditions for mussels (De los Rios et al. 2013; Tsangaris et al. 2011). In the present study this phenomenon was seen in mussels caged at contaminated sites close to the oil terminal in Porvoo (II) and in the WWTP discharge area in Helsinki (III).

However, by feeding actively the mussels were largely exposed to hydrophobic contaminants adhered to food particles (Okay et al. 2006), resulting in other biomarker responses detected in these mussels, including, e.g., genotoxic effects. Decreased GR and CI were observed in mussels caged at the contaminated site in Sundsvall which was probably reflected in the depletion of GSH due to the multiple stressors present in that area (IV; see 3.3.4 and 3.4).

3.3 Specific biomarker patterns observed in different caging experiments

The integrated biomarker response index (IBR) was used as a simple tool to combine the observed biomarker responses into one stress index value. IBR clearly demonstrated the contamination gradients (I, II) and showed the highest combined responses at the most impacted sites (III, IV). The method has been successfully applied in accordance with more complex health index calculation methods to assess the level of stress in organisms (Raftopoulou and Dimitriadis 2010). Information provided in this thesis would help to employ other more complex integration methods such as the Expert System (Dagnino et al. 2007) also for the Baltic mussels, thus allowing more efficient classification of the observed stress levels in mussels (from "healthy" to "pathological stress") in the future studies.

Relations between the different biomarker responses were investigated using multivariate principal component analysis (PCA), which showed the capability of biomarkers to distinguish between contaminated sites and their reference sites in all the caging experiments (Fig. 6) discussed more thoroughly below.

3.3.1 Archipelago Sea, Turku (I)

Biomarker responses were generally higher in mussels caged at the most contaminated site (C3) along the assumed contamination gradient outside the city and harbor of Turku and the outlet of the river Aurajoki (Fig. 4). For an unknown reason the mussels at C2 exhibited a remarkably different soft tissue growth rate and tissue contaminant concentration pattern compared to the other two stations. Therefore, the contaminant induced effects are examined here by comparing mussels from the reference site C1 and the most contaminated site C3 only. The observed responses at C2 are discussed in the paper (I).

Site C3 was under the greatest influence of various types of contaminants and especially of organotins, this being due to the numerous large passenger ferries and other vessels entering and leaving the Turku city harbor several times a day as well as the proximity of a dumping site of dredged harbor sediments. In July, the CI of mussels had increased markedly from the very low April values measured at all sites; however, significantly higher soft tissue growth was observed at C1 compared to C3. Furthermore, GR was elevated in mussels at C3, coinciding with the highest measured levels of PCBs, OCPs and TBT and thus indicating higher contaminant induced antioxidant activity and GSH metabolism at this site.

In August, GST and SOD activities were higher at C3 where mussels also showed higher tissue contaminant concentrations compared to C1. AChE and CAT activities demonstrated the seasonal effect with higher levels measured in July compared to August without any differences between the sites. The observed decrease in the overall level of AChE in August could be related to the intensive bloom of the toxic blue-green algae *Nodularia spumigena* occurring regularly in late summer in the Baltic Sea, and is known to induce AChE inhibition in bivalves (Lehtonen et al. 2003, Kankaanpää et al. 2007).

The elevated CAT activity was probably related to temperature-associated changes in the patterns of food uptake and energy metabolism (Devier et al. 2005, Farcy et al. 2013), and possibly also to the reproductive activity of the mussels close to the peak season of spawning. PCA conducted using the biomarkers mentioned above was able to distinguish between the different seasons as well as to indicate the highest stress effects at site C3 (Fig. 6).

Shell growth rate measured in individuals at C1 suggested that the mussels first allocated the energy obtained from the abundant food originating from the spring phytoplankton bloom to the build-up of energy reserves, and only then, during the late summer, used a part of the assimilated energy to shell growth. Shell and tissue growth patterns

recorded at the cleanest site C1 are likely to represent a more-or-less "normal" situation in this sea area (Westerbom et al. 2002); therefore, the differing growth pattern observed at C3 could be caused by stress induced physiological disturbances due to the elevated chemical pollution levels.

In conclusion, the antioxidant enzyme response, GST activity, and impaired soft tissue and shell growth demonstrated an increased level of

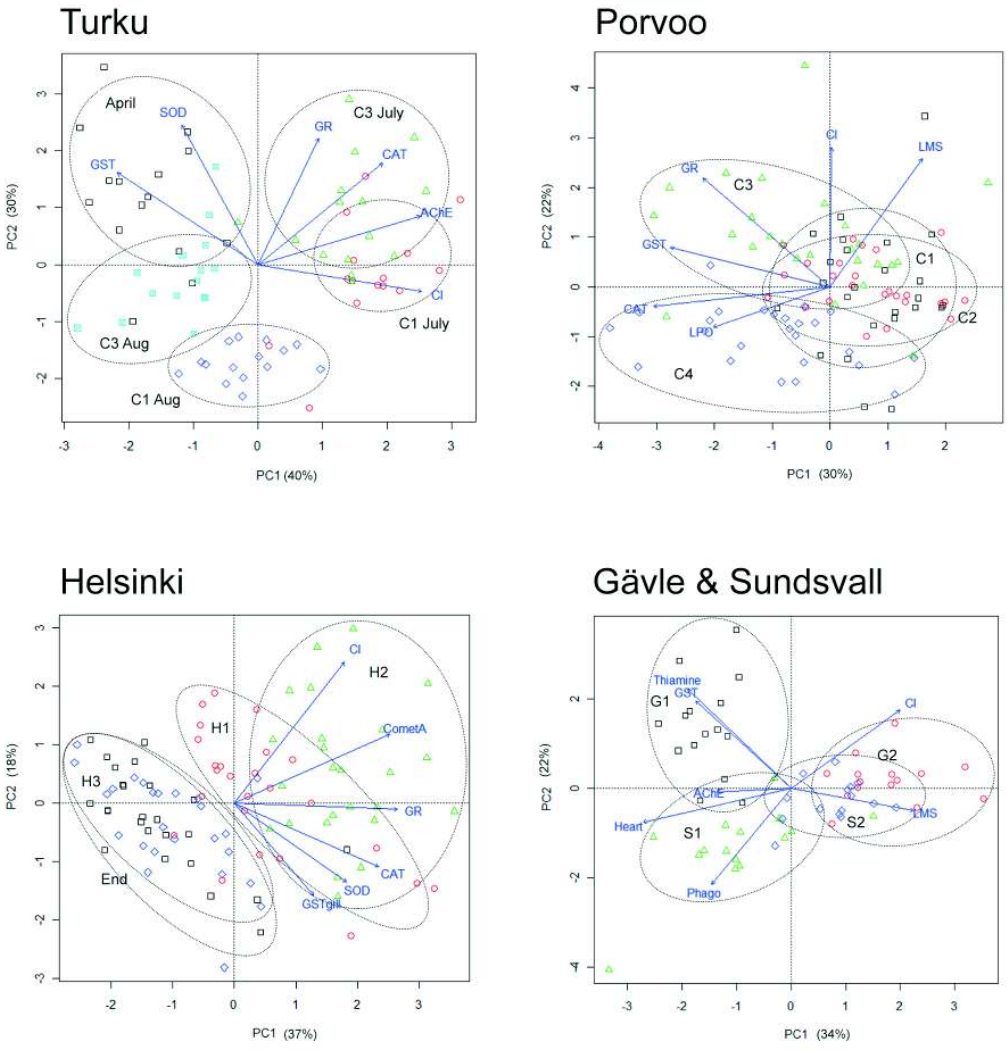


Figure. 6. Principal component analysis (PCA) performed using different biomarker batteries in the different caging experiments: Turku (I), Porvoo (II), Helsinki (III) and Gävle and Sundsvall (IV). PCA distinguished the contaminated sites from the reference sites in each experiment.

biological effects coinciding with elevated tissue concentrations of especially PCBs, OCPs and TBT in mussels caged close to the city and harbor of Turku in the Archipelago Sea.

3.3.2 Gulf of Finland, Porvoo (II)

The highest levels of biomarker responses and tissue contaminant concentrations were observed in mussels caged at the two innermost sites (C3 and C4) close to the Kilpilahti oil refinery and harbor (Fig. 4). Exposure to organic pollutants was indicated by an elevated GST activity at both of these sites compared to the mussels caged further away from the apparent contamination point sources. Severe impairment in the health status of mussels was observed only at site C4 where an increased antioxidant enzyme activity (CAT) and level of LPO indicated contaminant induced oxidative damage in mussels. Furthermore, LMS was significantly reduced at this site (see 3.2.1), confirming the high level of stress suggested here by the other biomarkers.

A significantly elevated GR activity and the highest CI value were observed in mussels caged at the contaminated site C3, showing the critical role of GR and GSH in protecting the cells against contaminant induced damage. PCA distinguished a higher proportion of mussels at C3 and C4 with elevated biomarker responses compared to sites C1 and C2 (Fig. 6).

Compared to the mussels caged at the two outermost sites, those at the innermost sites showed higher tissue concentrations of TBT (30.8-40.0 ng Sn g dw^{-1}), which is regarded as a level of concern according OSPAR criteria (Table 3). The TBT biodegradation index (BDI) suggested recent exposure to the parent compound at these sites, most likely originating from contaminated sediments due to the heavy tanker traffic in the area. Moreover, increased tissue concentrations of HMW PAHs such as B(a)P at sites C3 and C4 as well as an elevated PCB concentration at site C4 were detected (Table 3).

All these contaminants are recognized as inducers of the biotransformation phase I (cytochrome P-450 system) and phase II enzymes (mainly GST in mussels). Adverse effects on hemocyte function and viability has been observed in bivalves exposed to TBT (Hagger et al. 2008). Therefore, the reduced LMS measured in hemocytes, elevated antioxidant response and GST activity are considered here as sensitive responses to the chemical mixture present in the Kilpilahti oil terminal area, with TBT probably as a major effect-causing component.

3.3.3 Gulf of Finland, Helsinki (III)

Elevated antioxidant enzyme activities and DNA damage in mussels caged close to a WWTP discharge site suggested high pro-oxidant and

genotoxic capacity of the chemical mixture present in the treated waste water. Notably higher CI values observed at sites H1 and H2 compared to H3 (Fig. 4) and to the values recorded at the Hanko collection site reflected active food intake during the caging period, leading to rapid growth of the soft tissues. Subsequently, the mussels were exposed both to waterborne dissolved chemicals as well as contaminants attached to the particles filtered as food. PCA distinguished the highest biomarker effects at H1 and H2 as well as an increased variability in the responses of individual mussels at H2 (Fig. 6).

Apart from the measured different tissue concentration ranges, the distinct biomarker response patterns observed in mussels at H1 and H2 may be caused by the presence of other chemicals (e.g., particle bound, water soluble, non-bioaccumulative, metabolites), and different exposure routes (respiration, food intake) at these sites. Passive samplers attached to the cages showed a clear decrease in concentrations of pharmaceuticals from H1 to H3 (Table 4 in III). Moreover, according to the performed 3D hydrodynamic modelling of contaminant dispersal, intensity of the exposure at the study sites fluctuated differently during the experiment (Fig. 6 in III), which resulted in exposure to different chemical cocktails at the different sites potentially affecting the biomarker responses.

CAT activity measured in the digestive gland was increased at site H2, indicating enhanced production of H_2O_2 and contaminant induced oxidative stress in the caged mussels (Box et al. 2007). CAT located in the peroxisomes breaks down H_2O_2 formed by SOD mediated dismutation of $O2^{\bullet-}$ or in spontaneous and peroxisomal reactions (Halliwell and Gutteridge 2001). However, exposure to strong peroxisome proliferators such as PAHs, phthalates, PCBs, alkylphenols, pesticides and estrogens generally present in WWTP effluents (Vieno 2014) leads to the production of H_2O_2 by peroxisomal enzymes and altered CAT activity (Orbea and Cajaraville et al. 2006), possibly explaining the lack of the CAT response at site H1. Moreover, the elevated GR activity observed both at H1 and H2 could indicate an increased antioxidant activity of glutathione peroxidase (GPx, see Fig. 3) and other possible GSH dependent antioxidant reactions (e.g., Ault and Lawrence 2003) for the elimination of excess H_2O_2.

GST activity was induced in the gills of mussels at H2 and H3, suggesting a higher detoxification rate of water-soluble chemicals (especially at H2); however, according to the results on pharmaceuticals obtained using passive samplers and the dispersion model, also mussels at the local reference site H3 were exposed to some degree of contamination originating from the WWTP discharge.

An increased GST activity in gills has previously been reported in mussels exposed to sublethal concentrations of trace metals and organic pollutants (Canesi et al. 1999; Gowland et al. 2002), and has also been associated with moderate to high pollution and oxidative stress in wild mussels (Vidal-Liñan et al. 2010). Furthermore, the elevated GST activity in gills (182.4-214.8 nmol min^{-1} mg $protein^{-1}$, Fig. 3 in III) could be related to a higher level of nutrients and phytoplankton at sites H1-H3, indicated by an increased soft tissue growth and elevated chlorophyll a concentrations of 5.1-16.4 μg^{-1} L^{-1} measured in the area (Riisgård et al. 2014).

Lima et al. (2007) showed significant positive correlations between GST activity in gills and nitrate and ammonia levels in water. Similarly, GST activity in gills was possibly induced due to a combined effect of eutrophication and contamination in mussels caged outside Gävle (195.8-230.5 nmol min^{-1} mg $protein^{-1}$, Table 2 in IV), in the coastal area of Pori in the Gulf of Bothnia receiving nutrients from the river Kokemäenjoki (117.4-180.4 nmol min^{-1} mg $protein^{-1}$, unpublished data), and in the coastal area of the Gulf of Gdansk in the Southern Baltic Sea (104.8-171.2 nmol min^{-1} mg $protein^{-1}$, Dabrowska et al. 2012). The GST response also can be affected by competition between endogenous and toxic substrates, especially at high toxicant concentrations (Fernández et al. 2010; including algal toxins: Kankaanpää et al. 2007, Turja et al. 2014).

CAT activity measured in gills was not induced in any of the above mentioned studies conducted in the Baltic Sea and showed a generally lower level of activity compared to that measured in the digestive gland. It has been suggested that a low CAT activity and a low production of H_2O_2 in gills (Soldatov et al. 2006) is compensated by the peroxidase activity of GST (Lima et al. 2006). Constant ventilation of the gills leads to higher formation of $O2^{\bullet-}$ and therefore high SOD activity is of major importance for this tissue type (Manduzio et al. 2004; Soldatov et al. 2006), and possibly more efficient biomarker of the effects of contaminants than CAT.

In the present study, mussels exposed to the WWTP discharge showed increased antioxidant enzyme activities, which most likely prevented lipid peroxidation (at least in the digestive gland); however, a reduced LMS and increased genotoxic effects revealed the severity of the detected effects. Since LMS was measured in hemocytes, the observed response pattern could be explained so that the exposure to the WWTP discharge constituted of an extensive mixture of chemicals as well as abundant bacteria, which challenged especially the haemocytic lysosomal function. De los Rios et al. (2013) reported no effect in LMS determined in the digestive gland of caged mussels exposed to urban

discharges, and suggested, similarly to study (III), that the increased amount of food particles at the impacted site was able to increase the CI in mussels and therefore counteract the adverse effects of contaminants.

Many biomarker responses and other mechanisms to maintain homeostasis are highly energy consuming processes. The increased energy consumption and reduced lipid reserves indicated that a longer exposure to the WWTP effluent might lead to chronic effects resulting in reduced energy stores and growth in mussels, as suggested also by Smolders et al. (2004); these are impacts critical for the fitness and survival of the mussels and therefore definitely worth of future investigations.

In conclusion, the mussel caging and biomarker approach was demonstrated to be a sensitive method to assess biological effects induced by a one month field exposure to a complex mixture of chemicals present in a treated WWTP effluent. Furthermore, assessment of the presence and ecological impact of trace concentrations of emerging contaminants such as pharmaceuticals in water greatly benefit from the application of passive samplers (Bayen et al. 2014, Devier et al. 2011) attached to mussel cages (Ahkola 2014).

3.3.4 Bothnian Sea, Gävle and Sundsvall (IV)

In general, mussels caged at all the Gulf of Bothnia study sites showed elevated stress effects indicated by increased antioxidant response (SOD), mitochondrial respiration (ETS activity), and DNA damage (measured here as MN) (Table 2 in IV). All these effects can be linked to excessive ROS formation and coincided with higher tissue levels of trace metals (Cd and Cr) and organic pollutants (PAHs and PCBs) in caged mussels compared to the native mussels in Hanko (Table 3).

In high quantities, ROS are able to inhibit the antioxidant system (Regoli et al. 2002), and in this study that possibly resulted in the observed low responsiveness of the other antioxidant enzymes (CAT and GR) that in turn might have led to a marked cellular production of $HO^{\bullet}$, which is the most potent mediator of DNA damage. Moreover, the significantly elevated ETS activity suggested that the contaminants present in the caging area were able to interfere with the mitochondrial function, possibly resulting in enhanced intracellular production of ROS (Murphy 2009).

Interpretation of the above mentioned effects is made more difficult by the possible confounding effect of low salinity (discussed in more detail in 3.4) as well as the unexpectedly high tissue concentrations of PAHs and PCBs detected in native "Day 0"

mussels collected for this study in early June (i.e., seasonal effects, discussed in more detail in 3.1 and I).

Despite of the above described "background stress", mussels caged to the more impacted sites close to the city, harbor and industrial areas of Gävle (G1) and Sundsvall (S1) showed yet higher levels of adverse biological effects compared to the mussels deployed at the local reference sites (Fig. 4). Mussels caged at G1 showed a reduced LMS, and higher levels of GST and AChE activities as well as an elevated thiamine (vitamin B1) concentration compared to those at the other caging sites. Conversely, tissue concentrations of OCPs, which are traditionally regarded as the most potent neurotoxic agents able to induce AChE inhibition, were highest at G1 (Table 3).

AChE inhibition can be induced by direct neurotoxic action or through oxidative stress mediated toxicity, and therefore also other types of substances may have caused the observed neurotoxic responses. At G1, the significantly increased GST activity may have prevented AChE inhibition by eliminating neurotoxic or redox-cycling compounds (e.g., Tsangaris et al. 2010). Another protective mechanism recorded in mussels at G1 compared to the other caging sites was the elevated level of thiamine, which is involved in several key functions related to the other measured biomarkers including antioxidant defense, mitochondrial function, glycogen metabolism and cholinergic system (Depeint et al. 2006).

The highest tissue concentrations of free thiamine recorded at the allegedly most eutrophicated site G1 (1654±731 ng g^{-1} ww) were in the same range observed in mussels caged in the nutrient rich coastal area off Helsinki (1366-1654 ng g^{-1} ww) in the study (III). Somewhat lower thiamine levels were observed in "End" mussels in Hanko (976±208 ng g^{-1} ww) while clearly the lowest levels were detected at G2 and Sundsvall (S1 and S2) (528-660 ng g^{-1} ww).

Animals need to obtain thiamine from their food, and applied here as a marker of food quality the measured thiamine concentrations in mussels were in line with the nutrient levels in water. Due to the marked antioxidant properties of thiamine (Lukienko 2000) it may also have affected some of the potential contaminant induced responses observed in studies III and IV.

Finally, due to the complex and multiple functions of the measured biomarkers, LMS was able to show most clearly the adverse effects of contamination on mussels at G1, supported also by the higher tissue concentrations of some of the contaminants (Table 3).

High tissue concentrations especially of Hg and HMW PAHs were observed at site S1 in Sundsvall (Table 3) where the mussels were characterized by severe health effects

including increased phagocytic activity and heart rate recovery time, and decreased CI. The increased phagocytic activity was most likely caused by mixture toxicity effects on the immune system, supported by the further observations on these mussels including a low haemocyte count, increased vacuolation and nuclear deformities in the haemocytes (Höher et al. in prep.). Haemocyte function represents the vital first line of immune defense against various pathogens in mussels (Pipe and Coles 1995).

Moreover, the observed slow recovery of the heart rate after exposure to environmental or chemical stressors characterizes an organism's poor ability to restore normal functions and a decreased physiological fitness (IV, Kholodkevich et al. 2009). Conclusively, S1 served as an example of a high stress situation where multiple biomarker responses reflected a critically impaired physiological status at the individual level linked to low salinity (see 3.4) and contamination.

3.4 The effects of low salinity (II, IV)

In parts of the present work, mussels were caged under critically low salinity conditions (ca. 4.5) in the Gulf of Finland (II) and the Bothnian Sea (IV). In contrast to the negligible effects of low salinity observed in the Gulf of Finland study (II), possible effects were recorded in the Bothnian Sea where also a wider biomarker battery was applied (IV). Moreover, the timing and length of the exposures (September-October vs. June-September), and interannual differences in the condition of mussels at the Hanko collection site (CI values of 17 and 9 in September 2007 and 2010, respectively) could have influenced the sensitivity of the mussels to exposure to low salinity.

In the Bothnian Sea (June-September) experiment the caged mussels were exposed to natural fluctuations with marked occasional reductions in ambient salinity (Fig. 1 in IV). Considering study IV, some biomarker responses and contaminant levels discussed below could be interpreted as effects of low salinity, or more likely, the combined effect of stress induced by contamination (Table 3) and low salinity (< 4.5).

The high tissue concentrations of Hg, Cd and Cr were observed in mussels caged in the Gulf of Bothnia (Table 3). Metal ions have higher bioavailability at low salinities (Hall and Anderson, 1995). Trace metals are important components of cellular ROS-generating reactions contributing to the increased formation of ROS, which results in elevated SOD activity (Livingstone 2001) and potentially to DNA damage (Fernández et al. 2011, Prevodnik et al. 2006) also detected in caged mussels in the Bothnian Sea (IV). However, regardless of salinity the general tendency for bioaccumulation of metals in mussels has been

ranked as follows: Hg > Cd > Cu > Pb (Karbe et al. 1994).

AChE inhibition has been suggested as an indicator of general physiological condition (e.g., Lehtonen et al. 2006a), and in the Gulf of Bothnia study it was most likely influenced both by contaminant and low salinity stress (except at G1, see 3.3.4). Previous studies have showed AChE inhibition under high chemical stress conditions in Baltic mussels (Kopecka et al. 2006, Schiedek et al. 2006). Despite of the recorded contaminant gradients neurotoxicity was neither observed in studies I-III nor detected in native mussels in previous studies (Lehtonen et al. 2006b). The ability to sustain energy consuming stress responses is closely linked to the prevailing energetic status of the mussels (see discussion on CI below). Beneficial environmental factors such as "normal" salinity and favorable food conditions (e.g., indicated by high CI and good growth) may explain the lack of an AChE response in caged mussels (I-III).

Sufficient energy reserves are required to mount and maintain a successful stress response when challenged by exposure to damaging agents. In the study by Prevodnik et al. (2007) mussels were collected along a south-north salinity gradient in the Baltic Sea and then exposed to Cu and oil The "southern" mussels showed low levels of protein carbonyls and a high scope for growth (SfG; corresponding to CEA measured here) indicating that they had more energy to counteract the contaminant induced stress compared to the "northern" mussels that showed low SfG and high protein damage.

Osmoregulation is achieved by adjusting the intracellular concentration of organic osmolytes, particularly the free amino acids as well as ion and amino acid transporters in the cell membranes (Lockwood and Somero 2011). Osmoregulation has been shown to result in high excretion of amines leading to an increased energy consumption in Baltic *M. trossulus* (Riginos and Cunningham 2005). In the present work, high energy consumption was observed in mussels caged in the low salinity Bothnian Sea but no drastic effects could be detected in CEA (Table 2 in IV).

The total energy budget in mussels seems to be relatively flexible, and the energy stored in lipids, proteins and carbohydrates is reallocated for osmoregulatory purposes and required stress mechanisms (Sokolova et al. 2010). However, this is ultimately achieved only at the expense of a decline in soft tissue growth as observed in mussels at S1 (IV, Riisgård et al. 2014), and under such high stress situations mussels are not able to survive for extended time periods (Sokolova et al. 2010).

In general, *M. trossulus* has a good capability to tolerate changes in ambient salinity and therefore it outcompetes other mytilid species in many estuarine habitats influenced

by high riverine inputs of freshwater (Fly and Hilbish 2013). In contrast to *M. trossulus* occupying high salinity areas the Baltic mussels have a remarkable capability to adjust their filtration rate when exposed to salinity changes between 6.5 and 20 (Riisgård et al. 2013). Adaptation to low salinity impedes the production of shell material restricting the size of the mussels to a maximum of ca. 3.5 cm. Shell length is related to the gill area, which in turn has an effect on filtration capacity and, therefore, on soft tissue growth (Riisgård et al. 2013). Soft tissue growth up to 2.6% day^{-1} was recorded in Baltic mussels fed with algae or transplanted in cages under favorable food conditions; however, mussels exposed to a salinity below 4.5 showed weight loss at a rate of -0.3% day^{-1} (Riisgård et al. 2014) indicating that a decrease in ambient salinity due to climate change is a significant threat to mussel populations in the northern Baltic Sea.

4. CONCLUSIONS

The results obtained in this thesis showed that the difficulties in assessing mixture toxicity effects under varying environmental conditions can be at least partly tackled by applying the mussel caging method and the use of biomarkers. In all the studies performed here the biomarkers successfully discriminated contaminated sites from their local reference sites. The general health indicator LMS proved to be a powerful biomarker of contaminant induced stress also in Baltic mussels. In regard to the antioxidant defense biomarkers the activity of SOD was linked to the level of nutrition, energy consumption (mitochondrial function) and osmoregulation, all of which alter intracellular ROS formation and are affected by contamination. CAT activity exhibited natural variability linked to changing environmental conditions such as increasing temperature and food availability in early spring and summer; however, in mussels caged at contaminated sites the increased CAT activity was most likely associated with contaminant induced oxidative challenge. An elevated GR activity correlated markedly with soft tissue growth, thus indicating the general role of GSH in energy metabolism in mussels as well as in the antioxidant response by scavenging the contaminant induced ROS. Even though these multifaceted mechanisms are usually present behind the formation of ROS, the antioxidant response observed in this study was generally linked to the level of contaminants measured in the tissues of mussels. The bioenergetic status of individuals, indicated

by CEA demonstrating the amount of available energy and CI, was important in determining the functioning of the energetically costly defense responses such as elevated enzymatic activities towards contaminant induced stress.

The marked variability in biomarker responses clearly demonstrated the importance of using a battery of biomarkers encompassing different biological functions and to examine closely the response profiles. In addition, to minimize the effects of a number of seasonal factors interfering with the assessment of contaminant induced effects in caged mussels, the experiments in the northern Baltic Sea are recommended to be conducted between late summer and autumn (e.g., August to October) when the mussels are in their most stable biochemical and physiological condition.

Further studies on the interaction of environmental factors, cellular regulation pathways and catalytic functions in mussels are required to be able to explain more thoroughly some of the controversial results related to the apparent mismatches between the measured tissue contaminant concentrations and biomarker responses in order to facilitate the establishment of biological effects methods in environmental monitoring. Due to the presence of various types on non-bioaccumulating chemicals and contaminants not measured here, as well as the unpredictable mixture effects occurring in the specific physico-chemical environments, these apparent mismatches should have a true ecotoxicological background after all.

Since salinities below 4.5 affect the physiology of Baltic *M. trossulus* and thus also several biomarker responses, the development of a "biomarker toolbox" requires further attention in the Baltic Sea to establish biomarker methods in suitable sentinel species along the salinity gradients.

This thesis provides more profound understanding of biochemical and physiological characteristics related to the health status of Baltic mussels influenced by environmental factors and contaminant exposure, which is a prerequisite to include biomarkers measured in caged mussels in Baltic Sea biomonitoring programs.

5. ACKNOWLEDGEMENTS

My PhD work was carried out in the SYKE Marine Research Centre (Merikeskus). This work was funded by Onni Talas Foundation and I'm very grateful of the good care I received as your stipendiate.

First of all, Kari, I'm grateful that you have been my supervisor and I have learned so much from you on my way to grown-up scientist. I cannot thank you enough for your guidance and dedication in my work and for all your time helping with manuscripts even on your (every) holiday! I also enjoy our discussions, which are not solely science based due to our shared interest in many things, such as Spanish language and Latin American countries as well as during the last years training swimming in our small but powerful swimming group. You also know well all the international top scientists in our field and I feel privileged that from the beginning of my PhD studies you have introduced me to everybody. I'm happy that many of them have become good colleagues and friends of mine.

Thank you for all the current and former members of our "Biomarker team" Anna S, Anna N, Anu, Anna J, Susku, Tanja, Anna K and Jukka of your valuable help in the lab & field & Aranda and more importantly your friendship during these years. Caging experiments would not have happened without especially Jussi Rapo and Tuomo Roine as well as many others working on Aranda fixing the cages no matter how stormy it was. Very special thanks to Anna S for all the crazy hours we have been together in the field diving the mussels and in the lab being optimistic on how many samples it is ok to analyze during one day…which often ended up including the night as well. I would not have survived without you!

Thank you for many PhD students and Post-docs Eeva, Jonna, Anne-Mari, Laura, Anna R, Pirjo, Aura, Noora, Anu V and others for all the friendship, discussions and support during these years.

Many thanks belong to various researchers in Merikeskus for all the help, valuable conversations and a great environment to work in. I wish to thank Kirsten and Harri for all ideas, I have enjoyed a lot in our brainstorming and project planning sessions and I hope all that will continue in the future. Make, thank you for your participation and sincere interest in my work. Ripa, thank you for always asking how things are going and it is great to have you as a very motivated and competitive member of our swimming group. Ville, you saved me by making the maps, thank you! Aranda cruises to deploy and sample the caged mussels have been prerequisite for my

experiments. I wish to thank especially Roope for taking care of Aranda and your interest in my work.

I also wish to thank my co-authors for all the expertise and enthusiasm you've shared with me. Thank you to Professors Jussi Kukkonen and Ketil Hylland for reviewing this thesis and giving valuable comments.

Scientific diving course meant a lot to me personally to be part of the scientific diver community and to get many new friends, as well to my scientific career to master the underwater work and ecology. Jouni, I'm privileged to be one of your divers! Thank you TuSu:t for the great work and fun dives and help with my mussels with special thanks to Iiris and Paula for being my buddy in and out of water and science.

A big hug to you Maija, thank you for being such an amazing friend, it is always so much fun with you. Tanja, Mellu, Karo and Allu, our long friendship means so much to me and I always enjoy meeting with you girls and discuss all the possible things which have taken my mind out of science. I have been missing to spend more time with you all!

The scientific work of this thesis has involved participation of my friends and family. Thank you so much for that! Especially, Tanja, you have been irreplaceable life savior designing and fixing all the possible graphic design related issues for this thesis as well as to my manuscripts and presentations.

Mum, thank you for your endless support in my life and during my scientific career. You have never doubted that this is not a real job, not even when we packed your car with canisters filled with gammarids and mussels and drove together to Norway to make some experiments. I will never forget that!

O-P you have helped in all possible ways like diving and sampling the mussels, computer stuff, bringing pizza to Kumpula in the middle of the night, marking endless amount of eppendorf tubes and many other things. Thank you for your love and patience and sharing your life with me.

6. REFERENCES

Ahkola, H., Herve, S., Knuutinen, J., 2014. Study of different Chemcatcher configurations in the monitoring of nonylphenol ethoxylates and nonylphenol in aquatic environment. Environ. Sci. Poll. Res. 21, 9182-9192.

Andrady, A.L., 2011. Microplastics in the marine environment. Mar. Pollut. Bull. 62, 1596-1605.

Ault, J.G., Lawrence, D.A., 2003. Glutathione distribution in normal and oxidatively stressed cells. Exp. Cell Res. 285, 9-14.

Backer, H., Leppänen, J., Brusendorff, A.C., Forsius, K., Stankiewicz, M., Mehtonen, J., Pyhälä, M., Laamanen, M., Paulomäki, H., Vlasov, N., Haaranen, T., 2010. HELCOM Baltic Sea Action Plan - A regional programme of measures for the marine environment based on the Ecosystem Approach. Mar. Pollut. Bull. 60, 642-649.

Backhaus, T., 2014. Medicines, shaken and stirred: a critical review on the ecotoxicology of pharmaceutical mixtures. Phil. Trans. R. Soc. B 369: 20130585.

Backhaus, T., Faust, M., 2012. Predictive Environmental Risk Assessment of Chemical Mixtures: A Conceptual Framework. Environ. Sci. Technol. 46, 2564-2573.

Backhaus, T., Snape, J., Lazorchak, J., 2012. The impact of chemical pollution on biodiversity and ecosystem services: the need for an improved understanding. Integr. Environ. Assess. Manag. 8, 575-576.

Baršienė, J., Lehtonen, K.K., Koehler, A., Broeg, K., Vuorinen, P.J., Lang, T., Pempkowiak, J., Syvokiene, J., Dedonyte, V., Rybakovas, A., Repecka, R., Vuontisjarvi, H., Kopecka, J., 2006. Biomarker responses in flounder (*Platichthys flesus*) and mussel (*Mytilus edulis*) in the Klaipeda-Butinge area (Baltic Sea). Mar. Pollut. Bull. 53, 422-436.

Bartosz, G., 2009. Reactive oxygen species: Destroyers or messengers? Biochem. Pharmacol. 77, 1303-1315.

Bayne, B., 1976. Marine Mussels: Their Ecology and Physiology. Cambridge University Press.

Beliaeff, B., Burgeot, T., 2002. Integrated biomarker response: A useful tool for ecological risk assessment. Environ. Toxicol. Chem. 21, 1316-1322.

Bhattacharya, S., Zhang, Q., Carmichael, P.L., Boekelheide, K., Andersen, M.E., 2011. Toxicity testing in the 21(st) century: Defining new risk assessment approaches based on perturbation of intracellular toxicity pathways. Plos One 6, e20887.

Bocchetti, R., Fattorini, D., Pisanelli, B., Macchia, S., Oliviero, L., Pilato, F., Pellegrini, D., Regoli, F., 2008. Contaminant accumulation and biomarker responses in caged mussels, *Mytilus galloprovincialis*, to evaluate bioavailability and toxicological effects of remobilized chemicals during dredging and disposal operations in harbour areas. Aquat. Toxicol. 89, 257-266.

Bocquene, G., Galgani, F., 1998. Biological effects of contaminants: cholinesterase inhibition by organophosphate and carbamate compounds. ICES Techniques in Marine Environmental Sciences 22, 12.

Bolognesi, C., Cirillo, S., 2014. Genotoxicity biomarkers in aquatic bioindicators. Current Zoology 60, 273-284.

Box, A., Sureda, A., Galgani, F., Pons, A., Deudero, S., 2007. Assessment of environmental pollution at Balearic Islands applying oxidative stress biomarkers in the mussel *Mytilus galloprovincialis*. Comp. Biochem. Physiol. C-Toxicol. Pharmacol. 146, 531-539.

Boxall, A.B.A., Rudd, M.A., Brooks, B.W., Caldwell, D.J., Choi, K., Hickmann, S., Innes, E., Ostapyk, K., Staveley, J.P., Verslycke, T., Ankley, G.T., Beazley, K.F., Belanger, S.E., Berninger, J.P., Carriquiriborde, P., Coors, A., DeLeo, P.C., Dyer, S.D., Ericson, J.F., Gagné, F., Giesy, J.P., Gouin, T., Hallstrom, L., Karlsson, M.V., Larsson, D.G.J., Lazorchak, J.M., Mastrocco, F., McLaughlin, A., McMaster, M.E., Meyerhoff, R.D., Moore, R., Parrott, J.L., Snape, J.R., Murray-Smith, R., Servos, M.R., Sibley, P.K., Straub, J.O., Szabo, N.D., Topp, E., Tetreault, G.R., Trudeau, V.L., Van Der Kraak, G., 2012. Pharmaceuticals and personal care products in the environment: What are the big questions? Environ. Health Perspect. 120, 1221-1229.

Bradford, M., 1976. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. Anal. Biochem. 72, 248-254.

Brian, J.V., Harris, C.A., Scholze, M., Backhaus, T., Booy, P., Lamoree, M., Pojana, G., Jonkers, N., Runnalls, T., Bonfa, A., Marcomini, A., Sumpter, J.P., 2005. Accurate prediction of the response of freshwater fish to a mixture of estrogenic chemicals. Environ. Health Perspect. 113, 721-728.

Broeg, K., Lehtonen, K.K., 2006. Indices for the assessment of environmental pollution of the Baltic Sea coasts: Integrated assessment of a multi-biomarker approach. Mar. Pollut. Bull. 53, 508-522.

Cajaraville, M.P., Bebianno, M.J., Blasco, J., Porte, C., Sarasquete, C., Viarengo, A., 2000. The use of biomarkers to assess the impact of pollution in coastal environments of the Iberian Peninsula: a practical approach. Sci. Total Environ. 247, 295-311.

Canesi, L., Viarengo, A., Leonzio, C., Filippelli, M., Gallo, G., 1999. Heavy metals and glutathione metabolism in mussel tissues. Aquat. Toxicol. 46, 67-76.

Carlberg, I., Mannervik, B., 1975. Purification and characterization of flavoenzyme glutathione reductase from rat liver. J. Biol. Chem. 250, 5475-5480.

Carr, R.S., Linden, O., 1984. Bioenergetic responses of *Gammarus salinus* and *Mytilus edulis* to oil and oil dispersants in a model ecosystem. Mar. Ecol. Prog. Ser. 19, 285-291.

Claiborne, A., 1985. Catalase activity, in Greenwald, R.A. (Ed.), *Handbook of Methods for Oxygen Radical Research*. CRC Press, Boca Raton, pp. 283-284.

Collins, A., Koppen, G., Valdiglesias, V., Dusinska, M., Kruszewski, M., Moller, P., Rojas, E., Dhawan, A., Benzie, I., Coskun, E., Moretti, M., Speit, G., Bonassi, S., ComNet Project, 2014. The comet assay as a tool for human biomonitoring studies: The ComNet Project. Mutat. Res. - Rev. Mut. Res. 759, 27-39.

Dabrowska, H., Kopko, O., Turja, R., Lehtonen, K.K., Gora, A., Polak-Juszczak, L., Warzocha, J., Kholodkevich, S., 2012. Sediment contaminants and contaminant levels and biomarkers in caged mussels (*Mytilus trossulus*) in the southern Baltic Sea. Mar. Environ. Res. 84, 1-9.

Dachs, J., Mejanelle, L., 2010. Organic pollutants in coastal waters, sediments, and biota: A relevant driver for ecosystems during the anthropocene? Estuaries and Coasts 33, 1-14.

Dagnino, A., Allen, J.I., Moore, M.N., Broeg, K., Canesi, L., Viarengo, A., 2007. Development of an expert system for the integration of biomarker responses in mussels into an animal health index. Biomarkers 12, 155-172.

Damiens, G., Gnassia-Barelli, M., Loques, F., Romeo, M., Salbert, V., 2007. Integrated biomarker response index as a useful tool for environmental assessment evaluated using transplanted mussels. Chemosphere 66, 574-583.

Davies, I.M., Vethaak, D., 2012. Integrated marine environmental monitoring of chemicals and their effects. ICES Cooperative Reseach Report 315.

De Coen, W.M., Janssen, C.R., 2003. The missing biomarker link: Relationships between effects on the cellular energy allocation biomarker of toxicant-stressed Daphnia magna and corresponding population characteristics. Environ. Toxicol. Chem. 22, 1632-1641.

de los Rios, A., Perez, L., Ortiz-Zarragoitia, M., Serrano, T., Barbero, M.C., Echavarri-Erasun, B., Juanes, J.A., Orbea, A., Cajaraville, M.P., 2013. Assessing the effects of treated and untreated urban discharges to estuarine and coastal waters applying selected biomarkers on caged mussels. Mar. Pollut. Bull. 77, 251-265.

Decleves, X., Jacob, A., Yousif, S., Shawahna, R., Potin, S., Scherrmann, J., 2011. Interplay of drug metabolizing CYP450 enzymes and ABC transporters in the blood-brain barrier. Curr. Drug Metab. 12, 732-741.

Depeint, F., Bruce, W.R., Shangari, N., Mehta, R., O'Brien, P.J., 2006. Mitochondrial function and toxicity: Role of the B vitamin family on mitochondrial energy metabolism. Chem. Biol. Interact. 163, 94-112.

Depledge, M.H., 1994. The Rational Basis for the use of Biomarkers as Ecotoxicological Tools. In Fossi, M.C., Leonzio, C. Nondestructive Biomarkers in Vertebrates. Lewis Publishers, CRC Press Boca Raton, USA.

Depledge, M.H., Fossi, M.C., 1994. The Role of Biomarkers in Environmental Assessment .2. Invertebrates. Ecotoxicology 3, 161-172.

Devier, M.H., Augagneur, S., Budzinski, H., Le Menach, K., Mora, P., Narbonne, J.F., Garrigues, P., 2005. One-year monitoring survey of organic compounds (PAHs, PCBs, TBT), heavy metals and biomarkers in blue mussels from the Arcachon Bay, France. J. Environ. Monit. 7, 224-240.

Devier, M., Mazellier, P., Ait-Aissa, S., Budzinski, H., 2011. New challenges in environmental analytical chemistry: Identification of toxic compounds in complex mixtures. Comptes Rendus Chimie 14, 766-779.

Diaz de Cerio, O., Hands, E., Humble, J., Cajaraville, M.P., Craft, J.A., Cancio, I., 2013. Construction and characterization of a forward subtracted library of blue mussels *Mytilus edulis* for the identification of gene transcription signatures and biomarkers of styrene exposure. Mar. Pollut. Bull. 71, 230-239.

Dondero, F., Banni, M., Negri, A., Boatti, L., Dagnino, A., Viarengo, A., 2011. Interactions of a pesticide/heavy metal mixture in marine bivalves: a transcriptomic assessment. BMC Genomics 12, 195.

Dondero, F., Piacentini, L., Marsano, F., Rebelo, M., Vergani, L., Venier, P., Viarengo, A., 2006. Gene transcription profiling in pollutant exposed mussels (*Mytilus* spp.) using a new low-density oligonucleotide microarray. Gene 376, 24-36.

Erk, M., Ivankovic, D., Strizak, Z., 2011. Cellular energy allocation in mussels (*Mytilus galloprovincialis*) from the stratified estuary as a physiological biomarker. Mar. Pollut. Bull. 62, 1124-1129.

EU, 2009. Council of the European Union: Council conclusion on combination effects of chemicals, 2988th Environment Council Meeting, Brussels, 22 Dec. 2009.

Farcy, E., Burgeot, T., Haberkorn, H., Auffret, M., Lagadic, L., Allenou, J., Budzinski, H., Mazzella, N., Pete, R., Heydorff, M., Menard, D., Mondeguer, F., Caquet, T., 2013. An integrated environmental approach to investigate biomarker fluctuations in the blue mussel *Mytilus edulis* L. in the Vilaine estuary, France. Environ. Sci. Poll. Res. 20, 630-650.

Fernández, B., Campillo, J.A., Martínez-Gómez, C., Benedicto, J., 2010. Antioxidant responses in gills of mussel (*Mytilus galloprovincialis*) as biomarkers of environmental stress along the Spanish Mediterranean coast. Aquat. Toxicol. 99, 186-197.

Fernández, B., Antonio Campillo, J., Martínez-Gómez, C., Benedicto, J., 2011. Micronuclei and other nuclear abnormalities in mussels (*Mytilus galloprovincialis*) as biomarkers of cyto-genotoxic pollution in Mediterranean waters. Environ. Mol. Mutagen. 52, 479-491.

Ferrao-Filho, A.d.S., Kozlowsky-Suzuki, B., 2011. Cyanotoxins: Bioaccumulation and effects on aquatic animals. Marine Drugs 9, 2729-2772.

Fly, E.K., Hilbish, T.J., 2013. Physiological energetics and biogeographic range limits of three congeneric mussel species. Oecologia 172, 35-46.

Forbes, V.E., Palmqvist, A., Bach, L., 2006. The use and misuse of biomarkers in ecotoxicology. Environ. Toxicol. Chem. 25, 272-280.

Gagné, F., Blaise, C., André, C., Salazar, M., 2006. Effects of pharmaceutical products and municipal wastewaters on temperature-dependent mitochondrial electron transport activity in *Elliptio complanata* mussels. Comp. Biochem. Physiol. C-Toxicol. Pharmacol.143, 388-393.

Garmendia, L., Soto, M., Ortiz-Zarragoitia, M., Orbea, A., Cajaraville, M.P., Marigómez, I., 2011. Application of a battery of biomarkers in mussel digestive gland to assess long-term effects of the Prestige oil spill in Galicia and Bay of Biscay: Correlation and multivariate analysis. J. Environ. Monit. 13, 933-942.

Gillis, P.L., Higgins, S.K., Jorge, M.B., 2014. Evidence of oxidative stress in wild freshwater mussels (*Lasmigona costata*) exposed to urban-derived contaminants. Ecotoxicol. Environ. Saf. 102, 62-69.

Giuliani, M.E., Benedetti, M., Arukwe, A., Regoli, F., 2013. Transcriptional and catalytic responses of antioxidant and biotransformation pathways in mussels, *Mytilus galloprovincialis*, exposed to chemical mixtures. Aquat. Toxicol. 134, 120-127.

Goldberg, E.D., 1975. The mussel watch—a first step in global marine monitoring. Mar. Pollut. Bull. 6, 111.

Gorbi, S., Regoli, F., 2012. Total Oxyradical Scavenging Capacity Assay. In: Abele, D., Vázquez-Medina, J.P. and Zenteno-Savín, T. Oxidative stress in aquatic ecosystems. Blackwell Publishing Ltd. Ch. 26, 359-366.

Gorokhova, E., Löf, M., Halldorsson, H.P., Tjärnlund, U., Lindström, M., Elfwing, T., Sundelin, B., 2010. Single and combined effects of hypoxia and contaminated sediments on the amphipod *Monoporeia affinis* in laboratory toxicity bioassays based on multiple biomarkers. Aquat. Toxicol. 99, 263-274.

Gowland, B.T.G., McIntosh, A.D., Davies, I.M., Moffat, C.F., Webster, L., 2002. Implications from a field study regarding the relationship between polycyclic aromatic hydrocarbons and glutathione S-transferase activity in mussels. Mar. Environ. Res. 54, 231-235.

Granskog, M.A., Kaartokallio, H., 2004. An estimation of the potential fluxes of nitrogen, phosphorus, cadmium and lead from sea ice and snow in the northern Baltic Sea. Water Air Soil Poll. 154, 331-347.

Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione S-transferases - first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249, 7130-7139.

Hagger, J.A., Depledge, M.H., Galloway, T.S., 2005. Toxicity of tributyltin in the marine mollusc *Mytilus edulis*. Mar. Pollut. Bull. 51, 811-816.

Hagger, J.A., Jones, M.B., Lowe, D., Leonard, D.R.P., Owen, R., Galloway, T.S., 2008. Application of biomarkers for improving risk assessments of chemicals under the Water Framework Directive: A case study. Mar. Pollut. Bull. 56, 1111-1118.

Hall, L.W., Anderson, R.D., 1995. The Influence of salinity on the toxicity of various classes of chemicals to aquatic biota. Crit. Rev. Toxicol. 25, 281-346.

Halliwell, B., Gutteridge, J., 1999. Free Radicals in Biology and Medicine. Oxford University Press.

Heiss, E.H., Schachner, D., Zimmermann, K., Dirsch, V.M., 2013. Glucose availability is a decisive factor for Nrf2-mediated gene expression. Redox biology 1, 359-65.

HELCOM, 2010. Ecosystem Health of the Baltic Sea 2003–2007: HELCOM Initial Holistic Assessment. Balt. Sea Environ. Proc. No. 122.

HELCOM, 2010. Hazardous substances in the Baltic Sea – An integrated thematic assessment of hazardous substances in the Baltic Sea. Balt. Sea Environ. Proc. No. 120B.

HELCOM, 2009. Hazardous substances of specific concern to the Baltic Sea - Final report of the HAZARDOUS project. Balt. Sea Environ. Proc. No. 119.

HELCOM, 2007. HELCOM Baltic Sea Action Plan. www.helcom.fi/Documents/Baltic sea action plan/BSAP_Final.pdf, last visited: 10 Dec 2014.

Höher, N., Regoli, F., Dissanayake, A., Nagel, M., Kriews, M., Koehler, A., Broeg, K., 2013. Immunomodulating effects of environmentally realistic copper concentrations in *Mytilus edulis* adapted to naturally low salinities. Aquat. Toxicol. 140, 185-195.

ICES, 2011. Report of the Study Group on Integrated Monitoring of Contaminants and Biological Effects (SGIMC), 14–18 March 2011, Copenhagen, Denmark.

Johannesson, K., Smolarz, K., Grahn, M., André, C., 2011. The future of Baltic Sea populations: Local extinction or evolutionary rescue? Ambio 40, 179-190.

Kaitala, S., Hällfors, S., 2008. Phytoplankton biomass and species succession in the Gulf of Finland, Northern Baltic Proper and Southern Baltic Sea in 2008. HELCOM Baltic Sea Environment Fact Sheet. www.helcom.fi/Documents/Baltic sea trends/Environment fact sheets/BSEF_Phytoplankton biomass and species succession in the Gulf of Finland 2008.pdf, last visited: 10 Dec 2014.

Kankaanpää, H., Leiniö, S., Olin, M., Sjövall, O., Meriluoto, J., Lehtonen, K.K., 2007. Accumulation and depuration of cyanobacterial toxin nodularin and biomarker responses in the mussel *Mytilus edulis*. Chemosphere 68, 1210-1217.

Karbe, L., Aletsee, L., Durselen, C., Heyer, K., Kammann, U., Krause, M., Rick, H., Steinhart, H., 1994. Bioaccumulation and effects of plankton and benthos on the fate of contaminants, in Sundermann J (Ed.), Circulation and Contaminant Fluxes in the North Sea. Springer-Verlag, Berlin, pp. 555-597.

Kautsky, L., Kautsky, N., 2000. Baltic Sea, including Bothnian Sea and Bothnian Bay, In: Sheppard, C.R.C.(Ed.), Seas at the Millennium: An Environmental Evaluation. Elsevier Science Ltd.

Kautsky, N., 1982. Growth and size structure in a Baltic *Mytilus edulis* population. Mar. Biol. 68, 117-133.

Kautsky, N., 1982b. Quantitative studies on gonad cycle fecundity reproductive output and recruitment in a Baltic *Mytilus edulis* population. Mar. Biol. 68, 143-160.

Kholodkevich, S.V., Kuznetsova, T.V., Trusevich, V.V., Kurakin, A.S., Ivanov, A.V., 2009. Peculiarities of valve movement and of cardiac activity of the bivalve mollusc *Mytilus galloprovincialis* at various stress actions. J. Evol. Biochem. Phys. 45, 524-526.

Klaine, S.J., Alvarez, P.J.J., Batley, G.E., Fernandes, T.F., Handy, R.D., Lyon, D.Y., Mahendra, S., McLaughlin, M.J., Lead, J.R., 2008. Nanomaterials in the environment: Behavior, fate, bioavailability, and effects. Environ. Toxicol. Chem. 27, 1825-1851.

Koivisto, M.E., Westerbom, M., 2010. Habitat structure and complexity as determinants of biodiversity in blue mussel beds on sublittoral rocky shores. Mar. Biol. 157, 1463-1474.

Kopecka, J., Lehtonen, K.K., Baršienė, J., Broeg, K., Vuorinen, P.J., Gercken, J., Pempkowiak, J., 2006. Measurements of biomarker levels in flounder (*Platichthys flesus*) and blue mussel (*Mytilus trossulus*) from the Gulf of Gdansk (southern Baltic). Mar. Pollut. Bull. 53, 406-421.

Krewski, D., Acosta, D.,Jr., Andersen, M., Anderson, H., Bailar, J.C.,III, Boekelheide, K., Brent, R., Charnley, G., Cheung, V.G., Green, S.,Jr., Kelsey, K.T., Kerkvliet, N.I., Li, A.A., McCray, L., Meyer, O., Patterson, R.D., Pennie, W., Scala, R.A., Solomon, G.M., Stephens, M., Yager, J., Zeise, L., Staff Comm Toxicity Testing Assess, 2010. Toxicity Testing in the 21st Century: a Vision and a Strategy. J. Toxicol. Environ. Health. B Crit. Rev. 13, 51-138.

Lam, P.K.S., 2009. Use of biomarkers in environmental monitoring. Ocean Coast. Manage. 52, 348-354.

Lange, F.T., Scheurer, M., Brauch, H., 2012. Artificial sweeteners-a recently recognized class of emerging environmental contaminants: a review. Anal. Bioanal. Chem. 403, 2503-2518.

Lee, R.F., Steinert, S., 2003. Use of the single cell gel electrophoresis/comet assay for detecting DNA damage in aquatic (marine and freshwater) animals. Mutat. Res. - Rev. Mut. Res. 544, 43-64.

Lehtonen, K.K., Leiniö, S., Schneider, R., Leivuori, M., 2006. Biomarkers of pollution effects in the bivalves *Mytilus edulis* and *Macoma balthica* collected from the southern coast of Finland (Baltic Sea). Mar. Ecol. Prog. Ser. 322, 155-168.

Lehtonen, K.K., Schiedek, D., 2006. Monitoring biological effects of pollution in the Baltic Sea: Neglected - but still wanted? Mar. Pollut. Bull. 53, 377-386.

Lehtonen, K.K., Schiedek, D., Koehler, A., Lang, T., Vuorinen, P.J., Forlin, L., Barsiene, J., Pempkowiak, J., Gercken, J., 2006. The BEEP project in the Baltic Sea: Overview of results and outline for a regional biological effects monitoring strategy. Mar. Pollut. Bull. 53, 523-537.

Lehtonen, K.K., Sundelin, B., Lang, T., Strand, J., 2014. Development of Tools for Integrated Monitoring and Assessment of Hazardous Substances and Their Biological Effects in the Baltic Sea. Ambio 43, 69-81.

Leiniö, S., Lehtonen, K.K., 2005. Seasonal variability in biomarkers in the bivalves *Mytilus edulis* and *Macoma balthica* from the northern Baltic Sea. Comp. Biochem. Physiol. C-Toxicol. Pharmacol. 140, 408-421.

Leppäranta, M., Myrberg, K., 2009. Physical Oceanography of the Baltic Sea. Springer-Verlag, Berlin-Heidelberg-New York.

Lima, I., Moreira, S.M., Rendon-Von Osten, J., Soares, A.M.V.M., Guilhermino, L., 2007. Biochemical responses of the marine mussel *Mytilus galloprovincialis* to petrochemical environmental contamination along the North-western coast of Portugal. Chemosphere 66, 1230-1242.

Livingstone, D.R., 2001. Contaminant-stimulated reactive oxygen species production and oxidative damage in aquatic organisms. Mar. Pollut. Bull. 42, 656-666.

Livingstone, D.R., Martinez, P.G., Michel, X., Narbonne, J.F., Ohara, S., Ribera, D., Winston, G.W., 1990. Oxyradical production as a pollution-mediated mechanism of toxicity in the common mussel, *Mytilus edulis* L, and other mollusks. Funct. Ecol. 4, 415-424.

Lockwood, B.L., Somero, G.N., 2011. Transcriptomic responses to salinity stress in invasive and native blue mussels (genus *Mytilus*). Mol. Ecol. 20, 517-529.

Lowe, D.M., Fossato, V.U., Depledge, M.H., 1995. Contaminant-induced lysosomal membrane damage in blood cells of mussels *Mytilus galloprovincialis* from the Venice Lagoon: An *in vitro* study. Mar. Ecol. Prog. Ser. 129, 189-196.

Lowe, D.M., Pipe, R.K., 1994. Contaminant induced lysosomal membrane damage in marine mussel digestive cells - an *in vitro* study. Aquat. Toxicol. 30, 357-365.

Lukienko, P.I., Mel'nichenko, N.G., Zverinskii, I.V., Zabrodskaya, S.V., 2000. Antioxidant properties of thiamine. Bull. Exp. Biol. Med. 130, 874-876.

Lushchak, V., 2011. Environmentally induced oxidative stress in aquatic animals. Aquat. Toxicol. 101, 13-30.

Lyons, B.P., Thain, J.E., Stentiford, G.D., Hylland, K., Davies, I.M., Vethaak, A.D., 2010. Using biological effects tools to define Good Environmental Status under the European Union Marine Strategy Framework Directive. Mar. Pollut. Bull. 60, 1647-1651.

Machado, A.A.d.S., Wood, C.M., Bianchini, A., Gillis, P.L., 2014. Responses of biomarkers in wild freshwater mussels chronically exposed to complex contaminant mixtures. Ecotoxicology 23, 1345-1358.

Manduzio, H., Monsinjon, T., Galap, C., Leboulenger, F., Rocher, W., 2004. Seasonal variations in antioxidant defences in blue mussels *Mytilus edulis* collected from a polluted area: major contributions in gills of an inducible isoform of Cu/Zn-superoxide dismutase and of glutathione S-transferase. Aquat. Toxicol. 70, 83-93.

Marigómez, I., Garmendia, L., Soto, M., Orbea, A., Izagirre, U., Cajaraville, M.P., 2013. Marine ecosystem health status assessment through integrative biomarker indices: a comparative study after the Prestige oil spill "Mussel Watch". Ecotoxicology 22, 486-505.

Marigómez, I., Zorita, I., Izagirre, U., Ortiz-Zarragoitia, M., Navarro, P., Etxebarria, N., Orbea, A., Soto, M., Cajaraville, M.P., 2013. Combined use of native and caged mussels to assess biological effects of pollution through the integrative biomarker approach. Aquat. Toxicol. 136, 32-48.

Martínez-Gómez, C., Vethaak, A.D., Hylland, K., Burgeot, T., Koehler, A., Lyons, B.P., Thain, J., Gubbins, M.J., Davies, I.M., 2010. A guide to toxicity assessment and monitoring effects at lower levels of biological organization following marine oil spills in European waters. ICES J. Mar. Sci. 67, 1105-1118.

Matozzo, V., Giacomazzo, M., Finos, L., Marin, M.G., Bargelloni, L., Milan, M., 2013. Can ecological history influence immunomarker responses and antioxidant enzyme activities

in bivalves that have been experimentally exposed to contaminants? A new subject for discussion in "eco-immunology" studies. Fish Shellfish Immunol. 35, 126-135.

Mccord, J.M., Fridovich, I.,1969. Superoxide dismutase an enzymic function for erythrocuprein (Hemocuprein). J. Biol. Chem. 244, 6049.

McVeigh, A., Moore, M., Allen, J.I., Dyke, P., 2006. Lysosomal responses to nutritional and contaminant stress in mussel hepatopancreatic digestive cells: A modelling study. Mar. Environ. Res. 62, S433-S438.

MED POL, 2007. UNEP/MAP/MED POL Biological Effects Monitoring Programme: Achievements and future orientations: Proceedings of the workshop, Alessandria, Italy 20-21 December 2006. MAP Technical Report Series 166.

Moore, M.N., 2002. Biocomplexity: the post-genome challenge in ecotoxicology. Aquat. Toxicol. 59, 1-15.

Moore, M.N., Depledge, M.H., Readman, J.W., Leonard, D.R.P., 2004. An integrated biomarker-based strategy for ecotoxicological evaluation of risk in environmental management. Mutat. Res. -Fundam. Mol. Mech. Mutag. 552, 247-268.

Moore, M.N., Viarengo, A., Donkin, P., Hawkins, A.J.S., 2007. Autophagic and lysosomal reactions to stress in the hepatopancreas of blue mussels. Aquat. Toxicol. 84, 80-91.

Murphy, M.P., 2009. How mitochondria produce reactive oxygen species. Biochem. J. 417, 1-13.

Newman, M.C., Clements, W.H., 2008. Ecotoxicology: A Comprehensive Treatment. CRC Press Taylor & Francis Group.

Nikinmaa, M., Rytkönen, K.T., 2011. Functional genomics in aquatic toxicology-Do not forget the function. Aquat. Toxicol. 105, 16-24.

Nizzetto, L., Macleod, M., Borga, K., Cabrerizo, A., Dachs, J., Di Guardo, A., Ghirardello, D., Hansen, K.M., Jarvis, A., Lindroth, A., Ludwig, B., Monteith, D., Perlinger, J.A., Scheringer, M., Schwendenmann, L., Semple, K.T., Wick, L.Y., Zhang, G., Jones, K.C., 2010. Past, present, and future controls on levels of persistent organic pollutants in the global environment. Environ. Sci. Technol. 44, 6526-6531.

Ohkawa, H., Ohishi, N., Yagi, K., 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal. Biochem. 95, 351-358.

Okay, O.S., Tolun, L., Tufekci, V., Telli-Karakoc, F., Donkin, P., 2006. Effects of pyrene on mussels in different experimental conditions. Environ. Int. 32, 538-544.

Omstedt, A., Elken, J., Lehmann, A., Leppäranta, M., Meier, H.E.M., Myrberg, K., Rutgersson, A., 2014. Progress in physical oceanography of the Baltic Sea during the 2003-2014 period. Prog. Oceanogr. 128, 139-171.

Orbea, A., Cajaraville, M.P., 2006. Peroxisome proliferation and antioxidant enzymes in transplanted mussels of four Basque estuaries with different levels of poly cyclic aromatic hydrocarbon and polychlorinated biphenyl pollution. Environ. Toxicol. Chem. 25, 1616-1626.

OSPAR commission, 2013. Background document and technical annexes for biological effects monitoring, Update 2013. Monitoring and Assessment Series, www.ospar.org/documents/dbase/publications/p00589/p00589_biologicaleffectstechniquesbackgrounddocuments.pdf, last visited: 24 Nov 2014.

OSPAR commission, 2012. Levels and trends in marine contaminants and their biological effects - CEMP Assessement report 2012. Monitoring and Assessment Series.

OSPAR commission, 2008. 2007/2008 CEMP Assessment - Trends and concentrations of selected hazardous substances in sediments and trends in TBT-specific biological effects. Monitoring and Assessment Series.

Peakall, D.B., 1994. The role of biomarkers in environmental assessment .1. Introduction. Ecotoxicology 3, 157-160.

Piña, B., Barata, C., 2011. A genomic and ecotoxicological perspective of DNA array studies in aquatic environmental risk assessment. Aquat. Toxicol. 105, 40-49.

Pipe, R.K., Coles, J.A., 1995. Environmental contaminants influencing immune function in marine bivalve mollusks. Fish Shellfish Immunol. 5, 581-595.

Prevodnik, A., Gardeström, J., Lilja, K., Elfwing, T., McDonagh, B., Petrovic, N., Tedengren, M., Sheehan, D., Bollner, T., 2007. Oxidative stress in response to xenobiotics in the blue mussel *Mytilus edulis* L.: Evidence for variation along a natural. Aquat. Toxicol. 82, 63-71.

R Core Team, 2013. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. www.R-project.org/.

Raftopoulou, E.K., Dimitriadis, V.K., 2010. Assessment of the health status of mussels *Mytilus galloprovincialis* along Thermaikos Gulf (Northern Greece): An integrative biomarker approach using ecosystem health indices. Ecotoxicol. Environ. Saf. 73, 1580-1587.

Rank, J., Lehtonen, K.K., Strand, J., Laursen, M., 2007. DNA damage, acetylcholinesterase activity and lysosomal stability in native and transplanted mussels (*Mytilus edulis*) in areas close to coastal chemical dumping sites in Denmark. Aquat. Toxicol. 84, 50-61.

Regoli, F., Frenzilli, G., Bocchetti, R., Annarumma, F., Scarcelli, V., Fattorini, D., Nigro, M., 2004. Time-course variations of oxyradical metabolism, DNA integrity and lysosomal stability in mussels, *Mytilus galloprovincialis*, during a field translocation experiment. Aquat. Toxicol. 68, 167-178.

Regoli, F., Gorbi, S., Frenzilli, G., Nigro, M., Corsi, I., Focardi, S., Winston, G.W., 2002. Oxidative stress in ecotoxicology: from the analysis of individual antioxidants to a more integrated approach. Mar. Environ. Res. 54, 419-423.

Regoli, F., Principato, G., 1995. Glutathione, glutathione-dependent and antioxidant enzymes in mussel, *Mytilus galloprovincialis*, exposed to metals under field and laboratory conditions - implications for the use of biochemical biomarkers. Aquat. Toxicol. 31, 143-164.

Regoli, F., 2012. Chemical pollutants and the mechanisms of reactive oxygen species generation in aquatic Organisms.

Regoli, F., Benedetti, M., Krell, A., Abele, D., 2012. Oxidative challenges in polar seas. In: Abele, D., Vázquez-Medina, J.P. and Zenteno-Savín, T. Oxidative stress in aquatic ecosystems. Blackwell Publishing Ltd. Ch. 12, 20-40.

Regoli, F., Giuliani, M.E., 2014. Oxidative pathways of chemical toxicity and oxidative stress biomarkers in marine organisms. Mar. Environ. Res. 93, 106-117.

Regoli, F., Giuliani, M.E., Benedetti, M., Arukwe, A., 2011. Molecular and biochemical biomarkers in environmental monitoring: A comparison of biotransformation and antioxidant defense systems in multiple tissues. Aquat. Toxicol. 105, 56-66.

Regoli, F., Pellegrini, D., Cicero, A.M., Nigro, M., Benedetti, M., Gorbi, S., Fattorini, D., D'Errico, G., Di Carlo, M., Nardi, A., Gaion, A., Scuderi, A., Giuliani, S., Romanelli, G., Berto, D., Trabucco, B., Guidi, P., Bernardeschi, M., Scarcelli, V., Frenzilli, G., 2014. A multidisciplinary weight of evidence approach for environmental risk assessment at the Costa Concordia wreck: Integrative indices from Mussel Watch. Mar. Environ. Res. 96, 92-104.

Riginos, C., Cunningham, C.W., 2005. Local adaptation and species segregation in two mussel (*Mytilus edulis* x *Mytilus trossulus*) hybrid zones. Mol. Ecol. 14, 381-400.

Riisgård, H.U., Bjornestad, E., Mohlenberg, F., 1987. Accumulation of cadmium in the mussel *Mytilus edulis* - kinetics and importance of uptake via food and sea-water. Mar. Biol. 96, 349-353.

Riisgård, H.U., Luskow, F., Pleissner, D., Lundgreen, K., Lopez, M.A.P., 2013. Effect of salinity on filtration rates of mussels *Mytilus edulis* with special emphasis on dwarfed mussels from the low-saline Central Baltic Sea. Helgol. Mar Res. 67, 591-598.

Riisgård, H.U., Larsen, P.S., Turja, R., Lundgreen, K., 2014. Dwarfism of blue mussels in the low saline Baltic Sea—growth to the lower salinity limit. Mar. Ecol. Prog. Ser. 517, 181-192.

Rudel, H., 2003. Case study: bioavailability of tin and tin compounds. Ecotoxicol. Environ. Saf. 56, 180-189.

Schiedek, D., Broeg, K., Baršienė, J., Lehtonen, K.K., Gercken, J., Pfeifer, S., Vuontisjärvi, H., Vuorinen, P.J., Dedonyte, V., Koehler, A., Balk, L., Schneider, R., 2006. Biomarker responses as indication of contaminant effects in blue mussel (*Mytilus edulis*) and female eelpout (*Zoarces viviparus*) from the southwestern Baltic Sea. Mar. Pollut. Bull. 53, 387-405.

Schmitt, H., Boucard, T., Garric, J., Jensen, J., Parrott, J., Pery, A., Rombke, J., Straub, J.O., Hutchinson, T.H., Sanchez-Arguello, P., Wennmalm, A., Duis, K., 2010. Recommendations on the environmental risk assessment of pharmaceuticals: Effect characterization. Integr. Environ. Assess. Manag. 6 Suppl, 588-602.

Schwarzenbach, R.P., Escher, B.I., Fenner, K., Hofstetter, T.B., Johnson, C.A., von Gunten, U., Wehrli, B., 2006. The challenge of micropollutants in aquatic systems. Science 313, 1072-1077.

Serafim, A., Lopes, B., Company, R., Cravo, A., Gomes, T., Sousa, V., Bebianno, M.J., 2011. A multi-biomarker approach in cross-transplanted mussels *Mytilus galloprovincialis*. Ecotoxicology 20, 1959-1974.

Shaw, J.P., Dondero, F., Moore, M.N., Negri, A., Dagnino, A., Readman, J.W., Lowe, D.R., Frickers, P.E., Beesley, A., Thain, J.E., Viarengo, A., 2011. Integration of biochemical, histochemical and toxicogenomic indices for the assessment of health status of mussels from the Tamar Estuary, UK. Mar. Environ. Res. 72, 13-24.

Smith, K.E.C., Schmicdt, S.N., Dom, N., Blust, R., Holmstrup, M., Mayer, P., 2013. Baseline toxic mixtures of non-toxic chemicals: "solubility addition" increases exposure for solid hydrophobic chemicals. Environ. Sci. Technol. 47, 2026-2033.

Smolders, R., Bervoets, L., De Coen, W., Blust, R., 2004. Cellular energy allocation in zebra mussels exposed along a pollution gradient: linking cellular effects to higher levels of biological organization. Environ. Poll. 129, 99-112.

Sokolova, I.M., Frederich, M., Bagwe, R., Lannig, G., Sukhotin, A.A., 2012. Energy homeostasis as an integrative tool for assessing limits of environmental stress tolerance in aquatic invertebrates. Mar. Environ. Res. 79, 1-15.

Soldatov, A.A., Gostyukhina, O.L., Golovina, I.V., 2007. Antioxidant enzyme complex of tissues of the bivalve *Mytilus galloprovincialis* Lam. under normal and oxidative-stress conditions: A review. Appl. Biochem. Microbiol. 43, 556-562.

Storey, K.B., Wu, C., 2013. Stress response and adaptation: A new molecular toolkit for the 21st century. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 165, 417-428.

Strelitz, J., Engel, L.S., Keifer, M.C., 2014. Blood acetylcholinesterase and butyrylcholinesterase as biomarkers of cholinesterase depression among pesticide handlers. Occup. Environ. Med. 71, 842-7.

Tedengren, M., Arner, M., Kautsky, N., 1988. Ecophysiology and stress response of marine and brackish water gammarus species (Crustacea, Amphipoda) to changes in salinity and exposure to cadmium and diesel-oil. Mar. Ecol. Prog. Ser. 47, 107-116.

Tsangaris, C., Kormas, K., Strogyloudi, E., Hatzianestis, I., Neofitou, C., Andral, B., Galgani, F., 2010. Multiple biomarkers of pollution effects in caged mussels on the Greek coastline. Comp. Biochem. Physiol. C. Toxicol. Pharmacol. 151, 369-378.

Turja, R., Höher, N., Snoeijs, P., Baršienė, J., Butrimavičienė, L., Kuznetsova, T., Kholodkevich, S.V., Devier, M., Budzinski, H., Lehtonen, K.K., 2014. A multibiomarker approach to the assessment of pollution impacts in two Baltic Sea coastal areas in Sweden using caged mussels (*Mytilus trossulus*). Sci. Total Environ. 473, 398-409.

Turja, R., Guimarães, L., Nevala, A., Kankaanpää, H., Korpinen, S., Lehtonen, K.K., 2014. Cumulative effects of exposure to cyanobacteria bloom extracts and benzo[a]pyrene on antioxidant defence biomarkers in Gammarus oceanicus (Crustacea: Amphipoda). Toxicon 78, 68-77.

Turja, R., Soirinsuo, A., Budzinski, H., Devier, M.H., Lehtonen, K.K., 2013. Biomarker responses and accumulation of hazardous substances in mussels (*Mytilus trossulus*) transplanted along a pollution gradient close to an oil terminal in the Gulf of Finland (Baltic Sea). Comp. Biochem. Physiol. C. Toxicol. Pharmacol. 157, 80-92.

Väinolä, R., Strelkov, P., 2011. *Mytilus trossulus* in Northern Europe. Mar. Biol. 158, 817-833.

Valavanidis, A., Vlahogianni, T., Dassenakis, M., Scoullos, M., 2006. Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants. Ecotoxicol. Environ. Saf. 64, 178-189.

Vidal-Liñán, L., Bellas, J., Campillo, J.A., Beiras, R., 2010. Integrated use of antioxidant enzymes in mussels, *Mytilus galloprovincialis*, for monitoring pollution in highly productive coastal areas of Galicia (NW Spain). Chemosphere 78, 265-272.

Vieno, N. 2014. Haitalliset aineet jäteveden puhdistamoilla – hankkeen loppuraportti. Envieno ky (Report in Finnish). www.vvy.fi/files/3739/Haitta-aineet_loppuraportti_1.6.2014.pdf, last visited: 10 Dec 2014.

Walker, C.H., 2009. Organic Pollutants: An Ecotoxicological Perspective. Second edition. CRC Press Taylor & Francis Group.

Walker, C.H., Hopkin, S.P., Sibly, R.M., Peakall, D.B., 2001. Principles of Ecotoxicology. Second edition, Taylor & Francis Group.

Westerbom, M., Kilpi, M., Mustonen, O., 2002. Blue mussels, *Mytilus edulis* at the edge of the range: population structure, growth and biomass along a salinity gradient in the north-eastern Baltic Sea. Mar. Biol. 140, 991-999.

Widdows, J., Donkin, P., 1992. Mussels and environmental contaminants: Bioaccumulation and physiological aspects. In Gosling, E., The Mussel *Mytilus*. Elsevier Press, Amsterdam, 383-424.

Widdows, J., Donkin, P., 1991. Role of physiological energetics in ecotoxicology. Comp. Biochem. Physiol. C. Toxicol. Pharmacol. Endocrinol. 100, 69-75.