Doctoral Thesis
For the degree of Doctor of Philosophy in
Applied Environmental Science

Does Fish Health Matter?

The Utility of Biomarkers in Fish for Environmental Assessment

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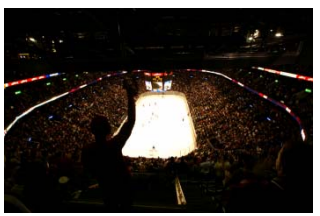

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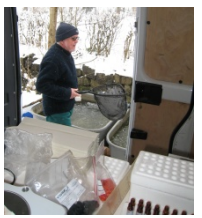

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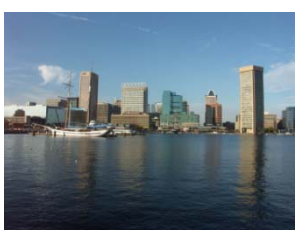

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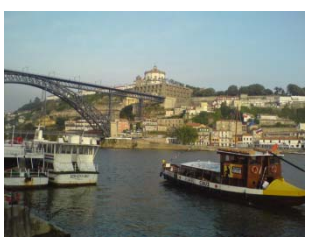

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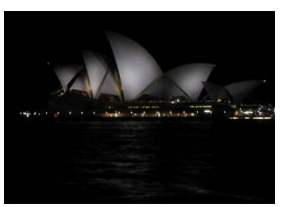

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Abstract

Two common strategies to assess exposure to environmental toxicants are to measure chemical concentrations in the environment and to examine the presence of certain species that are known to be sensitive to pollution. The two strategies can sometimes give conflicting results. It is, e.g., possible that there are elevated levels of contaminants but no biological effect due to low bioavailability. Furthermore, chemical measurements only give information about those chemicals that are included in the analysis and abundance of species can vary due to many factors that are not linked to pollution. An alternative, or complementing, strategy for environmental assessment is to examine sub-organism responses (biomarkers), which includes physiological and biochemical variables. Biomarkers indicate exposure to contaminants and health impairment in individuals. Because the initial response to pollution is assumed to occur at low levels of biological organization, biomarkers are expected to act as early warning signals. Furthermore, biomarkers may provide a mechanistic link between exposure and effects. There is, however, little evidence that link biomarker responses to effects at higher levels. In addition, confounding factors, such as migration and age, can complicate the interpretation of results.

In the present thesis, which is based on six scientific papers [I-VI], the utility of biomarkers in fish for assessing the environmental status was evaluated. Fish are suitable for assessing the environment as they can be found in most aquatic environments and play a major ecological role in aquatic food webs. In [I-IV], a methodology with farmed rainbow trout (*Oncorhynchus mykiss*), which were reared in net cages and/or plastic tanks, was used and evaluated. This was done as it was expected that caging would increase the precision of biomarker measurements by, e.g., preventing migration and thereby achieve a standardized exposure. In [V-VI], biomarker responses from a 20-year data set on feral perch (*Perca fluviatilis*) from national reference areas on the Swedish Baltic coast were examined. During this period, an increasing trend in detoxification enzyme activity (ethoxyresorufin-O-deethylase, EROD) in the liver and a reduction in gonad size (gonadal somatic index, GSI) have been observed, and potential explanations for these trends were evaluated in [V-VI].

The potential confounding effect of different holding conditions (net cages or plastic tanks) and differences in feeding was examined in [I-II]. The results suggested that the methodology with caged fish was robust to differences in holding conditions but that differences in feeding can affect the responses for several variables. The methodology was used to assess the exposure to pollutants in two water systems [III-IV] with, presumed, high and low anthropogenic impact, respectively. The methodology worked well in the water with high anthropogenic impact, and it was shown that rainbow trout at certain sites were exposed to increased levels of pollutants. Furthermore, this information was linked to observations of fin and skeletal damage on feral brown trout (*Salmo trutta*). In the water system with low impact, however, interpretation of results was more complicated and little useful information could be retrieved. A possible explanation to this is that the relative impact of confounding factors became more important when the exposure to contaminants was low.

In [V], biomarker responses in perch and water temperatures were analyzed at the national reference station Kvädöfjärden as well as the flow rate in a nearby small river. The

results showed that the flow rate in the river correlated with EROD activity in perch liver. It is, therefore, likely that contaminants are brought to the area by runoff from land. Furthermore, it was found that fish that lived during years with higher EROD activity also had lower GSI, which may affect the reproductive capacity of the perch. The responses in Kvädöfjärden were further investigated in [VI] by analyzing frozen bile from perch that were collected in two years with high and low EROD levels, respectively. It was found that increasing levels of polycyclic aromatic hydrocarbons (PAHs) is a likely contributor to increasing biomarker responses in Kvädöfjärden.

Biomarkers in fish can be a useful tool to evaluate environmental pollution in many, although not all, situations. The use of farmed fish can improve the precision in the measurements, but some ecological relevance will be lost and certain confounding factors may be introduced. The choice between farmed or feral fish depends on the situation and what questions that needs to be answered. The results from biomarker analyses are most useful when data from other methodologies can be included in the interpretation. Future work should focus on how different methods can be integrated to get an effective and more reliable assessment of environmental conditions.

Sammanfattning

Två vanliga strategier för att bedöma miljögiftsexponeringen in naturen är att mäta koncentrationer av kemikalier i miljön samt att undersöka förekomsten av vissa arter som är känsliga för miljögifter. Dessa två strategier kan i vissa fall ge motstridiga resultat. Det är, t.ex., möjligt att det finns förhöjda halter av föroreningar samtidigt som inga biologiska effekter kan påvisas på grund av att biotillgängligheten är låg. Kemiska mätningar visar dessutom endast halter av de kemikalier som är med i analysen och förekomsten av olika arter kan påverkas av andra faktorer än miljögifter. En alternativ, eller kompletterande, strategi för att bedöma miljögiftsexponeringen är att undersöka responser på sub-organism nivå (biomarkörer), vilket inkluderar fysiologiska och biokemiska variabler. Biomarkörer påvisar exponering för föroreningar och hälsopåverkan på individer. Eftersom de första responserna för miljögifter uppträder på låga biologiska organisationsnivåer antas biomarkörer fungera som tidiga varningssignaler samt visa en koppling mellan exponering och effekt. Det är dock ont om bevis för att biomarkörer faktiskt kan förvarna om relevanta ekologiska effekter och resultaten kan påverkas av andra faktorer som försvårar tolkningen (t.ex. skillnader i migration och ålder).

I den här avhandlingen, som baseras på sex vetenskapliga artiklar [I-VI], har nyttan av biomarkörer hos fisk för att undersöka förekomsten av miljögifter utvärderats. Eftersom fisk finns i de flesta akvatiska miljöer och har en viktig roll i näringskedjor är de lämpliga att använda för att undersöka förekomsten av miljögifter i vatten. I [I-IV] utvärderades en metodik med odlad regnbåge (*Oncorhynchus mykiss*) som hölls i nätkassar och/eller plastbassänger. Metoden förväntades öka jämförbarheten i mätningarna genom att bland annat förhindra migration och därigenom uppnå en kontrollerad exponering. I [V-VI] undersöktes biomarkörresponser i en 20-årig dataserie för abborre (*Perca fluviatilis*) från nationella referensområden på den svenska Östersjökusten. Under denna period har en ökande trend i avgiftningsaktivitet i levern (etoxyresorufin-O-deetylas, EROD) och en minskande trend i gonadstorlek (gonad somatiskt index, GSI) observerats och möjliga förklaringar till dessa trender utvärderades i [V-VI].

Påverkan på biomarkörer hos odlad fisk beroende på olika experimentella betingelser samt effekten av olika matningsnivåer undersöktes i [I-II]. Resultaten indikerade att metodiken är robust för skillnader mellan nätkassar och bassänger, men att variationer i matransoner kan påverka resultaten för flera biomarkörer. Metodiken användes för att bedöma exponeringen för miljögifter i två vattensystem [III-IV] med, förväntad, hög respektive låg föroreningsbelastning. Metodiken fungerade tillfredsställande i vattensystemet med hög belastning och visade att regnbåge på vissa platser var exponerade för förhöjda miljögiftshalter. Påvisade effekter kunde dessutom kopplas till observationer av skelettskador på vild öring (*Salmo trutta*). I vattensystemet med låg mänsklig påverkan var resultaten mer svårtolkade, och informationen som erhölls var mindre användbar. En möjlig förklaring till detta är att påverkan av andra faktorer blir relativt sett större då halten av miljögifter är låg.

I [V] analyserades biomarkörer hos abborre och vattentemperatur vid den nationella referensstationen Kvädöfjärden samt vattenflödet i en närbelägen å. Resultaten visade att vattenflödet i ån korrelerade till EROD aktivitet i levern hos abborre. Det är därför troligt att miljögifter transporteras till områden genom avrinning från land. Resultaten visade också att abborre som levt under år med höga EROD nivåer hade lägre GSI, vilket skulle

kunna påverka reproduktionskapaciteten för abborrarna. Kvädöfjärden undersöktes ytterligare i [VI], där fryst galla från abborrar som provtagits under två år med hög respektive låg avgiftningsaktivitet analyserades. Dessa analyser visade att en ökad förekomst av polyaromatiska kolväten (PAHer) är en trolig bidragande orsak till de ökade biomarkörsresponserna i Kvädöfjärden.

Biomarkörer i fisk kan vara användbara för att undersöka miljöföroreningar i många, men inte alla, situationer. Genom att använda odlad fisk som placeras i nätkassar eller bassänger kan precisionen i mätningarna ökas. Samtidigt går dock viss ekologisk relevans förlorad och nya störande faktorer kan introduceras. Valet mellan odlad eller vild fisk beror på vad som skall undersökas och vilken typ av frågor som skall besvaras. Resultat från biomarkörstudier är som mest användbara när data från andra typer av miljöövervakning kan inkluderas i tolkningen. Framtida forskning bör inriktas på att undersöka hur olika metoder kan integreras för att ge en effektiv och mer tillförlitlig bedömning av tillståndet i miljön.

List of papers

This thesis is based on the following papers, which are referred to in the text by their roman numerals:

I. Hanson N, Guttman E, Larsson Å. 2006. The effect of different holding conditions for environmental monitoring with caged rainbow trout (*Oncorhynchus mykiss*). *Journal of Environmental Monitoring* 8(10):994-999.

II. Hanson N, Larsson Å. 2007. Influence of feeding procedure on biomarkers in caged rainbow trout (*Oncorhynchus mykiss*) used in environmental monitoring. *Journal of Environmental Monitoring* 9(2):168-173.

III. Hanson N, Larsson Å. 2008. Experiences from a biomarker study on farmed rainbow trout used for environmental monitoring in a Swedish river. *Submitted Manuscript*

IV. Hanson N, Larsson Å. 2008. Biomarker analyses in fish suggest exposure to pollutants in an urban area with a landfill. *Submitted Manuscript*

V. Hanson N, Förlin L, Larsson Å. 2008. Evaluation of long term biomarker data from perch (*Perca fluviatilis*) in the Baltic Sea suggest increasing exposure to environmental pollutants. *Environmental Toxicology and Chemistry*. *In press* DOI:10.1897/08-259.1

VI. Hanson N, Persson S, Larsson Å. 2008. Analyses of perch (*Perca fluviatilis*) bile suggest increasing exposure to PAHs and other pollutants in a reference area on the Swedish Baltic coast. *Journal of Environmental Monitoring. In press* DOI:10.1039/b817703a

Facts are meaningless.
You could use facts to prove
anything that's even remotely true!

Matt Groening (The Simpsons)

1. Introduction

1.1 Environmental pollution

Global environmental problems have emerged during the 20th century as a result of increased per capita consumption of resources and a growing human population. For the last three decades global consumption of ecological resources has exceeded the regeneration rate, in 2003 by approximately 25% (WWF 2006). This is possible as the earth has built up ecological assets over time. The global human population size has more than doubled during the past 40 years, to more than 6 billion people, and is expected to reach 9 billion within the next 40 years. The demand for ecological services can therefore be expected to put an even higher pressure on the world's ecosystems in the future. Adjusting the consumption to a sustainable level is aggravated by what was described by Hardin (1968) as "the tragedy of the commons". This describes the problem of overexploitation of common resources that arises when several individuals try to maximize their own harvest. A classic example of this is a pasture which is used by several herdsmen. Each herdsman will try to maximize his own yield by putting as many animals as possible on the common. Once the total number approaches the carrying capacity of the system, the total yield of the pasture will start to decrease (**Figure 1**). The rational behaviour of each herdsman is, however, still to put more animals on the pasture as the extra yield is received by the owner while the negative component by overgrazing is shared by all herdsmen who use the pasture.

The tragedy of the commons can also be applied when pollutants are released into the common. An industry that acts rationally will, most often, find that the cost of treating the waste is higher than the share of the cost of degrading the recipient. In fact, if the industry is not dependent on the ecological status of the recipient, there is no real cost. This has led to widespread contamination of the world's ecosystems and today even remote areas like the Polar Regions are affected by anthropogenic

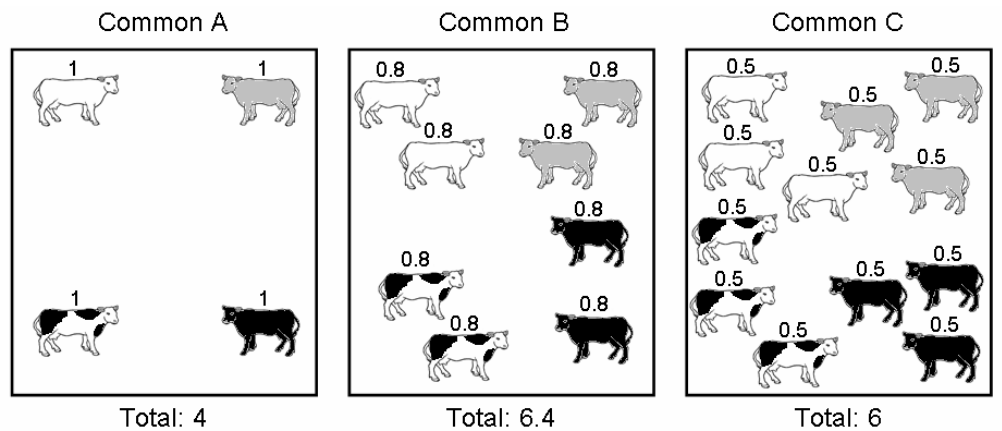

Figure 1. An example of the tragedy of the commons for a pasture that is used by four herdsmen (who have differently coloured cattle). When each herdsman put only one cow on the commons (Common A), the productivity of each cow is 1 (e.g., 1 litre of milk). When the herdsmen introduce more cattle (Common B), the production per animal is reduced due to increased density but the total production is higher. When the carrying capacity is approached (Common C), also the total productivity is reduced. Each individual herdsman will, however, gain nothing from reducing his stock unless other herdsmen do the same.

chemicals (Chapman and Riddle 2005).

There are numerous examples of chemicals that have been beneficial for mankind through, e.g., increasing production in agriculture (pesticides), treatment of diseases (pharmaceuticals) and improved durability of food products (preserving agents). There are, however, also examples of chemicals that have caused adverse effects on human health as well as on ecosystems. Reduced biodiversity is one of the adverse effects that may be caused by pollution (Preston and Shackelford 2002) and global biodiversity has been decreasing during the past 30 years (WWF 2006). However, there are other anthropogenic factors that contribute to reduced biodiversity, e.g. habitat destruction (Lawton et al. 1998), overfishing (Allan et al. 2005) and introduced species (Vitousek et al. 1997). In some cases, a reduced use of chemicals may result in increased impact due to other factors. Several studies have, e.g., shown that crops that are grown without the use of pesticides produce lower yields than conventionally produced crops (Mäder et al. 2007; Rembialkowska 2007). This means that a reduction in pesticide use may call for larger areas to be used for farming (habitat destruction) and that other food sources need to be used more intensively (overfishing). Furthermore, chemicals can be an important tool to fight the establishment of introduced species and thereby help to save native species from being outcompeted.

Considering the present overconsumption of resources and the predicted increase in global population size during this century, it is obvious that we can not afford to degrade important ecosystems by pollution. However, chemicals will no doubt play an important role in feeding and providing welfare for the growing population. Today, more than 100 000 different chemical substances are in use and the annual world wide production has increased from 1 million tonnes in 1930 to more than 400 million tonnes today (European Commission 2001). From this, it is obvious that good scientifically based approaches will be necessary to evaluate the risks as well as benefits of chemicals in the future.

1.2 Ecological risk assessment

When chemicals occur at concentrations above the background level, it is termed contamination. When contamination results in adverse biological effects, it is called pollution. Hence, contamination is not automatically pollution (Chapman 2007), and does thereby not automatically degrade ecological resources. Contamination levels can, in most cases, easily be measured with analytical instruments. There is, however, no universally applicable instrument to measure adverse biological effects (toxicity). Instead, ecotoxicologists are forced to rely on a battery of tests with living organisms (Cairns and Mount 1990), which is referred to as toxicity testing. One of the most commonly used organisms for toxicity testing is the small crustacean *Daphnia magna*. To increase the precision and repeatability of the measurements, the tests are performed under standardized conditions. For example the toxicity tests with *D. magna* are standardized by the International Organization for Standardization (ISO 1996). This means that, e.g., the water is standardized by adding certain constituents to distilled water. However, the environment is variable and can therefore not easily be described by standardized tests. Photo-induced toxicity may, e.g., be missed in the laboratory but have a significant impact in the environment (Hatch and Burton 1999). Factors such as water turbidity, suspended

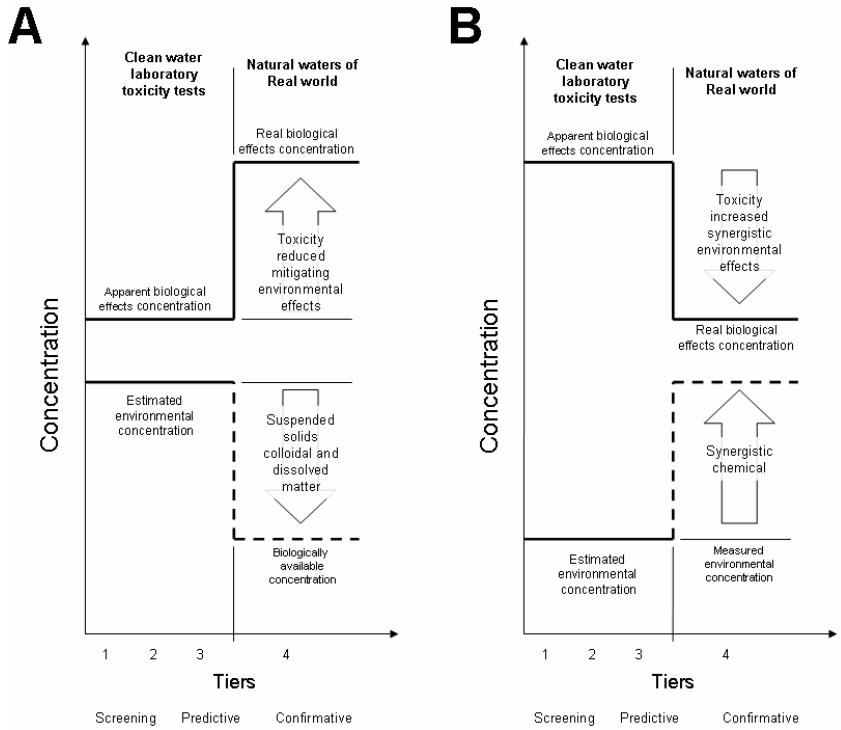

Figure 2. The margin of safety that is estimated from laboratory experiments can be increased (A) or reduced (B) when the exposure occurs under natural conditions. The figure is modified from Cairns and Mount (1990).

solids and dissolved organic matter, on the other hand, may reduce the toxicity due to reduced bioavailability (Smith and Lizotte 2007). **Figure 2** shows two examples where standardized laboratory tests underestimate (A) or overestimate (B) the margin of safety.

Another problem is that ecotoxicologists often have to rely on acute toxicity tests (often 24 or 48 h) as chronic tests are expensive and time consuming. A shortcut to this problem can be to use multigeneration tests on species with short life cycles. However, results from species with short life cycles can not easily be used to assess the effects on species with longer life cycles. When reliable data on chronic effects are missing, an extrapolation factor of 10 is suggested by the Technical Guidance Document (TGD) of the European Commission (2003) when moving from acute toxicity data on three trophic levels to at least one chronic test (Forbes and Calow 2002b). This factor is assumed to be sufficiently protective regardless of the life history of the species or the properties of the chemical compound. In a study on a total of 102 chemicals, Roex et al (2000) found an average acute-to-chronic ratio (ACR) of 6.03, i.e. below 10 and, hence, conservative. However, looking at specific groups of chemicals the mean ACR may vary strongly. Ahlers et al (2006) found that although most ACRs were below the safety factor recommended by the TGD, individual ACRs varied considerably and went up to 4 400. From this it is obvious that fixed extrapolation factors will result

in errors, and sometimes in large errors. In most cases the factor is conservative, which may result in higher tier analyses or in unnecessary bans or restrictions of beneficial chemicals. In other cases, chemicals may be released to the environment in quantities that can cause ecological damage.

The process of ecological risk assessment determines the probability that a negative effect (hazard) will occur due to a certain action, such as the release of pollutants. This allows weighing the benefits of a specific chemical to its environmental hazard. An ecological risk assessment consists of an effects assessment (toxicity) and an exposure assessment (predicted environmental concentration) (Suter 1993). In both the effect and the exposure assessments, assumptions are made and errors may occur. A schematic representation of the work flow in a *predictive ecological risk assessment* is shown in **Figure 3**. Predictive ecological risk assessments are used to quantify the probability for a negative effect when a certain action is performed, e.g., the introduction of a pesticide. The outcome of the predictive risk assessment can thereby be used to aid decision makers. Predictive risk assessment can be distinguished from *retrospective ecological risk assessment*, which deals with hazards that began in the past and that may have ongoing and future effects (e.g. waste disposal sites or oil spills) (Suter 1993).

1.3 Environmental monitoring

Because of the uncertainties in the process of ecological risk assessment, there is always a risk that chemicals will cause adverse ecological effects. Chemicals may

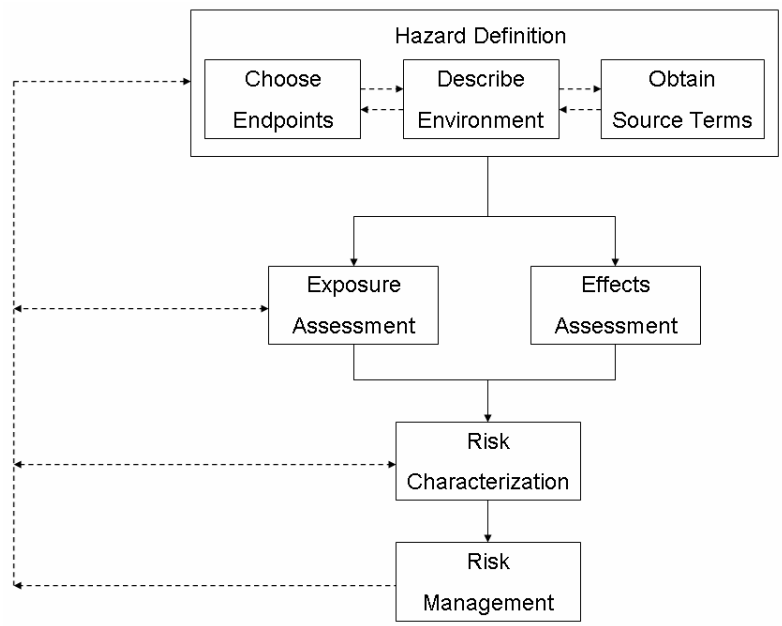

Figure 3. A diagrammatic representation of predictive ecological risk assessment. After the Hazard definition, which includes choosing endpoints, describing the environment and obtaining information about the source(s), the two parallel assessments of exposure and effects are performed. The scientific output is summarized in the risk characterization, which serves as input to the risk manager(s). The figure is modified from Suter (1993).

also be released without proper risk assessment as well as from unexpected sources. In many cases, observations by the public have put the light on environmental damage that has been caused by chemical substances. However, for a reliable guard against mistakes in ecological risk assessment, well designed programmes for environmental monitoring are needed. Environmental monitoring aims at finding information on spatial as well as temporal variations in contamination load and biological effects. The results of monitoring studies can be used to assess the current status of the environment and to evaluate the results of ongoing measures to reduce discharges of contaminants.

The most common method for environmental monitoring is to measure concentrations of specific contaminants, often in a recipient to a known discharge. A drawback with chemical monitoring is that it only provides information about those chemicals that are included in the analyses. As the emphasis is shifting from known point sources to diffuse pollution (Crathorne et al. 1996) and mixtures of known and unknown pollutants, chemical monitoring may not be useful in all situations. Furthermore, if contaminants are present but not taken up by organisms, little (or no) damage will be caused to the ecosystem (Whitfield 2001). The extent to which a contaminant is taken up by organisms, the *bioavailability*, may depend on environmental factors such as water hardness and pH (Walker et al. 2001). An alternative to chemical monitoring is biological monitoring, or *biomonitoring*. This means that effects on living organisms are monitored and that bioavailability therefore automatically is included. An example of biomonitoring is to examine the species composition of a certain community. In the aquatic environment, community structure for benthic macrofauna has been widely used to determine environmental stress (Ingole et al. 2006) and biotic indices based on the presence or absence of certain indicator species are often used to simplify the interpretation (Bustos-Baez and Frid 2003; Roberts et al. 1998). As indicator species are not equally sensitive to all contaminants, this approach will respond differently to different kinds of stressors (Rand et al. 1995). Another methodology with benthic indicators is the Sediment Quality Triad (Chapman 1990) where benthic indicators (e.g. species indices) are integrated with bioassays to test toxicity and chemical measurements to determine the presence of contaminants. This means that bioavailability and structural redundancy of the community are included in the assessment as well as measurements of specific chemicals. The results can then be interpreted using a tabulated decision matrix (**Table 1**).

Table 1. Examples of possible outcomes and interpretations of the Sediment Quality Triad. The table is based on Chapman (1996).

Contamination	Toxicity	Community	Conclusion
Elevated	High	Altered	Strong evidence for pollution induced degradation
Low	Low	Normal	Strong evidence against pollution induced degradation
Low	Low	Altered	Alteration is not due to toxic contamination
Elevated	Low	Normal	Contaminants are not bioavailable
Low	High	Altered	Unmeasured toxic contaminants are causing degradation

The bioindicator approach can be useful for monitoring of pollution and environmental degradation when the types of contaminants are not known. However, the use of bioindicators and indicator species will only respond once changes have become measurable at the population and community level. It can be argued that environmental monitoring should respond before effects are seen at these levels. This leads to another aspect of environmental monitoring, early warning signals and identification of new threats.

1.4 Biomarkers, the sub-organism level

The approach with biological indicators can be expanded to go beyond the presence or absence of certain species by including health related measurements. The biological levels of organization can be considered as a hierarchical system where 'processes at one level take their mechanisms from the level below and find their consequences at the level above' (Caswell 1996). Effects can be manifested through the levels of organization until populations are affected and species decline or disappear (**Figure 4**). By performing monitoring at the sub-organism level, e.g. by using physiological or biochemical measurements, it may therefore be possible to get an early warning signal for effects at higher levels. Furthermore, the need for experts to define indicator species and reference communities will be reduced as physiological and biochemical measurements tend to be more quantitative. Sub-organism indicators for exposure are often referred to as biological markers, or *biomarkers* (Shugart 1996). The use of biomarkers has its origin in human toxicology where they have proved to be very useful as measures of exposure to chemicals as well as to provide early warning signals for specific diseases (Timbrell 1998). In

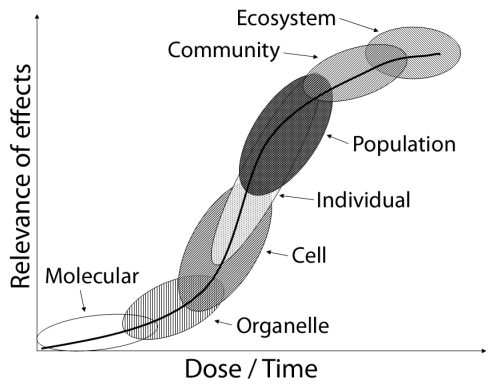

Figure 4. Hierarchical relationship between ecotoxicological responses at different levels of organization. A higher dose will have effects at higher, and more relevant, levels. A lower exposure can, however, cause the same effect given enough time. The figure is modified from Spurgeon et al. (2005).

ecotoxicology, however, the primary concern is populations, communities and ecosystems, and only rarely the health of individual organisms. Moving up the ladder of biological organization, the distance to the biomarker level increases and the predictability should, in theory, decrease. Forbes et al (2006) concluded that biomarkers should not be expected to provide useful predictions of relevant ecological effects. A similar critical view was held by McCarty and Munkittrick (1996), who said that 'much biomarker-related work is poor science because useful information cannot be generalized from it'.

Effects at higher levels have high ecological relevance, while the specificity, in terms of determining the cause of the effects, will be poor. For lower levels of organization, such as biomarkers, the specificity will be higher, while the ecological relevance can be questioned. This antagonistic relationship between ecological relevance and specificity is shown in **Figure 5**.

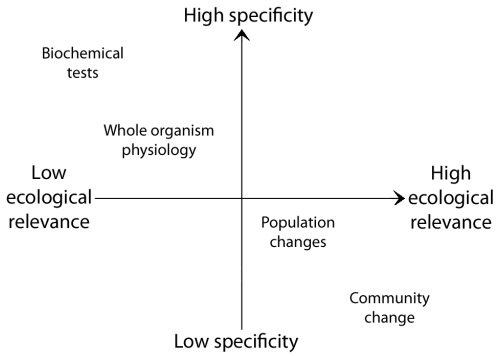

Figure 5. The conflict between specificity and ecological relevance. When higher levels of organization are studied, the ecological relevance is higher but the specificity of the measurements is lower. The figure is modified from Addison (1996).

Several definitions have been given to the term 'biomarker'. Peakall (1994) defined a biomarker as 'a biological response to a chemical or chemicals that gives a measure of exposure and sometimes, also, of toxic effect'. This includes effects from the molecule and cell level to the function and structure of the ecosystem. McCarty and Munkittrick (1996) had a similarly wide definition: 'A biomarker is an anthropogenically-induced variation in biochemical, physiological, or ecological components or processes, structures, or functions that is measurable in a biological sample or system'. A much more narrow definition was given by Lagadic et al. (1994), who defined biomarkers as biochemical sublethal changes from individual exposure to xenobiotics. In the present thesis, the definition for the term biomarker is based on the suggestions of van Gestel and van Brummelen (1996), who linked the different terms to the level of biological organization at which they are measured. According to this definition, *biomarkers* are measured below the individual level, *bioindicators* are organisms that give information on the environmental condition by their presence (or absence) and behaviour, and *ecological indicators* are parameters that describe the structure and function of the ecosystem.

Biomarkers are often subdivided into *biomarkers of exposure* and *biomarkers of effect*. This terminology is to some extent misleading as all biomarkers, by definition, demonstrate an *effect* caused by an *exposure* (Peakall and Walker 1994; van der Oost et al. 2003). However, the subdivision between biomarkers of exposure and biomarkers of effect can still be useful for the discussion and interpretation of results from biomarker studies. Biomarkers of exposure cover measurements of exogenous substances, metabolites of exogenous substances and interactions between exogenous substances and target molecules, while biomarkers of effect include measurements that can be associated with health impairments (van der Oost et al. 2003). In this thesis, ethoxyresorufin-O-deethylase (EROD) activity and PAH (polycyclic aromatic hydrocarbon) metabolites in bile are examples of biomarkers that fall under the category biomarkers of exposure. EROD activity measures the detoxification activity (Phase I) in the liver, i.e. the interaction between the exogenous substance and a target molecule. Examples of biomarkers of effects are relative numbers of white blood cells (WBC) and gonadal somatic index (GSI). McCarty and Munkittrick (1996) compared biomarkers of exposure and biomarkers of effect to the dose-response paradigm of toxicology, where biomarkers of exposure and effect can be considered as surrogates for dose and response, respectively. In [V], this view was applied to the correlation between EROD activity and GSI, where EROD represented the dose and GSI was considered as a measurement of response. Many biomarkers fit somewhere between the defini-

tions of biomarkers of exposure and biomarkers of effect. As a rule of thumb, it can be said that effects at higher levels of organization fit better to the definition of biomarkers of effect while biomarkers of exposure are more often found at lower levels of organization. The location on the ladder for hierarchical levels of organization can also be linked to ecological relevance and specificity (**Figure 6**). A third group of biomarkers that is sometimes mentioned is *biomarkers of susceptibility*, which can be defined as measurements of the ability of an organism to respond to exposure from a certain stressor (van der Oost et al. 2003). However, this definition is not very useful in ecotoxicology and therefore the term is not often used.

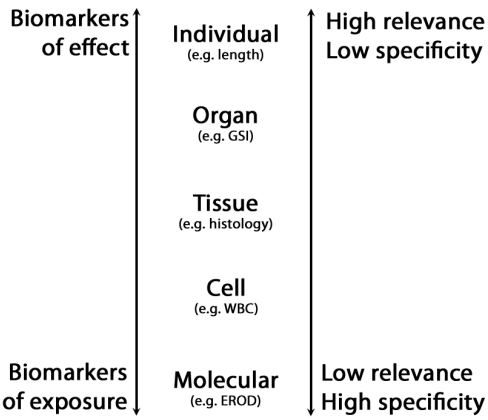

Figure 6. Biomarkers of effect are often found at higher hierarchical levels, where the ecological relevance is higher but specificity is lower. Biomarkers of exposure are often found at lower levels with high specificity but reduced ecological relevance.

1.5 Biomarkers in fish

As most contaminants ultimately end up in water, the aquatic environment is of highest interest in environmental monitoring. Environmental monitoring with biomarkers in the aquatic environment can be performed with various groups of organisms, but the most common ones are mussels and fish (Viarengo et al. 2007). Fish can be found in most aquatic environments and they play a major ecological role in aquatic food webs to transport energy (as well as pollutants) from lower to higher trophic levels (Beyer 1996). Furthermore, as fish is an important food resource for humans, there is a risk that pollutants that accumulate in fish also reach humans. A well known example of transport of pollutants to humans via the aquatic food web is the mercury poisoning that occurred in Minamata bay, Japan, in the 1950s (Walker et al. 2001). In the Baltic Sea, most environmental pollutants have decreased in concentrations during the last decades. There is, however, still a risk that women of childbearing age will exceed the tolerable intake levels of dioxins and polychlorinated biphenyls (PCBs) if they eat fatty fish from the Baltic Sea regularly (Becker et al. 2007). As fish populations are of ecological as well as economical importance, monitoring of pollutants is necessary to retrieve the information that is needed for good management of the resource. Besides the economical and ecological importance of fish populations, exposure levels in fish are likely to reflect the exposure level for other organisms that live in the same environment.

Biomarkers in fish have been used to investigate polluted areas since the 1970's. Examples of sources of pollution that have been investigated are pulp mills (Larsson and Förlin 2002; Larsson et al. 2003; McMaster et al. 1995; Munkittrick et al. 1994), sewage treatment works (Jessica et al. 2007; Larsson et al. 1999), mining areas (Martin and Black 1998; Schmitt et al. 2007), pesticide contaminated farmland (Whitehead et al. 2004) and pollution from urban areas (Hansson et al. 2006b; Linderoth et al. 2006; Webb et al. 2005). As

physiological and biochemical responses in fish can be affected by factors other than pollution, it is important to use valid reference sites with similar conditions. Alternatively, by selecting sampling sites along a gradient to the suspected discharge, causal relationships between the source and the biological responses can be established (Andersson et al. 1988).

Another strategy for environmental monitoring is to examine reference areas without significant point sources or large population centres. Such areas can give information on large scale changes in the environment rather than changes in local effluents. Furthermore, the results can provide information about natural biomarker levels and variation and, hence, act as references to more contaminated sites. When the focus is shifting from point to diffuse pollution, this type of monitoring may become more important. An example where such a biomarker strategy has been used is the monitoring of feral coastal fish within the Swedish Environmental Monitoring Programme (Hansson et al. 2006a; Sandström et al. 2005).

Most field studies with biomarkers have been performed with feral fish. This means, if stationary fish species are used, that the fish have a life time exposure to the pollution load in the area. However, suitable fish species are not always available, and the life history of feral fish, including earlier exposure, can never be guaranteed. Besides the risk of misleading results due to migratory behaviour of the fish, transport of contaminants from other areas through migration of prey species may occur. Other factors that could affect the results are differences in nutritional status, age or reproductive stage, genetic differences and disease outbreaks. Because of these factors, the validity of the results can often be questioned regardless if the responses are positive or negative. Such weaknesses have previously been used to question the validity of results from biomarker studies in recipients to pulp and paper mills (Munkittrick et al. 2003).

The use of caged fish (feral or farmed) allows some degree of control for these confounding factors. The risk for migration is clearly eliminated by caging, and factors like age and feeding status can be controlled if farmed fish is used. Furthermore, monitoring is not restricted to areas where suitable species for biomarker analysis are available. In a review by Oikari (2006) a number of advantages with studies on caged fish were discussed (**Table 2**).

There are, however, also drawbacks with caging experiments. The use of feral fish has an obvious advantage in that the fish represents the ecosystem and has a life time exposure (hopefully) to the water that is assessed, thereby increasing the ecological relevance. Furthermore, natural feeding is reduced or eliminated during caging experiments. Therefore, exposure through the food web may be missed in the analyses. For chemicals with log K_{OW} above 5.6, food is the predominant rout of exposure for fish (Barber 2008). Examples of chemicals that are mainly taken up by food

Table 2. Advantages of caging in fish experiments. After Oikari (2006).

Known site of exposure (downstream vs. upstream; gradients)
Known time of exposure (e.g. before vs. after)
Desired species (cultured or feral)
Known age and size
Controllable for sediment contact
Integrates the ambient conditions to the chemical exposure
Repeatable (e.g., monitoring)
Can be started quickly on demand
Cost and time-use is more predictable
Can be standardized

are dioxins and PCBs, while others, such as PAHs, are mainly taken up *via* the gill tissues. Most studies with caged fish have been performed in recipients with known discharges of pollutants and reference cages in low polluted areas (Abrahamson et al. 2007; Larsson et al. 1999) or with several cages along an exposure gradient (Förlin and Hylland 2006). It is, however, unclear how caging studies perform in situations where diffuse pollution is monitored and references or pollution gradients can not easily be identified. In such situations, confounding factors may become more important. If the use of caged fish is chosen, e.g. because it is important to eliminate migration, a reliable methodology is necessary.

1.6 Aim of the thesis and specific aims of the six papers

The aim of this thesis has been to evaluate the use of biomarkers in fish for environmental assessments of aquatic ecosystems. A methodology with farmed rainbow trout (*Oncorhynchus mykiss*), reared in net cages or flow through tanks, has been developed and tested, and the data from these studies as well as from long term studies on feral perch (*Perca fluviatilis*) have been analyzed statistically. The majority of the studies with caged rainbow trout was performed in the river *Göta älv* and connected waters in western Sweden during 2004-2007, while one study was performed in the brook *Vallkärrabäcken* in southern Sweden during 2008. Data on feral perch were retrieved from the Swedish National Marine Monitoring Programme. The monitoring programme on coastal fish is funded by the Swedish Environmental Protection Agency and biomarker data has been collected in perch since 1988.

The thesis is based on six papers [I-VI]. In [I-IV], the use of biomarkers in caged rainbow trout is assessed, while [V-VI] examines biomarker responses in feral perch. The specific aims of the six papers were as follows:

I. Evaluate the effect of different holding conditions on caged rainbow trout.

II. Evaluate the effect of different feeding levels on caged rainbow trout.

III. Evaluate the methodology with caged rainbow trout in a water body with low anthropogenic impact.

IV. Evaluate the methodology with caged rainbow trout in a water body with high anthropogenic impact.

V Evaluate long term biomarker responses in feral perch at national reference areas.

VI. Evaluate the presence of PAHs and other pollutants in bile from feral perch at a national reference area.

2. Methods

The results presented in this thesis are mainly based on biomarker analyses of fish. In [I-IV], the results have been obtained from analyzing biomarker responses in caged rainbow trout, and in [V-VI] data from a feral population of perch were used. The methods that have been used are presented shortly here and described in more detail in the different papers. The most important biomarkers that have been used in [I-VI] are summarized in **Table 3**.

Table 3. Biomarkers used in [I-VI] and their interpretation. The interpretations are based on Larsson et al (2000) and Sandström et al (2005).

Biomarker	Interpretation	Used in
Condition factor (CF) and body mass index (BMI)	Feeding status and metabolic disturbances.	I, II, III, IV
Liver somatic index (LSI)	Reflects nutritional and metabolic status. Increased liver size indicates high metabolic activity. Reduced liver size can be caused by nutritional deficiency	I, II, III, IV
Gonadal somatic index (GSI)	Reduced GSI indicate lower fecundity, possibly caused by reduced energy allocation for reproduction.	V
Ethoxyresorufin-O-deethylase (EROD)	Measures detoxification activity. Increased EROD indicates exposure to organic pollutants.	I, II, III, IV, V, VI
Glutathione reductase (GR), Glutathione S-transferase (GST) and catalase	Antioxidant enzymes that indicate oxidative stress and exposure to oxygen radicals.	I, II
PAH metabolites in bile	Indicates exposure to 2-, 4- and 5-ringed PAHs.	I, II, III, IV, VI
Relative abundance of white blood cells (lymphocytes, granulocytes and thrombocytes)	Indicates effect on the immune defence system.	I, II, III, IV
Blood glucose and lactate in blood plasma	Indicates metabolic disturbances but changes can also be caused by sampling stress.	I, II, III, IV
Hematocrit (HT) and Hemoglobin (Hb)	Reflects the oxygen carrying capacity of the blood. Low values can be caused by gill damage or impaired osmoregulation, high values indicate increased oxygen demand or acute stress.	I, II, III, IV
Metallothionein (MT)	Metal binding protein. Increased MT indicates exposure to certain metals.	IV
Blood plasma ions (Cl^-, Ca^{2+}, Na^+, K^+)	Changes in plasma ions may indicate disturbed osmoregulation or ion regulation, kidney damage, gill damage or impaired intestinal uptake.	I, II, III, IV

2.1 Caged fish (Papers I-IV)

2.1.1 Experimental set-up

The caging studies with rainbow trout [I-IV] took place in western and southern Sweden during the years 2004 to 2008. The majority of the studies was conducted in the river Göta älv and connected waters, while one study was performed in the brook Vallkärrabäcken (**Figure 7**).

In most cases, two PVC tanks ($1\times1\times0.5$ m=0.5 m^3) or net cages ($1\times1\times1$m =1 m^3) were used as replicate units of each treatment (with the exception of [II]). Replicate units, rendering a nested experimental design, were used to reduce the risk that random environmental factors or hierarchical feeding would affect the results (Ling and Cotter 2003). The water flow rate in the plastic tanks ranged between 10 and 15 l/min. In most experiments, the fish were fed 4% (feed weight/fish weight) weekly. However, experiments were also performed with other feeding rations or no feeding at all. The pumps that were used to transport water through the PVC tanks allowed particles of up to 8 mm to pass. The mesh size of the net cages was 10 mm. This may allow some degree of natural feeding when small organisms pass through the cages. The fish were delivered from local fish farms and transported in aerated tanks to the experimental sites. Transportation times were in the range 45 to 90 minutes. **Figure 8** shows the set-up with plastic flow through tanks at two sites.

Figure 7. Experiments with caged fish were conducted in Göta älv in western Sweden and in Vallkärrabäcken in southern Sweden. The figure is modified from [III] and [IV].

2.1.2 Sampling and analytical procedures

The fish was killed by a blow to the head and blood was taken into a syringe. Weight and length were recorded before dissection for bile and liver samples. The gall bladder

Figure 8. Experimental flow through tanks at the experimental sites Lärjeholm (left) and southern Vallkärrabäcken (right).

was emptied with a syringe and the bile was frozen on dry ice. The blood values HT, Hb and glucose were measured directly on site and blood smears were prepared for later differential counting of blood cells. Blood was centrifuged for plasma which was frozen on dry ice. The liver was weighed and liver samples were frozen in liquid nitrogen for later determination of EROD, GR, GST, catalase and MT. The total sampling time for each fish was approximately 5 minutes.

Liver samples were stored in liquid nitrogen until homogenization and centrifugation. Resulting S9 fraction was stored at -80°C until EROD activity was measured. Liver samples for GR, GST, catalase and MT were stored at -80°C until processed or sent for analysis. Bile and plasma samples were stored at -20°C until analyzed. Sampling procedure and analytical methods were essentially according to ISO (2007) and are described in more detail in [I], with variations between studies described in the corresponding papers [II-IV].

2.1.3 Statistical treatments

Differences between treatments were tested using Analysis of Variances (ANOVA). In [I, III, IV], nested experimental designs were used to avoid pseudoreplication (Hurlbert 1984). In [II], however, no replicate tanks or net cages were used. Instead, the study was replicated at two sites using a two-way ANOVA. The risk that differences should occur due to random environmental factors is, thus, not higher than for a nested experimental design. When ANOVA is used, equal variances within samples are required. This was tested using Cochrans C. When significant differences were found for factors with more than two levels, the Student-Newman-Keul (SNK) procedure was used to determine which treatments that differed. ANOVA, Cochrans C and the SNK procedure are described in detail by Underwood (1997).

2.2 Feral fish (Papers V-VI)

2.2.1 Experimental set-up

The data on feral perch were retrieved from the Swedish National Marine Monitoring Programme, which is funded by the Swedish Environmental Protection Agency. The main area of focus in [V-VI] was *Kvädöfjärden*, located on the Swedish Baltic coast. In this area, biomarker measurements have been performed every year since 1988. Furthermore, *Holmöarna,* in the Bothnian bay, has been included in the analyses. In Holmöarna, biomarker measurements have been performed since 1993. The locations for Kvädöfjärden and Holmöarna are shown in **Figure 9**.

At each station, 25 females in the size range 20-30 cm have been sampled each year, with a few exceptions when catches were to low. The author of this thesis has participated in sampling of perch at these three stations during the period 2003-2008.

Figure 9. Location of the National reference sites Holmöarna and Kvädöfjärden used in [V-VI].

In addition to biomarker data, individual and population level measurements as well as concentrations of metals and organic pollutants have been analyzed yearly in Kvädöfjärden since 1989. The use of perch in the Swedish National Marine Monitoring Programme is described in more detail by Sandström et al (2005).

2.2.2 Sampling and analytical procedures

Perch for biomarker analyses were caught using gill nets and left to recover for 2-4 days in fish chests. The fish were killed by a blow to the head before body length and weight were recorded. The weight of the gonads was recorded and a central piece of the liver was frozen in liquid nitrogen for later determination of EROD activity. Liver samples were stored at -80°C until processed. Sampling is performed essentially as described in ISO (2007) and takes place in early September in Holmöarna and late September in Kvädöfjärden. The biomarker measurements are described in more detail by Hansson et al (2006a).

Perch that were used for individual and population data in Kvädöfjärden were also caught with gill nets. All female perch found in the nets were measured for length and divided into length groups of 2.5 cm. A number of perch from each length group were later analyzed for age. The age distribution within length classes can then be used to estimate the age distribution of the catches. Sampling for individual and population level data takes place in August each year. This data set is described in more detail in [V] and by Sandström et al (2005).

2.2.3 Statistical treatments

In [V], correlation analyses were used to establish relationships between environmental factors and biomarkers as well as between different biomarkers. In most cases, partial correlation was used to remove the effect of one or several potential confounding factors. In [VI], hypotheses were tested using ANOVA as well as correlation analyses.

3. Results & Discussion

The most important findings in [I-VI] are summarized and discussed here along with a concluding discussion of the six papers.

3.1 Biomarker studies on caged fish

3.1.1 Methodological considerations (Papers I-II)

For environmental assessments with caged fish, it is important that the methodology can be applied to many different locations. However, there are limitations when using different rearing systems such as net cages and flow through tanks. Examples of such limitations are rapidly streaming water that prevents net cages to be used, or lack of electricity, which makes it impossible to run flow through tanks. There is an obvious risk that biomarkers will be affected if holding conditions differ between treatments. It has previously been shown that factors such as lighting conditions (Head and Malison 2000; Volpato and Barreto 2001), tank colour (Papoutsoglou et al. 2005; Papoutsoglou et al. 2000) and shape of the rearing tanks (Ross et al. 1995) can affect growth and stress levels in farmed fish. It is, thus, important that the methodology is tested for effects of different holding conditions.

Fish that are kept in net cages or tanks will not be able to feed like in nature. Therefore, exposure to contaminants through the food web will be eliminated or strongly reduced. Instead, the major route of exposure will be *via* gill tissues. If the fish is fed during the experiment, there is a risk that the feed will be a source of contaminants. Chemical analyses of farmed salmon have shown high levels of dioxins and PCBs, probably caused by contaminated fish feed (Hites et al. 2004; Jacobs et al. 2002). The effect of holding conditions and feeding levels were examined in [I] and [II], respectively.

In [I], biomarker responses were compared for rainbow trout that were reared in net cages placed outdoors and flow through tanks placed inside a building. These two treatments were chosen as they represented the largest difference in holding conditions that could be expected when the methodology is used for environmental assessments. For the biomarkers that were analyzed, there were no differences between treatments. For catalase, however, differences between two plastic tanks were observed. This could be an effect of differences in light conditions (Khessiba et al. 2005) as one of the tanks was placed next to a window, while the other tank was about two meters from the window.

In [II], two treatments with feed rations of 8% and 2% (feed weight/fish weight), respectively, were used. The results of the experiment showed that over a period of four weeks, eight variables differed significantly between the two treatments (**Figure 10**). Among the differences

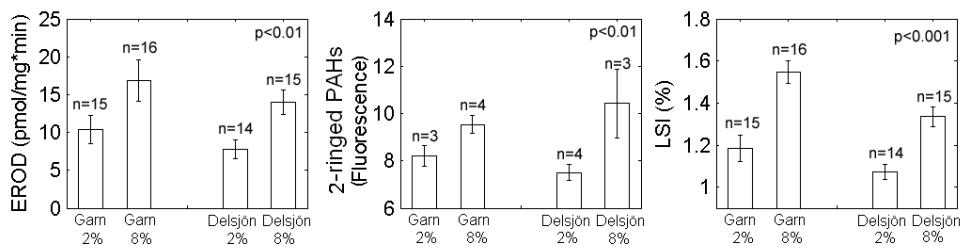

Figure 10. Three examples of biomarkers that differed significantly between feeding levels (2% and 8%) at the sites Garn and Delsjön. Error bars are standard error. The figure is modified from [II].

was a higher EROD activity for the treatment that received more feed. This could be caused by inhibition of EROD due to low nutritional status in the low feeding group. Another potential explanation is that the fish with a higher feeding ration have higher metabolic rate and oxygen consumption and thereby take up more pollutants from the water *via* the gills. This assumption is supported by an observed increase in 2-ringed PAH metabolites in bile for the higher feeding group. However, it can not be excluded that the feed itself contained EROD inducing chemicals, such as dioxins and PCBs. For other variables, such as the liver somatic index (LSI), differences are more likely a physiological response to the different feeding levels.

The results of the studies performed in [I-II] showed that net cages and plastic tanks can be used and compared for environmental assessments, but that measures must be taken to keep the environmental conditions and feeding rations as similar as possible. A simple method to standardize feeding and secure that all fish receive equal amounts could be to starve the fish during the exposure period. This would, however, limit the possible exposure time for the fish.

3.1.2 Experiences from field studies (Papers III-IV)

Two studies were performed where the methodology with farmed fish, reared in flow through tanks, was used to examine the exposure to environmental pollutants in the field. In [III], four sites in the water system of the river Göta älv in western Sweden were analyzed along with an external reference site at the fish farm from which the fish were delivered (**Figure 11**). The experiments were conducted during October and November in 2007, and two

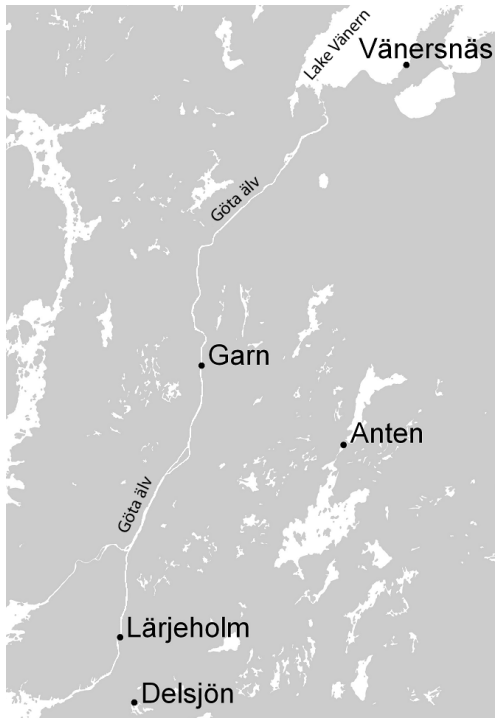

Figure 11. Map of experimental sites in the river Göta älv. *Vänersnäs* in located in the lake Vänern, which is the origin of the river Göta älv. *Garn* and *Lärjeholm* are located in Göta älv. Lake *Delsjön* receives water that is pumped from Göta älv. Lake Anten is not connected to the other sites and was used as an external reference. The figure is modified from [III].

of the sites were examined at approximately the same time of the year in 2006. The contamination load in this water system can be expected to be low due to the relatively low human population density in the area in combination with the high water flow rate (mean flow: 550 m^3/s) in the river.

Significant differences between sites were found for nine variables, including CF, PAH metabolites in bile and EROD activity (**Figure 12A**). The differences in CF complicate the interpretation of the results as this could be taken as evidence that feeding differed between sites, even

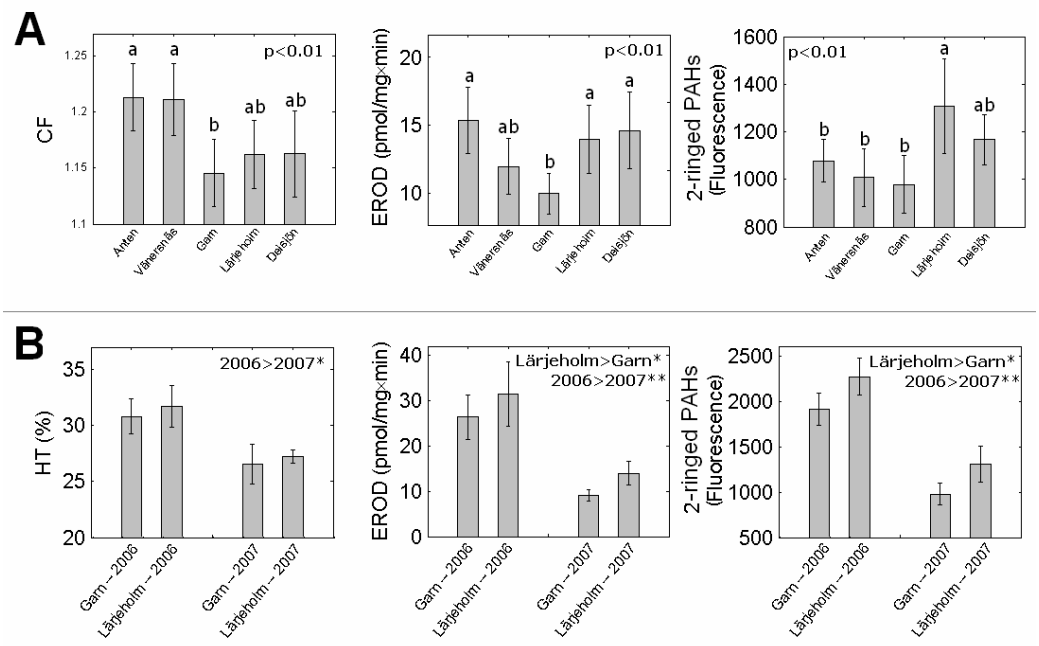

Figure 12. In 2007 there were differences between sites for several biomarkers, including CF, EROD activity and PAHs in bile (A). For several biomarkers, the response patterns for the sites Lärjeholm and Garn were similar for the years 2006 and 2007 (B). Error bars are 95% confidence limits and letters show significant differences (a>b). * p<0.05, **p<0.01. The figure is modified from [III].

though the feeding rations where equal. A possible explanation to this could be that the ability for the fish to find the feed was affected by differences in water turbidity between sites. In 2007, EROD as well as the concentration of PAH metabolites in bile were found to be higher at the sites Lärjeholm and Delsjön than at the site Garn, which is located further upstream. A logical interpretation to this could be that there is a source of PAHs between Garn and Lärjeholm. However, with the results from [II] in mind, it can not be excluded that the differences were caused by differences in feeding. The site Anten differed from the other sites in that the level of PAHs was low while the EROD activity was high. In this case, the increase in EROD can not be explained by increased uptake of PAHs due to higher feeding. However, as the CF was high at this site, other EROD inducers may have been taken up as a result of higher feeding level, e.g. due to contaminants in the fish feed (Hites et al. 2004). The increase in EROD activity and PAH metabolites in bile between Garn and Lärjeholm was also seen for 2006 (**Figure 12B**). During this year, however, there was no difference in CF between the two sites. EROD activity, the concentration of PAH metabolites in bile and HT were all significantly higher in 2006, which suggest that exposure to pollutants was higher during this year. Overall, the biomarker responses in Göta älv were weak and the influence of factors other than pollution might be important.

In [IV], three sites were analyzed in the brook Vallkärrabäcken in southern Sweden. The study was performed to ex-

amine the cause of earlier observations of skeletal damage in feral brown trout (*Salmo trutta*) in Vallkärrabäcken and can therefore be considered as a retrospective risk assessment rather than environmental monitoring. The damaged brown trout were observed during electro fishing studies in the southern branch of the brook, while no damaged fish were found in the northern branch (Eklöv 2002). The southern and northern branches of Vallkärrabäcken differ in that the northern branch drains an area that mainly consists of farmland, while the southern branch drains an urban area which includes an old landfill (**Figure 13**). Numerous pollutants can therefore reach the brook by urban storm water or leachate water from the landfill. In such a scenario, chemical measurements of a few known contaminants may not be sufficient. However, biomarker responses can help to identify groups of chemicals that are taken up by fish, thereby providing a link between observed effects and the responsible contaminants. Fish were placed in flow through tanks in February 2008 at the site in the southern branch of Vallkärrabäcken where skeletal damages had been observed some years earlier (site 2). Furthermore, tanks were placed at a surface water pond (site 3) located upstream from the brook (closer to the urban area and the landfill) and at a site in the northern branch of the brook (site 1). Besides rainbow trout, feral brown trout were sampled by electro fishing in the northern as well as the southern branch.

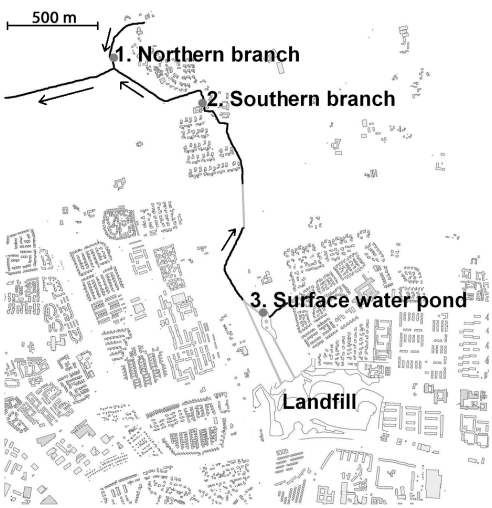

Figure 13. Map of sampling sites in Lund. The black lines show the brook Vallkärrabäcken and the grey line shows stretches with culverts. The map shows buildings (urban area) as well as the landfill. Arrows indicate the direction of water flow. The figure is modified from [IV].

The study provided clear evidence for high exposure levels in the branch of Vallkärrabäcken where skeletal damage previously had been observed in feral brown trout. For EROD activity, the concentration of PAH metabolites in bile and the ratio between 2- and 4-ringed PAH metabolites, there were significant differences between sites. The higher levels of EROD activity and PAH metabolites were found in the southern branch of the brook and at the surface water pond. There was also a shift towards proportionally more 4-ringed PAHs at these sites (**Figure 14**). The surface water pond differed from the other two in higher LSI and reduced Hb and HT (see [IV]). This suggests that the exposure level was even higher at this site. Compared to the reference sites, EROD levels in rainbow trout were 5-6 times higher in the branch where skeletal deformations had been observed. The study on feral brown trout showed the same increase in EROD activity in the southern compared to the northern branch.

Because the rainbow trout were not fed during the exposure period, problems with variations between sites caused by differences in feeding were avoided. The study was performed during the winter when feeding levels are naturally low in rainbow trout. From the results it is obvious that

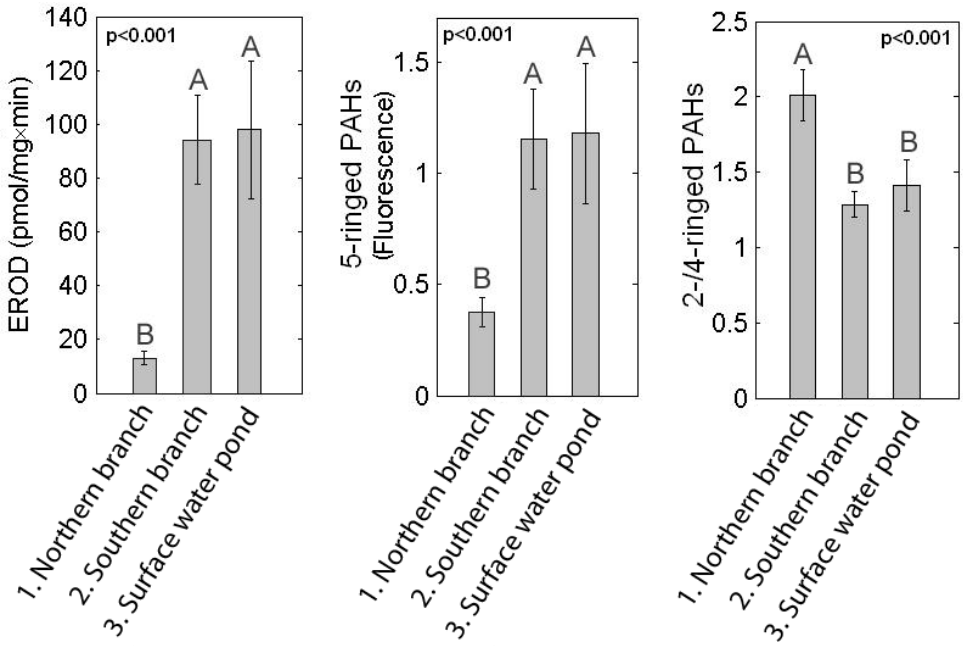

Figure 14. EROD activity and the concentration of PAH metabolites in bile were higher in the southern branch of Vallkärrabäcken and in the surface water pond. The ratio between 2- and 4-ringed PAHs was lower at these stations. Error bars are 95% confidence limits and letters show significant differences (A>B). The figure is modified from [IV].

PAHs contribute to the increase in EROD activity. The decrease in the ratio between 2- and 4-ringed PAHs suggests that the exposure is shifted towards combustion processes, such as road traffic. Therefore, it is likely that pollutants are brought to the brook by urban storm water. Previous studies suggest that leachate water from landfills is not an important source of PAHs (Ahel and Tepić 2000; Marttinen et al. 2003). However, the landfill may still contribute to the observed differences by releasing other EROD-inducing pollutants, such as dioxins and PCB.

When the two studies [III & IV] are compared some important differences can be seen. The study in Vallkärrabäcken gave important information about the pollution in the river and possible explanations to the observed skeletal damage in feral brown trout. The study in Göta älv, however, gave little useful information. The major reason for this is probably that there were only small differences between sites and that the pollution level was relatively low at all sites. The low pollution level leads to small responses in the biomarkers, which are then likely to be more easily affected by confounding factors such as differences in feeding between sites. In Vallkärrabäcken, however, the responses were so strong that a small effect by differences in feeding would not have changed the results. This can easily be seen by comparing the levels of EROD activities in **Figure 12A** and **Figure 14**. Another important difference is that for the study in Vallkärrabäcken, effects (fin and skeletal damage) had been observed that could be linked to the biomarker responses. Thereby, the relevance of the findings in Vallkärrabäcken was increased.

3.2 Biomarker studies on feral fish

3.2.1 What is causing the biomarker responses on feral perch in Kvädöfjärden? (Papers V-VI)

In [V-VI], potential explanations to an increasing trend in EROD activity and a negative trend in GSI (**Figure 15**) observed in perch at Kvädöfjärden on the Swedish Baltic coast (Hansson et al. 2006a; Sandström et al. 2005) were examined.

Kvädöfjärden was chosen as a coastal reference site in the Swedish National Marine Monitoring Programme as it is located far from known point sources and large population centres. The observed time trends in perch could be taken as warning signals for increased exposure to pollutants and, potentially, negative effects for the population. This is, however, contradicted by increasing trends in abundance and body size of perch in the area (Sandström et al. 2005). As biomarkers were measured in fish within a certain length interval, the increase in body size has resulted in reduced mean age of the sampled fish.

Chemical measurements in fish tissue from Kvädöfjärden suggest that known EROD inducers, such as PCBs and dioxins, have been decreasing in the area during the same period as EROD activity has been increasing (Bignert and Nyberg 2007). If the effects on perch are caused by increasing pollution, it must be by chemicals that are not monitored in the area. One group of known EROD inducers that have not been monitored in fish tissues from Kvädöfjärden is PAHs as they are metabolized quickly in fish and do not bioaccumulate to a large extent.

An alternative explanation that has been suggested is that successively increasing water temperature in the area has affected the physiology of the fish, including indirect effects on biomarkers. The two alternative explanations are shown schematically in **Figure 16**, including secondary effects such as increasing catches for abundance estimates due to increasing body size of fish.

In [V], biomarker data and water temperature from Kvädöfjärden were evaluated to investigate if increasing temperature and/or exposure to pollutants are likely explanations for the observed time trends. In addition, the water flow rate in the nearby river Vindån was tested for correlation with EROD activity and GSI to

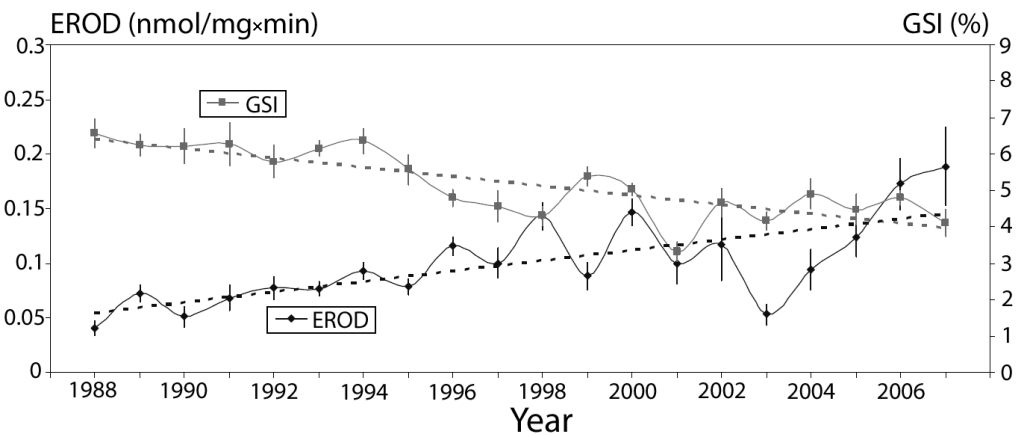

Figure 15. Time trends in EROD and GSI for female perch in Kvädöfjärden. Error bars are 95% confidence limits. The figure is modified from Naturvårdsverket (Swedish EPA) (2008).

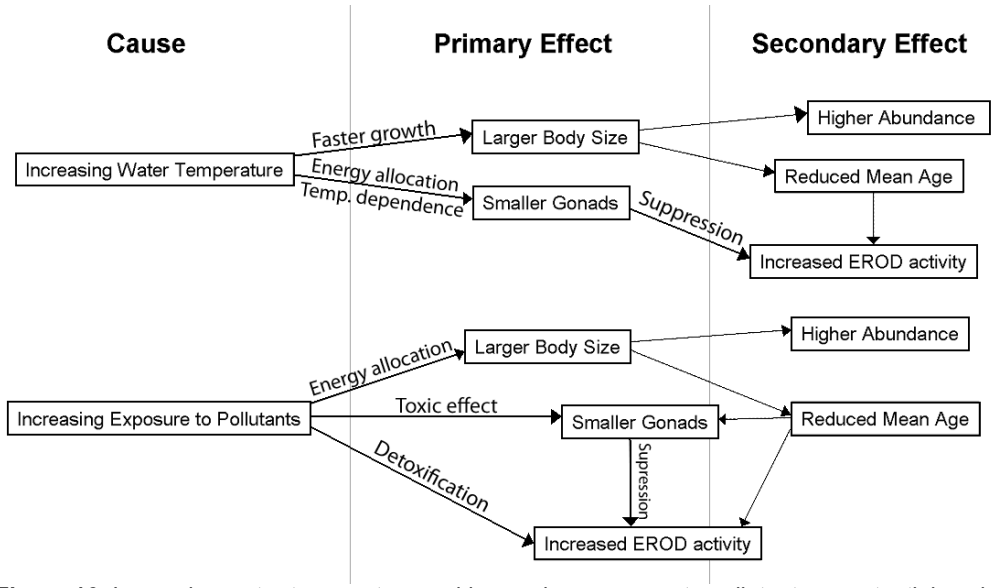

Figure 16. Increasing water temperature and increasing exposure to pollutants as potential explanations to observed time trends in biomarkers in perch at Kvädöfjärden. The figure is modified from [V].

investigate if this could be a source of pollutants. Furthermore, data on perch from another national coastal reference site, Holmöarna, were also included in the study.

When the factor age was controlled for, the time trends in EROD activity and GSI were still significant for the period 1988 – 2007 ($p<0.001$). This means that the trends are not caused by the decrease in mean age of sampled fish. A similar significant trend in GSI could be seen at Holmöarna ($p<0.05$).

Water temperatures during year of sampling and during the perch life were tested for correlations with EROD, GSI and body length. The temperature correlations were also compared to the correlation to time to investigate if it was likely that an increase in temperature contributed to the time trends in these variables. For EROD and GSI, the time trend was stronger than the correlation to water temperature. Therefore, it is not likely that these time trends were caused by increasing temperature. For body length, however, the correlation to water temperature during the perch life was stronger than the correlation to time (**Figure 17A**). It is, therefore, likely that increasing water temperature drives the time trend in body length for perch in Kvädöfjärden.

If the reduction in GSI is caused by increasing exposure to pollutants, EROD activity may serve as a marker of exposure level. EROD activities during the year of birth, year of sampling and the sum of EROD activities during the perch life were tested for correlations with GSI in Kvädöfjärden and Holmöarna, and body length in Kvädöfjärden. It was found that the sum of EROD activities during the perch life correlated negatively to GSI in Kvädöfjärden as well as in Holmöarna, and that these correlations were stronger than the time trends at these areas (**Figure 17B**). This means that perch that have lived during years with high EROD activities have

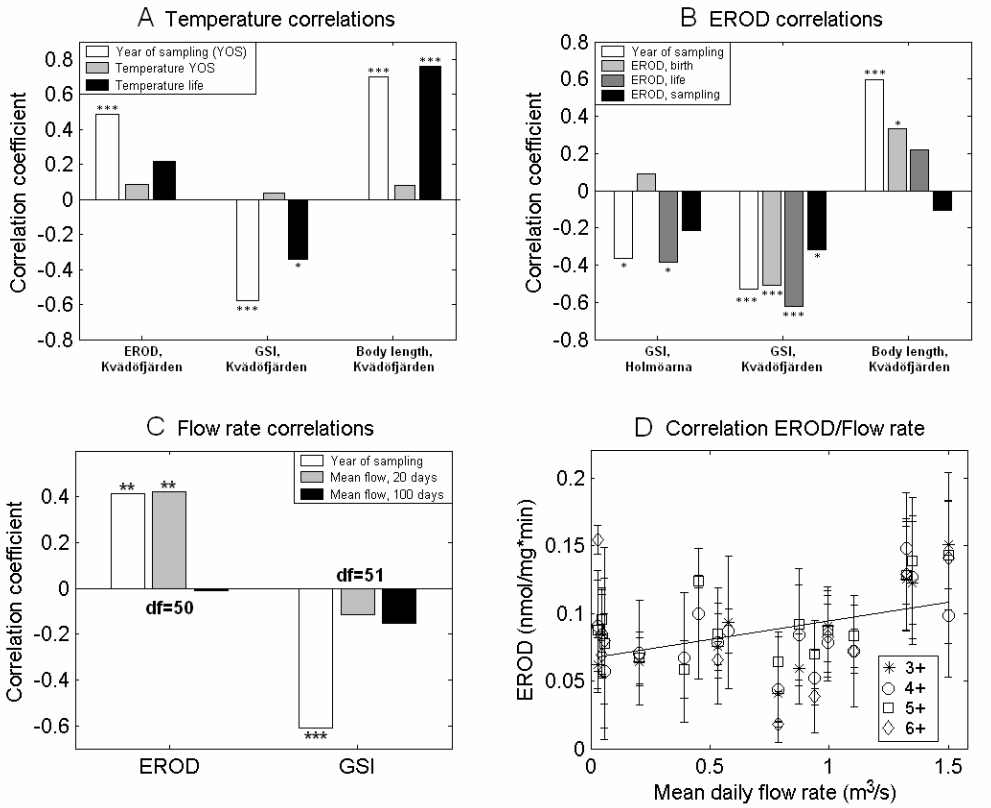

Figure 17. A: Correlation coefficients for EROD, GSI and body length to time (year of sampling) and temperature. B: Correlation coefficients for GSI and body length to time and EROD. C: Correlation coefficients for EROD and GSI to time and flow rate in the river Vindån. D: The correlation between EROD activity and flow rate in Vindån the last 20 days prior to sampling for age classes 3+ to 6+. * p<0.05, **p<0.01, *** p<0.001. The figure is modified from [V].

smaller gonads and that the increase in EROD activity may be causing the time trend in GSI. As Kvädöfjärden and Holmöarna are located more than 700 km apart, these results may indicate large scale environmental changes in the Baltic Sea. For body length, the correlations to EROD activity were poor compared to the correlation to time (**Figure 17B**).

Data on mean daily flow rate in a nearby small river were correlated to EROD activity and GSI in perch at Kvädöfjärden. The mean daily flow rate for a longer period of 100 days and a shorter of 20 days prior to sampling were used in the analysis. EROD activity correlated significantly to the mean flow rate the last 20 days before sampling (Figure **17C&D**). Therefore, it is likely that EROD inducing pollutants are brought to Kvädöfjärden by runoff from land.

In [VI], perch in Kvädöfjärden were further examined by studying frozen bile from a year with high (2006) and a year with low (2003) EROD activity. It was shown that the concentration of PAH metabolites in bile was higher in 2006 than in 2003. Furthermore, the ratio between 2-

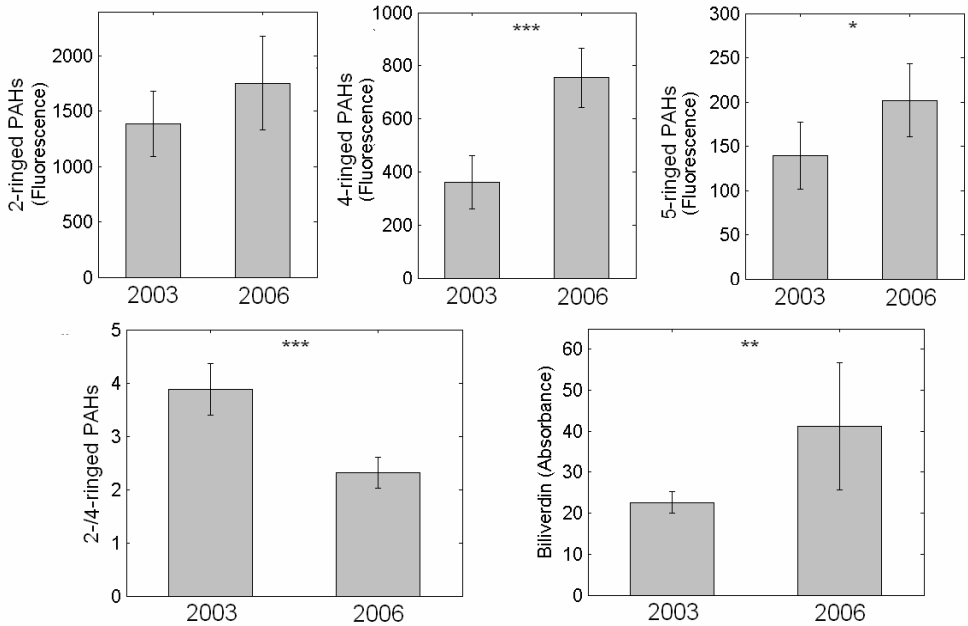

Figure 18. The concentrations of PAH metabolites were higher in bile from 2006 than in bile from 2003 (top). The ratio between 2- and 4-ringed PAHs was higher in 2003 than in 2006 (lower left). The biliverdin concentration was higher in 2006 than in 2003 (lower right). Error bars are 95% confidence limits. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. The figure is modified from [VI].

and 4-ringed PAH metabolites was lower in 2006 (**Figure 18**). This shows that the exposure to PAHs from combustion processes (4-ringed) was higher during the year with high EROD activity. An obvious source for this type of PAHs is motor traffic. With the correlation between EROD activity and flow rate in Vindån in mind [V], it is likely that increasing runoff from roads contribute to this difference in PAH concentration. Furthermore, PAHs with four or five rings are more potent EROD inducers than those with fewer rings (Bosveld et al. 2002; Skupinska et al. 2007). Besides differences in PAH concentration and composition, the bile density, based on biliverdin measurements, was significantly higher in 2006 (**Figure 18**).

Because the bile density was higher in bile from 2006, there is a risk that the observed difference in PAH concentration could be caused by accumulation of PAHs in bile during a longer period of time. However, there was a significant ($p<0.05$) correlation between EROD activity and the concentration of PAH metabolites within years (**Figure 19**), but no correlation between biliverdin and PAH concentration. This suggests a link between recent exposure to PAHs and increased EROD activity in perch in Kvädöfjärden. The change in PAH composition (more 4-ringed PAHs) further strengthens the conclusion that there are real differences in exposure between 2003 and 2006. A study on Atlantic croaker (*Micropogonias undulates*) has previously shown that PAHs can cause reduced gonad development (Thomas and Budiantara 1995). This is in agreement with the finding that perch that have lived during years with high EROD levels have smaller gonads.

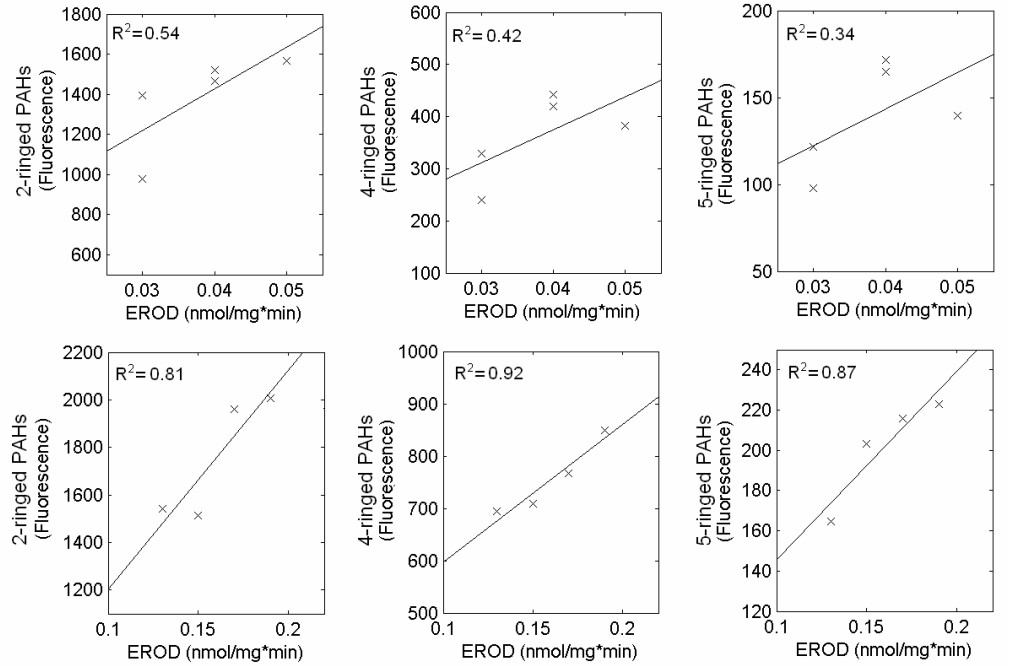

Figure 19. Correlations between EROD activity and concentration of PAH metabolites in bile samples from 2003 (top) and 2006 (bottom). The correlations were stronger in 2006, when the PAH concentrations were highest. The figure is modified from [VI].

3.3 Concluding discussion

3.3.1 The utility of biomarkers in fish for environmental assessment (Does fish health matter?)

The use of biomarkers in fish that are reared in net cages or flow through tank systems offers a number of advantages. Those advantages were apparent in [IV], where fish could be exposed to water at sites of concern without the risk of migration between polluted and clean areas. The fact that all fish came from the same batch and had the same life history, including exposure to pollutants, strengthens the evidence that differences between sites were caused by pollution. For feral fish, there are always potential confounding factors such as feeding status, migration and genetic differences. The methodology with fish reared in tanks also allowed studying a site where feral fish were not available, i.e. the surface water pond. Furthermore, all variables could not have been analyzed if only feral fish were used. One example is the analyses of PAH metabolites in bile that could not be performed on feral brown trout due to the insufficient amounts of bile in the fish.

In all situations, however, the use of caged fish may not be equally successful. The results in [III] show small but significant differences between sites in Göta älv for several biomarkers. The interpretation of the results is, however, complicated by differences in CF between sites, which suggest that there were differences in feeding level. Differences in EROD activity and PAH concentration between sites in Göta älv could, therefore, be caused by differences in feeding status rather then by

differences in exposure level. Furthermore, the responses that can be seen in caging studies are restricted to changes that occur relatively fast (within the exposure period). This means that effects that are caused by chronic exposure will be missed in such studies. Therefore, the ecological relevance of the studies will be reduced unless there are observed effects from higher levels of organization to relate to. The study with farmed rainbow trout in Vallkärrabäcken is a good example of a situation where studies with caged fish can be useful by linking observed effects in wild fish to exposure to pollutants.

In [I] and [III], there were significant differences between replicate tanks for several variables. This shows that the risk of pseudo-replication (Hurlbert 1984) is substantial and must be considered in caging experiments with rainbow trout. This complicates the experimental design and reduces the statistical power achieved from a limited number of fish (Hanson and Larsson 2007). A methodology that avoids the problem is to cage the fish individually. This was done by Vermeirssen et al (2005), who placed brown trout in individual mini cages of stainless steel that were anchored to the bottom of a river. Besides avoiding the risk of pseudoreplication, the method provides a number of other advantages. There is no need for electricity, stress at sampling from chasing the fish is reduced, the equipment is robust and the risk for vandalism is minimized as the cages are below the water surface. The method would, however, not have worked at all sites used in [I-IV]. For example, the bottom of the surface water pond in [IV] was covered with soft mud. Furthermore, the results may be affected by differences in water flow rate between sites, which may lead to differences in exposure as well as in physical stress of the fish. The method could, however, be further developed to avoid these problems. In [IV], where the fish were starved during the exposure period, no differences between replicate units were seen. This suggests that hierarchical feeding behaviour may be one of the most important reasons for differences in biomarker responses between replicate tanks.

In [V] and [VI], biomarker data from perch in reference areas were used. Because the biomarkers were analyzed in feral fish, effects of life time exposure could be seen. It was shown that exposure to PAHs by increasing runoff from land is a probable explanation both to the increase in EROD activity, and to the observation that female perch that have lived during years with high EROD levels have reduced gonad size. In this case, the reduction in gonad size has high ecological relevance and may affect the reproductive capacity. There is an ongoing debate regarding the use of biomarkers in ecotoxicology and the ecological relevance of sub-organism responses (Forbes et al. 2006). Few studies have been able to show a link between biomarkers of exposure and ecologically relevant effects. In [V], exposure to EROD inducing chemicals for several years could be linked to decreased gonad size. This was possible because of the unique 20-year data set that was used. A reduction in gonad size does not automatically reflect a reduction in population size as factors such as density dependence can compensate by increasing survival of recruits as well as of adults. It is, however, probable that a population with impaired reproduction will be more sensitive to other disturbances, such as unfavourable environmental conditions or high fishing pressure.

Munkittrick et al (2003) examined the criticism of biomarker studies in receiving waters for pulp and paper effluents and

found three valid concerns; 1) the appropriateness of reference sites, 2) that unknown factors can affect responses in feral populations and 3) variability between sites complicates interpretations. The methodology with caged fish will reduce many problems linked to these three concerns by controlling confounding factors such as age and migration of the sampled fish. The ecological relevance of results from caging experiments is, however, inevitably lower than for results from experiments with feral fish. The major reasons for this are that responses that reflect life time exposure to pollutants are missed in caging studies and that exposure through the food web is limited. It is clear that neither the use of feral fish nor the use of caged fish is the best method in all situations. The major questions that have to be answered before the methodology is chosen are; 1) Are there suitable feral species available? 2) Is there a risk that migration will be a confounding factor? and 3) What kind of questions do we want to answer? If suitable feral species are available and there is no risk of migration, the use of feral fish is preferable as the results have higher ecological relevance. If, however, the major questions that have to be answered are linked to exposure rather than effects, and the exposure is expected to be mainly from water, the use of caged fish may still be a good alternative.

3.3.2 Proposals for future research

Biomarker responses, in fish as well as in other organisms, suffer from a number of disadvantages such as questionable ecological relevance and that results sometimes are difficult to interpret. There are, however, drawbacks with all methods for environmental assessment. Higher level measurements, such as community indices, can also be difficult to interpret due to increased noise and the specificity of the results is low. Chemical measurements, on the other hand, require knowledge about which contaminants to measure and do not include environmental factors such as bioavailability. If the strengths of different strategies could be combined, a clearer picture of the situation may be achieved. A path for future research could therefore be to focus on how different levels of organization can be used and analyzed together (multiple lines-of-evidence) to get the best assessment of the state of the environment. In reality, however, research is often more focused on finding new methods rather than to understand those that are already in use (Forbes et al. 2006).

Biomarkers in fish can be an important component in weight-of-evidence based approaches (where multiple lines-of-evidence are used) to assess the environmental status. In the Sediment Quality Triad (SQT) three endpoints are used to determine sediment quality. The three endpoints are sediment chemistry (contamination), toxicity tests, and benthic community alterations. The results can be interpreted with a tabulated decision matrix (Table 1, from Chapman (1996)). The SQT can be used as a basis for constructing a weight-of-evidence tool to assess water quality. An example of a strategy that can be used in small streams is to use passive samplers to investigate contamination, biomarkers in caged fish to investigate bioavailability and total pollution load (exposure), and electro fishing to investigate age and community structure (Hughes et al. 2002) as well as visible damage on individuals (effect). If a larger water body is investigated, such as a lake, biomarkers in feral fish can be used to determine exposure, and fishing with gill nets may be preferable to examine effects on the population. The outcome of the study can be

Table 4. Examples of possible outcomes when passive samplers, biomarkers in fish and visible damage to feral fish are used and analyzed together. The interpretations are based on Chapman (1996).

Passive sampler (increased contamination)	Biomarkers (elevated responses)	Damage (observed effects)	Interpretation
Yes	Yes	Yes	Strong evidence for pollution induced degradation.
No	No	No	Strong evidence against pollution induced degradation.
No	No	Yes	Alteration is not due to toxic contamination.
Yes	No	No	Contaminants are not bioavailable.
No	Yes	Yes	Unmeasured toxic contaminants are causing degradation

interpreted in a similar way as the SQT (**Table 4**).

The strategy can also be used in retrospective risk assessment when, e.g., there is a known source of chemicals. This can be done with a tiered approach, starting with measurements of the chemicals in the environment. If the chemical is found at elevated levels, biomarkers and community structure can be investigated to determine if the chemical is bioavailable and has effects in the environment. The tiered approach can also be performed in the opposite direction, starting with an observation (by the public or within routine monitoring) of adverse effects in the environment. Biomarkers can then be used to establish which group of contaminants that are causing the effects and chemical measurements can point out the chemicals that are responsible and, possibly, identify the source. A more complex, weight-of-evidence based, procedure for retrospective ecological risk assessment is described by Forbes and Calow (2002a). Here, biomarkers are recommended to answer the question 'Is there effects in the target known to be specifically caused by exposure to the agent?', which is one of seven questions that are used in the assessment.

Future research should focus on developing integrated methodologies, where information from several techniques and several levels of biological organisation are used to provide a better assessment of the ecosystem.

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That makes sense to me, doesn't it?

George Walker Bush

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