Application and interpretation of biomarkers in ecotoxicology – from molecular to individual level responses

Sara Furuhagen
Application and interpretation of biomarkers in ecotoxicology

From molecular to individual level responses

Sara Furuhagen
Abstract

The use of biomarkers is considered a promising alternative, or complement, to traditional ecotoxicological assays. Toxic effects are often initially manifested at the molecular or biochemical level, biomarkers are therefore used as sensitive indicators of toxic exposure. Ideally, biomarkers would also indicate reduced fitness and possible later effects at the individual or population levels. However, implementing biomarkers in ecotoxicology is challenging and few biomarkers have an established connection to reduced individual fitness. The aim of this thesis was to increase the value and improve the interpretation of biomarker responses in ecotoxicological studies by examining the impact of confounding factors and the relationship between oxidative biomarkers and reproductive effects in crustaceans.

The sensitivity of biomarkers was confirmed in paper I as toxic effects of pharmaceuticals with conserved drug target orthologs were observed at the molecular and biochemical levels both earlier and at lower concentrations than effects on mortality and reproduction. No toxic effects were observed for the pharmaceutical without identified drug target orthologs, thus stressing the importance of considering toxic mechanisms and being aware of the most likely target when evaluating toxic effects also in non-target species. Many xenobiotics and environmental stressors interfere with oxidative processes, making oxidative biomarkers interesting to study in ecotoxicology and stress ecology. Still, feeding rate was identified as a confounding factor for antioxidant capacity (assayed as oxygen radical absorbance capacity, ORAC) and lipid peroxidation in ecotoxicological studies (paper II). However, ORAC normalized to protein was independent of altered feeding rates, hence it can be applied as a suitable exposure biomarker without considering alterations and effects of feeding rate. The connection between reproduction and oxidative stress is dual, as reproduction both can be inhibited by oxidative stress and induce pro-oxidative processes. Further, a positive association was found between ORAC and the occurrence of embryo aberrations in the benthic amphipod Monoporeia affinis (paper III). An association between antioxidant defense and reproduction was also observed for Daphnia magna (paper IV). Threshold values for identification of exposed individuals and prediction of possible later reproductive effects were established for ORAC.
This thesis has contributed to diminishing some of the knowledge gaps limiting the use of oxidative biomarkers in ecotoxicology, by contributing to increased understanding of how oxidative biomarkers relate to important life-traits. Moreover, ORAC has been identified as a suitable biomarker of not only exposure, but also reproductive effects. Future research should continue to establish connections between biomarker responses and effects at higher levels, and focus on providing defined threshold values to enable predictions about later effects.
Biomarkörer anses vara lovande alternativ och komplement till traditionella ekotoxikologiska tester. Toxiska effekter tar sig vanligtvis först uttryck genom molekylära och biokemiska förändringar och biomarkörer används därför som känsliga indikatorer av toxisk exponering. Biomarkörer som indikerar effekter på individers hälsa, tillväxt och reproduktion är av stort värde i riskbedömningar av kemikalier. Tyvärr är det få biomarkörer i ekotoxikologin som har kopplats till effekter på individnivå. Tolkningen av effekter på biomarkörer försvåras av att molekylära och biokemiska processer påverkas av många andra faktorer än bara kemikalieexponering. Målsättningen med denna avhandling var att öka värdet och förbättra tolkningen av biomarkörer i ekotoxikologiska studier genom att studera yttre påverkande faktorer och kopplingen mellan oxidativa biomarkörer och effekter på reproduktion i kräftdjur.

I paper I bekräftades biomarkörers känslighet, då effekter på molekylära- och biokemiskanivåer observerades tidigare och vid lägre koncentrationer i *Daphnia magna* efter exponering av läkemedel. De två läkemedel, vars målmolekyl i människan även fanns i testorganismen, var mer toxiska än det läkemedel vars målmolekyl inte fanns bevarad i testorganismen. Resultaten från denna studie tydliggjorde hur viktigt det är att förstå och vara medveten om vilka toxiska mekanismer som är mest troliga att orsaka oönskade effekter i organismer, när man använder biomarkörer i ekotoxikologiska studier. Många kemikalier påverkar oxidativa processer i organismer och biomarkörer kopplade till oxidativ stress har därför fått stor uppmärksamhet i ekotoxikologi och stress-ekologi. I paper II identifierades dock födointag som en yttre påverkande faktor för oxidativa biomarkörer i ekotoxikologiska studier, då både antioxidativ kapacitet (måttes som oxygen radical absorbance capacity, ORAC) och lipidperoxidering påverkades av födointag. Däremot var ORAC, normaliserat till proteinmängd, opåverkat av födointag och kan således anses vara en lämplig biomarkör för exponering. Kopplingen mellan reproduktion och oxidativ stress är tadelad då reproduktion dels kan minska som ett resultat av oxidativ stress och även vara en orsak till störningar i den oxidativa balansen. Vi visade att förekomsten av embryoskador i amphipoden *Monoporeia affinis* var kopplat till ökad ORAC (paper III). Vi observerade även en koppling mellan antioxidativ kapacitet och reproduktion i *D. magna* och tog fram gränsvärden för ORAC, vilka kan användas för identifiering av
exponerade individer och för att förutse möjlig minskad reproduktion (paper IV).

Den här avhandlingen har ökat förståelsen om oxidativa biomarkörer i ekotoxikologi genom att bidra med ny kunskap om hur oxidativa biomarkörer är kopplade till reproduktion. Vidare identifierades ORAC som en lämplig biomarkör för att påvisa både exponering och effekter på individnivå. Framtida forskning bör fortsätta att fokusera på att beskriva kopplingar mellan biomarkörer och effekter på individnivå då detta är en viktig faktor som begränsar användandet av biomarkörer i ekotoxikologi.
List of papers

**Paper I**
Furuhagen S., Fuchs A., Lundström Belleza E., Breitholtz M., Gorokhova E.
Are pharmaceuticals with evolutionary conserved molecular targets more potent to cause toxic effects in non-target organisms?
*PLOS ONE 2014*, 9 (8), DOI 10.1371/journal.pone.0105028

**Paper II**
Furuhagen S., Liewenborg B., Breitholtz M., Gorokhova E.
Feeding activity and xenobiotics modulate oxidative status in *Daphnia magna*: Implications for ecotoxicological testing.

**Paper III**
Reutgard M., Furuhagen S.
Linking sub-cellular biomarkers to embryo aberration in the benthic amphipod *Monoporeia affinis*.
Manuscript to be submitted

**Paper IV**
Furuhagen S., Liewenborg B., Breitholtz M., Gorokhova E.
Oxidative biomarkers as indicators of reproductive effects.
Manuscript

All published papers were reproduced with permission from the publishers.
Paper II was reproduced with permission from Environmental Science and Technology 48 (21), 12886-12892
Copyright 2014 American Chemical Society
Statement

I have made following contributions to the papers included in this thesis:

**Paper I**
I participated in the acute and reproduction tests, as well as in the qPCR analysis. I conducted the exposure for biomarker analyses and carried out the RNA and DNA measurements. I conducted the RNA statistics and took the lead role in writing the paper.

**Paper II**
I planned and performed the study. I did all laboratory work and conducted most of the data analyses. I took the lead role in writing the paper.

**Paper III**
I performed the analyses of protein, ORAC and TBARS. I participated in the data interpretation and had a chaired responsibility for writing of the manuscript.

**Paper IV**
I planned and performed the study and did all biochemical analyses, except for CAT activity. I conducted most of the statistics and took the lead role in writing the manuscript.
Abbreviations

AChE  Acetylcholinesterase
AOP  Adverse outcome pathway
ATP  Adenosine triphosphate
CAT  Catalase
DNA  Deoxyribonucleic acid
ERA  Environmental risk assessment
ETC  Electron transport chain
FRAP  Ferric iron reducing antioxidant parameter
GPX  Glutathione peroxidase
GSH  Glutathione
GSSG  Glutathione disulfide
HPLC  High performance liquid chromatography
hsp  Heat shock proteins
MDA  Malondialdehyde
OECD  Organization for economic co-operation and development
ORAC  Oxygen radical absorbance capacity
qPCR  Quantitative real-time polymerase chain reaction
RNA  Ribonucleic acid
ROS  Reactive oxygen species
rRNA  ribosomal RNA
SOD  Superoxide dismutase
TOSC  Total oxygen scavenger capacity
TBA  Thiobarbituric acid
TBARS  Thiobarbituric acid reactive substances
USEPA  US Environmental protection agency
UV  Ultraviolet
Contents

1 Introduction ........................................................................................................... 1
2 Aim ......................................................................................................................... 3
3 Background ............................................................................................................ 4
  3.1 Ecotoxicological assays ....................................................................................... 4
  3.2 Biomarkers ......................................................................................................... 4
  3.3 Oxidative stress .................................................................................................. 6
    3.3.1 Antioxidant defense ...................................................................................... 7
    3.3.2 Lipid peroxidation ....................................................................................... 7
    3.3.3 Oxidative stress, health and longevity ....................................................... 8
    3.3.4 Oxidative stress induced by xenobiotics ................................................... 8
4 Material and Methods ........................................................................................... 10
  4.1 Test organisms .................................................................................................. 10
    4.1.1 Daphnia magna ......................................................................................... 11
    4.1.2 Monoporeia affinis .................................................................................... 11
  4.2 Molecular and biochemical assays ..................................................................... 11
    4.2.1 Gene expression analysis ............................................................................ 11
    4.2.2 RNA content ............................................................................................ 12
    4.2.3 Antioxidant assays .................................................................................... 12
    4.2.4 Lipid peroxidation ..................................................................................... 12
    4.2.5 Acetylcholinesterase ................................................................................ 13
5 Results and Discussion ......................................................................................... 14
  5.1 Targets of xenobiotics, toxic mechanisms and biomarker response ............... 14
  5.2 Oxidative biomarkers and effects on reproduction ........................................... 15
  5.3 ORAC and TBARS – suitable biomarkers of oxidative stress? ......................... 16
  5.4 Confounding factors for oxidative biomarkers ................................................ 17
6 Conclusions ............................................................................................................ 19
7 Future perspectives ............................................................................................... 20
8 Acknowledgements ............................................................................................... 22
9 References ............................................................................................................. 24
1 Introduction

Many researchers have set their hope and trust in the use of biomarkers as a promising alternative, or complement, to time consuming and work intensive traditional ecotoxicological assays. Toxic effects at the molecular and biochemical levels became more frequently studied in aquatic organisms about 30 years ago. Since then, enhanced knowledge about physiological processes and development of analytical methods has led to increased implementation of biomarkers in ecotoxicology. The biological system has different organizational levels (Figure 1) that are connected to each other by numerous physiological processes. Effects at one level may, therefore, have consequences higher or lower in the biological organization. The rationale for using biomarkers is based on the notion that toxic effects are manifested first at the molecular and biochemical levels as these often are most sensitive and reactive. These initial changes may translate into effects at the individual, population or even ecosystem levels. The physiology of organisms is amazing; with the aid of repair and adaptation mechanisms they can – at least to a certain extent - cope with increasing stress and minimizing the impact on individual fitness. Biomarkers are therefore not always indicative of adverse effects at higher organizational levels, even if they indicate events of environmental stress.

Figure 1 Illustration of the structure of the biological organization levels.

The number of chemicals in the society is constantly growing, and it would be an endless task to test all these substances using traditional ecotoxicological assays, which largely rely on mortality or reproduction as endpoints. Even though it would be an almost equally difficult task to test effects of all existing
chemicals using biomarkers, this could be a more efficient approach to identify the most potent chemical substances (xenobiotics), as it reduces the costs and workload of the testing procedure. Moreover, biomarkers are more likely to detect effects at lower and more environmentally relevant concentrations. Several chemical substances interfere with oxidative processes in organisms and cause an imbalance in the oxidative status that may result in oxidative stress, a potentially harmful condition for organismal fitness. Biomarkers of oxidative stress are therefore common in ecotoxicological studies to demonstrate exposure. While biomarkers, in theory, can facilitate environmental risk assessments (ERA), there are still considerable knowledge gaps surrounding confounding factors and connections between biomarker response and effects at higher biological levels. These gaps limit the full potential of using biomarkers in ecotoxicological studies and ERA. The purpose of the current thesis has been to fill some of these gaps by studying confounding factors of biomarkers as well as the relationships between biomarker response and individual- and population level effects, with a special focus on biomarkers associated with oxidative stress.
2 Aim

The overall aim of this thesis was to increase the value and interpretation of biomarker response in ecotoxicological studies. This was done by establishing relationships between biomarkers and effects at the individual level, and by studying the effect of confounding factors on biomarker responses in ecotoxicological studies. I have had a special focus on biomarkers of oxidative stress in this thesis.

- The first objective was to study the influence of conserved drug targets on the toxicity of pharmaceuticals in non-target organisms using a battery of individual endpoints and biomarkers (paper I). It was hypothesized that pharmaceuticals for which drug target orthologs have been identified in non-target organisms would be more toxic than pharmaceuticals without identified drug target orthologs.

- The second objective was to study the impact of feeding activity as a confounding factor for oxidative biomarkers in ecotoxicological assays (paper II). It was hypothesized that feeding rate would affect oxidative biomarkers and that this relationship would be altered by xenobiotic exposure.

- The third objective was to study the connection between oxidative biomarkers and reproductive effects in crustaceans (papers III-VI). It was hypothesized that reproductive effects would be reflected in oxidative biomarker responses and that changes in oxidative biomarkers could be used as predictors of reproductive effects.

Addressing these objectives would allow oxidative biomarkers to be applied as both exposure and effect biomarkers, thus allowing these biomarkers to be applied in a wider context.
3 Background

3.1 Ecotoxicological assays

Traditionally, ecotoxicological assays have been focused on effects of xenobiotics at the individual level and assaying endpoints like mortality, reproduction and individual growth. Many of the ecotoxicological assays standardized by organizations like OECD and USEPA focus on individual level effects. An optimal ecotoxicological assay should fulfill the criteria of reliability, cost-effectivity, sensitivity and relevance [3]. It is difficult to design an assay that fulfills all these criteria as one often comes at a cost of another. Even though most standardized ecotoxicological assays meet some of these criteria, they are often very time-consuming and labor intensive, making them relatively expensive. Additionally, they seldom provide information regarding toxic mechanisms. However, assays at this level are often reproducible in terms of obtaining similar results when repeating the assay, thus making the results comparable between laboratories and they can easier be implemented in regulatory hazard- and risk assessments. Even though endpoints like mortality and reproduction are ecologically relevant, they rarely provide information about effects at environmentally relevant concentrations as these endpoints often are assessed after acute, or semi-acute, exposure, using relatively high concentrations of xenobiotics. Two of the questions addressed in this thesis were, therefore, whether biomarkers can

- be used to identify the most toxic xenobiotic earlier and at lower concentrations than individual endpoints (paper I) and
- predict later individual and population effects (papers III-IV).

3.2 Biomarkers

There are several definitions of a biomarker available in the literature [4-7]. In this thesis, I use a modified definition from Peakall [8]:

*a biomarker is a low-level biological response to environmental stress that gives a measurement of exposure and sometimes adverse physiological effect*
This definition is in agreement with what is generally referred to as a biomarker in ecotoxicology.

Many ecotoxicological biomarkers originate from biomedical sciences and are developed and validated in humans and mammalian model species before making their way to the field of ecotoxicology. Our knowledge about these biomarkers is, therefore, largely based on studies on animals that are not necessarily closely related to the organisms that are of interest in ecotoxicology. However, many of the biomarkers are measuring effects on conserved mechanisms and can thus be used in ecotoxicology as indicators of the same or similar processes as in mammals. The conservation of toxic mechanisms and the sensitivity of biomarker responses were studied in paper I. The molecular, biochemical and cellular systems are often the most sensitive and there is a fast response at these levels to changes in the surrounding environment. These lower level processes are responsible for detoxification, adaptation, reparation and defending the integrity of the cell and, ultimately, organismal fitness in response to stress. By assaying biomarkers connected to these processes, environmental stress can be diagnosed earlier and at lower stress levels.

Depending on how they are used, biomarkers can be divided into different classes. Biomarkers of susceptibility indicate an organism’s ability to respond to a specific xenobiotic. Exposure biomarkers are indicative of an event of exposure to a certain stressor, whereas effect biomarkers are associated with effects on the fitness and health of the organism. This thesis focuses on the two latter classes. Most biomarkers used in ecotoxicology would be classified as exposure biomarker as only a few of them have well described connections with alterations at higher biological levels. As mentioned earlier, the biomarkers used in ecology and ecotoxicology have mainly evolved from the fields of medicine and human toxicology. However, the incitement for using biomarkers differs between these areas, due to different protection goals in human and environmental risk assessment. In human clinical studies and risk assessments, the aim is to protect individuals and make predictions about an individual’s future health and/or response to a medical treatment. By contrast, ERA aims to protect the integrity and function of the ecosystem. This makes it desirable to use biomarkers for making predictions about effects at population and community levels [9, 10]. Hence, it is the biomarkers connected to effects at the higher biological levels that are of greatest value in environmental monitoring and assessment [5, 11]. The focus of the studies included in this thesis has, therefore, been on the connections between the biomarker responses and effects at higher biological levels (papers I-IV).

Some biomarkers are specific to a certain chemical or type of stressor, whereas others are unspecific and respond to a wide range of different stressors and changes. Heat shock proteins (hsp) are examples of general biomarkers. Their
function is to prevent protein denaturation [12], a common effect of many environmental stressors; hence, induction of hsp is considered a general stress response. Other responses are only induced by a certain group of xenobiotics or stressors. Acetylcholine esterase (AChE) is considered a specific biomarker as it responds to organophosphates and carbamate pesticides and not environmental stressors or xenobiotics in general [13, 14]. General biomarkers often have more confounding factors interfering with the toxic response. These biomarkers respond to not only the xenobiotic of interest, but also numerous other stressors and environmental factors, e.g. nutritional status, temperature and UV irradiation. The issue of confounding factors was addressed in paper II.

3.3 Oxidative stress

Oxygen is the fuel in the mitochondrial electron transport chain (ETC) that ultimately generates ATP, the cells’ main energy source. Oxygen is thus essential for almost all organisms. The ETC leaks electrons that react with dissolved oxygen and together generates superoxides \( \text{O}_2^- \). This molecule is unstable and will further react with water and form hydrogen peroxides \( \text{H}_2\text{O}_2 \) and hydroxyl radicals \( \text{OH}^- \). Due to the free (unpaired) electron and the unstable molecular structure, these molecules are highly reactive; collectively, they are called reactive oxygen species (ROS). The ETC is the primary source of endogenous ROS production in aerobic species [15]. The reactivity of these molecules makes them react extremely fast with almost anything in their surrounding and can, therefore, cause oxidative damage to biochemical structures and molecules, such as lipids, proteins and DNA. Fortunately, cells have a defense against ROS, the antioxidant defense. During normal physiological conditions, there is a balance between the production of ROS and the antioxidant defense (Figure 2A). This balance can shift (Figure 2B and 2C) and when an organism is subjected to increased stress, this balance may shift in favor of

![Figure 2](image.png)

*Figure 2* At normal physiological conditions, a balance between ROS concentration and the antioxidant defense is maintained (A). This balance can be shifted either in the favor of the antioxidant defense (B) or in favor of ROS (C). It is the latter situation (C) that is referred to as oxidative stress.
ROS, causing oxidative stress (Figure 2C). Alterations in ROS production is a normal physiological response that can have positive effects on the cells and health of the organism, as ROS is important in the intracellular signaling system [16] and for the adaptation of the antioxidant defense response [17, 18].

3.3.1 Antioxidant defense

The antioxidant defense consists of both enzymes, which catalyze the reactions that ultimately convert ROS to water, and a number of non-enzymatic molecules with antioxidative properties, which help to eliminate ROS. The primary enzymes involved in the detoxification of ROS are superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX). Together with iron (Fe), these enzymes catalyze specific parts of the conversion of ROS to water (Figure 3). The non-enzymatic part of the defense consists of low-molecular weight compounds that are synthesized in vivo or obtained through diet. The most commonly studied of these are glutathione, vitamin E, vitamin C, carotenoids and uric acid, among others.

![Figure 3](image)

*Figure 3* Summary of the reactions of ROS-generation and the enzymes responsible for the different steps in the generation and detoxification chain. SOD – Superoxide dismutase, GPX – Glutathione peroxidase, CAT – Catalase.

3.3.2 Lipid peroxidation

Lipids are the primary biomolecular targets for ROS, and these oxidative attacks result in lipid peroxidation. An increased peroxidation of the membrane-associated lipids decreases the fluidity of the mitochondrial membrane, resulting in increased leakage of molecules across the membrane and damage to the membrane-associated proteins [15, 19]. Products of lipid peroxidation can also be reactive and have cytotoxic effects [20]. The concentration of lipid peroxidation has been shown to rise with age [21, 22] and with decreased antioxidant capacity [20]. Several chemicals have also been found to enhance
lipid peroxidation in a number of organisms, such as bivalves [23], fish [24] and daphnids [25].

3.3.3 Oxidative stress, health and longevity

As mentioned earlier, the production of ROS fluctuates due to normal physiological events, such as exercise, feeding, and reproduction, which increase the oxygen demand and metabolism. However, an extensive increase in ROS due to additional stressors can induce oxidative stress. This can be harmful as it may result in increased levels of oxidative damaged biomolecules, which could compromise their functions. In medical science, oxidative stress has been related to various diseases, such as infections, cancer and Alzheimer’s disease. Much research is focused on the connections between oxidative stress and decreased health as this may result in new ways to prevent and cure diseases. To provide a firm basis for these studies, biomedical research addressed molecular response and regulation of oxidative stress [26, 27], but also the potential health benefits of antioxidants in diet [28, 29]. During the last two decades, the interest in oxidative stress has grown in stress ecology, fueled by exciting findings in medicine, particularly clinical studies in oncology, aging, and immune diseases as well as in environmental medicine. In ecology, oxidative stress and antioxidants have been connected with reproductive dysfunction [30] and mating success [31] as well as longevity and processes of aging [32]. The studies of oxidative stress in ecology have also identified fluctuations in ROS production and antioxidant defense in response to e.g. seasonal variability [33], food sources [34] and developmental stage [22]. If we are to use biomarkers of oxidative stress in ecotoxicological studies, these confounding factors must be considered, as they modulate biomarker responses to xenobiotic exposure.

3.3.4 Oxidative stress induced by xenobiotics

The production of ROS fluctuates in response to many different factors, both natural and anthropogenic, such as hypoxia, temperature, ultraviolet (UV) irradiation and pollutants. A vast number of ecotoxicological studies, applying oxidative biomarkers, have been published demonstrating effects on oxidative processes after exposure to nano-particles [35], pesticides [36] and polycyclic aromatic hydrocarbons (PAHs) [37], among others [38]. The most common mechanisms through which pollutants induce oxidative stress are by acting as an electron donor/acceptor or entering redox cycles. However, substances can also cause oxidative stress by interfering with the antioxidant defense, either by inactivating antioxidant enzymes or by causing depletion of substances involved in the defense, for example by conjugation with glutathione in phase II metabolism. Moreover, the antioxidant defense can respond to pro-oxidative exposure by increasing the activity of the enzymes responsible for ROS
detoxification. This dual response of the antioxidant defense sometimes makes interpretation of these biomarkers difficult. Individual enzymes and antioxidant compounds may respond differently which further complicates the interpretation. In this thesis, I have evaluated the response of the antioxidant defense mostly by measuring the oxygen radical absorbance capacity (ORAC) of animals (papers II-IV).

The connections between oxidative processes and life-history traits, e.g. reproduction, makes environmental researchers hope that oxidative biomarkers could be used as predictors of effects on individual and population levels. Biomarkers that are used in clinical trials to make early predictions of clinically meaningful endpoints are called surrogate endpoints [1, 2, 39]. By validating the predictive capacity of oxidative biomarkers, they could reach a similar status as surrogate endpoints in ecotoxicology and stress ecology. The theoretical connection between oxidative biomarkers and individual effects in ecotoxicology is illustrated in Figure 4, based on the conceptual idea of surrogate endpoints. For oxidative biomarkers to act as ideal predictors of individual effects, toxic effects should be mediated only through oxidative stress, illustrated by the orange boxes in Figure 4. However, many xenobiotics also act through other toxic mechanisms. This, together with confounding factors, make the connection between the response of oxidative biomarkers and individual effects more difficult to establish. Nevertheless, the connections between biomarker responses and toxicological effects on the individual level have been identified as important information that should be available for biomarkers applied in ERA [6]. I therefore studied the connection between biomarker response and reproductive effects, as well as the potential of oxidative biomarkers to be applied as effect biomarkers in papers III-IV. Feeding rate as a confounding factor for oxidative biomarkers was studied in paper II.

Figure 4 Conceptual model of the link between exposure, oxidative stress and individual effects. The figure also illustrates that individual effects can be manifested through other toxic mechanisms, and the influence of confounding factors on oxidative stress. Modified from De Gruttola et al. [1] and Fleming and DeMets [2].
4 Material and Methods

Test organisms, stressors, individual endpoints and biomarkers used in this thesis is summarized in Table 1.

Table 1. Summary of test organisms, stressors, individual endpoints and biomarkers used in this thesis.

<table>
<thead>
<tr>
<th>Paper</th>
<th>Test organism</th>
<th>Stressors</th>
<th>Individual endpoints</th>
<th>Biomarkers</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td><em>D. magna</em></td>
<td>Pharmaceuticals (levonorgestrel, miconazole and promethasine)</td>
<td>Mortality</td>
<td>RNA content</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Number of offspring</td>
<td>Gene expression</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>vitellogenin and</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>cuticle protein</td>
</tr>
<tr>
<td>II</td>
<td><em>D. magna</em></td>
<td>Haloperidol (pharmaceutical) and lindane (pesticide)</td>
<td>Feeding rate</td>
<td>ORAC</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>TBARS</td>
</tr>
<tr>
<td>III</td>
<td><em>M. affinis</em></td>
<td>Multiple stressors. Exposed to field-collected sediments.</td>
<td>Mortality</td>
<td>AChE</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Embryo aberrations</td>
<td>ORAC</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>TBARS</td>
</tr>
<tr>
<td>IV</td>
<td><em>D. magna</em></td>
<td>UVB irradiation</td>
<td>Mortality</td>
<td>ORAC</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Individual growth rate</td>
<td>CAT</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Age at first reproduc-tion</td>
<td>TBARS</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Number of offspring</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Population growth rate</td>
<td></td>
</tr>
</tbody>
</table>

4.1 Test organisms

Toxic effects on all species present in the environment cannot be tested as this would be an endless task. Model species are therefore often used to represent effects on the environment, or a specific part of the ecosystem. Such species should be sensitive to toxic events since it would otherwise be a risk of under-estimating effects of pollutants. Invertebrates represent the lower trophic levels of an ecosystem, making them important as a limitation for primary producers and as a food source for consumers at higher trophic levels. Using model species that are well studied makes it also easier to understand and
mechanistically explain observed effects. In this thesis, two crustaceans were chosen as model species: the cladoceran *Daphnia magna*, one of the most commonly used crustacean in ecotoxicology, and the amphipod *Monoporeia affinis*, a sentinel species used for environmental monitoring in the Baltic Sea.

4.1.1 Daphnia magna

In papers I, II and IV *Daphnia magna* was used as a model species. *D. magna* is a fresh water microcrustacean with a wide geographic distribution. It possesses several traits that make it suitable as a model species in ecological and ecotoxicological assays. It is easy to keep and culture in the laboratory and has a short generation time and a well-studied physiology and morphology. There are several standard assays for *D. magna* developed by different organizations (e.g. OECD, USEPA) that are mandatory in ERA of chemical substances. In addition to its use in studies assaying individual endpoints, such as mortality, reproduction, growth and feeding, several methods for measuring molecular and biochemical endpoints have been developed. The genome of the closely related species *Daphnia pulex* has been mapped [40], thus making it possible to study genetic effects also in *D. magna*.

4.1.2 Monoporeia affinis

*Monoporeia affinis* is a benthic, deposit-feeding amphipod that is abundant in the Baltic Sea. In this thesis, it was used in paper III to study the connection between embryo aberrations and biomarkers. *M. affinis* is a semelparous species, meaning that it only reproduces once, usually at an age of 1–4 years, before it dies. Several environmental stressors, e.g. hypoxia, temperature and contaminants, have been shown to induce embryo aberrations in *M. affinis* [41, 42]. Currently, this amphipod is used as an indicator of biological effects of contaminants in the Baltic Sea within the Swedish National Monitoring Program.

4.2 Molecular and biochemical assays

4.2.1 Gene expression analysis

Gene expression analysis is a good technique for studying toxic effects related to specific molecular mechanisms. Alteration in gene expression is often a fast response to changes in the surrounding environment and can have downstream effects on protein transcription and translation, which ultimately will translate into metabolic processes. While other genomic techniques, e. g., microarrays,
reveal semi-quantitatively the up- or down-regulation of multiple genes, quantitative PCR (qPCR) provides more precise information about how much a specific genes is up- or down-regulated [43]. In paper I, qPCR was applied to two genes related to reproduction and somatic growth in *D. magna* to study the effect of pharmaceuticals at the molecular level.

4.2.2 RNA content

Organismal RNA content was used in paper I as a proxy for total protein production as the major part of the RNA in a cell is ribosomal RNA (rRNA), a key component in protein synthesis [44]. The protein synthesis can be affected both by physiological factors, such as age and feeding rate, as well as by altered environmental conditions. In ecotoxicology, protein synthesis can increase as a result of the enhanced need for protective proteins and proteins involved in the metabolism of xenobiotics.

4.2.3 Antioxidant assays

It is not an easy task to measure the antioxidant defense as there are a number of different enzymes and compounds with antioxidative properties. Most likely, no single measurement will provide the full measure of the capacity of the antioxidant defense. In ecotoxicology, the most common approach is to measure the specific activity of the antioxidative enzymes, often CAT, SOD and GPX. In this thesis, the CAT activity was measured in paper IV and total antioxidant capacity was assayed as oxygen radical absorbance capacity (ORAC) in papers II-IV. The ORAC assay was developed by Ou et al. [45] and has been used to measure antioxidant capacity in food as well as in biota and human samples. The assay is based on the probe fluorescence that declines when it reacts with the added peroxyl radicals. Samples with high antioxidant capacity detoxify the peroxyl radicals and the fluorescence of the probe is maintained longer.

4.2.4 Lipid peroxidation

Oxidative damaged lipids were measured in papers II-IV by monitoring the formation of MDA [46]. This is the most common way to measure lipid peroxidation in ecotoxicological studies [20]. Lipids react with TBA and then form MDA that can be quantified fluorometrically. This assay demands a relatively large sample size (approximately 15 juvenile daphnids per sample) and as a result, a large experimental set up is needed to obtain sufficient material for measurements and replicates to statistically detect potential effects.
4.2.5 Acetylcholinesterase

Alterations in the activity of AChE, a biomarker of neurotoxicity, was used in paper III. AChE is an enzyme that is involved in controlling synaptic nerve transmissions, where it cleaves acetylcholine that is released at the synaptic cleft. This is a well-established biomarker that has been used in ecotoxicological studies for decades as organophosphates and carbamate pesticides have been shown to inhibit the AChE activity [13]. In this thesis, the activity of AChE was measured in a spectrophotometric assay in which thiolcholine, produced by AChE, is let to react with 5,5´-dithiobis(2-nitrobenzoic acid). Together, these two molecules produce a colometric anion that can be quantified and that is directly proportional to the activity of AChE [47].
5 Results and Discussion

All papers included in this thesis address stress responses at different organizational levels with the common aim of increasing the interpretability and understanding of biomarker response in ecotoxicological studies. The sensitivity of biomarker response was studied in paper I, reflecting the first objective of this thesis. Addressing the second objective, paper II focused on the impact of feeding activity as a confounding factor on biomarker responses. The two final papers (papers III-IV) connected biomarkers with effects at the individual and population levels, thus addressing the third objective of this thesis.

5.1 Targets of xenobiotics, toxic mechanisms and biomarker response

The responsiveness of biomarkers at low exposure concentrations was demonstrated in paper I. It was concluded that the toxicity of pharmaceuticals is highly dependent on the presence of a human drug target ortholog in an organism. One of the pharmaceuticals, levonorgestrel, has previously been shown to cause adverse reproductive effects in fish and amphibians at very low and environmentally relevant concentrations (ng/l) [48, 49]. These species have identified drug target orthologs for vitellogenin, the primary target for levonorgestrel. However, in D. magna it is lacking, and, despite rather high concentrations (mg/l), no adverse effects on either biomarkers or individual endpoints were found in this species after the exposure. Even though it was concluded that the most toxic pharmaceuticals could be identified using immobilization or reproduction as endpoints, biomarker responses occurred not only at lower concentrations but also within shorter time span (paper I).

The toxicity of a chemical substance is often manifested in initial reactions with molecular, biochemical or cellular targets. Pharmaceuticals are designed to react with specific biological targets at low concentrations [50] and, therefore, pose greater risk to cause adverse effects in biota. Many other chemical substances also have the capacity to react with molecular or biochemical components in living cells. These properties could be intentional (as for e.g. pesticides) or undesirable consequences of the chemical design. When studying the effect of xenobiotics on biomarkers, there should be an awareness of the most likely target and what toxic mechanisms that are most probable to be
activated. To randomly measure all possible biomarkers is not a good strategy. Potentially, the results would then provide more questions than answers, and be more confusing than measure the responses of a few biomarkers, selected based on their mechanistic connections with the stressor. Statistically, if 100 biomarkers would be measured, 5 biomarkers of these would by chance be significantly affected by, or correlated with, the stressor or individual endpoint of interest, using a significance level of \( p=0.05 \). The adverse outcome pathway (AOP) is a conceptual model that links chemical properties to molecular responses within an organism and then further connecting these initial changes to possible effects higher up in the biological organization [51]. The AOP stresses the importance of implementing existing knowledge about the toxic mechanisms of a xenobiotic when choosing relevant biomarkers and endpoints to study. Interference with oxidative processes are common toxic mechanisms for xenobiotics and the interest for implementing oxidative biomarkers in ecotoxicological studies have therefore increased during the last decades. However, very few xenobiotics pose their toxicity via one single mechanism. Hence, it is important to consider whether oxidative biomarkers need to be complemented by other biomarkers or endpoints. The toxic effects may otherwise be underestimated and individual effects disregarded.

5.2 Oxidative biomarkers and effects on reproduction

One of the most important factors affecting population growth is reproduction, and the connection between oxidative stress and reproduction has therefore received great attention [52-54]. However, this is a complex relationship, as reproduction can both be inhibited by oxidative stress and act as an inducer of pro-oxidative processes [53]. The relationship between reproduction and oxidative stress is important to understand, if we are to use oxidative biomarkers as biomarkers of effects. Therefore, I studied this relationship in papers III-IV. As shown in paper III, the occurrence of embryo aberrations and, in particular, arrested development and malformed embryos in *M. affinis* were significantly associated with increased ORAC and AChE activity. The only type of embryo aberration that was associated with increased lipid peroxidation were dead broods. Even though the exact mechanisms of these embryo aberrations still are to be unraveled, our results indicate that the underlying mechanisms differ for the different types of aberrations. This hypothesis is supported by the established connection between the different classes of aberrations with specific environmental stressors [55]. The occurrence of malformed embryos has been shown to increase in response to many xenobiotics [41], whereas arrested embryo development and dead broods appear to be mostly related to increased temperature and oxygen deficiency [42]. In paper IV we tested whether oxidative biomarkers could be used as predictors of reproductive effects in *D. magna* since predictive biomarkers are of great value in ecotoxicological studies and ERA. We exposed daphnids to UVB irradiation,
sampled animals for biomarker analysis at different life-stages and followed their reproductive success. An early negative effect on ORAC in juveniles and increasing ORAC in adults due to pro-oxidative exposure, together with observed negative effects on reproduction, suggest that ORAC is a suitable predictor of decreased reproductive success. We have thus validated ORAC, as not only a good biomarker of exposure, but also as a good biomarker for later reproductive effects. Additionally, we provided threshold values for diagnosing pro-oxidative exposure. This brings ORAC closer to what can be considered an equivalent to surrogate endpoints in medicine, as it is a suitable indicator of decreased fitness and reproductive success in crustaceans.

The increased antioxidant capacity in exposed daphnids also caused transgenerational effects, as offspring from exposed females had lower antioxidant capacity (paper IV). The decreased allocation of antioxidants makes offspring from exposed daphnids more vulnerable to oxidative damages that may have further consequences on their individual fitness, as juveniles have a higher ontogenetic antioxidant requirements [22]. Alterations in oxidative biomarkers can thus translate to effects on both reproduction and on the next generation.

5.3 ORAC and TBARS – suitable biomarkers of oxidative stress?

What are the advantages of using total antioxidant capacity instead for individual antioxidative enzymes, which is the most common approach in ecotoxicology? As enzymes may respond differently, some are up-regulated, whereas others are down-regulated or unaffected, it is often difficult to make any conclusions about the overall antioxidant response. There are also many assays required to cover all enzymes and still there is very little information about the non-enzymatic part of the defense. Several assays, besides ORAC, have been developed to measure total antioxidant capacity, e.g. total oxygen scavenger capacity (TOSC) [56] and ferric iron reducing antioxidant parameter (FRAP) [57]. The ORAC assay has been applied to measure the antioxidant capacity in food [45], blood and tissue samples [58] and – to some extent – in ecotoxicology and stress ecology [59-61]. The ORAC assay measures the capacity of the water soluble antioxidants, both enzymatic and non-enzymatic. Moreover, it has been shown that ORAC is positively correlated with the activity of both CAT and SOD and the GSH/GSSG ratio [59], all commonly assayed biomarkers for antioxidant defense. Results included in this thesis show that ORAC is responsive to many different stressors (papers II-IV) and that ORAC normalized to protein is unaffected and thus independent of altered feeding rates (paper II). The disadvantage of using ORAC is that information
about the response of specific antioxidants is lost, information that could help elucidate mechanisms that interfere with oxidative processes.

In papers II-IV, we found that TBARS is a rather poor predictor of exposure and reproductive effects in *D. magna*. The difficulty in detecting response in this variable is probably due to low replication as a consequence of the large number of animals needed for the analysis (15 juvenile daphnids in comparison to a single animal for ORAC). There have also been criticism towards the TBARS assay as it is unspecific and tend to react with other components in the sample, such as proteins [20]. Measuring lipid peroxidation using HPCL, increases the sensitivity and specificity as MDA is separated from interfering substances [62, 63].

5.4 Confounding factors for oxidative biomarkers

Oxidative stress is considered a general stress response as it can be induced by a variety of stressors. A number of confounding factors can affect the biomarker response when using oxidative biomarkers as exposure biomarkers in ecotoxicology. Caloric intake is one of the factors affecting oxidative processes in many organisms [64]. As feeding rate in planktonic species is a sensitive endpoint of xenobiotic exposure [65, 66], we studied the impact of feeding on biomarker response in *D. magna* exposed to pro-oxidative xenobiotics (paper II). Daphnids were exposed to chemicals that are known to cause feeding inhibition and to interfere with oxidative processes. We found that alterations in oxidative biomarkers is not only a direct response to xenobiotics but can also be a response to feeding inhibition induced by toxicity. Therefore, it is important to consider alterations in food intake when interpreting oxidative biomarker response. Moreover, feeding *ad libitum* may interfere with oxidative processes as high feeding rates alone led to increased antioxidant levels and lipid peroxidation (paper II). Based on these results, we suggested that moderate food levels are the most suitable in ecotoxicological assays employing oxidative biomarkers.

Experimental setups such as microcosms and mesocosms, aim to mimic environmentally realistic scenarios and thus include factors that are difficult to control. In papers II-IV, the objective was to elucidate the relationship between biomarker responses and effects at higher biological levels. To do this, interfering factors, that may affect the biomarker response, had to be minimized. As elaborated in paper III, it is complex to predict and interpret biomarkers in response to xenobiotics when potentially confounding factors, such as salinity, organic content and different composition of sediment, are present. In this study, the two sediments with highest chemical burden were not the sediments showing the most pronounced effect on either reproduction
or biomarker response. This was probably due to different sediment composition, affecting the bioavailability of the sediment-bound chemicals. However, in this study, the biomarkers provided information about the health of the amphipods and the occurrence of embryo aberrations. Hence, even though there are multiple factors affecting biomarker response in the field, the biomarkers could be used as health indicators.
I started this thesis by saying that there were knowledge gaps limiting the use of biomarkers in ecotoxicological studies and ERA. With the research presented in this thesis, I have contributed to diminishing some of these gaps by establishing connections between biomarker responses and effects at individual level. More specifically, the research presented in this thesis has shown that:

- The toxic potential of a pharmaceutical on non-target organisms is highly dependent on an identified human drug target ortholog. This increased toxic potential is observed throughout the biological organization, but effects at the individual level is observed first at higher concentrations than biomarker responses.

- Altered feeding rate is a confounding factor for the response of ORAC and TBARS. However, when normalized to protein, ORAC was independent of feeding rate. This makes ORAC/protein ratio a suitable biomarker for evaluating effects on oxidative processes without considering feeding rate as a confounding factor.

- The occurrence of embryo aberrations in M. affinis is reflected by increased ORAC, lipid peroxidation and AChE activity. Different types of embryo aberrations correlated to different biomarkers, thus indicating differences in underlying mechanistic causes for the aberrations.

- ORAC/protein ratio in daphnids is a suitable biomarker for diagnosing pro-oxidative exposure, delayed reproductive effects, and trans-generational effects. We provided age-specific threshold values for such diagnostics, which can be used in field and experimental studies on mixed populations.

These findings have increased the value of oxidative biomarkers in ecotoxicology as confounding factors and weak connections to effects at higher biological levels have been presented as two of the major factors limiting the implementing biomarkers in ERA [6]. The results have increased the understanding of how oxidative biomarkers relate to important life-traits, such as reproduction and how these connections can be studied.
7 Future perspectives

There is an increasing employment of molecular and biochemical endpoints in ecotoxicological studies led by the development of new techniques and analytical methods. We can observe a gradual shift from ecotoxicological studies, based on individual endpoints, toward studies applying biomarker assays based on the understanding of the biological pathways perturbed by xenobiotics. Novel biomarkers need to be evaluated based on their ability to respond to toxic exposure, but also based on their ability to indicate physiological impairments. A general agreement regarding methods to validate biomarkers in ecotoxicology would greatly facilitate comparison and interpretation of biomarker responses.

In this thesis, biomarkers have been employed using knowledge and statistical methods frequently used in stress ecology and medicine, but less common in ecotoxicological practice. Many of these approaches can be adopted to improve the validation and predictions of effect biomarkers in ecotoxicology, thus enabling quantitative linkages in the AOP models. In particular, statistical methods and models can connect molecular and biochemical responses to changes in life-history traits and, further, to population-level responses. For a wide range of organisms, a number of individual-based and population models have been developed and integrated with each other to extrapolate and predict stress outcome at higher levels of the biological organization [67]. Yet, the integration of subcellular responses in such models is still in the early phase, which hampers development and application of the AOP framework in ecotoxicology. One way to approach this, is to develop, not mechanistic, but statistical models linking exposure biomarkers to life history or physiological traits (as in paper IV); the latter can further be used as target variables in individual-based or bioenergetics models.

To facilitate interpretation of biomarker responses in the assessment of biological effects of environmental contaminants in the field, the nutritional status of the animals needs to be taken into account as shown in paper II. This can be done by incorporating biomarkers of growth status (RNA:DNA ratio, protein content, etc.; papers I-II) in the sampling regime. In the search for new, species- and stage-specific markers, valuable insights can be gained from the biomedical studies [68] but also from the genomics of the test species (pa-
per I) and molecular docking for the substance in question [69]. When validating biomarkers, it is imperative to define threshold values (paper IV) that can be used in the modeling and risk assessment and there is much room for improvement of the numerical methods that can be applied.
Först vill jag tacka min handledare Elena Gorokhova för att du ständigt kommer med nya infallsvinklar och väcker nya idéer och tankar. Du har fått mig att utmana mitt eget tänkande många gånger och fått mig att inse att jag kan mycket mer än vad jag trodde innan.

Det är ganska precis 10 år sen jag började läsa miljövetenskap på universitetet och jag vet inte om du, Magnus Breitholtz, trodde på mig redan då (jag vet inte om jag själv skulle ha gjort det...), men du har litat och trott på mig och varit ett stort stöd under mina år på ITM/ACES. Utan dig hade det här blivit en betydligt tuffare tid.

Tack till min externa mentor, Clare Bradshaw, för att du visat intresse för både min forskning och mitt välmående som doktorand.

Jag har haft två labb-mammor som lärt mig ALLT jag kan på labb och som tog hand om mig så att jag inte kom bort i den stora vida världen (läs Norge). Karin E har visat mig hur man tar hand om den perfekta Daphnia-odlingen och Birgitta är min förebild när det kommer till perfekta standardkurvor. Ni har varit ovärderliga i det praktiska arbetet av den här avhandlingen! Karin N är ”huvud-morsan” på enheten och den som håller ordning på oss alla andra. Utan dig skulle vi vara som yra höns!


Min doktorandtid har handlat om så mycket mer än bara forskning, den har även fyllts med resor, fester, löptävlingar och mycket mer. Tack till Kim, Marko, Lukas, Dimitri, Seth, Leena och många andra för oförglömliga minnen och många skratt.

Johannes och Bruce, ni visade vägen och nu är det min tur. Sist ut att disputera, men nu är doktorerna äntligen i majoritet i ”mellanstadie-gänget”. Filippa och Alex, nu slipper ni lyssna på långa utläggningar om livet som doktorand och vi kan börja prata om roligare saker. Jag ser fram emot många fler middagar och resor tillsammans med er!


Tack till Ina och Anna som aldrig är mer än ett telefonsamtal bort.

Tack till alla vänner och familj som har varit med på den här resan och hjälpt mig att koppla bort forskningen på fritiden och fått mig att fokusera på allt annat som är viktigt i livet.

Min familj finns alltid i närheten och har supportat mig otroligt mycket under den här tiden. Tack mamma, pappa och Christin. Ett stort tack, en stor kram och många pussar till Mats som surfade in i mitt liv och som har varit ett ovärderligt stöd och trygghet!
9 References


13. Payne, J.F., et al., Acetylcholinesterase, an old biomarker with a new future? Field trials in association with two urban rivers and a paper


42. Wiklund, A.K.E. and B. Sundelin, *Impaired reproduction in the amphipods Monoporeia affinis and Pontoporeia femorata as a result*


