Toxic effects of multiple chemical exposures in the three-spined stickleback, *Gasterosteus aculeatus*: single and mixed interaction of cadmium, benzo-α-pyrene and bisphenol A

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To my parents and grandma, who have supported me all the way since the beginning of my studies.
Abstract

There is great number of compounds that end up in the marine environment and the resulting chemical cocktail is potentially responsible for adverse impacts. This study evaluates the effects of three different types of chemicals: cadmium (Cd), benzo-α-pyrene (BαP) and bisphenol A (BPA) in three-spined stickleback (Gasterosteus aculeatus). Brackish acclimated fishes were exposed for five days in a semi-static experiment to the single compounds (low dose and high dose) and to a mixture of them at three different concentrations. Biological responses were explored both through analyses of the hepatic genes expression, metallothionein (heavy metal), cyp1A (PAH), vitellogenin (estrogen), nrf2 (regulator of an array of antioxidant response element) using real-time PCR and through standard biochemical tests of oxidative stress in the liver, catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), glutathione S-transferase (GST). In addition, 7-ethoxyresorufin-O-deethylase (EROD) was measured both in gill and liver. MT induction in females was detectable both at low dose and high dose of Cd. BαP in females and BPA in males did not induce expression of cyp1A and vtg, respectively. All three chemical upregulated the expression of nrf2 gene in the liver. Furthermore, in female stickleback a strong dose-dependent induction of EROD in gills and liver, as well as CAT, GPx and GR was observed in Cd and BαP exposures. Gene expression in the mixture was showed weaker or higher in comparison to the responses of the single exposures depending on the concentration tested; similar patterns were observed in the enzymatic activities. In conclusion, when multiple compounds are present together, responsiveness of the standard biomarkers set is altered; further studies are needed to understand the cascade of effects that are triggered.
Abbreviation list

AhR= Aryl hydrocarbon receptor
ARβ = Androgen receptor beta
ARNT= Aryl hydrocarbon receptor nuclear translocator
BPA= Bisphenol A
BαP= Benzo-α-pyrene
βact= beta-actin
βtub= beta tubulin
CA= Concentration addition
CAT= Catalase
Cd= Cadmium
cDNA= Complementary DNA
CYP= cytochrome
DbA= 1,2:5,6-dibenzanthracene
DTNB= 5,5'-Dithiobis-(2-Nitrobenzoic Acid)
EDTA= Ethylenediaminetetraacetic acid
EFSA= European Food Safety Authority
ELISA= Enzyme-linked immunosorbent assay
EROD= Ethoxyresorufin-O-deethylase
ERα= Estrogen receptor alpha
GOI= Gene of interest
GR= Glutathione reductase
GPx= Glutathione peroxidase
GSH= Glutathione
GSSG= Glutathione disulfide
GST= Glutathione-S-transferase
IA= Independent Action
mRNA= Messenger RNA
MT= Metallothionein
NADPH= Nicotinamide adenine dinucleotide phosphate
NaN₃= Sodium azide
Nrf2= Nuclear factor (erythroid-derived 2)-like 2
PAHs= Polycyclic aromatic hydrocarbon
PCA= Principal component analysis
PCBs= Polychlorinated biphenyl
PFOS= perfluorooctane sulfonate
qPCR= real time PCR
ROS= Reactive oxidative species
Vtg= Vitellogenin
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2. Introduction

2.1 Background

The ecosystem health depends on reducing resources exploitation and enhancing the conservation of the global environment which is continuously loaded with a range of chemical compounds. Aquatic animals are exposed to a wide mixture of xenobiotics and that has attracted the public attention toward the development of new strategies to counter more effectively the deterioration of the marine ecosystem (Depledge and Galloway, 2005).

According to the World Health Organization, more than 100,000 compounds are discharged into the environment every year from different industrial and commercial activities as a consequence of their manufacture, use and disposal. Chemical substances or contaminants released into the environment are “natural” or “manmade”; they include a broad range of compounds: metals, pesticide, herbicide, PCBs, PAHs, PFOS, pharmaceuticals from both point source (e.g. municipal wastewater discharges, sewage sludge) and diffuse (e.g. agricultural runoff) inputs (http://www.who.int).

It should be noted that there is a distinction between the terms “contaminant” and “pollutant”, the former is defined as a substance detected in levels above those that normally happen in an environment as a result of human actions but it does not necessarily cause a measurable damage on living organisms. When the increase in concentration of the substance leads to a damage of the biological system whether at biochemical or cellular level, at whole organism or population, the contaminant becomes a pollutant (Castilhos Ghisi, 2012).

Moreover, it is not the mere presence of a contaminant that makes it potentially responsible for adverse effects, but its concentration and bioavailability. Many of these chemicals are particularly stable and persistent, can resist degradation, can bioaccumulate both in terrestrial and aquatic ecosystems and can be transported for long distances through different pathway (including water, soil, sediment, air-water interface, or air) far from the place of their spill. Xenobiotics impact the health of organisms both directly or indirectly modifying their habitats and producing measurable alterations in the composition of the pristine components of the biosphere (Forbes et al., 2006; Lehtonen and Schiedek, 2006).

2.2 Mixture

The typical situation found in ecosystems is characterized by a cocktail of different classes of chemicals that act simultaneously on wildlife organisms; a
screening project along Swedish costs shows a list of over 100 contaminants in the sea, including phthalates, metals, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), organochlorine, chlorophenols and other pesticides (Broeg and Lehtonen, 2006; Lehtonen and Schiedek, 2006).

While a great deal of laboratory research has been carried out to comprehend the toxicity and modes of action of single compounds and has tried to predict their harmful effects under realistic ecological condition, the dynamics of mixture are still poorly understood. There is a lack of knowledge of how interactions between different compounds affect toxicity on fishes due to the multiple levels on which chemicals act (at enzyme activity, at receptor or at transcription factor level).

Preliminary studies indicates that the exposure to chemical cocktails lead to a much stronger impairment of animal health than the toxicity of each compound alone even when all components are in low concentrations (Backhaus et al., 2000; Faust et al., 2003).

The impacts of complex, ill-defined mixtures are hard to foresee in the marine environment and the combined effects potentially being either more or less toxic than their components alone. Two concepts, from pharmaceutical and toxicological literature, have been used in the attempt of explaining the mechanisms of interaction in chemical cocktails: Concentration Addition (CA) and Independent Action (IA, or Response Addition) (Fig. 1; Altenburger, 2004; Cedergreen et al., 2007). Both models assume no interaction between the mixture components and all compounds are toxic if applied singly. CA is generally regarded as a reasonable expectation for the joint toxicity of similarly acting substances whereas IA is expected to be more appropriated when toxicants have dissimilarity mode of actions (chemicals are interacting with different molecular targets) (Cedergreen et al., 2007; Walter et al., 2002).

Supporting evidences from both concepts are found in literature but no generally conclusion can be drawn, in some scenario CA is better at estimating the joint toxicity of the mixture in other it underestimates the actually observed toxicity and vice versa with IA (Altenburger, 2004).

### Dissimilarly acting substances: Independent Action

$$E_{Mix} = 1 - \prod_{i=1}^{n} (1 - E_i)$$

- $E_{Mix}$ = Effect of the mixture of $n$ compounds
- $E_i$ = Effect of substance $i$, when applied singly

### Similarly acting substances: Concentration Addition

$$ECX_{Mix} = \left( \sum_{i=1}^{n} \frac{P_i}{ECX_i} \right)^{-1}$$

- $P_i$ = Concentration of component $i$ in the mixture ($i = 1...n$)
- $ECX_i$ = Concentration of substance $i$ provoking a certain effect $x$ when applied alone
- $ECX_{Mix}$ = Predicted total concentration of the mixture, that provokes $x\%$ effect.
- $p_i$ = relative fraction of component $i$ in the mixture

**Fig. 1** Equations to calculate the expected mixture toxicity (modified from Altenburger, 2004)
2.3 Biomonitoring and sticklebacks

Over the last century, research on a wide number of organisms has played a crucial role in advancing our understanding of numerous biological and ecological processes.

As stated above, with the continuous production and release of chemicals, the aquatic ecosystems in many regions are polluted. The use of wild species as bioindicators or sentinels has provided a base to comprehend a direct linkage between the ecological effects of pollution and organism health (Minissi et al., 1996).

When a species or group of species are used to assess the quality of the environment, the impacts and the change on a habitat, community or ecosystem through changes over time and observations of their presence or absence, behavior, or some other characteristic, and it’s possible to extrapolate information on the environmental conditions of that habitat, these organisms can be defined as bioindicators (Katsiadaki et al., 2007a; Srogi, 2007).

Fish species have brought attention as bioindicators in projects evaluating the biological and biochemical responses to environmental contaminants due to the fact that relying on chemical criteria alone rather than on animal responses to stressors, may inaccurately represent the biological and ecological condition of aquatic systems. Fishes play a number of roles in the aquatic food-web, their physiology integrates several biological responses, they can bioaccumulate toxic substances and they possess detoxification enzymes. The complications of studying fish are that they show seasonal movements, ample home range, they feed across a broad range of habitats and may not live long.

Sticklebacks (Gasterosteus aculeatus, meaning “bony stomach with spines”) are small fishes (5-11cm) characterized by a laterally compressed body with three spines in front of the dorsal fin. Dorsal coloration is dark whereas the flanks and belly are silvery. Breeding season is in spring and summer when males belly change to orange-red with blue-green flank and eyes while the throat and belly of females turn pink (Fig.2; http://www.fishbase.org).

Sticklebacks show complex breeding behavior, males are territorial and in breeding season they build, take care and aerate nests in shallow areas (Caspers, 1976).

Fig.2 Three-spined stickleback, Gasterosteus aculeatus (Caspers, 1976)
Most populations are anadromous, they inhibit coastal seas, lagoons, estuaries and fresh waters; marine fishes are pelagic and very tolerant of changes in salinity. The three-spine stickleback is widely distributed in the Northern Hemisphere, it can be found throughout Europe between 35°N and 70°N, along North Sea coasts of Scotland and Scandinavia; it occurs on the shores of Baltic Sea, Iceland and White Sea. It has been introduced to northern Italy. In eastern Asia, the distribution stretches from Japan and the Korean peninsula to the Bering Straits. In North America, it ranges along the East Coast from Chesapeake Bay northward and on the Pacific Coast from California to northwestern Alaska (Fig.3; http://www.fishbase.org).

![Fig. 3 Distribution map of Gasterosteus aculeatus (http://www.fishbase.org)](http://www.fishbase.org)

There are several traits that make the three-spined sticklebacks suitable for environmental monitoring and laboratory studies, primarily they have a wide distribution in freshwater, brackish and marine ecosystems and they are endemic in Scandinavian country giving a great potential as sentinel organism. Sticklebacks are easy to find in nature, they have a limited home range, they show limited migration behavior thus being representative of the environment from which they are sampled. Secondly they are present in large amount and are easily caught with the minimum of equipment and resources (hand-nets, traps). Furthermore, their small dimension allows rearing a large numbers of individual in laboratory aquaria, they are easy to handle and they can be used in long term experiments; reproduction is readily induced and they have short generation time (eggs hatch in 7-9 days) (Katsiadaki et al., 2005; Pottinger et al., 2002; Sanders et al., 2008).

The three-spined stickleback has been use as a test organism for several years in many disciplines: biology, ecology, behavior, genetics, evolution, endocrinology and physiology (Katsiadaki et al., 2007b). Moreover the entire genome is sequenced and, although not annotate, OMICs investigation techniques are relatively straightforward to implement in near future (http://www.ensembl.org/index.html).

Regarding its use in ecotoxicology studies, sticklebacks have been exposed to different classes of contaminant as reported in table 1.
Table 1 Examples of toxicity studies conducted using three-spined stickleback

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Endpoints</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cadmium</td>
<td>Whole-body levels</td>
<td>(Woodworth and Pascoe, 1983a)</td>
</tr>
<tr>
<td>Cadmium sulfite nanoparticles</td>
<td>Gill, liver and muscle</td>
<td>(Bervoets et al., 2001)</td>
</tr>
<tr>
<td>PAH</td>
<td>Dibenzanthracene (DbA)</td>
<td>(Williams et al., 2009a)</td>
</tr>
<tr>
<td>PC1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAH</td>
<td>CYP1 mRNA expression</td>
<td>(Gao et al., 2011)</td>
</tr>
<tr>
<td>PCB 126</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCBs, PBDEs, PCNs</td>
<td>EROD, LSI, GSI</td>
<td>(Holm et al., 1994; Holm et al., 1993)</td>
</tr>
<tr>
<td>Androgens and estrogens</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methyltestosterone, estradiol</td>
<td>Spiggin, kidney epithelial cell height</td>
<td>(Katsiadaki et al., 2002a)</td>
</tr>
<tr>
<td>(EE2), 5alpha-dihydrotestosterone (DHT)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flutamide, vinclozolin, linuron, fenitrothion</td>
<td>Spiggin</td>
<td>(Katsiadaki et al., 2005)</td>
</tr>
<tr>
<td>Methyltestosterone, estradiol</td>
<td>Spiggin mRNA expression</td>
<td>(Nagae et al., 2007b)</td>
</tr>
<tr>
<td>(E2), progesterone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Estradiol (E2), dibenzanthracene</td>
<td>EROD, cyp1A, vtg, spiggin mRNA expression</td>
<td>(Geoghegan et al., 2008)</td>
</tr>
<tr>
<td>Methyltestosterone, estradiol</td>
<td>Spiggin and vitellogenin mRNA expression</td>
<td>(Hogan et al., 2008)</td>
</tr>
<tr>
<td>(E2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sewage effluent</td>
<td>Spiggin, reproductive behaviour, nest building</td>
<td>(Beijer et al., 2013)</td>
</tr>
</tbody>
</table>

2.6 Biomarkers

All organisms can be exposed to the concentrations of xenobiotics and natural toxins and other compounds with possible toxicological adverse effects. One of the main problems in ecotoxicological research is the evaluation of the effect in terms of quantify the exposure to chemical stressors.

To address this problem, the study of biological monitoring based on the chemical analysis of pollutants in the waters has been combined with an approach based on changes in physiological, behavior and evolutionary responses of organisms, populations or communities. A biomarker is defined as "a biochemical, cellular, physiological or behavioral variation that can be measured in biological systems such as tissues, cells and biological fluids or at the level of the whole organism (either individuals or populations) that provides evidence of exposure (exposure biomarkers) to and/or effects (effect biomarkers) of one or more chemical pollutants" (van der Oost et al., 2003b).

After exposure to contaminants biological responses can be triggered; at first the toxicity is identified at the biochemical and molecular level (change in enzymatic activities, DNA) and only subsequently at cellular, tissue or organ level, and at the end at population level (Fig. 4). The magnitude of change is measurable and often related to the severity of the exposure (Bayne et al., 1985).
In addition to the measurement of contaminants accumulating in tissues, biomarkers provide information about the health status of organisms and can be used as warning signals of general or particular stress thus enabling the detection of undesirable effect much earlier than one can observe changes in the whole organism itself (van der Oost et al., 2003b).

Besides, monitoring the parameters of an initial change caused by the interaction of organism and xenobiotic compound, biomarkers can be also used as diagnostic or prognostic tools for monitoring environment contamination (Sarkar et al., 2006).

An ideal biomarker should be sensitive to chemical stress as well as an indicator of sub-lethal ecological effects, show biological significance, relative easy to measure, allowing quantification in numerous individuals without being expensive and time consuming. An ideal biomarker also responds in a dose- or time-dependent manner in order to determine the magnitude of the effect/exposure (Livingstone, 1993).

Biomarkers can be classified in two categories: the first one are exposure, they indicate if an organism has experienced exposure to a toxicant; the second class is of effect, they are specifically associated with the toxicant’s mechanism of action.

Biomarkers of exposure are: “all the molecular and biochemical markers that show the detection and measurement of an exogenous substance or its metabolite or the product of an interaction between a xenobiotic agent and some target molecules or cell that is measured in a compartment within an organism” (van der Oost et al., 2003b).

Gene expression biomarkers are new sensitive markers that are growing popularity in ecotoxicology studies due to the ability to detect toxicity signals more sensibly than conventional markers.

However, the expression of single biomarker genes cannot provide a detailed profile of biological responses to a compound or a cocktail of them. The use of genomic biomarkers to assess the effects of chemical pollutants on wildlife is still, at times, confusing and not significant enough because complex changes and
interactions can happen at the same time. A key to the process of understanding how the different chemical classes influence gene expression is to compare responses of individual toxicants and mixtures in laboratory.

Exposure of organisms to compounds, either as individual chemicals or as mixtures, may simplify the understanding of modes of action on biological systems, the feedback mechanisms triggered and what pathways result changed most.

In fish, induction of 7-ethoxyresorufin O-deethylase (EROD) activity and synthesis of vitellogenin in male or juvenile fish are common biomarkers for dioxin-like chemicals and estrogenic substances, respectively. Additional examples of biomarkers are metallothionein, proteins that are produced upon exposure to heavy metals. Quantification of mRNA levels of this biomarkers are presented in this thesis using quantitative PCR (qPCR).

2.4 Biotransformation

Numerous contaminants that may be present in water bodies are known to induce damage and oxidative stress in fishes impairing in the long run the overall health of the organisms.

Many chemicals are lipophilic and readily enter the body through skin, gill or food where they can they can accumulate. Evolutionary pressure has led the organisms to develop sufficient mechanisms to avoid lethal effects.

Biotransformation reactions are cellular mechanisms to defend against the toxic effects of chemical compound. The detoxification is carried out by a battery of enzymes and is usually divided in two phases: phase I and II. The actions of these proteins is converting the xenobiotic into a more water-soluble form, facilitating excretion with urine or bile and thereby preventing harmful accumulation in the body.. The most important organ for detoxification is the liver (van der Oost et al., 2003a).

The first phase includes oxidase reactions with mixed function catalyzed mainly by heme proteins called cytochromes P450. In some case the metabolized compounds become more activated, e.g. benzo-α-pyrene (Celander, 2011).

The intermediates are further metabolized in the second phase of biotransformation where they are bound with endogenous molecules to form more soluble products. Aminoacids or glutathione conjugation reactions are the most important of this phase.

Glutathione-S-transferase (GST) enzymes are responsible for conjugation of glutathione, a tripeptide, to the phase I intermediate. GSTs enzymes can be cytosolic or bound to a membrane (mitochondria, endoplasmic reticulum) and plays a key role in detoxification of many organic xenobiotics (Bayne et al., 1985).

2.5 Oxidative stress and nrf2 transcriptional factor

Frequently when xenobiotics enter into the environment they exert damage through their ability to create redox cycle, as well as damage to proteins, membrane lipids and DNA molecules (van der Oost et al. 2003). Oxidative stress is explained as a
consequence of an imbalance between production and elimination of reactive oxygen species (ROS). These physiological state could be cause by a rapid and transient oxidative burst or a long-lasting production of ROS that exceed the defense mechanisms of the cell. “Reactive oxygen species” which are very reactive molecules possessing an unpaired electron, include both oxygen radicals and non-radicals forms, which are oxidizing agents and/or are readily transformed into radicals. The three main activated oxygen forms are superoxide radical (O$_2^-$), hydrogen peroxide (H$_2$O$_2$) and hydroxyl radical (OH$^-$)(van der Oost et al., 2003a).

ROS are produced continuously in aerobic cells as byproducts of aerobic metabolism, e.g. leakage from the electron transport chain in mitochondria, oxygen demanding enzymes in hepatocytes and peroxisomes, phagocytosis by leukocytes. Experimental evidences show that, at moderate concentrations, organisms take advantages of these radicals, for examples ROS play an important role in physiological functions as mediators in signaling processes (Kazlauskiene et al., 2012).

On the other hand, exposure to anthropogenic compounds can stimulate an over-production of oxy-radicals, leading to sublethal effects, deterioration of macromolecules loss of cellular structure and membrane integrity, DNA damage, enzyme inactivation, lipid peroxidation and protein degradation (Carney Almroth et al., 2008; Livingstone, 1993).

The oxidative phenomenon can be induced directly by pollutants or indirectly by their metabolisation if not not adequately remove (Droege 2002); organic compounds (PAHs PCBs) and heavy metals (Cd, Cu, Hg) are shown to be pro-oxidants and to accelerate the formation of ROS (Förlin et al., 1995).

The cells have evolved defenses against the formation and removal of radicals with a set of enzymatic and non-enzymatic mechanisms and scavenger molecules. Effects of xenobiotic exposure on antioxidant enzyme are all potential biomarker for oxidative stress.

Among the enzyme systems there are: glutathione peroxidases, glutathione reductases and catalases; they all metabolize oxidative toxic intermediates.

Glutathione peroxidase enzymes (GPx) are broadly distributed in animal tissues and they neutralize hydrogen peroxide by coupling its reduction to H$_2$O$_2$ with oxidation of reduced glutathione.

$$2\text{GSH} + \text{H}_2\text{O}_2 \rightarrow \text{GS–SG} + 2\text{H}_2\text{O}$$ (glutathione peroxidase)

GPx can also act on peroxides other than hydrogen peroxide (Sanchez et al., 2005).

Glutathione reductase (GR) is an enzyme that converts glutathione disulphide GSSG back to GSH.

$$\text{GSSG} + \text{NADPH} + \text{H}^+ \rightarrow 2\text{GSH} + \text{NADP}^+$$

Catalases (CAT) are enzymes catalyzing the conversion of hydrogen peroxide to water and oxygen:

$$2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2$$ (catalase)

In fishes, catalase is present in peroxisomes and is especially concentrated in liver
tissues. The rate of hydrogen peroxide removal catalyzed by catalase is proportional to the hydrogen peroxide concentration, which is used for quantification in the catalase assay (Aebi 1984).

Catalase, together with superoxide dismutase and peroxidases, are so-called first level of antioxidant enzymes in that they are able to limit the formation of radical species by converting the ROS molecules in less reactive species. Other molecules, such as glutathione and vitamins have antioxidant action of the second level, because they limit the spread of radical forms.

When the production of ROS exceeds the antioxidant capacity of the cell, different types of cellular mechanisms are turned on to maintain homeostasis, a specific powerful transcription factor that is activated is nuclear factor (erythroid-derived 2)-like 2 (nrf2)(Ma and He, 2012).

Under normal conditions, the protein is normally latent, bind to actin filament in the cytosol through a Kelch-like ECH-associated protein 1 (Keap1), a cysteine-rich protein(He et al., 2008).

Under oxidative and electrophilic stresses, the complex Nrf2-Keap1 dissociates followed by ubiquitination of Keap1 and nuclear translocation of Nrf2. Within the nucleus, it binds to its transcriptional partner, Maf proteins, forming a heterodimer able to recognize the antioxidant responsive elements (ARE) and promote transcriptional activation of a multitude of antioxidant and phase II enzymes (FIG. 5)(Kaspar et al., 2009).

**Fig. 5** Keap1-Nrf2 Stress Response Pathway: the picture show the key function of nrf2 as transcription factor controlling homeostatic antioxidant system in presence of oxidative stress (modified from http://www.frontiersin.org/)

### 2.6 CYP1A and EROD

The cytochromes P450 are a diverse multigene family of monooxygenase enzymes that catalyze up to 95% of all phase I reaction with the role of oxidizing,
hydrolyzing, or reducing a number of endogenous and exogenous substances to maintain homeostasis. The aim of the reaction is to detoxify xenobiotic chemicals increasing their water solubility and their elimination rate. However some contaminants may bio-activate, becoming more toxic than the parent compound (Huggett R.J., 1992; Uno et al., 2012).

In fish, these enzymes are concentrated mainly in the liver, but have been also measure in brain, gill, kidney tissues and gastrointestinal tract (Snyder, 2000); in literature five different cyp1 genes are reported for teleost: cyp1A, cyp1B, cyp1, cypC and cyp1D, but only the first four isoforms have been described so far in stickleback (Gao et al., 2011; Goldstone and Stegeman, 2008).

Induction of the CYP1A subfamily is widely used as a biomarker to assess aquatic pollution due to the enzyme’s tendency to increase in concentration upon exposure to a variety of chemicals and chemical mixtures. Examples are: polycyclic aromatic hydrocarbons (PHHs and PAHs) such as benzo-α-pyrene (BaP), 2,3,7,8-tetraklordiben-p-dioxin, TCDD and aromatic amines. Other substrates include coplanar polychlorinated biphenyls (PCBs), polychlorinated dibenzodioxins and dibenzofurans (Whyte et al., 2000; Williams et al., 2009b).

At molecular level, expression of cyp1A is mediated through the cytosolic aryl hydrocarbon receptor (AhR) that binds to the xenobiotics. The complex is then translocated into the nucleus where it dimerizes with the AHR nuclear translocator (ARNT) and recognizes a specific DNA sequences called “dioxin responsive elements” (DRE). The final effect is the expression of genes known as the “Ah-gene battery” involved in the homeostasis of toxic AhR ligands (Fig. 6; Whyte et al., 2000) The measurement of the induction of cyp1A in terms of 7ethoxyresorufin O-deethylase (EROD) activities is an established enzimatic biomarker in environmental bimonitoring (Whyte et al. 2000).

Fig. 6 The AhR mediates the CYP1A induction (modified from Whyte et al., 2000).

Ethoxyresorufin-O-deethylase (EROD) activity describes the rate of the CYP1A-mediated deethylation of the substrate 7-ethoxyresorufin (7-ER) to form the
product resorufin. The resultant substrate is an indication of the amount of enzyme present (Whyte et al., 2000). EROD induction is found in more than 150 species of fish and its activity has been demonstrated to be influenced by a variety of endogenous and exogenous factors such as sexes, fish size, age, temperature and pH (Geoghegan et al., 2008).

2.6 AhR ligands and benzo-α-pyrene

Increased EROD activity and cyp1A mRNA level are often associated with exposure to AhR-ligands. Most of these chemicals are hydrophobic and planar molecules such as dioxins, PCBs and PAHs and they are mainly by-products of various industrial processes and other anthropogenic activity.

In particular, benzo-α-pyrene (BaP) is a polyaromatic hydrocarbon formed during incomplete combustion processes; it is not manufactured and has no industrial uses. BaP is persistent and tend to bioaccumulate in fatty tissues. BaP is a very potent cyp1A inducer and substrate for the enzyme in many mammalian and fish species. When BaP is biotransformed during phase, I a highly mutagenic and carcinogenic epoxides is activated (Andersson et al., 2010; Verma et al., 2012; Wang et al., 2009; Willett et al., 1995).

2.7 Heavy metals and cadmium

Among the countless toxic substances that are found in the marine environment, heavy metals are one of the categories widely distributed and that cause harmful effects on all living creatures.

Cadmium (Cd), lead (Pb), mercury (Hg) and copper (Cu) are among the non-essential metal, (except for Cu), which can cause health problems in animals. It is known that over the past few decades, the amount of contaminants released into the environment by human activities, have gone worse and worse, until reaching levels of alert that required in-depth studies (e.g. EMEP European Monitoring and Evaluation Programme) and significant legislative interventions (WHO, 2007).

Most of the metals such as cadmium, mercury and lead are natural contaminants. There are areas on Earth's surface whose background values are naturally higher than in other parts, resulting in contamination of bottom fauna and local flora. Still, human activities can produce and release a large amount of naturally-occurring minerals into the environment. Regarding cadmium, 50% of this metal found in the sea originates from anthropogenic activities; it is common in surface waters and can cause adverse effects on fish and other organisms (WHO, 2007).

Cd is still found in the environment in larger amounts because of its intensive use in mining, metallurgical and smelting industries, the use of fertilizer products, agriculture, household waste production, the industries of paints and coatings. The prevalent form of emission is dusts and aerosols into the atmosphere, effluents into rivers and lakes (Bervoets et al., 2001).

For example, northern Sweden is one of the most polluted areas of heavy metal due to sulfide and smelter industries. The factories released about 684 ton lead, 13.4
ton cadmium, 154 ton arsenic and 3.5 ton mercury per year during the late 1960s (Borell M., 2006).

In the aquatic environment cadmium is found abundantly in the forms of ions which form rather stable complexes with chloride ions. Cd is persistent in sediments and can accumulate in aquatic organisms including fishes, phytoplankton, macrophytes, shellfishes and mollusks that tend to concentrates it in amounts significantly higher than the other species. In fish, bioaccumulation factors are lower and metals are mainly concentrated in the kidney, liver and in inedible parts as the gills thus not threatening human health (Kovarova et al., 2009).

Cadmium causes both severe sublethal and lethal effects at relatively low environmental concentrations, in literature Cd exposures to fishes are reported to alter various level of organization from changes in behavior and reproduction to damages at cellular level (Kovarova et al., 2009; Sanders et al., 2008). Among the latter, Cd exposure increases the formation of reactive oxygen species (ROS), reduces lysosomal stability, induces lipid peroxidation and hepatic metallothioneins; it also compromises the activities of various enzyme both phase I and phase II like EROD, cytochrome P450 and glutathione-S-transferase (Table 2;Bouraoui et al., 2008; Kovarova et al., 2009; Oronsaye, 1987b; Sanders et al., 2008; Woodworth and Pascoe, 1983a). Cadmium does also interact with chlorine cells interfering with efflux pumps in gills and modifying the ultrastucture of the lamellae(Oronsaye, 1987a; Oronsaye, 1983; 1989; 1997; Oronsaye and Brafield, 1984; Thphon et al., 2004).

![Table 2 Effect of cadmium in humans and fishes (WHO, 2007(Sevekova et al., 2011)).](image)

**2.8 Metallothioneins**

Among the various strategies that fishes have developed to protect themselves against heavy metal poisoning are metallothionen (MT). They are a group of low molecular mass (6–7 kDa) single-chain, heat stable proteins localized in the membrane of the Golgi apparatus that have been validated as biomarkers for metal exposure. They are rich in cysteine (25–35% of the amino acidic residues due to
which they are able to bind metal ions through metal-thiolate bonds (Huggett R.J., 1992; Sanchez et al., 2005).

MT are widely distributed in nature and have been identified in all major classes of vertebrates, though, there are some differences in structure. In mammals four MT isoforms (MT-1 – MT-4) and 13 MT-like human proteins are known, in fish only two main isoforms, MT-1 and MT-2 are identified (Scudiero et al., 2005).

Experimental data show that MT plays a variety of functions other than heavy metal detoxification, they are involved in regulation of physiological homeostasis, transporting essential metals to place of need (Zn and Cu, being able to bind both) and they also provide protection from stress conditions and against oxidative species (Bouraoui et al., 2008).

An extensive amount of literature has been published on the level of MT in animals, including marine fishes (Barbus graellsii, Hemiborbus mylodon, Takifugu obscurus, Oryzias latipes), mammals (seal pups) and molluscs (cockle, Mytilus galloprovincialis, Crassostrea gigas and Crassostrea virginica) (Sevcikova et al., 2013).

The synthesis of MT varies with fish species and age. The isoforms are expressed differentially in different tissue, and the expression is regulated at both transcriptional and translational levels. MT expression is induced by a variety of stimuli including metal exposure, oxidative stress, glucocorticoids, hydric stress; other factors are diet, season, ultraviolet light and temperature. The level of the response to these inducers depends on the MT gene (Bouraoui et al., 2008; Kvarova et al., 2009).

In fishes, MTs are typically synthesized in the liver, the main detoxification organ of the body and the first one to respond to exposure to contaminants. MT has also been found in kidneys, muscle, gills and digestive tract (Carginale et al., 2002; Hauser-Davis et al., 2012; Kvarova et al., 2009; Sevcikova et al., 2013).

Various analytical techniques have been developed for detection of MT including capillary electrophoresis, liquid chromatography, saturation methods, spectrometry and electrochemistry. Electrochemical assays are the most sensitive ones. Other analyzes utilize immunochemical methods as enzyme-linked immunosorbent assay, radioimmunoassay or blotting techniques. Beside the direct detection of MTs as biomarkers, MT mRNA expression is also recently used in studies with fish (Sevcikova et al., 2013). So far there’s only one article that assesses mRNA expression of the MT gene in stickleback (Santos et al., 2010).

2.9 Endocrine disrupting chemicals and estrogens

Endocrine disruption is the alteration of the normal endocrine system by natural or man-made compounds that mimic the activity, alter the synthesis or the metabolism of endogenous hormones leading to physiological disturbances. These kinds of chemicals are called endocrine disrupting chemicals (EDCs) (Larkin et al., 2003a).

The main sources of release into the aquatic environment are sewage treatment plants, effluents from industrial and municipal wastes, pulp mills and run-off from animal farms. Examples of compounds that can trigger the endocrine system both in laboratory studies and in the environment are pharmaceuticals and synthetic
hormones (eg. ethinylestradiol (EE2) used in oral contraceptives), pesticide like DDT and its breakdown products, tributyltin, industrial compound like bisphenols and even metals (mercury) (Hogan et al., 2008; Larkin et al., 2003a).

Some of the impairments on fish associated with EDCs exposure are altered sexual behavior, adverse development of reproductive system (female ovaries and male testes), reduced fertility, hatchability and survival of offspring (Larkin et al., 2003a; Nagae et al., 2007a).

To detect EDCs in aquatic environment the most common endpoints are: vitellogenin induction in male (feminization); androgenic (masculinizing) effects in female; intersex and imposex (especially in gastropods); gonadosomatic index and change expression of key genes and proteins responsible for hormone signaling (Larkin et al., 2003b; Milla et al., 2011; Scholz and Mayer, 2008).

Bisphenol A (BPA; 2,2-bis(4-hydroxyphenyl)propane, Fig.7c) is an industrial chemical mainly used in the production of epoxy resins and polycarbonate plastics.

BPA is a known endocrine disruptor and is acutely toxic to aquatic organisms related to resistance to degradation. Due to intensified usage of these products, exposure of organisms to BPA via several routes, such as the environment and food, has increased. Currently, an assessment on risks to human health is being carried out by EFSA (European Food Safety Authority)(Huang et al., 2010; Jolly et al., 2009; Pettersson et al., 2007).

2.10 Vitellogenin

Vitellogenin (vtg) is an egg-yolk protein and has been widely used as a specific biomarker of exposure to estrogens or estrogen mimics in several species of fishes including sticklebacks (Larkin et al., 2003a).

The molecule is synthesized by the liver upon binding of estrogens to estrogen receptor (ER) and secreted in the blood stream and is normally present at high levels only in females undergoing oogenesis. The gene is normally suppressed in males and juveniles; however in response to estrogens exposure, vtg synthesis is induced. Thus, presence of vtg in male or juvenile fish has been used widely as a biomarker for exposure to estrogenic chemicals (Hogan et al., 2008).

Sensitive assays to quantify vtg levels have been established for several fish species, for example the protein can be quantified with measurement of the level of alkaline-labile phosphorus (ALP) and calcium in plasma (vtg is characterized by a large number of serine-associated phosphate groups). The method is based on the correlation between plasma levels of ALP and circulating vitellogenin content. (Pottinger et al., 2002).

Other methods are ELISA assay on plasma (Katsiadaki et al., 2002b; Naderi et al., 2013; Sabo-Attwood et al., 2004) and measure vtg mRNA levels in liver through qPCR (Hogan et al., 2008; Sabo-Attwood et al., 2004). With this technique, it has been proved even in stickleback that during normal conditions, female livers have
higher levels of gene transcripts when compared to levels in the male liver. When the fish are exposed to estrogenic model compounds, results showed significantly induced hepatic vtg expression in males (Hogan et al., 2008).

In summary, the expression of vtg in male liver is an excellent indicator of exposure to estrogenic compound. The standardization of method to simultaneous measure the transcripts levels could have significant application in short-term laboratory bioassays and field-based biomonitoring of sticklebacks.
2.14 Aims of this thesis

The aims of this project are to study the effects of chemical mixture in aquatic organisms and to understand the links between toxicants with known modes of action and their effects on sticklebacks.

This dissertation thesis characterizes the responses of selected biomarkers of exposure and effects under the influence of three different classes of anthropogenic compounds (cadmium, estrogen and PAH) both individually and in mixture.

The following biological response will be measured: ethoxyresorufin-O-deethylase (EROD) activity as phase I biotransformation parameter, phase II biotransformation enzymes (glutathione-S-transferase) and antioxidants (catalase, glutathione peroxidase and glutathione reductase).

The project also aims to assess changes in the expression of four biomarker genes on a mRNA level (cyp1A, metallothionein, vitellogenin and nrf-2) as new sensitive markers to detect toxicity signals in experimental studies. This is carried out by using the qPCR in liver tissues.

The essential goal of the dissertation work is to study how these biomarkers act in chemical cocktails, to determine which of them respond most and frequently due to the exposures and thus identified the ones that could be used as the most appropriate early-warning signals of mixture toxicity.
3. Materials and Methods

3.1 The three-spined stickleback

Three-spined sticklebacks (*Gasterosteus aculeatus*) were obtained from a wild population in Fjällbacka and Kristineberg on the south-west coast of Sweden in June 2013 and brought to the Department of Biological and Environmental Sciences at University of Goteborg, Sweden. The sites are considered as having a low level of contamination by anthropogenic activities. After capture, external examination was performed to identify and exclude individuals exhibiting parasitism or morphological alterations.

The fishes were in late breeding season, adults were housed in two 150l holding tanks supplied with artificial brackish water (25‰) at 16°C and light regime of 12h light/12h dark. One third of the water volume was renewed every four days and debris was removed from the tanks. The fishes were fed *ad libitum* five times a week with a combination of frozen red mosquito larvae and tubifex (Aleds Akvarium AB, Sweden).

Three-spined sticklebacks were kept under these conditions to acclimate until the experiments started (three weeks after catching). All the fish experiments were carried out according to the ethical permit DNR 274-2011.

3.2 Tested substances

The three model compounds used in thesis were:

Cadmium (Cd) (Fig.7a) was used as a model compound for MT induction. Fishes were exposed to the form of cadmium chloride salt (CdCl$_2$). The average cadmium content along the coast varies in relation to the amount of surface waters coming from urban and industrialized areas. Depending on specific location levels range from 10ng/l to 4000ng/l (WHO, 2007).

Benzo-α-pyrene (BaP, Fig.7b) was the model compound for AhR agonists and cyp1A inducer.

BPA (BaP, Fig.7b) was chosen as the vtg inducer. Reports on the presence of BPA in the environment are lacking. The U.S. Environmental Protection Agency, in 2010 reported a concentration between 0.012 to 0.14µg/l on surface water, <0.1µg/l in wastewater and 3.5µg/l in marine sediments (http://www.epa.gov/).

All chemicals were obtained from Sigma–Aldrich (Sweden). All other reagents were of analytical grade.
3.3 Experimental design: semi-static exposures

Adult male and female three-spined sticklebacks (0.8 ± 0.2mm, 4.9 ± 0.4g and 1.3 ± 0.4 mm, 5.4 ± 0.5g respectively) were separated and randomly transferred from holding tanks to 15l glass aquariums one day before the experiments started (each tank containing 15 fishes). The females were gravid and ready to spawn, while the males were in a breeding condition.

The fishes were exposed for five days to either copper-free tap water (control), copper-free tap water with acetone (solvent control) or the substances CdCl₂, BaP (dissolved in acetone), BPA (dissolved in acetone). Maximum acetone concentration in the aquaria was less than 0.001%. A mixture exposure of CdCl₂, BaP and BPA was also performed. A single tank of fish was exposed to each concentration. The nominal concentrations are reported in table 3, these concentrations were determined from a literature research. Only specimen weighing more than 0.5g and showing no external signs of parasitic infections were used in the experiment. Every 24h, half of the water was changed and chemical solutions were renewed. At the same time water sample (10ml) were collected from each aquarium for analysis of the actual concentrations of chemicals and stored in −20 °C until analyzed.

Over the period of the experiment the light regime was 12h light/12h dark, the water in all aquaria was aerated constantly, and temperature (16.4±0.5°C), pH (8±0.06) and salinity (25±0.5ppm) monitored daily. The fish were not fed during the exposures.

Table 3 Exposure concentrations of cadmium chloride (CdCl₂, mg/l), benzo-a-pyrene (BaP, μg/l) and bisphenol A (BPA, μg/l) in laboratory experiments with three-spined sticklebacks (Gasterosteus aculeatus).

<table>
<thead>
<tr>
<th>Concentrations</th>
<th>Single exposure</th>
<th>Mixture set-up</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CdCl₂ (mg/l)</td>
<td>BaP (μg/l)</td>
</tr>
<tr>
<td>Low dose</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>High dose</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>10%</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>100%</td>
<td>10</td>
</tr>
</tbody>
</table>

Mixture 1% = CdCl₂ (0.1mg/l) + BaP (0.1μg/l) + BAP (0.1μg/l)
Mixture 10% = CdCl₂ (1mg/l) + BaP (1μg/l) + BAP (1μg/l)
Mixture 100% = CdCl₂ (10mg/l) + BaP (10μg/l) + BAP (10μg/l)
3.4 Tissues sampling

At the end of the exposure period, fishes from all aquaria were numbered, stunned by a blow to the head and sacrificed; wet weight (mg) and fork length (mm) of each individual were recorded. The sex of each fish was verified macroscopically and breeding color was noted. A ventral incision was made using dissecting scissors and liver and gonads were excised, placed in eppendorf tubes and immediately frozen at -80°C.

One gill arch was also dissected and the remainder of each fish was packed in aluminum foil and stored in liquid nitrogen for later uses. Gills were collected in order to measure biochemical responses, liver tissues for both gene expression and biochemical analysis.

3.5 Gene expression

Sticklebacks were exposed to Cd, BαP and BPA to induce heavy metal-, PAH- and estrogen-responsive genes, respectively. Quantitative real-time PCR (qPCR) was applied on mRNA isolated from the liver and kidney of the fish to investigate gene expression and detect any correlation with the concentration of the compounds employed.

Metallothionein was chosen because of its role as protector from metal poisoning, cyp1A was selected for being extensively involved in metabolism of PAHs, vitellogenin was picked as biomarker for exposure to estrogenic chemicals and nrf2 was tested because of its involvement in the oxidative stress response.

Prior to the real experiment, five male and female individuals were sacrificed and kidney and liver tissues were collected in order to validate the qPCR protocol and compare relative expression between sexes.

3.5.1 Isolation of RNA and quantification

Total RNA was isolated from half of the frozen livers in order to produce cDNA and run qPCR. During the screen projects also kidney was tested. The purification was performed using the RNeasy® Plus Mini Kit from QIAGEN GmbH (Hilden, Germany) according to manufacturer's instructions “RNeasy® Plus Mini Handbook, September 2010”.

Briefly, the procedure was carried out in the RNA-extraction room where the fume hood and the bench were kept RNase free with RNaseZAP® (Ambion®, Life Technologies, Stockholm, Sweden). Around 10mg of sample was transferred into an eppendorf with 350ml of Buffer RLT Plus containing β-mercaptoethanol and lysed with a Pellet Pestle® Motor Kontes (Sigma-Aldrich Sweden AB, Stockholm, Sweden). According to recommendations from QIAGEN when extracting hepatic RNA, 50% ethanol was used, when extracting kidney RNA 70% ethanol, thus having a higher yields with different samples. The RNA was eluated in RNase-free water (30µl) and stored at -80°C.
The purity and quantity of RNA were determined spectrophotometrically employing a Nanodrop 1000 spectrophotometer (Nanodrop Wilmington, De, United States) (260/280nm ratio and 260/230nm ratio were around 2). The RNA concentration (1µl per sample) was measured two times, the average annotated and used in further calculations. RNA integrity and purity of 12 random samples were analyzed with Experion™ automated electrophoresis system from Bio-Rad with the Experion™ RNA stdsens chip (Bio-Rad Laboratories Inc., Hercules, United States) (Fig. 8). All samples had a RQI value ranging between 8.8-10 and a 28S:18S ratio around 2.

![Fig. 8 Electropherogram of a total RNA sample from Gasterosteus aculeatus liver-BaP, low dose. The relative positions of the lower alignment marker, 5S rRNA, 18S rRNA, and 28S rRNA are indicated.](image)

3.5.2 cDNA synthesis

RNA was reverse transcribed to cDNA using iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories Inc., Hercules, United States). Each tube of a 8-tubes strip was filled in with 5x iScript reaction mix, reverse transcriptase, nuclease-free water and RNA template. According to the protocol, the master mix volume per reaction was 5µl (4µl of 5x iScript reaction mix plus 1µl of reverse transcriptase enzyme). The amount of RNA solution used depended on the RNA concentrations and the final concentration of cDNA was equal to 50ng/µl in a volume of 20µl. Two tubes were not filled in with RNA reverse transcriptase enzymes but with nuclease-free water and used to control for genomic DNA contamination (noRT controls). The strips were let incubate in MyCycler™ Thermal Cycler (Bio-Rad Laboratories Inc., Hercules, United States) following the scheme: 25°C for five minutes, 42°C for thirty minutes, 85°C for five minutes, 4°C ad libitum.

Once the cDNA was ready, it was aliquoted into 4µl×5 tubes and stored in -20°C until use. cDNA samples were diluted 1/8 (by adding 28µl of RNA free water) before use in quantitative qPCR analysis, resulting in a final concentration of 25 ng/µl.
3.5.3 Primers

In this study some primers were taken from previously published coding sequences for *Gasterosteus aculeatus*: 18S mRNA, β-actin (βact), β-tubulin (βtub), cyp1A, metallothionein (MT), vitellogenin (vtg), estrogen receptor α (ERα) and spiggin. Other primers, androgen receptor β (ARβ) and nrf2 were designed with the software Beacon designer 2.0 (PREMIER Biosoft International, USA). These latter sequences were obtained from NCBI and tested for specify using the stickleback genome in Ensemble Genome Browser. Primers analysis was performed using default settings and a temperature of 60±5°C (which is the annealing temperature tested for the other primers). Forward and reverse sequences with less probability of formation of secondary structures were chosen. Primers were synthesized by Eurofins MWG GmbH, Germany. Primers and amplicon information are listed in Table 4.

The gene coding for the 18S mRNA, β-actin and β-tubulin were used as reference gene; whereas cyp1A, MT, vtg and nrf2 were the target genes of the study.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence (5´-3´)</th>
<th>Amplicon length (bp)</th>
<th>Response Site Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S rRNA</td>
<td>18s-f</td>
<td>TAGCACATCCAAAGAAGGCA</td>
<td>244</td>
<td>Liver</td>
<td>Geoghegan et al., 2008</td>
</tr>
<tr>
<td></td>
<td>18s-r</td>
<td>TCGATCCGAGATCACAATGA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Tubulin</td>
<td>β-Tub-f</td>
<td>ACCAGATCCGGCCAAAGT</td>
<td>129</td>
<td></td>
<td>Williams et al., 2009</td>
</tr>
<tr>
<td></td>
<td>β-Tub-r</td>
<td>ACACGGATCGCCATGGAC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Act</td>
<td>β-Act-f</td>
<td>CTGTTGTTTGCCAGAGAA</td>
<td>196</td>
<td>Liver</td>
<td>Geoghegan et al., 2008</td>
</tr>
<tr>
<td></td>
<td>β-Act-r</td>
<td>CTCTTGCTCTGGGCTTCATC</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Cyp1A</td>
<td>Cyp1a-f</td>
<td>ACGTGCGAGATGCAGAGGAG</td>
<td>196</td>
<td>Liver</td>
<td>Geoghegan et al., 2008</td>
</tr>
<tr>
<td></td>
<td>Cyp1a-r</td>
<td>TTGGGTGTTGCGAGAGAA</td>
<td></td>
<td></td>
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<tr>
<td>Metallothionein</td>
<td>Mt-f</td>
<td>CCCCCCTGCACCGACTG</td>
<td>137</td>
<td>Liver</td>
<td>Santos et al., 2010</td>
</tr>
<tr>
<td></td>
<td>Mt-r</td>
<td>TTGGCAACCTGGCCCATCTC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitellogenin</td>
<td>Vtg-f</td>
<td>GTGAGGACGCAAAGAGTAGG</td>
<td>100</td>
<td>Liver</td>
<td>Hogan et al., 2008</td>
</tr>
<tr>
<td></td>
<td>Vtg-r</td>
<td>TCCGGTGTAAGTCAAACCTTG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nrf2</td>
<td>nrf2-f</td>
<td>AAGAAGGAAGAGAGCACATC</td>
<td>184</td>
<td>Liver</td>
<td></td>
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<tr>
<td></td>
<td>nrf2-r</td>
<td>ATGGCATCATCGGCTTCCAG</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Estrogen receptor α</td>
<td>ER α-f</td>
<td>AGTGTCCGTCTCCTCGCGAC</td>
<td>103</td>
<td>Liver</td>
<td>Geoghegan et al., 2008</td>
</tr>
<tr>
<td></td>
<td>ER α-r</td>
<td>AAGAAGGAAGAGAGCACATC</td>
<td></td>
<td></td>
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<tr>
<td>Androgen receptor β</td>
<td>AR β-f</td>
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<tr>
<td></td>
<td>AR β-r</td>
<td>ACCCTGGCAATCCCTTCCAG</td>
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<tr>
<td>Spiggin</td>
<td>Spg-f</td>
<td>GCTGGCTCTTGAGTGTGCTG</td>
<td>95</td>
<td>Kidney</td>
<td>Hogan et al., 2008</td>
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<tr>
<td></td>
<td>Spg-r</td>
<td>TGACCATTTCCCACATC</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3.5.4 Optimization

In order to check the reliability of amplified targets and detect possible primer dimers formation, PCRs were conducted on pooled cDNA (5 females and 5 males and from both livers and kidneys) with a suitable temperature gradient. The products were run for 55 min at 75volt on an electrophoresis gels, 2% agarose (Sigma-Aldrich, Stockholm, Sweden). In each PCR reaction, 50ng of cDNA and 400nM of each
primer (forward/reverse) in a final volume of 25µl were used (illustra PuReTaq Ready-To-Go PCR Beads, GE Healthcare Life Sciences, England).

The method was optimized for the best primer concentration and annealing temperature to obtain a minimum cycle threshold (Ct) values by performing both a primer concentration gradient and a temperature gradient on a 96-well qPCR plate (FrameStar® 96, 4titude, UK). The three different concentrations were 300nM, 500nM, 700nM. The temperatures were 55-57-59°C or 58-60-62°C. See Table 5 and Table 6 for details.

To evaluate amplification efficiencies a standard curve of 6 points for each gene of interest was constructed using serial dilutions (25ng, 18.75 ng, 12.5ng, 9.375ng, 6.25ng, 3.125ng). Reaction efficiencies ranged from 85% to 110%. All standards and all experimental samples were assayed in duplicate.

The melting curve analysis showed a single sequence-specific peak thus confirming the identity of each PCR product, absence of primer dimers and other nonspecific products.

| Table 5 Primer sequences for candidate normalization genes. |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| **Gene symbol** | **Function**     | **Primer conc. (nM)** | **Amplification efficiency** | **Temperature °C** |
| 18S rRNA        | 18S ribosomal RNA | 700              | 100.7            | 60              |
| ACT-β           | beta-actin       | 700              | 96.9             | 60              |
| TUB-β           | beta-tubulin     | 500              | 98.8             | 60              |

| Table 6 Primer sequences for target genes. |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| **Gene symbol** | **Function**     | **Primer conc. (nM)** | **Amplification efficiency** | **Temperature °C** |
| CYP1A           | Phase I enzyme  | 700              | 100.7            | 60              |
| MT              | Heavy metal detoxification | 500         | 84.9             | 60              |
| VTG             | Egg yolk precursor protein | 700         | 110.4            | 60              |
| Nrf2            | Transcription factor regulating the stress response | 700          | 100.8            | 57              |
| ERα             | Estrogen receptor | 500           | 103.9            | 60              |
| ARβ             | Androgen receptor | 500          | 95.7             | 60              |
| Spiggin         | Glued glycoprotein | 500          | *                | 60              |

a: not tested

3.5.5 Quantification and data analysis of qPCR

Quantitative PCR was conducted by using Bio-Rad’s iQ™ SYBR® Green Supermix and iCycler™ Thermal Cycler, (Bio-Rad Laboratories Inc., Hercules, United States). cDNA corresponding to 25ng of RNA was used in each PCR reaction.

The cycling conditions were as follows: initial denaturation at 95°C for 1 min, followed by 50 cycles of 20s at 94°C (denaturing step), 20s at optimized temperature for the specific primer (annealing) and 30s at 72°C (extension).
Following the final cycle, melting curve analysis was performed to examine the specificity in each reaction. The temperature rose in 0.5°C increments with a 30s hold at each degree in the range from 65°C to 95°C (FIG. 9).

The qPCR plates were then saved in +4°C fridge.

All samples were loaded on a 96-well plate (FrameStar® 96, 4titude, UK) and each reaction contained in order: 4μl cDNA followed by 16μl master mix (10μl of the SYBR Green MasterMix, 2μl of forward and reverse primers, 2μl of RNA-free water) to a final volume of 20μl. The cDNA templates were diluted eight times (1:8 RNA dilution factor) before being added in the wells, final concentration was 6.25 ng/μl. Each sample was amplified in duplicate.

When samples for a single gene did not fit on one plate, interplate "calibrators" were used to compare separate plates.

In addition, each assay included negative control or no-template control (NTC) where RNase-free water was added to the reaction instead of the template (cDNA), acting as a reference of non-contaminated master mix and a no reverse transcriptase control (noRT) where RNase-free water was added to the cDNA synthesis reaction (previously described) instead of the enzyme to check cDNA transcription.

Furthermore, aliquots of each reaction mixture were analyzed by agarose gel electrophoresis to evaluate amplification of non-specific products.

Before the qPCR started, the loaded plates were closed with a Microseal® B Film (Bio-Rad Laboratories Inc., Hercules, United States) and spun with Hermle Z 360 K Refrigerated Centrifuge (American Laboratory Trading, Inc., United States) for one minute to eliminate air bubbles and to guarantee mixing in the bottom of the well.

Baselines and cycle threshold values were automatically calculated by the Bio-Rad iQ5 software for all tested samples in a given run. Dissociation curves were plotted for all primer pairs to ensure that single products were produced.

Moreover, the qPCR products were confirmed via gel electrophoresis after which the single band of MT and nrf2 were isolated, purified and sequenced to confirm that the correct gene was amplified (MWG-Biotech, Germany) and results were blasted against known sequences.

Relative mRNA expression of target genes was calculated by $E^{-\Delta CT}$ (Livak and Schmittgen, 2001). For all target genes the $E^{-\Delta CT}$ value (ECTreference − CTtarget) of each sample was divided by the mean $E^{-\Delta CT}$ value of the controls, i.
18S mRNA, β-actin and β-tubulin were used to normalize results of the target genes with the following formula:

\[
\text{Normalized expression (\(\Delta\Delta C_t\))}_{\text{sample (GOI)}} = \frac{\text{RQ}_{\text{sample (GOI)}}}{\text{RQ}_{\text{sample (18s)}} \times \text{RQ}_{\text{sample (β tub)}} \times \text{RQ}_{\text{sample (β act)}}}^{1}
\]

RQ= Relative quantity of a sample  
GOI= gene of interest (MT, cyp1A, vtg, nrf2)

Where relative quantity (Ct) of any sample was calculated as:

\[
\text{Relative quantity}_{\text{sample (GOI)}} = E^{Ct_{\text{(min)}} - Ct_{\text{(sample)}}}
\]

E= efficiency of primers. It was set at 100%, so that: 
\[E = (\%\text{Efficiency} * 0.01) + 1 = 2\].

Ct_{\text{(min)}}= Average Ct for the sample with the lowest average for the GOI.

Ct_{\text{(sample)}}= Average Ct for the sample.

Normalized values where then scaled down to the controls to show fold change in the graphs (Hellemans et al., 2009, Bustin et al., 2009).

3.6 Biochemical assays

Half of the frozen livers were used for homogenization and consecutive biochemical analysis. The samples were put in 200µl of ice-cold sodium-phosphate buffer (0.1M, pH 7.8) with 10% glycerol and homogenized using an ultrasound (Branson Ultrasonics, United States) for 7 seconds. Samples were always kept on ice. The homogenates were centrifuged at 10 000 g at 4°C, for 20 min. After that, approximately 180µl of supernatant was collected avoiding the pellet and the floating lipid layer, aliquoted and stored in a -80°C freezer until required for assays. These S9 fractions contain cytosol and microsomes. Hepatic biomarker includes EROD, CAT, GR, GPx and GST and were conducted according to different methods, adapted to microplates and optimized for stickleback tissue to fit the linearity of each assay.

As in the liver, all gills samples were homogenized and centrifuged to obtain the post-mitochondrial fraction and total protein concentration was determined.

The number of fishes measured in each assay was four or five, except in some treatment (as reported later). The two sexes were analyzed separately.

All spectrophotometric assays were carried out on the SpectraMax® 190 Absorbance Microplate Reader (Molecular Devices, LLC, United States) if not otherwise specified.

3.6.1 Liver EROD assay

A modified method from Forlin et al. 1994 was used to measure EROD activity
with rhodamine as standard. 50µl of microsomes were added to a fluorimeter cuvette and mix with 1.84ml of 0.1M Na/K buffer (pH 7.4) and 100µl ethoxyresorufin solution (the substrate of the reaction made up of 50µM 7-ethoxyresorufin in methanol, diluted in 4 volumes of Na/K buffer). The reaction started after addition of 10µl of cold NADPH 10mM.

The fluorescence was determined with the PTI (Photon Technology International) Photomultiplier Detection System (PhotoMed GmbH, Germany) at 585nm after excitation at 530nm. For each reaction, the software displays the amount of ethoxyresulfin (y-axis) used over time (on the x-axis). A time interval of the curve was selected at an appropriate linear fit and EROD activity was expressed as µmol resorufin formed per mg protein and minute according to the formula:

$$\text{EROD activity (µmol/mg protein*min)} = \frac{0.8 \times \text{Standard} \times \text{Time (min)} \times \text{Slope}}{\text{Sample (ml)} \times \text{Protein content (mg/ml)}}$$

0.8= correlation factor to µmol rhodamine in the standard sample;
Standard= mean value;
3= rhodamine values are three time higher than resorufin ones;
Time= minutes of observed reaction;
Sample= 0.05ml.

3.6.2 Gills EROD assay

The details of the gill EROD assay are described in Forlin et al. (1994) and the assay adapted from the liver assay. In short, gill filaments were placed in cuvette with a reaction buffer consisting of Na/K buffer (0.1M, pH 7.7) and 7-ethoxyresorufin. 10µl of NADPH 10mM was added to the content to initiate the reaction. The fluorescence of resorufin was determined with a PTI (Photon Tecnology International) Photomultiplier Detection System (PhotoMed GmbH, Germany) at 585nm after excitation at 530nm and EROD activity was expressed as µmol resorufin formed per mg of protein and minute. The same formula of EROD activity in liver was applied.

3.6.3 Catalase assay

Catalase activity was determined by the method of Aebi (1984) and modified to microplate reader by Sturve et al. (2005). Briefly, a mix consisted of potassium phosphate buffer (0.08M, pH 6.5) and H2O2 (0.08M) was used. Samples were first diluted 20-time with 0.08M KPO4 buffer and then 10µl were pipetted in triplicate in the UV light 96-wells microplate (Greiner Bio-One GmbH, Germany). The total volume of the reaction was 200µl. Change in absorbance was recorded at 240nm.

CAT activity was calculated in terms of mmol min⁻¹mg⁻¹protein, using bovine erythrocyte catalase as standard.
3.6.4 Glutathione peroxidase assay

Glutathione peroxidase activity was measured in liver microsomes according to the method described by Sturve et al. (2005). 4µl of diluted sample (1:10) or buffer reference was loaded in triplicate in a 96-wells microplate, where 176µl of the reaction mixture were later added. The latter is made of GR 40units/ml, GSH 70mM, K-phosphate buffer (0.1M, pH 7.7), milliQ, NADPH 2.4mM and NaN₃ 20mM. The reaction was started adding 20µl of H₂O₂ 8mM. Three blank wells were present in each run as well as a calibrator sample. Progressive increase in fluorescence was monitored at 340nm (excitation) and 490nm (emission).

\[
\text{GPx activity (µmol/mg protein*min) = } \frac{\text{Slope per min} \times 10^{-3} \times \text{Height} \times 1000}{\text{Ext.coeff.} \times \text{Sample ml} \times \text{Protein content mg} \times \text{Dilution}}
\]

Slope per min= median slope;
10⁻³= correlation factor from mOD to OD;
Height of the well= 0.59cm;
1000= mmol to µmol;
Extinction coefficient= 40 cm⁻¹ mM⁻¹;
Sample= 0.01ml;
Dilution= 20 times.

3.6.5 Glutathione reductase assay

Glutathione reductase activity was measured in liver S9 fraction as described by Cribb et al. (1989) and adapted to microplate reader by Stevenson et al. (2002).

A reaction buffer consisting of NADPH 0.6mM and DTNB (4mg/100ml Na₂HPO₄ (0.1M, pH 7.5)-EDTA (1mM)) was added to six wells of a microplate containing 20µl of diluted sample (1:10 in Na₂HPO₄ (0.1M, pH 7.5)). The reaction was initiated by adding GSSG 0.083mM to only three well, reference wells contained no GSSG.
Change in absorbance at 415 nm was monitored during 3 minutes. The results were expressed as nmol/mg of proteins * min according to the formula:

\[
\text{GR activity (nmol/mg protein*min)} = \frac{\text{Slope per min} \times 10^{-3} \times \text{Total volume (\mu l)} \times 1000000000}{\text{Height} \times \text{Ext. coeff.} \times \text{Sample \mu l} \times \text{Protein content (mg)} \times 1000 \times \text{Dilution}}
\]

Slope per min = mean sample slope – mean reference slope
10^{-3} = correlation factor from mOD to OD
Total volume = 200\mu l
1000000000 = mol to nmol
Height of the well = 0.59 cm
Extinction coefficient = 14150 cm^{-1} mM^{-1}
Sample = 20\mu l
1000 = protein contents from ml to \mu l
Dilution = 10 times

3.6.6 Glutathione-S-transferase assay

Glutathione-S-transferase activity was analyzed in liver cytosol according to Habig et al. (1974) and adjusted to a microplate reader by Stephenson et al. (2002). For each reaction, three wells contained a sample (1/10 diluted in Na_2HPO_4 (0.1 M, pH 7.5)) while three others contained 10\mu l of buffer, acting as a reference. 190\mu l of a mix solution of GSH (1mM solved in the buffer) and CDNB (2mM, dissolved in DMSO) was added to initiate the reaction. Change in absorbance was monitored at 350nm.

\[
\text{GST activity (\mu mol/mg protein*min)} = \frac{\text{Slope per min} \times 10^{-3} \times \text{Total volume (\mu l)} \times 1000}{\text{Height} \times \text{Ext. coeff.} \times \text{Sample \mu l} \times \text{Protein content (mg)} \times 1000 \times \text{Dilution}}
\]

Slope per min = mean sample slope – mean reference slope
10^{-3} = correlation factor from mOD to OD
Total volume = 200\mu l
1000 = mmol to \mu mol
Extinction coefficient = 9.6 cm^{-1} mM^{-1}
Height of the well = 0.59 cm
Sample = 10\mu l
1000 = protein contents from ml to \mu l
Dilution = 10 times

3.6.7 Protein determination assay

The protein measurement was determined according to Lowry et al. (1951) by adding microsome suspension (dilute 10 times in millQ), in triplicate for each sample, in the wells of a 96-well plate. A standard curve with five different concentrations points
(0.1, 0.3, 0.5, 0.7, 0.9 mg/ml) was made from bovine serum albumin (BSA) (Sigma-Aldrich Sweden AB, Stockholm, Sweden) (Fig.10). The fluorescence was determined at 750 nm.

**FIG.10** Total protein concentrations in a 96-wells microplate. BSA standard curve in the top row.

### 3.7 Statistical analyses

Statistical calculations were performed using different software. First, normal distribution and homoscedasticity of data were verified using Shapiro-Wilk test and Bartlett’s test, respectively. Outliers were highlighted with QQ-plot and Mahalanobis distance. These tests were computed with the “R v3.0.2 statistical programming language” (R Development Core Team, 2013), using the “HH”, “mvnormtest” and “mvoutlier” packages.

The software GenEx (BioMCC, Germany) was used for qPCR data analysis (normalizing gene expressions, intercalibration runs) prior to the statistical analyses in R. Basal gene expressions between sexes were analyzed using an unpaired t-test followed by Bonferroni correction. Consequently, male and female data were treated separately. In the concentration-response studies, target gene expressions were compared using one-way analysis of variance (ANOVA) and Tukey’s Multiple Comparison Tests. The carrier solvent treatment was set as the control.

Biomarker responses were also evaluated using one-way analysis of variance (ANOVA) followed by pairwise comparisons (Tukey's test) between treatment and control group.

To get a general picture of the trends and groupings in the exposure study, the data from all fish individuals were tested with Principal Component Analysis (PCA) (multivariate data analyses) using the software PRIMER 6 (PRIMER-E Ltd, UK). A PERMANOVA is carried out to assess differences among the groups with the R package “vegan 2.0-10”.

All data are reported as mean±standard error and P-values lower than 0.05 were considered significant.
4. Results

4.1 Genetic biomarker

In the kidney the extraction of mRNA was not consistent; thus spiggin and androgen receptor β mRNA expressions were not quantified. In addition mRNA expression of estrogen receptor α was not measured in the liver.

Gel electrophoresis of PCR products showed that all primers were specific (FIG. 11).

![Gel electrophoresis of PCR products in liver of stickleback. The length of the products is reported in different colors.](image)

**Fig. 11** Gel electrophoresis of PCR products in liver of stickleback. The length of the products is reported in different colors.

4.1.1 Sex differences in mRNA expression

Our results showed that mRNA expression of some genes and some of the biotransformation enzymes and antioxidants activity were gender dependent parameters, therefore males and females are presented separately.

A comparison of the basal expression of cyp1A, nrf2 and vtg in reproductively mature male and female sticklebacks revealed significant sex differences (Fig. 12). No differences were observed in MT levels ($t(8)=0.133, p=0.897$, unpaired t-test).

In the liver, vtg mRNA was 618 orders of magnitude higher in the females when compared to levels in the males ($t(8)=-3.7896, p<0.001$). Conversely, there was higher cyp1A expression (2.9-fold) in male stickleback liver ($t(8)=-2.30, p<0.05$). Basal nrf2 expression was significantly higher ($t(8)=2.345, p<0.01$) in control males compared to control females.
4.1.2 Effects of single compounds on mRNA expression

In females, a significant induction in MT was observed following exposure to 1 mg/l and 10 mg/l of Cd (f(2,12)= 4.96, p= 0.02, ANOVA) (FIG.13A).

Exposure to BaP did not induce cyp1A expression in the liver of females but a growing trend can be observed (f(2,12)= 2.76, p= 0.09, ANOVA) (FIG.13B).

Vtg expression in males was no significantly higher than expression in control(f(2,12)= 1.18, p= 0.33, ANOVA) (FIG.13C).

The results of the nrf2 analyses in female exposure are given in FIG.14, mRNA levels were substantially increased after exposure to all low doses of single compounds (f(6,40)= 6.54, p= 0.05, ANOVA). However, the high doses of Cd and BPA showed no up-regulation.
4.1.3 Effects of mixture on mRNA expression

In female, a significantly induced hepatic MT expression at both 10% and 100% was detected ($f(3,16)= 54.98, p < 0.001$, ANOVA) (Fig. 15A). A down-regulation at 1% and 10% can be observed in the hepatic cyp1A expression; no effect at the highest concentration ($f(3,16)= 8.46, p < 0.05$, ANOVA) (Fig. 15B). Vtg expression in males showed elevate levels at 10%, (26-fold) ($f(3,14)= 3.83, p < 0.01$, ANOVA) (Fig. 15C).

Fold change of nrf2 in the 10% and 100% mixture are statistically different from the control. No effect was observed at 1% ($f(3,16)= 14.25, p < 0.01$, ANOVA) (Fig. 15D).
4.2 Biochemical assays

4.2.1. Hepatic EROD activity in females

Exposure to Cd (1–10 mg/l) caused a dose-dependent induction of EROD activity ($f(2,12)= 8.23, p<0.001$, ANOVA) (Fig.16A). The same pattern is observed with BaP ($f(3,16)= 11.27, p<0.001$, ANOVA) (Fig.16B). BPA depressed the EROD activity but not statistically significant ($f(3,10)= 1.26, p=0.33$, ANOVA) (figure16.C).

After co-exposure with Cd, BaP and BAP; EROD activity was significantly higher both at 1% and 100% than in controls. Exposure to 10% of the mixture was not significantly influenced ($f(4,20)= 21.54, p<0.001$, ANOVA) (fig.16D).

![Fig. 16](image) EROD activity (mean± S.E.M.) in livers in female threespined sticklebacks after exposure for 5 days to different concentration of cadmium (A), benzo(a)pyrene (B), bisphenol A (C) or a combination of them at different % (D). Number of observations is stated within brackets. Different letters above bars indicate significant differences, $p<0.05$, (one-way ANOVA followed by Tukey’s Multiple Comparison Post-Test). All groups are statistically tested against each other.

4.2.2. Hepatic EROD activity in males

High doses of cadmium ($f(2,10)= 10.07, p<0.01$, ANOVA) and BaP ($f(3,9)= 16.18, p<0.001$, ANOVA) induced EROD activity but not the low doses (Fig. 7A,B).

Also, exposure to BPA did not affect EROD response ($f(3,16)= 1.79, p=0.89$, ANOVA) (Fig.17C).

On the other hand, the mixture shows a 6-fold induction at 100%. Suppression is seen at 10% ($f(4,18)= 16.02, p<0.001$, ANOVA) (Fig17D).
4.2.3 Gill EROD activity in females

Compared to controls, gill EROD activity was significantly higher in individuals exposed to Cd (f(2,12)= 8.90, p < 0.01, ANOVA) and BaP (f(3,16)= 18.57, p < 0.001, ANOVA), at both concentrations. Exposure to 10μg BaP/l caused a significant 73-fold induction of EROD activity (Fig.18A,B). BPA had no substances influence in this study (f(3,10)= 7.45, p < 0.01, ANOVA) (Fig.18C).

A significant trend that EROD activities increased with increasing % of mixture was observed (f(4,20)= 3.91, p < 0.01, ANOVA) (Fig.18D).
4.2.4. Gill EROD activity in males

Both Cd (f(2,10)= 26.95, p< 0.001, ANOVA) and BαP (f(3,9)= 739.8, p< 0.001, ANOVA) significantly induced EROD activity in the gills (Fig. 19A,B). The gill responded with a lower activity than the liver but with a higher induction rate, respectively, 44-fold to 5-fold after exposure to 10mg Cd/l and 7-fold to 27-fold after exposure to 10μg BαP/l. EROD activity is not induced in the BPA exposure (f(3,16)= 8.90, p< 0.01, ANOVA) (Fig.19C).

No induction was observed in gills after exposure to Cd, BαP and BPA in combination at 1% and 10% (f(2,12)= 1.73, p= 0.2, ANOVA) (Fig.19D).

![Fig. 19 EROD activity (mean+ S.E.M.) in gills in male three spined sticklebacks after exposure for 5 days to different concentration of cadmium (A), benzo(a)pyrene (B), bisphenol A (C) or a combination of them at different % (D). Number of observations is stated within brackets. Different letters above bars indicate significant differences, p<0.05, (one-way ANOVA followed by Tukey's Multiple Comparison Post-Test). All groups are statistically tested against each other.]

4.3 Enzyme activity and oxidative stress biomarker

The results of the detoxifying enzymes (GST) and antioxidant (CAT, GPx and GR) are reported with the respective p-values in table 6 for females and table 7 for male in the Appendix. GST activity showed no significance at both low dose and high dose in all the three single exposure experiments (p> 0.05). Unexpected and relatively high activity was observed at 10% mixture.

In the cadmium treatment, CAT, GPx and GR are induced in female liver in a concentration dependent manner. The same pattern is observed in males.

In the BαP exposure significant increase of CAT activity (p< 0.01) was noticed in female at low dose, but no significant alterations with respect to controls were shown.
at the highest concentration. In contrast, males showed no differences at all doses in for this enzyme.

GPx and GR had a similar trend at both concentrations of BaP, following significant induction in a dose-dependant relationship. Males displayed a significant increase ($p < 0.01$) for both these antioxidant at $1\mu g/l$ ($p< 0.05$).

BPA pattern are the same in both sexes. At both concentrations of BPA, no induction of CAT activity ($p> 0.05$) was observed whereas GPx and GR activity increase at both doses.

Effect of the mixture is different in the sexes. In females, maximal induction for all the parameters were observed at 10%, and the higher concentrations caused no further effect. Effects of the 1% are observable only in GST(Fig. 20). In male, no significant increase was found in CAT and GST. GPx is induced at all concentration whereas GR only at the highest (Fig. 21).

Fig. 20 Enzymatic activity (mean± S.E.M.) in liver in female three spined sticklebacks after exposure for 5 days to a mixture of different concentration of Cd, BaP and BPA. Number of observations is 5. Different letters above bars indicate significant differences, $p<0.05$, (one-way ANOVA followed by Tukey's Multiple Comparison Post-Test). All groups are statistically tested against each other.
Fig. 21 Enzymatic activity (mean± S.E.M.) in liver in male three spined sticklebacks after exposure for 5 days to a mixture of different concentration of Cd, BaP and BPA. Number of observations is 5. Different letters above bars indicate significant differences, p<0.05, (one-way ANOVA followed by Tukey's Multiple Comparison Post-Test). All groups are statistically tested against each other.
4.4 Multivariate analysis

The global response pattern in the data set was examined using a principal component analysis (PCA) based on a dataset that included all the oxidative stress parameters as variables.

The Fig. 22A shows that 74.1% of overall variance in female sticklebacks is explained by the first two principal components. The first principal component (PC1, 53.9% of variance) was mainly built by CAT and EROD gill that were found to be strongly correlated. The PC2 (20.1% of overall variance) was mostly formed by EROD liver and EROD gill.

The PCA showed that individuals exposed to the same treatment were grouped together, in particular for the exposure to Cd Hd, BaP Hg, Mix 1% and Mix 100% (Fig.22B). In the single exposure a concentration-dependent relationship was evident along the principal component 1 (PC1) axis. Result from the multivariate permutational analysis (PERMANOVA) was statistically significant ($f(10,42)=2.379, p=0.019$, PERMANOVA).

Fig. 22 Female (A) Scores plot from a principal component analysis (two components) of the individual oxidative stress biomarker profiles. ($n=49$) exposed to varying concentrations of Cd (1-10 mg/l), BaP (1-10 µg/l) and BPA (1-10µg/l) and a mixture of them (1%, 10%, 100%). The corresponding loading plot include the variables: EROD liver, EROD gills, CAT, GPx, GR, GST. (B) PC1 scores versus sample number plot from the principal components analysis. To highlight the concentration-dependent effect of compounds, sample numbers are presented in terms of their corresponding concentrations.
Another PCA was run on males to explore the overall relationships between the different variables. On the basis of the first three components, 75.8% of the total variance is explained. In the loading plot (Fig. 23A) it can be seen that several variables were correlated. For instance, GST variable was negatively correlated to the other enzymatic antioxidants, meaning that an individual with high GST had low GR and GPx. In addition, a positive correlation between EROD activities in gills and livers and CAT on PC2 is showed. The model presented a clear trend with increasing concentration only in the cadmium exposure. In these case concentration-related effects for BaP, BA and the mixture are less evident (Fig. 23B). Individuals in the same groups showed similar response patterns. Result from the multivariate permutational analysis (PERMANOVA) was statistically significant ($f(10,48)= 3.49$, $p= 0.003$, PERMANOVA).

![Fig. 23 Male (A) Scores plot from a principal component analysis (two components) of the individual oxidative stress biomarker profiles. ($n = 43$) exposed to varying concentrations of Cd (1-10 mg/l), BaP (1-10 $\mu$g/l) and BPA (1-100$\mu$g/l) and a mixture of them (1%, 10%, 100%). The corresponding loading plot include the variables: EROD liver, EROD gills, CAT, GPx, GR, GST. (B) PC1 scores versus sample number plot from the principal components analysis. To highlight the concentration-dependent effect of compounds, sample numbers are presented in terms of their corresponding concentrations.](image-url)
5. Discussion

Chemical compounds accidently or intentionally are released into the environment through different sources, emissions and spills from industries, agricultural and domestic wastes. Many anthropogenic activities are related or dependent on water resources; consequently all of these compounds and/or their transformation products can potentially wash into waterways, rivers, lakes, lagoons, and the ocean where their presence is a threat towards the aquatic ecosystem. Aquatic organisms are exposed to varying degrees of this contamination, thus making them suitable to understand the adverse environmental effects.

In several studies it has been reported the involvement of pollutants in the process of declining health of aquatic species, associated with both changes in gene expression and/or exerting their toxicity via their prooxidant nature (Livingstone, 2001).

Therefore it was of interest to investigate the potentially effects of a mixture of three-model compound found constantly in water samples along Swedish coast through a set of standard biomarkers and determine which responses were triggered most in the three spined stickleback, (*Gasterosteus aculeatus*).

5.1 Quantification of mRNA levels and enzymatic assays

The qPCR assay is an efficient method to quantify target mRNA, it is sensitive and required only small amount of initial sample. One limitation of the analysis is the assumption of linear production between mRNA synthesis, protein translation and functional proteins. Complementary measurement of protein levels and enzyme activities helped verifying the molecular results.

5.2 Cadmium exposure

Metals such as cadmium (Cd$^{2+}$) and copper (Cu$^{2+}$) are inducers of metallothioneins in different species of fish. Cadmium is a non-essential metal with no known biological function that enters into the aquatic ecosystem mainly through anthropogenic activities and is bioaccumulated and a threat to various trophic levels. For some aquatic organism a relationships between hepatic Cd accumulation and MT protein content have been observed (Kovarova et al. 2009), however in other species it has been reported that MT levels is not a reliable bioindicator of heavy metals pollution. Kovarova et al. (2009) observed no significant correlation between liver content and MT concentration in a field study and similar result are reported by
Sevcikova et al., (2013) where high metal contamination did not indicated induced metallothionein.

(Oronsaye, 1983) demonstrated that uptake of Cd is related to the exposure concentration and 100 mg/l was found to be lethal to sticklebacks. According to (Woodworth and Pascoe, 1983b) Cd accumulate in liver, gills and gut of sticklebacks in a dose-dependent matter following both waterborne exposure and intraperitoneal injection. In both cases, the highest concentration of the heavy metal was detected in the liver of the fish; gall bladder and kidney were secondary tissues of bioaccumulation. A significant bioaccumulation of copper was observed in liver of stickleback exposed to 200μg of Cu/l for 8 and 12 days and to 100 μg/l for 12 days by (Sanchez et al., 2005).

In stickleback, concentration between 2 mg/l and 6 mg/l has been reported to damage both kidneys and gills tissue structure after 30 days exposure (Oronsaye, 1989). In the kidney modifications of tubular cell form were observed with a consequently impairment of fluid and ion balance mechanisms, in particular disrupting the calcium metabolism and leading to hypocalcaemia and hyperglycemia. In gills, numbers of clorine cell increased and a partial detachment of epithelium layer, swelling on the lamella and vacuolation of the gill filaments was observed. The extent of tissue breakdown is related to concentration and time of exposure. Similar ultrastructural alterations are reported in the white-sea bass, Lates calcarifer, after 10 cadmium mg/l exposure (Thophon et al., 2004).

CdCl₂ is a model compound used in laboratory experiments to induce MT, and this process is clearly organ-specific). The induction of de-novo synthesis of MTs following cadmium exposure has been reported in several studies water exposure and intraperitoneal injection (Kovarova et al., 2009; Livingstone, 1993; Sevcikova et al., 2013; Sevcikova et al., 2011) both after.

In our exposure, an elevated expression of MT transcription in both treatments was observed is both sexes, however these doses are not environmentally relevant concentration. No differences in basal expression of MT genes was found between the sexes, thus suggesting that this response is not sex related, though no final conclusion can be drawn without obtained results of MT protein content of the liver (future analysis).

In rainbow trout (Oncorhynchus mykiss) hepatic and branchial MT mRNA levels were significantly altered after 28 days of exposure to 10 μg Cd/l (Lange et al., 2002).

(Tiwari et al., 2012b) quantified MT transcripts level in freshwater murrel, (Channa punctate) on different tissue, liver, kidney and gill after exposure to waterborne cadmium (3.74mg/l) for 8 h. In the liver a significant up regulation of the gene was observed at all the time points (1, 2, 4 & 8 h). Contradictory a 14 days exposure with the same fish and experimental setup, no heightened MT mRNA expression was seen in liver, MT gene expression decreased to the basal level (Tiwari et al., 2012a). As in our result, the liver seems to play a major role for metal uptake in
the initial defence mechanism. Further studies on MT response in stickleback with a time-course study will be necessary to confirm these hypotheses.

5.3 Benzo-α-pyrene exposure

BaP has been shown to be a strong AhR agonist and cyp1A up-regulator, EROD inducer and oxidative stress promoter in fish (van der Oost et al., 2003a).

In the gene expression study, contrary to previous results, BaP did not induce cyp1A at both concentration used in this study, only a small non-significant trend is observed. A variable to consider is the high inter-individual variation between the 5 fish at high dose of BaP. Nevertheless, our result could imply that mature female and male do not respond to this concentration of the chemical or it could take less time to reach the induction of the cyp1A gene.

Study using shorter exposure have showed cyp1A gene up regulation in sticklebacks, for example (Geoghegan et al., 2008) observed a statistically significant up-regulation of cyp1A in both male and female fish following exposure to 40μg/l of 1,2:5,6-dibenzanthracene (DbA) over a period of 48 h. DbA is a polycyclic aromatic hydrocarbon (PAH) known to act via the aryl-hydrocarbon receptor. Interestingly these significant responses happen at environmentally relevant concentrations of the chemical. Furthermore (Williams et al., 2009a) measured changes in the gene expression in male after four days, at concentrations of 10μg/l DbA. Accordingly, cyp1A mRNA was markedly up-regulated in gill, liver and brain of Gasterosteus aculeatus exposed for 24h to three concentrations of effluent from a treatment plant receiving wastewater (Beijer et al., 2013).

(Gao et al., 2011) exposed stickleback to two aryl hydrocarbon receptor (AhR) agonists, PCB 126 (32μg/l) and indigo (226ng/l). After 24h expression pattern of cyp1A gene in brain, gill, kidney and liver were analyzed. Their result showed a strong gene expression in all tissue and the highest induction was observed in the brain and kidney. Over a nine days exposure test the authors found a high and persistent induction of cyp1A in gills and liver with PCB 126 (3.2 μg/l) and a transient induction in both organs with indigo (226ng/l), supporting the idea that stickleback can use other way to cope with the pollutant.

5.4 Bisphenol A exposure

Several studies have shown the estrogen-compound BPA to be a potent ER-receptor ligand and vitellogenin (vtg) inducer. Vtg has been proposed as an early indicator of xenoestrogens in the environment due to its highly induced gene expression when exposed to various estrogen-like compounds (Huang et al., 2010). In the present study sticklebacks were exposed to BPA for 5 days, and no induction of vtg gene expression in males was observed in the single exposures. No record of experiments with BPA and stickleback were found in literature, so it is premature to exclude the possibility that the vtg transcripts peaked earlier in the exposure. Possibly, the levels of vtg protein in the fish was higher but was not possible to measure levels
of vtg in the plasma. Our result could also be explained speculating that the vtg primers used for the qPCR align with an isoform of vtg that need higher concentration to be induced. There are no reported different vtg splicing forms in stickleback; in rainbow trout (Oncorhynchus mykiss) three forms of Vtg (A, B and C) are present with different acute response, different concentration for activation and transcription time (Van der oost, 2001). In particular in an E2 exposure experiment, vtgA gene responds more rapid to high levels of chemical, but needs higher concentration for maintenance (Van der oost, 2001).

Other studies have shown that vtg production is not only affected by hormones (eg. E2 and EE2) but also by other estrogenic compounds such as pesticides, detergents, plasticizers. (Porte et al., 2006) To our knowledge, no study has investigated the effect of BPA on the biomarker responses in three-spined stickleback.

Geoghegan et al., (2008) reported statistically significant increases in vtg mRNA expression for both sexes treated with estradiol 40μg/l. And, as in our study vtg protein expression was higher in female controls than in males. When exposed to E2 for 7 days vtg induction in male stickleback liver was significant at 10ng/l (Hogan et al., 2008), whereas Katsiadaki et al., (2008) reported significant transcriptomic induction in male stickleback expose to environmentally relevant concentrations (0.1–100ng/l) of E2 for 4 days.

Li et al., (2013) demonstrated in Cynoglossus semilaevis that both BPA and E2 at different concentration administered through intraperitoneal injection induced changes in vtg gene expression, although the estrogenic effects of E2 were stronger.

5.5 EROD activity as a biomarker

The measured catalytic activity of the Cyp1A gene (EROD activity) is a common tool in biomonitoring, increased EROD activity is related to exposure with AhR-ligands, such as PAHs, PCBs and TCDDs, representing polluted areas.

In the present study EROD activity was analyzed in gill and liver after single exposure to two concentrations of cadmium, BαP, BPA and to combination of three concentrations of them.

In livers of females, only the highest tested concentrations of Cd (10mg/l) caused obvious EROD induction and a significant effect was also only found after exposure to 10 mg/ l of Cd in males. The induced gene expression indicates that Cd exposure give a physiological response at a molecular as well as biochemical level, making both assays suitable in biomonitoring studies. Gill EROD activity was also elevated in fish exposed to both concentration for females, moreover the highest activity in males was found at the high dose. Previous laboratory studies exposing Cd to fish has mixed responses (Bouraoui et al., 2008).

Even if cyp1A gene expression was not induced by BαP, the compounds gave rise to cytochrome P450 1A-dependent ethoxyresorufin-O-deethylase (EROD) activity in both sexes. Exposures resulted in a dose-dependent increase in gill and liver. Although in male at low doses, due to only one fish sampled the trend could not be
observed. The temporal variation between mRNA and the protein could suggest that the induction in the liver reflects the water concentration of inducers during a limited time period, after 5 day exposure there could be a saturation of the AhR ligand, and there is not up-regulation of the gene anymore. (Gao et al., 2011) found a delay between mRNA and protein synthesis using PCB 126 as the inducer compound.

Our findings are in accordance with a previous study of (Andersson et al., 2010) showed that exposure to BaP for 48 h at 2.5µg/l, 12µg/l and 63 µg/l induced EROD activity in the liver of stickleback.

Kirby et al., (2007) reported that, contrary to expectations, BaP failed to produce any elevation in EROD activity in flounder (Platichthys flesus) at 10 µg/l concentration waterborne exposure. No EROD response was induced both in the short and long period (21 days). 10 µg/l over a 10-day test of two other PAHs tested, dibenzanthracene and benzo[k]fluoranthene, exhibited significant effects on EROD over a shorter, 10-day, test. In the study of Gao et al., (2011) administration of both indigo and PCB 126 after 6h resulted in the strongest induction response of EROD, indicating that both chemicals rapidly penetrated in gills cell at an enough intracellular concentrations.

Beije and colleagues (2013) exposed gill filaments of stickleback to diluted effluent, EROD activity was strongly induced in a concentration-dependent manner after 24h. The observation that estrogens can affect hepatic EROD activity is documented in several fish species. The common described effect is suppression after exposure to estrogens such as E2 or nonylphenol (Kirby et al., 2007).

The observed results were fairly conflicting compared to those of many other studies, the tested concentrations of BPA did not significantly reduces hepatic EROD activity nor in females or males, however it can be noticed that EROD activity in the liver of females is apparently lower, but not statistically significant, unexpectedly only two fishes for treatment were measured. On the other hand EROD activity in the gill assay in males is significant increased at high dose.

No significant response in EROD activity was observed in stickleback following treatment with 17β-oestradiol E2 at 40µg/l over 48hr by Geoghegan et al., (2008).

The main conclusions from the quantification of EROD were that the liver showed a higher biotransformation capacity (significant higher basal activity in both sexes) than the gill, nevertheless that inductions of EROD activity were consistently higher in the gill than in liver, consequently it seems like gill EROD activity is a more sensitive biomarker than hepatic EROD activity. These results are in accordance with what Anderson et al., (2008) found exposing sticklebacks to humic substance, though they used a different gill filament assay.

5.6 Antioxidant biomarkers and nrf2 gene expression

In the present study, we also studied the enzymatic responses of a battery of antioxidant enzymes and the gene expression of a transcription factor that regulates their expression (nrf2)
Nrf2 gene expression was strongly activated in the single exposures of cadmium and benzo-α-pyrene, but the gene expression was not activated by BPA. This is the first study that has quantified the expression of nrf2 through qPCR in three-spined stickleback. It was noted that females generally had a stronger response towards oxidative stress than males. Therefore studies should always consider gender and make sure that both sexes are present in sufficient numbers or focus on only one sex. The molecular results are in accordance with most of the enzymatic assays. Notably, CAT, GPX and GR are significantly elevated in the high dose of cadmium confirming the heavy metal as an inducer of oxidative stress. GR seemed to be the most responsive among the enzymatic endpoints. GST activity was not induced by any of the single compounds studied regardless of the sex.

(Sanchez et al., 2007) used a similar multi-biomarker approach with three-spined sticklebacks to distinguish between streams with different levels of pollution in France. The biomarkers used were EROD, glutathione-S-transferase (GST), total glutathione content (GSH) and lipid peroxidation. It appears from the multivariate analysis carried out on all the responses that the highly contaminated areas were distinguished from the references ones. The same authors used a multi-biomarker approach to analyses also the effects of the herbicide Diquat and nonylphenol polyethoxylate adjuvant (Agral90). When the chemicals were tested alone Diquat inhibited basal hepatic EROD activity and in combination, more important oxidative effects were shown (Sanchez et al., 2006).

Pereira et al., 2010 quantified a set of biochemical biomarkers (acetylcholinesterase, 7-ethoxyresorufin-O-deethylase, carboxylesterase, catalase, glutathione peroxidase and glutathione S-transferase) in Nile tilapia (Oreochromis niloticus) exposed to BaP and the organophosphate pesticide diazinon (DZ), at a concentration of 0.5 mg/l. The exposure was 7 days long. BaP caused the induction of phase I and II enzymes, and DZ caused carboxylesterase inhibition in gills but not in liver. No significant modulation was observed in antioxidant enzymes. When in combination with BaP, DZ caused a significant decrease of EROD and GST induction.

5.7 Mixture

In the aquatic ecosystem, chemicals are rather present in mixtures than alone and their impacts on organism can be additive or synergistic, or effects can be masked by competition between antagonistic chemicals. Latest research has revealed that mixtures of chemicals can act in an additive manner and elicit responses at ineffective concentrations when present individually (Altenburger, 2004; Altenburger et al., 2012; Celander, 2011). To date, rather few studies have focused on effects caused by mixtures of different classes of contaminant with different properties.

Our gene expression study in the fish exposed to the mixture, showed elevated MT expression in a dose-dependent manner, and statistically significant change were observed at 10% and 100%. MT was strongly activated in the single exposure with Cd, but the same level of stimulation in the gene expression was found in the mixture.
Costa et al., (2007) analyses the effect of co-exposure of cadmium (Cd) and BαP on *Solea senegalensis* through the 2-D gels of cytosolic proteins in the liver. They found variation in the expression of different protein especially the ones linked to apoptosis and tissue regeneration. Single exposure activated different antioxidative stress response, BαP gave rise to up-regulation of GPx, Cd exposure led to increase in peroxiredoxins, whereas the interaction of the chemicals induced an antagonistic response and inhibited apoptosis. The paper showed that the mixture is acting on “impaired specific responses rather than to a combined effect of BαP and Cd” (Costa et al., 2007).

Hepatic levels of cyp1A were not significantly different from controls at the highest concentration of the mixture; however a significant down-regulation of the gene was present at 1% and 10%. Even in this case, the gene expression at 100% of the mixture has a fold change in the same range of the single exposure.

A study from Geoghegan et al., (2008) showed that following exposure to binary treatment of DbA (40μg/l) and E2 (50ng/l) a statistically significant up-regulation of cyp1A in both male and female sticklebacks was observed, accordingly with elevation of EROD activity. In addition, the response of the mixture revealed significantly greater levels of cyp1A gene than the one recorded in the single compounds exposure in both sexes.

Up-regulation of vtg mRNA expression was detected at both 10% and 100%, notably a 27- fold and 9-fold induction, respectively. Interestingly the high up-regulation of vtg coincides with the low expression of cyp1A; speculation can be made regarding a cross-talk between the two receptors (AhR and ER). Carrying out qPCR over ERα could help interpreting the results.

Significant up-regulation of vtg was also observed in male stickleback exposed to a combination of DbA (40μg/l) and E2 (50ng/l) and, as in our results, the interaction of DbA/E2 revealed to be significantly greater than the single treatments, though the impact seemed to be no more than additive (Geoghegan et al., 2008).

Örn, and co-workers (2006) exposed zebrafish (Danio rerio) to a binary mixtures of the estrogen 17-ethynylestradiol (EE2) and the androgen 17-trenbolone (Tb) observing altered vitellogenin concentrations, both reductions and increases compared to exposure of the chemicals alone.

Female’s nrf2 mRNA levels in the mixture tended to increase in a dose-dependent matter and are significantly elevated at all three concentrations, meaning the antioxidant response pathway has been activated and the expression of several antioxidant enzymes increased. As a consequence alterations in antioxidant and detoxifying enzymes were found. The activities in the single exposures of all three chemicals are comparable to the one measured at 10% and 100% of the mixture, no additive or synergistic interaction is shown.

EROD activity in liver of fish treated simultaneously with the three compound showed a dose- response pattern. In both sexes at 10% there is no induction. In gills, this study reports an antagonistic influence of BPA on BαP, the activity values, at all
three mixture concentrations are lower from the value obtain when compounds are administered alone. The results have been observed before in literature. Mdege et al. (2006) exposed african sharpooth catfish (Clarias gariepinus) for 24h to different concentration of BaP, 17 alpha-ethynylestradiol (EE2) alone or to a combination of both compounds and measured cyp1). The biomarker showed dose dependent effects in the single exposure. Significant EROD activities both in gill filaments and liver were observed at all doses after exposure to BaP alone or co-administration with EE2. As in our result EROD induction was inhibited by co-administration of the mixture in comparison to the single treatment of BaP. The authors concluded that chemical mixtures may affect biomarker responses differently when no interaction among the pollutants is present, that the sensitivity of cyp1A is different in liver and gills. It appears from the study that EROD activity, especially in the gill respond to very low concentrations of tested inducers.

In the current study, we also measured a battery of oxidative stress parameters, but no significant differences in the highest dose of the mixture were found, except for GPx in both sexes at the highest doses. There could be an underestimation of the activity due to the possibility that the chemical we added to measure the spectrophotometric reaction bound just to the available site of the enzymes and not to those occupied by the toxic chemicals, which resulted in lower measured enzyme activity. On the other hand, there could be a biological inhabitation at that concentration due to overloading or competition of the enzymes or the chemicals could have bound together in the water tank due to the high concentration, therefore becoming ineffectively bioavailable to the fish. In order to determine the actual concentration of the test compounds, chemical analysis of the water sample are needed. In females the highest responses for all the biochemical biomarkers is observes at 10%, even GST that is not elevated in single exposure is significant different.

The impact of the interaction of the three chemicals is highlighted in the PCA, showing that interactive chemicals act on different pathways, and the overall results are not easy to understand.

Lever et al., (2010) used transcriptomic analysis of liver of flounder (Platichthys flesus) to outlined different response in hepatocytes treated with benzo(a)pyrene (BAP), estradiol (E2), copper or a mixture of them. BAP and E2 promoted expected changes in the cDNA microarray analyses, whereas the expression of biomarkers changed in the mixture exposure, in particular, contrary to expectation, the expressions of common mRNAs used as environmental exposure biomarkers of were not changed. The results also indicate different pattern of gene expression in the mixture compared to administration of single compounds.

5.8 Conclusion

One of the major aims of this thesis was to further characterize the response of the hepatic biomarkers, cyp1A, metallothionien, nrf2 and vitellogenin on a gene
expression level. In addition, liver and gill EROD activities and enzymatic activities in the antioxidant battery were analyzed. These studies were performed in a mixture of three compounds in order to understand interaction between these three model classes of compounds. The results suggest that no mechanism for synergy of toxicity is present in our mixture, and an antagonist effect is present in gill EROD activity.

However in my studies, differences in reproductive and endocrine status of the wild fish could be responsible for some observed variations, such as modified gene expressions. Therefore, to get clearer results it is important to exclude season variability and new laboratory studies with a more synchronized reproductive status and/or with reproductively quiescent fish should be carried out.

In addition complementary studies on hepatocytes should be performed to further understand the mechanics of the responses of these biomarkers and find a correlation between in vitro and in vivo exposure.

Ultimately, the use of the multi-biomarker approach described in this thesis could certainly improve our knowledge of interaction in mixture.
6. Acknowledgement

Many people have contributed to the existence of this thesis, I particularly want to thank:

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“Container” friends thanks for making life so much more fun.
Family, thank you for being there when needed!

And lastly, my utmost gratitude goes to the sticklebacks: “I will leave you in peace now….”
7. References


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http://www.who.int.org
8. Appendix

Table 6 Enzymatic activity (mean±S.E.M) in liver in female three-spined stickleback (Gasterosteus aculeatus) exposed for five days to different concentration of cadmium (Cd), benzo-α-pyrene (BαP), bisphenol A (BAP) or to a combination of them at different %.

<table>
<thead>
<tr>
<th>Nominal concentration</th>
<th>Control</th>
<th>Control acetone</th>
<th>Cd (mg/l)</th>
<th>BαP (μg/l)</th>
<th>BPA (μg/l)</th>
<th>Mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAT activity&lt;sup&gt;1&lt;/sup&gt;</td>
<td>46±126&lt;sup&gt;a&lt;/sup&gt;</td>
<td>57±126&lt;sup&gt;a&lt;/sup&gt;</td>
<td>646±130&lt;sup&gt;b&lt;/sup&gt;</td>
<td>960±131&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1027±135&lt;sup&gt;b&lt;/sup&gt;</td>
<td>557±99&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>GST activity&lt;sup&gt;2&lt;/sup&gt;</td>
<td>199±29&lt;sup&gt;a&lt;/sup&gt;</td>
<td>210±22&lt;sup&gt;a&lt;/sup&gt;</td>
<td>171±60&lt;sup&gt;a&lt;/sup&gt;</td>
<td>211±49&lt;sup&gt;a&lt;/sup&gt;</td>
<td>251±43&lt;sup&gt;a&lt;/sup&gt;</td>
<td>166±22&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>GR activity&lt;sup&gt;3&lt;/sup&gt;</td>
<td>5.19±0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.49±1.35&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13.44±2.01</td>
<td>20.21±5.56</td>
<td>10.14±1.30</td>
<td>6.28±0.50</td>
</tr>
<tr>
<td>GPx activity&lt;sup&gt;4&lt;/sup&gt;</td>
<td>2.1±0.38&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.33±0.36&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.27±3.77&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.55±3.77&lt;sup&gt;a&lt;/sup&gt;</td>
<td>19.29±3.74&lt;sup&gt;a&lt;/sup&gt;</td>
<td>19.44±4.07&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Number of observations varied between 4 and 5.

<sup>1</sup> mmol/mg protein*min  
<sup>2</sup> mmol/mg protein*min  
<sup>3</sup> nmol/mg protein*min  
<sup>4</sup> mmol/mg protein*min  

Letters = significantly different from each other's, p<0.01, one-way ANOVA followed by Tuckey's Multiple Comparison Post-Test.

Table 7 Enzymatic activity (mean±S.E.M) in liver in male three-spined stickleback (Gasterosteus aculeatus) exposed for 5 days to different concentration of cadmium (Cd), benzo-α-pyrene (BαP), bisphenol A (BAP) or to a combination of them at different %.

<table>
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<th>Cd (mg/l)</th>
<th>BαP (μg/l)</th>
<th>BPA (μg/l)</th>
<th>Mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAT activity&lt;sup&gt;1&lt;/sup&gt;</td>
<td>461±28&lt;sup&gt;a&lt;/sup&gt;</td>
<td>480±39&lt;sup&gt;a&lt;/sup&gt;</td>
<td>662±177&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1236±52&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>376±35&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>GST activity&lt;sup&gt;2&lt;/sup&gt;</td>
<td>0.2±0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.2±0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.23±0.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.12±0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.11±n/a&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.16±0.01&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>GR activity&lt;sup&gt;3&lt;/sup&gt;</td>
<td>9.3±0.66&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.35±1.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12.43±2.12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12.23±4.95&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.52±n/a&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.31±0.30&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>GPx activity&lt;sup&gt;4&lt;/sup&gt;</td>
<td>2.14±0.18&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.83±0.37&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.79±2.16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>19.18±2.33&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.79±n/a&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.29±0.31&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Number of observations varied between 4 and 5.

<sup>1</sup> mmol/mg protein*min  
<sup>2</sup> mmol/mg protein*min  
<sup>3</sup> nmol/mg protein*min  
<sup>4</sup> mmol/mg protein*min  

Letters = significantly different from each other's, p<0.01, one-way ANOVA followed by Tuckey's Multiple Comparison Post-Test.